THE UNIVERSITY *of* EDINBURGH

EDINBURGH CELL WALL GROUP
INSTITUTE OF MOLECULAR PLANT SCIENCES
SCHOOL OF BIOLOGICAL SCIENCES

Presence of oligosaccharides in seed-coat mucilage of Lepidium sativum: role in allelopathy

By

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Declaration

This thesis has been composed by my self and the work, of which it is recorded, has been carried out by myself. All sources of information have been specifically acknowledged by means of reference.

Amjad Iqbal

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I am most thankful to Almighty 'Allah' who gave me the energy to utilize in the supreme interest of mankind.

My heartfelt respect and appreciation goes to my supervisors Prof S.C. Fry, leader of the Edinburgh Cell Wall Group, and Dr. C. French for their invaluable assistance throughout my study. Without their keen interest and constant effort it would not have been possible to complete this research project.

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Amjad Iqbal.

Dedication To

My Parents Mr. and Mrs. M. Iqbal

For their love, financial assistance and best teaching

My wife Nadia and son MAM. Iqbal

Abstract

Lepidimoide is a naturally occurring disaccharide reported to be an oligosaccharin, i.e. to exhibit 'hormone-like' biological activity. It was found in cress (*Lepidium sativum*) root exudates and exerts apparently allelopathic effects on neighbouring *Amaranthus* seedlings. In the present study the effect of cress root exudates on hypocotyl and root length of *Amaranthus caudatus* and *Lactuca sativa* was studied. The seedlings of both species grown with *Lepidium sativum* seedlings had longer hypocotyls and shorter roots as compared to the control. In this study I found an active principle with biological effects similar to those of lepidimoide to be more abundant in cress seed-coat mucilage than in root exudates. The active principle peaked 24 hours after seed soaking, and thereafter plateaued. I also for the first time confidently proved that the bioactive compound(s) were exuded by cress and were not microbial digestion products or seed treatment chemicals. Quantitative tests of cress root exudates and cress seed-coat mucilage showed the presence of hexoses, pentoses, uronic acids and unsaturated uronic acid. The presence of unsaturated uronic acid might be of interest because the known structure of lepidimoide includes an unsaturated uronic acid.

Active principle from mucilage was partitioned into the aqueous phase when shaken with ethyl acetate at pH 2, 6.5 and 12, showing it to be hydrophilic, unlike auxins and gibberellins. The mucilage was also heated at 130°C for 48 h and severe heating did not affect its biological activity, suggesting that if the compound is lepidimoide then it is heat-resistant.

In an attempt to test whether the compound is of high or low M_r, the mucilage was partitioned into 75% ethanol-precipitated and non-precipitated fractions. The biological activity in the non-precipitated fraction was very high, and was further separated by gel-permeation chromatography (GPC). GPC on Bio-Gel P-10 and P-2 suggested that the active principle had M_r ~500–750, compatible with oligosaccharide(s), suggesting that a particular oligosaccharide may be the active principle. TLC separation of bioactive fractions from P-2 showed that the bioactive compound migrated between GalA and Gal but co-migrated with sucrose; however, paper chromatography separation proved that the compound is not sucrose and might be a different disaccharide (lepidimoide).

From the structure of lepidimoide, Fry *et al*. (1993) proposed that lepidimoide is formed by the lyase-catalysed cleavage of a pectic polysaccharide, rhamnogalacturonan-I (RG-I). So I tried to prepare lepidimoide or lepidimoide-like compounds by the action of RG-I lyase from *Pichia pastoris* on purified potato RG-I. The lyase showed its activity but the digest did not demonstrate biological activity, which might be due to presence of tris-HCl buffer in the solution. An attempt was also made to prepare lepidimoide by methyl esterification and β-elimination of purified potato RG-I but again the product did not show any biological activity, which might be due to presence of borate buffer in the solution. This part of research might be useful for future work on preparation of lepidimoide and lepidimoide-like compounds.

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Abbreviations

Ara	Arabinose
BAW	Butane-1-ol: acetic acid: water
BPAW	Butane-1-ol: Propane-2-ol: water: acetic acid
cpm	Counts per minute
Fru	Fructose
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GGM	Galactoglucomannan
GalR	Galactaric acid
Glc	Glucose
GlcA	Glucuronic acid
GlcN	Glucoseamine
GlcR	Glucaric acid
GPC	Gel permeation chromatography
HG	Homogalacturonan
HOAc	Acetic acid
HPLC	High performance liquid chromatography
k_{av}	Elution coefficient
kV	Kilovolts
M_2	Maltose
M_3	Maltotriaose
M_4	Maltotetraose
M_5	Maltopentaose
M_6	Maltohexaose
M_7	Maltoheptaose
ManA	Manuronic acid
Man	Mannose
MDA	Malondialdehyde
NMR	Nuclear magnetic resonance
PC	Paper chromatography
Py	Pyridine
RBCs	Root border cells
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
Rha	Rhamnose
TBA	Thiobarbituric acid
TFA	Trifloroacetic acid
TLC	Thin layer chromatography
XyG	Xyloglucan
Xyl	Xylose

1 Introduction

1.1 Plant exudates

Plants are sources of diverse classes of natural products, some of which have biological activity such as phytotoxins, antimicrobial agents, phytoalexins and signalling agents (Deng *et al.,* 2004; Muscolo *et al.,* 2004; Hegab *et al.,* 2008), some of which provide nutrition to soil microbes (Fry *et al.,* 1993; Braga *et al.,* 1998a and 1998b; Brigham *et al.,* 1999; Beninger *et al.,* 2004; Meepagal *et al.,* 2005; Isfahan and Shariati, 2007). The exudates from various plant organs contain low molecular weight compounds (such as sugars, inorganic ions, vitamins, nucleotides, amino acids and phenolics), higher molecular weight substances (polysaccharides and enzymes and other proteins) and root border cells (RBCs) (Campbell *et al.,* 1995a; Dakora and Phillips, 2002; Prakash *et al.,* 2003; Macario *et al.,* 2003; Muscolo *et al.,* 2005; Bias *et al.,* 2006).

Many of the compounds that exude from various plant organs e.g. seeds, stems, leaves, flowers and roots have a positive or negative effect on environment (Rice, 1984; Barthomeuf *et al.,* 1996). For example, root exudate was observed to contribute to the complex set of chemical, physical and biological interactions in the rhizosphere, including root–root, root–nematode, and root–microbe interactions (Ruess *et al.,* 1998; Brigham *et al.,* 1999; Hirsch *et al.,* 2003; Walker *et al.,* 2003; Bais *et al.,* 2006; Weir *et al.,* 2004; Rudrappa *et al.,* 2008).

Root–root, root–nematode and root–microbe interactions are categorized as either positive or negative (Bais *et al.,* 2006). For example when root exudate

provide nutrition to the beneficial microorganisms then the interactions are regarded as positive while root exudate containing nematocides or antimicrobial agent against nematodes or microorganism the interactions are said to be negative. In short, plant exudates consist of complex mixtures of large and small molecules, which might have an effect on neighbouring organisms.

1.2 Allelopathy

Allelopathy, which means the effect of one plant species on the germination, growth and development of other plant species, has been known for over 1000 years. The allelopathic effect of chick pea (*Cicer arietinum*) and barley (*Hordeum vulgare*) on weeds and other plant species were known even before 300 BC (Rice, 1984).

The word allelopathy was first introduced by Molish in 1937 and is derived from Greek words allelon 'of each other' and pathos 'to suffer', which means the injurious effect of one species upon the other species. In 1996 allelopathy was defined by the International Allelopathy Society as "Any process involving secondary metabolites produced by plants, micro-organisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals)" (Torres *et al.,* 1996).

The secondary metabolites that are released by plant organs such as roots, rhizomes, leaves, stems and seeds into the environment and that affect other plants in the rhizosphere are known as allelochemicals (Fig. 1.1). These allelochemicals might enter the environment through different routes like leaching, volatilisation, root exudation, seed-coat exudation after imbibition and decomposition of different parts

of the plant (Rice, 1984; Higashinakasu *et al.,* 2004). When susceptible plant species are exposed to allelochemicals, germination might be inhibited and if they germinate they might show abnormal growth and development. The most visible effects observed are retarded germination, short or no roots, lack of root hairs, abnormally long or short shoots, swollen seeds and low reproductive ability (Rice, 1979).

Leaf extracts of both *Eucalyptus camadulensis* and *Juglans regia* and seed extracts of *Onobrychis sativa* contain allelopatheic compounds which inhibited the germination and seedling growth of *Coronilla varia* seeds and seedlings (Isfahan and Shariati, 2007). Noguchi (2003a) also showed that some putative allelochemicals (*cis*-xanthoxin and *trans*-xanthoxin) from the leaf extracts of *Pueraria thumbergiana* caused growth inhibition of *L. sativum* roots (Fig. 1.2). Extracts from the flowers of *Chrysanthemum cinerariefolium* contain a biologically active compound known as pyrethrin with herbicidal effect (Barthomeuf *et al.,* 1996). Pisatin 1 (Fig. 1.3) is also a good example of an allelopathic compound found in the extracts of pea stem residues that inhibited the root and hypocotyls growth of *L. sativum* at a concentration of 10 µM (Noguchi, 2003b).

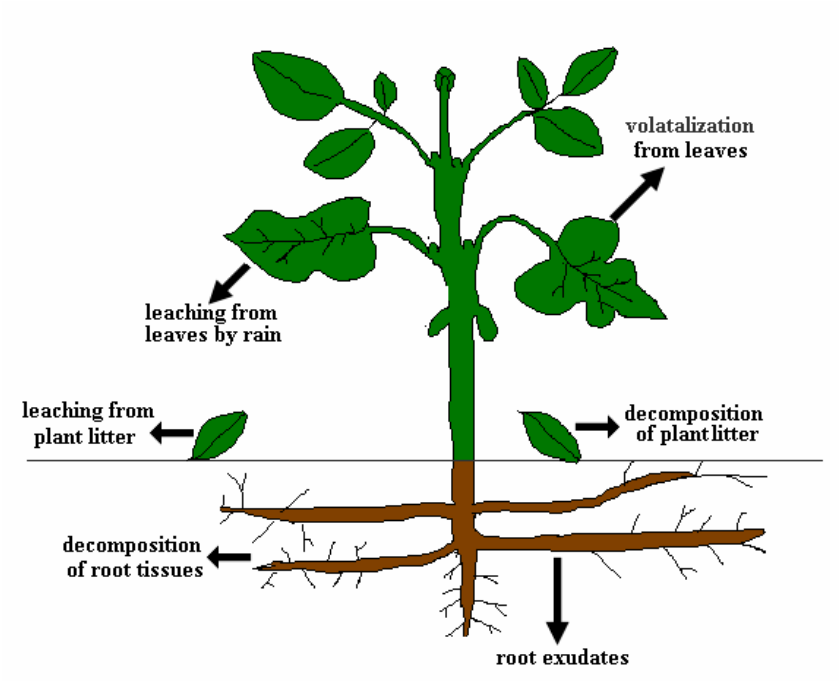
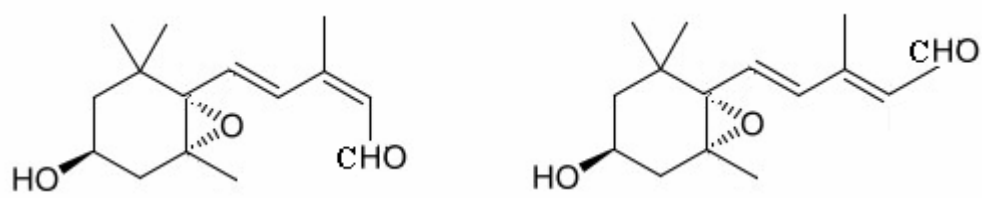
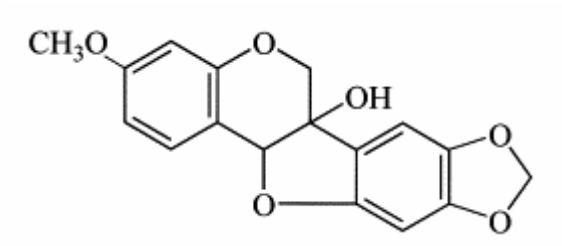


Figure 1.1: Various routes through which allelopathic compounds interact with the environment



cis-Xanthoxin *trans*- Xanthoxin

Figure 1.2: Structure of allelopathic *cis* and *trans*-xanthoxin
Obtained from Noguchi (2003a).



Pisatin 1

Figure 1.3: Pisatin 1, allelopathic compound from pea
Obtained from Noguchi (2003b).

1.3 Agricultural use of allelochemicals

One of the most studied aspects of allelopathy is its role in agriculture. Much current research is focused on the effects of weeds on crops, crops on weeds, crops on micro-organisms, micro-organisms on crops, nematodes on crops and crops on crops (Friebe *et al.,* 1995; Ruess *et al.,* 1998; Brigham *et al.,* 1999; Colpas *et al.,* 2003; Meepagala *et al.,* 2005; Mareggiani *et al.,* 2005). The aim behind much of this research is the possible use of allelochemicals as growth regulators and natural herbicides (a number of them are either commercially available or in the process of large-scale manufacture) to promote sustainable agriculture. Allelochemicals released from allelopathic crops can be used for weed control if the allelopathic crop is grown in a crop rotational sequence (Macias, 1995). Phenolic compounds from allelopathic crops can be used as herbicides and against seed borne fungi (Colpas *et al.,* 2003). Meepagala *et al.* (2005) observed a high algicidal and antifungal activity of a natural compound rutacridone epoxide from *Ruta graveolens* root. Rutacridone epoxide has shown high fungicidal activity even compared with the commercially available fungicides captan and benomyl. Leaf extracts from *Empetrum hermaphroditum* and *Betula pubescens* can be used as a potential nematocide in soils that are rich in parasitic nematodes (Ruess *et al.,* 1998).

1.4 Allelopathy and competition

It is very important to distinguish between allelopathy and competition. In allelopathy a plant exudes into the environment a compound or compounds which affect the rhizosphere, while in competition a plant has the ability to remove or

reduce the available resources (e.g. nutrients, water, and light) in the environment (Einhellig, 2002).

Nillson (1994) attempted to differentiate between allelopathy and competition. She used the boreal dwarf shrub *Empetrum hermaphroditum* as donor species and Scots pine (*Pinus sylvestris*) as receiver species. The seedlings of *P. sylvestris* were grown under four different treatments in a greenhouse and field for 27 months in *E. hermaphroditum* vegetation. The different treatments used were: (a) *P. sylvestris* were grown in PVC (polyvinyl chloride) transparent plastic tubes (root-exclusion tubes), which minimized the effect of resource competition by roots, (b) activated carbon was used to adsorb the allelochemicals that were released by the *E. hermaphroditum*, (c) PVC tubes and activated carbon was used to minimize the effect of both resource competition and allelopathic effect of *E. hermaphroditum*, and (d) seeds of *P. sylvestris* were grown in untreated *E. hermaphroditum* vegetation in order to evaluate the effect of both allelopathy and resource competition on the growth inhibition and weight gain of the *P. sylvestris* seedlings.

After 27 months of plant growth, Nilsson (1994) studied shoot length and dry weight of the *P. sylvestris* seedlings. The seedlings from treatments a, b, and d had shorter shoots and lower dry weight than those from c. The effect of root competition was higher than that of toxic compounds because seedlings from root-exclusion tubes with no activated carbon (a) were affected less than seedlings from the treatment with activated carbon to adsorb the allelochemicals but without reducing the competition (b). The results of the experiment led Nilsson (1994) to conclude that two different interference mechanisms, i.e. below-ground competition and

allelopathy by *E. hermaphroditum,* were responsible for inhibition of shoot elongation and failure to gain weight of the *P. sylvestris* seedlings.

Wardle and his co-workers in 1996 also set up an experiment to differentiate between allelopathy and competition. In their experiment they selected six species of grass: *Dactylis glomerata* (cocksfoot), *Phalaris aquatica* (phalaris), *Bromus wildenowii* (prairie grass), *Lolium perenne* (perennial ryegrass), *Festuca arundinacea* (tall fescue) and *Holcus lanatus* (Yorkshire fog) as donor species and *Carduus nutans* or nodding thistle (a major weed in many temperate regions) as an acceptor species. The seedlings of the donor species and acceptor species were grown from commercially available seeds in the local market. The collected root and shoot leachates from the donor species were analysed for their nutrient content (i.e. ammonium, phosphate and nitrate) and were tested for their effect on the germination and growth of *C. nutans*. The collected root leachates from the six grass species had no significant effect on the germination and growth of *C. nutans*. However, both hypocotyl and root growth of the *C. nutans* were significantly reduced by the shoot leachates of *P. aquatica, L. perenne, H. lanata* and *F. arundiancea*. As the nutrient concentration in both root and shoot leachates of the six species was not significantly different, it was concluded that the differential effect was purely due to allelochemicals present in the shoots of the four donor species and not to competition.

1.5 Factors interacting with allelochemicals in soil

Many factors interact with allelochemicals such as soil pH, organic matter content, nutrient and moisture content. An allelochemical can either retain or lose its

effectiveness at a given concentration according to the availability or non-availability of more readily available carbon sources to soil microbes. Soil microbes may preferentially metabolize readily available carbon sources other than organic allelopathic compounds and thus leave the soil relatively enriched in allelochemicals, which are available for the uptake by plant roots (Blum *et al*., 1993; 1995; 1996). The concentration of released allelochemicals in the rhizosphere also depends upon the age of the allelopathic plant. A young plant releases more allelochemicals into surroundings, which might help it in establishing themself among well-established plants (Indejit, 1996).

The transformation of allelochemicals in the soil can result in more or less toxic compounds. Some allelochemicals are labile while others such as some alkaloids resist changes because of their anti-microbial activity (Wink *et al*., 1998). Some of the transformed products from allelochemicals become more potent than the original compound: for example the hydroxamic acids DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and MBOA (6-methoxy-benzoxazolin-2-one) from maize and wheat become more active when transformed into the corresponding derivatives, DIBOA (2,4-dihydroxyl-1,4-benzoxazin-3-one) and BOA (2-benzoxazolinone) by removal of formaldehyde from the parent molecule (Nair *et al*., 1990).

Allelopathy also has a close relationship with plant stresses, because most stressed plants have the ability to release allelochemicals into the environment to a greater concentration than normal. By doing so they are targeting the stress-producing competitor plants, nematodes or microorganisms with allelochemicals.

Measuring the effects of allelochemicals and the concentration released might help to establish the relationship between allelopathy and stress (Reigosa *et al.,* 2002). Other factors which are responsible for the release or effectiveness of allelochemicals are light, nutrient availability, water availability, pesticide treatment and diseases (Einhellig, 1996, Reigosa *et al*., 1999).

Kobayashi (2004) also concluded that allelopathy is a complex phenomenon which can be affected by soil factors (organic matter, moisture, pH and micro-organisms), age (young vs. old plant) of donor and receiver plants and climatic factors (temperature and rain) (Fig. 1.4). The released allelochemicals adsorbed on soil solids and further metabolized biologically (micro-organisms) and chemically (oxidation or reduction) with time.

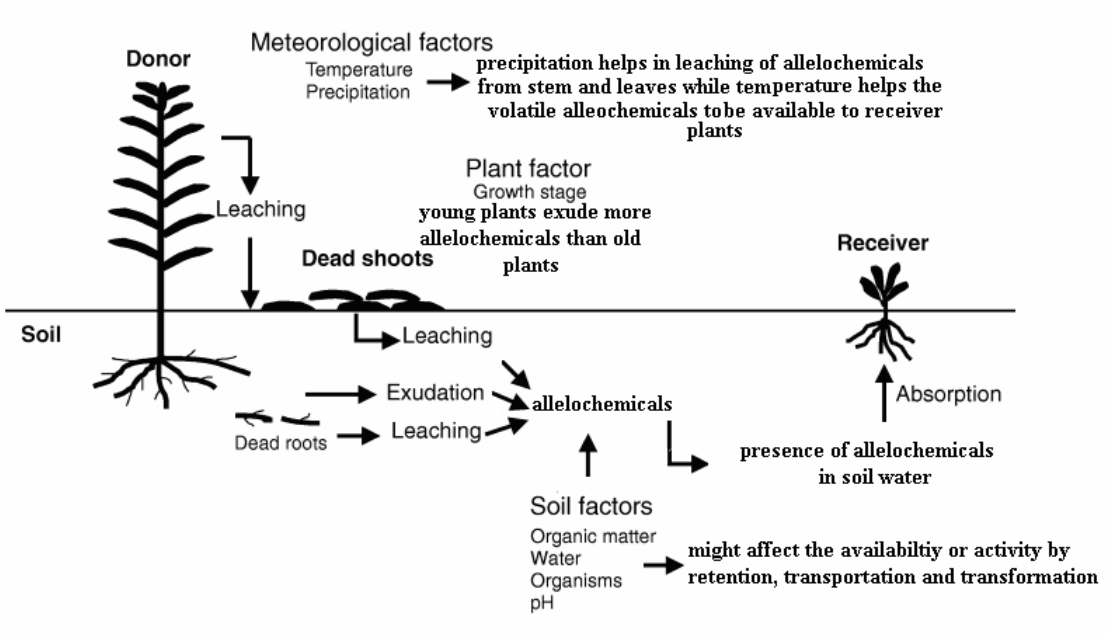


Figure 1.4: Factors affecting behaviour and the phytotoxic activity of allelochemicals in soil
Modified from Kobayashi (2004).

1.6 Allelochemicals from trees

The study of compounds that are produced by some plants to affect the environment is of great importance to understanding the mechanism of ecological interaction (Feo *et al.,* 2003). In many studies allelopathy has been demonstrated to play a crucial role in forests, influencing the composition of the flora and providing an explanation for the patterns of forest regeneration. The black walnut (*Juglans nigra*) produces juglone (Fig. 1.5), an allelopathic substance that interferes with the growth of other plants species (Davis, 1928). Under natural conditions juglone enters the soil through various process including root exudation, litter decay and rain throughfall. Since root exudation is a continuous process, it is assumed that juglone is continuously added to the soil, affecting the neighboring plants (Jose and Gillespie, 1998a). Although Davis (1928) observed that plants grown in the vicinity of black walnut died because of the presence of juglone, the physiological action of juglone was not well understood until the study conducted by Hejl *et al.,* (1993). This study showed that juglone at a concentration of 10 μM inhibited the growth of duckweed (*Lemna minor*) by decreasing the chlorophyll content and net photosynthesis rate. The results were further confirmed by an experiment in which leaf discs of soya bean (*Glycine max*) were placed in 10 μM juglone: the rate of photosynthesis was reduced.

In North America both maize (*Zea mays*) and soya bean (*G. max*) are often planted with black walnut but had not been studied for the adverse effect of black walnut until Jose and Gillespie (1998b). They studied the effect of juglone on these crops in detail. Seedlings of both species (maize and soya) were exposed to various concentrations of juglone (10^{-6} M, 10^{-5} M and 10^{-4} M) and a control (0 M). Within

three days juglone significantly inhibited the root and shoot growth, leaf photosynthesis, transpiration, and leaf and root respiration of both species but soya was found more sensitive than maize (Jose and Gillespie,1998b).

Another good example of an allelopathic tree is tree of heaven (*Ailanthus altissima*), which is native to China and was introduced in Europe around the 18th century. The tree of heaven is now widely distributed in the world and can thrive among a wide variety of floras, which might be due to its ability to produce several classes of allelochmicals (Shah, 1997). The first investigation on the phytotoxic activity of *A. altissima* extract was that of Mergen (1959); it was confirmed by Voigt and Mergen (1962). In both studies the authors found that water extracts from foliage and stems of A. *altissima* were toxic to neighbouring plants.

A bioassay experiment to study the allelopathic effect of aqueous extract of *A. altissima* and based on seed germination and subsequent radicle growth was done by Feo *et al.,* 2003. The surface-sterilized seeds of radish (*Raphanus sativus*), garden cress (*Lepidium sativum*) and purslane (*Portulaca oleracea*) were incubated in Petri dishes containing filter paper, impregnated with aqueous extract from *A. altissima* or distilled water as a control. The *A. altissima* extract inhibited the root growth of radish, garden cress and purslane by 100, 80 and 72% respectively. The active compounds were isolated from the crude extract and identified as terpenoids: ailanthone, ailanthinone, chaparrine and ailanthinol B (Fig. 1.6). Feo *et al.* (2003) suggested that the active principle from *A. altissima* could be used as a possible natural herbicide.

Phytochemical studies of *A. altissima* revealed triterpenoids (Kubota *et al.,* 1996), lipids and fatty acids (Kucuk *et al.,* 1994), phenolics (El-Baky *et al.,* 2000) and volatile compounds (Mastelic and Jerkovic, 2002) are the main allelopathic compounds.

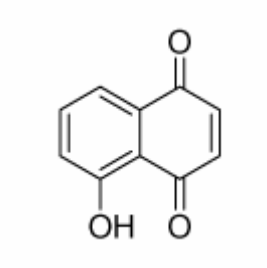
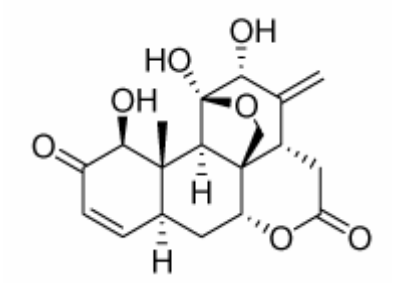
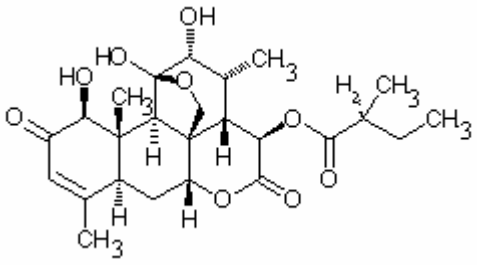


Figure 1.5: Structure of juglone
Obtained from Macias (2007).



Ailanthone



Ailanthinone

Figure 1.6: Structure of allelopathic terpenoids from *A. altissima*
Obtained from Macias (2007).

1.7 Allelopathic crop species

1.7.1 Wheat

Wheat (*Triticum aestivum*), one of the essential staple food-crops, has been studied extensively for its allelopathic potential. Aqueous extracts of wheat residue (dried leaves and stem) and seedlings are autotoxic (Wu *et al.,* 2007) and showed an allelopathic effect against weeds (Wu *et al.,* 1998). The inhibitory effect of an aqueous extract from wheat straw on the germination and growth of rye grass (*Lolium rigidum*) increased interest in separating and identifying the active compounds (Wu *et al.,* 1998). The phytotoxic compounds identified in extracts were mainly phenolic acids and benzoxazinone (Fig. 1.7) (Wu *et al.,* 2000).

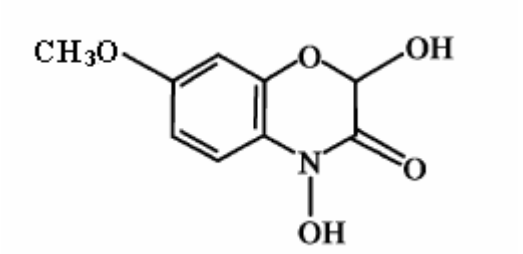
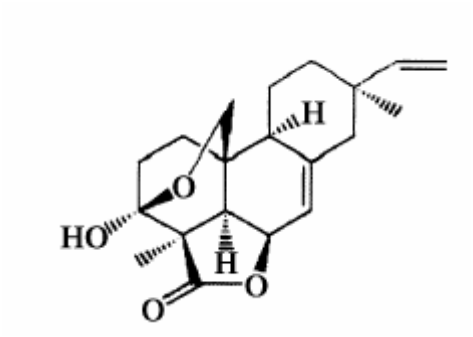


Figure 1.7: The allelopathic compound, DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) from wheat
Benzoxazinone consists of a benzene ring and an oxazine ring. Oxazine is a heterocyclic ring having 1 oxygen, 1 nitrogen and 4 carbon atoms in a ring. A methoxy group is attached to the benzene ring and ene is because of a double bond at C3 position. Obtained from Belz (2007).

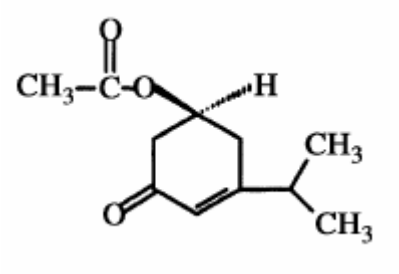
1.7.2 Rice

Various staple crops have been screened for their weed suppressive field performances and/or laboratory allelopathic potential, with rice being the most thoroughly studied staple crop species (Belz, 2007). Various classes of compounds have been identified as allelochemicals from rice exudates including phenolics, fatty acids, benzoxazinoids and terpenoids (reviewed by Belz, 2007). Olofsdotter *et al.* (2002) showed that the phenolic acid profile of allelopathic and non-allelopathic cultivars were not significantly different from each other. Further to that, the concentration of phenolics in the aqueous exudates was not enough to cause allelopathy. Seal *et al.* (2004) identified 15 phenolic acids in rice exudates but in very low concentrations. The non-significant effect of phenolic acids from rice on root growth of arrowhead (*Sagittaria montevidensis*) in a bioassay experiment revealed that phenolic acids were not a major class of allelopathic compounds, confirming the observations of Olofsdotter *et al.* (2002).

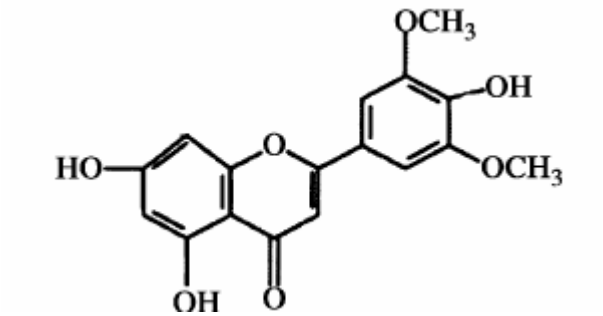
Compounds putatively responsible for growth inhibition isolated from rice root exudates were the diterpenoid, momilactone B (3,20-epoxy-3α-hydroxy-9β-primara-7,15-dien-16,6β-olide); a flavone (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone); and a cyclohexenone (3-isopropyl-5-acetoxycyclohex-2-enone) (Fig.1.8)(reviewed by Blez, 2007). Generally, different classes of allelochemicals are distributed among different tissues of rice seedling with root tissues containing large quantities of phenolic acids while momilactone B, the flavone and the cyclohexenone are abundant in both root and shoot tissues (Kong *et al.,* 2004).



A terpenoid, momilactone B (3,20-epoxy-3α-hydroxy-9β-primara-7,15-dien-16,6β-olide)



A cyclohexenone (3-isopropyl-5-acetoxycyclohex-2-enone)



A flavone (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone)

Figure 1.8: Allelochemicals identified in rice exudates
Obtained from Belz (2007).

1.7.3 Barley

Barley (*Hordeum vulgare*) is a smother crop (one sown to suppress persistent weeds) that possesses allelopathic potential to inhibit weed growth (Lovett and Hoult, 1995). The secondary metabolites (alkaloids: gramine and hordenine, Fig. 1.9) that are released by the barley root are responsible for the inhibition of *Sinapis alba*, a weed plant. The alkaloids from barley root exudates were separated and quantified by HLPC. Hordenine was a major component of barley root exudates with a production rate of 2 μg/plant/day in hydroponic solution. Purified barley alkaloids were tested for allelopathy in a laboratory bioassay. Hordenine and gramine were applied at three different concentrations (0, 15 and 50 ppm) to surface-sterilized *Sinapis alba* seeds on filter paper and incubated in the dark for three days. Both hordenine and gramine inhibited radicle growth of *Sinapis alba* by increasing both size and number of vacuoles, disorganizing organelles and damaging the cell wall. The cell wall from the control treatment was uniform while cell wall from alkaloid treatment was irregular (i.e. in some part it was thinner while in other it was very thick) (Liu and Lovett, 1993).

Other phytotoxic compounds were also released by barley including vanillic acid, chlorogenic acid, *p*-coumaric acid and ferulic acid. These phenolic acids significantly inhibited seed germination and radicle and shoot growth of green foxtail (*Setaria viridis*), which is a typical grass weed of arable land (Asghari and Tewari, 2007). The evidence of morphological changes caused by barley allelochemicals suggests that the presence and release of those biologically active metabolites from

barley may contribute to the control of weeds on arable land (Liu and Lovett, 1993; Asgahri and Tewari, 2007).

Hordine Gramine

Figure 1.9: Structure of allelochemicals from barley
Obtained from Belz (2007).

1.7.4 Rye

Natural compounds from rye (*Secale cereale*) with high allelopathic activity can also be used as a good source of natural herbicides (Burgos *et al.,* 2004). The weed-suppressive ability of rye is due to the presence of the benzoxazinoid, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its breakdown product, 2-benzoxazolinone (BOA) (Barnes *et al.,* 1987). The effectiveness and mode of action of DIBOA and BOA (Fig. 1.10) on cucumber (*Cucumis sativus*) seedlings was checked by electron and light microscopy. BOA inhibited the root growth and reduced the number of lateral roots by 77-100%. DIBOA also slowed root growth but had no effect on the number of lateral roots. Both compounds reduced the regeneration of root cap cells, increased cytoplasmic vacuolation, reduced ribosome density and number of mitochondria and interfered with lipid catabolism. Disturbance in cell ultrastructure revealed that both DIBOA and BOA reduce root growth as a result of disrupting lipid

metabolism, reducing protein synthesis and reducing transport or secretory capabilities because of underdeveloped dictyosomes (Burgos *et al.,* 2004).

DIBOA
(2,4-dihydroxy-1,4-benzoxazin-3-one)

BOA
(2-benzoxazolinone)

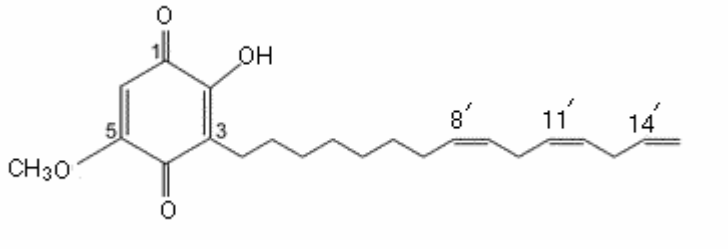
Figure 1.10: Structure of allelochemicals from rye
Obtained from Macias (2007).

1.7.5 Sorghum

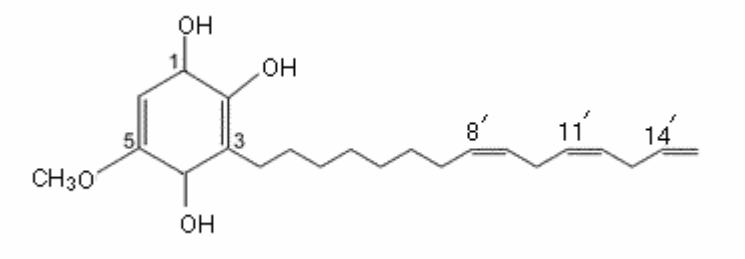
The allelopathic proerties of sorghum have been studied for decades (Kagan *et al.,* 2003) because of its inhibitory effect on weed growth by releasing hydrophobic compounds into the rhizosphere (Einhellig, 1992). The bioactive compounds isolated from sorghum root exudates mainly consist of a dihydroquinone that was quickly oxidised to a *p*-benzoquinone known as sorgoleone (Fig. 1.11) (Kagan *et al.,* 2003).

Sorgoleone when tested at 50 μM against a wide range of weeds (*Abutilon theophrasti, Datura stramonium, Amaranthus retroflexus, Setaria viridis, Digitaria sanguinalis* and *Echinochloa crusgalli*) in a nutrient medium for 10 days caused growth inhibition. The effect of 10 μM sorgoleone on weeds in a laboratory bioassay suggested that sorgoleone is a potential herbicide and contributes to sorghum allelopathy (Einhellig *et al.,* 1992). Further studies by Einhellig *et al.* (1993) showed

that the stunted growth of weeds in the presence of sorgoleone is due to its interference with photosynthesis.



(1)



(2)

Figure 1.11: Structures of (1) sorgoleone and (2) dihydroquinone of sorgoleone (Chang et al., 1986; Netzly et al., 1988; Fate et al., 1990).

1.7.6 Sunflower

Sunflower (*Helianthus annuus*) has a high level of secondary metabolites that are of commercial interest. The crude extract of shoots showed an inhibitory effect on germination of lettuce (*Lactuca sativa*) seeds (Macias *et al.,* 1999). In both greenhouse and laboratory experiments the water- soluble extract from sunflower leaf, stem, flower and root inhibited seed germination, radicle elongation, radicle weight increase, hypocotyl elongation and hypocotyl weight increase of wild barley (*Hordeum spontaneum*) (Ashrafi *et al.,* 2008). Plant height and weight of wild barley was also reduced significantly when it was grown in soil previously used by

sunflower. Incorporation of fresh sunflower roots and shoots into soil released allelopathic compounds that inhibited the germination, and the height and weight increase of barley (Ashrafi *et al.,* 2008). Batlang and Shushu (2007) observed similar effects when they treated bambara groundnut (*Vigna subterranea*) seeds with sunflower (shoot and root) extracts on a filter paper in the dark for 5 d under laboratory conditions. Both radicle and shoot lengths were negatively affected by sunflower exudates. The water-soluble allelochemicals of sunflower could be used in weed management programmes (Ashrafi *et al.,* 2008).

