Analysis of polysaccharides released by plant roots

By

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The candidate confirms that the work submitted is his own, expect where work, which has formed a part of jointly-authored publications has been included. The contribution of the candidate and other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within this thesis where reference has been made to the work of others.

In Chapter 4, liverworts were grown and maintained by Ms Bev Merry. The initial ELISA screen on liverwort agar gel extracts, and liverwort imaging and tissue printing was performed by Ms Sue Marcus. This contributed to Figure 4.15.

In Chapter 4, work leading to the monosaccharide composition and monosaccharide linkage analyses included in Tables 4.1 and 4.2, and Figures 4.16 and 1.7 was undertaken by Mr Bernhard Jaehrig based at the Complex Carbohydrate Research Centre, US. Data was analysed and summarised by the candidate.

This work is dedicated to my parents, Debra and Glen Galloway, who without their continual support this work would not be possible.

Abstract

Plant roots have a dynamic relationship with the surrounding soil, which forms a vital interface for the terrestrial biosphere. Without a strong interface with soil, plants could not extract the necessary resources needed for growth. As a part of a multifaceted strategy, plant roots release a variety of high and low molecular weight compounds into the soil. This exudate is believed to increase water and nutrient uptake, form the first barrier of defence, and aid in the symbiosis with fungi and bacteria. This investigation reports on the identity and biochemistry of the polysaccharides released from the roots of several crops and one basal land plant, and explores their possible functions. Crops were grown hydroponically in order to isolate the polysaccharides released by their roots. After growth, the hydroponic media were screened with a library of monoclonal antibodies (MAb). The MAbs revealed the presence of arabinogalactan-protein (AGP), extensin, xylan and xyloglucan. Signatures of these polysaccharides were also determined by monosaccharide linkage analysis. By using anion-exchange Epitope Detection Chromatography, polysaccharides released into the hydroponic medium of the crops were separated for further immunochemical analysis. This analysis demonstrated that the polysaccharides released by wheat were part of a multi-polysaccharide complex, Root Exudate Complex 1 (REC1). A similar polysaccharide complex, formed of AGP-xyloglucan (REC2) was also found to be released by liverworts, which were not previously known to secrete polysaccharides. Novel soil analytics were developed in this study to decipher the effects of polysaccharides released by roots on soil aggregate status. Tamarind seed xyloglucan, xylan from birchwood, and isolated REC1 from wheat were each demonstrated to increase the abundance of soil aggregates, with REC1 shown to be most effective. This increase in the abundance aggregates may help plants to bioengineer the rhizosphere resulting in increased uptake of resources required for growth.

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List of Abbreviations

AGP	Arabinogalactan-protein
AIR	Alcohol Insoluble Residue
ANOVA	Analysis of variance
APAP	Arabinoxylan Pectin Arabinogalactan-Protein
BG	Blue Green
brb	Bald Root Barley
BSA	Bovine serum albumin
CBM	Carbohydrate-binding Module
CDTA	Cyclohexane Diamine Tetraacetic Acid
Cv	Cultivar
CT	Computerised tomography
dH_2O	Deionised water
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
EDC	Epitope Detection Chromatography
ELISA	Enzyme Linked Immuno-Sorbent Assay
FITC	Fluorescein isothiocyanate
GalA	Galacturonic acid
GAX	Glucuronoarabinoxylan
GlucA	Glucuronic acid
GPS	Global Positioning System
HG	Homogalacturonan
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
JIM	John Innes Monoclonal
LM	Leeds Monoclonal
MAb	Monoclonal antibody
MAC	Monoclonal Antibody Centre
MP/PBS	Milk powder in phosphate buffered solution
MSD	Mass Selective Detector
m/z	Mass-to-charge
$NaIO_4$	Sodium periodate
NASA	National Aeronautics and Space Administration
NMR	Nuclear Magnetic Resonance
PBS	Phosphate buffered solution

Rha	Rhamnose
REC	Root Exudate Complex
RG	Rhamnogalacturonan
RT	Room temperature
SEM	Scanning Electron Microscopy
TMS	per-O-trimethylsilyl
V0	Void volume
XGA	Xylogalacturonan
XG	Xyloglucan
xxt	Xylosyltransferase

Chapter 1

Introduction

1.1 The root and soil environment

1.1.1 Role of root exudates

Plant roots are highly dynamic organs, which serve as the sole interface between the plant and the soil. The root-soil interface not only serves to gather water and nutrients for the plant but involves a complex series of interactions with soil-dwelling microbiota, and the soil itself. These below ground interactions are fundamental for maintaining soil structure, soil ecology and plant growth (Kibblewhite *et al*. 2008; Lehmann and Kleber 2015), which are vital to the terrestrial biosphere. As a part of these complex interactions with the soil, plant roots release a range of low and high molecular weight compounds into the soil, which include: organic acids, amino acids, phenolics, enzymes, deoxyribonucleic acid (DNA), proteins, sugars, and polysaccharides (Neumann and Romheld 2001; Bais *et al*. 2006; Dennis *et al*. 2010). The exuded organic compounds have been demonstrated to be widespread amongst plants. However, it is unknown what proportion each molecule makes of the total root exudate. This array of released molecules is known as root exudate (Oades 1978; Foster 1982; McCully 1999).

Root exudates form the basis of a multifaceted response that plants may use to bioengineer the surrounding soil known as the rhizosphere. Root exudates can alter rhizosphere chemistry to gain more nutrients for the plant by acidifying or changing the redox conditions through chelating agents such as phytosiderophores, which can liberate nutrients, particularly iron that are bound to soil particles. Root exudates can also aid in mycorrhizal fungi associations, increasing the uptake of phosphorous and nitrogen for plants (Haichar *et al*. 2014), sequester toxic metals (particularly Al^{3+}), and form the initial point of contact for invading pathogens before they enter the root body through degrading the cell walls (Badri and Vivanco 2009; McNear 2013; Baetz and Martinoia 2014). Furthermore, exudates released by a competitor plant have been shown to induce defence mechanisms in other species through genetic cues (Bais *et al*. 2006; Semchenko *et al*. 2014). However, the identity of these genetic cues and how they are recognised by the plant remain undetermined. Through scattered evidence, the organic substances and their proportions within root exudate are thought to be unique to a particular plant species, reflecting the biochemistry of their cells (Bacic *et al*. 1986; Moody *et al*. 1988; McCully and Sealey 1996; Read and Gregory 1997; Narasimhan *et al.* 2003; Biedrzycki *et al*. 2010).

Root exudates are believed to be released by two mechanisms, the first mechanism, is the passive transportation of exudates across the cellular

membranes of the root cells into the surrounding soil due to osmotic differences between the soil and the cell (Bais *et al*. 2006). The second mechanism is through pre-programmed detached root cells, border and border-like cells (Driouich *et al*. 2013; Mravex *et al*. 2017). These border cells detach as the root caps penetrate through the soil and remain alive for up to 90 days, secreting root exudates independently of the root body (Miyasaka and Hawes 2001; Driouich *et al*. 2013). The exudates from these cells can blanket the root caps and protect them from friction as they burrow deeper into the soil (Figure 1.1; Ray *et al*. 1988; Bengough and McKenzie 1997; McCully 1999). Border cells are also at the forefront of the defence network of the plant. These cells can form cellular traps with actively released DNA that attract pathogens, engulfing them before invading the root body (Hawes *et al*. 1998; Gunawardena and Hawes 2002).

1.1.2 Role of root mucilage

Polysaccharides, long repeating subunits of sugars, and glycoproteins, proteins encapsulated in polysaccharide, are released by roots and form the high molecular weight components of root exudate. These components relate to the cell wall components of the root body. Released polysaccharides and glycoproteins form a thick mucilaginous layer surrounding the root caps and tips of plants (Schwartz 1883; Chaboud 1983; Bacic *et al*. 1986; Moody *et al*. 1988). These released polysaccharides and glycoproteins are collectively referred to as root mucilage. This root mucilage can be visualised as it forms a viscous droplet on exposed root caps and tips (Guinel and McCully 1986; Bacic *et al*. 1986). No other root structures have been reported to be involved in the release of these components. The mechanisms involved in the release of root mucilage from the root caps and tips remain unclear. The leading hypothesis is that they derive from sloughed off root cap and tip cells that lyse, releasing their cell wall components into the rhizosphere, due to continual friction whilst penetrating through soil (Read and Georgy 1997). As roots penetrate further into the soil, root caps are faced with an extraordinary amount of pressure (>7 kg/cm^2; McNear 2013). Mucilage serves to lubricate the root caps to reduce the pressure faced by the caps as they penetrate through deeper layers of soil (Guinel and McCully 1986; Read and Gregory 1997; Figure 1.1).

Similar to that of the low molecule weight compounds of root exudate, root mucilage has also been implicated as a part of the defence network of plants, serving as a

bait molecule, attracting beneficial microorganisms and sequestering heavy metals including aluminium, cadmium and lead (Morel *et al*. 1986; Ray *et al*. 1988; Dennis *et al*. 2010). Root mucilage that envelops root caps and tips also provides protection from desiccation, ensuring that they remain hydrated (Greenland 1979; McCully and Sealey 1996). It has even been proposed that root mucilage could bind soil particles together to form aggregates (Ray *et al*. 1988; Morel *et al*. 1991; Traore *et al*. 2000). This aggregation effect strengthens and maintains the root-soil interface otherwise known as the rhizosheath, which in turn increases water and nutrient acquisition for the plant, and enhances soil quality by increasing water infiltration and soil pore size, and thus aeration (Oades 1984; Hartnett *et al*. 2012; Sun *et al*. 2015). Some species of grass have been demonstrated to have some regulation over the thickness of their rhizosheath. During periods of drought some grasses can increase the thickness of their rhizosheath in an attempt to secure the uptake of water from the soil (Hartnett *et al.* 2012).

Research has alluded to the identity of the polysaccharides present within the root mucilages of several species including maize (*Zea mays*), wheat (*Triticum aestivum*), cowpea (*Vigna unguiculata*) and lupin (*Lupinus angustifolius*). These studies indicate the presence of arabinogalactan-protein (AGP), pectin, heteroxylan and xyloglucan within the root mucilages of the plants studied (Bacic *et al.* 1986; Moody *et al.* 1988; Guinel and Sealey 1996; Read and Gregory 1997). The release of these polysaccharides and glycoproteins consumes a large proportion of the photosynthetically fixed-carbon by plants. Research suggests that these macromolecules can consume up to 40% of the photosynthetic production of a plant (Newman 1985; McNear 2013). Although, the proportion of photosynthetically fixed-carbon released as mucilage remains controversial with numbers ranging between 10% and 40% (Walker *et al*. 2003; Jones *et al*. 2007). The release and amount of mucilage is thought to be dependent on a plethora of factors including; species, climate, nutrient availability, and the biological, chemical and physical properties of the local soil (Miki *et al*. 1980; Jones *et al*. 2009). There are many unanswered questions about the released polysaccharides and glycoproteins, and it is an area of research which is neglected as it is a hidden process of a plant, despite the high energy demand of releasing these molecules.
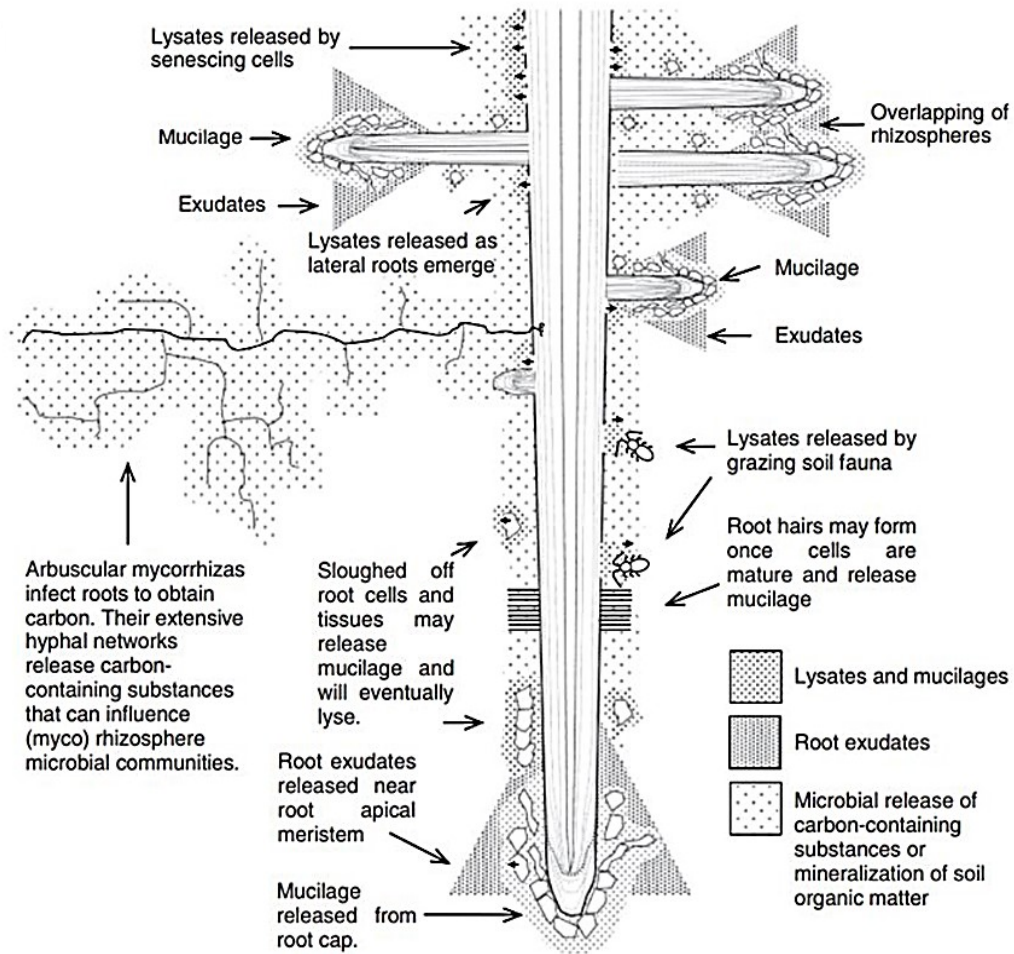
Figure 1.1 | **Overview of the complex interactions of the rhizosphere, taken from Dennis *et al.* 2010**
Roots release an array of low and high molecular weight substances, known as root exudate, which may be involved in nutrient acquisition, plant defence, attraction of beneficial microbiota, and the modification of the structure of the rhizosphere. Some of the released polysaccharides and glycoproteins form a thick gel surrounding root caps and tips, known as root mucilage.

1.2 Related-cell wall components of released polysaccharides

1.2.1 The plant cell wall

Cell walls maintain the structural integrity of plant cells (Albersheim *et al*. 2010), and act as a decentralised skeleton which maintains the integrity of plant's shape, and prevents it from collapsing under its own weight. Cell walls that are formed of a highly heterogeneous combination of polysaccharides (Figure 1.2; Bacic *et al*. 1988; Keegstra 2010; Fry 2011). There are two types of cell walls present in plants, primary and secondary. The primary cell walls are a flexible barrier that encapsulates each cell within plants. Primary cell walls provide mechanical support to each cell, maintain cell shape, regulate internal turgor pressure, pressure caused by water influx within the cell, and protect against pathogens, dehydration and other environmental factors (Somerville *et al*. 2004). The secondary cell wall, forms between the plasma membrane and primary cell wall of supporting tissues including sclerenchyma (fibrous cells), which develops after a cell has constructed its primary cell wall and has stopped expanding (Carpita and McCann 2000). Secondary cell walls provide mechanical support to many land plants. The secondary cell wall consists of cellulose microfibrils that form in parallel with non-pectic polysaccharides for instance, xylan and xyloglucan which crosslink with them (Carpita and McCann 2002). One of the key differences between primary and secondary cell walls is the inclusion of lignin, which is a phenolic that crosslinks with polysaccharides, making secondary cell walls less permeable to water and more rigid. Primary and secondary cell walls contain cellulose, non-pectic polysaccharides and pectin, although in different proportions (Carpita and McCann 2002; Albersheim *et al*. 2010).

In primary cell walls, cellulose microfibrils are extremely tough supporting structures which are crosslinked with non-pectic polysaccharides; examples include xylan, xyloglucan, heteromannan, and mixed-linkage glucans which are only found in Poaceae cell walls. These cellulose-non-pectic polysaccharides crosslinks form a strong interconnected framework (Cosgrove 2005; Knox 2008; Burton *et al*. 2010). This framework is embedded in a pectin matrix, which adds to the plasticity of cell walls (Figure 1.2; Cosgrove 2005). Pectin is a heterogeneous polysaccharide that is formed of four domains; homogalacturonan (HG), rhamnogalacturonan II (RG-II), xylogalacturonan, and rhamnogalacturonan I (RG-I). Pectin is rich with galacturonic acid making this complex macromolecule highly acidic. This acidity attracts calcium ions that bind the HG together, forming a gelatinous pectin matrix (Somerville *et al*.

2004). The outer layer of cell walls in plants are layered with pectin, forming the middle lamella, which acts like flexible gum, adhering neighbouring cells together and allowing them to stretch (Somerville *et al*. 2004; Albersheim *et al*. 2010). Cell walls are found across the entire plant kingdom with degrees of variation on the type and proportion of each polysaccharide.
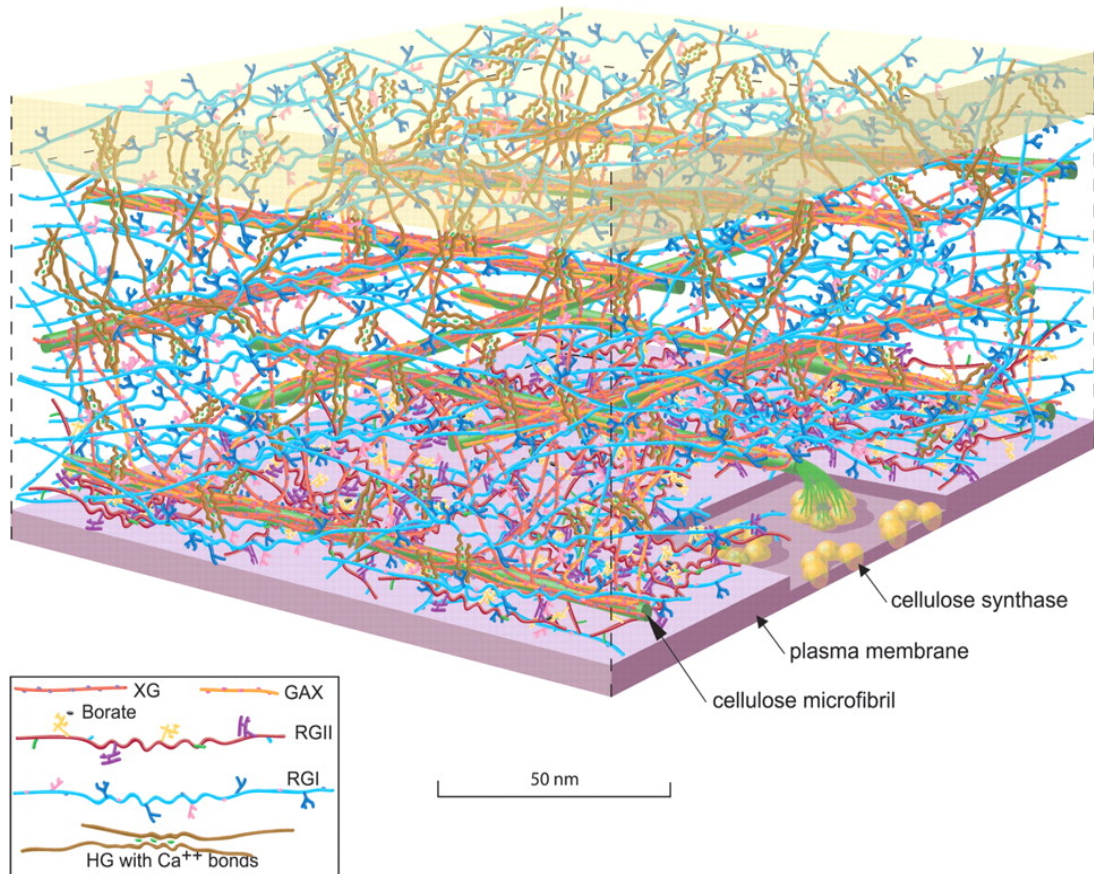
Figure 1.2 I **Current model of primary cell wall matrix, based on known Arabidopsis configuration, taken from Somerville *et al.* 2004**
Three major polysaccharides are present in primary cell walls, cellulose, non-pectic polysaccharides and pectin. Cellulose microfibrils are woven together to form microfibrils, which provide the tensile strength of the cell wall. The non-pectic polysaccharides, including xyloglucan (XG), form interwoven tethers linking the cellulose microfibrils together. This cellulose-non-pectic polysaccharide framework is embedded within a pectin matrix.

1.2.2 Major polysaccharides within plant cell walls

Cellulose is the most abundant polysaccharide on Earth. It is a ubiquitous polysaccharide, accounting for a third of the total mass of a typical plant (Somerville 2006). Cellulose is a homogenous polysaccharide formed of linear chains of many thousands of 1,4-β-linked glucose residues (Figure 1.3). Cellulose microfibrils are formed of cellulose chains that are hydrogen bonded in parallel to each other (Somerville *et al*. 2004; Zhang *et al.* 2017). Cellulose microfibrils are the key structural support of plant cell walls, and are found in numerous everyday products such as paper, textiles and insulation.

Non-pectic polysaccharides are a broad description of any non-cellulosic cell wall polysaccharides; for instance, heteroxylan, xyloglucan, heteromannan and mixed-linkage glucan. Heteroxylan is a heterogeneous polysaccharide, which has a backbone of 1,4-β-linked xylose residues (Figure 1.3). Heteroxylan can be in three main forms depending on the inclusion of arabinosyl or glucuronyl side chains, glucuronoxylan, arabinoxylan and glucuronoarabinoxylan (GAX). When heteroxylan has a single 1,2-α-linked glucuronic acid residue linked to the backbone, for about every 10 xylosyl residues, it is glucuronoxylan (Albersheim *et al*. 2010). Glucuronoxylan is a charged form of heteroxylan that is mainly found in the secondary walls of eudicotyledons such as in birchwood (Albersheim *et al*. 2010; Hao and Mohnen 2014). When heteroxylan has been substituted with α-L-arabinofuranose residues, attached at positions 2, 3 or 2,3 on the backbone, it is arabinoxylan. Arabinoxylan is particular abundant in the secondary cell walls of plants, and within the endosperm of cereal grains (Albersheim *et al*. 2010). GAX is similar to its counterpart arabinoxylan but includes a single glucuronic acid residue for every 5 to 6 xylosyl residues on its backbone (Albersheim *et al*. 2010; Hao and Mohnen 2014). GAX is only present within the cell walls of commelinid monocotyledons such as Bromeliads and Poaceae. The GAX in the secondary cell wall of Poaceae contain fewer side chains than the GAX found in primary cell walls, which is believed to result in a more robust GAX-cellulose interaction (Vogel 2008; Burton *et al*. 2010). Heteroxylan is a structural supporting polysaccharide within the cell wall, forming hydrogen bonds on cellulose microfibrils (Simmons *et al*. 2016).

Xyloglucan comprises of a backbone of 1,4-β-linked glucose residues carrying various side chains, such as 1-α-linked xylose, 1,2-β-linked galactose, 1,2-α-L-arabinofuranose. Within cereals xyloglucan can be fucosylated through a 1,2-α-linked L-fucose linked to a 1,2-β-linked galactose residue. The backbone, which is

the same as cellulose, is responsible for the strong crosslinking between xyloglucan and cellulose microfibrils within cell walls (Knox 2008; Scheller and Ulskov 2010; Burton *et al.* 2010; Figure 1.3). One feature of xyloglucan is that there is a unique regularity in the distribution of the side chains, which occurs in different species. This results in repetitive subunits on the structure of xyloglucan. This variation in branching patterns is denoted by a one letter code, for instance, XXXG represents three 1-α-linked xylose residues followed by one 1,4-β-linked glucose residue which are all linked to the 1,4-β-linked glucose backbone (Hayashi 1989; York *et al*. 1996; Figure 1.3). The primary cell walls of a range of eudicotyledons, non-cereal monocotyledons and gymnosperms contain fucosylated xyloglucans with a XXFGF structure. Whereas in Solananceae, which includes potato, fucose residues are rarely present but have short arabinofuranosyl side chains (Hayashi 1989; York *et al*. 1996; Albersheim *et al*. 2010).

Generally, xyloglucan does not carry a charge, however, there have been reports that demonstrate the presence of a charged form of xyloglucan within the cell walls of non-vascular land plants such as liverworts (Peña *et al*. 2008), and in the root hairs of Arabidopsis (Peña *et al.* 2012a). This rare xyloglucan has xylose side chains that contain a 1,2-β-linked galactosyluronic residue, which gives this xyloglucan a charge (Peña *et al.* 2012a). This galacturonic acid residue typically forms a xyloglucan structure where a 1,2-β-linked galacturonic acid residue is linked to a 1-α-linked xylose, which is in turn linked to the 1,4-β-linked glucose backbone. Within the root hairs of Arabidopsis a 1,2-α-linked L-fucose residue is able to link with the galactosyluronic residue (Peña *et al.* 2012a). Despite the variability within the structure of xyloglucan, xyloglucan has been demonstrated to be widespread throughout plants and is considered unique to plants (Fry 1989; Peña *et al*. 2008).

Mannans are a highly heterogeneous polysaccharide, which have a backbone of 1,4-β-linked mannose residues (Figure 1.3). This heteromannan can be in four forms, mannan only formed on 1,4-β-linked mannose residues, glucomannan, galactomannan and galactoglucomannan (Albersheim *et al*. 2010). Glucomannan consist of an 1,4-β-linked mannose and 1,4-β-linked glucose backbone. Glucomannan is a minor polysaccharide within the primary cell walls of eudicotyledons and cereals (Scheller and Ulskov 2010). In galactomannan there is a 1,4-β-linked mannose backbone which has side chains of 1,6-α-linked galactose. In galactoglucomannan the backbone consist of mannose and glucose residues, which has side chains of 1,6-α-linked galactose. These side chains of galactose residues are mostly linked to the mannose on the backbone but some galactose can

linked to the glucose on the backbone (Benova-Kaosova *et al*. 2006; Goubet *et al*. 2009). Within the secondary cell walls of gymnosperm there is a galactose: glucose: mannose ratio of 1:1:3 for the composition of heteromannan. Whereas, this ratio is 1:1 (glucose: mannose) within the primary cell wall of plants. Furthermore, the proportion of galactose is much lower in primary cell walls (Benova-Kaosova *et al*. 2006; Scheller and Ulskov 2010). Heteromannans are widely spread throughout the plant kingdom, functioning as a structural support as well as a means of storage (Goubet *et al*. 2009).

Mixed-linkage glucans are only found in cereals and ferns. Mixed-linkage glucans are formed of a mixture of 1,4-β-linked and β-1,3 linkage glucose residues (Woodward *et al*. 1983). Each segment of mixed-linkage glucan contains between two and three continuous 1,4-β-glucan residues attached by a single 1,3-β-glucan residue (Woodward *et al*. 1983; Buckerigde *et al.* 1999; Figure 1.3). These 1,3-β-glucan linkages twist the structure of the polysaccharide, which gives the mixed-linkage glucan more flexibility as it prevents the aggregation of cellulose hydrogen bonds. These linkages also increase the solubility of mixed-linkage glucan compared to a continuous chain of 1,4-β-glucan residues, which forms cellulose (Kiemle *et al*. 2014; Figure 1.3). Mixed-linkage glucans are most abundant in the cell walls of the endosperm, and young tissues (Kiemle *et al*. 2014).

Pectin is a complex heterogeneous macromolecule formed of four domains: HG, xylogalacturonan, RG-I and RG-II. Pectin is a highly acidic polysaccharide due to the backbone containing an abundance of 1,4-α-linked galacturonic acid residues (Caffall and Mohnen 2009; Figure 1.3). The ratios of the pectin domains is highly variable, with HG being the most abundant, constituting typically 65% of the total polysaccharide, RG-I constitutes between 20% and 35%, RG-II and xylogalacturonan each constituting less than 10% (Zandleven *et al*. 2007; Mohnen 2008). HG consists of a backbone of 1,4-α-linked galacturonic acid residues. A number of galacturonic acid residues can be methyl esterified on the carboxyls or acetylated on the hydroxyls (Carpita and McCann 2000; Figure 1.3). The backbone of galacturonic acids enables HG to form gels by crosslinking divalent ions such as calcium. Modifications, methyl and acetyl esterification, influence the gelling properties of pectin by lowering the density of the gels (Cosgrove 2005). Similar to HG, xylogalacturonan is formed of a backbone of 1,4-α-linked galacturonic acid with side chains of 1,3-β-linked xylose attached to the hydroxyl of the galacturonic acid residues (Zandleven *et al*. 2007; Mohnen 2008; Figure 1.3).

RG-I consists of a backbone of alternating 1,2-α-linked rhamnose and 1,4-α-linked galacturonic acid residues. RG-I is a highly branched polysaccharide with many terminal residues of galactosyl and arabinosyl residues on the hydroxyls of the terminal rhamnosyl residue (Figure 1.3). The 1,4-α-linked galacturonic acid backbone is frequently acetylated on the hydroxyls (Moller *et al.* 2008; Verhertbruggen *et al.* 2009). The backbone of RG-I can also be substituted on the C-4 position with 1,5-α-linked arabinans, 1,5-α-linked galactans or arabinogalactans (Carpita and Gibeaut 1993).

RG-II is considered to be the most complex component of the cell wall that consists of 12 monosaccharides linked by 20 different types of glycosidic linkages as determined by enzyme treatment (Ralet *et al.* 2008; Lee *et al.* 2013). RG-II typically consists from eight 1,4-α-linked galacturonic acid residues within its backbone. Some of the galacturonic acid on the backbone can be methylated (Figure 1.3). The number of side chains of RG-II is highly dependent on the species (Ishii *et al.* 1999; Harholt *et al.* 2010). Rare sugars including D-apiose are also present on RG-II, which makes RG-II hard to degrade by cell wall enzymes. RG-II is also a highly conserved structure of plant cell walls, which uniquely contains a borate (BO_3) that gives RG-II the ability to link with other RG-II (Kobayashi *et al.* 1996; Roach *et al.* 2012). Pectin is a gelling agent that is primarily involved in forming the matrix of the cell wall. Commercial uses of pectin vary but take advantage of its gelling properties, and are used as binding agents in food including jams (Crandall and Wicker 1986).

Cell walls also contain various proteins, which have enzymatic or structural roles. Cell wall proteins have many functions including signalling, remodelling or constructing the architecture of the cell wall, and defence (Albersheim *et al*. 2010). Structural cell wall proteins can be classified into three groups based on their amino acid content, glycine-rich proteins, proline-rich proteins and hydroxyproline-rich proteins. One group of proteins, AGPs are rich in both proline and hydroxyproline (Clarke *et al*. 1979; Kieliczewski 2001). AGPs are typically formed of 90% glycan and 10% protein (Bacic *et al.* 1997). The protein domain is typically heavily glycosylated and varies in length between 5 KDa and 30 KDa, and rich in alanine and hydroxyproline amino acids (Bacic *et al*. 1997; Kieliczewski 2001). The glycan domain of AGP is formed of arabinogalactan that is O-linked to the hydroxyproline residues in the protein core. The glycan backbone of AGP is typically formed of 1,3 and 1,6-β-linked galactose residues with side chains of a single 1,6-β-linked galactose that holds 1,3-α-linked L-arabinose residues, 1,6-β-glucuronic acid

residues and a single 1,4-α-linked L-arabinose residues (Ellis *et al*. 2010; Figure 1.3). The presence of glucuronic acids can vary between species making the glycoprotein more charged, for instance, the AGP in gum Arabic is rich in glucuronic acid making the polysaccharide highly acidic (Gane *et al*. 1995). AGPs have a variety of functions from cell differentiation, reproduction, cell signalling and programmed cell death (Bacic *et al*. 1997; Kieliczewski 2001; Cosgrove 2005; Pereira *et al*. 2013).

Another example of a structural cell wall protein are extensins, which are a type of hydroxyproline-rich proteins, which contain low amounts of repeating serine amino acids (Borner *et al*. 2002). These chains of repeating one serine and four hydroxyprolines form a rod-like shape of extensin. Extensin are glycosylated proteins with short (3-4 residues) repeating chains of 1,4, 1,2 1,2 and 1-3-α-linked L-arabinose residues that are attached to the hydroxyprolines. Attached to the serine is a single 1,3-β-linked galactose residue (Borner *et al*. 2002; Figure 1.3). Extensins are widespread throughout the plant kingdom, and are a key component that is responsible for cell wall rigidity. Production of extensins can be induced as a wounding response (Hirsinger *et al*. 1997).
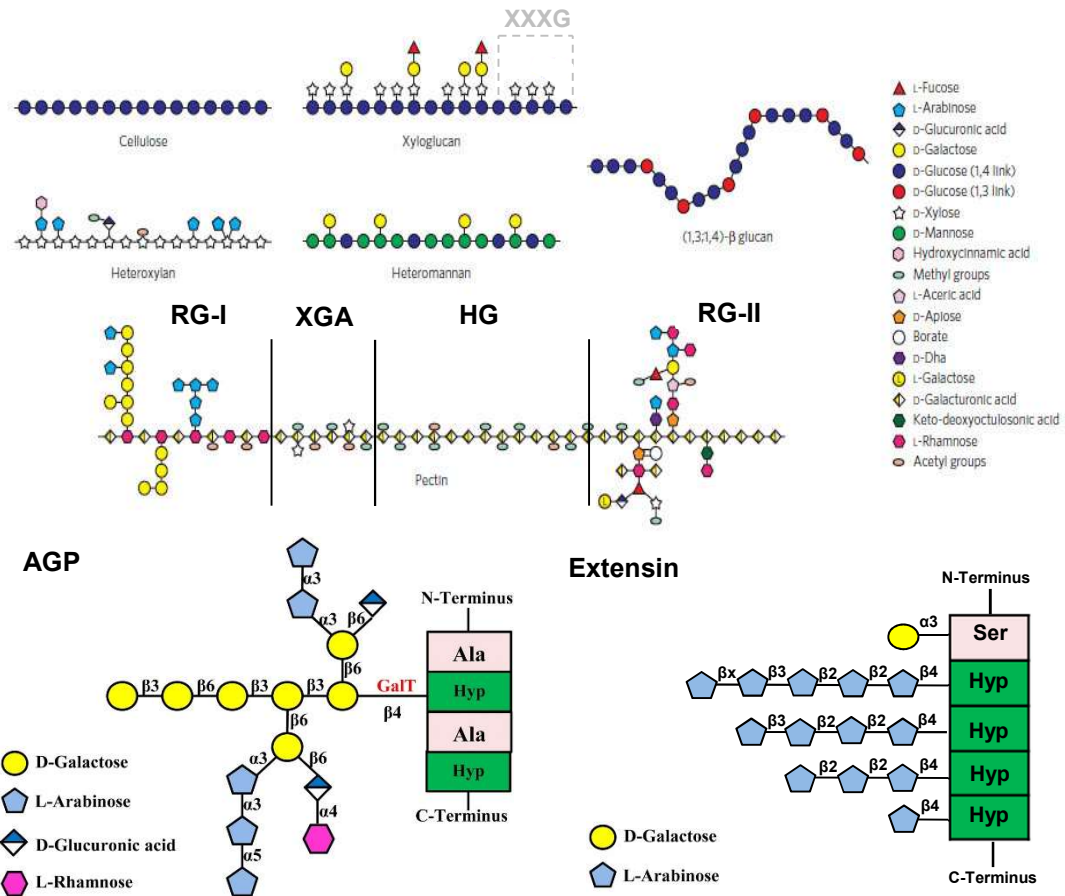
Figure 1.3 I **Schematic of the major cell wall polysaccharides, adapted from Burton *et al*. 2010 and Liang *et al*. 2010**

The major macromolecules of the cell wall can be placed into four categories: cellulose, non-pectic polysaccharides, pectin and glycoprotein. Cellulose is formed of a 1-4-β-D-glucan backbone. When the backbone of cellulose has been decorated with xylose, galactose and fucose residues it forms the non-pectic polysaccharide, xyloglucan. When the backbone of cellulose is twisted by having a 1-3-β-D-glucan substitute, forming mixed-linkage glucan. Xylan is a heterogeneous non-pectic polysaccharide, which generally has a backbone formed of 1-4-β-D-xylose with residues of arabinose and glucuronic acid residues. Hetromannan is another heterogeneous non-pectic polysaccharide that generally has a backbone of mannose and glucuronic acid residues that is decorated with galactose residues. Pectin is a complex macromolecule formed of four domains, RG-I, HG, xylogalacturonan (XGA) and RG-II. The backbone of pectin is rich in galacturonic acid residues. AGP are formed of a protein core rich in hydroxyproline that has been enclosed in a glycan rich in galactose, arabinose, glucuronic acid and rhamnose residues. Extensin is a rod shaped hydroxyproline-rich glycoprotein, which contains high amounts of arabinoses with one galactose residue.

Based on cell wall biochemistry and architecture, angiosperm or flowering plants can be placed into two basic categories, Type I and Type II. Type I primary cell walls are found in eudicotyledons and non-commelinid monocotyledons, which include the families of Solananceae and Brassicaceae. Type I cell walls contain cellulose microfibrils and high amounts of xyloglucan crosslinks (Figure 1.2; Carpita and Gibeaut 1993), which are embedded within a pectic matrix (Ishii 1997). Type II cell walls, found in grasses such as Poaceae, which include wheat, oat (*Avena sativa*) and barley (*Hordeum vulgare*), contain cellulose microfibrils that are encased within a matrix of GAX and mixed-linkage glucans, with trace levels of xyloglucan crosslinks (Nishitani and Nevins 1989; Carpita 1996; Vogel 2008). In addition, Type II cell walls contain less pectin and glycoproteins such as AGP, and contain higher crosslinks between lignin and other polysaccharides, which form rigid interconnections when the cell has stopped expanding (Iijima *et al*. 1990; Burton *et al*. 2010). An example of a phenolic crosslink in Type II cell walls is feruloylated arabinoxylan. Feruloylated arabinoxylan is formed of arabinoxylan that has been decorated by ferulic acid, which mediates linkages between arabinoxylan chains and between arabinoxylan and lignin in grasses (Chateigner-Boutin *et al*. 2016).

1.2.3 Cell wall multi-polysaccharide complexes

The current view of the cell wall matrix is that the polysaccharides are separate structures that are tethered together non-covalently, forming a complex framework (Gibeaut and Carpita 1994; Keegstra 2010; Cosgrove and Park 2012). Recently, this view has begun to change, as some protein, non-pectic and pectic polysaccharides have been shown to form covalent complexes. This finding adds an additional layer of complexity to the cell wall architecture of plants. This type of bond can also form linkages with other polysaccharides, therefore, forming multi-polysaccharide complexes. Covalent attachments between polysaccharide-protein were initially hypothesized in the 1990s (Ryden and Selvendran 1990), but very little evidence had been uncovered until covalent bonds between xyloglucan-pectin (RG-I) were reported in Arabidopsis callus cells (Renard *et al.* 1997; Duan *et al*. 2004; Popper and Fry 2008). It was proposed that the negatively charged pectin domain of this xyloglucan-pectin complex promoted the integration of xyloglucan into the cell wall matrix (Popper and Fry 2008). After the synthesis of xyloglucan-pectin, within the Golgi apparatus, the complex remained highly stable, being transported to the cell wall through vesicles until its integration into the cell wall (Popper and Fry

2008). It was also observed that approximately 30% of this complex was released into the medium of the cells in the form of sloughed cell wall components (Popper and Fry 2008). However, the function of this complex remains unclear. It is assumed that the xyloglucan-pectin complex aided in cell wall assembly and acted as structural support within the cell wall. Potential linkages of xylan-pectin and xylan-AGP have also been reported when using immunochemical techniques (Cornuault *et al.* 2015). Another form of polysaccharide complex has also been described, where xylan could fold onto the surface of cellulose microfibrils through hydrogen bonding in a two-fold helical screw formation (Simmons *et al*. 2016). This folding, similar to the xyloglucan-pectin, is also assumed to contribute to the structural integrity of the cell wall.

Evidence for a covalently linked multi-polysaccharide complex had not been detected until four years ago. Research using Arabidopsis (*Arabidopsis thaliana*) callus cells has uncovered a multi-polysaccharide complex, Arabinoxylan Pectin Arabinogalactan-Protein (APAP1; Tan *et al*. 2013), which was released into the liquid medium. This complex contains an AGP core with branching subdomains of covalently attached arabinoxylan and RG-I (Tan *et al*. 2013). The AGP core is acting as a cross-linker holding the pectic and arabinoxylan subdomains together (Tan *et al*. 2013). Within the cell wall, this crosslinking could provide a continuous network of cell wall polysaccharides and structural proteins, thereby increasing the strength of the cell wall. However, this multi-polysaccharide complex was only found to be a minor component of the cell wall when compared to the other polysaccharide components of the callus cells. On further analysis, the pectic and arabinoxylan subdomains were shorter within the complex that was exuded compared to the complex that was detected within the cell wall (Tan *et al*. 2013). This suggests that this multi-polysaccharide complex was tightly woven through hydrogen bonding within the fabric of the cell wall matrix. Although, the mechanisms underpinning how the subdomains are assembled into a complex, and the location of its assembly both remain undetermined. One hypothesis suggests that the subdomains are formed in the Golgi apparatus and translocated to the plasma membrane where it is assembled before being integrated into the cell wall matrix (Tan *et al*. 2013).

1.3 Plant cell wall polysaccharide analysis

1.3.1 Monosaccharide linkage analysis

For monosaccharide linkage analysis to be undertaken, polysaccharides within a sample are methylated, typically using methyl iodide, uncovering all open hydroxyls on the polysaccharide (Lisec *et al*. 2006). The sample is then hydrolysed using a strong acid, trifluoroacetic acid, which breaks down the glycosidic linkages holding the polysaccharide together (Björndal *et al*. 1970; Lindberg 1972), thereby, generating monosaccharides. The hydroxyls that were involved in the glycosidic linkages remain unmethylated. The free monosaccharides are then reduced, typically with sodium hydroxide, to open their rings. Once reduced, these open rings are then acetylated with the unmethylated hydroxyl groups, which were involved in linkage, forming stable acetyl groups (Hakan *et al*. 1970; York *et al.* 1985). The samples are then analysed by gas chromatography, liquid chromatography or gel chromatography that is combined with mass spectrometry (Hakan *et al*. 1970; Pettolino *et al*. 2012). NMR can be used instead of mass spectrometry to explore in more detail the conformational structure within polysaccharides (Pettolino *et al*. 2012), rather than molecular weights. To identify monosaccharides linkages, the retention times from the chromatography separation are combined with the molecular weights determined from mass spectrometry (Bauer 2012; Pettolino *et al*. 2012). Altogether, the hydroxyls linking the polysaccharide and types of glycosidic links can be determined, and therefore, the identity of the polysaccharide can be inferred (Björndal *et al*. 1970; Lisec *et al*. 2006; Pettolino *et al*. 2012). Using this established technique, many studies have determined the identity of the polysaccharides released by plant roots and within the cell walls of many species. However, fully understanding the biochemistry of plant cell walls using monosaccharide linkage analysis is challenging and time consuming. Moreover, these physio-chemical methods require large amounts of material for analysis, typically a minimum of 1 mg is needed (Moller *et al.* 2008; Pattathil *et al.* 2012). This can be a limiting factor of deciphering smaller components of the cell wall.

1.3.2 Monoclonal antibodies and Carbohydrate Binding Modules

A complementary approach to detect a range of cell wall-related polysaccharides and glycoproteins are monoclonal antibodies (MAb) and carbohydrate-binding modules (CBMs), which are highly specific and sensitive molecular probes. When a foreign substance or antigen (for example bacteria) enters an animal's body, the white blood cells or lymphocytes within the lymphatic system, which acts like a blood filter, detects the antigen and in response increases the production of antibodies (Medzhitov and Janeway 1997). These antibodies recognise and bind to the foreign substance for other lymphocytes to target them (Willats *et al.* 2002).

MAbs used to detect cell wall polysaccharides are cloned from a single lymphocyte (removed from the spleen which stores them) that generates antibodies in response to a polysaccharide (antigen) that has been injected into an animal such as mice or rat (Willats *et al.* 2000; Willats *et al.* 2002). The antibodies generated from these lymphocytes solely bind to one epitope of that antigen (Moller *et al*. 2008; Lee *et al*. 2011). An epitope is defined as a specific oligosaccharide motif on a polysaccharide, usually between 3 and 6 monosaccharides in length (Lee *et al*. 2011). For instance, the MAb LM2 binds to the epitopes of β-glucuronic acid which is present on AGP (Smallwood *et al*. 1996; Yates *et al*. 1996). These MAbs are highly versatile, and are used within many *in situ* and *in vitro* immuno-assays as diagnostic tools including: Enzyme Linked Immuno-sorbent Assay (ELISA), immunoblotting and chromatography. There is also a large library of MAbs specific to many different types of glycan and glycoprotein available from a variety of sources including: Leeds Monoclonal (LM), John Innes Monoclonal (JIM), Complex Carbohydrate Research Centre Monoclonal (CCRCM) and Monoclonal Antibody Centre, Cambridge (MAC).

As an alternative to MAbs, CBMs are also highly specific molecular probes, which derive from microbial cell wall polysaccharide hydrolases. These molecular probes are non-catalytic protein domains from bacterial carbohydrate-active enzymes such as glycoside hydrolases, which specifically bind to their target polysaccharide (Boraston *et al*. 2004; Knox 2008), comparable to that of MAbs. There is a diverse range of CBMs available on the Carbohydrate-Active enZYmes database that bind to many polysaccharides such as CBM2b1-2, specific to xylan (McCartney *et al.* 2006), CBM3a specific to xyloglucan and cellulose (Blake *et al.* 2006), which are used within many immuno-assays including ELISA, as with MAbs.

Most techniques that do not use MAbs or CBMs, such as gas chromatography and monosaccharide linkage analysis, require large amounts of material, between 1 mg and 10 mg for one run (Pettolino *et al.* 2012). This need for large amounts is problematic to dissecting small tissues or samples containing low concentrations of polysaccharides. These MAbs and CBMs are highly advantageous at exploring polysaccharides contained in low amounts within cell walls, and can be used on mass to generate cell wall profiles. Molecular probes are also widely used to dissect the architecture of cell walls. The cell walls of tissues can be sequentially solubilised using alkali treatments for example, sodium carbonate (Na_2CO_3) and potassium hydroxide (KOH) to release the components of the cell wall. Cation collators are also used to release polysaccharides within the cell walls that are bound by cations for instance, Cyclohexane Diamine Tetraacetic Acid (CDTA) binds to Ca^{2+} that holds pectic polysaccharides (specifically HG) together (Cornuault *et al*. 2014; Pose *et al*. 2015). Upon treatment, either MAbs or CBMs can efficiently identify which polysaccharides have been released through immuno-based techniques including ELISA. Using the outlined technique, research that solubilised cell walls with CDTA and KOH uncovered potential linkages between pectin and xylan in potato tubers (*Solanum tuberosum*), and xylan and AGP in commercial oat grain cell walls (Cornuault *et al*. 2015).

