Cell wall involvement in desiccation tolerance in the resurrection plant *Craterostigma wilmsii*

Maïté VICRE

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Supervisors: **Pr. J.M. FARRANT**

Pr. A. DRIOUICH (Université de Rouen, FRANCE)

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Resurrection plants have the unique capacity to revive from an air-dried state. In order to cope with desiccation, resurrection plants have to overcome a number of stresses, mechanical stress being one. This occurs when the cytoplasm shrinks creating tension between the plasma membrane and the cell wall. In leaves of the *Craterostigma* species, an extensive shrinkage occurs during drying as well as a considerable wall folding. It is thought that this folding is a well controlled process rather than a simple collapse and that the ability of the wall to fold is important for the viability of the tissues upon drying.

The aim of this study was to characterize the cell wall architecture and composition in hydrated and dry leaves of *C.wilmsii* using microscopical and biochemical techniques. Calcium and hormone contents were also determined during drying. The development of anhydrous fixation for microscopy confirmed the important folding of the wall previously observed with chemical fixation. Using immunocytochemical techniques and a variety of well characterized antibodies, the nature and composition of wall polymers was investigated. There was nothing unusual in the wall composition of *C.wilmsii* leaves as compared with other dicotyledonous plants. The results show a significant increase of the hemicellulosic polysaccharide xyloglucan and of the unesterified pectins during drying with levels declining again during rehydration. In contrast no increase was observed in others polysaccharides such as β (1-4) galactans and methylesterified pectins. Biochemical analysis allowed further characterization of cell wall composition of *C.wilmsii*. The data demonstrate marked changes in the pectic and hemicellulosic wall fraction from dry plants compared to hydrated ones. The most conspicuous change was a decrease in glucose content in the hemicellulose fraction of the dry plant. Together these findings show that dehydration causes important alteration of polysaccharides content in the cell wall of *C.wilmsii*. Such modifications might be involved in the modulation of the mechanical properties of the wall during dehydration. Furthermore calcium ions content was shown to increase in the cell wall of dry plants, this could also have a role in stabilizing the wall architecture. All these alterations might be under the control of auxin, an hormone whose content was shown to increase during dehydration.

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CONTENTS

ABBREVIATIONS

ABA	Abscisic Acid
AceA	Aceric Acid
AGPs	ArabinoGalactan Proteins
AIR	Alcohol-Insoluble residue
Ala	Alanine
AP	Ascorbate Peroxidase
Api	Apiose
Ara	Arabinose
BSA	Bovine Serum Albumin
CDTA	Diamino cyclohexane tetraacetic acid
Cpm	Counts per minute
CWM	Cell Wall Material
Cyt	Cytochrome
DA	Degree of Acetylation
DFA	Diferulic Acid
DM	Degree of Methylation
DMP	Dimethoxy 2-2 propane
DMSO	Dimethylsufoxide
EDTA	Ethylenediamine tetraacetic acid
EDS	Energy Dispersive Spectrometry
ER	Endoplasmic Reticulum
FAD	Flavine adenine dinucleotide
FeS	Non-heme iron sulfur center
FMN	Flavine mononucleotide
FTIR	Fourier Transform Infrared
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic Acid
GC	Gas Chromatography
Glc	Glucose
GlcA	Glucuronic Acid
Gly	Glycine
GR	Glutathione Reductase
HDT	Homoiochlorophillous desiccation tolerant
His	Histidine
HPLC	High Performance Liquid Chromatography
HRGP	Hydroxyproline-Rich GlycoProtein
Hyp	Hydroxyproline
IAA	Indole-3 Acetic Acid
KDO	3-deoxy-manno-octulosonic acid
LEA	Late Embryogenesis Abundant
LRW	London Resin White
Lys	Lysine
Man	Mannose
MHDP	M-Hydroxyphenyl Method
NMR	Nuclear Magnetic Resonance
NSB	Non-Specific Binding

4-Ome-GlcA	4-methyl glucuronic acid
PDT	Poikilochlorophillous desiccation tolerant
PEG	Polyethylene Glycol
PG	Endopolygalacturonase
PGA	Polygalacturonic Acid
PIXE	Proton Induced X-Ray Emission
PM	Plasma Membrane
PME	Pectin Methylesterase
PO	Propylene Oxide
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
RH	Relative Humidity
Rha	Rhamnose
RIA	Radioimmunoassay
RWC	Relative Water Content
SEM	Scanning Electron Microscopy
Ser	Serine
SIMS	Secondary Ion Mass Spectrometry
SOD	Superoxide Dismutase
SPS	Sucrose-Phosphate-Synthase
SuSy	Sucrose Synthase
Ta	Tracer Activity
TBS	Tris Buffer Saline
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
TGN	Trans Golgi Network
Thr	Threonine
TLC	Thin Layer Chromatography
TTBS	Tween Tris Buffer Saline
Tyr	Tyrosine
Val	Valine
XET	Xyloglucan Endotransglycosylase
XG	XyloGlucan
Xyl	Xylose
Z	Zeatin
ZR	Zeatin Riboside

CHAPTER 1 :

LITTERATURE REVIEW

CHAPTER 1: LITERATURE REVIEW

Water is essential for the life of the plants. It is involved in major functions such as the maintenance of cell turgor, transport of solutes and nutrients or as a solvent. Water provides hydrophobic and hydrophilic interactions essential for macromolecular structure and is involved in all aspects of plant metabolism. Most of the plants can not survive even mild water deficit and are killed due to irreversible damages when water is lost (Bohnert *et al.,* 1995; Whitsitt *et al.,*1997).

Over one third of the earth's surface is considered to be arid or semi arid because it is subjected to permanent drought. Plants found in dry areas have developed astonishing ways to survive water deficit stress.

Three main strategies have been recognized whereby plants are able to cope with water stress: evasion, avoidance or tolerance (Levitt, 1980). Water deficit stress **evasion** occurs in plants which accomplish their cycle of growth and reproduction when moisture is available. Dry periods are survived by the production of desiccation tolerant seeds. Plants with water deficit stress **avoidance** strategy retard the loss of water and/or increase their capacity for water retention (Bewley and Krochko, 1982). Such is the case of many desert succulents such as Euphorbia or Cacti which can survive months and even years without water. Water-deficit **tolerance** is a completely different strategy in which the plant will be able to experience protoplasmic dehydration without permanent injury (Bewley and Krochko, 1982). Except for seeds and pollen, this last mechanism is extremely rare in higher plants. However, many lower plants and a few angiosperms have the capacity to withstand severe water loss or desiccation. These plants are called "resurrection plants".

1. THE RESURRECTION PLANTS

Definition

Desiccation tolerance is defined as the ability to revive following the loss of all the protoplasmic water (Bewley, 1979). Resurrection plants, also named poikilohydrous plants or poikiloxerophytes, (Bewley and Krochko, 1982) have this unique ability to revive from an air-dried-state (Gaff, 1971; Oliver, 1996). In this study these plants will be referred to desiccation tolerant or resurrection plants. In contrast, plants which do not survive desiccation are termed desiccation-sensitive, desiccation-intolerant or homoiohydrous plants (Bewley and Krochko, 1982).

Taxonomic Origins of Desiccation tolerant plants

Most of higher plants are able to produce structures such as seeds or pollen, which are tolerant of desiccation. Although a small number of plants possess desiccation tolerant vegetative tissues, these plants are widespread and found in most major classes of plants except gymnosperms (Gaff, 1971; Oliver, 1996). Desiccation tolerance is found in numerous bryophytes, fungi, algae, lichens, in approximately 60-70 species of ferns and ferns allies and at least 60 species of angiosperms (Bewley and Krochko, 1982). Most of the angiosperms are herbs or small shrubs rarely taller than 1 m high. Since it is so widely represented it has been proposed that desiccation tolerance has arisen several times during evolution (Bewley and Krochko, 1982).

Ecology and geographical distribution

The majority of resurrection plants were originally described in the 1970's (Gaff, 1971; Gaff and Ellis, 1974; Gaff and Churchill, 1976; Gaff and Latz, 1978). They are essentially found in seasonally arid subtropical and tropical regions. Most of the desiccation tolerant angiosperms as *Myrothamnus flabellifolius* (Child, 1960), *Xerophyta* spp or *Craterostigma* spp (Gaff, 1977) are native to Southern Africa (including South Africa, Namibia and Zimbabwe). Some of them as *Borya nitida* Labill. a species from the Liliaceae family occurs in Australia (Gaff and Churchill, 1976). Very few are found in

Southern America, Southern Europe and/or Mediterranean and none of them are found in Northern America (Bewley and Krochko, 1982).

Ecologically, desiccation-tolerant angiosperms are predominantly pioneer species colonizing rock surfaces and shallow soils. Most of the angiosperms are growing in full sunlight and are thus also subjected to the drying effect and high light intensities of the sun. In contrast a lot of ferns are more likely found in shaded area as in rock crevices (Gaff, 1977).

Water deficit stress and mechanisms to overcome desiccation.

During dehydration, desiccation tolerant plants have to face numerous stresses as the water is lost from the cells (Figure 1). Stress also occurs on rehydration when the water rushes into the cells. The stress that resurrection plants have to overcome in order to survive desiccation can be classified into 3 main categories:

1) oxidative stress related to the metabolism disruption (Seel *et al.*, 1992 a,b; Smirnoff, 1993; Farrant, 2000)
2) destabilization or loss of membrane integrity (Vertucci and Farrant, 1995)
3) mechanical stress associated with loss of turgor (Iljin, 1957).

The mechanisms developed by resurrection plants to survive desiccation often differ between lower plants and angiosperms. Lower plants more rely on a repairing system whereas angiosperms adopt a strategy of protections to prevent damage (Farrant, 2000). Although some comparison might be done with mosses, this literature review will mainly focus on angiosperm mechanisms.

Oxidative stress

Free radical formation and its effects on sensitive cells.

As the plant dries, its metabolism is severely altered (Bewley and Krochko, 1982; Gaff, 1989; Schwab *et al.*, 1989; Tuba *et al.*, 1996 a,b). Photosynthesis which is particularly disrupted by water stress declines even under mild water stress (Bradford and Hsiao, 1982; Smirnoff, 1993) whereas respiration rates decline only at low water content (Bewley and Krochko, 1982; Seel *et al.*, 1992 a; Tuba *et al.*, 1998; Farrant, 2000).

Interruption of the metabolism due to water stress causes a number of perturbations, the most critical being the oxidative stress. Oxidative stress is the result of drying-induced disruption of the electron transport which causes oxygen radical free production (Figure 2). When desiccation occurs in the light, there is an active production of singlet oxygen by transfer of excitation energy from chlorophyll to oxygen. Since this energy cannot be dissipated *via* photosynthetic pathways it is used to photo-reduce oxygen, initiating the free radical generating process (Seel *et al*, 1992 a,b; Smirnoff, 1993). A number of biological oxidations, both enzymatic and spontaneous, generate the free superoxide radical ($O_2^{\cdot-}$) which is cytotoxic. $O_2^{\cdot-}$ can react with H_2O_2 to produce singlet oxygen and the hydroxyl radical ($OH^{\cdot}$) with a high potential for oxidation (see for reviews Bewley and Krochko, 1982; Leopold, 1990; Winston, 1990). This in turn results in damages for nucleic acids, polysaccharides, proteins and membrane lipids. A loss of proteins, chlorophyll and carotenoids (Seel *et al.*, 1992 a) and an increased in the rate of lipid peroxidation has been commonly noticed in desiccation-sensitive tissues (Dhindsa and Matowe, 1981; Seel *et al.*, 1992 a; Smirnoff, 1993). Light-induced oxidative damages of the photosynthetic apparatus occurs in desiccation-sensitive plants but not in desiccation-tolerant plants. Singh *et al.* (1984) using ^{31}P NMR and ultrastructural analyses suggested that, even at very low water content, the membranes of the desiccation tolerant moss *Tortula ruralis* retained their phospholipid bilayers.

How resurrection plants cope with oxidative stress

Plants prevent excess light energy absorption by different strategies: leaf movements, the production of pigments and/or production of quenching molecules and enzymes.

In desiccation tolerant plants, photosynthesis activity declines at relatively high water contents (Tuba *et al.*, 1998; Farrant *et al.*, 1999; Farrant, 2000) whereas photosynthesis is maintained until low relative water content in desiccation-sensitive species (Farrant and Sherwin, 1997; Sherwin, 1995; Walters *et al.*, 2001). It has been suggested that this shut down in metabolism is a way for resurrection plants of protecting membranes from photo-oxidation (Farrant, 2000; Koonjul *et al.*, 2000). Mechanisms involved in this phenomenon vary among plant species but it has been hypothesised that, for angiosperm

resurrection plants, these mechanisms are more preventive during drying than repairing mechanisms upon rehydration (Oliver and Bewley, 1997; Oliver *et al.*, 1998).

- The poikilochlorophyllous desiccation tolerant plants (PDT)

Some plants like *Xerophyta* species lose their chlorophyll and thylakoid membranes are dismantle during desiccation. These resurrection plants are termed poikilochlorophyllous plants (Hetherington *et al.*, 1982; Tuba *et al.*, 1994, 1996 b; Sherwin and Farrant, 1996). Many of the monocotyledons resurrection plants have this unique strategy (Gaff, 1977). The photosynthetic system in PDT plants has to be resynthesized upon rehydration which retards recovery rate. Dace *et al.*, (1998) have shown in *Xerophyta humilis* that while these degradative processes are occurring, messenger RNA encoding for their resynthesis, is transcribed from the genome. However, these mRNA's are prevented from being translated into chlorophyll and the photosynthetic enzymes until the plants are rehydrated. These results suggest that the breakdown of the photosynthetic apparatus is not only a consequence of drying but it is rather a well-controlled process.

- The homoiochlorophyllous desiccation tolerant plants (HDT).

Such plants retain chlorophyll and maintain their photosynthetic apparatus during dehydration (Sherwin and Farrant, 1998; Farrant *et al.*, 1999; Farrant, 2000; Koonjul *et al.*, 2000). They tend to undergo morphological changes during drying to protect their tissues from oxidative stress. For example, leaves of the shrub *Myrothamnus flabellifolius* fold vertically against the stem shading abaxial structures from light, whereas *Craterostigma* species are characterised by a folding of their rosette leaves such that only the abaxial surfaces of the older outermost leaves are exposed to sunlight (Gaff, 1977; Sherwin and Farrant, 1998; Farrant, 2000). During drying, all inner leaves are protected from light and thus photo-oxidation.

- Anti-oxidant systems.

Anti-oxidant systems can be classified into 3 categories:

1) water-soluble reductants: ascorbate, urate, catechol, glutathione 2) fat soluble vitamins: carotene 3) enzymatic anti-oxidant: glutathione peroxidase, catalase, superoxide dismutase (SOD).

Cells exposed to direct sunlight of both HDT and PDT plants accumulate pigments as carotenoids or anthocyanins when drying (Sherwin and Farrant, 1998; Farrant, 2000). It has been hypothesised that for HDT plants as *Craterostigma* species, these pigments could act as a "sun-screen" masking the chlorophyll from excessive radiation (Gaff, 1989; Sherwin and Farrant, 1998). Furthermore by acting as antioxidants, they can minimise damage from free radicals which are produced (Smirnoff, 1993; Farrant, 2000). Carotenoids are efficient singlet oxygen scavengers (Smirnoff, 1993). Seel *et al.*, (1992 a) have suggested that the lack of photobleaching in *T.ruraliformia* could be due to its high content of carotenoids compared to chlorophyll.

Enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (AP) and glutathione reductase (GR) are known to regenerate antioxydant (ascorbate, glutathione) and also accumulate in leaves of these plants (Table 1) during drying (Ingram and Bartels, 1996; Sherwin and Farrant, 1998). These antioxydant systems are complex and varied among plant species. For example, in both hydrated and dehydrated *T.ruraliformia*, Seel *et al.* (1992 b) have shown SOD and catalase activities were higher than in the sensitive specie *Dicranella palustris*. Light or dark desiccation in *T.ruraliformia* lead to an increase in SOD activity but does not affect peroxydase or catalase activities.

Based on chlorophyll fluorescence studies, correlation has been made between desiccation and UV-B tolerance in the desiccation tolerant moss *T.ruralis*. This plant does not respond to UV-B stress. The ability to withstand both stresses involves similar protection mechanisms (Takács *et al.*, 1999).

Maintenance of integrity of the subcellular milieu

Membranes and macromolecules are held in active configuration due to the fact that they are in aqueous solution. Removal of water results in their loss of integration (Koster and Leopold, 1988; Caffrey *et al.*, 1988). Furthermore the loss of water leads to considerable

increase in ions concentrations which can result in considerable metabolic perturbations and also membrane damages in sensitive plants (Gaff, 1989).

Solute leakage measurements during dehydration and rehydration has been shown to be a useful tool to estimate the extent of lesions in membranes following a desiccation stress (Leopold *et al.*, 1981). In desiccation sensitive species the extent of leakage increases with the extent of desiccation whereas the leakage of desiccation tolerant species does not increase on drying (Dace *et al.*, 1998).

The ability of resurrection plants to protect the integrity of their membrane is thought to be due to the accumulation of "compatible solutes" such as sugars or amino acids (Tymms and Gaff, 1979; Crowe *et al.*, 1993; Bianchi *et al.*, 1991, 1993; Dure, 1993; Schneider *et al.*, 1993; Ingram and Bartels, 1996).

Sugars as protectants of membranes and cytoplasm.

Accumulation of polyols such as mannitol, sorbitol, myo-inositol and its methylated derivatives is often associated with tolerance to drought and/or salinity in many species including bacteria, yeast, marine algae, higher plants and animals (Figure 3, reviewed by Bohnert *et al.*, 1995).

During drought stress all the resurrection plants have been shown to accumulate carbohydrates such as trehalose or sucrose (Bianchi *et al.*, 1991; Drennan *et al.*, 1993). Trehalose occurs predominantly in desiccation-tolerant lower organisms including some lower vascular plants whereas sucrose is found in higher plants (Kaiser, 1985; Drennan *et al.*, 1993; Ghasempour *et al.*, 1998; Whittaker *et al.*, 2001). One hypothesis to explain the role of sugars in desiccation tolerance is the water replacement hypothesis. Hydroxyl groups of sugars could act as a substitute for water then maintaining the required hydrophilic interactions for membrane and proteins stabilisation (Crowe *et al.*, 1993; Hartung *et al.*, 1998). The second hypothesis is the glass formation. The sugars could protect the cytoplasm by forming a glass structure, a supersaturated liquid with the mechanical properties of a solid. This is proposed to prevent denaturation of proteins, protect against pH modifications, restrict the molecular diffusion and minimise free radical damages (for reviews see Leopold, 1990; Ingram and Bartels, 1996).

Different resurrection plants appear to accumulate different types and quantities of soluble carbohydrate. For example, in *Boea hygroscopica,* all sugars except sucrose, decline during dehydration and sucrose levels increase significantly (Bianchi *et al.*, 1991; Marinone Albini *et al.*, 1999). High amounts of galactinol and oligosaccharides of the raffinose family have been reported in leaves of this species. It has been proposed that these sugars might have a role in restoring the pre-drying functions upon rehydration (Marinone Albini *et al.*, 1999). The leaves of *Myrothamnus flabellifolius* contain high amounts of trehalose (which is exceptional in higher plants) and sucrose. The amount of these two sugars is considerably increased during dehydration, suggesting they are probably involved in desiccation tolerance (Bianchi *et al.*, 1993; Drennan *et al.*, 1993). Bianchi *et al.* (1993) indicated that glucopyranosyl-β-glycerol may also act as osmoprotectant in *Myrothamnus flabellifolius* leaves.

In *C.Plantagineum*, an extremely high concentration of the unusual C8 sugar 2-octulose occurs in hydrated leaves (Bianchi *et al.*, 1992). During drying, the level of this sugar declines and this is inversely proportional to the accumulation of sucrose (Ingram and Bartels, 1996). These authors have proposed that 2-octulose is converted into sucrose upon drying. Although both the Glc and Fuc configurations are contained in the octulose molecule, the pathway of conversion remains unclear. However Norwood *et al.* (1999) have shown that the amount of 2-octulose is insufficient to make it the major source of carbon for sucrose synthesis during drying. These authors suggest that sucrose is more likely to be mobilised from root reserves or even from the older leaves of plants which do not survive desiccation.

Dehydrins and Late Embryogenesis Abundant Proteins (LEA proteins).

Dehydrins or LEA proteins have been shown to accumulate in plants in response to water stress, reduced temperatures and/or salinity and have been identified in resurrection plants during drying (Bartels *et al.*, 1993; Bray, 1993; Reynolds and Bewley, 1993; Michel *et al.*, 1994; Velasco *et al.*, 1994; Alamillo and Bartels, 1996; Close, 1996; Bockel *et al.*, 1998). The exact role of these proteins in desiccation tolerance is still unclear. Dehydrins are thought to be mainly structure stabilizers and possess chaperonin-like properties. Dure *et al.*, (1989) proposed that the hydroxyl groups on the surface of

these polypeptides might be a substitute for water, so maintaining the integrity of macromolecules and membranes. Charged amino acids in such proteins serve to neutralise the increasing concentration of ions during desiccation (Dure, 1993). Schneider *et al.*, (1993) reported the presence of three desiccation-related proteins in *C.plantagineum* localised in the cytosol and two others in the chloroplast, one in the stroma, the other one associated with thylakoids membranes. Recently Mundree *et al.* (2000) have reported the presence of dehydrin-like proteins in the resurrection plant *Xerophyta Viscosa*.

One of the earliest response to stress by plants is the accumulation of the plant hormone abscissic acid (ABA). ABA is playing a major role in desiccation and more especially by regulating genes expression encoding for LEA proteins and dehydrin (Gaff and Loveys, 1984; Bartels *et al.*, 1990; Piatowski *et al.*, 1990; Reynolds and Bewley, 1993; Michel *et al.*, 1994; Nelson *et al.*, 1994). However, the role of plant growth regulators in desiccation tolerance will be further detailed in chapter 6.

Mechanical stress

One of the major stress plants have to overcome in order to survive desiccation is the mechanical stress occurring as the water is lost from the cells (Iljin, 1957). A very similar stress happens upon rehydration when the water rushes into the cells. In hydrated state, plant cells are characterised by a large vacuole. When plants dry, the cytoplasm shrinks creating tensions between the plasmalemma and the more rigid cell wall. This can results in a tearing of the plasmalemma and so irreversible damage to the cells. Microscopical studies have shown that on drying a fragmentation of the main vacuole into several small vacuoles occurs in many of the resurrection plants (Gaff *et al.*, 1976 b; Hallam and Luff, 1980 a, b; Farrant and Sherwin, 1997). It is proposed that accumulation of osmolytes like sugars in the vacuoles during drying might be a way to preserve a normal osmotic pressure in the cells and prevent mechanical stress (Bohnert *et al.*, 1995; Farrant, 2000). For some of the resurrection plants such *Selaginella lepidophylla* and the *Craterostigma* species, upon drying, the cell walls folds in along with the cell contents becoming highly convoluted (Hallam and Luff, 1980 a, b; Sherwin, 1995; Thomson and Platt, 1997). When the plant is rehydrated, cells return to their original volume without apparent

injury. It has been proposed by Farrant and Sherwin (1997) that the folding of the cell wall could be a strategy developed by the plants to avoid the tearing of the plasmalemma from the cell wall during dehydration and maintain its integrity. If this is the case, it would be a more a controlled phenomenon rather than a simple collapse of the cells. **As no studies have been carried out concerning the cell wall of resurrection plants, its role in desiccation tolerance remains speculative. Considering the various and major role of cell walls in the life of the plants, it is of great interest to learn more about its possible role in desiccation tolerance.**

2. THE CELL WALL

Plant cell walls are involved in major functions. Cell walls confer the shape to the cell, are responsible for the maintainance of osmotic pressure, controls the cell expansion and growth (Brett and Waldron, 1990; Mc Cann and Roberts, 1991; Knox, 1997; Pennel, 1998). It is also involved in cell to cell adhesion and cell separation. The cell wall is not simply a box surrounding the cell. It is a highly dynamic cell component in which the composition and structure is strictly regulated during the plant development (Carpita, 1984; Lorences *et al.*, 1987; Arribas *et al.*, 1991; Piro and Dalessandro, 1998). Cell wall composition can be modified upon cold acclimation (Weiser *et al.*, 1990; Fujikawa *et al.*, 1999; Kubacka-Zebalska and Kacperska, 1999; Stefanowska *et al.*, 1999), osmotic (Hohl and Schopfer, 1995; Wakabayashi *et al.*, 1997; Marshall *et al.*, 1999) or salinity stress (Iraki *et al.*, 1989 a,b,c ; Neumann, 1993; Neumann *et al.*, 1994; Snir and Neumann, 1997; Murai and Yoshida, 1998), drought (Zwiazek, 1991), wounding (Cardemil and Riquelme, 1991) or pathogen attacks (Boudart *et al.*, 1998). The functional properties of cell walls are determined both by their chemical composition, the linkages between the polymers and by their three-dimensional arrangement of these components. In order to understand the role of the cell wall in stress such as desiccation it is essential to know the composition and the structure of the walls. The following section will give an overview of the structure and composition of the cell wall and present some examples of cell wall modifications during plant development.

Structure and composition of the cell wall

Primary cell walls are composed of cellulose microfibrils embedded in an amorphous matrix consisting primarily of pectins and hemicelluloses (Table 2). Additional networks of structural proteins (*e.g* extensins, arabinogalactan proteins) and enzymes are also present. Some phenolics are found although in minor amounts. The development of new tools and techniques (e.g. immunocytochemistry) has shown that the matrix composition varies among plant species, tissues or during the growth of the plant (McCann and Roberts, 1996).

The cellulose framework

Cellulose consists of unbranched β1,4 glucan chains (Figure 4) of at least 15 000 sugar residues per molecule (Brett and Waldron, 1990; Rose and Bennett, 1999). Cellulose is organized in long and thin microfibrils and can be distinguished from the matrix phase by its high degree of crystallinity and its relative homogeneous composition (Vian and Roland, 1991; Delmer and Amor, 1995; Delmer, 1999). Cellulose chains are held together *via* both intramolecular and intermolecular hydrogen bonds.

Cellulose microfibrils provide the cell wall with mechanical support and tensile strength. A passive reorientation of cellulose microfibrils has been observed by electron microscopy in elongating cells (Iwata and Hogetsu, 1989; Wolters-Arts and Sassen, 1991). Cellulose is synthesized by an enzyme, cellulose synthase, which is believed to be associated with a sucrose synthase (SuSy) complex in the plasma membrane (Figure 5).

The cell wall matrix

The non-crystalline matrix phase of the cell wall consists largely of a variety of non-cellulosic polysaccharides, proteins and phenolic compounds. In contrast with the cellulose phase, the matrix is highly heterogeneous and its composition depends on the species, the organs, the cell type and the stage of plant development (Brett and Waldron, 1990).

Pectins

Pectins play a major role in the cell wall hydration, cell adhesion, wall porosity and plasticity during growth (Carpita and Gibeaut, 1993).

Pectins are also involved in cell signalling, overall charge of the cell wall and are involved in the plant defence mechanisms. These numerous functions can be correlated to the complexity and variability in the carbohydrate composition of the pectins. Pectins are heterogeneous polysaccharides with a highly hydrated structure. Homogalacturonans consists of D-galacturonic acid (GalA) chains linked *via* α- (1→4) bonds (Figure 6). Pectins are organised in blocks with "smooth parts" comprised of long chains of unbranched α-D GalA linking to "hairy" blocks containing rhamnose (Rha) and GalA. Many of the GalA residues might be methyl esterified or acetyl esterified on their hydroxyl groups. In the Rhamnogalacturonan I (RG-I), the ratio of GalA to Rha is about 1:1 and forming an alternating regular sequence (Brett and Waldron, 1990). Numerous side chains containing sugars such as arabinose (Ara) and galactose (Gal) are carried by the Rha residues (Figure 7).

The Rhamnogalacturonan II (RG-II) is a complex pectic polysaccharide and its structure is conserved in the primary walls of all higher plants (Figure 8). RG-II molecule has homogalacturonan backbone with side chain containing unusual residues such as 3-deoxy-manno-octulosonic acid (KDO), aceric acid (AceA), apiose (Api) (Fleisher *et al.*, 1999; Hervé Du Penhoat *et al.*, 1999). RG-II can dimerize through a borate diester cross-link. RG-II is also present in fruit-derived juice and wines (Vidal *et al.*, 2000).

A RG1-like pectic polysaccharide (Figure 9) with anti-ulcer properties has been detected in the root of *Bupleuran falcatum* (Sakurai *et al.*, 1996). This polysaccharide contains several side chains rich in Ara, Gal and methyl esterified glucuronic acid (4-Ome-GlcA). Some primary cell wall might contain pectins whose side chains are rich in β1,4-linked galactans (John *et al.*, 1997). There are often branched blocks of more than one type in pectins from one cell wall or even within a single pectin molecule (Jarvis, 1984). Ester groups, acetyl substituents are known to make the cell wall structure more flexible (Jarvis, 1984).

Pectins are also known to have a composition which is dependent on the cell types, the plant species and it can be modified with the age of the plant (Freshour *et al.*, 1996; Vicré *et al.*, 1998 a; Mogami *et al.*, 1999).

Hemicelluloses

Hemicelluloses are a class of components including xylans, mannans, glucomannans, galactomans and xyloglucans (Figure 10). Depending of the cell type, one of the hemicellulose predominates whereas the others are found in lesser quantities.

Xylans are made up of a backbone of β(1→4) xylose (Xyl) substituted by 4-0-methylglucuronic acid (4-O-Me-β-GlcA), arabinose (Ara) and acetyl esters (Figure 10a). Side chains in xylans are not distributed following a regular pattern. Glucomannans are found in large quantities in the secondary wall of gymnosperms. They consist of a chain of β(1→4) linked Glc and mannose (Man) in a ratio of about 1:3 (Figure 10b). Mannans and galactomannans are essentially found in the cell walls of some seed endosperms. They are known to play a role in storage nutrients providing a source of soluble carbohydrates to the young seedlings during germination. They consist of a backbone of β(1→4) Man where side chains containing Gal are linked (Figure 10c and d). The numerous Gal side chains of the galactomannans made the molecule hydrophilic and play a role in water retention. It facilitates the water uptake when the seed is in a moist environment and protect the embryo from desiccation when the seed is under water stress (Brett and Waldron, 1990).

Xyloglucan (XG) is the major hemicellulose found in the primary wall of dicotyledonous plants (Roberts, 1990). As it is the case for cellulose, XG has a backbone of β1,4 glucans but possesses side chains varying among the species (Hayashi and Maclachlan, 1984; Brummel and Maclachlan, 1989; Edashige and Ishii, 1998; Kakegawa *et al.*, 1998). Mature xyloglucans of dicot plants possess three xyloses linked out of four sequentially linked glucans. At regular intervals, the trisaccharide xylose, galactose and fucose are linked to the backbone (Figure 10e). Side chains consisting of galactosyl-xylose disaccharide have been found in seeds. Xyloglucans from monocotyledons are usually lacking fucose and are less substitued with xylose and galactose than dicots (Brummel and Maclachlan, 1989). Partial hydrolysis of pea, sycamore, soybean and mung bean

xyloglucan revealed the presence of a nonasaccharide (Glc4. Xyl3. Gal2. Fuc) and a heptasaccharide (Glc4. Xyl3) unit. In mung bean small amounts of the decasaccharide (Glc4. Xyl3. Gal.2. Fuc) was also found. Some XG serve as "temporary" storage reserve of sugars in cotyledons and their structure differs from primary cell wall XG by being nonfucosylated (Desveaux *et al.*, 1998).

Proteins

Although proteins are not a major component of cell wall structure, a number of structural proteins or enzymes are present. Most of the structural proteins are glycoproteins which belong to the hydroxyproline-rich glycoprotein (HRGPs) class.

Extensins (Figure 11) represent the best-known family of the HRGPs class (Fry, 1991). Extensins are encoded by multigene families and have cell type-specific patterns. They are characterized by a high content of hydroxyproline (Hyp) which generally occurs together with serine (Ser) to give the characteristic sequence Ser-Hyp4. A combination of others amino acids such as valine, tyrosine, lysine and histidine are also very common (Stafstrom and Staehelin, 1987). Due to their high level of proline, extensins are basic proteins. Most of the hydroxyproline residues are glycosylated with arabinose (Ara) and some of the serine residue are glycosylated with galactose (Gal) (Showalter, 1993). Carbohydrates consist of more than half of the mass of the glycoprotein with Ara (90% of total sugars) as the major sugar (Stafstrom and Staehelin, 1987). Van Holst and Varner, (1984) have shown that the carbohydrate moiety plays a role in the conformation of the peptide backbone by reinforcing its cohesion. Extensins are abundant in dicotyledons but have also been found in some graminaceous monocotyledons such as *Zea mays* (Kieliswekski *et al.*, 1990). However, during the desiccation phase of seed maturation, some irreversible changes in the cell wall conformation make extensin extraction and thus analysis impossible (Cassab and Varner, 1988). Extensins have been shown to be expressed under stress conditions especially wounding or pathogen attacks. Extensins have been thought to participate in the tensile strength of mechanical cells such as sclerenchyma cells (Cassab and Varner, 1988).

Expansins are another class of cell wall proteins. Their role in making the cell wall more flexible have been demonstrated during cell expansion and thus the growth of the plant.

These proteins act by disrupting hydrogen bonds within the cell wall matrix polymers (McQueen-Mason 1995, McQueen-Mason and Cosgrove, 1995; Civello *et al.*, 1999). To date, two families of expansins are identified, the α- and β- expansins (Cosgrove, 1999).

Several enzymes are associated with cell wall. Most of them play an important role in the modification of cell wall polymers *in muro* such as pectin-methylesterases (Goldberg *et al.*, 1992; Stephenson and Hawes, 1994); xyloglucan endotransglycosylases (XET) (Pritchard *et al.*, 1993; Potter and Fry, 1994; Antosiewicz *et al.*, 1997) endo and exo-β-D-glucanase (Huber and Nevins, 1981).

Arabinogalactan proteins (AGPs) are highly glycosytaled proteins found in all higher plants. Their protein content represents between 2 to 10% (wt/wt) and contain more than 90% (wt/wt) carbohydrate. The protein moiety is rich in Hydroxyproline (Hyp), Serine (Ser), Alanine (ala), Threonine (Thr), and Glycine (Gly) (Chasan, 1994). Carbohydrates, the major constituent of AGPs consist mostly in D-galactose and L-arabinose. The extent of glycosylation varies among the AGPs, this is reflected by the various molecular weight of AGPs. AGPs are localized on the plasmalemma, bound to the cell wall or soluble in the cell wall space (Fincher and Stone, 1983; Showalter, 1993; Majewska-Sawka and Nothnagel 2000). The specific interaction between AGPs and the Yariv reagent is the most used criteria for AGPs identification and isolation (Fincher and Stone, 1983; Cassab, 1986; Classen *et al.*, 2000). The functions of AGPs are still a matter of debate, however there is evidence that they are involved in many developmental processes. It is thought that AGPs have a role in cell communication, in the control of plant cell proliferation or as lubricants (Fincher and Stone, 1983; Kreuger and Van Holst, 1993; Schindler *et al.*, 1995 a; Yates *et al.*, 1996). Knox *et al.* (1991) have shown using immunocytochemistry a change in AGPs expression in root tissues during cell development and maturation. It has also be shown that AGPs could serve as developmental markers for future cell differentiation (Schindler *et al.*, 1995 a).

How the cell wall polymers interact to provide cell wall strength.

Cell wall polymers are held together by numerous and diverse linkages including covalent, ionic or hydrogen bounding. The nature of these linkages is highly complex and two main networks can be described, the pectic network and the cellulose/hemicellulose

network. Proteins also participate in the architecture of the cell wall by forming crosslinking between polymers.

The pectic network

Jarvis, (1982) using different techniques for the pectins extraction, has suggested that pectins are both ionically and covalently bonded.

In pectins, unbranched non esterified galacturonic blocks are bonded together via calcium ions forming the "egg-box" model (Figure12), (Jarvis, 1984; Goldberg *et al.*, 1996). In this structure, two galacturonan chains are held together in the twofold (2_1) helical conformation with calcium ions assuring a locked structure (figure 13a). When dehydration occurs, it has been suggested that there is a conformational transition to the right-handed (3_1) helical form (Figure 13b) (Goldberg *et al.*, 1996). Two chains form a primary structure. If calcium is in excess, this can lead to the formation of an aggregate with different layers (Jarvis, 1984). The presence of side chains or methyl groups makes the structure more flexible, and aggregation is avoided by preventing calcium bonding. The size of the aggregates is related to the quantity of calcium available. The strength of the binding depends on the length of uninterrupted pectate segments and if there is enough calcium present to link the structure. Linear molecules with low degree of esterification and high calcium concentration may be expected to form a structure with the greatest rigidity (Goldberg *et al.*, 1996). Roy *et al.*, (1994) have concluded that an exogenous excess of calcium in parenchyma cells of apple modifies the anionic sites in the cell wall and leads to the formation of calcium bridges. The softening of the fruit is prevented due to a strengthening of the cell walls. Interactions between pectin chains are complex and depend on the pH or the degree of esterification (Vreeland, 1989).