1.7.7 Allelochemicals of other plant species

Vulpia myros (silver grass), an annually growing winter grass, is one of the best examples that was extensively studied for an allelopathic effect on other plant species. An *et al.* (1997) started a series of experiments which provided support for the concept of *V. myros* allelopathy. In the first set of experiments, the aqueous extracts of decomposed plant residue collected at various time-points from *V. myros* were applied to wheat seeds. The water-soluble extract at high concentrations significantly inhibited germination while at low concentration it delayed germination of wheat seeds. The residues also showed an inhibitory effect on coleoptile and especially on growth (An *et al.,* 1997). After that successful experiment the authors tried to purify, quantify and characterise the compounds of interest by gas chromatography – MS (GC–MS). The extracts from *V. myros* were fractioned by gas chromatography (GC) through a wide-bore packed column. The fractions showed bioactivity towards wheat were collected and were identified by MS (An *et al.,* 2000). In pursuit of identification they have characterised 20 active compounds

(Table 1.1), out of which syringic acid, dihydroferulic acid, vanillic acid, *p*-hydroxybenzenepropanoic acid and succinic acid were present in much higher amounts than dihydrocinnamic acid and catechol. The identified compounds accounted for 0.05% of the dry weight of *V. myros* residues.

The identified compounds were then utilised in bioassays to test for their biological role. Each individual compound exhibited characteristic behaviour towards the test plant, wheat. Each of the compounds caused greater reduction of root elongation than coleoptile elongation. Dihydrocinnamic acid and catechol had high allelopathic effects even at very low concentrations (An *et al.,* 2001).

Plants in the genus *Ophiopogon* are also famous for a wide varity of biologically active compounds; one of the best-studied examples is *Ophiopogon japonicus* commonly known as dwarf lily turf. *O. japonicus* was initially known for its medicinal value but was latterly discovered to be an allelopathic plant. The methanolic extract from the roots of *O. japonicus* reduced root and hypocotyl growth in lettuce. Salicyclic acid and *p*-hydroxybenzoic acid were identified as allelochemicals in *O. japonicus* by NMR (Iqbal *et al.,* 2004). Gallic acid, hydroquinone and caffeic acid were also identified as allelochemicals in *Polygonella myriophylla* and sweet potato (Jeffrey and John, 2004; Howard *et al.,* 2003).

Root exudates of buckwheat (*Fagopyrum esculentum*) are also a good source of allelopathic compounds including vanillic acid, gallic acid 4-hydroxyacetophenone. All three compounds have inhibitory effects on the growth and development of other plant species (Kalinova *et al.,* 2007). Kushumi *et al.* (1998) also found that vanillic acid from germinating water melon seeds at a

concentration of 30-300 mg/l has inhibited the shoot growth of lettuce and tomato but stimulated the shoot growth of amaranth and barnyard grass in a laboratory bioassay.

Table 1.1: Dried yield and phytotoxic strength of allelochemicals isolated from an ether extract of V. myros plant residue.

Chemical name	Dry weight (%)	Threshold concentration (ppm)		I_{50} (ppm)	
		Coleoptile	Root	Coleoptile	Root
Coniferyl alcohol	0.0005	500	500	> 1000	775
Protocatechuic acid	0.0018	500	250	> 1000	712
Succinic acid	0.0080	500	100	> 1000	631
3,4-Dimethoxyphenol	0.0002	250	250	> 1000	585
Dihydrocaffeic acid	0.0004	250	100	941	576
Syringic acid	0.0140	500	10	932	602
Hydroquinone	0.0002	500	250	924	542
p-Hydroxybenzoic acid	0.0018	250	500	886	463
Vanillic acid	0.0081	500	100	800	373
p-Hydroxyphenyl acetic acid	0.0008	10	1	886	97
3-(4-Hydroxyphenyl) propanoic acid	0.0044	1	1	729	458
Gentisic acid	0.0011	1	100	712	441
p-Coumaric acid	0.0005	100	250	678	373
Pyrogallol	0.0007	100	100	729	275
Ferulic acid	0.0008	250	1	534	352
Catechol	0.0002	100	1	644	200
2-Hydroxy-3-phenyl propanoic acid	0.0017	10	250	483	246
Dihydrocinnamic acid	0.0001	100	1	288	85
Salicylic acid	0.0037	10	100	273	169
Benzoic acid	0.0012	100	100	309	200

Total dry weight of ether extract was 306.1 mg from 100 g of *V. myros,* whole plant residue. % dry weight: dry weight of a compound relative to total dry weight of whole plant residue.

Threshold concentration: the concentration at which a compound started growth inhibition of wheat coleoptiles and roots.

I_{50} is the concentration of compound at which it caused 50% inhibition of coleoptile and root growth in the test plant (modified table from An *et al.,* 2000 and An *et al.,* 2001)

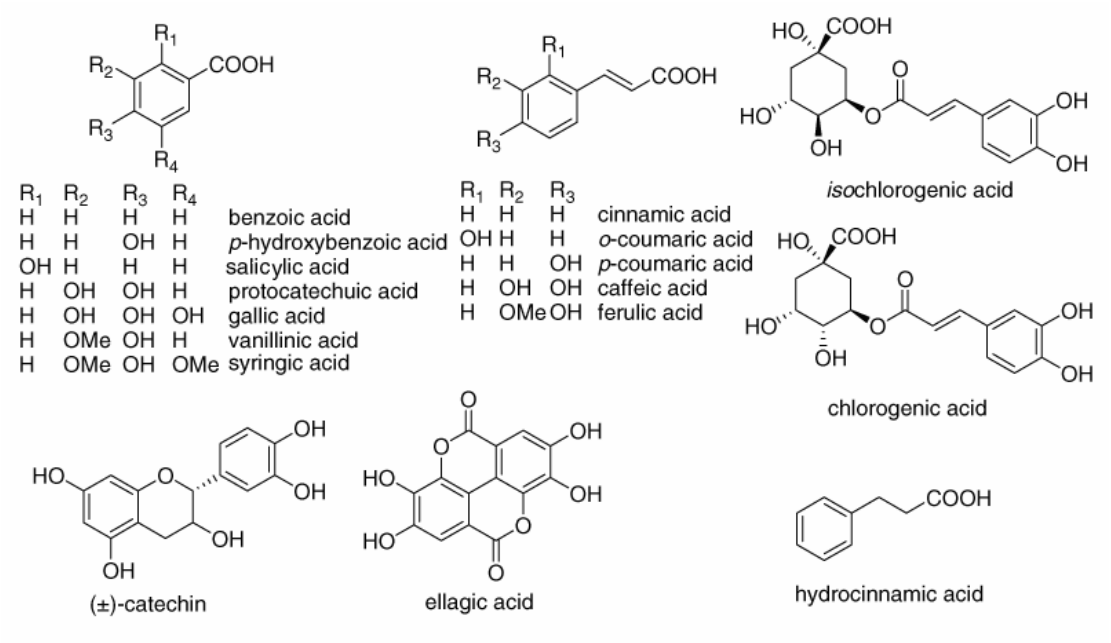


Figure 1.12: Structure of allelopathic phenolics
Obtained from Macias (2007).

1.8 Oligosaccharins

Some oligosaccharides obtained from plant cell wall degradation exhibit allelopathic effects. Zabotina *et al.* (2002) isolated a physiologically active compound from shoot cell walls of *Pisum sativum* after partial acid hydrolysis. The hydrolyzed fraction showed inhibiting effect on root formation in thin-cell- layer explants. The fraction mainly contained fragments of xyloglucan, galactan and arabinan with DP of 5-6.

A growth inhibitory effect has been demonstrated for xylem exudates from *Vitis vinifera* on *Lemna minor*. By ultrafiltration the crude exudate was fractioned and the inhibitory effect was observed in the 0.5-10 kDa fraction. This fraction was found to contain abscisic acid but not in a sufficient quantity to cause the observed inhibitory effect. The 0.5-10 kDa fraction was then separated on a Sephadex G-15 gel-permeation column and the fractions corresponding maltoheptaose to maltotriose were pooled together. The pooled fractions showed the same biological activity as the crude exudate, which provides evidence that the inhibitor was an oligosaccharide (Campbell *et al.,* 1995b).

The degradation product of sodium alginate formed by the action of alginate lyase (from *Altermonas macleodii*) showed root growth-promoting activity in barley seedlings. Sterilized barley seeds were germinated in dark for 16 h; the seedlings were then placed on an agar bed containing or lacking the alginate lyase-lysate. The lysate promoted root growth 1.7-fold relative to the control within 12 d. The lysate was then fractioned by HPLC (on a Carbo pac PA1 column) and each individual

fraction was tested for biological activity. The compounds showing biological activity were identified by NMR as trisaccharides 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid-(1→4)-α-L-gulopyranosyluronic acid-(1→4)-L-gulopyranuronic acid and 4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid-(1→4)-α-D-mannopyranuronic acid-(1→4)-D-mannuronic acid (Natsume *et al.,* 1994).

A saponin with a sugar moiety (Fig. 1.13) extracted from whole mature *Tagetes patula* plants with methanol was observed to have a strong allelopathic effect against *Ischaemum rugosum, Vicia sativa* and *Echinochloa colona* weeds (Sondhia, 2005). The author isolated the saponin from *T. patula* and characterized it by NMR and MS. The authentic compound was then utilized in a laboratory bioassay. Seeds of I. *rugosum, V. sativa* and *E. colona* were incubated on filter paper in Petri dishes containing 0-10% (w/v) saponin. Both root and shoot growth was inhibited by the saponin; roots were affected most, which might be because of the direct long-term contact with the active compound.

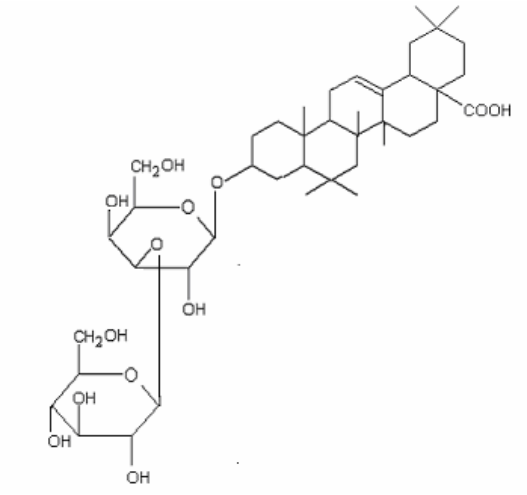


Figure 1.13: Structure of saponin glycoside (3-O-[β-d-glucopyranosyl-(1→3)-β-d-galactopyranosyl]-3-hydroxy olean-12-en-28-oic acid) from Tagetes patula.

1.9 Plant cell walls

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. Growing plant cell walls are predominantly composed of pectin, hemicellulose and cellulose (McNeill *et al.,* 1984) (Fig. 1.13). A function of cell walls is to maintain the size and shape of the cells and ultimately plant morphology. Growing plant cells expand by the modification of existing wall polymers and addition of newly formed polymers into the cell wall architecture. This modification of the cell wall might generate oligosaccharides, some of which might influence cell and tissue growth of the host plants or act as elicitors of phytoalexins. Such oligosaccharides are known as oligosaccharins (Albersheim *et al.,* 1983; Aldington and Fry, 1993). Various oligosaccharins were prepared from plant-derived pectins, hemicelluloses, glycolipids and glycoproteins (McDougall and Fry, 1989; McDougall and Fry, 1990; McDougall and Fry, 1994; Fry *et al.,* 1993a; Garcia-Romera and Fry, 1995; Smith *et al.,* 1999; Dumville and Fry, 2000; Dumville and Fry, 2003).

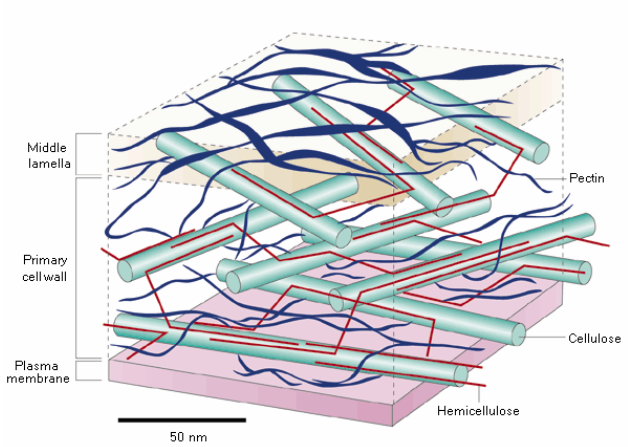


Figure 1.14: Structure of primary cell wall of a dicot
Obtained from Smith (2001).

1.9.1 Hemicelluloses

Hemicelluloses are the second most abundant class of polysaccharides in plant cell wall. Hemicelluloses of the plant cell wall are extractable with alkaline solutions. Hemicelluloses extracted from different plant sources are rarely identical. The major hemicelluloses extracted from cereals are xylans, which consist of a β-1,4 linked D-xylose backbone substituted by different side groups such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl and glacturonic acid residues (Wilkie and Woo, 1977). Another hemicellulose structure, found in soft and hard wood, was galactoglucomanan (Timell, 1967). Xyloglucan is a major component of hemicelluloses in cell walls of dicots and some monocots (e.g onion).

1.9.1a Galactoglucomannan (GGM) and GGM-derived oligosaccharins

Galactoglucomannan (GGM) is a major hemicellulose fraction of both primary and secondary cell wall of some higher plants, representing up to 15% of the cell wall dry mass (Aspinall, 1980). It is most commonly found in the cell walls of Leguminosae (Dea and Morison, 1975). GGM of both soft-wood and hard-wood consists of β-1,4-linked D-mannose and D-glucose with α-1,6-linked D-galactose as a side chain (Timell, 1967) (Fig. 1.13). It has been observed that some of the oligosaccharides derived from GGM are biologically active and are therefore oligosaccharins (Auxtova *et al.,* 1995; Slovakova *et al.,* 2000; Kollarova *et al.,* 2006).

Auxtova *et al.* (1995) isolated galactoglucomannan by a fractionation procedure as summarised in a flow chart (Fig. 1.14) from poplar (*Populus monilifera*). The GGM- derived oligosaccharides were obtained by partial acid

hydrolysis of GGM and purified compounds were obtained by GPC and PC (Kubackova *et al.,* 1992). The purified GGM oligosaccharides (GGMOs) at different concentration (10^{-5}-10^{-10} M) were then tested for their biological activity. In a laboratory bioassay several pea-stem segments were used to test the effect of GGMOs on the 2,4-D-induced elongation growth. A significant inhibition of growth was recorded after 18 h of assay. The effect was more significant at lowest concentration tested i.e.10^{-10} M than at high concentration (10^{-5} M). In a similar experiment when Auxtova *et al.* (1995) added 2,4-D after 1.5 h of total 18 h experiment same result was obtained. The results from both experiments revealed that the GGMOs (DP 4-8) inhibited the 2,4-D– stimulated elongation of pea stem segments. The high inhibition at a low concentration indicated that the resulting GGMOs from GGM were not phytotoxic but were oligosaccharins.

Further biological characteristics (structure–function relationship) of GGMOs were studied by Kollarova *et al.* (2006). After partial hydrolysis of GGM with TFA (0.4 M), the GGMOs (DP 4-8) were obtained by GPC on Bio-Gel P-2 column. The purified GGMOs consisted of galactose, glucose and mannose in the molar ratio 1:8:33. GGMOs-g (degalactosylated oligosaccharides) and GGMOs-r (reduced oligosaccharides) produced from GGMOs with α-galactosidase and $NaBH_4$ respectively (Bilisics and Kubackova 1989) were used in the pea stem bioassay. Both GGMOs-g and GGMOs-r inhibited the elongation of pea stem segments induced by 2,4-D but the effect of GGMOs-r was higher than GGMOs-g. The result indicated that probably the galactosyl side chain linked to glucomanno core played an important role in their biological activity.

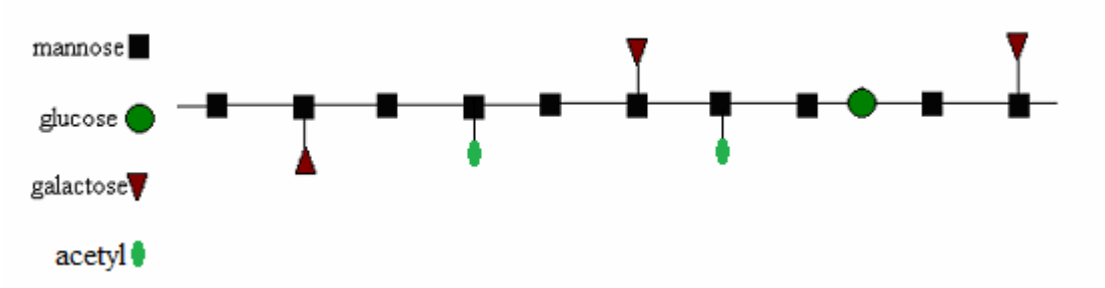


Figure 1.15: Schematic presentation of galactoglucomannan from spruce softwood
Obtained from De Vries and Visser (2001).

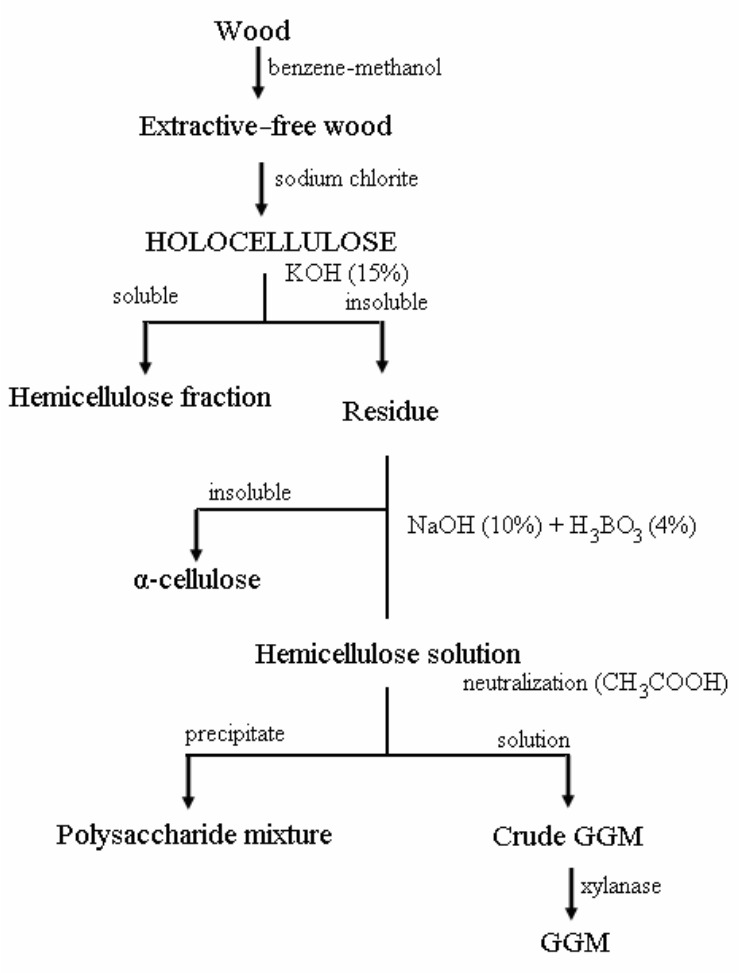


Figure 1.16: Isolation and purification of GGM from *Populus monilifera*
Obtained from Auxtova *et al*. (1995).

1.9.1b Xyloglucan (XyG) and XyG-derived oligosaccharins

Xyloglucan (XyG) is a structural polysaccharide found mainly in the primary cell wall of higher plants (Levy *et al.,* 1991). Two major types of XyG have been identified in the plant cell wall. The XXXG type consists of three repeating units of β-1,4-D-glucopyranose substituted with α-1,6-D-xylopyranose, which are separated by an un-substituted glucose residue. In type XXGG, two xylosylated glucose units are separated by two un-substituted glucose unit (De Vries and Visser, 2001) (Fig. 1.15). To the xylose residue is bound, in addition, fucose, galactose and arabinose residues as, for example, α-L-fucopyranosyl-β-(1→2)-D-galactopyranosyl-(1→2)- and α-L-galactopyranosyle-β-(1→)2-D-galactopyranosyl-(1→2)- disaccharides (Hantus *et al.,* 1997; Vincken *et al.,* 1997), and smaller amounts of α-L-arabinofuranosyl-(1→2)- (Hisamatsu *et al.,* 1992; Fry *et al.,* 1993b; York *et al.,* 1996; Huisman *et al.,* 2000). Xyloglucan chains bond to cellulose through hydrogen bonding and thus help in structural integrity of the cell wall and might play an important role in cell wall expansion (Fry, 1989a and 1989b; Hayashi, 1989).

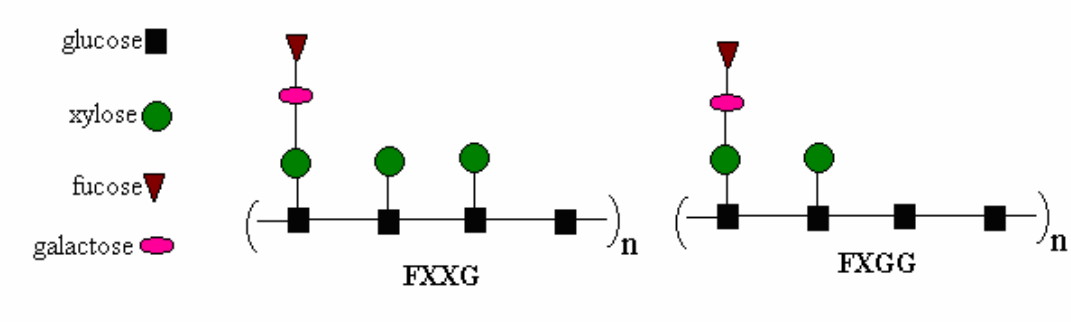


Figure 1.17: Structural representation of two types of xyloglucan
Modified from De Vries and Visser (2001).

The long chain of xyloglucan can tether cellulose microfibrils, may result in a strong structure. Xyloglucan can be hydrolysed by β-1,4-D-glucanase (cellulase), releasing oligosaccharides some of which might be biologically active and regarded as oligosaccharins (Fry, 1986; MacDougall and Fry, 1989; MacDougall and Fry, 1991; Warneck *et al.,* 1993; Cutillas-Iturralde *et al.,* 1998; Hoson and Masuda, 1991). Xyloglucan fragments can be obtained by enzymic hydrolysis and subsequent purification by GPC, PC and HPLC (Fry, 1986; McDougall and Fry, 1989; McDougall and Fry, 1991; McDougall and Fry, 1994). The biological activity of purified XXFG was tested for the inhibition of 2,4-D-induced elongation of pea stem segments (McDougall and Fry, 1989). Washed segments of pea were placed in Petri dishes with test material and 2,4-D (10^{-6} M). Controls were carried out without the addition of 2,4-D. XXFG showed an optimum antiauxin activity at a concentration of 10^{-9}. The same experiment was done with XXXG (lacking fucose and galactose side residues), XXLG (lacking fucose side chain) and XLFG (with an extra galactose side chain) (Fig. 1.16) but in all cases no antiauxin activity were observed. The results from those experiments suggested that the side chain residue (Fuc) of the XXFG is important for antiauxin activity.

The first experiment which showed the *in-vivo* production of biologically active xyloglucan-derived oligosaccharides was by Fry (1986). The author studied the *in-vivo* formation of the bioactive nanosaccharide (XXFG) from xyloglucan by spinach cell-suspension cultures. [^{3}H]Arabinose and [^{3}H]fucose were fed to the culture. The oligosaccharides from culture filtrate were examined by PC and GPC. One of the oligosaccharides was labelled with [^{3}H]fucose and [^{3}H]pentose residues, and was identical to XXFG. Later, McDougall and Fry (1991) fed L-[^{3}H]fucose to

cell-suspension cultures of spinach. The appearance of [^{3}H]oligosaccharides and [^{3}H]polysaccharides was monitored by PC. Soluble [^{3}H]polymers were released into medium after a lag period (~ 30 min) while [^{3}H]XXFG was observed at an accelerating rate from ~ 5-8 h. From the result it was concluded that XXFG arises from the partial hydrolysis of pre-formed xyloglucan rather than by *de-novo* synthesis of oligomers.

The heptasaccharide (XXXG) and octasaccharide (XXLG) produced from xyloglucan were reduced with $NaBH_4$ to form XXXGol and XXLGol. XXLGol at a concentration of 0.5 to 100 nM showed a growth-promoting activity towards wheat coleoptiles in the absence of 2,4-D. In the presence of 2,4-D, XXXGol increased the auxin induced reponses even at nanomolar concentration suggesting that it is a signalling molecule (Vargas-Rechia *et al.,* 1998).

The XXFG produced from xyloglucan acts as an antiauxin in etiolated pea stems (McDougall and Fry, 1988). The promoting effect on excised shoots is more pronounced when growth is already enhanced by gibberellic acid (Warneck *et al.,* 1993). Substitution of the XXXG core with one or two Gal residues to give XXLG and XLLG resulted in growth promotion and in-vitro stimulation of cellulase (McDougall and Fry, 1990). The reduced form of XXXG and XXLG also showed growth promotion of wheat coleoptiles (Vargas-Rechia *et al.,* 1998).

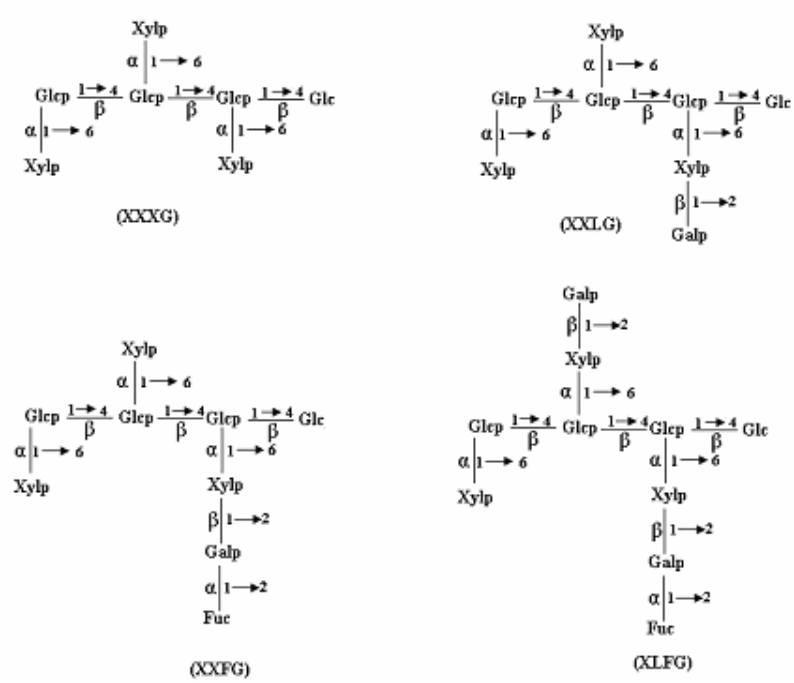


Figure 1.18: Structure of xyloglucan oligosaccharides
Obtained from McDougall and Fry (1989).

1.9.2 Pectin

Pectins or pectic substances are a mixture of heterogeneous, branched, and highly hydrated polysaccharides (Fishman *et al.,* 2001) composed of 17 different monosaccharides (Ridley *et al.,* 2001; Voragen *et al.,* 2001) (Fig. 1.17). This class of heteropolysaccharides is one of the major components of plant cell wall and consists of several distinct regions (Perez *et al.,* 2000). The 'smooth' region consists of homogalacturonan (HG) and the 'hairy' region or ramified region consists of xylogalacturonan (XGA), rhamnogalacturonan I, rhamnogalacturonan II and polysaccharides composed of mainly neutral sugars (arabinan, galactan and arabinogalactan) (Sakamoto and Saki, 1995; Bacic *et al.,* 1988). The composition and chemical structure of the individual segments of pectin varies significantly depending on the source from which it is extracted (Huisman *et al.,* 2001). Unlike cellulose, pectin is largely restricted to the primary cell wall and accounts for

approximately 1/3rd of primary cell wall macromolecules in dicotyledonous and non-gramineous monocotyledonous plants. Pectin plays an important role in cell wall physiology, determining the wall porosity, control of ion transport and permeability of the cell wall, regulation of cell growth and differentiation and determining the water holding capacity of the cell wall (Fishman *et al.,* 2001). Various enzymes act on pectic polysaccharide backbones releasing oligosaccharides, some of which exhibit biological activity (Fry *et al.,* 1993; Cutsem and Messiaen, 1994; Spiro *et al.,* 1998).

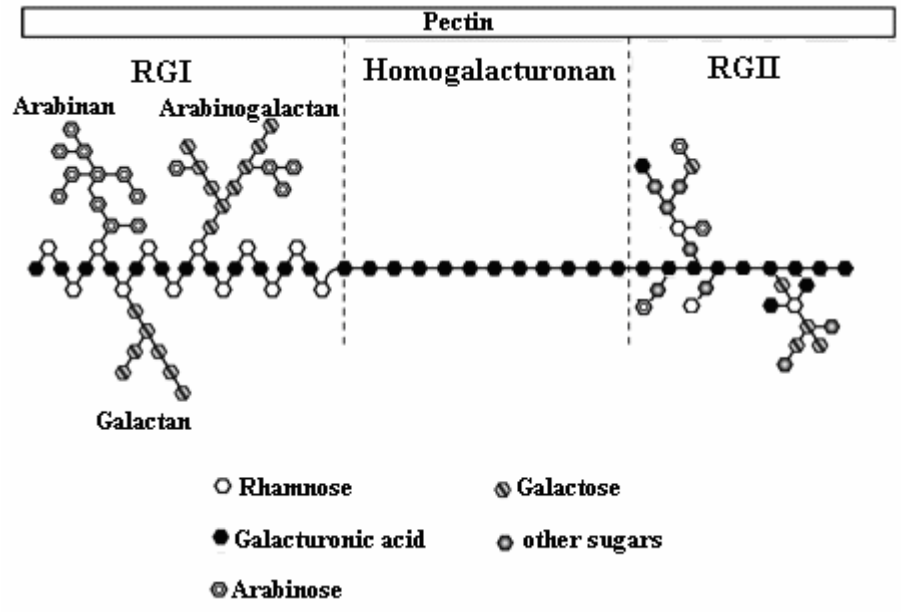


Figure 1.19: Structure of pectin
Modified from Ochiai *et al*. (2007).

1.9.2a Homogalacturonan (HG) and HG-derived oligosaccharins

HG is a straight chain of α-1,4-D-galacturonic acid (GalA), with the carboxylic acid groups methyl-esterified at various degrees as high as 70-80% (Ishii, 1997) (Fig. 1.18). HG has been reported to consist of 70-100 GalA units (Thibault *et al.,* 1993). Pure HG as an individual discrete polymer is not common in native pectin but commercial pectin primarily contains HG segments (Wong, 2008).

Some oligogalacturonide fragments of HG act as bioactive molecules like hormones (Ridley *et al.,* 2001). A mixture of oligoglacturonides produced by the action of TFA on HG was tested for their effect on development of strawberry explants *in vitro* by Miranda *et al.* (2007). When the mixture of oligogalacturonides was added at 0.1 and 1 mM concentration to strawberry explants it stimulated shoot and root number and root elongation. The result of high-performance anion exchange chromatography revealed that the mixture of bioactive compounds consisted of galacturonides with a degree of polymerization from 1-5 (Miranda *et al.,* 2007).

Suzuki *et al.* (2002a) also studied the effect of hot water and hot acid extracts from tomato juice waste on root and shoot growth of cockscomb (*Celosia argentea*), an ornamental dicot from family Amaranthaceae . A potent shoot growth promoting activity was observed in the acid extract whereas the water extract did not show any activity. A mixture of biologically active oligosaccharides (DP 2-12) was separated by chromatographic and electrophoretic steps, including anion-exchange chromatography. The individual fractions were then utilised in a bioassay. Seeds of cockscomb on wet filter paper responded to the oligomer mixture by exhibiting growth promoting activity. The active principle was identified as an octamer of

galacturonic acid ($GalA_8$). This oligomer showed stimulatory effect on shoot growth at 10 µM while at 300 µM it inhibited root growth of cockscomb (Suzuki, 2002b).

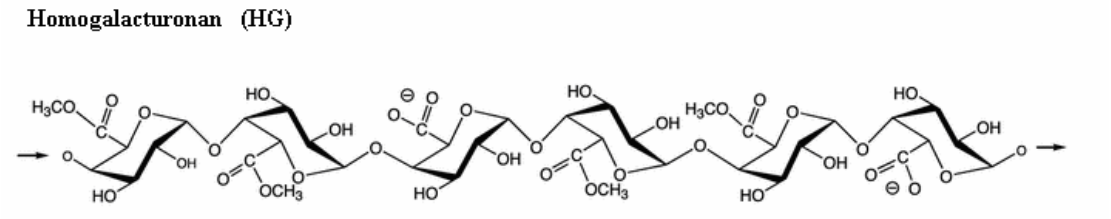


Figure 1.20: Partially methylesterified homogalacturonan
Obtained from Wong (2008).

1.9.2b Rhamnogalacturonan II as oligosaccharin

Rhamnogalacturonan II (RGII) is a highly substituted oligogalacturonan with a variety of side chains (Vidal *et al.,* 2000) (Fig. 1.19). RGII side chains consist of four well-defined oligosaccharides units attached to a block of at least nine Gal*p*A units (O'Neill *et al.,* 1996). The side chain consists of several different sugars including α- and β-D-Gal*p*A, α- and β-L-Rha*p*, α-D-Gal*p*, α-L-Fuc*p*, α-L-Ara*p*, β-L-Ara*f*, β-D-Api*f*, β-D-Glc*p*A, α-D-KDO, β-L-Ace*f*A, α-D-Xyl*p* and β-D-DHA (Puvanesarajah *et al.,* 1991). Acetylation on side chain residues, 2-*O*-methylfucose and aceric acid was reported by Whitcombe *et al.* (1995). RGII also forms a cross linked dimer on account of a covalent borate-diol diester between OH-2 and OH-3 of apiose residues (Pellerin *et al.,* 1996).

RGII from pectin was found to interfere with the uptake of [^{14}C]leucine by tomato cell-suspensions (Aldington and Fry, 1994). Small galacturonides (GalA and $GalA_2$), had no significant effect on uptake of [^{14}C]leucine, while $GalA_3$ and $GalA_4$ inhibited the incorporation of leucine by 25%. The authors proposed that RGII was

affecting the uptake by binding the [^{14}C]leucine extracellularly and in this way preventing its contact with the cells. The unusual sugar moieties such as KDO and apiose also inhibited the uptake of [^{14}C]leucine by tomato cells but weakly. This suggested that these two unusual sugar units are integral part of RGII bioactivity. RGII also inhibited the incorporation of [^{14}C]glutamate, [^{14}C]histidine, [^{14}C]proline, [^{14}C]arginine, [^{14}C]tyrosine (Aldington and Fry, 1994).

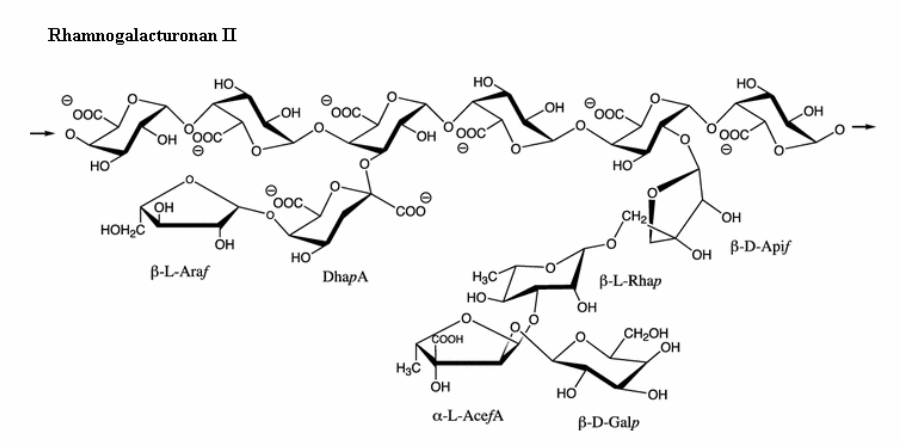


Figure 1.21: Partial structure of RGII
Obtained from Wong (2008).

1.9.2c Rhamnogalacturonan I

Rhamnogalacturonan I (RGI) is a heteropolymeric domain of pectin, consists of about 100 repeated units of [→4)-α-D-galacturonic acid-(1→2)-α-L-rhamnose-(1→] in pyranose form (Renard *et al.,* 1998). The C2 and / or C3 position of Gal*p*A may be substituted with an acetyl group depending on the origin of the cell wall (Ros *et al.,* 1996). Polysaccharides including arabinogalactan, galactan and arabinan are often attached to the rhamnose residue of the RGI backbone at the C4 position (Fig. 1.20). Arabinans are homopolymers of α-1,5-L-arabinofuranose units with substitution of 1 to 3 arbinofuranose residues at the C2 and C3 positions. Galactan is common in

various plant tissues and consists of β-1,4-D-galactose residues. Hairy regions of apple pectin also have β-1,3-D-galactosyl residues and β-1,6-D-galactose side chains (De Vries *et al.,* 1982). Galactan substituted with α-L-arabinofuranose side chains are known as arabinogalactan I, the backbone of which is made up of β-1,4-D-galactose residues and has α-1,5-L-arabinofuranose residues as a side chains (Aspinall *et al.,* 1972). Various enzymes act on RGI including RG hydrolases, RG lyases and galactanase to produce oligosaccharides, some of which are of great interest in regard to their biological activities. In this context, Fry *et al.* (1993) suggested that the bioactive disaccharide (lepidimoide), which consists of rhamnose and an unsaturated uronic acid might formed by the action of RGI lyases on the RGI backbone.