MAbs and CBMs can also be used for *in situ* fluorescence labelling to reveal locations of specific polysaccharides within the cell wall (Knox 2008). This fluorescence labelling has enabled a plethora of studies exploring the architecture, composition, plasticity and re-modelling of the cell walls in many tissues including: leaves and stems (Hanford *et al.* 2003; Blake *et al.* 2006; Verhertbruggen *et al.* 2009; McCartney *et al*. 2009), pollen (van Aelst and van Went 1992), fruits (Ordaz-Ortiz *et al.* 2009) and roots (Willats *et al*. 2004) within a wide range of species. These MAbs have enabled researchers to reveal where each major polysaccharide is within the cell wall matrix with precision, which had previously been unavailable. Prior to the development of MAbs and CBMs, a limited number of dyes were used to localise cell wall components. However, these dyes were not as specific or quantitative as MAbs. For instance, Calcofluor white binds to 1-4-β-D-glucan which forms cellulose but this also forms the backbone of xyloglucan; as a result the dye could bind to both polysaccharides (Herth and Schnepf 1980). Consequently this led to difficulties interpreting their signals. As a direct result of the development of MAbs and CBMs, many new techniques have been established such as, high-throughput glycan microarrays. These microarrays use small dots of cell wall extract that are

placed onto nitrocellulose sheets, and are screened using a large library of MAbs or CBMs (Moller *et al*. 2008). This allowed the rapid identification of polysaccharides within an extract, which was previously unavailable. Additionally as these microarrays use MAbs or CBMs, small components of the cell wall could be assayed.

1.4 Profiles of polysaccharides released by roots

A limited number of studies examining polysaccharides released by plant roots suggest a close relationship between the polysaccharides present within the root body and polysaccharides released by roots (Bacic *et al.* 1986; Guinel and McCully 1986; Moody *et al*. 1988; Read and Gregory 1997). Investigations using monosaccharide linkage analysis have determined that wheat and maize released high amounts heteroxylan, and low amounts of xyloglucan and pectic polysaccharides (Chaboud, 1983; Chaboud and Mireille 1984; Bacic *et al*. 1986; Table 1.1). Whereas, cowpea cress (*Lepidium sativum*), and Indian rhododendron (*Melastoma malabathricum*) released trace amounts of xyloglucan, and high proportions of pectic polysaccharides including, RG-I (Moody *et al*. 1988; Sims *et al*. 2000; Watanabe *et al*. 2008). The released polysaccharides are similar to their respective eudicotyledons and grasses cell wall biochemistries (Vogel 2008). However, one investigation using callus cells of Timothy-grass (*Phleum pratense*) found that there was more arabinogalactan-protein (AGP) and xyloglucan exuded by cells compared to that of their cell wall (Sims *et al*. 2000). This differential in released and cell wall polysaccharides may be due to the undifferentiated state of the cells. It is probable that these polysaccharides would be released in similar proportions to that of the cell wall. However, there is no evidence that polysaccharides are released in equal proportions. Many studies have not directly detected these polysaccharides, and as a result they cannot calculate their proportions in and outside of the cell wall. For a summary of work to date see Table 1.1.

Prior to analysis, polysaccharides released from roots had to be isolated. There were three widely employed methods. One method involved teasing roots from the soil to collect root mucilage *in situ* over a number of days (Morel *et al*. 1986; Mounier *et al.* 2004). Another method involved growing seedlings (defined as ≤7 days old) on moist filter paper in the dark (McCully and Sealey 1996; Read and Gregory 1997; Narasimhan *et al*. 2003) or in the light (Ray *et al*. 1988) and directly

pipetting the mucilage that was secreted from the root caps of seedlings (Read and Gregory 1997). Alternatively, seedlings could be submerged in sterile liquid media and grown on (Bacic *et al*. 1986; Moody *et al*. 1988; Osborn *et al*. 1999). However, submerging seedlings in liquid medium may result in difficulties interpreting data as the whole plant has been submerged, not just the roots. Furthermore, plants grown using liquid culture must be agitated to oxygenate the medium. It has been documented that this agitation can damage plants particularly the leaves, which may release particular cell wall components such as extensin as a stress response (Smallwood *et al*. 1995). As yet, no research has used hydroponics to collect the polysaccharides released from the roots of plants.

Table 1.1 Released polysaccharides as detected by monosaccharide linkage analysis

Plant	Polysaccharide	Collection method	Reference
Maize	AGP, arabinoxylan, pectin, heteroxylan and xyloglucan	Liquid culture Moist filter paper Direct pipetting	Bacic *et al*. 1986 Guinel and McCully 1986 Moody *et al*. 1988 Read and Gregory 1997
Timothy-grass callus cells	AGP, Arabinoxylan, glucomannan and xyloglucan	Agar extract	Sims *et al*. 2000
Wheat	AGP, arabinoxylan, heteroxylan and xyloglucan	Liquid culture	Moody *et al*. 1988
Cowpea	AGP, arabinan and RG-I	Liquid culture Moist filter paper	Moody *et al*. 1988 Read and Gregory 1997
Arabidopsis	Pectin	Moist filter paper	Osborn *et al*. 1999
Cress	AGP, arabinan and pectin	Liquid culture Moist filter paper Agar extract	Moody *et al*. 1988 Ray *et al*. 1988 Osborn *et al*. 1999 Sims *et al*. 2010
Indian rhododendron	AGP	Liquid culture	Moody *et al*. 1990
Lupin	AGP, arabinan and RG-I	Moist filter paper	McCully and Sealey 1996 Read and Gregory 1997
Pea	AGP, pectin and xyloglucan	Moist filter paper	Narasimhan *et al*. 2003

1.5 Mechanisms involved in the release of polysaccharides and from roots

The mechanisms underpinning the release of polysaccharides and glycoproteins by roots have yet to be determined. However, there are two hypotheses that have been proposed. The first hypothesis proposes that released polysaccharides originate from lysed root cap cells (Read and Gregory 1997). As roots penetrate through the soil, root cap cells and tips are regularly lysed due to the continual friction that the soil exerts on the root caps. When the cells undergo lysis they release the polysaccharides that were contained within the cell wall matrix (Read and Gregory 1997; Iijima *et al*. 2002; Iijima *et al*. 2004). The second hypothesis proposes that the released polysaccharides are actively secreted through root cell walls (Guinel and McCully 1986). Plant polysaccharides are synthesised in the Golgi apparatus, and then are packaged into vesicles. The vesicles fuse to the plasma membrane and are deposited to the cell wall (Northcote and Pickett-Heaps 1966). The hypothesis suggests that the polysaccharides to be released travel through the cell wall matrix until they are secreted through specialised pores within the cell wall (Guinel and McCully 1986). However, no direct evidence for these specialised pores or the active secretion of polysaccharides has been observed. The active transportation of polysaccharides through this complex matrix would demand high amounts of energy. It has been shown that as roots penetrate through the soil; root cap cells are regularly lysed, thereby releasing their components which are made of polysaccharides that are sticky (Millar *et al*. 1989; Iijima *et al*. 2004). These gummy polysaccharides would then form the mucilage that surrounds the caps where they had originated (Bacic *et al*. 1986; Morel *et al*. 1987).

Root border cells are cells that are programmed to detach themselves from the root cap, and divert all their energy reserves into the production of released polysaccharides (Hawes *et al*. 1998; Cannsean *et al.* 2012; Mravec *et al*. 2017). Prior to their separation, the cement holding cell walls together, pectin, is degraded through the enzyme polygalacturonase (Driouich *et al*. 2007; Durand *et al*. 2009). The process of separation also loosens the cell wall matrix, resulting in the solubilisation of cell walls (Hadfield and Bennet 1998). It was proposed that the degradation of pectin (HG, XGA and RG-I) and arabinan within the middle lamella were involved in the detachment of cells on the root caps, which form root border cells (Willits *et al.* 2004; Mravec *et al.* 2017). It was also suggested that pectic polysaccharides could significantly contribute to root mucilage. Using a MAb that specifically binds to XGA (LM8; Willats *et al.* 2004), it was uncovered that XGA was secreted into the root mucilage of pea through either secretory vesicles or by

aggregates (Mravec *et al*. 2017). After detachment, root border cells continually form new polysaccharides to replace their cell wall matrix, which is continually being lost to the rhizosphere (Figure 1.1; Stephenson and Hawes 1994; Driouich *et al*. 2007; Driouich *et al*. 2013). Once root border cells become free, the enzymatic activity that resulted in their detachment continues to degrade attached cells. However, these activities are not as effective, which results in semi-detached cells with a loosened cell wall matrix (Hawes *et al*. 2002; Driouich *et al*. 2013). Once loosened, the weakened cells could release their components into the rhizosphere. The semi-detached root cap cells would then have to produce more polysaccharides, to ensure their structural integrity, to replace their cell wall matrix, which was being lost to the rhizosphere.

Some research indicates that polysaccharides are not solely released from the root caps and tips of plants. It was hypothesized that these polysaccharides are also released from the epidermal layers behind the root caps and tips (Guinel and McCully 1986). An AGP-specific binding reagent, β-glycosyl Yariv reagent, and an AGP-specific antibody, α-L-arabinofuranosyl were used to stain the roots of maize, and could bind to the root caps, tips and epidermal layers behind the caps and tips (Bacic *et al*. 1986). Perhaps, as the root caps penetrate through the soil, epidermal cells are also subjected to mechanical pressures but to a lesser amount. This idea was supported by the lower levels of AGP that were detected being exuded from the epidermal layers compared to the root caps and tips (McCully and Sealey 1996; Read and Gregory 1997). The areas (how far along the root epidermis) that are involved in the release of polysaccharide behind the caps and tips remain unknown. Abundant on the epidermal layer of roots are root hairs, which are single celled elongated outgrowths. The primary role for root hairs is to increase the surface area of roots to secure the rhizosheath, and to extract water and nutrients from the soil (Gilroy and Jones 2000; Hartnett *et al*. 2012; Huang *et al*. 2017). Root hairs have not been observed to release molecules such as cell wall-related polysaccharides; with no research being undertaken on root hairs with regards to exudation.

Despite the mechanisms underpinning secretion, pectic polysaccharides, specifically homogalacturonan, and AGP have been detected from the root caps of several crop species such as maize and wheat (Table 1.1; Bacic *et al*. 1986; Guinel and McCully 1986; Morel *et al*. 1987; Moody *et al*. 1988; Read and Gregory 1997). Within plants, pectic polysaccharides are involved with the adhesion of plant cells (Willats *et al*. 2004; Driouich *et al*. 2007); AGPs are also present on the surface of cells (Albersheim *et al*. 2010). Since both these polysaccharides are present on the

cellular surface, it is probable that they would be present in root mucilage. Pectic polysaccharides and AGP have a high water binding capacity that can form highly viscous gels within the cell wall matrix (Albersheim *et al*. 2010). Due to their gelling properties these polysaccharides are used as food additives to produce jams and gums. It is reasonable to suggest that these gelling agents result in the gelatinous properties of root mucilage.

1.6 Soil organic matter and aggregate status

1.6.1 Soil organic matter

Root mucilage is continually released into the rhizosphere. It is hypothesized that root mucilage can influence the abundance of soil aggregates (Metha *et al.* 1960; Tisdall and Oades 1982; May *et al*. 1993). This form of bioengineering ensures that the roots have a strong interface with the soil so that they can extract the necessary resources required for plant growth. Soil is a vital component of the terrestrial biosphere, which supports ecosystems that are crucial to all land-based life. Soil is a highly complex and dynamic substrate, which is estimated to contain between 10^{10} and 10^{26} microorganisms per gram (Prosser 2015). As well as holding a multitude of life, soil also supports all crop production that the world population depends on (Prosser 2015). Prior to land-dwelling life, the land would have been formed of fragmented rock caused by atmospheric chemical weathering, and mechanical processes (Huggett 1998; Bateman *et al*. 1998).

The structure and composition of soil is complex, and highly heterogeneous. Soil particles can be placed into three basic categories, clay particles (≤2 μm), silt particles (between 2 μm and 60 μm) and sand particles (between 60 μm and 2,000 μm; Day 1965). Various concentrations of these particles exist in nature, which generate different soil types that have distinctive characteristics (Brown 2008). For instance, if a soil contains a high proportion of sand particles, the soil becomes more efficient at draining excess water. However, this results in the soil being poor at retaining water (Day 1965). Plants grown in this soil type will require frequent irrigation compared to soils with high proportions of clay and silt particles, which retain more water but are less effective at draining excess water (Lal 1997). The smaller particles in soil including silt and clay, collectively have a greater surface area compared to larger particles such as sand. The larger surface area allows the smaller particles to hold onto higher amounts of water (Cheshire 1979; De Booth *et*

al. 1984; Brown 2008). Additionally, soils that contain higher amounts of aggregates have greater porosity. Soils with larger and more frequent pores have a greater capacity to bind to water due to the larger surface area, which would otherwise drain away (Lal 1997). This increase in water holding capacity is also influenced by the levels of organic matter within the soil. A larger amount of organic matter in soils also increases the retention of water (Kibblewhite *et al.* 2008). Having a high water holding capacity results in more water being available to plants.

One of the most complex facets of soil is the organic matter. One of the largest contributors of soil organic matter is from decaying plant matter such as leaves and roots. Other sources include excretions and decaying matter from animals and microbes (Kibblewhite *et al*. 2008; McNear 2013). These sources of organic matter significantly contribute to the stability of soils by gluing soil particles together forming aggregates (Cheshire 1979; De Booth *et al.* 1984; Ebringerová and Heinze 2000; Carrizo *et al.* 2015). Without this glue, the structure of the soil would breakdown and become easily eroded. The gradual decay of organic matter in soils is mostly caused by microbes, turning freshly fallen leaves into humus. Humus is the dark brown or black substance found in the upper layer of soil (0-30 cm depth), where the soil is very crumbly and biologically active (Brown 2008). Soil-dwelling invertebrates, including earthworms regularly churn up this humus with the mineral fragments of the soil. Humus has many beneficial aspects to soil, from maintaining the structure of soil through particle aggregation, thereby increasing porosity, water infiltration and nutrient cycling (Haynes and Francis 1993; Lal 1997; Kibblewhite *et al.* 2008).

The decomposition of biomass in soil by microorganisms results in the release of carbon dioxide due to microbial respiration. Only a small proportion of carbon is retained within soil through the formation of humus. Humus is highly resistant to decomposition, whereas freshly laid plant debris is more prone to decomposition (Cheshire 1979; De Booth *et al.* 1984; McNear 2013). As a result of this deposition and release of carbon, soils potentially hold a vast amount of carbon. Some estimates suggest there is up to 2,500 billion tonnes of carbon within soils, up to 80% of global organic carbon (Houghton 2007; Kibblewhite *et al*. 2008). This sink is increasingly under scrutiny to develop more accurate climate models and as a tool in climatic imitation strategies.

Humin is said to be the principal component of humus, which is a collective term used to describe a heterogeneous mixture of macromolecules, derived from

decaying organic matter in soils (Cheshire 1979; Cheshire 1990). Humin is said to be the most resistant organic substance within humus to breakdown. It is assumed that this humin derives from plant cell wall components of decaying matter (Haynes and Francis 1993; Lal 1997). However, this has not yet been demonstrated. Isolated humin, derived from alkali treatments (using KOH) of humus, could form aggregates between the mineral fragments of soil with organic matter, which were water stable (Metha *et al.* 1960; Chenu and Guerif 1989; Cheshire 1990). However, the term humin remains vague with no characterisation of the macromolecules within humin, or a standard method of isolation. Furthermore, the effects of humin in aggregating soil remains controversial with issues of repeatability in published work due to the heterogeneity of the humus sampled, and the resulting humin extracted (Lehmann and Kleber 2015).

1.6.2 Polysaccharide-derived soil aggregates

It has been proposed that decomposing organic matter from plant debris, bacterial, algal and fungal debris and secretions can form water stable aggregates in soil (Oades, 1984; Six *et al*. 1999; Carrizo *et al*. 2015). Polysaccharides are thought to be the adhesive binding soil particles together through weak hydrogen bonds between the carboxyls (negatively charged), and to a lesser degree the hydroxyls on the released molecules and the cations (typically Ca^{2+}, K^+ and Mg^{2+}) that adsorb to negatively charged surface of clay minerals (Figure 1.4; Foster 1981; Traore *et al*. 2000; Olsson *et al*. 2011). Clay particles (defined as: ≤2 µm) are formed from amorphous Fe and Al oxides and hydroxides, or from naturally occurring sheet-like structures formed from repeating silicate or aluminium oxide units (Greenland 1965; Fitz Patrick 1993). Without the presence of organic deposits, the binding of clay particles is reduced, highlighting the importance of organic residues in the formation of aggregates (Oades 1993; Rasse *et al*. 2005). In addition to hydrogen bonds, polysaccharides may even interact with clay particles through electrostatic bonds or Van der Waal interactions (Cheshire *et al.* 1985; Cheshire 1990). Once more polysaccharides become available, more clay particles can bind together forming microaggregates (defined as: ≤250 µm), which then adhere to other microaggregates and larger particles, forming larger more stable macroaggregates (defined as: ≥250 µm; Tisdall and Oades 1982; Vaughan and Malcolm 1985; Foster 1981; Tisdall 1994).
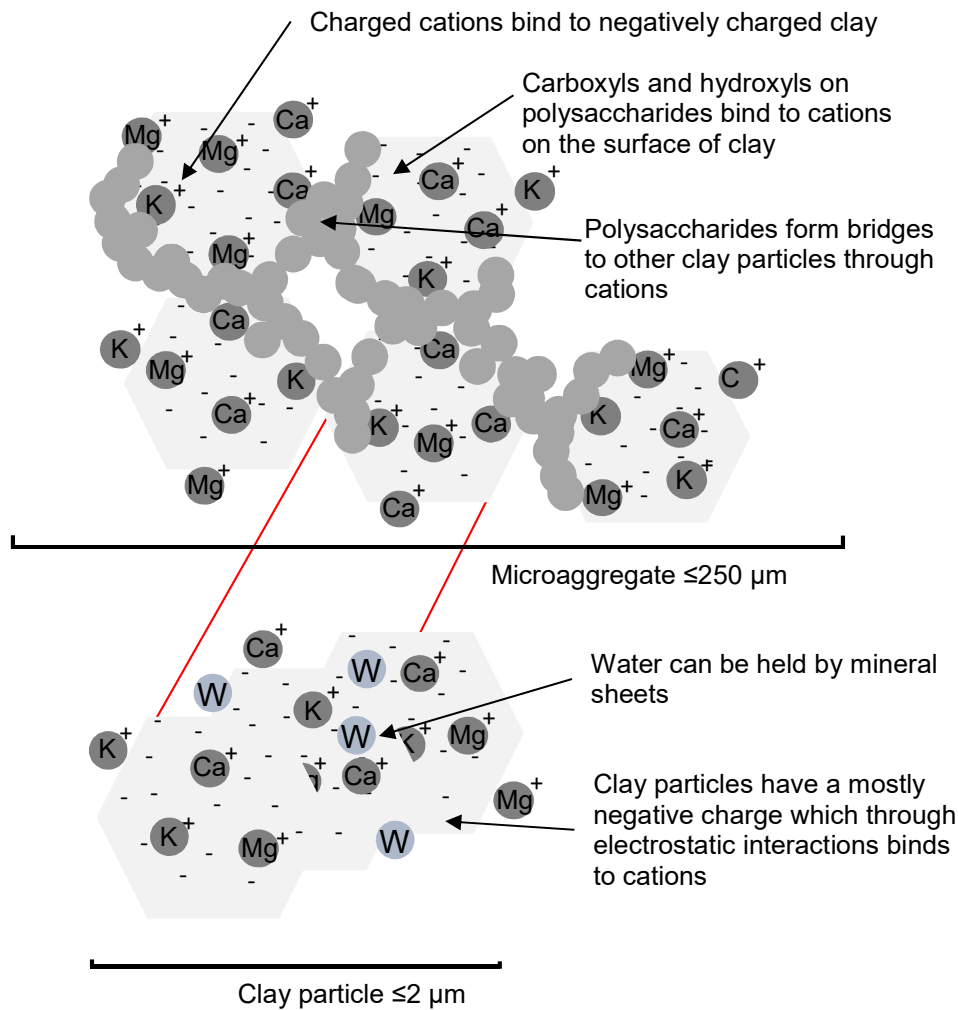
Figure 1.4 I **Schematic of polysaccharide-clay binding leading to the formation of microaggregates**
Clay particles have a mostly negative charge which attracts cations (typically Ca^{2+}, K^+ and Mg^{2+}) in soil through electrostatic interactions. These cations then form a bridge with the negatively charged carboxyl and hydroxyl groups present on polysaccharides released by plant roots. After sticking, polysaccharides bond other clay particles together, bridging the gaps between each particle; thus increasing soil particle aggregation.

Within most fertile soils, the presence of acidic polysaccharides is well documented (Edwards and Bremner 1967; Clapp and Davis 1970; Cheshire 1990). Acidic polysaccharides are more charged, containing higher levels of carboxyls, compared to their neutral counterparts, and therefore, possess a stronger capacity to bind to clay particles (Figure 1.4; Edwards and Bremner 1967; Cheshire 1990). This increases the capacity for polysaccharide-clay particle binding. Neutral polysaccharides, derived from bacterial exopolysaccharides have been shown to have weak capacity for polysaccharide-clay particle binding (Clapp and Davis 1970;

Cheshire 1990). Using transmission electron microscopy, these neutral polysaccharides, could readily bind to the flat surface of clay particles but did not contain any carboxyls for clay binding (Olness and Clapp 1973; Chenu and Guerif 1989). The stability of aggregates is thought to be proportional to the molecular weight of the polysaccharides (Cheshire 1990). The larger the molecule the more carboxyls there are to form more polysaccharide bridges that link clay particles, thus stabilising their attachment (Figure 1.4; Chenu and Guerif 1989).

Polysaccharide adherence to clay particles is believed to prevent enzymatic degradation by soil microorganisms (De Booth *et al*. 1984; Cheshire 1990). However, the carboxyls and hydroxyls that are not bound to the cations on clay particles may be vulnerable to enzyme degradation (Cheshire *et al*. 1983). This would reduce aggregate stability. The gradual degradation of the glycosidic bonds within the polysaccharides would correspond to a gradual loss of aggregate stability (De Booth *et al*. 1984). One of the key factors for polysaccharide-derived aggregates is the viscosity and the solubility of the polysaccharide. For aggregate formation to occur, polysaccharides within soils must be water-soluble so that they can diffuse to the clay particles.

Limited evidence has shown that root mucilage sourced directly from the root caps of maize *in situ* was able to form stable soil aggregates when added to fresh soil (Tisdall and Oades 1982; Morel *at al*. 1991; Watt *et al*. 1993). Once formed, these aggregates caused by maize root mucilage were found to be water stable, as determined by wet sieving. Wet sieving fractionates soil into defined sizes through sieves that are shaken and flooded with tap water (Brown 2008). When sodium periodate ($NaIO_4$), which oxidises the glycosidic bonds within polysaccharides, was introduced to the soil the isolated mucilage was rapidly degraded. There was also a significant reduction in the abundance of soil aggregates (Tisdall and Oades 1982). Other studies using a modelled form of root mucilage, polygalacturonic acid (commercial standard of pectin), uronic acid and glucose, have also demonstrated an increase in the abundance of soil aggregates (Metha *et al.* 1960; Reid and Goss 1981; May *et al*. 1993). However, this modelled root mucilage was less effective compared with the isolated maize root mucilage (Tisdall and Oades 1982; Morel *at al*. 1991; Watt *et al*. 1993). This suggests that as well as decaying matter, plants could be continually adding carbon into soils without the need for decay. The identities of the polysaccharides present within the isolated root mucilage were not determined nor the total glycan concentrations of each root mucilage collected. These initial investigations show a potential new source of carbon that could be

being continually released into soils. Although the proportion of a plant's carbon which is generated and then released into the soil remains hotly debated with some figures suggesting between 10% and 40% (Walker *et al*. 2003; Jones *et al*. 2007).

An investigation determined that cellulose, from decaying matter, was a strong source of soil macroaggregates (Mizuta *et al*. 2015). As cellulose is not charged (containing no carboxyls) contact between the hydroxyls, which have a weaker bind affinity to the cations on the silica sheets of clay, makes cellulose a poor microaggregator (Cheshire *et al*. 1979; Cheshire 1990). This suggests that not all polysaccharides released by decaying plant matter may be responsible for aggregate formation. The identity of polysaccharides released from decaying matter, which can increase micro- and macroaggregates remains undetermined. Furthermore, there has been no study to determine the binding affinities of the polysaccharides known to be released from plants, in the form of root mucilage, to clay particles. The only major known polysaccharides released by roots, in the form of root mucilage, are AGP and pectin (Bacic *et al*. 1986; Ray *et al*. 1998, Osborn *et al*. 1999). It may be possible that these polysaccharides are involved soil aggregation.

Soil microbiota such as bacteria, fungi and algae exude exopolysaccharides into the bulk soil, which is outside the influence of roots (Oades 1978; Tisdall and Oades 1982; Oades 1993). These microorganisms release a matrix of extracellular polysaccharide that they can also potentially aggregate soil mineral particles (Griffiths and Burns 1972; Tisdall 1994; Flemming and Wingender 2010). It is believed that the porosity caused by microaggregates in soil forms a refuge for microorganisms, which are vulnerable to predation (Rillig *et al.* 2015; Rillig *et al*. 2017). Additionally the interior of these microaggregates have been show to differ to that of the bulk soil, forming microenvironments favourable for microbe growth (Rillig *et al*. 2017. Therefore, it is in the interests of the microorganisms to maintain microaggregates.

It is hypothesized that microorganisms release exopolysaccharides so that they can adhere to microaggregates, and even help to form them as a secondary effect by binding to clay particles (Griffiths and Jones 1965; Griffiths and Burns 1972; Rillig 2004). Interestingly, microorganisms including rhziobacteria (*Rhizobium leguminosarum* and R. *fluorescens*) and arbuscular mycorrhizal fungi (*Glomus perpusillum*) have been demonstrated to use root mucilage isolated from maize and pea as a source of energy (May *et al*. 1993; Knee *et al*. 2001; Jones *et al.* 2000;

Gunina and Kuzyakov 2015; Sun *et al*. 2015). In effect these microorganisms were found to increase their production of exopolysaccharides when their medium supplemented with maize root mucilage. This increase in secretion could enhance the abundance of soil microaggregates.

There is only one clear example of an exopolysaccharides released from a microorganism that forms soil aggregates. The bacteria *Xanthomonas campestri* has been reported to release xanthan to form an extracellular matrix, adhering cells together in order to shuttle resources required growth (Czarnes *et al*. 2000; Chang *et al*. 2015). When xanthan, which is formed of long repeating subunits of glucose, mannose and glucuronic acid was collected from cultures of *Xanthomonas* and added to soil, it was demonstrated to increase the abundance of microaggregates (Chang *et al*. 2015). The increase in microaggregates was shown to be comparable to that of isolated root mucilage, modelled root mucilage, uronic acid and glucose which were also added to soil (Metha *et al.* 1960; Reid and Goss 1981; Tisdall and Oades 1982; Morel *at al.* 1991; May *et al.* 1993). Another similarity to root-derived aggregates is that bacterial-derived aggregates remain stable for weeks after the bacteria have undergone senescence (Griffiths and Jones 1965; Chang *et al*. 2015). This aggregation effect increases the uptake of water and nutrients from the soil to bacteria, as in the case of roots, and stabilises the microaggregates that bacteria inhabit (Cheshire 1979). Another example of an exopolysaccharide from a microorganism that has been shown to cause soil aggregate is the disputed glycoprotein glomalin (Wright and Upadhayya 1997). Some believe glomalin is secreted by the hyphae and spores of arbuscular mycorrhizal fungi (*Glomus* spp; Rillig 2004; Rillig *et al*. 2015). It is suggested that glomalin adheres the hyphae network to the surface of roots as well as the surrounding soil resulting in microaggregates (Rillig 2004; Spohn and Giani 2010; Rillig *et al*. 2015). However, glomalin has not been defined and nothing as yet is known about its component parts or structure.

1.7 Aims and objectives

To date, the hydroponic medium of plants has never been probed using MAbs. This investigation aims to develop an understanding of the biochemistry and function of polysaccharides released from the roots of crop species, with a focus on wheat (*Triticum aestivum*). A hydroponic system was developed in order to isolate the polysaccharides in preparation for immunochemical analysis. There are three key objectives that were followed throughout this investigation:

1. Identify the polysaccharides released from the roots of a variety of crops using a hydroponic system and monoclonal antibodies.
2. Study the biochemistry of the major polysaccharides released by crop species.
3. Determine possible functions of polysaccharides released by roots in relation to soil aggregate status.

Chapter 2

Materials and Methods

2.1 Plant materials

2.1.1 Growth of crop species prior to hydroponics

Wheat (*Triticum aestivum* L. cvs Avalon, Cadenza and Skyfall), maize (*Zea mays* L. F1 cv. Earlibird), barley (*Hordeum vulgare* L. cvs Golden Primrose and Pallas Andrew), pea (*Pisum sativum* L. cv. Avola), rapeseed (*Brassica napus* L. cv. Extrovert) and tomato (*Solanum lycopersicum* L. cv. Ailsa Craig) were initially grown in a Sanyo growth cabinet (MLR-352-PE; Sanyo, Japan). Seeds were not sterilised before planting. Seeds were grown in a mixture of vermiculite and perlite (50:50) for 7 days with a photoperiod of 16 h, a temperature of 22°C and an average light level of 634 µmol m^{-1} s^{-1}. Plants were watered every two days using 300 mL deionised water (dH$_2$O). Twenty seeds were assigned to one 10 cm plant pot. Seeds were evenly planted on the surface of the vermiculite and perlite, which was then covered with a 2 cm layer of substrate. Plant pots then were watered and placed into the growth cabinet. After 7 days, seedlings were placed into a glasshouse overnight to acclimatise.

2.1.2 Liverwort growth conditions and agar gel extraction

Liverwort growth and agar extraction was performed by Ms Bev Merry. Liverwort (*Marchantia polymorpha* L.) samples were obtained locally (GPS coordinates 53.804°N/1-.55517°E) in May 2016 by Dr Katie Field. After collection, liverworts were grown on 0.9% plant agar (Biochemie, Netherlands) with full-strength BG11 medium without sucrose (Rippka *et al*. 1979) within the Sanyo growth cabinet. Liverworts were grown with a photoperiod of 12 h, a temperature of 22°C and an average light level of 634 µmol m-1 s-1. After collection, liverworts were grown on agar by removing each thallus using tweezers under a compound microscope, and placing them individually onto the agar in a 10 cm^2 Petri dish. These liverworts were grown for two weeks until sub-culturing. Ten liverwort gemmae (cup-like appendage that form clones) were removed using tweezers, and then grown on a Petri dish for 30 days. Some liverworts were grown on 0.9% agar with dH$_2$O only for 30 days. One Petri dish containing 10 gemmae formed one biological replicate. After 7 days, some liverworts were imaged with a Leica MZ12 light microscope with a GXCAM-5 camera (Leica Microsystems, UK) using a 4x objective. After 30 days of growth, gemmae were removed from agar. Where the gemmae were grown, agar was finely cut, 5 cm^2 round location of each gemmae, into small cubes and placed into a 15 mL Falcon tube with 10 mL dH$_2$O. The agar gels with the water were agitated on a

see-saw rocker at 50 osc/min (Stuart Gyro-Rocker: SSL4, UK) overnight. After agitation, liquid was removed and stored in a fresh 15 mL Falcon tube at -20°C until assaying.

2.2 Hydroponic system for the isolation of polysaccharides released by roots

Seedlings grown in vermiculite and perlite (as previously outlined in section 2.1.1) were transferred to the hydroponic system, which used 9 L half-strength Hoagland's solution (7.1 g of H2395-10L; Sigma-Aldrich, UK; Arnon 1938). Twelve plants were assigned to each 9 L bucket to form one biological replicate. A standard of three biological replicates of each species and cultivars was grown within a hydroponic system (Figure 2.1). The plants were grown for a further 14 days until harvesting. The glasshouse had a 16 h photoperiod with a constant temperature of 22°C, and an average light level of 1,382 µmol m^{-1} s^{-1}. A constant air flow (1 L/min) was added to the medium using an air compressor (12 v; Corning Limited, UK) with a limit of four buckets per compressor. In order to transfer the plants into the hydroponic system, the plants pots were tipped onto their side with the plants gently being removed from the substrate. After removing plants, the roots were gently rubbed down using a thumb and index finger to remove any loose vermiculite and perlite. Two plants were assigned to each foam holder by placing the foam holder around the base of the stems. The foam holder with the plants was fitted into the hole of the polystyrene cover. Every two days each hydroponic bucket was topped up with dH$_2$O if the medium level had dropped below 9 L.

After 14 days of growth, plants were harvested, and their total fresh root weights and longest root lengths were measured. The 9 L buckets containing the hydroponate (hydroponic medium with plant exudate) were concentrated on the same day of harvest. Post-harvest, all hydroponic equipment including buckets, aerating tubing, polystyrene covers were thoroughly cleaned using household bleach (Domestos, UK), and stored until further use. The foam holders were autoclaved for 45 minutes at 121°C using a benchtop autoclave (Prestige Classic Medical, UK).
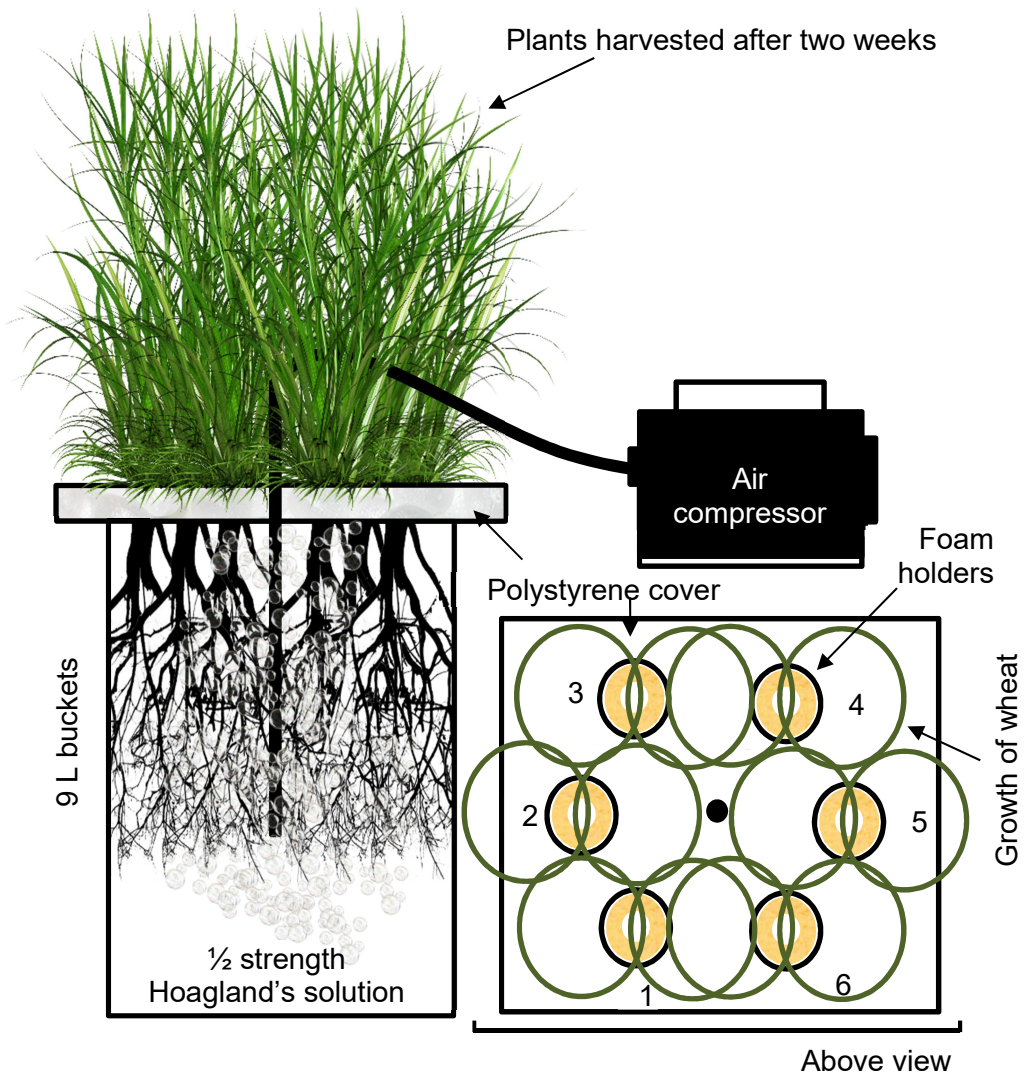
Figure 2.1 I **Hydroponics system developed for growing crop species**
Crops were grown for one week in 50:50 mixture of perlite and vermiculite prior to hydroponic culture. After pre-hydroponic growth, plants were transferred into 9 L ½ strength Hoagland's solution. Twelve plants were grown in each bucket. An air compressor constantly pumped air into the hydroponic medium. Foam holders prevented plants from falling, and prevented the hydroponic medium being exposed to the atmosphere. This hydroponics system was placed within a glasshouse, which had a 16 h photoperiod with a constant temperature of 22°C, and an average light level of 1,382 µmol m-1 s-1. After two weeks of growth, plants are harvested and the hydroponic medium concentrated. Images included are from an open source platform. Numbers on above view indicated where each pair of plants were placed.

2.3 Concentrating hydroponic media

After 14 days of growth, hydroponates were harvested and concentrated using an ultrafiltration system. To determine which filter cut-off point to use to concentrate the released polysaccharides, a range (100 KDa, 50 KDa, 30 KDa and 10 KDa) of

Vivaspin 2 mL tubes (Sartorius, Germany) were used. One millilitre of hydroponate was pipetted into a tube, which was subsequently spun down for 5 min at 3,893 x g.

Prior to concentrating, the hydroponates were filtered using pre-folded paper filters (Whatman Grade 2V, 240 mm; GE Healthcare, Germany) to prevent any particles, such as plant debris, from blocking the concentrator. After filtering, the hydroponates from each bucket were not pooled but kept as biological replicates. A Centramate ultrafiltration system (FScentr005K10; PALL Life Sciences, US) which used a 30 KDa cut-off point cassette was utilised to concentrate each 9 L volume to ~200 mL. An Easy-Load pump (Masterflex, US) was used to circulate the liquids within the ultrafiltration system (Figure 2.2). Prior to use, the cassette was stored in 0.1 M NaOH which was cleared using 5 L dH$_2$O. This was performed by securing the cassette into the ultrafiltration system by tightening the screws (torque 10 Nm), and then placing the inlet tube into the water, and the retentate and filtrate tubes into a large container kept on the floor. The water was run through the cassette using a flow rate of 200 mL/min. After passing the water though the system, the pH was measured by removing the retentate and filtrate tubes individually, and placing the pH paper onto the flow of water. Once the pH had become neutral the ultrafiltration system was ready to use.

The inlet and retentate tubes were placed and secured in the hydroponate with the filtrate tube being retained in the container on the floor (Figure 2.2). The container holding the hydroponate was covered using aluminium foil. A flow rate of 500 mL/min (1 bar) was set with each sample being concentrated from 9 L to 200 mL. After concentrating, the hydroponates were dialysed in dH$_2$O (200x sample volume) for 3 days, with two changes per day, at RT using a 3.5 KDa cut-off point. Spectra/pro membrane (Spectrumlabs, US). The subsequent dialysed concentrated hydroponates were transferred to 4 x 50 mL Falcon tubes (Falcon, US), which were placed at -80°C for 24 h. After 24 h, concentrated hydroponates were freeze-dried using a lyophiliser (Heto LyoPro 6,000, US) for 4 days at -90°C. The dried concentrated hydroponates were transferred to 15 mL Falcon tubes.

Once the ultrafiltration system had concentrated each 9 L hydroponate the system was washed using 5 L dH$_2$O with a flow rate of 500 mL/min. The inlet tube was placed into the water with the retentate and filtrate placed into the container kept on the floor. This washing step was repeated between each biological replicate. A further washing step was required between each species and cultivar. For this additional step, 0.5 M NaOH was run though the system for 5 min after the water

was passed through. A further 0.1 M NaOH was run through the system for 15 min. Once complete, 5 L dH$_2$O was passed through the system. When the pH returned to neutral the next hydroponates were concentrated. When the ultrafiltration system was no longer going to be used, the above step was repeated without the final wash of 5 L dH$_2$O, and was stored in 0.1 M NaOH at 4°C until future use. The cassette was also stored in 0.1 M NaOH at 4°C.
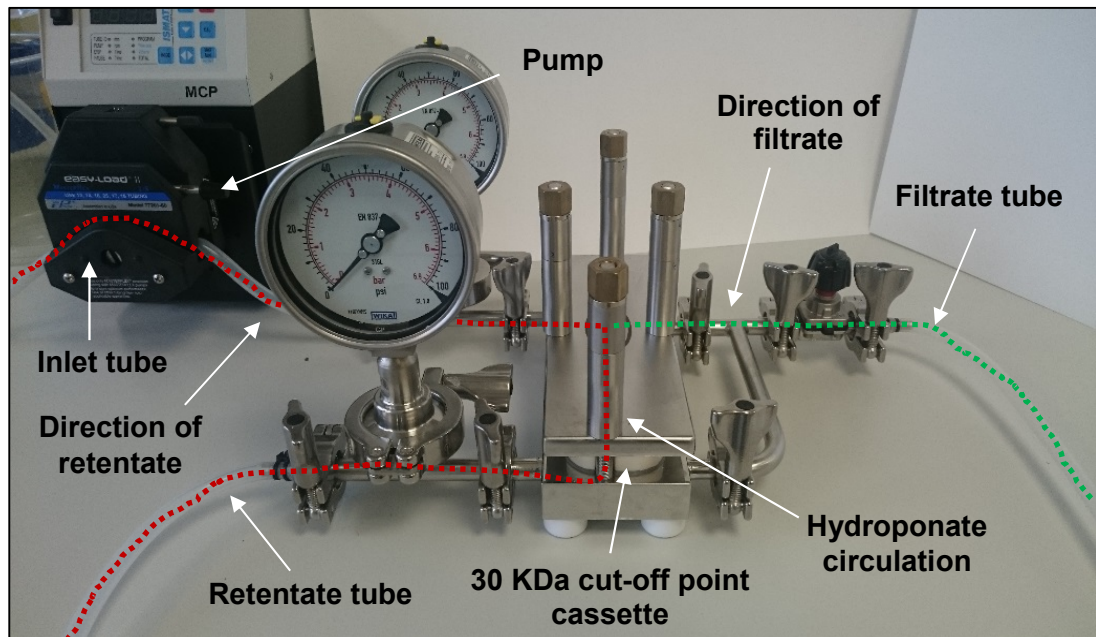
Figure 2.2 I H**ydroponate was concentrated by 45x using the ultrafiltration system**
Hydroponate was passed through a 30 KDa cut-off point cassette, which was circulated through the inlet and retentate tubes (red). Any molecules that were less than 30 KDa was passed through the filtrate tube (green). Direct hydroponate was passed through the ultrafiltration system at 500 mL/min with 1 bar of pressure. Once 9 L of direct hydroponate was concentrated to 200 mL, the cassette was purged with dH2O and NaOH by removing the retentate tube and placing it with the filtrate tube.

2.4 Enzyme Linked Immuno-Sorbent Assay (ELISA)

ELISA was performed (Engvall and Perlmann 1971) using 96 well microtitre plates (NUNC Maxisorp, Thermo Fisher Scientific, Denmark). Samples were dissolved in 1x phosphate buffered solution (PBS; Severn Biotech Limited, UK) as standard for coating. During titration the bottom row of each microtitre plate did not contain any sample; this was to act as a no antigen control. All microtitre plates were incubated at 4°C overnight.

After overnight incubation, plates were hand washed using tap water by shaking them three times under water. After washing, plates were dried by tapping them on

a small pile of dry paper towels. Plates were incubated using 300 µL per well of MP/PBS (5% w/v milk powder (Marvel, UK) in 1x PBS that had been filtered using a fine tea strainer to remove milk aggregates) for 2 h at room temperature (RT). The plates were double wrapped in aluminium foil during each incubation. After incubation, the washing step was repeated. Subsequently, the plates were incubated with a 1:10 dilution of a chosen anti-rat MAb (hybridoma cell supernatant; Table 2.1) in MP/PBS (100 µL per well) for 1 h at RT. The washing step was repeated with the plates being shaken six times under water before drying. After washing, a 1:1,000 dilution of anti-rat secondary antibody coupled with horse radish peroxidase (HRP; A9552; Sigma-Aldrich, US) secondary antibody was added to MP/PBS (100 µL per well) for 1 h at RT. For Complex Carbohydrate Research Centre (CCRC) antibodies, a 1:1,000 dilution of anti-mouse coupled with HRP was used (A9044; Sigma-Aldrich, US). For CBMs, a 1:100 dilution of anti-polyHistidine peroxidase (A7058; Sigma-Aldrich, US) was used followed by an additional incubation step, which used a 1:1,000 dilution of anti-rat coupled with HRP. The washing step was repeated after incubation. The following substrate (100 µL per well) was added to the wells for 8 min: 9 mL dH_2O, 1 mL 1 M sodium acetate pH 6.0, 100 µL 3,3',5,5'-tretamethylbenzidine (10 mg/mL Dimethyl sulphoxide, DMSO; T-2885; Sigma-Aldrich, US) and 10 µL H_2O_2. After 8 min, 50 µL 2.5 M sulphuric acid was added to the wells. The plates were subsequently read using the absorbance at 450 nm in a Multiskan FC plate reader (Thermo-Scientific, US) that used SkanIt software (Thermo-Scientific, US).

Table 2.1 MAbs and CBMs used within this investigation

Antigen	MAb/CBM	Reference
Arabinogalactan-protein	LM2	Smallwood *et al.* 1996; Yates *et al.* 1996
	JIM4	Knox *et al.* 1989; Stacey *et al.* 1990; Yates *et al.* 1996
	JIM14	Knox *et al.* 1991; Yates *et al.* 1996
	JIM15	Knox *et al.* 1991; Yates *et al.* 1996
	MAC207	Pennell *et al.* 1989; Yates 1996
Callose	1,3-β-glucan	Meikle *et al.* 1991
β-linked Galactose	JIM1	Knox and Roberts 1989
Extensin	LM1	Smallwood *et al.* 1995
	JIM20	Smallwood *et al.* 1994
Heteromannan	CCRCM-170	Pattathil *et al.* 2010
	CBM27	Boraston *et al.* 2003
	LM21	Marcus *et al.* 2010
	LM22	Marcus *et al.* 2010
Mixed linkage glucan	1,3:1,4-β-glucan	Meikle *et al.* 1994
Pectic polysaccharides **Homogalacturonan**	LM7	Willats *et al.* 2001; Clausen *et al.* 2003
	JIM7	Clausen *et al.* 2003
	LM18	Verhertbruggen *et al.* 2009a
	LM19	Verhertbruggen *et al.* 2009a
	LM20	Verhertbruggen *et al.* 2009a
Rhamnogalacturonan-I	LM5	Jones *et al.* 1997
	LM6	Willats *et al.* 1998a
	LM9	Clausen *et al.* 2004
	LM13	Moller *et al.* 2008; Verhertbruggen *et al.* 2009b
	LM16	Verhertbruggen *et al.* 2009b
	LM26	Unpublished
Xylogalacturonan	LM8	Willats *et al.* 2004
Xylan	CBM2b1-2	McCartney *et al.* 2006
	CCRCM-108	Pattathil *et al.* 2010
	LM10	McCartney *et al.* 2005
	LM11	McCartney *et al.* 2005
	LM12	Pedersen *et al.* 2012
	LM27	Cornuault *et al.* 2015
	LM28	Cornuault *et al.* 2015
Xyloglucan	CBM3a	Blake *et al.* 2006
	LM15	Marcus *et al.* 2008
	LM24	Pedersen *et al.* 2012
	LM25	Pedersen *et al.* 2012

2.5 Sandwich-ELISA analysis

Microtitre plates were coated with 100 µL of 10 µg/mL of the capturing probe CBM2b1-2 (xylan-binding CBM; McCartney *et al*. 2006) or 10 µg/mL CBM3a (Crystalline cellulose/xyloglucan-binding; Hernandez-Gomez *et al.* 2015) in 1x PBS. For negative controls, wells just containing 100 µL 1x PBS were coated. Both treatments were doubled wrapped in aluminium foil and incubated overnight at 4°C (Cornuault and Knox 2014). After incubation, plates were washed using tap water by shaking them three times under water. To dry the plates, plates were tapped on a small pile of paper towels. Plates were incubated with 250 µL MP/PBS in each well (Cornuault and Knox 2014). Plates were then doubled wrapped in aluminium foil (this occurred during each incubation), and incubated for 3 h at RT. After incubation washing was repeated. Once plates were dried, MP/PBS with dissolved concentrated hydroponate was pipetted (100 µL per well) into the wells. The plates were then incubated at 4°C overnight. After incubation washing was repeated. Plates were then incubated using a 1:10 in dilution of anti-rat MAb (Table 2.1) in MP/PBS (100 µL per well) for 2 h at RT. After incubation washing was again repeated. Plates were then developed following ELISA as outlined above.

