

Immunocytochemistry of the mucilage cells of *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae)

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ABSTRACT – (Immunocytochemistry of the mucilage cells of *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae)). The use of monoclonal antibodies for specific pectic epitopes is an important tool in the study of the cell wall. Throughout the development of mucilage cells of *Araucaria angustifolia* (Bertol.) Kuntze, a gradient of distribution was observed in relation to the pectic de-esterification, as well as to the increase of galactan and arabinan epitope distribution, and to the reduction of arabinogalactans proteins (AGPs) epitope at maturity. AGP and methyl-esterified homogalacturonan (HGA) were present in the mucilage. Galactans and arabinans were also observed in the mucilage, though with weak labelling. Degradation of AGP in the maturity of mucilage cells, in cell wall, as well as in the secretion, could be involved in the programmed cell death (PCD). Different labellings found among parenchyma and mucilage cells suggested differences in the cell wall properties of the mucilage cells.

Key words - *Araucaria angustifolia*, cell development, cell wall, monoclonal antibodies, mucilage cells, pectin

RESUMO – (Imunocitoquímica das células de mucilagem de *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae)). A utilização de anticorpos monoclonais para epitopos pécticos específicos é uma importante ferramenta no estudo da parede celular. Ao longo do desenvolvimento das células de mucilagem de *Araucaria angustifolia* (Bertol.) Kuntze foi observado um gradiente de distribuição com relação à de-esterificação de pectinas, bem como um aumento na distribuição dos epitopos de galactanos e arabinanos e uma redução na distribuição de arabinogalactanos proteínicos (AGPs) na maturidade. AGP e homogalacturonanos (HGA) metil-esterificados foram detectados na mucilagem. Galactanos e arabinanos foram também observados na mucilagem, embora com fraca marcação. A degradação de AGPs na maturidade das células de mucilagem, tanto na parede celular, como na secreção, poderia estar envolvida no processo de morte celular programada (MCP). Diferentes marcações encontradas entre as células de parênquima e células de mucilagem sugerem diferenças nas propriedades da parede celular das células mucilaginosas.

Palavras-chave - anticorpos monoclonais, *Araucaria angustifolia*, células de mucilagem, desenvolvimento celular, parede celular, pectinas

Introduction

Several authors had described certain unusual cells in the mesophyll of the genus *Araucaria* Juss. as cells of large volume with a rare presence of chloroplasts, suggesting a function of water storage (Baker & Smith 1910, Griffith 1950, Vasiliyeva 1969, Monteiro *et al.* 1977). Bamber *et al.* (1978) observed that these cells had pectic partitions, which subdivided the cell lumen, forming numerous compartments. Owing to these reticulated partitions, the authors described them as “compartmented cells.”

Nevertheless, the cytological characteristics observed during the developmental stages described in ultrastructural studies by Mastroberti & Mariath (2003) suggested that compartmented cells of *Araucaria*

angustifolia (Bertol.) Kuntze could be a type of mucilage cell, confirming its function of water storage through apoplastic tracers and histochemical tests. Mastroberti & Mariath (2008) described four developmental stages for these cells. In stage 1, the shoot apex volume increased in some mucilage cells showing that the cells were very young. In stage 2, these cells reached greater volume and became abundant. Mucilage was continuously deposited between the cytoplasm and the vacuole. A cavity was formed by the shrinkage of the tonoplast. In stage 3, the mucilage cells were still abundant and the cytoplasmic amount decreased. In stage 4, the cells were completely filled with pectic mucilage that led to the formation of denser regions. The nucleus and the cytoplasm degenerated, but the cell wall and mucilage integrity were maintained.

Mucilage cells are known to occur in several families, *e.g.*, Lauraceae (Bakker & Gerritsen 1989, Bakker *et al.* 1991), Cactaceae (Trachtenberg & Fahn 1981, Trachtenberg & Mayer 1981a, b, 1982a, b), Malvaceae (Bakker & Gerritsen 1992), and others. The secreted

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mucilage accumulates between the cell wall and the protoplast in *Cinnamomum burmanni* Blume, *Opuntia ficus-indica* (L.) Mill. and *Hibiscus schizopetalus* (Mast.) Hook., which is not identical to the secretory process observed in *A. angustifolia*, in which the mucilage accumulates between the cytoplasm and the vacuole (Mastroberti & Mariath 2008).

Mucilages are complex acidic or neutral polysaccharide polymers of high molecular weight. They may be associated with various functions: serving as a food reserve, helping in water retention, acting as a lubricant of the growing root tip, as adhesive in seed dispersal, and aiding in capturing of insects in carnivorous plants. They also regulate the germination of seeds, and may probably be useful for other unknown purposes (Fahn 1979).

According to Esau (1965) and Fahn (1979), polysaccharides contribute to the plants' resistance to dry climate. It is well known that the pectins are polysaccharides of hydrophilic nature, and these substances are the base of mucilage composition.

Pectic polymers are classified into three major domains: homogalacturonans (HGA), which have a methyl-esterification variation, responsible for the ionic regulation, hydration, and gel states of the cell wall matrix; rhamnogalacturonans II (RG-II), which occur in all primary cell walls in the form of complex side chains of the HGA backbone; and the rhamnogalacturonans I (RG-I) formed by alternation of HGA by the rhamnoses (Ridley *et al.* 2001). Approximately 80% of the residues of rhamnoses, in RG-I undergo substitutions by side chains, rich in arabinans and galactans (Krishnamurthy 1999, Scheller *et al.* 1999, Pérez *et al.* 2000). It is generally accepted that pectins are synthesized in a highly methyl-esterified form (Micheli 2001). The degree of pectin esterification results in varying states of pectin. In the same wall of a cell, there are many domains where this degree is modified. Thus, the methyl-esterification degree and the change in the pectic molecule are important in the functional properties of the plant cell wall (Knox 1997).

In general, pectins have an important physiological role, contributing to the cell wall hydration, strength and flexibility of non-lignified bodies, fruit maturing, signaling to wound and to pathogen-host interactions, morphogenetic effects (regulatory effect in hormonal action and synthesis) and intercellular adhesion. Consequently, pectin could be considered not only as a structural polymer, but also as the main informative polymer of the plant cell wall, because of its influence in the calcium entrance regulation, which in turn regulates the plasmalemma permeability (Van Cutsem & Messiaen 1994).

In addition to the major polysaccharides, the growing plant cell wall also contains structural proteins (Showalter 1993). Several classes of cell wall structural proteins have been described in the plants, classified into hydroxyproline-rich glycoprotein (HRGP), glycine-rich protein (GRP), proline-rich protein (PRP), etc. Unlike the structural proteins listed above, arabinogalactan proteins (AGPs) are soluble and heavily glycosylated (Fincher *et al.* 1983, Showalter 1993). Multiple AGP forms are found in plant tissues, either in the cell wall or associated with the plasmalemma, and they display tissue- and cell-specific expression patterns (Pennel *et al.* 1989). The AGPs have a sticky quality and show specific binding to pectins, being involved in the cell wall adhesion (Cosgrove 1997). The AGPs are also implicated in the growth, nutrition, and other developmental processes (Pennel & Roberts 1990).