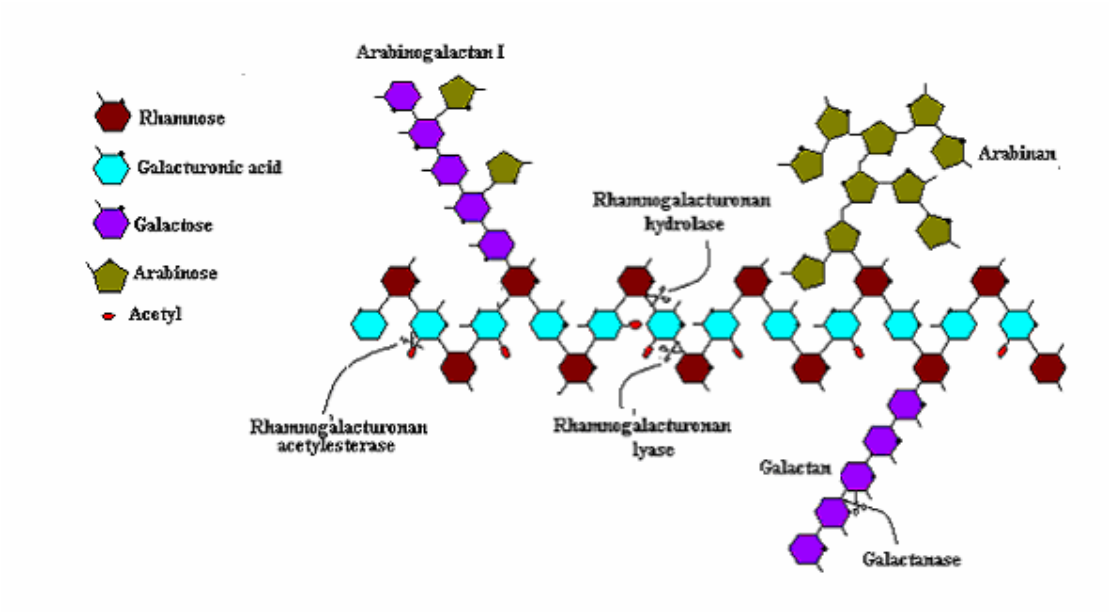


Figure 1.22: Structure of RGI and sites of enzyme action
Obtained and modified from University of Copenhagen, Department of Chemistry website.

1.10 Lepidimoide

Lepidimoide (Fig. 1.21) was noted as an unusual unsaturated disaccharide exuded from 2-day cress seedlings (*Lepidium sativum*) (Hasegawa *et al*., 1992). The authors observed it as a potent allelopathic compound in a laboratory bioassay. *A. caudatus* seeds were placed on a filter paper wetted with the concentrated cress seed exudates. A significant stimulation of hypocotyl growth and inhibition of root growth were recorded. The cress seed exudate was fractioned to purify and characterise the active principle. The exudate was fractionated into three size classes (i.e. M_r above 10^5, from 10^5 to 5×10^3, and below 5×10^3) by gel-permeation chromatography. The fraction with M_r lower than 5×10^3 stimulated the hypocotyl and inhibited the root growth and was further purified by reverse-phase HPLC. After spectral (IR, UV, NMR and MS) and polarimetric analysis, the purified bioactive compound was identified as 4-deoxy-β-L-*threo*-hex-4-enopyranuranosyl-(1→2)-L-rhamnose. Later the interest in lepidimoide was increased because of its high allelopathic activity and was isolated from sunflower (*Helianthus annuus*), buckwheat (*Eriogonum douglasii*) (Yamada *et al*., 1995 and 1997) and *Arabidopsis thaliana* (Tomita *et al*., 1998 and 2003).

As the structure of lepidimoide consists of an unsaturated uronic acid glycosidically attached to rhamnose, Fry *et al*. (1993a) proposed that lepidimoide might be formed enzymically by the action of a lyase on the RG-I backbone in cress cell walls. Polysaccharide lyases cleave sugar polymers via a β-elimination mechanism resulting in the formation of a double bond between C-4 and C-5.

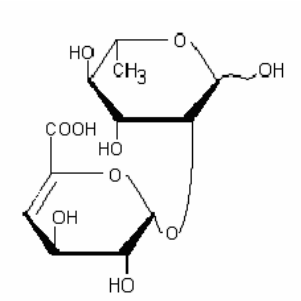


Figure 1.23: 4-Deoxy-β-l-threo-hex-4-enopyranuranosyl-(1→2)-l-rhamnose (lepidimoide)
Obtained from Hirose *et al.* (2004).

1.10.1 Laboratory synthesis of lepidimoide

Lepidimoide was first prepared in the laboratory from D-glucose and L-rhamnose, chemically in 22 steps by Kosemura & Yamamura (1993). The method was complicated and only a small quantity of lepidimoide was obtained. Because of the allelopathic effect and large interest in oligosaccharins, large-scale production of lepidimoide in a short period of time was demanded.

Hirose *et al*. (2004) first produced lepidimoide from okra mucilage in large quantities by a simpler method. The reason to start with okra seed mucilage was the structure of the polysaccharide from okra which has the same repeating unit as RG-I (Tanaka *et al*., 2002). The extracted polysaccharide contained residues of rhamnose, galacturonic acid, galactose, glucose and glucuronic acid. The next step was to remove the side chain sugar (galactose and glucose) which was done by partial acid hydrolysis. As a result, a polysaccharide backbone composed of repeating units of rhamnose and galacturonic acid (-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-) was produced. The resulting heteropolysaccharide unit was then acetylated with acetic anhydride–sodium acetate and methyl esterified with trimethylsilyldiazomethane and the resulting polysaccharide derivative was treated with sodium methoxide in the

presence of methanol. During this process, β-eliminative degradation of the polysaccharide occurs. Lepidimoide was finally obtained by the mild acid hydrolysis of the reaction mixture and was purified by HPLC. The purified compound was identified by spectral and polarimetric analysis. The biological activity of the purified compound was measured by the plumed cockscomb (*Celosia argenta*) elongation test. The purified lepidimoide from okra mucilage showed the same biological effects as lepidimoide from cress mucilage.

Tanaka *et al.* (2002) also prepared lepidimoide from okra mucilage by the action of endophytic fungal enzymes. The spectral analysis showed same results as for authentic lepidimoide. The purified compound formed by the action of enzyme from fungal strain AHU9748 showed similar properties as authentic lepidimoide on TLC.

1.10.2 Effect of lepidimoide on hypocotyl and root growth

Lepidimoide serves as a potent allelopathic substance that over-stimulated the shoot growth and inhibited the root growth of various plant species. For example, at a concentration of 3 μM, it promoted hypocotyl growth in etiolated *A. caudatus*, while having little effect on the roots; it inhibited root growth while stimulating increasingly the hypocotyl growth at a concentration higher than 100 μM. The growth-promoting activity at 100 μM in *A. caudatus* hypocotyls was higher than that of gibberellic acid (Hasegawa et al., 1992).

1.10.3Effect of lepidimoide on leaf senescence and light-induced chlorophyll accumulation

The effect of lepidimoide on senescence in oat (*Avena sativa*) leaf segments in the dark was studied (Miyamoto *et al.,* 1997a). Lepidimoide inhibited loss of total chlorophyll in excised oat leaf segments during senescence, especially in cooperation with cytokinin. Yamada *et al*. (1998) also studied the effect of lepidimoide on the light-induced chlorophyll accumulation in sunflower seedlings. Besides lepidimoide, they also studied the effect of sucrose and an analogue of lepidimoide with (saturated) GalA in place of unsaturated UA. Hydrogenated lepidimoide (lacking the double bond in the uronic acid) showed 2 times lower activity. Sucrose on the other hand had no activity. In addition to the effect of lepidimode on light-induced chlorophyll accumulation it is also observed a promoting effect on light-induced 5-aminolaevulinic acid content in sunflower seedlings. The result suggests that lepidimoide enhanced light-induced chlorophyll accumulation by affecting the level of 5-aminolaevulinic acid (ALA) (Yamada *et al,*. 1998). ALA is an essential precursor of tetrapyrrole compounds and is known as an essential biosynthetic precursor of all porphyrin compounds, including chlorophyll and haem (von Wettstein *et al.,* 1995).

1.10.4 Inhibition of abscission of bean petiole explants by lepidimoide

Plant 'hormones' such as cytokinins, indole acetic acid (IAA), ethylene and abscisic acid are known to have an important role in leaf senescence (Osborne 1968; Reddy *et al.,* 1988; Uedda *et al.,* 1991 and 1992). In the case of IAA a strong leaf abscission inhibition in bean petiole explants has been reported (Ueda *et al*., 1992). Both

lepidimoide and IAA inhibited root growth in *Amranthus caudatus* to the same extent (Hasegawa *et al*. 1992). Therefore, Miyamoto *et al.* (1997b) tested the effect of lepidimoide on abscission in bean petiole explants. The authors incubated bean petiole explants in various concentrations of lepidimoide for 60 h in constant light. In the presence of 1 μM to 100 μM of lepidimoide, only 30% of the explants abscised, compared with 80% of the control plants treated with only water. The results showed that lepidimoide mimicked IAA significantly inhibiting the abscission of bean petiole explants.

In short lepidimoide acts as an oligosaccharin that promotes hypocotyl growth, and inhibits root growth in *Amaranthus* (Hasegawa et al., 1992), leaf senescence in *Avena* (Miyamoto *et al.,* 1997a), abscission of bean petiole explants (Miyamoto *et al.,* 1997b) and light-induced chlorophyll accumulation in sunflower (Yamada *et al.,* 1998).

1.11 Project aims

1.11.1 Evidence of cress bioactivity

The study of Hasegawa *et al.* (1992) revealed that the exudate of cress roots significantly affected the hypocotyl and root elongation of *Amaranthus* seedlings. The bioactive exudate substance was purified and identified as 4-deoxy-β-L-*threo*-hex-4-enopyranuranosyl-(1→2)-L-rhamnose (lepidimoide) by MS, NMR and polarimetric techniques. This was the first report that the exudates of cress seedlings have an ability to stimulate the hypocotyl elongation and inhibit root elongation of

neighbouring plant species, and one of the few examples of an oligosaccharide with allelochemical activity.

However, it was not known whether the cress root exudate contained the bioactive compound or if the cress seedlings released a polysaccharide into solution, which could then be digested by microorganisms and converted into bioactive lepidimoide. An experiment was therefore designed to study whether lepidimoide is exuded by cress or formed by the action of microbes. To achieve this objective I incubated cress seeds with *Amaranthus* seeds in both aseptic and non-aseptic conditions.

There was also a possibility that cress does not exhibit allelopathic effects but that the effect on *Amaranthus* seedlings is due to competition between the two species for available oxygen in the sealed Petri dishes. Therefore, I designed an experiment in which *Amaranthus* seeds were not incubated with cress seeds but in the exudate of cress seedlings.

Another possibility was that the effect on hypocotyl and root elongation was due to seed-treatment chemicals and not natural cress exudates. To answer that question, I incubated *Amaranthus* seeds in exudate of 'organic' cress seedlings. Although 'organic' seeds were free of synthetic chemicals, there was still a possibility that the seeds had been treated with natural hormones, auxin and/or gibberellins. Therefore, *Amaranthus* seeds were incubated with exudate of home grown cress seeds (free of both synthetic and natural exogenous chemicals).

1.11.2 Effect of cress seed-coat mucilage

Hasegawa *et al.* (1992) only tested the effect of 3-d old cress roots exudate but it appeared possible that the effect of mucilage from non-germinated imbibed cress seeds was greater. Therefore, I carried out an experiment to test the effect of mucilage from non-germinated but imbibed cress seeds on *Amaranthus* seedlings**.**

1.11.3 Physical properties of active principle

In order to identify some of the properties of the bioactive compound(s), I heated/boiled an aqueous solution of cress seed-coat mucilage for several hours to determine whether the bioactive compound(s) is/are heat labile or heat resistant. *Amaranthus* seeds were then incubated in the boiled mucilage.

To see whether the bioactive compound(s) are polar or non-polar, I partitioned the mucilage between ethyl acetate and water phases at various pH values. *Amaranthus* seeds were then incubated with compounds from the ethyl acetate or water phase and hypocotyl and root length were monitored.

1.11.4 Estimation of size of bioactive compound(s) in cress seed-coat mucilage

The bioactive compound from germinated cress seed exudate proposed by Hasegawa *et al.* (1992), Tanaka *et al.* (2002) and Hirose *et al.* (2004) is a disaccharide, but there is a possibility that cress seed-coat mucilage has bioactive compound(s), other than lepidimoide, with different molecular sizes. Thus, I separated the mucilage into ethanol-precipitated and non-precipitated fractions. The bioactive non-precipitated

fraction was further separated on a Bio-Gel P-10 column. The pooled fractions from the partially included volume were finally separated on Bio-Gel P-2 column. Each fraction was then tested for biological activity.

1.11.5 Preparation of active principle

Lepidimoide is a highly potent allelopathic compound that exhibits multiple functions in the growth and development of plants. It was extracted from mucilage of germinating cress in small quantities and needs to be produce in large quantities. Therefore, experiments were done in attempt to produce lepidimoide and lepidimoide- like compounds with biological activity in the laboratory chemically and/or microbially.

Kosemura and Yamamura (1993) prepared lepidimoide by a series of complex chemical reactions with low yield while Hirose *et al.* (2004) prepared lepidimoide from okra mucilage by a series of chemical reactions. Deng *et al.* (2006) prepared lepidimoide by methyl esterification and β-elimination of *Arabidopsis* mucilage. This latter method was the simplest to carry out in this laboratory so was followed with the only change being the use of commercially available potato RGI.

Tanaka *et al*. (2002) produced lepidimoide from okra mucilage by the action of endophytic fungus but their strain, which has the ability to secrete lyases, was not readily available. Therefore, the synthesis of lepidimoide was attempted by the action of lyases secreted by *Pichia pastoris* (strain 10108).

1.11.6 Long-term goals

The overall aim of the project, to be achieved through the above approaches, is to discover a potent allelopathic compound(s) that might be used as potential herbicides to help in sustainable agriculture.

2 Materials and methods

The chemicals used in different experiments were bought from Sigma-Aldrich (Poole, United Kingdom), BDH Analar Chemicals Limited (Poole, United Kingdom), Acros Organics (Geel, Belgium) and Fisher Chemicals (Fisher Scientific, Loughborough, United Kingdom).

Potato RG-I was purchased from Megazyme International Ireland Ltd. Bio-Gels were provided by Bio-Rad. TLC Silica gel 60 (plastic sheets 20 × 20 cm) were ordered from Merck (Darmstadt, Germany), and Petri plates were obtained from Sterlin Ltd. (Caerphilly, United Kingdom). *Pichia pastoris* strains were kindly provided by Fungal Genetic Stock Center, School of Biological Sciences, University of Missouri, USA.

2.1 Seed viability test

Cress (*Lepidium sativum*) seeds (Sutton Seeds, UK) were tested for viability by placing 20 seeds in duplicate in 9-cm Petri plates containing Whatman No.1 filter paper wetted with water. The Petri plates were then placed in an incubation room at 23°C.

2.2 Disinfection of cress seeds

A 10-ml aliquot of ethanol (70% v/v) or sodium hypochlorite (NaOCl) solution (containing 0.13% or 0.65% active chlorine) was added to 20 seeds in duplicate and shaken for 10, 30, 90, 270 min or 24 h with constant agitation. HCl (0.01 M) was

then added with shaking for 10 min to remove the traces of NaOCl and the seeds were washed thoroughly with sterile deionised water. They were then transferred into 9-cm sterile Petri plates for testing microbial colonies (see section 2.3). Seeds treated with sterile deionised water were used as a control. The experiment was carried out in a laminar flow hood (Bassaire, I S clean air) to avoid a non-sterile environment.

2.3 Sterility test

The seeds in 9-cm Petri plates were incubated for two days at 23°C, after which 2 ml of sterile nutrient broth (see section 2.4) was added in a laminar flow hood and the samples were further incubated at 23°C for two days. Any bacterial colony formation was recorded.

2.4 Preparation of nutrient broth

CM1 nutrient broth (13 g) was dissolved in 850 ml of water and the pH was adjusted to 7.0 with 0.1 M NaOH. De-ionised water was added to make the final volume to 1 litre and the medium was autoclaved.

2.5 Selection of the growth medium

The seeds were germinated on or in different media for determination of the best possible sterile environment and convenient collection of the root exudates. Seeds (20, 40 and 80 in number) were germinated in sterile sand (5 mm deep), on sterile filter paper, in shaking water (80 rpm) or in still water, all in 9-cm Petri plates. The seeds were incubated at 23°C for two days and were then checked for hypocotyl and root growth.

2.6 Collection and analysis of cress root exudate

2.6.1 Root exudate

The seeds were germinated in both aseptic and non-aseptic conditions. Forty seeds were disinfected with NaOCl solution (containing 0.13% active chlorine) for 30 min and were further treated as described in panel 2.2. The seeds were then placed in 9-cm sterile Petri plates containing 15 ml of water and incubated with shaking (3 d, 23°C and 80 rpm). The exudates were collected, dried and assayed for carbohydrates. The experiment was conducted in triplicate.

The above protocol was later scaled up to 1000 seeds in a litre of water with the seeds being spread out on stainless steel gauze. The steel gauze was then placed in a 10 × 4 cm tray containing 1 litre of water in order to submerge the seeds. Aseptic air as a source of oxygen was bubbled into the water via sterile tubing from a pressure pump. The collected cress root exudates after 3 d were lyophilised and re-dissolved at 0.5% w/v in sterile deionised water.

2.6.2 Cress seed-coat mucilage

Cress seeds (20 g) were imbibed in water (1 l) at 4°C for 24 h. The seed-coat mucilage obtained upon rocking was then centrifuged at 6800 rpm for 30 min in a Sorvall Evolution Ultra centrifuge and the supernatant was freeze dried. The dried mucilage was re-dissolved in sterile de-ionised water at 0.5% (w/v) and kept frozen at -80°C to avoid any microbial contamination. Diverse qualitative and quantative assays and bioassays were carried out on aliquots of this material.

2.7 Assays for carbohydrates

2.7.1 Total carbohydrate assay, soluble + insoluble

To 0.4 ml of an aqueous sample containing 2 – 15 µg carbohydrate, phenol (80% w/w; 10 µl) was added, followed by 98% concentrated H_2SO_4 (1 ml). The solution was vortexed and allowed to stand at room temperature for 10 min. The sample was cooled in a water bath for 10 min and the absorbance was read (485 nm) with a spectrophotometer (CECIL, series 8000). Standards that contained known amounts of dextran (1 – 20 µg) were used to make a calibration curve (Dubois *et al*., 1956).

2.7.2 Hexoses, free and polymer-bound

To 0.5 ml of an aqueous solution containing 5–50 µg hexose, an anthrone solution (0.2% w/v anthrone in conc. H_2SO_4; 1 ml) was added. The tubes were mixed and incubated in a boiling water bath (5 minutes). The tube was then cooled and the absorbance was read (620 nm) with the spectrophotometer. Standards that contained known amounts of glucose were used to make a calibration curve (Dische, 1962).

2.7.3 Pentoses, free and polymer-bound

To 0.5 ml of the aqueous solution (containing 1–10 µg pentose), an orcinol solution (6% w/v orcinol in EtOH; 67 µl) was added followed by an iron chloride solution ($FeCl_3.6H_2O$ in conc. HCl, 0.1% w/v; 1 ml). The tubes were mixed and incubated in a boiling water bath (20 min). The tubes were then cooled, mixed and the absorbance was read (665 nm) with the spectrophotometer. Standards that contained known amounts of xylose were used to make a calibration curve (Dische, 1962).

2.7.4 Uronic acids, free and polymer-bound

A solution of borax ($Na_2B_4O_7.10H_2O$, 0.5% w/v; 1 ml) in 98% concentrated H_2SO_4 was added to a 0.2 ml solution/suspension of the sample that contained 1 – 20 µg of uronic acid. The solution was incubated in a boiling water bath (5 min), cooled and the absorbance (520 nm) was read on the spectrophotometer. *m*-Hydroxybiphenyl (0.15% w/v; 20 µl) in NaOH (1 M) was added to the solution, incubated at room temperature (5 min) and the absorbance (520 nm) was read. The difference between the two readings indicated the uronic acid content. A calibration curve produced with galacturonic acid standards was used (Blumenkrantz and Asboe-Hansen, 1973).

2.7.5 Unsaturated uronic acids

To 0.5 ml of aqueous solution (containing the mucilage sample or authentic malondialdehyde (MDA); 5 – 20 µM), a thiobarbituric acid (TBA) solution (0.04 M TBA, pH adjusted to 2 with NaOH; 0.3 ml) was added followed by HCl (0.075 M; 0.2 ml) and dH_2O (50 µl). The tubes were mixed, incubated in a boiling water bath (30 min), cooled in ice for 5 – 10 min and the absorbance was read (550 nm) on the spectrophotometer (Payasi and Sanwal, 2003).

2.8 Separation of the compounds present in the cress root exudates and seed-coat mucilage

2.8.1 Gel-permeation chromatography

Sample (7.5 ml; 0.25% w/v) was applied to a Bio-Gel P-2 column (1.5 × 85 cm) (Yamashita *et al.,* 1982), previously calibrated with dextran (to indicate void volume) and tritiated water (^3H_2O) (included volume). Each fraction (2.4 ml) was

collected in an 18×100 mm culture tube by drop-counting with the help of a fraction collector. Initially, 80 fractions were collected and were assayed for carbohydrates while later on the number of fractions was reduced to 40. The column was further calibrated with a number of markers (GalA, $GalA_2$ and $GalA_3$).

2.8.2 Thin-layer chromatography (TLC)

TLC (Stahl, 1969) was carried out on pre-coated silica gel plastic sheets (20×20 cm, silica gel 60, Merck, Darmstadt, Germany). The samples were loaded about 2 cm above the bottom of the plate and dried with a hair drier. The plate was then placed in a clean glass tank containing 90 ml of the solvent. The tank was covered with a lid to prevent air currents. Solvent systems used were butanol:acetic acid:water (BAW, 2:1:1 v/v) or butanol:propanol:acetic acid:water (BPWA, 7:5:4:2 v/v) (Tanaka *et al*., 2002). For BAW the plate was removed when the solvent front was approximately 2–3 cm from the top of the plate while for BPWA the plate was subject to two developments of 12 cm.

2.8.3 Descending paper chromatography

Samples were dispensed onto Whatman No.1 paper. The paper was placed into a glass chromatography tank and hung from a trough situated at the top of the tank that contained the solvent (butanol/acetic acid/water (BAW) 12:3:5 or ethyl acetate/pyridine/water (EPW, 8:2:1)). The tank was then sealed with a glass lid and the chromatogram removed after the appropriate time (Hais and Macek, 1963).

2.8.4 Paper electrophoresis

Samples were loaded onto Whatman No.1 paper. The paper was then wetted with either pH 2 (FAW, 1:35:355 by vol.), pH 3.5 (PyAW, 1:10:189 by vol.) or pH 6.5

(PyAW, 33:1:300 by vol.) buffer. It was then placed in a glass tank and hung from a trough situated at the top of the tank that contained the buffer. The bottom of the tank contained the same buffer to dip the opposite end of the paper. The tank was filled with white spirit (pH 2 and pH 3.5 buffer tanks) or toluene (pH 6.5 buffer tank) as an immiscible coolant. A voltage of 2.5 kV was then applied through the buffer for a period of half an hour in order to separate the compounds of interest. Orange G and methyl green was used as an internal visible marker (Smith, 1960).

2.9 Stains

2.9.1 Thymol stain (general purpose stain)

Thymol (0.5 g) was dissolved in 95 ml of ethanol. Concentrated H_2SO_4 (98%, 5 ml) was added slowly to the solution. TLC plates were dipped into the stain, taken out quickly and placed on aluminium foil for drying in a fume hood. After drying the plate was placed in an oven at 110°C for 10 minutes to stain (Stahl, 1969).

2.9.2 p-Anisaldehyde stain (for acidic sugars)

To 135 ml of ethanol, 98% concentrated sulphuric acid (5 ml) was added, followed by acetic acid (1.5 ml) and *p*-anisaldehyde (3.7 ml). The solution was then stirred vigorously. TLC plates were dipped into the stain and completely dried in a fume hood. The plates were then incubated in an oven for 3 min at 130°C; acidic sugars stain orange-brown while neutral sugars stain green (Stahl, 1969).

2.9.3 Silver nitrate stain (for monosaccharides and oligosaccharides)

Silver nitrate stain was used to visualize the sugars (Trevelyan *et al.,* 1950). Silver nitrate staining requires three solutions.

Solution A: This was prepared by the addition of 2 ml of saturated aqueous silver nitrate to 260 ml of acetone. The solution was mixed thoroughly. The precipitate formed was dissolved by the drop-wise addition of water with mixing till the solution became clear.

Solution B: This was prepared by the addition of 12.5 ml 10 M NaOH to 987.5 ml of ethanol to make the final vol. of 1 litre.

Solution C: 10% (w/v) solution of $Na_2S_2O_3$ in water.

The paper from the electrophoresis tank was hung overnight in a fume hood to dry. It was then pulled through solution A and was left to dry for 15 minutes. The paper was then pulled through solution B twice with an interval of 15 minutes between each dip. It was finally pulled through solution C and was placed in a tank of water for an hour. The paper was then dried and examined for the compounds stained.

2.9.4 Aniline hydrogen-phthalate stain (for monosaccharides and reducing sugars)

To a solution containing acetone (245 ml), diethyl ether (245 ml), water (10 ml) and 8 g of phthalic acid was added aniline (0.5% (v/v)). The paper was dipped through this solution, dried and placed in an oven (105°C, 5 min) (Fry 2000; adapted from Partridge, 1949).

2.10 Determination of $^{3}H_2O$ by scintillation counting

Radioactivity was assayed in a Beckam LS 6500 multipurpose scintillation counter. Aqueous samples were assayed with a 10:1 (v/v) ratio of "OptiPhase HiSafe" scintillation fluid to aqueous sample. The samples were analysed twice to avoid any false reading by the counter.

2.11 Autoradiography

^{14}C-Labelled GalA on a paper electrophoretogram was detected by autoradiography (Yeung, 1984) on Kodak BioMax MR-1 film. The film was placed in the dark for 3 d exposure.

2.12 Bioassays

2.12.1 Bioassays in big Petri plates (9 cm)

2.12.1a Growing cress with other species

Twenty seeds of love-lies-bleeding (*Amaranthus caudatus*) and 20 seeds of cress (*Lepidium sativum*) were randomly placed in a 9-cm Petri plate (containing 15 ml of aseptic deionised water) in aseptic conditions. Forty seeds of *A. caudatus* alone were used as a control. The seeds were incubated for 5 d at 23°C in the dark. After 5 d the seedlings were dried with paper towels and were placed on cardboard. A sheet of glass was positioned on top of the seedlings carefully to avoid any damage to them. Images were taken with a Doc-It, Bioimaging System (Ultra Violet Products Ltd.). The length of hypocotyl and root was measured by use of LabWorks software. Similar experiments were repeated for lettuce (*Lactuca sativa*).

LabWorks is a program used to eliminate the common, time-consuming steps connected with manual measurement of images in biological laboratories. A variety of functions such as drawing a line along a straight or curved path helps in precise measurements of hypocotyls and root lengths in bioassay experiments.

2.12.1b Growing *Amaranthus caudatus* in cress exudates

Forty seeds of cress were incubated in a 9-cm Petri plate containing 15 ml of sterile water for 5 d in the dark at 23°C. The seedlings were then removed and the exudates were left in the Petri plate. *Amaranthus caudatus* (20 seeds) were placed in the collected cress exudates and were incubated for next 5 d in the dark at 23°C. The seedlings were removed, dried with towel paper and the lengths of hypocotyl and root were measured.

In another experiment, 40 commercially available non-organic cress seeds, 'organic' (free of sprayed pesticides or fungicides) cress seeds and cress seeds collected from private garden in Edinburgh (free of natural and synthetic sprayed with, inhibiting and stimulating agents) were imbibed in a separate 9-cm Petri plate containing 15 ml of sterile water in each plate for 24 h at 4°C to avoid any contamination from micro-organisms. The seeds were then removed in a laminar flow hood and 20 seeds of *Amaranthus caudatus* were placed in the same water (containing cress seed-coat mucilage) and incubated for 5 d at 25°C in dark. Lengths of hypocotyl and root of *Amaranthus caudatus* were measured the same way as mentioned earlier.

2.12.2 Bioassays in small Petri plates

The bioassay experiments were later on carried out in triplicate in deep 5-cm Petri plates in order to apply small volumes of samples. Paper disks (4.8 cm in diameter) were cut out of Whatman No.1 paper and two disks were placed in each Petri plate. *Amaranthus caudatus* (10 seeds) were incubated in 850 µl of the solution being assayed for bioactivity for 5 d in the dark at 23°C. The lengths of hypocotyl and root were then measured. Seeds incubated in water only were used as controls.

2.13 Solvent phase partitioning of cress seed-coat mucilage

The pH of three 3-ml aliquots of seed-coat mucilage solution was adjusted to pH 2 with TFA, pH 12 with sodium hydroxide (NaOH) or left unadjusted at pH ~6.5 (Figure 2.1). Ethyl acetate (EtOAc) (3 ml) was added to each tube, followed by vortexing. The tubes were left to stand for 30 min and the two phases were separated, dried, re-dissolved in 2 ml of water and lyophilised. The lyophilised samples were re-dissolved in 3 ml of sterile de-ionised water and utilised in bioassays as described in section 2.12.2.

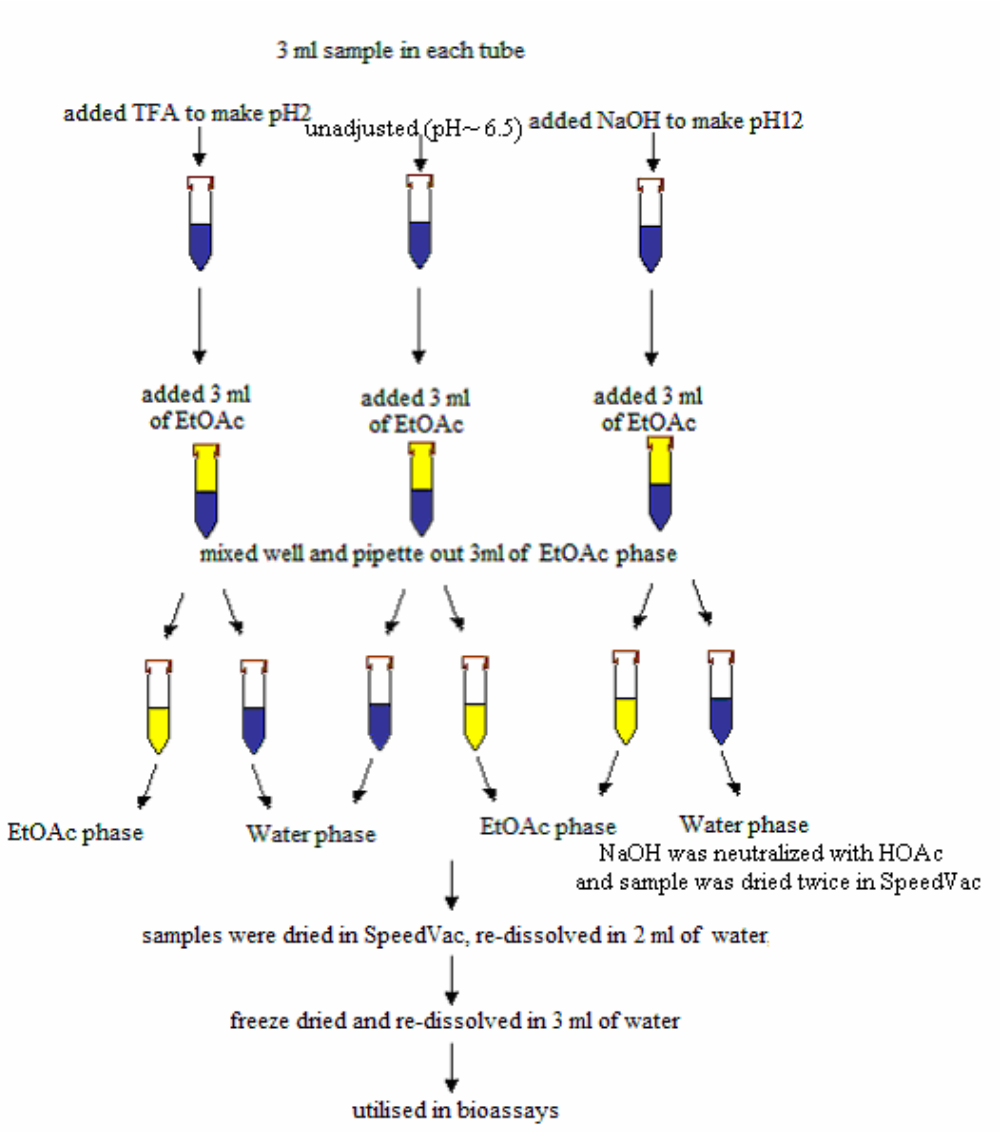


Figure 2.1: Separation of active principle between polar and non-polar phase

2.14 Heat stability of active principle in seed-coat mucilage

A seed-coat mucilage solution (0.5%, w/v) was incubated in tightly capped Sarstedt tubes in an oven (110°C) for 1, 2, 4, 8, 16, 32 or 48 h. Samples were then cooled in an ice-bath and used in bioassay as described earlier. A control containing water was treated in the same way as samples.

2.15 Ethanol fractionation of cress seed-coat mucilage

To 3 ml of 0.5% (w/v) cress seed-coat mucilage, ethanol (final conc. 75%, v/v) was added. The sample was mixed thoroughly and left to stand for an hour at room temperature. The precipitate was separated by centrifugation (4000 rpm, 20 min). The precipitated fraction was washed three times with 75% ethanol and centrifuged (4000 rpm, 20 min). Both supernatant and precipitate were twice re-dissolved in de-ionised water (1 ml) and dried at 50°C in SpeedVac and finally freeze dried to remove any traces of ethanol. The dried samples were then re-dissolved in sterile de-ionised water (3 ml) and utilised in bioassays as mentioned in section 2.12.2.

2.16 Attempt to synthesise lepidimoide and related oligosaccharides

2.16.1 Methyl esterification and beta-elimination of RG-I

Potato RG-I (Megazyme International Ireland Ltd, Co. Wicklow, Ireland) was found to be contaminated with oligosaccharides and was therefore purified on a Bio-Gel P-10 column. The void volume fractions were pooled while the included volume fractions were discarded. The purified RG-I (20 mg) was dissolved in 0.32 ml of water in a 5-ml capped tube and was then mixed with a solution containing tetrabutyl

ammonium fluoride (40 mg), 4 ml of dimethyl sulphoxide (DMSO) and 20 µl of methyl iodide (MeI). The mixture was stirred at room temperature for 16 – 18 h. The reaction mixture was then poured into ice-cold water (12 ml) and centrifuged (4000 rpm). The supernatant was collected and dialysed (12-kDa MWCO tubing, Medicell International Ltd, 239 Liverpool Road, London) against deionised water for 48 h. The solution was lyophilised and the methylesterified RG-I was re-dissolved in PyAW (1:1:98 v/v), purified on a Bio-Gel P-10 column as void volume fractions and freeze dried.

The product (10 mg) was dissolved in aqueous sodium borate (0.2 M; 5 ml; pH adjusted to 7.3 with boric acid) and heated (125°C, 150 min) in a 5-ml tube. The methylesterified RG-I solution containing β-eliminated compounds was then cooled, dried, re-dissolved in PyAW (1:1:98 v/v) and fractioned through a Bio-Gel P-2 column (Chenghua *et al*., 2005).

2.16.2 Treatment of potato RG-I with lyases from transgenic Pichia pastoris

Pichia pastoris clone 10108 (strains kindly provided by Fungal Genetic Stock Center, School of Biological Sciences, University of Missouri, USA) was grown in 50 ml of buffered glycerol-complex medium (BMGY) in a 250-ml conical flask at 28°C with constant shaking for 48 h. Minimum methanol (MM) medium (50 ml) was then added and incubated for the next three days (Bauer *et al.,* 2006). Each day an additional 0.3 ml of MeOH was added. The suspension was centrifuged and the supernatant was dialysed against deionised water for 48 h at 4°C. The dialysed supernatant (containing lyase) was freeze dried and then re-dissolved in 1 ml of Tris buffer (50 mM, pH 7.5) (Bauer *et al.,* 2006).