2.6 Tissue printing on nitrocellulose

The nitrocellulose printing and imaging was performed by Ms Sue Marcus. After liverworts were grown (as previously outlined in section 2.1.2) they were removed from the agar using tweezers. A nitrocellulose sheet was then placed on top of the agar, ensuring that the surface area was fully covered. The nitrocellulose and agar were incubated overnight at 4°C uncovered in aluminium foil. After overnight incubation, nitrocellulose was gently washed with tap water. Nitrocellulose was then blocked with MP/PBS with 0.0025% sodium azide to inhibit bacterial growth. The sheet was then incubated at RT for 1 h. After 1 h, a 1:5 dilution of LM25 was added to the nitrocellulose, and incubated for a further 1 h. After incubation, nitrocellulose was gently washed with tap water, and washed again in 1x PBS three times for 5 mins each. Subsequently, block with 1:1,000 dilution of anti-rat HRP was added to the nitrocellulose, and incubated for a further 1 h. Washing was repeated with tap water and 1x PBS. After washing, the following substrate was added to the nitrocellulose, and incubated for 30 min: 25 mL dH$_2$O, 5 mL chloronaphthol (5 mg/mL in EtOH) and 30 µL of H$_2$O$_2$, which was added at the last minute. Once developed, the nitrocellulose was thoroughly washed in a container full of dH$_2$O for

5 min. Nitrocellulose was then dried in between two sheets of blotting card for 30 min. Subsequently, nitrocellulose was imaged using an Epson Perfection V750 Pro scanner (Epson, Japan).

2.7 Isolation of polysaccharides from root body cell walls

Root cell walls of wheat cultivar, Cadenza were isolated by preparation of Alcohol Insoluble Residue (AIR; Fry, 1988). After weighing fresh root material, the material was immediately placed into liquid nitrogen for 2 min. After liquid nitrogen, the root material was freeze-dried for 3 days prior to AIR. Fifteen milligram of freeze-dried root material was placed into a 2 mL Qiagen tube with two iron beads. The tube was then frozen in liquid nitrogen. The tube was then homogenised into a Tissue lyser (Qiagen, UK) for 2 min at 50 Hz. After grinding, 10 mg of ground root material was placed into a 2 mL Eppendorf tube along with 1 mL 70% EtOH (v/v), which was agitated on a see-saw rocker at 50 osc/min for 1 h. The samples were centrifuged for 1 min at 400 x g. After centrifugation the supernatant was removed. Subsequently, 1 mL solutions of 80%, 90% and 100% EtOH, 100% acetone, and methanol and chloroform (2:3) were added to the samples and each agitated for 1 h at 50 osc/min. Centrifugation was repeated after each incubation. After the methanol and chloroform was added, the samples were left within a laminar flow hood overnight at RT to dry.

One milligram of dried root AIR was placed into a fresh 2 mL tube with 400 µL 4 M KOH and 1% $NaBH_4$. The tube was then placed into the Tissue lyser for 20 min at 50 Hz. The sample was centrifuged at 3,893 x g for 15 min. Subsequently, the supernatant was removed and placed into a 5 mL Eppendorf tube. The sample was then diluted by 10 times in 1x PBS. The diluted supernatant was neutralised using 80% (v/v) acetic acid. The pH was tested by using pH paper. The neutralised samples were dialysed and freeze-dried as outlined in concentrating hydroponic media. The resulting solutions then were assayed by ELISA, anion-exchange EDC and sandwich-ELISA.

2.8 Chromatography analyses of concentrated hydroponates

2.8.1 Anion-exchange Epitope Detection Chromatography (EDC)

EDC analyses were performed by injecting samples, which had been dissolved in 20 mM sodium acetate (pH 4.5) buffer into separate 1 mL weak anion-exchange chromatography columns (Hi–Trap ANX FF, GE Healthcare, UK). A Bio-Rad BioLogic LP System (Bio-Rad, US; Cornuault *et al*. 2014) was used to undertake the anion-exchange chromatography. Two gradients, step and neutral, of NaCl were used to elute the samples at 1 mL/min.

For gradient 1, Buffer A (20 mM sodium acetate pH 4.5) eluted the samples for 13 min, a linear gradient from 0-20% using Buffer B (50 mM sodium acetate + 0.6 M NaCl) was used for 16 min, 30% Buffer B for 16 min, 40% Buffer B for 16 min, 50% Buffer B for 16 min, followed by 100% Buffer B for 19 min. In order to isolate sufficient fractions for detailed biochemical analysis, gradient 1 was scaled up by fifteen times, using a 15 mL column. This resulted in the programme running Buffer A for 540 min, followed by a linear gradient from 0% to 100% using Buffer B for 540 min, followed by a further 360 min of 100% Buffer B; each fraction became 15 mL.

For gradient 2, samples were eluted with Buffer A for 36 min, followed by a linear gradient from 0% to 100% using Buffer B for 72 min, followed by a further 24 min of 100% Buffer B.

For both gradients, 96 fractions of 1 mL volumes were collected within a 96 well megablock (2.2 mL wells; Sarstedt, Germany). The collected fractions were adjusted to pH 9.0 by adding 40 µL 1 M sodium carbonate into each fraction (1 mL), and thoroughly mixing using an automated pipette. This was confirmed by removing 2 µL of five randomly selected fractions, and pipetting them onto pH paper. Aliquots (100 µL) of each 1 mL fraction were incubated per well on a microtitre plate (Cornuault *et al*. 2014), and were developed using ELISA. The BioLogic LP data view software (Bio-Rad, US) was used to monitor and log the conductivity of the NaCl gradient during chromatography. Between each run, the column was washed by reducing the salt levels with Buffer A and by injecting 1 mL 0.1 M NaOH. Once NaOH was injected, the column was equilibrated using Buffer A. Once the columns had been washed they were stored at 4°C wrapped in aluminium foil. The tubing was kept in 20% EtOH prior to each use. For the start of each run, the tubing was purged using dH_2O with a flow rate of 6.5 mL/min (Cornuault *et al*. 2014). After purging, the column was added to the system. Once the column was in place, Buffer

B was added at 1 mL/min to increase the salt concentration within the column. After the gradient of salt had reached its maximum peak, Buffer A was added to lower the salt concentration (Cornuault *et al*. 2014). When the salt concentration was lowered the column had been calibrated for use.

2.8.2 Size-exclusion EDC

A modified version of size exclusion chromatography was followed (Pfannkoch *et al*. 1980). Samples were each dissolved into 1 mL of vacuum filtered (0.45 µm pore size; Supelco Nylon 66 membrane; Sigma-Aldrich, US) 20 mM sodium acetate with 1 M NaCl, pH 4.5 (buffer). This buffer was injected into a 120 mL HiPrep (16/60 Sephacryl S400H; GE Healthcare, UK) size exclusion chromatography column. An AKTA start chromatography system (GE Healthcare, UK) was used. Unicorn 5.0 Manager Software (Amersham Biosciences Corp, US) was used to monitor column conductivity. Prior to use the column was cleaned by selecting the lowest flow through rate, passing 20 mM sodium acetate with 1 M NaCl pH 4.5 through the column overnight at a flow rate of 0.2 mL/min. After cleaning, 2.5 mL of sodium acetate buffer was passed through the loop where the sample would be injected. Subsequently, samples, dissolved in 1 mL of buffer, were injected into the column with a flow rate of 0.7 mL/min. Ninety-six fractions of 1 mL were collected in total. After size exclusion, the pH of the collected fractions was adjusted to pH 9.0 by adding 40 µL 1 M sodium carbonate. One-hundred microliters of each fraction was added into each well of a 96 microtiter plate. The microtitre plate was incubated overnight at 4°C. A standard ELISA protocol followed, as previously outlined, with the exception of a 1:20 dilution for all MAbs used. After size exclusion, the column was eluted with 20% EtOH at 0.2 mL/min overnight in order to clean it. The column was then wrapped in aluminium foil and stored at 4°C until future use.

A gel filtration HMW and LMW calibration kit (28-4038-42; GE Healthcare, UK) which contained the following protein standards: carbonic anhydrase 29 KDa (bovine erythrocytes), conalbumin 75 KDa (chicken egg white), aldolase 158 KDa (rabbit muscle), ferritin 440 KDa (horse spleen) and thyroglobulin 669 KDa (bovine thyroid) was used to calculate KDa values. Four milligrams of each protein standard was dissolved into 1 mL buffer and individually run on the size exclusion chromatography. Where the signals of protein standards peaked, the KDa values were mapped onto the chromatogram.

2.9 Total carbohydrate content assay

A Phenol-Sulphuric Acid assay was followed to determine the total carbohydrate content of samples (Masuko *et al*. 2005). Aliquots (50 µL) of each 15 mL fractions from the anion-exchange EDC were removed, and placed into 1.5 mL Eppendorf tubes. Concentrated sulphuric acid (18 M) was rapidly pipetted into the 50 µL sample followed by 30 µL 5% (w/w) phenol in dH_2O. The solutions were then incubated for 5 min at 90°C. The subsequent samples were pipetted into a 96 microtiter plate and read at an absorbance of 450 nm using the plate reader.

2.10 Enzyme digests of concentrated hydroponate

The following enzymes and buffers were made for the enzyme digests: β-xylanase M1 (*Trichoderma viride*; Megazyme, Ireland) was dissolved in 100 mM sodium acetate (pH 4.5), xyloglucanase (GH5, *Paenibacillus* sp.; Megazyme, Ireland) was dissolved in 100 mM sodium acetate (pH 5.0) with 0.5 mg/mL BSA. Concentrated hydroponate (1 mg) was dissolved directly into 10 mL of each buffer (as outlined in above). Aliquots (50 µL) containing 50 µg of concentrated hydroponate were further diluted into 950 µL of each buffer. This solution was used for each digest. The β-xylanase M1 digest samples were incubated at 40°C for 2 h. The xyloglucanase digest samples were incubated at 60°C for 2 h. Forty units of xyloglucanase (60 µg of enzyme) was required to breakdown the xyloglucan within the 50 µL sample of concentrated hydroponate. For β-xylanase M1, 480 U (120 µg of enzyme) was required to digest xylan within the concentrated hydroponate. After samples were incubated with the enzymes treatments, the samples were left to cool at RT for 10 min before EDC.

2.11 Monosaccharide composition and monosaccharide linkage analyses

2.11.1 Monosaccharide composition analysis

The monosaccharide composition analysis was undertaken by Mr Bernhard Jaehrig based at the Complex Carbohydrate Research Centre, US. A sample of REC1 (300 µg) was placed into an Eppendorf tube (2 mL), and hydrolysed using 2 M trifluoroacetic acid for 4 h within a sealed tube at 100°C. The trifluoroacetic acid was removed by heat-assisted evaporation, which used a gentle flow of liquid nitrogen. The sample was then reduced in 1 mL (10 mg/mL) sodium borohydride prepared in

1 M ammonium hydroxide, which was incubated for 1 h at RT (Peña *et al.* 2012b). The resulting residue was removed by adding 300 µL of methanol to the sample. The sample was then dried using heat-assisted evaporation. Aliquots (125 µl) of 9:1 (v/v) methanol in acetic acid were added to the sample for 1 h at RT. Heat-assisted evaporation was repeated (Peña *et al.* 2012b). The resulting alditol acetates were analysed on a 7890A gas chromatography (Agilent Technologies, US) with a SP2330 bonded phase fused silica capillary column (30 m x 0.25 cm; Sigma-Alchrid, US). The gas chromatography was interfaced to a 5975C Mass Selective Detector (MSD) mass spectrometer that was operating on the electron impact ionization mode (Agilent Technologies, US; York *et al.* 1985). Inositol (20 µg) was used as an internal standard.

2.11.2 Monosaccharide linkage analysis

The monosaccharide linkage analysis was undertaken by Mr Bernhard Jaehrig based at the Complex Carbohydrate Research Centre, US. A sample of REC1 (1 mg) was suspended in 200 µL of DMSO and agitated (120 rpm) at RT for 1 week. Methylation was undertaken by agitating (120 rpm) the dissolved REC1 in 200 µL of 1 M sodium hydroxide for 15 min, and then in 100 µL of methyl iodide for 45 min, both at RT. An aliquot (2 mL) of dH_2O was then added to the sample. Removal of excess methyl iodide was carried out by sparging small aliquots of liquid nitrogen into the sample (Heiss *et al.* 2009). Subsequently, the methylated sample was hydrolysed using 400 µL of 2 M trifluoroacetic acid for 2 h within a sealed Eppendorf tube at 120°C. The trifluoroacetic acid was removed by heat-assisted evaporation, which used a gentle flow of liquid nitrogen. Isopropanol (400 µL) was added to the sample and immediately dried using liquid nitrogen. This was repeated twice. The sample was then reduced with the addition of 1.2 mL of sodium borodeuteride prepared in 1 M ammonium hydroxide (10 mg/mL), which was incubated for 3 h at RT. Glacial acetic acid (300 µL) was added to the sample and vortexed. Methanol (300 µL) was then added to the sample, vortexed and dried using liquid nitrogen. This was repeated twice. Three-hundred microliters of 9:1 (v/v) methanol in acetic acid was added to the sample, and vortexed. Heat-assisted evaporation was repeated. Methanol (600 µL) was added to the sample, vortexed and dried. This was repeated twice. Subsequently, the dried sample was acetylated using 250 µL of 16:234 (v/v) dH_2O in acetic anhydride, which was thoroughly agitated to re-suspend the sample (Heiss *et al.* 2009).

Concentrated trifluoroacetic acid (13 M; 230 μL) was added to the sample, thoroughly agitated, and incubated for 10 min at 50°C. Once cool, 2 mL of isopropyl alcohol was added to the sample, and dried. Two millilitres of 0.2 M sodium carbonate was added to the sample along with 1 mL of dichloromethane, which was vortexed for 30 sec. Centrifugation followed with the top layer of the reaction removed. The bottom layer was washed with dH_2O, centrifuged and removed. This was repeated twice. Heat-assisted drying was repeated. The resulting pellet was dissolved in 1 mL of dichloromethane. The resulting partially methylated alditol acetates were analysed on a 7890A gas chromatography (Agilent Technologies, US) with a SP2330 bonded phase fused silica capillary column (30 m x 0.25 cm; Sigma-Aldrich, US), which separated the polysaccharide products by size. The gas chromatography was interfaced to a 5975C MSD mass spectrometer that was operating on the electron impact ionization mode (Agilent Technologies, US). This mode separated the polysaccharide products by their mass-to-charge (m/z) ratio. Both the retention times determined by gas chromatography and the m/z ratios determined by the mass selective detector were used to calculate the linkages through the Agilent 7890 Series Software (Agilent Technologies, US; Heiss *et al.* 2009).

2.12 Soil sourcing and preparation

Sandy loam soil for the aggregate analyses was obtained locally (GPS coordinates 53°48'17.4"N 1°33'18.2"W) in May 2015. The soil was collected at a depth of 10 cm. Once removed, all visible containments including plant material and stones were discarded from the test soil. Soil was sieved using a 2,000 μm analytical sieve and sterilised by autoclaving at 121°C for 45 min. For optimal sterilisation, soil was thinly spread on a metal tray. After sterilisation, soil was stored in a large glass beaker, which was doubled wrapped in aluminium foil, and kept the dark at 4°C. To determine soil texture, a typical manual soil texture analysis, widely used in horticulture was undertaken (Brown 2008). Other soil types were sourced and prepared using the same method: clay loam soil (coordinates 53°48'15.3"N 1°33'16.4"W), and slit loam (coordinates 53°48'17.3"N 1°33'18.8"W), both collected in June 2015. Sand (22.5 Kg Blooma Play Sand; B&Q, UK) was also collected and prepared in the same manner. Glacial rock (Fox Glacier, New Zealand; coordinates 43°28'14.4"S 169°56'53.3"E) was collected by Dr Katie Field, and prepared in the same manner.

2.13 Measuring polysaccharide adherence to soils

Commercial polysaccharide standards, gum Arabic from Acacia tree (G975; Sigma-Aldrich, US), xylan from birchwood (P-XYLNBE; Megazyme International, Ireland) and tamarind seed xyloglucan (P-XYGLN; Megazyme International, Ireland), were used. Each standard polysaccharide was added to 1 mg of soil (ratio: 1:100), resulting in a 10 μg/mL solution. After each standard polysaccharide was added, the solution containing both the soil and polysaccharide was agitated for 2 h on a see-saw rocker at 50 osc/min. For a negative control, 10 μg/mL of each commercial standard was placed into tube without any soil. After agitation, samples were centrifuged at 3,856 x g (4°C) for 10 min. Once spun down, supernatant was gently removed and assayed by ELISA. The remaining soil was dried for 48 h at 60°C for dry dispersion analyses, SEM, and immuno-staining. A standard of six technical replicates per analysis was used. This resulted in six rows of wells per plate being used for each group. Results of the replicates were averaged.

To calculate the amounts (μg) of polysaccharide remaining within the supernatant, standard curves were generated (EnCor Biotechnology Inc., 2016) by using commercial polysaccharides (see above) that were titrated (10-fold). Once developed by ELISA, the absorbances were plotted onto a scatter graph (with a maximum of 1.0 OD). The linear part of the scatter graph was removed and re-plotted. Once plotted, the Y and X values of the linear plot were gathered along with R^2 (minimum value of 0.98 was used). After collecting the outlined values the following formula was used to convert ELISA absorbance into total μg (a), a =((au*Y)-X)*d, where d = dilution factor of the absorbance value used.

2.14 Wet sieving soil analyses of aggregate status

Aliquots (100 mL) of gum Arabic, xylan from birchwood and tamarind seed xyloglucan solutions (10 mg/mL) were added to 100 g of sandy loam soil (ratio: 1:100). These samples were then thoroughly agitated for 2 h at 50 osc/min prior to wet sieving. An Octagon (200 series; Endecotts, UK) mechanical sieve shaker (located in the School of Geography, University of Leeds) was set to 1.8 mm/g for 5 min for each round with a contestant flow of cold tap water. Five sieves were stacked in order of deceasing sizes: 1,000 μm, 500 μm, 250 μm, 90 μm and ≤90

μm. After sieving, sieves were initially dried at 95°C for 30 min. Soil was then transferred using a fine brush into blotting card envelopes that had been weighed before soil was added, and placed at 40°C overnight. After the sieves were cleared of soil they were ready to be used again. Once dried, envelopes with soil were re-weighed, and the weight gained calculated, weight of envelope before soil minus weight after soil. Each fraction was then expressed as a percentage relative to each other. Each treatment was repeated three times; the results were then averaged.

2.15 Dry dispersion analysis of soil aggregation

Dry dispersion particle analyses were carried out using a Morphologi G3 (Malvern, UK) automated particle characterisation microscope. This microscope was based within the Institute of Particle Science and Engineering, School of Chemical Engineering (University of Leeds). Solutions with or without the added polysaccharides (ratio 0.1:100) were agitated for 2 h on a see-saw rocker at 50 osc/min. After agitation, samples were centrifuged (4°C) for 10 min at 3,856 x g. The supernatants were removed and assayed. The pelleted soils were dried for 48 h at 40°C. Soil samples (18 mm^3) were added into the dispersal unit within the blast chamber of the microscope. Soil was then dispensed onto a glass slide with a burst (1 bar) of liquid nitrogen. Before and after each analysis the dispersal unit, blast chamber and glass slide were cleaned with antistatic spray. The Morphologi G3 imaged 150,000 particles per soil using a 5x objective. After the microscope had measured all aggregate volumes, any outlining aggregates which did not conform to known aggregate dimensions were manually removed, for instance long strands of fibres or dust particles. The volumes of each aggregate were calculated using the 18.2 Morphologi G3S (Malvern, UK) software, which took three Z-stacks of each aggregate and determined volume (v) by using the following formula, $v = 4/3\pi(d/2)^3$, where d = diameter of aggregate (μm).

2.16 Scanning electron microscopy (SEM) of sandy loam soil

SEM was undertaken using a Quanta Scanning Electron Microscope (200 FEG; FEI Company, US), which used xT microscope control software (FEI Company, US). For sample preparation refer to dry dispersion analysis of soil aggregation. After soil was dried for 48 h, a thin layer of soil was spread onto a glass slide. Stubs with

carbon-rich tape were dipped into the soil. The stubs were immediately coated with a 5 nm thick layer of platinum under a vacuum using a mini sputter coater (SC7620; Polaron Equipment Limited, UK) prior to imaging. Each treatment was repeated three times; representative images were displayed. All samples were imaged with a vacuum pressure of 30 Pa, and with an electrical potential of 3 kV; images of REC1 used an electrical potential of 20 kV.

2.17 Immuno-labelling of soils

Aliquots (100 µL) of dH_2O containing 50 µg of xylan from birchwood and tamarind seed xyloglucan were added to 50 mg of sterile sandy loam (ratio 0.1:100). As controls, soil that had no commercial polysaccharide, only containing dH_2O, was screened with MAbs. Furthermore, soil with the commercial polysaccharide was screened without MAbs. These solutions were agitated for 2 h on a see-saw rocker at 50 osc/min. After agitation, samples were centrifuged (4°C) for 10 min at 3,856 x g. The supernatants were removed. A small scoop (~500 µg) of this soil was then placed into the wells of a Vectabonded eight well microscope slide (MP Biomedicals LLC, Germany), and then gently pressed down. Slides were then covered with aluminium foil, and left to dry overnight at RT. Once dried, excess soil was removed by gently tapping the slide onto a bench.

Wells containing soil were blocked with 10 µL filtered 5% milk powder with 1x PBS for 30 min at RT. Slides were covered during each incubation. A 1:5 dilution of MAb (hybridoma cell supernatant) was directly added to each well, which already contained the block. The slides were then incubated for 1.5 h at RT. MAb solutions were removed, and the wells were each washed three times with 1x PBS for 5 min. After washing, anti-rat (Immunoglobulin G; IgG) coupled with fluorescein isothiocyanate (FITC; F1763; Sigma-Aldrich, US) was added to block using a dilution of 1:100. Aliquots (10 µL) of this solution were added to each well. The slides were incubated for 1 h at RT. The wells were each washed three times with 1x PBS for 5 min. After washing, each well had been treated with 10 µl 0.1% Toluidine Blue O (Sigma-Aldrich, US), which was in 0.2 M sodium phosphate (pH 5.5) for 5 min. Washing was repeated extensively. After washing, a small drop of Citifluor in glycerol/PBS (AF-1; Agar Scientific, UK) anti-fade was placed onto the top of each well before glass cover slides were added. The slides were then imaged using an Olympus Optical GX Microscope (BX61; Olympus, US), Olympus BX-UCB control unit, and a Hamamatsu ORCA publisher camera that were tethered to a PC.

An X-Cite 120Q (120 watt; Excelitas Technologies, US) was used as the excitation light source (456 nm). Volocity 4 (PerkinElmer, US) image analysis software was used to merge the resulting bright-field and FITC channels. Each treatment was repeated six times.

2.18 Statistical and phylogenetic tree analyses

The statistical significance of differences between mean or median values was determined using Student's t-test or the non-parametric equivalent, Mann-Whitney U test. For data sets with three or more groups a One-way Independent ANOVA or the non-parametric equivalent, Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests, were used. Differences were considered significant when the P-values were below 0.05. Minitab 17 (Minitab, UK) statistical software was used for all statistical analyses. For phylogenetic trees, plant genus and species were written in a list format, and pasted onto the PhyloT taxonomy-based phylogenetic tree generator (2015.1; BioByte Solutions, Germany).

Chapter 3

Characterisation of polysaccharides released from the roots of hydroponically grown crops

3.1 Introduction

Little is known about the identity of polysaccharides released from plant roots and their function. MAbs are highly efficient and sensitive molecular probes that can be used to detect epitopes of polysaccharides (Lee *et al*. 2011). Physio-chemical methods of identifying polysaccharides are time consuming, and usually require a large amount of material which has been purified, for instance, a minimum of ~1 mg is required for monosaccharide linkage analysis (Moller *et al.* 2008; Pattathil *et al.* 2012). Whereas, immuno-based methods such as ELISA require approximately 100-fold less material, which does not require as much purification (Lee *et al*. 2011) to reveal the epitopes of cell wall polysaccharides. Previous attempts that have isolated polysaccharides released from roots (Chaboud and Rougier 1984; Bacic *et al.* 1986; Moody *et al.* 1988; Osborn *et al*. 1999) produced little material (up to tens of micrograms) for physio-chemical analysis. Prior to this investigation, no studies have used an immuno-based approach to screen the root exudate for the epitopes of cell wall polysaccharides released by plant roots. There is a large library of MAbs raised against a range of cell wall polysaccharides, and which may aid in deciphering the complexity of polysaccharides released from plant roots. Although MAbs are efficient, they may not identify all the polysaccharides that are released by plant roots. If there is a polysaccharide released by plant roots that does not contain epitopes that the current MAb library recognise, then it will not be detected. Whereas, for a more physio-chemical approach, such as monosaccharide composition and monosaccharide linkage analysis, can identify all polysaccharides present.

This investigation focused on growing plants hydroponically so that the polysaccharides released from roots could be efficiently isolated for immunochemical and biochemical analysis. Furthermore, by growing plants hydroponically it could be assured that the polysaccharides detected originated from the root network, and not from the seed coats or leaves. This study has also selected to grow a range of crop species, wheat, barley, maize, pea, tomato and rapeseed, which develop large root networks, maximising the probability of collecting sufficient material for biochemical analysis.

The first aim of this project was to identify the major cell wall polysaccharide epitopes released into the hydroponic medium of various crop species using MAbs, with a particular focus on three wheat cultivars, Avalon, Cadenza and Skyfall. Focusing on three cultivars of wheat can determine if there is a genetic factor that is

involved in the release of polysaccharide through any differences observed between the polysaccharides detected in their growth medium. By using MAbs in conjunction with ELISA, the epitopes released by wheat roots were determined, and compared in the form of epitope profiles. From this initial exploration, these epitope profiles were compared to the cell walls of the root body of the same wheat cultivar screened. Cell wall polysaccharide epitope profiles from three major eudicotyledon crops, pea, tomato and rapeseed, and two other major cereal crops, barley and maize, were then compared.

3.2 Results

3.2.1 There were no differences in root growth between the wheat cultivars

To assess the effects of the hydroponic system on the growth of wheat, the root lengths and root fresh weights of three wheat (*Triticum aestivum*) cultivars, Avalon, Cadenza and Skyfall were compared. These cultivars represent a range of wheat varieties, Avalon and Skyfall are both widely grown winter varieties whereas, Cadenza is an older and less popular spring variety (Kumar *et al*. 2011). Prior to hydroponics, wheat were germinated in vermiculite and perlite (50:50) for one week in a growth cabinet until, they were strong enough to be transferred to the hydroponic system. Once in the hydroponic system wheat were grown for an additional two weeks. Two weeks was found to be an effective period to allow polysaccharides to accumulate in the hydroponates to be readily detected. After two weeks of hydroponics, the hydroponates of the wheat cultivars were each filtered using Whatman filter paper to remove any plant debris and then concentrated (from 9 L to ~200 mL). Once a bucket had been filtered the wheat were harvested with their longest root lengths and total fresh root weights measured. After measuring, it was determined that the longest root lengths of the cultivars were not significantly different (One-Way Independent ANOVA, F= 0.67, P= <0.05), being on average 45 cm long (Figure 3.1, A). The total fresh root weights were also not significantly different (One-Way Independent ANOVA, F= 0.87, P= 0.544), being on average 4.42 g (Figure 3.1, B). Overall, the growth of the three wheat cultivars was not statistically different.
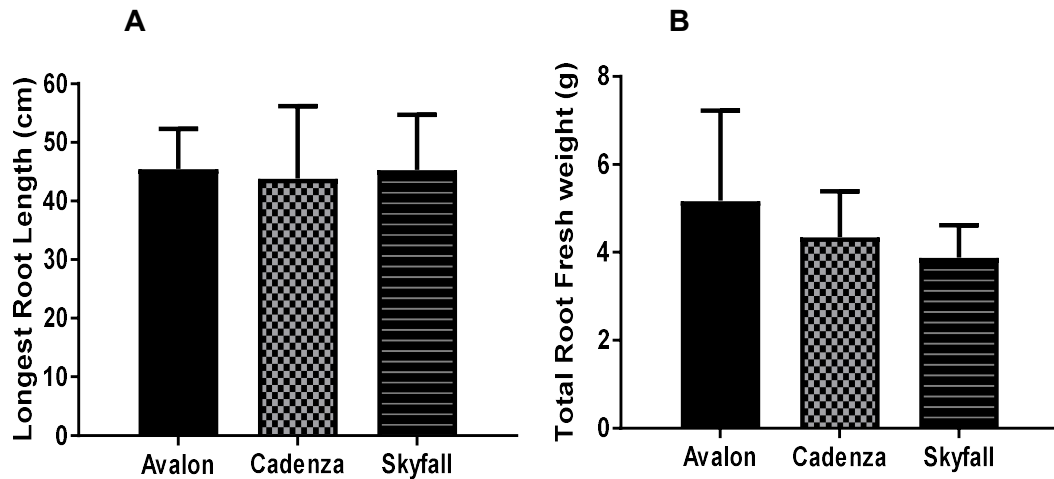
Figure 3.1 I **Mean root lengths and root fresh weights of the wheat cultivars**
After two weeks of hydroponics, the longest root lengths and total fresh weights were recorded. There was no significant difference between the mean longest root length and total fresh weights of the wheat cultivars, Avalon, Cadenza and Skyfall. Twelve wheats were grown in one 9 L bucket to form one biological replicate. A total of three biological replicates were used. Standard deviation bars shown.

3.2.2 Wheat roots release AGP, extensin, xylan and xyloglucan epitopes

To reveal the identity of the polysaccharide epitopes released by wheat roots, the hydroponates of wheat were probed with a range of MAbs. After two weeks of hydroponics, plants were harvested, and the hydroponate directly assayed using an ELISA. An array of 29 MAbs was used to probe the hydroponate to reveal the types of cell wall-related polysaccharides being released by the roots of the wheat cultivars (Table 2.1). The resulting heat map revealed that a diverse mixture of polysaccharide epitopes were present within each of the hydroponates of the wheat cultivars (Table 3.1). The top ranking MAb signals were epitopes of xyloglucan, xylan, extensin and AGP. Unexpectedly, signals from the epitopes of pectic-related polysaccharides were either very low or not present across the cultivars (Table 3.1). An interesting observation was made when LM8 (XGA) was not detected within the hydroponate of the cultivars. The LM8 epitope is specific to root cap cells of a range of angiosperm including pea and maize (Willats *et al.* 2004).

LM2 had the highest signal from the AGP-specific MAbs across the cultivars. This indicates that the AGP has a β-linked glucuronic acid residues (Table 3.1). The signals of MAbs, which were specific to one polysaccharide varied, suggesting that there may be different forms of those polysaccharides being released from each cultivar. For example, LM25 was the only signal from a xyloglucan-binding MAb that

was present in the hydroponate of Cadenza. Within the hydroponates of Avalon and Skyfall, there was a clear signal from LM15, suggesting that the xyloglucan may be less galactosylated compared to Cadenza (Table 3.1).

Antigen	MAb	Wheat cultivars (au)		
		Cadenza	Avalon	Skyfall
	LM2	0.68	0.67	0.61
Arabinogalactan-protein	JIM4	-	0.07	0.06
	JIM14	-	-	-
	JIM15	-	-	-
	MAC207	-	-	-
β-linked Galactose	JIM1	-	-	-
Extensin	**LM1**	1.35	1.24	1.07
	JIM20	1.69	1.12	1.08
Heteromannan	LM21	-	-	-
	LM22	-	-	-
Pectic polysaccharides	LM7	-	0.01	-
Homogalacturonan	JIM7	-	-	0.09
	LM18	-	0.01	-
	LM19	-	0.03	-
	LM20	-	-	-
Rhamnogalacturonan-I	LM5	-	0.03	-
	LM6	-	0.06	0.12
	LM9	-	-	-
	LM13	-	-	-
	LM16	0.16	0.09	0.05
	LM26	-	0.01	-
Xylogalacturonan	LM8	-	-	-
Xylan	LM10	-	-	-
	LM11	1.03	2.17	1.7
	LM12	-	0.01	-
	LM28	0.23	0.06	-
Xyloglucan	LM15	-	0.37	0.63
	LM24	-	0.08	0.31
	LM25	2.22	1.85	1.82

Scale						
0.1	0.25	0.5	1	1.5	2	2.5

Table 3.1 I **Heat map of polysaccharide epitopes released from the roots of wheat cultivars, Cadenza, Avalon and Skyfall as determined by ELISA**
Twenty-nine monoclonal antibodies raised against cell wall polysaccharides were used to screen the hydroponates of three-week-old wheat cultivars through ELISA. Form the heat map, MAb signals from LM2 (AGP), LM1 (extensin), LM11 (xylan) and LM25 (xyloglucan) were ranked the highest across the wheat cultivars. An aliquot (9 mL) from each bucket was removed for one replicate, 1 mL 10x PBS was then added to each aliquot for the ELISA. The 1:25 dilution of the hydroponate was used for this analysis. Each data point is a mean of three biological replicates. Twelve wheat plants were grown per cultivar per replicate. A cut off point of 0.1 au was removed, any values below this level were regarded as having no signal (-), shading of heat map was across the cultivars screened.

3.2.3 A 30 KDa cut-off point membrane was suitable to concentrate polysaccharides

In order to explore the polysaccharides released by roots, the hydroponic media were concentrated from 9 L volumes to a more manageable volume (~200 mL). In order to determine which membrane size would be used for ultrafiltration, aliquots of wheat cv. Cadenza hydroponate were pipetted into a range of molecular weight cut-off point centrifuge tubes (10 KDa, 30 KDa, 50 KDa and 100 KDa). After centrifugation, the filtrate was removed. Aliquots of deionised water were placed on top of the filter, mixed and the retentate removed. Both the filtrate and retentate were assayed using an ELISA. LM25 was used as a marker to assay the hydroponate due to having the highest signal (Table 3.1).

When the hydroponate was passed through the 100 KDa and 50 KDa membranes, the LM25 signal was not retained by the membrane. When the hydroponate was passed through the 30 KDa membrane, the LM25 signal was retained (Figure 3.2). When using the larger 30 KDa cut-off point membrane within the ultrafiltration system, the MAbs with the highest signals (LM1, LM2, LM11 and LM25; Table 3.1) were used to assay the retentate and filtrate of the hydroponate. The signals from these MAbs were only present within the retentate, confirming that the 30 KDa cut-off point filter was retaining the high molecular weight compounds present within the hydroponate (Figure 3.3). This demonstrates that the 30 KDa membrane would retain four major polysaccharide epitopes within the hydroponate, and therefore, would be used to concentrate the high molecular weight compounds within the hydroponate.
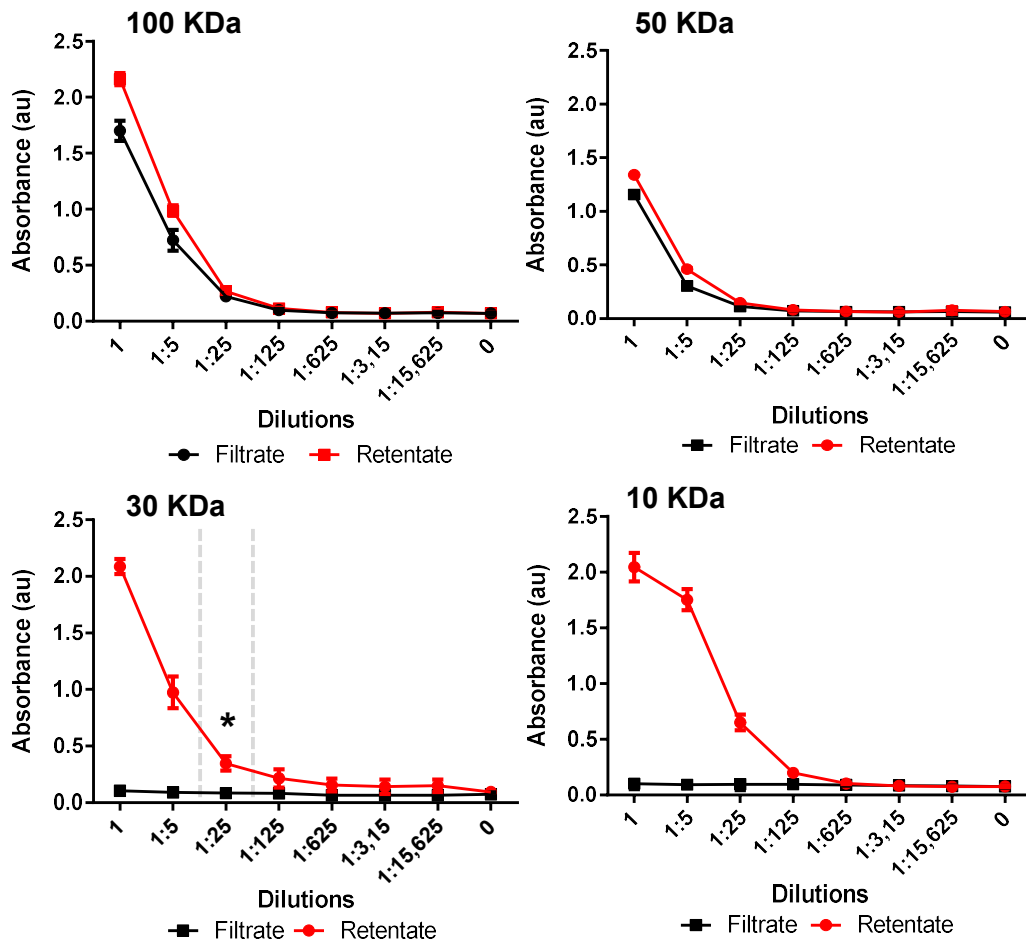
Figure 3.2 I **Determining the molecular weight cut-off point to use to concentrate the released polysaccharides within the hydroponate**
Aliquots of wheat cv. Cadenza hydroponate (1 mL) were added to a range of 2 mL Vivaspin tubes with the following cut-off points, 100 KDa, 50 KDa, 30 KDa and 10 KDa. Tubes were centrifuged for 5 mins at 3,893 x g. The resulting filtrate was then collected. Aliquots (1 mL) of dH_2O were then used to wash the filters to remove retentate. Both the filtrate and retentate were assayed using ELISA. As LM25 (xyloglucan) had the highest signals within the hydroponates of wheat, it was used to explore which membrane cut-off point to use for concentration. The signal of LM25 was retained when using the 30 KDa cut-off point. The filtrate fraction of the 30 KDa membrane contained no detectable amounts of xyloglucan epitopes, whereas, the retentate fraction contained high amounts of xyloglucan epitopes. Standard deviation bars are shown; asterisk indicates significant difference P= <0.05.

3.2.4 The four major epitopes were still present within the concentrated hydroponate of wheat cultivars

To determine the effects of concentrating the hydroponate on the polysaccharide epitopes, the major epitopes were re-assayed. Each bucket of hydroponate was filtered using Whatman paper filter to remove any particulates. Hydroponates were then concentrated (9 L to ~200 mL) using an ultrafiltration system that used a 30 KDa cut-off point membrane. After concentrating, the hydroponates were dialysed in dH_2O with six changes, and then freeze-dried. Known quantities (10 µg/mL) were re-suspended in 1x PBS, and assayed using an ELISA. To confirm that the concentrated hydroponate retained the major epitopes, LM1, LM2, LM11 and LM25 were used to probe the newly concentrated hydroponates of the wheat cultivars. A similar profile of the major epitopes was evident (Table 3.1 and Figure 3.3); LM25 had the highest signals followed by LM11, LM2 and LM1. This demonstrated that by concentrating the hydroponate the four majority polysaccharides were being retained (Figure 3.3).
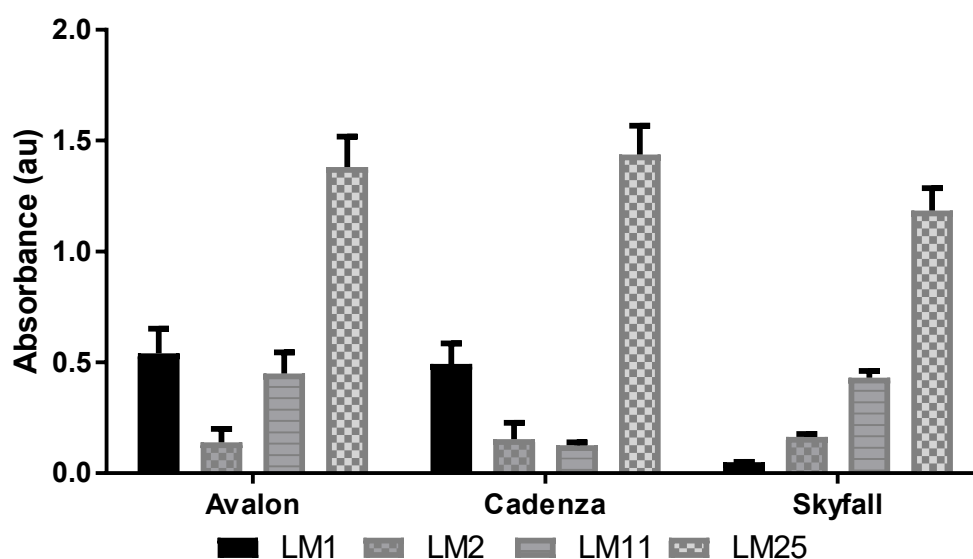
Figure 3.3 I **The four major polysaccharide epitopes detected within the hydroponate of the wheat cultivars were retained after concentrating**
A similar profile of released epitopes was detected after concentrating 9 L of hydroponate to ~200 mL. After concentrating, hydroponates were dialysed in dH2O with six changes, and then freeze-dried. Quantities of total dried hydroponate that was above 30 KDa (10 µg/mL) were re-suspended in 1x PBS for assaying. MAb signals of LM25 (xyloglucan) were the highest across the cultivars followed by LM11 (xylan), LM1 (extensin) and LM2 (AGP). Overall Skyfall had the lowest MAb signals when compared to the other cultivars. Data are a mean of three biological replicates. Twelve plants were grown per bucket, per cultivar, per replicate. Error bars indicate standard deviation.

3.2.5 Cell wall polysaccharide profile of released polysaccharides differs to the root body

After examining the released polysaccharides within the directly sampled and concentrated hydroponates of wheat, the cell wall polysaccharides of the root body were extracted. The resulting relative epitope levels of the root body were compared to the concentrated hydroponate. Cadenza was selected for further analysis due to the hydroponate of the cultivar containing the highest signals of the MAbs screened (Table 3.1). Once Cadenza had been grown hydroponically for two weeks, the roots were harvested, frozen in liquid nitrogen and freeze dried. Subsequently, the root material was homogenised and underwent a sequential series of dehydration steps. Once a sample of AIR was attained the material was extracted using 4 M KOH and 1% $NaBH_4$. The four major polysaccharide epitopes detected in the hydroponate of wheat, AGP, extensin, xylan and xyloglucan (Table 3.1) were used for this analysis. The epitope profiles of extensin (LM1) and xyloglucan (LM25) were higher within the hydroponate compared with the root body (Figure 3.4). The epitope profile of AGP (LM2) was similar in the hydroponate and the root body. However, the epitope profile of xylan was higher within the root body compared to the hydroponate (Figure 3.4).
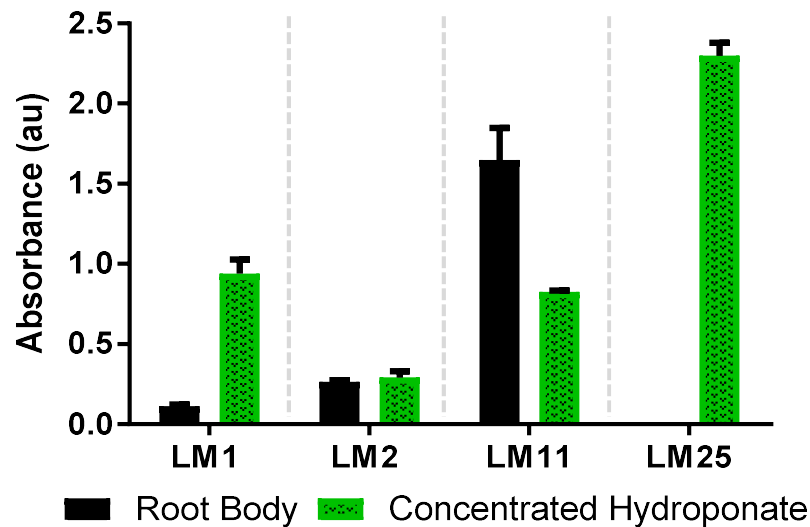
Figure 3.4 I **ELISA analysis of the root body and concentrated hydroponate of Cadenza** Wheat cv. Cadenza was grown hydroponically for two weeks. After two weeks, one wheat was taken from each bucket with the roots being cut from the stem and leaves. Roots were then frozen in liquid nitrogen and freeze dried. After drying, roots were homogenised with 10 mg of material being dehydrated using a sequential step of EtOH, acetone and methanol:chloroform. After dehydration, 1 mg of material was extracted using 4 M KOH and 1% $NaBH_4$. The epitope profiles of LM1 (extensin) and LM25 (xyloglucan) were higher within the hydroponate of wheat compared to the root body. However, the epitope profile of LM11 (xylan) was higher within the root body compared to the hydroponate. The epitope profile of LM2 (AGP) was similar when comparing the hydroponate with the root body. The 1:125 dilution was used for the analysis. Data are a mean of three biological replicates; one root system was selected per bucket. Relative ELISA absorbance values were determined by measuring the absorbance at 450 nm. Standard deviation bars are shown.

3.2.6 Cereal crops release relatively more xyloglucan compared to eudicotyledons

To expand knowledge of cell wall epitopes released by roots, epitope profiles of wheat were compared to five additional crop species, which were also grown hydroponically, barley (*Hordeum vulgare* cv. Golden Primrose), maize (*Zea mays* F1 cv. Earlibird), pea (*Pisum sativum* cv. Avola), tomato (*Solanum lycopersicum* cv. Ailsa Craig) and rapeseed (*Brassica napus* cv. Extrovert). These species represent a range of plant types including other cereals (barley and maize), Fabaceae or legumes (pea), Solananceae (tomato), and Brassica (rapeseed) which is a relative of Arabidopsis. Additionally, each of these species was found to have produced a strong root system within the hydroponic system chosen.

Each crop was germinated in 50:50 mixture of vermiculite and perlite for one week before being transferred to the hydroponics system. After two weeks, hydroponates were directly sampled and assayed using an ELISA. Two weeks was also found to be an effective period to allow polysaccharides to accumulate in the hydroponates of the species to be readily detected. The MAbs demonstrated that each crop released a unique profile of epitopes. Across the species, the relative epitopes of xyloglucan, extensin, xylan and AGP were present within the hydroponates screened (Table 3.2). For barley and maize, LM25 dominated the xyloglucan-binding MAb signals (Table 3.2). The only detectable AGP-related MAb relative signal within the hydroponates of barley and maize was LM2. This indicates that the AGP released by the roots of barley and maize contained β-linked glucuronic acid. Within the hydroponate of barley, LM11 was the only signal from the xylan-specific MAbs, indicating the presence of heteroxylan. For maize, there were signals from LM11 and LM12. The binding of LM12 within the hydroponate of indicates that the xylan released by the roots of maize is possibly feruloylated (Table 3.2; Pedersen *et al*. 2012). A signal from LM28, which recognises the epitopes of glucuronosyl substituted xylans, was also present in barley and maize hydroponates. Barley hydroponate had a high a signal of LM6, which indicated the present of RG-I (arabinan) or an AGP glycan (Table 3.2; Willats *et al.* 1998a).

