UNIVERSITY OF
LEADING
THE WAY
WESTMINSTER⌗

WestminsterResearch

http://www.westminster.ac.uk/research/westminsterresearch

Cell-walls of growing plant cells

Azharul Islam

School of Life Sciences

CELL - WALLS OF GROWING PLANT CELLS

By

AZHARUL ISLAM

UNIVERSITY OF
LEADING
THE WAY
WESTMINSTER

A thesis submitted in partial fulfilment of the requirements of
the University of Westminster for the degree of
Doctor of Philosophy

January 2013

Abstract

The plant primary cell wall is a three-dimensional interwoven network of cellulose microfibrils, cross-linked by xyloglucan and dispersed in a pectin matrix. It has been suggested that in the wall of growing plant cells, xyloglucan is bound to the rigid cellulose microfibrils by hydrogen bonds and holds the microfibrils together by forming molecular tethers, which is referred to as the 'sticky network' model. Plant growth occurs when these tethers are peeled from the microfibrils by expansins or broken by glycosidases or transglycosylases. A number of researchers have presented theoretical difficulties and observations inconsistent with this model and a new hypothesis has been proposed, claiming that the cellulose – xyloglucan cross-links may act as 'scaffolds' holding the microfibrils apart. Analogies with synthetic polymers suggests that the spacing between the cellulose microfibrils may be an important determinant of the mechanical properties of the cell wall and the results presented in this thesis support this hypothesis. Water contents of *Acetobacter xylinus* synthesized cellulose based cell wall analogues (as a mimic of primary cell wall) and sunflower hypocotyl cell walls were altered using high molecular weight polyethylene glycol (PEG) solution, and their extension under a constant load was measured using a creep extensiometer and showed that there were clear reduction (30-35%) in extensibility suggesting that water content of the wall and therefore the cell wall free volume directly influence wall extensibility. When hydration of *A. xylinus* cellulose composite pellicles was reduced using PEG 6000 solution and re-hydrated in buffer solution, followed by treatment with α-expansin or snail acetone powder extract, it was found that expansin and snail powder extracts caused a rapid rehydration of the composites and that the pellicles only returned to their original weights after these treatments, suggesting that expansin and snail powder can increase the free volume of the wall perhaps contributing to the increases in extensibility that they cause. Assays on cell wall fragments also indicated that expansin increased the cell wall free volume, demonstrated by changes of the turbidity of fragment suspensions. The role of pectic polysaccharide, RG-II, in cell wall biomechanics

was also investigated using mechanical and biochemical testing of available *Arabidopsis thaliana* cell wall mutants and by incorporating RG-II (purified from red wine) with *Acetobacter* cellulose. It was demonstrated that RG-II significantly increased the hydration of cellulose composite; hydration rate was 15 -16% more than the composite without RG-II and thus increased the pellicle extensibility.

From the results, it is evidenced that cell wall extension is not only the consequences of breaking hydrogen bonds between cellulose microfibrils and xyloglucan by expansins or glycosidases and transglycosylases, but also a wider range of factors are involved including cell wall water content, cell wall free volume and the pectic polymers, especially RG-II.

Author's Declaration

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster.

This thesis is entirely my own work and that where any material could be construed as the work of others, it is fully cited and referenced, and/or with appropriate acknowledgement given.

Until the outcome of the current application to the University of Westminster, the work will not be submitted for any such qualification at another university or similar institution.

Signed: Azharul Islam   **Date: January, 2013**

Dedication

To my beloved Father and Mother

Quotation

" Education is not preparation for life; education is life itself "

John Dewey, 1859-1952 AD

Acknowledgments

Firstly, I would like to thank my supervisor Dr. Stuart Thompson for his valuable advice and guidance in every aspect of my research throughout the program and for providing valuable discussions on this thesis. I would also like to thank Dr. Ipsita Roy, a member of my supervisory committee, for her support and encouragement and Professor John Hedger for his support during the transfer review.

I would like to express my gratitude to my friends and technical staff at the School of Life Sciences, especially to Dr. Thakor Tendel for his continuous technical supports. I would like to thank Professor Simon McQueen-Mason (University of York, UK) for giving us the expansin as a generous gift, Professor Malcolm A O'Neill and Dr. Stefan Eberhard of the Complex Carbohydrate Research Center, University of Georgia, USA, for the RG-II sample and their advice during the red wine RG-II purification process and Dr. Phil A P Jackson of the New University of Lisbon, Portugal for the grapevine extensin binding assay.

I would like to acknowledge 'Biosciences and Biotechnology Research Scholarship' at the University of Westminster for the financial support of my research.

I would like give a special thanks to Professor Taj Keshavarz, the Research Director at the School of Life Sciences for his time to time challenges and encouragements that were really needed to complete my research program.

Finally, I would like to thank my family, my parents for their encouragement and especially to my wife Shadia, for her sacrifices and supports throughout the course of my PhD.

Abbreviations

Abs	absorbance
ATCC	American Type Culture Collection
$CaCO_3$	calcium carbonate
$CaCl_2$	calcium chloride
CsExp1	cucumber α-expansin
dicot	dicotyledonous
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis tetraacetic acid
Fuc	fucose
g	gram
g	gravitational force
Gal	galactose
GalA	galacturonic acid
GRPs	glycine-rich proteins
GvP1	grapevine extensin1
h	hour
HG	homogalacturonan
HRGPs	hydroxyproline-rich glycoproteins
H_2O_2	hydrogen peroxide
KCl	potassium chloride
kDa	kilodalton
KOH	potassium hydroxide
L	litre

LVDT	Linear Variable Displacement Transducer
M	molar
Mbar	megabar
min	minute
mL	millilitre
mM	millimolar
mm	millimeter
MPa	mega Pascal
MW	molecular weight
MWCO	molecular weight cut off
MES	morpholinoethane sulfonic Acid
N	newton
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
nm	nanometer
OD	optical density
PEG	polyethylene glycol
PgA	polygalacturonic acid
PRPs	proline-rich proteins
RG-I	rhamnogalacturonan I
RG-II	rhamnogalacturonan II
rpm	revolutions per minute
SP	snail powder
UDP	uridine diphosphate
w/v	weight in grams per volume in millilitres

XET	xyloglucan endotransglycosylase
XG	xyloglucan
µg	microgram
µL	microlitre
µm	micrometer

Abbreviations for the composites produced using *Acetobacter xylinus* synthesized cellulose

C	cellulose (produced by *Acetobacter xylinus* culture)
C/B	cellulose / β-glucan
C/L	cellulose / lichenan
C/P	cellulose / pectin
C/PgA	cellulose / polygalacturonic acid
C/P/X	cellulose / pectin / xyloglucan (cell wall analogue)
C/RG-II	cellulose / rhamnogalacturonan-II
C/X	cellulose / xyloglucan

Table of Contents

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 4: General Discussion

Chapter 5: Conclusion

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

1.1 – The plant cell wall

Plant cell walls are a distinguishing feature of plant cells and are crucially important for many processes in growth, development and reproduction of plants. The plant cell wall largely consists of a rigid layer of complex polysaccharides on the outer surface of the plasma membrane that encases the plant cell (Dey and Brinson, 1984; Fry, 2000), and it is one of the most important characteristic features of plant cells as well as being the main system for osmoregulation. It provides the strength and rigidity to the cell and therefore to the plant but at the same time allows growth and expansion of the cell (Brett and Waldron, 1996; Dey and Brinson, 1984; Reiter, 2002). The thickness of plant cell walls ranges from 0.1 to over 10 µm (Fry, 2001). Cell walls are not uniform structures, but vary in chemical composition, shape and structure. The mature cell wall is a complex laminate structure of three distinct layers, the middle lamella, the primary cell wall and sometimes a secondary cell wall (Brett and Waldron, 1996; Fry, 2000; Popper, 2008). The earliest layer, deposited soon after mitosis as a boundary of two adjacent cells, is called the middle lamella (Brett and Waldron, 1996) and is primarily composed of pectic polysaccharides. Once the cell plate is completed between two daughter nuclei, a new thin layer is deposited surrounding the growing cell called the primary cell wall (Brett and Waldron, 1996; Dey *et al.*, 1997; Popper, 2008). This thesis mainly focuses on the primary cell wall, the details of which will be discussed later in this chapter. Primary cell walls continue to be deposited throughout the growing stage of the cell and the secondary cell walls are then formed between the primary wall and the cell membrane after growth has ceased (Dey *et al.*, 1997; Fry, 2000). Secondary cell walls are not present in all cell types.

The cell wall plays a significant role in providing mechanical strength to plant cells. Based on the thickness of the wall plants cells are grouped into three different types: parenchyma, collenchyma and sclerenchyma. Parenchyma and collenchyma cells possess only a primary wall but sclerenchyma cells have both a primary and a secondary wall. The vertically orientated wall of stem parenchyma, containing unthickened primary wall, permits the cells to bend without breaking. Collenchyma cells, with thickened primary walls containing cellulose, hemicelluloses and pectin but little lignin, provide structural support to herbaceous stems, petioles and leaves. Sclerenchyma cells are lignified cells with a secondary cell wall laid over the primary wall. When matured, both primary and secondary walls are lignified and extremely thick and provide the main supporting strength to the plants (Brett and Waldron, 1996; Popper, 2008).

Plant cell walls are considered to be the most abundant biomass on earth with huge economic importance world-wide. Plant cell walls, or their derivatives, ultimately make up a large variety of industrial and agricultural products, including cotton, linen, paper, lumber, textiles, dietary fibre, nitrocellulose and a variety of other essential products that we use on a daily basis. Most recently, multimillion dollar research work has been started around the World for the commercial production of biofuels from lignocellulosic biomass. Because of their tremendous economic importance, plant cell walls have been studied for many years by specialist research groups. Approaches employing diverse areas of research, including biochemistry, biophysics and molecular biology have begun to be applied to plant cell walls, but even these multidisciplinary approaches have not yet achieved certainty about the exact structure and functions of primary cell wall components in the context of plant growth and development (O'Neill and York, 2003). My research has focused on the

3

mechanical and biochemical properties of the primary cell wall components of dicotyledonous plants with regards to the cell wall extension during plant growth.

1.2 – The primary cell wall

The primary cell wall is a heterogeneous polymeric structure comprising a three-dimensional interwoven network of cellulose microfibrils, cross-linked by xyloglucan and dispersed in a pectin matrix (Fig. 1.01). The nature of the interactions and bonds that keep the wall together is still a mystery, as are the exact roles of its individual polymer components (Chanliaud *et al.*, 2002). The mechanical properties of plant tissues are mostly dependent on the structure of the cell wall and plant cells are prevented from rapidly and uncontrollably increasing in volume due to their internal turgor pressure by their walls (Darley *et al.*, 2001).

Therefore, it is generally accepted that the mechanical properties of the primary cell wall have substantial importance in regulating plant growth (Lockhart, 1965; Thompson, 2005).

In general, the primary cell wall is a composite polymeric structure in which crystalline cellulose microfibrils are embedded in a matrix of complex and highly hydrated polysaccharides with a smaller amount of structural protein (Cosgrove, 1997; Taiz and Zeiger, 2002). The thickness of primary cell walls is typically only 0.1–1.0 μm (Brett and Waldron, 1996; O'Neill and York, 2003). The thickness and morphology of primary walls vary and depends on cell type (Cosgrove, 1997) and the thickness can be up to 10 μm in collenchyma cells (Fry, 2001).

Based on polysaccharide composition primary cell walls can be separated into two distinct types: Type-I and Type-II (Carpita and Gibeaut, 1993; Brett and Waldron, 1996; Carpita, 1996). In type-I cell walls xyloglucan is the main hemicellulose

component that inter-links the cellulose microfibrils and creates a polymer network embedded in pectic polymers (Carpita and Gibeaut, 1993; Darley *et al.*, 2001). The dicotyledonous plants and most of the non-graminaceous monocotyledonous plants cell walls are type-I. The Gramineae family and some other monocotyledonous plants have cell walls of type-II with a lower content of pectin and where the major hemicelluloses are arabinoxylans, and mixed linkage β1-3, 1-4 glucan (Brett and Waldron, 1996).

Cellulose fibrils   Xyloglucan   Pectin matrix   Extensin
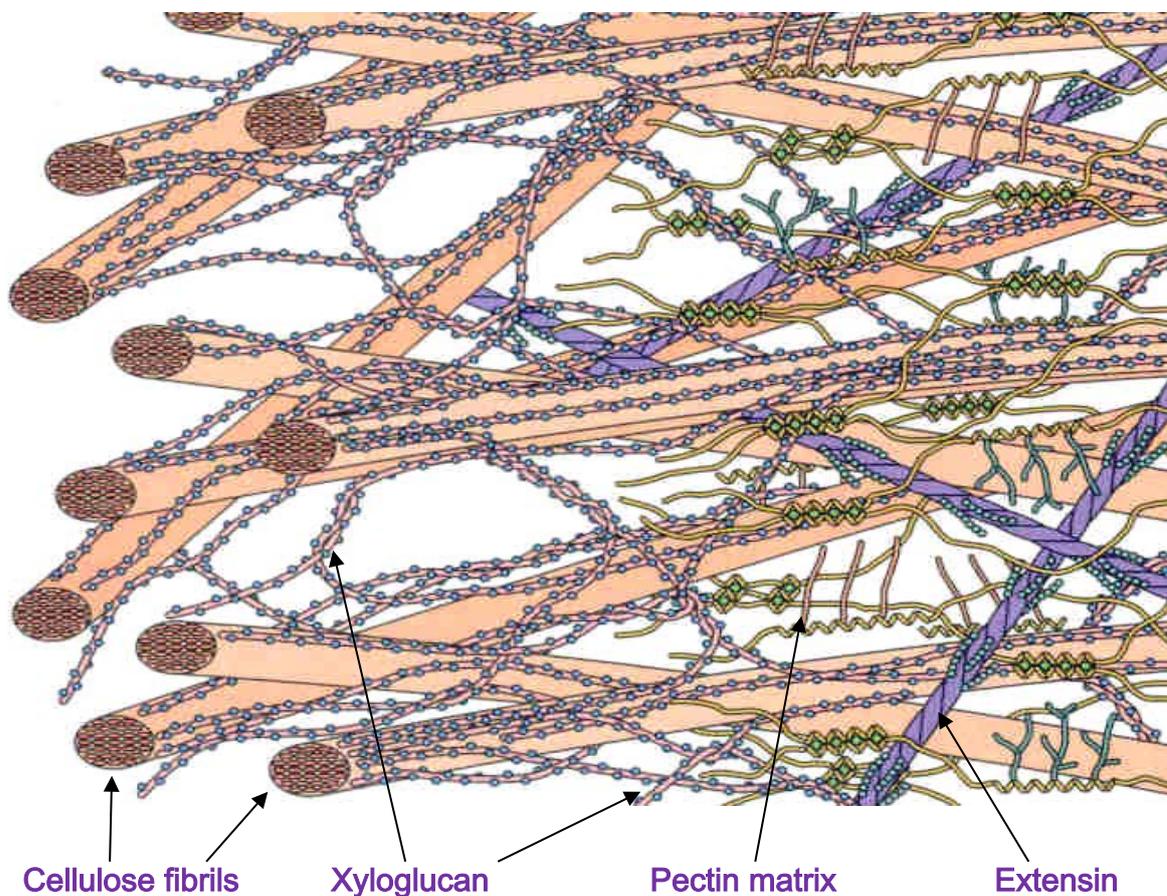
Figure 1.01: Model of the primary cell wall in most of the higher plants (Carpita and Gibeaut, 1993)

The type-I cell wall is composed of approximately 30% cellulose, 30% hemicelluloses 35% pectin and 1-5% of structural protein on a dry weight basis (Carpita and Gibeaut, 1993; Cosgrove, 1997). The composition is substantially different in cell

5

walls of type-II, where the amount of pectin can be as low as 10%, with up to 55% hemicelluloses and 25% cellulose. Water is the most abundant component of the cell wall and makes up more than two thirds of the growing cell wall mass (75-80%) (Cosgrove, 1997; Taiz and Zeiger, 2002) and up to 70 % of the cell wall volume (Monro *et al.*, 1976; O'Neill and York, 2003), given which, its role in the wall has received surprisingly little attention.

1.3 – Primary cell wall components

1.3.1 – Cellulose

Among the cell wall components, cellulose plays the major role in providing the strength and is the structural basis of the wall. Cellulose, the most abundant biopolymer on earth, is a tightly packed aggregate of simple unbranched chains of β - (1→4) D-glucan (McCann and Roberts, 1991; Carpita and Gibeaut, 1993) (Fig. 1.02) with a degree of polymerization from about 2000 to more than 25000 (Brown *et al.*, 1996; Cosgrove, 1997) and each chain can be 1-7 µm long (Dey *et al.*, 1997). About 36-70 such chains are held together by intra and intermolecular hydrogen bonds to form a flat ribbon-like structure, referred to as microfibril (Brown *et al.*, 1996; Dey *et al.*, 1997).
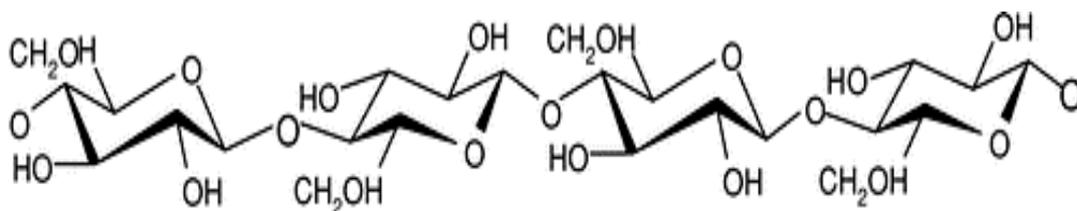
Figure 1.02: Chemical structure of cellulose, a fragment of β- (1→4) linked glucan chain (Somerville, 2006)

The molecular structure of the microfibril is not entirely clear but it seems that it has a structure of highly crystalline domains linked to less organized amorphous regions, as illustrated in Fig. 1.03 (Carpita and Gibeaut, 1993; Taiz and Zeiger, 2002). The width of the cellulose microfibrils of plant cell wall are 5 to 12 nm but indeterminate in length with a separation of 20 to 40 nm between adjacent microfibrils (McCann *et al.*, 1990; McCann and Roberts, 1991; Taiz and Zeiger, 2002).
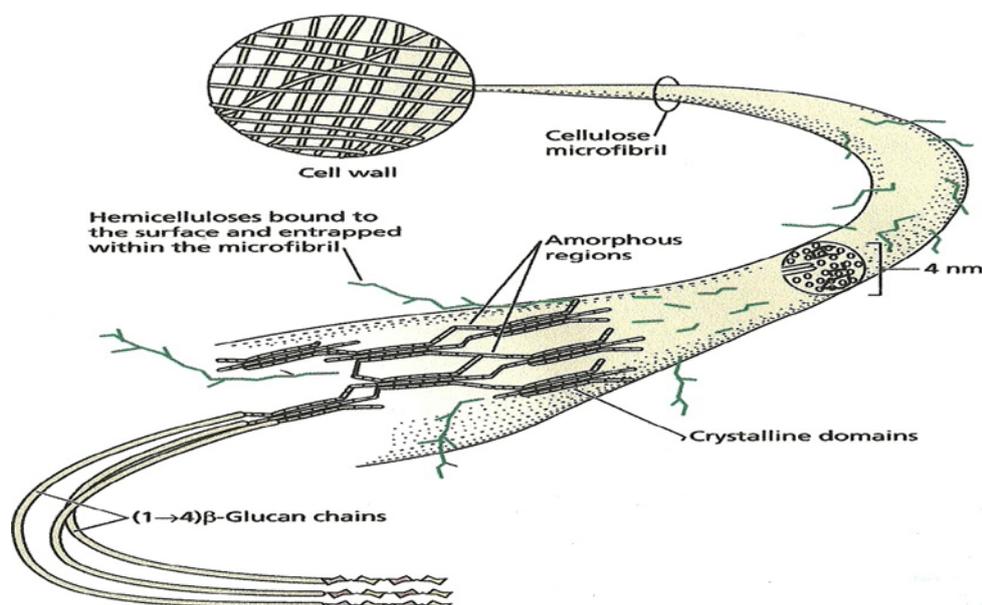
Figure 1.03: Structural model of cellulose microfibril showing the highly crystalline and less organized amorphous regions with hemicellulose bound to the microfibril surface (Taiz and Zeiger, 2002)

Tight non-covalent bonding between adjacent glucans within cellulose microfibrils gives the structural properties of cellulose, notably a very high tensile strength comparable to that of steel (Cosgrove, 1997; Taiz and Zeiger, 2002). Additionally, its chemical stability and insolubility make cellulose suited for use as the primary

structural component of the wall (as well as for many commercial and industrial applications).

Cellulose biosynthesis involves a sophisticated membrane complex (cellulose synthase complex) containing a number of protein subunits (Brown *et al.*, 1996; Saxena and Brown, 2005; Guerriero *et al.*, 2010; Keegstra, 2010). These proteins form a complex rosette-like structure of six complexes arranged hexagonally, each in turn consisting of six hexagonally arranged cellulose synthase (CESA) subunits embedded in the plasma membrane that transfer glucose from cytoplasmic UDP-glucose to produce multiple glucan chains that eventually hydrogen bond to form the cellulose microfibril (Brown and Montezinos, 1976; Brown *et al.*, 1996; Somerville, 2006; Keegstra, 2010).

1.3.2 – Xyloglucan

Xyloglucan is the main hemicellulose in the primary cell walls of most dicotyledonous plants. Xyloglucan is a branched polysaccharide with a backbone, like cellulose, of β-(1- 4) linked D- glucan but unlike cellulose it has short side chains consisting of xylose, galactose and often, but not always, a terminal fucose, as illustrated in Fig 1.04 (McNeil *et al.*, 1984; Fry, 1989; Cosgrove, 1997; Scheller and Ulvskov, 2010). The chain length of the xyloglucan has been reported to range from 200 to more than 1000 residues and therefore the length of the xyloglucan molecule may range from ~100 nm to more than 500 nm (Fry, 1989). Xyloglucan adheres to the cellulose microfibrils *via* extensive hydrogen-bonding and coats the microfibrils' surface with a monolayer (McCann *et al.*, 1990; McCann and Roberts, 1991). This cellulose-

xyloglucan network is generally considered to be the major load-bearing element of the primary wall. However, it also seems that xyloglucan prevents microfibril self-association (McCann *et al.*, 1990; Carpita and Gibeaut, 1993; Reiter, 2002; Somerville *et al.*, 2004). The length of the xyloglucan is much longer than the spacing between adjacent cellulose microfibrils (typically 20-40 nm in dicotyledonous plants) so that some of the xyloglucan appears to continue across several microfibrils (McCann and Roberts, 1991; Cosgrove, 1997). Amounts of xyloglucan and cellulose are almost equal in the primary wall. A part of the xyloglucan can be bound to the cellulose and the rest span the gaps between microfibrils and it has been hypothesised to hold the microfibrils together as tethers or to hold them apart against the tendency of the cellulose to aggregate, acting like supportive 'pit-props' (McCann *et al.*, 1990).
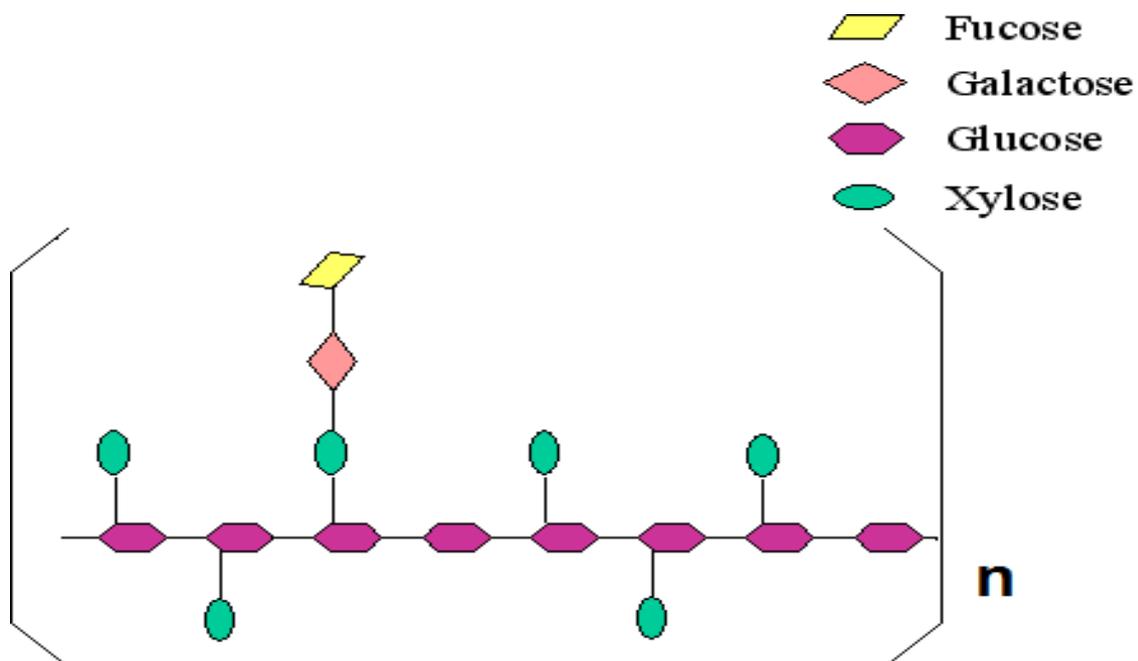
Figure 1.04: Schematic representation of xyloglucan structure. (The figure is taken from http://plantbiology.ucr.edu/faculty/raikhel.html)

The biosynthesis of xyloglucan occurs in the Golgi membranes and at least four types of enzymatic activity are needed to synthesize this complex polymer (Faik *et al.*, 2002; Liepman *et al.*, 2010). The enzymatic activities involve; using UDP-glucose β-(1,4) glucan synthase to synthesize the glucan backbone, α-(1,6) xylosyltransferase to attach xylose residues to the glucan backbone, β-(1,2) galactosyltransferase to attach galactose residues to the xylose residues and a α-(1,2) fucosyltransferase to attach fucose residues to specific galactose residues. Once synthesized, it is transported to the cell surface and deposited to the wall matrix (Faik *et al.*, 2002; Liepman *et al.*, 2010; Scheller and Ulvskov, 2010).

1.3.3 – Pectins

Pectins are the most structurally complex group of polysaccharides in nature and most soluble in the wall, making up about 35% of the primary wall of dicots and non-graminaceous monocots and up to 10% of the primary wall of grass and other commelinoid plants (Brett and Waldron, 1996; Ridley *et al.*, 2001; Scheller *et al.*, 2007; Mohnen, 2008). It is almost the sole component of the middle lamella which is responsible for cell-cell adhesion (Jarvis *et al.*, 2003). As many as 17 different monosaccharides are organised in a number of polysaccharide structures to form the pectin network in plant cell wall, as illustrated in Fig. 1.05 (Ridley *et al.*, 2001; Vincken *et al.*, 2003), which coexists with the cellulose-xyloglucan network of the primary wall but is generally thought to be structurally independent and can be removed from the wall without affecting it. Pectins are a covalently linked galacturonic acid (GalA) rich cell wall polysaccharide family (Albersheim *et al.*, 1996; Mohnen, 2008) consisting of three different types of polysaccharides domains; (a)

homogalacturonan (HG), a linear chain of 1,4 linked α-D- galacturonic acid residues that makes up about 65% of pectin. The carboxylic groups of HG are partially methylesterified, (b) rhamnogalacturonan - I (RG-I), a branched polymer consisting of a backbone of a repeating disaccharide of [- 4)-α-D-galacturonic acid- (1,2)-α-L-rhamnose-(1-] with side chains predominantly comprising arabinofuranosyl and galactopyranosyl residues (Ridley *et al.*, 2001) and representing 20 - 35% of wall pectin (Mohnen, 2008), and (c) rhamnogalacturonan - II (RG-II) (Darvill *et al.*, 1978; O'Neill *et al.*, 2001; O'Neill *et al.*, 2004), the most structurally complex pectin consisting of a backbone of linear 1,4-linked α-D- GalA residues as in HG but highly branched, with four different side chain consisting of 12 different glycosyl residues linked by more than 20 different linkages (Ishii *et al.*, 1999; O'Neill *et al.*, 2004; Mohnen, 2008), and representing only about 10-15% of the pectin. It has been demonstrated that RG-II exists in the primary cell wall as a dimer that is cross linked by a borate diester (Kobayashi *et al.*, 1996). See section 1.3.4 for further details.

The interaction of the domains within the pectin network is highly complex. It is generally thought that the backbones of RG-I and RG-II are covalently bonded to HG and perhaps to each other (Willats *et al.*, 2001) but it is not completely understood how these polysaccharides are linked (Mohnen, 2008). The ionic "egg-box" cross-linking of calcium cross-linked HG and the datively bonded borate dimer of RGII are also believed to contribute to the three-dimensional pectic network (O'Neill *et al.*, 2004).
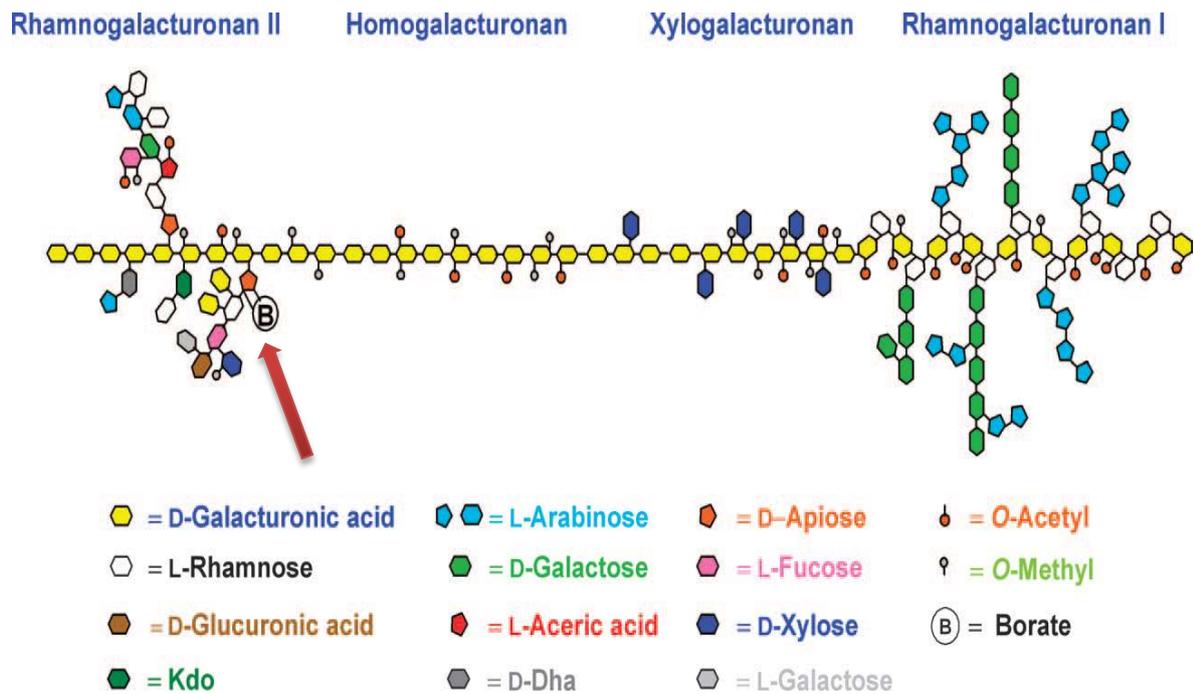
Figure 1.05: A schematic representation of pectin structure. The figure illustrates different domains of pectin and the side chains in RGII (side chain A; arrowed) contains an apiose residue that is linked by a borate ester that usually form RG-II dimer by linking to an apiose residue in the side chain A of another RG-II (Scheller *et al.,* 2007).

Pectin controls the wall porosity and therefore the movement of macromolecules (including enzymes) and also provides charges on the wall surface for cell-cell adhesion (Jarvis *et al.*, 2003) and within the wall. The pH and ionic status of the wall matrix therefore make an important contribution to the cell wall mechanical properties (Brett and Waldron, 1996; Willats *et al.*, 2001). The function of cell wall pectin in the extensibility of the wall is still not certain, but it is abundant in the primary cell wall and may play some role during cell wall extension. It has been suggested that calcium chelators, such as EDTA, promote the creep of plant tissue (Thompson, 2005). Recently It has also been reported that chelation of calcium by polygalacturonic acid or EGTA (ethylene glycol-bis (beta-aminoethyl ether)

N,N,N',N'-tetraacetic acid) accelerate growth of live *Chara* cells in calcium containing culture medium (Proseus and Boyer, 2012).

Crosslinking of pectic polysaccharides within the cell wall is not the only interaction that affects mechanical properties but the extent of hydration of the pectin matrix is also important (Tibbits *et al.*, 1998). Research has been shown that Ca^{2+} and Mg^{2+} counterions reduced the swelling and increased the stiffness of pectin film by controlling the hydration of the film (Zsivanovits *et al.*, 2004).

It can be noted that the amount of pectin content in Type-ll cell walls (5-10%) is much lower than that of Type-I cell wall (more than 30%), indicating that if pectin has a role in wall extension, it is not necessarily dependent on abundance. High pectin concentrations might give a high hydration potential to the wall, which may help the compressive strength of the wall (Darley *et al.*, 2001).

Pectin is synthesized in the Golgi vesicles by membrane bound or associated glycosyltransferases and secreted into the cell wall (Baydoun *et al.*, 2001; Willats *et al.*, 2001; Scheller *et al.*, 2007; Mohnen, 2008). Because of the structural complexity of pectin a large number of enzymes must be involved in its production, and it has been reported that about 54 different enzymatic activities are required for pectin synthesis (Scheller *et al.*, 2007).