Pectins contain ferulic and coumaric acids which are subjected to some oxidative coupling leading to the formation of dimers like diferulic acids (DFA) (Fry, 1986). Through this coupling, pectin molecules become cross-linked in the cell wall via diferulate bridges. This reaction is catalysed within the cell walls by extracellular peroxydase (Figure 14). In spinach cell walls, Fry, (1983) has demonstrated that the principal feruloylation sites are the arabinopyranose termini and the galactopyranose termini of the pectic polysaccharides. Wall bound DFA have been shown to confer

strength to the cell wall (Wakabayashi *et al.*, 1997). An increase in the amounts of DFA has been correlated to a decrease in the cell wall extensibility caused by ageing or light irradiance (Kamisaka *et al.*, 1990). Direct ester linkages involving the carboxyl groups of uronic acid residues in a pectin and the hydroxyl group of a neighbouring pectin chain have also been proposed (Iiyama *et al.*, 1994). Borate cross-links the apiose residues of RG-II and produce dimers. Recent studies suggested that borate, calcium and RG-II have a major role in influencing the porosity of the cell wall (Fleischer *et al.*, 1999; Hervé du Penhoat *et al.*, 1999).

The cellulose- hemicellulose network

Xyloglucan plays a key role in the architecture of the cell wall by linking together cellulose microfibrils (Brummel and Maclachlan, 1989). Xyloglucans are associated with the cellulose microfibrils by hydrogen bonds (Figure 15) in a pH-dependant manner (Hayashi *et al.*, 1987; McCann and Roberts, 1991). It is possible that the ends of an individual xyloglucan molecule could be hydrogen- bonded to two different microfibrils (Fry, 1989 a). Xyloglucan is important in regulating the spacing and the maintainance of cellulose microfibrils. The association between xyloglucan and cellulose results in such a strong network such that only concentrated alkali can extract the xyloglucan from the cellulose framework (Brummel and Maclachlan, 1989). This network is responsible for the structure and rigidity of the cell wall. Whitney *et al.*, (1999) using tensile testing methods demonstrated that xyloglucan/cellulose network provides a balance for extensibility and strength which is not possible with cellulose microfibrils alone. Ferulic and coumaric residues also occur in gluco-arabino xylans the major hemicellulosic polysaccharides of monocotyledons (Schooneveld-Bergmans *et al.*, 1999).

The interactions between the pectin and cellulose-hemicellulose networks

It has been suggested that pectins form an independent network from the cellulose-hemicellulose network (McCann and Roberts, 1991, 1996). However, many interactions may occur between the two networks making the cell wall a complex architecture (Figure 16). Some of the pectin molecules can be linked to the xyloglucan chain (Jarvis, 1984).

Chambat *et al.*, (1984) suggested that glycosidic linkages occur between xyloglucan and pectins but Fry, (1986) has hypothesised non covalent linkages between these molecules. Brady *et al.*, (1998) have demonstrated the presence of pulcherosine, an oxidatively coupled trimer of tyrosine, in cell wall proteins of tomato. Pulcherosine could be involved in interpolypeptide bridge contributing to the cross linking of the polypeptide chains. It has been shown that extensins can be ionically linked to the acidic polysaccharides (Brady *et al.*, 1998).

The cell wall is a dynamic structure

Cell walls as, mentioned previously, are very dynamic compartments. Cell wall composition is subjected to many modifications in their chemistry or in the linkages influencing the strength of the cell wall and its overall architectural organisation. Such modifications can occur in events as growth and development. The following section presents some examples and illustrates how the cell wall can cope with such events.

Plant growth

Cell growth consists of an irreversible increase in volume occurring by expansion or elongation. Tugor pressure and cell wall loosening are major factors regulating the cell elongation (Cosgrove, 1997). An extensive turnover of cell wall polysaccharides has been associated with the cell growth (Lorences *et al.*, 1987).

During cell growth the existing cell wall has to undergo major modification in order to incorporate new material and to allow the water uptake from the protoplast. At this stage cell wall is characterised by a high extensibility to permit microfibril separation and insertion of new wall polymers.

A transverse reorientation of the cellulose microfibrils has been observed during cell elongation (Pritchard *et al.*, 1993). It is accompanied by a reorientation of others matrix component but in a tissue specific manner (McCann and Roberts, 1994). Possible rearrangements of microfibrils within and between layers require enzymes that act on the xyloglucans that cross-link them into the network. Two proteins acting on the cellulose/xyloglucan network have been characterised. The first one is the xyloglucan endotransglycosylase (XET) able to cleave xyloglucans allowing movement of the

microfibrils and then catalyse the bounding of the XG fragments to maintain wall strength (Potter and Fry, 1994; Campbell and Braam, 1999). The second protein, expansin, could also be involved in displacing xyloglucans from cellulose microfibrils by disrupting the hydrogen bounding (Cosgrove, 1997). One of the major changes concerning the pectin composition during growth consists of the modification of their degree of esterification. This has been identified using mainly FTIR spectroscopy. Lorences and Zarra, (1986) have studied growth in a gymnosperm species *Pinus pinaster*. Gas liquid chromatography study indicated that a glucose rich polysaccharide was involved during growth and a partial degradation of xyloglucan has been suggested.

Fruit ripening and cell wall polymers

Fruit ripening is a highly controlled process requiring multiple factors such as the age, environmental signals and endogenous hormones (Civello *et al.*, 1999). Ripening is accompanied by a loss of flesh firmness mainly due to the dissolution of the cell wall. Pectic polysaccharides and hemicelluloses are actively involved in this process (McCollum *et al.*, 1989; Redgwell et *al.*, 1990; Chin *et al.*, 1999). Redgwell *et al.*, (1992) have noticed a considerable solubilisation of the pectic polymers during ripening of kiwifruit. After solubilisation these polysaccharides are subjected to some degradation such as depolymerisation and degalactosidation. It has been thought that the cell wall breakdown during ripening is a consequence of the activity of polysaccharide hydrolases such as the endopolygalacturonase (PG) or β-galactosidase (Crookes and Grierson, 1983; Redgwell *et al.* 1992; Chin *et al.*, 1999; Rose and Bennett, 1999). Mc Collum *et al.*, (1989) have shown a large increase in solubility and decrease in molecular weight of polyuronides during the ripening of muskmelon fruit. However these modifications were not due to a polygalacturonase activity. A decrease in the molecular size of hemicellulosic components was also found and this decrease was correlated with a large loss in galactose and glucose content. Expression of expansin genes have been shown to occur in ripening strawberry fruits and could be involved in the cell wall disassembly (Civello *et al.*, 1999). Almeida and Huber, (1999) proposed that the decline in apoplastic pH and the increase in ionic strength during fruit ripening could be the factor regulating the cell wall hydrolases. The synthesis of new cell wall polymers has been described by

Greve and Labavitch, (1991) in ripening tomato pericarp tissue. A gas chromatographic-mass spectrometric technique utilising D-[U-13C] glucose indicated an increased incorporation in xylosyl and mannosyl residues into hemicellulosic cell wall fraction during ripening. The synthesis of certain polymers and their incorporation into the cell wall could induce its relaxation and its weakening.

Stress responses

Changes in the composition of the cell wall polysaccharides and proteins has been shown to be an important event of plant adaptive responses to many stresses including dehydration (Iraki *et al.*, 1989 a,b,c). It has been reported that cell wall polysaccharides and proteins alterations occur in cold acclimated pea seedlings (Weiser *et al.*, 1990). Accumulation of extensin mRNA and incorporation of extensin into the cell wall is suggested to increase cell wall rigidity which is proposed to be necessary to prevent a cell collapse due to cold-associated dehydration (Weiser *et al.*, 1990; Murai and Yoshida, 1998). In cold acclimated leaves of winter oilseed rape, exposure to low temperature results in an increase especially in non-covalently bound pectins. The galactose, arabinose and glucose content in these pectins increased whereas a high level of galactose and arabinose was detected in the hemicellulosic fraction. Kubacka-Zebalska and Kacperska, (1999) suggested that the enrichment of hemicellulose in galacatose and arabinose might be explained by a linkage of these components to the xylosyl units of the xyloglucan. If this is the case xyloglucan would play a role in cold acclimation of leaves of winter oilseed rape. The low temperatures bring important modifications in cell wall contents. The response to chilling depends on whether the plant has been subjected to temperature above or below 0°C. In freezing adaptations, the cell walls of oilseed rape are different in their content of bound proteins, the amount of xylose and glucose of the hemicellulosic component declined. These adaptations could be explained by an increase in cell wall extensibility. In drought-affected spruce needles, a decrease in pectin content and an increase in hemicellulose level were observed and proposed to play a role in cell tolerance to dehydration (Zwiazek, 1991). Iraki *et al.*, (1989 b) have shown that adaptation of tobacco cells to water and saline stress resulted in the formation of a loosely bound shell of polygalacturonic acid and rhamnogalacturonan. Marshall *et al.*, (1999)

have shown that roots of jack pine subjected to osmotic stress had adjusted plasticity of the cell wall to maintain the cell turgor. This process requires synthesis and incorporation of cell wall proteins as well as an oxidative insolubilization of these proteins within the wall. Overexpression of peroxidase was correlated to salt tolerance of tomato cells growing in suspension culture (Medina *et al.*, 1999), to the improved germination under osmotic stress of tobacco plants (Amaya *et al.*, 1999) or in drought response of *Lolium temulentum* (Bacon *et al.*, 1997). The variability in the modifications of cell wall components suggest a different role of cell walls in plant adjustement to water or to cold stress and thus to dehydration (Iraki *et al.*, 1989 a,b; Kubacka-Zebalska and Kacperska,1999; Stefanowska et al. 1999). Depending on the nature of the stress plants could react either by a relaxation or an increase in the rigidity of its wall.

3. *CRATEROSTIGMA* SPECIES : A MODEL OF CHOICE TO UNDERSTAND THE MECHANISM OF DESICCATION TOLERANCE.

The production of transgenic crop plants with desiccation tolerant properties would be particularly useful for cultivation in semi arid areas in Africa. Although some attempts to get transgenic plants with drought tolerance properties have already been realised, only minor improvements were obtained (Table 3). The production of these plants is highly complex as desiccation tolerance is not conferred by a single gene but by gene products from different pathways (Ingram and Bartels, 1996). In order to obtain such plants, the complex and various mechanisms allowing the resurrection plants to withstand desiccation must be fully understood.

The resurrection plant *C.plantagineum* has been largely studied as an experimental model for understanding the molecular basis of desiccation tolerance (Piatkowski *et al.*, 1990; Bianchi *et al.*, 1992; Schneider *et al.*, 1993; Velasco *et al.*, 1998; Kleines *et al.*, 1999). The following part will summarise the previous works performed on *Craterostigma* species.

Molecular and biochemical studies revealed accumulation of transcripts and proteins upon dehydration or rehydration. The cellular processes involved include major changes in the carbohydrate composition during the dehydration/rehydration cycle (Bianchi *et al*, 1992; Bernacchia *et al.*, 1996) and an accumulation of desiccation-specific proteins in dried and ABA-treated tissues (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990; Nelson *et al.*, 1994; Velasco *et al.*, 1994; Alamillo and Bartels, 1996).

Sugar metabolism play a major role in desiccation tolerance of *C.plantagineum* (Bianchi *et al.*, 1992; Ingram *et al.*, 1997; Norwood *et al.*, 1999, 2000). This resurrection plant accumulates large amount of octulose (a rare sugar in higher plant) in fully hydrated leaves tissues whereas upon dehydration octulose level decreases and sucrose concentration increases up to 90% of the total sugar content (Bianchi *et al.*, 1992). It would appear that *Craterostigma* species have evolved a capacity to accumulate sucrose very rapidly from carbohydrate sources already present in the leaf. In roots there is no conversion from 2-octulose to sucrose during dehydration but large quantities of stachyose are presents. In more recent studies, the activity of enzymes implied in sugar metabolism was investigated according to the drying rate (Ingram *et al.* 1997, Kleines *et al.*, 1999; Norwood *et al.*, 1999, 2000). The activity of sucrose-phosphate-synthase (SPS), a key enzyme in the regulation of sucrose metabolism responsible for the synthesis of sucrose 6-phosphate is increasing in leaves and roots upon dehydration. These observations suggest a role for SPS in the sugar interconversions related to desiccation in *C.plantagineum* (Ingram *et al.*, 1997). Sucrose synthase (SS) transcripts and protein levels are also regulated by dehydration and rehydration. The accumulation of this enzyme involved in the breakdown of sucrose could be explain by an increased glycolytic demand during stress and early phases of recovery in the plant (Kleines *et al.*, 1999).

A number of desiccation-related transcripts have been cloned, they are highly expressed in dried leaves but also in callus treated by ABA (Bartels *et al.*, 1990, Piatkowski *et al.*, 1990). Accumulation of both cytosolic and chloroplastic proteins upon dehydration have been reported as protectant for the cytoplasm and chloroplast structures during desiccation stress (Schneider *et al.*, 1993).

C.wilmsii, a species similar to *C.plantagineum* has been less studied although recent studies have focused on the protection developed by the plant against free radical damages (Sherwin and Farrant, 1998) and in its ability to tolerate rapid drying (Farrant *et al.*, 1999). In this plant dehydration leads to an increase in anthocyanin content and a decline (30%) in chlorophyll content. Light-chlorophyll interactions are reduced as the chlorophyll becomes masked by anthocyanins but also due to leaves curling. Ascorbate peroxidase (AP) increased during dehydration and then decreased when the plant is desiccated. Superoxide dismutase (SD) and glutathione reductase (GR) activities increased during rehydration as the leaves uncurled preventing free radical damages until full hydration and metabolic recovery had occurred. Electrolyte leakage measurements have shown no difference among hydrated, dry and rehydrated leaves suggesting that the plasma membrane integrity is maintained during the dehydration/rehydration cycle (Sherwin and Farrant, 1996). *C.wilmsii* is characterised by the ability to rapidly put protection mechanisms into place during drying and also a rapid recovery upon rehydration. Leaves can put protection into place within 4 –8 hours (Farrant *et al.*, 1999) and become fully hydrated after 48h of rewatering (Sherwin and Farrant, 1996) and the the chloroplasts become functional after only 36h of rehydration.

A consequence of dehydration in resurrection plants is the considerable shrinkage of cells as the water is lost from them. In the resurrection plant *C.wilmsii* a diminution of 78% of the initial cell volume occurs when the plant is dried (Farrant *et al.*, 2000). This reduction in cell size **is due entirely to an extensive folding of the cell wall**. Such wall folding was also noticed in some seeds (Webb and Arnott, 1982). It is believed that this phenomena is a controlled process, rather that a simple collapse of the cell wall (Sherwin, 1995). If this is the case, the active participation of the cell wall would involve a change in its composition or structure as the plant dries. Although cell wall is known to play a major role in dehydration caused by factors such osmotic, cold, salinity or drought stresses, no studies have been done on the role of the cell wall in desiccation tolerance in resurrection plants.

4. AIMS OF THIS THESIS

The aim of the study was to do a detailed analysis of the cell wall composition of the resurrection plant, *C.wilmsii*. The walls of leaves were analysed in the hydrated and dried states and comparisons drawn. As the time of drying does not affect the response of *C.wilmsii* to desiccation (Farrant *et al.*, 1999) rate of drying was not considered in this study.

The cell wall of *C.wilmsii* leaves folds considerably upon drying. On rehydration, cell walls expand once more without apparent damages. How is this achieved?
These following questions will be addressed in this study:

1. Is the wall composition of *C.wilmsii* leaves differ from other species? Immunocytochemical techniques will allow mapping of the wall polymers distribution and determine if there is any unusual repartition of different molecules in *C.wilmsii* compared to previous models described in cell wall literature.

2. The cell wall is known to be a metabolically active compartment. Changes can occur rapidly in response to environmental stress. In the case of *C.wilmsii* what is the nature of the changes allowing the cell wall to folds upon dehydration without any apparent damages? Biochemical techniques would allow a quantitative and qualitative analyze of wall polymers in both hydrated and dry plants. The results should give indications whether this folding is due to active metabolic modifications (changes in polymer composition between hydrated and dry leaves) or due only to physical changes as the plant dehydrates (water, pH...).

3. Ions, and more especially calcium ions, are major factors in wall architecture. Does the removal of water from the plant induces a relocation or change in concentration of calcium in the wall?

4. The following plant hormones zeatin (Z), zeatin riboside (ZR), absisic acid (ABA) and the auxin indole-3 acetic acid (IAA) have been shown to be involved either in drought stress or in the regulation of cell wall mechanical properties. Do these hormones play a role in desiccation tolerance in *C.wilmsii*?

The results of these studies will be organised as followed:

Chapter two presents microscopical observations of *C.wilmsii* performed on leaves using different techniques.

Chapter three deals with immunocytochemical analyses using well characterised antibodies directed against various wall polymers.

Chapter four presents biochemical analysis of wall composition after sequential extraction of cell wall polymers. The qualitative and quantitative data are also presented.

Chapter five focuses on calcium distribution using different analytical methods.

Chapter six deals with hormones quantification such as zeatin, zeatin riboside, abscisic acid and auxin.

Chapter seven correspond to a general discussion of the major results obtained with different approaches.

CHAPTER 2 :

UlTRASTRUCTURAL STUDY

CHAPTER 2: ULTRASTRUCTURAL STUDY

1. INTRODUCTION

Transmission electron microscopy has been widely used to study the ultrastructural changes in tissues of resurrection plants during drying and rehydration (Gaff *et al.*, 1976; Wellburn and Wellburn, 1976; Schneider *et al.*, 1993; Bewley, 1995; Sherwin, 1995; Sherwin and Farrant, 1996; Dace *et al.*, 1998; Farrant *et al.*, 1999). The use of such techniques gave good results with resurrection plants and many structural changes have been identified between hydrated and dry tissues. In the majority of studies, chemical fixatives were used with apparently good preservation. However the investigation of the fine structure of dried plants is very complex especially in the case of resurrection plants because they are ultra-dried tissues. Fixation with aqueous buffers can lead to rehydration of dried tissues before the stabilisation of molecules is achieved. This can result in artefacts such as the swelling of dry cells and detachment of the protoplast from the cell wall (Opik, 1980; Thomson and Platt, 1997). These problems have been partially overcome by the use of anhydrous fixations. The fine structural changes in the leaves of the desiccation tolerant plant *Talbotia elegans* has been investigated using both aqueous and dimethylsufoxide (DMSO) fixation (Hallam and Luff, 1980 b). Cell expansion was noticed in dried-aqueous-fixed-material and to a lesser extent with DMSO fixation. Vigil *et al.*, (1984) studied the structure of cotton seed radicles after fixation with vapour phase acrolein or osmium tetroxide but water was not entirely absent in these conditions. Opik, (1985) has developed a completely anhydrous fixation to stabilise dry seed tissues using acrolein or osmium vapour phase fixation. The best results were obtained with acrolein fixation. Acrolein is a compound of low molecular weight and thus it penetrates the tissues more easily than the more commonly used fixative osmium tetroxide. Dried tissues were characterised by shrivelled cells and highly convoluted walls when anhydrous fixation was used. Chemical fixation results in the swelling of the dried tissues and detachment of the protoplast from the cell wall.

Whereas cryopreparation followed by resin embedding have been widely reported with bacteria (Armbruster *et al.*, 1981) or animal tissues (Carlemalm *et al.*, 1982; Sitte

Figure 1: Map of South Africa showing the area where *C.wilmsii* plants were collected (▲). Lydenberg is located in the Mpumalanga region (North East of RSA).

et al., 1986; Wroblewski *et al.*, 1990; Robertson *et al.*, 1992), few successful applications have been reported for plants. High pressure freezing followed by freeze substitution and low temperature embedding have been shown to be the best method for the preservation of root tips of *Nicotiana* and *Arabidopsis* (Kiss *et al.*, 1990), wheat and *Arabidopsis* leaves (Hippe-Sanwald, 1995) and suspension cells of sycamore (Driouich *et al.*, 1993 b, 1997). However many methodological problems are incurred with older tissues and tissues rich in phenolics and secondary metabolites.

In order to examine the ultrastructural status of hydrated and dried leaves of *C.wilmsii*, several methods have been attempted and compared. The results are presented in this chapter.

2. MATERIAL AND METHODS

Material

The desiccation-tolerant plants *C.wilmsii* are endemic to South Africa. They occur in the Mpumalanga province localized in the northern part of the country where the climatic conditions are semi-arid. Plants were collected from the Buffelskloof Nature Reserve near Lydenberg (Figure 1). They were grown in a mixture of peat, river sand and potting soil and were maintained in a green house under 30% shadecloth with no supplementary lighting. To maintain the capacity of the plants to tolerate desiccation, regular cycles of drying and rehydration are necessary. Whole plants were dried by withholding water and allowing the plant to dry out naturally. The plants were left in the dry state exposed to natural sunlight. Rehydration was effected by watering the plants to simulate rainfalls. These conditions were followed in all the experiments reported in this manuscript.

Methods

Sample preparations for light microscopy and transmission electron microscopy (TEM).

Chemical fixation (conventional method).

Figure 2: *C. wilmsii* hydrated (a) and dry (b). Pieces in the mid leaves area (purple) were taken from the central leaves of the rosette and cut into smaller fragments (about $2mm^2$). These small pieces of tissue were prepared for microscopical observations as described in material and methods.

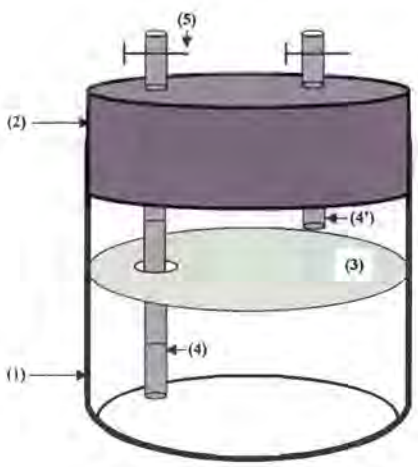

Figure 3: Schematic representation of the container used for vapour phase fixation. **(1) glass**; (2): silicon tap; (3): plastic grid; (4) and (4'): longer and shorter pipe; (5):on-off system.

- Fixation

Small samples (1-2 mm) were excised from the mid-blade of the plant rosette (figure 2). These were immersed in the caffeinated fixative buffer (glutaraldehyde 2.5%; phosphate buffer 0.1M, caffeine 0.5%; pH 7.4) overnight at room temperature according to the protocol of Sherwin (1995). Caffeine allows a better preservation in tissues containing phenolic components (Mueller and Greenwood, 1978). A post-fixation in 0.1% osmium tetroxide (OSO_4) for one hour occurred at room temperature.

- Dehydration

Samples were progressively dehydrated with diluted ethanol (concentration (V/V): 30%, 50%, 70%, 80%, 90% and 95%) then with absolute ethanol.

- Resin infiltration and polymerisation

Samples were placed in acetone and then were progressively infiltrated in Spurr's resin (Spurr, 1969). The samples were infiltrated overnight in a mixture (v/v) acetone / Spurr (75/25), one day in acetone/ Spurr (50/50), one night in acetone/ Spurr (25/75) and 24h in 100% Spurr's resin. Polymerisation occurred in the oven (60°C) for 16 hours.

Vapour phase fixation

The method of vapour phase fixation used was adapted from the protocol developed by Follet-Gueye *et al.*, (1999). Dried leaves were placed on a plastic grid in a glass container sealed with a silicon tap (Figure 3). Two plastic pipes allowed the replacement of solutions without opening the container. The plastic grid holding the samples was placed in a manner such that the samples were never in contact with any of the liquids. A volume of 10 ml of acrolein solution was placed *via* the pipe to the bottom of the container. The leaves were fixed by acrolein vapour for 17 days. The acrolein solution was then replaced with 10 ml of dimethoxy 2-2 propane (DMP) acidified by a drop of concentrated (36% w/w) chloric acid (HCl). The vapour of acidified DMP allows the dehydration of the leaves. Exposure was for intervals of 5 min (2 times) and then for one hour, the solution being renewed each time. After dehydration, leaves were transferred to a petri dish filled with propylene oxide (PO).

Figure 4: *C.wilmsii* plants during drying. (a) plants fully hydrated have green expanded leaves; (b) and (c) when drying the leaves are progressively curling to finally form a tightly bud where only older leaves are exposed to the sunlight (d). Note the purple colour in dried leaves indicating the presence of anthocyanin on the abaxial surface. This pigment functions as a "sun screen" to protect cell structures against radiations.

Small pieces of tissues (approximately 3 mm^3) were excised from the middle of the leaves while submerged in PO. The samples were embedded in Spurr's resin as described above.

Cryopreparation

Pieces from dried leaves were hand sectioned as thin as possible. These samples were plunged into propane precooled by liquid nitrogen (-180°C). The freeze drying was performed over 10 days until the temperature reached -40°C. Infiltration with the lowicryl resin HM20 was performed at -40°C under vacuum for 10 days. Polymerisation was by exposure to UV light for 24 hours following by curing at room temperature (2 days). The blocks were very brittle and the sectioning was easier at lower temperature (-40°C) than at room temperature. Thick sections (200nm) were mounted on copper-formwar grids.

Light microscopy

Semi-thin sections (0.25μm) were cut with glass knives using an automatic microtome (LEICA). Sections were stained either with basic fuchsin methylene blue or with toluidine blue (0,5%) only . After being washed thoroughly with distilled water, the sections were viewed with an Axioskop microscope (ZEISS).

Transmission Electron Microscopy

Ultrathin sections (95nm) are collected on copper grids and stained 10 min in uranyl acetate followed by 10 min in lead citrate (adapted from Reynolds, 1963). Pellets of NaOH were used to trap the CO_2 and so prevent lead citrate precipitation. The grids were carefully washed with water and dried on filter paper. Samples were observed at 80 kV on a ZEISS transmission electron microscope (EM 109).

Sample preparations for scanning electron microscopy (SEM)

Leaves from both hydrated and dry plants were cryofixed in liquid nitrogen slush and sublimed at –70°C (frozen hydrated samples). They were coated with gold palladium and viewed using the cooled stage in Hitachi s-570 scanning electron microscope.

Leaves from dried plants were also dissected in small pieces and coated with gold paladium without any fixation.

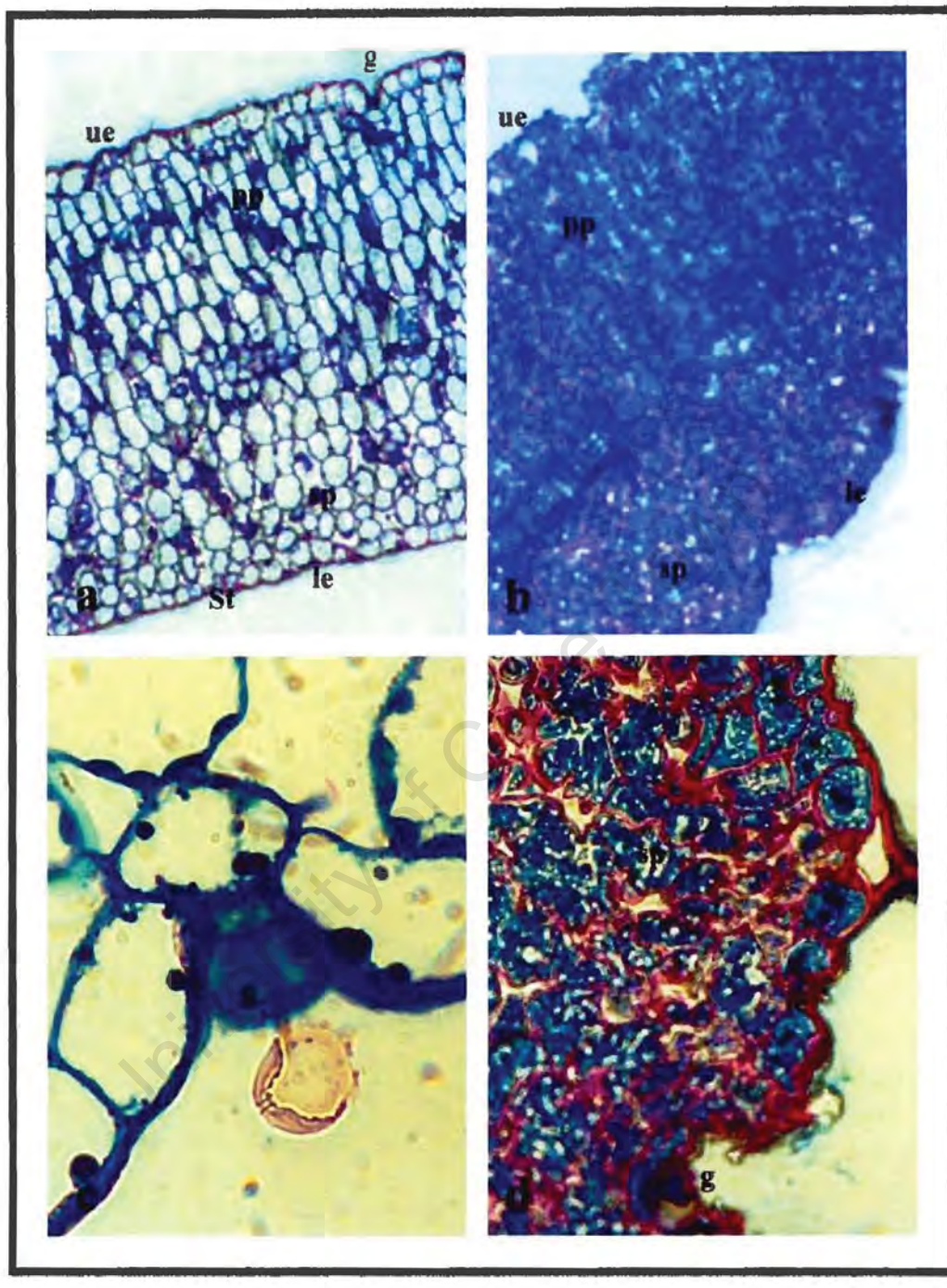

Figure 5: Transversal sections of leaves from hydrated (a,c) and dry (b,d) leaves of *C.wilmsii* stained with basic fuchsin methylene blue. The cytoplasm presents a blue colour whereas the walls appear pink. Palisade and spongy parenchyma are visible in hydrated leaves (a) but they are less distinguishable in dry leaves (b). Note that the vacuole which occupied the whole cell in hydrated leaves (c) has disappeared in dried cells (d). Glands are present on the epidermal surface (c). (g) gland; (h) hair; (le) lower epidermis; (sp) spongy parenchyma; (pp) palisade parenchyma; (ue) upper epidermis. Magnification: (a),(b) X 20; (c) X 100, (d) X 40.

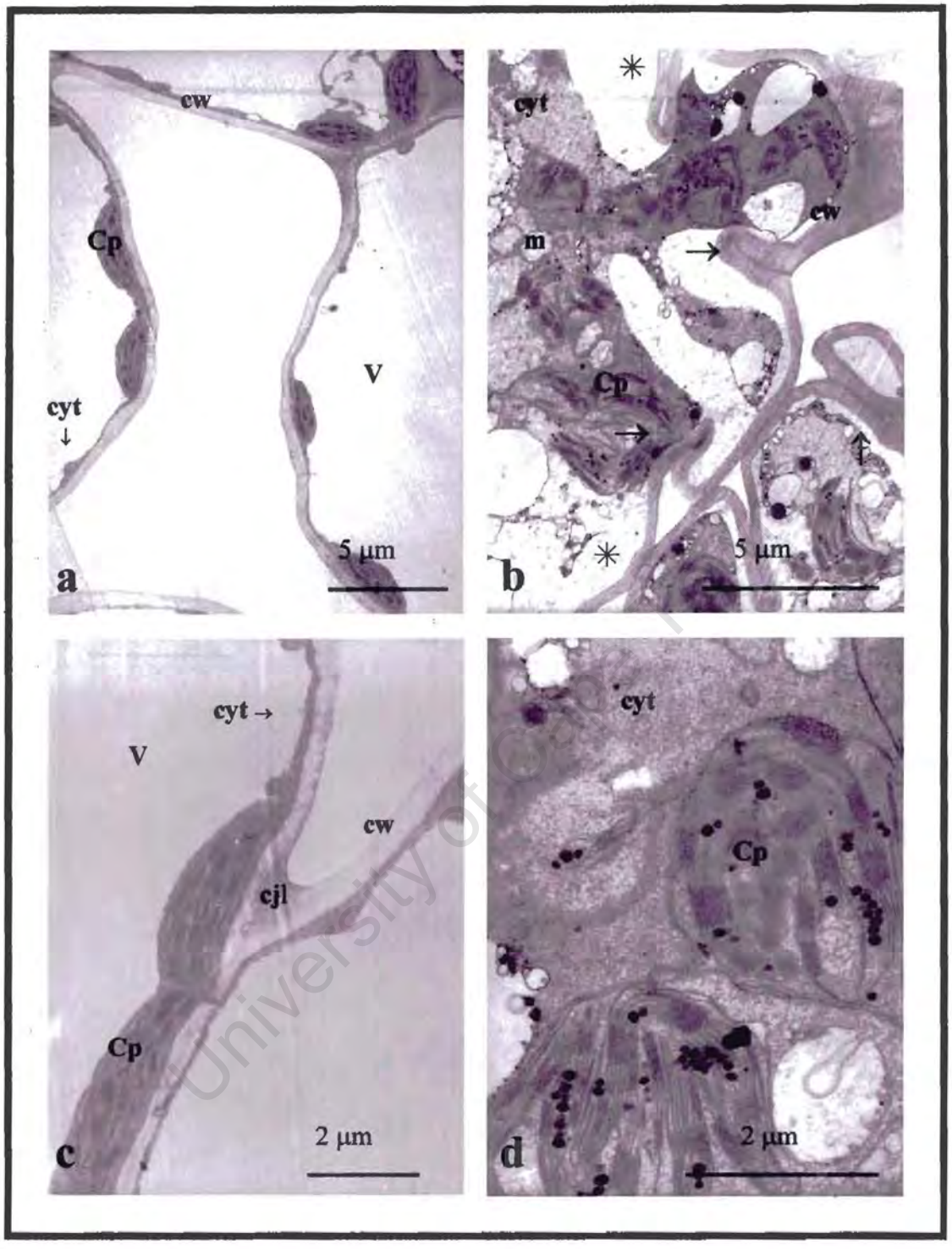

Figure 6: TEM micrographs of hydrated (a,c) and dry (b,d) leaves of *C.wilmsii*. In hydrated leaves, cells are rounded shape and the cytoplasm forms a thin layer close to the cell wall (a). Chloroplasts are elongated (c). In dry cells, cell walls are folding the large vacuole is not apparent (b). Note the plasmalemma withdrawal from the cell wall in dry cells (*). Chloroplasts are rounded with well-defined membranes (d). (cj) corner junction; (Cp) chloroplast; (cw) cell wall; (cyt) cytoplasm; (m) mitochondria; (v) vacuole; (→) cell wall folding.

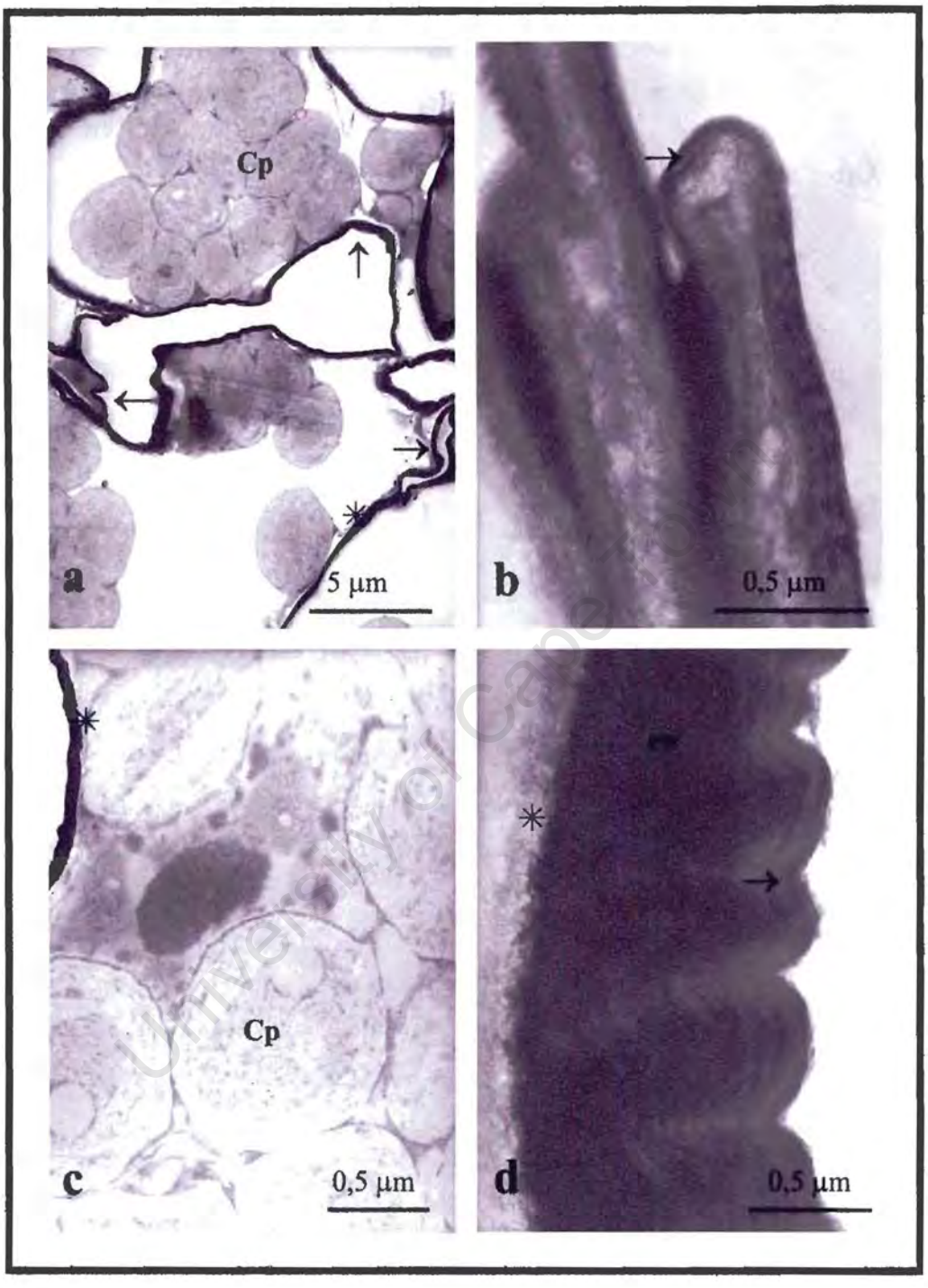

Figure 7: Vapour phase fixation of dried leaves. Chloroplasts present a rounded shape (a) and are located in the middle of the cell. This anhydrous fixation confirmed the cell wall folding apparent with chemical fixation. (b) Cell wall folding at higher magnification; (c) Chloroplasts; (d) epidermal folds. Note the close association between the plasma membrane and the cell wall (→).(cp) chloroplast; (cw) cell wall; (ew) epidermal wall; (✳) contact between plasmalemma and cell wall.

