STRUCTURE, COMPOSITION AND DEGRADATION OF THE CELL WALLS OF FORAGE CHICORY (*Cichorium intybus* L.) LEAVES

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Abstract

Chicory (*Cichorium intybus* L.), a valuable forage for ruminant livestock in temperate regions, appears highly degradable in the rumen. Fundamental reasons for the rapid breakdown of chicory cell walls in the rumen were studied.

Cell walls were isolated from laminae and midribs of chicory (cv. Grasslands Puna II) leaves. The walls, which, except for the walls of xylem tracheary elements in vascular bundles, were non-lignified, were fractionated progressively with 50 mM CDTA, 50 mM Na_2CO_3, 1 M KOH, 4 M KOH, 4 M KOH + 3.5% H_3BO_3, and hot water. The polysaccharides were similar to those in nonlignified walls of other dicotyledons, but with high proportions of pectic polysaccharides (67% of the total wall polysaccharides in the laminae). These included homogalacturonans (HGs, 50% of the total wall polysaccharides in laminae) and rhamnogalacturonan I (RG I). In contrast, the proportions of cellulose, xyloglucans, heteroxylans and glucomannans were low.

The locations of different pectic polysaccharides were determined using the monoclonal antibodies JIM5 and JIM7 against HGs with low and high degrees of methyl esterification, respectively, LM6 against arabinan and LM5 against galactan. All primary walls were labelled with all the antibodies used. However, the middle lamella, tricellular junctions and the corners of intercellular spaces were labelled with JIM5 and JIM7, but not with LM5. The middle lamella was labelled with LM6, but not the corners of intercellular spaces. These results support the involvement in cell adhesion of HGs with low degrees of methyl esterification.

A preparation of endopolygalacturonase (endo-PG) was used to investigate cell adhesion, and its effect on forage particle breakdown was determined using weight loss, chemical analysis and immunofluorescence labelling. The preparation dramatically reduced particle size. Cell separation was accompanied by a loss of HGs with low degrees of methyl esterifcation from the middle lamella and corners of intercellular spaces. A consequential loss of cell adhesion evidently caused leaf breakdown.

The degradation of fresh chicory leaves by rumen bacteria was investigated by measuring weight loss, monosaccharide release and immunocytolabelling. Two bacteria, the pectolytic *Lachnospira multiparus* D32 and the cellulolytic *Fibrobacter succinogenes* S85, effectively degraded chicory. Pectic polysaccharides were degraded

faster than other wall polysaccharides, with uronic acid released faster and more completely than neutral monosaccharides.

The preponderance of non-lignified primary walls and abundance of pectic polysaccharides may account, in part, for the rapid degradation of forage chicory in the rumen. The HGs in the middle lamellae and corners of intercellular spaces probably have a role in cell adhesion, and their degradation is probably responsible for the rapid reduction in the particle size of chicory leaves in the rumen.

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ABBREVIATIONS

α	anomeric configuration/ or cellulose polymorph
β	anomeric configuration/ or cellulose polymorph
ADF	acidic detergent fibre
AG-I	Arabinogalactan I
AG-II	Arabinogalactan II
AGP	Arabinogalactan Proteins
AGX	arabino(glucurono)xylan
AIR	alcohol-insoluble residues
Ara	arabinose
BSA	bovine serum albumin
CDTA	*trans*-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid
D-	optical isomer of the sugar
DM	dry matter
DMD	dry matter digestibility
DPX	dibutyl phthalate xylene medium
EC	Enzyme Commission
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
endo-PG	endopolygalacturonase
f	furanose form of the monosaccharide
FID	flame ionisation detector
Fuc	fucose
g	gravity
GalA	galacturonic acid
GAX	glucuronoarabinoxylan
GC	gas chromatography
GC-MS	gas chromatography-mass spectra
GGM	galactoglucomannan
GI	gastrointestinal
Glc	glucose
GlcA	glucuronic acid
HG	homogalacturonan

IgG	Immunoglobulin G
IVDMD	*in vitro* dry matter digestibility
IVOMD	*in vitro* organic matter digestibility
KV	kilo voltages
L-	optical isomer of the sugar
Man	mannose
min	minute
MOPS	3-(N-morpholino)propanesulfonic acid
NDF	neutral detergent fibre
NSP	non-starch polysaccharide
OM	organic matter
OMD	organic matter digestibility
p	pyranose form of the monosaccharide
PBS	phosphate buffered saline buffer
PBS-T	phosphate buffered saline buffer with Tween 20
Phl-HCl	phoroglucinol-HCl
PIPES	piperazine-1,4-bis (2-ethanesulfonic acid)
PMAA	partially methylated alditol acetate
PME	pectin methylesterase
PRG	perennial ryegrass
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
Rha	rhamnose
SEM	standard error of mean
t-	terminal
TFA	trifluoroacetic acid
UA	uronic acids
UV	ultraviolet (radiation)
v/v	volume : volume ratio
VFI	voluntary feed intake
w/v	weight : volume ratio
w/w	weight : weight ratio
WC	white clover
Xyl	xylose

Chapter one

Literature review

1.1 Introduction

Forages are the basis of grazing ruminant production systems. Chicory (*Cichorium intybus* L.) (Fig. 1, Fig. 1.2 and Fig. 1.3) is a relatively new cultivated forage herb (family Asteraceae) with high yield (Rumball, 1986) and high feeding value compared with other forage crops (Niezen et al., 1993; Hoskin et al., 1995, 1999a; Barry, 1998; Fraser et al., 1999; Foster et al., 2002; Neel et al., 2002) under grazing conditions. The studies of Hoskin et al. (1995) and Kusmartono et al. (1997) have shown that chicory (cv. Grasslands Puna) is more easily degraded in the rumen, is more highly digestible and elicits higher voluntary feed intake, leading to greater animal production than more traditional forages. Although the content of neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin, have been determined by Van Soest's methods (Kusmartono et al., 1997), little is known about the chemical structure of chicory cell walls and their degradation.

Forage plants include the forage grasses (family Poaceae), which are monocotyledons, and the forage legumes (family Fabaceae), brassicas (family Brassicaceae) and herbs, including members of the families Asteraceae and Plantaginaceae, which are all eudicotyledons (Barnes et al., 1995). The feeding value of forages is affected by differences in cell wall compositions among taxa and consequent differences in cell wall degradation by rumen bacteria. The cell wall compositions of grasses such as perennial ryegrass, forage legumes such as lucerne and forage brassicas such as kale have been well studied (Lam et al., 1990). However, this is not so for the forage herbs such as forage chicory.

In order to clarify the fundamental reasons for the rapid digestion of chicory by ruminant animals, this thesis will describe a study of the composition and degradation of chicory cell walls.

To achieve the above two purposes in the study, a literature survey was carried out to review the use of chicory as a forage, the structure of forage cell walls and the degradation of forage cell walls by rumen bacteria.

Figure 1.1 Chicory field

Figure 1.2 Chicory in vegetative stage

Figure 1.3 Chicory in reproductive stage (above), chicory flowers (left) (from Simone Hoskin)

1.2 Forage chicory

1.2.1 Biology and origins

Chicory is a perennial herb with stiff, erect, angled, branched stems up to 120 cm tall. Although leaves occur on the stem, most occur as a rosette in the vegetative stage. These leaves are lance shaped, normally shiny and deep green in colour. They are toothed and normally hairless. The lower leaves in the rosette are up to 30 cm long and 7 cm wide. A sticky white juice often secretes from the stems and leaves. Rosette leaves are the main parts of the plants consumed by ruminants. Chicory has long, thick, fleshy, vertical taproots. Its flowers, which appear in New Zealand from December to March, are bright pale blue or occasionally pink and have a diameter of 25-40 mm. The seeds are short, angled, top-shaped and are up to 3 mm long (Roy, 2004).

Chicory prefers to grow in well-drained or moderately drained soils with a pH of 5.5 or higher. Chicory grows from early spring to late autumn with rapid summer growth even in light drought conditions, but it is dormant in winter (Rumball, 1986).

Chicory originated in Europe, western Asia and northern Africa (Roy, 2004). It has been used as a vegetable "witloof" in European countries for over 300 years (George, 1985; Ryder, 1999). The extract of its roots is also used as a coffee substitute (Barry, 1998) and for fructose and inulin production (Volesky, 1996). Recently, it has been used for the extraction of oligofructans and inulin for use as prebiotics (Hughes and Rowland, 2001) and chicory extracts from its roots are potential antifungals (Mares et al., 2005).

In New Zealand, Cockayne (1915) first mentioned chicory as a component of grass-seed mixtures. But the amount of herbage provided by chicory was considered to be too minor to warrant attention. O'Brien (1955) conducted fifteen trials, including an animal trial with sheep between 1948 and 1950, concluding that chicory is not a valuable forage because of low persistence and yield. Lancashire (1978) reported chicory of value for forage under rotational grazing in dry summer conditions. It has recently received attention as a new cultivated forage crop for grazing in New Zealand (Rumball, 1986) and in many other regions of the world such as North America (Jung et al., 1996), South America (Maddaloni et al., 1985), Europe (Jones, 1990; Jones and Haggar, 1994), Australia (Hopkins et al., 1995), and Asia (Liu and Zhang, 1998; Xu and Wang, 1999).

1.2.2 Cultivars

In 1985, the first forage chicory cultivar in the world "Grasslands Puna" was approved for commercial release (Rumball, 1986). Now, about 100,000 to 200,000 hectares of chicory are planted annually for finishing red deer, sheep and cattle in New Zealand (Allan Stewart, 10 April 2005, personal communication).

Since 1985, a series of cultivars, including "Puna II" and "Choice", have been bred from the original Puna (Rumball et al., 2003a; Rumball et al., 2003b). "Puna II" forage chicory has a tolerance to the fungus *Sclerotinia* and has increased growing activity during the cool season (Rumball et al., 2003b). "Choice" forage chicory, which has lower levels of sesquiterpene lactones (lactucin and lactucopicrin), was bred for dairy farms (Rumball et al., 2003a). Another cultivar, "Grouse", released in 2000 for improved winter growth, was bred from crosses of a number of cultivars from New Zealand (Puna), Germany, Argentina and Italy by Dr. Allan Stewart at Pyne Gould Guinness Ltd (Allan Stewart, 10 April 2005, personal communication). "Chico" was imported from Italy to New Zealand by Cropmark Seeds Limited.

In the rest of the world, many other forage cultivars, including "Lacerta" and "Forage Feast" (Foster et al., 2002), have been bred.

1.2.3 Chemical composition

The chemical composition of a forage contributes to its feeding value (see Section 1.2.4).

1.2.3.1 Minerals

Chicory contains high concentrations of minerals (Shukla and Talpada, 1977; Kusmartono, 1996), as has been also reported for plantain (Table 1.1). Generally, chicory is higher in mineral content than alfalfa or cool-season grasses (Belesky et al., 2001). Minerals in chicory meet or exceed the recommended dietary mineral requirements of lactating dairy cows (Jung et al., 1996). Compared to perennial ryegrass (PRG)/white clover (WC) pasture, chicory contains higher concentrations of most minerals (Crush and Evans, 1990; Barry et al, 2001, 2002; Hoskin et al., 2005). The concentration of silicon is lower than in either PRG or WC (Hoskin et al., 1995). The

liver copper status of lambs and deer has been shown to increase after grazing chicory compared with PRG/WC pasture (Clark, 1995; Barry et al., 2002).

1.2.3.2 Crude protein

The crude protein concentration in forage chicory ranges from 12-18% (Crush and Evans, 1990; Jackson et al., 1996; Volesky, 1996) and is low compared with that of PRG which ranges from 18-19% and red clover which ranges from 27-29%.

1.2.3.3 Soluble and structural carbohydrates

In contrast to crude protein, the concentrations of soluble sugars and pectin are higher in chicory than in PRG. On the other hand, the concentrations of cellulose and "hemicellulose" in chicory determined using the detergent analysis system (Roberston and Van Soest, 1981) are lower than those in PRG, and are similar to those of red clover (Table 1.1).

The ratio of readily-fermentable carbohydrate (water-soluble sugars and pectins) to structural carbohydrate ("hemicelluloses" and cellulose) in chicory is higher by up to approximately three times than that in PRG (Kusmartono et al., 1997) and is even higher than that in red clover (Barry, 1998). The metabolizable energy (ME) concentration is higher in vegetative chicory forage than in PRG (Table 1.1). These contribute to the higher nutritive value of chicory.

1.2.3.4 Secondary compounds

The lignin content of chicory is about 20 g kg^{-1} (Barry, 1998), which is lower compared with that in lucerne (75 g kg^{-1}, young orchard grass(43 g kg^{-1}) and the average lignin content of forage (41 g kg^{-1}) (Van Soest, 1994).

The concentration of condensed tannins in chicory is low, only 4.2 g kg^{-1} DM (Table 1.1), and is lower than the minimum concentration (5 g kg^{-1} DM) required to prevent bloat in cattle and to increase wool growth in sheep (Jackson et al., 1996).

The sesquiterpene lactones, cichoriin and cichoric acid occur in chicory at low concentrations, and are present at the highest levels in the most actively growing tissue (Rees and Harborne, 1985). They are part of the natural plant's defense against insect

attack (Rees and Harborne, 1985), but result in tainted milk when cows are fed chicory (Barry, 1998). These components have been implicated in the reduction of gastrointestinal (GI) parasitism in young ruminants grazing chicory (Hoskin et al, 1999b; Schreurs et al, 2002).

Table 1.1 Chemical composition of vegetative chicory and other forages (g kg^{-1} DM)

	Perennial ryegrass (*Lolium perenne*)	Red clover (*Trifolium pratense*)	White clover (*Trifolum repens*)	Chicory (*Cichoriu m intybus*)	Plantain (*Plantago lanceolata*)
Ash	105	104	110	149	
Calcium	6.6			14.9	19
Phosphorus	3.6			3.4	3.9
Sodium	0.8			2.1	
Potassium	25.5			36.4	25
Magnesium	1.8			2.8	3.5
Total N	45.2	46.9	43	19.7	17.5
Soluble sugar(a)	74	95	79	111	170
Pectin(a)	10	39	51	98	60
Cellulose(b)	184	115	213	104	115
Hemicelluloses(b)	212	54	33	44	65
Ratio (a : b)	0.21	0.79	0.53	1.41	1.28
Lignin	10	12	6	20	51
Metabolizable energy	12.3	13.4	10.6	13.7	
Secondary phenolic compounds:					
Condensed tannin	1.8	1.7	3.1	4.2	14.1
Sesquiterpene lactones				3.6	
Cichoriin				0.5	
Chicoric acid				5.8	

From Barry (1998), Jackson et al. (1996), Beever et al. (1985), Sanderson et al. (2003), Barry et al. (2002), Ramirez-Restrepo and Barry (2005).

Pectin was determined using the method of Bailey (1967). Pectin was extracted with 0.5% (w/v) ammonium oxalate from the residue after treatment with boiling 80% ethanol and water. Soluble sugars were defined as sugars soluble in boiling 80% ethanol and water. Pectin in white clover was measured by the method of McComb and McReady (1952).

Cellulose and hemicelluloses were determined using the method of Robertson and Van Soest (1981).

1.2.4 Feeding value

1.2.4.1 Factors affecting feeding value

In the grazing animal production system, feeding value has been defined as the animal production obtained from grazing forage under unrestricted conditions (Ulyatt, 1973). It is a function of voluntary feed intake (VFI) and nutritive value per unit dry matter eaten. VFI contributes more than nutritive value *per se* to feeding value (Waghorn and Barry, 1987).

1.2.4.1.1 Voluntary feed intake

The VFI of ruminants grazing chicory is higher than that of ruminants grazing PRG/WC pasture. Kusmartono et al. (1996b) found that for deer this VFI was higher by 56% during summer, 26% during autumn and 15% during spring. Min et al. (1997) also found the VFI of deer grazing chicory was higher than on PRG/WC pasture during autumn, but was similar in spring. For castrated male red deer kept indoors, the wilting of chicory increased voluntary DM intake from 49 to 57g DM/kg $W^{0.75}$/day without significant changes in the apparent digestibility of DM, organic matter (OM), hemicellulose and cellulose (Tinworth et al., 1999). Preference studies with deer and sheep showed that chicory is highly palatable (Foster et al., 2002).

1.2.4.1.2 Nutritive value

Nutritive value is a composite of the following: the chemical composition of the forage, the availability (digestibility and absorption) of nutrients, the efficiency of use of absorbed nutrients and the effect of chemical composition on VFI (Coleman and Henry, 2002).

i) Chemical composition

The chemical composition of chicory has been described in Section 1.2.2.

ii) Availability of nutrients (digestibility and absorption)

In vivo

In vivo digestibilities of DM, organic matter (OM) and gross energy were higher for chicory than in PRG (Hoskin et al., 1995; Kusmartono et al., 1997) or PRG/WC (Min et

al., 1997), but fibre digestibility (NDF and ADF) was lower in vegetative chicory than in vegetative PRG in fistulated deer fed indoors (Hoskin et al., 1995; Kusmartono et al., 1997). Organic matter *in vivo* digestibility (OMD) of chicory (88.4%) was greater than that of the forage legume sulla (*Hedysarum coronarium*) (78.3%) and PRG/WC pasture (83.8%) during spring for deer although during autumn OMD of all forages was similar (Hoskin et al., 1999a).

In vitro

Volesky (1996) found that the *in vitro* dry matter digestibility (IVDMD) of chicory planted in Oklahoma, USA was 70.9%. Kusmartono et al. (1996) found that organic matter *in vitro* digestibility (IVOMD) of vegetative Puna chicory was high (85%) and relatively constant throughout the growing season in New Zealand.

In sacco

Both proportions of the degradable water-soluble matter (42%) and the degradable water-insoluble matter (52%) in chicory did not differ greatly from WC and PRG. However, the degradation rate of the insoluble matter of chicory was 33% higher than that of WC, and was 128% higher than that of PRG. The lag time before degradation was much shorter for chicory than for PRG and WC (Burke et al., 2000).

iii) Efficiency of use of absorbed nutrients

Energy metabolism

There have been no studies to directly determine the energy utilisation of digested nutrients from chicory. However, the efficiency of energy utilization varies inversely with the ratio of acetic acid to propionic acid in the rumen (MacRae and Lobley, 1986). Hoskin et al. (1995) found that the acetate: propionate ratio in the rumen of chicory-fed deer was higher than that in perennial ryegrass-fed deer, suggesting that the energy released from chicory may not be better utilised than energy from perennial ryegrass-based pasture.

Table 1.2 *In vivo* and *in vitro* DM, OM digestibilities of chicory

Chicory	Other forages	Animals or method	Sources	
		in vivo		
DMD 75.2%	PRG 68.5%	deer		
75.9%	wilted chicory 72.4%	deer	Tinworth et al., 1999	
78.5%	PRG/WC 72.7%	deer	Kusmartono et al., 1997	
OMD 82.0%	PRG/WC 74.4%	deer	Kusmartono et al., 1996	
78.9%	wilted chicory 75.5%	deer	Tinworth et al., 1999	
80.6%	PRG 72.3%	deer	Hoskin et al., 1995	
81.2%	PRG/WC 66.6%, lotus 75%, autumn	deer	Min et al., 1997	
85.3%	PRG/WC 80.2%, lotus 75.3%, spring	deer	Min et al., 1997	
		in vitro		
DMD 71.9-74.6%		a	McCoy et al., 1997	rumen fluid
79.0%		a	Clark et al., 1990	rumen fluid
70.9%		b	Volesky, 1996	rumen fluid
OMD 89.7%	PRG 84.5%, plantain 85.9%	c	Jackson et al., 1996	enzymes
57.6-62.1%		d	Belesky et al., 2000	rumen fluid
56.7%	orchardgrass 65.1%, alfalfa 56.4%	d	Turner et al., 1999	rumen fluid
86.8%	PRG/WC 83.3%	c	Hoskin et al., 1999a	enzymes
59.4-66.9%		d	Belesky et al., 1999	rumen fluid

Note: a, Marten and Barnes (1980) modified from Tilley and Terry (1963); b, Monson et al. (1969) modified from Tilley and Terry (1963); c, Roughan and Holland (1977); d, Moore (1970) modified from Tilley and Terry (1963). PRG/WC, perennial ryegrass/white clover. DMD, dry matter digestibility; OMD, organic matter digestibility.

Nitrogen metabolism

Due to the lower nitrogen content of chicory, lambs grazing chicory had a lower N intake and a lower rumen ammonia concentration than those grazing cocksfoot (Komolong, 1994). However, the loss of N across the rumen was reduced because of higher N utilisation in the rumen. For example, lambs fed cocksfoot and chicory lost

Seasonality

In New Zealand, the traditional forages, PRG and WC, are widely used by grazing livestock. However, the supply of herbage from PRG/WC pasture is unevenly distributed throughout the year because growth rate slows in mid-summer, or even earlier (late spring/early summer) if insufficient moisture is available (Smetham, 1994). This causes a mismatch between forage supply and the feed requirements of grazing ruminants lactating in summer (Barry et al., 2002). Chicory, which has a different seasonal growth pattern to that of PRG/WC, grows rapidly in summer and in spring (Li and Kemp, 2005). This growth pattern matches the strong demand for forage supply from a range of ruminants in late spring and summer (Li and Kemp, 2005).

Drought tolerance

Chicory is tolerant of drought conditions (Lancashire, 1978) because of its relatively deep, thick taproot (Gentile et al. 2003), and produces a higher yield of dry matter than other forages during a summer drought (Sanderson et al., 2005; Li and Kemp, 2005). Chicory also has a salt tolerance similar to that of lucerne (Boyd and Rogers, 2004).

1.3 Forage cell walls

This section of the literature review focuses on the cell-wall structure and composition of leaves and stems of forages, which are the plant parts consumed by grazing animals. The cell-wall compositions of eudicotyledon forages are quite different from those of grasses (Harris, 2005). In each forage plant species, the composition of tissues depends upon the proportions of differing cell types and their degree of lignification. The composition can also be influenced by environmental factors such as temperature and light (Lam et al., 1990).

1.3.1 Plant anatomy

There are a range of different cell types in forage plants with different sizes and shapes and with walls of different structures and compositions. Anatomical knowledge of forages improves our understanding of cell wall degradation in the rumen and hence feeding value. The walls of different cell types differ in their degradability by rumen bacteria (Akin, 1989). The arrangement of the different cell types in an organ also

influences forage degradability (Wilson and Hatfield, 1997). The outer walls of the epidermis are covered with a cuticle and a waxy layer, which make a barrier for rumen bacteria to access and digest the underlying cell wall (Wilson, 1993). The main types of cells and tissues in the parts of forage plants consumed by grazing ruminants are cuticle, epidermis, parenchyma, phloem, xylem, and sclerenchyma.

1.3.2 Structures of the cell walls of forage plants

Plant cell walls may consist of three layers: the middle lamella, primary wall and secondary wall. All the wall layers consist of two phases: a cellulose microfibrillar phase and a matrix phase composed of other polymers. Cellulose microfibrils can be seen by transmission electron microscopy and have a high degree of crystallinity (Brett and Waldron, 1996). They are long and thin structures, normally with an average width of 4-6 nm (Davies and Harris, 2003). The microfibril is composed of cellulose molecules, which lie side-by-side and align parallel to the long axis of the microfibril. In contrast, the matrix phase is not crystalline and appears relatively featureless under the electron microscope (Brett and Waldron, 1996). The matrix consists of a variety of polysaccharides, proteins, lignin and esterified or etherified phenolic compounds such as hydroxycinnamic acids (Bacic et al., 1988; Carpita and Gibeaut, 1993).

1.3.2.1 Middle lamella

The middle lamella, the earliest-formed layer at cell division, is in the middle of the wall between two adjacent cells (Brett and Waldron, 1996). The middle lamella is frequently thin because it is derived from the cell plate, formed during cell division, and is often stretched during cell growth. It is often thickest at cell corners. The middle lamella can be detected with the electron microscope by its staining characteristics.

1.3.2.2 Primary cell walls

The primary cell wall is deposited while the cell is growing (Brett and Waldron, 1996). Primary cell walls are usually thin, unlignified and 0.1-1.0 μm thick, but are often lignified if a secondary wall is present.

1.3.2.3 Secondary cell walls

The secondary cell wall is deposited on the inner surface of the primary cell wall towards the cytoplasm with different patterns and thicknesses for various walls once cell differentiation begins. It is normally thicker than the primary cell wall and often contains the complex, three-dimensional phenolic polymer, lignin. Lignin also permeates the middle lamella and primary wall of cells with secondary walls.

Some cell types such as parenchyma cells, collenchyma and cambial initials have only a primary wall, other cell types such as tracheids have both a primary and a secondary wall (Harris, 1990).

1.3.3 Compositions of cell walls

The main components of plant cell walls are listed in Table 1.4 and are described in the following sections. In addition, cell walls contain minor amounts of suberin, cutin, tannins, waxes and minerals (Lam et al., 1990).

The primary walls of dicots and non-commelinid monocots contain large amounts of pectic polysaccharides, smaller amounts of xyloglucans and minor amounts of heteroxylans, glucomannans and/or galactoglucomannans (Harris, 2005). Poaceae primary walls contain large amounts of glucuronoarabinoxylans (GAXs), variable amounts of (1→3),(1→4)-β-D-glucans, and small amounts of xyloglucans and pectic polysaccharides (Harris, 2005).

In the lignified secondary walls of dicotyledons, 4-O-methylglucuronoxylans are usually the major non-cellulosic polysaccharides. Glucomannans are usually present in smaller proportions, but in some cases they are major components, for example in the walls of bast fibres of *Crotalaria juncea* (Bacic et al., 1988). In the lignified secondary walls of the Poaceae, a large amount of glucuronoarabinoxylans, a small amount of (1→3),(1→4)-β-glucans and a minor amount of (galacto-)glucomannans are present (Harris, 2005).

Table 1.4 Plant cell wall components

Phase	Components	
Microfibrillar	Cellulose	(1→4)-β-glucan
Matrix*	Pectic polysaccharides	Rhamnogalacturonan I Arabinan Galactan Arabinogalactan I Homogalacturonan Rhamnogalacturonan II
	Non-cellulosic, non-pectic polysaccharides	Heteroxylan glucomannan and/or galactoglucomannan Xyloglucan Callose ((1→3)-β-glucan) (1→3), (1→4)-β-glucan Arabinogalactan Type II
	Proteins and glycoprotein	Extensin Arabinogalactan-proteins Enzymes
	Phenolic compounds	Lignin Ferulic acid *p*-coumaric acid, truxillic acid, etc.

Adapted from Brett and Waldron, 1996

* not all these matrix components are found in all cell walls

1.3.3.1 Cellulose

Cellulose, a simple linear polymer of (1→4)-linked β-D-glucopyranosyl residues (Franz and Blaschek, 1990), is the most abundant plant polysaccharide and forms the basic structure of all plant cell walls (Gibeaut and Carpita, 1994). Primary cell walls contain

~20-30% and lignified secondary walls contain ~40-60% cellulose (Stephen, 1980). Cellulose typically accounts for about 35~50% of plant dry matter (Lynd et al., 2002).

1.3.3.2 Pectic polysaccharides

Pectic polysaccharides are present in the primary walls of all land plants (O'Neill et al., 1990), and are located particularly in the middle lamella (Bacic et al., 1988; Cosgrove, 2005). The location of pectins has been extensively studied using immunolabelling techniques (see Section 1.3.4.). The pectic polysaccharides in the primary walls of the Poaceae have a similar structure to those in dicot primary walls, but they occur in much lower proportions (Chesson, et al., 1995). Galacturonic acid, rhamnose, arabinose and galactose are the principal monosaccharide components of pectins (Brett and Waldron, 1996). There are three main types of pectic polysaccharide domains: homogalacturonan (HG), rhamnogalacturonan I (RG I) and rhamno-galacturonan II (RG II) (O'Neill et al., 2004). However, a small amount of a fourth xylogalacturonan (XGA) domain also occurs in some walls (Schols et al., 1995; Ishikawa et al., 2000; Harris, 2005). The structure of pectic polysaccharides has been reviewed recently (Ridley et al., 2001; Willats et al., 2001a; Vincken et al., 2003; O'Neill et al., 2004). HG, RG I and RG II are covalently linked to one another (Ridley et al., 2001; Willats et al., 2001a). It has been considered that the pectin backbone is made of HG and RG I (Visser and Voragen, 1996). However, this view has been challenged recently by Vincken et al. (2003) who proposed that HGs occur as side chains of RG I.

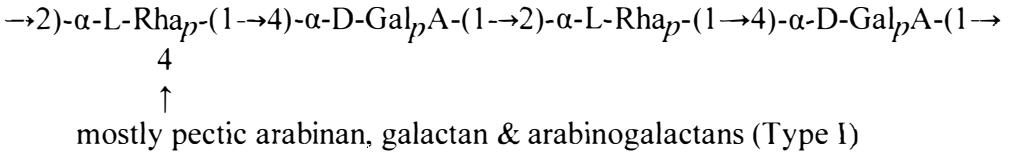
HG is a linear homopolymer of 4-linked α-D-galactosyluronic acid residues (Fig. 1.4). Methyl-esterification of galactosyluronic acid residues often occurs at the C-6 carboxyl group; acetyl groups are also present at C-2 and/or C-3 in some plants (O'Neill et al., 1990; Ishikawa et al., 2000). Two HG chains with blocks of more than 10 unesterified galacturonic acid (GalA) residues can form a dimer via Ca^{2+}-cross-linking (Daas et al., 2001), often referred to as an "egg-box" model.

→4)-α-D-Gal$_p$A-(1→4)-α-D-Gal$_p$A-(1→4)-α-D-Gal$_p$A-(1→4)-α-D-Gal$_p$A-(1→

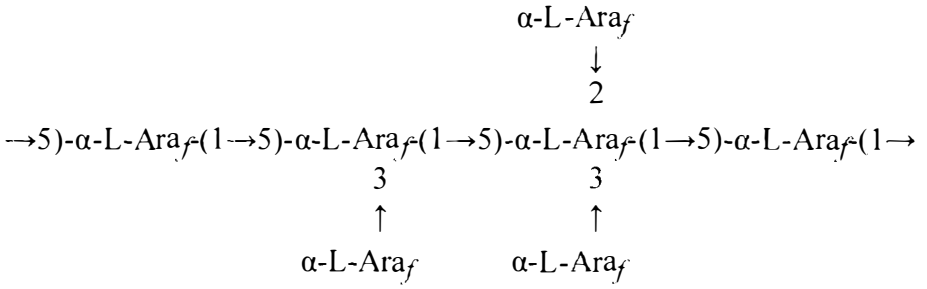
Figure 1.4 The structure of homogalacturonan

XGA is composed of a backbone of 4-linked α-D-galactosyluronic acid residues to which xylose residues are attached at the C(O)3 position of about half of the galacturonic acid residues (Schols et al., 1995).

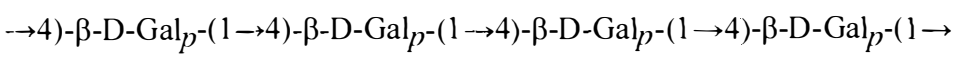
Rhamnogalacturonan I (RG I) backbone



Arabinan



Galactan



Arabinogalactan (Type I)

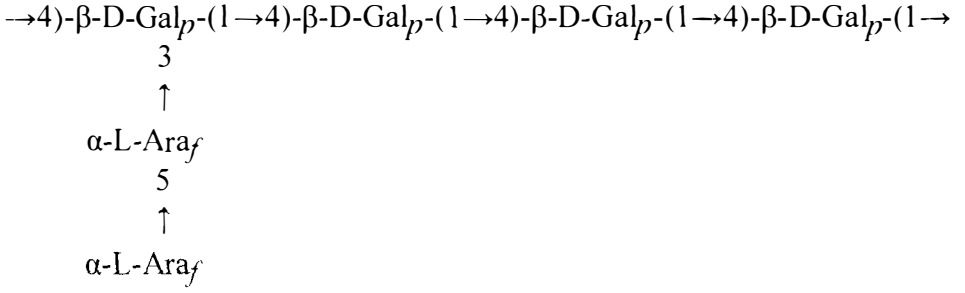


Figure 1.5 The structure of rhamnogalacturonan I (from Harris, 2005)

RG I consists of a backbone of alternating (1→4)-linked α-D-galacturonic acid and (1→2)-linked α-L-rhamnose residues. It also has several different side-chains attached to the C4 of rhamnose (Fig. 1.5). These side-chains are mainly composed of arabinose and galactose. Arabinose chains (arabinans) are linked by (1→5)-linkages, galactose chains (galactans) by (1→4)-linkages (Brett and Waldron, 1996). Type I arabinogalactan side-chains have (1→4)-β-galactan chains with limited substitution by short arabinose-containing oligomers (Øbro et al., 2004). Arabinans, arabinogalactans and galactans are probably present as the side-chains of RG I (O'Neill et al., 1990; Geshi et al., 2000; Øbro et al., 2004).

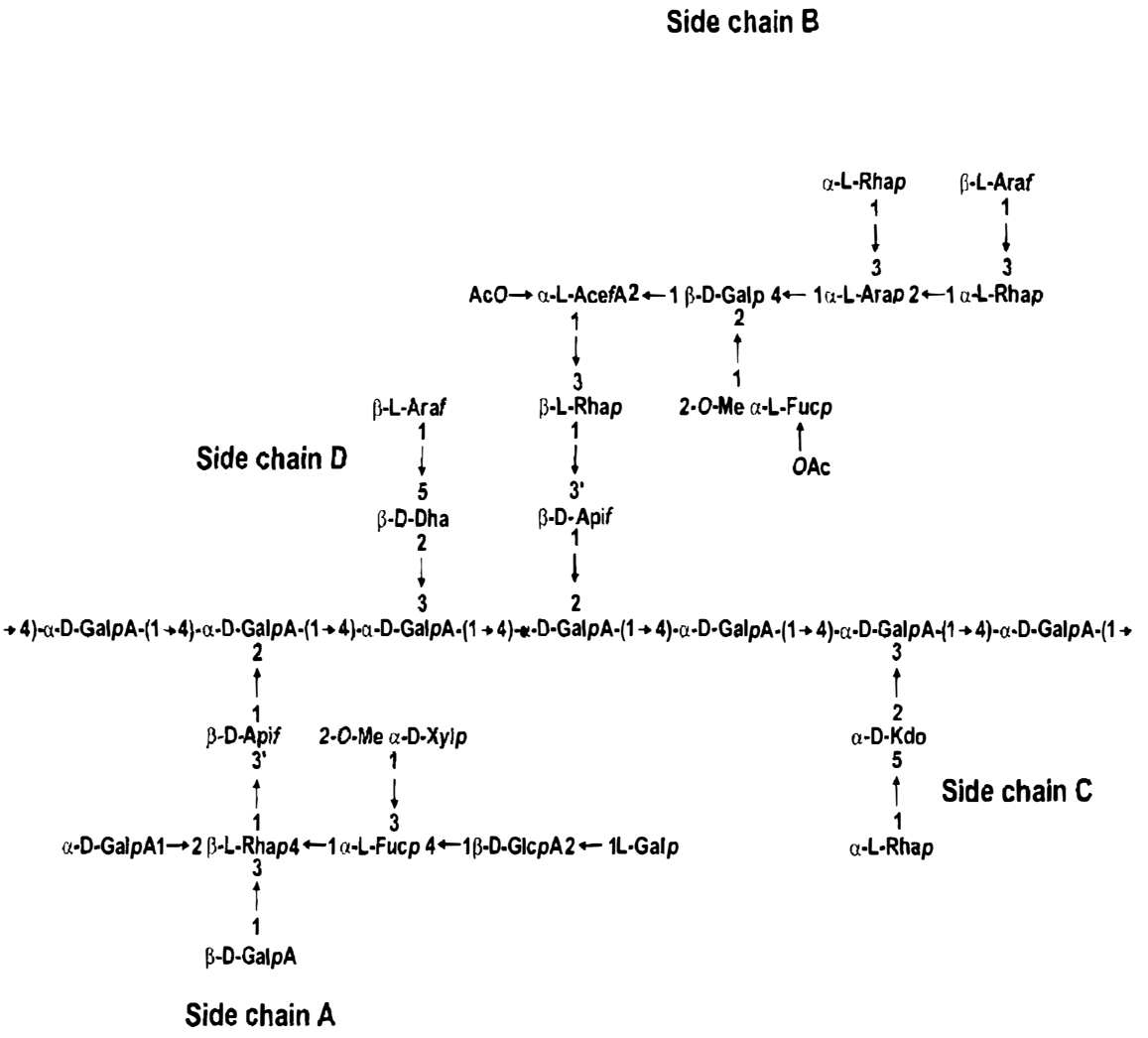


Figure 1.6 Structure of rhamnogalacturonan II (adapted from O'Neill et al., 2004)

RG II was first identified in 1978 (Darvill et al., 1978). It is quantitatively a minor constituent of primary cell walls in dicotyledons (O'Neill et al., 2004), and has also been isolated from the primary cell walls of the Poaceae (Kaneko et al., 1997). It is released as low-molecular-weight polysaccharides, containing about 30 different glycosidic linkages with at least 12 different glycosyl residues, after treating with endo-polygalacturonase (Vidal et al., 2000). RG II is composed of a backbone of at least eight 4-linked α-D-galacturonic acid residues to which are attached four structurally distinct oligosaccharide side chains (Vidal et al., 2000; O'Neill et al., 2004). Its structure is shown in Fig. 1.6 (O'Neill et al., 2004). RG II exists in cell walls mainly as a dimer (Vidal et al., 2000). The cross-linking of RG II forms a covalently cross-linked pectic polysaccharide matrix (O'Neill et al., 1996). The RG II dimer is formed via borate esters which cross-link two apiosyl residues (O'Neill et al., 1996).

Three domains of pectic polysaccharides can be isolated for *in vitro* analysis, but *in vivo* pectic macromolecules exist with three domains covalently cross-linked (Ridley et al., 2001; Willats et al., 2001a).

1.3.3.3 Other non-cellulosic polysaccharides ("hemicelluloses")

These have traditionally been called hemicelluloses (see Table 1.1) and comprise a group of polysaccharides that are extracted from cell walls with alkaline solution after initial treatments with water and chelating reagents (Selvendran and O'Neill, 1987). They include xyloglucans, heteroxylans and heteromannans. They are bound to cellulose by hydrogen bonds and hence can only be extracted with strong alkali or other chaotropic agents. Their structures vary greatly in the walls of different cell types and different species.

Xyloglucans (XGs) are the principal other non-cellulosic polysaccharides of dicotyledonous primary cell walls (Brett and Waldron, 1996; O'Neill and York, 2003), accounting for up to 20-25% of the primary cell walls (Hayashi, 1989; Fry, 1989). The main backbone is composed of 4-linked β-D-glucopyranosyl residues, most of which are linked to α-D-xylopyranosyl residues of side chains with α-(1→6)-glycosidic linkage and these xylopyanosyl residues can be linked to fucose, galactose and less commonly arabinose (Hayashi, 1989). There are two main types of xyloglucans in

primary cell walls, the XXXG type and the XXGG type, distinguished by the number of branched glucose residues (Vincken et al., 1997).

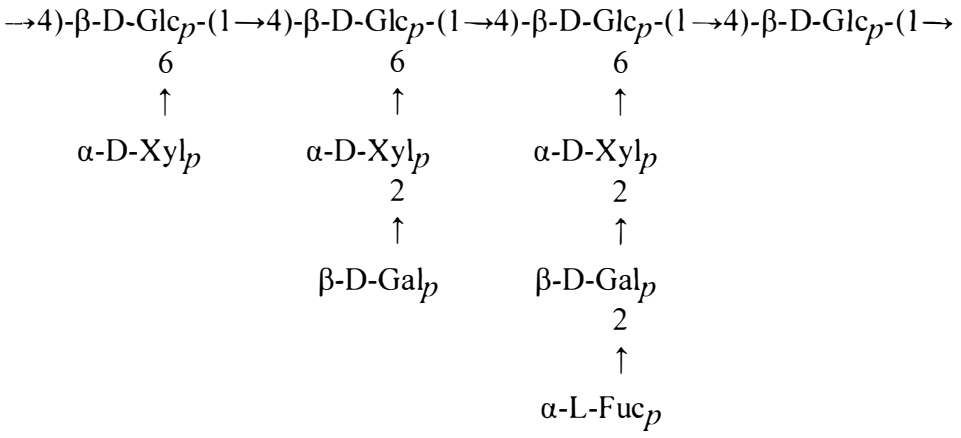


Figure 1.7 Fucogalactoxyloglucan (from Harris, 2005)

Fucogalactoxyloglucans (Fig. 1.7) have been found in the primary walls of most dicotyledonous plants except for those of solanaceous plants which contain arabinoxyloglucan (XXGG) (Vincken et al., 1997). The primary walls of Poaceae contain small amounts of xyloglucan in which arabinose and fucose are not present (Carpita, 1996).

Heteroxylans (Fig. 1.8) include arabinoxylans, glucuronoxylans and glucurono-arabinoxylans. They have a backbone of 4-linked xylose residues with short sidechains of arabinose, glucuronic acid and 4-O-methyl-glucuronic acid residues (Ebringerova and Heinze, 2000). The xylose residues in the backbone may be O-acetylated and in the Poaceae the arabinose residues may be esterified with ferulic or *p*-coumaric acid (Ebringerova and Heinze, 2000). Heteroxylans are present in small proportions in the primary walls of dictyledons, but they are abundant in the Poaceae. Cellulose microfibrils in the walls of Poaceae are interlocked mainly by glucuronoarabinoxylans (GAXs). Unbranched GAXs are cross-linked with each other, or to cellulose, via hydrogen bonds. Branched GAXs are unable to form crosslinking between two GAXs or GAX to cellulose since arabinose and glucuronic acid side groups prevent the formation of hydrogen bonds (Carpita, 1996).

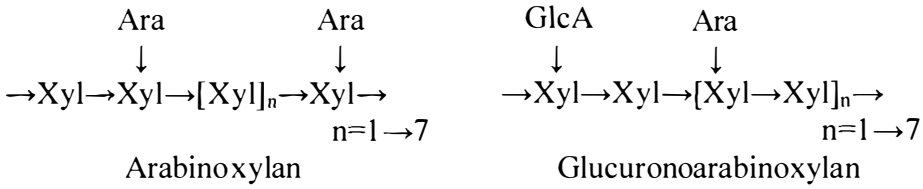


Figure 1.8 The structures of heteroxylan (from Hatfield, 1989)

Heteromannans Galactoglucomannans (GGM) are found widely in small amounts. They have a backbone of (1→4)-linked β-D-Man and β-D-Glc residues (Fig. 1.9). Mannans and galactomannans, although abundant in the endosperms of certain legumes and members of the Palmales (Li and Wu, 1993), are not normally found in vegetative cell walls and will not be discussed in this review.

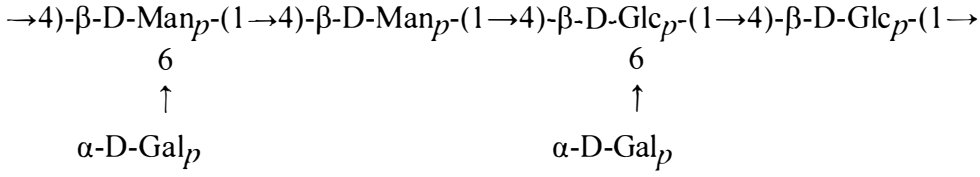


Figure 1.9 The structure of galactoglucomannans (from Harris, 2005)

(1→3),(1→4)-β-Glucans are also known as mixed-linked glucans (Carpita, 1996). They have been found in the primary cell walls of Poaceae, and contain about 30% 3-linked residues and 70% 4-linked residues (Åman, 1993; Li and Wu, 1993).

Callose is a (1→3) linked β-glucan present in the cell plates, and phloem sieve plates. It is also formed in response to special situations such as wounding. Its helical conformation results from its (1→3) linkage (Brett and Waldron, 1996).

1.3.3.4 Proteins and glycoproteins

The protein content of cell walls depends on the tissue type and the maturity of the plant, but it is normally less than 10% in the primary walls. Proteins in cell walls include enzymes, some of which have roles in wall formation. For example, several enzymes are involved in the process of lignification (Cassab, 1998). Structural proteins also occur in cell walls and have been classified into three groups: extensins, proline-

rich proteins and glycine-rich proteins (Harris, 2005). They play critical roles in cross-linking wall components, particularly in primary walls.

1.3.3.5 Phenolic components

The most common phenolic component in plant cell walls is lignin. There are three common precursors of lignin, coumaryl, coniferyl and sinapyl alcohols, from which the *p*-coumaryl, guaiacyl and sinapyl propane subunits, respectively, are formed. The formation of lignin occurs first at the cell corner and middle lamella and then spreads to the primary walls after the S1 layer of the secondary walls starts to form. Lignification of the rest of the secondary cell wall occurs after this has been fully formed (Terashima et al., 1993).

Other phenolic components in the wall include ferulic and *p*-coumaric acid. In the Cronquist (1981)-defined order Caryophyllales, ferulic acid is esterified to arabinose and galactose in pectins and may be important in the cross-linking of pectins (Brett and Waldron, 1996; Mathew and Abraham, 2004; Harris, 2005). In sugar beet (*Beta vulgaris*) root cell walls, ferulic acid residues, by forming dehydrodiferulates, provide diferulic bridges for pectic arabinans and galactans covalent cross-linking (Ralet et al., 2005). In the Poaceae and other commelinid monocotyledonous families, ferulic acid is ester linked to arabinose residues of glucuronoarabinoxylans (Harris and Hartley, 1980; Harris, 2005).

1.3.4 Location of polysaccharides in cell walls

Understanding of the functions of cell wall polymers is advanced by studies on their location within the walls, and significant contributions in recent years have been made regarding pectic polysaccharides and xylans (Willats et al., 2000; Guillon et al., 2004).

Pectic polysaccharides are major components of forage legumes and may well contribute to their high rumen digestibility (Van Soest, 1994). They are laid down in the cell plate at cell division and become the major part of the middle lamella joining two adjacent cells. Cell separation, as in fruit ripening (Sutherland et al., 1999) and in maceration by pathogenic bacteria (Murdoch et al., 1999), involves degradation of pectic polysaccharides by enzymes, and this might account for their rapid digestion by rumen microbes. The intra-wall location of pectic polysaccharides has been

demonstrated histochemically by reagents such as Ruthenium Red and Alcian Blue, which interact with polyanionic polymers (Benes, 1968; Scott et al., 1964). However, more detailed studies have recently been made using monoclonal antibodies, which allow identification of the specific location of members of the pectic polysaccharide complex with different structural features (Willats et al., 2000). Early studies used the antibody 2F4 (Liners et al., 1989; Liners and Van Cutsem, 1992), but most recent work has used JIM5 and JIM7 for homogalacturonans with low and high degrees of methyl esterification, respectively (Knox et al., 1990) and a variety of other antibodies for RG I and RG II motifs.

Glucuronoarabinoxylans are the main non-cellulosic polysaccharides in the cell walls of grasses. Antibodies against xylans have been generated by several research groups (Migne et al., 1994; Guillon et al., 2004; McCartney et al., 2005). For example, LM10 can label unsubstituted xylans and xylans with low degrees of substitution and LM11 can bind both unsubstituted xylans and arabinoxylans (McCartney et al., 2005). Although both LM10 and LM11 epitopes occur in the secondary walls of xylem, they are differentially present in walls of other cell types (McCartney et al., 2005).

1.3.5 Models of cell walls

To facilitate the study of how wall components are organised and how they interconnect with each other in the wall, a number of models have been proposed for the primary cell walls of dicotyledons (Fig. 1.10, Fig. 1.11) and forage grasses (Fig. 1.12) (McCann and Roberts, 1991; Carpita and Gibeaut, 1993; Hatfield, 1993; Carpita, 1996; Vincken et al., 2003; Fry, 1986, 2004; Somerville et al., 2004).

In the models of dicotyledon primary walls it is proposed that cellulose is linked to xyloglucan by hydrogen bonding to form a cellulose-xyloglucan framework. This framework is embedded in a pectin matrix (Willats et al. 2001a). The pectin matrix phase controls some cell wall properties, for example, wall porosity, charge density, and microfibril spacing. In these models the pectic network is independent of the cellulose-xyloglucan network, without significant interactions. However, covalent linkages between xyloglucan and pectin were found in the primary cell walls of suspension-cultured rose cells (Thompson and Fry, 2000). Brett et al. (2005) reported more evidence for covalent pectin-xyloglucan linkages in walls of growing pea (*Pisum*

sativum) cells. The pectin-xyloglucan complex is synthesised in the Golgi apparatus of pea cells (Baydoun et al., 2005; Cumming et al., 2005). On the other hand, it has been suggested that pectins attach to cellulose (Zykwinska et al. 2005). RG I was found to bind to cellulose and was suggested to bind through the galactan side chains (Oechslin et al., 2003). Therefore, the original model of dicotyledon primary walls needs modification (Cosgrove, 2005).

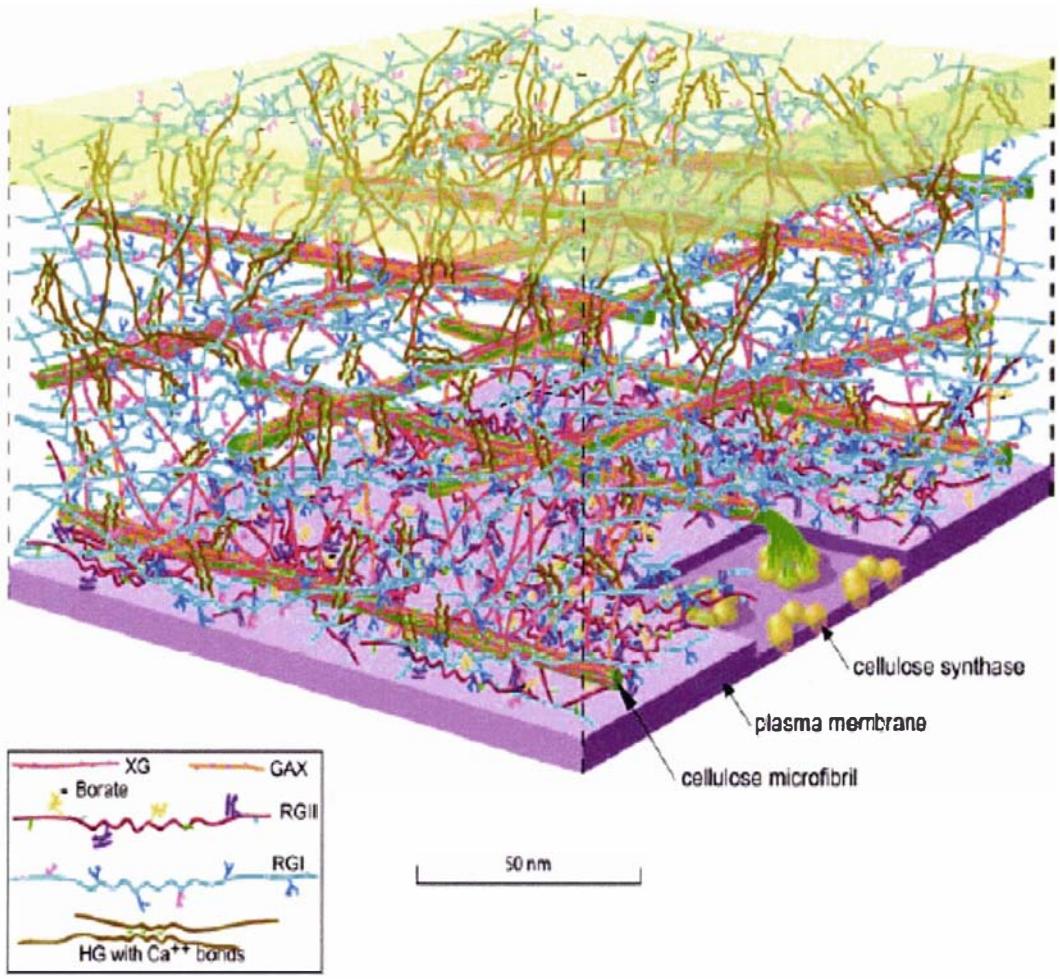


Figure 1.10 Model of the primary cell walls of dicotyledonous plants. From Somerville et al. (2004) elaborated from McCann and Roberts (1991). Because of the exaggerated distance between microfibrils, the "hemicellulose" cross-links [shown in dark orange (xyloglucan, XG) or light orange (glucoronoarabinoxylan, GAX)] are abnormally extended.

Within pectin, there are three pectic polysaccharide domains, HG, RG I and RG II, which are thought to be linked to one another covalently (Ridley et al., 2001; Cosgrove, 2005) (Fig. 1.11). Willats et al. (2001a) suggested that HG, RG I and RG II are covalently linked to form a linear backbone. However, Vincken et al. (2003) have suggested that HG and RG II are side-chains of RG I. In addition, there are two kinds of cross-links interconnecting pectic molecules, one involving calcium and the other boron. Two RG IIs can covalently cross-link by a borate ester to form a dimer (Ridley et al., 2001). Two unesterified HG chains can cross-link via calcium cations to form stiff gels; this is the so-called "egg-box" model (Vincken et al., 2003; Section 1.3.3.2). This cross-linking via calcium is considered to be located in the middle lamella and to have a role in cell adhesion (Jarvis et al., 2003).

A conceptual model of grass primary walls is shown in Fig. 1.12. The wall has a tight structure from the polysaccharides which pack together densely (Hatfield, 1993).

There is less information on the structures of the lignified secondary walls of forage dicotyledons than on those of the woody dicotyledons (Terashima et al., 1993). Grabber (2005) concluded that in the dicotyledons the cellulose structure and deposition patterns in secondary walls are different from those in primary walls, and lignin deposition and secondary wall formation occur concurrently.

During the lignification of grass cell walls, coniferyl alcohol and small amounts of *p*-coumaryl alcohols are first incorporated into the primary walls, then coniferyl alcohol and increasing amounts of sinapyl alcohol are deposited in the secondary walls (Grabber, 2005). Lignin is covalently linked to polysaccharides via hydroxycinnamate interactions in the secondary walls (Hatfield et al., 1999).

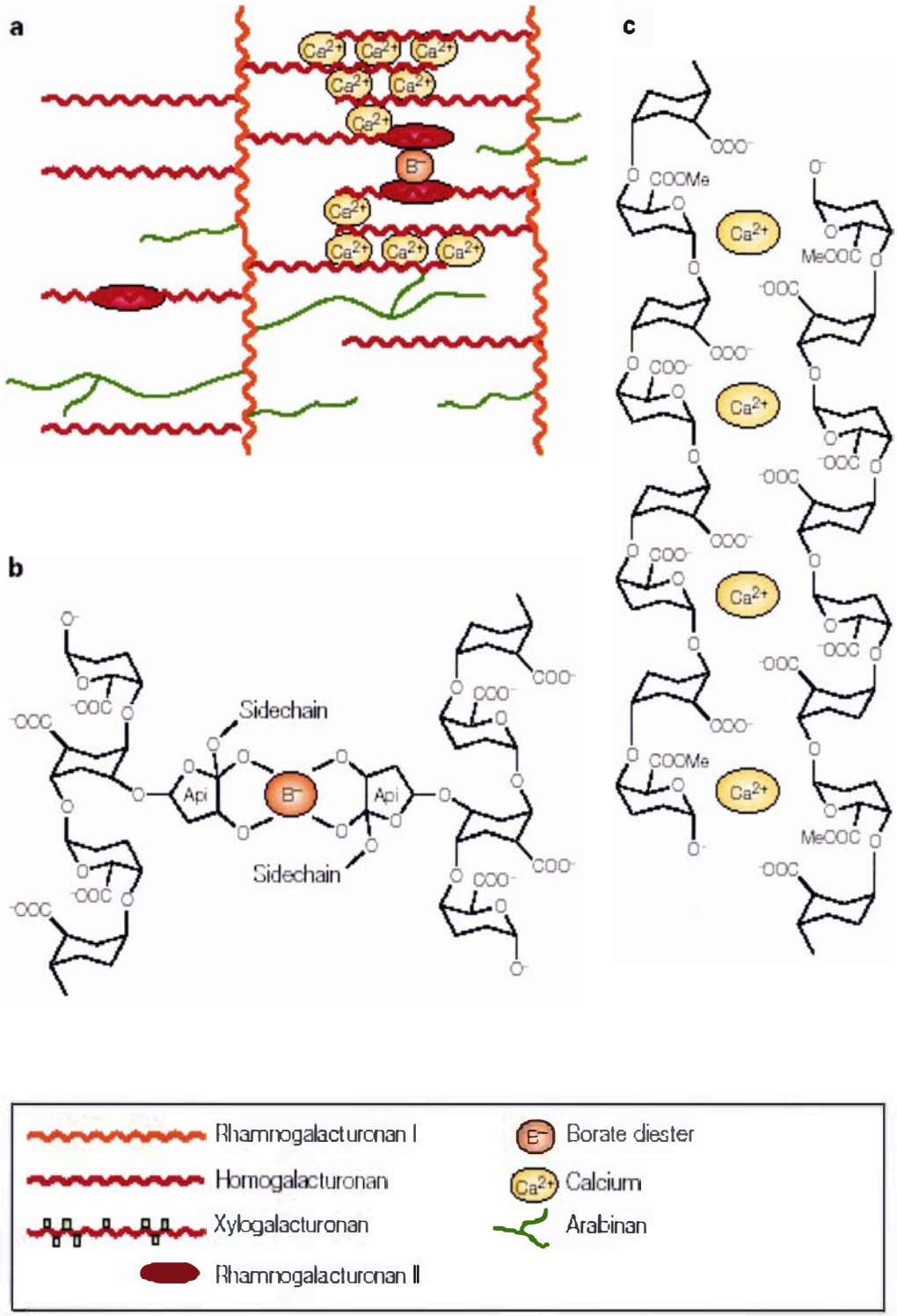


Figure 1.11 Cross-links between pectic polysaccharides. a) cross-links among pectin domains, b) cross-links between RG II molecules via borate ester, c) cross-links between HG molecules via calcium cations (Cosgrove, 2005).

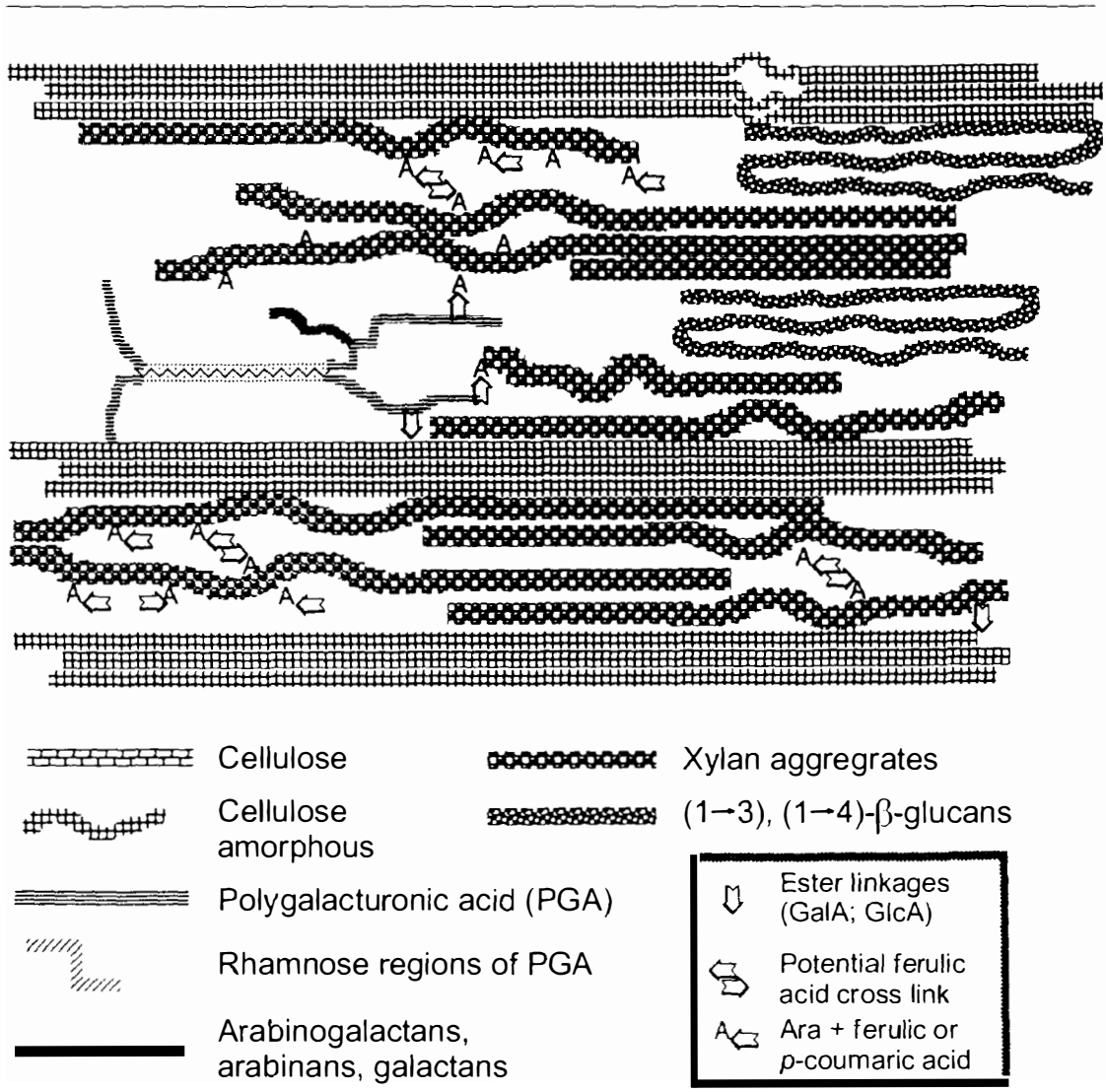


Figure 1.12 Conceptual model of polysaccharide interactions in the cell wall of a grass (adapted from Hatfield, 1993)

1.4 Ruminal degradation of forage plant cell walls

Forages ingested into the rumen are first subject to a reduction in particle size before further degradation by rumen microbes. The reduction in particle size results mainly from chewing during rumination and eating (Ulyatt et al., 1986). However, Hoskin et al. (1995) and Kusmartono (1996b) found that with deer fed chicory, there was little or no time spent ruminating, suggesting that reduction in particle size had occurred without rumination.

After the reduction in particle size, rumen microbes play a major role in forage cell-wall degradation. Rumen microbes include many different species of bacteria, protozoa and fungi. These can be divided into several groups according to their functions (Hobson and Stewart, 1997). Fibrolytic microbes refer to the group involved in the breakdown of forage cell walls. Proteolytic microbes refer to those which breakdown dietary protein. Others are responsible for the breakdown of lipids. In the following sections, fibrolytic microbes will be discussed because they are responsible for forage cell-wall degradation.

Increasing the digestion of forage cell walls by ruminal microbes is a key factor in improving animal performance. Scientists from many disciplines such as plant anatomy, plant biochemistry, forage breeding, ruminant nutrition and ruminal microbiology have been involved in this area. This topic has been exhaustedly reviewed (Jung et al., 1993; Chesson and Forsberg, 1997; Krause et al., 2003) and this section only briefly outlines fibrolytic rumen microbes and their activities in cell wall degradation.

1.4.1 Rumen microbes

1.4.1.1 Bacteria

The main rumen microbes involved in degrading plant cell walls are listed in Table 1.5 (Dehority, 1993). Bacteria and fungi account for about 80% of plant cell wall degradation in the rumen; protozoa account for approximately 20% (Dijkstra and Tamminga, 1995).

In the rumen, there are a few dominant cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus*, *R. flavefaciens* and *Butyrivibrio fibrisolvens* (Dehority, 1993; Miron and Ben-Ghedalia, 1993). However, the cellulolytic bacteria in the rumen vary with the substrate. In New Zealand, perennial ryegrass/white clover pasture is the main feed for grazing ruminants and the dominant cellulolytic bacteria in the rumen of these ruminants are *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens* (Keith Joblin, 2003, personal communication).

Table 1.5 Identity and enzyme activities of ruminal microbes involved in the degradation of plant cell walls in the rumen

Organism	Degradative activity		
	Cellulolytic	Hemicellulolytic	Pectinolytic
Bacteria			
Fibrobacter succinogenes	+	+	+
Ruminococcus albus	+	+	+
Ruminococcus	+	+	+
flavefaciens	+	+	+
Butyrivibrio fibrisolvens	+		+
Eubacterium cellulosolvens	+		+
Clostridium longisporum	+		
Clostridium locheadii		+	+
Prevotella ruminantium		+	+
Eubacterium xylanophilum		+	
Ruminobacter amylophilus		+	
Succinimonas amylolytica		+	
Succinivibrio dextrinosolvens		+	
Selenomonas ruminantium		+	
Selenomonas lactilytica		+	
Lachnospira multiparus			+
Streptococcus bovis		+	+
Megasphaera elsdenii		+	+
Fungi			
Neocallimastix frontalis	+	+	+
Neocallimastix patriciarum	+	+	+
Neocallimastix joyonii	+	+	
Caecomyces communis	+	+	+
Piromyces communis	+	+	+
Orpinomyces bovis	+	+	
Ruminomyces elegans	+	+	
Protozoa			
Eudiplodinium maggii	+	+	+
Ostracodinium dilobum	+	+	+
Epidinium caudatum	+	+	
Metadinium affine	+	+	+
Eudiplodinium bovis	+	+	+
Orphryoscolex caudatus	+	+	+
Polyplastron multivesiculatum	+	+	+
Diplodinium pentacanthum	+		
Endoploplastron triloricatum	+		
Orphyroscolex tricoronatus	+		
Ostracodinium gracile	+		
Entodinium caudatum	+	+	
Isotricha intestinalis	+	+	+
Isotricha prostoma	+	+	+

Adapted from Dehority (1993). + indicates activity. The term hemicellulolytic refers to mainly xylanolytic activity.

1.4.1.2 Fungi

Rumen fungi have been less studied than rumen bacteria. Typical fungi associated with cell wall degradation are *Neocallimastix frontalis*, *N. patriciarum*, *Piromyces communis* and *Orpinomyces bovis* (Wubah et al., 1993; Trinci et al., 1994). They have enzymatic and physical roles in the degradation of cell walls in the rumen. The cellulases and xylanases from fungi are highly active, and rumen fungi produce a wider range of polysaccharidases than bacteria (Trinci et al., 1994). The removal by fungi of wall components that limit access by bacteria to the substrate facilitates further degradation. Forsberg and Cheng (1992) reported that fungal growth is apparently limited to the recalcitrant lignified walls of sclerenchyma fibres. Fungi can penetrate the cuticle at the plant surface as well as the cell walls of lignified tissue (Akin, 1989). Fungi can also mechanically destroy forage plant structure (Orpin and Joblin, 1997; Gordon and Phillips, 1998). This suggests that fungi play a different role from bacteria in the degradation of cell walls. Fungi appear to play an important role in the degradation of the resistant components of forages, but a lesser role in the digestion of forages with a low lignocellulosic component (Orpin and Joblin, 1997). This may be due to the rapid clearance of the forage digesta from the rumen.

1.4.1.3 Protozoa

Because cell wall digestion in the rumen was found to decrease when protozoa were absent (Williams and Coleman, 1991) and fibrolytic enzyme activities have been detected in protozoa (Wang and McAllister, 2002), protozoa are believed to contribute significantly to the digestion of plant cell walls. Studies to further define their contribution have been hindered by difficulties in culturing protozoa.

1.4.2 Cell wall degradation by rumen microbes

1.4.2.1 Fibrolytic enzymes

The major fibrolytic enzymes secreted by rumen microbes which hydrolyse forage cell walls are listed in Table 1.6 (White et al., 1993; Wang and McAllister, 2002). The polysaccharide hydrolases and their encoding genes in microbes have been reviewed in detail by Warren (1996).

Table 1.6 Major enzyme activities present in the rumen and required for the hydrolysis of plant cell wall polymers

Wall polymer	Target bond for hydrolysis	Enzyme effecting hydrolysis
Cellulose		
Cellulose	(1→4)-β-glucose linkage	Endo-(1→4)-β-glucanase
Cellulose (non-reducing end)	(1→4)-β-glucose linkage	Exo-(1→4)-β-glucanase
Cellobiose	(1→4)-β-glucose linkage	(1→4)-β-glucosidase
Soluble cellooligomers	(1→4)-β-glucose linkage	Cellodextrinase
Cellulose or xylan	(1→4)-β-glucose linkage or xylose linkage	Xylocellulase
Pectic polysaccharides		
HG	(1→4)-α-galacturonide linkages	Pectate lyase
Methyl-esterified HG	(1→4)-α-galacturonide linkages	Pectin lyase
Other non-cellulosic polysaccharides		
Xylan	(1→4)-β-xylose linkage	Endo-(1→4)-β-xylanase
Xylobiose	(1→4)-β-xylose linkage	(1→4)-β-xylosidase
Arabinoxylan	α-Ara*f*-(1→3)-xyl-linkage	Arabinofuranosidase
Glucuronoxylan	4-O-methyl-α-D-glucuronic acid-(1→2)-α-D-xylose linkage	Glucuronidase
Acetylxylan	Acetylester bond	*O*-Acetyl xylan esterase
Ferulic acid	Feruloylester bond	Ferulic acid esterase
p-Coumaric acid	*p*-Coumaryl ester bond or linkage	*p*-Coumaric acid esterase

Adapted from White et al., 1993 and Wang and McAllister, 2002

Plant cell walls contain cellulose, pectic polysaccharides and a range of non-cellulosic polysaccharides. Rumen microbes usually produce multiple enzymes to degrade these materials. Cellulolytic enzymes are often active agaist xyloglucans, and specific xyloglucan-degrading enzymes are commonly coproduced by cellulolytic rumen microbes (Lynd et al., 2002). Due to the insolubility of cellulose and xyloglucans, these

fibrolytic enzymes must be either extracellular and free in the solution or on the cell surface. As a consequence, fibrolytic enzyme systems can be divided into cell associated or extracellular classes (Chesson and Forsberg, 1997).

Extracellular enzymes, non-complexed cellulase systems (Lynd et al., 2002), are synthesized and secreted separately (Chesson and Forsberg, 1997), but appear less important in the rumen than cellulase enzymes present in cellulosomes. Three major types of enzymatic activities occur: a) endoglucanases including (1→4)-β-D-glucan-4-glucanohydrolases, b) exoglucanases, including (1→4)-β-D-glucan glucohydrolases (cellodextrinases) and (1→4)-β-D-glucan cellobiohydrolases (cellobiohydrolases), c) β-glucosidases or β-glucoside glucohydrolases (Lynd et al., 2002). The endoglucanases hydrolyse internal amorphous regions of cellulose chains, generating reducing and non-reducing ends of oligosaccharides of various lengths. Exoglucanases attack the reducing or non-reducing ends in a progressive manner, liberating glucose (glucohydrolases) or cellobiose (cellobiohydrolases). Exoglucanases hydrolyse crystalline cellulose, whereas β-glucosidases hydrolyse soluble cellodextrins and cellobiose to glucose (Lynd et al., 2002).

In addition to extracellular enzymes, complexes of cellulases occur as cellulosomes. These complexes include both catalytic and carbohydrate-binding modules (CBMs), bringing the catalytic domain close to the insoluble cellulose, so facilitating cellulose hydrolysis and shortening the distance for the host bacteria to take up hydrolysis products. Carbohydrate-binding modules are particularly important for the initiation and processing of exoglucanases (Lynd et al., 2002).

The degradation of pectic polysaccharides involves three major enzymes, polygalacturonate lyase, polygalacturonase and pectin methyesterase (White et al., 1993).

The degradation of other non-cellulosic polysaccharides requires a range of enzymes. For example, enzymes for the degradation of heteroxylans include endo-(1→4)-β-xylanase, exo-(1→4)-β-xylanase, (1→4)-β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, O-acetyl xylan esterase and ferulic acid esterase (White et al., 1993).

1.4.2.2 Cell-wall polysaccharide degradation

Various models of cell-wall polysaccharide degradation in the rumen have been proposed (Van Soest, 1993). Lignification has been taken into consideration in almost all of these models. Chesson (1993) proposed a mechanistic model of wall polysaccharide degradation for forages with lignified cell walls based on knowledge that the residue composition of barley straw after degradation was similar to the original substrates before degradation (Gordon et al., 1983). The degradation of cell walls is a process of erosion of the surface of cell walls and all wall components are released at the same rate.

A model for the degradation of non-lignified primary walls has not been proposed. Chesson et al. (1986) found that the degradation rates of different polysaccharides in the mesophyll and epidermis isolated from ryegrass did not differ. A study on the maceration of white clover during incubation with the pectinolytic bacterium *Lachnospira multiparus* suggested that pectin hydrolysis was the initial step in degradation (Cheng et al., 1979). Similar maceration has been found in fruits during ripening (Sutherland et al., 1999) and in some plants after bacterial infection (Murdoch et al., 1999).

1.5 Summary and research objectives

Chicory has been planted for use as forage since 1985 and is a promising feed for grazing livestock in temperate regions (Barry, 1998). Voluntary feed intake, digestibilities of the dry matter, organic matter and energy, and consequent animal performance are higher in ruminants fed chicory than in ruminants fed perennial ryegrass or perennial ryegrass/white clover pasture (Section 1.2). The structural polysaccharides in forage cell walls are the main energy source for ruminants (France and Siddons, 1993) and a knowledge of forage plant cell walls is essential in understanding factors involved in forage digestion. At present, little is known about the composition and anatomy of cell walls of forage chicory or their degradation.

The cell-wall compositions of by-products from vegetable chicory (witloof) have been investigated (Femenia et al., 1998) and forage chicory cell walls have previously been analysed (Kusmartono, 1996). However, the analysis on forage chicory involved

proximate analytical methods, for pectin by a method of Bailey (1967) and for fibre by a method of Robertson and Van Soest (1981). The pectin analysis by the method of Bailey (1967) involved a process in which prior to pectin extraction, samples were treated with hot water. The hot-water extracts which contain water-soluble polysaccharides were excluded from the pectin analysis. Despite this, chicory was found to be rich in pectins (Kusmartono, 1996). Fibre analysis by the method of Robertson and Van Soest (1981) was unable to distinguish individual polysaccharides, so polysaccharides present in chicory cell walls were not determined.

Several families of flowering plants are used as forages for ruminant livestock. The principal families are the dicotyledon families Fabaceae (legumes) and Brassicaceae (brassicas), and the monocotyledon family Poaceae (grasses). The non-lignified primary walls of dicotyledons, including those of legumes and brassicas, contain large proportions of pectic polysaccharides. Smaller proportions of xyloglucans, and minor proportions of heteroxylans, glucomannans and/or galactoglucomannans are also present. The major non-cellulosic polysaccharides of lignified secondary walls of dicotyledons are usually 4-O-methylglucuronoxylans, with smaller proportions of glucomannans (Bacic et al., 1988; Carpita and Gibeaut, 1993; Harris, 2005). In contrast, in the non-lignified primary walls of the grasses, the most abundant non-cellulosic polysaccharides are glucuronoarabinoxylans (GAXs) (Bacic et al., 1988; Carpita, 1996; Harris, 2005). These are associated with variable proportions of (1→3),(1→4)-β-glucans together with small proportions of pectic polysaccharides and xyloglucans. GAXs with similar structures to those of the primary walls, but with a lower degree of substitution by glycosyl residues, are the major non-cellulosic polysaccharides of lignified secondary walls of the grasses. Forage chicory, as a dicotyledonous plant, would be predicted to have a cell wall structure similar to other dicotyledons with a high pectin content.

Forage cell-wall degradation in the rumen is slower when the forage contains lignin (Wilson and Hatfield, 1997). Chicory is low in lignin (Kusmartono, 1996). However, the location of lignin in chicory tissue has not been reported.

Therefore, the first objective of this study was to determine the structure and composition of cell-wall polysaccharides of forage chicory, and to examine the

distribution of lignin in chicory. This knowledge should help explain the high apparent digestibility of chicory.

Although the methods used in previous studies to determine the pectin content of chicory were rough, they did establish that chicory has a relatively high pectin content (Barry, 1998). Pectin is known to be readily degradable by bacterial enzymes in the rumen (Hatfield and Weimer, 1995) and hence the high pectin content of chicory may contribute to its high digestibility. Particle breakdown of chicory in the rumen is rapid (Kusmartono et al., 1997). Pectins are associated with cellular junctions for cell adhesion (Cosgrove, 2005) and the degradation of such pectins could be responsible for chicory cell separation. Therefore, the second and third objectives of this study were to investigate the location of pectic polysaccharides in chicory leaves (Objective 2) and to examine the role of pectin degradation in cell separation (Objective 3).

As noted above, pectin in chicory cell walls is probably important for the rapid degradation of chicory in the rumen. Pectin-rich forage white clover can be macerated by *L. multiparus* (Cheng et al., 1979), some plant cells are macerated by bacteria pathogenic to plants (Murdoch et al., 1999), and fruits and vegetables are macerated by pectin-degrading enzymes (Chesson, 1980). Maceration of chicory possibly also occurs because of pectin-degrading enzymes produced by rumen bacteria. Degradation of forage pectins is not well documented, so the fourth objective of this study was to investigate chicory pectin degradation and maceration by rumen bacteria (Objective 4).

In summary, because of the lack of knowledge of the structure and degradation of forage chicory cell walls, this study aimed to determine the structure and location of polysaccharides in chicory cell walls, and then examine cell wall degradation by enzymes and rumen bacteria. A diagram of the research objectives is shown in Fig. 1.13.

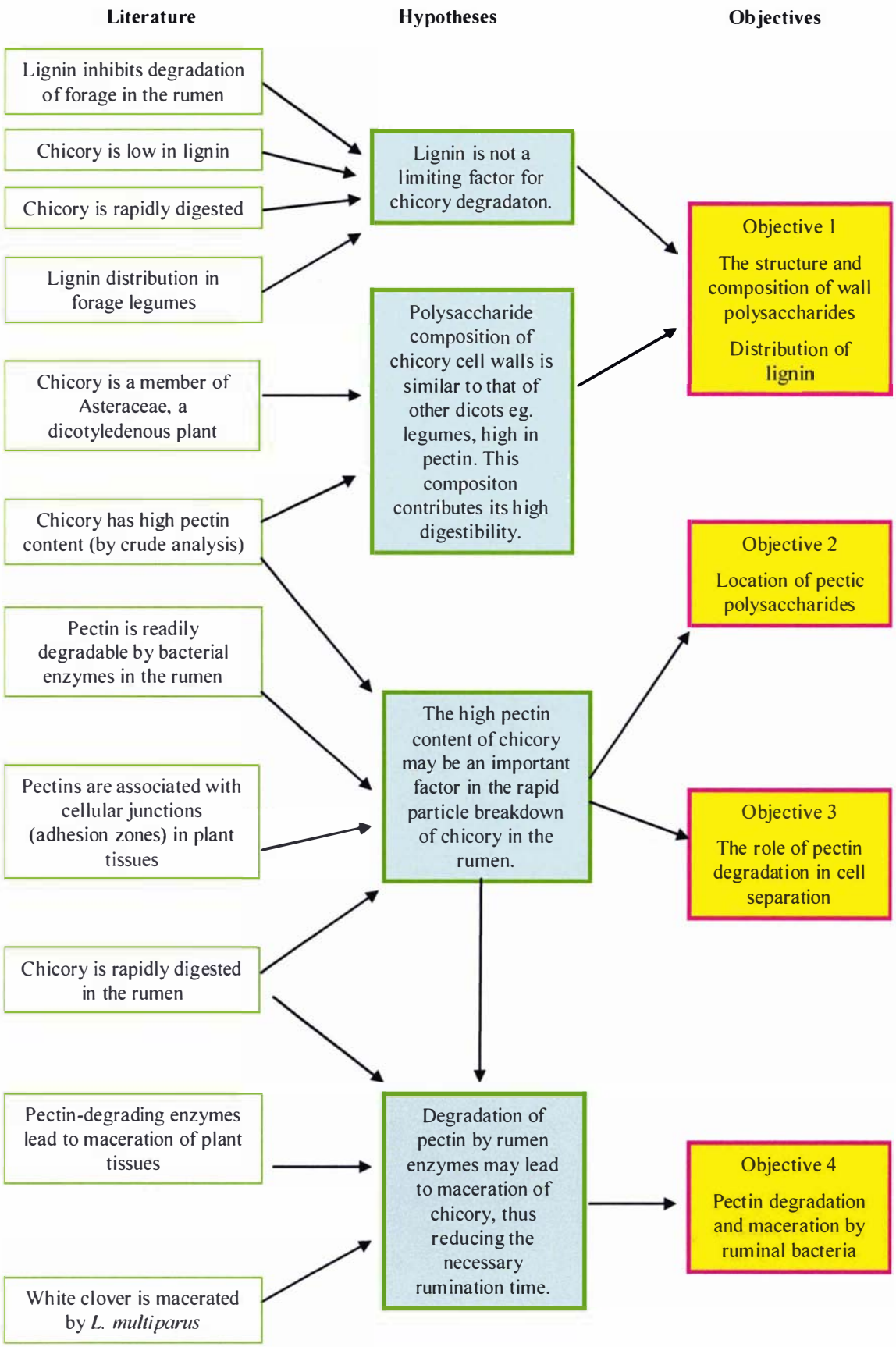


Figure 1.13 The proposed hypotheses and objectives

Chapter two

Polysaccharide compositions of leaf cell walls of forage chicory (*Cichorium intybus* L.)