1.3.4 – Rhamnogalacturonan-II (RG-II)

RG-II is a structurally complex low molecular mass (5 – 10 kDa) pectic polysaccharide, with four different side chains (A-D), and is present in the plant primary cell wall. It was first identified in 1978 as a minor component of suspension cultured sycamore cell walls (Darvill *et al.*, 1978). Subsequently, RGII has been isolated from several different plant species, for instance; from suspension cultured rice cells (Thomas *et al.*, 1989), from sugar beet pulp (Ishii and Matsunaga, 1996), from radish cell walls (Kobayashi *et al.*, 1996) and it was also isolated from red wine in appreciable quantities (Doco and Brillouet, 1993). RGII contains twelve different glycosyl residues including some rare monomers such as apiose, 2-0-methyl-L-fucose, 2-0-methyl-D-xylose, L-aceric acid, 2-keto-3-deoxy-D-*lyxo*-heptulosaric acid (Dha) and 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo), and linked together by more than 20 different types of linkages (Pellerin *et al.*, 1996; O'Neill *et al.*, 2004). Since its discovery, it has been the subject of intensive research by various groups and it has been determined that cell wall RG-II exists as a dimer that is covalently cross-linked by a borate diester as illustrated in Fig. 1.06 (Kobayashi *et al.*, 1996; O'Neill *et al.*, 1996).
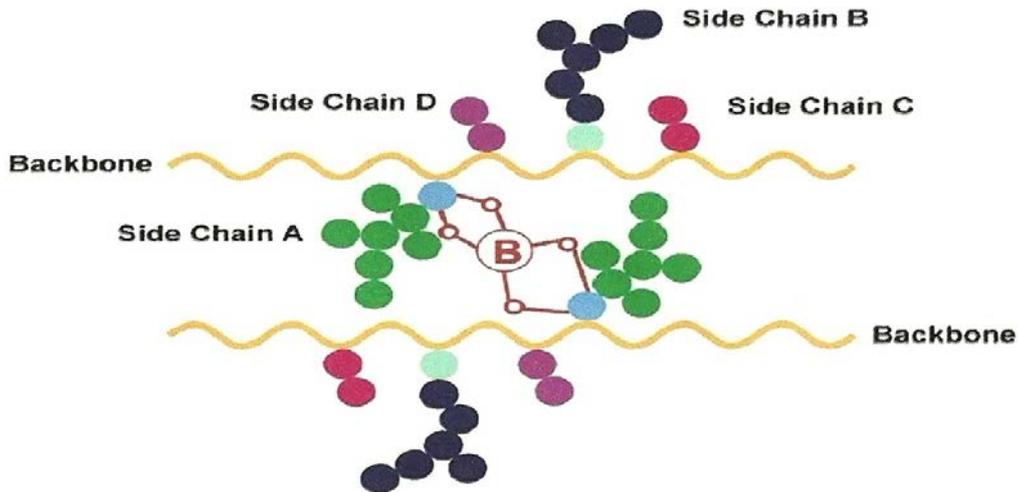
Figure 1.06: Schematic representation that shows two RG-II molecules covalently cross-linked to one another by a 1:2 borate diester. The apiosyl residue of side chain A of each monomer is cross-linked by the borate ester (O'Neill *et al.*, 2004)

The exact function of RG-II is unknown, but it is the only boron-containing polysaccharide in the plant cell wall and boron is considered to be an essential micronutrient for plant growth. Although boron is an essential nutrient, its exact function in plant growth has been uncertain but it has been reported that boron deficiency causes plant growth with abnormal morphology (O'Neill *et al.*, 1996). It has been demonstrated that *mur1 Arabidopsis* mutant plants are dwarfed and have brittle stems. In *mur1* mutants the L-Fuc and 2-O-methyl L-Fuc residues of RG-II are replaced by L-Gal and 2-O-methyl L-Gal and unexpectedly only 50% of the RG-II is cross linked by borate. Supplying these mutants with L-Fucose or boric acid rescued their normal growth (O'Neill *et al.*, 2001) therefore indicating that RG-II that linked with boron, plays an important role during plant growth. Because of its presence in the primary wall and its role in plant growth, the function of RG-II in cell wall extension was investigated, using *Arabidopsis* cell wall mutants and the *Acetobacter* cell wall model (see section 1.4 for further details).

1.3.5 – Cell wall proteins

In addition to the polysaccharides, the plant cell wall contains small amounts of protein. Cell wall proteins are generally glycoproteins and include both enzymes and structural proteins. The structural wall proteins are classified according to their amino acid composition, as hydroxyproline-rich glycoproteins (HRGPs) most notably extensin, glycine-rich proteins (GRPs), proline-rich proteins (PRPs) and a few others. Among the structural proteins, HRGPs, especially extensin, are the best-studied. They have a backbone containing a repeated Ser-Hyp$_{(4-6)}$ sequence (Kieliszewski and Lamport, 1994; Dey *et al.*, 1997). Extensin is water soluble when it is secreted at the plasma membrane but once it is in the wall it becomes insoluble by forming a network through covalent links between extensin monomers and with other cell wall components (Dey *et al.*, 1997; Johnson *et al.*, 2003). HRGPs present, with greatest quantities, in the phloem, cambium and schlerenchyma tissues cell wall and GRPs and PRPs most often present in the xylem tissues. Extensins are induced by numerous physiological and environmental stresses such as water stress, wounding, elicitors and pathogen attack (Kawalleck *et al.*, 1995; Johnson *et al.*, 2003). The cross-linked extensin network is thought to provide rigidity by strengthening the cell wall in response to wounding and infection of tissues and creates a physical barrier against pathogen attack. It has been proposed that peroxidase mediated extensin cross-linking may be an important factor in restricting plant growth (Brownleader *et al.*, 2000).

Besides these structural proteins, cell walls also contain some functional proteins (enzymes). Important enzymes in the plant cell wall include – oxidative enzymes (peroxidases), hydrolytic enzymes (pectinases, cellulases), transglycosylases and

expansins (Brett and Waldron, 1996) and see section 1.3.6 for further details. There is evidence that peroxidase could alter the mechanical properties of cell wall, and it has been demonstrated that the stiffness of the tomato exocarp cell wall increased significantly when peroxidase, isolated from matured tomato fruit skin, was applied (Andrews *et al.*, 2002).

1.3.6 – Expansins

Expansins do not appear to be 'classic' enzymes, and were first isolated from young cucumber seedlings in 1992 as the mediators of 'acid-growth' (Rayle and Cleland, 1992; McQueen-Mason *et al.*, 1992). Expansins stimulate cell wall creep in response to a unidirectional extensive force, and also seem to play diverse roles in plant development, such as leaf organogenesis, fruit softening and wall disassembly (Cosgrove, 2000b; Darley *et al.*, 2001).

Cosgrove (1989) reported that under a constant load at neutral pH the plant cell walls soon stop extending, but they rapidly extend if the pH is then lowered. He showed that for long term creep the optimum pH was 2.5 to 4.0 and at pH 6.8 extension stops (Cosgrove, 1989). This phenomenon was well known, but Cosgrove demonstrated that it was eliminated by heat treatment, suggesting that acid induced expansion is not only due to the physical properties of cell wall polysaccharides but also the activity of wall functional proteins. The active proteins, responsible for acid induced expansion were named 'expansins' when they were identified (McQueen-Mason and Cosgrove, 1995). The molecular mass of the two related proteins identified from the active components of the cucumber hypocotyls were initially reported to be about 29 kDa (Cosgrove, 1997) although subsequent work has

suggested that molecular weight of expansins is 25-27 kDa (Keller and Cosgrove, 1995; Cho and Kende, 1997a; Cho and Kende, 1997b). The cucumber expansin encoded by the gene consists of three domains, a signal peptide with 22-25 amino acids that directs the nascent polypeptide into the ER/Golgi and is removed before the protein enters to the cell wall, a carboxy-terminal domain, with ~ 98 amino acids, which appears to function as a polysaccharide-binding domain and between these two domains a cysteine–rich EG 45- like domain with sequence similarity to family-45 endoglucanases (Darley *et al.*, 2001). The sequence analysis and molecular cloning of cucumber hypocotyls expansins (Shcherban *et al.*, 1995) led to identification of expansins from different plant varieties including grass pollens with a sequence similarity identified between cucumber expansins and grass pollen proteins previously described as Group 1 pollen allergens (Knox and Suphioglu, 1996).

On the basis of substrate specificity and sequence similarities, expansins have been classified into two subgroups (i) α- expansins and (ii) β- expansins (Cosgrove, 2000a; Cosgrove, 2000b; Darley *et al.*, 2001). It has been reported that α-expansins were more effective in inducing creep and stress relaxation in Type-I cell walls, with little effect on grass cell wall but β-expansins mostly affect grass cell walls (osmola *et al.*, 1997; Cosgrove, 2000a). Expansins loosen the cell wall, but have no identifiable glycosidase or transglycosylase activity. It has been hypothesised that expansins allow turgor driven creep (extension) by weakening the non-covalent bonding of the wall polysaccharides by a unique and novel mechanism, but their exact biochemical mechanism and their site of action are uncertain. One of the hypotheses is that expansin facilitates creep or stress relaxation by breaking load bearing bonds between cellulose and xyloglucan of the cell wall and thus allows cell wall extension

to occur (Cosgrove, 2000b; Darley *et al.*, 2001; Cosgrove *et al.*, 2002). It has been claimed that β-expansins are members of the C1 (papain-like proteases) family of cysteine proteases and that their proteolytic activity degrades cell wall structural proteins and thus enhances wall extension (Grobe *et al.*, 1999; Grobe *et al.*, 2002) but this was refuted by Li and Cosgrove (2001) after evaluating β- expansins using a range of proteinase assays. Additionally, expansins have been shown to act on bacterial cellulose composites that do not contain structural protein (Whitney *et al.* 2000 and work presented in this thesis). In contrast to the prevalent theories of the mode of expansin action it has been proposed that expansins not only break the non-covalent bonding between the polysaccharides but also increase the hydration of cell walls and thus increase the cell wall free volume reducing constraints upon microfibril movements and facilitating turgor-driven cell wall creep and this thesis will examine this hypothesis. Cucumber α- expansin, kindly provided by Simon McQueen-Mason (CsExp1 expressed in tomato; Rochange and McQueen-Mason, 2000) and an extract of an acetone powder from the visceral hump of *Helix pomatia* L. (the edible snail) obtained from Sigma (Sigma S-9764, now discontinued) that contains an expansin like protein (Cosgrove and Durachko, 1994), have been used in this research.

1.3.7 – Snail acetone powder

Snail acetone powder (SP) from the visceral hump of *Helix pomatia* includes a variety of proteins, including β-glucuronidase, sulfatase and β-D-mannosidase. It has been claimed that snail acetone powder made from snail digestive tract induced

long-term extension of heat-inactivated cell wall in the same way as expansins (Cosgrove and Durachko, 1994). Further investigation revealed that SP contained a protein with a similar molecular weight to expansin (29 kDa). An antibody raised against the expansin-29 bound to a protein in the snail extract with a molecular weight of about 26 kDa suggesting that this expansin-like protein might be responsible for the creep activity on cell walls (Cosgrove and Durachko, 1994; Cosgrove *et al.*, 1999).

1.4 – Cell wall analogue based on *Acetobacter* cellulose

Cellulose, xyloglucan and pectin are three major polysaccharide components of the plant primary cell wall and constitute the majority of the wall's dry weight. Understanding how these molecular components are associated in the cell wall network is central to understanding the molecular basis of cell wall extension and plant growth. Researchers have attempted to assemble the components of the cell wall *in vitro,* but these studies failed to produce composites with correct relative amounts of the polymers (Darley *et al.*, 2001). However, an alternative, more effective way to produce cell wall-like composites has been developed (Whitney *et al.*, 1995; Chanliaud and Gidley, 1999). *Acetobacter xylinus* produces cellulose microfibrils similar to those in plant cell wall. In static culture the microfibrils become entangled and form a mat known as a pellicle. When *A. xylinus* is grown in the presence of xyloglucan and pectin in the medium, they become incorporated into the pellicle and form cellulose composite that have many biochemical and mechanical characteristics analogous to those of the cellulose-matrix network of plant cell walls (Chanliaud and Gidley, 1999; Whitney *et al.*, 1999; Chanliaud *et al.*, 2002). Research has showed that *A. xylinus* can allow the production of synthetic analogues of plant

primary cell walls as a model for cell wall study (Whitney *et al.*, 1995; Chanliaud and Gidley, 1999; Whitney *et al.*, 1999; Whitney *et al.*, 2000; Chanliaud *et al.*, 2002). Uniaxial tension measurements revealed that the cellulose alone is strong and stiff, but the composites produced with xyloglucan and xyloglucan / pectin are weaker and more extensible. The strength of the xyloglucan containing composites is about 20% of the cellulose strength and the effects of expansin on xyloglucan containing composites are similar to its effect on intact plant cell wall (Whitney *et al.*, 2000). In this research project, cell wall analogue was produced by incorporating tamarind xyloglucan and apple pectin with *A. xylinus* subsp. *sucrofermentans* cellulose to simulate the plant primary cell wall. Also novel composites were produced by incorporating polygalacturonic acid or RG-II with *A. xylinus* cellulose to determine the role of RG-II in cell wall extensibility and hydration, and also with lichenan and oat mixed linkage β-glucan to compare the effects of hemicelluloses from type II walls.

1.5 – Cell wall models and wall extension

Cell walls of growing plant cells are extended by the physical force generated by cell turgor pressure. The wall of growing cells is a thin (0.1-1.0 μm) and flexible layer consisting of complex carbohydrates and small amounts of structural proteins. The turgor pressures of growing cells typically range from 0.3- 1.5 MPa (Thompson, 2005) and the turgor generated stress to the thin wall layer are reported to be 10 to 100 MPa (Cosgrove, 2003; Thompson, 2005). The cell wall has to resist this huge pressure and enlarge at the same time. The organisation and interaction between cell wall components is crucial for the normal growth and development of a plant and knowledge of cell wall architecture is central to understanding how the cell wall

expands during growth and many cell wall models have been proposed. Keegstra *et al.* (1973) suggested that covalently linked xyloglucan, pectic polysaccharides and structural proteins formed a giant macromolecular complex and cross-linked cellulose microfibrils together. This model was subsequently abandoned because there is little evidence of cross-links between pectic polymers and structural proteins (Talbott and Ray, 1992). An alternative, referred to as the multicoat model, suggests that cellulose microfibrils are coated with xyloglucans which do not directly tether them together but interact with other layers of hemicelluloses and pectins in the space between the microfibrils (Talbott and Ray, 1992). Another model proposes that cellulose microfibrils may be tethered together by xyloglucan chains which are non-covalently bonded and entangled by independent networks of pectic polysaccharides and structural proteins (Fry, 1989; Hayashi, 1989; Pauly *et al.*, 1999).

Based on this tethered model Passioura and Fry (1992) proposed a model, which has come to be widely accepted and is often referred to as the 'sticky network' model, assuming that xyloglucan is bound to the cellulose microfibrils by hydrogen bonds and forms molecular tethers holding the microfibrils together and plant growth occurs when these bridging polysaccharides are broken, or removed from the microfibril surface by expansins (Cosgrove, 2000a). In contrast, most recently Thompson (2005) stated, with evidence, that the work done during the cell wall extension is greater than the total hydrogen bond energy of interactions between cellulose microfibrils and xyloglucan, so that these interactions cannot limit the growth rate, as predicted by the sticky network model (Thompson, 2005). Another model has been proposed in which xyloglucans are cross-linked to the microfibrils but keep them apart rather than together. Cellulose and xyloglucan are embedded in

a pectin matrix and the xyloglucan, and perhaps pectin, act as supportive pit-props that hold the cellulose microfibrils apart (McCann *et al.*, 1990; McCann and Roberts, 1991). Walls collapse if the xyloglucan is removed from the cell wall (McCann *et al.*, 1990) and there is evidence that elimination of xyloglucan in *Arabidopsis* mutants caused little difference in mechanical behaviour between wild type and the xylosyltransferase mutants (Cavalier *et al.*, 2008) and was not lethal, as might be expected if xyloglucan was as fundamental as proposed by the sticky network model.

The free space separating polymer molecules is critical in determining the properties of synthetic polymers (Ward and Hadley, 1993), and so it is conceivable that the cellulose-xyloglucan cross-links may act as 'scaffolds' regulating the space available for microfibril movement and increasing wall extensibility, rather than holding them together (Thompson, 2005). Therefore, cell wall volume and spacing, as well as the viscosity of the matrix between the microfibrils might play a significant role in plant growth regulation (Thompson, 2005).

Following from this, it has been suggested that cell wall water content directly affects cell wall extensibility (Evered *et al.*, 2007). Water is the most abundant component of the cell wall, making up more than two thirds of the wall mass and the hydration state of the matrix has been shown to be an important determinant of the physical properties of the wall; particularly removal of water makes the wall stiffer and less extensible (Edelmann, 1995; Evered *et al.*, 2007; Thompson, 2008). This stiffening effect of dehydration may play a role in growth inhibition by water deficits (Evered *et al.*, 2007).

It has been noted that hydrogen bonds are not directional, so although they should be able to affect cell wall volume and alterations in hydrogen bonding seem likely to

lead to wall swelling or compaction, it is not clear that they can determine shape (Veytsman and Cosgrove, 1998). It is thus possible that hydrogen bonding between microfibrils and tethers regulates growth by controlling free space in the wall, rather than by directly contributing to its mechanical strength, and that breakage of hydrogen bonds (perhaps by expansins) could modify wall properties by releasing steric constraint on microfibril movement irrespective of any effect on load bearing bonds.

1.6 – Aims and Objectives

The overall aim of this project was to investigate and establish a model to describe how the plant cell wall extends during plant growth and to relate this to the physical and biochemical properties of plant cell wall components in order to clarify their functions in the regulation of plant growth. *Acetobacter* cellulose and cellulose-based composites, sunflower hypocotyls and *Arabidopsis* mutants were used as models for this project. It was hypothesised that cell wall extension is not only a consequence of breaking hydrogen bonds between cellulose microfibrils and xyloglucan by expansins, but that a wider range of factors are involved including wall water content and cell wall free volume and that pectic polymers are also important, especially RG-II. Additionally the project aimed to study the behaviour of cell wall polymers in order to evaluate the 'sticky network' and 'scaffolded' models of plant cell wall. This was to be done by investigating factors affecting cell wall water content and the water binding capacity of the wall polysaccharides.

The main aspects of the work were:

(i) Effects of water and space on wall extensibility.

- Investigation of how cell wall components affect the water content and consequences of this effect on wall extensibility.

- Understanding of the action of expansin on water uptake and wall swelling.

- Investigation of the water potential of cell wall polymers.

(ii) The roles of specific polymers and expansin that comprise the cell wall in regulating growth and development.

- Ascertaining the contributions of individual wall components to the mechanical properties of the cell wall by incorporating them into *Acetobacter* cellulose.

- The role of Rhamnogalacturonan II (RG-II) in plant growth was investigated using different *Arabidopsis* cell wall mutants and by cellulose / RG-II composites.

CHAPTER 2

MATERIALS AND METHODS

2.01 – Materials

The bacterial strain, *Acetobacter xylinus* subsp. *Sucrofermentans* (ATCC® 700178)*, was obtained from the LGC Promochem, London - a partner of American Type Culture Collection, USA. This bacterium recently renamed as *Gluconacetobacter xylinus* but it will be termed as *Acetobacter xylinus* in this thesis as this name is well known in the literature.

Sunflower (*Helianthus annuus*) seeds, Giant Yellow variety, were obtained from Suttons seeds, UK. All the *Arabidopsis thaliana* seeds were obtained from The Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, UK. Perlite was obtained from Silvaperl, Gainsborough, UK.

Xyloglucan was obtained from Megazyme International Ltd, Ireland. The RG-II sample used in preliminary experiments, purified from red wine, was a generous gift from Professor Malcolm O'Neill, Complex Carbohydrate Research Center (CCRC), University of Georgia, USA. The α-expansin (CsExp1) was a gift from Professor Simon McQueen-Mason, University of York, UK which had been isolated from cucumber hypocotyls and expressed in tomato plants (Rochange and McQueen-Mason, 2000). Vantage L Laboratory Column VL 32 x 1000 was obtained from Millipore (UK) Ltd (Watford Hertfordshire, UK) and red wine (Cité de Carcassonne Merlot 2009, France) was purchased from Sainsbury's supermarket UK.

All other chemicals and materials were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) and VWR International Ltd, UK.

2.02 – Production of bacterial cellulose

The production of bacterial cellulose involved culturing of the organisms in a suitable medium, followed by harvesting the pellicle from the medium. The composition of the medium (referred to as YGC medium) for the growth and maintenance of *A. Xylinus* is shown in Table 2.01 below. The components were mixed using a magnetic stirrer and heater. To dissolve the Calcium Carbonate ($CaCO_3$) the medium was mixed thoroughly and cooled rapidly.

For the production of cellulose, *A. xylinus* was cultured in static conditions in 250 mL Erlenmeyer flasks. 100 mL of the YGC medium was poured into the flask and, to minimize contamination, a rubber sponge and aluminium foil was placed on top of the flask before autoclaving at 121° C for 15 min. Maintaining aseptic conditions, each flask was then inoculated with 1 mL of seed culture from a previously prepared universal bottle. The flasks were then incubated at 26° C for 96 to120 hours in static condition.

Table 2.01: The composition of YGC medium.

Compounds	Quantity (g)
Glucose	50.0
Yeast extract	5.0
$CaCO_3$	12.5
Distilled water	1.0 L

The cellulose formed a thin film on the air-liquid surface within 48 hours and after a further 24 hours, a distinct pellicle was present (Fig. 2.01a). The pellicles were initially transparent, but as growth continued they became thicker and opaque white in colour. After the incubation period cellulose pellicles were harvested and washed by gentle agitation in excess of distilled water to remove the loosely attached cells from the surface. The cellulose was then placed in Petri dishes full of de-ionised water and stored at 4° C.
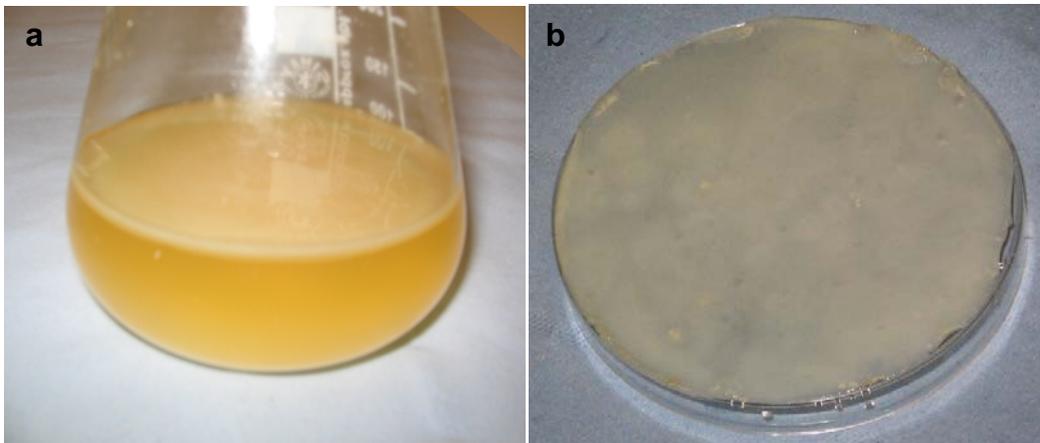
Figure 2.01a: Formation of cellulose film on the surface of a 72 hours liquid culture.
Figure 2.01b: Harvested smooth and glossy cellulose pellicle from a 84 hours culture.

2.03 – Composite preparation

A. xylinus was cultured in YGC medium to produce composites of cellulose. Different types of composites have been produced by dissolving different components into the medium. Initially four types of composites were produced; those were (a) cellulose alone, (b) cellulose / pectin (c) cellulose / pectin / xyloglucan and (d) cellulose / xyloglucan composites. Table 2.02 below shows the amount and components that supplemented to the YGC medium (Table 2.01) to produce different types of composites.

Table 2.02: *Amount and components that added to the YGC medium to produced different composites*

Composites	Components added to the YGC medium
Cellulose (C)	YGC medium only
Cellulose / pectin composite (C/P)	0.5% (w/v) pectin (Apple pectin, Sigma – Aldrich, cat. No. 76282,) 70-75% esterified.
Cellulose / xyloglucan composite (C/X)	0.5% (w/v) xyloglucan (Tamarind seed xyloglucan, Megazyme International, Ireland, Cat. No. P-XYGLN)
Cellulose / pectin / xyloglucan composite (C/P/X)	0.25% (w/v) pectin and 0.25% (w/v) xyloglucan

To dissolve the pectin and xyloglucan properly a magnetic stirrer and low heating was applied to the medium. It took about 30 minutes to completely dissolve. The pH of the medium ranged from 6.2 - 6.5. The medium was prepared in flasks and followed the procedures as described in section 2.02. During the culture, pectin and xyloglucan incorporated into the cellulose and formed a composite pellicle. By adding pectin and xyloglucan to the medium, it produced a synthetic analogue of the primary cell wall and the composite pellicle became more soft and flexible. This composite was distinguished as "cell wall analogue".

2.04 – Growing sunflower plants

Helianthus annuus seeds were imbided in tap water in a beaker overnight. The seeds were then sown in pots containing water-saturated perlite. The pots were fully covered with a plastic pot lid wrapped with aluminium foil and the plants were grown for 5 days at about 30° C and in complete darkness to obtain etiolated hypocotyls.

2.05 – Extensiometry

2.05.01– Sunflower hypocotyls

20 mm long segments were cut from the growing part of the sunflower hypocotyls and were longitudinally split into two using a double edged razor blade. The bisected hypocotyls were then frozen using freezing spray (RA Lamb, Eastbourne, UK). After 60 s the segments were thawed using 10 mM MES buffer containing 5 mM KCl and

1 mM CaCl$_2$, the pH of the buffer was titrated to 5.0 by adding 1M NaOH drop by drop. This buffer will be referred to as MES buffer for the remainder of the thesis and the pH value will be 5.0 unless otherwise stated. The hypocotyl segments were then pressed between microscopes slides wrapped with absorbent paper using about 2 kg of weight for 60 s and then placed them in to MES buffer or other experimental solution for 10 min.

2.05.02 – Bacterial cellulose and bacterial cellulose composites

Pieces of 2x1 mm in cross section and about 10 mm in length were cut from the cellulose or cellulose composite pellicle with a pair of razor blades held in a spaced block. The pieces were then incubated in MES buffer at pH 5.0 for 10 min at room temperature.

As described by Thompson (2001), one end of the segment or piece of pellicle was clamped to the bottom of reservoir tubes of the extensiometer (Fig. 2.02) by inserting the end into a slot. The other end of the segments was clamped to a similar slot which was hooked to one end of a cantilever arm. The clamps were constructed of nylon rod of 10 mm diameter with a central slot of 3 mm wide. The hypocotyl segment or piece of pellicle was placed into the slots and a small piece of 1.5 mm wide epoxy-glass board held the segment to the side of the slot and then by advancing a stainless steel screw pressing the epoxy board the segment was held firmly between the epoxy board and the side of the slot. About 10 mm of material was exposed between the clamps and the reservoir tubes were then filled with

experimental solution. The exact initial length of the exposed portion of the segment or piece of pellicle was measured using a magnifying eyepiece with a graticule.
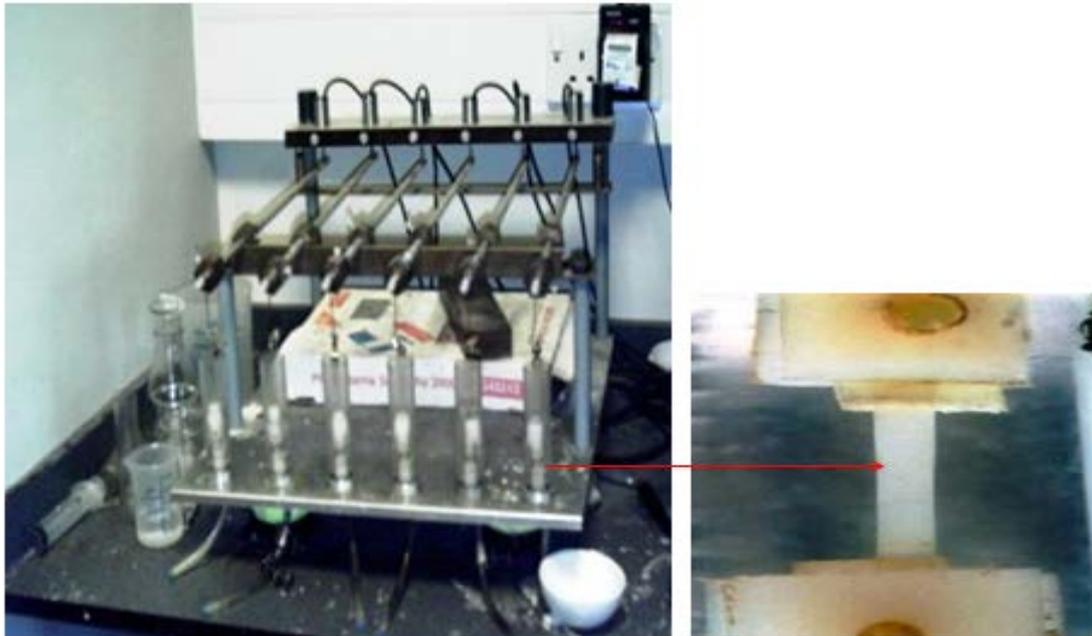
Figure 2.02 : An extensiometer with strips of *Acetobacter* cellulose composites clamped (arrowed) inside the tubes, filled with MES buffer or buffer containing PEG to determine the extensibility under constant load. It was custom built by the Biological Sciences workshop at the University of Lancaster, UK.

The arms of the cantilever to which the segments were clamped were one third of the length of the opposite arms so that it could amplify the extension of the segments mechanically. A Linear Variable Displacement Transducer (LVDT) core (Schlumberger DFG 2.5 – from RS Components Ltd., Corby, Northants, UK) was balanced at the end of the longer arm of each cantilever. A computer attached to the extensiometer recorded the displacement data from the LVDT core via a Bede PC-ADH24 analogue input card (Bede Technology, Jarrow, Tyne and Wear, UK). Every 30 s, each LVDT core position was determined 1000 times and the average was recorded. The cantilever was counterweighted, which made the hypocotyls pieces

slack until the LVDT core was put into its place, so that the initial weight was less than that of the LVDT core. The machine consisted of six reservoir tubes and clips, and six cantilevers and LVDT cores mounted on a steel base-plate which was placed on tennis balls to dampen the vibration. The clamped samples were left for 20 min to settle before any force was applied. The segments were stressed by sliding the brass weights along the cantilever arms (a schematic diagram of how the extensiometer works is shown on Fig. 2.03). The force was applied step by step beginning with 0.098 N (10 g of load) and increased further 0.098 N after 2 h and the segments were left for a minimum of 4 h to extend at the maximum force. The creep extension of the segments was calculated based on true strain (see section 3.01 for the description of true strain).
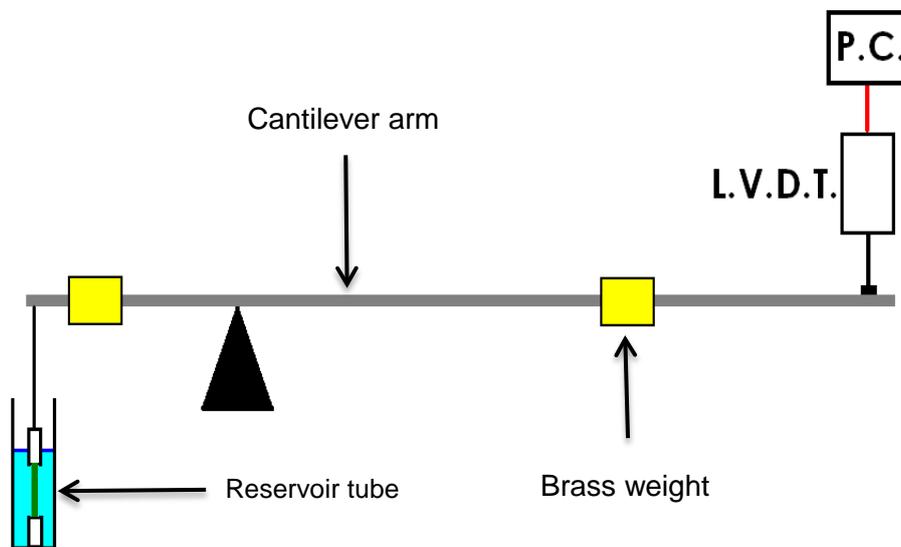
Figure 2.03: Schematic representation of the mechanism of works of the extensiometers.

2.06 – Effects of water on cell wall mechanical properties

Longitudinally bisected frozen and thawed sunflower hypocotyls or 20 mm long strips of bacterial cellulose or cellulose composites 2x1 mm in cross section were clamped in the extensiometer and the reservoir tube was filled either with control buffer (10 mM MES containing 5 mM KCl and 1 mM $CaCl_2$ at pH 5.0) or control buffer containing polyethylene glycol (PEG) 6000 (0.27 g/g) to give an osmotic pressure of ~ 0.62 MPa. The osmotic pressure of PEG was determined by vapour pressure deficit osmometry, using a Vapor$^®$ 5520 osmometer (Wescor Inc, USA) as this method is most commonly reported in the plant physiology literature (Evered *et al.*, 2007). It has been reported that the osmotic pressure of PEG solution measured by melting point depression gives higher values than the vapour pressure method (Kiyosawa, 2003) but has plausibly been argued that this is because higher order virial coefficients of the Van't Hoff equiation for PEG are affected by temperature so that measurement at low temperature (as is necessary for melting point determination) introduces artefacts (Winzor, 2004).

