The mode of action of the herbicide oxaziclomefone: an inhibitor of cell expansion

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Declaration

I hereby declare that I have composed this thesis, which is a record of my own work (unless otherwise stated).

Nichola O'Looney

Edinburgh 2002

To Vincent, Kathleen and Veronica,

my love

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Abstract

Oxaziclomefone [OAC; IUPAC: 3-(1-(3,5-dichlorophenyl)-1-methylethyl)-2,3-dihydro-6-methyl-5-phenyl-4H-1,3-oxazin-4-one] is a new herbicide recently released onto the market by Aventis, in Japan. OAC is very effective in controlling *Echinochloa* sp., a weed which is particularly prevalent in paddy fields and which can substantially reduce the yield of rice. OAC inhibits growth of graminaceous monocots but is relatively ineffective on most dicots.

OAC was found to inhibit cell expansion in maize cell suspension cultures (CSCs) and roots within 8 h of exposure and has an ID_{50} (inhibition dose) of ~7 nM. Cell expansion is promoted and regulated by turgor pressure, cell wall loosening and tightening. OAC did not reduce turgor pressure in maize CSCs or impede water transport across cell membranes. The herbicide did not promote the activity, apoplastic action or secretion of peroxidase, which can effect cell wall tightening by catalysing the formation of diferulate and di-isodityrosine. OAC did not inhibit the activity, action or secretion of XET, which may promote cell wall loosening via transglycosylation of xyloglucan chains. The herbicide did not reduce the consumption of extracellular ascorbate by maize CSCs, suggesting that OAC did not inhibit the formation of $^{\bullet}OH$ (generated via the Fenton reaction which, requires the presence of ascorbate) believed to cause polysaccharide scission and therefore effect cell wall loosening.

OAC did reduce acid-dependent creep in methanol-killed maize silks (though not consistently) but not in methanol-killed celery petiole vascular bundles. OAC also reduced acid-dependent creep in frozen, thawed maize roots but again not consistently. However, OAC did inhibit the action of β-expansin (aqueously extracted from *Zea mays* pollen) on boiled maize silks and roots, assayed using an extensiometer.

OAC may inhibit cell expansion by suppressing β-expansin-mediated cell wall loosening. As graminaceous monocots are more susceptible to OAC and believed to have a higher β-expansin activity than dicots, it is plausible that the herbicide might inhibit β-expansin action.

Abbreviations

A	Dehydroascorbate
AIR	Alcohol insoluble residue
ABP	Auxin binding protein
AGPS	Arabinogalactan proteins
AGT	Acid growth theory
AH_2	Ascorbate
ALS	Acetolactate synthase
BAEE	Nα-benzoyl-L-arginine ethyl ester
CPM	Counts per minute
CrO_3	Chromium trioxide
CSC	Cell suspension culture
DCB	Dichlobenil or 2,6-dichlorobenzonitrile
DCMU	N'-(4-chlorophenyl)-N,N-dimethylurea
DCPIP	Dichloroendophenol
D-Gal*p*A	Galacturonic acid
D-Glc*p*A	Glucuronic acid
D-Glc*p*	D-Glucose
DMSO	Dimethylsulphoxide
D-Xyl *p*	D-xylose
EDTA	Ethylenediamine-tetraacetic acid
EPSP	5-enolpyruvylshikimate 3-phosphate
g	Gravity
GAX	Glucuronoarabinoxylan
GHCs	Glycerinated hollow cylinders
GIA	Group I allergen
GRPs	Glycine rich proteins
HCl	Hydrochloric acid
HNO_3	Nitric acid
HPO_3	Metaphosphoric acid
HPRGs	Hydroxyproline rich glyco proteins (extensin)

HS GAXs	Highly substituted glucuronoarabinoxylans
IAA	Indole acetic acid
ID_{50}	Inhibition dose
L-Ara*f*	L-Arabinose
LVDT	Linear voltage displacement transducer
MLG	Mixed linkage glucan
OAC	Oxaziclomefone or 3-(1-(3,5-dichlorophenyl)-1-methylethyl)-2,3-dihydro-6-methyl-5-phenyl-4H-1,3-oxazin-4-one
$^{\bullet}OH$	Hydroxyl radical
P	P value
PCV	Packed cell volume
PEP	Phosphoenolpyruvate
PIP	Protoplast intrinsic protein
POIP	Point of incipient plasmolysis
PRPs	Proline rich proteins
RG 1	Rhamnogalacturonan 1
S.E.	Standard error
SCV	Settled cell volume
TIP	Tonoplast intrinsic protein
WL	White light
XET	Xyloglucan endotransglycosylase
XGO-SRs	Xyloglucan oligosaccharide–sulforhodamine conjugate
2,4-D	(2,4-dichlorophenoxy)acetic acid

1. Introduction

1.1 Cell wall function

Plant cells, unlike animal cells, possess a wall. This feature confers shape, mechanical strength and rigidity on the cell and therefore the plant. An important, though seemingly paradoxical, function of the cell wall is the role it plays in plant cell growth. It has the ability to 'relax' its structure to accommodate turgor-induced cell expansion, while simultaneously upholding its overall structural integrity (Cosgrove, 1986; Cosgrove, 1993a; Taiz, 1984; Cosgrove, 1993b). The cell wall dictates the direction of cell elongation (McNeil *et al.*, 1984; Carpita and Gibeaut, 1993; Cosgrove, 1999; Cosgrove, 2000a) and can control its growth rate (Lockhart, 1965). Other functions of the cell wall include acting as a defence system by preventing even the smallest pathogenic organisms, such as viruses, from penetrating into the cell (Brett and Waldron, 1996). It is also thought that oligosaccaharides produced by pathogenic attack on the cell wall can move to neighbouring cells and trigger defence responses (Brett and Waldron, 1996). The cell wall also features in fruit ripening as pectin breakdown leads to the softening of fruit tissue (Fischer and Bennett, 1991), facilitating seed dispersal.

This study focuses on the mode of action of the novel herbicide oxaziclomefone (OAC), developed by Aventis Crop Science U.K. Ltd. OAC inhibits cell expansion, especially in graminaceous monocotyledons, particularly in the root. As one of the functions of the cell wall is to control the rate of cell expansion, it is possible that OAC affects the cell wall so that it can no longer fulfil or carry out this function. The herbicide has been commercialised as a method for controlling *Echinochloa* sp. (barnyard grass) in paddy fields in Japan.

1.2 Plant cell walls

The cell wall has a complex molecular structure, reflecting the wide range of functions with which it is associated. There are two different types of cell walls formed: primary and secondary. After the cell plate (formed during cell division) has been laid down, the primary cell wall is formed. Primary cell walls are formed while the cell is growing unlike secondary cell walls, which are laid down between the protoplast and primary cell wall after cell growth has ceased. Secondary cell walls are generally thicker than primary cell walls and are more likely to be lignified (Turner *et al.*, 2001).

1.2.1 Primary plant cell wall structure

There are two main types of primary cell wall: type I and type II. The type II cell wall (found in graminaceous monocots) differs in general composition from the type I cell wall, which refers to the primary cell wall of dicotyledons. However, both type I and type II cell walls have a similar microfibrillar structure consisting of cellulose (Carpita and Gibeaut, 1993). The following description of the primary cell wall will focus on the type II cell wall, as the plant material predominantly used throughout this project was maize (a graminaceous monocot). The type II cell wall is composed of ~35% cellulose, ~43% hemicellulose, ~10% pectin and 7–17% protein (dry weight) (Burke *et al.*, 1974; Carpita, 1983).

1.2.1.1 Microfibril network

Primary cell walls have a microfibrillar network, each microfibril being composed of long, thin strands of cellulose. Cellulose itself consists of (1→4)-β-D-Glc*p* residues arranged into linear chains where every other glucose residue is rotated 180^0 (Delmer and Amor, 1995) **(Fig. 1.2.1.1)**. Cellulose strands (glucan chains) are hydrogen bonded together to form either a crystalline or paracrystalline rod-like structure known as a microfibril, the crystalline structure being the more prevalent. Cellulose is not synthesised as single strands in nature but as crystalline microfibrils. These

microfibrils are extruded by large, ordered structures, named rosettes, embedded in the plasma membrane (Brown *et al.*, 1996). These rosettes consist of a hexagonal arrangement of subunits also containing the cellulose synthase catalytic subunits (Kimura *et al.*, 1999). Each of the subunits is believed to be responsible for the polymerisation, secretion and alignment of a single glucan chain, which comprises the microfibril (Delmer and Amor, 1995). Microfibrils then form a stratified structure, cross-linked with hemicelluloses, and acts as the skeleton of the cell wall. They are 5–15 nm in diameter and spaced 20–40 nm apart (McCann *et al.*, 1990).

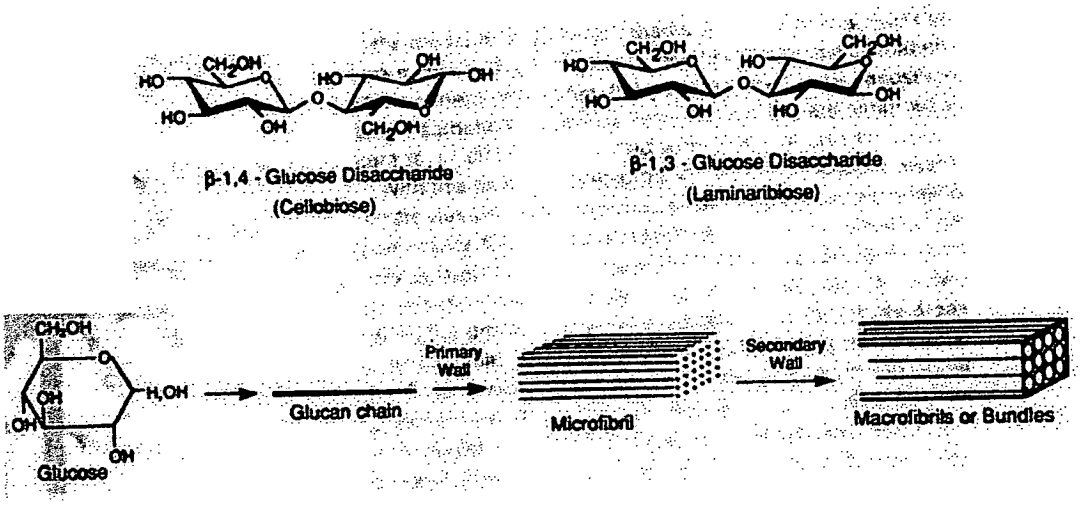


Fig. 1.2.1.1 The basic repeating unit of cellulose is the disaccharide cellobiose. Each glucose is rotated ~180° forming a glucan chain and eventually native cellulose (Delmer and Amor, 1995).

1.2.1.2 Non-cellulosic polysaccharide synthesis

Unlike cellulose and callose, which are synthesised at the plasma membrane, non-cellulosic polysaccharides such as pectins and hemicelluloses are synthesised in the Golgi apparatus and secreted into the cell wall as high molecular weight polymers (Darvil *et al*, 1980; Carpita, 1996). Sugar nucleotides are the substrates for polysaccharide synthesis. They are formed in the cytosol or Golgi cisternae through various complex biochemical pathways. Sugar nucleotides consist of a monosaccharide, which is glycosidically attached to a phosphate-containing base. The sugar nucleotides are transported via Golgi vesicles to the plasma membrane

where polysaccharide synthases transfer the monsaccharides from the sugar nucleotide to the non-reducing ends of sugar residues in growing polysaccharide chains (Brett and Waldron, 1996). Two types of enzymes that are involved in non-cellulosic polysaccharide biosynthesis are glycan synthase and glycosyltransferase (Perrin *et al.*, 2001). Little is known about glycan synthases but they are believed to be located in the Golgi and to link sugars together to make up the backbone of any given polysaccharide (Perrin *et al.*, 2001). Glycosyltransferases are thought to transfer the sugar residue from a sugar nucleotide to a specific location on an acceptor molecule. Gylcosyltransferases are believed to be type II integral membrane proteins (Perrin *et al.*, 2001). There are several types of sugar nucleotides found in plants for e.g. one of which is UDP-Glc*p*. UDP-Glc*p* can lead to the formation of glucan and xyloglucan - depending upon enzyme specificity. However, UDP-Xyl*p* can also lead to the formation of xyloglucan as well as xylan. Other sugar nucleotides include UDP-Gal*p*, GDP-Man*p*, UDP-Ara*f* and UDPGal*p*A.

1.2.1.3 Hemicelluloses

Hemicelluloses are proposed to tether microfibrils by hydrogen bonding along or to adjacent microfibrils. The hemicelluloses are therefore thought to play an important role in maintaining the structural integrity of the cell wall and in restraining cell expansion (Fry, 1989; Cosgrove, 1999). Disruption of hemicellulose, which tethers microfibrils, is believed to be instrumental in cell growth by allowing cell expansion to occur. Unlike type I primary cell walls, which have xyloglucan as their main hemicellulose, type II primary cell walls have glucuronoarabinoxylans (GAXs) (Darvill *et al.*, 1980; Carpita, 1983; Carpita, 1996). Even though the amount of xyloglucan present in graminaceous monocotyledons is low (~ 2% dry weight of the cell wall) (Darvill *et al.*, 1980; McNeil *et al.*, 1984), it is still found to bind to cellulose in type II cell walls (Labavitch and Ray, 1978).

The GAX backbone is a series of (1→4)-β-D-Xyl*p* residues, with many side groups, the most commonly occurring of which are: L-α-Ara*f*, D-α-Glc*p*A and Me-α-D-

Glc*p*A, which can be attached at the O2 or O3 position of the (1→4)-β-D-Xyl*p* residues (Carpita and Gibeaut, 1993). No regulatory functions have yet been attributed to these side groups, in contrast to xyloglucan where the presence of side chains are believed to prevent xyloglucan from hydrogen-bonding to itself and can influence its binding to cellulose (Hayashi *et al.*, 1994; Vinken *et al.*, 1995). GAXs with fewer side chains form stronger bonds with cellulose than do the heavily substituted GAXs (HS GAXs); this is because the presence of side chains makes the formation of hydrogen bonds between GAXs and microfibrils difficult (Carpita, 1983; Carpita, 1996).

An important component of some type II primary cell walls is mixed linkage glucan (MLG). MLG is believed to be present only in the Gramineae (Mc Neil *et al.*, 1984) and is an unbranched homopolymer of glucose containing (1→4)-β-D-Glc*p* linear oligomers interconnected by (1→3)-β-D-Glc*p* residues, which introduce 'kinks' into the polymer. During cell expansion, MLG is synthesised in parallel with GAX (Carpita and Gibeaut, 1993; Carpita, 1996). It is believed that MLG may play a role similar to that of the xyloglucan found in type I cell walls (Carpita, 1996). MLG increases during cell wall growth and disappears when the growth phase is completed (Luttenger and Nevins, 1985; Carpita and Kanabus, 1988). It has been demonstrated, using gravistimulated oat pulvini, that the rapidly growing lower half of the organ accumulates more MLG than the slower growing upper half (Gibeaut *et al.*, 1990).

1.2.1.4 Pectin

Pectin includes the most complex known polysaccharides in the cell wall. Type II cell wall pectin is composed of two main polysaccharide domains: the acidic rhamnogalacturonan (RG I), comprising [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] repeat units, and homogalacturonan, which is a helical homopolymer of (1→4)-α-D-Gal*p*A residues (Carpita, 1996). RG I is described as the 'hairy' region of the pectin owing to the presence of arabinogalactan side chains.

The acidic pectins (non-methyl esterified) are highly hydrated owing to their negative charge which strongly binds cations. Ca^{2+} ion cross-links are believed to cause the formation of pectin gels via 'egg box' junctions (Jarvis, 1984).

Rhamnogalacturonan II (RG II) is found as a minor component both of type I and type II cell walls. It has a complex chemical structure comprising at least 12 different sugars including β-L-aceric acid, β-D-apiose, α-D-Gal*p*A, α-L-Ara*f*, α-L-Rha*p* and β-D-Gal*p* (Stevenson *et al.*, 1988). Chains of RG II are believed to be cross-linked by borate-diol ester bonds and therefore may play a role in cell wall 'tightening' (Kobayashi, 1996). It is believed that a borate-diol ester cross-link is formed via the β-D-apiose residue of a Me-β-D-Xyl*p*-containing side chain of two RG II molecules (Ishii *et al.*, 1999). Ishii and Matsunga (2001) has recently suggested that borate-diol esters cross-link homogalacturonan and RG II. O'Neil *et al.*, (2001) have demonstrated that borate-diol ester cross-linking of RGII is necessary for the growth of *Arabidopsis*. In the *Arabidopsis* mutants *mur1-1* and *1-2* the L-Fuc and 2-*O*-Me L-Fuc are replaced with L-Gal and 2-*O*-Me L-Gal. These mutants, which have dwarfed phenotypes and abnormal rosette leaves, contain normal amounts of RGII but only half of them exist as borate cross-linked dimers. O'Neil *et al.*, (2001) showed that when borate was exogenously added to the *mur1-1* and *1-2* mutants that they continued to grow as noraml. This suggests that the function of borate-diol ester cross-linking in RGII is not just to 'tighten' the cell wall and that relatively minor changes within the pectic polysaccharides can have dramatic consequences for plant growth.

However, type II cell walls do not have a high pectin content (McNeil *et al.*, 1984), though antibodies against methyl esterified and unesterified homogalacturonans have detected their presence in the cortex, stele, cortical cells and intercellular spaces of oat root apexes (Knox *et al.*, 1990).

Other pectic polysaccharides are the highly branched neutral arbinans composed of a backbone of (1→5)-α-L-Ara*f* residues with side chains of α-L-Ara*f* residues; galactans which contain (1→4)-α-D-Gal*p* with no other type of sugar residues present; and arabinogalactans, composed of a (1→4)-β-D-Gal*p*-rich backbone with (1→5)-α-L-Ara*f* side chains (Brett and Waldron, 1996).

1.2.1.5 Phenolics

Ferulic acid (4-hydroxy-3-methoxycinnamic acid), a cinnamic acid derivative, is ester linked to some of the α-L-Ara*f* residues of GAX and can be oxidatively coupled to form diferulic acid **(Fig.1.2.1.4)** (Fry, 1983; Grabber *et al.*, 1995; Wende and Fry, 1997).

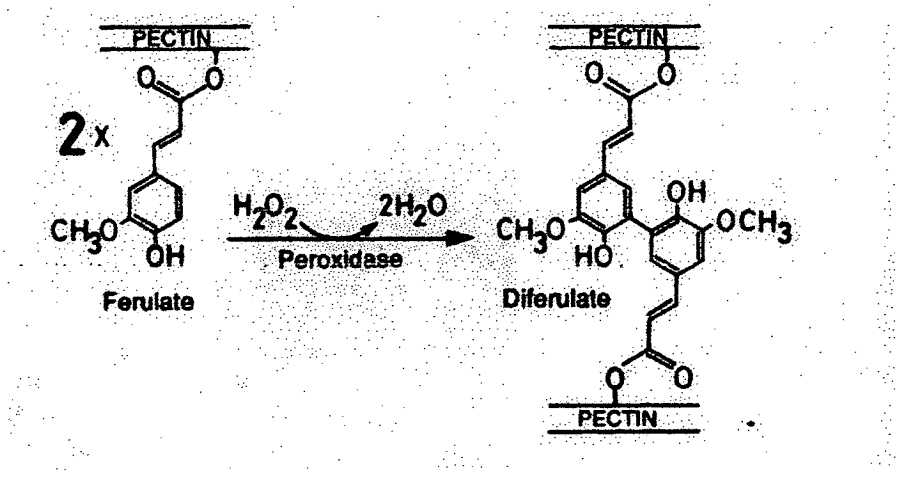


Fig. 1.2.1.4 The peroxidase catalysed cross-linking of ferulate to form diferulate (Brett and Waldron, 1996).

Feruloyl GAX side chains, which type II cell walls have in abundance (Kato and Nevins, 1985), cross-link part of the GAX matrix (Scalbert, 1985; Carpita, 1986) by forming diferulate esters in the presence of peroxidase. Fry *et al.* (2000) reported that some polysaccharide-bound ferulate was dimerised to form diferulates intra-protoplasmically, before being secreted into the cell wall. They also reported that trimers and larger coupling products of ferulate were more abundant in the cell wall

than diferulate and were consequently largely responsible for cross-linking the GAX matrix. *p*-Coumaroyl GAX, another derivative of cinnamic acid found in the cell wall, can be oxidised by peroxidase in the presence of H_2O_2 to form dimers *in vitro* (Wallace and Fry, 1999). However, *in vivo* feruloyl esters appear to be preferentially oxidised over *p*-coumaroyl esters (Fry *et al.*, 2000). Other phenols found in the cell wall are syringldehyde, vanillin, *p*-hydroxybenzaldehyde and lignin (Hartley and Ford, 1989).

Lignin surrounds cells with a hydrophobic network and acts as a barrier against pathogens. Lignin is believed to be a constituent of secondary cell walls, which are essentially dead and incapable of growth (Brett and Waldron, 1996). However, Müsel *et al.* (1997) reported the presence of lignin, which correlated with the presence of proline-rich protein (PRP) epitopes, in maize coleoptiles. Müsel *et al.* (1997) noted that lignin content increased when the maize coleoptiles were irradiated with white or far-red light, both of which can inhibit growth, and in the presence of H_2O_2, which is believed to facilitate cross-linking of phenolic compounds. This led to the suggestion that lignin might be linked to PRP and may be a mechanism for controlling or restraining cell expansion.

Basic lignin is derived from three monolignols, *p*-coumaroyl alcohol, coniferoyl alcohol and sinapyl alcohol (Boudet, 1998). The conventional lignin biosynthetic pathway is described in **Fig 1.2.1.5** (Boudet, 1998), though the exact pathway of lignin biosynthesis has not yet been categorically elucidated. Ralph *et al.*, (2001) reported that downregulation of CAD and COMT resulted in significant structural changes in lignin. CAD downregulation lead to the incorporation of hydroxycinnamyl aldehyde monolignol precursors immediately into the polymer, while COMT downregulation resulted in the production of novel benzodioxane units in lignin.

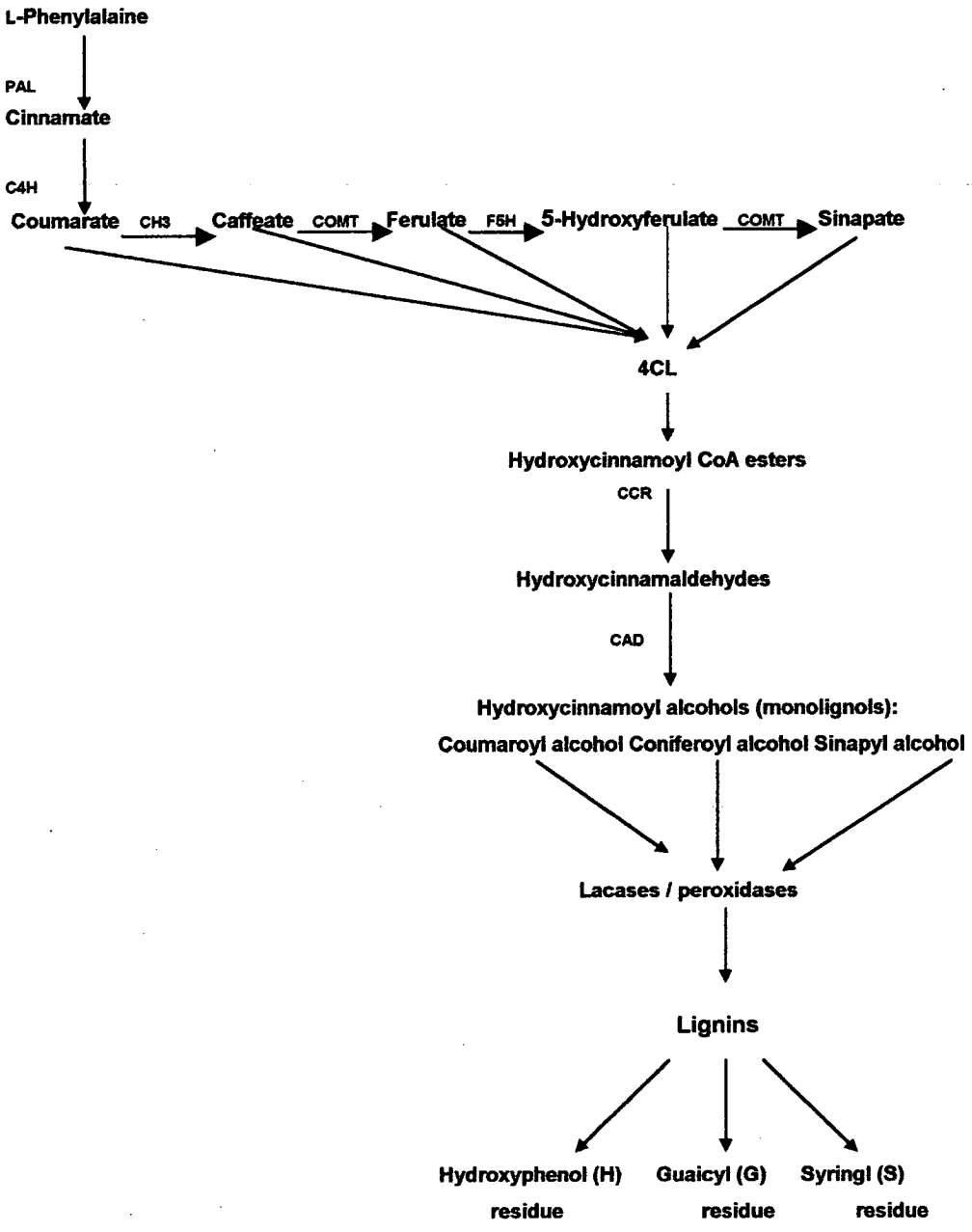


Fig. 1.2.1.5 The conventional biosynthetic lignin pathway (Boudet, 1998). PAL = phenylalanine ammonia-lyase; C4H = cinnamate 4-hydroxylase; C3H = 4-hydroxycinnamate 3-hydroxylase; COMT = S-adenosyl-methionine:caffeate/5-hydroxyferulate-o-methyltransferase; F5H = ferulate 5-hydroxylase; 4CL = hydroxycinnamate:CoA-ligase; CCR = hydroxycinnamoyl-CoA:NADPH oxidoretuctase; CAD = hydroxycinnamoyl alcohol dehydrogenase.

1.2.1.6 Proteins

Excluding enzymes, there are several cell wall-related proteins, including hydroxyproline-rich glycoproteins (HRGPs) or extensins, arabinogalactan proteins (AGPs), glycine-rich proteins (GRPs) and proline-rich proteins (PRPs). The most widely studied structural proteins in the cell wall are extensins, which are tyrosine-rich (Fry, 1987). Extensin is found in abundance in dicots but to a lesser extent in graminaceous monocots (Cassab, 1998). Peroxidase catalyses the formation of isodityrosine between two tyr residues **(Fig. 1.2.1.5)**. Extensins are believed to form peroxidase-catalysed cross-links between two Tyr residues of isodityrosine to form di-isodityrosine (Fry, 1982a; Brady and Fry, 1997).

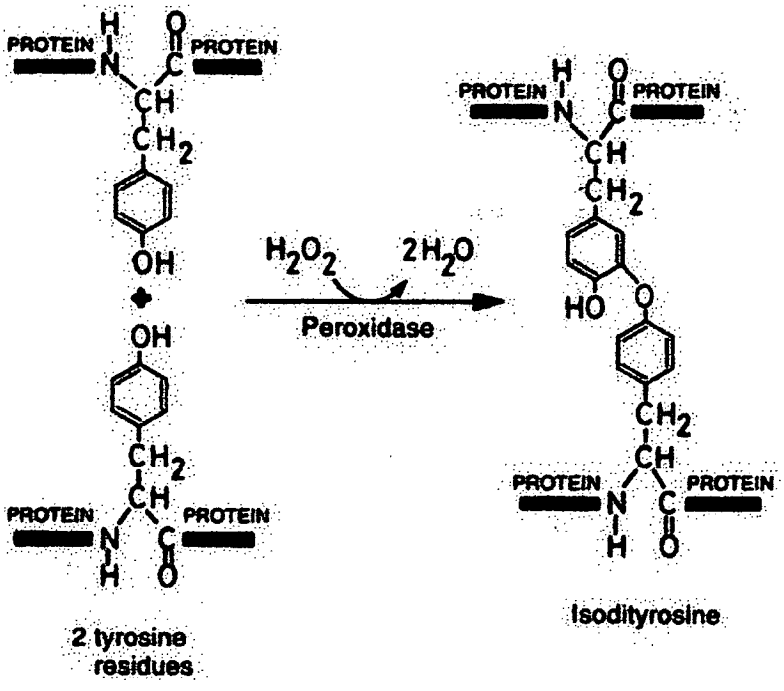


Fig. 1.2.1.5 The peroxidase catalysed cross-linking between 2 Tyr residues to form isodoityrosine (Brett and Waldron, 1996).

Epstein and Lamport (1984) reported that isodityrosine formed intramolecular cross-links that stabilised the extensin protein while maintaining its rod-like shape. An increase in isodityrosine cross-linking is believed to be induced upon wounding, and is thought to help form a barrier against pathogenic attack (Bradley *et al.*, 1992). The tetramer di-isodityrosine and the trimer pulcherosine are also believed to form

interpolypeptide cross-links in the cell wall (Brady *et al.*, 1996; Brady *et al.* 1998). Cross-links between pectin (RG I) and extensin have been reported by Qi *et al.* (1995). Graminaceous monocots contain threonine-rich proteins instead of Hyp-rich proteins (Carpita and Gibeaut, 1993; Carpita, 1996). They are highly basic like extensin but contain more threonine, less valine and tyrosine and are less glycoyslated (Kieliszewski, 1990). However, Hood *et al.* (1991) reported the presence of extensin in popcorn pericarp.

AGPs are believed to be situated either extraprotoplasmically or on the plasma membrane (Cassab, 1998). AGPs are 90–95% carbohydrate, composed mainly of α-D-Gal*p* and α-L-Ara*f* residues (Jose-Estanyol and Puigdomenech, 2000). AGPs have been attributed a wide range of functions including facilitating adhesion between pollen and stigma, controlling water balance in the cell, cell differentiation, cell-to-cell signalling and defence against pathogenic attack (Schultz, 1998; Cassab, 1998; Jose-Estanyol and Puigdomenech, 2000).

There are two classes of GRPs, one of which is located as a structural component of the cell wall and the other in the cytoplasm (Showalter, 1993). The GRPs located in the cell wall are believed to be developmentally regulated and involved in wound healing; they are also believed to act as nucleation sites for lignin deposition (Showalter, 1993). Lei and Wu (1991) proposed that GRPs found in rice might be covalently linked (via Tyr residues) to each other, forming a network-like structure and therefore playing a role similar to that of extensins in the cell wall.

PRPs may also act as nucleation sites for lignin deposition (Jose-Estanyol and Puigdomenech, 2000). They may also play a role in the regulation of cell growth in a similar manner to that of GRPs (Bradley *et al.*, 1992). They might be involved in nodule formation and are thought to form an oxygen barrier around oxygen sensitive nodules (Showalter, 1993).

1.3 Primary cell wall models

Models of the structure of the cell wall have been proposed to explain interactions between various cell wall components (Keegstra *et al.*, 1973; Talmadge *et al.*, 1973; Valent and Albersheim, 1974; Fry, 1986; McCann and Roberts, 1991). A comprehensive literature review on the various structural aspects and interactions of the cell wall, by Carpita and Gibeaut (1993), enabled them to describe in detail a model of a dicot and monocot primary cell wall. Structural knowledge of the components of the cell wall and how these components might be interconnected helps to further our understanding of what aspects of the cell wall are critical in growth.

1.3.1 A chemical model emphasising glycosidic cross-links (Albersheim)

One of the first models **(Fig. 1.3.1)** to describe interactions between cell wall polysaccharides and between polysaccharides and proteins was proposed by Albersheim's group (Keegstra *et al.*, 1973; Talmadge *et al.*, 1973). They proposed that xyloglucan was covalently linked to pectic polymers through arabinan and galactan side-chains. They also suggested that pectic polymers were covalently attached via terminal galactan side-chains to the OH group of serine residues of structural proteins (extensin).

They also proposed that xyloglucan was hydrogen-bonded along cellulose microfibrils and suggested that auxin effected cell expansion by lowering the pH of the cell wall, the resulting protons competitively weakening the hydrogen-bonding between the cellulose and xyloglucan. This disruption of hydrogen bonding was thought to allow cell wall 'slippage' whereby the hemicellulose and cellulose were able to slip past one another allowing growth, before new hydrogen bonds were reformed; this was described as 'xyloglucan creep'.

However, Valent and Albersheim (1974) later reported that cell wall extension was unlikely to be solely as a result of acid-mediated 'xyloglucan creep' when it was found that the percentage of xyloglucan binding to cellulose *in vitro* did not vary in response to changes in pH in the range of 2 to 7.

Through this model, Albersheim's group attempted to demonstrate how various cell wall components interacted in order to control cell expansion and, from this perspective, it was an important development in this area of research. However, in general the model was of limited value, as it did not include cell wall components that are currently well known. It was also later called into question by Darvill *et al.* (1980) as the HPRG present in the culture medium was shown to be structurally different from the HPRG in the cell wall and Monro *et al.* (1976) found no evidence of a covalent linkage between the cell wall HPRG and pectic polysaccharide.

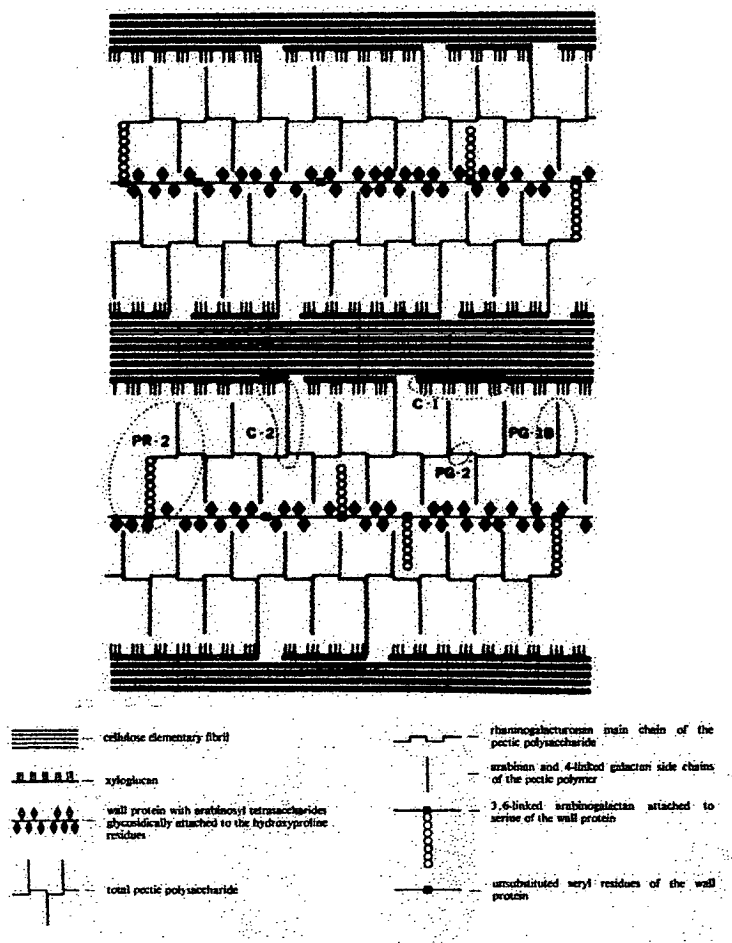


Fig. 1.3.1 The chemical model proposed by Albershiem and colleagues highlighting glycosidic linkages (Keegstra *et al.*, 1973).

1.3.2 A chemical model emphasising diverse cross-links (Fry)

An important aspect of a cell wall model is the chemical nature of the cross-links formed between cell wall components. Fry (1986) described the cell wall as a biphasic structure, consisting of cellulose and a gel-like matrix comprised of non-cellulosic polysaccharides and glycoproteins. In this model **(Fig. 1.3.2)**, specific cross-links found in the cell wall were described in detail using the following techniques to characterise them: isolation of individual cross-links, solubilisation of polymers using a range of extraction procedures, inhibition of cross-link formation *in vivo* and promotion of cross-link formation *in vitro*. Techniques used and reported by Fry (1986) provided evidence for a range of cross-links. One of several cross-links proposed was cross-linking of extensin, via the oxidative coupling of tyrosine residues forming isodityrosine cross-links (Fry, 1982a). Fry (1986) also described cross-links found between hemicelluloses and attributed them to oxidative coupling of ferulate side chains, thereby producing diferulate bridges (Fry, 1982b; Fry, 1983; Fry, 1984). Also described were non-covalent attachments (hydrogen bonding) between xyloglucan and cellulose. Solubilisation of pectin, using calcium-chelating agents, provided evidence for cross-linking of pectin through the formation of Ca^{2+} ion bridges (Jarvis, 1984).

This model was amongst the first to describe the diverse covalent and non-covalent cross-links found in the cell wall as well as to highlight the possibility of phenolic cross-links. This model enhanced the understanding of mechanisms involved in controlling cell expansion. However, as presented, this model of the cell wall does not fully describe the spatial arrangement of cell wall components.

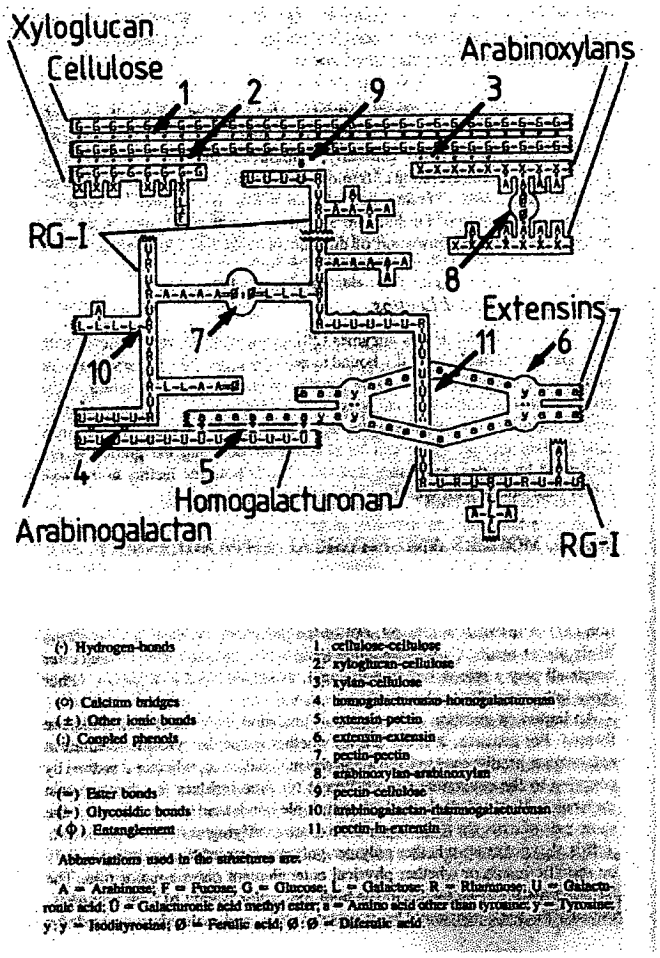


Fig. 1.3.2 A chemical model (Fry, 1986) describing a variety of cross-links.

1.3.3 A model based on EM observation (McCann and Roberts)

McCann *et al.* (1990) described the cell wall (as viewed under a transmission electron microsope) using a fast-freeze, deep-etch technique that gave the wall a three-dimensional perspective. They showed physical evidence of links between cellulose microfibrils. They proposed that cellulose microfibrils were 5–15 nm in diameter (McCann and Roberts, 1991) and were tethered by xyloglucan. They estimated the xyloglucan tethers to be 20–40 nm in length, in comparison to

extracted xyloglucan molecules which were measured 30–<700 nm. They also estimated the thickness of the primary cell wall, middle lamella and cellulose microfibril to be ~75 nm, ~20 nm and ~5–15 nm respectively.

McCann *et al.* (1990) believed that this technique provided evidence that an onion cell wall was composed of four layers (lamellae) of parallel-running microfibrils, the space between which was maintained by xyloglucan. As the pectin was alkali extractable, they suggested that non-methyl ester-linked pectins formed a network that was co-extensive with but also independent of the cellulose–hemicellulose network. This contrasted with the Albersheim group's finding (Keegstra *et al.*, 1973) which suggested that pectin was covalently attached to xyloglucan.

This model detailed a useful and comprehensive description of a three-dimensional cell wall. However, the nature or range of cross-links found in the cell wall was not described.

1.3.4 Review-based cell wall model (Carpita and Gibeaut)

Carpita and Gibeaut (1993) described the most recent and widely used (review-based) model of the cell wall **(Fig. 1.3.4)**. In this model they compared and highlighted differences found between type I and type II cell walls. They proposed that the type I cell wall comprised three domains that were structurally distinct and independent of, yet interactive with, one another. They described the three domains as the xyloglucan–cellulose network, the pectin matrix and the structural proteins.

However, the type II cell wall model was not as clearly defined. Carpita and Gibeaut (1993) proposed that the function of GAX reflected to some extent the function of xyloglucan, which is found more abundantly in type I cell walls. Carpita (1996) suggested that the degree of branching of GAX influenced the ability of GAX to bind to cellulose and that unbranched GAX, hydrogen bonded to cellulose, tethered layers of microfibrils. Carpita and Gibeaut (1993) noted that highly substituted GAX (HS-

GAX), i.e. GAX with many side chains **(1.2.1.2)**, accumulated during cell division and elongation but that these side chains were removed by hydrolysis after the cell ceased to elongate. The transient presence of MLG during elongation was proposed to interlock the cellulose microfibrils. Because MLG can be hydrolysed by endo-β-glucanase (Hatfield and Nevins, 1987), it was suggested that MLG–microfibril interactions provided a controlling point for cell expansion in graminaceous monocots (Carpita, 1996).

Carpita and Gibeaut (1993) also described the presence of threonine-rich, structural proteins in type II cell walls but noted the absence of extensin, found in type I cell walls. Carpita and Gibeaut (1993) suggested that structural proteins effected a controlling mechanism in type I cell walls, as they believed that the structural proteins 'tightened' the wall by forming cross-links. However, in the case of type II cell walls, Carpita and Gibeaut (1993) suggested that phenolic cross-links (diferulate bridges) restrained cell expansion by cross-linking to the GAX matrix.

This model provides a comprehensive description of a three-dimensional type II cell wall as well as taking into account the nature and range of cell wall components and how they might interact with each other. It also suggests a way in which cell expansion in grasses might be regulated, which was not clearly defined in previous models.

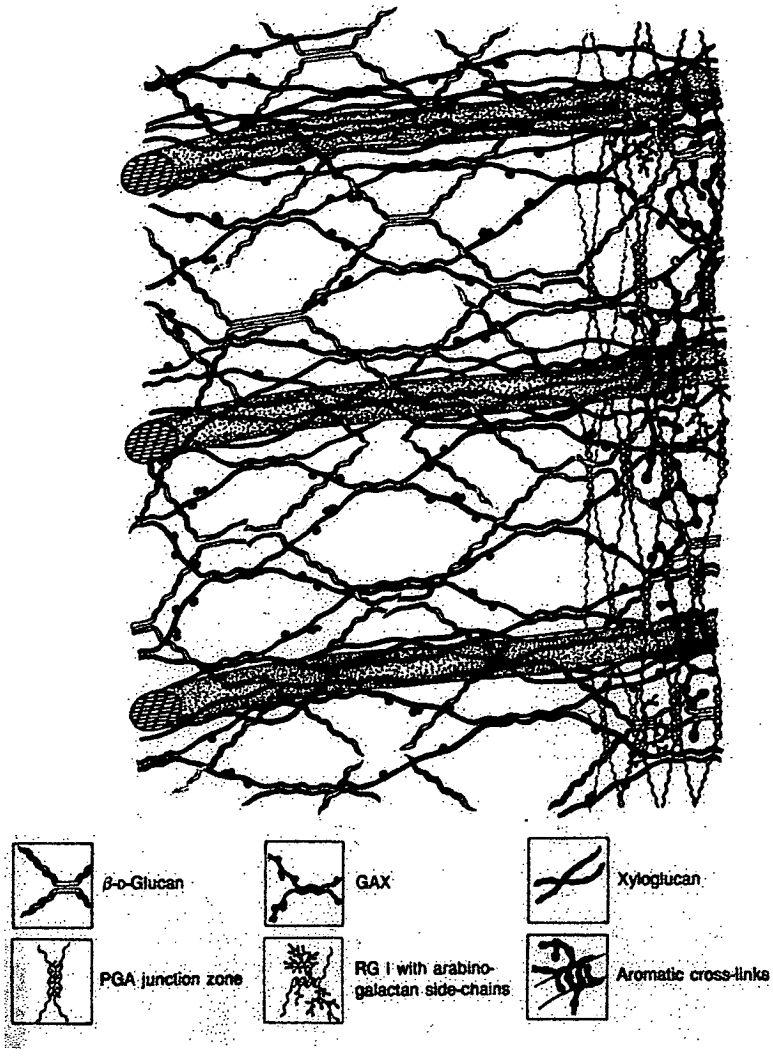


Fig. 1.3.4 Review based model described by Carpita and Gibeaut (1993)

1.4 Factors involved in regulating plant growth

Growth is defined by Thimann (1969) as an irreversible increase in volume; which may or may not be accompanied by cell division. Plant growth is influenced by a wide spectrum of factors. This section will include a brief overview of some of the regulatory mechanisms used by plants to sustain or control growth rate in response to internal or environmental cues.