A paper based on this chapter has been published in Plant Science.
Sun, X.Z., Andrew, I.G., Joblin, K.N., Harris, P.J., McDonald, A., Hoskin, S.O., 2006. Polysaccharide compositions of leaf cell walls of forage chicory (*Cichorium intybus* L.). Plant Science, 170(1), 18-27.

2.1 Abstract

The polysaccharide compositions of cell walls isolated from the laminae and midribs of chicory (cv. Grasslands Puna II) leaves were investigated. The walls were fractionated progressively with 50 mM CDTA, 50 mM Na_2CO_3, 1 M KOH, 4 M KOH, 4 M KOH + 3.5% H_3BO_3, and hot water. Monosaccharide and linkage analyses of the polysaccharides in the walls and wall fractions indicated that the polysaccharides were similar to those in the non-lignified walls of other dicotyledons. However, the proportions of pectic polysaccharides were particularly high (67% of the total wall polysaccharides in the laminae, 58% in the midribs). These included homogalacturonans (50% of the total wall polysaccharides in the laminae, 40% in the midribs) and rhamnogalacturonan I (RG I). In contrast, the proportions of cellulose, xyloglucans, heteroxylans and glucomannans were particularly low. The xyloglucans were probably fucogalactoxyloglucans. The wall polysaccharides from the leaf laminae differed from those from the midribs in having higher proportions of homogalacturonans, lower proportions of non-pectic polysaccharides, and RG I with a higher ratio of galactan to arabinan. Lignification was also examined. A histochemical study showed that chicory leaf cell walls are all non-lignified primary walls, apart from the low proportion of xylem tracheary elements in vascular bundles. These elements have lignified wall thickenings. The preponderance of non-lignified primary walls and the abundance of pectic polysaccharides in these walls, may account, at least in part, for the rapid degradation of forage chicory in the rumen.

2.2 Introduction

Rumen microbial digestion of forage cell walls produces volatile fatty acids, which are absorbed across the rumen wall, and provide the main energy source for ruminant animals (France and Siddons, 1993). The chemical compositions of the cell walls of forage plants are important in relation to digestibility in ruminant animals (Jung and Allen, 1995; Krause et al., 2003). Lignin is not only undegradable in the rumen, but also interferes with degradation of forage cell walls by rumen microbes (Chesson and Forsberg, 1997). In contrast, pectins are rapidly and readily degradable in the rumen (Moore and Hatfield, 1994). However, the degradation of cellulose and alkali-

extractable polysaccharides are variable and incomplete due to their association with lignin and other wall polymers.

Because of the importance of wall compositions of forage plants, the cell-wall structure of species in the principal families Fabaceae (legumes), Brassicaceae (brassicas) and the Poaceae (grasses) have been well studied (Lam et al., 1990). Chicory, a member of the dicotyledon family Asteraceae, has recently received attention as a forage plant for ruminants (Rumball et al., 2003b). Chicory is high yielding, can be grown throughout the temperate world, and has a high feeding value for grazing livestock (Barry, 1998). Although the content of neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin in chicory, have been determined by the Van Soest method (Kusmartono et al., 1997), little is known about the detailed chemical structure of chicory cell walls.

Polysaccharides are major components of the cell walls of flowering plants and consist of cellulose, in the form of microfibrils, and a variety of non-cellulosic polysaccharides. The proportions and structures of these non-cellulosic polysaccharides may vary with cell type and plant taxon (Bacic et al., 1988; Carpita and Gibeaut, 1993; Harris, 2005). Other wall components may be present, such as phenolic components, including the complex polymer lignin, as well as small proportions of proteins and glycoproteins.

In mature organs of a particular plant species, two types of walls are often recognized: non-lignified primary walls and lignified secondary walls. These frequently have different non-cellulosic polysaccharide compositions. In leaves, cell types with non-lignified primary walls include the mesophyll and epidermal cells; whereas cell types with lignified secondary walls often include xylem tracheary elements and sclerenchyma fibres. Thus, when comparing the wall polysaccharide compositions of different plant taxa, it is important to compare the compositions of wall preparations that contain predominantly either non-lignified primary, or lignified secondary walls.

The main source of feed from chicory for grazing animals is the primary leaves, together with the secondary and axillary leaves (Li et al., 1997). A leaf is composed of a lamina and a midrib each of which has quite different anatomical structures. The midrib contains more vascular tissues with more secondary walls, compared with the lamina which predominates mesophyll cells with only primary walls (Esau, 1977).

Consequently, the midrib and laminae should have distinctly different chemical compositions. In this study, the midrib and laminae were treated as separate entities.

The aims were to examine the anatomy, wall histochemistry and polysaccharide compositions of the laminae and midribs of rosette leaves of forage chicory.

2.3 Materials and methods

2.3.1 Plant material

Plants of forage chicory (*Cichorium intybus* L. cv. Puna II) (Rumball et al., 2003b) were grown from seed in containers at AgResearch Grasslands, Palmerston North, New Zealand. For the first four weeks, containers were in an unheated, naturally-lit glasshouse. Then, they were placed outside for a further four weeks. Eight weeks after sowing, the three youngest, fully-expanded but actively-growing leaves (ca. 20 cm long) were harvested (see Appendix 1). For wall isolations, the laminae were separated from the midribs using a razor blade. Any leaves that showed yellowing were discarded.

2.3.2 Anatomy

For anatomical studies, segments (ca. 1 × 5 mm) were cut from the lamina and midrib midway along the leaf, fixed, postfixed, dehydrated, embedded in resin, and sectioned as described by Trethewey and Harris (2002). Sections were stained with 0.05% w/v Toluidine Blue in 100 mM sodium phosphate buffer (pH 7.2) for 2 min, washed in water and mounted in dibutyl phthalate xylene medium (DPX, BDH Laboratory supplies, Poole, UK). Examination under bright-field was carried out using a microscope (Olympus BX51, Japan) fitted with a 100-W halogen quartz lamp. Photomicrographs were taken with a digital microscope camera (Optronics, Olympus U-TV 0.5XC, Japan).

2.3.3 Histochemistry

For histochemical studies, transverse sections were sectioned by hand using a razor blade. Sections of midrib and lamina were stained with phloroglucinol-HCl (2 ml of concentrated HCl and 1 ml of 95% (v/v) ethanol containing 20mg of phloroglucinol) to detect lignin (Harris et al., 1980; Pomar et al., 2002). The Mäule reagent (consecutive

treatments with 0.5% $KMnO_4$ for 10 min, 10% HCl for 5 min and mounting in 29% aqueous ammonia) was used to detect lignin containing syringl units (Chapple et al., 1992). Syringyl lignin was also detected by chlorine-sodium sulphite (consecutive treatments with a mixture of 1 ml 30% calcium hypochlorite and 0.75 ml concentrated HCl for 5 min, washed twice with water, treated with 10% w/v sodium sulphite for 3 min, and mounted in water) (Wardrop, 1971).

The metachromatic stain Toluidine Blue O (0.05% w/v in sodium benzoate buffer 20 mM, pH 4.4)) was used to distinguish between lignified and non-lignified walls (Feder and O'Brien, 1968; O'Brien and McCully, 1981). Staining with Alcian Blue 8GX [1% w/v in aqueous acetic acid (3% v/v, pH 2.5)] was used to detect polyanionic polymers, such as homogalacturonans (Benes, 1968; Scott et al., 1964).

The autofluorescence from phenolic components in cell walls under UV irradiation was determined by fluorescence microscopy using sections mounted in water and in 0.1 M ammonia solution (Harris & Hartley, 1976).

Wall preparations were also stained by all of the above procedures.

A solution of I_2 (0.2% w/v) in KI (2% w/v) was used to detect starch in sections, wall preparations and wall fractions (Jensen, 1962).

2.3.4 Isolation of walls

The cell-wall isolation procedure was based on that of Carnachan and Harris (2000) and Newman et al. (1996). All isolation procedures were performed at 4°C.

Forty seven plants (113 leaves) were harvested for cell-wall isolation. Midribs (about 6 g in each batch) and laminae (about 6 g in each batch) were separated from freshly harvested leaves as described in Section 2.3.1 after washing first with tap water and then with reverse-osmosis water. The material was cut into small pieces (3 mm × 3 mm) and homogenized in 300 ml of 3-(N-morpholino)-propanesulfonic acid (MOPS)-KOH buffer (20 mM, pH 6.8) containing 10 mM 2-mercaptoethanol (Newman et al., 1996; Carnachan and Harris, 2000) using a blender (Breville Super blender 2, Model BLR-2 Q92144, China) for approximately 4 min.

The samples (10 ml per batch) were further homogenised using a Tenbroeck ground-glass homogeniser until there were less than ~ 1% unbroken cells. Unbroken cells were identified histochemically using Ponceau 2R, a specific stain for proteins (Harris, 1983). After centrifuging (750 g, 4 min), the pellets were washed onto nylon mesh (pore size 11 μm) with cold water and further washed until the filtrates were clear (about 1000 ml of water). The walls on the mesh were dried by solvent exchange by washing successively with ethanol, methanol, and *n*-pentane (150 ml of each) and stored in a vacuum desiccator over silica gel. Cell-wall preparations (see Appendix 2 and 3) were assessed for lignification as described for leaf sections (Section 2.3.3). The starch content of wall preparations were determined by the method of McCleary et al. (1994) using a Megazyme total starch kit (Megazyme International Ireland Ltd, Co. Wicklow, Ireland). Because only very low concentrations of starch were found (0.05% and 0.12% w/w of the wall preparations from the midribs and laminae, respectively), no attempt was made to remove starch. No starch granules were detected histochemically in the wall preparations.

2.3.5 Fractionation of the wall preparations

Fractionations were performed using a modification of the procedures of methods of Selvendran (1985), Selvendran and O'Neill (1987) and Coimbra et al. (1996). Extractions were carried out in triplicate. During extraction, tubes were shaken gently on an orbital shaker to ensure mixing of reagents and cell walls. All extractions were at room temperature (20°C) unless stated otherwise. Tubes were shaken with a gentle orbital rocking motion to achieve thorough mixing.

CDTA extraction

Accurately-weighed wall preparations (125 mg per replicate) were extracted twice (6 h and 2 h) with 50 mM *trans*-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid (CDTA, 15 ml, adjusted to pH 6.5 with 1 M KOH). After centrifuging (450 g, 5 min) and washing with water (3 times) the supernatants and washings were filtered through a glass-fibre filter to give the 'CDTA fraction'.

Na_2CO_3 extraction

The pellet was extracted with a solution of 50 mM Na_2CO_3 in 25 mM $NaBH_4$ (12.5 ml, 16 h, 1°C) under nitrogen and centrifuged (450 g, 5 min). The pellet was then extracted with a solution of 50 mM Na_2CO_3 in 25 mM $NaBH_4$ (12.5 ml, 2 h) under nitrogen, centrifuged (450 g, 5 min), and washed twice with 50 mM Na_2CO_3 (10 ml) and twice with water (10 ml) by centrifuging (450 g, 5 min). The extracts and washings were combined to give the 'Na_2CO_3 fraction'.

1M KOH extraction

The pellet was extracted twice (2 h each) with a solution of 1 M KOH in 25 mM $NaBH_4$ (12.5 ml) under nitrogen, and the two extracts combined to give the '1M KOH fraction'.

4M KOH extraction

The pellet was extracted with a solution of 4 M KOH in 25 mM $NaBH_4$ (12.5 ml, 2 h) under nitrogen. This extract constituted the '4M KOH fraction'.

4M KOH+H_3BO_3 extraction

The pellet was extracted with a solution of 4 M KOH in 25 mM $NaBH_4$ and 3.5% H_3BO_3 (12.5 ml). This extract constituted the 'KOH/borate fraction'.

Hot water extraction

The pellet was resuspended in water (5 ml) and the pH adjusted to 7.0 with 18 M acetic acid. After 12 h at 4°C, the suspension was made up to 12.5 ml with water and heated at 100°C for 2 h. After cooling, the suspension was centrifuged (450 g, 5 min), and the pellet washed twice with water. The extract and washes were combined to give the 'Hot water fraction'.

Residue

The pellet remaining after the hot water extraction was the 'Residue fraction'.

After each of the extraction steps, the supernatants and washing were filtered through a glass-fibre filter. Toluene (3-4 drops) was added to all fractions to prevent microbial growth. The CDTA fraction was dialysed against ammonium acetate buffer (100 mM,

pH 5.2, 1°C) for 36 h with three changes of buffer (Mort et al., 1991), followed by further dialysis against running tap water for 3 days and then with three changes of reverse osmosis water (1°C, 24 h). The Na_2CO_3 and KOH fractions were adjusted to pH 5.2 with 18 M acetic acid and dialysed against running tap water for 2 days followed by three changes of reverse osmosis water (1°C, 24 h). The dialysed fractions were freeze-dried.

2.3.6 Hydrolysis of wall preparations and wall fractions

Weighed fractions and wall preparations (6-10 mg) were suspended in 12 M H_2SO_4 (125 µl, 35°C, 30 min), diluted to 1 M H_2SO_4 and heated (100°C, 2 h) for hydrolysis (Englyst et al., 1994).

2.3.7 Determination of the neutral monosaccharide compositions of wall preparations and fractions

Neutral monosaccharides were estimated by the method of Albersheim et al. (1967) as modified by Englyst et al. (1994).

Aliquots (0.3 ml) of hydrolysed samples were used for analysis, with allose (0.1 ml, 2 mg ml^{-1}) added as an internal standard. The samples were neutralized with 0.15 ml concentrated ammonia solution (pH>10 by pH paper after neutralization), and the monosaccharides reduced with 0.1 ml $NaBH_4$ (freshly prepared, 100 mg ml^{-1} in 3 M aqueous NH_3) at 40°C for 60 min. To stop the reaction, acetic acid (HOAc, 0.1 ml) was added to destroy residual borohydride. The samples were then acetylated using acetic anhydride (Ac_2O, 4 ml) for 10 min with 0.2 ml of 1-methyl imidazole as a catalyst. Ethanol (1 ml) was added, left for 5 min, and then 8 ml of water added to destroy surplus Ac_2O. After 5 min, bromophenol blue (0.04%, 0.15 ml) was added for distinguishing layers in the next step and the tubes placed in ice for 5 min. To promote phase separation, KOH (7.5 M, 4 ml) was added and the tubes left on ice for 5 min. A further 4 ml of KOH was then added and the tubes mixed by inversion. After phase separation, the upper ethyl acetate layer was cleanly transferred to an Eppendorf tube for analysis by gas chromatography (GC).

The alditol acetates were separated and quantified by gas chromatography (using a Hewlett-Packard GC HP3890 fitted with a flame ionisation detector) on a BPX70

megabore column (70% cyanopropyl polysilphenylene-siloxane; length 30 m; ID 0.53 mm; film thickness 0.25 μm; SGE, Melbourne, Australia) using hydrogen as the carrier gas. The following conditions were used: injector temperature 220°C; column temperature 150°C to 220°C increased at 5°C per min; detector temperature 220°C.

2.3.8 Determination of the acidic monosaccharide compositions of wall preparations and fractions

2.3.8.1 Paper chromatography

The uronic acids in the wall polysaccharides were identified by acid hydrolysis followed by paper chromatography. Wall preparations were hydrolysed with trifluoroacetic acid (TFA) (2 M, 121°C, 1 h), the hydrolysates evaporated to dryness, an aqueous ammonium carbonate solution (0.2 M) (pH 8.5, 15 μl) added, incubated for 3 min to convert lactones to uronic acids, and the final residue dissolved in water (50 μl). Galacturonic and glucuronic acids were separated by descending paper chromatography using ethyl acetate : pyridine : H_2O : acetic acid (5:5:3:1, v/v/v/v) (Hickman and Ashwell, 1966). The uronic acids were located using aniline hydrogen phthalate (Fry, 1988). The aniline hydrogen phthalate spray reagent was made by mixing of 0.93 g of aniline and 1.66 g of phthalic acid in 100 ml of water-saturated *n*-butyl alchohol (Bailey, 1968).

2.3.8.2 Determination of uronic acids

Uronic acids were determined spectrophotometrically by the method of Scott (1979) as modified by Englyst et al. (1994), using galacturonic acid as a standard.

Aliquots (0.15 ml) of hydrolysed sample (after dilution to about 100 μg uronic acids per ml) were mixed with 0.15 ml of sodium chloride-boric acid solution (2 g NaCl and 3 g boric acid in 100 ml water), followed by adding 2.4 ml concentrated sulfuric acid. The samples were then heated at 70°C for 40 min. After cooling to room temperature in water, 3,5-dimethylphenol (0.1 ml, 1 mg ml^{-1}) was added to each tube at approximately one-minute intervals. The absorbances at 400 nm and 450 nm were measured in a spectrophotometer against the reagent blank exactly 15 min after adding the 3,5-dimethylphenol reagent. The readings at 400 nm were subtracted from those at 450 nm to overcome interference from hexoses. The concentration of uronic acids was

calculated from the difference in absorbance at 450 nm and 400 nm against a standard curve.

2.3.9 Linkage analysis of the wall polysaccharides using methylation analysis

Methylation

Methylation was based on the method of Needs and Selvendran (Needs and Selvendran, 1993).

Weighed freeze-dried samples (walls or fractions) (about 5-20 mg) in 10 ml vials with Teflon-lined screw caps were heated at 60°C for 3-4 h and stored overnight in a desiccator over P_2O_5 to get very dry before methylation. For at least 2 days before use dimethyl sulphoxide (DMSO, dry, Aldrich) was stored over approximately one-quarter of its volume of molecular sieves (Type 3A) which had been activated by heating at 300°C overnight (Needs and Selvendran, 1993).

Samples were dissolved in DMSO (0.5 ml) under nitrogen with sonication for 120 min. If necessary, samples with low solubility were dissolved by prolonged heating (120 min or more) at 40°C. Sodium hydroxide (NaOH) was finely ground in a mortar and pestle, inside a plastic bag, with a blanket of nitrogen, and was stored under nitrogen. Finely powdered NaOH (1-5 mg) was added under nitrogen and the solution sonicated for 60 min. Methyl iodide (CH_3I) (50 µl) was added under nitrogen and the solution sonicated for 60 min at room temperature; this was repeated a further three times. The reaction was quenched by careful dropwise addition of about 2 ml of water with cooling and constant shaking to lessen the effects of the highly exothermic reaction. Glacial acetic acid (100 µl) and chloroform ($CHCl_3$, 1ml) were then added, and mixed thoroughly. Centrifugation was carried out to allow the mixture to settle into two layers. The lower chloroform layer was washed 3-4 times with water (3-4 ml each) until the aqueous layer being discarded was neutral (pH=7.0). Any trace of water left in the chloroform layer was removed by sodium sulphate. The sample was then filtered through a Pasteur pipette containing cotton wool. The sample was evaporated down under a stream of air.

Subsequent steps for conversion to the partially methylated alditol acetates (PMAAs) were conducted by a modification of the method of Harris et al. (1984).

Hydrolysis

The methylated preparations were hydrolysed by TFA (2 M, 0.5 ml, 121°C, 1 h) and the TFA removed by evaporation in a stream of dry air at 40°C.

Reduction

The partially methylated neutral monosaccharides were reduced for 1 h using an aqueous solution (1 ml) of sodium borodeuteride ($NaBD_4$) (3 mg ml^{-1}). Acetic acid (0.1 ml) was added with cooling to destroy excess sodium borodeuteride, and the mixture left for 3 min. Borate was removed by adding 1 ml of methanol and the tube contents evaporated to dryness at 40°C under a stream of air. Methanol (1 ml, containing 1% acetic acid) was added and evaporated to dryness. This was repeated 3 times.

Acetylation

The dry samples were treated with Ac_2O (1 ml, 100-120°C, 2 h), then cooled and 5 ml of water added, with 100 µl of methylimidazole as catalyst for conversion of acetic anhydride to acetic acid. The reaction product was taken up in 1 ml of dichloromethane and washed once with water prior to analysis by gas chromatography.

Partially methylated alditol acetate identification and quantification

PMAAs were separated and quantified on a BPX70 column (30 m × 0.53 mm) using a gas chromatograph (GC, HP3890) fitted with a flame ionisation detector (FID) with the oven temperature programmed from 150 to 220°C at 5°C per min, injector temperature 220°C, detector temperature 220°C.

The PMAAs were identified by their retention times (Lau and Bacic, 1993) and mass spectra (Janssön et al., 1976). Combined GC-mass spectrometry (MS) was performed in a Polaris Q iontrap (Thermofinnigan, San Antonio, Texas, USA) in positive ei-mode. For separation, a BPX70 column (30 m, 0.25 mm ID, 0.25 µm film thickness) was used, with helium as the carrier gas with helium flow at 1.2 ml min^{-1} (constant flow rate) and a split flow rate of 12 ml min^{-1}. The temperature program was as follows: held at 150°C for 1 min, then raised to 200°C at 2°C min^{-1}, held for 15 min, then raised to 220°C at 5°C min^{-1}, and held at this temperature for 5 min. The injector temperature was 225°C. The PMAA peaks were monitored between 9 and 35 min (total run of 50 min) and

Xcalibur (Thermofinnigan, San Antonio, Texas, USA) software was used to record the data. Confirmation of some peaks was obtained with an SP2340 column (Supelco, Sigma-Aldrich, Bellefonte, Pennsylvania, USA).

For quantification, the response factors of Sweet et al. (1975) were used with the FID data from the GC (HP3890). For overlapping peaks, quantification was based on integration of peaks on selected ion chromatograms on the GC-MS data. For co-eluted PMAAs such as obtained from 2-linked and 4-linked xylopyranose, their relative amounts were determined using their unique ions in the mass spectra.

2.3.10 Determination of the polysaccharide compositions of wall preparations and fractions

The proportions of different polysaccharides in the wall preparations were estimated using the monosaccharide compositions and the linkage structure characteristic of individual polysaccharides (Bacic et al., 1988). The proportions of heteroxylans were determined by summing the amounts of 4-Xyl (4-linked xylose), 2,4-Xyl and the branch on the 2,4-Xyl, assumed to be a uronic acid. Any arabino(glucurono)xylans (AGX), would be expected to have Ara*f* (arabinofuranose) linked to xylose residue in position 3 (Bacic et al., 1988), this appearing as 3,4-Xyl in the linkage analysis. Since this was not found, AGX was excluded from the calculations. Furthermore, in the fraction containing most heteroxylan, the midrib 1 M KOH fraction, there was sufficient uronic acid to account for the branches, but insufficient t-Ara*f*. Therefore, t-Ara*f* is not included in the xylan. The proportions of galactoglucomannan were determined by summing the amounts of 4-Man (4-linked mannose), 4,6-Man, part of the 4-Glc (4-linked glucose) and part of the t-Gal (terminal galactose), where the proportion of 4-Glc was based on a Man:Glc ratio of 2:1 and the proportion of t-Gal was assumed to be equal to the amount of 4,6-Man (Bacic et al., 1988). Some Gal may also be attached to Glc, but this was considered to be only minor and the 4,6-Glc was attributed to xyloglucan. The linkages in the 4 M KOH fractions suggested that fucogalactoxyloglucans were present. Accordingly, the proportion of xyloglucan was calculated by summing the amounts of t-Fuc (terminal fucose), 2-Gal, 2-Xyl, part of t-Xyl, part of t-Gal, 4,6-Glc and part of 4-Glc, where 4,6-Glc is equal to the sum of 2-Xyl and t-Xyl. In the midrib 4 M KOH fraction, which lacks detectable galactan, the t-Gal left after assigning some to galactoglucomannan was assumed to belong to xyloglucan; 2-Xyl should be equal to the

sum of this part of t-Gal and 2-Gal, and the latter should be equal to t-Fuc. The remaining 4-Glc in the Residue fraction and total walls was assumed to be cellulose.

For pectic polysaccharide components, the proportion of backbone RG I was determined by summing 2-Rha (2-linked rhamnose), 2,4-Rha, and an amount of uronic acid equimolar to the sum of 2-Rha and 2,4-Rha. The arabinans were quantified as the sum of 5-Ara*f*, t-Ara*f*, 3-Ara*f* and 3,5-Ara*f*. The galactans were quantified as the sum of 4-Gal, t-Ara*p* (terminal arabinopyranose), 3-Gal, 6-Gal, 2,4-Gal, 3,4-Gal, 3,6-Gal, 4,6-Gal and any remaining t-Gal. The remaining uronic acids were assigned to homogalacturonan. Some t-Xyl in the pectic polysaccharide fractions was attributed to xylogalacturonan, where there was no evidence to suggest the presence of a heteroxylan or xyloglucan. The proportion of RG II was not estimated as the only characteristic linkages found in significant quantity were 2,4-Gal and t-Rha.

2.4 Results

2.4.1 Leaf anatomy

Fig. 2.1A and Fig. 2.1B show transverse sections of the lamina and midrib, respectively, of a chicory leaf. The lamina has an adaxial and an abaxial epidermis, each one-cell thick, separated by mesophyll cells. The lamina is wider where there are vascular bundles which contain xylem tracheary elements and phloem. The walls of the epidermal cells above and below the vascular bundles are thicker than in the rest of the lamina. In the midrib, there is a distinct hypodermis beneath both the ab- and adaxial epidermis. The midribs contain several vascular bundles which are larger and better defined than in the lamina. Large parenchyma cells occupy most of the region between the two hypodermal layers.

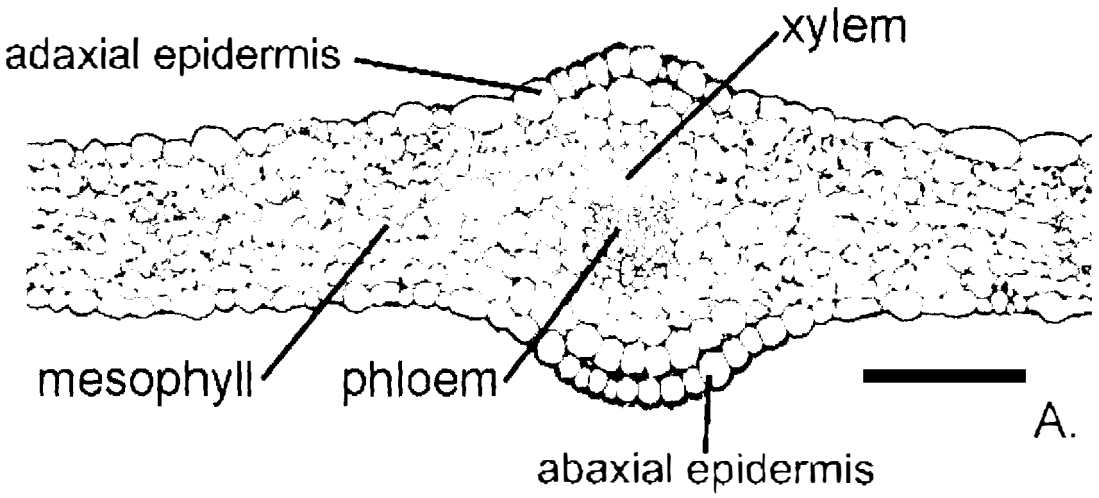


Figure 2.1A Photomicrograph of a transverse section of the lamina midway along a rosette leaf of chicory. Bar = 100 μm.

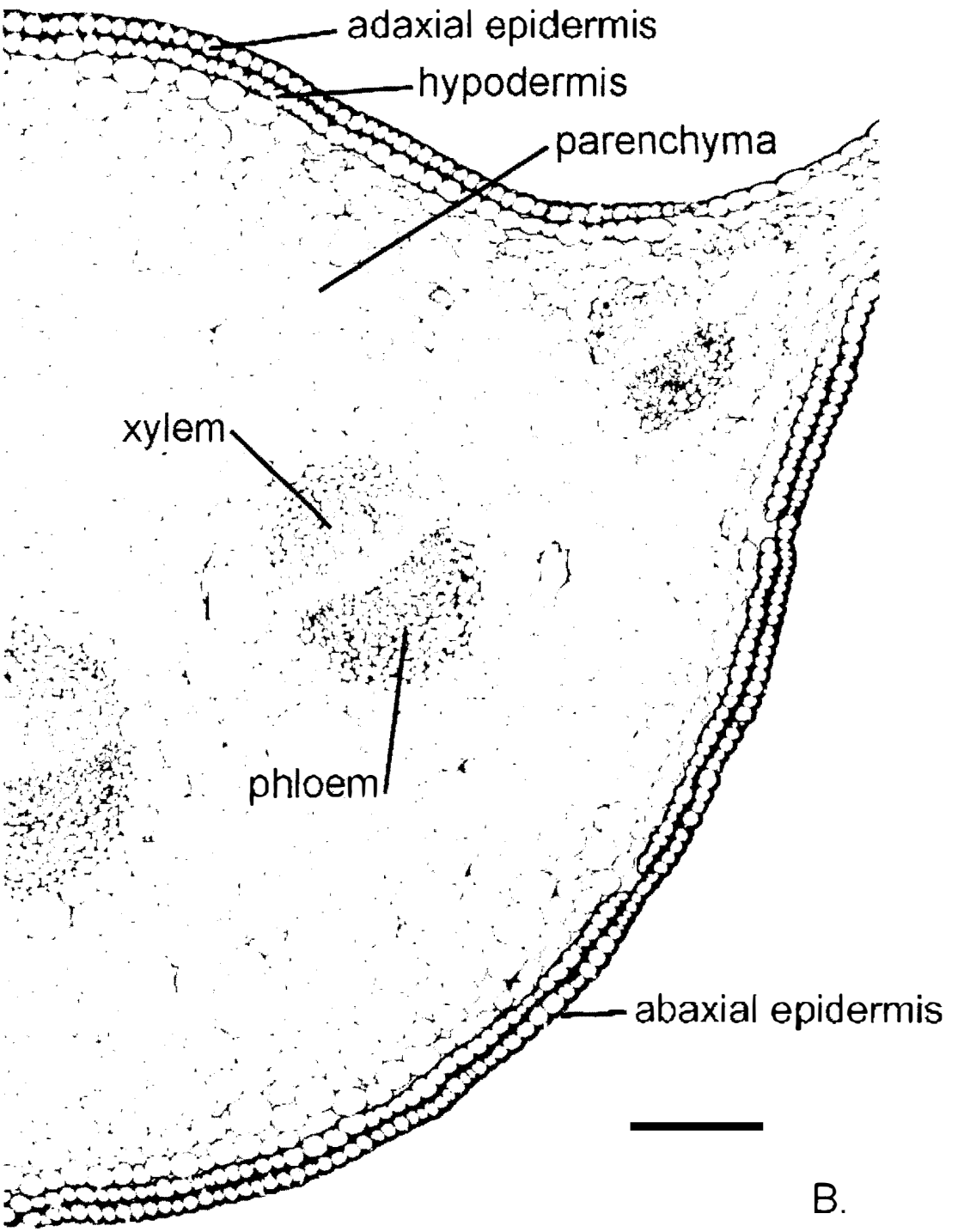


Figure 2.1B Photomicrograph of a transverse section of the midrib midway along a rosette leaf of chicory. Bar = 200 μm.

2.4.2 Wall histochemistry

The staining properties of the walls of different cell types in transverse sections of chicory midrib and lamina are summarized in Table 2.1.

Table 2.1 Autofluorescence and histochemical staining of leaf cell walls of chicory

Cell/tissue type	Cell wall staining/autofluorescence properties						
	UV fluorescence (water)	UV fluorescence (NH_4OH)	Phoroglucinol-HCl	Toluidine Blue O	Mäule reagent	Alcian Blue	Chlorine-sodium sulphite
Midrib							
Cuticle	+++	+++	-	-	-	+++	+/-
Epidermis	-	-	-	+++ (purple)	-	+++	-
Parenchyma	-	-	-	++/+ (purple)	-	++	-
Phloem	-	-	-	-	-	+++	-
Xylem	+++	+++	+++	+++ (blue)	+/- or -	-	++(large cells) -(small cells)
Lamina							
Cuticle	+++	+++	-	-	-	+++	+/-
Epidermis	-	-	-	+++ (purple)	-	+++	-
Parenchyma	-	-	-	++/+ (purple)	-	++	-
Phloem	-	-	-	-	-	+++	-
Xylem	+++	+++	+++	+++ (blue)	+/- or -	-	++(large cells) -(small cells)

Notes: - no stain, +/- very weak, + weak, ++ medial, +++ intense stain

Specificity: autofluorescence in UV radiation shows the presence of phenolic components; phoroglucinol-HCl stains lignin; Toluidine Blue O stains lignin and other phenolic components a green or blue-green colour, and polyanions (e.g. rhamnogalacturonans) a pink or reddish purple; the Mäule reagent gives a red colour with lignin containing syringl units; Alcian Blue stains polyanions (e.g. rhamnogalacturonans) blue; chlorine-sodium sulphite gives a red or pink colour reaction with syringyl lignin.

The only walls in sections that gave a positive colour reaction for lignin with phloroglucinol-HCl were those of the xylem tracheary elements. The walls of these cells also stained blue with Toluidine Blue and red with chlorine-sodium sulphite, and showed a blue autofluorescence in UV light, which increased in intensity after treatment with aqueous ammonia. These observations are consistent with the walls being lignified. The walls of the other cell types, as well as the primary walls of the xylem tracheary elements, stained pink-purple with Toluidine Blue, indicating they contained polyanions such as homogalacturonans. In these walls, ammonia did not enhance autofluorescence, suggesting that ferulate esters are very minor components if present at all. These walls stained blue with Alcian Blue again indicating the presence of homogalacturonans. The secondary walls of the xylem tracheary elements did not stain with Alcian Blue.

The staining properties of isolated cell walls were similar to those of walls in sections.

2.4.3 Yields of isolated walls and fractions

Yields of isolated walls are shown in Table 2.2. Chicory leaves are composed of 50% midrib and 50% lamina on a fresh weight basis and 54% midrib and 46% lamina on a dry weight basis. The walls constitute 48% of the total midrib dry weight and 33% of the lamina dry weight.

Table 2.2 Yields of isolated walls from chicory leaves

	Midrib	Lamina
Fresh weight as a % of whole leaves	49.7	50.3
Dry weight as a % of whole leaves	54.1	45.9
Dry cell walls as a % of fresh weight	5.0	4.2
Dry cell walls as a % of dry weight	48.2	32.6
Cell contents as a % of dry weight	51.8	67.4

The proportions of the wall fractions obtained are shown in Table 2.3. The largest two fractions were the Residue and CDTA fractions. The proportions of both fractions as percentages of the total wall dry weight were greater from the midrib walls (Residue 34.5%, CDTA 30.8%) than from the lamina walls (Residue 27.5%, CDTA 28.6%). In

contrast, the next two largest fractions, the hot-water soluble and Na_2CO_3 fractions, were larger from the lamina walls (hot water 16.9%, Na_2CO_3 16.3%) than from the midrib walls (hot water 13.2%, Na_2CO_3 10.8%). The smallest fractions were the 1 M KOH, 4 M KOH and KOH/borate fractions, together representing a total of 12.8% and 11.4% from the midrib and lamina walls, respectively.

2.4.4 Starch concentrations in the wall preparations and wall fractions

Starch granules were not detected in isolated wall preparations or wall fractions. Starch concentrations of wall preparations were less than 0.2% (Section 2.3.4), and those of wall fractions were all less than 1%, except the 1M KOH fractions, which were about 1.4% for both lamina and midrib.

2.4.5 Monosaccharide compositions of unfractionated wall preparations

On a dry weight basis, the uronic acid content of the chicory walls was 27.5% for the midrib and 36.2% for the lamina (Table 2.3). In acid hydrolysates of the midrib and lamina walls, Glc was the most abundant neutral monosaccharide, accounting for about 50% of the total neutral monosaccharides. The second most abundant neutral monosaccharide was Gal, followed by Ara (arabinose) and Xyl. The ratio of Gal to Ara was 1:1 in the midrib walls and 1.5:1 in the lamina walls. Galacturonic acid (GalA) was the only uronic acid detected in acid hydrolysates of midrib and lamina walls.

2.4.6 Monosaccharide compositions of the wall fractions

The percentages of the neutral and acidic monosaccharides in acid hydrolysates of wall fractions are shown in Table 2.3 and Fig. 2.2.

The highest proportion of uronic acid was in the CDTA fractions of both lamina and midrib, in which it constituted over 90% of the total carbohydrate, followed by the Na_2CO_3 and Hot water fractions, which had similar proportions, and a significant amount in the Residue fractions (Table 2.3). In the CDTA, Na_2CO_3 and Hot water fractions, Ara and Gal were the most abundant neutral monosaccharides, followed by Rha. In these fractions, there was more uronic acid and a higher ratio of Gal to Ara from the lamina compared with the midrib.

Table 2.3 Percentage recoveries, the content of uronic acids and anhydrous neutral monosaccharides in wall preparations from the lamina and midrib of chicory leaves

Walls or fractions	Percentage recovery[a]		Uronic acids[b]		Neutral monosaccharides[c]													
					Rha		Fuc		Ara		Xyl		Man		Gal		Glc	
	L.	M	L.	M	L.	M	L.	M	L.	M	L.	M	L.	M	L.	M	L.	M
Walls			361.6	275.2	21.7	14.8	3.8	3.6	41.0	51.8	28.8	34.9	21.0	23.7	74.0	62.7	187.7	218.0
			±33.8	±4.2	±3.0	±0.3	±0.3	±0.0	±2.9	±0.2	±1.4	±0.1	±1.6	±0.6	±4.7	±0.5	±13.3	±0.3
CDTA	28.6	30.8	166.1	177.2	2.8	2.9	0.5	0.6	4.4	8.2	0.3	0.5	0.1	0.2	3.4	4.0	0.5	1.3
	±0.7	±1.8	±3.3	±0.3	±0.3	±0.2	±0.1	±0.1	±0.3	±0.2	±0.0	±0.1	±0.1	±0.0	±0.1	±0.2	±0.1	±0.1
Na_2CO_3	16.3	10.8	75.8	42.9	6.4	2.8	0.4	0.3	9.0	7.9	0.8	0.5	0.2	0.1	14.7	6.3	0.3	0.3
	±1.5	±1.1	±15.6	±0.1	±2.6	±0.1	±0.2	±0.0	±2.2	±0.6	±0.1	±0.1	±0.0	±0.0	±3.2	±0.0	±0.0	±0.1
1 M KOH	3.5	3.6	6.8	3.9	1.1	0.5	0.2	0.3	1.8	1.3	5.8	11.2	1.0	2.2	4.5	2.5	2.8	4.3
	±0.3	±0.7	±1.2	±0.2	±0.3	±0.1	±0.0	±0.0	±0.4	±0.0	±0.3	±0.9	±0.0	±0.6	±1.0	±0.2	±0.2	±0.5
4 M KOH	5.0	6.1	1.1	0.8	0.2	0.2	1.1	1.4	0.5	0.6	8.1	12.0	5.4	8.5	5.8	6.5	15.2	20.4
	±0.3	±0.5	±0.3	±0.1	±0.0	±0.0	±0.1	±0.1	±0.2	±0.1	±1.5	±0.9	±0.6	±0.4	±0.6	±0.3	±1.6	±1.0
4 M KOH + H_3BO_3	2.9	3.1	6.0	3.0	0.9	0.4	0.2	0.2	1.2	1.1	2.4	1.4	0.9	0.8	3.7	2.5	2.2	2.1
	±0.8	±0.9	±1.2	±0.4	±0.2	±0.0	±0.0	±0.0	±0.2	±0.2	±0.2	±0.1	±0.0	±0.2	±0.7	±0.3	±0.1	±0.3
Hot water	16.9	13.2	76.3	37.1	7.1	4.6	0.4	0.2	14.5	16.9	3.3	1.1	0.2	0.1	30.0	20.8	0.9	1.2
	±0.7	±0.8	±8.2	±6.3	±2.7	±0.4	±0.0	±0.1	±2.2	±2.5	±0.5	±0.2	±0.0	±0.0	±3.6	±3.0	±0.2	±0.8
Residue	27.5	34.5	12.0	15.8	1.4	2.7	0.5	0.8	4.5	10.3	3.9	5.6	7.5	7.1	7.6	15.9	153.9	215.3
	±0.9	±1.3	±1.0	±0.2	±0.2	±0.6	±0.1	±0.3	±0.1	±0.4	±0.2	±0.3	±0.5	±0.1	±0.0	±0.1	±2.4	±7.0

Values are means of three determinations ± standard errors.

[a] Recoveries of the wall fractions are expressed as percentages of total wall and are means from three fractionations (± standard errors).

[b] Amounts of uronic acid (mg) in 1 g (dry weight) of wall preparations and fractions (L. = lamina, M = midrib). Values are from three fractionations, each assayed twice (± standard errors).

[c] Amounts of neutral monosaccharides (mg) in 1 g (dry weight) wall preparations and fractions (L. = lamina, M = midrib). Values are from three fractionations, each assayed twice (± standard errors).

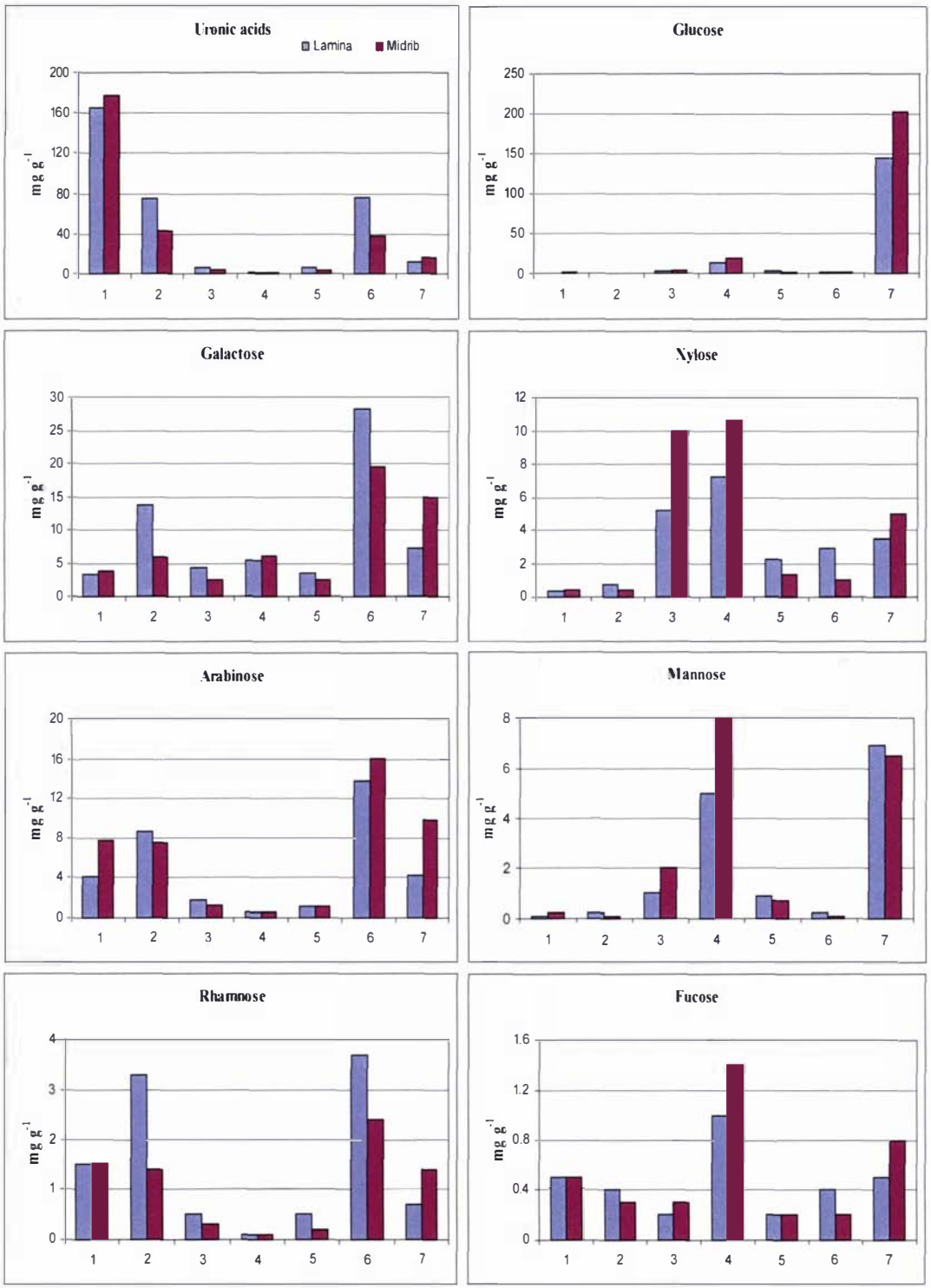


Figure 2.2 Distribution of monosaccharides in wall fractions of lamina and midrib of chicory leaves. 1, CDTA fractions; 2, Na_2CO_3 fractions; 3, 1 M KOH fractions; 4, 4 M KOH fractions; 5, 4 M KOH + H_3BO_3 fractions; 6, Hot water fractions; 7, residue fractions.

In hydrolysates of the 1 M KOH fractions, the most abundant neutral monosaccharides were Xyl, followed by Gal in the lamina, and Xyl, followed by Glc in the midrib. In hydrolysates of the 4 M KOH fractions, Glc was the major neutral monosaccharide, followed by Xyl. In both these fractions, the Xyl, Glc and Man contents were higher in the midrib than in the lamina. The recovery percentages of the KOH/borate fractions were smaller than the other two alkali fractions, with Gal the most abundant neutral monosaccharide and significant uronic acid. Ara and Rha were present in only small amounts in the alkali fractions. Glc was the most abundant neutral monosaccharide in the Residue, with more in the midrib than in the lamina. The Residue contained about 90% of the total wall Glc, the rest being mostly in the 4 M KOH fraction. Over 50% of the total Gal and Ara were recovered in the hot water and Residue fractions. Xyl was predominantly in the 1 M KOH and 4 M KOH fractions. Man was mostly distributed equally between the 4 M KOH and Residue fractions.

2.4.7 Identification of uronic acids in wall preparations

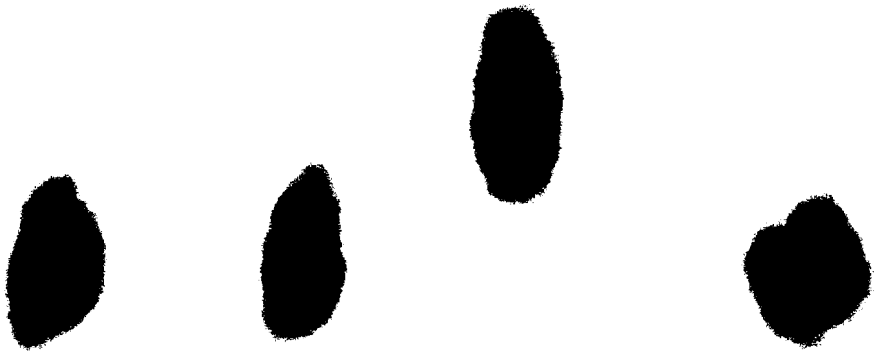


Figure 2.3 Paper chromatograph of hydrolysates of chicory cell wall preparations from lamina and midrib. GalA, galacturonic acid; GlcA, glucuronic acid.

From the paper chromatograph (Fig. 2.3), the form of uronic acids present in wall preparations is mainly galacturonic acid (GalA). Glucuronic acid (GlcA) was not present or present in only very tiny amounts.

2.4.8 Linkage analysis of the wall polysaccharides

The glycosidic linkage compositions of the wall preparations and fractions are shown in Table 2.4. The proportions of particular polysaccharides in the wall preparations and fractions have been inferred from the glycosidic linkage compositions (Table 2.5).

The most abundant polysaccharides in the wall preparations were the pectic polysaccharides, especially the homogalacturonans. RG I was present, with galactan side chains being more abundant than arabinan side chains. The linkage composition indicated the presence of a pectic galactan or arabinogalactan I (AG-I), but with a rather high proportion of 6-Gal. On the other hand, 3,4-Gal, characteristic of AG-I, was found in only small amounts, while there were only small amounts of the 3-Gal and 3,6-Gal typical of arabinogalactan II (AG-II). Some of the 2,4-Gal and t-Rha may arise from RG II, whereas t-Xyl in the pectic fractions possibly represents xylogalacturonan. The most abundant other non-cellulosic polysaccharides were xyloglucans, followed by galactoglucomannans and heteroxylans. Cellulose was the second most abundant polysaccharide in the walls.

A comparison of the proportions of polysaccharides in the wall preparations from the lamina and midrib showed that the midrib walls contained more cellulose than the lamina walls and more of each of the other three non-pectic polysaccharides. However, the lamina walls had higher proportions of homogalacturonan. In the RG I, the ratio of galactan to arabinan was higher in the lamina walls than in the midrib walls.

Table 2.4 Glycosyl linkage composition of the polysaccharides in the walls and wall fractions from the lamina and midrib of rosette chicory leaves (mol%)

Deduced glycosidic linkage	Walls		Wall fractions													
			CDTA		Na_2CO_3		1 M KOH		4 M KOH		4 M KOH + H_3BO_3		Hot water		Residue	
	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M
t-Ara*f*	3.3	4.2	10.9	18.7	7.0	16.3	4.0	2.2	0.4	0.6	3.3	4.3	7.2	8.5	0.6	1.0
t-Ara*p*	0.2	0.2	1.1	0.9	0.3	0.5	---	---	---	---	0.2	0.3	---	0.4	---	---
2-Ara*f*	---	---	---	---	---	---	---	---	0.4	---	---	---	---	---	---	---
3-Ara*f*	0.5	0.1	---	---	0.3	1.0	0.3	0.1	0.2	0.2	---	---	0.6	0.1	0.3	0.2
5-Ara*f*	4.9	5.4	20.1	18.6	14.5	17.2	4.2	1.9	0.3	0.3	4.6	5.1	11.2	13.9	0.9	1.3
3,5-Ara*f*	3.0	4.0	6.5	10.4	8.1	10.8	2.8	2.0	0.2	0.2	3.2	4.3	8.7	17.4	1.0	2.0
t-Fuc	0.9	0.9	4.3	3.4	1.3	1.7	1.2	1.4	3.2	3.0	1.8	2.5	0.6	0.4	0.3	0.3
3,4-Fuc	0.2	0.1	---	---	---	---	---	---	---	---	---	---	0.1	---	---	---
t-Gal	4.6	2.5	4.6	4.0	7.8	5.6	4.4	2.5	7.4	5.6	7.9	6.9	9.3	6.5	0.5	0.8
2-Gal	0.9	1.0	---	---	---	---	4.2	4.1	5.7	6.1	4.8	4.1	0.5	0.6	0.5	0.4
3-Gal	0.8	0.4	1.5	---	1.7	2.0	0.2	---	---	---	---	---	1.2	0.4	0.1	0.1
4-Gal	8.9	7.1	14.5	11.3	25.6	15.5	10.2	2.3	0.9	---	8.9	11.4	27.0	24.9	2.0	3.7
6-Gal	2.6	1.6	3.9	4.2	5.1	4.6	4.3	0.8	0.9	0.4	8.5	4.8	10.0	7.5	0.5	0.5
2,4-Gal	0.7	1.3	1.1	1.3	0.7	1.2	0.8	0.6	0.3	0.5	0.4	0.8	0.4	0.5	0.4	0.5
3,4-Gal	---	---	---	---	0.8	0.2	---	---	---	---	---	---	0.5	---	---	---
3,6-Gal	0.1	0.1	0.3	---	---	1.5	0.1	---	---	---	---	---	0.1	---	---	---
4,6-Gal	0.4	0.8	0.1	---	1.6	1.4	0.2	---	0.1	0.1	---	---	1.2	3.0	0.2	0.3
t-Glc	0.3	0.3	0.1	0.1	0.9	0.1	1.3	1.3	1.0	0.7	1.0	0.7	0.2	0.1	0.6	0.2
4-Glc	43.2	44.8	3.6	6.2	---	1.4	9.0	10.7	19.5	19.7	8.0	13.0	1.2	2.3	81.3	78.9
3,4-Glc	0.4	1.1	---	---	---	---	---	---	---	---	---	---	---	---	0.9	1.1
4,6-Glc	4.2	5.2	0.1	0.4	---	---	4.9	5.9	19.8	19.2	9.2	9.8	0.1	0.1	2.4	2.5
4-Man	5.0	4.7	0.8	1.0	---	0.5	4.6	7.4	12.1	12.9	6.3	6.5	0.3	0.2	3.9	2.4
4,6-Man	0.4	0.9	---	---	---	---	0.9	1.7	2.3	3.6	1.1	2.5	---	---	0.2	0.4
t-Rha	0.9	0.8	3.6	5.1	2.1	3.9	---	---	---	---	0.4	0.7	0.9	1.7	---	---
2-Rha	1.7	1.4	8.6	5.5	8.6	6.6	1.8	1.7	0.1	0.1	1.6	1.3	2.9	3.5	0.3	0.4
3-Rha	---	---	4.0	---	---	---	0.3	---	---	---	---	---	---	---	---	---
4-Rha	0.7	0.1	---	---	---	---	---	---	---	---	---	---	---	---	---	---
2,3-Rha	0.1	---	---	---	---	---	---	---	---	---	---	---	0.1	---	---	---
2,4-Rha	2.8	1.4	7.4	6.1	10.2	5.3	4.5	0.6	0.5	0.3	6.2	3.0	9.2	5.3	0.6	0.7
2,3,4-Rha	---	---	0.3	---	---	---	---	---	---	---	---	---	0.1	0.2	---	---
t-Xyl	3.8	3.0	1.1	2.2	2.7	2.3	4.8	3.2	10.9	9.4	10.8	6.8	5.3	0.7	1.2	1.2
2-xyl	1.2	0.8	---	---	---	0.1	2.8	3.2	5.7	6.4	5.2	1.5	---	0.1	0.7	0.3
4-Xyl	2.6	5.2	1.6	0.8	---	0.5	24.2	40.5	6.8	9.8	5.8	8.0	0.5	1.2	0.6	0.7
2,4-Xyl	0.7	0.4	---	---	---	---	4.1	6.0	1.0	1.0	0.8	1.5	0.5	0.6	---	0.2

L, lamina; M, midrib; ---, not detected.

Table 2.5 Estimated proportions of polysaccharides in the walls and wall fractions from the lamina and midrib of rosette chicory leaves

Polysaccharides	Walls		Wall fractions													
			CDTA		Na_2CO_3		1 M KOH		4 M KOH		4 M KOH + H_3BO_3		Hot water		Residue	
	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M
HG/XGA	49.5	39.4	26.7	28.2	11.1	7.5	0.9	0.3	0.1	0.1	1.0	0.8	9.6	5.7	1.6	1.8
RG I[a]	1.9	2.1	0.4	0.4	1.1	0.5	0.2	0.1	0.0	0.0	0.2	0.1	1.0	0.8	0.3	0.4
Arabinan	5.6	7.8	0.8	1.4	1.5	1.5	0.3	0.2	0.1	0.1	0.2	0.3	2.1	2.9	0.7	1.3
Galactan	9.5	8.4	0.6	0.7	2.5	1.2	0.6	0.1	0.4	0.1	0.5	0.6	4.0	3.5	0.8	1.8
Xyloglucans	6.0	7.6					0.5	0.7	2.8	3.3	0.6	0.7	0.1	0.1	1.5	1.8
Heteroxylans	1.9	3.2	0.0				0.8	1.7	0.4	0.7	0.1	0.3	0.1	0.2	0.1	0.3
Galactoglucomannans	4.5	5.8	0.0	0.0		0.0	0.3	0.5	1.3	1.9	0.3	0.4	0.0	0.0	1.7	1.5
Cellulose	21.1	25.8													20.8	25.6

[a]RG I backbone only (arabinan and galactan not included); L, lamina; M, midrib.

The content of pectic polysaccharides was 67% of the total lamina wall polysaccharides (Table 2.5), with about 50% as homogalacturonan and 17% RG I, whereas in the midrib the total was about 58% (39% HG, 18% RG I). Over half the homogalacturonan was extracted by CDTA, with a uronic acid to Rha ratio in these fractions of about 60:1. Only about 15% of the arabinan and 7% of the galactan were extracted by CDTA. Na_2CO_3 extracted more RG I together with further homogalacturonan, but half of the RG I remained even after all the alkali extractions. About 60-80% of this resistant RG I was extracted by hot water, along with about 80-85% of the remaining uronic acid. Of the total homogalacturonan in the lamina walls, 19% was in the Hot water fraction (14% in the midrib walls). For the RG I backbone, 50% of the total was in the Hot water fraction from the lamina walls (40% from the midrib walls). The corresponding percentages for the arabinan were 33% (lamina walls) and 37% (midrib walls), and for the galactan 39% (lamina walls) and 42% (midrib walls) in the Hot water fraction. The pectic polysaccharides in the Hot water fractions differ structurally from those in the CDTA and Na_2CO_3 fractions. For example, the ratio of Gal/Ara was higher in the Hot water fractions from both lamina and midrib walls.

In the alkali fractions (1 M and 4 M KOH and KOH/borate), the heteroxylan was predominant in the 1 M KOH fractions, whereas the xyloglucans and galactoglucomannans were predominant in the 4 M KOH fractions; the KOH/borate fractions also contain pectic polysaccharides. The Residue contained xyloglucans and galactoglucomannans, but only traces of heteroxylans. Cellulose was confined to the Residue fractions.

2.5 Discussion

2.5.1 Overall compositions of non-lignified primary walls

Although the walls of forage chicory have not previously been analysed in detail, Femenia et al. (1998) studied the walls of chicory grown as a vegetable (witloof). These walls, like those of the rosette leaves of forage chicory, are probably predominantly non-lignified, primary walls; lignified walls make vegetables unpalatable. Femenia et al. (1998) determined the monosaccharide compositions of the polysaccharides in alcohol-insoluble residues (AIR) from the outer leaf, inner bud, upper stem and root of witloof; they also determined the glycosyl-linkage composition of the polysaccharides in the

AIR from roots. Most of the glycosyl linkage types they found in the wall polysaccharides were also present in forage chicory. Femenia et al. (1998) found that the cellulose content of "leaf bud" walls was 24.8% of the total non-starch polysaccharide (NSP), which is similar to the 20.6% and 24.9% found in the present study for the walls of forage chicory lamina and midrib, respectively. The corresponding percentage for pectic polysaccharides in the walls of witloof "leaf bud" was 63.6%, compared with 67% and 59% for the walls of forage chicory lamina and midrib, respectively. However, although Barry (1998) reported that chicory had higher pectin content than other forage species, only 30-33% pectic polysaccharides were in the walls of the total dry matter of forage chicory determined by Kusmartono et al. (1996a). This low percentage is probably because the authors quantified only the ammonium oxalate fraction after extraction of carbohydrates soluble in hot water.

Overall the composition of the non-lignified primary walls of chicory is similar to that of the non-lignified primary walls of other dicotyledons, including forage plants, that have been analysed (O'Neill and York, 2003; Harris, 2005). However, the proportions of pectic polysaccharides, cellulose and other polysaccharides in primary walls do vary widely with species. Because of the complex structures of the pectic polysaccharides and the different methods that have been used to quantify them, comparisons of the proportions of pectic polysaccharides in the walls of different species are not straightforward. However, O'Neill and York (2003) indicated a range of 32-45% (dry weight) for total pectic polysaccharides in the primary walls of dicotyledons. The equivalent percentages for forage chicory walls are ~50% for the lamina (equal to 67% of wall carbohydrate) and ~40% for the midrib, indicating they are at the high end of the range. A similar high percentage of pectic polysaccharides (40.5% of AIR) has been reported for cabbage (*Brassica oleracea*) walls (Stevens and Selvendran, 1980).

The cellulose content of primary walls of vegetative organs of dicotyledons ranges from ~20 to ~40% (Bacic et al., 1988). The cellulose content of forage chicory walls, 20.6% of total polysaccharides in the lamina walls and 24.9% in the midrib walls, is at the low end of this range. The xyloglucan content of primary walls of dicotyledons ranges from <2% in parenchyma walls of celery (*Apium graveolens* L.) (Thimm et al., 2002) to ~21% in the walls of suspension cultured sycamore (*Acer pseudoplatanus*) (Talmadge et al., 1973). The xyloglucan content of the walls of forage chicory is at the low end of this range. The proportion of heteroxylans in forage chicory walls (2-3%) is similar to

that reported for primary walls of cabbage (Stevens and Selvendran, 1980), celery (Thimm et al., 2002) and carrot (*Daucus carota*) (Stevens and Selvendran, 1984). Thus overall, forage chicory leaf walls contain low proportions of cellulose and xyloglucan and relatively high proportions of pectic polysaccharides. This composition is similar to that reported for the walls isolated from cabbage, except that cabbage walls have a lower Gal/Ara ratio (Stevens and Selvendran, 1980).

2.5.2 Lamina vs midrib

The current analyses of leaf walls of forage chicory showed there were differences in the polysaccharide compositions of the walls of the lamina and midrib. The proportions of cellulose and the other three non-pectic polysaccharides in the midrib walls were higher than those in the lamina walls. The ratio of galactans to arabinans in midrib walls was lower than that in lamina walls. These differences in wall composition probably reflect the different cell types in the two structures. Although parenchyma cells predominated in both lamina and midrib, in the midrib, the cells are much larger in cross-section area (~10 times) and have very thin walls (Fig. 2.1A and Fig. 2.1B). In the midrib, cells of the vascular bundles, and especially the xylem tracheary elements with lignified secondary walls, appear to make a much larger contribution to the total cell walls than is the case for lamina. Furthermore, the midrib walls include hypodermal walls which are absent from the lamina.

Table 2.6 The ratio of galactose to arabinose in plant cell wall preparations

Plants	Gal/Ara		Sources
Chicory	1.80 (lamina)	1.21 (midrib)	This study
Witloof chicory	0.78 (outer leaf)	0.42 (upper stem)	Femenia et al., 1998
Lucerne	0.94 (leaf)	0.79 (stem)	Bourquin and Fahey, 1994
Lucerne	1.28 (leaf)	0.95 (stem)	Hornstein et al., 1989
Lucerne	1.32 (young[a])	0.99 (old[b])	Jung and Engels, 2002
Red clover	1.81 (leaf)	1.05 (stem)	Hornstein et al., 1989

[a, b] The seventh internode counting from the base after [a]12 or [b]87 days of regrowth.

The high proportion of galactan to arabinan found in the present study for lamina walls compared with midrib walls has also been reported for leaf compared with stem walls in witloof chicory (Femenia et al., 1998) and in lucerne (*Medicago sativa*) and red clover (*Trifolium pratense*) (Hornstein et al, 1989) (see Table 2.6).

2.5.3 Pectic polysaccharides

The pectic polysaccharides of forage chicory walls are particularly rich in homogalacturonans. The uronic acid to rhamnose ratio of the CDTA fraction was about 60, indicating that this fraction was rich in homogalacturonans. This compares with ratios of 20-50 reported by Ryden and Selvendran (Ryden and Selvendran, 1990) in CDTA fractions from walls of runner bean (*Phaseolus coccineus*) pods. The polysacccharides in the walls of witloof chicory were also reported to have a high ratio of uronic acid to rhamnose, similar to that found in the CDTA fractions in the present study (Femenia et al., 1998). In the present work, small amounts of xylose in the CDTA fraction of chicory may indicate the presence of xylogalacturonan.

In the forage chicory walls, it was found that about 20% of total pectic polysaccharides were in the Hot water and Residue fractions. These pectic polysaccharides, which were resistant to extraction by alkalis, were enriched in RG I and most were soluble in hot water. The uronic acid to rhamnose ratios in the Hot water fractions were 7-10, whereas in the final residue they were about 5. Pectic polysaccharides resistant to extraction by alkalis have been reported in the walls of other species, including the dicotyledons runner beans (Ryden and Selvendran, 1990) and apple (*Malus domestica*) (Oechslin et al., 2003), and the monocotyledon taro (*Colocasia esculenta*) (Quach et al., 2001). These pectic polysaccharides from runner beans and apple were enriched in RG I. Those from runner beans were treated with acid-chlorite, which preferentially solubilized RG I (Ryden and Selvendran, 1990), and those from apples were treated with a crude cellulase, which left RG I (uronic acid to rhamnose ratio of 1) (Quach et al., 2001).

The structure of the RG I varied in different fractions from forage chicory leaf walls. The RG I from the hot water and Residue fractions mostly had a higher gal/ara ratio than did the RG I from other fractions. The "arabinan" and "galactan" are unlikely to be distinct entities, but rather parts of AG-I side chains. This is borne out by the linkage analysis, which showed that the RG I in the Hot water and Residue fractions contained

more 3,5-Ara*f* branch points than t-Ara*f*, and it is probable that t-Gal is an essential part of at least some arabinan side chains. In contrast, the t-Ara*f* content in the CDTA fractions exceeded that of 3,5-Ara*f*. Although 6-Gal was found in the chicory leaf walls, this is unlikely to have arisen from AG-II (Bacic et al., 1988) because very little 3- and 3,6-linked Gal, which are characteristic of these polysaccharides, was present. Femenia et al. (Femenia et al., 1998) also found 6-linked galactose in the wall polysaccharides of witloof chicory root, although in smaller proportions to that in forage chicory leaves (Table 2). Interestingly, 6-Gal was the major galactose linkage reported in pectic polysaccharides from carrot embryogenic and non-embryogenic callus (Kikuchi et al., 1996).

2.5.4 Xyloglucans

The present linkage analyses of the wall polysaccharides of forage chicory leaves indicated that the xyloglucans are fucogalactoxyloglucans, which are the most common type of xyloglucans in the primary walls of dicotyledons. Another type of xyloglucan, arabinoxyloglucans, occurs in the primary walls of species in the families Solanaceae and Oleaceae (O`Neill and York, 2003; Harris, 2005). Interestingly, in a recent classification of the flowering plants based mostly on the nucleotide sequences of genes, these families are placed in the same major group, the asterids, as the Asteraceae, but in a different subgroup (APG, 2003). The Solanaceae and Oleaceae are placed in euasterids I, whereas Asteraceae is in euasterids II. The xyloglucans in another species of Asteraceae, burdock (*Arctium lappa*), have also been found to be fucogalactoxyloglucans (Kato and Watanable, 1993).

2.5.5 Connection of pectins to cellulose

In current structural models of the primary cell wall of dicots (Carpita and Gibeaut, 1993), it is proposed that pectins are non-covalently linked to cellulose. Therefore, pure cellulose should be obtained after extracting pectins and xyloglucan. However, in the present study, significant quantities of pectic substances were left after consecutive extraction with CDTA, Na_2CO_3 and KOH. For homogalacturonan, 22 and 19% remained in the residues of the lamina and midrib, respectively, after extraction, whilst most of the RG I backbone remained (67% in the lamina, 65% in the midrib). Much

arabinan (44% in the lamina, 55% in the midrib) and galactan (48% in the lamina, 65% in the midrib) was not extracted.

Pectin remaining in residues after extensive extraction has been reported for several other species. A residue obtained from cabbage walls after extraction with hot water, oxalate, 1 M KOH and 4 M KOH contained pectins with Ara 46 mg g^{-1}, Gal 32 mg g^{-1} and uronic acid 113 mg g^{-1} (Stevens and Selvendran, 1984). In addition, wall residues containing pectins associated with cellulose have been reported for *Rosa glauca* (suspension-cultured cells) (Chambat and Joseleau, 1980; Chambat et al., 1981, 1984), runner bean (O'Neill and Selvendran, 1983), potato (*Solanum tuberosum*) (Selvendran and Ryden, 1990) and apple (Oechslin et al., 2003). Arabinan- and galactan-containing pectic polymers have also been found in the alkali-insoluble α-cellulose residue from tomato (*Solanum lycopersicum*) fruit (Seymour et al., 1990; Redgwell et al., 1997). Oechslin et al. (2003) obtained pectic polysaccharides using enzymic hydrolysis from the cellulosic residue of apple walls that remained after sequential extraction with CDTA, Na_2CO_3, 1 M NaOH, and 4 M NaOH. These reports all support the existence of cellulose-associated pectic polysaccharides in walls, as suggested by the data from this study.

So, how are pectic substances associated with cellulose? A definitive model of a connection between cellulose and pectins has not been proposed, but several speculative possibilities have been presented.

The first hypothesis is that as "the cellulose fibrils are supported by a cellulose-xyloglucan network, the fibril could collapse after the alkali-extraction of xyloglucans, resulting in an embedding of the pectins in cellulose" (Oechslin et al., 2003). Booten et al. (2004) observed by solid-state NMR that only some xyloglucans in the primary cell walls of mung beans (*Vigna radiata*) interacted with cellulose on the surface of cellulose microfibrils, while most xyloglucans were mobile and most of the surface area of the microfibrils was unoccupied. Thus, it would appear unlikely that alkali extraction could cause fibril collapse.

It is well established that cellulose can interact with xyloglucans via hydrogen bonding (Cosgrove, 2005), and covalent links have recently been found between xyloglucans and pectins (Thompson and Fry, 2000; Abdel-Massih et al., 2003; Brett et al., 2005;

Cumming et al., 2005). So, the second possibility is that pectins are associated with cellulose via xyloglucans, with a complex of cellulose-xyloglucan-pectin possibly present. Indeed, Iwai et al. (2001) found that arabinose-rich pectins in cell walls of *Nicotiana plumbaginifolia* are strongly associated with cellulose-xyloglucan complexes. Since the hydrogen bonds between cellulose and xyloglucans will be extensively broken by strong alkali (Brett and Waldron, 1996), alkali would release xyloglucan and pectins together from such a complex. However, in the present study, pectins in the residues left after extraction with CDTA and Na_2CO_3 were released by alkali in only very small proportions, compared with a much greater release of xyloglucans. Therefore, it is unlikely that xyloglucan provided the link between cellulose and pectin in a cellulose-xyloglucan-pectin complex of chicory cell wall residues after CDTA and Na_2CO_3 extractions.

Given that it appears that a cellulose-xyloglucan-pectin complex is unlikely, an alternative explanation is needed for the fact that in the present study about 25% of the total xyloglucans remained after solubilization with strong alkali, referred to alkali-resistant xyloglucans in this chapter. Therefore, a third hypothesis is proposed.

This hypothesis is that alkali-resistant xyloglucans may be associated with pectins, and alkali-resistant xyloglucans and pectins could be present as a cellulose-pectin-xyloglucan complex, in which pectin provides the link between cellulose and xyloglucan.

A diagram of the postulated structure of a cellulose-pectin-xyloglucan complex is presented in Figure 2.4. It is postulated that when KOH is used for extraction it cleaves hydrogen bonds, removing the free xyloglucan that is not linked to pectins, but not the xyloglucan that is part of the cellulose-pectin-xyloglucan complex, since this xyloglucan is associated with pectins. The alkali would partially release xyloglucans, but would be unable to release pectins from the residues after CDTA and Na_2CO_3 extraction.

Support for this hypothesis comes from the work of Thompson and Fry (2000) who separated neutral and anionic xyloglucans from the walls of suspension-cultured rose (*Rosa* sp., cv. Paul's Scarlet). They confirmed that the anionic xyloglucan was covalently attached to the pectic network through arabinan/galactan of RG I, since a part

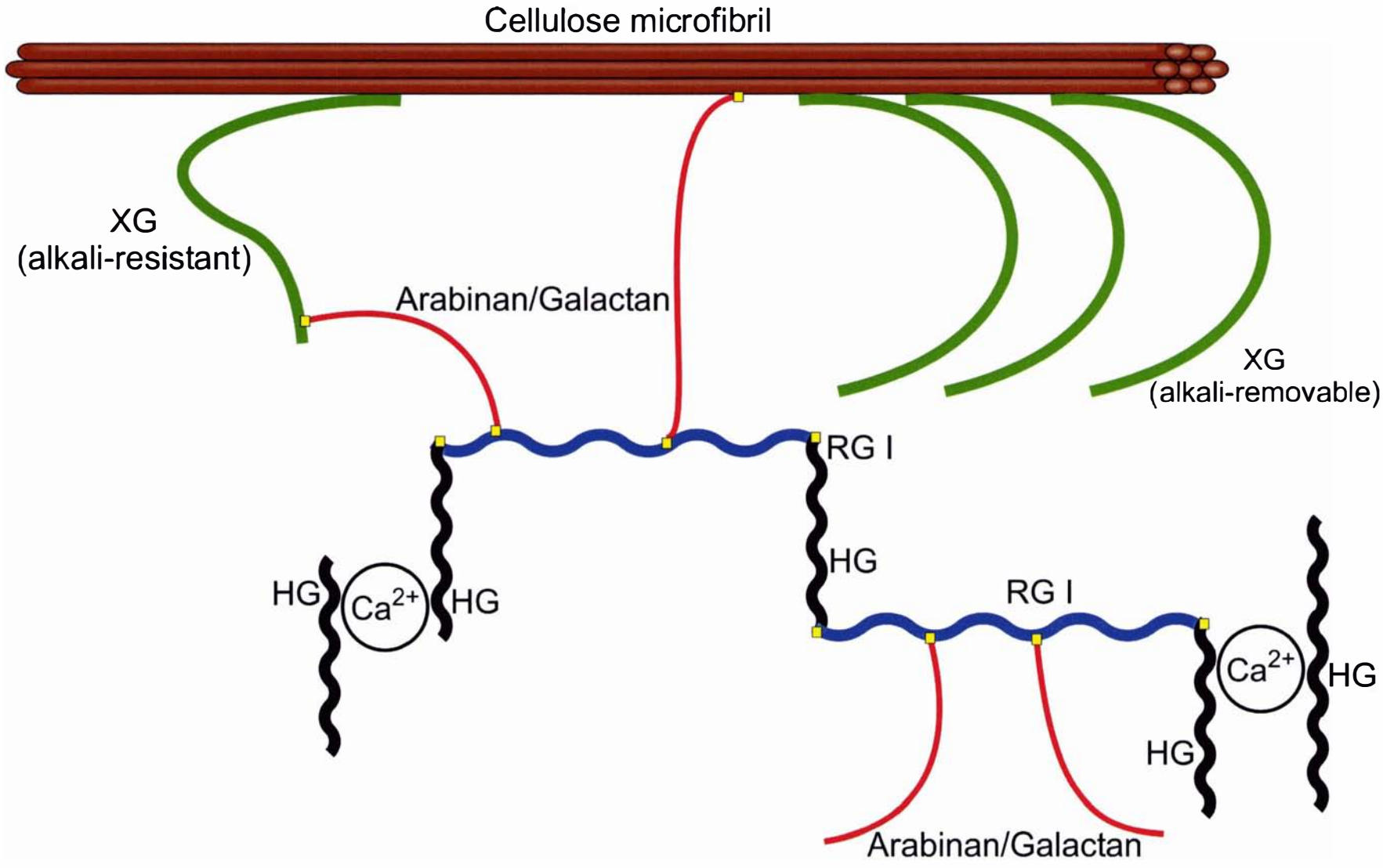


Figure 2.4 A diagram of the putative structure of a cellulose-pectin-xyloglucan complex in chicory cell walls

of the anionic xyloglucan could be converted to neutral material by treatment with endo-polygalacturonase, arabinanase or galactanase. Abdel-Massih et al. (2003) also found that in etiolated pea (*Pisum sativum* L.) epicotyls the newly formed β-(1→4)-D-galactan was present wholly or partially as a complex with xyloglucan, suggesting that the side-chains of RG I could be linked to xyloglucans. In contrast, Cosgrove (2005) suggested HG as a possible candidate for the linkage since acidic residues were found to covalently attach to xyloglucan. In the current study, 76-86% of HG, 67-77% of RG I core, 69-75% of arabinan and 66-83% of galactan, but only about 5% of xyloglucan were released into Hot water fractions from the residues after alkali extraction. This indicates that some of the RG I in alkali is solubilised by hot water without releasing HG, while some remains bound. Thus, less than half of the RG I can be involved in linking XG to cellulose. Similar results were reported in the studies of Quach et al. (2001) and Ratnayake et al. (1999). After alkali extraction, water, at room temperature, solubilised pectin with more uronic acid than Rha, Ara and Gal from the residues of taro cell walls (Quach et al., 2001) and buttercup squash (*Cucurbita maxima*) walls (Ratnayake et al., 1999). These water-extractable pectins may possibly result from β-elimination by alkali.

The above paragraph has discussed the possible linkages between pectins and xyloglucan in the cellulose-pectin-xyloglucan complex. But how are pectins linked to cellulose? A direct covalent binding between them has been proposed (Oechslin et al., 2003; Vignon et al., 2004; Zykwinska et al., 2005), but it is not clear which pectic domains are involved. Oechslin et al. (2003) failed to detect the structural units of RG II from their cellulosic residue from apple cell wall preparations, so they excluded RG II in the association of pectin and cellulose. As RG II is a quantitatively minor polysaccharide, there may have been too little to detect in their study.

From NMR studies on the residue of onion cell walls after extensive extractions with 4M KOH, Foster et al. (1996) suggested that RG I backbones, rather than their side-chains, are strongly associated with cellulose. However, most reports have supported the association of pectins with cellulose via the RG I side-chains galactan or arabinan (Oechslin et al., 2003; Vignon et al., 2004; Zykwinska et al., 2005). Vignon et al. (2004) isolated an arabinan-cellulose complex from the alkali-extracted residue of cactus (*Opuntia ficus-indica*) spine fibres. On the other hand, Oechslin et al. (2003) suggested

that galactans play a role in an interaction between cellulose and pectins. In a recent study by Zykwinska et al. (2005), both arabinans from sugar beet (*Beta vulgaris*) and galactans from potato were observed to bind *in vitro* to cellulose and xyloglucan. The absorption affinity of debranched arabinans to cellulose microfibrils was higher than those of galactans and branched arabinans. In the present study, more galactans than arabinans remained in the residues after extensive alkali extractions. This suggests that both arabinans and galactans are linked to cellulose, with galactans possibly more closely associated than arabinans.

In view of the lack of direct evidence for covalent linkage between pectin and cellulose, it is possible that some pectic polysaccharides remain associated with cellulose because they are physically entangled in the wall.

Chapter 3

Location of pectic polysaccharides in the cell walls of forage chicory (*Cichorium intybus*) leaves using immunolabelling

3.1 Abstract

Pectic polysaccharides are abundant in chicory cell walls, but little is known about their distribution among walls of different cell types/tissues and within walls. Their location was investigated with sections, both fresh and resin-embedded, cut from the laminae and midribs of chicory (*Cichorium intybus* cv. Grasslands Puna II) rosette leaves using immunofluorescence and immunogold labelling with antibodies specific to homogalacturonan with a low proportion of methyl esters (JIM5), homogalacturonan with a high proportion of methyl esters (JIM7), (1→4)-β-D-galactan (LM5) and (1→5)-α-L-arabinan (LM6). Although the JIM7 epitope was more uniformly distributed in the cell walls of chicory leaves than the JIM5 epitope, both were concentrated in the walls of the epidermis, hypodermis and vascular bundles of the midrib and in the walls of the epidermis of the lamina. The JIM5 epitope was concentrated in the middle lamella, at the corners of intercellular spaces and the tricellular junctions, where the JIM7 epitope was less intense, the LM5 absent and the LM6 epitope present only in the middle lamella, not at the corners of intercellular spaces. The JIM5 epitope was more concentrated in the middle lamella of the radial walls than in the middle lamella of the tangential walls of the epidermis. In thin-walled cortical parenchyma and inner phloem cells of the midrib, the LM5 epitope occurred in the wall near the plasma membrane. In thick-walled outer phloem parenchyma, the LM5 labelling was confined to the extreme inner part of the wall adjacent to the plasma membrane. In the epidermal walls of the midrib, the LM5 labelled a zone much wider than in the walls of the phloem parenchyma, but labelling did not extend to the cuticle and labelling intensity increased towards the plasma membrane. In a few mesophyll cells of the lamina and some small phloem parenchyma cells of the midrib, the LM5 epitope was undetectable. The LM5 labelling of the primary walls of the outer phloem fibres of the midrib was particularly intense, whereas labelling with LM6 was not observed. The thick secondary walls of these phloem fibres were not labelled with any of the antibodies used. The walls of the stomatal guard cells of the lamina were rich in the LM6 epitope, but poor in the LM5 epitope. The transfer cell walls in the minor vein of the lamina and their wall ingrowths were labelled with all four antibodies used. The specific location of pectic polysaccharides is discussed in relation to their possible functions.

3.2 Introduction

In Chapter 2, the polysaccharide compositions of the cell walls of rosette chicory leaves were examined for the lamina and midrib, separately. Preliminary histochemical investigation showed that most of the walls were primary and non-lignified. The cell wall polysaccharide compositions were found to be similar to those of primary walls of other eudicotyledons examined, but the walls had a particularly low content of cellulose and a particularly high content of pectic polysaccharides. The abundance of pectic polysaccharides may contribute to the high nutritive value of chicory, as they are readily degraded wall components (Hatfield and Weimer, 1995).