Force was applied to the hypocotyls or composite strips by sliding the brass weight along the cantilever arms of the extensiometer. After about 180 min, the initial bathing solution was drained from the reservoir tubes and gently replaced with buffer with a greater or lower osmotic pressure using a syringe. After a further 180 min, the bathing solution was again exchanged and the changes of extension of the hypocotyls / composites strips were recorded for a further period of time at constant load.

2.07 – Investigation of the effect of expansin on sunflower hypocotyl or cellulose composite mechanical properties

To determine the effects of expansin on cell wall extensibility 20 mm long bisected sunflower hypocotyls were boiled for 90 s then pressed between microscope slides wrapped with absorbent paper, and for the cellulose composites strips of 1x1 mm cross section and 20 mm length were boiled for a short time (30 s) and incubated in MES buffer (pressing between microscope slides process was omitted in this case).
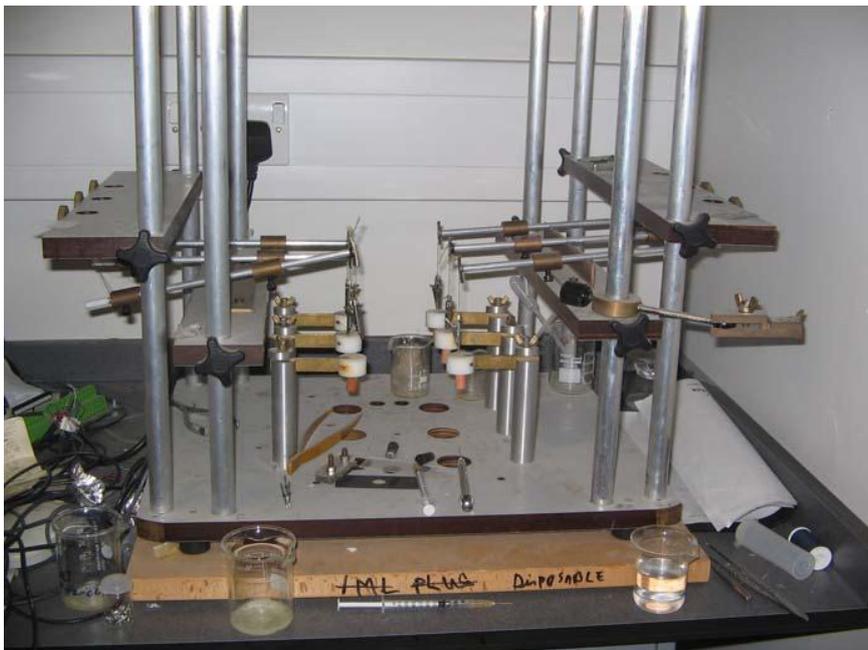
Figure 2.04: A purpose built extensiometer to observe the effects of proteins such as 'expansin' or any other solution of interest on cell wall extensibility (custom built by the Biological Sciences workshop at the University of Lancaster, UK.).

The strips were clamped into a purpose built extensiometer (Fig. 2.04) with a smaller reservoir volume of 120 µL. One end of the hypocotyl or composite strip was glued

between two small pieces of plastic (made by cutting up weighing boats) and was held in place by a cork at the bottom of the reservoir and the other end was clipped at one end of a cantilever arm. About 10 mm of the strip or plant material was exposed inside the reservoir, which contained pH 5.0 MES buffer. After loading into the extensiometer the strips were left to settle for about 20 min. Then a force of 0.098 N was applied to the hypocotyls or strips which were left to extend for an hour. Force was increased to 0.147 N and left for further one hour after which force was increased to 0.172 N for 5 min and then reduced to 0.147 N again. The reservoirs were topped up with distilled water every hour to compensate for evaporation. The strips were allowed to extend for about 2 hours and then the bathing solution was removed using a syringe and replaced with buffer containing extract (protein) or control buffer without protein and the test material was allowed to extend under a constant load for at least 2 h. The computer recorded the displacement of the LVDT core throughout.

2.08 – Snail acetone powder extract

50 mg of Snail acetone powder (Sigma-Aldrich, Dorset, UK, Cat. No # S 9764, and the product has been discontinued), from the visceral hump of *Helix pomatia*, was mixed into 1 mL of pH 5.0 MES buffer in an Eppendorf tube and mixed thoroughly using a laboratory vortex and incubated for 10 min at room temperature to maximize the protein in the extract. The mixture was centrifuged for 10 min at 7200 *g* using a table top micro centrifuge. The supernatant was collected as snail powder extract and the pellet was discarded. The collected extract was stored at 4° C for further use.

2.09 – Dehydration and re-hydration of cellulose and composites

Pieces of 2x1 mm in cross section and about 10 mm in length were cut from the cellulose and cellulose composites with a pair of razor blades held in a spaced block. The pieces were then incubated in MES buffer at pH 5.0 for 10 min at room temperature. The pieces were then blotted using absorbent paper to remove the surface water. The initial weight of the pieces was measured quickly using a laboratory microbalance. The pieces were then transferred into Eppendorf tubes containing 800 µL of PEG 6000 solution (0.27 g/g of MES buffer) giving an osmotic pressure of ∼ 0.62 MPa and the weight changes were measured. As the PEG solution was quite viscous, the pieces were rinsed into buffer as quickly as possible

and blotted using absorbent paper before measuring the weight and returned to the tubes for further readings. The changes of weight were recorded at regular intervals up to 180 min. At this time, the pieces were transferred to Eppendorf tubes containing 800 µL of control buffer (MES buffer, pH 5.0) to rehydrate them and the changes of weight were recorded at intervals. The changes were recorded until the rehydration had almost stopped. Then 11.2 µg of pure alpha-expansin (CsExp1) was added to the control buffer solution and the further changes of weight were recorded. The expansin was dissolved in pH 4.5 35 mM MES / 35 mM Acetate buffer containing 200 mM NaCl.

With the same procedure, experiments were also carried out to observe the effects of snail acetone powder extract. 200 µL of snail powder extract (50 mg / mL of MES buffer, pH 5.0) was added to the buffer solution when the rehydration had almost stopped with the control buffer, and the further changes were recorded.

The experiments were carried out at room temperature. While it was necessary to blot pieces using absorbent paper before measuring their weight, in every case the pieces were blotted the same number of times and same way and they were rinsed in buffer the same number of times before blotting.

2.10 – Turbidity assay

Turbidity of suspensions of cell wall fragments was observed by light scattering. Changes in cell wall free volume were observed *via* alterations of turbidity of suspensions of cell wall fragments.

Cell wall fragments suspension was prepared from approximately 1 g of the top 2 cm of sunflower hypocotyls or 1 g of small pieces of cellulose composites. The hypocotyls and composites pieces were boiled for 90 s and then homogenized in 10 mL of 10 mM MES buffer, containing 5 mM KCl, 1mM $CaCl_2$ at pH 5.0, using a laboratory mixer emulsifier at full speed for 5 min (Silverson Machines Ltd, Waterside, UK). The homogenized suspension was then centrifuged for 2.5 min at 650 *g* to remove un-fragmented tissue pieces. The supernatant was centrifuged again for 10 min at 1450 *g* and the pellet was re-suspended in 10 mL of fresh MES buffer pH 5.0 and the supernatant was discarded. The optical density of the resuspended fragments was determined using a spectrophotometer at a wavelength of 750 nm (*UV / VIS* Spectrophotometer, Lambda 20, Perkin Elmer). 1.0 mL of suspension was placed into a plastic cuvette and the extinction of the suspension was recorded for around 30 s to measure initial optical density and ensure that the reading was stable. After 30 s a known volume of expansin (CsExp1) or snail acetone powder solution was added to the cuvette and was gently inverted to mix the solution. The cuvette with suspension was returned to the spectrophotometer and the extinction was recorded for a further 120 s. Turbidity assays also carried out using *Trichoderma viride* cellulase, peroxidase from horseradish (Sigma-Aldrich, P6140) and H_2O_2.

2.11 – Expansin activities at different pH values

Strips of boiled sunflower hypocotyls or cell wall analogue were clamped into the micro-extensiometer and bathed into MES buffer of different pH values. The strips were then treated with expansin (CsExp1) or snail powder extracts.

Also, as described in section 2.10, boiled hypocotyls pieces or cell wall analogue pieces were homogenized into MES buffers covering a range of pH values to obtain cell wall fragment suspensions. The optical density of the fragment suspensions of different pH values was then measured and the changes of optical density after treatment with expansin or snail powder extracts were recorded.

2.12 – Comparing the mechanical properties of composites analogous to dicotyledonous and monocotyledonous cell walls

Cellulose composites were prepared using medium viscosity oat β-glucan (Megazyme International, Ireland, Cat. No. p-BGOM.) and Lichenan, from Icelandic moss (Megazyme International, Ireland, Cat. No. p-LICHN) to give composites C/ β-G and C/L respectively, in order to compare them with the composites prepared with xyloglucan (C/X) and xyloglucan and pectin (C/P/X). The composites were prepared using the method described in section 2.03. 0.5% (w/v) of β-glucan or Lichenan was added to the YGC medium to produce C/ β-G or C/L composites. The creep of the

C/X, C/P/X, C/ β-G and C/L composites was measured and compared using creep extensiometer as described on section 2.05.

2.13 – Growing *Arabidopsis thaliana* plants

Arabidopsis thaliana plants were cultured on half strength Murasige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.7% agar.

2.13.01 – Medium preparation

2.15 g of MS basal salt mixture (Sigma, Cat. No. M5524) was dissolved into 800 mL of distilled water in 2.0 L Erlenmeyer flask. A stirring bar was placed into the flask and powder was dissolved by stirring the medium on a stirring plate. The pH of the medium was adjusted to 5.7 by adding 1 M KOH drop by drop. 10 g of sucrose was added to the medium with stirring and 7 g of agar once the sucrose had dissolved. 200 mL of water was then added to adjust the final volume of the medium to 1000 mL.

The top of the flask was covered with aluminum foil before sterilizing by autoclaving. The autoclaved medium was placed on a stirring plate and stirred while it cooled. Sterilized medium was then poured into a sterilized Magenta™ vessel (GA-7). About 40-50 mL of medium was poured in each vessel under a laminar flow hood. When the medium was cooled and the gel was formed, the vessels were kept at 4° C.

2.13.02 – Seed preparation and plant growth conditions

About 50-60 seeds were placed in an Eppendorf tube and 1 mL of 70% ethanol was added. The seeds were mixed well and incubated for 2 min. Seeds were spun down using a micro centrifuge and the ethanol was carefully removed and discarded using a sterilized pipette tip. 1 mL of 5% sodium hypochlorite (NaOCl) solution was then added to the seeds and mixed for 10 min. Seeds were again spun down and the NaOCl solution was discarded. Seeds were then washed 5 times with 1 mL of sterile water and spun down again. About 20 seeds were spread onto the medium in each of the vessels. The vessels were capped, tightly covered with aluminium foil and kept at 4° C for 4 days in complete darkness for stratification. Seeds were washed and spread onto agar under a laminar flow cabinet to avoid contamination. Plants were grown for about 14 days under 16 h light / 8h dark photoperiod and 19º C / 23º C temperature regime.

Plants were grown by sowing the following *Arabidopsis* seed types:

(i)   Wild type (*Col 0*)

(ii)   *mur 1*   (N6244)

(iii)   *mur 2*   (N8565)

2.14 – Extensiometry on *A. thaliana* cell wall material

20 mm inflorescence stems from the growing region of 3 weeks old plants were cut using a razor blade. Stems were taken from three different plant varieties; *col-0*, *mur1* and *mur2*. Plant materials were frozen using freezing spray and thawed by dropping then into MES buffer before fixing them into the extensiometer. Stems were bathed in pH 5.0 MES buffer and force was applied in a series of steps and the LVDT positions were recorded. As the plants were very small and the stems are fragile, a lower force was applied starting with 0.049 N (5.0 g) with an increase of 0.049 N after 2 h and a further 0.0245 N (2.5 g) after another 2 h to a maximum of 0.123 N (12.5 g). Stems were stressed for longer time at the maximum force and the longer term creep was recorded for the various mutant plants.

2.15 – Turbidity assays on *A. thaliana* cell wall fragments suspensions

A. thaliana cell wall fragments suspensions were prepared from *Col 0* (wild type), *mur1 and mur2* mutant plants. Plants about two weeks old were taken from the vessels and roots were cut off then the rest of the plants materials were boiled for 60 s to inactivate endogenous expansins. The plant materials were then homogenised in pH 5.0 MES buffer, using 10 mL of buffer per gram fresh weight of plant tissues, using a lab mixer emulsifier for 5 min at full speed. The homogenates were centrifuged for 2 min at low speed and the supernatants were collected and

transferred to different centrifuge tubes and centrifuged again at 1400 g for 10 min. Pellets from the second centrifugation were re-suspended using the same buffer and centrifuged again at same speed for 10 min. This process was repeated 2 times to wash out the pigments. After the final centrifugation the pellets were suspended in fresh buffer and the rest of the procedures of turbidity assay were followed as section 2.10.

2.16 – Rhamnogalacturonan- II (RG-II) purification from red wine

French red wine (Cité de Carcassonne Merlot 2009), was purchased from a local supermarket (Sainsbury's Plc. UK). The RG-II purification protocol followed was the 'wine RG-II purification Scheme' developed at the Complex Carbohydrate Research Center, University of Georgia, USA (Pellerin *et al.*, 1996).

1 L of wine was taken and concentrated to 200 mL using Rotaevaporator (Rotavepor). The colloids from the concentrated wine were precipitated by 60% ethanol (EtOH) precipitation (with 60 mM Hydrochloric acid) for 24 h at 4° C. 200 mL of concentrated wine along with 300 mL of 100% EtOH and 2.5 mL of concentrated HCl were mixed in a 500 mL centrifuge bottle and left for 24 h at 4° C for precipitation.

The precipitate was then separated by centrifugation at 4570 *g* for 30 min at $4^{°}$ C and supernatant was discarded. The pellet was washed with 500 mL EtOH / 60 mM HCl (2.5 ml conc. HCl) in a blender in a cold room and was centrifuged again for 30 min. The supernatant was discarded and the pellet was dissolved in distilled water (dH_2O).

The wine polysaccharide solution was dialyzed in a cold room against dH_2O with 12-14000 MWCO (Spectra / Por 2) dialyzing tube. The dialyzed solution was then filtered with glass microfiber filter (Whatman 934-AH). The filtrated solution was lyophilized for further separation. The yield from an initial volume of wine of 1 L was about 450 mg (wine pellet).

2.17 – Size Exclusion Chromatography

A Vantage L Laboratory Column VL 32 x 1000 was packed with Sephadex G-75 in pH 5.2 50 mM Acetate buffer (containing 50 mM NaOAc and 0.02% sodium azide). The column was packed to a bed height of 70 cm. The 450 mg wine pellet was dissolved in 10 mL of acetate buffer (the same buffer as in the column but without the sodium azide) and loaded carefully onto the column to separate the RG-II from other components of the wine pellet. When the loaded volume had nearly moved into the bed, running buffer (acetate buffer) was poured gently into the column from the top, the running buffer was kept topped up until fraction collection was complete. The flow rate was about 2 mL / min and was controlled using a peristaltic pump. In the first instance, a void volume of 100 mL was allowed to elute from the column before fraction collection commenced, 20 fractions of 10 mL were collected using 10 mL centrifuge tubes. Fractions that contained pectins were identified by performing uronic acid assays.

2.17.01 – Uronic acid assay

Uronic acid assays were performed to determine which fractions might contain RG-II by quantifying uronic acid colorimetrically. As described by Taylor & Buchanan-Smith (1992), galacturonic acid was used as standard. For a standard curve six solutions of different concentrations ranging from 0 - 500 µg ml^{-1} were prepared.

***Preparation of reagents*:**

Acid borate reagent: For 100 mL of reagent (80% Sulphuric acid)

(i) 0.80 g of Sodium borate, (ii) 16.67 mL water and (iii) 83.33 mL concentrated sulphuric acid (H_2SO_4) (96%). Sodium borate was dissolved in the water in glass bottle and the concentrated H_2SO_4 was added to the bottle to carefully form a layer. The bottle was left over night to mix properly and cooled to room temperature.

Carbazole reagent: Carbazole reagent was prepared in absolute ethanol. 100 µg of carbazole was dissolved in 10 mL of ethanol to give a 0.1% (w/v) carbazole solution.

From all the collected fractions and galacturonic acid standard solutions, 200 µL of sample was transferred to borosilicate tubes and 3 mL of the acid-borate reagent was added to each. 100 µL of carbazole reagent was then added and the tube was thoroughly mixed. The solution became pink in colour. The tubes were then incubated in a preheated water bath at 60$^{°}$ C for 1 h and then cooled to room temperature using another water bath. The absorbance of the samples was read at 530 nm using a spectrophotometer, using the standard with no galacturonic acid as a blank. Samples containing uronic acid changed in colour from pink to red.

Fractions identified as containing uronic acid were dialyzed against dH_2O using 2000 MWCO (Spectra / Por 7) dialysis tubing. The fractions identified as containing RG-II were then concentrated using the rotary evaporator and lyophilized to obtain RG-II powder.

2.18 – Producing cellulose / RG-II and cellulose / polygalacturonic acid (PgA) composites

As described in section 2.03, cellulose composites were produced by incorporation of dissolved polysaccharides with the cellulose during culture, in these cases RG-II and PgA. The YGC *Acetobacter* culture medium was enriched with 0.5% (w/v) RG-II to produce cellulose / RG-II composite, 0.5 % (w/v) of PgA to produce cellulose / PgA composite and 0.25% (w/v) of xyloglucan and PgA each to produce cellulose/PgA/xyloglucan composite. RG-II was readily soluble in water but to completely dissolve the PgA the powder was added to the water in stages (three sequential additions). PgA was dissolved by heating the water to 50° to 60° C and by stirring the medium in a hot plate. The composites were harvested after 7 days incubation.

2.19 – Determining the mechanical properties of composites incorporating RG-II

To determine the effects of RG-II on cell wall mechanical properties, RG-II containing and RG-II free cellulose composites strips were clamped into the extensiometer, bathed into pH 5.0 MES buffer, and stressed to observe their mechanical behaviour. The applied load was increased in increments, starting with 0.29 N (30 g) and increasing to a maximum of 0.88 N (90 g) while the long term creep behaviour was measured by the extensiometer LVDTs.

2.20 – Dehydration and rehydration of cellulose / RG-II composites

As described in section 2.09, pieces of hydrated cellulose / pectin, cellulose / PgA and cellulose / RG-II composites pellicles were saturated in pH 5.0 MES buffer at room temperature. Pieces were blotted using absorbent paper and their initial weight was recorded before immersing them into PEG 6000 solution (0.27 g/g giving an osmotic pressure of ~ 0.62 MPa) to reduce their water content. The dehydrated weight was recorded at regular intervals up to 180 min. The pieces were then transferred into control buffer (MES buffer, pH 5.0) to rehydrate and the changes in weight were recorded at intervals up to 300 min.

2.21 – Comparing the hydrated weight and the dry weight of different composites

Small pieces were cut from different cellulose composite pellicles. They were blotted to remove the surface water before transfer to different centrifuge tubes. The initial weight of the empty tubes was recorded and the weight with the composites pieces was measured to obtain the wet weight of the composites. The tubes were then frozen at -80º C for 24 h. The frozen composites were then lyophilised (Savant Modulyo D Freeze drier, Thermo electron Corp, UK) for up to 48 h in a freeze dryer at -47°C and at a pressure of 133 mbar. After freeze drying the tubes were weighed again to obtain the dry weight of the composites.

2.22 – Measuring the water potential of cell wall materials

The osmotic pressures of pectin and xyloglucan solutions were measured using vapor pressure deficit osmometry. Pectin and xyloglucan solutions of a range of concentrations were tested;

1. 100 mg/kg of water
2. 200 mg/kg of water
3. 1.00 g/kg of water and
4. 5.00 g/kg of water.

Measurements were taken from fresh solution at 23 ±1° C. All the samples were transferred to Eppendorf tubes and closed tightly to protect possible evaporation.

The osmolality value of the samples was measured using Vapro® 5520, Vapor Pressure Osmometer (Wescor Inc., USA). The machine was calibrated using the 290 mmol/kg, 1000 mmol/kg and 100 mmol/kg Opti-mole™ osmolality standards (Wescor Inc., USA).

10 µL samples were taken using a micropipette and carefully dropped into the paper sample disc placed in the sample chamber to measure the osmolality of the solution. After the measurement cycle of 80 s was complete, the reading was recorded. Then the sample chamber was opened and the wet disc carefully removed and discarded. The sample holder was cleaned of any residual liquid using a lint free tissue. A series of readings were taken for every sample. During the experiments the osmometer was recalibrated between measuring different types of samples to reduce errors.

2.23 – Statistical analysis

All experimental data are presented as mean ± standard deviation. The data were compared using Student's *t*-test to assess the significance of differences between the selected factors. The differences were considered using the p-values as follows: significant when the $p < 0.05$, very significant when $p < 0.01$, highly significant when $p < 0.001$ and when the p value is higher than 0.05 it was considered there was no significant difference. The statistical analysis was performed using Microsoft Office Excel 2007 (Microsoft Corporation, USA).

CHAPTER 3

RESULTS AND DISCUSSION

3.01 – Effects of water on cell wall mechanical properties

Water is the most abundant component of the cell wall, making up more than two third of the growing cell wall mass, but its contribution to wall behaviour has often been overlooked. Considering the abundance of this water component, it has recently been hypothesised that water must be a key role player in plant growth and that the water content of cell wall directly affects the extensibility of growing cell wall (Evered *et al.*, 2007). To examine this hypothesis the water contents of sunflower hypocotyls and cellulose composites were altered using high molecular weight PEG solution and their mechanical behaviour under a constant load was observed using a creep extensiometer. The changes in extension under different water availability conditions were determined from the LVDT data recorded by the computer connected to the extensiometer. The relative lengths of the hypocotyls segments and the composite strips were calculated for comparison of their linear extension under constant load over time. After the stress was applied to the strips, it resulted in a characteristic extension (which would be described as strain in the material sciences). In previous work, the relative length of the hypocotyls or strips was calculated by dividing their length by their initial length before the stress was applied (Thompson, 2001), which is related to the engineering strain. This was done so that the degree of deformation of the strips could be compared even if the initial lengths were not the same (either because of variation in the starting material or differences in extension after previous increases in stress).

Relative length = L_t / L_0

Engineering strain (e) = $\Delta L_t / L_0$

where L_0 is the initial length and L_t is the length at time t.

However, in this work the relative length of the strips after the increase of stress was calculated based on 'true strain' or logarithmic strain, which is more realistic as engineering strain is calculated only based upon the initial lengths of the strips whereas for true strain the continuous variation of lengths over time is taken into account as it is the integral of change of length with time. The natural log of the engineering strain is true strain and for uniaxial extension, engineering strain is converted to true strain by the following equation;

True Strain (e_1) = Ln (L_t / L_0)

The relative length of the strips was calculated as;

$$L_{rel} = Ln\ (L_t / L_0) + 1 \qquad\qquad (1)$$

where L_{rel} is relative length, L_t is the length at time t, L_0 is the initial length just before the stress increased. One is added to the value so that the value can be used as a relative length as opposed to a relative extension and can be considered as a constant of integration (Evered *et al.*, 2007).

Results showed that increasing and decreasing water availability in hypocotyl segments and cell wall analogue strips under constant load also increased and decreased the extensibility (Fig. 3.01 and Fig. 3.02). Fig. 3.01 shows the effect of replacing control buffer with the same volume of the buffer containing PEG with an osmotic pressure of 0.62 MPa and buffer containing PEG with control buffer. The hypocotyls segments and strips were initially bathed in control buffer or buffer

containing PEG while the stress was increased from an initial value of 0.004 N to 0.098 N and it was kept for long enough (180 min) to observe the long-term pattern of extension. The initial bathing solution was then replaced. When the control buffer was replaced with PEG solution with an osmotic pressure of 0.62 MPa there was a rapid extension for a short period, but after that the extension rate was slower (30-35%) than before the exchange. However when the osmotic pressure was reduced by replacing the PEG solution with control buffer, there was a significant increase (50-60%) of creep extension rate which started almost immediately after the exchange and the long-term extension was higher than that of before the exchange.
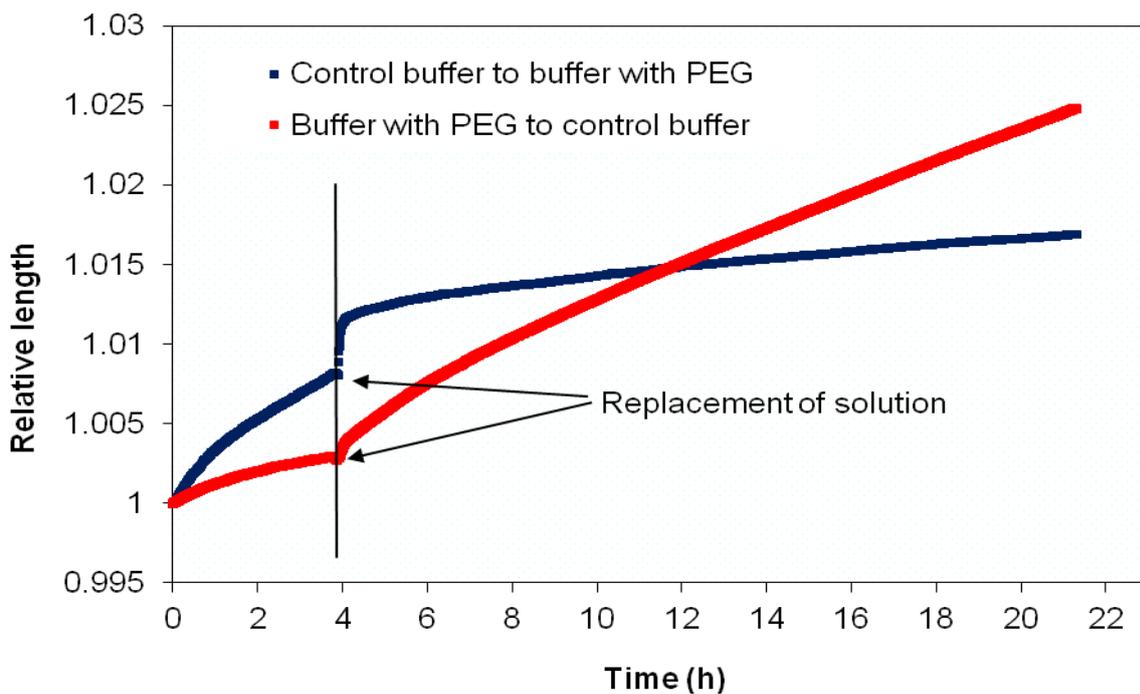
Figure 3.01: Changes in extensibility of sunflower hypocotyls, under a constant load of 0.098 N. The control buffer was replaced with buffer containing PEG 6000 (0.27g/g), to give an osmotic pressure of ~0.62 MPa, at about 4 hours (arrowed). The buffer containing PEG was replaced with control buffer. Both control buffer and buffer containing PEG solutions were pH 5.0

In the case of cell wall analogue, similar behaviour to the sunflower hypocotyls were observed after the exchange of bathing solution under a constant load of 0.098 N (Fig. 3.02). It is interesting to note that the changes of extension rate were reversible if the osmotic pressure was altered further in later stages of the experiments, so both cell wall material and the cell wall composites have the capacity to rehydrate and generate the water potential gradients required to do so.
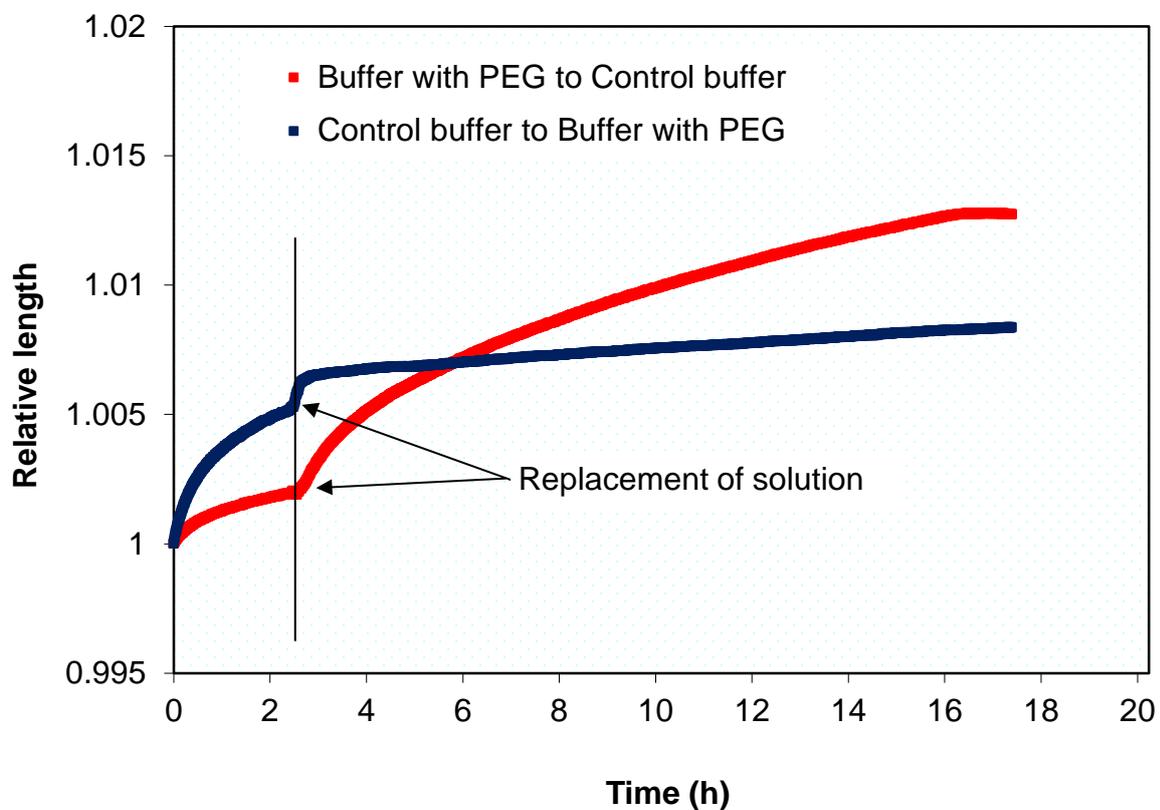
Figure 3.02: Changes in extensibility of the strips of cell wall analogue(C/P/X), under a constant load of 0.1 N. The control buffer was replaced with buffer containing PEG (0.27g/g), to give an osmotic pressure of 0.62 MPa at 2.5 h (arrowed). The buffer containing PEG was replaced with control buffer. Both control buffer and buffer containing PEG solutions were pH 5.0

The results confirmed that increasing the water content of the cell wall increased the rate of extension. The physical properties of synthetic polymers are affected by space constraints and different plasticizers are added to control these properties (Thompson, 2008). Similarly, it appears that the space between the cellulose microfibrils of plant primary cell walls is important, as hypothesised by Thompson, (2005) and water content, together with cross-linking polysaccharides, within the cell wall may have an important role in determining wall properties. The increased water content increased the rate of extension, which is a clear indication that as water molecules have increased the spacing between the cellulose microfibrils, this has influenced the mechanical behaviour of the wall. Conversely, limitations of water may have reduced the space within the cell wall and led to the reduced cell wall extensibility.

PEG 6000 was used to alter the water potential of the sunflower hypocotyls and cellulose composites as PEG with molecular weight higher than 4000 cannot penetrate into the plant cell wall (Carpita *et al.*, 1979) and PEG 6000 also appears to be excluded from the *Acetobacter* cellulose composites to some degree (see section 3.03). Evered *et al.,* (2007) confirmed that PEG solution with an osmotic pressure of ~ 0.62 MPa reduced the thickness of cortical cell walls of sunflower hypocotyls and thus the spacing within the cell wall by cryo-scanning electron microscopy. Although the spacing changes were not directly examined, the reduction in thickness logically indicates the reduction in spacing.