1.4.1 Plant cell growth

Kutschera (2000) described plant growth as involving three separate processes: (a) cell division (production of new cells), (b) cell expansion (irreversible enlargement of the cell) and (c) cell maturation (the formation of cross-links in the cell wall and the generation of a secondary cell wall). However, cell division alone does not contribute to growth (Fry, 1988). Using Thimann's (1969) definition, growth describes an irreversible increase in volume. Cell growth (expansion) is an increase in cell volume and therefore plant mass. As cell division results in the formation of two daughter cells, generally equal in size, plant mass does not increase by continued cell division alone (as the volume occupied by two daughter cells is the same as that occupied by the original mother cell) but does increase with the expansion of the newly divided cells. Heupel and Kutschera (1997) reported that more cells are present in an etiolated sunflower hypocotyl than in a white light (WL) irradiated hypocotyl (which is shorter in length) and therefore believed that cell division must account for some of the increase in mass. However, it could be interpreted that, if there are more cells available for expansion (in comparison to the WL irradiated hypocotyl) in etiolated hypocotyls, it does not necessarily follow that the extra number of cells present resulted in increased cell mass.

Kutschera (2000) describes the formation of cross-links in the cell wall and the generation of a secondary cell wall as part of plant growth. However, this does not result in an irreversible increase in volume as described by Thimann (1969) and is therefore not plant growth.

In conclusion, both cell division and cell expansion are necessary to effect continued plant growth but cell division, independent of cell expansion, does not in itself result in plant growth. Plant growth is therefore considered to be effected by cell expansion.

1.4.2 Developmental regulation

As embryogenesis dictates only a basic plant form, consisting of a shoot and root, continued developmental regulation of these organs is necessary to produce an adult plant. The root and shoot, formed in the plant embryo, each initiate their development as axial plant organs (which when combined eventually form a fully mature plant) from a group of unspecialised cells called the apical meristem. The apical meristem is located at the tip of each of these organs.

1.4.2.1 Root development

In the root tip, there are three recognised zones: the zone of cell division (meristem), the zone of cell elongation and the zone of cell maturation. The development of specialised cells is not completely understood but they originate from the meristem and their fate is believed to be decided by their spatial position in the root (Salisbury and Ross, 1992). The meristem is a group of actively dividing cells. They are unspecialised, non-photosynthetic, contain only primary cell walls and small vacuoles (Taiz and Zeiger, 1998). The centre of the meristem is known as the 'quiescent centre'. This is where cells divide very slowly and periclinally (longitudinal to root axis) (Barlow, 1987), typically once every 200 hours (Macleod, 1991). These cells appear to be very resistant to damage from toxins and radiation. They are believed to act as reserve cells that can be utilised if the meristem becomes damaged.

Lineages of cells radiate from the behind the meristem. The proximal meristem, lying behind the quiescent centre, is the region where ~ 10,000–20,000 new cells are formed daily. In maize roots this region extends 1–3 mm back from the root tip (Feldman, 1994). Just behind the proximal meristem (further away from the root tip), three concentric cylinders of cells are formed. These cylinders of cells continue to divide, forming the primary meristems, which are the protoderm, procambium and ground meristem. These primary meristems eventually form three primary tissue systems in the root: the epidermis, the stele (vascular system) and the ground tissues

(parenchyma cells which make up the cortex, lying between the epidermis and the stele), respectively.

In maize roots the zone of elongation, which begins 1–3 mm back from the root tip, is 7–8 mm in length in actively growing roots at 25°C. The rate of elongation varies in this zone, the highest rate being at 3–4 mm from the root tip; further back from this, the elongation rate decreases to zero. Final cell length depends upon cell type, with epidermal and cortical cells elongating to about 40–60 times their original length (Feldman, 1994). As cells become displaced from the meristem they stop dividing and form different cell types. The main types of cells formed are the parenchyma, water transport cells and sieve-tube members. The parenchyma cells are not specialised and have only primary cell walls. They conduct most of the plant's metabolic functions. The water transport cells comprise tracheids and vessel element cells, both of which have lignified secondary cell walls (Salisbury and Ross, 1992). Behind the elongation zone, in the maturation zone, cells cease to elongate though this is not believed to be distance-related but may be influenced by the plant hormone auxin (Doerner, 2000). Cell growth stops when the cell wall 'tightens' preventing further cell wall extension.

1.4.2.2 Cell division and expansion in cultures

Cells in suspension culture are acquired from callus and are mainly spherical, but can also be elongated in shape (Street, 1977). In spherical cells, division occurs by placement of a wall midway through the cell. The next cell division results in the formation of three cells, a small central cell flanked by two other cells (Street, 1977). This set of three cells continues to divide at right angles to and along the axis eventually developing into a mass of dense, actively dividing cells (moruloid). New moruloid masses arise from cells which are released from the original moruloid mass. Elongated cells do not form moruloid masses but instead continue to divide into a row or mass of cells by internal division without undergoing cell expansion. *Melilotus alba* cell cultures form aggregates of up to 200 cells (Hinnawy, 1974). Cell expansion commences when the rate of cell division slows down or ceases and can

be measured by calculating the packed or settled cell volume of the CSC (Street, 1977).

1.4.3 Light and dark

Photoperiodism is a term used to describe the response of plants to varying lengths of light and dark. One of these responses is flowering. The length of time a flowering plant experiences darkness (over a 24-h period) determines when the plant will flower.

In a phenomenon called skotomorphogenesis, etiolated seedlings exhaust their seed food supply to reach light. An etiolated sunflower hypocotyl shows an increase in length almost double that of WL irradiated hypocotyls (Kutschera, 2000). In the absence of light, seedlings promote stem growth to reach light while simultaneously minimising leaf growth (Hart, 1988). However, this process is reversed after exposure to light. A deceleration of stem growth is accompanied by an increase in leaf expansion. Exposure to light induces chloroplast development and the synthesis of enzymes and pigments involved in photosynthesis. These developmental responses to light and dark enable the plant to receive the maximum amount of light possible for photosynthetic purposes, resulting in an autotrophic plant (Hart, 1988). The presence and wavelength of light is detected by photoreceptors (Frankhauser and Chory, 1997). A higher ratio of far red light to red light promotes hypocotyl, coleoptile or stem growth. This phenomenon occurs in plants that are growing in shady environments because they are surrounded by other plants. The shading plants reflect or absorb red light but transmit far red light. This is detected by plants growing in a shaded environment, which respond by growing longer stems towards light.

1.4.4 Plant hormones

Hormone is a general term used to describe a chemical, synthesised in one specific organ, which is transported to a different organ where it induces or controls a specific function. There is some controversy as to whether the mechanism by which plant 'hormones' induce or regulate specific events can actually be described as hormonal, as their mode of action and effects are very different from those observed in mammals (Weyers and Paterson, 2001). However, there is little doubt concerning the importance of 'hormonal' regulation in plants. Plants contain a variety of hormones with a range of different functions. All of the hormones, including cytokinins, gibberellins and auxins, influence plant growth.

1.4.4.1 Cytokinins

Cytokinins are produced in growing tissues and are found most abundantly in the meristematic regions. They are believed to be synthesised in the roots and translocated to the shoots via the xylem. Cytokinins are derivatives of the purine adenine (Matthews and Van Holde, 1995). They have numerous functions including stimulating cell division, leaf expansion, morphogenesis and the conversion of etioplasts into chloroplasts through stimulation of chlorophyll synthesis.

Cytokinin and auxin work in concert to affect cell division and differentiation. In the absence of cytokinin cells grow to a large size but do not divide. However, cell division will not take place in the presence of cytokinin only, as auxin is also required for this process. Differentiation is controlled by different ratios of auxin to cytokinin (Moore, 1979). Experiments using tobacco callus have shown that in equal concentrations of auxin and cytokinin, cell growth continues with the absence of differentiation. However, a higher ratio of cytokinin to auxin results in bud development whereas a reverse ratio initiates root formation. Cytokinins also effect cell expansion. Cortical cells, in tobacco roots, have been shown to enlarge up to four times their original size in the presence of kinetin (a cytokinin). Kinetin can also

induce cell elongation of tobacco pith, however, it is thought to favour the promotion of lateral expansion over elongation (Fox, 1969).

1.4.4.2 Gibberellins

Gibberellins are diterpenes synthesised from acetyl-CoA via the mevalonic acid pathway. They are found in meristematic regions of growing tissue, young fruits and germinating seeds. Gibberellins have a wide range of effects but were first recognised for the role they played in cell division and elongation in stems. The effects of gibberellins were observed in Japan. Japanese scientists were investigating a disease, which affected rice plants by making them pale, easily broken and extremely fast growing. Gibberellins were first isolated from a fungus found on diseased rice crops and the effects of the disease were attributed to their presence (Leopold and Kriedemann, 1975). Radley (1956) identified similar substances in higher plants. Other functions of gibberellins include promoting, flowering in biennial plants, α-amylase production in germinating cereal grains to mobilise seed reserves, parthenocarpic fruit development and delaying of senescence and breaking of dormancy. Gibberellins have also been reported to play a role in cell wall synthesis and expansion in *Avena* shoots (Montague, 1995). Inada *et al.* (2000) reported that in the presence of uniconazole (a gibberellin biosynthesis inhibitor) the length and diameter of *Lemna minor* root cells were significantly smaller than in control plants. Matsukura *et al.* (1998) measured the mitotic index and cell size in the elongation zone of the leaf sheath of rice and found that gibberellic acid increased the extensibility of the cell wall but did not promote cell division.

1.4.4.3 Auxin

Auxin is largely synthesised from tryptophan. Auxin influences seedling morphology, geotropism, phototropism, apical dominance, leaf senescence and abscission, flowering, fruit ripening and growth. However, the exact growth promoting mechanism of auxin is not fully understood and many questions remain unanswered. Auxin initiates two types of growth response: (a) a rapid increase in cell

elongation after 7–10 min and (b) a long-lasting growth response starting after 30–45 min (Cobb, 1992). The most rapid and obvious effect of auxin is the stimulation of cell elongation in coleoptiles (Rayle and Cleland, 1992; Taiz, 1984).

Auxin is transported into and out of the cell via the 'influx carrier' and the 'efflux carrier'. The influx carrier, which is encoded by the AUX 1 gene, is evenly distributed along the surface of the plasmalemma and the efflux carrier, which is encoded by the PIN and PIN 2 genes (Muday and DeLong, 2001), is located at the apical end of the cell (Devine *et al.*, 1993). These two types of carriers effect the polar transport of auxin through the plant (independent of gravity), at a rate faster than that of diffusion. In the acidic environment of the cell wall, auxin picks up a proton so that it becomes electrically neutral and able to pass through the plasma membrane. Once inside the cell, auxin becomes ionised and can only be carried out through the apical end of the cell by efflux carrier proteins, which simultaneously pump protons into the cell wall. Auxin is believed to indirectly stimulate ATPase enzymes to pump protons into the cell wall thereby lowering the pH of the cell wall (Brett and Waldron, 1996). A lower or acidic cell wall pH is in turn believed to stimulate cell elongation via the acid growth response, which is described by the acid growth theory and will be discussed later **(1.4.5)**.

The later growth response induced by auxin is mediated by auxin binding to receptors in the cell. Löbler and Klämbt, (1985) identified an auxin-binding protein (ABP) which is located in the endoplasmic reticulum. Brummell and Hall (1987) suggest that after auxin binds to ABP it induces changes in the concentration of Ca^{2+} ions. The Ca^{2+} ions can bind to calmodulin, which they believe stimulates protein kinases, which in turn can phsophorylate and activate key enzymes involved in cell wall metabolism. Brummell and Hall (1987) also suggest that auxin binds to a receptor in the cytoplasm which forms a complex that migrates to the nucleus. This complex is believed to induce the derepression of specific genes and specific mRNA sequences are synthesised and translated into products involved in cell wall synthesis.

1.4.5 The acid growth theory (AGT)

The AGT states that auxin promotes cell elongation by causing the cell to pump protons in to the apoplast at an enhanced rate, resulting in cell wall acidification, which promotes cell wall loosening (Rayle and Cleland, 1992). The exact nature by which acidification occurs is unclear. The AGT has been controversial since its inception; critics of the AGT generally do not disagree with the theory in principle, but argue that the results to-date do not prove the AGT, mainly because of the methodology employed to test the theory.

The main concern associated with the AGT was the effect that removing the epidermal layer (so that the apoplastic pH could be altered) might have upon cell expansion (Kutschera and Schopfer, 1985). Kutschera and Schopfer (1985) believed that subsequent cell elongation was merely in response to the removal of a 'confining layer' (epidermis) from around the tissue as they believed that epidermal layer alone was sensitive to H^+ secretion .

Rayle and Cleland (1992) argued that the AGT had merit, citing the following reasons: (1) abraded coleoptiles treated with auxin secreted more protons and lowered the apoplastic pH value by a full unit (Hager *et al.*, 1991), (2) treatment of auxin-sensitive tissues with buffers of pH 5.0 caused the elongation of cells at rates comparable to or greater than that induced by auxin (Rayle and Cleland, 1992), (3) auxin-induced growth was inhibited by neutral buffers in the apoplast (Rayle, 1973) and (4) a fungal toxin, fusicoccin, which causes acidification of the apoplast, also induced rapid cell elongation (Rayle and Cleland, 1977).

In relation to the removal of the epidermal layer, Rayle and Cleland (1992) argued that attempts to demonstrate that growth was not inhibited by neutral buffers were unsuccessful because of incomplete abrasion of the cuticle resulting in less than a third of the apoplast being bathed in neutral buffers. However, they acknowledged that complete removal of the epidermal layer could potentially damage underlying

tissue and could alter the ionic composition of the apoplast as well as effecting a loss of solutes.

Rayle and Cleland (1992) cited what they perceived to be flaws of experimental design by opponents of the AGT, in particular, work carried out by Kutschera and Schopfer (1985) and Schopfer (1989). Kutschera and Schopfer (1985) had preincubated tissue in distilled water for a 1-h period; the tissue underwent extension at a rate comparable to that induced by a pH 5.0 buffer. When the tissue was placed in pH 5.0 buffer no extension occurred; however, Rayle and Cleland (1992) believed that because the tissue had already undergone extension, it was no great surprise that it failed to extend in pH 5.0 buffer. They concluded that the reason tissue extension occurred in distilled water was because dissolved CO_2 had acidified the water or that cations, available in the Donnan free space (where cell wall loosening enzymes are believed to be located), were diffusing into the distilled water and were consequently replaced with H^+ which acidified the cell wall, thereby promoting extension. They argued that the main reason the AGT was in dispute was because of the widely held belief that the epidermis was the unique target of auxin action and therefore H^+ secretion.

Kutschera and Schopfer (1985) believed that the epidermis played a pivotal role in the elongation of tissue in response to a lower pH. They were of the opinion that its removal resulted in cell elongation simply because it had been confining the underlying tissue and that elongation was merely a response to the removal of a constrictive force. Rayle and Cleland (1992) believed that elongation was not merely a response to the removal of a constrictive force and cited experimental work by Peters *et al.* (1992) who demonstrated that auxin-induced H^+ secretion could occur in tissues lacking an epidermal layer. Peters *et al.* (1992) showed that tissues with and without the epidermal layer were able to effect similar changes in pH when placed in weakly buffered solutions and therefore the epidermal layer was not essential for the induction of a pH response. These experiments feature as Rayle and Cleland's (1992) main support for the acid growth theory.

Cosgrove (2000b) proposed that expansin (discussed in detail in section **1.5.2.3**), a protein that is believed to be involved in cell wall 'loosening' at an acidic pH, mediated to some extent the acid growth response.

1.5 Cell growth mechanisms

1.5.1 Turgor pressure

Turgor pressure has an important role to play in plant cell expansion (Kutschera and Kohler, 1993; Kutschera and Kohler, 1994). Turgor pressure (regulated by differences in solute concentrations between the protoplast and the apoplast) causes the protoplast to push against the cell wall. The cell wall exerts an equal and opposite force against the protoplast to prevent the cell from bursting. During cell expansion, the wall has to 'relax' while simultaneously maintaining the overall structural integrity of the cell wall. Cell expansion can be described using the Lockhart (1965) equation.

$$(dV/dt) \times (1/V) = m(P - Y) \quad \text{for } P > Y$$

Where: $(dV/dt) \times (1/V)$ = relative growth rate

m = cell wall extensibility

P = turgor pressure

Y = yield threshold (the turgor pressure above which plastic expansion occurs)

However, it has been shown (by applying the Lockhart equation to experimental results) that m and Y are not constants but variables (Cosgrove *et al.*, 1984).

Cell expansion is dependent upon the turgor pressure of a cell exceeding the threshold turgor pressure providing that m has a value above zero so that the rate at

which cells elongate or expand is controlled by m (the extensibility or ability of the cell wall to 'relax') and not turgor pressure (Pritchard *et al.*, 1993). Consequently, according to this equation an increase in turgor pressure alone does not decree an increase in growth rate (Cosgrove, 1993b). According to the Lockhart equation a linear relationship would be expected between turgor pressure and growth rate i.e. as turgor pressure decreases there should be a linear decrease in growth rate. However, a non-linear relationship between growth rate and turgor pressure has been demonstrated using pea epicotyls (Cosgrove *et al.*, 1984; Van Volkenburgh and Cleland, 1984), suggesting that turgor pressure does not control the rate of growth and that Lockhart's equation does not always hold.

Cell expansion is believed to be initiated by 'relaxation' of the cell wall followed by an influx of water to maintain turgor (Boyer, 1985; Cosgrove, 1993a; Cosgrove, 1993b). Jarvis and McCann (2000) describe cell expansion as 'turgor driven' but note that 'under constant turgor, cell expansion is permitted and controlled by the mechanical behaviour of the cell wall'.

Turgor pressure results from movement of water into a plant cell and this is dependent upon hydraulic conductivity and the water potential gradient (Salisbury and Ross, 1992). Turgor pressure is estimated by subtracting the apoplastic osmotic pressure from the osmotic pressure in the cell (protoplast). Turgor pressure is an estimation of the pressure that protoplasm exerts on the cell wall, and which the cell wall must withstand to prevent the cell from bursting.

$$P = \pi_i - \pi_e$$

Where: P = Turgor pressure

π_i = Internal (protoplasmic) osmotic pressure

π_e = External (apoplastic) osmotic pressure

This equation is used to calculate the turgor pressure of a cell (the pressure that the wall must exert upon the cytoplasm to prevent the cell from bursting). It is assumed

that the hydraulic conductivity of a growing cell is very high and does not influence the system or the equation (Cosgrove, 1986).

A factor that could affect the hydraulic conductivity of water across the plasma membrane and tonoplast is the presence of water channel proteins —aquaporins. Water moves across the plasma membrane and tonoplast by the simple process of diffusion. However, recent work by Chaumont *et al.*, (1998) and Chrispeels and Maurel (1994) suggests that the presence of intrinsic membrane proteins facilitate the passage of water across the plasma membrane and tonoplast 10–20 fold. These proteins have been named aquaporins. Aquaporins are not pumps but are believed to increase the passage of water across membranes when the hydrostatic pressure is higher on one side than the other. They are 26–28 kDa and belong to the membrane intrinsic protein (MIP) family which also includes glycerol channels and ion channels (Chrispeels *et al.*, 1999). Aquaporins are highly expressed in meristematic regions suggesting a role in the rapid uptake of water for expanding cells. They are also highly expressed in parenchyma cells (surrounding the xylem vessels) indicating that they might have a role to play in transcellular water flow (Chrispeels *et al.*, 1999), though there is no evidence to support this hypothesis.

1.5.2 Cell wall loosening

Extension of the cell wall is growth. It is believed to be promoted by cell wall 'loosening', which is counterbalanced by cell wall 'tightening' to prevent over-extension of the cell wall. Loosening of the type II primary cell wall could result from disruption of the hydrogen bonding between GAX and cellulose or by the cleaving of GAX (the tether). Hydrogen bonding within the GAX–cellulose or xyloglucan–cellulose framework could be disrupted by the protein expansin (Cosgrove, 1996). Xyloglucan endotransglycosylase (XET) (Fry *et al.*, 1992) is another candidate believed to promote cell wall loosening by cleaving xyloglucan chains and rejoining them to the non-reducing terminus of another xyloglucan molecule. These and other possible cell wall loosening candidates such as $^{\bullet}OH$ and

hydrolases will be discussed next. Essentially, cell wall extension could be prevented by OAC inhibiting any of the following cell wall loosening mechanisms.

1.5.2.1 Hydrolases

Hydrolases are enzymes that cause the cleavage of bonds using water. An example of an endo-hydrolase in the cell wall is cellulase [endo-β-(1→4)-D-glucanase]. Cellulase cleaves MLG and xyloglucan, perhaps thereby helping to effect cell wall loosening. There are also exo-hydrolases present in the cell wall, such as β-D-xylosidase, α-D-galactosidase and β-D-glucosidase, though their function *in vivo* has not been established. They remove monosaccharides from the non-reducing termini of polysaccharides *in vitro* (Fry, 1999), the effect of which on a large polysaccharide is probably insignificant in terms of direct cell wall loosening.

Exo-β-(1→3)-D-glucanase has been shown to promote growth in *Avena* coleoptiles (Taiz, 1984), while Hoson and Masuda (1995) showed that Concanavalin A inhibits β-glucanase activity, thereby, reducing growth in excised rice coleoptiles. Hayashi *et al* (1984) have also demonstrated that endo-β-(1→4)-D-glucanase is expressed in growing tissues.

However, Cosgrove (1997) believes that there is a lack of evidence to support the direct role of hydrolases in cell wall loosening. Cosgrove and Durachko (1994) demonstrated that when hydrolases were applied directly to heat-inactivated cell walls and stretched using an extensiometer that little extension was exhibited.

1.5.2.2 Xyloglucan endotransglycosylase (XET)

Fry *et al.* (1992) proposed that the enzyme, XET, was involved in cell wall growth. They suggested that XET cleaved xyloglucan chains (which may tether microfibrils in the cell wall), causing localised cell wall weakening (permitting growth) as well as rejoining the cut portion to the non-reducing terminus of another xyloglucan chain, thereby maintaining overall cell wall integrity. XET activity was assayed on the

basis that XET transfers a portion of a large non-radioactive xyloglucan molecule to a [^{3}H]nonasaccharide (e.g. XLLGol), thus forming a [^{3}H]polymer that binds tightly to cellulose (Fry, 1997). It has been demonstrated that tissue extension occurs when there is high XET activity in maize roots (Pritchard *et al.*, 1993), barley leaves (Smith *et al.*, 1996), pea stems (Potter and Fry, 1993) and *Arabidopsis* stems (Aubert and Herzog, 1996). It has also been shown that XET mRNA production correlates with the application of growth-promoting substances such as gibberellins (Aubert and Herzog, 1996), auxin (Xu *et al.*, 1995) and brassinosteroids (Zurek and Clouse, 1994). Vissenberg *et al.* (2000) developed an *in vivo* assay for XET action using sulphorhodamine conjugates of xyloglucan oligosaccharides (XGO–SRs) which act as acceptor substrates for XET. The incorporation of XGO–SRs into the cell wall (denoted by an orange fluorescence) can therefore be monitored. Vissenberg *et al.* (2000) reported a high degree of fluorescence in the cell elongation zone of *Arabidopsis* root, supporting XET's role in cell wall loosening. Vissenberg *et al.* (2001) have also reported that XET action increases at the site where root hair formation is initiated. Thompson and Fry (2001) have recently reported that XET can effect transglycosylation of newly synthesised xyloglucan molecules to cell wall bound xyloglucan (that had already been incorporated into the cell wall) and/or transglycosylation of 'older' cell wall-bound xyloglucan molecules to one another by density- and radioactive-labelling of cell wall polysaccharides. They described the effects of these two forms of transglycosylation as 'integrational' and 'restructuring' transglycosylation, respectively. They proposed that both forms of transglycosylation facilitate cell expansion and are involved in cell wall assembly. The pH at which XET works most efficiently is usually about 5.5, not fitting in very well with the AGT, where 4.0 is believed to be the optimum pH for cell wall loosening to occur. This suggests that there are independent systems of cell wall loosening mechanisms in operation in the cell wall.

1.5.2.3 Expansins

Cosgrove (1989) identified proteins that were able to cause extension or creep in boiled cucumber hypocotyl walls at pH 4.5. After pre-incubation with proteinases or

boiling in water, the activity of these proteins was irreversibly inhibited. The proteins were unable to effect creep at a neutral pH. Metals such as Cu^{2+}, Hg^{+} and Al^{3+} inhibited the activity and/or action of the proteins. Interestingly boiling methanol did not inhibit protein action unlike water at the same temperature or boiling ethanol. McQueen-Mason *et al.* (1992) determined that two of these proteins were 29 and 30 kDa. Li *et al.* (1993) called these proteins expansins.

The proteins appeared to be restricted to the growing region of the hypocotyls and when added back to heat-inactivated mature (non-growing) walls, no creep was observed. When these proteins were extracted from cucumber hypocotyls and were exogenously added to heat-inactivated pea, radish and tomato, cell wall creep was observed. However, they had little effect on maize and barley. McQueen-Mason *et al.* (1992) also demonstrated that expansins probably bound to inhibitory metals because walls that were pre-incubated with Cu^{2+}, Hg^{+} and Al^{3+} and then subsequently washed had their creep restored. They suggested that the proteins acted catalytically as activity persisted for many hours without synthesis or addition of new material, resulting in cell wall extension of more than 40%, and only very low concentrations of protein (~0.3 μg) were required.

The possibility of XET and expansins effecting cell wall loosening through similar means was ruled out by McQueen-Mason *et al.* (1993). McQueen-Mason and Cosgrove (1994) demonstrated that expansins weakened filter paper (almost pure cellulose), which derives its mechanical strength from hydrogen bonding, and that this result was not due to the presence of cellulases. They noted that 2 M urea (which is known to disrupt hydrogen bonding) enhanced expansin activity and that deuterated water (deuterium bonds are 20% stronger than those formed in H_2O) decreased expansin activity. They hypothesised that expansins used mechanical energy (from stress caused by turgor pressure) rather than chemical energy to disrupt hydrogen bonding.

McQueen-Mason (1995) observed that there was no detectable release of sugars from the cell wall after long periods of incubation in the presence of expansins suggesting that they were not exo-hydrolases. It was also observed that heat-inactivated walls incubated with exogenous expansin showed the same rates of extension at 10 and 40 minutes, indicating that no additional changes in wall mechanical properties occurred. McQueen-Mason and Cosgrove (1995) demonstrated that expansins did not alter the molecular mass or viscosity of solutions of matrix polysaccharides, indicating that expansins did not hydrolyse pectins or hemicelluloses.

Expansins were found to bind weakly to pure cellulose but binding was greatly enhanced when cellulose was coated with hemicellulose (xyloglucan). It was hypothesised that expansins effected creep by acting at the interface between cellulose and xyloglucan, reversibly disrupting non-covalent bonds (McQueen-Mason, 1995; Cosgrove, 2000a). Whitney *et al.* (2000) showed that α-expansin (CsEXP1) effected extension in cellulose-based composites containing xyloglucan but not mannan-based polysaccharides. It was noted that the effect of expansin was more pronounced on composites with longer, rather than shorter, xyloglucan chains. Whitney *et al.* (2000) suggested that these results further support the theory that expansins act at the cellulose–xyloglucan interface.

It is believed that expansins mediate, to some extent, the acid growth response (Cosgrove, 2000b; Cosgrove, 2000c; Cosgrove, 2001). Cosgrove *et al.* (1997) reported that group I allergens (GIAs) in maize pollen (which cause hay fever) were structurally related to expansins and effected creep. However, while there were structural similarities between α- and β-expansin, GIAs appeared to be more effective at causing creep in grass cell walls than in type I cell walls. The GIA form of expansin was called β-expansin and the previously identified expansins were renamed α-expansins. β-Expansins were found to be highly glycosylated unlike α-expansins. They were also more easily extractable and loosely bound to cell wall. While NaCl could inhibit the action of both types of expansin, lower concentrations were required to inhibit β-expansins. It was also observed that β-expansins did not

bind to cellulose. As α-expansins are highly expressed in dicots and β-expansins are highly expressed in monocots, Cosgrove (1997, 1998) proposed that α-expansins effected cell wall loosening in dicots while β-expansins effected cell wall loosening in grasses, probably as a result of differences in their hemicelluloses; however, both forms of expansins have been found in each of the two cell wall types. Pena *et al.* (1999) noted a correlation between expansin activity and MLG degradation and suggested that this was because expansin rendered MLG available to hydrolytic enzymes. Link and Cosgrove (1998) reported the presence of expansin in tobacco (Bright yellow 2) cell suspension cultures and demonstrated that exogenously added expansin (extracted from cucumber) promoted cell expansion in the tobacco culture in comparison to the control.

Grobe *et al.* (1999) reported that GIAs (β-expansins) were structurally similar to papain-related proteinases and that a GIA from *Phleum pratense* (Phl pl) had a cysteine proteinase function. They demonstrated that when a GIA from Phl pl was expressed in *Pichia pastoris* it had papain-like properties and could cleave Api-zym and Chromozym PL. However, Li and Cosgrove (2001) challenged the structural similarity between GIAs and proteinases and reported that GIAs had no proteinase activity. They suggested that the proteinase activity reported by Grobe *et al.* (1999) was probably caused by contamination by a proteinase induced upon expression of Phl pl in *Pichia pastoris.*

Downes and Crowell (1998) reported that the protein Cim 1 belonged to the β-expansin family and was regulated in soybean cell suspension cultures by cytokinin. Expansins are also believed to have a function in the regulation of fruit ripening in tomatoes (LeEXP1) and strawberries (FaEXP2) (Rose *et al.*, 1997; Civello *et al.*, 1999), initiation of root hair formation (Baluska, 2000) and pollen tube penetration (Cosgrove, 1997; Cosgrove *et al.* 1997). Active expansins have also been identified in maize roots, rice internodes and oat coleoptiles (Wu *et al.*, 1996; Zhang and Hasenstein, 2000; Cho and Kende, 1997; Li *et al.*, 1993). Wu *et al.* (2001a) suggested that β-expansins were more abundantly expressed (based on northern blots) in maize

than α-expansins and that in *Arabidopsis* only 5 out of 31 expansin genes belonged to the β-expansin family. They also reported that a particular form of expansin, *ExpB7*, was highly expressed in maize silk as well as in maize roots. α- and β-Expansin were also reported by Wu *et al.* (2001b) to be expressed in maize roots with low water potential.

A recent paper by Reidy *et al.* (2001) suggested that α- and β-expansin were involved in tissue differentiation; however, they noted that mRNA expression of both forms of expansin did not always correlate with an increase in growth rate. This supports an earlier observation by Caderas *et al.* (2000), where a lack of *LeExp18* mRNA expression in growing regions of tomato hypocotyls was reported.

1.5.2.4 Yieldins

Okamoto-Nakazato *et al.* (2000) purified two proteins (30 and 32 kDa) from *Vigna unguiculata* hypocotyls and called the proteins yieldin 30 and yieldin 32. They incubated the proteins with heat denatured-glycerinated hollow cylinders (GHC) from *Vigna unguiculata* overnight at 10°C and then measured the rate of extension (using an extensiometer) of the GHC bathed in either an acetate buffer at pH 4 or a histidine buffer at pH 6.2. After demonstrating that the proteins did not recognise expansin antibodies, Okamoto-Nakazato *et al.* (2000) suggested that they facilitated cell wall loosening by lowering the yield threshold tension of the wall. Okamoto-Nakazato *et al.* (2000) also reported that yieldin mRNA was expressed mainly in the rapid and moderate elongation region of *Vigna unguiculata.* Using yieldin antibodies Okamoto-Nakazato *et al.* (2001) recently reported that yieldin was present in aerial organs (plumule, hypocctyl and epicotyl) but not in roots. The most intense yieldin signal was found in the apical pre-elongation or hook region of *Vigna unguiculata.*

1.5.2.5 Hydroxyl radicals (•OH)

The non-enzymic scission of polysaccharides is effected *in vitro* by hydroxyl radicals (•OH) and could be another mechanism for cell wall loosening *in vivo.* •OH are

extremely reactive oxidants which will react with almost any organic molecule within ~ 1 nm of its site of production (Halliwell and Gutteridge, 1999). $^{\bullet}OH$ can be generated in different ways, including γ-radiolysis of water or the action of light on periodate. Hydroxyl radical formation in the cell wall may occur via the Fenton reaction. Fry (1998) introduced the idea that Hydroxyl radical formation, via the Fenton reaction, could induce cell wall loosening. He proposed that the non-enzymic action of ascorbate on Cu^{2+} and O_2 gave rise to Cu^{+} and H_2O_2 respectively, which are necessary for the Fenton reaction to occur:

$$O_2 + AH_2 \rightarrow H_2O_2 + A$$

$$2\,Cu^{2+} + AH_2 \rightarrow 2\,Cu^{+} + A + 2H^{+}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + {}^{\bullet}OH \qquad \text{(Fenton reaction)}$$

A = dehydroascorbate

AH_2 = ascorbate

The presence of ascorbate in the cell wall has been reported by Takahama and Oniki (1992) and Castillo and Greppin (1988), while Walker and Webb (1981) reported the presence of Cu^{2+}. Recent work by Fry *et al.* (2001) reported a technique, developed to detect the action of $^{\bullet}OH$ on polysaccharides *in vivo*. Driselase digestion of pectin and xyloglucan, previously incubated *in vitro* with an ascorbate–H_2O_2–Cu^{2+} mixture (to generate $^{\bullet}OH$) followed by treatment with NaB^3H_4, resulted in characteristic ^3H-labelled pectin and xyloglucan products. The ^3H-labelled products (some of which were separable by electrophoresis) provided a 'fingerprint' for hydroxyl radical scission of pectin and xyloglucan. Using this method Fry *et al.* (2001) demonstrated that polysaccharides reacted with $^{\bullet}OH$, during the softening process, in ripening pear fruit. Schweikert *et al.* (1999) also demonstrated the action of $^{\bullet}OH$ (generated from O_2 by horseradish peroxidase using NADH or dihydroxyfumarate) causing scission of polysaccharides *in vitro*. Schopfer (2001) demonstrated that $^{\bullet}OH$ induced irreversible cell wall extension in coleoptiles and hypocotyls selected from a range of plants. Abraded coleoptiles and hypocotyls were pre-loaded with Cu and Fe ions and incubated with AH_2 and H_2O_2 (to generate $^{\bullet}OH$). Cell wall extension, induced by this

treatment, was measured using an extensiometer. Schopfer (2001) reported that auxin induced cell-wall extension could be inhibited by hydroxyl radical scavengers (adenine, thiourea and histidine) and that auxin promoted the production of superoxide (O_2^-), a precursor to the formation of $^{\bullet}OH$.

1.5.3 Cell wall tightening

Cell wall tightening is an important feature in the control of cell growth. A cell wall that lacked the ability to tighten would become too weak to fulfil its function and would not be able to maintain sufficient strength to provide the plant with the support it needs (Cosgrove, 1993a). During the continued process of cell growth the cell wall requires mechanisms or agents to loosen the cell wall and tightening could therefore be considered a controlling factor of cell expansion. Another aspect of cell wall tightening which could act as a controlling factor in cell growth is that cell wall tightening can arrest cell growth after cells have reached maturity.

The cell wall can be tightened by the formation of cross-links. Examples of covalent cross-links are isodityrosine, diferulate and amide linkages. Non-covalent cross-links also occur in the form of calcium bridges and hydrogen bonds. It is possible that OAC could promote cell wall 'tightening', thereby inhibiting cell expansion.

1.5.3.1 Diferulate cross-links

Fry (1983) proposed that ferulic and coumaric acid were esterified with D-Gal*p* and L-Ara*f* residues of pectic polysaccharides and that they could play a structural role in the cell wall by forming cross-links. It had been previously suggested that ferulate could undergo an oxidative coupling reaction to form diferulate after treatment with peroxidase and H_2O_2 (Hartley and Jones, 1975; Fry, 1979). Kamisaka *et al.* (1990) also suggested that ferulate could be oxidised to form diferulate. They noted that a decrease in the growth rate of etiolated coleoptiles was accompanied by an increase in the ferulate and diferulate content in the cell wall, which was correlated with a decrease in cell wall extensibility. Tan *et al.* (1991) demonstrated that rice

coleoptiles grew faster when submerged under water than in air or in oxygenated water. They hypothesised that oxygen promoted the formation of diferulic acid which caused the cell wall to become less extensible, therefore inhibiting cell elongation. Tan *et al.* (1992) also demonstrated that the ferulic and diferulic acid content of coleoptiles irradiated with WL (which inhibits coleoptile growth) increased.

Grabber *et al.* (1995) suggested that the formation of diferulate was limited by H_2O_2 rather than by peroxidase activity or by the abundance of feruloyl residues. Wallace and Fry (1995) demonstrated *in vitro* the formation of diferulic acid esters via the peroxidase-catalysed oxidation of ferulic acid esters. It has been shown that peroxidase inhibitors, such as azide and ascorbate, inhibit cell wall tightening, and that H_2O_2 induces the accumulation of fluorescent, insoluble material in the cell wall (Schopfer, 1996). Wallace and Fry (1999) reported that peroxidases were more effective than laccases at promoting diferulate formation. However, Fry *et al.* (2000) reported that the addition of exogenous H_2O_2 did not always promote the dimerisation of feruloyl arabinoxylans.

It has also been shown that feruloyl coupling occurs intra-protoplasmically as well as in the cell wall (Fry *et al.*, 2000). In summary, it is believed that through a peroxidase-catalysed reaction, ferulate becomes cross-linked to form diferulate, which helps tighten the cell wall. However, intraprotoplasmic coupling of feruloyl residues could help promote growth in young cells by preventing cross-linking of feruloyl esters in the cell wall (through competition) which effect cell wall tightening.

1.5.3.2 Isodityrosine and amide cross-links

Isodityrosine is believed to cross-link extensin in the cell wall via a peroxidase-catalysed reaction between two tyrosine residues of isodityrosine (Fry, 1987; Brady and Fry, 1997); however, the presence of extensin in monocot primary cell walls is scarce. Interpolypeptide links are formed by tetramers of di-isodityrosine and the trimer pulcherosine (Brady *et al.*, 1998). Similarly, pectin molecules may be able to

form amide cross-links with the ε-NH_2 group of lysine residues in extensins (Perrone *et al.*, 1998). Threonine-rich glycoproteins, GRPs and PRPs are found in graminaceous monocots, which may be able to undergo peroxidase catalysed cross-linking (Hood *et al.*, 1991).

1.6 Cell wall regulation of cell expansion

Turgor pressure and cell wall extensibility are considered to be the driving and controlling factors in plant cell growth. However, while turgor pressure is essential for cell growth, the extensibility and the alignment of microfibrils in the cell wall determine the size and shape of the cell (Taiz, 1984; Carpita and Gibeaut, 1993; Cosgrove, 1993a). The extensibility of the cell walls depends on a plethora of factors that either 'loosen' or 'tighten' the cell wall (**1.5.2** and **1.5.3**, respectively). Regulation of cell expansion is an important feature in plant development as it establishes cell shape and polarity. In axial organs cell expansion is anisotropic i.e. cells elongate rather than expand uniformly in all directions. Anisotropic expansion is believed to be determined by the alignment of cellulose microfibrils in a transverse or helical orientation to the axis of elongation (Roelofson and Houwink, 1953; Preston, 1982). Viewing the cell wall as a series of lamellae composed of microfibrils, Roelofson and Houwink (1953) put forward the 'multinet growth hypothesis' to explain the orientation and role of microfibrils during cell elongation. They suggested that newly synthesised microfibrils were laid down on the inner surface of the cell wall in a helical manner, transverse to the axis of cell elongation and under zero stress. They proposed that in a growing cell, a series of new lamellae were continually laid down under zero stress in the same orientation, pushing older lamellae towards the outer surface of the cell wall. They believed that the older lamellae realigned their microfibrillar structure to a more random orientation as a result of the strain driven by stress (turgor pressure) during cell elongation. As the lamellae progressed towards the outer surface of the cell wall (still under stress), the microfibrils realigned to become more nearly longitudinal to the axis of elongation. Preston (1982) agreed with the multinet growth hypothesis in principle but favoured

the term 'passive reorientation' over the 'multinet growth hypothesis' as a means of explaining microfibrillar re-orientation during growth.

Passive reorientation of microfibrils allows cells to expand longitudinally with little increase in the diameter of the cell. Despite the fact that the turgor pressure experienced by the cell is uniform in all directions, the cell elongates, i.e. expands anisotropically (Kutschera, 2000) rather than isotropically. Hauser *et al.* (1995) identified *Arabidopsis* mutants, in which cell expansion was affected at maximal growth rates. The direction of expansion of the cells was altered from anisotropic to isotropic which affected root shape in comparison to the wild type. Tsuge *et al.* (1996) also identified mutants which were unable to regulate polarity of cell elongation, leading to a change in the morphology of leaves, in comparison to wild types.

The microfibrillar orientation of an anisotropically expanding cell exhibits the same reaction to multidirectional force as a coiled spring, except that the cell does not fully return to its original size after removal of the force. That is, like a spring experiencing this type of force, it will stretch in one direction only (longitudinally) with little or no stretch in any other direction.

Cell growth is regulated by the extensibility of the cell wall and the shape is determined by the direction in which the microfibrils are laid down. However, continued synthesis of cell wall polymers is necessary to maintain a uniform cell wall thickness throughout the growth process of the plant cell.

1.7 Herbicides

A dramatic growth in herbicide research followed the discovery of auxin-type herbicides during the 2nd world war. The first time a herbicide was used on a large scale was in 1947, when 2,4-D [(2,4-dichlorophenoxy)acetic acid] was applied to fields (Klingman and Ashton, 1982). Since then a wide range of compounds have

been employed to eliminate broad- or narrow-leaved weeds from fields. Most of these compounds have been discovered through the systematic screening of novel compounds and the identification of active compounds (Kirkwood, 1991). The benefits of herbicides are numerous, but generally speaking they have greatly improved the life of many people whose staple diet is affected by weeds.

There are thousands of different herbicides on the market today, each of which is selective for a range of weed types or for a particular mode of action. The following is a general overview of the major target sites of herbicides.

1.7.1 Auxin mimics

Auxin mimics are a synthetic form of the naturally occurring indole-3-acetic acid (IAA), which is synthesised from tryptophan and found in plants. One of the main functions associated with IAA is the promotion of plant growth. There are five different families of auxin mimics: phenoxyacetic acids, benzoic acids, pyridine-carboxylic acid, aromatic carboxymethyl derivatives and quinoline carboxylic acids (Cobb, 1992).

Dicots are more susceptible to auxin-mimic type herbicides than graminaceous monocots. The reason for this is unclear though it has been suggested that the monocots' leaves retain less herbicide than dicot leaves and because monocots lack a layer of cambium (cells capable of giving rise to secondary xylem and phloem) in comparison to dicots (Cobb, 1992). IAA is actively transported into cells by an influx carrier and leaves the cells via an efflux carrier. Auxin mimics bind to auxin-binding sites and affect the hormone balance of the plant. The exact mode of action of auxin mimics is unknown but it is believed that the plant is unable to control the concentration of auxin in the cell. The 'correct' balance between auxin and other hormones, necessary to regulate metabolism and morphogenesis, is then affected. 2,4-D effects a rapid and continued cell elongation and division as well as increasing

RNA concentrations. Excessive cell division and growth eventually crushes the plant's vascular system, leading to plant death (Zimdahl, 1993).

1.7.2 Inhibitors of photosynthesis

The similarity of photosynthesis between crop and weed (except C4 plants) means that for a herbicide to discriminate between weeds and crops, herbicide selectivity must be based upon differential uptake, movement or metabolism (Dodge, 1991). There are two main ways in which photosynthesis is disrupted by herbicides. The first is through the inhibition of photosynthetic electron transport. Several compounds were found to effect this action, the first being N'-(4-chlorophenyl)-N,N-dimethylurea (DCMU), followed by phenylureas, triazines, uracils, phenylcarbamates, triazinones and many more. The second main way in which photosynthesis can be inhibited by herbicides is the diversion of electron flow at photosystem I (PS I) (Dodge, 1991). The herbicide can act as an alternative electron acceptor to ferredoxin, thereby diverting electrons from $NADP^+$, preventing NADPH formation and in turn preventing CO_2 being reduced to form carbohydrate. Examples of such compounds are diquat, paraquat and other related compounds (Dodge, 1991).

