EVALUATION OF CELL WALL COMPONENTS AS POTENTIAL RECOGNITION FACTORS IN SOLANACEOUS GRAFTS USING A MODEL SYSTEM.

BY

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DECLARATION

I declare that the composition and substance of this thesis are my own work except where otherwise stated.

ELIZABETH WADE-ROYLE, 1994

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DEDICATION

For Nick; the best result of these Ph. D. years.

"It is the glory of God to conceal a matter;

to search out a matter is the glory of kings."

Proverbs 25^{v2}, N.I.V.

ABSTRACT

The *Nicandra physaloides/Lycopersicon esculentum* heterograft consistently fails to produce functional vascular reconnections across the graft union (GU). Evidence supplied by earlier workers culminated in the hypothesis that a recognition system may exist between stock and scion (Yeoman, 1984). Thus incompatibility might be attributed to the absence of a compatibility signal, or to the receipt of an incompatibility signal (Parkinson, Jeffree, and Yeoman, 1987). Recent evidence suggests that the putative recognition factor(s) is/are located on the surface of callus cells (which are produced into the GU from the cut faces of stock and scion), possess limited mobility and reside in the insoluble fraction of the cells (Jeffree, Yeoman, Parkinson and Holden, 1987).

The postulated recognition system has at least two control points in the development of a compatible graft, i.e.

[1] the cessation of callus cell proliferation in the GU (Holden, Jeffree and Yeoman, 1987), and

[2] the differentiation of callus cells into xylem and phloem wound vessel members (WVMs) organised to form functional conduits across the GU only in compatible grafts.

Incompatible combinations do not exhibit stage [1], but proceed directly to stage [2], and produce disorganised arrangements of WVMs which form no functional re-connections. Therefore it appears that stage [1] is a prerequisite for the ordered and successful recombination of the stock and scion vascular systems.

This thesis documents attempts to identify the stage [1] "off" signal for cell division in a model GU provided by actively-dividing suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* cv. Ailsa Craig (*L. esculentum* AC) by monitoring protein synthesis. The application of deproteinated cell walls of *N. physaloides*, *L. esculentum* AC, and *L. esculentum* x *peruvianum* consistently inhibited incorporation of [^{14}C] leucine into protein, as did hemicelluloses from all sources at pH 4.5. The effects of hemicelluloses were found to be pH-dependant. No consistent effects could be established when pectins or pectic fragments were added to the cells.

pH measurements of apoplastic fluid from the GU of homografts and heterografts demonstrated a minimum value 4 d after grafting. In homografts GU pH increased thereafter, but in incompatible heterografts pH rose and then dropped.

[^{14}C]-labelled cell wall components were used to trace the fate of pectins and hemicelluloses applied to suspension-cultured cells; for cells of *L. esculentum* x *peruvianum* about 75% of the associated exogenous pectin was ionically bound to the cell surface, while 25% was internalised or bound by non-ionic means. At pH 6.0 about 73% of the associated hemicellulose bound to the cell surface while approximately 27% was internalised, but at

pH 5 these proportions were almost 100% and 0% respectively. Experiments with cells of *L. esculentum* AC revealed that at pH 6.0 approximately 44% of the associated hemicellulose had bound to the cell surface, while about 55% had been internalised; when incubation occurred at pH 4.5 these percentages remained almost unchanged.

The behaviour of the two types of suspension-cultured cells to identical stimuli is compared, and the relevance of the responses discussed in relation to graft compatibility. The applicability of suspension-cultures as model systems for the investigation of grafting phenomena is evaluated.

In summary, some evidence is provided that components of the normal internodal cell walls have effects on cell metabolism which may be consistent with their involvement in cell signalling leading to graft incompatibility or compatibility. However, little support is provided for the suggestion of Jeffree, Yeoman, Parkinson *et al.* (1987) that a pectic fragment might be the signalling molecule. The activity of hemicellulose fractions of the cell walls in slowing protein synthesis and the relationship of this effect with pH are significant findings in that few oligosaccharin effects involving hemicelluloses are documented.

LIST OF ABBREVIATIONS

UNITS

±	plus or minus
≤	less than or equal to
>	greater than
<	less than
%	percent
A_{490}	Absorbance at 490 nano meters
cm	centimetre
°C	degrees centigrade
Ci	curie
cpm	counts per minute
d	day
g	gram
h	hour
l	litre
psi	pounds per square inch
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mm^3	cubic millimetre
mmol	millimolar
nm	nanometre
pH	$-\log_{10}$ of the concentration of hydrogen ions
rpm	revolutions per minute
µCi	microcurie
µg	microgram
µl	microlitre
µmol m^{-2} s^{-1}	photon flux density in microEinsteins per metre squared, per second
µM	micromolar
v/v	volume to volume
w/v	weight to volume

GENERAL

AGP	arabinogalactan protein
AIR	alcohol insoluble residue
autograft	graft constructed with tissue taken from an individual plant
BSA	Bovine serum albumen
$[Ca^{2+}]_i$	internal calcium concentration
chlorbutol	1,1,1-trichloro-2-methyl-2-propanol
CHS	chalcone synthase
(conc)	concentrated
CPA	p-chlorophenoxyacetic acid
c.v.	cultivar
D-	dextro
2,4-D	2,4-dichlorophenoxyacetic acid
DP	degree of polymerisation
DTT	Dithiothreitol
F1	first generation produced by cross-breeding
F2	second generation produced by cross-breeding
FDA	fluoresceine diacetate
Fuc	fucose
Gal	galactose
GalA	galacturonic acid
Glu	glucose
GU	graft union
H-bonds	hydrogen bonds
heterograft	graft constructed between plants of different species
homograft	graft constructed between plants of the same species
HRGP	hydroxyproline-rich glycoprotein
HR	hypersensitive response
Hyp	hydroxproline
$[^{125}I]$	iodine with molecular weight 125
IAA	indole-3-acetic acid
L-	laevo
L. esculentum AC	Suspension-cultured cells of *Lycopersicon esculentum* cultivar Ailsa Craig
PAW	solution of phenol, acetic acid and water; proportions as stated in text
pcv	packed cell volume
PI	proteinase inhibitor

PIIF	proteinase inhibitor inducing factor
POPOP	1,4-di-2(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
Pro	proline
RGI	rhamnogalacturonan 1
RGII	rhamnogalacturonan 2
MES	2-(N-morpholino) ethanesulfonic acid
MOPS	3-(N-morpholino) propane-sulfonic acid
NAA	α napthaleneacetic acid
PAL	Phenylalanine ammonia lyase
scv	settled cell volume
Ser	serine
TCA	trichloroacetic acid
TCL	thin-cell layer
TFA	trifluroacetic acid
TIBA	tri-iodo benzoic acid
Tyr	tyrosine
[U-^{14}C]	universally labelled with carbon 14
var.	variety
WVM	wound vessel member
Xyl	xylose

MEDIA ± ADDITIONS

Prefixes

Control	No additions made to the incubation medium,
+HYBcw	+ *L. esculentum* x *peruvianum* deproteinated cell walls
+NPcw	+ *N. physaloides* deproteinated cell walls
+ACcw	+ *L. esculentum* AC deproteinated cell walls
+ACsolps	+ *L. esculentum* AC soluble polysaccharides
+NPsolps	+ *N. physaloides* soluble polysaccharides
+HYBpec	+ *L. esculentum* x *peruvianum* pectin
+NPpec	+ *N. physaloides* pectin
+ACpec	+ *L. esculentum* AC pectin
+HYBpfrags	+ *L. esculentum* x *peruvianum* pectic fragments
+NPpfrags	+ *N. physaloides* pectic fragments
+HYBhemi	+ *L. esculentum* x *peruvianum* hemicellulose

+NPhemi	+ *N. physaloides* hemicellulose
+AChemi	+ *L. esculentum* AC hemicellulose
+CITpec	+ *Citrus* pectin
+PGA	+ Polygalacturonic acid

Suffixes

/8-d TOM-I	8-d spent TOM-I culture medium obtained from *L. esculentum* x *peruvianum* suspension cultures
/TOM-I	Fresh TOM-I culture medium
/SH	Fresh SH culture medium
/MES	10 mM MES at pH 6.0
/TART	10 mM Tartaric acid at pH 4.5

NOTE

In all graphs where "Cpm associated with cells x 1000" appears it should be noted that this is the convention adopted for stating " x 10^{-3}".

Chapter 1: INTRODUCTION TO THE HYPOTHESIS THAT CELL WALL-DERIVED RECOGNITION FACTORS MIGHT CONTROL GRAFT DEVELOPMENT IN THE SOLANACEAE

1.1: THE OLIGOSACCHARIN CONCEPT

"A spoonful of sugar helps the medicine go down"

"Mary Poppins", Walt Disney, 1964.

Albersheim, Darvill and McNeil *et al.* (1983) defined oligosaccharins as "particular oligosaccharides that, at low concentrations, exert biological effects on plant tissue other than as carbon or energy sources." The biological effects attributed to oligosaccharins are diverse and include inhibition of flowering in *Lemna gibba* G3 (Gollin, Darvill and Albersheim, 1984), inhibition of auxin-induced growth in pea stem segments (York, Darvill and Albersheim, 1984; McDougall and Fry, 1988; McDougall and Fry, 1989a, b) and in wheat coleoptiles in the presence of exogenous indole acetic acid (IAA) (Tran Van Than and Mutaftschiev, 1990). Oligosaccharins have also been implicated in several aspects of plant disease resistance, notably the stimulation of phytoalexin accumulation (Ayers, Ebel, Finelli *et al.*, 1976a; Ayers, Ebel, Valent *et al.*, 1976b; Ayers, Valent, Ebel *et al.*, 1976c; Ebel, Ayers and Albersheim, 1976), initiation of the hypersensitive response (HR) (Keenan, Bryan and Friend, 1985; Yamazaki, Fry, Darvill *et al.*, 1983), promotion of phenylalanine ammonia lyase (PAL), an enzyme involved in lignin synthesis (Ebel *et al.*), and callose deposition (Ride, 1978). Active oligosaccharide fragments can be derived from host (Hahn, Darvill and Albersheim, 1981; Nothnagel, McNeil, Albersheim *et al.*, 1983) or pathogen (Ayers *et al.* 1976b), though a combination of the two can produce synergistic effects which, in one system, increased the magnitude of the plant response by a factor of 35 (Davis, Darvill and Albersheim, 1986c). Oligosaccharins implicated in plant defence reactions are termed "elicitors", however this term can be applied to any substance that evokes plant defence responses and does not imply that the active principle is a carbohydrate moiety.

1.1.1: DEFENCE REACTIONS ELICITED BY OLIGOSACCHARINS

1.1.1.1: Induction of phytoalexins

Phytoalexins are anti-microbial compounds which are produced *de novo* in plants in response to pathogen attack, but can also be induced artificially by wounding, surface applications of heavy metal compounds, (Weinstein, Hahn and Albersheim, 1981), ultra-violet light (Bridge and Klarman, 1970; Hadwiger and Schwochau, 1971) and organic solvents (Albersheim, Darvill Sharp *et al.*, 1986). The first steps towards understanding the

precise nature of the plant-pathogen interactions responsible for phytoalexin induction were taken by Ayers *et al.* (1976 a, b, and c), who showed that an oligosaccharide, extracted from the mycelial walls of *Phytophthora megasperma* var. *sojae* would elicit production of glyceollin, a soybean phytoalexin, when applied in nano-molar concentrations to healthy soybean seedlings in the absence of pathogens. Further investigations identified the active oligosaccharide as a β, 1-3 glucan. This elicitor was also able to promote the specific activity of PAL in suspension-cultured cells of soybean, parsley and sycamore (Ebel *et al.*, 1976), suggesting that it affected several metabolic processes, e.g. lignification and production of hydroxyproline-rich glycoproteins (HRGPs) in addition to phytoalexin synthesis, possibly in a general non-specific manner.

Later work by Hahn *et al.* (1981) revealed that phytoalexin production could also be elicited by oligosaccharides released from the plant cell wall of soybeans by partial acid hydrolysis. This elicitor was identified as a dodeca-α-1,4-D-galacturonide (Nothnagel *et al.*, 1983) derived from the pectic fraction of the plant cell wall. Previously an endo-α-1,4-polygalacturonase produced by *Rhizopus stolonifer*, a fungal pathogen of castor bean, was shown to elicit phytoalexin production (Lee and West, 1981) by liberating elicitor-active oligogalacturonides from the host plant cell wall (Bruce and West, 1982). These active plant cell wall-derived elicitors can be released by the action of microbial pathogenesis-related enzymes during attack (Davis, Lyon, Darvill *et al.*, 1982; Davis, Darvill, Albersheim *et al.*, 1986a; Davis, Darvill, Albersheim *et al.*, 1986b), and also as a result of physical or microbial damage to the plant when host cell wall-degrading enzymes are brought into direct contact with the plant cell wall (Lyon and Albersheim, 1982).

1.1.1.2: Induction of the hypersensitive response

The hypersensitive reaction, or response, (HR), was first named by Stakman (1915) who observed localised necroses at penetration sites on cereal leaves attacked by *Puccinia graminis*. Mansfield (1986) defines the HR as, "the rapid and localised death of challenged plant cells associated with the restriction of microbial colonization". The HR is difficult to delineate further since the overall events which accompany it vary with the system under investigation. Consequently it may or may not involve phytoalexin accumulation, lignification, synthesis of HRGPs etc.. During the HR the spread of biotrophic microbial pathogens within the plant is limited by the auto-necrotic sacrifice of cells in the immediate vicinity of pathogen penetration; thus the pathogen becomes isolated from healthy living host tissues and further colonisation is prevented. Klement (1982) considers that the HR consists of six stages, namely,

i) recognition of the pathogen,

ii) induction of response,

iii) events leading to cell collapse,

iv) necrosis,

v) formation of anti-microbial substances, and

vi) limitation of pathogen.

HR can be induced by elicitors including pathogen-derived cell wall factors, lipids (Kurantz and Zacharius, 1981), glycoproteins (de Wit and Kodde, 1981), oligopeptides (Schottens-Toma and de Wit, 1988) and host cell wall fragments, probably pectic oligosaccharides, released by microbial enzyme action (Fushtey, 1957; Hopper, Venere, Brinkerhoff *et al.*, 1975; Fry, Darvill and Albersheim, 1983; Yamazaki *et al.*, 1983). The available evidence suggests that the HR is not induced by wounding. Details concerning the series of events, and the order in which they occur, are currently matters of conjecture, particularly because of the lack of agreement as to the exact moment of plant cell death (Bailey, 1983; Mansfield and Brown, 1986), but also because it is unclear whether anti-microbial compounds are synthesised prior to cell death and released at its onset (for example chitinase, which accumulates in the vacuole (Boller and Vogeli, 1984) and emerges once the plant cell membranes have deteriorated), or whether synthesis occurs *post mortem*.

Treatment of challenged cells with cyclohexamide, which prevents transcription, blocks the HR (Masuta, Van Den Bulcke, Bauw *et al.*, 1991) suggesting that *de novo* protein synthesis is a necessary part of the process. Work by Slusarenko, Longland and Friend (1986) on mRNA activities has shown that prior to HR cell collapse (20 - 25 h after inoculation of *Phaseolus vulgaris* by *Pseudomonas syringae* pv. *phaseolicola*) a cascade of changes in gene activity was observed in tissues in which the HR had been induced. This evidence suggests that HR is a controlled and ordered sequence of self-destructive events, and not death attributable to pathogenic toxins or damage.

The physical cause of cell death appears to be membrane dysfunction, demonstrated by the loss of electrolytes from the cells (Lyon and Wood, 1975). This damage may result from peroxidation of lipids in the host membranes by active oxygen generating systems (Hitchcock and Nichols, 1971; Keppler and Novacky, 1986) released by lipoxygenase activity. Alternatively, these radicals may disrupt the action of membrane transport proteins and so destroy the ionic homeostatic integrity of the cell (Wolff, Garner and Dean, 1986). The normal turnover rate of proteins in plant cell membranes is believed to be rapid, measured in hours rather than days. Slusarenko *et al.* (1986) hypothesised that if the mRNA activities which decrease during the HR (the changes mentioned earlier include increases and decreases in activities) encode for membrane proteins, then loss of membrane integrity may be due to natural turnover continuing in the absence of

replacement activity. However, the possibility remains that the three mechanisms outlined above may provide a joint contribution towards membrane disintegration.

1.1.1.3: Induction of proteinase inhibitors

Plants contain proteinase inhibitors (Ryan, 1968) in aerial tissues, seeds and tubers, which apparently protect against insect attack (Green and Ryan, 1972). In tomato plants the levels of these inhibitors were shown to increase after damage by insects or physical wounding. These observations led to the exposition of the hypothesis that a putative proteinase inhibitor inducing factor (PIIF) was released from plant tissues as a result of insect or physical damage. PIIF appears to act systemically, promoting synthesis of proteinase inhibitor (PI) in tissues at a distance from the damaged area. The main PI produced, inhibitor 1, was effective against chymotrypsin and trypsin. High concentrations would render plants unpalatable and indigestible to grazing insects and possibly fungi. Generation of inhibitor 1 was completed *in planta* 10 h after initiation (Green and Ryan, 1972) although the rate of PIIF induction, synthesis and accumulation of inhibitor were shown to be light- and temperature-mediated (Green and Ryan, 1973).

A phylogenetic survey (McFarland and Ryan, 1974) revealed that macerates of species from the four major divisions of plants showed PIIF activity, and that this was particularly high for some fungal extracts. Early experiments were mainly performed on tomato plants, using a crude PIIF extract obtained from autoclaved tomato leaves which were then freeze-dried (Ryan, 1974). Later work indicated that PIIF was a pectic polysaccharide with a molecular weight of approximately 5 000 (Ryan, Bishop, Pearce *et al.*, 1981) which retained its activity after digestion by fungal endopolygalacturonase and pectinesterase (Bishop, Makus, Pearce *et al.*, 1981), implying that large and small homologues of the original PIIF would still elicit the response and that, in this case, size was not a determining factor of biological activity. Not all pectic polysaccharide preparations showed PIIF activity (Ryan *et al.*, 1981). Sycamore rhamnogalacturonan I (RGI) and some commercial pectin preparations showed PIIF activity although sycamore rhamnogalacturonan II (RGII) and other commercially-available pectin preparations did not. Neutral polysaccharides including starch, dextran, cellobiose and sucrose also showed no activity (Walker-Simmons and Ryan, 1984). Chitosan, a β-1,4-linked glucosamine polymer extracted from shrimp chitin, which elicits accumulation of the phytoalexin pisatin in pea pods, induced maximal levels of inhibitor I when applied to tomato plants at a tenth of the concentration of purified PIIF required to produce the same effect (Walker-Simmons, Hadwiger and Ryan, 1983). Reciprocal tests showed that PIIF would elicit pisatin, at a comparable rate to chitosan, when applied to pea pods. Therefore a common mechanism of gene activation sensitive to stimulation by a variety of polysaccharide molecules may operate in plants of different

species, enabling them to respond defensively to a perceived threat (Walker-Simmons *et al.*, 1983).

It has been suggested that PIIF is transported around the plant via the phloem (Ryan and Huisman, 1970; Zuroske, Makus and Ryan, 1980). However, since experiments by Baydoun and Fry (1985) indicated that the pectic polysaccharide remained at the site of injury and was not translocated beyond the point of application, logically, a secondary messenger substance must carry the stimulus through the plant. Alternatively, PIIF is not pectic in nature, but is, itself, induced by pectic polysaccharides. The putative existence of secondary messenger molecules increases the possibility that a single stimulus is capable of eliciting a multitude of defence responses, which will only be detected *en-masse* if simultaneous bioassays for each are performed.

1.1.1.4: Induction of lignification

Lignin is a very complex cell wall polymer composed chiefly of trans-coniferyl, trans-sinapyl and trans-p-coumaryl alcohols (Sarkanen and Ludwig, 1971) which polymerise in free radical reactions (Ride, 1978) believed to be mediated by a variety of enzymes including many peroxidase isosymes (Bruce and West, 1989), PAL and chalcone synthase (CHS) (Lamb, Lawton, Dron *et al.*, 1989). Lignin imparts structural rigidity to cells, prevents the passage of water and solutes through cell walls, and appears to provide resistance to attack by micro-organisms by impeding penetration of pathogenesis-related enzymes into the cell wall (Sarkanen and Ludwig, 1971).

Many instances have been reported of lignin synthesis by plants in response to both saprophytic and incompatible pathogenic fungi (Vance, Kirk and Sherwood, 1980). Different types of lignin may occur in the same plant (Wardrop, 1971) and analysis of wounded wheat leaves suggests that the lignin produced in response to an encounter with non-pathogenic fungi does not have the same structure as that found in healthy leaves (Ride, 1975). Known elicitors of lignification include pectic oligogalacturonides (Bruce and West, 1989), chitosan (Kohle, Young and Kauss, 1984), extracts from fungal cell walls and fungal lipids (Moerschbacher, Kogel, Noll *et al.*, 1986), suspensions of chitin or fungal cell walls (Pearce and Ride, 1982) and mercuric ions (Pearce and Ride, 1978). Although it may take 3 h for an increase in cell lignin levels to be detectable (Bruce and West, 1989), genes encoding for enzymes involved in the lignin synthesis pathway, PAL and CHS, are activated within 2 - 3 minutes of treatment with an elicitor (Lamb *et al.*, 1989) leading to changes in the activities of a variety of peroxidases within 2 - 6 h (Bruce and West, 1989). Pearce and Ride (1978) reported that the induction of lignin synthesis was much more specific with respect to the nature of the elicitor than was the accumulation of phytoalexins, however most elicitors of lignification are also capable of eliciting phytoalexin production.

The mechanism by which lignin hinders pathogen progress is not known, but several possibilities exist: i) reinforcement of cell walls may make them resistant to mechanical penetration and so isolate the point of infection, ii) a layer of lignin may render the cell wall inert or alter its chemical composition so that it becomes indigestible to fungal enzymes, and iii) lignin deposition may physically restrict the passage of enzymes and toxins between pathogen and plant, and also hinder the reverse flow of water and nutrients (Ride, 1978) effectively disarming the pathogen and starving it to death.

1.1.1.5: Induction of HRGP synthesis and cross-linking

HRGPs compose about 1 - 10% of the cell wall in healthy plants (Rumeau, Mazau and Esquerré-Tugayé, 1986; Lamport, 1978), and are thought to improve the structural integrity of cell walls under attack since levels rise in challenged cells (Wilson and Fry, 1986). These glycoproteins consist of a polypeptide backbone (Lamport, 1967) containing a frequently repeated unit of one serine (Ser) residue adjacent to four hydroxyproline (Hyp) residues $Ser(Hyp)_4$ (Lamport, 1973), as well as some lysine (Lys) and tyrosine (Tyr) (Lamport, 1969). Short arabinoside chains, attached via glycosidic links to the Hyp residues (Lamport 1967), constitute the major sugar component, with lesser amounts of galactose linked to the Ser residues (Lamport, Katona and Roerig, 1973). This class of compounds was originally thought to control cell expansion via links to the carbohydrate portions of the cell wall (Lamport, 1967), however lack of firm evidence for this (Albersheim, 1976) and later evidence that the molecules form inter- and intra-molecular bonds amongst themselves, via isodityrosine cross-links (Fry, 1982b), has led to an abandonment of the original concept of function, although the original name, extensin (Lamport, 1965), remains in use.

The proportion of HRGPs in cell walls increases in dicotyledons (Rumeau *et al.*, 1986) as a result of wounding (Chrispeels, 1969), extreme environmental conditions including tissue culture, red light, and temperature (Stermer and Hammerschmidt, 1984), exposure to a wide variety of pathogens (Esquerré-Tugayé and Mazau, 1974; Giebel and Stobiecka, 1974), fungal elicitors (Roby, Toppan and Esquerré-Tugayé, 1985) and ethylene (Esquerré-Tugayé , Lafitte, Mazau *et al.*, 1979). HRGP levels in elicited cells can rise by a factor of 10 (Esquerré-Tugayé and Lamport, 1979). Experiments with [^{14}C]-labelled proline (Pro) (Pro being the precursor of Hyp) indicate that considerable amounts of HRGPs are synthesised *de novo* in elicited cells (Toppan, Roby and Esquerré-Tugayé, 1982) within approximately 10 h (Rumeau *et al.*, 1986), but there is also significant evidence that a rapid cross-linking of soluble HRGPs already present in the cell wall - thus rendering them insoluble - may also occur within 10 minutes of elicitation (Bradley, Kjellbom and Lamb, 1992). Elicited cells do show a burst of oxidative activity in the cell walls during which hydrogen peroxide (H_2O_2) is generated (Apostol, Heinstein and Low, 1989). H_2O_2 is

required for two important steps in HRGP metabolism; firstly in the production of Hyp, and secondly in the formation of the isodityrosine bridges which immobilise HRGPs in the cell wall. It is likely that the combination of these two events which hedge transcription reinforce the overall effectiveness of this defence strategy.

Accumulation of HRGP is thought to toughen the cell wall (Bradley *et al.*, 1992) and so confer resistance against certain pathogens providing that sufficient enhancement of HRGP levels occurs in the early stages of infection. Accumulation is also stimulated in susceptible plants, but this appears to be due to physical damage incurred during colonisation (Esquerré-Tugayé and Lamport, 1979; Benhamou, Mazau and Esquerré-Tugayé, 1990). Interestingly, plants which show raised levels of HRGP after elicitation sometimes exhibit a simultaneous increase in lignification (Hammerschmidt, Lamport and Muldoon, 1984), a process which is also dependant upon the availability of H_2O_2 (Van Huystee, 1987).

1.1.2: OLIGOSACCHARIN CONTROL OF GROWTH AND MORPHOGENESIS

1.1.2.1: Xyloglucan growth effects

1.1.2.1.a: Xyloglucan structure and location

Xyloglucan is a major hemicellulose polysaccharide of primary cell walls known to provide 20 - 25% (dry weight) of cell wall material in dicotyledons (McNeil *et al.*, 1984), 2 - 5% in grasses (Kato and Matsuda, 1985) and 10% in cultured gymnosperm cells (Thomas, McNeil, Darvill *et al.*, 1987). This polysaccharide is a substituted cellulose, i.e. a β-1,4-linked D-glucopyranose backbone (Kato and Matsuda, 1980a) heavily xylosylated with a variety of short side chains (Bauer, Talmadge, Keegstra *et al.*, 1973; Kato and Matsuda, 1976; Hayashi, Kato and Matsuda, 1980; Joseleau and Chambat, 1984). Every fourth glucose residue in the backbone remains free of substitution and it is at these points that xyloglucan is susceptible to hydrolysis by cellulase (Kato and Matsuda, 1980b; Hayashi and Maclachlan, 1984a), wherein lies the basis of its activity (Fry, 1989a).

Xyloglucan is bound very tightly in the wall, and can only be removed completely by prolonged treatment with cold alkali (Kato and Matsuda, 1976; Edelmann and Fry, 1992). This tenacity is due, in part, to the formation of H-bonds between xyloglucan and cellulose microfibrils (Hayashi, Marsden and Delmer, 1987). Estimations of length of xyloglucan molecules vary (Hayashi *et al.*, 1980; Nishitani and Masuda, 1982, 1983) but it is likely that a single xyloglucan molecule can H-bond to more than one cellulose microfibril (Hayashi and Maclachlan, 1984a), thus tethering cellulose microfibrils together and contributing an element of resilience to the cell wall (Fry, 1989b).

1.1.2.1.b: Anti-auxin activity of xyloglucan oligosaccharides

Auxin treatment enhances cell expansion by loosening primary cell walls (Cleland, 1981). This may be because auxin treatment induces *de novo* synthesis of cellulase (Hayashi and Maclachlan, 1984b) and stimulates H^+ extrusion, thus providing the acidic pH conditions required in the apoplast for the activation of cellulase in the wall (Byrne, Christou, Verma *et al.*, 1975). Cellulase acts preferentially upon xyloglucan (Hayashi and Maclachlan, 1984a) (possibly because the cellulose microfibrils are completely encased in xyloglucan and therefore not readily available as a substrate) as an endoglycosylase and cleaves strands at unsubstituted glucose residues. This action releases a predictable variety of oligosaccharides, some of which are biologically active, and one in particular which exhibits various concentration-dependant effects.

The anti-auxin effects of a xyloglucan nonasaccharide, XG9, (Glc4 . Xyl3 . Gal. Fuc) shown to be produced *in vivo* (Fry, 1986), were the first to be investigated. This nonasaccharide inhibited 2,4-dichlorophenoxyacetic acid stimulated growth in pea stem segments, and showed distinct concentration effects, with maximum inhibition at approximately 10^{-8} M (York *et al.* 1984) or 10^{-9}M (McDougall and Fry, 1988). Similar oligosaccharides were either inactive, (XG7) (York *et al.*, 1984), or did not show the concentration-dependence of XG9, (XG5 and 2'-fucosyl-lactose) so may have been somewhat cytotoxic (McDougall and Fry, 1989a). XG9 did not inhibit growth in the absence of exogenous auxin at the concentrations tested (York *et al.*, 1984).

1.1.2.1.c: Auxin-mimicking activity of xyloglucan oligosaccharides

An analogue of XG9, XG9n showed auxin-mimicking activity in the pea stem assay in the absence of exogenous auxins, at concentrations of 10^{-6} - 10^{-4} M (McDougall and Fry 1990) (later amended to 10^{-7} - 10^{-6} M [Fry, McDougall, Lorences *et al.*, 1990]). XG9 showed a similar but less marked effect over this range. This growth promoting ability is reduced at 10^{-5} M; the oligosaccharides may become cytotoxic at such high concentrations, or induce the HR (Yamazaki *et al.*, 1983).

1.1.2.1.d: Structural specifications for anti-auxin activity

The structural requirements of these biologically active xyloglucan oligosaccharides are remarkably precise; the activity of XG9 depends upon the presence of the terminal α-L-fucopyranose residue (McDougall and Fry, 1989b), as does that of XG5 and the human milk trisaccharide, 2'-fucosyl-lactose (McDougall and Fry, 1989a). Augur, Yu, Sakai *et al.* (1992) confirmed that the terminal α-fucose residue bestowed activity on XG9, and that a similar undecasaccharide (XG11) which contained two fucosyl-galactosyl side chains, but was otherwise identical to XG9, was more effective than XG9 at inhibiting auxin-induced growth in the pea stem assay. McDougall and Fry (1989b) assayed an almost identical

inactive decasaccharide (XG10) which lacked the second terminal fucose of XG11, but had one more Gal residue than XG9, and concluded that the proximity of the additional Gal residue caused steric crowding of the essential terminal fucose and impeded recognition of the active portion of the molecule. It would seem that the addition of a terminal Fuc to this Gal residue reinstates the correct conformation to the oligosaccharide and allows it to bind to, or fit into the receptor site more effectively. Adjustments to the remainder of the XG9 molecule, including reduction of the reducing terminus, and the omission of some, or most, of the other residues, have little effect on the biological potency of the oligosaccharin (Augur *et al.*, 1992; McDougall and Fry, 1989b).

1.1.2.2: Growth effects of pectic oligosaccharides

There is only one reported instance of pectic oligomers affecting auxin-induced elongation in the pea stem assay (Branca, de Lorenzo and Cervone, 1988). Experiments showed that the inhibition of growth was proportional to the concentration of the pectic fragments, i.e. that the oligogalacturonides behaved as competitive antagonists of IAA, and possibly inhibited growth by interfering with IAA uptake. The fragments tested were generated from sodium polypectate by enzymic hydrolysis over a range of incubation times. The greatest biological activity was associated with the fraction that had been incubated for 30 minutes. This contained an uncharacterised heterogeneous collection of oligogalacturonides with a mean DP = 12.8 (minimum DP = 8) which falls within the size range expected by other workers for pectic oligosaccharins (Messiaen, Read, Van Cutsem *et al.*, 1993).

An unsubstantiated report of a potentially-pectic oligosaccharin promoting elongation of wheat coleoptiles exists (Tran Thanh Van and Mutaftschiev, 1990), however the identity of the fragment preparation is not clear. This is disappointing, since otherwise this would be an unusual example of oligosaccharin activity for the following reasons: a) that the susceptibility of monocotyledons to oligosaccharins is not well documented, b) that the effect is one of growth-promotion and not the more common inhibition, and c) that pectic oligomers are more often implicated in a disease resistance scenario than growth effects.

1.1.2.3: Control of morphogenesis

Oligosaccharides have also been reported to influence certain morphogenic events which include the inhibition of flowering in *Lemna gibba* by unidentified fragments generated from cell walls by acid hydrolysis (Gollin *et al.*, 1984); the induction of roots, vegetative shoots or flowers (governed by indole-3-butyric acid/kinetin balance) in tobacco thin-cell-layer explants (TCL) by pectic fragments (Eberhard, Doubrava, Marfà *et al.*, 1989; Marfà, Gollin, Eberhard *et al.*, 1991); and the differentiation of strawberry callus cultures in the absence of exogenous plant growth regulators by uncharacterised whole cell and cell wall extracts (Bois, 1992). Albersheim's laboratory have completed the most extensive research into

these phenomena and concluded that in their tobacco TCL system the active principle, which was resistant to extensive pronase digestion and heat treatment, was carbohydrate in nature (Eberhard *et al.*, 1989). Further investigations showed that RGI and RGII were inactive in this system but that both methyl-esterified and non-methyl-esterified oligogalacturonides with DP 6 - 14 were active, with those in the DP 12 - 14 range being most effective. Other polyanions tested included oligomannuronides, oligoguluronides, polyglutamic acid and dextran sulphate, none of which were active (Marfà *et al.*, 1991).

Tran Thanh Van, Toubart, Cousson *et al.*(1985) reported a variety of organogenic effects on tobacco TCL s caused by pectic and hemicellulosic extracts; the nature of organs differentiated depended upon the medium composition and pH. Both Tran Thanh Van *et al.* (1985) and Marfà *et al.* (1991) estimated that the pectic oligosaccharins operate at concentrations between 10^{-8} - 10^{-9}M, which agrees with previous values attributed to xyloglucan oligosaccharins e.g. XG9 (York *et al.*, 1984; McDougall and Fry, 1988), but differs noticeably from the 2×10^{-4}M figure previously specified for another pectic effect, the inhibition of auxin-induced growth by pectic fragments (Branca *et al.*, 1988).

1.1.3: MODE OF ACTION FOR OLIGOSACCHARINS

1.1.3.1: Fragment size and determination of mode of action for pectic oligomers

It is difficult to know:

i) to what extent the size of an oligogalacturonide fragment determines its function

ii) whether the biological effects described really are as size-specific as they at first appear, and,

iii) if apparently inactive oligomers, which lie outside the recognised size range for a particular effect, are biologically inert, or exert an unmonitored influence in another direction.

It is possible that in an assay for phytoalexin production, which usually requires oligosaccharides in the DP 9< range (Davis *et al.*, 1986a; Jin and West, 1984), elicitation of proteinase inhibitor or a degree of hypersensitive death in the test population by smaller "inactive" phytoalexin elicitors with DP 2 - 6 (Bishop, Pearce, Bryant *et al.*, 1984; Cervone, de Lorenzo, Degrà *et al.*, 1987) would go undetected. However, there seems to be a certain amount of overlap in the responses instituted by oligogalacturonides of particular sizes. Lignification seems to be attributable to fragments of DP 7 - 20 (Bruce and West, 1989; Robertsen, 1986) with maximum activities attributed to oligogalacturonides of DP 7 and DP 10 - 11 respectively; this coincides with the reported values for elicitation of

phytoalexins, ie. DP 10 (Davis *et al.*, 1986a), DP 12 (Nothnagel *et al.*, 1983), DP >9 with maximum activity at DP 13 (Jin and West, 1984); inhibition of auxin-induced growth where fragments with DP 6 - 11 show low and intermediate activity, those with DP 12 -14 exert maximum effect, while those with DP 16 - 20 are inactive (Branca *et al.*, 1988); and induction of flowering in tobacco TCL s where the maximum activity is associated with oligogalacturonides of DP 12 - 14 (Marfà *et al.*, 1991).

Messiaen *et al.* (1993) report that only double-stranded oligogalacturonides with DP $\geqslant$9 raised internal Ca^{2+} concentrations ($[Ca^{2+}]_i$) in carrot protoplasts (Ca^{2+} has been postulated as a secondary messenger of oligosaccharin signals), so, for pectic oligosaccharins, the inter-molecular conformation might determine the exact mode of action. Alternatively, since most workers establish the effective DP of the elicitors, but do not elucidate the precise structure further, specificity of function may be attributable to particular side-chains or residues as seen for the xyloglucan oligosaccharin, XG9, (McDougall and Fry, 1989b). The reported range of concentrations over which oligogalacturonide effects have been reported is quite wide, 10^{-9}M - 10^{-4}M (Marfà *et al.*, 1991; Branca *et al.*, 1988), so it is possible that the concentration could decide the mode of action of the molecules in a similar manner as that described for XG9 between 10^{-9}M and 10^{-5}M (Fry *et al.*, 1990).

1.1.3.2: Location and identity of oligosaccharin receptors

Once the identity and role of biologically active oligosaccharides has been established the sequence of events which they initiate can be investigated, starting with the nature and location of the oligosaccharin receptors. The majority of the evidence available relates to elicitors of fungal origin which induce phytoalexin synthesis and/or anti-fungal enzymes, which consistently purport to bind to specific receptors, probably proteins or glycoproteins, located in the plasmalemma (Schmidt and Ebel, 1987; Cosio, Pöpperl, Schmidt *et al.*, 1988; Cosio, Frey, Verduyn *et al.*, 1990; Cheong and Hahn, 1991; Liénart, Gautier and Driguez, 1991). This seems reasonable given that most elicitors are too large to pass through the plasmalemma (Horn, Heinstein and Low, 1989).

Liénart *et al.*(1991) demonstrated that passage through the plasmalemma was not required for induction of the defence-related enzyme, laminarinase, in *Rubus* protoplasts when they immobilised the elicitors on silica beads. The addition of these beads to the protoplast medium stimulated rapid increases in the levels of the enzyme in the preparations, thus showing that recognition of the elicitors and signal transduction occurred while the elicitors were physically retained outside the cell membranes, and providing a strong indication that the binding sites, or receptors, for these elicitors are located in the plasmalemma.

Schmidt and Ebel (1987) also observed that a β-1,3[^{3}H] glucan elicitor from *Phytophthora megasperma* bound to membrane fractions extracted from soybean roots, especially a

plasmalemma enriched fraction. Cosio *et al.* (1988) showed a correlation between the binding affinity of radioactively-labelled fungal glucans to soybean protoplasts and their elicitor activities. A 1,3-1,6-hepta-β-glucoside displayed the highest binding affinity and was the most potent elicitor (Cosio *et al.*, 1990). Tests with a radioactively-labelled ligand and membrane preparations revealed a linear relationship between the amount of membrane protein present and the amount of glucan that bound to the membranes (Cosio *et al.*, 1988). Cheong and Hahn (1991) investigated the binding of a fungal hepta-β-glucoside phytoalexin elicitor in some detail and discovered that membrane binding was impaired by pH values <6 and >8. Subjection of membranes to protease or heat treatment (60°C for 10 minutes) inhibited binding completely, indicating that the receptor might be a heat-labile protein or glycoprotein. In tests with structurally-related glucans and glucosides the membranes exhibited extraordinary binding specificity, and a strong correlation emerged between binding ability and ability to induce phytoalexin accumulation. Experiments with a fluorescein-labelled *Citrus* pectin-derived elicitor also indicated that elicitors of plant origin bind to the plasmalemma of suspension-cultured cells of soybean (Horn *et al.*, 1989).

1.1.3.3: Immediate effects of oligosaccharins upon plant cells

Several very rapid responses have been reported in elicited cells. Mathieu, Kurkdjian, Xia *et al.* (1991) listed the following immediate reactions in suspension-cultured tobacco cells exposed to a mixture of α-1,4-D-oligogalacturonides, DP 6 - 15:

i) a rapid and transient stimulation of K^+ efflux,

ii) the alkalinisation of the external medium,

iii) acidification of the cytoplasm, and iv) an influx of Ca^{2+}.

1.1.3.3.a: pH effects

Some of the above processes probably occur in tandem, for instance an efflux of K^+ ions will induce a corresponding reverse flow of H^+ ions so that the membrane charge differential is maintained. The H^+ influx culminates in a simultaneous decrease in internal pH and increase in external pH, while the rise in the internal calcium concentration $[Ca^{2+}]_i$ may balance the charge short-fall evident in the K^+/H^+ movements since only 0.8 H^+ ions cross the membrane for every K^+ ion. These $[Ca^{2+}]_i$ increases have also been documented by Messiaen *et al.* (1993) as occurring within 20 minutes of addition of oligogalacturonides of a similar DP to carrot protoplasts. Ca^{2+} has been recognised as a secondary messenger of a number of metabolic processes (Gilroy, Fricker, Read *et al.*, 1991; Shacklock, Read and Trewavas, 1992) but at present there is little evidence of its relevance in

oligosaccharin-induced signal hierarchy, although this is a field which may well burgeon in the near future (Kurosaki, Tsurusawa and Nishi, 1987).

Observations from investigations of the intracellular pH decrease observed by Horn, Meadows, Apostol *et al.* (1991) in elicited cells contradict those of Mathieu *et al.* (1991). The observations of Horn and colleagues indicated that cytoplasmic and vacuolar pH changes could be induced in cells, in the absence of elicitors, by adjustments to the pH of the cell-bathing solution alone. In addition, if care was taken to keep the external pH constant, elicitors could be introduced to the medium without causing any internal pH fluctuations in the cells.

1.1.3.3.b: Depolarisation of the plasmalemma

Rapid depolarisations of the plasmalemma by oligogalacturonides have been reported from two sources (Thain, Doherty, Bowles *et al.*, 1990; Mathieu *et al.*, 1991). The former achieved almost instantaneous reversible depolarisation of tomato leaf disc membranes with PI-inducing oligogalacturonide mixtures, DP 1 - 7 and DP 10 - 20, at pH 5.5 and pH 7.5 at a concentration of 1 mg/ml. Mathieu *et al.* (1991) employed a variety of oligogalacturonide preparations, (with DP s of 2 - 6, 6 - 15 and 12) at a maximum concentration of 20 µg/ml on suspension-cultured cells of tobacco, and discovered that the depolarising potential of the preparations closely paralleled their phytoalexin-inducing ability, with the DP 12 preparation showing the most activity for both, which agrees well with the earlier results of Nothnagel *et al.* (1983). Mathieu *et al.* (1991) observed a lag period of 2 - 4 minutes between applications of elicitor and depolarisation which has been reported by other workers (Low and Heinstein, 1986) and may imply that a threshold level of elicitor binding must occur prior to action. In both instances the depolarisations appear to have been energy-dependent, but it is not known whether such elicitor-induced depolarisations increase the permeability of the plasmalemma to ions, or whether they are just a measure of the ion fluxes across it.

1.1.3.3.c: Protein phosphorylation

There is a growing body of evidence that protein phosphorylation may be required for signal transduction of some oligosaccharin-induced signals to occur. Certain tomato and potato membrane proteins were phosphorylated after exposure to tomato oligogalacturonides which induce PI synthesis (Farmer, Pearce and Ryan, 1989), as were some tomato proteins after cells were exposed to a yeast elicitor (Felix, Grosskopf, Regenass *et al.*, 1991). The introduction of protein kinase inhibitors to the system prevents this protein phosphorylation together with elicitor-induced extracellular alkalinisation and production of ethylene. Continuous phosphorylation of the relevant proteins is needed to maintain the elicited state; if protein kinase inhibitors are added after the initiation of elicitation, the previously

phosphorylated [^{32}P]-labelled proteins "disappear" within 1 - 4 minutes and the cells revert to an unelicited, apparently normal state. Monitoring of extracellular pH reveals that the elicitor-induced alkalinisation ceases within 20 - 40 seconds of the addition of protein kinase inhibitors (Felix *et al.*, 1991). Protein kinase inhibitors have also been shown to block elicited increases in ethylene and PAL metabolism, but do not impede elicitor-induced callose formation (Grosskopf, Felix and Boller, 1991).

1.1.3.3.d: Generation of H_2O_2

A rapid burst of hydrogen peroxide (H_2O_2) generation by oligosaccharin-elicited cells has been identified (Apostol *et al.*, 1989) and commented on earlier with respect to the cross-linking of HRGPs as a preliminary defence precaution (sect. 1.1.1.5). H_2O_2 can stimulate glyceollin biosynthesis directly in soybean cells and so may be a secondary messenger in the phytoalexin pathway. Pretreatment of the cells with catalase, an inhibitor of the oxidative burst, reduces glyceollin production by 80% (Apostol *et al.*, 1989). Peroxidase activity on membrane lipids may account for the observation of membrane depolarisation already described, and the leaky membranes associated with the HR (Lyon and Wood, 1975; Hitchcock and Nichols, 1971; Keppler and Novacky, 1986). Peroxidase action appears to be restricted to the cell wall, but this does not preclude the plasmalemma as a substrate. H_2O_2 appears to have a finger in every metabolic pie; it has been implicated in lignification and IAA regulation, the production of Hyp, toxic quinones (Apostol *et al.*, 1989) and as a secondary messenger in animal cells (Ramasarma, 1982). H_2O_2 shows several properties of a defence-related signal transducer since it is rapidly formed in response to elicitation and can be destroyed with equal rapidity (Apostol *et al.*, 1989).

1.1.3.3.e: Mechanism for clearance of oligosaccharins from binding sites

Once a metabolic signal has been received and acted upon there is a requirement to clear the binding sites to ensure that the response is finite in duration and that the receptors are re-sensitised. Auxins, gibberellins and cytokinins are small enough to pass through the plasmalemma to receptor sites inside plant cells, but oligosaccharins are too large and polar to do this (Horn *et al.*, 1989) and appear to bind to receptors on the outside of the membrane. Currently the mechanism proposed for the removal of oligosaccharin messenger molecules from these putative plasmalemma-located receptors is endocytosis (Horn *et al.*, 1989). The traditional view is that this type of transport across the membrane is not energetically feasible for the plant (Cram, 1980) which is almost certainly true with regard to the uptake of "housekeeping" inorganic ions, but probably deserves reconsideration and recalculation of the energy quotients in the light of the present day knowledge about elicitors.

Cram (1980) made several relevant points which are worth remembering when considering whether endocytosis can be diagnosed for biologically-active oligosaccharides in plants. The first is that if charge was removed from the cell surface on a pinocytotic vesicle then the plasmalemma would be depolarised, and that even the return of membrane material by reverse pinocytosis would not restore the charge. Such depolarisations have been observed in elicited cells after lag periods of 1 - 4 minutes (Thain *et al.*,1990; Mathieu *et al.*, 1991) and the membrane charge is not recovered until the elicitor is removed from the bathing solution (Thain *et al.*, 1990).

The second of Cram's criteria was that the binding sites would have to be on the membrane and would be complex, with internal allosteric sites sensitive to several internal factors which would appear to be the case for oligosaccharin receptors of all types (McDougall and Fry 1989a, 1989b; Messiaen *et al.*, 1993; Marfà *et al.*,1991). Thirdly, with regard to the energy required for the cell to ingest endocytotic vesicles, Cram stipulated that any pinocytosis that occurred in plants would be an active process and therefore would be expected to cease at 4 °C.

The possibility of a receptor-mediated endocytotic mechanism deployed in the disposal of spent oligosaccharins was investigated in some detail by Horn and colleagues (1989) who were able to visualise the binding of fluorescein-labelled elicitors of both plant and fungal origin to the plasmalemma of suspension-cultured soybean cells, and the subsequent endocytotic relocation of the fluorescence to the major vacuole over the next few hours. Exposure of the cells to fluorescein-labelled bovine serum albumin and insulin failed to exhibit any association of fluorescence with the cells, and suggested that the endocytotic process must be receptor-mediated. Elicitor movements were suspended when the cells were treated with 1mM KCN or chilled to 4 °C indicating a definate energy requirement for the process. Thus Cram's original stipulations appear to have been fulfilled and a case for selective endocytosis in plant cells can be developed. It would appear that elicitors may be sequestered intact within the tonoplast since extraction and analysis of an [^{125}I]-labelled polygalacturonic acid elicitor (prepared through linking [^{125}I] to tyrosine residues on derivatives of polygalacturonic acid) from the vacuole showed that it had not been broken down within 40 minutes of the commencement of vacuolar accumulation, although this does not rule out degradation at a later stage (Horn *et al.*, 1989).

The apparent differences in the lag times for membrane depolarisation and visualisation of fluorescein-labelled elicitors within the tonoplast (4 minutes versus 1 hour) have been reconciled by experiments with [^{125}I]-labelled elicitors which showed that internalization of oligosaccharin occurred instantaneously. Therefore it is highly likely that the fluorescent compounds were also ingested immediately, in which case the lag period is probably an artifact introduced by the detection limits of the system (Horn *et al.*, 1989).

1.2: GRAFTING, AND THE NATURE OF A RECOGNITION EVENT

...After all, if you were cut out of an olive tree that is wild by nature, and contrary to nature were grafted into a cultivated olive tree, how much more readily will these, the natural branches, be grafted into their own olive tree!"

St. Paul's Letter to the Romans, Ch. 11 ^{v24}, circa 57 AD.

A graft is formed when plant tissues, brought into contact either naturally or artificially, develop a functional union. The tissues involved in the graft can originate from a single plant (autograft), or include another member of the same species (homograft), or even a member of a different species or genus (heterograft). If the graft is successful vascular continuity will be established between the stock, or rooted portion, and the scion, the apical section, and the resulting unit will function as a single entity (Yeoman, Kilpatrick, Miedzybrodzka *et al.*, 1978). However if it is not successful the premature death of one or both partners will ensue, rapidly where herbaceous species are concerned, but following several years of decline in the case of certain fruit trees (Serr and Forde, 1959; Miller, 1965). The tissues of stock and scion are in contact only at the graft union (GU), and, with the exception of this region, the tissues remain separate from one another throughout the life of the plant (Hartmann and Kester, 1975).

1.2.1: HISTORICAL, NATURAL AND ECONOMIC PERSPECTIVE

1.2.1.1: Grafting in History

Grafting is an ancient practice which has been used for several thousand years and was well recognised in ancient Greece (Garner, 1967). The basic principles of the technique, i.e. that plants will graft more readily to tissue of their own species than to that of a related species, and that support from the rootstock is essential if the branches are to survive, were well established by the time the apostle Paul (C.57) used the analogy of the wild and cultivated olive trees in his writings.

1.2.1.2: Spontaneous grafting in nature

Spontaneous grafts by herbaceous plants have been reported (Millner, 1932; Piehl, 1962) but natural grafts between branches and roots of neighbouring trees in a stand is much more common (Neilson-Jones, 1969; Loehle and Jones, 1990), and probably occurs when limbs become fixed spatially next to one another. Graham and Bormann (1966) stated that root grafting has been reported in 150 different tree species which included grafts with self, with members of the same species, and with different species. The adaptive significance of this phenomenon is not fully understood. Grafts usually form between members of the same species, which gives the interactions a co-operative appearance; intergrafting between individuals in a population may permit the sharing of inter-specific competition and

environmental stress by enlarging the water and nutrient "catchment area", and facilitating the even dispersal of water and nutrients throughout the population. At the same time the spread of disease may be assisted (Kuntz and Riker, 1956). The existence of interspecific grafts suggests that this may have been a starting-point for the evolution of parasitism, since it would appear that the exchange of water and minerals between partners is of less importance than the exchange of photoassimilate (Bormann, 1966). Root grafts may confer additional stability to a group of trees (Coutts, 1983; Warren, Black, Eastmond *et al.*, 1988) but there is a paucity of hard evidence for this benefit. Adjacent trees appear to graft selectively and not at random (Eis, 1972). Root grafts are thought to form in areas abraded by mutual contact and so may constitute a form of wound healing which prevents an injury from being repeated.

1.2.1.3: Grafting in horticulture

In horticulture grafting is an important technique which permits reliable and rapid propagation of elite clones and is especially useful in situations where varieties do not breed true, e.g. certain apple varieties, when propagation from seed takes too long, or in situations where seed production is unreliable. It can also be employed as a means of combining the most sought-after features of two plant varieties, for example in vineyards across Europe grape varieties with roots resistant to *Phylloxera* infection are used as stocks for high yielding scions. On the continent in commercial tomato production, elite clones are grafted onto stocks resistant to eelworms. A wide variety of cutting and binding procedures are employed to maximise the chances of success. These are covered in detail in several publications to which the reader is referred (Hartmann and Kester, 1975; Garner, 1967).

1.2.2: GRAFT COMPATIBILITY/INCOMPATIBILITY

1.2.2.1: Working definition and guiding principles

Graft combinations which achieve the re-establishment of vascular union and continue to grow and flourish on a regular and predictable basis are said to be compatible (Yeoman *et al.*, 1978). Graft failure is often due to technical problems resulting from poor fit of stock and scion, desiccation, lack of adequate mechanical support, or subsequent pathogenic infections (Hartmann and Kester, 1975), but in interspecific grafts failure can also result from physical and physiological factors inherent in the plants themselves, in which case the combination will fail consistently and is said to be incompatible. Incompatibility is physiologically determined at some level in all incompatible grafts, and can be manifest either as physical disparity between stock and scion, for instance when one partner outgrows the other (Hartmann and Kester, 1975), or as fundamental irreconcilable cellular differences which are more difficult to fathom.

Development of compatible grafts is restricted almost entirely to dicotyledonous species (Moore and Walker, 1981a) and gymnosperms (Copes, 1969) because these produce callus readily in the GU which monocotyledons, which lack a vascular cambium, do not. Monocotyledonous species graft only rarely (Muzik and LaRue, 1954; Muzik, 1958).

In general, grafts between plants from genetically related sources tend to be accepted, while those from genetically diverse sources tend to be rejected. This implies that either a closer taxonomic relationship imparts greater metabolic and developmental similarities which are conducive to heterograft development (Moore, 1984), or that the somatic tissues possess the capacity to recognise tissue that is non-self in origin (Clarke and Knox, 1978). Indeed both may be possible, since if closely related species exhibit few biochemical differences there would be little to alert the putative recognition system to the presence of foreign tissue; conversely, by virtue of greater biochemical deviance, distantly related species would be perceived more readily.

1.2.2.2: Reasons for incompatibility

1.2.2.2.a: Toxicity

Certain instances of incompatibility are attributable to toxic interactions between stock and scion initiated by the enforced propinquity of the other. In some instances, for example the pear/quince (Gur, Samish and Lifshitz, 1968) and peach/almond heterografts (Gur and Blum, 1973), a diffusable toxic substrate is produced by one partner and is broken down to its constituent parts by enzymes produced in the other, this releases quantities of poison into the GU which causes abnormal development and leads to graft failure. Alternatively, one graft partner may produce a substance noxious to the other, which is probably the situation in the *Sedum telephoides/Solanum pennellii* heterograft, where *Sedum* cells exhibit extensive necrosis whilst *Solanum* cells remain overtly healthy (Moore and Walker, 1981b). The accumulation of dead cells in the GU, and to a lesser extent, the appearance of physical deformities at the interface are features indicative of toxic activity between the participants.

1.2.2.2.b: Unknown

There are certain interspecific and intergeneric grafts for which the causes of incompatibility are unknown (Parkinson, 1983; Hossain, 1986). In the Solanaceous system investigated by Yeoman and colleagues *Lycopersicon esculentum* will form successful grafts to itself and with *Datura stramonium*, as will *Nicandra physaloides*, however, the *N. physaloides/L. esculentum* heterograft, in which stock and scion are sufficiently close taxonomically for compatibility to be predictable, fails repeatedly despite the absence of major physical differences between the plants. There is no evidence of any known cause of incompatibility in operation; the lack of necrotic tissue in the GU certainly negates the

possibility of incompatibility due to toxic effects. This combination and reasons for its incompatibility will be discussed in detail at a later stage.

1.2.3: EVENTS DURING GRAFT FORMATION

The initial events during graft development occur in both compatible and incompatible grafts and form part of the normal plant wound-healing responses and not a specific reaction to the graft partner (Moore and Walker, 1981b). This non-specific phase includes the adhesion of stock and scion within a few hours caused by the agglutination of severed cell walls and pectic materials secreted into the GU by intact cells adjacent to the wound (Yeoman and Brown, 1976; Jeffree and Yeoman, 1983; Hossain, 1986; Jeffree, Gordon and Yeoman, 1989; Moore and Walker, 1981a, 1981b; Tiedemann, 1989). Callus proliferation occurs in the peripheral tissues of the GU, and in the *L. esculentum* homograft newly-formed cells from stock and scion come into contact by 3 - 4 d after grafting (Jeffree and Yeoman, 1983). Adhesion similar to that seen early on between stock and scion has been demonstrated by cut stems of *S. telephoides* and *S. penellii* when confronted with a wooden stick in place of a graft partner (Moore and Walker, 1981a, 1981b).

1.2.3.1: Compatible graft development

The distal ends of the callus cells bear pectic beads in association with tightly packed membrane complexes (Jeffree and Yeoman, 1983; Jeffree *et al.*, 1989). Similar beads have been reported on parenchymatous cells of carrot (Davies and Lewis, 1981). When the callus cells touch, the beads coalesce and establish a mechanical union between them (Jeffree and Yeoman, 1983). In *L. esculentum* autografts a period of wall thinning ensues when the dead cell debris and secreted pectic material which built up in the GU is dissolved (Jeffree and Yeoman, 1983; Stoddard and McCully, 1980); a similar thinning is displayed during development of *Glycine max* autografts (Hossain, 1986) and in compatible epidermal grafts of Solanaceous species (Holden, 1985). It is thought that this might bring the cells into greater proximity and so permit the establishment of plasmadesmata observed between cells of stock and scion (Jeffree and Yeoman, 1983; Holden, 1985). Development of plasmadesmata between stock and scion has also been reported for the compatible heterograft *Vicia faba/Helianthus annus* heterograft (Kollmann and Glockmann, 1985; Kollmann, Yang and Glockmann, 1985).

Once the cells of stock and scion meet, the development of compatible and incompatible grafts diverges noticeably. In a compatible autograft such as *L. esculentum* or *S. telephoides*, division of callus cells stops (Jeffree and Yeoman, 1983; Moore and Walker, 1981a). Experiments on *D. stramonium* epidermal grafts to investigate the stimulus which prompts this cessation showed that the application of pressure alone to the

actively dividing cells would not arrest division, but that cell-cell contact with the tissue explant would (Holden, Jeffree and Yeoman, 1987). Therefore it appears that cell proliferation will continue until specifically signalled to stop (Lipetz, 1970), and that this "off" signal originates from the cell-cell contact achieved 3 - 4 d after grafting (Holden, 1985). Cells in the GU, stock and scion proceed to differentiate into organised files of xylem wound vessel members (WVMs) and wound phloem sieve tubes which re-establish vascular continuity across the GU (Moore and Walker, 1981a, 1981b; Jeffree and Yeoman, 1983; Hossain, 1986; Tiedemann, 1989).

There are differences in the minutiae of available accounts of compatible graft formation, chiefly with respect to time-scales, but also with respect to certain details, for instance dictyosome activity. In the *S. telephoides* homograft dictyosome activity peaked during the first two days and was interpreted as the wholesale export of cell wall precursors from the endoplasmic reticulum to the wall for secretion into the GU "necrotic layer" (Moore and Walker, 1981a). In *L. esculentum* autografts however, dictyosome activity is at a maximum during the thinning of the dead and pectic material in the GU around the third and fourth days after grafting (Jeffree and Yeoman, 1983).

1.2.3.2: Incompatible graft development

The purpose of this study is to investigate the factors other than toxicity which might be responsible for incompatibility in grafts. Consequently the development of the incompatible heterograft *S. telephoides/S. penellii*, where incompatibility is accompanied by classic symptoms indicative of toxins at work, (i.e. formation of an isolation layer of dead, suberised cells and the rapid decline and death of the *S. telephoides* cells (Moore and Walker, 1981b; Moore, 1982) will not be covered here.