3.02 – Expansin and cell wall extension

To determine the effects of expansin on the mechanical properties of cell wall analogue, strips of composite pellicle were clamped into the micro-extensiometer bathed in pH 5.0 MES buffer and their creep was recorded. The composite was from a 7 days bacterial culture. The maximum force of 0.172 N (17.5 g) was applied to the strips for about 5 min and then reduced to 0.147 N (15.0 g). This has been found to give a less complex pattern of extension, often a linear increase in length with time, so that the effect of expansin was easier to identify. While there was little extension after the stress was reduced from 0.172 N to 0.146 N, after strips were treated with either 10 µL (2.8 µg) of α-expansin (CsExp1), a significant increase in extension was observed for the strips treated with CsExp1, while the control treatment with a similar volume of control buffer had almost no effect (Fig. 3.03). Expansin induced extension immediately and the strips elongated for an extended period of time.
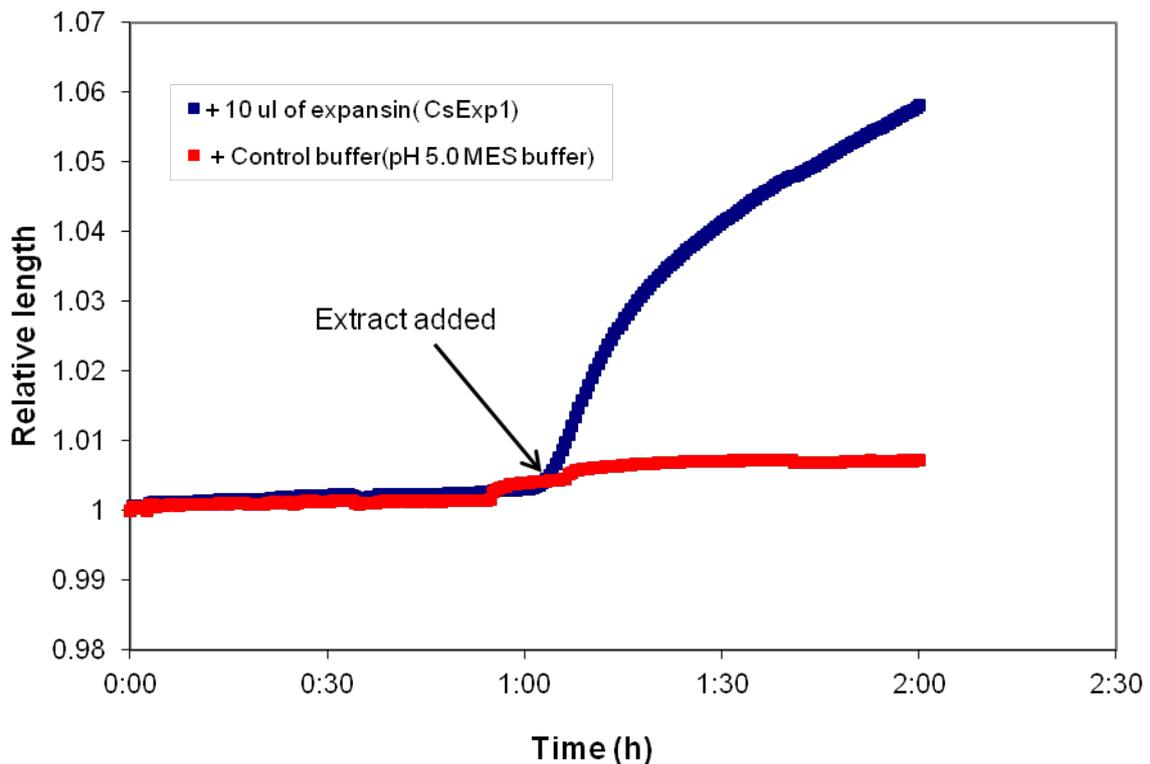
Figure 3.03: Graph showing the changes of extension of boiled strips of cell wall analogue (cellulose / pectin / xyloglucan composite) after addition of ~ 2.8μg of α-expansin. The strips were bathed into pH 5.0 MES buffer under a constant load of 0.147 N. There was no change observed in the case of addition of control buffer.

Strips of cellulose itself and composites of cellulose and pectin (C/P) were also clamped while bathed in to MES buffer at pH 5.0 and the effect of expansin on creep was observed. In the case of cellulose itself expansin showed no effect on extensibility while a slight increase occurred in strips of C/P composites (Fig. 3.04).
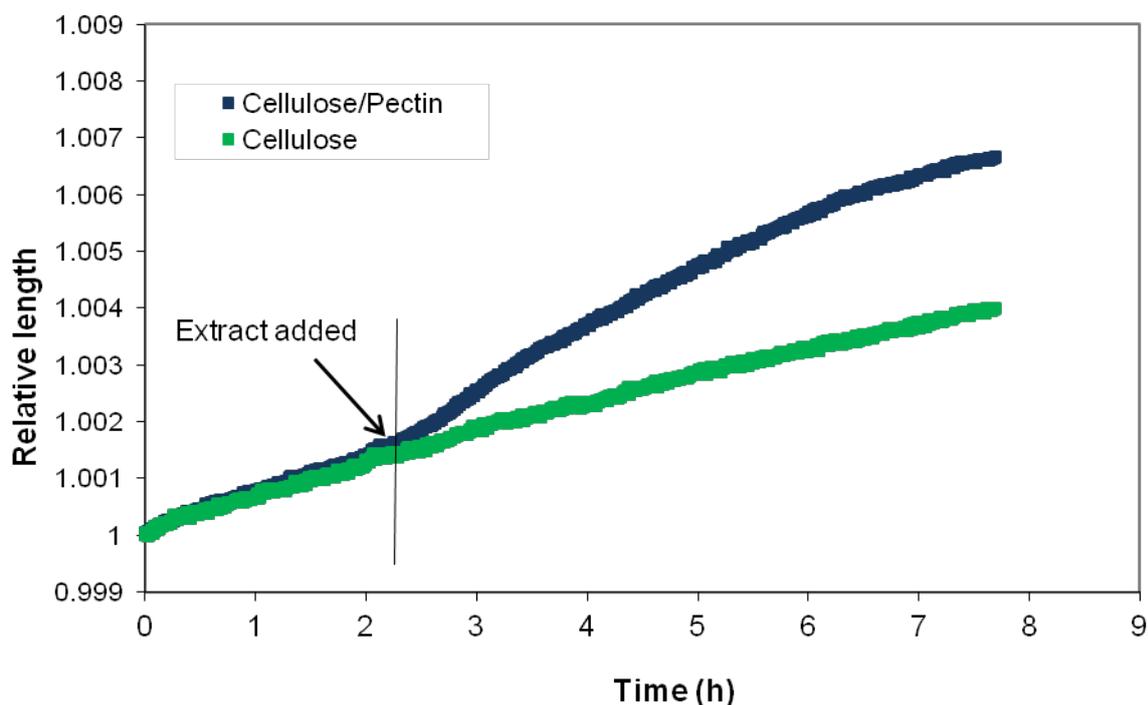
Figure 3.04: Graph showing the changes of extension of boiled strips of cellulose and cellulose / pectin composites after addition of ~ 2.8µg of α-expansin. The strips were bathed into pH 5.0 MES buffer under a constant load of 0.196 N.

LVDT data from another extensiometry experiment on the strips of cell wall analogue bathed in to a range of pH values MES buffer showed that expansin induced creep extension in a range of pH environments although the highest rate of extension was observed at the lowest pH (pH 4.5, which was the lowest in this experiment). Strips were tested under three different pH conditions; pH 4.5, 5.0 and 6.6. Strips were under the highest applied force of 0.123 N (12.5 g) for a short period of time then stress was reduced to 0.098 N and allowed to extend for a period of almost 2 h before they were treated with 10 µL of expansin (~ 2.8µg) and their change of extension under the constant load was observed.
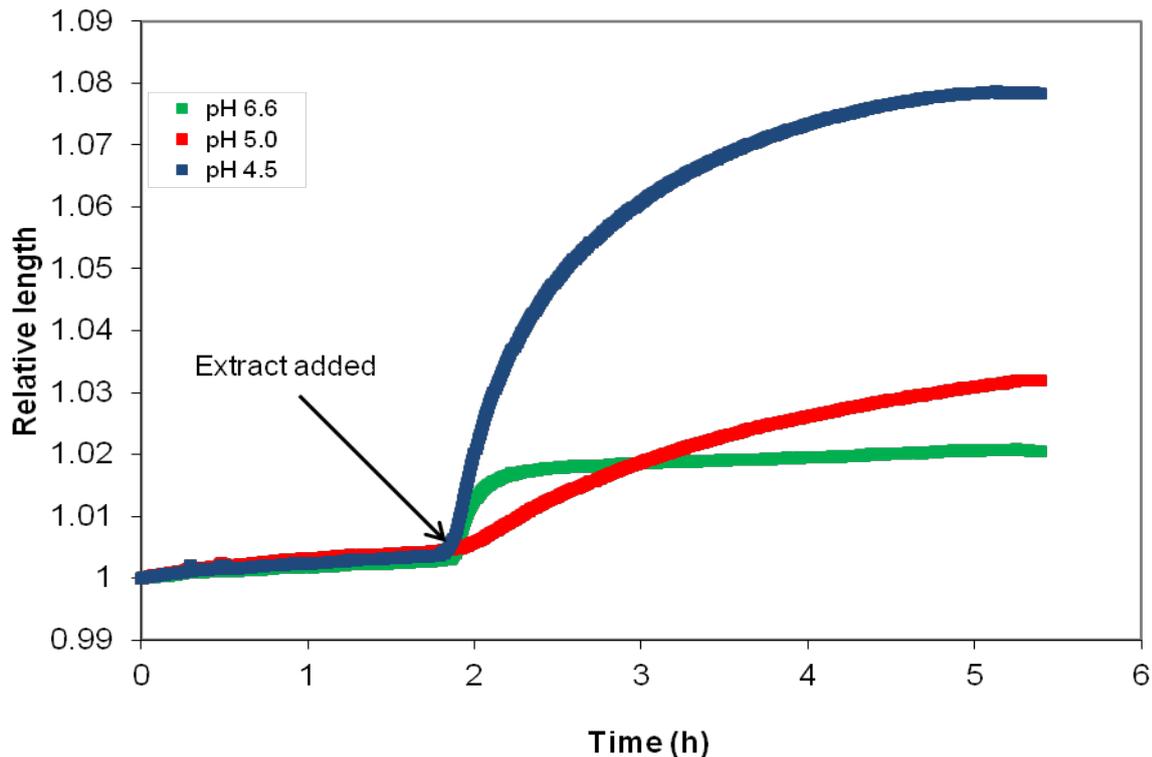
Figure 3.05: Graph showing the changes of extension of strips of cellulose / pectin / xyloglucan composite after addition of ~ 2.8µg of α-expansin. The strips were bathed into MES buffer in three different pH values (4.5, 5.0, and 6.6) under a constant load of 0.098 N. Although the highest increase in extension was occurred at lowest pH (4.5), expansin also induced extension at pH 6.6.

Results showed that at pH 4.5 the strip extended at a higher rate for a prolonged period (Fig. 3.05). The strip at pH 5.0 also exhibited prolonged extension but extension rate was significantly lower than at pH 4.5, but in pH 6.6 the strip extended at a moderate rate only for a short period of time before it stopped extending (this short initial extension is unusual). The cell wall analogue in all three different pH conditions exhibited viscoelastic properties. The mechanical behaviour of the analogue strips could be categorised using the rheological model (equation-2) proposed for plant cell walls by Thompson (2001). It has been reported that the extension of plant tissues after an increase of stress using constant load extensiometer is close to the equation-2. This model is composed of a number of

different elements, a log-time function element (LTE), two Kelvin elements with different retardation times (KE1 and KE2) and a viscous flow element (VFE).

$$L_t = L_0 + N_{creep} \, log \, t + k + f_1 \, \tau_1 \, (1- exp(- t / \tau_1)) + f_2 \, \tau_2 \, (1-exp(- t / \tau_2)) + f_3 t \qquad (2)$$

Where, L_0 and L_t are the initial length and length at time t. $f1$, $f2$ and $f3$ are the initial flow rates of KE1, KE2 and viscous flow element (VEF), τ_1 and τ_2 are Kelvin element retardation times, N_{creep} is the constant associated with a log-time function (as described by Buntemeyer $et \, al.,$ 1998) and k is a correction factor, because it is impossible to extrapolate LTE at $t = 0$ (Thompson, 2001).

The change in extension in strips at pH 6.6 appeared to be dominated by short timescale processes corresponding to Kelvin Element 1 (KE1) and the Log-Time Function in Thompson's model (2001), but in the case of the strips at pH 4.5, the behaviour was primarily due to Kelvin Element 2 (KE2) (where the retardation time was much higher than the KE1) and viscous flow. The typical retardation time for KE1 is 10-20 min and for KE2 is 70-120 min.

As expansin induced the extension of the composite strips under a range of pH environments extensiometric experiments were also carried out with heat inactivated sunflower hypocotyls strips and cellulose composites strips under neutral and acidic buffer condition to find out if the pH played any role on cell wall extension independently of expansin.
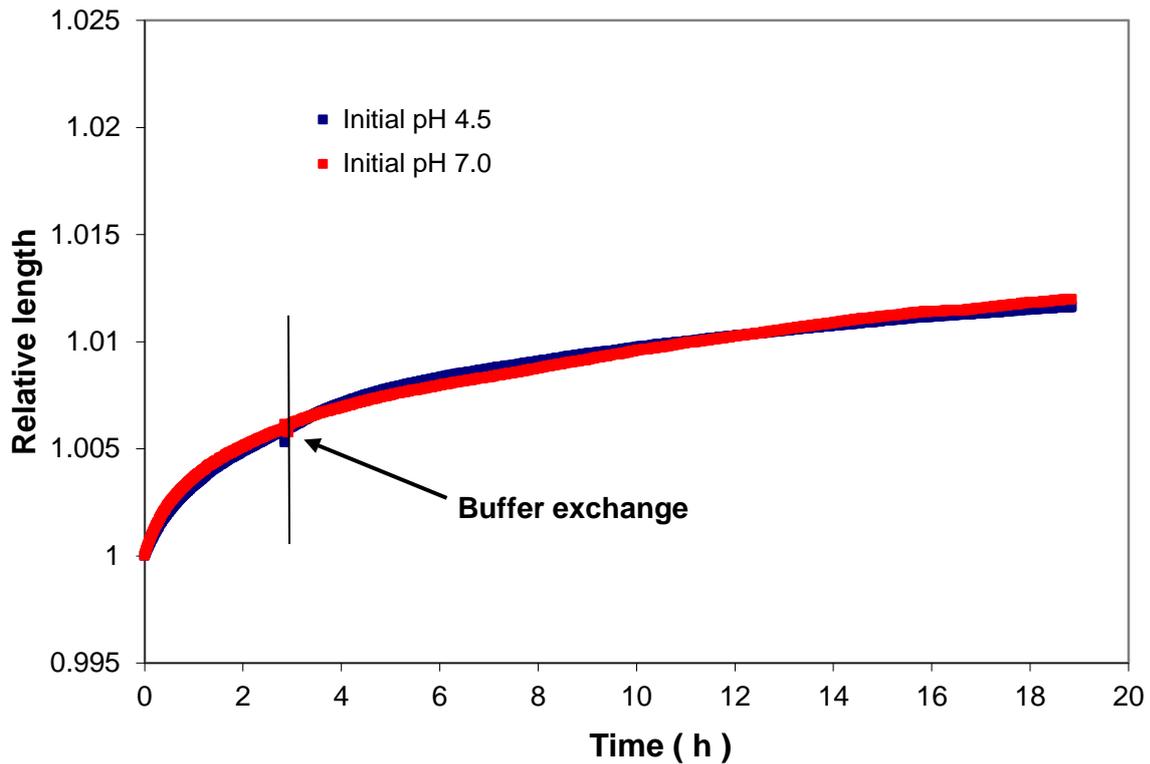
Figure 3.06: Extension of boiled sunflower hypocotyls bathed in two different pH environments (4.5 and 7.0), under a constant load of 0.294 N. There was no difference in extension rate observed during the initial pH condition. The initial buffer was exchanged after about 2:40 h (arrowed) to change their pH environment and no changes in extension were observed.

Strips of sunflower hypocotyls were stressed under a constant load of 0.294 N bathed in either pH 4.5 or pH 7.0 MES buffer initially for about 3 h and their extension was recorded. After that time the initial buffer was drained and replaced *vice versa* and any changes of extension was observed. Similarly, boiled strips of cellulose / xyloglucan composite were stressed at 0.098 N forces under acidic and neutral pH conditions and after about 1:30 h the initial pH conditions were altered.
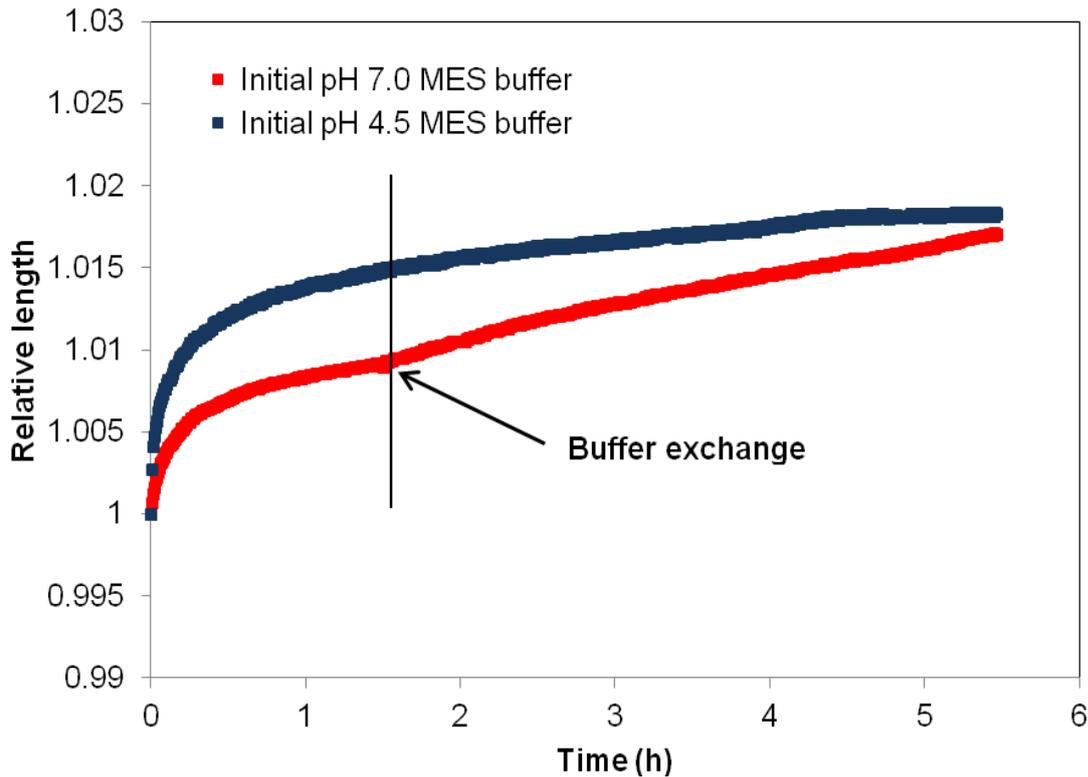
Figure 3.07: Extension of boiled cellulose / xyloglucan composites bathed into two different pH environments (4.5 and 7.0), under a constant load of 0.098 N. There was no difference in extension rate observed during the initial pH condition. The initial buffer was exchanged after about 1:30 h (arrowed) and no changes in extension were observed after changing from acidic to neutral pH but slight changes were observed while the buffer was changed from neutral to acidic pH.

The results (Fig. 3.07) indicated that the changes in extension of the composites strips (Fig. 3.07) after changing the buffer from pH 7.0 to pH 4.5 is slight but striking, and perhaps does suggest that there was some effect of pH on extensibility in the absence of expansin, although no changes on extension due to pH alteration were observed in the case of sunflower hypocotyls segments (Fig. 3.06). It was also worth noting that the change in extension rate occurred in composite without pectin, the polysaccharide that might be expected to be primarily affected by pH.

Since expansins were discovered, it has been well known that their effect on cell wall extension is acid induced (McQueen-Mason *et al.*, 1992; Cosgrove, 1996; Cosgrove, 1997) with rapid cell wall extension induced in acidic solutions, usually at pH values of less than 5.5 (Cosgrove, 1997). It had long been known that native cell walls extend rapidly at acidic pH (so called "acid growth") and the extension ceased soon after they are placed into neutral buffer but in the case of denatured cell wall there was generally no effect of pH on wall extension (Cosgrove, 1998; Ezaki *et al.*, 2005) As a consequence of this observation it was concluded that acid growth was dependent on proteins, leading to the discovery of expansins. It has been hypothesised that the mode of action of the expansin is that at acidic pH (< 5.5) they become active and break non-covalent binding between cellulose and xyloglucan and allow turgor driven wall extension (Cosgrove, 1997; Cosgrove, 2000a). In contrast, the extensiometric data with synthetic cell wall analogue suggested that expansin has some effects over a wide pH range, inducing composite extension at a pH of 6.6 although for a short period of time. It was also interesting that expansin caused a steady increase of the creep of cellulose / pectin composites, even though it has been suggested that expansin loosens the cellulose – xyloglucan network (Cosgrove, 2000a), and so this observation is inconsistent with the 'sticky network' model. These results may indicate that expansin doesn't only loosen the cell wall by breaking hydrogen bonds between cellulose and xyloglucan also other factors may involve.

3.02.01 – Comparing the mechanical properties of dicotyledonous and monocotyledonous cell wall

To understand the cellulose - hemicellulose crosslinks and the mechanical behavior of the grass and commelinoid cell walls using *Acetobacter* cellulose model, composites were prepared using β-glucan or lichenan. The prepared composites were as soft and pliable as those with xyloglucan. The extensibility of the composites was compared with the composites prepared with xyloglucan and/or pectin. In creep extensiometry using a constant load of 0.098N (Fig. 3.08), the C/X and C/BG composites exhibited a rapid initial extension rate that decayed sharply. The C/P/X strips showed much lower initial extension but slightly greater than the C/L strips.
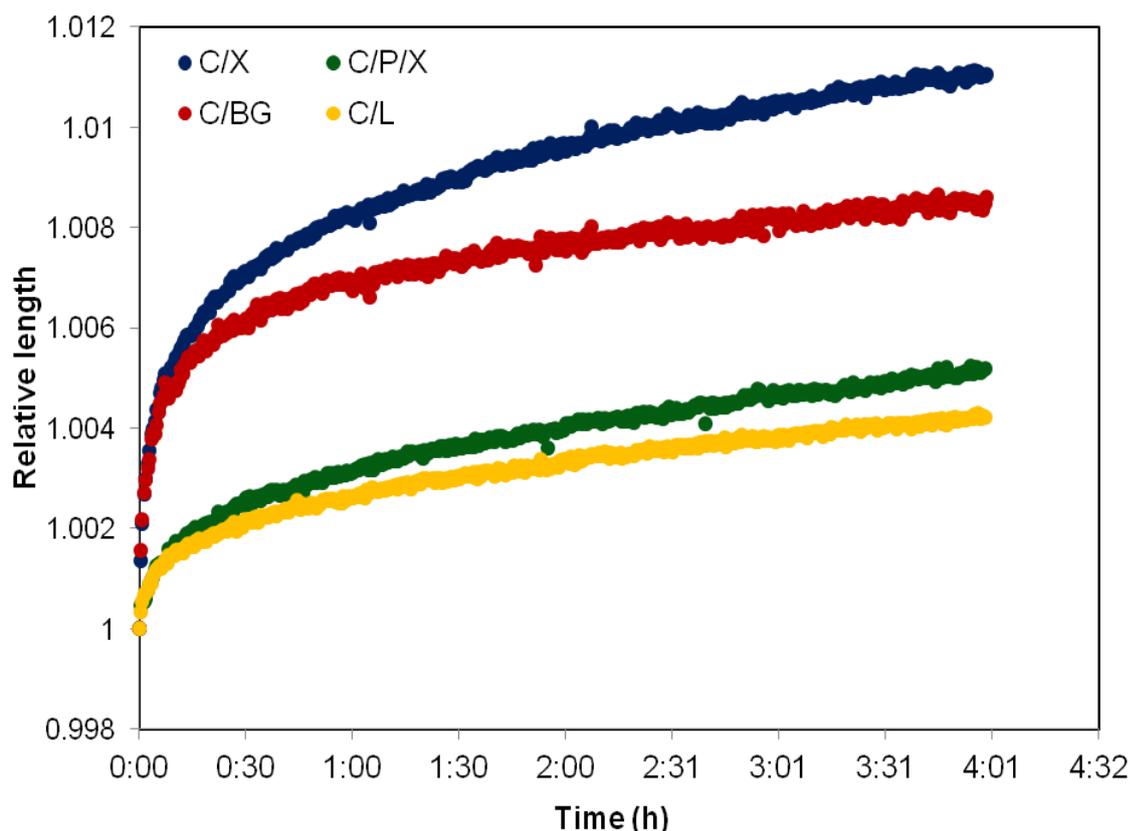
Figure 3.08: Extension of different cellulose composites bathed in MES buffer pH 5.0 under a constant load of 0.098 N. The graph showing the comparative extension pattern of composites made with xyloglucan and pectin (C/X and C/P/X) and the composites made with β-glucan (C/BG) or lichenan (C/L).

The data was noisy and only the first 45 min of data was statistically analyzable and comparable. T-tests assuming the significance are $p > 0.95$ did not exhibit significant differences between the materials, except that the elasticity of the C/BG was significantly greater than that of C/L and C/P/X but not C/X. These results can only be regarded as preliminary but it may show that altering the 1-4, 1-3 ratio can alter the behavior (1-4, 1-3 ratio of oat β-glucan range from 2.2 : 1 to 2.6 : 1 and the ratio is 2 : 1 in the case of lichenan) and might cause some of the same changes as addition of pectin, as if it is stretched the C/X flow rate is greater than the C/P/X with

p>0.9. Intriguingly this is consistent with the low levels of pectin in type II walls as the linkage ratio can modulate wall behaviour in a similar way to pectin.

3.03 – Effects of expansin on cell wall water content

To determine the effects of expansin on cell wall water contents, cellulose and cellulose composites were dehydrated using hypertonic solutions made with high molecular weight PEG, to give an osmotic pressure of 0.62 MPa and re-hydrated in control buffer, followed by treatment with α-expansin (CsExp1) or snail acetone powder extract. Results showed that PEG solution reduced the water content of cellulose and the cellulose composites at different rates.

All the dehydration and rehydration measurements were performed in at least three replicates and the average values were taken and the standard deviation of the mean value was calculated using MS Excel.

After 180 min of incubation at pH 5.0, cell wall analogues had lost as much as 72.33 ± 5.17 % of their initial weight through loss of water. Cellulose alone had lost about 44.63 ± 1.70 % and cellulose/pectin composites also lost about 44.11 ± 4.61% (see Fig. 3.08 and Fig. 3.10). The reduction in weight suggests that PEG 6000 was excluded from the composites to some degree. During re-hydration in control buffer, cell wall analogues (C/P/X) recovered almost 29.16 ±5.66 % of their weight by taking up water over a period of 120 min. The recovery was much lower with 23.61 ± 3.38% and 22.73 ±4.34% in the case of cellulose and cellulose/pectin composites respectively, although this was a slightly greater proportion of the water they had lost during the initial period of the experiment. After adding 11.2 μg of α-expansin to the buffer (800μL) there was a dramatic increase in water uptake in all cases. The cell

wall analogue recovered a further 24% of its weight within 5 min and cellulose and cellulose/pectin composites recovered about 10% and 12% respectively. After 65 min of incubation with expansin the cell wall analogue recovered 43.43 ± 1.51 % of its weight and was restored to its initial weight. In the case of cellulose and cellulose/pectin composites over the same period, recovery was 20.70 ± 5.51% and 22.50 ± 4.77% respectively and reached about 99-100% of their initial weight (Fig. 3.10).
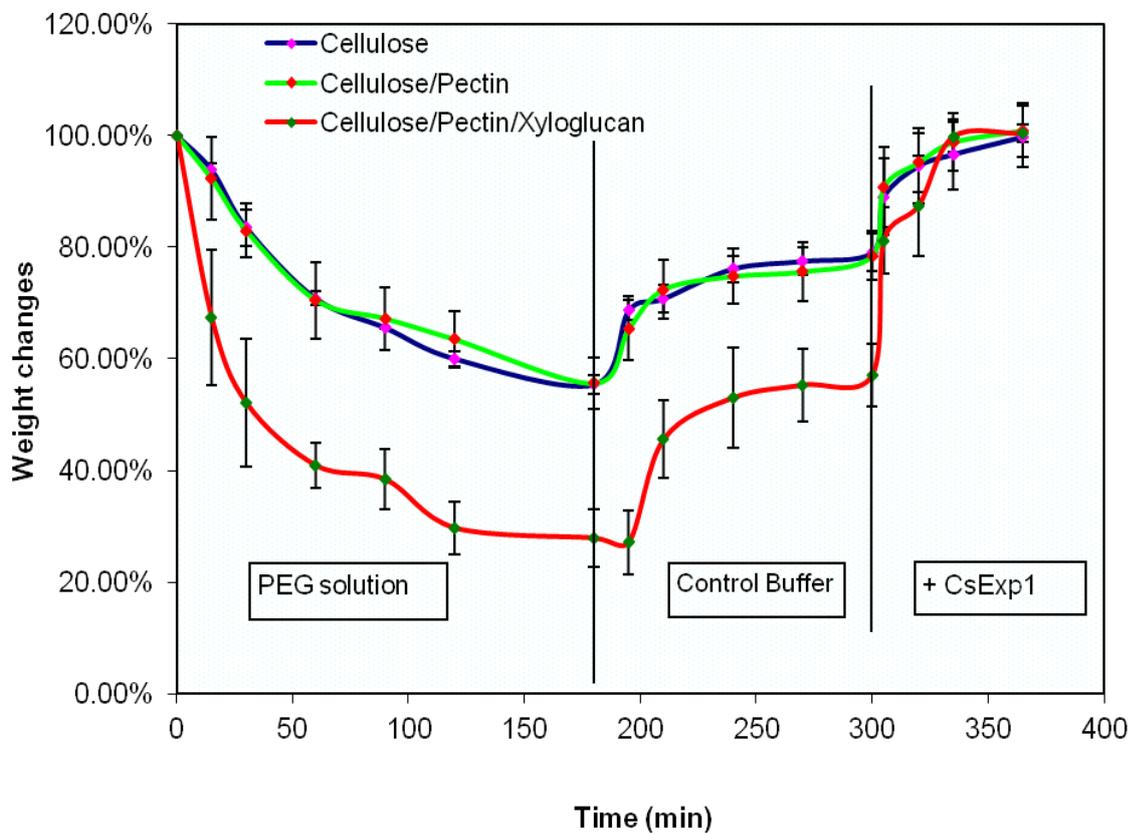
Figure 3.09: Changes in weight of cellulose and cellulose composites exposed to high molecular weight PEG solution with an osmotic pressure of 0.62 MPa. The composites pieces were transferred to control buffer followed by addition of α-expansin (11.2 µg) to the buffer (800 µL). Both control buffer and buffer containing PEG solutions were pH 5.0.

The experiment was repeated but instead of expansin, snail powder extract was added to the control buffer. During exposure to PEG solution cellulose, cellulose/pectin and cell wall analogue lost water and their weights were reduced by 43.03 ± 2.59%, 31.02 ± 2.92% and 66.89 ± 0.33%.

Rehydration of C, C/P, C/P/X in MES buffer lead to an increase in weight of 28.41±1.89%, 21.97±1.86% and 34.59 ± 4.00% over a period of 120 min while recovery with the addition of 200 μl of snail powder extract (50 mg / ml) was by about a further 4.0%, 3.5% and 14% respectively in the first 10 min. After a total of 65 min incubation with the extract, cell wall analogue recovered about 33.29 ± 1.48% of the water and almost returned to its initial weight. Cellulose and cellulose/pectin recovered about 12.95 ± 1.07% and 7.74 ± 1.39% respectively and reached around 97.32 ± 1.07% and 98.70 ± 1.39% of their initial weight (Fig. 3.12).
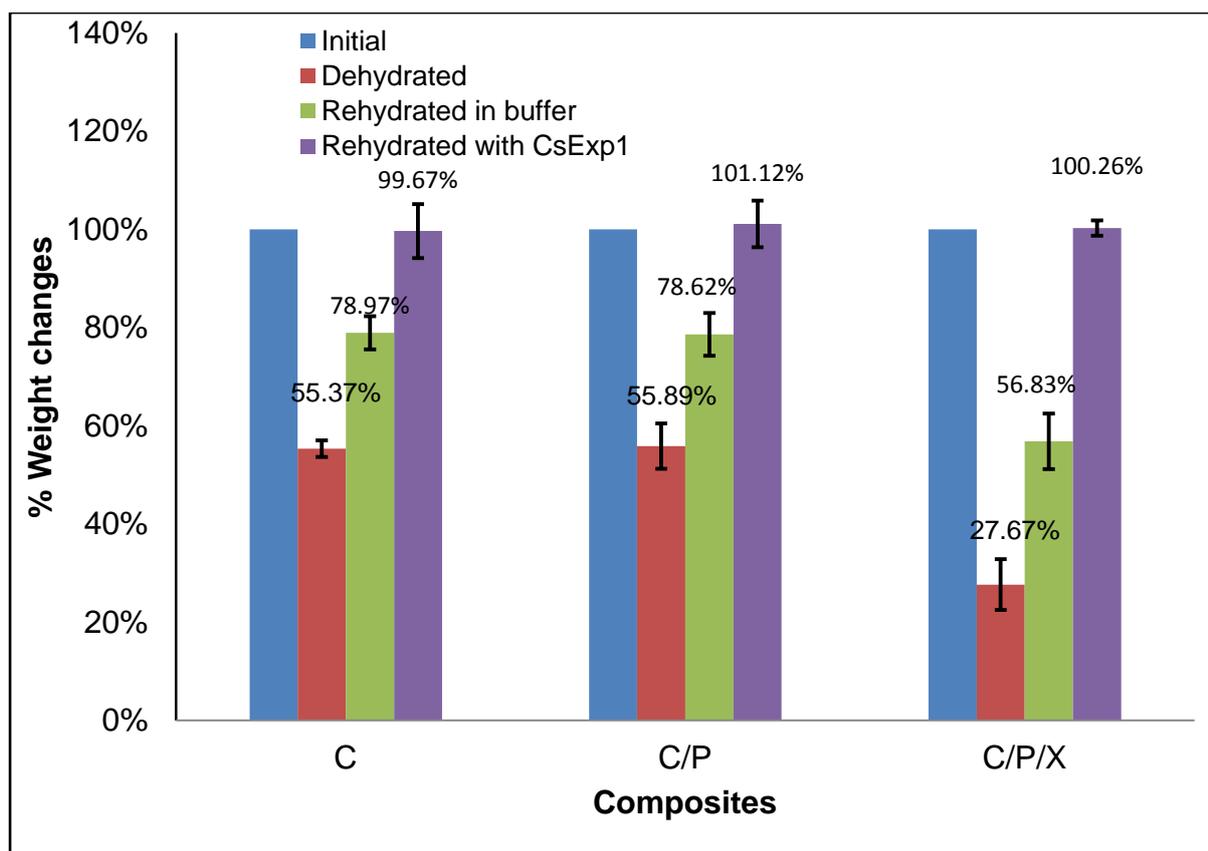
Figure 3.10 :   Graph showing the % weight changes of different cellulose composites exposed to buffer containing PEG 6000 (0.27 g/g) solution and then rehydrated in control buffer (pH 5.0 MES buffer) followed by addition of α-expansin (11.2 µg). C = Cellulose, C/P = Cellulose / pectin, C/P/X = Cellulose / pectin / xyloglucan composites.