Pectic polysaccharides have a domain structure consisting of homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II) and sometimes a small amount of xylogalacturonan (Willats et al., 2000a; Ridley et al., 2001). HG is primarily an α-(1→4)-linked polymer of galacturonic acid (GalA) residues, which may be methyl esterified (Ishikawa et al., 2000). The degree of methyl esterification has important implications in regard to location in, and function of, the walls. RG I is a branched polymer with a backbone of alternating GalA and rhamnose (Rha) residues, the latter bearing arabinan and/or galactan side-chains (Lau et al., 1985). RG II is a complex polymer involved in linking of chains by way of borate diester bridges (Vidal et al., 2000; O'Neill et al., 2004).

Different polysaccharides are recognised by different specific monoclonal antibodies, which can be used to locate the epitopes within the cell walls. The monoclonal antibodies JIM5 and JIM7 are specific for homogalacturonans that have low and high degrees of methyl esterification, respectively. The epitopes are more precisely described by Willats et al. (2000a) and Clausen et al. (2003). The monoclonal antibodies LM5 and LM6 recognise, respectively, the (1→4)-β-D-galactan and (1→5)-α-L-arabinan side-chains of RG I. LM5 is specific for an epitope consisting of four (1→4)-β-D-galactosyl residues (Jones et al., 1997) and LM6 for an epitope consisting of five (1→5)-α-L-arabinosyl residues (Willats et al., 1998).

Understanding the distribution of pectic polysaccharides is essential for clarification of their biological functions and their degradation by rumen bacteria. The previous chemical studies (Chapter 2) of cell-wall polysaccharide compositions using isolated

cell walls of forage chicory leaves gave no information about the distribution of the different polysaccharides in the walls of the various cell types or about their location within particular walls. Therefore the aim of the current study was to extend the previous work by using immunofluorescence and immunogold labelling to determine the distribution of HG and RG I in the walls of different cell types and within specific walls. In particular, the location of HG with various degrees of esterification was determined using the monoclonal antibodies JIM5 and JIM7, and the location of (1→4)-β-D-galactan and (1→5)-α-L-arabinan side chains of RG I was determined using LM5 and LM6.

3.3 Material and methods

3.3.1 Plant material

Plants of forage chicory (*Cichorium intybus* L. cv. Puna II) (Rumball et al., 2003b) were grown as described in Chapter 2.

3.3.2 Monoclonal antibodies

The monoclonal antibodies JIM 5 and JIM 7 were kindly provided by Professor K Roberts, John Innes Centre, Norwich, UK, and LM5 and LM6 were provided by Professor JP Knox, Leeds University, UK.

3.3.3 Fixation and embedding

Transverse segments (0.5-1 mm wide) were cut midway along the midrib harvested from the three youngest, fully-expanded, but actively growing leaves. Segments (0.5-1 mm wide, 5-8 mm long) were also taken from the lamina midway along the leaf half-way between the midrib and the edge of the leaf. The segments were fixed and embedded using the procedure described by Trethewey and Harris (2002). Briefly, the samples were cut and fixed in a freshly-prepared solution containing 2% (w/v) paraformaldehyde and 1% (w/v) glutaraldehyde in 100 mM NaOH-PIPES (sodium hydroxide-piperazine-1,4-bis [2-ethanesulfonic acid]) buffer (pH 7.2) for 4 h at room temperature, followed by five washings in the buffer solution without fixative and then postfixed with 1% (w/v) osmium tetroxide in 50 mM NaOH-PIPES buffer (pH 7.2) for

1 h at room temperature. The segments were then washed five times in 50 mM NaOH-PIPES buffer solution without the fixative, and dehydrated using a graded ethanol series (30, 60, 90, 95, 100%), leaving them for 10 min in each solution. Prior to infiltration, the segments were left in 100% ethanol for 1 h for further dehydration. The segments were infiltrated with LR White Resin (London Resin G Ltd, Basingstoke, UK) in a lid-opened bottle using first a mixture of ethanol and resin (1:1 v/v, 16 h) and then 3 changes (12 h each) of pure resin. Mixing was done by rotating the containers. Finally, the segments were embedded using fresh resin in a pre-dried gelatin capsule at 60°C for 2 days.

3.3.4 Light microscopy and immunofluorescence labelling

Semithin sections (0.5 μm thick) of leaf midrib and lamina embedded in resin were cut with a diamond knife using an ultramicrotome (Model Ultracut E, Reichert-Jung), collected on a slide coated with polylysine, and dried at 60°C.

For bright-field light microscopy, sections were stained with Toluidine Blue (0.05% w/v) in 100 mM sodium phosphate buffer (pH 7.2) for 2 min, washed in water, mounted in DPX (BDH Laboratory Supplies, Poole, UK), and examined using a compound microscope (Olympus BX51, Japan) fitted with a 100-W halogen quartz lamp. Photomicrographs were taken with a digital microscope camera (Optronics, Olympus U-TV 0.5XC, Japan).

For immunofluorescence labelling, sections were washed in 10 mM phosphate buffered saline (PBS) buffer (pH 7.2, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl) for 2 min. Non-specific binding sites were blocked by incubating the sections with 1% (w/v) bovine serum albumin (BSA) (Fraction V, Sigma, USA) in PBS buffer (BSA/PBS) for 20 min. After washing three times with PBS, the sections were incubated with primary antibodies. JIM5, JIM7, LM5 and LM6 were diluted 10-, 10-, 4- and 4-fold, respectively, in BSA/PBS before use. Sections were incubated with JIM5, JIM7 and LM5 for 2 h at 20°C, and with LM6 for 16 h at 4°C in a humidity chamber, an anatomy dissection tray (20 cm × 10 cm × 5 cm deep) with tissue paper moistened with distilled water on the bottom, glass rods to support the slides and a sheet of Perspex as the lid. The sections were washed again with 3 changes of PBS, 1 min per change. They were then incubated with the secondary antibody, biotinylated anti-rat IgG (Amersham

International plc, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK), for 1 h and subsequently in streptavidin fluorescein isothiocyanate (FITC) (Amersham Life Science) for 30 min. Both anti-rat IgG and streptavidin fluorescein isothiocyanate were used at dilutions of 1/200 in BSA/PBS. After washing in PBS 3 times and in tap water once, sections were mounted in Vectashield mounting medium for fluorescence microscopy (Vector Laboratories, Burlingame, CA 94010, USA) and examined with a fluorescence microscope (Olympus, Japan) fitted with a BP 460-490 nm excitation filter, a DM 505 nm chromatic beam splitter, and a BA 510-550 nm barrier filter. Controls were done in which incubation with the primary antibodies and the secondary antibody was omitted.

Fresh, transverse hand-cut sections were prepared from the three youngest, fully-expanded but actively growing leaves by cutting with a razor blade and mounting directly in water. Sections were taken from midway along the midrib and from the lamina half-way between the midrib and the edge of the leaf. Sections were labelled with antibody as described for semithin sections, except that incubation with primary antibody LM6 was for 2 h at 20°C.

3.3.5 Immunogold labelling

Ultrathin sections (70-100 nm thick) were cut with a diamond knife using an ultramicrotome and collected on Formvar/carbon-coated or uncoated nickel grids (150 mesh). The sections were washed with PBS-T buffer (pH 7.4, 16mM Na_2HPO_4, 4 mM KH_2PO_4, 0.1% (w/v) BSA, 0.1% (v/v) Tween 20, 150 mM NaCl and 15 mM NaN_3, filtered through a 0.22 μm pore-size filter), then preincubated for 30 min at room temperature with PBS-T buffer containing 1% (w/v) BSA (BSA/PBS-T) to block nonspecific binding sites. The sections were then incubated at 4°C overnight with the primary antibodies (dilution 1:10, 1:10, 1:4 and 1:4 (v/v) for JIM5, JIM7, LM5 and LM6, respectively). After washing with PBS-T (5 times, 2 min for each wash), the sections were incubated for 2 h at 20°C with goat anti-rat IgG (H & L) conjugated to 1 nm colloidal gold (Electron Microscopy Sciences, Fort Washington, PA, USA) at 1:50 dilution in BSA/PBS-T. The sections were then washed with PBS-T and water (5 times with PBS-T, 5 times with water, 2 min for each wash). After drying, the sections were silver enhanced with silver enhancement kit (BBInternational Ltd, UK) for 3 min in the dark at 20°C, followed by washing with water 3 times for 1 min each time in darkness

with a weak red safelight. Section washing was carried out by running water from a wash bottle through grid-held shaking forceps. After drying, the sections were examined using a transmission electron microscope (Philips TEM 201C, Eindhoven, The Netherlands) with operating voltage 60 KV.

Controls were done by either omitting the incubation with the primary monoclonal antibody or by omitting the incubation with the secondary antibody.

3.4 Results

3.4.1 Immunolabelling with JIM5

Immunofluorescence labelling of hand-cut sections

In transverse, fresh, hand-cut sections from the midrib (Fig. 3.1F, 3.1E), JIM5 bound strongly to the walls at tricellular junctions, the corners of intercellular spaces and the outer walls of cells facing these spaces. However, JIM5 bound only weakly or not at all with the middle lamella regions of most cells (including cortical parenchyma, phloem parenchyma and hypodermis) and showed only spasmodic labelling of the radial walls of epidermal and hypodermal cells. It also labelled the most inner parts of cell walls of cortical parenchyma (double arrow, Fig. 3.1F). The outer tangential wall of the epidermis of the midrib showed a narrow zone of fluorescence, which dipped inward toward the radial walls between cells (single arrow, Fig. 3.1F).

Immunofluorescence labelling of resin-embedded sections

In the midrib, with thin sections of fixed, resin-embedded tissue, JIM5 labelling was intense in the walls of the epidermis, hypodermis and vascular bundles, but weak in the walls of the cortical parenchyma (Fig. 3.1A). The labelling of the walls of the cortical parenchyma was more intense in the outermost layer adjacent to the hypodermis and in the ring of cells immediately surrounding each vascular bundle. Labelling with JIM5 was again intense at the corners of intercellular spaces (Fig. 3.1H), but was also strong for the walls of cells in most tissues and the outer wall of the epidermis (Fig. 3.1A). Labelling was confined to primary walls and middle lamellae and was not observed in the secondary walls of the xylem, although it did occur in the middle lamella between adjacent vessels (Fig. 3.1D). In vascular bundles, the thick primary walls of the xylem

and phloem parenchyma were labelled throughout, although more intensely in the walls of the phloem (Fig. 3.1G).

In the lamina, the JIM5 epitope was mainly located in the outer walls of the epidermis (Fig. 3.1B, 3.1C). Labelling was somewhat less intense in the guard cell walls than in the neighbouring epidermal cell walls (Fig. 3.1C), but was intense in the outermost regions of the cell walls and throughout the outer ledge. Compared to the epidermal walls, the labelling of the mesophyll walls was much weaker (Fig. 3.1C). As with the walls of the midrib parenchyma, the mesophyll walls were labelled most intensely at the cell corners.

Immunogold labelling

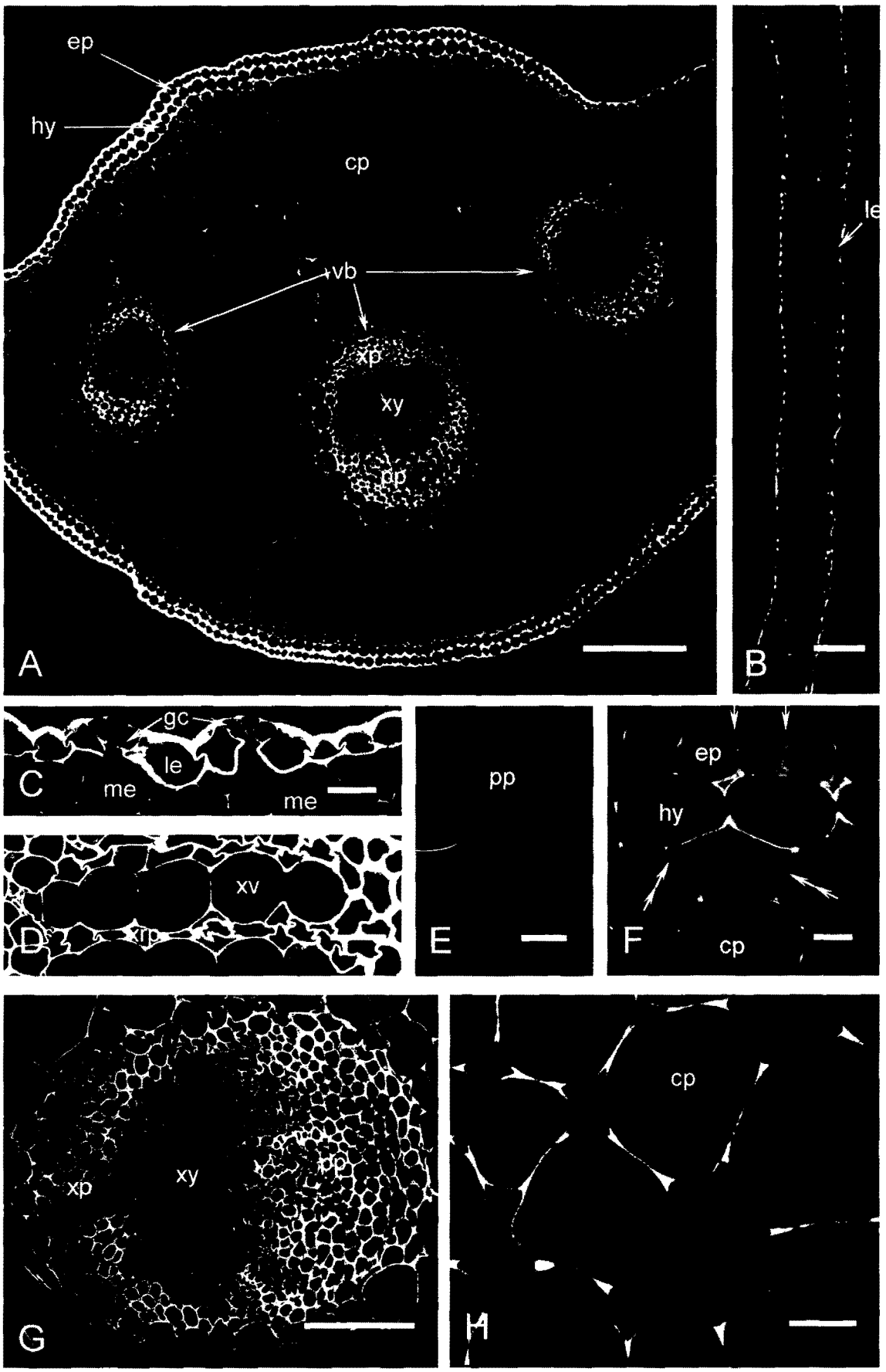
Immunogold labelling of embedded sections from the midrib gave similar results to immunofluorescence. The labelling was intense in the middle lamella region of most cell types (Fig. 3.1K to 3.1M) and cell walls adjacent to intercellular spaces (Fig. 3.1M). The labelling was often more intense at the corners of the intercellular spaces (Fig. 3.1M). The middle lamella region of the radial walls of the epidermis and hypodermis also labelled conspicuously, but the labelling of the contiguous tangential walls of the epidermis and hypodermis was more diffuse, spreading unevenly throughout the walls (Fig. 3.1K). The labelling was less in the middle lamella region and more intense in the region closer to the cytoplasm in the tangential walls. Labelling was particularly intense throughout the outer walls of the epidermis, with a definite narrow zone of higher intensity immediately adjacent to the cuticle (Fig. 3.1K).

In the lamina, the JIM5 epitope was present in all mesophyll cell walls, where it was concentrated in the middle lamella and at all the corners (Fig. 3.1J). Labelling was most intense in the outer walls of the epidermis (Fig. 3.1I), especially in the outer half (Fig. 3.1N) and where the wall thickened next to the radial walls. In guard cell walls (Fig. 3.1I), labelling was intense in the outermost regions of the cell walls and throughout the outer ledge, but absent from the cuticle. In minor veins of the lamina, labelling was most intense at the tricellular junctions among sieve cells and companion cells (transfer cells), but labelling extended throughout the middle lamella region of sieve cell and transfer cell walls, as well as the wall ingrowths of the latter (Fig. 3.1L).

Figure 3.1 Micrographs of transverse sections from the midrib and lamina of 8-week-old rosette chicory leaves labelled with the JIM5 antibody. Fluorescence micrographs (A, B, C, D, E, F, G, H). Transmission electron micrographs (I, J, K, L, M, N). Micrographs from the lamina (B, C, I, J, L, N), from the midrib (A, D, E, F, G, H, K, M). (E, F) Midrib sections cut from fresh chicory, others cut from resin-embedded chicory. (A) Midrib, showing that labelling was in all primary cell walls, but mainly in epidermis and hypodermis, and vascular bundle, xylem and phloem parenchyma cells. (B) Lamina, showing the labelling was mainly in outer walls of epidermis. (C, I) Guard cells, showing staining in guard cell walls was lighter than in neighbouring epidermis walls. (D) Xylem, showing the middle lamella and the primary walls between two xylem vessels were stained. (E) Phloem collenchyma, showing that the labelling was heavy at the corners of intercellular spaces and the outer walls of cells facing these spaces were heavily stained, but weakly or not at all with the middle lamella regions of most cells. (F) Epidermis, hypodermis and cortical parenchyma. (G) Vascular tissue, all parenchyma cell walls of phloem and xylem, including the corners of conjunction among several cells were labelled. (H, M) Parenchyma, JIM5 epitope was present in the cortical parenchyma everywhere (H), but particularly more concentrated at the corners of intercellular spaces (M). (J) Mesophyll, JIM5 epitope was present in all mesophyll cell walls with more in adhesive area. (K) Epidermis and hypodermis of midrib, the middle region of walls between two epidermis cells or between two hypodermis were heavily stained. (L) Lamina vein, showing wall ingrowths stained. (N) Lamina epidermis, JIM5 epitope gradually increased from inner to outer face of outer wall.

cp, cortical parenchyma; ep, epidermis; gc, guard cell; hy, hypodermis; le, lamina epidermis; me, mesophyll; pp, phloem parenchyma; sc, sieve cell; tc, transfer cell; vb, vascular bundle; wi, wall ingrowths; xp, xylem parenchyma; xrp, xylem ray parenchyma; xv, xylem vessel; xy, xylem.

Bars, (A), 200 μm; (B, G), 100 μm; (C, D, E, F, H), 20 μm; (C, I, J, K, L, M, N), 5 μm.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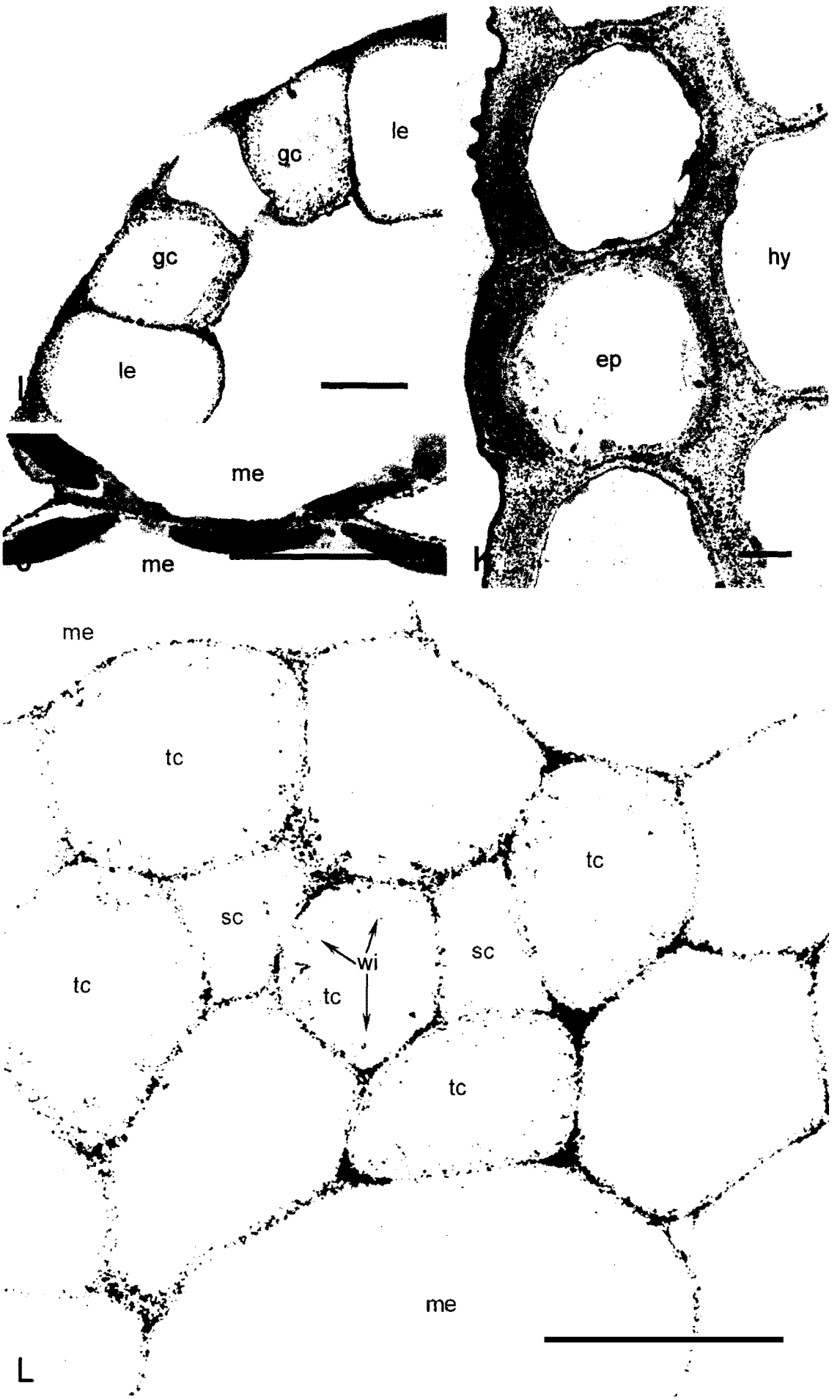
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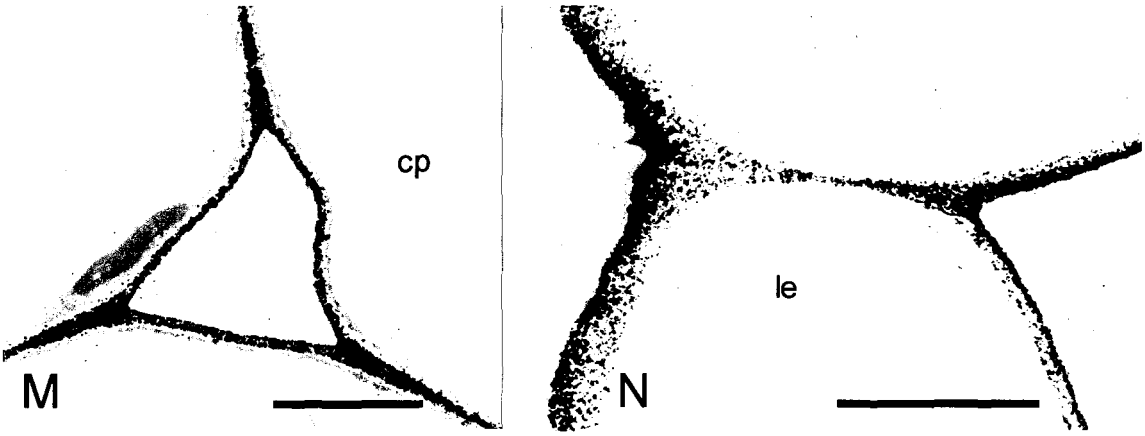
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3.4.2 Immunolabelling with JIM7

Immunofluorescence labelling of hand-cut sections

In transverse fresh, hand-cut sections from the midrib, JIM7 labelling occurred on most primary walls, but often gave a bright region along the extreme outer and inner faces of the walls (Fig. 3.2D). The fluorescence along the outer face of the epidermis was brighter than autofluorescence observed in the control.

Immunofluorescence labelling of resin-embedded sections

The JIM7 epitope was present in the primary walls of all cell types, but was especially intense in the walls of the epidermis (Fig. 3.2A. Fig. 3.2C). The JIM7 epitope was more uniformly distributed among the walls of the different cell types than the JIM5 epitope. In the cortical parenchyma, the labelling was usually uniform throughout the primary wall, but was less intense in the middle lamella (Fig. 3.2B). The intensity was slightly greater at tricellular junctions and corners of intercellular spaces (Fig. 3.2B), but this concentration was lower than with JIM5 (Fig. 3.1H).

Immunogold labelling

The epidermal cell walls of the midrib were heavily and almost uniformly stained. Staining of the outer wall of the epidermis was less intense opposite the radial walls (Fig. 3.2N). In contrast to the labelling with JIM5, the labelling of the walls of the cortical parenchyma cells of the midrib appeared uniform throughout. The JIM7 epitope was distributed throughout the walls but tended to be more intense away from the middle lamellae and the corners of the intercellular spaces (Fig. 3.2M). A similar pattern was observed for the labelling of the walls of the phloem parenchyma of the midrib (Fig. 3.2H). Labelling was absent from the secondary walls of xylem vessels (Fig. 3.2I) and phloem fibres (Fig. 3.2E).

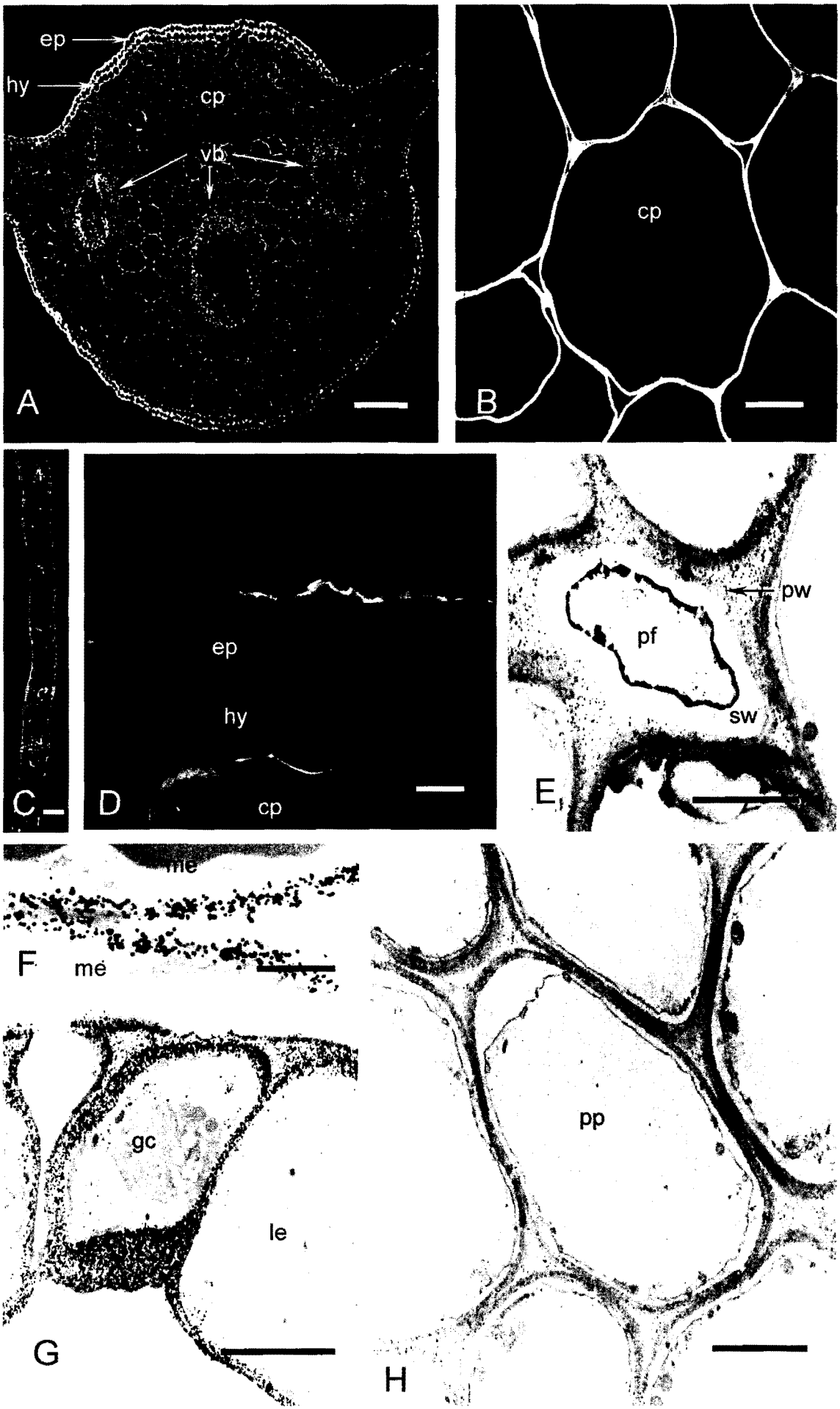
In contrast to the labelling in the lamina with JIM5, JIM7 labelling was dense and fairly uniformly distributed over the lamina epidermal walls (Fig. 3.2C), but in the outer wall it was less dense opposite the conjunction of two cells (Fig. 3.2J). Guard cell walls exhibited a dense, but non-uniform labelling with JIM7, with the epitope also present in the cuticular layer (Fig. 3.2G).

In phloem cells of the lamina, the JIM7 epitope was distributed throughout the walls of sieve cells and transfer cells as well as in the wall ingrowths of the latter (Fig. 3.2K, 3.2L).

Figure 3.2 Micrographs of transverse sections from the lamina and midrib of 8-week-old rosette chicory leaves labelled with the JIM7 antibody. Fluorescence micrographs (A, B, C, D). Transmission electron micrographs (E, F, G, H, I, J, K, L, M, N). Micrographs from the lamina (C, F, G, J, K L), from the midrib (A, B, D, E, H, I, M, N). (A) Midrib, JIM7 epitope was present in walls of all cell types also except xylem vessel secondary walls, especially strong in epidermis, indicating the presence of high-esterified HG throughout all walls. (B, M) parenchyma, JIM7 epitope was distributed throughout the walls in the cortical parenchyma (B), including in the middle lamella and intercellular spaces (M). (C) Lamina, JIM7 epitope mainly in epidermis. (D) Epidermis and hypodermis in midrib section cut from fresh chicory. (E) Phloem fibre cells, showing JIM7 epitope was present in whole walls as they were in other walls. (F) Mesophyll cells, JIM7 epitope did not accumulate at the corners of intercellular spaces. (G) Guard cells, JIM7 epitope was present in guard cell walls, slightly less in the wall facing towards another guard cell. (H) Vascular tissue, JIM7 epitope was present in phloem parenchyma walls, but only weakly in the corners among three cells. (I) Middle lamella between two xylem vessels was stained with JIM7. (J) Lamina epidermis, JIM7 epitope evenly distributed in epidermis walls. In outer wall in the conjunction of two epidermis cells JIM7 tend to be less heavily stained. (K) Transfer cells in lamina vein, showing transfer cell walls and wall ingrowths stained. (L) Lamina vein. (N) Epidermis and hypodermis, Epidermis walls were heavily and evenly stained with JIM7.

cp, cortical parenchyma; ep, epidermis; gc, guard cell; hy, hypodermis; le, lamina epidermis; me, mesophyll; pf, phloem fibre; pp, phloem parenchyma; sc, sieve cell; tc, transfer cell; vb, vascular bundle; wi, wall ingrowths; xp, xylem parenchyma; xrp, xylem ray parenchyma; xv, xylem vessel; xy, xylem.

Bars, (C), 500 μm; (A), 200 μm; (B, D), 20 μm; (E, G, H, I , L, M, N), 5 μm; (F, J, K) 1 μm.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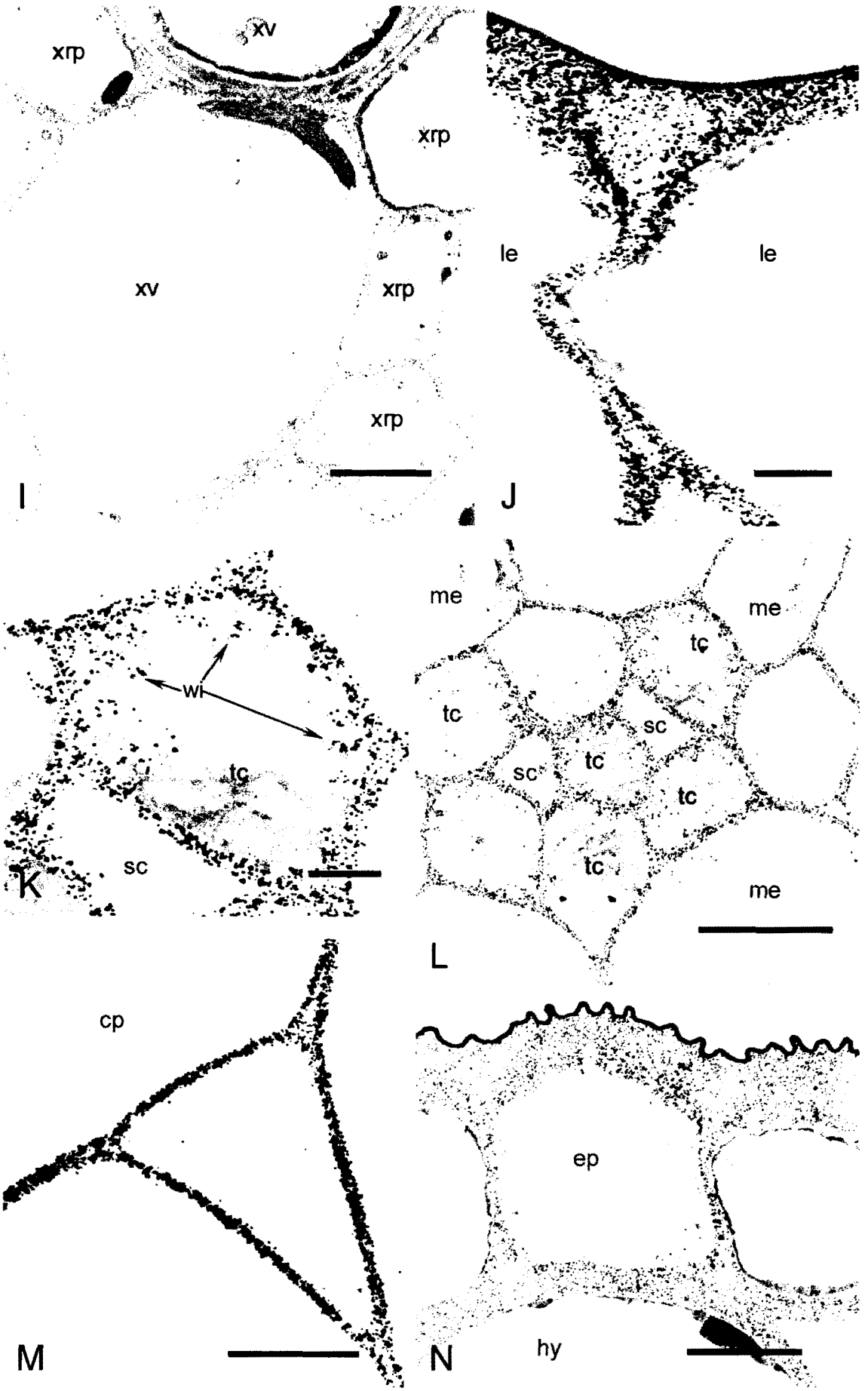
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3.4.3 Immunolabelling with LM5

Immunofluorescence labelling of hand-cut sections

In transverse, fresh, hand-cut sections from the midrib, the LM5 label was largely confined to the inner faces of primary cell walls (Fig. 3.3I), and a dense labelling in a broken ring of outer phloem fibres (Fig. 3.3G).

Immunofluorescence labelling of resin-embedded sections

The LM5 epitope was present in the primary walls of most cell types in the midrib (Fig. 3.3A), with the greatest intensity of labelling over the walls of the epidermis, hypodermis and outer phloem fibres (Fig. 3.3H), whereas the walls of the cortical parenchyma and vascular bundles had relatively uniform labelling (Fig. 3.3A). LM5 labelling of the walls between the contiguous cells generally took the form of a double line, with a gap down the middle lamella region (arrows in Fig. 3.3B, 3.3F), in contrast to the single line with JIM5. There was no labelling at corners of intercellular spaces (Fig. 3.3B). In the phloem parenchyma of the midrib, the LM5 labelling was confined to the extreme inner part of the walls adjacent to the plasma membrane (Fig. 3.3H). In the cortical parenchyma of the midrib, regions at the corners of cell junctions were seen where no LM5 epitope was present (arrows in Fig. 3.3F). The labelling was not observed in the pit fields (double arrows in fig. 3.3F).

In the lamina, the LM5 epitope was present in the walls of most cells, but labelling was most intense in the outer epidermal walls, and was largely absent from the walls of the guard cells (Fig. 3.3C). In the veins of the lamina, intense LM5 labelling was present in the walls and wall ingrowths of transfer cells (Fig. 3.3E, 3.3D).

Immunogold labelling

Immunogold labelling showed the LM5 epitope concentrated in the regions of wall near the cytoplasm of most cell types, including the cortical parenchyma (Fig. 3.3O), phloem (Fig. 3.3M) and xylem parenchyma (Fig. 3.3S), and xylem ray parenchyma (Fig. 3.3P shows a thin line of antibody on the edge of the narrow unstained wall, next to the dark-staining cytoplasm). However, the LM5 epitope was absent from the middle lamella region and corners of intercellular spaces. There was a gradation of intensity of labelling

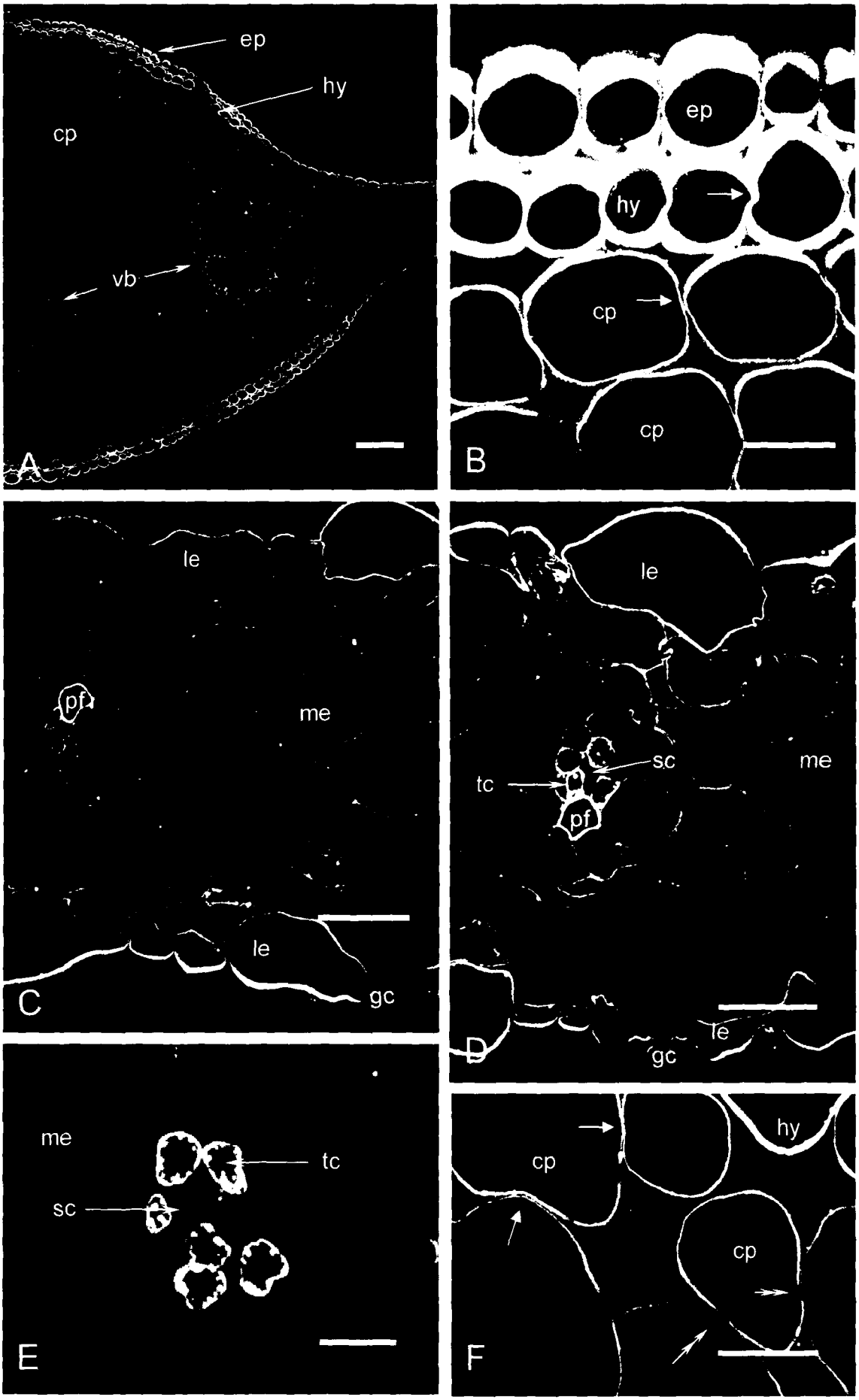
in the outer epidermal walls increasing from the cuticle to the plasma membrane (Fig. 3.3K). In the parenchyma, the labelling generally took the form of two parallel lines are the walls of adjacent cells (Fig. 3.3R, 3.3Q). In the phloem, a broken ring of outer fibre cells showed very dense labelling in their primary walls (Fig. 3.3J) adjacent to the plasma membrane. These cells had thick secondary walls that were not labelled. In the thick walls of the outer phloem parenchyma, the LM5 epitope was confined to the extreme inner edge of the wall adjacent to the plasma membrane (Fig. 3.3Q). Most inner phloem cell walls, including those of sieve cells and transfer cells showed strong labelling, largely confined to the inner wall, but walls of some (mostly smaller) inner phloem cells did not react (Fig. 3.3M). In the xylem, LM5 epitope was found in the primary walls of tracheary elements, but was sparse in the secondary walls (Fig. 3.3P). It was usually present in walls of immature vessels retaining some cytoplasmic content, but was absent altogether from walls of some more mature vessels. In the cortical parenchyma (Fig. 3.3R) and inner phloem (Fig. 3.3M), pit fields (double arrows) were observed in regions of cell contact with plasmodesmata (Fig. 3.3M), and these always lacked the LM5 epitope.

In the lamina, immunogold labelling showed the LM5 epitope was less dense in guard cell walls than in those of the adjacent epidermal cells (Fig. 3.3L). Mesophyll walls were generally uniformly labelled with LM5, but a few lacked the epitope (Fig. 3.3N).

Figure 3.3 Micrographs of transverse sections from the lamina and midrib of 8-week-old rosette chicory labelled with the LM5 antibody. Fluorescence micrographs (A, B, C, D, E, F, G, H, I). Transmission electron micrographs (J, K, L, M, O, P, Q, R, S). Micrographs from the lamina (C, D, E, L, N), from the midrib (A, M, F, G, H, I, K, M, O, P, Q, R, S). (A, B) Midrib, LM5 epitope was present in primary walls of most cell types (A), mainly located in epidermis and hypodermis (B). (C) Lamina, except guard cells, the walls of most cells were labelled. (D) Lamina with a minor vein. (E) Lamina vein, showing transfer cell walls and their wall ingrowths strongly labelled. (F, O) Cortical parenchyma, LM5 epitope was present in the primary walls, but was absent from the middle lamella and the corners of intercellular spaces. (G, H) Phloem fibre cells, LM5 epitope present in phloem fibre cell walls. (I) Fresh, hand-cut midrib section, showing labelling on walls of epidermis, hypodermis and cortical parenchyma. (J) Phloem fibre cells, showing that the LM5 labelling was heavy and in a narrow region of the primary walls. (K) Epidermis in midrib, showing that LM5 epitope was gradually increased concentration from cuticle to plasma membrane in the outer epidermis wall of midrib, but was largely from in the middle lamella. (L) Guard cells, showing absence of LM5 epitope in the walls compared with abundance in adjacent epidermal cell walls. (M) Phloem parenchyma, LM5 epitope was absent from walls of some small phloem parenchyma cells. (N) Mesophyll, walls were stained with LM5, but a few were devoid of stain. (P) Xylem, LM5 epitope occurred in the primary walls of some xylem vessels, not all. (Q) Outer phloem parenchyma, showing thick walls, with labelling confined the region near plasma membrane. (R) Cortical parenchyma, LM5 epitope absent from pit field. (S) Xylem parenchyma, showing the same pattern of LM5 epitope location as cortical parenchyma.

cp, cortical parenchyma; ep, epidermis; gc, guard cell; hy, hypodermis; le, lamina epidermis; me, mesophyll; pp, phloem parenchyma; sc, sieve cell; tc, transfer cell; vb, vascular bundle; wi, wall ingrowth; xp, xylem parenchyma; xrp, xylem ray parenchyma; xv, xylem vessel; xy, xylem.

Bars, (A), 100 μm; (B, C, D, F, G, H, I), 20 μm; (E, J, K, L, M, N, O, P, S), 5 μm; (Q, R), 1 μm. ⟶ A gap in the middle lamella region, ➙ pit field; ▸ labelling absent.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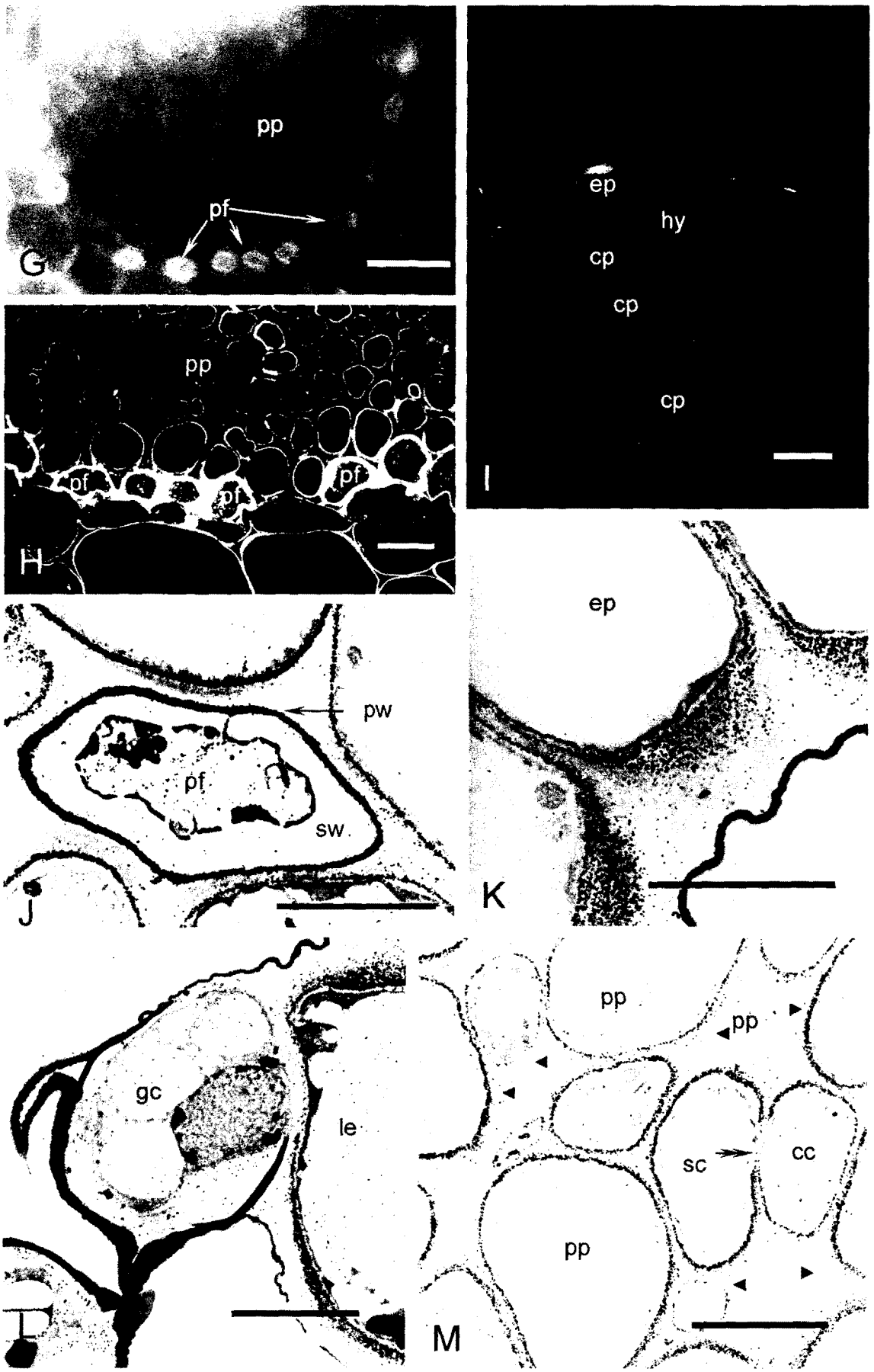
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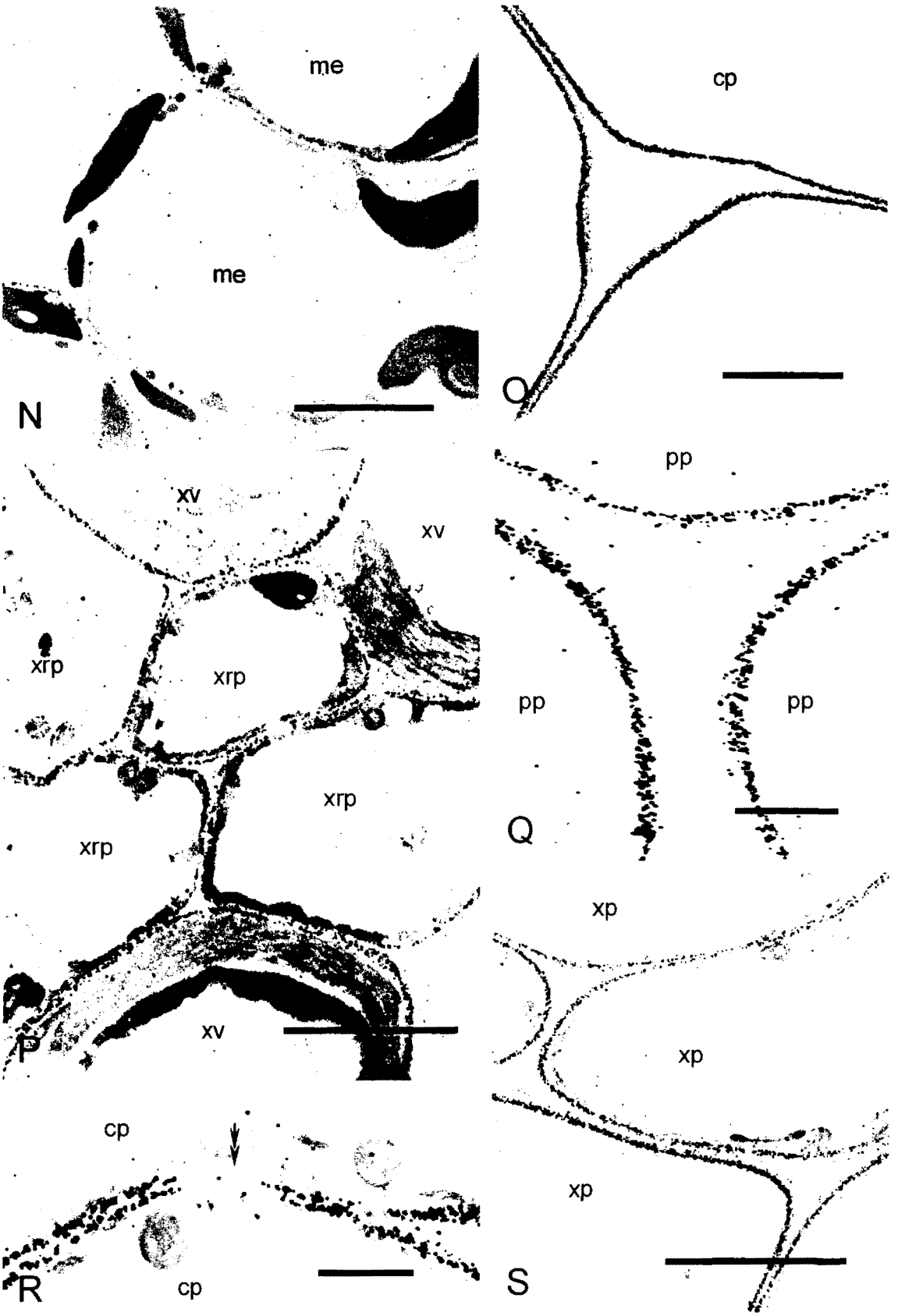
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3.4.4 Immunolabelling with LM6

Immunofluorescence labelling of hand-cut sections

In fresh, transverse, hand-cut sections, LM6 labelling was similar to that of JIM5, with a strong concentration on corners of intercellular spaces and the outer walls of cells facing these spaces. This was especially noticeable in outer phloem parenchyma (Fig. 3.4H).

Immunofluorescence labelling of resin-embedded sections

In embedded sections of midrib (Fig. 3.4A), LM6 labelling was most intense in the epidermis and phloem tissue. It was also moderately intense in the thick walls of parenchyma cells in the outer xylem (cf Fig. 3.4F, showing fresh section). Labelling of cortical parenchyma walls was more intense in the outer regions of the midrib. Inner cortical parenchyma and xylem parenchyma were only weakly labelled. In contrast to LM5, LM6 showed relatively weak labelling of the hypodermal walls (Fig. 3.4B). In addition to the labelling at intercellular spaces that was prominent in fresh sections, the epitope was present in the middle lamella. The fluorescence formed a single line between cortical parenchyma cells, not a double line as with LM5. In the xylem, the LM6 epitope continued along the middle lamella between vessels (Fig. 3.4D). LM6 labelling was particularly strong in the thick walls of outer phloem parenchyma (Fig. 3.4G) and the thinner walls of some inner phloem parenchyma. These latter are probably companion cells as evidenced by their size and distribution within the inner phloem, their cell contents and their wall epitopes compared with those of the leaf minor vein companion cells. The epitope was also found as inclusions in many inner phloem cells (Fig. 3.4E) and in epidermal cells (Fig. 3.4B). The walls of the phloem fibres that labelled with LM5 labelled only weakly with LM6 (Fig. 3.4G, cf Fig. 3.3H).

In the lamina, the most intensely stained cell walls were the outer walls of epidermal cells and stomatal guard cell walls, as well as those of phloem transfer cells (Fig. 3.4I). In addition to cell walls, the LM6 epitope was found in cytoplasmic inclusions in a number of cell types such as epidermis and phloem parechyma. It was also found in the wall ingrowths of transfer cells in the minor veins of laminae (Fig. 3.4I, 3.4J).

Immunogold labelling

Immunogold labelling showed that the LM6 epitope was evenly distributed in epidermis walls of the midrib (Fig. 3.4N). In the cortical parenchyma, it was present in the middle lamella, but, unlike the JIM5 epitope, was not specifically concentrated in the corners of intercellular spaces (Fig. 3.4P). In the inner phloem parenchyma (Fig. 3.4K), some cell walls were heavily stained with LM6. Many cells also had LM6-reactive internal membranes (Fig. 3.4N, 3.4K).

In the lamina, the LM6 epitope was found in the outer face of spongy mesophyll walls, but in some cell walls a second line of epitope was found along the plasma membrane (Fig. 3.4O). In the outer wall of the epidermis, LM6 labelling was dense, except at the corner of the radial walls (Fig. 3.4M). Dense LM6 labelling also occurred in stomatal guard cell walls (Fig. 3.4L). In minor vein phloem cells, the transfer cell walls and wall ingrowths were densely labelled, but walls of other cell types were much more sparsely labelled with LM6(Fig. 3.4Q).

3.4.5 Specificity of labelling

No labelling was observed when the primary antibody or the secondary antibody was omitted.

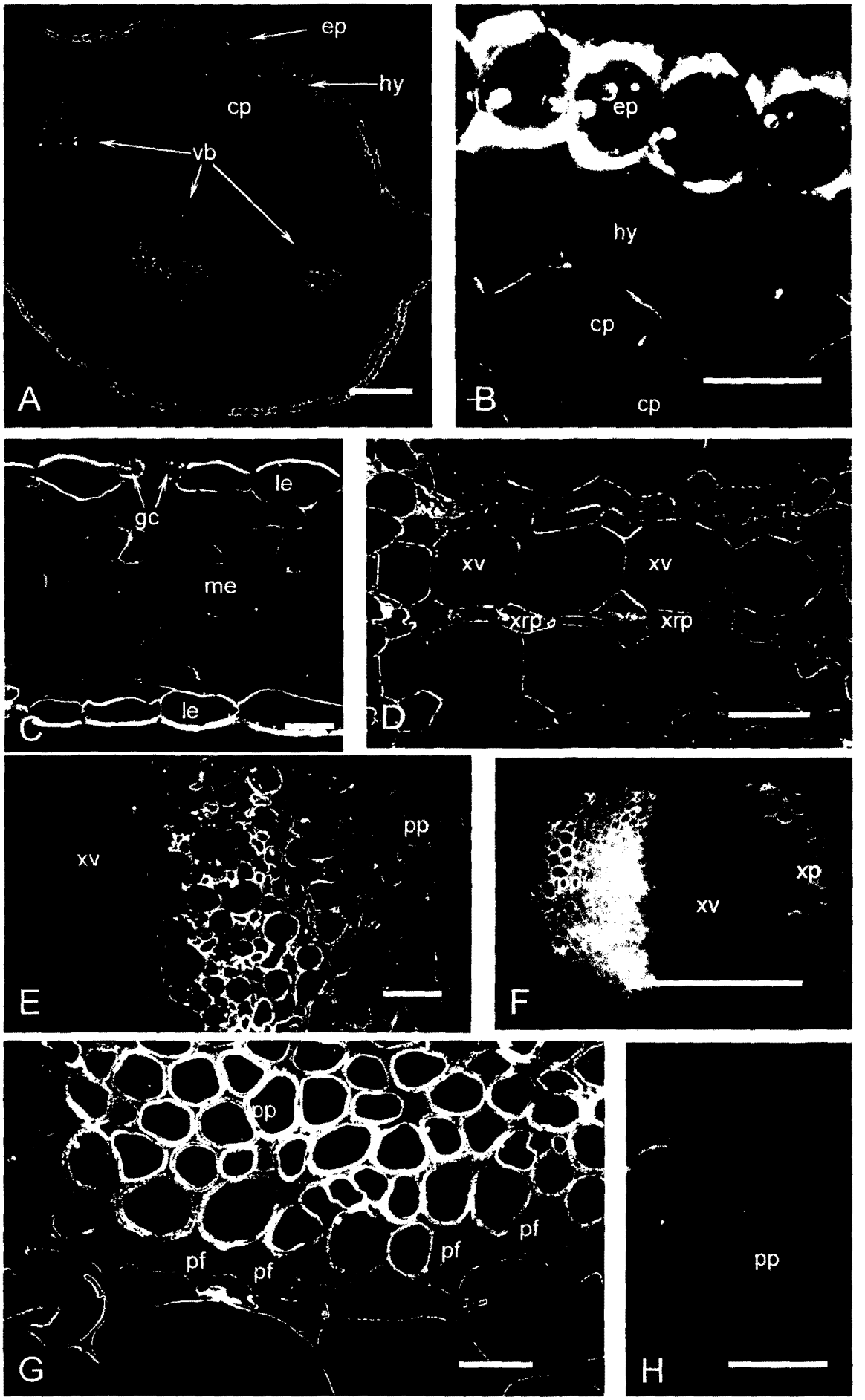
3.4.6 Effect of postfixation with osmium tetroxide

Some sections (not shown) were prepared without osmium tetroxide postfixation. There were no obvious differences in the intensity of immunogold labelling between sections with and without postfixation.

Figure 3.4 Micrographs of transverse sections from the lamina and midrib of 8-week-old rosette chicory labelled with the LM6 antibody. Fluorescence micrographs (A, B, C, D, E, F, G, H, I, J). Transmission electron micrographs (K, L, M, N, O, P, Q). Micrographs from the lamina (C, I, J, L, M, N, O, Q), from the midrib (A, B, D, E, F, G, H, K, P). (A) Midrib, LM6 epitope was present in primary walls of most cell types, mainly located in epidermis and vascular tissues. (B) Epidermis and hypodermis, showing that epidermis was more heavily stained than hypodermis. (C) Lamina, the outer walls of epidermis cells and guard cell walls were intensely stained. (D) Xylem, LM6 epitope continued along the middle lamella between vessels. (E) Phloem, LM6 epitope was found as inclusions in many inner phloem cells. (F) Vascular bundle in midrib section cut from fresh chicory, walls of phloem tissue were more intensely labelled than those of the outer xylem. (G) Outer phloem, showing intensely-stained thick-walled parenchyma and phloem fibres lacking LM6 epitope. (H) Phloem parenchyma in midrib section cut from fresh chicory, the corners of intercellular spaces and the outer walls of cells facing these spaces were intensely labelled. (I) Lamina. (J) Minor vein of lamina, showing that walls and ingrowths of transfer cells were heavily stained. (K) Phloem parenchyma, some cell walls were heavily stained with LM6 in phloem parenchyma. (L) Guard cells, heavily labelled with LM6 antibody. (M, N) Epidermis of lamina and midrib, LM6 epitope was evenly distributed in epidermis walls of lamina (M) and midrib (N). (O) Lamina mesophyll, the label occurred in mesophyll walls, but appeared as two lines in some cell walls. (P) Cortical parenchyma, LM6 epitope was present in the outer side region of primary walls, but is absent from the corners of intercellular spaces. (Q) Minor vein in the lamina, transfer cell walls were heavily stained.

cp, cortical parenchyma; ep, epidermis; gc, guard cell; hy, hypodermis; le, lamina epidermis; me, mesophyll; pf, phloem fibre; pp, phloem parenchyma; sc, sieve cell; tc, transfer cell; vb, vascular bundle; wi, wall ingrowths; xp, xylem parenchyma; xrp, xylem ray parenchyma; xv, xylem vessel; xy, xylem.

Bars, (A, F), 200 μm; (B, C, D, E, G, H, I), 20 μm; (J, L, M, N, Q), 5 μm; (K, O, P), 2 μm.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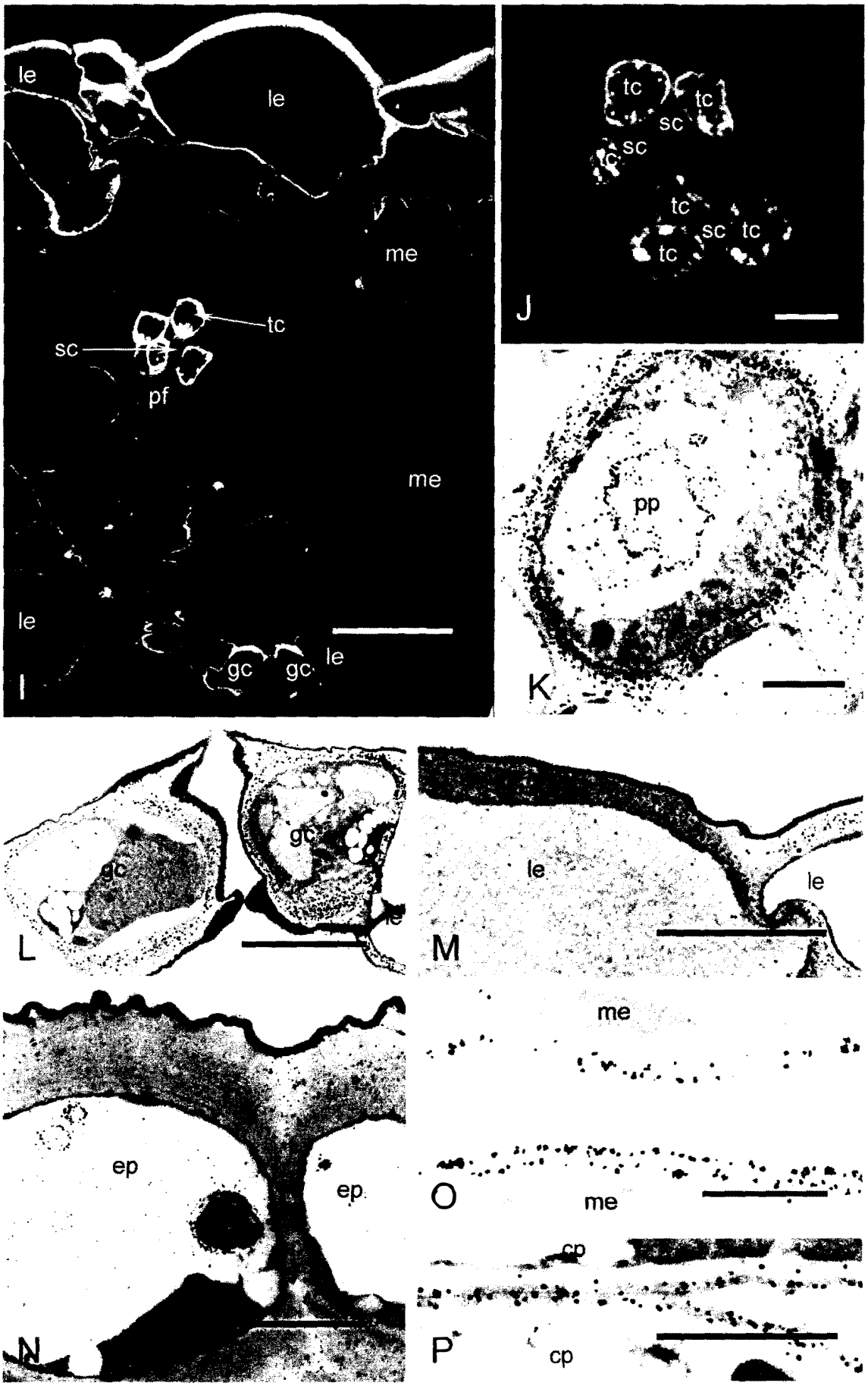
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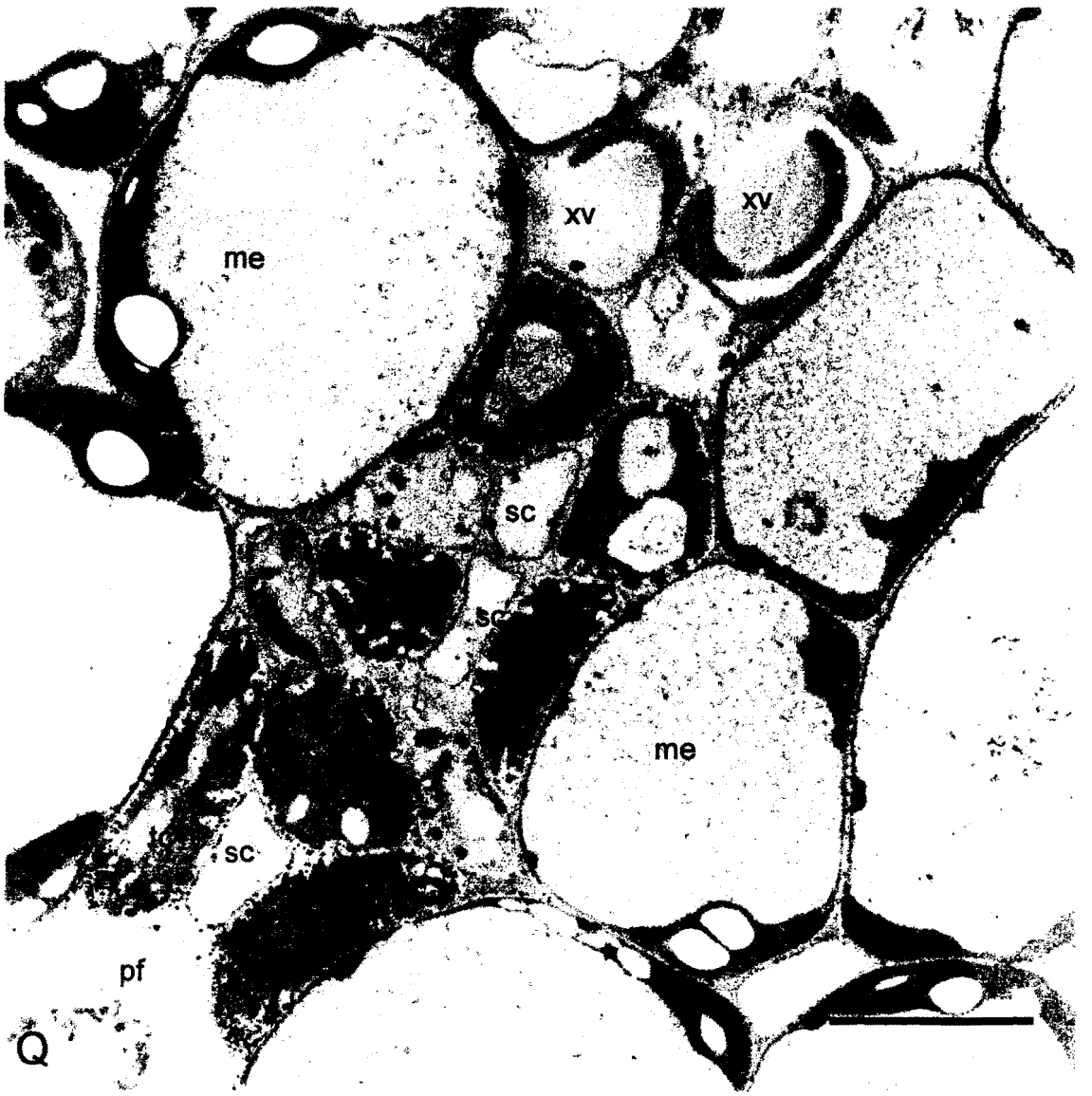
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3.5 Discussion

As reported in Chapter 2, the principal pectic polysaccharides of chicory leaf cell walls are HG and RG I, the latter having side chains of (1→4)-β-D-galactan and (1→5)-α-L-arabinan. The immunocytochemical results reported here show the distribution of these polymers.

3.5.1 Homogalacturonan (JIM5 and JIM7)

The labeling with JIM5 and JIM7 showed the distribution of HG with low and high degrees of methyl esterification, respectively.

3.5.1.1 Distribution, deposition and de-methylesterification

The immunofluorescence and immunogold results showed that the highly-esterified HG epitope recognised by JIM7 in the walls of chicory leaves was distributed relatively uniformly throughout the primary walls of most cell types, as described by other workers in a range of eudicotyledons (Knox, 1997; Bush and McCann, 1999; McCartney and Knox, 2002). However, the labelling tended to be less intense in the region of the middle lamella and intercellular spaces.

In contrast, the HG with a low degree of methyl esterification, recognised by JIM5, was more localised in distribution. JIM5-labelling of the cortical parenchyma of midrib was much less intense than that of JIM7 when compared to that of vascular bundles and epidermis. Although JIM5 epitope was detected throughout most walls, the labelling was frequently stronger in the middle lamella and cell corner regions. Labelling of the corners of intercellular spaces was particularly noticeable in the cortical parenchyma and phloem tissue. Thus, the HG with a low degree of methyl esterification was present in all primary walls, but concentrated in the middle lamella and outer walls facing intercellular spaces. This finding is consistent with that of Knox et al. (1990) who, using JIM5 epitope, found that relatively unesterified pectins were mainly located in the middle lamella and the cell corners in tomato (*Solanum lycopersicum*) and sugar beet (*Beta vulgaris* L.). Unesterified pectins recognised by the 2F4 antibody (Liners and Van Cutsem, 1992; Bush and McCann, 1999) had a similar pattern of location to the JIM5 epitope in the present study.

In general, the JIM7 epitope is distributed more uniformly than the JIM5 epitope. Thus, the question is raised as to why these two epitopes have different patterns of location.

Steele et al. (1997) showed that in the pericarp of ripening tomato fruit, the JIM5 epitope was initially confined to the middle lamella region, but that de-esterification of the HG led to a spread in distribution of the JIM5 epitope throughout the wall. This finding leads us to consider deesterification. HG is generally believed to be secreted from the Golgi in the form of highly methylesterified HG (Dupree and Sherrier, 1998) and is recognised by JIM7 in all primary cell walls, whereas the low-esterified HG is formed by the subsequent action of intra-wall pectin methylesterase (PME). Once highly esterified HG is deposited in the wall, de-methylesterification occurs in selected sites, through the action of PME (Quentin et al., 1997; Saher et al., 2005). Thus, the JIM7 epitope is present throughout the primary walls and is distributed relatively uniformly, whereas the JIM5 epitope is present in the site where de-methylesterification by PME occurred.

The present study shows that regions in chicory leaves particularly rich in HG with a low degree of methyl esterification were the middle lamella and corners of intercellular spaces of most types of cells, and the outer walls of the epidermis and the thick-walled outer phloem parenchyma. The PME may be preferentially distributed in these regions.

3.5.1.2 Cell adhesion

The PME activity in the middle lamella probably causes de-methylesterification of highly methylesterified HG to HG with a low degree of methyl esterification. Roy et al. (1994b) showed that in tomato fruit, the distribution of wall calcium paralleled that of the JIM5 epitope, in middle lamellae and intercellular spaces. The low esterified and unesterified HGs in the middle lamellae can crosslink with Ca^{2+} (Goldberg et al., 1996). This Ca^{2+}-HG crosslinking may be partly responsible for cell adhesion (Jarvis et al., 2003).

That Ca^{2+}-HG crosslinking plays a role in cell adhesion is supported by other some studies. Parker et al. (2001) observed in potato (*Solanum tuberosum*) tubers that cell adhesion is strongest at the edges of the conjoined faces of walls, and that this adhesive strength is correlated with a high JIM5 label. These edges appear as corners in sectional views. These corners also have large amounts of the high LM7 and PAM1 epitopes

(Jarvis et al., 2003). The binding requirements of LM7 and JIM5 are similar, except that LM7 requires GalA residues with non-block-wise pattern of methyl deesterification (Willats et al., 2001b). PAM1 recognises about 30 contiguous non-esterified GalA residues (Willats et al., 1999a).

In the present studies, the accumulation of JIM5 label in the middle lamella between chicory cells, at tricellular junctions and the corners of intercellular spaces (Fig. 3.1H) observed in the present study suggest that JIM5 epitope may also have a role in adhesion.

The PMEs that produce the form of HG with a low degree of methyl esterification are possibly most active at the corners where adhesion is greatest, but the JIM5 epitope apparently remains on the non-adherent outer walls in the intercellular spaces as the spaces enlarge in response to the turgor pressure (Jarvis, 1992b). Such calcium bridging would be prevented by high degrees of methyl esterification. Highly esterified pectins were restricted to the outer region of the wall in the mesophyll and palisade cells in *Zinnia elegans* leaves (Stacey et al., 1995). It was suggested that highly esterified pectins may restrict cell adhesion (Stacey et al., 1995). However, in chicory leaves, the spongy mesophyll cells in the lamina generally showed a similar distribution of JIM5 and JIM7 epitopes to that found in the cortical parenchyma of the midrib, with JIM7 throughout the wall and with little accumulation at cell corners, contrasting with JIM5 on the outer part of the primary wall and middle lamella and concentrating at cell corners.

It is of interest that Chapman et al. (2000) found that during somatic embryogenesis in chicory roots a network of low esterified HG accumulated on the surface of the proembryo cells. Their finding of JIM7 confined to the middle lamella of embryos reflects the finding of Stacey et al. (1995) for mesophyll and palisade cells of *Zinnia* leaves, but in the present study the JIM7 was usually spread more evenly through the primary walls. The walls of two adjacent mesophyll cells intensely labelled with JIM7 suggest that highly esterified HG may not interfere with cell adhesion.

Thus, the JIM5 epitope concentrated in the middle lamella is consistent with Ca^{2+}-HG crosslinking being involved in cell adhesion. However, the tangential walls of epidermis and hypodermis showed a rather different pattern, with the JIM5 epitope lower in the

middle lamella region. This pattern of JIM5 distribution may suggest that adhesion is low in this region or that alternatively other molecules may be involved in adhesion.

3.5.1.3 Structural support

In the collenchyma and epidermis, the thick wall is important in holding the leaf rigid and requires a strong wall, possibly strengthened by the pectin hydrogel, requiring calcium bridging of low esterified HG (Jarvis, 1992a). A pectin hydrogel can swell and shrink according to ionic conditions (Zwieniecki et al., 2001), and so regulate the porosity and flow of water, which may be important in maintaining turgor pressure.

The current study showed the JIM5 epitope to be more abundant in the walls of the tissues where cell adhesion is important such as epidermis, hypodermis and vascular bundles in the midrib and in the epidermis of the lamina (Fig. 3.1A). However, the JIM5 epitope is also particularly abundant in the outer walls of the epidermis, where no cell adhesion is involved. Therefore, the role of the JIM5 epitope may relate to maintenance of a strong hydrogel and a turgid leaf surface.

Similarly, in chicory leaf midribs, the high concentration of the JIM5 epitope in the thick walls of the outer phloem parenchyma may increase the mechanical strength of these cell walls against turgor pressure, as postulated in celery collenchyma (Jarvis, 1992a). Jarvis (1992a) suggested pectin has a role in controlling the thickness of collenchyma cell walls. The outer zones of thick-walled parenchyma on both phloem and xylem of vascular bundles in chicory leaf midrib probably have a role in strengthening the tissues and supporting the leaf.

3.5.1.4 Homogalacturonan in fresh sections

In the present study it was found that the JIM5 labelling was more restricted in sections of fresh tissue than noted with the embedded sectins, being concentrated around the intercellular spaces and not in the middle lamellae. This result is similar to that observed in fresh sections of pea stems by Willats et al. (2001b). In contrast to their work, this study did observe occasional labelling in the middle lamella region between cells and a narrow zone of labelling in the outer walls of the epidermal cells was observed. It is probable that the results with embedded sections reflect the true distribution of low esterified HG, but that the JIM5 epitope is partially masked in fresh sections. This is

borne out by Toluidine Blue staining of fresh sections, in which the pattern of pink staining indicative of polyanionic polymer (in this case presumably deesterified HG) was similar to that found with JIM5 in resin-embedded sections, including pronounced labelling in the middle lamella region. Willats et al. (2001b) stated that LM7 epitope was detectable only in fresh sections and suggest that they are labile to the processing involved in preparation of embedded sections. Jauneau et al. (1998) suggest that JIM5 epitope in apple (*Malus domestica*) fruit may be masked even in resin-embedded sections.

Knox et al. (1990) showed that JIM5 epitope in the root apex was restricted in some species (such as spinach) to the lining of intercellular spaces. A similar restricted distribution in fresh sections of chicory leaf was found in the current study, but not in resin-embedded sections, suggesting that the apparent variation in distribution of low esterified HG could be due, in part, to differences in masking of the JIM5 epitope. Therefore, caution should be exercised when comparisons between results from fresh tissues and those from resin-embedded tissues are made.

3.5.2 (1→5)-α-L-arabinan (LM6) and (1→4)-β-D-galactan (LM5)

3.5.2.1 (1→5)-α-L-arabinan and (1→4)-β-D-galactan in cell adhesion

The current study has shown that the LM5 epitope ((1→4)-β-D-galactan) was absent from the middle lamella, the tricellular conjunction and the corner of the intercellular spaces. The distribution of LM6 epitope within walls resembled that of JIM5 epitope, but the LM6 did not localise so specifically to the middle lamella or intercellular region. These data accord with the previous suggestion (Jarvis et al., 2003) that RG I does not have a key role in cell adhesion.

3.5.2.2 (1→5)-α-L-arabinan and (1→4)-β-D-galactan during cell development

The current study showed that the LM6 epitope ((1→5)-α-L-arabinan) was present in the primary wall near the middle lamella. Majewska-Sawka and Munster (2003) found that the LM6 epitope was particularly prominent in the newly formed walls of sugar beet generated from mesophyll-derived protoplasts. They showed that the walls of leaf mesophyll and protoplast-derived callus were particularly rich in LM6 epitope. An association of (1→5)-α-L-arabinan with meristematic cells has also been observed in

Pinus radiata (Ian Andrew, unpublished). The results of these authors and the observations in the present study on its localisation to the outer primary wall regions of most walls except those of the outer phloem parenchyma in chicory leaf suggest that it was laid down early in development.

In contrast to the distribution of LM6 epitope, the current study showed that the LM5 epitope (1→4)-β-D-galactan was in the wall near the plasma membrane, and was absent from the middle lamella, the tricellular conjunction and the corners of the intercellular spaces. This distribution was consistent with the results of Jones et al. (1997) for tomato, Bush and McCann (1999) for potato tuber, McCartney et al. (2000) for pea cotyledons and Vicré et al. (1998) for flax roots. These workers found that the (1→4)-β-D-galactan epitope was restricted to a thin layer of wall close to the plasma membrane. Therefore, the LM6 epitope appears to be located in the lately-formed regions of the walls. Vicré et al. (1998) found the (1→4)-β-D-galactan was only in the walls of peripheral cells of the flax root cap. The LM5 epitope was completely absent in newly formed walls from mesophyll-derived protoplasts of sugar beet (Majewska-Sawka and Munster, 2003). In the walls of pea cotyledons, the (1→4)-β-D-galactan was laid down in a late stage of seed development and evidently did not diffuse through the wall (McCartney et al. 2000). These observations, and the pattern of LM5 epitope in chicory leaves, suggested that the (1→4)-β-D-galactan was laid down at a particular stage of development, but the localisation to the inner margin of the wall could also be due to turnover of the (1→4)-β-D-galactan. Thus, the thick walls of midrib outer phloem parenchyma were presumably derived from thin walls of inner phloem cells of younger leaves (Esau, 1969), yet the (1→4)-β-D-galactan is still confined just to the extreme inner part of the wall.