In the *L. esculentum/N. physaloides* heterograft the opposing callus cells of stock and scion meet 3 - 4 d after grafting (Parkinson, 1983) and continue to divide (Holden, 1985). The cells produced at this stage are smaller than at first, so despite their numbers, the width of the GU remains more or less constant and is comparable in size to that of compatible grafts (Jeffree, pers. comm.). A similar scenario is seen in the leguminous incompatible heterograft *Vigna sinensis/G. max* (Hossain, 1986). Differentiation of vascular cells accompanies the continuation of division; WVMs differentiate through stock, scion and GU in unorganised, scattered clumps, and files from stock and scion fail to meet and make connections. In whole-plant *N. physaloides/L. esculentum* heterografts few connections have been found (Yeoman and Brown, 1976; Yeoman *et al.*, 1978) and these were non-functional, a pattern repeated when internode segments were cultured as explant grafts using the method devised by Parkinson (1983) (and, Parkinson and Yeoman, 1982). Evidence concerning these *in vitro* connections (Jeffree, unpublished results) indicated that

they might be blocked by tyloses, did not appear to form plasmadesmata, and so could be considered non-functional (Parkinson, Jeffree and Yeoman, 1987).

Interestingly the inversion of an inter-stock of the same species in a homograft (Yeoman and Brown, 1976), or the application of the auxin inhibitor tri-iodo benzoic acid (TIBA) to the GU (Parkinson, 1983), produces a distribution of regenerated WVMs that is virtually indistinguishable from that in an incompatible graft; the random differentiation of WVMs produces only a few non-functional tylosed connections (Yeoman, 1984) and local vascular "whirlpools" (Sachs and Cohen, 1982), conversely, addition of the artificial auxin α naphthaleneacetic acid (NAA) to the GU of *L. esculentum/N. physaloides* heterografts induces a pattern of WVM development similar to that of compatible grafts (Parkinson, 1983). While the auxin supply may not be responsible for initiation of WVM differentiation (Fosket and Torrey, 1969; Torrey and Fosket, 1970) it seems likely that auxin gradients or fluxes may "steer", or guide, the orderly differentiation of WVMs in compatible grafts (Sachs, 1969), and therefore, that breakdown or interruption of auxin transport between stock and scion may be a contributory factor in incompatible reactions. Sachs (1991) considers that the blockage of auxin or other long-distance signals may be entirely responsible for incompatible graft reactions.

The interruption of auxin transport is a potential source of the incompatibility exhibited between *L. esculentum* and *N. physaloides*, especially since *N. physaloides* exhibits lower levels of acropetal auxin movements than *L. esculentum* (Parkinson, 1983). This difference might account for incompatibility in grafts where *N. physaloides* constitutes the stock, however, since the combination is still incompatible when stocks of *L. esculentum* are used, it is more likely to be a facet of more complex interactions between the species which are activated by the presence or absence of a stimulus, or stimuli, inherent in the graft combination itself. In short, some form of recognition system must be in operation in this grafting system which either encourages acceptance of a compatible partner by delivery of a "compatibility" signal, or promotes rejection of a partner upon receipt of an "incompatibility" signal (Yeoman and Brown, 1976; Yeoman *et al.*, 1978; Yeoman, 1984).

Parkinson's (1983) experiments indicated that it was likely that an incompatibility signal was involved in the *L. esculentum/N. physaloides* graft interactions. Release and/or receipt appeared to occur upon the contact of opposing callus cells 4 d after grafting. Receipt of an incompatible signal programmed development of the graft irreversibly, so that failure would ensue even if the stock and scion were disassembled and reassembled with a compatible partner. Further investigations showed that a substance or substances present in the solid fraction of a crude macerate of cells of one species could induce symptoms of incompatibility when added to the GU of homografts of the incompatible partner; this suggested that the putative recognition factors were located in the cell walls. Attempts to

transfer the incompatibility factor(s) on agar blocks between homografts of *L. esculentum* and *N. physaloides* failed, suggesting that the factor(s) possessed low mobility and high molecular weight (Parkinson, 1983). Molecules which might fulfil the required specifications for the putative recognition factor(s) for this system, and have been identified as participants in other recognition systems, include proteins in pollination self-incompatibility reactions (Heslop-Harrison, 1975), lectins in the establishment of symbiotic nodular complexes between *Rhizobium* species and leguminous hosts (Hamblin and Kent, 1973) and cell wall oligosaccharides in pathogen detection systems (Ayers *et al.*, 1976a; Keenan *et al.*, 1985; Hahn *et al.*, 1981). Before the suitability of each of these molecular species is considered the relevance of a vegetative recognition system for whole plants will be discussed.

1.2.4: RELEVANCE OF A VEGETATIVE RECOGNITION SYSTEM

The circumstances in which vegetative cells from different higher plants come into contact are reasonably frequent, but are of limited types, namely infection of angiosperms by angiosperm parasites e.g. *Cuscuta* (Schofield, 1988), and natural grafting of roots and shoots (Neilson-Jones, 1969; Loehle and Jones, 1990). There is an explicit expression of parasitism in the former and an implicit potential for it in the latter. Plants able to recognise the invasion of their tissues by parasitic non-self cells may be able to mobilise defence strategies to prohibit further development of the invader; the existence of certain resistant host plants which exhibit a hypersensitive-like reaction stimulated by contact with the parasite's tissues during the initial stages of colonisation (Gäumann, 1950) indicates that such a system may well exist. The tale-bearing molecules which alert the host defences are most probably host cell wall fragments released during the attempted penetration in a fashion analogous to that seen in some instances of angiosperm resistance to fungal pathogens (Hahn *et al.*, 1981; Nothnagel *et al.*, 1983), especially since pectinesterase and cellulase, and possibly other cell wall lysing enzymes are released by parasitic angiosperms to assist in haustorial invasion (Schofield, 1988). However, the ability of parasites to avoid detection should not be underestimated; it is reported that *Cuscuta* does not show any polygalacturonase activity in its tissues (Schofield, 1988), which implies that successful parasites may abandon the oligosaccharin-releasing enzymes of betrayal and rely on other elements of their parasitic arsenal to achieve haustorial contact and establishment.

It is imperative that parasitic angiosperms are able to recognise suitable host plants, as time wasted attempting to tap the resources of unsuitable plants may deprive the parasite of precious energy reserves and preclude a later successful colonisation bid. Experiments by Atsatt, Hearn, Nelson *et al.* (1978) showed that formation of haustoria could be induced on roots of *Orthocarpus purpurascens in vitro* by water soluble diffusates from cotton fibres

and extracts of host-plant seeds, which suggests that these plants possess a very specific recognition system. Correct alignment of the haustoria inside the host tissues, to ensure an adequate supply of photoassimilate, water and mineral salts, is also very important (Tsivion, 1978) and exhibits a precision that implies an ability on the part of the parasite to interpret tissue patterns and fluxes; appropriate signals may be generated by concentration gradients of plant growth regulators, sugars, water and mineral salts, the translation of which provides the parasite with the "spatial awareness" required for a successful foray into the host's tissues.

The occurrence of natural grafts has already been described, and its ecological implications considered. A means of determining the identity and symbiotic/parasitic capability of a potential graft partner would be a very valuable competitive tool for all plants, and might be an essential component of the overall defence strategy, permitting mutually beneficial associations to form and possibly sabotaging attempts to graft by parasitic or partially-parasitic plants by disruption of graft development thus impeding the translocation of photoassimilate and water between the plants.

1.2.5: NATURE AND INVESTIGATION OF POSSIBLE RECOGNITION FACTORS AND EVENTS

1.2.5.1: Potential candidates for the role of recognition factor

1.2.5.1.a: Proteins

Dual-labelling experiments with radioactive amino acids have shown that internodes produce certain distinct proteins during grafting that are not produced as a result of wounding (Yeoman *et al.*, 1978; Miedzybrodzka, 1981). The high specificity of structure and function of protein molecules, and the time-frame in which they are produced fit the requirements of recognition factors very well. However, proteins should probably be excluded as recognition messenger molecules on the basis of their relative mobility. The relevance of these proteins to the process has not yet been established, and while, it is unlikely that they might be involved in the initiation of recognition phenomena, it would be feasible for them to amplify or propagate the original compatible/incompatible signal, thus forming part of the "succession or hierarchy of recognition events" envisaged by Jeffree and Yeoman (1983).

1.2.5.1.b: Lectins

The specificity of binding of lectins is best characterised in the *Rhizobium*/host arena (Hamblin and Kent, 1973; Bohool and Schmidt, 1974; Wolpert and Albersheim, 1976). Research on *D. stramonium* (Jeffree and Yeoman, 1981) showed that although lectin molecules are distributed throughout the tissues and cell membranes, the levels remain

unaffected during graft formation, this may imply non-involvement of lectins in the recognition process, however, the potential of lectins as receptors of messenger molecules rather than the active molecules themselves should not be ruled out. While it is likely that messenger molecules will be synthesised *de novo* or increase in concentration during grafting, it is not a prerequisite that binding sites should do likewise, and it is therefore sensible to consider the type of substrate which would bind to this putative receptor site with a high degree of specificity; substrates for these candidates must be present in the GU, the active products should increase in concentration after grafting, have been shown to possess limited mobility, and, if lectins act as receptors, probably contain combinations of sugar molecules.

1.2.5.1c: Cell wall fragments

Yeoman (1984) postulated that cell wall fragments might fulfil all the requirements for messenger molecules within the graft complex. The capacity of complex cell wall carbohydrates for metabolic and physiological regulation has been well documented in recent years (Gollin *et al.*, 1984; Eberhard *et al.*, 1989; McDougall and Fry, 1988, 1990) and certain biologically active oligosaccharides show a very advanced level of structural specificity (McDougall and Fry, 1989a, 1989b). The substrates from which these oligosaccharins can be generated are present in the cell walls of both intact and grafted tissue, and would remain inert until active oligomers were specifically cleaved from the parent polymers at particular points. Biological activity has been attributed to oligosaccharins of pectic (Bruce and West, 1982; Branca *et al.*, 1988), and hemicellulosic origins (Fry, 1986).

The evidence available thus far shows oligalacturonides released from pectic polymers to be the most likely candidates for the role of messenger molecules within the grafting system. Pectin is present in the GU in great abundance, as a normal cell wall constituent of the cells involved, and also as the secreted "glue" which holds stock and scion together in the early stages of development. It has been suggested that the "beads" observed on the distal surfaces of GU callus cells prior to contact may be pectinaceous and contain enzyme complexes which digest the pectin-agglutinated debris layer to enable formation of plasmodesmata (Jeffree and Yeoman, 1983). Staining and blotting experiments showed that pectinase was present in the GU during grafting (Jeffree, Gordon and Yeoman, 1989), which, during wall thinning would be capable of releasing potentially active oligosaccharide fragments. In addition, experiments in which incompatible pectin was introduced into the GU of homografts on lens tissue resulted in a large reduction in the number of connections formed compared to the number formed when compatible pectin was present (Jeffree, Yeoman, Parkinson *et al.*, 1987) which would suggest that pectic fragments may exert some form of developmental regulation during grafting, which may stem from a recognition

reaction. Pectic fragments are relatively immobile (Baydoun and Fry, 1985), effective at very low concentrations (Tran Thanh Van *et al.*, 1985; Marfà *et al.*, 1991) and could be cleared from receptor sites once the message had been imparted by means of endocytosis (Horn *et al.*, 1989); thus oligogalacturonides fulfil the required criteria for the putative recognition factor(s).

There is a paucity of information regarding possible hemicellulosic recognition factors; hemicellulose is present in the GU as a normal cell wall constituent, but there is no evidence to suggest that fragments are released during the process of graft development. Cellulase is the enzyme most likely to cleave the xyloglucans, and has been reported as being present in the GU (Jeffree *et al.*, 1989). Hemicellulosic oligosaccharins tend to affect growth processes (York *et al.*, 1984; McDougall and Fry, 1988; McDougall and Fry, 1989) and have not been implicated in plant protection and wound-healing processes as have the pectic oligosaccharides (Nothnagel *et al.*, 1983), so are possibly not involved in this type of reaction.

1.2.5.2: Nature of a possible recognition event

Interpretation of events leading to the development of a compatible graft has suggested (Holden *et al.*, 1987; Jeffree *et al.*, 1987) two recognition mediated events may be involved; i) the cessation of prolific wound-stimulated cell division in the GU after callus cells from graft partners make surface contact and, ii) the commencement of organised differentiation of xylem and phloem wound vessel members into functional conduits across the GU, although it is possible that the latter follows on automatically from the former.

A variety of different models have been proposed to account for the compatibility or incompatibility observed in the Solanaceous model system. The first, started from a proposal that a catalytic complex was formed by the newly formed cells in the graft union, during stock-scion adhesion in the first phase of graft development (Lindsay, Yeoman and Brown, 1974), although the adhesion is not compatibility-specific. Later the idea was introduced that a recognition system in which the cells growing into the GU were highly polar and produced different compatibility factors, probably proteins, at their distal ends (Yeoman and Brown, 1976). The required catalytic complex would only be formed when unlike compatibility complexes came into contact. This hypothesis was based on evidence from experiments in which an apparently incompatible response had been induced in compatible homografts through inversion of stock or scion or a compatible bridge. Parkinson's experiments with TIBA (sect. 1.2.3.2) suggested that these phenomena might be attributable to interuption of the auxin flux.

In a later model 'Yeoman *et al.*,(1978) proposed that incompatibility factors might prevent the graft combination from proceeding to a compatible conclusion. It

was suggested that recognition factors might be macromolecules, possible candidates including proteins, glycoproteins, glycolipids or glycopeptides, whose progress to the binding site, probably located on the plasmalemmas of opposing the callus cells, might be facilitated by the dissolution of the adhesive layer, thereby establishing contact between plasmalemmas.

Subsequently, in the light of early reports of oligosaccharin effects in other fields, the broad spectrum of cell wall-derived fragments produced during the dissolution of the adhesive pectic layer began to be considered as a source of suitable molecules for the proposed recognition factors (Yeoman, 1984). In compatible combinations these fragments would probably be perceived as "self" regardless of their source, and grafting would proceed by default, while in incompatible combinations the recognition of "non-self" fragments would cause grafting to fail. Most recently, the question of compatibility *vs.* incompatibility factors has been addressed by Jeffree *et al.* (1987), who concluded that a compatibility factor must operate between viable graft partners, as cell division leading to callus proliferation continues unabated when an inert substance is substituted for stock or scion (Moore and Walker, 1981b; Holden, 1985; Holden *et al.*, 1987). Furthermore, incompatibility factors also operate, and eventually over-ride compatibility factors, since, when stocks and scions originally grafted to an incompatible partner for four days, were reformed into homografts, the incompatible response was transferred (Parkinson, 1983; Jeffree *et al.*, 1987). Jeffree *et al.* (1987) determined that while only self-molecules were detected graft formation would proceed in an orderly fashion, but that detection of non-self factors, possibly enzyme-released cell wall fragments, would hijack development.

1.2.5.3: Advantages of using suspension cultures

The GU is composed of a large number of different cell types which complicate analysis of events. Experiments performed with epidermal grafts (Holden, 1985; Holden *et al.*, 1987) showed that the restriction of tissue types was of great assistance in evaluating graft phenomena, especially as it was accompanied by a reduction in events i.e. in the absence of vascular regeneration. Suspension-cultured cells provide a controlled model of wound-induced callus proliferation analogous to that found in the GU and could be used as a tool to screen potential mediators of recognition phenomena for biological activity. In particular it would be useful to be able to identify the elicitor of the "off" signal for cell division in compatible grafts, which should slow or stop cell division in cultured cells too. The metabolic activity of these cells could be more easily assessed than that of the entire GU area, dosage of cells would be more uniform, and evaluation of potential elicitors facilitated by the absence of release of other wound- and graft-related metabolites. Suspension cultures may provide a means to screen the activity of a large number of potentially potent

substances in a comparatively short time, which is particularly desirable since the current graft evaluation system of counting WVMs is extremely labour intensive and lengthy.

Chapter 2: MATERIALS AND METHODS

2.0: SUPPLIERS

The names and addresses of suppliers of chemicals and equipment will be found in Appendix I, and a detailed composition of the media used in Appendix II.

2.1: DEVELOPMENT OF SUSPENSION-CULTURES

2.1.1: Establishment of callus cultures

Plants of *Lycopersicon esculentum* cv. Ailsa Craig and *Nicandra physaloides* were grown for six to eight weeks in a heated greenhouse at about 20 °C. The seeds were planted in John Innes No. 1 compost and watered as required; daylength was extended to 16 h with 400 W mercury vapour lamps. Primary tissue was excised from the second and third internodes, counting from the cotyledons upwards, and the ends sealed with molten paraffin wax (melting point 49 °C).

Under sterile conditions, provided by a laminar flow-cabinet, the internodes were placed in sterilising tubes, immersed for 10 min in a solution of commercial sodium hypochlorite (A & J Beveridge Ltd) which had been diluted to give a final solution 10% (v/v) of its original, commercial concentration (10-14% available chlorine). The internodes were washed with three rinses of sterile distilled water to remove the hypochlorite solution. The washed internodes were transferred to sterile petri dishes, cut into small segments approximately 2 - 3 mm long and placed, with the apical end uppermost, on plates of Murashige and Skoog (Sigma Chemicals Ltd.) callus initiation medium [CI (s)] (sect. 2.10.2); the waxed ends were discarded. The explants were incubated at 25 °C in continuous light at 35 µmol m^{-2} s^{-1}.

Newly-formed callus cells were excised from the parent tissue 14 d after culture initiation and transferred to fresh plates of CI (s) medium. After 2 - 3 further subcultures at 16 - 21 d intervals onto fresh CI (s) medium the calluses were transferred to Schenk and Hildebrandt (Imperial Laboratories) callus maintenance medium (SH) (s), (sect. 2.10.3), 9 weeks after culture initiation. Calluses of *L. esculentum* cv. Ailsa Craig (*L. esculentum* AC) were maintained on SH (s) medium for the duration of the project, calluses of *N. physaloides* remained on this medium but did not survive the first year.

2.1.2: Establishment of suspension-cultures

Attempts were made to introduce calluses of both species to suspension-culture from both CI (s) and SH (s) media (sects. 2.10.2 and 2.10.3), the results of these attempts is

described in section 3.1. Suspension-cultures of *L. esculentum* AC in SH (liq) medium (sect. 2.10.4) were established and maintained throughout the project. These were incubated in the same conditions (sect. 2.1.3) as suspension-cultures of *L. esculentum* x *peruvianum* which were grown in TOM-I medium (sect. 2.10.1). Attempts to produce suspension-cultures of *N. physaloides* were not successful.

The culture of a hybrid tomato, *L. esculentum* x *peruvianum*, was obtained from Dr. M. Koorneef, The Agricultural University, Wageningen, The Netherlands. The hybrid had been produced by crossing *L. esculentum* with *L. peruvianum*, then back-crossing the F1 generation with *L. peruvianum* to generate a population which was 25% *L. esculentum* and 75% *L. peruvianum*. The hybrid suspension culture used was produced from a single member of this F2 population, and had excellent growth rates, suitable texture and cells of robust character.

2.1.3: Standard maintenance conditions for suspension-cultures of *L. esculentum* x *peruvianum* and *L. esculentum* AC

Cultures were maintained in 75 - 80 ml sterile medium in 250 ml conical flasks rotated at 95 rpm, with an amplitude of radius 1 cm, at 25 °C and continuously illuminated with 7.5 - 10.5 µmol m^{-2} s^{-1}. Cultures were subcultured into fresh medium every 2 - 3 weeks, or as required. Cells of *L. esculentum* x *peruvianum* were cultured in TOM-I medium, while cells of *L. esculentum* AC were cultured in SH medium (liq).

2.2: DEVELOPMENT OF GROWTH CURVES

2.2.1: Method for determining settled cell volume and packed cell volume (scv & pcv)

5 ml samples of suspension-cultures were placed in graduated centrifuge tubes. The cells were allowed to settle for 15 min, then the settled cell volume, i.e. the volume occupied by cells in the sample, was recorded. The tubes were centrifuged at 1 500 rpm for 5 min in an MSE Centaur 2 benchtop centrifuge, and the new volume occupied by cells, i.e. the packed cell volume, was recorded.

2.2.2: Method for counting suspension-cultured cells

The method used was adapted from that of Brown and Rickless (1949). Suspension-cultured cells for counting were filtered. 50 mg of the cells were added to 0.5 ml of 10% aqueous chromic acid (w/v) (BDH Chemicals Ltd.) and left overnight at room temperature. Prior to counting, the cell suspension was agitated by being passed through a Pasteur pipette approximately 20 times. This helped to separate aggregations into individual cells. Aliquots of the resulting cell suspension were diluted with distilled water to give a cell

density of 100 - 200 cells/grid. The volume of the haemocytometer grid was 18 µl (1.8 mm^3), and a minimum of 6 grids were counted for each cell sample.

2.2.3: Fluoroscein diacetate (FDA) test for cell viability

The method used was adapted from that used by Heslop-Harrison and Heslop-Harrison (1970) to assess the viability of pollen grains. A 0.5% (w/v) solution of fluorescein diacetate (FDA) (Sigma) in acetone was used as a stock solution, maintained at 4°C and shielded from light. To make a test solution drops of the stock solution were added to 5 ml of incubation medium until it became cloudy; the resulting solution was used to assess cell viability and was always made up immediately prior to a test. The FDA test solutions were always made up in the medium in which cells would be incubated when tested.

Several drops of the FDA test solution were added to an equal number of drops of a cell suspension on a microscope slide and examined in blue light (FITC filter, peak transmittance = 485 nm) on an Olympus 1M inverted microscope. Living cells emitted a bright green fluorescence. Generally three samples of about 200 cells each were assessed and the viability expressed as a percentage, except where otherwise stated. The FDA solution was protected from light at all times, and a fresh batch was made up every hour that examinations continued.

2.3: SUMMARY OF FINAL PROTOCOL DEVELOPED FOR L-[U-^{14}C] LEUCINE INCORPORATION ASSAY

8-d old suspension-cultured cells of *L. esculentum* x *peruvianum* or *L. esculentum* AC were sieved through a 0.4 mm mesh, filtered almost to dryness under vacuum, and added to the test media, fresh TOM-I or SH with, or without, test substances, to give a final concentration of 0.1 g cells/1 ml medium. 0.5 ml aliquots of the cell suspensions were dispensed with a Gilson pipette, on which the blue-tip attachment had been slightly shortened to allow cells to pass through easily (1 - 2 mm cut off the mouth end with a heated scalpel), into 5 ml polypropylene cryotubes (Greiner Labortechnik Ltd.).

Racks of sample tubes were wrapped in aluminium foil to block out all light, incubated at 25 °C and shaken at 200 rpm on a Certomat M shaker. After 1 h, 20 µl L-[U-^{14}C] leucine solution (Amersham International Plc.), 0.05 µCi (specific activity = 310 mCi/mmol), was added to each tube and the mouths of the tubes blocked with non-absorbent cotton wool. All racks, except 0 h samples, were re-wrapped in aluminium foil and incubated as before for up to 3 h. At the end of the incubation period, 4.5 ml of 4 °C 10% tri-chloroacetic acid (TCA) (w/v) (BDH) was added to each tube to kill the cells, thus preventing further incorporation of L-[U-^{14}C] leucine into protein, and causing the precipitation of intracellular proteins thereby minimising loss through leaching. In samples taken at 0 h, TCA was

added immediately after the L-[U-^{14}C] leucine. Tubes were sealed with screw caps, shaken gently, and kept at 4 °C for 16 h. Individual samples were filtered through a Millipore 12-port filtration assembly (Millipore (UK) Ltd.), onto Whatman GF/C 2.5 cm glass microfibre filters (Whatman LabSales Ltd.). Sample tubes and cells were rinsed with 2 x 5 ml of 10% TCA, at 4 °C, and 5 ml of 0.1% non-radioactive leucine solution (w/v) (Sigma), at room temperature, to elute unincorporated L-[U-^{14}C] leucine. The filters were placed into individual Zinsser polyvials (Zinsser Analytic (UK) Ltd.) and oven-dried at 40 - 50 °C. Non-Triton scintillant (sect. 2.11.3) (2 ml) was added to each vial and the samples were scintillation-counted on a Beckman LS 5000CE scintillation-counter (Beckman Instruments (UK) Ltd.). Any variations to this standard procedure are specified in the text.

The mean incorporation of L-[U-^{14}C] leucine, and standard errors, of each treatment at each time point were calculated and compared to those of the control treatment to assess the effect of test substances on L-[U-^{14}C] leucine incorporation into protein, and hence the rate at which protein metabolism proceeded.

2.4: EXTRACTION OF CELL AND CELL WALL COMPONENTS FOR USE IN L-[U-^{14}C] LEUCINE INCORPORATION ASSAYS

2.4.1: Extraction of deproteinated cell walls from suspension-cultured cells and plant tissues

The method used was based on that described by Fry (1988) for producing an alcohol-insoluble residue (AIR) of deproteinated cell walls. Cultured cells, or tissues from whole plants, were weighed, placed in a blender and homogenised in 7x (tissue weight in g) ml of phenol/acetic acid (5:2) (phenol 80% w/w aqueous [BDH], glacial acetic acid [Fisons Scientific Equipment]), and stirred for a minimum of 2 h at 25°C. The resulting suspension was then filtered on sintered glass and resuspended in 5 x the original weight (as before) of a phenol/acetic acid/water solution (PAW) (5:2:1). The residue was washed in fresh rinses of this PAW solution until the filtrate was shown to be free of protein by the ammonium formate (BDH) test (sect. 2.8.3). This usually required between three and six rinses of 2 h duration. The final residue was washed in 100% ethanol until clear of PAW, judged by nasal analysis of the fumes rising from the mixture, freeze-dried in an LSL Secfroid (LSL, Laboratory Sales (UK) Ltd.), and stored at room temperature.

The cell wall material obtained from whole plants was much coarser than that from cell cultures, and, to reduce particle size, was ground under liquid nitrogen with a pestle and mortar prior to use.

2.4.2: Extraction of pectins from deproteinated cell walls

The method used was that described by Fry (1988) for the rapid extraction of pectic polysaccharides from cell walls by autoclaving, which causes partial degradation of the polysaccharides obtained. To each 5 g of deproteinated cell walls 250 ml pyridine (Fisons)/glacial acetic acid/water (1:1:23) was added and the mixture stirred until the cell walls entered suspension. This suspension was autoclaved at 121°C and 1.4 bars (20 psi) for 30 min in a tightly sealed screw-cap bottle, cooled and filtered through a sintered glass funnel. The residue was rinsed with 2 x 50 ml H_2O; the filtrate and rinses were pooled, and freeze-dried in an LSL Secfroid freeze-drier. To remove the final traces of pyridinium acetate, the resulting pectic material was redissolved in H_2O, freeze-dried once more, then stored at room temperature. The solid cell wall residue was retained for later extraction of hemicelluloses.

2.4.3: Extraction of hemicelluloses from residue of deproteinated cell walls

The method used was that described by Fry (1988) for the extraction of a high proportion of total hemicellulose from the residue of deproteinated cell walls from which the majority of the pectic components have already been removed. This procedure causes the de-acylation of some of the hemicellulosic material, and can extract some de-esterified pectins. The starting material was the solid deproteinated cell wall residue, originally from 5 g deproteinated cell walls, which remained after pectins had been extracted (sect. 2.4.2). This residue was suspended in 100 ml of 6 M NaOH containing 1% $NaBH_4$ (Sigma) and stirred at 25 °C for 16 h. The suspension was centrifuged at 3 000 rpm for 10 min, and the supernatant was removed and stored. The solid fraction was resuspended in 6 M NaOH 1% $NaBH_4$ solution and stirred as before. Centrifugation and resuspension of the solids continued until the rinses no longer contained polysaccharide material, as shown by the ethanol test (sect. 2.8.1).

The washings were pooled, centrifuged at 3 000 rpm for 5 min to pellet any solid cell wall particles, then neutralised by the addition of glacial acetic acid, in the proportion of 120 ml acetic acid:200 ml 6 M NaOH washings. The neutral extract was dialysed to excess at 4 °C in pre-soaked 1½" wide Visking dialysis tubing (The Scientific Instrument Centre Ltd.), in 0.05% 1,1,1-trichloro-2-methyl-2-propanol (chlorbutol) (BDH) solution. The samples were freeze-dried, and stored at room temperature.

2.4.4: Preparation of pectic fragments from pectins

1 ml of 2 M tri-fluroacetic acid (TFA) was added to each 20 mg pectin from which fragments were to be generated, and the mixtures were heated at 85 °C for 2 h in hydrolysis tubes. After cooling the samples were dried in a Savant Speed Vac Concentrator used in conjunction with a Savant Refrigerated Condensation Trap (Savant,

c/o International Equipment Co. Ltd.) and a GeneVac CVP100 vacuum pump (Genevac Sales Development Ltd.). Each sample of pectic fragments was re-dissolved, as far as possible, in 3 ml water, 1 ml 30 mM MES, and 3 ml 100 mM MES, then re-dried in the speed-vac. The fragments were used as a suspension in leucine incorporation assays since they were not completely soluble in aqueous solution or in dilute MES solutions.

2.4.5: Extraction of soluble polysaccharides from apoplastic fluid of calluses

Calluses of *L. esculentum* AC and *N. physaloides* were rinsed separately, drop-wise, with fresh SH medium. An appropriate volume of 100% ethanol was added to the washings to attain a final concentration of 75%, at which soluble polysaccharides precipitate out. The ethanol + washings solutions were centrifuged at 1 500 rpm for 15 min, the supernatants were discarded, and the precipitated polysaccharides were extracted. Gentle heating of the precipitates removed traces of ethanol. The polysaccharide products were redissolved in a minimal volume of H_2O to form stock solutions.

20 µl of each solution was placed on a separate pre-weighed piece of aluminium foil, dried, then weighed to establish the amount of polysaccharide in each solution, and permit a suitable volume of each to be added to the assay medium for testing.

2.4.6: Extraction of membranes from internodes of *L. esculentum* AC and *N. physaloides*

The procedure was performed on ice throughout, all measurements refer to quantities required to process 15 g of starting material. Internodes were finely chopped, then liquidised in 25 ml homogenisation buffer (sect. 2.11.1) using an Ultra Tarrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik). The suspension was poured into pre-cooled centrifuge tubes which were balanced and spun on an MSE Mistral 4L centrifuge at 14 000 rpm for 30 min to pellet the solid matter. The supernatants were transferred to pre-chilled ultracentrifuge tubes and spun on a Sorvall Ultracentrifuge OTD 65B (DuPont UK Ltd.) at 26 000 rpm for 17 min. The supernatants were discarded, the pelleted membranes resuspended in rinsing buffer (sect. 2.11.2), and the tubes centrifuged again at 26 000 rpm for 17 min. The supernatants were discarded, the membranes resuspended in 2 ml fresh SH medium and frozen until required.

2.4.7: Fractionation of hemicelluloses from *L. esculentum* x *peruvianum* on a size basis

35 mg hemicellulose from suspension-cultured cells of *L. esculentum* x *peruvianum* was dissolved in 3.5 ml 10 mM NaOH and run on a Sepharose CL-6B column (Sigma), with a bed volume of approximately 100 ml, at approximately 7 ml/h. 80 fractions of approximately 2 ml each were collected on a BioRad Model 2110 fraction collector (BioRad Laboratories Ltd.). These were tested for carbohydrate content using the phenol-sulphuric

acid test (Dubois, Gilles, Hamilton *et al.*, 1956; sect. 2.8.2). The profile obtained for carbohydrate distribution is shown in Figures 4.4.5.3.a.i and ii. The fractions from the column were pooled to equalise the carbohydrate content of the hemicellulosic fractions tested in the L-[U-^{14}C] leucine incorporation assay. The fractions were pooled as follows:

Pooled sample	Fraction number
A	11 - 18
B	19 - 24
C	25 - 33
D	34 - 43

A rough calibration for the column was provided by running 35 mg total mass of blue dextran (Sigma), prepared from dextran with an average molecular weight of approximately 2 000 000, and glucose in 3.5 ml 10 mM NaOH on the column, under similar conditions, and comparing the profile that emerged from the phenol-sulphuric acid tests on the fractions collected, Figures 4.4.5.b.i and ii, with that from the hemicellulose run. The polymeric dextran eluted in a comparatively broad peak in fractions 8 - 18, while the monosaccharide, glucose, eluted in a much sharper peak in fractions 24 - 27.

2.5: CHROMATOGRAPHY

2.5.1: Enzymic digestion of pectins for comparative chromatography.

2.5.1.a: Purification of Driselase

The enzyme mixture used was Driselase (Sigma), an unpurified enzyme preparation from the fungus *Irpex lacteus* which contains an extensive selection of exo-hydrolases and endo-hydrolases (Fry, 1988) that permit complete digestion of pectins and most other cell wall components. The enzyme mixture was purified before use; the quantities specified below were sufficient to process 5 g of crude Driselase (Fry, 1982a). Driselase was mixed in 50 ml of sodium acetate buffer, consisting of 50 mM of acetic acid adjusted to pH 5 with 1 M NaOH, for 15 min. The mixture was centrifuged for 10 min at 3 000 rpm and the supernatant collected. $(NH_4)_2SO_4$ (s), at 52% w/v, was added to the supernatant which was stirred until the crystals dissolved. The resulting solution was left at 0 °C for 15 min, then centrifuged for 10 min at 3 000 rpm, the pellet resuspended in 5 ml H_20 and desalted on a Sephadex G-25 (Sigma) column. The desalted proteins were freeze-dried, and stored dry below 0 °C. A fresh working solution was made up immediately prior to use and consisted of 0.02 g purified Driselase in 4 ml pyridine/acetic acid/water (1:1:23).

2.5.1.b: Driselase digestion of pectins for chromatography

To each pectin sample of approximately 4 mg, 0.4 ml of the Driselase working solution (sect. 2.5.1.a) was added. The samples were shaken gently at 95 rpm, at 25 °C overnight. The incubation period was terminated by the addition of 100 µl formic acid (Fisons) to each sample to prevent digestion of the chromatography paper during loading. The samples were centrifuged at high speed for 5 min in an MSE Micro Centaur microfuge and the supernatant drawn off. The solid residues were washed with 200 µl H_2O and centrifuged again at high speed for 5 min; the supernatants were retrieved, pooled with the original ones, and loaded onto the chromatogram (sect. 2.5.1.c).

2.5.1.c: Chromatography system for pectin digests

Aliquots (225 µl) of the pooled supernatants (sect. 2.5.1.b) were loaded onto Whatman 3MM chromatography paper. The chromatogram was run first in butanol(Fisons)/acetic acid/water (12:3:5), dried, then run in ethyl acetate(Fisons)/pyridine/water (8:2:1) and allowed to dry. The developed chromatogram was sprayed with methanol, dried, then stained with "Wilson's Dip" (aniline hydrogen-phthalate stain, Fry, 1988) (sect. 2.5.1.e), and heated at 105 °C for 5 min to develop the colours.

2.5.1.d: Analysis of pectin digest chromatograms

The density of the monosaccharide spots which appeared on the developed chromatogram after staining with "Wilson's Dip" (sect. 2.5.1.e) were assessed visually on a scale of 0 - 5 according to the intensity of staining, which was indicative of the concentration of sugar present: 0 indicated that a sugar was absent, while 5 indicated a heavy deposit.

2.5.1.e: Composition of aniline hydrogen-phthalate stain ("Wilson's Dip") for monosaccharides and reducing disaccharides

The stock solution used contained the following reagents in the proportions shown below:

Phthalic acid	16 g
Acetone	490 ml
Diethyl ether	490 ml
H_2O	20 ml

The stock solution was kept in a tightly stoppered bottle; immediately prior to use 100 ml of this solution was mixed with 0.5 ml aniline.

2.5.1.f: Chromatography and counting systems for [^{14}C]-labelled pectins and hemicelluloses

1 ml aliquots of the samples, original media and subsequent rinses, were loaded as 4 cm streaks on Whatman 3MM chromatography paper, dried, and run overnight in ethyl

acetate/acetic acid/water (10:5:6). The chromatogram was dried, cut into sections and scintillation-counted. The sections were 5 cm wide and were cut as follows: Section 1: lcm behind, to 1.5 cm in front of the origin line; Sections 2 - 7, 7 cm lengths forward of Section 1; and Section 8, approximately 4 cm long and, due to the serrations at the distal end of the chromatogram, of variable size.

2.6: *IN VITRO* GRAFTING

2.6.1: Procedure for making split-plates for grafting

The procedure used was that of Parkinson and Yeoman (1982): 12 mm wide aluminium dividers were individually wrapped in foil and autoclaved in a metal box. The box was transferred to a sterile environment where all further manipulations were performed with sterile techniques and equipment. Each divider was unwrapped and placed across the centre of a sterile triple-vented 9 cm 101VR20 Sterilin petri dish; the petri dishes were closed and a mark made on the outside of the dish to show which half would contain +IAA agar. The IAA solution had previously been filter-sterilised into a sterile bottle and was added to half the agar after autoclaving.

Approximately 3 - 5 ml of the medium was poured into the appropriate side of each dish, and encouraged to seal the join between the edge of the divider and the surface of the plate; once this had cooled the process was repeated with the other medium on the other side of the plate. Once both sides had set, the two halves of each plate were filled with the appropriate medium. After cooling the dividers were removed, leaving a gap into which grafts would be placed.

2.6.2: *In vitro* grafting protocol

The procedure followed was the split-plate system developed by Parkinson (Parkinson and Yeoman, 1982; Parkinson, 1983). Plants of *L. esculentum* AC and *N. physaloides*, grown under greenhouse conditions in trays of John Innes No. 1 compost, were suitable for use once the first five true leaves had emerged, about 4 - 6 weeks after planting. The internode between the nodes of the cotyledons and first true leaf was excised with a razor blade, and the cut ends occluded with molten paraffin wax (melting point 49 °C). The internodes were immersed in 10% (v/v) solution of commercial hypochlorite (10 - 14% free chlorine) for 10 min and transferred to a sterile environment for the rest of the operation; at all times manipulations were performed with sterile equipment and procedures. The internodes were washed by immersion in three rinses of sterile water, and transferred to sterile petri dishes.

The wax ends of the internodes were cut off, the internodes were cut into 7 mm long segments which were inserted down either end of a 12 mm length of versilic rubber tubing, which provided mechanical support until the grafts became established. Five grafts were

placed in each split-plate petri dish (sect. 2.6.1) with the apices embedded in the indole-3-acetic acid (IAA)-containing agar and the distal ends in the non-IAA agar; the polarity of the internodes and segments was maintained at all times. The plates were sealed with Parafilm (Sigma)and transferred to a growth room where they were maintained in an upright position with the graft apices uppermost and incubated at 25 °C in continuous light at 8 µmol m^{-2} s^{-1}.

2.6.3: Measurement of the pH of apoplastic fluid in the GU

Measurements of the pH of apoplastic fluid from the GU were taken between 1 - 7 d after grafting. Grafts were removed individually from split-plates, and the versilic rubber tubing was cut away carefully, with a scalpel, to release the graft. Forceps were used to separate stock and scion. 5 µl deionised water was applied to one exposed grafting face, retrieved and placed in the sampling-well of a pH Boy-C1 (CamLab Ltd.). A sample was taken from the other grafting face, pooled with the original sample, and the pH measured. Readings could be obtained with a minimum sample volume of 3 µl provided that the droplet was in contact with the reference electrode in the sampling-well.

In the early stages of development of this technique some difficulty was experienced in retrieval of samples from segments of *N. physaloides*, since the deionised water droplets would flow into the hollow stem. Loss of sample was prevented by ensuring that the distal end of stock or scion was blocked thoroughly by the application of a thumb prior to the application of deionised water to the grafting face. The grafting faces were not touched, except by the deionised water, during the sampling procedure.

2.7: EXPERIMENTS WITH [^{14}C]-LABELLED CELL WALLS OF *L. ESCULENTUM X PERUVIANUM*

2.7.1: Manufacture of [^{14}C]-labelled cell walls of L. *esculentum* x *peruvianum*

1.5 ml of D-[U-^{14}C] glucose (Amersham) at a radioactive concentration of 200 µCi/ml (specific activity = 292 mCi/mmol), in 3% ethanol was dried down in a 250 ml conical flask. 50 ml TOM-I medium, containing 10% of the normal level of glucose, was added to the flask. 100 µl of the resulting radioactive medium was removed and scintillation-counted to determine its activity. The medium was then autoclaved at 121°C and 1.4 bars (20 psi) for 19 min.

Under sterile conditions, the D-[U-^{14}C] glucose medium was inoculated with suspension-cultured cells of *L. esculentum* x *peruvianum*, which had been filtered and rinsed with glucose-free TOM-I medium to remove any normal strength TOM-I medium. The cells were incubated under standard conditions (sect. 2.1.3) for 60 h, then filtered and plunged into phenol/acetic acid (5:2). Deproteinated cell walls, pectins and hemicelluloses were

then extracted as described in sections 2.4.1, 2.4.2 and 2.4.3. Calculations based on the number of counts in 100 µl unused [^{14}C] glucose TOM-I and the counts extracted in PAW rinses indicated that a minimum of 40 µCi of the 250 µCi available in the D-[U-^{14}C] glucose had become incorporated into the cell walls.

2.7.2: General protocol for polysaccharide binding assays with [^{14}C] cell wall components on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC

Suspension-cultured cells (50 mg ± 2 mg) of *L. esculentum* x *peruvianum*, or *L. esculentum* AC were weighed into 0.5 ml aliquots of fresh TOM-I, fresh SH, or 10 mM tartaric acid (TART) medium containing [^{14}C]-labelled cell wall components (0.5 mg/ml). These were incubated for 3 h in darkness, at 25 °C on a Certomat M shaker rotating at 200 rpm. At the end of the incubation 3 ml of [^{14}C] polysaccharide-free medium, fresh TOM-I, fresh SH, or TART as before was added to each sample as the first of three rinses to elute unassociated [^{14}C]-labelled polysaccharides from the cells (sect. 2.7.3). The cells were pelleted by centrifugation for 5 min at 3 000 rpm. The supernatant was removed carefully, and the cells rinsed twice more in the same fashion: the individual supernatants were retained.

Cells were either sampled intact, or were fractionated into soluble and insoluble fractions. Pelleted cells which were to be sampled intact were resuspended in 2 ml of incubation medium free from [^{14}C]-labelled polysaccharides, and washed by filtration on a Millipore 12-port filtration assembly. Each sample tube was rinsed out with a further 2 ml medium: these rinses removed the cells from the tubes onto the Whatman GF/C 2.5 cm glass microfibre filters and did not serve a specific elution function, so were not retained. The filters were dried and scintillation counted in Non-Triton scintillant on a Beckman LS 5000CE scintillation-counter. Aliquots of 1 ml of the supernatants retained from the rinses were also counted in Triton scintillant (sect. 2.11.4).

Cells which were to be fractionated into soluble and insoluble fractions were frozen in a -40^{0}C deep-freeze for a minimum of 1 h, thawed and sonicated on ice for 1.5 min (3 x 30 s). The ruptured cells were rinsed in 3 x 3 ml of 10 mM MES (pH 6.0) (or other eluant as specified in the text), and pelleted by centrifugation at 3 000 rpm for 5 min. Supernatant fractions were retained as before, and were considered to contain the bulk of membranes and soluble cell contents. The remaining pellet of solid matter, composed primarily of cell walls, plasmalemmas and larger organelles, was washed by filtration as before, oven-dried at 40 - 50 °C and scintillation counted. Aliquots of 1 ml of each supernatant from the rinses were counted in Triton scintillant. In all cases the means were obtained from three replicates.

In addition to the measurements already indicated, 2 x 0.5 ml of the [^{14}C]-polysaccharide media were scintillation counted to provide an independent assessment of the total number of cpm supplied to the cells, which could then be compared with the number of counts retrieved. The number of counts contained in 0.5 ml of the media are shown below:

[^{14}C]-pectic TOM-I	22 336 cpm
Centrifuged [^{14}C]-pectic TOM-I	15 637 cpm
[^{14}C]-pectic SH	20 024 cpm
[^{14}C]-hemicellulosic TOM-I	1 409 cpm
[^{14}C]-hemicellulosic SH	1 500 cpm
[^{14}C]-hemicellulosic TART	2 474 cpm

2.7.3: Rinsing procedure for cells and cell walls incubated with [^{14}C]-labelled cell wall components in polysaccharide binding assays

At the end of the incubation period the substrate, suspension-cultured cells or cell walls, which had been incubated with [^{14}C]-labelled cell wall components, was rinsed to remove unattached [^{14}C]-labelled cell wall components. 3 ml of polysaccharide-free incubation medium was added to each sample tube, tubes were gently agitated, and centrifuged in an MSE Centaur 2 benchtop centrifuge at 3 000 rpm for 5 min. A maximal amount of each supernatant was removed and retained. A further 3 ml of polysaccharide-free medium was added to the sample tubes and the process repeated twice more. Cells which were to be sampled intact were then filtered, oven-dried at 40 - 50 °C and scintillation-counted in Non-Triton scintillant (sect. 2.11.3) on a Beckman LS 5000CE scintillation-counter.

Rinses performed after any form of pre-incubation, or further processing, e.g. rupturing and washing with 10 mM MES, followed the pattern outlined above.

2.7.4: Method for wetting deproteinated cell walls for use as substrates in experiments with [^{14}C]-labelled cell wall components

A mass of deproteinated cell walls was weighed into a 5 ml round-bottomed polypropylene cryotube (Greiner) to which 3 ml of a distilled water and polyoxyethylene-sorbitan monolaurate (Tween 20) (Sigma) mixture (100 ml:1 drop) were added. The tubes were shaken overnight at 18 °C on a Luckham Model 802 Suspension Mixer (Luckham Ltd.). Once the cell walls had been thoroughly wetted the tubes were centrifuged at 3 000 rpm for 10 min, the supernatant (water + Tween) was removed. The cell walls were rinsed four times in 3 ml aliquots of distilled water. Each time the tubes were centrifuged, as before, to pellet the cell walls, and the rinsing water pipetted off. All supernatants were discarded.

2.7.5: Method for producing centrifuged [^{14}C]-pectic TOM-I medium

~ 1 ml aliquots of [^{14}C]-pectic TOM-I medium were placed in 1.5 ml Eppendorf tubes and spun in an MSE Micro Centaur microfuge at high speed for 10 min. Undissolved pectic solids pelleted out and the supernatant was carefully drawn off and pooled before use in [^{14}C]-labelled polysaccharide binding assays.

2.8: CHEMICAL TESTS

2.8.1: Ethanol test for the detection of polysaccharides

The test used was that of Fry (1988). 0.2 ml of the aqueous solution to be tested was mixed with 10 µl 10% ammonium formate solution (w/v) and 1 ml ethanol, and left to stand for 1 h. The presence of a precipitate or turbidity in the solution after 1 h was indicative of the presence of polysaccharides. When used during the extraction of hemicellulose (sect. 2.4.3) the 0.2 ml of 6 M NaOH washings were diluted with 0.8 ml H_2O, prior to the addition of ammonium formate and ethanol, since NaOH concentrations greater than 3 M can induce a false positive result.

2.8.2: Phenol-sulphuric acid test for total carbohydrate

The method used was that of Dubois and colleagues (Dubois, Gilles, Hamilton *et al.*, 1956): to each 0.4 ml aliquot of the fractions to be tested 10 µl of phenol (80% w/w) was added, followed by 1 ml of H_2SO_4 (conc) (BDH), the latter was pipetted directly into the sample to maximise mixing. The solutions were shaken cautiously and left to cool for 30 min. The A_{490} of the fractions was measured using a Corning Colorimeter 252 (Evans Electroselenium Ltd.); absorbance was directly proportional to the carbohydrate concentration of the sample.

2.8.3: Ammonium formate test for detection of protein

1 ml of the filtrate to be tested was added to a test-tube containing 5 ml acetone plus 50 µl 10% ammonium formate solution in water (final concentration ammonium formate 0.0001% w/v). After thorough mixing the tube was incubated on ice for 1 h. The presence of a white precipitate after 1 h indicated that protein was present in the filtrate and that further PAW washes would be required (Fry, 1988).

2.9: METHODS FOR INTRODUCING CELL WALL COMPONENTS TO AQUEOUS MEDIA

2.9.1: Deproteinated cell walls

A mass of deproteinated cell walls was weighed out and placed in an empty flask. A minimal volume of ethanol, <1% of final volume, was used to "wet" the cell walls, and the remaining total volume of medium added. The medium was stirred until the cell walls formed a smooth suspension in the liquid.

2.9.2: Pectins

A minimal volume of ethanol, <1% of final volume, was used to "wet" the freeze-dried pectic material prior to addition of the aqueous medium. Prolonged stirring, 1 - 2 h, assisted passage of pectins into solution, but, since the final solution was always cloudy it is possible that not all the pectic material dissolved.

2.9.3: Pectic fragments

Pectic fragments did not dissolve readily in aqueous media, but prolonged stirring ensured that the maximum possible did enter solution.

2.9.4: Hemicelluloses

Hemicelluloses dissolve very readily in aqueous media, therefore, the required mass of hemicellulose was simply added to the final volume of medium. After a brief period of stirring the medium could be used.

2.10: COMPOSITION OF MEDIA FOR CELL CULTURES AND GRAFTING

2.10.0: Note about media

All quantities outlined below are for 1 L final volumes of media. In all cases the volume was made up to 1L with distilled water, pH was adjusted to 5.7 with NaOH, and the medium autoclaved at 121°C and 1.4 bars (20 psi) for 19 min.

2.10.1: TOM-I medium for suspension-cultured cells of *L. esculentum* x *peruvianum*

The TOM-I medium for suspension-cultured cells of *L. esculentum* x *peruvianum* contained the following reagents in the proportions shown below:

	L^{-1}
Murashige and Skoog basal salt mixture Sigma (cat. no. M5524)	4.4 g
Glucose	20 g

IAA (Sigma)	10 mg
Kinetin (Sigma)	0.5 mg
Nicotinic acid (BDH)	0.5 mg
Pyridoxine hydrochloride (BDH)	0.5 mg
Thiamine hydrochloride (Sigma)	0.1 mg
Glycine (Sigma)	2.0 mg

2.10.2: CI medium for initiation of *L. esculentum* AC and *N. physaloides* calluses

The MS medium for initiation of *L. esculentum* AC and *N. physaloides* calluses contained the following reagents in the proportions shown below:

	L^{-1}	
Murashige and Skoog salts (Flow Labs. cat. no. 26-100-24)	4.7	g
Sucrose	20	g
2,4-dichlorophenoxyacetic acid (2,4-D) (Hopkins & Williams Ltd.)	0.221	mg
Kinetin	0.2142	mg
Oxoid Bacteriological Agar no.1 (Unipath Ltd.)	12	g

2.10.3: SH medium for maintenance of *L. esculentum* AC and *N. physaloides* calluses

The SH medium for maintenance of *L. esculentum* AC and *N. physaloides* calluses contained the following reagents in the proportions shown below:

	L^{-1}	
Schenk and Hildebrandt salts (Imperial Laboratories Prod. no. 9-650-50)	4.46	g
Sucrose	30	g
2,4-D	0.5	mg
p-chlorophenoxyacetic acid (CPA) (Sigma)	2.0	mg
Kinetin	0.1	mg
Oxoid Bacteriological Agar no.1	10	g

2.10.4: SH medium for suspension-cultured cells of *L. esculentum* AC

The liquid medium used for *L. esculentum* AC suspension-cultures was the same as that used for callus maintenance (sect. 2.10.3), but with the agar omitted.

2.10.5: Split-plate grafting media

Equal volumes of +IAA and -IAA agar are required to make split-plates. The medium contained the following reagents in the proportions shown below:

	L^{-1}	
Murashige and Skoog salts (Flow Labs. cat. no. 26-100-24)	4.7	g
Sucrose	20	g
Kinetin	0.215	2 mg
Oxoid Bacteriological Agar no.1	12	g
+/- IAA (made into solution, filter-sterilised and added to autoclaved medium under sterile conditions)	0.2	mg

2.11: COMPOSITION OF BUFFERS AND SCINTILLATION FLUIDS

2.11.1: Composition of Homogenisation Buffer for membrane extraction

The following reagents were mixed to make 100 ml of the homogenisation buffer used in membrane extraction:

	g/100 ml
3-(N-morpholino)propane-sulfonic acid (MOPS) (Sigma)	1.046
Dithiothreitol (DTT) (Sigma)	0.0462
Ethylenediaminetetra-acetic acid (EDTA)	0.0336
Bovine serum albumen (BSA)	0.5
Sucrose	8.55

The volume was made up to 100 ml with distilled water, and the pH adjusted to 7.2. The solution was chilled to 0 °C before use.

2.11.2: Composition of Rinsing Buffer for membrane extraction

The following reagents make 100 ml of the rinsing buffer used in membrane extraction:

	g/100 ml
MOPS	1.046
DTT	0.0462
Sucrose	8.55

The volume was made up to 100 ml with distilled water, and the pH adjusted to 7.2. The solution was chilled to 0 °C before use.

2.11.3: Composition of Non-Triton scintillant for dry samples

The Non-Triton scintillation fluid used to count dry samples contained the following reagents in these proportions:

PPO (2,5-diphenyloxazole) (BDH)	5 g
POPOP (1,4-di-2(5-phenyloxazolyl)-benzene (BDH)	0.5 g
Toluene	1 L

The constituents were stirred together for 4 h or more until they had dissolved, then stored in darkness.

2.11.4: Composition of Triton scintillant for aqueous samples

The Triton scintillation fluid used to count aqueous samples contained the following reagents in these proportions:

PPO	3.3 g
POPOP	0.33 g
Toluene	667 ml
Triton X-100 (BDH) (iso-octylphenoxypolyethosyethanol)	333 ml

The constituents were stirred together for 4 h or more until they had dissolved, then stored in darkness.

Chapter 3: DEVELOPMENT OF THE ASSAY TO MEASURE L-[U-^{14}C] LEUCINE INCORPORATION INTO PROTEIN

3.0: GENERAL INTRODUCTION

A key hypothesis in this study was that oligosaccharides from Solanaceous species might provide the "off" signal for cell division required for the organised development of compatible grafts. To facilitate manipulation, dosage, and accurate monitoring of events, it was decided to use suspension-cultures as models of the callus cells produced in the GU during grafting. An assay was developed in which rates of cell division were measured indirectly by determination of the incorporation of L-[U-^{14}C] leucine into protein by cells during the test period. At the start of the study the only *L. esculentum*-containing suspension-culture available was one of *L. esculentum* x *peruvianum* (sect. 2.1.2). Callus cultures of *L. esculentum* c.v. Ailsa Craig (*L. esculentum* AC) and *N. physaloides* were initiated so that, once suspension-cultures had been generated, these could be substituted into the assay in place of the hybrid cells.

3.1: ESTABLISHMENT OF *L. ESCULENTUM* AC AND *N. PHYSALOIDES* SUSPENSION-CULTURES

3.1.1: **Summary:** Establishment of suspension-cultures

Suspension-cultures of *L. esculentum* AC and *N. physaloides* were required to provide biological material on which to perform the proposed L-[U-^{14}C] leucine incorporation assays. Callus cultures of both species were established (sect. 2.1.1) and maintained on Schenk and Hildebrant callus maintenance medium (sect. 2.10.3). Callus of *L. esculentum* AC was successfully transferred to the liquid version of the Schenk and Hildebrant callus maintenance medium, however, attempts to transfer it to other liquid media failed, as did all attempts to introduce callus of *N. physaloides* to suspension culture. The *N. physaloides* callus cultures inexplicably declined and died. Further attempts to create new cultures failed, and consequently no suspension-cultures of *N. physaloides* were produced.

3.1.2: **Introduction:** (see General Introduction above)

3.1.3: **Materials and Methods:** Establishment of suspension-cultures

Callus of *L. esculentum* AC and *N. physaloides* established on Murashige and Skoog callus initiation medium (sect. 2.10.2) and SH callus maintenance medium (sect. 2.10.3) were

introduced to a variety of liquid media, under sterile conditions, using a generous level of inoculum.

3.1.4: Results: Establishment of suspension-cultures

3.1.4.a: Establishment of *L. esculentum* AC tissue and cell cultures

3.1.4.a.i: Transfer of *L. esculentum* AC from CI (s) to TOM+I (liq)

Callus of *L. esculentum* AC was introduced to the standard suspension-culture medium, TOM -I (sect. 2.10.1), with the addition of inositol at 100 mg/l (= TOM+I). Initially, the cultures grew well, but within a week the cells began to produce considerable quantities of phenolic substances and to decline. Cells of the parent tissue did not separate in solution, so it was not possible to perform viability tests. No evidence of growth and division was visible after the first week, and death occurred within 6 weeks of the initiation of the suspension culture.

3.1.4.a.ii: Transfer of *L. esculentum* AC from CI (s) to CI (liq)

An agar-free version of the CI (s) medium (sect. 2.10.2) on which callus of *L. esculentum* AC had been initiated was tested as a suspension culture medium. Addition of tissue to the CI (liq) medium appeared to cause cell division to cease, induced production of phenolics and deterioration in the condition of the cells.

Production of phenolics had been a problem in *L. esculentum* AC callus grown on solid medium, but was exacerbated in liquid culture. Two weeks after culture initiation in CI (liq) medium cells were resuspended in CI (liq) medium containing 1 mM ascorbic acid [TOM CI (liq)] in an attempt to control the production and deleterious effects of the phenolics, but to little avail, as the cultures died shortly after.

3.1.4.a.iii: Transfer of *L. esculentum* AC from SH (s) to various liquid media

After several months of culture a phenolic-free cell line of *L. esculentum* AC arose spontaneously from callus growing on SH (s) callus maintenance medium. This cell line, 8b, was isolated, bulked up and used to inoculate flasks of TOM+I (liq), TOM CI (liq) and SH (liq) media, an agar-free version of SH(s) medium (sect. 2.10.4).

Suspensions of cell line 8b in TOM CI (liq) medium suffered from over-production of phenolics within the first two weeks, and were discarded. 8b tissue incubated in TOM+I (liq) medium survived and was transferred to TOM-I medium, however growth rates were very slow in both TOM-I and TOM+I media.

Tissue of 8b transferred to SH (liq) displayed the best growth rates, colour and texture, so it was decided to continue attempts to establish suspension-cultures of *L. esculentum* AC in this medium and to abandon the others. Further phenolic-free lines of *L. esculentum* AC

tissue arose. These adapted to culture in SH (liq) medium better than the earlier line, and displayed much more vigorous growth rates, similar to those exhibited by cell cultures of *L. esculentum* x *peruvianum*. All assays performed on cells of *L. esculentum* AC used one of the later cell lines, 3bb.b., which required subculturing every 10 - 21 d depending upon the level of the original inoculum.

3.1.4.b: Establishment of *N. physaloides* tissue and cell cultures

3.1.4.b.i: Transfer of *N. physaloides* from CI (s) into TOM+I (liq) medium

Callus of *N. physaloides* grown on CI (s) medium was introduced to TOM+I (liq) medium. Early evidence of cell division was not sustained, and the tissue died after several weeks, despite regular replenishment of nutrients. FDA viability tests (sect. 2.2.3) performed within the first week of culture revealed no live cells.

3.1.4.b.ii: Transfer of *N. physaloides* from CI (s) into CI (liq) medium

Callus of *N. physaloides* grown on CI (s) medium was introduced to CI (liq) medium. Survival was higher in CI (liq) medium than in TOM+I (liq) (sect. 3.1.4.b.i), but there was no evidence of growth or cell division, despite regular changes of medium. The cells died within three months.

3.1.4.b.iii: Further attempts to introduce *N. physaloides* to suspension culture

Further attempts to introduce *N. physaloides* to suspension culture ceased due to the decline and death of the callus cultures. All measures adopted to revive the culture were unsuccessful. Several attempts to re-initiate *N. physaloides* callus cultures failed, and the resulting lack of inoculum meant that this part of the experimental approach had to be abandoned.

3.1.5: Discussion: Establishment of suspension-cultures

The failure to develop *N. physaloides* suspension-cultures was a disappointment, since it meant that a range of planned reciprocal experiments involving cells of the graft-incompatible species was impossible to perform. However, valuable information could still be obtained with the *L. esculentum* AC culture, which would indicate whether it would be worthwhile pursuing the establishment of suspension-cultured *N. physaloides* at a later date.