Table 3.01: Statistical analysis of weight differences on dehydration and rehydration values of different cellulose composites using Student's t-test. Composites were dehydrated in PEG 6000 solution and rehydrated in control buffer followed by treatment with CsExp1. The p-values in bold numbers indicate significant differences.

	Non paired t-test p-values (level of significance was set at p= 0.05)		
Comparing Composites	Dehydration in PEG 6000 solution	Rehydration in MES buffer	Rehydration with CsExp1
C and C/P	0.866452	0.754087	0.603867
C and C/P/X	**0.006701**	0.112142	**0.004464**
C/P and C/P/X	**0.002237**	**0.050023**	**0.011451**

It is well established that expansins promote cell wall extension (Cosgrove, 1997), but there are questions as to how they bring about changes in the mechanical properties of the cell wall. Present observations suggest that expansins and proteins in the snail acetone powder increase the water content of the cell wall and therefore the spacing between cellulose microfibrils and that this contributes the higher extensibility.
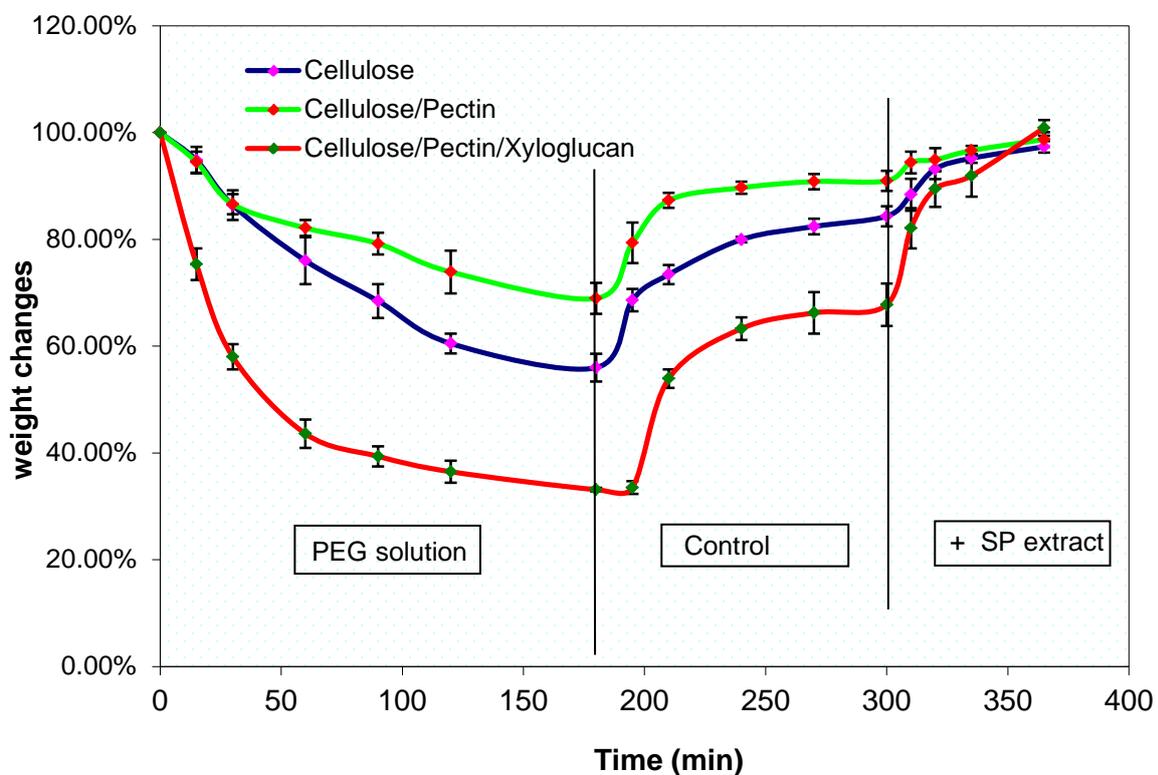
Figure 3.11: Changes in weight of cellulose and cellulose composites exposed to high molecular weight PEG solution with an osmotic pressure of 0.62 MPa. The composite pieces were transferred to control buffer followed by addition of 200 µL of snail powder extract (50 mg/mL) to the buffer (800 µL). Both control buffer and buffer containing PEG solutions were pH 5.0.
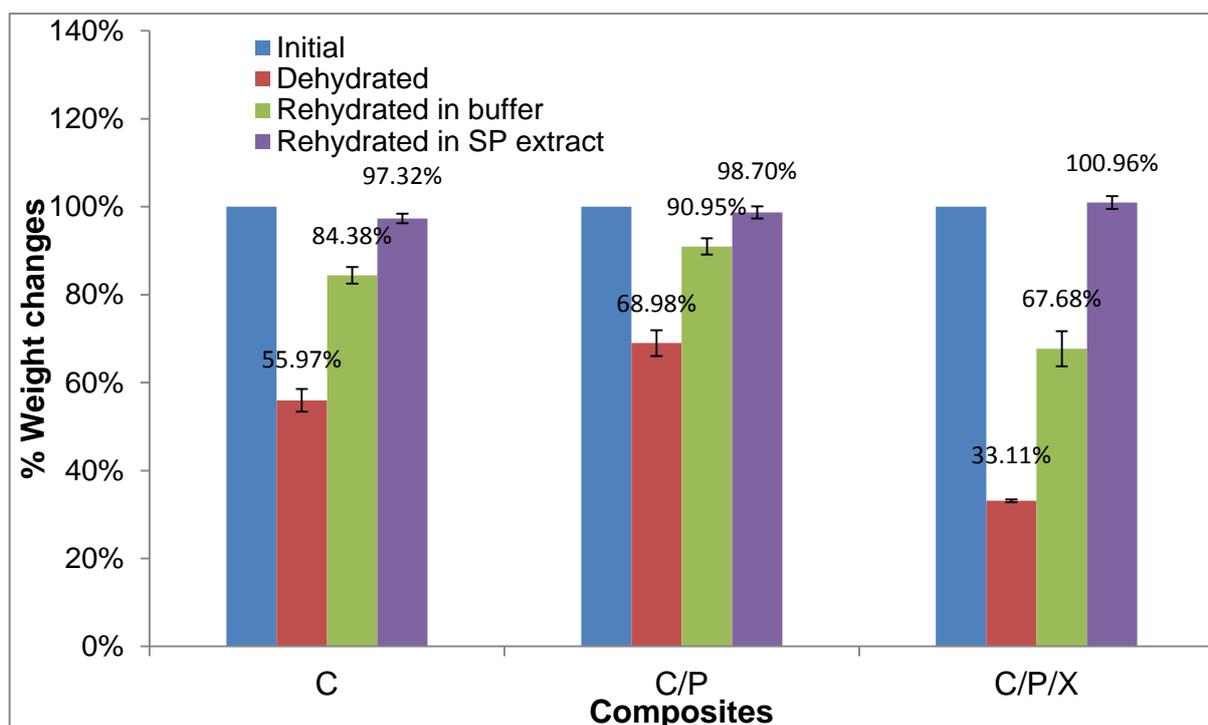
 Figure 3.12: Graph showing the % of weight changes of different cellulose composites exposed to buffer containing PEG 6000 (0.27 g/g) solution followed by rehydration in control buffer (pH 5.0 MES buffer) followed by addition of 200 µL of snail powder extract (50 mg /mL of control buffer). C = Cellulose, C/P = Cellulose / pectin, C/P/X = Cellulose / pectin / xyloglucan composites.

Table 3.02: Statistical analysis of weight differences on dehydration and rehydration values of different cellulose composites using Student's t-test. Composites were dehydrated in PEG 6000 solution and rehydrated on control buffer followed by treatment with snail powder extract. The p-values in bold indicate significant differences.

Comparing Composites	Non paired t-test p-values (level of significance was set at p= 0.05)		
	Dehydration in PEG 6000 solution	Rehydration in MES buffer	Rehydration with snail powder extract
C and C/P	**0.004658**	**0.035851**	0.087908
C and C/P/X	**0.000380**	0.086148	**0.001802**
C/P and C/P/X	**0.001974**	**0.016213**	**0.005586**

Previous studies on the effect of expansins on cell wall thickness (Islam, 2006) showed that expansins (CsExp1) significantly increased the thickness of cellulose and cellulose composites (This data is shown in Fig. 3.13). It was found that when the pieces of different composites pellicle were treated with expansin, the thickness of the hydrated pellicle increased immediately. Thus, it appears possible that expansin increases extensibility by increasing the spacing within the cell wall.
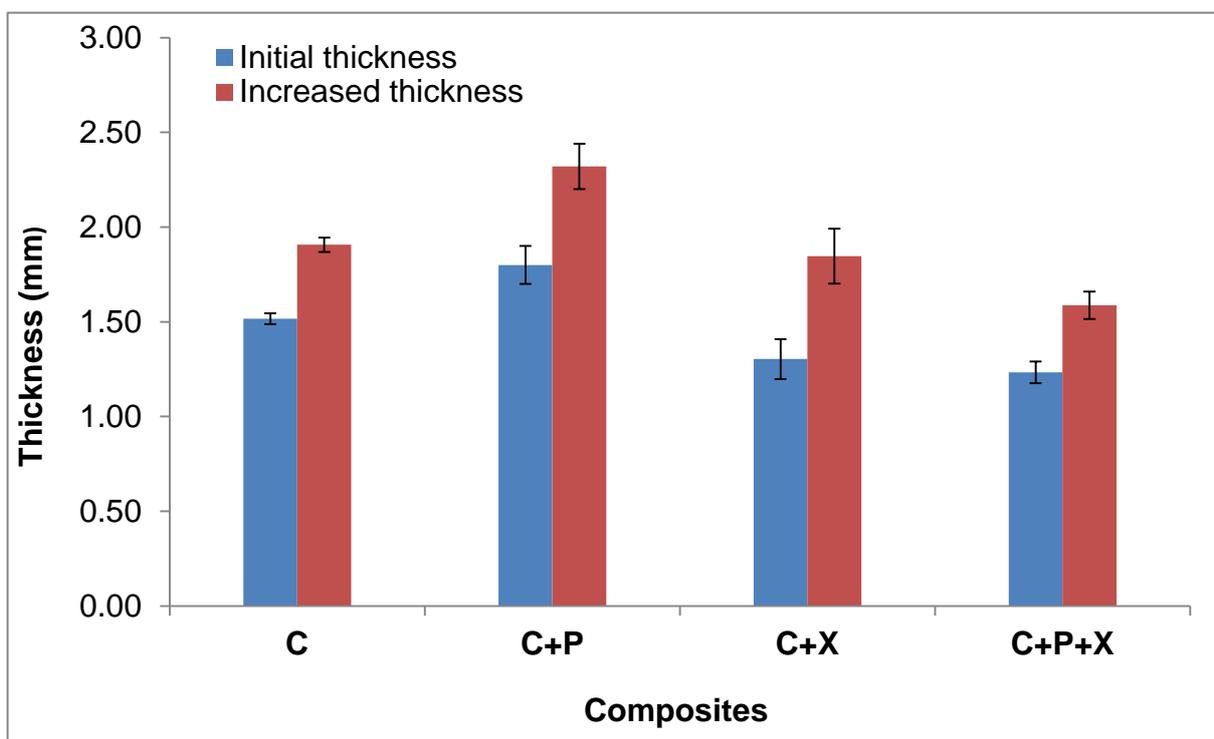
Figure 3.13: Effect of expansin on swelling of Acetobacter cellulose and cellulose composites. It shows the initial thickness and expansin treated thickness of different pellicles. C = Cellulose, C+P = Cellulose+pectin, C+X = Cellulose + xyloglucan, C+P+X = Cellulose + pectin + xyloglucan composites (Islam, 2006).

3.04 – Effects of expansin on cell wall free volume

It has been proposed that the free volume within the plant primary cell wall separating the cellulose microfibrils may affect the cell wall mechanical properties (Thompson, 2005). Also, it was observed that alteration of water content altered the mechanical behaviours of sunflower hypocotyls tissues and the synthetic analogue of plant primary cell wall. Considering the effect of alteration of the free volume of the cell wall on tissue mechanical properties it was assumed that cell wall free volume may contribute to regulation of plant growth. As expansin induced the long term extension of cell wall and also increased the hydration rate of cell wall analogue, it appears that expansin may increase the cell wall free volume by increasing the water content of the cell wall and eventually promote wall extension. To test this hypothesis, the effect of expansin on cell wall swelling was investigated using a turbidity assay, based on the light scattering by cell wall fragments in suspension. Changes of the free volume of the fragments caused by expansin were observed by the alteration of the optical density of the suspension.

All the measurements presented are means of at least 3 replicates. The increases in optical density were calculated as a percentage of values before the expansin or other extracts were added to the suspension. The dilution factor and any absorbance of the treatment solution were corrected during processing of the raw data.

Turbidity assays on cell wall analogues and sunflower hypocotyls cell wall fragments suspended in MES buffer pH 5.0, showed that addition of 14 μg of expansin to 1.0 mL of fragments in suspension caused a rapid increase in extinction. The majority of the effect was complete within the first 30 s but the optical density of the suspension

increased for up to 3 min. The final optical densities of the sunflower hypocotyl cell wall fragments suspensions and cell wall analogue fragments suspensions were increased by 12.12 ± 1.76 % and 12.45 ± 0.5 % respectively in a period of 2.5 min (Fig. 3.14 & Fig. 3.15).   Addition of 50 µL of snail powder extract (extract from 50 mg/mL) to 1.0 mL of sunflower hypocotyl cell wall fragment suspension at pH 5.0 showed a similar effect to expansin, with an increase of 18.34 ± 1.11 % in 2.5 min (Fig. 3.16).   No effects were observed when an equivalent or a greater volume of control buffer was added to the suspension.
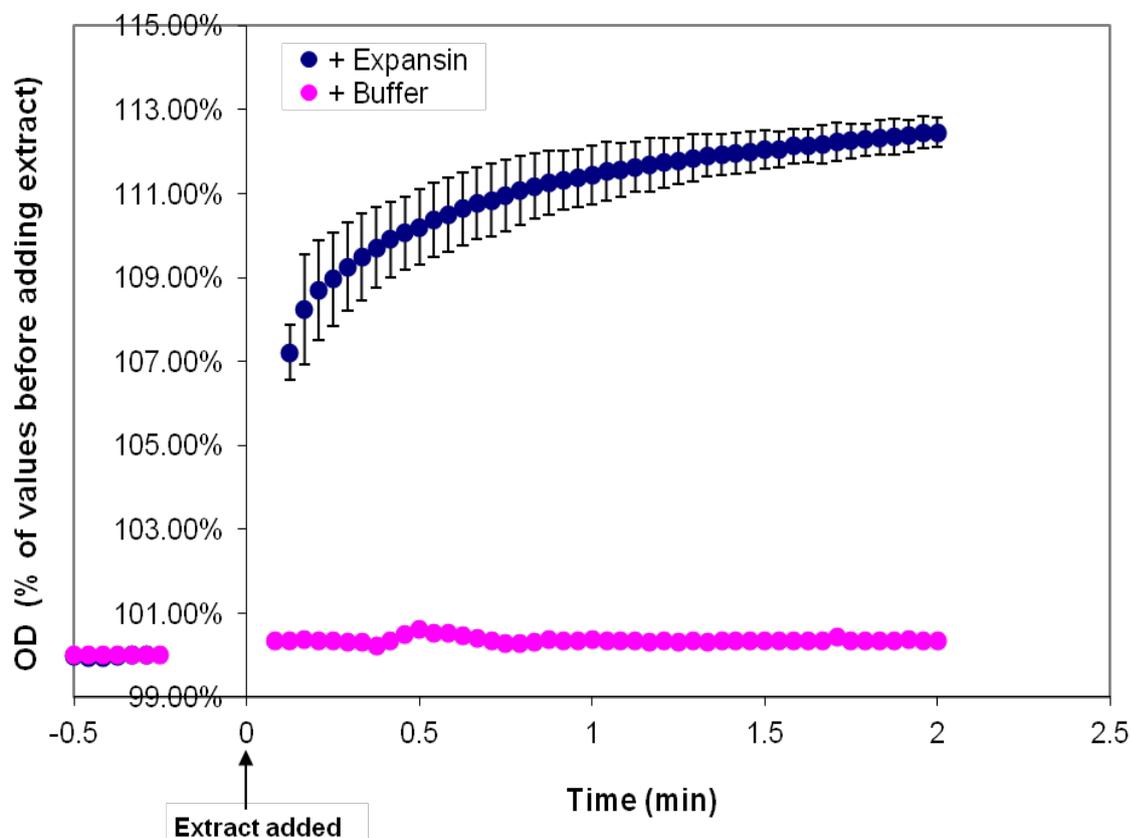
Figure 3.14: Effect of addition of 14 µg of expansin on the optical density of 1.0 mL of cell wall analogue fragment suspension in MES buffer at pH 5.0. Addition of expansin (arrowed) caused significant increase on OD values and no effect on OD was observed when 50 µL of control buffer was added.

Addition of 200 µg of *Trichoderma viride* crude cellulase extract to 1 mL of cell wall fragments suspension caused no changes of the optical density.
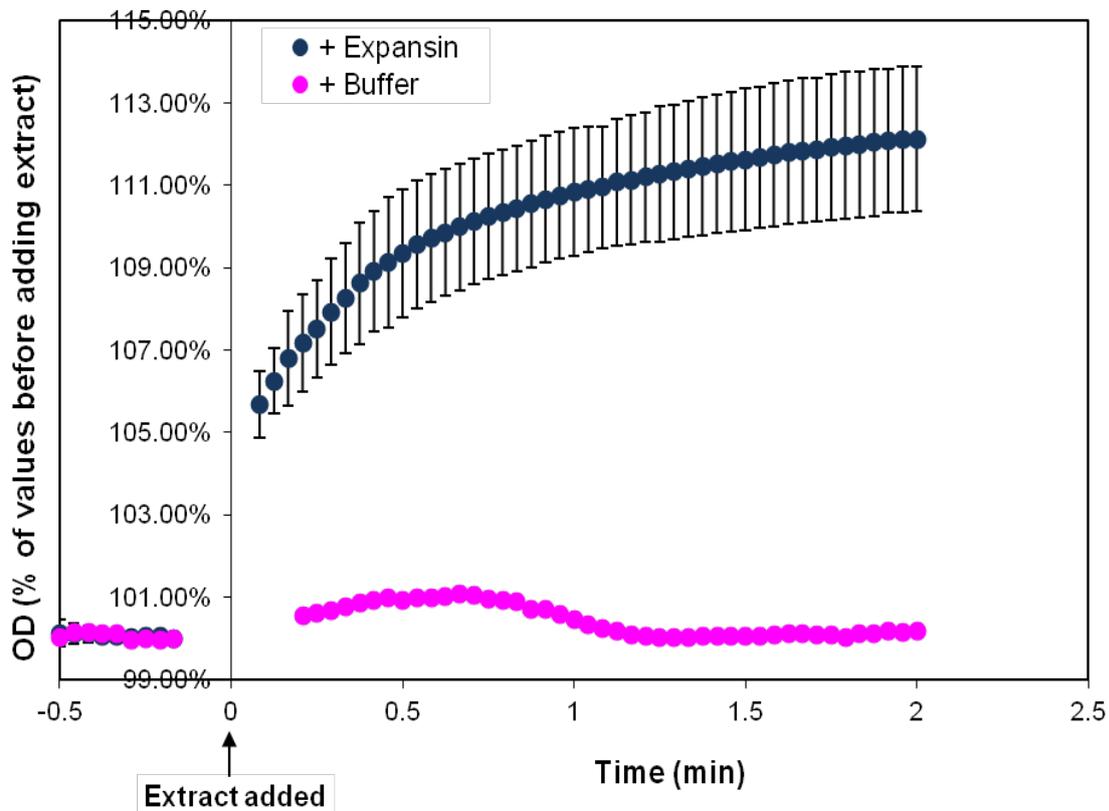
Figure 3.15: Effect of addition of 14 µg of expansin on the optical density of 1.0 mL of sunflower hypocotyls cell wall fragment suspension in MES buffer at pH 5.0. Addition of expansin (arrowed) caused a significant increase in OD values but no effect on OD values was observed after addition of 50 µL of control buffer.

Another set of turbidity experimental data showed that treating cell wall fragments with peroxidase and hydrogen peroxide (H_2O_2) caused cell wall compaction rather than swelling. 1 mL of sunflower hypocotyls cell wall fragments suspended in pH 5.0 MES buffer was treated with 100 µL of diluted (80 µL / 800 µL of MES buffer) peroxidase from horseradish (Sigma-Aldrich, P6140) and the changes of optical density was recorded. In a period of 2.5 min the final optical density was reduced by 4.45 ± 1.54 % (Fig. 3.17). Similarly, addition of 50 µL of diluted (500 µL / 2 mL of buffer) H_2O_2 in to 1 mL of hypocotyls cell wall fragments suspension caused a slight

decrease (2.16 ± 0.26 %) of the optical density (Fig. 3.17). The results indicated that H_2O_2 and peroxidise reduce the hydration of cell wall and thus reduce the thickness of cell wall. Similar phenomena have also been reported with grapevine callus primary cell wall, indicating that H_2O_2 cause a rapid decrease in hydration and the thickness of the primary cell wall (Pereira *et al.*, 2011)
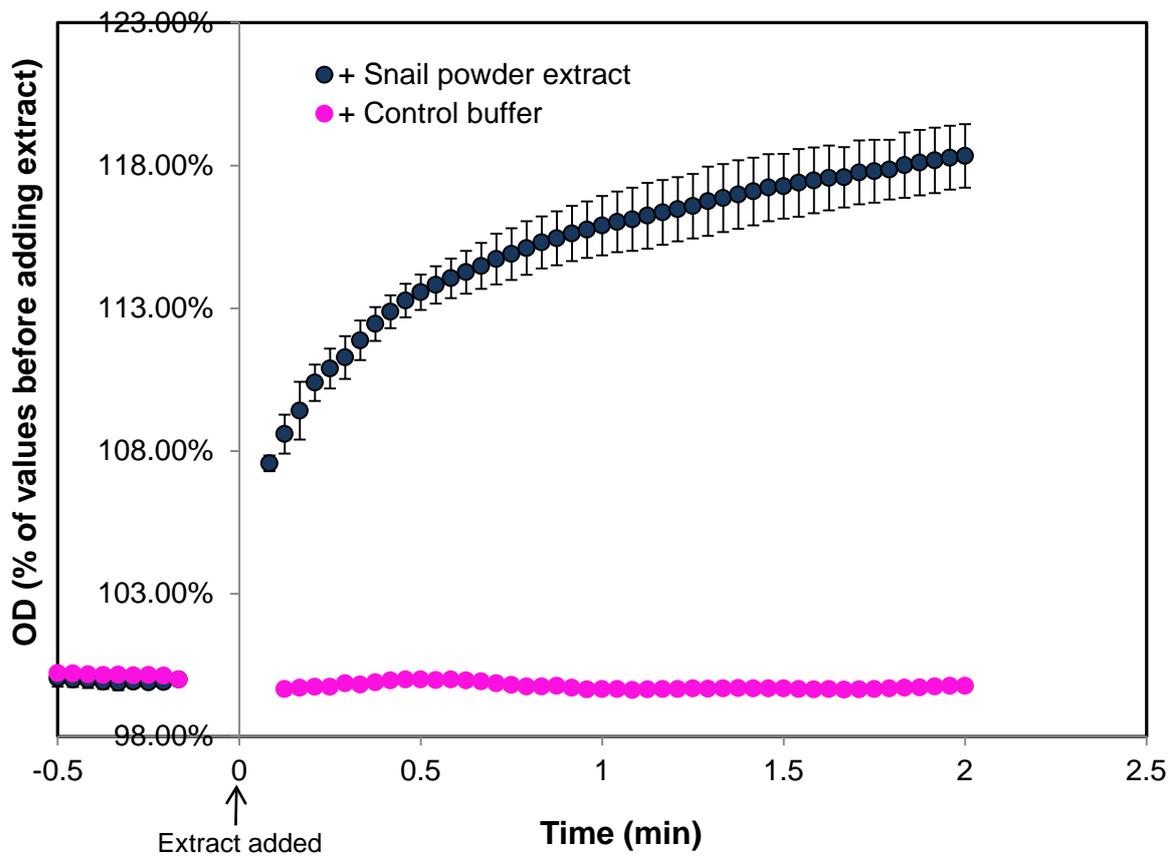
Figure 3.16: Effects of addition of 50 µL (50mg / mL) of snail powder extract on the optical density of 1.0 mL of sunflower hypocotyls cell wall fragment suspension in MES buffer at pH 5.0. Addition of snail powder extract (arrowed) caused significant increase on OD values but no effect on OD values was observed after addition of 50 µL of control buffer.

Expansin action is believed to be pH dependent and is reported as becoming active at acidic pH of < 5.5 (Cosgrove, 1997; Cosgrove, 2000a; Cosgrove, 2000b) and so turbidity assays were carried out to determine the pH dependence of expansin induced increases in the cell wall free volume.
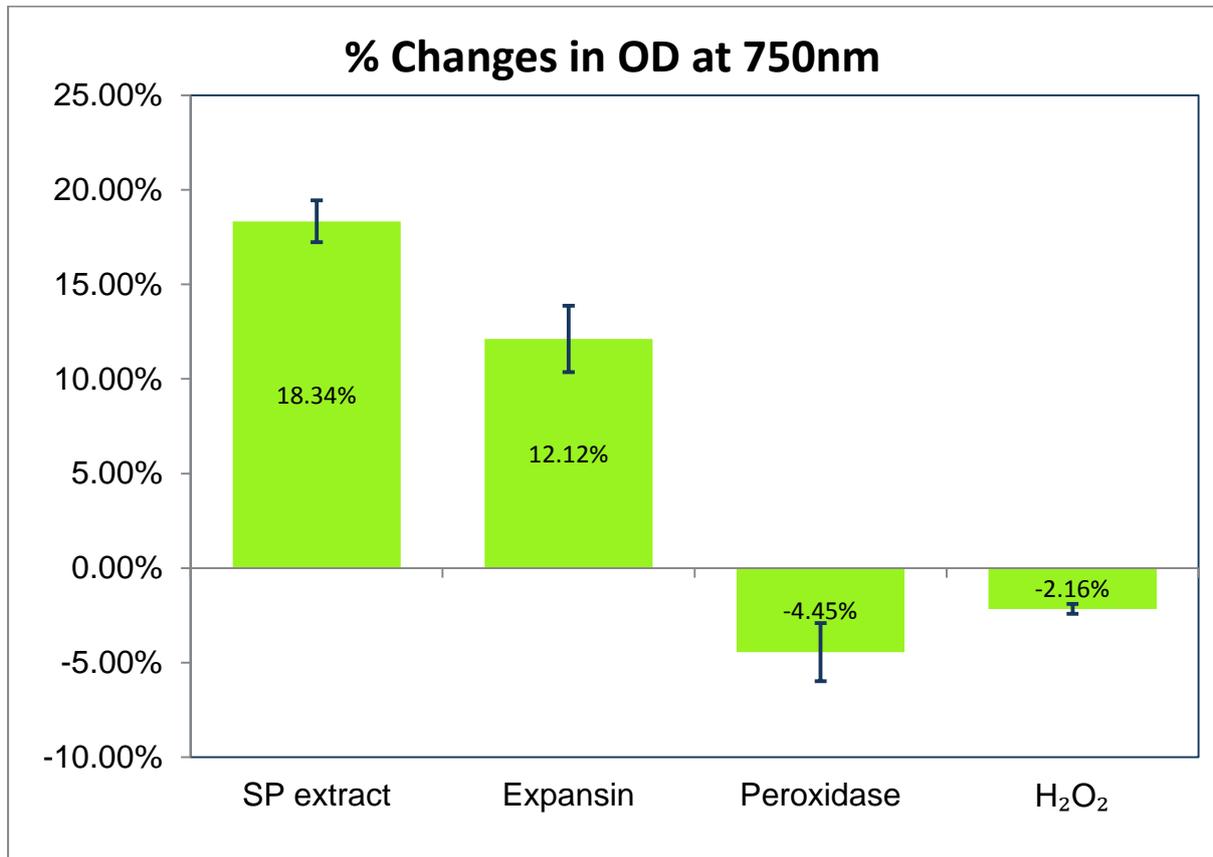
Figure 3.17: Graph showing the effects of addition of 50 μL (50mg / mL) of snail powder extract, 14 μg of expansin, and 100 μL of (80 μL / 800 μL of buffer) diluted peroxidase and 50 μL of H2O2 (500 μL / 2mL of buffer) on the optical density of 1.0 mL of sunflower hypocotyls cell wall fragments suspended in pH 5.0 MES buffer.

Results showed that addition of 14 μg of expansin to 1 mL of boiled sunflower hypocotyls cell wall fragments suspended into MES buffer with pH values of (a) 4.7, (b) 5.16, (c) 5.55, (d) 5.8 and (e) 6.2 increased the optical density in all cases, with the greatest increases at lower pH. The optical density was increased by 17.82 ±

1.54 % at pH 4.70, 16.12 ± 0.53% at pH 5.16, 14.45 ± 0.67 % at pH 5.55, 13.23 ±

0.51% at pH 5.80 and 9.41 ± 0.81% at pH 6.20 (Fig. 3.18).
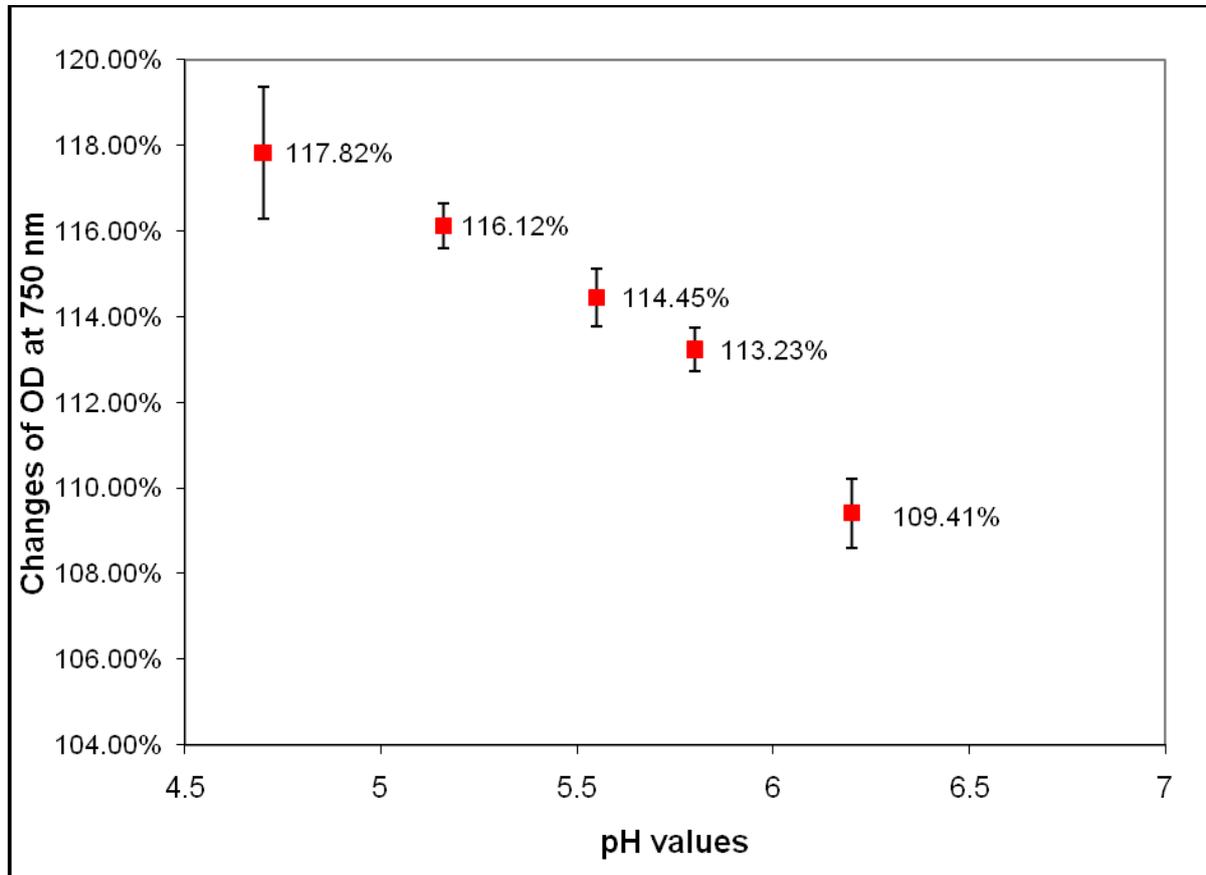
Figure 3.18: Graph showing the effects of addition of 14 µg of expansin on the optical density of 1.0 mL of sunflower hypocotyls cell wall fragments suspended in MES buffer with the pH values of 4.7, 5.16, 5.5, 5.8 and 6.2. Results show that expansin increased the optical density of the suspension by altering the turbidity in every case with the greatest effect at lower pH.

A similar experiment was carried out with the fragments of cell wall analogue

suspended in MES buffer with pH values of (a) 4.5, (b) 5.0, (c) 6.6 and (d) 7.0.