1.7.3 Inhibitors of amino acid synthesis

The biosynthetic pathways of amino acids present ideal targets for herbicide action as it is relatively simple to inhibit a key enzyme of a pathway. One of the best known herbicides, glyphosate (Roundup), developed by Monsanto, affects the shikimate pathway by inhibiting 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, which catalyses the reversible transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to shikimate 3-phosphate (Mousdale and Coggins, 1991). The inhibition of EPSP synthase prevents the synthesis of tryptophan, tyrosine and phenylalanine. Acetolactate synthase (ALS) initiates the synthesis of the branched chain amino acids, valine, leucine and isoleucine. There are three classes of herbicide which affect the action of this enzyme: sulphonylureas, imidazolinones and

triazolopyrimidine sulphonamides. The synthesis of two other amino acids, histidine and glutamine, can also be the target of herbicides (Mousdale and Coggins, 1991).

1.7.4 Inhibitors of lipid synthesis

Initially, fatty acid synthesis inhibitors were researched as a means of reducing lipid levels in man, which proved unsuccessful but worked in plants (Harwood, 1991). *De novo* formation of fatty acids in plants requires the combined action of acetyl-CoA carboxylase and fatty acid synthetase. Acetyl-CoA carboxylase catalyzes the first step for fatty acid synthesis. Aryloxyphenoxypropionates and cyclohexanediones are two important classes of herbicide which have been shown to affect the function of acetyl-CoA carboxylase, by binding to one of the enzyme's active sites. However, in some plants, these herbicides, an example of which is diclofop methyl, prove to be ineffective at inhibiting growth on the plant level, even if they have been successful at inhibiting enzyme activity (Harwood, 1991). Other compounds which affect lipid biosynthesis are pyridazinone, which is believed to affect levels of fatty acid desaturation, as well as inhibiting the formation of trans-Δ3-hexadecadienoate (an unusual acyl group of chloroplast phosphatidylglycerol), and thiocarbamates, which inhibit lipid metabolism by altering the proportions of long-chain fatty acids. There are several other herbicides which affect lipid biosynthesis which have not been mentioned (Harwood, 1991).

1.7.5 Inhibitors of carotenoid biosynthesis

Carotenoids are isoprenoids, generally consisting of eight isoprene units (Bramley, 1991). Their main function is the photoprotection of the photosynthetic apparatus. They are capable of quenching triplet chlorophyll (chlorophyll in a state of excitation which is closer to ground state than first state due to reverse spin of electrons effecting longer half life and phosphoresence (Lawlor, 1993)) and singlet oxygen, which is a highly reactive species of oxygen. Phenylpyridazinone herbicides such as metflurazon, norflurazon, fluridone and halyoxydine, inhibit carotenoid synthesis.

There are also several other mechanisms by which herbicides inhibit carotenoid synthesis and function (Bramley, 1991).

1.7.6 Inhibitors of cellulose synthesis

Polysaccharide synthesis involves the transfer of monosaccharide residues from sugar-nucleotides to growing polysaccharide chains, generally at the non-reducing ends. The enzymes that carry out this process are normally known as polysaccharide synthases. At the present time little is known about the synthesis of cellulose (1→4)-D-Glc*p*) in plants, though its sugar-nucleotide precursor is believed to be uridine-diphosphate-D-glucose (UDP-D-Glc*p*). The catalytic subunit (an 83-kDa polypeptide) of cellulose synthase has been identified in *Acetobacter xylinum* (Delmer, 1999). Plants were then screened for homologues of this gene (the polypeptide sequence) which were identified in *Gossypium hirsutum*, *Arabidopsis thaliana* and *Oryza sativa* (Delmer, 1999). Pear *et al.* (1996) characterised two cotton cDNA clones and identified one rice cDNA that are homologues of bacterial *celA* genes (encoding the catalytic subunits of cellulose synthase) and may encode plant *celA*. The cotton genes are expressed at high levels during secondary wall cellulose synthesis; however, they show little homology at the N- and C-termini with bacterial *celA* and they also contain two internal insertions of sequence, one conserved, and one hypervariable unlike the bacterial *celA* gene.

Although the exact mechanism of cellulose synthesis has not yet been determined, there are herbicides on the market which block cellulose synthesis. One of the first such herbicides was 2,6-dichlorobenzonitrile (DCB or dichlobenil). The inhibitory effect of DCB was measured by its ability to inhibit the incorporation of [U-^{14}C]glucose into the cell wall polymers of cotton fibres (*Gossypium hirsutum*) actively engaged in cellulose synthesis. Specificity was demonstrated by the lack of inhibitory effect of DCB on [U-^{14}C]glucose incorporation into non-cellulosic polysaccharides. High specificity indicated that incorporation of [U-^{14}C]glucose into

non-cellulosic glycans was uninhibited relative to the inhibition of cellulose synthesis (Montezinos and Delmer, 1980).

Further studies, using a photoreactive, tritium-labelled analogue of DCB [2,6-dichlorophenylazide (DCPA)], revealed an 18-kD [^{3}H]DCPA-labelled protein, indicating a binding site of DCPA. However, it is not thought to be the catalytic polypeptide of cellulose synthase; instead it is believed to be a regulatory protein of β-glucan synthesis. On SDS–PAGE the 18-kD polypeptide ran close to the regulatory protein camodulin (Ca^{2+}-binding protein); although it has been shown not to be calmodulin, it is believed to have similar properties such as being relatively small and acidic. It has been shown that the amount of radiolabelled 18-kD polypeptide increased at the onset of cellulose synthesis and was barely detectable in elongating cotton fibres, when the rate of cellulose synthesis was low (Delmer *et al.*, 1987).

Recently, Nakagawa and Sakurai (1998) reported that DCB-habituated tobacco cells had elevated amounts of celA1 protein in comparison to normal tobacco cells, but only 1/10 the amount of cellulose. This led them to suggest that DCB stabilised celA1 protein by binding it while simultaneously inhibiting the formation of cellulose microfibrils.

Measurment of [U-^{14}C]glucose incorporation into cell wall polymers showed that isoxaben, N-[3-(1-ethyl-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide inhibited cellulose synthesis (Heim *et al.*, 1990; Sabba and Vaughn, 1999). This conclusion was further demonstrated through the use of isoxaben-resistant *Arabidopsis thaliana* mutants DH1 (Heim *et al.*, 1989), DH47 and DH48 (Heim *et al.*, 1990), which are believed to be altered at the isoxaben interaction site. Uptake and accumulation of isoxaben showed little difference between the wild type and three mutants. However, [U-^{14}C]glucose incorporation into the cell wall polymers by mutant *Arabidopsis* was shown to be unaffected unlike that by the wild-type, which showed severely inhibited incorporation of [U-^{14}C]glucose into cellulose (Heim *et al.*, 1991).

Two other herbicides, triazofenamide (1-(3-methylphenyl)-5-phenyl-1H-1,2,4-triazole-3-carboximide) and thiazolidinone (5-tert-butyl-carbamoyloxy-3-(3-trifluoromethyl)phenyl-4-thiazolidinone), also inhibit cellulose biosynthesis in a manner similar to isoxaben (Heim *et al.*, 1998; Sharples *et al.*, 1998). However, triazofenamide is believed to have a unique binding site because of its lack of cross-resistance with isoxaben or DCB in mutants unlike thiazolidinone. Another noteworthy difference between these two types of herbicide is that the triazofenamide does not inhibit lateral root formation, unlike the thiazolidinone. Heim *et al.* (1998) suggest the lack of lateral root formation is indicative of the inhibition of microtubule polymerisation. Similar symptoms have been observed in maize roots, shortly after addition of OAC (personal communication by Dr. Sarah Miller, Aventis).

Another herbicide believed to inhibit cellulose synthesis is quinclorac (3,7-dichoro-quinolinecarboxylic acid). Koo *et al.* (1996) showed that it differed from the other cellulose synthesis-inhibiting herbicides because it also appeared to inhibit hemicellulose synthesis (GAX and MLG) in maize, though the authors were unsure if this was a primary or secondary effect.

In general, of all the herbicides currently released onto the market, few are known to be cell wall-related and none appears to inhibit specifically cell wall expansion.

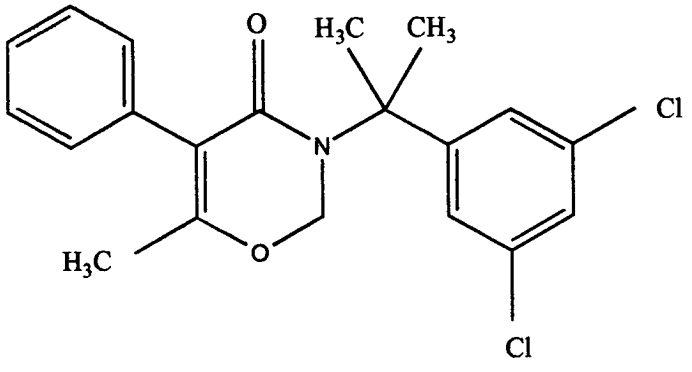
1.8 Oxaziclomefone

Oxaziclomefone (OAC; **Fig. 1.8**) is a new herbicide discovered by Aventis Crop Science U.K. Ltd. It is very effective in controlling the graminaceous monocot, *Echinochloa* sp. (barnyard grass), which is otherwise difficult to control owing to its high percentage seed settling and germination. Oxaziclomefone has a long residual activity and a wide application window, but no carry-over effects into following crops such as wheat, barley, Chinese cabbage, radish and onion.

Earlier work by Jikihara (1997) indicated that OAC might inhibit meristem cell expansion in a manner dissimilar from that of any known herbicide. It does not appear to affect *Echinochloa* through any of the previously explained modes of action of herbicides. OAC also inhibits growth of *Arabidopsis thaliana* (personal communication from Dr. David Cole).

Prof. S.C. Fry, The University of Edinburgh, (unpublished) showed that an analogue of OAC, RPA 800298, did not inhibit the uptake of β-D-Glc, α-L-Ara, β-D-GlcA, *trans*-cinnamic acid and L-methionine by freshly subcultured maize cells. The incorporation of the previously mentioned precursors and that of acetate, into cell wall polymers, also remained unaffected in the presence of RPA 800298. Fry (unpublished) monitored the partitioning of total carbon between the major monosaccharide residues of the wall matrix and cellulose. Paper chromatography of the matrix monosaccharides, which were quantified for ^{14}C, indicated that RPA 800298 had no effect on incorporation of ^{14}C from [^{14}C]glucose into uronic acid, D-β-Gal, D-β-Glc, D-β-Man, L-Ara and D-Xyl residues of the cell wall matrix. Paper chromatography of Driselase-digests of cellulose (to release D-Glc*p*) clearly showed that RPA 800298 did not affect the ^{14}C content of cellulose. This result indicates that the primary mode of action of RPA 800298 is not through inhibition of the biosynthesis of cellulose or of any of the major wall matrix polysaccharides. Fry (unpublished) also monitored the affect of OAC upon the partitioning of (hydroxy)cinnamoyl groups into the cell wall and their formation of oxidatively cross-linked products. Alcohol insoluble residues (AIRs) from cell suspension culture fed with [U-^{14}C]cinnamate were treated with NaOH to saponify ester bonds and then neutralised with trifluoroacetic acid (TFA). The suspension was partitioned against ethyl acetate. The ethyl acetate phase was concentrated, dissolved in acetone and loaded onto a TLC silica gel plate. Autoradiography of the plate indicated the presence of an unknown compound in OAC treated cells which was not present in the control. This suggested that OAC might have been promoting the formation of phenolic cross-links. This could be achieved by promoting the secretion of

peroxidase into the cell wall, promoting the formation of H_2O_2 or by decreasing the levels of anti oxidants which can prevent phenolic cross-links. However, these results were not reproducible.



IUPAC: 3-(1-(3,5-dichlorophenyl)-1-methylethyl)-2,3-dihydro-6-methyl-5-phenyl-4H-1,3-oxazin-4-one

The structure of oxaziclomefone

1.9 Aim

The aim of this project is to identify the means by which OAC prevents plant growth. This aim requires investigation of the effect of OAC upon any or all of the agents or mechanisms related to cell wall growth, such as turgor pressure and cell wall loosening and tightening.

2. Materials and Methods

2.1 General Methods

2.1.1 Chemical Reagents

All chemical reagents were obtained from Fisher Chemicals (Fisher Scientific), Sigma-Aldrich (Poole, U.K.), or BDH AnalaR Chemicals Ltd. (Poole, U.K.) unless otherwise stated.

2.1.2 Culture medium

Cell suspension culture (CSC) medium was made up in four-litre batches containing 18.8 g of Murashige and Skoog salts (Murashige and Skoog, 1962) (5519; Sigma catalogue reference), 80 g sucrose and 40 ml of 442 μM 2,4-D. The pH was adjusted to 5.6–5.8 with NaOH (1 M). The medium (1 litre) was dispensed into 500-ml flasks at 200 ml per flask and autoclaved for 15 min at 121°C.

2.1.3 Maintenance of cultures

CSCs referred to throughout this thesis are *Zea mays* ("Black Mexican"). CSCs were inoculated each week, and subcultured fortnightly. From a 200-ml, two-week-old CSC, 20 ml was aseptically transferred into a 500-ml flask containing 200 ml of fresh, autoclaved medium.

2.1.4 Hydroponically grown maize seedlings

All maize seeds (*Zea mays*) were of the Tuxedo variety and were purchased from E.W. King and Co. Ltd., Monks Farm, Kelvedon, Essex. Maize seeds were placed between two layers of wet tissue paper and germinated in the dark. Approximately 2 days after germination the seedlings were transferred to perforated polystyrene 'rafts'. The rafts were floated on water (gently aerated by means of an air pump

(Elite 800)) in the dark at room temperature. The seedlings were grown until they were of a suitable length.

2.1.5 Radioisotope assay

Radiolabelled aqueous solutions were assayed using a 1:10 ratio of aqueous sample to OptiPhase scintillation fluid (Wallac, Milton Keynes, Bucks, U.K.). The isotopes were assayed twice, over a 10 min period.

Initial experiments revealed that the scintillation counter, when counting both ^{14}C and ^{3}H simultaneously, 'mistook' low-energy ^{14}C for ^{3}H and therefore reported an inflated presence of ^{3}H. To correct for this, serial dilutions of known ratios of ^{14}C and ^{3}H were counted with both isotopes either mixed together or separate, using the dual channel **(Table 2.1.5.1)**. From this table a correction factor was calculated (0.623) and applied to the ^{3}H counts. All results, reported in this thesis, have had their background radioactivity subtracted and where necessary the ^{3}H has been corrected for the presence of ^{14}C.

Table 2.1.5.1 Calculation of correction factor, used to estimate the correct levels of ^{3}H present in a mixture of ^{14}C and ^{3}H

Vial No.	Isotopes in vial		Recorded ^{14}C	Recorded ^{3}H	Ratio $^{3}H/^{14}C$
	^{14}C (Bq)	^{3}H (Bq)			
1	333	1665	13740	38944	2.83
2	33	166.5	1410	3952	2.80
3	3	16.65	136	393	2.89
4	0.30	1.66	14	40	2.86
5	0.03	0.16	1	5	5
6	0	1665	0	30630	0
7	0	166.5	0	3070	0
8	0	16.65	0	308	0
9	0	1.66	0	28	0
10	0	0.16	0	0	0
11	333	0	14066	8793*	0.625
12	33	0	1419	884*	0.623
13	3	0	132	82*	0.621
14	0.30	0	13	11*	0.846
15	0.03	0	0	3*	0

*** falsely recorded presence of ^{3}H. To determine the correction factor for ^{3}H, ^{14}C that was wrongly counted as ^{3}H (green) was divided by the ^{14}C (blue) counts for each of the dilutions. This factor (an average of $^{3}H/^{14}C$ ratio (red)) was then multiplied by the ^{14}C (blue) and subtracted from the inflated ^{3}H (purple).**

2.1.6 Application of OAC

OAC, which has a water solubility of 0.18 ppm at 25°C (180 μg l^{-1} or 478 nM) (Jikihara, 1997), was dissolved at 10 mM in dimethylsulphoxide (DMSO) and then added to aqueous solutions to give a final concentration of 478 nM, unless otherwise stated. All experimental controls contained the same concentration of DMSO (typically <1%) as was used to dispense OAC.

2.1.7 pH measurements

pH was measured using a Mettler Delta 320 pH meter with a Mettler Toledo probe ~ 4 mm in diameter.

2.2 To assay the physiological effects of OAC upon maize CSCs and seedlings

2.2.1 To determine the ID_{50} of OAC

The ID_{50} (inhibition dose) was defined as the concentration of OAC which inhibits 50% of cell growth. CSCs of different ages were incubated with OAC (added as 20 μl of a concentrated solution in DMSO per 4-ml culture) for 6 days. Triplicate cultures were set up for each concentration. After six days the settled cell volume (SCV) and packed cell volume (PCV) of each CSC were estimated as follows: a known volume of CSC was decanted into a 15-ml graduated centrifuge tube, the CSC was allowed to settle for 15 min and the volume to which the cells settled was expressed as a percentage of the initial volume of culture. The PCV was measured in a similar manner, except that the cells were centrifuged (Centaur 2) for 5 min at ~ 850 *g*.

2.2.2 To determine the physiological effects of OAC upon etiolated maize seedlings

Maize seeds (20) were sown in four boxes of vermiculite ((a), (b), (c) and (d)). To initiate germination, the seeds in boxes (b) and (c) received only water, box (a) was used as a control (~1% DMSO in water) and box (d) received OAC. All of the boxes were placed in the dark at 25°C. The seedlings in each box were irradiated with red light (to make the coleoptiles grow longer) for a 10-min period 72 h after sowing and at the same time OAC was added box (b). At 96 h (after seeds had been sown) OAC was added to box (c). The root, coleoptile and mesocotyl length of each seedling from each box were measured 120 h after sowing.

2.2.3 To determine the effect of OAC upon excised coleoptile growth in the presence of auxin

Maize seedlings were grown as described in section **2.2.2 (box (a))**. The coleoptiles (4-day-old) were excised at the base and lightly abraded with a light emery cloth (Kutschera and Schopfer, 1985). The coleoptiles (18) were measured to the nearest 0.5 mm. Each coleoptile was placed upside down in a well of a 'Cell Well' plate (Falcon), ~ 2.5 cm in diameter and ~2 cm deep; a third of the wells contained H_2O (~1.5 ml), another third contained 20 μM indole 3-acetic acid (IAA) (~ 1.5 ml) and the remaining third contained 20 μM IAA and 100 nM OAC (~ 1.5 ml). The coleoptiles were aerated by gentle shaking at 60 rpm, in the light at 25°C. The length of each coleoptile was measured hourly using a ruler, over a 7-h period.

2.2.4 To determine the effect of OAC upon cell elongation (by longitudinal sectioning of maize roots) and the length of time it takes OAC to inhibit root growth

Maize seeds were germinated between two layers of wet tissue paper in the dark as in **2.1.4**. Approximately 2 days after being sown, a portion of the seedlings were blotted dry. An ink dot was placed on the hypocotyl of each seedling with a permanent marker. The distance between the ink dot and root tip was measured. The seedlings

were then transferred to grow hydroponically as described in section **2.1.4** except that they were divided into two separate containers of water. Approximately 18 h after transfer, the distance between the ink dot and root tip was measured again and either OAC or DMSO was added to each container. After a further 2, 4, 8, 24 and 48 h the roots were re-measured.

Additional roots in the same containers were harvested and 10-mm tip zones were vacuum-infiltrated with FAA, formalin, acetic acid and 70% ethanol 1:1:18. After 15 min the FAA was gradually replaced by incubation of the roots in increasing concentrations of EtOH (60%, 70% and 85%), each for 1 h at 60°C. The 85% EtOH was replaced by 95% EtOH containing 1% eosin (w/v) overnight, which was subsequently replaced by 100% EtOH (containing 1% (w/v) eosin) for 30 min while shaking at room temperature. The EtOH and 1% eosin (w/v) was replaced by a series of increasing concentrations of Histoclear (CellPath plc, Hemel Hempstead, U.K.) (25%, 50% and 75%) mixed with decreasing concentrations of EtOH (75%, 50% and 25%) each for 1 h at room temperature. The Histoclear and EtOH mixture was replaced by incubation of the roots for 1 h in 100% Histoclear, which was replaced with fresh 100% Histoclear and again incubated for 1 h at room temperature. The Histoclear was then replaced by 100% Histoclear and one quarter volume of Paraplast chips (Gibco BRL) (wax) and left overnight. The roots were then embedded in blocks of melted wax at 42°C. Thin sections (~ 8 μm) were cut from each block of wax using a microtome (Leicia RM 2025) and placed on microscope slides. The roots were dewaxed with Histoclear and stained with 0.1% toluidine O blue (in acetate buffer) and washed with H_2O to remove the stain. The root sections were observed under a light microscope and photographed. I thank Dr. Andrew Hudson, The University of Edinburgh, for use of the microtome and Jill Harrison for advice.

2.3 To determine the effect of OAC upon turgor pressure in CSC's and roots

2.3.1 Comparison of the elution rates of ^3H_2O and [^{14}C]mannitol from CSCs

Two 45-ml volumes of 6-day-old maize CSC were placed into 60-ml cylindrical Sterilin containers (Bibby Sterilin Ltd., Stone, Staffs., U.K.). The 45-ml CSCs were allowed to settle and a 30-ml volume of medium was removed from each Sterilin container. ^3H_2O (5 μCi; 0.5mCi mmol^{-1}) and [^{14}C]mannitol (1 μCi; 55mCi mmol^{-1}) were added to each container and incubated at room temperature, for 1 h, on a shaker in the fume hood. A 5-ml volume of CSC was removed from each pot and passed through a Polyprep column (Chromatography column, Bio Rad) to form a bed volume of cells of ~1.8 ml. Using a peristaltic pump, fresh medium was passed through the bed of cells so that the flow rate of medium in approximately equalled the flow rate out. Fractions were collected every 10 s over a 15-min period and assayed for both ^{3}H and ^{14}C simultaneously **(2.1.5)**.

2.3.2 To determine the effect of OAC upon water transport across cell membranes

Section **2.3.1** was repeated except that CSCs were treated with or without OAC overnight prior to assaying or 3 h after addition of radioisotopes, fractions were collected over a 150-s period and Triton-X 100 was added (to 0.1%) to 45-ml CSC prior to addition of radioactive isotopes.

2.3.3 To determine the effect of OAC upon the point of incipient plasmolysis (POIP) of CSCs

The POIP is the osmotic pressure at which 50% of cells become plasmolysed. [^{3}H]Maltitol (~20Mbq μmol^{-1}) was dried down in a Speed Vac (Savant; using refrigerated condensation trap, Stratech Scientific, London; Genevac VP100 pump),

dissolved in 100 µl of H_2O and added (3.75 µCi) to 300 ml of CSC (4-day-old). Also added to the same culture was 0.8 µCi [^{14}C]dextran (18500–1370000 MBq g^{-1}; dissolved in 100 µl H_2O). The 300 ml CSC was equally divided into two flasks, OAC was added to one flask and DMSO was added to the remaining flask. A 4-ml volume was dispensed from each of the flasks into each of sixty six, 60-ml Sterilin cylindrical containers, which contained solid D-glucitol (to give a final concentration of 0–1 M). Each concentration was carried out in triplicate. The CSCs were incubated for 2 h and then transferred into centrifuge tubes. The medium was pipetted off and thin strips of Whatman 3MM paper were placed into each pot to draw off excess medium. The cells were assayed for radioactivity.

This experiment was repeated, except that instead of pipetting off excess medium the CSC was pipetted into a centrifuge tube containing a 1-ml silicone fluid (Dow Corning, BDH) layer placed above a 4-ml layer of glycerol and centrifuged for 10 min at ~ 850 *g*.

2.3.4 To determine if OAC affects osmotic pressure of maize CSC, by measurement of protoplastic volume

CSCs (6-day-old) contained in sixteen flasks (at 200 ml per flask) were treated with or without OAC (eight flasks for each treatment). After 2, 6 and 10 h incubation, four flasks of CSC (two with OAC and two without OAC) were filtered through Miracloth (Calbiochem) and the cells were retained. Surplus medium surrounding the cells was gently squeezed off in a uniform fashion for each of the four sets of cells. The fresh weight of the cells (combined for each treatment) was measured. At each time point, the cells were added to a volume (in ml) of mannitol solution (0, 0.2, 0.4, 0.6, 0.8 or 1.0 M) equal to their fresh weight (in g) and incubated for 15 min. Each concentration was carried out in triplicate. The cells were regularly agitated throughout this incubation period and then poured through a 5-ml syringe barrel (Plastipak, 5-ml syringe barrels, Becton Dickinson) (plugged with glass wool). The plunger was re-inserted into each barrel and depressed until it was just above the level of the cells to force out the remaining liquid. The amount of eluate collected from the cells (with and without OAC) at each mannitol concentration was recorded.

2.3.5 To determine if OAC affects osmotic pressure of maize roots, by measurement of the angle of root curvature

Hydroponically grown 5-day-old maize seedlings were treated with or without OAC. After 2 and 6 h the roots were excised and placed in 3 ml of 0, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40 or 0.45 M NaCl, for ~ 20 min. Each concentration was carried out in triplicate. The apical section of each root (~ 80 mm from the root tip) was cut and stuck onto a block using BluTack (Bostik) **(Fig. 2.3.4.1)** so that there was an overhang of root of about 50 mm. A 150-mg weight (small piece of rubber tubing) was placed on the overhanging root tip. Lockhart (1959) stated that when the POIP is reached it is accompanied by an abrupt increase in tissue flexibility. Consequently, the angle of curvature (away from the horizontal) traced by the roots treated with OAC (at each NaCl concentration) was measured and compared to the angle traced by the control.

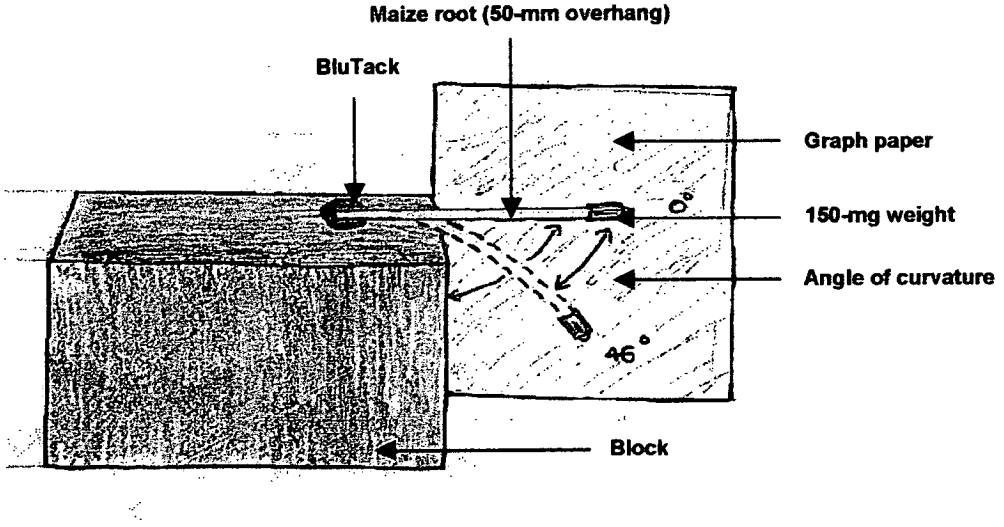


Fig.2.3.4.1 Method for measuring the angle of curvature of treated roots. Each root was placed on a block with a 150-mg weight on its tip. The angle through which the root curved away from the horizontal was traced and recorded.

2.3.6 To determine the effect of OAC upon turgor pressure in CSCs, measured using a depression of freezing point osmometer

In this experiment, the turgor pressure of CSCs was assayed by use of a depression of freezing point osmometer. A depression of freezing point osmometer supercools a sample solution down to –80°C, at which point it freezes. The temperature, which is measured by a thermocouple, is then slowly raised until the sample melts. The osmometer records the osmolarity of the sample at the melting/freezing point in mosmol l^{-1}. The higher the concentration of solutes present in the sample the lower the freezing/melting point of the sample.

CSCs (5-day-old) contained in flasks (200 ml per flask) were treated with or without OAC (done in duplicate). After 2, 4, 8, 24 and 48 h, CSCs (with and without OAC) were filtered through glass fibre paper which was inserted into the outlet of a 2-ml syringe barrel. The plunger was inserted into the barrel and depressed to remove any excess medium, care was taken not to squash the cells. The culture filtrate (1 ml) was collected and frozen. The cells (in the syringe barrel) were frozen at –80°C overnight then thawed and squashed (by depressing the syringe plunger). The sap (1 ml) from the squashed cells was collected, filtered through glass wool and frozen. The osmotic pressure of 100 µl of each sample was measured using a depression of freezing point osmometer (CAMLAB, Roebling) (many thanks to Dr. Wieland Fricke, The University of Paisley for the use of his osmometer and helpful advice).

The turgor pressure of the cells was estimated by subtracting the osmotic pressure of the cell sap from the osmotic pressure of the medium (apoplastic fluid).

2.4 To determine the effect of OAC upon cell wall tightening

2.4.1 To determine the effect of OAC upon peroxidase action in CSCs

A flask containing 200 ml of 4-day-old CSC was dispensed into 30 cylindrical Sterilin containers (at 4 ml per container). OAC was added to 15 of the containers and DMSO was added to the remaining containers. After 1, 2, 4, 8 and 24 h, 600 µl of buffer containing 50 mM tartaric acid (Na^+, pH 4.5) and 0.8 mM *o*-dianisidine di-HCl, was added to 6 containers (three of which had received OAC) to give final concentrations of ~ 6 mM tartrate and 0.1 mM *o*-dianisidine. Change in colour due to oxidation of *o*-dianisidine was observed by eye.

2.4.2 To assay the effect of OAC upon peroxidase activity *in vivo*

Approximately 400 ml of 5-day-old CSC was filtered through Miracloth, washed with fresh medium and weighed (fresh weight ~23 g). The cells were dispensed (3.1–3.2 g per flask) into six 250-ml flasks containing 25 ml of fresh medium. Three of the flasks received OAC and the remaining three were used as controls. The flasks were then placed on a shaker at room temperature. After 1, 2, 7 and 13 h the CSCs were allowed to settle and 20 µl of medium was removed from each flask and added to 2 ml of 50 mM tartrate buffer (Na^+, pH 4.5) containing 0.8 mM *o*-dianisidine. A 600-µl volume of 0.8 mM H_2O_2 was added to the mixture. At 0, 15, 25, 45, 60 and 90 min after addition of H_2O_2 the samples were read A_{430} on a spectrophotometer (CECIL 8000 series).

2.4.3 To determine the effect of OAC upon the cross-linking of exogenous coumaroyl esters

CSCs (4 days old), treated with or without OAC for 24 h, were dispensed (in 10-ml volumes) into six cylindrical Sterilin containers and fed 50 Bq ml^{-1} and 75 Bq ml^{-1} of [U-^{14}C]coumaroyl esters, M1B and M2B, respectively (kindly prepared by Dr. Antonio Encina, The University of Edinburgh). The coumaroyl esters were not fully characterised but one was probably a glucosyl ester and the other a low-$_{Mr}$ coumaroyl ester. The CSCs were shaken continuously at room temperature. At various times after addition of [U-^{14}C]coumaroyl esters, the CSCs were allowed to settle and 500 µl of the medium was removed. A 200-µl volume of each sample was assayed for total radioactivity. The remaining 300 µl was mixed with 1.3 ml of 100% EtOH, placed in the fridge overnight, then centrifuged and assayed for radioactivity **(2.1.4)**.

2.5 To determine the effect of OAC upon cell wall loosening

2.5.1 XET assay

XET transfers a portion of a large non-radioactive xyloglucan molecule to [^{3}H]XLLGol, thus forming a [^{3}H]polymer that binds tightly to cellulose. The activity of XET can therefore be determined by assaying the presence of ^{3}H bound to the cellulose.

2.5.1.1 To determine the effect of OAC *in vitro* upon XET activity extracted from maize roots

Maize roots (1 week old) were ground up in buffer 350 mM succinate, Na^+, pH 5.5, 16.7 mM calcium chloride and 1.67 mM dithiothreitol and sand with a mortar and pestle at 4°C, and centrifuged for 10 min at ~ 850 *g*. XET (10 µl) extracted from the maize roots was added to 20 µl substrate solution, which contained tamarind xyloglucan (0.3%) and [^{3}H]XLLGol (80 Bq μl^{-1}; 100 MBq µmol l^{-1}), and incubated in an Eppendorf vial for a specified period of time. To stop the reaction, 20 µl of

50% formic acid was added. The reaction mixture was dried onto cellulose (Whatman 3MM) paper (marked out in 4 × 4 cm squares). The Whatman 3MM was left overnight in running cold water to remove unbound radiolabelled oligosaccharide. The paper was oven dried at 80°C and assayed for ^{3}H using 2 ml OptiScint HiSafe scintillation fluid (Wallac, Milton Keynes, Bucks, U.K.). The xyloglucan was a gift from Mr. K. Yamatoya, Dainippon Pharmaceutical Co., Osaka, Japan. The reducing end of XLLG was reduced with $NaB^{3}H_4$ to give [^{3}H]XLLGol (Hetherington and Fry, 1993). This experiment was repeated except that XET was pre-incubated with OAC for 1 h.

2.5.1.2 To determine the effects of OAC *in vivo* upon XET activity secreted into the medium and extractable XET from cells

CSC (4 days old) was treated with or without OAC over a 24-h period. After 24 h the cells were filtered through Miracloth and weighed. From each flask, 3 g of CSC was ground up in 4.5 ml of buffer (composition as before in **2.6.1.1**). The medium and cell extract were assayed for XET activity as before except that 20 µl of sample was added to 10 µl of substrate mixture and the incubation times were 0, 30, 90, 150 and 210 min.

2.5.2 To determine the effect of OAC upon ascorbate consumption in CSC

CSC (4 day old) was dispensed (in 100-ml volumes) into six 250-ml flasks, three of which received OAC and three of which were used as controls. Ascorbic acid (to 200 µM) was added to each flask at 0 and 320 min. The flasks were placed on a shaker (~ 100 rpm) at room temperature. At various times after addition of ascorbic acid, the CSCs were allowed to settle and 1 ml of medium was removed from each flask. Each 1-ml sample of medium was added to 500 µl of formic acid. A third of this mixture was added to 500 µl of 10% HPO_3, which was then titrated against 0.1% DCPIP (blue) until it turned, and stayed, colourless for a minimum of 3 s.

2.5.3 To determine the effect of OAC upon endogenous acid 'creep' in methanol-killed maize silks

Maize silks (harvested by Dr. V. Walbot, Stanford University, California), that had been stored in methanol at 4°C, were briefly rehydrated in water. Two ink dots were marked on each silk a distance of 45 mm apart (measured with a ruler) using a permanent marker. One end of each silk was attached (by rubber tubing) to a plastic cocktail stick and threaded through a glass tube (~ 6 mm in diameter) which had a piece of rubber tubing attached at one end. The free end of each silk was trapped in the rubber tubing (attached to the glass tube) by means of a medi-clip (Fisher). Each tube was filled with either, 25 mM succinate buffer (Na^+, pH 4 with or without OAC to 10 nM), or 25 mM phosphate buffer (Na^+, pH 7 with or without OAC to 10 nM). The cocktail stick was then suspended from a line by means of a bull-dog clip **(Fig. 2.6.3)**.

The force required for creep was provided by the weight of the entire apparatus, i.e. buffer, cocktail stick, glass tube, rubber tubing and medi-clip, which was ~ 8 g. The silks were 'stretched' overnight, after which the 'relaxed' distance between the two ink dots was measured again.

Variations of this experiment were carried out in bulk solution, blind or both. For experiments done in bulk solution, maize silks were not individually suspended from a line; instead they were suspended from glass rods traversing the top of a large container of buffer (containing ~12.5 l of 25 mM succinate buffer (Na^+) at pH 4, with or without a range of concentrations of OAC) so that they were completely submerged. The force applied on each silk was in the form of a bull-dog clip (~ 5 g) attached to the free end of each silk (with a small piece of rubber tubing surrounding the end of the silk to prevent the bull-dog clip from breaking it).

In blind experiments, colleagues added a known concentration of OAC or a known volume of DMSO to a series of labelled test tubes containing 25 mM succinate buffer (Na^+, pH 4). Colleagues recorded what each labelled test tube received, which they disclosed after the silks had been measured (many thanks to Dr. Jo Dumville

and Ms. Martha Green). Therefore, when the silks were measured after stretching there was no way of my knowing whether or not they had been stretched in the presence of OAC.

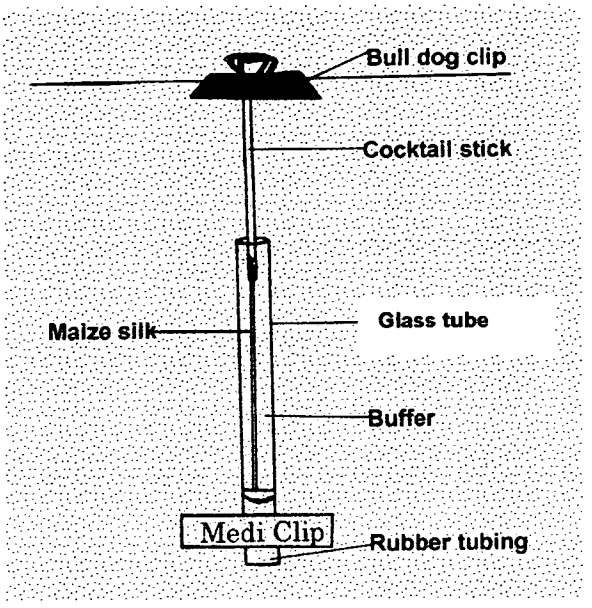


Fig. 2.5.3 Apparatus used to assay for the affect of OAC upon acid-mediated creep

2.5.4 To determine the effect of OAC upon endogenous acid 'creep' in methanol-killed celery petiole vascular bundles

Vascular bundles were removed from celery (purchased from Sainsbury's) petioles (young, ~ 100 cm in length) and methanol-killed. The vascular bundles were then stretched in 25 mM succinate buffer, (Na^+) pH 4, with or without OAC (100 nM) as described in section **2.6.3**, except that a force of ~ 10 g was applied.

2.5.5 Extraction of β-expansins from maize pollen

Maize pollen (Greer Laboratories Inc, Lenoir, N.C. 28645, U.S.A.), shipped frozen, was thawed and 15 g was added to 60 ml of 50 mM acetate (Na^+, pH 4.5) and stirred continuously at 4°C for 1 h. The mixture was centrifuged at 850 *g* for 10 min and the supernatant was collected. The pellet was resuspended in 40 ml of the above buffer and centrifuged again. The combined supernatants were dialysed twice at 4°C against 20 mM acetate (Na^+, pH 4.5) for 4 h and against 0.5% (w/v) chlorobutanol in water

for 4 h. The extract was dried in a freeze-dryer (Modulyo). The extract (0.598 mg) was dissolved in 12 ml of water.

2.5.6 To determine the effect of OAC upon acid creep mediated by β-expansin extract on boiled maize silks

Methanol-killed maize silks were boiled in water for 30 s to denature endogenous proteins. The silks were stretched as previously described in section **2.6.3** except that 0.02 vols of β-expansin extract was added to the succinate buffer with or without OAC. Unboiled silks were also stretched in succinate buffer (pH 4) as a control.

2.5.7 To determine the effect of OAC upon acid creep in maize roots

Maize seedlings were grown hydroponically until the roots were ~ 8 cm in length and then harvested. The roots were frozen at –80°C overnight and thawed. They were then placed between two pieces of Whatmann 3MM paper (sandwiched between two sheets of rayon acetate) and squashed by running the top of a ruler along the lengths of the roots. Two small squares of glossy (magazine) paper were covered with Super Glue (containing cyanoacrylate, purchased from Sainsbury's); one square of paper was glued to the root tip so that it covered < 2 mm of the root tip and the other was glued ~ 1 cm back from the root tip. Paper was glued to the roots, which were then laid out on moistened graph paper (Whatman No. 1 paper with scaled markings printed on it with a laser printer) and photographed. The root tip (and paper) was then dipped in carborundum powder and placed into a small cork that had been partly split in half (with a razor). The root tip was firmly gripped in the partly split cork by being wedged between the two halves, and then forced (root first) through an Eppendorf vial (with the bottom cut off). The root was then threaded through a glass tube (with rubber tubing attached at one end). The Ependorf tube fitted inside the rubber tubing, which formed a seal around the Eppendorf. The glass tube was then filled with 50 mM acetate (Na^+) buffer at pH 4, with or without OAC. The entire apparatus was suspended from a line via a cocktail stick by means of a bull-dog clip, and the glass tube, buffer, cork, Eppendorf and five metal paper clips provided the

force required (~ 4 g) to drive creep. The roots were stretched overnight then taken down and placed on Whatman No. 1 'graph paper' and re-photographed. The photographs were scanned and the images were magnified 10-fold, using Adobe PhotoShop. The distance between the two squares of paper before and after stretching was measured on Adobe PhotoShop. The results, in arbitrary units, were converted to cm (calculated by measuring the length of 1 cm of Whatman No. 1 graph paper on Adobe PhotoShop).

2.5.8 To determine the effect of OAC upon acid creep mediated by β-expansin extract on boiled maize roots

Frozen maize roots were boiled in water for 30 s to denature endogenous expansins. The roots were stretched as described in section **2.5.7** except that β-expansin extract was added to the 50 mM (pH 4) acetate (Na^+) buffer, with or without OAC. As controls, unboiled roots were stretched in sodium acetate buffer (pH 4) without β-expansin extract and boiled roots were stretched in the presence of β-expansin, with or without 200 mM NaCl, which is reported (Cosgrove, 1997) to inhibit the action of β-expansin.

2.5.9 To determine the effect of OAC upon acid creep mediated by exogenous β-expansin extract on boiled maize silks and roots, measured using an extensiometer

Maize silks (grown and harvested in The University of Edinburgh green house) and roots (from hydroponically grown seedlings) were frozen at –80°C, thawed and then boiled in water for ~30 s to denature endogenous proteins. Silks were stretched, using a 12-g force, in an extensiometer (many thanks to Prof. S.J. McQueen-Mason, The University of York, for the use of his extensiometer) for 10 min prior to addition of 0.05 vols β-expansin extract, with or without OAC. The roots (which were squashed and had a small square of paper glued to the root tip, covering <2 mm) were stretched, using an 8-g force, for 11 min prior to addition of 0.05 vols β-expansin extract, with or without OAC. The extensiometer measured cell wall

extension by measuring change in voltage with an LVDT (Linear Voltage Displacement Transducer). The change in voltage was then converted into extension rate by multiplying by 250.

2.5.10 To determine the effect of OAC upon pollen grain germination and subsequent tube growth

A few drops of a solution containing sucrose (5% w/v), H_3BO_3 (0.01%), $Ca(NO_3)4H_2O$ (0.03%), $MgSO_47H_2O$ (0.02%) and KNO_3 (0.01%), with or without OAC (100 nM) was placed on a microscope slide containing *Tradescantia* pollen. After 1 h incubation the percentage of germinated pollen grains (judged by eye) was estimated by counting 100 individual grains.

2.5.11 To determine the effect of OAC upon the proteinase activity of papain

Papain (from papaya latex, Sigma) (10 mg) was dissolved in 1 ml of 50 mM acetate buffer (Na^+, pH 4). A 75-µl aliquot of this mixture (containing 5.95 units of activity; 1 unit will hydrolyse 1.0 µmole of BAEE per min at pH 6.2 at 25°C) was added to 425 µl of H_2O and 250 µl of azoalbumin solution (5 mg ml^{-1} in 0.1 M Tris buffer (pH 7.5)) in a test tube. A blank of 2 ml of H_2O and 1 ml of azoalbumin solution was also prepared. The test tubes were incubated in a water bath at 30°C over a range of time periods, after which, 500 µl of 8% trichloroacetic (TCA) acid was added to stop the reaction and precipitate unhydrolysed azoalbumin. The content of each test tube was then centrifuged at ~ 850 *g* and 500 µl of supernatant was added to 500 µl of 0.5 M NaOH (to intensify colour) and read A_{400} on a spectrophotometer.

As the papain had proteinase activity, the experiment was repeated except that OAC was present in the papain (dissolved in 50 mM sodium acetate (pH 4)): 0, 1, 10, 100 and 1000 nM. The papin was then incubated with azocaesin solution (containing 5 mg ml^{-1} in 0.1 M Tris buffer (pH 7.5)) for 1 h.