Purified potato RG-I (0.1 ml, 0.5% w/v) was added to 0.1 ml of enzyme and incubated at 30°C for up to 48 h. The reaction was terminated at 1, 2, 4, 8, 16, 24 and 48 h by placing the sample mixture in boiling water for 5 min. The compounds produced were then tested quantitatively and qualitatively for carbohydrates (as described in previous sections) and utilised in bioassays as mentioned in panel 2.12.2.

3 Results

3.1 Selection of disinfectant

Cress seeds were disinfected before incubation in order to make them free of microbes, which may produce enzymes to digest the polysaccharides in cress exudate. In a preliminary experiment cress seeds were treated with NaOCl solution (containing 0.13% or 0.65% active chlorine) or 70% (v/v) ethanol for various times (10, 30, 90, 270 minutes and 24 h). Seeds treated with NaOCl solution (containing 0.13% active chlorine) for 30 minutes showed the best result in terms of maximum germination and minimum microbial growth after the treatment. Treatment with 70% ethanol for 30 minutes had no effect on microbial growth, while germination was stopped when seeds were treated for a longer time. However, 90 minutes of treatment with 70% ethanol disinfected the seeds. Cress seeds disinfected with NaOCl solution (containing 0.65% active chlorine) even for a short time (10 minutes) germinated well without any infection but the seedlings were bleached. Therefore, I decided to disinfect seeds with NaOCl solution (containing 0.13% active chlorine) for 30 minutes for future experiments.

3.2 Selection of the germination and growth medium for cress

This experiment was designed to select a medium for the best possible seedling growth and convenient collection of exudates of disinfected cress seeds. Twenty, forty and eighty disinfected cress seeds were incubated in or on different sterile media (wet sand, wet filter paper, still water, shaking water) at 25ºC for 3 d. Seeds

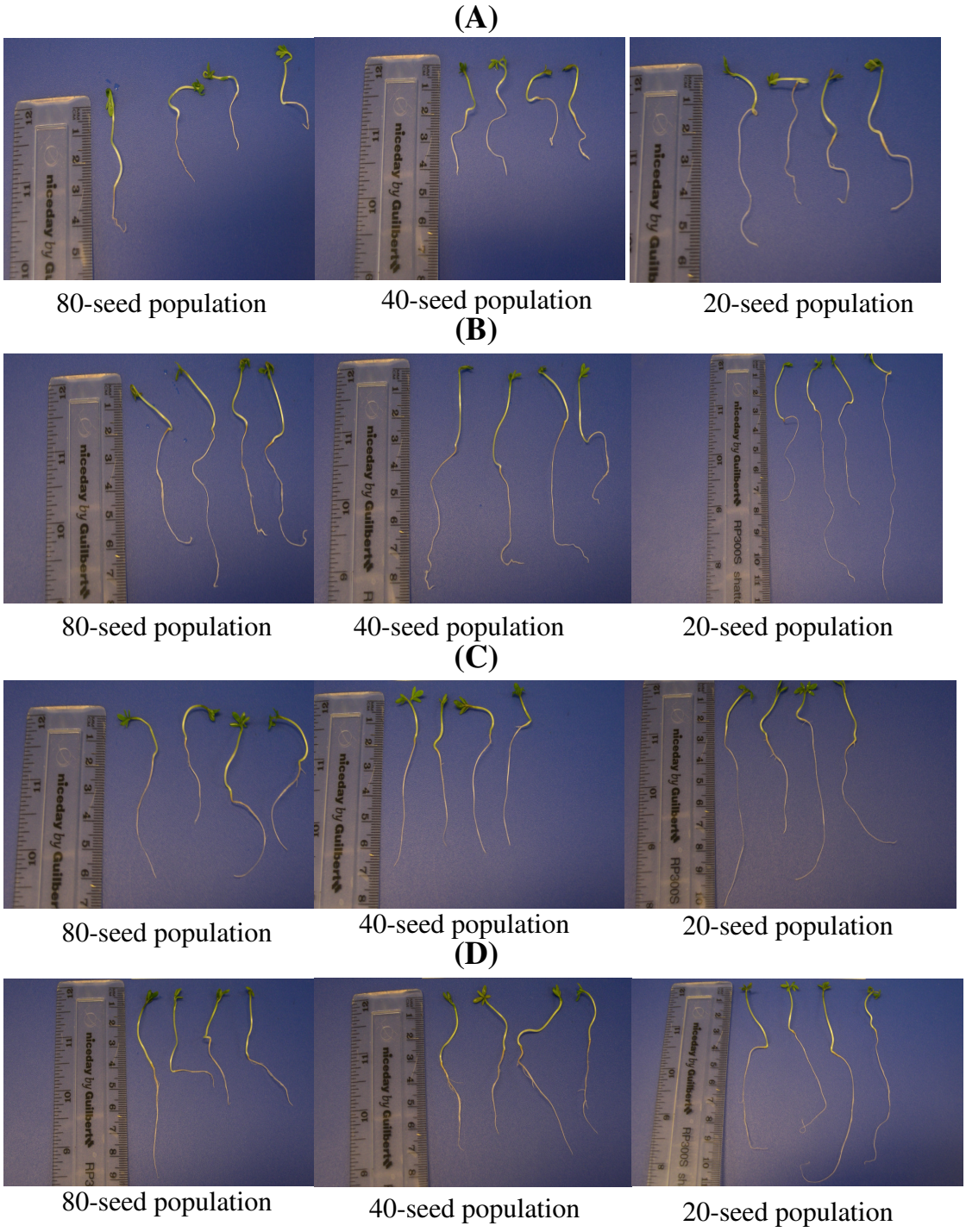


Figure 3.1: Representative seedlings from a population of 80, 40 and 20
Cress seeds were incubated on or in various media at 25ºC for 3 d in dark. (A) Wet sand, (B) wet filter paper, (C) still water, (D) shaking water.

germinated successfully in all media and with different seed numbers (20, 40, and 80) (Fig. 3.1). The length of the seedlings from the 80-seed population in all four

media was shorter than from 20-seed and 40-seed populations, presumably because of the dense population.

3.3 Evidence of cress bioactivity

To see the effect of cress exudates on germination and development of other species, cress seeds were grown with other species in the same Petri plate. In this bioassay 20 seeds of either *Amaranthus caudatus* or *Lactuca sativa* were incubated with 20 seeds of *Lepidium sativum*. As a control 40 seeds of each species were incubated without cress seeds. The seedlings of both species that were incubated with *Lepidium sativum* had longer hypocotyls and shorter roots than the control (Fig. 3.2).

The results were repeated for the sake of statistical analysis. Dishes of seeds were incubated under the same conditions in triplicate. The hypocotyls and roots of the seedlings incubated with cress were found significantly longer and shorter, respectively, than those incubated without cress (Fig. 3.3).

3.4 Effect of aseptic and non-aseptic conditions

I designed this experiment to determine whether the bioactivity was due to the cress itself or a breakdown product formed by the action of microbes. Cress plus *Amaranthus* seeds were incubated in both aseptic and non-aseptic conditions; *Amaranthus* seeds were incubated alone as a control. After 5 d of incubation in dark at 25ºC, *Amaranthus* seedlings from both aseptic and non-aseptic treatments had longer and thinner hypocotyls and shorter and thicker roots when grown with cress than when grown alone (Fig. 3.4). The bioassay showed that the cress might exudate

allelopathic compounds or might compete with *Amaranthus* for dissolved oxygen in the medium.

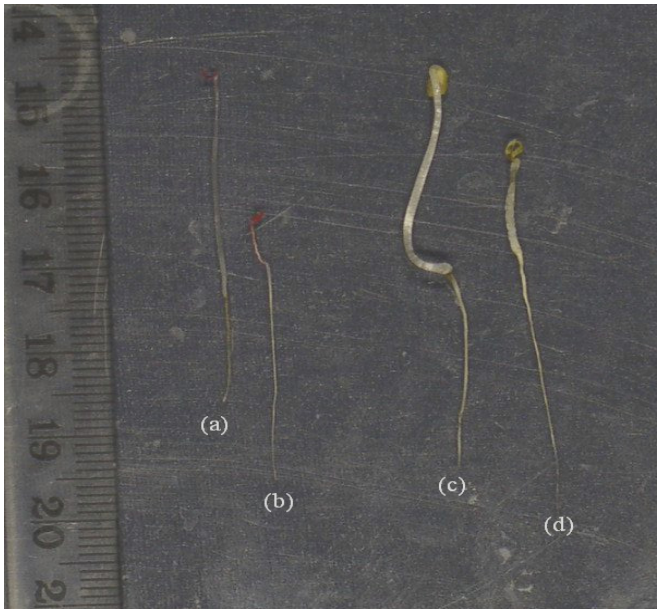


Figure 3.2: Interaction of cress seedlings with *Amaranthus* and lettuce seedlings

(a) *Amaranthus* incubated with cress seeds, (b) *Amaranthus* alone as a control, (c) lettuce incubated with cress, (d) lettuce alone as a control. All seeds were incubated for 5 d at 25ºC in dark. The above seedlings are representative of their respective populations.

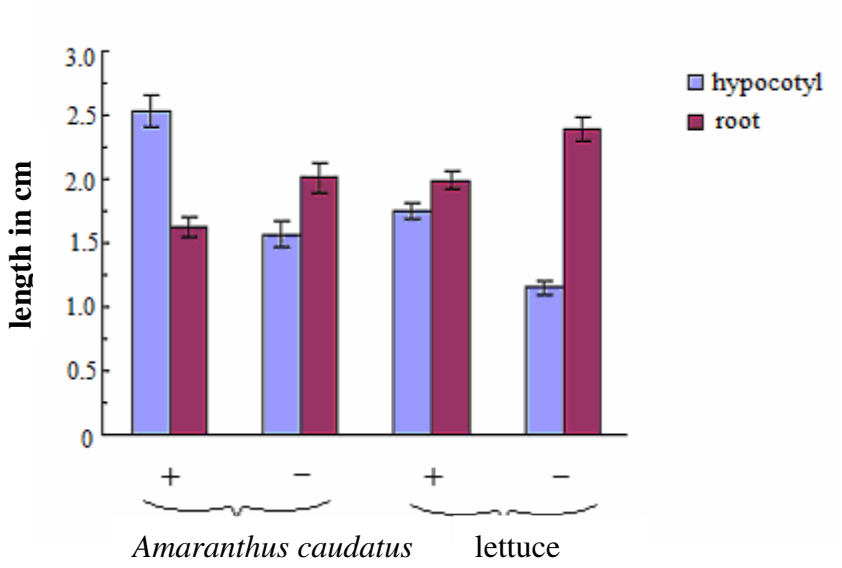
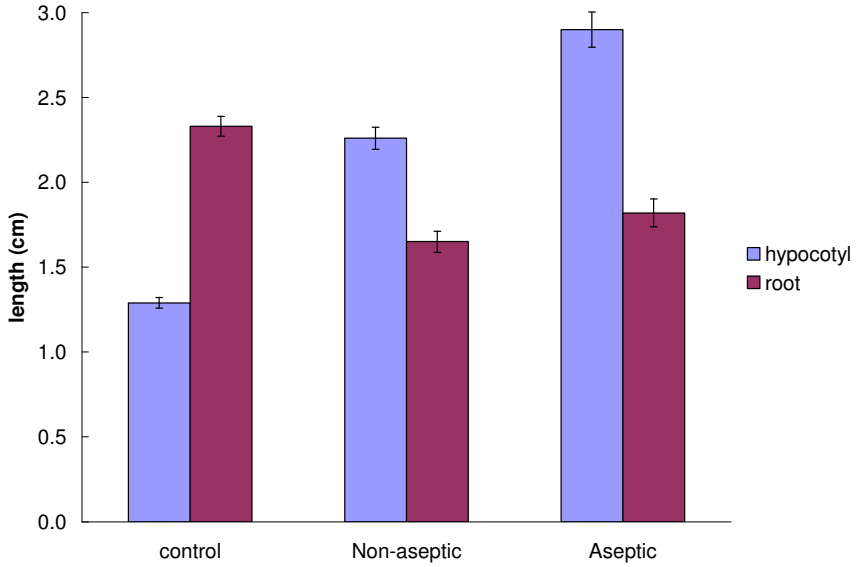
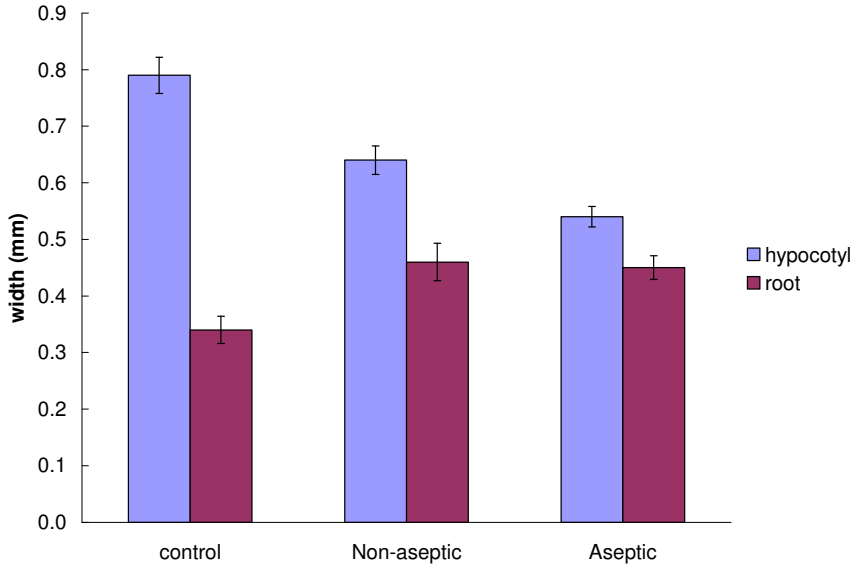


Figure 3.3: Effect of cress on Amaranthus and lettuce

+ = grown with cress, − = grown without cress. All seeds were incubated for 5 d at 25ºC in dark. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.



(a)



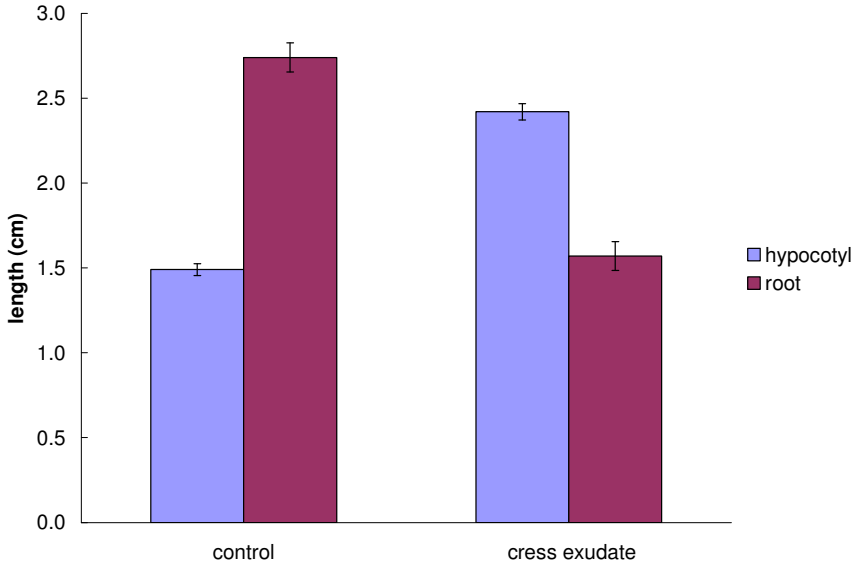
(b)

Figure 3.4: Growth of Amaranthus incubated with cress in aseptic and non-aseptic conditions

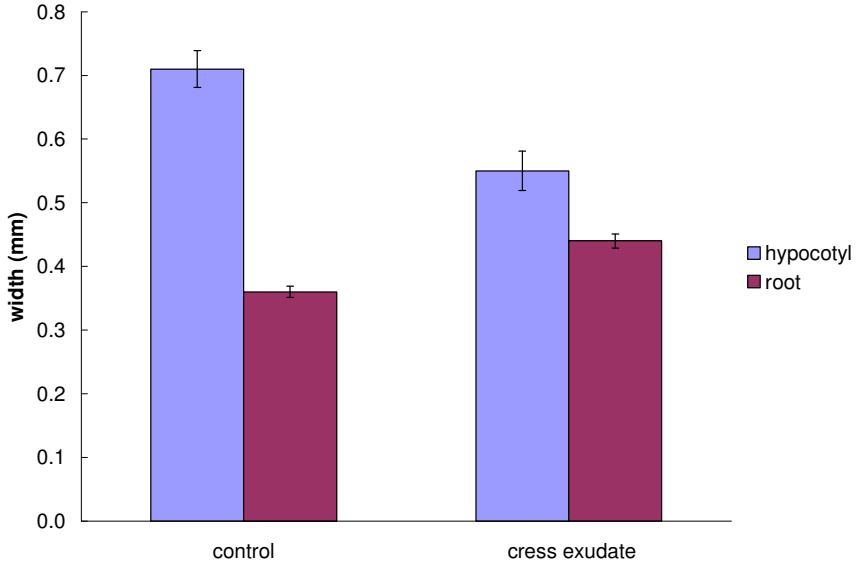
Amaranthus seeds were incubated with cress in aseptic and non-aseptic conditions for 5 d at 25ºC in the dark in 9-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. The length and width of hypocotyls and roots were measured by use of Labwork software. (a) Length; (b) width. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

3.5 Effect on *Amaranthus* seedlings: due to competition or cress exudates?

To study whether the effect of cress seedlings on growth of *Amaranthus* seedlings was due to competition or was an effect of cress exudate, I grew 20 *Amaranthus* seedlings in the exudate of 20 cress seedlings after removal of the cress seedlings. After 5 d of incubation, the length and width of *Amaranthus* hypocotyls and roots were measured. *Amaranthus* seedlings incubated with cress exudates had longer and thinner hypocotyls and shorter and thicker roots even though no living cress tissue was present, which meant that the effect of cress on other species is not due to competition but an effect of the cress exudates (Fig. 3.5).



(a)



(b)

Figure 3.5: Effect of cress exudate on *Amaranthus* seedlings in the absence of competition

Amaranthus seeds were incubated in collected cress exudates for 5 d at 25ºC in the dark in 9-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. The length and width of hypocotyls and roots were measured by use of Labwork software. (a) Length; (b) width. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

3.6 Quantitative tests for carbohydrates in cress root exudate

To test for the presence of (possibly biologically active) oligosaccharides in cress root exudate, I incubated one thousand cress seeds in sterile de-ionised water at 25ºC in the dark for 3 days and assayed the collected root exudate by various carbohydrate tests (Table 3.1). The results indicated quite high amount of sugars, sufficient to be used in various experiments. Unsaturated uronic acid was also found in the cress exudate, which might be an important residue of the active principle.

Table 3.1: Amount of carbohydrates present cress exudate
Cress seeds (1000) were placed on a stainless steel mesh and incubated aseptically for 3 days at 25ºC in dark. The exudates were analysed for carbohydrates.

Total CHO (µg/ seedling)	Pentoses (µg/ seedling)	Hexoses (µg/ seedling)	Uronic acids (µg/ seedling)	Unsaturated uronic acids (µg/ seedling)
120	79.5	45.6	43.2	0.098

* The average dry mass of seed was 2.7 mg that exudates 0.2 mg of soluble mucilage after three days of incubation in sterile de-ionised water.

3.7 Qualitative tests for carbohydrates in cress root exudate

3.7.1 Thin-layer chromatography

The quantitative results showed the presence of various classes of carbohydrates. The next step was to separate qualitatively the carbohydrates present in the sample of 1000-seed exudate. The sample along with a range of standards was fractioned by TLC.

Six migrating bands were seen, while polysaccharide stayed at the origin (Fig. 3.6). One of the bands, which ran between galactose and galacturonic acid, may have been lepidimoide in comparison to the results of Tanaka *et al.* (2002).

TLC produced in present work TLC by Tanaka *et al.* (2002)

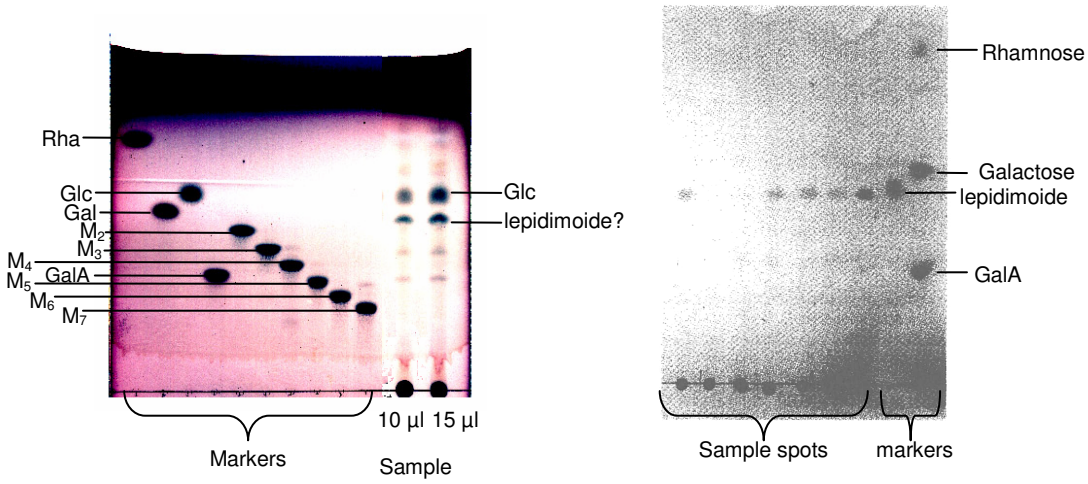


Figure 3.6: Separation of cress exudate by TLC

Rha = rhamnose, Gal = galactose, Glc = glucose, GalA = galacturonic acid, M_2 = maltose, M_3 = maltotriose, M_4 = maltotetraose, M_5 = maltopentaose, M_6 = maltohexaose, M_7 = maltoheptaose. The solvent system used was BPWA (7:5:4:2, double development of 12 cm). The plate was stained with *p*-anisaldehyde. The right hand image was copied from the Tanaka *et al.* (2002). The last two spots of Tanaka *et al*. (2002), consist of authentic lepidimoide, GalA, Gal and Rha. Lepidimoide migrated faster than GalA but slower than Gal. The sample spots were the digestion products from okra pectic polysaccharide by various strains of endophytic fungus.

3.7.2 Electrophoresis

The exudate from cress seedlings was loaded in the middle of a paper before electrophoresis to determine whether the carbohydrates were acidic, basic or neutral. Both external and internal markers were used to characterise the mobility of the unknown compounds. The $AgNO_3$-stained compounds present in the exudate were acidic and neutral at pH 3.5; in addition white-'staining' spots migrated towards the cathode, which might be a non-carbohydrate cation (Fig. 3.7). Although one of the compounds in the exudate approximately co-migrated with GalA, this was not enough to demonstrate that it was GalA.

Since no cationic carbohydrates were present, the sample was loaded near the cathode end of the paper and electrophoresed for a longer time at the same pH. The anionic compound in the cress exudate that approximately co-migrated with GalA in the previous electrophoretogram ran faster than GalA when electrophoresed for a longer time (Fig. 3.8). The sample compound migrated close to GlcA. To determine if the cress compound was GlcA, a trace of [^{14}C]GlcA was added to the sample. The exudate compound migrated more slowly than the internal [^{14}C]GlcA (Fig. 3.9) and therefore was not GlcA. It is concluded to be an acidic sugar (possibly lepidimoide?) other than GalA and GlcA.

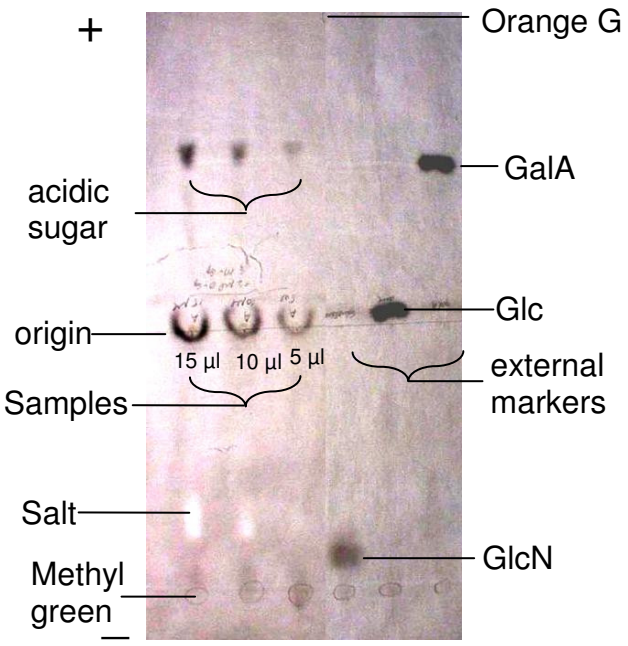


Figure 3.7: Paper electrophoresis for the separation of neutral, acidic and basic sugars present in cress exudate

Cress root exudate samples were loaded in the middle of Whatman No.1 paper and subjected to electrophoresis (pH 3.5, 3 kV, 30 min.). Orange G and methyl green were used as internal markers. The external markers and separated compounds were stained with silver nitrate.

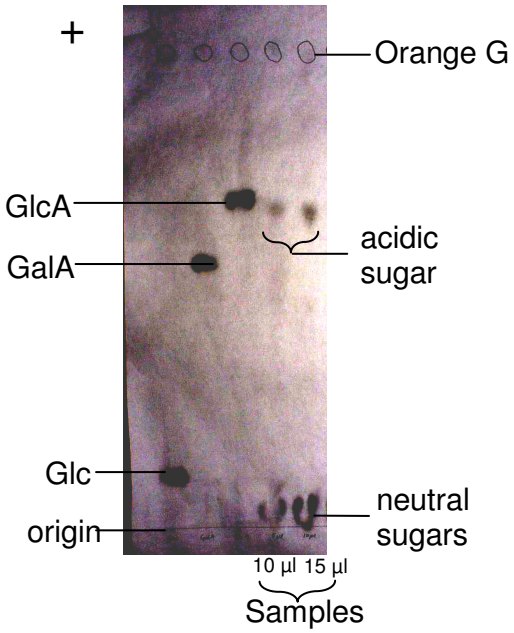


Figure 3.8: Further characterisation of the acidic sugar by electrophoresis

Glc, GalA and GlcA were added as external markers. The samples (cress root exudate) were loaded near the bottom of Whatman No.1 paper and subjected to electrophoresis (pH 3.5, 3 kV, 1 h). Orange G was used as internal marker. The external markers and separated compounds were stained with silver nitrate.

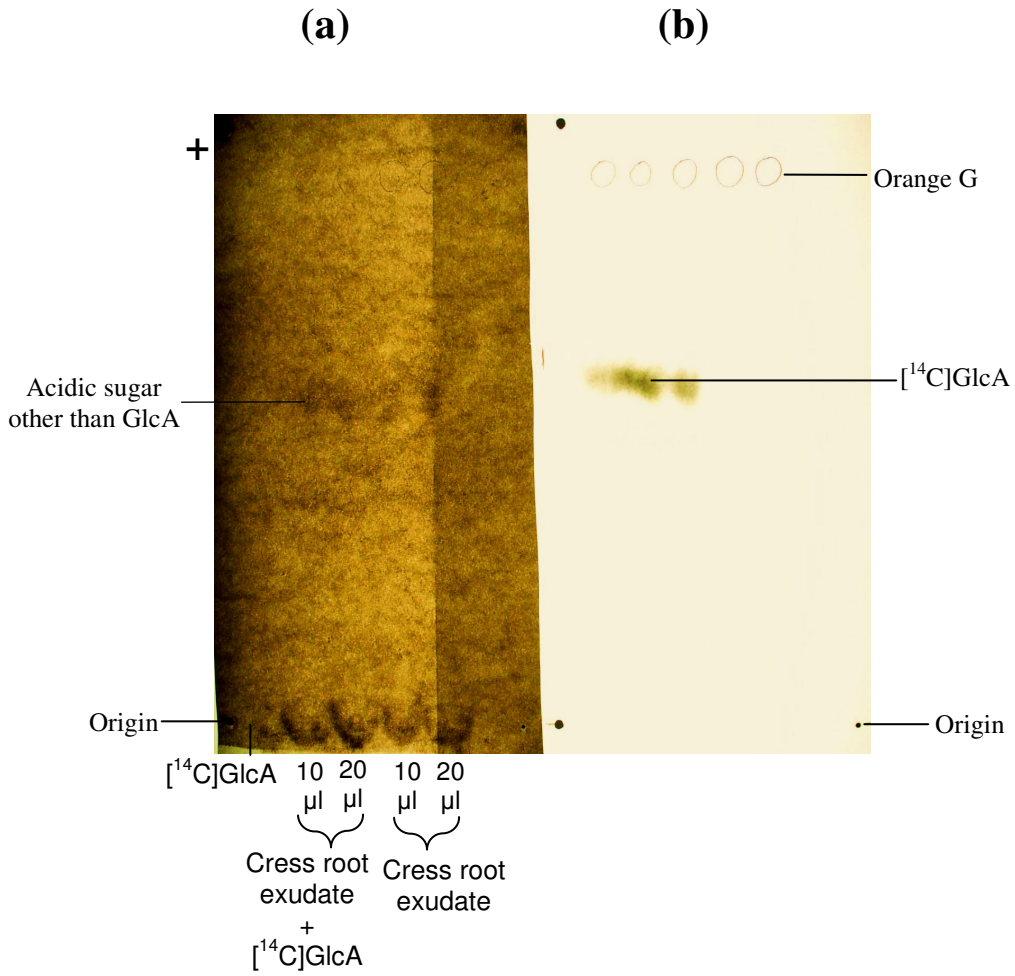


Figure 3.9: Autoradiogram developed from electrophoretogram for the comparison of exudate spot with [^{14}C]GlcA

(a) = Electrophoretogram after staining with silver nitrate, (b) = autoradiogram developed from the electrophoretogram before staining. Cress root exudate mixed with or without [^{14}C]GlcA and loaded on Whatman No.1 paper and electrophoresed (pH 3.5, 3 kV, 1 h). Orange G was used as internal marker. The autoradiogram (b) was developed from the un-stained electrophoretogram (a). Electrophoretogram (a) after staining with silver nitrate and autoradiogram (b) was then placed side by side for the comparison of unstainable [^{14}C]GlcA and stainable acidic sugar. The black spots are radioactive ink used to place the electrophoretogram equally with the autoradiogram.

3.8 Fractionation of cress root exudate on Bio-Gel P-2

Cress root exudate that contained an internal marker of ^3H_2O was loaded onto a Bio-Gel P-2 to obtain an estimate of the molecular weight of compounds present in cress exudate. Compound containing unsaturated uronic acid residues eluted in fractions 16-24 with a peak at k_{av}=0.4 (Fig. 3.10). To determine if the ΔUA-containing compound of interest was likely to be an oligosaccharide, the column was then calibrated with dextran, GalA, $GalA_2$, $GalA_3$, and ^3H_2O (Fig. 3.11). As $GalA_3$, $GalA_2$ and GalA were not completely resolved by this method, I used TLC to determine accurately where those compounds eluted. TLC showed that $GalA_3$, $GalA_2$ and GalA eluted with k_{av} = 0.7, 0.6 and 0.4, respectively (Fig. 3.12).

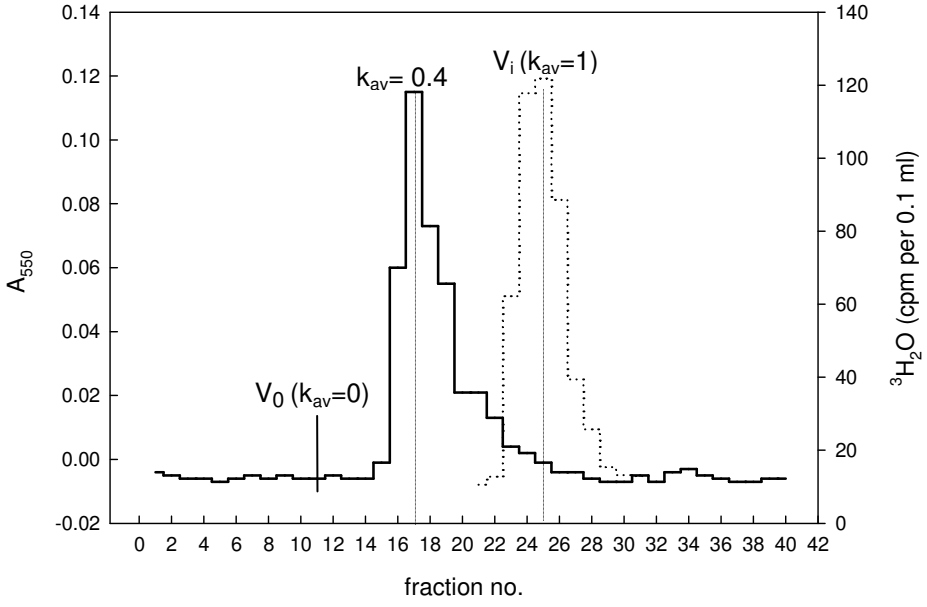


Figure 3.10: Fractionation of compounds in cress root exudate on Bio-Gel P-2
V_o indicates where polysaccharide eluted (tested by phenol/sulphuric acid test) and the V_i indicates where ^3H_2O eluted (assayed by scintillation counter). TBA test was done for the analysis of ΔUA-containing carbohydrates.

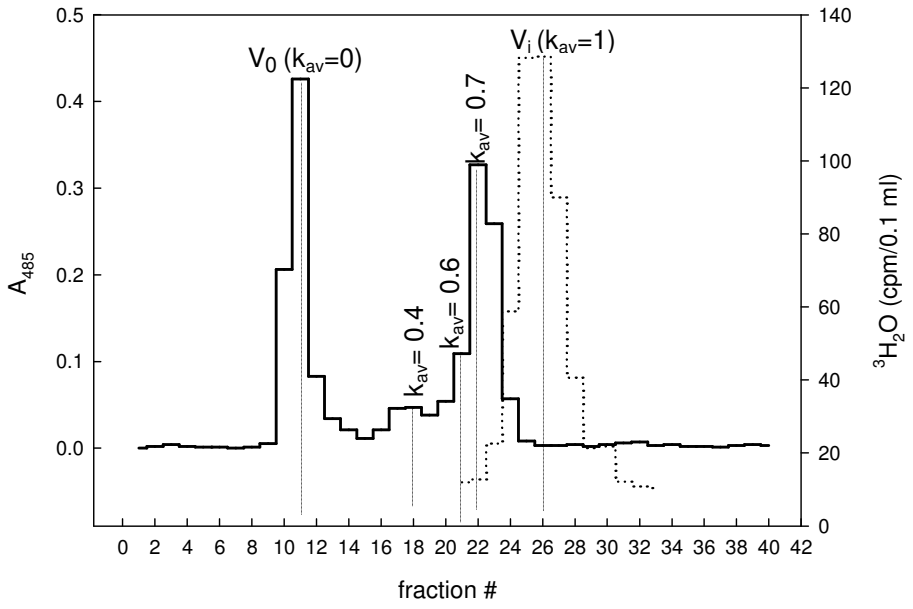


Figure 3.11: Calibration of Bio-Gel P-2 column with markers
V_o indicates dextran with k_{av}=0 and V_i indicates ^3H_2O with k_{av} =1, assayed by scintillation counter and represented by dotted line. GalA, $GalA_2$ and $GalA_3$ eluted at k_{av}= 0.7, 0.6 and 0.4, respectively. Carbohydrates were detected by phenol/sulphuric acid assay, represented by solid line.

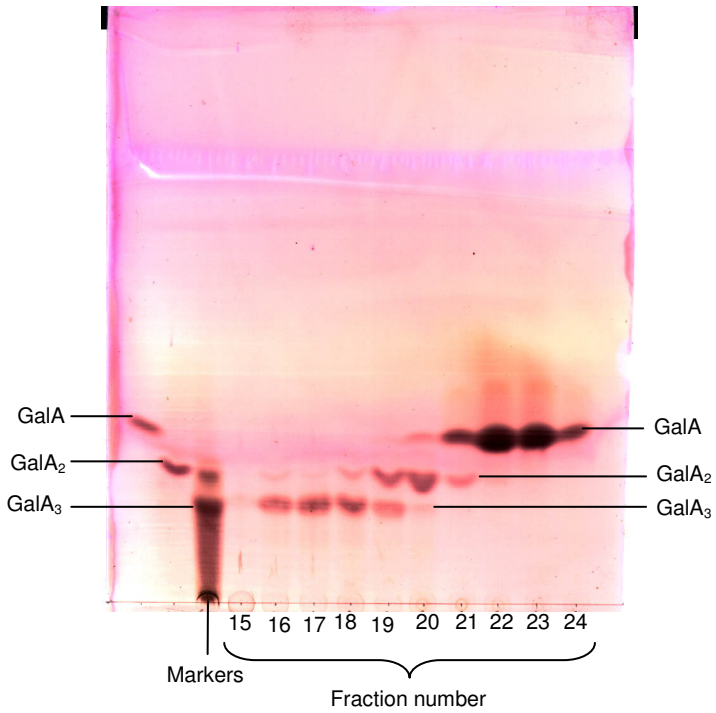


Figure 3.12: TLC showing the calibrated Bio-Gel P-2 column fractions
Markers (5µg) and Bio-Gel P-2 column fractions (10 µl) were loaded on the TLC plate and developed in BAW (2:1:1, 8 h). The plate was stained with thymol/ sulphuric acid.