Addition of 14 µg of expansin to 1 mL of fragment suspension increased the optical

density by 15.23 ± 1.04% at pH 4.5, 12.46 ± 0.36% at pH 5.0, 10.93 ± 0.84% at pH

6.6 and 7.71 ± 0.48% at pH 7.0 (Fig. 3.19).
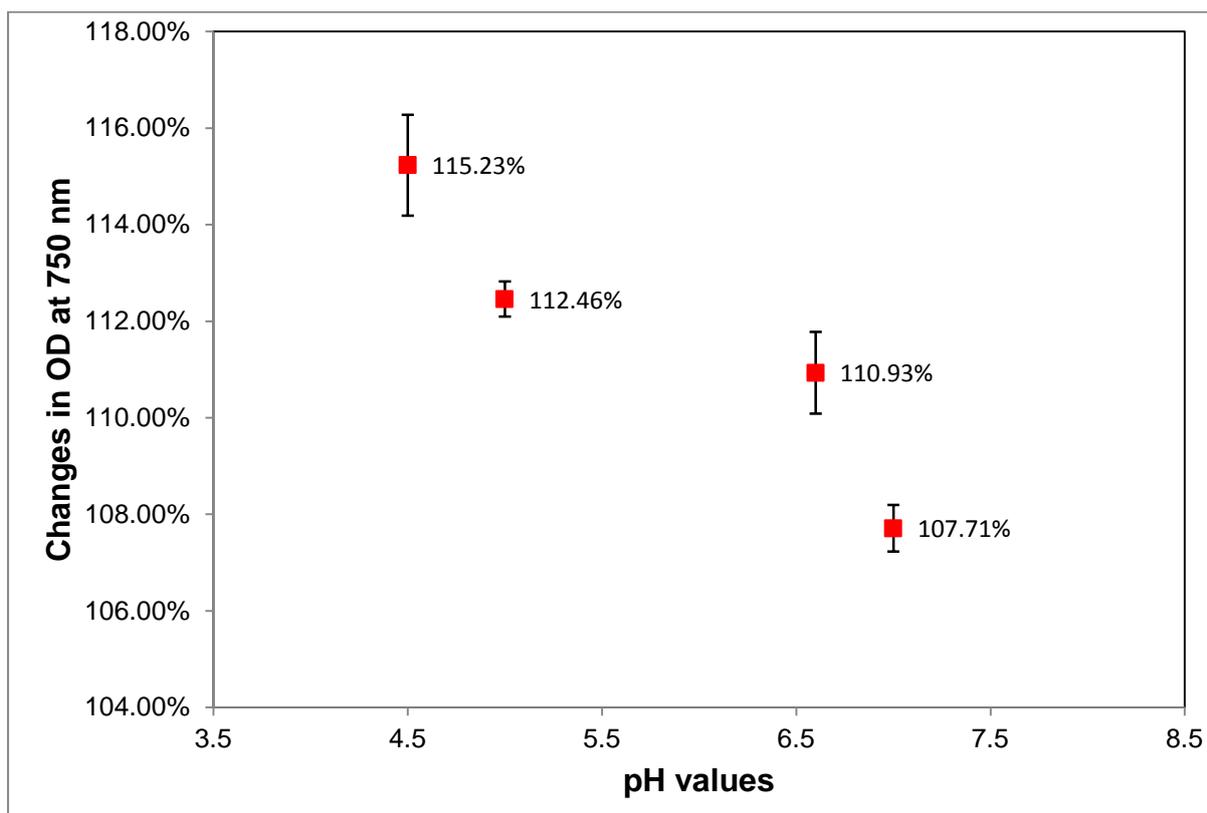
Figure 3.19: Graph showing the effects of addition of 14 µg of expansin on the optical density of 1.0 mL of cell wall analogue fragments suspended in MES buffer with pH of 4.5, 5.0, 6.6 and 7.0. Expansin increased the optical density of the suspension by altering the turbidity in every case with the greatest effect at lower pH.

Addition of expansin or snail powder caused an increase in the optical density of both cell wall analogues and hypocotyls. This effect is presumably due to the swelling of the suspended fragments. It is logical that when the fragment swells the free volume must be increased, and if the free volume increases the spacing within the cell wall should increase (Evered *et al.,* 2007). *Trichoderma viride* crude cellulase extract did not cause the same effect (which suggests that neither cellulose binding nor non-specific protein interactions cause the increase in optical density).

Previous researchers have shown that expansin loosened the cellulose in filter paper without hydrolysing it and increased the rate of extension in extensiometric assays, whereas long term incubation of cellulose with cellulase released soluble sugars by hydrolysis of the cellulose and caused mechanical failure rather than long term creep (McQueen-Mason and Cosgrove, 1994; Cosgrove, 1997). The turbidimetric experiments evidenced that expansin and snail powder extract increase the spacing within the cell wall which is hypothesised to be an important determinant in plant growth regulation

3.05 – Role of RG-II in the cell wall

The role of pectic polysaccharides in the mechanical strength and properties of plant primary cell wall is well evidenced (Jarvis and McCann, 2000; Wilson *et al.*, 2000; Zsivanovits *et al.*, 2004). Since its discovery, considerable attention has been given to the pectic polysaccharide RG-II, because of its complex structure and its presence in the primary cell wall as a dimer that is covalently cross-linked by a borate diester (Matoh *et al.*, 1993; Ishii and Matsunaga, 1996; Kobayashi *et al.*, 1996; O'Neill *et al.*, 1996; O'Neill *et al.*, 2004). To contribute further understanding of the role of RG-II, experiments were carried out using mutants of the model plant *Arabidopsis thaliana* with modified cell wall composition and the bacterial cellulose based cell wall model.

3.06 – Cell wall properties of *A. thaliana* cell wall mutant plants

L-fucose is a constituent of RG-II and xyloglucan, and the fucose deficient *A. thaliana* cell wall mutants *mur1(1-2)* and *mur2* were grown and their cell wall mechanical properties were compared with their background wild type (*Col 0*) plants cell wall. Plants were grown in MS-agar medium as described in section 2.13, no visible phenotypic variations were observed between wild type and *mur2* plants but *mur1* plants were dwarfed with abnormal growth of leaves and petioles and more fragile tissues (Fig. 3.20), also reported by O'Neill *et al.,* (2001).

Figure 3.20: Pictures showing the phenotypic variations of different *Arabidopsis* mutant plants. Three week old plants were initially grown in MS medium and then transferred to compost. A = wild type (*Col 0*) B = *mur1* and C = *mur2*. It shows that *mur2* mutant plant had normal growth but the *mur1* mutant was dwarfed with more fragile tissue.

To determine the mechanical strength and cell wall extensibility, growing tissues from the inflorescences stems of the three different types of plants were clamped into the extensiometer and their creep measured under constant load (as described in section 2.13).

Results showed that the stem segment of *mur2* mutant and wild type plant extended more than the segment of *mur1* mutant plant. When the applied force was increased from 0.049 N to 0.123 N the *mur2* and wild type plant tissue segments extended in a similar fashion. They extended almost linearly up to 4 h before the creep rate slightly decreased. However, in the case of *mur1* mutant tissue, the extension rate was substantially lower compared to the wild type and *mur2*. Under a constant load of 0.123 N the *mur1* tissue segment extended 16 to 20 % less than the segments of wild type and *mur2* mutant segments (Fig. 3.21), and also it was interesting to observe that strips were much more fragile than the other two types.
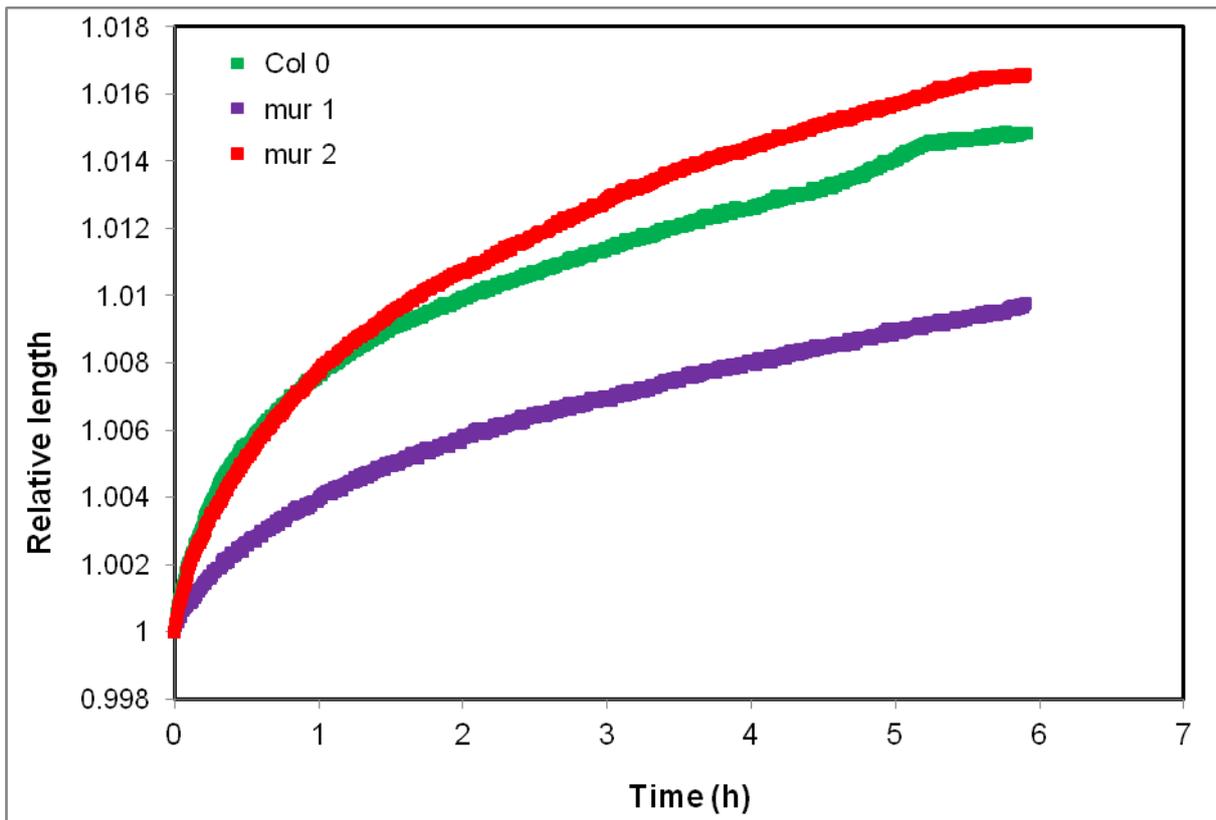
Figure 3.21: The graph illustrates the creep of the growing Inflorescences stems of different *A. thaliana* plants under a constant load of 0.123 N (12.5 g). The tissue strips were cut from the top growing portion of the inflorescences stems and frozen and thawed before being clamped and were incubated in pH 5.0 MES buffer.

To determine the effects of expansin on *Arabidopsis* mutant cell wall properties turbidity assays were carried out with wild type plants and two fucose deficient mutants, *mur1* and *mur2*. Cell wall fragments were prepared from the leaves and petioles of two weeks old plant (as described in section 2.14). The fragments were washed twice to reduce the pigments. The final suspension was slightly green in colour but had no absorbance at a wavelength of 750 nm after centrifugation to pellet the fragments (750 nm was selected for these measurements as photosynthetic pigments do not absorb light of this wavelength).

All the measurements were repeated a minimum of three times and the average values were plotted and the standard deviation calculated using Microsoft Excel.
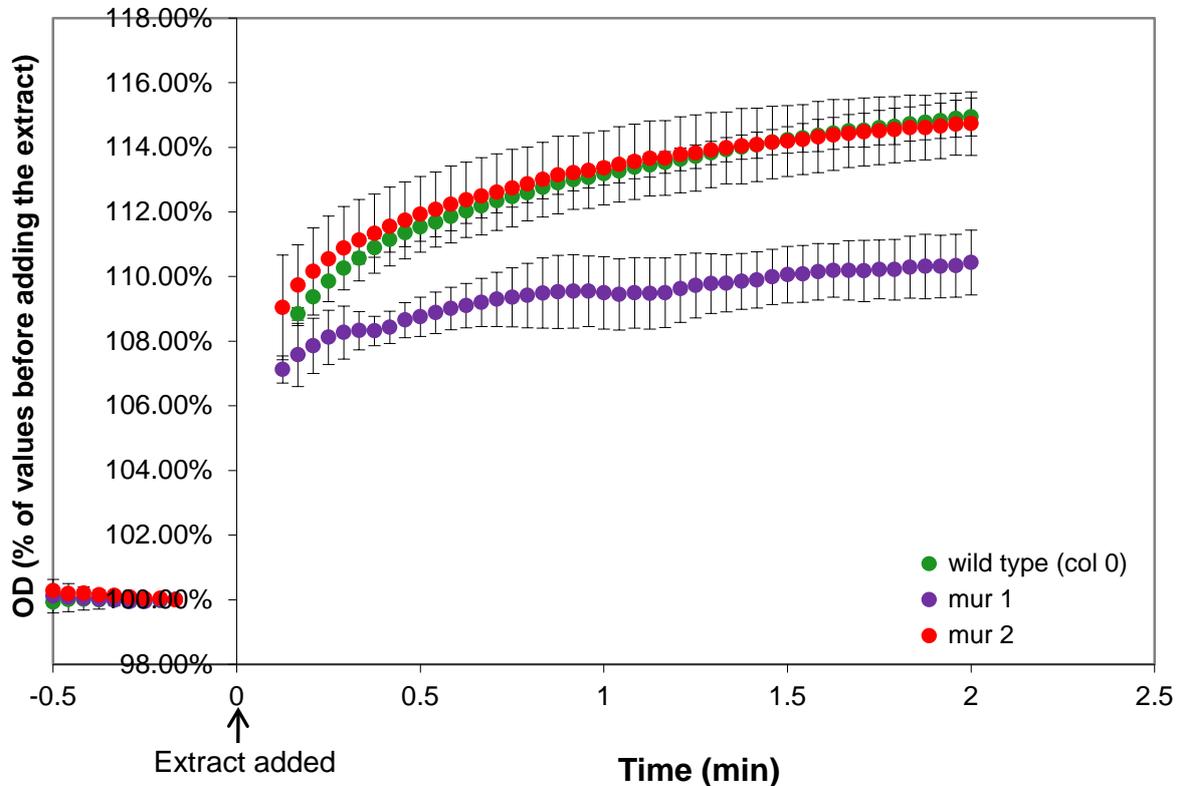
Figure 3.22: Effect of addition of 14 µg of expansin on the optical density of 1.0 mL of different *A. thaliana* plant cell wall fragment suspensions in MES buffer at pH 5.0. Addition of expansin (arrowed) caused a similar increase on the OD values of the wild types and *mur2* cell wall fragments suspension but a much lower change occurred with the *mur1* fragment suspension.

Addition of 14 µg of expansin caused an immediate increase of optical density of the all three fragment suspensions. The turbidity of the wild type and *mur2* cell wall suspensions were altered very similarly with the optical density increasing by almost 15 % whereas the optical density of the *mur1* cell wall fragment suspension only increased by 10%**.**

87
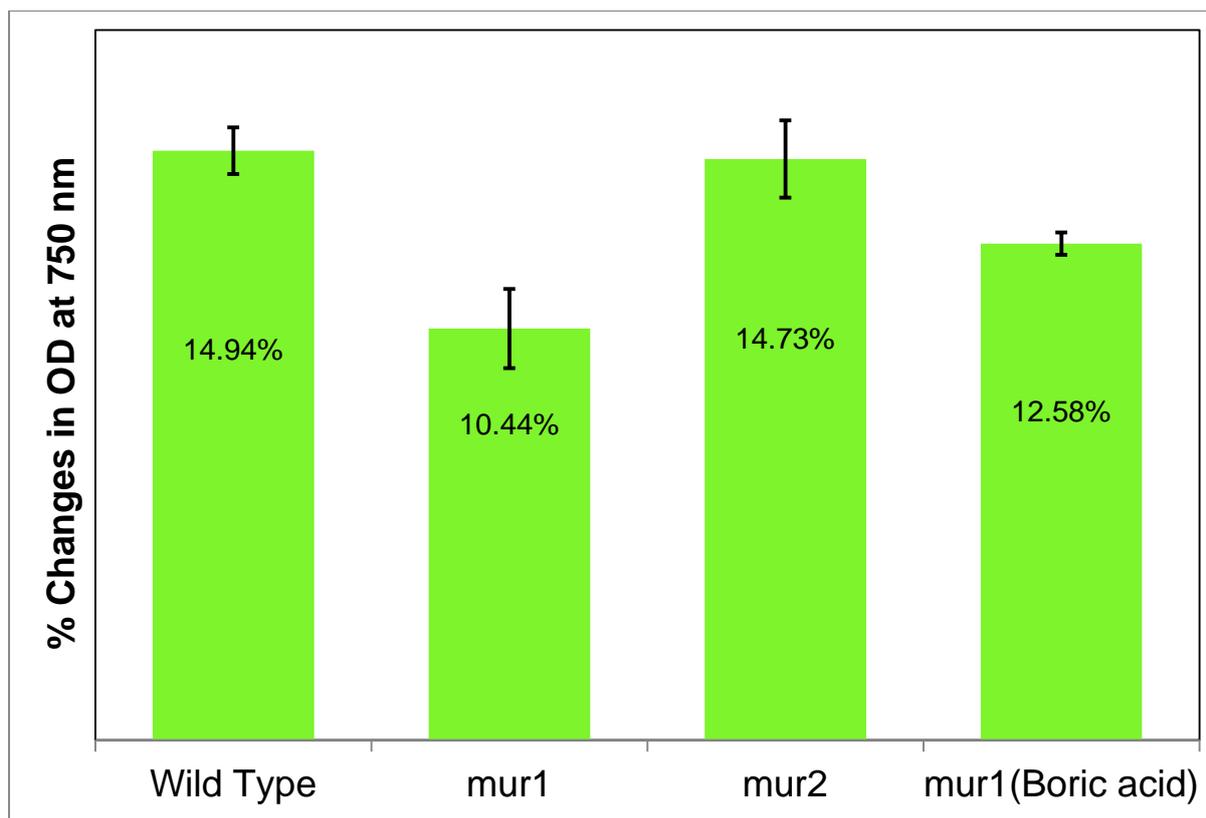
Figure 3.23: Absorbance of suspensions of cell wall fragments of *Arabidopsis* mutant cells showing the effect of addition of 14 µg of expansin on the optical density of 1.0 mL of cell wall fragment suspended in MES buffer at pH 5.0 treated with 150 µL of expansin buffer before the initial optical density was measured. The data represents the changes of absorbance after 2 min of addition of the expansin.

From the extensiometric experiment along with the turbidity data it is clear that *mur1* mutant plant tissues have a lower extensibility which is due to the altered cell wall composition. It was reported that in *mur1* mutant the primary structure of the rhamnogalacturonan-II is changed by alteration of the side chains (Reiter *et al.*, 1997; O'Neill *et al.*, 2001; Ryden *et al.*, 2003; Reuhs *et al.*, 2004) so that although the leaf cell wall of *mur1* mutant contains normal amounts of RG- II, only half of the sections form the borate cross-linked dimer whereas almost 95% of the RG- II forms the borate dimer in wild type plants (O'Neill *et al.*, 2001) indicating that normal growth in *Arabidopsis* depends on the organization of RG- II. The L-fucose and 2-O-methyl

L-fucose residues of RG-II of *mur1* mutant are replaced by L-galactose and 2-O-methyl L-galactose, which would have caused the reduction in dimer formation and the *mur1* mutant may have a high boron requirement as the phenotype can be rescued by treatment with elevated levels of boron (O'Neill *et al.*, 2001; O'Neill *et al.*, 2004). As *mur1* plants grow almost normally in presence of L-fucose or boric acid, turbidity assays were carried out using *mur1* cell wall fragment suspensions treated with boric acid.

The *mur1* cell wall fragments were treated with 100 mM boric acid and incubated for 60 min at room temperature then pelleted by centrifugation and re-suspended in MES buffer and after their initial optical density had been recorded, 14 μg of expansin was added and any changes were observed. Results showed that expansin increases the optical density of the suspension of fragments treated with boric acid almost by 2% more than the normal *mur1* cell wall fragment suspensions indicating that addition of boron has altered the cell wall properties, presumably by changing the organization of RG-II.

Although most research work on cell wall mechanical properties had been concentrated on the cellulose-xyloglucan network, recent examination of the role of RG- II has indicated that this polysaccharides may play a significant role in the cell wall biomechanics (Vanzin *et al.*, 2002). It is worth mentioning that the *mur2* mutant showed normal growth and similar creep behavior and turbidity responses in fragments suspension treated with expansin to the wild type. Although both *mur1* and *mur2* mutants are fucose deficient, *mur2* mutant cell walls contain less than 2% of fucosylated xyloglucan compared to wild type and perhaps more importantly, xyloglucan is the only affected polysaccharide in *mur2* mutants (Vanzin *et al.*, 2002),

which is a clear indication that fucosylation of xyloglucan is not critical for the tensile properties of cell wall. To determine the role of the pectic polysaccharide RG- II in determining cell wall mechanical properties, further investigation was carried out using *Acetobacter* cellulose model.

3.07 – Purification of RG- II from red wine

The uronic acid assay indicated that four fractions collected from the chromatographic separation contained the majority of the RG- II (Fig. 3.24). These fractions were combined and dialyzed using 2000 MWCO (Spectra/Por 7) dialysis membranes to eliminate the buffer salts and other impurities in the fractions.
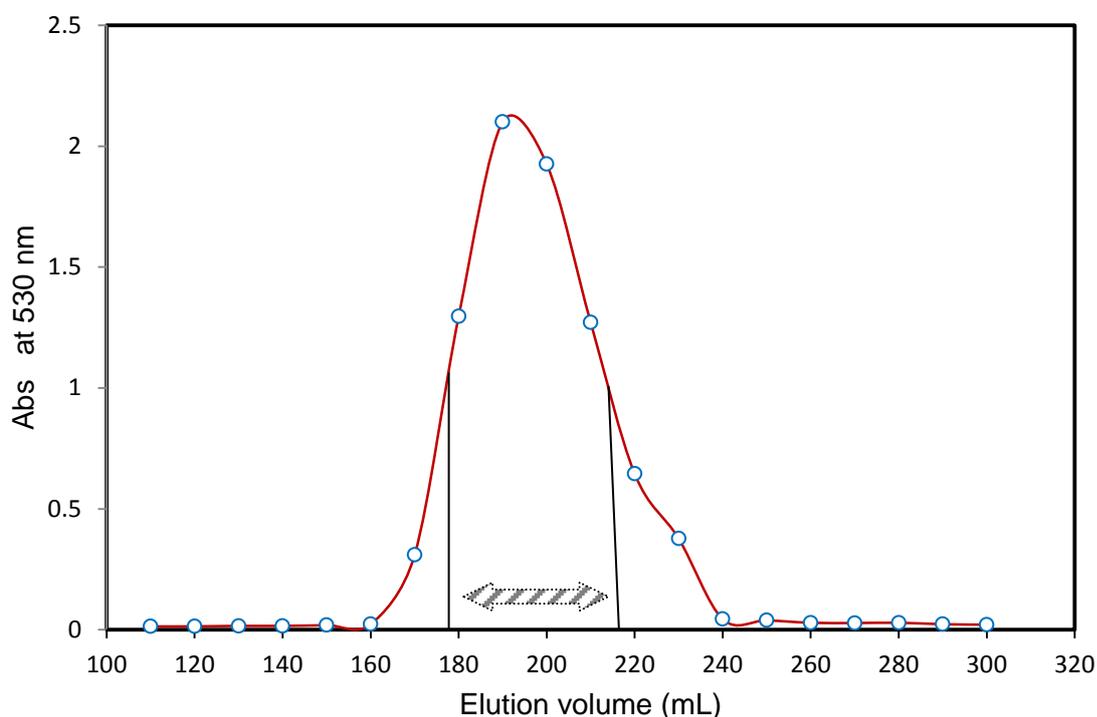
Figure 3.24: Purification of red wine RG-II by size exclusion chromatography on a Sephadex G-75 column. 10 mL fractions were collected and assayed for the presence of uronic acid. Uronic acid assay confirmed that 4 fractions (area marked by arrows) contained uronic acid and were retained for further purification.

From a total of 12 L red wine ~ 5.40 g of lyophilized total wine polysaccharides were collected by ethanol precipitation of the concentrated wine. From the collected four fractions of chromatographic separation a total of ~1.21 g lyophilized RG-II was recovered, accounting for about 22% of the total ethanol precipitated

polysaccharides. The isolated RG-II was slightly purplish in colour, presumably due to the pigments of the wine.

3.08 – Cellulose / RG-II composites

To determine the effects of RG-II on cell wall properties, RG-II was dissolved in the *Acetobacter* culture medium and incorporated with the cellulose to form a polysaccharide composite. This composite was compared with cellulose composites prepared with polygalacturonic acid (PgA, i.e. RG-II free pectic acid) and also with cellulose / pectin composite. RGII was compared with PgA and pectin as because apple pectin contains RG-II and as well as other acidic polysaccharides such as RG-I and homogalacturonan (HG), but and PgA is an RG-II free pectic polymer.

Composites were collected from 7 day cultures and the C/P and C/RG-II composites were noticeably more pliable than the C/PgA composites. Creep extensiometry and de-hydration and re-hydration measurements were carried out to determine the effects of RG-II on composite mechanical behavior.

Results from the creep experiments showed that when under a constant load, C/P composites strip extended more rapidly than the C/PgA composite strip (Fig. 3.25). When a force of 0.686 N was applied to the strips, CP composite extended for a prolonged period of time but in the case of C / PgA composite there was a rapid extension for a short time, after which extension almost ceased.
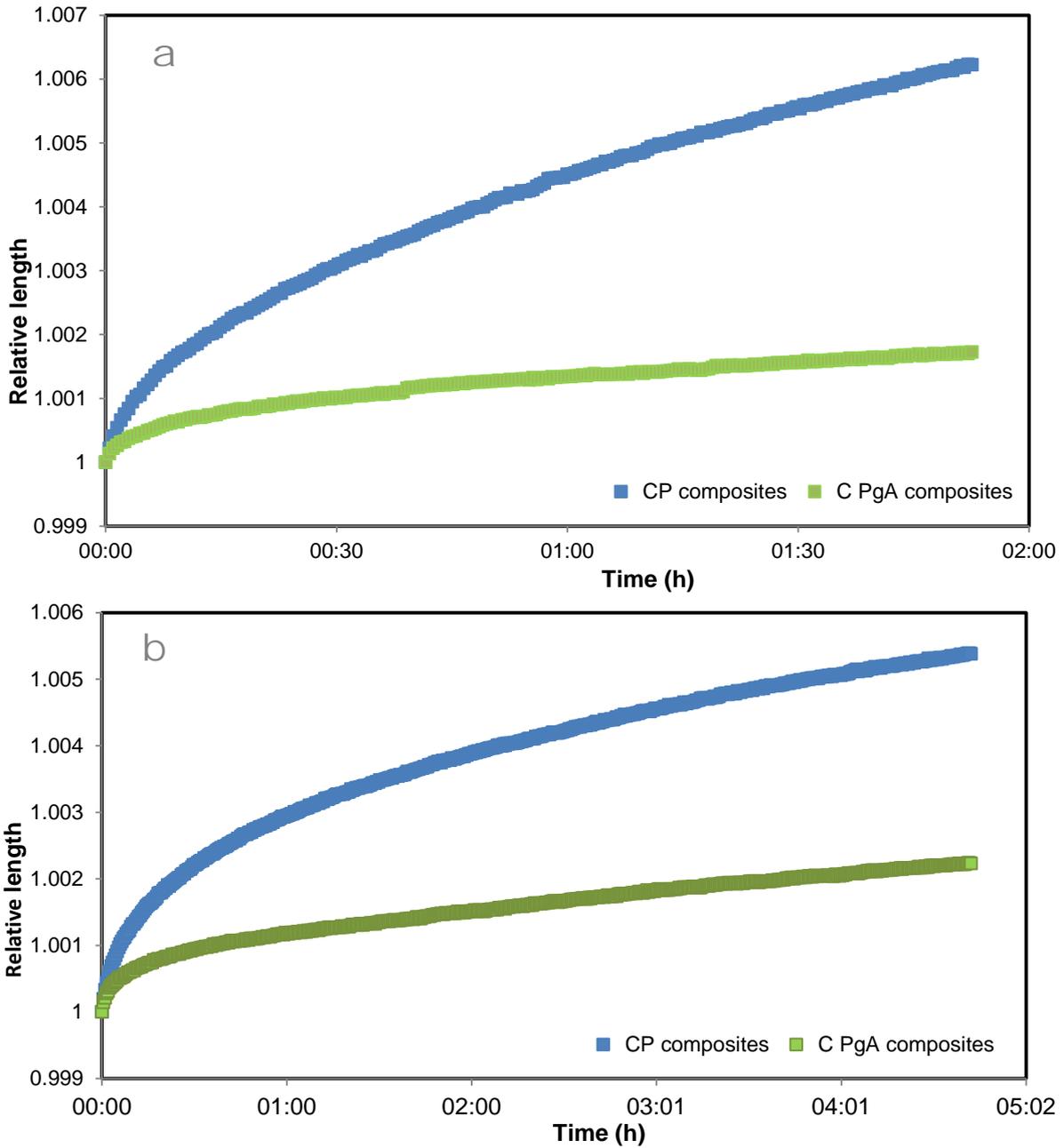
Figure 3.25 (a,b): The extension of strips of cellulose/pectin (C/P) and cellulose/PgA (C/PgA) composites after the applied force was increased from 0.294 to 0.686 N (a) and from 0.686 to 0.882 N (b). The strips, 2x1 mm in cross-section with an initial length of 10 mm, were bathed in pH 5.0 MES buffer. The LVDT data was taken from the same strips in both cases.

When the force was increased from 0.686 N to 0.882 N similar behaviour was observed as in lower stress conditions. The C/P composite extension seemed to exhibit behaviour with long retardation times while the C/PgA composite showed only an initial extension which appeared to have a much shorter retardation time.
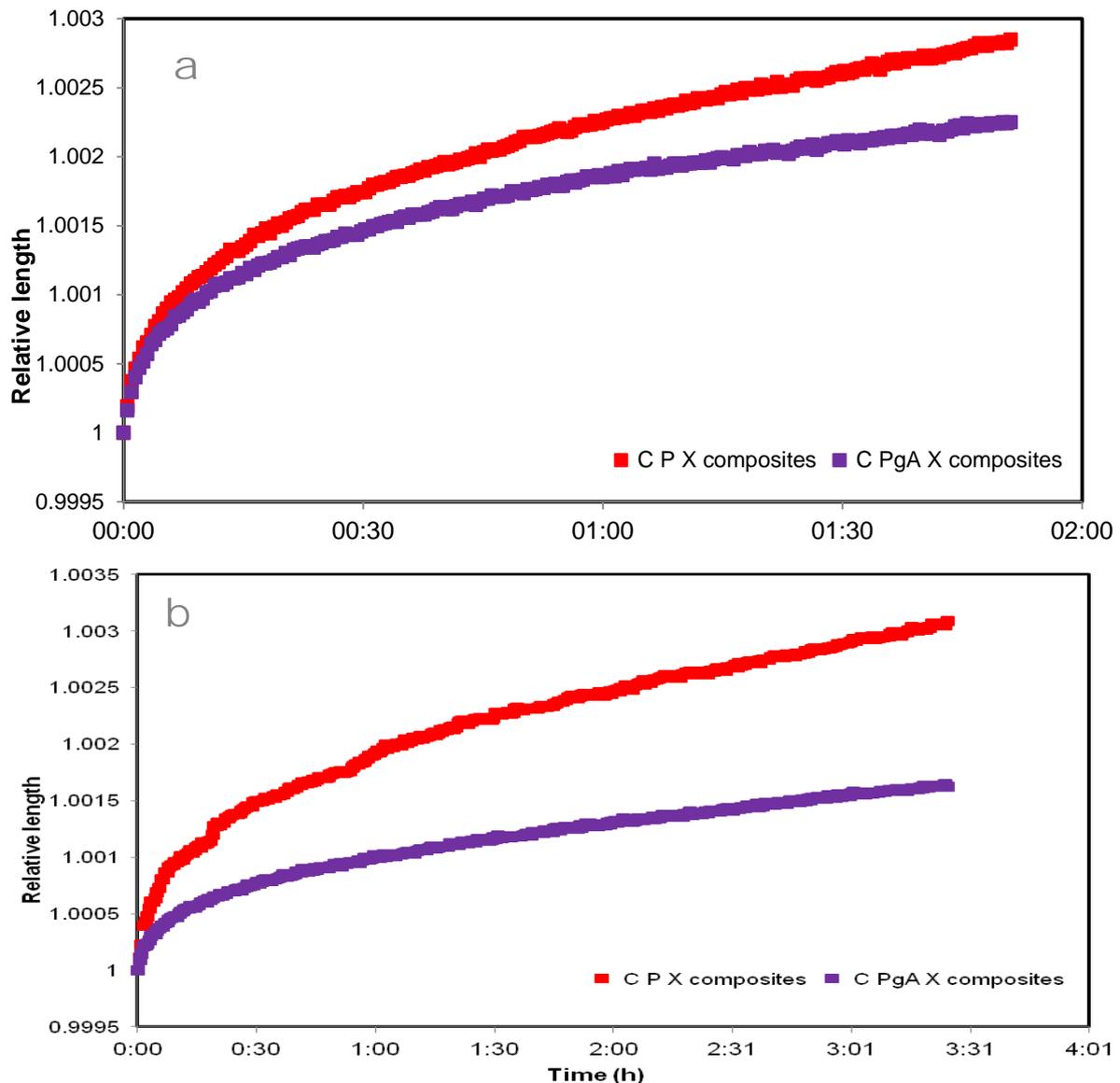
Figure 3.26 (a, b): The extension of strips of cellulose / pectin / xyloglucan (C/P/X) and cellulose /PgA /xyloglucan (C/PgA/X) composites after the force was increased from 0.294 N to 0.686 N (a) and from 0.686 N to 0.882 N (b). The strips, 2x1 mm in cross-section and with an initial length of 10 mm, were bathed in pH 5.0 MES buffer.