The hydroponates of the eudicotyledons, pea, tomato and rapeseed, crops had relatively lower signals of LM25 compared to the cereals (Table 3.2). The highest xyloglucan-specific MAb signal was from LM25. Relatively weak signals of LM11 were detected across the eudicotyledons, indicating that they released low amounts of heteroxylan epitopes. The hydroponate of tomato had the highest relative signal of LM2, indicating the presence of β-linked glucuronic acid AGP epitopes. Whereas, for pea and rapeseed MAC207 had the highest signal, suggesting that they released different types of epitopes on the arabinogalactan domain present within AGP (Smallwood *et al*. 1996; Yates *et al*. 1996). Generally, there were higher signals from pectic and heteromannan MAbs within the eudicotyledon hydroponates compared to the cereal hydroponates. In particular, tomato had a high signal from JIM7 that binds to the epitopes of pectin (HG; Clausen et al. 2003), and LM6 that recognises the epitopes of RG-I (arabinan; Willats *et al.* 1998a; Table 3.2).

Antigen	MAb	Grasses (au)		Eudicotyledons (au)		
		Barley	Maize	Pea	Tomato	Rapeseed
Arabinogalactan-protein	**LM2**	0.61	0.23	0.23	2.73	0.03
	JIM4	-	-	0.05	0.06	-
	JIM14	-	-	0.06	0.08	-
	JIM15	-	-	0.05	0.09	-
	MAC207	-	-	0.06	0.98	0.63
β-linked Galactose	JIM1	-	-	0.06	0.08	-
Extensin	**LM1**	1.09	0.11	0.5	0.39	-
	JIM20	0.68	-	1	0.17	0.05
Heteromannan	LM21	-	0.01	0.07	0.1	0.03
	LM22	-	-	0.18	0.07	-
Pectic polysaccharides Homogalacturonan	LM7	0.02	-	0.08	0.09	0.01
	JIM7	-	-	0.05	0.81	0.02
	LM18	-	0.01	0.09	0.12	0.02
	LM19	0.01	0.02	0.08	0.2	0.1
	LM20	-	-	0.05	0.11	-
Rhamnogalacturonan-I	LM5	0.02	0.01	0.1	0.17	0.03
	LM6	1.48	-	0.07	0.62	-
	LM9	-	-	0.05	0.1	-
	LM13	-	-	0.06	0.1	-
	LM16	0.03	0.01	0.15	0.24	0.08
	LM26	-	-	0.08	0.07	0.02
Xylogalacturonan	LM8	-	-	0.07	0.1	-
Xylan	LM10	-	-	0	0.02	-
	LM11	0.59	0.29	0.11	0.19	0.5
	LM12	-	0.22	0.07	0.09	-
	LM28	0.14	0.45	0.08	0.15	0.12
Xyloglucan	LM15	-	0.01	0.08	0.07	0.05
	LM24	-	-	0.05	0.07	-
	LM25	1.04	1.15	0.19	0.15	0.47

Scale							
0.1	0.25	0.5	1	1.5	2	2.5	3

Table 3.2 l **Heat map of polysaccharides released from the roots of barley, maize, pea, tomato and rapeseed as determined by ELISA**
Twenty-nine cell wall monoclonal antibodies were used to screen the hydroponates of three-week-old barley, maize (F1 cv. Earlibird), pea, tomato and rapeseed through ELISA. From the heat map of the hydroponates, each species released a unique epitope profile. The relative epitope levels of AGP (LM2), extensin (LM1 and JIM20), xylan (LM11) and xyloglucan (LM25) were present across the crops. The relative signals of the pectic polysaccharide-related MAbs were overall higher within the eudicotyledons. The relative epitope levels of HG (JIM7) and RG-I (LM6) were high within the hydroponate of tomato. The epitope levels of RG-I (LM6) were also high within the hydroponate of barley. An aliquot (9 mL) from each bucket was removed for one replicate, 1 mL 10x PBS was then added to each aliquot for the ELISA. The 1:25 dilution of the barley and maize hydroponates was used for the analysis; 1:5 dilution was used for pea, tomato and rapeseed. Data are a mean of three biological replicates. Twelve wheat plants were grown per bucket, per species, per replicate. A cut off point of 0.1 au was removed, any values below this level were regarded as having no signal (-), shading of heat map was across the species screened.

3.2.7 Polysaccharide epitopes released by the roots of crops remained present after concentrating

To evaluate the effects of concentrating the hydroponate of other species, the major epitopes were re-assayed after concentrating. Each bucket of hydroponate was filtered using Whatman paper filter to remove any particulates. The hydroponates of barley, maize, pea, tomato and rapeseed were concentrated from 9 L to ~200 mL using an ultrafiltration system that used a 30 KDa cut-off point membrane. After concentrating, the hydroponates were dialysed in dH_2O with six changes, and then freeze-dried. Hydroponates (10 µg/mL) were re-suspended in 1x PBS, and assayed using an ELISA. A similar profile of polysaccharide epitopes across the species was apparent when comparing the hydroponates to the concentrated hydroponates (Table 3.2 and Figure 3.5). Barley and maize concentrated hydroponates contained the highest relative epitopes of xyloglucan, xylan and extensin (Figure 3.5). The concentrated hydroponate of tomato contained the highest relative signal of AGP. The concentrated hydroponate of pea had the lowest epitope levels across the hydroponates screened. The concentrated hydroponates of barley and tomato had the highest relative epitopes of RG-I, whereas, for the other species the relative epitopes of RG-I were low (Figure 3.5).
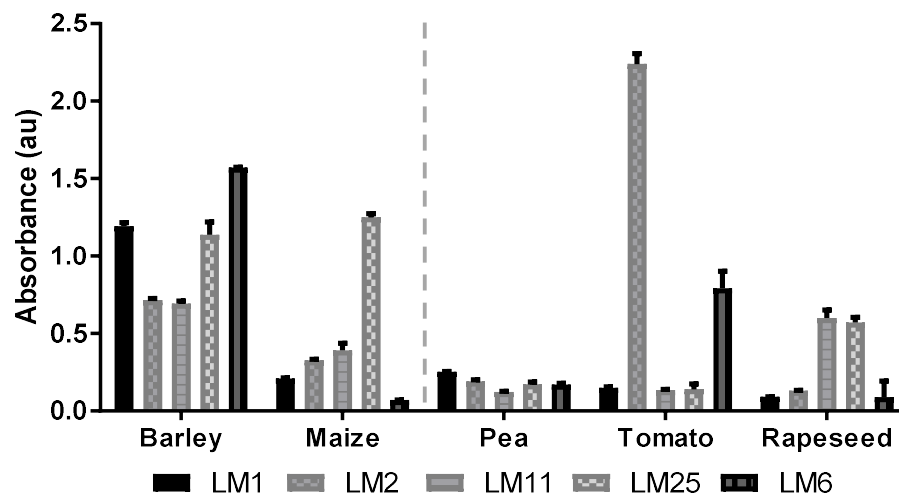
Figure 3.5 I **The major epitopes detected within the hydroponates of barley, maize, pea, tomato and rapeseed were retained after concentrating**
Hydroponic medium was concentrated from 9 L to ~200 mL prior to ELISA. Quantities of total dried hydroponate that was above 30 KDa (10 μg/mL) were re-suspended in 1x PBS for assaying. Across the species screened, there was high variation within the released epitope profiles. The epitopes of xyloglucan were the highest in barley and maize. Barley had the most epitopes of xylan and extensin. RG-I (arabinan/AGP) related epitopes were high within barley and tomato hydroponates. Epitopes of AGP were highest in the hydroponate of tomato. Pea had the overall lowest MAb signals. In the hydroponate of rapeseed, the epitopes of xylan and xyloglucan dominated. Data shown are a mean of three biological replicates. The 1:25 dilution of barley and maize concentrated hydroponates, and the 1:5 dilution of pea, tomato and rapeseed concentrated hydroponates were used for the analysis. Error bars show standard deviation.

3.2.8 The epitope profiles of barley wild type and a root hairless mutant differed

Barley wild type (*Hordeum vulgare* cv. Pallas Andrew*)* and the bald root barley mutant (brb; Gahoonia *et al.* 2001), which lacks root hairs, were grown in perlite and vermiculite (50:50) for a week prior to hydroponics. After a week, barley were transferred to the hydroponics system, and grown for a further two weeks. The resulting hydroponates were concentrated, and assayed using ELISA. Twenty-nine antibodies raised against cell wall polysaccharides were used to probe the concentrated hydroponates. The only polysaccharide epitopes detected within the hydroponates were LM2 and MAC207, AGP-specific; LM1 and JIM20, extensin-specific; LM11 and LM28, xylan-specific and LM25-specific, xyloglucan. In general, the heat map demonstrated that the MAb signals were lower within the hydroponate of the barley root hairless mutant compared to the wild type (Table 3.3). The only MAb signal that was higher within the hydroponate of brb compared to the wild type was LM25, that recognises the epitopes of xyloglucan, was 4.7x higher (Table 3.3).

	MAb	Barley	
		WT	**brb**
Arabinogalactan-protein	LM2	0.99	0.46
	JIM4	-	-
	JIM14	-	-
	JIM15	-	-
	MAC207	0.37	-
β-linked Galactose	JIM1	-	-
Extensin	LM1	1.67	1.28
	JIM20	1.37	0.86
Heteromannan	LM21	-	-
	LM22	-	-
Pectic polysaccharides	LM7	-	-
Homogalacturonan	JIM7	-	-
	LM18	-	-
	LM19	-	-
	LM20	-	-
Rhamnogalacturonan-I	LM5	-	-
	LM6	-	0.01
	LM9	-	-
	LM13	-	-
	LM16	-	-
	LM26	-	-
Xylogalacturonan	LM8	-	-
Xylan	LM10	-	-
	LM11	0.35	0.12
	LM12	-	-
	LM28	0.04	0.06
Xyloglucan	LM15	-	-
	LM24	-	-
	LM25	0.50	2.33

Scale						
0.1	0.25	0.5	1	1.5	2	2.5

Table 3.3 I **Heat map of the polysaccharides released from barley wild type (WT) and the bald root barley (brb) mutant as determined by ELISA**
Twenty-nine monoclonal antibodies raised against cell wall polysaccharides were used to screen the hydroponates of three-week-old barley through ELISA. Hydroponate of barley was concentrated from 9 L to ~200 mL prior to ELISA. Overall, the signals from the MAbs are lower within the barley brb mutant compared to the wild type (cv. Pallas Andrew). The signal of LM25 is much higher within the concentrated hydroponates of the barley mutant. An aliquot (9 mL) from each bucket was removed for one replicate, 1 mL 10x PBS was then added to each aliquot for the ELISA. The 1:25 dilution of the respective concentrated hydroponates was used for the analysis. Data are a mean of three biological replicates. Twelve barley plants were grown per bucket, per species, per replicate. A cut off point of 0.1 au was removed, any values below this level were regarded as having no signal (-), shading of heat map was across the wild type and brb screened.

3.3 Discussion

3.3.1 Monoclonal antibodies and hydroponic systems are useful tools to explore polysaccharides released by roots

For the first time, MAbs have been extensively used to directly probe the hydroponic medium of crop species. These highly sensitive molecular probes detected an abundance of AGP, extensin, xylan and xyloglucan within the hydroponates of the cereals screened, particularly from the roots of wheat. Within the hydroponic media of the eudicotyledons screened, there was more variation between the species for instance; there was an abundance of AGP within the hydroponate of tomato. However, within the hydroponate of pea the signals from the AGP-specific MAbs were low. The epitope profiles of the root body and concentrated hydroponate of wheat differed, suggesting that these polysaccharides released from roots were being produced to be secreted. Interestingly, the root growth of the wheat cultivars did not significantly differ, yet the release of polysaccharides from their roots did. This suggests that there is a possible genetic factor behind the secretion of these molecules. By growing plants hydroponically and concentrating their hydroponate (Table 3.1 and Figure 3.3), this investigation has developed a novel method of attaining sufficient material for detecting the presence of a wide range of polysaccharides released by roots. Screening the hydroponic medium of the crops used in this study has led to the understanding that each plant released a unique epitope profile (Table 3.1 and Table 3.2).

Prior to this study, time consuming methods had to be undertaken to identify the polysaccharides that were being released by roots. One commonly used method, involved excavating plant roots from the soil in order to collect root mucilage (Morel *et al*. 1986; Mounier *et al*. 2004). Another method involved collecting released polysaccharides from seedlings that were grown in the dark on moist filter paper (Read and Gregory 1997). After a set number of days, released polysaccharides could be collected by pipetting the droplets of mucilage that percolated through the root caps. Alternatively roots were suspended in sterile deionised water (Bacic *et al*. 1986; Moody *et al*. 1988; Osborn *et al*. 1999). These methods of collection would have placed plants under stress, which could inadvertently affect the rate and types of polysaccharides released by roots. After collection, monosaccharide composition and monosaccharide linkage analyses would have to occur to reveal which polysaccharides were present. Monosaccharide composition and monosaccharide linkage analyses are the standard physio-chemical methods employed to determine what monosaccharides were present and how they were linked together. MAbs are

highly sensitive molecular probes that are able to detect minor components of which these physio-chemical methods may not detect (Lee *et al.* 2011). Additionally, immuno-based assays such as ELISA require less purification than physio-chemical methods (Lee *et al.* 2011). Thus these methods can be combined to develop a more detailed understanding of polysaccharides released by roots.

A major limitation of MAbs and CBMs is that the resulting signals must be carefully interpreted as the binding affinities of each molecular probe can vary (Pattathil *et al*. 2012; Gilbert *et al.* 2013). However, using commercial standards to quantify absorbance units generated by MAbs and CBMS could reduce this effect. Another limitation is that if a sample is abundant in molecules they can begin to compete the binding sites of MAbs and CBMs within an ELISA (Marcus *et al*. 2008). This molecular crowding can mask epitopes of polysaccharides giving a distorted understanding of what polysaccharide is present and in what amount. It has been reported that xyloglucan, xylan and mannan can be effectively masked by the present of pectin during fluorescence microscopy of the primary cell walls (Hervé *et al.* 2009; Marcus *et al.* 2010). Using higher dilutions of the sample can reduce the concentration of polysaccharides, and thus the masking of the epitopes. An additional factor to consider when using these probes is that there might be a molecule that is present within a sample that neither a MAb nor CBM recognises, which may lead to a narrow understanding of that sample. Thus careful interpretation is required when using these molecular probes.

Using a hydroponic system ensured that the crops were subjected to close to as natural conditions as possible, nevertheless, ensuring strict control conditions, and ease of isolating released polysaccharides. Hydroponics also ensures that only the roots are in contact with the medium. This makes certain that these polysaccharides are released from roots and not released from other sources, including the seed coat (Haughn and Western 2012). Despite the efficiency and ease of isolating polysaccharides, hydroponics still remains an unnatural system to grow plants. Nevertheless, studies have optimised hydroponic systems for growing many crops (du Toit and Labuschagne 2007; Giecornmelli *et al*. 2015). Organisations such as Thanet Earth are already successfully contributing to UK food production; frequently growing crops, including tomato, lettuce, cucumber and peppers, hydroponically that are more cost effective compared to field grown crops.

3.3.2 Polysaccharides released from the roots of wheat may be produced to be secreted into the rhizosphere

A diverse range of polysaccharides were released into the hydroponates of crops screened. Crops were selected because of their large root networks, maximising the probability of collecting sufficient material for biochemical analysis, but also as some of the species had already been identified to release polysaccharides (Bacic *et al*. 1986; Moody *et al*. 1988; Read and Gregory 1997). Crops were also selected due to their tolerance for hydroponic culture (Arnon 1983; du Toit and Labuschagne 2007), and for the potential applications of this research on their production. There was an abundance of xyloglucan, xylan, extensin and AGP with β-linked glucuronic acid in the hydroponate of wheat (Table 3.1). This confirms previous work that had detected the presence of AGP, xylan and xyloglucan through the use of monosaccharide linkage analysis (Moody *et al*. 1988). The glycoprotein extensin has not previously been detected within the exudates of plants. The role of extensin within exudate remains unclear, however, they are abundant within the primary cell walls of roots, vascular bundle, and at the surface of epidermal cells (Smallwood *et al*. 1995), making more prone to be sloughed off and released into the rhizosphere.

Reasons for the abundance of xyloglucan within the hydroponates of cereals remain unclear. When the hydroponates of three wheat cultivars were screened, there were differences in their epitope profiles (Table 3.1). This was also observed when growing two cultivars of barley (Table 3.2 and Table 3.3). These observations suggest that there is a genetic factor behind the release of these molecules, further alluding to their importance. Perhaps, this indicates these polysaccharides, particularly xyloglucan act as functional molecules outside of the root body. Another interesting observation is the difference in the epitope profiles of the root body and hydroponate of wheat (Figure 3.4). If these polysaccharides solely derived from lysed cell wall components then the epitope profiles of the polysaccharides would not differ within the root body, and the hydroponate. This suggests that wheat roots, in particular, are producing these polysaccharides to be released whether that is through constant release through active secretory mechanisms or border cells (Northcote and Pickett-Heaps 1966; Guinel and Gregory 1986; Stephenson and Hawes 1994; Mravec *et al*. 2017).

As root caps and tips release root mucilage to aid soil penetration, it may then be possible that the root hairs also release root mucilage to aid their penetration into soil as well as secure the rhizosheath. If root hairs release polysaccharides then this

would greatly increase the surface area of which these molecules are secreted. This may explain the difference in epitope profiles of the root body and hydroponate of wheat. These single celled hairs have not been demonstrated to be involved in the secretion of any organic substances. However, there is a strong indication that root hairs play a role in releasing polysaccharides into the soil. From the barley wild type and root hairless mutant heat map (Table 3.3), the MAb signs were mostly lower within the mutant, suggesting they may be releasing polysaccharide. The only signal that was higher in the hydroponate of the mutant was from xyloglucan. Reasons for the higher signals of xyloglucan remain unclear, potentially highlighting the importance of xyloglucan as a released polysaccharide. Perhaps, polysaccharides released from roots are secreted by different regions of the root system for example, xyloglucan may be released by the root hairs, whereas, AGP and pectin are release by the root caps and tips. It would be interesting to examine the temporal dynamics of polysaccharides released from plant roots. Monitoring the levels of the major polysaccharide epitopes within the hydroponate of a plant, from seedling to senescence, would enable a glimpse into how their secretion alters over time.

Growing plants hydroponically does not reflect plants that are growing in soils. When plants are grown hydroponically their roots are not faced with high friction as they grow deeper into the medium. However, within soils the roots are subjected to high amounts of friction as they penetrate through the medium to get to the resources that they require. It would be interesting to determine how hydroponics affects the release of polysaccharides from roots and their composition. Since no other studies have used hydroponics to explore released polysaccharides, more research is required to understand these effects. These effects could be studied by growing wheat in an invert transparent soil such as glass beads, which could then be washed with KOH to extract the polysaccharides released from the roots.

Other polysaccharide epitope profiles were developed on two other cereal crops. These profiles indicate that the major polysaccharides within the hydroponate of barley and maize were of AGP, extensin, xylan and xyloglucan (Table 3.2). These polysaccharides (excluding extensin) released from maize roots have also been detected by other investigations, which use maize as a model for root mucilage (Bacic *et al*. 1986; Moody *et al*. 1988). This investigation determined that there was a lack of pectin within the hydroponates of the crops, with the exception of barley where RG-I was found. In general, a lack of pectin has also been determined within the root growth media of cereals (Table 1.1; Moody *et al*. 1988; Guinel *et al*. 2000;

Sims *et al*. 2000), which conform to known grass cell wall biochemistry, where pectic polysaccharides are present in trace amounts (Nishitani and Nevins 1989; Carpita 1996; Vogel 2008). This supports the lack of pectin in the hydroponates of cereals tested. However, previous work has determined the presence of pectin within the root growth media of a number of eudicotyledons (Table 1.1, Ray *et al.* 1988; Osborn *et al.* 1999; Narasimhan *et al.* 2003). This investigation uncovered the presence of HG and RG-I within the hydroponate of tomato. However, the signals from the pectin-related MAbs were generally very low to not present across the other eudicotyledons. Additional research is required to further understand the differential in the detection of pectin uncovered in the hydroponates of eudicotyledons, and the detection of pectin within previous investigations. More research is also needed to understand the detected of RG-I (LM6; Table 3.2) within the hydroponate of barley.

There were no signals of LM8 (XGA) in the hydroponates probed. This is an interesting observation as LM8 has been shown to be specific to the root cap cells of pea and maize (Willats *et al.* 2004), which have also been used for this investigation. This may indicate that there were issues related to the solubility of XGA, which could be tightly attached to root cap cells. This may also be an indication that the root cap cells are not lysing within the hydroponic medium as roots are not subjected to friction caused by soil. This could support the hypothesis that these polysaccharides are being produced to be released. Further research is required to immuno-label the hydroponically grown roots using LM8 to determine if XGA is present within the root cap cells, as previously determined (Willats *et al.* 2004). If LM8 labels the root cap cells but is not detected within the hydroponate, this is a good indication that the roots are releasing polysaccharides not as a secondary effect but as a direct means. This would support their possible functional importance outside of the root body. If root mucilage, which contains AGP and pectin (Miki *et al*. 1980; Morel *et al.* 1987; Read and Gregory 1997), forms a highly viscous layer surrounding root caps and tips, it is conceivable that it remains at the root caps and tips of plants when grown hydroponically. Droplets of root mucilage were visible when plants were lifted from the hydroponic medium, as previously described (McCully and Sealey 1996; Read and Gregory 1997), but were disturbed when plants were harvested. Perhaps, some polysaccharides released, such as xyloglucan, are more soluble upon release compared to their more viscous counterparts, AGP and pectin. To confirm this hypothesis, plants could be grown on nitrocellulose sheets, which are highly absorbent, could be used to absorb

polysaccharides released by roots. These nitrocellulose sheets could then be screened by MAbs to determine how far these molecules diffuse from the root body.

3.3.3 Eudicotyledons release different polysaccharide epitope profiles to their cell walls

The hydroponates of eudicotyledons typically contained relatively more pectic polysaccharides, corresponding with their cell wall biochemistry (Table 3.2; Carpita 1996; Vogel 2008). AGP, extensin and xyloglucan were also present in the hydroponate of eudicotyledons (Table 3.2). However, AGP was predominately detected by a different MAb in rapeseed (MAC207). There was also a high signal from MAC207 from the hydroponate of tomato (Table 3.2). One possible explanation for the different AGP-specific MAb signals is that the glycan proportion of the AGP detected is heterogeneous, which is well documented (Bacic *et al.* 1997; Kieliczewski 2001; Cosgrove 2005). Another study had also reported the presence of AGP, pectin and xyloglucan from pea root mucilage (Narasimhan *et al*. 2003), supporting the released epitope profile of pea developed by this study. The eudicotyledons appeared to have greater diversity of released polysaccharides within their hydroponates compared to the hydroponates of cereal. The hydroponates of the eudicotyledons contained polysaccharides that were similar to their cell wall components (Table 3.2; Moody et al. 1988; Vogel 2008). This was reinforced by previous research, which undertook monosaccharide linkage analysis on the released polysaccharides of cowpea (Moody *et al*. 1988; Read and Gregory 1997), Arabidopsis (Chaboud and Rougier 1984), cress (Moody *et al*. 1988; Ray *et al*. 1988; Osborn *et al*. 1999; Sims *et al*. 2010) Indian rhododendron (Moody *et al*. 1988) and lupin (McCully and Sealey 1996; Read and Gregory 1997).

3.4 Conclusion

Monoclonal antibodies are useful diagnostic tools for exploring polysaccharides released from plant roots into a hydroponic medium. For the first time, crops have been grown hydroponically to isolate their released polysaccharides. The major polysaccharides uncovered within the hydroponates of the crops screened were xyloglucan, xylan, extensin and AGP. Other polysaccharides were uncovered but were not represented across the species for instance; there was an abundance of RG-I within the hydroponates of barley and tomato. There was also a high detection

of pectin (HG) within the hydroponate of tomato. There was a higher relative signal of xyloglucan within the hydroponates of cereal. On further analysis, the relative epitopes of AGP, extensin and xyloglucan were more abundant within the hydroponate of wheat compared to the root body. Additionally, there were was no detection of LM8 (XGA), which is specific to root cap cells, within the hydroponates screened. These results support the hypothesis that these molecules are being formed to be released by roots. When comparing the relative epitopes levels of barley wild type and the root hair-less mutant, xyloglucan was found to be higher within the hydroponate of the mutant. This suggests that the root hairs may play a role in the secretion of polysaccharide. The root growth of the three wheat cultivars grown did not significantly differ, yet the polysaccharides released into their hydroponates differed. This was also observed within the hydroponates of two cultivars of barley. These observations indicate that there may be a genetic factor involved in the release of polysaccharides by roots.

Chapter 4

A multi-polysaccharide complex is released by the roots of cereal

4.1 Introduction

Our understanding of the biochemical properties of polysaccharides released by plant roots remains largely uncomplete. Difficulty in collecting sufficient material from roots has remained a limiting factor in deciphering the biochemistry and structure of these polysaccharides. In order to explore micro-structures, polysaccharide complexes, within the cell wall matrix highly sensitive techniques have been developed, namely, Epitope Detection Chromatography (EDC) amongst other immunochemical assays. EDC utilises chromatography, including anion-exchange chromatography and size-exclusion chromatography, to elute fractions of polysaccharides, which are then probed with a range of monoclonal antibodies through ELISA (Cornuault *et al*. 2014). A gradient of sodium chloride is used during anion-exchange EDC to separate polysaccharides by charge. For instance polysaccharides that elute prior to sodium chloride have no charge as no salt was required to elute them. Highly charged polysaccharides, including pectin require high amounts of salt to be eluted (Figure 4.1). During size-exclusion EDC polysaccharides are eluted by size. Large polysaccharides have less interaction with the column matrix as they cannot pass through the small pores, thus eluting early. Small polysaccharides and oligosaccharides are able to pass through the pore spaces and thus elute later.

EDC can also be used to explore intra-molecular interactions, sub-families of polysaccharides, namely pectin, and multi-polysaccharide complexes. This technique can also be used on the nanogram and microgram scales, which is useful for deciphering polysaccharide-polysaccharide and polysaccharide-protein links as well as multi-polysaccharide complexes that may be present as minor components of the cell wall architecture (Cornuault *et al*. 2015). In addition to EDC, sandwich-ELISA can be used to explore co-linked polysaccharides. Within this technique, a CBM is used to coat a microtitre plate, which binds to one co-linked partner. Samples containing this hypothesized intra-molecular interaction are then added to the plate. MAbs are then used to probe the sample (Cornuault and Knox 2014). If there is a link between the partners then there will be a signal. These tools when combined form a powerful approach to determine the presence of these nanogram to microgram scale multi-polysaccharides complexes.

This chapter explores the biochemical properties of polysaccharides released into the concentrated hydroponate of wheat cv. Cadenza (hereon referred to as Cadenza) using these highly sensitive techniques, with a focus on anion-exchange

EDC. A survey on the root cell walls as well as the polysaccharides released by wheat roots was conducted. To confirm the observations made by the antibody-based approach a glycan sample was provided to the Complex Carbohydrate Research Centre (CCRC) to undertake monosaccharide composition and monosaccharide linkage analyses. The biochemical properties of other cultivars of wheat, Avalon and Skyfall were compared to Cadenza along with other species of cereal, maize and barley, and eudicotyledons, pea, rapeseed and tomato, and a basal land plant, liverwort.
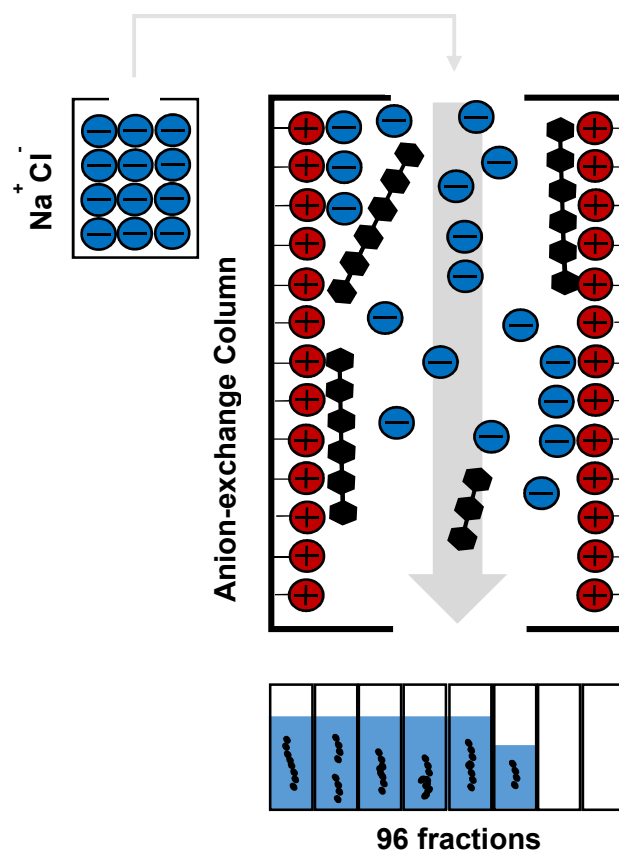
Figure 4.1 I **Schematic diagram that shows anion-exchange EDC**
A salt gradient (0.6 M) is run after a sample containing polysaccharides is injected into an anion-exchange column. Within the column, the column matrix is positively charged, which attracts the polysaccharides that have a negative charge due to the presence of carboxyls. As the salt gradient is increased, the chloride ions, which are negatively charged, elute the polysaccharides that are retained within the column. This elution is caused by the chloride ions outcompeting with the polysaccharides bound to the matrix. Polysaccharides that are slightly negative are eluted with little salt, and polysaccharides that are highly negative are eluted with high amounts of salt. The resulting fractions are collected and then transferred into 96 microtitre plates. The microtitre plates are subsequently developed using the ELISA protocol.

4.2 Results

4.2.1 Four released epitopes co-eluted during anion-exchange EDC

To explore the biochemistry of the released polysaccharides, Cadenza concentrated hydroponate (50 µg) was suspended in 20 mM sodium acetate buffer (Figure 4.2). The buffer with the hydroponate was injected into a weak 1 mL anion-exchange chromatography column. A step gradient of sodium chloride (0.6 M) was used to elute the polysaccharides within the concentrated hydroponate, into 1 mL fractions. Aliquots (40 µL) of 1 M sodium carbonate were then placed into each fraction to increase the pH. This facilitated the binding of the molecules within the sample to the wells of a microtitre plate for ELISA. MAbs were used to screen the wells of the ELISA plate. There were two peaks of LM25 (xyloglucan) neutral (arrow **1**) which eluted before the salt gradient, and acidic (arrow **2**) which eluted as the salt gradient was increased. Within the second acidic peak of xyloglucan epitopes, there was a clear co-elution of the AGP, extensin and xylan epitopes (Figure 4.2, **A**). When comparing the commercial standard of xyloglucan, tamarind seed xyloglucan, with the xyloglucan in the concentrated hydroponate, the xyloglucan within the hydroponate appeared to be acidic (Figure 4.2, **B**). As tamarind seed xyloglucan eluted prior to the salt gradient it indicated that the commercial standard was neutral unlike the xyloglucan from the hydroponate which required ~300 mM NaCl to elute from the column.
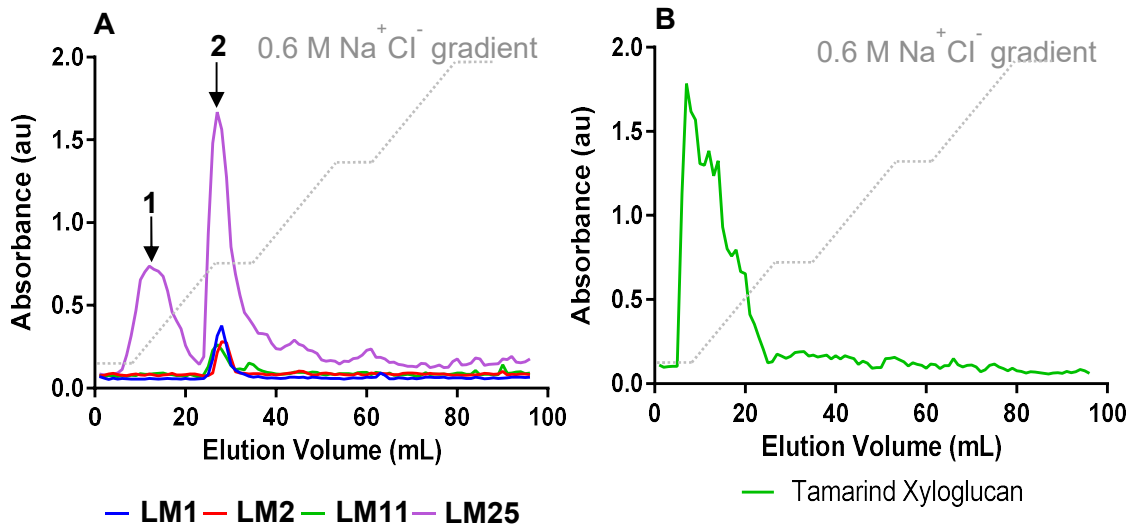
Figure 4.2 I **Anion-exchange EDC analysis of polysaccharides released from the roots of Cadenza**
Fifty micrograms of concentrated Cadenza hydroponate was injected into a 1 mL weak anion-exchange chromatography column. Aliquots of 100 µL (containing ~5 µg) of each collected fraction were assayed for each MAb. A step gradient of 0.6 M of NaCl was used (**A**). Two forms of xyloglucan, neutral (**1**) and acidic (**2**), were detected from the released polysaccharides. A co-elution of AGP, extensin and xylan was present within the second peak of xyloglucan (**2**) which was acidic. Data shown are a mean of three biological replicates. (**A**). Tamarind seed xyloglucan (100 ng) was injected into the same 1 mL anion-exchange column (**B**). The tamarind seed xyloglucan eluted before the salt gradient, indicating that the polysaccharide was neutral (**B**). Data shown are a mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm.

4.2.2 Delaying the gradient of salt retained the acidic and neutral forms of xyloglucan within the anion-exchange column

To examine the co-elution in more detail, samples (50 µg) of Cadenza concentrated hydroponate were passed through an anion-exchange chromatography column (1 mL). Samples were eluted using a delayed gradient of sodium chloride (0.6 M). This gradient had a steeper increase in salt compared to the gradient previously utilised (Figure 4.2) in an attempt to tease a part the neutral and acidic fractions. The neutral (arrow **1**) and acidic (arrow **2**) forms of xyloglucan remained for longer within the column when using the delayed gradient of salt. The acidic form of xyloglucan was retained for longer within the column compared to using the step gradient. Additionally, the amount of salt needed to elute the co-elution remained the same, ~300 mM. The co-elution of AGP, extensin and xylan epitopes also remained within the acidic form of xyloglucan (Figure 4.3). The signal of LM11, which binds to the epitopes of xylan, had slightly increased compared to the other gradient used (Figure 4.2, **A** and Figure 4.3). This increase was observed throughout the three biological replicates. Delaying the gradient of salt divided the neutral and acidic peaks, perhaps reducing molecular crowding on the ELISA plates, which probably account for this increase in signals.
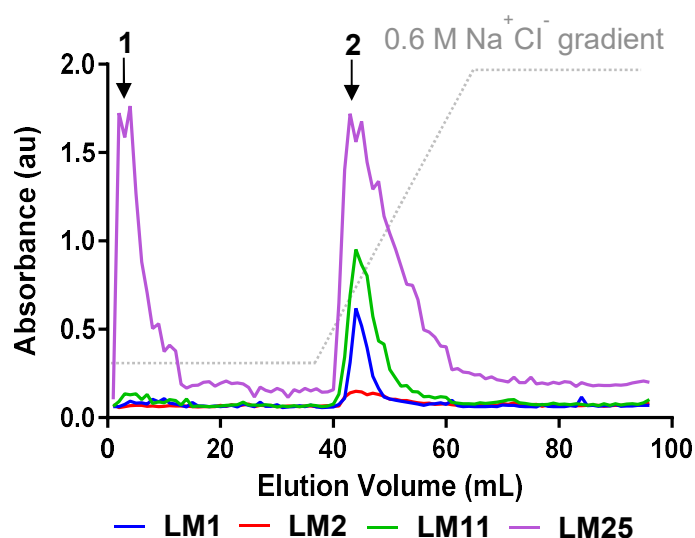
Figure 4.3 I **The co-elution of AGP, extensin, xylan and xyloglucan was retained for longer within the column when the gradient of salt was delayed**
Epitope detection chromatogram of 50 µg concentrated Cadenza hydroponate. The chromatogram reveals that the two forms of xyloglucan; neutral xyloglucan (fractions 0-16; **1**) and an acidic xyloglucan (fractions 40-60; **2**) that was detected using LM25 was retained in the column for longer before the salt gradient. The co-elution of extensin (LM1), AGP (LM2) and xylan (LM11) also remained stable when delaying the salt gradient. The acidic peak of xyloglucan was retained for longer within the anion-exchange column under the longer neutral gradient. Data shown are a mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm.

4.2.3 Acidic and neutral forms of xyloglucan were isolated

Using the delayed gradient of salt, the neutral and acidic forms of xyloglucan could be isolated (Figure 4.4). Samples of concentrated hydroponate (3 mg) were suspended in 20 mM sodium acetate buffer, and injected into a larger, 15 mL weak anion-exchange chromatography column. Fractions of 15 mL were collected, and assayed using LM1 (extensin), LM2 (AGP), LM11 (xylan) and LM25 (xyloglucan). Increasing the throughput by 15x enabled the isolation of these two forms of xyloglucan. The co-elution remained with what appeared to be the acidic xyloglucan (Figure 4.4, **B**). Furthermore, similar patterns of the epitopes within the co-elution remained stable after isolation. The neutral form of xyloglucan also remained unchanged when compared to previous assays (Figure 4.2, **A** and Figure 4.4, **A**). Further examination of the anion-exchange chromatograms (Figure 4.2, Figure 4.3 and Figure 4.4), including shallower salt gradients (not included), revealed that the co-elution of REC1 was eluted with a salt concentration of ~300 mM. This co-elution of four epitopes, AGP, extensin, xylan and xyloglucan from the roots of Cadenza is referred to as Root Exudate Complex 1 (REC1).
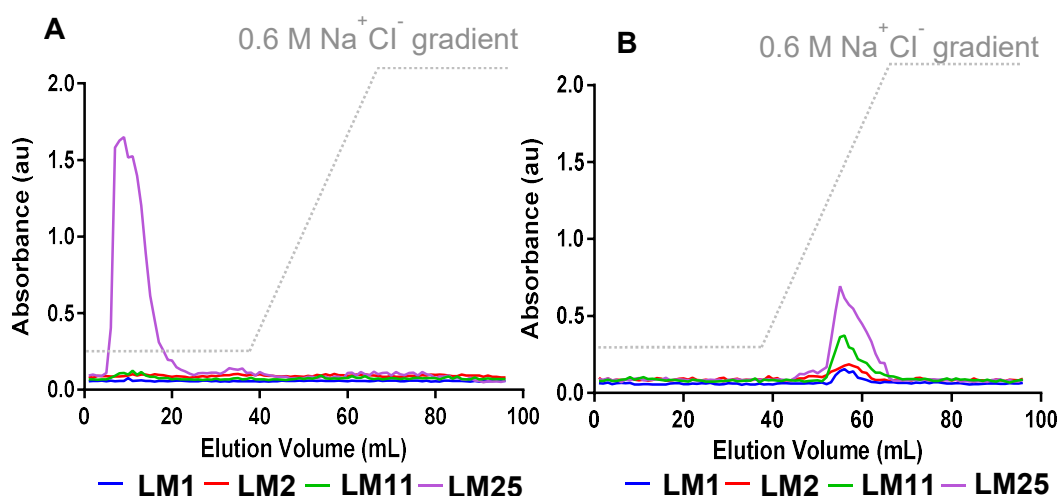
Figure 4.4 I Isolation of the neutral and acidic forms of xyloglucan
Anion-exchange EDC of 5 μg isolated neutral fractions (**A**) and 5 μg acidic fractions (**B**). Three milligrams of concentrated wheat hydroponate was injected into a 15 mL column. After collecting, samples were freeze-dried, dialysed and re-run through anion-exchange EDC. Fifty micrograms of isolated fractions were injected into a 1 mL column for this analysis. After the re-run both the neutral fractions (**A**) and acidic fractions (**B**) were detected independently. This co-elution of AGP (LM2), extensin (LM1), xylan (LM11) and xyloglucan (LM25) within the second acidic peak is referred to Root Exudate Complex 1 (REC1). Each data point is a mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm.

4.2.4 Size-exclusion EDC demonstrated high heterogeneity within the sizes of the polysaccharides in REC1

To examine the sizes of the polysaccharides released by Cadenza roots (REC1), and within its root body, size-exclusion EDC was undertaken that used a different column matrix. Isolated REC1 (100 µg; Figure 4.4) as well as extracted polysaccharides from the root body (50 µg) were injected into a 120 mL size exclusion column, and eluted using 1 M NaCl. The resulting elutions were collected into 96 1 mL fractions. Aliquots (40 µL) of 1 M sodium carbonate were then placed into each fraction to increase the pH, which helped to facilitate the binding of the molecules within the sample to microtitre plate wells for ELISA. The larger the molecule, the lower amount of interaction that molecule will have with the column as it percolates, thus large molecules will elute and appear early on the chromatogram. From the analysis, there was a large range of sizes within the polysaccharides of REC1 (Figure 4.5, **A**) and the root body (Figure 4.5, **B**). In particular, there was a wide size range of xyloglucan molecules that were in REC1 when compared to the commercial standard, tamarind seed xyloglucan (Figure 4.5). This was also reflected by the range of xylan molecules detected released by wheat roots. There is a small signal of LM2 (AGP), which eluted just before fraction 40. There is also a large signal of LM1 (extensin), between fractions 40 and 65 (Figure 4.5). When examining the root body, there were two clear peaks of xylan, which was reflected by a lower signal trace of xyloglucan (Figure 4.5, B). There were no signals from LM1 and from LM2 in the root body. The xyloglucan that was contained within the root body and the hydroponate considerably varied to tamarind seed xyloglucan (Figure 4.5). Unexpectedly, the chromatographic trace of tamarind seed xyloglucan (Figure 4.5, **B**) appeared similar to that of the anion-exchange EDC. This shows that the xyloglucan within tamarind seed tested was very high in size (Figure 4.2).
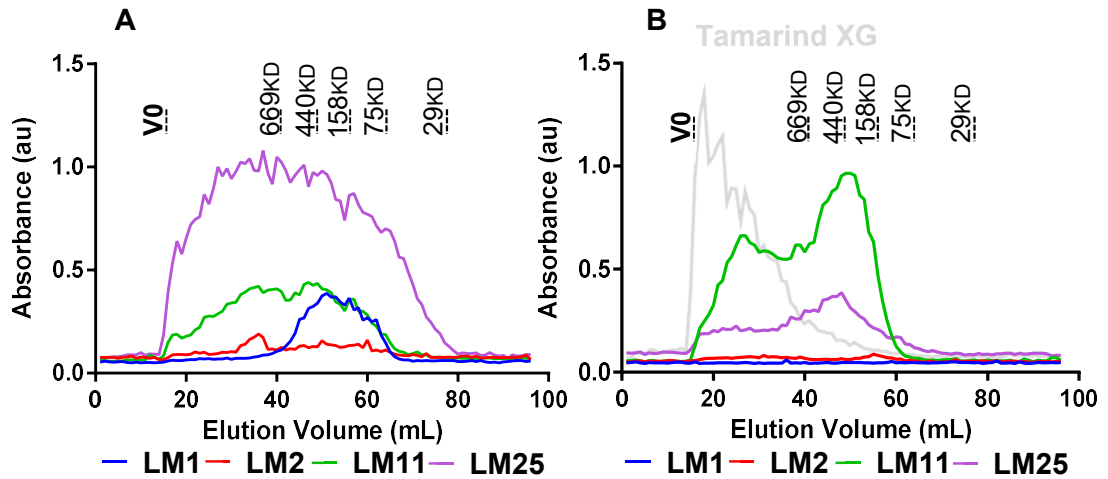
Figure 4.5 I **Complex heterogeneity in polysaccharides size is present within REC1 and the root body**
Size-exclusion EDC revealed a large range of differentially sized AGP (LM2), extensin (LM1), xylan (LM11) and xyloglucan (LM25) molecules in REC1 (**A**) and root body (**B**). This range of differentially sized polysaccharides also differs between what is present within the root cell walls (**B**) compared to what is released (**A**). Tamarind seed xyloglucan (100 ng) screened using LM25 was also injected into the size exclusion column with the same data inserted onto the above. Tamarind seed xyloglucan (grey) is a large molecule that contains fewer smaller domains compared to the hydroponate (**A**) and root body (**B**). Samples of the acidic co-elution (100 µg) and root body cell wall extract (50 µg) were each dissolved into 1 mL of high salt buffer, and injected into a 120 mL size exclusion column. The resulting fractions were directly probed with the MAbs. Each data point is a mean of two biological replicates. A gel filtration calibration kit containing five protein standards was used to calculate KDa values displayed. ELISA absorbance values were determined by measuring the absorbance at 450 nm, V0 = void volume.

4.2.5 Total carbohydrate analysis revealed the presence of undetected glycan within the anion-exchange EDC

To determine the total glycan content of the concentrated hydroponate of Cadenza, a Phenol-Sulphuric Acid assay was carried out on fractions of the anion-exchange EDC analysis (Figure 4.3). For this assay, 50 µL from each of the 96 fractions collected during anion-exchanged EDC were removed. Aliquots (50 µL) of concentrated sulphuric acid and 5% (w/w) phenol in dH_2O (30 µL) were sequentially added. Glucose was used to convert the absorbance units into total µg per fraction. When all fractions of the anion-exchange EDC were totalled, there was 300 µg of glycan detected. As there was 300 µg used for the anion-exchange EDC analysis, this indicates that the total concentrated hydroponate of Cadenza is formed of glycan (Figure 4.6). Within the neutral region of the chromatogram there was a high detection of total carbohydrate. This total carbohydrate was present within the neutral xyloglucan fractions (1-15), and between the neutral xyloglucan fractions and REC1 fractions, marked X (Figure 4.6). This second region of total neutral glycan (16-35) did not follow the MAb peaks on the chromatogram trace. There was also a third peak of total carbohydrate (40-60), which was within the acidic region of the chromatogram (Figure 4.6).
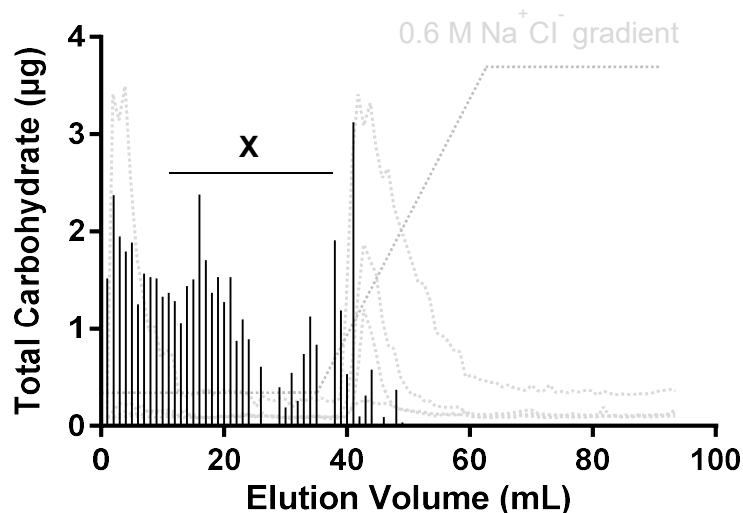
Figure 4.6 I **Total carbohydrate analysis of the concentrated hydroponate of Cadenza using the anion-exchange EDC system**
Aliquots (50 µL) of concentrated hydroponate were collect from each 1 mL fraction from the same anion-exchange EDC assay within Figure 4.3. An overlay of the anion-exchange EDC results from Figure 4.3 has been added. The Phenol-Sulphuric Acid fractions were read using the absorbance at 450 nm. From this analysis, there were three forms of total carbohydrate. Fractions 1 to 15, and fractions 16-35 show high concentrations of carbohydrate within the neutral region. The second neutral form of total carbohydrate does not match the two peaks detected within the EDC (**X**). The third form of total carbohydrate, between fractions 40 and 60, reveals the presence of acidic carbohydrates. Data are a mean of three biological replicates.