3.2: DEVELOPMENT OF GROWTH CURVES

3.2.1: **Summary:** Growth curves

A series of measurements were made on suspension-cultures of *L. esculentum* x *peruvianum* and *L. esculentum* AC to determine the time after subculture at which rates of cell division rates were maximal, when the effects of test substances in the proposed L-[U-^{14}C] leucine incorporation assay was expected to be most pronounced. Measurements on *L. esculentum* x *peruvianum* of settled and packed cell volumes, cell mass, cell numbers and viability indicated that this logarithmic phase occurred 5 - 7 d after subculture and was followed by a period of rapid cell growth between days 7 - 9. Measurements of cell numbers on cells of *L. esculentum* AC also suggested that the logarithmic phase occurred between 5 and 7 d after subculture. Eight-day old cells were therefore used in the L-[U-^{14}C] leucine incorporation assays in order to combine maximal rates of cell division with rapid growth.

3.2.2: **Introduction:** Growth curves

It was necessary to determine when, after subculturing, the cultures of *L. esculentum* x *peruvianum* and *L. esculentum* AC would enter the logarithmic phase of cell division, when division rates peak, as any effects of exogenous substances on the rate of cell division, and presumably protein synthesis, would be most pronounced at this point.

3.2.3: **Materials and Methods:** Growth curves

3.2.3.a: **Cultures of *L. esculentum x peruvianum***

Five flasks containing 75 - 80 ml TOM-I (liq) medium were inoculated with 10 ml cell suspension from a single culture of *L. esculentum* x *peruvianum* and grown under standard conditions (sect. 2.1.3) for the duration of the investigation. Approximately 5 ml of culture fluid and cells were removed from each flask at various times after sub-culturing with a glass syringe; maintaining sterile conditions. The volume, settled and packed cell volumes (scv and pcv) (sect. 2.2.1) were measured for each of the 5 ml samples. Cells in each sample were resuspended, filtered, weighed and the measured mass, of approximately 50 mg, added to chromic acid for cell counting (sect. 2.2.2). Towards the end of the monitored period the viability of sampled cells was assessed (sect. 2.2.3).

The measurements were carried out over a 14 d period, 0, 1, 2, 3, 5, 7, 9, 11 and 14 days after subculture. From 9 d onwards samples taken by syringe were clearly not representative of the composition of the culture, since at high cell densities the mouth of the syringe became blocked by the rush of cells into it, resulting in the preferential extraction of medium and consequent under-representation of cells. In situations where

adjustments to calculate the correct values could not be made any obvious anomalies were excluded from analysis (see sects. 3.2.4.a.i, ii and iii).

3.2.3.b: Cultures of *L. esculentum* AC

For analysis of suspension-cultured cells of *L. esculentum* AC, four cultures in SH (liq) medium, were subcultured to the same starting density in fresh medium. The zero time samples (t = 0) were then taken from the parent cultures with a sterile tissue-culture draining-spoon, which was used to obtain all samples. Care was taken to maintain the sterility of the cultures for the 20-d duration of the test.

The assessment procedures for suspension-cultured cells of *L. esculentum* x *peruvianum* had proved lengthy and certain sampling problems had been encountered. For evaluation of suspension-cultures of *L. esculentum* AC the only criteria monitored were the number of cells in 50 mg samples of filtered cells, and the viability.

3.2.4: Results: Growth curves

3.2.4.a: Suspension-cultures of *L. esculentum* x *peruvianum*

3.2.4.a.i: Settled cell volume and packed cell volume measurements

The scv and pcv were assessed (sect. 2.2.1). Disparities in the original sample volumes extracted by glass syringe were compensated for by standardisation of the values obtained using equation 1, which could normalise variations in sample volumes to the 5 ml equivalent, but could not correct for under-representation of cells and over-representation of medium:

Equation 1:

$$\text{adjusted scv or pcv for 5 ml sample (ml)} = \frac{\text{measured scv or pcv (ml)}}{\text{actual volume of sample (ml)}} \times 5 \text{ (ml)}$$

The means of the adjusted figures obtained with cells of *L. esculentum* x *peruvianum* are displayed in Fig. 3.2.4.a.i. Scv and pcv paralleled each other closely throughout the duration of the test. The Sigmoid curve obtained from the measurements indicated that *L. esculentum* x *peruvianum* growth followed the normal lag-log-lag pattern for suspension-cultures. The rate of increase was most rapid between 5 - 9 d after subculture, and slowed abruptly between 9 - 11 d, which coincided with the commencement of the second lag phase and was the point at which extraction of representative samples became difficult. In addition, differences in uniformity of cell packing between samples, which persisted after centrifuging, rendered the data less accurate than desired.

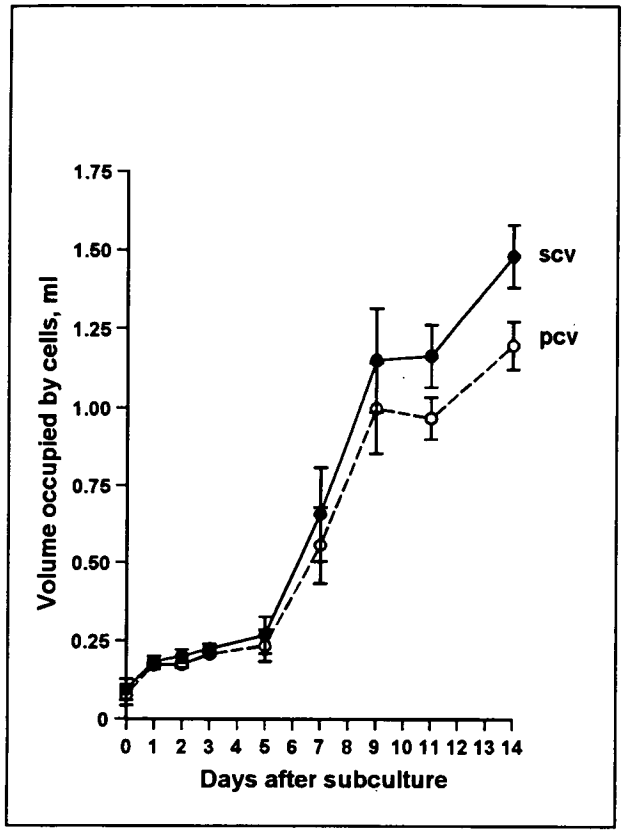


Figure 3.2.4.a.i:

Settled and packed cell volumes (scv and pcv) of 5 ml culture samples taken from suspension-cultures of *L. esculentum* x *peruvianum* 0 - 14 days after subculture.

Means were obtained from single samples taken from 5 cultures at each time point.

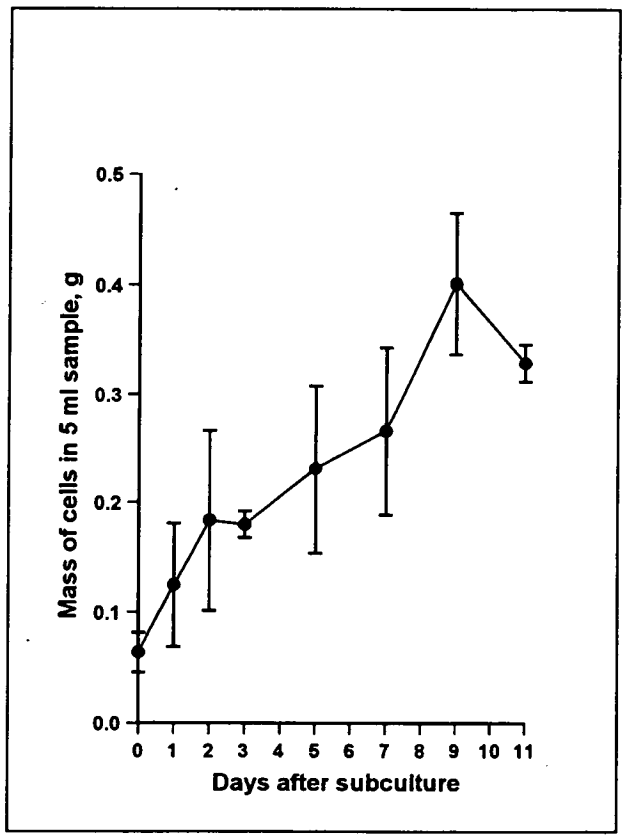


Figure 3.2.4.a.ii:

Mean mass of suspension-cultured cells of *L. esculentum* x *peruvianum* present in 5 ml samples of culture fluid.

Means were obtained from single samples from each of 5 cultures at each time point, except for the 5 d and 11 d values which were obtained from 3 samples.

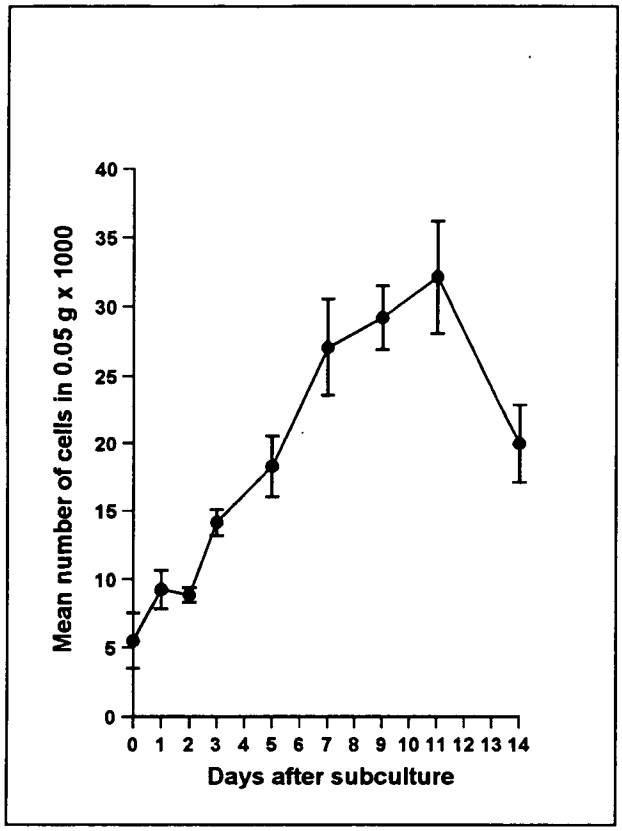


Figure 3.2.4.a.iii:

Cell number in 0.05 g sample of cells of *L. esculentum* x *peruvianum* 0 - 14 days after subculture.

Means were obtained from a mimimum of 6 and maximum of 8 counts of a haemocytometer grid for each of 5 suspension-cultures at each time point.

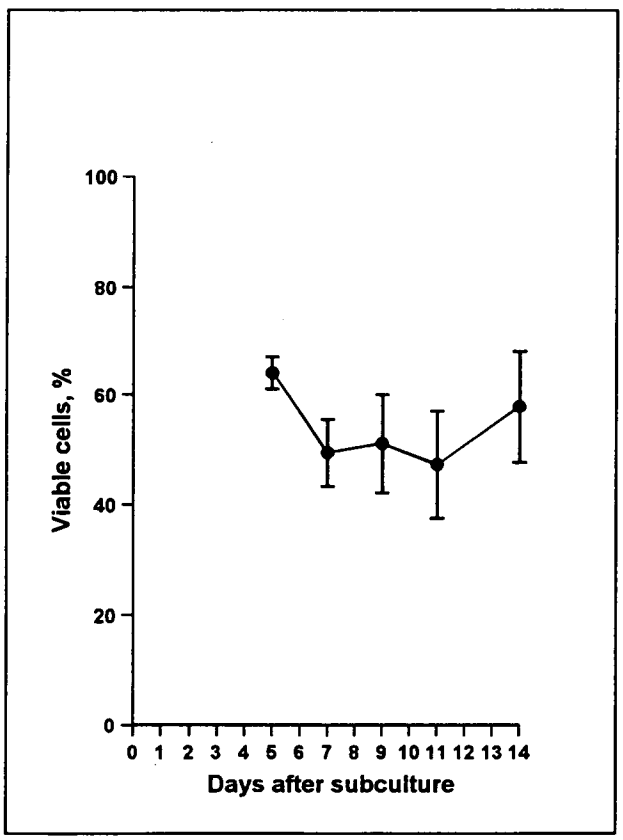


Figure 3.2.4.a.iv:

Viability of suspension-cultured cells of *L. esculentum* x *peruvianum* sampled 5 - 14 days after subculture.

Means were obtained from samples from 5 cultures except for days 5 and 7, which were obtained from 3 cultures.

3.2.4.a.ii: Cell mass in 5 ml samples

The mean mass of cells of *L. esculentum* x *peruvianum* extracted in 5 ml culture fluid was standardised to compensate for errors in the sample volumes using equation 2, which was subject to the same limitations as equation 1 (sect. 3.2.4.a.i):

Equation 2:

$$\text{adjusted mass for 5 ml sample (g)} = \frac{\text{mass of original sample (g)}}{\text{actual volume extracted (ml)}} \times 5 \text{ (ml)}$$

The standardised mean values are shown in Fig. 3.2.4.a.ii. The mass of cells in the samples increased steadily until day 9. The apparent drop in weight at 11 d was probably an artefact introduced by the sampling problems previously mentioned, or due to the difficulties involved in filtering all cell samples to the same degree of dryness. Visual appraisals indicated that the cell density increased steadily throughout the monitoring period, despite the mean obtained at 11 d.

3.2.4.a.iii: Cell numbers

The mass of cells of *L. esculentum* x *peruvianum* added to chromic acid for maceration was weighed and the corresponding cell number for a sample of exactly 50 mg was calculated using equation 3; this was not subject to the limitations of equations 1 and 2 (sects. 3.2.4.a.i and ii) since in this case the ratio of cells to medium was not relevant:

Equation 3:

$$\text{adjusted cell no. for 50 mg sample} = \frac{\text{number of cells in original sample}}{\text{actual mass of cells in cell count sample (mg)}} \times 50 \text{ (mg)}.$$

The mean cell numbers obtained are presented in Fig. 3.2.4.a.iii. Rapid increase in cell number occurred between 3 - 11 d after subculturing. Cell number dropped to ~60% of its peak by day 14. Between days 3 - 11 the average size of cells decreased (cf. pcv + cell number), but by 14 d it had started to increase once more. Some problems were experienced obtaining standard samples for this assessment since cells did not always filter to the same degree of dryness.

3.2.4.a.iv: Cell viability

Viability was measured using the FDA test (sect. 2.2.3) for samples were taken between 5 and 14 d. Mean values for cell viability are shown in Fig. 3.2.4.a.iv. Peak cell viability, 64%, occurred at 5 d after subculturing decreasing slightly thereafter, but remaining around 50%, with values from 49% to 58% between days 7 and 14.

3.2.4.b: Suspension-cultures of *L. esculentum* AC

3.2.4.b.i: Cell number

Adjustments for variation in the mass of filtered cells added to the 0.5 ml chromic acid were calculated as for the suspension-cultured cells of *L. esculentum* x *peruvianum* (sect. 3.2.4.a.iii: equation 3) to obtain cell number for 50 mg samples. The mean cell numbers are illustrated in Fig. 3.2.4.b.i. The cell number increased until day 7, with the most marked increases occurring during days 5-7. An immediate rapid drop in cell number was evident between days 7-9, after which fluctuations in cell number levelled off at about 12 000/50 mg.

3.2.4.b.ii: Cell viability

Cell viability of the cultures was measured on 7 d, 14 d and 20 d after subculturing, by scoring a minimum of 3 x 200 cells extracted from each suspension culture. Daily means for each culture were obtained and pooled to calculate overall means. The results obtained are displayed in Table 3.2.4.b.ii below.

Table 3.2.4.b.ii: Cell viability in *L. esculentum* AC suspension-cultures assessed at 7, 14 and 20 d after subculturing; the percentages of viable cells for each culture at each time point, and means obtained by excluding extreme data are shown.

Percentage viable cells			
Culture/Sample time	**7 d**	**14 d**	**20 d**
A	62.3	87.5	81.0
B	63.0	87.7	84.0
C	70.8	85.5	82.6
D	66.0	88.0	8.8
Mean	65.5	87.2	82.5
± Standard error	±1.9	±0.6	±0.9
n	4	4	3

The general level of viability increased up to 14 d after subculture, and there was no marked decline in viability up to 20 d after culturing.

3.2.5: Discussion: Growth curves

The measurements of scv, pcv, cell mass, and cell number all indicated a similar time frame for the logarithmic phase of suspension-cultures of *L. esculentum* x *peruvianum*.

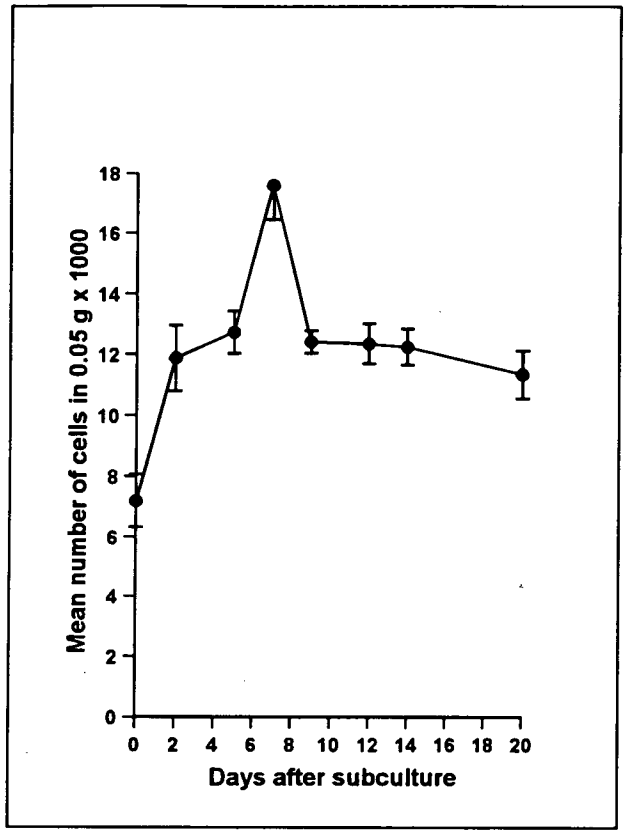


Figure 3.2.4.b.i:

Cell number in 0.05 g sample of cells of *L. esculentum* AC 0 - 20 days after subculture.

Means were obtained from a minimum of 6 and maximum of 8 counts of a haemocytometer grid for each of 4 suspension-cultures at each time point.

Cell number results indicated maximal rates of cell division between 5 and 7 d after subculturing, while those from cell mass measurements indicated that the cell division peak was followed by a peak of cell growth, between 7 and 9 d after subculture. Scv and pcv measurements provided a broader estimate of 5 - 11 d after subculture as the period of maximal division. The constant proportion of viable cells observed throughout the latter part of the experiment indicated that nutrient levels in the medium were not limiting growth or division towards the end of the monitored period.

After careful consideration of the evidence it was decided to use 8 d old suspension-cultured cells of *L. esculentum* x *peruvianum* in the L-[U-^{14}C] leucine incorporation assays, since the combination of high division and growth rates at that point would be expected to be accompanied by high rates of protein metabolism, and would maximise the sensitivity of the assay to changes in protein metabolism induced by test substances.

Cell number measurements on suspension-cultures of *L. esculentum* AC also indicated that the logarithmic phase lay 5 - 7 d after subculture. Cell viability at 7 d after subculturing was not ideal, but was judged to be acceptable, and improved with time. Consequently, it was decided to perform experiments with cultures of *L. esculentum* AC 8 d after subculturing since, as with cultures of *L. esculentum* x *peruvianum*, the division and growth rates would be high and would presumably be accompanied by intense protein metabolism. Furthermore, if 8 d old cultures were used in assays, rather than 7 d old ones, there might be some improvement in the percentage of viable cells present.

3.3: DEVELOPMENT OF THE L-[U-^{14}C] LEUCINE INCORPORATION ASSAY

3.3.1: **Summary:** Development of leucine incorporation assay

The objective was to develop an assay to assess the effects of cell wall polysaccharides upon the rate of cell division in suspension-cultured cells. It was hoped that suspension-cultured cells would provide a suitable model for wound-induced callus cells and that results obtained in this assay could be extrapolated to predict the response of those cells in the GU to these substances. Suspension-cultures of *L. esculentum* x *peruvianum* and *L. esculentum* AC cells were chosen for use, and the fine detail of the procedure defined. It was decided to dispense the cells in suspension, and to incubate them in darkness in fresh native medium (TOM-I or SH) to which test substances would be added.

3.3.2: **Introduction:** Development of leucine incorporation assay

The general outline of a L-[U-^{14}C] leucine incorporation assay to determine the effects of cell wall-derived polysaccharides upon suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC had been developed (see below). Results from the

assay would be extrapolated back to the situation in the GU. However, the assay procedure had to be tuned to ensure the production of consistent results, and to maximise differences resulting from treatments. The growth curves of these cultures (sect. 3.2) had indicated that 8 d old cells would be in an ideal growth phase for this type of assay, but the best choice of incubation conditions or incubation medium had yet to be established.

Initially the medium used in assays was obtained from 8 d old suspension-cultures of the type of cell being tested, i.e. 8-d TOM-I medium for cells of *L. esculentum* x *peruvianum* and 8-d SH medium for cells of *L. esculentum* AC. These "spent" media were employed to minimise the environmental change experienced by the cells on introduction to the assay system, but meant that synergistic and antagonistic action between cell wall fragments present in the "spent" media and the test substances was possible.

Time-saving measures in the setting up of assays were required and were assessed.

3.3.3: Materials and Methods: Development of leucine incorporation assay

3.3.3.i: Original procedure for L-[U-^{14}C] leucine incorporation assay

The original procedure used in development of the L-[U-^{14}C] leucine incorporation assay is presented here, the standard procedure which resulted is described in detail in section 2.3. Samples of approximately 50 mg of suspension-cultured cells were weighed into 0.5 ml of culture medium, and incubated with or without added test substances (concentrations stated in relevant section for individual experiments). Incubation conditions were as follows: sample tubes were wrapped in aluminium foil to avoid differences in intensity of illumination (preliminary assays were performed in the light, but these are specified where included), and rotated at 200 rpm at 25 °C. Routinely, cells were allowed 1 h to acclimatise to the test media prior to the addition of 20 µl of L-[U-^{14}C] leucine containing 0.05 µCi (specific activity = 310 mCi/mmol) to each sample, after which sample tubes were plugged with non-absorbent cotton wool and incubated for 0, 1, 2, and 3 h.

At the end of the incubation period further incorporation of L-[U-^{14}C] leucine into protein was prevented by killing the cells with the addition of excess 10% tri-chloroacetic acid (TCA) (4.5 ml). Samples were left overnight. This procedure precipitated L-[U-^{14}C] leucine-labelled proteins inside the cells. Cells were filtered onto Whatman GF/C 2.5 cm glass microfibre filters. Unincorporated L-[U-^{14}C] leucine was eluted with two 5 ml rinses with TCA and one 5 ml rinse with "cold", non-radioactive leucine (1 g/L). The filters were oven-dried and scintillation-counted in Non-Triton scintillant (sect. 2.11.3) in a Beckman LS 5000CE scintillation-counter for 1 minute. The mean incorporation of L-[U-^{14}C] leucine, and standard errors, of each treatment at each time point were calculated and compared to those of the control treatment to assess the effect of the test substances on leucine incorporation into protein, and hence the rate at which protein metabolism proceeded.

3.3.3.ii: Comparison of culture media used in the leucine incorporation assay

Suspension-cultured cells of *L. esculentum* x *peruvianum* were incubated under standard conditions (sect. 2.3) in either 8-d TOM-I, fresh TOM-I, or 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) (Sigma) buffer at pH 5.8. After the addition of 0.05 µCi L-[U-^{14}C] leucine to the cells, samples were taken at hourly intervals, and were processed according to the standard procedure (sect. 2.3). Results are presented in section 3.3.4.iii.

3.3.4: Results: Development of leucine incorporation assay

3.3.4.i: Inoculation of sample tubes; weighing *versus* cell suspension

In initial experiments filtered cells were weighed into each sample tube and the scintillation counts were adjusted to 50 mg equivalent using equation 4:

Equation 4:

$$\text{adjusted cpm for 50 mg cells} = \frac{\text{cpm for recorded mass of cells (cpm)}}{\text{recorded mass of cells (mg)}} \times 50 \text{ (mg)}$$

This time-consuming process was stream-lined by taking a slight excess of the total volumes of the various media required in an assay (± test substances), to which appropriate masses of cells were added to give a final concentration of 50 mg cells/0.5 ml. Aliquots of the resulting suspensions, 0.5 ml, were pipetted into individual sample tubes using a Gilson pipette, on which the mouth of the C200 (blue) tip had been widened, by cutting off the distal 1 - 2 mm, to allow cells to pass through easily. The cell suspensions were gently agitated during distribution into the sample tubes to ensure uniformity. This method of cell distribution reduced processing times for both assays and analysis of data, and, in repeated experiments (results not presented here), provided results indistinguishable from those produced when cells were weighed into sample tubes.

3.3.4.ii: Comparison of culture media used in the leucine incorporation assay

The comparison of incubation media was made to evaluate any effects on L-[U-^{14}C] leucine incorporation activity in cells of *L. esculentum* x *peruvianum* induced by incubation in 8-d TOM-I medium. The results obtained are shown in Fig. 3.3.4.ii.

Cells incubated in 8-d TOM-I incorporated considerably more L-[U-^{14}C] leucine into protein over the entire 3 h period of the assay than cells incubated in either 10 mM MES or fresh TOM-I, which, 3 h after the addition of L-[U-^{14}C] leucine, had incorporated 77% and 73%, respectively, of the amount of L-[U-^{14}C] leucine incorporated by cells in the 8-d TOM-I medium. Cells incubated in 10 mM MES and fresh TOM-I media showed less variance than those in 8-d TOM-I, and hence consistently smaller standard errors.

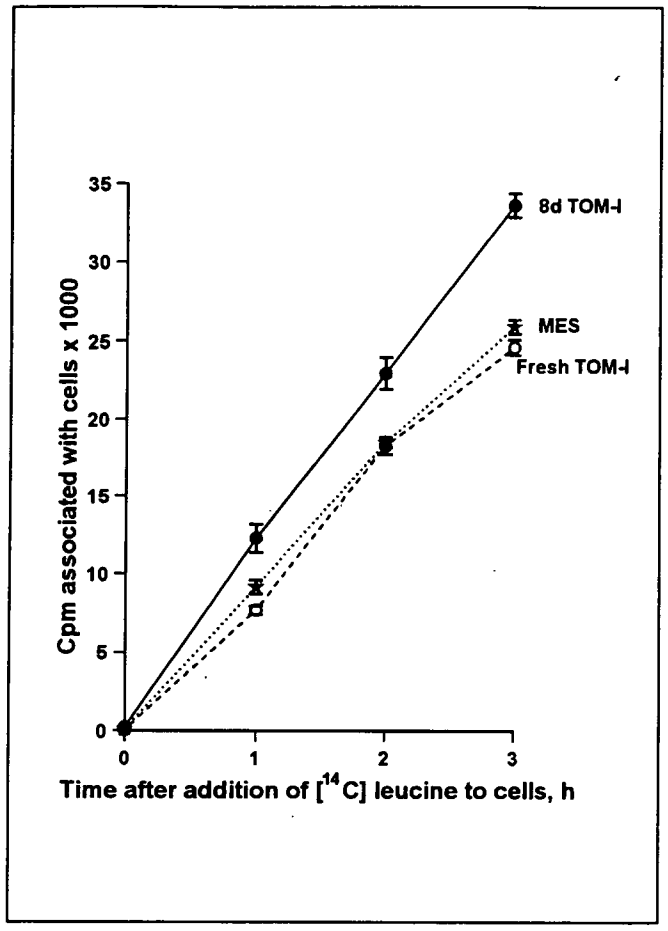


Figure 3.3.4.ii:

Effect of 8-d TOM-I, 10 mM MES and Fresh TOM-I media on the incorporation of [^{14}C]-leucine into protein by suspension-cultured cells of *L. esculentum* x *peruvianum*.

Means were obtained from 4 replicates at 0 h, and 10 replicates at 1 h, 2 h and 3 h.

3.3.5: **Discussion:** Development of leucine incorporation assay

The comparison of media used in the L-[U-^{14}C] leucine incorporation assay reinforces the possibility that oligosaccharides secreted into the medium during culture growth may exert effects, in this case promotional, on the rate of protein metabolism of the cells. The composition of each batch of endogenous cell wall components in 8-d "spent" medium was not known, could not be regulated, and might vary in effect upon cells. Potential interference of this nature could lead to problems in attributing the cause of any change in protein metabolism, either promoted by the test substance alone, or due to synergistic interactions between it and polysaccharides already present in the medium.

Consequently, it was decided to discard 8-d TOM-I medium in favour of fresh TOM-I in further experiments with cells of *L. esculentum* x *peruvianum*, despite the fact that the cells incorporated L-[U-^{14}C] leucine at the greatest rate when incubated in this medium. The use of 8-d SH medium in experiments on cells of *L. esculentum* AC was also abandoned in favour of fresh SH. However, a certain number of L-[U-^{14}C] leucine incorporation assays had been performed by this stage, for which, where presented in this thesis, the fact that 8-d spent medium was used is mentioned, and unless stated otherwise, it should be assumed that fresh TOM-I or SH media were used.

3.4: LOSSES OF L-[U-^{14}C] LEUCINE BY ADSORPTION TO SURFACES OF THE CELLS AND DEPROTEINATED CELL WALLS

3.4.1: **Summary:** Adsorption of L-[U-^{14}C] leucine to cells and cell walls

The adsorption of L-[U-^{14}C] leucine to the suspension-cultured cells and deproteinated cell walls in the L-[U-^{14}C] leucine incorporation assays was measured. About 1% of the amount of L-[U-^{14}C] leucine associated with live cells (less than 0.4% of the mean total counts applied) became absorbed to dead cells, while a maximum of 2% of the amount of L-[U-^{14}C] leucine incorporated by live cells (less than 0.7% of the total mean cpm applied) became adsorbed to deproteinated cell walls. It was concluded that the levels of free L-[U-^{14}C] leucine in the medium would not limit the accuracy of the assays.

3.4.2: **Introduction:** Adsorption of L-[U-^{14}C] leucine to cells and cell walls

For a L-[U-^{14}C] leucine incorporation assay to be useful and accurate the L-[U-^{14}C] leucine must remain freely available to the cells at all times and not be lost by adsorption to the surfaces of the apparatus used. The following experiments were carried out to evaluate the adsorption of free, unincorporated L-[U-^{14}C] leucine to various components of the L-[U-^{14}C] leucine incorporation assay to ensure that the leucine remained available to the cells for the duration of the assay. Unless otherwise specified all experimental details are as outlined in the final protocol for the leucine incorporation assay (sect. 2.3).

3.4.3: Materials and Methods: Adsorption of [^{14}C] leucine to cells and cell walls

3.4.3.i: Adsorption of L-[U-^{14}C] leucine to cells of *L. esculentum* x *peruvianum*

Suspension-cultured cells of *L. esculentum* x *peruvianum* were filtered, killed by immersion for 35 min in 1% glutaraldehyde solution (Agar Scientific Ltd.), refiltered and 50 mg ± 2 mg weighed into sample tubes containing 0.5 ml 8-d TOM-l medium. A control was provided by living cells of *L. esculentum* x *peruvianum* in 8-d TOM-l incubation medium. After the addition of L-[U-^{14}C] leucine the tubes were incubated for 3 h only before the reaction was stopped with TCA, thereafter cells were processed as usual (sect. 2.3).

3.4.3.ii: Adsorption of L-[U-^{14}C] leucine to cell walls

L-[U-^{14}C] leucine was added to tubes containing 0·5 ml 8-d TOM-l medium plus 0·5 mg deproteinated cell walls from either *L. esculentum* x *peruvianum* (+HYBcw) or *N. physaloides* (+NPcw) (sect. 2.4.1). Tubes of 8-d TOM-l containing no cell walls provided the control. Following addition of L-[U-^{14}C] samples were incubated for 3 h, then processed as usual (sect. 2.3).

3.4.4: Results: Adsorption of L-[U-^{14}C] leucine to cells and cell walls

3.4.4.i: Adsorption of L-[U-^{14}C] leucine to cells of *L. esculentum* x *peruvianum*

The means of the counts retained on the filters in association with the dead and live cells are shown in Fig. 3.4.4.i. These data reveal a mean of 302 cpm, maximum 447 cpm, of L-[U-^{14}C] leucine became associated with the dead cells. A mean of 32 035 cpm were associated with the live cells; this mean was accompanied by a greater variance than was seen for the glutaraldehyde-treated dead cells. The radioactivity retained by the dead cells amounted to only 1% of that associated with live cells, and less than 0.4% of the mean total counts, 90 551 cpm, originally applied to each sample.

3.4.4.ii: Adsorption of L-[U-^{14}C] leucine to cell walls

The mean values obtained are shown in Fig. 3.4.4.ii. No major differences were evident in the counts retained on the filters for any of the three treatments. The means retained were 273, 455, and 365 cpm for +HYBcw, +NPcw, and the control media respectively. A maximum of 610 cpm was retained by a +NPcw sample, which equates to approximately 2% of the activity incorporated into protein by live cells during a 3 h incubation and less than 0.7% of the total mean cpm present in the L-[U-^{14}C] leucine added to the tubes initially.

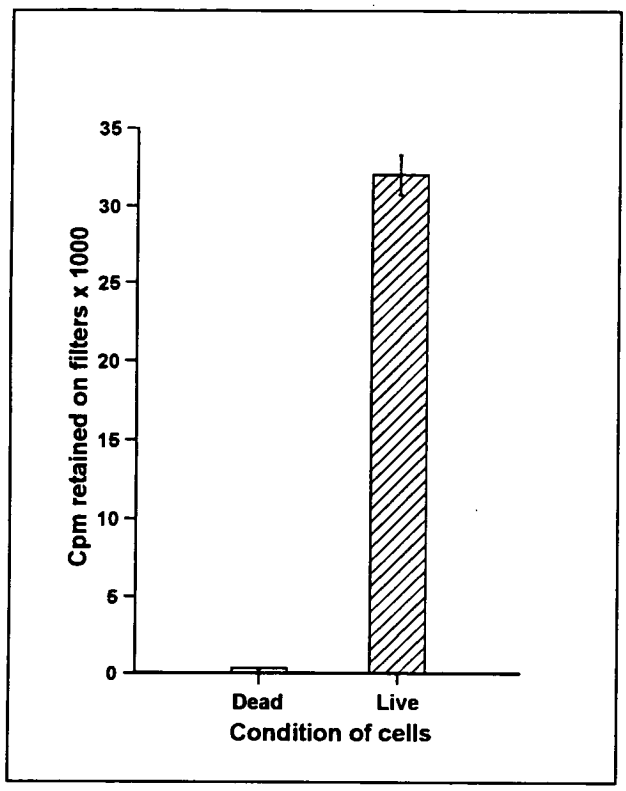


Figure 3.4.4.i:

Adsorption of L-[U-^{14}C] leucine to live and dead suspension-cultured cells of *L. esculentum* x *peruvianum*.

Means were obtained from 6 samples of each treatment.

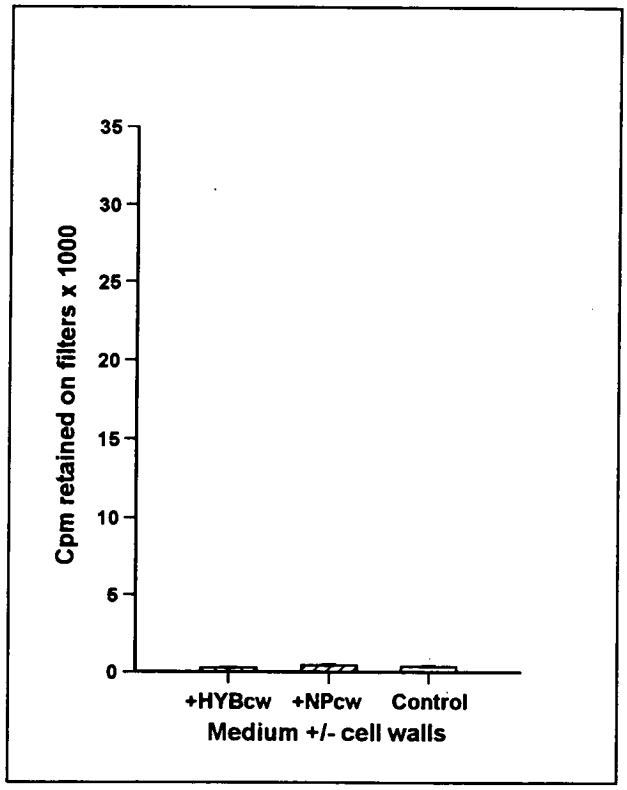


Figure 3.4.4.ii:

Adsorption of L-[U-^{14}C] leucine to cell walls of *L. esculentum* x *peruvianum* and *N. physaloides*.

Means were obtained from 6 samples of each treatment.

3.4.5: **Discussion:** Adsorption of L-[U-^{14}C] leucine to cells and cell walls

The results from these experiments indicate that the amounts of L-[U-^{14}C] leucine which adsorbed passively to cells of *L. esculentum* x *peruvianum* and to deproteinated cell walls were minimal and were not likely to interfere with the interpretation of assays.

In the experiment in sect. 3.4.4.ii, 365 cpm were retained on the filters in the control treatment, from which cell walls were absent. This suggests that the L-[U-^{14}C] leucine which associates with filters may constitute a large part of the radioactivity interpreted as being associated with the cell walls. Therefore, the counts actually associated with the cell walls were probably in the region of 0 - 100 cpm, and even less likely to interfere with the results of future assays than was predicted with the maximal adsorption figures.

3.5: ADSORPTION OF CELL WALL POLYSACCHARIDES TO ASSAY TUBES

3.5.1: **Summary:** Adsorption of polysaccharides to assay tubes

Previously it was shown that L-[U-^{14}C] leucine would remain available to cells throughout a L-[U-^{14}C] leucine incorporation assay (sect. 3.4.4). The availability, to the cells, of the test substances under investigation is of equal importance. Experiments with radioactively-labelled [^{3}H-arabinosyl] pectin and [^{3}H-fucosyl] xyloglucan were performed to establish the ability of two major classes of cell wall polysaccharides to bind to the 5 ml round-bottomed polypropylene cryotubes used in the L-[U-^{14}C] leucine incorporation assay. Very low levels of radioactively-labelled polysaccharides adsorbed to the sides of the tubes: [^{3}H-arabinosyl] pectin adsorbed to a slightly greater degree, 2.9% maximum of total counts retrieved, than [^{3}H-fucosyl] xyloglucan, 2.8% maximum. Radioactively-labelled polysaccharides adsorbed to a considerably lesser degree in 8-d SH medium, 0.54% maximum of total counts retrieved, compared to 2.9% in 8-d TOM-I medium, possibly because of preferential binding by the 8-d SH medium endogenous polysaccharide population. However, the maximum levels of binding were not of a degree likely to interfere with the biological activity, or availability of the cell wall polymers tested in the L-[U-^{14}C] leucine incorporation assays.

3.5.2: **Introduction:** Adsorption of polysaccharides to assay tubes

Test substances must remain available to the cells throughout the incubation period of an assay for their effect upon protein metabolism of suspension-cultured cells to be determined accurately. The strong adsorption of cell wall polysaccharides to a variety of blotting polymers has been documented (Jeffree, 1993), therefore the extent of adsorption of these test substances to the surfaces of the polypropylene assay tubes was evaluated to ensure that adsorption would not interfere with assay results.

3.5.3: **Materials and Methods:** Adsorption of polysaccharides to assay tubes

The polysaccharides tested were [^{3}H-arabinosyl] pectin and [^{3}H-fucosyl] xyloglucan (a major constituent of hemicellulose), which had been extracted from suspension-cultured Rose cells fed with tritiated precursors; these were the kind donation of Dr. C.E. Jeffree.

7 µl [^{3}H-arabinosyl] pectin solution and 10 µl [^{3}H-fucosyl] xyloglucan solution were added separately to 0.5 ml 8-d TOM-I and 8-d SH in 5 ml polypropylene cryotubes (Greiner Labortechnik Ltd.). The tubes were incubated in the dark for 3 h under standard conditions (sect. 2.3). Tubes containing only 8-d TOM-I and 8-d SH provided controls.

At the end of the incubation period the contents of all the tubes were removed and reserved for scintillation counting. One tube from each combination was allowed to dry, then cut into three parts, i.e. the base - the area covered by fluids during incubation, the middle and the top, these latter segments were each half of the remainder of the tube after the base had been removed. The tube sections were scintillation-counted in Non-Triton scintillant on a Beckman LS 5000CE scintillation-counter without further processing.

After the incubation medium had been removed, the remaining tubes were washed with 2 x 2 ml rinses of the appropriate spent medium, free from [^{3}H-arabinosyl] pectin and [^{3}H-fucosyl] xyloglucan. The washings were kept and scintillation-counted, and the tubes were sectioned and counted as above. The original [^{3}H-arabinosyl] pectin-containing and [^{3}H-fucosyl] xyloglucan-containing media (8-d TOM-I, or 8-d SH), and the 2 x 2 ml rinses were counted in Triton scintillant in their entirety; adjustments were made for counting efficiency and subtraction of background levels determined from the results from the control tubes. As the tube segments were scintillation-counted in a non-aqueous medium only background levels were subtracted from the counts obtained.

3.5.4: **Results: Adsorption of polysaccharides to assay tubes**

3.5.4.a: **Controls**

The results from the control tubes which contained only 0.5 ml 8-d TOM-I or 8-d SH media are displayed in Table 3.5.4.a. A maximum of 467 cpm remained in association with the control tubes. This assessment of background counts was used to adjust the number of counts obtained from tubes to which radioactively-labelled polysaccharides had been added.

Table 3.5.4.a: Summary of counts obtained from tube contents, rinses and tubes when 0.5 ml aliquots of 8-d TOM-I and 8-d SH were incubated in Greiner polypropylene 5 ml cryotubes in the absence of radioactively-labelled cell wall components.

Medium only	Rinses (self) (8-d TOM-I or 8-d SH)	Total cpm detected in medium and rinses	Total cpm detected on tube	Total cpm for tube and contents
8-d TOM-I	2 x 2 ml	286.9	85.3	372.1
8-d SH	2 x 2 ml	391.6	75.8	467.4

3.5.4.b: [^{3}H-arabinosyl] pectin solution in 8-d TOM-I medium

The results from tubes which contained [^{3}H-arabinosyl] pectin solution in 8-d TOM-I medium are displayed in Table 3.5.4.b. The total number of counts accounted for from both the rinsed and unrinsed tubes were comparable. 5.4 fold more radioactivity remained on the unrinsed tube than on the rinsed tubes, however this constituted a maximum of 2.9% of the total counts retrieved from the tube. The number of counts retained on the sides of rinsed tubes was equivalent to a maximum of 0.64% of the radioactivity retrieved from them.

Table 3.5.4.b: Summary of counts obtained from tube contents, rinses and tubes when 7 µl aliquots of [^{3}H-arabinosyl] pectin in 0.5 ml 8-d TOM-I medium were incubated in Greiner polypropylene 5 ml cryotubes.

Medium +[^{3}H]-pectin	Rinses (8-d TOM-I)	Cpm detected in medium and rinses minus background at 286.86	Total cpm detected on tube minus background at 85.25 cpm	Total with background subtracted at 372.11 cpm
1. 8-d TOM-I	No rinses	15 427	462.0	15 889
2. 8-d TOM-I	2 x 2 ml	13 952	90.3	14 043
3. 8-d TOM-I	2 x 2 ml	14 550	80.0	14 630
Mean (n = 3)	---	14 643	210.8	14 854
± Std. Error	---	±428.3	±125.7	±554.7

3.5.4.c: [^{3}H-arabinosyl] pectin solution in 8-d SH medium

The results from tubes which contained [^{3}H-arabinosyl] pectin solution in 8-d SH medium are displayed in Table 3.5.4.c.

Table 3.5.4.c: Summary of counts obtained from tube contents, rinses and tubes when 7 µl aliquots of [^{3}H-arabinosyl] pectin solution in 0.5 ml 8-d SH were incubated in Greiner polypropylene 5 ml cryotubes.

Medium +[^{3}H]-pectin	Rinses (8-d SH)	Cpm detected in medium and rinses minus background at 391.61 cpm	Total cpm detected on tube minus background at 75.75 cpm	Total with background subtracted at 467.36 cpm
1. 8-d SH	No rinses	13 897	75.5	13 972
2. 8-d SH	2 x 2 ml	19 732	58.3	19 791
3. 8-d SH	2 x 2 ml	13 793	50.0	13 843
Mean (n = 3)	---	15 837	61.2	15 868
± Std. Error	---	±1 963	±7.5	1 961

The aqueous samples removed from rinsed and unrinsed tubes contained broadly similar numbers of counts. Importantly, more counts remained on the unrinsed tube than on the rinsed tubes amounting to 0.54% of the total counts retrieved from that tube. The number of counts on the sides of the rinsed tubes was equivalent to a maximum of 0.36% of the total retrieved from those tubes.

3.5.4.d: [^{3}H-fucosyl] xyloglucan solution in 8-d TOM-I medium

The results from tubes which contained [^{3}H-fucosyl] xyloglucan solution in 8-d TOM-I medium are displayed in Table 3.5.4.d. The counts remaining on the unrinsed tube equated to 2.8% of the total retrieved from that tube. The maximum percentage associated with the rinsed tubes was 0.4%.

Table 3.5.4.d: Summary of the data obtained when 10 µl aliquots of [^{3}H-fucosyl] xyloglucan solution in 0.5 ml 8-d TOM-l medium in Greiner polypropylene 5 ml cryotubes.

Medium +[^{3}H] xyloglucan	Rinses (8-d TOM-l)	Cpm detected in medium and rinses minus background at 286.86 cpm	Cpm detected on tube minus background at 85.25 cpm	Total with background subtracted at 372.11 cpm
1. 8-d TOM-l	No rinses	52 243	1 514.8	53 758
2. 8-d TOM-l	2 x 2 ml	54 949	240.0	55 190
3. 8-d TOM-l	2 x 2 ml	55 591	137.3	55 728
Mean (n = 3)	---	54 261	630.7	54 892
± Std. Error	---	1 026	443.0	558.8

3.5.4.e: [^{3}H-fucosyl] xyloglucan solution in 8-d SH medium

The results from tubes which contained [^{3}H-fucosyl] xyloglucan solution in 8-d SH medium are displayed in Table 3.5.4.e. Less of the total amount of [^{3}H-fucosyl] xyloglucan accounted for adsorbed to the sides of the unrinsed tube, 0.38%, than to the rinsed tubes, 0.47% maximum.

Table 3.5.4.e: Summary of the data obtained when 10 µl [^{3}H-fucosyl] xyloglucan solution was incubated in cryotubes in 8-d SH medium.

Medium +[^{3}H] xyloglucan	Rinses (8-d SH)	Cpm extracted in medium and rinses minus background at 391.61 cpm	Cpm remaining on tube minus background at 75.75 cpm	Total with background subtracted at 467.36 cpm
1. 8-d SH	No rinses	48 857	184.5	49 041
2. 8-d SH	2 x 2 ml	54 677	258.8	54 936
3. 8-d SH	2 x 2 ml	55 360	205.3	55 565
Mean (n = 3)	---	52 965	216.2	53 181
± Std. Error	---	2 063	22.1	2 078

3.5.5: **Discussion:** Adsorption of polysaccharides to assay tubes

The data from the control tubes, which contained 8-d TOM-I and 8-d SH but no [^{3}H]-labelled pectin or xyloglucan, show that the background counts detected for media and tubes were considerable. Consequently, once the background levels were subtracted from the counts for all other tubes it was seen that the adsorption of [^{3}H]-labelled pectin and xyloglucan to the polypropylene cryotubes was minimal. However, the possibility remains that pectins and other cell wall polysaccharides present in the spent medium might have bound to the available sites on the tubes, and that the radioactively-labelled polysaccharides reveal only a proportion of the actual adsorption which occurred. If, however, the ratio of labelled and unlabelled polysaccharides present in the media were reflected in the proportions of each adsorbed, then these results still provide an indication of the extent of polysaccharide binding to the tubes used in the L-[U-^{14}C] leucine incorporation assays.

The results obtained when [^{3}H]-labelled pectin was incubated in 8-d TOM-I medium suggest that very little [^{3}H]-labelled pectin, 0.64%, remained adsorbed to the internal surfaces of Greiner cryotubes after rinsing. The activity associated with the unrinsed tube demonstrated the maximal extent of binding possible in this system, 2.9%, which is probably an overestimation of the actual level. The extent to which the polysaccharide was cleared from the rinsed tubes by 8-d TOM-I medium suggests that the pectic material remained in solution and that practically all of it would have been available to cells during an assay.

Comparison of the results summarised in Tables 3.5.4.b and 3.5.4.c shows that less [^{3}H-arabinosyl] pectin remained in the unrinsed tube when 8-d SH was used as an incubating medium, 0.54%, than when 8-d TOM-I was used, 2.9%, although the amounts which associated with rinsed tubes in both media were negligible, 0.36% and 0.64% respectively. This phenomenon, with respect to incubation medium, was also observed in the [^{3}H] xyloglucan tests, see Tables 3.5.4.d and 3.5.4.e, where 0.38% of the total counts retrieved remained adsorbed to the unrinsed tube when 8-d SH had been used as the incubating medium, but 2.8% remained on the tubes when 8-d TOM-I was used: once again, the amounts associated with rinsed tubes were negligible, 0.47% and 0.4% respectively. At present there is no explanation available for this phenomenon, unless the polysaccharides naturally present in 8-d SH medium bind preferentially to the tubes and thereby prevent adsorption of the radioactively-labelled polysaccharides. Fortunately, even if this were the case, it seems that the maximum percentages of bound polysaccharides are still low enough to ensure that an adequate amount will remain available to the cells.

Generally the adsorption of xyloglucan to the sides of tubes was minimal, in both media, and probably not sufficient to interfere with the availability of hemicellulosic material to the

cells during the L-[U-^{14}C] leucine incorporation assays. Surprisingly, especially in comparison with the results from all the other tests with [^{3}H-fucosyl] xyloglucan and [^{3}H-arabinosyl] pectin, less [^{3}H-fucosyl] xyloglucan adsorbed to the sides of the unrinsed tube, 0.38%, than to the rinsed tubes, 0.47% maximum when it was applied in 8-d SH medium. This cannot be explained at present and was probably due to experimental error.

Chapter 4: L-[U-^{14}C] LEUCINE INCORPORATION ASSAYS FOR THE EVALUATION OF THE ACTIVITY OF CELL WALL-DERIVED POLYMERS.

4.0: GENERAL INTRODUCTION TO L-[U-^{14}C] LEUCINE INCORPORATION ASSAYS

The first distinct event in a compatible graft, and one which may indicate receipt of a "compatibility" signal, is the cessation of cell division at graft interfaces. The investigation to identify the putative "off" signal for cell division has been assisted by the development of a L-[U-^{14}C] leucine incorporation assay (Chapter 3) in which suspension-cultured cells provide a model of wound-generated callus cells present in the GU, and are used in tests to determine the effect of potential signal substances upon protein metabolism. Extrapolation of results from the L-[U-^{14}C] leucine incorporation assay to the graft itself is required, but use of such a system enabled a wider range of possible trigger-substances to be tested within the time available.

Cell wall components had been proposed as potential candidates for the role of recognition factors (Yeoman, 1984) or controlling substances during graft development (Jeffree, Yeoman, Parkinson *et al.*, 1987) and so the effects of various classes of cell wall-derived compounds were examined on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC using L-[U-^{14}C] leucine incorporation assays.

4.1: EFFECTS OF EXOGENOUS DEPROTEINATED CELL WALLS ON INCORPORATION OF L-[U-^{14}C] LEUCINE INTO PROTEIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC

4.1.1: **Summary:** Effects of cell walls

Polysaccharides present in deproteinated cell walls were thought to be too large to be "read" by cells, consequently, application of deproteinated cell walls to suspension-cultured cells would provide an indication of the base-line variation present in L-[U-^{14}C] leucine incorporation assays. However, the application of cell walls induced sizeable reductions in L-[U-^{14}C] leucine incorporation into protein in cells incubated in the light, or in darkness. 3 h after the addition of L-[U-^{14}C] leucine to the medium, cells of *L. esculentum* x *peruvianum* exposed to self and non-self cell walls exhibited inhibitions of 48% and 25% respectively in leucine incorporation, while cells of *L. esculentum* AC exhibited inhibitions of up to 43% and 42% respectively.

In order to exert an effect the component of deproteinated cell walls responsible for the inhibition of L-[U-^{14}C] leucine incorporation must either have been readily soluble in aqueous media, or have been cleaved enzymically from the exogenous cell walls . Evidence of a species-specific effect for magnitude of inhibition was obtained for cells of *L. esculentum* x *peruvianum*, but not *L. esculentum* AC. However, the use of cultures of *L. esculentum* x *peruvianum* cells, in the initial absence of *L. esculentum* AC material, appears to have been justified.

4.1.2: Introduction: Effects of cell walls

The assays of the effects of cell wall components on L-[U-^{14}C] leucine incorporation into protein were commenced with the application of deproteinated cell walls, which, it was anticipated, would not exert any effect on the protein metabolism of suspension-cultured cells, and consequently would provide a convenient base-line for effects noted in future assays for tests on specific classes of cell wall polysaccharides.

4.1.3: Materials and Methods: Effects of cell walls

4.1.3.i: Summary of protocol for L-[U-^{14}C] leucine incorporation assays

Briefly, the protocol for L-[U-^{14}C] leucine incorporation assays (sect. 2.3) was as follows: suspension-cultured cells suspended in fresh medium (TOM-I or SH) to which test substances had been added, were dispensed into sample tubes in 0.5 ml aliquots at a concentration of 100 mg/ml. Tubes were incubated under standard conditions, i.e. wrapped in aluminium foil, maintained at 25 °C, and shaken at 200 rpm. Cells were permitted 1 h to acclimatise before the addition of 0.05 µCi L-[U-^{14}C] leucine to each tube. Cells were incubated for 0, 1, 2 and 3 h then further incorporation of L-[U-^{14}C] leucine was stopped by killing the cells with an excess of 10% tri-chloroacetic acid (TCA) at 4 °C, which also precipitated out the proteins. TCA was added to the 0 h samples directly after the addition of L-[U-^{14}C] leucine. Samples were kept at 4 °C overnight, then the cells were filtered onto Whatman GF/C glass microfibre filters and rinsed with 2 x 5 ml 10% TCA at 4 °C and 1 x 5 ml "cold" leucine solution [1 g/l]. The filters were dried and scintillation-counted.

4.1.3.ii: Particulars relating to assays with entire deproteinated cell walls.

These experiments with exogenous cell walls were performed before the L-[U-^{14}C] leucine incorporation assay procedure had been finalised, consequently, throughout this section cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC were incubated in 8-d TOM-I and 8-d SH, respectively, to which deproteinated cell walls of *L. esculentum* x *peruvianum* (+HYBcw) or *L. esculentum* AC (+ACcw) and *N. physaloides* (+NPcw) were added at 1 mg/ml. In each case the control was provided by cells in 8-d TOM-I or 8-d SH to which no additions had been made. Cell walls were extracted from suspension-cultured cells of

L. esculentum x *peruvianum* and from internodes of plants of *N. physaloides*, and *L. esculentum* AC (sect. 2.4.1).

Any additional variations from the standard procedure in these assays are detailed in the appropriate sections.

4.1.4: Results: Effects of cell walls

4.1.4.a: Effect of entire deproteinated cell walls on [^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* x *peruvianum*.

Cells were not dispensed as a suspension in this experiment, but were weighed into each sample tube. The results are displayed in Figure 4.1.4.a.

Sizeable differences emerged in the quantity of L-[U-^{14}C] leucine incorporated into protein by cells in the different treatments. The addition of cell walls of both *L. esculentum* x *peruvianum* (+HYBcw) and *N. physaloides* (+NPcw) to cells of *L. esculentum* x *peruvianum* reduced the rate at which L-[U-^{14}C] leucine was incorporated into protein. After 3 h, cells incubated in +NPcw had incorporated 75% of the L-[U-^{14}C] leucine incorporated by cells in control treatment, while cells incubated in +HYBcw medium had incorporated only 52%. The differences in incorporation by cells in each treatment became apparent after 1 h of incubation and persisted until the end of the experimental period.

4.1.4.b: Effect of entire deproteinated cell walls on [^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* AC.

Incubation took place in the light, otherwise all procedures were as specified (sect. 2.3). The results obtained are displayed in Figure 4.1.4.b. The rates of incorporation of L-[U-^{14}C] leucine by the cells were uniformly low, with a maximum of <4 000 cpm compared to one of <60 000 cpm for the corresponding experiment (4.1.4.a) with suspension-cultured cells of *L. esculentum* x *peruvianum*.

The addition of both *L. esculentum* AC (+ACcw) and *N. physaloides* (+NPcw) cell walls to the medium caused a reduction in the final amount of L-[U-^{14}C] leucine incorporated into protein by the cells. After 3 h, cells incubated in +ACcw medium had incorporated 89% of the L-[U-^{14}C] leucine incorporated by cells from the control treatment, while cells incubated in +NPcw medium had incorporated 86%.

The reduction in L-[U-^{14}C] leucine incorporation in cells incubated with cell walls, +ACcw and +NPcw treatments, was apparent 1 h after the addition of L-[U-^{14}C] leucine to the media. However, at the 2-h sample point, cells incubated in +ACcw medium had incorporated the greatest levels of L-[U-^{14}C] leucine, while cells incubated in +NPcw medium had incorporated the least; cells from the control treatment had incorporated an intermediate amount of leucine.

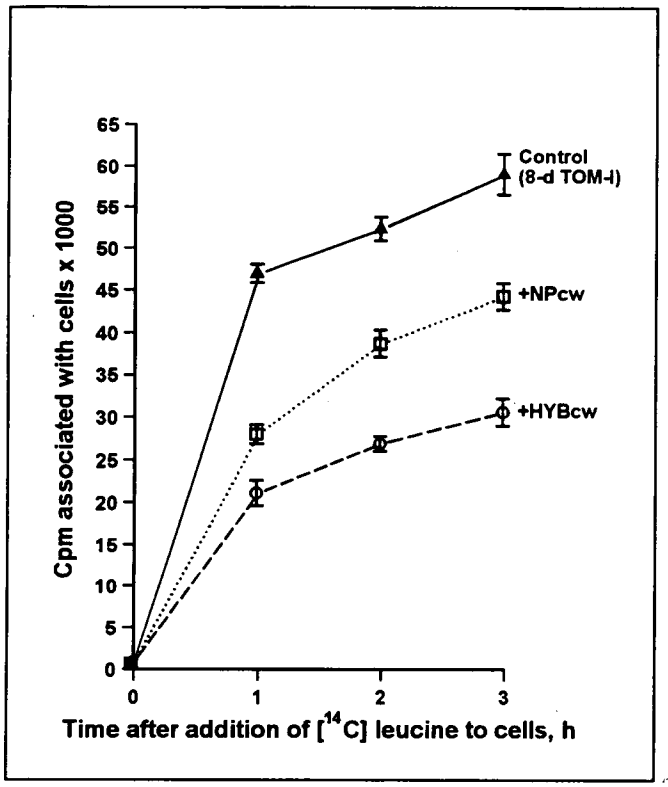


Figure 4.1.4.a:

Effect of exogenous deproteinated cell walls on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 8-d TOM-I medium.

Means were obtained from 10 samples.