It was also observed that when those two composites (C/P and C/PgA) were prepared in presence of xyloglucan in the culture medium, the extensibility of the PgA containing composite was slightly increased but was still much lower than the equivalent material containing pectin (the degree of methyl esterification of the apple pectin 70-75 %) (Fig. 3.26), indicating that, some of the pectic polysaccharide components contribute towards the cell wall extensibility. As RG-II accounts for 3-4% of plant cell wall composition, there should be more than 10 % in the pectin (apple pectin) that had been used for the composite preparation, which aroused further interest as to whether RG-II contributes to cell wall physical properties and if so how it does so.

To test this, creep extensiometry was carried out using C/RG-II composite and compared with C/P and C/ PgA composite creep data. Results confirmed that incorporation of red wine RG-II with *Acetobacter* cellulose substantially increased the extensibility of the composites compare to its counterpart cellulose/PgA composite (Fig. 3.27). Most interestingly, RG- II containing composite strips exhibited a slightly higher rate of extension than Cellulose/pectin composites and both composites exhibited viscoelastic rheological properties with greater long-term extension. When the applied force was increased from 0.004 N to 0.294 the C/RG- II composites strip extended rapidly for about half an hour, after which the extension rate progressively decreased but extension continued for a long period of time. With a further increase of the force from 0.294 N to 0.588 N, RG- II containing composite strips exhibited similar types of creep behaviour with a higher extensibility and RG-II free composites exhibited much lower extensibility with rapid retardation (Fig. 3.27).
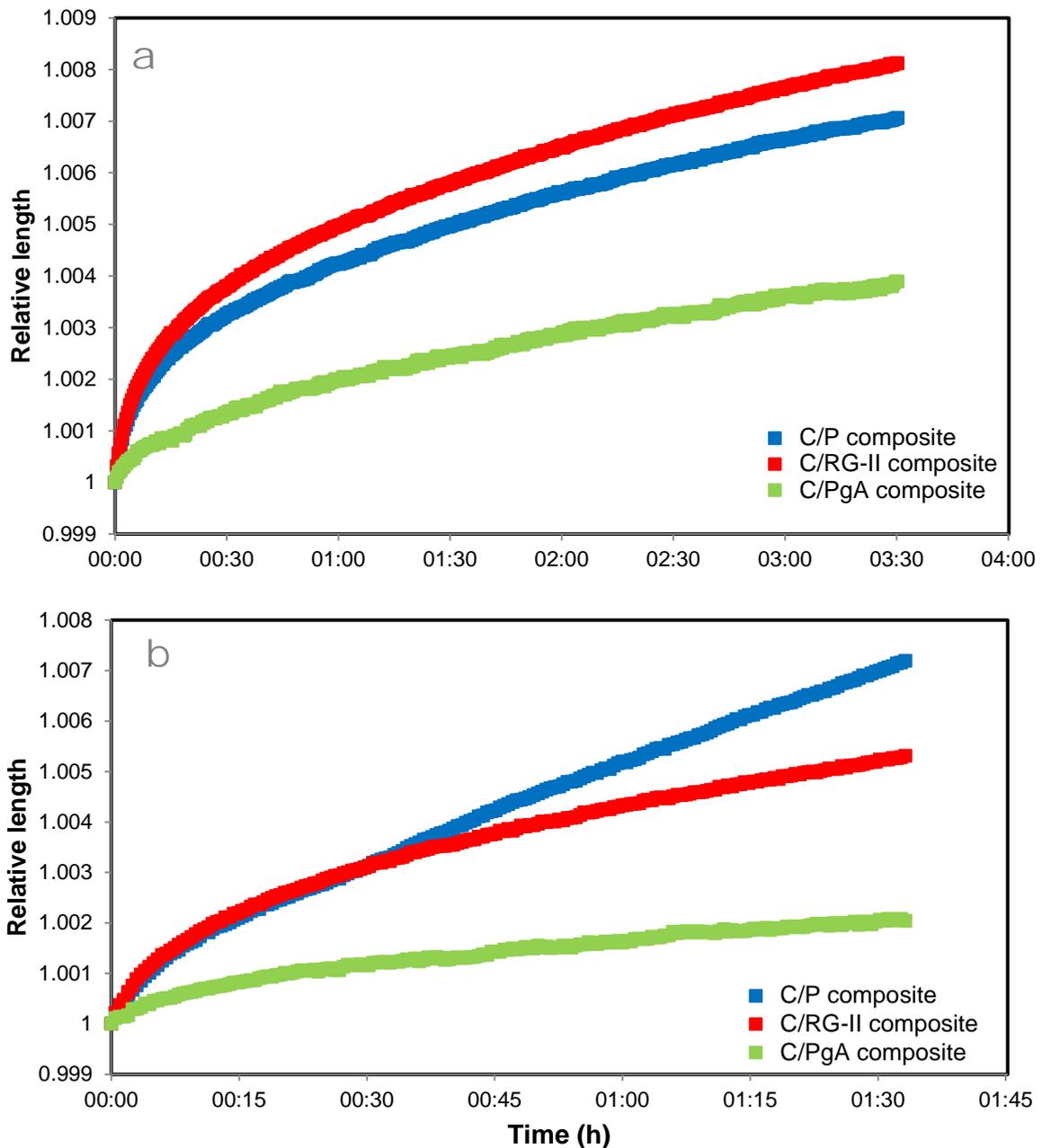
Figure 3.27 (a,b): The extension of strips of cellulose/pectin (CP), cellulose/RG-II and cellulose / PgA (CPgA) composites after the applied force was increased from 0.004 to 0.294 N (a) and from 0.294 to 0.588 N (b). The strips, 2x1 mm in cross-section with the initial length of ~ 10 mm, were bathed in to pH 5.0 MES buffer. The LVDT data was taken from the same strips at each applied force.

To determine the role of this complex polysaccharide on cell wall hydration properties, a dehydration and rehydration assay was carried out using C/RG- II composites and compared with C/P and C/PgA material.
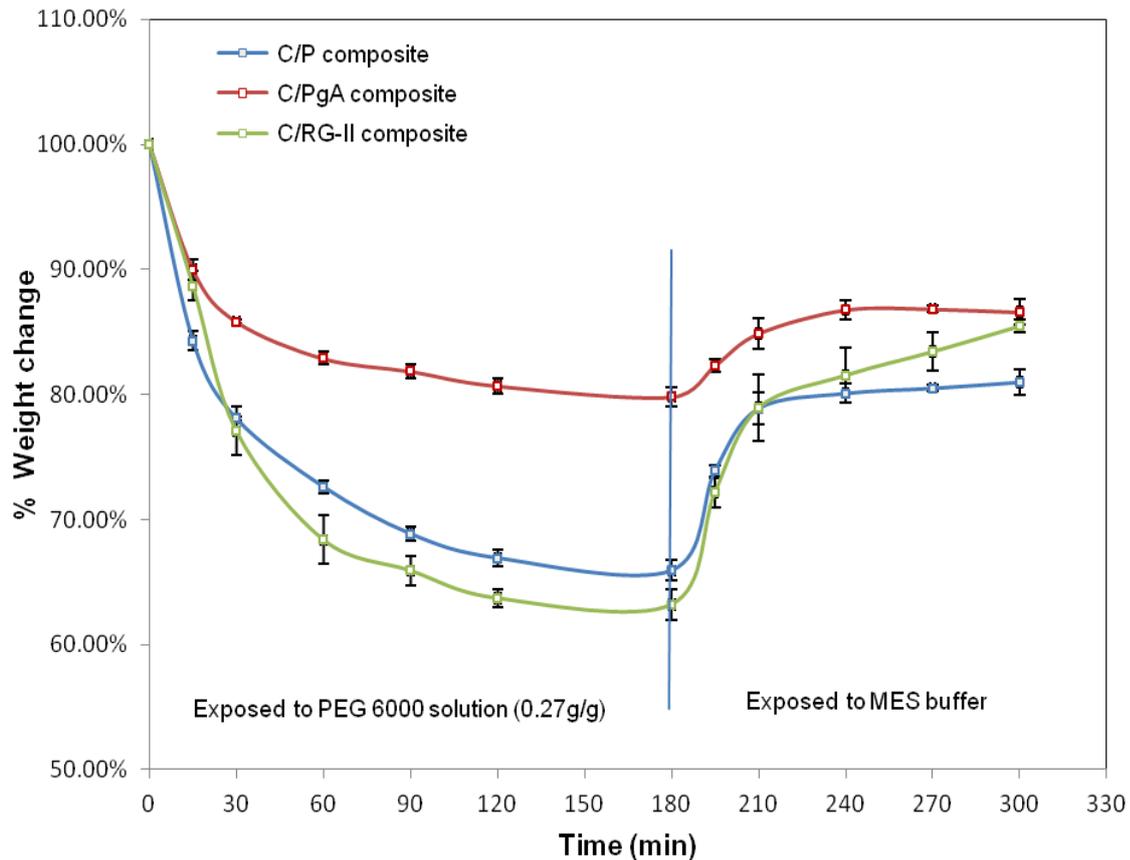
Figure 3.28: Changes in weight of C/P, C/RG-II and C/PgA composites pieces exposed to PEG 6000 solution (0.27g/g) to give an osmotic pressure of ~ 0.62 MPa and after returning them to control buffer (800 µL) to rehydrate. Both control buffer and buffer containing PEG solutions were pH 5.0.

Upon exposure to PEG 6000 solution, C/RG-II exhibited a rapid decrease of weight due to loss of water. Within a period of 180 min C/RG-II lost 36.79 ± 1.16 % of its initial weight while C/P and C/PgA composites lost about 34.11 ± 2.26 % and 20.16 ± 2.61 % respectively. The majority of these losses occurred during the first 30 min of the incubation period in all cases. During that period, C/RG-II, C/P and C/PgA composites lost 22.92 ±1.54, 21.84 ± 3.91 and 14.17 ± 2.41 % of their weight respectively. Similarly, during the rehydration cycle C/RG-II composites recovered highest amounts of water with 22.27 ± 1.15% in a period of 120 min and the recovery was much lower with 6.79 ± 2.89% in the case of C/PgA composite, which represents only 33.60 % of the total loss of water, and 14.96 ± 1.50 % with C/P composite. Interestingly, there was a rapid recovery observed during the first 30 min with 15.74 ± 1.71 (42.76% of total loss), 5.04 ± 2.48 (24.26% of total loss) and 12.87 ± 1.43 % (38.03% of total loss) with C/RG-II, C/PgA and C/P composites respectively. The C/RG-II composites showed a higher rate of dehydration as well as a higher rate of rehydration and opposite results had been found in the case of C/PgA composites. Therefore, it could be possible that the RG-II substantially increase the hydration capacity of the composite that might lead to the higher extensibility of the strips and the results also well indicate that the pectins, that provide resistance against wall compression, which is primarily due to the PgA. From this complex set of results it seems that the mechanical behaviour of apple pectin and RG-II are quite similar, suggesting two possibilities. The first is that the RGII substantially contributes to the properties of pectin and the second is that the RGII and apple pectin have similar degrees of esterification (the apple pectin was 70-75% esterified but data was not available for the PgA). The RG-II was clearly less effective than PgA in preventing the loss of water, but a greater proportion of the

water loss was recovered. Interpreting this is not easy, but an influence on the relationship between water potential and water content seems clear although whether this is due to esterification, the RG-II or both is hard to say.

Table 3.03: Comparison of the statistical difference on dehydration and rehydration of different cellulose composites. Dehydration was carried out with PEG 6000 solution (0.27g/g) and rehydration was carried out with MES buffer, pH 5.0. The p-values in bold indicate significant differences.

	Non paired t-test p-values (level of significance was set at p= 0.05)	
Composites	Dehydration in PEG 6000 solution (0.27g/g)	Rehydration in MES buffer, pH 5.0
C/P and C/ PgA	**0.00031**	**0.02448**
C/P and C/ RG- II	0.05870	**0.02937**
C/PgA and C/ RG- II	**0.00010**	**0.00001**
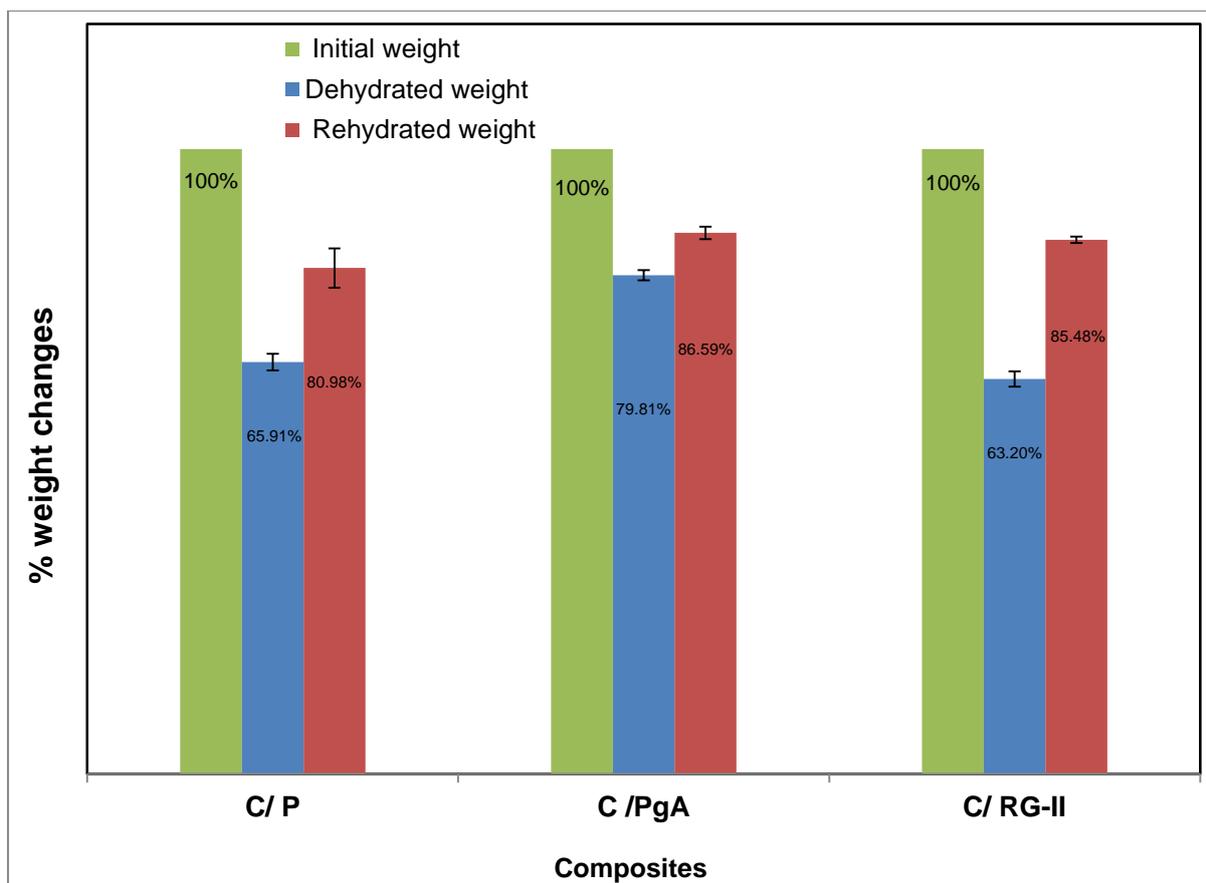
Figure 3.29: Graph showing the % of weight changes of different cellulose composites exposed to buffer containing PEG 6000 (0.27 g/g) solution and after returning them to MES buffer to rehydrate at pH 5.0.

Extensiometry on *Arabidopsis* mutant tissues and the *Acetobacter* cellulose composites confirm that RG-II contributes to cell wall extensibility. It has been reported that *mur1* mutants contain normal amounts of RG-II but only a half of them form the borate cross link dimer, and that this was sufficient to make the plant dwarfed with fragile tissue (O'Neill *et al.*, 2001). All the RG-II in red wine is present as borate cross-linked dimer due to the presence of significant amounts of boric acid in the highly acidic red wine (typical pH between 3.0 and 3.5) maintaining the dimeric form of RG-II (Pellerin *et al.*, 1996; Pellerin and O'Neill, 1998) and composites with those isolated dimeric RG-II exhibited higher extensibility than composites with PgA or apple pectin, indicating that RG-II dimerization with boron, perhaps

counterintuitively, makes plant cell walls more extensible. Boron is an essential micronutrient for the normal growth and development of plants although the role of boron is not well understood. Previous researchers have reported that boron plays an important role in cell wall synthesis and integrity as boron deficiency resulted in changes in the structure of cell wall (Matoh *et al.*, 1992; Kaneko *et al.*, 1997; Matoh *et al.*, 2000; Brown *et al.*, 2002). As RG-II is the only boron containing polysaccharide in plant cell walls and is covalently attached to a linear polygalacturonic acid chains, the boron cross-linked dimer may increase the formation of the cross-linked pectin network and therefore may control the cell wall porosity (Hofte, 2001; Brown *et al.*, 2002) and thus influence the hydration properties of the plant primary cell wall. The paradoxical increase in extensibility associated with an increased capacity for cross-linking suggests that the cross links organise the architecture of the wall and are not solely load bearing.

3.09 – The hydrated weight and the dry weight of different composites

The results of the hydrated weight and dry weight of different cellulose composites showed a clear pattern that the dry weights of all the composites that contained xyloglucan were higher than the cellulose/pectin ones (Table 3.04). The highest percentage of additional dry matter appeared to be in the C/RG-II/X composites and the lowest with the C/P composites. Xyloglucan particularly clearly increased the dry matter of the composites, presumably by incorporation into the pellicle between the microfibrils so that there was simply more material in the pellicle.

Table 3.04: *Table showing the percentages of dry weight and water contents of Acetobacter cellulose and different cellulose composites.*

Composites	% Dry weight	% Water content	% Dry weight compare to Cellulose
C	1.26%	98.74%	100.00%
C / P	1.60%	98.40%	127.17%
C / PgA	1.81%	98.19%	143.81%
C / RG-II	1.83%	98.17%	145.44%
C / P / X	2.08%	97.92%	165.39%
C / X	2.17%	97.83%	172.45%
C / PgA / X	2.23%	97.77%	176.84%
C / RG-II / X	2.71%	97.29%	215.28%

In conjunction with the dehydration experiments it seems probable that pectins, particularly polygalacturonic acid, provide resistance against compression of the wall through holding water. From the morphological characteristics of different composites it appears that the composites without xyloglucan were more rigid but including xyloglucan or other hemicelluloses into the composites caused them to become softer and more pliable, and it seems possible that xyloglucan incorporated between the microfibrils makes it easier for the microfibrils to realign under applied stress or hypertonic conditions so that the material is more able to extend longitudinally and narrow laterally.

3.10 – Water potential of cell wall polymers

It is well known that water deficiency often reduces plant growth. Water deficient plant cells can lose turgor and become unable to impose pressure on the cell wall (Passioura, 2001), which prevents wall extension leading to reduced growth rates. As plant cell growth is the result of turgor driven cell wall enlargement, the mechanical properties of the rigid, but at the same time slightly extensible cell wall are important for plant growth (Nonami and Boyer, 1990a; Nonami and Boyer, 1990b; Passioura, 2001; Evered *et al.*, 2007). However in addition, hydration of the cell wall may directly affect the rheological behaviour of the wall which in turn affects cell wall extension (Edelmann, 1995; Evered *et al.*, 2007). In relation to the hypothesis that the water potential of the cell wall components is important for the hydration status of the cell wall the water potentials of pectin and xyloglucan solution were determined using a vapor pressure deficit osmometer.

The osmolality value of the solutions measured by the osmometer was converted to the osmotic pressure (π) using the van't Hoff equation;

$$\pi = iR\,T\,c$$

where, c is the measured osmolality of the solution in moles kg^{-1} (or osmoles/kg), R is the universal gas constant and T is the temperature in K and i = Vant Hoff factor (i=1 for solutes that do not dissociate). This simplified form of the equation assumes ideality, but is sound as it is simply being used to convert an osmolality obtained from the vapour pressure (also assuming ideality) to an osmotic pressure.

Gas constant (R) = *8.314 J K^{-1} mol^{-1}*

[1 J = 1 N m and 1 Pa = 1 N m^{-2} and so 1 N = 1 Pa m^{2}. $\therefore$ 1 J = 1 Pa m^{3},

$\therefore$ *1 J mol^{-1} = 1 Pa m^3 mol^{-1}]*

$$= 8.314 \text{ Pa } m^3 \text{ } K^{-1} \text{ mol}^{-1}$$

[1 m^3 contains 1000 L of water and 1 L of water is equivalent to 1 kg for low solute concentrations. Therefore molal concentrations can be used to obtain osmotic pressures in Pa]

$$= 8.314 \text{ x } 1000 \text{ kg Pa } K^{-1} \text{ mol}^{-1}$$

$$= 8314 \text{ Pa (kg mol}^{-1}) \text{ } K^{-1}$$

mol kg^{-1} is the unit for molal concentration

At 23 ^{o} C the value of RT = 296.15 K x 8314 Pa kg mol^{-1} K^{-1}

$$= 2.462 \text{ MPa kg mol}^{-1}$$

From the RT value and osmometer reading (c), the osmotic pressure of the polysaccharides solution was calculated as shown on Table 3.05 below.

Table 3.05: The osmolality and the osmotic pressure of pectin and xyloglucan at different concentrations measured using vapour pressure osmometry. The p-values in bold indicate significant differences.

g/kg of water	Pectin		Xyloglucan		
	Osmolality (c = moles/kg) (data shown in mmol / kg)	Osmotic pressure, π (MPa)	Osmolality (c = moles/kg) (data shown in mmol / kg)	Osmotic pressure, π (MPa)	t-test (p=0.05)
0.1	55.67 ± 0.58	0.137	57.00 ± 1.00	0.140	0.13358
0.2	57.00 ± 1.00	0.140	58.33 ± 0.58	0.144	0.13358
1.0	58.67 ± 0.58	0.144	61.33 ± 0.58	0.151	**0.00481**
5.0	60.33 ± 0.58	0.149	63.33 ± 0.58	0.156	**0.00312**

These results showed that the osmotic pressure of xyloglucan was slightly higher; and therefore gave a lower water potential than the pectin solution although the difference was not significant in the case of lower concentrate solutions. It should also be noted that all values are quite low compared to common water potentials in plant tissues (the range of water potentials, as opposed to osmotic pressure, experienced by plant tissues are between 0 and -1.0 MPa). From the turbidity assay and dehydration and rehydration assays it was revealed that composite made with xyloglucan hydrated faster than the composite only with pectin and retained water less than the pectin composites as well.

Water is the most abundant component of cell wall, making up almost two thirds of the wall mass (Cosgrove, 1997) and wall extensibility can be limited by wall water deficits. The water potential of the cell wall components is potentially important for maintaining the hydrated status of the cell wall, but these data suggest that the wall as a whole may behave differently to its individual components. This would not be surprising as the matrix seems likely to become distorted and strained as water is drawn out of it so that the relationship between water content and water potential is determined by the physical behaviour of the wall and the arrangement of its components to a much greater degree than the osmotic behaviour of the individual components.

CHAPTER 4

GENERAL DISCUSSION

4.01 – *Acetobacter* cell wall model

To mimic the plant primary cell wall, *Acetobacter* cellulose based cell wall analogues can be an important tool for the studies of cell wall mechanical and biochemical properties. The compositional analysis of the cellulose composites showed that the composites produced with three major cell wall polysaccharides, cellulose, pectin and xyloglucan, form a reasonably complete model of plant cell wall polysaccharide structure. Chanliaud *et al.,* (2002) reported that the cell wall analogue consisted of 63% cellulose, 22% pectin and 15% xyloglucan, which represent a composition reasonably similar to type-I primary cell walls (McCann and Roberts, 1991).
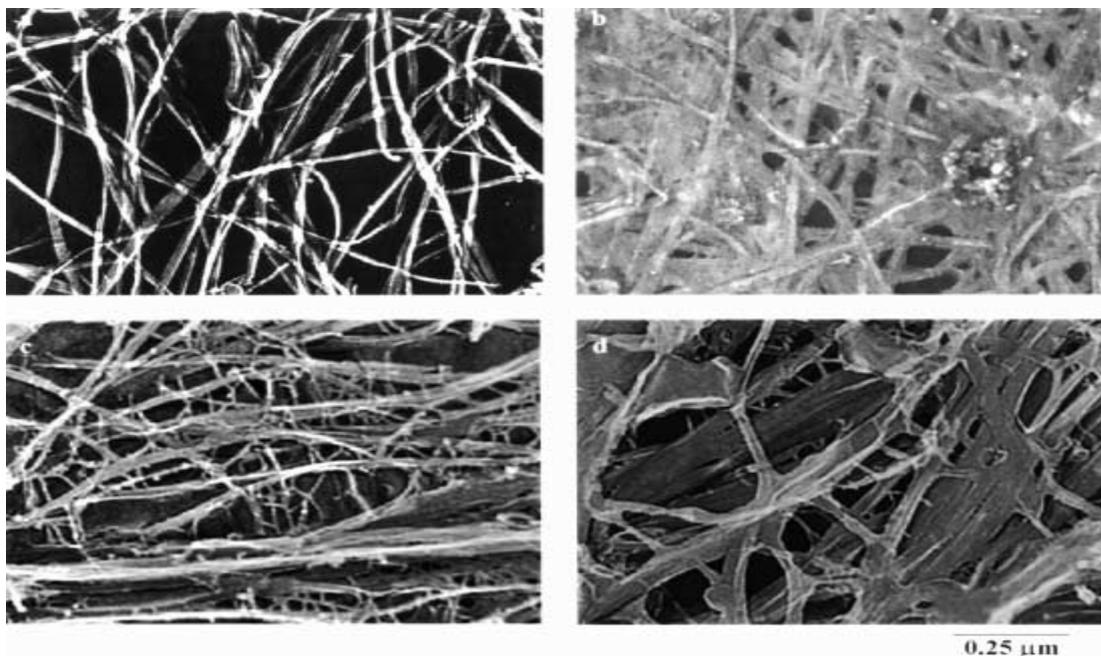
Figure 4.01: Transmission electron micrographs of cellulose (a), cellulose / pectin (b), cellulose / xyloglucan (c) and cellulose / pectin / xyloglucan (d) composites (Chanliaud *et al.,* 2002).

While only the pectin or xyloglucan was added to the medium 39% of pectin was incorporated into the cellulose composites and 22% of xyloglucan was incorporated. Transmission electron micrographs (Fig. 4.01) of the cell wall analogue showed that a pectin layer covered the cellulose/xyloglucan structure and that the bridges of xyloglucan between cellulose fibrils were visible, which is similar to the structure present in plant primary cell wall (Chanliaud *et al.*, 2002). To further investigate how effectively the *Acetobacter* model corresponds to plant cell walls, the capacity of the cell wall analogue to bind the grapevine extensin GvP1 (Pereira *et al.*, 2011) was examined, and it was found that the binding capacity of the cell wall analogue was significantly (t-test; p≥0.05) lower than that of freeze/thawed onion pellicules (OP) and purified grapevine callus cell walls. The variation in the composite components, including the use of cellulose alone, cellulose with pectin or polygalacturonic acid, cellulose with xyloglucan and cell wall analogue had only minor quantitative effects on the apparent binding capacity for GvP1. Surprisingly, the presence of pectin or polygalacturonic acid did not significantly enhance binding, whereas a non-significant but interesting increase in binding was detected at the $p \leq 0.1$ level in composites containing xyloglucan (Jackson, 2011). Despite this limitation of the protein binding capacity, the mechanical behaviours and the structural properties of the *Acetobacter* cellulose composites as presented previously (Whitney *et al.*, 1995; Chanliaud and Gidley, 1999; Whitney *et al.*, 1999; Whitney *et al.*, 2000; Astley *et al.*, 2001; Chanliaud *et al.*, 2002; Astley *et al.*, 2003) and the results demonstrated in this thesis show that this system is a powerful tool for study of the plant cell wall.

4.02 – Water and cell wall extension

It is well established that water is essential for plant growth. Well watered plants grow faster than plants experiencing water deficit because well watered plant cells are turgid (Passioura, 2001) and therefore exert higher pressures against their walls and growth of plant cells occurs when the, rigid but slightly plastic, cell wall is stretched by this pressure. Therefore, the growth of a plant is related to the cell turgor pressure and the cell wall extensibility (Nonami and Boyer, 1990b). It has been hypothesized that hydration of the cell wall directly affects its extensibility (Evered *et al.*, 2007). In this research it was observed that changing the water potential of sunflower hypocotyls cell wall (as found by Evered *et al.*; 2007) or cell wall analogues substantially changed their creep behavior. Evered *et al.* (2007) also confirmed that the thickness of the cell wall was reduced significantly in sunflower hypocotyls treated with PEG 6000 solutions with an osmotic pressure of ~0.62 MPa, Similarly, PEG treatment reduced the thickness of the pellicle of cell wall analogue, which was easily visible with the naked eye and reduced thickness must correspond with a decrease in the space within the cell wall. This appears to be a clear indication that the space within the cell wall is an important determinant of cell wall extensibility as proposed in the 'scaffolded model' of cell wall extension (Thompson, 2005).

The 'sticky network' model (Passioura and Fry, 1992; Cosgrove, 2000a) proposes that the cross-linked cellulose - xyloglucan network is the main load bearing component of primary cell wall in which xyloglucan chains hydrogen bonded to the microfibril surfaces tether them together and wall extension occurs when these tethers are broken by glycosidases or transglycosylases or xyloglucans are stripped off from the microfibril surfaces by expansins (Cosgrove, 2000a). In contrast,

Thompson (2005) proposed with analytical evidence that work done during cell wall extension is greater than the total hydrogen bond energy of interactions between cellulose microfibrils and xyloglucan and therefore that the hydrogen bonds cannot limit the wall extension as in the sticky network model.

It has already been proved that cell wall water content directly affects wall extensibility and therefore it is apparent that cell wall extension is not only the function of breaking the bonds between microfibrils and xyloglucan but also the hydration of cell wall is important and the factors affecting the cell wall water content become relevant to our understanding of wall biomechanics. Although the exact mode of action of expansins is uncertain, it has been hypothesised that expansins act on the cellulose-xyloglucan cross-link (Cosgrove, 1996; Cosgrove, 1997; Cosgrove, 1998; Cosgrove, 1999; Cosgrove, 2000a). However, results presented in this thesis showed that α-expansin (CsExp1) increases the extension of cellulose / pectin composites and raising questions about this. The scaffolded model of the cell wall suggested the possibility that expansins may increase extensibility in part by increasing wall spacing, and this was supported by the observation by Yennawar *et al,*. (2006) that β-expansins cause swelling of maize cell walls.

Therefore, experiments were carried out to investigate dehydration and rehydration of *Acetobacter* cellulose and cellulose composites, the results of which demonstrated that expansins and the expansin-like proteins from snail powder significantly increased the hydration of cell wall analogues. Although the greatest hydration occurred in xyloglucan containing composites, it also increased the hydration of the cellulose / pectin composites and in pure cellulose pellicles, strongly suggesting expansins can increase cell wall hydration, which is an important determinant of wall extensibility. It has also previously been reported that CsExp1 substantially increased

the thickness of cellulose and cellulose composites made with pectin and / or xyloglucan (Islam, 2006), another indication that expansin can increase the space within the cell wall by increasing the water content of the wall, presumably also affecting extensibility. Given the experimental evidence clearly suggesting that the hydration of the matrix polysaccharides that keeps the microfibrils separated is also important for wall extensibility, it was interesting to find that expansin and snail powder extract increased the rehydration and thickness of every composite, although highest increase was with the xyloglucan containing composite. While it is possible that expansins break xyluglucan - cellulose hydrogen bonds, it seems that at the same time they increase the space and hydration of the wall, although this effect could also be mediated by effects on non-covalent interactions in the cell wall. It appears certain that expansins do not act as cellulases or as endoglucanases that hydrolyse xyloglucan (McQueen-Mason and Cosgrove, 1994) as no polysaccharide hydrolytic activities have been detected. Also, the claim that β-expansins have proteolytic activities (Grobe *et al.*, 1999) has been refuted (Li and Cosgrove, 2001, as well as the effects observed on composites containing no structural proteins reported in this thesis).

Further confirmation of the hypothesis, that expansins increase the free space within the cell wall by increasing the water content was provided by the results of the turbidity assays. Addition of α-expansin or snail protein extract to the sunflower hypocotyl cell wall fragments and cell wall analogue fragments in suspension significantly increased the optical density (OD) of the suspension, which was presumably due to an increase in light scattering by the fragments because of an increase in their surface area. The initial rapid increase of OD suggested that the expansin caused an immediate swelling of the cell wall fragments, although the OD

kept increasing up to 3 min which was consistent with the dehydration – rehydration experiments. It could be predicted that if the surface area of the fragments is increased the cell wall free volume must have increased accordingly. Therefore the space between the microfibrils must have increased with water molecules acting as spacers in a manner analogous to the effect of plasticisers in synthetic polymers.

The 'sticky network' model is focused upon tethering polysaccharides with cell wall extension occurring when those tethering bonds are disrupted or tethers are cleaved, but the experimental evidence presented in this thesis appears to show that non-cellulosic wall components and the space within the wall are also important in determining cell wall extensibility. Veytsman and Cosgrove (1998) stated that the hydrogen bond strength affects cell wall volume but not shape, so alterations in hydrogen bonding seem at least as likely to lead to wall swelling or compaction as to extension. Therefore it is possible that bonding between microfibrils and tethers regulate growth by altering free space of the wall, not by directly contributing to the mechanical strength. Perhaps hydrogen bonds prevent wall swelling and expansins modify the wall properties by breaking the bonds to release steric constraint on microfibrils movement, rather than by breaking load bearing bonds (Thompson, 2005).