Immunocytochemical studies have contributed to the study of identifying, defining, and understanding the special distribution of pectins in the cell and in the apoplast. Several immunocytochemical studies have been conducted in many plants, showing that during cell development, modifications were observed in pectin esterification (Fujino & Itoh 1998, Sutherland *et al.* 1999, Bush *et al.* 2001), in the presence of galactans and arabinans (Jones *et al.* 1997, Bush *et al.* 2001, Willats *et al.* 2001) and in the calcium binding (Liners *et al.* 1994, His *et al.* 1997).

Monoclonal antibodies (MAbs) are important tools to show the spatial location and distribution of different pectic and AGPs epitopes of the cell wall (Knox 1997, Bush *et al.* 2001). However, very little is known about the mucilage immunolocalization besides those secreted in seeds, as in *Arabidopsis thaliana* (Willats *et al.* 2001).

A. angustifolia is a native species from Brazil and anatomical studies pertaining to this species should be encouraged. Besides, immunocytochemical studies in gymnosperms and in mucilage cells are uncommon (Mogami *et al.* 1999). Hence, it is important to point out that this is the first report on the immunocytochemistry of an idioblast cell wall, as well as investigation of the process of mucilage formation in the stages of leaf development.

In this work, five MAbs have been used to investigate the distribution of different pectic epitopes and AGPs during leaf development in *A. angustifolia*, using epifluorescence microscopy.

Material and methods

Seeds of Brazilian Pine (*A. angustifolia* (Bertol.) Kuntze) were collected from São Francisco de Paula National Forest

(FLONA de São Francisco de Paula, 29°23' and 29°27' S and 50°23' and 50°25' W) in the state of Rio Grande do Sul, Brazil. The seeds were cultivated in a seedbed in full sunlight. The leaves were collected when the young plants reached about 4 months of age (100-210 mm height). Planting was undertaken in the green house at the Genetic Department of the Federal University of Rio Grande do Sul (UFRGS). Three stages of leaf development were observed and studied. Immature leaves (10-15 mm length) were collected from the shoot apex to observe stages 1 and 2. Mature leaves (30-45 mm length) were collected from the middle portion of the stem.

Material processing for immunofluorescence – The middle portions of the immature and mature leaves from young plants were fixed in 2.5% glutaraldehyde, 2% formaldehyde, in 0.1 M sodium phosphate buffer, pH 7.2 (Roland & Vian 1991), dehydrated in an ethanol-graded series (20, 30, 50, 60, 70, 80, 90, and 100%), and embedded in LR White “Hard Grade” (London Resin Company). The semithin sections (350-500 nm) were cut on a Leica Ultracut UCT microtome with a glass knife, and adhered to glass slides recovered with poly-L-lysine (Sigma). The sections observed in the bright field microscope were stained with 1% Toluidine Blue O (C.I. 52040), pH 8-9 (Souza 1998). Sections were incubated with the MAbs JIM5, JIM7, JIM13, LM5, and LM6 (provided by Dr. Keith Roberts, John Innes Centre, and Dr. Paul Knox, Centre for Plant Sciences, University of Leeds, UK), which recognize the epitopes described in table 1.

Incubation of the sections for immunofluorescence – Sections were hydrated with saline phosphate buffer (PBS) of pH 7.1 (Harris 1994), blocked with a centrifuged solution with 3% of no-fat milk powder in PBS, and incubated with the primary antibody in PBS for 1-2 h. Primary antibody incubation was left out for controls. The material was washed in PBS and then incubated with the secondary antibody Goat anti-rat FITC (Sigma) in PBS for 1-2 h in a dark room. After washing with PBS, sections were mounted in 0.1% para-phenylenediamine (PPD) (Sigma), 10 mM of PBS with 10% of 0.15 M NaCl and 90% glycerol anti-fade solution,

and observed on a Leica DMR microscope equipped with epifluorescence (excitation filter 450-490 nm).