Therefore, (1→5)-α-L-arabinan is laid down early in development, but (1→4)-β-D-galactan later in development. Vincken et al. (2003) also noted that (1→4)-β-D-galactan epitope is deposited later than (1→5)-α-L-arabinan during tuberisation in potato. They proposed a model in which pectic molecules cannot move freely as they are anchored in cell walls and they suggest this may involve a linkage of (1→4)-β-D-galactan side-chains to cellulose. Therefore, later deposition of (1→4)-β-D-galactan causes the (1→4)-β-D-galactan to be highly concentrated near the plasma membrane. In support of this model, the data in Chapter 2 showed that in chicory cell walls, much of the (1→4)-

β-D-galactan was tightly associated with cellulose residues after extraction of most of the pectic polysaccharides. The present study showed that epidermal cell walls of the midrib have a much broader distribution of LM5 epitope than those of other cell types. This suggests that (1→4)-β-D-galactan was deposited over a much more extended period in the epidermal walls than in the walls of most cell types. Alternatively, the (1→4)-β-D-galactan in the epidermal outer wall may have been more mobile than in other walls, allowing it to diffuse part-way across the wall before being anchored onto the cellulose. The (1→4)-β-D-galactan was also absent from the primary walls of mature xylem vessels, suggesting a secondary loss. The turnover of (1→4)-β-D-galactan has also been suggested in the walls of flax (*Linum ustatissimum* L.) phloem fibres, but this is a soluble galactan in the secondary walls (Gorshkova et al., 2004).

The differential deposition of (1→5)-α-L-arabinan to (1→4)-β-D-galactan could be a result of cell development. Willats et al. (1999b) found that, in the carrot (*Daucus carota*) root apex and suspension cultures, walls of meristematic or proliferating cells lacked the LM5 epitope but had abundant LM6 epitope, whereas in elongated cells the reverse was the case. They suggested a developmental switch in the suspension culture, where withdrawal of growth hormone led to both elongation and a change from (1→5)-α-L-arabinan to (1→4)-β-D-galactan being deposited.

3.5.2.3 (1→5)-α-L-arabinan and (1→4)-β-D-galactan in the walls of different cell types

Phloem fibres

One of the most conspicuous findings of this work was the intense specific LM5 labelling of a (1→4)-β-D-galactan in the primary walls of outer phloem fibres of the midrib, which in transverse section appeared as irregularly-shaped cells forming a disconnected ring between the outer phloem parenchyma and the cortical parenchyma. These fibres had thick secondary walls, but the (1→4)-β-D-galactan was confined to the primary walls. This cell-specific labelling recalls that of bast (phloem) cell fibres of flax stems (Gorshkova et al., 2004), but in the latter, the (1→4)-β-D-galactan was mostly extractable by phosphate buffer (pH 7) and was in the secondary wall. The chicory fibre (1→4)-β-D-galactan was not extracted by various buffers at pH 4.5 and 7.0 (Chapter 4).

It is notable that (1→4)-β-D-galactan was laid down before the secondary wall deposition as the secondary walls were not labelled with LM5.

McCartney et al. (2000) found that (1→4)-β-D-galactan increased the mechanical strength of cell walls of developing pea cotyledons, as the firmness of cotyledons was two-fold higher in those with the (1→4)-β-D-galactan-rich cell wall layer than in those without detectable (1→4)-β-D-galactan. The high (1→4)-β-D-galactan concentration in peripheral phloem fibres in the present study may relate to the mechanical strength of phloem. In the bast fibres of developing flax, cell-specific (1→4)-β-D-galactan only occurred right below the snap point where the secondary cell walls were intensively thickening (Gorshkova et al., 2004). The flax fibre (1→4)-β-D-galactan may thus have a role in strengthening the fibre. However, the majority of the flax (1→4)-β-D-galactan was in the secondary wall (Gorshkova et al., 2004), as is the (1→4)-β-D-galactan of lupin (*Lupinus albus* L.) cotyledons, where it has been assigned a storage function (Buckeridge et al., 2005).

It was found that the primary walls of phloem fibres had small amounts of HG (both JIM5 and JIM7 epitopes) and almost no LM6 arabinan. This provided a stark contrast to the outer phloem parenchyma, with its intense JIM5 and LM6 reactivity. The thick secondary walls of these phloem fibres were not labelled with antibodies against pectic polysaccharides, consistent with their identification as fibre.

Phloem

The walls of most inner phloem cells of midrib, including sieve cells, showed both LM5 and LM6 epitopes, indicating the presence of (1→4)-β-D-galactan and (1→5)-α-L-arabinan. However, some inner phloem cells in midrib lacked the LM5 epitope. These appeared to be developing cells as they had a relatively small size. In contrast, companion cells in both midrib and minor veins (lamina) showed intense LM6 labelling of both wall and, in some cases, cytoplasmic contents. The companion cells of lamina minor veins in chicory, as in other members of the Asteraceae (Turgeon et al., 2001), are transfer cells, with wall ingrowths to facilitate apoplasmic loading of the sieve cells (Oparka and Turgeon, 1999). It was observed that these transfer cell walls were strongly labelled with LM6 (in comparison to the much weaker labelling of mesophyll cells and sieve cells) and the label extended into the wall ingrowths. The ingrowths appeared to

be similar to the peripheral walls in their response to JIM5, JIM7, LM5 and LM6. JIM7 epitope has been reported in the walls and wall ingrowths of pea root nodule transfer cells (Dahiya and Brewin, 2000; Sherrier and Vandenbosch, 1994), but JIM5 epitope was absent from both walls and ingrowths of these cells. No reports of pectic polysaccharides in transfer cell wall ingrowths of other plants appear to have been published and the report is therefore the first for LM5 and LM6 epitopes.

The thick-walls of phloem outer parenchyma showed high (1→5)-α-L-arabinan content and low (1→4)-β-D-galactan. The (1→4)-β-D-galactan (LM5 epitope) was confined to the extreme inner edge of the wall. Thus, if these cells were derived from thin-walled inner phloem parenchyma in the developing leaf, there must have been a turnover of (1→4)-β-D-galactan or a redistribution in the wall.

In active shoots of aspen (*Populus tremula* L. × *P. tremuloides* Michx.), Ermel et al. (2000) found that (1→5)-α-L-arabinan became "prevalent at a very early stage in cells committed to differentiate into phloem" and was particularly abundant in walls of sieve cells, but (1→4)-β-D-galactan was absent from sieve cell walls. In sugar beet root, the LM5 epitope was found to be mainly present in the cambial zone (Guillemin et al., 2005). These observations may suggest that (1→5)-α-L-arabinan is related to phloem cell differentiation. Although this could also be true in chicory, the finding in the present study is rather different, in that the companion cell walls in chicory leaf were much more heavily labelled than the sieve cell walls. Oomen (2003) found LM5 epitope in sieve cells of potato stolons but not in those of tubers.

Guard cell walls

Stomatal guard cells have low (1→4)-β-D-galactan and high (1→5)-α-L-arabinan content in the walls. They also have cytoplasmic LM6-positive inclusions, similar to those in inner phloem, and their JIM7 and LM6 wall epitopes were markedly resistant to extraction by sodium carbonate. Guard cell walls need to be particularly strong and flexible since they undergo stresses and reversible changes during stomatal opening and closing. Jones et al. (2003) reported that degradation of cell wall arabinans prevented either stomatal opening or closing. They suggested that (1→5)-α-L-arabinan side chains of RG I separate two neighbouring stretches of homogalacturonan to impede the formation of tight associations, which results in the flexibility of these walls. The low

(1→4)-β-D-galactan content could result in greater flexibility, in view of the reported association of (1→4)-β-D-galactan with cellulose (Vincken et al., 2003).

In guard cells, the distribution of the JIM5 epitope, mostly in the outermost part of the cell walls, but not in the cuticle, contrasts with a report from sugar beet leaves (Majewska-Sawka et al., 2002), where the JIM5 epitope was restricted to the cuticle of guard cells. In the present study, JIM7 epitope was dense throughout the guard cell walls, including the cuticular layer. The JIM7 epitope may form a more flexible structure suited to guard cell function, while JIM5 epitope provides rigidity (Jones et al., 2005).

Pit field

LM5 label was also completely absent in some parts of cortical parenchyma cell walls. They appeared to be pit fields crossed by plasmodesmata. Orfila and Knox (2000) found that (1→5)-α-L-arabinan epitope was present at the inner face of cell walls surrounding the pit fields, while (1→4)-β-D-galactan epitope was absent from the pit field in the pericarp walls of the tomato.

Lamina

In a few lamina mesophyll cells, LM6 epitope was observed along the plasma membrance as well as in the outer wall. Cytoplasmic (1→5)-α-L-arabinan may represent pools in transit or storage. Alternatively, it could be associated with AGP molecules since LM6 antibody can bind to some AGPs in the moss *Physcomitrella patens* (Lee et al., 2005).

Ara/Gal

LM5 labelling of midrib cell walls was relatively uniform apart from intense labelling of a ring of outer phloem fibres. On the other hand, LM6 labelling of midrib appeared much more intense in epidermis and phloem parenchyma than in hypodermis and cortical parenchyma, recalling the distribution of JIM5 epitope. This suggests that the galactan/arabinan ratio was much higher in cortical parenchyma than in epidermis and phloem parenchyma. The galactan/arabinan ratio was higher in lamina of chicory leaf than in midrib (Chapter 2), but no quantitative information is available from the

immunocytochemical studies, and the difference between lamina and midrib may relate to subtle differences in the concentrations in the epidermis and vascular bundles, regions which contain the bulk of the label.

Opposing distribution

Although the (1→4)-β-D-galactan showed relatively uniform distribution among some cell types, other cell types had either (1→4)-β-D-galactan or (1→5)-α-L-arabinan as the predominant RG I epitope. For example, stomatal guard cell walls had high (1→5)-α-L-arabinan and low (1→4)-β-D-galactan. The pit fields of cortical parenchyma cell walls had no (1→4)-β-D-galactan. Some phloem cells had either high (1→5)-α-L-arabinan or low (1→4)-β-D-galactan or both. The thick-walled outer phloem parenchyma showed low LM5 eptitope (compared to cortical parenchyma) and high LM6 epitope. In contrast, outer phloem fibres had high (1→4)-β-D-galactan and almost no (1→5)-α-L-arabinan. The opposing distributions of arabinans and galactans, together with their differential deposition during development (Section 3.5.2.2), may suggest that these two RG I side-chains have opposing functions. Several cell wall properties could be influenced by the presence or absence of (1→4)-β-D-galactan.

The high (1→5)-α-L-arabinan content in cell walls of outer phloem parenchyma, as well as those of companion cells, guard cells and meristematic cells suggests that this polysaccharide may have several distinct functions in cell walls. Its role in midrib outer phloem parenchyma is unlikely to be one of maintaining flexibility as postulated for guard cells, as this thick-walled tissue is probably important in maintaining rigidity of the leaf.

3.5.3 Significance of distribution

This is the first report on the immunolocalisation of pectic polysaccharides in the chicory leaf, and it extends the work published by various other authors on a range of tissues in other plant species. One previous paper (Chapman et al., 2000) reports on the use of JIM5 and JIM7 in immunolocalisation of HG polymers in chicory, but it relates to somatic embryogenesis from root tissue.

The data reported herein extend the findings of others on the role of low esterified HG hydrogel formation in cell adhesion and in supporting plant structures such as leaves by thick walls of epidermis and phloem parenchyma. The suggestions that HG is secreted in a highly methylated state are supported and discrete roles for the (1→4)-β-D-galactan and (1→5)-α-L-arabinan side chains of RG I have been proposed. Evidence from phloem fibres and from guard cells support a role for (1→4)-β-D-galactan in cell wall rigidity and this could also account for the thick deposit in the outer epidermal walls. The roles of (1→5)-α-L-arabinan in guard cell function and in the early stages of wall formation are supported. In addition, likely roles for (1→5)-α-L-arabinan in phloem cell differentiation have emerged.

Chapter 4

The degradation of pectins in chicory leaf cell walls by endopolygalacturonase preparations

4.1 Abstract

Ruminants fed chicory spend little or no time ruminating, indicating a quick reduction in forage particle size in the rumen. For a better understanding of the reasons for this rapid particle breakdown, cell adhesion in chicory leaves was studied with endopolygalacturonase preparations (endo-PG) and some chemical agents. Endo-PG caused extensive maceration and more than 60% of chicory dry material was solubilised or broken down to less than 1 mm particle size. Cell separation in the cortical parenchyma of the midrib was induced with endo-PG, accompanied by the loss of HG from the middle lamella, the corners of intercellular spaces and the tricellular junctions. This supports the role of calcium-associated homogalacturonan (HG) in the cell adhesion. Although *trans*-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid (CDTA) removed the JIM5 and JIM7 epitopes from the regions of cell adhesion, cortical parenchyma cells were not separated. This suggests that other substances, possibly including arabinan, are involved. The adjacent tangential walls of the epidermal and hypodermal layers were sometimes separated by all reagents tested, although only slowly with acetate buffer, but the radial walls of the epidermal and hypodermal layers were generally not separated by CDTA, Na_2CO_3 or endo-PG treatments. These results imply other mechanisms in addition to Ca^{2+}-HG crosslinking are involved for cell adhesion with these walls.

4.2 Introduction

Chapter 2 showed that a high pectin content is a dominant feature of the polysaccharide composition of chicory cell walls. Chapter 3 reported on the location of the pectic polysaccharides in the cell walls and the important finding that homogalacturonans with low degrees of esterification were concentrated in the middle lamella between two neighbouring cells and at the corners of intercellular spaces. This finding is consistent with studies in other plants such as potato (Parker et al., 2001) and pea (Willats et al., 2001b). Both the findings in Chapter 3 and those of other workers support a prevalent hypothesis that calcium-linked gels of homogalacturonans with low degrees of esterification might be responsible for cell adhesion (Jarvis et al., 2003). In this hypothesis, calcium and homogalacturonan are key components leading to cell adhesion. Therefore, removal of calcium or solubilisation of HG may cause disruption of cell adhesion, leading to cell separation and further tissue maceration.

Endopolygalacturonase (endo-PG), which randomly splits the homogalacturonan chain (Singh and Rao, 2002; Pilnik and Voragen, 1993), has been found to be involved in pod dehiscence in Arabidopsis and oilseed rape (*Brassica napus*) (Jenkins et al., 1996, 1999) and in cell separation during fruit ripening (Roy et al., 1994a, 1995). It has also been used for flax (*Linum usitatissimum*) retting (Zhang et al., 2000) and for macerating vegetables and fruits (Chesson, 1980). Pectic enzymes in the rumen of grazing animals could likewise have a role in maceration of high-pectin forages such as chicory.

Since edges of conjoined faces between walls are key sites for cell adhesion (see Section 3.4.1.2, Chapter 3), enzymes such as endo-PG could induce artificial cell separation by solubilising HG of low esterification from the middle lamella and also from the corners of tricellular junctions. On the other hand, chelating reagents, such as *trans*-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid (CDTA), and other chemical reagents (Parker and Waldron, 1995) could cause cell separation by removal of calcium, and it has been found that cell walls incubated in sodium acetate buffer or potassium citrate-phosphate buffer can release some HG (Recio et al., 2003). If these enzymes and other reagents disrupt cell adhesion, the role of HG with a low degree of methyl esterification in cell adhesion would be further supported, hence reinforcing the hypothesis that pectic enzymes have a role in ruminal digestion of chicory.

In this chapter, the effects of endo-PG and chemical reagents on cell adhesion have been investigated. In the first experiment, the action of endo-PG on chicory leaves was compared with that on leaves of other forage plants. The second experiment then investigated a series of chemical reagents, based on those used by Parker and Waldron (1995), on cell separation. In the third experiment, the release of carbohydrate by incubation with endo-PG was measured. Finally, a detailed investigation into the effects of endo-PG at a microscopic level is reported. This enables us to see the extent of cell separation in different specific tissues and the concomitant changes in pectic epitope distribution. In this experiment, the effects of CDTA and of citrate-phosphate buffer are also studied, as examples of non-enzymic processes, whereby wall-wall adhesion might be expected to be affected by calcium removal. Sodium carbonate is known to promote hydrolysis of ester bonds, such as the methylester bond in pectin. Hence, sodium carbonate is also tested to investigate its possible effects on pectic epitopes and cell adhesion.

4.3 Materials and methods

4.3.1 Plant materials

Chicory (*Cichorium intybus* L. cv. Puna II), perennial ryegrass (*Lolium perenne* L. cv. Ruanui) and white clover (*Trifolium repens* L.) were used in this study.

Chicory plants were grown in a tray and harvested at eight weeks from emergence as described in Chapter 2. In some cases the midribs and laminae were separated as described in Chapter 2.

Perennial ryegrass and white clover were grown outdoors in pots. Perennial ryegrass leaf blades and fully expanded white clover leaflets were harvested 4 weeks after regrowth for experiments.

4.3.2 Experiment 1 (forage particle breakdown by endo-PG treatment)

In this experiment the effects of enzymes on different forages were studied qualitatively (i) and quantitatively (ii).

(i) Chicory leaves, perennial ryegrass leaf blades and white clover leaflets were harvested, and then immediately incubated with or without endopolygalacturonase (endo-PG, 1 unit ml^{-1}) from *Aspergillus niger* (see Appendix 4, Megazyme International Ireland Ltd., Wicklow, Ireland) for 16 h at 39°C in an sodium acetate buffer (pH 4.0, 200 mM) with shaking (150 rpm) using a controlled environment incubator shaker (New Brunswick Scientific Co., Inc. Edison, N.J., USA). After incubation the sample was transferred to a Petri dish for photographing.

(ii) (a) Fresh chicory leaves, chicory laminae, chicory midribs, perennial ryegrass leaf blades and white clover leaflets were harvested and washed clean with tap water and reverse osmosis water consecutively. They were dried with tissue paper to remove surface water and cut into 3 cm lengths. Accurately weighed fresh chicory leaves, dissected chicory midribs, chicory laminae, ryegrass leaf blades and white clover leaflets (about 2 g in fresh weight) were incubated with or without crude pectinase (1 unit ml^{-1}, Sigma No. 5146, polygalacturonase preparations, E.C. 3.2.1.13, from *A. niger*) for 16 h at 39°C in a citrate-phosphate buffer (40 ml, pH 4.0; Gomori, 1955)

containing sodium azide (0.02%) with shaking as described above [Section 4.3.2 (i)] in triplicate. Samples were then filtered using two sieves (pore sizes 4 mm and 1 mm) consecutively and gently washed on the sieves with 40 ml and then 60 ml water, and the filtrates centrifuged (450 g, 5 min). The residues and pellets were dried at 65°C. Four fractions (>4 mm, 1-4 mm, <1 mm, and soluble material) were obtained. Unincubated samples for each treatment were dried at 65°C to calibrate the weight of forage particles on a dry matter basis. All samples were weighed except the soluble fraction which was determined by difference.

(b) Chicory midribs and laminae were incubated and macerated fractions sieved according to the methods described above (a), except that endo-PG (1 unit ml^{-1}) was substituted for crude pectinase.

4.3.3 Experiment 2 (effect of chelating reagents, acids and bases on maceration)

Midrib sections (3 cm in length) and lamina sections (3 cm in length and 1 cm in width) of chicory leaves were treated with a series of reagents described below and selected from those used by Parker and Waldron (1995). These reagents included chelating reagents, acids and bases. CDTA and ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA) were chosen for their ability to chelate calcium, causing the release of unesterified HGs or HGs with low degrees of methyl esterification. Na_2CO_3 was chosen to extract esterified homogalacturonan. KOH was selected since KOH can release alkali-extractable polysaccharides. TFA was selected because TFA at low concentrations can rapidly hydrolyse furanosidic linkages (Parker and Waldron, 1995). H_2SO_4 and HCl at low concentrations can partially hydrolyse polysaccharides.

Midrib and lamina samples were treated with the following solutions:

- CDTA (adjusted to pH 6.5 with NaOH, 10, 50, 100 mM) at 39°C for 24 h,
- EGTA (adjusted to pH 7.4 with NaOH, 5, 10, 50, 100, 200 mM) at 39°C for 24 h,
- Na_2CO_3 (50 mM) containing 25 mM $NaBH_4$ at 20°C for 24 h with gentle shaking,
- KOH (0.05, 0.1, 0.5, 1, 4 M) for 20 h at 20°C with gentle shaking,
- TFA (0, 0.01, 0.05, 0.1 M) at 100°C for 25 min and TFA (0.1 M) at 20°C for 24 h,
- H_2SO_4 (0.25 M at 100°C for 20 min, 0.5 M at 100°C for 15 min),
- HCl (0.25 M at 100°C for 30 min, 0.5 M at 100°C for 15 min),
- Endo-PG (1 unit ml^{-1} in 200 mM sodium acetate buffer (pH 4.0) containing 0.02% sodium azide) at 39°C for 16 h,
- Control was heated in water at 100°C for 90 min.

The degree of maceration was monitored using a vortex-induced cell separation (VICS) method modified from Parker and Waldron (1995). Treated samples were transferred into screw-capped 10 ml test tubes, and vortexed for 1 min. Prior to examination, the tubes were shaken by hand vigorously 10 times. Subjective scores were assigned for the degree of maceration (see Table 4.2).

4.3.4 Experiment 3 (the release of monosaccharide residues after endo-PG treatment)

Fresh green chicory leaves were washed clean with tap water and reverse osmosis water consecutively. After removing any water on the surface with tissues, they were separated into midrib and lamina. Midrib and lamina (about 2 g fresh weight accurately weighed) were cut into 3 cm lengths for incubation. Four midrib and lamina samples were freeze dried for calculation of dry matter (DM) content. Incubation was conducted in a conical flask (60 ml) with or without endogalacturonase preparations (1 unit ml^{-1}) for 16 h at 39°C in 40 ml of 200 mM sodium acetate buffer (pH 4.0) containing sodium azide (0.02%) with shaking (150 rpm). The experiment was carried out with 4 replicates.

After 16 h incubation, the samples were adjusted to pH 7.0 with 1 M NaOH and placed in an icebox to stop the reaction. Samples were then filtered using a 1 mm sieve by gentle washing with 100 ml water and the filtrates were centrifuged. Three fractions (>1 mm, <1 mm, and soluble material) were obtained and, after freeze-drying, were weighed and retained for monosaccharide analysis. Prior to monosaccharide analysis, samples were hydrolysed with H_2SO_4 as described in Chapter 2. Neutral monosaccharides were measured using the method of Albersheim et al. (1967) modified by Englyst et al. (1994), and uronic acids were measured using the method of Scott (1979) modified by Englyst et al. (1994) as described in Chapter 2.

The proportions of monosaccharides in macerated fractions was calculated according to the following equation.

$$\text{Monosaccharide proportion in fractions} = \frac{\text{monosaccharide concentration content in fraction} \times \text{fraction dry wt after incubation}}{\text{fresh wt before incubation} \times \text{DM\% of fresh material}} \times 100$$

After calculation, the sum of fractions was adjusted to 100%.

4.3.5 Experiment 4 (immunolabelling with JIM5, JIM7, LM5 and LM6)

i) endo-PG treatment

Transverse segments from the midrib or lamina of a chicory leaf (ca. 1 mm wide, 4-5 mm long for lamina; ca. 1 mm long for midrib) were cut in incubation buffer for incubation. Incubation was carried out in a 10 ml bottle with a gentle orbital rocking motion with or without endopolygalacturonase preparations (1 unit ml^{-1}) in sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) in an incubation room at 37°C for 1 h or for 12 h. Incubated segments were washed with the sodium acetate buffer twice and NaOH-PIPES (100 mM, pH 7.2) buffer once, and then fixed and embedded as described in Chapter 3.

Semi-thin sections (0.5 μm) were cut with a glass knife using a microtome. These sections were stained with 0.05% Toluidine Blue in sodium phosphate buffer (pH 7.2, 0.1 M) for 2 min. After thoroughly washing and drying, they were mounted in DPX

Mountant (DPX, BDH Laboratory supplies, Poole, UK) for observation under a bright-field light microscope. Photographs were taken with a digital camera (Optronics, Olympus U-TV 0.5XC, Japan) with the same exposure time unless indicated otherwise.

Semi-thin sections were stained immunocytochemically with JIM5, JIM7, LM5 and LM6 antibodies and examined using an epifluorescence microscope as described in Chapter 3.

ii) CDTA treatment

Midrib segments (ca. 2 cm in length) cut from midway along the midrib were treated overnight (16 h) with 50 mM CDTA (pH adjusted to 6.5 with NaOH) at room temperature (20°C) with slight shaking (50 rpm) in a 10 ml bottle.

These segments were washed with water (3 times), fixed and embedded using the procedure described above for (i). They were then treated as described Section 4.3.5 (i).

iii) Na_2CO_3 treatment

The Na_2CO_3 treatment was carried out in the same way as the CDTA treatment (Section 4.3.5 ii), except 50 mM Na_2CO_3 was used for incubation instead of CDTA.

iv) Acetate treatment

Midrib and lamina sections of chicory leaves as described above (Section 4.3.5 i) were incubated in a sodium acetate buffer (200 mM, pH 4.0) containing 0.02% sodium azide in an incubation room at 37°C for 12 h. All procedures were carried out as described in Section 4.3.5 (i).

v) Citrate-phosphate treatment

The sections and experimental procedures were the same as described in Section 4.3.5 (iv), except that a citrate-phosphate buffer (pH 4.0, 30 mM citric acid and 40 mM Na_2HPO_4) containing sodium azide (0.02%) was used instead of acetate buffer.

This treatment was run separately from the others above and a separate control was run at the same time.

4.4 Results

4.4.1 Experiment 1 (forage particle breakdown by endo-PG treatment)

(i) Qualitative comparison

Chicory leaves incubated in sodium acetate buffer without endo-PG showed no sign of maceration, even after vigorous shaking (Fig. 4.1A), indicating that any autolysis did not cause maceration. However, chicory leaves incubated in 1 unit ml^{-1} endo-PG solution for 16 h showed significant maceration (Fig. 4.1B). The midrib was more resistant to degradation than the lamina (Fig. 4.1B).

Ryegrass leaves were not macerated after control or endo-PG treatments (Fig. 4.1C and 4.1D). White clover leaflets with endo-PG treatment showed some maceration, but to a lesser extent than for the chicory leaves (Fig. 4.1E and 4.1F).

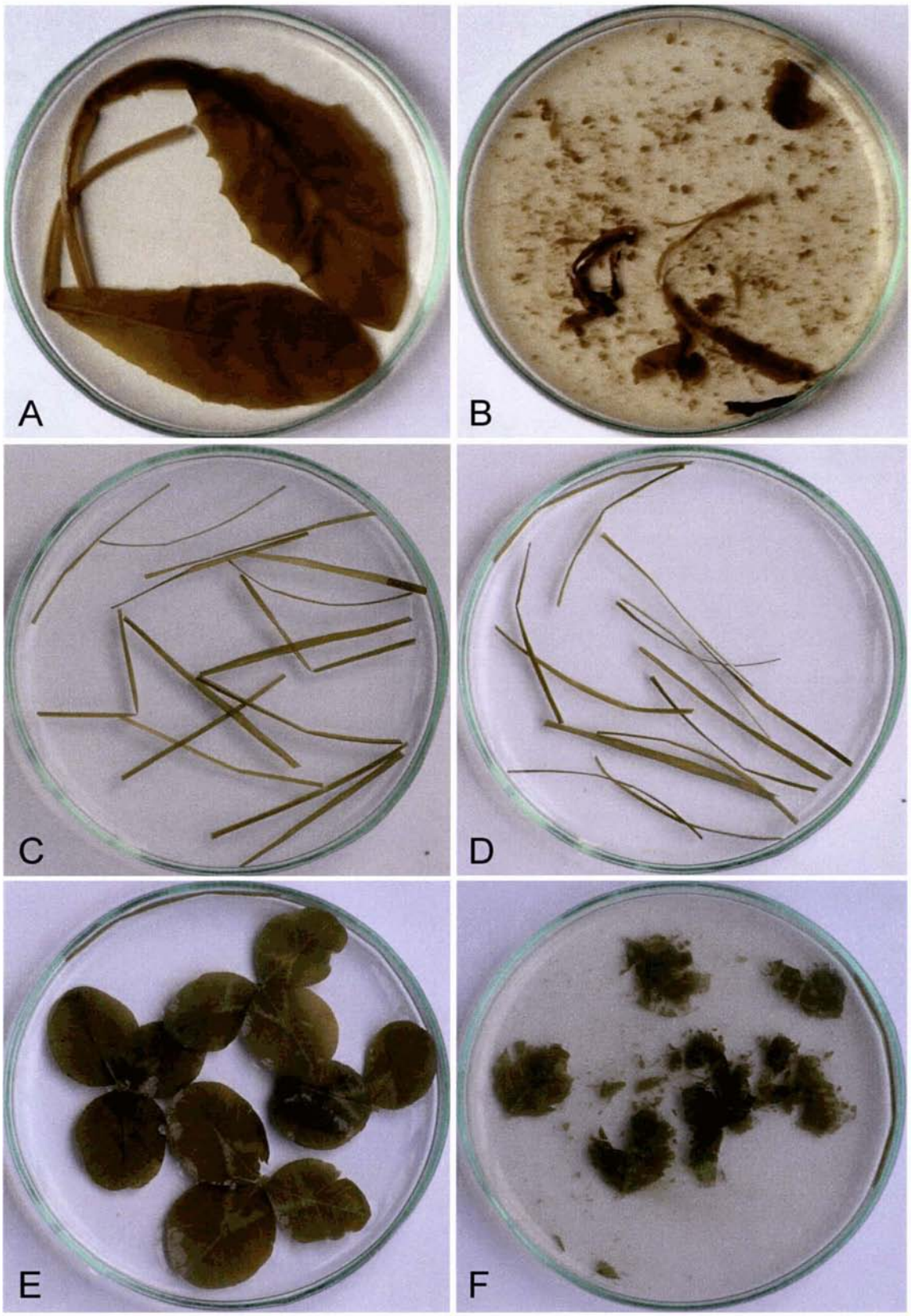


Figure 4.1 Chicory leaves (A, B), perennial ryegrass leaf blades (C, D) and white clover leaflets (E, F) incubated without (A, C, E) or with (B, D, F) endo-PG (1 unit ml^{-1}) for 16 h at 39°C in acetate buffer (pH 4.0, 200 mM) with rotary shaking (150 rpm).

(ii) Quantitative comparison

The proportions of particles sized 1-4 mm and less than 1 mm were zero in all controls with citrate-phosphate as a buffer, indicating that the forages did not break down without crude pectinase or endo-PG regardless of the forage species or tissue type (Table 4.1). This, along with the results shown in Fig. 4.1, suggested that any autolysis in the citrate-phosphate buffer in the incubation period did not cause significant maceration of forage leaves.

In contrast to untreated controls, samples treated with pectinase were macerated in the case of chicory (both lamina and midrib) and white clover, but not ryegrass (Table 4.1). Endo-PG caused both midrib and lamina of chicory to be macerated (Table 4.1 and 4.3).

The proportions of unmacerated chicory leaves (>4 mm in size) were higher for the midrib than for the lamina in the endo-PG treatment ($P<0.01$) (Table 4.1), showing that the midrib was more resistant to maceration than the lamina. Following pectinase treatment, there was no significant difference in the proportions of macerated tissue between the lamina and midrib.

For midrib, the total proportion of small particles (<1 mm in size) and the soluble fraction recovered following crude pectinase treatment was higher than that recovered following endo-PG treatment ($P<0.01$), suggesting that crude pectinase was more effective than endo-PG in breaking down the midrib, but this difference was not observed for the lamina ($P>0.05$). This difference between pectinase and endo-PG treatments probably results from the fact that the pectinase was not a pure pectin-degrading enzyme.

Table 4.1 Effect of crude pectinase and endopolygalacturonase preparations on forage breakdown

Plant material	Treatment	Fractions (% of total forage materials)(dry weight basis)			
		> 4 mm	1 – 4 mm	< 1 mm	Soluble
	Crude pectinase				
Chicory	Control	81.4±2.2^{ab}			18.6±2.2^{f}
	Pectinase	26.4±8.6^{c}	0.4±0.2^{c}	17.7±5.2^{c}	55.4±3.2^{b}
Chicory lamina	Control	89.7±3.2^{a}			10.3±3.2^{g}
	Pectinase	24.7±1.9^{c}	0.8±0.4^{c}	26.8±1.5^{b}	47.7±0.3^{cd}
Chicory midrib	Control	88.0±5.1^{a}			12.0±5.1^{f}
	Pectinase	14.6±0.8^{d}	0.1±0.1^{c}	13.3±1.2^{c}	72.1±0.5^{a}
White clover	Control	80.1±2.6^{b}			19.9±2.6^{ef}
	Pectinase	19.1±2.9^{d}	3.5±1.4^{b}	27.2±0.8^{b}	50.1±0.8^{bc}
Perennial ryegrass	Control	91.0±4.4^{a}			9.0±4.4^{g}
	Pectinase	88.4±0.7^{a}	0.0±0.0^{c}	0.9±0.2^{d}	10.6±0.9^{g}
	Endo-PG				
Chicory lamina	Control	72.6±0.9^{b}			27.4±0.9^{e}
	Endo-PG	8.1±4.0^{e}	6.7±4.5^{b}	41.1±1.6^{a}	44.1±2.1^{d}
Chicory midrib	Control	76.7±4.4^{b}			23.3±4.4^{ef}
	Endo-PG	31.3±0.7^{c}	20.1±0.7^{a}	23.8±1.9^{bc}	24.8±0.5^{ef}

Mean ± standard error of mean (n=3).

$^{a,b,\cdots,f}$ Values with different superscripts differ significantly ($P < 0.05$) between rows within columns.

Forages were incubated in crude pectinase (1 unit ml^{-1}) or endopolygalacturonase preparations (endo-PG) (1 unit ml^{-1}) in a citrate-phosphate buffer at pH 4.0 at 39°C for 16 h with shaking (150 rpm). Samples treated with endo-PG have been shaken for cell separation before filtering.

The soluble fraction was determined by difference. Soluble = 100 minus solid fraction proportions.

4.4.2 Experiment 2 (effect of chelating reagents, acids and bases on maceration)

Maceration is a result of the loss of cell adhesion. A series of chemical reagents were used to study their effects on cell adhesion (Parker and Waldron, 1995).

Neither CDTA nor EGTA caused vortex-induced cell separation (Table 4.2), although both reagents were able to release homogalacturonans, resulting from the loss of calcium caused by the chelating function of CDTA and EGTA (Vian and Roland, 1991; Hoyos et al., 1996).

Na_2CO_3 also did not cause vortex-induced cell separation.

Visible maceration was not observed with TFA. However, the tissue strength appeared to be reduced because the chicory tissue fell apart when it was picked up with forceps. The leaf colour had also changed from green to reddish brown. It was unlikely that the slight maceration by TFA was caused by high temperature (100°C) during the treatment since samples in the control at 100°C for 90 min were not macerated. Parker and Waldron (1995) found furanosidic linkages were broken using TFA (0.1 M), suggesting these linkages may have a role in maceration.

Treatments with KOH did not cause maceration, suggesting that alkali-extractable polysaccharides or phenolics are not related to cell separation in chicory.

Both HCl and H_2SO_4 caused vortex-induced cell separation. This may be due to the release of pectins as Kar and Arslan (1999) found that even very dilute HCl (3 mM, pH 2.5, 90°C, 90 min) was able to extract pectins. H_2SO_4 (0.5 M) at 100°C for 15 min will partially hydrolyse most polysaccharides, thus causing cell separation.

Endo-PG introduced a complete vortex-induced cell separation.

Table 4.2 Effect of chelating reagents, acids, base on maceration of chicory leaves

Agents and concentrations	Maceration scores[a]	
	Midrib	Lamina
Control in H_2O at 100°C for 90 min	0	0
CDTA (10, 50, 100 mM) at pH 6.5 at 39°C for 24 h	0	0
EGTA (5, 10, 50, 100, 200 mM) pH 7.4 at 39°C for 24 h	0	0
0.25 M H_2SO_4 at 100°C for 20 min	4	3
0.5 M H_2SO_4 at 100°C for 15 min	4	4
0.25 M HCl for 30 min or 0.5 M HCl for 15 min at 100°C	4	5
0.01 M TFA at 100°C for 90 min	1	1
0.05 M TFA at 100°C for 90 min	3	3
0.1 M TFA at 100°C for 25 min	4	4
0.1 M TFA at 20°C for 24 h	0	0
KOH (0.05, 0.1, 0.5, 1, 4 M) at 20°C for 20 h	0	0
Na_2CO_3 (50 mM) containing 25 mM $NaBH_4$ at 20°C for 24 h	0	0
Endo-PG (1 unit ml^{-1} in 200 mM sodium acetate buffer (pH 4.0) containing 0.02% sodium azide) at 39°C for 16 h	5	5

[a] Note: 0, tissue section intact, and the solution clear; 1, tissue section intact, but the solution was not clear, and some cells were dispersed into the solution; 2, tissue section broken into large clumps (>4 mm); 3, tissue sections broken into medium clumps (1-4 mm); 4, tissue sections broken into small clumps (<1 mm), but vascular bundles or sheets of epidermis can be seen; 5, tissue sections broken into small clumps (<1 mm), no visible vascular bundles or epidermal sheets.

4.4.3 Experiment 3 (the release of monosaccharide residues after endo-PG treatment)

The release of monosaccharide residues and particle breakdown of chicory lamina and midrib after endo-PG treatment in sodium acetate buffer is shown in Table 4.3. The results of particle breakdown with the sodium acetate buffer were similar to those with the citrate-phosphate buffer. Neither the lamina nor the midrib was broken down in the acetate buffer, although there was some soluble material released, largely from the cell contents. After incubation with endo-PG, the chicory tissues were broken down, indicating that the particle breakdown resulted from the action of endo-PG.

After endo-PG treatment, xylose, mannose and glucose were present in the soluble fraction to the same extent as in the control treatment, indicating that alkali-extractable polysaccharides and cellulose have not been affected by endo-PG. This was not surprising as endo-PG acts only on homogalacturonan (Pilnik and Voragen, 1993; Singh and Rao, 2002). In contrast, arabinose, galactose and uronic acid were released into the soluble fraction significantly more ($P < 0.05$) with the endo-PG treatment than with the control treatment for both midrib and lamina. As arabinose and galactose are mainly associated with RG I and uronic acid with HG (Chapter 2), this showed that both RG I and HG were released from chicory leaves. RG I released by endo-PG may result from the covalent linkage of RG I to HG (Ridley et al., 2001).

The net percentage solubilisation (i.e. percentage released into the soluble fraction on endo-PG treatment minus that of the control) of uronic acid, arabinose and galactose were, in the lamina, 52.3, 38.9, 6.4%, and, in the midrib, 46.7, 28.1, 17.4%, respectively. These results show the release of UA was greater than that of the Ara, which in turn, was greater than that of the Gal, suggesting that the three monosaccharides were released at different rates.

In the control, less than 4% of the uronic acid, arabinose and xylose were released from both the midrib and lamina (Table 4.3), showing autolysis was not significant for these monosaccharide residues. The mannose and glucose reported in the table were probably derived from the fructose of fructans found in the vacuoles of chicory leaves in a water-soluble form (De Roover et al., 2000; Van den Ende et al., 2002) as fructose can be converted into mannose and glucose derivatives during monosaccharide analysis (Fry,

1988). The percentage of glucose released from the midrib was higher than that for the lamina, whereas for galactose nearly twice as much was released from the lamina as from the midrib.

Table 4.3 The particle breakdown (%, w/w) and the distribution of monosaccharides in fractions from endo-PG-treated chicory lamina and midrib (%, w/w)

Sugars	Enzyme	Fractions		
		> 1 mm	< 1 mm	Soluble
Particle				
Lamina	Control	77.9±1.3[a]	0	22.1±1.3[c]
	Endo-PG	38.8±2.1[c]	21.7±1.1[a]	39.6±2.2[b]
Midrib	Control	63.5±1.4[b]	0	36.5±1.4[b]
	Endo-PG	39.8±2.1[c]	15.3±0.6[b]	45.0±1.5[a]
Rhamnose				
Lamina	Control	97.6±2.4[a]	0	2.4±2.4[c]
	Endo-PG	23.4±6.6[d]	11.0±2.4[a]	65.6±5.3[a]
Midrib	Control	77.8±6.6[b]	0	22.2±6.6[b]
	Endo-PG	49.2±5.9[c]	13.9±1.5[a]	36.9±7.3[b]
Fucose				
Lamina	Control	96.6±3.4[a]	0	3.4±3.4[b]
	Endo-PG	47.4±3.8[b]	35.0±1.8[a]	17.6±2.7[a]
Midrib	Control	70.0±12.8[b]	0	30.0±12.8[a]
	Endo-PG	58.3±3.3[b]	33.9±5.8[a]	7.8±4.4[b]
Arabinose				
Lamina	Control	97.0±0.4[a]	0	3.0±0.4[c]
	Endo-PG	43.3±4.5[c]	14.9±0.8[a]	41.9±5.2[a]
Midrib	Control	97.2±1.2[a]	0	2.8±1.2[c]
	Endo-PG	57.1±1.1[b]	12.0±0.5[b]	30.9±1.1[b]
Xylose				
Lamina	Control	96.5±0.6[a]	0	3.5±0.6[a]
	Endo-PG	52.7±3.3[c]	42.3±1.4[a]	5.0±1.9[a]
Midrib	Control	96.8±1.0[a]	0	3.2±1.0[a]
	Endo-PG	66.0 0.7[b]	29.8±1.2[b]	4.2±0.8[a]
Mannose				
Lamina	Control	88.0±0.8[a]	0	12.0±0.8[b]
	Endo-PG	45.3±3.0[b]	39.5±3.1[a]	15.2±0.8[ab]
Midrib	Control	79.4±3.9[a]	0	20.6±3.9[a]
	Endo-PG	54.0±2.1[b]	27.7±1.2[b]	18.4±2.9[ab]
Galactose				
Lamina	Control	87.9±0.3[b]	0	12.1±0.3[c]
	Endo-PG	55.0±2.8[c]	26.5±2.4[a]	18.5±1.0[b]
Midrib	Control	93.3±1.1[a]	0	6.7±1.1[d]
	Endo-PG	56.5±0.9[c]	19.4±1.3[b]	24.1±0.5[a]
Glucose				
Lamina	Control	87.7±1.5[a]	0	12.3±1.5[b]
	Endo-PG	45.9±4.3[c]	42.5±3.4[a]	11.6±0.9[b]
Midrib	Control	64.3±5.2[b]	0	35.7±5.2[a]
	Endo-PG	44.3±1.2[c]	25.6±1.4[b]	30.2±1.8[a]
Uronic acid				
Lamina	Control	97.1±0.3[a]	0	2.9±0.3[c]
	Endo-PG	37.5±0.2[c]	7.3±0.4[a]	55.2±0.4[a]
Midrib	Control	98.5±0.3[a]	0	1.5±0.3[c]
	Endo-PG	43.1±1.5[b]	8.7±0.8[a]	48.2±1.6[b]

Mean ± standard error of mean (n = 4)

[a,b,c] Values with different superscripts differ significantly ($P < 0.05$) within columns

The concentration of rhamnose and fucose were low compared with the measuring sensitivity, which resulted in high standard errors.

The soluble fractions were calculated by subtracting the > 1mm and < 1mm fractions from 100%.

4.4.4 Experiment 4 (immunocytolabelling with JIM5, JIM7, LM5 and LM6)

Cell separation occurred at the middle lamella between the walls of adjacent cells. The change in cell shape after treatment of chicory lamina and midrib tissues with endo-PG, CDTA, Na_2CO_3, citrate-phosphate buffer and acetate buffer are reported first below, followed by the results of immunolabelling.

4.4.4.1 Cell separation and cell shape change

The epidermal and hypodermal walls in the midrib of unincubated leaves of chicory were intact and adherent. After 12 h incubation in the sodium acetate buffer, the epidermal layer was sometimes separated from the hypodermal layer (Fig. 4.6C) although the walls were still intact. This separation was not observed after 1 h incubation (Fig. 4.6B), but 1 h was sufficient to cause separation of the layers with citrate-phosphate buffer. CDTA also caused a separation of the epidermis from the hypodermis and of the hypodermis from cortical parenchyma and, in addition, the epidermal and hypodermal cells were grossly distorted. Most of the radial walls within these two layers still adhered sufficiently to preclude total cell separation within the layers (Fig. 4.2D), but occasional breakage within the layers was observed, with some cells falling apart (Fig. 4.14D). Na_2CO_3 (Fig. 4.2E) and endo-PG (Fig. 4.2G) caused similar effects to CDTA.

All these treatments also caused separation of phloem cells (Fig. 4.4, Fig. 4.8). The separation was more significant in the outer phloem parenchyma than in the inner phloem parenchyma. Phloem outer parenchyma were all intact and had thick walls in unincubated leaves, but after incubation in an acetate buffer (Fig. 4.8B) or a citrate-phosphate buffer (Appendix 5O) for just 1 h, some cells were separated and the walls rounded. A similar effect was also observed with CDTA treatment. However, after treatments with Na_2CO_3 or endo-PG, these cells not only separated, but also became angular (Fig. 4.4E, 4.4F, 4.4G), suggesting a further loss of integrity.

4.4.4.2 Immunolabelling

Sections from treated segments were stained with the following antibodies: JIM5 against HG with a low degree of methyl esterification, JIM7 against HG with a high degree of methyl esterification, LM5 against galactan and LM6 against arabinan. The

results are presented in the following sequence: epidermis, parenchyma, vascular bundle from the midrib, and cells from lamina. The figures for citrate-phosphate are given separately, in appendix 5, since they were run with a different batch of chicory, with different controls.

4.4.4.2.1 JIM5 labelling

Epidermis from the midrib

In sodium acetate buffer, the density of JIM5 labelling had not changed after 1 h (Fig. 4.2B), but decreased slightly and evenly by 12 h (Fig. 4.2C).

Both CDTA and Na_2CO_3 treatments solubilised the JIM5 epitope in the outer regions of the epidermal and hypodermal walls, including the middle lamella (Fig. 4.2D and 4.2E). JIM5 labelling appeared stronger in CDTA-treated sections compared with Na_2CO_3-treated sections in walls of all cell types, suggesting that Na_2CO_3 treatment caused a greater loss of the epitope than CDTA.

Endo-PG degraded most of the JIM5 epitope in epidermal walls apart from in the inner regions of the walls near the cytoplasm (Fig. 4.2F, 4.2G).

Citrate-phosphate caused a loss of some of the JIM5 epitope (Appendix 5E), significantly from a narrow region between two adjacent cells opposite the radial walls in epidermis or hypodermis, but released the epitope evenly from other regions of walls (Appendix 5I).

Each of the treatments with endo-PG (Fig. 4.2F, Fig. 4.2G), CDTA (Fig. 4.2D) and Na_2CO_3 (Fig. 4.2E) caused a loss of the JIM5 epitope from the outer region of epidermal and hypodermal walls. These walls were intact, indicating that it is just a loss of the JIM5 epitope. The effect of Na_2CO_3 on the loss of the JIM5 epitope appeared greater than that of either CDTA or endo-PG. The differential loss of the JIM5 epitope between inner and outer regions of both epidermal and hypodermal walls did not occur with the sodium acetate buffer and citrate-phosphate buffer. The distribution of the JIM5 epitope in these two treatments was similar to that in unincubated leaves.

Parenchyma from the midrib

In the cortical parenchyma walls of the unincubated leaves, the JIM5 epitope was concentrated at the corners of intercellular spaces and in the middle lamella regions (Fig. 4.3A). This epitope was partially lost from the corners of intercellular spaces and the middle lamella regions after incubation in an acetate buffer for 12 h (Fig. 4.3C), but not for 1 h (Fig. 4.3B). This loss was greater after incubation in a citrate-phosphate buffer for 12 h (Appendix 5J). CDTA also caused this loss and the JIM5 epitope appeared as two separated parallel zones between pairs of adjacent cells (Fig. 4.3D inset). Na_2CO_3 had a stronger effect than CDTA, with the two zones separated more widely (Fig. 4.3E inset). Endo-PG also released the JIM5 epitope from the corners of intercellular spaces (Fig. 4.3F, 4.3G). This occurred in as little as 1 h (Fig. 4.3F).

Vascular bundles from the midrib

The JIM5 epitope in the walls of all the cell types in the vascular bundle was resistant to CDTA extraction although the epitope was lost from the conjunction regions of the walls among phloem and xylem parenchyma cells and in the middle lamella regions of the walls of these cells (Fig. 4.4D). In contrast to CDTA, relative to the walls of some small cells in the inner phloem which remained unseparated, the walls of some larger separated cells in the outer phloem were less bright after Na_2CO_3 extraction (Fig. 4.4E). A trace amount of the JIM5 epitope in the primary walls of xylem tracheary elements was resistant to Na_2CO_3 extraction also (see arrows in Fig. 4.4E). Phloem fibre walls still gave a bright fluorescence with JIM5 labelling after CDTA treatment (Fig. 4.4D1), but were dim after Na_2CO_3 treatment (Fig. 4.4E1). After endo-PG incubation for 1 h (Fig. 4.4F), the JIM5 epitope was released slightly, with further degradation being evident after 12 h (Fig. 4.4G). The JIM5 epitope did not change much after incubation in the sodium acetate buffer (Fig. 4.4B, 4.4 C), but was greatly decreased after treatment with citrate-phosphate (Appendix 5K).

Lamina

The JIM5 labelling in the walls of the stomatal guard cells after Na_2CO_3 treatment was more prominent than that in other cell types, indicating that the JIM5 epitope in guard cell walls was more resistant to Na_2CO_3 extraction than the walls of other cells (Fig. 4.5E). Similarly, this epitope in guard cell walls was also resistant to endo-PG treatment

(Fig. 4.5G, 4.5F). In addition, the JIM5 epitope in the walls of the mesophyll and leaf minor veins showed some resistance to endo-PG, but it was largely degraded from lamina epidermal walls. Acetate buffer (12 h) led to a slight loss of the JIM5 epitope (Fig. 4.5C), but a greater loss was apparent with the citrate-phosphate buffer (Appendix 5L).

Figure 4.2 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with JIM5 antibody, in the region of epidermis and hypodermis, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, 50 μm.

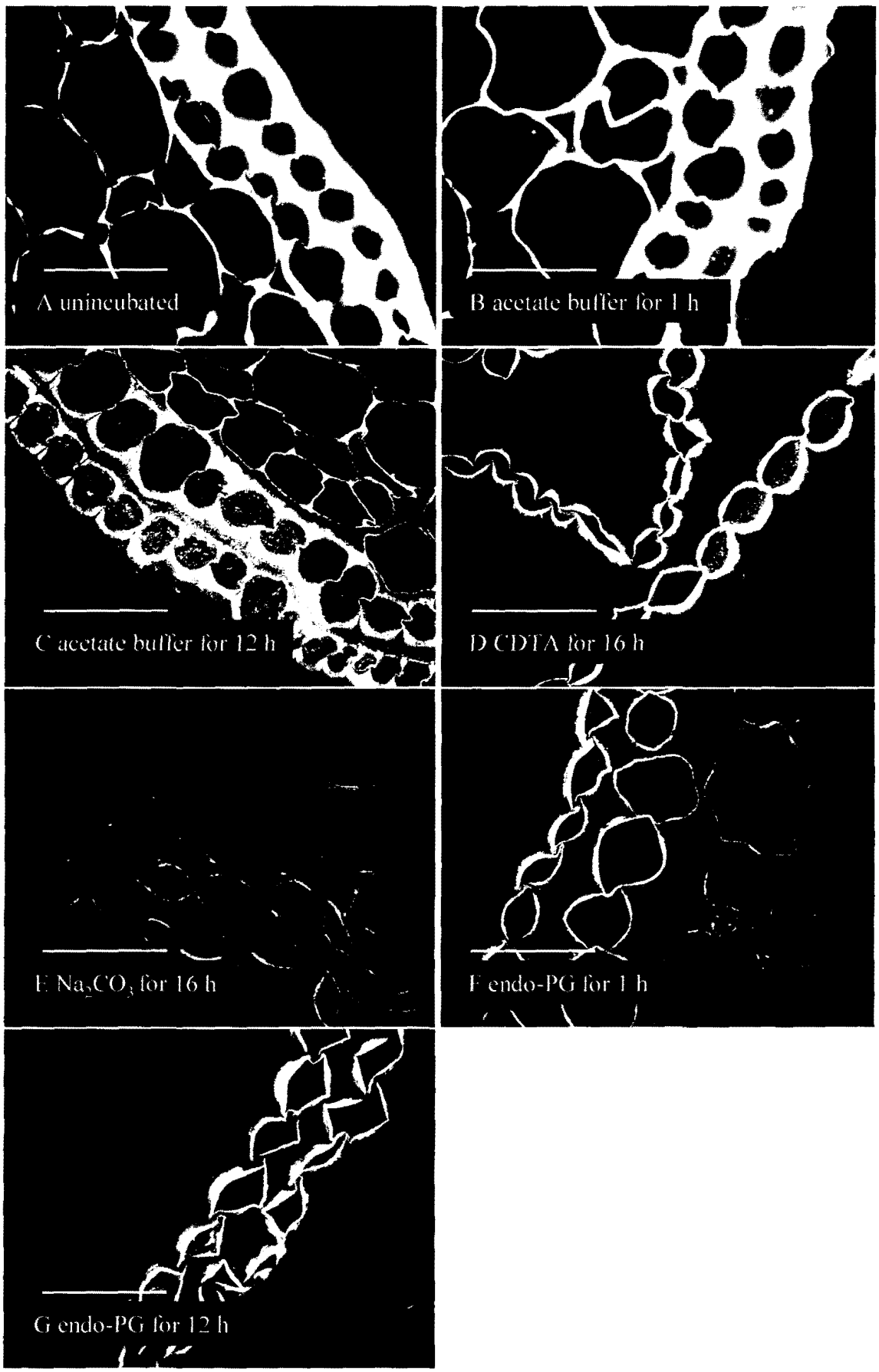

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.3 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with JIM5 antibody, in the region of cortical parenchymas, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Insets show enlargements. Bar, 50 μm.

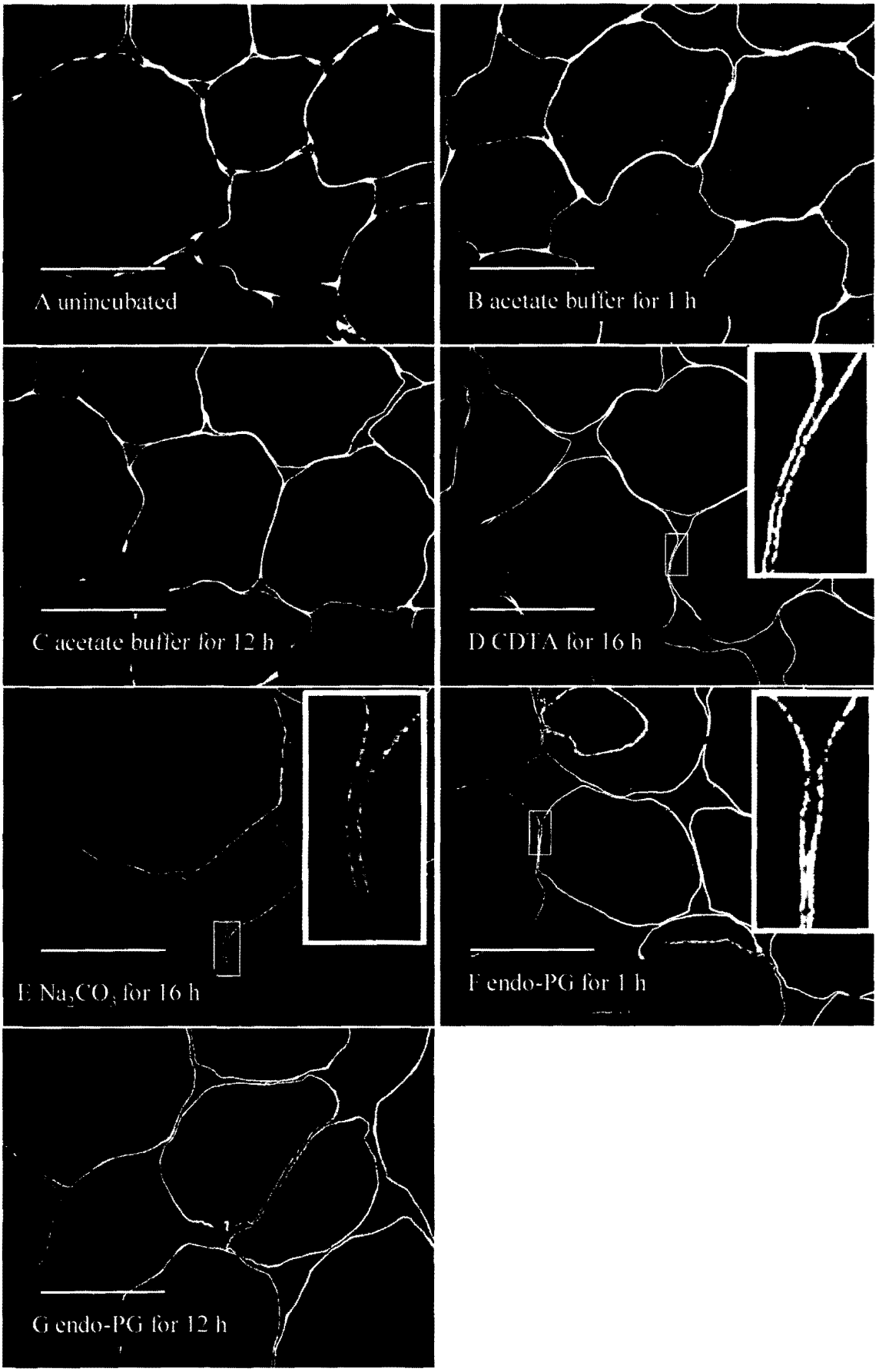
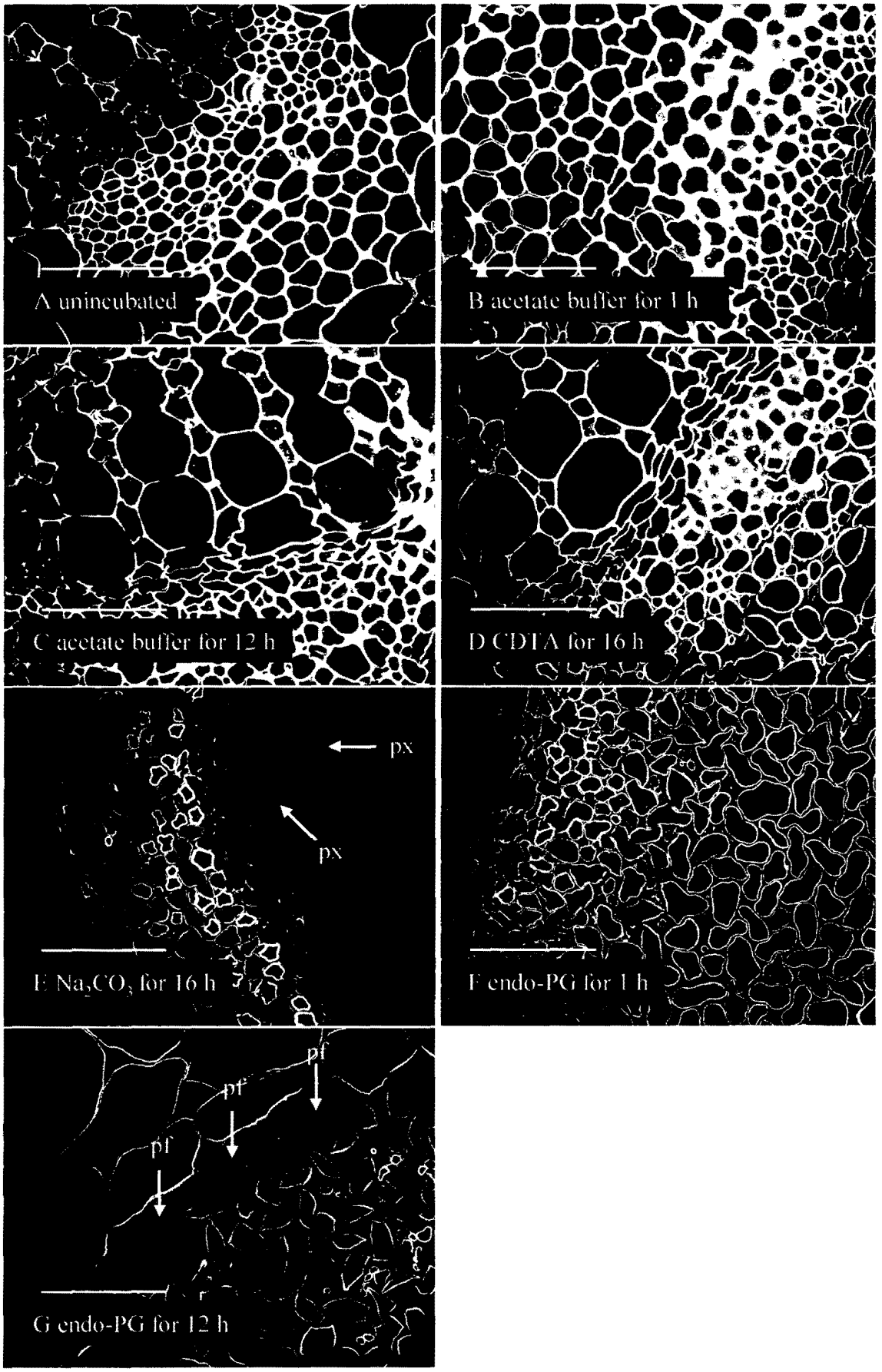

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.4 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with JIM5 antibody, in the region of vascular bundle, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** and **D1.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** and **E1.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, 50 μm. pf, phloem fibres; px, the primary walls of xylem tracheary elements.


A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
px
px
E Na_2CO_3 for 16 h
F endo-PG for 1 h
pf
pf
pf
G endo-PG for 12 h

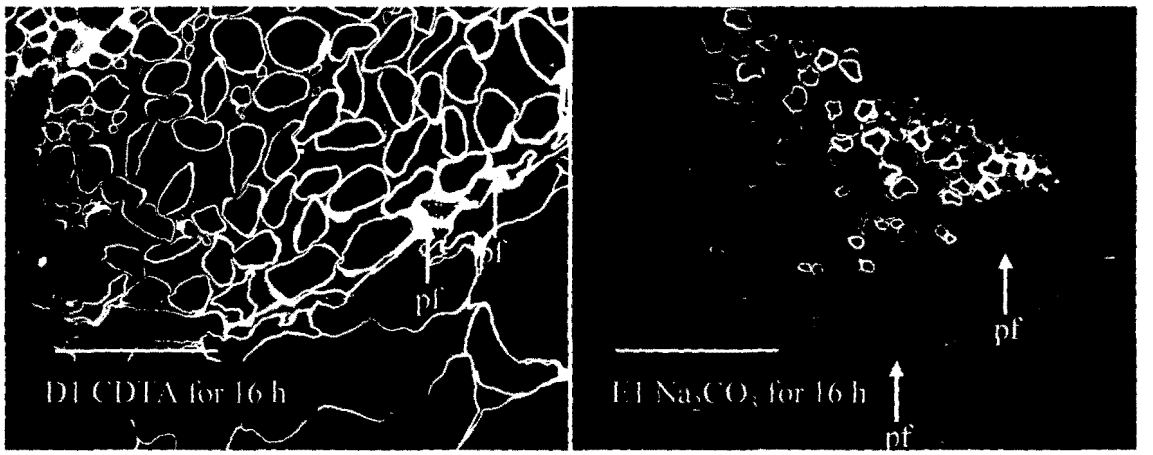
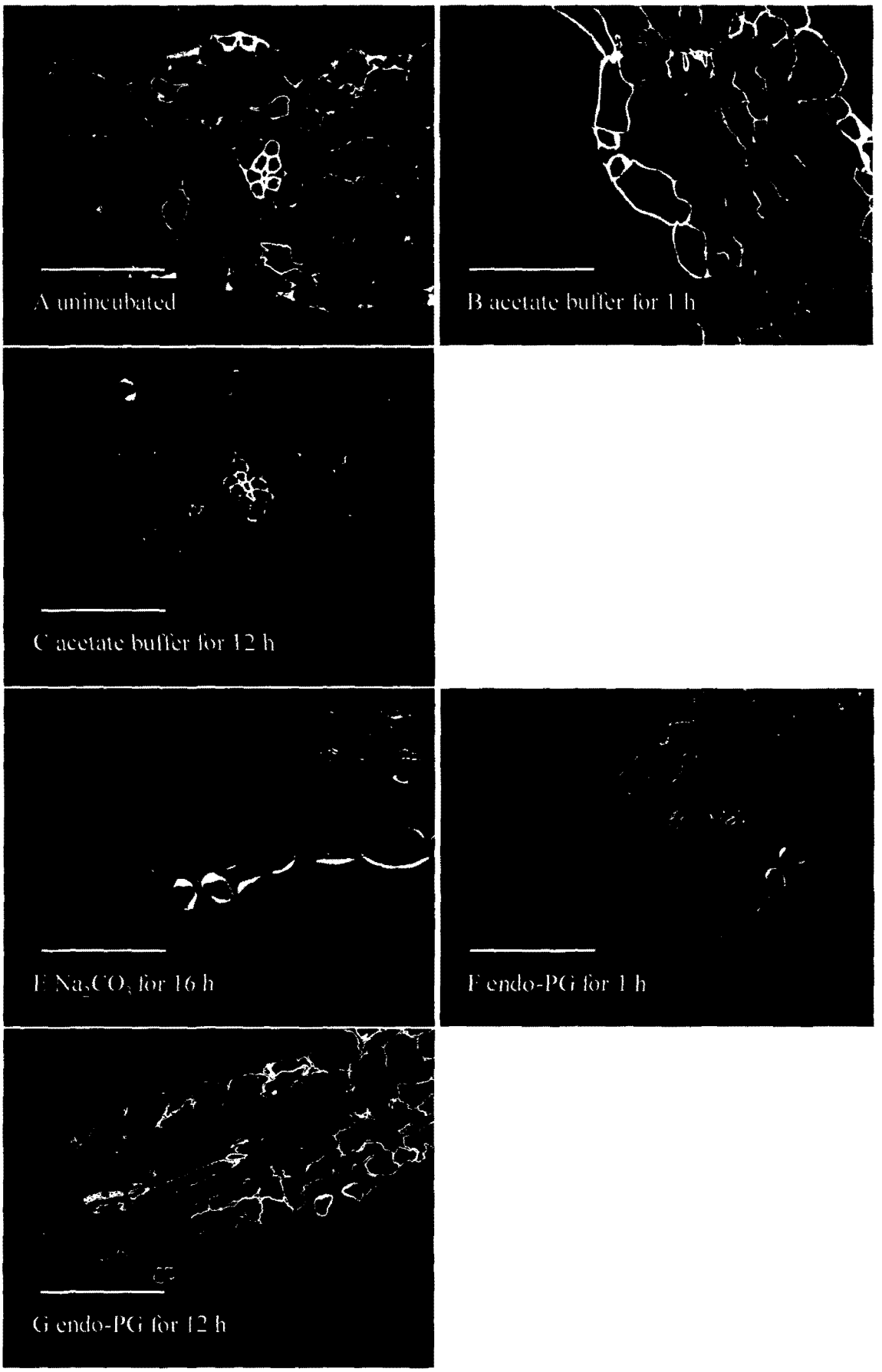

pf
pf
D1 CDTA for 16 h
pf
E1 Na_2CO_3 for 16 h
pf

Figure 4.5 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf lamina with JIM5 antibody, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in an sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, (E, H), 50 μm; (A, B, C, F, G), 150 μm.


A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

4.4.4.2.2 JIM7 labelling

Epidermis from the midrib

The JIM7 epitope was largely released from epidermal walls after either CDTA or Na_2CO_3 treatment, but Na_2CO_3 released more of the epitope than CDTA (Fig. 4.6D, Fig. 4.6E). As with the JIM5 epitope, a small amount of the JIM7 epitope was left in the inner layer of the epidermal and hypodermal walls after either CDTA or Na_2CO_3 treatment. After endo-PG treatments, the labelling intensity of the JIM7 epitope in the epidermal and hypodermal walls decreased, even after as little as 1 h. But a small amount of the JIM7 epitope in the inner layer of the epidermal and hypodermal walls was also resistant to endo-PG (Fig. 4.6F, Fig. 4.6G). This inner layer of these walls with JIM7 was thinner than that with JIM5 (cf Fig. 4.2). The epidermal walls in the sodium acetate buffer did not change significantly (Fig. 4.6C, Fig. 4.6B). In contrast to the JIM5 epitope, the JIM7 epitope was slightly released by citrate-phosphate buffer (Appendix 5F), but the epitope was completely lost from walls in the middle lamella region along the radial walls of epidermis or hypodermis, causing a rounded appearance of the cell in Appendix 5M (contrast, the rectangular epidermis cells in Appendix 5Q).

Parenchyma from the midrib

After incubation with the sodium acetate buffer, no loss of the JIM7 epitope was seen (Fig. 4.7B) even after 12 h (Fig. 4.7C). CDTA, Na_2CO_3 and citrate-phosphate all extracted the JIM7 epitope from the cortical parenchyma walls and completely from the middle lamella regions, and the corners of intercellular spaces (Fig. 4.7D, Fig. 4.7E, Appendix 5N). After 1 h of incubation with endo-PG, the JIM7 epitope in the cortical parenchyma walls, as observed with JIM5, was completely degraded in the middle lamella regions, and at the corner of intercellular spaces (Fig. 4.7F). In contrast to JIM5, the JIM7 epitope in the primary walls was greatly degraded within 1 h (Fig. 4.7F) and nearly completely after 12 h (Fig. 4.7G).

Vascular bundles from the midrib

The labelling intensity of the JIM7 epitope in the vascular bundles did not change much after treatment with the sodium acetate buffer, but the fluorescence intensity showed that the JIM7 epitope was lost from the corners of intercellular spaces in the phloem

(Fig. 4.8B, Fig. 4.8C). This was not observed with the JIM5 epitope. After either CDTA or Na_2CO_3 treatment, the JIM7 epitope remained in the walls of the sieve elements and their companion cells. This epitope also remained in the walls of the phloem fibre cells and the tracheary elements after CDTA extraction (Fig. 4.8D), but was lost after Na_2CO_3 extraction (Fig. 4.8E).

After endo-PG treatment (1 h), the JIM7 epitope was mostly lost from the vascular bundle walls, but the fibre cells and some of the inner phloem parenchyma were more resistant to endo-PG than others (Fig. 4.8F). After 12 h of endo-PG treatment, the JIM7 epitope was almost completely lost except for the walls of a few small cells in the inner phloem (Fig. 4.8G, Fig. 4.8G1).

Citrate-phosphate released very little JIM7 epitope from the cell walls of the phloem, but completely from the middle lamella regions, and the cell corners of intercellular spaces (Appendix 5O).

Lamina

The JIM7 epitope was more resistant to Na_2CO_3 extraction in the walls of the stomatal guard cells than in those of other cells in the lamina (Fig. 4.9E).

Compared to incubation in the sodium acetate buffer, endo-PG removed most of the JIM7 epitope from the walls of the epidermis and mesophyll. The loss of epitope was greater after 12 h (Fig. 4.9G) than 1 h (Fig. 4.9F), especially from the epidermal wals, where label was virtually absent after the 12 h treatment except for the walls of the guard cells, which were markedly resistant.

Citrate-phosphate had very little effect on the intensity of the JIM7 epitope on the walls of all cell types in the lamina (Appendix 5P).

Figure 4.6 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with JIM7 antibody, in the region of epidermis and hypodermis, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, 50 μm.

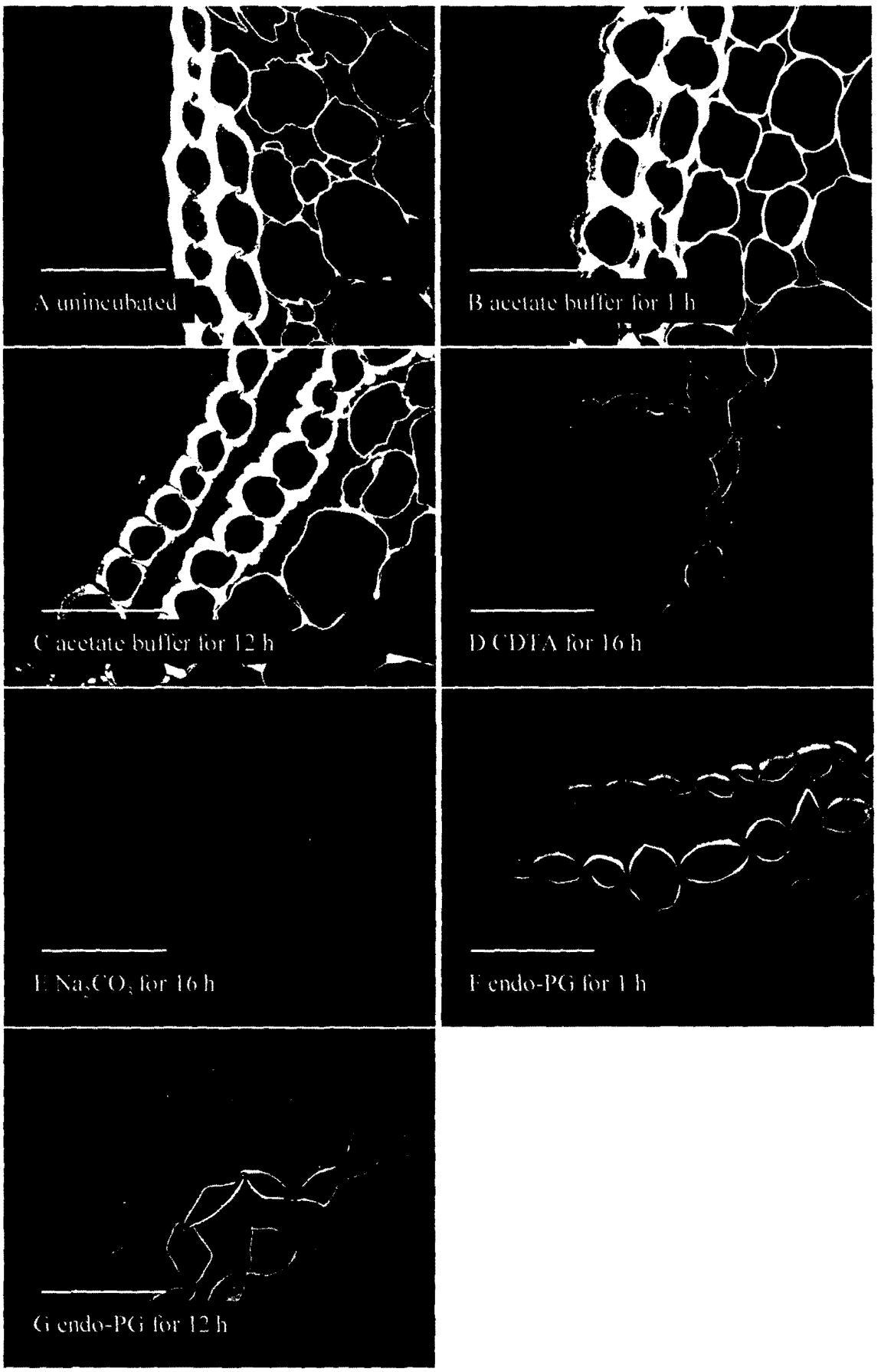

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.7 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with JIM7 antibody, in cortical parenchyma, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, 50 μm.

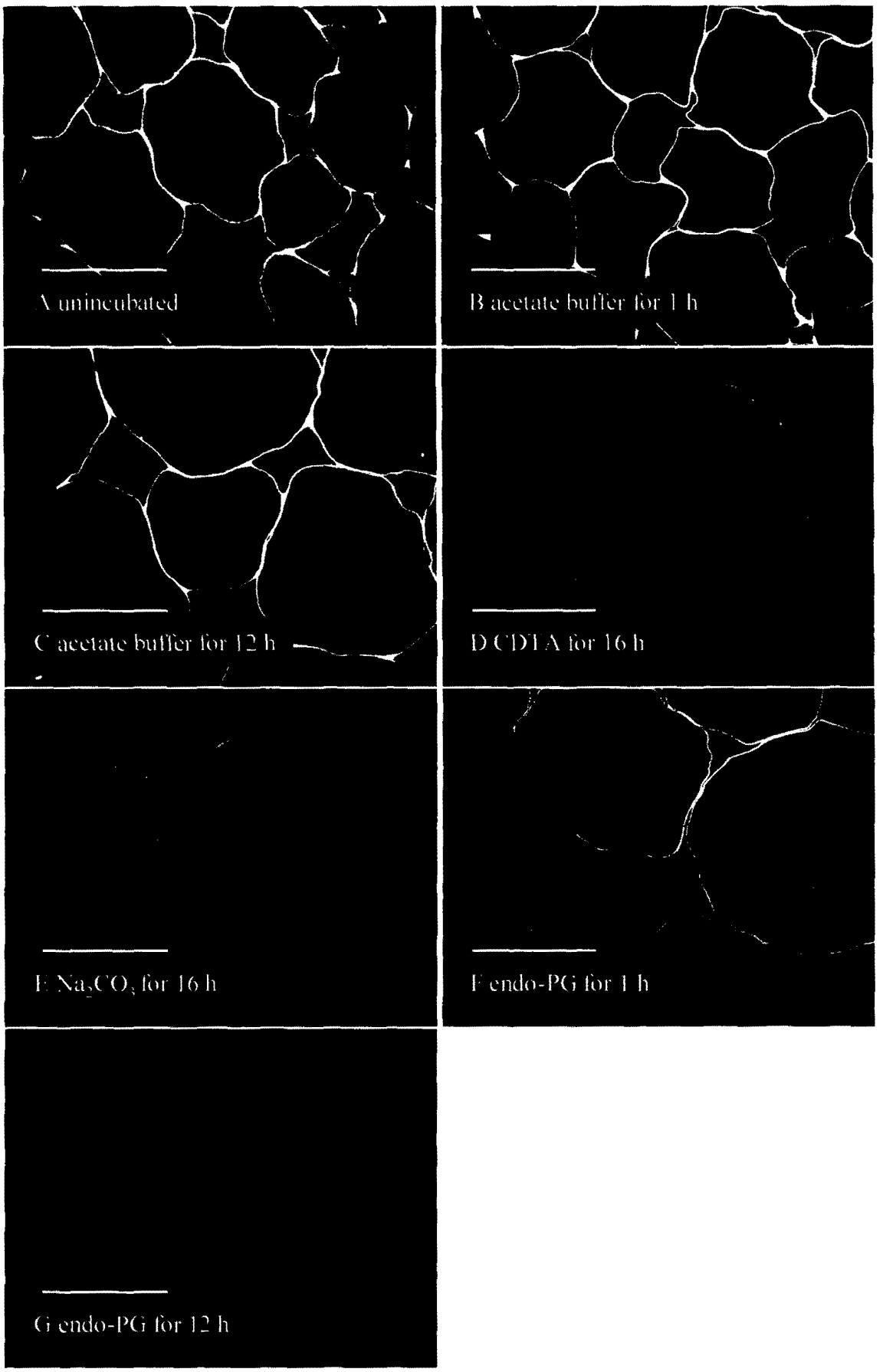

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.8 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with JIM7 antibody, in the region of vascular bundle, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (B.), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit/ml) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. **G1.** Same as (**G.**), but exposure time prolonged. Bar, (A, B, C, F, G, H, G1), 50 μm; (D, E), 125 μm.