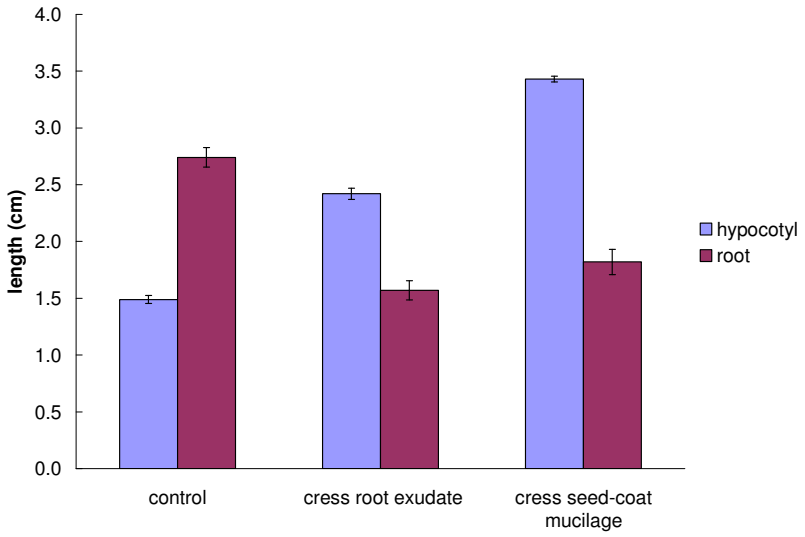
3.9 Effect of cress seed-coat mucilage on Amaranthus seedlings

In previous experiments when I was disinfecting cress seeds with NaOCl solution, I found the disinfectant very viscous, suggesting that some of the mucilage from seed-coat released into the solution might have bioactivity. Because of this, I imbibed cress seeds in sterile deionised water for 24 h and collected seed-coat mucilage rather than three-day root exudate. The collected seed-coat mucilage was then used in bioassays, which showed a higher bioactivity towards *Amaranthus* hypocotyl elongation, while the effect on root was not different from that of root exudate (Fig. 3.13), suggesting that seed-coat mucilage might have new potent compound(s).

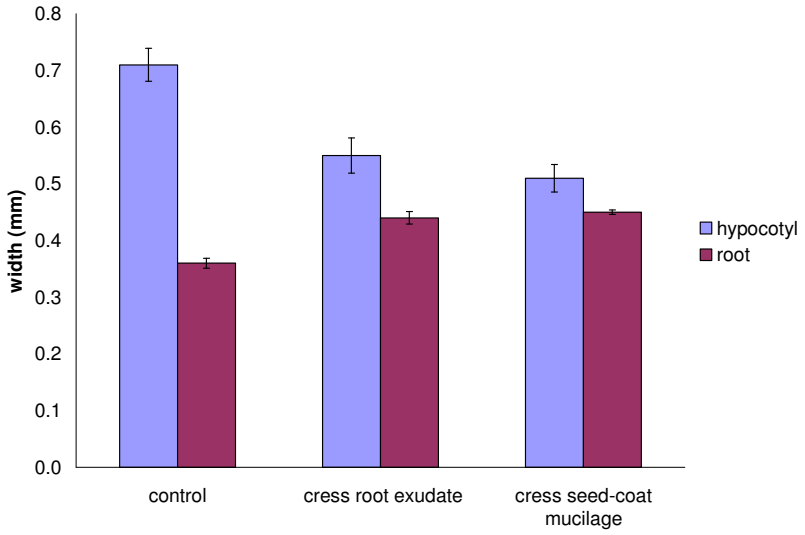
3.10 Bioassay of 'organic' cress seed-coat mucilage

Previously I had shown that the effects on *Amaranthus* were not due to competition with cress (see section 3.5). The next question was whether it might be an effect of seed treatment chemicals (e.g. pesticides, fungicides and other synthetic chemicals). Therefore, I used 'organic' cress seeds (free of synthetic chemicals) in this experiment. 'Organic' cress seed-coat mucilage solution was collected after imbibition in water. *Amaranthus* seedlings grown in it had hypocotyl and root length and width significantly affected as compared to the control (Fig. 3.14). The result showed that the effect of mucilage was not due to seed treatment chemicals. Although 'organic' seeds were free from synthetic chemicals there was still a chance

that they had been treated with sources of natural hormones (e.g. auxin and gibberellins).



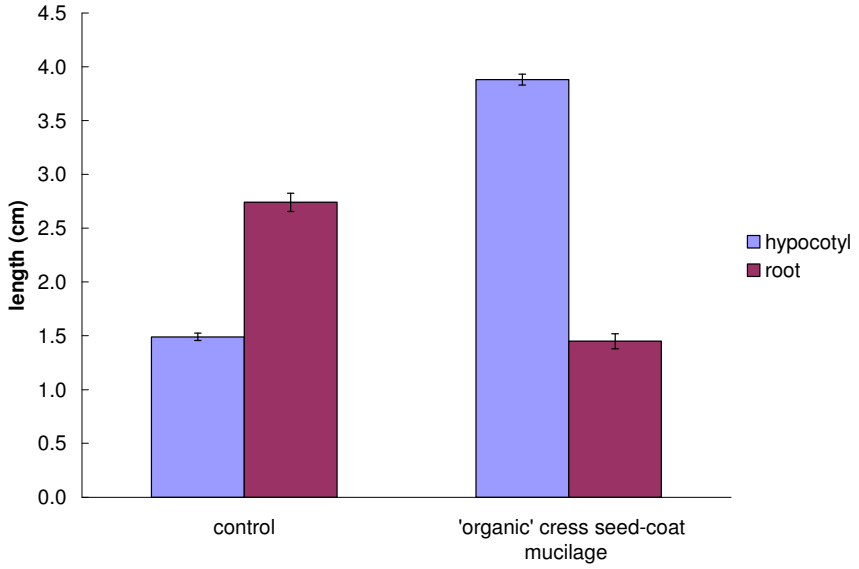
(a)



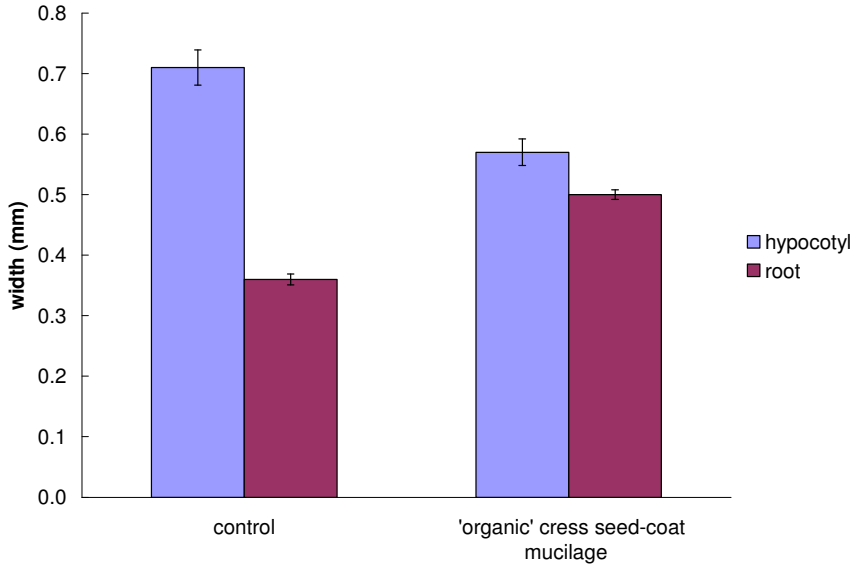
(b)

Figure 3.13: Comparing the effect of cress root exudate and cress seed-coat mucilage on Amaranthus seedlings

Cress seeds were imbibed in sterile water for 24 h, while cress exudate was collected after incubating cress seedlings for 3 days in sterile water. *Amaranthus* seeds were then incubated in the collected cress seed-coat mucilage or cress exudate for 5 d at 25ºC in the dark in 9-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. The length and width of hypocotyls and roots of *Amaranthus* were measured by use of Labwork software. (a) Length; (b) width. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.



(a)



(b)

Figure 3.14: Effect of 'organic' cress seed-coat mucilage on Amaranthus seedlings

'Organic' cress seeds were imbibed in sterile water for 24 h. *Amaranthus* seeds were then incubated in the collected mucilage for 5 d at 25ºC in the dark in 9-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. The length and width of hypocotyls and roots of *Amaranthus* seedlings were measured by use of Labwork software. (a) Length; (b) width. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

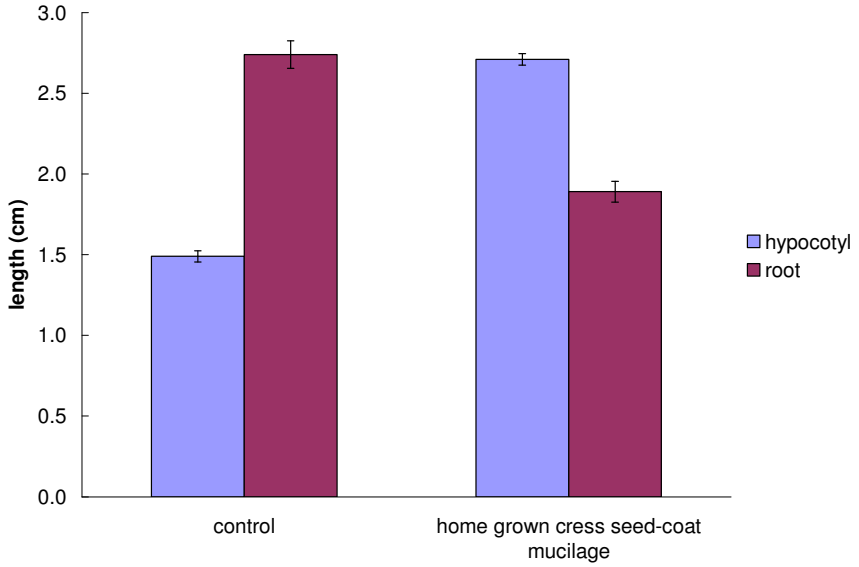
3.11 Bioassay of home-grown cress seeds

I did this experiment to clarify whether the effect of cress seed-coat mucilage on *Amaranthus* seedlings was due to the presence of natural hormones in the mucilage solution. 'Organic' cress seeds were sown in a private garden in Edinburgh supplied only with rain water and the next generation of seeds was collected after flowering. The collected seeds were imbibed and the seed-coat mucilage after centrifugation was kept for bioassay experiments with *Amaranthus*. Hypocotyl and root length and width of the *Amaranthus* seedlings were significantly different from those of controls grown in water (Fig. 3.15). From the results it was concluded that neither competition nor seed treatment chemicals are responsible for the apparent allelopathic effect. So the effect on *Amaranthus* seedlings was due to the compound or compounds from cress.

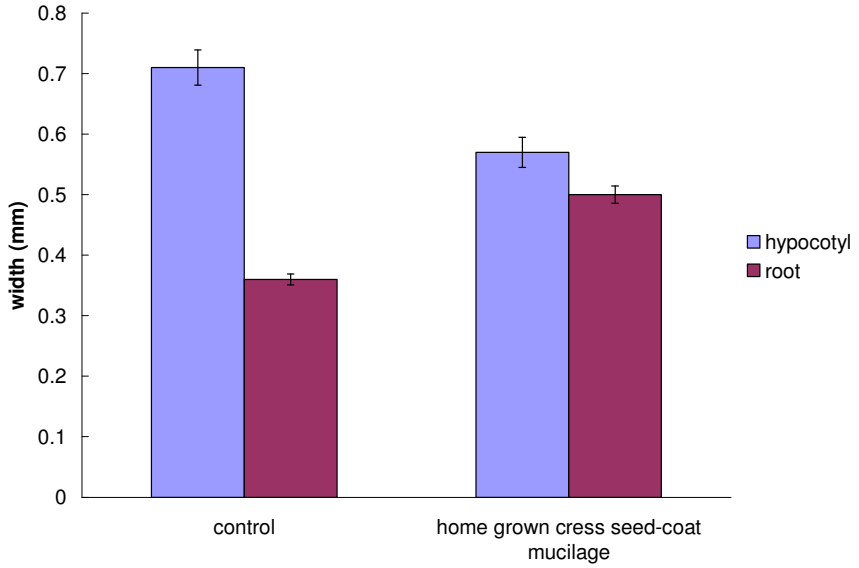
3.12 Collection of cress seed-coat mucilage at various imbibition times

To discover the time point at which cress seed-coat mucilage shows maximal bioactivity, I imbibed cress seeds in sterile water for various times. Each time point seed-coat mucilage was centrifuged and *Amaranthus* seeds were incubated in the supernatant. The result (Fig. 3.16) showed that even within the first few minutes cress seeds release the bioactive compound or compounds into their surroundings. Both hypocotyl and root length and width were affected by cress-seed coat mucilage collected even 10 min. The bioactivity of the compound or compounds towards *Amaranthus* hypocotyls reached its maximum within 24 h and thereafter started

losing activity, while the activity towards *Amaranthus* roots reached its maximum within 6 h and remained constant till at least 48 h.



(a)



(b)

Figure 3.15: Effect of home-grown cress seed-coat mucilage on Amaranthus seedlings

Home-grown cress seeds were imbibed in sterile water for 24 h. *Amaranthus* seeds were then incubated in collected mucilage for 5 d at 25ºC in the dark in 9-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. The length and width of hypocotyls and roots of *Amaranthus* seedlings were measured by use of Labwork software. (a) Length; (b) width. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

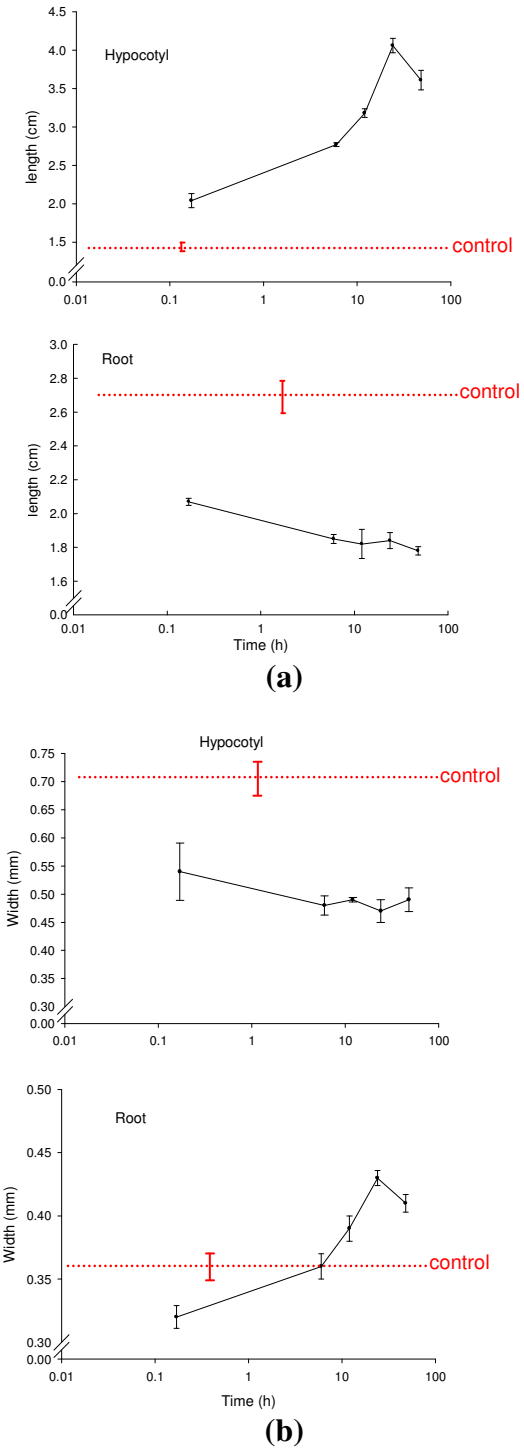
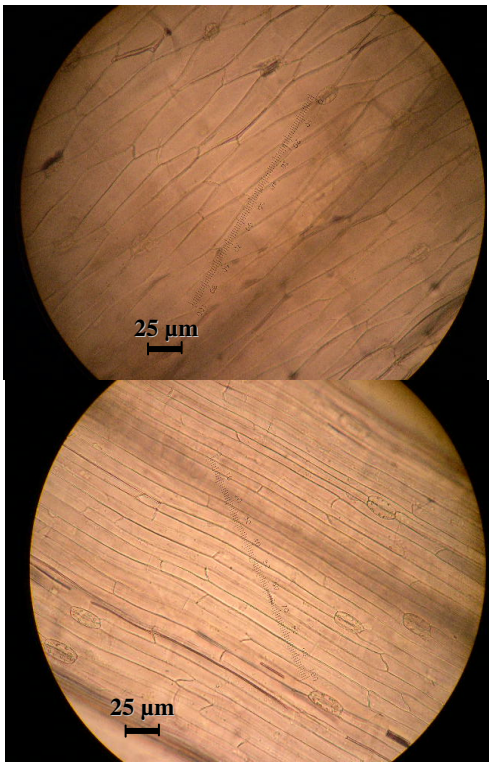


Figure 3.16: Effect of cress seed-coat mucilage collected at various time points on Amaranthus seedlings

Cress seeds were imbibed in sterile water at 4ºC for various times. *Amaranthus* seeds were then incubated in collected mucilage for 5 d at 25ºC in the dark in 9-cm Petri plates. Seeds incubated in sterile water alone were used as a control represented by dotted red line. Hypocotyl and root were measured by use of Labwork software (a) Length; (b) width. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

3.13 Effect of cress seed-coat mucilage on epidermal cell expansion

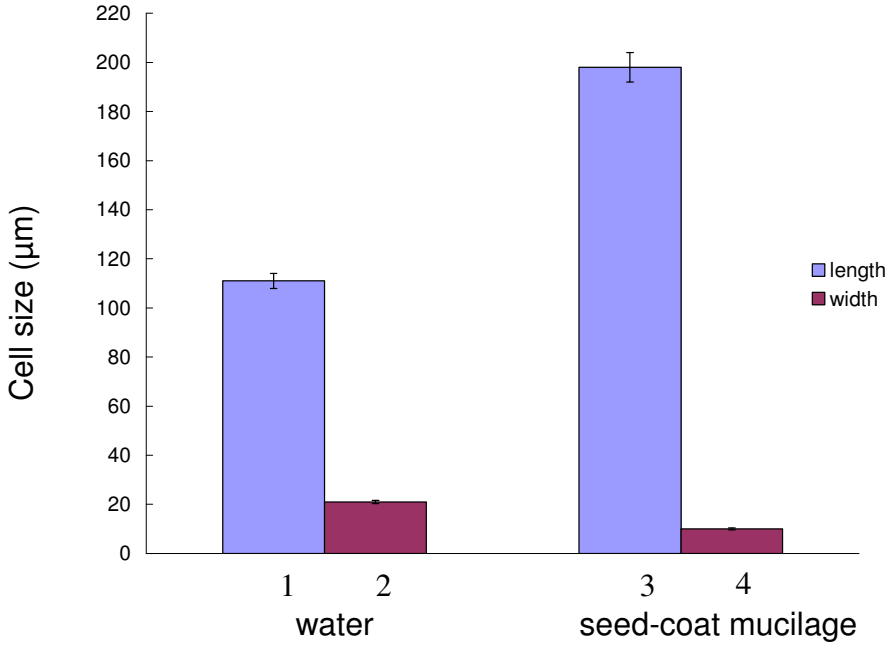
Once I had established that cress seed-coat mucilage contains bioactive compound(s) that stimulated hypocotyl and inhibited root elongation in *Amaranthus*, I tested whether the mucilage affects *Amaranthus* epidermal cell expansion or division or both (Fig. 3.17). Epidermal cells of *Amaranthus* hypocotyls that had been incubated in mucilage were narrower and longer than water controls. Also the ratio of hypocotyl length to cell length was higher in the mucilage-treated sample than in the control, which revealed mucilage promotes cell division by cross wall formation. Similarly, the ratio of hypocotyl width to cell width was higher in the treated than control samples revealing that cell division by long-wall formation was observed as well. The results suggest that the bioactive compound(s) promote both cell expansion and division.



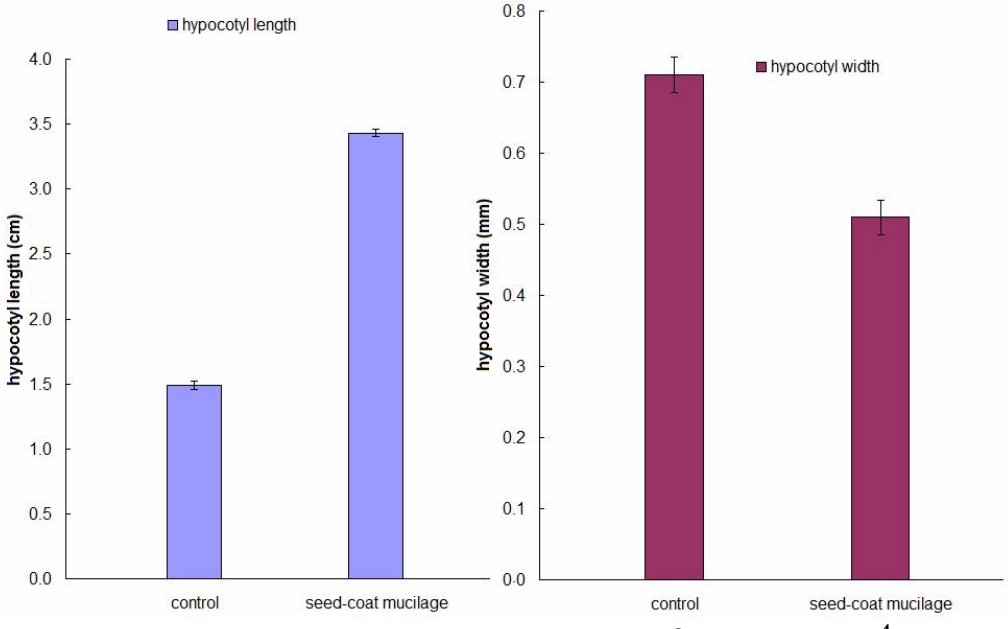
Control

Treated

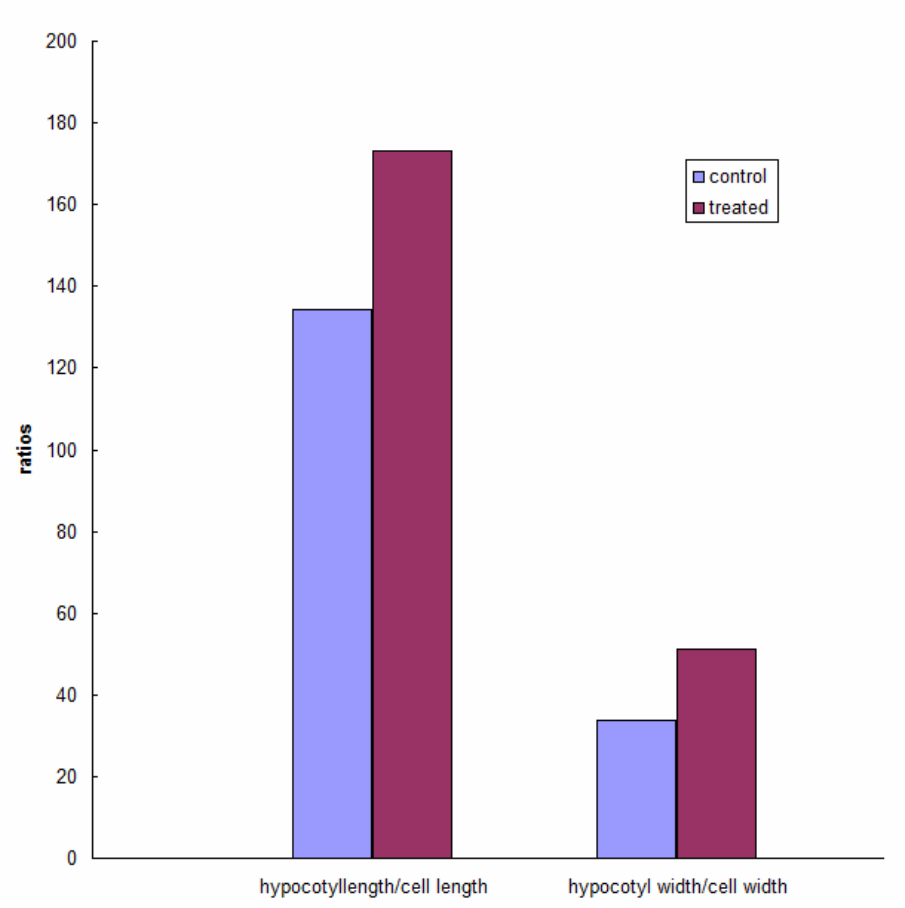
(a)


Cell size (μm)
220
200
180
160
140
120
100
80
60
40
20
0
length
width
1
2
3
4
water
seed-coat mucilage

(b)


hypocotyl length
hypocotyl length (cm)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
seed-coat mucilage
1
3
hypocotyl width
hypocotyl width (mm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
control
seed-coat mucilage
2
4

(c1) **(c2)**



(d)

Figure 3.17: Effect of cress seed-coat mucilage on epidermal cell size and shape in Amaranthus hypocotyls

Amaranthus seeds were incubated in cress seed-coat mucilage for 5 d at 25ºC in the dark in 9-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. The length and width of hypocotyl epidermal cells were examined under 40x power of a compound microscope. Photographs of hypocotyl epidermal cells were measured by use of Labwork software. (a) Photographs of cells representative of their respective populations; (b) mean length and width of the cells from triplicate Petri plates with error bars representing inter-plate S.E.; (c1 & c2) hypocotyl length and width from triplicate Petri plates with error bars representing inter-plate S.E.; and (d) ratios of hypocotyl length to cell length, and hypocotyl width to cell width.

3.14 Bioactivity of cress seed-coat mucilage at various concentrations

Dried cress seed-coat mucilage was dissolved in sterile de-ionised water at various concentrations to find the concentration of the mucilage at which it shows maximum activity towards hypocotyl and root growth in *Amaranthus*. The results (Fig. 3.18) showed that both hypocotyl and root length were increasingly affected as the concentration of mucilage was increased. At low concentrations up to 40 µg per 0.9 ml the mucilage had a small effect on the hypocotyl but no effect on root growth. There was also a small observable effect of mucilage on roots at concentrations of 156 and 625 µg but a larger effect on hypocotyls. A larger effect on both hypocotyl and root length was observed at high concentrations of mucilage.

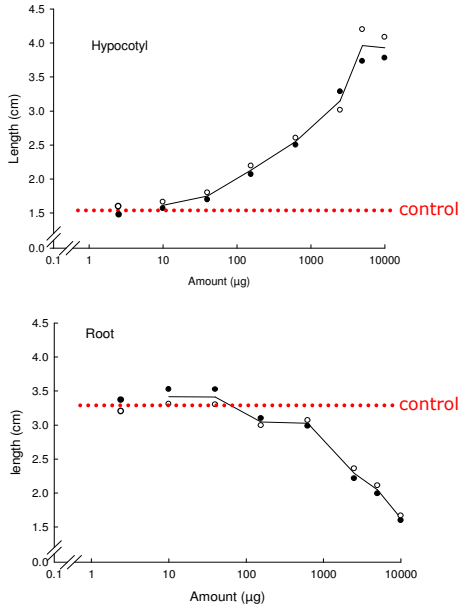


Figure 3.18: Effect of various concentrations of cress seed-coat mucilage on *Amaranthus* seedlings
Dried cress seed-coat mucilage was dissolved in sterile de-ionised water at various concentrations. *Amaranthus* seeds were then incubated in 0.9 ml of mucilage for 5 d at 25ºC in the dark in 3-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control and represented by dotted red lines. The length of hypocotyls and roots were measured. Data represent from duplicate Petri plates.

3.15 Quantitative tests for carbohydrates in cress seed-coat mucilage

To provide material for an investigation of the presence of biologically active oligosaccharides in cress seed-coat mucilage, 25 g of cress seeds were incubated in sterile de-ionised water at 4ºC in dark for 24 h. The collected mucilage was assayed by various carbohydrate tests (Table 3.2). The results indicated that the mucilage was rich in sugars, which were easily enough to be used in various experiments. Unsaturated uronic acid residues were also found and might be important components of the active principle.

Table 3.2: Amount of carbohydrates present in cress seed-coat mucilage
Cress seeds (25 g) were imbibed in sterile de-ionised water for 24 h at 4ºC in dark. The seed-coat mucilage was centrifuged at 4500 rpm and the supernatant was analysed for carbohydrates.

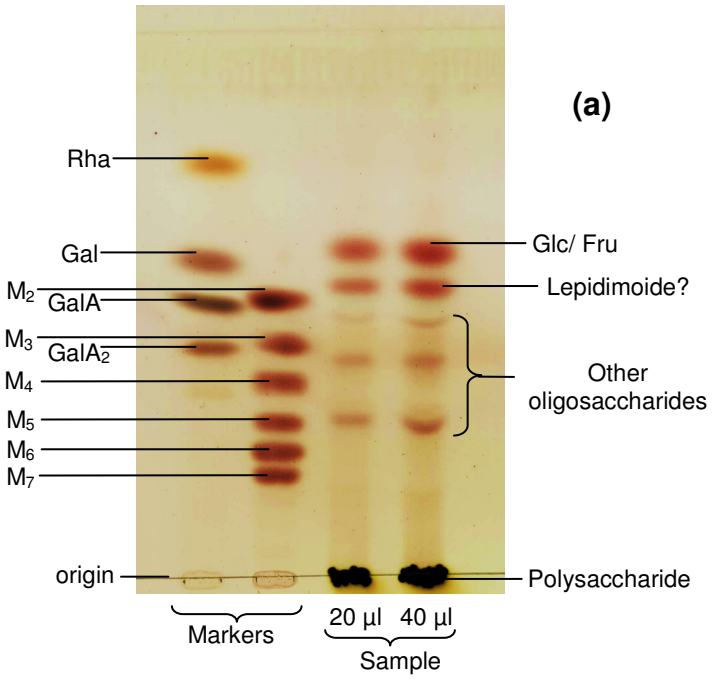
Total CHO (µg/ seed)	Pentoses (µg/ seed)	Hexoses (µg/ seed)	Uronic acid (µg/ seed)	Unsaturated uronic acid (µg/ seed)
116	57.1	41.9	49.1	0.104

* The average dry mass of a seed was 2.7 mg that released 0.15 mg of soluble seed-coat mucilage after 24 h imbibition in sterile de-ionised water.

3.16 Qualitative tests for carbohydrates present in cress seed-coat mucilage

3.16.1 Thin-layer chromatography

As the quantitative test results showed the presence of various classes of carbohydrates, I performed TLC in three different solvent systems to separate qualitatively the carbohydrates present in the mucilage. At least five migrating bands were seen, while polysaccharide stayed at the origin (Fig. 3.19). One of the bands, which ran between galactose and galacturonic acid may have been lepidimoide, a bioactive compound. The band approximately co-migrated with sucrose on TLC (a) and (b) but ran slow on TLC (c). The other slow-migrating bands were probably oligosaccharides that might be bioactive.



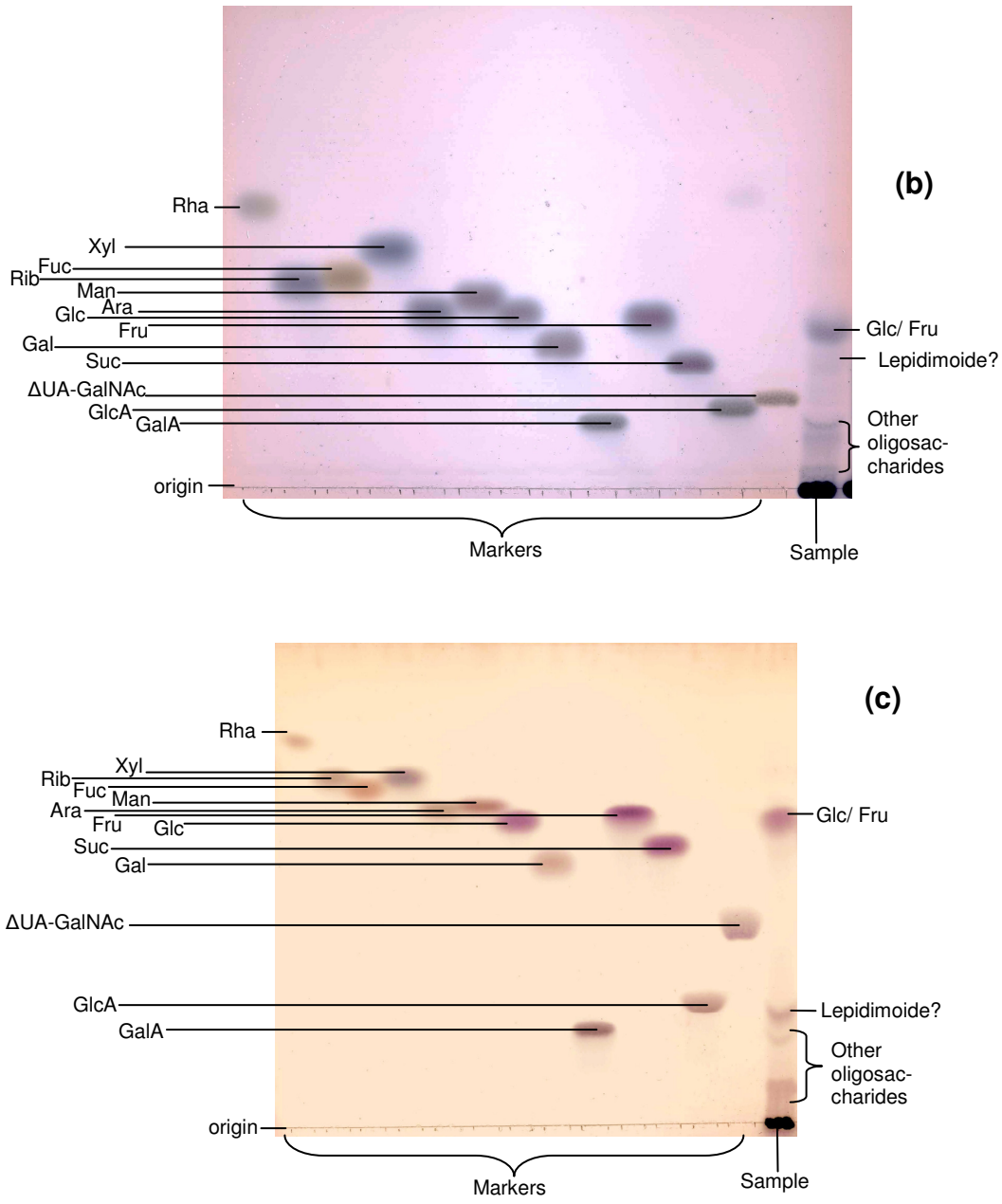


Figure 3.19: Separation of compounds present in cress seed-coat mucilage
Markers (5 µg each) and cress seed-coat mucilage (100 µg) were loaded on TLC plates developed in (a) BAW (2:1:1) for 8 h, (b) BAW (3:1:1) for 8 h, or (c) BAW (3:1:1) for 8 h followed by EPW (10:4:3) for 3 h. The plates were stained with thymol.

3.16.2 Paper chromatography

Paper chromatography was also done to separate and identify the compounds in the cress seed-coat mucilage. Mucilage along with a range of sugars were loaded on Whatman No.1 papers and developed in two solvent systems (Fig. 3.20). One of the sample bands was identified as fructose and the other glucose. A streak of slow-migrating compounds was also observed, which might include oligosaccharides with biological activity.

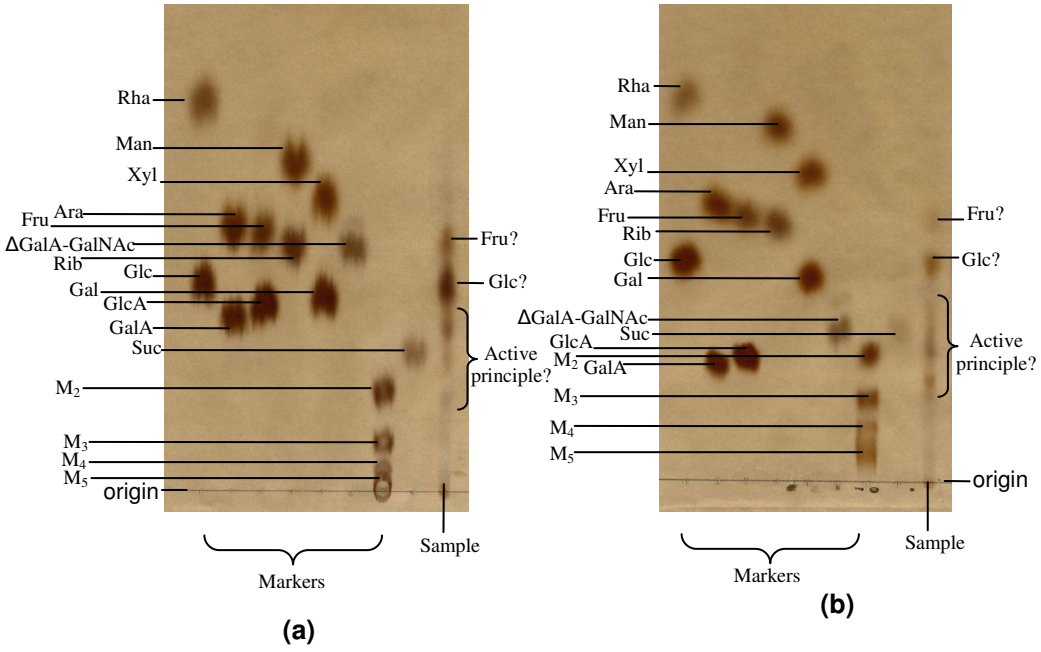


Figure 3.20: Separation of the compounds in cress seed-coat mucilage by paper chromatography

Markers of monosaccharides and oligosaccharides (10 µg each) were loaded along with cress seed-coat mucilage (150 µg) on Whatman No.1 paper. The paper was placed in solvent system (a) BAW (12:3:5) for 24 h or (b) BAW (12:3:5) for 24 h followed by EPW (10:4:3) for 8 h. The sugars were stained with silver nitrate.