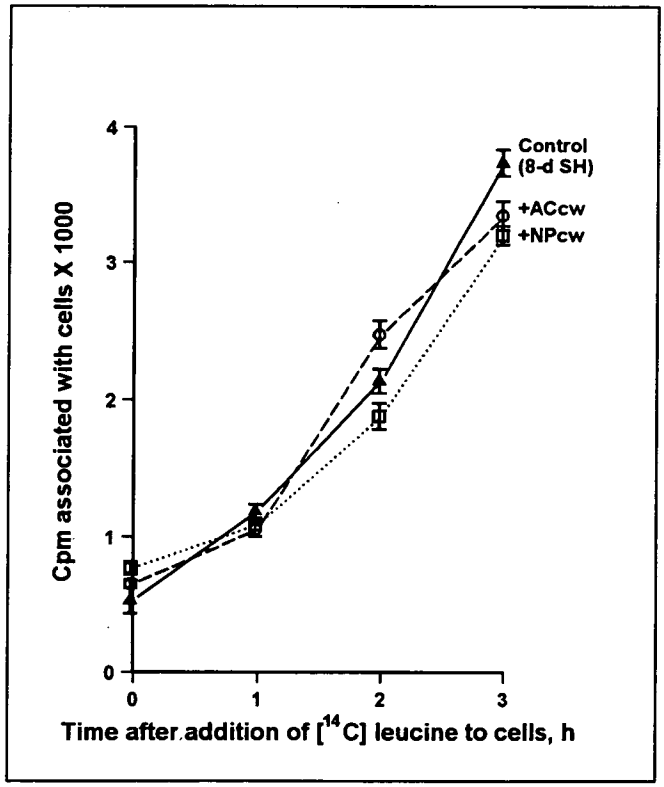


Figure 4.1.4.b:

Effect of exogenous deproteinated cell walls on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* AC incubated in 8-d SH medium.

Means were obtained from 5 samples at 0 h, 1 h and 2 h, and 10 samples at 3 h.

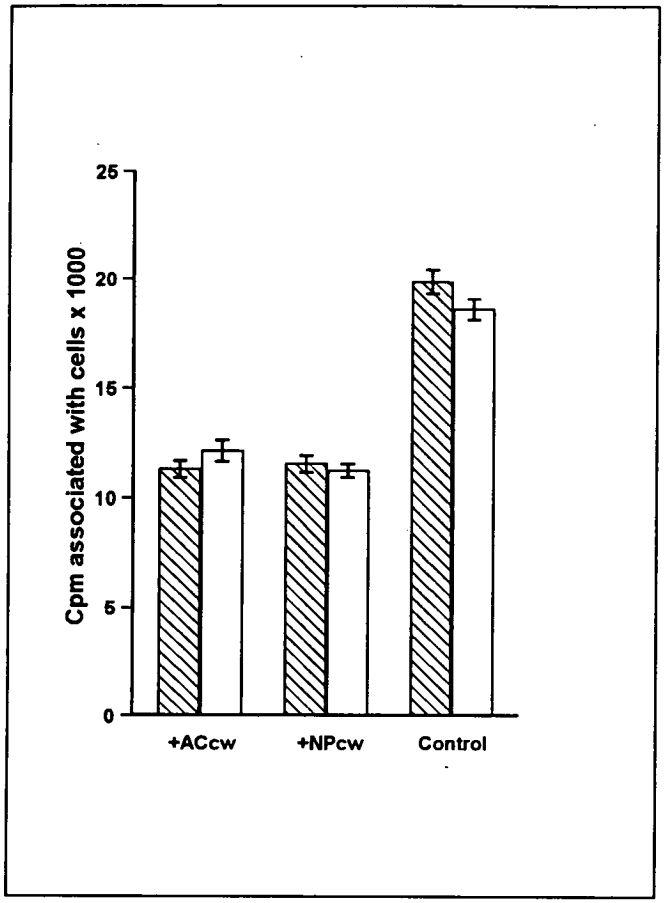


Figure 4.1.4.c:

Effect of deproteinated cell walls on L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* AC after dark and light incubations of 3 h.

Means were obtained from 10 samples.

KEY

Dark incubated

Light incubated

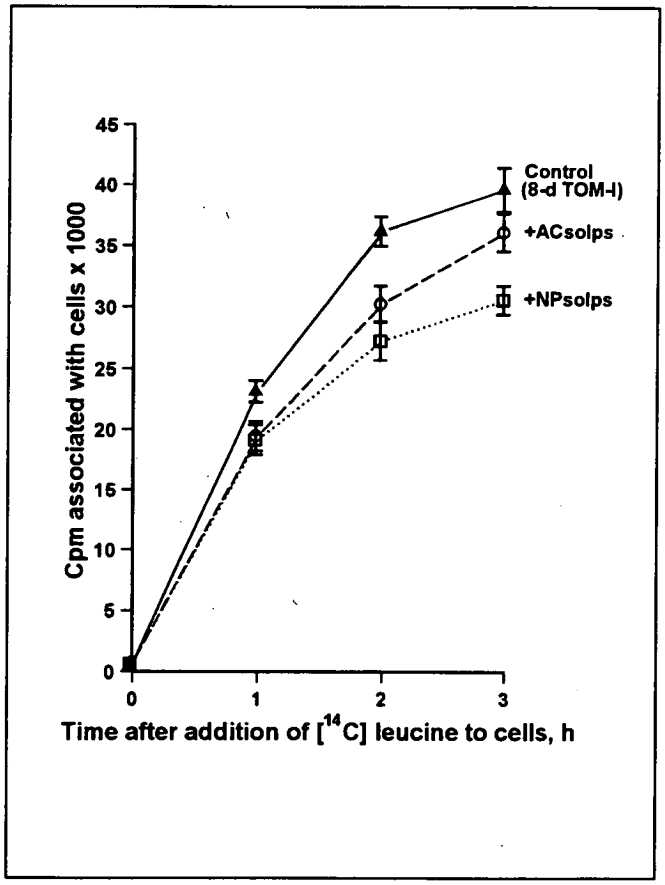


Figure 4.2.4:

Effect of exogenous, apoplastic, soluble polysaccharides on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum*.

Means were obtained from 10 samples.

In this experiment inter-sample variance was low, however, the inconsistency of effect observed across the duration of sampling suggests that an uncontrolled element of variation may have been introduced to the system at some point. There is no indication as to what this factor might have been.

4.1.4.c: Effect of entire deproteinated cell walls on [^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* AC incubated in the dark and the light.

An L-[U-^{14}C] leucine incorporation assay was performed to assess the effect of exogenous deproteinated cell walls on the incorporation of L-[U-^{14}C] leucine into protein by suspension-cultured cells of *L. esculentum* AC incubated in the light at 7.5 µmol m^{-2} s^{-1} and in darkness (improvised by wrapping the rack of sample tubes in aluminium foil prior to incubation). Incorporation was stopped 3 h after the addition of L-[U-^{14}C] leucine to the cells; all other procedures were as specified (sect. 2.3). The results are displayed in Figure 4.1.4.c.

Cells incubated with cell walls of both *L. esculentum* AC (+ACcw) and *N. physaloides* (+NPcw), under light and dark conditions, exhibited reduced levels of incorporated L-[U-^{14}C] leucine by comparison with cells from the control treatment. There was little difference in the magnitude of the reductions: dark-incubated cells from the +ACcw and +NPcw treatments incorporated 57% and 58%, respectively, of the amount of L-[U-^{14}C] leucine incorporated by cells in the dark-incubated control treatment, while light-incubated cells from +ACcw and +NPcw treatments incorporated 62% and 60% of the amount of leucine incorporated by cells in the light-incubated control treatment.

Cells from the control treatments incorporated different amounts of L-[U-^{14}C] leucine: after 3 h, light-incubated cells had fixed 94% of the amount of L-[U-^{14}C] leucine fixed by dark-incubated cells. It was decided that, since incubation in darkness appeared to enhance leucine incorporation rates, all future experiments would be conducted in the dark to maximise differences in inhibition of L-[U-^{14}C] leucine incorporation, and to reduce variation between replicates.

4.1.5: Discussion: Effects of cell walls

The addition of *L. esculentum* x *peruvianum*, *L. esculentum* AC or *N. physaloides* deproteinated cell walls to the medium reduced the rate of L-[U-^{14}C] leucine incorporation by suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC. Such an effect could be interpreted as, and is consistent with, a slowing of the rate at which protein metabolism proceeds. The identity of the component(s) of the walls which induce this effect is not clear; presumably the active principle must be directly soluble in 8-d TOM-I and/or 8-d SH medium, or, alternatively, becomes biologically active, and/or metabolically available to the cells, as a result of the cleavage of active fragments from the entire cell

walls by *in situ* enzymic action by the cells. The factor operates with equal efficiency in light or darkness on cells of *L. esculentum* AC (sect. 4.1.4.c), although results from sections 4.1.4.b and c suggest that a lower metabolic rate prevailed with these cells.

The inhibition of protein metabolism in suspension-cultured cells of *L. esculentum* x *peruvianum* by a component of *L. esculentum* x *peruvianum* cell walls (sect. 4.1.4.a), and in cells of *L. esculentum* AC by cell walls of *L. esculentum* AC (sects. 4.1.4.b and c), is particularly important with respect to graft development, where such an effect would be consistent with the slowing, or cessation of cell division, observed in compatible homografts prior to the differentiation of newly-formed callus cells into xylem wound vessel members (WVMs) and formation of functional vascular conduits (Moore and Walker, 1981a; Jeffree and Yeoman, 1983; Holden *et al.*, 1987). Therefore, evidence for the slowing of protein metabolism, and by analogy cell division, might be anticipated in the assays in the presence of self cell wall components. However, it is noteworthy that a considerable reduction was detected regularly in the presence of deproteinated cell walls of non-self, *N. physaloides*, which could imply that the capacity to induce this reduction is not a species-specific characteristic of cell wall constituents.

In cells of *L. esculentum* x *peruvianum* the inhibition caused by cell walls of *N. physaloides* was less marked than that caused by self cell walls. However, in cells of *L. esculentum* AC the application of cell walls of *L. esculentum* AC and *N. physaloides* inhibited leucine incorporation to the same degree. This evidence indicates that applications of non-self, *N. physaloides*, cell walls cause a slowing of cell division in suspension-cultured cells of both *L. esculentum* x *peruvianum* and *L. esculentum* AC. This contradicts previous evidence obtained from observations of the GU region of incompatible heterografts of *N. physaloides*/*L. esculentum* , where cell division did not cease once stock and scion came into contact, but continued, accompanied by the re-differentiation of vascular elements in the GU (Jeffree, pers. comm.). It could be expected then, while cell division continued unabated in the GU, that the rate of protein metabolism would remain constant; this juxtaposition of contradictory phenomena has yet to be explained.

The principle responsible for slowing protein metabolism may be released from deproteinated cell walls via enzyme action by the cells. This inhibitive product might be present at a low concentration throughout, especially during the initial stages, which would be indicated by less intense inhibition of protein metabolism at the start of the assay. The incorporation of L-[U-^{14}C] leucine by suspension-cultured cells of *L. esculentum* x *peruvianum* (sect. 4.1.4.a) was more intense between 0 - 1 h than between 1 - 3 h for all treatments, however, although the required pattern for concentration-dependent inhibition was observed in the +cell wall treatments the appearance of the same pattern in the control

treatment suggests that there is no evidence for the concentration-dependence of the suppression of protein metabolism by entire cell walls.

The results of these experiments indicate that suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC responded in a similar manner when incubated in the presence of exogenous deproteinated cell walls. Therefore, the use of suspension-cultured cells of *L. esculentum* x *peruvianum*, which was the closest material to the *L. esculentum* AC tomato used in the Solanaceous model grafting system (Lindsay *et al.*, 1974; Yeoman and Brown, 1976; Parkinson and Yeoman, 1982; Parkinson, 1983, Parkinson *et al.*, 1987), that was available at the start of the project, was justified.

Although there is no direct evidence for this, the possibility of the occurrence of synergistic effects between endogenous polysaccharide fragments, already present in the 8-d TOM-I and 8-d SH media, and components of the exogenous deproteinated cell walls should not be forgotten. So far, no evidence for synergistic effects between two or more oligosaccharins of plant origin has been recorded, although synergistic effects between elicitors of fungal origin and elicitors of plant origin, leading to the induction of phytoalexins (Davis *et al.*, 1986c), have been reported. Although phytoalexin production might be characterised by an increase in L-[U-^{14}C] leucine incorporation in suspension-cultured cells, it is unlikely that fungal elicitors would have been present in these assays. Furthermore, the ubiquitous endogenous cell wall components, which introduce an element of uncertainty to the effectiveness of individual media, were also present in the control treatments, and, though the results of a previous experiment (3.3.4.iii), indicated that the incorporation of L-[U-^{14}C] leucine was enhanced when suspension-cultured cells of *L. esculentum* x *peruvianum* were incubated in 8-d TOM-I rather than fresh TOM-I and 10 mM MES, all treatments in these experiments (sects. 4.1.4.a, b and c) would have benefited from promotional activity of this nature. Consequently the inhibition of protein metabolism exhibited by cells in 8-d spent media suggests that the effect attributed to the presence of cell walls operated independently, or over-ruled any others initiated by components already present in the media.

4.2: EFFECT OF SOLUBLE POLYSACCHARIDES FROM CALLUSES OF *L. ESCULENTUM* AC AND *N. PHYSALOIDES* ON [^{14}C] LEUCINE INCORPORATION IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM*

4.2.1: **Summary:** Effects of apoplastic polysaccharides

Wound-generated callus cells from both graft partners meet in the GU approximately 4 d after graft establishment. At this point exchange and/or receipt of soluble polysaccharides present in the apoplast of these cells may determine the further development of the graft.

Soluble polysaccharides from *L. esculentum* AC and *N. physaloides* calluses were tested on cells of *L. esculentum* x *peruvianum* in a L-[U-^{14}C] leucine incorporation assay. Application of *L. esculentum* AC-derived soluble polysaccharides reduced L-[U-^{14}C] leucine incorporation by the cells by 9%, while application of *N. physaloides*-derived polysaccharides reduced it by 23%. The active soluble polysaccharides might be pectic or hemicellulosic in nature: biological activities have been reported for oligosaccharides in both groups, and both would be present in the apoplastic fluid, or could be cleaved by enzymes present on the surfaces of the calluses.

4.2.2: Introduction: Effects of apoplastic polysaccharides

4 d after graft-establishment wound-generated callus cells situated on opposing faces of the graft meet, and it is after this that the divergence in future development patterns between compatible and incompatible graft combinations emerges (Jeffree and Yeoman, 1983; Moore and Walker, 1981a; Parkinson and Yeoman, 1982; Holden, 1985; Hossain, 1986). It is possible that cell wall components active in determination of graft development will be readily soluble, and present in the apoplastic fluid of these callus cells. Soluble polysaccharides obtained from calluses of *L. esculentum* AC and *N. physaloides* tissue were tested in a L-[U-^{14}C] leucine incorporation assay on suspension-cultured cells of *L. esculentum* x *peruvianum*.

4.2.3: Materials and Methods: Effects of apoplastic polysaccharides

Soluble polysaccharides were obtained from apoplastic fluid rinsed from the surfaces of month old calluses of *L. esculentum* AC (+ACsolps) and *N. physaloides* (+NPsolps) (sect. 2.4.5). These were applied to cells of *L. esculentum* x *peruvianum* in 8-d TOM-I medium at 0.5 mg/ml, and a L-[U-^{14}C] leucine incorporation assay performed. The cells were weighed into the tubes manually, otherwise the standard procedure (sect. 2.3) was followed throughout. This experiment was not repeated with cells of *L. esculentum* AC.

4.2.4: Results: Effects of apoplastic polysaccharides

The results are displayed in Figure 4.2.4. The addition of soluble extracellular polysaccharides from *L. esculentum* AC (+ACsolps), and *N. physaloides* (+NPsolps), to the medium accompanied a reduction in the rate of L-[U-^{14}C] leucine incorporation by cells of *L. esculentum* x *peruvianum*, which became apparent 1 h after the addition of L-[U-^{14}C] leucine, and persisted until the 3-h sample point. After 3 h, cells incubated in +ACsolps medium had incorporated 91% of the amount of L-[U-^{14}C] leucine incorporated by cells in the control treatment, and cells incubated in +NPsolps medium had incorporated 77%.

4.2.5: **Discussion:** Effects of apoplastic polysaccharides

The inhibitions in L-[U-^{14}C] leucine incorporation observed in cells incubated with exogenous soluble apoplastic polysaccharides, were not as marked as the inhibitions observed in the previous experiments (sects. 4.1.4.a, b and c) with deproteinated cell walls. The identity of the substance(s) released by rinsing the calluses, and responsible for these effects is not known. However, in order to be extracted by SH medium it must be soluble, and located in the apoplast or on the cell surface. Proteins and additional non-polysaccharide metabolites extracted in the rinses would have remained in solution in the 70% ethanol solution used to precipitate the oligo- and polysaccharides prior to centrifuging, therefore the inhibitory agent must be polysaccharide in nature.

The solubility of pectic polymers is governed by the concentration of Ca^{2+} ions present in the medium which determines whether pectic strands conglomerate in the "egg-box" formation or remain as single strands (Powell, Morris, Gidley *et al.*, 1982; Jarvis, 1984). There is evidence that pectic material is located in beads on cells on the surface of calluses of carrot (Davis and Lewis, 1981), *L. esculentum*, *D. stramonium* and *N. physaloides* (Jeffree *et al.*, 1989) grown on solid media. This material would probably be extractable in the SH rinses used in this experiment. In addition Jeffree *et al.*,(1989) stated that free pectinase was detected on the cell surfaces of calluses of *L. esculentum*, *D. stramonium* and *N. physaloides*. Therefore it is feasible that biologically-active pectic oligosaccharins could be produced *in situ*, in which case pectic fragments might have inhibited protein metabolism in this instance.

Hemicellulosic molecules and fragments might be present in the apoplast as a result of sloughing, post-secretion transglycosylation of monomerics, or cellulase activity. Jeffree, Gordon and Yeoman (1989) indicated that cellulase, the enzyme which cleaves xyloglucan polymers into potentially active oligosaccharides, is present on the surfaces of calluses of *L. esculentum*, *D. stramonium* and *N. physaloides*. Therefore, it is possible that hemicellulosic oligosaccharides were present in the apoplastic fluid of these calluses.

Polymers of the hemicellulosic polysaccharide xyloglucan might also be present in the apoplastic fluid. These molecules are highly soluble and, after manufacture, may be extruded into the apoplast by a process called reptation, which permits the secretion of linear molecules of high molecular weight, endwise through pores in the cell wall matrix (Kieliszewski and Lamport, 1994). As yet there is no evidence that hemicelluloses cause the cessation of cell division, however, certain xyloglucan oligomers have been shown to inhibit auxin-induced growth (York *et al.*, 1984; McDougall and Fry, 1988).

Interestingly, soluble polysaccharides of *N. physaloides* inhibited protein metabolism to a greater extent than those from *L. esculentum* AC, which is a reversal of the species-specific

effect observed with cell walls (sect. 4.1). This may be due to the exogenous self component in this experiment not having been sufficiently self to produce the full effect, as it was derived from *L. esculentum* AC material and not from *L. esculentum* x *peruvianum*.

4.3: DETERMINATION OF THE EFFECTS OF PECTINS UPON THE INCORPORATION OF [^{14}C] LEUCINE INTO PROTEIN

4.3.0: GENERAL INTRODUCTION TO THE BIOLOGICAL POTENTIAL OF PECTINS IN GRAFTING

The precise function of the plentiful pectic secretions found in the GUs of various grafting combinations is not known, although it appears that the pectic layer permits the adhesion of stock and scion in the early stages of graft development before newly-formed callus cells, generated from the cut faces of both graft partners, make contact (Yeoman *et al.*, 1978; Moore and Walker, 1981a, b). Electron microscope studies of compatible *L. esculentum* homografts, have shown that once contact has been made the pectic layer is thinned, and plasmadesmata are formed, thus re-establishing cytoplasmic continuity between stock and scion (Jeffree and Yeoman, 1983). Similar thinning of the "necrotic layer" has also been observed in homografts of *Coleus* (Stoddard and McCully, 1980), but no evidence for the thinning of the pectic layer has been reported for incompatible combinations, possibly because adequate examinations have not been made. Biologically active pectic fragments, which could identify each graft partner, and confer compatibility or incompatibility to the combination, may be cleaved from the pectic layer during the thinning process, especially if thinning is a "non-specific" process.

In vitro grafting experiments had suggested that the compatibility/incompatibility factor was probably a cell wall component (Parkinson, 1983). Experiments in which pectins, loaded in solution onto disks of Whatman lens cleaning tissue, were sandwiched in the GUs of compatible Solanaceous homografts showed that the introduction of pectins from incompatible sources substantially reduced the final number of connections of WVMs formed across the GU (Jeffree *et al.*, 1987). Consequently pectins appeared ideal candidates to test in L-[U-^{14}C] leucine incorporation assays.

4.3.1: EFFECTS OF EXOGENOUS PECTINS ON THE INCORPORATION OF [^{14}C] INTO PROTEIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM*

4.3.1.1: **Summary:** Effects of pectins on *L. esculentum* x *peruvianum*

Previous studies indicated that pectin, or pectic fragments, were the strongest candidates for a cell wall-derived recognition molecule operative in the GU (Parkinson, 1983; Jeffree *et*

al. 1987). Pectins from cells of *L. esculentum* x *peruvianum* and internodes of *N. physaloides* were tested in L-[U-^{14}C] leucine incorporation assays on suspension-cultured cells of *L. esculentum* x *peruvianum*. Consistent effects could not be established and a sample of the results obtained are presented.

The application of self, *L. esculentum* x *peruvianum*, pectins caused inhibitions in leucine incorporation of between 9 - 24%, while the application of non-self, *N. physaloides*, pectins, on separate occasions, caused an acceleration of leucine incorporation by 5%, and inhibitions of 7 - 26%. The spread of these results may be due to variations in the pectic media applied to cells caused by differences in crystallisation patterns, related to the Ca^{2+} content of the media, or some other cause. The application of pectins did not affect the viability of the cells.

4.3.1.2: **Introduction:** Effects of pectins on *L. esculentum x peruvianum* (see General Introduction sect. 4.3.0)

4.3.1.3: **Materials and Methods:** Effects of pectins on *L. esculentum x peruvianum*

L-[U-^{14}C] leucine incorporation assays were performed on suspension-cultured cells of *L. esculentum* x *peruvianum* (sect. 2.3). 8-d TOM-I-based media were used throughout. *L. esculentum* x *peruvianum* (+HYBpec) and *N. physaloides* pectins (+NPpec) were extracted from entire deproteinated cell walls (sect. 2.4.2) obtained from suspension-cultured cells and internodes respectively. Pectins were applied at 0.5 mg/ml.

4.3.1.4: **Results:** Effects of pectins on *L. esculentum* x *peruvianum*

4.3.1.4.a: **Effect of exogenous pectins on [^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* x *peruvianum*.**

4.3.1.4.a.i: **L-[U-^{14}C] leucine incorporation assay**

The results are shown in Figure 4.3.1.4.a. From the 1-h sample point onwards, cells incubated with pectins from *N. physaloides* (+NPpec) exhibited an increased incorporation of L-[U-^{14}C] leucine into protein, while cells incubated with pectins from *L. esculentum* x *peruvianum* (+HYBpec) displayed a reduced rate of incorporation. 3 h after the addition of L-[U-^{14}C] leucine to the media, cells incubated in +NPpec medium had incorporated 105% of the amount of leucine incorporated by cells in the control treatment, while cells incubated in +HYBpec medium had incorporated 91%.

4.3.1.4.a.ii: **Viability assessments**

An FDA viability test (sect. 2.2.3) was performed concurrently with the L-[U-^{14}C] leucine incorporation assay to assess the effect of exogenously applied pectins on cell viability. This test was performed 3.5 h after the cells had been added to the various media. The fluorescence exhibited by viable cells was strong throughout. There were no significant

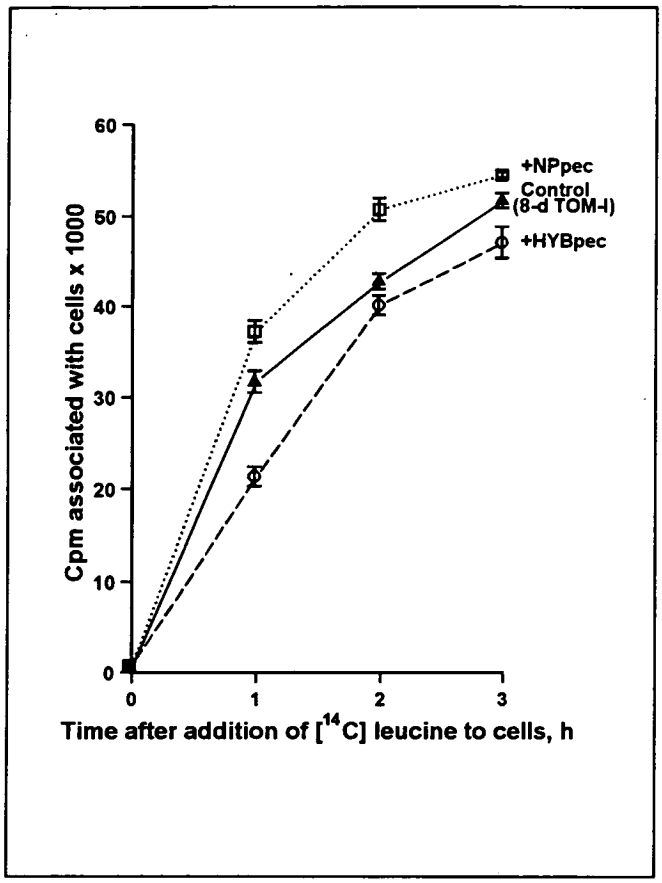


Figure 4.3.1.4.a:

Effect of exogenous pectin on rate of incorporation of L-[U-^{14}C] leucine into protein by suspension-cultured cells of *L. esculentum* x *peruvianum*.

Means were obtained from 5 samples at 0 h, and from 10 samples at 1 h, 2 h and 3 h.

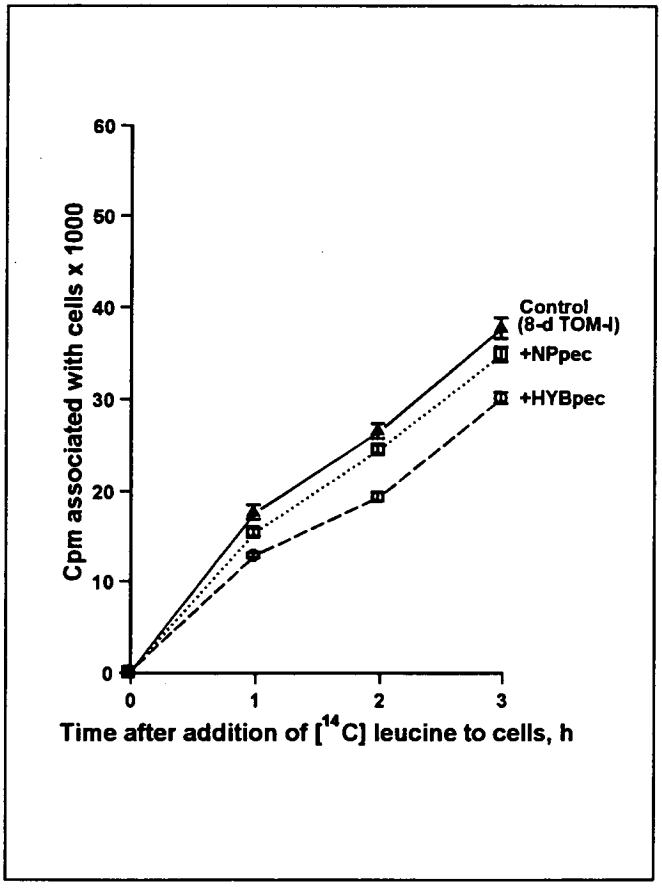


Figure 4.3.1.4.b:

Effect of exogenous pectin on rate of incorporation of L-[U-^{14}C] leucine into protein by suspension-cultured cells of *L. esculentum* x *peruvianum*.

Means were obtained from 4 samples at 0 h, 8 samples at 1 h, and 10 samples at 2 h and 3 h.

differences in the viability rates exhibited by cells of *L. esculentum* x *peruvianum* incubated in the different media ± pectins. The results obtained are displayed in Table 4.3.1.4.a.ii.

Table 4.3.1.4.a.ii: Percentage of viable suspension-cultured cells of *L. esculentum* x *peruvianum* after 3.5 h incubation in +HYBpec, +NPpec or Control (8-d TOM-l) media. Each mean value was obtained from 3 separate assessments of 200 cells; fluorescence of cells was strong throughout.

Viability/Treatment	+HYBpec medium	+ NPpec medium	Control (8-d TOM-l) medium
Mean (n = 3)	97%	96%	95%
±Std. Error	1.3%	0.9%	0.8%

4.3.1.4.b: Effect of exogenous pectins on [^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* x *peruvianum* using same batch of media as used in section 4.3.1.4.a

A double batch of incubation media, 8-d TOM-l ± pectin extracts, had been made up for the previous L-[U-^{14}C] leucine incorporation assay (sect. 4.3.1.4.a). Half was frozen and used in an almost identical L-[U-^{14}C] leucine incorporation assay: the cells used came from a different flask and no assessment of cell viability was made.

The results are illustrated in Figure 4.3.1.4.b. Throughout, cells of *L. esculentum* x *peruvianum* incubated in the control treatment maintained the highest levels of L-[U-^{14}C] leucine incorporation and showed the greatest rate of incorporation between 0 h and 1 h, thereafter the rate of incorporation was closely paralleled by cells incubated in +NPpec medium. Throughout, cells incubated in +HYBpec medium exhibited considerably reduced levels of L-[U-^{14}C] leucine incorporation. 3 h after the addition of L-[U-^{14}C] leucine, cells incubated in +NPpec had incorporated 93% of the amount of L-[U-^{14}C] leucine incorporated by cells in the control treatment, while cells incubated in +HYBpec medium had incorporated 80%. From the 1 h sample point onwards the mean values for each treatment differed visibly from the other treatments.

4.3.1.4.c: Effect of exogenous pectin on the incorporation of [^{14}C] leucine into protein in suspension-cultured cells of *L. esculentum* x *peruvianum* using a fresh, different batch of incubation media

A L-[U-^{14}C] leucine incorporation assay (sect. 2.3) was performed on cells of *L. esculentum* x *peruvianum* using a new batch of 8-d TOM-l ± pectin media, made in the same manner and to the same specifications as before. The sample tubes were incubated for 3 h only after the addition of L-[U-^{14}C] leucine. The results obtained are displayed in Figure 4.3.1.4.c.

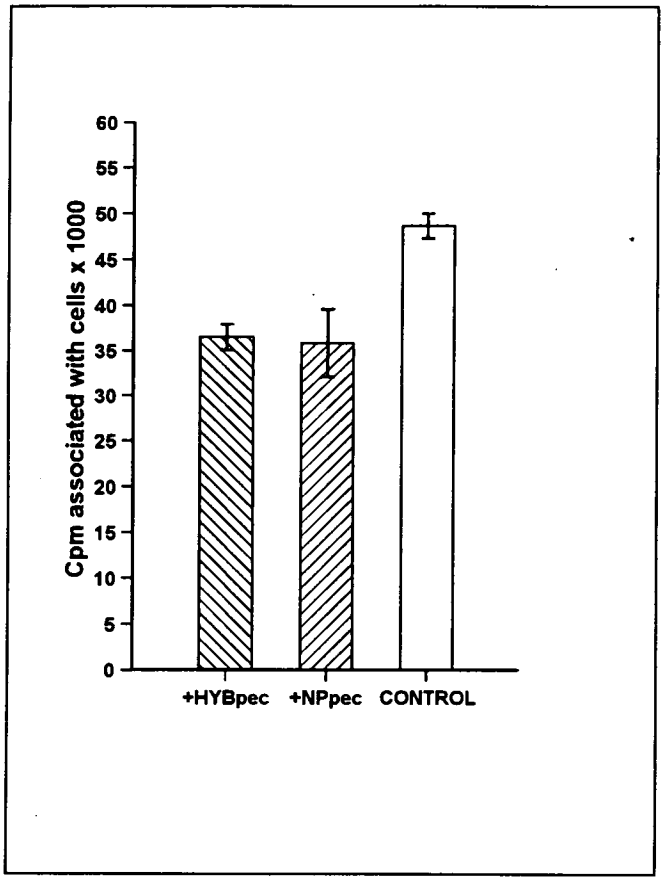


Figure 4.3.1.4.c:

Effect of exogenous pectins on the final incorporation of L-[U-^{14}C] leucine into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* after a 3 h incubation in the presence of L-[U-^{14}C] leucine.

Means were obtained from 10 samples.

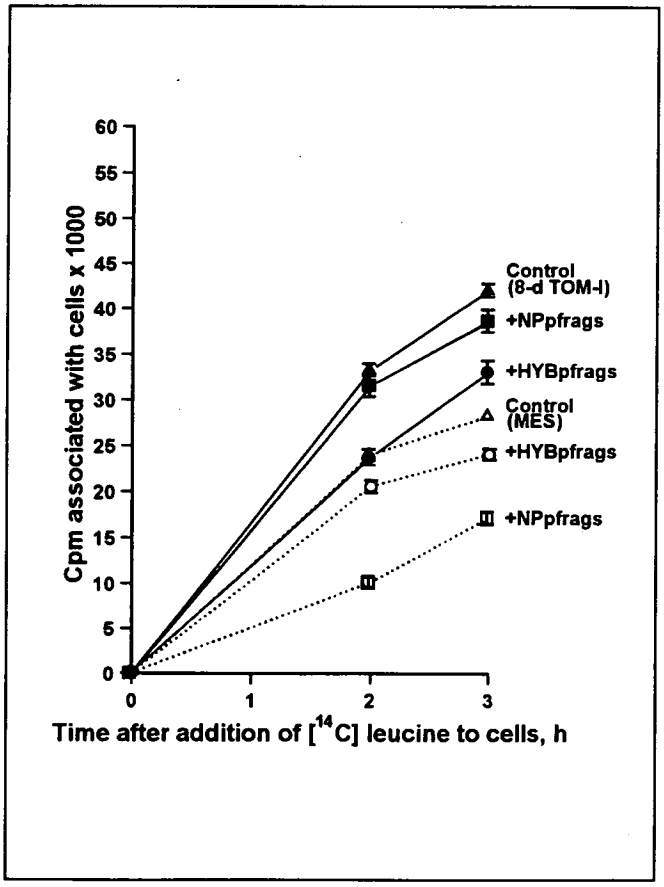


Figure 4.3.2.4.i:

Effect of exogenous pectic fragments on rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 8-d TOM-I medium (solid lines and symbols) or 10 mM MES (dotted lines and open symbols).

Means were obtained from 4 samples at 0 h, 8 samples at 2 h (except for the +NPpfrags and Control 8-d TOM-I treatments which had 7 samples), and 10 samples at 3 h.

Suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in +HYBpec or +NPpec medium exhibited almost equally reduced levels of L-[U-^{14}C] leucine incorporation after 3 h incubation; cells incubated in +HYBpec medium had incorporated 75% of the amount of leucine incorporated by cells in the control treatment, while cells incubated in +NPpec medium had incorporated 74%.

4.3.1.5: Discussion: Effects of pectins on *L. esculentum* x *peruvianum*

Several generalisations can be made from the variety of results which emerged from this group of experiments with suspension-cultured cells of *L. esculentum* x *peruvianum*. Firstly, that the presence of self pectins (+HYBpec) consistently accompanied a reduction in the amount of L-[U-^{14}C] leucine incorporated by the cells of between 9 - 24%. Secondly, that the presence of non-self pectins from *N. physaloides* induced a variety of responses from promotion of L-[U-^{14}C] leucine incorporation by +5% to inhibition by -26%, but that no reliable effect upon leucine incorporation could be attributed to pectins from this source.

These observations suggest that although statistically significant differences (at 5%) appeared in all experiments, the variation between experiments was not completely controlled. The underlying reasons for this variation are not understood yet, but since tissue cultures are particularly prone to change between generations (Aldington and Fry, 1993) fluctuations in the metabolism of *L. esculentum* x *peruvianum* cultures used on different days may have been wholly or partly responsible for the variety of responses detected.

However, it is unlikely that all uncontrolled variation could be attributed to inherent metabolic differences in the cells, since in multiple assays, described later (sects. 4.1 and 4.4), performed to determine the effects of hemicelluloses and deproteinated cell walls on protein metabolism, highly consistent results were achieved. The inhibitory effects observed in those assays were of greater amplitude than these associated with pectins and so may have been affected less by metabolic differences in the cells. However, responses to a uniform mixture of substances do not usually vary to the extent, or as persistently as those observed in these pectic assays - of which the assays reported here are but a sample. Therefore the possibility that the distribution of pectins within the media was not consistent must be considered.

Potential causes of non-uniformity of these pectic media, for which there is no direct evidence, might include the grouping together and crystallisation of pectins of different sizes and/or branching patterns during the freeze-drying process (sect. 2.4.2), which would cause the small amounts used in assays to be unrepresentative of the original population of molecules, and would allow the composition of successive batches of media to vary.

Messaien and colleagues (1993) found, in experiments using a variety of pectic strands of known size and bonding arrangements, that the internal Ca^{2+} concentrations of carrot protoplasts were affected only by Ca^{2+}-bound double-stranded oligogalacturonides with DP $\geq$9. Since Ca^{2+} bonding, in the "egg-box" formation, is generally restricted to blocks of 12 or more consecutive un-esterified GalA residues in pectin molecules (Yamaoka and Chiba, 1983), Ca^{2+} bridge formation is most likely to occur in the portion of the pectic population which is biologically-active. Aggregation of such oligogalacturonides might render them inactive, un-recognisable or unavailable to the cells and limit the proportion of molecules capable of affecting metabolism present in the media. Differences in concentrations of Ca^{2+} between successive batches of used medium, caused by variations in the metabolic activities of cells previously cultured therein, might also have produced inconsistent bonding patterns amongst this section of the pectic population, and contributed to the disparity in results.

Synergistic effects determined by the variety of cell wall products present in the 8-d TOM-l medium may have contributed to the spread of results, however, later experiments which utilised fresh TOM-l medium (not detailed here) also failed to produce any definite patterns of activity for pectins, which suggests that the use of 8-d media may not have produced the inconsistencies observed.

The evidence that incubation in the presence of exogenous pectins did not affect the viability of cells of *L. esculentum* x *peruvianum* agrees with earlier observations from examinations of the GU of incompatible *N. physaloides*/*L. esculentum* heterografts where no necrosis of cells, other than those directly wounded in establishment of the graft (Yeoman, 1984), was observed. Yeoman's observations implied that the putative recognition factor is not toxic to either species, and while these viability results show that pectins from *N. physaloides* and *L. esculentum* x *peruvianum* are not toxic to suspension-cultured cells of *L. esculentum* x *peruvianum*, the results from the L-[U-^{14}C] leucine incorporation assays do not substantiate the proposed role for pectins as recognition factors in grafting.

4.3.2: EFFECT OF EXOGENOUS PECTIC FRAGMENTS, IN 8-d TOM-l AND 10 mM MES MEDIA, ON THE INCORPORATION OF [^{14}C] LEUCINE INTO PROTEIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM*

4.3.2.1: **Summary:** Effects of pectic fragments on *L. esculentum* x *peruvianum*

The failure to establish a consistent effect with entire pectins applied to cells of *L. esculentum* x *peruvianum* (sect. 4.3.1) prompted an investigation into the effects of pectic fragments. Simultaneous L-[U-^{14}C] leucine incorporation assays were performed

which revealed that incubation with *L. esculentum* x *peruvianum* (+HYBpfrags) and *N. physaloides* fragments (+NPpfrags) in 8-d TOM-I medium caused inhibitions of 21% and 8%, respectively, in the amount of L-[U-^{14}C] leucine incorporated into protein by cells of *L. esculentum* x *peruvianum*, while cells incubated in 10 mM MES-based media containing +HYBpfrags and +NPpfrags exhibited inhibitions of 15% and 39% respectively, by comparison with the MES control.

Cells in all MES treatments incorporated less L-[U-^{14}C] leucine than cells in the 8-d TOM-I treatments. Exposure to +HYBpfrags/8-d TOM-I medium caused a slight reduction in cell viability, cells in all other treatments remained unaffected. A repeat of this protocol revealed inexplicable variations in the effects noted, although cells in MES media continued to fix less L-[U-^{14}C] leucine than those in 8-d TOM-I treatments.

4.3.2.2: Introduction: Effects of pectic fragments on *L. esculentum* x *peruvianum*

The application of entire pectins to suspension-cultured cells of *L. esculentum* x *peruvianum* had failed to produce consistent effects on the incorporation of L-[U-^{14}C] leucine into protein. Evidence from Messaien (1993) suggested that the biological activity of pectins is related to fragment size and the extent of inter-fragment bonding. It was decided to compare the effects of pectic fragments, produced by partial acid hydrolysis, in a L-[U-^{14}C] leucine incorporation assay with those produced with entire pectins.

4.3.2.3: Materials and Methods: Effects of pectic fragments on *L. esculentum* x *peruvianum*

A L-[U-^{14}C] leucine incorporation assay was performed on suspension-cultured cells of *L. esculentum* x *peruvianum* to test the effect of pectic fragments, produced from *L. esculentum* x *peruvianum* (+HYBpfrags) and *N. physaloides* pectins (+NPpfrags) by partial acid hydrolysis (sect. 2.4.4), on the incorporation of leucine into protein. The fragments were added to 8-d TOM-I medium or 10 mM MES at 0.5 mg/ml. The pH of all media was adjusted to 6.0 - 6.1 before the addition of cells from a single suspension-culture of *L. esculentum* x *peruvianum*. Samples were taken 0, 2 and 3 h after the addition of L-[U-^{14}C] leucine. An FDA viability test (sect. 2.2.3) was performed on cells in all six incubation media (+HYBpfrags, +NPpfrags in 8-d TOM-I and MES, with controls provided by 8-d TOM-I and MES media).

4.3.2.4: Results: Effects of pectic fragments on *L. esculentum* x *peruvianum*

4.3.2.4.i: L-[U-^{14}C] leucine incorporation assay

The results from the L-[U-^{14}C] leucine incorporation assay are shown in Figure 4.3.2.4.i. Cells of *L. esculentum* x *peruvianum* incubated in +HYBpfrags/8-d TOM-I medium exhibited reduced levels of incorporation of L-[U-^{14}C] leucine throughout the incubation period and,

after 3 h, had incorporated 79% of the amount of L-[U-^{14}C] leucine incorporated by cells in the Control/8-d TOM-I treatment. Cells incubated in +NPpfrags/8-d TOM-I exhibited reduced incorporation by the 3-h sample point, when cells had fixed 92% of the final amount of L-[U-^{14}C] leucine incorporated by cells in Control/8-d TOM-I medium.

L-[U-^{14}C] leucine incorporation levels were universally reduced in MES-based media. However, by comparison with the Control/MES treatment, cells of *L. esculentum* x *peruvianum* incubated in +pectin treatments showed definite reductions in leucine incorporation throughout the experiment. 3 h after the addition of L-[U-^{14}C] leucine, cells incubated in +HYBpfrags/MES had incorporated 85% of the amount of L-[U-^{14}C] leucine incorporated by cells in the Control/MES treatment, while cells incubated in +NPpfrags/MES medium had incorporated 61%.

Throughout the experiment cells incubated in 8-d TOM-I media exhibited much higher levels of L-[U-^{14}C] leucine incorporation than those incubated in 10 mM MES media. The final level of incorporation exhibited by cells in Control/MES medium was 76% of the final level achieved by cells in Control/8-d TOM-I medium.

4.3.2.4.ii: Viability assessments

Table 4.3.2.4.ii: Percentage of viable *L. esculentum* x *peruvianum* cells incubated in 8-d TOM-I and 10 mM MES-based incubation media to which pectic fragments have and have not been added at a concentration of 0.5 mg/ml, as show by the FDA test (sect. 2.2.3)

8-d TOM-I Medium	Mean (n = 3)	Std. Error	MES Medium	Mean (n = 3)	Std. Error
+HYBpfrags	73%	±3.0%	+HYBpfrags	76%	±3.0%
+NPpfrags	82%	±5.0%	+NPpfrags	78%	±3.7%
Control	82%	±3.0%	Control	76%	±6.3%

Cells incubated in +HYBpfrags/8-d TOM-I showed a slightly lower viability rate than cells incubated in either +NPpfrags/8-d TOM-I or Control/8-d TOM-I media, this was the only notable difference which appeared in this test.

4.3.2.5: Discussion: Effects of pectic fragments on *L. esculentum* x *peruvianum*

The cells for this experiment came from a single culture, however, those incubated in 8-d TOM-I media incorporated greater amounts of L-[U-^{14}C] leucine than those incubated in 10 mM MES media. These results may provide evidence of synergistic effects between cell wall components already present in 8-d TOM-I medium and those added to the assay for testing. The overall promotion of L-[U-^{14}C] leucine incorporation observed in 8-d TOM-I

media implies that an endogenous component of the media must be responsible for the heightened activity exhibited by the cells. This might be only a promotional effect and not a true example of synergism, but the possibility of synergism should not be overlooked.

Cells from both Control/8-d TOM-I and Control/MES treatments exhibited greater levels of L-[U-^{14}C] leucine incorporation than cells in the corresponding pectic media. The degree of inhibition caused by pectic media was reversed between 8-d TOM-I- and MES-based media, with respect to the species origins of the pectins. All the cells came from a single culture, so the reversal of comparative inhibition should not be due to metabolic variations, but might be attributable to any variation in pectin samples that might arise during freeze drying (sect. 4.3.1). Alternatively, this effect may be due to oligosaccharin(s) in the 8-d TOM-I media and pectic fragments from *N. physaloides* combining to alleviate the extent of the inhibitory effect induced by the pectic fragments alone.

Different results were obtained in a repeat run of this experiment (results not presented here) in which the second half of this batch of media, which had been frozen in the meantime, were used. Cells incubated in MES media again incorporated lower levels of L-[U-^{14}C] leucine than cells incubated in 8-d TOM-I media, and cells incubated in +HYBpfrags/8-d TOM-I medium again exhibited reduced levels of L-[U-^{14}C] leucine incorporation by comparison with those in Control/8-d TOM-I medium. However, in this later run, cells incubated in +NPpfrags/8-d TOM-I exhibited no inhibition of leucine incorporation, cells incubated in +NPpfrags/MES exhibited slightly enhanced leucine incorporation, and cells incubated in +HYBpfrags/MES incorporated a similar amount of leucine to cells in Control/MES medium.

Therefore, an element of variation appears to have been present in the L-[U-^{14}C] leucine incorporation assays to test the effects of pectins and pectic fragments on cells of *L. esculentum* x *peruvianum*. In these assays the application of self, *L. esculentum* x *peruvianum*, pectins and pectic fragments never promoted leucine incorporation. The causes of variation described above for assays with non-fragmented pectins (sect. 4.3.1), i.e. the presence of endogenous oligosaccharides in 8-d spent medium, and Ca^{2+} bonding between oligogalacturonides are here only applicable to cells incubated in 8-d TOM-I media and not those incubated in MES-based media which were free from both endogenous cell wall components and Ca^{2+} ions. However, since the same batch of MES-based media was used in both runs of this experiment, unless the effects of pectins vary, in which case the variation is inherent in the pectins added to the media, the disparity observed in incorporation rates of cells incubated in MES media can only be attributed to metabolic fluctuations in the different suspension-cultures used on each day.

4.3.3: EFFECTS OF EXOGENOUS PECTINS ON THE INCORPORATION OF [^{14}C] INTO PROTEIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* AC

4.3.3.1: **Summary:** Effects of pectins on *L. esculentum* AC

The effects of Solanaceous pectins and other pectic compounds on suspension-cultured cells of *L. esculentum* AC were assessed using the L-[U-^{14}C] leucine incorporation assay in both 8-d SH and fresh SH incubation media. Previously, no consistent effect of Solanaceous pectins had been established with suspension-cultured cells of *L. esculentum* x *peruvianum*; a similar pattern of varied responses emerged for cells of *L. esculentum* AC.

In 8-d SH media, cells incubated with *L. esculentum* AC pectins (+ACpec) and with *N. physaloides* pectins (+NPpec) exhibited reductions in L-[U-^{14}C] leucine incorporation of 31% and 21%, respectively. However, in fresh SH media, cells incubated in +ACpec and +NPpec media exhibited increases of 12% and 17% in L-[U-^{14}C] leucine incorporation compared to cells in the control, although a reduction of 44% was exhibited by cells incubated with *L. esculentum* x *peruvianum* pectins (+HYBpec). Furthermore, in the same experiment, cells incubated with commercially-available *Citrus* pectin (+CITpec) and polygalacturonic acid (+PGA) in fresh SH medium exhibited increases of 11% in the amount of L-[U-^{14}C] leucine incorporated.

The application of pectins in different media and differences in cell densities between experiments may have contributed to the disparity of effects observed.

4.3.3.2: **Introduction:** Effects of pectins on *L. esculentum* AC (see General Introduction sect. 4.3.0)

4.3.3.3: **Materials and Methods:** Effects of pectins on *L. esculentum* AC

L-[U-^{14}C] leucine incorporation assays (sect. 2.3) were performed on suspension-cultured cells of *L. esculentum* AC, in both 8-d SH and fresh SH media. Pectins were obtained from cell walls from internodes of *L. esculentum* AC (+ACpec) and *N. physaloides* (+NPpec), from suspension-cultured cells of *L. esculentum* x *peruvianum* (+HYBpec) (sect. 2.4.2), and from a commercial preparation of *Citrus* pectin (+CITpec). Polygalacturonic acid (+PGA) was also tested. Pectins were added to the media at 0.5 mg/ml.

4.3.3.4: **Results:** Effects of pectins on *L. esculentum* AC

4.3.3.4.a: **Effect of exogenous pectins on [^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* AC.**

A L-[U-^{14}C] leucine incorporation assay was performed on suspension-cultured cells of *L. esculentum* AC incubated in 8-d SH medium ± *L. esculentum* AC (+ACpec) or

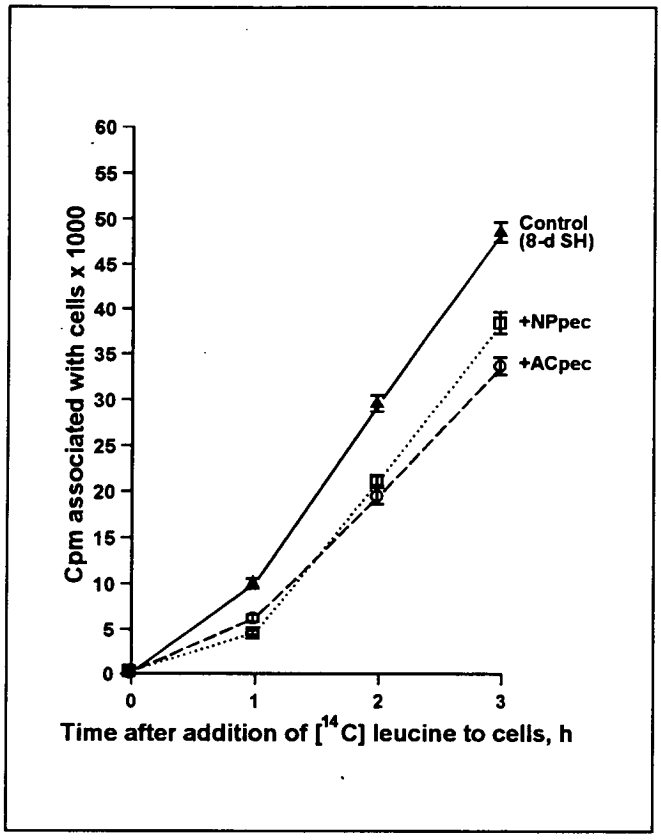


Figure 4.3.3.4.a:

Effect of exogenous pectins on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* AC.

Means were obtained from a minimum of 5 and maximum of 6 samples at 0 h and 1 h, and 10 samples at 2 h and 3 h.

N. physaloides pectins (+NPpec). Cells were added to the media at a concentration of 135 mg/ml instead of the usual 100 mg/ml. The results are displayed in Figure 4.3.3.4.a.

Cells incubated in +ACpec/8-d SH and +NPpec/8-d SH media exhibited reduced levels of incorporation of L-[U-^{14}C] leucine into protein from the 1-h sample point onwards and, 3 h after the addition of L-[U-^{14}C] leucine, had incorporated 69% and 79%, respectively, of the amount of leucine incorporated by cells in the Control/8-d SH treatment.

4.3.3.4.b: Effect of Solanaceous and other exogenous pectins on the incorporation of [^{14}C] leucine into protein in suspension-cultured cells of *L. esculentum* AC.

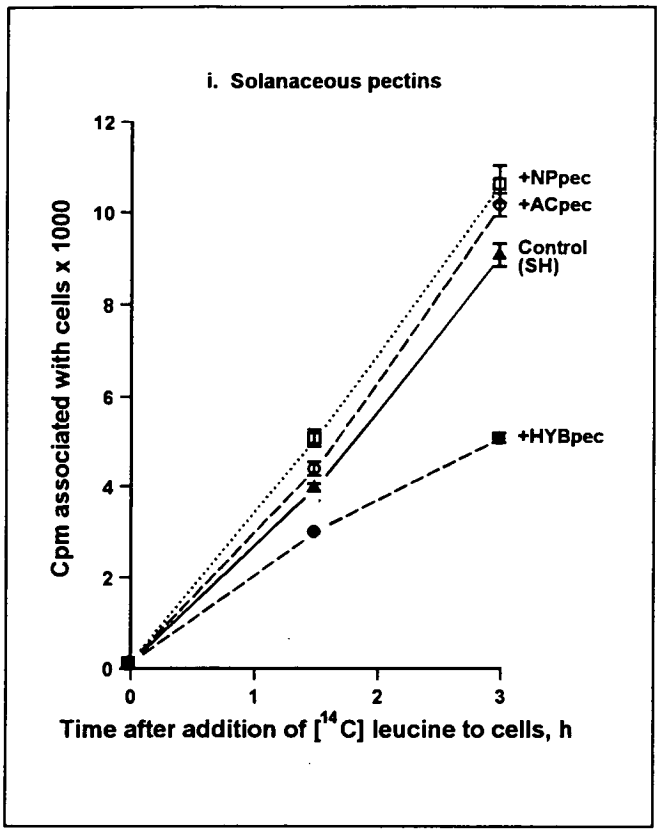
A L-[U-^{14}C] leucine incorporation assay (sect. 2.3) was performed with suspension-cultured cells of *L. esculentum* AC incubated in fresh SH-based media with the following pectic additions: +ACpec, +NPpec, *L. esculentum* x *peruvianum* (+HYBpec), *Citrus* (+CITpec), and polygalacturonic acid (+PGA), at 0.5 mg/ml; the control treatment was provided by unadulterated fresh SH medium. The addition of pectins lowered the pH of the medium from 5.7 - 5.8 to between 4.0 - 5.4. The pH of each incubation medium was readjusted to 5.7 - 5.8 with 1 M NaOH prior to the addition of cells. Samples were taken at 0, 1.5 and 3 h. For clarity, the results of the assay are shown in two figures, Figures 4.3.3.4.b.i and ii; the Control/SH medium line appears on both graphs to aid comparison of treatments.

The results obtained with Solanaceous pectins are shown in Figure 4.3.3.4.b.i. In this instance cells of *L. esculentum* AC incubated in +NPpec/SH and +ACpec/SH media exhibited increased rates of leucine incorporation, and after 3 h had incorporated 17% and 12% more L-[U-^{14}C] leucine than cells in the Control/SH treatment. Cells incubated in +HYBpec/SH medium exhibited a very marked reduction in leucine incorporation throughout, and by the 3-h sample point had incorporated only 56% of the amount of L-[U-^{14}C] leucine incorporated by cells in the Control/SH treatment.

The results obtained with non-Solanaceous pectin and PGA are shown in Figure 4.3.3.4.b.ii. After 3-h cells incubated in +CITpec/SH and +PGA/SH media had incorporated 11% more L-[U-^{14}C] leucine into protein than cells in the control treatment. These increases were similar in size to those measured in cells incubated in +ACpec/SH and +NPpec/SH media. At the 1.5-h sample point cells incubated in +CITpec/SH medium had incorporated 17% more L-[U-^{14}C] leucine than those incubated in +PGA/SH but the size of this difference did not persist to the 3-h sample point.

4.3.3.5: Discussion: Effects of pectins on *L. esculentum* AC

The results from section 4.3.3 showed a degree of variation similar to that observed in the parallel set of experiments with suspension-cultured cells of *L. esculentum* x *peruvianum* (sect. 4.3.1). However, in this case the variation may be due to identical compounds



Note: Figures 4.3.3.4.b.i and ii show results from a single experiment which could not be displayed adequately on a single axis.

Figure 4.3.3.4.b.i:

Effect of exogenous Solanaceous pectins on L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* AC.

Means were obtained from 4 samples at 0 h and 8 samples at 1.5 h and 3 h.

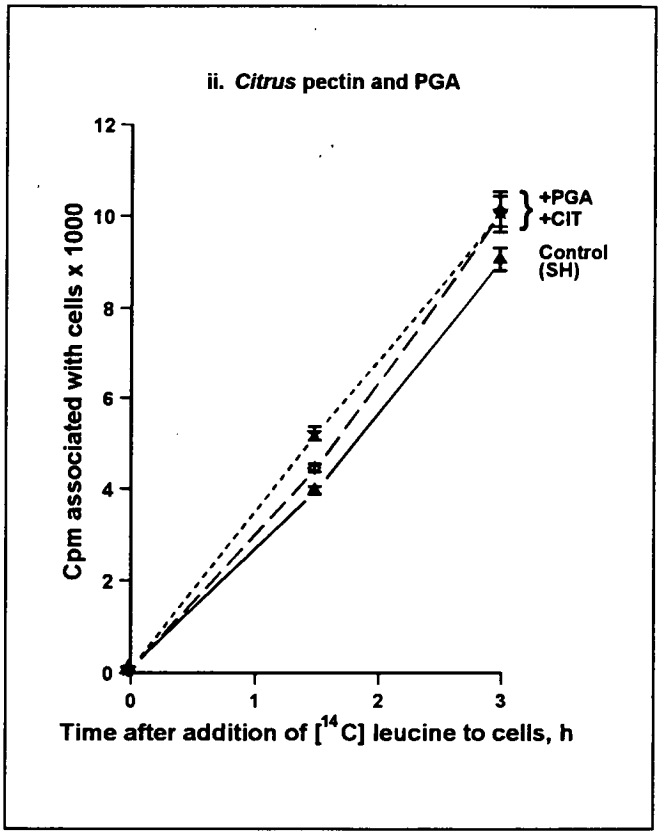


Figure 4.3.3.4.b.ii:

Effect of exogenous *Citrus* pectin (CIT) and polygalacturonic acid (PGA) on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* AC.

Means were obtained from 4 samples at 0 h and 8 samples at 1.5 h and 3 h.

producing different effects when supplied to cells in different media, particularly when one medium contains an unspecified background population of oligo- and polysaccharides (this will be elaborated in overall discussion sect. 7.1.2). When 8-d SH-based media were used (sect. 4.3.3.4.a), the addition of pectins from *L. esculentum* AC and *N. physaloides* reduced leucine incorporation by the cells. However, when fresh SH media were used (sect. 4.3.3.4.b), exogenous Solanaceous and non-Solanaceous pectins and PGA, with the exception of +HYBpec, increased levels of incorporation in cells of *L. esculentum* AC. Interestingly, Yamazaki and colleagues (1983) reported an increase in L-[U-^{14}C] leucine incorporation when acid-generated pectic fragments of *Citrus* were supplied to suspension-cultured cells of *Acer*, which agrees well with these observations with cells of *L. esculentum* AC.

If the variations detected were not attributable to the different media in which the pectic compounds were supplied, then it is most likely that they were caused by differences in the final pH of the medium (since the pH of fresh SH media, but not 8-d SH media, was adjusted prior to use) or metabolic activity of the cells. pH might well be the factor governing the riddle of pectin behaviour with both types of cells, since generally the pHs of media were not adjusted. Furthermore, the behaviour of hemicellulosic cell wall polysaccharides was shown to be pH-dependant in this assay (sect. 4.4).

4.3.4: COMPOSITION OF PECTINS FROM CELL WALLS OF DIFFERENT SPECIES.