2.5.12 Attempts to develop an alternative *in vitro* assay for expansin activity

^3H_2O (4 μCi; 0.5mCi $mmol^{-1}$) was added to 100 mg of crystalline cellulose (Avicel) in a hydrolysis tube and placed on a heating block at 100°C overnight. The theory behind this was that some of the H of the OH groups of the D-Glc*p* residues in the cellulose backbone would become ^3H. The [*hydroxyl*-^{3}H]cellulose (50 mg) was then washed five times, over a 2-min period, with 5 ml of water for each wash,.

The [*hydroxyl*-^{3}H]cellulose (100 mg) was then incubated in 25 ml of H_2O, 6 M NaOH, 8 M urea, acetone or 0.3 M cellobiose on a rotary shaker, for 32 h to determine if or how much ^3H_2O was released. After 1, 2, 4, 8 and 32 h, 1 ml of supernatant from each treatment was assayed for radioactivity. After 32 h the cellulose was also assayed for radioactivity.

A new batch of [*hydroxyl*-^{3}H]cellulose (0.5 mCi 50 mg^{-1}; 166Bq mg^{-1}) was incubated in 5 ml of 50 mM acetate (Na^+, pH 4) on a rotary shaker to determine how much ^3H_2O was released. After 0, 15, 45, 90, 180, 360, 720, 1440 and 2880 min, 100 μl of supernatant was removed and assayed for radioactivity. After 48 h all the remaining supernatant was removed, and the [*hydroxyl*-^{3}H]cellulose was dried off overnight in the fume hood and resuspended in 4.5 ml of 50 mM acetate (Na^+, pH 4) (continuously stirred using a magnetic stirrer to maintain a homogenous mixture). Five 90-μl aliquots were removed from the mixture and placed into five Sarstedt tubes (Sarstedt microtubes, Aktiengesellschaft & Co. Nümbrecht) labeled (a), (b), (c), (d) and (e). A 10-μl aliquot of 'batch 1' α-expansin (Cs-exp 1, expressed in tomato) solution (stored in the freezer at –80°C for < 6 months; a gift from Prof. S.J. McQueen-Mason) was added to tube (a). A 10 μl aliquot of 'batch 2' α-expansin (Cs-exp 1, expressed in tomato) solution (stored in the freezer at –80°C for >6 months, a gift from Prof. S.J. McQueen-Mason) was added to tube (b). A 10 μl aliquot of 0.5% Driselase (a mixture of endo- and exo-enzymes from a basidiomycete fungus (*Irpex lacteus*)) was added to tube (c), 10 μl of β-expansin extract **(2.5.5)** was added to tube (d) and 10 μl of H_2O was added to tube (e). The Sarstedt tubes were then placed on a Griffin shaker (100 rpm) at room temperature,

then microfuged (Micro Centaur), and a 10-µl supernatant sample was removed and assayed for radioactivity **(2.1.4)** 0, 15, 45, 90, 180, 360, 720, 1440 and 2880 min after addition of protein.

This experiment was repeated except that 36.9 mg of [*hydroxyl*-^{3}H]cellulose was placed in a Polyprep column and 2 l of water was passed through the column over a 48-h period. The [*hydroxyl*-^{3}H]cellulose was not allowed to dry out before addition of the expansins, 5% cellulase (Megazyme, endo-cellulase, EC.3.2.1.4; *Trichoderma longibrachtiatum*) or 0.5% Driselase. The 0.5% Driselase was assayed in triplicate and α-expansin (batch 1 and 2) and β-expansin were assayed in duplicate; 5% cellulase was also assayed (in triplicate), with five sets of control (water). The samples were then centrifuged and the supernatant was assayed for solubilised radioactivity as previously described.

3. Physiological effects of OAC upon maize CSCs and maize roots

The effect of OAC upon maize CSC and seedlings was assayed in order to determine whether the herbicide inhibited (a) cell division and/or (b) cell expansion or (c) was organ specific. The ID_{50} of OAC (concentration of OAC which inhibits 50% of cell growth) as well as the length of time it took to inhibit cell expansion was also investigated.

3.1 The ID_{50} of OAC

Maize CSCs of various ages were incubated in a range of OAC concentrations. The aim was to determine if the susceptibility of CSC to OAC was age-dependent. The SCV and PCV of CSC treated with or without OAC were used to estimate the ID_{50} of the herbicide. The ID_{50} **(Table 3.1)** was calculated from graphs of SCV and PCV (measured after 6 days) plotted against OAC concentration **(Figs. 3.1.1 and 3.1.2).** The 'fold growth' or the amount of growth that CSCs underwent 6-days subculturing was estimated by dividing the control (DMSO treated) final SCV and PCV (measured after 6 days) by the initial SCV and PCV **(Table 3.1)**.

Table 3.1 The ID_{50} of OAC (on CSCs of various ages) and fold growth of CSCs

CSC age (days)	'Fold-growth'		ID_{60} (nM)	
	SCV	PCV	SCV	PCV
0 (i) low-density	~4	~3	~0.18	~0.25
1	~4.5	~3.5	~2.1	~2.1
3	~2	~1.5	~7	~8
5	~1	~1.5	~410	~7*
0 (ii) high-density	~2	~2	~6	~8

The ID_{50} was estimated from the SCV and PCV of CSCs of varying ages treated with a range of concentrations of OAC for 6 days. *Estimated from line of best fit.

CSC growth was negligible at highest concentration of OAC tested. Initially it appeared that low-density 0-day-old CSCs (i) **(Fig. 3.1.1 (a))** were most susceptible to OAC as shown by the ID_{50} of ~ 0.18 nM. To clarify whether this was due to the

young age of the CSC or whether it was because the cell density was lower, therefore resulting in more OAC being available per cell, the cell density of a 0-day-old CSC was increased approximately five-fold so that it was similar to that of older CSCs **(Fig. 3.1.2)**.

The ID_{50} of OAC, calculated from the high-density 0-day-old CSC (ii) was ~7 nM which is more than 5 × the estimated ID_{50} of the low-density 0-day-old CSC (i) (0.9 nM). However, the 3-day-old and high-density 0-day-old (ii) CSCs, which had similar initial SCVs and PCVs, also had similar ID_{50}s at ~6 and 7 nM respectively **(Table 3.1 and Fig. 31.1.1 (c) and 3.2)**. These results suggested that CSC susceptibility to OAC was not age-dependent, as high-density 0-day-old (ii) CSC had a higher ID_{50} than low-density 0-day-old (i) CSC, but that the effect of OAC was not determined by OAC dose per cell alone. The low- and high-density 0-day-old CSCs (i) and (ii) increased ~ 3.5- and 2-fold in size respectively **(Fig. 31.1.1 (a) and 3.2)**, while the 3-day-old cells increased ~ 1.5-fold **(Fig. 31.1.1 (c)) (Table 3.1)**. This suggests that the ID_{50} was dependent upon the amount of growth each CSC had undergone after the initial SCV and PCV had been measured.

The highest 'concentration' of OAC used was 1 μM, which is above the saturated concentration of OAC (478 nM) (Jikihara, 1997). However, **Fig. 3.1.1 (c), (d)** and **3.1.2** indicated that CSC growth was more inhibited in 3- and 5-day-old CSC at 1 μM OAC than at 478 nM, despite the fact that a 1μM concentration of OAC is higher than the saturated solution. It is possible that the cells were taking up OAC from the medium allowing the remaining insoluble OAC to dissolve and then be taken up by the cells. This suggests that OAC (non-polar) was more soluble in the cells (perhaps by accumulating in the membranes) than in the medium. It is also possible that OAC was metabolised into a less insoluble form which was taken up by the cells and then transported into the vacuole.

The results indicate that the inhibitory effect of OAC on cell growth was neither age- nor density- dependent. High-density 0-day-old cells (ii) showed lower growth than low-density 0-day-old cells (i) (~2- and 3.5- fold, respectively) suggesting that a higher cell density affected the cell growth independently of OAC perhaps due to a

limiting supply of nutrients. It seems that low concentrations of OAC inhibit CSCs that are capable of undergoing a high degree of growth (have a high fold-growth). This is apparent in low-density 0-day-old CSC (i) and 1-day-old CSC **(Table. 3.1)**, however, 3- and 5-day old CSC and high-density 0-day-old CSC (ii), which have a lower fold-growth, require higher concentrations of OAC **(Table. 3.1)**. It could be interpreted that low concentrations of OAC are required to inhibit long-term growth in CSCs, but in CSCs where growth has already started, higher concentrations of OAC are required.

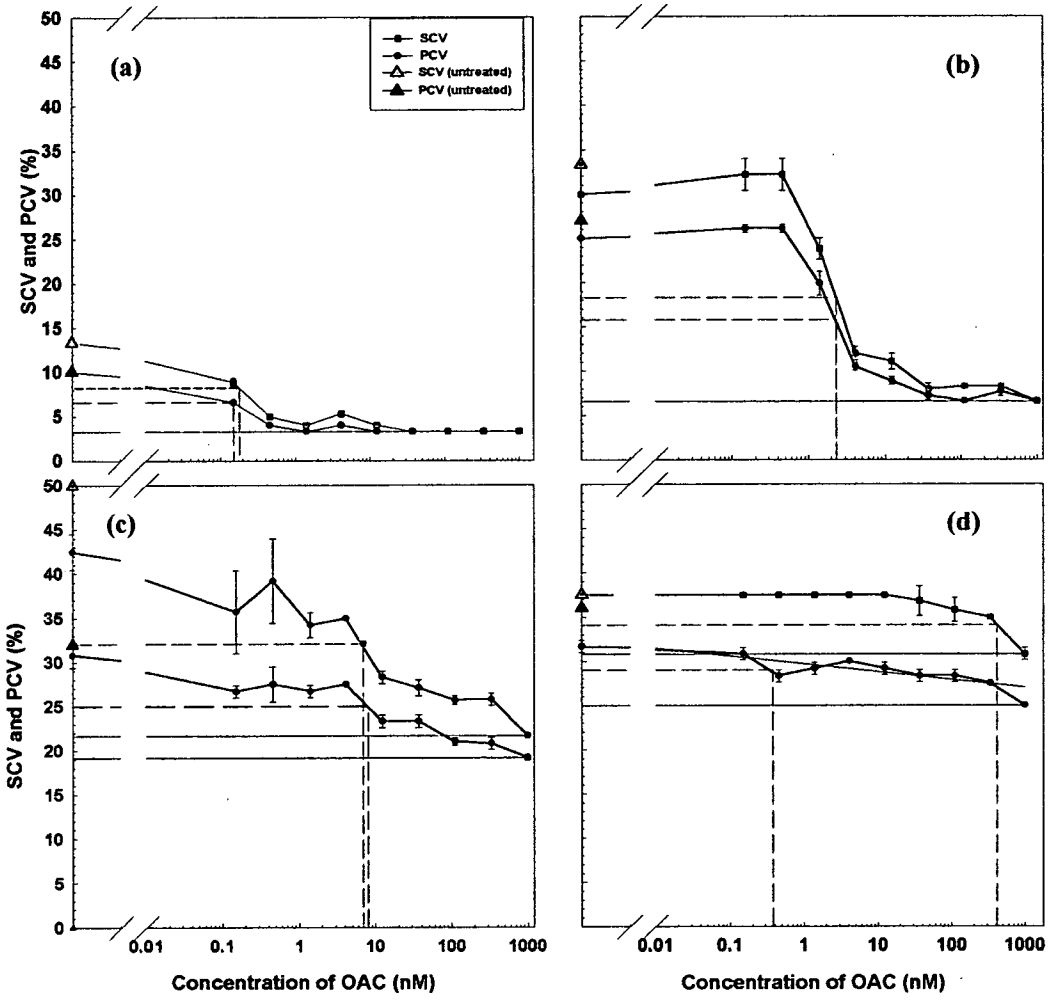


Fig. 3.1.1 Dose-response curves for the effect of OAC on the SCV and PCV of maize CSCs; (a) 0-day-old, (b) 1-day-old, (c) 3-day-old and (d) 5-day-old. The SCV and PCV of a range of ages of CSCs were measured after 6 days treatment with a range of concentrations of OAC. The horizontal solid lines represent the initial SCV and PCV of the CSCs. The ID_{50} of OAC, for each CSC age, was calculated from these graphs (dashed lines). The green line in 3.1.1 (d) represents the line of best fit. The triangles represent untreated CSC i.e. did not receive either OAC or DMSO. N=3

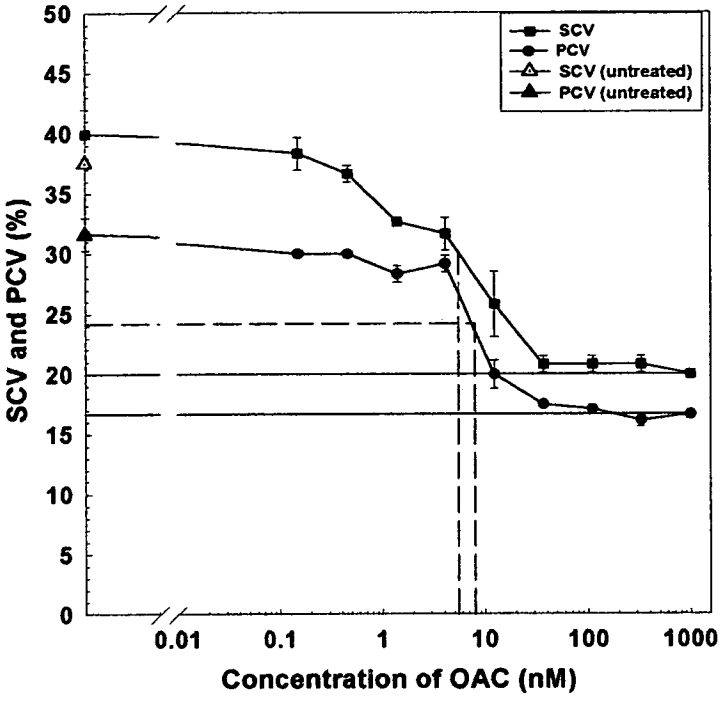


Fig 3.1.2 The dose–response curve for the effect of OAC on the SCV and PCV of 0-day-old maize CSC inoculated at ~ 5 times normal cell density. The SCV and PCV of 0-day-old (ii) CSC was measured after 6 days' treatment with a range of concentrations of OAC. The horizontal solid lines represent the initial SCV and PCV of the CSCs. The ID_{50} of OAC, for each CSC age, was calculated from these graphs (dashed lines). The triangles and squares represent CSCs treated with DMSO. The triangles represent untreated CSC i.e. did not receive either OAC or DMSO. N=3

3.2 The physiological effects of OAC upon etiolated maize seedlings

Previous work had shown that CSC growth was inhibited by OAC at low concentrations **(Fig. 3.1.2)**. However, it was important to assay the effects of OAC on whole plants to determine if there was an obvious inhibitory effect of the herbicide on a specific tissue type or organ.

Maize seeds were germinated and grown in the dark over a 120-h period. Some of the seeds were germinated in a saturated solution of OAC; others received a saturated solution of OAC after 72 or 96 h. The remaining seedlings received only DMSO (0 h after sowing) and were used as a control. The mesocotyl, coleoptile and root lengths, of each seedling, were measured 120 h after sowing.

Mesocotyl growth was significantly promoted ($P = 0.01$), 96 h after addition of OAC **(Fig. 3.2.1 (a))**. The growth of coleoptiles (treated with OAC) continued as normal **(Fig. 3.2.1 (b))**. However, root growth was dramatically decreased ($P < 0.0001$) when seeds were germinated in OAC **(Fig. 3.2.1 (c))**. Even after a lower exposure period (24 and 48 h) to the herbicide, root growth was still significantly inhibited ($P = 0.022$ and 0.002, respectively). Since OAC did not inhibit coleoptile growth and appeared to promote mesocotyl growth, inhibition of growth by OAC may be specific to the root. The lack of inhibition of coleoptile and mesocotyl growth could be because OAC was not transported to the mesocotyl or coleoptile, and was therefore unable to inhibit their growth. It is possible that the seedlings used their resources to promote shoot growth because their roots were not growing as normal and therefore did not require as much energy as would be expected.

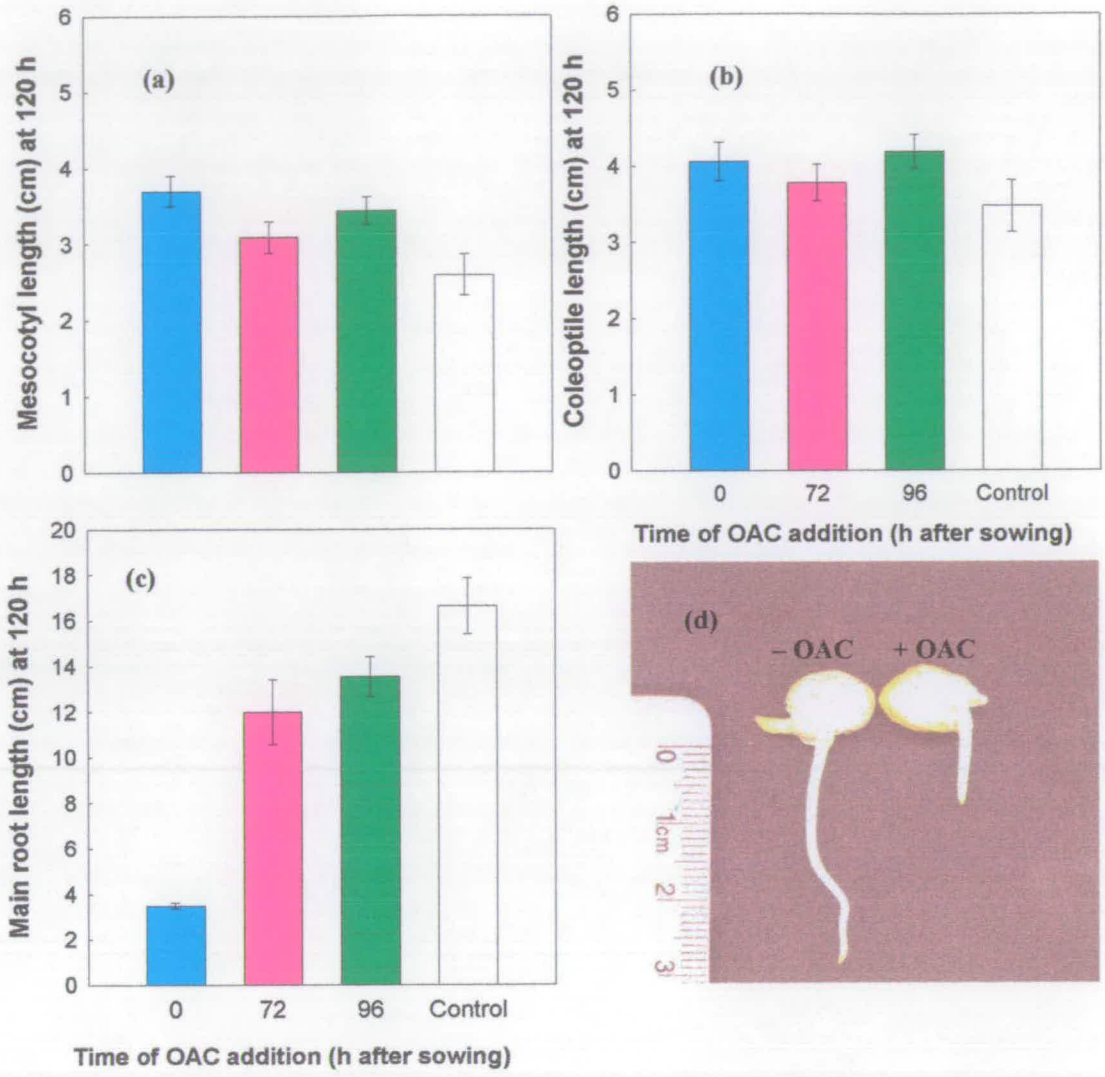


Fig. 3.2.1 The effect of OAC upon etiolated maize seedlings. (a) Mesocotyl, (b) coleoptile and (c) root length 120 h after sowing. Maize seeds were germinated in or later exposed to a saturated solution of OAC. A control set of seeds received only DMSO (0 h after sowing). All seedlings were briefly exposed to red light (72 h after sowing). N = 20, I = S.E. (d) Photograph of 3-day-old maize seedlings treated with or without OAC added 0 h after sowing.

3.3 The effects of OAC upon excised coleoptile growth in the presence of auxin

Saturated solutions of OAC appeared to have no inhibitory effects on coleoptile growth **(Fig. 3.2.1 (b))**. The aim of this experiment was to test whether coleoptiles (independent of the rest of the maize seedling), when in direct contact with OAC,

had their growth inhibited. Coleoptile segments were incubated in IAA, IAA and OAC, or H_2O, and measured hourly.

Coleoptile growth was promoted by IAA, but OAC did not inhibit IAA-induced growth **(Fig. 3.3.2).** This suggests that the inability of root-supplied OAC to inhibit coleoptile growth was not due to an inability to transport OAC **(Fig. 3.2.1 (b))**. It is more likely that the agents or mechanisms (such as XET, expansins, cross-linking of ferulic acid) by which OAC inhibited growth were more sensitive to OAC in roots than in coleoptiles.

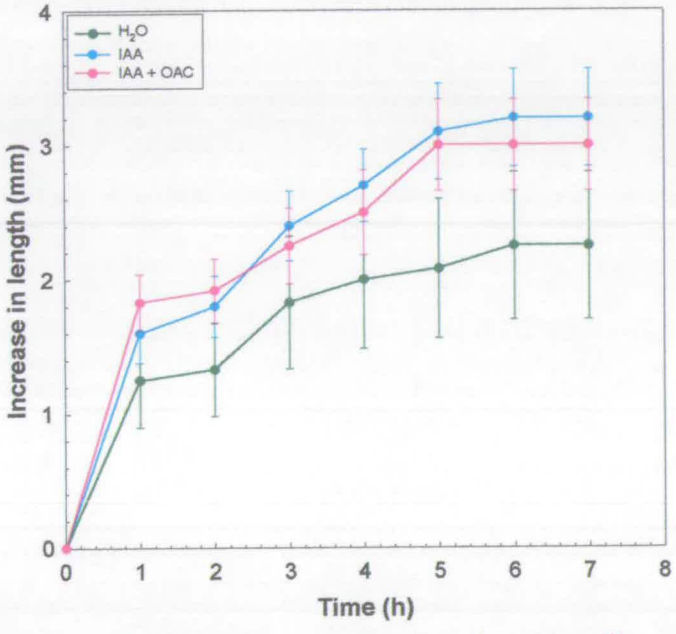


Fig. 3.3.2 Effect of IAA and OAC on the growth of maize coleoptile segments. Coleoptile segments ~ 28.0–29.5 mm in length, from 5-day-old maize seedlings, were incubated in H_2O, 20 μM IAA and 100 nM OAC, or 20 μM IAA.

3.4 To determine the effect of OAC upon cell division and expansion in maize CSC

Cell division and expansion are two important features of plant growth, so it was important to determine if OAC was inhibiting cell division as well as cell expansion. A simple way to determine this was to count cells treated with or without OAC. However, several attempts to separate the cells to make them countable were

unsuccessful. Cells are held together by the middle lamella, which is believed to be pectinaceous (Knox, 1990); consequently, CSC was incubated with pectinase. This treatment proved unsuccessful so a succession of treatments were employed to attempt to separate the cells **(Table 3.4.1)**.

Table 3.4.1 A range of treatments employed to attempt to separate maize CSCs

Treatment	Concentration	Time	Temperature	pH*
Pectinase (EC 3.2.1.15), *Aspergillus* sp., Koch	0.014% w/v 0.1% w/v 1.0% w/v	24 h 24 h 48 h	Room	4.5
EDTA	300, 500 mM	2 h	100°C	5.0
Pectinase and EDTA	0.1%, 300 mM	24 h	Room	4.5
Cellulase (EC3.2.1.4) *Trichoderma longibrachtiatum*, Megazyme	10%, 1%, 0.1%	2 h	37°C	5.0
Cellulase and pectinase	1% and 0.1%	1 h	37°C	5.0
Pectolyase, *Aspergillus japonicus*, Sigma	40 units ml^{-1}	4 h	25°C	5.5
Pectolyase and pectinase (sequential)	40 and 50 units ml^{-1}	24 h	25°C	5.5 4.5
Sodium hypochlorite	15% v/v	24 h	Room	
HCl (pretreatment), CrO_3, (sequential)	11.47 M 5% v/v	5 min 25 min	60°C 70°C	
CrO_3 and HNO_3 (mixture)	10% v/v and 10% v/v	24 h	Room	
H_2O_2 and HOAc (mixture)	30% v/v, 3.5 M	24 h	60°C	
NaOH then brought to pH 4 and treated with pectinase (shaking)	1 M 1%	3 h 6 h	100°C Room	
Sonicator (ultra-sonic disintegrator) Soniprep 150 MSE, Sanyo		30 min	Room	

Several different treatments were used in an attempt to separate maize cells so that they were countable in order to determine whether OAC inhibited cell division.

***Enzymes were buffered in 25 mM succinate buffer.**

None of these treatments were successful at separating the cells, which remained uncountable as viewed under a light microscope. The failure of these treatments to separate the cells suggested that there may be a difference between the middle

lamella of CSC and whole plants and/or that the exact chemical nature of the middle lamella has not yet been fully determined.

3.5 Longitudinal sections of maize roots

Owing to the difficulties encountered in separating maize CSC, longitudinal sections of maize roots treated with or without OAC were taken and examined under a light microscope.

Maize seedlings, which had been growing hydroponically for ~ 2 days without OAC, were harvested 2, 4, 8, 24 and 48 h after treatment with or without OAC (478 nM), embedded in wax, longitudinally sectioned and stained with 0.1% toluidine blue O to emphasise the cell walls **(Fig. 3.5.)**.

Cells ceased to elongate after a 24 h period of treatment with OAC **(Fig. 3.5.1 (b))**. A zone of elongation in the cortex of the control root was visible ~1.5 mm from the tip **(Fig. 3.5.1 (a))**. In the root treated with OAC **(Fig. 3.5.1 (b))** there was no obvious zone of cortical cell elongation, and within 1.5 mm of the tip the cells were noticeably smaller. These and similar longitudinal sections indicated that OAC inhibited cell expansion between 8 and 24 h.

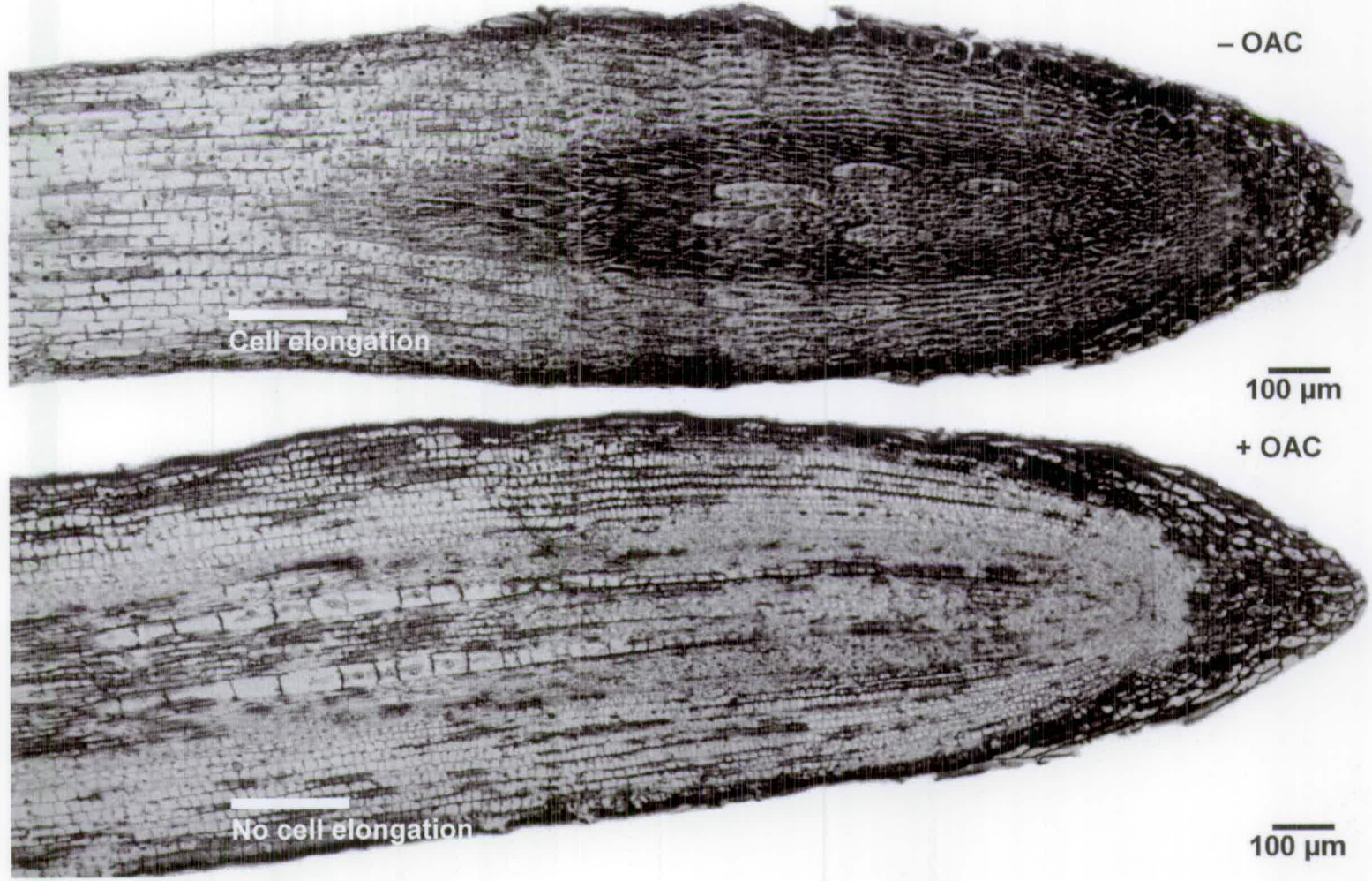


Fig 3.5.1 The effect of OAC upon cell elongation in maize roots after a 24 h incubation period. Longitudinal sections of roots (treated with or without OAC 478 nM) were embedded in wax and stained with 0.1% toluidine blue O.

3.6 To determine when OAC begins to inhibit root growth

Alongside the maize roots used for longitudinal sectioning in the previous experiment, a control set of roots was also grown. Root measurements were taken 18 h prior to treatment with or without OAC, after which measurements were taken at intervals. OAC did not start to inhibit growth significantly until between 4 and 8 h ($P = 0.007$) **(Fig. 3.6)**. The growth rate of roots dropped significantly after addition of DMSO, which was not expected. DMSO has been shown in previous experiments not to have any effect on root growth **(Fig.3.2.1 and 3.5.1 (a))**. This drop in growth rate was probably because of the roots being surface-dried so that ink dots could be applied. The low growth rate could also be because the roots were disturbed by taking them out of the water to be measured.

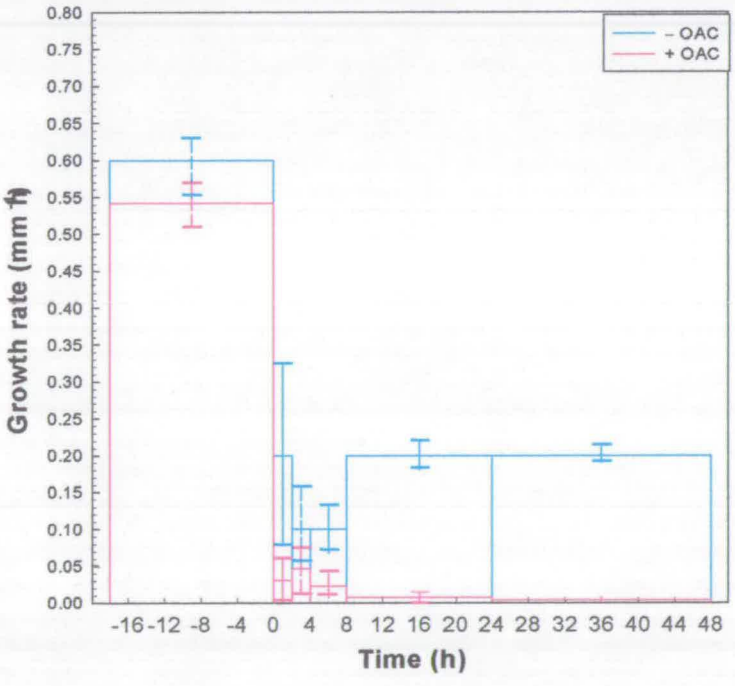


Fig. 3.6 The growth rate of maize roots before and after treatment with OAC (478 nM). At time 0 the roots were treated with or without OAC and then measured at intervals.

A brown ring formed ~3 mm back from the root tip (in hydroponically growing maize seedlings) 24–48 h after addition of OAC. When jabbed against a firm surface, the root tip broke off near or at this brown colour 3.00 mm ($N = 19$, $S.E = 0.34$) from

the tip, with very little resistance in comparison to the control, which broke 8 mm (N = 21, S.E. = 0.63) from the tip. This brittleness or colour formation was not always observed in roots treated with OAC. However, these observations could indicate a promotion of cell wall tightening (as the cell wall would be less flexible therefore breaking instead of bending) by the herbicide. This possibility was tested and the results will be discussed in chapter 5.

3.7 Conclusion

OAC is a very effective inhibitor of cell growth (in maize CSCs) at low concentrations such as ~ 7 nM **(Fig. 3.1.2)**. It starts to inhibit growth (in roots) between 4 and 8 h after exposure **(Fig. 3.6)**. Cell expansion —which defines plant growth— appears to be inhibited by OAC **(Fig. 3.5.1 (b))**. In particular, OAC inhibits maize root growth **(Fig. 3.2.1 (c) and (d))**, but it does not affect coleoptile growth **(Fig.3.3 (b) and 3.4)** and even seems to promote mesocotyl growth **(Fig. 3.2.1 (a))**.

Roots may be more susceptible to OAC because they contain specific targets of OAC not present in the coleoptile or mesocotyl. These targets could be a family of cell wall loosening enzymes such as XETs and/or expansins or a specific type of peroxidase which is central in promoting cell wall tightening and is found only in the root.

4. The effect of OAC upon turgor pressure in maize CSCs and roots

Turgor pressure plays a central role in cell growth as explained in section **1.5.1**. In brief, turgor pressure builds up in the protoplast (owing to differences in solute concentrations inside and outside the protoplast) causing the protoplast to push against the cell wall. Upon reaching threshold turgor pressure (the turgor pressure above which plastic extension occurs), the cell wall extends while simultaneously maintaining its overall structural integrity. This process leads to plastic or irreversible extension, i.e. growth. OAC could theoretically prevent cells from reaching their threshold turgor pressure, thereby inhibiting cell growth.

4.1 The effect of OAC upon water transport in maize CSC

Initial experiments to test the effect of OAC upon water transport focused on whether the herbicide inhibited the transport of water across cell membranes. Inhibition of the passage of water into the protoplast or vacuole could prevent cells from reaching their threshold turgor pressure, therefore preventing cell growth. Water passes through the permeable plasma membrane and tonoplast by diffusion. However, it is believed that water transport is enhanced 10–20-fold by the presence of proteins called aquaporins, which were first reported by Preston *et al.* (1992) in *Xenopus* oocytes and are expressed in the plasma membrane (protoplast intrinsic protein, PIP) and in the tonoplast (tonoplast intrinsic protein, TIP) of plants (Barrieu *et al.*, 1998). Maurel (1997) reported that high tonoplast permeability was related to the presence of Hg^{2+}-inhibitable aquaporins. As aquaporins are highly expressed in meristematic regions (Chrispeels *et al.*, 1999), it was proposed that they feature in the biogenesis of new vacuoles. It is believed that the activity of some aquaporins, PM28A, is regulated by phosphorylation whereby phosphorylated aquaporins increase apoplastic water transport whereas dephosphorylated aquaporins cause a lower increase in water transport (Johansson, 1998). Ohshima *et al.*, (2001) reported that *Graptopetalum paraguayense* had a lower content (~1%) of aquaporin in the leaf

plasma membrane as well as lower water permeability than the radish leaf. It was also reported that radish leaf protoplasts, placed in hypotonic solutions, increased in volume from 100 to 135% over a 2-min period in contrast to *Graptopetalum paraguayense* protoplasts which increased from 100 to 115% over the same period of time. Tonoplast aquaporins are highly expressed in maize root epidermis, endodermis, and parenchyma cells (Barrieu *et al.*, 1998; Chaumont *et al.*, 1998).

Aquaporins are therefore a plausible target of OAC as the herbicide has a significant inhibitory effect upon root growth, which requires water uptake. However, the effect of OAC upon aquaporin function could not be directly assayed, so the following experiment was an attempt to assay, in general, the effect of OAC upon water transport out of (and, by implication, also into) the cell.

Preliminary experiments were set up in order to determine if it was possible to assay water transport in CSCs. The aim of the following experiment was to determine if [^{14}C]mannitol passed out of a 'bed' of cultured cells faster than $^{3}H_2O$. It was expected that the bed of cells would act like a gel-permeation column, whereby the hydrophilic [^{14}C]mannitol would be unable to enter the cells and would have to pass in between the cells, therefore eluting relatively quickly. However, $^{3}H_2O$ was expected to enter the cells and then to exit from them (possibly via aquaporins), therefore eluting more slowly. If the results showed the above assumption to be correct, then it would be possible to determine whether OAC inhibited water transport by comparing the elution of ^{3}H:^{14}C in CSC treated with or without OAC. $^{3}H_2O$ transport across the membrane, if inhibited by OAC, would be expected to take more time than the control i.e. $^{3}H_2O$ would elute more slowly from the bed of cells than in the control.

4.1.1 A comparison of the elution rates of $^{3}H_2O$ and [^{14}C]mannitol from a bed of maize CSC

Inhibition of water transport out of (and thus presumably also into) cells by OAC could repress cell growth. [^{14}C]Mannitol and $^{3}H_2O$ were added to maize CSCs and left for ~1 h. The CSCs was then passed through a Polyprep column. The medium

was allowed to drain off and fresh medium was added so that the flow rate in and out was equal and the cells were permanently wet.

After the ^{3}H count was corrected for ^{14}C falsely recorded as ^{3}H **(2.1.5)**, the ratio of $^{3}H_2O$ to [^{14}C]mannitol in the eluate was calculated and plotted against time. The results **(Fig. 4.1.1)** indicated that [^{14}C]mannitol eluted more quickly from the column than $^{3}H_2O$. This experiment supports the idea that $^{3}H_2O$ had entered the cells so that its elution was retarded relative to that of [^{14}C]mannitol. Consequently, [^{14}C]mannitol was used as a control in subsequent experiments to show whether any impedance or promotion in the elution of $^{3}H_2O$ by OAC was due to an effect in the transport of water out of the cells.

The results of the experiment **(Fig. 4.1.1)** also showed that, after ~ 140 s, the [^{14}C]mannitol had almost disappeared, making the ^{3}H:^{14}C ratio redundant. Consequently, subsequent experiments were run for ~ 200 s instead of 15 min.

Triton X-100 was also used as a control in similar experiments. Triton X-100 is a detergent that solubilises some membrane-integral proteins, making the plasma membrane permeable to both ^{14}C and ^{3}H. As a result, the relative elution rates of [^{14}C]mannitol and $^{3}H_2O$ would be expected to be the same, which would be correlated by no increase in the ratio of ^{3}H to ^{14}C in the eluate. This control was important, as it was possible that some of the D-Glc*p* residues of polysaccharides in the cell wall became reversibly radiolabelled during incubation with $^{3}H_2O$. If this had occurred then the non-radiolabelled medium passing through the bed of CSC might have gradually become radiolabelled so that it appeared that ^{3}H was eluting more slowly than the ^{14}C **(Fig. 4.1.1)**, rendering the assay invalid. However, if the relative elution rates of [^{14}C]mannitol and $^{3}H_2O$ were similar after CSCs had been incubated with Triton X-100, it would further support the interpretation of the increasing ^{3}H:^{14}C ratio reported in **Fig. 4.1.1** as a direct result of $^{3}H_2O$ having to pass (slowly) through the cell membrane.

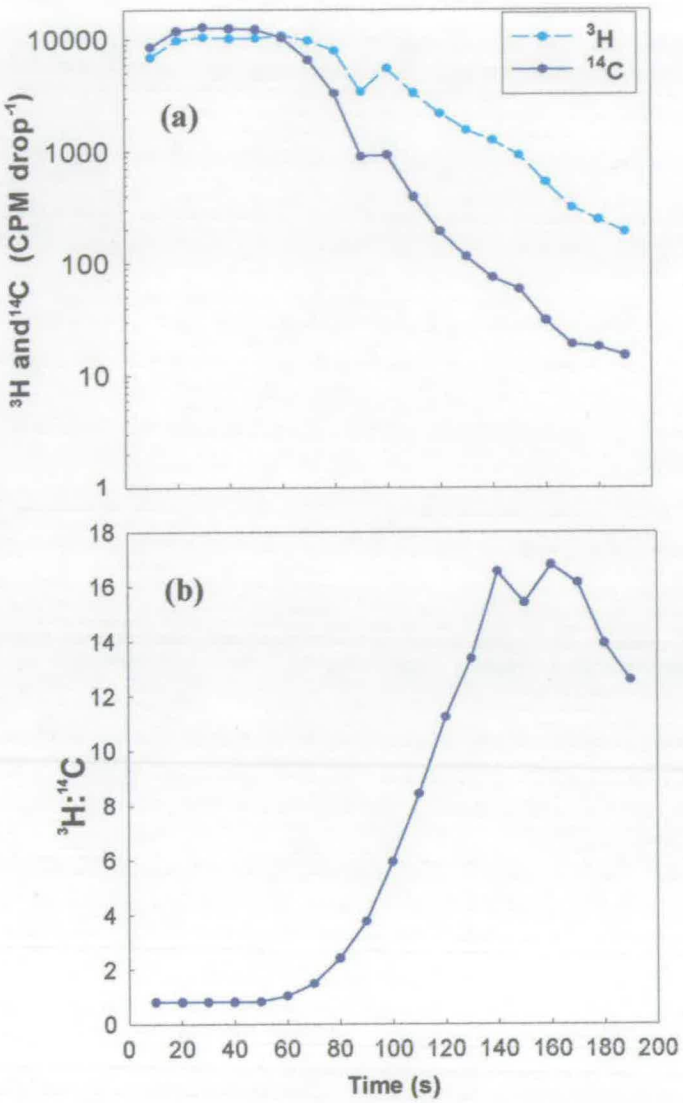


Fig. 4.1.1 The elution of ^3H_2O and [^{14}C]mannitol out of a bed of maize CSC. (a) Represents the elution and (b) a ratio of the elution of ^3H_2O and [^{14}C]mannitol. CSCs (4-day-old) were incubated with ^3H_2O and [^{14}C]mannitol and then passed through a Polyprep column forming a 1.8-ml CSC bed volume. Fresh medium was passed through the column at ~ 0.35 drops s^{-1} and fractions of eluate were collected at 10-s intervals.

4.1.2 To determine whether OAC affects transport of water across cell membranes

Maize CSCs were incubated with or without OAC overnight. [^{14}C]Mannitol and ^3H_2O were added to each of the CSCs; in addition, 1% Triton X-100 was added to a CSC which had not received either DMSO or OAC. Approximately 1 h after addition of radioactivity (+/–Triton X-100), the CSCs were passed through a Polyprep

column. Additionally, OAC was added to a separate CSC upon addition of ^{3}H and ^{14}C for a 3-h period, to determine whether or not pre-incubation with OAC (without radioactivity) prevented the transport of $^{3}H_2O$ into the cells. The elution of $^{3}H_2O$ and [^{14}C]mannitol was assayed as before **(4.1.2 (b))**.

The transport of water out of the cells treated with OAC was unimpeded **(Fig. 4.1.2 (a) and 4.1.2 (b)).** $^{3}H_2O$ eluted more quickly from OAC-treated cells than from cells which received only DMSO.

The water output from ~ 1.7 g of maize CSC not treated with OAC was ~ 180 ml day $^{-1}$, as estimated from the results **(Fig. 4.1.2.(a))** by calculating the amount of ^{3}H collected in a known volume of eluate in the first 60 s after passing cold medium through the bed of CSC. A growing cell needs to take up its own volume of water to double its volume, i.e. 1 g of CSC needs to take up ~1 ml of water. Therefore, if OAC's primary mode of action was to inhibit water transport, it would have to effect a 99.4% reduction in the uptake capacity of the CSC in order to have any effect on cell expansion. The water output from ~1.7 g of OAC-treated CSC was ~ 220 ml day $^{-1}$ as estimated from **Fig. 4.1.2. (a)**, indicating that OAC did not act by inhibiting water transport. The presence of Triton X-100 (which makes the plasma membrane highly permeable) caused a dramatic difference in the ratio of ^{3}H:^{14}C when compared to cells which had received only DMSO, confirming that the rise in ^{3}H:^{14}C ratio in the control reflected the relatively slow transport of $^{3}H_2O$ across membranes. The results indicated that OAC was not affecting transport of water out of the cells and was therefore unlikely to inhibit water uptake or cell expansion.