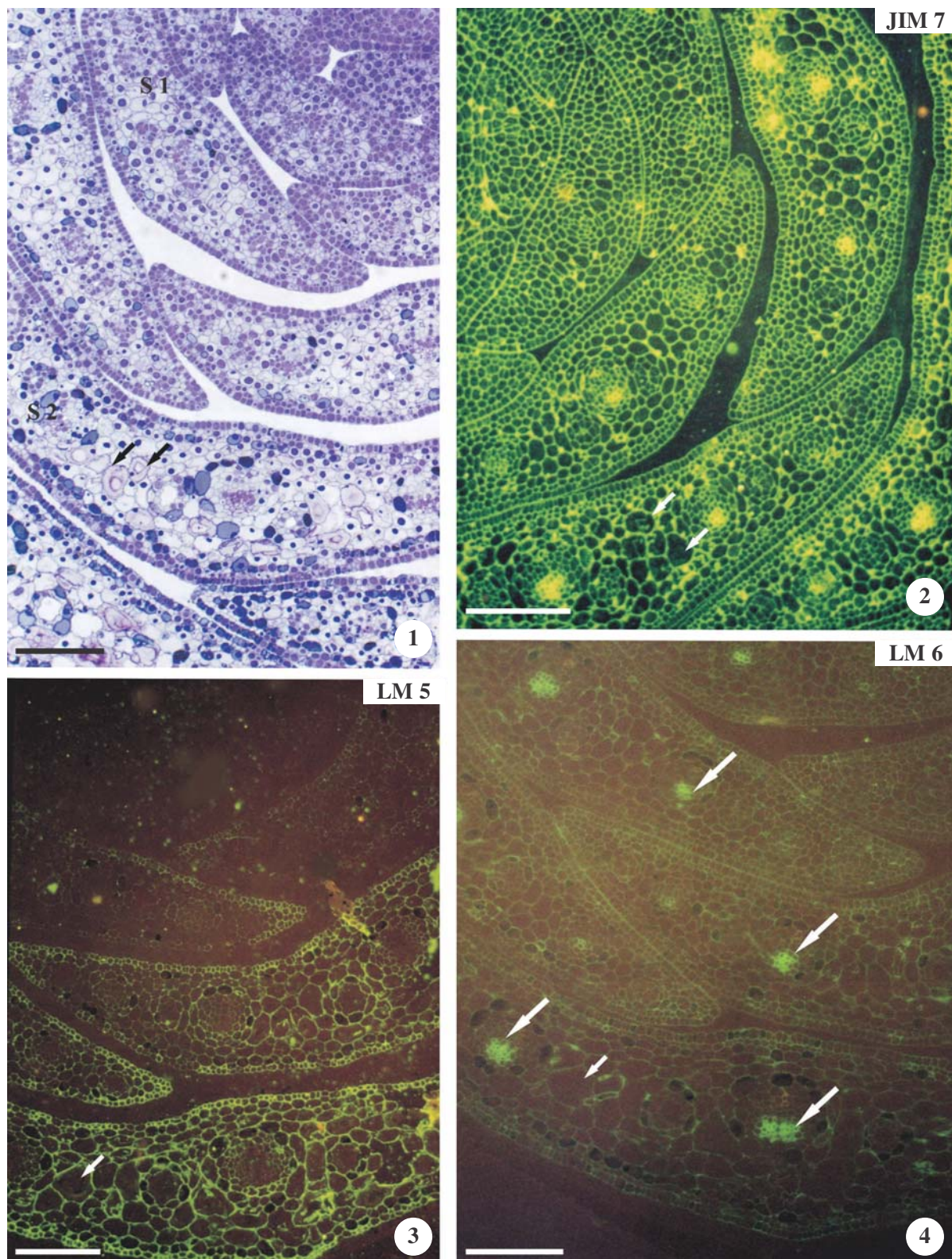
Results

Stages 1 and 2 (immature leaves) were observed in transverse section of the shoot apex (figure 1). In stage 1, immature leaves had an undistinguished mesophyll, and mucilage cells were not observed (data not shown). In stage 2, the immature leaf possessed mucilage cells. Material incubated with JIM7 (methyl-esterified pectins) had strong and homogeneous labelling (figure 2). With LM5 (galactans), there was a gradient observed throughout the leaf development in which the galactan epitope increased with the leaf maturity (figure 3). On the other hand, labelling with LM6 (arabinans) had a homogeneous, low distribution of these chains throughout the leaf development. However, a strong labelling of the arabinan epitope was observed in the region of the phloem (figure 4, arrows).

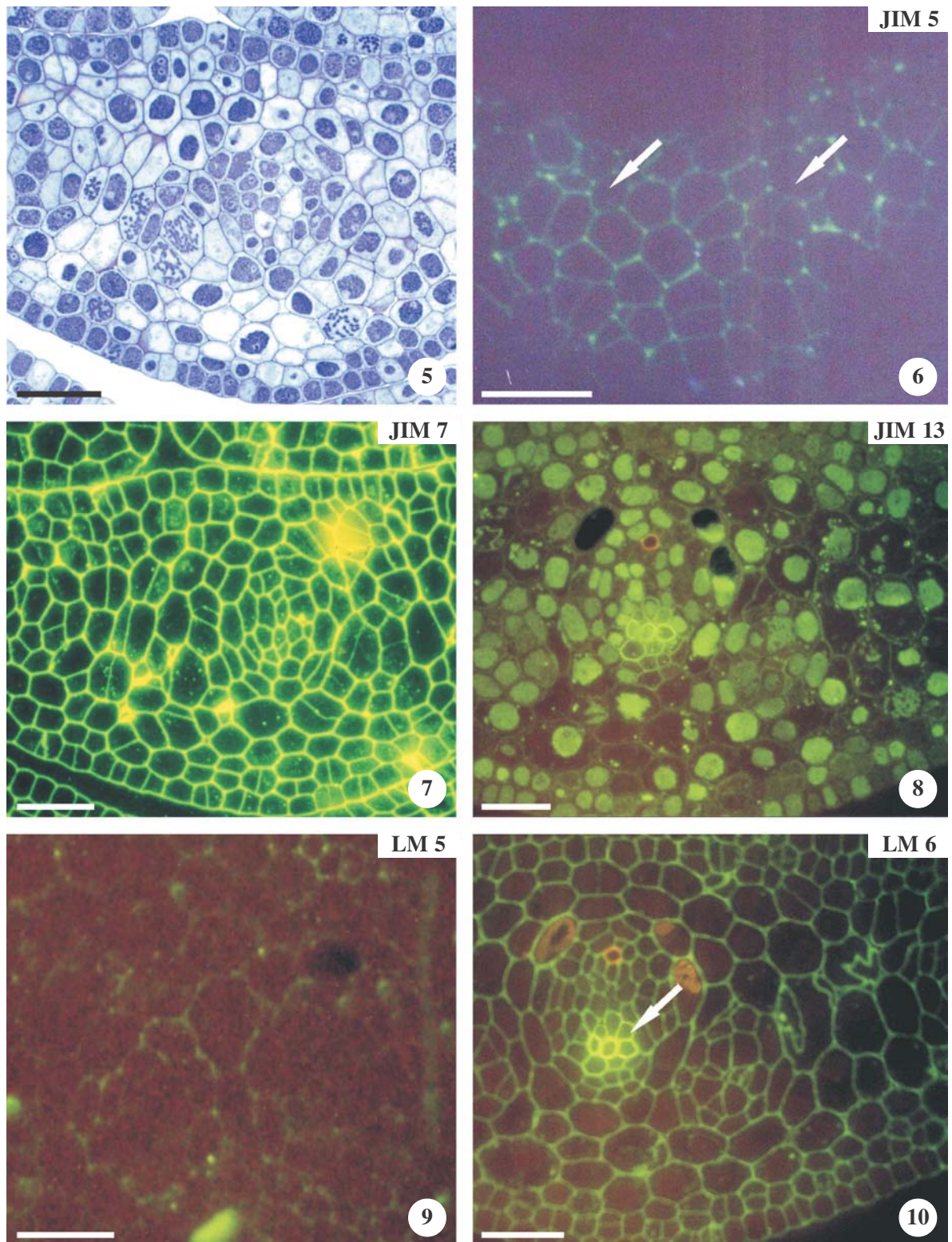
In the transverse section, a leaf in stage 1 (figure 5), when incubated with JIM5 (pectins with low methyl-esterification) resulted either in a weak or no labelling in the mesophyll, making it impossible to distinguish the differences in labelling of other mucilage cells. Epidermal and hypodermal cells were not labelled with JIM5. These chains of low methyl-esterification, however, were abundant in the cell vertices (figure 6, arrows). Observations with JIM7, differently from JIM5, showed that the cell walls were strong and uniformly labelled in all tissues (figure 7). With JIM13 (AGPs), cells were weakly labelled, except the phloem cells. Nevertheless, we observed that the cell lumen had high labelling with AGPs (figure 8). The anti-AGP MAb, JIM13, binds to plasmalemma, but in the figures, this epitope in the plasmalemma and cell wall cannot be easily distinguished.

Table 1. Recognition of the monoclonal antibodies to the different epitopes

Monoclonal antibody	Epitopes	References
JIM 5	Homogalacturonan (HGA) methyl-esterified up to 40% in a specific but undefined pattern	VandenBosch <i>et al.</i> (1989), Knox <i>et al.</i> (1990), Willats <i>et al.</i> (2000), Clausen <i>et al.</i> 2003
JIM 7	HGA methyl-esterified to 15-80%	Knox <i>et al.</i> (1990), Willats <i>et al.</i> (2000), Clausen <i>et al.</i> 2003
JIM 13	Arabinogalactan proteins	Knox <i>et al.</i> (1991)
LM 5	(1 → 4)-β-D-galactan	Jones <i>et al.</i> (1997)
LM 6	(1 → 5)-α-L-arabinan	Willats <i>et al.</i> (1998)



Figures 1-4. Transverse sections of the shoot apex. Small arrows indicate the presence of mucilage cells. 1. Light microscopy in bright field showing stage 1 (S1) and stage 2 (S2). 2. Immunofluorescence with JIM7 labelling methyl-esterified pectins. 3. With LM5, a gradient is observed in the galactans. 4. Labelling with LM6 showed higher distribution of arabinan in the phloem (larger arrows). Bars = 100 μ m



Figures 5-10. Transverse sections of immature leaves in stage 1. 5. Light microscopy in bright field. Mucilage cells not yet distinct. 6. Immunofluorescence with JIM5 showed high distribution of low methyl-esterified pectins in the cell vertices (arrows). 7. JIM7 showed the presence of methyl-esterified pectins in cell walls. 8. With JIM13, note the AGP presence in the lumen and wall cell. 9. Weak labelling with LM5 (galactans). 10. LM6 showed the presence of arabinans in the walls and high deposition in the phloem (arrow). Bars = 25 μ m.