4.2.6 Sandwich-ELISA confirms potential linkages of REC1

To further explore if the co-elution of the major polysaccharides released by the roots of Cadenza, as seen in anion-exchange EDC, were linked a sandwich-ELISA was undertaken. Microtitre plates were coated with a xylan-specific CBM (CBM2b1-2; McCartney *et al*. 2006), which also bound to the xylan released by Cadenza (Figure 4.7, **A**). Other polysaccharides that were linked with the xylan released by Cadenza would bind to the CBM-coated wells, generating MAb signals. The MAbs, LM1 (extensin), LM2 (AGP), LM11 (xylan) and LM25 (xyloglucan) along with the secondary anti-rat HRP were used to reveal the presence of any linked polysaccharides. Negative controls, wells with no CBM2b1-2, were directly compared to wells with CBM2b1-2 to verify the signal for a possible link between polysaccharides. The sandwich-ELISA used 10 µg/mL of the isolated acidic fractions (Figure 4.7, **B**), and determined that the major epitopes released by Cadenza were linked as a part of a putative multi-polysaccharide complex (Figure 4.7, **B**).

The signals from the top MAbs in the wells containing CBM2b1-2 were all significantly higher compared to the negative controls (Two-Sample T-Test, T= 31.43, P= <0.05). Therefore, supporting the presence of REC1 (Figure 4.7, **B**). The signal from LM11 confirmed that the CBM2b1-2 was binding to xylan.
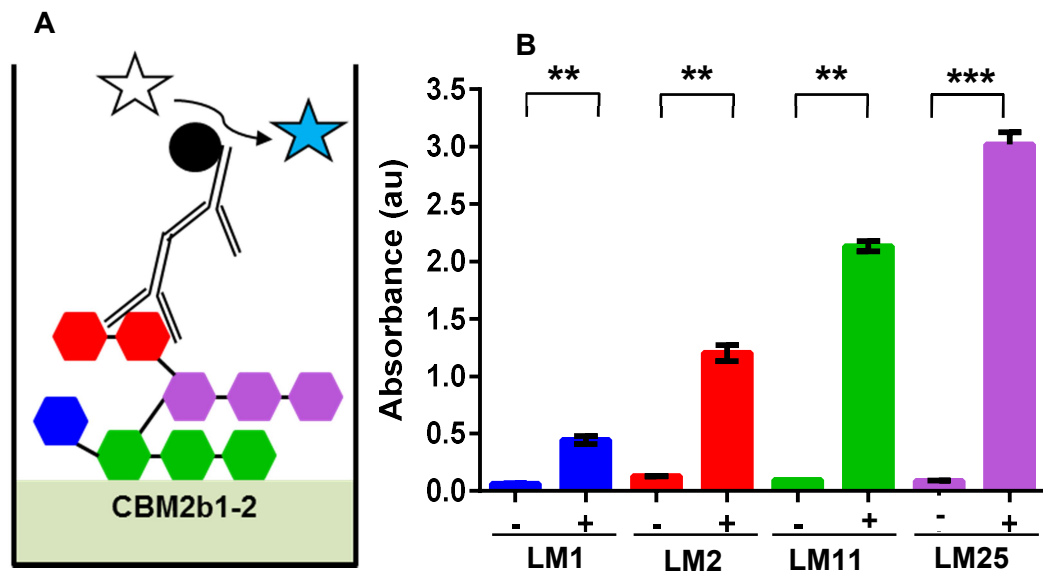
Figure 4.7 I **Sandwich-ELISA confirms the presence of REC1**
Schematic diagram representing a sandwich-ELISA analysis (**A**). The xylan-specific carbohydrate binding module, CBM2b1-2, is coated onto a 96 well microtitre plate. After coating, a sample of hydroponate is incubated on the CBM2b1-2 coated plate. Any xylan present within a multi-polysaccharide complex binds to the CBM. The primary anti-rat MAb along with the secondary anti-rat HRP antibody are incubated onto the CBM coated wells revealing the presence of other epitopes that are bound to the xylan epitopes (**A**). The sandwich-ELISA demonstrates that AGP, extensin, xylan and xyloglucan epitopes were a part of a complex (**B**). CBM2b1-2, which binds to xylan, was used to coat the microtitre plates, negative = no CBM control. Concentrated hydroponate (10 µg/mL) was used for the analysis (**B**). Each data point is a mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm. Standard deviation bars are shown; asterisks indicates significant difference (**P= 0.001 and ***P= 0.0001).

4.2.7 The epitope profile on anion-exchange EDC of the root body differs to that of the hydroponate

In order to see if REC1 was present within the root body of Cadenza, the biochemical properties of the cell walls were explored. To determine the profile of the root body, root cell walls were extracted. Roots were frozen by liquid nitrogen and freeze-dried. Fifteen milligrams of freeze-dried root material was dehydrated by adding increasing amounts of EtOH (v/v; 70%, 80%, 90% and 100%), and then acetone (100%) and methanol and chloroform (2:3), which removed the non-polysaccharide components of the root body. After removing these soluble materials, 1 mg of root cell walls was extracted using 4 M KOH and 1% sodium borohydride. This final material was assayed using LM1 (extensin), LM2 (AGP), LM11 (xylan) and LM25 (xyloglucan). For the EDC analysis 50 µg was injected into an anion-exchange column, and for the sandwich-ELISA analysis 10 µg/mL was used. The EDC chromatogram revealed that there was a range of xylan forms within the cell walls of Cadenza roots (Figure 4.8). Within the first peak of xylan, there is a co-elution of neutral xyloglucan epitopes. Within the second peak, there is a co-elution of AGP, extensin, xylan and xyloglucan epitopes, which is similar to that of REC1 that was detected within the concentrated hydroponate (Figure 4.2). There are no co-elutions of the major epitopes within the other peaks of xylan. The sandwich-ELISA analysis demonstrates that the putative multi-polysaccharide complex may also present within the root cell walls (Figure 4.8, **2**). Signals from the MAbs in the wells containing CBM2b1-2 were all significantly higher to that of the negative controls (Two-Sample T-Test, T= 27.33, P= <0.05), supporting the presence of REC1 within the root body of Cadenza (Figure 4.4). The signal from LM11 confirmed that the CBM2b1-2 was binding to xylan.
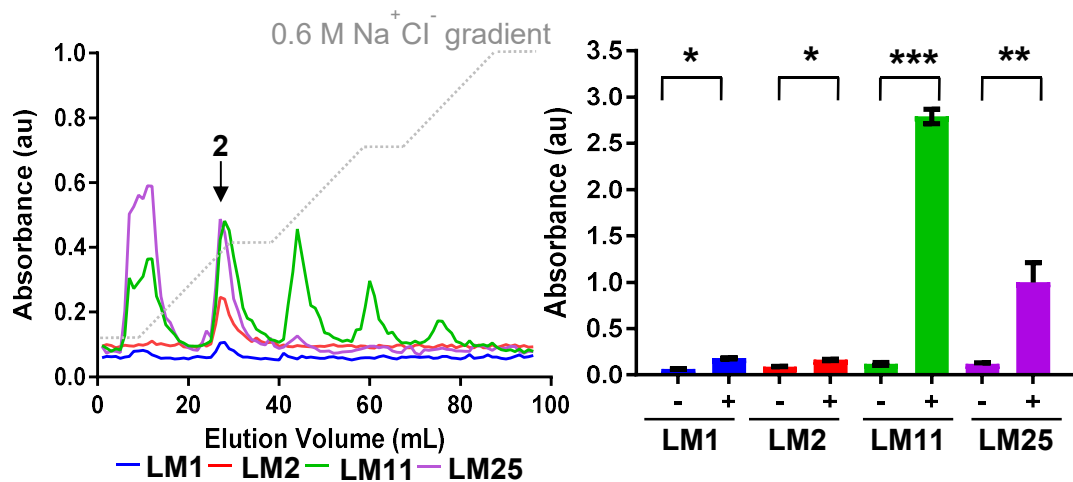
Figure 4.8 I **EDC and sandwich-ELISA analyses of Cadenza root cell walls reveal a range of acidic forms of xylan**

Fifty micrograms of root cell wall material was injected into a 1 mL anion-exchange chromatography column for EDC. Aliquots of 100 µL (containing ~5 µg) of each collected fraction were assayed for each MAb (**A**). The chromatogram revealed a peak of LM25 that was co-eluting with AGP (LM2), extensin (LM1) and xylan (LM11; **2**). Five clear peaks of LM11 dominate the chromatogram, which range from neutral to highly acidic (**A**). Data are a mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm. The sandwich-ELISA reveals that REC1 released by wheat roots is also present within the root body but at potentially lower levels compared to the concentrated hydroponate (**B**). CBM2b1-2, which binds to xylan, was used to coat the microtitre plates, negative = no CBM control. Root cell wall material (10 µg/mL) was used for the analysis (**B**). The antibody LM11 which binds to xylan was included as a control for CBM2b1-2. There was strong binding of xyloglucan (LM25) to xylan (CBM2b1-2) along with weaker binding of AGP (LM2) and extensin (LM1) to xylan (CBM2b1-2; **B**). Each data point is a mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm. Standard deviation bars are shown; asterisks indicates significant difference (*P= <0.05, **P= 0.001 and ***P= 0.0001).

4.2.8 REC1 was not disrupted after xylanase and xyloglucanase digests

As REC1 putatively contains xylan and xyloglucan it was decided to degrade them in order to explore their potential linkages, and how removing them would affect the behaviour of REC1 on the chromatographic system. Xylan and xyloglucan were selected as there are a range of enzymes, xylanase and xyloglucanase, which are available to degrade them unlike the other domains of REC1, AGP and extensin. Aliquots (50 µL) containing 50 µg of concentrated hydroponate was dissolved in 100 mM sodium acetate buffer. Hydroponates were incubated at 40°C and 60°C for 2 h with β-xylanase (480 U) and xyloglucanase (40 U). After digesting, samples of hydroponate were assayed with the MAbs. When the enzymes were added to the concentrated hydroponate their respective targets were successfully digested. This was shown by the absence of the signals of LM11 (xylan), and LM25 (xyloglucan; Figure 4.9). Surprisingly the co-elution of the AGP, extensin, xylan and xyloglucan epitopes remained unaffected after the enzyme treatments, which digested the xylan and xyloglucan domains of REC1 (Figure 4.9). This demonstrates that degrading the xylan and xyloglucan did not affect the co-elution that forms REC1, suggesting that they were not acidic domains.
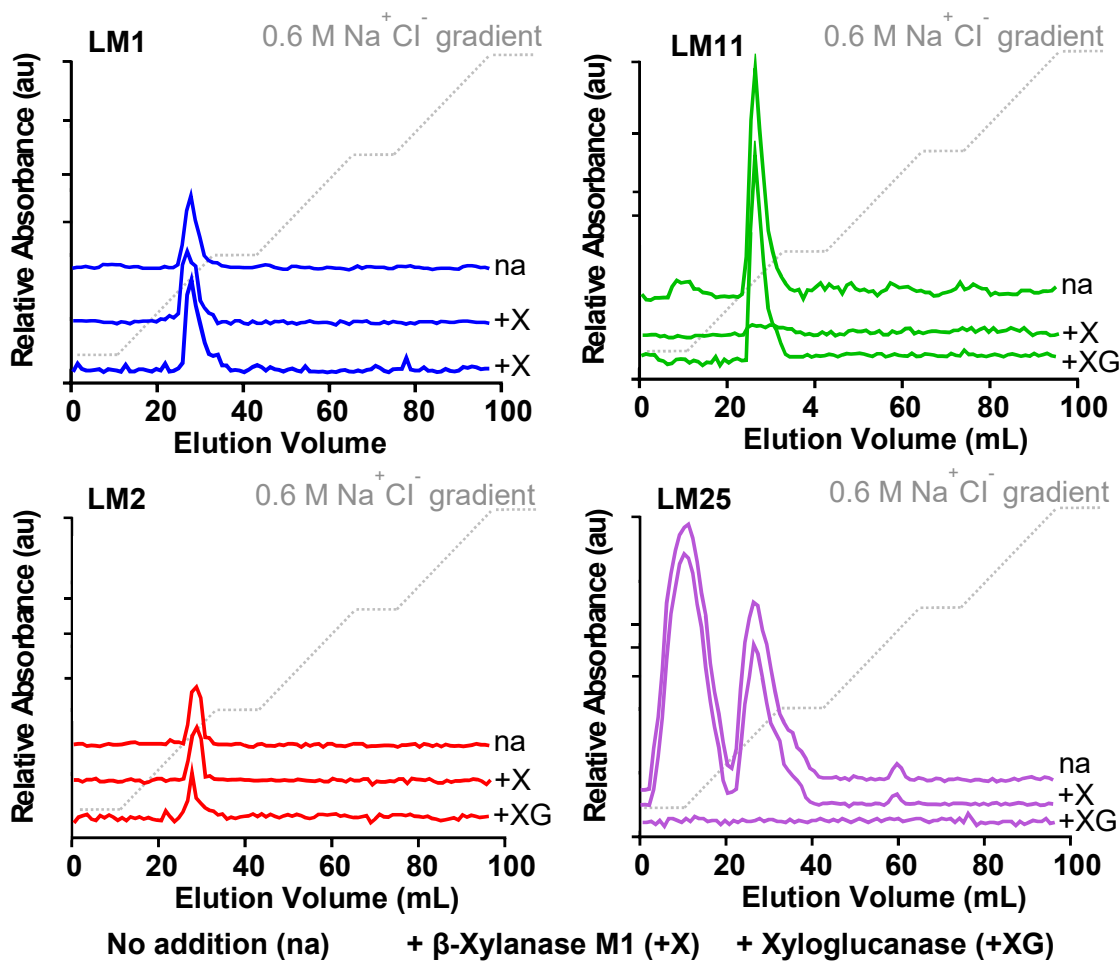
Figure 4.9 I **Xylanase and xyloglucanase digests of Cadenza concentrated hydroponate**

Concentrated hydroponate (50 µg) was dissolved in sodium acetate (100 mM) buffer along with BSA (1 mg/mL). Enzymes, β-Xylanase M1 (60 µg/mL), which degrades (1,4)-β-D-xylosidic linkages in xylan and xyloglucanase (120 µg/mL), which degrades 1,4-β-D-glucosidic linkages in xyloglucan were added to 1 mL of sample for 10 mins. Signals of LM11 (xylan) and LM25 (xyloglucan) were not detected when their respective enzymes were added showing that the treatments were effective. Each treatment had a maximum absorbance of 1.0 au. A 1:10 dilution of antigen was used to get signals of 1.0 au for LM25. Each mark on the y axis represents 0.5 au relative to each group. Each data point is a mean of three biological replicates. Relative ELISA absorbance values were determined by measuring the absorbance at 450 nm.

4.2.9 Co-elutions of the major epitopes were detected within the hydroponates of other wheat cultivars

After investigating some aspects of the biochemical properties of REC1 released by Cadenza, two other cultivars of wheat were analysed. Wheat cultivars, Avalon and Skyfall, were grown using the same parameters and hydroponic system as used for Cadenza. These cultivars were chosen as they are widely grown winter varieties whereas, Cadenza is an older and less popular spring variety (Kumar *et al*. 2011). After two weeks of hydroponic culture, the hydroponates were concentrated, dialysed and freeze-dried. Fifty micrograms of Avalon and Skyfall concentrated hydroponates were then assayed using EDC. The chromatograms reveal that were are two forms of xyloglucan, neutral and acidic released into the hydroponates of Avalon and Skyfall. Within the neutral form of xyloglucan no other epitopes were detected from the initial survey (Figure 4.10). The fractions containing the second, acidic form of xyloglucan contained a co-elution of AGP, extensin and xylan (Figure 4.10), as previously observed in the hydroponate of Cadenza (Figure 4.3).

When the salt gradients are delayed the acidic peaks of LM25 (xyloglucan), along with the co-elution were retained for longer within the anion-exchange column (Figure 4.10). This demonstrates that the co-elution was unaffected when the salt gradient was delayed (Figure 4.10), as previously observed in Cadenza (Figure 4.3). The highest signal detected from the MAbs was from LM25 followed by LM1, LM11 and LM2 (Figure 4.10). There were increases in the signals of the MAbs within the co-elution of AGP, extensin, xylan and xyloglucan when the gradient of salt was delayed for both Avalon and Skyfall (Figure 4.10). This was also observed from the hydroponate of Cadenza (Figure 4.3), perhaps due to reducing molecular crowding. Additionally, there were slight differences in the hydroponic profiles between the cultivars, for example, the signals of LM1 were higher, ranking the second highest, within the concentrated hydroponates of Avalon and Skyfall (Figure 4.10) compared to Cadenza where LM11 was the second highest signal (Figure 4.3). These slight differences suggest that Avalon and Skyfall released a similar molecule to REC1, which contained different components.
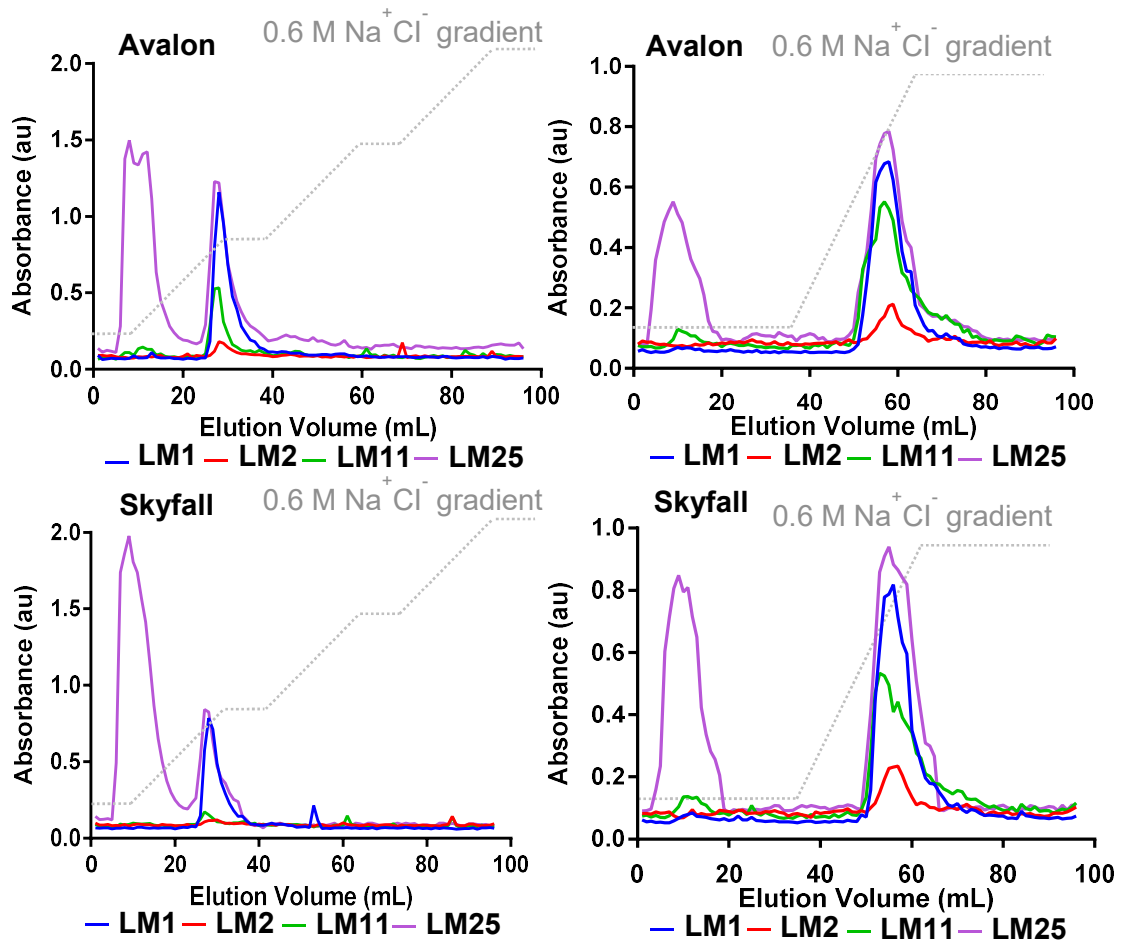
Figure 4.10 I **Two forms of xyloglucan are released by the wheat cultivars Avalon and Skyfall as determined using Anion-Exchange EDC**

Concentrated hydroponates of Avalon and Skyfall (50 µg) were injected into a 1 mL anion-exchange chromatography column. Five micrograms of this was assayed for each MAb. A step gradient of 0.6 M of Na^+Cl^- was used (figures on the left) followed by a long neutral gradient of 0.6 M of Na^+Cl^- (figures on the right). Two forms of xyloglucan (LM25), neutral and acidic were detected in both cultivars along with a co-elution of AGP (LM2), extensin (LM1) and xylan (LM11) within the acidic form of xyloglucan (figures on the left). When the gradient was altered to have a longer neutral step the co-elution of LM1, LM2, LM11 and LM25 were retained in the column for longer (figures on the right). Each data point is a mean of three biological replicates. Relative ELISA absorbance values were determined by measuring the absorbance at 450 nm.

4.2.10 REC1-like molecule is released by the other cultivars of wheat

As a co-elution of AGP, extensin, xylan and xyloglucan was determined within the hydroponates of Avalon and Skyfall, which was similar to Cadenza, a sandwich-ELISA was undertaken to determine if they were linked. This was to determine if the co-elution of epitopes, previously determined were linked (Figure 4.10). Microtitre plates were coated with the xylan-specific carbohydrate-binding module, CBM2b1-2 (McCartney *et al*. 2006). LM1 (extensin), LM2 (AGP), LM11 (xylan) and LM25 (xyloglucan) were used to screen the microtitre wells with and without the CBM coating. Isolated acidic fractions (from Figure 4.10; 10 µg/mL) were added to each well. The signals with the CBM coating were all significantly higher to that of the wells that lacked CBM (Figure 4.11); for Avalon (Two-Sample T-Test, T= 36.47, P= <0.05), and for Skyfall (Two-Sample T-Test, T= 24.49, P= <0.05). These significant differences demonstrate that these cultivars of wheat release molecules similar to REC1. The signal from LM11 confirmed that the CBM2b1-2 was binding to xylan (Figure 4.11).
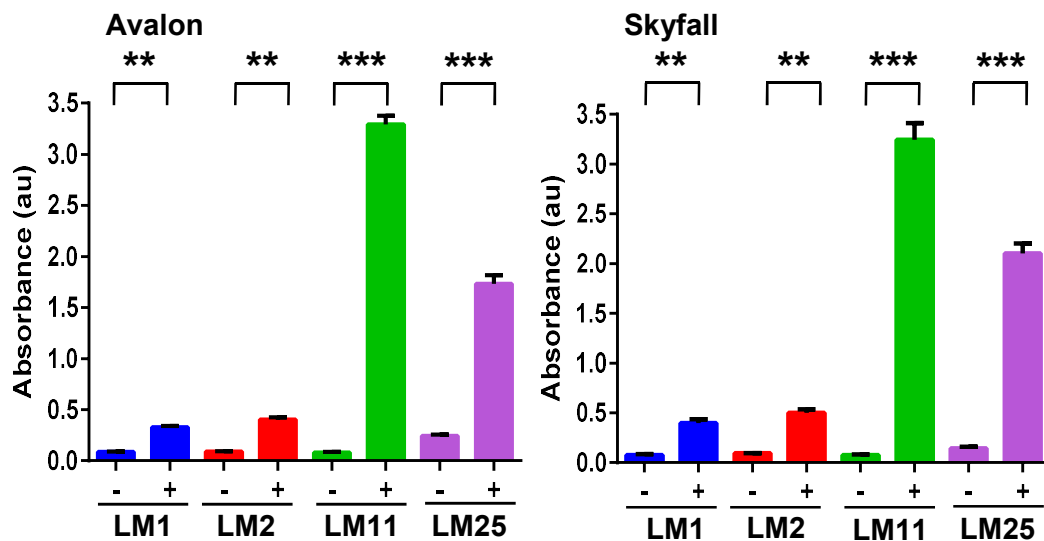
Figure 4.11 I **Sandwich-ELISA reveals REC1-like macromolecules are released by Avalon and Skyfall**
CBM2b1-2, which binds to xylan, was used to coat the microtitre plates, negative = no CBM control. Ten micrograms a millilitre of concentrated hydroponate was used. The sandwich-ELISAs reveal that xyloglucan (LM25), AGP (LM2) and extensin (LM1) epitopes were strongly bound to xylan (CBM2b1-2). LM11 that binds to the epitopes of xylan had the highest signal, confirming that the CBM was binding to xylan. Each data point is a mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm. Standard deviation bars are shown; asterisks indicates significant difference (**P= 0.001 and ***P= 0.0001).

4.2.11 Similar hydroponic profiles were observed between wheat and other cereals

After uncovering a putative multi-polysaccharide complex released by wheat roots, other species of cereal were grown hydroponically to investigate the taxonomic occurrence of REC1 and REC1-related molecules. Barley and maize hydroponates were initially screened with 29 MAbs and found to contain the four epitopes, similar to that of wheat (Table 3.1 and Table 3.2). After concentrating, 50 µg of each species hydroponate was suspended in 20 mM sodium acetate buffer, and injected into a 1 mL weak anion-exchange chromatography column. Two gradients were used to explore alterations in the elution of the epitopes. The concentrated hydroponates of both barley and maize contained two forms of xyloglucan; neutral and acidic (Figure 4.12). Furthermore, both concentrated hydroponates contained a co-elution of AGP, extensin and xylan with the second acidic form of xyloglucan (Figure 4.12). There were slight differences in the signal strengths of each MAb within the co-elution. For barley, extensin epitopes ranked the highest followed by xyloglucan, xylan and AGP epitopes. Whereas, for maize, xyloglucan epitopes ranked the highest followed by xylan, AGP and extensin. These differences suggest that barley and maize release a macromolecule similar to REC1. When the salt gradient was extended the co-elution was retained for longer within the column (Figure 4.12). The rankings of the MAb signals remained the same during the delayed gradient of salt.
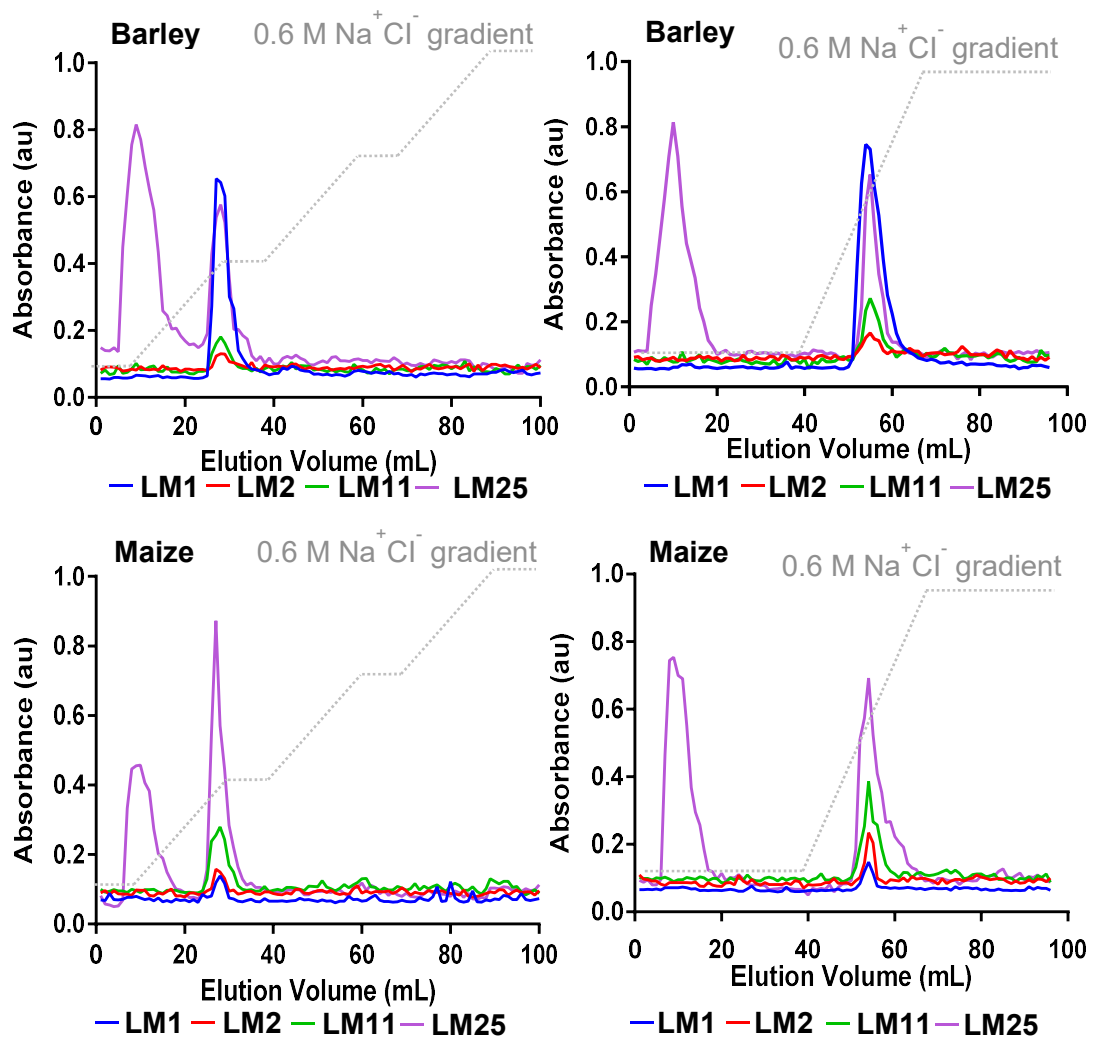
Figure 4.12 | **Two forms REC1-like macromolecules were detected within the hydroponates of barley and maize**
Fifty micrograms of concentrated hydroponate of barley and maize were injected into a 1 mL anion-exchange chromatography column. Five micrograms of this was assayed for each MAb. A step gradient of 0.6 M of Na^+Cl^- was used (figures on the left) followed by a long neutral gradient of 0.6 M of Na^+Cl^- (figures on the right). Two forms of xyloglucan (LM25), neutral and acidic were detected in both species along with a co-elution of AGP (LM2), extensin (LM1) and xylan (LM11) within the acidic form of xyloglucan. Relative ELISA absorbance values were determined by measuring the absorbance at 450 nm.

4.2.12 Sandwich-ELISA evidence of a REC1-like macromolecule released by other cereals

As there was a co-elution of the four major epitopes detected within the hydroponates of barley and maize, a sandwich-ELISA was undertaken to determine if these co-eluting polysaccharides were linked. Microtitre plates were coated with CBM2b1-2 (McCartney *et al*. 2006), a xylan-specific binding agent. Samples of both barley and maize isolated acidic fractions (from Figure 4.12; 10 µg/mL) were incubated on the plates. Wells with no CBM coating were used as a negative control. MAbs were used to reveal the presence of any linked polysaccharides. The sandwich-ELISAs reveal that the four polysaccharide epitopes were linked as a part of a REC1-like complex, released by the roots of both barley and maize (Figure 4.13). The MAb signals were all significantly higher than their respective controls; for barley (Two-Sample T-Test, T= 27.43, P= <0.05), and for maize (Two-Sample T-Test, T= 20.66, P= <0.05). LM11 signals, in both species hydroponate, were significantly higher to their respective controls, indicating that the CBM was indeed binding to xylan (Figure 4.13). REC1 (Figure 4.3) and similar macromolecules to REC1 (Figure 4.13) have been detected within the concentrated hydroponates of three cultivars of wheat and three species of cereal (Figure 4.7 and Figure 4.11), which not only supports the release of REC1 macromolecules but highlights its potential biological importance as these macromolecules are being released by a range of grasses.
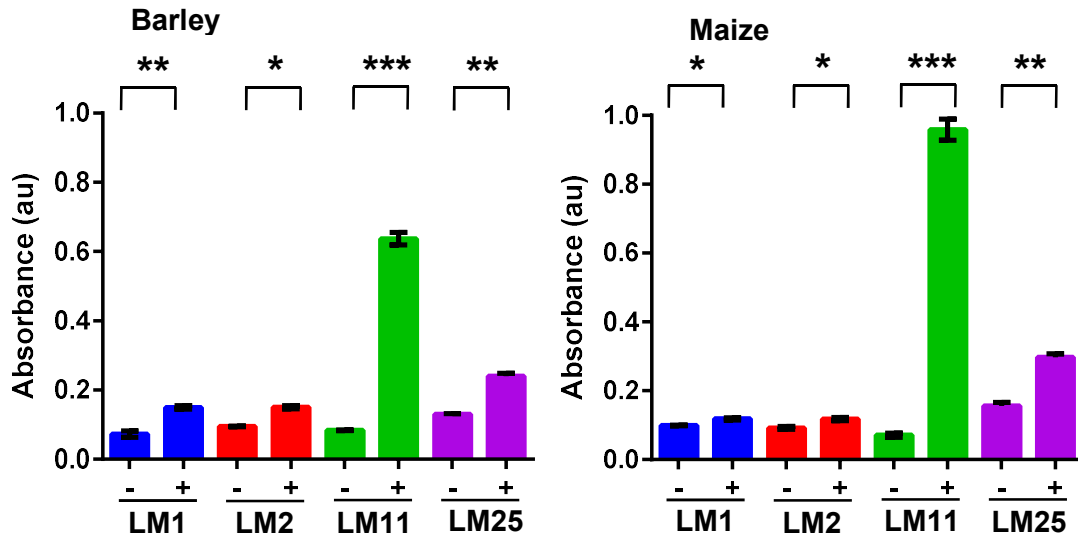
Figure 4.13 I **Sandwich-ELISA analyses reveal the presence of REC1-like component released by barley and maize roots**

CBM2b1-2, which binds to xylan, was used to coat the microtitre plates, negative = no CBM control. Ten micrograms a millilitre of concentrated hydroponate was incubated on the microtitre plates. The sandwich-ELISAs show for both barley and maize, xyloglucan (LM25), extensin (LM1) and AGP (LM2) were bound to the xylan-specific CBM, CBM2b1-2. For both species hydroponate, LM11 strongly bound to CBM2b1-2, confirming that the CBM was binding the xylan. Each data point is a mean of three biological replicates. Relative ELISA absorbance values were determined by measuring the absorbance at 450 nm. Standard deviation bars are shown; asterisks indicates significant difference P= <0.05.

4.2.13 Some evidence of co-eluting polysaccharide epitopes from eudicotyledons

Exploring the biochemical properties of cereal hydroponates revealed that AGP, extensin, xylan and xyloglucan were linked to form REC1. The major epitopes in the hydroponates of eudicotyledons (Table 3.2 and Figure 3.5) were examined to determine if these polysaccharides were linked to form a similar complex. Commonly cultivated eudicotyledon crops, pea, rapeseed and tomato, were grown in a 50:50 mixture of vermiculite and perlite. Seedlings were transferred into hydroponics for two weeks. After two weeks, their hydroponates were concentrated. Quantities of concentrated hydroponate, 100 µg of tomato, 200 µg of pea and rapeseed, were suspended in sodium acetate buffer, and injected into an anion-exchange column. Subsequent sandwich-ELISAs followed using 10 µg/mL of each concentrated hydroponate. The chromatograms of each eudicotyledon revealed highly diverse relative levels of the four major polysaccharide epitopes, AGP (LM2), extensin (LM1), xylan (LM11) and xyloglucan (LM25; Figure 4.14). From anion-exchange EDC, no such strong co-elutions were evident within the hydroponates of the eudicotyledons compared to the cereals (Figure 4.14). For all sandwich-ELISAs, LM11 was significantly higher compared to wells that contained no CBM2b1-2 (McCartney et al. 2006), demonstrating that CBM was binding to xylan.

The chromatogram of pea hydroponate reveals the presence of three forms of xyloglucan epitopes, neutral, acidic and highly acidic. There appears to be slight indications of three co-elutions of AGP, extensin and xylan with the three forms of xyloglucan (Figure 4.14). When the sandwich-ELISA was undertaken, the wells coated with CBM2b1-2 were all significantly higher compared to their respective controls (Two-Sample T-Test, T= 28.27, P= <0.05). However, when combined with the low signals on the chromatogram, there is weak evidence of a REC-like complex within the hydroponate of pea (Figure 4.14).

The concentrated hydroponate of rapeseed contained two distinct peaks of xyloglucan, neutral and acidic. The acidic form of xyloglucan was lower compared to the neutral form. This acidic peak of xyloglucan maybe present due to the root hairs of Arabidopsis, which is within the same family as rapeseed (Brassicaceae), containing an acidic form of xyloglucan, which contains a galacturonic acid residue (Peña et al. 2012a). There were also two peaks of xylan one of which slightly co-eluted with the acidic xyloglucan (Figure 4.14). AGPs were also present but did not co-elute with the other polysaccharide (Figure 4.14). A very low signal from LM1

(extensin) was present across the chromatogram. The sandwich-ELISA indicated that AGP, extensin and xylan could be linked as a part of a complex (Two-Sample T-Test, T= 45.53, P= 0.05). However, the signals from the sandwich-ELISA are only just significant, and since there are little evidence of co-eluting polysaccharides it cannot be confidently established that these polysaccharides were linked.

The chromatogram of tomato hydroponate reveals the presence of two forms of xyloglucan epitopes, neutral and acidic. There was a lower signal of LM25 (xyloglucan) detected within the acidic region compared to the neutral region of the chromatogram (Figure 4.14). There was a large signal of LM2 (AGP), which dominates the chromatogram of the concentrated hydroponate of tomato. Xylan was also present in a highly acidic form, eluting just before the AGP. There was a small indication that the four major polysaccharides may be linked by the weak signals that appear just before fraction 60. However, no clear co-elutions were evident across the chromatogram. This result was reflected by the lack of a strong signal of LM1, LM2 and LM25 within the sandwich-ELISA (Figure 4.14). The only signal that was significantly higher to that of the respective control is LM11, which demonstrates that CBM2b1-2 is binding to xylan.
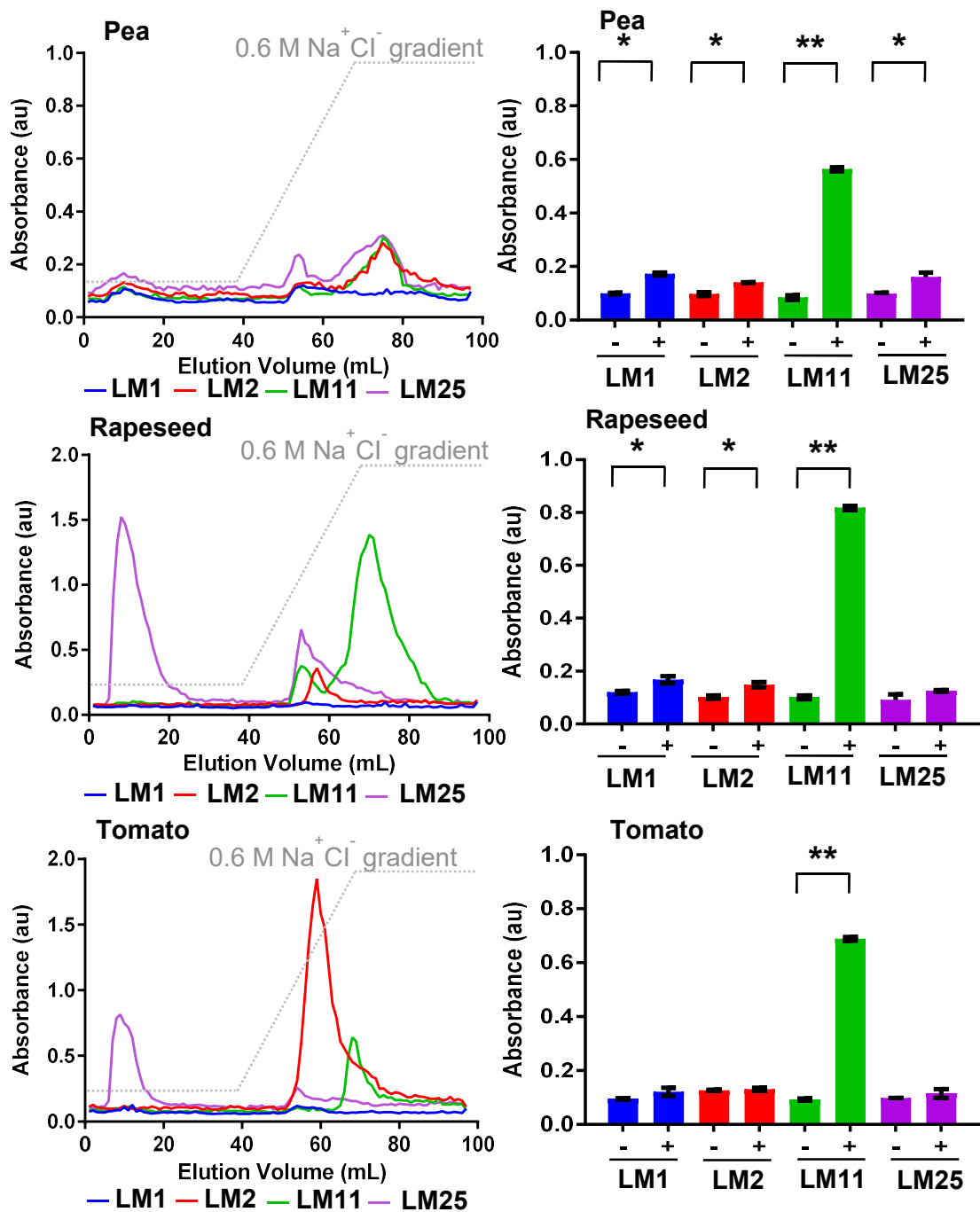
Figure 4.14 | **Anion-exchange EDC analysis of released polysaccharides from pea, rapeseed and tomato differs to that of grasses**

Two hundred micrograms concentrated hydroponate of pea and rapeseed, and 100 μg of tomato were injected into a 1 mL anion-exchange chromatography column (figures on the left). Twenty micrograms of pea and rapeseed, and 10 μg of tomato hydroponates were assayed for each MAb; a long neutral gradient of 0.6 M of Na^+Cl^- was used. There are three weak co-elutions between AGP (LM2), extensin (LM1), xylan (LM11) and xyloglucan (LM25) within the pea hydroponate. Within the hydroponate of rapeseed, AGP (LM2) and xylan (LMM11) weakly to co-elute within the acidic region. AGP (LM2) dominates the hydroponate of tomato followed by two forms of xyloglucan and xylan (LM11). CBM2b1-2, which binds to xylan, was used to coat the microtitre plates of pea and tomato concentrated hydroponate (figures on the right). Ten micrograms a millilitre of concentrated hydroponate was incubated on the microtitre plates. Negative = no CBM control; standard deviation bars are shown. The sandwich-ELISAs show that xyloglucan (LM25), AGP (LM2) and extensin (LM1) binds to

xylan (CBM2b1-2) within the pea hydroponate. The sandwich-ELISA demonstrates that the released polysaccharides from rapeseed, AGP (LM2) and extensin (LM1) bind to xylan (LM11). For tomato, no co-elution was demonstrated. Each data point for both the EDC and sandwich-ELISA results were a mean of three biological replicates. Relative ELISA absorbance values were determined by measuring the absorbance at 450 nm. Asterisks indicates significant difference (*P= <0.05 and **P= 0.001).

4.2.14 Rhizoids of liverwort release an putative AGP-xyloglucan complex designated REC2

The opportunity arose to examine the liquid medium of liverwort, which is an early diverging form of land plant. The ancestors of these liverworts were the first forms of plants to colonise the land over 470 millions of years ago (Kenrick and Crane 1997; Bateman *et al*. 1998). Comparing the biochemical properties of the liquid medium of liverworts to the concentrated hydroponates of more recent plants has provided a greater insight into the polysaccharides released by plants. Liverworts (*Marchantia polymorpha*) were grown and the biochemical proprieties of their exudates examined. Ten liverwort gemmae, cup-like appendage that form clones, were grown on agar with full BG11 medium for 30 days. After 30 days, gemmae were removed and the surrounding agar finely cut, and placed into an Eppendorf tube. Deionised water was placed into the tube, and the mixture agitated overnight to remove the exudates released into the agar. This water gel extract was then screened with 29 MAbs only revealing the presence of LM2, which bind to the epitopes of AGP and LM25 that binds to the epitopes of xyloglucan. There were no signals detected from LM11 (xylan), JIM7 and LM19 (HG), and LM6 (RG-I; work was undertook by Ms Bev Merry). After initially screening the agar gel extracts of liverworts, a tissue print was carried out using a nitrocellulose sheet, which was undertaken by Ms Sue Marcus. The nitrocellulose was probed with LM25, and revealed that xyloglucan was released along the rhizoids of liverworts as well as the rhizoid tips. Higher signals of LM25 (xyloglucan) were detected at the tips of rhizoids (Figure 4.15, **D**). Signals of LM2 (AGP) were also found to be higher at the tips of the rhizoids.