3. RESULTS

The morphological changes undergone by the resurrection plant *C.wilmsii* during drying are presented in Figure 4. When the plant is fully hydrated (Figure 4a) leaves are green and expanded. As the plant dried, leaves curled inward (figure 4b-c) and a purple colour characteristic of anthiocyanin pigments was visible on the abaxial surface. In the dry state the leaves were tightly folded with abaxial surfaces of the outermost leaves exposed to sunlight (Figure 4d). Note that in the dry leaves, chlorophyll is still present as indicated by the green colour of the leaves. This is characteristic of homoiochlorophyllous plant.

Light microscopical transverse sections are shown in Figure 5. It can be seen that the boundary between the palisade parenchyma and the spongy parenchyma is less clear that what is generally observed in the classic leaf morphology. This has also been reported for the related *C.nanum* leaves (Sherwin, 1995). The cell volume of hydrated leaf cells was occupied by a large central vacuole which does not retain the basic fuchsin methylene blue colorant (Figure 5a). The cytoplasm, shown by a thin blue layer, was compressed between the cell wall and the vacuole. Stomata were present in both lower and upper epidermis. Glands were present in the lower and upper epidermis. As it is seen in Figure 5c these glands stained in blue with the basic fuchsin methylene blue are composed of several units.

The Figure 5-b indicates that the loss of water during dehydration leads to severe alterations in the cellular anatomy of the *C.wilmsii* leaves. The cells were more compact, and had lost their rounded shape and it was difficult to make a clear distinction between palisade and spongy parenchyma. The large central vacuole had disappeared and the cytoplasm, stained in blue, occupied the whole cell volume. The epidermis was convoluted and the epidermal glands observed in the hydrated leaves seem to be trapped in the folding of the epidermis.

The ultrastructure of chemically fixed hydrated and dry leaf cells are given in Figure 6. In hydrated leaves, the TEM observations confirm that the cytoplasm, including organelles such as chloroplasts and mitochondria were peripherally located along the cell wall (Figure 6a). The endoplasmic reticulum (ER) and the Golgi apparatus were

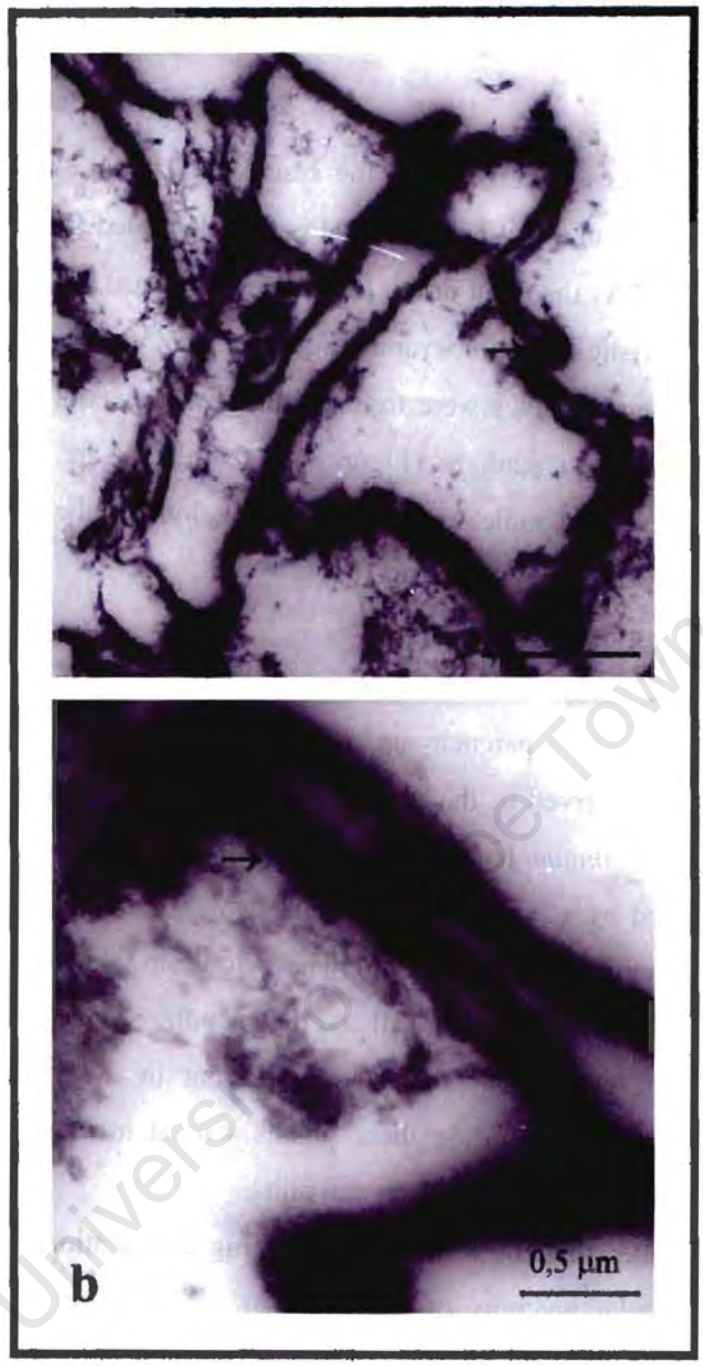

Figure 8: Dry leaves of *C.wilmsii* prepared by freeze-drying and HM20 embedding. Cryofixation followed by resin embedding at low temperature are difficult to achieve with plant cells. Although cells are badly preserved, cell folding is apparent. (cj) corner junction; (cw) cell wall; (→) cell wall folding.

rarely seen. Chloroplasts were typically elongated, the thylakoid membranes were well defined and starch was often present.

In dried leaf tissues, considerable cell wall folding was observed (Figure 6b). The central vacuole, which completely filled the cell in the full turgor state, had disappeared and, as observed with the light microscope, cytoplasm occupied the entire volume of the cell. In some cells several little vacuoles were observed. Most chloroplasts were rounded in shape and were localised in the middle of the cell. Membranes, including the thylakoids were well defined. Plastoglobuli were present in the stroma. The plasmalemma from dry cells was not always immediately adjacent to the cell wall, with some withdrawal evident in some cells.

Attempts at anhydrous fixation of dry leaf material for observation with TEM are shown in Figure 7. Detailed ultrastructure observation revealed that fixation was not successful numerous cells being damaged. However there were few cells with good preservation and in this case, cell wall folding was confirmed and the plasmalemma remained adjacent to the cell wall. Epidermis cell wall also had large undulations.

Tissues prepared by cryofixation and low temperature embedding in HM20 resin were very difficult to section because they were very brittle. This technique was not successful and the preservation was not good. However it was possible to see the folding of the cell wall in the dry leaves (Figure 8).

Figure 9 is the SEM images of frozen hydrated sections of hydrated and dry leaves with no chemical fixation. Transverse sections from hydrated leaves confirms the previous observations with chemical fixation concerning the organisation of parenchyma cells (Figure 9a). Spongy and palisade parenchyma cells were observed and cells possessed a rounded shape. In cells from dried leaves, there was again the observation of a considerable decrease in cell volume associated with dehydration (Figure 9b). Cells were shrunken and it becomes impossible to delimit a boundary between palisade and spongy parenchyma. Note the presence of the numerous hairs at the surface of the lower epidermis.

Figure 9c-d are surface views of epidermis from hydrated and dry tissues respectively. These figures are at the same magnification in order to show the level of contraction of the dried cells compared to the hydrated cells. In Figure 9c stomata and glands are

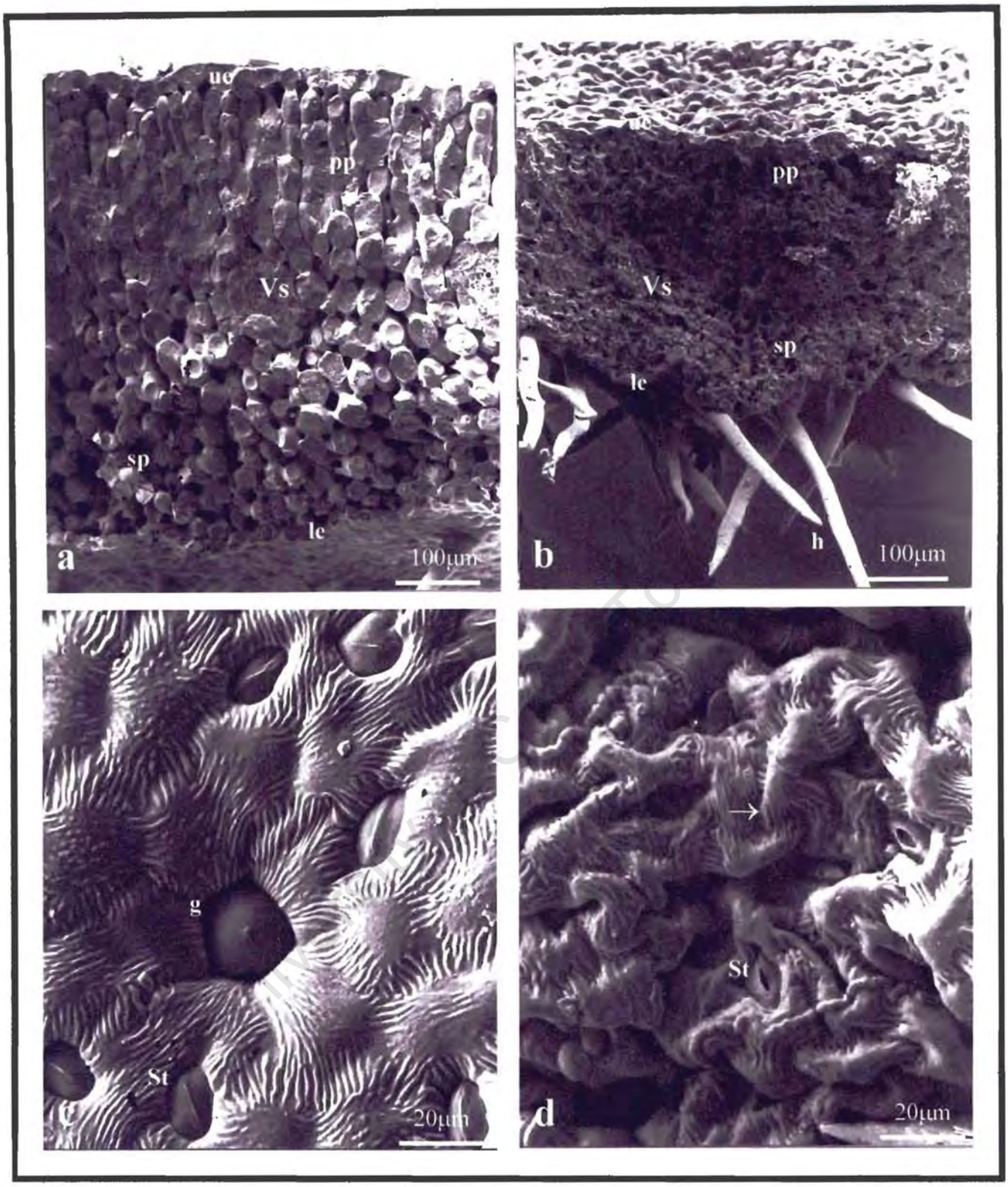

Figure 9: Scanning electron (SEM) micrograph of hydrated and dried *C. wilmsii* leaves. Transversal section of hydrated (a) and dried leaves (b). Observation of the epidermal surface of hydrated (c) and dried (d) leaves. Glands are present in the epidermis. Note that in the dry leaves, the stomata are largely opened. (le) lower epidermis; (ue) upper epidermis; (h) hair; (s) stoma; (g) gland; (→) folding, (v) vessels; (pp) palisade parenchyma; (sp) spongy parenchyma.

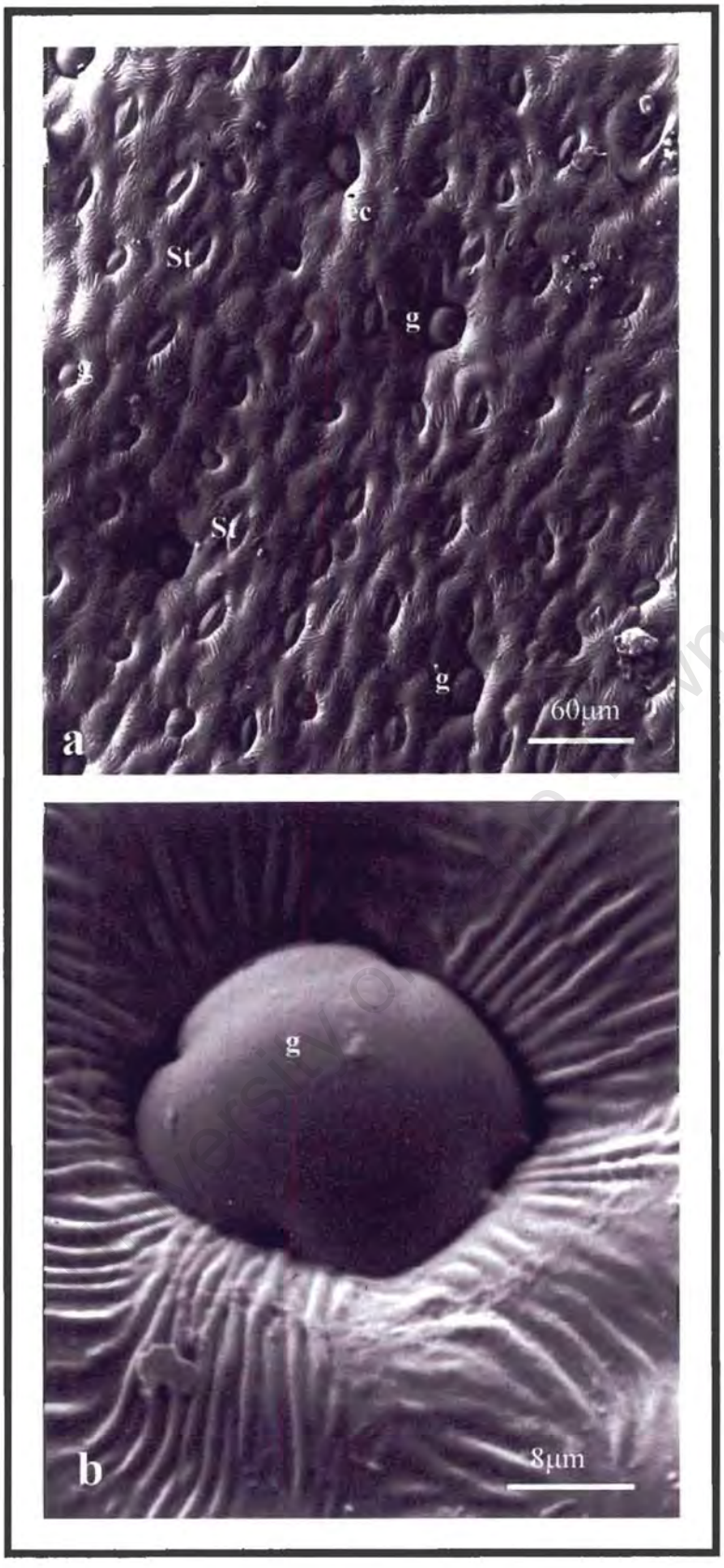

Figure 10: Scanning electron micrographs showing the upper epidermal surface of hydrated leaves of *C.wilmsii*. (a) Epidermis presents numerous glands and stomata. (b) gland at higher magnification. (g) gland; (s) stoma; (ec) epidermal cell.

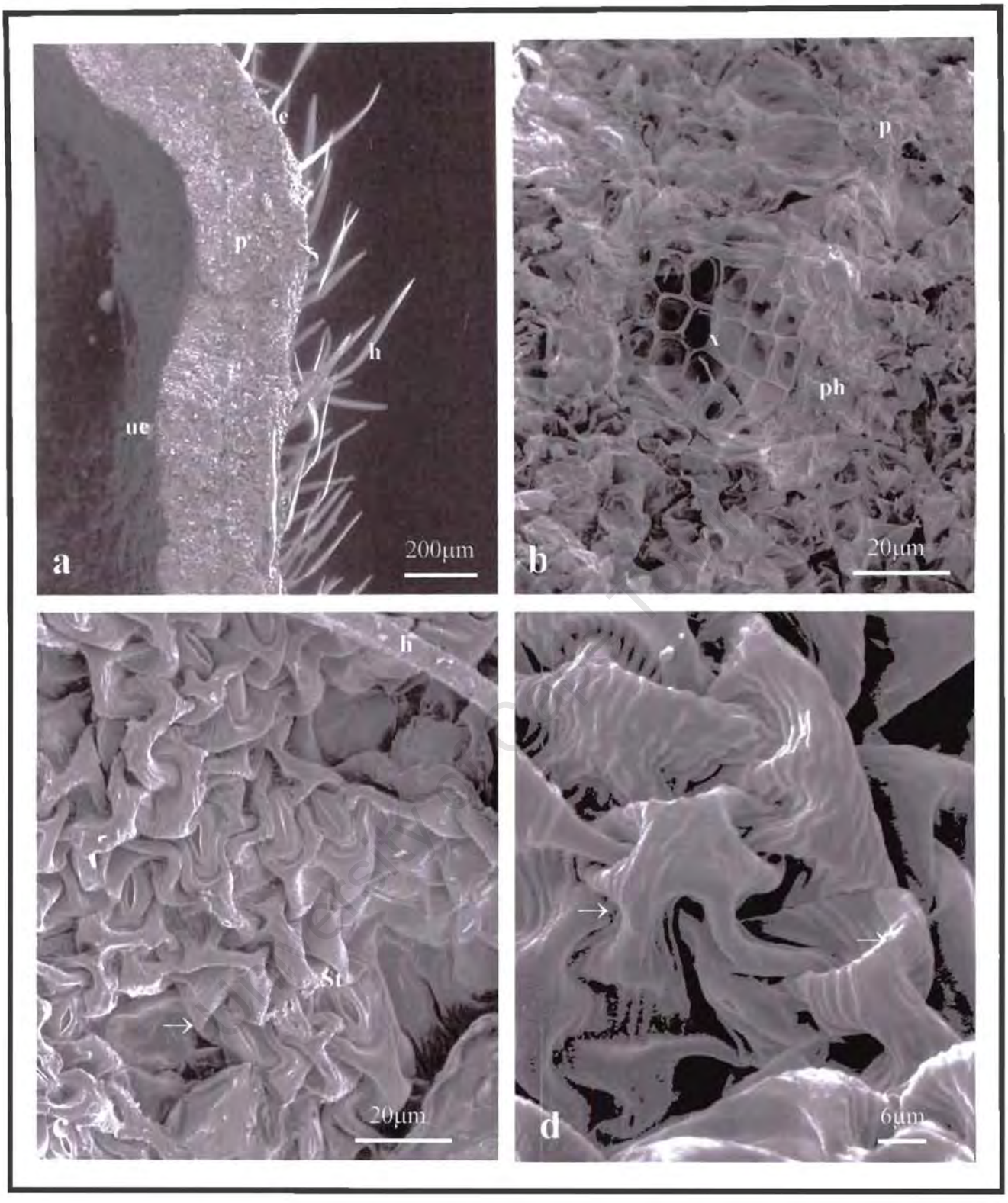

Figure 11: Scanning electron micrograph of dried leaves of *C.wilmsii* without fixation. (a) transversal section; (b) transversal section showing the folded cells of parenchyma. The xylem maintains its original shape whereas the phloem (less rigid) appears collapsed; (c) epidermal surface; (d) epidermis at higher magnification showing the important cell folding (→).
(le) lower epidermis; (ue) upper epidermis; (h) hair; (s) stoma; (p) parenchyma (x) xylem; (ph) phloem.

visible. Two interesting features arise from the observations of epidermis from dried cells (Figure 9d). First, stomata were open. Drought usually induces stomata closure to avoid water loss by transpiration (Zhang *et al.* 1987; Trejo and Davies, 1991). A second important observation is that the glands were not apparent at the epidermal surface and they seem to be masked in the epidermis folding. Because the presence of such glands have not been reported to date, a detailed observation was made in this study. From Figure 10a it can be seen that they were quite abundant at the epidermal surface. At higher magnification, it is evident that these glands are comprised of 4 sub-units.

SEM observation of dried plants without fixation revealed a structure for dry leaves similar those dried leaves preserved by freezing (Figure 11). Cells appeared highly shrunken except for the xylem (Figure 11b). The study of epidermis surface confirmed that some stomata were open and the epidermal glands could be seen inside the folding (Figure 11c).

4. DISCUSSION

The use of chemical fixation for dry tissue has been highly criticised as it has been suggested that the use of aqueous fixative buffer induce rehydration of sample before the fixation occurs. To date, ultrastructural studies on *Craterostigma* species were only performed by using aqueous fixative and TEM observation (Schneider *et al.*, 1993; Sherwin, 1995; Sherwin and Farrant, 1996; Farrant *et al.*, 1999). As indicated by Sherwin, (1995) even with chemical fixation folding of the cell wall can be seen in dry cells.

This study was the first attempt to get anhydrous fixation of *Craterostigma* plants followed by resin embedding.

Vapour phase fixation

Vapour phase fixation was not completely successful with the dry leaves of *C.wilmsii* although different times of exposure to the acrolein vapour were tried up to 17 days. Vapour phase fixation for dry material several of weeks to months has been reported

(Thomson and Platt, 1997). Singh *et al.*, (1984) found good preservation of hydrated *Tortula ruralis* moss with osmium vapour fixation but two weeks exposure on dried moss was still unsuccessful. Opik, (1985) obtained good fixation of mung bean radicals and wheat embryos with acrolein vapour after only 8 days exposure. To this aim the organs taken from the seeds were dissected to allow a better penetration of the chemicals in the tissues. Thomson and Platt, (1997) reported that infiltration of resins in anhydrously fixed dry plants is often difficult. Problems occur during sectioning and only small fragments of the sectioned material are good enough for observation with electron microscopy (Hallam, 1976; Opik, 1980; Thomson and Platt, 1997). Exposure of dry leaves of *C.wilmsii* to acrolein fixation might not have been long enough to allow a good fixation of the tissues. Due to restricted time, it was unfortunately not possible to further improve this technique.

However it is important to note that this technique gave important indications in the cells well preserved:

1) The close association between cell wall and plasmalemma was visible in dry cells with vapour phase fixation. When chemical fixation was used on dried cells the withdrawal of the plasmalemma often occurred. This is thought to be an artefact of preparation rather than a proper response to dehydration. Moore, (1989) has reported a similar phenomenon in leaf parenchyma cells of clover. The author suggests that it is due to the plasmolysis resulting from fixation, dehydration and resin embedding. When chemical fixation is used on dried material, a differential rehydration of cell wall and cytoplasm cause the pulling away of the plasmalemma from the cell wall.

2) Vapour phase fixation confirmed the folding of cell walls in parenchyma and epidermis in dry leaves of *C.wilmsii*. This is important for the rest of the study to note that this folding is not an artefact of chemical fixation. Vapour phase fixation was the first anhydrous technique to confirm this folding for *C.wilmsii*.

Cryofixation

Cryofixation techniques followed by low temperature embedding are now commonly used with animal tissue preparation for TEM observation (Carlemalm *et al.*, 1982; Sitte *et al.*, 1986; Wroblewski *et al.*, 1990; Robertson *et al.*, 1992). Only high pressure freezing, freeze substitution and low temperature embedding are reported in the

CHAPTER 3: IMMUNOCYTOCHEMICAL STUDY

1. INTRODUCTION

In order to understand how cell wall polymers change during growth and during active defense against diseases and environmental stresses, it is important to learn where the polymers are located within the cell wall. Immunocytochemistry has been a powerful tool to investigate cell wall architecture (for review see Knox, 1997). The development of antibodies against cell wall components allowed a better understanding of the existence of molecular domain in the cell wall. This type of information can not be gained with biochemical techniques. Biochemistry analysis requires large amount of material which usually includes a diversity of cell types. Immunocytochemistry is a good complement to biochemical analysis in the way that it can give information at the scale of a single cell or even at the cell compartment level. The following section will present the main applications of immunolocalisation techniques in investigating cell wall structure, biosynthesis and functions. All these data could only be gained with the generation of antibodies recognizing specific epitopes of the cell wall molecules.

Mapping the architecture of the cell wall

The first immunocytochemistry study concerning the immunolocalisation of plant cell wall antigens was the work of Knox in 1970 with a pollen wall component (See Knox, 1997). In the subsequent 30 years, antibodies have been generated to various wall components. The use of these antibodies has allowed the mapping of the cell wall and specific domains have been established in cell wall architecture depending of the nature of the components. Cell wall matrix components were found in complementary areas of the cell wall. Using antibodies against homogalacturonans, Knox *et al.*, (1990) established that pectins were localized in different cell wall sites depending on their degree of methylesterification. In the root apex of carrot, the low methylesterified pectins detected by JIM5 antibodies were located essentially in region of the cell wall close to middle lamella and at the outer surface of the intercellular space. Highly methylesterified

pectins recognized by JIM7 antibodies were distributed more uniformly throughout the cell wall. Bush and McCann (1999) observed with *Solanum tuberosum* that cell walls from the parenchyma were uniformly labeled with both JIM5 and JIM7 antibodies. A similar location was found with the vascular tissues for JIM5 and JIM7. The antibodies LM5 recognizing the β (1→4) galactans were located essentially in the wall close to the plasma membrane in tomatoes (Jones *et al.*, 1997), flax roots (Vicré *et al.*, 1998 a) and in fiber cell walls from flax (Andeme-Onzighi *et al.*, 2000-b) . Moore and Staehelin (1988) found that xyloglucan was more located in the cellulose microfibril-containing region of the cell wall in clover root tip and leaf cells. Detection of xyloglucan was very scarce in the middle lamella area and in corner junction (Moore and Staehelin, 1988; Freshour *et al.*, 1996). However in cell walls of suspension-cultured sycamore cells XG was present throughout the cell wall including middle lamella (Moore *et al.*, 1986). A similar distribution was noticed with anti-extensin antibodies. Stafstrom and Staehelin (1988) found that extensin was excluded from the middle lamella and more especially associated with the region of the cell wall containing cellulose microfibrils.

Antibodies against arabinogalactan proteins (AGPs) have also been generated. Immunogold labeling shown that AGPs were associated within the cell walls (Kikuchi *et al.*, 1993; Schindler *et al.*, 1995) and essentially with the wall domain associated with the plasma membrane area (Herman and Lamb, 1992; Schindler *et al.*, 1995; Freshour *et al.*, 1996; Ferguson *et al.*, 1999).

Cell wall polymers are species- and/or tissue-specific

Labeling of the developing root apex of carrot (*Daucus carota L.*) with JIM5 and JIM7 antibodies showed that these epitopes carried by low or high methylesterified pectins were present in the walls of cells in all tissues (Knox *et al.*, 1990). Mogami *et al.*, (1999) have shown using immunolocalisation that cell wall formation in gymnosperm pollen was different from angiosperm. Furthermore they demonstrated using JIM5 antibody that in *P.dendiflora*, no label was found in the cell wall of the pollen tube tip whereas the outer layer of the intine and the intine of the germinal aperture were strongly labeled.

In *Arabidopsis* root tip, the rhamnogalacturonan I epitope recognized by the antibody CCRCM2 was only detected in cell walls of cells from at least 1 mm from the root tip

extremity (Freshour *et al.*, 1996). Its distribution, however, was restricted to the epidermal and cortical layers, no labeling was found in endodermal pericycle, vascular tissues or in the parts of the root closest to the hypocotyl. These results suggest that cells are able to modify the structure of their cell wall polysaccharides depending on their developmental stage. Lynch and Staehelin (1995) noticed the absence of PGA/RG1 labeling in any cell wall of the root tip of *Avena sativa*. The mucilage of *Avena sativa* was weakly labeled but in root cap mucilage of clover PGA/RG1 labeling was very dense. In the coleoptile and leaf tissues of oat seedlings unesterified pectins revealed by JIM5 antibodies were abundantly present in cells whereas methylesterified pectins detected by JIM7 were not present (Knox *et al.*, 1990).

Vicré *et al.* (1998) showed, using immunofluorescence, that β-(1→4)-D-galactan epitopes were restricted to peripherical cells of flax root cap. This epitope was not detectable in the columnella, cortex or meristematic cell walls. In contrast β- (1→6)-D-galactan epitopes were present in all cell types of flax root. These data demonstrate that the synthesis and location of β-(1→4)-D-galactan epitopes were highly regulated in developing flax roots and that different β-linked D-galactans associated with cell wall polysaccharides were expressed in a cell type-specific manner.

Arabinogalactan epitopes detected with CCRCM7 in *Arabidopsis* roots were present only in the mature part of the root but not in the root tips (Freshour *et al.*, 1996). The monoclonal antibodies raised against the various carbohydrate epitopes of AGPs were of great interest to elucidate the complex distribution of AGPs (Schindler *et al.*, 1995; Ferguson *et al.*, 1999; Gao *et al.*, 1999). The presence of AGPs is highly regulated and linked to the determination of cell identity (Schindler *et al.*, 1995).

Cell wall polymer biosynthesis

Immunocytochemistry yielded important information on the cell wall biosynthesis. Cel lulose is synthesized at the plasma membrane by cellulose synthesizing complex (Delmer and Amor, 1995; Kudlicka and Brown, 1997; Delmer, 1999) whereas wall matrix components are assembled in the Golgi apparatus and transported to the cell surface in Golgi derived secretory vesicles (Brett and Waldron, 1990; Driouich and Staehelin, 1997). Kimura *et al.*, (1999) demonstrated for the first time using immunocytochemistry

and antibodies directed against cellulose synthase that the complex cellulose synthase was located in the rosette structures present at the plasma membrane. Although numerous studies have been realized concerning the cellulose synthase, there was no direct evidence to date to show that these rosette structures were really involved in the synthesis of cellulose. Immunogold localization was used to investigate the precise site of synthesis of cell wall polymer and their pathway trough the endomembran system of secretion. The data obtained by Moore and Staehelin (1988) and Mogami *et al.*, (1999) confirmed that the site of synthesis of non cellulosic wall polymers was restricted to the Golgi apparatus, no labeling was found in the rough ER. In *Vicia* root hair cells, Sherrier and VandenBosch (1994) shown a specific pattern of labeling for Golgi apparatus depending of the type of polysaccharides studied. The monoclonal antibody JIM7 recognizing mostly methylesterified pectins indicated that the epitopes were associated with the *median* and *trans* cisternae of the Golgi apparatus as well as in the secreted vesicles. These immunocytochemical data suggest that in *Vicia* root hair cells, these pectins are assembled in the *median* and *trans* cisternae and are secreted in a neutral form into Golgi vesicles to the cell wall compartment. Concerning the xyloglucans, only the *trans* cisternae and the secreted vesicles were labeled by gold particles. None of the epitopes were detected in the *cis* cisternae or in the rough ER. Double labeling experiments demonstrated that these two polysaccharides could be synthesized simultaneously in the same cisternae and be transported in the same vesicles to the cell wall. Similar results were found by Zhang and Staehelin (1992) in sycamore suspension cells.

Lynch and Staehelin (1992) found PGA/RG1 located in the *trans* cisternae and trans Golgi network (TGN) for epidermal and peripheral root cap cells of clover. Moore *et al.* (1991) and Zhang and Staehelin (1992) noticed a different location of these epitopes in cortical cells of onion and clover root tips as well as in sycamore maple cell suspension culture. These epitopes were found only in *cis* and *median* Golgi cisternae whereas highly methylesterified PGA recognized by JIM7 antibody was detected in the *median*, the *trans* cisternae, the TGN and secreted vesicles. It was suggested by Zhang and Staehelin (1992) that PGA could be synthesized in unesterified form and being methylesterified only in the *trans* cisternae and TGN. This is consistent with the results obtained by Liners and Van

Cutsem (1992) using a monoclonal 2 F4 antibody specific for a Ca^{2+} dependant conformational epitope of homogalacturonans. Discrepancy in these localizations might be due to a species-specific or tissue-specific differences in Golgi cisternae function. In a same way differences were obtained concerning the xyloglucan localization in the Golgi cisternae. A location of XG in the *trans* cisternae and TGN of several plant tissues (Moore *et al.*, 1991; Lynch and Staehelin, 1992; Sherrier and VandenBosch, 1994) whereas Zhang and Staehelin (1992) obtained labeling in the *trans* cisternae of Golgi apparatus in sycamore maple suspension culture cells. Vicré *et al.*, (1998 a) detected the presence of β-(1→4) and β-(1→6)- D-galactan epitopes in Golgi cisternae as well as in secreted vesicles showing the involvement of the Golgi apparatus in the synthesis and transport of these cell wall polymers. The β-(1→4)- D-galactan epitopes detected with the LM5 antibody were mainly associated with the *trans* cisternae of the Golgi complex. The synthesis and secretion of β-D-glucans is highly regulated during cell development and regulation and post-secretory modifications can occur in cell wall as shown in the root tip of *Avena sativa* (Lynch and Staehelin, 1995).

Andeme-Onzighi *et al.* (2000 a) using an antibody specific to the β-(1→3, 6) -D-galactosyl epitopes containing uronic acids associated with pectins demonstrated that this cell wall polymer was assembled in Golgi apparatus in a very specific manner. In flax root cells this epitope was detected in the *cis*, *median*, *trans* and TGN Golgi cisternae. The heavier labeling of the *trans* cisternae and TGN indicated the epitopes were essentially assembled in these two specific Golgi compartments. More recently, Samaj *et al.* (2000) detected the presence of the arabinogalatan proteins recognized by the antibody LM2 in the endoplasmic reticulum (ER) and the ER-derived vesicles in the growing pollen tube.

Immunocytochemistry: a tool to understand the role of the cell wall.

The understanding of cell wall role has been largely improved by the use of immunocytochemistry. Vian and Roland (1991) used the enzyme-gold complex technique and immunocytochemistry to locate respectively cellulose and polygalacturonans in different growing plant cells. By this technique, the authors showed that cellulose and polygalacturonans were closely associated during growth. The co-

localization of pectins and AGPS in the cell wall of pollen grain and pollen tube in *Nicotiana tabacum* L. might play an important role, not only in maintenance of cell shape, but also in cell-cell interaction during pollen tube growth and development (Li *et al.*, 1995). Roy *et al.* (1998) confirmed with immunogold labeling after Yariv phenylglycoside incubation the importance of AGPs in pollen tube growth and its possible role in the deposition of cell wall polymers.

The interactions between the higher plants and organisms such as fungi involve cell wall and plasmamembrane participation. This includes communication or exchange for symbioses or pathogenic attack. Immunocytochemistry brought important knowledge concerning this role of interface played by the cell wall and more especially enabled researchers to determine whether the structures developed at the interface of infection were coming from the plant or invaders (see for review Knox, 1997). Bonfante-Fasolo *et al.* (1990) investigated the composition of cell walls associated with the mycorrhizal process in roots of mycorrhiza *Allium porrum*. They found that cellulose and pectins were localised in a complementary area in roots involved in the mycorrhizal symbiosis. A similar pattern was noticed around the vesicular-arbuscular mycorrhizal suggesting the interfacial material was coming from the plant. Harrak *et al.* (1999) demonstrated that a proline-threonine-and glycine-rich protein (PTGRP) involved in drought tolerance in *Lycopersicon chilense* was located in cell walls. The synthesis of this protein was down regulated in pit membrane of xylem elements. This was the first example of a drought-regulated protein that has been clearly localized in the cell wall.