4.3.4.1: **Summary:** Composition of pectins

Variations in the basic structure or sugar composition of pectins from the different species tested in L-[U-^{14}C] leucine incorporation assays might account for the differences of effects on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC. Samples of all pectic preparations used in the project were completely digested by the enzyme mixture "Driselase". Samples were separated by paper chromatography and the profiles of monomeric sugars and some disaccharides compared. The profile for *Citrus* pectin displayed considerable similarity to the Solanaceous pectins and was clearly identifiable as pectin, but greater differences existed between it and the Solanaceous pectins, than between the Solanaceous pectins,which resembled each other closely. Any identifiable differences in the effects which these pectins exert on suspension-cultured cells is probably attributable to the production of particular oligosaccharides which are structurally specific and not to any basic differences in the proportion of particular monosaccharides within the polymers.

4.3.4.2: **Introduction:** Composition of pectins

The relative concentration of sugar residues in Solanaceous and other pectic extracts were compared to ascertain whether the variety of responses observed when pectins were applied to suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC could be attributed to differences in the ratio of monomers in the overall composition of the polymers. If the profiles obtained contained only minor differences in composition any identifiable biological effects attributable to pectins would probably not be because of a disproportionate representation of certain monomers within the polymers, but be related to the specific structure and arrangement of residues within oligosaccharides cleaved from the polymers.

4.3.4.3: **Materials and Methods:** Composition of pectins

4 mg of pectin extracts from *L. esculentum* x *peruvianum*, *L. esculentum* AC and *N. physaloides* were incubated with the enzyme Driselase (sect. 2.5.1) for 16 h to achieve digestion of the polysaccharides into composite monomers, a galacturonic acid dimer and pectic sidechains of digestion-resistant rhamnogalacturonan II. Controls were provided by 4 mg samples of a) a commercially-available non-Solanaceous pectin, *Citrus* (Sigma), b) polygalacturonic acid (PGA), which if digestion was complete should yield only galacturonic acid, but would yield oligomers thereof if digestion was incomplete, and c) Driselase incubated in the absence of exogenous polysaccharides, to permit identification of any endogenous sugars not of pectic origin.

The sugars in the resulting digest were separated by paper chromatography (PC) first in BuOH/HOAc/H_2O (12:3:5), then in EtOH/Pyr/H_2O (8:2:1) (sect. 2.5.1.c). (Prior to loading samples onto the chromatogram a volume of formic acid [Fisons Scientific Equipment] was added to each sample to prevent "Driselase" digestion of the paper.) After staining with "Wilson's Dip" (sect. 2.5.1.e) the sugars in each preparation were identified by mean of comparison with a parallel lane in which marker sugars had been run.

The intensity with which the sugars stained was directly proportional to their concentration. Identical masses of starting material had been used in digests of each polysaccharide sample, therefore the proportions of monomers in each sample could be determined qualitatively by direct comparison of the intensity of all spots of that monomer. The intensity of these spots was graded on a scale of 0 - 5, where 0 indicated that no trace of that particular sugar were observed for the sample, and 5 indicated that the sugar showed the heaviest level of staining observed.

4.3.4.4: **Results:** Composition of pectins

The results are shown in Figures 4.3.4.4.i and ii. For the Solanaceous pectins, *L. esculentum* AC, *L. esculentum* x *peruvianum*, and *N. physaloides* (Fig. 4.3.4.4.i) two

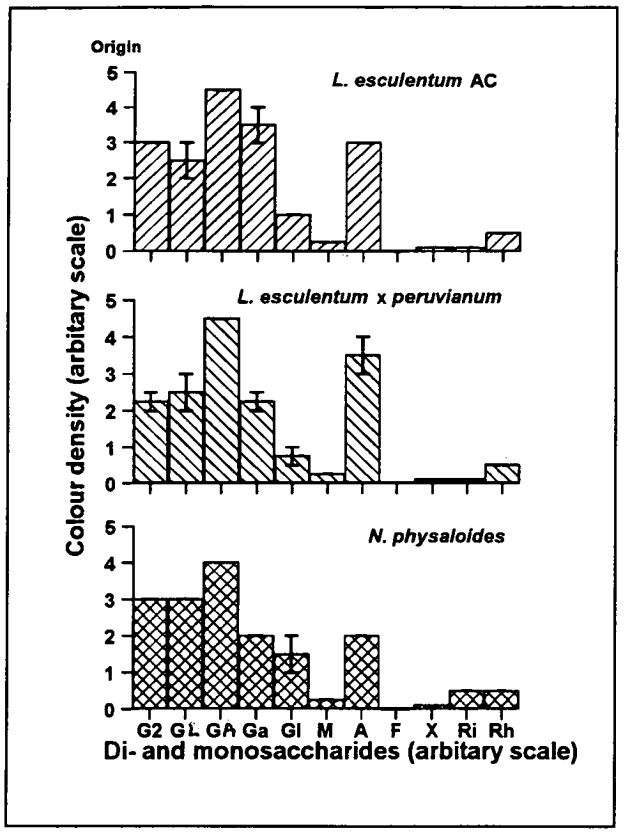


Figure 4.3.4.4.i:

Chromatogram to compare the sugar composition of pectins isolated from *L. esculentum* AC, *L. esculentum* x *peruvianum* and *N. physaloides*, after complete digestion with Driselase. Intensity of stained of di- and monosaccharide spots graded from 0 - 5 (i.e. absent - most intense) on arbitary scale.

Means were obtained from 2 samples.

<u>Key</u>

G2	dimer of galacturonic acid
GA	galacturonic acid
GL	glucuronic acid
Ga	galactose
Gl	glucose
M	mannose
A	arabinose
F	fucose
X	xylose
Ri	ribose
Rh	rhamnose.

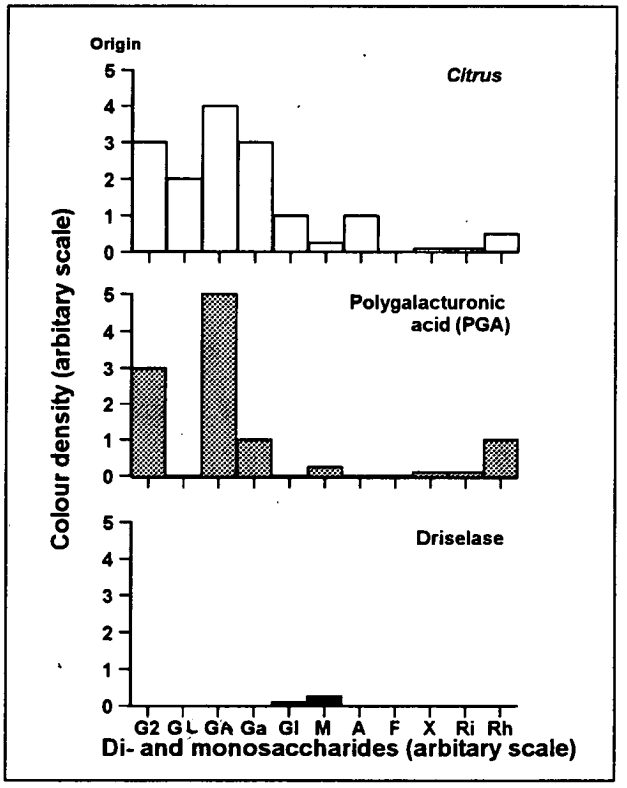


Figure 4.3.4.4.ii:

Chromatogram to compare the sugar composition of commercially-available *Citrus* pectin and polygalacturonic acid (PGA), after complete digestion with Driselase, and Driselase alone. Intensity of staining of di- and monosaccharide spots was graded from 0 - 5 (i.e. absent - greatest intensity) on an arbitary scale.

Single samples of these substances were run, hence the absence of error bars.

<u>Key</u> - as above.

samples from different pectic preparations were run in parallel, the means of these are shown, with error bars to indicate the variation observed. Single samples of *Citrus* pectin, PGA and Driselase (Fig. 4.3.4.4.ii) were run, therefore only absolute values are presented.

In all samples, including the Driselase control, a spot of intensity 1 - 3 remained at the origin. Once the Driselase component had been taken into account, the sugar component of these residues would have registered 1 - 2. It is probable that this consisted chiefly of pectic sidechains of rhamnogalacturonan II (RGII), the nature of which will be discussed later. The Driselase control also demonstrated that, in addition to the material which remained at the origin, the enzyme contributes a certain amount of mannose (Man) and glucose (Glu) to the digest. All samples showed comparable levels of Man, indicating that Man was not initially present in any sample, but was introduced by incubation with the enzyme preparation. Glu was present in higher levels in all other samples, so only a small proportion appears to have been contributed by the enzyme.

Overall there was a great similarity in the sugar profiles obtained for the composition of Solanaceous and *Citrus* pectins. *Citrus* showed lower levels of glucuronic acid (GluA) and arabinose (Ara) than the Solanaceous samples, but the overall spectra are highly comparable. Within the Solanaceous pectins more similarities than differences were observed; the levels of the galacturonic acid dimer (GalA2), GluA, galacturonic acid (GalA), xylose (Xyl) and rhamnose (Rha) detected were almost identical for the three species. The *N. physaloides* samples displayed the highest levels of Glu and ribose (Rib), and the lowest levels of Ara. The levels of galactose (Gal) detected were the highest in the *L. esculentum* AC sample. No fucose (Fuc) was detected in any sample.

The commercial PGA sample yielded GalA2, GalA, and Gal in the greatest quantities. Man appeared to be present, but was probably due to contamination by the enzyme preparation. Xyl, and Rib were present in trace amounts, and a small quantity of Rha was detected.

4.3.4.5: Discussion: Composition of pectins

PC of Driselase digests of Solanaceous and *Citrus* pectins and PGA indicated that Man was not present initially in any polysaccharide sample, but was produced in each by the autolytic action of the enzyme mixture (Fry, 1988) which also generated low levels of Glu. These sugars were not products of the enzymic degradation of the chromatography paper itself since the addition of formic acid to each sample solution stopped all enzyme activity prior to loading the chromatogram.

Generally the PC profiles for the Solanaceous and *Citrus* pectins were very similar, and there were no major variations between pectins from different species, or between different preparations of pectins from the same species. The sugars detected were characteristic of pectin and appeared in the expected proportions (Fry, 1988).

The profile for PGA apparently lacked Glu although a trace amount would have been contributed from the autolysis of the enzyme mixture. This apparent absence was probably due to the crowding of monomer spots along the chromatogram path causing Glu to be over-looked; Glu chromatographs closely with Gal and Man, so can be difficult to detect if present only at a very low concentration. The Rha was probably contributed by the pectin from which PGA was de-esterified with alkali (Fry, 1988).

4.3.5: GENERAL DISCUSSION OF ALL EXPERIMENTS IN WHICH PECTINS OR PECTIC FRAGMENTS WERE APPLIED TO SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC

The effects of pectins and pectic fragments extracted from suspension-cultured cells of *L. esculentum* x *peruvianum* or internodes of *L. esculentum* AC and *N. physaloides* on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC show that either the applications of pectins to the cells were not uniform, in sample or pH, or the effects of these pectins, especially that from *N. physaloides*, were highly variable under the conditions of the assay. PC of the constituent monosaccharides of Solanaceous pectins and *Citrus* pectin indicated that there were no obvious differences in the sugar content of the pectins and that, although the profiles were broadly similar, there was greater similarity within Solanaceous pectins than between Solanaceous and *Citrus* pectins.

If the effects noted in the leucine incorporation assays were attributable to the exogenous pectins it is possible that enzymes secreted by the cells may have cleaved biologically-active fragments from the polysaccharides. Pectins are thought to possess a high degree of structural similarity, so it is likely that enzyme action would release identical fragments from pectins extracted from different species. These would be expected to exert a single effect upon rates of cellular L-[U-^{14}C] leucine incorporation, but this has not been detected, although some generalisations of effect have been outlined. Therefore it is possible that discrete differences in structure exist in pectins from *L. esculentum* x *peruvianum* and *N. physaloides* sources, which, when subjected to cellular enzyme activity produced distinct fragments with different effects. However, the difficulty experienced in attempting to establish a repeatable pattern for pectic effects indicates that even if this were so, identical enzyme-cleaved fragments were not produced regularly from each source, or did not produce identical effects upon the cellular activity on each occasion.

An area in which a greater element of structural specificity may occur are the pectic polysaccharide side-chains known as rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII), which, despite their names, are structurally unrelated to each other. RGI has a DP of ~ 2 000 and consists of a frequently branching backbone in which

Rha and Gal A residues alternate (McNeil, Darvill and Albersheim, 1980, 1982; Lau, McNeil, Darvill *et al.*, 1985). Up to 30 different side chains, attached to the backbone by a variety of linkages (Fry, 1988), have been detected; it is thought that the DP of most is about 7 residues and that they may be structurally specific (Darvill, Albersheim, McNeil *et al.*, 1985).

Investigations with antibody-labelling have shown RGI to be located on the outer surface of primary cell walls and in the middle lamella (Moore, Darvill, Albersheim *et al.*, 1986) of mature cells, although it is largely absent from the cell plate of recently divided cells (Moore and Staehelin, 1988), which might preclude a recognition role in newly formed GU callus cells. Furthermore, recent work by Aldington and Fry (1994), in a very similar assay to the L-[U-^{14}C] leucine incorporation assay used here, demonstrated that RGI does not inhibit leucine incorporation in suspension-cultured cells of *L. esculentum* x *peruvianum*. This might also mean that RGI is not involved in grafting communications.

RGII sidechains are also comparatively large structures, DP of ~ 60, made up from blocks of two different, complex heptasaccharides, and several smaller oligosaccharides attached to a GalA-rich core. (Spellman, McNeil, Darvill *et al.*, 1983; Melton, McNeil, Darvill *et al.*, 1986). These units consist of normal pectic monosaccharides and low concentrations of a rare sugar, ketodeoxy-octulosonic acid (KDO), which has been detected only in RGII and prokaryotes (Darvill, McNeil and Albersheim, 1978). RGII is released from the pectic backbone by pectinase, which cleaves sites of contiguous α-(1$\rightarrow$4)-GalA resides, however pectinases do not attack the backbone of RGII itself. The complexity and resistance to digestive breakdown do not appear to confer any recognised advantage or perform any function in pectic molecules or the cell wall itself, but may mean that RGII side-chains, in which the structure might be species-specific, remain intact once cleaved from the main molecule, and could be capable of eliciting specific and different effects in cells.

Aldington and Fry (1994) demonstrated that the application of exogenous fractions of low DP RGII to suspension-cultured cells of *L. esculentum* x *peruvianum* inhibited incorporation of [^{14}C]-labelled leucine, proline, arginine, histidine and glutamate. The uptake and incorporation of tyrosine and phenylalanine remained unaffected, suggesting that RGII may have affected the membrane transport systems. The available evidence indicates that RGII might be capable of playing an active role in graft development, but further work would need to be done to corroborate this proposal.

4.4: DETERMINATION OF THE EFFECTS OF HEMICELLULOSES UPON INCORPORATION OF [^{14}C] LEUCINE INTO PROTEIN

4.4.0: GENERAL INTRODUCTION TO THE BIOLOGICAL POTENTIAL OF HEMICELLULOSES IN GRAFTING

Although previous experiments with the in-vitro grafting system had implicated pectins as the major contenders for the role of recognition/signalling molecules in graft development (Jeffree *et al.*, 1987), failure to establish a repeatable pattern of activity for pectins in L-[U-^{14}C] leucine incorporation assays led to the examination of the activity of another class of cell wall polymers, the hemicelluloses.

Hemicelluloses, particularly xyloglucans, have been shown to possess biological activities (York *et al.*, 1984; Fry, 1986; McDougall and Fry, 1988; McDougall and Fry, 1989a) which seem to be restricted to growth phenomena. The structure of those oligosaccharides which affect auxin-induced growth has been well characterised (McDougall and Fry, 1989a, b; Augur *et al.*, 1992) and shows a strong specificity that might be expected to be associated with recognition molecules. *In-vitro* grafting tests, comparable to those performed with pectins, had not been performed on hemicelluloses, however, to remedy this lack of knowledge, the effects of Solanaceous hemicelluloses were determined with suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC in L-[U-^{14}C] leucine incorporation assays.

4.4.1: EFFECT OF EXOGENOUS HEMICELLULOSE, IN 8-d TOM-l AND 10 mM MES MEDIA BUFFERED TO pH 6.0, ON THE INCORPORATION OF [^{14}C] LEUCINE INTO PROTEIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM*

4.4.1.1: **Summary:** Effects of hemicelluloses at pH 6

Hemicellulosic fragments, particularly xyloglucan oligomers, have been shown to exert biological effects (York *et al.*, 1984; Fry, 1986; McDougall and Fry, 1988; McDougall and Fry, 1990; Fry *et al.*, 1990). The activity of hemicelluloses, from *L. esculentum* x *peruvianum* and *N. physaloides*, was evaluated in a L-[U-^{14}C] leucine incorporation assay with suspension-cultured cells of *L. esculentum* x *peruvianum*.

Cells incubated in 8-d TOM-l-media containing *L. esculentum* x *peruvianum* (+HYBhemi) and *N. physaloides* hemicelluloses (+NPhemi) exhibited reductions in L-[U-^{14}C] leucine incorporation of 28% and 50%, respectively. In a parallel assay using MES-based hemicellulosic-media cells incubated in +HYBhemi and +NPhemi media exhibited reductions of 6% and 13%, respectively. Cells in the MES control treatment incorporated

22% less L-[U-^{14}C] leucine than cells in the 8-d TOM-I control treatment. Use of 8-d TOM-I media appeared to maximise the inhibitory capacity of the hemicelluloses, which might result from synergistic interactions with the population of poly- and oligosaccharides already present in the medium. The application of *N. physaloides* hemicelluloses consistently caused the greater inhibitions, which may imply an element of species specificity in the effects of hemicelluloses.

4.4.1.2: **Introduction:** Effects of hemicelluloses at pH 6 (see General Introduction sect. 4.4.0)

4.4.1.3: **Materials and Methods:** Effects of hemicelluloses at pH 6

Hemicelluloses were extracted (sect. 2.4.3) from the cell wall residues of *L. esculentum* x *peruvianum* and *N. physaloides* after the pectic component had been removed (sect. 2.4.2). A L-[U-^{14}C] leucine incorporation assay (sect. 2.3) was performed on suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 8-d TOM-I and 10 mM MES media ± *L. esculentum* x *peruvianum* (+HYBhemi) or *N. physaloides* hemicelluloses (+NPhemi) at 0.5 mg/ml. The pH of the media was adjusted to 6.0 - 6.2 prior to the addition of cells. Cells were obtained from two suspension-cultures of *L. esculentum* x *peruvianum*, which were mixed prior to use to ensure a uniform mixture of cells. Samples were taken 0, 2 and 3 h after the addition of L-[U-^{14}C] leucine. This experiment was not repeated with cells of *L. esculentum* AC.

4.4.1.4: **Results:** Effects of hemicelluloses at pH 6

The results are shown in Figures 4.4.1.4.i and ii. At the 2-h and 3-h sample points cells of *L. esculentum* x *peruvianum* incubated in +HYBhemi/8-d TOM-I and in +NPhemi/8-d TOM-I media showed notable reductions from cells in the Control/8-d TOM-I treatment in the amounts of L-[U-^{14}C] leucine incorporated into protein (Fig. 4.4.1.4.i); cells incubated in +NPhemi/8-d TOM-I exhibited the minimum rate and level of incorporation throughout. By the 3-h sampling point cells incubated in +HYBhemi/8-d TOM-I medium had incorporated 72% of the amount of L-[U-^{14}C] leucine incorporated by cells in the Control/8-d TOM-I treatment, while cells incubated in +NPhemi/8-d TOM-I medium had incorporated 50%.

Cells of *L. esculentum* x *peruvianum* incubated in MES-based media (Fig. 4.4.1.4.ii) exhibited much smaller differences in the final levels of L-[U-^{14}C] leucine incorporated than cells incubated in 8-d TOM-I media (Fig. 4.4.1.4.i). At the 3-h sample point cells incubated in +HYBhemi/MES medium had incorporated 94% of the amount of L-[U-^{14}C] leucine fixed by cells incubated in Control/MES medium, while cells incubated in +NPhemi/MES medium had incorporated 87%. Greater differences between treatments were measured at the 2-h sample point.

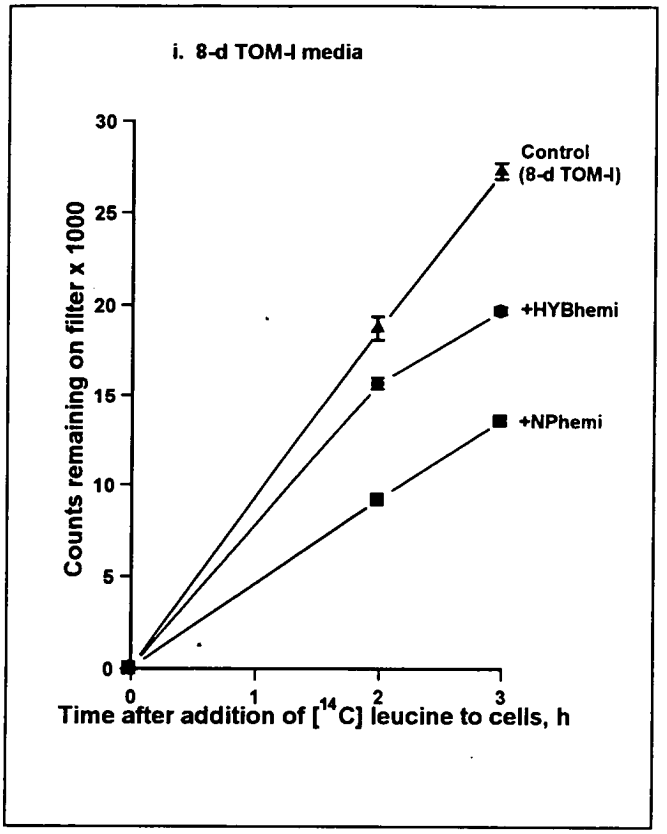


Figure 4.4.1.4.i:

Effect of exogenous hemicellulose on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 8-d TOM-I-based media.

Means were obtained from 4 samples at 0 h, 8 samples at 2 h and 10 samples at 3 h.

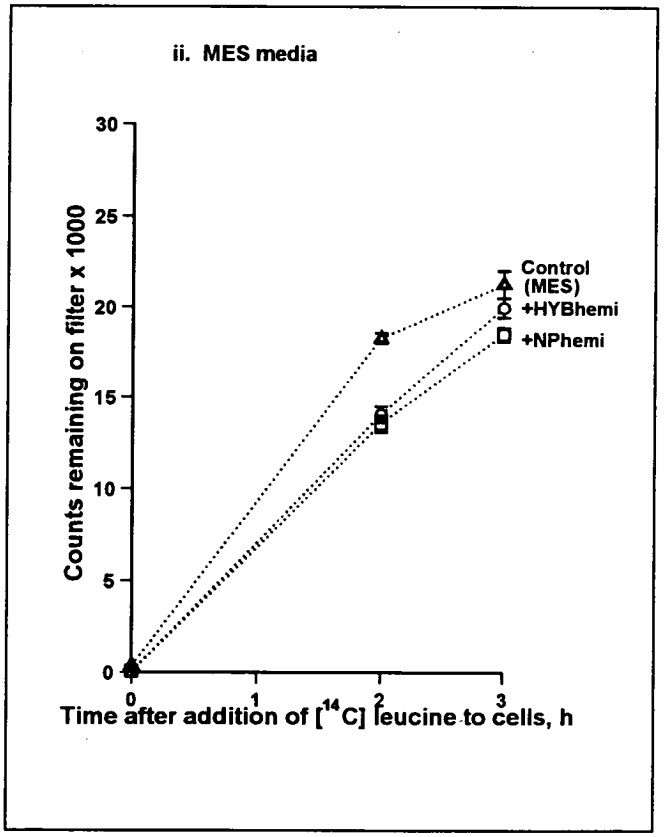


Figure 4.4.1.4.ii:

Effect of exogenous hemicellulose on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 10 mM MES-based media.

Means were obtained from 4 samples at 0 h, 8 samples at 2 h and 10 samples at 3 h.

Comparison of the amounts of L-[U-^{14}C] leucine incorporated into protein by cells in the control treatments reveals that cells incubated in Control/MES medium had incorporated 78% of the amount of L-[U-^{14}C] leucine incorporated by cells in the Control/8-d TOM-I treatment by the end of the assay. After 3 h, cells incubated in +NPhemi/8-d TOM-I had incorporated 73% of the amount of L-[U-^{14}C] leucine incorporated by cells incubated in the corresponding +NPhemi/MES medium, while cells incubated in +HYBhemi/8-d TOM-I had incorporated 99% of the amount incorporated by cells incubated in +HYBhemi/MES medium. The difference between the 8-d TOM-I and MES-based media was small for the +HYBhemi treatments, but was considerable for the +NPhemi treatments.

4.4.1.5: **Discussion:** Effects of hemicelluloses at pH 6

Cells of *L. esculentum* x *peruvianum* incubated with hemicelluloses exhibited reductions in L-[U-^{14}C] leucine incorporation, and, hence, in the rate of protein metabolism. Since hemicelluloses from both sources inhibited protein metabolism, the effect may not be species-specific, however, hemicelluloses from *N. physaloides* induced the greater reductions in protein metabolism in both 8-d TOM-I and MES media, which may indicate that an element of specificity was present.

Differences in the effect of hemicelluloses, apparent in MES media, were enhanced when 8-d TOM-I medium was used, possibly because of synergistic effects between the applied hemicelluloses and the endogenous cell wall polysaccharides naturally present in 8-d TOM-I medium. These differences may be attributable to variable interpretation of particular oligo- or polysaccharide signals by cells in the presence or absence of a background population of cell wall components (this idea is developed in section 7.1.2).

4.4.2: EFFECTS OF EXOGENOUS HEMICELLULOSES IN A MEDIUM AT pH 4.5 ON THE PROTEIN METABOLISM OF SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC

4.4.2.1: **Summary:** Effects of hemicellulose at pH 4.5

The addition of hemicelluloses to 8-d TOM-I or MES media caused a reduction in pH, which, if not adjusted, produced results which were noticeably different to those produced previously at pH 6.0 - 6.2 (sect. 4.4.1.4). L-[U-^{14}C] leucine incorporation assays were performed to assess the effects of hemicelluloses, under controlled conditions of low pH, on cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC.

Cells of *L. esculentum* x *peruvianum* incubated at pH 4.5 with *L. esculentum* x *peruvianum* (+HYBhemi) and *N. physaloides* hemicelluloses (+NPhemi) exhibited severe and immediate reductions in L-[U-^{14}C] leucine incorporation of 92% and 96%, respectively.

Concurrent viability tests showed that cells of *L. esculentum* x *peruvianum* incubated at pH 4.5 in the absence of hemicelluloses were 77% viable initially, and 63% viable after more than 3 h incubation. 55% of cells incubated at pH 4.5 with +HYBhemi were viable initially, dropping to about 49% after 3 h incubation. Cells incubated at pH 4.5 with +NPhemi were 37% viable initially, but only 20% viable after 3 h. Cells incubated in TOM-I medium throughout retained approximately 77% viability. Plasmolysis was observed in cells exposed to low pH, but appeared more frequently when hemicelluloses were present.

Cells of *L. esculentum* AC exhibited comparable and severe reductions in the levels of L-[U-^{14}C] leucine incorporated when incubated at pH 4.5, regardless of whether or not hemicelluloses were present; the effect appeared to be attributable to low pH and independent of exogenous hemicellulose. After an incubation of 1 h, no viable cells were detected in medium containing +AChemi or +NPhemi, and only 0.75% of cells incubated at low pH in the absence of hemicellulose showed signs of life. This suggested that the cessation of protein synthesis could be attributed largely to cell death.

4.4.2.2: Introduction: Effects of hemicellulose at pH 4.5

In preliminary experiments, which are not documented here, it had been noticed that if 10 mM MES incubation medium was not carefully buffered at pH 6.0 - 6.2 the observed effects of hemicellulose on the protein metabolism of the cells were quite different to those previously described in section 4.4.1.4. Consequently the effect of exogenous hemicelluloses on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum AC* were investigated under controlled conditions of low pH. The incubation medium chosen for the experiment was 10 mM tartaric acid (TART), which is an effective buffer around pH 4.5.

4.4.2.3: Materials and Methods: Effects of hemicellulose at pH 4.5

L-[U-^{14}C] leucine incorporation assays were performed (sect. 2.3) using 10 mM tartaric acid, adjusted to pH 4.5, as the incubation medium to which hemicelluloses extracted from cell wall residues of *L. esculentum* x *peruvianum* (+HYBhemi), *L. esculentum* AC (+AChemi) or *N. physaloides* (+NPhemi) (sect. 2.4.3) were added at 0.5 mg/ml.

The viability of suspension-cultured cells of *L. esculentum* x *peruvianum* in each of the tartaric acid-based media was determined twice, and that of suspension-cultured cells of *L. esculentum* AC determined once, during the assays, by means of the FDA viability test (sect. 2.2.3), to assess whether any reduction in protein metabolism observed in the cells might be due to cell death induced by conditions of low pH.

4.4.2.4: Results: Effects of hemicellulose at pH 4.5

4.4.2.4.a: Effect of exogenous hemicellulose, in 10 mM tartaric acid buffer at pH 4.5, on the incorporation of [^{14}C] leucine into protein in suspension-cultured cells of *L. esculentum* x *peruvianum*, in conjunction with an FDA viability test.

4.4.2.4.a.i: L-[U-^{14}C] leucine incorporation assay

The results of the L-[U-^{14}C] leucine incorporation assay are displayed in Figure 4.4.2.4.a.i. The incorporation of L-[U-^{14}C] leucine into protein by cells of *L. esculentum* x *peruvianum* was generally reduced under conditions of low pH: the final counts fixed by cells in the Control/TART treatment in this experiment were <7 000 cpm, and in repeat experiments, which are not documented here, did not exceed 12 500 cpm, which was considerably lower than the minimum final levels previously observed for cells incubated at more neutral pHs in Control/8-d TOM-I and Control/MES media, i.e. >25 000 cpm and >20 000 cpm respectively. The levels of L-[U-^{14}C] leucine incorporated by cells incubated in +HYBhemi/TART and +NPhemi/TART media were exceedingly low, <600 cpm throughout. At the 3-h sample point, cells incubated in +HYBhemi/TART medium had incorporated 8% of the amount of L-[U-^{14}C] leucine incorporated by cells incubated in Control/TART medium, while cells incubated in +NPhemi/TART medium incorporated only 4%. 2 h after the addition of L-[U-^{14}C] leucine cells incubated in +NPhemi/TART medium had incorporated a maximum 371 ± 63 cpm. The amount of L-[U-^{14}C] leucine fixed by cells incubated in +HYBhemi/TART medium fluctuated between 374 - 500 cpm during the assay.

4.4.2.4.a.ii: Viability assessments

The results of the cell viability test (sect. 2.2.3) are shown in Figure 4.4.2.4.a.ii. Initial assessments were completed 0.5 - 2 h after the cells were added to the media, and the second assessments completed after 3.5 - 4.25 h. Controls were provided by 8 d old cells of *L. esculentum* x *peruvianum* in Control/TART medium, and in their original culture fluid, which had not been extracted from another culture, so was described simply as TOM-I medium.

The mean viability of suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in TOM-I medium remained at 76 - 77% during the 4.25 h of the observation period; there was no real change in the viability recorded at 0.5 h and 4.25 h. The FDA solution used to determine the viability of these control cells was made up in fresh TOM-I medium, however, viability assessments for cells incubated in tartaric acid media were performed using an FDA solution which had been made up in 10 mM tartaric acid at pH 4.5.

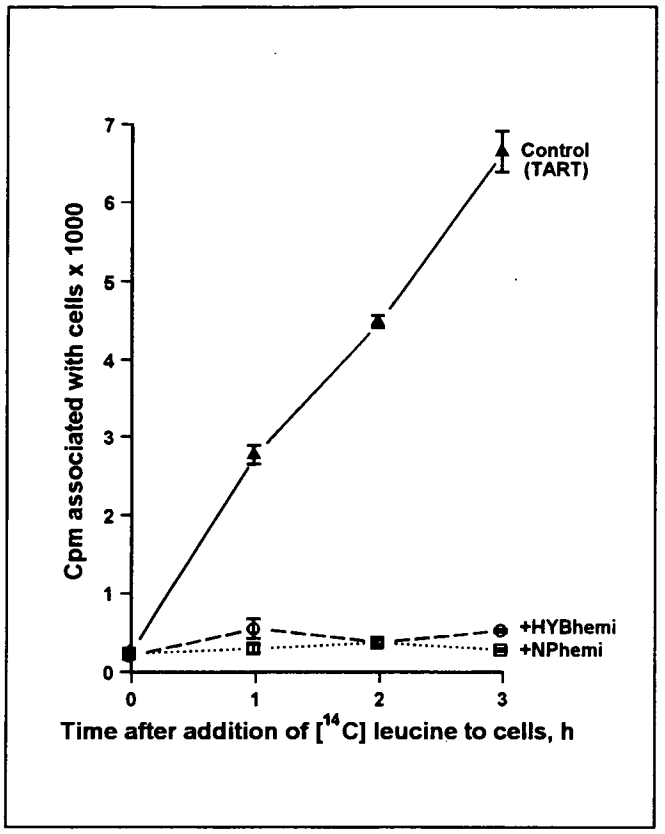


Figure 4.4.2.4.a.i:

Effect of exogenous hemicellulose on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 10 mM tartaric acid (TART) at pH 4.5.

Means were obtained from 4 samples at 0 h, and 8 samples at 1 h, 2 h and 3 h.

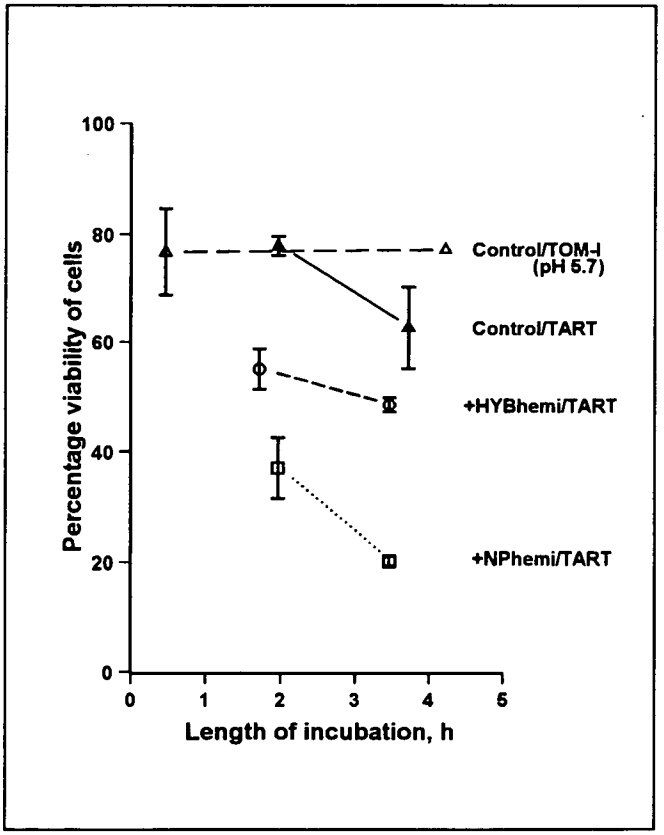


Figure 4.4.2.4.a.ii:

Viability of suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 10 mM tartaric acid media at pH 4.5.

Means were obtained from 3 counts of 200 cells incubated in each medium at each time point.

The viability of cells incubated in Control/TART medium was 78% at the first assessment, which was directly comparable to that of cells in TOM-I medium, however, a significant reduction to 63% had occurred by the time of the second assessment.

In the initial assessment, cells incubated in +HYBhemi/TART and +NPhemi/TART treatments showed rapid and sizeable reductions in viability to 55% and 37% respectively. The second assessment showed that the viability of cells incubated in +HYBhemi/TART medium had fallen to 49%, and that the viability of cells incubated in +NPhemi/TART medium had fallen to 20%. The maximum reduction in viability of cells of *L. esculentum* x *peruvianum* incubated in tartaric acid media ± hemicelluloses for 3.75 h was approximately -57% ± 9%, given the original viability figure of 77% for cells in Control/TOM-I, and the final viability figure of 20% for cells in +NPhemi/TART medium.

By the second assessment many, but not all, cells which failed to accumulate fluorescein in +HYBhemi/TART and +NPhemi/TART media were seen to be plasmolysed. Plasmolysis was also observed in the Control/TART cells, but to a lesser extent. No plasmolysis was observed in dead cells in the Control/TOM-I medium. A few plasmolysed cells retained fluorescein within the plasmalemma, indicating that membrane integrity had not been lost (Heslop-Harrison and Heslop-Harrison, 1970).

4.4.2.4.b: Effect of exogenous hemicellulose, in 10 mM tartaric acid buffer at pH 4.5 on the incorporation of [^{14}C] leucine into protein in suspension-cultured cells of *L. esculentum* AC, in conjunction with an FDA viability test.

4.4.2.4.b.i: L-[U-^{14}C] leucine incorporation assay

Figure 4.4.2.4.b.i shows that cells in all treatments displayed exceedingly low rates of protein metabolism throughout the monitored period. Previous maximal leucine incorporation levels in other L-[U-^{14}C] leucine incorporation assays with cells of *L. esculentum* AC ranged from 12 000 cpm to ~ 50 000 cpm, which were 10 - 40 fold greater than the maximum 3-h incorporation level of ~ 1 250 cpm recorded here.

The assay showed two unusual features; firstly a wide spread of data at 0 h, followed by an apparent drop in the levels of incorporated leucine at 1 h. Thereafter the levels of incorporated L-[U-^{14}C] leucine increased steadily in all treatments. At the end of the assay the levels incorporated were comparable with those measured at 0 h.

At the 1-h sample point, cells incubated in +AChemi/TART had incorporated 41% more L-[U-^{14}C] leucine than cells incubated in either +NPhemi/TART or Control/TART media, which had incorporated similar amounts. At 2 h, cells incubated in +AChemi/TART and Control/TART media had incorporated roughly the same amount of L-[U-^{14}C] leucine, while the amount incorporated by cells incubated in +NPhemi/TART medium was 28% less. At the 3-h sample point no notable differences were apparent between treatments.

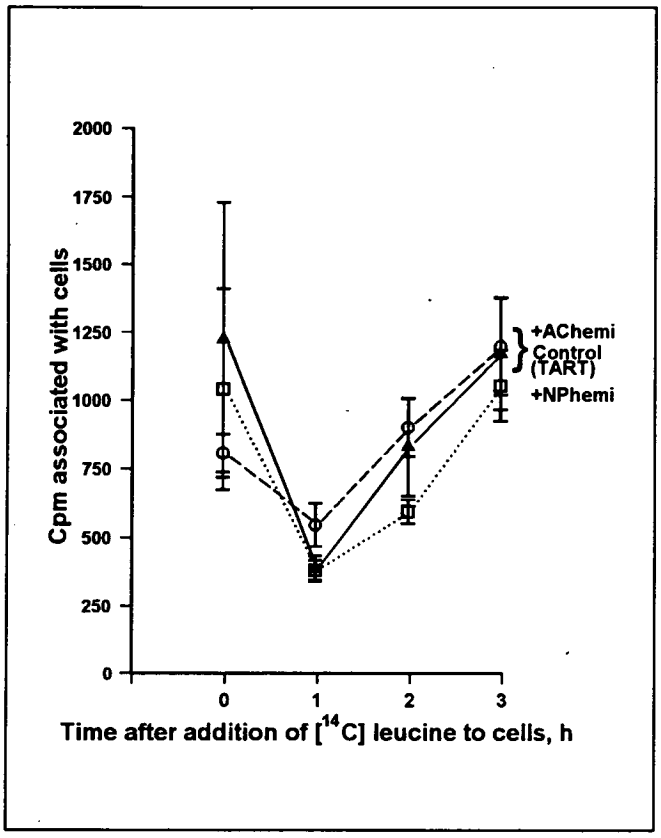


Figure 4.4.2.4.b.i:

Effect of exogenous hemicellulose on L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* AC incubated in 10 mM tartaric acid (TART) at pH 4.5.

Means were obtained from 4 samples at 0 h, and 8 samples at 1 h, 2 h, and 3 h.

4.4.2.4.b.ii: Viability assessments

Table 4.4.2.4.b.ii: Mean viability of suspension-cultured cells of *L. esculentum* AC incubated in 10 mM tartaric acid media ± hemicellulose [0.5 mg/ml], at pH 4.5.

MEDIUM	Length of incubation, h	Mean % viability	Comments
+AChemi/TART	1	0	50-60% of cells plasmolysed
+NPhemi/TART	1	0	50-60% of cells plasmolysed
Control/TART	1	0.75	30-40% of cells plasmolysed

After 1 h incubation in tartaric acid media nearly all cells of *L. esculentum* AC appeared to be dead, since the FDA viability test (Table 4.4.2.4.b.ii) detected no live cells in +AChemi and +NPhemi media and only 0.75% viable cells in the Control/TART medium. Plasmolysis was widespread; after 1 h of incubation 50 - 60% of cells incubated in +AChemi/TART and +NPhemi/TART media exhibited plasmolysis, while 30 - 40% of cells incubated in Control/TART medium appeared to be affected.

4.4.2.5: Discussion: Effects of hemicellulose at pH 4.5

These results indicate that L-[U-^{14}C] leucine incorporation, and hence protein metabolism, almost ceased very early on in the incubation of cells of *L. esculentum* x *peruvianum* in low pH hemicellulosic media. Despite the massive inhibition of protein metabolism in both hemicellulosic media, leucine incorporation was inhibited to a slightly greater degree in cells incubated in +NPhemi/TART than in +HYBhemi/TART, indicating some element of specificity with respect to the source of hemicellulose. A similar situation was observed in section 4.4.1.4 with both 8-d TOM-I and MES-based pectic media.

Viability of cells of *L. esculentum* x *peruvianum* incubated in +NPhemi/TART and +HYBhemi/TART media fell to 20% and 49%, while L-[U-^{14}C] leucine incorporation was inhibited by 96% and 92% respectively. The inhibition of leucine incorporation does not, therefore, appear to be entirely attributable to cell death, but may be due to impairment of anabolic processes by the combination of low pH and hemicellulose. If so, hemicelluloses from *N. physaloides* appear to be more noxious to cells of *L. esculentum* x *peruvianum* than self hemicelluloses from *L. esculentum* x *peruvianum*. Alternatively, the cells might have

adopted a metabolically subdued state from which protein manufacture could recommence if conditions improved.

The results obtained with cells of *L. esculentum* AC in section 4.4.2.4.b.ii suggest that brief incubation at low pH killed most, if not all, cells of *L. esculentum* AC within one hour, indicating that cells of *L. esculentum* AC are more sensitive to conditions of low pH than those of *L. esculentum* x *peruvianum*, in which loss of active protein metabolism was not linked with massive cell death. Furthermore, the amounts of L-[U-^{14}C] leucine incorporated by cells of *L. esculentum* AC did not increase consistently with time, as cells from the 0-h sample point, which were in the presence of L-[U-^{14}C] leucine for only a few seconds prior to death by TCA, incorporated a greater amount than cells incubated for a further hour (see Fig. 4.4.2.4.b.ii). This should not have been possible, since cells incubated for longer in the presence of L-[U-^{14}C] leucine should have fixed an equal if not greater amount of leucine. This drop between 0 h and 1 h is difficult to explain since, once incorporated, L-[U-^{14}C] leucine could only be lost if secreted or excreted from the cells, which is most unlikely. Therefore it is possible that this anomaly resulted from experimental error.

Interestingly, the structural and temporal framework for the hypersensitive response (HR) in pepper plants, developed by Brown, Mansfield and Irlam *et al.* (1993), showed that damage to the plasmalemma, and vesiculation of the cytoplasm, preceded death. In this experiment, cells of *L. esculentum* AC sampled after incubation for an hour in conditions of low pH ± hemicellulose, may have had a brief burst of protein metabolism when exposed momentarily to L-[U-^{14}C] leucine at 0 h. Further incubation of cells, sampled at 1, 2 and 3 h, under these conditions, may have initiated the onset of membrane disintegration, thus permitting radioactively-labelled proteins to be washed away with the unincorporated L-[U-^{14}C] leucine during the filtering procedure. Therefore, cells killed with TCA at 0 h may have retained radioactively-labelled proteins which were released from cells incubated for longer, in which loss of membrane integrity may have been induced.

Data from the 2-h and 3-h sample points in section 4.4.2.4.b.i indicate that, despite the evidence to the contrary from the viability tests, not all cells of *L. esculentum* AC were killed by the prevailing conditions; those that survived continued to incorporate low levels of L-[U-^{14}C] leucine into protein, which accumulated with time. The results from this experiment indicate that incubation of suspension-cultured cells of *L. esculentum* AC in low pH media inhibited protein metabolism severely, and that, under these conditions, protein metabolism was not affected by the presence of exogenous hemicelluloses.

Yamazaki *et al.*(1983) devised a test, from which this leucine-incorporation assay was derived, which provided evidence of phytotoxicity of sycamore cell wall fragments generated by acid hydrolysis on suspension-cultured cells of sycamore. The authors stated that their assay was not a direct measure of cell death, but speculated that inhibition of

protein synthesis would eventually cause death. However, since then this type of assay has generally been accepted as a form of viability test (Bucheli, Doares, Albersheim *et al.*, 1990). The results from the L-[U-^{14}C] leucine-incorporation assays presented in sections 4.4.2.4.a and b could easily be interpreted as evidence of mass cell death caused by a combination of low pH and self or non-self hemicelluloses. However, the viability tests performed during the assay period suggest that, while this may be true for cells of *L. esculentum* AC, the loss in viability which accompanied inhibition of protein metabolism in cells of *L. esculentum* x *peruvianum* did not account fully for the extent to which leucine incorporation was reduced.

It is not known whether the adoption of a plasmolysed state confers any advantage to the cells. Certainly, viable protoplasts can be generated by digestion of cell walls with pectic-degrading enzymes, which are normally toxic to cells, if the cells are plasmolysed prior to treatment (Takebe, Otsuki and Akio, 1968; Basham and Bateman, 1975), and the effect on [^{14}C] leucine incorporation in sycamore cells by cell wall fragments is much reduced after plasmolysis of cells with sorbitol solutions of up to 0.7 M (Yamazaki *et al.*, 1983). Therefore, plasmolysis may restrict the interaction of cell wall fragments with protoplasts and limit physical damage to cells.

Cells of *L. esculentum* x *peruvianum* sustained smaller losses in viability, and appeared to be more sensitive to hemicelluloses than to the low pH, therefore, the employment of plasmolysis as a protective strategy for survival under these conditions may be advantageous, as might the concurrent adoption of a temporary quiescent state of metabolism, which may have been detected here. All documented evidence for plasmolysis as a protective mechanism thus far has been obtained from situations in which it was induced artificially. Therefore, if plasmolysis in this situation is for defensive purposes, this may be the first documented occurrence of spontaneous plasmolysis as a natural defence strategy *in vivo* (this is discussed further in sect. 7.1.6).

4.4.3: PERMANENCE OF EFFECTS OF HEMICELLULOSES AT LOW pH ON THE VIABILITY AND ESTERASE ACTIVITY OF SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC

4.4.3.1: **Summary:** Permanence of hemicellulose effect on viability

To determine whether the loss of viability detected in cells incubated in tartaric acid ± hemicellulose (sect. 4.4.2.4.) was due to low pH or whether hemicellulose becomes toxic under such conditions, cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC were incubated for 3 h in tartaric acid ± hemicellulose, and the viability of cells assessed with FDA. Viability of cells was reassessed 1 h after their removal from hemicellulosic media

and resuspension in either fresh native medium at pH 5.7, or fresh 10 mM tartaric acid at pH 4.5 (TART).

L. esculentum x *peruvianum* cells incubated in native medium throughout remained 85 - 88% viable. After 3 h, cells incubated in +HYBhemi/TART, +NPhemi/TART and Control/TART media were 66%, 51% and 84% viable, respectively. In all treatments cells transferred to fresh TOM-I medium exhibited no notable changes in viability, but the viability of cells transferred to TART continued to decline.

Cells of *L. esculentum* AC incubated in SH medium throughout exhibited viability levels of 88% at 3 h, and 76% at 7 h. Cells incubated in +AChemi/TART were 6% viable after 3 h; this fell to 4% in cells transferred to TART, while indiscriminate fluorescence, suggestive of membrane damage, surrounded cells transferred to fresh SH medium and made it impossible to reassess viability. About 9% of cells incubated in +NPhemi/TART were viable after 3 h; cells exhibited an apparent increase in viability to 17%, upon transfer to fresh SH medium, viability fell to around 4% in cells transferred to TART. After 3 h, cells incubated in Control/TART medium were 37% viable; transfer to TART did not affect viability, but transfer to fresh SH medium apparently increased it to 46%.

The FDA viability test appears to be accurate for cells of *L. esculentum* x *peruvianum*, but less so for cells of *L. esculentum* AC which experience an apparent revival of esterase activity upon transfer to a hemicellulose-free medium with a more neutral pH.

4.4.3.2: Introduction: Permanence of hemicellulose effect on viability

Previously (sect. 4.4.2.4), apparent loss of viability, as assessed by the FDA viability test, accompanied incubation of cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC in tartaric acid ± hemicellulose media. It was important to determine whether the reduction in the number of viable cells was due to low pH (possibly exacerbated by the presence of hemicellulose) or whether hemicellulose becomes toxic under such conditions. The loss of viable cells could be attributed either to their death or to adoption of reversible metabolic quiescence, expressed as a reduction in esterase activity, which would be reflected by the FDA test. The following experiments were performed to determine whether cells previously classified as non-viable were capable of recovery and whether the prevailing conditions exceeded the limits of sensitivity of the FDA viability test.

4.4.3.3: Materials and Methods: Permanence of hemicellulose effect on viability

Briefly the protocol was as follows: three tubes containing 0.5 ml suspension-cultured cells of *L. esculentum* x *peruvianum* or *L. esculentum* AC at 0.1 g/ml in 10 mM tartaric acid at pH 4.5 were incubated with, and without, exogenously applied hemicelluloses at 0.5 mg/ml. After 3 h the viability of the cells in one tube was measured using the FDA test (sect. 2.2.3), the tube was then discarded. Cells in the remaining two tubes were rinsed in the medium to

which they were to be transferred (sect. 2.7.2), to remove all free hemicellulose, and resuspended in either their native medium (fresh TOM-I or fresh SH), or in fresh 10 mM tartaric acid (TART) for a minimum of 1 h, after which viability was assessed again. Additional control treatments were provided by cells incubated in their original culture fluid throughout, which were subjected to the same treatment, including centrifuging, as cells originally incubated in tartaric acid media, although the bathing medium remained the same throughout.

4.4.3.4: **Results:** Permanence of hemicellulose effect on viability

4.4.3.4.a: **Experiment to determine, by means of the FDA viability test, whether incubation in tartaric acid media ± hemicellulose kills, or renders suspension-cultured cells of *L. esculentum* x *peruvianum* quiescent.**

The experiment was performed using 7d old suspension-cultured cells of *L. esculentum* x *peruvianum*. A maximum of 8.5 h elapsed between setting up the sample tubes at t = 0 and the final measurement of viability performed upon the Control/TOM-I sample. The results are displayed in Figures 4.4.3.4.a.i - iv.

Cells of *L. esculentum* x *peruvianum* initially incubated in +HYBhemi/TART medium (Fig. 4.4.3.4.a.i) were 66% ± 6% viable after 3 h incubation. After elution of hemicellulose, the viability of cells resuspended in fresh TOM-I showed little change at 63% ± 2%. A reduction in the viability of cells resuspended in TART was observed, but at 58% ± 5% it was not noteworthy.

Cells of *L. esculentum* x *peruvianum* in +NPhemi/TART medium (Fig. 4.4.3.4.a.ii) were 51% ± 9% viable after 3 h incubation; the viability was considerably lower than the 3 h levels recorded for all other treatments, though the difference between this measurement and that for cells in +HYBhemi/TART is only just statistically significant (at 5%). After elution of hemicellulose, 54% ± 4% of cells transferred to fresh TOM-I medium were viable, compared to 43% ± 4% of those transferred to TART. The differences between the final viability of cells resuspended in fresh TOM-I and TART were statistically significant with respect to each other (at 5%), but not with respect to the earlier 3 h value.

After 3 h incubation in Control/TART medium (Fig. 4.4.3.4.a.iii) 84% ± 4% of cells of *L. esculentum* x *peruvianum* appeared to be viable. After rinsing, the viability of cells transferred to fresh TOM-I medium remained almost unchanged at 82% ± 2%, however a notable decline in viability, to 69% ± 3%, was observed in cells transferred to TART.

The control treatment provided by cells of *L. esculentum* x *peruvianum* incubated in the original TOM-I medium throughout (Fig. 4.4.3.4.a.iv) showed practically no changes in viability at 3 h and at 8 h, with measurements of 85% ± 2% and 88% ± 3% respectively.

Figure 4.4.3.4.a.i:

Viability of suspension-cultured cells of *L. esculentum* x *peruvianum* incubated for 3 h in +HYBhemi/TART medium both before and after transfer to Fresh TOM-I and Fresh TART media.

Figure 4.4.3.4.a.ii:

Viability of suspension-cultured cells of *L. esculentum* x *peruvianum* incubated for 3 h in +NPhemi/TART medium both before and after transfer to Fresh TOM-I and Fresh TART media.

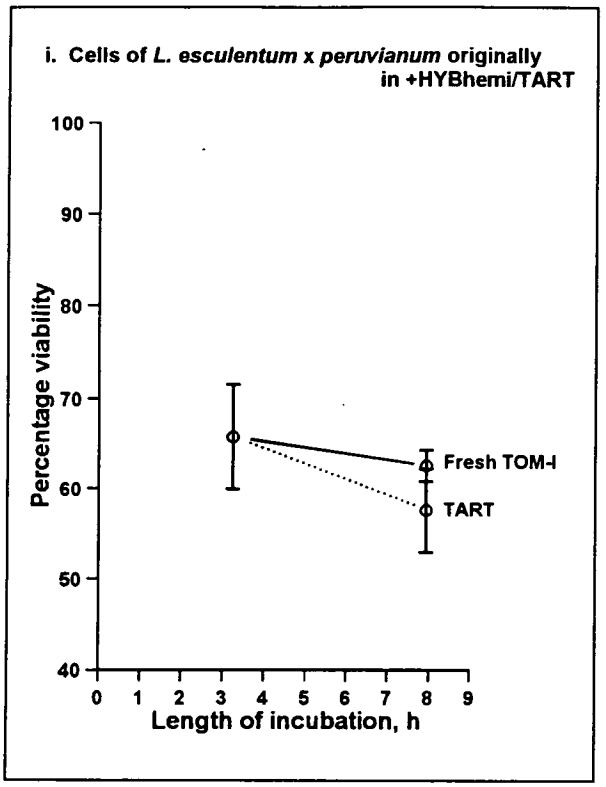
Figure 4.4.3.4.a.iii:

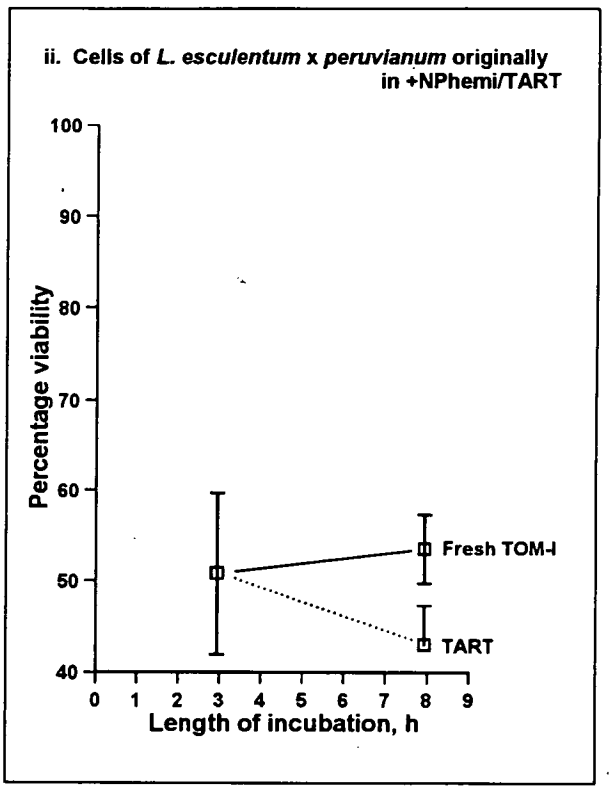
Viability of suspension-cultured cells of *L. esculentum* x *peruvianum* incubated for 3 h in TART medium, to which no additions had been made, both before and after transfer to Fresh TOM-I and Fresh TART media.

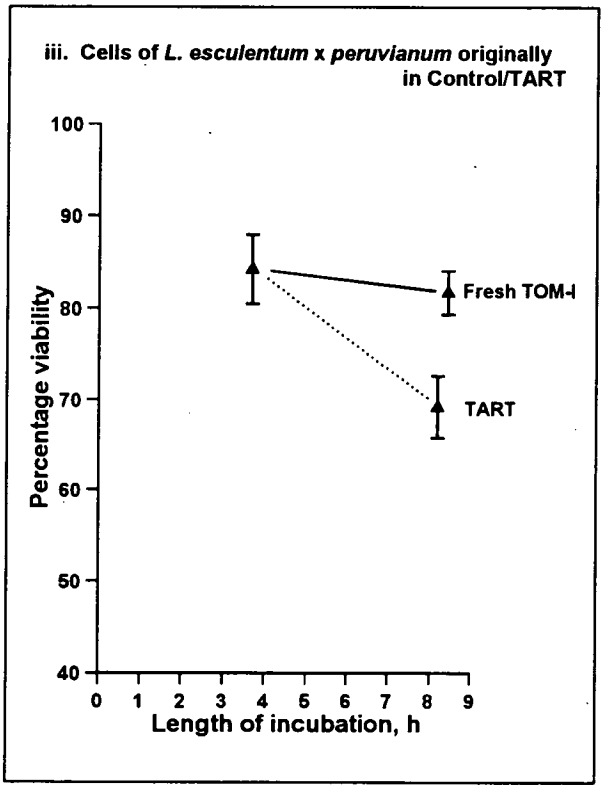
Figure 4.4.3.4.a.iv:

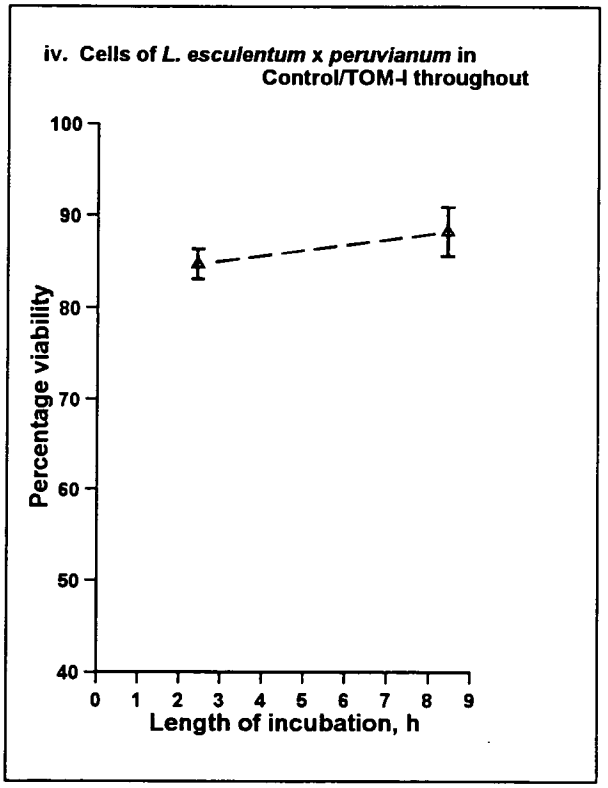
Viability of suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in their original TOM-I medium throughout.