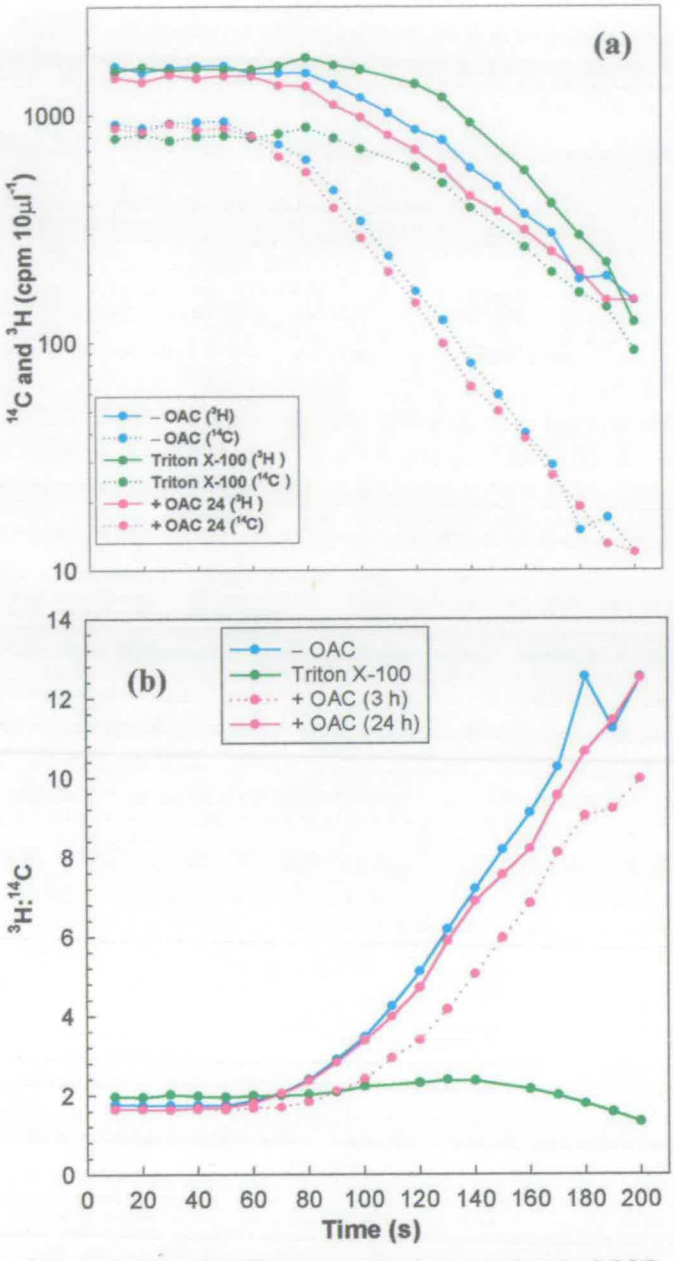


Fig. 4.1.2 The effect of OAC and Triton X-100 upon water transport out of CSCs. (a) Represents the elution and (b) a ratio of the elution of $^{3}H_2O$ and [^{14}C]mannitol. CSCs (4-day-old) treated with or without OAC (478 nM) or Triton X-100 (1%) were incubated for 3 or 24 h with $^{3}H_2O$ and [^{14}C]mannitol and then passed through a Polyprep column forming a 1.8-ml CSC bed volume. Fresh medium was passed through the column at ~ 0.69 drops s^{-1} and fractions of eluate were collected at 10-s intervals.

4.2 To determine if OAC affects the point of incipient plasmolysis (POIP) of CSCs

This experiment focused on using [^{3}H]maltitol and [^{14}C]dextran to compare the POIP (concentration of glucitol at which 50% of the cells are plasmolysed) of CSC treated with or without OAC. Direct microscopic examination for plasmolysis was impossible, because of the difficulties associated with visualising individual cells **(3.4)**.

The theory behind this experiment was as follows **(Fig. 4.2)**: as cells become plasmolysed by 0–1 M glucitol, the protoplast pulls away from the cell wall and the newly created space becomes filled with [^{3}H]maltitol solution, while the high-M_r [^{14}C]dextran remains outside the cell wall; this causes an increase in the ratio of ^{3}H to ^{14}C in the harvested cells. By comparison of results from control and OAC-treated CSCs, it would be possible to determine whether the POIP had been affected by OAC. A graph indicating that osmotic pressure was decreased in the presence of OAC would show the POIP occurring at a lower concentration of glucitol. This could prevent cell expansion, as the cells would be unable to reach threshold turgor pressure, which is necessary for expansion.

[^{3}H]Maltitol and [^{14}C]dextran were added to CSCs pre-treated with or without OAC. A quantity of CSC from each flask was incubated with solid glucitol calculated to give various final concentrations. After a 2-h incubation period the CSC was sedimented at 1 × g in a centrifuge tube. The supernatant was removed by pipetting and the remaining medium was drawn off, in a reproducible method, by placing thin strips of blotting paper into the CSC.

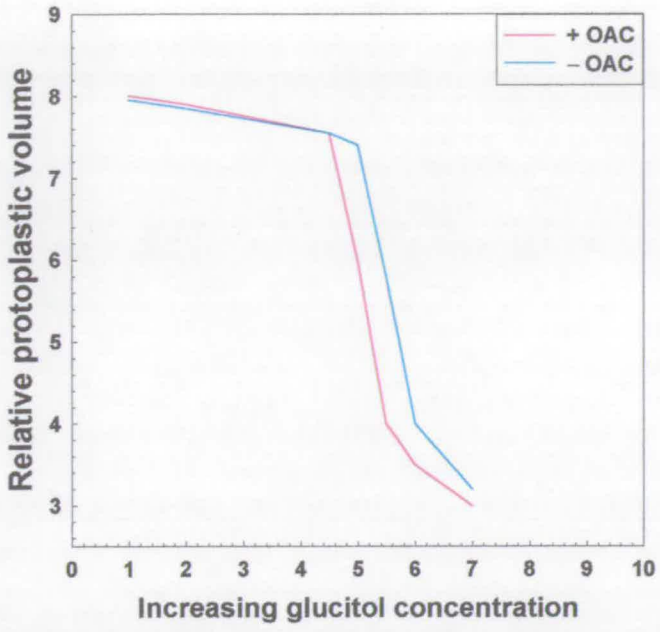


Fig. 4.2 A theoretical graph showing what would happen if OAC reduced the cells' osmotic pressure. The POIP of the cells would be shifted to the left, i.e. they would become plasmolysed by lower glucitol concentrations than normal.

Results from this experiment were, however, inconclusive. It proved too difficult to remove excess medium (containing [^{3}H]maltitol and [^{14}C]dextran) surrounding the cells. The presence of ^{14}C and ^{3}H in the excess medium surrounding the cells interfered with calculation of the ratio of ^{3}H:^{14}C in the cells. It appeared to lower the ^{3}H:^{14}C ratio in the cells because the ^{14}C and ^{3}H present in the excess medium was counted as though it was inside the cell. Another approach used in an attempt to remove the excess medium was to pass the CSC through a layer of silicone oil. It was expected that after centrifugation the cells (being dense) would pass though the silicone layer while the medium remained above the silicone. However, after centrifugation only some of the cells passed though the silicone layer and in some cases created a 'hole' in the silicone allowing the excess medium to pass through. This method was abandoned after several unsuccessful attempts.

4.3 To determine if OAC affects osmotic pressure in maize CSC by measurement of protoplastic volume

In order to determine if OAC affected osmotic pressure of maize CSCs, the POIP of CSCs pre-treated with or without OAC was assayed by estimation of protoplastic volume in media of various osmotic pressures. The theory behind this experiment was as follows: if OAC was affecting osmotic pressure then the POIP of cells, incubated in a range of concentrations of mannitol, would occur at a lower concentration of mannitol. This could be tested by comparing, the protoplastic volumes of cells treated with or without OAC as measured by the volume of solution eluted from CSCs after incubation in each concentration of mannitol. If OAC affected the POIP of the cells then a higher volume of solution would elute from the CSCs at a given concentration of mannitol, i.e. the cells would have a lower protoplastic volume at that mannitol concentration than the control.

CSCs were pre-treated with or without OAC. After 2, 6 and 10 h the cells were collected on muslin, placed into an equal volume of a range of concentrations of mannitol, incubated for 15 min and then collected. The volume of medium that eluted was recorded.

It was expected that similar volumes would elute from cells that were not under any osmotic stress; however, this was not reflected in the results **(Fig. 4.3. (a))**. There was no obvious plateau in the results followed by a steep rise in the curve, which would have indicated that the cells had reached their POIP **(Fig. 4.3.)**. This made it impossible to observe the POIP of the cells and therefore to decide whether or not the herbicide affected the osmotic pressure of the cells. As the results gave no clear indication of the POIP (suggesting that the mannitol concentrations used were too high and should have been confined to a lower range of mannitol concentrations) they were inconclusive. It was, therefore, impossible to determine if the herbicide was affecting osmotic pressure from this experiment. The experiment was repeated (graphs not shown) using lower concentrations of mannitol; however, the POIP was still not obvious in the repeat experiments.

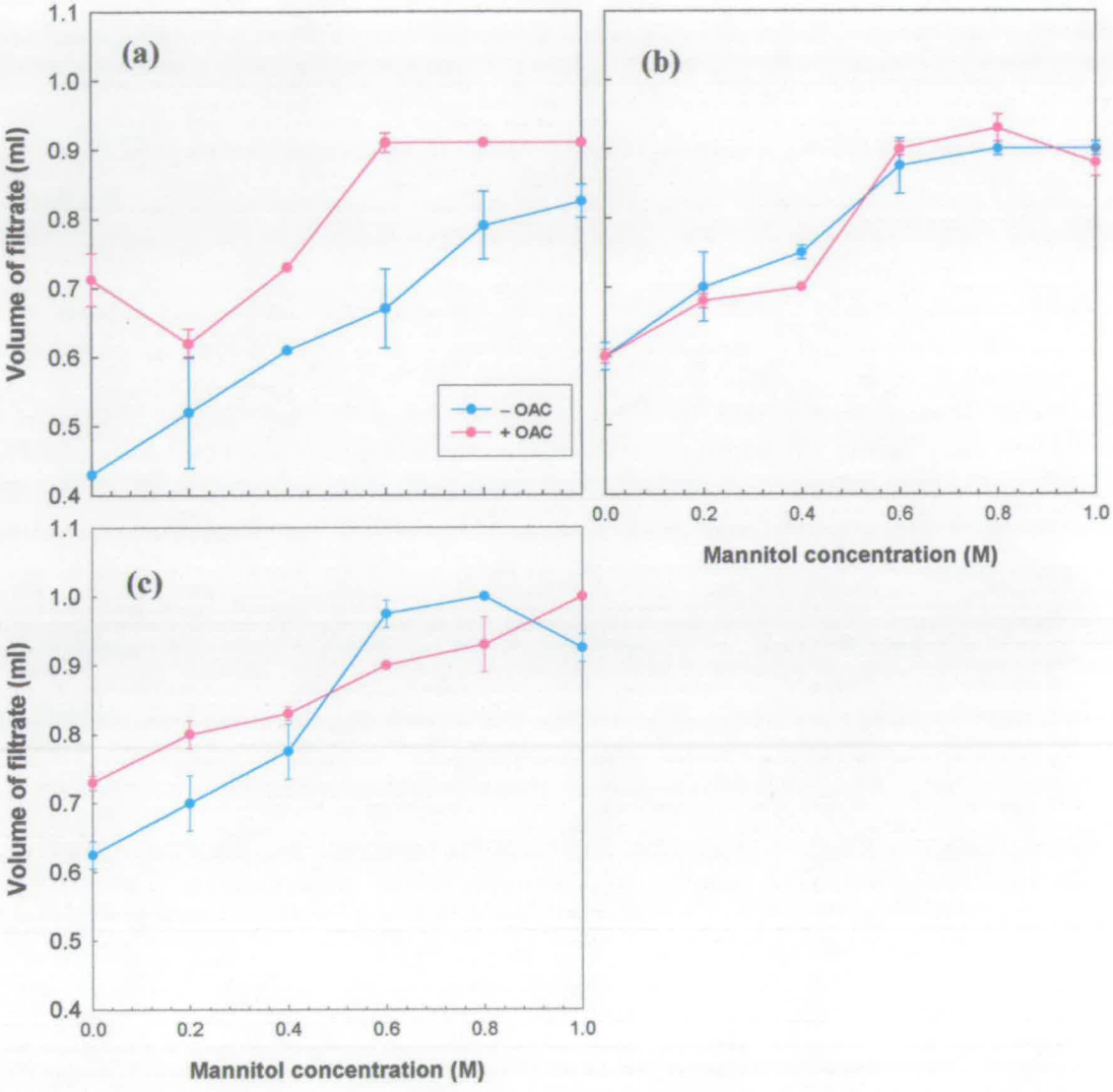


Fig. 4.3 The effect of OAC upon the protoplastic eluate volumes of CSCs. CSCs were treated with or without OAC (478 nM) for 2 h (a), 6 h (b) and 10 h (c). The cells were then incubated in a range of concentrations of mannitol, filtered, and the volume eluted from the CSCs was measured.

4.4 To determine if OAC affects the osmotic pressure of maize roots tested by measurement of angle of curvature

Lockhart (1959) stated that when the POIP is reached it is accompanied by an abrupt increase in tissue flexibility. As in the previous experiment, the effect of OAC upon the POIP was assayed but this time using maize roots. Hydroponically grown maize seedlings were treated with or without OAC for 2 and 6 h. They were then placed in a range of concentrations of NaCl for ~ 20 min. Their roots were cut and held horizontal with a 150-mg weight placed on the root tip. The angle of curvature was recorded for each root. It was expected that, if OAC had decreased the osmotic pressure of the cell sap, the roots would have exhibited their abrupt increase in flexibility at lower concentrations of salt.

As in the previous experiment, the POIP was not obvious; however, a line of best fit (averaged +/– OAC) indicated that both treated and untreated roots reach their POIP between 0.06 and 0.07 M NaCl **(Fig. 4.4)**. Also a direct comparison of the angles of root curvature (with or without OAC) **(Fig. 4.4)** indicated that OAC did not affect the angle of curvature of the roots in comparison to the control. After 2 h +/– OAC, the results **(Fig. 4.4 (a))** showed that at 0.15 and 0.2 M NaCl, the presence of OAC appeared to make the roots slightly less flexible than the controls, suggesting that OAC may be effecting cell wall tightening; this will be discussed in chapter 5. This was not reflected in the 6-h time point **(Fig. 4.4 (b))**, which indicated that the herbicide had no effect upon the angle of root curvature.

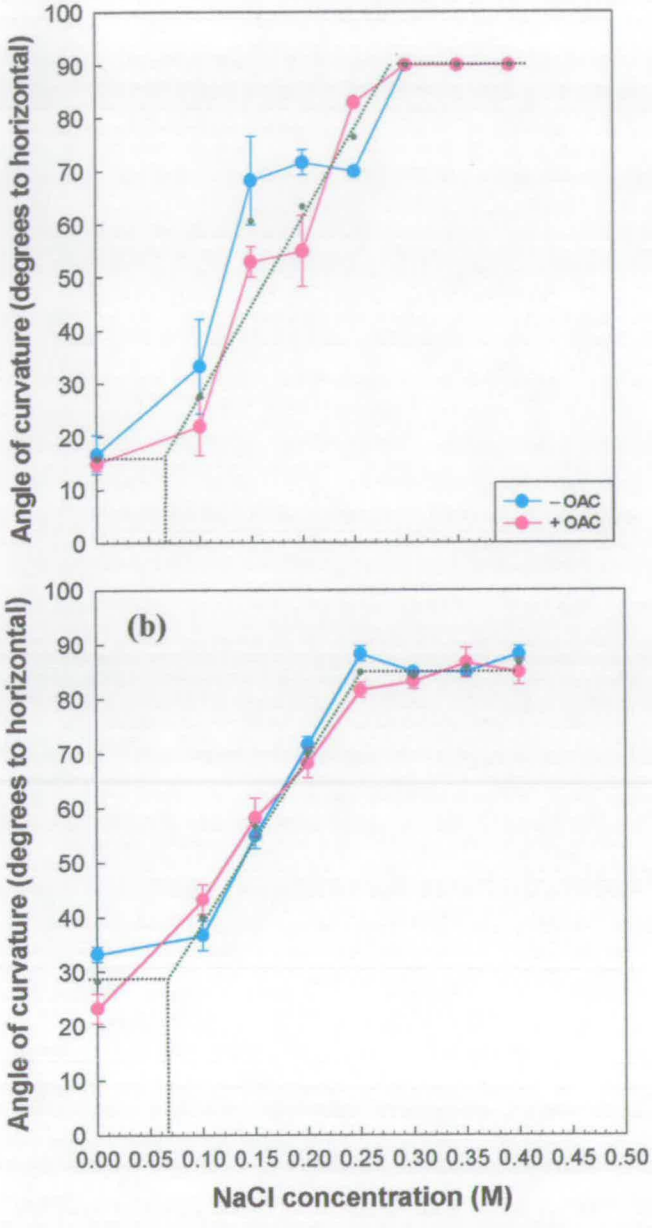


Fig. 4.4 The effect of OAC upon the flexibility of maize roots treated with a range of NaCl concentrations. Roots (from hydroponically grown seedlings) were incubated with or without OAC for 2 h (a) or 6 h (b) and treated with a range of concentrations of NaCl. The angle of curvature, from the horizontal, made by each root was traced and plotted against concentration. The green dashed line represents the line of best fit (average of + and – OAC).

4.5 To estimate the effect of OAC upon turgor pressure in CSC using a depression of freezing point osmometer

The effect of OAC upon turgor pressure in CSC was assayed using an alternative approach to the previous two methods. In this experiment at intervals after treatment with or without OAC, cells and media were collected and frozen independantly. The frozen cells were thawed and squashed; the cell sap was collected and the osmotic pressure of both the cell sap and medium was measured using a depression of freezing point osmometer. It has been shown experimentally (Nobel, 1991) that 1 MPa corresponds to ~ 407.5 mosmol l^{-1} solutes, and this relationship was used here.

The turgor pressure was estimated as osmotic pressure of cell sap minus osmotic pressure of culture filtrate. This assumes that the osmotic pressure gradient between cells and medium is the only factor determining turgor pressure, i.e. that the hydraulic conductivity of the tissue is very high. The results **(Fig. 4.5)** indicate that turgor pressure remained unaffected by the presence of OAC even after a 48-h incubation period. **Table 4.5.1** contains a summary of the osmotic pressures of the cell sap and the medium, with or without OAC.

Table 4.5.1 A summary of the osmotic pressure of the cell sap and the medium

Time (h)	Osmotic pressure (mosmol/kg^{-1})			
	– OAC		+ OAC	
	Media	Cell Sap	Media	Cell Sap
2	164.0	271.0	175.0	272.5
4	150.0	240.0	164.5	254.0
8	166.0	254.5	158.5	256.0
24	155.5	247.0	143.5	217.0
48	129.5	211.0	119.0	207.0

The osmotic pressure of the cell sap and the medium were measured using a depression of freezing point osmometer

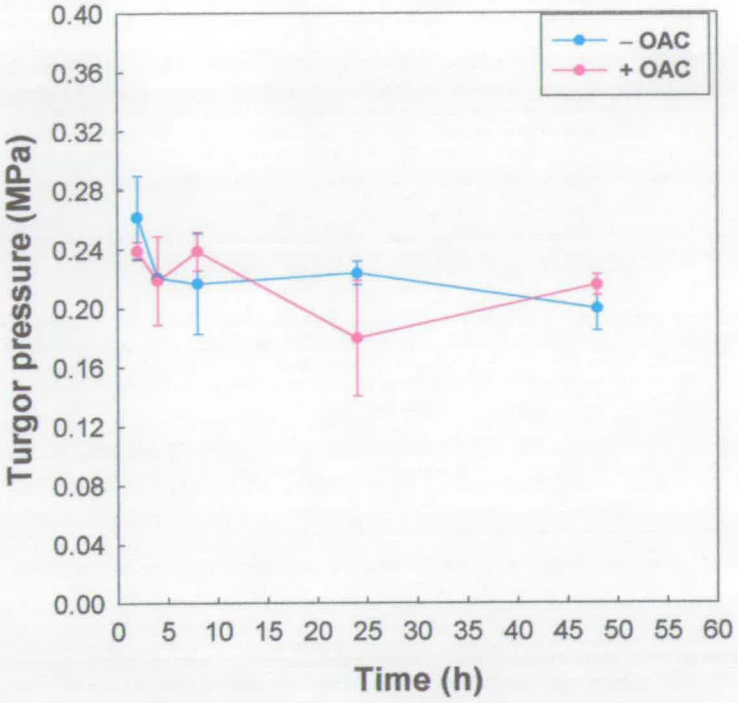


Fig. 4.5 The effect of OAC upon the turgor pressure of CSC. CSC was treated with or without OAC for the duration indicated on the x-axis. A depression of freezing point osmometer was used to estimate the osmotic pressure of the cell sap and the culture medium. The turgor pressure of the cells was calculated as osmotic pressure of cell sap minus medium.

4.6 Conclusion

$^{3}H_2O$ eluted from a bed of CSC after [^{14}C]mannitol **(Fig. 4.1.1)** and was therefore useful in assaying any effect of OAC upon water transport though the plasma membrane. OAC did not inhibit water transport out of the cells suggesting that OAC did not inhibit water transport across cell membranes **(Figs. 4.1.2. (a) and 4.1.2.(b))**. In **Fig. 4.3** the point of incipient plasmolysis of the CSC estimated by shrinkage of protoplastic volume was not obvious from the graphs and the results were inconclusive. After incubation of maize roots (pre-treated with or without OAC) in a range of concentrations of NaCl, OAC did not affect the angle of curvature of the maize roots **(Figs. 4.4)**, indicating that the herbicide did not affect the osmotic pressure of the cells. This result was supported in the next experiment where OAC did not affect turgor pressure in maize CSC, as estimated from the osmotic pressure of cell sap and culture medium using a depression of freezing point osmometer **(Fig. 4.5)**.

This set of experiments ruled out one of the most important factors of cell growth, turgor pressure, as the primary target of OAC. Consequently, future experiments focused on the effect of OAC upon cell wall tightening and loosening mechanisms.

5. The effect of OAC upon cell wall tightening mechanisms

Cell wall tightening is responsible for establishing and maintaining the overall structural integrity of the wall during growth as well as arresting growth. Ferulate and tyrosine are oxidatively cross-linked in the presence of peroxidase and H_2O_2 to form diferulates and isodityrosine. These potential cross-links are believed to cause cell wall tightening (Fry, 1983; Brady *et al.*, 1996; Grabber *et al.*, 2000). Therefore, OAC could induce cell wall tightening thereby, preventing cell growth, by enhancing the secretion or activity of peroxidase or by promoting H_2O_2 production.

5.1 To determine the effect of OAC upon peroxidase secretion and action in CSC

The effect of OAC upon peroxidase action and/or activity was assayed. Peroxidase action describes an *in vivo* catalytic reaction: it depends on not only the presence of (potentially) active enzyme but also on the presence of H_2O_2 in the same cellular compartment and the absence of inhibitors (e.g. ascorbate, cysteine). However, peroxidase activity describes the rate at which the cell-free enzyme catalyses a reaction *in vitro* in the presence of optimal [H_2O_2] and at an optimal pH.

5.1.1 To assay peroxidase action *in vivo*

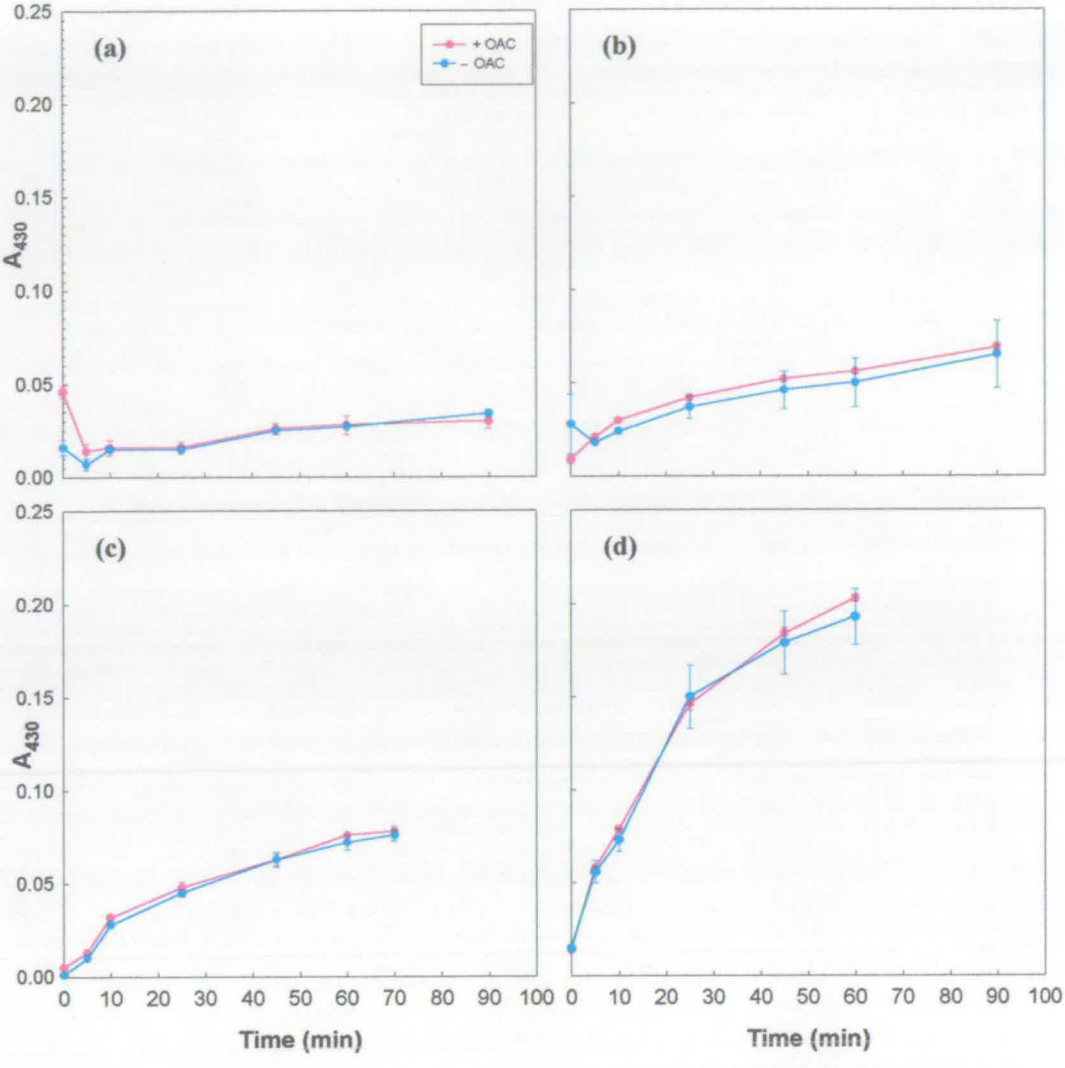
To assay peroxidase action *in vivo*, a peroxidase electron-donor substrate (0.1 mM *o*-dianisidine) was added to 4.5-day-old CSC at a range of intervals after the addition of OAC. *o*-Dianisidine, which is positively charged, is less likely to enter the protoplast and may remain predominantly in the apoplast.

Peroxidase action upon *o*-dianisidine resulted in the formation of a brown colour in the cells approximately 1 h after addition of *o*-dianisidine (regardless of the duration of OAC pretreatment). If OAC promoted cell wall tightening by promoting peroxidase action (e.g. upon ferulic acid, forming diferulic acid), a darker brown

colour would be observed in OAC-treated cells than in the control. However, the intensity of the brown colour observed (by eye) was the same, with or without OAC pre-treatment, with little or no colour observed in the medium. OAC did not promote, on a model aromatic substrate in the apoplast, peroxidase action *in vivo*.

5.1.2 To determine if OAC promotes secretion of peroxidase activity in CSCs

The activity of peroxidase, secreted from CSC treated with or without OAC, was also assayed. CSC (4-day-old), was filtered, washed, resuspended in fresh medium and then treated with or without OAC. Samples of medium were taken at intervals and assayed for peroxidase activity with 0.1 mM *o*-dianisidine + 0.8 mM H_2O_2. No significant effect of OAC was observed **(Figs. 5.1.2 and 5.1.3)**. Therefore, OAC did not appear to promote the secretion of peroxidase into the medium but might have promoted its secretion into the wall which would not be detected by an assay of the medium.



Figs. 5.1.2. The effect of OAC upon peroxidase secretion into the medium. CSCs were washed with fresh medium and then treated with or without OAC. A sample of medium, after (a) = 1 h, (b) = 2 h, (c) = 7 h and (d) = 13 h, was assayed for peroxidase activity with 0.1 mM *o*-dianisidine + 0.8 mM H_2O_2. I = standard error. N = 3

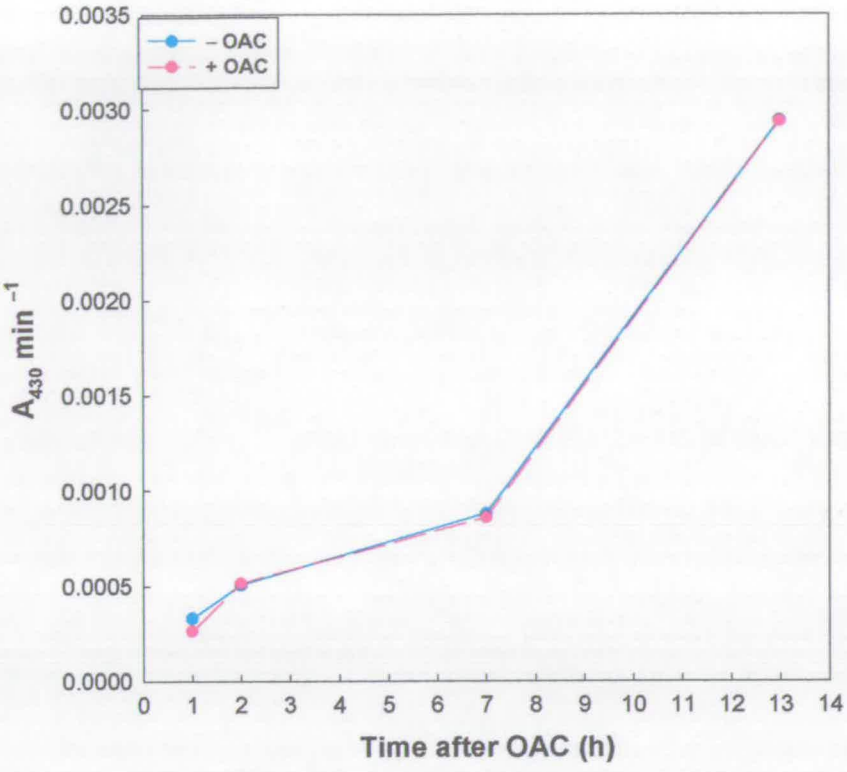


Fig. 5.1.3 The effect of OAC upon peroxidase activity. Peroxidase activity was calculated from Fig. 5.1.2. Other details as in Fig. 5.1.2.

5.2 To determine the effect of OAC upon the cross-linking of exogenous coumaroyl esters

To support the finding that OAC did not promote peroxidase action in the apoplast, the cross-linking of coumaroyl esters by maize CSCs in the presence and absence of the herbicide was also assayed. Coumaroyl esters may be oxidatively cross-linked in the presence of peroxidase and H_2O_2. Endogenous coumaroyl esters are not readily cross-linked in maize cell walls (Fry *et al.*, 2000), but exogenous coumaroyl esters behave similarly to feruloyl esters and cross-link readily in the presence of peroxidase and H_2O_2 (Wallace and Fry, 1999).

For the preparation of [^{14}C]coumaroyl esters, [U-^{14}C]cinnamic acid was fed to 4-day-old maize CSC and low-M_r compounds were extracted from the cells in ethanol. These compounds were separated by paper chromatography, located by autoradiography and further purified by paper chromatography. A small amount of

two of these compounds (M1B and M2B) was alkaline hydrolysed; the products were chromatographed and thereby identified as free *p*-coumaric acid. Thanks to Dr. Antonio Encina, The University of Edinburgh, for preparing the [^{14}C]coumaroyl esters.

The two *p*-[^{14}C]coumaroyl esters (M1B and M2B), were fed to 4-day-old CSC pre-treated overnight with or without OAC. If OAC promoted peroxidase action in the cell wall (apoplast), then an increase in the formation of dimers or higher M_r compounds of coumaroyl esters (coumaroyl esters cross-linked to soluble polysaccharides or wall bound components) could be expected. The higher M_r compounds could then be ethanol-precipitated (polymeric fraction) and the non-polymeric fraction (low M_r or ethanol-soluble fraction) could then be assayed for radioactivity **(Fig. 5.2.1)**. It is unlikely that dimers of *p*-[^{14}C]coumaroyl esters would be ethanol precipitated.

A decrease in the amount of ethanol-soluble ^{14}C over time indicated that the cross-linking of coumaroyl esters, to form larger cross-linked compounds, had occurred **(Fig. 5.2.1)**. OAC did not promote the cross-linking of the coumaroyl esters, suggesting that OAC does not inhibit cell expansion by the formation of peroxidase promoted cross-links in the apoplast. The results indicated that OAC was significantly preventing cross-linking of the coumaroyl esters ethanol-insoluble partners, which was unexpected.

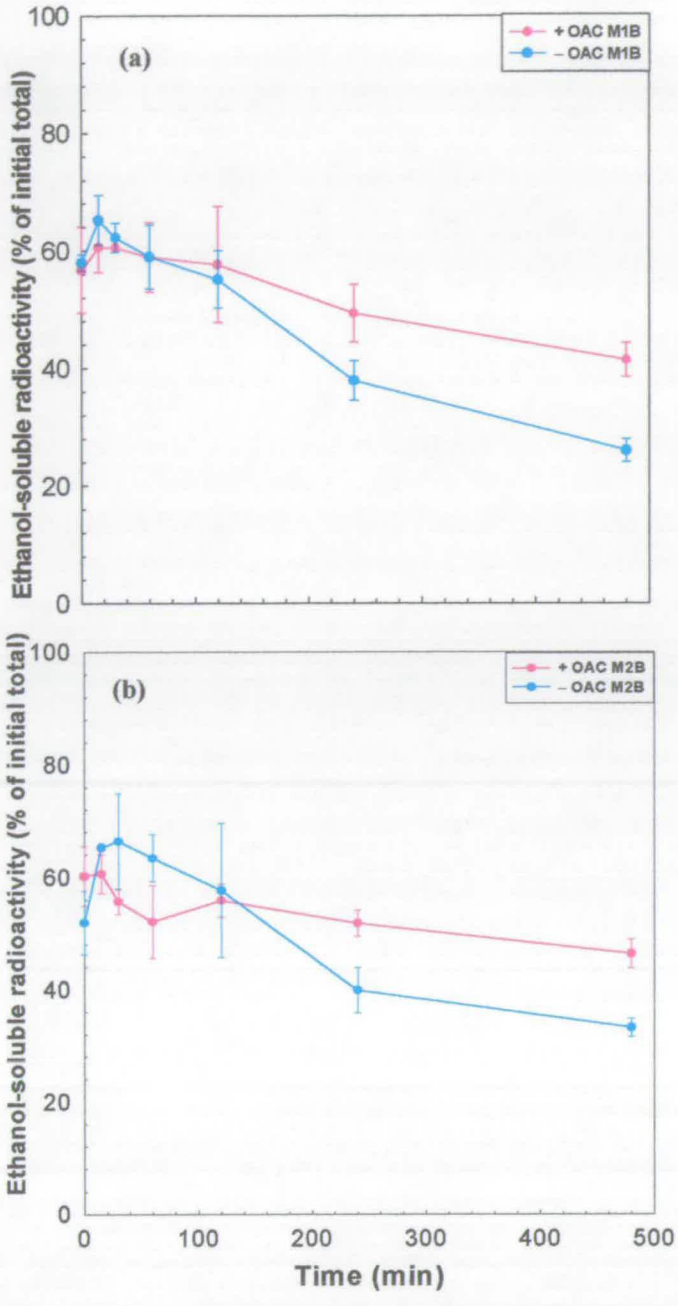


Fig 5.2.1 The effect of OAC upon the cross-linking of coumaroyl esters. CSC, treated with or without OAC (478 nM) over a 24-h period, was fed with [^{14}C]coumaroyl esters, M1B (a) and M2B (b). At intervals over a 480-min period, samples of medium were added to ethanol and centrifuged. The supernatant was assayed for radioactivity. N = 3. I = standard error

5.3 Conclusion

OAC did not affect the action of peroxidase on coumaroyl esters *in vivo* **(5.1.1 and Fig. 5.2.1)**. OAC did not promote the secretion of peroxidase activity into the

medium **(Fig. 5.1.2 and 5.1.3)**. The above results indicate that OAC did not inhibit cell expansion by increasing peroxidase activity, secretion or action in the cell wall, which can induce the cross-linking of feruloyl esters, coumaroyl esters and tyrosine. These results suggest that OAC does not inhibit cell expansion by promoting cell wall tightening via peroxidase-catalysed cross-linking. Consequently, subsequent experiments focused on whether OAC was inhibiting cell wall loosening mechanisms.

6.The effect of OAC upon cell wall loosening mechanisms

Cell wall loosening plays an important role in plant growth as it promotes cell expansion. The cell wall is believed to be loosened by a number of different agents including transglycosylases (XET), hydrolases, $^{\bullet}OH$ radicals and expansins. The effect of OAC upon each of these cell wall loosening agents was assayed, to determine if the herbicide inhibited its action.

6.1 XET

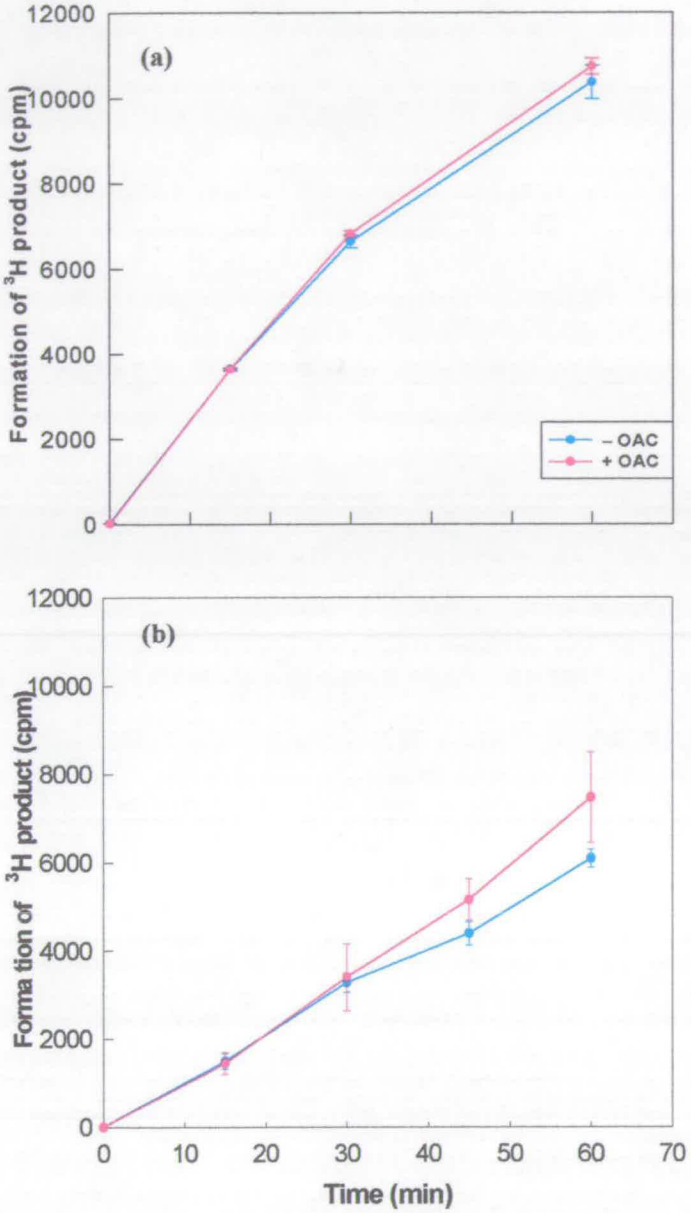
XET is believed to promote cell wall loosening by cleaving xyloglucan chains (which may tether microfibrils in the cell wall), causing localised cell wall weakening and allowing cell expansion. XET also rejoins the cut portion of a xyloglucan chain to the non-reducing terminus of another xyloglucan chain, thereby maintaining overall cell wall integrity (Fry *et al.*, 1992; Thompson and Fry, 2001).

6.1.1 To determine the effect of OAC upon XET activity extracted from maize roots

The effect of OAC on the activity of XET (extracted from roots) *in vitro* was assayed to ascertain whether OAC might exert its *in vivo* effect on growth by affecting transglycosylation of xyloglucan chains. As XET may be a wall-loosening enzyme, a reduced XET activity could lead to a reduction in cell wall expansion.

XET and substrate were incubated with or without OAC over a range of time periods and the solution was then assayed for products. The results show that the activity of XET was undiminished by OAC **(Fig. 6.1.1 (a))**. In a different experiment, XET was incubated with OAC for 1 h, prior to addition to substrate, to determine whether OAC inactivated XET in the absence of substrate. The results clearly show that XET activity remained unaffected **(Fig. 6.1.1 (b))**. In conclusion, total maize root XET

activity was not affected by OAC and a direct effect of OAC on the catalytic action of XET can be ruled out as a possible mode of action of OAC.

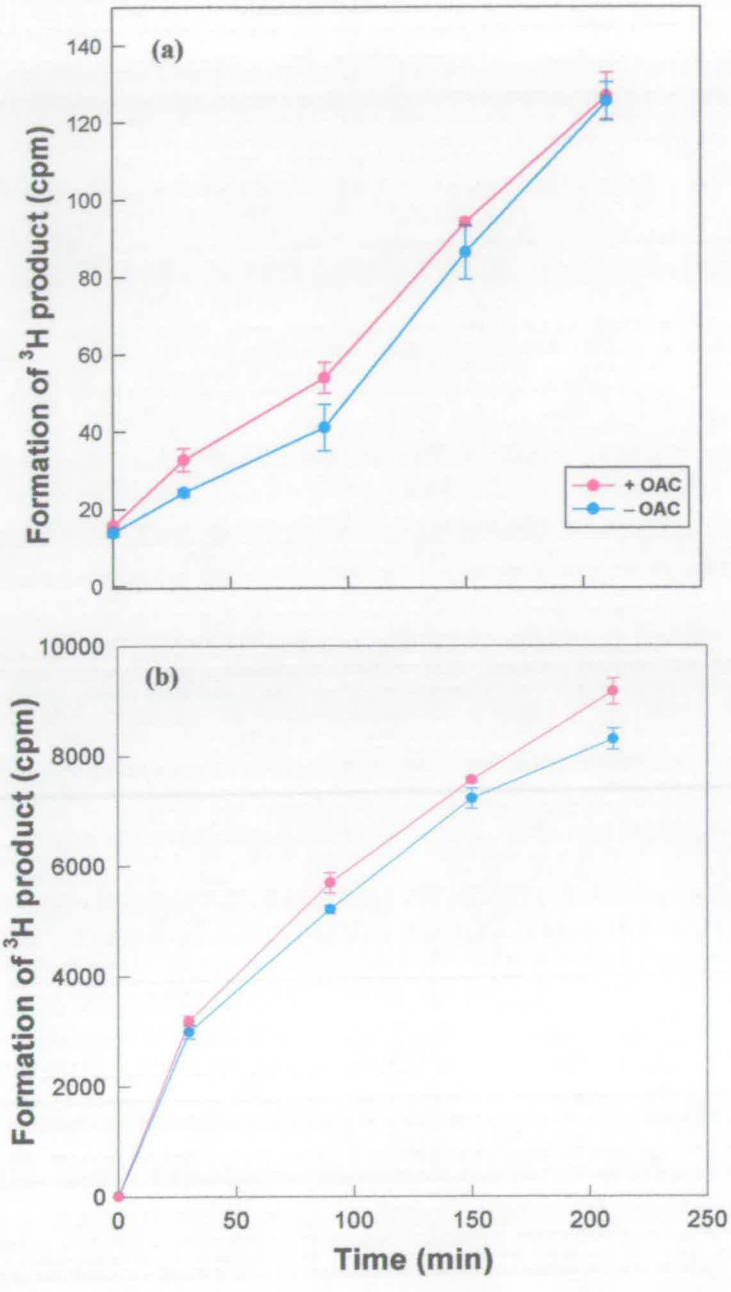


Figs. 6.1.1 The effect of OAC upon XET activity. OAC (to 478 nM) was added either to (a) to XET and substrates (tamarind xyloglucan) or (b) incubated with XET prior to addition to substrates. XET reaction products were then assayed over a range of time intervals.