Results with LM5 (galactans) showed weak or no labelling in the leaf tissues in stage 1 (figure 9). The LM6 (arabinans) showed that labelling in the walls was moderate and uniform, except for the phloem cells, which had strong labelling (figure 10, arrow).

In stage 2, the leaf was still immature, although young mucilage cells were already observed (figure 11). The control was demonstrated without the primary antibody (figure 12). With JIM5, mesophyll cell wall, epidermal cells were labelled only in some regions (figure 13). With JIM7, the labelling was similar as in stage 1 (figure 14). Results with JIM13 showed an increase in AGP labelling in the cell wall of different cell tissues (figure 15). With LM5, the tissues were strongly labelled, increasing the galactan epitope from stage 1 (figure 9) to stage 2 (figure 16). The absence of galactans was observed in the epidermal cell wall. On the other hand, galactans occurred in varied distributions in the hypodermis, in the zones of decreased cell elongation (figure 17, arrows). The LM6 showed an arabinan distribution similar to the galactans (figure 18), except for the presence of arabinans that is observed in the epidermal cell wall (figure 19).

Mucilage cells and the neighbouring parenchyma cells had the same strong labelling in the cell wall when the antibodies JIM5 (figure 13), JIM7 (figure 14), and LM6 (figure 17) were used, but with JIM13 and LM5, the mucilage cell wall showed stronger labelling than the parenchyma cell wall (figures 15 and 16).

With JIM7, mucilage of the mucilage cells had moderate labelling only in some regions of the cell lumen (figure 14). With JIM13, there was a moderate detection of AGP in the mesophyll cell wall, which was stronger in the mucilage cell wall (figure 15). Moreover, with this antibody, a strong mucilage labelling was also observed in the denser regions of the mucilage (figure 15, arrows). With JIM5, LM5, and LM6, the pectin with low esterification, galactans, and arabinans, respectively, presented weak labelling in the mucilage (figures 13, 16, and 18).

The observed mature leaves (stage 3) were those in which the mucilage cell lost its polygonal outline and in which the protoplast degenerated (figure 20). A control test was conducted without the primary antibody and this can be observed in figure 21. The JIM5 showed weak labelling of low methyl-esterified pectins in the leaf tissue walls, except in specific regions, where the presence of these chains was strong (figure 22). Nevertheless, cell walls of all tissues were labelled strongly with JIM7, where the mucilage was also labelled with this antibody, thus revealing a clear presence of methyl-esterified chains (figure 23, arrow). When incubated with JIM13, a decrease

in AGP in the mucilage cell wall and an increase in this compound in the neighbouring parenchyma cell wall (figure 24, arrow) was observed, showing an inversion in the AGP labelling when compared with stage 2. Results with LM5 showed that mucilage cells also had a strong labelling, compared with the neighbouring cells with moderate labelling of galactan chains in the spongy parenchyma cell wall and an absence in the palisade parenchyma (figure 25). With LM6, there was a strong and uniform distribution of arabinans in the cell wall of different leaf tissues (figure 26).

In relation to the mucilage, the occurrence of methyl-esterified pectins (figure 23, arrow) and a severe reduction in the AGP distribution were also observed (figure 24, arrow). Moderate labelling with LM5 and LM6 showed that galactans and arabinans, respectively, were present in the mucilage of these cells and that they increased following the leaf maturity (figures 25 and 26, arrow).

The unstructured secretion stage lasts until a denser organized net stage is resulted from the cell development owing to biochemical alterations that the mucilage undergoes.

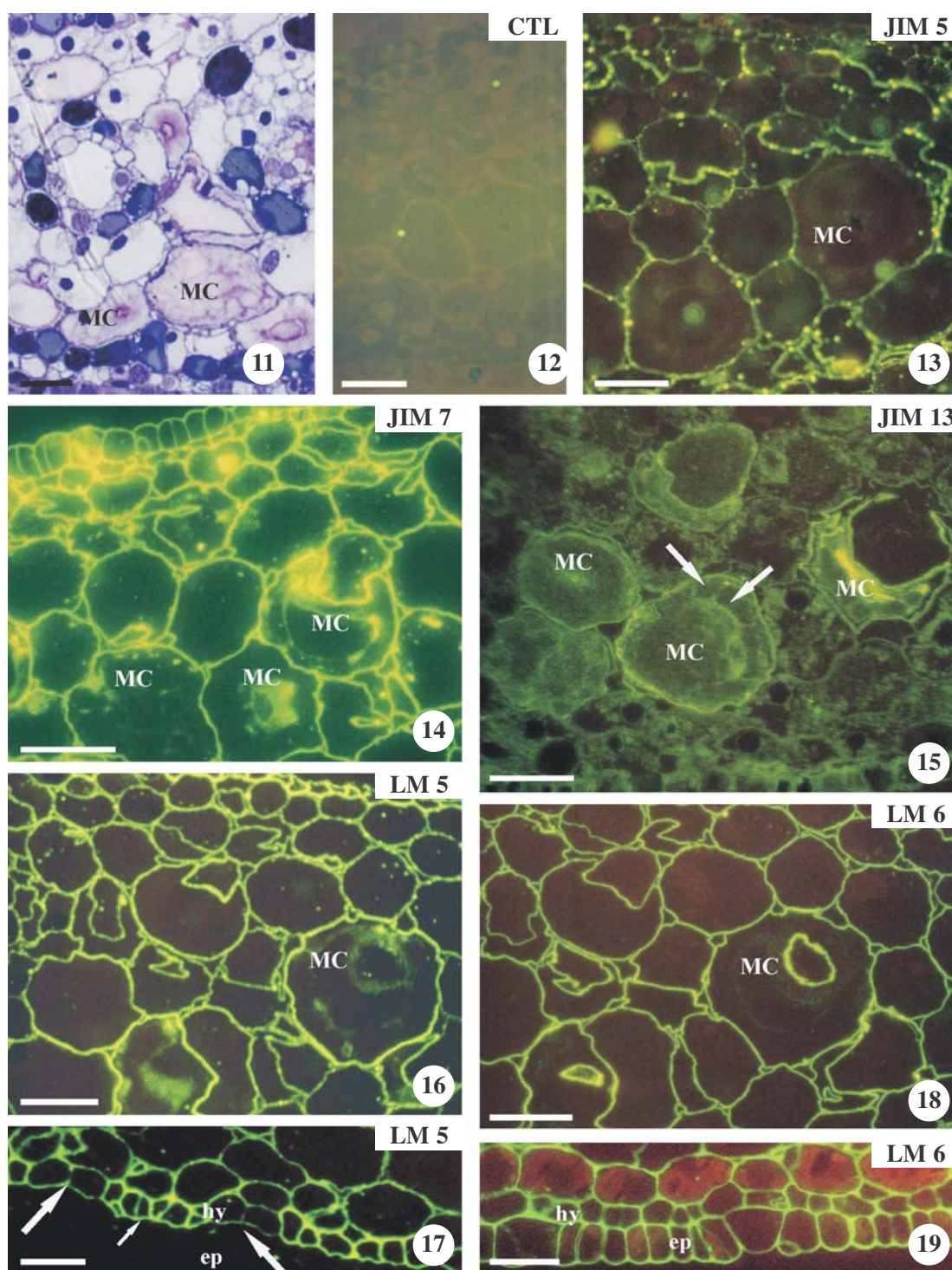
Table 2 shows the synthesis of different labellings obtained in the analyzed stages of the leaves.