3.16.3 Paper electrophoresis

Cress seed-coat mucilage was subjected to electrophoresis at pH 3.5 to determine whether the carbohydrates were acidic, basic or neutral. Three $AgNO_3$-staining compounds in mucilage migrated towards the anode (Fig. 3.21). Two of the compounds migrated slower than GlcA, while one migrated a little faster. A large amount of carbohydrate in mucilage stayed at the origin. In an attempt to identify the acidic sugars, a range of authentic acidic sugars were loaded along with the sample (Fig. 3.22) and the electrophoresis was conducted for twice as long. The acidic sugars in cress seed-coat mucilage were well separated from each other. The acidic sugars did not co-migrate with any of the authentic sugar, which means that the mucilage contains unfamiliar acidic sugars that might possibly have biological activity.

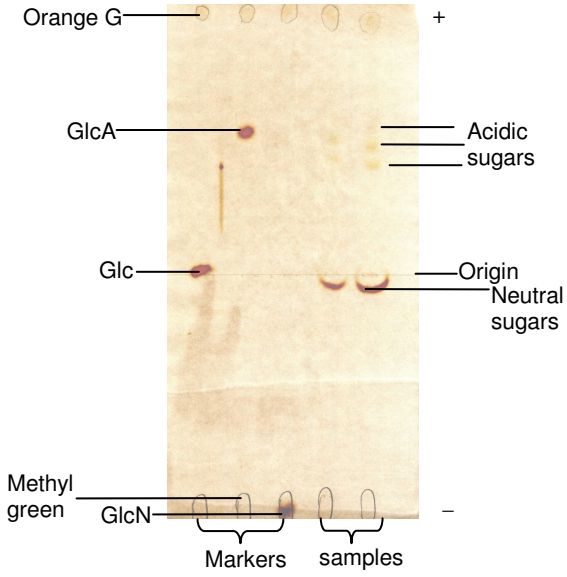


Figure 3.21: Paper electrophoresis for the separation of acidic, neutral and basic sugars in cress seed-coat mucilage
Cress seed coat mucilage (100 µg and 200 µg) and markers (10 µg) were loaded in the centre on Whatman No.1 paper. Both orange G and methyl green were used as internal markers. Electrophoresis was conducted at pH 3.5, 3 kV for 30 min and the paper was stained with silver nitrate.

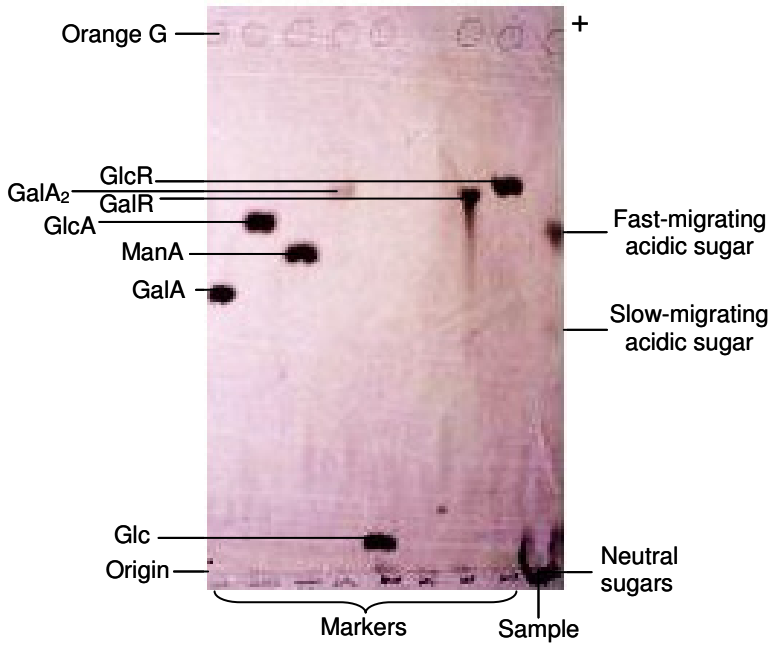


Figure 3.22: Paper electrophoresis for the separation of sugars in cress seed-coat mucilage for longer time

Cress seed-coat mucilage (400 µg) and markers (10 µg) were loaded on Whatman No.1 paper. Orange G was used as internal marker, while various authentic acidic sugars were used as external markers. Electrophoresis was done for 1 h at pH 3.5 and 3 kV. The paper was stained with silver nitrate stain.

3.17 Solvent phase portioning of compounds present in cress seed-coat mucilage.

The compounds in cress seed-coat mucilage were partitioned between polar and non-polar phases to indicate the polarity of the bioactive compound. The desired pH of aqueous solutions of mucilage was achieved with acetic acid or sodium hydroxide. Ethyl acetate was then added to them and the contents of the tube were vigorously shaken and left to stand. The two phases were then separated, dried and re-dissolved in sterile de-ionised water and were loaded with markers on a TLC. At all tested pH values the stainable compounds in mucilage were present in the aqueous phase (Fig. 3.23). It was concluded that the stainable compounds were highly hydrophilic.

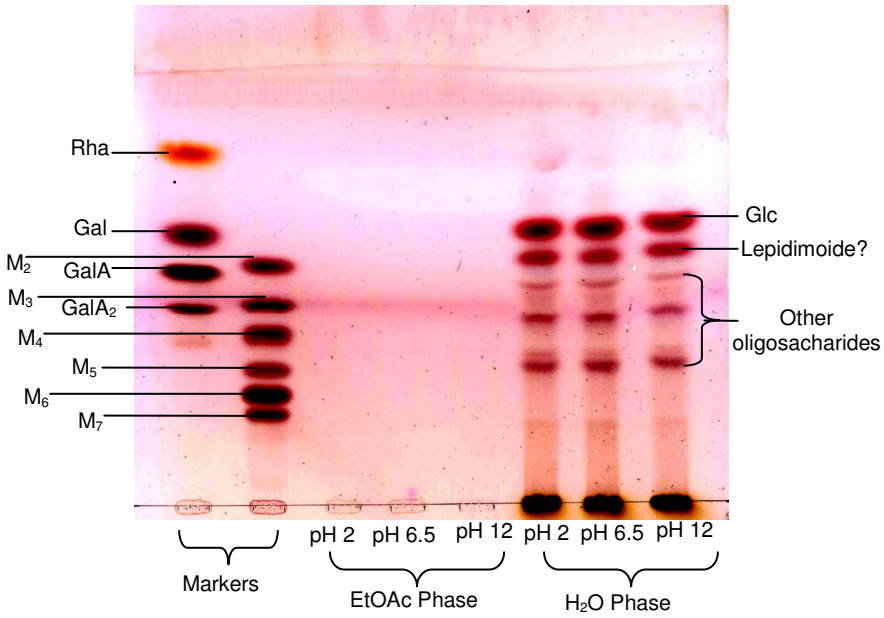


Figure 3.23: TLC showing partitioning of sugars present in mucilage between water and ethyl acetate phases at various pH values

Cress seed-coat mucilage (10 mg/ml) was partitioned between water and ethyl acetate phases at pH 2, pH 6.5 and pH 12. Samples (40 µl) from separated phases at various pH values, and markers, were loaded on a TLC plate and were developed in BAW (2:1:1) for 8 h. The plates were stained with thymol.

3.18 Bioassay of ethyl acetate (EtOAc) and H_2O partitioned fractions

The compounds separated between water and EtOAc phases at various pH values were used in a bioassay experiment to test at what pH the bioactive compound or compounds were recovered in the polar phase. The result revealed at all tested pH values the bioactivity partitioned into the water phase (Fig. 3.24) and are hydrophilic. It was concluded that the compound(s) of interest were not auxin or gibberellin.

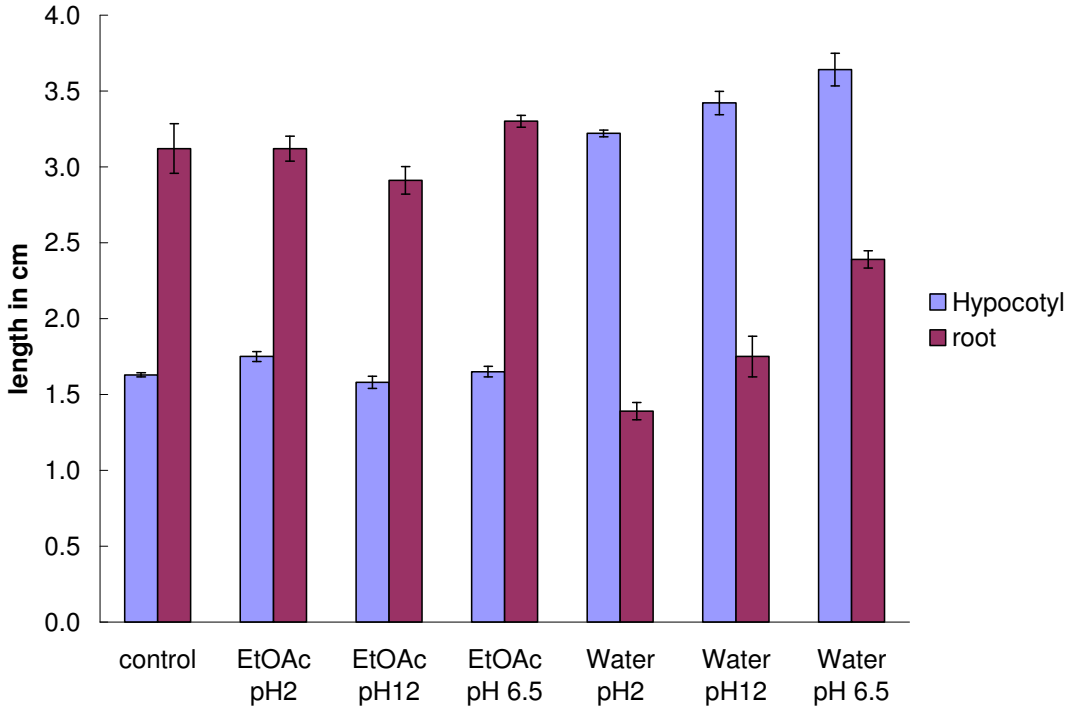


Figure 3.24: Effect of compounds in cress seed-coat mucilage partitioned into water or ethyl acetate phases at various pH values on *Amaranthus* seedlings
Cress seed-coat mucilage was fractioned between H_2O and EtOAc phases at various pH values. *Amaranthus* seeds were then incubated in those fractions for 5 d at 25ºC in the dark in 3-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

3.19 Effect of heat on stability of compounds in cress seed-coat mucilage

To test whether the bioactive compound(s) were heat-labile or heat-resistant, I incubated cress seed-coat mucilage at 100ºC for various times. A portion of mucilage was loaded on a TLC plate with markers. After development and staining the TLC showed the spots unaffected by boiling (Fig. 3.25). To test the bioactivity of the boiled samples the remaining portion of the sample was utilised in bioassays.

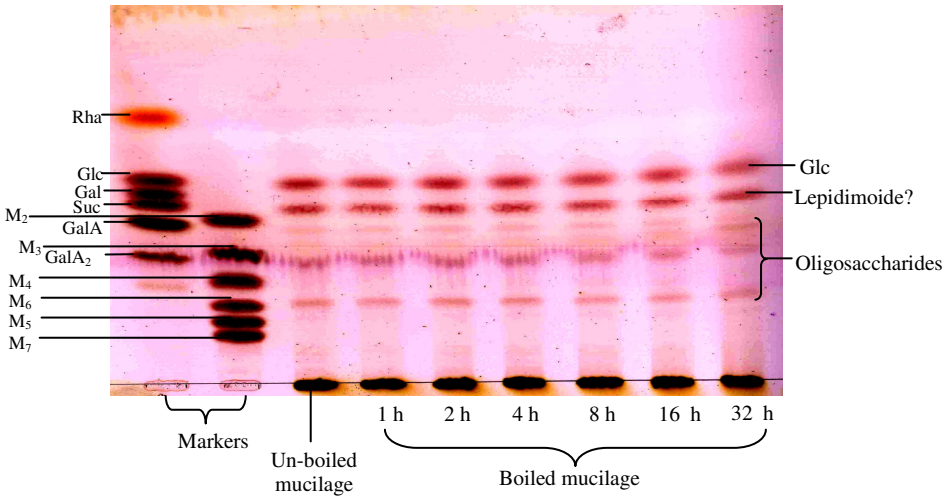


Figure 3.25: TLC showing the effect of heat on sugars present in cress seed-coat mucilage

Cress seed-coat mucilage (5mg/ml) was treated at 100ºC for various times. The boiled samples (40µl) and markers were then loaded on a TLC plate and were developed in BAW (2:1:1) for 8 h. The plates were stained with thymol.

3.20 Effect of heat on stability of active principle in seed-coat mucilage

Cress seed-coat mucilage was heated at 100ºC for various times. The boiled mucilage was then cooled to room temperature and *Amaranthus* seeds were incubated in it. The bioactive compound(s) were clearly heat-stable (Fig. 3.26).

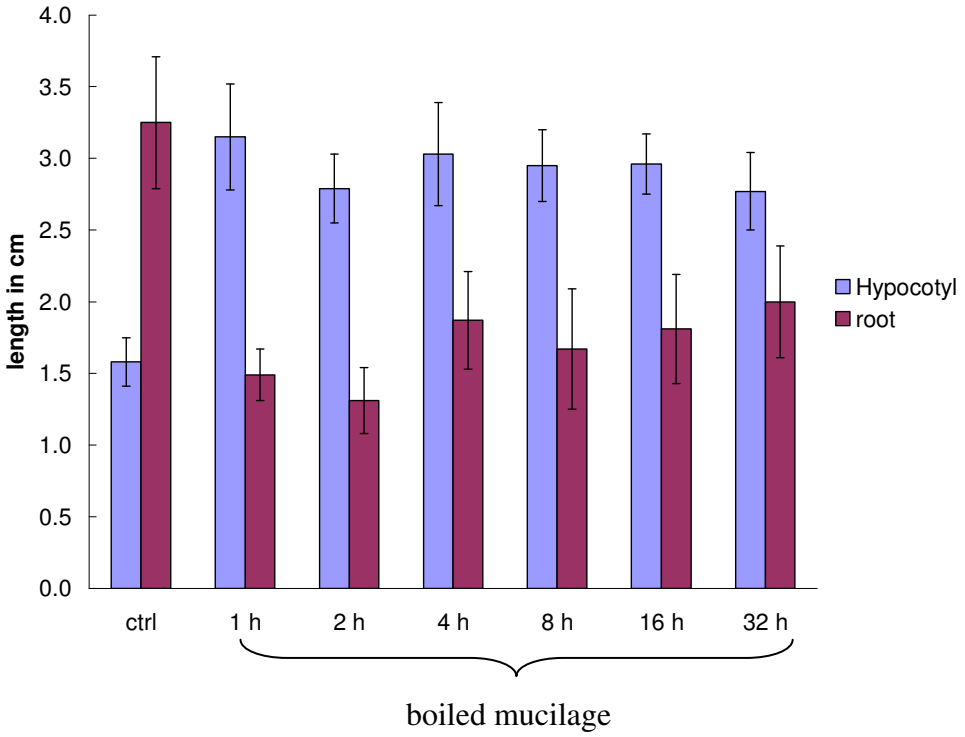


Figure 3.26: Effect of boiled cress seed-coat mucilage on *Amaranthus* seedlings
The samples were as in Fig. 3.25. *Amaranthus* seeds were then incubated in them for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent mean of 10 seedlings from a single Petri plate with error bar representing intra-plate S.D.

3.21 Effect of 48 h heat treatment on compounds in seed-coat mucilage

Although the previous experiment on heat-treated mucilage was successful, the experiment had not been done in triplicate. So for the sake of statistical validity the experiment was repeated in triplicate. In this experiment an aqueous solution of cress seed-coat mucilage was heated at higher temperature and longer time, 130ºC for 48 h, to see whether the compound with stand the harsh conditions. A small portion of the mucilage was then loaded on a TLC plate with markers. The compounds were unaffected by high temperature and long-time treatment (Fig. 3.27).

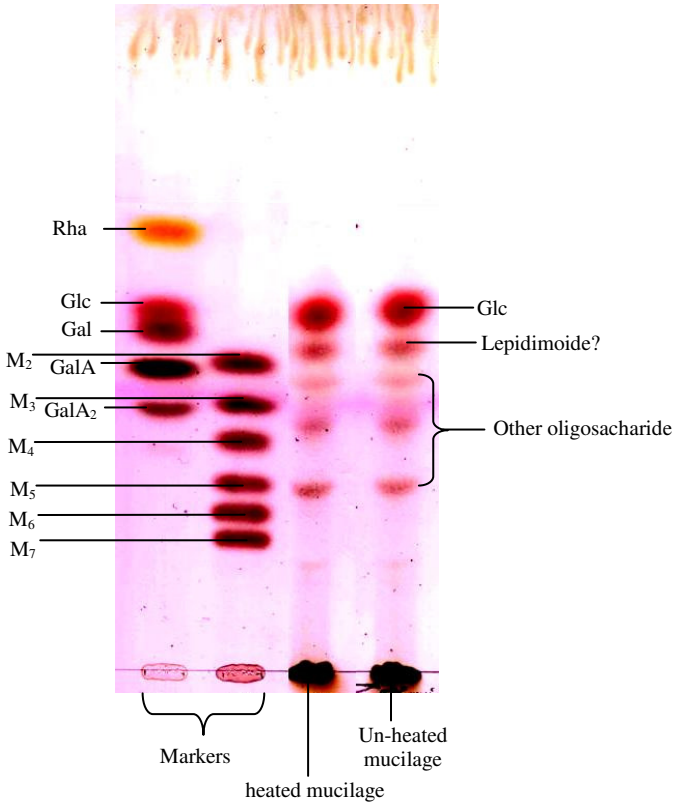


Figure 3.27: TLC showing the effect of 48 h heat treatment on sugars present in cress seed-coat mucilage
Cress seed-coat mucilage was heated at 130ºC for 48 h. The heated samples (40µl) and markers were then loaded on a TLC plate and were developed in BAW (2:1:1) for 8 h. The plates were stained with thymol.

3.22 Effect of 48 h heat treatment on stability of active principle in seed-coat mucilage

The cress seed-coat mucilage that had been heated for 48 h at 130ºC retained bioactivity even after this harsh temperature and long time treatment (Fig. 3.28).

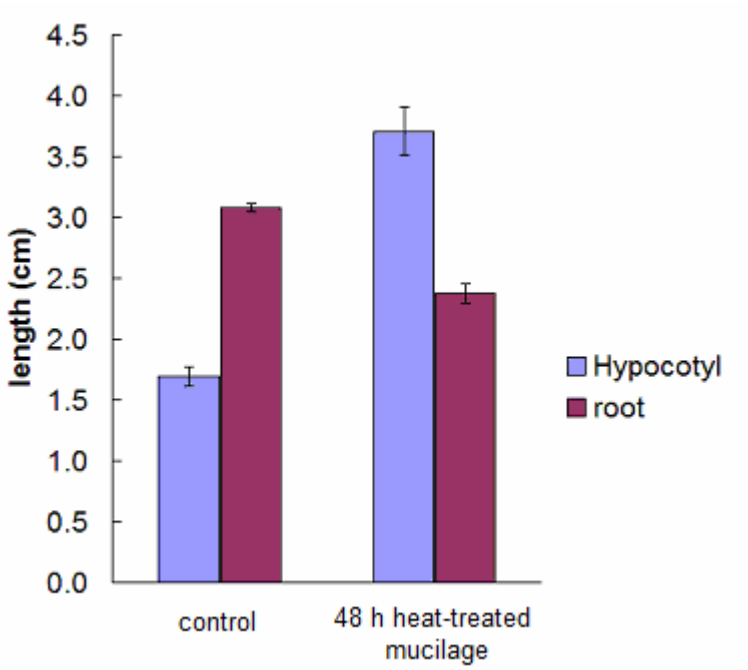


Figure 3.28: Effect of 48 h heated cress seed-coat mucilage on *Amaranthus* seedlings
The sample was same as in Fig. 3.27. *Amaranthus* seeds were incubated in it for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

3.23 Fractionation of cress seed-coat mucilage on GPC columns

3.23.1 Fractionation of cress seed-coat mucilage on Bio-Gel P-10

Cress seed-coat mucilage that contained an internal marker of ^3H_2O was loaded on Bio-Gel P-10 to separate the oligosaccharides and small polysaccharides from bigger polysaccharides. Fractions were collected and tested for total carbohydrates. The void volume (V_o) containing polysaccharides peaked in fraction 22 while tritiated water as included volume (V_i) peaked in fraction 66. The mono, oligosaccharides and some small polysaccharides were assumed to be eluted between fractions 39 and 76 (Fig. 3.29).

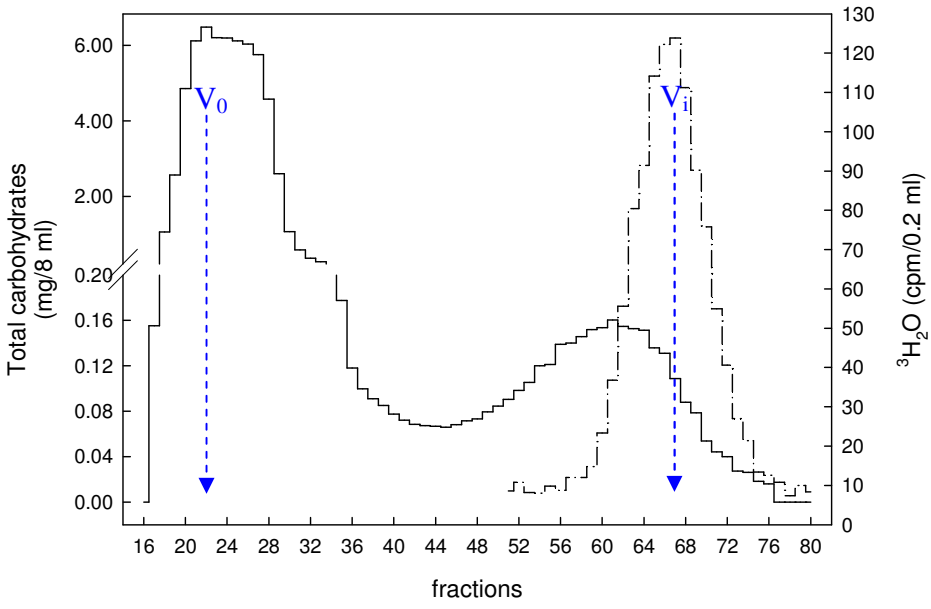


Figure 3.29: Separation of compounds in cress seed-coat mucilage on Bio-Gel P-10

Cress seed-coat mucilage (70 mg) and ^3H_2O was loaded on a1 litre bed volume Bio-Gel P-10 column. Eighty 8-ml fractions were collected. Collected fractions were then tested for carbohydrates by phenol/ sulphuric acid (—) and for ^3H_2O (.—.—).

A small portion of each eluted fraction from Bio-Gel P-10 was combined initially into three pools, which were loaded along with markers on a TLC plate. Pool 1 contained polysaccharides, pool 2 mainly contained polysaccharides and a small amount of oligosaccharides, and pool 3 contained monosaccharides, oligosaccharides and polysaccharides that might be of interest (Fig. 3.30).

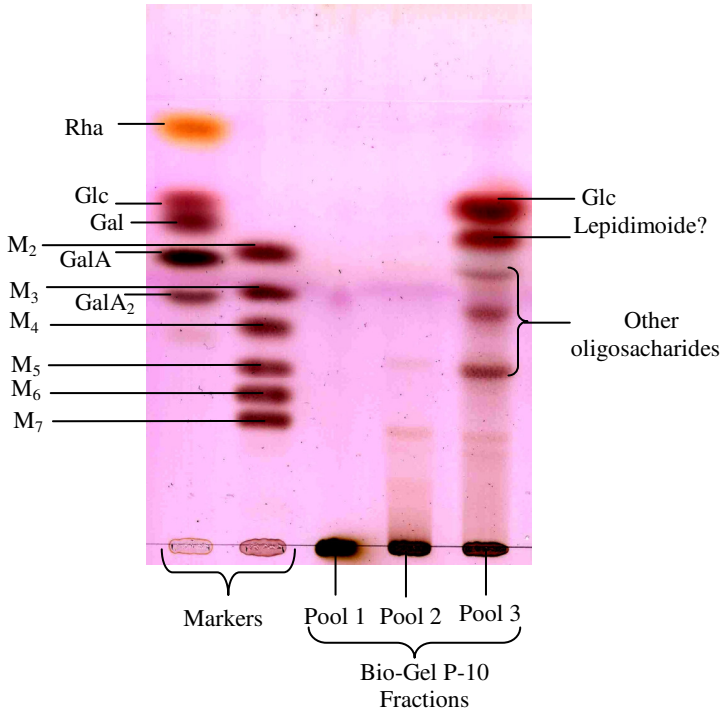


Figure 3.30: TLC showing the presence of various classes of carbohydrates in large pools of fractions from Bio-Gel P-10

A small portion from fractions (16-75) shown in Fig. 3.29 were combined in three pools. The pooled fractions (20 µl) and markers were then loaded on a TLC. The plate was developed in BAW (2:1:1) for 8 h and stained with thymol. Pool 1 = fractions 16-35, pool 2 = 36-55, pool 3 = 56-75.

To refine the pooling procedure, I analysed 15 smaller pools (instead of 3 larger pools) and markers by TLC. The first seven pools mainly contained polysaccharides. Small amounts of chromatographically mobile compound(s) started eluting in the 8th pool. Higher concentrations of monosaccharide and oligosaccharides were eluted in pools 10 to 14 (Fig. 3.31).

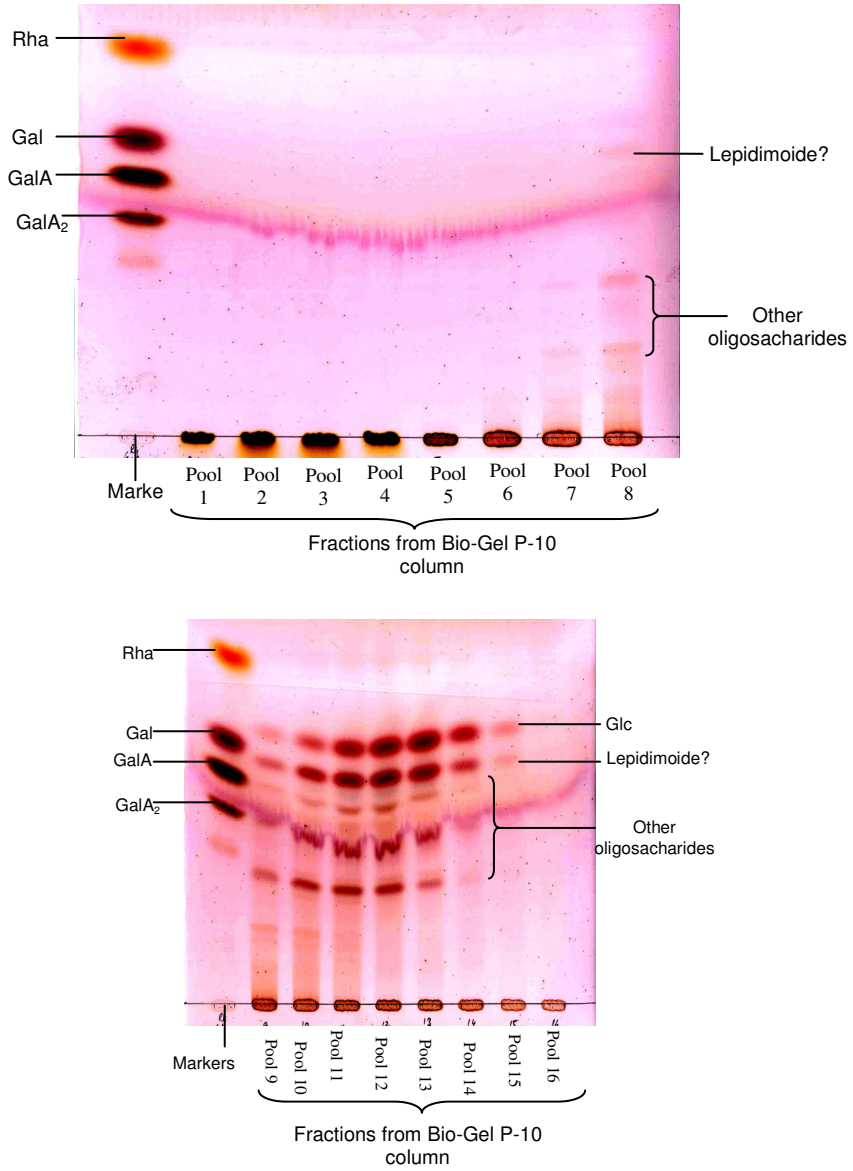


Figure 3.31: Separation of compounds present in smaller pools of fractions from Bio-Gel P-10 by TLC

Pooled fractions (20 µl) from Bio-Gel P-10 and markers were loaded on TLC. The plate was then developed in BAW (2:1:1) for 8 h and stained with thymol. Each pool contains 4 fractions starting from fraction no. 16.

Each of the 15 pools was then utilised in a triplicated bioassay. The bioactivity started to elute in the 9th pool and peaked in about the 13th. The root inhibition in pools 2-4 was probably due to low pH because of the acidic sugars in these pools (Fig. 3.33). It was concluded that Bio-Gel P-10 is good for an initial separation of bioactive compound(s) from the large amounts of polysaccharide present.

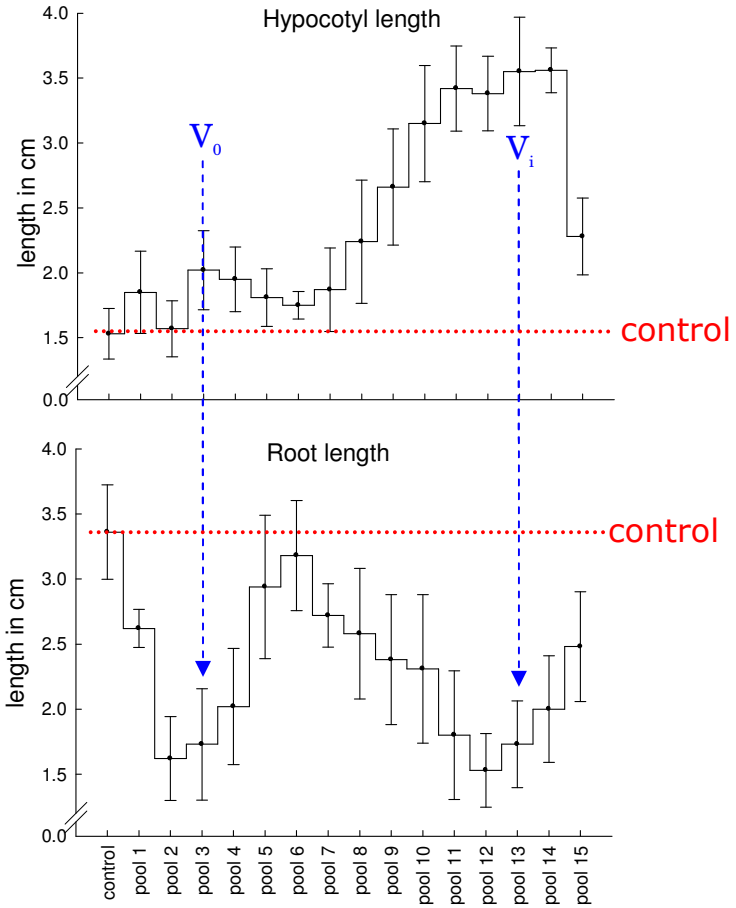


Figure 3.32: Effect of fractions of cress seed-coat mucilage from Bio-Gel P-10 on *Amaranthus* seedlings

Cress seed-coat mucilage was fractionated on Bio-Gel P-10 (see Fig. 3.29). The collected fractions were then pooled into 15 pools. Each pool consisted of 4 fractions starting from fraction no. 16. The pools were freeze dried and re-dissolved in 6 ml of sterile de-ionised water. *Amaranthus* seeds were incubated in these pools (0.9 ml each) for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent mean of 10 seedlings from a single Petri plate with error bar representing intra-plate S.D.

3.23.2 Further fractionation of partially included fractions from Bio-Gel P-10 on Bio-Gel P-2

For further fractionation and partial purification of bioactive compound(s), the included volume fractions (bioactive fractions) from the P-10 column were pooled and loaded with $^{3}H_2O$ on a Bio-Gel P-2 column. The sample gave three carbohydrates peaks which meant that the pooled P-10 fractions were further resolved on the P-2 column (Fig. 3.33).

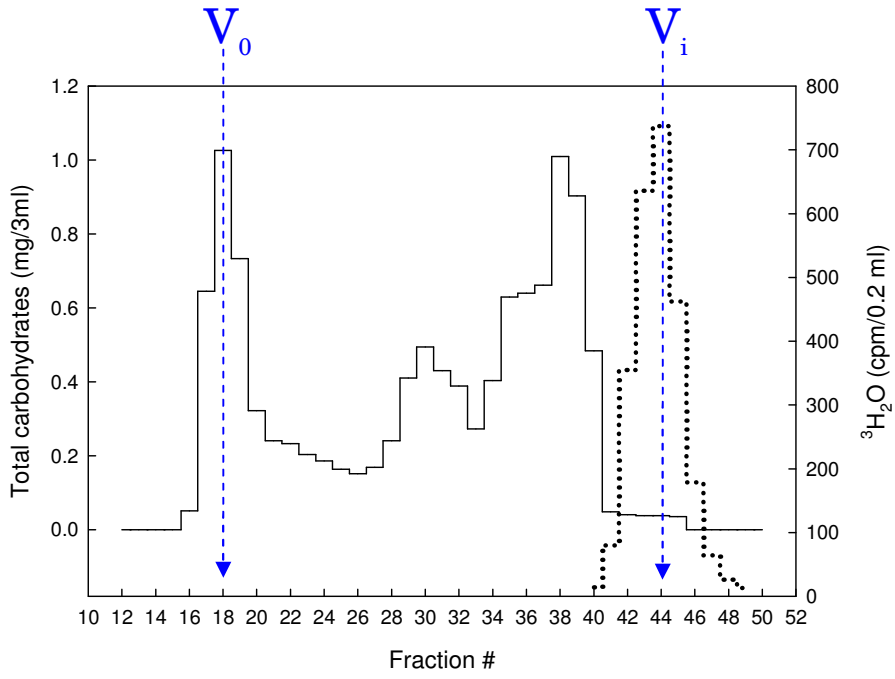


Figure 3.33: Separation of compounds in bioactive fractions from Bio-Gel P-10 on Bio-Gel P-2

Pooled partially included volume (fractions 44-75) (15 mg) from the Bio-Gel P-10 shown in Fig. 3.29, and $^{3}H_2O$, were loaded on a Bio-Gel P-2 column. Sixty 3-ml fractions were collected and tested for carbohydrates by the phenol/ sulphuric acid assay (—) and for $^{3}H_2O$ (….).

A small portion from each P-2 fraction was pooled and utilised in a bioassay, which showed that the biological activity had been successfully recovered from Bio-Gel P-2 (Fig. 3.34). The P-2 fractions (30-fold diluted) were then individually utilised in a triplicated bioassay to show in which fractions the bioactivity eluted. Several partially included fractions had bioactivity but most of these fractions showed a ('ceiling') maximum bioactivity when 0.9 ml of each fraction was used in bioassay (Fig. 3.35). In order to reveal the peak of bioactivity, a 100-fold dilution was bioassayed. Fraction 35 from P-2 column had the peak of biological activity (Fig. 3.36).