The generation of mutants with altered wall composition is particularly useful to understand the role of cell wall polymers (Sorensen *et al.*, 2000). The Arabidospsis qrt1 and qrt2 mutants produce tetrad pollen in which microspores fail to separate during pollen development. Using immunofluorescence, Rhee and Sommerville (1998) found that in wild type species pectic components were no longer detectable at the time of microspore release whereas in these mutants pectic components persisted. A failure in pectin degradation in the pollen mother cell wall could be associated with lethal pollen formation in qrt mutants. The authors suggested that QRT1 and QRT2 might be required for cell-type-specific pectins degradation in order to separate the microspores. Nickle and Meinke (1998) found by immunogold detection that the cytokinesis-defective mutant of

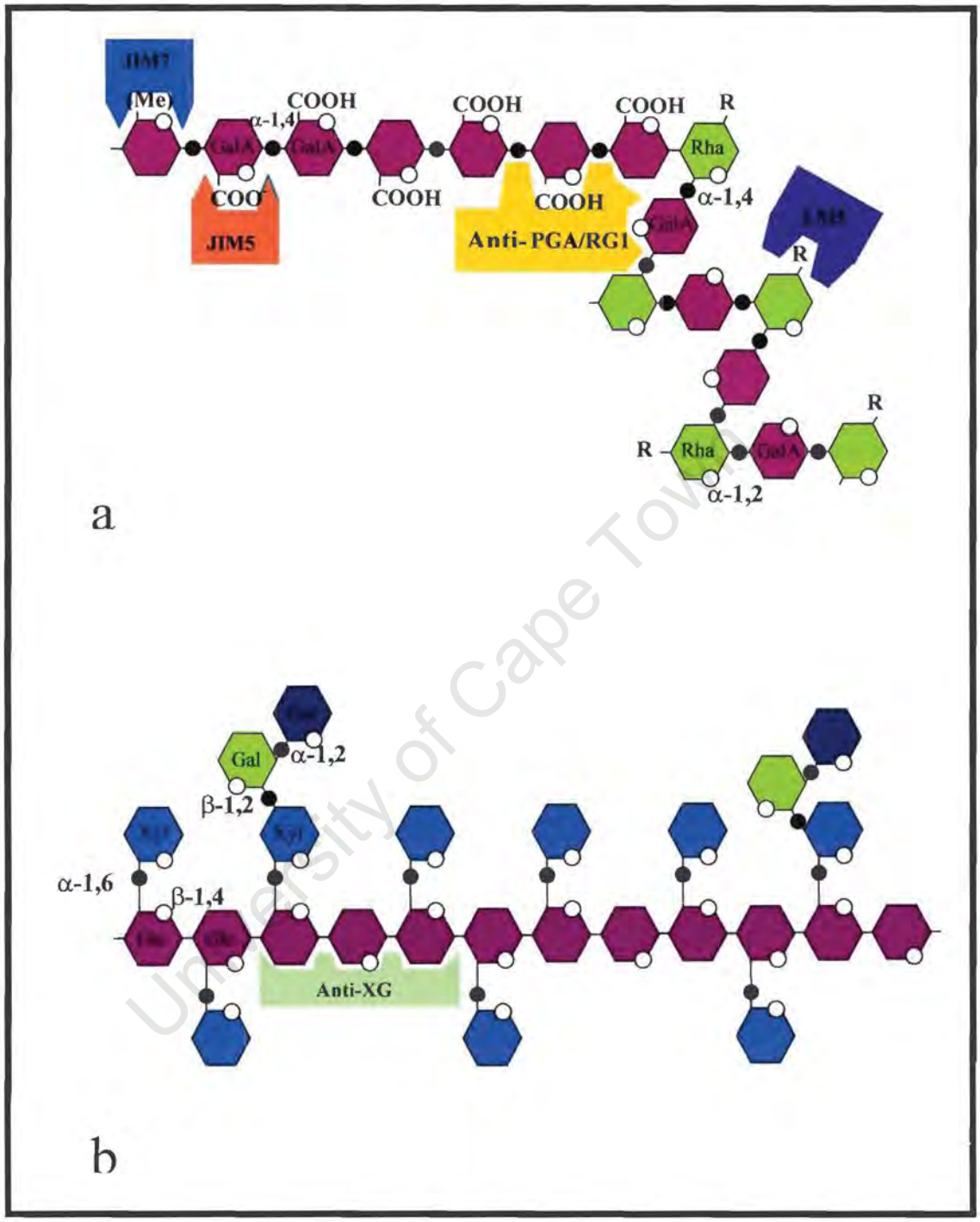

Figure 1 : Representation of the epitopes recognized by the antibodies JIM5, JIM7, anti-PGA/RG1, LM5 (a) and the antibodies anti-XG (b)

Arabidopsis Cyt1, characterized by embryonic lethality possess incomplete cell walls and an excessive callose deposition. More recently His *et al.* (2001) studied the cell wall composition of *Korrigan*, a dwarf mutant of *Arabidopsis*, deficient in a membrane-bound endo 1,4-β-glucanase. The authors found that a deficiency in an endo 1,4-β-glucanase, which is in principle not directly implied in pectin metabolism induced significant changes in the pectin composition. They identified the existence of feedback mechanisms controlling the synthesis and/or deposition of pectic polysaccharides in primary cell walls.

The aim of this part of my work was to use immunocytochemistry to analyze the cell wall architecture in leaves of *C.wilmsii*. The location of the detected epitopes (associated with various wall polymers) was compared to the results found in literature to determine if the cell wall composition of *C.wilmsii* is common to most of dicotyledonous leaves or is atypical. This study focused on pectin (pectins are involved in many stress responses), xyloglucan and protein (laccase, peroxidase) occurrence and distribution within the wall. Some antibodies were selected and further investigation were undertaken to quantify the labeling in the walls of leaves of dry and hydrated plants.

2. MATERIAL AND METHODS

The plants were collected and grown as described in chapter 2 (see Material and methods). *P.sativum* plants used as control were germinated in soil and the seedlings were grown under the same conditions as *C.wilmsii*. Plants of 2 weeks old were dried by the same procedure used for *C. wilmsii*. Leaves from 3 different plants of *C. wilmsii* were also sampled at intermediate stages of dehydration and rehydration. The relative water content of the leaves were estimated according to Sherwin (1995):

When samples were taken for immunocytochemistry, the weight of the remainder of the leaf was determined (fresh weight) before being drying in a 70°C oven overnight and then weighed again (dry weight). The relative water content was calculated according to the following relation:

Table 1: Characteristics of the antibodies used in this study. The antibodies, anti PGA/RG1, JIM5, JIM7, anti-RGII, LM5, anti-bupleuran and anti-XG recognise non cellulosic polysaccharide and the antibodies anti-laccase and anti-peroxydase recognised enzymes involved in cell wall metabolism.

Antibody	**Antigen/epitope**	**Characteristics**	**References**
Anti-PGA/RG1	Mostly unesterified Homogalacturonan	Polyclonal	Moore *et al.*, (1986) Lynch and Staehelin, (1992)
JIM5	Unesterified homogalacturonans	Monoclonal	VandenBosch *et al.*, (1989) Knox *et al.*, (1990)
JIM7	Methyl-esterified homogalacturonan	Monoclonal	Knox *et al.*, (1990)
Anti-RGII	Rhamnogalacturonan II	Polyclonal	Matoh *et al.*, (1998)
LM5	β(1→4) glucan	Monoclonal	Jones *et al.*, (1997)
Anti-Bupleuran 2IIc epitope	(1→3,6)-β-D-galactosyl epitope rich in uronic acids associated with pectins	Polyclonal	Sakurai *et al.*, (1996)
Anti-XG	β(1→4) glucose of the main backbone of the hemicellulose, xyloglucan	Polyclonal	Moore *et al.*, (1986) Moore and Staehelin, (1988) Lynch and Staehelin, (1992)
Anti-peroxydase	Deglycosylated horseradish peroxydase	Polyclonal	Driouich *et al.*, (unpublished)
Anti-laccase	Deglycosylated laccase	Polyclonal	Driouich *et al.*, (1992)

$$\text{Relative Water Content (\%)} = \frac{\text{(fresh weight-dry weight) / dry weight}}{\text{(full turgor weight-dry weight) dry weight}} \times 100$$

Full turgor was obtained by placing a detached leaf in water for 12 h and then weighed.

Tissue preparation

Samples were chemically fixed as described in the Material and Methods of Chapter 2. The choice of the resin was dependent on the nature of the epitopes (see discussion for details). When LRW resin was used, the fixation and dehydration procedures were similar to those used for Spurr's resin. However, no acetone were used for infiltration and LRW was progressively introduced with ethanol in v/v proportions (1/3,1/1, 3/1) for 30 min and then 1h in pure LRW. Resin polymerisation conditions were similar to that used for the Spurr's resin.

Antibodies

The monoclonal antibodies JIM5 and JIM7, specific for acid pectins and methylesterified pectins respectively, (Figure 1a) were characterized by Knox *et al.*, (1990). These antibodies were used pure for immunocytochemistry. The polyoclonal antibody anti-PGA/RG1 (dilution 1:10 in TTBS) produced in rabbit, mainly recognize the nonesterified form of the pectins (Moore *et al.*, 1986; Moore and Staehelin 1988). LM5 monoclonal antibody produced in rats (Jones *et al.*, 1997) recognizes the β(1-4) galactans of polysaccharides. This antibody was not diluted. A polyclonal antibody (anti-bupleuran 2II C epitope) was raised against the ramified region of the polysaccharide bupleuran 2II C (Sakurai *et al.*, 1999). The antigenic epitope is composed of 6-linked β-galactosyl chains carrying terminal glucuronic acid (GlcA) or 4-methyl glucuronic acid (4-Ome-GlcA) attached to (1→3)- β-D-galactosyl chains (Sakurai *et al.* 1998). This polyclonal antibody was produced in rabbit and used in this study at a 1:100 dilution. Anti-RGII was generated by Matoh *et al.* (1998) and used at a dilution of 1:50. The anti-XG polyclonal antibody is specific of the β(1-4) glucose of the xyloglucan backbone (Figure 1b). This antibody was produced in rabbit (Moore *et al.*, 1986) and was used diluted 1:20 in TTBS buffer.

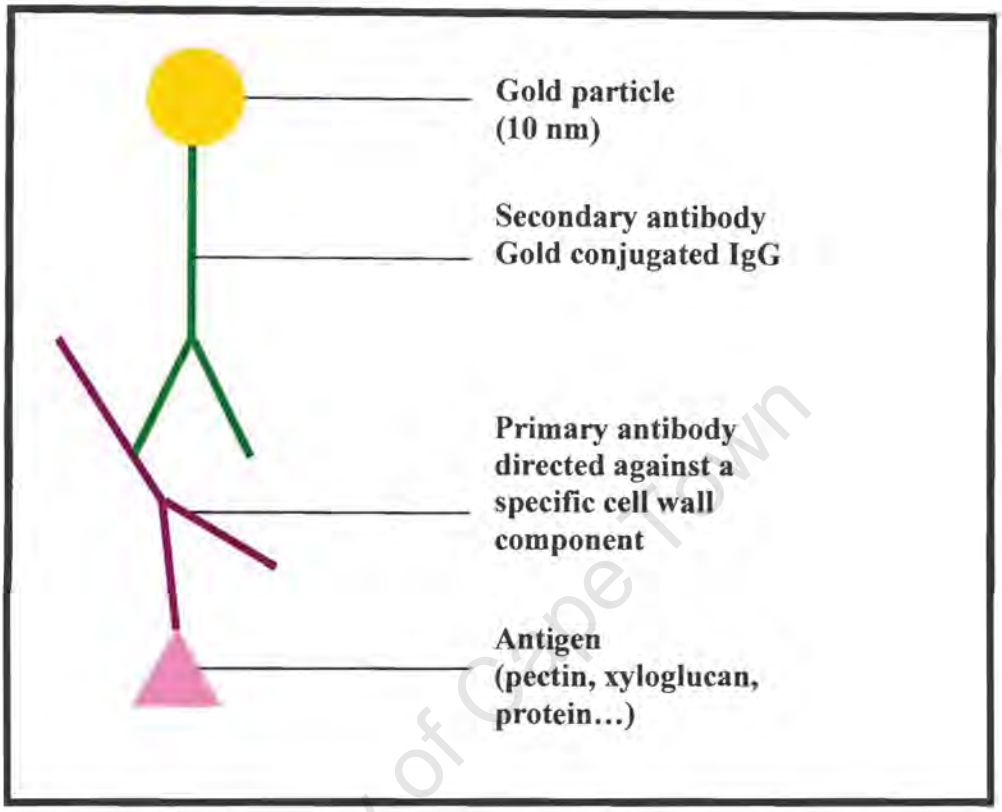

Figure 2: Schematic drawing of indirect method of immunologold labeling for localization of cell wall antigens.

The antibodies used to locate cell wall proteins were: anti-laccase (diluted 1:20 in TTBS) (Driouich *et al.*, 1992) and anti-horseradish peroxidase (diluted 1:100 in TTBS). These polyclonal antibodies were raised in rabbits.

The characteristics of the antibodies used in this study are summarized in Table 1.

Immunocytochemistry

A schematic drawing of the immunogold localisation of cell wall polymers is presented in Figure 2.

Ultra-thin sections (90-100 nm) were mounted on nickel grids. Non-specific binding sites were blocked by incubating the sections in 1% bovine serum albumin (BSA) or 0,1 % low fat milk made up in Tris-buffer saline (TBS-50 mmol/L Tris HCl and 150 mmol/L NaCl, pH 7,2). Sections were then incubated either:

1) overnight at 4°C in the antibodies anti-PGA/RG1, anti-XG, JIM7 or LM5

2) 4 hours at 30°C in the antibodies anti-bupleuran 2IIc epitope, anti-RGII or JIM5

3) 1h30 at 30°C in the antibodies anti-laccase or anti-peroxidase.

The sections were then washed with TTBS and incubated in 1:20 dilution of secondary antibody (either goat anti-rat or goat anti-rabbit, coupled to 10 nm colloidal gold (Sigma) for 1h. The sections were washed with TTBS and distilled water. Sections in Spurr's resin were stained with uranyl acetate (10 min) and lead citrate (10 min) and sections in LRW resin were stained respectively 10 min and 1 min. The sections were observed at 80 kV on a Zeiss transmission electron microscope (EM 109).

Controls were carried out by omitting the primary antibodies. The preimmune antibodies were previously tested by the authors who produced them (see references in table 1) on different plant species.

Quantification of the labeling

The number of gold particles was counted on up to 10 electron micrographs and the density of gold labeling was expressed as the average number of gold particles per μm2. StudentT test was realized to determine if the values were significantly different or not.

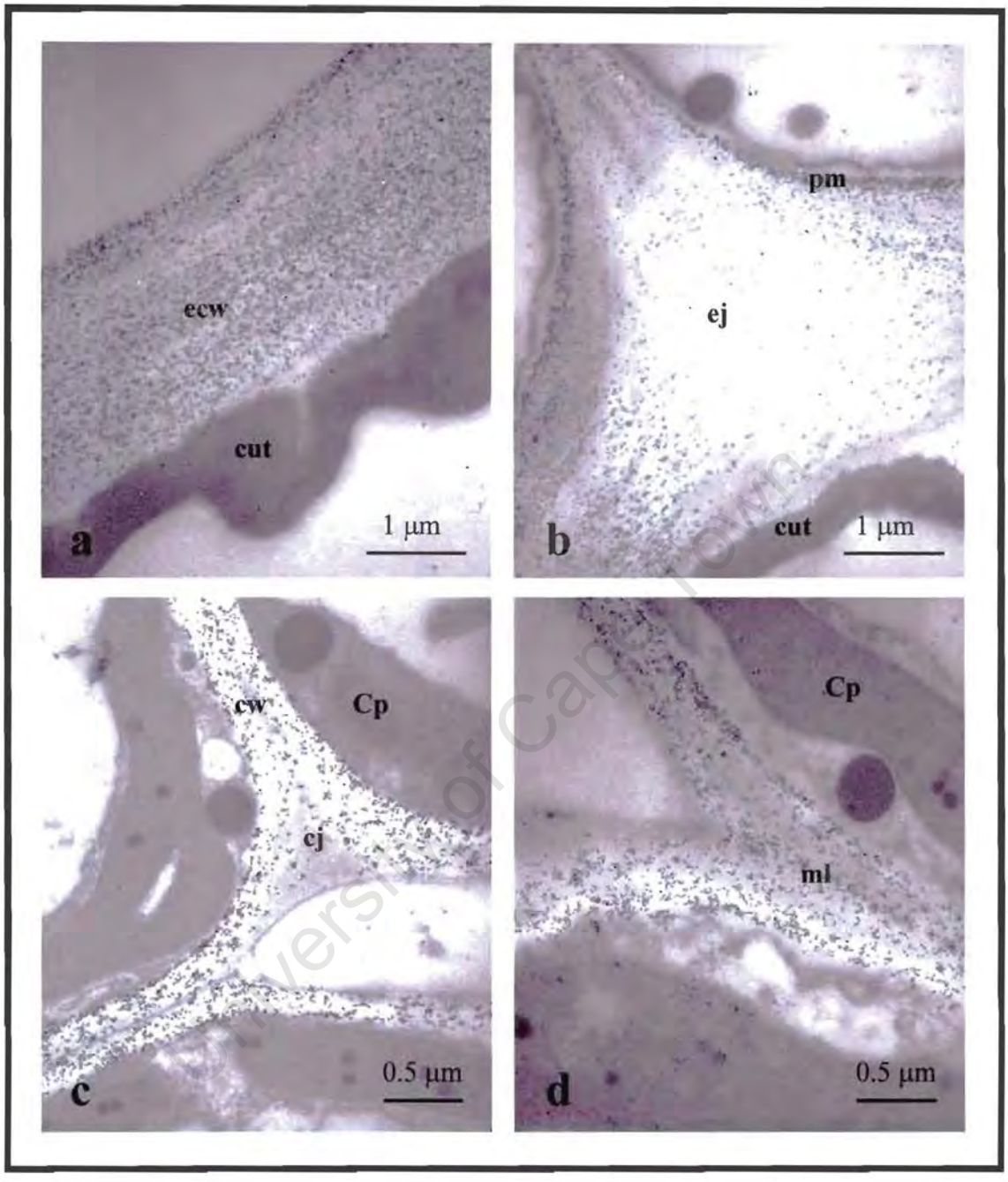

Figure 3: Immunolocalization of the bupleuran 2-IIC epitope in the cell wall of hydrated leaves of *C.wilmsii*. (a) epidermis; (b) epidermal junction; (c) corner junction in parenchyma cells; (d) parenchyma cells. (cj) corner junction; (Cp) chloroplast; (cut) cuticle; (cw) cell wall; (cyt) cytoplasm; (ej) epidermal junction; (ecw) epidermal cell wall; (ml) middle lamellea; (pm) plasma membrane.

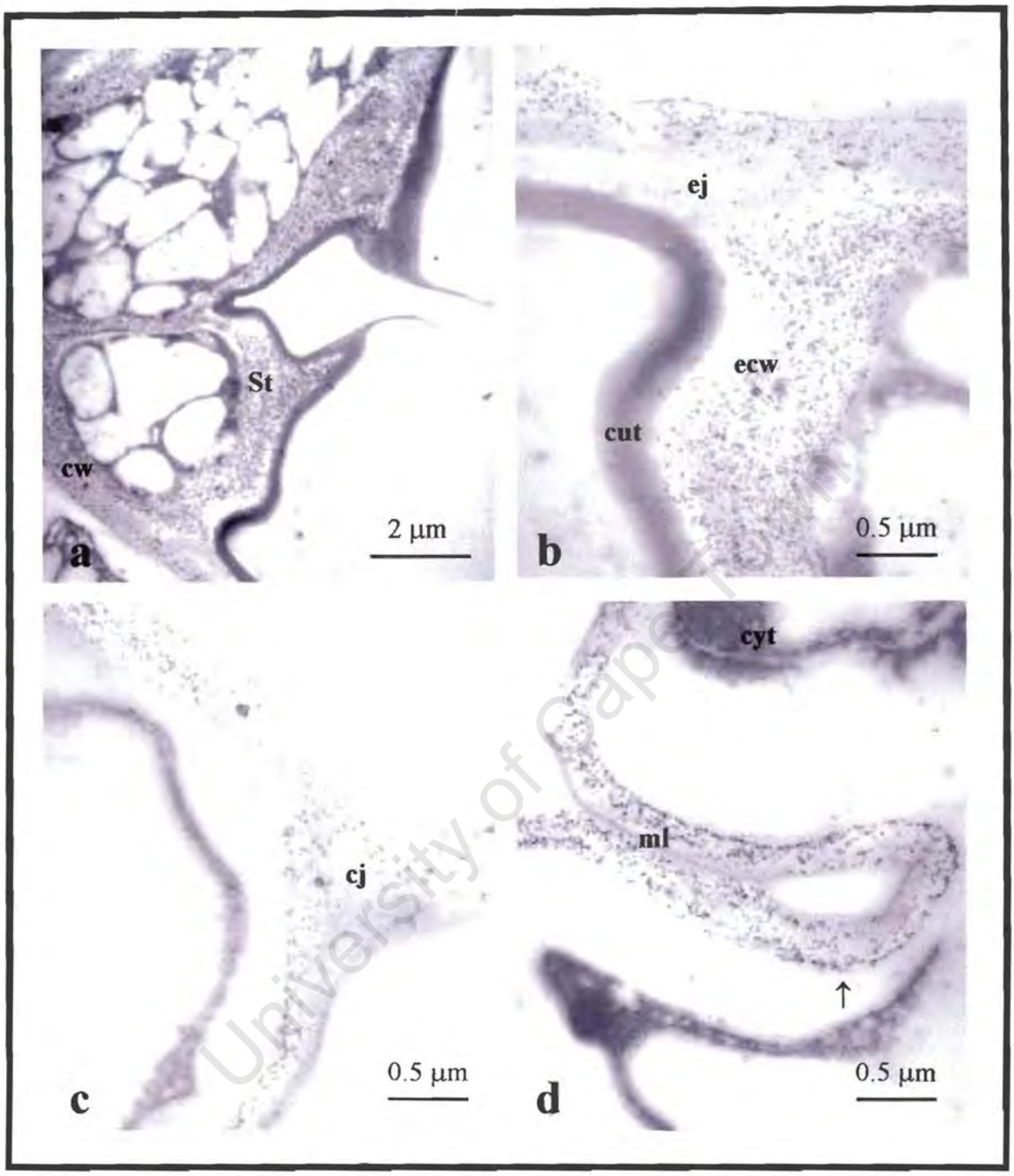

Figure 4: Immunolocalization of the bupleuran 2IIC epitope in the cell wall of dry leaves of *C.wilmsii*. (a) stoma; (b) epidermal junction; (c) corner junction in parenchyma cells; (d) cell wall folding in parenchyma cells. (cj) corner junction; (cut) cuticle; (ecw) epidermal cell wall; (ml) middle lamella; (St) stoma; (↑) cell wall folding.

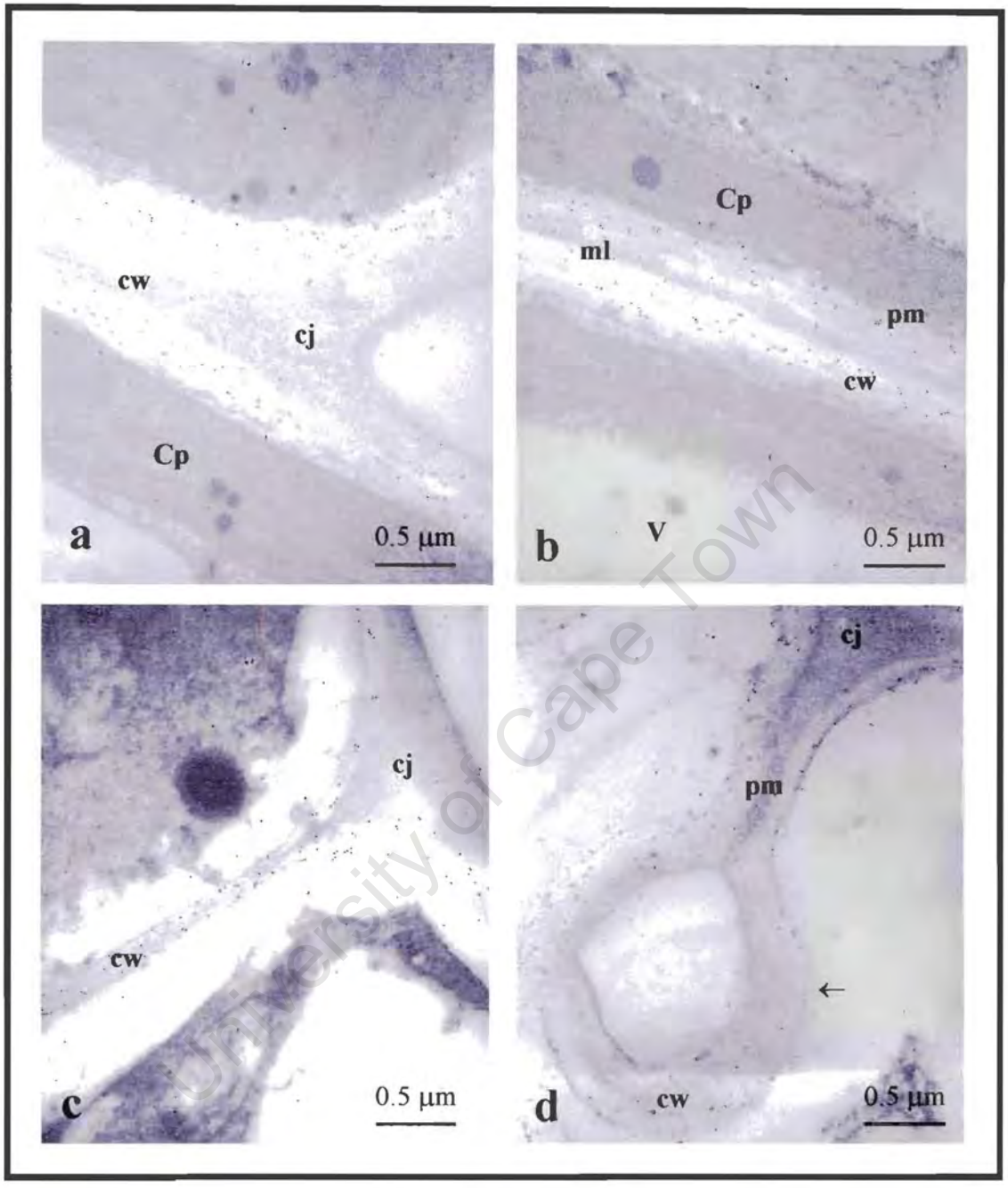

Figure 5: Immunolocalization of the polysaccharide RGII in the cell wall of hydrated (a,b) and dry (c,d) leaves of *C.wilmsii*. Corner junctions and middle lamella were deprived of labeling. (cj) corner junction; (Cp) chloroplast; (ml) middle lamella; (pm) plasma membrane; (V) vacuole, (←) cell wall folding.

3. RESULTS

Most of the labeling experiments were performed using tissue section which had been embedded in LRW and Spurr's resin. When no differences in labeling were observed among the resins, Spurr's resin was chosen because a better morphological preservation was obtained with this resin. Labeling with, JIM7, anti-PGA/RG1, anti-XG and LM5 were carried out on Spurr's embedded samples whereas JIM5, anti bupleuran, anti-RGII, anti-peroxidase and anti-laccase labeling were performed on LRW embedded plants.

Pectin localization

A polysaccharide called bupleuran 2II C contains a complex pectic epitope recognized by the anti-bupleuran 2IIc epitope antiboby. The epitope detected by the antibody consists of 6-linked β-D galactosyl chains carrying terminal glucuronic acid (Glc A) or 4-O-methyl glucuronic acid (4-OMe-GlcA) attached to (1→3) β-D-galactosyl chain. Figure 3 represents the labeling obtained with the anti-bupleuran antibodies in the different cell types of hydrated leaves. In epidermal cells (Figure 3a) the cell wall was heavily labeled but the cuticle, the cytoplasm and the resin had only few or no gold particles. The epidermal corner junction was deprived of gold labeling (Figure 3b). In parenchyma cells, the cell wall labeling was not homogeneous (Figure 3c). The corner junction had little labeling whereas in the area close to the plasma membrane (Figure 3d), the density of labeling was often relatively high. Anti-bupleuran 2IIc epitope labeling of dry cells is presented in Figure 4. Epidermal cell walls presented a very dense labeling (Figure 4a) but the corner junctions were less labeled (Figure 4b). In parenchyma cells, the corner junction was also less abundantly labeled and the area adjacent to the plasma membrane was often underlined by gold particles (Figure 4d).

Rhamnogalacturonan II was located with the anti-RGII antibodies. Parenchyma cell walls were characterized by a very specific labeling (Figure 5). In hydrated leaves, the corner junction (Figure 5a) and the middle lamella (Figure 5b) were deprived of gold particles. A same distribution was obtained with parenchyma cell wall of dry leaves. Few gold particles were detected in the corner junction (Figure 5c). Figure 5d illustrates that the middle lamella in a wall folding was often deprived of gold particles.

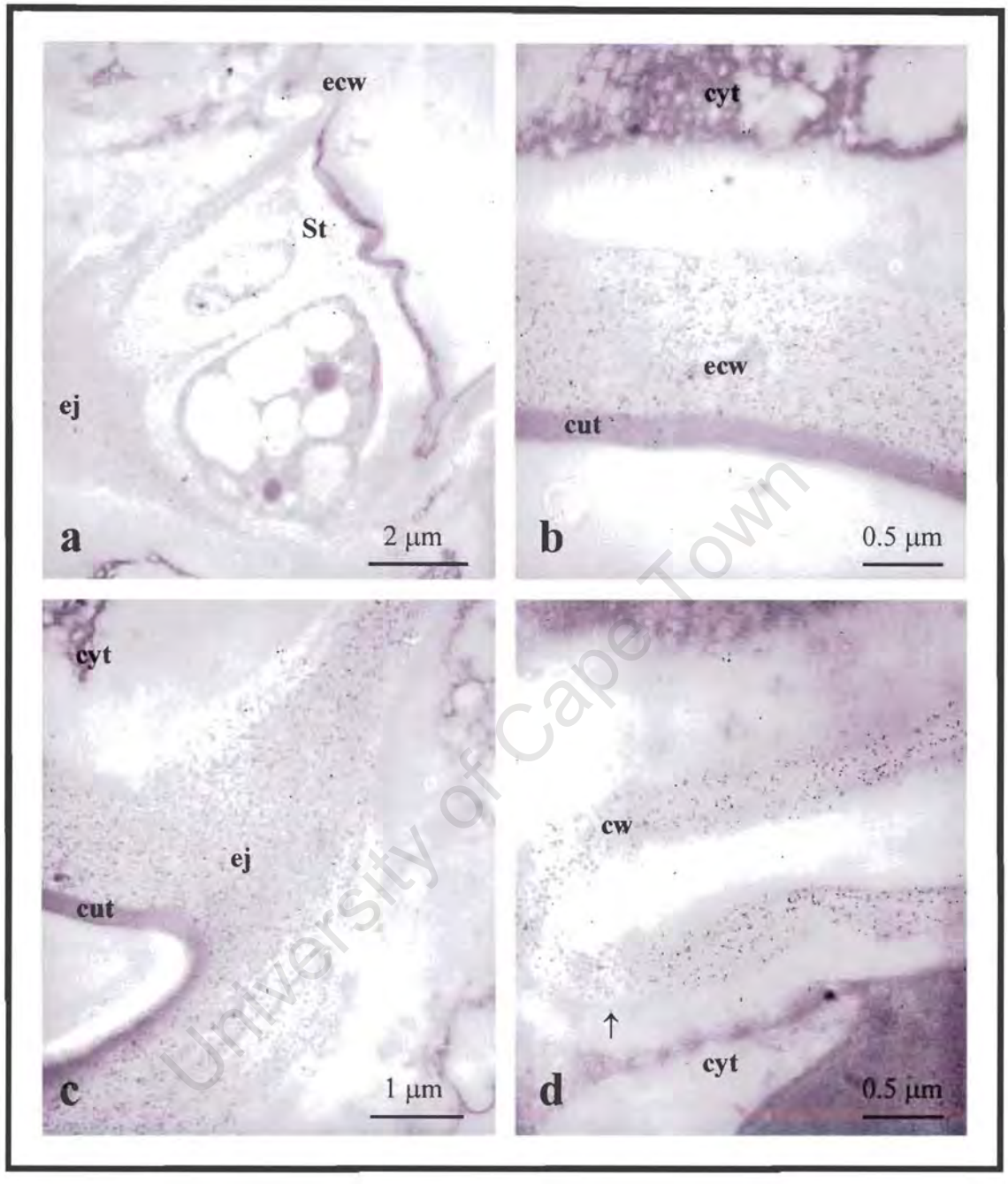

Figure 6: Immunolocalization of pectins with low degree of methylesterification using the monoclonal antibody JIM5 on dry cell walls of *C.wilmsii*. (a) stomata; (b) epidermal cell wall; (c) epidermal junction; (d) folding of the parenchyma cell wall. Labeling with JIM5 was homogeneous in the whole cell wall including corner junction, middle lamella and the area close to the plasma membrane. (cut) cuticle; (cw) cell wall; (cyt) cytoplasm; (ej) epidermal junction; (ecw) epidermal cell wall; (St) stoma; (↑) cell wall folding.

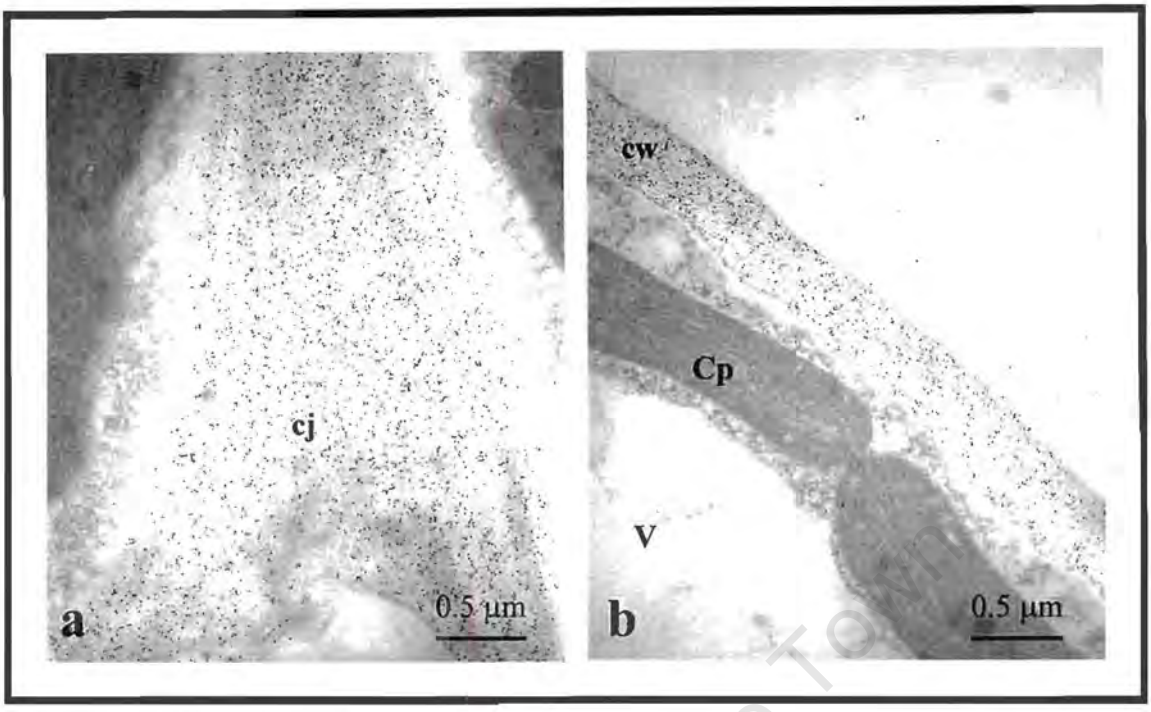

Figure 7: Immunolocalization of low-esterified pectins with the antibodies JIM5 in the cell wall of hydrated parenchyma leaves of *C.wilmsii*. Labeling was uniform in corner junction (a) and the whole cell wall (b). (cj) corner junction; (Cp) chloroplast; (cw) cell wall; (V) vacuole.