← phloem fibres

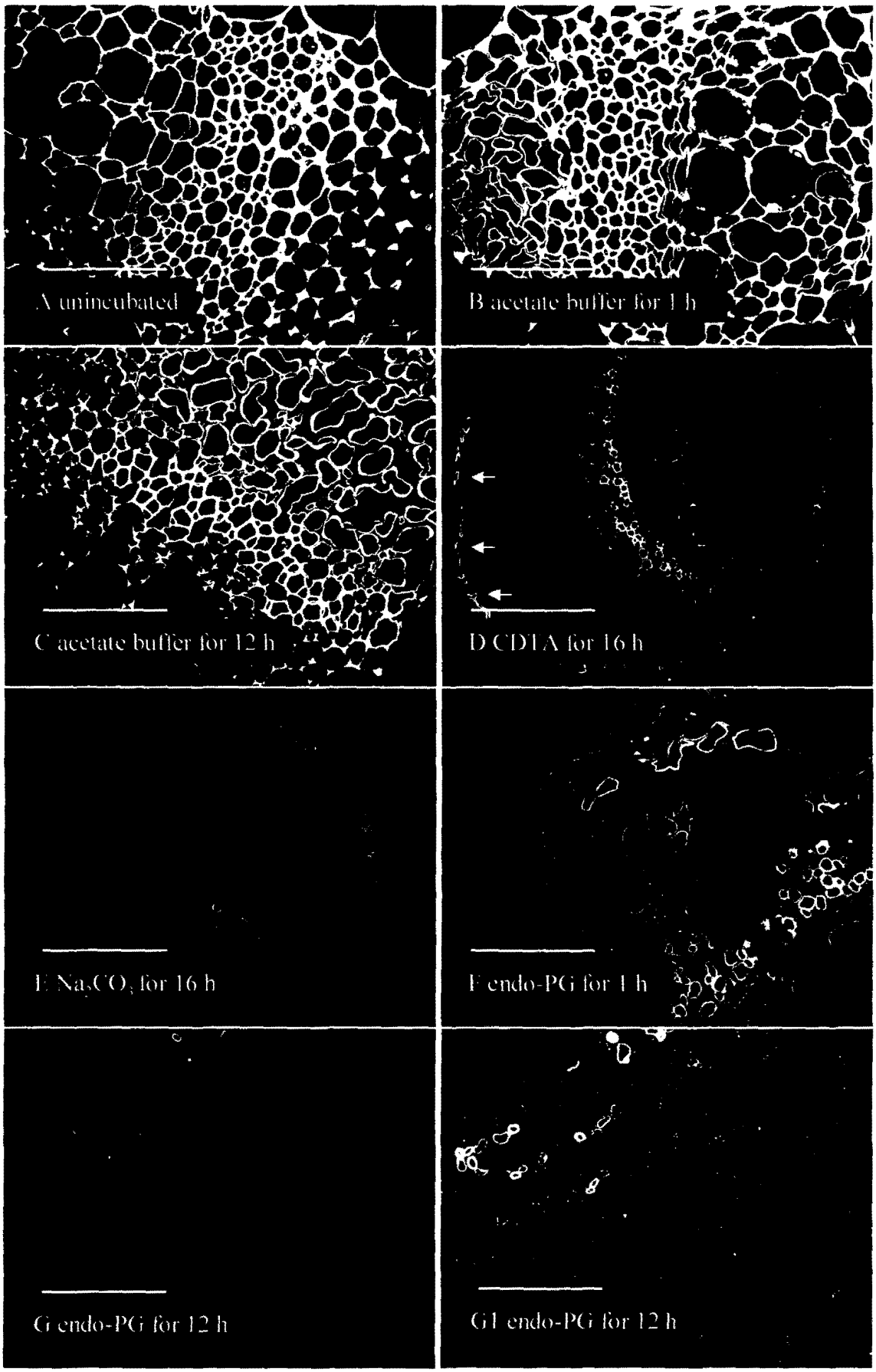
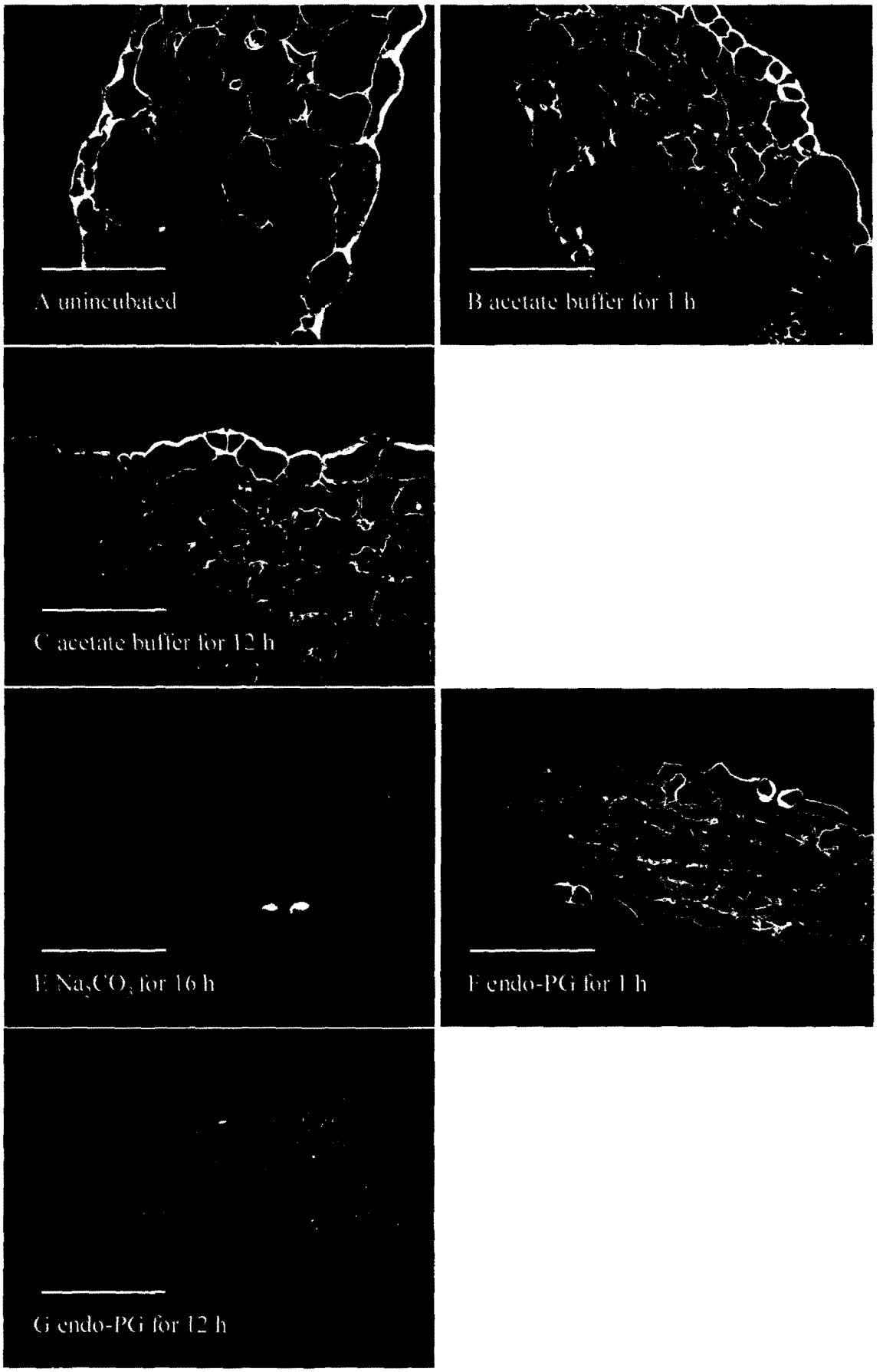

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h
G1 endo-PG for 12 h

Figure 4.9 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf lamina with JIM7 antibody, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 ^{o}C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 ^{o}C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 ^{o}C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 ^{o}C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, (C, E), 50 μm; (A, B, F, G), 125 μm; (H), 250 μm.


A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

4.4.4.2.3 LM5 labelling

Epidermis from the midrib

In the walls of the epidermis and hypodermis, the LM5 epitope was still prominent after endo-PG treatment (Fig. 4.10F, 4.10G), CDTA treatment (Fig. 4.10D) and citrate-phosphate treatment (Appendix 5Q), but the labelling decreased after Na_2CO_3 treatment (Fig. 4.10E).

Parenchyma from the midrib

The labelling intensity of the LM5 epitope in the walls of the cortical parenchyma did not markedly change after CDTA treatment (Fig. 4.11D), but it significantly decreased after Na_2CO_3 treatment (Fig. 4.11E). Endo-PG treatment for 1 h caused no loss of the LM5 epitope and only a slight decrease after 12 h compared with control (Fig. 4.11A, 4.11F, 4.11G). LM5 labelling decreased only slightly with citrate-phosphate (Appendix 5R), and not at all with the sodium acetate buffer (Fig. 4.11B, 4.11C).

Vascular bundles from the midrib

The LM5 epitope in vascular bundles was largely resistant to CDTA extraction, except for phloem fibre walls where the labelling was similar to that in walls of phloem parenchyma (Fig. 4.12D), not as bright as that in unincubated leaves. In contrast, the LM5 epitope in the walls of fibre cells was not affected by Na_2CO_3 treatment, while fluorescence of the walls of xylem parenchyma and xylem ray parenchyma was quite dim (Fig. 4.12E).

No significant effect on the LM5 epitope was observed with endo-PG treatment (Fig. 4.12F, 4.12G), or with citrate-phosphate (Appendix 5S).

Lamina

The LM5 labelling in lamina did not significantly change after endo-PG treatment for 1 h (Fig. 4.13F), but after 12 h treatment the intensity in epidermis was greatly reduced (Fig. 4.13G). Na_2CO_3 slightly decreased the LM5 labelling in lamina (Fig. 4.13E), but citrate-phosphate did not cause any loss of the LM5 epitope (Appendix 5T).

Figure 4.10 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with LM5 antibody, in the region of epidermis and hypodermis, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, 50 μm.

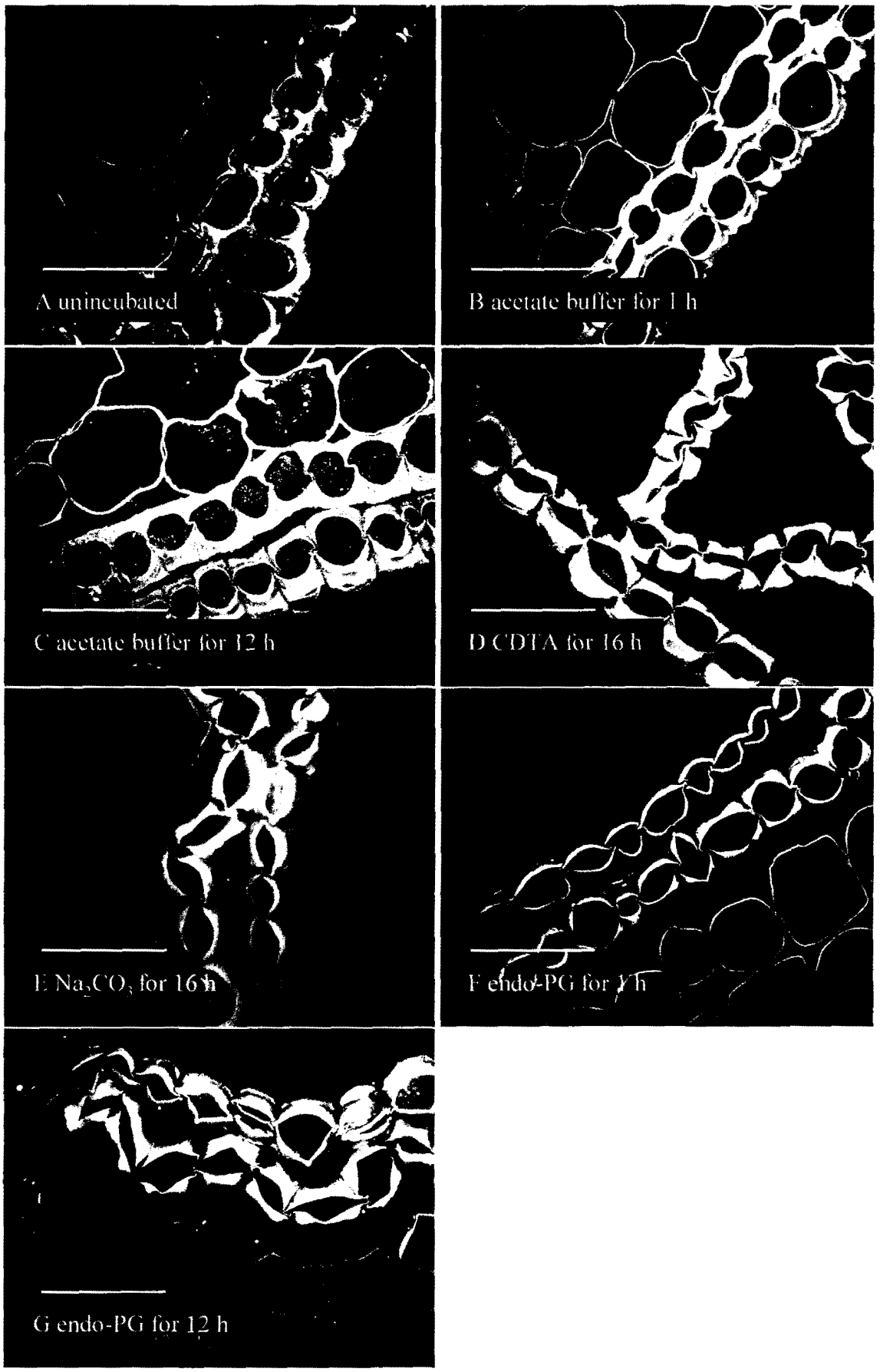

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.11 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with LM5 antibody, in cortical parenchyma, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3 and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, 50 μm.

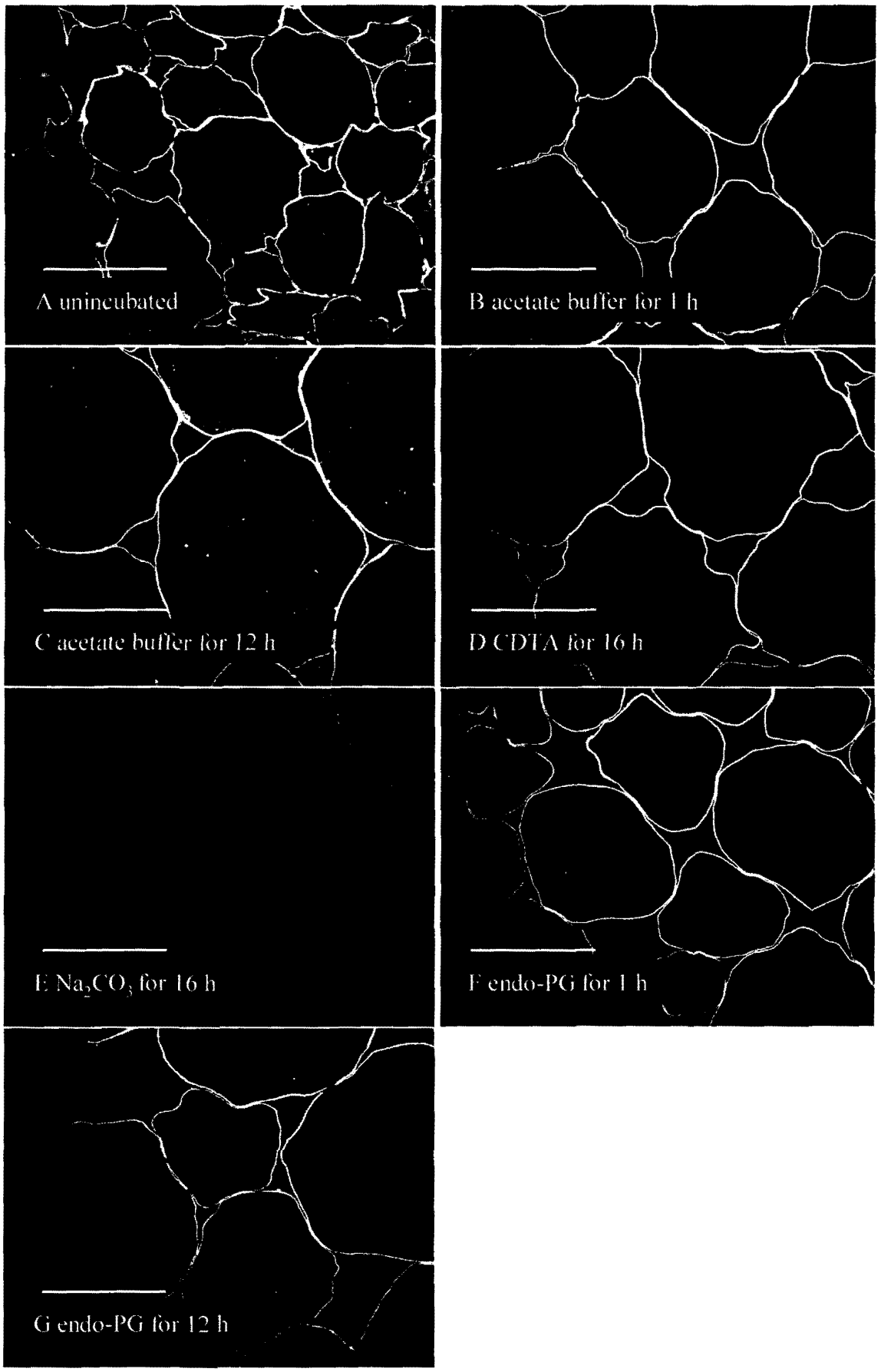

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.12 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with LM5 antibody, in the region of vascular bundle, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3, and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, (A, B, C, F, G, H), 50 μm; (D, E), 125 μm.

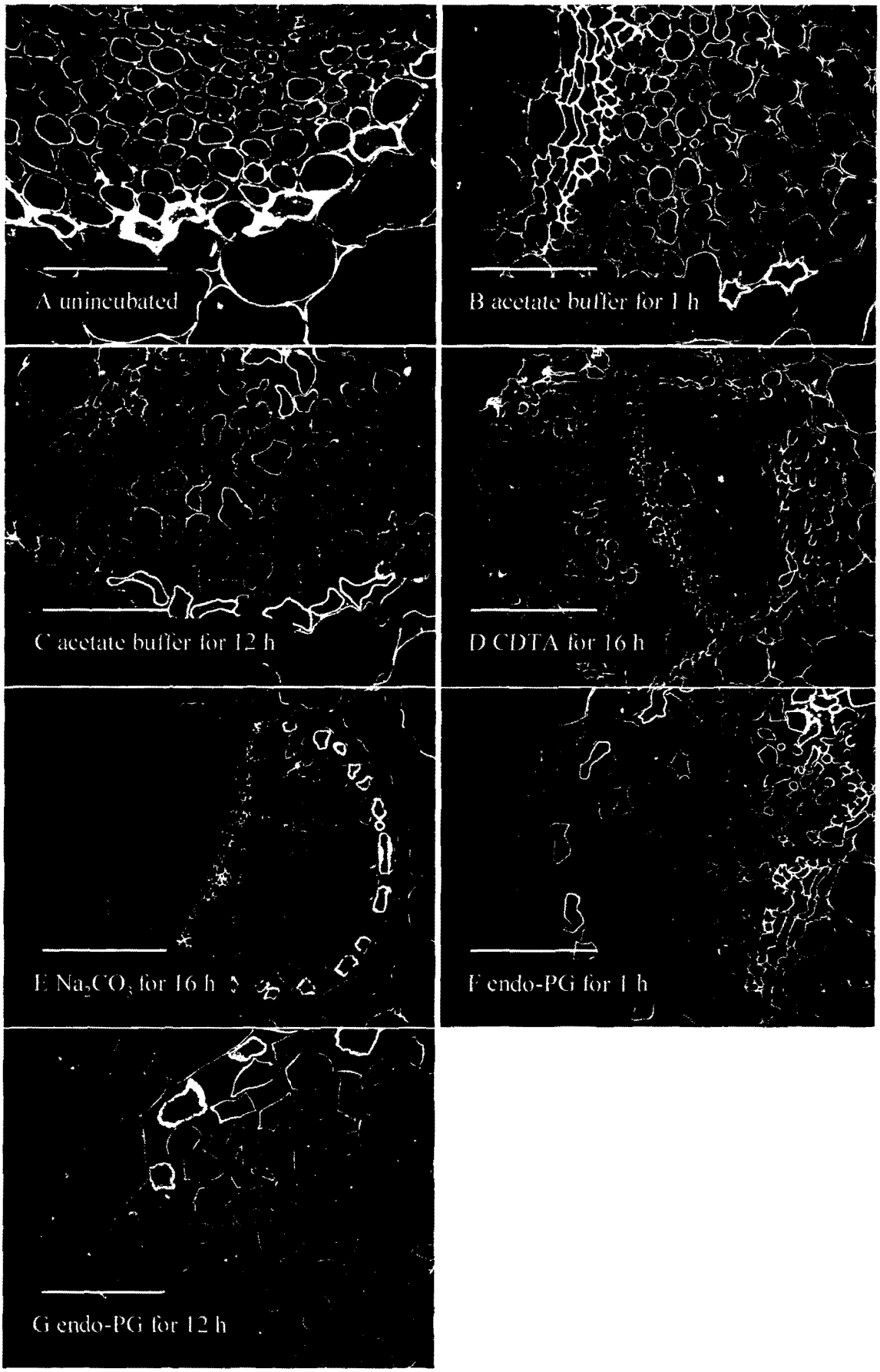
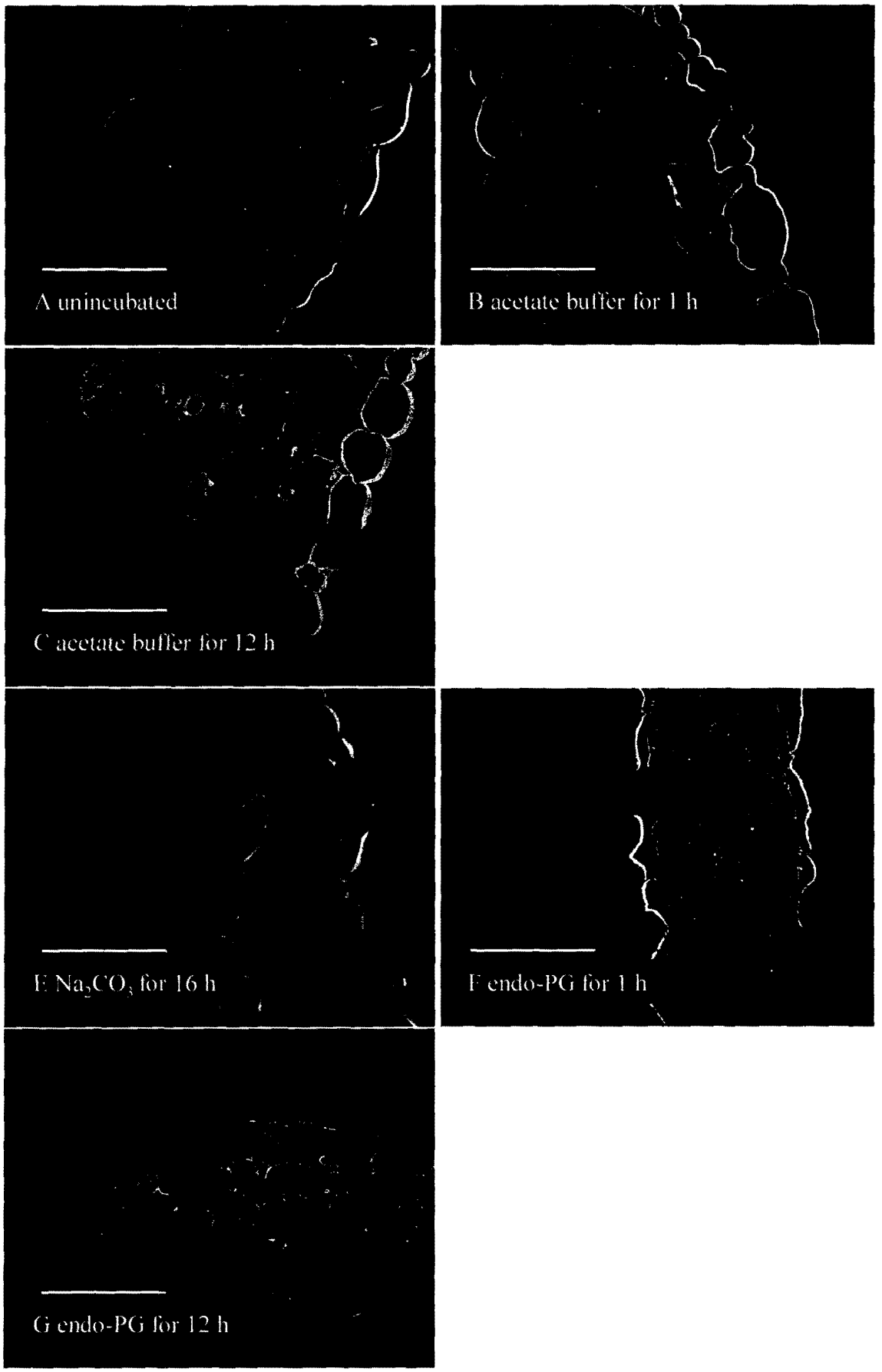

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.13 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf lamina with LM5 antibody, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3, and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, (E, H), 50 μm; (A, B, C), 125 μm; (F, G), 250 μm.


A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

4.4.4.2.4 LM6 labelling

Epidermis from the midrib

The LM6 epitope was appreciably released from the epidermal and hypodermal walls by endo-PG (1 h and 12 h) (Fig. 4.14F, 4.14G) and by Na_2CO_3 (Fig. 4.14E), but not by CDTA (Fig. 4.14D), citrate-phosphate (Appendix 5U) or sodium acetate buffer (Fig. 4.14B, 4.14C).

Parenchyma from the midrib

The loss of the LM6 epitope was not significant in an acetate buffer (Fig. 4.15C, Fig. 4.15B). Both CDTA and Na_2CO_3 released the LM6 epitope in the cortical parenchyma (Fig. 4.15D, Fig. 4.15E). After prolonging exposure time, it was observed that there was a single line of LM6 label after CDTA treatment (inset in Fig. 4.15D), whereas there was a double line after Na_2CO_3 treatment (inset in Fig. 4.15E). In contrast to LM5, endo-PG caused considerable loss of the LM6 epitope in parenchyma walls, especially from the region of the middle lamella to give a double line (Fig. 4.15F, Fig. 4.15G).

Vascular bundles from the midrib

The LM6 epitope in the walls of sieve elements and their companion cells showed resistance to CDTA to some extent, but sensitivity to Na_2CO_3 (Fig. 4.16D, Fig. 4.16E). As stated at the beginning of section 4.4.4, phloem parenchyma cells were separated and became angular with Na_2CO_3. This was obvious also in inner phloem except in the small rounded cells which still maintained their shape and had bright walls with both CDTA and Na_2CO_3. Inner phloem and xylem differed markedly in their relative reactions to CDTA and Na_2CO_3. With CDTA, some loss of LM6 epitope was apparent from sieve elements and their companion cells, but not from in the primary walls of xylem vessels and xylem parenchyma (Fig. 4.16D1). With Na_2CO_3 the epitope was obviously lost from the primary walls of xylem vessels and xylem parenchyma, but remained on the walls of sieve elements and their companion cells (Fig. 4.16E1). The LM6 epitope in vascular bundle walls exhibited a similar response to endo-PG when compared with those in parenchyma and epidermis (Fig. 4.16G, Fig. 4.16F), but a small amount of the LM6 epitope in some phloem parenchyma was resistant to endo-PG (Fig. 4.16G1).

Lamina

LM6 labeling of guard cell walls was still strong after Na_2CO_3 treatment, whereas that of other cell walls was weak (Fig. 4.17E). LM6 fluorescence was mostly lost after endo-PG treatment in all walls of leaf lamina except in guard cell walls (Fig. 4.17F, Fig. 4.17G) and the LM6 epitope in guard cell walls of midrib was also resistant to endo-PG (Fig. 4.14F). Citrate-phosphate buffer had no effect on the labelling density (Appendix 5X).

Overall, endo-PG removed much LM6 label except from guard cell walls. Na_2CO_3 also removed much LM6 label, but the epitope largely remaining in walls of guard cells and the small rounded inner phloem cells. CDTA had very little effect, as did citrate-phosphate and acetate.

Figure 4.14 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with LM6 antibody, in the region of epidermis and hypodermis, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3, and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, 50 μm.

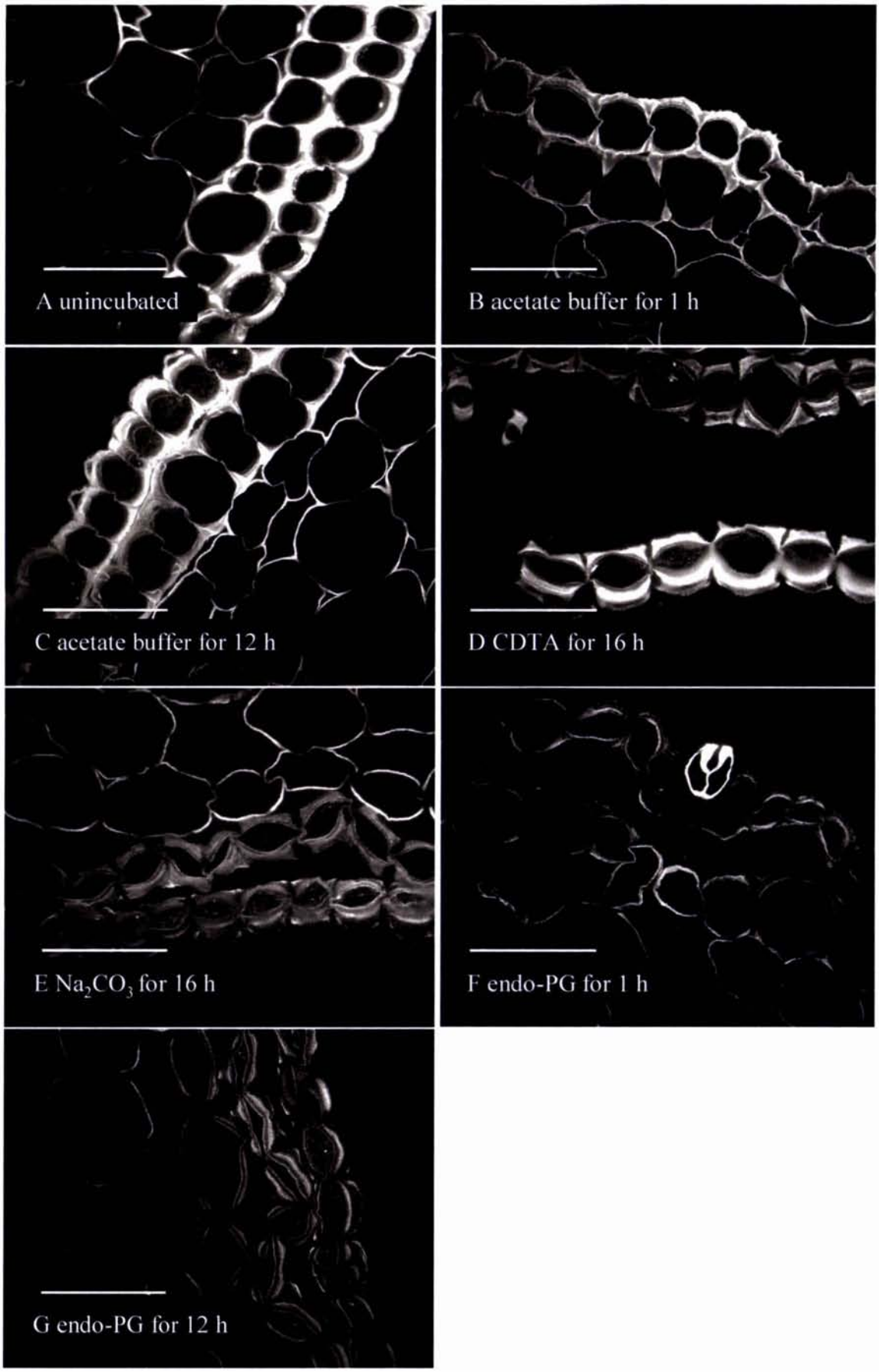

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.15 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with LM6 antibody, in the cortical parenchyma, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3, and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 ^{o}C for 1 h. **C.** Same as (B.), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 ^{o}C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 ^{o}C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 ^{o}C for 1 h. **G.** Same as (a.), but incubated for 12 h. Bar, 50 μm.

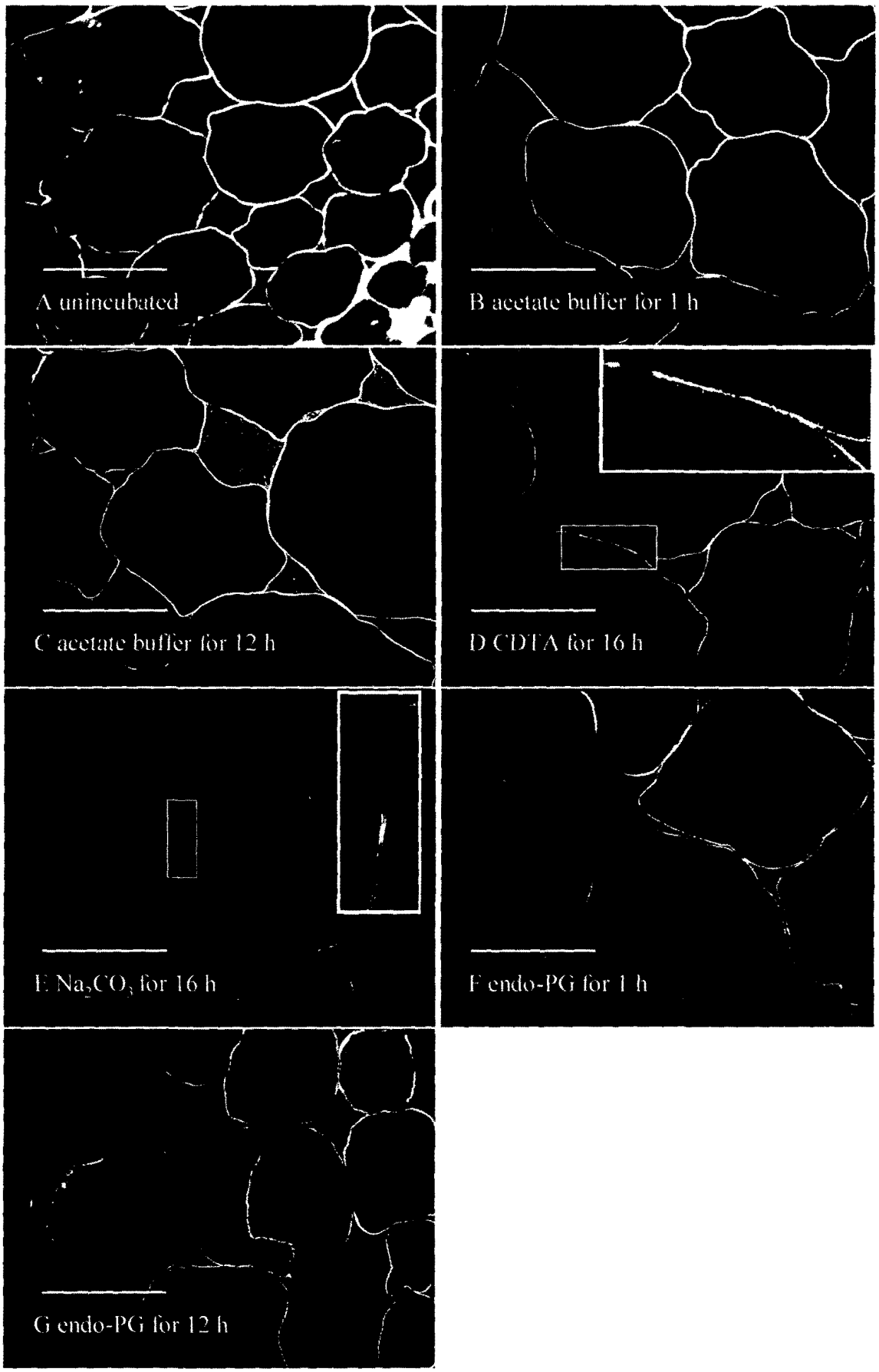
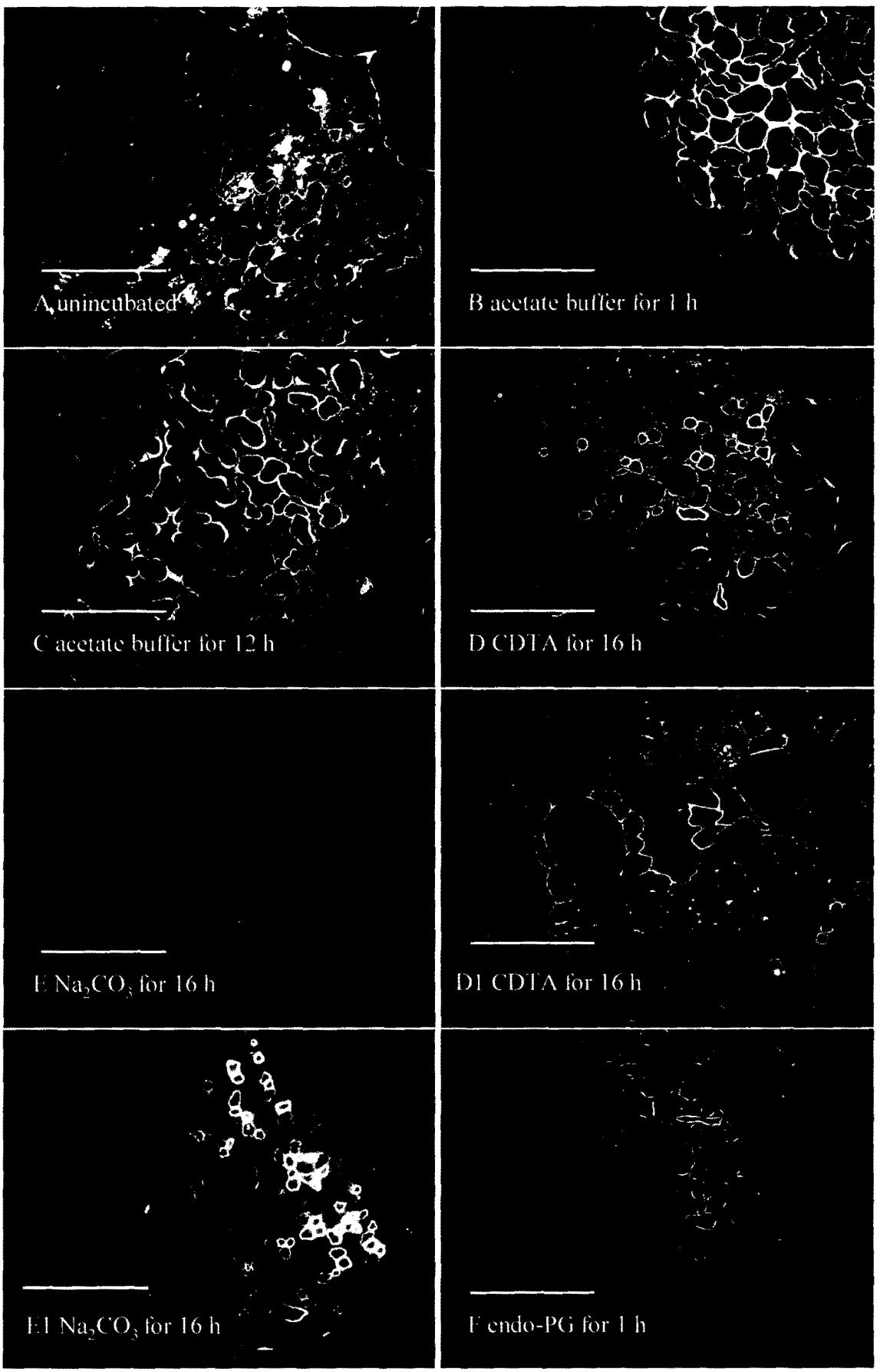

A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

Figure 4.16 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with LM6 antibody, in the region of vascular bundle, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3, and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (B.), but incubated for 12 h. **D., D1.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **E1.** Same section as (**E.**), but with prolonged exposure time. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same treatment as (**F.**), but incubated for 12 h. **G1.** Same section as (**G.**), but with prolonged exposure time. Bar, (A, B, C, D, F, G, H, D1), 50 μm; (E, E1), 125 μm.


A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
D CDTA for 16 h
E Na_2CO_3 for 16 h
D1 CDTA for 16 h
E1 Na_2CO_3 for 16 h
F endo-PG for 1 h

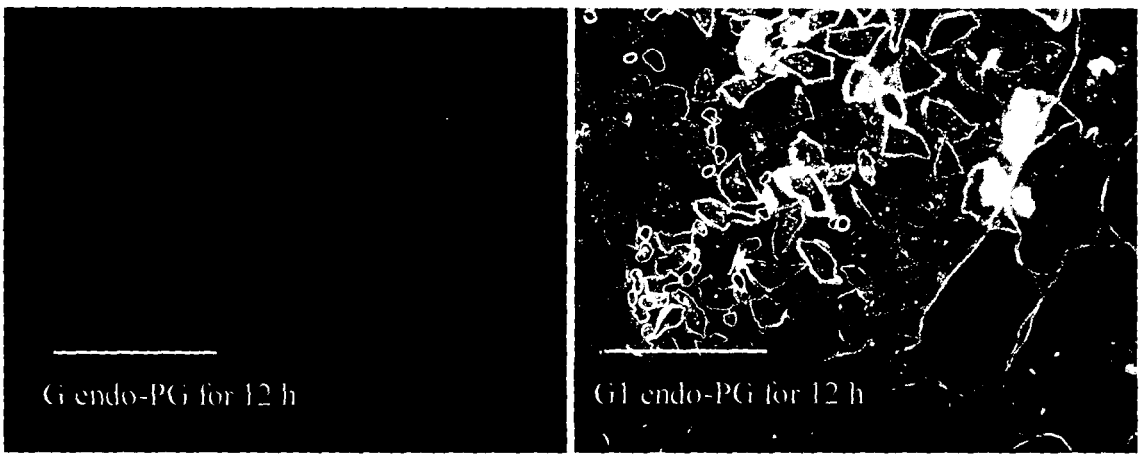
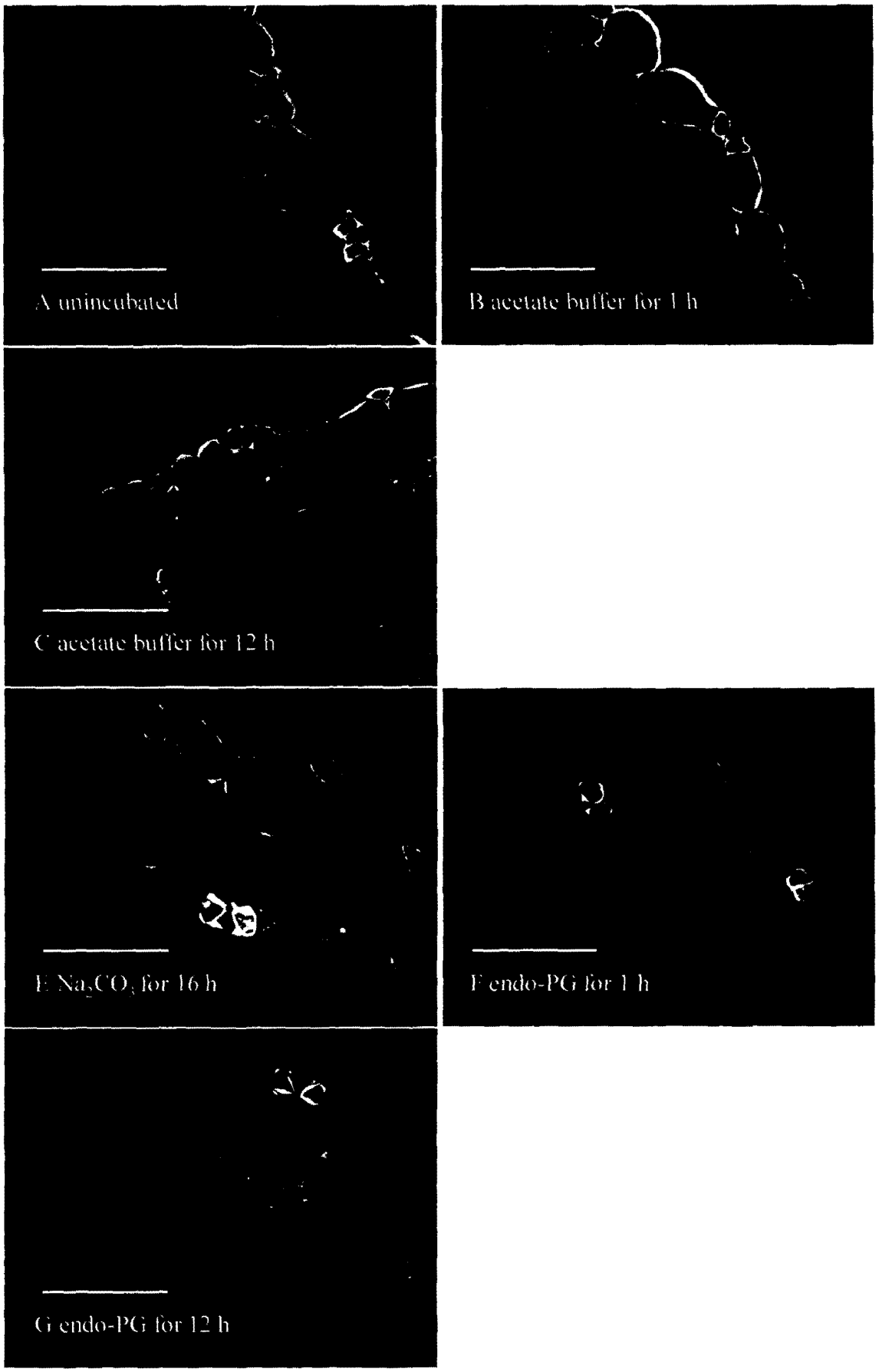

G endo-PG for 12 h
G1 endo-PG for 12 h

Figure 4.17 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf lamina with LM6 antibody, before and after treatments with sodium acetate buffer, CDTA, Na_2CO_3, and endo-PG. **A.** Before incubation. **B.** Incubated in a sodium acetate buffer (200 mM, pH 4.0) containing sodium azide (0.02%) at 37 °C for 1 h. **C.** Same as (**B.**), but incubated for 12 h. **D.** Incubated in CDTA (50 mM, pH 6.5) solution at 20 °C for 16 h. **E.** Incubated in Na_2CO_3 (50 mM) solution at 20 °C for 16 h. **F.** Incubated with endo-PG (1 unit ml^{-1}) in an acetate buffer (200 mM, pH 4.0) at 37 °C for 1 h. **G.** Same as (**F.**), but incubated for 12 h. Bar, (A, B, C, E, G, H), 50 μm. (F), 125 μm.


A unincubated
B acetate buffer for 1 h
C acetate buffer for 12 h
E Na_2CO_3 for 16 h
F endo-PG for 1 h
G endo-PG for 12 h

4.5 Discussion

One feature of chicory degradation in the rumen is its rapid particle breakdown. In order to better understand this rapid breakdown, the present study focused on cell adhesion and separation. The results show that endo-PG and some chemical reagents can cause chicory cell separation and further break down to small particles *in vitro*, which helps our understanding of cell adhesion and particle breakdown in the rumen. Some implications on structural functions of pectic polysaccharides were also obtained from results on the change of pectic epitopes after the treatments. Cell adhesion, particle breakdown and structural functions of pectic polysaccharides will be discussed in the following sections.

4.5.1 Cell adhesion

Cell adhesion is a fundamental function of plant cells and is generally thought to occur in the middle lamella of the wall. Different mechanisms for cell adhesion have been proposed for dicotyledons and the Poaceae.

4.5.1.1 Cell adhesion in dicotyledons

The destruction of cell adhesion will cause cell separation. CDTA, Na_2CO_3 and endo-PG all caused a separation between the epidermal and hypodermal layers observed by microscopy. But with vortex-induced cell separation, CDTA and Na_2CO_3 had no effect, whilst endo-PG had a dramatic effect. This difference indicates that cell separation in some tissues did not necessarily cause vortex-induced cell separation. Cell separation and vortex-induced cell separation are different concepts. The latter could be referred to as tissue disintegration. From this point, cell separation in the discussion refers to cell disconnection observed by microscopy.

Cell separation was observed with endo-PG or some chemical reagents. However, different effects of these treatments on cell separation were found amongst the different cell types. For example, separation between epidermal and hypodermal layers was found after 12 h incubation in sodium acetate buffer, but separation did not occur between cortical parenchyma cells. This suggests that different mechanisms for cell adhesion could be involved for different cell types. Therefore, the different cell types

will be discussed separately below. As the cortical parenchyma has been the subject of most research on cell adhesion, it will be discussed first.

4.5.1.1.1 Cortical parenchyma

Several mechanisms have been proposed for cell adhesion in dicotyledons. A prevalent hypothesis is that gels formed by linking calcium and unesterified homogalacturonans in the middle lamella have a role in cell adhesion (Jarvis et al., 2003). Calcium and unesterified HG are two important components for this hypothesis. CDTA can chelate calcium, whilst endo-PG can degrade unesterified HG (Chen and Mort, 1996). Therefore, both of them should cause cell separation in the middle lamella if this hypothesis is valid for chicory cell adhesion.

After endo-PG treatment, a double line in labelling with LM6 was observed, whereas the LM6 epitope was found in the middle lamella *in situ* (Chapter 3). Were these cells separated or just the LM6 epitope lost from the middle lamella? The distance between two LM6-labelled walls was wider compared with other treatments such as Na_2CO_3, indicating that cells were separated by endo-PG. In Chapter 3, the JIM5 epitope was found to be present more abundantly in the middle lamella, at the corners of intercellular spaces and at the tricellular junctions of cortical parenchyma compared with its labelling in the primary wall. In the current study, the JIM5 epitope was released from these regions by endo-PG. It can be concluded that endo-PG removed the JIM5 epitope from the regions involved in cell adhesion and caused cell separation. These results give support to the so-called "egg box" model whereby unesterified HG has a role in cell adhesion by forming dipolymer links via calcium (Jarvis et al., 2003).

In contrast to the endo-PG, the LM6 epitope remained in the region of the middle lamella of cortical parenchyma after CDTA treatment, indicating that these cells were not separated. The immunocytochemical study found that the JIM5 epitope was removed by CDTA from the region of the middle lamella and at the corners of intercellular spaces. CDTA is able to release HG with low degree of esterification by chelating Ca^{2+} (Jarvis et al., 1981) and about 50% of the total wall HG is solubilised by CDTA (Chapter 2). CDTA would disrupt calcium bridges and consequently separate cells. However, cell separation did not occur. This suggests that in addition to HG with low degree of esterification, other substances which are not solubilised in CDTA,

perhaps HG with high degree of esterification and/or RG I, could be involved in cell adhesion.

Highly esterified HG can be recognised by JIM7. The JIM7 epitope was found in the present study to be released from the region of the middle lamella, the corners of intercellular spaces and the primary walls after CDTA treatment; hence the JIM7 epitope for cell adhesion cannot be responsible for cell adhesion on its own. The loss of the JIM7 epitope may be due to the release of highly esterified HG fragments formed from the removal of highly unesterified HG by CDTA.

RG I remains for consideration as a candidate substance for cell adhesion. RG I can crosslink with diferulate ester (Fry, 1986). This linkage may play a role in chicory cell adhesion, but there is no evidence for significant amounts of ester-linked ferulate found in chicory walls (Section 2.4.2). McCartney and Knox (2002) working with pea testa also found vortex-induced cell separation did not occur with CDTA treatment. The LM5 epitope can be simply excluded since it is not located in the middle lamella and at the corners of intercellular spaces (Chapter 3). Therefore, the RG I backbone and/or arabinan or arabinogalactan could play a role in chicory cell adhesion. Peña and Carpita (2004) found that "*loss of highly branched arabinans and debranching of rhamnogalacturonan I accompany loss of firm texture and cell separation during prolonged storage of apple*". Therefore, arabinan may be more likely than the RG I backbone or arabinogalactan to be involved in cell adhesion, at least in apples. The possible role of RG I in cell adhesion does not conflict with the present result with endo-PG since endo-PG cleaves the HG backbone and this would cause release of RG I fragments, as shown by the arabinose and rhamnose in the present study.

A double line in the middle lamina region was observed with LM6 labelling in the Na_2CO_3 treatment. Since the middle lamina was labelled before the treatments (Chapter 3), the double line probably suggests cell separation. A double line was also seen with JIM5 and JIM7 labelling with Na_2CO_3 treatment. Pectic polysaccharides can be released by β-elimination action with Na_2CO_3 (Albersheim, 1959). This action may result in disruption of "egg-box" in the middle lamella and at the corners of intercellular spaces. Ester linkages can be broken with Na_2CO_3 (Jarvis et al., 1981). Therefore, Na_2CO_3 would break both calcium bridges for "egg-box" and ester linkages and further cause cell separation.

4.5.1.1.2 Epidermis and hypodermis

Cell adhesion in the epidermis and hypodermis is different from that in cortical parenchyma. A separation between the epidermal and hypodermal layers was sometimes observed in midrib tissue even after incubation in the sodium acetate buffer in the present study. Using the same conditions, no cell separation occurred with the cortical parenchyma. This difference suggests that the mechanism of cell adhesion between the tangential walls of the epidermis and the hypodermis is different from that between the cortical parenchyma walls. What might be this mechanism?

JIM5 labelling was low in the middle lamella that occurs between the tangential walls of the epidermal and hypodermal layers. In the present study, the sodium acetate buffer treatment caused some separation of these layers. From these observations, several possibilities can be considered: 1) there is no calcium bridge between two layers, 2) a weak calcium bridge exists, 3) calcium was removed by the sodium acetate buffer, 4) autolysis occurred. Since endo-PG promoted the separation between the two layers, calcium-associated HG could also be involved in the cell adhesion of the tangential walls between epidermal and hypodermal layers. As CDTA separated the tangential walls, calcium should be involved. The first possibility can be excluded, but the second possibility may be more likely because low concentrations of the JIM5 epitope were present in the tangential walls and the JIM5 epitope would be expected to be associated with calcium. Following incubation with acetate buffer, cell separation did not occur in other cell types apart from the tangential walls between the epidermis and hypodermis and between the cells of the outer phloem. Monosaccharide analysis with chicory tissues showed that only small amounts (about 3% or less) of arabinose and uronic acid were released following a 16 h incubation with acetate buffer (Table 4.3), suggesting that overall autolysis is minor, but in these particular regions autolysis could not be excluded. Therefore, possibilities 2-4 remain for discussion.

Recio et al. (2003) treated isolated cell walls from bean calli (*Phaseolus vulgaris* L.) with Tris-buffered phenol (pH 7.0) or boiling in 80% ethanol to obtain walls with a low enzymatic activity. These walls were then incubated in 50 mM sodium acetate buffer (pH 4-6) containing 150 mM NaCl or in 50 mM potassium citrate-phosphate buffer (pH 4-6) at 35°C for 12 h. They found the cell walls lost about 3% of their dry weight after incubation. Of the released weight, 90% was pectic polysaccharides, mainly composed

of CDTA-soluble HG, 10% in the form of monosaccharides. Thus the release of pectic polysaccharides occurred non-enzymatically. Therefore, it is quite possible that a weak calcium bridge exists in the middle lamella of two adjacent tangential walls of the epidermis and hypodermis that is susceptible even to reagents such as acetate buffer and/or autolysis.

Although cell separation occurred between the epidermal and hypodermal layers after incubation with the sodium acetate buffer, CDTA, Na_2CO_3, and endo-PG, cell separation within these layers was not observed with these reagents since the middle lamella can be detected with LM6 labelling. This observation indicates that adhesion between the radial walls within the epidermis and hypodermis is stronger than between the tangential walls between these two layers. This is consistent with the finding in Chapter 3 that the JIM5 epitope was concentrated in the middle lamella between the radial walls of the epidermis and hypodermis, but was less concentrated in the middle lamella of the tangential walls. As the JIM5 epitope was released from the radial walls with CDTA, Na_2CO_3 and endo-PG, Ca^{2+}-HG crosslinking could have a role in the adhesion. But the release of the JIM5 epitope did not result in cell separation; other mechanisms would be involved in cell adhesion in the radial walls. Since endo-PG did not cause cell separation with the radial walls, the additional mechanisms would be different from those for the cortical parenchyma. The present study did not provide enough evidence to suggest possible mechanisms.

4.5.1.1.3 Outer phloem parenchyma

The data in Chapter 3 show that, in contrast to the adjacent walls of the epidermal and hypodermal layers, the walls and the tricellular conjunctions of the outer phloem parenchyma were particularly rich in the JIM5 epitope. It can be postulated that the outer phloem parenchyma would exhibit stronger cell adhesion. However, in the present study, the outer phloem parenchyma cells were observed to be similar to the tangential walls between the epidermal and hypodermal cells in that the cells were separated after incubation with acetate buffer. Furthermore, the separation was more significant in the outer phloem parenchyma as it started after just 1 h incubation with the sodium acetate buffer. Why is cell adhesion between the outer phloem parenchyma cells less whereas the JIM5 epitope is concentrated there? CDTA and citrate-phosphate buffer had a more significant effect on these cell walls than acetate buffer, suggesting that Ca^{2+}-HG

crosslinking is also involved in cell adhesion of these cells. The Ca^{2+}-HG crosslinking between the outer phloem parenchyma cells is apparently weaker.

4.5.1.2 Cell adhesion in the Poaceae

In contrast to white clover and chicory, perennial ryegrass was not macerated by enzyme treatments. How might dicotyledonous leaves be macerated by pectic enzymes, whereas Poaceae leaves might not?

The structure and composition of dicotyledonous cell walls are different from those of Poaceae walls. Perennial ryegrass mesophyll walls contain only about 4.6% pectin (Chesson et al., 1995), and pectin is not a principal macromolecule in the middle lamella of the Poaceae (Jarvis et al., 2003). It is considered that cross-linking of GAXs via diferuloyl residue have a role for cell adhesion in the Poaceae (Jarvis et al., 2003). It would therefore follow that pectinolytic enzymes acting alone cannot lead to maceration in perennial ryegrass.

4.5.2 Structural function of pectins

4.5.2.1 Structural support from pectins

In addition to loss of HG from the walls of the epidermis and hypodermis by treatments with endo-PG, CDTA and Na_2CO_3, their cell shapes were greatly distorted, possibly due to the removal of HG. Cell distortion also occurred in the phloem parenchyma where cells became angular after both Na_2CO_3 and endo-PG treatments. In the epidermal walls of lamina, both the JIM5 and JIM7 epitopes were more degraded by endo-PG than those in the guard cells, mesophyll and minor leaf veins. These results support the hypothesis proposed in Chapter 3 regarding the role of a pectin hydrogel, formed from calcium bridging of low methyl HG, in maintaining the mechanical strength of cell walls. According to this hypothesis, the removal of HG by endo-PG, CDTA or Na_2CO_3 would cause disruption of the pectin hydrogel, consequently destroying wall integrity, as appears to have occurred in the epidermis and outer phloem in the present study.

4.5.2.2 Differential distribution of pectic polysaccharides within a wall

Data presented in this chapter indicated that the epidermal and hypodermal walls remained intact after treatments with endo-PG, CDTA or Na_2CO_3. However, labelling

with JIM5 and JIM7 showed that HG was removed more from the outer region than from the inner region of the walls. This suggests that the HG in the inner region of epidermal and hypodermal walls was more resistant to these treatments. The HG in the inner regions of these walls may therefore be structurally different from that in the outer regions, or shielded from endo-PG or CDTA treatment.

4.5.2.3 Connection of pectic polysaccharides to cellulose

The finding that the monosaccharide residues of cellulose and alkali-extractable polysaccharides were not released by endo-PG was predictable, as endo-PG degrades only pectins. It is also not surprising that uronic acid was partially released and that most JIM5 and JIM7 epitopes were lost after treatment with endo-PG, since endo-PG catalyses the hydrolysis of (1→4) linkages of homogalacturonan (Singh and Rao, 2002). However, why would arabinose and galactose be released also? Arabinose and galactose are mainly derived from arabinan and galactan, sidechains of RG I. HG and RG I are likely connected together in chicory leaf walls, as suggested by Ridley et al. (2001). Therefore, the degradation of homogalacturonan by endo-PG would cause solubilisation of RG I, with consequent loss of arabinan and galactan.

On the other hand, the present study found that about half of the total uronic acid was not released by endo-PG and there was a greater release of uronic acid than of arabinose or galactose. Immunocytochemical study also indicated that the LM6 epitope was more readily extracted by endo-PG than the LM5 epitope. These results are consistent with those in Chapter 2 where substantial amounts of pectic polysaccharides remained in the residue, with more galactan than arabinan remaining after alkali extraction. The results in the present study also support the hypothesis regarding the existence of a cellulose-pectin-xyloglucan complex in chicory cell walls, as proposed in Chapter 2. In that hypothesis, pectins are thought to partially bind to cellulose and xyloglucan, with cellulose and xyloglucan not degraded by endo-PG. This could be a reason why pectin remains after endo-PG treatment. In the cellulose-pectin-xyloglucan complex, galactan was proposed to be more tightly associated with cellulose than arabinan. This could explain why the release of arabinan is faster than that of galacatan.

4.5.2.4 Pectic polysaccharides in the walls of phloem sieve cells and companion cells

In the vascular bundles, the JIM7 epitope in the walls of the primary and secondary sieve elements and their companion cells was resistant to both CDTA and Na_2CO_3 treatments. The JIM5 epitope in the walls of sieve elements and companion cells in phloem cells was resistant to Na_2CO_3 extraction. The LM6 epitope in these walls was also resistant to Na_2CO_3 and to some extent to CDTA. Sieve tubes in *Asclepias speciosa* were specifically labelled by LM2, a monoclonal antibody binding to β-glucuronic acid residues in AGPs and also by *Ricinus communis* agglutinin, a lectin binding to terminal *β*-D-galactosyl residues (Serpe et al., 2001). The LM5 epitope was totally absent from sieve-tube cell walls in aspen (*Populus tremula* L. x *P. tremuloides* Michx.) (Ermel et al., 2000). The results in the present study, together with these authors' results, show that the walls of sieve elements and companion cells are structurally different from other cell walls.

4.5.2.5 Pectins in guard cell walls

In Chapter 3, it was found that the JIM5 epitope was located mostly in the outermost parts of stomatal guard cell walls, whereas the JIM7 epitope was dense throughout the walls and the LM6 epitope was particularly prominent in the stomatal guard cell walls. Polysaccharides containing these epitopes are considered important for the function of guard cells (Jones et al., 2005). On the other hand, the LM5 epitope was absent. That the JIM5, JIM7 and LM6 epitopes in guard cell walls were more resistant to endo-PG treatment and Na_2CO_3 extraction than in the walls of other cells in the lamina further supports the importance of these epitopes for guard cell function. These results also suggest that these epitopes in guard cell walls are more tightly bound to other polymers in the walls than are those in the walls of other lamina cells as they were resistant to endo-PG treatment or Na_2CO_3 extraction.

4.5.2.6 Pectins in the walls of phloem fibres

In Chapter 3, it was found that the primary walls of phloem fibre cells are distinguished from walls of other cells by their pectic polysaccharides. The primary walls are rich in the LM5 epitope and deficient in the LM6 epitope, but the distributions of the JIM5 and JIM7 epitopes are similar to those in other cells.

In the present studies, fluorescence in phloem fibre primary walls labelled with JIM5 and JIM7 did not significantly change following CDTA treatment. The labelling with JIM5 was still bright after CDTA treatment, indicating that Ca^{2+}-binding is not alone responsible for holding the HG in the walls. Large amounts of the JIM7 epitope remaining may result from little loss of low esterified HG as the JIM5 and JIM7 epitopes may occur in a linear molecule. The intensity of LM5 labelling in phloem fibre walls was similar to that in the walls of other cell types after CDTA treatment, suggesting partial loss of the LM5 epitope. Both the JIM5 and JIM7 epitopes were released from fibre walls by treatments with endo-PG and Na_2CO_3, but the LM5 epitope in these walls was somewhat more resistant to endo-PG and Na_2CO_3 treatments than in walls of other cell types. The resistance of the LM5 epitope to endo-PG and Na_2CO_3, and its loss after CDTA treatment suggests that some RG I and HG of phloem fibre walls are not linked covalently.

4.5.3 Chicory particle breakdown

As discussed in Section 4.5.1, it has been suggested that endo-PG removes HG from the middle lamella and the corners of intercellular spaces, resulting in cell separation. This mechanism could be responsible for the maceration of chicory leaves by the endo-PG reported in Section 4.4.1 and could be a basis for the rapid particle breakdown of chicory in the rumen.

Polygacturonases, pectin lyase and pectate lyase are able to degrade homogalacturonan. The major pectinolytic enzyme in the rumen, secreted from the principal pectinolytic rumen microbe *Lachnospira multiparus*, is pectate lyase (Preston et al., 1991). Although endo-PG hydrolyses (1→4) linkages next to free carboxyl groups whereas pectate lyases act by β-elimination, both of them are able to randomly split the homogalacturonan chain (Singh and Rao, 2002; Pilnik and Voragen, 1993). It is believed that the results obtained from endo-PG can adequately represent the function of pectate lyase. Since the optimum pH of commercial pectate lyase is 8, whereas pectins are most stable at pH 4 and deesterification and depolymerisation can occur below or above this value (Eriksson et al., 1997), endo-PG with the optimum pH at 4.0 was employed for the current study instead of pectate lyase.

Cell walls of dicotyledonous leaves are mainly composed of cellulose, alkali-extractable polysaccharides and pectin, together with some proteins, glycoproteins and lignin. Pectins are distributed in primary cell walls and the middle lamella between adjacent cell walls (Section 1.3.3.2, Chapter 1). The polysaccharides in the middle lamella are mainly pectins.

In this study it is obvious that both white clover and chicory were macerated with pectic enzymes. Immunolabelling work in this study showed that pectin in the region of the middle lamella and at cell corners of chicory was removed after pectic enzyme treatment, suggesting that maceration in chicory and white clover was caused by the removal of pectin in the middle lamella and at cell corners. This supported the suggestion that these forages could be broken down just with an enzyme, without mechanical action.

In the rumen, pectins are more rapidly and completely degraded than other components in cell walls. The half time of pectin degradation in rumen fluid is 4 h (Hatfield and Weimer, 1995). Therefore, pectins will be removed from cell walls before alkali-extractable polysaccharides and cellulose. Thus, pectin removal may cause tissue disintegration, i.e. maceration. Cheng et al. (1979) first reported maceration in white clover leaflets after incubation with *Lachnospira multiparus*. There is a wide range of bacteria in the rumen. The dominant species are *Fibrobacter succinogenes*, *Ruminococcus albus*, *R. flavefaciens* and *Butyrivibrio fibrisolvens* (Dehority, 1993; Miron and Ben-Ghedalia, 1993). They also secrete pectinolytic enzymes. Forages with a high pectin content will be susceptible to degradation by pectinolytic enzymes. Therefore, the removal of pectins from walls, leading to maceration, may partially explain the quick forage particle breakdown in the rumen and subsequently less rumination. Maceration is probably the primary event of cell wall degradation for pectin-rich forages in the rumen.

As for perennial ryegrass, there is a different situation as discussed in Section 4.5.1. Since cross-linking of GAXs appears involved in ryegrass cell adhesion (Jarvis et al., 2003), this may be why pectic enzymes were not able to macerate ryegrass leaves.

The present study showed that the crude pectinase preparation was more effective at particle size breakdown than pure endo-PG, probably because the crude pectinase

preparation containing other enzymes capable of hydrolysing cell-wall polysaccharides. In a preliminary experiment, some cellulase activities were found in the preparation. Spaguolo et al. (1997) reported synergistic effects of cellulolytic and pectinolytic enzymes in degrading sugar beet pulp. These authors suggested that the hydrolysis of pectins on the surface of the lignocellulosic substrate favours the degradation of cellulose and alkali-extractable polysaccharides by the respective enzymes. From the present study, it can be concluded that maceration, caused by the removal of pectin in the middle lamella, would probably give more cell wall surface area for attack by enzymes and consequently facilitate the degradation of cellulose by cellulase.

In conclusion, pectic enzymes appear to remove pectin in the middle lamella, which is associated with subsequent maceration and particle size reduction. This could explain why ruminants fed chicory require shorter or minimal rumination time compared with ruminants fed ryegrass. However, this hypothesis needs to be confirmed by a further study with rumen bacteria which will be explored in Chapter 5.

Chapter 5

The degradation of intact fresh chicory by rumen bacteria

5.1 Abstract

Little is known about the degradation of fresh chicory leaves by rumen bacteria. Large fragments of fresh chicory leaf midrib or lamina tissues were incubated with each of the following rumen bacteria: *Lachnospira multiparus* (pectinolytic), *Ruminococcus flavefaciens* (cellulolytic), *Butyrivibrio hungatei* (xylanolytic) and *Fibrobacter succinogenes* (cellulolytic and pectinolytic). Weight-loss measurements showed that *L. multiparus* and *F. succinogenes* degraded chicory leaves whereas *R. flavefaciens* and *B. hungatei* did not. *L. multiparus* degraded midrib more rapidly than lamina, but midrib was degraded most extensively by *F. succinogenes*. The rates and extent of monosaccharide release during incubation with *F. succinogenes* and *L. multiparus* were measured, showing that *L .multiparus* did not release glucose, xylose or mannose from midrib or lamina tissues but degraded pectins extensively (95%) and rapidly in both tissues and released uronic acids. *F. succinogenes* also released uronic acids extensively but at a slower rate than *L. multiparus* and also released glucose, xylose and mannose. *L. multiparus* released the pectin-associated monosaccharides arabinose, galactose and rhamnose simultaneously with uronic acids, whereas *F. succinogenes* released these monosaccharides sequentially in the order uronic acid, rhamnose, and then arabinose and galactose together. An immunocytochemical study on degraded tissues labelled with targeted monoclonal antibodies showed that the JIM5 and JIM7 epitopes were almost completely, the LM5 and LM6 epitopes largely, lost from the walls of all cell types after incubation with *L. multiparus* for 24 h. These epitopes in the walls of vascular bundles, such as the walls of some phloem parenchyma and xylem parenchyma, remained after degradation with *F. succinogenes* for 24 h, but the labelling strength was weak. The study also showed that the cortical parenchyma cells were separated after incubation with *L. multiparus*.

5.2 Introduction

In previous chapters, an analysis of structural polysaccharides and an investigation into the location of pectic polysaccharides in the cell walls of chicory leaves showed that an abundance of pectins is a feature. Pectic polysaccharides accounted for 67% of the total wall polysaccharides in the laminae and 58% of those in the midribs (Chapter 2). The location of some pectic polysaccharides varied between cell types (Chapter 3). Results from incubation tests with endopolygalacturonase (Chapter 4) supported the hypothesis

that pectin degradation is likely to be important in the breakdown of chicory in the rumen. There have been no studies on the degradation of pectin in chicory by rumen bacteria. During an investigation into chicory as a forage for ruminants (Hoskin et al., 1995, 1999; Kusmartono et al., 1997; Min et al., 1997; Burke et al., 2000), the breakdown of pectins received little attention.

The degradation of pectins has been studied in the forage legumes lucerne (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) (Chesson and Monro, 1982; Hatfield and Weimer, 1995). Chesson and Monro (1982) freeze-dried lucerne and red clover and ground them to 1 mm particles before incubation in nylon bags in the rumen of a sheep. They found that the hot-water and oxalate-soluble galacturonans were lost faster than other structural polysaccharides from whole plants and from leaf and stem fractions. The two types of galacturonans were lost at approximately the same rate. Hatfield and Weimer (1995) incubated isolated cell walls from lucerne leaf and stem tissues with mixed cow ruminal microbes, and found that more total uronosyl residues were released from leaves than from stems, and that pectin-associated monosaccharide residues were lost at different rates from leaves and stems. These studies using rumen fluid *in vitro*, and previous *in vivo* studies on chicory digestion (Hoskin et al., 1995, 1999; Kusmartono et al., 1997; Min et al., 1997; Burke et al., 2000), have measured digestion by mixed rumen microbes, with the result that the effects of different types of rumen bacteria on pectin degradation could not be distinguished.

In the rumen, the most well-known pectinolytic bacterium is *Lachnospira multiparus*. This bacterium, first recorded by Bryant and Small (1956), does not degrade cellulose. Bryant et al. (1960) found that the rumen of cattle fed pectin-rich forage legumes contained a large population of *L. multiparus*. *Prevotella* (formerly *Bacteroides*) *ruminicola*, *Streptococcus bovis* (Hobson and Stewart, 1997) and *Butyrivibrio fibrisolvens* (Dehority, 1993) are also known to degrade pectins. *S. bovis* has been shown to have polysaccharide depolymerases for the degradation of pectic polysaccharides, but does not have the glycosidases necessary for utilising oligogalacturonides (Dehority, 1993). Some cellulolytic ruminal bacteria have been found to degrade, but not utilise, pectic polysaccharides (Dehority, 1993).

In their *in vitro* studies on pectin degradation (Chesson and Monro, 1982; Hatfield and Weimer, 1995), forage materials were freeze dried, ground to small particles and

sterilised by autoclaving. These pre-treatments are expected to change the physical properties of the tissues. To better simulate the normal conditions *in vivo*, in the present study, fresh, intact chicory tissues were chosen and efforts made to retain tissues in a near natural state. Substrates were not autoclaved but were sterilised using antibiotics in a pre-treatment process.

Work in Chapter 2 has revealed that the lamina and midrib portions of chicory leaf are different in their anatomical and chemical structure, and the lamina and midrib tissues have been found to be different with respect to degradation by endopolygalacturonase (Chapter 4). Therefore, incubation studies with pure culture of ruminal bacteria were carried out with laminae and midribs separately.

Immunocytochemical studies have been carried out to monitor changes in plant cell walls during degradation by rumen bacteria (Migne et al., 1996a, 1996b, 1996c, 1998, 1999). These studies focused on changes in the feruloyl-arabinose, *p*-coumaric acid and arabinoxylan moieties in the degradation of maize-stem cell walls using immunogold techniques. There have been no immunocytochemical studies on the degradation of pectic polysaccharides in forage cell walls during breakdown by ruminal bacteria. In this study, efforts were made to apply fluorescence labelled antibodies to obtain information on the degradation of pectic substances in forage chicory cell walls.

The aim of this study was to investigate the susceptibility of intact chicory leaf lamina and midrib tissues to degradation by cell-wall degrading bacteria from the rumen growing *in vitro*, and to identify species-specific effects on the breakdown of pectins in chicory leaves.

5.3 Materials and methods

5.3.1 Plant material

Chicory plants were grown in a glasshouse for 4 weeks and then outdoors for 4 weeks. The first to the third expanded leaves (ca. 20 cm in length x 4 cm in width), as described in Chapter 2, were harvested and immediately used for experiments.

5.3.2 Rumen bacteria

Four cell-wall degrading bacteria were selected for study. The bacteria were the pectinolytic bacteria *Lachnospira multiparus* D32 and *Butyrivibrio hungatei* Su6C, and the cellulolytic bacteria *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* YL228. The bacteria were obtained from the culture collection of the Rumen Microbiology Unit at AgResearch Grasslands, Palmerston North, New Zealand.

5.3.3 Incubation media

The anaerobic techniques of Hungate (1966) were used in the preparation of media, inoculation and incubation. The basal anaerobic medium (BY) was based on that of Joblin et al. (2002). This medium consisted of, per litre: salt solution A (170 ml), salt solution B (170 ml), centrifuged bovine rumen fluid (300 ml), $NaHCO_3$ (5 g), distilled water (360 ml), 10 drops resazurin (0.1%, w/v), yeast extract (1 g) and L-cysteine hydrochloride (500 mg, L-cyst-HCl). All components, except L-cyst-HCl, were added to an Erlenmeyer flask and mixed thoroughly. The solution was boiled under O_2-free CO_2 for 3-5 min, cooled under O_2-free CO_2 by immersing in an ice bath and L-cyst-HCl added. The medium (10 ml) was dispensed into CO_2-filled Hungate tubes (Bellco Biotechnology, Vineland, NJ) and autoclaved for 20 min at 15 psi (121°C). Before incubation, vitamin solution (0.1 ml, see Appendix 6) was added anaerobically and aseptically to each culture tube.

Salt solution A consisted of, per 2.5 litre, NaCl (15 g), KH_2PO_4 (7.5 g), $(NH_4)_2SO_4$ (3.75 g), $CaCl_2.2H_2O$ (1.98 g), $MgSO_4 \cdot 7H_2O$ (3.0 g). Salt solution B consisted of, per 2.5 litres, $K_2HPO_4 \cdot 3H_2O$ (19.65 g) or K_2HPO_4 (15 g). Frozen rumen fluid collected from a fasted cow was thawed and centrifuged twice at 22,000 g for 20 min before addition.

F. succinogenes and *R. flavefaciens* were sub-cultured and maintained in BY medium containing 0.5% cellulose, whereas *L. multiparus* and *B. hungatei* were sub-cultured and maintained in BY medium containing 2% pectin (potassium polygalacturonate, Sigma, St. Louis, USA).

5.3.4 Weight loss and monosaccharide residue release

To prepare tissues for fermentation tests, chicory leaves were washed with tap water and distilled water and dried with tissue to remove surface water. The midrib (tapering from about 8 mm to about 1 mm wide) was carefully removed by cutting it from the lamina and both tissues were cut transversely into 3 cm long pieces. After the samples were accurately weighed (fresh weight about 500 mg), they were transferred into pre-weighed tubes, and the tubes were flushed with O_2-free CO_2 for 10 min. For sterilization, plant tissues were treated with an anaerobic solution of penicillin and streptomycin (Joblin, 1981). BY medium (10 ml) and a solution of penicillin and streptomycin (0.1 ml) were added anaerobically and aseptically to each tube under O_2-free CO_2, and the tubes kept at 39°C for 3 days. The penicillin and streptomycin solution contained 80,000 IU ml^{-1} penicillin and 8 mg ml^{-1} streptomycin sulphate. The antibiotic solution was removed aseptically and anaerobically by syringe and replaced with 8 ml BY medium. After 24 h, the solution was removed and the wash process repeated a further 2 times with 10 ml BY medium. The samples were then inoculated with rumen bacteria (0.3 ml) and incubated at 39°C, together with uninoculated tubes as controls. Before inoculation, inoculates were washed twice by centrifuging in a bench centrifuge at 1,100 g for 15 min for the removal of carbohydrates in the sub-culture medium.

Cultures were incubated for specified times (0, 24, 36, 48, 72, 144 h) and were then centrifuged at 1,100 g for 15 min. The final pH of the cultures was measured with the electrode of the pH meter near the bottom in the tube. The samples were washed twice with 10 ml distilled H_2O by centrifugation, then the residues were freeze dried and stored in a desiccator over P_2O_5.

After weighing, the residue was dissolved in 12 M H_2SO_4 (750 µl, 35°C, 30 min), diluted with water to 9 ml (yielding a final concentration of 1 M H_2SO_4) and heated (100°C, 2 h) for hydrolysis (Englyst et al., 1994). Neutral monosaccharide residues were estimated using GC by the method of Englyst et al. (1994) modified from Albersheim et al. (1967), as described in Chapter 2. Uronic acid was determined spectrophotometrically by the method of Englyst et al. (1994) modified from Scott (1979), with galacturonic acid as a standard, as described in Chapter 2.

both lamina and midrib tissues were partially disintegrated (data not shown), with more small particles observed for 144 h *F. succinogenes* incubation.

5.4.2 Weight loss and culture pH

In uninoculated control cultures, 47.2 ± 2.4% of lamina and 53.4 ± 3.5% of midrib by dry weight was solubilised during pre-treatment with antibiotics and during incubation. This soluble material was excluded in the calculation of net weight loss from inoculated samples. The insoluble weight in the uninoculated control after pre-treatment was set as the weight before degradation.

The mean net weight losses from chicory leaf lamina and midrib after incubation for 144 h with the four bacterial species are shown in Table 5.1. The data show that *B. hungatei* and *R. flavefaciens* were unable to degrade either lamina or midrib. However, *L. multiparus* and *F. succinogenes* significantly degraded both tissues. *F. succinogenes* has significantly greater activity compared with *L. multiparus*. It solubilised 49% of lamina and 83% of midrib, whereas *L. multiparus* solubilised 40% of both lamina and midrib.

Table 5.1 Net dry weight losses (%) from chicory leaf lamina and midrib after incubation with ruminal bacteria for 144 h

Bacteria	Lamina	Midrib
Control	0	0
L. multiparus	39.5 ± 0.3^{b}	40.1 ± 1.8^{b}
B. hungatei	0.6 ± 1.3^{a}	0 ± 1.8^{a}
F. succinogenes	49.0 ± 1.8^{c}	82.6 ± 2.1^{c}
R. flavefaciens	0.4 ± 3.2^{a}	1.4 ± 3.6^{a}

Mean ± SEM (n=4)

$^{a.b.c}$ Values with different superscripts differ significantly ($P < 0.05$) between rows within columns

The time courses of degradation of lamina and midrib fragments during incubation with *L. multiparus* and *F. succinogenes* are given in Fig. 5.2. Midrib was degraded most

rapidly by *L. multiparus* with around 40% of the tissue solubilised within 48 h. After 48 h, midrib was little further degraded. In contrast, with *F. succinogenes*, weight loss of midrib was slow until about 36 h of incubation. After this time, midrib degradation was rapid with around 50% degraded within 72 h.