Means were obtained from 3 separate counts of 200 cells from each sample at each time point.


i. Cells of L. esculentum x peruvianum originally in +HYBhemi/TART
Percentage viability
100
90
80
70
60
50
40
0 1 2 3 4 5 6 7 8 9
Length of incubation, h
Fresh TOM-I
TART


ii. Cells of L. esculentum x peruvianum originally in +NPhemi/TART
Percentage viability
100
90
80
70
60
50
40
0 1 2 3 4 5 6 7 8 9
Length of incubation, h
Fresh TOM-I
TART


iii. Cells of L. esculentum x peruvianum originally in Control/TART
Percentage viability
100
90
80
70
60
50
40
0 1 2 3 4 5 6 7 8 9
Length of incubation, h
Fresh TOM-I
TART


iv. Cells of L. esculentum x peruvianum in Control/TOM-I throughout
Percentage viability
100
90
80
70
60
50
40
0 1 2 3 4 5 6 7 8 9
Length of incubation, h

These levels were directly comparable with the 3 h viability level of 84% ± 4% recorded for the Control/TART treatment.

4.4.3.4.b: Experiment to determine, by means of the FDA viability test, whether incubation in tartaric acid media ± hemicellulose kills, or renders suspension-cultured cells of *L. esculentum* AC quiescent.

This experiment was performed on 7d old suspension-cultured cells of *L. esculentum* AC. A maximum of 7 h elapsed between setting up the sample tubes at t = 0 and the final measurement of viability performed upon the Control/SH medium sample. The results are presented in Figures 4.4.3.4.b.i - iv.

Cells of *L. esculentum* AC initially incubated in +AChemi/TART (Fig. 4.4.3.4.b.i.) exhibited the lowest viability of all treatments with 6% ± 2.5% of cells viable after 3 h incubation. After elution of hemicellulose, the viability of cells resuspended in fresh SH medium could not be established due to an excess of general fluorescence around the cells, and a complete absence of specific fluorescence within the cells. Cells resuspended in TART showed a slight reduction in viability to 4% ± 1.2%.

After 3 h, the viability of cells incubated in +NPhemi/TART (Fig. 4.4.3.4.b.ii) was 9.2% ± 2.5%, which was considerably higher than the corresponding viability of cells incubated in +AChemi/TART, and notably lower than that of cells originally incubated in Control/TART or SH medium. After rinsing, cells which were resuspended in fresh SH medium exhibited an apparent increase in viability to 17% ± 4.8%, while the viability of cells resuspended in TART fell to 3.5% ± 0.9%. The final viabilities of cells from +NPhemi/TART medium resuspended in fresh SH and TART media were statistically different to each other at 5%.

After 3 h incubation in Control/TART medium cells of *L. esculentum* AC were 37% ± 1% viable (Fig. 4.4.3.4.b.iii.). After rinsing, there was an apparent increase in the viability of cells transferred to fresh SH medium to 46% ± 5%. The viability of cells resuspended in TART medium remained virtually unchanged at 37% ± 4%. In the additional control, provided by cells of *L. esculentum* AC incubated in the original SH medium throughout (Fig. 4.4.3.4.b.iv), viability declined from 88% after 3 h incubation to 76% after 7 h. Approximately twice as many of the cells incubated in SH medium throughout were recorded as viable than those initially incubated in Control/TART medium, even after the latter were transferred to fresh SH medium.

4.4.3.5: Discussion: Permanence of hemicellulose effect on viability

Results from sections 4.4.3.4.a and b suggest that the viability data supplied by the FDA test were accurate across a range of pH values for cells of *L. esculentum* x *peruvianum*, but were less accurate for cells of *L. esculentum* AC. Inhibition of esterase activity at low pH,

Figure 4.4.3.4.b.i:

Viability of suspension-cultured cells of *L. esculentum* AC incubated for 3 h in +AChemi/TART medium both before and after transfer to Fresh SH and Fresh TART media.

Figure 4.4.3.4.b.ii:

Viability of suspension-cultured cells of *L. esculentum* AC incubated for 3 h in +NPhemi/TART medium both before and after transfer to Fresh SH and Fresh TART media.

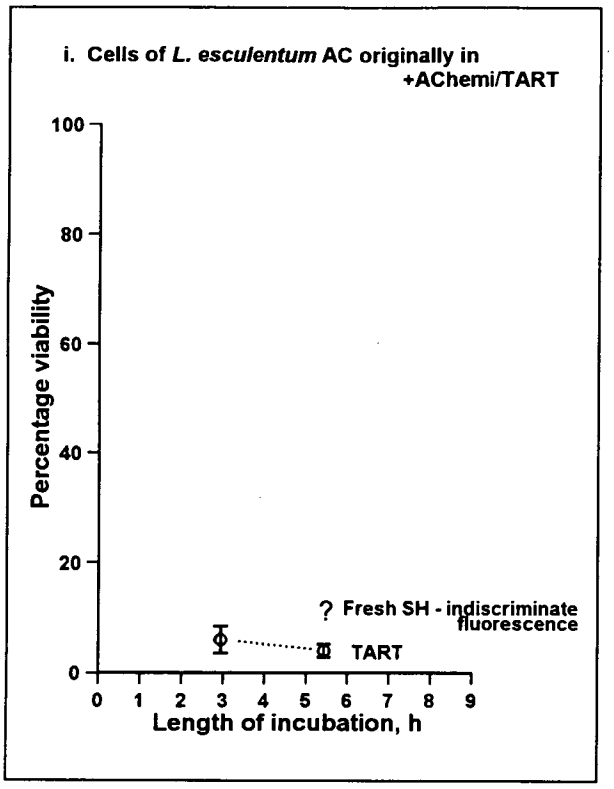
Figure 4.4.3.4.b.iii:

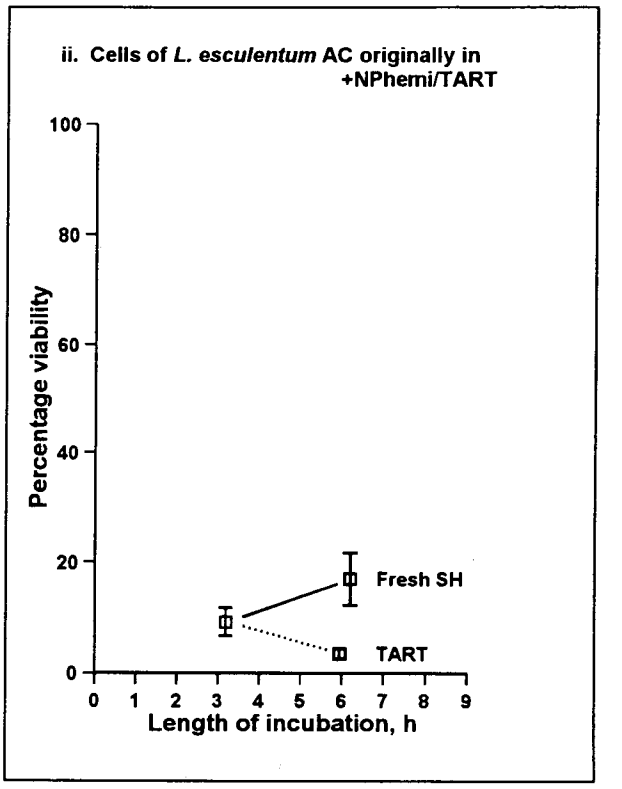
Viability of suspension-cultured cells of *L. esculentum* AC incubated for 3 h in TART medium, to which no additions had been made, both before and after transfer to Fresh SH and Fresh TART media.

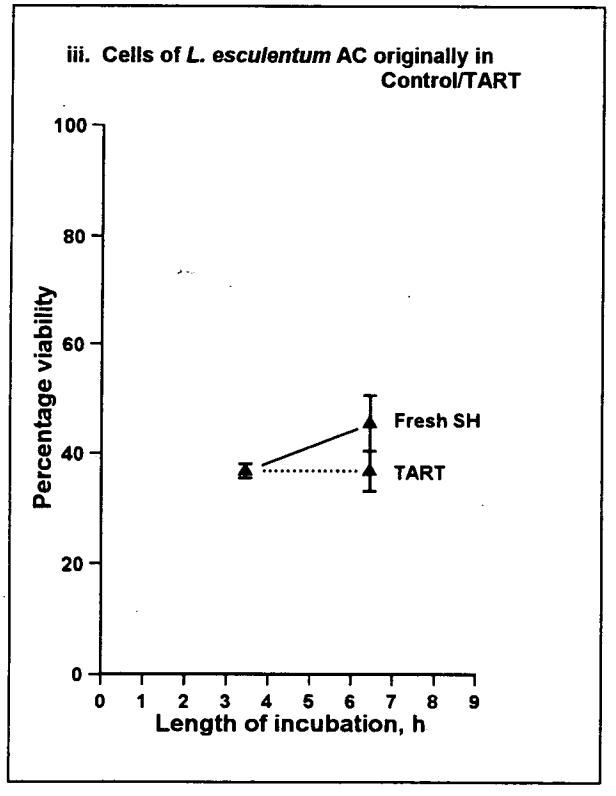
Figure 4.4.3.4.b.iv:

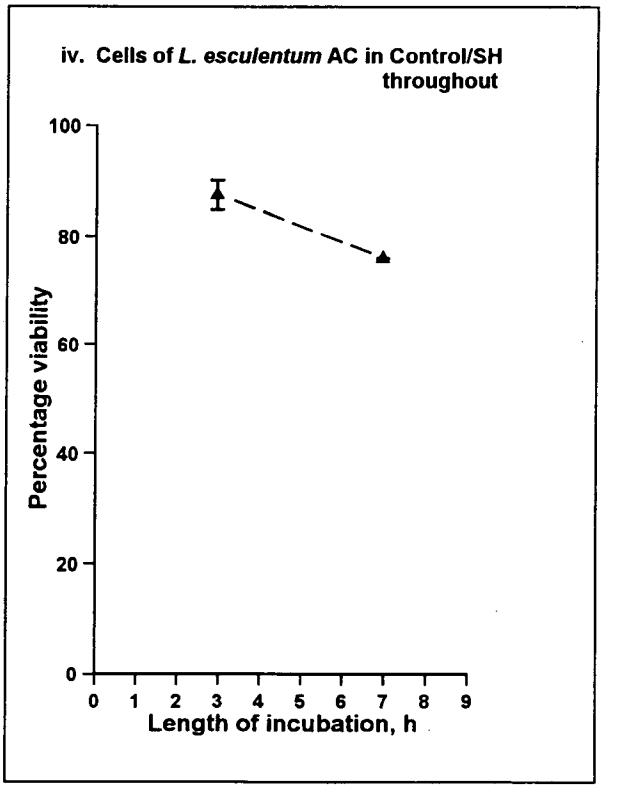
Viability of suspension-cultured cells of *L. esculentum* AC incubated in their original SH medium throughout.

Means were obtained from 3 separate counts of 200 cells from each sample at each time point.


i. Cells of L. esculentum AC originally in +AChemi/TART
Percentage viability
100
80
60
40
20
0
? Fresh SH - indiscriminate fluorescence
TART
0 1 2 3 4 5 6 7 8 9
Length of incubation, h


ii. Cells of L. esculentum AC originally in +NPhemi/TART
Percentage viability
100
80
60
40
20
0
Fresh SH
TART
0 1 2 3 4 5 6 7 8 9
Length of incubation, h


iii. Cells of L. esculentum AC originally in Control/TART
Percentage viability
100
80
60
40
20
0
Fresh SH
TART
0 1 2 3 4 5 6 7 8 9
Length of incubation, h


iv. Cells of L. esculentum AC in Control/SH throughout
Percentage viability
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9
Length of incubation, h

or alternatively, insensitivity of the FDA test to esterase activity at that pH, would result in an apparent recovery of the cells in a medium with a more neutral pH. Such a recovery could not be attributable, in the time available, to the generation of viable cells by division. Therefore, the absence of such recovery in cells of *L. esculentum* x *peruvianum* indicates that cells which failed to exhibit esterase activity during that incubation were, in fact, dead. However, the detection of small, but definite, recoveries in cells of *L. esculentum* AC originally incubated in +NPhemi and Control/TART media, suggests that the cells may have undergone a period of subdued esterase activity while in the low pH media. The apparent increase in the viability, upon transfer, would indicate that normal esterase activity had recommenced once the adverse conditions ameliorated.

Earlier, results from section 4.4.2.4.b suggested that the presence of hemicellulose in tartaric acid did not affect viability of cells of *L. esculentum* AC, since low pH alone was sufficient to kill almost 100% of them. However, the results from section 4.4.3.4.b suggest that the application of exogenous hemicelluloses from *L. esculentum* AC and *N. physaloides* reduced the viability of cells incubated in low pH medium from the 37% measured for cells in hemicellulose-free Control/TART medium to 6% and 9.2% respectively, and that self, *L. esculentum* AC, hemicelluloses exerted a slightly more deleterious effect than non-self, *N. physaloides*, hemicelluloses.

Cells of *L. esculentum* AC transferred from Control/TART to fresh tartaric acid showed no change in viability, although viability was reduced in those transferred to fresh tartaric acid from hemicellulosic media. This suggests that incubation with hemicellulose from either source weakened the resistance of suspension-cultured cells of *L. esculentum* AC to continued exposure to low pH. Furthermore, since the viability of cells transferred from +NPhemi/TART to fresh SH increased, but that of those transferred from +AChemi/TART to fresh SH did not, the implication is that incubation with self, *L. esculentum* AC, hemicelluloses caused more harm to the cells than incubation with non-self, *N. physaloides*, hemicelluloses. Indeed, the loss of discrete, cell-retained, fluorescence visible in cells transferred from +AChemi/TART medium to fresh SH implies that the damage inflicted by *L. esculentum* AC hemicellulose at low pH was quite serious, and may have resulted in loss of membrane integrity. The FDA test was originally designed to monitor membrane integrity of pollen grains (Heslop-Harrison and Heslop-Harrison, 1970) and has since been used to assess viability. Interestingly, the development of leaky membranes is one of the characteristic features of the HR (Lyon and Wood, 1975). Furthermore, it appears that medium pH is a critical factor in the development of the HR (Salzwedel, Daub and Huang, 1989).

It is probable that cells incubated in conditions of low pH will suffer a degree of membrane damage due to an increase in activity and occurrence of active oxygen species. During

normal metabolism most plant cell organelles, including the plasmalemma and cell wall, produce superoxide anion radicals (O_2^{-1}) which are especially harmful to DNA, proteins and membrane lipids (Tzeng and DeVay, 1993). Superoxide dismutase (SOD) and other detoxifying and scavenging compounds including peroxidase, catalase and β-carotene combat O_2^{-1} ions, and are essential for cell survival. The pH optimum for SOD lies between pH 7.0 - 8.0, its action is severely restricted below pH 6.0 (Tzeng, 1989), which makes maintenance of normal cellular pH of critical importance. Below pH 5.6 SOD is ineffectual and releases an Fe co-factor, which catalyses an increase in production of oxygen radicals (Halliwell and Gutteridge, 1984). The leaky membranes characteristic of the HR may be partially attributable to an increase in superoxide production which has been shown to occur within one hour of inoculation in suspension cultured soybean cells treated with *Pseudomonas syringae* pv. *glycinea* (Glazener, Orlandi, Harmon *et al.*, 1991).

The results from sections 4.4.3.4.a. and b provide further comparison of the responses of the two types of suspension-cultured cells, *L. esculentum* x *peruvianum* and *L. esculentum* AC, to conditions of low pH ± exogenous hemicelluloses. Cells of *L. esculentum* x *peruvianum* did not exhibit subdued esterase activity, alleviated by transfer to medium with a more neutral pH, but cells of *L. esculentum* AC did. Cells of *L. esculentum* x *peruvianum* did not exhibit signs of physical damage after incubation in low pH media, unlike cells of *L. esculentum* AC incubated in +AChemi/TART which exhibited symptoms indicative of loss of membrane integrity. Both cell types exhibited significant losses in viability as a result of exposure to low pH media, for both cell types these losses were enhanced when exogenous hemicelluloses were present; hemicelluloses from *N. physaloides* exerted a more noxious effect on cells of *L. esculentum* x *peruvianum* than *L. esculentum* x *peruvianum* hemicelluloses, while those from *L. esculentum* AC appeared to be more deleterious to cells of *L. esculentum* AC than those extracted from *N. physaloides*.

4.4.4: EFFECTS OF INCUBATION IN TARTARIC ACID ± HEMICELLULOSE ON [^{14}C] LEUCINE INCORPORATION IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* BEFORE AND AFTER TRANSFER TO HEMICELLULOSE-FREE TARTARIC ACID OR TOM-I MEDIA

4.4.4.1: **Summary:** Permanence of hemicellulose effect on leucine incorporation

Previous results indicated that at low pH the FDA test assessed the viability of suspension-cultured cells of *L. esculentum* x *peruvianum* accurately, but was less accurate for cells of *L. esculentum* AC. Using a similar experimental protocol the FDA results were supplemented with L-[U-^{14}C] leucine incorporation data. Cells of *L. esculentum* x

peruvianum transferred from low pH hemicellulosic media to hemicellulose-free TOM-I or TART medium exhibited increases in L-[U-^{14}C] leucine incorporation of between +129% and +987%. Cells of *L. esculentum* x *peruvianum* transferred to TOM-I from Control/TART exhibited an increase of +13%, while cells transferred to TART exhibited a decline of -55%.

Cells of *L. esculentum* AC, exhibited limited recovery of L-[U-^{14}C] leucine incorporation of +23% and +258%, in cells transferred from +AChemi/TART and Control/TART media to SH medium. Cells of *L. esculentum* AC from all treatments exhibited decreases in incorporation between -21% and -47% when transferred to tartaric acid medium.

Results suggested that L-[U-^{14}C] leucine incorporation in cells of *L. esculentum* x *peruvianum* underwent a reversible period of reduced protein metabolism when subjected to hemicelluloses at low pH, and was inhibited more by exogenous hemicelluloses than by low pH alone. Inhibition of L-[U-^{14}C] leucine incorporation in cells of *L. esculentum* AC exposed to hemicellulosic low pH media was not reversible.

4.4.4.2: Introduction: Permanence of effect on leucine incorporation

Results from earlier experiments (sects. 4.4.3.4.a and b) indicated that the FDA viability test provided an accurate assessment of the viability of suspension-cultured cells of *L. esculentum* x *peruvianum* under conditions of low pH, but underestimated the viability of suspension-cultured cells of *L. esculentum* AC in +NPhemi/TART and Control/TART media. This may have been due to suspension of intra-cellular esterase activity under adverse conditions, which revived once the cells were transferred to more favourable conditions. These experiments (sect. 4.4.4.) complemented those in section 4.4.3 by providing data about the metabolic activity of the cells by measuring the amount of L-[U-^{14}C] leucine incorporated into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC during incubation in tartaric acid media ± hemicelluloses, and after transfer to either their native medium, or to tartaric acid free from exogenous hemicelluloses. It was hoped to clarify whether results obtained from the L-[U-^{14}C] leucine incorporation assay used thus far were merely a reflection of cell viability of the samples, or were an indication of actual metabolic activity.

4.4.4.3: Materials and Methods: Permanence of effect on leucine incorporation

7d old suspension-cultured cells of *L. esculentum* x *peruvianum* or *L. esculentum* AC were incubated (sect. 2.3), in self and *N. physaloides* hemicellulosic tartaric acid media, and hemicellulose-free tartaric acid medium at pH 4.5, and native medium, TOM-I or SH, at pH 5.7. After 2 h, 0.05 µCi L-[U-^{14}C] leucine was added to the tubes, an hour later incorporation was stopped with 10% TCA.

A second batch of cells was incubated concurrently, in identical media and conditions to the first, for 3 h. Cells were rinsed with 3 x 3 ml of the medium to which they were to be transferred and resuspended in 0.5 ml of fresh native medium (TOM-I or SH) or fresh tartaric acid (TART). 0.05 µCi aliquots of L-[U-^{14}C] leucine were added to the tubes, an hour later incorporation was stopped with 10% TCA. A control batch of cells was incubated concurrently in fresh native medium for 2 and 3 h prior to the addition of L-[U-^{14}C] leucine, and 10% TCA added an hour later. After the addition of TCA, all cells were processed according to the L-[U-^{14}C] leucine incorporation assay procedure (sect. 2.3).

These experiment were designed to complement the experiments in section 4.4.3 and although performed on a different day with different cells, the results of the two sets of experiments should be comparable.

4.4.4.4: Results: Permanence of hemicellulose effect on leucine incorporation

4.4.4.4.a: L-[U-^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in tartaric acid ± hemicellulose before and after transfer to hemicellulose-free tartaric acid or TOM-I media

The results obtained are shown in Figures 4.4.4.4.a.i - iv in terms of actual counts incorporated by the cells in one hour, and in Figures 4.4.4.4.a.v - viii as a percentage of the leucine fixed in one hour by cells of *L. esculentum* x *peruvianum* incubated in the original TOM-I medium for 2 h and 3 h prior to L-[U-^{14}C] leucine addition. Comparisons of the amounts of L-[U-^{14}C] leucine incorporated by cells in different treatments always refer to corresponding measurements made at the same time.

All cells of *L. esculentum* x *peruvianum* incubated in tartaric acid media exhibited reduced incorporation of L-[U-^{14}C] leucine by comparison with the control cells in fresh TOM-I medium. Cells incubated in Control/TART for 2 h prior to the addition of L-[U-^{14}C] leucine (Figs. 4.4.4.4.a.iii and vii) incorporated 56% of the amount of L-[U-^{14}C] leucine incorporated by control cells incubated in fresh TOM-I. Cells transferred to fresh TOM-I from Control/TART medium exhibited a small increase of +13% in the rate of leucine incorporation, while those transferred to TART from Control/TART showed a substantial decrease in the rate of leucine incorporation in the following hour of -55%.

Cells of *L. esculentum* x *peruvianum* incubated in +HYBhemi/TART (Figs. 4.4.4.4.a.i. and v) exhibited very reduced rates of leucine incorporation, and the amount incorporated equated to 14% of the corresponding Control/TART level and 7.8% of the fresh TOM-I level. Transfer of cells to hemicellulose-free fresh TOM-I medium was followed by an increase of +522% in the amount of L-[U-^{14}C] leucine incorporated; the amount incorporated equated to 60% of that incorporated by control cells in fresh TOM-I medium. Cells transferred from +HYBhemi/TART to TART showed an increase of +129% in the

Figure 4.4.4.4.a.i:

Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* x *peruvianum* originally incubated in +HYBhemi/TART medium immediately before and immediately after transfer to Fresh TOM-I and Fresh TART media.

Figure 4.4.4.4.a.ii:

Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* x *peruvianum* originally incubated in +NPhemi/TART medium immediately before and immediately after transfer to Fresh TOM-I and Fresh TART media.

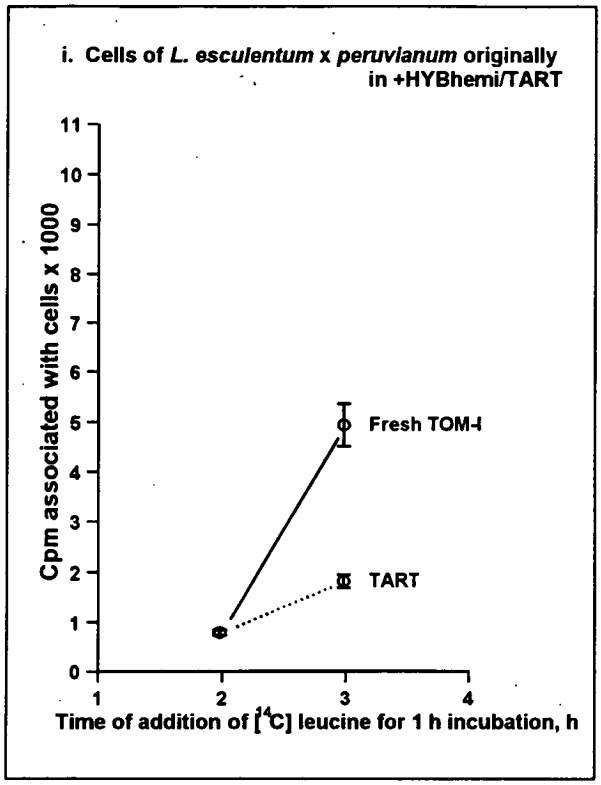
Figure 4.4.4.4.a.iii:

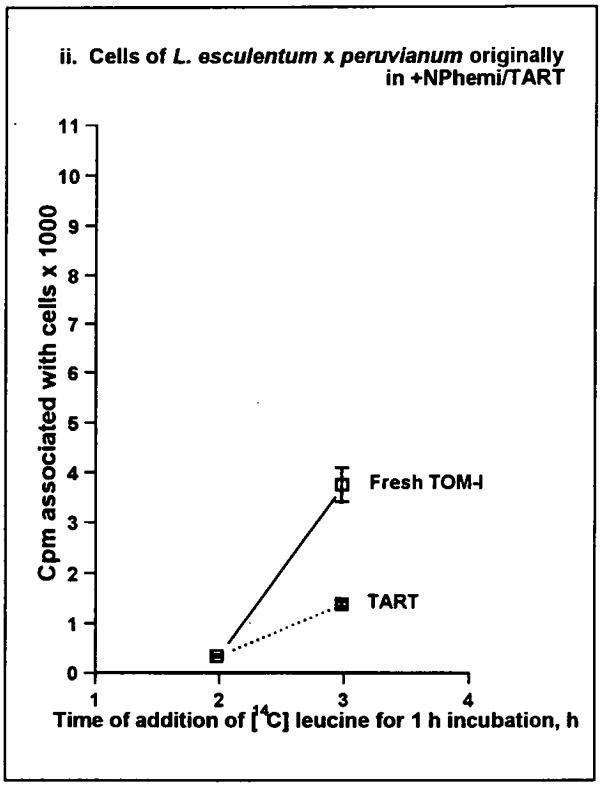
Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* x *peruvianum* originally incubated in Control (TART) medium immediately before and immediately after transfer to Fresh TOM-I and Fresh TART media.

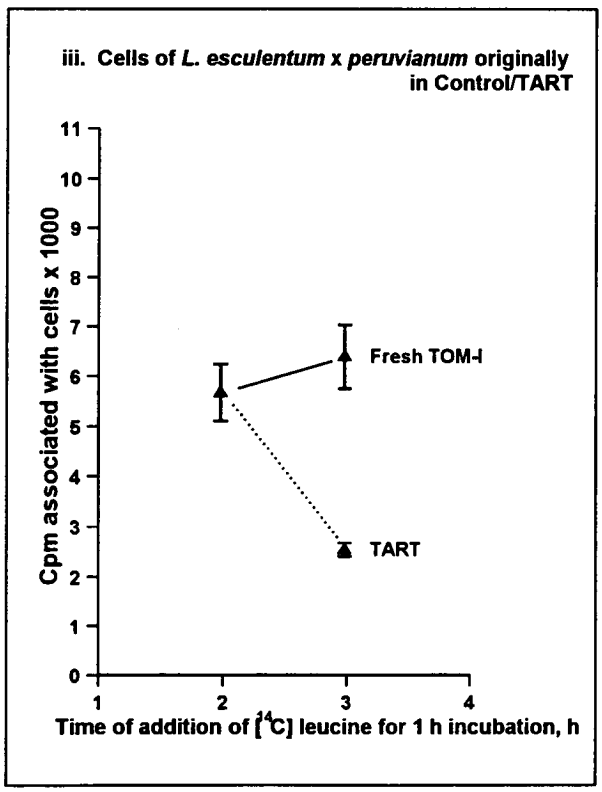
Figure 4.4.4.4.a.iv:

Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* x *peruvianum* maintained for two or three hours in the original TOM-I medium prior to the addition of L-[U-^{14}C] leucine.

Means were obtained from 6 samples of each treatment at each time point.


i. Cells of L. esculentum x peruvianum originally in +HYBhemi/TART
Cpm associated with cells x 1000
Fresh TOM-I
TART
Time of addition of [14C] leucine for 1 h incubation, h


ii. Cells of L. esculentum x peruvianum originally in +NPhemi/TART
Cpm associated with cells x 1000
Fresh TOM-I
TART
Time of addition of [14C] leucine for 1 h incubation, h


iii. Cells of L. esculentum x peruvianum originally in Control/TART
Cpm associated with cells x 1000
Fresh TOM-I
TART
Time of addition of [14C] leucine for 1 h incubation, h

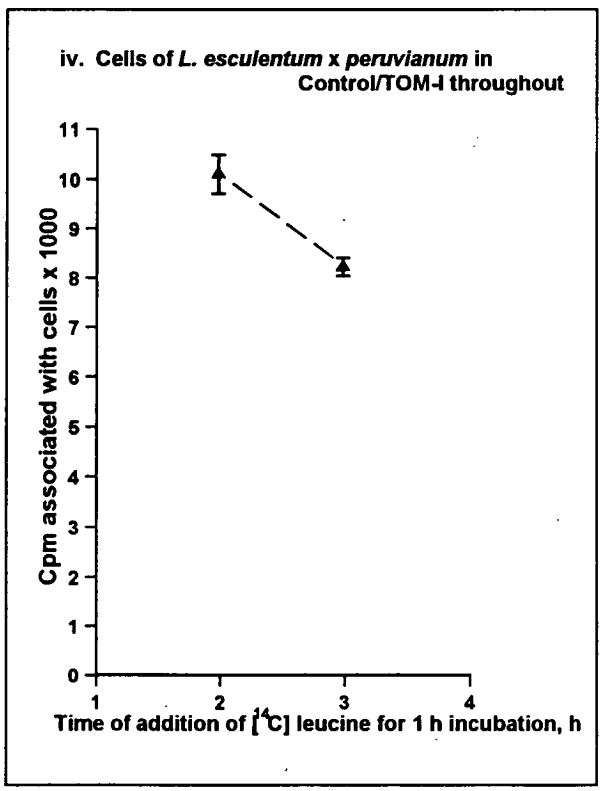

iv. Cells of L. esculentum x peruvianum in Control/TOM-I throughout
Cpm associated with cells x 1000
Time of addition of [14C] leucine for 1 h incubation, h

Figure 4.4.4.4.a.v:

Comparison of the percentage of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in +HYBhemi/TART medium immediately before and immediately after transfer to Fresh TOM-I and Fresh TART media with the total incorporated by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in the original TOM-I medium throughout.

Figure 4.4.4.4.a.vi:

Comparison of the percentage of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in +NPhemi/TART medium immediately before and immediately after transfer to Fresh TOM-I and Fresh TART media with the total incorporated by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in the original TOM-I medium throughout.

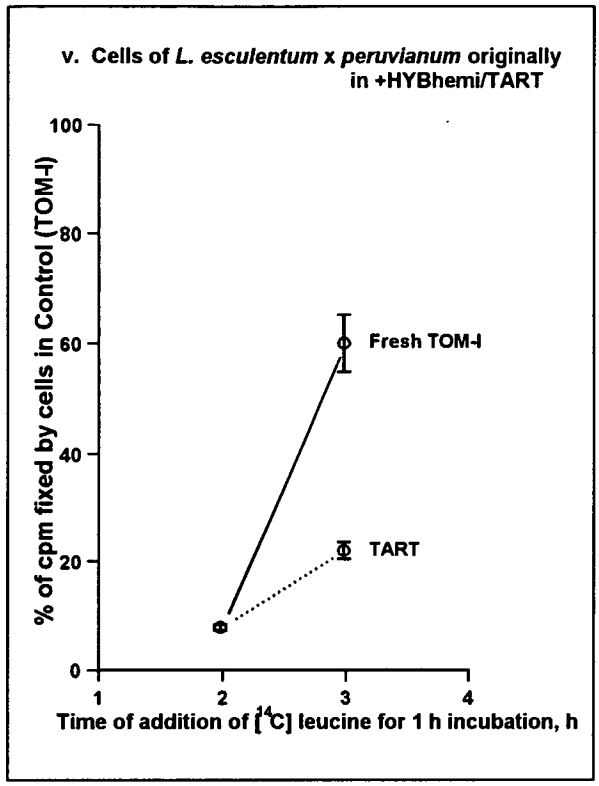
Figure 4.4.4.4.a.vii:

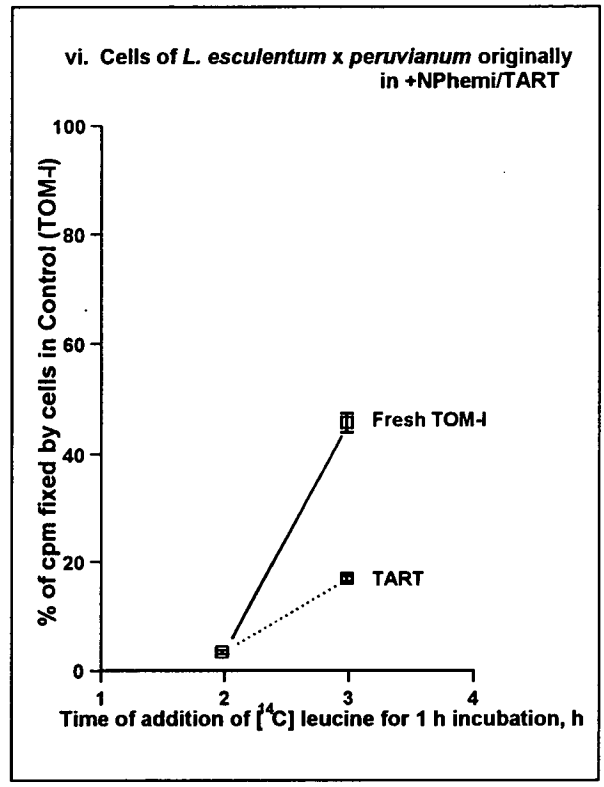
Comparison of the percentage of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in Control (TART) medium immediately before and immediately after transfer to Fresh TOM-I and Fresh TART media with the total incorporated by suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in the original TOM-I medium throughout.

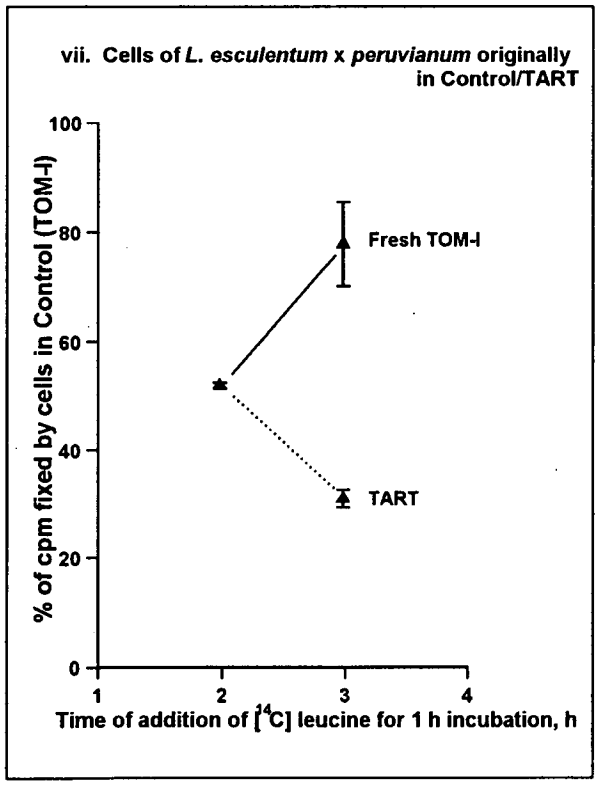
Figure 4.4.4.4.a.viii:

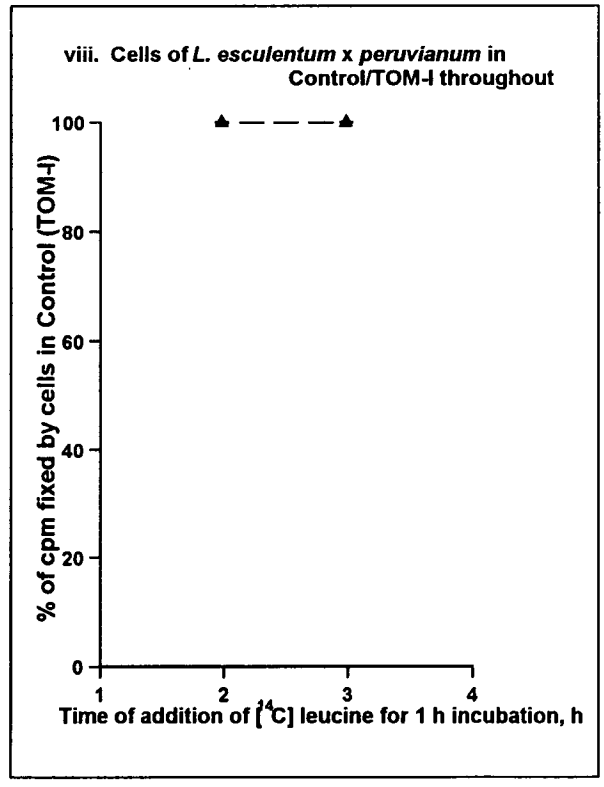
Suspension-cultured cells of *L. esculentum* x *peruvianum,* incubated in the original TOM-I medium throughout, which provided the Control 100% L-[U-^{14}C] leucine incorporation level.

Means were obtained from 6 samples of each treatment at each time point. The same data were used to produce Figures 4.4.4.4.a.i - iv and Figures v - viii; the former express the data in terms of actual counts incorporated, while the latter express the data as percentages of L-[U-^{14}C] leucine incorporated by cells which were incubated in Control (TOM-I) medium throughout.


v. Cells of L. esculentum x peruvianum originally in +HYBhemi/TART
% of cpm fixed by cells in Control (TOM-I)
100
80
60
40
20
0
Fresh TOM-I
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


vi. Cells of L. esculentum x peruvianum originally in +NPhemi/TART
% of cpm fixed by cells in Control (TOM-I)
100
80
60
40
20
0
Fresh TOM-I
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


vii. Cells of L. esculentum x peruvianum originally in Control/TART
% of cpm fixed by cells in Control (TOM-I)
100
80
60
40
20
0
Fresh TOM-I
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


viii. Cells of L. esculentum x peruvianum in Control/TOM-I throughout
% of cpm fixed by cells in Control (TOM-I)
100
80
60
40
20
0
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h

quantity of L-[U-^{14}C] leucine fixed within one hour; the amount incorporated equated to 71% of that incorporated by cells in Control/TART medium and to 22% of that incorporated by control cells in fresh TOM-I medium. Cells transferred from +HYBhemi/TART to TART incorporated 37% of the amount of L-[U-^{14}C] leucine incorporated by cells transferred from +HYBhemi/TART to TOM-I.

Cells of *L. esculentum* x *peruvianum* incubated in +NPhemi/TART (Figs. 4.4.4.4.a.ii and vi) exhibited reduced rates of leucine incorporation; the amount incorporated equated to 6.1% of that incorporated by cells in Control/TART medium and to 3.4% of that incorporated by control cells in fresh TOM-I. Cells transferred to fresh TOM-I exhibited an increase of +987% in the amount of leucine incorporated within one hour; the amount incorporated equated to 46% of that incorporated by control cells in fresh TOM-I medium. Cells transferred from +NPhemi/TART to TART exhibited an increase of +302% in L-[U-^{14}C] leucine incorporation; the amount incorporated equated to 17% of that incorporated by control cells in fresh TOM-I medium. Cells transferred from +NPhemi/TART to TART incorporated 37% of the amount of L-[U-^{14}C] leucine incorporated by cells transferred to fresh TOM-I medium.

Control cells incubated in fresh TOM-I medium (Figs. 4.4.4.4.a.iv and viii) displayed a statistically significant reduction of -19% in the rate at which leucine was incorporated after 3 h of incubation. However, throughout, the rates exhibited by cells in this medium were significantly higher than all other treatments.

4.4.4.4.b: L-[U-^{14}C] leucine incorporation in suspension-cultured cells of *L. esculentum* AC incubated in tartaric acid ± hemicellulose before and after transfer to hemicellulose-free tartaric acid or SH media

Figures 4.4.4.4.b.i - iv display the data in terms of amounts of leucine incorporated during the hour-long incubation period, while Figures 4.4.4.4.b.v - viii express the same data as a percentage of the leucine fixed in one hour by cells of *L. esculentum* AC incubated in the original SH medium for 2 h and 3 h prior to L-[U-^{14}C] leucine addition. Comparisons of the amounts of L-[U-^{14}C] leucine incorporated by cells in different treatments always refer to corresponding measurements made at the same time.

While control cells incubated in fresh SH medium throughout (Figs. 4.4.4.4.b.iv and viii) showed a decrease of -13% in the amount of L-[U-^{14}C] leucine incorporated during the third and the fourth hours, the amounts incorporated by cells incubated in this medium were 2 - 29 fold greater than those incorporated by cells originally incubated in tartaric acid media.

Cells of *L. esculentum* AC incubated in Control/TART (Figs. 4.4.4.4.b.iii and vii) exhibited a reduced rate of L-[U-^{14}C] leucine incorporation and incorporated 15% of the amount of L-[U-^{14}C] leucine incorporated by control cells in fresh SH medium. Cells transferred from

NOTE: The scales on the y-axies of Figures 4.4.4.4.b.i and ii are not identical to those on Figures 4.4.4.4.b.iii and iv.

Figure 4.4.4.4.b.i:

Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* AC originally incubated in +AChemi/TART medium immediately before and immediately after transfer to Fresh SH and Fresh TART media.

Figure 4.4.4.4.b.ii:

Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* AC originally incubated in +NPhemi/TART medium immediately before and immediately after transfer to Fresh SH and Fresh TART media.

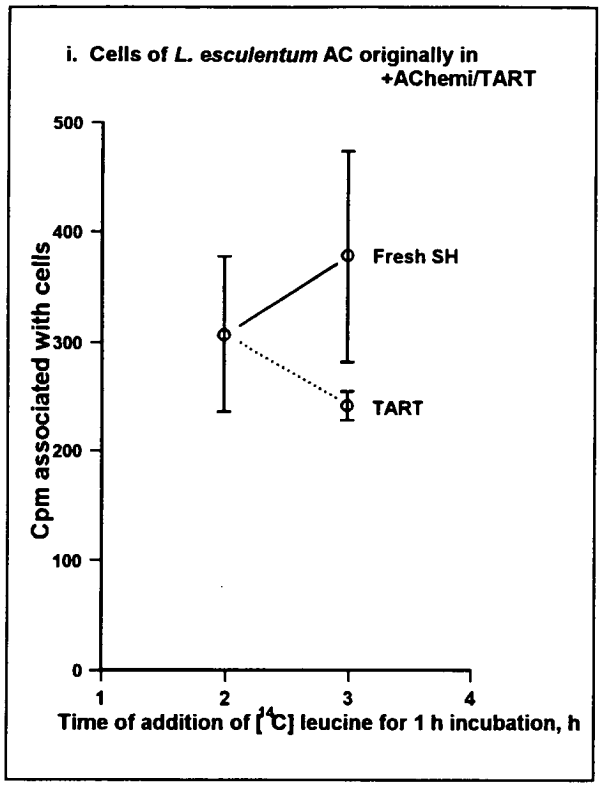
Figure 4.4.4.4.b.iii:

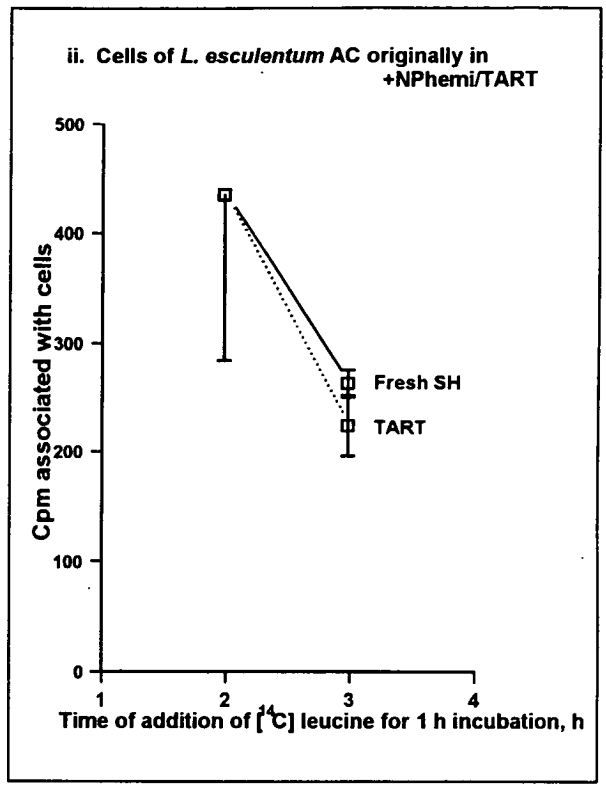
Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* AC originally incubated in Control (TART) medium immediately before and immediately after transfer to Fresh SH and Fresh TART media.

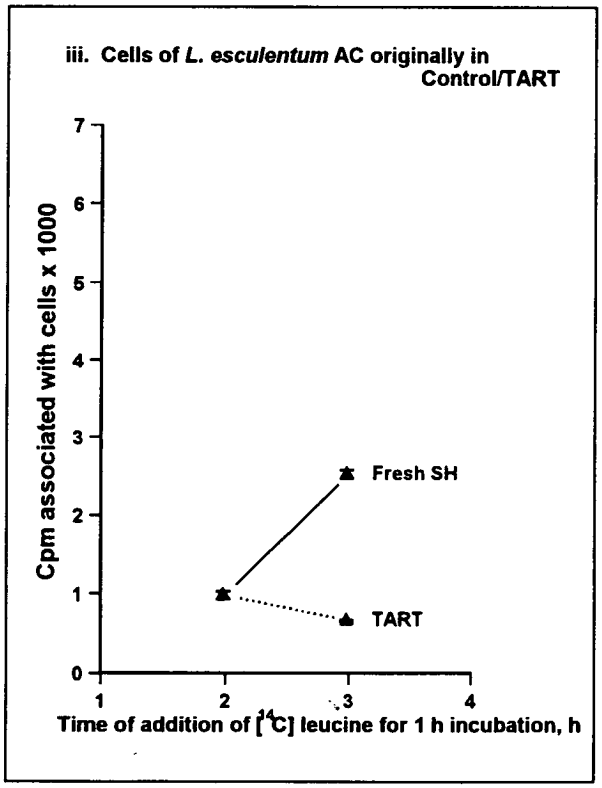
Figure 4.4.4.4.b.iv:

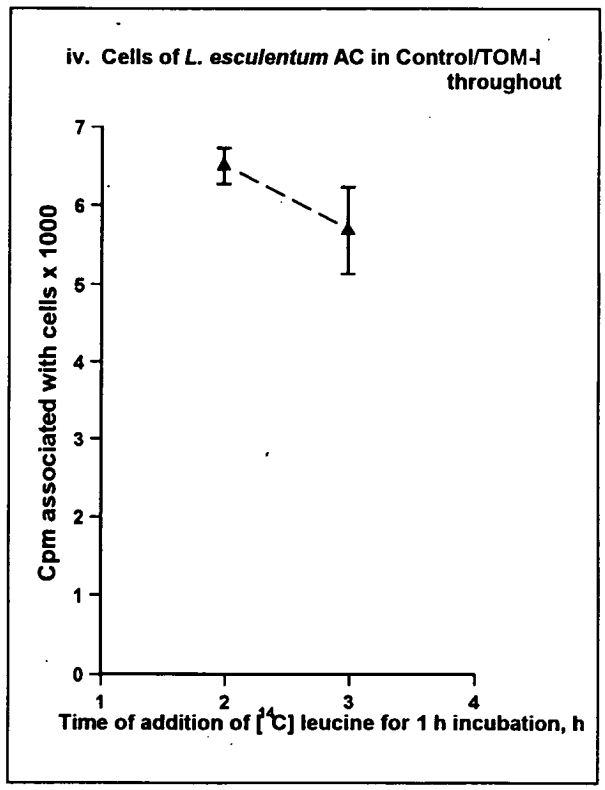
Amount of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* AC maintained for two or three hours in the original SH medium prior to the addition of L-[U-^{14}C] leucine.

Means were obtained from 6 samples of each treatment at each time point.


i. Cells of L. esculentum AC originally in +AChemi/TART
Cpm associated with cells
500
400
300
200
100
0
Fresh SH
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


ii. Cells of L. esculentum AC originally in +NPhemi/TART
Cpm associated with cells
500
400
300
200
100
0
Fresh SH
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


iii. Cells of L. esculentum AC originally in Control/TART
Cpm associated with cells x 1000
7
6
5
4
3
2
1
0
Fresh SH
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


iv. Cells of L. esculentum AC in Control/TOM-I throughout
Cpm associated with cells x 1000
7
6
5
4
3
2
1
0
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h

NOTE: The scales on the y-axies of Figures 4.4.4.4.b.v and vi are not identical to those on Figures 4.4.4.4.b.vii and viii.

Figure 4.4.4.4.b.v:

Comparison of the percentage of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* AC incubated in +AChemi/TART medium immediately before and immediately after transfer to Fresh SH and Fresh TART media with the total incorporated by suspension-cultured cells of *L. esculentum* AC incubated in the original SH medium throughout.

Figure 4.4.4.4.b.vi:

Comparison of the percentage of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* AC incubated in +NPhemi/TART medium immediately before and immediately after transfer to Fresh SH and Fresh TART media with the total incorporated by suspension-cultured cells of *L. esculentum* AC incubated in the original SH medium throughout.

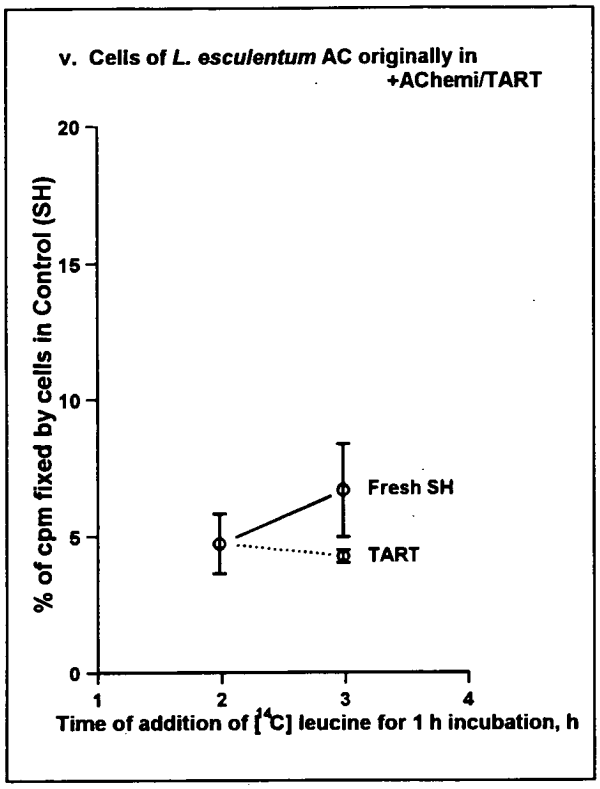
Figure 4.4.4.4.b.vii:

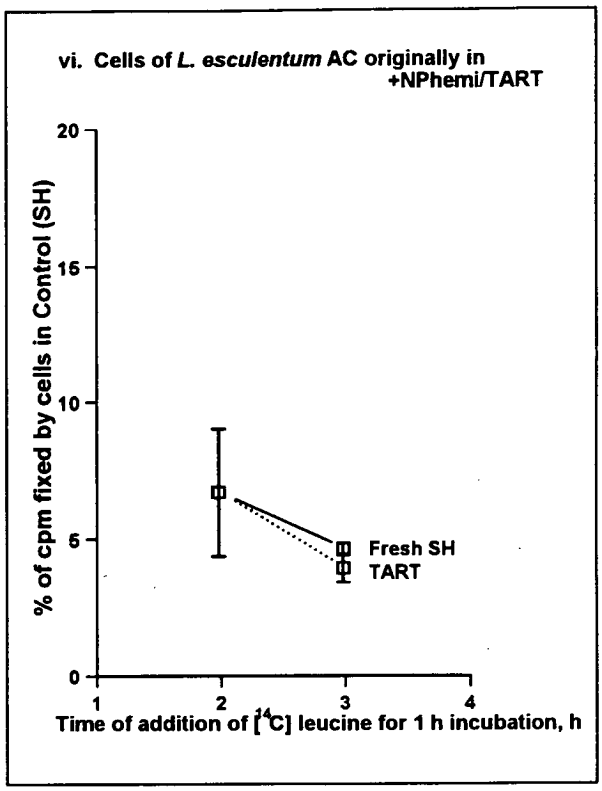
Comparison of the percentage of L-[U-^{14}C] leucine incorporated during a single hour by suspension-cultured cells of *L. esculentum* AC incubated in Control (TART) medium immediately before and immediately after transfer to Fresh SH and Fresh TART media with the total incorporated by suspension-cultured cells of *L. esculentum* AC incubated in the original SH medium throughout.

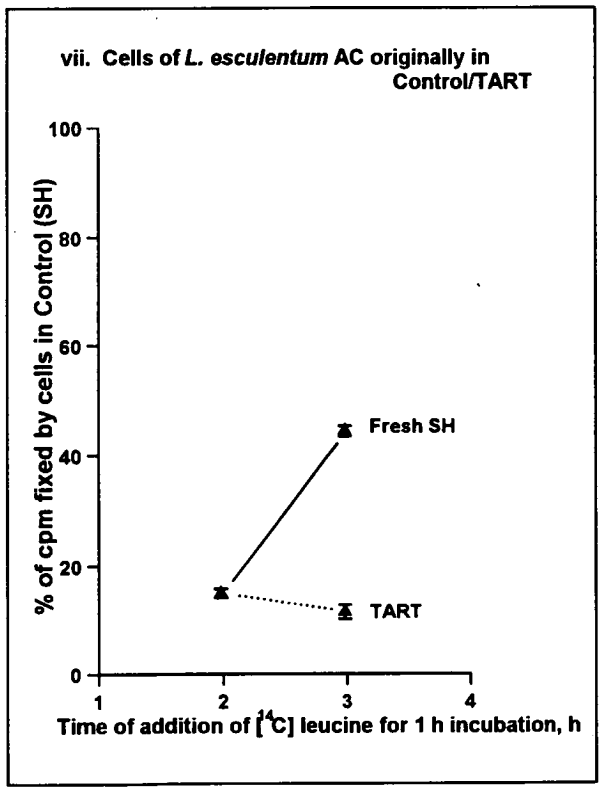
Figure 4.4.4.4.b.viii:

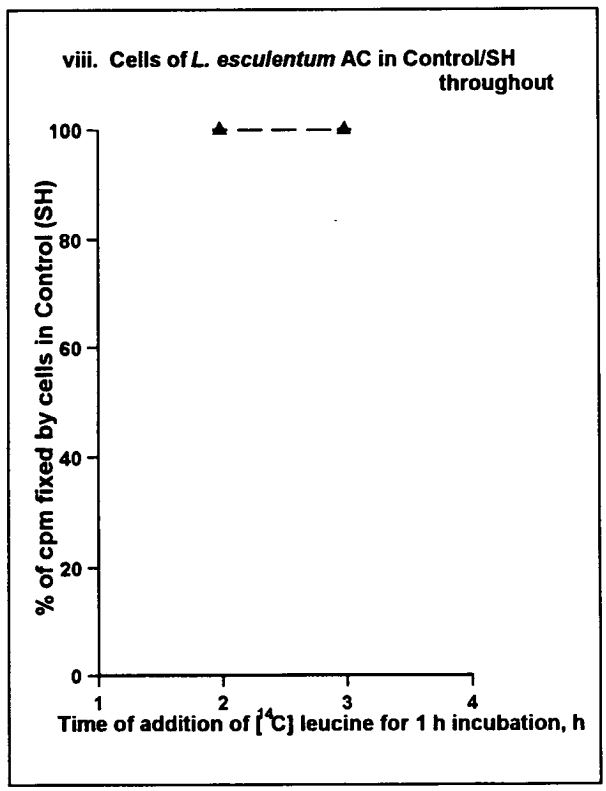
Suspension-cultured cells of *L. esculentum* AC, incubated in the original SH medium throughout, which provided the Control 100% L-[U-^{14}C] leucine incorporation levels.

Means were obtained from 6 samples of each treatment at each time point. The same data were used to produce Figures 4.4.4.4.b.i - iv and Figures v - viii; the former express the data in terms of actual counts incorporated, while the latter express the data as percentages of L-[U-^{14}C] leucine incorporated by cells which were incubated in Control (SH) medium throughout.


v. Cells of L. esculentum AC originally in +AChemi/TART
% of cpm fixed by cells in Control (SH)
20
15
10
5
0
Fresh SH
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


vi. Cells of L. esculentum AC originally in +NPhemi/TART
% of cpm fixed by cells in Control (SH)
20
15
10
5
0
Fresh SH
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


vii. Cells of L. esculentum AC originally in Control/TART
% of cpm fixed by cells in Control (SH)
100
80
60
40
20
0
Fresh SH
TART
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h


viii. Cells of L. esculentum AC in Control/SH throughout
% of cpm fixed by cells in Control (SH)
100
80
60
40
20
0
1
2
3
4
Time of addition of [14C] leucine for 1 h incubation, h

Control/TART to fresh SH medium exhibited a +157% increase in the amount of L-[U-^{14}C] leucine incorporated within one hour which equated to 45% of that incorporated by control cells in SH medium. Cells transferred from Control/TART to TART incorporated -33% less L-[U-^{14}C] leucine in the following hour, which was 12% of that incorporated by control cells.

Leucine incorporation in cells of *L. esculentum* AC incubated in +AChemi/TART (Figs. 4.4.4.4.b.i and v) was reduced to 31% of the corresponding amount incorporated by cells in Control/TART medium and to 4.7% of that incorporated by the control cells in fresh SH medium. Transfer of cells from +AChemi/TART to fresh SH medium led to an increase in incorporation of +23%; the amount incorporated equated to 6.7% of that incorporated by control cells in fresh SH medium. This +23% increase was not statistically different (at 5%) compared to the original level of incorporation in +AChemi/TART, or when compared to the -21% reduction in incorporation in cells transferred from +AChemi/TART to TART. The amount incorporated by cells transferred from +AChemi/TART medium to TART corresponded to 4.3% of that incorporated by the control cells in fresh SH medium.

Cells of *L. esculentum* AC incubated in +NPhemi/TART (Figs. 4.4.4.4.b.ii and vi) exhibited reduced rates of L-[U-^{14}C] leucine incorporation and incorporated 44% of the amount incorporated by cells in Control/TART medium and 6.7% of that incorporated by control cells in fresh SH medium. Cells transferred to fresh SH medium exhibited a decrease of -39% in the amount of leucine incorporated within an hour; the amount incorporated equated to 4.6% of that incorporated by control cells in fresh SH medium. Cells transferred to TART exhibited a decrease of -48% in the amount of L-[U-^{14}C] leucine incorporated; the amount was equivalent to 4% of that incorporated by control cells in fresh SH medium. There were no notable differences between the amounts of leucine incorporated by cells transferred from +NPhemi/TART to fresh SH or TART.

Control cells incubated in fresh SH medium for 2 h prior to the addition of L-[U-^{14}C] leucine (Figs. 4.4.4.4.b.iv and viii) incorporated 13% more L-[U-^{14}C] leucine during one hour than cells incubated in the same medium for 3 h. Throughout the experiment cells incubated in this medium incorporated significantly higher amounts of L-[U-^{14}C] leucine than cells in all other treatments.

4.4.4.5: Discussion: Permanence of hemicellulose effect on leucine incorporation

The decline in the amount of L-[U-^{14}C] leucine incorporated by cells of *L. esculentum* x *peruvianum* (sect. 4.4.4.4.a) would have been due to decreases in cell viability measured earlier (sect. 4.4.3.4.a), and to incubation with hemicelluloses at low pH, since partial recovery of synthetic capacity was achieved by transferring cells to hemicellulose-free TART or fresh TOM-I medium. The increased incorporation rates in cells of *L. esculentum* x *peruvianum*, transferred from hemicellulosic /TART to fresh TOM-I and TART

media show that protein metabolism was slightly inhibited by low pH. Cells transferred to fresh TOM-I medium exhibited greater recovery than those transferred to tartaric acid possibly because two inhibiting factors, low pH and hemicellulose, were removed.

Cells of *L. esculentum* x *peruvianum* from Control/TART medium resuspended in tartaric acid exhibited a -55% decrease in leucine incorporation. This suggests that some damage may have been sustained by cells during the rinsing process. Since this sizeable decrease was not paralleled in cells from hemicellulosic-tartaric acid media transferred to fresh tartaric acid, it would appear that the alleviation of inhibition produced by the elution of hemicellulose more than compensated for any restriction of protein metabolism resulting from the rinsing process. This implies that the inhibition of protein metabolism attributable to hemicelluloses was greater than that attributable to low pH alone.