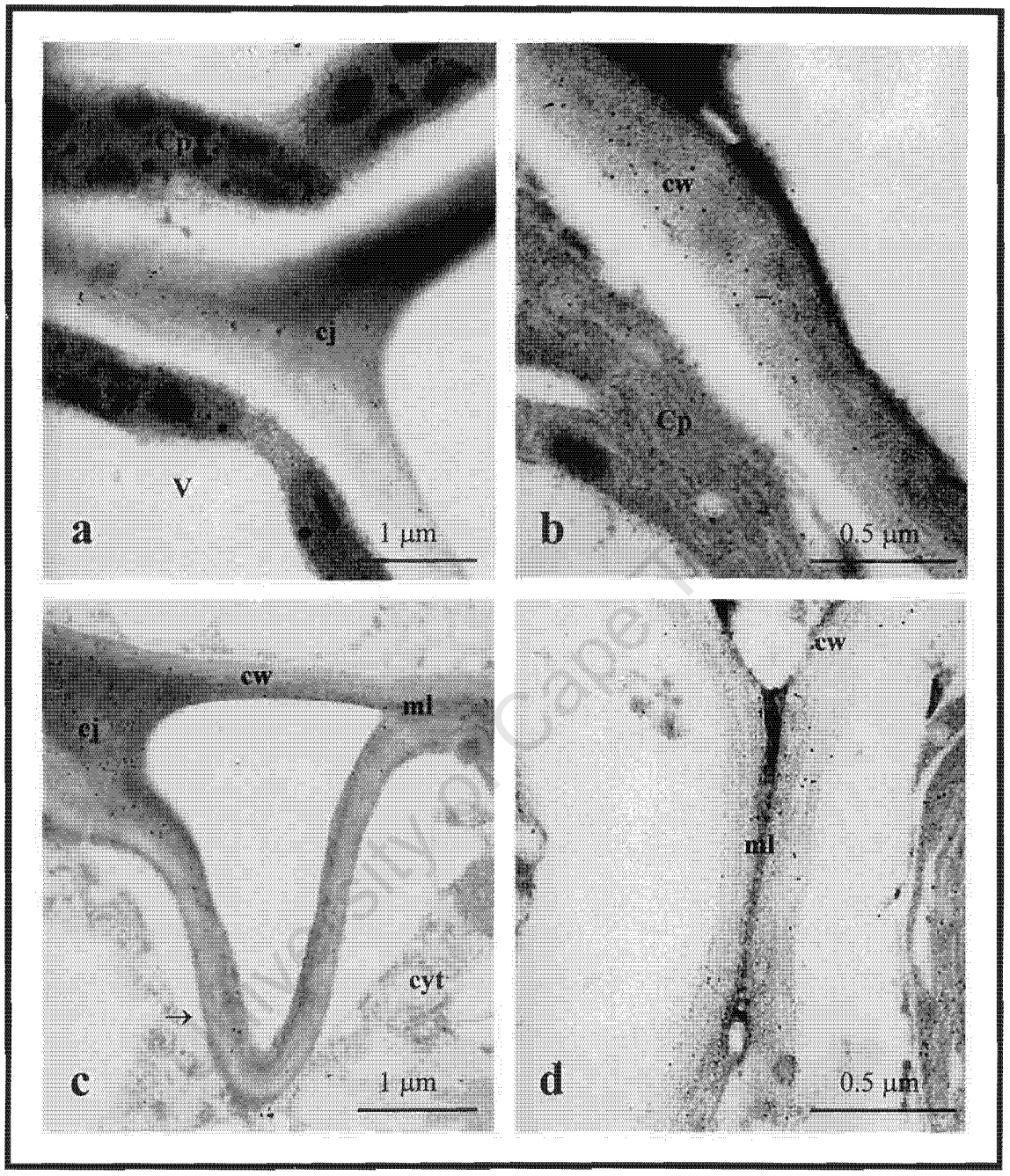

Figure 8: Immunolocalization of pectins with a high degree of methylesterification ranging between 35% and 90% with the monoclonal antibody JIM7 in hydrated (a,b) and dry (c,d) leaves. (cj) corner junction; (Cp) chloroplast; (cw) cell wall; (cyt) cytoplasm; (ml) middle lamella; (→) cell wall folding.

The labeling obtained with JIM5 antibodies specific for low esterified pectins dry leaves are presented in Figure 6. Epidermal as well as parenchyma cell walls were labeled. Figure 6a-b and c show the pattern of labeling for the epidermal cell walls in a dry plant. The localization of the gold particles was homogeneous throughout the cell wall (Figure 6b). Note the absence of gold particles on the cuticle, the resin or the cytoplasm. A very homogenous labeling was found on the cell wall of stomata (Figure 6a), and the epidermal corner junction (Figure 6c). The distribution of the gold particles in the middle lamella and the area close to the plasma membrane was uniform, no distinct cell wall domains could be distinguished. The parenchyma cell walls were uniformly labeled as shown on the folded wall (Figure 6d). A very homogenous pattern of labeling was obtained for hydrated epidermal cell wall (data not shown). The labeling obtained on parenchyma cells is represented in Figure 7. The corner junction was abundantly labeled (Figure 7a) and no particular wall domain was distinguishable (Figure 7b).

Pectins with a relatively high degree of methylation ranging between 35% and 90% are usually detected with the monoclonal antibody JIM7 (Knox *et al.*, 1990). In hydrated parenchyma leaves, unlike JIM5, the labeling with JIM7 weak, scarce and not regularly distributed along the cell wall (Figure 8b). Few gold particles were associated with the corner junction (Figure 8a). For dry leaves a similar pattern was observed with gold particles mainly present in the corner junctions (Figure 8c) and more irregularly dispersed in the wall (Figure 8d).

Density of labeling was estimated with JIM7 antibodies (Table 2). In hydrated cell walls the density of gold particles per μm^2 detected was 38 ±14 and in the dry cell wall the density of labeling was 48 ±19 gold particles per μm^2. Analysis of these data with student T-test did not reveal any significant differences for the JIM7 labeling between hydrated and dry cell walls.

The LM5 antibody recognizes β1-4 galactans of RGI side chains. The distribution of these epitopes in the cell wall showed a very specific pattern (Figure 9). No labeling was found in the middle lamella area or in the corner junction. Gold particles were located in a thin layer along the plasma membrane. This typical distribution was observed in the parenchyma cell wall of hydrated and dry plants. The density of label for hydrated cell

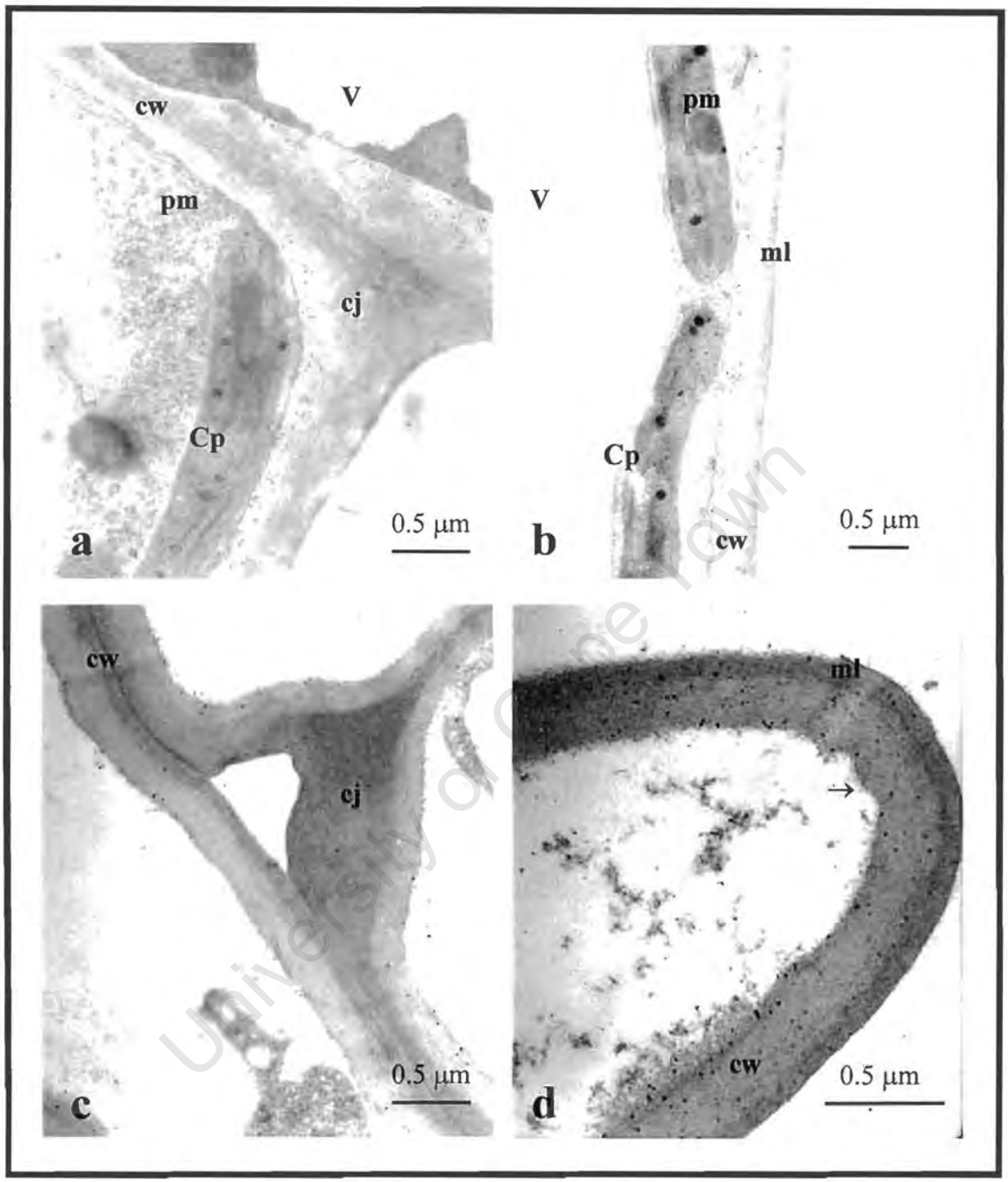

Figure 9: Immunolocalization of β(1-4) galactans with the monoclonal antibody LM5 in the cell wall of hydrated (a,b) and dry (c,d) leaves. Gold particles are mainly associated with the cell wall zone close to the plasma membrane. (cj) corner junction; (Cp) chloroplast; (cw) cell wall; (cyt) cytoplasm; (ml) middle lamella; (pm) plasma membrane; (V) vacuole; (→) cell wall folding.

Table 2: The density of labeling expressed as the number of gold particles per μm^2 for hydrated and dry leaves of *C. wilmsii*. The number of gold particles per μm^2 was counted on 10 micrographs taken from three different labeling studies. The means and the standard deviations are indicated. Significant differences were tested for between the hydrated and dry treatments using a t-test. Significant differences (95% confidence limit) are given by different letters in superscript.

Antibody	Hydrated leaves	Dry leaves
Anti PGA/RG1	51 ± 15^a	288 ± 66^b
Jim 7	38 ± 14^a	48 ± 19^a
LM 5	62 ± 12^a	58 ± 18^a
Anti XG (*C.wilmsii*- chemical fixation)	209 ± 40^a	1002 ± 91^b
Anti XG (*C.nanum*- cryofixation and freeze substitution)	419 ± 145^a	770 ± 124^b

Table 3: The density of labeling expressed as the number of gold particles per μm^2 for hydrated and dry leaves of *P. sativum*. The number of gold particles per μm^2 were counted on at least 10 micrographs taken from three different labeling studies. Significant differences were tested for between the hydrated and dry treatments using a t-test. Note that no increase in the density of labeling occurred in the dry leaves for the antibodies anti-XG and anti-PGA/RG1.

Antibody	Hydrated leaves	Dry leaves
Anti-XG	867 ± 221^a	734 ± 194^b
Anti-PGA/RG1	284 ± 101^a	230 ± 90^b

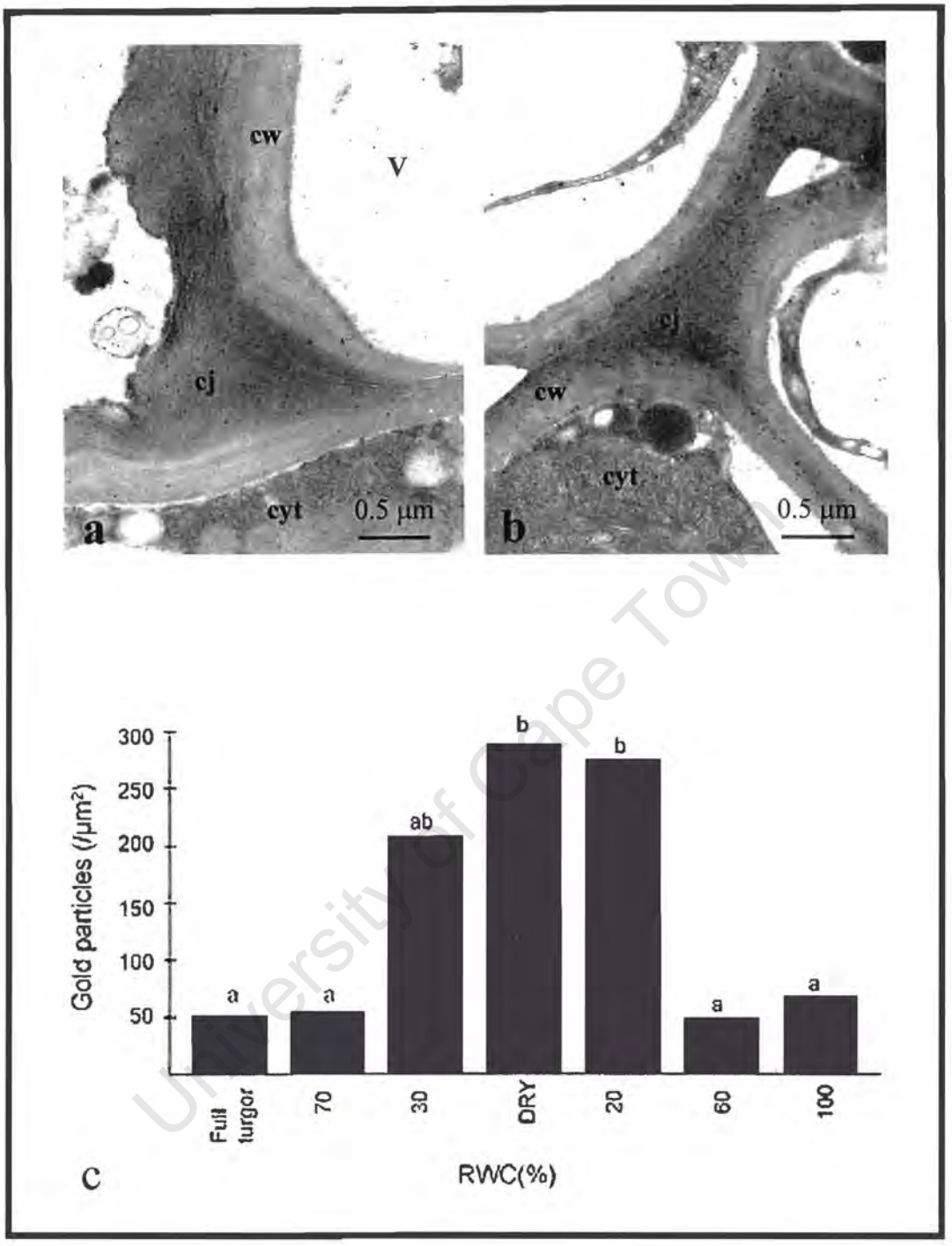

Figure 10: Immunolocalization of pectins with the antibody anti-PGA/RG1 in the cell wall of hydrated (a) and dry (b) leaves of *C.wilmsii*. (c) Density of gold labeling (per μm^2 of cell wall) estimated during dehydration and rehydration of *C.wilmsii* leaves. (cj) corner junction; (Cp) chloroplast; (cyt) cytoplasm; (cw) cell wall; (V) vacuole.

walls was 62 ±12 gold particles per μm^2 and 58 ±18 gold particles per μm^2 for dry cell walls (Table 2).

Anti-PGA/RG1 antibody was used to locate the blocks polygalacturonic acids with low degree of esterification (Figure 10). In the parenchyma cell walls labeling was mainly associated with the corner junctions and the middle area of the cell wall (Figure 10 a and b). As difference in the density of labeling was visible for hydrated and dry cell wall, quantification of gold particles was done. In dry parenchyma cell walls, the density of label was higher (288 ±66 gold particles per μm^2) than in hydrated cell wall (51 ±15 gold particles per μm^2). In order to determine when this increase of density of label occurred during dehydration, labeling with anti-PGA/RG1 antibodies was performed at different stages of dehydration and rehydration (Figure 10c). In the earlier part of dehydration at RWC 70%, the density of label was still similar to the hydrated one (55 ±28 gold particles per μm^2). At RWC 30% the intensity of labeling (208 ±53 gold particles per μm^2) was higher than the hydrated cell wall. In dry leaves, the density of label was higher and this high density was maintained during the first stages of rehydration. Upon rehydration to 20% RWC the density level was similar to dry tissues (275 ±74 gold particles per μm^2) but a large decrease occurred at leaves rehydrated to RWC 60% (50 ±18 gold particles per μm^2). Finally, the intensity of label upon further rehydration was equivalent to the density of label observed at the full turgor stage (68 ±31 gold particles per μm^2). Labeling with the polyclonal antibody PGA/RGI was also performed on leaves of the desiccation sensitive plant *P.sativum*. The number of gold particles in this experiment was estimated at 284 ± 101 in the hydrated cell wall compared to 230 ± 90 in the dry cell wall. There was no increase of labeling detected in the dry cell wall compared to the hydrated cell wall in *P.sativum* (Table 3).

Xyloglucan immunolocalization

To examine the distribution of the major hemicellulose of primary walls in *C.wilmsii*, xyloglucan was detected by the anti-XG antibodies. In the parenchyma cell wall, the xyloglucan labeling was spread evenly over the entire cell walls. In the hydrated cell wall it can be noticed that corner junctions were less labelled than the rest of the cell wall (Figure 11). There was a significant increase in the density of labeling in dry cell wall (1002 ±91 gold particles per μm^2) compared to the hydrated cell wall (209 ±40 gold

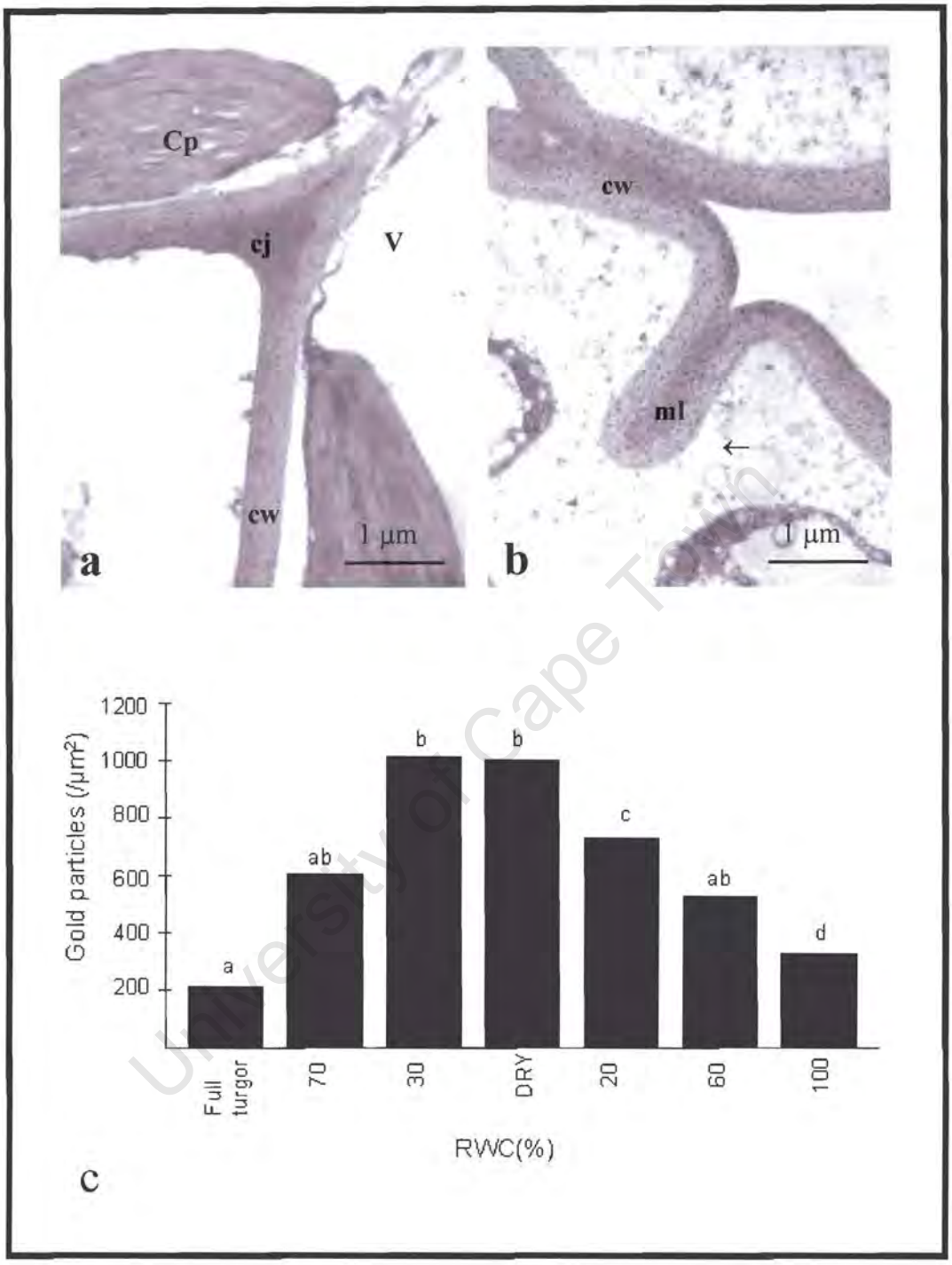

Figure 11: Immunolocalization of the hemicellulose xyloglucan with the antibody anti-**XG** in the cell wall of hydrated (a) and dry (b) leaves of *C.wilmsii*. (c) Density of gold labeling (per μm^2 of cell wall) estimated during dehydration and rehydration of *C.wilmsii* leaves. (cj) corner junction; (Cp) chloroplast; (cyt) cytoplasm; (cw) cell wall; (ml) middle lamellea; (V) vacuole; (←) cell wall folding.

particles per μm^2). Labeling with anti-XG antibodies was performed with an other Craterostigma species *C.nanum* cryofixed and freeze substituted. Even if the fixation was not optimal, cell walls were well preserved and showed folding (Sherwin, 1995). In these samples, quantification of the cell wall labeling indicated a density of 419 ± 145 μm^2 gold particles in hydrated plants compared to a density of 770 ± 124 gold particles per μm^2 for the dry plants (Table 2). Labeling of *C.wilmsii* with anti-XG was performed on plants at different relative water contents. Results are presented in Figure 11c. The density of label was higher at the onset of dehydration at RWC 70% and increased until RWC 30%, the labeling intensity remained constant to air dryness. During rehydration the density of labeling decreased progressively to reach a value close to those obtained at full turgor. Anti-XG labeling on *P.sativum* showed no increase in dry leaves compared to hydrated leaves (Table 3).

Immunolocalization of two wall proteins

The occurrence and distribution of two important wall enzymes, laccase and peroxidase in dry and hydrated plants was also examined with specific antibodies.

Laccase, an enzyme involved in cell wall lignin biosynthesis, was located using the anti-laccase antibodies. In the hydrated plant the epidermis was uniformly labeled but the area close to the plasma membrane had far less gold particles (Figure 12a). The secondary walls of vessel elements were heavily labeled (Figure 12b). In parenchyma cell walls, label was mostly associated with the region close to the plasma membrane, the corner junctions (Figure 12c) and the middle lamella (Figure 12d) being devoid of gold particles. Laccase was detected in epidermal cell wall of dry plants (Figure 13a). Labeling was distributed evenly in the whole epidermal cell wall. An intense labeling was observed in the secondary walls of vessels of dry plant (Figure 13b). This labeling was uniformly distributed. In the corner junction of parenchyma cell wall the labeling was very low (Figure 13c). This labeling was relatively dispersed and gold particles were most of the time excluded from the middle lamella area as shown in Figure 13d.

Peroxidase, involved in proteins cross-linkage, was located in the cell wall using the polyclonal antibodies anti-peroxidase (Figure 14). The peroxidase was detected in the parenchyma cell walls and the labeling was relatively intense. In hydrated cells the

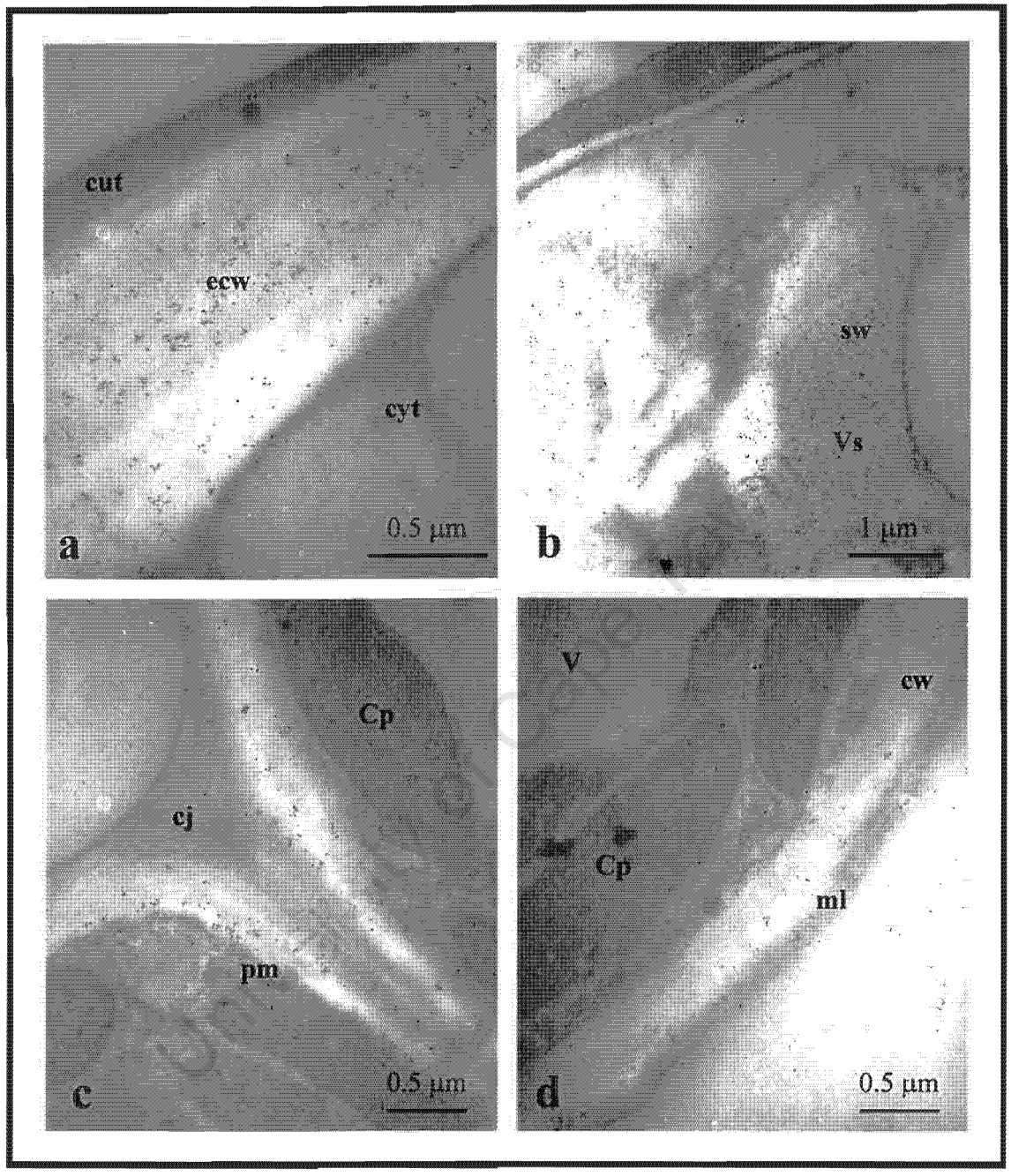

Figure 12:Immunolocalization of laccase in the cell wall of hydrated leaves. (a) epidermis; (b) vessels; (c) junction in parenchyma cells; (d) parenchyma cells. (cj) corner junction; (Cp) chloroplast; (cut) cuticule; (cw) cell wall; (cyt) cytoplasm; (ecw) epidermal cell wall; (ml) middle lamellea; (pm) plasma membrane; (sw) secondary wall; (V) vacuole; (Vs) vessels.

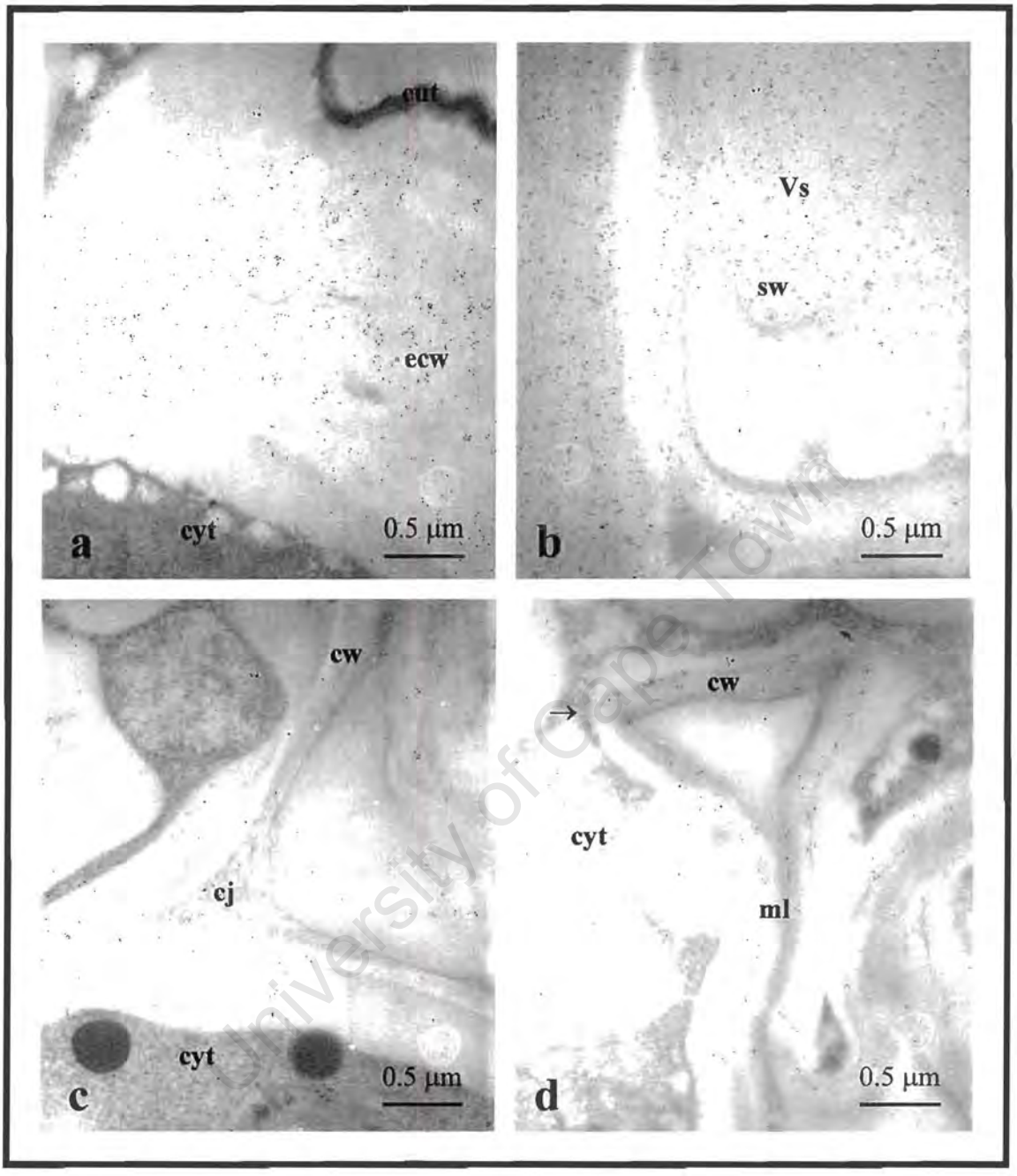

Figure 13: Immunolocalization of laccase in the cell walls of dry leaves of *C.wilmsii*. (a) epidermis; (b) vessels; (c) corner junction in parenchyma cells; (d) cell wall folding in parenchyma cells. (cj) corner junction; (cut) cuticle; (cw) cell wall; (cyt) cytoplasm; (ecw) epidermal cell wall; (ml) middle lamellea; (sw) secondary wall; (Vs) vessels; (→) cell wall folding.

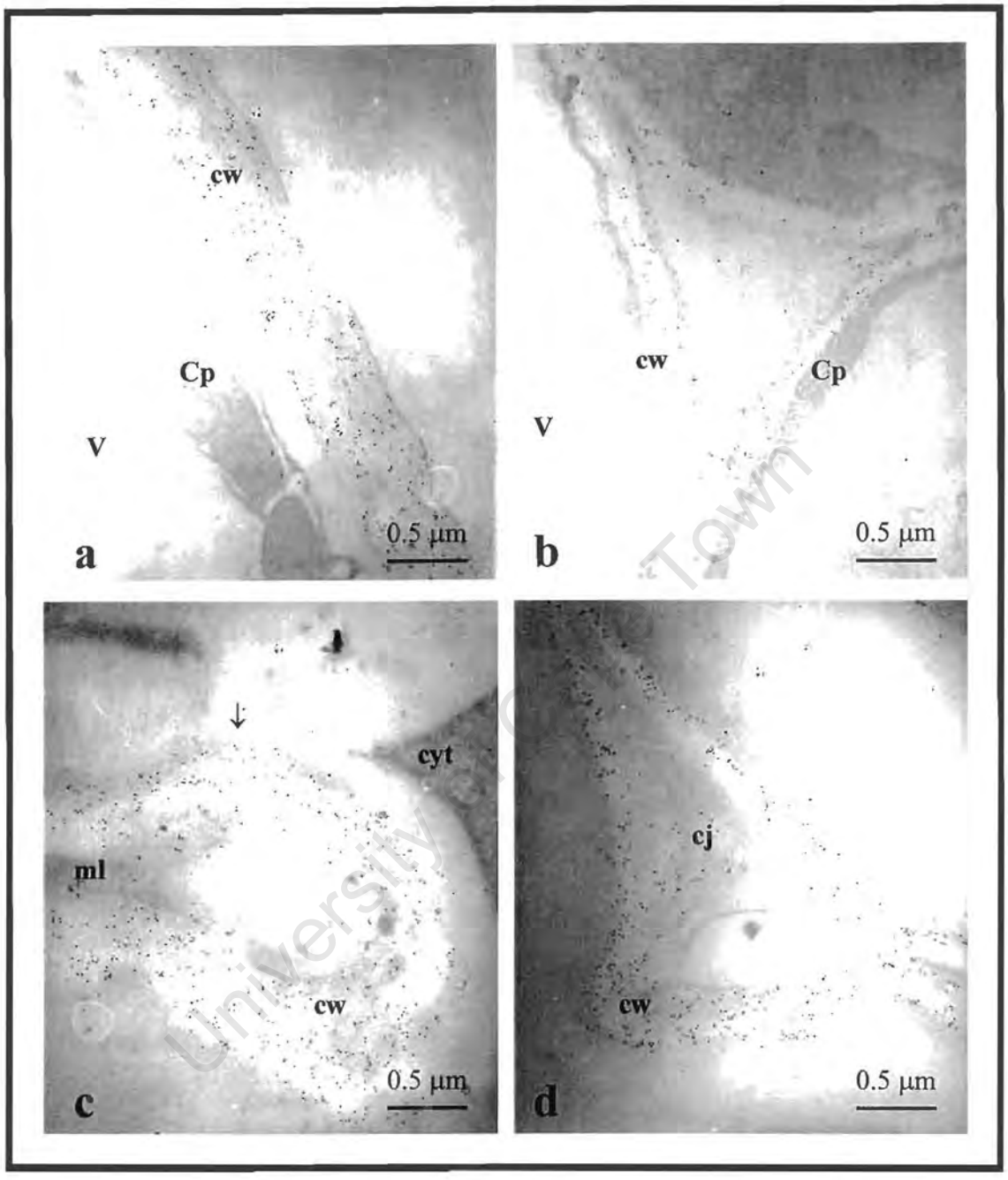

Figure 14: Immunolocalization of peroxydase in the cell wall of hydrated (a,b) and dry (c,d) leaves of *C.wilmsii*. (cj) corner junction; (Cp) chloroplast; (cw) cell wall; (cyt) cytoplasm; (ml) middle lamellea; (pm) plasma membrane; (V) vacuole; (↓) cell wall folding.

labeling was less abundant in the middle lamellea area (Figure 14a) and corner junctions (Figure 14b). A similar pattern has been observed in parenchyma cell wall of dry leaves. The middle lamella was less labeled as seen where wall folding occurred (Figure 14c) and gold particles were absent from the corner junctions (Figure 14d).

4. DISCUSSION

Tissue preparation

The choice of tissue preparation is of importance in order to preserve the antigenicity site for immunocytochemistry. Often material embedded in resins allowing a better structural preservation are less compatible with antigen maintenance. The preservation of the epitopes is variable and the choice of the resin depends on the nature of the epitopes. Immunocytochemistry studies have been performed with Spurr's resin (Berg *et al.*, 1988; Mueller *et al.*, 1993; Ferguson *et al.*, 1999; Mogami *et al.* 1999) or with LRW resin (Moore *et al.*, 1986; Peretto *et al.*, 1990; Lynch and Staehelin, 1995; His *et al.*, 1997; Zeier *et al.*, 1999). In this study labeling experiments were carried out either with Spurr's resin or LRW resin. Osmium tetroxide (OSO_4) was used as post fixative. This chemical is known to mask some protein epitopes but can be used for immunolabeling of carbohydrate epitopes associated with pectins or arabinogalactan proteins (Lynch and Staehelin, 1995; His *et al.*, 1997; Quentin *et al.*, 1997; Vicré *et al.*, 1998 a; Mogami *et al.*, 1999). In this study good labeling was obtained even with OSO_4 so we decided to keep this fixative in order to get a better preservation of the cells.