From the wet and dry weight of cellulose composites it appeared that the dry weights for all the composites that contained xyloglucan are higher than the cellulose/pectin ones intermediate. It is possible that C/P/X lost more weight in PEG simply because the structure was more closed and the PEG was more effectively excluded, although this appears improbable as substantial penetration of the matrix by PEG would be expected to lead to a slow increase in weight as the PEG diffuses into the matrix leading to rehydration. An alternate explanation is that incorporation of hemicellulose

reduces the lateral stability of the XG containing materials. It has also been noted that the composites with XG exhibited a much greater tendency to become thinner as they were extended. This phenomenon was even clear during blotting them or positioning them for cutting. Although it wasn't calculated, this observation suggests that the xyloglucan containing composites have a higher Poisson ratio than that of the composites without xyloglucan. It could be hypothesised that the cellulose is spaced during synthesis but the xyloglucan spans have very little rigidity so the material collapses as soon as there is any shear, including osmotic pressure drawing water out of the structure, so that the "pit-props" (McCann *et al.*, 1990) metaphor looks somewhat misleading. An alternative to this could be that if xyloglucan becomes incorporated into the microfibril surface (as proposed by Pauly *et al.*, 1999) and if this could make some sections of the microfibril less rigid, the whole structure could flex more easily and facilitating extension. It certainly looks as though there is some correlation between the dehydration caused by PEG and the pellicle extensibility and perhaps this connects to the way that hemicellulose containing composites pull out of shape so easily. Interestingly composites prepared with RGII behaved in a similar fashion to xyloglucan containing composites to some degree.

4.03 – RG-II and Cell wall properties

RG-II is one of the most complex and unusual polysaccharides in nature. Considerable interest has been given to this polysaccharide in recent years because of its structural complexity and its ability to interact with borate and also the extreme phenotypes observed in RG-II deficient mutants of *Arabidopsis* (O'Neill and York,

2003). Several reports have suggested that boron is associated with cell wall formation so that boron deficiency caused abnormal primary cell wall formation (Matoh *et al.*, 1993; Matoh, 1997; O'Neill *et al.*, 2001; Ishii, 2006). Results in this research using the *mur1* mutant of *Arabidopsis*, which has reduced levels of RG-II – borate dimer, showed that the mutant plants were dwarfed with abnormal leaves and petioles, also reported earlier by O'Neill *et al.* (2001). It has also been reported that the leaf and petiole of boron deficient pumpkin plants are smaller and more irregular than those of normal plants (Ishii *et al.*, 2001). The creep rate of *mur1* inflorescences stem cell walls was found to be much lower than that of their wild type counterparts. *Mur1* plants grown in the presence of boric acid exhibited normal growth (O'Neill *et al.*, 2001) which suggested that it is the RG-II – boron dimer that is essential for normal cell wall extension rather than RGII itself as the *mur1* mutant has reduced borate cross-linking dimerisation relative to the wild-type plants (Reiter *et al.*, 1997; O'Neill *et al.*, 2001). The results of the turbidity assays on *Arabidopsis* cell wall fragment suspensions suggested that RG-II-borate dimer may also play a role in the expansin mediated cell wall swelling by hydration, as the effect of expansin on the optical density of the *mur1* cell wall fragments suspension was much less than that of the cell wall fragments suspension of wild-type plants, but pretreating the *mur1* cell wall fragments with 100 mM boric acid increased the swelling of the wall fragments almost equal to the wild type levels, presumably because the RG-II dimer was formed *in vitro* in the presence of boric acid by reaction with what *mur1* RG-II monomer was present in the mutant cell walls. This result is consistent with the report that boron treatment rescued the normal phenotype of the *mur1* plants (O'Neill *et al.*, 2001).

Together with RG-II, pectin contains other acidic polysaccharides, so dimeric RG-II purified from red wine was incorporated with bacterial cellulose by dissolving it into the culture medium to get a clear answer as to whether RG-II dimer is essential for cell wall extension. The C/RG-II composite showed higher extensibility than the C/PgA and was also slightly more extensible than the C/P composites. The C / RG-II and C / P composites were prepared using equal amount of apple pectin or red wine RG-II so that if they incorporated in an equal rate then the C / RG-II composite should have much higher amount of RG-II than C / P but the creep rate was not substantially greater, which indicated that the abundances of RG-II is not necessarily important, although it was not feasible to determine the percentage of RG-II incorporation into C / RG-II composite.

It was observed that the cellulose composite prepared with RG-II was more pliant and extended faster than the composite with PgA which was much harder and more rigid and/or holds water more tightly than the C/RG-II composite so that any extra space isn't easily accessible, whereas RG-II makes the structure more flexible. The wet and dry weight of the composites suggested that PgA composite hold more water than the RG-II composites, although the difference was quite small, and the dehydration and rehydration experiments demonstrated that RG-II composites dehydrated much quicker than the PgA composites. As with the hemicellulose composites, this may mean the PEG molecules are excluded more effectively in the case of RG-II composites and indicate the composite has a smaller pore size than the PgA containing composites, or that RGII reduces resistance to lateral compression. The results also showed that the creep rate of C / P composites was substantially higher than the C / PgA but when those composites were prepared in

presence of xyloglucan, the creep rate of PgA containing composite (C / PgA / X) was increased indicating that the mechanical properties of cell walls depend both on xyloglucan cross-linking and RG-II, as has previously been reported in the case of *Arabidopsis* cell wall (Ryden *et al.*, 2003).

The dehydration and rehydration assays results indicated that composites with RG-II dimer lost and gained water much more rapidly than the C / PgA composite. The reduced rate of water loss by the C / PgA might be caused by Ca^{++} cross-linking of PgA and increased rigidity of the pellicle causing increased water retention.

CHAPTER 5

CONCLUSION

5.01 – Concluding remarks

In conclusion, the results in this research clearly showed that alteration of water content of plant cell walls and cell wall analogues alter their extensibility and that expansin and expansin-like proteins in snail acetone powder extract significantly increase the water content of cell wall analogue and increased their thickness. Expansin also increased the water content of cellulose / pectin composite as well as its extensibility and elicited rapid swelling of sunflower hypocotyl cell wall and cell wall analogue fragments in suspension. Taken together, these observations suggest that expansin increases the cell wall free volume by hydration of the wall and it is logical that if the cell wall swells the spacing between the microfibrils is increased. A causal relationship between these phenomena has not been confirmed but appears a strong possibility. Likewise, reduction of water content may collapse the wall structure and thus make the wall rigid and less extensible. It has been reported that oxidative cross-linking in the wall by H_2O_2 decreases the hydration of cell walls and significantly decreases the thickness of the wall (Pereira *et al.*, 2011) and it seems plausible that this would contribute to the reduction of extensibility caused by these reactions, which are frequently associated with reductions in growth rate or pathogen responses.

Although it has been argued that disruption of hydrogen bonding between cell wall polymers by protein such as expansin allows cell wall extension to occur, results of this study show that at the same time expansins increase the hydration of cell wall and therefore the space available for microfibril movement, and that water molecules and the matrix polysaccharides may act as spacers and plasticisers, facilitating wall extension.

It was also shown that, perhaps paradoxically, RG-II- borate dimer increased the extensibility of composites produced with bacterial cellulose. *In vitro* dimer formation using boric acid increased the swelling of *mur1* cell wall fragments. Perhaps RG-II dimer increases and controls the hydration and fluidity of the wall to some extent, while PgA cross-linked with Ca^{++} reduces the freedom of movement in the wall.

5.02 – Further studies

A number of possible lines of further enquiry are suggested by this work:

1. Incorporation of monomeric RG-II with *Acetobacter* cellulose and comparison of their mechanical properties with the composite prepared with the RG-II – borate dimer and composites with monomeric RGII after dosing with boron may shed further light on the role of the RG-II – borate dimer in determining cell wall extensibility.

2. Observation of how extraction or digestion of xyloglucan and RG-II affect the composites behaviour would shed some light on the structural complexity of the cell wall macromolecular structure.

3. It would be desirable to obtain scanning electron micrographs for different cellulose composites, or to visualise them using atomic force microscopy to identify differences visually would also give a better idea about their structure and cellular shapes. Also Immunolocalisation of the incorporated polysaccharides

of the composites using monoclonal antibody will contribute further knowledge to understand the structure more effectively.

4.   Further studies how expansins increase the hydration of the cell wall will contribute knowledge towards the expansin mode of action.

5.   Quantification of the effects of incorporation of hemicelluloses and RG-II on the Poisson ratio of the materials would be desirable.

References

Albersheim, P., Darvill, A. G., O'Neill, M. A., Schols, H. A. & Voragen, A. G. J. (1996) An hypothesis: The same six polysaccharides are components of the primary cell walls of all higher plants. *In:* Visser, J. and Voragen, A. G. J. (eds.) *Progress in Biotechnology,* Elsevier, **14**, 47-55.

Andrews, J., Adams, S. R., Burton, K. S. & Edmondson, R. N. (2002) Partial purification of tomato fruit peroxidase and its effect on the mechanical properties of tomato fruit skin. *Journal of Experimental Botany*, **53**, 2393-2399.

Astley, O. M., Chanliaud, E., Donald, A. M. & Gidley, M. J. (2001) Structure of Acetobacter cellulose composites in the hydrated state. *International Journal of Biological Macromolecules*, **29**, 193–202.

Astley, O. M., Chanliaud, E., Donald, A. M. & Gidley, M. J. (2003) Tensile deformation of bacterial cellulose composites. *International Journal of Biological Macromolecules*, **32**, 28-35.

Baydoun, E. A. H., Abdel-Massih, R. M., Dani, D., Rizk, S. E. & Brett, C. T. (2001) Galactosyl- and fucosyltransferases in etiolated pea epicotyls: product identification and sub-cellular localisation. *Journal of Plant Physiology*, **158**, 145-150.

Brett, C. T. & Waldron, K. W. (1996) *Physiology and Biochemistry of Plant Cell*, London: Chapman & Hall.

Brown, P. H., Bellaloui, N., Wimmer, M. A., Bassil, E. S. & Ruiz, J. (2002) Boron in plant biology. *Plant Biology*, **4**, 205.

Brown, R. M., Jr. & Montezinos, D. (1976) Cellulose microfibrils: visualization of biosynthetic and orienting complexes in association with the plasma membrane. *Proceedings of the National Academy of Sciences, USA*, **73**, 143-147.

Brown, R. M. J., Saxena, I. M. & Kudlicka, K. (1996) Cellulose biosynthesis in higher plants. *Trends in Plant Science*, **1**, 149-156.

Brownleader, M. D., Hopkins, J., Mobasheri, A., Dey, P. M., Jackson, P. & Trevan, M. (2000) Role of extensin peroxidase in tomato (Lycopersicon esculentum Mill.) seedling growth. *Planta*, **210**, 668-676.

Büntemeyer, K., Lüthen, H. & Böttger, M. (1998) Auxin-induced changes in cell wall extensibility of maize roots. *Planta*, **204**, 515-519.

Carpita, N., Sabularse, D., Montezinos, D. & Delmer, D. P. (1979) Determination of the Pore Size of Cell Walls of Living Plant Cells. *Science*, **205**, 1144-1147.

Carpita, N. C. (1996) Structure and biogenesis of the cell walls of grasses. *Annual review of Plant Physiology and Plant Molecular Biology*, **47**, 445-476.

Carpita, N. C. & Gibeaut, D. M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant Journal*, **3**, 1-30.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V. & Keegstra, K. (2008) Disrupting Two Arabidopsis thaliana Xylosyltransferase Genes Results in Plants Deficient in Xyloglucan, a Major Primary Cell Wall Component. *The Plant Cell Online*, **20**, 1519-1537.

Chanliaud, E., Burrows, K. M., Jeronimidis, G. & Gidley, M. J. (2002) Mechanical properties of primary plant cell wall analogues. *Planta.*, **215**, 989–996.

Chanliaud, E. & Gidley, M. J. (1999) *In vitro* synthesis and properties of pectin / Acetobacter xylinus cellulose composites. *The Plant Journal*, **20**, 25-35.

Cho, H. T. & Kende, H. (1997a) Expansins in deepwater rice internodes. *Plant Physiology*, **113**, 1137-1143.

Cho, H. T. & Kende, H. (1997b) Expansins and Internodal Growth of Deepwater Rice. *Plant Physiology*, **113**, 1145-1151.

Cosgrove, D. J. (1989) Characterization of long-term extension of isolated cell walls from growing cucumber hypocotyls. *Planta*, **177**, 121-130.

Cosgrove, D. J. (1996) Plant cell enlargement and the action of expansins. *BioEssays*, **18**, 533-540.

Cosgrove, D. J. (1997) Assembly and enlargement of the primary cell wall in plants. *Annual Review of Cell and Developmental Biology*, **13**, 171-201.

Cosgrove, D. J. (1998) Cell Wall Loosening by Expansins. *Plant Physiology*, **118**, 333-339.

Cosgrove, D. J. (1999) Enzymes and other agents that enhance cell wall extensibility. *Annual review of Plant Physiology and Plant Molecular Biology*, **50**, 391-417.

Cosgrove, D. J. (2000a) Expansive growth of plant cell walls. *Plant Physiology and Biochemistry*, **38**, 109-124.

Cosgrove, D. J. (2000b) Loosening of plant cell walls by expansins. *Nature.*, **407**, 321-326.

Cosgrove, D. J. (2003) Expansion of the plant cell wall. *In:* Rose, J. K. C. (ed.) *The Plant Cell Wall.* Oxford: Blackwell Publishing Ltd.

Cosgrove, D. J., Bedinger, P. & Durachko, D. M. (1997) Group I allergens of grass pollen as cell wall-loosening agents. *Proceedings of the National Academy of Sciences*, **94**, 6559-6564.

Cosgrove, D. J. & Durachko, D. M. (1994) Autolysis and extension of isolated walls from growing cucumber hypocotyls. *Journal of Experimental Botany*, **45**, 1711-1719.

Cosgrove, D. J., Li, L. C., Cho, H.-T., Hoffmann-Benning, S., Moore, R. C. & Blecker, D. (2002) The Growing World of Expansins. *Plant Cell Physiology*, **43** 1436-1444.

Cosgrove, D. J., McQueen-Mason, S., Guiltinan, M., Shcherban, T. & Shi, J. (1999) Proteins catalyzing the extension of plant cell walls. *United States Patent and Trademark Office.* USA.

Darley, C. P., Forrester, A. M. & McQueen-Mason, S. J. (2001) The molecular basis of plant cell wall extension. *Plant Molecular Biology*, **47**, 179–195.

Darvill, A. G., McNeil, M. & Albersheim, P. (1978) Structure of Plant Cell Walls: VIII. A New Pectic Polysaccharide. *Plant Physiology*, **62**, 418-422.

Dey, P. M. & Brinson, K. (1984) Plant Cell-Walls. *In:* Tipson, R. S. and Derek, H. (eds.) *Advances in Carbohydrate Chemistry and Biochemistry.* London: Academic Press.

Dey, P. M., Brownleader, M. D. & Harborne, J. B. (1997) The plant, the cell and its molecular components. *In:* Dey, P. M. and Harborne, J. B. (eds.) *Plant Biochemistry.* London: Academic Press.

Dey, P. M., Brownleader, M. D., Pantelides, A. T., Trevan, M., Smith, J. J. & Saddler, G. (1997) Extensin from suspension-cultured potato cells: a hydroxyproline-rich glycoprotein, devoid of agglutinin activity. *Planta*, **202**, 179-187.

Doco, T. & Brillouet, J.-M. (1993) Isolation and characterisation of a rhamnogalacturonan II from red wine. *Carbohydrate Research*, **243**, 333-343.

Edelmann, H. G. (1995) Water potential modulates extensibility of rye coleoptile cell walls. *Botanica Acta*, **108**, 374–380.

Evered, C., Majevadia, B. & Thompson, D. S. (2007) Cell wall water content has a direct effect on extensibility in growing hypocotyls of sunflower (*Helianthus annuus* L.). *Journal of Experimental Botany*, **58**, 3361-3371.

Ezaki, N., Kido, N., Takahashi, K. & Katou, K. (2005) The Role of Wall Ca2+ in the Regulation of Wall Extensibility During the Acid-induced Extension of Soybean Hypocotyl Cell Walls. *Plant and Cell Physiology*, **46**, 1831-1838.

Faik, A., Price, N. J., Raikhel, N. V. & Keegstra, K. (2002) An Arabidopsis gene encoding an Î±-xylosyltransferase involved in xyloglucan biosynthesis. *Proceedings of the National Academy of Sciences*, **99**, 7797-7802.

Fry, S. C. (1989) The Structure and Functions of Xyloglucan. *Journal of Experimental Botany*, **40**, 1-11.

Fry, S. C. (2000) *The growing plant cell wall: Chemical and metabolic analysis*, New Jersey, USA: The Blackburn Press.

Fry, S. C. (2001) Plant Cell Walls. *In: Encyclopedia of Life Sciences(ELS).* Chichester: John Wiley & Sons, Ltd.

Grobe, K., Becker, W.-M., Schlaak, M. & Petersen, A. (1999) Grass group I allergens (β-expansins) are novel, papain-related proteinases. *European Journal of Biochemistry*, **263**, 33-40.

Grobe, K., Poppelmann, M., Becker, W. M. & Petersen, A. (2002) Properties of group I allergens from grass pollen and their relation to cathepsin B, a member of the

C1 family of cysteine proteinases. *European Journal of Biochemistry*, **269**, 2083-2092.

Guerriero, G., Fugelstad, J. & Bulone, V. (2010) What Do We Really Know about Cellulose Biosynthesis in Higher Plants? *Journal of Integrative Plant Biology*, **52**, 161-175.

Hayashi, T. (1989) Xyloglucans in the Primary Cell Wall. *Annual review of Plant Physiology and Plant Molecular Biology*, **40**, 139-168.

Hofte, H. (2001) A Baroque Residue in Red Wine. *Science*, **294**, 795-797.

Ishii, T. (2006) Rhamnogalacturonan II- Borate complex in plant cell wall. *In:* Hayashi, T. (ed.) *The science and lore of the plant cell wall Biosynthesis, structure and function.* Florida: BrownWalker Press.

Ishii, T. & Matsunaga, T. (1996) Isolation and characterization of a boron-rhamnogalacturonan-II complex from cell walls of sugar beet pulp. *Carbohydrate Research*, **284**, 1-9.

Ishii, T., Matsunaga, T. & Hiyash, N. (2001) Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness of pumpkin tissue. *Plant Physiology*, **126**, 1698 - 1705.

Ishii, T., Matsunaga, T., Pellerin, P., O'Neill, M. A., Darvill, A. & Albersheim, P. (1999) The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. *Journal of Biological Chemistry*, **274**, 13098-13114.

Islam, A. (2006) Production of composites of *Acetobacter* cellulose with other biopolymers*. MSc. Thesis*, University of Westminster, London.

Jackson, P. A. (2011) An analysis of artificial cell wall composites produced with the Acetobacter system for their capacity to bind the grapevine extensin, GvP1. *Unpublished observation*

Jarvis, M. C., Briggs, S. P. H. & Knox, J. P. (2003) Intercellular adhesion and cell separation in plants. *Plant, Cell & Environment*, **26**, 977-989.

Jarvis, M. C. & McCann, M. C. (2000) Macromolecular biophysics of the plant cell wall: concepts and methodology. *Plant Physiology and Biochemistry*, **38**, 1-13.

Johnson, K. L., Jones, B. J., Schultz, C. J. & Bacic, A. (2003) Non-enzymic cell wall (glyco)proteins. *In:* Rose, J. K. C. (ed.) *The Plant Cell Wall.* Oxford: Blackwell Publishing

Kaneko, S., Ishii, T. & Matsunaga, T. (1997) A boron-rhamnogalacturonan-II complex from bamboo shoot cell walls. *Phytochemistry*, **44**, 243-248.

Kawalleck, P., Schmelzer, E., Hahlbrock, K. & Somssich, I. E. (1995) Two pathogen-responsive genes in parsley encode a tyrosine-rich hydroxyproline-rich glycoprotein (hrgp) and an anionic peroxidase. *Mol Gen Genet*, **247**, 444-452.

Keegstra, K. (2010) Plant Cell Walls. *Plant Physiology*, **154**, 483-486.

Keegstra, K., Talmadge, K. W., Bauer, W. D. & Albersheim, P. (1973) The Structure of Plant Cell Walls: III. A Model of the Walls of Suspension-cultured

Sycamore Cells Based on the Interconnections of the Macromolecular Components. *Plant Physiology*, **51**, 188-197.

Keller, E. & Cosgrove, D. J. (1995) Expansins in growing tomato leaves. *Plant Journal*, **8**, 795-802.

Kieliszewski, M. J. & Lamport, D. T. (1994) Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *Plant Journal*, **5**, 157-172.

Kiyosawa, K. (2003) Theoretical and experimental studies on freezing point depression and vapor pressure deficit as methods to measure osmotic pressure of aqueous polyethylene glycol and bovine serum albumin solutions. *Biophysical Chemistry*, **104**, 171-188.

Knox, B. & Suphioglu, C. (1996) Environmental and molecular biology of pollen allergens. *Trends in Plant Science*, **1**, 156-164.

Kobayashi, M., Matoh, T. & Azuma, J. (1996) Two Chains of Rhamnogalacturonan II Are Cross-Linked by Borate-Diol Ester Bonds in Higher Plant Cell Walls. *Plant Physiology*, **110**, 1017-1020.

Li, L.-C. & Cosgrove, D. J. (2001) Grass group I pollen allergens (β-expansins) lack proteinase activity and do not cause wall loosening via proteolysis. *European Journal of Biochemistry*, **268**, 4217-4226.

Liepman, A. H., Wightman, R., Geshi, N., Turner, S. R. & Scheller, H. V. (2010) Arabidopsis – a powerful model system for plant cell wall research. *The Plant Journal*, **61**, 1107-1121.

Lockhart, J. A. (1965) An analysis of irreversible plant cell elongation. *Journal of Theoretical Biology*, **8**, 264-275.

Matoh, T. (1997) Boron in plant cell walls. *Plant and Soil*, **193**, 59-70.

Matoh, T., Takasaki, M., Kobayashi, M. & Takabe, K. (2000) Boron Nutrition of Cultured Tobacco BY-2 Cells. III. Characterization of the Boron-Rhamnogalacturonan II Complex in Cells Acclimated to Low Levels of Boron. *Plant and Cell Physiology*, **41**, 363-366.

Matoh, T. r., Ishigaki, K.-i., Mizutani, M., Matsunaga, W. & Takabe, K. (1992) Boron Nutrition of Cultured Tobacco BY-2 Cells. *Plant and Cell Physiology*, **33**, 1135-1141.

Matoh, T. r., Ishigaki, K.-i., Ohno, K. & Azuma, J.-i. (1993) Isolation and Characterization of a Boron-Polysaccharide Complex from Radish Roots. *Plant and Cell Physiology*, **34**, 639-642.

McCann, M. C. & Roberts, K. (1991) Architecture of the primary cell wall. *In:* Lloyd, C. W. (ed.) *The Cytoskeletal Basis of Plant Growth and Form.* London: Academic Press.

McCann, M. C., Wells, B. & Roberts, K. (1990) Direct visualization of cross-links in the primary plant cell wall. *Journal of Cell Science*, **96**, 323-334.

McNeil, M., Darvill, A. G., Fry, S. C. & Albersheim, P. (1984) Structure and Function of the Primary Cell Walls of Plants. *Annual Review of Biochemistry*, **53**, 625-663.

McQueen-Mason, S. & Cosgrove, D. J. (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proceedings of the National Academy of Sciences*, **91**, 6574-6578.

McQueen-Mason, S., Durachko, D. M. & Cosgrove, D. J. (1992) Two Endogenous proteins that induce cell wall extension in Plants. *Plant Cell*, **4**, 1425-1433.

McQueen-Mason, S. J. & Cosgrove, D. J. (1995) Expansin Mode of Action on Cell Walls - Analysis of Wall Hydrolysis, Stress Relaxation, and Binding. *Plant Physiology* **107**, 87-100.

Mohnen, D. (2008) Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, **11**, 266-277.

Monro, J. A., Penny, D. & Bailey, R. W. (1976) The organization and growth of primary cell walls of lupin hypocotyl. *Phytochemistry*, **15**, 1193-1198.

Murashige, T. & Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, **15**, 473 - 497.

Nonami, H. & Boyer, J. S. (1990a) Primary events regulating stem growth at low water potentials. *Plant Physiology*, **93**, 1601-1609.

Nonami, H. & Boyer, J. S. (1990b) Wall extensibility and cell hydraulic conductivity decrease in enlarging stem tissues at low water potentials. *Plant Physiology*, **93**, 1610-1619.

O'Neill, M. A., Eberhard, S., Albersheim, P. & Darvill, A. G. (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science*, **294**, 846-849.

O'Neill, M. A., Ishii, T., Albersheim, P. & Darvill, A. G. (2004) Rhamnogalacturonan II: Structure and Function of a Borate Cross-Linked Cell Wall Pectic Polysaccharide. *Annual Review of Plant Biology*, **55**, 109-139.

O'Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G. & Albersheim, P. (1996) Rhamnogalacturonan-II, a Pectic Polysaccharide in the Walls of Growing Plant Cell, Forms a Dimer That Is Covalently Cross-linked by a Borate Ester. IN VITRO CONDITIONS FOR THE FORMATION AND HYDROLYSIS OF THE DIMER. *Journal of Biological Chemistry*, **271**, 22923-22930.

O'Neill, M. A. & York, W. S. (2003) The composition and structure of plant primary cell walls. *In:* Rose, J. K. C. (ed.) *The Plant Cell Wall.* Oxford: Blackwell Publishing Ltd.

Passioura, J. B. (2001) Plant–Water Relations. *In: Encyclopedia of Life Sciences(ELS).* John Wiley & Sons, Ltd.

Passioura, J. B. & Fry, S. C. (1992) Turgor and Cell Expansion: Beyond the Lockhart Equation. *Functional Plant Biology*, **19**, 565-576.

Pauly, M., Albersheim, P., Darvill, A. & York, W. S. (1999) Molecular domains of the cellulose/xyloglucan network in the cell walls of higher plants. *The Plant Journal*, **20**, 629-639.

Pellerin, P., Doco, T., Vida, S., Williams, P., Brillouet, J.-M. & O'Neill, M. A. (1996) Structural characterization of red wine rhamnogalacturonan II. *Carbohydrate Research*, **290**, 183-197.

Pellerin, P. & O'Neill, M. A. (1998) The interaction of the pectic polysaccharide Rhamnogalacturonan II with heavy metals and lanthanides in wines and fruit juices. *Analusis*, **26**, 32-36.

Pereira, C. S., Ribeiro, J. M., Vatulescu, A. D., Findlay, K., Macdougall, A. J. & Jackson, P. A. (2011) Extensin network formation in Vitis vinifera callus cells is an essential and causal event in rapid and H2O2-induced reduction in primary cell wall hydration. *BMC Plant Biology*, **11**, 106.

Popper, Z. A. (2008) Evolution and diversity of green plant cell walls. *Current Opinion in Plant Biology*, **11**, 286-292.

Proseus, T. E. & Boyer, J. S. (2012) Calcium deprivation disrupts enlargement of Chara corallina cells: further evidence for the calcium pectate cycle. *Journal of Experimental Botany*, 1-6.

Rayle, D. L. & Cleland, R. E. (1992) The Acid Growth Theory of auxin-induced cell elongation is alive and well. *Plant Physiology*, **99**, 1271-1274.

Reiter, W. D. (2002) Biosynthesis and properties of the plant cell wall. *Current Opinion in Plant Biology*, **5**, 536-542.

Reiter, W. D., Chapple, C. & Somerville, C. R. (1997) Mutants of Arabidopsis thaliana with altered cell wall polysaccharide composition. *The Plant Journal*, **12**, 335-345.

Reuhs, B. L., Glenn, J., Stephens, S. B., Kim, J. S., Christie, D. B., Glushka, J. G., Zablackis, E., Albersheim, P., Darvill, A. G. & O'Neill, M. A. (2004) l-Galactose replaces l-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the l-fucose-deficient mur1 Arabidopsis mutant. *Planta*, **219**, 147-157.

Ridley, B. L., O'Neill, M. A. & Mohnen, D. (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, **57**, 929-967.

Rochange, S. F. & McQueen-Mason, S. J. (2000) Expression of a heterologous expansin in transgenic tomato plants. *Planta*, **211**, 583-586.

Ryden, P., Sugimoto-Shirasu, K., Smith, A. C., Findlay, K., Reiter, W.-D. & McCann, M. C. (2003) Tensile Properties of Arabidopsis Cell Walls Depend on Both a Xyloglucan Cross-Linked Microfibrillar Network and Rhamnogalacturonan II-Borate Complexes. *Plant Physiology*, **132**, 1033-1040.

Saxena, I. M. & Brown, R. M. (2005) Cellulose Biosynthesis: Current Views and Evolving Concepts. *Annals of Botany*, **96**, 9-21.

Scheller, H. V., Jensen, J. K., Sørensen, S. O., Harholt, J. & Geshi, N. (2007) Biosynthesis of pectin. *Physiologia Plantarum*, **129**, 283-295.

Scheller, H. V. & Ulvskov, P. (2010) Hemicelluloses. *Annual Review of Plant Biology*, **61**, 263-289.

Shcherban, T. Y., Shi, J., Durachko, D. M., Guiltinan, M. J., McQueen-Mason, S. J., Shieh, M. & Cosgrove, D. J. (1995) Molecular cloning and sequence analysis of expansins--a highly conserved, multigene family of proteins that mediate cell wall extension in plants. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 9245-9249.

Somerville, C. (2006) Cellulose Synthesis in Higher Plants. *Annual Review of Cell and Developmental Biology*, **22**, 53-78.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S. & Youngs, H. (2004) Toward a Systems Approach to Understanding Plant Cell Walls. *Science*, **306**, 2206-2211.

Taiz, L. & Zeiger, E. (2002) *Plant Physiology*, Sunderland: Sinauer Associates.

Talbott, L. D. & Ray, P. M. (1992) Molecular size and separability features of pea cell wall polysaccharides : implications for models of primary wall structure. *Plant Physiology*, **98**, 357-368.

Taylor, K. A. & Buchanan-Smith, J. G. (1992) A colorimetric method for the quantitation of uronic acids and a specific assay for galacturonic acid. *Analytical Biochemistry*, **201**, 190-196.

Thomas, J. R., Darvill, A. G. & Albersheim, P. (1989) Isolation and structural characterization of the pectic polysaccharide rhamnogalacturonan II from walls of suspension-cultured rice cells. *Carbohydrate Research*, **185**, 261-277.

Thompson, D. S. (2001) Extensiometric determination of the rheological properties of the epidermis of growing tomato fruit. *Journal of Experimental Botany,* **52,** 1291 – 1301.

Thompson, D. S. (2005) How do cell walls regulate plant growth? *Journal of Experimental Botany*, **56,** 2275 – 2285.

Thompson, D. S. (2008) Space and Time in the Plant Cell Wall: Relationships between Cell Types, Cell Wall Rheology and Cell Function. *Annals of Botany*, **101**, 203–211.

Tibbits, C. W., MacDougall, A. J. & Ring, S. G. (1998) Calcium binding and swelling behaviour of a high methoxyl pectin gel. *Carbohydrate Research*, **310**, 101-107.

Vanzin, G. F., Madson, M., Carpita, N. C., Raikhel, N. V., Keegstra, K. & Reiter, W.-D. (2002) The mur2 Mutant of Arabidopsis thaliana Lacks Fucosylated Xyloglucan Because of a Lesion in Fucosyltransferase AtFUT1. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 3340-3345.

Veytsman, B. A. & Cosgrove, D. J. (1998) A Model of Cell Wall Expansion Based on Thermodynamics of Polymer Networks. *Biophysical Journal*, **75**, 2240-2250.

Vincken, J.-P., Schols, H. A., Oomen, R. J. F. J., McCann, M. C., Ulvskov, P., Voragen, A. G. J. & Visser, R. G. F. (2003) If Homogalacturonan Were a Side Chain of Rhamnogalacturonan I. Implications for Cell Wall Architecture. *Plant Physiology*, **132**, 1781-1789.

Ward, I. M. & Hadley, D. W. (1993) *An introduction to the mechanical properties of solid polymers*, Chichester: John Wiley & Sons.

Whitney, S. E. C., Gothard, M. G. E., Mitchell, J. T. & Gidley, M. J. (1999) Roles of Cellulose and Xyloglucan in Determining the Mechanical Properties of Primary Plant Cell Walls. *Plant Physiology*, **121**, 657-664.

Whitney, S. E. C., Jennifer, E. B., Arthur, H. D., Reid, J. S. G. & Michael, J. G. (1995) *In vitro* assembly of cellulose/xyloglucan networks: ultrastructural and molecular aspects. *The Plant Journal*, **8**, 491-504.

Whitney, S. E. C., Mike, J. G. & Simon, J. M.-M. (2000) Probing expansin action using cellulose/hemicellulose composites. *The Plant Journal*, **22**, 327-334.

Willats, W. G. T., McCartney, L., Mackie, W. & Knox, J. P. (2001) Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology*, **47**, 9-27.

Wilson, R. H., Smith, A. C., Kacurakova, M., Saunders, P. K., Wellner, N. & Waldron, K. W. (2000) The Mechanical Properties and Molecular Dynamics of Plant Cell Wall Polysaccharides Studied by Fourier-Transform Infrared Spectroscopy. *Plant Physiology*, **124**, 397-406.

Winzor, D. J. (2004) Reappraisal of disparities between osmolality estimates by freezing point depression and vapor pressure deficit methods. *Biophysical Chemistry*, **107**, 317-323.

Yennawar, N. H., Li, L.-C., Dudzinski, D. M., Tabuchi, A. & Cosgrove, D. J. (2006) Crystal structure and activities of EXPB1 (Zea m 1), a β-expansin and group-1 pollen allergen from maize. *Proceedings of the National Academy of Sciences*, **103**, 14664-14671.

Zsivanovits, G., MacDougall, A. J., Smith, A. C. & Ring, S. G. (2004) Material properties of concentrated pectin networks. *Carbohydrate Research*, **339**, 1317-1322.