Discussion

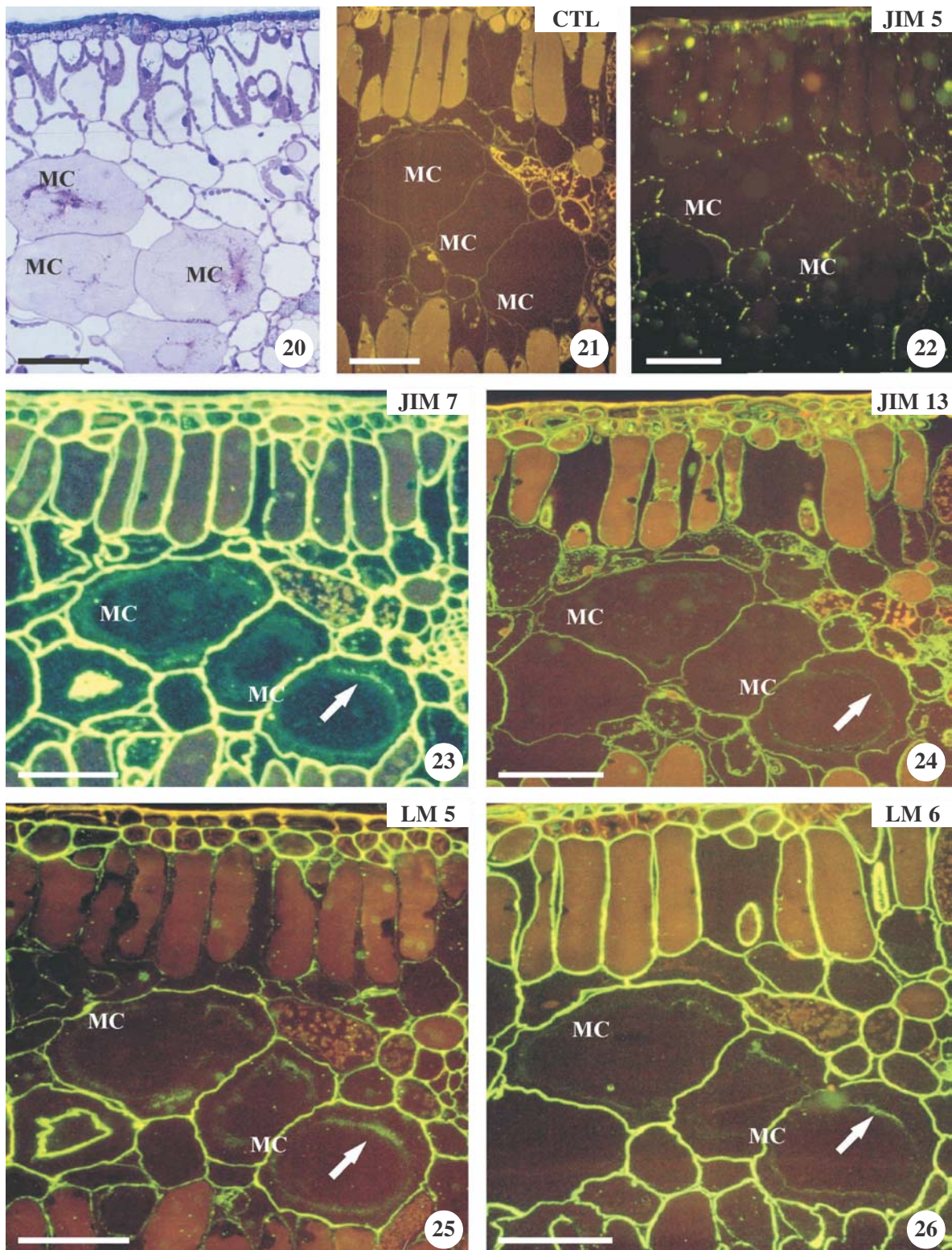
The results indicated the differences in the distribution of pectic epitopes between immature and mature leaves. In the mucilage cell wall, a gradient was observed throughout the development, in relation to methyl-esterification of pectins and distribution of galactans, arabinans, and AGP. Spongy parenchyma cells (neighbouring cells) also showed a gradient in relation to the reduction of these pectic epitopes.

There was an increase in the presence of low and high methyl-esterification pectins (JIM5 and JIM7), not only with regard to the mucilage cells, but also the other cells of the spongy parenchyma, observed in stages 1 and 2 (shoot apex). The high methyl-esterification (JIM7) did not present any variation during leaf or cellular maturation. These data are in accordance with previous findings that showed that pectins are synthesized in a highly methyl-esterified type in the Golgi (Knox 1997, Bush *et al.* 2001).

The JIM5 and JIM7 antibodies had been characterized by Willats *et al.* (2000) and Clausen *et al.* (2003), who found that JIM5 showed a binding with antigens of more than 40% methyl-esterified chains. Thus, the results with JIM5 should be considered with caution in terms of an indicator for low esterification.



Figures 11-19. Transverse sections of immature leaves in stage 2. 11. Light microscopy in bright field showed the mucilage cells (MC). 12. Control. 13. With JIM5, the presence of low esterification is observed in certain cell wall regions. 14. JIM7 showed the labelling of methyl-esterification in all cell walls and in the mucilage. 15. With JIM 13, AGPs are present in the mucilage cell walls and they are abundant in the mucilage. The more dense regions are also labelled (arrows). 16. With LM5 (galactans), there is a strong labelling in the mucilage cell walls, including in the mucilage. 17. Abaxial face. Weak (large arrows) and strong labelling (small arrow) in the hypodermis (hy). 18. With LM6, a homogeneous arabinan distribution is observed in the cell walls. 19. Abaxial face. Labelling in the epidermis (ep) and homogeneous labelling in the hypodermis (hy). Bars = 25 μ m (11-16 and 18). Bars = 20 μ m (17 and 19).



Figures 20-26. Transverse sections of mature leaves in stage 3. 20. Light microscopy in bright field showing enucleated mucilage cells (MC). 21. Control. 22. JIM5 showed the presence of low esterification in certain regions of the cell wall. 23. JIM7 (methyl-esterification) labelled cell walls and mucilage (arrow). 24. JIM13 labelling in the cell walls and weak labelling in the mucilage (arrow). 25. LM5. Intensive labelling in the mucilage cell walls. Presence of galactans in the mucilage (arrow). 26. LM6. Presence of arabinans in the cell walls and in the mucilage (arrow). Bars = 50 μ m.