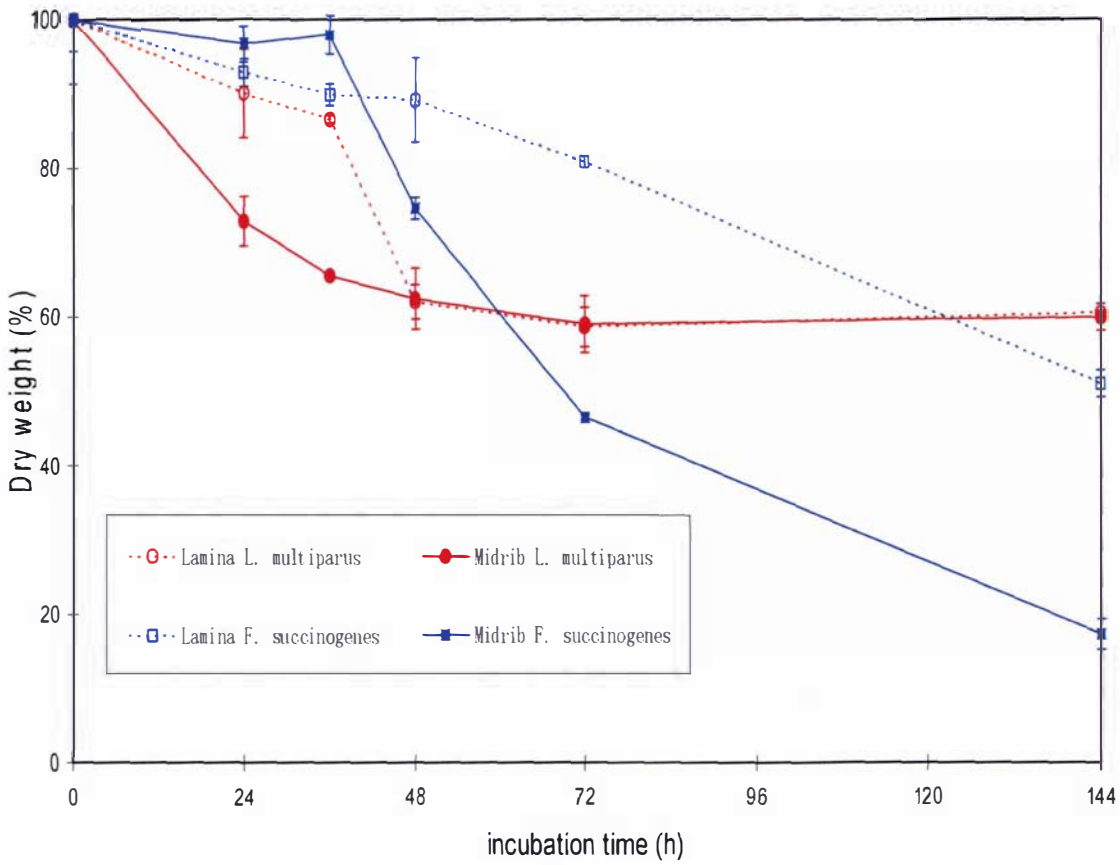


Figure 5.2 Net dry weight loss (%) (mean ± SEM) in chicory leaf lamina and midrib incubated with *L. multiparus* or *F. succinogenes*.

In the case of leaf lamina tissue, weight loss was minor, but measurable for both *L. multiparus* and *F. succinogenes* for the first 36 h of incubation. Solubilisation continued at a faster rate for *L. multiparus*, but after 48 h, further weight loss was not observed. For *F. succinogenes*, there was little weight loss during further incubation until around 48 h with most of the degradation occurring after 48 h.

The pH of the culture fluid at the completion of incubation was consistent with the weight loss data found. For the lamina, the pH of the culture fluid of *B. hungatei*

(6.42±0.01) or *R. flavefaciens* (6.43±0.01) after 144 h was similar to those of uninoculated cultures (6.42±0.01). However, the pH decreased with *L. multiparus* (pH 6.33±0.03) ($P < 0.0001$) and *F. succinogenes* (pH 6.30±0.00) ($P < 0.0001$). For the midrib a similar tendency was observed (Fig. 5.3). Since the decrease of culture pH occurred in parallel with the increase in bacterial growth and substrate degradation (Joblin et al., 2002), these results indicated that there was no growth with either *B. hungatei* or *R. flavefaciens*, but growth with *L. multiparus* and *F. succinogenes*.

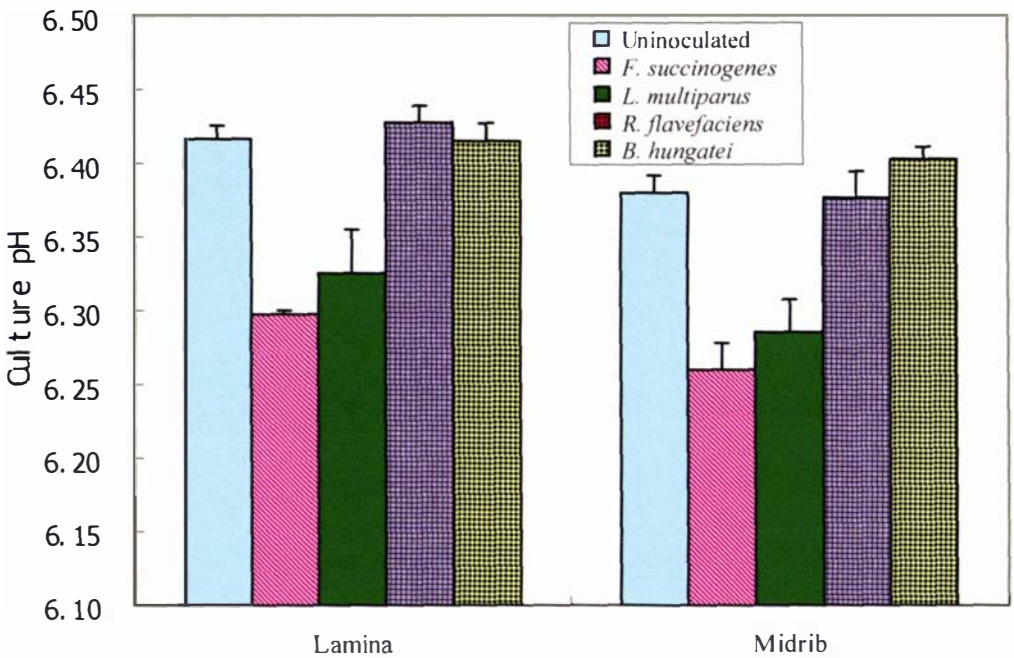


Figure 5.3 pH (mean ± SEM) of the uninoculated control and after incubating chicory lamina and midrib with rumen bacteria for 144 h.

5.4.3 Monosaccharide residue release

Uronic acid (UA), galactose (Gal), arabinose (Ara) and rhamnose (Rha) are components of pectins in chicory cell walls (Chapter 2). The time courses of release of these monosaccharide residues by *L. multiparus* or *F. succinogenes* during their degradation of chicory leaf lamina and midrib are shown in Fig. 5.4 (UA), Fig. 5.5 (Gal), Fig. 5.6 (Ara) and Fig. 5.7 (Rha), respectively.

UA (Fig. 5.4) was released from both the lamina and midrib in the early period of incubation with *L. multiparus* with around 60% of UA released from the midrib, and

around 40% released from laminae within 24 h. This rapid degradation lasted for 36 h for midribs with 93% of UA released and 48 h for laminae with 96% of UA released. Loss of UA from chicory tissues was slower with *F. succinogenes* than with *L. multiparus*, but *F. succinogenes* caused the remaining UA to decrease almost linearly until 144 h incubation.

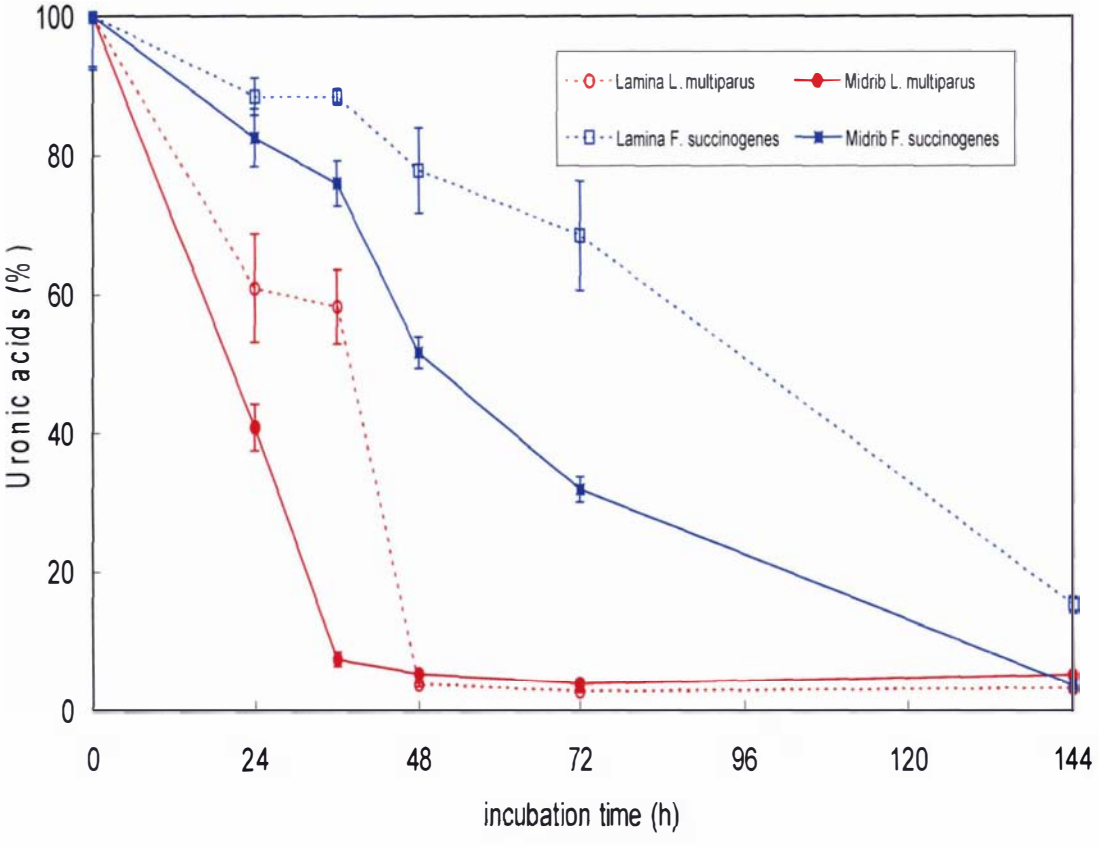


Figure 5.4 Loss of uronic acids (mean ± SEM) from chicory leaf lamina and midrib during incubation with *L. multiparus* or *F. succinogenes*.

As can be seen in Fig. 5.4, the rate of release of UA from the midrib was faster than that from the lamina for both bacterial species. Although the percentages of UA loss from the lamina and midrib were nearly the same after 48 h incubation with *L. multiparus*, the loss from the lamina was less than from the midrib within 48 h. Following incubation with *F. succinogenes*, the loss of UA from the lamina was more than from the midrib during the whole incubation period. For example, with 48 h incubation, 50% of midrib and 20% of lamina were degraded.

Galactose (Fig. 5.5) in chicory tissues was released by *L. multiparus* most rapidly within 48 h incubation. After 48 h, little further Gal was released. In contrast, during incubation with *F. succinogenes*, little Gal was released during the first 36 h. After 36 h, Gal was released at a rate similar to that observed with *L. multiparus*. Although the release of Gal was less by *F. succinogenes* than by *L. multiparus* within 72 h incubation, the rate of Gal release was rapid after 36 h with *F. succinogenes*. More Gal was released from both lamina and midrib by *F. succinogenes* than *L. multiparus* by the end of incubation.

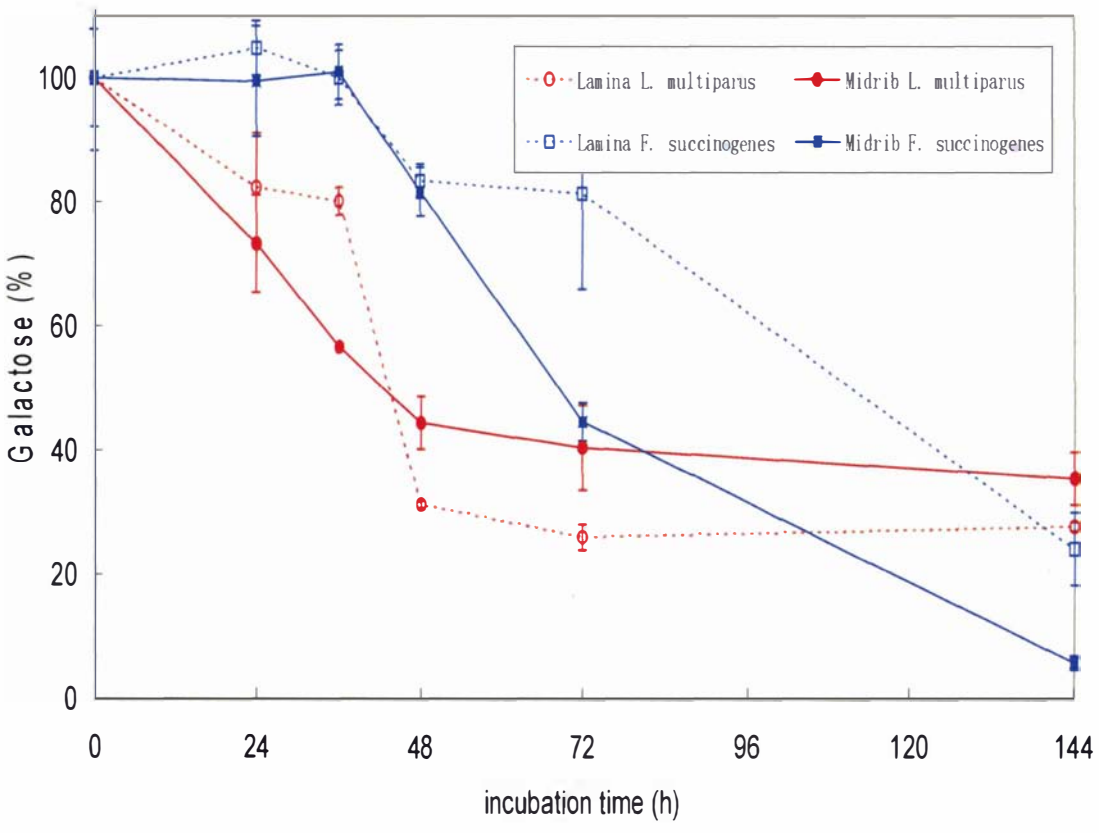


Figure 5.5 Loss of galactose residues (mean ± SEM) from chicory leaf lamina and midrib during incubation with *L. multiparus* or *F. succinogenes*.

Galactose release was influenced by tissue type. As with UA, Gal was released more slowly from laminae than from midribs with *L. multiparus* for the first 36 h incubation, but a rapid release after 36 h led to a greater ultimate release from lamina. In the case of *F. succinogenes*, Gal release was negligible over the first 36 h, but significant at 48 h, with release from midribs proceeding more rapidly thereafter.

Arabinose (Fig. 5.6) was rapidly released from midribs in the first 36 h incubation by *L. multiparus*. In contrast, Ara was not released from lamina by *L. multiparus* in the first 36 h, but from 36 h to 48 h, was rapidly released, with 23% remaining after 48 h. After the rapid release period, little remaining Ara was released from either tissue. The release of Ara from tissues by *F. succinogenes* was similar to that of Gal. Release of Ara started after a lag of 36 h and continued until the end of the experiment. As with Gal, the eventual release of Ara from midrib (95%) was greater than that with *L. multiparus*.

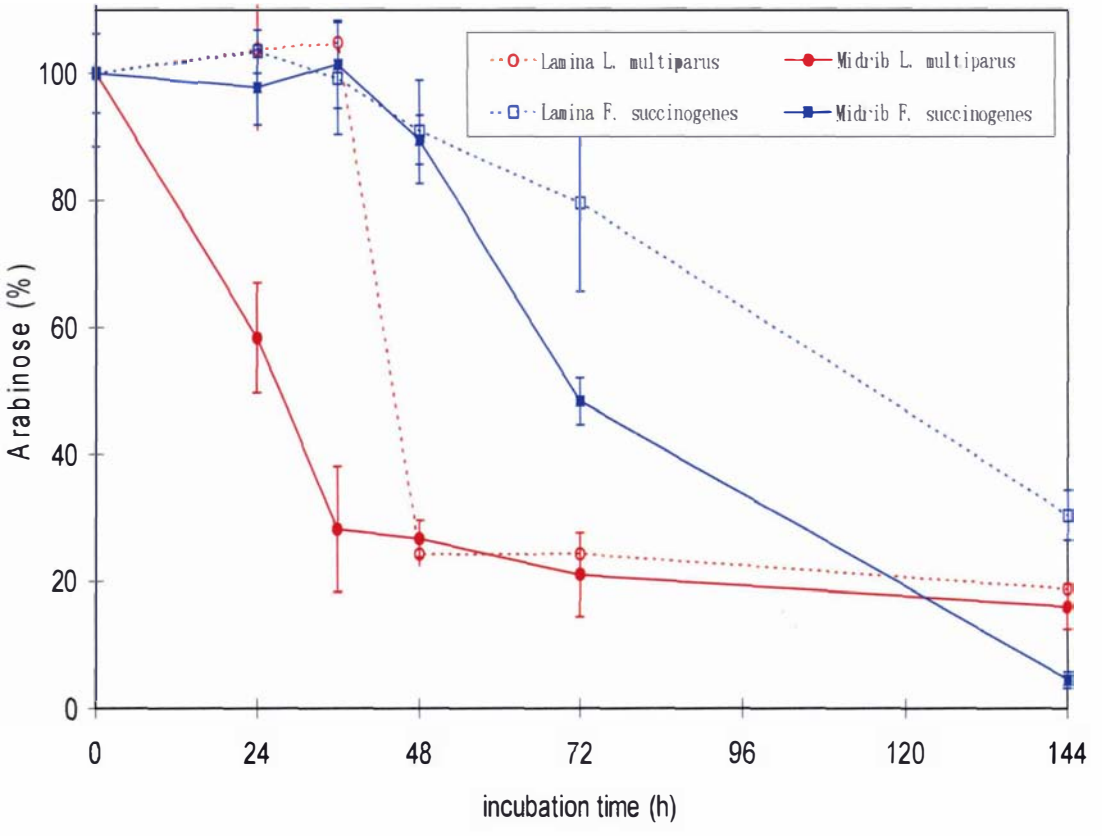


Figure 5.6 Loss of arabinose residues (mean ± SEM) from chicory leaf lamina and midrib during incubation with *L. multiparus* or *F. succinogenes*.

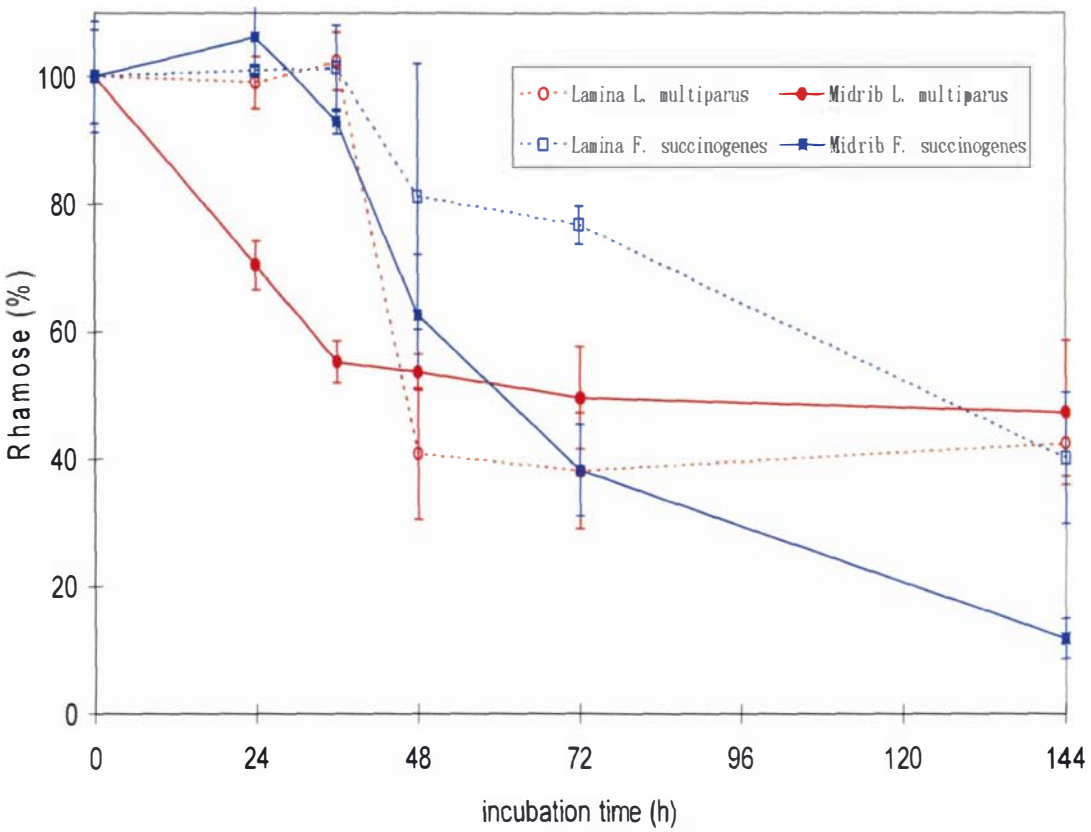


Figure 5.7 Loss of rhamnose residues (mean ± SEM) from chicory leaf lamina and midrib during incubation with *L. multiparus* or *F. succinogenes*.

Rhamnose (Fig. 5.7) was released in a pattern similar to that of Ara. Rha release from the midrib by *L. multiparus* occurred mostly in the first 36 h of incubation. The release from the lamina occurred mostly during 36 h to 48 h incubation. With *F. succinogenes*, release of Rha from midribs began after 24 h and from laminae after 36 h. After this lag period, release of Rha proceeded more rapidly from midribs than from laminae.

The rates of release of each pectin-associated monosaccharide residue from midribs are presented in Figures 5.8 and 5.9.

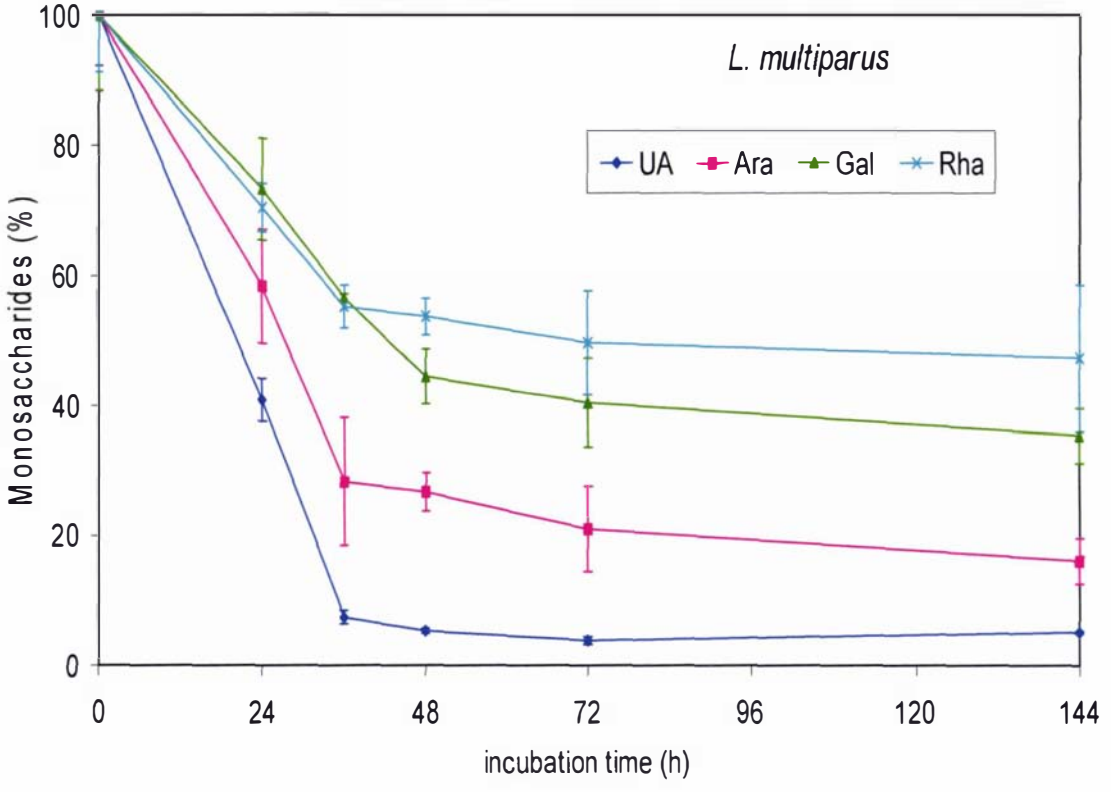


Figure 5.8 Release of pectin-associated monosaccharide residues (mean ± SEM) from chicory leaf midribs incubated with *L. multiparus*.

Following incubation with *L. multiparus* (Fig. 5.8), UA was released from midribs more quickly than other pectin-associated monosaccharide residues. The latter were released at different rates in the order Ara > Gal = Rha. However, the pattern of release for these four residues was similar. All residues were released most rapidly in the first 36 h, except Gal in which the rapid release period extended to 48 h. Further release was much slower.

In the case of *F. succinogenes* (Fig. 5.9), UA was also released the most quickly, but the relative rates of the release of the neutral residues were reversed (Rha > Gal > Ara) to those from incubation with *L. multiparus*. UA was released from the beginning of incubation, whereas pectin-associated neutral monosaccharide residues were mostly released only after the first 36 h of incubation.

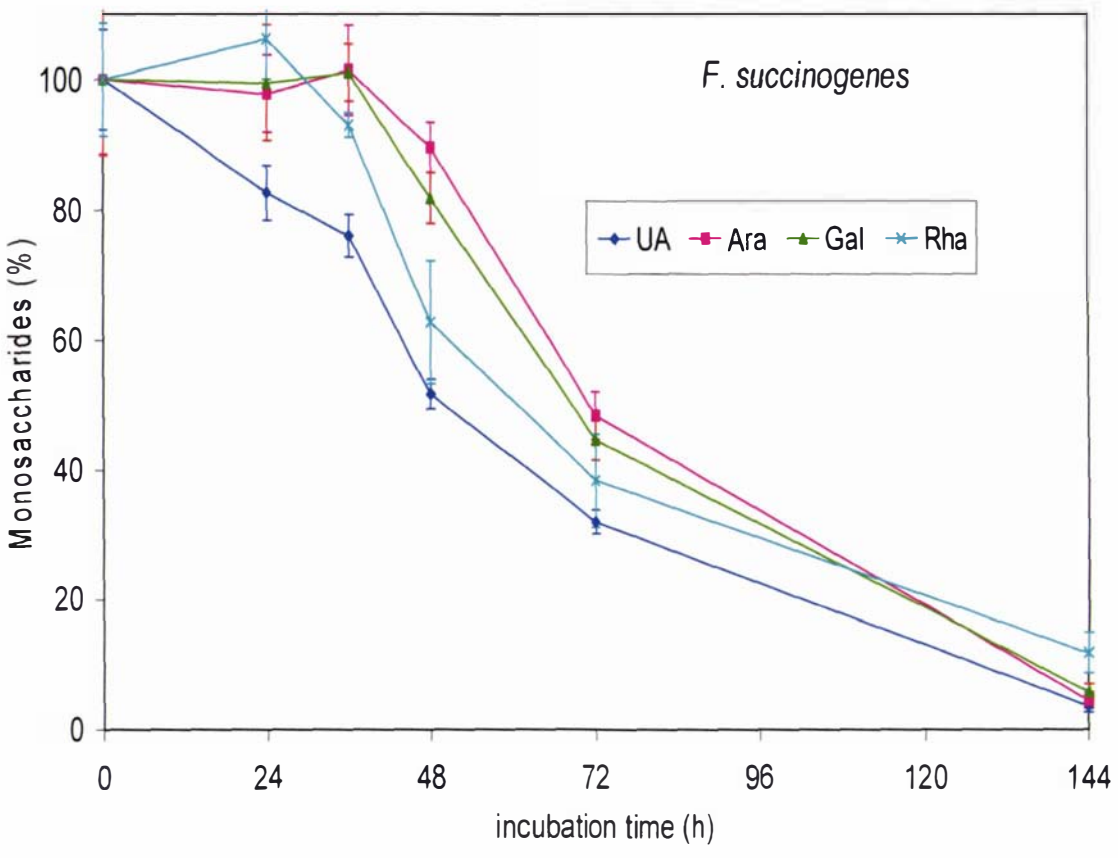


Figure 5.9 Release of pectin-associated monosaccharide residues (mean ± SEM) from chicory leaf midribs incubated with *F. succinogenes*.

Glucose (Glc), xylose (Xyl), mannose (Man) and fucose (Fuc) are associated with the cellulose or alkali-extractable polysaccharides in chicory (Chapter 2). Their release from chicory tissues by *L. multiparus* and by *F. succinogenes* is shown in Figs. 5.10, 5.11 and 5.12. Most fucose was derived from alkali-extractable polysaccharides (Chapter 2), but it is not included in figures because it formed only a very small proportion of the chicory cell-wall monosaccharide composition (Chapter 2). In contrast to the pectin-associated monosaccharide residues (52-96%), only very small amounts of Glc, Man and Xyl were released by *L. multiparus* ($P > 0.3$). However, the cellulolytic *F. succinogenes* was able to release these monosaccharide residues, which are components of cellulose or alkali-extractable polysaccharides, but only after a lag of 36 h ($P > 0.3$). After 48 h incubation, these monosaccharide residues were released to a greater extent (70-90%) from midribs than from laminae ($P < 0.05$).

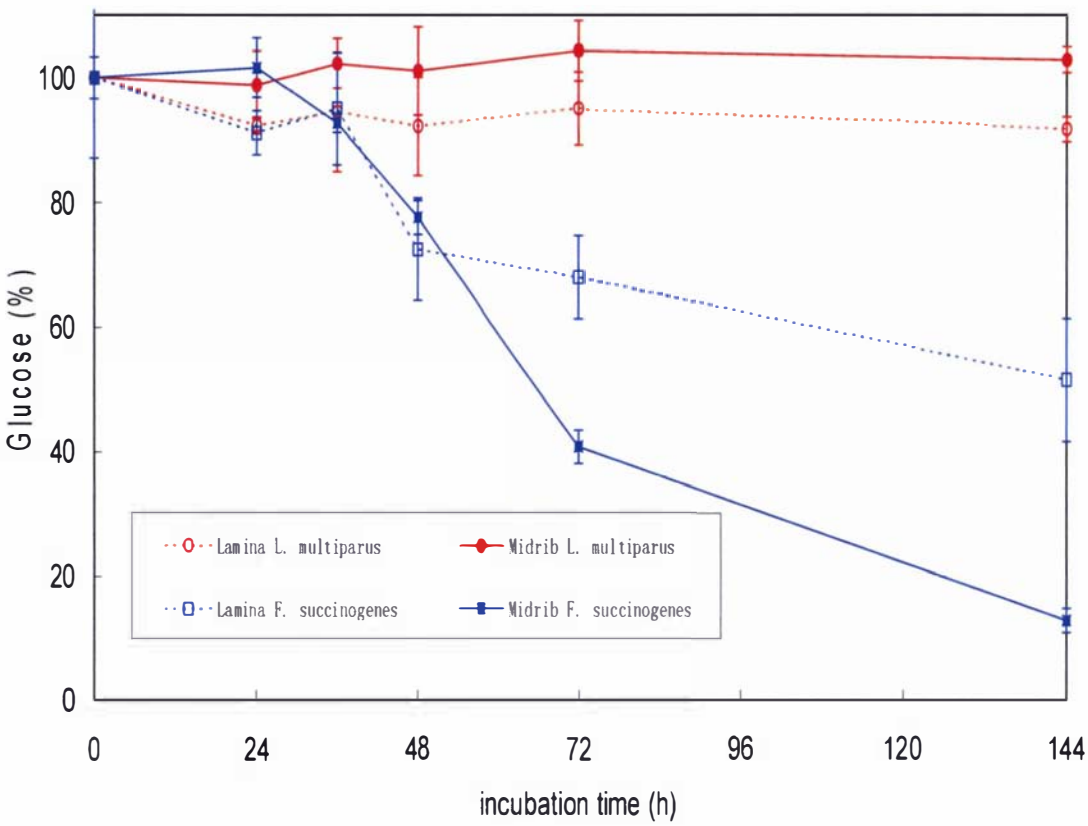


Figure 5.10 Release of glucose residues (mean ± SEM) from chicory lamina and midrib incubated with *L. multiparus* and *F. succinogenes*.

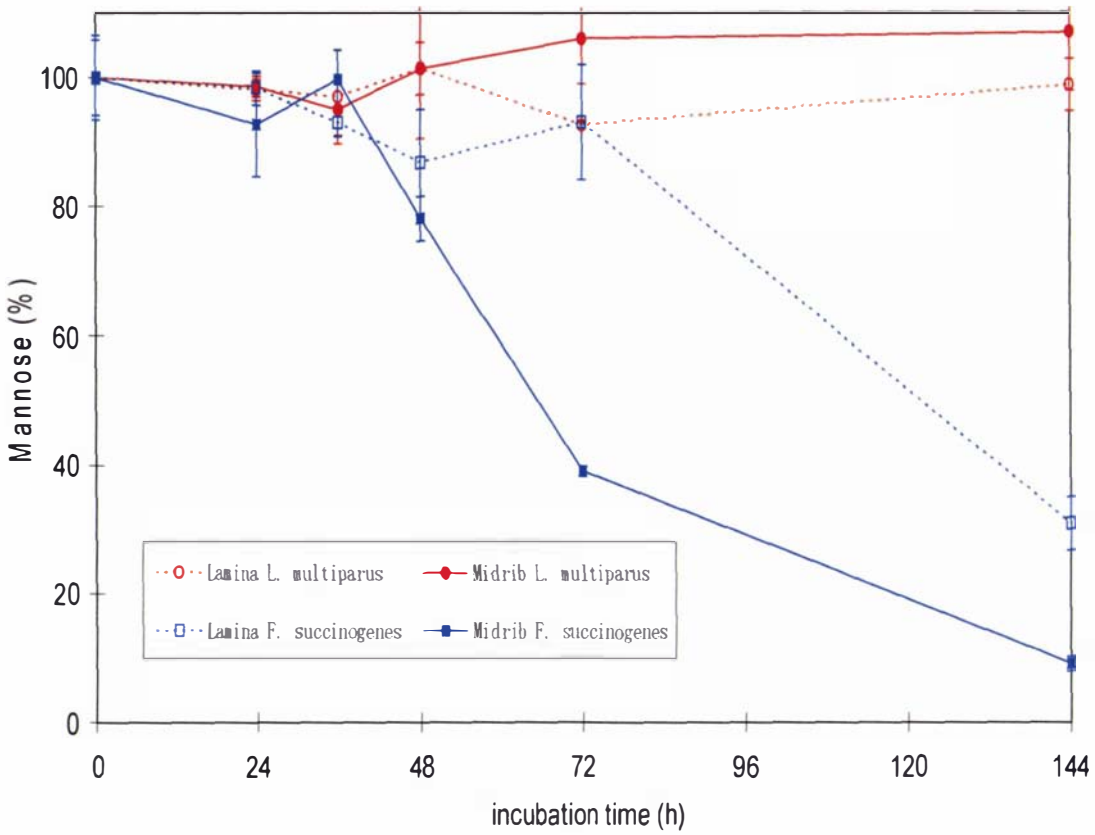


Figure 5.11 Release of mannose residues (mean ± SEM) from chicory lamina and midrib incubated with *L. multiparus* and *F. succinogenes*.

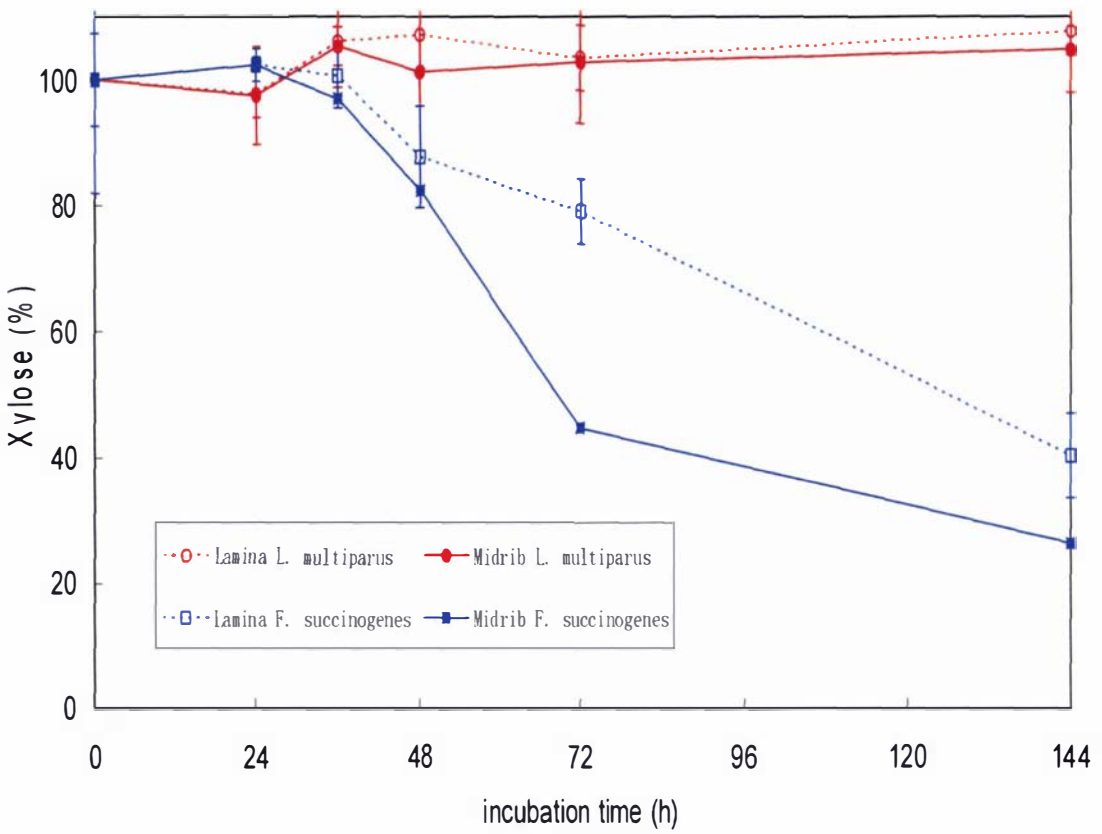


Figure 5.12 Release of xylose residues (mean ● SEM) from chicory lamina and midrib incubated with *L. multiparus* and *F. succinogenes*.

The sample data after 144 h incubation were analysed to determine the maximal extent of degradation of individual polysaccharides (Table 5.2). The maximal extent of release of each pectin-associated monosaccharide residue was 95-96% for UA, 79-84% for Ara, 65-72% for Gal, 53-58% for Rha, respectively, by *L. multiparus*. In contrast, Glc, Man or Xyl were released in only minor amounts, if at all. The percentages of dry weight loss and the release of UA and neutral monosaccharide residues by *L. multiparus* were similar for both lamina and midrib tissues of chicory.

In the case of *F. succinogenes*, the pectin-associated monosaccharide residues released were 85-96% for UA, 70-96% for Ara, 76-94% for Gal and 60-88% for Rha, respectively (Table 5.2). Glc, Man and Xyl, three residues associated with cellulose and alkali-extractable polysaccharides, were released by *F. succinogenes* at levels of 48-87%, 69-91% and 60-74%, respectively. These indicate the extent of degradation of cellulose and alkali-extractable polysaccharides. Compared with *L. multiparus*, *F.*

succinogenes degraded midrib to a greater extent than laminae. *F. succinogenes* released significantly more dry weight and all monosaccharide residues, except fucose, from midrib than from laminae.

Table 5.2 Loss (%) of dry weight (DM), uronic acids (UA) and neutral monosaccharide residues from chicory tissues after 144 h incubation with bacteria

Bacteria	*L. multiparus*		*F. succinogenes*	
Tissue	Lamina	Midrib	Lamina	Midrib
Dry weight	39.5±0.3^{c}	40.1±1.8^{c}	49.0±1.8^{b}	82.6±2.1^{a}
Uronic acids	96.7±0.1^{a}	94.9±0.2^{a}	84.7±1.2^{b}	96.3±1.0^{a}
Rhamnose	57.5±5.1^{b}	52.6±11.3^{b}	59.8±10.3^{b}	88.2±3.1^{a}
Fucose	51.6±1.7^{ab}	24.3±7.4^{c}	87.4±3.9^{a}	70.0±11.1^{a}
Arabinose	81.2±0.5^{b}	83.9±3.6^{b}	69.5±3.9^{c}	95.5±1.2^{a}
Xylose	0±3.8^{b}	0±6.8^{b}	59.6±6.7^{a}	73.6±2.3^{a}
Mannose	1.2±4.1^{c}	0±9.0^{c}	69.1±4.2^{b}	90.8±1.2^{a}
Galactose	72.2±0.7^{bc}	64.5±4.2^{c}	75.8±5.9^{b}	94.2±1.2^{a}
Glucose	8.3±2.0^{c}	0±2.1^{c}	48.4±9.9^{b}	87.1±2.0^{a}

Mean ± SEM (n=4)

^{a,b,c} Values with different superscripts differ significantly ($P < 0.05$) within rows

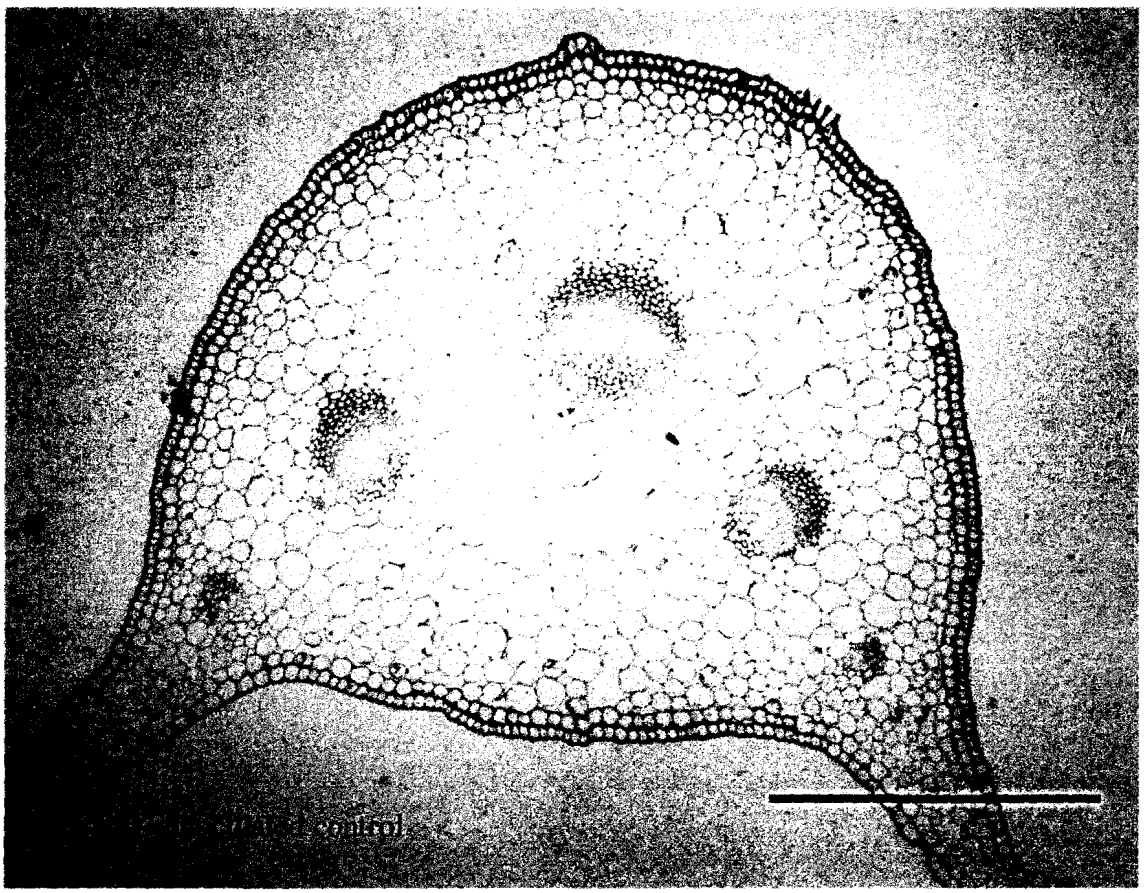
5.4.4 Histochemical staining and immunocytochemical labelling

In an effort to obtain information on the individual pectic polysaccharides and on the cell types and cell walls preferentially degraded by *L. multiparus* and *F. succinogenes*, cultures were incubated for 24 h, tissue samples embedded in resin and sections examined after staining with Toluidine Blue or with specific antibodies targeted to various pectic polysaccharide components.

5.4.4.1 Histochemical staining

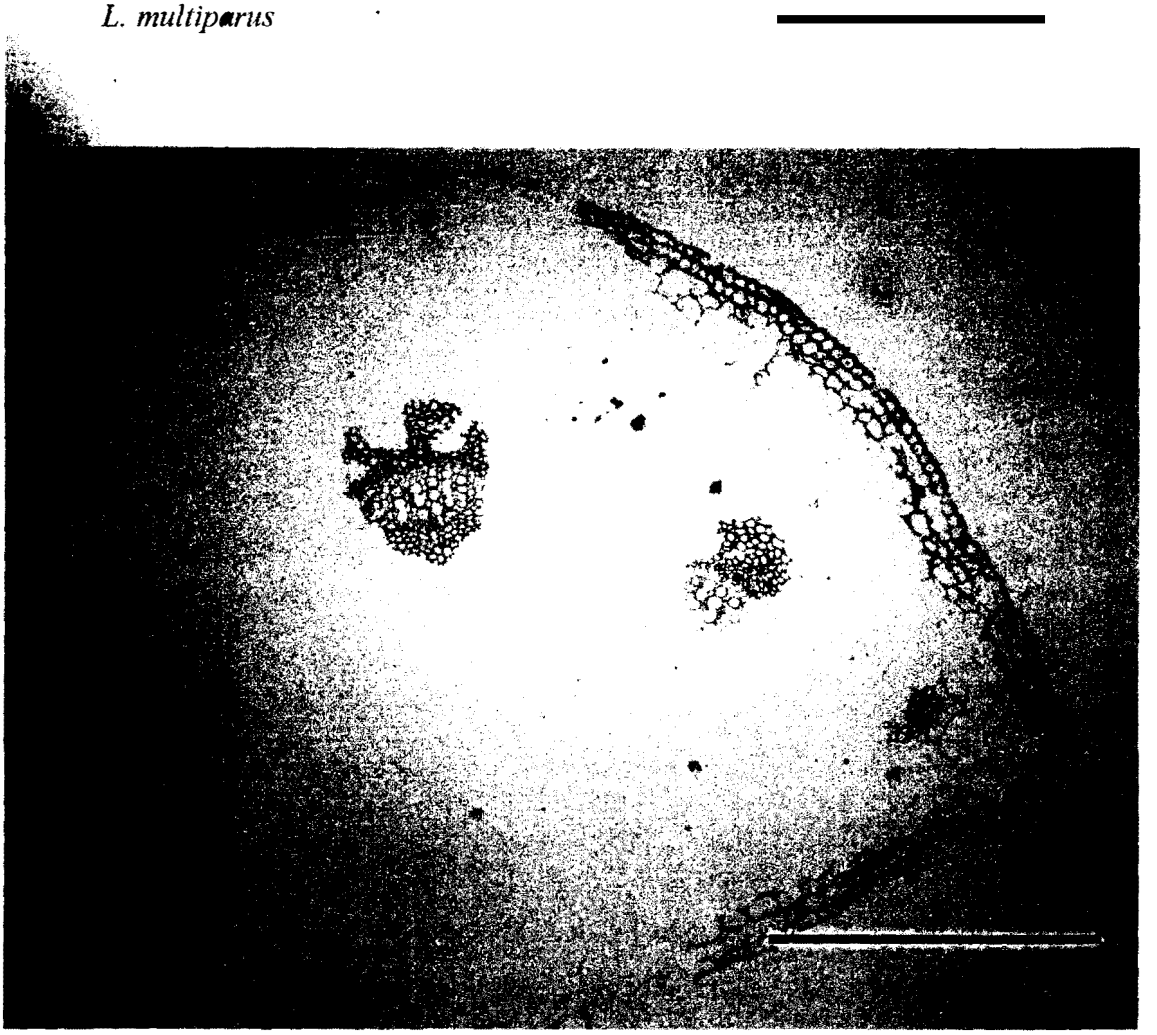
Fig. 5.13 shows transverse sections of chicory midribs stained with Toluidine Blue to reveal pectic substances and lignin (Feder and O'Brien, 1968; O'Brien and McCully, 1981) before and after incubation with *L. multiparus* or *F. succinogenes*. The results

Figure 5.13 Micrographs of resin-embedded transverse sections of chicory midrib after incubation with *L. multiparus* or with *F. succinogenes* for 24 h. The sections were stained with toluidine blue. (A) Uninoculated control. (B) Incubated with *L. multiparus*. (C) Incubated with *F. succinogenes*. Bar, (A, C) 500 μm, (B) 100 μm.



B

L. multiparus



showed that after 24 h incubation *F. succinogenes* had extensively degraded the walls of cortical parenchyma cells. The walls of the epidermis and vascular bundles were only partly degraded (Fig. 5.13C). For *L. multiparus*, a higher magnification was required compared with *F. succinogenes*, but Fig. 5.13B showed that *L. multiparus* extensively degraded walls of all cell types except the xylem tracheary elements. It appears that although chicory was extensively degraded by both *L. multiparus* and *F. succinogenes*, the patterns of degradation were quite different.

5.4.4.2 JIM5 labelling

Fig. 5.14 shows micrographs of resin-embedded transverse sections from chicory midrib stained with JIM5 antibody targeted at homogalacturonan with a low degree of methyl esterification. The results showed that homogalacturonan with a low degree of methyl esterification on the walls of all cell types including in the vascular bundles was almost completely released from midrib incubated with *L. multiparus* for 24 h (Fig. 5.14B). It is worthy of mention that homogalacturonan with a low degree of methyl esterification in the primary walls of xylem tissue was completely degraded by *L. multiparus* after 24 h (Fig. 5.16A).

In the case of *F. succinogenes*, although the walls of some phloem parenchyma and xylem parechyma remaining after degradation reacted with the JIM5 antibody, the labelling was weak (Fig. 5.14C). Homogalacturonan with a low degree of methyl esterification on the walls of other cell types, such as cortical parenchyma, was almost completely released.

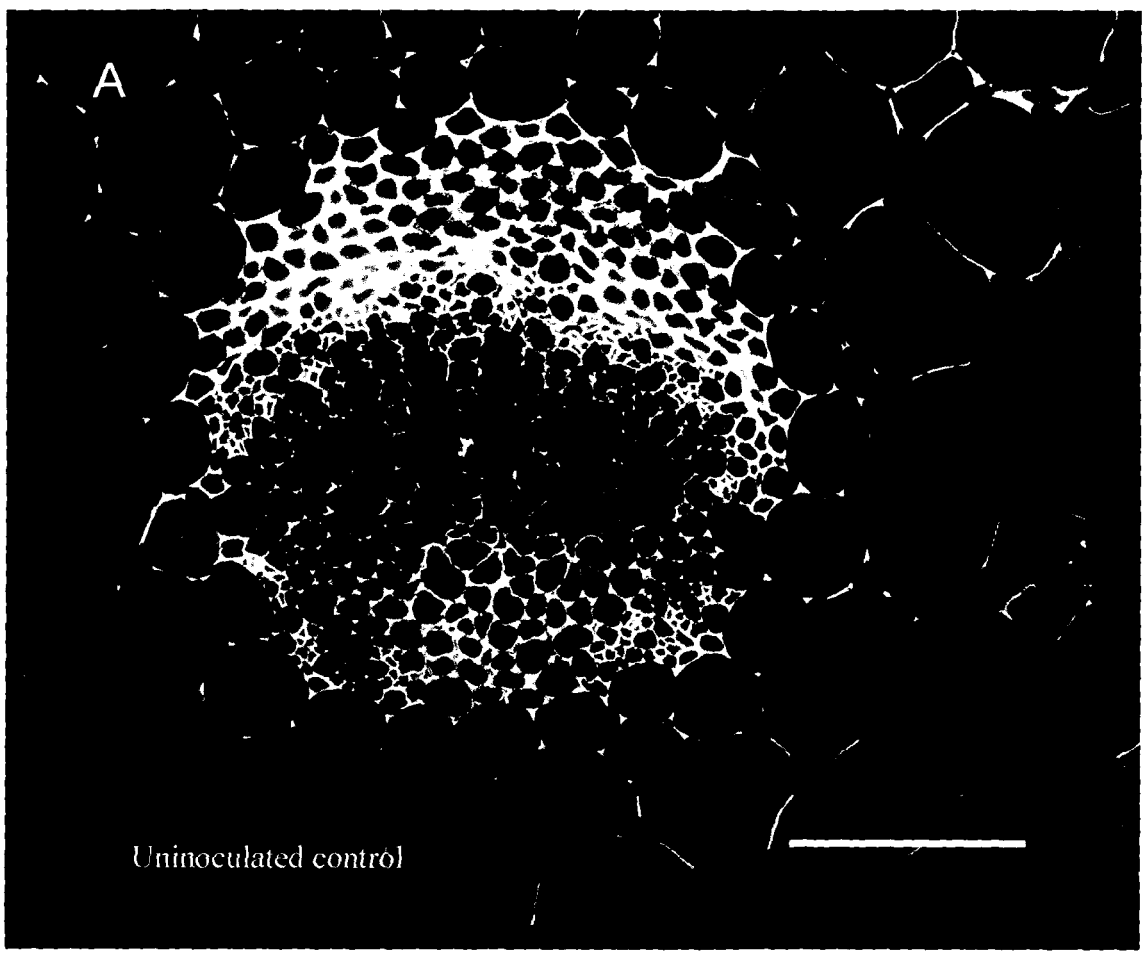
5.4.4.3 JIM7 labelling

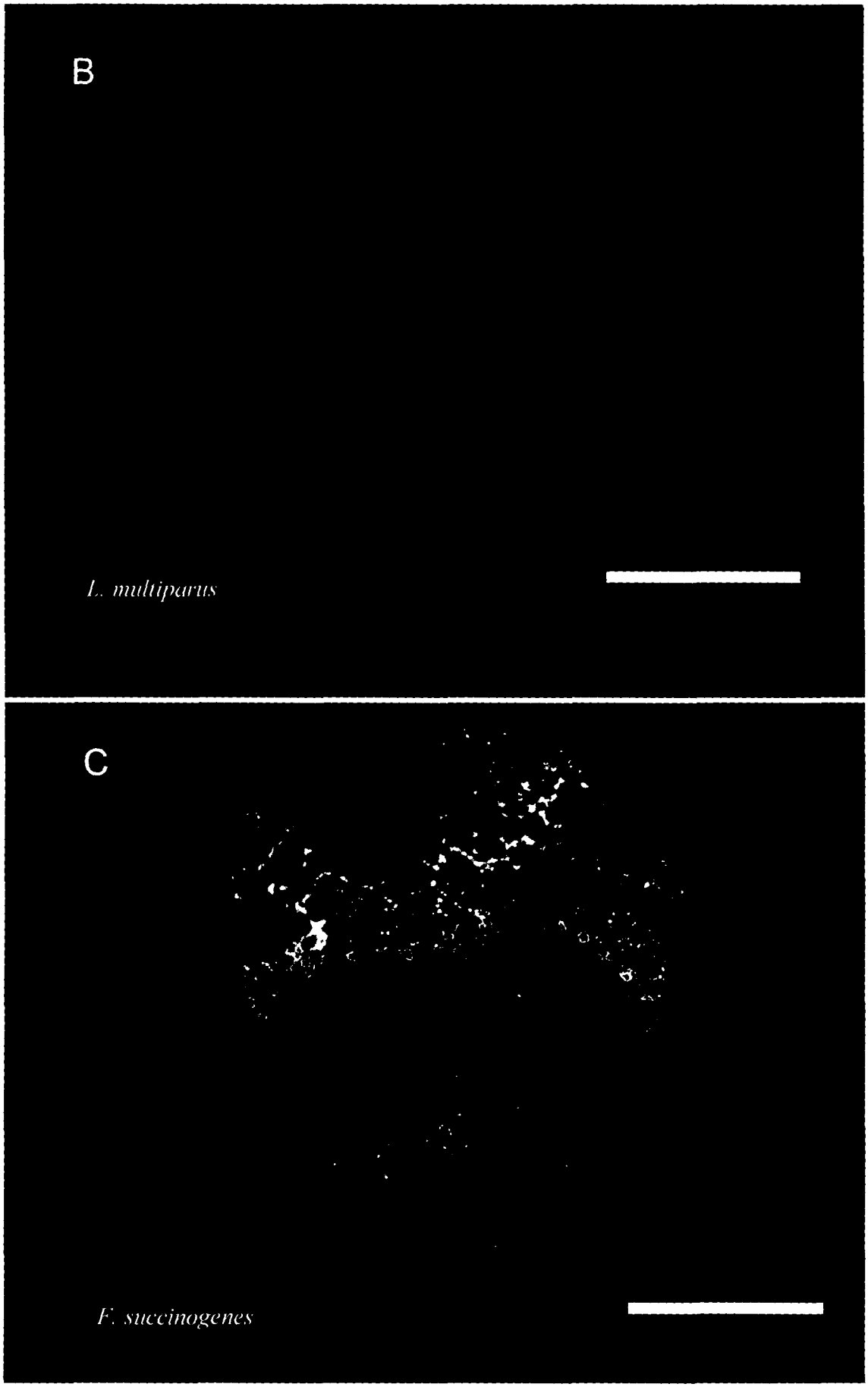
Fig. 5.15 shows micrographs of resin-embedded sections from chicory midrib stained with JIM7 targeted at homogalacturonan with a high degree of methyl esterification. The results with JIM7 were similar to those with JIM5. After incubation with *L. multiparus*, the residual midrib tissue did not label with JIM7 (Fig. 5.15C, 5.15D). But homogalacturonan with a high degree of methyl esterification in the primary walls of xylem tissue was partially resistant to degradation (Fig. 5.16B) even after 72 h incubation (Fig. 5.16C). The fluorescence from samples incubated for 72 h was weaker than that from samples incubated for 24 h, showing that homogalacturonan with a high

degree of methyl esterification is degradable, but is more resistant to degradation than homogalacturonan with a low degree of methyl esterification.

Some cell walls, such as those of the epidermal and hypodermal cells, phloem parenchyma and xylem parenchyma remained after incubation with *F. succinogenes*. These were labelled at low intensity with JIM7 (Fig. 5.15E, 5.15F).

Figure 5.14 Micrographs of resin-embedded transverse sections of chicory midrib labeled with JIM5 targeting HG with a low degree of methyl esterification. after incubation with *L. multiparus* or with *F. succinogenes* for 24 h. The sections were (A) Uninoculated control. (B) Incubated with *L. multiparus* for 24 h. (C) Incubated with *F. succinogenes* for 24 h, exposure time twice prolonged. Bar, 100 μm.




B
L. multiparus
C
F. succinogenes

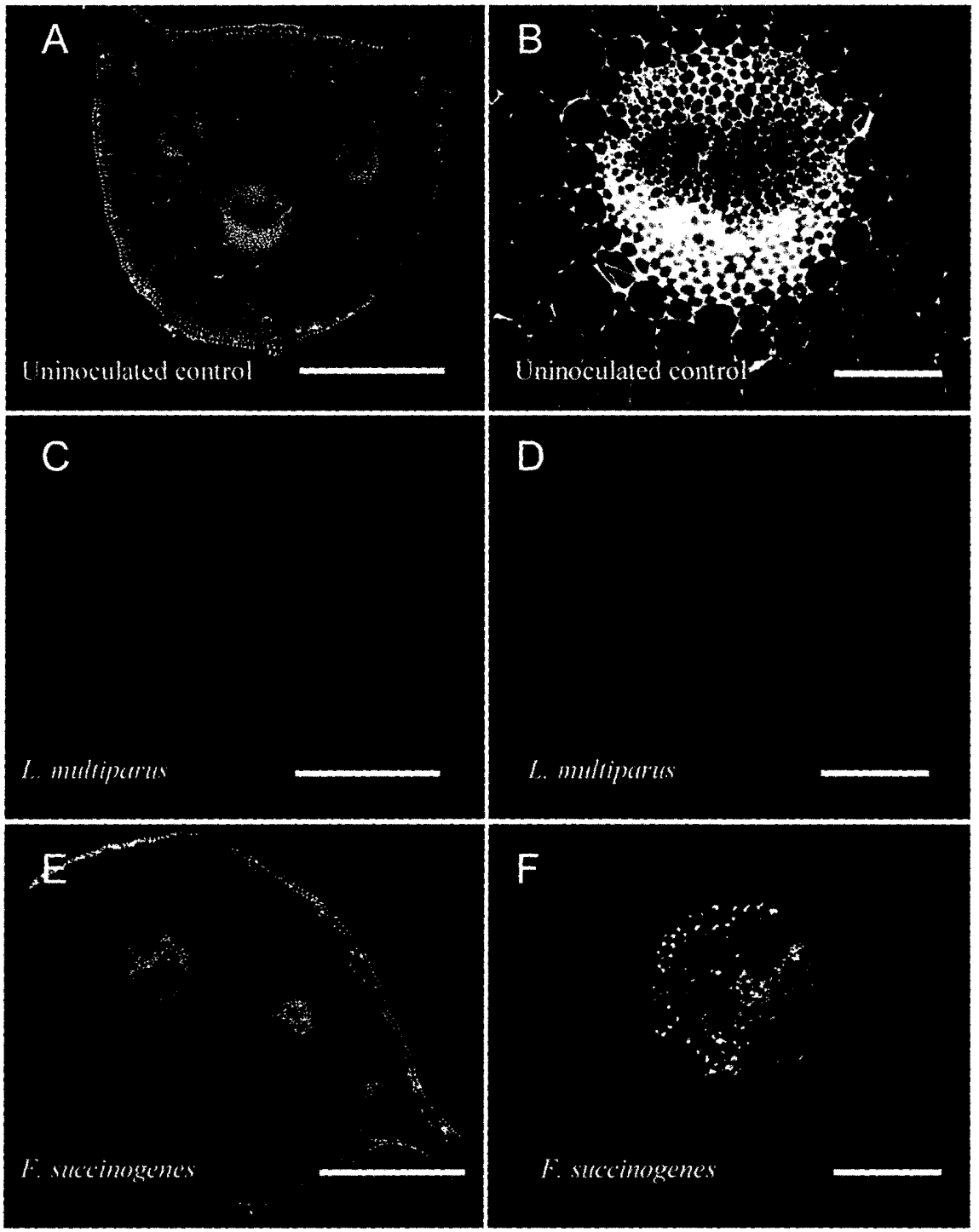
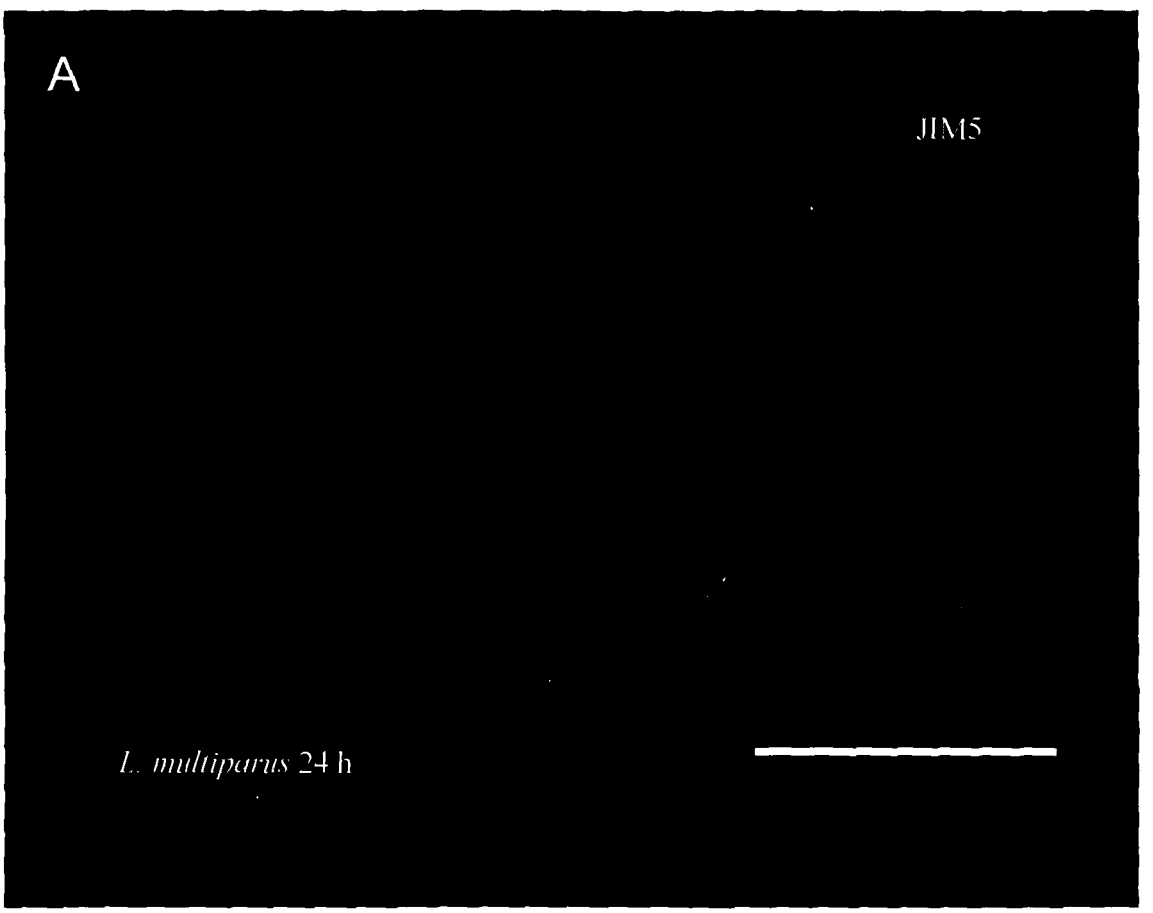
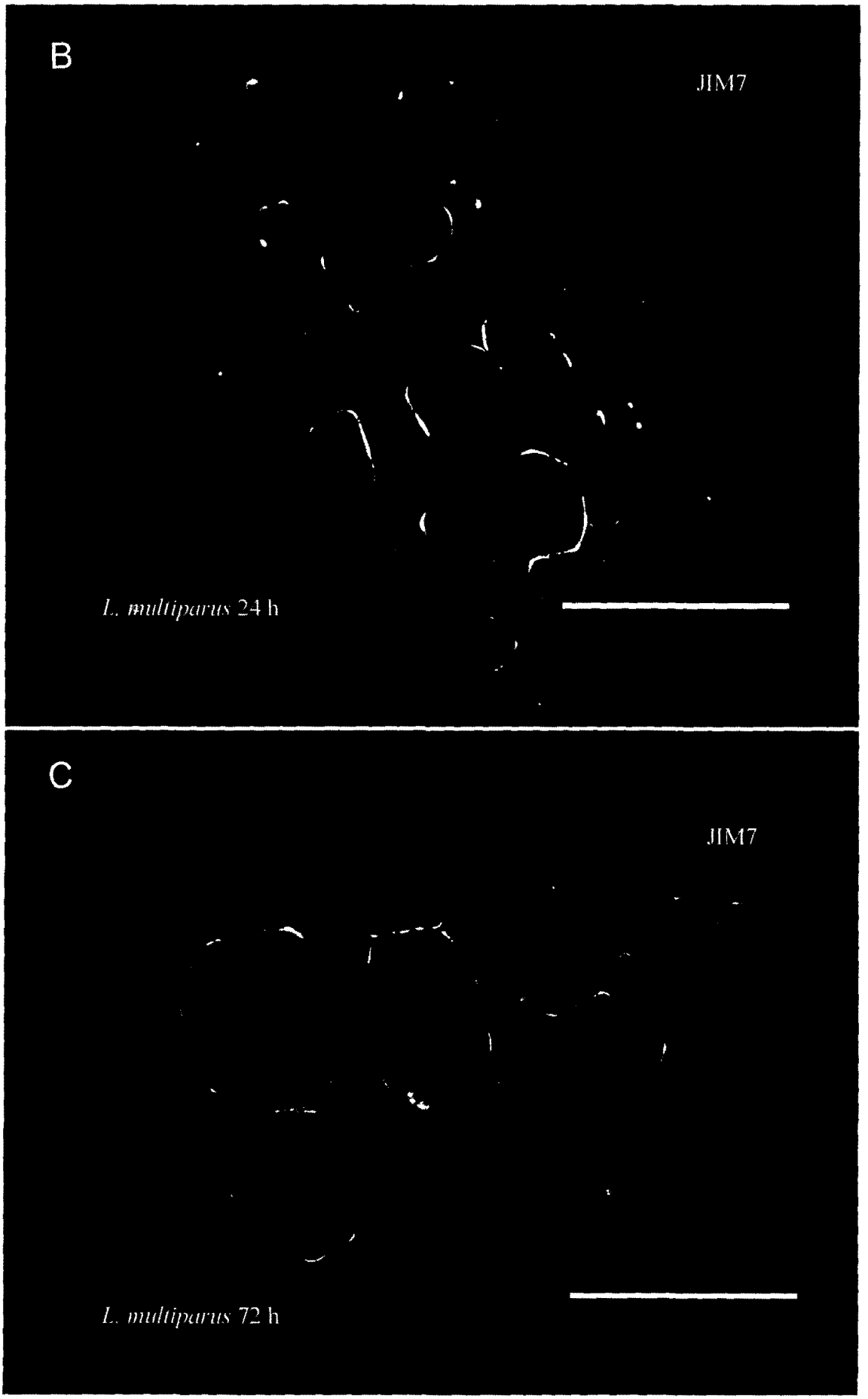


Figure 5.15 Micrographs of resin-embedded transverse sections of chicory midrib labeled with JIM7 targeting HG with a high degree of methyl esterification. The sections were. (A, C, E) Whole midrib section. (B, D, F) Vascular bundle. (A, B) Uninoculated control. (C, D) After incubated with *L. multiparus* for 24 h. (E, F) After incubated with *F. succinogenes* for 24 h. Bar, (A, C, E) 500 μm, (B, D, F) 100 μm.

Figure 5.16 Micrographs of vascular bundles from chicory midrib after incubation with *L. multiparus* for 24 h (A, B) or for 72 h (C). The sections were labeled with antibodies JIM5 (A) and JIM7 (B, C) targeting HG with a low and high degree of methyl esterification, respectively. (A) Incubated with *L. multiparus* for 24 h staining with JIM5. (B) Incubated with *L. multiparus* for 24 h staining with JIM7. (C) Incubated with *L. multiparus* for 72 h staining with JIM7. Bar, 50 μm.




B
JIM7
L. multiparus 24 h
C
JIM7
L. multiparus 72 h

5.4.4.4 LM5 labelling

Sections of LM5 in chicory midrib before and after incubation with *L. multiparus* or *F. succinogenes* were labelled with LM5 antibody specific for (1→4)-β-D-galactan. The results are given in Figure 5.17. After 24 h, *L. multiparus* had degraded most of the (1→4)-β-D-galactan from walls of all cell types, except phloem fibre walls (see Chapter 3). Phloem fibre walls were partially resistant to degradation (Fig. 5.17B). These walls were still labelled after incubation for 72 h (Fig. 5.17E). The HG with a high degree of methyl esterificatione in the phloem fibre walls was also largely resistant to degradation after 72 h incubation (Fig. 5.17D). A small amount of (1→4)-β-D-galactan remained in the primary walls of xylem vessels after 72 h incubation as was shown by some detectable labelling with LM5 (Fig. 5.17E).

After incubation with *F. succinogenes* for 24 h, sections of the remaining tissue were examined. This showed that the vascular bundles were labelled with LM5 (Fig. 5.17C), but the labelling intensity was much weaker than that in the walls of uninoculated tissue (Fig. 5.17A).

5.4.4.5 LM6 labelling

Sections of chicory midrib before and after incubation were labelled with LM6 antibody targeting (1→5)-α-L-arabinan. The results shown in Figure 5.18 were similar to those found wtih LM5.

The (1→5)-α-L-arabinan was partly degraded by *L. multiparus* after 24 h from walls of the epidermis, hypodermis, cortical parenchyma (Fig. 5.18B) and vascular bundle (Fig. 5.18E) and completely degraded after 72 h (Fig. 5.18H). Unexpectedly, a ring of cells in the vascular bundles that gave positive staining with LM6 after incubation with *L. multiparus* for 24 h was observed in a photograph with a prolonged exposure time (Fig. 5.18G). The labelling was over the cell contents and not the cell walls.

In the cortical parenchyma, two lines, representing two primary walls, between neighbouring cells after incubation with *L. multiparus* for 24 h were observed after labelling with LM6 (Fig. 5.18I). The distance between these two lines was wider than that between adjacent primary walls of two cells in the control, indicating that these cells were separated.

On incubation with *F. succinogenes*, (1→5)-α-L-arabinan was released from cell walls as indicated by the decreased labelling with LM6 (Fig. 5.18C, Fig. 5.18F). This occurred to a lesser extent than that found with *L. multiparus*.

Figure 5.17 Micrographs of resin-embedded transverse sections of chicory midrib labeled with antibodies LM5 targeting galactan (A, B, C, E) and JIM7 targeting HG with a low degree of methyl esterification (D). (A) Uninoculated control. (B) Incubated with *L. multiparus* for 24 h. (C) Incubated with *F. succinogenes* for 24 h. (D) Incubated with *L. multiparus* for 72 h. (E) Incubated with *L. multiparus* for 72 h, exposure time prolonged. Bars, (A, D) 50 μm, (B, C, E) 100 μm.

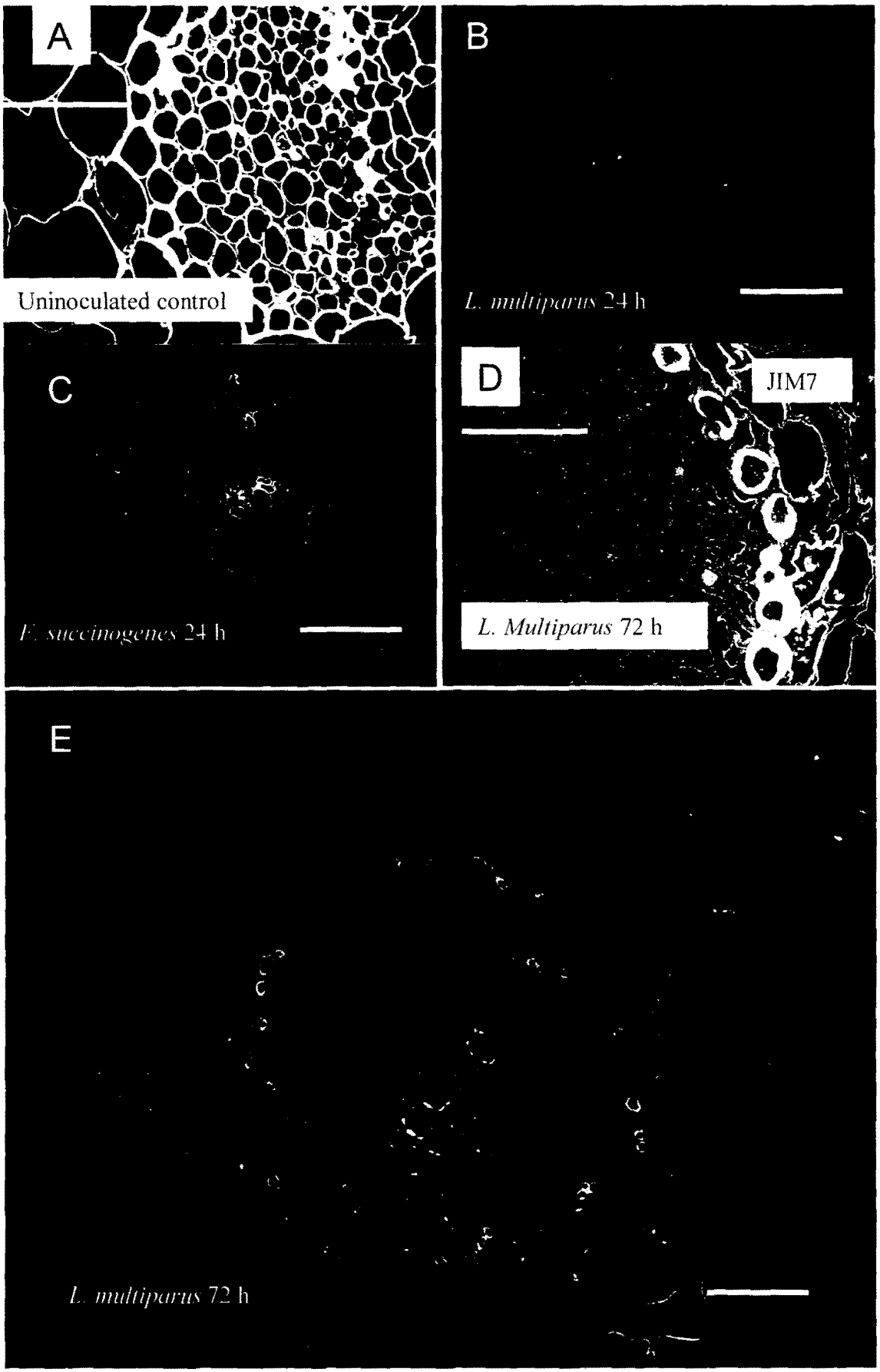
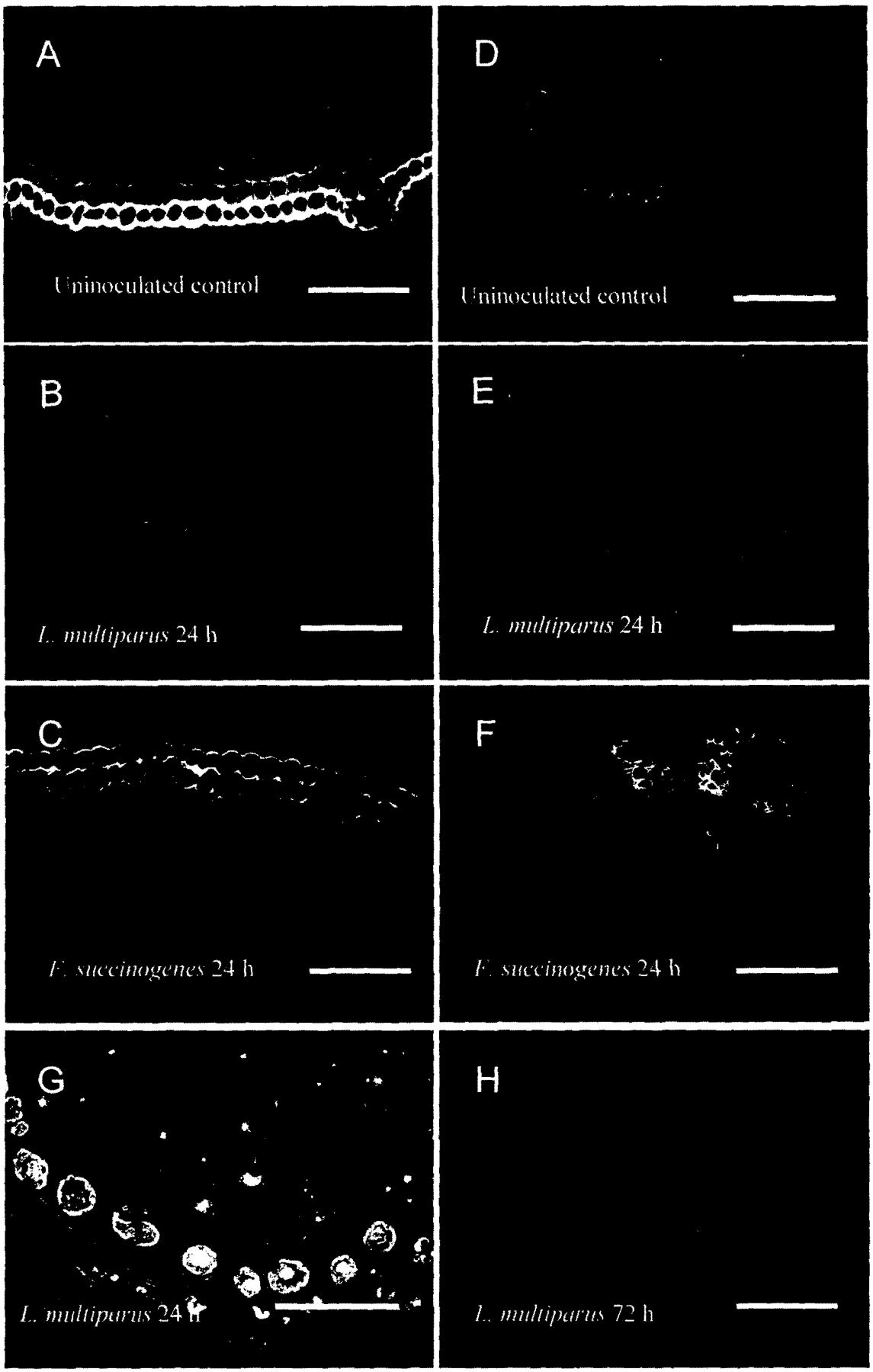
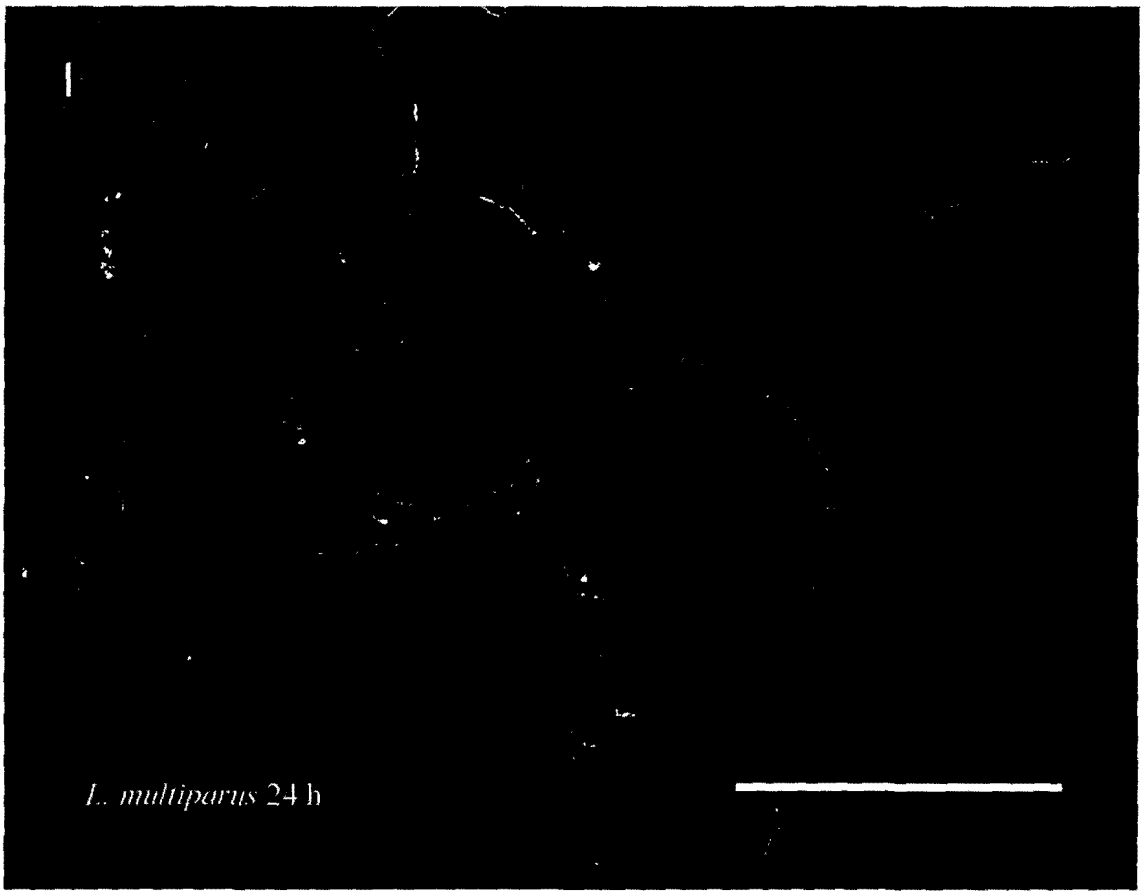

A
Uninoculated control
B
L. multiparus 24 h
C
F. succinogenes 24 h
D
JIM7
L. Multiparus 72 h
E
L. multiparus 72 h

Figure 5.18 Micrographs of resin-embedded transverse sections of chicory midrib after incubation with *L. multiparus* or with *F. succinogenes*. The sections were labeled with LM6 targeting arabinan. (A, D) Uninoculated control; (B, E, I) *L. multiparus* for 24 h; (C, F) *F. succinogenes* for 24 h; (G) *L. multiparus* for 24 h with a prolonged exposure time; (H) *L. multiparus* for 72 h. bar, (A, B, C, D, E, F, H) 100 μm; (G, I) 50 μm.