To further explore the biochemical properties of the AGP and xyloglucan released by liverwort into liquid medium, anion-exchange EDC and sandwich-ELISA were undertaken. After the liverworts have been grown in agar for a month they were removed and the agar extracted with dH2O. One millilitre of this wash was directly injected into a weak anion-exchange column (1 mL). An additional 1 mL of this wash was also incubated in microtitre wells coated with a xyloglucan- and cellulose-binding CBM, CBM3a (Blake *et al*. 2006). The anion-exchange EDC clearly shows

two distinct forms of xyloglucan, neutral and acidic, released by the rhizoids of liverwort (Figure 4.15, **B**). The chromatogram also reveals a clear co-elution of AGP with the second acidic form of xyloglucan. This suggests that there is a possible complex formed of AGP and xyloglucan that was being released by the liverworts (Figure 4.15, **B**). This possible link between AGP and xyloglucan was confirmed by the strong MAb signals with the CBM3a coated wells, within the sandwich-ELISA (Two-Sample T-Test, T= 19.37, P= <0.05; Figure 4.15, **C**). Once determining the presence of a REC2, a basic phylogenetic analysis was carried out based on taxonomy, which determined that the last common ancestor between liverworts and cereals, which release these multi-polysaccharide complexes, lived over 400 million years ago (Figure 4.15, **A**). This indicates that releasing these putative complexes may be wide spread across the plant kingdom. Further examination of the anion-exchange chromatograms, revealed that the co-elution of AGP-xyloglucan was eluted with a salt concentration of ~500 mM. It was observed that the amount of salt required to elute REC2 (Figure 4.15, **C**) was more than what it was required to elute wheat REC1 (Figure 4.7, **B**) and REC1-like complexes released from the roots of barley and maize (Figure 4.12). This was most likely due to the presence of an acidic xyloglucan, which contains galacturonic acid residues found within liverworts (Peña *et al.* 2008).
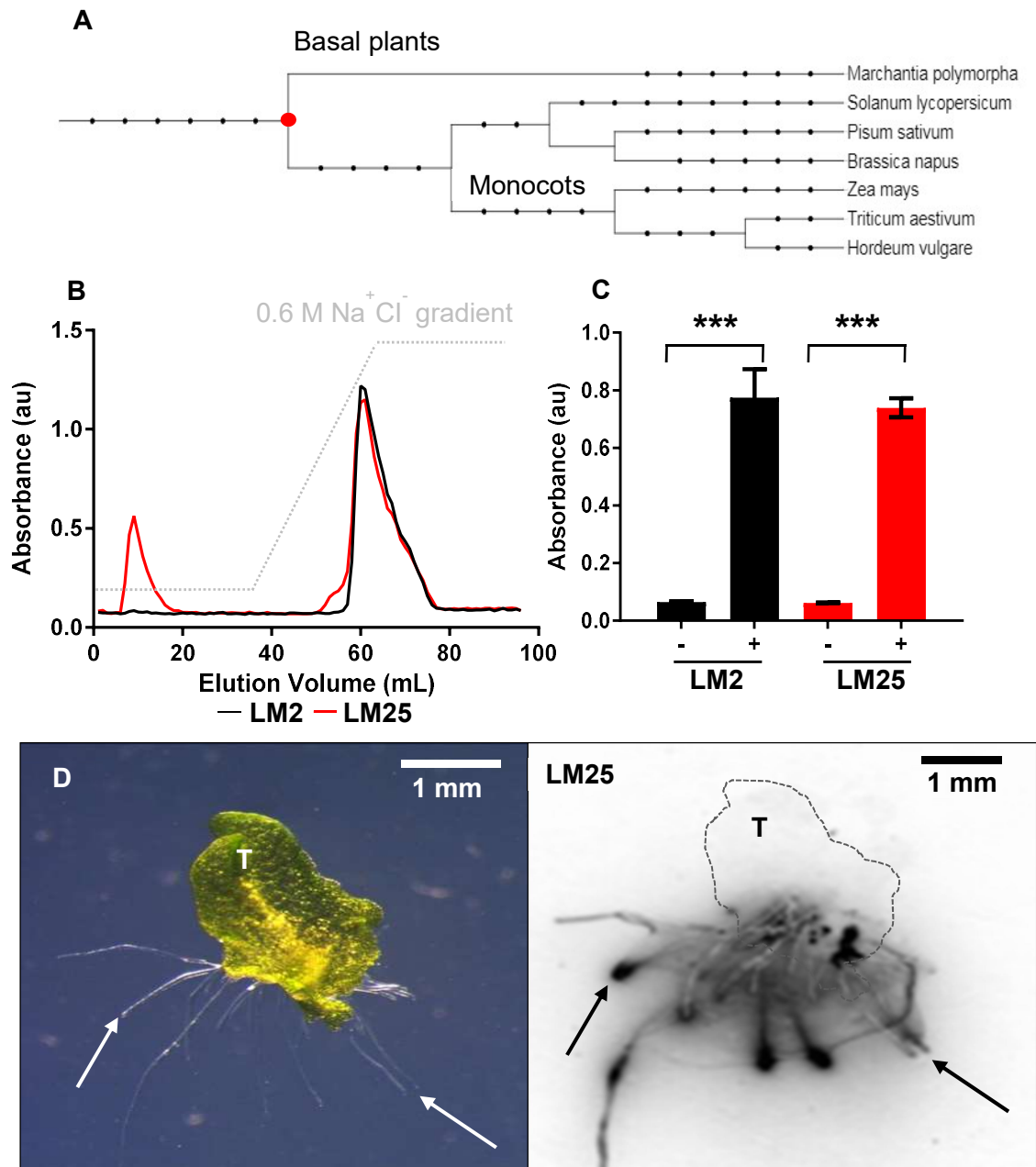
Figure 4.15 | **Analysis of liverwort (*Marchantia polymorpha*) agar gel extracts reveals a REC1-like complex, REC2**

Phylogenetic tree analysis of the species used within this study including liverwort (**A**). The last common ancestor of the species used was the clade Embryophyta, which were the first land plants that evolved from green algae, specifically the Charophyte, which occurred 470 million years ago, indicated by red circle. This phylogenetic tree was created using the Phylot phylogenetic tree taxonomy generator (**A**). One millilitre of agar gel extract was injected into a 1 mL anion-exchange chromatography column. One hundred microliters of liverwort gel extract were assayed for each MAb. A long neutral gradient of 0.6 M of Na+Cl- was used (**B**). A clear co-elution of AGP (LM2) and xyloglucan (LM25) can be seen between fractions 58 and 80. Two peaks of xyloglucan (LM25) are also visible (**B**). CBM3a, which binds to xyloglucan-cellulose, was used to coat the microtitre plates of liverwort gel extracts (**C**). One millilitre of liverwort gel extract was incubated on the microtitre plates. Negative = no CBM control; standard deviation bars are shown (**C**). The sandwich-ELISA clearly demonstrates that AGP (LM2) and xyloglucan (LM25) are bound to each other in a REC1-like complex (**C**). Each data point for both the EDC and sandwich-ELISA results were a

mean of three biological replicates. ELISA absorbance values were determined by measuring the absorbance at 450 nm. Asterisks indicates significant difference P= <0.001. Liverworts were grown for 7 days on agar with no nutrients by Ms Sue Marcus. Liverwort was imaged using a compound microscope (**D**, left). After growth, nitrocellulose sheets were placed onto the agar without the liverwort. The nitrocellulose sheet was then probed with LM25 (xyloglucan). The nitrocellulose print revealed the location of xyloglucan released along the rhizoids and tips of rhizoids (**D**, right). Scale = 1 mm; T = thallus.

4.2.15 REC1 contains monosaccharides that are abundant within the four major polysaccharides determined by MAbs

In order to verify the observations made within the immunochemical analyses, a preparation of REC1 was provide to the Complex Carbohydrate Research Centre (CCRC) for monosaccharide composition and monosaccharide linkage analyses. These biochemical analyses determined the major monosaccharides within the preparation. The CCRC hydrolysed the sample using 2 M trifluoroacetic acid, then reduced the sample using sodium borohydride (10 mg/mL) prepared in 1 M ammonium hydroxide, and finally, acetylated the sample using 9:1 (v/v) methanol and acetic acid. The resulting alditol acetates were analysed using gas chromatography combined with mass spectrometry. From the analysis, the sample was found to contain, in descending order of abundance, mannose, glucose, xylose, arabinose, rhamnose, galactose and fucose (Figure 4.16). The most abundant monosaccharide was mannose, forming 30.6% of the sample analysed (Figure 4.16), which was not previously determined by mannan-specific MAbs (Table 3.1). No acidic monosaccharide residues were detected within the analysis, which is also an unexpected result due to previous immunochemical analyses that suggest the presence of glucuronic acid present in AGP.
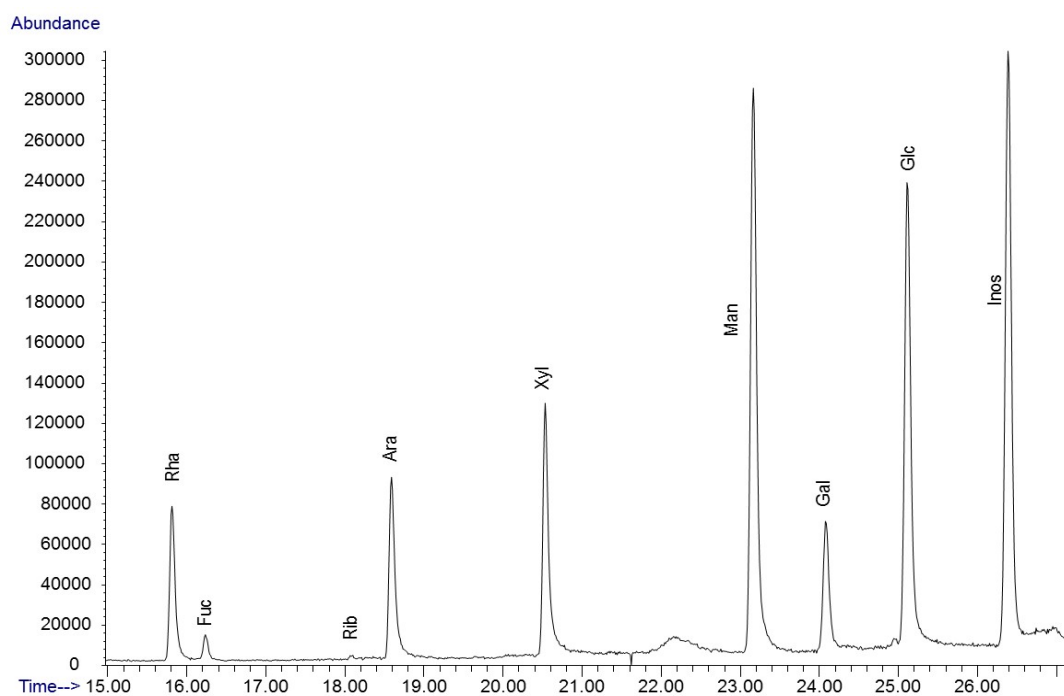
Deduced monosaccharide	Mol (%)
Mannose	30.6
Glucose	26.3
Xylose	16.7
Arabinose	10.5
Rhamnose	8.3
Galactose	6.6
Fucose	0.9

Figure 4.16 I **Total ion chromatogram and summary of monosaccharide composition analysis of REC1**

The preparation of REC1 (300 μg) was hydrolysed with 2 M trifluoroacetic acid for 4 h at 100°C. REC1 was then reduced with sodium borohydride (10 mg/mL) prepared in 1 M ammonium hydroxide for 1 h at RT. The preparation was then acetylated using 9:1 (v/v) methanol in acetic acid for 1 h at RT. The resulting alditol acetates were analysed using total ion chromatography combined with mass spectrometry. Inositol (20 μg) was used as an internal standard. The analysis reveals that mannose had the largest proportion (%) compared to the other monosaccharides within REC1. Glucose had the second highest proportion of REC1 followed by xylose, arabinose, rhamnose, galactose and fucose. This work was undertaken at the CCRC.

4.2.16 Monosaccharide linkage analysis reveals a high diversity of glycosidic linkages within REC1

To further explore the biochemistry of the REC1 preparation, the CCRC undertook a monosaccharide linkage analysis to determine which glycosidic linkages were present. From these linkages, signatures of polysaccharides were determined, which could verify the presence of the polysaccharides detected by the MAbs. The sample was suspended in DMSO, and thoroughly agitated for a week. Aliquots (200 µL) of 1 M sodium hydroxide and methyl iodide (100 µL) were used to methylate the free hydroxyls on the polysaccharides within the preparation. The preparation was then hydrolysed using 2 M trifluoroacetic acid, and reduced using sodium borohydride (10 mg/mL) in 1 M ammonium hydroxide. The resulting partially methylated alditol acetates were analysed using gas chromatography combined with mass spectrometry, which was undertaken at the CCRC. The chromatogram revealed a high diversity of glycosidic linkages within the preparation (Figure 4.17). The major linkages were formed of mannose (35.6%), glucose (34.7%) and xylose (9.5%), which verified with the monosaccharide composition analysis (Figure 4.16). There was a particular abundance of 1,4-linked mannose, which forms the backbone of mannan. There was also an abundance of 1,4-linked glucose residues, which forms the backbone of many plant cell wall polysaccharides including cellulose, xyloglucan and mixed linkage glucan, amongst others. The third most abundant linkage was 1,3-linked glucose, which is present within mixed linage glucan and callose (Figure 4.17).

On further examination, many linkages were uncovered that were present within the four major polysaccharides previously determined by the MAbs. Strong signatures of AGP and extensin, terminal, 3-, 4-linked Ara*p*, terminal, 5-linked Ara*f*, terminal Gal*p*, terminal Rha*p*, were detected. Strong signatures of xylan, 4-linked Xyl*p*, and xyloglucan, terminal Fuc*p*, terminal Gal*p*, 4- and 4,6-linked Glc*p*, terminal and 2-linked Xyl*p* were detected. Other polysaccharide signatures were also detected including arabinan, cellulose, galactomannan, glucomannan, mannan, mixed linkage glucan, RG-I and RG-II (Table 4.1). Possible signatures of fungal cell wall polysaccharides may have also been detected within the sample of REC1. Many other linkages were present within the preparation of REC1, which could not be assigned to a clear polysaccharide signature (Table 4.1).
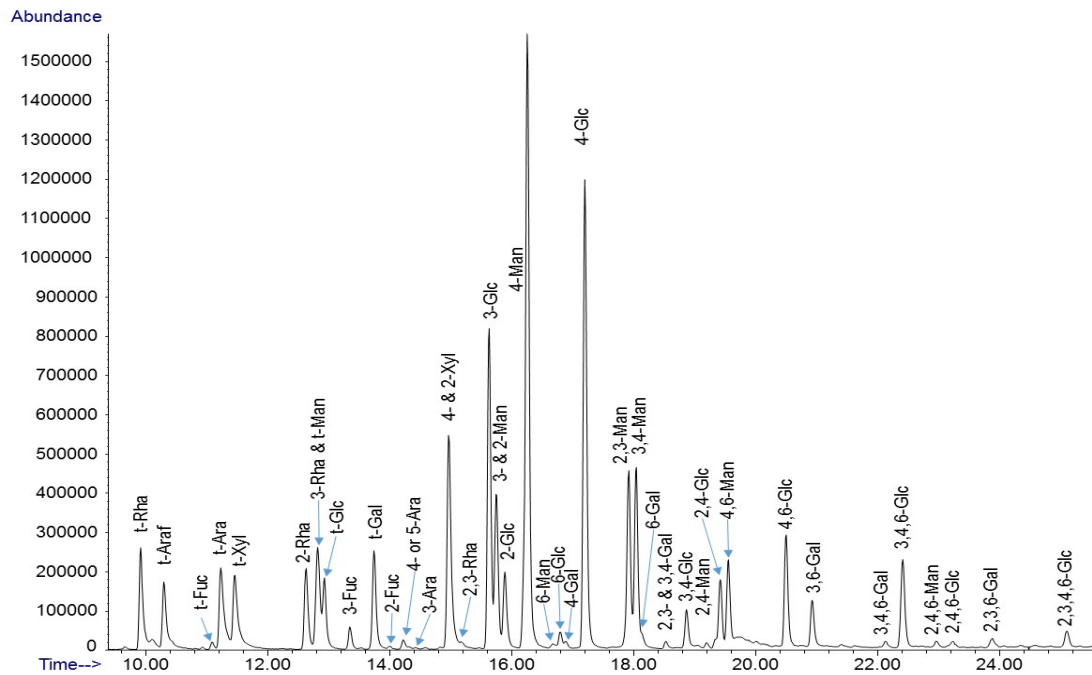
Figure 4.17 I **Total ion chromatogram of monosaccharide linkage analysis of REC1**
The sample (1 mg) was suspended in DMSO and agitated for 1 week. Methylation was undertaken by using 200 µL of 1 M sodium hydroxide, and then 100 µL methyl iodide. The sample was then hydrolysed using 400 µL trifluoroacetic acid at 121ºC. This preparation was then reduced using 1.2 mL sodium borodeuteride (10 mg/mL) prepared in 1 M ammonium hydroxide. The resulting partially methylated alditol acetates were analysed using gas chromatography combined with mass spectrometry. There was a high amount and diversity of linkages within the preparation. A large amount of 1,4-linked mannose, which forms the backbone of mannan was determined. The next most abundant linkage was 1,4-linked glucose that forms the backbone of many plant cell wall polysaccharides including, cellulose, xyloglucan, mixed linkage glucan and others. The third most abundant linkage was 1,3-linked glucose, which is present in mixed linkage glucan. Many other linkages involving arabinose, fucose, galactose, rhamnose and xylose that were detected are found in the four polysaccharides, AGP, extensin, xylan and xyloglucan, determined by the MAbs. This work was undertaken at the CCRC.

Table 4.1 Monosaccharide linkage analysis of REC1

Monosaccharide	Deduced linkage		Mol (%)	Plant Polysaccharide
Ara*p*	Terminal		3.0	AGP/Extensin
	3-		0.1	AGP/Arabinoxylan/Extensin/RG-II
	4-		0.2	AGP/Extensin
		Total	**3.3**	
Ara*f*	Terminal		2.3	AGP/Extensin/Heteroxylan
	5-		0.1	Arabinan
		Total	**2.4**	
Fuc*p*	Terminal		0.2	Arabinan/Xyloglucan
	2-		0.1	AGP/RG-II
	3-		0.6	-
		Total	**0.9**	
Gal*p*	Terminal		2.7	AGP/Extensin/RG-I/Xyloglucan
	4-		0.2	AGP/RG-I
	6-		0.5	AGP/Galactomannan/RG-I/RG-II
	2,3-		0.1	AGP
	3,4-		0.1	AGP
	3,6-		1.4	AGP/RG-I
	2,3,6-		0.5	AGP
	3,4,6-		0.3	AGP
		Total	**5.8**	
Glc*p*	Terminal		2.0	Xyloglucan
	2-		2.2	AGP
	3-		8.0	Callose/Mixed linkage glucan
	4-		12.1	Cellulose/Glucomannan/Mixed linkage glucan/Starch/Xyloglucan
	6-		0.5	-
	2,4-		1.9	-
	3,4-		1.1	*
	4,6-		3.2	Starch/Xyloglucan
	2,4,6-		0.3	*
	3,4,6-		2.7	*
	2,3,4,6-		0.7	*
		Total	**34.7**	
Man*p*	Terminal		0.9	*
	2-		3.7	*
	3-		0.5	*
	4-		17.5	Mannan
	6-		0.2	*
	2,3-		5.0	*
	2,4-		0.2	*
	3,4-		4.8	Galactomannan
	4,6-		2.5	Mannan
	2,4,6-		0.3	-
		Total	**35.6**	
Rha*p*	Terminal		2.9	AGP
	2-		2.3	RG-I
	3-		2.3	RG-II
	2,3-		0.3	-
		Total	**7.8**	
Xyl*p*	Terminal		3.0	Xyloglucan
	2-		1.5	Xyloglucan
	4-		5.0	Xylan
		Total	**9.5**	

Table 4.1 I **Summary of monosaccharide linkage composition of REC1**

The sample (1 mg) was suspended in DMSO and agitated for 1 week. Methylation was undertaken by using 200 μL of 1 M sodium hydroxide, and then 100 μL methyl iodide. The sample was then hydrolysed using 400 μL 2 M trifluoroacetic acid at 121ºC. The sample was then reduced using 1.2 mL sodium borodeuteride (10 mg/mL) prepared in 1 M ammonium hydroxide. The resulting partially methylated alditol acetates were analysed using gas chromatography combined with mass spectrometry. This work was undertaken at the CCRC. The major linkages uncovered in the preparation were mannose (pyranose) based including 1,4-linked mannose, which forms the backbone of mannan. The other mannose linkages were not determined within plants. Glucose-based linkages were the second most abundant linkages found in REC1. In particular, 1,4-linked glucose was uncovered in the sample that forms the backbone of many polysaccharides including xyloglucan, cellulose, glucomannan and mixed linkage glucan. Another glucose linkage was 1,4,6-glucose which is found in xyloglucan. Other linkages uncovered within the preparation of REC1 were rhamnose-based, which can be found in AGP and rhamnogalacturonan, arabinose-based linkages found in AGP and extensin, and xylose-based linkages which form the backbone of xylan, and the decorations on xyloglucan. Abbreviations stand for: Ara, arabinose; Fuc, fucose, Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose; P, pyranose, F, furanose. All values (Mol %) ≥3 have been underlined. Any deduced linkage that cannot be assigned a clear polysaccharide signature has been marked (-). Signatures of fungal cell wall polysaccharides (*) may have been detected within the sample (Pettolino *et al.* 2009).

4.2.17 There was no mannan detected when screening REC1 with a wide range of mannan-specific probes

To explore further the presence of mannan within REC1, as deduced by the monosaccharide composition and monosaccharide linkage analyses, a range of mannan-specific probes (LM21, CCRCM-170; Pattathil *et al.* 2010 and CBM27; Boraston *et al.* 2003) were used to screen a sample of REC1. Other MAbs specific to AGP (LM2), extensin (LM1), xylan (CCRCM-108; Pattathil *et al.* 2010, LM11, LM27 and LM28) and xyloglucan (LM25) were included as these polysaccharides have been detected before. Additionally, callose (1,3-β-D-glucan; Meikle *et al.* 1991) and mixed linkage glucan (1,3:1,4-β-D-glucan; Meikle *et al.* 1994) specific MAbs were included. The screen revealed the presence of AGP, extensin, xylan and xyloglucan (Table 4.2). There was also a slight signal from CCRCM-108 which recognises the epitopes of methylated xylan. Furthermore, there was no detection of callose, mixed linkage glucan or mannan (Table 4.2). The lack of signals from the mannan-specific MAbs appears to contrast the monosaccharide composition and monosaccharide linkage analysis (Table 4.1 and Table 4.2).

Antigen	MAb	Absorbance (au)
Arabinogalactan-protein	**LM2**	0.15
Callose	1,3-β-glucan	-
Extensin	**LM1**	0.22
Heteromannan	CCRCM-170	-
	LM21	-
	CBM27	-
Mixed Linkage Glucan	1,3:1,4-β-glucan	-
Xylan	CCRCM-108	0.11
	LM11	0.61
	LM27	1.95
Xyloglucan	LM28	-
	LM25	2.46

Table 4.2 I **No mannan was detected when screening REC1 with a range of mannan-specific probes through ELISA**

After screening a sample of REC1 (10 µg/mL) through ELISA with a range of molecule probes specific to mannan (CCRCM-170, LM21 and CBM27), there was no mannan detected. This contrasts with the high levels of mannan detected within the monosaccharide composition and monosaccharide linkage analyses. The mannan detected may be novel to the roots of Cadenza or fungi cell walls. Callose (1,3-β-glucan) and mixed linkage glucan (1,3:1,4-β-glucan) were also not detected within REC1. AGP (LM2), extensin (LM1), xylan (CCRCM-108, LM11 and LM27) and xyloglucan (LM25) where detected within REC1. All LM and CCRCM antibodies were used at a 1:10 dilution, CBM was used at a concentration of 10 µg/mL, and callose and mixed linkage glucan antibodies were used at a 1:1,000 dilution. ELISA absorbance values were determined by measuring the absorbance at 450 nm. Data are a mean of two biological replicates. A cut off point of 0.1 au was removed, any values below this level were regarded as having no signal (-).

4.3 Discussion

4.3.1 Released polysaccharides have a complex biochemistry and potential linkages

There has been a renewed effort to explore the cell wall architecture with a particular focus on how polysaccharides form continues interconnected networks. The current view of the cell wall matrix is that cell wall constituents have a singular function (Keegstra 2010; Cosgrove and Park 2012). However, this concept is increasingly becoming more nuanced as more polysaccharides have been shown to be linked (Renard *et al.* 1997; Duan *et al.* 2004; Popper and Fry 2008) some of which have been shown to form a multi-polysaccharide complex (Tan *et al*. 2013). For the first time, this investigation has used EDC and sandwich-ELISA to explore the biochemical properties of polysaccharides released by roots. Using these tools, AGP, extensin, xylan and xyloglucan, were demonstrated to be linked as a part of a multi-polysaccharide complex released by cereal roots. Uncovering this released complex has expanded our understanding of polysaccharides released by roots. Prior to this investigation released polysaccharides were thought to be released as individual polysaccharides rather than the more interconnected structures extrapolated within this research. As it is believed that polysaccharides released from roots come from the cell walls of the root body (Bacic *et al.* 1986; Moody *et al.* 1988), they reflect the widening understanding of the cell wall architecture.

The initial EDC screens revealed the presence of two forms of xyloglucan, neutral and acidic, within the concentrated hydroponate of wheat (Figure 4.2 and Figure 4.3). The majority of literature suggests that xyloglucan is neutral (Somerville *et al*. 2004; Cosgrove 2005; Knox 2008; Albersheim *et al*. 2010). Acidic forms of xyloglucan have been determined, which include galacturonic acid residues, within basal land plants including liverworts (Peña *et al.* 2008), and within the root hair of Arabidopsis (Peña *et al.* 2012a). Acidic xyloglucan has not been previously determined in or being released by other plants particularly, cereals. The xyloglucan released by wheat as determined by this investigation may be novel or may not be acidic. The hydroponates of cereal indicate that there is a link between AGP, extensin, xylan and xyloglucan (Figure 4.3 and Figure 4.12). A comparison between the standard tamarind seed xyloglucan, which is neutral, was compared to the xyloglucan released by wheat roots (Figure 4.2). This comparison confirmed that the released xyloglucan was acidic. When taking all of this into account, the acidic xyloglucan uncovered in the concentrated hydroponate may be being retained within the anion-exchange column due to linkages with AGP. An AGP

macromolecule was detected co-eluting with the acidic xyloglucan. This AGP contained a glucuronic acid residue, inferred by the binding of LM2, which specifically recognises glucuronic acid within AGP. The presence of glucuronic acid makes AGP released by plants highly acidic (Smallwood *et al*. 1996; Yates *et al*. 1996). AGP characteristically consists of a small protein core (typically between 5-30 KDa) that has a series of highly heterogeneous arabinogalactans sidechains, which represents 90-95% of the total glycoprotein (Stacey *et al*. 1990; Showalter 2001; Geshi *et al*. 2013). This makes AGP a complex molecule, which may have attached the xyloglucan to the matrix within the anion-exchange column until enough salt had eluted the AGP, thus releasing the xyloglucan (Figure 4.2 and Figure 4.4). This indicates that the xyloglucan would remain neutral but is held in the column by the AGP, thus giving the appearance of an acidic xyloglucan. This AGP may act as a crosslinker within REC1, comparable to APAP1 (Tan *et al.* 2013), which holds the subdomains of extensin, xylan and xyloglucan together. To confirm this hypothesis degrading the AGP with galactanases that hydrolysed the β-1,3- and β-1,6-Gal*p* links that formed the backbone (Ellis *et al*. 2010), or using β-glucuronidase to target the β-glucuronic acid residue, as detected by LM2 (Smallwood *et al*. 1996; Yates *et al*. 1996) would be a key experiment. If degrading the AGP disrupts the co-elution of REC1 during anion-exchange EDC, then this would confirm the hypothesis. As a consequence, AGP could be the key polysaccharide within REC1.

Size-exclusion EDC (Figure 4.5) reveals a highly complex heterogeneous matrix of polysaccharides released from wheat roots. REC1 may not be comparable to APAP1 that has a defined AGP core that linked to the arabinoxylan and pectin subdomains (Tan *et al*. 2013). REC1 could be formed of different subpopulations of xyloglucan and xylan, which are linked to an AGP protein core with an extensin subdomain. Instead of neatly defined subdomains; REC1 may be formed of an aggregate of differentially sized polysaccharides. The chromatograms of the root body and hydroponate differed (Figure 4.5, A and B), suggesting the polysaccharides may aggregate after they are released by wheat roots forming these elongated traces. The xyloglucan released by wheat roots differed from the commercial standard, tamarind seed xyloglucan. The tamarind seed xyloglucan eluted from the column at the same time as the xyloglucan within REC1 (Figure 4.5, A). However, the tamarind seed xyloglucan was fully eluted at fraction 60, whereas, the xyloglucan from REC1 continued until it eluted at fraction 80. This suggests that

the xyloglucan released by wheat roots differs in size compared to the commercial sample.

When comparing polysaccharides that were contained within the root body to the hydroponate, xylan had the strongest signal, revealing two populations (Figure 4.5, B). This has been determined in previous work, which shows that xylan is highly abundant and heterogeneous in both the primary and secondary cell walls of grass species (Ebringerová and Heinze 2000; Vogel 2008; Simmons *et al*. 2016). The trace of xyloglucan on the anion-exchange chromatogram of the root body had a similar pattern to xylan, which suggests that there could be two populations of xyloglucan within the roots of wheat. Perhaps, containing higher amounts of a particular polysaccharide increases the chances of that polysaccharide becoming more diverse in size and structure. The factor that determines the size of each polysaccharide produced in a plant cell is unknown. There could be a great variation in the size of each polysaccharide, therefore, the differently sized polysaccharides could play a specific function or have a specific binding affinity to other polysaccharides.

The binding of LM11 within the concentrated hydroponate of wheat suggests the presence of heteroxylan (Figure 4.2). Furthermore, there was no signal of LM10 within the hydroponates of the cereal crops (Table 3.1 and Figure 3.3). LM10 is known to bind to the non-reducing terminal of xylan (Ruprecht *et al.* 2017). There was no binding of LM12, which indicates that the released xylan lacked ferulic acid, therefore, indicating that released xylan was neutral (McCartney *et al*. 2005). This is further supported by the lack of a signal from LM28 (Table 3.1), which binds to the epitopes of glucuronoxylan, which is an acidic form of xylan (Cornuault *et al*. 2015). If the released xylan was neutral then the signal of LM11 should have eluted earlier within the anion-exchange chromatogram, similar to that of the acidic xyloglucan. This further supports the hypothesis that xylan is linked to an acidic molecule (AGP). When samples of concentrated hydroponate were treated with xylanase and xyloglucanase there were no alterations within the co-elution (Figure 4.9). This verifies that xylan and xyloglucan do not affect the acidity of REC1.

When the onset of the salt gradient was delayed during anion-exchange EDC, the co-elution of the polysaccharides was retained for longer within the column (Figure 4.3). Additionally, there was no separation of the co-elution or any new peaks described. This demonstrates that the polysaccharides within this co-elution were strongly enough linked to be teased apart from the neutral peak of xyloglucan by

delaying the gradient of salt. If there was a weaker link between the polysaccharides the co-elution would have been disrupted as the salt gradient was altered. More detailed research using a shallow gradient, which do not have a sudden increase in salt (similar appearance to an exponential growth curve) is required to determine if the co-elution remains stable.

The total carbohydrate assay uncovered that there was a complex glycan content in the concentrated hydroponate of Cadenza. The neutral (fractions 0-25) and acidic (fractions 39-45) clusters of glycan generally confirm the observations made in anion-exchange EDC (Figure 4.3). However, there was a second mass of neutral glycan, which was unexpected. This second mass of neutral glycan had not been detected by the MAbs. This suggests that there could be another body of glycans being released by the wheat roots that are not detected by MAbs (Figure 4.6). This factor may play an important role in the polysaccharide matrix released by roots. There was a slight increase in MAb signals that were within the co-elution as the gradient of salt was delayed. This increase in signal suggests that this factor could potentially be masking the binding of the polysaccharides within this co-elution. More research exploring the total glycan and protein content is required to identify and characterise this mass of neutral glycan.

4.3.2 Complex heterogeneity occurred within the linkages of REC1

The monosaccharide composition and monosaccharide linkage analyses detected residues and linkages that are found in AGP, extensin, xylan and xyloglucan (Table 4.1) detected by the immunochemical assays. The detection of similar polysaccharide signatures verifies the observations made from the MAbs, which determined the presence of AGP, extensin, xylan and xyloglucan within the concentrated hydroponate of Cadenza. One surprise from these structural tests was the absence of acidic residues, particularly glucuronic acid found in AGP and galacturonic acid found in acidic xyloglucan (Figure 4.16). Previous work within this study determined that REC1 was acidic through the binding of LM2, which specifically recognises the epitopes of β-linked glucuronic acid present in AGP (Table 3.1 and Figure 3.3). Furthermore, the results from the anion-exchange chromatography highlighted the acidic property of REC1 (Figure 4.2). Past investigations have also determined the presence of glucuronic acid within the liquid medium of wheat (Moody *et al.* 1988). The investigation uncovered that glucuronic

acid formed 24% (total Mol) of the wheat exudate (Moody *et al*. 1986). The medium of maize has also been demonstrated to contain high levels of glucuronic acid (Bacic *et al.* 1986). Furthermore, galacturonic acid has also been detected within the root mucilage of wheat, representing a minor component (3% of the total monosaccharides detected; Moody *et al.* 1988). There are only two sources of galacturonic acid within the concentrated hydroponate of Cadenza as determined by MAbs, xyloglucan and xylan. Galacturonic acid found in xyloglucan has only been detected within the root hairs of Arabidopsis (Peña *et al.* 2012a) and basal plants such as liverworts (Peña *et al*. 2008). Additionally, galacturonic acid is also present within heteroxylans, glucuronoxylan and GAX, which are only present within cereals. This absence of galacturonic acid within the concentrated hydroponate of Cadenza indicates that the xylan and xyloglucan released by Cadenza are neutral. The discrepancy between this research and past investigations with the monosaccharide composition analysis (Figure 4.16) may be due to issues related to the methods employed by the CCRC or may suggest that REC1 is not acidic but could be highly sticky within the anion-exchange column.

During linkage analysis, the CCRC reported that there were issues with hydrolysing the sample. This may have been due to freeze-drying the sample, which has been previously reported to cause difficulties in rewetting (Pettolino *et al.* 2012). Perhaps, the glucuronic acid within the sample was insoluble or was somehow degraded during the analysis. Several gradients of salt were used through anion-exchange chromatography to analyse REC1 which all demonstrated that REC1 was eluting within the acidic region (Figure 4.2 and Figure 4.3). REC1 could be sticking to the anion-exchange columns until sufficient salt has dislodged the complex and eluted it. More work using different column matrixes is required to verify if REC1 is being retained in the column due to the complex being acidic or highly sticky. Using a cation-exchange column or different anion-exchange matrixes may help to expose if REC1 is acidic or highly sticky. Moreover, isolating more REC1 is required to undertake more structural tests that may further elucidate the acidity of the complex.

Previous examinations of root mucilage have detected similar monosaccharide residues and linkages, as well as their amounts to that of this investigation (Table 4.1; Bacic *et al.* 1986; Moody *et al.* 1988). Signatures of AGP (terminal Ara*f*, 6-linked Gal*p*, 3,6-linked Gal*p* and terminal Rha*p*), xylan (4-linked Xyl*p*) and xyloglucan (terminal Gal*p*, terminal Glc*p*, 4-linked Glc*p*, terminal and 2-linked Xyl*p*) have also been detected within the liquid medium of wheat seedlings (Moody *et al.* 1988). As well as these signatures, previous research has determined the presence

of linkages that cannot be assigned a clear polysaccharide signature, similar to this investigation. Past research examining the sterile liquid medium of wheat determined the presence of 6-linked Glc*p* and 2,4-linked Glc*p* (Moody *et al.* 1988). These unassigned linkages confirm the observations made in this investigation. Research examining the linkages within the media of other cereal crops such as maize and rice have also determine similar signatures of AGP, xylan and xyloglucan (Table 1.1; Bacic *et al.* 1986; Chaboud and Rougier 1984; Moody *et al.* 1988; Read and Gregory 1997). Other linkages uncovered within the liquid medium of maize seedlings grown in the dark, have also been detected within this investigation, namely 3-linked Fuc*p*, 2,3-linked Gal*p*, 3,6-linked Gal*p* and 2,3-Man*p* (Table 1.1; Bacic et al. 1986). Furthermore, 3,4-linked Gal*p* was detected within the liquid medium of cowpea seedlings (Table 1.1; Moody *et al.* 1988; Read and Gregory 1997). Although these linkages have been uncovered within several studies, how they fit together remains unknown.

This investigation has detected novel signatures of arabinan, cellulose (could also be xyloglucan; Figure 1.3), callose, extensin, galactomannan, mannan, mixed linkage glucan, RG-I and RG-II (Table 4.1) within the hydroponate of wheat, which have not previously been determined (Table 1.1). Much more research is needed to fully understand how these linkages are connected to determine which structures (oligo- or polysaccharide) they form. From the monosaccharide linkage analysis (Table 4.1), the preparation of REC1 is more heterogeneous and complex than initially thought. Considerably more research using NMR-based detection techniques are required to figure out how these linkages fit together. Is REC1 a complex or a highly heterogeneous aggregate or matrix of cell wall polysaccharides released by roots? Why is this complex so heterogeneous? These are amongst the many questions that remain unanswered about REC1.

Another unexpected result of the monosaccharide composition and linkage analyses was the high amount of mannose and 1,4-linked Man*p* linkages, which form the backbone of mannan (Figure 4.16; Figure 4.17; Table 4.1). From antibody-based approaches (Table 3.1 and Figure 3.3) there was no detection of mannan. Furthermore, there was an additional screen of a sample of REC1 using several mannan-specific molecular probes, which also did not detected any mannan (Table 4.2). This may suggest that there were issues with the methods employed by the CCRC or that the mannose residues and linkages are heavily masked by other molecules within the preparation. Screening a sample of REC1 with a range of mannan-specific MAbs and CBM revealed there was no signal of mannan (Table

4.2). This suggests that the mannan detected within the monosaccharide composition and monosaccharide linkage analyses may be novel to the exudate of Cadenza or fungi cell walls (Pettolino *et al.* 2009). Another explanation is that there was an issue with the structural analysis. Furthermore, the mannose residues and linkages may be modified in such a way that the mannan-specific MAbs could not recognise or that the mannose in REC1 is novel. There were also no signals from the callose and mixed linkage glucan MAbs when screening REC1 (Table 4.2). This may suggest that the deuced 1,3-linked Glcp (Table 4.1) may not belong to either callose or mixed linkage glucan but may be a linked novel in REC1.

Several mainly glucose and mannose linkages, for instance 3,4-linked Glc*p*, 2,4,6-linked Glc*p*, 2-linked Man*p*, 4-linked Man*p*, 2,3-linked and Man*p*, were detected within the preparation of REC1. These signatures may be from fungal cell wall polysaccharides (Pettolino *et al.* 2009) or from other possible sources of contamination, including from starch or paper (Pettolino *et al.* 2012). It may also be conceivable that these linkages (except for 4-linked Man*p*) are novel in plants. Wheat were grown in non-sterile conditions, which could have been contaminated with fungi, bacteria or a combination. Research exploring the major linkages within the cell walls of *Rhynchosporium secalis*, which causes barley leaf scald, determined that there were high levels of mannose and glucose linkages (Pettolino *et al.* 2009), which were also uncovered within this investigation (Table 4.1). It is known that barley leaf scald can infect wheat by entering the roots and causing symptoms such as greyish-green spots, dry lesions and chlorosis, primarily on the leaves (Pettolino *et al.* 2009). However, such symptoms were not apparent on the leaves of the wheat grown for this study. It is conceivable that other fungi or bacteria could have infected the hydroponics system without presenting any obvious indications of infection. This may account for the high amounts of mannose and mannose-based linkages detected. The MAbs may not bind to the epitopes of a mannan-like molecule released by fungi as they have been raised against plant cell wall polysaccharides. Within other research that grew wheat seedlings in sterile conditions, these linkages were not detected (Moody *et al.* 1988). One exception to the possible fungi cell wall linkages was 2,3-linked Man*p*, which had been detected within the sterilised liquid medium of maize (Bacic *et al.* 1986). How these possible fungal cell wall polysaccharides interact with the polysaccharides released from roots remains unclear. Future hydroponic systems would need to be grown in sterile conditions in order to prevent possible contamination issues.

When combining the signatures of polysaccharides detected within this investigation (Table 4.1), linkages described from past research (Bacic *et al.* 1986; Moody *et al.* 1988), and linkages present in the cell walls of barley leaf scald fungi (Pettolino *et al.* 2009) there are three linkages that have yet to be explained, 2,4-Glc*p* (1.9% of total linkages in REC1), 2,4,6-linked Man*p* (0.3%) and 2,3-linked Rha*p* (0.3%). These linkages appear to be specific to the REC1 preparation, and novel to wheat. Perhaps, these linkages play an important role within this putative complex.

4.3.3 Multi-polysaccharide complexes may share similar characteristics

Our understanding of the architecture of cell walls is becoming more complex with more emerging technologies used to explore it. The current paradigm of classifying cell wall polysaccharides into structurally defined groups, each with a single function (Keegstra 2010; Cosgrove and Park 2012) is increasingly becoming redundant. Recently, some cell wall polysaccharides have been found to be linked as a part of a multi-polysaccharide complex, APAP1 (Tan *et al*. 2013). Covalent linkages of xyloglucan and pectin have also been shown to occur (Duan *et al*. 2004; Popper and Fry 2008; Cornuault *et al.* 2015). These investigations have established a new understanding of cell wall architecture. Evidence thus far suggests that these multi-polysaccharide complexes may constitute a minor proportion of the cell wall (Tan *et al*. 2013). Furthermore, these complexes may be tissue-specific or specific to developmental stages of a plant. These complexes may also have crucial functions within the mechanical properties and dynamics of the cell wall matrix. The current understanding of these multi-polysaccharide complexes remains limited, with little evidence of their role, location and biosynthesis.

REC1 is the first multi-polysaccharide complex released by plant roots to be identified. The first complex uncovered in Arabidopsis callus, APAP1, is formed of an AGP core with covalently attached subdomains of RG-I and arabinoxylan (Tan *et al*. 2013). Prior to the discovery of these complexes, inter-glycan links between pectic polysaccharides and structural proteins were theoretically possible (Ryden and Selvendran 1990). Only until recently, covalent links between pectic polysaccharides and xyloglucan (Duan *et al*. 2004; Popper and Fry 2008), and hydrogen links between xylan and cellulose (Simmons *et al*. 2016) had been observed. Potential linkages of xylan-pectin and xylan-AGP have also been reported using immunochemical techniques (Cornuault *et al.* 2015). One major similarity between REC1 and APAP1 is the presence of AGP and xylan

subdomains. Within APAP1, the AGP acts as a crosslinker, which forms the core of the complex, holding the other polysaccharide subdomains together (Tan *et al.* 2013). This was confirmed by undertaking a knockdown of the gene that formed the AGP core. Once the knockdown had occurred, APAP1 was not detected (Tan *et al*. 2013). It is conceivable that the AGP within REC1 is also acting as a crosslinker molecule, holding the other subdomains together. Further evidence is shown by the delay in the elution of xyloglucan within the anion-exchange column when the extended salt gradient used. It was postulated that the AGP was retaining the other polysaccharides within the column (Figure 4.2 and Figure 4.3).

A limitation of APAP1 is that it is lacking in significance as it was only detected within the callus cells of Arabidopsis rather than the whole plant. Exploring the biochemistry of callus cells, which do not solely occur in the wild, may not be representative of whole plants. Whereas, REC1 appears to be a major factor released by roots. Growing cells of these plants may have affected the biosynthesis, mechanisms of release, and location of secretion. It would be interesting to see if the presence of APAP1 is altered within and being released from Arabidopsis plants rather than callus cells. This study detected REC1 by growing crop species in hydroponics, which may have also affected the release of these polysaccharides. Hydroponics remains an unnatural place for these crops to be grown in. It would be interesting to grow wheat in soil, or extract polysaccharides from soils that have had wheat growing in, with strong alkalis to see if REC1 can be detected.

4.3.4 REC1 may be constructed in the cell wall matrix

The biosynthesis of these multi-polysaccharide complexes remains unclear, however, it was speculated that the subdomains of APAP1 were produced in the Golgi apparatus (Tan *et al*. 2013). The biosynthesis of xylan and pectin occurs within the Golgi apparatus (Gibeaut and Carpita 1994; Reiter 2002). The synthesis of APAP1 would have to occur spatially and temporally in sync, as AGP is synthesised in both the endoplasmic reticulum (protein domain) and Golgi apparatus (glycan domain; Fincher *et al*. 1983; Gladys 1998). The biosynthesis of APAP1 probably occurs *in situ* within the cell wall matrix. This would require less energy as the whole structure would not need to be passed through the plasma membrane to get into the cell wall. The pectic and arabinoxylan subdomains may become attached to the AGP core within the cell wall matrix through enzymatic

(transglycanase) activities (A. Galloway, 2017. *Email to L. Tan*, 9 May). A comparable process could also occur for the biosynthesis of REC1 (Figure 4.3 and Figure 4.7) and REC2 (Figure 4.15).

The biosynthesis of plant polysaccharides occurs within the Golgi apparatus, with protein synthesis occurring within the rough endoplasmic reticulum (Albersheim *et al*. 2010). After production, these glycans and glycoproteins are packaged into vesicles and transported to the plasma membrane mediated through the protein complex exocyst for cell wall integration (Guinel and McCully 1986; Somerville *et al*. 2004; Synek *et al*. 2014). It is probable that the subdomains of REC1 are constructed on the plasma membrane, either as a continual conveyer or en bloc, after vesicles have delivered sufficient supplies of glycans. It would be less energetically favourable for these complexes to be passed through the plasma membrane, and then transported and integrated into the cell wall architecture. This would be like transporting a skyscraper from a construction site to a large city, and integrating it. It would be less resource intensive to transport smaller modules of a skyscraper to a large city, and fixing the modules together *in situ*. It would be interesting to explore the energy demands of REC1 exudation on plants.

As roots penetrate through deeper layers of soil they are subjected to ever increasing friction. This friction causes root cap cells to undergo lysis (Read and Gregory 1997; Iijima *et al*. 2002). As well as lysed cells, some cells are pre-programmed to detach themselves and actively release polysaccharides (Hawes *et al*. 1998; Driouich *et al*. 2013). Detaching border cells results in the loosening of cell walls of neighbouring cells. Cells with loosened cell walls actively produce a continual amount of polysaccharides, to ensure their structural integrity (Hawes *et al*. 2002; Driouich *et al*. 2013). As a consequence, it is reasonable to suggest that these means are the ways in which REC1 is released. Although the mechanisms underpinning the release of these multi-polysaccharide complexes remains uncertain. As cell detachment as not been observed from liverworts, it may be that lysed rhizoid cells result in the release of REC2. Nonetheless, rhizoids have not previously been determined to release molecules, and more research is required to further develop our understanding of liverwort exudates.

4.3.5 Pea and rapeseed may release a multi-polysaccharide complex that could be related to REC1

There were striking differences between the chromatograms of the concentrated hydroponates of eudicotyledons and cereals (Figure 4.3, Figure 4.10 and Figure 4.14). These differences suggest that plant roots release a unique mixture of polysaccharides. There was a range of pectic polysaccharides detected within the hydroponates of pea, rapeseed and tomato. This may reflect the high levels of pectin within the cell walls of eudicotyledons compared to cereals of which pectin is a minor component (Table 3.2). Pectin may have contributed to the biochemical diversity of the released polysaccharides from eudicotyledons. Pectic polysaccharides may be influencing the released polysaccharides assayed. Additional anion-exchange EDCs are required to explore the biochemistry of the released pectin, and if they are linked with the other released polysaccharides. Despite the lack of strong evidence of a multi-polysaccharide complex released by eudicotyledons, there were small hints that these polysaccharides could be linked (Figure 4.14). Additionally, there were some similarities between eudicotyledon and cereal released polysaccharides. There were generally two forms of xyloglucan released; neutral and acidic (Figure 4.14). This was particularly noticeable in the concentrated hydroponate of rapeseed, which is related to Arabidopsis that contains an acidic xyloglucan within its root hairs (Peña *et al*. 2012). Further biochemical exploration will clarify if these polysaccharides are linked as a complex or not.