Table 2. Labelling of the pectic epitopes between immature and mature leaves of *Araucaria angustifolia* (Araucariaceae). (MC = mucilage cells, PC = parenchyma cells, + = weak labeling, ++ = moderate labeling, +++ = strong labeling, 0 = not observed, – = not labelling)

	MC cell walls	PC cell walls	Mucilage
JIM 5 (HGA low methyl-esterified)	stage 1	+	0
		++	0
	stage 2	+++	+
	stage 3	+++	–
JIM 7 (HGA high methyl-esterified)	stage 1	+++	0
	stage 2	+++	++
	stage 3	+++	++
JIM 13 (AGP: arabinogalactan proteins)	stage 1	+	0
	stage 2	++	+++
	stage 3	+	+
LM 5 (galactan)	stage 1	+	0
	stage 2	+++	+
	stage 3	+++	++
LM6 (arabinan)	stage 1	++	0
	stage 2	++	+
	stage 3	+++	++

The detection of high methyl-esterified pectins in primary walls by using the JIM7 MAb was already described by Knox *et al.* (1990). Also, Vandenbosh *et al.* (1989) and Knox *et al.* (1990) demonstrated that with JIM5, there was strong labelling in the cell vertices and intercellular spaces. This was observed in the immature leaves from stage 1 in *A. angustifolia*, where the labelling in the vertice regions was very clear. Furthermore, this process occurred quickly as de-esterification occurs in the primordium leaf.

Van Cutsem & Messiaen (1994) and Morris *et al.* (2000) observed that when the cell wall rigidity was lower, there was a higher degree of methyl-esterification, thus making the cell wall expansion easier during the growth process. The leaves of stage 1 presented a weak labelling with JIM5 in the elongation regions, with more intensity in the cell vertices. Furthermore, the leaf continued its growth, in the absence or rare occurrence of low methyl-esterified pectins in the epidermal and hypodermal cell walls of immature leaves. These results were in accordance with previous observations of the epidermal cells on the epicotyl of *Pisum sativum* L., where the JIM5 epitope was distributed in regions of the cell wall that did not elongate (Fujino & Itoh 1998).

In mature leaves (stage 3), labelling with JIM5 seemed to be similar to stage 2 (immature leaves) because the de-esterification process was observed in the shoot apex

(between stages 1 and 2). In addition, the distribution of pectins with low methyl-esterification (JIM5) was not uniform. Some regions seen through the wall with strong labelling could be pit regions. Casero & Knox (1995) and Sutherland *et al.* (1999) showed that JIM5 had relevant labelling in the pit regions, which were observed in the form of rays in the inner wall layer. Methyl-esterified pectins (JIM7) remain abundant and uniformly distributed in the mature leaves. Similarly, Bush *et al.* (2001) observed the development of the potato tubercle stolons (*Solanum tuberosum*) in which the cell walls were rich in highly methyl-esterified pectins.

Cell wall rigidity is also related to the presence of side chains of galactans and arabinans in the RG-I (Jones *et al.* 1997). In immature leaves of *A. angustifolia*, no labelling with LM5 in the epidermal cells was observed. In contrast, galactans occurred in different distributions in the hypodermis in the regions of decreased cell elongation. Jones *et al.* (1997) also observed that these pectic epitopes were absent in the epidermal and sub-epidermal cells in tomato fruits (*Lycopersicon esculentum* Mill.). The side chains decreased the ability of the pectins to form more resistant gels. Thus, the absence of galactans in the epidermis and the different distribution in the hypodermis of this epitope in stages 1 and 2 are believed to correlate with the maintenance of the wall integrity during leaf elongation. According to

Willats *et al.* (1999) and Bush *et al.* (2001), galactans were not detected in the cell walls of meristematic regions of roots or shoot apex, but appeared in certain stages of tissue development. In *A. angustifolia*, this aspect was clearly observed in the transverse section of the shoot apex, where there was an increase in galactan epitope between stages 1 and 2. In stages 2 and 3, the galactan epitope distribution was higher in the mucilage cell walls than in the parenchyma cells. McCartney *et al.* (2000) and McCartney & Knox (2002) suggested that the presence of these chains could be related to mechanical properties necessary for cell elongation. McCartney *et al.* (2003) also described that besides the elongation, this epitope marks the acceleration of this process, contributing to the elasticity of the cell wall. In the mucilage cells of *A. angustifolia*, there was a faster cell elongation process than in others, suggesting that the galactans responsible for the decrease in cell wall rigidity contribute to the increase in volume of these cells at maturity and also to their elasticity while storing water.

The LM6 is also linked to RG-I epitope of the side chains, or to the arabinans. In stages 1 and 2, the arabinan (LM6) epitope was distributed uniformly with moderate labelling in the cell walls of different tissues, when compared with the distribution of galactans. In stage 3, arabinan distribution was slightly higher in the parenchyma cells, in contrast to the LM5 labelling. Both galactan and arabinan epitopes may occur in different regions of the cell wall of one single cell (Orfila & Knox 2000). Moreover, they have been already observed separately in different developing cells (Willats *et al.* 1999, Willats *et al.* 2000). Bush *et al.* (2001) observed that the arabinan epitope was restricted to cell walls from the youngest regions of the stolon of *S. tuberosum*; and in mature cell walls the arabinan epitopes were dramatically reduced. The authors suggested that different side chains might be attached to RG-I backbones at different times, probably in response to cell development. In this way, it was possible to explain the differential distributions of these side chains between mucilage and other neighbouring cells in *A. angustifolia*, which showed differences in the development process, similar to the increase in volume of mucilage cells, cell wall elasticity at maturity, and senescence.

Most often, type II arabinogalactans (AG II) are associated with proteins forming AGPs. It has not yet been clarified if this polysaccharide is a part of the pectin complex. Knox *et al.* (1991) described an antibody with anti-AGP binding in the plasmalemma that had different distributions during cell development. In the figures presented in our work, the labelling of the plasmalemma and cell wall cannot be easily distinguished.

In immature leaves, a higher AGP presence was observed in the cell walls of mucilage cells in comparison with the mature mucilage cells, and an increase in distribution of the neighbouring parenchyma cells was also observed.

One of the most widespread functions of the AGP, mainly in the plasmalemma, would be the formation of the cell wall (Mogami *et al.* 1999). The AGPs are involved in different aspects of plant growth and development, including embryogenesis and cell proliferation (Majewska-Sawka & Nothnagel 2000), but the molecular basis of their actions is still unclear (Letarte *et al.* 2006).

Knox *et al.* (1989) used AGP as molecular label to observe future cells, which die in the meristem cell population. By using an anti-AGP for the tobacco leaf (*Nicotiana tabacum* L.) and corn coleoptiles (*Zea mays*), respectively, Herman & Lamb (1992) and Schindler *et al.* (1995) showed the autophagy of cells and proved AGP degeneration at maturity. In corn coleoptiles, high levels of AGP associated with plasmalemma have been found in young cells, while more mature cells showed low levels of AGP. This concept could also be applied to the mucilage cells of *A. angustifolia*. The increase in AGP in the plasmalemma in the mucilage cells during its secretion stage and the drastic reduction in the mature cells lead us to suggest that these epitopes would indicate a participation in the PCD process in accordance with the observations in previous ontogenetic studies of these cells (Mastroberti & Mariath 2003). Gao & Showalter (1999) stated that AGPs were possibly involved in the control of PCD in *Arabidopsis* cell culture. Letarte *et al.* (2006) suggested that AGPs could play an important role in the prevention of PCD of microspores. However, interaction of AGPs in the plasmalemma and extracellular matrix, to induce or to prevent the PCD, deserves more detailed attention as there are only a few studies pertaining to it.