6.1.2. To determine the effect of OAC upon XET activity secreted into maize CSC medium and XET extractable from cells (including walls)

The previous assays **(6.1.1)** did not establish whether synthesis or availability of XET had been affected by OAC *in vivo*. While OAC did not affect the catalytic action of previously extracted XET, the synthesis, activation or availability of XET for transglycosylation may have been reduced. Therefore, it was necessary to determine if OAC had affected XET so that it was unavailable or unable to catalyse transglycosylation. Examples of processes that may have been affected by OAC include a reduction in the amount of XET synthesised, a conformational change in the enzyme reducing XET activity (OAC could have changed the conformation of XET in the protoplast before being secreted into the cell wall), disruption of the transport mechanism of XET to the cell wall or inhibition of the activation of a pro-enzyme preventing formation of active XET. In order to establish such possible effects of OAC, the activity of XET was assayed after a 24-h incubation period of CSC with or without OAC. The results showed that OAC did not affect XET activity extractable from the cells (including walls) or activity secreted into the medium **(Figs. 6.1.2. (a) and (b))**.

As OAC did not appear to inhibit XET activity, synthesis or secretion, other cell wall loosening agents were assayed for their susceptibility to the herbicide.



Figs. 6.1.2. The effect of OAC upon XET activity extractable from cells (and walls) and activity secreted into the medium. OAC was added to 4-day-old CSC and left overnight. The activity of XET (a) secreted into the medium and (b) extractable from cells (and walls) was assayed over a range of time intervals.

6.2 Hydroxyl radicals ($^{\bullet}$OH)

Hydroxyl radicals are believed to effect cell wall loosening by causing polysaccharide scission < 1 nm from their site of production (Fry, 1998). They can be generated via the Fenton reaction, which is promoted by the presence of ascorbate

if O_2 and traces of Cu^{2+} or Fe^{3+} are present. Fry (1998) and Schweikert *et al.* (2000) demonstrated $^{\bullet}OH$-induced polysaccharide scission *in vitro* and Fry *et al.* (2001) showed that $^{\bullet}OH$ facilitated polysaccharide scission during fruit softening. OAC could inhibit $^{\bullet}OH$ formation thereby preventing cell expansion.

6.2.1 To determine the effect of OAC upon ascorbate consumption in CSC

In order to investigate whether OAC was inhibiting cell wall loosening by preventing apoplastic $^{\bullet}OH$ formation (via an ascorbate-driven Fenton reaction) the effect of OAC upon ascorbate consumption was assayed. If OAC was inhibiting the partcipation of ascorbate in $^{\bullet}OH$ generation, then the OAC-treated cultures could show slower consumption of ascorbate exogenously added to the medium.

Ascorbate was added to CSC treated with or without OAC. Over a range of time intervals, the CSC medium was assayed with DCPIP (which is reduced by ascorbate from a blue to colourless solution). The results indicated that there was no significant difference in concentration of ascorbate present in the medium of CSC between OAC- treated and -untreated CSC **(Fig. 6.2.1)**. As OAC did not reduce ascorbate consumption in maize CSC, it is unlikely therefore to suppress the formation of $^{\bullet}OH$ *in vivo* and therefore $^{\bullet}OH$-mediated cell expansion. However, $^{\bullet}OH$ can also be generated in the following, more complex, series of reactions (Schweikert *et al.*, 2000):

$$NADH + \tfrac{1}{2} H_2O_2 \rightarrow NAD^{\bullet} + H_2O$$
$$NAD^{\bullet} + O_2 \rightarrow NAD^{+} + {}^{\bullet}O_2^{-}$$
$$NADH + H^{+} + {}^{\bullet}O_2^{-} \rightarrow NAD^{\bullet} + H_2O_2$$
$${}^{\bullet}O_2^{-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + O_2$$

The possibility that OAC affected the above process of generating $^{\bullet}OH$ was not tested.

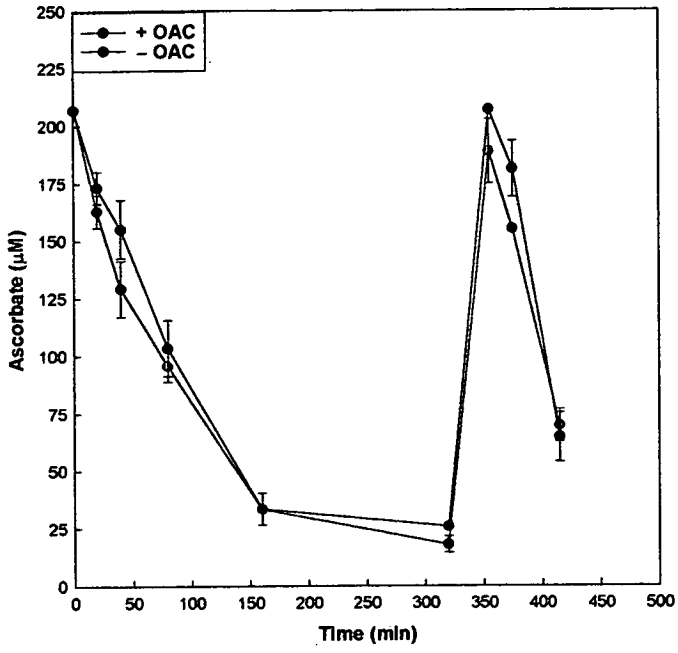


Fig. 6.2.1 The effect of OAC upon ascorbate consumption by maize CSC. Ascorbate (to 200 µM) was added to 4-day-old CSC at 0 and 320 min after being treated with or without OAC. The medium was assayed over a range of time intervals with DCPIP.

6.3 Expansins

Expansins are believed to promote cell wall loosening by disrupting hydrogen bonds formed between cellulose and hemicelluloses (Cosgrove, 1989, McQueen-Mason and Cosgrove, 1994). There are two types of expansin found in the cell wall: α- and β-expansin. Both forms of expansin are found in type I and II cell walls; however, α-expansin has a higher activity on type I cell walls, while β-expansin is more active on type II cell walls (Cosgrove, 1997, 1998). β-Expansins belong to the same family as the GIAs, which cause hay fever (Grobe *et al.*, 1999).

6.3.1 To determine the effect of OAC upon acid 'creep' in methanol-killed maize silks

Expansin action is widely assayed by measurement of the rate of cell wall creep or extension under a constant force using an extensiometer (Cosgrove, 1989). However, during this project a variation of this method was developed to accommodate a larger sample number. The basis of both forms of the assay involved measuring cell wall

extension; however, the assay developed during this project measured final plastic (irreversible) extension and not the rate of creep as measured by an extensiometer. Though this method was developed to assay expansin action, it is important to note that 'yieldin' a protein recently identified by Okamoto-Nakazato *et al.* (2000), which is believed to have a role in cell wall loosening (possibly by lowering the yield threshold of the wall), could also be assayed in this way. Consequently, throughout section **6.3** where it has been stated that endogenous expansin action has been assayed, the results should be interpreted as an effect (or otherwise) upon either expansin action or yieldin action.

In the assay developed during this project, methanol-killed maize silks (with presumably active endogenous expansins) were marked with two ink dots a set distance apart. Each silk was then stretched in either succinate buffer (pH 4), with or without OAC, or phosphate buffer (pH 7), with or without OAC, using the apparatus described in **Fig. 2.6.3**. The force on each silk equalled the weight of the stretching apparatus. The silks were stretched overnight, after which the relaxed distance between the two ink dots was measured again. If OAC was inhibiting expansin-mediated acid creep, then the increase in distance between the two dots would be expected to be lower in the presence of OAC than in the control.

Silks stretched at pH 4 without OAC showed greater extension than silks stretched at pH 7 with or without OAC, suggesting that the former had undergone expansin-mediated acid creep **(Fig. 6.3.1 and Table 6.3.1.2 (Exp. 1))**. They also showed greater extension than silks stretched at pH 4 with OAC, indicating that the herbicide might be inhibiting expansin-mediated acid creep ($P = 0.003$). A P value of 0.05 or less is considered to be significant.

This experiment was repeated a number of times, some of which were carried out 'blind' (a colleague added a known concentration of OAC to one of two volumes of pH 4 buffer) or in 'bulk' (stretched in a large container and not individually in glass tubes). The results **(Fig. 6.3.1 and Table 6.3.1.2)** from some repeats supported the preliminary findings. However, this was not observed for all the repeats. The results **(Table 6.3.1.2)** indicated that fifty percent of silks that were stretched at pH 4 in the

presence of OAC showed a lower plastic extension than the controls, with a P value below 0.05. Forty percent of the repeats indicated that silks, stretched in the presence of OAC, showed a lower plastic extension but with P values above 0.05 **(Fig. 6.3.1.1)** and **(Table 6.3.1.2 (Exp. 8)).** The remaining ten percent (**Table 6.3.1.2 (Exp. 12))** indicated that OAC-treated silks had a higher plastic extension than the controls but with a P value above 0.05. A summary of all the results is provided in **Table 6.3.1.2.**

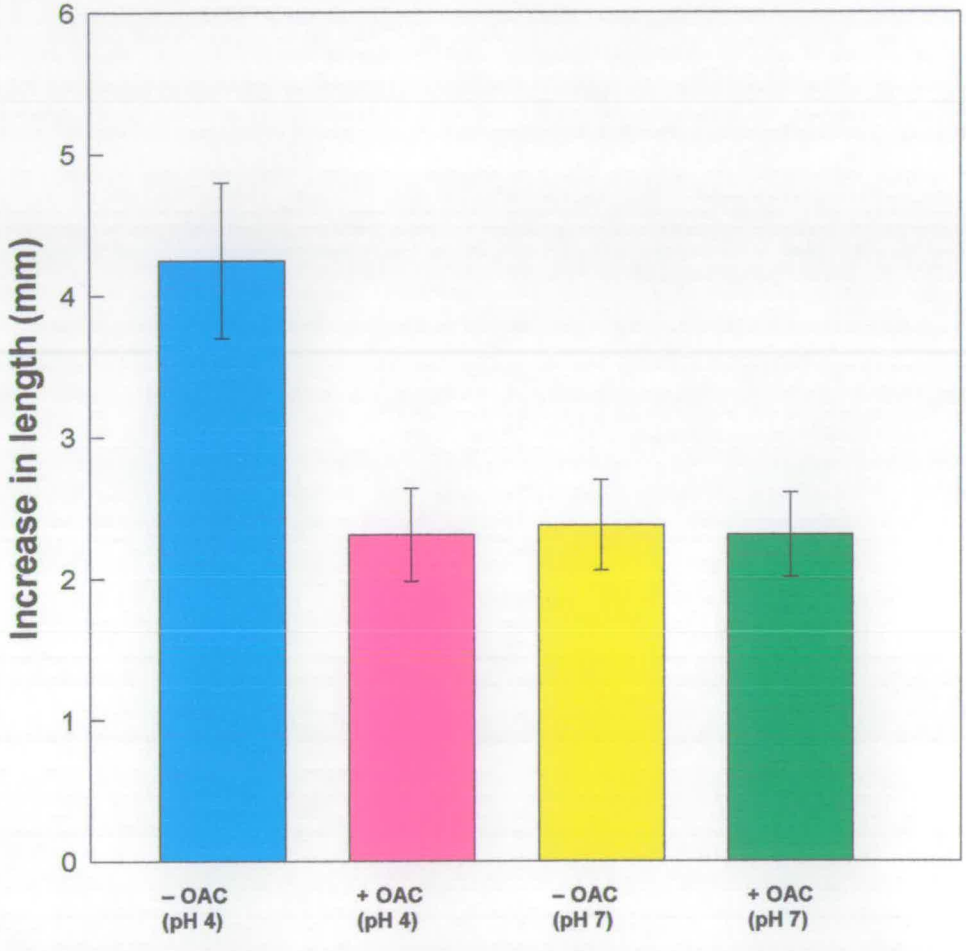


Fig. 6.3.1 The effect of OAC upon acid creep presumably mediated by endogenous expansin action in maize silks. Methanol-killed silks were stretched in either 25 mM succinate at pH 4 or 25 mM phosphate at pH 7, with or without OAC (10 nM), overnight. The distance between two ink dots marked on each silk was recorded before it was placed under tension and again after the incubation period when the weight was removed. The increase in (relaxed) length due to stretching is recorded. N = 16. P = 0.003 (– OAC, pH 4 vs. + OAC, pH 4). Error bars = standard error.

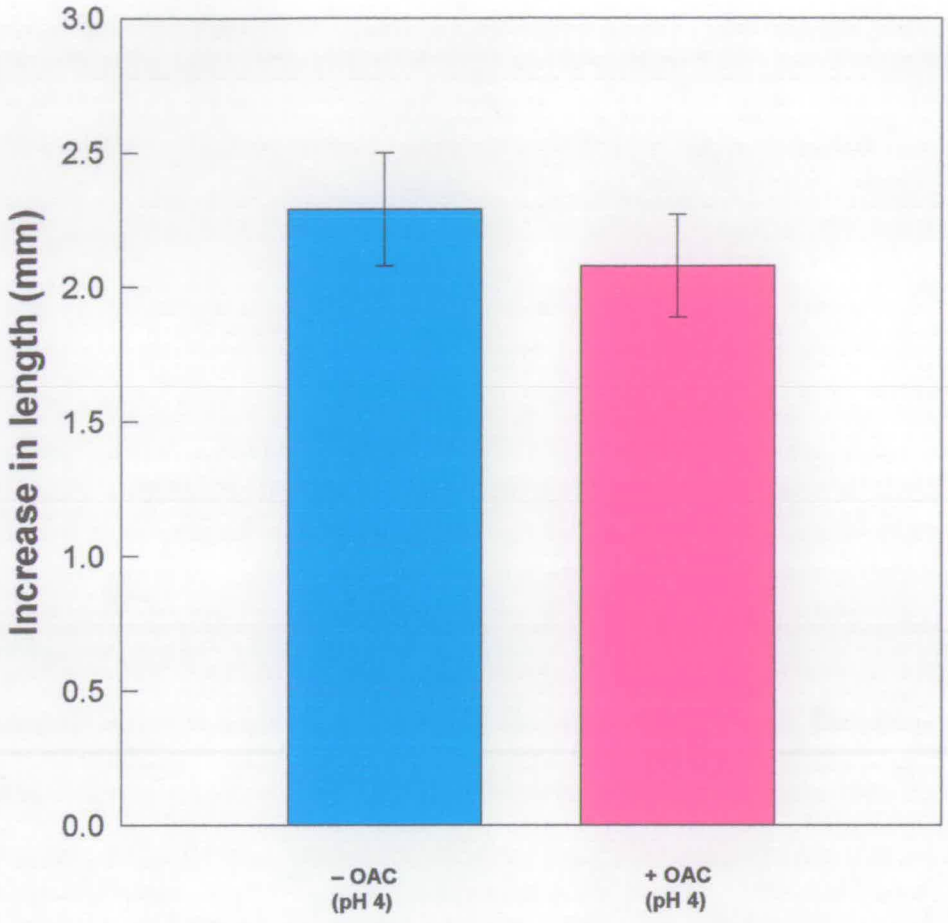


Fig. 6.3.1.1 The effect of OAC (repeat assay) upon acid creep presumably mediated by endogenous expansin action in maize silks. Methanol-killed silks were stretched in 25 mM succinate at pH 4, with or without OAC (478 nM), overnight. Other details as in Fig. 6.3.1. N = 19 (– OAC), 18 (+ OAC). P = 0.25. I = standard error.

Table 6.3.1.2 The effect of OAC upon acid creep in the fast growing region of maize silks — possibly expansin-mediated.

Exp.	[OAC] (nM)	N		Mean extension (mm)		S.E.		– OAC Ext / + OAC Ext	P	pH	Blind	Bulk
		– OAC	+ OAC	– OAC	+ OAC	– OAC	+ OAC					
1	10	16	16	4.25	2.31	0.55	0.33	1.84	**0.003**	4	No	No
	10	16	16	2.38	2.31	0.43	0.32	1.03	0.45*	7	No	No
2	100	17	15	2.53	1.60	0.28	0.33	1.58	**0.017**	4	No	No
3	100	25	23	2.68	1.74	0.26	0.23	1.54	**0.01**	4	No	No
4	100	26	20	2.92	2.10	0.13	0.17	1.39	**0.002**	4	No	No
5	0.1	18	10	4.33	4.35	0.28	0.30	1.00	0.43	4	Yes	Yes
	1.0		8		2.94		0.36	1.47	0.005			
	10.0		10		3.70		0.32	1.17	0.09			
6	0.1	16	10	2.31	2.20	0.23	0.31	1.05	0.39	4	Yes	Yes
	1.0		8		2.44		0.26	0.95	0.37			
	10.0		8		2.19		0.29	1.05	0.38			
7	0.1	18	10	2.50	2.25	0.18	0.26	1.00	0.22	4	Yes	Yes
	1.0		10		2.90		0.24	0.78	0.11			
	10.0		10		2.15		0.28	1.05	0.15			
8	478	19	18	2.29	2.08	0.21	0.19	1.10	0.25	4	Yes	No
9	478	24	29	4.15	3.57	0.24	0.22	1.16	**0.05**	4	Yes	No
10	478	29	28	3.52	3.04	0.15	0.16	1.16	**0.02**	4	No	No
11	478	25	26	2.90	2.85	0.21	0.23	1.02	0.43	4	Yes	No
12	478	14	14	4.18	4.50	0.56	0.37	0.93	0.32	4	Yes	No
13	100	9	9	4.22	3.39	0.32	0.25	1.24	**0.036**	4	Yes	No

A summary of results from all experiments assaying the effect of OAC upon endogenous expansin-mediated acid creep. All experiments were carried out under similar conditions except that herbicide concentration varied and a number of experiments were done either blind or in bulk solution or both.

Refer to legend on next page.

Exp. = experiment number
N = number of replicates
S.E. = standard error
P = P value (*t*-test)
Ext = extension
* P calculated from – OAC vs. + OAC at the same pH

The results from each individual experiment (with and without OAC), excluding **Exp. 1**, were combined. A *t*-test performed on this set of data gave a P value of 0.001 (mean = 3.07 and 2.69 mm, N = 222 and 199, S.E. = 0.09 and 0.08 mm (with and without OAC, respectively)).

6.3.2 To determine the effect of OAC upon acid creep in celery petiole vascular bundles

The effect of OAC upon acid creep (believed to be either expansin- or yieldin-mediated) in celery petiole vascular bundles was also assayed using the same method as previously described for silks **(6.3.1)**. The results indicate that OAC has no inhibitory effect upon expansin-mediated acid creep in celery vascular bundles, suggesting that OAC does not inhibit α-expansin action **(Fig. 6.3.2)**. However, as OAC might be inhibiting expansin-mediated creep in maize silks **(Table 6.3.1.1)** it is possible that OAC inhibits β-expansin action. In consideration of the fact that OAC is selective against graminaceous monocotyledons and that β-expansins have a higher activity in monocotyledons, it is plausible that OAC inhibits β- and not α-expansin. Both forms of expansin induce creep and belong to the same family but as previously mentioned in **1.6.3** there are structural differences between them.

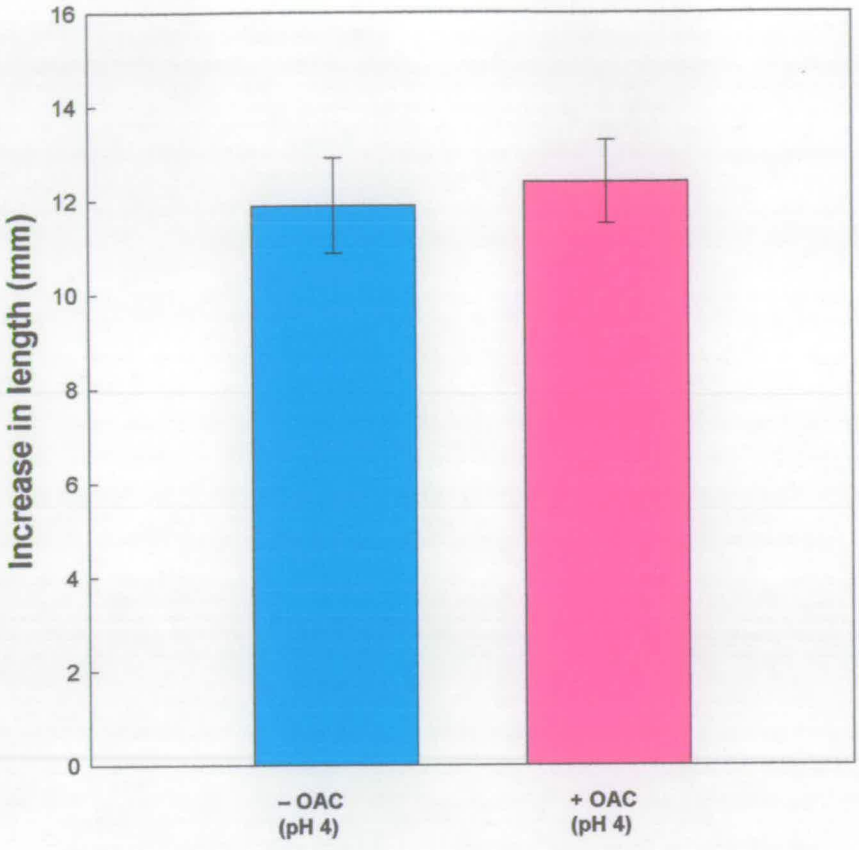


Fig. 6.3.2 The effect of OAC upon acid creep presumably mediated by endogenous expansin action in celery petiole vascular bundles. Methanol-killed celery petiole vascular bundles were stretched in 25 mM succinate (Na^+, pH 4, with or without OAC (478 nM), overnight. Other details as in Fig. 6.3.1. N = 10 (– OAC), 11 (+ OAC). P = 0.37. I = standard error.

6.3.3 To determine the effect of OAC upon acid creep in frozen, thawed, maize roots

The effect of OAC upon acid creep (believed to be either expansin- or yieldin-mediated) in maize roots was assayed, as the herbicide was observed to have a dramatic inhibitory effect upon root growth **(Fig. 3.2.1 (c) and (d))**. Frozen, thawed, maize roots were stretched in acetate buffer at pH 4, with or without OAC, in a similar manner to that described for maize silks **(6.3.1)**. However, any increase in length was measured by computer from enlarged scanned images of photographs taken before and after stretching. The results indicated that OAC was significantly inhibiting acid creep in maize roots (presumed to be expansin-mediated) **(Fig. 6.3.3**

and **Table 6.3.3.1 (Exp. 1)**). However, repeats of this experiment did not show significant inhibition of expansin-mediated creep **Table 6.3.3.1 (Exp. 2 and 3).**

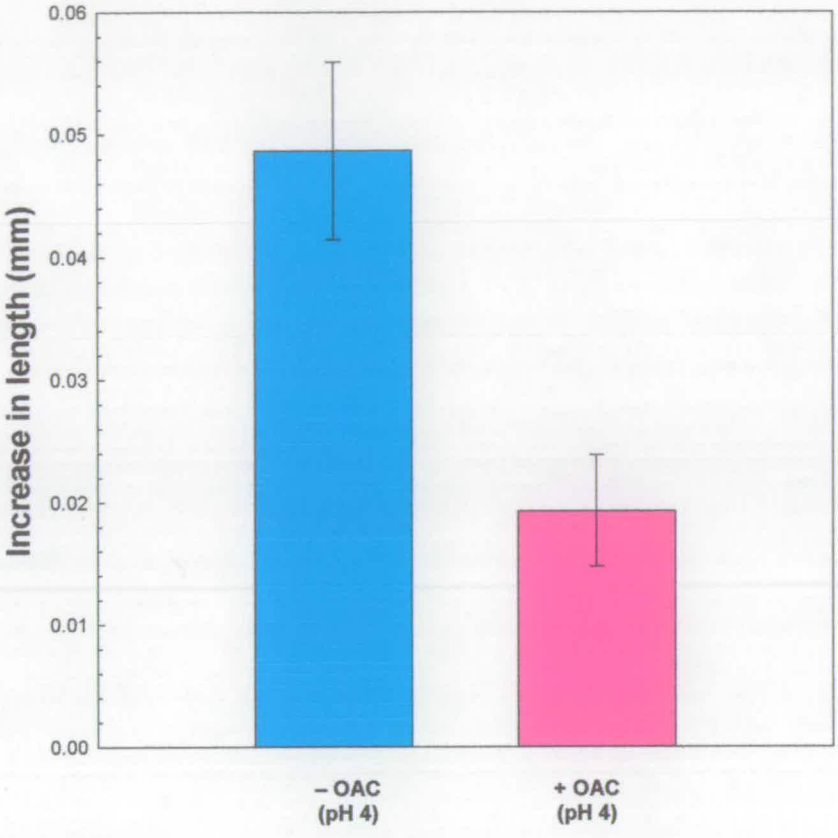


Fig. 6.3.3 The effect of OAC upon acid creep presumably mediated by endogenous expansin action in maize roots. Frozen, thawed, roots were stretched in 50 mM acetate (Na^+, pH 4), with or without OAC (478 nM), overnight. Other details as in Fig. 6.3.1. N = 4. P = 0.012. I = standard error.

Table 6.3.3.1 The effect of OAC upon acid creep in maize roots — possibly expansin-mediated.

Exp.	[OAC] (nM)	N		Mean extension (mm)		S.E.		– OAC Ext / + OAC Ext	P
		– OAC	+ OAC	– OAC	+ OAC	– OAC	+ OAC		
1	478	4	4	0.048	0.019	0.007	0.004	2.53	**0.012**
2	478	8	8	0.116	0.099	0.007	0.007	1.17	**0.077**
3	478	8	7	0.110	0.100	0.009	0.011	1.10	**0.275**

A summary of results from experiments assaying the effect of OAC upon expansin-mediated acid creep at pH 4.

Exp. = experiment number

N = number of replicates

S.E. = standard error

P = P value (*t*-test)

Ext = extension

Results from each individual experiment (+/– OAC) were combined and gave a P value of 0.098 (mean = 0.08 and 0.10 mm, N = 20 and 19, S.E. = 0.009 and 0.008 mm (with and without OAC, respectively)).

6.3.4 To determine the effect of OAC upon acid creep mediated by exogenous expansin action on boiled maize silks

β-Expansins are loosely bound to pollen and can be aqueously extracted (Cosgrove, 1997). Consequently, extract from pollen (believed to contain β-expansin) was added to boiled silks, with or without OAC, to determine whether exogenous expansin-mediated creep was inhibited by the presence of the herbicide. This pollen extract will be referred to as β-expansin extract, in the knowledge that it is a crude extract, which could contain other cell wall loosening proteins such as XET, cellulases or yieldin.

As a control, maize silks were boiled and then stretched, with or without OAC, to ascertain whether or not there was creep without active endogenous expansins. If

creep was observed, without the presence of endogenous expansins, then the aim was to determine if OAC affected the inherent creep.

The extent of creep in boiled silks (in the absence of expansin) and unboiled silks was determined after stretching them overnight in succinate buffer, pH 4. The average increase in length of boiled silks was 1.50 mm (N = 10, S.E. = 0.25) in comparison to 1.90 (N = 8, S.E. = 0.34) for unboiled silks. As the boiled silks extended in length, without the presence of active endogenous expansins, the extension of boiled silks, with or without OAC, and unboiled silks, with and without, was assayed to determine if OAC inhibited the inherent creep of silks **(Fig. 6.3.4. (a))**. Boiled silks stretched with or without OAC showed similar levels of extension with a P value of 0.361 **(Fig. 6.3.4. (a)).** Unboiled silks, without OAC, extended more than boiled silks, treated with or without OAC, with P values of 0.14 and <0.05, respectively. They also extended more than unboiled silks, treated with OAC, with a P value of <0.001.

Boiled and unboiled silks were then stretched in acetate buffer (pH 4) with or without β-expansin extract or OAC. OAC inhibited the action of exogenous β-expansin extract on boiled silks, though not significantly, with a P value of 0.12 **(Fig. 6.3.4. (b))**.

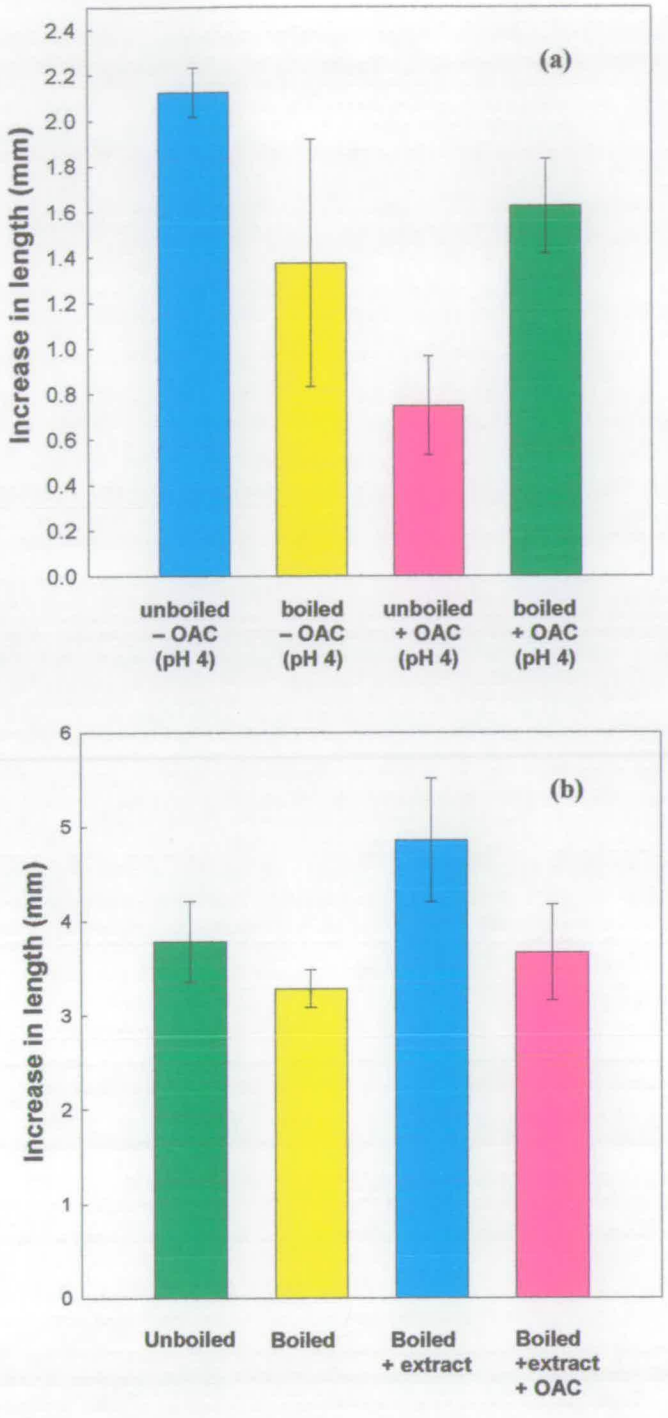


Fig. 6.3.4 The effect of boiling, exogenous β-expansin and OAC upon acid creep in maize silks. Silks, boiled and unboiled, were stretched in 50 mM acetate at (Na^+, pH 4) (a) with or without OAC overnight (N = 4) and (b) with or without OAC or β-expansin extract overnight. N = 7 (unboiled), N = 7 (boiled – extract), N =7 (boiled + extract) and N = 6 (boiled + extract + OAC). Other details as in Fig. 6.3.1. I = standard error.

6.3.5 To determine the effect of OAC upon acid creep mediated by exogenous β-expansin extract on boiled maize silks assayed using an extensiometer

The effect of OAC upon the rate of creep, mediated by exogenous β-expansin extract, was assayed on maize silks by use of an extensiometer. Boiled silks were stretched at pH 4.5 using an extensiometer. After a defined period of stretching, β-expansin extract, with or without OAC, was added to the silks. The results for each silk have been adjusted (as a percentage of the mean creep rate observed for the same silk before addition of β-expansin) so that average initial creep rate, prior to addition of β-expansin extract, equals one hundred percent. β-Expansin extract, without OAC, promoted the rate of creep. However, β-expansin extract with OAC promoted a lower rate of creep, suggesting that OAC inhibited β-expansin action **(Fig. 6.3.5 and Table 6.3.5.1)**. These results support previous findings, suggesting that OAC inhibits β-expansin-mediated creep.

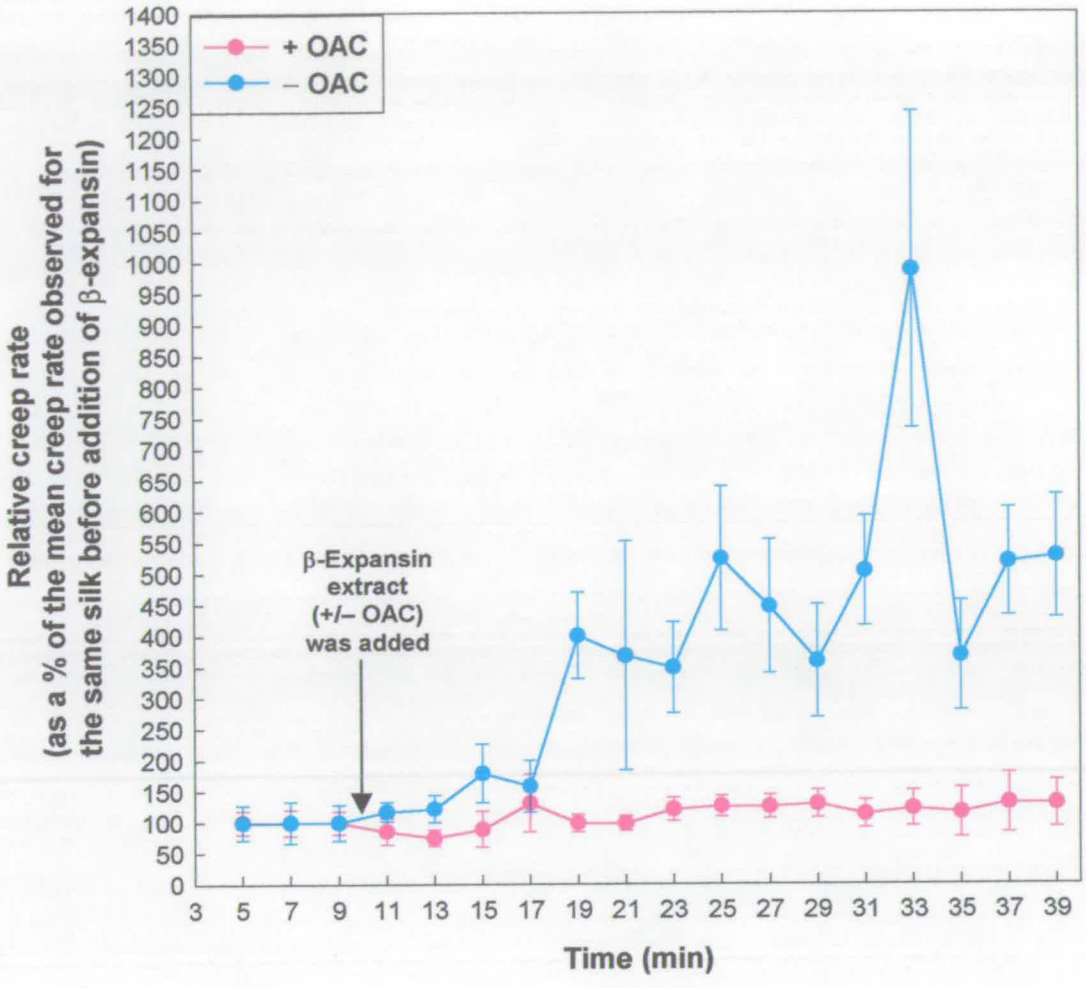


Fig. 6.3.5 The effect of OAC upon exogenous β-expansin-mediated creep in maize silks. Maize silks (boiled for 30 s) were stretched using a 12-g force, in 25 mM acetate (Na^+, pH 4) for 10 min, after which expansin extract (0.05 vols) was added, with or without OAC (to 478 nM). The voltage (measured by an LVDT) was recorded, at 4–s intervals, for 5 min prior to the addition of β-expansin. The rate of change of voltage (for each treatment) was calculated at two-min intervals; each time-point plotted here represents the mid-point of the two-min interval. N = 7. I = standard error.

Table 6.3.5.1 The effect of OAC upon the average rate of β-expansin-mediated creep

[OAC] (nM)	N		Mean relative creep rate: from 11–39 min (as a % of the mean creep rate observed for the same silk before addition of β-expansin)		S.E.		P
	– OAC	+ OAC	– OAC	+ OAC	– OAC	+ OAC	
478	7	7	424	109	60	1.2	**<0.001**

Exp. = experiment number

N = number of replicates

S.E. = standard error

P = P value (*t*-test)

6.3.6 To determine the effect of OAC upon acid creep mediated by exogenous β-expansin extract on boiled maize roots

Exogenous β-expansin (aqueously extracted from maize pollen using sodium acetate), with or without OAC, was added to boiled roots to determine whether exogenous expansin-mediated creep in maize roots was inhibited by the presence of the herbicide. After stretching overnight, the OAC-treated roots showed reduced extension in comparison to the control, though the effect was not significant **(Fig. 6.3.6 and Table 6.3.6.1)**.

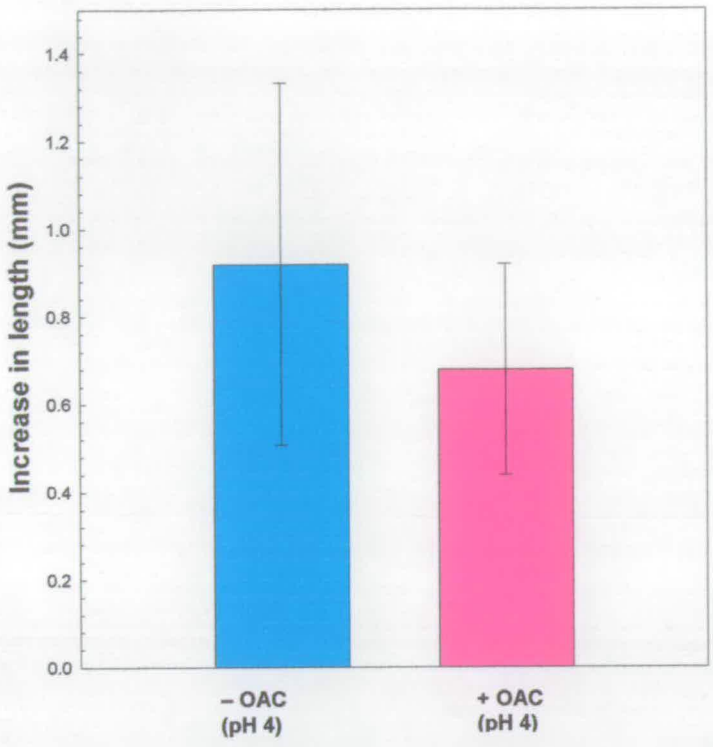


Fig. 6.3.6 The effect of OAC upon exogenous expansin-mediated creep in maize roots. Maize roots, boiled and unboiled, were stretched in 50 mM acetate (Na^+, pH 4), with or without OAC (478 nM), overnight. Other details as in Fig. 6.3.1. N = 5 (boiled + extract), N = 8 (boiled – extract). P = 0.19. I = standard error.

Table 6.3.6.1 The effect of OAC upon creep mediated by β-expansin extract in boiled maize roots

Exp.	Treatment	[OAC] (nM)	N		Mean extension (mm)		S.E.		– OAC Ext / + OAC Ext	P
			– OAC	+ OAC	– OAC	+ OAC	– OAC	+ OAC		
1	boiled + expansin	478	5	8	0.924	0.679	0.411	0.240	1.360	**0.14**
2	boiled + expansin unboiled – expansin	478	2 4	3	0.470 0.360	0.430	0.001 0.003	0.430	1.093	**0.27**
3	boiled + expansin unboiled – expansin	478	2 4	2	0.388 0.630	0.266	0.005 0.004	0.266	1.459	**0.15**
4	boiled + expansin boiled + expansin +NaCl	478	2 5	3	0.978 0.612	0.829	0.018 0.007	0.829	1.180 0.738	**0.37** **0.11**
5	boiled + expansin unboiled – expansin	478	2 3	3 4	0.280 0.37	0.215 0.500	0.000 0.016	0.215 0.500	1.302 0.740	**0.24** **0.27**

A summary of results from experiments assaying the effect of OAC upon expansin-mediated acid creep at pH 4 (method 2.6.3) overnight incubation. Roots were boiled for ~ 30 s to denature endogenous proteins.

Exp. = experiment number

N = number of replicates

S.E. = standard error

P = P value (*t*-test) of + OAC **vs.** OAC

Ext. = extension

Results from individual experiments were combined (boiled roots + OAC – β-expansin extract **vs.** boiled roots – OAC + β-expansin extract) giving a P value of 0.19 (mean = 0.059 and 0.071 mm, N = 20 and 15, S.E. = 0.035 and 0.040 mm (with and without OAC, respectively)).

6.3.7 To determine the effect of OAC upon acid creep mediated by β-expansin extract on boiled maize roots assayed using an extensiometer

The effect of OAC upon the rate of creep, mediated by β-expansin extract, was assayed on roots. Boiled maize roots were stretched at pH 4.5 using an extensiometer. After a defined period of stretching, β-expansin extract, with or without OAC, was added to the roots. The β-expansin extract, without OAC, significantly promoted the rate of creep in roots in comparison to the control. In the presence of OAC, β-expansin effected a lower rate of creep in the roots, suggesting that OAC inhibited β-expansin action **(Fig. 6.3.7 and Table 6.3.7.1)**, these results were not adjusted as the initial creep rate of the roots prior to addition of β-expansin were sufficiently similar. The β-expansin-mediated increase in creep rate was higher in silks than roots and OAC was less effective at inhibiting β-expansin-mediated creep in roots.

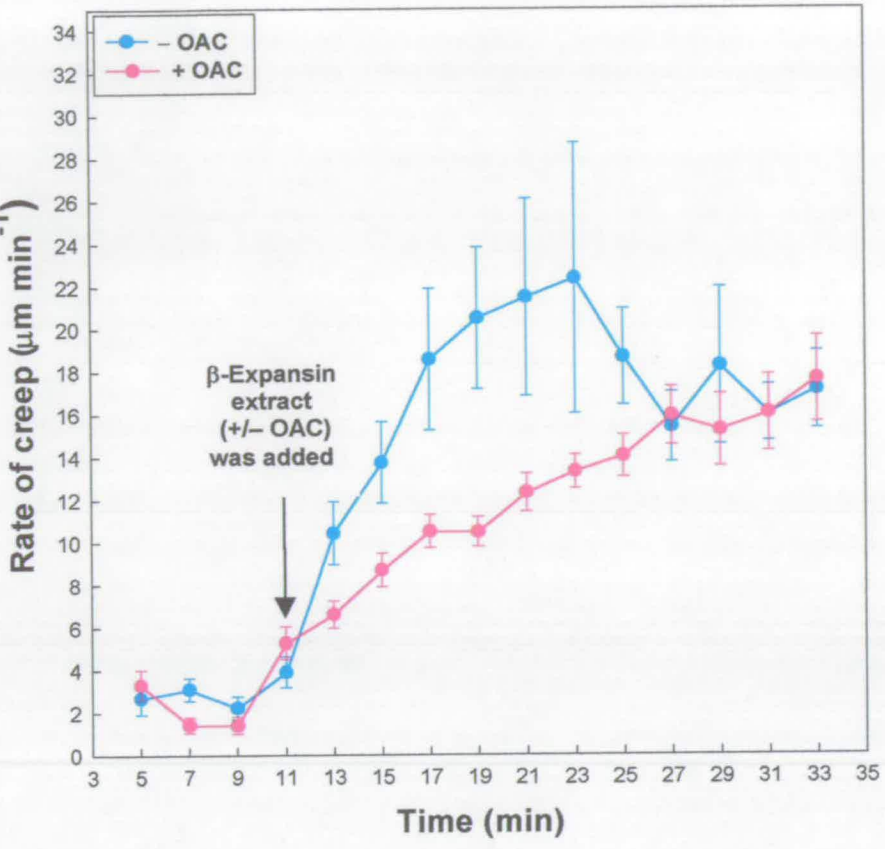


Fig. 6.3.7 The effect of OAC upon exogenous expansin-mediated creep in maize roots. Maize roots (boiled for 30 s) were stretched using an 8-g force, in 25 mM acetate (Na^+, pH 4) for 11 min, after which expansin extract (0.05 vols.) was added, with or without OAC (478 nM). The voltage (measured by an LVDT) was recorded, at 4–s intervals, for 5 min prior to the addition of β-expansin. The rate of change of voltage (for each treatment) was calculated at two-min intervals; each time-point plotted here represents the mid-point of the two-min interval. N = 13 (– OAC), N = 14 (+ OAC). I = standard error.