In cells of *L. esculentum* AC transferred from +NPhemi/TART to fresh SH medium (sect. 4.4.4.4.b), alleviation of the low pH environment was accompanied by a decrease in L-[U-^{14}C] leucine incorporation, although cells transferred from the other tartaric acid media to fresh SH exhibited increases.

The L-[U-^{14}C] leucine incorporation data from cells of *L. esculentum* AC transferred from hemicellulosic tartaric acid media to fresh SH medium covered a broader range of values than those transferred from Control/TART. This suggests that incubation with exogenous hemicelluloses increased variance, possibly by inflicting membrane damage which would facilitate the leakage of radioactively-labelled proteins from protoplasts into the medium during processing, resulting in differences in retention of labelled proteins. Evidence from the earlier FDA experiment (sect. 4.4.3.4.b) supports the possibility of membrane damage; in cells of *L. esculentum* AC incubated in +AChemi/TART, however, since cells incubated with *N. physaloides* hemicelluloses did not appear to be affected, the damage induced by hemicelluloses may depend the metabolic condition of the cells used in experiments involving stressful conditions (sects. 4.4.3.4.b and 4.4.4.4.b). Alternatively, fluorescein and L-[U-^{14}C] leucine-labelled proteins may be retained to differing extents at low pH.

The general decrease in leucine incorporation exhibited by cells of *L. esculentum* AC transferred to TART may have been due to the release of labelled proteins through leaky membranes after exposure to hemicelluloses and/or low pH. Alternatively, this may be due to a perpetuation of low pH-induced quiescence of protein metabolism, and/or an increase in the number of cells killed by prolonged exposure to conditions of low pH. This last option would agree with the data from the FDA viability tests from section 4.4.3.4.b.

The two suspension-cultures reacted quite differently to the common stimulus of low pH ± hemicellulose: cells of *L. esculentum* x *peruvianum* were the more resistant, and endured a

period of subdued protein metabolism which was substantially reversed by transferring the cells to hemicellulose-free media. There is some evidence that cells of *L. esculentum* AC incubated in Control/TART medium underwent a period of subdued reversible protein synthesis, although cells transferred from hemicellulosic tartaric acid media did not exhibit unequivocal increases in leucine incorporation, characteristic of the end of a quiet period, in either hemicellulose-free medium. Cells of *L. esculentum* AC may have suffered membrane, or other physical damage during incubation with hemicelluloses at low pH. However, there was no indication that cells of *L. esculentum* x *peruvianum* suffered any loss of membrane integrity under these conditions. The reasons for this disparity is not known, but the occurrence of plasmolysis and/or inhibition of esterase activity during incubation in adverse conditions may be important.

The differences in the FDA (sects. 4.4.3.4.a and b) and leucine-incorporation assays (sects. 4.4.4.4.a and b) show clearly that these assays produce assessments of separate phenomena, i.e. viability and protein metabolism, and are not interchangeable viability tests. Most importantly, these results (sect. 4.4.4.4) show that the L-[U-^{14}C] leucine incorporation assay provides a valid assessment of the metabolic condition of cells, rather than a reflection of the viability of the culture as a whole. This is particularly significant if the assay is to be used to gain insight into the reactions of callus cells in the GU of Solanaceous grafts to a range of cell wall-derived components and fragments, and to interpret grafting phenomena. These results suggest that this is a valid and relevant procedure with which to investigate putative recognition events in the GU.

4.4.5: EFFECTS OF HEMICELLULOSE FRACTIONS OF DIFFERENT SIZES ON INCORPORATION OF L-[U-^{14}C] LEUCINE INTO PROTEIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM*

4.4.5.1: **Summary:** Effects of hemicellulose fractions

Highly repeatable results had been obtained with hemicelluloses in L-[U-^{14}C] leucine incorporation assays, especially at low pH. Hemicellulose from *L. esculentum* x *peruvianum* was separated on a Sepharose CL-6B column and tested on cells of *L. esculentum* x *peruvianum* in the assay, at pH 5.7 and pH 4.5, to investigate the activity of four differently sized fractions (Fraction DP: A > B > C > D).

At pH 5.7, 2 h after the addition of L-[U-^{14}C] leucine, cells incubated in all fractions exhibited inhibitions in L-[U-^{14}C] leucine incorporation of between 4 - 33%, with Fraction D causing the greatest inhibition. Apparent anomalies appeared in the results from the 3-h samples; the cause of these is not yet known.

At pH 4.5, 3 h after the addition of L-[U-^{14}C] leucine, cells incubated with Fractions A and B exhibited enhancements in leucine incorporation of +7% and +15% respectively. Fraction C inhibited incorporation in cells by 17%. Fraction D produced no effect upon leucine incorporation.

Differences in results between assays performed at pH 5.7 and pH 4.5 suggest that the precise effects, and interpretation, of hemicellulosic fragments may be determined by pH. Fraction C appeared the most likely to contain the putative "off" signal, since cells exposed to it consistently exhibited inhibition of L-[U-^{14}C] leucine incorporation.

4.4.5.2: Introduction: Effects of hemicellulose fractions

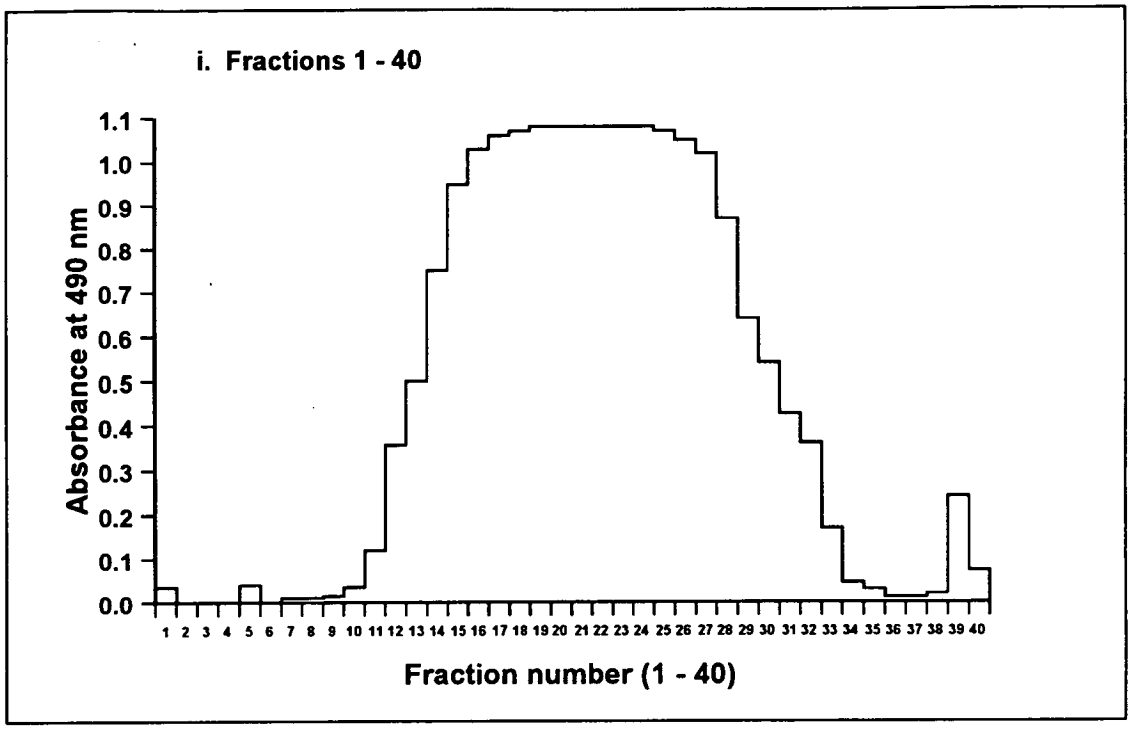
The effects of hemicelluloses on cells of *L. esculentum* x *peruvianum* in L-[U-^{14}C] leucine incorporation assays, were extremely consistent, particularly at low pH. Previous investigations have shown that specific structural requirements govern the biological activity of the hemicellulosic xyloglucan fragments which affect growth (McDougall and Fry, 1989a, b; Augur *et al.*, 1992). The identity of the hemicellulose fragment(s) responsible for the inhibition of L-[U-^{14}C] leucine incorporation detected by the L-[U-^{14}C] leucine incorporation assay was, as yet, completely unknown. Consequently an attempt was made to identify the size range of hemicellulose fragments in which this activity was located.

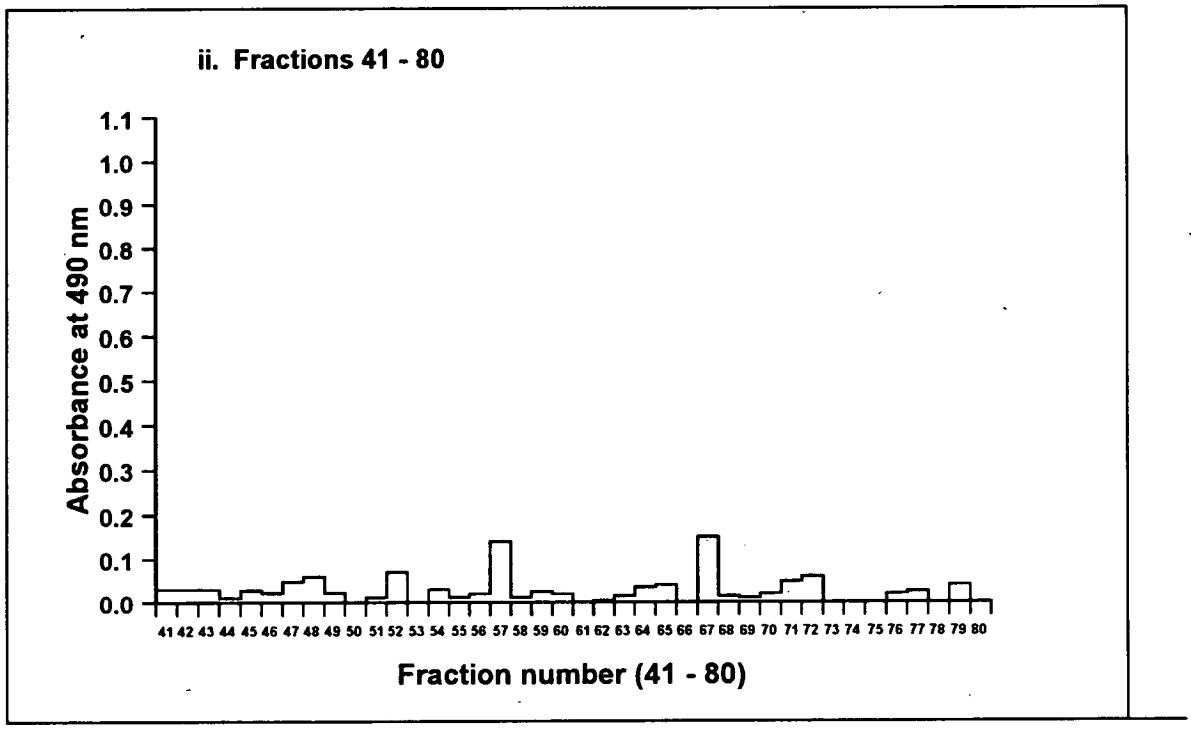
4.4.5.3: Materials and Methods: Effects of hemicellulose fractions

Hemicellulose from suspension-cultured cells of *L. esculentum* x *peruvianum* was fractionated on a size basis on a Sepharose CL-6B column (sect. 2.4.7). The A_{490} of the fractions was measured to assess carbohydrate content (sect. 2.8.2), the absorbance profiles for the hemicellulose samples are displayed in Figures 4.4.5.4.a.i and ii (Figs. 4.4.5.4.b.i and ii illustrate the absorbance when Blue Dextran and Glu were run as controls down the same column). Fractions were pooled into sample groups A - D, of which A - C contained approximately equal concentrations of carbohydrate (outlined below). All four were neutralised to pH 6.9 - 7.1 before being applied to cells of *L. esculentum* x *peruvianum*.

Pooled Sample	Numbers of Fractions
A	11 - 18
B	19 - 24
C	25 - 33
D	34 - 43

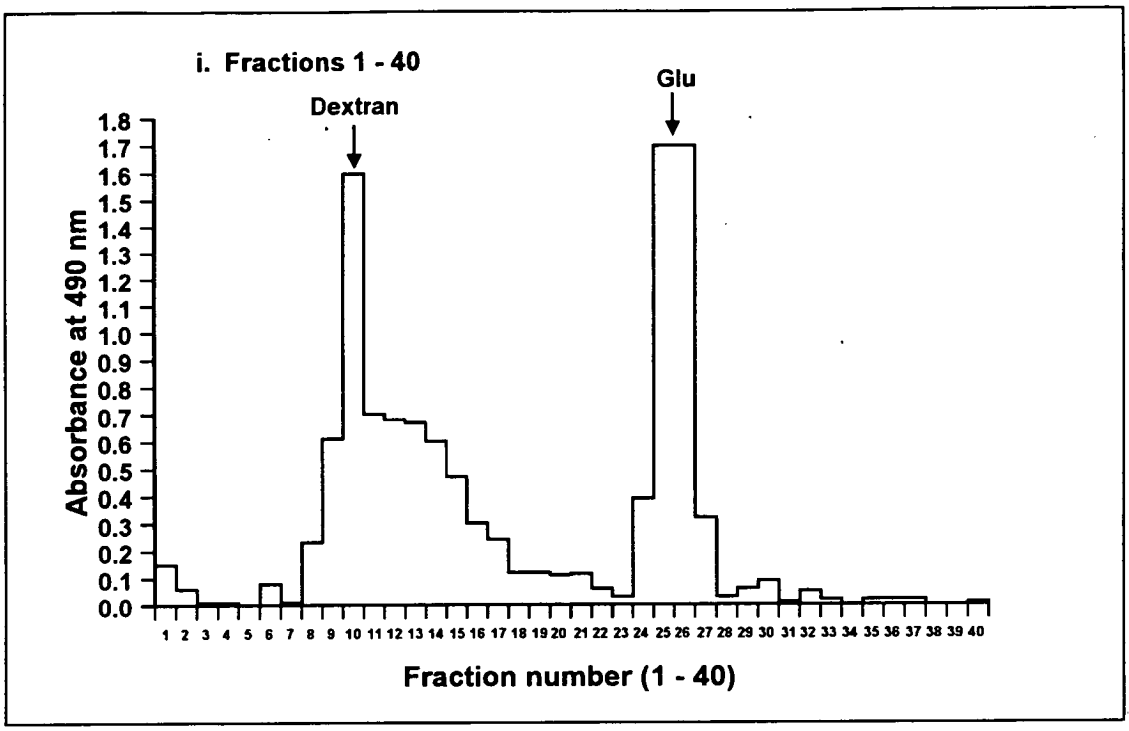
L-[U-^{14}C] leucine incorporation assays were performed with suspension-cultured cells of *L. esculentum* x *peruvianum* in fresh TOM-I at pH 5.7 and in 10 mM tartaric acid (TART) at pH 4.5. The concentration of the hemicellulose pooled samples was ~ 0.5 mg/ml. 30 µl

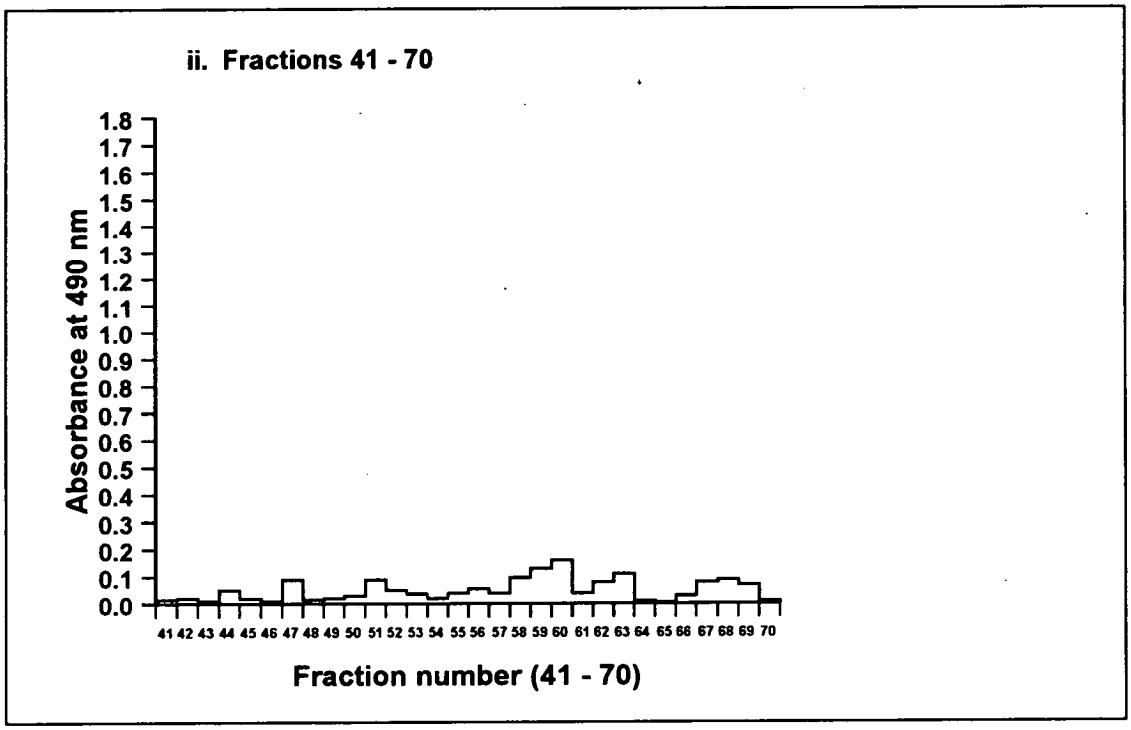




Figures 4.4.5.3.a.i and ii:

Absorbance at 490 nm of fractions 1 - 40 (Fig. i) and 41 - 80 (Fig. ii) of a hemicellulose preparation from suspension-cultured cells of *L. esculentum* x *peruvianum* which were separated on a Sepharose CL-6B column.





Figures 4.4.5.3.b.i and ii:

Absorbance at 490 nm of fractions 1 - 40 (Fig. i) and 41 - 70 (Fig. ii) of a control sample containing Blue Dextran and glucose run on a Sepharose CL-6B column.

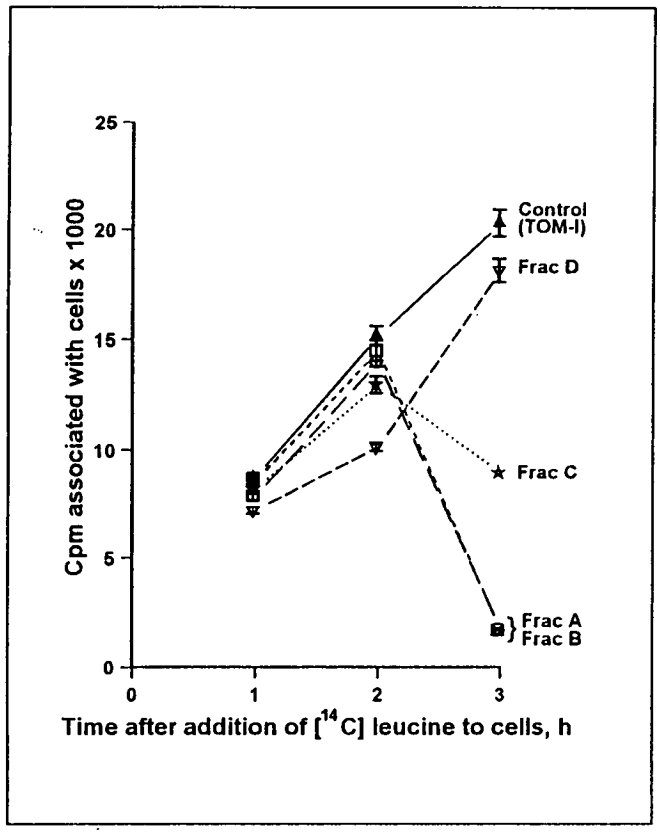
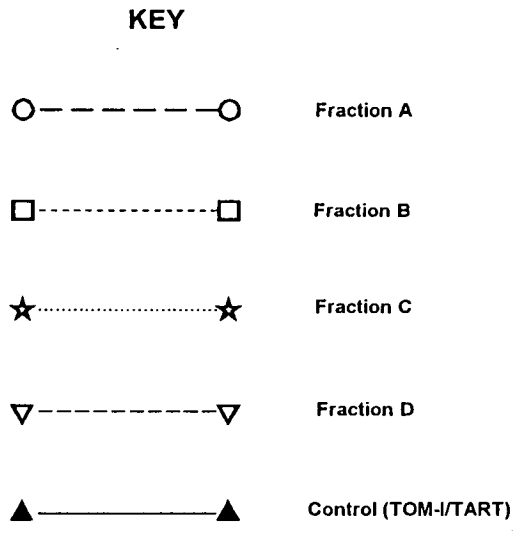


Figure 4.4.5.4.a:

Effect of exogenous hemicellulose fractions, separated on a size basis, on rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* at pH 5.7.

All means were obtained from 8 samples.



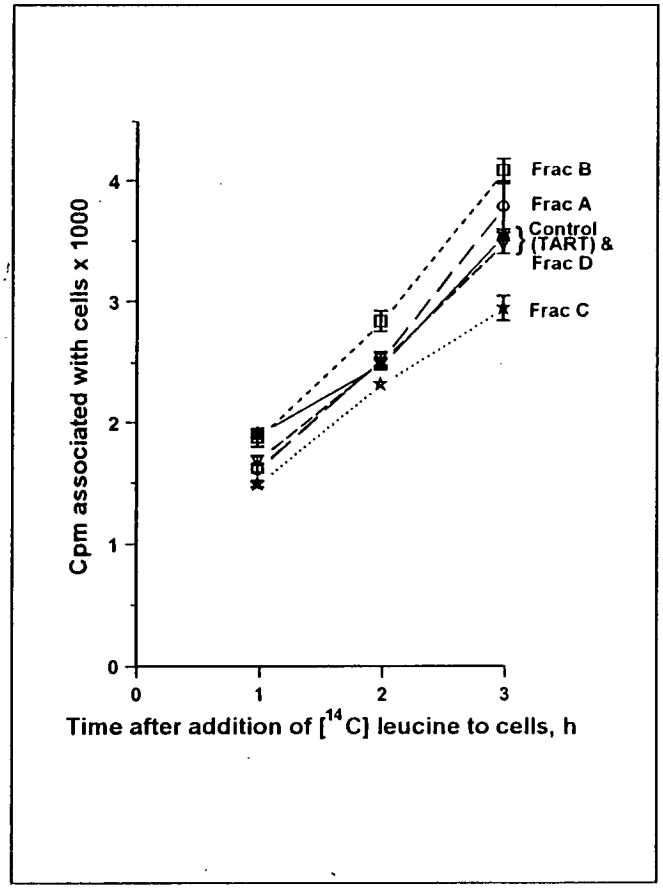


Figure 4.4.5.4.b:

Effect of exogenous hemicellulose fractions, separated on a size basis, on rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* x *peruvianum* at pH 4.5.

All means were obtained from 8 samples.

aliquots were added to each 0.5 ml cell sample, resulting in a final hemicellulose concentration of 30 µg/ml. To cells in the control treatment 30 µl aliquots of fresh TOM-I or TART were added in place of the hemicellulose solutions.

4.4.5.4.a: Effect of hemicellulose fractions A - D on protein metabolism at pH 5.7

The results obtained are displayed in Figure 4.4.5.4.a. By the 2 h sample point, cells incubated in the presence of Fractions A, B, C and D had incorporated 92%, 96%, 86%, and 67% respectively, of the amount of L-[U-^{14}C] leucine incorporated by cells in the control treatment. By the 3 h sample point cells in Fractions A, B, and C, had incorporated 8.7%, 8.4% and 44% of the amount of L-[U-^{14}C] leucine incorporated by cells in the control treatment. These amounts were lower in terms of cpm than had been recorded for these treatments at 2 h. Indeed, for Fractions A and B, the final recorded L-[U-^{14}C] leucine levels were considerably lower than those determined at the 1 h sample point.

4.4.5.4.b: Effect of hemicellulose fractions A - D on protein metabolism at pH 4.5

The results obtained are displayed in Figure 4.4.5.4.b. In this experiment the amounts of L-[U-^{14}C] leucine incorporated by cells, in all treatments increased steadily with time. By the 3-h sampling point cells incubated in Fractions A and B had incorporated 107% and 115% of the amount of L-[U-^{14}C] leucine incorporated by cells in the control treatment, cells incubated in Fraction C had incorporated 83%, and cells incubated in Fraction D had incorporated 98%. The inhibition of L-[U-^{14}C] leucine incorporation exhibited by cells incubated in Fraction C persisted from the 1-h sample point until the end of the experiment.

4.4.5.5: Discussion: Effects of hemicellulose fractions

The L-[U-^{14}C] leucine incorporation assay performed at pH 5.7 appeared to show anomalies in the results from the 3-h sampling point, where the clearest picture of any biological activity present would usually be found: cells treated with Fractions A, B, and C retained lesser amounts of L-[U-^{14}C] leucine-labelled proteins than at 2-h. This should not be possible and suggests an element of uncontrolled variation had been introduced to the system, therefore, the 2-h sample point probably provides a better comparison of effects.

There are two possible explanations for the apparent discrepancies in the results from the 3-h sample point. Firstly that the cell density in the suspension from which the sample tubes were inoculated did not remain constant throughout, and that the reduced levels of labelled-proteins retained on the filters resulted from a quantitative paucity of cells in the 3-h tubes for Fraction A and B. Secondly, incubation of cells of *L. esculentum* x *peruvianum* in hemicellulosic media containing a concentration of oligomers of a particular size range may have induced membrane disruptions (possibly also detected in cells of *L. esculentum* AC under conditions of low pH) previously absent only because of the biological dilution of the active oligomers by the rest of the hemicellulose population, or

because of synergistic activity. However, since there has been no other direct or indirect evidence of loss of membrane integrity in cells of *L. esculentum* x *peruvianum* at any pH this seems less likely.

At the 2-h sample point, all cells incubated in hemicellulosic treatments exhibited some degree of inhibition of L-[U-^{14}C] leucine incorporation, those in Fractions C and D were the most inhibited. Therefore, at pH 5.7 hemicelluloses of all sizes appear to elicit an element of inhibition of leucine incorporation in cells of *L. esculentum* x *peruvianum*, with the smaller oligosaccharides present in Fractions C and D being the most effective. However, at pH 4.5 application of the larger hemicellulose molecules in Fractions A and B had elicited increases in L-[U-^{14}C] leucine incorporation of up to 7% and 15% respectively at the 3-h sample point. Therefore the effects induced by hemicelluloses of different sizes, on the protein metabolism of cells of *L. esculentum* x *peruvianum,* appear to be pH-dependant.

At pH 5.7 cells from all hemicellulosic treatments exhibited inhibition to some degree, however, at pH 4.5 Fraction C was the only fraction to produce clear inhibition of L-[U-^{14}C] leucine incorporation. Since Fraction C consistently reduced L-[U-^{14}C] leucine incorporation, it is most probable that any hemicellulosic oligomers responsible for the "off" signal would be in the intermediate to small size range encompassed by this fraction. Unfortunately, estimations of actual DP cannot be made: results from a control column run with Blue Dextran and glucose as marker substances (Figs. 4.4.5.3.b.i and ii) which was designed to elucidate the size of molecules in each pooled fraction, indicated that Fraction C oligomers would have co-eluted with glucose, but, since it is highly unlikely that monosaccharides could exert any physiological effects of this nature, it is probable that the column did not run at the same rate on both occasions.

Fraction D contained the smallest hemicellulosic fragments and produced no discernible alteration in L-[U-^{14}C] leucine incorporation by cells of *L. esculentum* x *peruvianum* at pH 4.5. This suggests that the oligomers may have consisted of too few residues to be biologically active and were not "read" by the cells. Alternatively the oligomers in Fraction D may have been more diluted than fragments in the other fractions, and hence, less active. However, at pH 5.7 Fraction D had inhibited leucine incorporation by 33% at the 2-h sampling point, so this may be an example of the particular effects of certain oligomers being determined by the pH.

4.4.6: SUMMARY OF INFORMATION CONCERNING THE EFFECTS OF HEMICELLULOSE ON SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC

The results from L-[U-^{14}C] leucine incorporation assays with hemicelluloses were very consistent, particularly at pH 4.5, where hemicelluloses from *L. esculentum* x *peruvianum*

or *L. esculentum* AC, and *N. physaloides* inhibited protein metabolism in suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC and killed a proportion of the cells.

Cells of *L. esculentum* x *peruvianum* were more resistant to conditions of low pH ± hemicelluloses; fewer cells died, and protein anabolism became quiescent in those that survived, but was partially revived once cells were transferred to hemicellulose-free medium at pH 4.5, or pH 5.7. Essential metabolic functions, characterised by evidence of esterase activity, continued to function at low pH despite the subdued state of anabolic protein metabolism. Some cells exhibited plasmolysis when incubated in tartaric acid, but the proportion of plasmolysed cells was greater in hemicellulosic tartaric acid media.

Suspension-cultured cells of *L. esculentum* AC were more susceptible to the phytotoxic effects of hemicelluloses at pH 4.5 than cells of *L. esculentum* x *peruvianum*. There was evidence that essential metabolic functions such as esterase activity, were very subdued in cells of *L. esculentum* AC, but revived partially upon transfer to SH medium at pH 5.7. However, there was no evidence that anabolic protein metabolism was suspended under the same conditions. Cells incubated in hemicelluloses from *L. esculentum* AC at pH 4.5 exhibited visible signs of loss of membrane integrity during an FDA assessment. Indirect evidence of cellular damage during incubation of cells at low pH with hemicelluloses was also obtained.

The size of the biologically-active hemicellulosic fragment(s) is not yet known, however, experiments with hemicellulose from *L. esculentum* x *peruvianum*, fractionated on a size basis, indicated that oligomers, rather than polymers, mono- or disaccharides, are responsible.

These experiments with hemicellulose have established that the L-[U-^{14}C] leucine incorporation assay is a valid test with which to predict the effects of various cell wall-derived substances in the GU, and that it is a suitable vehicle to assess metabolic condition of cells, but is not appropriate for use as a viability test.

4.5: EFFECTS OF EXOGENOUS MEMBRANES ON INCORPORATION OF L-[U-^{14}C] LEUCINE INTO PROTEIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* AC

4.5.1: **Summary:** Effects of exogenous membranes

Parkinson (1983) concluded from indirect evidence that membranes from internodes of *L. esculentum* AC and *N physaloides* were not the active principles in the putative recognition process. To test this theory directly, membranes from *L. esculentum* AC and

N. physaloides were applied to cells of *L. esculentum* AC in a L-[U-^{14}C] leucine incorporation assay.

Cells in all treatments incorporated L-[U-^{14}C] leucine into protein at comparable rates and to almost identical levels. It was concluded that, since the application of membranes had caused no changes in L-[U-^{14}C] leucine incorporation in cells of *L. esculentum* AC, membranes did not act as active signalling molecules, although current evidence suggests that they are more likely to be involved in signal receipt and processing.

4.5.2: **Introduction:** Effects of exogenous membranes

Yeoman and colleagues (Yeoman *et al.*, 1978) postulated that contact between plasmalemmas of opposing callus cells might be permitted, when the pectic layer deposited in the GU was thinned, and that this contact might constitute the primary recognition event in the proposed recognition process. Later, Parkinson (1983) concluded from indirect evidence that membranes were not active progenitors of the recognition process. However, hard evidence that plasmalemmas were not actively involved in the primary recognition event had yet to be provided, so the activity of membranes from *L. esculentum* AC and *N. physaloides* internodes was investigated with a L-[U-^{14}C] leucine incorporation assay.

4.5.3: **Materials and Methods:** Effects of exogenous membranes

Cell membranes were extracted from internodes of *L. esculentum* AC and *N. physaloides* (sect. 2.4.6). The membranes were suspended in fresh SH medium, and 30 µl was added to each 0.5 ml aliquot of cells of *L. esculentum* AC in fresh SH medium at 0.1 g/ml. 30 µl of fresh SH medium was added to tubes in the control treatment. Samples were taken each hour for 3 h and processed according to the standard procedure (sect. 2.3).

4.5.4: **Results:** Effects of exogenous membranes

The results obtained are displayed in Figure 4.5.4. Throughout the assay cells incubated in the various media showed very similar rates and levels of L-[U-^{14}C] leucine incorporation with uniformly small standard error values. At 1 h and 2 h transient differences appeared between treatments, however, these were small and probably due to natural variation between samples. Importantly, at 3 h no significant differences were apparent between treatments.

4.5.5: **Discussion:** Effects of exogenous membranes

The absence of either suppression or promotion of protein metabolism in cells to which membranes had been added suggests that membranes, or membrane components, were not acting as signalling molecules in this system and remained undetected by the cells. Parkinson (1983) deduced from indirect evidence that membranes were not the active

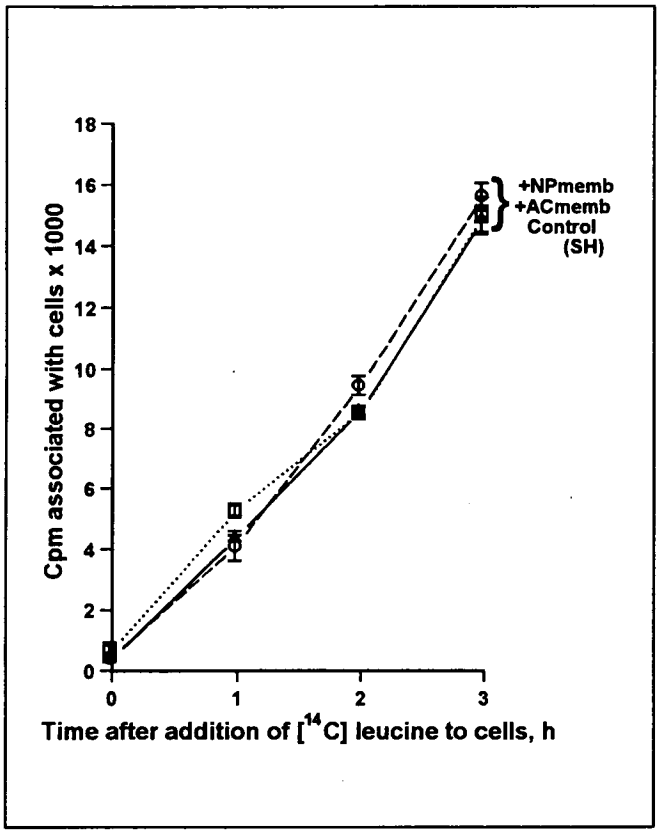


Figure 4.5.4:

Effect of exogenous membranes on the rate of L-[U-^{14}C] leucine incorporation into protein by suspension-cultured cells of *L. esculentum* AC.

Means were obtained from 4 samples at 0 h, and 8 samples at 1 h, 2 h and 3 h.

principles which controlled development in the GU, and the results from this experiment appear to corroborate his conclusions.

However, an absence of signalling agents *per se* in the membrane fraction does not render membranes redundant in the overall framework of intercellular-communications which are active during graft development; receptors for the putative recognition factors would probably be located on the plasmalemma, particularly if the factors are polysaccharide in nature. Work by Farmer *et al.* (1989) indicated that application of oligogalacturonide proteinase inhibitor (PI) elicitors to cells resulted in the phosphorylation of certain plasmalemma proteins. Prevention or interruption of phosphorylation caused cells to remain in, or revert to, the unelicited state. Furthermore, Horn *et al.* (1989) provided visual evidence of fluorescein-labelled elicitors of plant and fungal origin binding to the plasmalemma of suspension-cultured cells of soybean. Therefore, there is a strong argument for the receptors of oligosaccharins being located in the plasmalemma, and for the involvement of membrane components in the signalling process post-receipt of the initiating factor, the nature of which this thesis attempts to clarify.

The results from this experiment reinforce the fact that the L-[U-^{14}C] leucine incorporation assay appears to be a valid system for identifying compounds which would probably be active in recognition roles within the GU.

Chapter 5: FATE OF CELL WALL POLYSACCHARIDES APPLIED EXOGENOUSLY TO SUSPENSION-CULTURED CELLS

5.0: GENERAL INTRODUCTION TO EXPERIMENTS TO DETERMINE FATE OF CELL WALL POLYSACCHARIDES APPLIED EXOGENOUSLY TO SUSPENSION-CULTURED CELLS

The results obtained from L-[U-^{14}C] leucine incorporation assays (Chapter 4 and sect. 2.3) indicated that the protein metabolism of suspension-cultured cells, and possibly the cell division rates, were affected by the presence of cell wall-derived polysaccharides. Indirect evidence from the L-[U-^{14}C] leucine incorporation assays and direct evidence from FDA viability assays (sect. 2.2.3) indicated that, under certain circumstances, the viability and physical condition of the cells could also be affected by the polysaccharides. [^{14}C]-labelled cell wall components were generated (sect. 2.7.1) and applied to suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC in polysaccharide binding assays (sect. 2.7.2) to elucidate the fate, *modus operandi* and final location of cell wall substances previously applied to cells during L-[U-^{14}C] leucine incorporation assays.

Horn and colleagues (1989) showed that fluorescein-labelled elicitors applied to suspension-cultured cells bound initially to the plasmalemma and were subsequently internalised: the progress of the endocytotic process was quantified by use of an [^{125}I]-labelled elicitor. Internalisation of the elicitors was interpreted as a mechanism for clearing binding sites and re-sensitising the receptors after receipt of a signal. Post-incubation investigations revealed that the [^{125}I]- labelled elicitor had not been broken down up to 40 minutes after the commencement of vacuolar accumulation.

In the L-[U-^{14}C] leucine incorporation assays (sect. 2.3), pectins and pectic fragments produced highly variable effects when tested, while deproteinated cell walls and hemicelluloses produced more consistent effects (sects. 4.3, 4.1 and 4.4 respectively). The following experiments were performed to investigate whether cell wall polysaccharides become internalised by, or bound to, suspension-cultured cells during incubation and, whether any major structural breakdown of polysaccharides, or any preferential adsorption or ingestion could be detected.

The [^{14}C]-oligosaccharides generated from suspension-cultured cells of *L. esculentum* x *peruvianum* fed with D-[U-^{14}C]-glucose (specific activity = 292 mCi/mmol) (sect. 2.7.1) would have been labelled uniformly within each major group. The [^{14}C]-labelled cell wall polysaccharides were applied to suspension-cultured cells at the same concentrations as had their non-radioactive counterparts in the L-[U-^{14}C] leucine incorporation assays. Therefore, the percentage of the total [^{14}C]-polysaccharide applied which remained

adsorbed to the cells after rinsing, would provide a direct representation of the percentage of unlabelled polysaccharide which would have bound under the same conditions in the corresponding L-[U-^{14}C] leucine incorporation assay.

Generally, the following experiments are presented in the order in which they were performed, to illustrate the manner in which the results from one experiment were tested, and expanded with further experiments. While confirming results from initial experiments, it became clear that certain factors in the early experiments had been misleading, and the initial conclusions drawn were inaccurate. Consequently the following experiments provide information about the way in which the [^{14}C]-labelled polysaccharide binding assay was developed and honed to provide the desired information and improved accuracy of results.

5.1: THE FATE OF EXOGENOUSLY APPLIED [^{14}C]-PECTIN

5.1.1: LOCALISATION OF EXOGENOUS [^{14}C]-PECTIN APPLIED TO SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC

5.1.1.1: **Summary:** Localisation of exogenous [^{14}C]-pectin

To establish the fate and location of pectins incubated with suspension-cultured cells in earlier experiments (sect. 4.3), [^{14}C]-pectin from cell walls of *L. esculentum* x *peruvianum* was applied to suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC in [^{14}C]-labelled polysaccharide binding assays. Samples of unused [^{14}C]-pectic media, and the first rinses in native medium and in MES were run on a chromatogram to compare the populations of the pectin molecules in each.

Initial analysis of the results suggested that, of the total [^{14}C]-pectin applied, a maximum of 37% remained associated with cells of *L. esculentum* x *peruvianum*, and a maximum of 33% with cells of *L. esculentum* AC. Almost all of the associated pectin, 34% from *L. esculentum* x *peruvianum* and 28% from *L. esculentum* AC, was extractable in MES rinses from ruptured cells and seemed to have been internalised. The remaining associated [^{14}C]-pectin retrieved, 3.2% for *L. esculentum* x *peruvianum* and 4.5% for *L. esculentum* AC, was retained on the filters, ostensibly bound to the external cell wall. Although apparently clear-cut, further experiments which are presented later, showed these preliminary indications, that approximately 90% of the total associated pectin became internalised, while about 10% remained attached to the cell walls, to be inaccurate.

5.1.1.2: **Introduction:** Localisation of exogenous pectin (See General Introduction, sect. 5.0)

5.1.1.3: Materials and Methods: Localisation of exogenous pectin

5.1.1.3.i: Summary of protocol of [^{14}C]-labelled cell wall polysaccharide assays

The general protocol of the [^{14}C]-labelled cell wall polysaccharide assays is described in detail in sect. 2.7.2, however, a brief summary of the main points is outlined below.

Suspension-cultured cells of *L. esculentum* x *peruvianum*, or *L. esculentum* AC, were incubated for 3 h in 0.5 ml of native medium, TOM-I or SH, containing [^{14}C]-labelled cell wall components, then rinsed with 3 x 3 ml of native medium free from [^{14}C]-labelled compounds to elute unassociated [^{14}C]-labelled polysaccharides. The supernatants from each rinse were retained and 1 ml aliquots scintillation counted. Cells were sampled either in an intact state, or were fractionated into soluble and insoluble fractions: the former were filtered, dried and scintillation counted, while the latter were frozen, thawed, and sonicated on ice, then given 3 x 3 ml rinses in 10 mM MES (pH 6.0). Supernatant fractions were retained and 1 ml aliquots scintillation-counted. The remaining pellet of solid matter was filtered, dried and scintillation counted. In all cases the means were obtained from three replicates.

Intact cells were sampled to provide an overall figure for the amount of [^{14}C]-labelled polysaccharides which became associated with the cells. The soluble fraction of fractionated cells consisted mainly of the vacuolar and cytoplasmic contents of the cells which were eluted by MES rinses after cell rupture. The insoluble fraction retained on the filters was composed primarily of cell walls, with some plasmalemmas and larger organelles. Therefore, data from the soluble fraction permitted quantification of internalised [^{14}C]-polysaccharide, and data from the insoluble fraction permitted quantification of the proportion of [^{14}C]-polysaccharide adsorbed to the outside of the cells. Percentages are expressed in terms of "radioactivity retrieved" rather than "radioactivity applied", despite minimal variations between the two, because the former was measured directly in each experiment, while the latter was not assessed in the sample tubes.

5.1.1.3.ii: Chromatography of [^{14}C] pectic media and washings

After completion of the [^{14}C]-labelled polysaccharide binding assay, 1 ml aliquots of original unused [^{14}C]-pectic TOM-I or SH medium, the first TOM-I or SH rinse, and the first MES rinse from a fractionated sample were loaded onto Whatman 3MM chromatography paper and run in EtOAc/HOAc/$H_2$0 (10:5:6) overnight to permit identification of the portions of the pectic population which either remained unassociated with the cells or were internalised. Any major structural modifications to the pectic polysaccharides after incubation with the cells should have been visible. The chromatogram was dried, and cut into sections which were scintillation counted (sect. 2.5.1.f). A control track of 10 monomer marker sugars was also run on the chromatogram.

The samples of first rinses in TOM-I or SH and MES were taken from the same tube of fractionated cells, in order to provide consistency in the mixture of [^{14}C]-pectic compounds initially supplied to the cells. The figures obtained for 1 ml of the original unused pectic medium were divided by 2 prior to analysis to allow direct comparison of the actual levels of radioactivity added to the cell suspensions during assays, 0.5 ml, with the amounts extracted in the various rinses.

5.1.1.3.iii: Features peculiar to experiments in section 5.1.1: Localisation of exogenous pectin

In addition to the measurements already indicated in the [^{14}C]-labelled polysaccharide binding assay, 2 x 0.5 ml of the [^{14}C]-pectic media were scintillation counted to provide an independent assessment of the total number of cpm supplied to the cells, which could then be compared with the total number of counts retrieved.

5.1.1.4: Results: Localisation of exogenous pectin

5.1.1.4.i: Localisation of exogenously applied [^{14}C]-pectin after 3 h incubation with suspension-cultured cells of *L. esculentum* x peruvianum

A [^{14}C]-labelled polysaccharide binding assay (sect. 2.7.2) was performed with suspension-cultured cells of *L. esculentum* x *peruvianum* and [^{14}C]-labelled pectin in TOM-I medium at 0.5 mg/ml. Intact and fractionated cells were sampled. Chromatograms of samples of [^{14}C]-pectic medium, the first TOM-I rinse, and first MES rinse (from the same cell sample) were run as described above (sect. 2.5.1.f).

5.1.1.4.i.a: [^{14}C]-labelled polysaccharide binding assay

The results obtained, shown in Figure 5.1.1.4.i.a, reveal that 63% of the [^{14}C]-pectin applied to intact cells and 67% applied to fractionated cells, was eluted by the initial rinses in fresh TOM-I medium. With intact cells 33% remained on the filters, while with fractionated cells 3.2% remained on the filters and 34% was extracted in MES rinses.

The mean number of counts contained in 0.5 ml unused [^{14}C]-pectic TOM-I medium was 22 336 cpm. This was slightly more than the mean number of counts retrieved from intact and fractionated cells, i.e. 20 208 cpm and 20 765 cpm respectively.

5.1.1.4.i.b: Chromatography

The results of the counts are shown in Figures 5.1.1.4.i.b.i, ii, iii. The mobility of polysaccharides in this system is inversely proportional to their degree of polymerisation. The track of monomer markers was stained and the distribution was as follows: glucuronic acid (GlcA), galacturonic acid (GalA), galactose (Gal) and glucose (Glc) chromatographed to section 4, mannose (Man), arabinose (Ara), and xylose (Xyl), to section 5, and ribose (Rib) and rhamnose (Rha) to section 6.

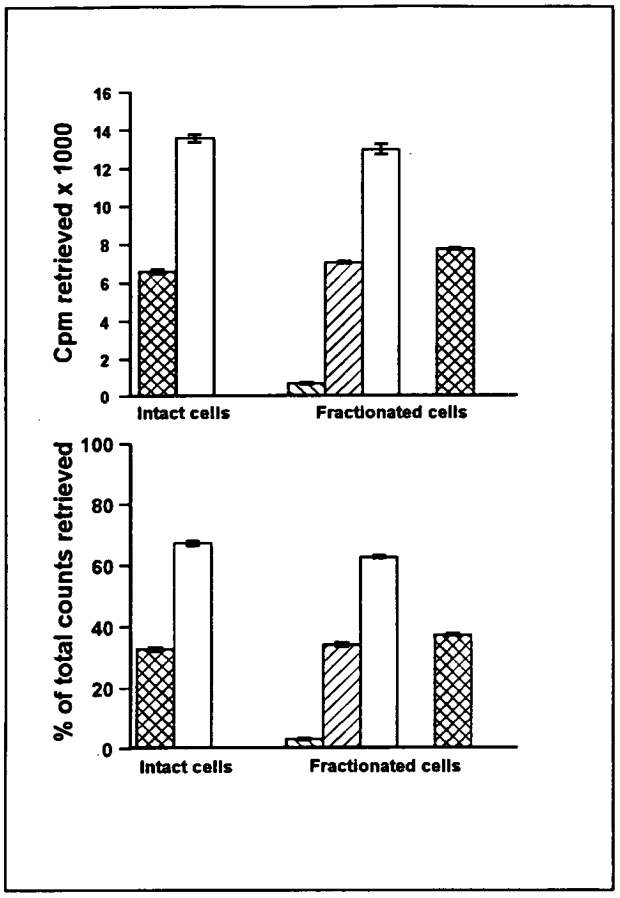


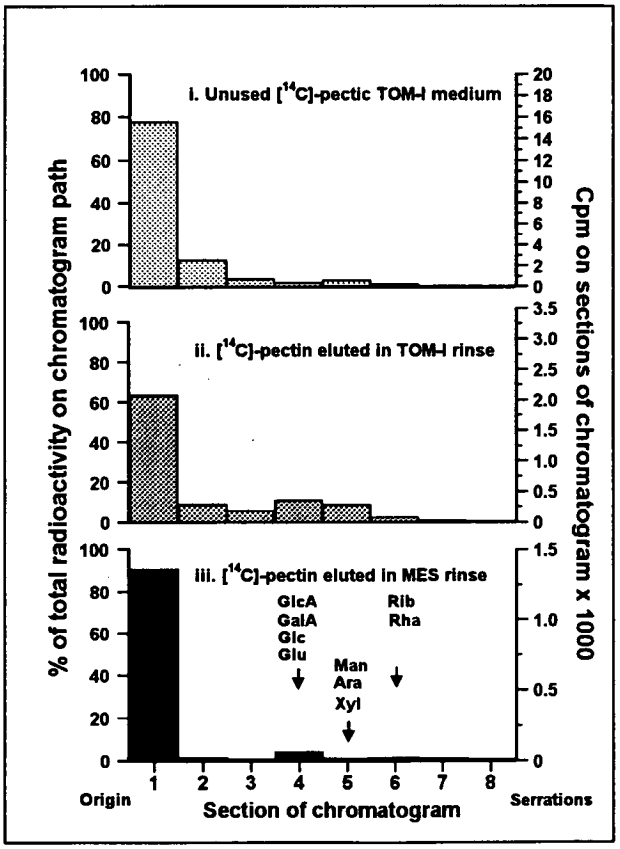
Figure 5.1.1.4.i.a:

Distribution of exogenously applied [^{14}C]-pectin after 3 h incubation with cells of *L. esculentum* x *peruvianum* expressed as activity (cpm) retrieved, and the corresponding percentage of the total activity retrieved.

Means were obtained from 3 samples.

KEY

- total associated with cells
- in TOM-I rinses
- on filter
- in MES rinses



Figures 5.1.1.4.b.i, ii, and iii:

Comparison of chromatographic profiles of [^{14}C]-pectic TOM-I medium before and after incubation with cells of *L. esculentum* x *peruvianum*.

Measurements shown are for 0.5 ml sample of unused [^{14}C]-pectic TOM-I medium (Fig. i), and 1 ml samples of [^{14}C]-pectin eluted in TOM-I and MES rinses (Figs. ii and iii).

Distance travelled by marker sugars along chromatogram:

Key

GlcA: glucuronic acid
GalA: galacturonic acid
Gal: galactose
Glu: glucose
Man: mannose
Ara: arabinose
Xyl: xylose
Rib: ribose
Rha: rhamnose

Figure 5.1.4.i.b.i shows the profile of pectic polymers supplied to the cells. Most of the radioactivity detected, 78%, remained at the origin (section 1). Section 2 exhibited the next largest population of pectic molecules with 13% of the total activity. The total activity for sections 3 and 5 were 3.6% and 2.9% respectively, the activity of the remaining sections lay between 0.08 - 1.7%.

The pectin extracted in TOM-I rinses (Fig. 5.1.4.i.b.ii) exhibited a polymer profile that was broadly similar to the pectic medium initially supplied to the cells. Again, the highest level of radioactivity, 63% of the total detected, was located at the origin. However, the next highest peak, 11%, lay at section 4, not at section 2 as previously. Comparatively high proportions of the radioactivity were detected on sections 2, 3, 5 and 6, i.e. 8.7%, 5.7%, 8.4% and 2.5% respectively. Very low levels of radioactivity were located on sections 7 and 8, 0.6% and 0.25% (8.25 - 19.8 cpm above background readings).

The chromatographic profile of the pectin recovered in MES rinses after cell rupture (Fig. 5.1.1.4.i.b.iii) differed both from that of the unused original medium and that of the TOM-I rinses in the ratio of molecules of different mobilities contained. A higher percentage of [^{14}C]-pectic polymers than previously recorded, 91%, remained at the origin. With the exception of section 4, which registered 4% of the total radioactivity detected, the levels recorded varied between 0.51% (8 cpm, section 3) and 1.3% (20.25 cpm, section 2).

5.1.4.ii: Localisation of exogenously applied [^{14}C]-pectin after 3 h incubation with suspension-cultured cells of *L. esculentum* AC

A [^{14}C]-labelled polysaccharide binding assay (sect. 2.7.2) was performed with suspension-cultured cells of *L. esculentum* AC and [^{14}C]-labelled pectin in SH medium, at 0.5 mg/ml. The sample tubes were rotated at 140 rpm instead of 200 rpm during incubation and only cells which had been fractionated were sampled. Chromatograms of samples of [^{14}C]-pectic medium, the first SH rinse, and first MES rinse (from the same cell sample) were run as described (sect. 2.5.1.f).

5.1.1.4.ii.a: [^{14}C]-labelled polysaccharide binding assay

The results are shown in Figure 5.1.1.4.ii.a. SH rinses eluted 67% of the total radioactivity retrieved, 33% of the [^{14}C]-pectin remained in association with the cells. After cell rupture, rinses in 10 mM MES eluted 28% of the total [^{14}C]-pectin retrieved, leaving 4.5% on the filters. Thus the maximum amount of [^{14}C]-pectin which remained in association with the cells after 3 h incubation was 33% of that retrieved. Scintillation-counting of 0.5 ml unused [^{14}C]-pectic SH medium showed activity of 20 024 cpm; at least this amount was retrieved from all cell samples.

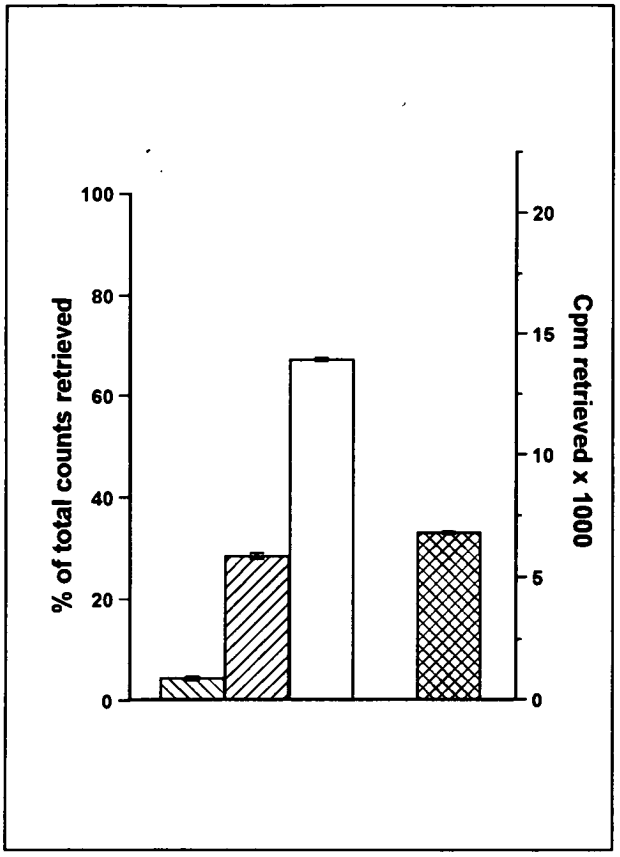
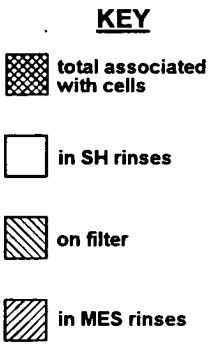
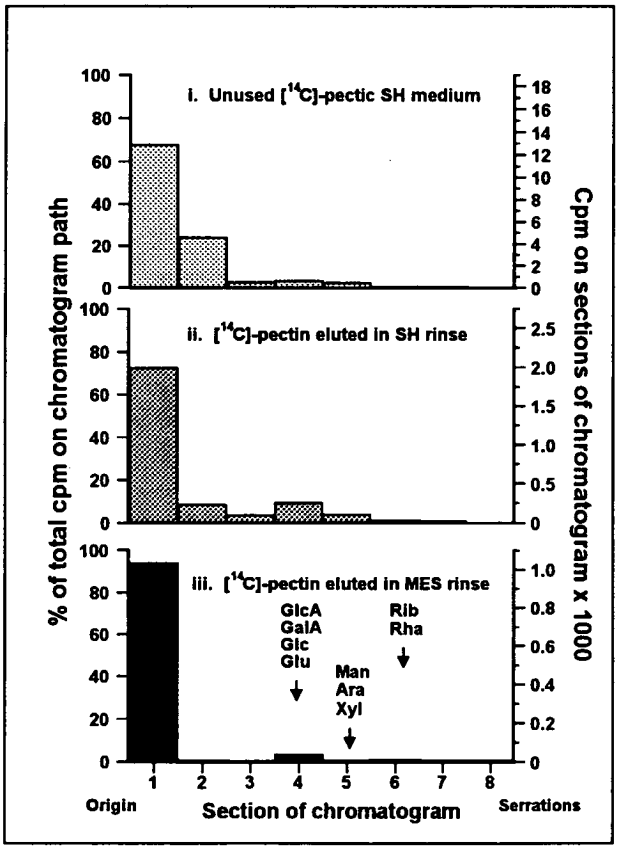


Figure 5.1.1.4.ii.a:

Distribution of exogenously applied [^{14}C]-pectin after 3 h incubation with cells of *L. esculentum* AC (fractionated cells only).

Means were obtained from 3 samples.





Figures 5.1.1.4.ii.b.i, ii, and iii:

Comparison of chromatographic profiles of [^{14}C]-pectic SH medium before and after incubation with cells of *L. esculentum* AC.

Measurements shown are for 0.5 ml sample of unused [^{14}C]-pectic SH medium (Fig. i), and 1 ml samples of [^{14}C]-pectin eluted in SH and MES rinses (Figs. ii and iii).

Distance travelled by marker sugars along chromatogram:

Key

GlcA: glucuronic acid
GalA: galacturonic acid
Gal: galactose
Glu: glucose
Man: mannose
Ara: arabinose
Xyl: xylose
Rib: ribose
Rha: rhamnose

5.1.1.4.ii.b: Chromatography

The results obtained from scintillation counting sections of the chromatogram are displayed in Figures 5.1.1.4.ii.b.i, ii, and iii.

In the overall polymer profile of unused [^{14}C] pectic/SH medium (Fig. 5.1.1.4.ii.b.i), the majority of the radioactivity detected, 68%, remained at the origin, with 24% on section 2. Thereafter there was a sudden drop in the radioactivity recorded per section; the remaining 8.5%, was spread more evenly across sections 3 - 8, with 2.1 - 3% recorded for sections 3, 4 and 5 and 0.06 - 0.62% (11.4 - 118.4 cpm) detected on sections 6, 7 and 8.

There was a high degree of similarity between the composition of the pectin in the unused and used media. In the SH rinse, 72% of the [^{14}C]-pectic polymers remained at the origin (Fig. 5.1.1.4.ii.b.ii). The proportion of the total activity attributed to section 2 was lower than the corresponding value for unused medium, 8.4% *vs.* 24% (Fig. 5.1.1.4.ii.b.i). Section 3 contained 3.6% of the total [^{14}C]-pectin. Sections 4, 5 and 6 exhibited higher proportions of radioactive polymers of these mobilities than the corresponding sections in the unused medium chromatogram, i.e. 9.3% *vs.* 3%, 4% *vs.* 2.1%, and 1.3% *vs.* 0.62% . Sections 7 and 8 recorded 0.79 and 0.1% of the total radioactivity respectively.

The amount of [^{14}C]-pectin in the 1 ml sample of the first MES rinse was comparatively small (Fig. 5.1.1.4.ii.b.iii). Most of the pectin extracted in these rinses, 94%, remained at the origin, section 1. Section 4 recorded the next highest levels with 3.6% (98 cpm). Trace quantities of radioactivity were located on sections 2, 3, 5, 6, 7 and 8, i.e. 1.25 - 8.75 cpm (0.11 - 0.77%), which constituted 3% of the total radioactivity measured. For the previous two samples of unused medium and [^{14}C]-pectin eluted in SH rinses, sections 2 and 3 recorded low, but considerable, proportions of the pectic polymer population, however with MES-eluted material very low levels of radioactivity were detected on sections 2 and 3 .