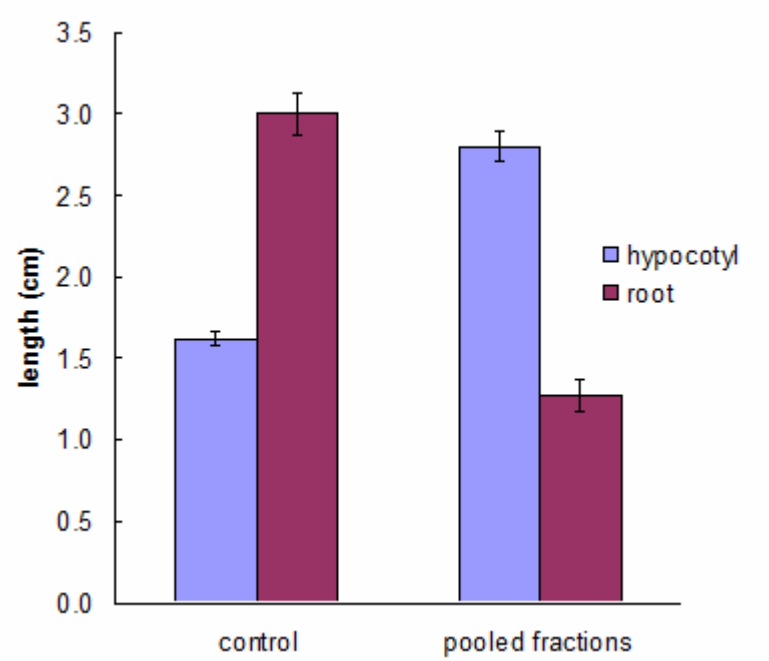


Figure 3.34: Effect of pooled fractions from Bio-Gel P-2 on *Amaranthus* seedlings

A 50-µl portion of all 60 collected (3 ml each) fractions from the P-2 run shown in Fig. 3.33 were pooled, dried and re-dissolved in 3 ml of water. *Amaranthus* seeds were then incubated in pools for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

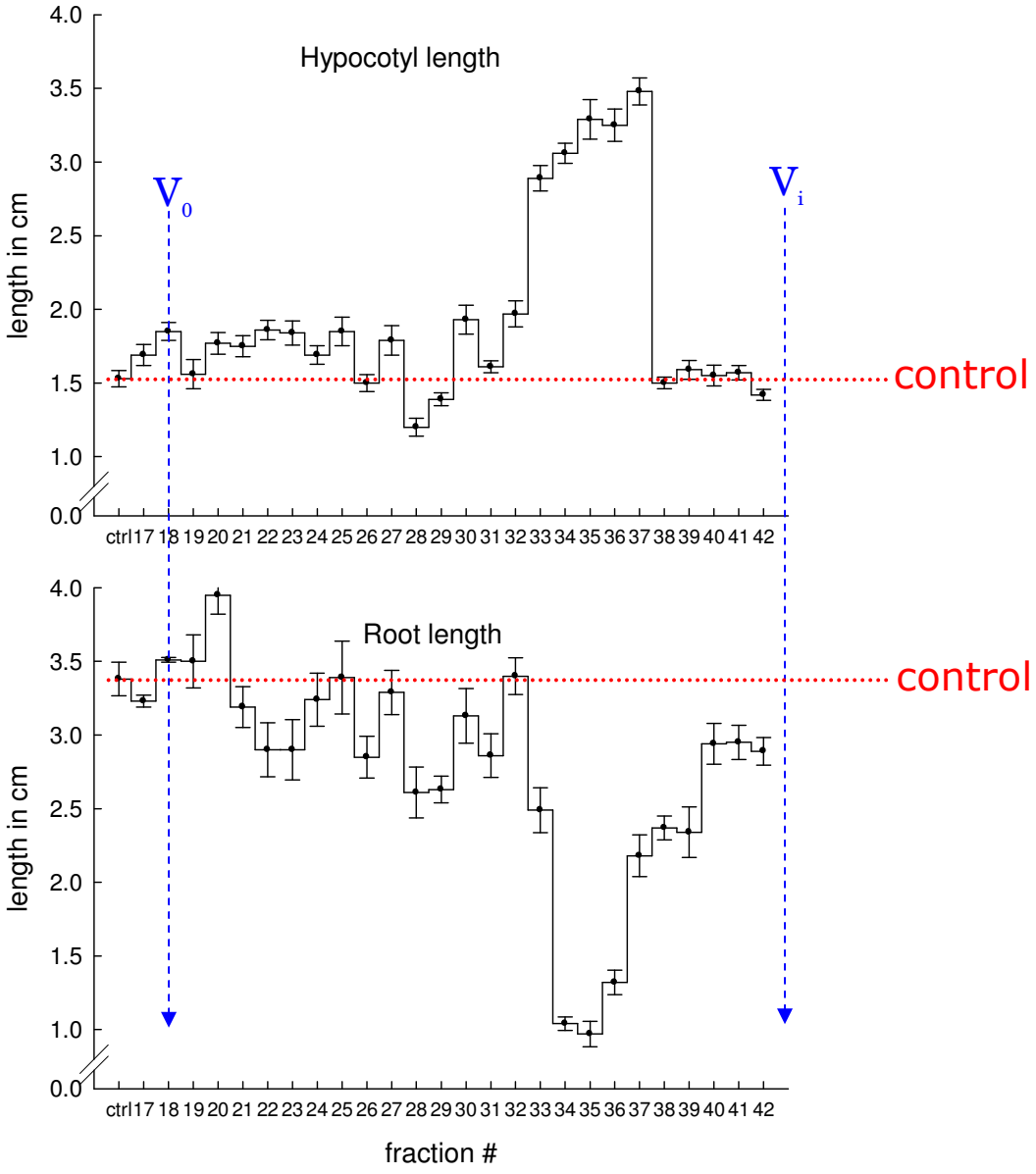


Figure 3.35: Effect of 30-fold diluted individual fractions from Bio-Gel P-2 on *Amaranthus* seedlings

Individual fractions from the P-2 run shown in Fig. 3.33 were freeze-dried and re-dissolved in 600 µl of sterile de-ionised water. 100 µl of each fraction was diluted to 3 ml with water. *Amaranthus* seeds were then incubated in the diluted fractions for 5 d at 25ºC in the dark in 3-cm Petri plates. *Amaranthus* seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

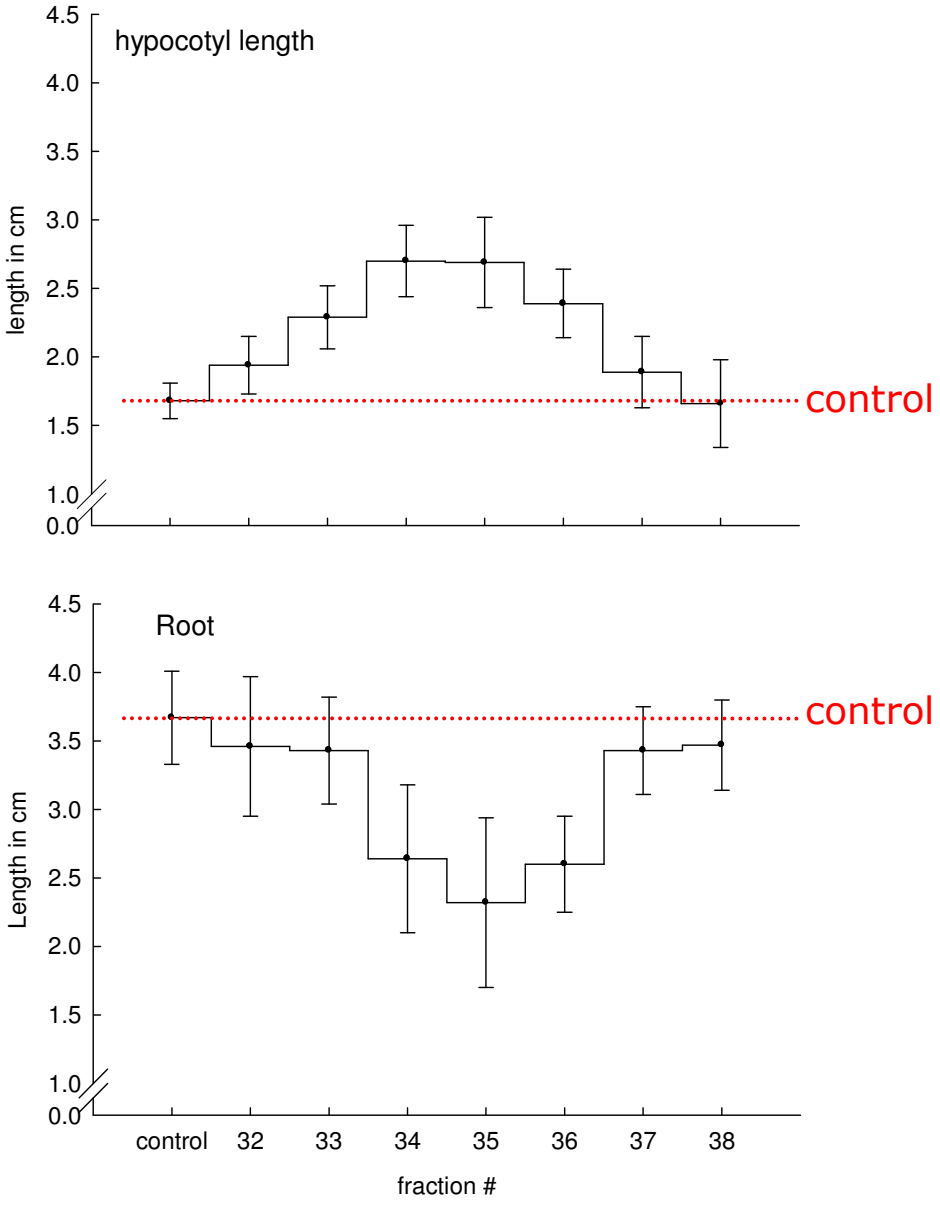


Figure 3.36: Effect of 100x diluted fractions from Bio-Gel P-2 on *Amaranthus* seedlings

Details are same as in Fig. 3.35 except that the bioactive fractions were diluted 100x and the experiment was not done in triplicate. Data represent mean of 10 seedlings from a single Petri plate with error bar representing intra-plate S.D.

I loaded fractions 29 to 39, including the bioactive fractions, from the Bio-Gel P-2 column on a TLC to see what compounds are present in the fractions of interest. Fractions 29 to 31 contained oligosaccharides which were not bioactive. A small amount of bioactivity was seen to start eluting in 32nd fraction. Fractions 33 to 36 showed highest bioactivity (as shown in Fig. 3.35) and have an intense band migrating between GalA and Gal. (Fig. 3.37), which might be the active principle (lepidimoide?).

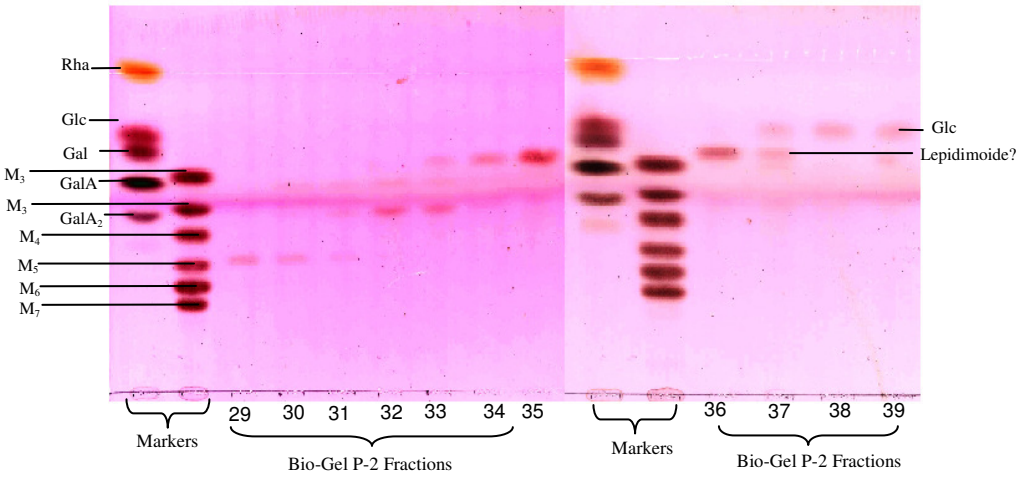


Figure 3.37: TLC showing the elution pattern of oligomers from Bio-Gel P-2
Fractions (10 µl each) from Bio-Gel P-2 column and markers were loaded on TLC. The plate was then developed in BAW (2:1:1) for 8 h and stained with thymol.

3.23.3 Fractionation of cress seed-coat mucilage by various concentrations of ethanol

Cress seed-coat mucilage was mixed with 75% of ethanol to precipitate the polysaccharides and to enable concentration of the bioactive compound. TLC showed that the ethanol-precipitated fraction had mainly polysaccharide with trace amounts of sugars, while the non-precipitated fraction had some soluble polysaccharide and most of the sugars (Fig. 3.38).

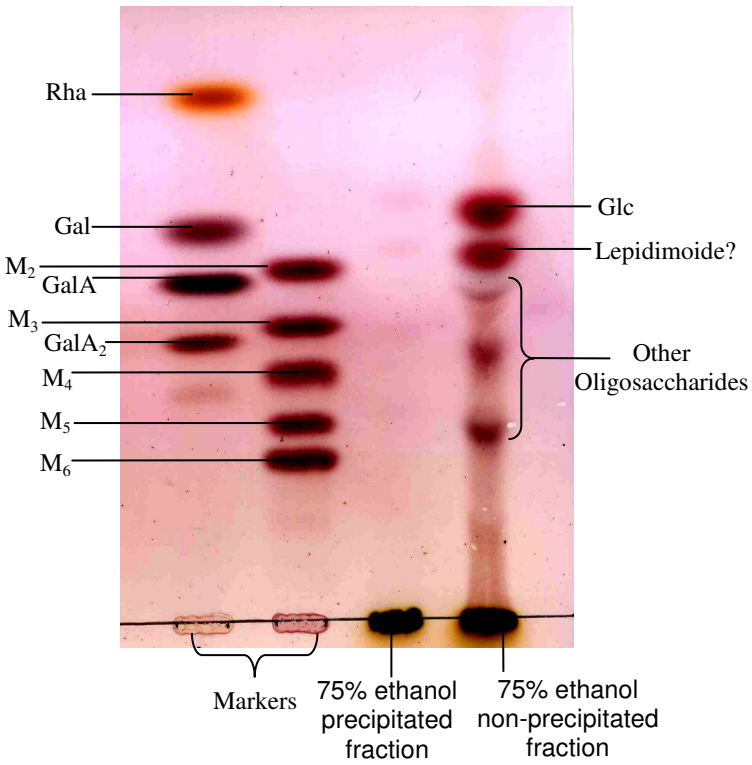


Figure 3.38: TLC showing 75% ethanol precipitated and non-precipitated fractions

Cress seed-coat mucilage was precipitated with 75% ethanol. The fractions were freeze dried and re-dissolved in sterile deionised water at 0.5% (w/v). The fractions (20 µl each) along with markers were analysed by TLC in BAW (2:1:1) for 8 h and stained with thymol.

Both 75% ethanol-precipitated and non-precipitated fractions were used in a bioassay experiment. The biologically active compound was mainly present in the non-precipitated fraction. The precipitated fraction also showed a little bioactivity which might be due to the presence of small amounts of sugars in it (Fig. 3.39).

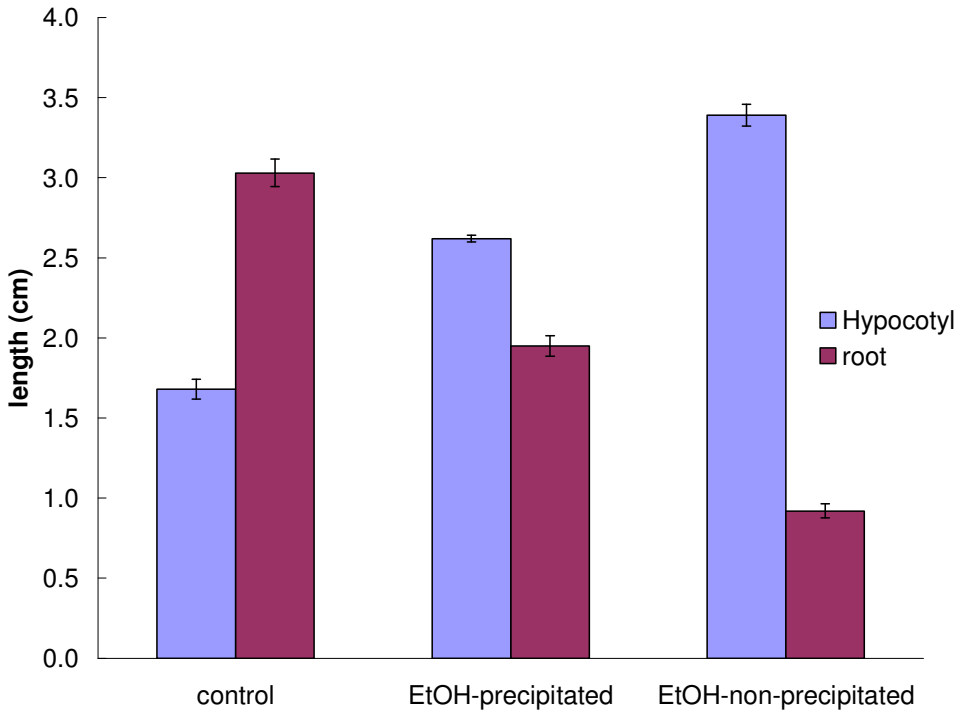


Figure 3.39: Effect of 75% ethanol-precipitated and non-precipitated cress seed-coat mucilage fractions on *Amaranthus* seedlings

EtOH-precipitated and non-precipitated fractions as shown in Fig. 3.38 were used in a bioassay. *Amaranthus* seeds were incubated in precipitated or non-precipitated fractions (2.5mg/ml) for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

Cress seed-coat mucilage was then fractioned by increasing concentrations of ethanol (70, 80 and 90% v/v) (Fig. 3.40). The 70% ethanol-precipitated fraction had polysaccharides as usual. Ethanol (80%) precipitated some polysaccharides as well as small amounts of possible oligosaccharides. The fraction precipitated by 90% ethanol did not show any compound on TLC, while the 90% ethanol-non-precipitated fraction showed large amounts of polysaccharides and sugars (Fig. 3.40).

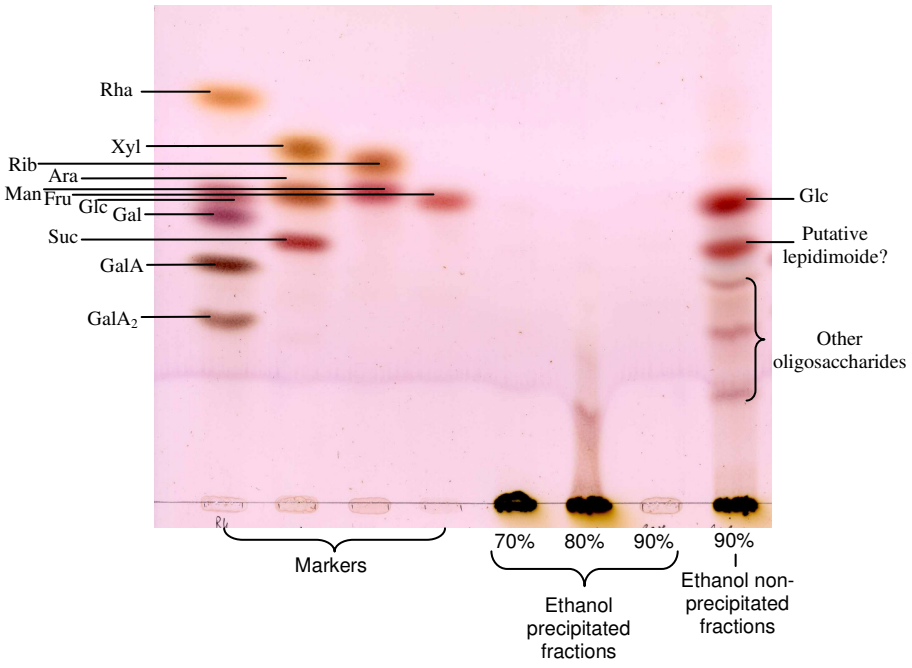


Figure 3.40: TLC showing fractionation of cress seed-coat mucilage by successive increases in ethanol concentration

Cress seed-coat mucilage (10 ml of 0.5% w/v) was fractioned with 70% ethanol and the non-precipitated fraction was adjusted to 80% ethanol followed by 90% ethanol. All fractions were freeze-dried and re-dissolved sterile de-ionised water (2.5 mg/ml) except 90% precipitated fraction, which contained lower amounts of carbohydrates and dissolved in 3-ml of water. Markers and 20 µl of each fraction were assayed by TLC. The plate was developed in BAW (2:1:1) for 8 h and stained with thymol.

All precipitated fractions from successive ethanol concentrations and the 90% ethanol-non-precipitated fraction were used in a bioassay experiment. The biological activity was mainly present in the 90% ethanol-non-precipitated fraction and might therefore be of low molecular weight. The 90% ethanol-precipitated fraction also showed a little bioactivity which might be due to the presence of small amounts of sugars that were not detected by thymol stain (Fig. 3.40). The result also indicated that ethanol fractionation is one of the good steps in initial purification of bioactive compound.

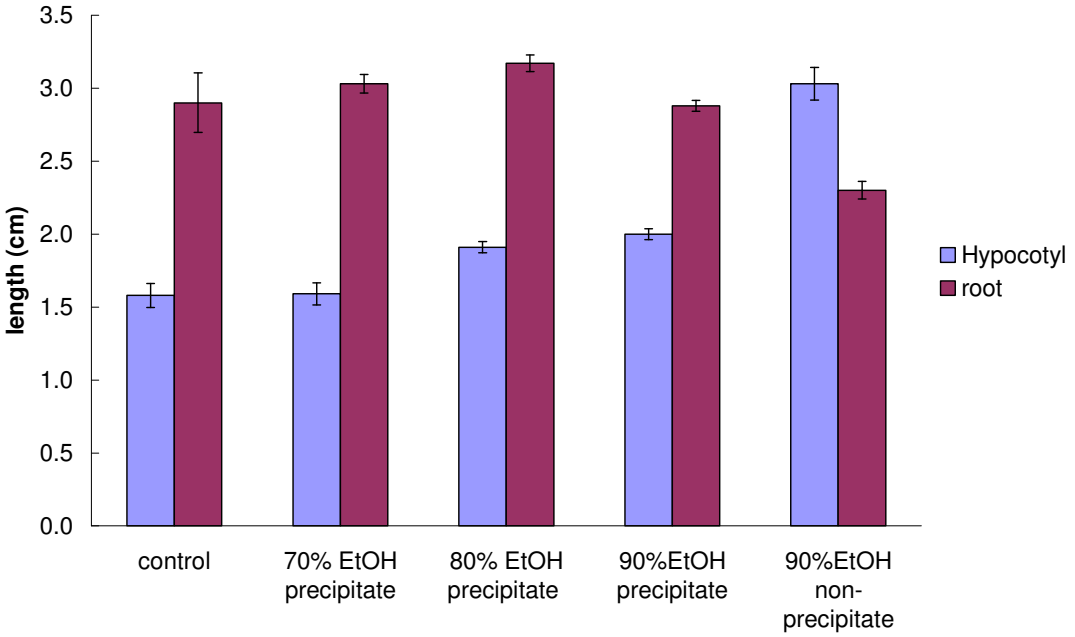


Figure 3.41: Effect of ethanol-precipitated and non-precipitated cress seed-coat mucilage fractions on *Amaranthus* seedlings
EtOH-precipitated and non-precipitated fractions as shown in Fig. 3.40 were used in a bioassay. *Amaranthus* seeds were incubated in precipitated or non-precipitated fractions (2.5 mg/ml) for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

3.23.4 Fractionation of ethanol non-precipitated fraction of cress seed-coat mucilage on Bio-Gel P-10

Because both 90% EtOH-precipitated and non-precipitated fractions showed bioactivity, the mucilage was fractioned into 80% precipitated and non-precipitated fractions. The 80% ethanol non-precipitated fraction of mucilage that contained an internal marker of $^{3}H_2O$ was then loaded on Bio-Gel P-10 to separate the oligosaccharides from the left-over polysaccharides. The void volume containing polysaccharides peaked in fraction 20 while tritiated water as included volume peaked in fraction 64. The oligosaccharides were assumed to be eluted between fractions 35 and 76 (Fig. 3.42).

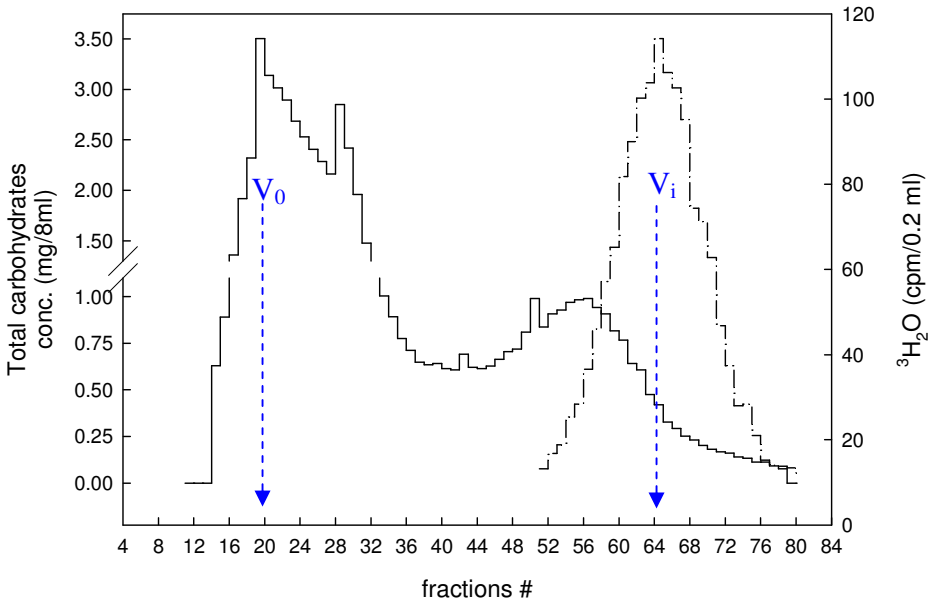


Figure 3.42: Separation of compounds in 80% ethanol-non-precipitated cress seed-coat mucilage on Bio-Gel P-10

Cress seed-coat mucilage was first fractioned by 80% ethanol into precipitated and non-precipitated fraction. The non-precipitated fraction (80 mg) after centrifugation and $^{3}H_2O$ were loaded on Bio-Gel P-10. Eighty 8-ml fractions were collected. Collected fractions were tested for carbohydrates by the phenol/ sulphuric acid assay (—) and for $^{3}H_2O$ (–.–).

Again because of the difficulty in utilising each of 80 fractions in a triplicate bioassay experiment individually, the fractions from the Bio-Gel P-10 column were combined into 15 pools. Each pool was then utilised in a triplicated bioassay. The bioactivity started to elute in the 9th pool and had its maximum in the 11th (Fig. 3.43). From the result it was concluded that Bio-Gel P-10 further purified the bioactive compound.

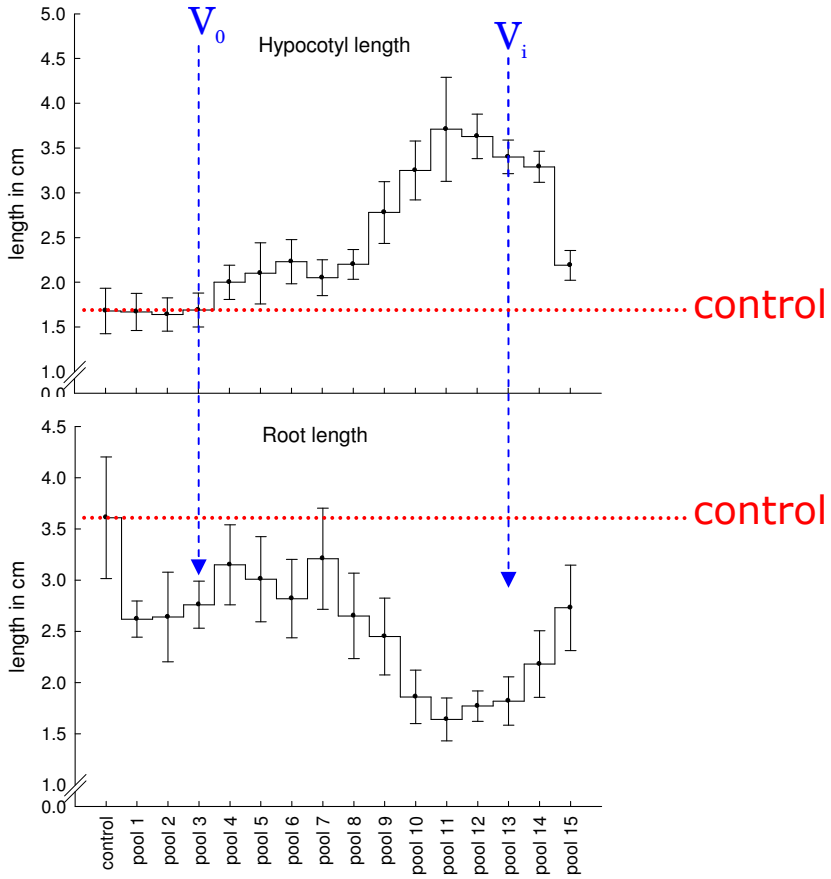


Figure 3.43: Effect of pooled fractions of ethanol non-precipitated cress seed-coat mucilage from Bio-Gel P-10 column on *Amaranthus*

Fractions of previous run (Fig. 3.42) were pooled. Each pool contains 4 fractions starting from fraction no. 16. The pools were freeze dried and re-dissolved in 6 ml of sterile de-ionised water. *Amaranthus* seeds were incubated in these pools (0.9 ml each) for 5 d at 25ºC in the dark in 3-cm Petri plates Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots of were measured by use of Labwork software. Data represent mean of 10 seedlings from a single Petri plate with error bar representing intra-plate S.D.

To see the separation pattern of compounds present in the fractions of interest, I analysed the pools and markers by TLC. The first five pools mainly contained polysaccharides. Small amounts of chromatographically mobile compounds were started being eluted in the 8th pool. Higher concentrations of monosaccharide and oligosaccharides were eluted in pools 9 to 12 (Fig. 3.44), which had been proved to be bioactive in the previous bioassay experiment (Fig. 3.43).

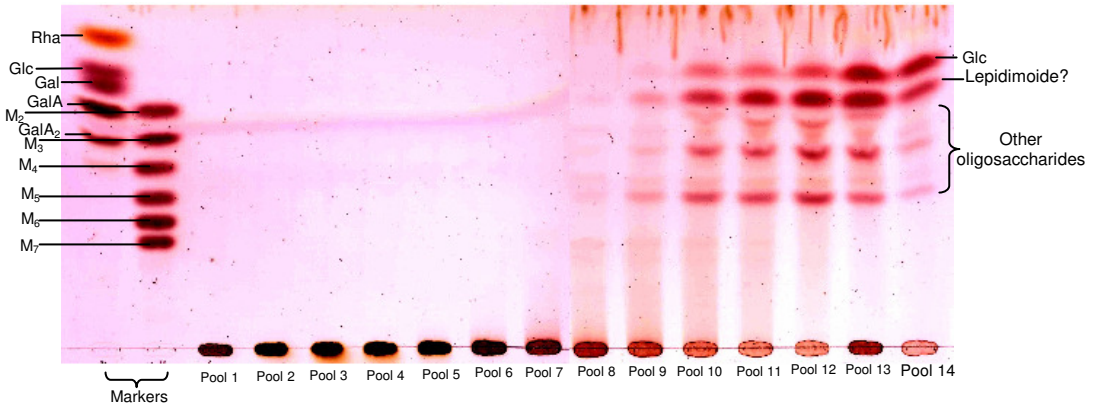


Figure 3.44: TLC showing the separation of compounds present in pooled fractions from Bio-Gel P-10

Pooled fractions (20 µl) as shown in Fig. 3.43, and markers were loaded on TLC. The plate was then developed in BAW (2:1:1) for 8 h and stained with thymol.

3.23.5 Further purification of partially included volume fractions (previously, 80% EtOH non-precipitated fraction) from Bio-Gel P-10 on Bio-Gel P-2

For the further fractionation and partial purification of bioactive compound(s), the included volume (bioactive) fractions from P-10 were pooled and re-run on Bio-Gel P-2. The sample gave three carbohydrates peaks which meant that the P-10 fractions were further resolved on the P-2 column (Fig. 3.45).

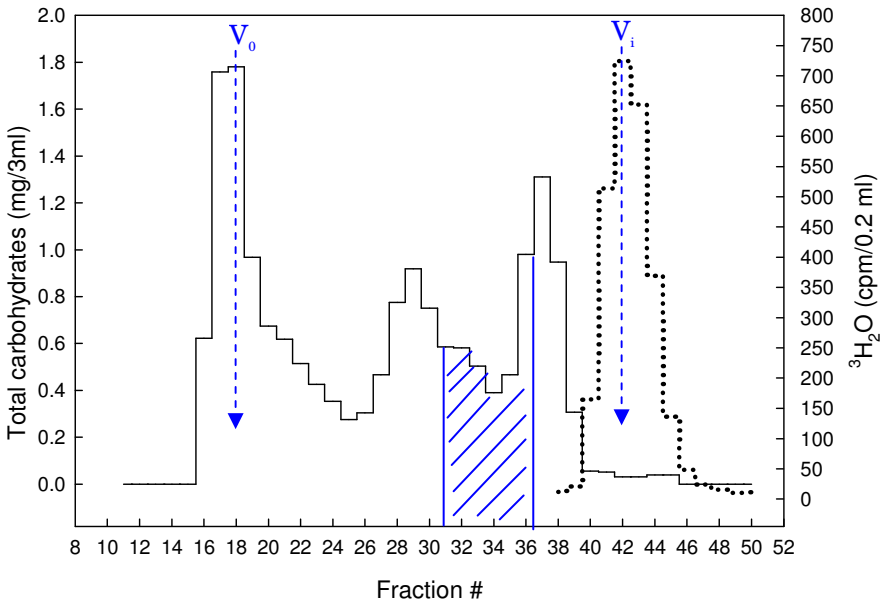


Figure 3.45: Separation of compounds present in included volume fractions (previously, 80% ethanol non-precipitated fraction) from Bio-Gel P-10 on Bio-Gel P-2

Pooled included volume fraction (44-75) (18 mg) from Bio-Gel P-10 (Fig. 3.42) and ^3H_2O was loaded on Bio-Gel P-2. Sixty 3-ml fractions were then tested for carbohydrates by phenol/ sulphuric acid (—) and for ^3H_2O (.....). The printed area was used for preparative TLC (Fig. 3.48) and bioassay (3.49).

These P-2 fractions were individually utilised in a triplicated bioassay. Fractions 31 to 36 had bioactivity, the peak being in fraction 33 (Fig. 3.46).

Fractions 29 to 39, including the bioactive fractions, from Bio-Gel P-2 were analysed by TLC with four combinations of solvent systems and stains. Fractions 29 and 30 contained oligosaccharides which were not bioactive. Fractions 32 to 35, which showed the highest bioactivity, had an intense band that migrated between GalA and Gal but close to sucrose (Fig. 3.47). To prove that the compound is not sucrose, paper chromatography and bioassay of the PC strips and TLC bands were done (Fig. 3.49, 3.50 and 3.51).

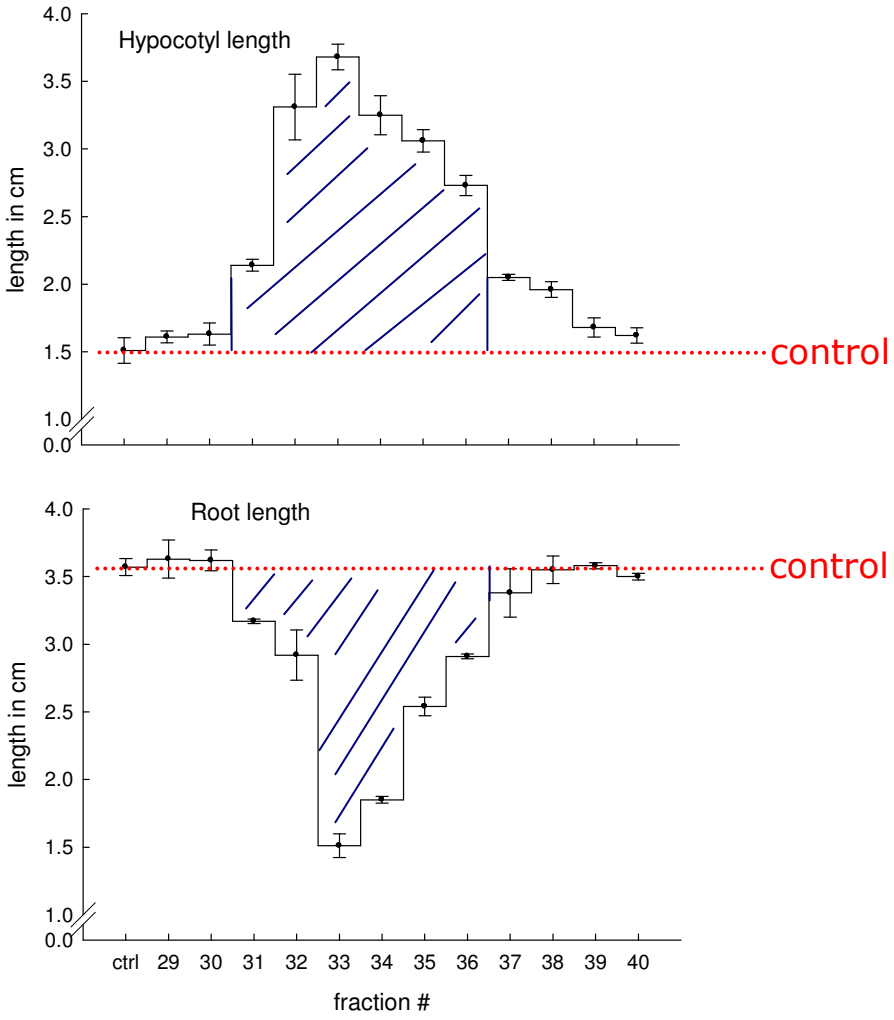


Figure 3.46: Effect of fractions from Bio-Gel P-2 column (previously non-precipitated fraction with 80% ethanol) on *Amaranthus* seedlings

Individual fractions from the P-2 run shown in Fig. 3.45 were freeze-dried and re-dissolved in 600 µl of sterile de-ionised water. 100 µl of each fraction was diluted to 3 ml with water. *Amaranthus* seeds were then incubated in diluted fractions from the P-2 for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E. The area refers to bioactive band of preparative TLC (Fig. 3.48) and bioassay (Fig. 3.49).