A
Uninoculated control
D
Uninoculated control
B
L. multiparus 24 h
E
L. multiparus 24 h
C
F. succinogenes 24 h
F
F. succinogenes 24 h
G
L. multiparus 24 h
H
L. multiparus 72 h


L. multiparus 24 h

5.5 Discussion

5.5.1 Background

In most published *in vitro* studies on forage plant degradation by rumen microoganisms, tissues have been substantially altered from their natural state as ingested by animals. Often materials are freeze dried, or ground to small particles. In many studies, tissues are also heat treated by autoclaving at 121°C under pressure to kill contaminating microbes. These pre-treatments yield forage tissues very different to natural ones and could cause physical and chemical changes (Joblin et al., 2002). In the present study, fresh, large intact chicory fragments were used as experimental materials. These materials were sterilized by antibiotics to keep the tissues and cell walls in as natural a state as possible. Bacterial contamination was not observed in the sample material with this pre-treatment, even after several months, in the present study.

5.5.2 Degradation of pectins

5.5.2.1 Degradation of different pectic polysaccharides

It was shown in Chapter 2 that pectic polysaccharides are major components of chicory cell walls. Pectin-associated monosaccharide residues in chicory cell walls are mainly uronic acid (UA), arabinose (Ara), galactose (Gal) and rhamnose (Rha) (Chapter 2). Pectic polysaccharides have been reported to be more readily and more completely degraded by mixed ruminal bacteria than other forage wall components (Hatfield and Weimer, 1995). A similar result was found in the present study in incubations of chicory leaf tissues with single bacterial species. UA, Ara, Gal, Rha residues were released faster than other monosaccharide residues. This suggests that the pectic polysaccharides were degraded preferentially.

UA was found to be released by *L. multiparus* more extensively and faster than neutral monosaccharide residues. This is probably because most UA was in the form of weakly bound (CDTA and Na_2CO_3 soluble) homogalacturonan (Chapter 2), hence easily accessible to *L. multiparus* enzymes. This finding is not consistent with results from studies on other forage plants (Table 5.3). For example, in the non-xylem tissues of lucerne internodes with 25% pectin, UA was released to a similar extent to arabinose and galactose (Grabber et al., 2002). The lack of agreement with the present study

probably reflects the different characteristics of pectins in chicory from those in alfalfa. Also, the bacterial enzymes secreted from *L. multiparus* differed from the fungal enzyme mixtures used by Grabber et al. (2002). The percentage UA residues solublised from lucerne and annual ryegrass (*Lolium rigidum*) in a study by Miron and Ben-Ghedalia (1993) were far less than those of arabinose and galactose residues. This was probably due to a loss of UA during the washing of their cell walls with neutral detergent before testing.

Table 5.3 Main forage cell wall pectin-associated monosaccharide residues released by rumen bacteria or enzymes

Forage	Tissue	UA	Ara	Gal	Rha	Bacteria or enzyme	Incubation time	Sources
Chicory	Lamina	96.7	81.2	72.2	57.5	*L. multiparus*	144 h	1
	Midrib	94.9	83.9	64.5	52.6	*L. multiparus*	144 h	1
	Lamina	84.7	69.5	75.8	59.8	*F. succinogenes*	144 h	1
	Midrib	96.3	95.5	94.2	88.2	*F. succinogenes*	144 h	1
Lucerne	Non-xylem	76.8	75.2	74.4	91.3	Fungal enzyme mixture	3 h	2
	Non-xylem	84.8	81.1	86.1	91.0	Fungal enzyme mixture	48 h	2
Lucerne	*	42.2	71.8	66.4		*F. succinogenes* S85	120 h	3
	*	41.9	67.8	66.6		*F. succinogenes* BL2	120 h	3
	*	21.4	36.4	24.2		*B. fibrisolvens* D1	120 h	3
Annual ryegrass	*	71.6	88.2	92.4		*F. succinogenes* S85	120 h	3
	*	71.2	83.6	87.3		*F. succinogenes* BL2	120 h	3
	*	59.3	71.6	66.4		*B. fibrisolvens* D1	120 h	3

* Stems and leaves ground to less than 1 mm (Miron and Ben-Ghedalia, 1993). 1, this study; 2, Grabber et al., 2002; 3, Miron and Ben-Ghedalia, 1993

In the present study, substantial amounts of pectin-associated monosaccharide residues were not released during degradation. After incubation of chicory lamina and midrib with *L. multiparus* for 144 h, although only 4-5% of UA remained, much Ara (16-21%),

Gal (28-35%), Rha (42-47%) were not released. This suggests that pectinolytic enzymes in the system on their own are unable to degrade RG I pectin completely. *L. multiparus* can secrete pectate lyase (Silley, 1985), but has little ability to degrade cellulose or alkali-extractable polysaccharides, as judged by the findings in this study that there were no significant releases of monosaccharide residues associated with cellulose and alkali-extractable polysaccharides. Osborne and Dehority (1989) have also found that the *L. multiparus* was unable to degrade cellulose forage plant cell walls.

In this study, *F. succinogenes* released significantly more pectin-associated monosaccharide residues from the midrib than did *L. multiparus.* Such differences between the two bacteria were not observed with lamina tissue. *F. succinogenes* has been shown to secrete enzymes degrading alkali-extractable polysaccharides, cellulose and pectins (Osborne and Dehority, 1989). These researchers found that when either the cellulolytic bacterium *F. succinogenes* or the "hemicellulolytic" bacterium *Bacteroides ruminicola* were co-cultured with the pectinolytic bacterium *L. multiparus*, pectin degradation from orchard grass (*Dactylis glomerata*) was greater than that with *L. multiparus* alone. This suggests that the degradation of pectins is facilitated by the degradation of other wall components. In the present study, a substantial amount of RG I was found in the alkali-resistant residue (Chapter 2), suggesting that RG I is possibly bound to cellulose. This could explain the finding that cellulose degradation resulted in increased release of neutral pectic monosaccharide residue (Ara, Gal and Rha) by *F. succinogenes.*

The degradation of pectin could affect the degradation of other wall components. It was shown in Chapter 4 that pectin degradation caused maceration and forage particle breakdown in chicory, hence increasing the surface area of forage particles. In a model of the primary cell walls of dicotyledons, the pectin network has been proposed to combine physically with the cellulose-xyloglucan network (McCann and Robert, 1991). Pectins determine the pore size of the cell wall matrix (Carpita and Gibeant, 1993). Pectins in lucerne (Hatfield and Weimer, 1995) and in chicory (this study) were degraded faster and more extensively than other wall polysaccharides. Thus the space available inside the network of cellulose and xyloglucan may increase after the release of pectins, and, as a result, the surface area for attack by bacteria might increase. This would facilitate the degradation of cellulose and xyloglucan. Spagnuolo et al. (1997) found synergistic effects between cellulolytic and pectinolytic enzymes during sugar

beet pulp saccharification and claimed that a barrier existed, whereby pectic substances prevented cellulose degradation. The possibility for pectin degradation facilitating the degradation of other wall polysaccharides remains.

Based on the above, a hypothesis regarding the degradation of polysaccharides in the nonlignified primary walls of dicotyledonous forages by rumen bacteria can be proposed. In these walls, homogalacturonan would be degraded first and then give access for rumen bacteria to attack the cellulose-xyloglucan network. Degradation of cellulose-xyloglucan would then facilitate the further degradation of pectins, mainly RG I. The hypothesis is illustrated as follows.

HG degradation→cellulose-xyloglucan degradation→RG I degradation

5.5.2.2 Effect of HG esterification on degradation

In the present study, homogalacturonans targeted by both JIM5 and JIM7 antibodies were almost completely degraded by *L. multiparus* in nonlignified primary walls of all cell types after 24 h. This indicated that HGs with both low and high degrees of methyl esterification were indistinguishably degraded in the primary wall, suggesting that the degree of esterification of HG was not limiting cell wall degradation. Grabber and Hatfield (2005) found that the amount of uronic acid released from nonlignified cell walls of *Zea mays* (L.) cell suspensions was not significantly enhanced after pectin methylesterase treatment. Chesson and Monro (1982) did not find that the presence of methyl esters influenced the degradation of boiling-water and ammonium-oxalate extractable homogalacturonan from red clover or lucerne.

In the primary walls of xylem vessels in chicory midrib, HG with a low degree of esterification was completely degraded after 24 h incubation with *L. multiparus*, whereas HG with a high degree of esterification was not completely removed. This indicates that HG with a high degree of methyl esterification in the primary wall of xylem vessels was somewhat resistant to degradation. A small amount of HG with a high degree of methyl esterification may be linked to resistant components such as lignin in such a way that deesterification to HG with a low degree of methyl esterification is not possible. Grabber et al. (2002) found that only 25% of total UA was released from relatively highly lignified alfalfa xylem tissue during degradation. They suggested that because a large amount of the UA was associated with xylans, and xylans

are severely restricted for degradation by lignification, HG degradation may be restricted by lignification. Grabber and Hatfield (2005) artificially lignified cell walls of *Zea mays* by adding coniferyl alcohol and H_2O_2 separately. They found that pectin methylesterase treatment before artificial lignification reduced uronosyl residue release by 55% after 4 h of degradation. Their results suggested that the degree of methylesterification of HG in lignified walls affects its degradation.

5.5.2.3 Effects of bacterial species on degradation

B. hungatei, a recently described rumen bacterium, produces xylanase, pectin hydrolase and endo–(1→4)-β-D-glucanase, and can utilize a wide range of carbohydrates (Kopecny et al., 2003). *R. flavefaciens* is regarded as one of the most active forage cell-wall-degrading bacteria in the rumen. It has been shown to degrade dewaxed fibre, a recalcitrant cellulose (Stewart et al., 1990; Hobson and Stewart, 1997), pure pectins, and pectins from forages (Gradel and Dehority, 1972). However, in the present study, neither *B. hungatei* nor *R. flavefaciens* degraded intact chicory, although both bacteria grew well on pure pectins and cellulose, respectively. These facts indicated that an effective degrader of pure substrate *in vitro* is not always effective in degrading forage materials.

On the other hand, the rumen bacterium *Prevotella ruminicola* was unable to degrade and utilize pure commercial pectin, but degraded and utilized considerable quantities of pectin from orchard grass (Osborne and Dehority, 1989). The characteristics of forage substrates can affect the degrading ability of rumen bacteria. *L. multiparus* was very efficient in degrading pectins in chicory leaves in this study, but had limited ability to degrade and utilize pectins in the orchard grass which had been finely ground to pass a screen with openings of 400 by 450 μm (Osborne and Dehority, 1989). *R. flavefaciens* did not degrade the large intact chicory tissue used in this study, but has been shown to solubilise pectins from finely ground alfalfa and bromegrass (*Bromus inermis*) (Gradel and Dehority, 1972). It can be concluded that bacterial characteristics determined from cultures grown on purified or semi-purified pectins are not always consistent with those obtained from pectins in intact forage tissues. Also, the results from one type of forage plant should not be extrapolated to other forage species. Experimental results obtained from *in vitro* studies with particular substrates or single bacterial cultures should not be extrapolated directly to overall rumen fermentation *in vivo*.

Both *L. multiparus* and *F. succinogenes* degraded intact chicory in this study, but showed different degradation patterns.

First, *L. multiparus*, a well-known pectinolytic bacterium, was found to release pectin-associated monosaccharide residues only, indicating that it acted exclusively on pectins. In contrast, *F. succinogenes*, one of the most important cellulotytic bacteria in the rumen (Hobson and Stewart, 1997), released acidic and neutral monosaccharides, indicating that it degraded pectic polysaccharides as well as cellulose and alkali-extractable polysaccharides.

Secondly, the results from immunofluorescence labelling of midribs indicated that *L. multiparus* significantly degraded pectin within 24 h and caused maceration through loss of pectin, but did not break down the walls; whereas *F. succinogenes* degraded pectin less extensively, but completely degraded some types of cell walls, resulting in the loss of the cortical parenchyma walls and the loss of some parenchyma walls in vascular bundles. *L. multiparus* degraded chicory tissue in a relatively uniform manner over time, whereas *F. succinogenes* selectively degraded cortical parenchyma first.

The third difference between *L. multiparus* and *F. succinogenes* is the lag time before degradation. In incubation with chicory, the lag time was much less for *L. multiparus* than for *F. succinogenes*. *L. multiparus* degraded chicory in the first 48 h of incubation and nearly all UA was lost within 24 h (midrib) or 48 h (lamina). Leaf fragments were highly macerated. It appears that *L. multiparus* may be involved in rapid reduction in particle size. In contrast, although immunofluorescent labelling showed degradation of midrib segments by *F. succinogenes*, there was little overall degradation of segments in the first 36 h. The rate of loss of UA by *F. succinogenes* was not as high as with *L. multiparus*. The differences in degradation may be due to differences in adhesion to substrate by the two bacteria. *F. succinogenes* is known to bind to substrates as part of the cellulolytic process, a process involving cellulosomes (Miron et al., 2001). Weimer (1993) has found that the most active pectinolytic bacteria are those that do not bind to substrates. Pectinolytic enzymes are extracellular and secreted into the medium (Warren, 1996). In the presence of *L. multiparus*, forages with an abundance of pectins would be expected to have a short lag time before degradation. From an *in sacco* degradation study, Burke et al. (2000) found that chicory had a shorter lag time than grasses and most legumes.

Although *L. multiparus* released pectin-associated monosaccharide residues earlier and faster than *F. succinogenes*, substantial amounts of Ara, Gal and Rha, which were associated with RG I, were not solubilised by *L. multiparus* even after 144 h incubation. These remaining pectins, inaccessible to *L. multiparus*, probably are in a complex of cellulose-pectin-xyloglucan, as proposed in Chapter 2. In contrast, *F. succinogenes* completely degraded pectic polysaccharides from midribs after 144 h incubation, indicating that *F. succinogenes* was able to degrade pectins not accessible to *L. multiparus*. This probably resulted from degradation of cellulose and xyloglucan in cellulose-pectin-xyloglucan in the complex. The results from this study suggest that *F. succinogenes* and *L. multiparus* have different modes of lyase action in terms of pectin degradation with *L. multiparus* degrading mainly homogalacturonan and *F. succinogenes* degrading mainly rhamnogalacturonan I.

5.5.2.4 Effects of tissue and cell types on degradation

Both lamina and midrib were solubilised to the same extent by *L. multiparus* after 48 h incubation, but in the first 36 h the rate of degradation was highest for the midrib. As noted above, pectin degradation depends mainly on extracellular pectinolytic enzymes. The slower degradation of laminae in the first 36 h is probably due to the protective effect of cuticles which lower access of the enzymes to cell walls. Galactose was released to a greater extent from the lamina than from the midrib by *L. multiparus*. This may be related to a higer Gal/Ara ratio in the lamina than in the midrib (Chapter 2).

In the case of *F. succinogenes*, the midrib lost more dry weight and pectin-associated monosaccharide residues than the lamina after 36 h. This was probably related to the relatively high exposed surface area of cut tissues which were greater in midrib than in laminae. Bacteria depending on attachment for degradation preferentially attack forage walls though the cut edges, rather than intact surfaces surrounded by cuticle (Cheng et al., 1979).

In terms of cell type, the walls of cells in the vascular bundles, such as phloem parenchyma and xylem parenchyma, were apparently less susceptible to degradation by *F. succinogenes*. The susceptibility of cell type to degradation by *L. multiparus* is nearly impossible to determine from the 24 h samples because most degradation was completed. But immunocytochemistry with JIM7 and LM5 antibodies targeted at HG

with a high degree of methyl esterification and (1→4)-β-D-galactan showed that these pectic polysaccharides were degraded by *L. multiparus* to lesser extent in phloem fibre walls than in other cell types.

Chapter 6

General discussion

6.1 Introduction

Forage chicory is a novel forage herb and a member of the family Asteraceae. It is increasingly used in New Zealand for grazing ruminants. Chicory can provide a large quantity of herbage, especially during summer conditions. Chicory also has a higher voluntary feed intake (VFI) and is more digestible, resulting in better animal performance (Hoskin et al., 1995, 1999; Kusmartono et al., 1997; Foster et al., 2002; Neel et al., 2002) compared with perennial ryegrass-based pasture, indicating that chicory has high feeding value (Barry, 1998). These findings have led to an increase in the use of chicory as a forage.

During investigation of chicory as a forage for ruminants, chicory was found to have a high pectin content (Kusmartono, 1996) measured by crude analysis (Bailey, 1967), to be rapidly degraded in the rumen and to have high apparent organic matter (OM) and energy digestibility (Hoskin et al., 1995). The polysaccharides of forage plant cell walls are important sources of energy for ruminant animals (France and Siddons, 1993). A knowledge of the structure of chicory cell-wall polysaccharides would be the basis for a better understanding of the rapid and ready degradation of chicory in the rumen. However, although the structures of the cell-wall polysaccharides in the dicotyledonous families Fabaceae (legumes) and Brassicaceae (brassicas), and the monocotyledonous family Poaceae (grasses) have been extensively studied, the structures of the polysaccharides in chicory cell walls and their degradation by rumen bacteria had not been examined prior to the studies described in this thesis.

The aim of this study was 1) to determine the structure and composition of chicory cell-wall polysaccharides, 2) to investigate the role of pectins in chicory degradation.

This thesis documents the structure of forage chicory cell-wall polysaccharides and their degradation by enzyme preparations and by rumen bacteria, and is the first report of this type on forage chicory. Four studies were conducted with leaves of eight-week-old chicory: the structure of the cell-wall polysaccharides, the distribution of pectic polysaccharides, and pectin degradation by enzyme preparations and by chemical agents and degradation by rumen bacteria.

The results obtained from the experiments described in this thesis supported the acceptance of hypotheses proposed at the beginning of the study (see Section 1.5). The polysaccharide compositions of the cell walls of forage chicory leaves are similar to those of the primary walls of other eudicotyledonous forages, although the pectin content is higher. The cell walls of forage chicory leaves are all nonlignified except for the walls of xylem vessels. These cell-wall characteristics may contribute to its high digestibility by ruminants. Lower-esterified HG is located in the middle lamina and at the corners of cellular spaces. The disruption of these pectic polysaccharides caused cell separation and forage particle breakdown. This distribution of HG, together with the high pectin content, could explain the rapid degradation of chicory in the rumen.

6.2 Chicory cell-wall composition

6.2.1 The proportions of cell contents and cell walls

The dry material of forage is composed of cell walls and cell contents. Cell contents can be nearly completely degradable (98%) in the rumen (Van Soest, 1994). In contrast, cell walls are only partially degraded (Moore and Hatfield, 1994). Therefore, high digestibility will be expected if a forage has a high proportion of cell contents. The cell contents in chicory constitute 52% of midrib dry weight and 67% of lamina dry weight. These are higher than those in all other forages in Table 6.1 except white clover, despite the fact that, for all other forages in the table (including white clover), NDF-soluble cell wall materials are included in the data for cell contents, suggesting that the high proportion of cell contents may contribute to the high digestibility of chicory.

Table 6.1 The proportions of cell contents and cell walls in chicory leaves and other forages

	Cell contents	Cell walls
Chicory midrib	51.8	48.2
Chicory lamina	67.4	32.6
Perennial ryegrass	42	58
Orchard grass	45	55
Bermuda grass	26	`74
Oat forage	34	66
White clover	64	36
Lucerne	49	51
Red clover	34	66

Note: Data on the proportions of cell walls in the table except chicory are the percentages of neutral detergent fibre (NDF) from Van Soest (1994).

6.2.2 Chicory cell-wall composition

6.2.2.1 Wall carbohydrates

Cell-wall polysaccharides of chicory grown as a vegetable "witloof" have been reported (Femenia et al., 1998). However, prior to this study the cell walls of forage chicory have only been analysed using a rough pectin extraction procedure (Bailey et al., 1967) and using the Van Soest fibre analysis procedure (Robertson and Van Soest, 1981). In these protocols, samples were first treated with boiling water, but the water-extractable substances including pectin and mono- and di-saccharides were not classed as pectins. Also, non-pectic polysaccharides were grouped as cellulose and "hemicelluloses" without information on the specific polysaccharides present. The present study is the first detailed report on forage chicory cell-wall polysaccharides.

6.2.2.1.1 Monosaccharide compositions of cell-wall polysaccharides

The monosaccharide compositions of cell-wall polysaccharides can be easily determined by acid hydrolysis of the cell wall and measuring the proportion of the different monosaccharides released. The monosaccharide compositions of cell walls can

be interpreted in terms of different polysaccharides present, although they are influenced by the proportions of the primary and secondary walls that depend on the organ and the stage of development. The monosaccharide compositions of the cell walls of forages, including grasses, legumes and brassicas, have been extensively investigated, and are summarised in Table 6.2. The monosaccharide compositions of chicory walls are also included. Chicory leaf cell walls have far more uronic acid than those of grasses and more than those of forage legumes and forage kale. Three neutral monosaccharides, rhamnose, galactose and arabinose, are also components mainly of pectins. The sum of these three monosaccharides in chicory is higher than in grasses, forage legumes and brassicas, suggesting that chicory is richer in pectic polysaccharides than grasses and legumes.

6.2.2.1.2 Polysaccharides

The major cultivated forage plants are, apart from grasses (monocotyledons), all dicotyledons, including Fabaceae, Brassicaceae and Asteraceae. The non-cellulosic polysaccharides in the cell walls of forage plants are summarised in Table 6.3. Cellulose is not listed in Table 6.3, since the basic structure of cellulose in all plants is the same, although it is acknowledged that the relative abundance could be different.

The non-cellulosic polysaccharide compositions of grasses are quite different from those of dicotyledonous forages. In grass primary cell walls, the main non-cellulosic polysaccharides are arabinoxylan and glucuronoarabinoxylan, with smaller amounts of xyloglucan and (1→3),(1→4)-β-glucans (Chesson et al., 1985); the predominant non-cellulosic polysaccharides in the secondary cell walls have similar structures but lower degrees of substitution of the backbone xylan (Åman, 1993). In contrast to this, in legumes, the predominant non-cellulosic polysaccharides in the primary walls are pectic polysaccharides, xyloglucans, and small proportions of heteroxylans and (galacto-) glucomannans; in the secondary walls, they are predominatly 4-O-methyl-glucuronoxylans and smaller proportion of glucomannans (Harris, 2005). Among dicotyledons, the wall polysaccharide compositions are similar. The chicory leaf cell walls examined in this study were, apart from those of xylem vessels, not lignified. Hence, the polysaccharide compositions of chicory walls appear similar to the primary walls of forage legumes.

Table 6.2 Monosaccharide compositions of the cell walls of grasses, forage legumes, brassicas and chicory

Forages	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Sources
				Poaceae (Grasses)					
Cocksfoot (*Dactylis glomerata*)	0.3	tr	7.5	32.4	0.6	4.0	55.2	7.7	Åman, 1993
Meadow fescue (*Festuca pratensis*)	0.3	tr	7.3	29.7	0.3	2.8	59.7	8.0	Åman, 1993
Tall fescue (*Festuca arundinacea*)	0.3	tr	7.5	35.3	0.5	2.4	53.9	8.4	Åman, 1993
Reed canarygrass (*Phalaris arundinacea*)	0.3	tr	8.3	33.9	0.5	3.2	53.8	7.7	Åman, 1993
Smooth bromegrass (*Bromus inermis*)	0.3	tr	6.9	30.3	0.5	2.4	59.6	7.2	Åman, 1993
Timothy (*Phleum pratense*)	0.6	tr	7.7	31.2	0.6	2.6	57.3	7.7	Åman, 1993
Perennial ryegrass (late cut) (*Lolium perenne*)	0.4	0.0	8.1	22.2	1.0	4.2	64.1		Gordon et al. 1983
Perennial ryegrass (early cut) (*Lolium perenne*)	0.5	0.2	7.5	19.9	0.9	3.7	67.2		Gordon et al. 1983
Perennial ryegrass (vegetative) (*Lolium perenne*)	0.4	1.0	5.1	27.2	0.6	2.7	63.0	5.1	Chesson and Forsberg, 1997
				Fabaceae (Legumes)					
Red clover (*Trifolium pratense*)	2.4	0.0	10.4	16.0	4.5	7.9	59.0	23.8	Åman, 1985
White clover (*Trifolium repens*)	2.7	0.0	9.3	8.9	5.4	9.0	64.8		Gordon et al. 1983
Lucerne (vegetative) (*Medicago safiva*)	1.4	1.1	7.1	16.5	2.8	5.1	65.8	17.6	Chesson and Forsberg, 1997
				Brassicaceae (Brassicas)					
Forage kale (*Brassica oleracea*)									
Parenchyma	2.4	0.5	10.6	13.3	0.0	16.3	56.9	20.0	Wilson et al. 1988
Secondary xylem	0.9	0.0	8.7	31.5	0.0	11.8	47.1	7.0	Wilson et al. 1988
				Asteraceae					
Forage chicory (*Cichorium intybus*)									
Midrib	3.6	0.9	12.6	8.5	5.8	15.3	53.2	40.2	This study
Lamina	5.7	1.0	10.8	7.6	5.6	19.6	49.7	48.9	This study

Note: neutral monosaccharides as % of total neutral monosaccharides, Uronic acids as % of non-starch polysaccharide.

Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acids; tr, trace.

Table 6.3 Cell wall non-cellulosic polysaccharides of chicory and other forages

Plant group		Non-lignified primary walls	Lignified secondary walls
Monocotyledons (commelinid)	Grasses	Glucurononarabinoxylan > pectic polysaccharides & xyloglucans	Glucurononarabinoxylans > (galacto-)glucomannans (minor)
Dicotyledons	Legumes	Pectin polysaccharides > xyloglucans > heteroxylans & (galacto-) glucomannans (minor)	4-O-methyl-glucurono-xylan > glucomannans
	Brassicas	Pectic polysaccharides > xyloglucans > glucomannans > heteroxylans	
	Chicory*	Pectic polysaccharides > xyloglucans > glucomannans > heteroxylans	

Modified from Lam et al. (1990) and Harris (2005); Brassica data calculated from Femenia et al. (1999).

*Chicory walls of all cell types, except xylem tracheary elements, were not lignified in this study.

The proportions of the various polysaccharides are different between grasses and dicotyledonous forages and are also different among dicotyledonous forages, although the difference is not as large as that between grasses and dicotyledons.

Pectic polysaccharides are the main non-cellulosic polysaccharides of the primary cell walls of dicotyledonous plants, but they are present at much lower levels in grasses (Jarvis et al., 1988; Brett and Waldron, 1996; Harris, 2005). Generally, the primary walls of dicotyledons contain 32-45% of pectic polysaccharides (O'Neil and York, 2003). The content of pectic polysaccharides is 10-30% in the cell walls of alfalfa (*Medicago sativa*) (Hatfield, 1992), 3-4% or less in the cell walls of grasses (Marounek and Duškovā, 1999). The proportion of pectic polysaccharides among total wall polysaccharides in chicory in the present work was 67% and 59% for the lamina and midrib, respectively. On the basis of total wall components, the content of pectic polysaccharides was 50% and 40% for lamina and midrib, respectively. Therefore the content in forage chicory appears higher than other dicotyledonous forages examined so far.

6.2.2.2 Lignin

It is well known that lignin inhibits cell wall degradation in the rumen. Chicory leaves have a low lignin content at only 20 g kg^{-1} (Table 1.1). Histochemical examination in the present study indicated that only the walls of xylem tracheary elements were lignified in chicory leaves (Chapter 2). Non-lignified walls in legumes can be nearly completely degraded in the rumen, whereas lignified secondary walls appear indigestible (Wilson and Hatfield, 1997). The distribution of lignin in chicory suggests that chicory cell wall degradation in the rumen is not inhibited by lignin except that of the walls of xylem tracheary elements. Therefore, it appears that lignin is not a main limiting factor for chicory degradation.

In summary,

- Forage chicory as a dicotyledon has cell-wall polysaccharides similar to those of forage legumes and brassicas and is quite different from those of grasses;
- But chicory has higher pectin content than other forage eudicotyledons;
- And lignin is restricted to the xylem tracheary elements;
- The abundant pectin in forage chicory, together with walls that are mainly non-lignified, may contribute to the high digestibility of chicory.

6.3 Functions of pectic polysaccharides in plants

Pectic polysaccharides in plant cell walls have multiple functions in plant growth and development (Cosgrove, 2005). For example, they contribute to cell adhesion, cell wall architecture, cell wall mechanical strength, control of cell wall porosity and cell wall ionic status. Their functions are related to their structures and spatial distribution (Willats et al., 2001a). Some of the results from the immunolabelling work on the location of pectic polysaccharides of chicory cell walls have not been well documented for other plants. These new findings contribute to knowledge on the functions of pectic polysaccharides.

6.3.1 Cell adhesion

Cell adhesion is an important and fundamental feature of plant cells and has been extensively studied (Jarvis et al., 2003; Sobry et al., 2005). Large forces generated from turgor pressure in non-woody plant tissues tend to separate cells (Jarvis et al., 2003) and plants have to develop various mechanisms to maintain cell adhesion.

In the Poaceae, it has been suggested that the cross-linking of arabinoxylans has a role in cell adhesion (Jarvis et al., 2003) and grasses contain little pectic polysaccharides. This is probably the reason why perennial ryegrass leaves were not macerated with endo-PG (Chapter 4). In dicotyledons, a network of specific pectic polymers has been widely accepted as playing a role in cell adhesion. Cell adhesion in dicotyledons will be

discussed from two aspects: the site of cell adhesion and the substances involved in cell adhesion.

Originally the middle lamella was considered to hold cells together (Knox, 1992). Moreover, Jarvis (1998) suggested that the cell corners (tricellular junctions) and the corners of intercellular spaces, defined as reinforcing zones, bear most turgor-imposed stresses. Furthermore, Parker et al. (2001) showed that these so-called reinforcing zones differ from both the primary cell walls and the middle lamella in pectic polymer composition, and can be distinguished under a scanning electron microscope. Therefore, rather than just the middle lamella, the tricellular junctions and the corners of intercellular spaces should also be considered in a discussion of cell adhesion.

Which substances in cell walls are involved in cell adhesion? Such substances should be found in the middle lamella, at the tricellular junctions and at the corners of intercellular spaces. Cellulose and alkali-extractable polysaccharides can be easily excluded since they have not been found in the middle lamella (Carpita and Gibeaut, 1993). This is supported by the observation that strong alkali did not cause vortex-induced cell separation in chicory leaves (Chapter 4). Pectic polysaccharides are the principal substances in the middle lamella and proteins are also present there (Carpita and Gibeaut, 1993). Hence, they are both candidate substances for cell adhesion.

Knox et al. (1990) found that in tomato and sugar beet relatively unesterified pectins are mainly located in the cell interface regions: the middle lamella and the cell corners. Liners and Van Cutsem (1992) showed that in suspension cultured carrot cells, unesterified pectins recognized by the 2F4 antibody are restricted to the tricellular junctions. Bush and McCann (1999) found, in potato tuber tissue, that HGs involved in Ca^{2+}-cross-linking (the epitope for the 2F4 monoclonal antibody) were located in the middle lamella and were abundant at the cell corners. It has been proposed that unesterified HGs are the main pectic components involved in cell adhesion (Jarvis et al., 2003). This hypothesis is supported by the recent evidence that the perturbation of HG biosysthesis leads to a reduction in the amount of HG (Bouton et al., 2002) and a reduction in cell adhesion in a QUA1 mutant phenotype of *Arabidopsis thaliana* (Orfila et al., 2005).

In the present studies, both the JIM5 and JIM7 epitopes have been found in the middle lamella, at the corners of intercellular spaces and at the tricellular junctions (Chapter 3). However, the JIM7 epitope is evenly distributed throughout the cell walls, whereas the JIM5 epitope is concentrated at the tricellular junctions and at the corners of intercellular spaces. Guillemin et al. (2005) found that the JIM5 epitope in sugar beet root was restricted to some cell junctions of the storage parenchymal cell walls and the lining of intercellular spaces, and at cell junctions in vascular tissues. The 2F4 epitope was detectable prior to de-esterification treatment. The 2F4 antibody binds to blockwise de-esterified HG, whereas JIM5 antibody binds to un-esterified HG or HG with a low degree of methyl esterification. Therefore, HG with a low degree of methyl esterification is probably also involved in cell adhesion as long as it contains at least nine consecutive unesterified GalA residues for the formation of an "egg-box" structure with calcium ions (Liners et al., 1989). The role of the "egg-box" structure in cell adhesion is also supported by evidence found in Chapter 4 that both CDTA and endo-PG caused cell separation between epidermis and hypodermis.

In Chapter 3, galactans, a side-chain of RG I, were not found in the middle lamella or at the corners of intercellular spaces in chicory leaves using immunogold labelling with LM5. This is consistent with the results of Jones et al. (1997) for tomato (*Lycopersicon esculentum*) pericarp, Bush and McCann (1999) for potato (*Solanum tuberosum*) tuber tissue and McCartney et al. (2000) for developing pea (*Pisum sativum*) cotyledons. It can be concluded that most galactan is not involved in cell adhesion.

Arabinans, another sidechain of RG I, were found in the middle lamella, but not at the corners of intercellular spaces in chicory leaves using immunogold labelling with LM6 (Chapter 3). McCartney et al. (2000) also found that α-(1→5)-arabinans were present in the region of the middle lamella in lupin cotyledon tissue. In overripened apple fruits with a soft texture, arabinans had been lost from the walls of the cortical cells (Pena and Carpita, 2004). Furthermore, the absence of arabinan side-chains was found to be strongly associated with loose cell association in *Nicotiana plumbaginifolia* non-organogenic callus (Iwai et al., 2001). However, although Bush and McCann (1999) found that arabinans were distributed throughout the entire wall of the cortex of potato tuber, they failed to find arabinans in the middle lamella of all parenchymal cell walls. Therefore, a role for arabinans in cell adhesion remains questionable. In chapter 4, loss

of cell adhesion on endo-PG treatment was accompanied by loss of HG and arabinan, but not galactan. This is consistent with a role for arabinan but not galactan in cell adhesion.

Using a polyclonal antibody, Moore and Staehelin (1988) found that the RG I backbone was restricted to the middle lamella in the root-tip and leaf tissues of red clover (*Trifolium pratense* L.) using immunogold labelling and showed that the label was mostly concentrated in the expanded regions of the middle lamella at the corner junctions. However, Moore and Staehelin (1988) found that the RG I backbone was not present in the forming of cell plates. The middle lamella originates from the cell plate. Therefore, there is a lack of confidence in the concept that the RG I backbone is involved in cell adhesion.

Using a polyclonal antibody, it was shown that RG II is absent from the middle lamella of radish and rice roots and from cultured tobacoo cells (Matoh et al., 1998). Williams et al. (1996) and O'Neill et al. (2004) also reported that RG II was absent from the middle lamella. These observations suggest that RG II cannot have a role in cell adhesion. However, Ridley et al. (2001) argued that RG II could be masked by a base-labile component and hence is not detectable. Iwai et al. (2002) claimed that a loss of cell adhesion in *Nicotinana plumbaginifolia* callus *nolac-H18*, characterised by a mutation in the gene NpGUT1 encoding a glucuronyl transferase, was due to the lack of β-glucuronic acid and α-L-galactose in the sidechains of RG II and necessary for the formation of a dimer via borate. Based on the findings of Iwai et al. (2002), Lord and Mollet (2002) suggested that RG II is involved in cell adhesion and the RG II dimer provides a "scaffold" for the assembly of other pectic polysaccharides. From the observation that endo-PG macerated chicory leaves and white clover leaflets (Chapter 4), RG II is not the sole molecule involved in cell adhesion, if it does have a role. Whether or not RG II plays a role in cell adhesion needs clarification, especially in regards to data on the role of RG II in cell plate formation (Lord and Mollet, 2002).

From the above discussion, it can be concluded that HG with a low degree of methyl esterification is often considered to have a role in cell adhesion. However, the polysaccharides in the middle lamella need to be firmly attached to the primary walls, otherwise cell adhesion cannot occur. One possibility is that these polysaccharides are linked to other polysaccharides as a complex of cellulose-pectin-xyloglucan, as

proposed in the Chapter 2. Another possibility is that RG II is linked to HG (Reuhs et al., 2004).

The mechanisms involved in cell adhesion in different cell types could be different (Section 4.5.1.1 in Chapter 4), and the mechanisms could also change during cell development. Leboeuf et al. (2005) found that, in *Arabidopsis*, JIM7 labelling was stronger in the wall of 'young' cells than in those of 'mature' cells, whereas 2F4 labelling was stronger in cell corners of 'mature' cells than in those of 'young' cells. Sobry et al. (2005) also found that Ca^{2+}-cross-linked HG was absent from the shoot meristem of *Sinapis alba*. This suggests that the "egg-box" mechanism of cell adhesion occurs in late development, but not in early development of cells.

6.3.2 Structural support

Pectic polysaccharides could have a function in structural support. HGs with a low degree of methyl esterification can form pectin hydrogels via calcium bridging (Jarvis, 1992a). The swelling and shrinking of the hydrogel can be adjusted according to the ionic conditions (Zwieniecki et al., 2001). This phenomenon may give structural support by maintaining turgor pressure. The abundance of the JIM5 epitope in the outer walls of the epidermis and in the thick walls of the outer phloem parenchyma of chicory leaf midrib probably has a role in strengthening the tissues. The possible function of pectic polysaccharides in structural support is evidenced by the collapse of these walls following treatment with endo-PG, CDTA and Na_2CO_3 (Chapter 4). For example, the removal of HG from the walls of the epidermis and hypodermis by endo-PG, CDTA and Na_2CO_3 distorted their cell shapes.

6.3.3 Phloem fibre cell walls

Prior to the present study, there had been no work using immunocytochemical methods on the distribution of pectic polysaccharides in the fibre cell walls of the vascular bundles in chicory leaves. The present study is the first to report that the primary walls of chicory fibre cells are rich in galactans, but lack arabinans (Chapter 3). Although the functions and characteristics of these pectic polysaccharides in these walls are unknown, it was found that the JIM5 epitope in these walls was resistant to CDTA extraction, the JIM7 epitope was resistant to CDTA extraction and endo-PG treatment, and the LM5

epitope was largely resistant to both CDTA and Na_2CO_3 extraction (Chapter 4). Endo-PG actively hydrolyses unesterified HG, but has less activity on HG with a high degree of methyl esterification (Daas et al., 2001a, 2001b). These results suggest that the JIM5 epitope may be not calcium bridged or that it is firmly bound to the JIM7 and LM5 epitopes, and therefore is resistant to CDTA extraction. These results also suggest that the JIM7 epitope in these fibre walls is extremely highly esterified preventing the action of endo-PG. In the thickened secondary walls of these fibre cells, pectic polysaccharides were not detectable using JIM5, JIM7, LM5 and LM6 antibodies (Chapter 3) and these walls were not lignified (Chapter 2).

Fibres in plants include those with lignin-rich walls (e.g. wood fibres) and those with non-lignified walls, such as tension wood fibres and some bast (phloem) fibres (e.g. flax, hemp, etc). Flax (*Linum usitatissimum* L.) fibres are primary phloem fibres (Gorshkova and Morvan, 2006) and are derived from procambial cells in the protophloem and encircle the vascular cylinder (Morvan et al., 2003). The walls of flax fibres can be compared with the walls of the phloem fibres of chicory leaves. Although the walls of flax fibres ares also rich in (1→4)-β-D-galactan (Andème-Onzighi et al., 2000), the location of this polysaccharide is completely different from that in the walls of chicory phloem fibres. In the immature fibre cells of the hypocotyl of flax seedlings (14-day-old), the LM5 epitope was in the secondary walls and the concentration gradually decreased from the plasma membrane towards the primary wall (Andème-Onzighi et al., 2000), whereas in chicory the epitope was present only in the primary wall of the phloem fibre (Chapter 3).

Based on a finding that the presence of galactans coincides with cellulose with a low degree of crystallinity, Gorshkova and Morvan (2006) suggested that soluble galactans delay the formation of crystalline cellulose. In Chapter 3, it was suggested that the galactans in the walls of phloem fibres may contribute to their mechanical strength, as McCartney et al. (2000) found that the firmness of cotyledons of peas is higher when galactans are present in high concentration in the walls. This hypothesis needs further testing.

6.3.4 Transfer cells and wall ingrowths

Transfer cells are cells with wall ingrowths for efficient nutrient distribution (Offler et aI., 2003). The wall ingrowths are deposited after primary wall deposition, and, although they are not lignified, they are described as secondary walls. Wall ingrowths result in an increase in the surface area and are associated with a high rate of solute transport. Histochemical and cytochemical studies showed the wall ingrowths contain cellulose and are rich in pectic polysaccharides (Offler et al., 2003). Prior to the present study, wall ingrowths in lamina minor veins had not been investigated using immunocytolabelling for pectic polysaccharides, although the wall ingrowths of transfer cells in the nodule vascular bundles of peas had been examined for the JIM5 and JIM7 epitopes (Dahiya and Brewin, 2000). In the present study, it was found that the antibodies JIM5, JIM7, LM5 and LM6 all bound to the wall and wall ingrowths of transfer cells. This was specially so for LM5 and LM6. These epitopes could have a function in wall ingrowth/plasma membrane complex for the enhancement of plasma membrane transport capacity.

6.3.5 Order of deposition of galactans and arabinans

In the present study, it was found that the LM6 epitope was present in the oldest regions of the wall near the middle lamella, whereas the LM5 epitope was present in only the more recently deposited regions and was absent from the oldest regions such as the middle lamella and the corners of intercellular spaces (Chapter 3). This is consistent with the finding of Vincken et al. (2003) with potato that (1→5)-α-L-arabinan is deposited earlier during tuberisation than (1→4)-β-D-galactan. These findings suggest that deposition of these polysaccharides is developmentally regulated. Alternatively, galactans could be turned over during cell wall formation. In some young phloem parenchyma walls, galactan was lacking, but in most inner phloem parenchyma galactan was present. The outer, thick-walled phloem parenchyma was presumably formed from a thin-walled precursor, yet the galactan was still confined to the inner, most recently laid down, region of the wall. This may suggest turnover.

Opposing distributions of LM5 arabinans and galactans were found in some instances (Chapter 3). Within a cell wall, the middle lamella, intercellular region and pit fields lacked the LM5 epitope, but contained the LM6 epitope. In walls of some cell types, the

LM5 epitope was absent or low in concentration, but the LM6 epitope was present or high in concentration, for example, the walls of certain younger, developing phloem parenchyma cells, and of guard cells; in contrast, LM5 epitope was present, LM6 epitope absent in some walls, for example, phloem fibre cell walls. These findings suggest that the LM5 and LM6 epitopes have quite different, temporally-segregated functions.

6.4 Importance of cell-wall pectins for forage degradation

6.4.1 Effect of cell-wall pectins on forage digestibility

As discussed earlier in Section 6.2, the cell walls of chicory leaves have a high pectin content and pectic polysaccharides accounted for 67% and 59% of the total wall polysaccharides of forage chicory lamina and midrib, respectively (Chapter 2). Pectins are reported to be readily degraded by bacterial enzymes in the rumen (Tamminga, 1993). Hatfield and Weimer (1995) found 97% of the pectin from the walls of lucerne leaves was degraded within 24 h by mixed ruminal microbes. In chapter 5, it was found that 84-96%, 70-96%, 76-94% of uronic acid, arabinose and galactose residues, repectively, were released from chicory tissues by *F. succinogenes*. Therefore, abundant pectin in forage chicory may be a reason for its high digestibility.

6.4.2 Effect of cell-wall pectin on forage particle breakdown

6.4.2.1 Importance of particle breakdown

In ruminant digestion, forage must be broken down to a critical particle size to have a high probability of leaving the rumen, subsequently allowing for further intake (Domingue et al., 1991b). Generally, the more quickly forage is broken down, the greater the VFI and hence feeding value.

6.4.2.2 Rapid breakdown of chicory in the rumen

On most diets, particle breakdown normally occurs during both eating and ruminating and depends on forage type (Ulyatt et al., 1986). Hoskin et al. (1995) reported that deer housed indoors spent a similar time eating chicory (361 min per 24 h) to perennial ryegrass (379 min per 24 h), but markedly less time ruminating chicory (33 min per 24

h) than perennial ryegrass (270 min per 24 h). Kusmartono (1996b) also found that deer grazing chicory were observed to spend less time ruminating than those grazing perennial ryegrass with half of the deer grazing chicory not observed to ruminate at all. The efficiency of particle breakdown during eating was slightly lower for deer fed chicory than perennial ryegrass (Kusmartono et al., 1996b). Thus, chewing during eating alone was not enough to break down chicory to the critical size for having a high probability of leaving the rumen. It could therefore be speculated that chicory continued to be rapidly broken down after swallowing, thus obviating the need for rumination.

6.4.2.3 Hypothesis for the rapid breakdown of chicory in the rumen

Barry (1998) proposed that the rapid breakdown of chicory in the rumen is "probably due to its high ratio of readily fermentable carbohydrate: structural carbohydrate and low content of silicon." In particular, this author suggested that the rapid degradation of pectin, an "intracellular cement", may result in the fast particle breakdown of chicory in the rumen. However, this hypothesis has not been tested and was not strongly supported by scientific evidence.

From the literature, it is known that pectin in the middle lamella plays a role in cell adhesion. In particular, the crosslinking of unesterified HG and calcium in the middle lamella contributes to cell adhesion (Jarvis et al., 2003). The concentration of pectin is high in the cell walls of chicory leaves (Kusmartono, 1996; Chapter 3) and pectic polysaccharides are readily and rapidly degraded in the rumen (Hatfield and Weimer, 1995). The failure of cell adhesion causing cell separation occurs in plants suffering from bacterial infection (Murdoch et al., 1999), during vegetable and fruit processing (Chesson, 1980) and as a result of flax retting (Zhang et al., 2000). Therefore, it is possible that degradation of the HG, which is responsible for cell adhesion, by pectolytic enzymes from the ruminal bacteria is a reason for the rapid breakdown of chicory leaves in the rumen.

Several questions need to be answered to support this hypothesis. First, are pectins important for chicory cell adhesion and which pectic polysaccharides have a role in cell adhesion? Secondly, does forage particle breakdown occur if the cell adhesion is destroyed by enzymes? Finally, do ruminal bacteria cause forage particle breakdown?

6.4.2.4 Support for the hypothesis on forage particle breakdown

From the study reported in Chapter 2, it is known that the cell walls of chicory leaves are particularly rich in pectin. From the study reported in Chapter 3 and Chapter 4 as discussed in Section 6.3.1, HG with a low degree of methyl esterification has a role in cell adhesion in chicory. Hence, the first question is answered.

The second question is answered by the study reported in Chapter 4. Endopolygalacturonase, which has the similar function to pectolytic enzymes secreted by rumen bacteria, was used to treat chicory leaves. The results showed that the particle size of chicory leaves was dramatically reduced after treatment with endopolygalacturonase (Table 4.1, Table 4.3), which resulted from cell separation after the treatment. Immunofluorescence work showed that degradation of homogalacturan in the middle lamella was accompanied by cell separation.

Chapter 5 reports a study in which the pectolytic ruminal bacterium *L. multiparus* caused significant chicory particle breakdown (Figure 5.1). Thus, the hypothesis proposed in Section 6.4.2.3 is supported.

In summary, HGs with a low degree of methyl esterification are located in the middle lamella and at the corners of intercellular spaces, play a role in cell adhesion. The degradation of HGs causes cell separation and subsequent forage particle breakdown. For this reason, along with the abundance of pectins and the walls being mainly non-lignified, forage chicory is rapidly degradable. This pathway of forage particle breakdown could be the same for other pectin-rich forages, such as white clover (Cheng et al., 1979). The diagram shown in Figure 6.1 summaries the role of a high concentration of pectins in the cell walls on the digestibility and rapid degradation of chicory leaves in the rumen.

6.4.3 Advantages of pectins as wall components for forages

Pectins are rapidly and extensively degraded in the rumen (Chesson and Monro, 1982; Hatfield and Weimer, 1995). With commercial pectins, Gradel and Dehority (1972) found that these polysaccharides were rapidly degraded by rumen microbes. Titgemeyer et al. (1992) and Hatfield and Weimer (1995) also found that pectins in lucerne cell walls were rapidly degraded. Furthermore, the pectins in lucerne cell walls were nearly

completely degradable in non-lignified tissues, with only about 10% of the pectin left in the highly lignified tissue after degradation by mixed rumen bacteria (Hatfield and Weimer, 1995). Unlike starch, the rapid degradation of pectin does not result in acidosis and consequently does not cause a drop in the rumen fluid pH (Hatfield and Weimer, 1995). Pectins are cell wall components and their metabolic turnover, if it occurs at all, is much slower than that of soluble sugars. For example, the content of water soluble carbohydrates in chicory herbage decreased after wilting for 24 h, but the pectin content remained unchanged (Tinworth et al., 1999).

From the above observations, it can be concluded that pectins are excellent nutrients in forages for ruminants. It is possible to increase the content of pectins in chicory and other forages. A great range in pectin concentrations was found in the leaf and stem cell walls among 33 alfalfa cultivars at Arlington, WI, and 36 alfalfa cultivars at Marshfield, WI (Hartnell et al., 2005). This suggests that the pectin content of forage cell walls could be a breeding target. In fact, the US Dairy Forage Research Centre has already increased the pectin concentration of lucerne stem by 15-20% through two cycles of selection (Hartnell et al., 2005).

6.5 Conclusions and recommendations for future work

6.5.1 Conclusions

- The pectin content of chicory leaves is high compared with other forages studied to date,
- Chicory leaf cell walls are all non-lignified primary walls, apart from a small proportion of xylem tracheary elements in vascular bundles,
- The homogalacturonan is located particularly in the intercellular junctions and adhesion zones (middle lamella and cell corners),
- A large proportion of homogalacturonans is also found throughout the thick walls of the epidermis, hypodermis and outer phloem parenchyma,
- Treatment with CDTA, endopolygalacturonanase preparations and pectolytic rumen bacteria leads to loss of homogalacturonan from the middle lamella and cell corners and also to cell separation, but some cells (e.g. rows of epidermal and hypodermal cells) still adhere,
- Loss of pectin from some walls is associated with loss of wall strength (e.g. collapse of hypodermal cells).

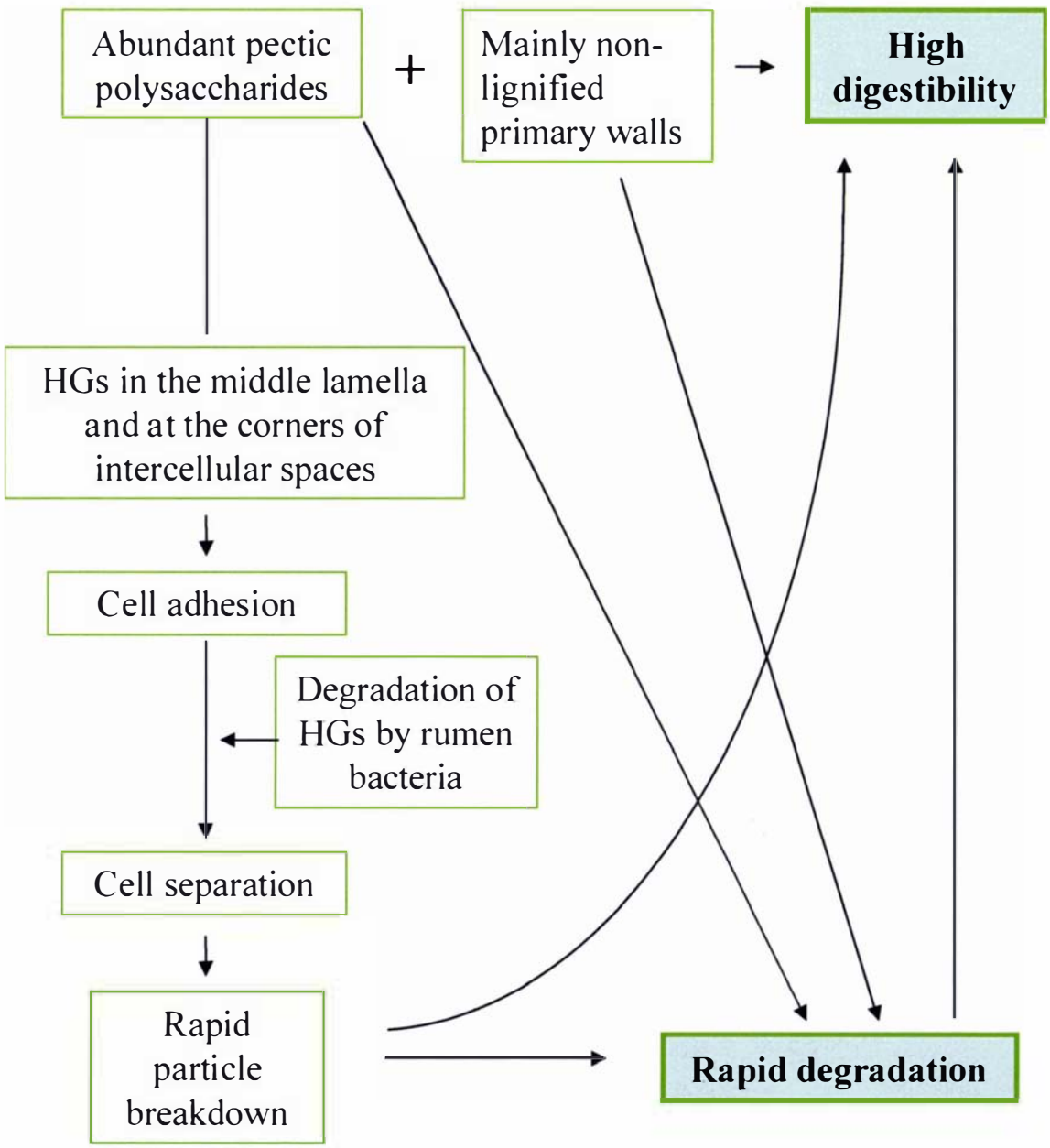


Figure 6. 1 The proposed role of pectins of the cell walls of chicory leaves in degradation in the rumen

6.5.2 Recommendations for future work

The present study has raised a number of questions for further study in relation to cell-wall structure and the location and degradation of pectic polysaccharides.

6.5.2.1 Cell-wall structure

Xylogalacturonan

Xylogalacturonan is a pectic polysaccharide normally found in the cell walls of fruits or roots, but not in the cell walls of leaves (Schols, H. A. 2004 personal communication). However, in the present study a small amount of terminal Xyl was present in the pectic fractions, according to methylation results (Chapter 2). Thus, it seems that xylogalacturonan may be present in chicory leaves. This needs to be confirmed with further study. LM8, an anti-xylogalacturonan antibody, has been used to examine sections from chicory leaves in a preliminary study, but the result was negative. However, the LM8 antibody does not bind to all xylogalacturonan (Knox, J.P. 2005 personal communication). In further work, the three pectic fractions (Chapter 2) need to be reduced to give GalA linkages before methylation analysis to check for presence or absence of 3,4-GalA in the galacturonan backbone. Another approach for determining xylogalacturonan is to subfractionate pectic fractions for NMR analysis.

6-linked Gal

Linkage analysis of the cell-wall polysaccharides of chicory leaves (Chapter 2) found that a substantial proportion of 6-linked Gal was present in the cell-wall preparations and their fractions (Chapter 2). The 6-linked Gal was arbitrarily assigned to galactans. Are β-(1→6)-galactans present in chicory leaf cell walls? This could be confirmed using an anti-β-(1→6) galactan antibody specific for AGP (Kikuchi et al., 1993). As Andème-Onzighi et al. (2000) found that the walls of the phloem fibres in hypocotyls of young flax were labelled with anti-β-(1→6)-D-galactan antibody, particular attention should be given to phloem fibre walls.

Cellulose-associated pectin

A substantial amount of cellulose-associated pectin was found in the present study. This type of pectin has also been found in apple fruit cellulosic residue (Oechslin et al.,

2003). Cellulose-associated pectin needs further study, as it is present in a range of plants. Two types of work should be initiated: determination of the fine structure of this pectin and the nature of the connection between pectin and cellulose. To analyse the fine structure of the cellulose-associated pectin, endo-α-(1→5)-L-arabinanase and endo-β-(1→4)-D-galactanase could be applied, individually or in combination to degrade the cellulosic residue from extensive alkali extraction. The supernatants and the residues after degradation should be analysed for monosaccharide and polysaccharide compositions and the content of methyl esters. LM5 and LM6 antibodies should also be applied to the supernatants and residues. The nature of the connection between pectin and cellulose could be investigated by NMR analysis on the residues after enzymatic degradation.

As discussed in Chapter 2, cellulose-associated pectin could also be present as a cellulose-pectin-xyloglucan complex. Hence, the linkage between pectin and xyloglucan also needs to be studied.

Homogalacturonan

The degree of methyl esterification and the distribution of the non-esterifed GalA residues in HG may have effects on cell adhesion. These can be measured using NMR.

Antibodies LM7, 2F4 and PAM1 can recognise HG with various patterns of methyl esterification. They should be employed in further work.

PME and calcium are associated with non-esterified HG. Their occurrence in cell walls could relate to cell adhesion and whould be monitored.

Acetylation of HG hase not been examined in this study. Acetylation could change the property of HG, subsequently having a role in cell adhesion. This is a good area to explore.

Plantain

Plantain (*Plantago lanceolata*) is another perennial herb suitable as a forage for grazing animals in temperate regions. Plantain also grows well under the stresses of drought and high temperature (Labreveux et al., 2004). Although the potential yields of plantain appear less than those of chicory in USA (Sanderson et al., 2003), but similar to or

greater than chicory in New Zealand (Simone Hoskin, unpublished data), plantain can be planted in a wider range of soils than chicory (Stewart, 1996). Thus, it can provide herbage for ruminants during a drought summer in lower quality soil. Plantain belongs to the family Plantaginaceae (Veronicaceae). Although the structures of xyloglucans from the cell walls of *Plantago major* have been examined in a comparison of structural diversity among different taxonomic orders (Hoffman et al., 2005), the polysaccharide compositions of forage cell walls from this family have not been reported. This proposed research would widen knowledge about forage cell-wall polysaccharides since currently only the families Poaceae (Grasses), Fabaceae (Legumes), Brassicaceae (Brassicas) (Lam et al., 1990) and Asteraceae (this study) have been studied.

6.5.2.2 Location of pectin

Phloem fibre cell walls

(1→4)-β-D-Galactan was found in particularly high concentration in the primary walls of phloem fibres in the vascular bundles of chicory leaves (Chapter 3). A series of questions need to be addressed about this polysaccharide. Does this galactan change during plant development? Is this galactan found in other plants? Materials from different developmental stages of chicory and from different plants such as legumes and grasses should be examined using immunolabelling. Is this galactan different in its properties from the galactans in the walls of other cell types ? A range of enzymes such as endo-β-(1→4)-D-galactanase and endo-α-(1→5)-L-arabinanase and a range of reagents which can liberate galactans could be used before immunolabelling. What are functions of these pectic polysaccharides? The modification of galactans in the wall of phloem fibres after they are secreted from Golgi apparatus could be different from that in the walls of other cell types. What are these differences? Which enzymes are involved in the modification?

The JIM5 epitope was not lost from phloem fibre walls on treatment with CDTA (Chapter 4), suggesting that calcium could be not present. To confirm this, the distribution of calcium in these walls needs to be examined. If the result shows calcium to be present, the JIM5 epitope may be bound to other polymer structures in the wall and hence not free to dissolve when the calcium is removed. The JIM7 epitope was resistant to CDTA and endo-PG treatments, suggesting that these epitopes are

particularly highly esterified. Esterification in these walls could be different. PME activity in these walls could be determined, and NaOH could be applied to deesterify HG before CDTA and endo-PG treatments for further study.

Fourier-transform infra-red micro spectroscopy can be used to compare wall composition at the level of single cells (Jones et al., 2005). Therefore, this technique would be ideal for distinguishing the differences between the walls of phloem fibres and other cells and differences before and after treatments with CDTA or endo-PG.

Cell adhesion

Although this study supports the hypothesis that crosslinking of calcium and HG with a low degree of methyl esterification is partly responsible for cell adhesion, the function of unesterified HG has not been elucidated. Several antibodies, including 2F4, which recognises a calcium-dependent conformation of HG with at least nine contiguous galacturonic acid residues (Liners et al., 1989, 1992), LM7, which requires at least four unesterified galacturonic acid residues (Clausen et al., 2003) and PAM1, which interacts with long stretches of unesterified HG of about 30 residues (Willats et al., 1999a, 2000a), should also be adopted for further study. The methyl groups in HG from fractions of isolated cell walls should also be determined using NMR. Calcium is essential for Ca^{2+} bridge fromation. The distribution of calcium can be investigated using secondary ion mass spectrometry (SIMS) analysis.

The roles of RG I and RG II in cell adhesion are not clear. This also needs to be clarified.

6.5.2.3 Degradation of pectins

Some pectin (mostly RG I) in the walls of chicory leaves was not degraded by *L. multiparus*. *F. succinogenes* degraded more pectin, but some still remained. Hatfield and Weimer (1995) also found undegraded pectin in the cell walls of lucerne leaves and stems after incubation by the mixed rumen bacteria. The characteristics of this undegraded pectin should be investigated for its structure and connection to other polysaccharides and lignin in chicory and other forages.

A hypothesis that the degradation of pectic polysaccharides could facilitate the degradation of the xyloglucan-cellulose network was proposed in this study (Chapter 5).

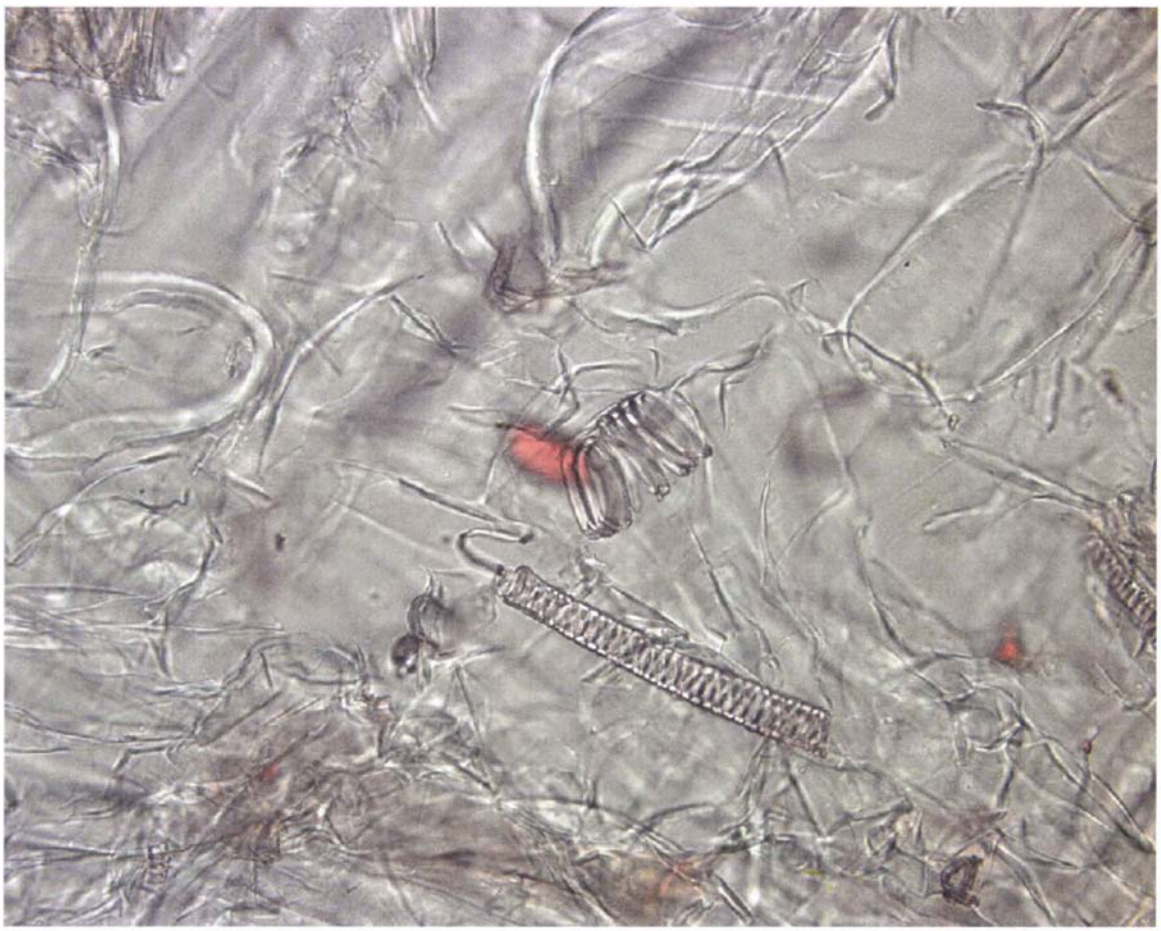
To test this hypothesis, a study is needed to compare depectinated walls with control walls without depectination.

Appendix 1

Eight-week old chicory leaves used in this study.

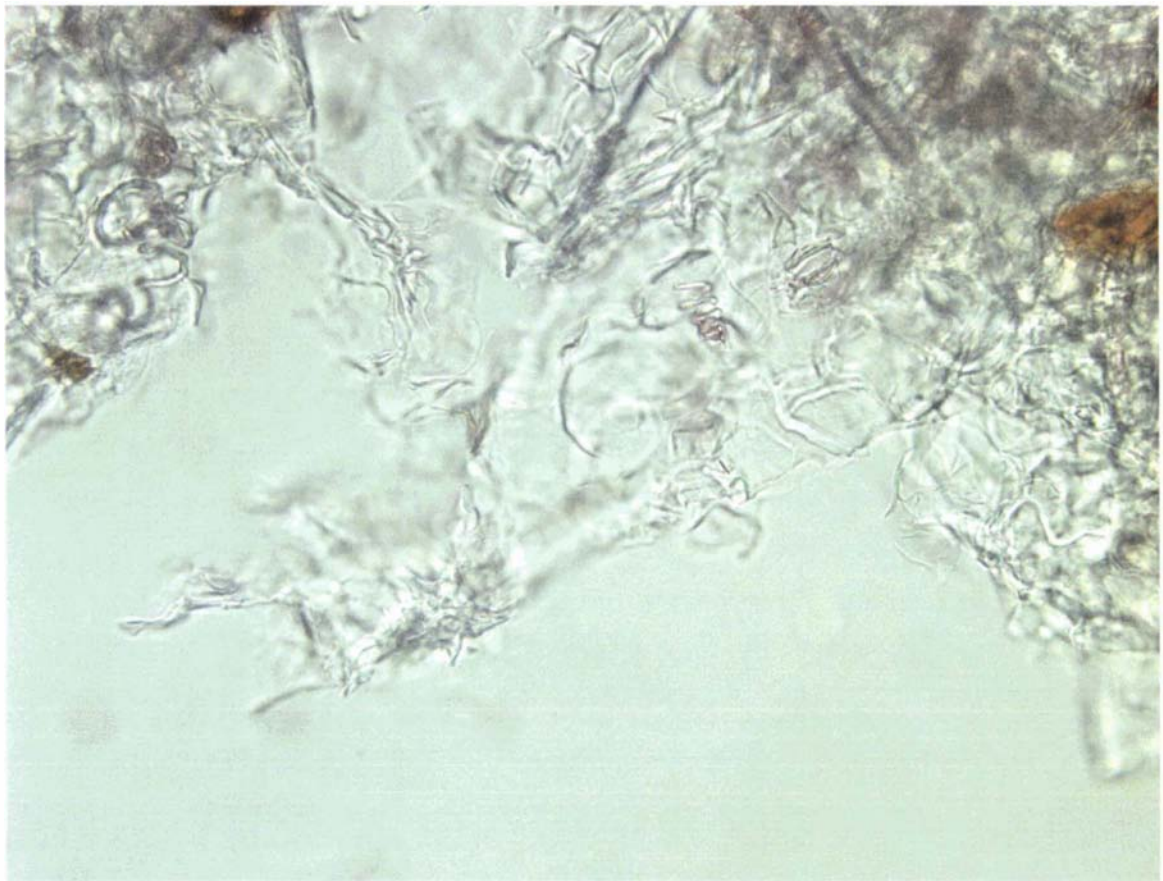
Appendix 2

Wall preparations from the midrib of chicory leaves stained with Ponceau 2R



Appendix 3

Wall preparations from the lamina of chicory leaves stained with Ponceau 2R



Appendix 4

Properties of endo-polygalacturonanase preparations from *Aspergillus niger* (Lot 00801)

1. Electrophoretic purity

Single major band on SDS-gel electrophoresis (MW = 24 000)

2. Specific activity and level of other activities

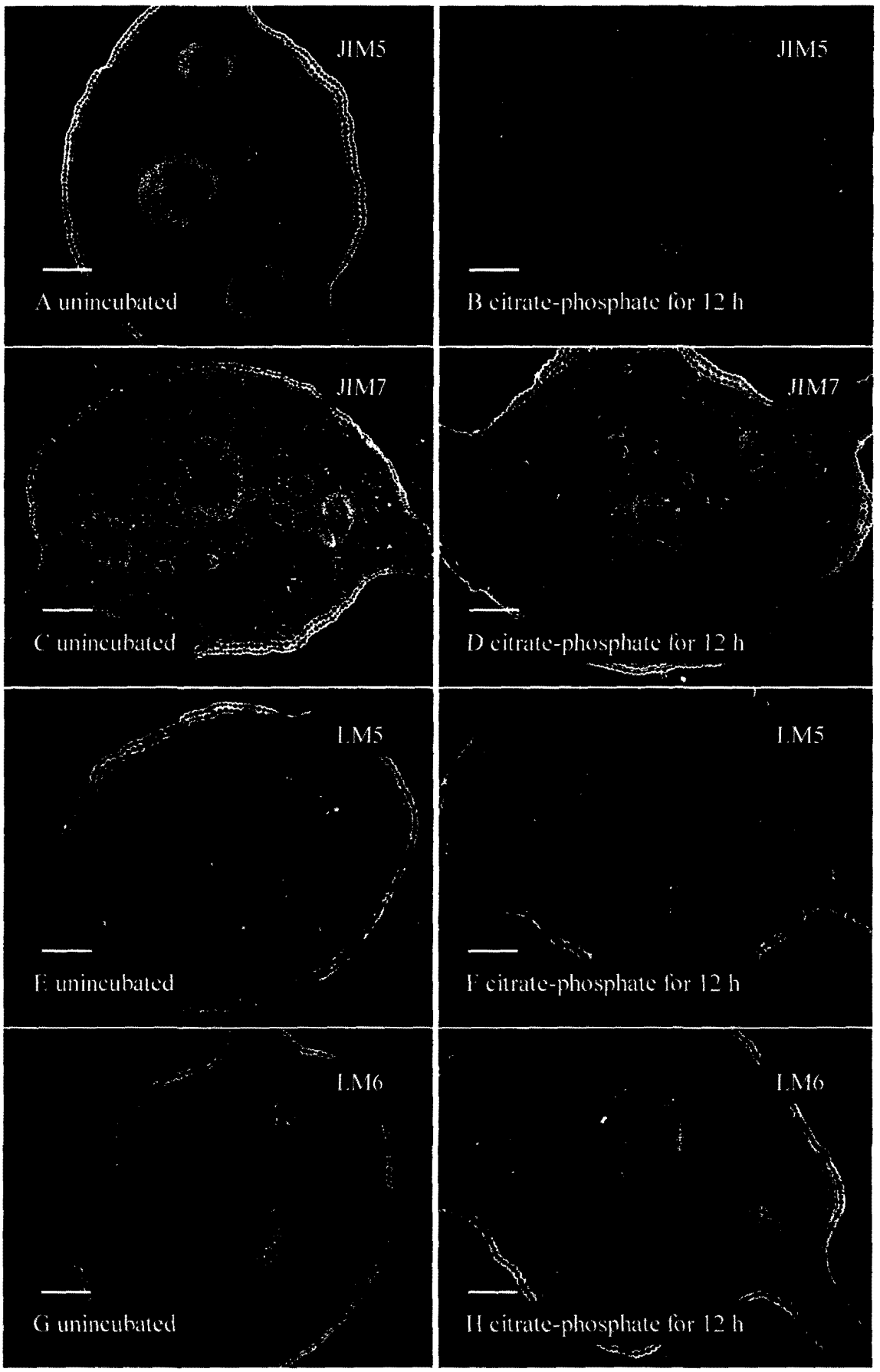
Substrate	Specific activity (U/mg protein)
Polygalacturonic acid (endo-polygalacturonanase)	1200
Galactan from potato fibre (endo-Galactanase)	0.010
CM-Linear Arabinan (endo-Arabinanase)	0.03
Citrus pectin (pectin transeliminase)	0.010
p-NP-α-L-arabinofuranoside (arabinofuranodidase)	0.002
p-NP-β-D-Galactoside (β-Galactosidase)	0.04

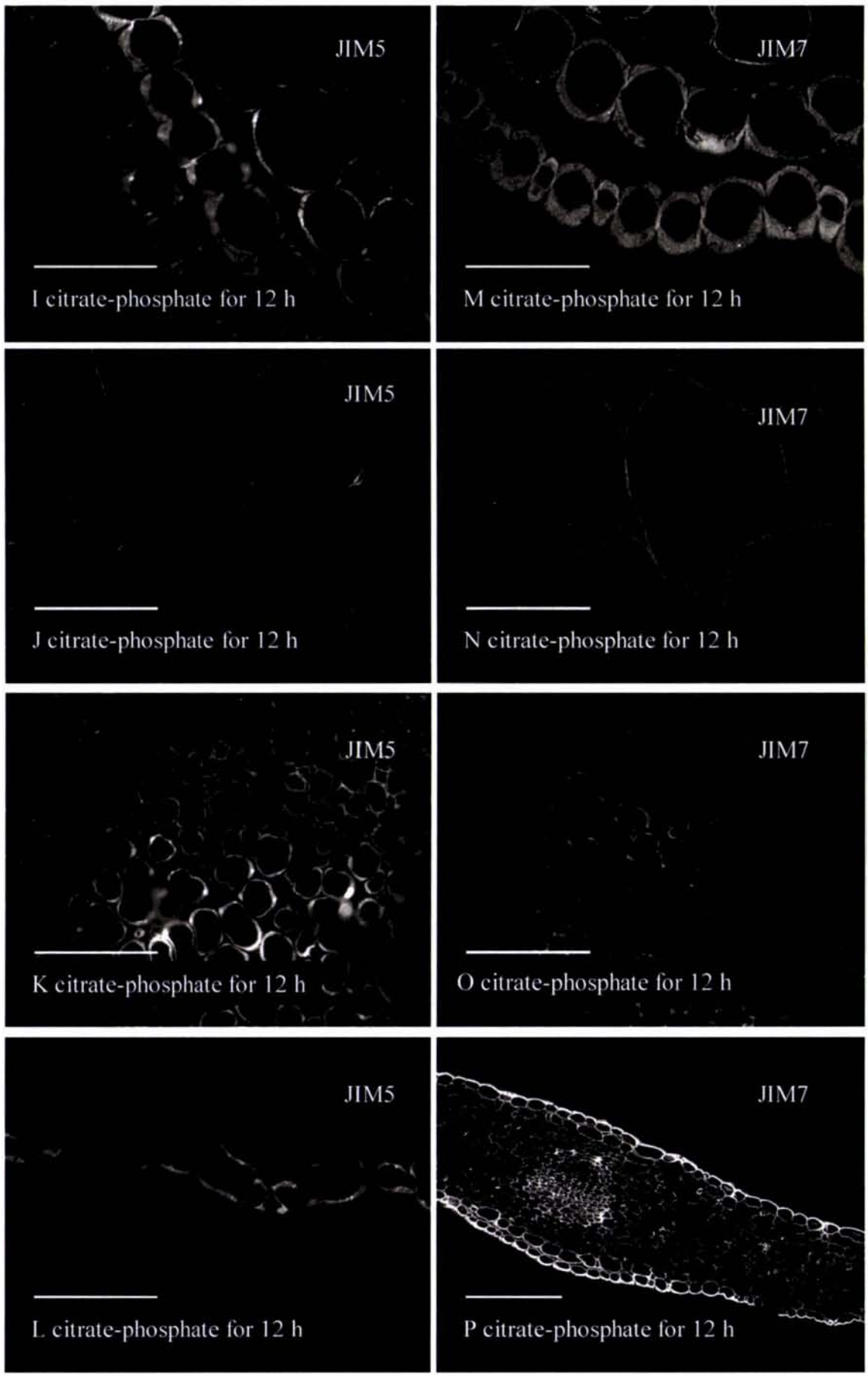
3. Physicochemical properties

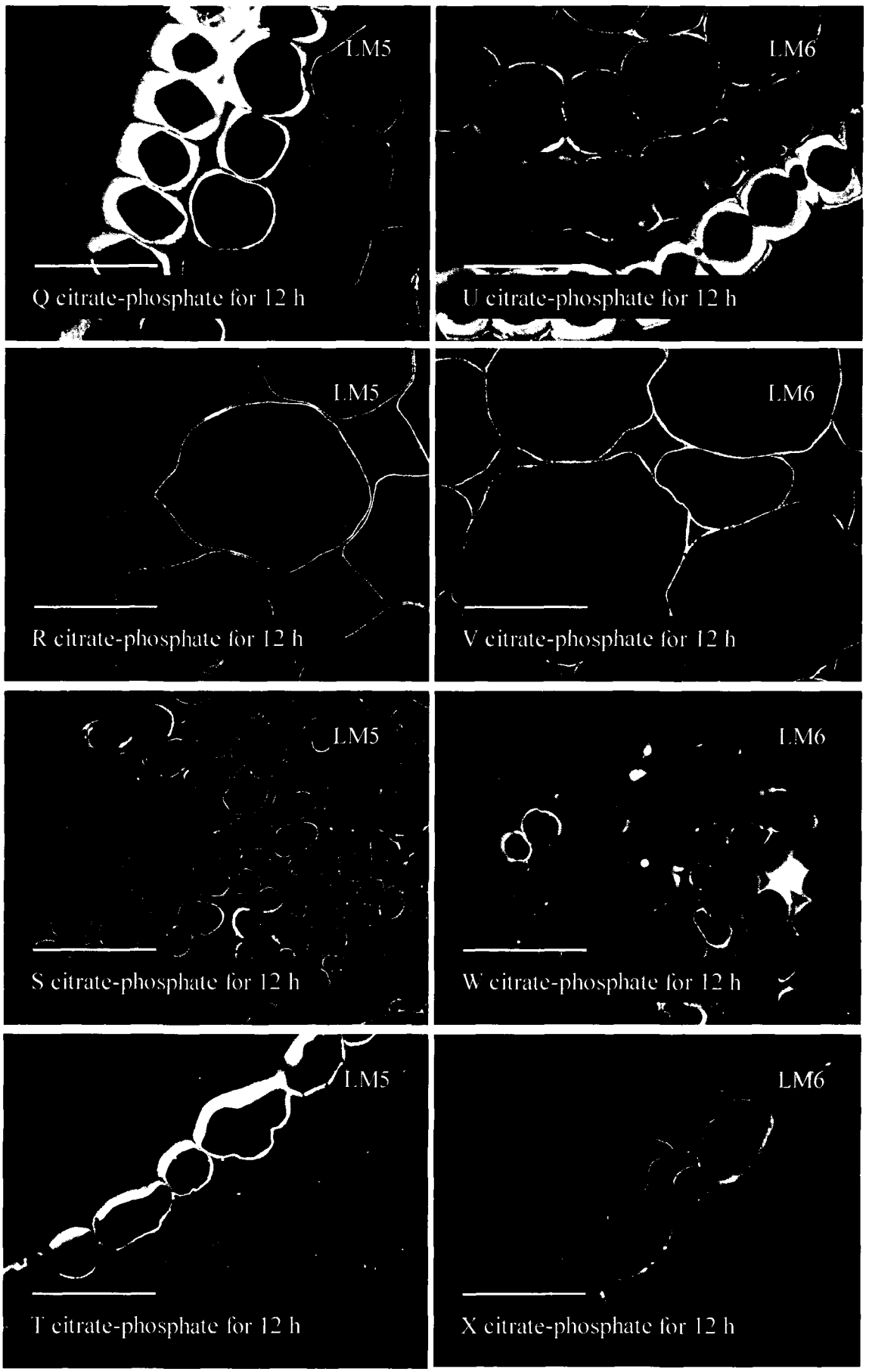
pH Opima	4.0	pH stability	3.0-7.0
Temp Opima	50 ^{o}C	Temp stability	< 50 ^{o}C

From product information, Megazyme International Ireland Ltd., Wicklow, Ireland

Appendix 5 Immunofluorescence labelling of transverse resin-embedded sections of chicory leaf midrib with JIM5, JIM7, LM5 and LM6 antibodies, before and after treatments with citrate-phosphate. **A.-D.** Unincubated. **E.-X.** Incubated in a citrate-phosphate buffer (pH 4.0, 30 mM citric acid and 40 mM Na_2HPO_4) containing sodium azide (0.02%) at 39 ^{o}C for 12 h. Bar, 50 μm.