Table 6.3.7.1 The effect of OAC upon the average rate of β-expansin-mediated creep

[OAC] (nM)	N		Mean creep rate ($\mu m\ min^{-1}$)		S.E.		P
	– OAC	+ OAC	– OAC	+ OAC	– OAC	+ OAC	
478	13	14	16.38	12.17	1.42	1.08	**0.017**

N = number of replicates

S.E. = standard error

P = P value (*t*-test)

6.3.8 To determine the effect of OAC upon pollen (*Tradescantia*) germination

As OAC inhibited β-expansin action it was expected that OAC might inhibit pollen germination or pollen tube growth. β-Expansin, which is loosely bound to pollen, is believed to facilitate the penetration of pollen tubes between cells of the style. Pollen from *Tradescantia* (a non-graminaceous monocotyledon) was placed in a 5% sucrose solution (to promote pollen tube germination) with or without OAC. However, the results indicated that OAC did not inhibit the germination of pollen grains **(Fig. 6.3.8)**.

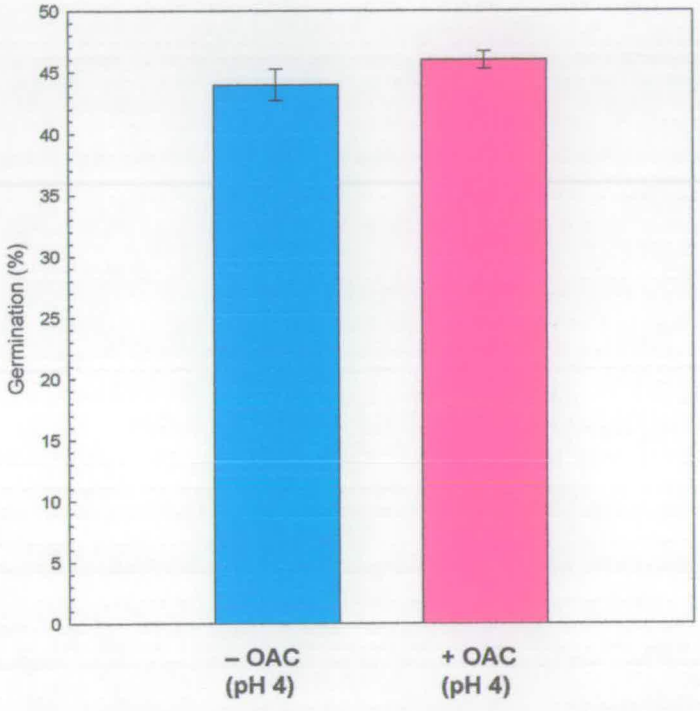


Fig. 6.3.8 The effect of OAC upon germination in *Tradescantia* pollen grains. *Tradescantia* pollen was placed in a 5% sucrose solution with or without OAC (478 nM). The percentage of germinated pollen grains (100) was measured after 1 h.

6.3.9 To determine the effect of OAC upon proteinase activity

Grobe *et al.* (1999) reported that GIAs, which belong to the same family as β-expansins, have proteinase activity. Consequently, the effect of OAC upon papain (a proteinase which belongs to the same (proteinase) family as GIAs (Cosgrove, 1997))

was assayed. For demonstration of proteinase activity, papain was incubated over a range of time periods with a chromogenic substrate. Unhydrolysed substrate was precipitated and the A_{400} of the supernatant was measured. After a 1-h incubation period the papain had suitable proteinase activity **(Fig. 6.3.9)**. Consequently, papain was then incubated with a range of concentrations of OAC for 1 h to determine if the herbicide inhibited the proteinase activity of papin. The proteinase activity of papin was not inhibited by OAC **(Fig. 6.3.10)** but a higher than expected absorbance reading after a 1-h incubation period was observed (in comparison to **Fig. 6.3.9**). This may have been because a different chromogenic substrate, azocasein instead of azoalbumin, was used.

Li and Cosgrove (2001) have recently suggested that the proteinase activity of Phl p1 (a GIA) reported by Grobe (1999) was attributable to contamination and that β-expansin did not have proteinase activity. This suggests that the inhibition of β-expansin action by OAC is not because the herbicide inhibits proteinase activity.

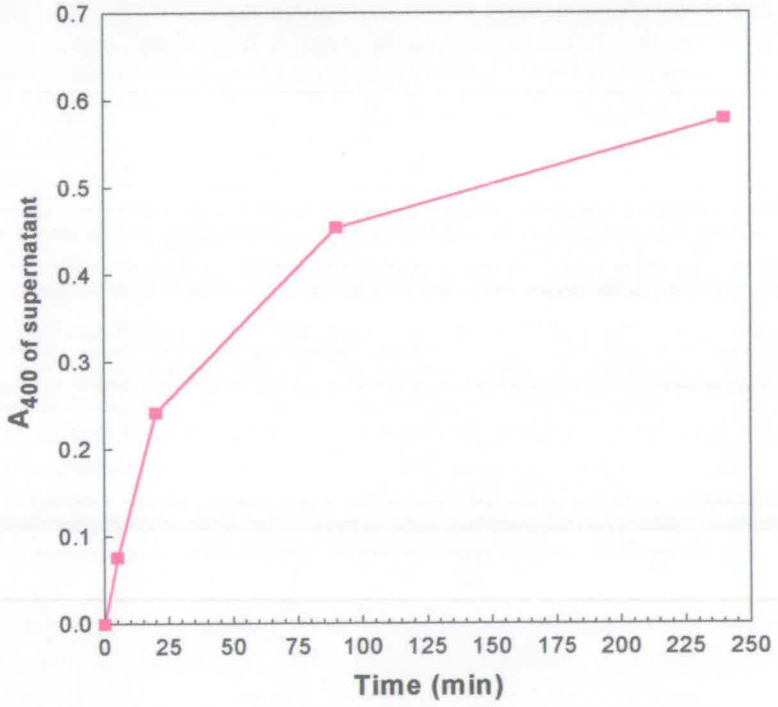


Fig. 6.3.9 Demonstration of the activity of papain on the chromogenic substrate azoalbumin. Papain (1 mg ml^{-1}) was incubated with azoalbumin (1.6 mg ml^{-1}) at pH 4 and at 30°C. Unhydrolysed protein was precipitated with 8% trichloroacetic acid and the A_{400} of the supernatant was measured.

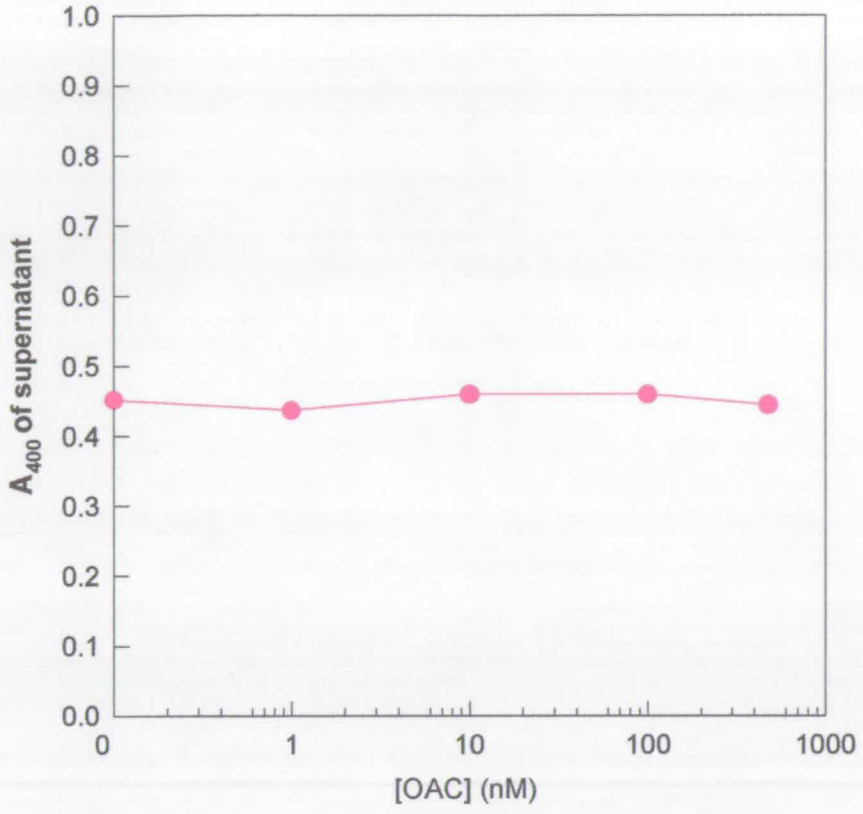


Fig. 6.3.10 The effect of OAC upon proteinase activity of papain. Papain (1 mg ml^{-1}) was incubated with azocasein (1.6 mg ml^{-1}) at pH 4 and a range of concentrations of OAC at 30°C. After a 1-h incubation period, unhydrolysed protein was precipitated with 8% TCA and the A_{400} of the supernatant was measured.

6.4 Development of an alternative assay for expansin action

An *in vitro* assay, enabling the detection of expansin action upon [*hydroxyl*-^{3}H]cellulose, was partially developed as an alternative to the conventional expansin-action assays currently used. Cellulose molecules (composed of D-Glc*p* residues) make up microfibrils. The theory behind this assay was that some of the H atoms of OH groups on the D-Glc*p* residues would become ^{3}H after incubation with ^3H_2O. Washing the [*hydroxyl*-^{3}H]cellulose in water would remove ^{3}H from the exposed D-Glc*p* residues but not the unexposed residues. Expansin action would then be detectable as disruption of hydrogen bonding within microfibrils would expose some of the remaining [^{3}H]Glc*p* residues allowing the formation and release of ^3H_2O.

The benefits of using [*hydroxyl*-^{3}H]cellulose as a substrate to detect expansin action include a relatively simple assay to perform and the production of a detectable and quantifiable product.

Crystalline cellulose (Avicel) was incubated in ^3H_2O at 100°C overnight and then washed with 0.5% chlorobutanol until the majority of the exposed ^{3}H was removed **(Fig. 6.4 (a))**. The washed [*hydroxyl*-^{3}H]cellulose was then incubated in water and various solvents which, were assayed over a range of time intervals for ^{3}H (results not shown), after which the [*hydroxyl*-^{3}H]cellulose was assayed for remaining levels of ^{3}H. Low levels of ^3H_2O were released after the [*hydroxyl*-^{3}H]cellulose had been washed for a total of five times with water and various solvents **(Fig. 6.4 (a))** whereas high levels of ^{3}H were still present in the washed [*hydroxyl*-^{3}H]cellulose **(Fig. 6.4 (b))**. This indicated that [*hydroxyl*-^{3}H]cellulose, after washing in water, could be a suitable substrate to detect expansin action. Water was the preferred solvent as similar amounts of ^3H_2O were released for each solvent apart from NaOH, which released higher levels of ^{3}H (results not shown).

A new batch of [*hydroxyl*-^{3}H]cellulose was washed with, and incubated in, water for ~ 2 days (to reduce further the expansin-mediated release of ^3H_2O). After this, β-expansin, α-expansin (from 2 fractions, one of which was more recently purified than the other), or Driselase were added. In the presence of β-expansin, α-expansin (both fractions) and Driselase, a greater amount of ^3H_2O was formed than in the control, suggesting that this method might be useful to detect expansin action. The release of high levels of ^3H_2O by Driselase reflects the ability of this mixture of enzymes to break down cellulose therefore exposing the inner, previously unexposed [*hydroxyl*-^{3}H]cellulose molecules.

This experiment was repeated except that the activity of pure cellulase was also assayed. The results indicated that Driselase, β-expansin, pure cellulase and one of the two α-expansin fractions promoted the release of ^3H_2O in comparison to the control, supporting the previous findings **(Fig. 6.4.2 and 6.4.3 (a) and (b))**.

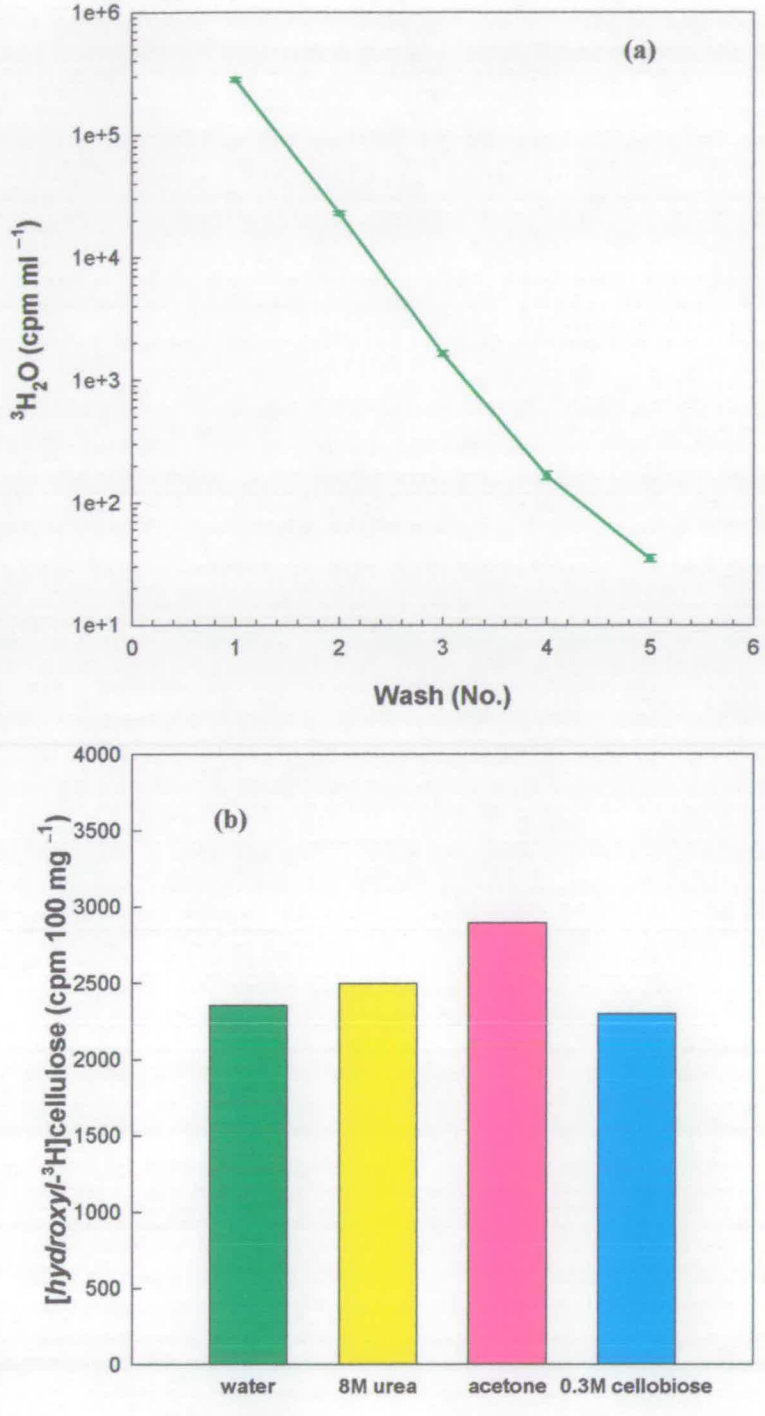


Fig. 6.4 The amount of ^{3}H present in [*hydroxyl*-^{3}H]cellulose rinses and in [*hydroxyl*-^{3}H]cellulose after rinsing with various solvents. Cellulose was incubated in ^3H_2O at 100°C overnight. The [*hydroxyl*-^{3}H]cellulose (100 mg) was incubated in 25 ml of water or of various other solvents (data not shown) overnight. The [*hydroxyl*-^{3}H]cellulose was then washed with 5 × 5 ml of water (a) or various solvents and the water washings were then assayed for ^{3}H. The total remaining ^{3}H in the [*hydroxyl*-^{3}H]cellulose after the five washes was also assayed (b).

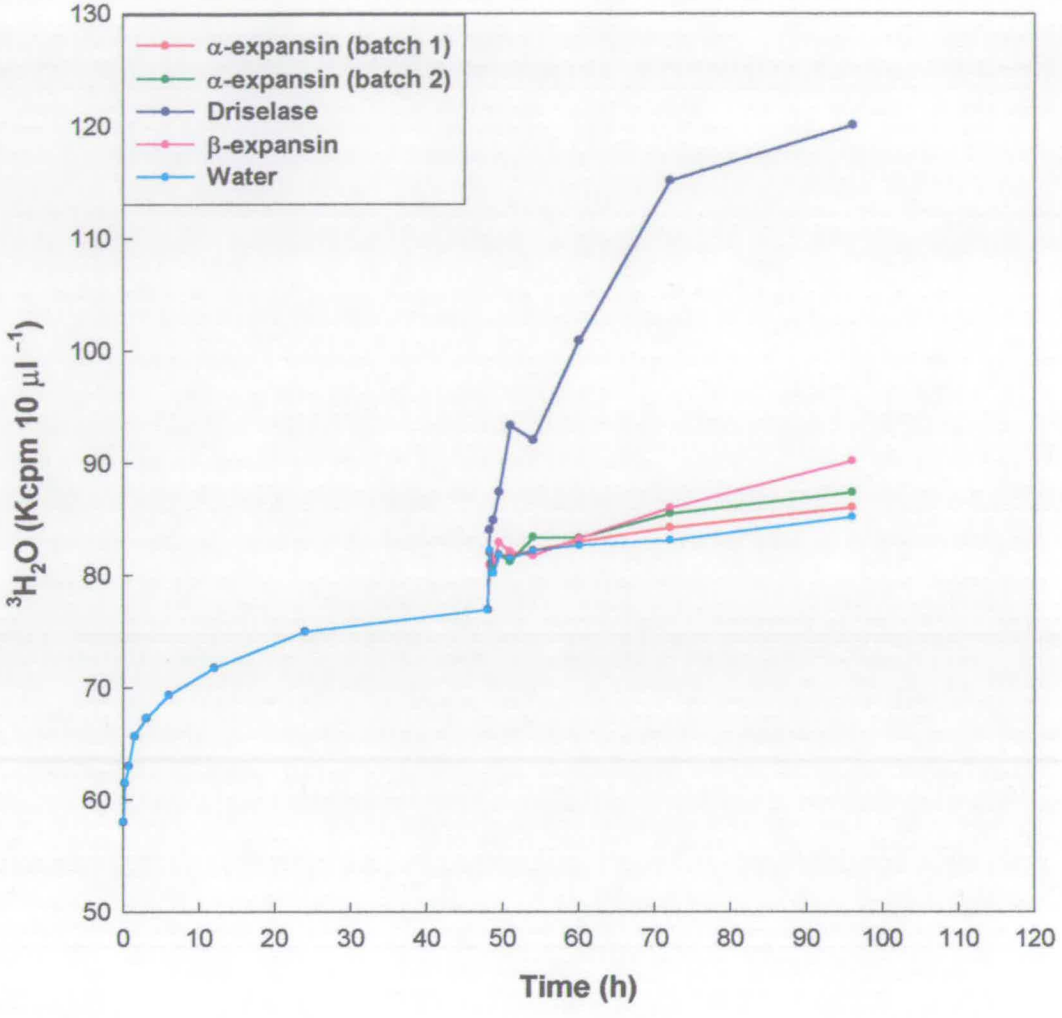


Fig. 6.4.1 The release of ^3H_2O by action of Driselase and α- and β-expansin on [*hydroxyl*-^{3}H]cellulose. [*hydroxyl*-^{3}H]Cellulose (50 mg) was incubated in 5-ml of water (0.5% chlorobutanol), which was then assayed at intervals over a 50-h period for ^{3}H. Driselase (0.5% final concentration) or α- or β-expansin (in 10 μl of 50 mM acetate (Na^+, pH 4)) was then added to 1 mg [*hydroxyl*-^{3}H]cellulose 90 μl^{-1}, which was assayed for ^{3}H, at intervals, for a further 46 h.

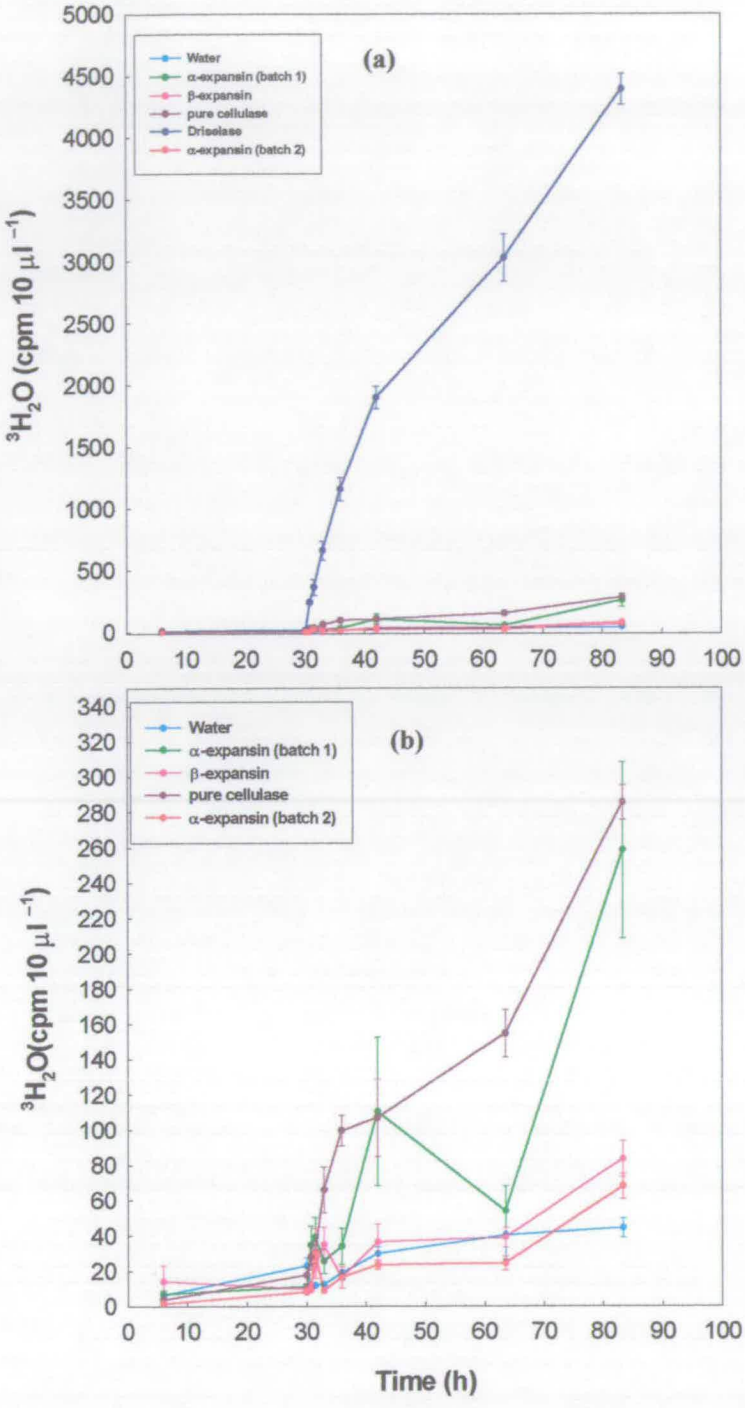


Fig. 6.4.2 The release of ^3H_2O by action of Driselase, pure cellulase and α- and β-expansin on [*hydroxyl*-^{3}H]cellulose. [*hydroxyl*-^{3}H]Cellulose (1 mg) was incubated in 100 µl of water (0.5% chlorobutanol), which was assayed at intervals over a 30-h period for ^{3}H. Driselase, pure cellulase or α-or β-expansin (in 10 µl of 50 mM acetate (Na^+, pH 4)) was then added to the remaining 90 µl of mixture, which was then assayed for ^{3}H at intervals for a further 54 h. (a) The effect of Driselase, pure cellulase and α- and β-expansin upon ^3H_2O formation and (b) a closer look at the effect of pure cellulase, α- and β-expansin upon ^3H_2O formation. N = 2 (α- and β-expansin), N = 3 (pure cellulase), N = 3 (Driselase) and N = 5 (water).

6.5 Conclusion

OAC did not inhibit maize root XET activity *in vitro* **(Fig. 6.1.1 (a) and (b))** or the amount of XET activity extractable from cultured maize cells (including walls) or the amount of XET activity secreted into the culture medium **(Fig. 6.1.2 (a) and (b))**. The herbicide also did not affect ascorbate consumption in maize CSC **(Fig. 6.2.1)**. As ascorbate can promote the Fenton reaction, which generates $^{\bullet}OH$ radicals, the lack of effect of OAC upon ascorbate consumption suggests that $^{\bullet}OH$-mediated cell wall loosening and therefore cell expansion is not inhibited by OAC. However, OAC did inhibit extension (believed to be either expansin- or yieldin-mediated) in methanol-killed maize silks **(Fig. 6.3.1)**, though not consistently **(Fig. 6.3.1.2)**.

OAC significantly reduced the rate of creep, mediated by β-expansin extract, in boiled maize silks **(Fig. 6.3.5)** and to a lesser extent in boiled maize roots **(Fig. 6.3.7)**. These results strongly suggest that OAC inhibited β-expansin-mediated creep. It should also be noted that OAC did not inhibit extension of young celery petiole vascular bundles (type I cell wall), believed to be mediated by α-expansins, which are thought to be more active in type I cell walls **(Fig. 6.3.2)**. One possible reason for preferential inhibition of β-expansin by OAC could be the structural differences between the two forms of expansin. β-Expansins, unlike α-expansins, are highly glycosylated, which could affect binding of OAC to the protein. The structural difference between the two forms of expansin and the fact that β-expansins have a higher activity in graminaceous monocots than in dicots (Cosgrove, 1997) could explain the selectivity of OAC against plant with type II, and not type I cell walls.

OAC might be more active against for β-expansin found in maize roots than against that found in pollen, as different forms of β-expansins are expressed in different tissues (Wu *et al.*, 2001). It would therefore be interesting to repeat the experiment using β-expansin extracted from maize roots or CSCs.

A number of 'bulk' experiments involved stretching methanol-killed maize silks in a large container of buffer to assay for creep. With hindsight, this method could have

diluted the concentration of β-expansin present in the silks (as β-expansin is highly soluble in water), thereby showing a reduction in plastic extension and masking the effect of OAC. This would be less likely to happen in 'non-bulk' experiments (with individual silks and roots stretched in a glass tube) as the volume of buffer was lower.

Grobe *et al.* (1999) suggested that GIAs (belonging to the same family as β-expansins) had proteinase activity: consequently, the effect of OAC upon papain (a proteinase which is believed to belong to the same family as GIAs) was assayed. It was possible that β-expansins effected creep by hydrolysing protein cross-links in the cell wall (such as threonine rich proteins found in type II cell walls) and thereby causing cell wall loosening. OAC could therefore have inhibited β-expansin action by inhibiting its proteinase activity but no such inhibition was observed **(Fig. 6.3.9)**. However, Li and Cosgrove (2001) have recently called the proteinase activity of β-expansins into question after observing no proteinase activity in Phl 1. Li and Cosgrove (2001) attributed the reported proteinase activity of Phl 1 (GIA) (Grobe *et al.* 1999) to the result of contamination by proteinases when Phl 1 was expressed in *Pichia pastoris.*

OAC had no effect upon the germination of *Tradescantia* pollen **(Fig. 6.3.8)**. This could be because *Tradescantia* is not a graminaceous monocotyledon, unlike maize, and therefore contained less β-expansin.

An alternative assay for expansin action using [*hydroxyl*-^{3}H]cellulose (which can also be used to detect and quantify cellulose and Driselase action) was also proposed **(6.4)**. The results **(Figs. 6.4.1 and 6.4.2 (a) and (b))** indicated that pure cellulase, Driselase, α-expansin and possibly β-expansin action can be detected using this method. However, these results need to be confirmed as the expansin extract may have been contaminated with cellulase, which could have been responsible for the release of ^3H_2O.

As β-expansins are believed to effect cell wall loosening and therefore cell expansion, inhibition of expansin action could result in a reduction of cell wall extension and therefore growth. Consequently, the primary mode of action of OAC may be to inhibit β-expansin action in maize roots or CSCs.

7. Discussion

Herbicides have been widely in use since the discovery of 2,4-D during the Second World War. Since then a broad range of herbicides with an array of biochemical sites of action have been marketed. Of all the major herbicides on the market those that inhibit amino acid synthesis, lipid biosynthesis, photosynthesis or carotenoid biosynthesis or act as auxin mimics corner ~10, 14, 28, 4 and 10%, respectively, of the market in comparison to <0.5% for herbicides that have been developed to inhibit cell-wall related processes such as cellulose biosynthesis (Worthing and Hance, 1991). These figures suggest that there is a huge market potential for cell wall-related herbicides.

As the cell wall controls the rate of cell expansion, (through a plethora of cell wall tightening and loosening mechanisms) a herbicide developed to interfere with cell wall loosening or tightening mechanisms could prove ideal at controlling or inhibiting weed growth. The Arabidopsis Genome Initiative (2000) reported that more than 420 genes have possible roles in the synthesis and modification of cell wall polymers including ~52 genes encoding putative polygalacturonases, 20 encoding putative pectate lyases, 79 encoding putative pectin esterases and 36 encoding putative expansins. These findings highlight the importance of the cell wall in plant growth and its involvement in other processes such as fruit ripening, indicating that there is great scope for developing herbicides that affect cell wall processes involved in promoting or restraining cell expansion. The differences in architecture between type I and II cell walls could be exploited as a basis for herbicide selectivity. Another significant advantage of developing a cell wall-related herbicide is that its mode of action may mean that it is less toxic to animals.

Aventis Crop Science U.K. Ltd. marketed OAC as a herbicide selective against graminaceous monocots. Amino acid synthesis, carotenoid biosynthesis, photosynthesis, lipid synthesis and cellulose synthesis did not appear to be inhibited by OAC (Dr. David Cole, personal communication). Therefore, the effect of the herbicide upon cell expansion was investigated. Regulation of cell expansion is dependent upon interplay between turgor pressure, cell wall loosening and cell wall

tightening. It was therefore possible that OAC inhibited cell expansion by effecting (a) reduced turgor pressure, (b) inhibition of cell wall loosening and/or (c) promotion of cell wall tightening.

Consequently, the effect of the herbicide upon factors effecting the promotion and regulation of cell expansion was investigated. This thesis provides new information about OAC and its possible mode of action.

7.1 Physiological effects of OAC upon maize CSCs and maize seedlings

It was important to establish the ID_{50} of OAC in CSCs and determine if there were any obvious physiological effects of the herbicide upon maize seedlings. The ID_{50} of OAC, estimated from a range of CSC ages, was ~ 7 nM. This is lower than the ID_{50} of the cellulose biosynthesis inhibitor DCB (400 nM) but comparable to that of triazofenamide (also a cellulose synthesis inhibitor) with an ID_{50} of 1 nM (Heim *et al.* 1998). A low ID_{50} is beneficial from an environmental perspective. Though the age or density of the CSC did not appear to influence the ID_{50} of OAC, a lower concentration of OAC was effective at inhibiting long-term growth of CSCs than the growth of CSCs that were already approaching stationary phase.

OAC significantly inhibited root growth in maize seedlings, to a degree which correlated positively with the period of exposure to OAC. Notably, coleoptile or mesocotyl growth was not inhibited by the herbicide. This could have been because the herbicide was not transported up to the shoots. However, even when excised coleoptiles were incubated with the herbicide, coleoptile growth remained uninhibited. It is more likely that coleoptiles were less susceptible to OAC because they did not contain a specific 'target' of OAC that is present in roots, e.g. an isoform of XET, expansin or peroxidase.

It was also important to determine whether OAC inhibited cell division and/or cell expansion, which could have been tested by counting cells treated with or without OAC. Several attempts were made to separate maize cells so that they could be

counted but none was successful. This was surprising as the treatments employed in attempts to separate the cells have been widely used either to chelate Ca^{2+} ions (EDTA), which cross-link pectin, to break glycosidic bonds (pectinase), or to extract pectin (NaOH), which is the main component of the middle lamella (Street, 1977; Fry, 1986; Knox *et al.*, 1990). These failed attempts at separating the cells shows that the exact nature of the middle lamella of maize CSCs is not completely known. Longitudinal sections of maize roots indicated that OAC inhibited cell elongation. Future work could include examination of OAC-treated and -untreated cells, to determine whether or not they undergo mitosis, to definitively rule out inhibition of cell division by OAC.

7.2 The effect of OAC upon turgor pressure in maize CSCs and roots

As turgor pressure plays an important role in cell expansion (Kutschera and Köhler, 1993) the effect of OAC upon turgor pressure was assessed. Turgor pressure could be affected by the impedance of water transport across plasma membranes in the cell. As the transport of water is believed to be facilitated by the presence of protein water-transport channels called aquaporins (Chrispeels and Maurel, 1994), an assay was developed to determine if the transport of water across the plasma membrane of CSCs was inhibited by OAC. The results indicated that OAC did not inhibit water transport out of (and thus presumably into) the cells. To prevent cell expansion a > 99% reduction in water transport into the cells would have been required. This makes inhibition of water transport by OAC a highly unlikely mode of action as the dramatic reduction in water transport required to prevent cell expansion would have been very easily detected. Also an effect of that magnitude would be visible in treated plants (wilting) though no such effect was observed. OAC did not appear to affect the osmotic pressure of maize root cells or the turgor pressure of maize CSC. These results suggested that the mode of action of OAC on cell expansion was more likely to involve inhibition of a cell wall loosening mechanism or the promotion of a cell wall tightening mechanism.

7.3 The effect of OAC upon cell wall tightening mechanisms

Cell wall tightening features in restraining cell expansion. OAC could have induced or promoted cell wall tightening, thereby preventing cell expansion. Peroxidase-catalysed cross-linking of diferulate and di-isodityrosine is believed to tighten the cell wall (Fry, 1983; Fry, 1987). However, OAC did not appear to promote peroxidase activity, action or secretion into the medium as would have been expected if OAC were promoting the peroxidase-catalysed formation of cross-links. OAC also did not promote the cross-linking of coumaroyl esters in the apoplast of maize CSCs. As the presence of extensin in type I primary cell walls has not been widely reported, it is unlikely that OAC inhibited expansion by promoting extensin cross-linking. Future work might include testing whether OAC enhanced the formation of Ca^{2+} ion cross-links in pectin by promoting the action, activity or secretion of methylesterase.

7.4 The effect of OAC upon cell wall loosening mechanisms

Cell wall loosening is an important feature of cell expansion. OAC did not appear to inhibit XET activity (believed to be involved in cell wall loosening (Fry *et al.*, 1992; Vissenberg *et al.*, 2000)) or its secretion into the apoplast. $^{\bullet}OH$ radicals are also believed to cause scission of polysaccharides and are therefore potentially capable of loosening the cell wall (Fry, 1998; Schweikert *et al.*, 2000; Fry *et al.*, 2000; Schopfer, 2001). $^{\bullet}OH$ can be generated via the Fenton reaction, which is promoted by the presence of ascorbate. The effect of OAC upon $^{\bullet}OH$ production was indirectly tested by determining whether OAC inhibited apoplastic ascorbate consumption by maize CSC; however, no effect was observed. $^{\bullet}OH$ can be generated via other mechanisms, which could be tested in the future to test the possibility that OAC prevents the formation of $^{\bullet}OH$.

7.4.1 Expansins

Expansin are proteins believed to effect cell wall loosening (by disrupting hydrogen bonding) (Cosgrove 1989; McQueen-Mason *et al.* 1992), as is yieldin, which has been reported to effect cell wall loosening at an acidic pH by lowering the yield threshold of the cell wall (Okamoto-Nakazato *et al.*, 2000). Consequently, the effect of OAC upon acid creep, believed to be mediated by either expansin or yieldin action, was assayed using methanol-killed maize silks, celery petiole vascular bundles and frozen, thawed maize roots. The results suggested that OAC inhibited extension of maize silks and roots but not celery petiole vascular bundles. However, repeats of these experiment did not consistently indicate that OAC significantly inhibited silk and root extension, though the extension measured in the majority of OAC-treated silks and roots was lower. The experiments were repeated under the same general conditions (similar pH, incubation period and force) but inconsistencies in the results may be due to an unknown variable inherent in either the plant material itself or its preparation.

The inhibition of extension by OAC in methanol-killed maize roots and silks could be an effect on either expansin or yieldin action. Both expansin and yieldin are pH-dependent and their action, i.e. ability to induce extension, can be assayed using the same method that was developed and used during this project. Therefore it was impossible to distinguish whether OAC was inhibiting expansin or yieldin action. As OAC did not inhibit acid creep of celery petiole vascular bundles it seemed likely that if OAC was inhibiting expansin action it would inhibit β- and not α-expansin action, because β-expansins have a higher activity in graminaceous monocots (Cosgrove *et al*, 1997). This could also explain the basis of the herbicide's selectivity against graminaceous monocots.

Consequently, the effect of OAC upon β-expansin action was assayed. As GIAs belong to the same family as β-expansins, pollen grains seemed a suitable source of β-expansins. β-Expansins are loosely bound to pollen grains and are therefore easily extractable. An extract from maize pollen, believed to contain β-expansins, was

added, with or without OAC, to boiled maize silks, which were then assayed for creep. The results suggested that OAC did inhibit β-expansin mediated extension.

Similarly, boiled maize roots and silks incubated with pollen extract and OAC showed a slower rate of creep than controls, as assayed using an extensiometer. However, yieldins are also relatively easy to extract so it is possible that along with β-expansin, yieldin was also extracted. This makes it difficult to determine whether OAC was inhibiting β-expansin or yieldin action. However, so far yieldin has only been reported to be extracted from cow pea hypocotyls and not pollen grains (Okamoto-Nakazoto *et al.*, 2000). To effect extension in cow pea hypocotyls yieldin needs to be pre-incubated with the GHCs overnight and Okamoto-Nakazoto *et al.* (2000) demonstrated that yieldin was unable to effect extension in GHCs if incubated for less than an eight-hour period. They noted that perfusion of pH 4 buffer containing yieldin into heat-denatured cow pea GHC did not effect extension. The method developed during this project to assay for expansin action included an overnight incubation period of silks with β-expansin extract; thus extension of the silks could be attributed to either β-expansin or yieldin action. However, the longest incubation period used during the assay for the effect of OAC upon the rate of creep of frozen, thawed, maize silks and roots incubated with β-expansin extract (assayed using an extensiometer) was ~ 30 min. As yieldin requires overnight incubation before being able to effect extension it seems likely that OAC was inhibiting the β-expansin action, at least in the extensiometer experiments.

Wu *et al.* (2000) reported that *ExpB7* is highly expressed in maize silks, roots and mesocotyls. This is interesting, as OAC has been shown to inhibit extension of frozen silks and of frozen and live roots, though no effect of the herbicide was observed in mesocotyls. The effect of OAC upon the action of ExpB1 (or GIA), reported to be expressed in pollen but not in vegetative tissue, on boiled maize silks and roots was assayed. However, it would be informative to test whether OAC inhibited the action of β-expansin (*ExpB7*; expressed in silks and roots) upon silks and roots, as this form of β-expansin may be more susceptible to OAC.

Other future work could include determining whether OAC binds to β-expansin. This could be achieved by passing radiolabelled OAC and β-expansin through a gel permeation column to determine whether or not they co-elute. It would also be interesting to determine if OAC inhibits β-expansin-mediated extension at concentrations of OAC where 50% cell growth is inhibited (~7 nM).

OAC had no effect upon the proteinase activity of papain, which is reported to belong to the β-expansin family (Grobe *et al.*, 1999). The proteinase activity of β-expansin has been disputed by Li and Cosgrove (2001) though it is possible that β-expansins are proteinases but that they effect cell wall loosening via a different mechanism.

A novel assay to detect expansin action was partially developed during this project as an alternative to the conventional assay(s) currently used. The alternative assay involved the radiolabelling of cellulose (Avicel) with ^3H (by incubating with ^3H_2O for a prolonged period at a high temperature) so that expansin action upon [*hydroxyl*-^3H]cellulose would result in the release of ^3H_2O. The amount of ^3H_2O released would reflect the effectiveness of expansin at disrupting hydrogen bonding. The results indicated that α-expansin and to some extent β-expansin action were detectable using this assay; however, the expansin extracts may have contained traces of cellulase which effected the release of ^3H_2O. The assay needs to be further developed to ascertain what concentrations of expansin are necessary to effect the formation of ^3H_2O. The assay proved to be effective at demonstrating both Driselase and pure cellulase action. Future work could also include testing whether OAC, if incubated in the presence of β-expansin and [*hydroxyl*-^3H]cellulose, released lower levels of ^3H_2O, than in the control.

This thesis has provided new information about OAC: it inhibits cell expansion — particularly in the root, it has a low ID_{50} and it starts to inhibit cell expansion 4–8 h after addition. This thesis also eliminates a range of cell-wall related processes involved in cell expansion as targets of OAC. A possible and plausible mode of

action for OAC has been suggested — OAC prevents cell expansion by inhibiting β-expansin-mediated cell wall extension.

Future work in this area could include focusing on the way in which OAC inhibits expansin action. As the mechanism by which β-expansin effects cell wall loosening is not known, understanding how OAC inhibits β-expansin action may enhance our understanding of β-expansin-mediated cell wall extension. Plant physiologists would also be able to inhibit β-expansin action, which could prove useful in deducing the role(s) of the protein in the life of a plant. OAC, which is currently used in paddy fields in Japan, is very effective at arresting graminaceous monocot growth. As OAC's mode of action is most likely to inhibit β-expansin action, I suggest that β-expansin action is fundamental to maize root growth.

8. References

AUBERT, D. and HERZOG, M. (1996) A new cDNA encoding a xyloglucan endo-transglycosylase-related polypeptide (AtXTR8) preferentially expressed in seedling, root and stem of *Arabidopsis thaliana*. *Plant Science*. **121**, 187–196

BALUSKA, F., SALAJ, J., MATHUR, J., BRAUN, M., JASPER, F., SAMAJ, J., CHUA, N.H., BARLOW, P.W. and VOLKMANN, D. (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Developmental Biology*. **227**, 618–632

BARLOW, P.W. (1987) The cellular organisation of roots and its response to the physical environment. *Root development and function* (Gregory, P.J., Lake, J.V. and Rose, D.A., eds). Cambridge University Press: Cambridge

BARRIEU, F., CHAUMONT, F. and CHRISPEELS, M.J. (1998) High expression of the tonoplast aquaporin *ZmTIP1* in epidermal and conducting tissues of maize. *Plant Physiology*. **117**, 1153–1163

BOUDET, A.M. (1998) A new view of lignification. *Trends in Plant Science*. **3**, 67–71

BOYER, J.S (1985) Water transport. *Annual Review of Plant Physiology*. **36**, 473–516

RALPH, J., LAPIERRE, C., MARITA, J.M., KIM, H., LU, F., HATFIELD, R.D., RALPH, S., CHAPPLE, C., FRANKE, R., HEMM, M.R., DOORSSELAERE, J.V., SEDEROFF, R.R., O'MALLEY, D.M., SCOTT, J.T., MacKAY, J.J., YAHIAOUI, N., BOUDET, A.M., PEAN, M., PILATES, G., JOUANIN, L. and BOERJAN, W. (2001) Elucidation of new structures in lignins od CAD- and COMT-deficient plants by NMR. *Phytochemistry*. **57**, 993–1003

BRADY, J.D. and FRY, S.C. (1997) Formation of di-isodityrosine and loss of isodityrosine in the cell walls of tomato cell-suspension cultures treated with fungal elicitors for H_2O_2. *Plant Physiology*. **115**, 87–92

BRADY, J.D., SADLER, I.H. and FRY, S.C. (1996) Di-isodityrosine, a novel tetrameric derivative of tyrosine in plant cell wall proteins: a new potential cross-link. *Biochemical Journal*. **315**, 323–327

BRADY, J.D., SADLER, I.H. and FRY, S.C. (1998) Pulcherosine, an oxidatively coupled trimer of tyrosine in plant cell walls: its role in cross-link formation. *Phytochemistry*. **47**, 349–353

BRADLEY, J.D., KJELLBOM, P. and LAMB, C. (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response. *Cell.* **70**, 21–30

BRAMLEY, P.M. (1991) *Target Sites for Herbicide Action* (Kirkwood, R.C., ed.). Plenum Press: New York.

BRETT, C. and WALDRON, K. (1996) *The Biochemistry of Plant Cell Walls.* Chapman & Hall: London.