The LM5 and LM6 epitope distribution showed a gradual increase of galactans and arabinans epitopes, respectively, throughout the mucilage cell development in *A. angustifolia*. This was in contrast to *Arabidopsis thaliana* seeds, which lacked these chains (Willats *et al.* 2001). Therefore, the presence of these side chains in the rhamnose would be feasible, forming a part of the mucilage constitution in *A. angustifolia*.

Willats *et al.* (2001) observed pectic polysaccharides in the mucilage secreted by *A. thaliana* seeds, proving the presence of HGA. These results were also observed for the mucilage cell secretions of *A. angustifolia*.

In contrast, AGP detection with JIM13 was strongest in the immature cell mucilage, with drastic reduction in the mature cells. The AGPs occurring in plant secretions

and mucilage have been extensively studied in terms of their highly varied carbohydrate chemistry, and possess great potential for carrying information (Fincher *et al.* 1983). Clarke *et al.* (1979) suggested that AGPs could contribute and protect against damage caused by low temperatures or freezing. According to these authors, AGPs could also be associated with water storage function and drought resistance, when found in gums and mucilage. For this reason, the presence of high methyl-esterification and AGP in the mucilage could act as a shield in young plants from desiccation in adverse periods.

In conclusion, the morphological aspects of the mucilage cell have been established by Bamber *et al.* (1978) and Mastroberti & Mariath (2003), showing an initial granular secretion evolving to a more organized structure, which reflects developmental stages of this particular cell, owing to biochemical alterations of the mucilage. Immunocytochemical studies showed such changes throughout the pectic epitopes and AGP analysis during the leaf development.

Moreover, the methyl-esterification of pectins was always present in the walls, and the de-esterification occurred throughout the leaf development, corroborating with other dicotyledon studies. However, arabinans and galactans showed different distributions during the leaf development. Their functions are still argued, but it is possible to outstand that pectic gradients were generated by the temporal difference in which galactans and arabinans side chains bind with RG-I. Besides, the increased galactans in these cells, in comparison with the neighbouring cells, indicated expansion acceleration on the mucilage cells. The AGPs decreased in these cells, which is associated to the expansion and cell death, as described for other cellular types of dicotyledons. Thus, different labelling found among parenchyma and mucilage cells suggested the differences in the cell wall properties of mucilage cells.

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References

- BAKER, R.T. & SMITH, H.G. 1910. A research on the pines of Australia. Government Printer, Sydney.
- BAKKER, M.E. & GERRITSEN, A.F. 1989. A suberized layer in the cell wall of the mucilage cells of *Cinnamomum*. *Annals of Botany* 63:441-448.
- BAKKER, M.E. & GERRITSEN, A.F. 1992. The development of mucilage cells in *Hibiscus schizopetalus*. *Acta Botanica Neerlandica* 41:31-42.
- BAKKER, M.E., GERRITSEN, A.F. & VAN DER SCHAAF, P.J. 1991. Development of oil and mucilage cells in *Cinnamomum burmanni*. An ultrastructural study. *Acta Botanica Neerlandica* 40:339-356.
- BAMBER, R.K., SUMMERVILLE, R. & GREGORY, J. 1978. Unusual cells in the mesophyll zone of leaves of *Araucaria*. *Australian Journal of Botany* 26:177-187.
- BUSH, M.S., MARRY, M., HUXHAM, I.M., JARVIS, M.C. & McCANN, M.C. 2001. Developmental regulation of pectic epitopes during potato tuberisation. *Planta* 213:869-880.
- CASERO, P.J. & KNOX, J.P. 1995. The monoclonal antibody JIM5 indicates patterns of pectin deposition in relation to pit fields at the plasma-membrane-face of tomato pericarp cell walls. *Protoplasma* 188:133-137.
- CLARKE, A.E., ANDERSON, R.L. & STONE, B.A. 1979. Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry* 18:521-540.
- CLAUSEN, M.H., WILLATS, W.G.T. & KNOX, J.P. 2003. Synthetic methyl hexagalacturonate hapten inhibitors of antihomogalacturonan monoclonal antibodies LM5, JIM5 and JIM7. *Carbohydrate Research* 338:1797-1800.
- COSGROVE, D.J. 1997. Assembly and enlargement of the primary cell wall in plants. *Annual Review of Cell and Developmental Biology* 13:71-201.
- ESAU, K. 1965. *Plant anatomy*. John Wiley, New York.
- FAHN, A. 1979. *Secretory tissues in plants*. Academic Press, London.
- FINCHER, G.B., STONE, B.A. & CLARKE, A.E. 1983. Arabinogalactan-proteins: structure, biosynthesis and function. *Annual Review of Plant Physiology* 34:47-70.
- FUJINO, T. & 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.
- GAO, M. & SHOWALTER, A.M. 1999. Yariv reagent treatment induces programmed cell death in Arabidopsis cell cultures and implicates arabinogalactan protein involvement. *Plant Journal* 19:21-331.
- GRIFFITH, M.M. 1950. A study of the shoot apex and leaf histogenesis in certain species of *Araucaria*. Tese de doutorado, University of California, Berkeley.
- HARRIS, N. 1994. Immunocytochemistry for light and electron microscopy. *In Plant Cell Biology. A practical approach* (N. Harris & K.J. Oparka, eds.). Oxford University Press, London, p.157-176.
- HERMAN, E.M. & LAMB, C.J. 1992. Arabinogalactan-rich glycoproteins are localized on the cell surface and in intravacuolar multivesicular bodies. *Plant Physiology* 98:264-272.