JIM5
A unincubated
JIM5
B citrate-phosphate for 12 h
JIM7
C unincubated
JIM7
D citrate-phosphate for 12 h
LM5
E unincubated
LM5
F citrate-phosphate for 12 h
LM6
G unincubated
LM6
H citrate-phosphate for 12 h


JIM5
I citrate-phosphate for 12 h
JIM7
M citrate-phosphate for 12 h
JIM5
J citrate-phosphate for 12 h
JIM7
N citrate-phosphate for 12 h
JIM5
K citrate-phosphate for 12 h
JIM7
O citrate-phosphate for 12 h
JIM5
L citrate-phosphate for 12 h
JIM7
P citrate-phosphate for 12 h


LM5
Q citrate-phosphate for 12 h
LM6
U citrate-phosphate for 12 h
LM5
R citrate-phosphate for 12 h
LM6
V citrate-phosphate for 12 h
LM5
S citrate-phosphate for 12 h
LM6
W citrate-phosphate for 12 h
LM5
T citrate-phosphate for 12 h
LM6
X citrate-phosphate for 12 h

Appendix 6

The formula for the vitamin solution used in bacteria incubation

Pyridoxine HCl (Vitamin B_6)	10.0 mg
L-Ascorbic acid (Vitamin C)	5.0 mg
Calcium pantothenate	5.0 mg
Choline chloride	5.0 mg
Lipoic acid (DL-6,8-Thioctic acid)	5.0 mg
i-Inositol (myo-inositol)	5.0 mg
Niacinamide	5.0 mg
Nicotinic acid	5.0 mg
p-Aminobenzoic acid	5.0 mg
Pyridoxal-HCl	5.0 mg
Riboflavin (Vitamin B_2)	5.0 mg
Thiamin-HCl (Vitamin B_1)	5.0 mg
D+ Biotin (Vitamin H)	2.0 mg
Folic acid	2.0 mg
Vitamin B_{12} (Cyanocobolamin)	0.1 mg
Distilled water	1000 ml

The components were added to the distilled water and the volume brought to 1 litre with aid of a magnetic stirrer for dissolving. The solution (200-300 ml) in a flask was bubbled with O_2-free CO_2 for 30 minutes, and was then filtered into sterile CO_2-filled Hungate tubes through a sieve (0.45 μm) for sterilisation. The tubes were frozen at -20 °C for storage. Before use, the solution was thawed.

References:

Abdel-Massih RM, Baydoun EAH, and Brett CT. 2003. *In vitro* biosynthesis of 1,4-β-galactan attached to a pectin-xyloglucan complex in pea. *Planta* **216:** 502-511.

Akin DE. 1989. Histological and physical factors affecting digestibility of forages. *Agronomy Journal* **81:** 17-25.

Albersheim P, Nevins DJ, English PD, and Karr A. 1967. A method for the analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. *Carbohydrate Research* **5:** 340-345.

Albersheim P. 1959. Instability of pectin in neutral solutions. *Biochemical and Biophysical Research Communications* **1:** 253-256.

Aman P. 1985. Chemical composition and in vitro degradability of major chemical-constituents in botanical fractions of red clover harvested at different stages of maturity. *Journal of the Science of Food and Agriculture* **36:** 775-780.

Aman P. 1993. Composition and structure of cell wall polysaccharides in forages. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility*. Madison, wisconsin, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 183-200.

Andeme-Onzighi C, Girault R, His I, Morvan C, and Driouich A. 2000. Immunocytochemical characterization of early developing flax fiber cell walls. *Protoplasma* **213:** 235-245.

APG II. 2003. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* **141:** 399-436.

Bacic A, Harris PJ, and Stone BA. 1988. Structure and function of plant cell walls. In: Preiss J, ed. *The biochemistry of plants*. San Diego, California: Academic Press. 297-371.

Bailey RW. 1967. Quantitative studies of ruminant digestion II. Loss of ingested plant carbohydrates from the reticulo-rumen. *New Zealand Journal of Agricultural Research* **10:** 15-32.

Bailey RW. 1969. Carbohydrate and related compounds. In: Dawson IRMC, Elliott DC, Elliott WH and Jones KM, eds. *Data for biochemical research.* UK: Oxford University Press. 544.

Barnes RF, Miller DA, and Nelson CJ. 1995. *Forages.* Iowa State University Press, Published Ames, Iowa, USA.

Barry TN, Hoskin SO, and Wilson PR. 2002. Novel forages for growth and health in farmed deer. *New Zealand Veterinary Journal* **50:** 244-251.

Barry TN, Molan AL, Wilson PR, Lopez-Villalobos N, Schreurs NM, and Duncan AJ. 2001. Chicory as an alternative forage for deer health. In: Wilson PR, ed. *Proceedings of a deer course for veterinarians no. 18.* Palmerston North, New Zealand: Deer Branch NZVA. 122-127.

Barry TN. 1998. The feeding value of chicory (*Cichorium intybus*) for ruminant livestock. *Journal of Agricultural Science* **131:** 251-257.

Baydoun E, Abdel-Massih RM, Cumming CM, Rizkallah HD, and Brett CT. 2005. Pectin is synthesised as a complex with xyloglucan in the Golgi apparatus in pea. *Abstracts of Papers of the American Chemical Society* **229:** U291.

Beever DE, Thomson DJ, Ulyatt MJ, Cammell SB, and Spooner MC. 1985. The digestion of fresh perennial ryegrass (*Lolium perenne* L. *cv* melle) and white clover (*Trifolium repens* L. *cv* blanca) by growing cattle fed indoors. *British Journal of Nutrition* **54:** 763-775.

Belesky DP, Turner KE, and Ruckle JM. 2000. Influence of nitrogen on productivity and nutritive value of forage chicory. *Agronomy Journal* **92:** 472-478.

Belesky DP, Turner KE, Fedders JM, and Ruckle JM. 2001. Mineral composition of swards containing forage chicory. *Agronomy Journal* **93:** 468-475.

Benes K. 1968. On the stainability of plant cell walls with alcian blue. *Biologia*

Plantarum **10:** 334-346.

Bootten TJ, Harris PJ, Melton LD, and Newman RH. 2004. Solid-state ^{13}C-NMR spectroscopy shows that the xyloglucans in the primary cell walls of mung bean (*Vigna radiata* L.) occur in different domains: A new model for xyloglucan-cellulose interactions in the cell wall. *Journal of Experimental Botany* **55:** 571-583.

Bourquin LD, and Fahey GC. 1994. Ruminal digestion and glycosyl linkage patterns of cell-wall components from leaf and stem fractions of alfalfa, orchardgrass, and wheat straw. *Journal of Animal Science* **72:** 1362-1374.

Bouton S, Leboeuf E, Mouille G, Leydecker MT, Talbotec J, Granier F, Lahaye M, Hofte H, and Truong HN. 2002. Quasimodo1 encodes a putative membrane-bound glycosyltransferase required for normal pectin synthesis and cell adhesion in *Arabidopsis*. *Plant Cell* **14:** 2577-2590.

Boyd DC, and Rogers ME. 2004. Effect of salinity on the growth of chicory (*Cichorium intybus* cv. Puna) - a potential dairy forage species for irrigation areas. *Australian Journal of Experimental Agriculture* **44:** 189-192.

Brett CT, and Waldron KW. 1996. *Physiology and biochemistry of plant cell walls*. Chapman & Hall, 2-6 Boundary Row, London SE1 8 HN, UK.

Brett CT, Baydoun EAH, and Abdel-Massih RM. 2005. Pectin-xyloglucan linkages in type I primary cell walls of plants. *Plant Biosystems* **139:** 54-59.

Bryant MP, and Small N. 1956. Characteristics of 2 new genera of anaerobic curved rods isolated from the rumen of cattle. *Journal of Bacteriology* **72:** 22-26.

Bryant MP, Barrentine BF, Sykes JF, Robinson IM, Shawver CV, and Williams LW. 1960. Predominant bacteria in the rumen of cattle on bloat-provoking ladino clover pasture. *Journal of Dairy Science* **43:** 1435-1444.

Burke JL, Waghorn GC, Brookes IM, Attwood GT, and Kolver ES. 2000. Formulating total mixed rations from forages - defining the digestion kinetics of contrasting species. *Proceedings of the New Zealand Society of Animal Production* **60:** 9-14.

Bush MS, and McCann MC. 1999. Pectic epitopes are differentially distributed in the cell walls of potato (*Solanum tuberosum*) tubers. *Physiologia Plantarum* **107:** 201-213.

Carnachan SM, and Harris PJ. 2000. Polysaccharide compositions of primary cell walls of the palms *Phoenix canariensis* and *Rhopalostylis sapida*. *Plant Physiology and Biochemistry* **38:** 699-708.

Carpita NC, and Gibeaut DM. 1993. Structural models of primary cell walls in flowering plants - consistency of molecular-structure with the physical properties of the walls during growth. *Plant Journal* **3:** 1-30.

Carpita NC. 1996. Structure and biogenesis of the cell walls of grasses. *Annual Review of Plant Physiology and Plant Molecular Biology* **47:** 445-476.

Cassab GI. 1998. Plant cell wall proteins. *Annual Review of Plant Physiology and Plant Molecular Biology* **49:** 281-309.

Chambat G, and Joseleau JP. 1980. Isolation and characterization of a homogalacturonan in the primary walls of rosa cells cultures *in vitro*. *Carbohydrate Research* **85:** C10-C12.

Chambat G, Barnoud F, and Joseleau JP. 1984. Structure of the primary cell walls of suspension-cultured *Rosa glauca* cells. 1. Polysaccharides associated with cellulose. *Plant Physiology* **74:** 687-693.

Chambat G, Joseleau JP, and Barnoud F. 1981. The carbohydrate constituents of the cell-wall of suspension cultures of *Rosa glauca*. *Phytochemistry* **20:** 241-246.

Chapman A, Blervacq AS, Hendriks T, Slomianny C, Vasseur J, and Hilbert JL. 2000. Cell wall differentiation during early somatic embryogenesis in plants. II. Ultrastructural study and pectin immunolocalization on chicory embryos. *Canadian Journal of Botany-Revue Canadienne De Botanique* **78:** 824-831.

Chapple CCS, Vogt T, Ellis BE, and Somerville CR. 1992. An *Arabidopsis* mutant defective in the general phenylpropanoid pathway. *Plant Cell* **4:** 1413-1424.

Chen EMW, and Mort AJ. 1996. Nature of sites hydrolyzable by

endopolygalacturonase in partially-esterified homogalacturonans. *Carbohydrate Polymers* **29:** 129-136.

Cheng KJ, Dinsdale D, and Stewart CS. 1979. Maceration of clover and grass leaves by *Lachnospira multiparus*. *Applied and Environmental Microbiology* **38:** 723-729.

Chesson A, and Forsberg CW. 1997. Polysaccharide degradation by rumen microorganisms. In: Hobson PN and Stewart CS, eds. *The rumen microbial ecosystem*. 2nd ed. London; New York: Blackie Academic & Professional. 329-381.

Chesson A, and Monro JA. 1982. Legume pectic substances and their degradation in the ovine rumen. *Journal of the Science of Food and Agriculture* **33:** 852-859.

Chesson A, Gordon AH, and Lomax JA. 1985. Methylation analysis of mesophyll, epidermis, and fiber cell-walls isolated from the leaves of perennial and Italian ryegrass. *Carbohydrate Research* **141:** 137-147.

Chesson A, Gordon AH, and Scobbie L. 1995. Pectic polysaccharides of mesophyll cell-walls of perennial ryegrass leaves. *Phytochemistry* **38:** 579-583.

Chesson A, Stewart CS, Dalgarno K, and King TP. 1986. Degradation of isolated grass mesophyll, epidermis and fiber cell-walls in the rumen and by cellulolytic rumen bacteria in axenic culture. *Journal of Applied Bacteriology* **60:** 327-336.

Chesson A. 1980. Maceration in relation to the post-harvest handling and processing of plant material. *Journal of Applied Bacteriology* **48:** 1-45.

Chesson A. 1993. Mechanistic models of forage cell wall degradation. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility*. Madison, Wisconson, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 347-376.

Clark RG. 1995. A study of high lamb liver copper concentrations on some farms in Otago and southland. *New Zealand Veterinary Journal* **43:** 141-145.

Clarke AE, Anderson RL, and Stone BA. 1979. Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry* **18:** 521-540.

Clausen MH, Ralet MC, Willats WGT, Mccartney L, Marcus SE, Thibault JF, and Knox JP. 2004. A monoclonal antibody to feruloylated-(1→4)-β-D-galactan. *Planta* **219:** 1036-1041.

Clausen MH, Willats WGT, and Knox JP. 2003. Synthetic methyl hexagalacturonate hapten inhibitors of antihomogalacturonan monoclonal antibodies LM7, JIM5 and JIM7. *Carbohydrate Research* **338:** 1797-1800.

Cockayne AH. 1915. Chicory. *New Zealand Journal of Agriculture* **11:** 468.

Coimbra MA, Waldron KW, Delgadillo I, and Selvendran RR. 1996. Effect of processing on cell wall polysaccharides of green table olives. *Journal of Agricultural and Food Chemistry* **44:** 2394-2401.

Coleman SW, and Henry DA. 2002. Nutritive value of herbage. In: Freer M and Dove H, eds. *Sheep nutrition.* London: CAB International Publisher. 1-26.

Cosgrove DJ. 2005. Growth of the plant cell wall. *Nature Reviews Molecular Cell Biology* **6:** 850-861.

Cruickshank GJ. 1986. *Nutritional constraints to lamb growth at pasture*, PhD dissertation, Lincoln University, Canterbury, New Zealand.

Crush JR, and Evans J. 1990. Shoot growth and herbage element concentrations of 'Grasslands Puna' chicory (*Cichorium intybus* L.) under varying soil pH. *Proceedings of New Zealand Grassland Association* **51:** 163-166.

Cumming CM, Rizkallah HD, McKendrick KA, Abdel-Massih RM, Baydoun EAH, and Brett CT. 2005. Biosynthesis and cell-wall deposition of a pectin-xyloglucan complex in pea. *Planta* **222:** 546-555.

Daas PJH, Boxma B, Hopman AMCP, Voragen AGJ, and Schols HA. 2001a. Nonesterified galacturonic acid sequence homology of pectins. *Biopolymers* **58:** 1-8.

Daas PJH, Voragen AGJ, and Schols HA. 2001b. Study of the methyl ester distribution in pectin with endo-polygalacturonase and high-performance size-exclusion chromatography. *Biopolymers* **58:** 195-203.

Dahiya P, and Brewin NJ. 2000. Immunogold localization of callose and other cell wall components in pea nodule transfer cells. *Protoplasma* **214:** 210-218.

Darvill AG, McNeil M, and Albersheim P. 1978. Structure of plant cell walls VIII. A new pectic polysaccharide. *Plant Physiology* **62:** 418-422.

Davies LM, and Harris PJ. 2003. Atomic force microscopy of microfibrils in primary cell walls. *Planta* **217:** 283-289.

De Roover J, Vandenbranden K, Van Laere A, and Van Den Ende W. 2000. Drought induces fructan synthesis and 1-sst (sucrose: Sucrose fructosyltransferase) in roots and leaves of chicory seedlings (*Cichorium intybus* L.). *Planta* **210:** 808-814.

Dehority BA. 1993. Microbial ecology of cell wall fermentation. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility.* Madison, Wisconsin, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 425-453.

Dijkstra J, and Tamminga S. 1995. Simulation of the effects of diet on the contribution of rumen protozoa to degradation of fibre in the rumen. *British Journal of Nutrition* **74:** 617-634.

Domingue BMF, Dellow DW, and Barry TN. 1991a. The efficiency of chewing during eating and ruminating in goats and sheep. *British Journal of Nutrition* **65:** 355-363.

Domingue BMF, Dellow DW, Wilson PR, and Barry TN. 1991b. Comparative digestion in deer, goats, and sheep. *New Zealand Journal of Agricultural Research* **34:** 45-53.

Dupree P, and Sherrier DJ. 1998. The plant Golgi apparatus. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1404:** 259-270.

Ebringerova A, and Heinze T. 2000. Xylan and xylan derivatives - biopolymers with valuable properties, 1 Naturally occurring xylans structures, procedures and properties. *Macromolecular Rapid Communications* **21:** 542-556.

Engels FM. 1989. Some properties of cell wall layers determining ruminant digestion. In: Chesson A and Orskov ER, eds. *Physico-chemical characteristics of plant research for industrial and feed use*. London: Applied Science Publishers. 80-87.

Englyst HN, Quigley ME, and Hudson GJ. 1994. Determination of dietary fibre as nonstarch polysaccharides with gas-liquid-chromatographic, high-performance liquid-chromatographic or spectrophotometric measurement of constituent sugars. *Analyst* **119:** 1497-1509.

Eriksson I, Andersson R, and Aman P. 1997. Extraction of pectic substances from dehulled rapeseed. *Carbohydrate Research* **301:** 177-185.

Ermel FF, Follet-Gueye ML, Cibert C, Vian B, Morvan C, Catesson AM, and Goldberg R. 2000. Differential localization of arabinan and galactan side chains of rhamnogalacturonan I in cambial derivatives. *Planta* **210:** 732-740.

Esau K. 1977. *Anatomy of seed plants*. John Wiley & Sons, Inc., New York, USA.

Feder N, and T.P. OB. 1968. Plant microtechniques: Some principles and new methods. *American Journal of Botany* **55:** 123-142.

Femenia A, Robertson JA, Waldron KW, and Selvendran RR. 1998. Cauliflower (*Brassica oleracea* L), globe artichoke (*Cynara scolymus*) and chicory witloof (*Cichorium intybus*) processing by-products as sources of dietary fibre. *Journal of the Science of Food and Agriculture* **77:** 511-518.

Femenia A, Waldron KW, Robertson JA, and Selvendran RR. 1999. Compositional and structural modification of the cell wall of cauliflower (*Brassica oleracea* L var Botrytis) during tissue development and plant maturation. *Carbohydrate Polymers* **39:** 101-108.

Forsberg CW, and Cheng K-J. 1992. Molecular strategies to optimize forage and cereal digestion by ruminants. In: Bills DD and Kung S-D, eds. *Biotechnology and nutrition*. Stoneham, UK: Butterworth Heinmann. 107-147.

Foster JG, Fedders JM, Clapham WM, Robertson JW, Bligh DP, and Turner KE. 2002. Nutritive value and animal selection of forage chicory cultivars grown in

central Appalachia. *Agronomy Journal* **94:** 1034-1042.

Foster TJ, Ablett S, Mccann MC, and Gidley MJ. 1996. Mobility-resolved ^{13}C-NMR spectroscopy of primary plant cell walls. *Biopolymers* **39:** 51-66.

France J, and Siddons RC. 1993. Volatile fatty acid production. In: Forbes JM and France J, eds. *Quantitative aspects of ruminant digestion and metabolism in ruminants*. Willingford, Oxon, UK: CAB International. 107–122.

France J, Beever DE, and Siddons RC. 1993. Compartmental schemes for estimating methanogenesis in ruminants from isotope dilution data. *Journal of Theoretical Biology* **164:** 207-218.

Franz G, and Blaschek. W. 1990. Cellulose. In: Dey PM and Harborne JB, eds. *Methods in plant biochemistry. Vol. 2 Carbohydrates*. London, UK: Academic Press. 291-322.

Fraser TJ, and Rowarth JS. 1996. Legumes, herbs or grass for lamb performance? *Proceedings of the New Zealand Grassland Association* **58:** 49-52.

Fraser TJ, Cosgrove GP, Thomas WJ, Stevens DR, and Hickey MJ. 1988. Performance of grasslands Puna chicory. *Proceedings of the New Zealand Grassland Association* **49:** 193-196.

Fraser TJ, Moss RA, Daly MJ, and Knight TL. 1999. The effect of pasture species on lamb performance in dryland systems. *Proceedings of the New Zealand Grassland Association* **61:** 23-30.

Fry SC. 1986a. Cross-linking of matrix polymers in the growing cell-walls of angiosperms. *Annual Review of Plant Physiology and Plant Molecular Biology* **37:** 165-186.

Fry SC. 1986b. *In vivo* formation of xyloglucan nonasaccharide - A possible biologically-active cell-wall fragment. *Planta* **169:** 443-453.

Fry SC. 1988. *The growing plant cell wall: Chemical and metabolic analysis.* Longman, Harlow,UK.

Fry SC. 1989. The structure and functions of xyloglucan. *Journal of Experimental Botany* **40:** 1-11.

Fry SC. 2004. Primary cell wall metabolism: Tracking the careers of wall polymers in living plant cells. *New Phytologist* **161:** 641-675.

Fujino T, and Itoh T. 1998. Changes in pectin structure during epidermal cell elongation in pea (*Pisum sativum*) and its implications for cell wall architecture. *Plant and Cell Physiology* **39:** 1315-1323.

Gentile RM, Martino DL, and Entz MH. 2003. Root characterization of three forage species grown in southwestern Uruguay. *Canadian Journal of Plant Science* **83:** 785-788.

George RAT. 1985. *Vegetable seed production.* Longmans, London.

Geshi N, Jorgensen B, Scheller HV, and Ulvskov P. 2000. *In vitro* biosynthesis of 1,4-β-galactan attached to rhamnogalacturonan I. *Planta* **210:** 622-629.

Gibeaut DM, and Carpita NC. 1994. Biosynthesis of plant cell wall polysaccharides. *Faseb Journal* **8:** 904-915.

Goldberg R, Morvan C, Jauneau A, and Jarvis MC. 1996. Methyl-esterification, de-esterification and gelation of pectins in the primary cell wall. In: Visser J and Voragen AGJ, eds. *Pectins and pectinases.* Amsterdam, The Netherlands: Elsevier. 561-568.

Gomori G. 1955. Preparation of buffers for use in enzyme studies. In: Colowick, S.P. and Kaplan NO, eds. *Methods in Enzymology.* 138-146.

Gordon AH, Lomax JA, and Chesson A. 1983. Glycosidic linkages of legume, grass and cereal straw cell-walls before and after extensive degradation by rumen microorganisms. *Journal of the Science of Food and Agriculture* **34:** 1341-1350.

Gordon GLR, and Phillips MW. 1998. The role of anaerobic gut fungi in ruminants. *Nutrition Research Reviews* **11:** 133-168.

Gorshkova T, and Morvan C. 2006. Secondary cell-wall assembly in flax phloem

fibres: Role of galactans. *Planta* **223:** 149-158.

Gorshkova TA, Chemikosova SB, Sal'nikov VV, Pavlencheva NV, Gur'janov OP, Stolle-Smits T, and Van Dam JEG. 2004. Occurrence of cell-specific galactan is coinciding with bast fiber developmental transition in flax. *Industrial Crops and Products* **19:** 217-224.

Grabber JH, and Hatfield RD. 2005. Methyl esterification divergently affects the degradability of pectic uronosyls in nonlignified and lignified maize cell walls. *Journal of Agricultural and Food Chemistry* **53:** 1546-1549.

Grabber JH, Panciera MT, and Hatfield RD. 2002. Chemical composition and enzymatic degradability of xylem and nonxylem walls isolated from alfalfa internodes. *Journal of Agricultural and Food Chemistry* **50:** 2595-2600.

Grabber JH. 2005. How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. *Crop Science* **45:** 820-831.

Gradel CM, and Dehority BA. 1972. Fermentation of isolated pectin and pectin from intact forages by pure cultures of rumen bacteria. *Applied Microbiology* **23:** 332-&.

Guillemin F, Guillon F, Bonnin E, Devaux MF, Chevalier T, Knox JP, Liners F, and Thibault JF. 2005. Distribution of pectic epitopes in cell walls of the sugar beet root. *Planta* **222:** 355-371.

Guillon F, Tranquet O, Quillien L, Utille JP, Ortiz JJO, and Saulnier L. 2004. Generation of polyclonal and monoclonal antibodies against arabinoxylans and their use for immunocytochemical location of arabinoxylans in cell walls of endosperm of wheat. *Journal of Cereal Science* **40:** 167-182.

Hare MD, and Rolston MP. 1987. Effect of time of closing and paclobutrazol (pp333) on seed yield of Grasslands Puna chicory (*Cichorium intybus* L). *New Zealand Journal of Experimental Agriculture* **15:** 405-410.

Harris PJ, and Hartley RD. 1976. Detection of bound ferulic acid in cell walls of Gramineae by ultraviolet fluorescence microscopy. *Nature* **259:** 508-510.

Harris PJ, and Hartley RD. 1980. Phenolic constituents of the cell walls of

monocotyledons. *Biochemical Systematics and Ecology* **8:** 153-160.

Harris PJ, Hartley RD, and Barton GE. 1982. Evaluation of stabilized diazonium salts for the detection of phenolic constituents of plant cell walls. *Journal of the Science of Food and Agriculture* **33:** 516-520.

Harris PJ, Henry RJ, Blakeney AB, and Stone BA. 1984. An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research* **127:** 59-73.

Harris PJ. 1983. Cell walls. In: Hall, JL and Moore, AJ eds. *Isolation of membranes and organelles from plant cell walls*. London: Academic Press. 25-53.

Harris PJ. 1990. Plant cell wall structure and development. In: Akin DE, Ljungdahl LG, Wilson JR and Harris PJ, eds. *Microbial and plant opportunities to improve lignocellulose utilisation by ruminants.* New York, USA: Elsevier. 114-126.

Harris PJ. 2005. Diversity in plant cell walls. In: Henry RJ, ed. *Diversity and evolution of plants: Genotypic and phenotypic variation in higher plants.* Wallingford, Oxon, UK: CAB International Publishing. 201-227.

Hartnell GF, Hatfield RD, Mertens DR, and Martin NP. 2005. Potential benefits of plant modification of alfalfa and corn silage to dairy diets. *Proceedings of Southwest Nutrition Conference***:** 156-172.

Hatfield RD, and Weimer PJ. 1995. Degradation characteristics of isolated and *in situ* cell-wall lucerne pectic polysaccharides by mixed ruminal microbes. *Journal of the Science of Food and Agriculture* **69:** 185-196.

Hatfield RD, Ralph J, and Grabber JH. 1999. Cell wall cross-linking by ferulates and diferulates in grasses. *Journal of the Science of Food and Agriculture* **79:** 403-407.

Hatfield RD. 1989. Structural polysaccharides in forages and their degradability. *Agronomy Journal* **81:** 39-46.

Hatfield RD. 1992. Carbohydrate composition of alfalfa cell-walls isolated from stem sections differing in maturity. *Journal of Agricultural and Food Chemistry* **40:** 424-430.

Hatfield RD. 1993. Cell wall polysaccharide interactions and degradability. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility.* Madison, Wisconsin, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 285-314.

Hayashi T. 1989. Xyloglucans in the primary-cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology* **40:** 139-168.

Hickman J, and Ashwell G. 1966. Isolation of a bacterial lipopolysaccharide from xanthomonas campestris containing 3-acetamido-3,6-dideoxy-D-galactose and D-rhamnose. *Journal of Biological Chemistry* **241:** 1424-1428.

Hobson PN, and Stewart CS. 1997. *The rumen microbial ecosystem.* Blackie Academic & Professional, Chapman & Hall, 2-6 Boundary Row, London SE1 8HN, UK.

Hoffman M, Jia ZH, Pena MJ, Cash M, Harper A, Blackburn AR, Darvill A, and York WS. 2005. Structural analysis of xyloglucans in the primary cell walls of plants in the subclass asteridae. *Carbohydrate Research* **340:** 1826-1840.

Hopkins DL, Holst PJ, Hall DG, and Atkinson WR. 1995. Carcass and meat quality of 2nd-cross cryptorchid lambs grazed on chicory (*Cichorium intybus*) or lucerne (*Medicago sativa*). *Australian Journal of Experimental Agriculture* **35:** 693-697.

Hornstein JS, Buxton DR, and Wedin WF. 1989. Cell-wall carbohydrates in leaves, stems, and herbage of alfalfa and red clover. *Crop Science* **29:** 1319-1324.

Hoskin SO, Barry TN, Wilson PR, Charleston WAG, and Hodgson J. 1999a. Effects of reducing anthelmintic input upon growth and faecal egg and larval counts in young farmed deer grazing chicory (*Cichorium intybus*) and perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture. *Journal of Agricultural Science* **132:** 335-345.

Hoskin SO, Barry TN, Wilson PR, Charleston WAG, and Kemp PD. 1999b. Growth and carcass production of young farmed deer grazing sulla (*Hedysarum coronarium*), chicory (*Cichorium intybus*), or perennial ryegrass (*Lolium*

perenne)/white clover (*Trifolium repens*) pasture in new zealand. *New Zealand Journal of Agricultural Research* **42:** 83-92.

Hoskin SO, Stafford KJ, and Barry TN. 1995. Digestion, rumen fermentation and chewing behavior of red deer fed fresh chicory and perennial ryegrass. *Journal of Agricultural Science* **124:** 289-295.

Hoskin SO, Wilson PR, Ondris M, and Bunod AH. 2005. Evaluation of forage herbs for deer: Feeding value and trace elements. In: Wilson PR, ed. *Proceedings of a deer course for veterinarians No. 22.* Palmerston North, New Zealand: Deer Branch NZVA. 85-88.

Hoyos ME, Stanley CW, He SY, Pike S, Pu XA, and Novacky A. 1996. The interaction of Harpin $_{Pss}$, with plant cell walls. *Molecular Plant-Microbe Interactions* **9:** 608-616.

Hughes R, and Rowland IR. 2001. Stimulation of apoptosis by two prebiotic chicory fructans in the rat colon. *Carcinogenesis* **22:** 43-47.

Hungate RE. 1966. *The rumen and its microbes.* Academic Press, New York.

Hunt WF. 1993. Maximising red deer venison production through high quality pasture. In: Baker MJ, Crush JR and Humphreys LR, eds. *Proceedings of the XVII International Grassland Congress*: Palmerston: Keeling and Mundy Ltd. 1497-1500.

Ishikawa M, Kuroyama H, Takeuchi Y, and Tsumuraya Y. 2000. Characterization of pectin methyltransferase from soybean hypocotyls. *Planta* **210:** 782-791.

Iwai H, Ishii T, and Satoh S. 2001. Absence o f arabinan in the side chains of the pectic polysaccharides strongly associated with cell walls of *Nicotiana plumbaginifolia* non-organogenic callus with loosely attached constituent cells. *Planta* **213:** 907-915.

Iwai H, Ishii T, and Satoh S. 2002. Necessity of the expression of pectin glucuronyltransferase for intercellular attachment in the meristem of higher plants. *Plant and Cell Physiology* **43:** S88.

Jackson FS, Barry TN, Lascano C, and Palmer B. 1996. The extractable and bound

condensed tannin content of leaves from tropical tree, shrub and forage legumes. *Journal of the Science of Food and Agriculture* **71:** 103-110.

Jansson P-E, Kenne L, Liedgren H, Lindberg B, and Lönngren J. 1976. A practical guide to the methylation analysis of carbohydrates. *University of Stockholm Chemical Communications* **8:** 1-74.

Jarvis MC, Briggs SPH, and Knox JP. 2003. Intercellular adhesion and cell separation in plants. *Plant Cell and Environment* **26:** 977-989.

Jarvis MC, Hall MA, Threlfall DR, and Friend J. 1981. The polysaccharide structure of potato cell-walls chemical fractionation. *Planta* **152:** 93-100.

Jarvis MC. 1988. Direct observation of the structure of mycelial cell-walls from the potato blight fungus phytophthora infestans by solid-state ^{13}C-NMR. *Letters in Applied Microbiology* **7:** 157-159.

Jarvis MC. 1992a. Control of thickness of collenchyma cell-walls by pectins. *Planta* **187:** 218-220.

Jarvis MC. 1992b. Self-assembly of plant cell walls. *Plant Cell and Environment* **15:** 1-5.

Jarvis MC. 1998. Intercellular separation forces generated by intracellular pressure. *Plant Cell and Environment* **21:** 1307-1310.

Jauneau A, Roy S, Reis D, and Vian B. 1998. Probes and microscopical methods for the localization of pectins in plant cells. *International Journal of Plant Sciences* **159:** 1-13.

Jenkins ES, Paul W, Coupe SA, Bell SJ, Davies EC, and Roberts JA. 1996. Characterization of an mrna encoding a polygalacturonase expressed during pod development in oilseed rape (*Brassica napus* L). *Journal of Experimental Botany* **47:** 111-115.

Jenkins ES, Paul W, Craze M, Whitelaw CA, Weigand A, and Roberts JA. 1999. Dehiscence-related expression of an *Arabidopsis thaliana* gene encoding a polygalacturonase in transgenic plants of *Brassica napus*. *Plant Cell and*

Environment **22:** 159-167.

Jensen WA. 1962. *Botanical histochemistry.* WH Freeman, San Francisco.

Joblin KN, Matsui H, Naylor GE, and Ushida K. 2002. Degradation of fresh ryegrass by methanogenic co-cultures of ruminal fungi grown in the presence or absence of fibrobacter succinogenes. *Current Microbiology* **45:** 46-53.

Joblin KN. 1981. Effect of urine on the elemental composition of spring regrowth herbage in a ryegrass pasture. *New Zealand Journal of Agricultural Research* **24:** 293-297.

Jones D, and Haggar RJ. 1994. Effect of grazing on the persistency of a newly sown forb-rich sward. In: Haggar RJ and Peel S, eds. *Grassland management and nature conservation: British grassland society occasional symposium No. 28*: Reading: British Grassland Society. 297-298.

Jones L, Milne JL, Ashford D, and Mcqueen-Mason SJ. 2003. Cell wall arabinan is essential for guard cell function. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 11783-11788.

Jones L, Milne JL, Ashford D, Mccann MC, and Mcqueen-Mason SJ. 2005. A conserved functional role of pectic polymers in stomatal guard cells from a range of plant species. *Planta* **221:** 255-264.

Jones L, Seymour GB, and Knox JP. 1997. Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1→4)-β-D-galactan. *Plant Physiology* **113:** 1405-1412.

Jones L. 1990. Forage chicory at a site in south-east England. In: Pollott GE, ed. *Milk and meat from forage crops: British grassland society occasional symposium, no. 24*: Maidenhead: British Grassland Society. 67-69.

Jung GA, Shaffer JA, Varga GA, and Everhart JR. 1996. Performance of 'Grasslands Puna' chicory at different management levels. *Agronomy Journal* **88:** 104-111.

Jung HG, and Allen MS. 1995. Characteristics of plant-cell walls affecting intake and

digestibility of forages by ruminants. *Journal of Animal Science* **73:** 2774-2790.

Jung HG, and Engels FM. 2002. Alfalfa stem tissues: Cell wall deposition, composition, and degradability. *Crop Science* **42:** 524-534.

Jung HG, Buxton DR, Hatfield RD, and Ralph J. 1993. *Forage cell wall structure and digestibility.* American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc., Madison, Wisconsin, USA.

Kaneko S, Ishii T, and Matsunaga T. 1997. A boron-rhamnogalacturonan-II complex from bamboo shoot cell walls. *Phytochemistry* **44:** 243-248.

Kar F, and Arslan N. 1999. Characterization of orange peel pectin and effect of sugars, L-ascorbic acid, ammonium persulfate, salts on viscosity of orange peel pectin solutions. *Carbohydrate Polymers* **40:** 285-291.

Kato Y, and Watanabe T. 1993. Isolation and characterization of a xyloglucan from gobo (*Arctium lappa* L). *Bioscience Biotechnology and Biochemistry* **57:** 1591-1592.

Kikuchi A, Edashige Y, Ishii T, Fujii T, and Satoh S. 1996. Variations in the structure of neutral sugar chains in the pectic polysaccharides of morphologically different carrot calli and correlations with the size of cell clusters. *Planta* **198:** 634-639.

Kikuchi S, Ohinata A, Tsumuraya Y, Hashimoto Y, Kaneko Y, and Matsushima H. 1993. Production and characterization of antibodies to the β-(1→6)-galactotetraosyl group and their interaction with arabinogalactan-proteins. *Planta* **190:** 525-535.

Knox JP, Linstead PJ, King J, Cooper C, and Roberts K. 1990. Pectin esterification is spatially regulated both within cell-walls and between developing-tissues of root apices. *Planta* **181:** 512-521.

Knox JP. 1992. Molecular probes for the plant-cell surface. *Protoplasma* **167:** 1-9.

Knox JP. 1997. The use of antibodies to study the architecture and developmental regulation of plant cell walls. *International Review of Cytology-A Survey of Cell*

Biology **171:** 79-120.

Komolong M, Nicol AM, Poppi DP, Fraser TJ, and Kirsopp S. 1992. Nutrient supply for lamb growth from Grasslands Puna chicory (*Cichorium intybus*) and Wana cocksfoot (*Dactylis glomerata*). *Proceedings of the New Zealand Society of Animal Production* **52**.

Komolong MK. 1994. Nutrient supply for lamb growth from Puna chicory and Wana cocksfoot: M Agric Sci thesis, Lincoln University, New Zealand.

Kopecny J, Zorec M, Mrazek J, Kobayashi Y, and Marinsek-Logar R. 2003. *Butyrivibrio hungatei* sp nov and *Pseudobutyrivibrio xylanivorans* sp nov., butyrate-producing bacteria from the rumen. *International Journal of Systematic and Evolutionary Microbiology* **53:** 201-209.

Krause DO, Denman SE, Mackie RI, Morrison M, Rae AL, Attwood GT, and Mcsweeney CS. 2003. Opportunities to improve fiber degradation in the rumen: Microbiology, ecology, and genomics. *Fems Microbiology Reviews* **27:** 663-693.

Kusmartono, Barry TN, Wilson PR, Kemp PD, and Stafford KJ. 1996a. Effects of grazing chicory (*Cichorium intybus*) and perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture upon the growth and voluntary feed intake of red and hybrid deer during lactation and post-weaning growth. *Journal of Agricultural Science* **127:** 387-401.

Kusmartono, Shimada A, and Barry TN. 1997. Rumen digestion and rumen outflow rate in deer fed fresh chicory (*Cichorium intybus*) or perennial ryegrass (*Lolium perenne*). *Journal of Agricultural Science* **128:** 87-94.

Kusmartono, Shimada A, Stafford KJ, and Barry TN. 1996b. Intra-ruminal particle size reduction in deer fed fresh perennial ryegrass (*Lolium perenne*) or chicory (*Cichorium intybus*). *Journal of Agricultural Science* **127:** 525-531.

Kusmotarno. 1996. Nutritive value of chicory (*Cichorium intybus*) as a special purpose forage for deer production. Palmerston North, New Zealand: PhD thesis, Massey University, New Zealand.

Labreveux M, Hall MH, and Sanderson MA. 2004. Productivity of chicory and plantain cultivars under grazing. *Agronomy Journal* **96:** 710-716.

Lam TB-T, Iiysm K, and Stone BA. 1990. Primary and secondary walls of grasses and other forage plants: Taxonomic and structural considerations. In: Akin DE, Ljungdahl LG, Wilson JR and Harris PJ, eds. *Microbial and plant opportunities to improve lignocellulose utilisation by ruminants.* New York: Elsevier Science Publishing Co., Inc. 43-69.

Lancashire JA. 1978. Improved species and seasonal pasture production. *Proceedings of the Agronomy Society of New Zealand* **8:** 123-127.

Lau E, and Bacic A. 1993. Capillary gas-chromatography of partially methylated alditol acetates on a high-polarity, cross-linked, fused-silica BPX70 column. *Journal of Chromatography* **637:** 100-103.

Lau JM, McNeil M, Darvill AG, and Albersheim P. 1985. Structure of the backbone of rhamnogalacturonan-I, a pectic polysaccharide in the primary-cell walls of plants. *Carbohydrate Research* **137:** 111-125.

Laurenzi M, Tipping AJ, Marcus SE, Knox JP, Federico R, Angelini R, and Mcpherson MJ. 2001. Analysis of the distribution of copper amine oxidase in cell walls of legume seedlings. *Planta* **214:** 37-45.

Leboeuf E, Guillon F, Thoiron S, and Lahaye M. 2005. Biochemical and immunohistochemical analysis of pectic polysaccharides in the cell walls of *Arabidopsis* mutant quasimodo 1 suspension-cultured cells: Implications for cell adhesion. *Journal of Experimental Botany* **56:** 3171-3182.

Lee KJD, Sakata Y, Mau SL, Pettolino F, Bacic A, Quatrano RS, Knight CD and Knox JP. 2005. Arabinogalactan proteins are required for apical cell extension in the moss *Physcomitrella patens*. *Plant Cell* **17:** 3051-3065.

Li GD, and Kemp PD. 2005. Forage chicory (*Cichorium intybus* L.): A review of its agronomy and animal production. In: Sparks DL, ed. *Advances in agronomy*. San Diego, California, USA: Elsevier Academic Press. 187-222.

Li GD, Kemp PD, and Hodgson J. 1997. Herbage production and persistence of Puna chicory (*Cichorium intybus* L) under grazing management over 4 years. *New Zealand Journal of Agricultural Research* **40:** 51-56.

Li XB, and Wu Q. 1993. *Plant cell walls.* Beijing University Press, Beijing, China.

Liners F, and Van Cutsem P. 1992. Distribution of pectic polysaccharides throughout walls of suspension-cultured carrot cells - an immunocytochemical study. *Protoplasma* **170:** 10-21.

Liners F, Letesson JJ, Didembourg C, and Van Cutsem P. 1989. Monoclonal antibodies against pectin - recognition of a conformation induced by calcium. *Plant Physiology* **91:** 1419-1424.

Liu DL, and Zhang WX. 1998. Studies on the seed production of chicory introduced from Europe. *Journal of grasses and animal husbandry*: 38-42.

Lord EM, and Mollet JC. 2002. Plant cell adhesion: A bioassay facilitates discovery of the first pectin biosynthetic gene. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 15843-15845.

Lynd LR, Weimer PJ, Van Zyl WH, and Pretorius IS. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* **66:** 506-577.

MacRae JC, and Lobley GE. 1986. Interactions between energy and protein. In: Milligan LP, Grovum WL and Dobson A, eds. *Control of digestion and metabolism in ruminants.* Englewood cliffs, New Jersey: Prentice-Hall. 367-385.

Maddaloni J, Bertin OD, and Josifovich J. 1985. *Cichorium intybus* and meat production. *Informe tecnico, estacion experimental regional agropecuaria pergamino no. 196.*

Majewska-Sawka A, and Munster A. 2003. Cell-wall antigens in mesophyll cells and mesophyll-derived protoplasts of sugar beet: Possible implication in protoplast recalcitrance? *Plant Cell Reports* **21:** 946-954.

Majewska-Sawka A, Munster A, and Rodriguez-Garcia MI. 2002. Guard cell wall:

Immunocytochemical detection of polysaccharide components. *Journal of Experimental Botany* **53:** 1067-1079.

Majewska-Sawka A, Munster A, and Wisniewska E. 2004. Temporal and spatial distribution of pectin epitopes in differentiating anthers and microspores of fertile and sterile sugar beet. *Plant and Cell Physiology* **45:** 560-572.

Mares D, Romagnoli C, Tosi B, Andreotti E, Chillemi G, and Poli F. 2005. Chicory extracts from *Cichorium intybus* L. as potential antifungals. *Mycopathologia* **160:** 85-92.

Marounek M, and Duskova D. 1999. Metabolism of pectin in rumen bacteria *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*. *Letters in Applied Microbiology* **29:** 429-433.

Marten GC, and Barnes RF. 1980. Prediction of energy digestibility of forages with in vitro rumen fermentation and fungal enzyme systems. In: Pidgen WJ, Balch CC, and Graham M. eds. *Proceedings of international workshop on the standardization of analytical methodology for feeds*. Ottawa, Canada: International Development Research Centre. 61-71.

Mathew S, and Abraham TE. 2004. Ferulic acid: An antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. *Critical Reviews in Biotechnology* **24:** 59-83.

Matoh T, Takasaki M, Takabe K, and Kobayashi M. 1998. Immunocytochemistry of rhamnogalacturonan II in cell walls of higher plants. *Plant and Cell Physiology* **39:** 483-491.

McCann MC, and Roberts K. 1991. Architecture of the primary cell wall. In: Lloyd CW, ed. *The cytoskeletal basis of plant growth and form*. London, UK: Academic Press. 109-129.

McCartney L, and Knox JP. 2002. Regulation of pectic polysaccharide domains in relation to cell development and cell properties in the pea testa. *Journal of Experimental Botany* **53:** 707-713.

McCartney L, Marcus SE, and Knox JP. 2005. Monoclonal antibodies to plant cell wall xylans and arabinoxylans. *Journal of Histochemistry & Cytochemistry* **53:** 543-546.

McCartney L, Ormerod AP, Gidley MJ, and Knox JP. 2000. Temporal and spatial regulation of pectic (1→4)-β-D-galactan in cell walls of developing pea cotyledons: Implications for mechanical properties. *Plant Journal* **22:** 105-113.

McCleary BV, Gibson TS, Solah V, and Mugford DC. 1994. Total starch measurement in cereal products - interlaboratory evaluation of a rapid enzymatic test procedure. *Cereal Chemistry* **71:** 501-505.

McComb EA, and McReady RM. 1952. Colourimetric determination of pectic substances. *Analytical Chemistry* **24:** 1630-1632.

McCoy JE, Collins M, and Dougherty CT. 1997. Amount and quality of chicory herbage ingested by grazing cattle. *Crop Science* **37:** 239-242.

Migne C, Grenet E, and Jamot J. 1996a. Microbial degradation of the apical internode of Co125 and W401 maize in the rumen. *Animal Feed Science and Technology* **58:** 165-185.

Migne C, Prensier G, and Grenet E. 1994. Immunogold labeling of xylans and arabinoxylans in the plant cell walls of maize stem. *Biology of the Cell* **81:** 267-276.

Migne C, Prensier G, Cornu A, and Grenet E. 1996b. Immunocytochemical localization of arabinoxylans in the cell wall of maize apical internode after microbial degradation in the rumen. *Biology of the Cell* **88:** 137-144.

Migne C, Prensier G, Cornu A, and Grenet E. 1998. Immunocytochemical localisation of para-coumaric acid in the cell walls of maize stem after rumen microbial degradation. *Journal of the Science of Food and Agriculture* **78:** 503-510.

Migne C, Prensier G, Cornu A, Utille JP, Angibeaud P, and Grenet E. 1999. Immunogold labelling of feruloyl-arabinose of maize stem after rumen microbial degradation. *Journal of the Science of Food and Agriculture* **79:** 1163-1170.

Migne C, Prensier G, Grenet E, and Cornu A. 1996c. Immunogold labelling of

feruloyl-arabinose on maize stems before and after rumen microbial degradation. *Abstracts of Papers of the American Chemical Society* **211:** 176-CELL.

Min BR, Barry TN, Wilson PR, and Kemp PD. 1997. The effects of grazing chicory (*Cichorium intybus*) and birdsfoot trefoil (*Lotus corniculatus*) on venison and velvet production by young red and hybrid deer. *New Zealand Journal of Agricultural Research* **40:** 335-347.

Miron J, and Ben-Ghedalia D. 1993. Digestion of cell-wall monosaccharides of ryegrass and alfalfa hays by the ruminal bacteria *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens*. *Canadian Journal of Microbiology* **39:** 780-786.

Miron J, Ben-Ghedalia D and Morrison M. 2001. Invited review: adhesion mechanisms of rumen cellulolytic bacteria. *Journal of Dairy Science* **84**: 1294-1309

Monson WG, Lowrey RS, and Forbes I. 1969. *In vivo* nylon bag vs two-stage *in vitro* digestion - comparison of two techniques for estimating dry-matter digestibility of forages. *Agronomy Journal* **61:** 587-589.

Moore JF. 1970. Procedures for the two-stage *in vitro* digestion of forages. In: Harris LE, ed. *Nutrition research techniques for domestic and wild animals*. Logan: Utah State University. 5001-5003.

Moore KJ, and Hatfield RD. 1994. Carbohydrates and forage quality. In: Fahey GC, Collins M, Mertens DR and Moser LE, eds. *Forage quality, evaluation, and utilization*. Madison, Wisconsin, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc. 229-280.

Moore PJ, and Staehelin LA. 1988. Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.: Implication for secretory pathways. *Planta* **174:** 433-445.

Mort AJ, Moerschbacher BM, Pierce ML, and Maness NO. 1991. Problems encountered during the extraction, purification and chromatography of pectic fragments and some solutions to them. *Carbohydrate Research* **215:** 219-227.

Morvan C, Andeme-Onzighi C, Girault R, Himmelsbach DS, Driouich A, and Akin DE. 2003. Building flax fibres: More than one brick in the walls. *Plant Physiology and Biochemistry* **41:** 935-944.

Murdoch L, Corbel JC, Reis D, Bertheau Y, and Vian B. 1999. Differential cell wall degradation by *Erwinia chrysanthemi* in petiole of *Saintpaulia ionantha*. *Protoplasma* **210:** 59-74.

Needs PW, and Selvendran RR. 1993. Avoiding oxidative degradation during sodium hydroxide methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide. *Carbohydrate Research* **245:** 1-10.

Neel JPS, Alloush GA, Belesky DP, and Clapham WM. 2002. Influence of rhizosphere ionic strength on mineral composition, dry matter yield and nutritive value of forage chicory. *Journal of Agronomy and Crop Science* **188:** 398-407.

Newman RH, Davies LM, and Harris PJ. 1996. Solid-state ^{13}C nuclear magnetic resonance characterization of cellulose in the cell walls of *Arabidopsis thaliana* leaves. *Plant Physiology* **111:** 475-485.

Niezen JH, Barry TN, Hodgson J, Wilson PR, Ataja AM, Parker WJ, and Holmes CW. 1993. Growth responses in red deer calves and hinds grazing red clover, chicory or perennial ryegrass white clover swards during lactation. *Journal of Agricultural Science* **121:** 255-263.

O'Brien AB. 1955. Chicory. *New Zealand Journal of Agriculture* **91:** 494.

O'Brien TP, and McCully ME. 1981. *The study of plant structure: Principles and methods.* Termarcarphi, Melbourne, Australia.

Obro J, Harholt J, Scheller HV, and Orfila C. 2004. Rhamnogalacturonan I in *Solanum tuberosum* tubers contains complex arabinogalactan structures. *Phytochemistry* **65:** 1429-1438.

Oechslin R, Lutz MV, and Amado R. 2003. Pectic substances isolated from apple cellulosic residue: Structural characterisation of a new type of rhamnogalacturonan I. *Carbohydrate Polymers* **51:** 301-310.

Offler CE, McCurdy DW, Patrick JW, and Talbot MJ. 2003. Transfer cells: Cells specialized for a special purpose. *Annual Review of Plant Biology* **54:** 431-454.

O'Neil MA, and York WS. 2003. The composition and structure of plant primary walls. In: Rose JKC, ed. *The plant cell wall*: Blackwell. 1-54.

O'Neill M, Alersheim P, and Darvill A. 1990. The pectic polysaccharides of primary cell walls. *Methods of Plant Biochemistry* **2:** 415-441.

O'Neill MA, and Selvendran RR. 1983. Isolation and partial characterization of a xyloglucan from the cell-walls of *Phaseolus coccineus*. *Carbohydrate Research* **111:** 239-255.

O'Neill MA, Ishii T, Albersheim P, and Darvill AG. 2004. Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology* **55:** 109-139.

O'Neill MA, Warrenfeltz D, Kates K, Pellerin P, Doco T, Darvill AG, and Albersheim P. 1996. Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester - *in vitro* conditions for the formation and hydrolysis of the dimer. *Journal of Biological Chemistry* **271:** 22923-22930.

Oomen RJFJ. 2003. *In planta* modification of the potato tuber cell wall: Wageningen University.

Orfila C, and Knox JP. 2000. Spatial regulation of pectic polysaccharides in relation to pit fields in cell walls of tomato fruit pericarp. *Plant Physiology* **122:** 775-781.

Orfila C, Seymour GB, Willats WGT, Huxham IM, Jarvis MC, Dover CJ, Thompson AJ, and Knox JP. 2001. Altered middle lamella homogalacturonan and disrupted deposition of (1→5)-α-L-arabinan in the pericarp of cnr, a ripening mutant of tomato. *Plant Physiology* **126:** 210-221.

Orfila C, Sorensen SO, Harholt J, Geshi N, Crombie H, Truong HN, Reid JSG, Knox JP, and Scheller HV. 2005. Quasimodo1 is expressed in vascular tissue of *Arabidopsis thaliana* inflorescence stems, and affects homogalacturonan and xylan

biosynthesis. *Planta* **222:** 613-622.

Osborne JM, and Dehority BA. 1989. Synergism in degradation and utilization of intact forage cellulose, hemicellulose, and pectin by 3 pure cultures of ruminal bacteria. *Applied and Environmental Microbiology* **55:** 2247-2250.

Parker CC, Parker ML, Smith AC, and Waldron KW. 2001. Pectin distribution at the surface of potato parenchyma cells in relation to cell-cell adhesion. *Journal of Agricultural and Food Chemistry* **49:** 4364-4371.

Parker ML, and Waldron KW. 1995. Texture of Chinese water chestnut - involvement of cell-wall phenolics. *Journal of the Science of Food and Agriculture* **68:** 337-346.

Pena MJ, and Carpita NC. 2004. Loss of highly branched arabinans and debranching of rhamnogalacturonan I accompany loss of firm texture and cell separation during prolonged storage of apple. *Plant Physiology* **135:** 1305-1313.

Perez S, Rodriguez-Carvajal MA, and Doco T. 2003. A complex plant cell wall polysaccharide: Rhamnogalacturonan II. A structure in quest of a function. *Biochimie* **85:** 109-121.

Pilnik W, and Voragen AGJ. 1993. Pectic enzymes in fruit and vegetable juice manufacture. In: Nagodawithana T and Reed G, eds. *Enzymes in food processing.* London: Academic Press. 363–399.

Pomar F, Merino F, and Barcelo AR. 2002. O-4-linked coniferyl and sinapyl aldehydes in lignifying cell walls are the main targets of the Wiesner (phloroglucinol-HCl) reaction. *Protoplasma* **220:** 17-28.

Preston JF, Rice JD, Chow MC, and Brown BJ. 1991. Kinetic comparisons of trimer-generating pectate and alginate lyases by reversed-phase ion-pair liquid-chromatography. *Carbohydrate Research* **215:** 147-157.

Puhlmann J, Bucheli E, Swain MJ, Dunning N, Albersheim P, Darvill AG, and Hahn MG. 1994. Generation of monoclonal-antibodies against plant cell-wall polysaccharides. 1. Characterization of a monoclonal-antibody to a terminal α-

(1→2)-linked fucosyl-containing epitope. *Plant Physiology* **104:** 699-710.

Quach M-L, Melton LD, Harris PJ, Burdon JN, and Smith BG. 2001. Cell wall compositions of raw and cooked corms of taro (*Colocasia esculenta*). *Journal of the Science of Food and Agriculture* **81:** 311-318.

Quentin M, Jauneau A, Morvan O, Mareck A, Gaffe J, and Morvan C. 1997. Immunolocalization of pectin methylesterases in the hypocotyl tissues of flax. *Plant Physiology and Biochemistry* **35:** 475-482.

Ralet MC, Andre-Leroux G, Quemener B, and Thibault JF. 2005. Sugar beet (*Beta vulgaris*) pectins are covalently cross-linked through diferulic bridges in the cell wall. *Phytochemistry* **66:** 2800-2814.

Ramirez-Restrepo CA, and Barry TN. 2005. Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. *Animal Feed Science and Technology* **120:** 179-201.

Ratnayake RMS, Hurst PL, and Melton LD. 1999. Texture and the cell wall polysaccharides of buttercup squash 'Delica' (*Cucurbita maxima*). *New Zealand Journal of Crop and Horticultural Science* **27:** 133-143.

Recio E, Encina A, Alvarez JM, and Acebes JL. 2003. Autolysis-like release of homogalacturonan from bean (*Phaseolus vulgaris* L.) callus cell walls. *Plant Science* **164:** 579-588.

Redgwell RJ, Fischer M, Kendal E, and Macrae EA. 1997. Galactose loss and fruit ripening: High-molecular-weight arabinogalactans in the pectic polysaccharides of fruit cell walls. *Planta* **203:** 174-181.

Rees SB, and Harborne JB. 1985. The role of sesquiterpene lactones and phenolics in the chemical defense of the chicory plant. *Phytochemistry* **24:** 2225-2231.

Reuhs BL, Glenn J, Stephens SB, Kim JS, Christie DB, Glushka JG, Zablackis E, Albersheim P, Darvill AG, and O'Neill MA. 2004. L-galactose replaces L-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the L-fucose-deficient mur1 *Arabidopsis* mutant. *Planta* **219:** 147-157.

Ridley BL, O'Neill MA, and Mohnen DA. 2001. Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **57:** 929-967.

Robertson JB, and Van Soest PJ. 1981. The detergent system of analysis and its application to human foods. In: James TWPT and Theander O, eds. *The analysis of dietary fiber in food*. New York: Marcel Dekker, Inc. 123-158.

Roughan PG, and Holland R. 1977. Predicting *in vivo* digestibilities of herbages by exhaustive enzymic hydrolysis of cell-walls. *Journal of the Science of Food and Agriculture* **28:** 1057-1064.

Roy B. 2004. *An illustrated guide to common weeds of New Zealand*. New Zealand Plant Protection Society (Inc), Lincoln, New Zealand.

Roy S, Conway WS, Watada AE, Sams CE, Pooley CD, and Wergin WP. 1994. Distribution of the anionic sites in the cell-wall of apple fruit after calcium treatment - quantitation and visualization by a cationic colloidal gold probe. *Protoplasma* **178:** 156-167.

Roy S, Jauneau A, and Vian B. 1994. Analytical detection of calcium-ions and immunocytochemical visualization of homogalacturonic sequences in the ripe cherry tomato. *Plant Physiology and Biochemistry* **32:** 633-640.

Roy S, Watada AE, Conway WS, and Wergin WP. 1995. Cell-wall autolysis during fruit ripening - visualization of calcium-ions and pectin sequences. *Journal of Cellular Biochemistry*: 460.

Rumball W, Keogh RG, Miller JE, and Claydon RB. 2003a. 'Choice' forage chicory (*Cichorium intybus* L.). *New Zealand Journal of Agricultural Research* **46:** 49-51.

Rumball W, Skipp RA, Keogh RG, and Claydon RB. 2003b. 'Puna II' forage chicory (*Cichorium intybus* L.). *New Zealand Journal of Agricultural Research* **46:** 53-55.

Rumball W. 1986. Grasslands Puna chicory (*Cichorium intybus* L.). *New Zealand Journal of Experimental Agriculture* **14:** 105-107.

Ryden P, and Selvendran RR. 1990. Cell-wall polysaccharides and glycoproteins of parenchymatous tissues of runner bean (*Phaseolus coccineus*). *Biochemical Journal*

269: 393-402.

Ryder EJ. 1999. *Lettuce, endive, and chicory*. CAB International Publisher, New York.

Saher S, Piqueras A, Hellin E, and Olmos E. 2005. Pectin methyl esterases and pectins in normal and hyperhydric shoots of carnation cultured in vitro. *Plant Physiology and Biochemistry* **43:** 155-159.

Sanderson MA, Labreveux M, Hall MH, and Elwinger GF. 2003. Forage yield and persistence of chicory and English plantain. *Crop Science* **43:** 995-1000.

Sanderson MA, Soder KJ, Muller LD, Klement KD, Skinner RH, and Goslee SC. 2005. Forage mixture productivity and botanical composition in pastures grazed by dairy cattle. *Agronomy Journal* **97:** 1465-1471.

SAS. 2003. *Statistical Analysis System*, version 9.1. SAS Institute Inc., Cary, NC, USA.

Scales GH, Knight TL, and Saville DJ. 1995. Effect of herbage species and feeding level on internal parasites and production performance of grazing lambs. *New Zealand Journal of Agricultural Research* **38:** 237-247.

Schols HA, Bakx EJ, Schipper D, and Voragen AGJ. 1995. A xylogalacturonan subunit present in the modified hairy regions of apple pectin. *Carbohydrate Research* **279:** 265-279.

Schreurs NM, Molan AL, Lopez-Villalobos N, Barry TN, and McNabb WC. 2002. Effects of grazing undrenched weaner deer on chicory or perennial ryegrass/white clover pasture on the viability of gastrointestinal nematodes and lungworms. *The Veterinary Record* **151:** 348-353, 356.

Scott JE, Quintarelli G, and Dellovo MC. 1964. The chemical and histochemical properties of alcian blue. I. The mechanism of alcian blue staining. *Histochemie* **4:** 73-85.

Scott RW. 1979. Colorimetric determination of hexuronic acids in plant materials. *Analytical Chemistry* **51:** 936-941.

Selvendran RR, and O'Neill MA. 1987. Isolation and analysis of cell walls from plant

material. *Methods of Biochemical Analysis* **32:** 25-153.

Selvendran RR, and Ryden P. 1990. Isolation and analysis of plant cell walls. In: Dey PM, ed. *Methods in plant biochemistry.* London: Academic Press. 549-578.

Selvendran RR. 1985. Developments in the chemistry and biochemistry of pectic and hemicellulosic polymers. *Journal of Cell Science***:** 51-88.

Serpe MD, Muir AJ, and Keidel AM. 2001. Localization of cell wall polysaccharides in non-articulated laticifers of *Asclepias speciosa* torr. *Protoplasma* **216:** 215-226.

Serpe MD, Muir AJ, Andeme-Onzighi C, and Driouich A. 2004. Differential distribution of callose and a (1→4)-β-D-galactan epitope in the laticiferous plant *Euphorbia heterophylla* L. *International Journal of Plant Sciences* **165:** 571-585.

Seymour GB, Colquhoun IJ, Dupont MS, Parsley KR, and Selvendran RR. 1990. Composition and structural features of cell-wall polysaccharides from tomato fruits. *Phytochemistry* **29:** 725-731.

Shukla PC, and Talpada PM. 1977. Nutritive value of chicory leaves (*Cichorium intybus* L.). *Gujarat Agricultural University Research Journal* **3:** 15-17.

Silley P. 1985. A note on the pectinolytic enzymes of *Lachnospira multiparus. Journal of Applied Bacteriology* **58:** 145-149.

Singh SA, and Rao AGA. 2002. A simple fractionation protocol for, and a comprehensive study of the molecular properties of, two major endopolygalacturonases from *Aspergillus niger. Biotechnology and Applied Biochemistry* **35:** 115-123.

Smallwood M, Beven A, Donovan N, Neill SJ, Peart J, Roberts K, and Knox JP. 1994. Localization of cell-wall proteins in relation to the developmental anatomy of the carrot root apex. *Plant Journal* **5:** 237-246.

Smallwood M, Martin H, and Knox JP. 1995. An epitope of rice threonine-rich and hydroxyproline-rich glycoprotein is common to cell-wall and hydrophobic plasma-membrane glycoproteins. *Planta* **196:** 510-522.

Smetham ML. 1994. Pasture management. In: Langer RHM, ed. *Pastures---their ecology and management*. Auckland, New Zealand: Oxford University. 197-240.

Sobry S, Havelange A, and Van Cutsem P. 2005. Immunocytochemistry of pectins in shoot apical meristems: Consequences for intercellular adhesion. *Protoplasma* **225:** 15-22.

Sobry S, Havelange A, Liners F, and Van Cutsem P. 2005. Immunolocalization of homogalacturonans in the apex of the long-day plant *Sinapis alba* at floral transition. The pectin content drops dramatically in the first hours of this transition. *Physiologia Plantarum* **123:** 339-347.

Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, Vorwerk S, and Youngs H. 2004. Toward a systems approach to understanding plant cell walls. *Science* **306:** 2206-2211.

Spagnuolo M, Crecchio C, Pizzigallo MDR, and Ruggiero P. 1997. Synergistic effects of cellulolytic and pectinolytic enzymes in degrading sugar beet pulp. *Bioresource Technology* **60:** 215-222.

Stacey NJ, Roberts K, Carpita NC, Wells B, and McCann MC. 1995. Dynamic changes in cell surface molecules are very early events in the differentiation of mesophyll cells from *Zinnia elegans* into tracheary elements. *Plant Journal* **8:** 891-906.

Steele NM, Mccann MC, and Roberts K. 1997. Pectin modification in cell walls of ripening tomatoes occurs in distinct domains. *Plant Physiology* **114:** 373-381.

Steffan W, Kovac P, Albersheim P, Darvill AG, and Hahn MG. 1995. Characterization of a monoclonal antibody that recognizes an arabinosylated (1→6)-β-D-galactan epitope in plant complex carbohydrates. *Carbohydrate Research* **275:** 295-307.

Stephen AM. 1980. Plant carbohydrates. In: Bell EA and Charlwood BV, eds. *Secondary plant products, encyclopaedia of plant physiology, new series*. Berlin: Springer-Verlag. 554-584.

Stevens BJH, and Selvendran RR. 1980. The isolation and analysis of cell-wall material from the alcohol-insoluble residue of cabbage (*Brassica oleracea* var capitata). *Journal of the Science of Food and Agriculture* **31:** 1257-1267.

Stevens BJH, and Selvendran RR. 1984. Structural features of cell-wall polysaccharides of the carrot *Daucus carota*. *Carbohydrate Research* **128:** 321-333.

Stewart AV. 1996. Plantain (*Plantago lanceolata* L.): A potential animal pasture species. *Proceedings of New Zealand Grassland Association* **58:** 77-86.

Stewart CS, Duncan SH, Mcpherson CA, Richardson AJ, and Flint HJ. 1990. The implications of the loss and regain of cotton-degrading activity for the degradation of straw by *Ruminococcus flavefaciens* strain-007. *Journal of Applied Bacteriology* **68:** 349-356.

Sutherland P, Hallett L, Redgwell R, Benhamou N, and Macrae E. 1999. Localization of cell wall polysaccharides during kiwifruit (*Actinidia deliciosa*) ripening. *International Journal of Plant Sciences* **160:** 1099-1109.

Sweet DP, Shapiro RH, and Albersheim P. 1975. Quantitative analysis by various G.L.C. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research* **40:** 217-225.

Talmadge KW, Keegstra K, Bauer WD, and Albersheim P. 1973. The structure of plant cell walls. I. The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of the pectic polysaccharides. *Plant Physiology* **51:** 158-173.

Tamminga S. 1993. Influence of feeding management on ruminant fiber digestibility. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility*. Madison, Wisconsin, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 571-602.

Terashima N, Fukushia K, He L-F, and Takabe K. 1993. Comprehensive model of the lignified plant cell wall. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility*. Madison, Wisconsin, USA: American

Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 247-270.

Thimm JC, Burritt DJ, Sims IM, Newman RH, Ducker WA, and Melton LD. 2002. Celery (*Apium graveolens*) parenchyma cell walls: Cell walls with minimal xyloglucan. *Physiologia Plantarum* **116:** 164-171.

Thompson JE, and Fry SC. 2000. Evidence for covalent linkage between xyloglucan and acidic pectins in suspension-cultured rose cells. *Planta* **211:** 275-286.

Tilley JMA, and Terry RA. 1963. A two-stage technique for the *in vitro* digestion of forage crops. *Journal of British Grassland Society* **18:** 104-111.

Tinworth TRH, Barry TN, and Wilson PR. 1999. The effect of wilting chicory on its voluntary feed intake and digestion by red deer. *Journal of Agricultural Science* **133:** 217-221.

Titgemeyer EC, Bourquin LD, and Fahey GC. 1992. Disappearance of cell-wall monomeric components from fractions chemically isolated from alfalfa leaves and stems following *in situ* ruminal digestion. *Journal of the Science of Food and Agriculture* **58:** 451-463.

Trethewey JAK, and Harris PJ. 2002. Location of (1→3)- and (1→3), (1→4)-β-D-glucans in vegetative cell walls of barley (*Hordeum vulgare*) using immunogold labelling. *New Phytologist* **154:** 347-358.

Trinci APJ, Davies DR, Gull K, Lawrence MI, Nielsen BB, Rickers A, and Theodorou MK. 1994. Anaerobic fungi in herbivorous animals. *Mycological Research* **98:** 129-152.

Turner KE, Belesky DP, and Fedders JM. 1999. Chicory effects on lamb weight gain and rate of *in vitro* organic matter and fiber disappearance. *Agronomy Journal* **91:** 445-450.

Ulyatt MJ, Dellow DW, John A, Reid CSW, and Waghorn GC. 1986. Contribution of chewing during eating and rumination to the clearance of digesta from the reticulo-rumen. In: Milligan LP, Grovum WL and Dobson A, eds. *Control of*

digestion and metabolism in ruminants. Prentice Hall, Englewood Cliffs, New Jersey: Reston Books. 488-517.

Ulyatt MJ. 1973. The feeding value of herbage. In: New Zealand Dairy Research Institute, ed. *Chemistry and biochemistry of herbage*. Palmerston North, New Zealand: New Zealand Dairy Research Institute. 36-40.

Van Cutsem P, and Liners F. 1993. The intercellular-adhesion in plants is not mediated by "egg boxes". *Journal of Cellular Biochemistry* Suppl. 17A**:** 23.

Van Den Ende W, Michiels A, Le Roy K, and Van Laere A. 2002. Cloning of a vacuolar invertase from *Belgian endive* leaves (*Cichorium intybus*). *Physiologia Plantarum* **115:** 504-512.

Van Soest PJ. 1993. Cell wall matrix interactions and degradation-session synopsis. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility*. Madison, Wisconsin, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 377-396.

Van Soest PJ. 1994. *Nutritional ecology of the ruminant*. Cornell University Press, New York, USA.

Varga G.A., and Kolver ES. 1997. Microbial and animal limitations to fiber digestion and utilization. *Journal of Nutrition* **127:** S819-S823.

Vian B, and Roland JC. 1991. Affinodetection of the sites of formation and of the further distribution of polygalacturonans and native cellulose in growing plant-cells. *Biology of the Cell* **71:** 43-55.

Vicré M, Jauneau A, Knox JP, and Driouich A. 1998. Immunolocalization of β-(1→4) and β-(1→6)-D-galactan epitopes in the cell wall and Golgi stacks of developing flax root tissues. *Protoplasma* **203:** 26-34.

Vidal S, Doco T, Williams P, Pellerin P, York WS, O'Neill MA, Glushka J, Darvill AG, and Albersheim P. 2000. Structural characterization of the pectic polysaccharide rhamnogalacturonan II: Evidence for the backbone location of the

aceric acid-containing oligoglycosyl side chain. *Carbohydrate Research* **326:** 277-294.

Vincken JP, Schols HA, Oomen RJFJ, McCann MC, Ulvskov P, Voragen AGJ, and Visser RGF. 2003. If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology* **132:** 1781-1789.

Vincken JP, York WS, Beldman G, and Voragen AGJ. 1997. Two general branching patterns of xyloglucan, XXXG and XXGG. *Plant Physiology* **114:** 9-13.

Visser J, and Voragen AGJ. 1996. *Progress in biotechnology 14: Pectins and pectinases*. Elsevier, Amsterdam.

Volesky JD. 1996. Forage production and grazing management of chicory. *Journal of Production Agriculture* **9:** 403-406.

Waghorn GS, and Barry TN. 1987. Pasture as a nutrient source. In: Nicol AM, ed. *Livestock feeding on pasture. New Zealand society of animal production occasional publication*. Hamilton, New Zealand: Ruakura Agriculture Centre. 21-38.

Wang Y, and McAllister TA. 2002. Rumen microbes, enzymes and feed digestion - a review. *Asian-Australasian Journal of Animal Sciences* **15:** 1659-1676.

Wardrop AB. 1971. Occurrence and formation in plants. In: Sarkanen KV and Ludwig CH, eds. *Lignins: Occurrence, formation, structure and reaction*. New York, USA: Wiley-Interscience. 19-42.

Warren RAJ. 1996. Microbial hydrolysis of polysaccharides. *Annual Review of Microbiology* **50:** 183-212.

Waugh CD, Clark DA, Harris SL, Thom ER, Copeman PJA, and Napper AR. 1998. Chicory for milk production. *Proceedings of the New Zealand Grassland Association* **60:** 33-38.

White BA, Mackie RI, and Doerner KC. 1993. Enzymatic hydrolysis of forage cell walls. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility*. Madison, Wisconsin, USA: American Society of

Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 455-484.

Willats WGT, Gilmartin PM, Mikkelsen JD, and Knox JP. 1999a. Cell wall antibodies without immunization: Generation and use of de-esterified homogalacturonan block-specific antibodies from a naive phage display library. *Plant Journal* **18:** 57-65.

Willats WGT, Limberg G, Buchholt HC, Van Alebeek GJ, Benen J, Christensen T, Visser J, Voragen A, Mikkelsen JD, and Knox JP. 2000a. Analysis of pectin structure part 2 - analysis of pectic epitopes recognised by hybridoma and phage display monoclonal antibodies using defined oligosaccharides, polysaccharides, and enzymatic degradation. *Carbohydrate Research* **327:** 309-320.

Willats WGT, Marcus SE, and Knox JP. 1998. Generation of a monoclonal antibody specific to (1 →5)-α-L-arabinan. *Carbohydrate Research* **308:** 149-152.

Willats WGT, McCartney L, Mackie W, and Knox JP. 2001a. Pectin: Cell biology and prospects for functional analysis. *Plant Molecular Biology* **47:** 9-27.

Willats WGT, Orfila C, Limberg G, Buchholt HC, Van Alebeek G, Voragen AGJ, Marcus SE, Christensen T, Mikkelsen JD, Murray BS, and Knox JP. 2001b. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls - implications for pectin methyl esterase action, matrix properties, and cell adhesion. *Journal of Biological Chemistry* **276:** 19404-19413.

Willats WGT, Steele-King CG, Marcus SE, and Knox JP. 1999b. Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. *Plant Journal* **20:** 619-628.

Willats WGT, Steele-King CG, McCartney L, Orfila C, Marcus SE, and Knox JP. 2000b. Making and using antibody probes to study plant cell walls. *Plant Physiology and Biochemistry* **38:** 27-36.

Williams AG, and Coleman GS. 1991. *The rumen protozoa.* Springer-Verlag, New York.

Williams MNV, Freshour G, Darvill AG, Albersheim P, and Hahn MG. 1996. An antibody FAB selected from a recombinant phage display library detects deesterified pectic polysaccharide rhamnogalacturonan II in plant cells. *Plant Cell* **8:** 673-685.

Wilson JR, and Hatfield RD. 1997. Structural and chemical changes of cell wall types during stem development: Consequences for fibre degradation by rumen microflora. *Australian Journal of Agricultural Research* **48:** 165-180.

Wilson JR. 1993. Organization of forage plant tissues. In: Jung HG, Buxton DR, Hatfield RD and Ralph J, eds. *Forage cell wall structure and digestibility*. Madison, WisconsinI, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc. and Soil Science Society of America, Inc. 1-30.

Wilson WD, Barwick JM, Lomax JA, Jarvis MC, and Duncan HJ. 1988. Lignified and non-lignified cell-walls from kale. *Plant Science* **57:** 83-90.

Wisniewski JP, Rathbun EA, Knox JP, and Brewin NJ. 2000. Involvement of diamine oxidase and peroxidase in insolubilization of the extracellular matrix: Implications for pea nodule initiation by *Rhizobium leguminosarum*. *Molecular Plant-Microbe Interactions* **13:** 413-420.

Wubah DA, Akin DE, and Borneman WS. 1993. Biology, fiber degradation, and Enzymology of anaerobic zoosporic fungi. *Critical Reviews in Microbiology* **19:** 99-115.

Xu QF, and Wang ZW. 1999. Experiments of the introduction of forage chicory. *Journal of Shanxi Agricultural University* **19:** 22-28.

Young OA, Cruickshank GJ, Maclean KS, and Muir PD. 1994. Quality of meat from lambs grazed on 7 pasture species in Hawkes bay. *New Zealand Journal of Agricultural Research* **37:** 177-186.

Zhang J, Henriksson G, and Johansson G. 2000. Polygalacturonase is the key component in enzymatic retting of flax. *Journal of Biotechnology* **81:** 85-89.

Zhou HW, Ben-Arie R, and Lurie S. 2000. Pectin esterase, polygalacturonase and gel formation in peach pectin fractions. *Phytochemistry* **55:** 191-195.

Zwieniecki MA, Melcher PJ, and Holbrook NM. 2001. Hydrogel control of xylem hydraulic resistance in plants. *Science* **291:** 1059-1062.

Zykwinska AW, Ralet MCJ, Garnier CD, and Thibault JFJ. 2005. Evidence for in vitro binding of pectin side chains to cellulose. *Plant Physiology* **139:** 397-407.