BROWN, R.M., SAXENA, .M. and KUDLICKA, K. (1996) Cellulose biosynthesis in higher plants. *Trends in Plant Science.* **1**, 149–156

BRUMMELL, D.A. and HALL, J.L. (1987) Rapid cellular responses to auxin and the regulation of growth. *Plant, Cell and Environment.* **10**, 523–543

BURKE, D., KAUFMAN, P., McNEIL, M. and ALBERSHEIM, P. (1974) The structure of plant cell walls. VI. A survey of the walls of suspension-cultured monocots. *Plant Physiology.* **54**, 109–115

CADERAS, D., MUSTER, M., VOLGER, H., MANDEL, T., ROSE, J.K.C., McQUEEN-MASON, S. and KUHLEMEIER, C. (2000) Limited correlation between expansin gene expression and elongation growth rate. *Plant Physiology.* **123**, 1399–1413

CARPITA, N.C. (1983) Hemicellulosic polymers of cell walls of *Zea mays* coleoptiles. *Plant Physiology.* **72**, 515–521

CARPITA, N.C. (1984) Cell wall development in maize coleoptiles. *Plant Physiology.* **72**, 205–212

CARPITA, N.C. (1986) Incorporation of proline and aromatic amino acids into cell walls of maize coleoptiles. *Plant Physiology.* **80**, 660–666

CARPITA, N.C. (1996) Structure and biogenesis of the cell walls of grasses. *Annual Review of Plant Physiology and Plant molecular Biology.* **47**, 44–476

CARPITA, N.C. and GIBEAUT, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the wall during growth. *The Plant Journal.* **3**, 1–30

CARPITA, N.C. and KANABUS, J. (1988) Chemical structure of the cell walls of dwarf maize and changes mediated by gibberellin. *Plant Physiology.* **88**, 671–678

CASSAB, G.I. (1998) Plant cell wall proteins. *Annual Review of Plant Physiology and Plant Molecular Biology.* **49**, 281–309

CASTILLO, F. and GREPPIN, H. (1988) Extracellular ascorbic acid and enzyme activities related to ascorbic-acid metabolism in *Sedum album* leaves after ozone exposure. *Environmental and Experimental Botany.* **28**, 231–238

CHAUMONT, F., BARRIEU, F., HERMAN, E.M. and CHRISPEELS, M.J. (1998) Characterization of a maize tonoplast aquaporin expressed in zones of cell division and elongation. *Plant Physiology.* **117**, 1143–1152

CHO, H-T. and KENDE, H. (1997) Expansins in deepwater rice internodes. *Plant Physiology.* **113**, 1137–1143

CHRISPEELS, M.J and MAUREL, C. (1994) Aquaporins: The molecular basis of facilitated water movement through living plant cells? *Plant Physiology.* **105**, 9–13

CHRISPEELS, M.J, HOLUIGUE, L., LATORRE, R., LUAN, S., ORELLANA, A., PENA-CORTES, H., RAIKHEL, N.V., RONALD, P.C. and TREWAVAS, A. (1999) Signal transduction networks and the biology of plant cells. *Biological Research.* **32**, 35–60

CIVELLO, P.M., POWELL, A.L.T., SABEHAT, A. and BENNETT, A.B. (1999) An expansin gene expressed in ripening strawberry fruit. *Plant Physiology.* **121**, 1273–1279

COBB, A. (1992) *Herbicides and Plant Physiology.* Chapman and Hall: U.K.

COSGROVE, D.J. (1986) Biophysical control of plant cell growth. *Annual Review of Plant Physiology.* **37**, 377–405

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

COSGROVE, D.J. (1993a) How do plant cell walls extend? *Plant Physiology.* **102**, 1-6

COSGROVE, D.J. (1993b) Wall extensibility: its nature, measurement and relationship to plant cell growth. *New Phytologist.* **124**, 1–23

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

COSGROVE, D.J. (1997) Creeping walls, softening fruit, and penetrating pollen tubes: The growing roles of expansins. *Proceedings of the National Academy of Sciences of the United States of America.* **94**, 5504–5505

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

COSGROVE, D.J. (1998) Cell wall loosening by expansins. *Plant Physiology.* **118**, 333–339

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

COSGROVE, D.J. (2000a) Loosening of plant cell walls by expansins. *Nature.* **407**, 321–326

COSGROVE, D.J. (2000b) New genes and new biological roles for expansins. *Current Opinion in Plant Biology.* **3**, 73–78

COSGROVE, D.J. (2000c) Expansive growth of plant cell walls. *Plant Physiology and Biochemistry.* **38**, 109–124

COSGROVE, D.J. (2001) Wall structure and wall loosening. A look backwards and forwards. *Plant Physiology.* **125**, 131–134

COSGROVE, D.J., BEDINGER, P. and DURACHKO, D.M. (1997) Group I allergens of grass pollen as cell wall-loosening agents. *Proceedings of the National Academy of Sciences of the United States of America.* **94**, 6559–6564

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

COSGROVE, D.J., VAN VOLKENBURGH, E. and CLELAND, R.E. (1984) Stress relaxation of cell walls and the yield threshold for growth: Demonstration and measurement by micro-pressure probe and psychrometer techniques. *Planta.***162**, 46–52

DARVILL, J.E., McNEIL, M,. DARVILL, A.G. and ALBERSHEIM, P. (1980) Structure of plant cell walls XI. Glucuronoarabinoxylan, a second hemicellulose in primary cell walls of suspension-cultured sycamore cells. *Plant Physiology.* **66**, 1135–1139

DELMER, D.P. (1999) Cellulose Biosynthesis: Exciting times for a difficult field of study. *Annual Review of Plant Physiology and Plant Molecular Biology.* **50**, 245–276

DELMER, D.P., READ, S.M. and COOPER, G. (1987) Identification of a receptor protein in cotton fibres for the herbicide 2,6-dichlorobenzonitrile. *Plant Physiology.* **84**, 415–420

DELMER, D.P. and AMOR, Y. (1995) Cellulose biosynthesis. *American Society of Plant Physiologists.***7**, 987–1000

DEVINE, M.D., DUKE, S.O. and FEDTKE, C. (1993) *Physiology of herbicide action.* Prentice-Hall: U.S.A.

DODGE, R.C. (1991). *Target Sites for Herbicide Action* (Kirkwood, R.C., ed.). Plenum Press: New York.

DOERNER, P. (2000) Root patterning: Does auxin provide positional cues? *Current Biology.* **10**, 201–203.

DOWNES, B.P. and CROWELL, D.N. (1998) Cytokinin regulates response of the leaf-sheath pulvinus of oat (*Avena sativa*). *Plant Physiology.* **94**, 411-416

EPSTEIN, L. and LAMPORT, D.T.A. (1984) An intramolecular linkage involving isodityrosine in extensin. *Phytochemistry.* **23**, 1241–1246

FELDMAN, L. (1994) In *The Maize Handbook* (Freeling, M. and Walbot, V., eds.). Springer-Verlag: New York.

FISCHER, R.L. and BENNET, A.B. (1991) Role of cell wall hydrolases in fruit ripening. *Annual Review of Plant Physiology and Plant Molecular Biology.* **42**, 675–703

FOX, J.E. (1969) *Physiology of Plant Growth and Development* (Wilkins, M.B., ed.). McGraw-Hill: England.

FRANKHAUSER, C. and CHORY, J. (1997) Light control of plant development. *Annual Review in Cell Developmental Biology.* **13**, 203–229

FRY, S.C. (1979) Phenolic components of the primary cell wall and their possible rôle in the hormonal regulation of growth. *Planta.* **146**, 343–351

FRY, S.C. (1982) Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. *Biochemical Journal.* **204**, 449–455

FRY, S.C. (1982) Phenolic components of the primary cell wall: feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. *Biochemical Journal.* **203**, 493–504

FRY, S.C. (1983) Feruloyated pectins from the primary cell wall: their structure and possible functions. *Planta.* **157**, 111–123

FRY, S.C. (1984) Incorporation of [^{14}C]cinnamate into hydrolase-resistant components of the primary cell wall. *Phytochemistry.* **23**, 59–64

FRY, S.C. (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annual Review of Plant Physiology.* **37**, 165-186

FRY, S.C. (1987) Formation of isodityrosine by peroxidase isozymes. *Journal of Experimental Botany.* **38**, 853–862

FRY, S.C. (1988) *The Growing Plant Cell Wall: Chemical and Metabolic Analysis.* Longman Scientific and Technical: Essex

FRY, S.C. (1989) The structure and function of xyloglucan. *Journal of Experimental Botany.* **40,** 1–11

FRY, S.C. (1997) Novel 'dot blot' assays for glycosyltransferases and glycosylhydrolases: optimization for xyloglucan endotransglycosylase (XET) activity. *The Plant Journal.* **11,** 1141-1150

FRY, S.C. (1998) Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochemical Journal.* **332,** 507-515

FRY, S.C. (1999) Plant cell walls. In *Encyclopedia of Life sciences,* http://www.els.net, Nature Publishing Group: London (Also paper edition in press)

FRY, S.C., DUMVILLE, J. C. and MILLER, J.G. (2001) Fingerprinting of polysaccharides attacked by hydroxyl radicals *in vitro* and in the cell walls of ripening pear fruit. *Biochemical Journal.* **357,** 729–737

FRY, S.C., SMITH, R.C., RENWICK, K.F., MARTIN, D.J., HODGE, S.K. and MATTHEWS, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochemical Journal.* **282,** 821–828

FRY, S.C., WILLIS, S.C. and PATERSON, A.E.J. (2000) Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. *Planta.* **211,** 679–692

GIBEAUT, D.M., KARUPPIAH, N., CHANG, S.R., BROCK, T.G, VADLAMUDI, B., KIM, D., GHOSHEH, N.S., RAYLE, D.L., CARPITA, N.C. and KAUFMAN, P.B. (1990) Cell wall and enzyme changes during the graviresponse of the leaf-sheath pulvinus of oat (*Avena sativa*). *Plant Physiology.* **94,** 411–416

GRABBER, J.H., HATFIELD, R.D., RALPH, J., ZON. J. and AMRHEIN, N. (1995) Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry.* **40,** 1077–1082

GRABBER, J.H., RALPH, R. and HATFIELD, R.D. (2000) Cross-link of maize walls by ferulate dimerization and incorporation into lignin. *Journal of Agricultural Food Chemistry.* **48,** 6106–6113

GROBE, K., BECKER, W-M., SCLAAK, M. and PETERSEN, A. (1999) Grass group I allergens (β-expansins) are novel, papain-related proteinases. *European Journal of Biochemistry.* **263,** 33–40

HAGER, A., DEBUS, G., EDEL, H-G., STRANSKY, H. and SERRANO, R., (1991) Auxin induces exocytosis and the rapid synthesis of a high turnover pool of plasma membrane H^+- ATPase. *Planta.* **185**, 527–537

HALLIWELL, B. and GUTTERIDGE, J.M.C. (1999) *Free Radicals in Biology and Medicine*, 3rd edition. Clarendon Press: Oxford

HART, J.W. (1988) *Light and Plant Growth.* Unwin Hyman: London.

HARTLEY, R.D. and JONES, E.C. (1975) Diferulic acid as a component of cell walls of *Lolium multiflorum. Phytochemistry.* **15**, 1157–1160

HARTLEY, R.D. and FORD, C.W. (1989) *Plant Cell Wall Polymers* (Lewis, N.G. and Paice, M.G., eds.). American Chemical Society: U.S.A.

HARWOOD, J.L. (1991). *Target Sites for Herbicide Action* (Kirkwood, R.C., ed.). Plenum Press: New York

HATFIELD, R. and NEVINS, D.J. (1986) Purification and properties of an endo-glucanase isolated from the cell wall of *Zea mays* seedlings. *Carbohydrate Research.* **148**, 265–278

HAUSER, M-T., MORIKAMI, H. and BENFEY, P.N. (1995) Conditional root expansion mutants of Arabidopsis. *Development.***121**, 1237–1252

HAYASHI, T.A., TAKEDA, T., OGAWA, K and MITUISHI, Y. (1994) Effects of the degree of polymerisation on the binding of xyloglucans to cellulose. *Plant Cell Physiology.* **35**, 893–899

HAYASHI, T.A., WONG, Y.S. and MACLACHLAN, G. (1984) Pea xyloglucan and cellulose. II Hydrolysis by pea endo-1,4-β-glucanases. *Plant Physiology.* **75**, 606–610

HEIM, D.R, ROBERTS, J.L., PIKE, P.D. and LARRINUA, I.M. (1989) Mutation of a locus of *Arabidopsis thaliana* confers resistance to the herbicide isoxaben. *Plant Physiology.* **90**, 146–150

HEIM, D.R., LARRINUA, I.M, MURDOCH, M.G. and ROBERTS, J.L. (1998) Triazofenamide is a cellulose biosynthesis inhibitor. *Pesticide biochemistry and Physiology.* **59**, 163–168

HEIM, D.R., ROBERTS, J.L., PIKE, P.D. and LARRINUA, I.M. (1990) A second locus, Ixr B1 in *Arabidopsis thaliana*, that confers resistance to the herbicide isoxaben. *Plant Physiology.* **92**, 858–861

HEIM, D.R., SKOMP, J.R., WALDRON, C. and LARRINUA, I.M. (1991) Differential response to isoxaben of cellulose biosynthesis by wild type and resistant strains of *Arabidopsis thaliana*. *Pesticide Biochemistry and Physiology*. **39**, 93–99

HEUPEL, T. and KUTSCHERA, U. (1997) Cell number and organ size in developing sunflower hypocotyls. *Journal of Plant Physiology*. **151**, 379–381

HINNAWY, E.E. (1974) Chelating compounds as cell wall-loosening agents in cell suspension cultures of *Melilotus alba*, desr. *Zeitschrift fur Pflanzenphysiology und Bodenkunde*. 71, 207–219

HOOD, E.E., HOOD, K.R. and FRITZ, S.E (1991) Hydroxyproline-rich glycoproteins in cell walls of pericarp from maize. *Plant Science*. **79**, 13–22

HOSON, T. and MASUDA, Y. (1995) Concanavalin A inhibits auxin-induced elongation and breakdown of (1→3), (1→4)-β-D-glucans in segments of rice coleoptiles. *Plant Cell Physiology*. **36**, 517–523

INADA, S., TOMINAGA, M. and SHIMMEN, T. (2000) Regulation of root growth by gibberellin. *Plant Cell Physiology*. **41**, 657–665

ISHII, T. and MATSUNAGA, T. (2001) Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry*. **57**, 969–974

ISHII, T., MATSUNAGA, T., PELLERIN, P., O'NEILL, M.A., DARVILL, A. and ALBERSHEIM, P. (1999) The plant cell wall polysaccharide rhamnogalacturonan II self assembles into a covalently cross-linked dimer. *Journal of Biological Chemistry*. **274**, 13098–13104

JARVIS, M.C. (1984) Structure and properties of pectin gels in plant cell walls. *Plant Cell and Environment*. 7, 153–164

JARVIS, M.C. and McCANN, M.C. (2000) Macromolecular biophysics of the plant cell wall: concepts and methodology. *Plant Physiology and Biochemistry*. **38**, 1–13

JIKIHARA, K., (1997) MY-100 A new herbicide for pre- and early post-emergence barnyard grass control in rice. *Brighton Crop Protection Conference* pp 73–80.

JOHANSSON, I., KARLSSON, M., SHUKLA, V.K., CHRISPEELS, M.J., LARSSON, C. and KJELLBOM, P. (1998) Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. *The Plant Cell*. **10**, 451–459

JOSE-ESTANYOL, M. and PUIGDOMENECH, P. (2000) Plant cell wall glycoproteins and their genes. *Plant Physiology and Biochemistry*. **38**, 97–108

KAMISAKA, S., TAKEDA, S., TAKAHASHI, K. and SHIBATA, K. (1990) Diferulic and ferulic acid in the cell-wall of *avena* coleoptiles—their relationship to mechanical properties of the cell-wall. *Physiologa Plantarum.* **78**, 1–7

KATO, Y. and NEVINS, D.J. (1985) Isolation and identification of O-(5-O-feruloyl-α-L-arabinosyl)-(1→3)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose as a component of Zea shoot cell walls. *Carbohydrate Research.* **137**, 139–150

KEEGSTRA, K., KENNETH, W., TALMADGE, K.W., BAUER, W.D. and ALBERSHEIM, P. (1973) The structure of plant cell walls.III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiology.* **51**, 188–197.

KIELISZEWSKI, M.J., LEYKAM, J.F. and LAMPORT, D.E. (1990) Structure of the threonine-rich extensin from *Zea mays. Plant Physiology.* **92**, 316–326

KIMURA, S., LAOSINCHAI, W., ITOH, T., CUI, X., LINDER, C.R. and BROWN Jr, R.M. (1999) Immunogold labeling of rosette terminal cellulose-synthesising complexes in the vascular plant *Vigna angularis. The Plant Cell.***11**, 2075–2085

KIRKWOOD, R.C. (1991) *Target Sites for Herbicide Action.* Plenum Press: New York.

KLINGMAN, G.C. and ASHTON, F.M. (1982) *Weed Sciences, Practices & Principles.* Wiley-Interscience: Canada.

KNOX, J.P, LINSTEAD, P.J., KING, J., COOPER, C. and ROBERTS, K. (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta.* **181**, 261–272

KOBAYASHI, M., MATOH, T. and AZUMA, J. (1996) Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiology.* **110**, 1017–1020

KOO, S.J., NEAL, J.C. and DiTOMASO, J.M. (1996) 3,7-Dichloro-quinolinecarboxylic acid inhibits cell-wall biosynthesis in maize roots. *Plant Physiology.* **112**, 1383–1389

KUTSCHERA, U. (2000) Review: Cell expansion in plant development. *R.Bras.Fisiol.Veg.* **1**, 65–95

KUTSCHERA, U. and KÖHLER, K. (1993) Turgor pressure and elongation growth in developing sunflower hypocotyls. *Plant Physiology.* **141**, 757–758

KUTSCHERA, U. and KÖHLER, K. (1994) Cell elongation, turgor and osmotic pressure in developing sunflower hypocotyls. *Journal of Experimental Botany.* **45**, 591–595

KUTSCHERA, U. and SCHOPFER, P. (1985) Evidence against the acid-growth theory of auxin action. *Planta.* **163**, 483–493.

LABAVITCH, J.M. and RAY, P.M. (1978) Structure of hemicellulosic polysaccharides of *Avena sativa* coleoptile cell walls. *Phytochemistry.* **17**, 933–937

LAWLOR, D.W. (1993) *Photosynthesis,* 2nd edition. Longman Scientific and Technical: Hong Kong

LEI, M. and WU, R. (1991) A novel glycine-rich cell wall protein gene in rice. *Plant Molecular Biology.* **16**, 187–198

LEOPOLD, A.C. and KRIEDEMANN, P.E. (1975) *Plant Growth and Development.* McGraw-Hill, INC: U.S.A.

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

LI, Z-C., DURACHKO, D.M. and COSGROVE, D.J. (1993) An oat coleoptile wall protein that induces wall extension *in vitro* and that is antigenically related to a similar protein from cucumber hypocotyls. *Planta.* **191**, 349–356

LÖBLER, M. and KLÄMBT, D. (1985) Auxin-binding protein from coleoptile membranes of corn (*Zea-mays*-L). 2. Localisation of a putative auxin receptor. *Journal of Biological Chemistry.* **260**, 9854–9859

LOCKHART, J.A. (1959) A new method for the determination of osmotic pressure. *American Journal of Botany.* **46**, 704–708

LOCKHART, J.A. (1965) *Cell extension*: Plant Biochemistry. Academic Press: New York and London

LUTTENGER, D.G. and NEVINS, D.J. (1985) Transient nature of a (1→3),(1→4)-β-D-glucan in *Zea mays* coleoptile walls. *Plant Physiology.* **77**, 175–178

LYNDON, R.F. (1990) *Plant Development*: The Cellular Basis (Black, M. and Chapman, J., eds.). Cambridge University Press: U.K.

MACLEOD (1991) *Plant roots: The hidden half.* Marcel Dekker, INC.

MAUREL, C. (1997) Aquaporins and water permeability of plant membranes. *Annual Review in Plant Physiology and Plant molecular Biology.* **48**, 399–429

MATSUKURA, C., ITOH, S-I., NEMOTO, K., TANIMOTO, E. and YAMAGUCHI, J. (1998) Promotion of leaf sheath growth by gibberellic acid in a dwarf mutant of rice. *Planta.* **205**, 145–152

MATHEWS, C.K. and VAN HOLDE, K.E. (1995) *Biochemistry*, 2nd edition. Benjamin Cummings, INC: U.S.A.

McCANN, M.C. and ROBERTS, K. (1991) *The Cytoskeletal Basis of Plant Growth and Form.* Academic Press: London and New York.

McCANN, M.C., WELLS, B. and ROBERTS, K. (1990) Direct visualization of cross-links in the primary plant cell wall. *Journal of Cell Science.* **96**, 323–334

McNEIL, M., DARVILL, A.G., FRY, S.C. and ALBERSHEIM, P. (1984) Structure and function of the primary cell wall of plants. *Annual Review of Biochemistry.* **53**, 625–663

McQUEEN-MASON, S. (1995) Expansins and cell wall expansion. *Journal of Experimental Botany.* **46**, 1639–1650

McQUEEN-MASON, S. and COSGROVE, D.J. (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Plant Biology.* **91**, 6574–6578

McQUEEN-MASON, S. and COSGROVE, D.J. (1995) Expansin mode of action on cell walls. *Plant Physiology.* **107**, 87–100

McQUEEN-MASON, S., DURACHKO, D.M. and COSGROVE, D.J. (1992) Two endogenous proteins that induce cell wall extension in plants. *The Plant Cell.* **4**, 1425–1433

McQUEEN-MASON, S.J., FRY, S.C., DURACHKO, D.M. and COSGROVE, D.J. (1993) The relationship between xyloglucan endotransglycosylase and in-vitro cell wall extension in cucumber hypocotyls. *Planta.* **190**, 327–331

MONTAGUE, M.J. (1995) Gibberellic acid promotes growth and cell wall synthesis in Avena internodes regardless of the orientation of cell expansion. *Physiologia Plantarum.* **94**, 7–18

MONTEZINOS, D. and DELMER, D.P. (1980) Characterization of inhibitors of cellulose synthesis in cotton fibers. *Planta.* **148**, 305–311

MONRO, J.A., PENNY, D. and WELLESLEY BAILEY, R. (1976) The organization and growth of primary cell walls of lupin hypocotyl. *Phytochemistry.* **15**, 1193–1198

MOORE, T (1979) *Biochemistry and physiology of plant hormones.* Springer-Verlag, INC: New York

MOUSDALE, D.M. AND COGGINS, J.R. (1991). T*arget Sites for Herbicide Action* (Kirkwood, R.C., ed.). Plenum Press: New York.

MUDAY, G.K. and DeLONG, A. (2001) Polar auxin transport: controlling where and how much. *Trends in Plant Science.* **6**, 635–642

MURASHIGE, T. and SKOOG, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Planatarum.* **15**, 473–497

MÜSEL, G., SCHINDLER, T., BERGFELD, R., RUEL, K., JACQUET, G., LAPIERRE, C., SPETH, V. and SCHOPFER, P. (1997) Structure and distribution of lignin in primary and secondary cell walls of maize coleoptiles analyzed by chemical and immunological probes. *Planta.* **201**, 146–159

NAKAGAWA, N. and SAKURAI, N. (1998) Increase in the amount of celA1 protein present in tobacco BY-2 cells by a cellulose biosynthesis inhibitor, 2,6-dichlorobenzonitrile. *Plant Cell Physiology.* **39**, 779–785

NOBEL, P.S. (1991) *Physicochemical and Environmental Plant Physiology.* Academic Press: San Diego.

OHSHIMA, Y., IWASAKI, I., SUGA, S., MURAKAMI, M., INOUE. and MAESHIMA, M. (2001) Low aquaporin content and low osmotic water permeability of the plasma and vacuolar membranes of a CAM plant *Graptopetalum paraguayense*:comparison with radish. *Plant Cell Physiology.* **42**, 1119–1129

OKAMOTO-NAKAZATO, A., NAKAMURA, T. and OKAMOTO, H. (2000) The isolation of wall-bound proteins regulating yield threshold tension in glycerinated hollow cylinders of cowpea hypocotyl. *Plant Cell and Environment.* **23**, 145–154

OKAMOTO-NAKAZATO, A., TAKAHASHI, K., KATOH-SEMBA, R. and KATOU, K. (2001) Distribution of yieldin, a regulatory protein of the cell wall yield threshold, in etiolated cowpea seedlings. *Plant Cell Physiology.* **42**, 952–958

OKAMOTO-NAKAZATO, A., TAKAHASHI, K., KIDO, N., OWARBIE, K. and KATOU, K (2000) Molecular cloning of yieldins regulating the yield threshold of cowpea cell walls: cDNA cloning and characterisation of recombinant yieldin. *Plant Cell and Environment.* **23**, 155–164

O'NEIL, M.A., EBERHARD, S., ALBERSHEIM, P. AND DARVIL, A.G. (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth.*Science*. **294**, 846–849

PEAR, J.R., KAWAGOE, Y., SCHRECKENGOST, W.E., DELMER, D.P. and STALKER, D.M. (1996) Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunits of cellulose synthase. *Proceedings of the National Academy of Sciences of the United States of America*. **93**, 12637–12642

PENA, M.J., ZARRA, I. and REVILLA, G. (1999) Autolysis promotes the extension capacity of *Zea mays* coleoptile cell walls in response to acid pH solutions. *Plant Cell Physiology*. **40**, 565–570

PERRIN, R., WILKERSON, C. and KEEGSTRA, K. (2001) Golgi enzymes that synthesize plant cell wall polysaccharides: finding and evaluating candidates in the genomic era. *Plant Molecular Biology*. **47**, 115–130

PERRONE, P., HEWAGE, C.M., SADLER, I. and FRY, S.C. (1998) N^{α}- and N^{ε}-D-galacturonoyl-L-lysine amides: properties and possible occurrence in plant cell walls. *Phytochemistry*. **49**, 1879–1890

PETERS, S.W., RICHTER, U. and FELLE, H.H. (1992) Auxin-induced H^{+}-pump stimulation does not depend on the presence of epidermal cells in corn coleoptiles. *Planta*. **186**, 313–316.

POTTER, I. and FRY, S.C. (1993) Xyloglucan endotransglycosylase activity in pea internodes. *Plant Physiology*. **103**, 235–241

PRESTON, G.M., CARROLL, T.P., GUGGINO, W.B., and AGRE, P. (1992) Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science*. **256**, 385–387

PRESTON, R.D (1982). The case for multinet growth in growing walls of plant cells. *Planta*. **155**, 356–363

PRITCHARD, J., HETHERINGTON, R., FRY, S.C. and TOMOS, A.D. (1993) Xyloglucan endotransglycosylase activity, microfibril orientation and the profiles of cell wall properties along growing regions of maize roots. *Journal of Experimental Botany*. **44**, 1281–1289

PRITCHARD, J., WYN JONES, R.G. and TOMOS, A.D. (1990) Measurement of yield threshold and cell wall extensibility of intact wheat roots under different ionic, osmotic and temperature treatments. *Journal of Experimental Botany*. **41**, 669–675

QI, X.Y., BEHERENS, B.X., WEST, P.R. and MORT, A.J. (1995) Solubilisation and partial characterisation of extensin fragments from cell walls of cotton suspension cultures. *Plant Physiology*. **108**, 1691–1701

RADLEY, M. (1956) Occurrences of substances similar to gibberellic acid in higher plants. *Nature.* **178**, 1070–1071

RAYLE, D.L (1973) Auxin-induced hydrogen-ion excretion in *Avena* coleoptiles and its implications. *Planta.* **114**, 63–73

RAYLE, D.L and CLELAND, R.E. (1977) Control of plant cell enlargement by hydrogen ions. *Current Topic Development Biology.* **11**, 187–214

RAYLE, D.L. and CLELAND, .R.E. (1992) The acid theory of auxin-induced cell elongation is alive and well. *Plant Physiology.* **99**, 1271–1274

REIDY, B., McQUEEN-MASON, S., NÖSBERGER, J. and FLEMING, A. (2001) Differential expression of α- and β- expansin genes in the elongating leaf of *Festuca pratensis. Plant Molecular Biology.* **46**, 491–504.

ROELOFSEN, P.A. and HOUWINK, A.L. (1953) Architecture and growth of the primary wall in some plant hairs and in the *Phycomyces* sporangiophore. *Acta Botanica Neerlandica.* **2**, 218–225

ROSE, J.K.C., LEE, H.H and BENNETT, A.B. (1997) Expression of a divergent expansin gene is fruit specific and ripening regulated. *Proceedings of the National Academy of Sciences of the United States of America.* **94**, 5955–5960

SABBA, R.P. and VAUGHN, K.C. (1999) Herbicides that inhibit cellulose biosynthesis. *Weed Science.* **47**, 757–763

SALIBURY, D. and ROSS, G. (1992) *Plant Physiology,* 4th edition. Wiley: London.

SCALBERT, A., MONTIES, B., LALLEMAND, J-Y., GUITTET, E. and ROLANDO, C. (1985) Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochemistry.* **24**, 1359–1362

SCHOPFER, P. (1989) pH-dependence of extension growth in *Avena* coleoptiles and its implications for the mechanism of auxin action. *Plant Physiology.* **90**, 202–207.

SCHOPFER, P. (1996) Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles. *Planta.* **199**, 43–49

SCHOPFER, P. (2001) Hydroxyl radical-induced cell-wall loosening in vitro and in vivo: implications for the control of elongation growth. *The Plant Journal.* **28**, 679–688

SCHULTZ, C., GILSON, P., OXLEY, D., YOUL, J. and BACIC, A. (1998) GPI-anchors on arabinogalactan-proteins: implications for signalling in plants. *Trends in Plant Science.* **3**, 1360–1385

SCHWEIKERT, C., LISZKAY, A. and SCHOPFER, P. (2000) Scission of polysaccharides by peroxidase-generated hydroxyl radicals. *Phytochemistry.* **53**, 565–570

SHARPLES, K.R., HAWKES, T.R., MITCHELL, G., EDWARDS, L.S., LANGFORD, M.P., LANGTON, D.W., ROGERS, K.M., TOWNSON, J.K. and WANG, Y. (1998) A novel thiazolidinone herbicide is a potent inhibitor of glucose incorporation into cell wall material. *Pesticide Science.* **54**, 368–376

SHOWALTER, A.M. (1993) Structure and function of plant cell wall proteins. *The Plant Cell.* **5**, 9–23

SMITH, R.C., MATTHEWS, R., SCHUNMAN, P. and CHANDLER, P. (1996) The regulation of leaf elongation and xyloglucan endotransglycosylase by gibberellin in 'Himalaya' barley. *Journal of Experimental Botany.* **47**, 1395–1404

STEVENSON, T.T., DARVILL, A.G. and ALBERSHEIM, P. (1988) Structural features of the plant cell-wall polysaccharide rhamnogalacturonan-II. *Carbohydrate Research.* **182**, 207–226

STREET, H.E. (1977) *Plant tissue and cell culture,* 2nd edition. Blackwell: Oxford

TAIZ, L. (1984) Plant cell expansion: Regulation of cell wall mechanical properties. Annual review *Plant Physiology.* **35**, 585–657

TAIZ, L. and ZEIGER, E. (1998) *Plant Physiology,* 2nd edition. Sinhauser Associates, INC: U.S.A

TAKAHAMA, U. and ONIKI, T. (1992) Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. *Plant Cell Physiology.* **33**, 379–387

TALMADGE, K.T., KEEGSTRA, K., BAUER, W.D. and ALBERSHEIM, P. (1973) The structure of plant cell walls: I, The macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of pectic components. *Plant Physiology,* **51**, 158–173

TAN, K.S., HOSON, T., MASUDA, Y. and KAMISAKA, S. (1991) Correlation between cell wall extensibility and the content of diferulic and ferulic acids in cell walls of *Oryza sativa* coleoptiles grown under water and in air. *Physiologa Planatarum.* **83**, 397–403

TAN, K.S., HOSON, T., MASUDA, Y. and KAMISAKA, S. (1992) Involvement of cell wall-bound diferulic acid in light-induced decrease in growth rate and cell wall extensibility of *Oryza* coleoptiles. *Plant Cell Physiology.* **33**, 103–108

THE ARABIDOPSIS GENOME INITIATIVE (2000) Analysis of the flowering plant *Arabidopsis thaliana*. *Nature*. **408**, 796–813

THOMPSON, J.E. and FRY, S.C. (2001) Restructuring of wall-bound xyloglucan by transglycosylation in living plant cells. *The Plant Journal*. **26**, 23–34

THIMANN, K.V. (1969) *Physiology of Plant Growth and Development* (Wilkins, M.B., ed.). McGraw-Hill: England.

TSUGE, T., TSUKAYA, H. and UCHIMIYA, H. (1996) Two independent and polarized processes of cell elongation regulate leaf blade expansion in Arabidopsis thaliana (L.) Heynh. *Development*. **122**, 1589–1600

TURNER, S.R., TAYLOR, N. and JONES, L. (2001) Mutations of the secondary wall. *Plant Molecular Biology*. **47**, 209–219

VALENT, B.S. and ALBERSHEIM, P. (1974) The structure of plant cell walls. *Plant Physiology*. **54**, 105–108

VAN VOLKENBURGH, E. and CLELAND, R.E (1984) Control of leaf growth by changes in cell wall properties. *Plant Physiology*. **15**, 25–28

VINKEN, J.P, DEKEIZER, A., BELDMAN, G. and VORAGEN, A.G.J (1995) Fractionation of xyloglucan fragments and their interaction with cellulose. *Plant Physiology*. **108**, 1579–1585

VISSENBERG, K., FRY, S.C. and VERBELEN, J-P. (2001) Root hair initiation is coupled to a highly localised increase of xyloglucan endotransglycosylase action in Arabidopsis roots. *Plant Physiology*. **127**, 1125–1135

VISSENBERG, K., MARTINEZ-VILCHEZ, I.M., VERBELEN, J-P, MILLER, J.G. and FRY, S.C. (2000) In vivo colocalisation of xyloglucan endotransglycosylase activity and its donor substrate in the elongation zone of Arabidopsis roots. *The Plant Cell*. **12**, 1229–1237

WALKER, C.D. and WEBB, J. (1981) *In, Copper in Soils and Plants* (Loneragan, J.F., Robson, A.D. and Graham, RD., eds.) Academic Press: Sydney.

WALLACE, G. and FRY, S.C. (1995) In vitro peroxidase-catalysed oxidation of ferulic acid esters. *Phytochemistry*. **39**, 1293–1299

WALLACE, G. and FRY, S.C. (1999) Action of diverse peroxidases and laccases on six cell wall-related phenolic compounds. *Phytochemistry*. **52**, 769–773

WENDE, G. and FRY, S.C. (1997) 2-*O*-β-D-Xylopyranosyl-(5-*O*-feruloyl)-L-arabinose, a widespread component of grass cell walls. *Phytochemistry*. **44**, 1019–1030

WEYERS, J.D. and PATERSON, N.W. (2001) Plant hormones and the control of physiological processes. *New Phytologist.* **152**, 375–407

WHITNEY, S.E.C., GIDLEY, M.J. and McQUEEN-MASON. (2000) Probing expansin action using cellulose/hemicellulose composites. *The Plant Journal.* **22**, 327–334

WORTHING, C.R. and HANCE, R.J. (1991) *The Pesticide manual: a world compendium.* British Crop Protection Council: Farnham.

WU, Y., MEELEY, R.B. and COSGROVE, D.J. (2001) Analysis and expression of α-expansin and β-expansin gene families in maize. *Plant Physiology.* **126**, 222–232

WU, Y., SHARP, R.E., DURACHKO, D.M. and COSGROVE, D.J. (1996) Growth maintenance of the maize primary root at low water potentials involves increases in cell-wall extension properties, expansin activity and wall susceptibility to expansins. *Plant Physiology.* **111**, 765–772

WU, Y., Meeley, R.B. and COSGROVE, D.J. (2001a) Analysis and expression of the α-expansin and β-expansin gene families in maize. *Plant Physiology.* **126**, 222–232

WU, Y., THORNE, E.T., SHARP, R.E. and COSGROVE, D.J. (2001b) Modification of expansin transcript levels in the maize primary root at low water potentials. *Plant Physiology.* **126**, 1471–1479

XU, W., PURUGGANAN, M.M., POLISENSKY, D.H., ANTOSIEWICZ, D.M., FRY, S.C. and BRAAM, J. (1995) Arabidopsis TCH4, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. *The Plant Cell.* **7**, 1555–1567.

ZHANG, N. and HASENSTEIN, K.H. (2000) Distribution of expansins in graviresponding maize roots. *Plant Cell Physiology.* **41**, 1305–1312

ZIMDAHL, R.D. (1993) *Fundamentals of Weed Science.* Academic Press, INC: U.S.A

ZUREK, D. and CLOUSE, S. (1994) Molecular-cloning and characterization of a brassinosteroid-regulated gene from elongating soybean (glycine max L) epicotyls. *Plant Physiology.* **104**, No.1, 161–170

9. Conference Abstracts

Poster presentation: Scottish Cell Wall Group meeting 30–31st March 1999 (Glasgow University and Paisley University)

Determination of the effects of a novel herbicide on cell wall expansion

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Introduction Oxaziclomefone [IUPAC: 3-(1-(3,5-dichlorophenyl)-1-methylethyl)-2,3-dihydro-6-methyl-5-phenyl-4H-1,3-oxazin-4-one] is a new herbicide discovered by Rhône Poulenc, which is very effective in controlling *Echinochloa* sp., a weed which is particularly prevalent in paddy rice fields. *Echinochloa* is a graminaceous monocot which is very difficult to control owing to its high percentage seed settling and germination. Oxaziclomefone has a favourable residual activity, a wide application window, but no carry over effects into following crops such as wheat, barley, Chinese cabbage, radish and onion. The target site of the herbicide has not yet been identified [1], though it is believed to inhibit meristem growth in a manner dissimilar from any known herbicides. The aim of this project is to elucidate the mode of action of oxaziclomefone, commencing with the determination of its effects on cell expansion in maize cell suspension culture.

Methods Maize cell suspension cultures, of different ages, were incubated in a range of concentrations of oxaziclomefone for a period of 6 days. Cell growth was then estimated using settled cell volume (SCV) and packed cell volume (PCV). Determination of SCV and PCV is not an exact method of quantifying cell expansion, as cells may change in shape or density, causing cells to pack differently, thereby giving an inaccurate impression of cell expansion. Therefore, an alternative assay is being developed to determine total extra-protoplasmic volume. A 1 day old maize cell suspension culture was adjusted to an SCV of ~70% and dispensed at 40 ml per flask. After an hour 100 µl of [^{14}C]mannitol solution (containing7.28 mg of 'cold' mannitol) was added to each flask. At 480 minutes oxaziclomefone (dissolved in 20 µl DMSO) was added to one of the flasks to give a final concentration of 100 nM. At the same time 20 µl of DMSO was added to a second flask to act as a control. At intervals throughout the experiment, 2.5 ml was removed from each flask and filtered. About 1 ml of filtrate was removed, weighed to get its exact volume and assayed for ^{14}C. The increase in intra-protoplasmic volume can be ascertained by calculating the difference between the initial and final concentration of [^{14}C]mannitol in the filtrate. This will indicate whether the herbicide is affecting cell growth by preventing cell expansion.

Results Calculated SCVs and PCVs indicate that oxaziclomefone is a potent inhibitor of plant cell growth even at low concentrations. Doses giving 50% maximal effect were typically ~ 4 nM. Experimental work concerning the [^{14}C]mannitol assay will be presented.

Acknowledgements: This research is supported by a BBSRC CASE studentship from Rhône-Poulenc Agriculture Ltd. Thanks to S.C.Fry, S.K Miller and D.J Cole for advice.

[1] JIKIHARA, K., et al (1997) MY-100 A new herbicide for pre- and early post-emergence barnyard grass control in rice. *Brighton Crop Protection Conference pp 73 - 80.*

Seminar presentation: Scottish Cell Wall Group meeting 10th April 2001 (Stirling University)

The novel herbicide oxaziclomefone: a specific inhibitor of wall extension?

Nick O'Looney The Edinburgh Cell Wall Group, ICMB, The University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JH, UK.

Background Oxaziclomefone (OAC) [IUPAC: 3-(1-(3,5-dichlorophenyl)-1-methylethyl)-2,3-dihydro-6-methyl-5-phenyl-4H-1,3-oxazin-4-one] is a new herbicide [1] recently released on to the market by Aventis, in Japan. OAC is very effective in controlling graminaceous monocots, and relatively ineffective on most dicots. Cell expansion is strongly inhibited in grasses. The aim of the project is to determine the primary mode of action of OAC, which is believed to be cell wall-related.

Results and discussion OAC inhibited the growth of maize cell suspension cultures but the treated cells did not change in colour and remained viable for at least a week. The ID_{50} (the concentration at which growth was 50% inhibited) of OAC in 3-day-old cultures was ~6 nM as estimated by cell volume measurements. In etiolated maize seedlings watered with saturated OAC (478 nM), root elongation was strongly inhibited whereas mesocotyl and coleoptile growth were slightly promoted.

OAC did not affect the turgor pressure of the cultured cells as calculated from the osmotic pressures (assayed using a depression of freezing point osmometer) of the cell sap and culture medium. Turgor pressure was also unaffected in maize roots as estimated by measuring the point of incipient plasmolysis. OAC did not affect the activity of extracellular peroxidase (assayed on *o*-dianisidine *in vitro*) from cultured maize cells. In addition, OAC did not affect the secretion of peroxidase into the culture medium. OAC also did not influence either the activity of maize XET assayed *in vitro* or the secretion of XET into the culture medium. The consumption of ascorbate, another potential wall loosening factor, measured by titration with DCPIP in cell cultures, was also unaffected by OAC.

However, OAC did appear too inhibit expansin activity, assayed by stretching (8 g wt) methanol-killed, rehydrated maize silks at pH 4 or pH 7, with or without OAC overnight. The increase in (relaxed) length of the silk due to stretching was recorded.

Conclusions OAC did not affect turgor pressure (the driving force of cell expansion), activity or secretion of peroxidase (a potential wall-tightening factor), activity or secretion of XET (a potential wall-loosening factor), and ascorbate consumption. It may be a specific inhibitor of expansin-mediated cell wall loosening.

Acknowledgements: This research is supported by a BBSRC CASE studentship from Aventis. Thanks to S.C.Fry and D.J Cole for advice. Thanks to W. Fricke for use of the osmometer.

[1] JIKIHARA, K., et al (1997) MY-100 A new herbicide for pre- and early post-emergence barnyard grass control in rice. *Brighton Crop Protection Conference pp 73 - 80.*

Poster presentation: 9th International Cell Wall Group Meeting 2–7th September 2001 (Toulouse, France).

The novel herbicide oxaziclomefone: a specific inhibitor of wall extension

Nick O'Looney and Stephen C. Fry, *The Edinburgh Cell Wall Group, ICMB, The University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh, EH9 3JH*

Background *Oxaziclomefone (OAC) [IUPAC: 3-(1-(3,5-dichlorophenyl)-1-methylethyl)-2,3-dihydro-6-methyl-5-phenyl-4H-1,3-oxazin-4-one] is a new herbicide [1] released on to the market by Aventis, in Japan. OAC is very effective in controlling graminaceous monocots, and relatively ineffective on most dicots. Cell expansion is strongly inhibited in grasses. The aim of the present work was to determine the primary mode of action of OAC, which is believed to be cell wall-related.*

Results and discussion In etiolated maize seedlings watered with a saturated OAC solution (478 nM), root elongation was strongly inhibited whereas mesocotyl and coleoptile growth were slightly promoted. Longitudinal sections of maize roots showed that OAC did not primarily affect cell division but strongly suppressed cell elongation. It also inhibited cell expansion in maize cell-suspension cultures at an estimated ID_{50} (concentration at which growth was 50% inhibited) of ~6 nM. An inhibitor of cell expansion could in principle act by (a) decreasing turgor pressure, (b) promoting wall-tightening, or (c) inhibiting wall-loosening. OAC did not affect the turgor pressure of cultured maize cells, as estimated from the osmotic pressures (assayed from depression of freezing point) of the cell sap and culture medium. OAC did not affect the *in vitro* activity of extracellular peroxidase (assayed on *o*-dianisidine) from cultured maize cells. OAC did not affect the consumption of ascorbate, as measured by titration with DCPIP, in cell cultures. In addition, OAC did not affect the secretion of peroxidase activity into the culture medium. The activity of maize xyloglucan endotransglycosylase (XET) assayed *in vitro* and the secretion of XET activity into the culture medium were also unaffected by the presence of OAC. However, OAC did inhibit expansin-like activity, assayed by stretching (8 g weight) methanol-killed, re-hydrated maize silks and roots at pH 4 or pH 7, with or without OAC for 18 h. The irreversible increase in length of the silk due to stretching was recorded.

Conclusions OAC did not affect cell division, turgor pressure (the driving force for cell expansion), the activity or secretion of peroxidase (a potential wall-tightening factor), the activity or secretion of XET (a potential wall-loosening factor), and the consumption of ascorbate (a potential wall-loosening factor). It may be a specific inhibitor of expansin-mediated wall loosening.

Acknowledgements: This research was supported by a BBSRC CASE studentship in collaboration with Aventis. Thanks to Dr. D.J Cole for advice.

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Seminar presentation: Scottish Cell Wall Group meeting 10th April 2001 (Stirling University)

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