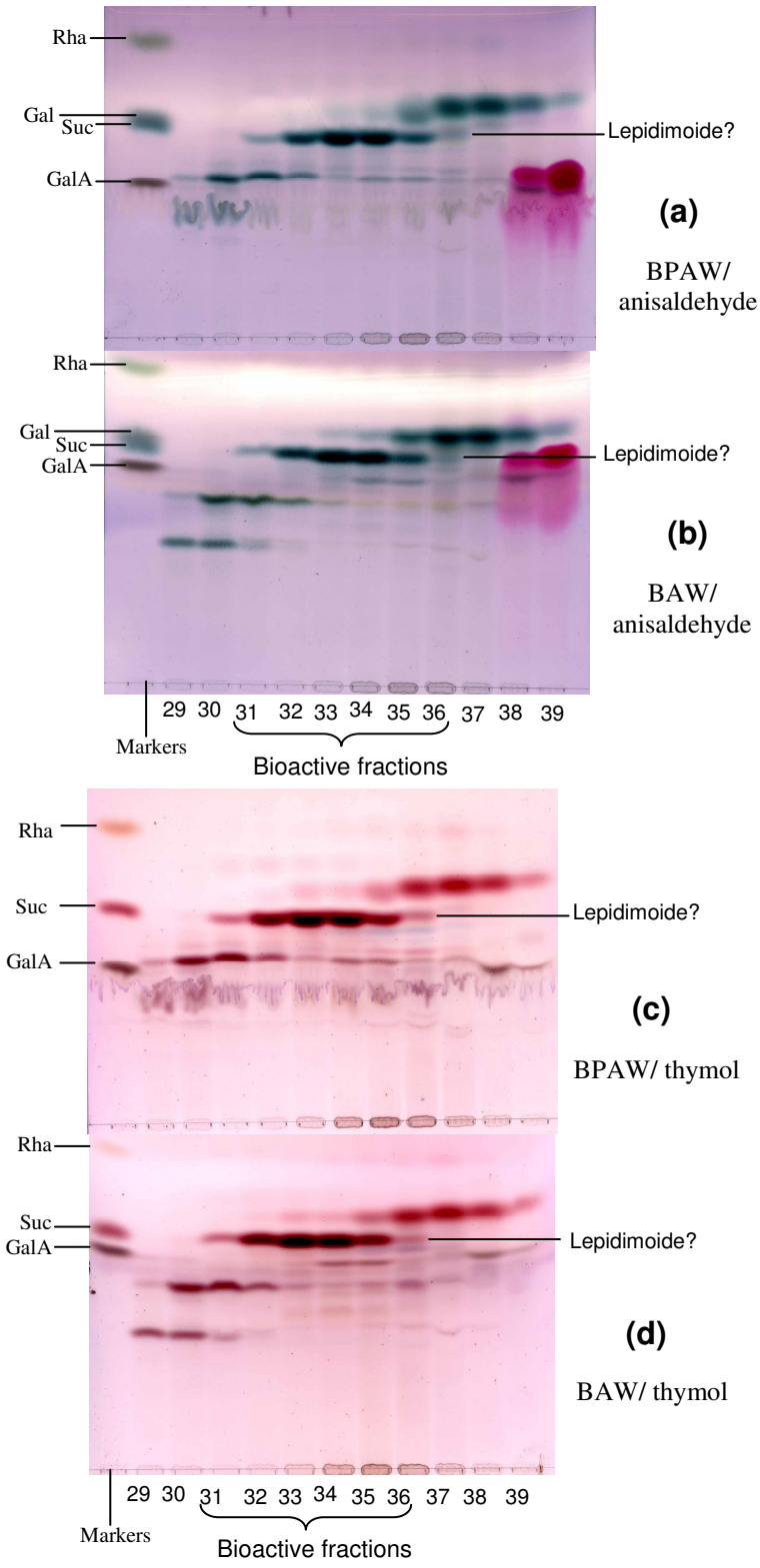


Figure 3.47: TLCs showing the separation of compounds present in Bio-Gel P-2

Partially included fractions (10 µl each) as shown in Fig. 3.45 were loaded on TLCs. Four combinations of solvent systems and stains were used. The plates were developed for 8 h, (a) BPAW (7:5:4:2), (b) BAW (2:1:1) and stained with *p*-anisaldehyde, (c) BPAW (7:5:4:2) and (d) BAW (2:1:1) and stained with thymol.

3.23.6 Preparative TLC of bioactive fractions from Bio-Gel P-2

In oredr to purify the bioactive compound(s) a preparative TLC was done. The bioactive fractions that were collected from Bio-Gel P-2 column (Fig. 3.47) were pooled and a portion was streak-loaded on a TLC. Sample spots on each side of the TLC acted as a guide marker. After the development of the TLC, the middle portion where the sample for bioassay had been loaded was cut out and the markers were stained. Individual bands, localised with reference to the stained guide markers, were eluted (Fig. 3.48) and bioassayed.

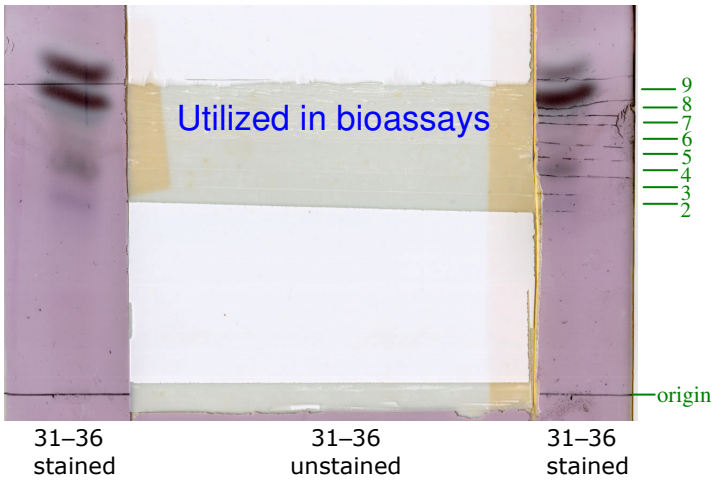


Figure 3.48: Preparative TLC of pooled bioactive fractions from Bio-Gel P-2
Bioactive fractions shown in Fig. 3.47 were pooled and 150 µl of this mixture was loaded on TLC. Two spots of same sample (20 µl) as a guide marker were also loaded on each side. After development in BPAW (7:5:4:2) the area designated for the bioassay experiment was cut out and the remaining parts containing guide markers were stained with *p*-anisaldehyde. The separated compounds from the cut part of TLC were scratched out with reference to the stained guide marker.

3.23.7 Bioassay of the separated compounds on TLC

The compounds present in bioactive fractions from Bio-Gel P-2 that had been further separated by TLC (Fig. 3.48) were eluted and tested individually for bioactivity. The compound or compounds in zones 8 and 9, which is where disaccharides normally migrate, were bioactive (Fig. 3.49).

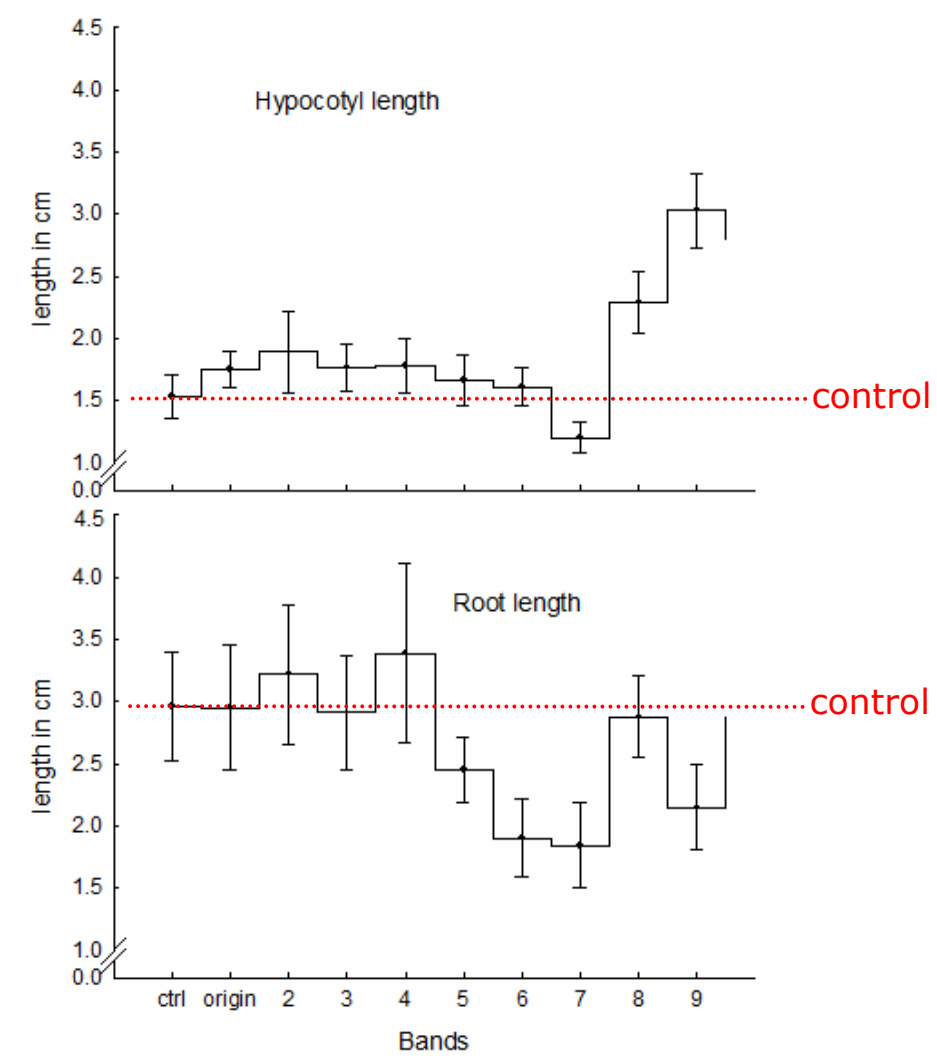


Figure 3.49: Effect of TLC,-separated compounds on *Amaranthus* seedlings
Pooled bioactive fractions (Fig. 3.46) were further separated on TLC (Fig. 3.48). The band were eluted individually, freeze dried and re-dissolved in 1 ml of sterile water. *Amaranthus* seeds were then incubated in eluted compounds for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent mean of 10 seedlings from a single Petri plate with error bar representing intra-plate S.D.

3.23.8 Preparative paper chromatography of pooled bioactive fractions from Bio-Gel P-2

This experiment was done to prove that the bioactive compound(s) are different than sucrose. Pooled bioactive fractions (Fig. 3.46) were streak-loaded on paper along with spot-loadings as a guide marker. After development, 5-cm strips of the middle part of the chromatogram were eluted and bioassayed, and the markers were stained. The guide markers showed various spots which migrated very close to each other (Fig. 3.50).

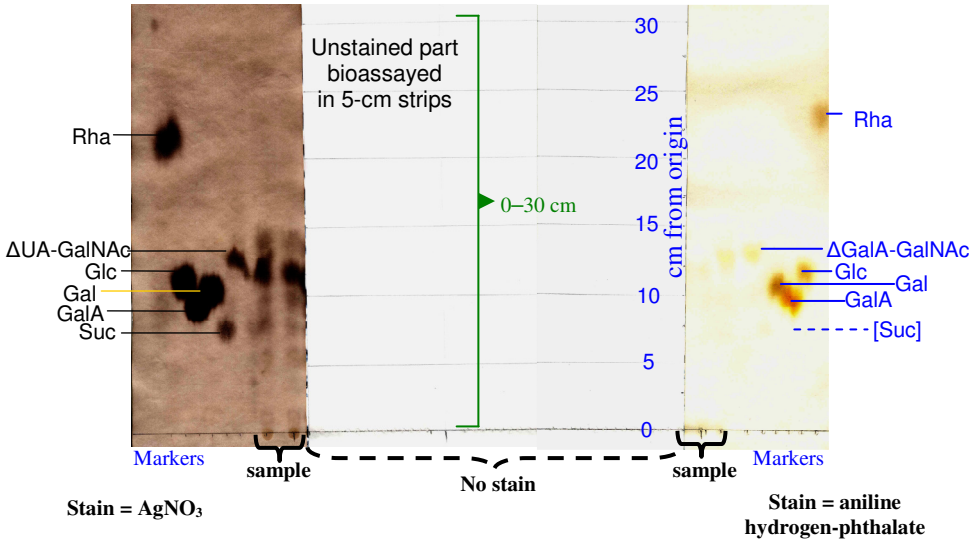


Figure 3.50: Preparative PC of pooled bioactive fractions from Bio-Gel P-2
Bioactive pool (250 µl) (as shown in Fig.3.46) was loaded on PC. Two spots of same sample (30 µl) as a guide markers and authentic markers (15 µg) were also loaded on each side of PC. After development in BAW (12:5:3) the designated 15-cm width for the bioassay was cut out, and the guide markers were stained with silver or Wilson's dip. The un-stained part of PC was cut into six 5-cm strips and the compounds from each strip were eluted.

3.23.9 Bioassay of the compounds from PC

The compounds from each of the 6 unstained 5-cm PC strip (Fig. 3.50) were eluted and used in a bioassay. The eluted compound(s) that migrated 10-15 and 15-20 cm on the PC were bioactive, which proved that the compound previously co-migrating with sucrose on TLC (Fig. 3.47) is not sucrose (Fig. 3.50). The compound(s) in at least 15-20 cm were not well stained with either silver or Wilson's dip so they were re-chromatographed on TLC.

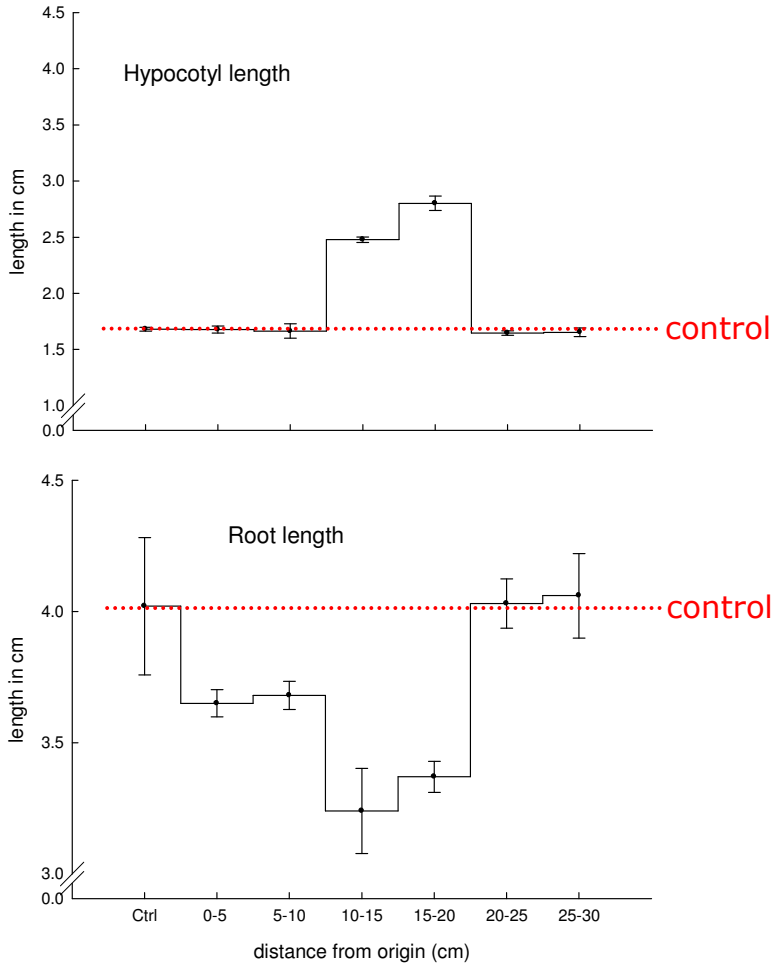


Figure 3.51: Effect of eluted compounds from PC on *Amaranthus* seedlings
Compounds from the PC (Fig. 3.51) were eluted, dried, re-dissolved in 200 µl of sterile water, 150 µl of which was diluted to 3 ml and used in bioassay. *Amaranthus* seeds were then incubated in it for 5 d at 25ºC in the dark in 3-cm Petri plates. Seeds incubated in sterile water alone were used as a control. Hypocotyls and roots were measured by use of Labwork software. Data represent means from triplicate Petri plates with error bars representing inter-plate S.E.

3.23.10 TLC of compounds from PC strips

The un-stained compounds from the 10-20 cm PC strips were combined and loaded on a TLC with markers. A thymol-stainable compound that had run faster than sucrose on PC (Fig. 3.50) was observed (Fig. 3.52), suggesting that the bioactive compound might be a sugar with unique properties.

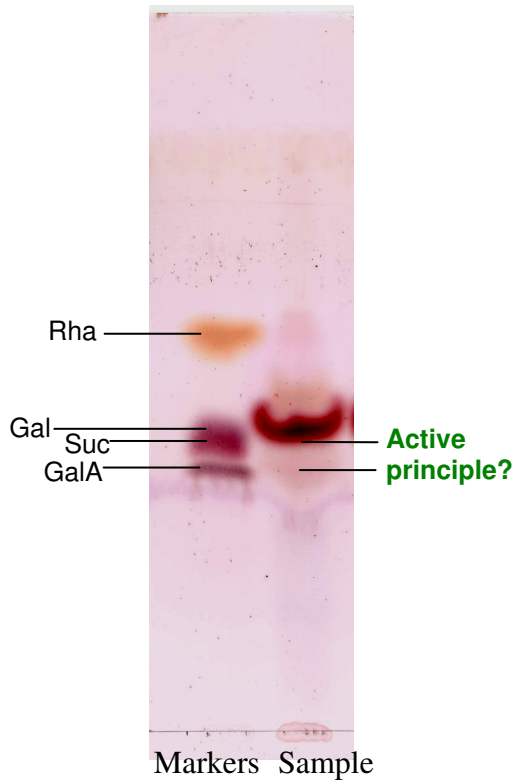


Figure 3.52: TLC showing the presence of compounds in the 10-20 cm strips of PC

Eluted bioactive compounds from PC strips (10-20 cm) and markers were loaded on TLC. The plate was then developed in BAW (2:1:1) for 8 h and stained with thymol.

3.24 Preparation of lepidimoide or lepidimoide-like compounds

3.24.1 Methyl esterification and β-elimination of RG-I

In order to produce lepidimoide and lepidimoide-like compounds in laboratory methyl esterification and β-elimination of Purified RG-I was done. Purified RG-I was dissolved in water, methylesterified with tetrabutylammonium fluoride, dimethyl sulphoxide and methyl iodide, and purified on Bio-Gel P-10 (void volume).

The product was then dissolved in aqueous sodium borate (pH 7.3) and heated. TLC indicated the formation of some β-eliminated oligosaccharides, one of which migrated between Gal and GalA and might be lepidimoide (Fig. 3.53). When I utilised the β-eliminated oligosaccharides (2.5 mg/ml) in an *Amaranthus* seeds bioassay, the seeds did not germinate, which might be due to high concentration of borate (results not shown).

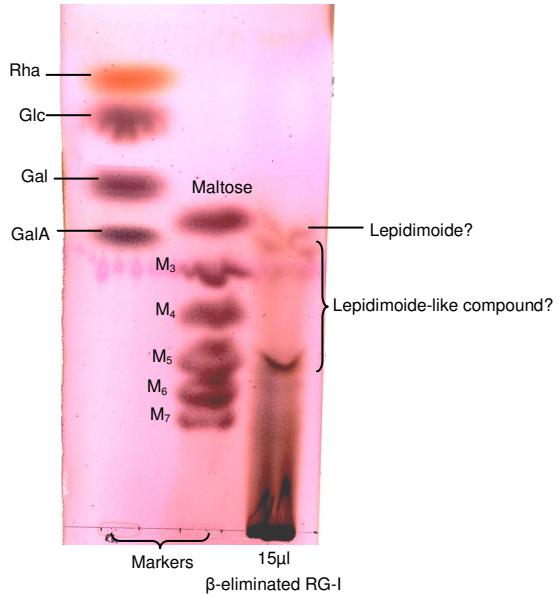


Figure 3.53: TLC showing the β-eliminated product of potato RG-I
Potato RG-I was methyl esterified and β-eliminated. The β-elimination product (15µl) and marker were loaded on a TLC plate and developed in BAW (2:1:1) for 8 h. The plate was stained with thymol.

3.24.2 Treatment of potato RG-I with lyases from transgenic Pichia pastoris

This experiment was followed to prepare lepidimoide or lepidimoide-like compounds by the action of lyase on potato RG-I. Purified potato RG-I was incubated with extracellular enzyme from *Pichia pastoris* clone 10108 (which secretes RG-I lyase). TLC showed that oligomers from the RG-I were produced by the action of RG-I lyase from *Pichia pastoris* clone 10108 (Fig. 3.54). The oligomers (2.5 mg/ml) were then utilised in a bioassay but *Amaranthus* seeds did not germinate, which might be due to the presence of tris-HCl buffer (result not shown).

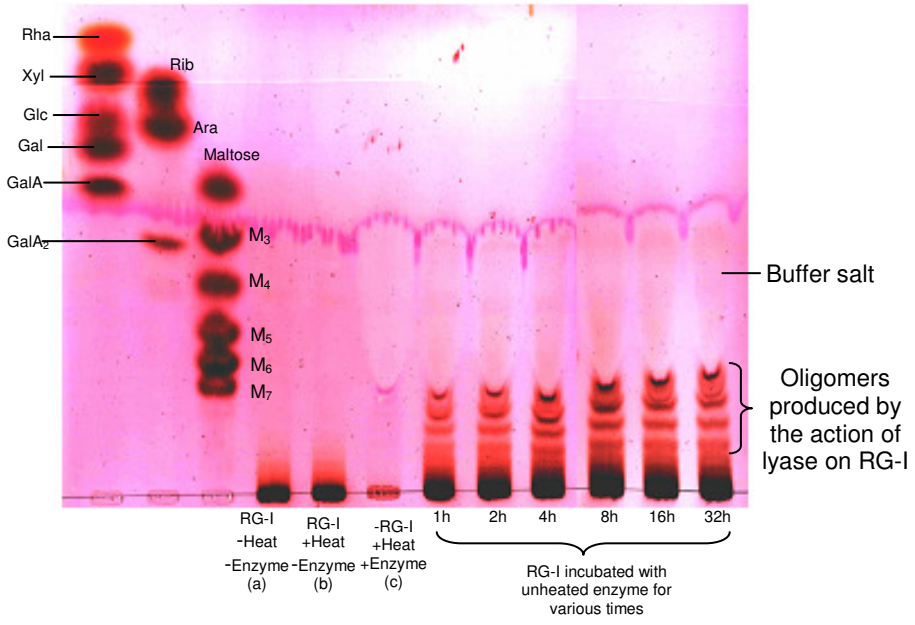


Figure 3.54: TLC showing the enzymic β-elimination products of potato RG-I
Potato RG-I was incubated at 30ºC with enzyme from *Pichia pastoris* clone 10108 for various time. After incubation the reaction was terminated by putting the tube in a boiling water bath for 5 min. The product (20µl) and markers were then loaded on a TLC plate and developed in BAW (2:1:1) for 8 h. The plates were stained with thymol. (a), (b) and (c) were used as a control.a=RG-I solution incubated without enzyme and heat treatment, b=RG-I incubated and heated without enzyme and c=enzyme incubated and heated without RG-I.

4 Discussion

4.1 Evidence of cress allelopathy

The starting point of this study was based on the allelopathic effect of cress root exudate on other plant species. Hasegawa *et al.* (1992) first discovered that cress root exudate has an allelopathic effect on *Amaranthus* seedlings. The purified and identified bioactive compound was a disaccharide and is known as lepidimoide. Tomita *et al.* (1998) and Hirose *et al.* (2004) also confirmed the biological activity of lepidimoide. For the first experiments of my research, I also selected *Amaranthus* as a receiver species because of its fast growth and easy availability, and to facilitate comparing the results with those of Hasegawa *et al.* (1992). My results confirmed that *Amaranthus* seedlings incubated with cress seedlings had excessively elongated hypocotyls and shorter roots than those incubated without cress (Fig. 3.3); the results were in close agreement with the findings of Hasegawa *et al.* (1992).

4.2 Possible role of surface microbes

Although the structure and biological activity of lepidimoide had been reported by both Hasegawa *et al.* (1992) and Tomita *et al.* (1998), it was not clear whether the compound was released by the cress itself or was a digestion product formed from cress by microbes. In order to determine this, I repeated the same experiment under both aseptic and non-aseptic conditions. The results showed that *Amaranthus* seedlings from both aseptic and non-aseptic treatments had longer and thinner hypocotyls and shorter and thicker roots when incubated with cress than when incubated alone (Fig. 3.4). From these results I concluded that the cress might exude

allelopathic compound(s) or might compete with *Amaranthus* for dissolved oxygen in the medium (there was no other nutrient present in the medium, de-ionised water, for potential competition).

4.3 Allelopathy vs competition

To study whether the effect of cress seedlings on the growth of *Amaranthus* seedlings was due to competition or was an effect of cress root exudates I incubated *Amaranthus* seedlings in cress root exudate. *Amaranthus* seedlings incubated with cress root exudate had longer and thinner hypocotyls as well as shorter and thicker roots, even though no living cress tissue was present (Fig. 3.5). This evidence meant that the effect of cress on another species is not due to competition but might be an effect of the cress root exudate or possibly of synthetic chemicals used in seed treatment such as fungicides, herbicides and pesticides.

4.4 Bioassay of 'organic' cress seed-coat mucilage

To test this latter possibility, I used 'organic' cress seed-coat mucilage, free of synthetic chemicals. It was observed that *Amaranthus* seedlings incubated in it had hypocotyl and root length and width significantly affected when compared to the control (3.14). Therefore, the results suggest that the effect of mucilage on *Amaranthus* seedlings was not due to seed treatment chemicals.

4.5 Bioassay of home-grown cress seed-coat mucilage

Although the 'organic' seeds were stated to be free from synthetic chemicals, there was still a possibility that they had been treated with sources of natural hormones

(e.g. auxins and gibberellins). Since the mucilage from home-grown seeds showed biological activity towards *Amaranthus* seedlings (Fig. 3.15), we may deduce that neither competition nor seed-treatment chemicals are responsible for the apparent allelopathic effect. So the effect on *Amaranthus* seedlings was due to the compound(s) which are naturally exuded from cress seeds.

4.6 Carbohydrate composition of cress root exudate

According to Emma *et al.* (2001) the mucilage of root exudate from most plants consists of 95-97% carbohydrates (dry weight basis) and 5-3% proteins, amino acids, vitamins and minerals. Ray *et al.* (1988) studied the carbohydrate composition of cress root exudate and suggested that it consists of high amounts of hexose, pentose and uronic acid residues. The results of my research also indicated a high amount of carbohydrates (84% of the exuded mucilage dry weight) and are in agreement with those of Emma *et al.* (2001). The exudate also contained high amounts of free and/or polymer-bound hexoses, pentoses and uronic acid (Table 3.1), confirming the findings of Ray *et al*. (1988). Unsaturated uronic acid was also found in the cress exudate, which might be an important residue of the active principle since the structure of lepidimoide suggested by Hasegawa *et al.* (1992) contains an unsaturated UA residue.

The qualitative results showed the presence of various classes of carbohydrates, most of which were polysaccharides. The sample was separated by TLC and six migrating bands were seen (Fig. 3.6). One of the bands, which ran between galactose and galacturonic acid might be lepidimoide because the results of

Tanaka *et al.* (2002) showed that authentic lepidimoide migrated between Gal and GalA.

The results from electrophoresis experiments (Fig. 3.7) suggested that the non-polymeric compounds present in the exudate included acidic and neutral species at pH 3.5. One of the compounds ran faster than GalA but slower than GlcA; that compound might be a bioactive oligosaccharide (lepidimoide?).

4.7 Bioactivity of cress root exudates vs cress seed-coat mucilage

Hasegawa *et al.* (1992) proposed the possibility that cress plants may protect themselves by exuding from roots an allelopathic substance (lepidimoide), which might affect the growth of other plant species. The results of the present study showed that cress seed-coat mucilage exhibits higher bioactivity towards *Amaranthus* hypocotyl elongation than cress root exudate, while the effect on root elongation was not different from that of the root exudate. The result was quite surprising and suggested that either the seed-coat mucilage contained a new bioactive compound(s) different from the one found by *Hasegawa et al.* (1992) in cress root exudate or that the amount of the same bioactive compound (lepidimoide) was higher in seed-coat mucilage.

4.8 Changes in allelochemical levels during imbibition

Collection of cress seed-coat mucilage at various imbibition times showed that the bioactive compound or compounds affecting hypocotyls and roots of *Amaranthus* seedlings were released into their surroundings within the first few minutes. This

quick release of bioactivity suggested that the allelochemical(s) might be produced and/or stored in the seed coat and released into the environment, possibly as a defence mechanism, as soon as the seeds are imbibed.

4.9 Effect of cress allelochemical(s) on extent and direction of epidermal cell expansion

Epidermal cells of *Amaranthus* hypocotyls that had been incubated in cress seed-coat mucilage were narrower and longer than water-treated controls (Fig. 3.17). Also, the ratio of hypocotyl length to cell length was higher in the treatment than in the control, and similarly the ratio of hypocotyl diameter to cell width was higher in treated than in control seedlings. These findings suggest that the bioactive compound(s) promoted both cell elongation and cell division in *Amaranthus* hypocotyl.

4.10 Carbohydrate composition of cress seed-coat mucilage

Total cress seed-coat mucilage contained high amounts of free and/or polymer-bound hexoses, pentoses and uronic acids, agreeing with the results of Ray *et al*. (1988). Unsaturated uronic acid, which might be an important residue of the active principle, was found to be higher in seed-coat mucilage than in cress root exudates, which is in agreement with bioassay experiment.

As the quantitative test results showed the presence of various classes of carbohydrates, I separated them by TLC in three different solvent systems. In all three systems a sample band that migrated between Gal and GalA was observed.

According to results of Tanaka *et al.* (2002), authentic lepidimoide migrated between the Gal and GalA, suggesting that the corresponding TLC band observed in this study may have been lepidimoide, a bioactive compound.

Two of the sample bands in mucilage, as separated on a paper chromatogram, were identified as fructose and glucose. A streak of slow-migrating compounds was also observed, which might be a mixture of mono- and oligosaccharides, some of which may exhibit bioactivity.

The results from electrophoresis experiments showed the presence of acidic sugars. The acidic sugars in cress seed-coat mucilage were well separated from each other and none of them co-migrated with the authentic markers, which means that the mucilage contains unfamiliar acidic sugars, some of which may have biological activity.

4.11 Properties of bioactive compounds present in cress seed-coat mucilage

Plants exude a vast array of secondary metabolites, which might include hormones or bioactive compounds (oligosaccharins?). To separate any classical plant hormones from the other exudates compounds, partitioning between polar and non-polar phase at various pH values is one of the most effective ways (Nakayama *et al.*, 1996). In this study, at all tested pH values (2, 6.5, 12), the stainable carbohydrates in the mucilage were hydrophilic, as judged by solvent partitioning (H_2O/ EtOAc), as were the bioactive compound(s). Although the exudates exhibit auxin- and gibberellin-like

activity, the major active principles present were not any of these because at pH 2 both auxin and gibberellins partitioned in to EtOAc phase rather than water phase (Kelen and Sanli., 2009; Nakayama *et al.*, 1996). In addition at pH 12, any auxins or gibberellins with hydrophilic ester-linked groups attached would have been hydrolysed to (hydrophilic) free auxins or gibberellins. Heat treatment of the bioactive compound(s) in mucilage showed that the compound(s) are stable at high temperatures for a long time and might be a useful method in future to sterilize the bioactive compound(s).

4.12 Mr of cress seed-coat mucilage components

Hasegawa *et al.* (1992) separated the cress root exudate into acetone non-precipitated and acetone-precipitated fractions. The acetone-precipitated fraction was further partitioned by GPC. The biological activity was observed in the fraction of M_r below 5×10^3. In this study, I initially separated the compounds present in cress seed-coat mucilage by various concentrations of ethanol and then by GPC (on Bio-Gel P-10 and P-2). The partially included volume fractions from P-2 were observed to be bioactive, suggesting that the bioactive compound(s) is of low M_r like lepidimoide and are in agreement with Hasegawa *et al.* (1992).

The bioactive fractions from P-2 were further purified on TLC. A prominent band exhibiting biological activity migrated between Gal and GalA (like lepidimoide) but closely co-migrated with sucrose. However, the bioactive compound(s) showed much faster migration on PC than sucrose, showing that it is not sucrose but a potent allelopathic compound other than sucrose.

4.13 Preparation of lepidimoide or lepidimoide-like compounds

Deng *et al*. (2006) prepared oligosaccharides by methyl esterification and β-elimination of mucilage from *Arabidopsis*. These oligosaccharides contained unsaturated UA and Rha and were therefore related to lepidimoide. I followed their procedure and tried to prepare lepidimoide from purified potato RG-I. The result of my experiment indicated the formation of some oligosaccharides, one of which migrated between Gal and GalA and may be lepidimoide. However, when I utilised these β-elimination products (2.5 mg/ml) in a bioassay experiment, no seeds were able to germinate, which might be due to the high borate concentration in the sample (results not shown).

Tanaka *et al.* (2002) also prepared lepidimoide from okra mucilage by the action of an endophytic fungal strain. In this study, I used a *Pichia pastoris* clone expressing RG-I lyase for the production of lepidimoide or lepidimoide-like compounds from purified potato RG-I. The results showed that enzymes from *Pichia pastoris* partially digested potato RG-I and produced oligomers from the polysaccharide. The mixture of oligomers and polymers (2.5 mg/ml) were then used in bioassay but *Amaranthus* seeds were unable to germinate, which might be due to the presence of tris-HCl buffer in the samples (results not shown).

4.14 Future work

The project clarified that the biologically active compound(s) present in cress seed-coat mucilage are oligosaccharide(s) of cress origin, and not classical hormones such as auxins or gibberellins. It also attempted to prepare lepidimoide or lepidimoide-like

compounds in the laboratory by methyl esterification and β-elimination or by the action of lyases on purified potato RG-I. The first part clarified that the bioactive compound(s) are indeed oligosaccharide(s), might be disaccharide(s) or disaccharide + trisaccharide that migrated from cress seed-coat mucilage but because of lack of time structural elucidation was not completed. Further purification and structural elucidation of the bioactive compound(s) from GPC, TLC and PC need to be done, for example by using HPLC and NMR.

As lepidimoide itself is known to be a potent allelopathic compound (Hasegawa *et al.,* 1992), more experiments need to be done for the preparation of lepidimoide and larger lepidimoide-like compounds. In this research both methyl esterification and β-elimination and action of RG-I lyases on purified RG-I showed some interesting results but unfortunately *Amaranthus* seeds did not germinate, perhaps due to high concentration of salts in the solution. So, on the basis of the results recorded in this thesis, future work should be done on removing the salts from the samples by ion-exchange chromatography and then checking the samples for bioactivity. The samples showing bioactivity should be further partitioned on GPC, TLC, PC and HPLC. The bioactive compounds should finally be identified by MS and NMR.

4.15 Conclusion

From the results of this research it is concluded that cress seed-coat mucilage release compound(s) with higher bioactivity than the root exudates. The bioactive compound(s) were also observed to be released from the cress and neither the microbial digest nor the seed treatment chemicals. The results of ethanol

precipitation, GPC, TLC, PC and ethyl acetate partitioning showed the compound of interest to be an oligosaccharide with unusual chromatographic properties and not an auxin or gibberellin. The compound(s) were not heat-labile.

The methyl esterification and β-elimination experiment showed the production of oligosaccharides from purified potato RG-I. Similarly, oligosaccharides were also produced by the action of lyase from *Pichia pastoris* on purified potato RG-I. Unfortunately *Amaranthus* seeds did not germinate in the solution of the oligosaccharides produced by both experiments, which might be due the presence of buffer salt in the solution.

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