- HIS, I., DRIOUICH, A. & JAUNEAU, A. 1997. Distribution of cell wall matrix polysaccharides in the epidermis of flax hypocotyl seedlings: calcium induced-acidification of pectins. *Plant Physiology and Biochemistry* 35: 631-644.
- JONES, L., SEYMOUR, G.B. & KNOX, J.P. 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.
- KNOX, J.P. 1997. The use of antibodies to study the architecture and the developmental regulation of plant cell walls. *International Review of Cytology* 171:79-120.
- KNOX, J.P., DAY, S. & ROBERTS, K. 1989. A set of surface glycoproteins forms an early marker of cell position, but not cell type, in the root apical meristem of *Daucus carota* L. *Development* 106:47-56.
- KNOX, J.P., LINSTEAD, P.J., KING, J., COOPER, C. & ROBERTS, K. 1990. Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta* 181:512-521.
- KNOX, J.P., LINSTEAD, P.J., PEART, J., COOPER, C. & ROBERTS, K. 1991. Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. *Plant Journal* 1:317-326.
- KRISHNAMURTHY, K.V. 1999. *Methods in cell wall cytochemistry*. CRC Press Boca Raton.
- LETARTE, J., SIMION, E., MINER, M. & KASHA, K.J. 2006. Arabinogalactans and arabinogalactan-proteins induce embryogenesis in wheat (*Triticum aestivum* L.) microspore culture. *Plant Cell Reports* 24:691-698.
- LINERS, F., GASPAR, T. & VAN CUTSEM, P. 1994. Acetyl- and methyl-esterification of pectins of friable and compact sugar-beet calli: consequences for intercellular adhesion. *Planta* 192:545-556.
- MAJEWSKA-SAWKA, A. & NOTHNAGEL, E.A. 2000. The multiple roles of arabinogalactan proteins in plant development. *Plant Physiology* 122:3-9.
- MASTROBERTI, A.A. & MARIATH, J.E.A. 2003. Compartmented cells in the mesophyll of *Araucaria angustifolia* (Araucariaceae). *Australian Journal of Botany* 51:267-274.
- MASTROBERTI, A.A. & MARIATH, J.E.A. 2008. Development of the mucilage cells of *Araucaria angustifolia* (Araucariaceae). *Protoplasma*. <http://www.springerlink.com/content/107724/?Content+Status-Accepted> (accessed 2008 February).
- McCARTNEY, L. & KNOX, J.P. 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., ORMEROD, A.P., GIDLEY, M.J. & KNOX, J.P. 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.
- McCARTNEY, L., STEELE-KING, C.G., JORDAN, E. & KNOX, J.P. 2003. Cell wall pectin (1-4) β-D-galactan marks the acceleration of cell elongation in the *Arabidopsis* seedling root meristem. *Plant Journal* 33:447-454.
- MICHELI, F. 2001. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends in Plant Science* 6:414-419.
- MOGAMI, N., NAKAMURA, S. & NAKAMURA, N. 1999. Immunolocalization of the cell wall components in *Pinus densiflora* pollen. *Protoplasma* 206:1-10.
- MONTEIRO, S.M., FERREIRA, A.G. & FLORES, F.E.V. 1977. Anatomia da plântula de *Araucaria angustifolia* (Bert.) O. Ktze. In XXI Congresso Nacional de Botânica, Curitiba, 393-399.
- MORRIS, G.A., FOSTER, T.J. & HARDING, S.E. 2000. The effect of the degree of esterification on the hydrodynamic properties of citrus pectin. *Food Hydrocolloid* 14:227-235.
- ORFILA, C. & KNOX, J.P. 2000. Spatial regulation of pectic polysaccharides in relation to pit fields in cell walls of tomato fruit pericarp. *Plant Physiology* 122:775-781.
- PENNELL, R.I. & ROBERTS, K. 1990. Sexual development in pea is presaged by altered expression of arabinogalactan protein. *Nature* 344:547-549.
- PENNELL, R. I., KNOX, J.P., SCOFIELD, G.N., SELVENDRAN, R.R. & ROBERTS, K. 1989. A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. *Journal of Cell Biology* 108:1967-1977.
- PÉREZ, S., MAZEAU, K., HERVÉ, C. & DU PENHOAT, C.H. 2000. The three-dimensional structures of the pectic polysaccharides. *Plant Physiology and Biochemistry* 38:37-55.
- RIDLEY, B.L., O'NEILL, M.A. & MOHNEN, D. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57:929-967.
- ROLAND, J.C. & VIAN, B. 1991. General preparation and staining of thin sections. In *Electron Microscopy of Plant Cells* (J.L Hall & E. Hawes, eds.). Academic Press, London, p. 2-66.
- SCHELLER, H.V., DOONG, R.L., RIDLEY, B.L. & MOHNEN, D. 1999. Pectin biosynthesis: a solubilized α-1,4-galacturonosyltransferase from tobacco catalyzes the transfer of galacturonic acid from UDP-galacturonic acid onto the non-reducing end of homogalacturonan. *Planta* 207:512-517.
- SCHINDLER, T., BERGFELD, R. & SCHOPFER, P. 1995. Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extension growth. *Plant Journal* 7:25-36.
- SHOWALTER, A.M. 1993. Structure and function of plant cell wall proteins. *Plant Cell* 5:9-23.

- SOUZA, W. 1998. Introdução à imunocitoquímica. In Técnicas básicas de microscopia eletrônica aplicadas às Ciências Biológicas (W. Souza, ed.). UENF, Rio de Janeiro, p. 104-105.
- SUTHERLAND, P., HALLET, I., REDGWELL, R., BENHAMOU, N. & MACRAE, E. 1999. Localization of cell wall polysaccharides during kiwifruit (*Actinidia deliciosa*) ripening. International Journal of Plant Science 160:1099-1109.
- TRACHTENBERG, S. & FAHN, A. 1981. The mucilage cells of *Opuntia ficus-indica* (L.) Mill.-development ultrastructure, and mucilage secretion. Botanical Gazette 142:206-213.
- TRACHTENBERG, S. & MAYER, A.M. 1981a. Calcium oxalate crystals in *Opuntia ficus-indica* (L.) Mill. Development and relation to mucilage cells – a stereological analysis. Protoplasma 109:271-283.
- TRACHTENBERG, S. & MAYER, A.M. 1981b. Composition and properties of *Opuntia ficus-indica* mucilage. Phytochemistry 20:2665-2668.
- TRACHTENBERG, S. & MAYER, A.M. 1982a. Biophysical properties of *Opuntia ficus-indica* mucilage. Phytochemistry 21:2835-2843.
- TRACHTENBERG, S. & MAYER, A.M. 1982b. Mucilage cells, calcium oxalate crystals and soluble calcium in *Opuntia ficus-indica*. Annals of Botany 50:549-557.
- VAN CUTSEM, P. & MESSIAEN, J. 1994. Biological effects of pectic fragments in plant cells. Acta Botanica Neerlandica 43:231-245.
- VANDENBOSH, K.A., BRADLEY, D.J., KNOX, J.P., PEROTTO, S., BUTCHER, G.W. & BREWIN, N. 1989. Common components of the infection thread matrix and the intercellular space identified by immunocytochemical analysis of pea nodules and uninfected roots. EMBO Journal 8:335-342.
- VASILYIEVA, G.V. 1969. A contribution to the comparative anatomy of leaves of the species of *Araucaria* Juss. Botanicheskij Zhurnal 54:448-459.
- WILLATS, W.G.T., MARCUS, S.E. & KNOX, J.P. 1998. Generation of a monoclonal antibody specific to (1-5)- α -L-arabinan. Carbohydrate Research 308:149-152.
- WILLATS, W.G.T., STEELE-KING, C.G., MARCUS, S.E. & KNOX, J.P. 1999. Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. Plant Journal 20:619-628.
- WILLATS, W.G.T., LIMBERG, G., BUCHHOLT, H.C., VAN ALEBEECK, G.-J., BENEN, J., CHRISTENSEN, T.M. I.E., VISSER, J., VORAGEN, A., MIKKELSEN, J.D. & KNOX, J.P. 2000. 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, W.G.T., McCARTNEY, L. & KNOX, J.P. 2001. In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of *Arabidopsis thaliana*. Planta 213:37-44.