Location of cell wall polymers in *C.wilmsii*

The major goal for this study was to investigate the cell wall architecture of *C.wilmsii* leaves. This has been never done before. Furthermore, we wished to determine whether the wall folding which occurs during dehydration and which recovers an apparently normal architecture upon rehydration, was due to a specific cell wall polymer composition. Immunogold labeling revealed a specific distribution within the cell wall according to the nature of the pectins. β(1-4) galactans recognized by the antibody LM5

were mostly associated with the cell wall domain close to the plasma membrane. Immunocytochemistry with LM5 antibodies was previously performed on developing flax root tip (Vicré *et al.*, 1998 a). Only peripheral cells were labeled by this antibody. In these cells gold particles were also restricted to the area close to the plasmamembrane. A similar location of this epitope was found in cambium from aspen shoot (Ermel *et al.*, 2000) and in *Arabidopsis* root cells (Driouich *et al.*, unpublished). Thus it seems that the wall distribution of β(1-4) galactans is common to most dicotyledon plants including the resurrection plant *C.wilmsii*.

A similar distribution was found with the antibody anti-bupleuran IIC. Although labeling was more spread over the wall than what was observed with LM5, labeling with anti-bupleuran antibody in corner junctions was sparse in epidermal and parenchyma junctions. This result occurred in cell walls from hypocotyl and root tissues of flax seedlings (Andeme-Onzighi *et al.*, 2000a). These authors have suggested that LM5 and bupleuran could be carried by the same polysaccharide, probably the RG1. The plasma membrane area contained also Rhamnogalacturonans II, corner junctions being deprived of gold particles. Low methylesterified pectins detected by JIM5 were abundant and equally distributed in parenchyma cell walls. This result might be surprising as JIM5 is often associated with middle lamella and corner junction (Knox *et al.*, 1990). However Bush and McCann (1999) found a similar pattern in cell walls of potato tuber tissue. The epitope recognized by the antibody anti-PGA/RG1 was essentially located in the middle lamella area and corner junction as was described for other tissues (Moore and Staehelin, 1988). The hemicellulose xyloglucan was found in the whole cell wall although corner junctions were sometimes less abundantly labeled.

Laccase was found essentially in the vessels, the parenchyma cell wall was characterized by a weaker labeling with this antibody. Laccase is involved in lignin biosynthesis pathways. Lignins are major components of secondary cell wall and it explains why laccase is essentially found in the vessel elements. In sycamore, Driouich *et al.*, (1992) found that laccase labeling was mostly associated with the vessels elements. Peroxidase was detected quite uniformly in the parenchyma cell walls. It can be a bit surprising to see so many gold particles in the parenchyma cell wall. The detection of peroxidase indicates that this enzyme is present in the cell wall but in any case it gives indication

about its activity. However the abundance of peroxidase in the cell wall of *C.wilmsii* parenchyma cells suggests that it may be involved in the cross-linking of some polymers in the wall (such as extensins).

Increase in xyloglucan and PGA/RG1 labeling during drying

Once the specificity of an antibody has been properly characterized, immunogold localization is a very accurate tool to locate epitopes on section surface. All the antibodies used in this study are now widely used and they have been well characterized by the authors who provided the antibodies (see Table 1 in Material and Methods). This technique gives major information about the architecture and the function of the cell. In this study an attempt has been made to quantify the density of cell wall labeling for some of the antibodies detected in the dry cell wall and the hydrated plants.

Immunocytochemistry revealed an increase in xyloglucan and PGA/RG1 labeling during drying. This was not seen in the desiccation-sensitive plant *Pisum sativum*. Both components are highly involved in giving strength to the cell wall. A primary role of xyloglucan is to bind cellulose microfibrils (Moore *et al.*, 1986; Fry, 1989 a). The cellulose/xyloglucan network is the major contributor to the tensile strength of the cell wall and largely determines its mechanical properties. Unesterified (acid) pectins bind calcium ions and in so doing also serve to strengthening the cell wall (Jarvis, 1984; Moore *et al.*, 1986; McCann and Roberts, 1991). The increase in density of labeling for these two components can be interpreted by two hypotheses: (1) The increase in gold labeling can reflect an increase in the synthesis of xyloglucan and pectins. In this case an increase in the cell wall strength can be expected. (2) As the plant dries, changes in wall architecture make these epitopes more accessible to the antibodies.

The results obtained by quantification of labeling gave a trend of what could happen in cell wall of *C.wilmsii*. However to confirm and complete accurately these data to desiccation tolerance, biochemical analyses are necessary (see Chapter 4).

Conclusion

There appears to be nothing particularly unusual about the architecture of the cell wall of *C.wilmsii* in terms of the location of the various cell wall components compared with other dicotyledonous plants. This is an important information as the composition of cell wall was never studied for resurrection plants. Immunocytochemistry shows an increase in xyloglucan and pectins recognized by the antibodies PGA/RG1 during dehydration. As this did not occur upon drying of *P.sativum*, a desiccation-sensitive plant, increase in these components in *C.wilmsii* plants could be related to desiccation tolerance. Two explanations for these observations can be advanced: (1) The increase in labeling might reflect an increase in synthesis of PGA/RG1 and xyloglucans upon drying. These components are particularly important in conferring strength to the cell wall. (2) Drying can results in modifications in cell wall architecture of *C.wilmsii* making these epitopes more accessible to the antibodies. Biochemical studies are necessary to confirm and elucidate these results.

CHAPTER 4 :

BIOCHEMICAL ANALYSIS

CHAPTER 4: BIOCHEMICAL ANALYSIS

1. INTRODUCTION

Biochemical techniques are essential in the elucidation of cell walls composition (Jarvis *et al.*, 1981 a, b; Gross, 1984; Fry, 1988; Goldberg *et al.*, 1989; Davis *et al.*, 1990; Dawson *et al.*, 1992; Zablackis *et al.*, 1995; Reiter *et al.*, 1997; Edelmann *et al.*, 1998; Girault *et al.*, 2000; Mollet *et al.*, 2000; Rizk *et al.*, 2000). These methods proved to be very helpful for plant biochemists and food scientists in order to determine the fine structure of wall polysaccharides and to understand their physiological and biochemical roles in plants or their physico-chemical functions in food.

Common biochemical procedures used to study cell wall polysaccharides composition consists of isolation of cell wall material from cell membranes and fractionation of the polysaccharides into monomers or oligomers. This is generally achieved by trifluoro acetic acid (TFA) hydrolysis which gives a TFA-soluble fraction containing non cellulosic polysaccharides and a residue consisting mainly of cellulose (Albersheim *et al.*, 1967; Lorences and Zarra, 1986; Weiser *et al.*, 1990; Morrison and Stewart, 1998). Sequential extractions separating polymers according to their properties are also performed. These usually consist of a series of extraction using successively water (to remove polysaccharides weakly bonds), a calcium chelator such as EDTA or CDTA (to remove pectins linked *via* calcium bridges) and finally an alkaline solution (to remove mainly hemicelluloses bound to the cellulose microfibrils *via* hydrogen linkage) such as NaOH or KOH (Mankarios *et al.*, 1980; Carpita, 1984; Zwiazek 1991; Zablackis *et al.*, 1995; Redgwell *et al.*, 1997; Edelmann *et al.*, 1998).

The mixture of monomers or oligomers can be analyzed by various techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC), Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) according to the type of information required.

Biochemical studies have revealed that cell wall metabolism is very sensitive to osmotic stresses such as drought, salinity or cold (Iraki *et al.*, 1989 a, b, c; Weiser *et al.*, 1990; Zwiazek, 1991; Wakabayashi *et al.*, 1997; Kubacka-Zebalska and Kacperska, 1999; Harrak *et al.*, 1999; Marshall *et al.*, 1999). Water deficit stress has been reported to induce various changes in cell wall compositions which vary depending on the type of wall materials present. Sakurai *et al.* (1987 a) reported that water stress reduced the cellulose content and most hemicellulosic polysaccharides. The polymerization of the hemicellulose, xyloglucan, was also suppressed under water stress (Sakurai *et al.*, 1987 b). Polyethylene glycol (PEG)–induced water stress resulted in growth suppression and reduced cellulose and hemicellulose contents in wheat coleoptiles (Wakabayashi *et al.*, 1997). White spruce needles subjected to severe drought stress had an increased hemicellulose and a decreased pectin content, whereas no changes occurred in the cellulose fraction (Zwiazek, 1991). Low temperature-induced modifications of cell wall content was studied in winter oilseed rape (Kubacka-Zebalska and Kacperska, 1999). Exposure of cold-acclimated leaves to a freeze/thaw treatment decreased the cell wall content. A decreased level of non-covalently bound pectins was found and the content of xylose and glucose from the hemicellulosic fraction was reduced. These cell wall modifications were dependent on the range of temperatures the plant was exposed to.

This chapter presents the biochemical analysis of the cell wall composition of hydrated and dry leaves of *C.wilmsii*. That is, the cell wall components of hydrated and dry *C.wilmsii* leaves were isolated, purified and chemically characterized. Attention was focused on carbohydrate composition and the aim of this study was to determine if:

1) the cell wall composition of *C.wilmsii* leaves was similar to that of other dicotyledonous plants
2) the sugar composition and proportions were affected by the desiccation of the plants.

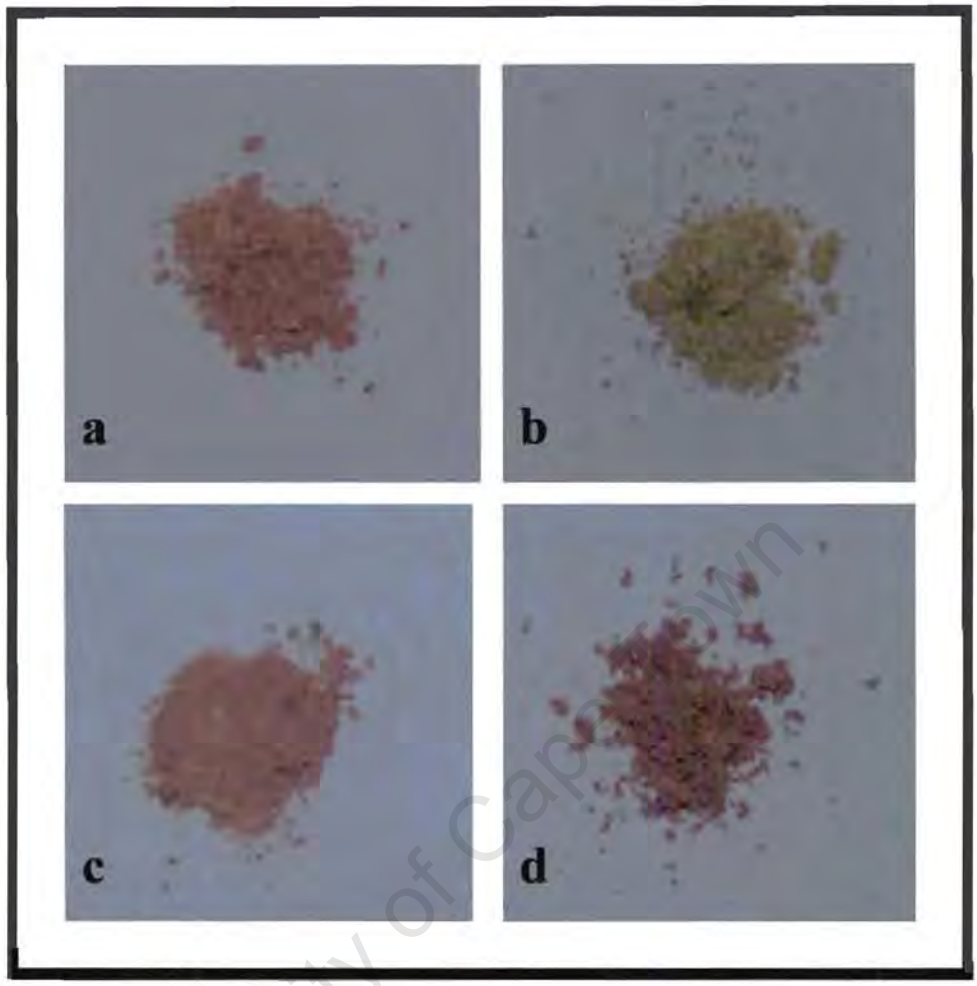

Figure 1: Cell walls from hydrated (a) and dry (b) plants after 1 hour washing in acetone. Dry cell walls presented a green colour whereas cell walls from hydrated plants were brown. This showed that chlorophyll was more difficult to extract from dry plants. Cell walls from hydrated (c) and dry (d) plants after one night in acetone (with fresh acetone). This was necessary to get clean dry cell walls.

2. MATERIAL AND METHODS

Leaves from hydrated and dry plants were frozen in liquid N2 and freeze dried for 72h in a Speed Vac SC110. After freeze drying, the leaves were stored at -80°C in presence of potassium, sodium alumino-silicate beads to avoid moisture absorption. This material was transported from South Africa to France (where I have performed the biochemical analyses) on ice.

Alcohol-insoluble residue (AIR)

The freeze dried leaves were separated into two sets of 2 grams (for both hydrated and dried plants). Leaves were ground to a powder in a mortar. The powder was weighed and the volume was measured using a conical flask. To prevent enzymatic cleavage of wall components during preparation, the leaves were suspended in boiling ethanol for 15 min and filtered. The filtrate was transferred into methanol/acetone (v/v) and agitated for 24h and then washed in 80% acetone. This type of preparation is called alcohol-insoluble residue preparation. The residue will be termed cell wall material (CWM). After 1h of incubation in acetone, the wall extract from dried plants were still a green color whereas hydrated cell walls were a brown color (Figure 1). The green color of the dry cell wall indicated that there was still some chlorophyll in the fraction. Both hydrated and dried cell walls were then placed in a fresh acetone solution and agitated overnight. The isolated walls were filtered and dried at 80°C overnight. The mass of the wall was material was determined and yields expressed the percentage of cell wall (% of dry weight) recovered from lyophilized leaves from hydrated and dry plants.

Preparation of cell wall material (CWM)

Boiling water: extraction of neutral pectins

Boiling water allowed mainly the extraction of neutral pectins. Cell walls were suspended in distilled water and pectins were extracted by three incubations in boiling water of 2 hours each. After filtration the soluble fractions were pooled together, concentrated by

rotavapor and lyophilised. Their dry mass was determined and the yield (expressed as the percentage of cell walls) calculated.

EDTA: extraction of acid pectins

The cell wall residue left after boiling water extraction were treated with EDTA to remove pectic substances linked *via* calcium bridges. Three extractions (2 hours each) were performed in boiling EDTA (0.1%, pH 7.5). The soluble fractions were pooled, filtered, concentrated and passed through amberlite IRH resin (previously equilibrated with H_2S0_4) for EDTA removal. Dialysis against distilled water occurred for 24 hours at room temperature (Mr cut-off 3500). The release of ions was checked by measuring water conductivity before and after dialysis. Fractions were lyophilized and the dry weight measured. Yield were expressed as a percentage of the cell wall.

NaOH: extraction of hemicelluloses.

The cell walls were further extracted in boiling NaOH (1.2M). The extraction occurred in presence of 1 % $NaBH_4$ in the dark and under bubbling N_2 to prevent oxidation. Soluble fractions were neutralized on amberlite IRH resin, concentrated and dialyzed before being lyophilized and weighed. Yield were expressed as a percentage of the cell wall.
The residue consisting mainly of **cellulose** was dried and its dry weight was recorded.

Trifluoroacetic acid (TFA): extraction of non cellulosic polymers from the final residue

In order to verify if some polysaccharides were still bound to cellulose, some aliquots from the residues were subjected to a mild hydrolysis with 2N trifluoro acetic acid (TFA) for 30 min, the tubes being placed in boiling water. TFA is generally used to hydrolyze non cellulosic polysaccharides, the soluble fraction being non cellulosic polysaccharides and the residue consisting of cellulose .
The insoluble residue (cellulose purified) was separated from the supernatant (hydrolyzed non cellulosic polysaccharides) by centrifugation. The supernatant was removed and the TFA was evaporated under a stream of air and a mild warming leaving a residue corresponding to hydrolyzed polysaccharides.

Characterization of polymers

Total sugars quantification

The total sugar content of each fraction was determined by the phenol sulfuric method using galacturonic acid (GalA) as standard (Dubois *et al.*, 1956). These experiments were performed 10 times. When solubilization was incomplete (EDTA and NaOH fractions), samples were first hydrolyzed with TFA or H_2S0_4 (for 2h at 80°C) and sonicated before phenol sulfuric assays. In this case, standards were prepared in the same conditions. Data were means of 2 values.

Uronic acid quantification

Total uronic acid contents were estimated colorimetrically by the m-hydroxyphenyl method (MHDP) according to Blumenkrantz and Absoe-Hansen (1973) using Gal A as standard.

Protein quantification

Protein content in each fraction was determined using the protein assay kit from Bio-Rad according to the method of Bradford (1976).

Determination of the degree of methylation and acetylation by HPLC

Degrees of methylation (DM) and acetylation (DA) of cell wall material were estimated according to Voragen *et al.* (1986). The HPLC column Aminex HPX-87H (elution H_2SO_4 5 mM 2 ml/min) allowed the separation of acetic acid and methanol. Pectins were saponified with a solution of isopropanol and NaOH (v/v) overnight at 4°C. The solution was centrifuged and the supernatant was injected onto the column (*via* a filter Millipore SJHVLO4 NS at the entrance of the injector). The values corresponding to the peaks of ethanol and acetic acid released from the pectins after saponification were compared to the standard. The degree of methylesterification and acetylation were determinated by reporting the quantity of methanol or acetic acid over the quantity of total extract injected. Results were means of 4 values.

Monosaccharide composition analysed by Gas Chromatography.

The monosaccharide composition of each fraction was determined by gas chromatography (GC). A solution of MeOH/HCl 4M was prepared using chloride acid (MeOH/HCl kit Altech) and anhydrous methanol precooled at –80°C. The solution was stored at –80°C and diluted with anhydrous MeOH to MeOH/HCl 1M before using.

Samples (0.5 mg of polysaccharide) were mixed with MeOH/HCl 1M (500 µl) for methanolysis 24h at 80°C. The solution was then evaporated under a stream of air and the samples were washed with MetOH and dried. This step was repeated three times. Samples were methylsilylated with 100 µl of Tri-Sil for 2h at 80°C. The solution was evaporated under a stream of air and the residue was suspended in 20 µl of pyridine and 1ml of cyclohexane. One µl was loaded on the Ross injector. The temperature conditions used for capillary column DB 225 (20m × 0.1 mm I.D) were 105°C for 1 min, 105°C to 155°C at 5C min-1, 155°C to 165°C at 0.5°C min-1, 165°C to 210°C at 5°C min-1, 210C for 10 min; FID:8 Range 11. Inositol was used as internal standard.

Sugars detected by CG were arabinose (Ara), rhamnose (Rha), xylose (Xyl), mannose (Man), galactose (Gal), glucose (Glc), glucuronic acid (GlcA) and galacturonic acid (GalA).

Thin layer chromatography

TLC analysis were performed to determine if other monosaccharides than those detected by GC were present in the fractions. Sugars consisted of Ara, Rha, Xyl, Man, Gal, Glc, GlcA, GalA and fucose (Fuc).

Samples (1mg of sugar/ml) were treated by 2N TFA for at 100°C in an oven. After being washed and dried, the samples were suspended into 100 µl of water and a volume of 10 µl was loaded at the bottom of aluminium sheets covered by Silica gel (Merk 5564). Standards were loaded following the same procedures. Migration occurred overnight with a solvant composed of butanol/acetic acid/H_2O (2v/v/v). The sheets were plunged in a solution of H_2SO_4 (20%) and orcinol (0.1%) in ethanol. The spots were revealed after being dried at least 5 min in a 100°C oven.

Table 1: Extraction of cell walls from hydrated and dry lyophilised leaves. Lyophilised leaves from hydrated plants occupied a larger volume compared to dry plants. The difference in volume observed for lyophilised leaves is not apparent once the walls are isolated. The yield is the percentage (W/W) of walls extracted from the lyophilised leaves. The ratio expresses the mass to volume of each fraction. Data are means of 2 extractions.

	Hydrated	Dry
Lyophilized leaves		
Mass (mg)	2024	2090
Volume (ml)	11.85	4.7
Ratio (mg/ml)	171	444
Cell walls		
Mass (mg)	649.5	735
Volume (ml)	6.6	7
Ratio (mg/ml)	98	105
Yield % (W/W)	31.7	35

AGPs detection by YARIV stained gel

The Yariv reagent is a specific dye that binds AGPs (Fincher and Stone, 1983; Girault *et al.*, 2000). A gel (agarose 1%, 0.15 M NaCl, 0.1M EDTA, 30 µg/ml Yariv) was placed in petri dish. A red wine purified AGPs (Pellerin *et al.*, 1996) was used as standard. The concentration of the standard was comprised in range between 0.2 mg/ml to 3 mg/ml. A volume of 5µl was loaded on the gel for both standards and samples (20mg/ml). The diffusion was performed for 24h at 37°C in a humid chamber. Orange spots characterized the presence of AGPs.

3. RESULTS

Cell wall isolation

The volumes and mass corresponding to the lyophilized leaves and extracted cell walls are reported in Table 1. A significant difference could be noticed between the volume of hydrated and dried lyophilized leaves. As indicated by the ratio (mass/volume) hydrated lyophilized leaves (171 mg/ml) occupied a larger volume than the dry lyophilized leaves (444 mg/ml) for a same mass. After cell wall extraction this ratio was identical for hydrated (98 mg/ml) and dry (105 mg/ml) plants. This ratio was also similar for hydrated and dry walls after sequential extractions with boiling water, EDTA and NaOH.

The content of cell wall (expressed as a percentage of lyophilized leaves) represented 31,7 % for hydrated plants and 35 % for dry plants. No significant differences were observed in cell wall yield between hydrated and dry plants.

Fractionation of cell wall polysaccharides

Quantities of polymers extracted by boiling water, the chelator EDTA and NaOH were estimated as a percentage of the isolated cell walls (Figure 2). No major differences in percentage were observed between hydrated and dry plants. There was a large proportion of pectins present in the walls of both hydrated and dry plants. Pectic substances extracted with boiling water represented 15 % of the total cell wall of hydrated and dry plants whereas pectins extracted by EDTA represented 28 % and 32 % of the cell wall

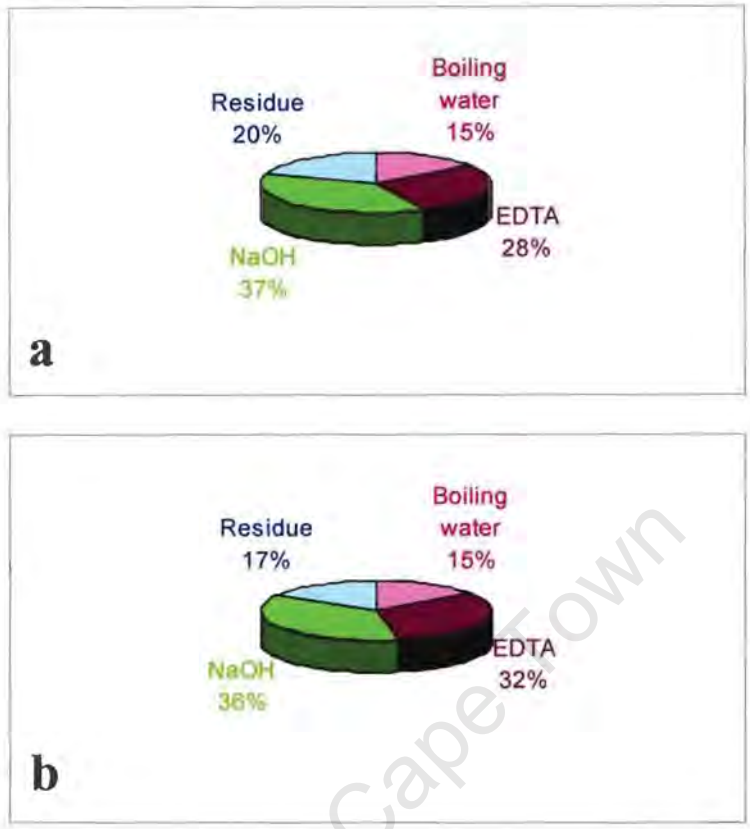

Figure 2: Quantities of polymers extracted successively with boiling water, EDTA and NaOH from cell walls of hydrated (a) and dry (b) plants. Data are the average of two extractions and are expressed as the percentage of cell wall (% of dry weight).

isolated from hydrated and dry plants respectively. Boiling water extracted mainly neutral pectins whereas EDTA removed pectins linked *via* calcium bridges. Hemicelluloses extracted with boiling NaOH comprised 37 % of the cell wall from hydrated plants and 36 % of the cell walls from dry plants. Residue which was not extracted by different treatments was regarded as the cellulose fraction. The residue represented 20 % of the walls from hydrated plants and 17 % of the walls from dry plants.

Total sugars and uronic acid content estimation

The results obtained for total sugars and uronic acid assays are presented in Table 2. These are expressed as a percentage (dry weight) of each fraction.

The sugar content of the polymers extracted with boiling water was estimated to 86 ± 7 % for hydrated plants. Concerning the dry plant the sugar content of the fraction extracted with boiling water consisted of 74 ± 11 %. The sugar content of the polymers extracted with boiling water was slightly higher for the hydrated plants as compared to the dry plants. In contrast, uronic acids contents were similar in both fractions represented 28 ± 8 % and 24 ± 5 % in hydrated and dry plants respectively.

The most important change was the increase observed in total sugar content in EDTA fraction from dry plants compared to hydrated plants. Sugars represented 50 ± 14% of EDTA polymers of hydrated plants. In dry plants sugars comprised 81 ± 9 % of the EDTA fraction. However it has to be noticed that EDTA fractions were not completely solubilized. Therefore, attempts were realized to overcome this problem and increase solubilization of the EDTA extracted polymers. To this end, samples were hydrolysed with either TFA or H_2SO_4 and sonicated before quantification of total sugars. After TFA treatment, total sugars were estimated at 84 % and 94 % for hydrated and dry plants respectively. However, after H_2SO_4 hydrolysis, values were still low representing 47 % for the hydrated walls and 54 % for the dry walls. This low recovery might be explained by a destruction of uronic acids and pentoses due to the experimental conditions (2h, 80°C). Thus TFA seemed to be more efficient in increasing the solubilization of EDTA polymers than H_2S0_4. Although the difference found between hydrated and dry plants was reduced, total sugar content was still slightly higher in the dry plants as compared to

Table 2: Analysis of carbohydrates solubilized with boiling water, EDTA and NaOH from cell walls of hydrated and dry plants. Assays for total sugars and uronic acid were repeated 10 and 6 times respectively and standard deviation calculated. Uronic acid were not quantified for NaOH fraction (nd1). Due to the incomplete solubilization with EDTA and NaOH fractions, these fractions were hydrolysed with TFA or H_2SO_4 and sonicated before sugar quantification by Dubois method. This was not performed on boiling water as the solubilization was complete (nd2: not determined). The data are means of two values. Degree of acetylation, DA and degree of methylation (DM) were estimated; the results are means of 4 values. Protein content was estimated according to Bradford assay.

Fractions	Boiling water		EDTA		NaOH	
	Hydrated	Dry	Hydrated	Dry	Hydrated	Dry
Total sugars % (w/w)	86 ± 7	74 ± 11	50 ± 14	81 ± 9	33 ± 6	27 ± 4
Uronic acids % (w/w)	28 ± 8	24 ± 5	18 ± 9	32 ± 8	nd1	nd1
Total sugars % (w/w) solubilized with TFA	nd2	nd2	84	94	43	39
Total sugars % (w/w) solubilized with H_2SO_4	nd2	nd2	47	54	37	20
DM %	45 ± 9	57 ± 10	35 ± 4	33 ± 4	nd1	nd1
DA %	16 ± 3	14 ± 2	10 ± 0.5	7 ± 0.7	nd1	nd1
Proteins % (w/w)	2.9	3.8	1.8	1.5	1.7	1.3

polymers than H_2S0_4. Although the difference found between hydrated and dry plants was reduced, total sugar content was still slightly higher in the dry plants as compared to hydrated plants. Discrepancies in these values might indeed arise from insufficient solubilization.

Uronic acid content was lower in the EDTA fraction from hydrated plants (18 ± 9 %) than in that of dry plants (32 ± 8 %). These data might be underestimated due to the incomplete solubilization. However, these values confirmed the lower content of total sugars obtained for the hydrated plants compared to the dry plants.

Total sugar content from the fraction extracted with NaOH was slightly higher in hydrated plants (33 ± 6 %) than in dry plants (27 ± 4 %). As solubilization appears to be incomplete attempts were performed to solubilize these fractions. After TFA hydrolysis and sonication, total sugars were estimated to 43 % for hydrated plants and 39 % for dry plants. H_2SO_4 treatment gave 37 % for hydrated fractions and 20 % for dry fractions.

Estimation of the degree of methylation (DM) and the degree of acetylation (DA)

The DM and DA of cell wall material were estimated for boiling water and EDTA-extracted fractions. The results are presented in table 2. In boiling water fractions DM was estimated to 45 ± 9 % and 57 ± 10 % for hydrated and dry plants respectively. DA was lower for both hydrated (16 ± 3 %) and dry (14 ± 2 %) plants. Although polymers extracted with EDTA are thought to correspond to acidic pectins, the DM was relatively high for hydrated (35 ± 4 %) and dry (33 ± 4 %) fractions. DA were respectively 10 ± 0.5 % and 7 ± 0.7 % for hydrated and dry fractions.

Chemical composition of the polymers extracted with boiling water.

Sugars detected by thin layer chromatography (TLC)

TLC was performed in order to determine the nature of the sugar content. The patterns obtained for hydrated and dry plants are given in Figure 3a. As expected all the sugars used as standard were recognized in the samples extracted with boiling water. A spot which does not correspond to any of the standard was detected in both hydrated and dried samples. This compound was probably a small sugar due to its position on the

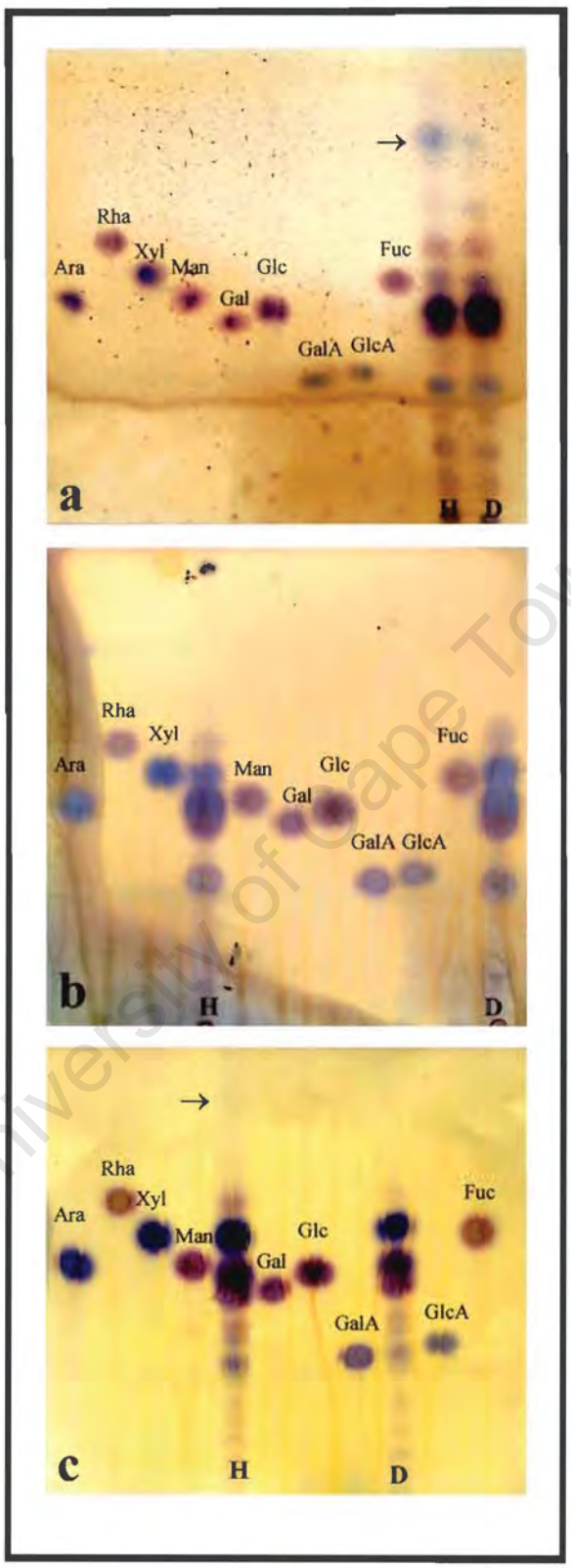

Figure 3: Sugars analysis of (a) boiling water, (b) EDTA and (c) NaOH fractions for hydrated (H) and dry (D) plants by TLC. → unknown sugar.

chromatogram. However its nature is still undetermined. This unknown spot could be in a suitable position for 2-O methyl xylose or 2-O-methyl fucose but it could also be deoxyribose derived from contaminating DNA.

Monosaccharide composition (Gas chromatography analysis)

The sugar composition of the boiling water fractions is presented in Figure 4a.

The boiling water fractions were mainly composed of GalA, Ara and Gal although Glc, GlcA and small amounts of Rha, Xyl and Man were also found. The major sugars found in these fractions are characteristic of pectins. In both hydrated and dry plants GalA was the predominant sugar. Its content was slightly lower in hydrated plants (32.1 ± 5 %) than in dry ones (38.9 ± 3.6 %). As GlcA content was higher in hydrated plants, this may explain why uronic acids content detected did not reveal significant differences between hydrated and dry plants (see Table 2). Ara was slightly lower in hydrated fraction representing 16.5 ± 4.9% as compared to 25.7 ± 7.7 % for the dry fraction. A slight loss of Glc occurred for dry plants.

Detection of Arabinogalactan proteins (AGPs)

The Yariv reagent revealed the presence of some AGPs in the boiling water fraction of both hydrated and dry plants. The gel obtained in presence of YARIV reagent is presented in Figure 5. As indicated by the red wine purified AGPs used as standard, the diameter of the spot was proportional to its concentration. The content of AGPs in the boiling fraction was relatively low. This was estimated to represent less than 0.2 mg / ml in hydrated and dry plants.

Protein detection

Protein contents are indicated in Table1. As expected the fractions extracted by boiling water contained low amounts of proteins. The contents estimated were 2.9% and 3.8% for hydrated and dry plants respectively.

Chemical composition of the polymers extracted with EDTA

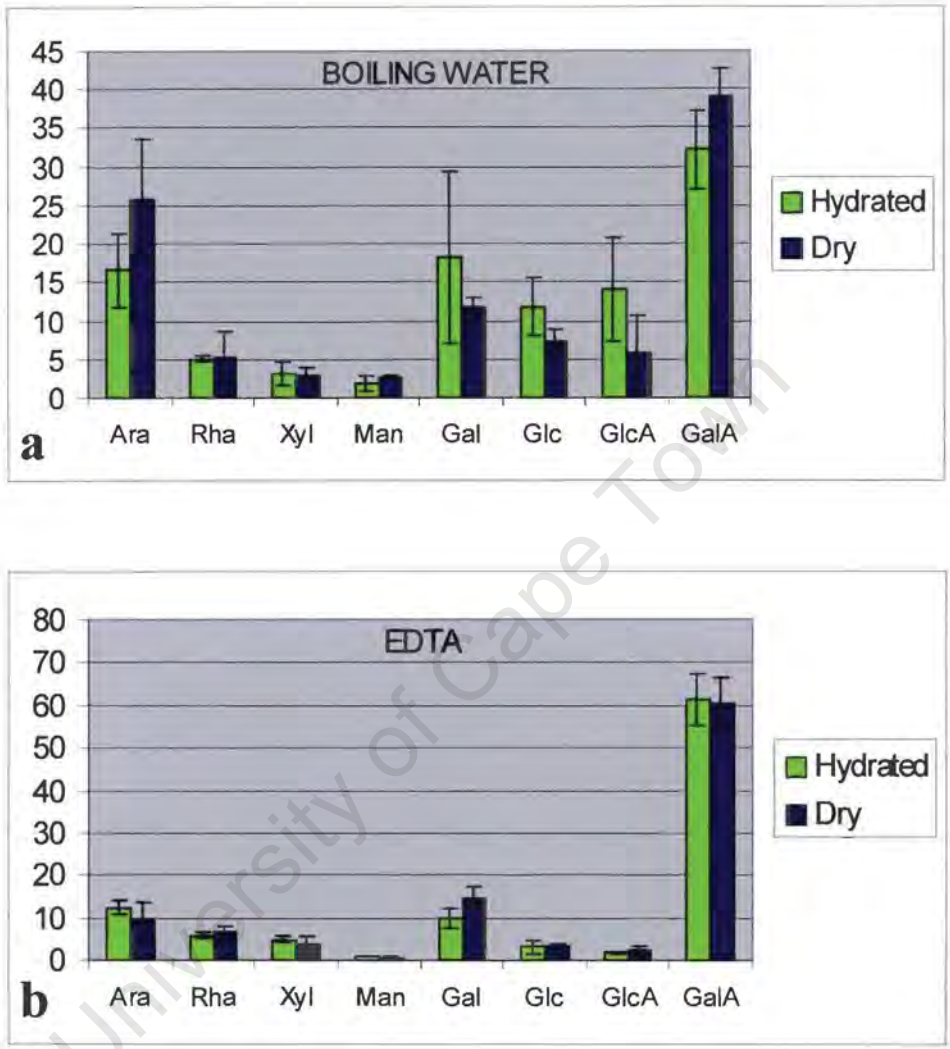

Figure 4: Chemical composition of the fractions extracted by boiling water (a) and EDTA (b) for hydrated and dry leaves. Data are means of 4 values and standard deviations are estimated. Note that no significant difference in the sugar composition of EDTA-extracted polymers is observed between hydrated and dry plants.