4.3.6 Releasing multi-polysaccharide complexes may occur across land plants

Early land plants would have been confronted with high water stress, low nutrient ability and serve weather conditions compared to that of living in the primordial oceans (Field *et al*. 2015; Mitchell *et al*. 2016). Despite the long evolutionary distance between modern-day plants and the relatives of early land plants (~470 million years; liverworts), much of their cell wall matrix composition remains similar to that of Type I primary cell walls (Popper and Fry 2003; Del Bem and Vincentz 2010; Popper *et al*. 2011). Despite the similarities in cell wall composition, the function of rhizoids and root hairs greatly differs. Rhizoids were thought to serve only to anchor the plants into the primordial soils, whereas, root hairs are primarily involved in water and nutrient uptake, as well as anchorage (Jones and Dolan 2012; Arteaga-Vazquez 2015). Furthermore, it has been uncovered that the root hairs of

Arabidopsis also released an acidic xyloglucan (Peña *et al*. 2012), comparable to that of xyloglucan in liverwort cell walls (Peña *et al*. 2008). As of yet it has not been demonstrated that the rhizoids of liverworts are involved in releasing cell wall components. This investigation uncovered that liverwort rhizoids release cell wall-relate polysaccharides, which form an AGP-xyloglucan complex, REC2. This finding may lead to a change in our understanding of the mechanics of rhizoids, as initial work present here suggests that they can release polysaccharide. By screening agar gel extracts of liverworts with 29 MAbs, AGP and xyloglucan, as detected by LM2 and LM25 respectively, ranked the highest (work undertaken by Ms Bev Merry). Furthermore, using nitrocellulose tissue prints the areas releasing these molecules were confirmed to be released by the rhizoids. Higher levels of xyloglucan were observed being released at the tips of the rhizoids (Figure 4.15, **D**). This observation is comparable to the polysaccharides released from the root caps and tips of higher plants (Bacic *et al*. 1986; Morel *et al*. 1988; Read and Gregory 1997; Watanabe *et al*. 2000). Perhaps, mechanisms which underpin the release of these polysaccharides in liverworts are comparable to higher plants (Figure 4.18). Interestingly, within the initial screen of the liverwort agar extract there were no xylan and pectin detected, which are components of the cell walls in liverworts (Konno *et al.* 1987; Popper and Fry 2003). The reason for the lack of pectin and xylan within the agar extract remains undetermined.

Liverwort xyloglucan is acidic due to the presence of galacturonic acid (Peña *et al*. 2008), which accounts for the second acidic peak of xyloglucan within anion-exchange EDC (Figure 4.15, **B**). However, there was a neutral form of xyloglucan detected. This neutral form of xyloglucan is also a new finding as the xyloglucan within liverwort cell walls was thought to be only acidic (Peña *et al*. 2008). It is interesting to observe that in cereal and liverwort exudates that there are consistently two forms of xyloglucan, neutral and acidic (Figure 4.3 and Figure 4.15, **B**). There could be a larger polysaccharide undetectable by MAbs screened, which is highly neutral, eluting a part of the xyloglucan. More work is required to understand liverwort exuded polysaccharides, and to characterise the structure of REC2 through monosaccharide composition and monosaccharide linkage analyses. This may be problematic as liverworts are slow growing, and it was found that they do not grow well within a hydroponic system. As AGP and xyloglucan may be linked within the hydroponates of cereal and the exudates of liverwort, it may be that releasing these polysaccharide complexes is a trait of land plants. The last common ancestor of both cereal and liverworts were the first land plants to have emerged

from Charophyte (green algae), over 470 million years ago (Kenrick and Crane 1997; Sorensen *et al.* 2010; Fangel *et al.* 2012; Figure 4.15, **A**). Perhaps, the first land plants that released their cell wall components were more able to colonise the land by developing a primitive root-soil interface. This interface would make it more effective to extract water and nutrients from the primordial soil. It would be interesting to trace the evolutionary development of REC1 by exploring the exudates of other basal plants such as hornworts, and other types of plants such as horsetails, ferns, cycads and gymnosperm such as conifers (Figure 4.18). Furthermore, cross-referencing the evolutionary development of AGP, extensin, xylan and xyloglucan with their release would also be of interest.
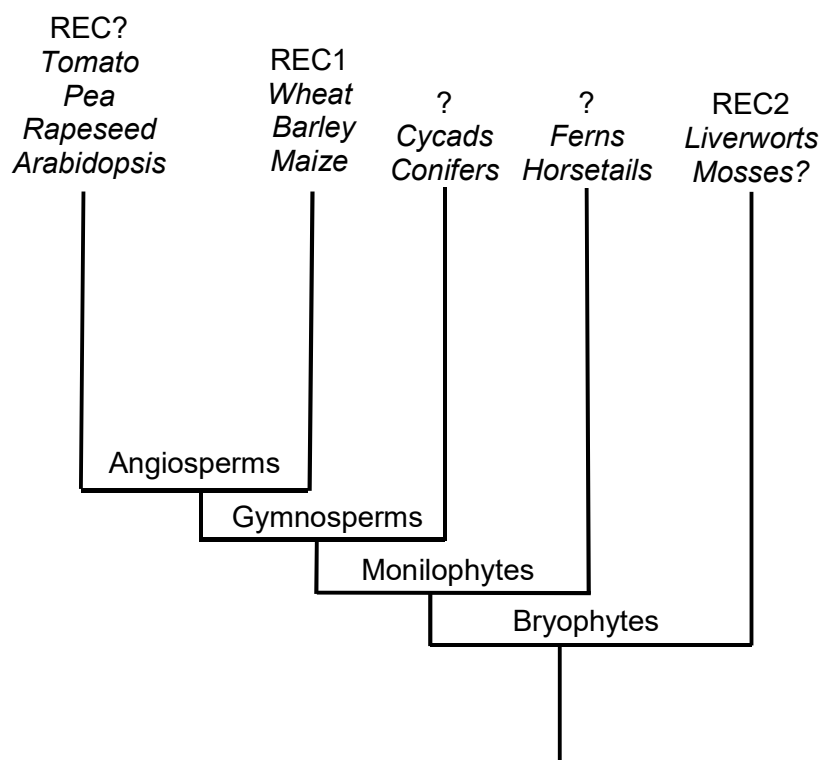
Figure 4.18 I **Schematic of REC1 detected across land plants**
REC1 was uncovered within the concentrated hydroponate of Cadenza. Further screening revealed the presence of a REC1-like molecule within the concentrated hydroponate of other wheat cultivars, Avalon and Skyfall. Expansion of the screening also revealed the presence of a REC1-like molecule released by the roots of barley and maize. Some evidence exists on a REC1-like molecule which may be released by the roots of pea, tomato and rapeseed. Liverworts were determined to release an AGP-xyloglucan putative complex, REC2, which may relate to REC1. Could other species across the plant kingdom from mosses to cycads release a complex similar to that of REC1?

The last common ancestor of liverworts and Arabidopsis was the first land plants, which emerged from fresh water green algae (Charophyte) over 470 million years

ago (Kenrick and Crane 1997; Sorensen *et al.* 2010). Prior to the colonisation of land, green algae were the dominant photosynthesising lifeforms. These algae had similar cell wall polysaccharides to that of early land plants, and modern-day plants. However, one major difference is that these green algae lacked xyloglucan (Fry 1989; Popper and Fry 2003). Taxonomic studies have determined that xyloglucan first appeared within the earliest land plants, particularly, liverworts (Popper and Fry 2003). This suggests that xyloglucan may have given early land plants an advantage that permitted the colonialisation of the land (Fry 1989; Popper and Fry 2008; Del Bem and Vincentz 2010). The acidic form of xyloglucan may be a relic of this pre-adaptive advantage or it may still play an important role for the establishment of roots in soils.

4.4 Conclusion

The current understanding of cell wall architecture is changing with the discovery of polysaccharide linkages, and multi-polysaccharide complexes. These changes reflect the growing complexity of released polysaccharides. Root Exudate Complex 1 was detected within the hydroponate of cereal. This complex is possibly formed of four subdomains, an AGP core with extensin, xylan and xyloglucan attachments. REC1 was initially uncovered when two forms of xyloglucan, neutral and acidic, were detected within the hydroponate of wheat. The charge of the AGP macromolecule may have been retaining the other subdomains within the anion-exchange column until enough salt eluted the AGP. This indicates that the xyloglucan was in fact neutral, and not acidic. Digesting the xylan and xyloglucan subdomains had no effects on the REC1 co-elution. This further supports the hypothesis that AGP is the major component of REC1. By extending the salt gradient, the REC1 fractions could be isolated for structural characterisation. The monosaccharide composition and linkage analyses verified the presence of the four major polysaccharides determined by the MAb. Furthermore, the linkage analysis demonstrated that REC1 contained a highly diverse mixture of linkages, some of which were novel to wheat. REC1 may be constructed within the cell wall matrix, and released through lysed root cap cells, actively released by border cells or lost from loosened cell walls of weakened root cells. The rhizoids of liverworts released an AGP-xyloglucan complex, REC2. By uncovering two multi-polysaccharide complexes in plants placed at opposite ends of the plant kingdom, these complexes may be wide spread in plants playing a vital function for plants.

Chapter 5

Influence of cell wall polysaccharides and REC1 on soil properties

5.1 Introduction

One of the aims of this study was to explore possible functions of released polysaccharides in relation to soil aggregate status. For this to occur, commercially equivalent components of the three major polysaccharides detected, AGP, xylan and xyloglucan, as well as the multi-polysaccharide complex, REC1 (Chapter 4), were added to soil. The soil was then assessed using various aggregate stability assays. This investigation uncovered that the commercial polysaccharide standards and subdomains of REC1 could readily adhere to soil, and increased the abundance of macroaggregates when supplemented to the soil. REC1 was the most effective soil aggregator when compared to the polysaccharide standards.

Plants secrete root mucilage to lubricate the root caps and tips to aid penetration through the soil. Once released, the polysaccharides within root mucilage contribute to carbon levels within the rhizosphere. This continual addition of carbon is thought to modify the structure of the surrounding soil (Tisdall and Oades 1982). In order to assess the structure of soil, aggregate status is measured and considered a good indicator of soil health (Kibblewhite *et al*. 2008). In uncultivated land, aggregates in soil are abundant, being formed of a mixture of organic and mineral particles, which increase the contact that the roots have with the soil and lowers the risk or erosion (Griffiths and Burns 1972; Tisdall and Oades 1982). An increase in the abundance of soil aggregates and strength of the adhesion of particles to the root surface, results in roots being able to extract more water and minerals from the rhizosphere. Other benefits include an increase in aeration, water infiltration and enhanced contact with beneficial soil-dwelling microorganisms, including mycorrhizal fungi (Haynes and Francis 1993; Lal 1997; Kibblewhite *et al*. 2008). In cultivated land, these aggregates are routinely broken down by systematic ploughing (Six and Paustian 1999; Ji *et al*. 2013). This agricultural treatment is resulting in ever increasing amounts of soil being eroded, and once productive land being lost (Hamilton *et al*. 2015).

To assess soil quality and determine how vulnerable a soil is to erosion, several techniques are employed. Various methods of sieving have been developed to examine aggregate stability (Day 1965; De Booth *et al.* 1984; Cheshire 1990; Brown 2008). One particular sieving method, wet sieving subjects soil to a constant flow of water to assess the water stability of aggregates (Brown 2008). Other techniques include visualising aggregates through optical microscopy (De Booth *et al.* 1984; Cheshire 1990; Hamilton *et al.* 2015). A novel application was developed for this

investigation, which used an automated particle characterisation microscope. As a part of this dry particle dispersion assay, soil was subjected to high mechanical forces through a burst of liquid nitrogen. This burst projects soil onto a glass slide where an automated particle characterisation microscope calculates the volume of each aggregate. If soil aggregates are strongly associated they will remain bound together after hitting the slide. If weakly associated with each other they will readily break apart after hitting the slide.

5.2 Results

5.2.1 Commercial polysaccharides can adhere to soil

To explore roles of polysaccharides released from plant roots on soil properties, a range of the most commonly available soil types were selected, including sandy, silt and clay loams. Commercially equivalent forms of known polysaccharides released by roots were added to soil to see if they could adhere to a heterogeneous mixture of particles within each soil type. The commercial polysaccharides, gum Arabic (AGP standard), xylan from birchwood (hereon referred to as xylan) and tamarind seed xyloglucan (hereon referred to as xyloglucan), were added (10 µg in 1 mL) to 1 mg sterile soil. The soil with the test polysaccharides was agitated for 2 h. After agitation, soil solutions were centrifuged (3,856 x g) for 10 min. After centrifugation, the supernatant from each sample was removed and screened using ELISA with the following MAbs (1:10 dilution), LM2 (AGP), LM11 (xylan) and LM25 (xyloglucan). Calibration curves were generated to convert the ELISA absorbances from the antibodies to µg/mL.

As expected the negative control (na) where test polysaccharides were only dissolved into dH_2O had the highest amounts detected. This also confirmed that the test polysaccharides were not adhering to the inner surface of the Falcon tubes (Figure 5.1, na). Adherence of the test polysaccharides was variable across the different soil types (Figure 5.1). Xyloglucan could readily adhere to all the soils. On average there was a reduction of 99% of xyloglucan when added to the substrates (One-Way Independent ANOVA, F= 351.12, P= 0.0001). There was a 43% reduction in gum Arabic when added to clay loam soil (One-Way Independent ANOVA, F= 40.32, P= <0.001). For xylan, there was a reduction of 97% when added to sandy loam soil (One-Way Independent ANOVA, F= 252.88, P= 0.0001). Overall, clay loam released the lowest amounts of test polysaccharides when the supernatant was assayed (Figure 5.1). Sand released the highest amounts of test polysaccharides when the supernatant was assayed.
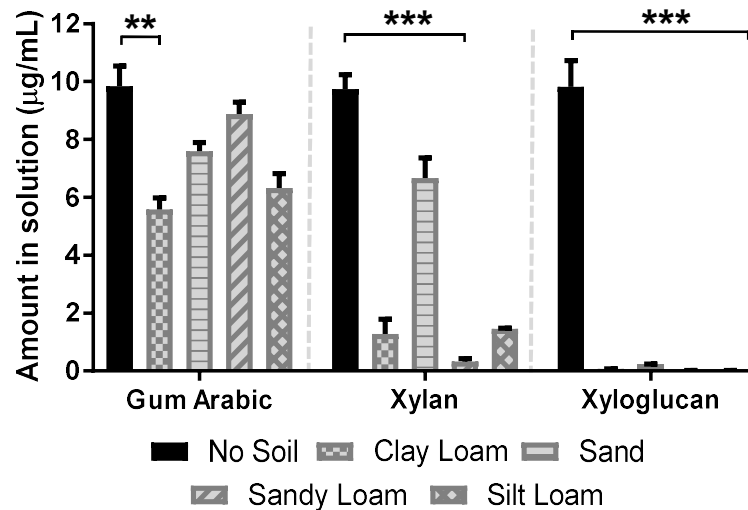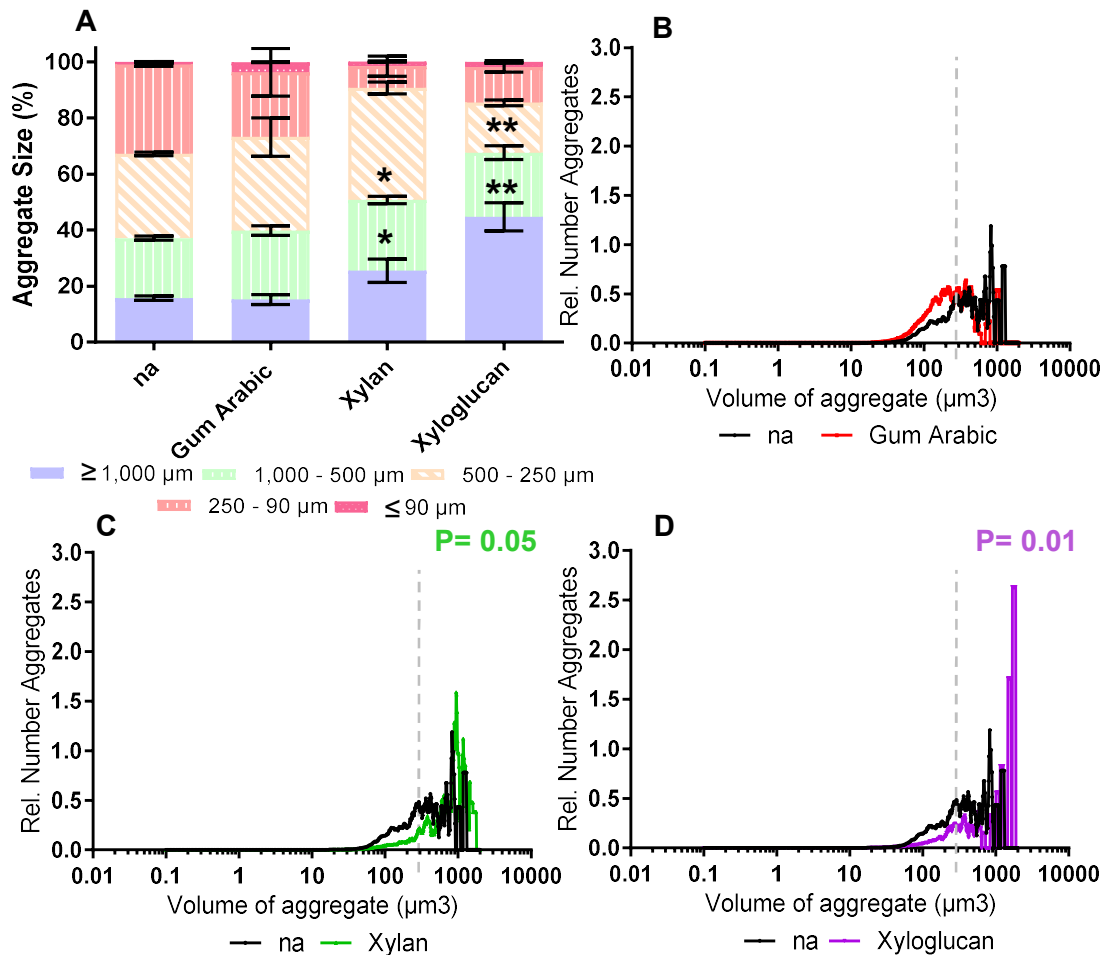
Figure 5.1 I **Commercial polysaccharides could adhere to a range of soil types**
Xyloglucan was the most effective commercial polysaccharide to adhere to the substrates. The most effective substrate to take up xyloglucan was the sandy loam soil. Gum Arabic was the least effective commercial polysaccharide to adhere to the substrates. However, the most effective substrate to take up gum Arabic was the clay loam soil. Xylan could readily adhere to clay, sandy and silt loam soils. The most effective substrate to take up xylan was the sandy loam soil. Commercial polysaccharides (10 µg in mL) were added to 1 mg of sterilised substrate, and incubated for 2 h. A 1:25 dilution of antigen was used; na = no substrate controls. Data are a mean of three biological replicates. Significant differences are indicated as follows: **P= 0.001 and ***P= 0.0001; standard deviation bars are displayed.

5.2.2 Xylan and xyloglucan increased the abundance of aggregates

To examine the effects of commercial polysaccharides (xylan and xyloglucan), equivalent to those released by plant roots on soil aggregate status, soil was subjected to wet sieving and dry particle dispersion analysis. One-hundred grams of sterile sandy loam soil was wetted with test polysaccharide solutions (10 mg/mL), and agitated for 2 h. After agitation, soil was added to a series of descending sized sieves, the highest sieve (1,000 µm) was placed on top, and the smallest (90 µm) placed at the bottom. Soil was subjected to a constant flow of tap water whilst being shaken for 5 min. After wet sieving, soil retained in each sieve was dried and weighed. The wet sieving analysis revealed that xylan and xyloglucan increased the abundance of macroaggregates ≥500 µm (Figure 5.2, **A**; Mann-Whitney U, W= 6.0, P= 0.01). As a result of the larger proportion of macroaggregates, the proportion of microaggregates was significantly lower when xylan and xyloglucan was added to the soil (Mann-Whitney U, W= 6.0, P= 0.01). When gum Arabic was added to the soil there was no significant alteration within the abundance of aggregates compared to the no soil control (Figure 5.2, **A**).

For dry dispersion analysis, test polysaccharide solutions (1 µg/mL) were added to sterile sandy loam soil, and agitated for 2 h. After agitation, soil was centrifuged with the supernatant removed. Soil was then dried for 48 h. A small scoop (18 mm^3) was added to a blast disk, which was placed within a sealed blast chamber. Soil was then projected onto a glass slide with high mechanical force through a burst of liquid nitrogen. An automated particle characterisation microscope determined the volumes of each aggregate that was on the slide. Dry dispersion analysis demonstrated an increase in the abundance of macroaggregates ≥500 µm^3 when test polysaccharides were added (Figure 5.2, **B, C** and **D**; Mann-Whitney U, W= 595, P= 0.01). This was most apparent when xyloglucan was added, which contained the most macroaggregates (Figure 5.2, **D**; One-way ANOVA, F= 670.52, P= 0.01). During dry particle dispersion analysis, gum Arabic contained the least amount of macroaggregates (≥250 µm^3) but contained a larger proportion of microaggregates (≤250 µm^3) compared to the other treatments (Figure 5.2, **B**; One-way ANOVA, F= 550.89, P= 0.01).

Figure 5.2 I **Commercial polysaccharides increase the abundance of aggregates in sandy loam soil**

Wet sieving analysis used 10 mg/mL of commercial polysaccharides which were added to 1 g of sterile sandy loam soil. Commercial standards included: gum Arabic, xylan and xyloglucan; na = no addition (**A**). Xyloglucan was the most effective polysaccharide to increase the abundance of aggregates ≥500 µm; standard deviation bars are displayed. Asterisks indicates significant difference P= <0.05 (**A**). Dry dispersion analysis used 18 mm^3 of sandy loam soil, which was subjected to a burst of liquid nitrogen, which projected the aggregates onto a glass slide (**B-D**). Commercial polysaccharides (1 µg in mL) were added to 1 mg of sterile substrate, and incubated for 2 h (**B-D**). Aggregates (≥500 µm^3) were more abundant within soils that contained xyloglucan and xylan (**C and D**). Soil with gum Arabic had fewer aggregates ≥500 µm^3 (**B**). na = no addition control (**A** and **B**); Rel. = Relative. Each data point is a mean of three biological replicates. Dashed line indicates where significance was calculated (aggregates ≥500 µm^3 where compared). Soil that contained xylan and xyloglucan could significantly increase the abundance of aggregates, whereas gum Arabic did not significantly increase the abundance of aggregates, P-values shown.

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5.2.3 Xyloglucan promoted aggregate formation in sandy loam soil

Scanning electron microscopy was used to visualise the effects that were previously observed when xyloglucan was added to sandy loam (Figure 5.1 and Figure 5.2, **D**). Xyloglucan was chosen to be imaged as it was the most effective polysaccharide to adhere to and increase the abundance of aggregates in the sandy loam soil. Xyloglucan (1 µg in 1 mL) was added to 1 mg sterile sandy loam soil, and agitated for 2 h. After agitation, soil was centrifuged with the supernatant removed. Soil was then dried for 48 h. Subsequently, soil was spread onto a glass slide with a stub containing a carbon-rich tape being dipped into the soil. A thin layer of platinum coated the particles that were stuck to the stub prior to imaging. SEM demonstrated that treating the soil with xyloglucan increased soil aggregation with small to medium sized particles (clay to slit) aggregating to each other (Figure 5.3, XG). Smaller soil particles could be seen aggregating onto the surface of the larger particles present in the sample (arrows), which has been supplemented with xyloglucan. Within the no addition control, low amounts of soil particles could be seen aggregating to larger particles (Figure 5.3, na; arrows).
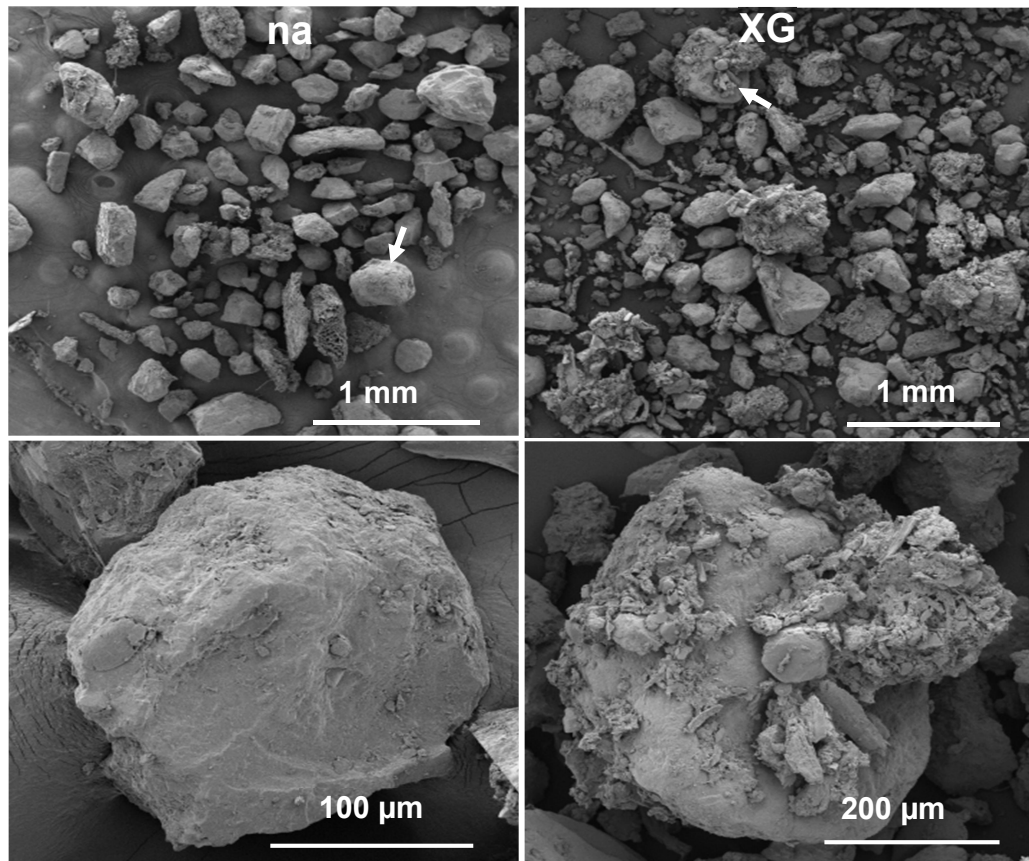
Figure 5.3 I **Scanning electron microscopy images of sandy loam soil with and without xyloglucan**
Xyloglucan (1 µg in 1 mL) was added to 1 mg of sterile sandy loam soil, and mixed in water for 2 h. After incubation, soil was dried for 48 h before imaging. Images on top are an overview of the soil samples using a magnification of 100x. Images below focus on a representative soil particle (shown by arrows) using a magnification of 500x. Adding xyloglucan increased soil particle binding, which enhanced soil aggregate formation, na = no addition.

5.2.4 Test polysaccharide increased aggregates in ground inert glacial rock

There was an opportunity to explore the aggregation effect caused by test polysaccharides using a young soil, glacial rock which contained no organic matter. This inert glacial rock was collected by Dr Katie Field from the Fox Glacier in New Zealand. After observing an increase in the abundance of aggregates in soil, inert ground glacial rock was selected (≥500 µm dry sieved) to determine if these test polysaccharides could aggregate particles without the presence of organic matter. Test polysaccharides (10 µg in 1 mL) were added to 1 mg sterile glacial rock, and agitated for 2 h. After 2 h, glacial solutions were centrifuged with the supernatant removed. Glacial rock samples were then dried for 48 h, and assayed using dry dispersion analysis. The analysis uncovered that the test polysaccharides could

increase the abundance of macroaggregates ≥500 µm^3 (Figure 5.4; Mann-Whitney U, W= 224.75, P= <0.01). Xylan ranked the highest test polysaccharide to accumulate the larger aggregates of glacial rock (Figure 4.5, **B**; One-way ANOVA, F= 124.32, P= 0.01). This was not observed when xylan was added to sandy loam soil which contained organic matter (Figure 5.2, **C**).
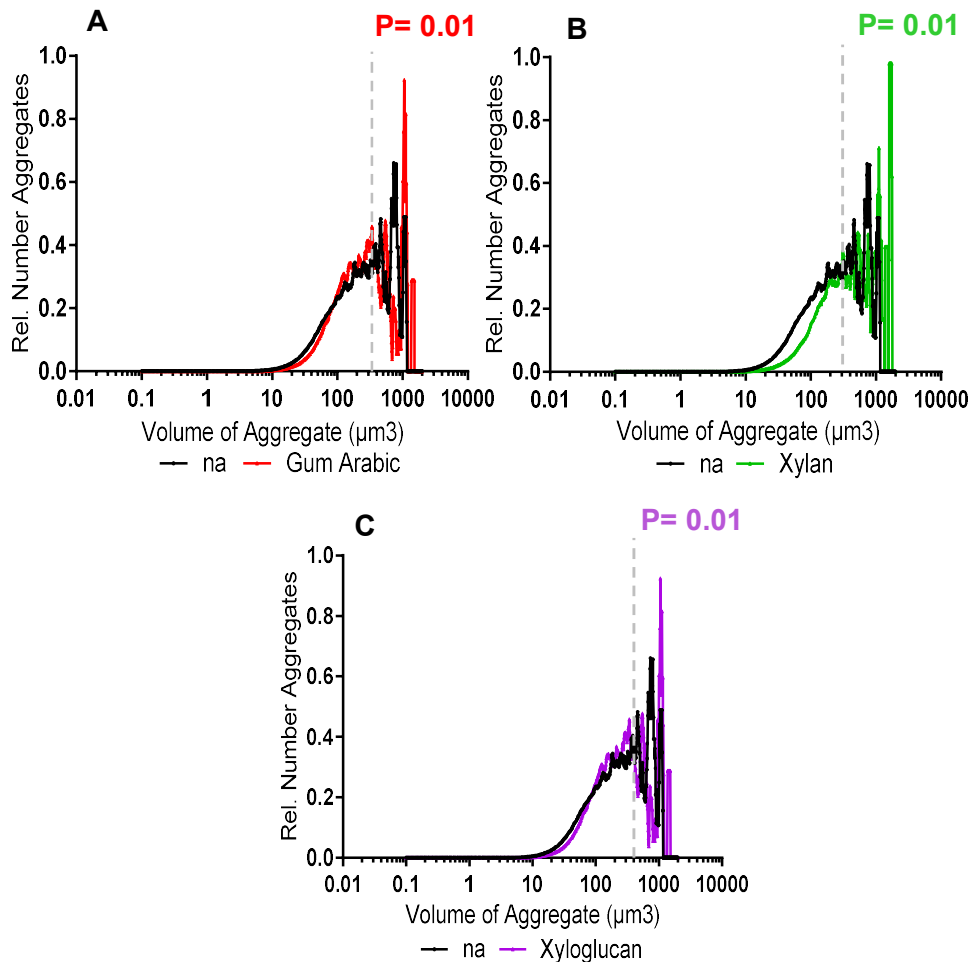
Figure 5.4 **I Commercial polysaccharides increased the abundance of aggregates of inert glacial rock**
Xylan (**B**) was the most effective at aggregating the glacial rock ≥500 µm^3 followed by xyloglucan (**C**) and Gum Arabic (**A**). Commercial polysaccharides (10 µg in 1 mL) were added to 1 mg (0.1% w/w) of sterile glacial rock, and incubated for two h. A 1:25 dilution of antigen was used; na = no substrate control. Each data point is a mean of three biological replicates. Dashed line indicates where significance as calculated (aggregates ≥500 µm^3 were compared). Glacial rock that contained gum Arabic, xylan and xyloglucan could significantly increase the abundance of aggregates, P-values shown. Rel. = Relative.

5.2.5 REC1 could adhere to soil and increase the abundance of aggregates

After exploring the commercially equivalent polysaccharides released by plant roots, a sufficient amount of REC1 (~1 mg) was collected from the concentrated hydroponate of Cadenza (Chapter 4). This was in preparation for soil adherence and aggregate status analyses. REC1 was added to soil with an ELISA being undertaken to determine if REC1 could adhere to soil. A dry particle dispersion analysis also occurred to see if REC1 could aggregate soils. Fifty micrograms of REC1 was dissolved into 100 µL of dH_2O and added to 50 mg soil. Once treated soil was agitated for 2 h, with the supernatant removed and assayed using an ELISA (Figure 5.5, **A**). The resulting pellet was dried and analysed using dry dispersion analysis (Figure 5.5, **B**). The subdomains of REC1 were detected by using LM1 (extensin), LM2 (AGP), LM11 (xylan) and LM25 (xyloglucan) MAbs within an ELISA, similar to the detection of the commercial polysaccharides that were added to soil. A control, where 50 µg of REC1 was dissolved in 100 µL of dH_2O without the soil, was used.

The ELISA demonstrates that all the subdomains of REC1 could adhere to the sandy loam soil (Figure 5.5, **A**). The MAb signals were significantly lower compared to the no soil control (One-way Independent ANOVA, F= 6124.21, P= 0.01). For the dry dispersion analysis, the control only consisted of 100 µL dH_2O and 50 mg soil. The analysis uncovered the REC1 treatment rapidly increased the abundance of macroaggregates ≥500 $µm^3$ compared to the no addition control (Figure 5.5, **B**; Two-Sample T-Test, T= 40.49, P= <0.001). The proportion of microaggregates within the REC1 treatment was significantly reduced (Figure 5.5, **B**; Two-Sample T-Test, T= 36.11, P= <0.05).
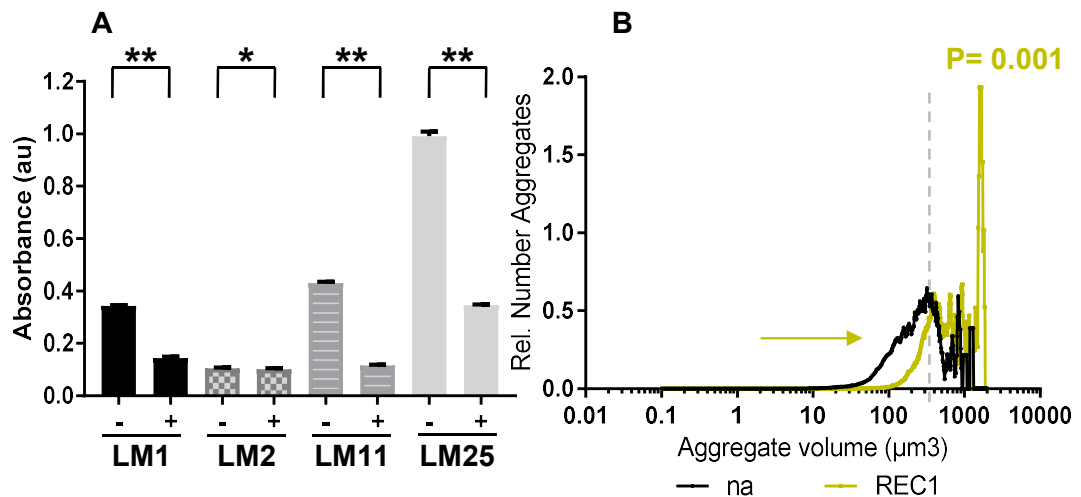
Figure 5.5 I **REC1 could adhere to soil and increased the abundance of soil aggregates**
Fifty micrograms of REC1 was added to 50 mg of sterile sandy loam soil (+), and incubated
with 100 µL dH$_2$O for 2 h (**A** and **B**). The signals of LM1 (extensin), LM2 (AGP), LM11
(xylan), and LM25 (xyloglucan) released from the sandy loam was lower compared to the no
addition control (-), which demonstrates that the components of REC1 could adhere to the
sandy loam soil (**A**). Data are a mean of three biological replicates. A 1:125 dilution of
antigen was shown; standard deviation bars are shown. Asterisks indicates significant
differences, *P= 0.05, **P=0.001 and ***P= 0.0001 (**A**). REC1 significantly increased the
abundance of aggregates (≥500 µm^3) compared to the no addition control (na), P-values
shown (**B**). The proportion of microaggregates was lower within the REC1 treatment as
indicated by the arrow (**B**). Each data point is a mean of three biological replicates. For dry
dispersion analysis only aggregates ≥500 µm^3 were used to test for significance, as shown
by dashed line (**B**). Rel. = Relative.

5.2.6 Application of REC1 resulted in more soil aggregates compared to the commercial polysaccharides

To gauge the effectiveness of REC1 at increasing the proportion of aggregates, a
comparative dry dispersion analysis was undertaken using commercially equivalent
polysaccharides, xylan and xyloglucan. Lower concentrations of each commercial
polysaccharide were used for this assay as they were found to be more effective at
aggregating soil using lower concentrations (Figure 5.4). Each polysaccharide
including REC1 (50 µg) was dissolved into 100 µL of dH$_2$O and then added to 50
mg sterile sandy loam soil. Soil was agitated for 2 h, and centrifuged to remove the
supernatants. Soil was then dried for 48 h, and examined using a dry dispersion
analysis. The analysis uncovered that xylan and xyloglucan increased the
abundance of aggregates ≥500 µm^3 compared to the no addition (Figure 5.6). Xylan
ranked the most effective commercial polysaccharide at increasing
macroaggregates (Kruskal-Wallis Test H= 9.36, P= <0.01). This was also observed
when xylan was added to inert glacial rock (Figure 5.4). Xylan became more
effective at aggregating soil particles compared to xyloglucan when used at a lower

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concentration (Figure 5.2, **C** and **D**, Figure 5.6; Mann-Whitney U, W= 1962.0, P= <0.05). When REC1 was added to the soil there was also an increase in the abundance of macroaggregates ≥500 μm^3 (Figure 5.6), as previously observed (Figure 5.5, B). When ranking all treatments, REC1 was the most effective aggregator of soil particles compared to commercial polysaccharides (Mann-Whitney U, W= 2608.0, P= 0.01). The portion of microaggregates was significantly less within the REC1 treatment compared to the others (Mann-Whitney U, W= 2974.0, P= 0.001). The portion of macroaggregates ≥1,000 μm^3 also greatly varied within the treatments. Macroaggregates ≥1,000 μm^3 represented 42% of the total soil fraction of REC1, whereas, for xylan this was 38%, and for xyloglucan this was 15% (Figure 5.6).
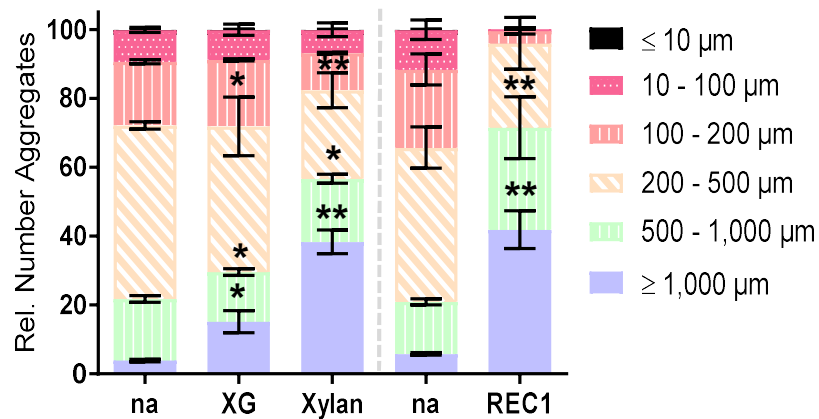
Figure 5.6 I **REC1 was the most effective soil aggregator compared to commercial polysaccharides**

Xylan, xyloglucan and REC1 (50 µg) were dissolved into 100 µL of dH_2O and added to 50 mg of sterile sandy, and incubated with for 2 h. Two no addition (na) controls were undertaken using three biological replicates each. REC1 was the most effective treatment that increased the abundance of aggregates ≥1,000 µm3. Xylan was the second most effective treatment followed by xyloglucan. Each data point is a mean of three biological replicates; standard deviation bars are shown; asterisks (*) indicates significant difference P= <0.05, and (**) P= 0.01. Rel. = Relative.

5.2.7 REC1 promoted aggregation within sandy loam soil

To visualise the effects of adding REC1 to soil SEM was used. REC1 (50 µg) was dissolved in 100 µL of dH$_2$O, and then added to 50 mg of soil. This soil solution was agitated for 2 h. After agitation, soil was centrifuged with the supernatant removed. Soils were then dried for 48 h. Soil with and without REC1 were spread onto glass slides with a stub containing a carbon-rich tape being dipped into the soil, and coated with a thin layer of platinum. Overall images of the soil are placed on top panel (Figure 5.7). Representative particles chosen from both groups are placed at the bottom. There are few clay and silt particles within the no addition sample, which can be seen forming aggregates. Mostly large smooth particles can be seen within the no addition (Figure 5.7; na). This may be due to the lack of substances retaining these particles on the surface of large organic or inorganic particles. Within the soil that has had REC1, lots of aggregates made of clay and silt particles are shown, forming aggregates (Figure 5.7; REC1). A larger proportion of these aggregates are probably sticking to large organic and inorganic particles. A large group of these aggregates can also be seen within the centre of the micrograph (Figure 5.7; REC1). REC1 appears to be acting as glue, retaining these smaller particles that are present within the SEM micrographs (arrows). Within the no addition there was a low amount of smaller particles (arrows). This may have been because they had been washed out during the removal of the supernatant. This was not observed in the soil that had REC1, where small particles can be seen sticking to larger particles.
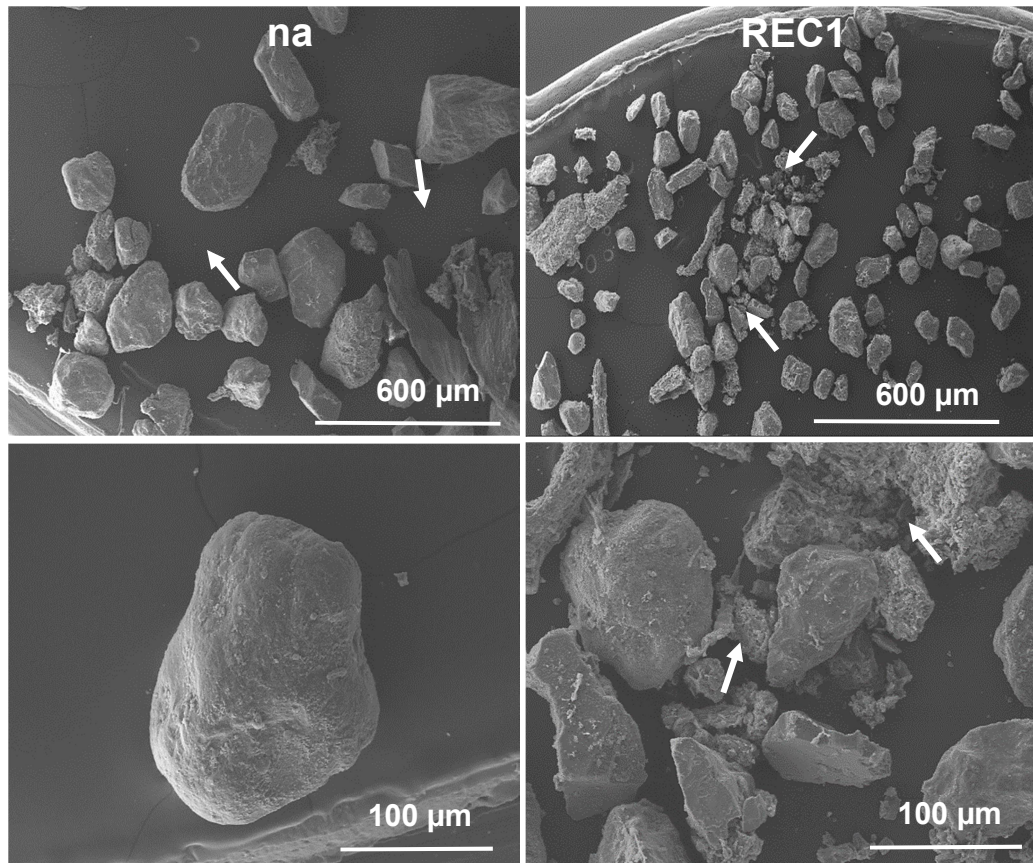
Figure 5.7 | **Scanning electron microscopy images of sandy loam soil with and without REC1**
REC1 (50 µg in 100 µL dH$_2$O) was added to 50 mg of sterile sandy loam soil, and incubated for 2 h. After incubation, soil was dried for 48 h prior to imaging. Images on top are an overview of the soil samples using a magnification of 70x. Images below focus on a representative soil particle using a magnification of 300x. Adding REC1 to soil increased soil aggregation, which in turn increased the appearance of soil particles that were rough denoted by arrows. Low amounts of small particles (arrow) can be seen within the no addition (na), which may have been lost during the removal of the supernatant prior to drying. Small clay particles can be seen clinging onto larger silt particles (arrows).

5.2.8 Immunofluorescence microscopy of sandy loam soil reveals xyloglucan adhering to soil particles

To visualise the locations where polysaccharides were binding to soil particles, immunofluorescence microscopy was used. Commercial polysaccharides, xylan and xyloglucan, were selected for immunofluorescence microscopy as there were MAbs which could be used to reveal where they were binding. This would not have been possible for REC1 as there are no MAbs raised against this multi-polysaccharide complex. Although four MAbs can be used to infer the presence REC1 this would result in more of the preparation being used for imaging particularly, when REC1 is in such short supply. Xyloglucan (10 µg in 1 mL) was added to 1 mg of soil, and agitated for 2 h. Once agitated, soil was centrifuged with the supernatant removed,

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and the pellet dried for 48 h. After drying, soil was placed into eight well glass slides that had been Vectabonded. Soil was then probed with LM25, and stained with anti-rat-FITC to visualise xyloglucan binding. Soil was imaged using a 40x objective. When xyloglucan was added to the soil, bright fluorescence was clearly shown where aggregates were present. This was demonstrated when the bright-field and FITC channels were combined (Figure 5.8; +LM25, +XG). When combined the bright-field and FITC channels there was no florescence when the xyloglucan was excluded from the soil (Figure 5.8; +LM25, -XG), and when LM25 was excluded from the soil (Figure 5.8; -LM25, +XG).
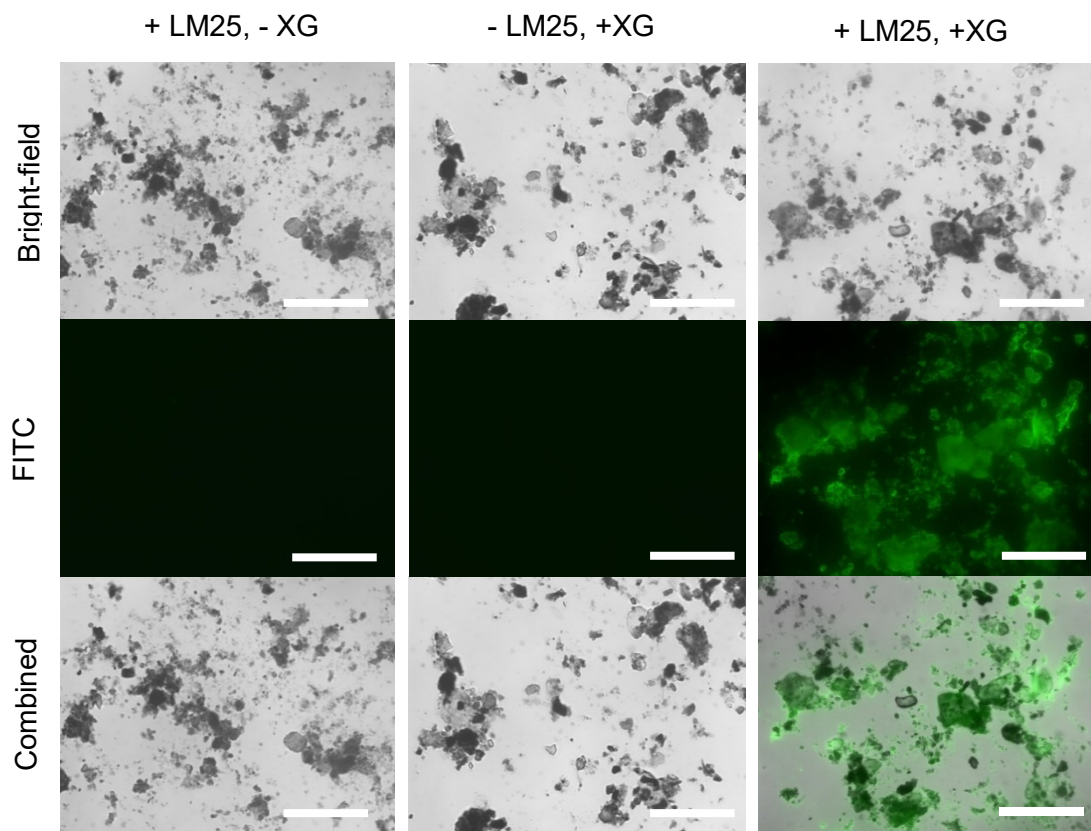
Figure 5.8 I **Detection of xyloglucan that was added to sterile sandy loam soil**
Xyloglucan was added at 10 µg in 1 mL to 1 mg of sterile sandy loam soil, and incubated for 2 h. Sandy loam soil then was centrifuged, and supernatant removed. Soil was then added to an eight well Vectabonded slide. Signals of LM25 can be clearly seen where soil aggregates were present. When removing either xyloglucan or LM25 from the soil no fluorescence was shown. Toluidine Blue O (0.1%; pH 5.5 0.2 M phosphate) was added to each well to remove autofluorescence. Scale bar = 50 µm.