Sugars detected by thin layer chromatography (TLC)

The chromatogram corresponding to EDTA fractions is presented in Figure 3b. All the sugars corresponding to the standard were present and no other spot were revealed. The spot revealed in the boiling water extracts was absent in the EDTA fraction (Compare the chromatogramm a, b on Figure 3).

Monosaccharide composition (Gas chromatography analysis)

The sugar composition of EDTA fractions is given in Figure 4b. The EDTA fraction was almost completely composed of GalA in both hydrated (61.3 ± 5.9 %) and dry (60.2 ± 6 %) plants. Small amounts of Gal were also detected in both fractions, 9.7 ± 2.4 % in the hydrated plants and 14.7 ± 2.6 % in the dry plants. Ara consisted of 12.4 ± 1.8 % of the total sugars in the hydrated plants and 9.2 ± 4.5 % in the dry plants. All the others sugar were present in minor proportions. It is interesting to note that no significant difference in the content of the sugars detected between hydrated and dry plants.

Detection of Arabinogalactan proteins (AGPs)

As for the boiling water fractions, the Yariv staining revealed the presence of AGPs in the EDTA fraction for both hydrated and dry plants (Figure 5). The slight diffusion which occurred with EDTA pectins from hydrated and dry plants indicated that the content of AGPs was extremely low and lower than in boiling water fraction.

Protein detection

Determination of the proteins content using Bradford test revealed that EDTA fractions contained low amounts of proteins. The percentages of proteins were 1.8% and the 1.5% for hydrated and dry plants respectively (Table 1).

<u>Chemical composition of the polymers extracted with NaOH</u>

Sugars detected by thin layer chromatography (TLC)

Sugars present in the standards were detected in the fraction extracted with NaOH. A sugar which does not correspond to any of the standards was detected for both hydrated

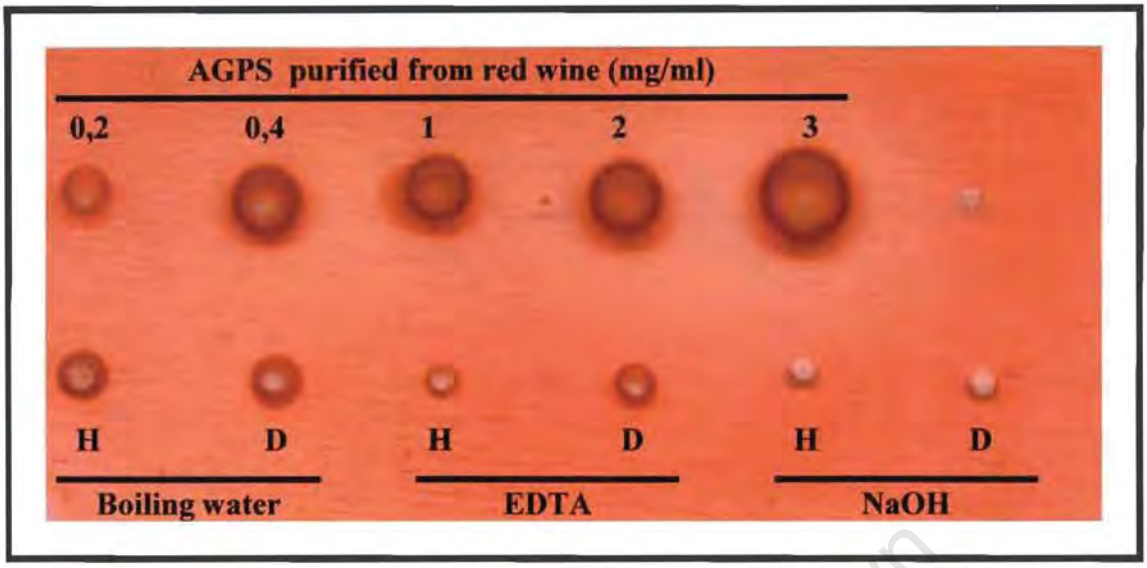

Figure 5: AGPs detection in hydrated and dry plants by YARIV stained gel. Orange spots characterizing the presence of AGPs were detected in boiling and EDTA fractions. A red wine purified AGPs (Pellerin et al; 1996) was used as standard.

and dry plant although extremely weak (Figure 3c). This spot corresponding to a small sugar was found at the same position as the one detected in the boiling water extract.

Monosaccharide composition (Gas chromatography analysis)

The results obtained with GC analyses are presented in Figure 6a. This fraction was characterized by the presence of many sugars. The major sugars detected were Glc, Gal and Xyl reflecting the presence of the hemicellulosic polysaccharide xyloglucan. The content of GalA and Ara were also quite high, although not as high as Glc content. This suggests that some pectins are also present in the NaOH fraction. Glc was the predominant sugar in hydrated plants. In these plants, Glc content represented 30.5 ± 3% whereas Gal and Xyl represented 17.4 ± 3 % and 16.2 ± 3 % respectively. Another major finding of this analysis is that unlike Glc, Gal content was lower in hydrated plants than in dry ones. It is interesting to note that the content of Glc was significantly higher in hydrated plants (30.5 ± 3%) as compared to dry plants (17.7 ± 1.9 %). Interestingly, in hydrated plants, Glc content was higher than Gal and Xyl contents which was not the case with dry plants.

Detection of Arabinogalactan proteins (AGPs)

To check for the presence of AGPs, NaOH fraction was tested using Yariv staining on agarose gel. The absence of staining by the Yariv reagent indicated that AGPs were probably not present among the polymers extracted with NaOH, either in hydrated or dry plants.

Protein detection

Quantification of the proteins using Bradford test revealed that NaOH fractions contained low amounts of proteins. The percentage of proteins was 1.7 % and the 1.3% for the hydrated and dry plants respectively (Table 1).

Chemical composition of the polymers extracted with TFA

The residue left after sequential extraction by boiling water, EDTA and NaOH consisted mainly of cellulose. The cellulosic fraction was treated to check if some polysaccharides

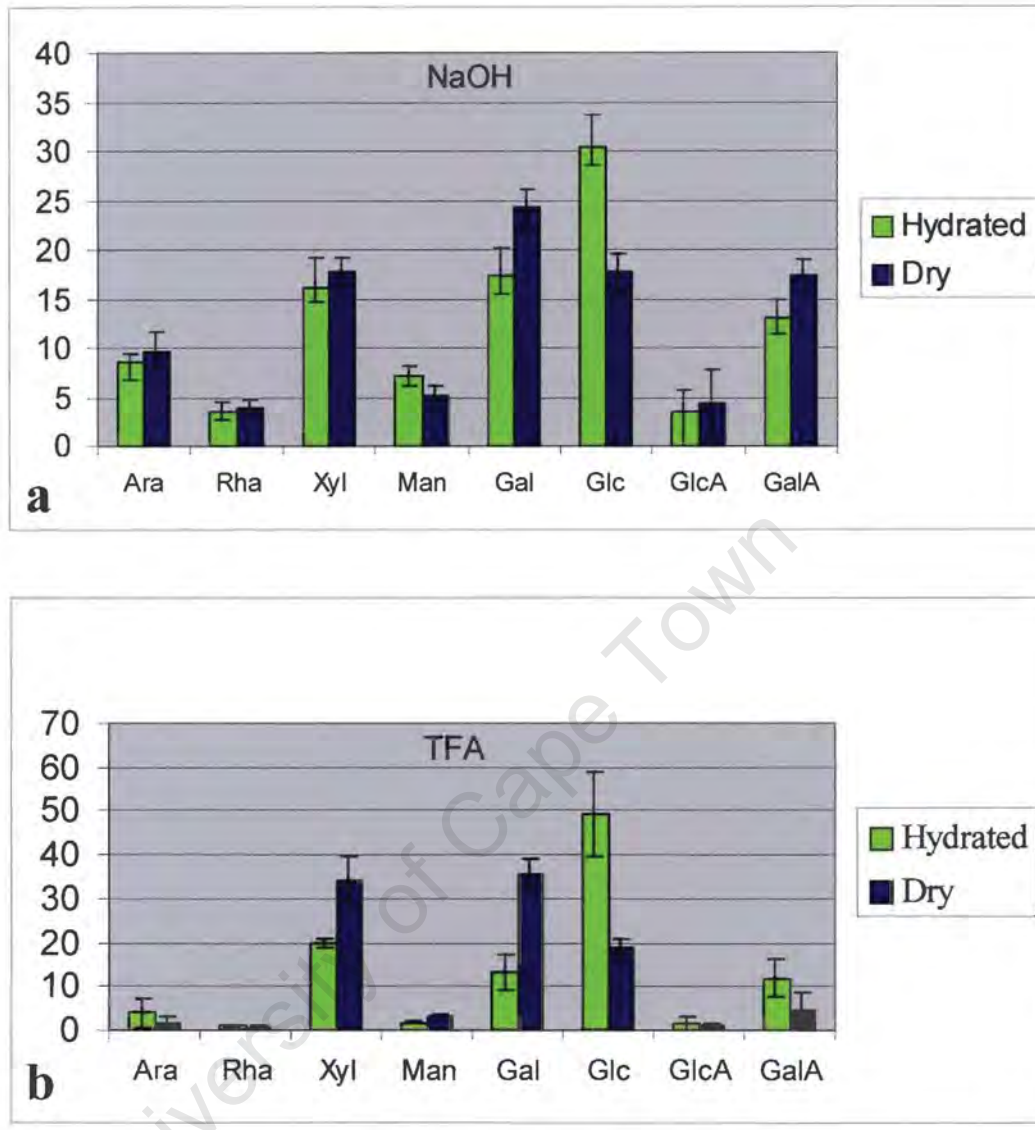

Figure 6: Chemical composition of the fractions extracted by NaOH (a) and TFA (b) for hydrated and dry leaves. Data are means of 4 values and standard deviations are estimated.

were still anchored to the cellulose. Cellulose fraction was treated by 2N TFA which is known to hydrolyze all polysaccharides except cellulose. Sugars present in the TFA soluble fraction are given in Figure 6b. The main components in the TFA soluble fraction were Glc, Gal and Xyl while Ara, GalA, Rha, Man, GlcA were present in very minor amounts. The presence of Glc, Gal and Xyl in quite important amounts suggest that some XG were still present with cellulose. Glc was the sugar which showed the most conspicuous change in the TFA soluble fraction. Compared to the hydrated plants the relative amount of Glc in the dry fraction decreased dramatically (43.2 ± 9.5 % versus 18.7 ± 2.1 %) while Xyl and Gal increased. In the dry fraction the predominant sugar were Xyl (34.2 ± 5.3 %) and Gal (35.5 ± 3.3 %), while Glc represented less than 20%.

4. DISCUSSION

The aim of this part of the study was to further characterize the cell walls of *C.wilmsii*. Thus the chemical nature of the cell wall polymers isolated from hydrated and dry leaves as well as their monosaccharide composition was studied using biochemical technique.

Overall composition of *C.wilmsii* leaves

C.wilmsii leaf cell walls appear to be typical of others dicotyledoneous plants except maybe for the high proportion of the hemicellulosic fraction. The typical dicotyledoneous cell wall contains about 20% of xyloglucan. In *C.wilmsii* the fraction extracted with NaOH comprise about 36% but some pectins are likely to be also present in this fraction. However, xyloglucans were also present in the cellulosic residue. Leaves from *C.wilmsii* plants were characterized by a large proportion of acidic pectins extracted by EDTA. Pectins released with boiling water and cellulosic residue were less important. The results reported here indicate that there was no significant changes in quantities of polymers extracted by boiling water, EDTA or NaOH, their percentage of cell wall being similar for hydrated and dry plants.

The polymers extracted with boiling water were mainly polysaccharides as indicated by the high content of total sugars obtained by phenol sulfuric assays. Monosaccharide

composition of boiling water extracts revealed the presence of GalA indicating the presence of pectins. According to the classification of pectins by Schols and Voragen (1996) which is based in part on the ratio of Rha to GalA rhamnogalacturonans are differentiated from homogalacturonans by having a ratio Rha/ GalA varying between 0.05 and 1. The ratio Rha / GalA in boiling water extract was 0.16 for hydrated plants and 0.13 for dry plants indicating that rhamnogalacturonan was present in this fraction. In addition, it seems that many of the GalA residues of RG1-like polysaccharide are methylesterified and some may be acetyl esterified. The presence of Ara and Gal suggested the occurrence of these polymers as side chain of RG1. However, this may also be the presence of AGPs in the boiling water extract. Indeed, gel diffusion on agarose and Yariv staining indicated the presence of AGPs in these fractions. Moreover, protein content of the boiling water fraction represented about 3%. Thus AGPs were also extracted with boiling water although in minor amount (less than 0.2 mg/ml). The sugar Glc A was found in quite important level in this fraction suggesting that it may be associated with the side chains of RGI-like polysaccharide. Indeed a polysaccharide of RGI type rich in GlcA (Bupleuran 2IIC) was recently detected in flax roots using purified antibodies (Andeme Onzighi *et al.*, 2000 a). The authors found this epitope essentially in the boiling water extract and in a low amount in the EDTA fraction, the NaOH fraction being deprived of this polysaccharide. The same epitope was also detected in *C.wilmsii* leaves by immunocytochemistry (see Chapter 3 in this thesis).

One of the most intriguing data is that TLC analysis revealed the presence of a small sugar in the boiling water extract whose nature was not determined. This might be one of the sugar present in the RGII polysaccharide.

Extractions using the calcium chelator EDTA are known to release mainly acidic polymers of low esterification levels. Although DM and DA were lower in EDTA fraction compared to those of boiling water extract, DM and DA remained quite important. The results show that the EDTA fraction was enriched in GalA indicating the presence of homogalacturonans. Rhamnogalacturonans were present in hydrated and dry plants as indicated by the Rha/GalA ratio 0.09 and 0.1 respectively.

This study showed that the sugar content in NaOH from hydrated and dry leaves amounted to low levels (about 30% of the dry weight). However the low recovery of

sugars from leaves cell walls might be due to the observed precipitation occurring during dialysis. This was also observed by Zablackis *et al.* (1995) with the hemicellulosic fraction extracted with 4M KOH with *Arabidopsis* leaves cell walls. This result may also be accounted for by the presence of components other than polysaccharide such as lipids and phenolics. High amounts of Glc, Xyl and Gal suggested that NaOH extract was essentially composed of the hemicellulose, xyloglucan. Other hemicelluloses such as xylans, glucomannans and type II arabinogalactans might also be present. Some pectins were also found in the NaOH fraction as judged by the presence of GalA and Rha.

Analysis of TFA soluble fraction indicated that non-cellulosic polysaccharides remained in association with cellulose even after NaOH extraction. These polysaccharides consisted mainly of Xyl, Gal and Glc. Xyloglucan is known to be able to form hydrogen bonds with cellulose so that xyloglucan was probably the component of the TFA soluble fraction.

The cell wall composition of *C.wilmsii* seems to be very similar to those of others dicotyledons.

Cell wall polysaccharides composition was affected by desiccation

Modifications in the composition of pectins were detected between hydrated and dry plants. The sugar content of EDTA soluble fraction was considerably higher in the dry than in hydrated plants. This was also the case for uronic acids content. However solubilization of EDTA fraction were not complete as compared to boiling water fraction. Attempts to further solubilize these fractions reduced the difference between hydrated and dry plants but a slight difference still occurred. These results showed that desiccation affected pectins composition and/or their organization. In *C.wilmsii* leaves, the organisation of pectins might be modify by desiccation and thus a difference in solubility might explain such results. Iraki *et al.* (1989 a) found that the proportions of total pectins in walls of adapted and unadapted cells to osmotic stress were about the same but the organization and composition of this material differs markedly. Water and saline stress induced changes in the organization of pectins whereby a fraction of the Ca^{2+}-insolubilized pectins is more loosely bond and hence more easily extracted from walls of adapted cells. Marshall *et al.* (1999) found that solubilization of cell wall proteins in roots

of *Pinus banksiana* was affected by osmotic stress. The authors suggested that the regulation of turgor, cell size, elasticity and diameter of jack pine roots is associated with the secretion and subsequent insolubilization of cell wall proteins.

However the most important finding is that Glc content was much lower in dry plants than in hydrated plants. This was observed in boiling water fraction but the most conspicuous changes occurred for polymers extracted with NaOH and this was confirmed by the analysis of the TFA-soluble fraction after hydrolysis of the cellulosic residue. The additional non-cellulosic Glc in hydrated plants is likely to be of cell wall origin. However minor contamination from starch can not be excluded. In theory any starch present should be solubilised at the hot water stage and at least the acid hydrolysable glucan in the cellulosic residue can be considered as cell wall origin.

Zwiazek (1991) showed a reduced content of cell wall Glc and Gal and an increase of Ara in drought-stressed white spruce seedlings with increased cell wall elasticity. Cell walls contain mainly carbohydrates which impart the mechanical properties to the walls. These changes in carbohydrate composition in desiccated *C.wilmsii* leaves could be responsible for changes in cell wall elasticity. Sherwin (1995) reported that during desiccation *C.wilmsii* leaves became more elastic and this could be due to an increase in the cell wall elasticity. Cell wall elasticity is important for turgor maintenance and cells with rigid walls can not undergo a large decrease in volume. The changes in non cellulosic Glc during desiccation might be due to a partial degradation of XG. A loss of Glc was observed in hypocotyl growth of *Pinus pinaster* seedlings (Lorences and Zarra, 1986). Non cellulosic Glc was involved in a rapid turnover during growth and a partial degradation of xyloglucan was suggested. Depolymerisation of XG has been postulated as one of the biochemical basis of cell wall loosening for both dicotyledons and monocotyledons. In *C.wilmsii*, a partial depolymerisation of XG resulting in a loss of Glc might be necessary to allow the cell wall being more extensible and thus folding upon desiccation.

These data also showed a significant increase of Xyl and Gal in dry plants as compared to the hydrated ones. This would suggest that the cell walls of dry plants contain a XG with a reduced Glc containing backbone but with more Xyl and Gal residues on the side chains. This xyloglucan is likely to form a strong association with cellulose in *C.wilmsii*

cell walls. The changes in XG structure may have exposed more antigenic sites for attachment of antibodies and may account for the apparent increase in antibody labeling reported in Chapter 3.

In the future, it should be interesting to further characterize cellulose fraction between hydrated and dry plants as xyloglucan/cellulose network seems to be highly modified during *C. wilmsii* dehydration. Iraki *et al.* (1989) found that cellulose/extensin framework was determinant for wall tensile strength with tobacco cells adapted to osmotic stress. Complete formation of this framework was apparently sacrificed to divert carbon to substances needed for osmotic adjustment. The same situation might be true of *C. wilmsii.* Furthermore, the glucose released from the cell walls might be an important energy source within the cytoplasm, for the induction of protection mechanisms involved in desiccation tolerance in this species.

CHAPTER 5 :

CALCIUM IONS

CHAPTER 5: CALCIUM IONS

1. INTRODUCTION

It is widely accepted that ions and more especially calcium ions (Ca^{2+}) play an important role in the architecture of the cell wall. This chapter provides information on the content and changes of Ca^{2+} in leaves of both hydrated and dry *C.wilmsii*.

Calcium and cell wall biosynthesis

The composition of the cell wall is highly dependant on the concentration of calcium which has a variety of roles in the metabolism of cell wall components, their secretion and their modification within the cell wall itself (Eklund and Eliasson, 1990; Jarvis, 1992; Goubet and Morvan, 1995). Demarty *et al.* (1984) proposed that Ca^{2+} could regulate wall pH by modifying H^+ linkage and thus regulate wall enzyme activity.

The concentration of calcium is a major factor in the regulation of the deposition of the cell wall. Eklund and Eliasson (1990) found that cell wall deposition in Norway spruce varies considerably depending on the exogenous calcium concentration. An increase in such calcium results in major modifications in the polysaccharides synthesised. Low concentrations of calcium inhibited the deposition of lignin and non-cellulosic polysaccharides whereas at high concentrations (> 1mM) the deposition of cellulose is stopped and callose production is increased (Eklund and Eliasson, 1990).

Calcium has been shown to be involved in cellulose synthesis. Callose and cellulose are synthesised by an enzymatic complex associated with the plasma membrane (Delmer, 1987). Girard and Maclachlan (1987) reported a calcium activated protease able to transform cellulose synthase into callose synthase in pea. Ripoll *et al.* (1993) reported that the ratio calcium to sodium is an important factor of the secondary wall deposits during fibre differentiation in flax.

Calcium modulates the activity of the enzymes involved in non cellulosic wall polysaccharides synthesis. Pectins are synthesized and methylesterified in the Golgi apparatus (Zhang and Staehelin, 1992). In flax, the pectin methylesterase enzyme responsible for this methylesterification has been shown to be inhibited *in vitro* by Ca^{2+} and Mg^{2+} (Vannier, 1994).

Calcium is also involved in the transport of the cell wall components from the Golgi apparatus to the plasma membrane. Non cellulosic polysaccharides (pectin and hemicellulose) are synthesised in the Golgi apparatus and then secreted in the cytoplasm in fusion vesicles (Driouich *et al.*, 1993 a, b, 1994; Andreeva *et al.*, 1998). The movement of the vesicles to the plasma membrane and the rate of vesicle fusion with the plasma membrane constitute important control points for the deposition of the material in the cell wall. Calcium is known to be involved in the regulation of the vesicle fusion which may be a limiting process for the rate of cell wall formation. The composition and amount of wall material deposited may respond very quickly to a stimulus at the cell surface which allows the rate of vesicle fusion to vary (Northcote, 1989).

Once the polysaccharides are deposited in the cell wall they are subject to modifications within the wall itself by enzymes. Pectins are secreted in methylesterification conformation and are only demethylesterified in the cell wall by a pectin-methylesterase (PME) (Zhang and Staehelin, 1992). PMEs are ionically bound to the cell wall and their activity has been shown to be regulated by calcium in mung bean hypocotyls (Goldberg *et al.*, 1992). Peroxidases are involved in polymer linkage formation (thus reducing the cell wall extensibility) and their activity has been proposed to controled by calcium (Shinkle and Jones, 1988). His *et al.* (1997) have shown that in flax hypocotyl seedlings, Ca^{2+} regulates wall acidification.

Calcium regulate cell wall architecture and mechanical properties

One of the most important function of calcium ions in the cell wall is to cross-link pectic polymers by forming a structure called "egg-box" (Jarvis, 1984). In this conformation, the calcium is ionically bound to the pectic polymers and a single calcium ion is attached to two negatively charged groups (e.g two COO^- groups of PGA). This arrangement could maintain cell wall strength by binding the polymers in the middle lamella (Jarvis, 1984; Roy et *al.*, 1994). Nakajima *et al.* (1981) have shown that the replacement of Ca^{2+} by Mg^{2+} in the cell wall of pea hypocotyl makes the wall more flexible.

Calcium is known to play an important role in growth regulation. Protons are believed to displace calcium from uronic acids (Sentenac and Grignon, 1981) which results in cell wall loosening due to the breaking of the calcium bridges. Such an hypothesis has been also invoked to explain gravitropic curvature (Slocum and Roux, 1983). The

results of Baydoun and Brett (1984) in pea epicotyl cell walls are consistent with the hypothesis that Ca^{2+} ions inhibits cell wall extension by forming ionic bridges between polygalacturonic acids. Their results support the hypothesis in which Ca^{2+} and H^{+} are competing for the same binding sites. They have shown than an increase in pH could displace Ca^{2+} from polygalacturonic acids.

Changes in cell wall Ca^{2+} are observable in fruit ripening (Jarvis *et al.*, 1984;Virk and Cleland, 1990; Roy *et al.*, 1994; Redgwell *et al.*, 1997). This mechanism of ripening is generally associated with a softening process. This alteration of fruit texture is thought to be due to alterations of cell wall components. It was suggested that calcium retards the rate of fruit ripening (Roy *et al.*, 1994). Exogenous calcium would increase the levels of calcium-bound pectin in cell walls preserving the cell integrity and adhesion.

This part of my thesis presents calcium ions distribution in *C.wilmsii* leaves using different techniques secondary ion mass spectrometry (SIMS), energy dispersive spectrometry (EDS) and proton induced X-ray emission (micro-PIXE) and different type of material preparation (chemical fixation, cryopreparation). Calcium ions were studied in cell wall of hydrated and dry plants.

2. MATERIAL AND METHODS

Secondary ion mass spectrometry (SIMS)

The leaves of *C.wilmsii* were prepared as described in Chapter 2 and samples were embedded in Spurr's resin. For SIMS analysis, semi-thin sections (2µm thick) were mounted on aluminium discs. The image of calcium distribution were acquired using a cameca IMS4F SIMS instrument. The images were treated by image processing (Optimas, Imasys Suresne France). In this study, the scanning images were acquired for 240s, using a beam of oxygen ions (O_2^{+}), 15keV in energy, 15-20pA in intensity and 0.6 µm in diameter.

X- ray analysis on cryofixed samples

Frozen hydrated leaves were prepared by plunging leaves from hydrated and dried plants in liquid nitrogen slush. Samples analysed in the frozen hydrated state. A

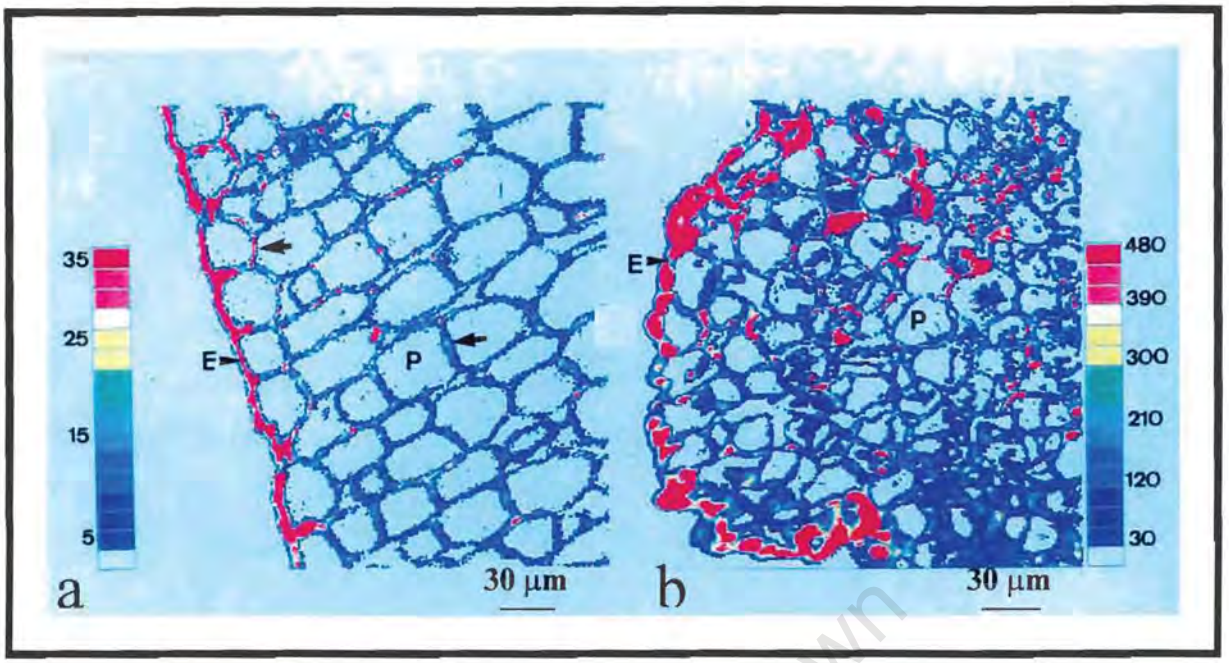

Figure 1 : Visualisation of calcium distribution in transverse sections of hydrated (a) and dry (b) *C.wilmsii* leaves using SIMS technique. Calcium is mostly associated with the cell walls (←). However the dry tissue has a much more higher signal compared with hydrated tissues (note the different values in the two colour scales). The false colour on the scales indicates the ions intensities (counts per second for an acquisition time of 5 min) within an image. (E) epidermis ; (P) parenchyma cells.

second batch of samples (leaves from hydrated and dry plants) was cryofixed in propane and then freeze dried for 24hours.

Samples were analysed by EDS (Energy Dispersive Spectrometry) at selected points with a Hitachi s-570 microscope (15kv).

Elemental distribution was analysed on frozen hydrated samples. Values represent the minima and the maxima obtained for 3 measures for parenchyma cell walls and 2 measures for parenchyma cytoplasm for hydrated plants. Because of the difficulty to distinguish the cytoplasm from the parenchyma cell wall in dry plants, the analyses were done only for parenchyma cells in hydrated plants. In both dry and hydrated plants 4 measurements were done on epidermal cell wall and 3 measures on epidermal cytoplasm. Data are expressed as weight percentage.

Elemental distribution was analysed on freeze dried samples. The measures were performed only on epidermal cells in both hydrated and dry plants. Values represent the minima and the maxima obtained for 3 measurements for the epidermal cell wall and 2 measurements for the cytoplasm of hydrated plants. Only one measurement was performed on the epidermal cytoplasm of the dry plants. Although this value is presented in Table 2, it was not be taken into consideration in the analyses of the data. Data are expressed as weight percentage.

Micro-PIXE (Proton-Induced-X-ray Emission)

Elemental distribution by micro-PIXE probe (0,6 MeV) was performed at the Van de Graaff Group National Accelerator Centre (Faure, South Africa). Micro-PIXE technique applied to botanical applications was described in previous papers (Przybylowicz *et al.*, 1997; Przybylowicz *et al.*, 1999; Prozesky *et al.*, 2000). Analyses occurred on freeze dried samples. The measurements are an average of a small region including cell walls and cytoplasm. Percentages were expressed as a weight percentage.

3. RESULTS

Localisation of calcium ions by secondary ion mass spectrometry

SIMS analysis allowed the detection of bound and free calcium. Figure 1 represents the calcium distribution in hydrated and dry leaves. In both hydrated and dry leaves,

Table 1: Elemental distribution analysed by EDS on frozen hydrated samples of wet and dry leaves of *C. wilmsii*. Values represent the minima and the maxima obtained for 3 measurements for parenchyma cell wall and 2 measurements for parenchyma cytoplasm for hydrated plants. Because of the difficulty to distinguish the cytoplasm from the cell wall in dry plants in parenchyma cells, analysis were done only for parenchyma cells in hydrated plants (Nd: not determined). In both dry and hydrated plants 4 measurements were done on epidermal cell wall and 3 measurements on epidermal cytoplasm. Data are expressed as weight percentage.

Elements	Tissue	Hydrated (%)	Dry (%)
Ca^{2+}	Parenchyma		
	Cytoplasm	0.51 - 0.6	Nd
	Cell wall	0.33 - 0.79	Nd
	Epidermis		
	Cytoplasm	0.11 - 0.46	Nd
	Cell wall	0.38 - 0.99	1.43 – 3.03
K^{+}	Parenchyma		
	Cytoplasm	1.26 - 1.58	Nd
	Cell wall	1.2 - 1.52	Nd
	Epidermis		
	Cytoplasm	0.53 - 2.49	Nd
	Cell wall	0.5 - 2.27	1.5 - 1.63
P	Parenchyma		
	Cytoplasm	0.39 - 0.44	Nd
	Cell wall	0.42 - 0.55	Nd
	Epidermis		
	Cytoplasm	0.06 - 0.37	Nd
	Cell wall	0.01 - 0.23	0.11 – 0.19

the calcium clearly occurs in the cell wall of the epidermis and parenchyma. In the hydrated leaves the concentration of Ca^{2+} was very low in the cytoplasm. Values detected were lower than 5 counts. The differences in scale shows that Ca^{2+} was spread heterogeneously in the cell wall. Ca^{2+} was mainly localised in the tri-corner-junction and in the outer epidermal cell wall. In dehydrated leaves, cytoplasmic concentration of Ca^{2+} was also very low. Ca^{2+} was found at a very high concentrations in the cell wall of epidermal cells. As indicated by false color scale, Ca^{2+} signal was higher in the epidermal walls of dry leaves that in those of hydrated ones.

EDS on frozen hydrated samples

Ca^{2+} was analyzed on frozen hydrated samples to avoid artifacts due to chemical fixation. Elemental distribution of K^+ and P was also investigated in order to determine if the results gained with Ca^{2+} were specific or just reflect the consequence of dehydration increasing ion levels in plants. Results are reported in Table 1.

In parenchyma cells from hydrated leaves, Ca^{2+} was detected in both cytoplasm and cell walls. Concentrations obtained for both compartments were similar although in cell walls a wider range of values were obtained than in the cytoplasm. In epidermal cells of hydrated plants, Ca^{2+} content of cytoplasm were slightly lower than those obtained for the cytoplasm of parenchyma cells. In epidermal cells, the level of Ca^{2+} in the cell wall was higher than that of the cytoplasm. The major finding here is that the Ca^{2+} in the cell wall was higher in dry than in hydrated plants.

In hydrated plant, the values of K^+ were similar in cytoplasm and cell walls of parenchyma cells. In epidermal cells of dry plants, values were also similar for cytoplasm and cell walls. There were no increase in K^+ content in dry epidermal cell walls compared to hydrated ones.

In hydrated plants, the P levels were similar between parenchyma cytoplasm and cell walls. In epidermal cells, values were also similar between cytoplasm and cell wall for hydrated plants. Values obtained in the cell wall of epidermal cells in dry plants were the same as the values obtained in hydrated plants.

EDS on freeze dried samples

Analyses were also carried out on freeze-dried samples. Data are reported in Table 2. Analyses were performed only on epidermal cells for both hydrated and dry plants.

Table 2: Elemental distribution analysed by EDS on freeze dried samples. The measures were performed only on epidermal cells in both hydrated and dry plants. Values represent the minima and the maxima obtained for 3 measurements for the epidermal cell wall and 2 measurements for the cytoplasm of hydrated plants. Only one measurement was performed on the epidermal cytoplasm of the dry plants. Even if this value is presented in this Table, it will not be taken into consideration in the analyses of the datas. Data are expressed as weight percentage.

Elements	Cell compartment	Hydrated	Dry
Ca^{2+}	Cytoplasm	0.2 - 0.24	0.59
	Cell wall	0.21 - 0.53	0.92 - 2.92
K^{+}	Cytoplasm	1.33 - 1.37	1.66
	Cell wall	0.59 - 1.78	1.08 - 2.33
P	Cytoplasm	0.26	0.25
	Cell wall	0.16 - 0.37	0.19 - 0.28

Table 3: Elemental distribution analysed by micro-PIXE probe on freeze dried samples. The measures are an average of a small region including cell walls and cytoplasm. Nd: not determined.

Elements	Tissues	Weight (%)	
		Hydrated	Dry
Ca^{2+}	Parenchyma	0.44	0.70
	Epidermis	Nd	1.24
K^{+}	Parenchyma	6.16	2.17
	Epidermis	Nd	1.92
P	Parenchyma	0.99	0.47
	Epidermis	Nd	0.27

In hydrated leaves, Ca^{2+} levels were slightly higher in the epidermal cell walls than in cytoplasm. The major finding was that the concentration of Ca^{2+} in epidermal cell walls was much higher in dry plants than in the hydrated plants.

There was not clear differences in K^+ content between epidermal cytoplasm and cell walls for hydrated plants. In dry cell walls the K^+ concentration was slightly higher than in the hydrated ones.

The P concentration was homogeneous in hydrated cytoplasm and cell walls in epidermal cells. There was no difference in P content in epidermal walls for hydrated and dry plants.

Micro-PIXE analyse on freeze dried samples

Using this technique, the value obtained is an average of a small region of cell walls and cytoplasm. Results are presented in Table 3.

In parenchyma cells the Ca^{2+}concentration was higher in dry plants than hydrated plants. The Ca^{2+}concentration was more plentiful in epidermal cells compared to parenchyma cells for dry plants. In hydrated parenchyma cells, K^+ levels were higher than in dry parenchyma cells. Values in parenchyma and epidermal cells are similar for dry plant. Concerning the element P there was a lower concentration in the dry epidermal cell compared to the hydrated cells.

4. DISCUSSION

SIMS analysis revealed the presence of calcium ions in cell walls of *C.wilmsii*. His *et al.* (1997) studied calcium distribution in flax hypocotyl using the same material preparation and SIMS analysis. The authors also found that calcium clearly outlines the walls of all the cells in the epidermis and parenchyma. The presence of calcium in walls of *C.wilmsii* parenchyma and epidermal cells seems usual as a substantial quantity of plant Ca^{2+} is known to be associated with cell walls and more especially low-ester galacturonans (review by Trewavas and Malho, 1997). Cell wall Ca^{2+} is known to be tightly bound to the cell wall and then can not be displaced by chemical fixation. In the past, chemical fixation was widely used for SIMS analysis (Jauneau *et al.*, 1992, Jauneau *et al.*, 1997, His *et al.*, 1997). However attempts are now made in order to avoid chemical fixation which can results in leakage of ions. Follet-Gueye *et*

al. (1998, 1999) developed a technique of vapor phase fixation to image Ca and Na in phloem fibres and xylem cells of hypocotyls of beech seedlings by SIMS analysis. This technique was recently applied on dry material by Lhuissier *et al.*, (2000) in order to locate nitrogen oxide in pollen grains. The ideal techniques to avoid ions redistribution would be to use cryopreparation of the samples. However this technique is still in developmental stages although promising results have been obtained by Dérue *et al.*, (1999).

It was noticed with SIMS analysis that the signal intensity was greater in the epidermal cell wall compared to the parenchyma cell walls in *C.wilmsii*. Karley *et al.*, (2000) found using X-ray microanalysis that Ca^{2+} content in barley leaves was more important in epidermal cells compared to mesophyll cells. A similar distribution was detected in flax hypocotyl seedlings using chemical fixation and SIMS microscopy (His *et al.*, 1997, Jauneau *et al.*, 1997). SIMS microscopy observations on flax hypocotyls showed that calcium ions are particularly concentrated in the epidermal and tricellular junctions (Jauneau *et al.*, 1997). Tri-cellular junction are supposed to be highly exposed to mechanical stress due to the turgor. The percentage of pectic components ionically bound to calcium is thought to confer a mechanical resistance in these junctions. Micro-PIXE on dry freeze dried plants also indicate that Ca^{2+} were more concentrated in epidermal cells of *C.wilmsii* leaves although it is not possible in this type of analyse to discern cytoplasm from cell walls. However in frozen hydrated samples analysed by EDX, differences between parenchyma cell walls and epidermal cell wall was very limited at least for hydrated leaves.

The quantification of Ca^{2+} in cell walls indicate an increase in calcium in cell walls in dry leaves compared to hydrated leaves. This was obtained with SIMS analysis with chemical fixation. EDX on both frozen hydrated and freeze dried samples also confirm this increase at least for epidermal cells although the increase was not as great as the one noticed with SIMS analysis. Micro-PIXE analyses of freeze dried leaves revealed an increase in parenchyma Ca^{2+} concentration in dry leaves compared to hydrated leaves. This result is an average of a small region of tissue and therefore it is not possible to attribute this increase for cell wall, cytoplasm or both compartments.

To determine if this increase in Ca^{2+} in dry cell walls was just a result of dehydration or if this is only specific of calcium, the concentration of others ions were also analyzed. Together the results for K^+ and P did not reveal a particular increase of these ions in the cell wall of dry plants compared to the hydrated ones. If Ca^{2+}

increase in cell walls of dry plants was only a consequence of the dehydration, this should be observable for all the ions. This was not the case. It could be hypothesized that the increase in Ca^{2+} observed in walls from dry plants could have an impact on the rigidity and mechanical properties of the walls during dehydration. More especially if the increase of PGA/RG1 labeling observed by immunocytochemistry (see Chapter 3 of this thesis) revealed an increase of acid pectins and / or a change in the wall organization, Ca^{2+} might be necessary to cross-link those polymers and maintain cell wall architecture in the dry state.

It was surprising to detect such a low level of Ca^{2+} in the vacuole using SIMS technique. This could be explained by two hypotheses: 1) These results could indicate that the calcium concentration in the vacuole is too low to be detected by this technique. 2) This result could be an artifact due to tissue preparation and redistribution of Ca^{2+} due to tissue preparation. Data obtained on frozen hydrated leaves in this study allowed the visualisation of Ca^{2+} presence both in cytoplasm and cell walls for parenchyma and epidermal cells. These results suggest that the absence of signal detection with SIMS technique in the vacuole was more due to the chemical fixation. The results obtained with freeze dried samples confirmed this hypothesis. One hypothesis is that unbound calcium fraction could be lost into solution during resin-embedding for SIMS and this may be why cytoplasmic calcium is not apparent in the SIMS images whereas cell wall bound calcium can be detected. The implication would be that the SIMS images reflect the distribution of sites at which calcium is strongly retained rather than the actual in vivo distribution of the calcium.

It can be postulated that in *C.wilmsii* calcium could play a role during dehydration even if this role remained unclear. Inconsistencies of these results could probably be due to different methods of sample preparation and different approaches to quantify the results. Future studies on single layers of cells prepared using cryotechniques should be the best way to get results in Ca localization in *C.wilmsii* leaves.

CHAPTER 6 :

HORMONES

CHAPTER 6: HORMONES

1. INTRODUCTION

Plant hormones are chemical messengers which regulate the normal progression of developmental changes as well as responses to environmental signals (Morgan, 1990). A complex regulatory system involving multiple hormones allows either to activate or repress genes. The concentration of the cytokinins (zeatin and zeatin riboside), absicissic acid, and auxin (IAA) was determined at different water content during dehydration and rehydration in order to understand whether changes in these might correlate with the cell wall changes reported on in previous chapters. Some of these hormones have been shown to regulate cell wall architecture and more especially the strength of the cell wall (Cleland, 1981; Thomas *et al.*, 1984; Miyamoto and Kamisaka, 1988).

Plant hormones are known to closely interact with each other that is why it is important not to look only at one hormone but to see how the different types change during the process of dehydration and rehydration. However in this study, each hormone is reported on separately.

Cytokinins

The cytokinins were first isolated in cultured tobacco cells and their name came from their property of inducing cytokinesis, *i.e* cell division. They comprise a relatively large number of species. The majority are derived from a purine and have been classified as N6 substituted adenine derivatives. Zeatin (Z) and zeatin riboside (ZR) are among the more prevalent species studied to date. Cytokinins are known to be synthesised in meristematic regions and areas with growth potential. In vegetative tissues, cytokinins are synthesised in root tips and redistributed to the upper part of the plant *via* xylem elements. Cytokinin metabolism is a dynamic balance between biosynthesis, formation of cytokinin conjugates and the catabolic reactions resulting in a loss of biological activity (Hare and Staden, 1994).

It has been shown that cytokinins regulate many responses that may or may not be a direct result of cell division (Horgan, 1984), cell and organ enlargement (Kappler and

Kristen, 1986; Bertell and Eliasson, 1992), root initiation and growth (Bourquin and Pilet, 1990; Bertell and Eliasson, 1992) and delayed senescence in leaves (Singh *et al.*, 1992). Among plant hormones the mechanism of action of cytokinins is probably the less understood. Furthermore cytokinin-regulated genes are also dependent on environmental factors and on other hormones. Such an interaction with other phytohormones makes understanding of cytokinin action more difficult.

Cytokinin levels have been shown to be regulated by environmental stress. Nutrient ion content affected cytokinin levels. Horgan and Wareing (1980) have shown that in birch a deficient supply of phosphate resulted in reduced levels of cytokinins. The cytokinins are also believed to play an active role in drought stress. Several reports have associated osmotic or drought stress with a decrease in cytokinin levels (Itai and Vaadia, 1965; Bano *et al.*, 1993). Cytokinins in conjunction with ABA have been shown to act as root signals modulating responses of shoot growth and stomatal aperture on drying (review in Spollen *et al.*, 1992).

ABA

ABA was discovered in the 1950s to be a phytochrome affecting leaf abscission and bud dormancy (reviewed in Skriver and Mundy, 1990). ABA is a sesquiterpene derived from mevalonate. It is synthesised predominantly in the mature leaf and it can be translocated *via* the phloem and the xylem. It is now well known that ABA synthesis can also occur in roots (Bano *et al.*, 1993).

ABA is involved in dormancy, inhibition of bud growth and shoot formation, seed development and germination, geotropism, stomatal closure and stress response (Zhang *et al.*, 1987). Increase in ABA has been reported in desiccation tolerant system such as maturing seeds (reviewed by King 1982, Farrant *et al.*, 1993). ABA has been reported to control various stages such as embryogenesis and seed formation including embryo morphogenesis, storage protein synthesis, desiccation tolerance and the maintenance of dormancy (reviewed by Skriver and Mundy, 1990).

It is now well accepted that one of the earliest responses to stress in plants is the accumulation of ABA. ABA is known to accumulate in higher plants in response to osmotic stress caused by desiccation, salt or cold (review in Skriver and Mundy, 1990).

In the case of drought stress ABA can influence a number of processes such as stomatal movement, phloem transport, ion and water transport in roots (Cornish and

Zeevaart, 1985; Guerrero and Mullet, 1986; Jones *et al.*, 1987; Zhang and Davies, 1987; Tardieu and Davies, 1992).

ABA has been shown to induce desiccation tolerance in carrot somatic embryos (Iida *et al.*, 1992). The viability of somatic embryos after desiccation increased gradually with the duration of ABA application. ABA has been shown to accumulate also in resurrection plants during water stress. Proline has also been shown to increase under water stress (Thymms and Gaff, 1979) and this increase was correlated to the increase of ABA in desiccation tolerant plants. ABA has been shown to play a major role in desiccation tolerance by inducing Late Embryogenesis Abundant (LEA) proteins (Galau *et al.*, 1986; Skriver and Mundy, 1990; Han and Kermode, 1996). LEA proteins are abundant and probably universal in plant seeds. However LEA mRNA and proteins can be induced at others stages of development or in others tissues in response to ABA application (Dure *et al.*, 1989). The liverwort *Exormotheca holstii* can lose their desiccation tolerance if the thalli were cultivated in well watered conditions for too long. However Hellwege et al. (1994) have shown that desiccation tolerance of these thalli can be restored after application of exogenous ABA.

Auxin

Auxins constitute a small group of phytohormomes. They were originally recognised as the hormones involved in tropic responses. The main auxin in plants is Indole-3-acetic acid (IAA) although others such as 4-CI-IAA and indole-3-butyric acid have been reported to occur naturally (reviewed by Sitbon and Perrot-Rechenmann, 1997). Auxin is known to promote numerous growth and developmental responses including cell extension, cell division, apical dominance, vascular differentiation, root formation. There are many cases where cytokinins and auxins are antagonistic: release of apical dormancy, root growth and formation and lateral bud growth. Guilfoyle *et al.* (1993) have cloned and sequenced a number of auxin-responsive cDNAs and their corresponding genes. Their results show that gene transcription in response to auxin occurs within minutes after hormones application and the increase in transcription level vary between 3 to 60 fold depending of the genes.

Most of the work on the role and the action of auxin has been performed with the phytohormone IAA (Thomas *et al.*, 1984; Normanly, 1997; Büntemeyer *et al.*, 1998; Wakabayashi *et al.*, 1999). It is well known that IAA is involved in growth and many studies have focused on its role in cell wall dynamics (Baker and Ray, 1964; Coartney

and Morre, 1980; Terry *et al.*, 1982, Thomas *et al.*, 1984; Miyamoto and Kamisaka, 1988). It is still believed that auxin can regulate the rate of cell elongation by acting on the cell wall extensibility. This is the growth acid theory in which auxin may loosen the primary walls by increasing the activity of glucanases. Cellulases, which have low pH optima, can be activated by the H^+ ions released by auxin (Fry, 1989 b).

Aim of the study

In the present study, radioimmunoassay (RIA) technique (Mac Donald *et al.*, 1981) was used to determine the changes in specific plant hormones during dehydration and rehydratation of *C.wilmsii* leaves. The free forms of the following plant hormones, zeatin, zeatin riboside, IAA and ABA were analysed.

The role of these hormones in desiccation tolerance and their impact on the cell wall architecture of *C.wilmsii* is discussed.

2. MATERIAL AND METHODS

Extraction and RIA was performed according to Farrant *et al.* (1993). Leaves were collected from plants during dehydration and rehydration. Leaves from two plants were pooled together to form one set. Analyses were performed on two separate samples for each RWC. Leaves were snap frozen in LN2 and stored at –80°C until required. Material was lyophilised and finely ground. Samples (0.2 g) were extracted in 5 ml of 70% methanol, containing 20 $mg.l^{-1}$ ascorbic acid. Extraction was performed in the dark for 24 h at 4°C with stirring. Particulate matter was removed by centrifugation at 20 000 g for 10 minutes. The supernatant was filtered using a 0.45 µm PTFE filter and then passed through a C18 Sep-Pac column. The resulting filtrate was reduced to dryness in a Savant vacuum concentrator.

A standard curve was generated for each plant hormone. Standards were assayed in triplicate.

100 µl aliquots of standard were put into Greiner tubes and the dried samples were dissolved in 100 µl of methanol and treated as follows:

1) 100 µl of dilute [^{3}H]- PGR (antigen-tracer) of known concentration and specific activity and 100 µl of antibody were added.

2) 500 µl bovine serum albumin (BSA) were added. The mixture was vortexed and then incubated at 37°C for 30 minutes.
3) The protein was precipitated by the addition of 850 µl 90% ammonium sulphate. Precipation was allowed to occur for 30 min at room temperature.
4) The protein was pelleted by centrifugation at 4000g for 10 min.
5) The pellet was washed with 1.5 ml 50% ammonium sulphate and recentrifuged.
6) The washed pellet was dissolved in 250 µl distilled water.
7) 2 ml of scintillation cocktail (Picofluor) was added. After mixing, the tubes were inserted into scintillation vials and counted in a Packard scintillation counter.

The amount of non-specific binding (NSB) was determined (in triplicate) by placing 100 µl antigen tracer and 100 µl water instead of antibody into a Greiner tube. This was subjected to steps 2 – 7.

The total tracer activity (Ta) was determined in triplicate by placing 100 µl [^{3}H]-PGR, 250µl water and 2 ml scintillant in a Greiner tube. After mixing this was counted in the scintillation counter. A Ta of 10 000 counts per minute (cpm) was used.

Quantifications were done using standard curves generated to each plant hormone. All data were corrected for non-specific binding and the antigen responses expressed as $B.B_0^{-1}$, where B is the antibody radioactivity in the presence of unlabelled antigen (either standard or unknown) and B_o is the antibody radioactivity in the absence of unlabelled antigen. In the presence study these calculations were done using an online computer and the Securia data reduction radioimmunoassay package (Packard Instrument Compagny, 1989 Publication Nn. 169-3016). Analyses were performed on two different sets of leaves for each RWC and triplicate were performed on each set. Results are expressed as ng of hormones per g dry weight of leaf material.

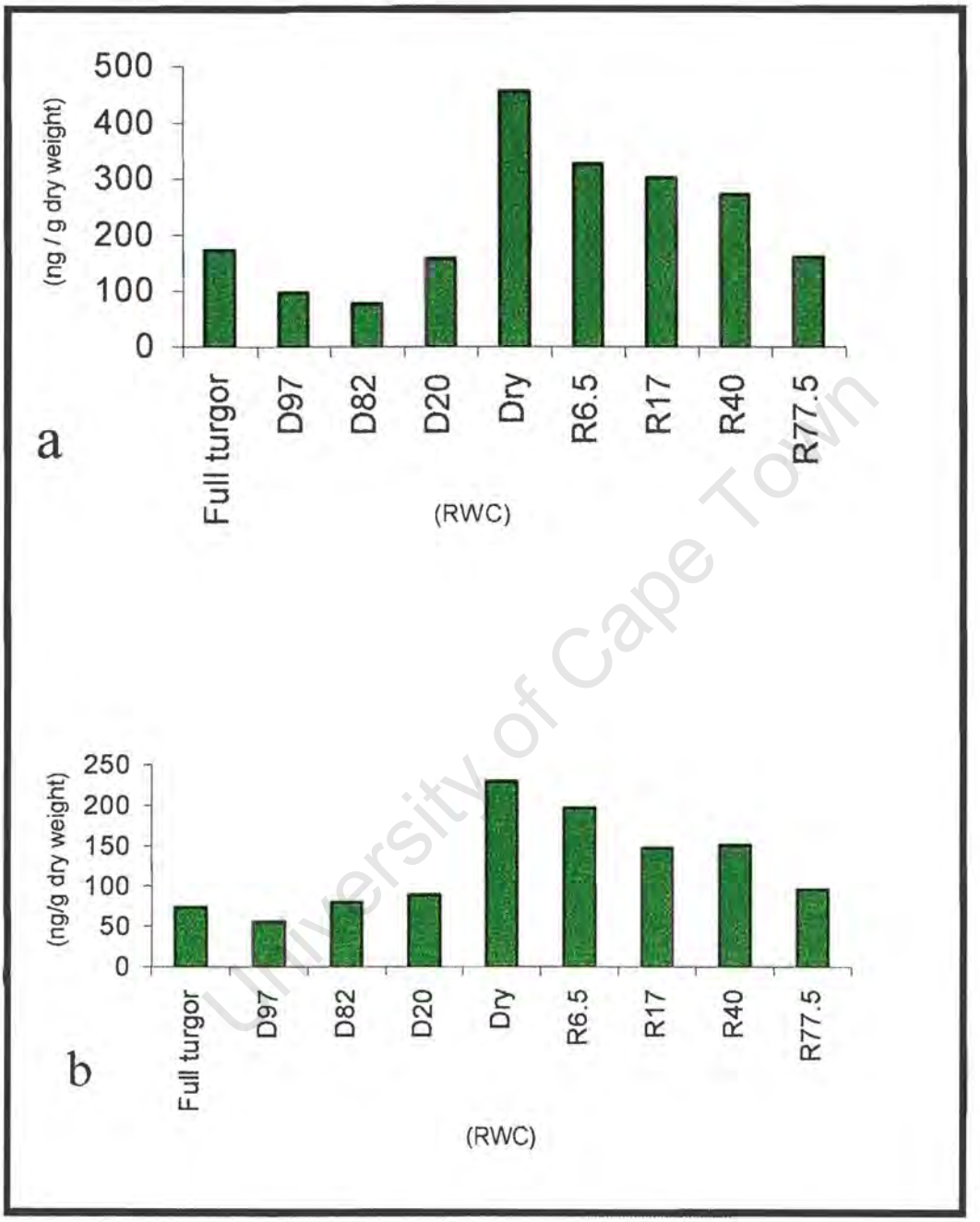

Figure 1: Cytokinin contents of *C.wilmsii* leaves during dehydration and rehydration. a) zeatin and b) zeatin riboside. The results are the average on triplicates performed on two different lots of plants.

3. RESULTS

Cytokinins

The changes in levels of the cytokinins zeatin and zeatin riboside during dehydration and rehydration of *C.wilmsii* leaves are presented in Figure 1 a and b respectively.

The zeatin content declined slightly during the initial stages of dehydration of the leaves then it started to increase at 20% RWC before reaching a maximum (454.5 ng/g) in the dry leaves. Upon rehydration the zeatin content declined gradually although it remained higher than during dehydration. At the end of rehydration the levels recovered to the value observed in fully hydrated leaves.

As shown in Figure1b, the content of zeatin riboside was lower than those obtained with zeatin. Values were low in fully hydrated plants (74 ng/g of dry weight) and were maintained during dehydration. A dramatic increase occurred however in dry leaves (228.5 ng/g of dry weight). During rehydration, zeatin riboside content were still high in the early stages and then progressively declined.

ABA

The changes in ABA content of leaves during dehydration and rehydration are presented in Figure 2a. Contents of ABA were low in fully hydrated leaves (44 ng/g of dry weight) but increased slightly at 97% RWC. The maximum of ABA content (437.5 ng/g of dry weight) was reached at RWC 82% after which it declined again and remained constant at 214 ng/g of dry mass in dry leaves. However, this value was still considerably higher than those initially detected in fully hydrated plants. Upon rehydration no changes in the ABA levels were detected.

IAA

The contents of IAA are presented in Figure 2b. IAA levels were low in fully hydrated leaves (100.5 ng/g of dry weight) but increased at an early stage of dehydration (RWC 97%). IAA content increased throughout dehydration to reach a maximum (927 ng/g of dry weight) at RWC 20%. A decline was then observed and the values for the dry leaves (592 ng/g of dry weight) dropped significantly. However, levels detected in dry leaves were still very high compared to those observed in fully hydrated plants. At the onset of rehydration the decline in IAA level was still

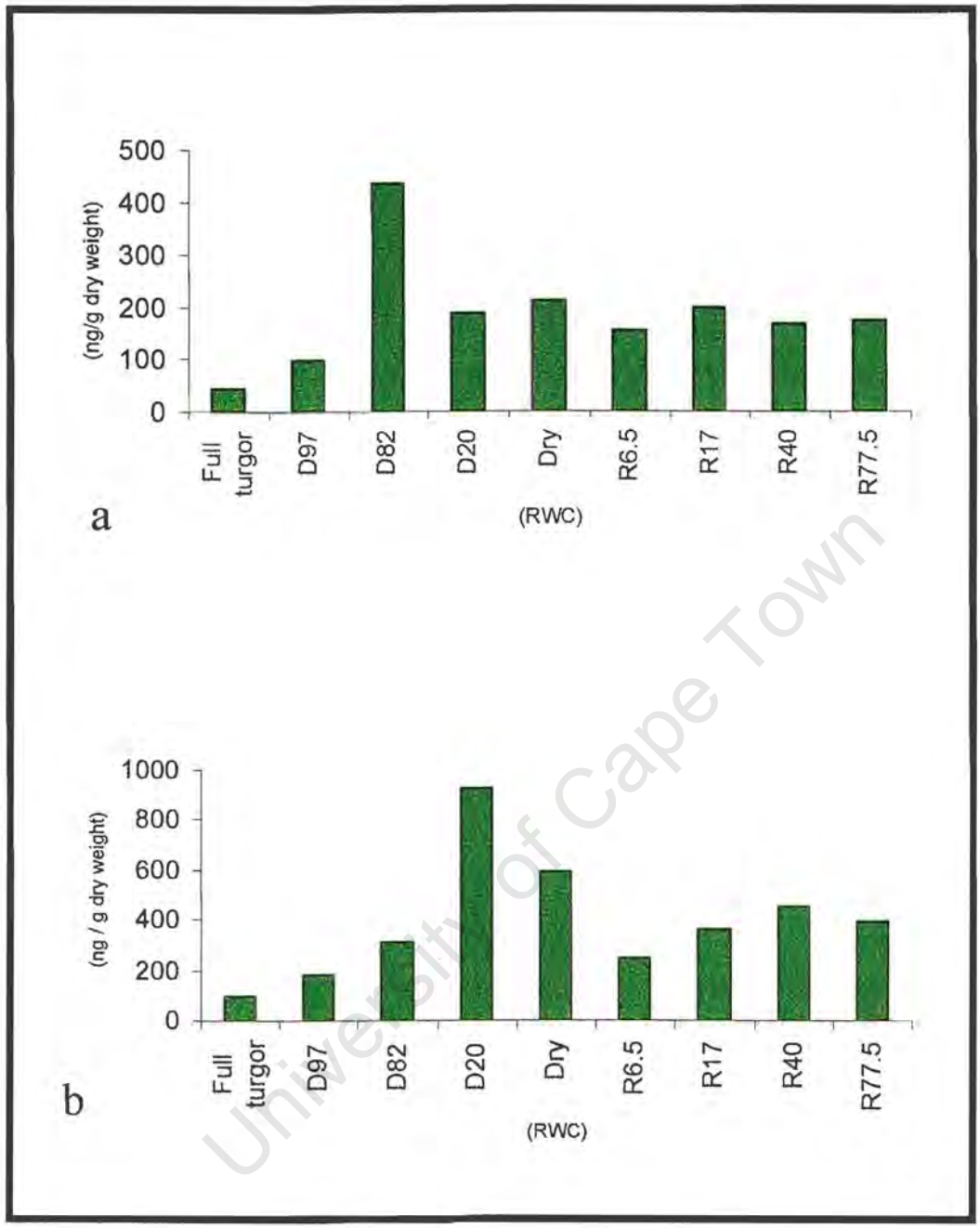

Figure 2: Hormones contents of *C.wilmsii* leaves during dehydration and rehydration. a) ABA and b) IAA of *C.wilmsii* leaves during dehydration and rehydration. The results are the average on triplicates performed on two different lots of plants.

apparent. After RWC 17% a second increase had occurred which remained constant at ca 400 ng/g dry mass until at least RWC 77.5%.

4. DISCUSSION

The changes in the cytokinins zeatin and zeatin riboside contents varied in a quite similar way. Dehydration induced first a slight decrease of zeatin and zeatin riboside contents and this effect was particularly evident with zeatin. Levels of both cytokinins were maintained low during dehydration and increased remarkably when the plants get completely dry. Upon rehydration both zeatin and zeatin riboside contents declined progressively to recover the initial level. These results suggest that zeatin and zeatin riboside are involved in desiccation tolerance of *C.wilmsii*. As the increase occurs at the end of dehydration and remained high during rehydration, these hormones might play a role during rehydration when the plant recovered its metabolism.

The response of ABA to water stress was extremely rapid in the case of *C.wilmsii*. A slight decrease in relative water content (RWC 97 %) in the leaves was enough to induce the synthesis of this hormone. This result support the data obtained by Gaff and Loveys (1984) with the leaves of the desiccation tolerant plant *Borya nitida*. The authors have found that ABA acts in tolerance induction mainly above water potential equivalent to 90% r.h (relative humidity). This increase in ABA levels in *C.wilmsii* leaves can be attributable either to a water-stress-induced increase in synthesis or by a release from the roots. Cornish and Zeevaart (1985) have suggested that root cells can synthesise ABA in response to stress before leaves become stressed.

These data suggest that ABA is involved in the desiccation tolerance in leaves of *C.wilmsii* as it is the case for *C.plantagineum* (Bartels *et al.*, 1990; Schiller *et al.*, 1997) or other resurrection plants such as *Borya nitida* and *Myrothamnus flabellifolius* (Gaff and Loveys, 1984). Its role on desiccation tolerance can be diverse, protein accumulation (dehydrin synthesis), regulation of the stomatal movement. However it is not excluded that ABA might also play a role in the cell wall. Pastor *et al.* (1999) have noticed an increase in ABA content in some water-stressed lavender (*Lavandula stoechas* L.), a plant well adapted to survive under water-stress conditions. In mildly stressed plants, a rapid increase in ABA was

specifically detected in cell wall compartment whereas longer stress reduced the ABA levels in cell walls. Bertrand *et al.* (1992) shown a specific accumulation of both ABA and polygalacturonic acid in the cell walls, the vesicles and the mucilage in tomato root cap cells. The authors suggested that this similarity in labeling distribution might reveal the relationship between pectic substances and ABA.

A large increase in IAA was detected during dehydration of *C.wilmsii*. This increase occurs very early during dehydration and the maximum was detected at a RWC of 20%. A second increase in IAA has been noticed during plant's rehydration. Many studies have shown the effect of IAA on cell wall enzymes (Inouhe and Nevins, 1991; Xu *et al.*, 1995; Wu *et al.*, 1996). IAA promotion of extension-growth is mediated by changes in cell wall chemistry (Thomas *et al.*, 1984). Although the precise role of IAA has been extensively discussed and is not always clear, the main effect noticed has been degradation of the hemicellulose XG. In Dicotyledonous plants auxin causes degradation (Fry, 1989 a), solubilization (Terry *et al.*, 1982) and a decrease in molecular mass of the polysaccharides (Nishitani and Masuda, 1981; Hoson *et al.*, 1991). Xu *et al.* (1995) have demonstrated that the *Arabidopsis* TCH4 gene encoding for a xyloglucan endotransglycosylase (XET) expression was dependent upon IAA and it was hypothesised by Fry (1989 b) that auxin regulates a cellulase activity. The action of IAA on the regulation of a glucanase has also been reported in maize coleoptiles (Inouhe and Nevins 1991). More recently Wu *et al.* (1996) have cloned an endo-β-(1,4) glucanase gene from pea epicotyl which activity was significantly increased by the application of the synthetic auxin analog 2.4-dichlorophenoxyacetic acid.

It should be expected that in response to environmental cues, the availability of cell wall-modifying enzymes would be regulated (Xu *et al.*, 1996). Regulation of genes encoding enzymes that alter cell wall property may underlie the rapid adaptation to environmental stress. IAA does not have to my knowledge a clear role in drought, desiccation or osmotic stress. However it could play a role by acting at the cell wall level by regulating some cell wall enzymes in *C.wilmsii* during desiccation. More especially IAA could be involved in the XG regulation by inducing its partial degradation. Nishitani and Masuda (1981) have postulated that IAA would induce a drop in pH in the cell wall which could break hydrogen bounds between XG and cellulose. Such a process could also be possible in *C.wilmsii* where IAA could

promote the sliding of XG molecules from cellulose and then allow the loosening of a cell wall thereby allowing cell wall folding observed during dehydration (see the general discussion).

CHAPTER 7 :

CONCLUSIONS AND FUTURE PROSPECTS

CHAPTER 7 : CONCLUSIONS AND FUTURE PROSPECTS

The leaves of the resurrection plant *C.wilmsii* respond to desiccation stress by an extensive cell shrinkage (78% reduction in cell area, Farrant, 2000) and considerable cell wall folding. Sherwin, (1995) suggested that cell wall folding in *C.wilmsii* was necessary to maintain plasma membrane / cell wall contacts and thus preserve the integrity of the tissue in the dry state. One important finding of the current work was the confirmation of the cell wall folding in desiccated leaves by the use of different fixation methods.

Such folded cell walls are also a common feature in dry seeds and the manner of the cell wall collapse is characteristic for a given species (Webb and Arnott, 1982). Webb and Arnott, (1982) suggested that this wall folding in seeds is essential for preserving the structural integrity of the tissue and to retain its viability upon rehydration. It was suggested that this folding depends on cell wall composition and structure. The results presented in this thesis have shown that for *C.wilmsii* there was an important difference in the cell wall composition of leaves of hydrated compared with dry plants.

Pectins were particularly altered by desiccation. Immunogold labeling indicated that the density of labeling with anti-PGA/RG1 antibodies was higher in the cell wall of dry plants compared to hydrated ones. Such result can reflect an increase in the synthesis of this component or a different accessibility of the epitope to the antibody in the cell wall of dry plants. Biochemical analysis confirmed that acid pectins were subjected to modification during drying. This was observed in the wall fraction extracted with the calcium chelator EDTA. A higher content of total sugars and Gal A were detected in the dry plants compared to hydrated plants. However further solubilization of these fractions tended to reduce the difference between hydrated and dry plants. Together these results indicate that changes in pectins occurred during dehydration. These changes could be due to an increase in pectin synthesis or to a modification in the organisation of the pectins when the plant dries. In the later case, the increased labeling could be explained by a general loosening and "opening" of the cell wall structure as the cell wall folded making PGA/RG1 epitopes more accessible for labeling in the dry plants. It is clear from these data that modifications occurred in pectin fraction as the plant dried. Pectin organisation has also been shown to be alter in stresses which incur cellular dehydration. Kubacka–Zebalska and Kacperska (1999) showed that

freezing treatment of winter oilseed rape leaves caused changes in the organisation of pectins. This was also proposed by Iraki *et al.* (1989) for tobacco cells subjected to saline and osmotic stress.

However the more remarkable change occur with the hemicellulosic polysaccharide. Immunocytochemical analysis revealed that xyloglucan (XG) labeling was greater in the cell walls of dry plants than hydrated ones. Biochemical analysis also indicated changes in the XG-containing fractions. A decrease in Glc content occurred in the dry plants compared to hydrated plants. These results suggest major modifications in XG composition during desiccation in *C.wilmsii* leaves.

An increase in xyloglucan synthesis could explain the increase in labeling obtained with immunocytochemical study. However this hypothesis seems unlikely since biochemical analysis did not reveal an increase in sugar content of the XG containing fraction in dry plants. Pectic matrix is known to be affected by hydration (Ha *et al.*, 1997). During drying, conformational changes in the pectic component to unmask the xyloglucan could be a possible explanation. The access to xyloglucan chains is likely to be hindered in all circumstances by pectins, and the conformation of the pectins may well modulate the extent of such changes. Another possible explanation is a change in the nature of XG. Interestingly the decrease in Glc content was not correlated with a decrease in Xyl or Gal content. Consequently, it seems that during dehydration a partial degradation occurs on the Glc backbone of XG, leaving containing-side chains parts intact. This is not necessarily in contradiction with results obtained with immunocytochemistry data, as the anti-XG antibodies are suspected to bind to the xyloglucan in certain conformation and such modifications in XG might increase the labeling. A partial degradation of XG would be able to modify the mechanical property of *C.wilmsii* cell walls, increasing its elasticity in response to dehydration. Consistent with this idea is the observation of Sherwin (1995) who showed that the leaves of *C.wilmsii* became more elastic during dehydration. Considering the large degree of shrinkage in cell volumes and the great capacity of the wall to fold, it seems reasonable to expect the cell wall of *C.wilmsii* being remarkably elastic during drying in order to prevent any irreversible damage.

Xyloglucans that hydrogen bond to cellulose microfibrils represent an important factor determining the strength of the cell wall. Changes in XG have been reported to play a role in many plant species in response to water stress. Kubacka-Zebalska and Kacperska, (1999) found that the hemicellulose fraction of cold acclimated leaves of winter oilseed rape

subjected to frost treatment had decreased Glc and Xyl contents. It was postulated that such modification might increase cell wall extensibility and cell wall stress relaxation in leaves of cold acclimated oilseed rape. Sakurai *et al.* (1987 b) proposed that water stress affected the polymerisation of XG in the hemicellulose fraction of squash hypocotyls. Hemicellulose was also found to be affected during drought acclimation. The proportion of hemicellulose was higher in the cell wall of water stress adapted cells of tobacco compared to unadapted cells (Iraki *et al.*, 1989 b). Zwiazek (1991) indicated that white spruce needles had increased hemicellulose content after preconditioning treatment or severe drought stress exposure. Changes in the proportion of hemicelluloses in the cell walls of drought-conditioned and drought-stressed plants could alter their physical properties including cell wall elasticity. It is thus likely that in *C.wilmsii* plants, xyloglucan is involved in the regulation of the cell wall elasticity during dehydration.

Furthermore, it has been reported that enzymes implied in the cell wall loosening were induced by drought stress. Xyloglucan endotransglycosylase (XET) has been associated with several loosening and hydrolysis processes, such as occur in fruit ripening (Redgwell and Fry, 1993), flooding (Saab and Sachs, 1996) or responses to water deficit (Wu *et al.*, 1994). Increase XET activity is associated with maintenance of root elongation at low water potential and this response requires increased ABA (Wu *et al.*,1994). It is suggested that XET induction after water deficit makes the wall more extensible. Wu *et al.* (1996) indicated that in maize roots subjected to low water potential, growth maintenance was due to an increase in cell wall extension properties correlated to an increase in expansin activity and a greater degree of wall susceptibility to expansins. It is possible that during the early stages of dehydration, some enzymes involved in XG hydrolysis or modification (glucanase, XET...) could be activated under specific conditions (pH, hormones) to increase wall yielding in *C.wilmsii*. However, this remains to be demonstrated. Auxin has been shown to regulate hydrolytic enzymes such as glucanase (Wu *et al.*, 1996) and XET (Xu *et al.*, 1995, 1996). It is not excluded that in *C.wilmsii* the increase in auxin during dehydration could have a role in wall enzyme regulation. Such modifications would allow cell wall relaxation under dehydration

Although it remains to be conclusively demonstrated that changes in XG or pectins could contributes to cell wall loosening in *C.wilmsii* leaves upon drying, these results provide strong evidence to suggest that xyloglucans and pectins are subjected to modifications during desiccation. To conclude, cell wall folding induced by desiccation stress is not a simple consequence of turgor loss. There is a rearrangement of the wall

architecture and XG and pectin play a key role in these process even if their exact role needs to be clarified.

This work allows new perspectives for future research. To gain a better understanding of the role of the cell wall in *C.wilmsii* during desiccation it would be of interest to focus our attention on these following points:

1 Structural characterisation of xyloglucan in hydrated and dry leaves of *C.wilmsii*. This analysis could be achieved by mass spectrometry and NMR spectroscopy.

2 It is well known that removal of water from the cell wall drastically alters its mechanical properties. This could mainly be expected for pectins as they are highly hydrophilic. Solid state magnetic resonance relaxation experiments have yielded novel information on the rigidity of individual molecules (Ha *et al.*, 1997). Such analysis on cell walls from hydrated and dry plants will provide important indications on the interactions between different polysaccharides as well as their mobility.

3 Cell wall proteins seem to play a key role in water stress. In some plant species, cell wall proteins are up (Covarrubias *et al.*, 1995; Weiser *et al.*, 1990; Garcia-Gomez *et al.*, 2000) or down regulated (Harrak *et al*,. 1999) by low water potential. Changes in the solubilization of cell wall proteins during osmotic stress have also been reported (Marshall *et al.*, 1999). It would be interesting to investigate the effect of dehydration on the content, composition and structure of wall proteins in *C.wilmsii*.

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