5.2.9 Immunofluorescence microscopy of soil reveals xylan adhering to soil particles

To demonstrate that xylan could also be seen adhering to soil aggregates, immunofluorescence microscopy was undertaken. Xylan (10 µg in 1 mL) was added to 1 mg of soil, and aggregated for 2 h. Once agitated, soil was centrifuged with the supernatant removed, and the pellet dried for 48 h. After drying, soil was placed into eight well glass slides that had been coated with Vectabonded adhesive agent. Soil was then probed with LM11, and stained with anti-rat-FITC to visualise xylan binding. Soil was imaged using a 40x objective. When xylan was added to the soil, strong fluorescence was apparent where the aggregates were present. This was demonstrated when the bright-field and FITC channels were combined (Figure 5.9; +LM11, +X). When combining the bright-field and FITC channels, there was no florescence when the xylan was excluded from the soil (Figure 5.9; +LM11, -X), and when LM11 was excluded from the soil (Figure 5.9; -LM11, +X).
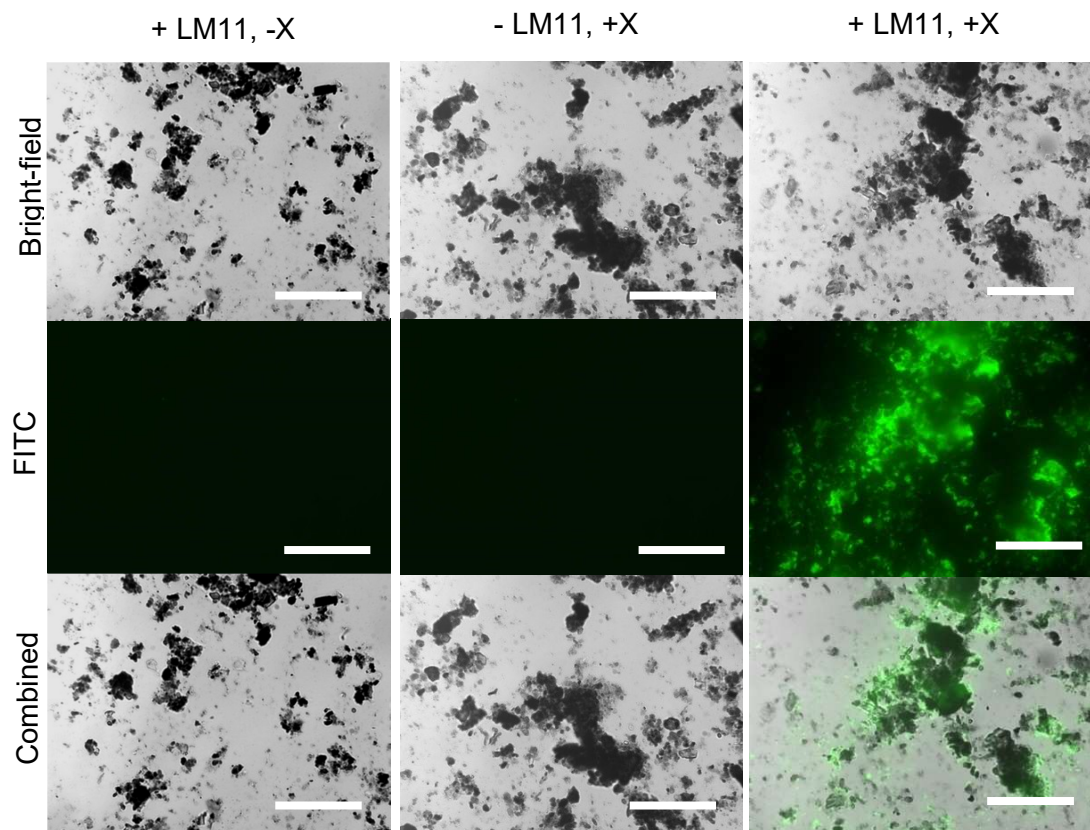
Figure 5.9 I **Detection of xylan that was added to a sterile sandy loam**
Xylan was added at 10 µg (in 1 mL) to 1 mg of sterile sandy loam soil, and incubated for 2 h. Sandy loam soil was centrifuged, and supernatant removed. Soil was then added to an eight well Vectabonded slide. Signals of LM11 were clear where soil aggregates were present. When removing either xylan or LM11 from the soil no fluorescence was shown. Toluidine Blue O (0.1%; pH 5.5 0.2 M phosphate) was added to each well to remove autofluorescence. Scale bar = 50 µm.

5.3 Discussion

5.3.1 Soil aggregation enhances root-soil contact

This research has shed a new light in the involvement of plant-derived polysaccharides in soil aggregate status. The current view of polysaccharides and glycoproteins released from plant roots is that they solely serve to lubricate roots to reduce friction as they penetrate through deeper layers of soil (Guinel and McCully 1986; Read and Gregory 1997). Research using isolated root mucilage from maize has hinted at the involvement of polysaccharide release by roots in aggregate status (Tisdall and Oades 1982; Morel *at al*. 1991; Watt *et al*. 1993). However, no detailed analysis of plant-derived polysaccharides involved in soil aggregate formation has been carried out.

This investigation has taken commercial plant-derived polysaccharides, xylan and xyloglucan, and has determined that they increase the abundance of aggregates within soil (Figure 5.2). Furthermore, these commercial polysaccharides increased the abundance of aggregates within glacial rock, without the presence of native organic residue (Figure 5.4). The abundance of soil aggregates had also rapidly increased when an isolated sample of Root Exudate Complex 1 (REC1) (Chapter 4) from the hydroponate of Cadenza was added to soil (Figure 5.5). When REC1 was compared to the commercial polysaccharides, REC1 was the most effective aggregator of soil (Figure 5.6). Increasing the abundance of aggregates enhances the rhizosheath, which in turns increases the extraction of water and nutrients from the soil, and provides a superior platform for initiating symbiotic associations with mycorrhizal fungi and nitrogen-fixing bacteria, and increases water infiltration capacity and soil aeration (Hartnett *et al.* 2012; Lehmann and Kleber 2015).

5.3.2 Plant-based polysaccharides cause soil aggregation

For the first time, immunofluorescence staining has been utilised on soil to enable the visualisation of xylan (Figure 5.8) and xyloglucan that was added to soil (Figure 5.9). When stained, both commercial polysaccharides were only located to aggregates. Previous studies were unable to visualise the binding of plant-derived polysaccharides within soils due to a lack of molecular probes (Oades 1978; Tisdall and Oades 1982; Oades 1984; De Booth *et al*. 1984; Cheshire 1990). Only the resulting effects of the polysaccharides could be documented using light or electron microscopy. This investigation determined that commercial plant-derived

polysaccharides added to soil had adhered differently to different soil types (Figure 5.1). It has been documented that polysaccharides initially adhere to the smallest soil particles, clay, to form microaggregates (Day 1965; Oades 1984; McCully 1999). Once microaggregates ($\leq$250 μm^3) form they can also bind to each other to form macroaggregates ($\geq$250 μm^3). As the amount of clay particles varies between soil types (Brown 2008), the binding of commercial polysaccharide would inevitably vary. The fewer clay particles available, the less a polysaccharide could bind. Clay loam, the substrate with the most clay particles, had the largest reduction of commercial polysaccharides detected within the supernatant (Figure 5.1). As sand contains no clay particles there should be no binding. However, there was a significant reduction in the commercial polysaccharides added.

The amount of polysaccharide added to the substrate was 1% (w/w), which may have saturated the substrate with commercial polysaccharide. The amount added may seem credibly high, and not presentative of a native soil, however, research suggests levels of polysaccharide released would be within this concentration close to the root cap and tips of plants (Oades 1984; Morel *et al*. 1991; Watt *et al*. 1993; Traore *et al*. 2000). Previous research adding commercial polysaccharides to soil, xanthan (Chang *et al*. 2015), polygalacturonic acid and glucose (Tisdall and Oades 1982; May *et al*. 1993) and guard gum (Metha *et al.* 1960) used concentrations of 1% (w/w), similar to this investigation (Figure 5.2). These studies also revealed that the commercial polysaccharides could aggregate soil particles. However, these studies did not investigate the stability of these aggregates.

It has yet to be determined if plant roots can regulate the rhizosphere depending on the needs of the plant for instance, to aid with water uptake during periods of drought. However, some research examining the rhizosheaths of various grasses, including spotted brachiaria (*Brachiaria nigropedata*) and canegrass (*Eragrostis rigidior*), has indicated that these plants could alter the thickness of their rhizosheaths in response to drought (Hartnett *et al.* 2012). Moreover, there were differences within the thickness of the rhizosheath between the subfamilies of Poaceae during drought. For instance, Arundinoideae (C3 grasses; including giant reed) had a significantly thicker rhizosheaths with a mean diameter of ~529 μm compared to Panicoideae (including maize and sugar cane), which had a mean diameter of ~280 μm (Hartnett *et al.* 2012). It may be possible that other grasses including cereals (particularly wheat) may form different rhizosheaths that could be strengthened during periods of drought. To date, no research has been undertaken

to explore the temporal dynamics of the rhizosheath. Plants may even a high burst of polysaccharides as a part of their attempts to establish their root network. Once established, plants may reduce the release of these polysaccharides as they begin to establish their rhizosheath.

5.3.3 Aggregates can be formed from plant-derived polysaccharides

Isolated maize root mucilage (Oades 1984; Watt et al. 1993; McCully and Sealey 1996) or modelled mucilages using polygalacturonic acid and glucose (Tisdall and Oades 1982; May *et al*. 1993) were the only examples of plant-based polysaccharides that were shown to be involved in the formation of soil aggregates. This limited use of plant-based polysaccharides had left a wide gap in knowledge. The only well documented example of a polysaccharide released by an organism, which caused soil to aggregate is xanthan gum from the bacteria *Xanthomonas campestri* (Chang *et al*. 2015). This study uncovered that the exuded polysaccharides from *Xanthomonas* acted in a similar way to that of modelled root mucilage and root mucilage isolated from maize. Microorganisms in soils are known to release exopolysaccharides in order to bind together, forming an extracellular matrix (Griffiths and Jones 1965; Chang *et al*. 2015). This matrix aids biochemical reactions between the cells, supports molecular signalling and resource shuttling (Flemming and Wingender 2010). As an indirect function, these exopolysaccharides enhance the interface that these organisms have with the soil (Flemming and Wingender 2010). This interface increases uptake of water and nutrients to cells just like the rhizosheath. Perhaps, the majority of soil dwelling organisms release polysaccharides either directly or indirectly to maintain this life supporting contact with the soil.

Wet sieving and dry particle dispersion analyses revealed that aggregates formed from commercial polysaccharides were highly stable (Figure 5.2). Subjecting these aggregates, that had been hydrated, with a high flow of water, and high mechanical pressure did not disrupt these aggregates. The abundance of macroaggregates ≥500 μm^3 was typically observed when the commercial polysaccharides, xylan and xyloglucan as well as REC1 were added to soil (each at 0.1% w/w). Furthermore, polygalacturonic acid, a commercial form of pectin, was also found to increase the abundance of macroaggregates ≥500 μm^3 in soil but to a lesser amount as to xylan and xyloglucan (data not included). These increases in aggregate size are twice the size of previously determined aggregates (typically ≤250 μm^3) formed from

modelled root mucilage, polygalacturonic acid and glucose, and isolated maize root mucilage (Tisdall and Oades 1982, May *et al*. 1993). The accumulation of macroaggregates due to the commercial polysaccharides and REC1 has until now only been reported to be caused by roots, hyphae networks and decaying organic matter such as leaves (Reid and Goss 1981; Tisdall 1994).

When using SEM, the binding of small particles to larger ones, producing aggregates was observed when xyloglucan and REC1 was added to soil (Figure 5.3). The sizes of the aggregates observed within SEM matched what was being observed within the aggregate stability assays. This supports previous research, which determined that soil aggregates formed by polysaccharides derived from decaying leaf matter are highly stable (Cheshire *et al*. 1985; Cheshire 1990). Aggregates formed by these polysaccharides could only be disrupted using sodium periodate which oxidises the bonds between adjacent carboxyl and hydroxyl-bearing carbons within the polysaccharide, which in turn degrades the polysaccharide strands holding clay particles together (Clapp and Emerson 1965; Cheshire *et al*. 1985). It would be of interest to explore the effects on soil structure and aggregate stability using a larger library of commercially available plant-derived polysaccharides. Perhaps, with more research a soil conditioner using a more cost effective form of commercial polysaccharide or with a blended combination of commercial polysaccharides could be developed to maintain or enhance soil aggregates. This product could help to prevent soil erosion, which is a major problem caused modern-day agricultural production.

5.3.4 Aggregation formation in soil is highly dynamic

Polysaccharides are thought to cause aggregation by binding to clay particles through weak hydrogen bonds between the carboxyl, and to a lesser degree the hydroxyl groups present on the molecule, to the cations attached to the surface of the clay particle (Foster 1981, Traore *et al*. 2000; Olsson *et al*. 2011; Figure 1.4). Once polysaccharides become bound to the surface of clay particles they form bridges between other clay particles, thus causing soil particles to form microaggregates ($\leq$250 μm^3; Foster 1981, Fitz Patrick 1993; Traore *et al*. 2000). These microaggregates then bind together to form macroaggregates ($\geq$250 μm^3; Tisdall and Oades 1982; Cheshire 1990). Inert glacial rock was also assayed within this study, which revealed a comparable aggregation effect to that observed using

sterile sandy loam soil (Figure 5.4). This supports the hypothesis of polysaccharide adhering to the inert surface of clay particles through cations, such as Ca^{2+}, K^+ and Mg^{2+}. If aggregation only occurred within sterile sandy loam soil, it would suggest that binding only occurred with the already present organic debris. Perhaps polysaccharides can adhere to both the organic and inorganic particles in soil. Previous work indicates that when polysaccharides are bound to clay particles, the molecules are inaccessible to soil dwelling microorganisms (Tisdall and Oades 1982; Cheshire 1990). It would be interesting to undertake xylanase and xyloglucanase treatments of soil and inert glacial rock with the addition of xylan and xyloglucan. Preliminary work suggests that these polysaccharides cannot be degraded by enzymes when bound to soil particles (Cheshire 1990). This adds to the growing body of evidence that these commercial polysaccharides form highly stable aggregates.

Polysaccharide aggregates are not held together indefinitely but degrade over time due to gradual degradation of the polysaccharides (De Booth *et al*. 1984). A continual release is, therefore, required to maintain the stability of these aggregates within the rhizosphere, which may account for the continual detection of release cell wall-related molecules (Greenland 1979; Tisdall and Oades 1982; Cheshire 1990). Moreover, if certain nutrients including Fe^{3+} become limited, roots can exude chelating agents. These agents target the ions bound to clay particles, which can disrupt the polysaccharide-clay particle binding (De Booth *et al*. 1984; McNear 2013). Once plants receive sufficient ions, roots stop exuding these agents. When sufficient ions have replaced the exposed surface of clay, released polysaccharides can then bind to the clay. This demonstrates that the turnover of aggregates is highly dynamic, and that plants have a rudimental control over aggregate turnover. Growing crops in nutrient deprived soils or by including heavy metals could help further explore this aggregate turnover.

Conditions that are optimum for the highest capacity for polysaccharide-clay binding are fertile soils with acidic polysaccharides (Cheshire 1979; Cheshire *et al*. 1979; Cheshire 1990). Acidic polysaccharides are generally more ionised compared to their neutral counterparts, making them possess greater anionic electrolyte properties (Edwards and Bremner 1967; Cheshire 1990). Neutral polysaccharides typically secreted from bacteria have a weaker capacity for soil aggregation (Clapp and Davis 1970). Releasing an acidic molecule into the soil would, therefore, be favourable to enhance soil aggregation, and the stability of these aggregates. As

REC1 is potential acidic multi-polysaccharide complex (Chapter 4; Figure 4.3), released by wheat, barley and maize, it would support this hypothesis. This also suggests that the AGP core of REC1 may be acting not just as a crosslinker for the other subdomains, but as a key determinate of soil binding. AGP may act as a multi-directional bridge between clay particles with the subdomains. It would be interesting to verify this multi-directional bridge hypothesis by using a purified sample of APAP1 (Tan *et al*. 2013), which also has an AGP core. As gum Arabic, a commercial form of AGP, was ineffective at increasing aggregate size during water saturation, and was least effective at increasing aggregate size during mechanical pressure. This supports the hypothesis that AGP is acting as a multi-directional bridge, which does not directly support polysaccharide-clay binding, but by itself AGP is a poor soil aggregator. This may explain the possible reason for the other subdomains of REC1. More research is required to explore this hypothesis. The stability of aggregates caused by polysaccharide is believed to be directly proportional to the molecular weight of the molecule binding them together (Cheshire 1990). This further supports why REC1 was the most effective soil aggregator, as REC1 is a putative multi-polysaccharide complex, having between four and five domains it is hypothesized to have a high molecular weight.

A common practice amongst farmers is to plough fields so that they can suppress weed growth, which hinders plant growth, prepare fields for seed planting and to incorporate fertiliser (Klute 1982). However, tillage, the process of agitating soil, breakdowns these micro- and macroaggregates that have been formed whilst crops were growing (Six and Paustian 1999; Ji *et al*. 2013). This not just disrupts the rhizosheath but other soil contact that soil-dwelling organisms have developed, thus eroding the soil. It has been demonstrated that restoring these aggregates within eroded soils can recover soil water infiltration capacity and aeration (Erktan *et al*. 2016). Polysaccharides derived from plants and other microbiota could be investigated for commercial use as a soil conditioner to reducing soil erosion due to tillage.

5.3.5 Released polysaccharides may have multiple roles

It is reasonable to suggest that released polysaccharides serve other purposes than root cap lubrication and soil aggregation. These polysaccharides could be used to cultivate microorganisms for plant growth. Several studies have used isolated root mucilage exuded by pea and alfalfa (May *et al*. 1993; Knee *et al*. 2001; Gunina and Kuzyakov 2015; Sun *et al*. 2015). These root mucilages were placed into agar, which had been inoculated with rhziobacteria. These bacteria proliferated in the presence of these high molecular weight compounds. Colonies of soil bacteria could also proliferate in the presence of isolated maize root mucilage (Knee *et al*. 2001; Walker *et al*. 2003); this was also shown for infectious bacteria (Zheng *et al*. 2015). Arbuscular mycorrhizal fungi have also been shown to contain plant-specific degrading enzymes, such as xyloglucanase (Rejon-Palomares *et al*. 1996). Evidence for polysaccharide catabolism of plant high molecular weight compounds in mycorrhizal fungi remains unclear. It may be possible that plants have some regulatory control over the amount, and which polysaccharides that they release. More research is required to explore how microorganisms use released polysaccharides for growth, and if a plant has regulatory control over their secretion.

5.3.6 Soil aggregation may have been vital for land colonisation

Without soil, most land plants would not be able to survive. The development of this medium was crucial for the development of complex plants that are present within many ecosystems, which are utilised for food, pharma and fabric production (Brown 2008; Prosser 2015). Prior to the colonisation of land plants, primitive soils would be comparable to glacial rock deposits that were formed by weathering processes (Huggett 1998). As early plants began to take the initial foot steps to complexity, over 470 million years ago (Kenrick and Crane 1997; Bateman *et al*. 1998; Popper *et al.* 2011), they had to develop a relationship with early soils. Early plants had to remove water and necessary nutrients from the inert rock minerals. Aggregating these inert minerals together could have developed a primitive rhizosheath as well as to anchor these early land plants (Huggett 1998; Konrat *et al*. 2008; Prosser 2015).

REC2 is an acidic macromolecule due to the presence of AGP, and acidic xyloglucan (Peña *et al*. 2008). This acidity would have maximised the binding of micro-particles within the early soils (Tisdall and Oades 1982; Chenu and Guerif 1989; Cheshire 1990). As well as being acidic, the molecular weight of REC2 would

have contributed to the binding of this multi-polysaccharide complex to inert minerals. More research is required to explore the effectiveness of REC2 to aggregate inert glacial rock. If aggregation occurs, it would support this hypothesis. As plants became more complex, comparable to today's cultivars, this released macromolecule may have developed more subdomains to increase the effectiveness of soil aggregation.

The development of a released multi-polysaccharide complex may have corresponded to the ever increasing complexity of soil. Plants may have further added more polysaccharide subdomains to increase the binding sites used in clay binding (Olness and Clapp 1973; Chenu and Guerif 1989). The appearance of early land plants corresponds to the development of xyloglucan (Popper and Fry 2003; Popper *et al.* 2011), which was acidic (Peña *et al*. 2008). Perhaps, AGP-xyloglucan (REC2 subdomains) was the most effective soil aggregator until AGP-xyloglucan-extensin-xylan (REC1 subdomains) evolved, superseding AGP-xyloglucan as the most effective soil aggregator. As more and more generations of plants colonised the land, a continual amount of organic matter was deposited into the evolving soil through decaying plant matter (Mitchell *et al*. 2016; Rimington *et al*. 2016). This matter would have greatly contributed to the development of macroaggregates, and soils (Kenrick and Crane 1997; Konrat *et al*. 2008). However, the ability to retain and maintain these aggregates surrounding roots within the rhizosphere would have been vital for safeguarding the root-soil interface. It would also be interesting to determine when plants evolved this ability to form a rhizosphere.

5.4 Conclusion

The rhizosheath serves as a vital interface for plants to extract water and nutrients from the soil, serve as a means of anchorage, and to develop symbiotic relationships with soil dwelling microorganisms. Commercial plant-derived polysaccharides, xylan and xyloglucan could readily bind to particles within various soil types that are commonplace throughout many ecosystems. After the commercial polysaccharides adhered to soil they increased the abundance of aggregates within sandy loam soil and glacial rock. Aggregates formed as a result of commercial polysaccharides, remained stable during high water stress, and high mechanical pressure testing. Given more research, a commercial polysaccharide or blend of commercial polysaccharides could be developed to produce a soil

conditioner. This conditioner could be used to fight against soil erosion. An isolated sample of REC1 was added to soil, which rapidly increased the abundance of macroaggregates during high mechanical pressure. The AGP within REC1 may act like a multi-directional bridge, with the subdomains binding to a large volume of clay particles. This would make REC1 an advanced aggregator compared to the commercial polysaccharides tested. A similar complex to REC1, REC2, was uncovered to be released from the basal plant liverwort. This finding suggests that these released multi-polysaccharide complexes may have played an important function in the initial formation of the rhizosheath, and modern-day soils.

Chapter 6

General Discussion

6.1 Towards a new understanding of polysaccharides released by plant roots

From this investigation, it is hypothesized that polysaccharides released by roots have a complex architecture, which would reflects the cell walls where they had been released from. These polysaccharides could be released from continually lysed cell wall polysaccharides of root caps, root tips (Bacic *et al.* 1986; Read and Gregory 1997) and root hairs, as well as root border cells or root border-like cells (Driouich *et al.* 2013). These areas of release could all contribute to the amount and distribution of polysaccharide released by roots. When seedlings are establishing themselves in soil, they may exude more polysaccharide to develop a strong rhizosphere required for future growth. As a plant begins to mature they may release fewer of these molecules, as they have established their rhizospheres, only needing to maintain their rhizosheath when new roots grow into the soil.

Preliminary work had grown wheat for two-and-a-half months, and uncovered a gradual decrease in the detection of polysaccharides after three weeks (data not included). This is supported by previous research which uncovered that seedlings released more root mucilage compared to older plants (Chaboud 1983; Bacic *et al*. 1986; Moody *et al*. 1988; Read and Gregory 1997; Osborn *et al*. 1999). Rapidly developing the rhizosphere would increase the uptake of resources, particularly, at a time when a plant requires a large amount of resources for growth. Future work exploring the role of these polysaccharides should aim to compare the rate of which these polysaccharides are released from young and mature plants.

This investigation demonstrated that there was a higher signal of LM25 (xyloglucan) within the hydroponate of the barley root hair-less mutant (brb; Gahoonia *et al.* 2001). This initial screen has provided tantalising results (Table 3.1) that indicate root hairs may release polysaccharide. Immunofluorescence microscopy could be used to determine if these root hairs release polysaccharide *in situ*. For instance, Cadenza could be grown in sand; once they reached three-weeks-old the plant roots could be carefully excavated so that it preserved the rhizosheath prior to microscopy. MAbs LM1 (extensin), LM2 (AGP), LM11 (xylan) and LM25 (xyloglucan) could then be used to screen the sample of rhizosheath. Some research has suggested that some grasses are able to increase the thickness of their rhizosheath during periods of drought (Hartnett *et al.* 2012). The mechanisms underpinning their ability to regulate the thickness of their rhizosheath were undetermined. However, it is feasible to suggest that polysaccharides play a role in rhizosheath thickening. As root hairs are abundant on roots (Gilroy and Jones 2000)

it may be possible that they aid with the thickening of the rhizosheath. Growing barley brb in sand and measuring the amount of particles adhered to their roots would be one way of exploring this hypothesis. Using such techniques as X-ray computerised tomography (CT; used at the University of Nottingham; Tracy *et al.* 2015; Daly *et al.* 2017) would enable accurate 3D imaging of the rhizosheath, and accurate quantification of the amount of soil adhering to roots.

Root mucilage may just be formed of AGP and pectin, which form viscous gels (Bacic *et al.* 1986; Moody *et al.* 1988; Read and Gregory 1997), whereas, other polysaccharides released by roots, as detected by this investigation, extensin, xylan and xyloglucan could be more diffuse in the soil. These more diffuse components could be involved in modifying the rhizosphere, enabling plants to extract more resources from soil. Modifications to the rhizosphere would include soil aggregation to secure the rhizosheath. While, the gel forming polysaccharides that produce root mucilage would solely lubricate roots as they penetrate through soil. The more soluble polysaccharides would be released across the entire root network, rather than the root caps and tips. To determine if the polysaccharides that are released by roots have different solubility, nitrocellulose sheets could be used to screen locations of secretion along the roots. Plants could be grown on nitrocellulose, which is highly absorbent for a few days. After growth, the nitrocellulose could be screened with MAbs to reveal where each polysaccharide is secreted. Attempts at growing Arabidopsis on nitrocellulose have demonstrated that pectin and AGP appear along the root surface, whereas xyloglucan and xylan appear more diffuse, moving away from the roots (data not included). However, developing nitrocellulose blots that had larger plants growing on including wheat was challenging. Issues included keeping roots in contact with the nitrocellulose, the moist nitrocellulose causing a run off effect where the polysaccharides covered the entire sheet, and determining an appropriate dilution to use for the MAbs which could quickly become overdeveloped.

6.2 Categorising the putative multi-polysaccharides uncovered in this study

An issue of categorising the putative multi-polysaccharide complexes uncovered within this investigation arose. REC1 was initially uncovered from the hydroponate of wheat cv. Cadenza. Further immuno-chemical and physio-chemical methods were used to explore the biochemistry of this complex. When screening the hydroponates of two other cultivars of wheat (Avalon and Skyfall) a REC1-like

molecule was detected. The term REC1-like was used as the immuno-based assays demonstrated that there were slight differences in the signal strengths of the MAbs between the cultivars. Whether REC1 released from Cadenza is the same as the other cultivars, more physio-chemical analysis through monosaccharide linkage analysis and possibly NMR is required. Moreover, REC1-like molecules were also uncovered within the hydroponates of barley and maize. Establishing whether REC1 released by wheat is the same (or not) as barley and maize is needed through physio-chemical methods, as outlined above. The exudates of more grass species needs to be screened to determine if releasing a REC1-like molecule is widespread across the grasses. Some evidence of REC1-like molecules was detected within the hydroponates of pea, tomato and rapeseed. More immuno-chemical and physio-chemical research is needed to determine if eudicotyledons release a REC1-like complex. Furthermore, a wider range of MAbs is needed to determine if eudicotyledons release a distinct multi-polysaccharide complex from grasses (REC1) and liverworts (REC2).

6.3 The possible structure of REC1

Results presented here have indicated to the presence of a multi-polysaccharide complex released by the roots of cereals. However, the structure of this complex remains to be fully understood. The monosaccharide linkage analysis undertaken by the Complex Carbohydrate Research Centre (CCRC) demonstrated that there was a high mixture of linkages and structural complexity within REC1. Clear signatures of AGP, extensin, xylan and xyloglucan were detected, confirming what the MAbs had been detecting. Previous investigations using wheat and maize grown in sterile conditions have also determined signatures from AGP, xylan and xyloglucan (Bacic *et al.* 1986; Moody *et al.* 1988). In addition to these signatures, past investigations had also determined signatures that do not have a clear polysaccharide signature, 6-linked Glc*p* and 2,4-linked Glc*p* (Moody *et al.* 1988). Other linkages detected within maize and cowpea, 3-linked Fuc*p*, 2,3-linked Gal*p*, 3,6-linked Gal*p* and 2,3-Man*p* and 3,4-linked Gal*p* (Moody *et al.* 1988) had also been described within this study that focused on wheat (Table 4.1). This investigation had determined novel signatures of arabinan, extensin, galactomannan, mannan, mixed linkage glucan, RG-I and RG-II. Considerably more work is necessary to understand how these linkages come together to form REC1 if indeed they are all part of a polysaccharide complex. Research using NMR-based techniques could be used to determine how

they are linked. However, a larger amount of REC1 would need to be isolated from Cadenza, thus a more efficient method of isolating these polysaccharides is required. Once determining what polysaccharides are present, the next stage of work would need to explore how these polysaccharides were linked. For instance, is AGP linked to extensin to form a protein core that crosslinks with the other polysaccharide domains (Figure 6.1)? What kind of bonding links REC1, for example covalent linkage? Is REC1 in the form of a complex (Figure 6.1), or could it be a heterogeneous mixture of polysaccharides released by roots?

Within the linkage analysis there were unexpectedly high levels of glucose and mannose linkages. These linkages were previously detected in the fungi (*Rhynchosporium secalis*), which causes barley leaf scald (Pettolino *et al.* 2009). As the wheat was grown in non-sterile conditions, and that these linkages have not been described before in plants, it is possible that these linkages show indications of fungi cell wall components. Perhaps, these fungal components are from another fungi or bacteria of which linkage surveys of their cell walls have not been conducted. One exception to this is 2,3-linked Man*p*, which had been described in the medium of maize (Bacic *et al.* 1986). Therefore, another possibility is that these linkages are novel polysaccharides released by wheat roots. When discounting all previously described linkages in wheat, maize, cowpea, and barley leaf scald there were two linkages within this study (2,3,6-linked Gal*p* and 2,4-Glc*p*), which had not previously been reported. Future work using the hydroponics system developed by this investigation would have to ensure that the hydroponate was sterile. Sealing the hydroponates within the buckets would reduce the risk of contamination by airborne fungi or bacteria. All equipment would also have to be autoclaved or chemically sterilised using bleach. Seeds would also have to be sterilised particularly, at the pre-hydroponics stage. A hydroponics system could also be grown within a sterile growth cabinet rather than a glasshouse. However, controlling humidity may be challenging.

A comparison of the linkages present in the root cell walls of wheat is required to fully understand the novelty of the polysaccharides released by roots. If a novel form of mannan is present within REC1 could an enzyme (endo-1,4-β-mannanase) be used to breakdown the backbone of this potential domain? If so what effect would this have on the structure of REC1? MAbs could be used to determine if there was an alteration in the four polysaccharide epitopes that form REC1, as determined by anion-exchange EDC. The consistent signal from LM2 throughout this investigation, which binds to β-glucuronic acid in AGP (Willats *et al.* 2004) has contrasted with the

lack acidic residues uncovered during monosaccharide linkage analysis at the CCRC. Could REC1 contain a minor proportion of β-glucuronic acid which is below the level of detection of the monosaccharide linkage analysis? If so could an enzyme that targets this residue (β-glucuronidase) be used to degrade this residue to determine if it makes REC1 acidic during anion-exchange EDC?
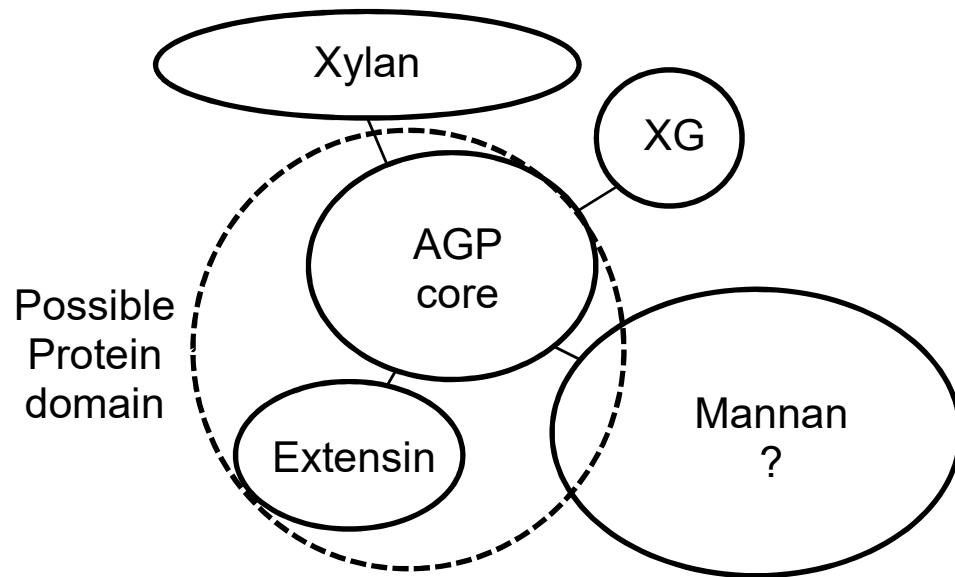

Figure 6.1 I **A schematic diagram of REC1**
After anion-exchange EDC, sandwich-ELISA, monosaccharide composition analysis and monosaccharide linkage analysis it is proposed that REC1 is formed of an AGP core, which crosslinks with the other domains. The AGP core could have a close attachment of extensin to form a possible protein domain (dashed line). Polysaccharide domains of xylan and xyloglucan may be attached to the AGP core branching out. It remains to be determined if mannan is a domain of REC1 or whether it drives from fungal cell wall components. XG = xyloglucan.

It would be interesting to conduct surveys on Arabidopsis exudates. Using this model species would open the possibilities of exploring the roles, structures and biosynthesis of these polysaccharides released by roots through the use of mutants. Such mutants as xxt1/2 (encodes two xylosyltransferase genes) which lack the ability to produce xyloglucan (Cavalier *et al.* 2008), and XUT which lacks the acidic xyloglucan from root hairs (Peña *et al.* 2012a), would enable research to explore the effects of removing xyloglucan from the polysaccharides released by roots. Attempts had been undertaken to grow Arabidopsis hydroponically; however, these attempts were not successful. Additionally, using the model species *Brachypodium distachyon* (purple false brome) would provide results more related to grass (Draper *et al.* 2001). Within the crops screened in this study, the plants were young (about

three-weeks-old). Research in the US and France have developed systems of growing Arabidopsis hydroponically, however, they only achieved this using plants close to maturity (A. Galloway, 2016. *In person A. Driouich*, 14 June). This research would require young plants (within three weeks) to maximise the collection of these polysaccharides for biochemical and physio-chemical analysis.

6.4 The possible roles for polysaccharides released by roots within the microbiome

Plant roots are a part of a complex ecosystem that contains a plethora of life. The microbiome of the rhizosphere has only begun to be described in detail. One such concept is the Wood Wide Web, which is a complex network of fungi hyphae and plant roots (Helgason *et al.* 1998). This interconnected network involves the shuffling of carbon to fungi in exchange for nutrients (phosphorous and nitrogen) and water, which is inaccessible to roots (Haichar *et al*. 2014; Rilling *et al*. 2015). These series of interactions have been shown to occur across a forest, where most of the plants are connected to this large network of fungal hyphae (Helgason *et al.* 1998; Haichar *et al*. 2014). It has even been demonstrated that these hypal networks can shuttle nutrients and carbon from plant to another, which are separated by tens of metres (Barea *et al.* 2002). It would be interesting to determine if polysaccharides released by plant roots have a role within this network. Could these polysaccharides be used as a source of energy for the microbiome of the soil?

Some research has suggested that polysaccharides released by plant roots can be accessed by the microbiome of soil (Mounier *et al*. 2004; Benizri *et al.* 2007; McNear 2013). Studies have demonstrated using the root mucilages of maize, pea, alfalfa (*Medicago truncatula*) and cowpea, as the sole carbon source, can be used to grow various species of rhziobacteria and arbuscular mycorrhizal fungi (May *et al.* 1993; Knee *et al.* 2001; Gunina and Kuzyakov 2015; Sun *et al.* 2015). One interesting observation of these microorganisms grown using root mucilage was that they increase the production of their exopolysaccharides (Gunina and Kuzyakov 2015; Sun *et al.* 2015). The numbers of bacterial cells also rapidly increased as a result of the increase in available energy (Knee *et al.* 2001; López-Gutiérrez *et al.* 2005). Plants that can undertake symbiosis with rhziobacteria such as pea and lupin, could release high amounts of glucomannan within their exudate to attract, and mediate the attachment of nitrogen-fixing bacteria to their roots (Hoflich *et al.*

1994; Knee *et al*. 2001). In response, rhziobacteria release various glucanases that breakdown parts of the cell wall of root hairs prior to symbiotic penetration (Peleg-Grossman 2007). These findings suggest that microorganisms can actively use these polymers as a carbon source. When isolated root mucilage from maize was added to agricultural soil, bacterial numbers rapidly increased followed by the numbers of bacterial species detected (Knee *et al.* 2001; Walker *et al.* 2003). Moreover, it has also been shown that there is a higher density of microorganisms within the rhizosphere (between 2% and 7% w/w) compared to the bulk soil (≤ 1% w/w; Dennis *et al*. 2010; Garcia-Salamanca *et al*. 2013). From the results of these studies, it is clear that polysaccharides from roots could be used by the microbiome of the soil as an energy source. However, it remains unclear whether this occurs in the rhizosphere. Future work should focus on addressing the following questions, what are the ecological consequences of plants releasing polysaccharides into soil? How will releasing polysaccharides be affected during climate change? Can plants regulate the amount and types of polysaccharide released into the rhizosphere?

It has been suggested that if infectious microorganisms evade border cells and defense molecules within the exudate, they could use polysaccharides released by roots as a source of energy (Cannsean *et al.* 2012). As an example, the plant pathogen Pythium blight (*Pythium aphanidermatum*) rapidly increased the uptake of polysaccharides from cucumber (*Cucumis sativus*) grown on agar in prior to infection (Zheng *et al.* 2000). When sufficient numbers of bacteria had developed infection occurred. Once infected, the rate of mucilage produced by the cucumbers was found to decrease, and is then re-directed to producing more Pythium (Zheng *et al.* 2000). However, there are still many unanswered questions between the interaction of infectious microorganisms and these released polysaccharides.

It has been speculated that different plant species release different levels and types of carbon into the rhizosphere (Morel *et al*. 1986; Ray *et al*. 1988; Sims *et al*. 2000). Furthermore, the diversity of bacteria was found to differ when the root mucilages of pea, alfalfa and maize were supplied to a mixture of bacteria (Mounier *et al*. 2004; Benizri *et al*. 2007). It may be possible that roots release a unique fingerprint of polysaccharide into the rhizosphere in order to modify the surrounding soil, to suit the particular needs of that species (Badri and Vivanco 2009; Miscallef *et al*. 2009). For instance, if a plant requires a particular interaction with a particular species of microbe, the plant could modify the amount, and type of polysaccharide it released. It is conceivable that these released polymers play a multitude of roles for the plant, from soil aggregation to developing symbiotic partnerships. It would be of interest to

determine if polysaccharides released by roots could be detected by MAbs within a native (non-sterile) soil. Glyco-typing the rhizosphere could have important ecological impacts such as in forest restoration, where a soil could be tested to see which plants had been growing prior to a natural disaster, such as wild fire. Furthermore, an experimenter could glyco-type a soil to determine the presence of invasive species before it spreads. For this to occur a large database would have to be developed in order to accurately glyco-type the polysaccharides released by a particular plant. This glyco-typing could also be expanded to explore the microbial polysaccharides of rhizospheres. For example, do the microbiomes of the rhizospheres of plants differ?

One major problem faced by agriculture is the increasing amount of soil becoming eroded (Erktan *et al*. 2016). As the global population increases the amount of food produced must increase in order to feed the growing population. Back in the 1960s the Green Revolution occurred which increased the production of food by using a combination of new intensive agricultural practices, developing shorter stem plant cultivars that produce more grain, and the use of chemical fertilisers and pesticides (Evenson and Gollin 2003). In order to feed a larger global population a second green revolution is needed. Although the successes of the Green Revolution are clear, the intensive agricultural practises have led to a rapid increase in soil erosion (Evenson and Gollin 2003; Erktan *et al*. 2016), which results in more land being used for agriculture. By reducing soil erosion, less land is required for food production. Perhaps, soil erosion could be reduced by growing plants that have a high release of polysaccharides. Growing these plants may help to increase the abundance of soil aggregates, and thus reduce the risk of erosion or even recover soil that has been damaged. Moreover, the development of a soil conditioner based on commercial plant polysaccharides could, firstly reduce the soil lost to erosion, and secondly protect soil that is already in use and vulnerable to erosion. Developing a soil conditioner could also act as a natural fertiliser feeding the microbiome, in order to help grow crops without the need for artificial fertilisers such as phosphate rock, which is an unsustainable resource. Within 50 years' phosphate rock is predicted to become depleted (Gilbert 2009).

Understanding how plants maintain and secure their rhizosheath may help future missions to the moon. By 2037 NASA aims to set up a Luna outpost, which must be self-sustaining, cultivating sufficient food for the inhabitants. Previous research which utilised moon regolith, comparable to glacial rock, collected from Apollos 11 to 15 uncovered that the Luna regolith could support plant growth when sufficient

water and nutrients were added (Walkinshaw *et al*. 1970; Walkinshaw and Johnson 1971; Baur *et al*. 1974; Ferl and Paul 2010). No examinations were conducted on the aggregate size and stability of the regolith post-plant growth (A. Galloway, 2017. *Email to J. Wheeler*, 9 May). It would be interesting to add released polysaccharides and commercial based-polysaccharides to Luna regolith to explore possible effects on regolith aggregate potential, which was observed when using different rock types on Earth.

6.5 Released polysaccharides may have given early plants an evolutionary advantage

Before plants colonised the land, early soils would have consisted of weathered rock fragments (Huggett 1998). Once plants had started to colonise the land, the turnover of early plants would have greatly added carbon into the rock fragments, which would come to form the first soils (Arteaga-Vazquez 2015; Mitchell *et al*. 2016; Harholt *et al*. 2016). To extract resources including water and nutrients, which would have been readily accessible within the sea, plants had to develop an interface with this rocky substrate. Perhaps, early plants with a higher rate of cell wall lysis resulted in a stronger interaction between rhizoids and early soils. This early rhizosphere may have given early plants an advantage over others, enabling them to access resources from rock fragments.

Liverwort rhizoids have been shown for the first time to release polysaccharide, similar to their modern-day relatives. This indicates that releasing polysaccharide may be widespread across the plant kingdom. Liverworts were found not just to release individual polysaccharides but release a complex, formed of AGP-xyloglucan. Considerably more research, using monosaccharide composition and monosaccharide linkage analyses is required to fully explore the physio-chemical properties of the polysaccharides released by liverworts. However, the amount of polysaccharide collected from liverwort is limited by the slow growth and small size of the plant. A more efficient method used to grow liverwort and to isolate the polysaccharides from the medium is needed in order to further explore the biochemistry of the polysaccharides released by liverwort. Future work should also focus on how widespread releasing multi-polysaccharide complexes are. Do mosses, hornworts or ferns (Figure 4.18) release a polysaccharide complex similar to that of REC2 or REC1?

6.6 Limitations

There are a few limitations to the techniques that were used in this investigation that should be noted. The hydroponic system developed by this investigation was effective at isolating the polysaccharides released by roots. However, this system does not reflect a natural environment. Plant roots were submerged in a liquid medium unlike soil which contains a heterogeneous mixture of particles with varying amounts of air pockets. Another difference between hydroponics and soil is that roots are not subjected to continual friction as they penetrate into the soil. Additionally roots in hydroponics are under a constant stream of air bubbles which is required for aeration. As a result roots are slowly and constantly being agitated which does not happen when plants are grown in soil where they are static. If plants were grown in as close to as natural conditions whether in sterile soil or in a field this would mean that polysaccharides could not be isolated in sufficient amounts required for biochemical and physio-chemical analysis.

MAbs have been instrumental in the biochemical analysis of polysaccharides within the hydroponate of plants. Although MAbs are highly sensitive probes their antigen may be masked by other polysaccharides that are in larger amounts. Furthermore, the ELISA plate wells will only bind to a narrow selection of molecules within the hydroponate which is further compounded by the washing steps that were undertaken during ELISA. Together this means that there was a narrow focus on the hydroponate of plants. There may be additional factors unexplored that may influence the polysaccharides released. Moreover, MAbs cannot discriminate between their polysaccharide antigens with their oligosaccharide counterparts. All together the issues reported here have been considered and a compromise between the advantages and disadvantages of the techniques was developed for this investigation.

6.7 Conclusion

Results presented here have expanded our understanding of polysaccharides released by plant roots. Grasses were uncovered to release REC1, a putative multi-polysaccharide complex that may be formed of a protein domain, containing AGP and extensin, as well as domains of xylan, xyloglucan and possibly mannan. REC1 was able to promote the aggregation of soil, more so than commercially available

polysaccharides, xylan and xyloglucan. A complex formed of AGP-xyloglucan (REC2) was also detected in the medium of liverwort. Despite this new appreciation of polysaccharides released by roots, much more work is needed to fully decipher the biochemical properties of these polysaccharides. Future research should focus on three key areas: the structure of REC1, how widespread releasing these multi-polysaccharide complexes are across the plant kingdom, and determine how these polysaccharides interact with the microbiome of the rhizosphere. There are still many unanswered questions that need to be address including, do root hairs release polysaccharide? How do plants regulate the rhizosheath? How is REC1 formed? What kind of bonds hold REC1 together? Can polysaccharides be detected within native soil? Growing plants with a high release of polysaccharide in land vulnerable to soil erosion could be a new method of preventing soil degradation. These plants may even regenerate soil that has succumbed to erosion. This soil conditioning, using *in situ* planting or commercial plant polysaccharides, may one day lead to sustainable food production on the moon, which NASA hopes to do by using the moon's regolith.

Major findings of this investigation

- Monoclonal antibodies are useful tools for exploring polysaccharides released from plant roots.

- Hydroponics is an effective method of growth for isolating polysaccharides released from plant roots.

- Profiles of the released polysaccharides from grasses and eudicotyledons differed.

- Major polysaccharides released from the roots of wheat and other cereals were AGP, extensin, xylan, xyloglucan and possibly mannan.

- Major polysaccharides released by cereals were a part of multi-polysaccharide complex, REC1.

- Liverwort rhizoids released an AGP-xyloglucan complex, REC2.

- Multi-polysaccharide complexes may be released across the plant kingdom.

- Commercial plant cell wall polysaccharides, xylan and xyloglucan, as well as REC1 isolated from Cadenza roots promote soil aggregation. REC1 was found to be the most effective at aggregating soil.

Chapter 7

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