5.1.1.5: Discussion: Localisation of exogenous pectin

The [^{14}C]-labelled polysaccharide binding assays with suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC produced very similar results. Initially interpretation of these assays appears perfectly clear, i.e. that for cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC respectively 37% and 33% of the total [^{14}C]-pectin applied became associated with fractionated cells; accordingly, 34% and 28% appear to have been internalised and were extracted in MES rinses following cell rupture. The remaining 3.2% and 4.5% of the total [^{14}C]-pectin retrieved associated with the cells were retained on the filters with the solid fraction of the fractionated cells, implying that a comparatively low proportion of the [^{14}C]-pectin had adsorbed to the external surface of the cells, the cell wall.

The comparison of composition of the [^{14}C]-pectin populations extracted in various rinses by paper chromatography will be discussed later in the light of further developments relating to the apparent sequestration of [^{14}C]-pectin by the cells.

5.1.2: QUANTIFICATION OF EXTERNALLY ADSORBED [^{14}C]-PECTIN BY ELUTION FROM SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* WITH LiCl (aq)

5.1.2.1: **Summary:** Elution of adsorbed pectin with LiCl (aq)

To obtain an independent assessment of the proportion of applied [^{14}C]-pectin which appeared to become adsorbed externally to the cell walls of cells of *L. esculentum* x *peruvianum*, i.e. <5% of that applied (sect 5.1.1.4.i.a), a [^{14}C]-labelled polysaccharide binding assay was performed. After the initial post-incubation rinses in TOM-I medium cells were processed as usual, or rinsed in 1M LiCl solution to elute externally adsorbed [^{14}C]-pectin, and then processed according to the standard protocol.

The results from intact and fractionated cells showed that 30 - 40% of the [^{14}C]-pectin applied remained in association with the cells after TOM-I rinses. Rinses with LiCl solution eluted 34 - 37% of the applied [^{14}C]-pectin from intact cells, and MES rinses, applied after LiCl rinses and cell rupture, eluted 1.3%. In the absence of LiCl rinses, MES rinses eluted 29% of the applied [^{14}C]-pectin from the cells.

The proportion of [^{14}C]-pectin applied which was eluted by LiCl rinses was greater than the 5% anticipated, which indicated that a considerable proportion of the [^{14}C]-pectin might not have been internalised, as previously thought, but became externally bound and vulnerable for elution with LiCl. Doubts arose concerning the suitability of MES as a suitable rinsing agent since it eluted a similar quantity of apparently externally adsorbed [^{14}C]-pectin from ruptured cells in the absence of LiCl rinses. Problems with the solubility of [^{14}C]-pectin in aqueous solutions may have contributed to the situation with pectic particles centrifuging down with cells, while not becoming adsorbed to them.

5.1.2.2: **Introduction:** Elution of adsorbed pectin with LiCl (aq)

Previous experiments (sect. 5.1.1) indicated that a large proportion, >30%, of the [^{14}C]-pectin applied to suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC became associated with the cells through internalisation or adsorption to the cell walls. An attempt was made to confirm the distribution of [^{14}C]-pectin during the incubation period with cells of *L. esculentum* x *peruvianum* using a modified [^{14}C]-labelled polysaccharide binding assay. If, after unadsorbed [^{14}C]-pectin had been eluted from the cells and before further processing such as rupturing occurred, the [^{14}C]-pectin adsorbed to

the cell walls of entire cells could be eluted for separate quantitative assessment a more accurate assessment of the ratio of surface-bound to internalised pectin could be made.

In primary cell walls pectins adsorb to xyloglucans in the cell wall by means of covalent bonds (Talmadge, Keegstra, Bauer *et al.*, 1973; Bauer *et al.*, 1973; Keegstra, Talmadge, Bauer *et al.*, 1973); to extensin and other pectic molecules via pH-dependent hydrogen-bonds (Lamport, 1986); and to pectin molecules through ionic bonds with divalent cations, principally Ca^{2+}, which produce the type of serial bonding known as the "egg-box formation" (Jarvis, 1984). The frequency of covalent and hydrogen-bonds between pectins and other cell wall components are limited (Smith, 1992), therefore "egg-boxing" is the main means of pectic adsorption.

Ionically-bound substances can be eluted from cell walls by rinsing the walls with aqueous salt solutions. (Nari, Noat, Ricard *et al.*, 1983). It was decided to use 1 M LiCl as the solution with which to elute pectin adsorbed to cells of *L. esculentum* x *peruvianum*.

5.1.2.3: Materials and Methods: Elution of adsorbed pectin with LiCl (aq)

This experiment was based on the [^{14}C]-labelled polysaccharide binding assay (sect. 2.7.2) and contained 4 treatments. Treatments 1 and 2 were performed on intact cells of *L. esculentum* x *peruvianum* throughout; Treatments 3 and 4 were performed on cells which were fractionated. In all treatments cells were incubated in [^{14}C]-pectic TOM-I [0.5 mg/ml], then rinsed with 3 x 3 ml of TOM-I medium according to the standard protocol (sect. 2.7.2). Treatments then diverged and are outlined below:

Treatment 1: cells were filtered and counted.

Treatment 2: cells were rinsed in 3 x 3 ml 1 M LiCl (Aldrich Chemical Co. Ltd.), filtered and counted.

Treatment 3: cells were rinsed in 3 x 3 ml 1 M LiCl, frozen, thawed, sonicated, rinsed in 3 x 3 ml 10 mM MES, filtered and counted.

Treatment 4: cells were frozen, thawed, sonicated, rinsed in 3 x 3 ml 10 mM MES, filtered and counted.

5.1.2.4: Results: Elution of adsorbed pectin with LiCl (aq)

In Treatments 1 (Figs. 5.1.2.4.i) and 2 (Fig. 5.1.2.4.ii) 31% and 39%, respectively, of the [^{14}C]-pectin retrieved remained associated with the intact cells. In Treatment 2, 37% of the [^{14}C]-pectin retrieved was eluted in LiCl rinses, leaving 1.7%, to filter out with the cells. For Treatments 2 and 1 respectively, the TOM-I rinses eluted 61% and 69% of the [^{14}C]-pectin retrieved, mostly in the first 2 rinses, with the final rinses showing comparatively low

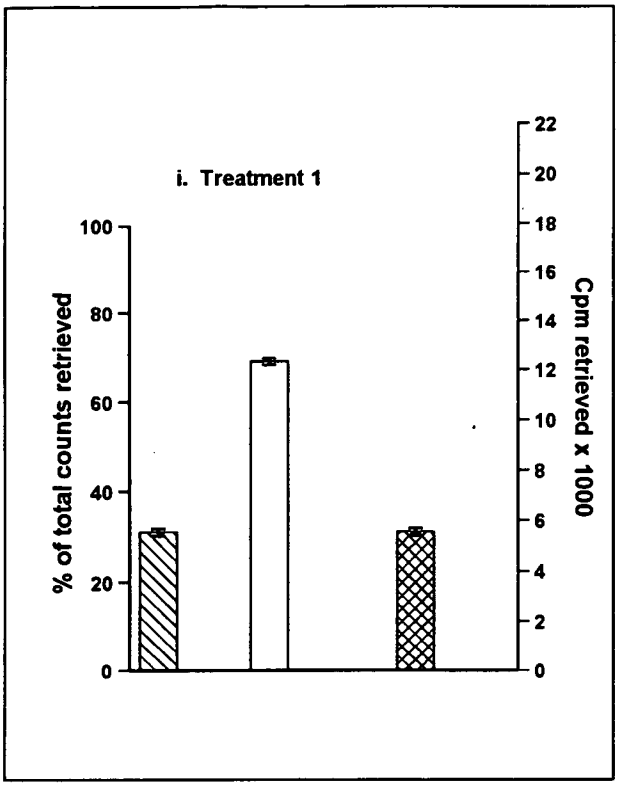


Figure 5.1.2.4.i, Treatment 1:

Distribution of exogenously applied [^{14}C]-pectin after 3 h incubation with cells of *L. esculentum* x *peruvianum* rinsed with only TOM-I. Cells were sampled intact.

Means were obtained from 2 samples.

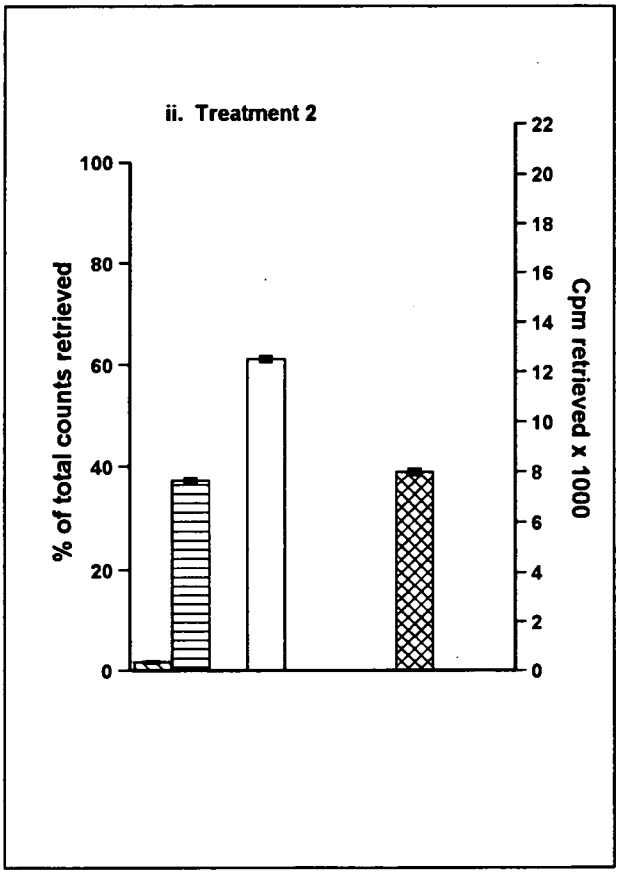
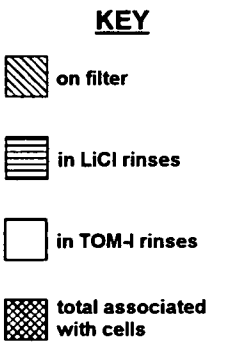


Figure 5.1.2.4.ii, Treatment 2:

Distribution of exogenously applied [^{14}C]-pectin after 3 h incubation with cells of *L. esculentum* x *peruvianum* rinsed with TOM-I and LiCl. Cells were sampled intact.

Means were obtained from 3 samples.



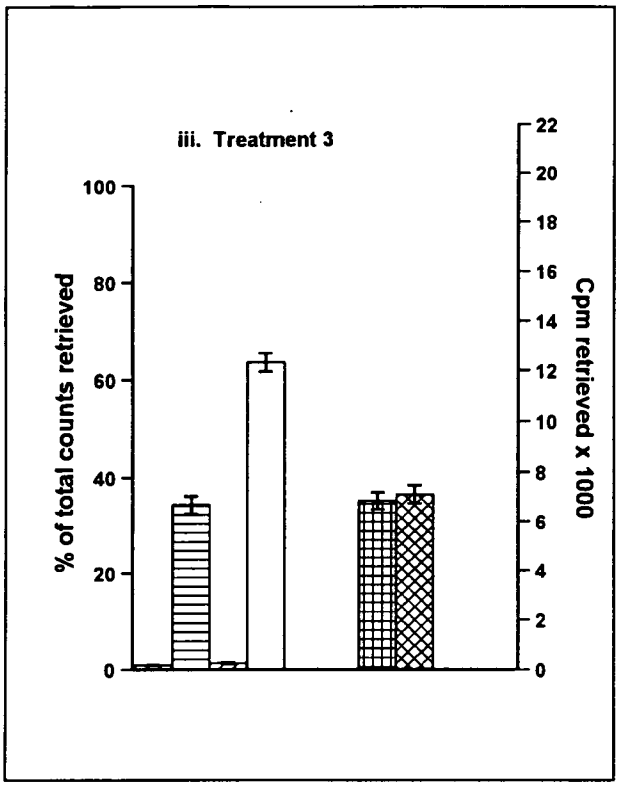


Figure 5.1.2.4.iii, Treatment 3:

Distribution of exogenously applied [^{14}C]-pectin after 3 h incubation with cells of *L. esculentum* x *peruvianum* rinsed with TOM-l and LiCl. Cells were fractionated, and rinsed with MES.

Means were obtained from 2 samples.

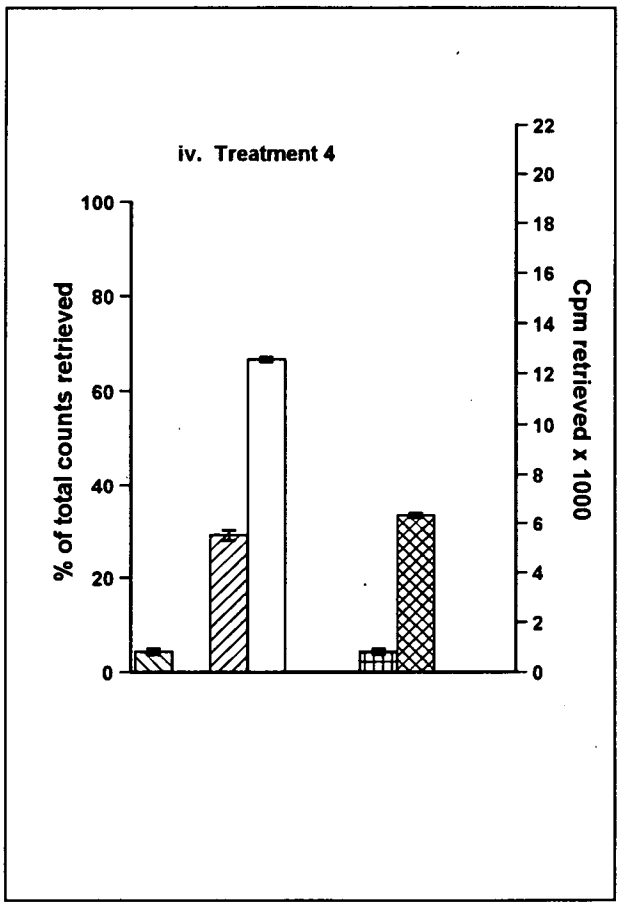
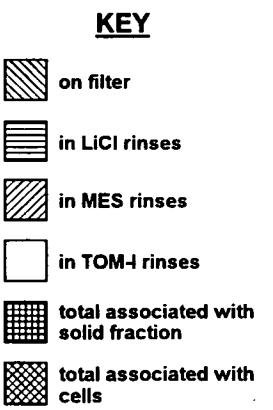


Figure 5.1.2.4.iv, Treatment 4:

Distribution of exogenously applied [^{14}C]-pectin after 3 h incubation with cells of *L. esculentum* x *peruvianum* rinsed with TOM-l. Cells were fractionated, and rinsed with MES.

Means were obtained from 2 samples.



activity. The initial LiCl rinse eluted approximately 150-fold more counts than the final TOM-I rinse.

In Treatment 3 (Fig. 5.1.2.4.iii) 37% of the [^{14}C]-pectin retrieved remained associated with the cells; LiCl rinses eluted 34%, post-rupture MES rinses eluted 1.3%, and 0.88% was retained on the filters. In Treatment 4 (Fig. 5.1.2.4.iv), in which cells were not rinsed in LiCl, 34% of the [^{14}C]-pectin retrieved remained in association with the cells; post rupture MES rinses eluted 29%, leaving 4.3% on the filters.

5.1.2.5: Discussion: Elution of adsorbed pectin with LiCl (aq)

Comparison of the total [^{14}C]-pectin retrieved which remained associated with cells in this experiment, i.e. 31 - 39%, with figures from the previous experiment (sect. 5.1.1.4.i), i.e. 33% and 37%, indicates that a large and consistent percentage of the total [^{14}C]-pectin appears to become associated with the cells.

Initial analysis of the results from this experiment (5.1.2) made in isolation from previous data would suggest that almost all, 34 - 37%, of the [^{14}C]-pectin retrieved cells was externally bound and could be eluted in LiCl rinses, leaving very low levels in the cell contents to be extracted by MES rinses. However, results from previous experiments (sect. 5.1.1) suggested that a maximum of 5% of the [^{14}C]-pectin retrieved remained externally-bound to the cells after incubation, and should have been eluted in the LiCl rinses. Therefore, the quantities of [^{14}C]-pectin eluted in LiCl rinses, >30%, were disproportionately large. Having said this, it is vital to note that the percentages of [^{14}C]-pectin retrieved eluted in LiCl rinses in Treatments 2 and 3, 37% and 34%, were directly comparable to the 29% eluted in MES rinses, applied in the absence of LiCl rinses, from cells in Treatment 4, and to the 34% eluted from ruptured cells in the previous experiment (sect. 5.1.1.4.i.a). This may suggest that the levels of [^{14}C]-pectin extracted by MES rinses after rupture, in the absence of LiCl rinses, might include more than just the pool of internalised [^{14}C]-pectin.

If the proportion of [^{14}C]-pectin originally thought to be located in the cell contents was, in fact, internalised, then LiCl showed an unexpected ability to elute [^{14}C]-pectin from the cytoplasm and vacuoles of intact cells. However, if the pectin was not internalised by the cells, but remained adsorbed to the external surfaces throughout, then it would have been available for elution by LiCl and the levels of [^{14}C]-pectin detected in the rinses could be explained.

Initially, the [^{14}C]-pectin extracted by MES rinses (sect. 5.1.1) was thought to originate from the cell contents, which became available after cell rupture. However, if most of the [^{14}C]-pectin extracted by MES rinses had not been internalised, but remained adsorbed to the external cell surfaces after rinses in TOM-I, then the subsequent rinses with 10 mM MES, a

buffer initially thought not to affect pectic bonding, appear to have been almost as effective as 1 M LiCl in eluting [^{14}C]-pectin from the external surfaces of the cells.

Even so, the ~ 30% apparently cell wall-bound pectin extracted by LiCl is an unusually large proportion of that retrieved to have adsorbed to the cell surfaces, especially if these polysaccharides do exhibit biological activity. It is possible that the [^{14}C]-pectin did not dissolve completely in the TOM-I medium in which it was applied to the cells, or in subsequent TOM-I rinses, but centrifuged down with the cells during rinsing, thus avoiding elution. If these pectins dissolved more readily in solutions of 10 mM MES or 1 M LiCl, then the latter rinses would extract a considerable proportion of the contaminating insoluble [^{14}C]-pectin, together with that which had genuinely been internalised or externally-bound to the cells, resulting in an overall misinterpretation of the situation.

This experiment would have been improved by the inclusion of three further control rinses in TOM-I for cells in Treatments 1 and 4 which did not receive LiCl treatment. However, later experiments of a similar nature in which the additional 3 x 3 ml TOM-I rinses were included in the control treatments (sect. 5.1.4.4.) indicated that only minimal amounts of [^{14}C]-pectin were eluted by these rinses, i.e. < 3% of the total radioactivity retrieved, and that these amounts were insignificant in relation to the quantities eluted by LiCl.

5.1.3: EXPERIMENT TO DETERMINE WHETHER FREEZING AND THAWING PRIOR TO SONICATION ENHANCES CELL RUPTURE RATES

5.1.3.1: **Summary:** Cell rupture techniques

A comparison of rupturing techniques was performed to maximise the release of internalised [^{14}C]-polymers during the [^{14}C]-labelled polysaccharide binding assay. After incubation with [^{14}C]-pectin cells of *L. esculentum* x *peruvianum* were either frozen and thawed prior to sonication, or sonicated immediately. Slightly more [^{14}C]-pectin was eluted from cells which had been sonicated immediately after incubation than from cells which had first been frozen and thawed. Initially, these results were interpreted as showing that both rupturing techniques were equally effective and should be used when convenient in the experimental protocol. However, in the light of previously mentioned doubts (5.1.2.5), and evidence from future experiments, concerning the suitability of MES as an elutant and the solubility of [^{14}C]-pectin in TOM-I medium, it was concluded that this experiment did not elucidate the extent to which cells were broken open by either technique.

5.1.3.2: **Introduction:** Cell rupture techniques

Cells which were to be fractionated in the [^{14}C]-labelled polysaccharide binding assay were routinely frozen and thawed prior to sonication on ice. The freezing and thawing process was thought to rupture the majority of cells, and the later sonication was to ensure that all

cells in a sample had been broken open, thereby ensuring a maximal volume of cell contents available for elution in MES rinses. The overall efficiency of this means of cell rupture was not known, and it was decided to evaluate the advantages conferred by the freezing and thawing process by comparing the amount of internalised [^{14}C]-pectin released from cells which had been frozen and thawed prior to sonication, with those released from cells which received immediate sonication only.

5.1.3.3: Materials and Methods: Cell rupture techniques

Suspension-cultured cells of *L. esculentum* x *peruvianum* were incubated for 3 h in [^{14}C]-pectic TOM-I medium, and rinsed in 3 x 3 ml of TOM-I (sect. 2.7.2). Cells were either frozen for an hour, thawed and sonicated on ice as previously described, or sonicated on ice immediately without prior freezing. 3 x 3 ml MES rinses were applied to elute the cell contents. Samples of all rinses were processed as described (sect. 2.7.2).

5.1.3.4: Results: Cell rupture techniques

The experiments described in sections 5.1.3 and 5.1.4 were performed simultaneously.

In terms of actual counts or percentages, there was virtually no difference between treatments in the quantities of [^{14}C]-pectin retained on the filters for cells ruptured by sonication alone (Fig. 5.1.3.4.i), 2.8% of total [^{14}C]-pectin retrieved (599 cpm), or for cells ruptured by freezing, thawing and sonication (Fig. 5.1.3.4.ii), 3.2% (671 cpm).

MES rinses eluted 33% of [^{14}C]-pectin retrieved from sonicated cells and 29% from cells frozen prior to sonication. Differences also emerged in the total amounts of [^{14}C]-pectin which appeared to be associated with the cells (i.e. filters + MES rinses); with 36% associated with sonicated cells, and 32% associated with cells which had been frozen prior to sonication.

5.1.3.5: Discussion: Cell rupture techniques

Initial analysis of this experiment, indicated that there was very little difference in the proportion of cells broken open by either treatment as shown by the quantities of internalised [^{14}C]-pectin released into MES rinses. However, in the light of the results from the experiment in sect. 5.1.4, which was performed simultaneously, it would be safer to say that the criterion chosen for determining the efficiency of rupturing method, i.e. levels of [^{14}C]-pectin eluted in MES rinses, was inappropriate, and did not constitute a specific measure of internal [^{14}C]-pectin levels, or of the proportion of cells ruptured.

There were small differences in the amounts of [^{14}C]-pectin eluted in the MES rinses between the treatments, but it is unlikely that these clarify questions about the proportion of pectin internalised by the cells, or the efficiency of the rupturing procedure, especially since

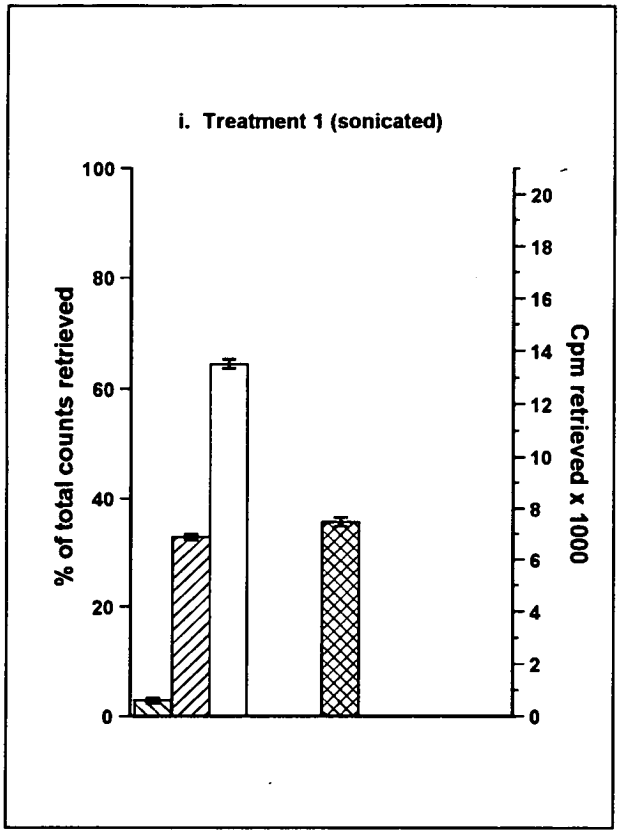


Figure 5.1.3.i, Treatment 1:

Distribution of exogenously applied [^{14}C]-pectin eluted from cells of *L. esculentum* x *peruvianum* sonicated immediately after the application of standard TOM-I rinses.

Means were obtained from 3 samples.

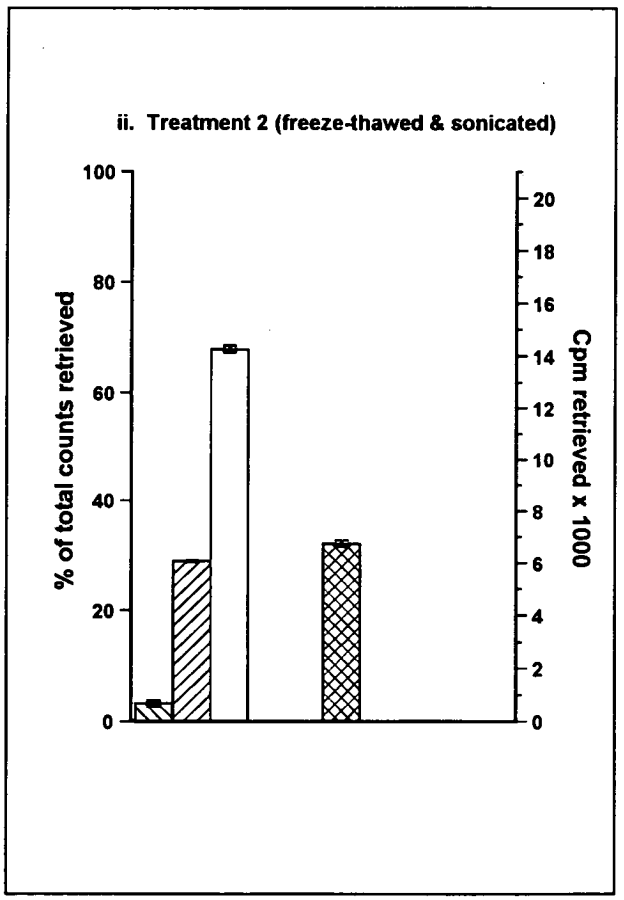
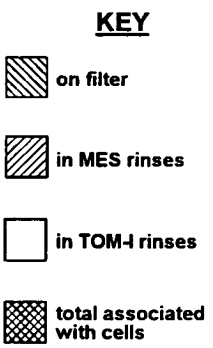


Figure 5.1.3.4.ii, Treatment 2:

Distribution of exogenously applied [^{14}C]-pectin eluted from cells of *L. esculentum* x *peruvianum* which were frozen, thawed and sonicated after the standard TOM-I rinses.

Means were obtained from 3 samples.



comparable differences were detected in the percentage of [^{14}C]-pectin eluted in TOM-I rinses prior to any divergence in treatment of the cells.

5.1.4: EXPERIMENT TO ASSESS THE CAPACITY OF 10 mM MES TO ELUTE EXOGENOUSLY-APPLIED [^{14}C]-PECTIN FROM THE SURFACES OF SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM*.

5.1.4.1: **Summary:** Elution of adsorbed pectin with MES

Results from earlier experiments (sect. 5.1.2) had indicated that the amounts of [^{14}C]-pectin eluted from intact and ruptured cells by solutions of LiCl and MES, alone, were very similar. To compare the extent to which 10 mM MES could elute adsorbed [^{14}C]-pectin from the external surfaces of intact cells of *L. esculentum* x *peruvianum* a [^{14}C]-labelled polysaccharide binding assay was performed in which the initial TOM-I rinses were followed either by further rinses of TOM-I medium, or rinses of MES. Further rinses with TOM-I medium eluted an extra 2.7% of the total [^{14}C]-pectin retrieved from the cells, while rinses with MES eluted 22%. It was concluded that MES is an effective eluant of [^{14}C]-pectin externally-bound to intact cells, and that MES was not the best choice of solution for elution of soluble cell contents after cell rupture, since [^{14}C]-pectin present in the solid fraction would also be vulnerable to elution.

5.1.4.2: **Introduction:** Elution of adsorbed pectin with MES

In an earlier experiment (sect. 5.1.2) it was noted that the percentages of [^{14}C]-pectin extracted from intact cells by LiCl solution and from ruptured cells by MES, in the absence of LiCl rinses, were very similar. This was confusing, since it was thought that LiCl solution eluted [^{14}C]-pectin from the external surfaces of the cells, while MES eluted only internalised [^{14}C]-pectin made available after cell rupture. MES had been chosen as an eluant for ruptured cells because it was thought that it would not elute [^{14}C]-labelled polysaccharides adsorbed to cell walls or plasmalemmas. However, if rinses with 10 mM MES could elute [^{14}C]-pectin from the surfaces of intact cells as effectively as LiCl solution, then all interpretations of previous results would have to be reconsidered, and the standard protocol for the assay adjusted. To establish the ability of 10 mM MES to do this the experiment described below was performed.

5.1.4.3: **Materials and Methods:** Elution of adsorbed pectin with MES

This experiment was performed only with intact cells to ensure that the cell contents would not be available for elution. Suspension-cultured cells of *L. esculentum* x *peruvianum* were incubated for 3 h in [^{14}C]-pectic TOM-I medium (0.5 mg/ml) (sect. 2.7.2). After the initial

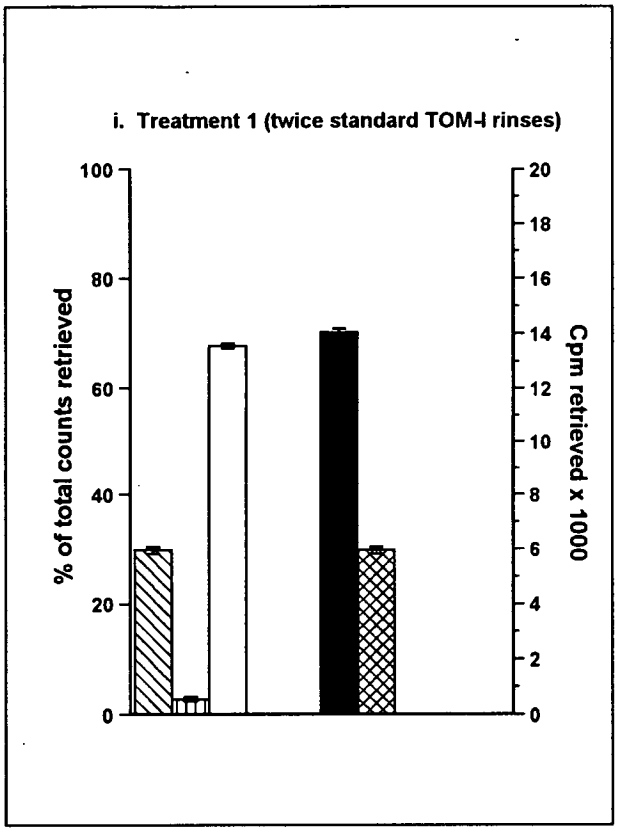


Figure 5.1.4.4.i, Treatment 1:

Distribution of exogenously applied [^{14}C]-pectin eluted from intact cells of *L. esculentum* x *peruvianum* by standard TOM-I rinses applied twice.

Means were obtained from 3 samples.

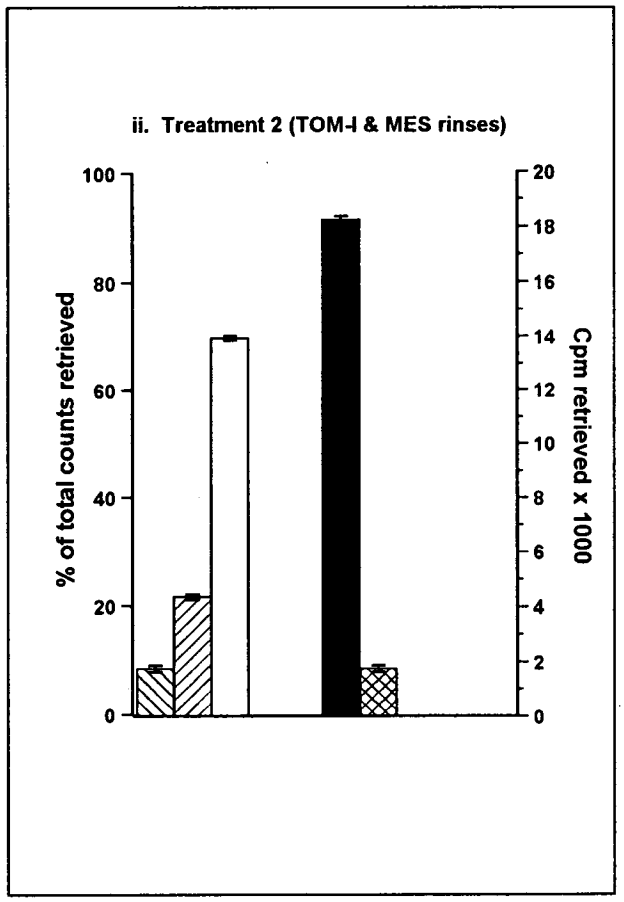
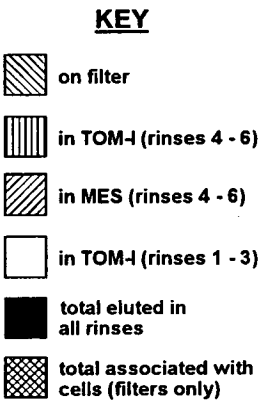


Figure 5.1.4.4.ii, Treatment 2:

Distribution of exogenously applied [^{14}C]-pectin eluted from intact cells of *L. esculentum* x *peruvianum* in standard TOM-I and MES rinses.

Means were obtained from 3 samples.



TOM-I rinses, the cells were rinsed again, either in a further 3 x 3 ml TOM-I, or in 3 x 3 ml 10 mM MES. All samples were filtered and counted (sect. 2.7.2).

5.1.4.4: Results: Elution of adsorbed pectin with MES

For cells rinsed only in TOM-I medium (Fig. 5.1.4.4.i), the initial TOM-I rinses (rinses 1 - 3) eluted 68% of the [^{14}C]-pectin retrieved, leaving 32% associated with the cells. Further rinses with TOM-I medium (rinses 4 - 6) eluted 2.7%, leaving 30% of the [^{14}C]-pectin on the filters.

70% of the [^{14}C]-pectin retrieved from Treatment 2 (Fig. 5.1.4.4.ii) was eluted in the initial TOM-I rinses (rinses 1 - 3), leaving 30% with the cells. MES rinses (rinses 4 - 6) extracted a further 22% of the [^{14}C]-pectin retrieved, leaving 8.5% on the filters.

Low levels of [^{14}C]-pectin were eluted in the third TOM-I rinse; the first, second and third MES rinses eluted 2.5-, 6- and 13-fold more [^{14}C]-pectin than the amount extracted in the last TOM-I rinse.

5.1.4.5: Discussion: Elution of adsorbed pectin with MES

These results suggest that most of the [^{14}C]-pectin which filtered out with intact cells remained outside the cells for the entire duration of the incubation period, and that 10 mM MES was reasonably effective at eluting externally located [^{14}C]-pectin. Access to internalised [^{14}C]-pectin could be gained only if exposure to 10 mM MES induced membrane disruption and caused the cells to become leaky, however, the available evidence is to the contrary, since in a previous experiment (sect. 3.3.4.iii) suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in 10 mM MES for 3 h showed no effects consistent with loss of membrane integrity.

Importantly, the very low proportion of [^{14}C]-pectin eluted by TOM-I rinses 4 - 6 indicates that TOM-I does not elute pectin bound to the cell wall. In addition it suggests that the choice of 10 mM MES as an "inactive" bathing medium to elute cell contents for analysis was a poor one, and that further rinses in TOM-I might have yielded less misleading results. These results strongly suggest that the amounts of [^{14}C]-pectin previously eluted from ruptured cells by MES, which were attributed to internal sequestration, more probably originated from external sources vulnerable to the action of MES.

5.1.5: LOCALISATION OF EXOGENOUSLY APPLIED PECTIN IN SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* INCUBATED IN CENTRIFUGED AND NORMAL [^{14}C]-PECTIC MEDIA.

5.1.5.1: **Summary:** Centrifuged *vs.* normal [^{14}C]-pectic medium

The results from previous experiments (sects. 5.1.2 and 5.1.4) highlighted potential sources of error in the [^{14}C]-labelled polysaccharide binding assay. To determine whether undissolved, suspended, solid particles of [^{14}C]-pectin suspended in TOM-I medium were responsible for producing misleading results, a [^{14}C]-labelled polysaccharide binding assay was performed with cells of *L. esculentum* x *peruvianum* in normal [^{14}C]-pectic TOM-I medium and in [^{14}C]-pectic TOM-I medium from which undissolved particles of [^{14}C]-pectin had been removed by centrifugation.

A greater total amount of [^{14}C]-pectin was retrieved from samples incubated in normal [^{14}C]-pectic TOM-I, than from ones in centrifuged medium. However, rinses in TOM-I medium eluted similar amounts of [^{14}C]-pectin, in terms of actual cpm, from cells in both media, leaving 11% of the total [^{14}C]-pectin retrieved from centrifuged medium on the filters with the cells, and 32% of that retrieved from normal [^{14}C]-pectic medium.

These results suggested that undissolved, solid particles of [^{14}C]-pectin had contributed significantly to the amounts of [^{14}C]-pectin thought to be associated with the cells - up to 5 000 cpm excess per sample. Comparison with results from section 5.1.4.4 indicates that [^{14}C]-pectic particles which originally failed to enter solution in TOM-I medium remained insoluble when rinsed with TOM-I, but dissolved when rinsed with 10 mM MES.

5.1.5.2: **Introduction:** Centrifuged *vs.* normal [^{14}C]-pectic medium

It had been observed that freeze-dried [^{14}C]-pectin did not dissolve readily in TOM-I and other aqueous media, but tended to form a suspension rather than a solution. This may have been caused by the pectic molecules "egg-boxing" to each other, in which case the presence of Ca^{2+} and other divalent ions in the TOM-I medium would have contributed to the problem of insolubility by permitting further egg-boxing to take place, rather than encouraging the separation of polymers into solution. It had also been observed that the proportion of [^{14}C]-pectin initially supplied to suspension-cultured cells of *L. esculentum* x *peruvianum* which then appeared to become associated with them was consistently and suspiciously high for a putative oligosaccharin effect, always lying between 30 - 40%. There was concern that the larger [^{14}C]-pectin particles in suspension might centrifuge down with the cells during rinses and be retained on the filters with the solid matter, thus increasing the population of labelled pectic molecules currently interpreted as being in association with the cells. Consequently, the distributions of exogenously applied pectins

was compared for suspension-cultured cells of *L. esculentum* x *peruvianum* incubated in either the normal [^{14}C]-pectic TOM-I medium or centrifuged [^{14}C]-pectic medium free from [^{14}C]-pectic solids.

5.1.5.3: Materials and Methods: Centrifuged *vs.* normal [^{14}C]-pectic medium

A [^{14}C]-labelled polysaccharide binding assay was performed with 9 d old cells of *L. esculentum* x *peruvianum*. Only intact cells were sampled. The incubation media were normal [^{14}C]-pectic TOM-I and what was referred to as "centrifuged medium", which was the supernatant from normal [^{14}C]-pectic TOM-I which had been centrifuged to pellet out any suspended solid matter (sect. 2.7.4). The pectin concentration was known for the normal TOM-I medium [0.5 mg/ml], but it was not known for the centrifuged medium [<0.5 mg/ml]. However, by comparison of scintillation counts of 0.5 ml aliquots of media it was estimated that about ¾ of the pectin originally added to the TOM-I medium remained in solution after centrifugation, giving an actual concentration in the region of [0.375 mg/ml], and that the rest formed the pellet.

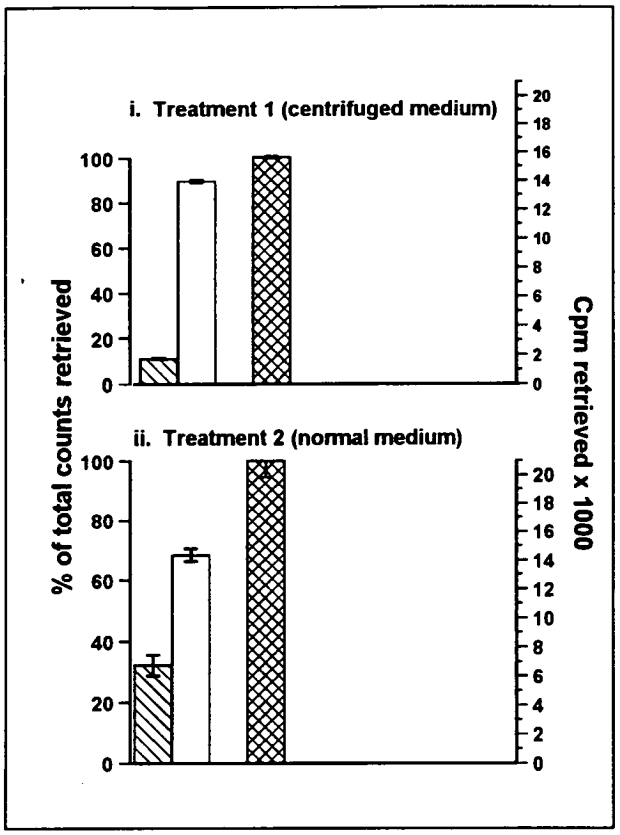
After 3 h incubation in the media the cells were rinsed in 3 x 3 ml TOM-I, then filtered and counted (sect. 2.7.2).

5.1.5.4: Results: Centrifuged *vs.* normal [^{14}C]-pectic medium

A greater number of counts were retrieved from cell samples incubated in normal [^{14}C]-pectic TOM-I medium (Fig. 5.1.5.4.ii), 21 123 cpm, than from centrifuged medium (Fig. 5.1.5.4.i), 15 637 cpm.

The quantity of [^{14}C]-labelled pectin retained on the filters with incubated with centrifuged pectic medium, 1 713 cpm, was considerably less than with normal, non-centrifuged pectin, 6 762 cpm, meaning that an excess of ~ 5 000 cpm was retained on the filters with cells incubated in normal medium. The relative proportions of [^{14}C]-pectin retained on the filters varied, partly as a result of the disparity between treatments of the total amount of [^{14}C]-pectin supplied to the cells and thus retrieved; 11% of the [^{14}C]-pectin retrieved from centrifuged medium was retained on the filters, and 32% from non-centrifuged medium.

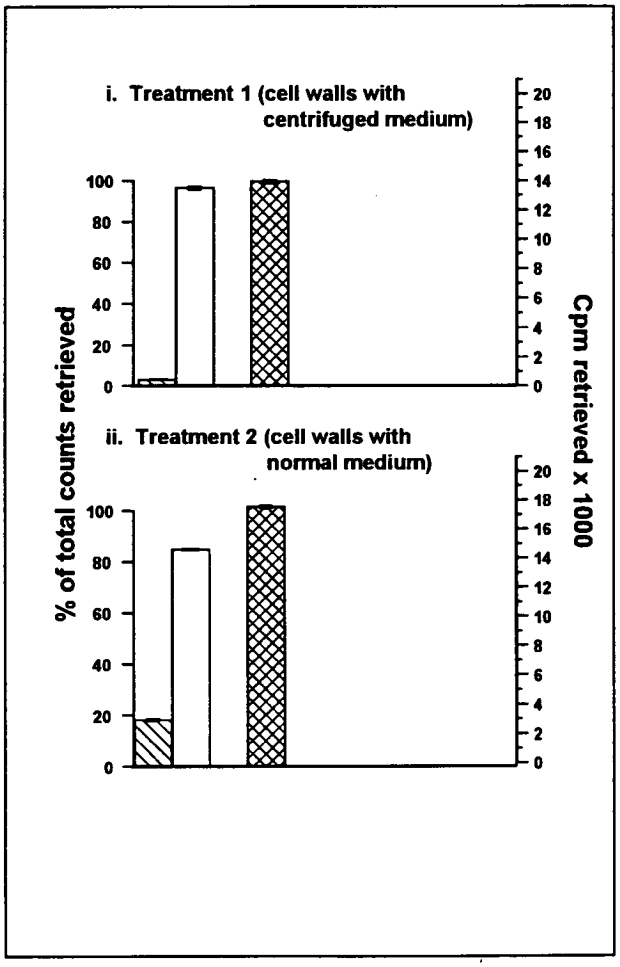
There were no major differences in the quantities of [^{14}C]-labelled pectin eluted in the TOM-I rinses; with 13 923 ± 100 cpm eluted from cells incubated with centrifuged [^{14}C]-pectic medium, and 14 361 ± 441 cpm eluted from those incubated in normal [^{14}C]-pectic TOM-I. However, proportionally speaking, TOM-I rinses eluted 89% of the [^{14}C]-pectin supplied to the cells in centrifuged medium, but only 68% of that in the normal medium (Figs. 5.1.5.4.i and ii).



Figures 5.1.5.4.i (Treatment 1), and ii (Treatment 2):

Distribution of exogenously applied [^{14}C]-pectin eluted from intact cells of *L. esculentum* x *peruvianum* incubated in centrifuged [^{14}C]-pectic TOM-I medium (Treatment 1) and normal [^{14}C]-pectic TOM-I medium (Treatment 2).

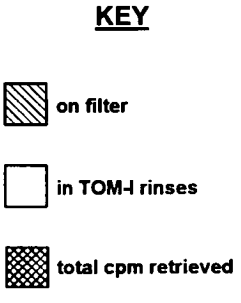
Means were obtained from 3 samples.



Figures 5.1.6.4.i (Treatment 1), and ii (Treatment 2):

Distribution of exogenously applied [^{14}C]-pectin eluted from deproteinated cell walls of *L. esculentum* x *peruvianum* incubated in centrifuged [^{14}C]-pectic TOM-I medium (Treatment 1) and normal [^{14}C]-pectic TOM-I medium (Treatment 2).

Means were obtained from 3 samples.



5.1.5.5: Discussion: Centrifuged *vs.* normal [^{14}C]-pectic medium

The results revealed a close correlation in the number of counts extracted in TOM-I rinses from cells in both media, 13 924 - 14 362 cpm, which suggests that a consistent amount of [^{14}C]-pectin dissolved in the TOM-I incubation medium, remained unassociated with the cells, and was extracted by TOM-I rinses.

Far fewer counts remained on the filters with cells which had been incubated in centrifuged medium, 1 714 cpm, than with cells incubated in non-centrifuged medium, 6 762 cpm. This difference indicated that solid pectic particles present in non-centrifuged medium were not eluted by TOM-I rinses, but were retained on the filters after centrifuging down with the cells. In this instance these contributed approximately 5 000 cpm to the quantity of [^{14}C]-pectin which would formerly have been interpreted as being in association with the cells. Therefore it would appear that the actual percentage of the [^{14}C]-pectin which does become associated with the cells is most probably in the region of 11% (centrifuged medium results) and not 32% as implied by the results obtained with non-centrifuged medium. This figure is closer to the concentration one might expect if any association between cells and polysaccharides were to produce biological effects.

Comparison of the amounts of [^{14}C]-pectin which remained in association with cells after incubation in centrifuged medium in this experiment, with those extracted by TOM-I rinses 4 - 6 and the equivalent MES rinses from non-centrifuged samples in experiment 5.1.4, indicate that rinses with TOM-I appear to extract only the pectic material already dissolved in the medium and do not solubilise *de novo*. However, rinsing with MES may increase the solubility of the excess undissolved, and unassociated, pectic particles and may also dislodge cell wall-bound pectin. The inability of TOM-I to dissolve further quantities of pectin is not particularly surprising given that the initial solubility problems were encountered while attempting to dissolve [^{14}C]-pectin in this medium. The comparative insolubility of [^{14}C]-pectin in TOM-I may be due to the presence of Ca^{2+} ions in the medium that encourage the aggregation of pectic polymers in the egg-box formation, which is less soluble than individual strands. In media which compete for Ca^{2+} and other divalent cations adoption of the egg-box formation is prevented and an increase in solubility ensues; MES appears to constitute such a solvent.

At this point it is possible to discuss the chromatograms from experiments 5.1.1.4.i. and ii critically. Experiments performed after these chromatograms were run indicated that solid particles of [^{14}C]-pectin which centrifuged down with the cells during assays must have affected these [^{14}C]-pectin profiles. This is particularly true of the MES samples which would contain both the part of the [^{14}C]-pectin population insoluble in native media, and the internalised [^{14}C]-pectin. Only a small percentage of [^{14}C]-pectin was internalised, so the profiles chiefly represent insoluble [^{14}C]-pectin. Interestingly, in addition to a peak at the

origin, both profiles exhibit small peaks at section 4, which may indicate two main pools of insoluble [^{14}C]-pectin, one highly polymeric and the other less so. Alternatively, the small peak may represent the internalised [^{14}C]-pectin, but this point cannot be clarified from the data available.

5.1.6: EXPERIMENT TO ASSESS DIFFERENCES IN DISTRIBUTION OF EXOGENOUSLY APPLIED PECTIN WHEN CELL WALLS OF *L. ESCULENTUM* X *PERUVIANUM* WERE INCUBATED IN CENTRIFUGED AND NORMAL [^{14}C]-PECTIC TOM-I MEDIA

5.1.6.1: Summary: Adsorption of pectin to cell walls

Some exogenous [^{14}C]-pectin will adsorb to the cell walls of suspension-cultured cells during incubation. This fraction may not be involved in any recognition processes or biological events since binding is to other cell wall polysaccharides and not to binding sites involved in signal receipt. A [^{14}C]-labelled polysaccharide binding assay was performed with deproteinated cell walls of *L. esculentum* x *peruvianum* as the substrate, using 10% of the normal mass present when cells are used in the assay, to quantify the amount of adsorption of [^{14}C]-pectin to the cell walls, and, by subtraction, to estimate the amount which becomes bound to signal receptors and/or internalised.

A smaller quantity of [^{14}C]-pectin remained on the filters with cell walls which had been incubated in centrifuged medium than with those incubated in normal [^{14}C]-pectic TOM-I medium both in terms of counts, 437.8 cpm and 2 935 cpm respectively, and percentages of the total [^{14}C]-pectin retrieved, 3.1% and 17% respectively. The level of binding of [^{14}C]-pectin to deproteinated cell walls appears to be <5%. The presence of undissolved [^{14}C]-pectic solids in the normal medium can inflate the estimate by more than 500%.

5.1.6.2: Introduction: Adsorption of pectin to cell walls

Earlier experiments had shown that the amounts of [^{14}C]-pectin which might be associated with suspension-cultured cells of *L. esculentum* x *peruvianum* were considerable (sects. 5.1.1; 5.1.2; 5.1.3 and 5.1.4), but that there was some doubt as to the accuracy with which these amounts and the cellular location of the [^{14}C]-pectin had been determined (sects. 5.1.2 and 5.1.5). When suspension-cultured cells are incubated with [^{14}C]-pectin a certain proportion will adsorb to the cell walls, a further proportion may be actively bound to the cell walls or plasmalemmas, probably via enzyme action, and a proportion may be internalised by the cells.

The following experiment was performed to clarify the percentage of [^{14}C]-pectin which adsorbs passively to the cell walls of suspension-cultured cells of *L. esculentum* x *peruvianum*. Deproteinated cell walls of *L. esculentum* x *peruvianum* were used as the substrate in order to exclude active surface binding and internal sequestration, both of

which are probably enzyme-mediated events, and to obtain a figure for passive binding alone. Cell walls usually constitute about 10% of the mass of the cell, therefore, in order to test a mass of cell walls similar to that naturally present in a standard 50 mg/ml sample of cells, 5 mg/ml of cell walls was added to the medium.

5.1.6.3: Materials and Methods: Adsorption of pectin to cell walls

A [^{14}C]-labelled polysaccharide binding assay was performed using deproteinated cell walls from suspension-cultured cells of *L. esculentum* x *peruvianum* [5 mg/0.5 ml TOM-l medium], prepared as described in sections 2.4.1 and 2.7.3, as the substrate. Samples were incubated in [^{14}C]-pectic TOM-l medium for 3 h on a rotary-tipping table at 18 °C, and rinsed only in 3 x 3 ml TOM-l medium prior to filtering (sect. 2.7.2).

5.1.6.4: Results: Adsorption of pectin to cell walls

A significantly greater amount of [^{14}C]-pectin was retrieved from cell wall samples incubated in normal TOM-l [^{14}C]-pectic medium (Fig. 5.1.6.4.ii), 17 520 cpm, than from centrifuged medium (Fig. 5.1.6.4.i), 13 933 cpm, indicating that approximately 3 500 cpm/0·5 ml had been lost in the pellet of solid pectin discarded after centrifuging.

Considerably less [^{14}C]-pectin was retained on filters with the cell walls after incubation in centrifuged medium, 438 cpm, than in normal TOM-l [^{14}C]-pectic medium, 2 935 cpm. These amounts equated to 3.1% and 17% respectively of the total [^{14}C]-pectin retrieved from each medium.

There was a small difference between the quantities of [^{14}C]-labelled pectin eluted in the TOM-l rinses; 13 494 ± 95 cpm were extracted from cell walls incubated in the centrifuged [^{14}C]-pectic medium, and 14 584 ± 20 cpm from those in the non-centrifuged pectic medium. The proportions of [^{14}C]-pectin eluted in TOM-l medium were 97% ± 0.1% from the centrifuged medium, and 83% ± 0.3% from the normal [^{14}C]-pectic TOM-l medium.

5.1.6.5: Discussion: Adsorption of pectin to cell walls

These results provide strong evidence that the level of adsorption of [^{14}C]-pectin to the deproteinated cell walls is comparatively low, in this case <5%, but that the presence of undissolved [^{14}C]-pectic solids inflates the estimate, in this case, by more than 500%.

Comparison of the amount of [^{14}C]-pectin associated with the deproteinated cell walls incubated in centrifuged medium in this experiment, and that associated with entire cells incubated in centrifuged medium in the previous one (Experiment 5.1.5, bearing in mind that these are not directly comparable having been performed on separate days and on different substrates), indicates that 3.1% of the total [^{14}C]-pectin applied bound passively to deproteinated cell walls, while 11% was associated with entire cells. Thus if the figure for passive cell-wall adsorption determined in this experiment (3.1%) holds fast when applied

to whole cells, and is subtracted from the total associated with whole cells, it appears that approximately 8% of the [^{14}C]-pectin initially available to the cells may bind to the plasmalemmas or be internalised.

5.1.7: CAPACITY OF EGTA AND MES TO ELUTE SURFACE-BOUND PECTIN FROM THE CELL WALL OF INTACT CELLS.

5.1.7.1: **Summary:** Elution of pectin with EGTA or MES

A proportion of [^{14}C]-pectin applied to suspension-cultured cells will adsorb to the cell surface by ionic interactions with Ca^{2+}, and other divalent ions. In order to quantify the degree of ionic bonding between [^{14}C]-pectin and cells of *L. esculentum* x *peruvianum*, and to compare the dissociating abilities of EGTA and 10 mM MES solutions towards such bonds, a [^{14}C]-labelled polysaccharide binding assay was performed with cells of *L. esculentum* x *peruvianum* incubated in centrifuged and normal [^{14}C]-pectic TOM-I medium. Rinses with 2 mM EGTA or 10 mM MES followed the initial TOM-I rinses, only intact cells were sampled.

The most accurate assessment of the degree of ionic bonding was obtained from cells incubated in centrifuged medium and rinsed with 2 mM EGTA, where 11% of the [^{14}C]-pectin was eluted in EGTA and appeared to be ionically-bound, 3.9% was retained on the filters and interpreted as having been internalised or actively bound to the cells.

EGTA rinses appeared to elute all ionically bound and undissolved [^{14}C]-pectin from cells incubated in normal, non-centrifuged [^{14}C]-pectic TOM-I medium; 3.5% of the [^{14}C]-pectin retrieved appeared to have been internalised and/or actively bound. This was directly comparable, in terms of actual cpm, to the corresponding amount determined for cells incubated in centrifuged medium.

MES was less effective at eluting [^{14}C]-pectin than EGTA, regardless of the incubation medium. The absence of thorough elution by MES prevented a further estimation of the degree of ionically bound [^{14}C]-pectin.

5.1.7.2: **Introduction:** Elution of pectin with EGTA or MES

Pectin is thought to exist in dimer or gel form in the presence of Ca^{2+} ions, when parallel strands of polymeric pectic residues are held together by the Ca^{2+} ions interposed between them, which permit the adoption of the "egg-box" formation, (Jarvis, 1984). The ability of EGTA to chelate metal ions, including Group 2 ions such as Ca^{2+}, was exploited here for two purposes. Firstly, the rinsing of cells, incubated in [^{14}C]-labelled pectin, with 2 mM EGTA would elute [^{14}C]-pectin which had become adsorbed to the cell surfaces by ionic bonds involving divalent. The elution of this pectin would permit quantification of the

degree of ionic binding of pectin to the cell surface. Secondly, the eluting properties of EGTA and MES solutions were to be compared.

5.1.7.3: Materials and Methods: Elution of pectin with EGTA or MES

This experiment was performed entirely upon whole cells, to avoid contamination with [^{14}C]-pectin from internal sources. Both centrifuged and normal, non-centrifuged [^{14}C]-pectic TOM-I media were used in parallel treatments. In all treatments cells of *L. esculentum* x *peruvianum* were incubated in centrifuged (Treatments 1 and 2) or non-centrifuged (Treatments 3 and 4) [^{14}C]-pectic TOM-I medium and rinsed with TOM-I medium (sect. 2.7.2). Thereafter the treatments diverged as shown below:

Treatment 1 (Centrifuged [^{14}C]-pectic TOM-I): 3 x 3 ml rinses in 2 mM EGTA
Treatment 2 (Centrifuged [^{14}C]-pectic TOM-I): 3 x 3 ml rinses in 10 mM MES
Treatment 3 (Normal [^{14}C]-pectic TOM-I): 3 x 3 ml rinses in 2 mM EGTA
Treatment 4 (Normal [^{14}C]-pectic TOM-I): 3 x 3 ml rinses in 10 mM MES

All cells were then filtered and scintillation-counted (sect. 2.7.2).

5.1.7.4: Results: Elution of pectin with EGTA or MES

In Treatment 1 (Fig. 5.1.7.4.i), TOM-I rinses eluted 85% of the [^{14}C]-pectin retrieved, and EGTA rinses eluted 11%, leaving 3.9% with the cells on the filter. The total quantity of [^{14}C]-pectin taken as being in association with the cells was the amount on the filter plus that eluted in EGTA rinses, which for Treatment 1 was 15%.

The quantity of [^{14}C]-pectin eluted in the third TOM-I rinse was fairly low. The amount eluted by the first EGTA rinse, was also low, but greater. The second EGTA rinse eluted most of the [^{14}C]-pectin eluted in EGTA rinses, and a moderately low count was detected in the third rinse.

In Treatment 2 (Fig. 5.1.7.4.ii) TOM-I rinses eluted 87% of the centrifuged [^{14}C]-pectin retrieved, and MES rinses eluted 7.1%, leaving 6.4% with the cells on the filter. The total quantity of [^{14}C]-pectin in association with the cells (filter + that in MES rinses) amounted to 14%.

A similar extraction pattern to that mentioned above for Treatment 1 was observed with respect to the MES rinses in Treatment 2, i.e. that the second rinse contained the highest levels of [^{14}C]-pectin. The differences in the quantities of [^{14}C]-pectin detected in each rinse were less than when EGTA was used, e.g. with MES the second rinse eluted five-fold more than the amount in the final TOM-I rinse, whereas EGTA eluted nine-fold more.

The distributions of radioactivity observed in Treatments 3 and 4, when cells were incubated in normal, non-centrifuged [^{14}C]-pectic TOM-I medium, were quite different to

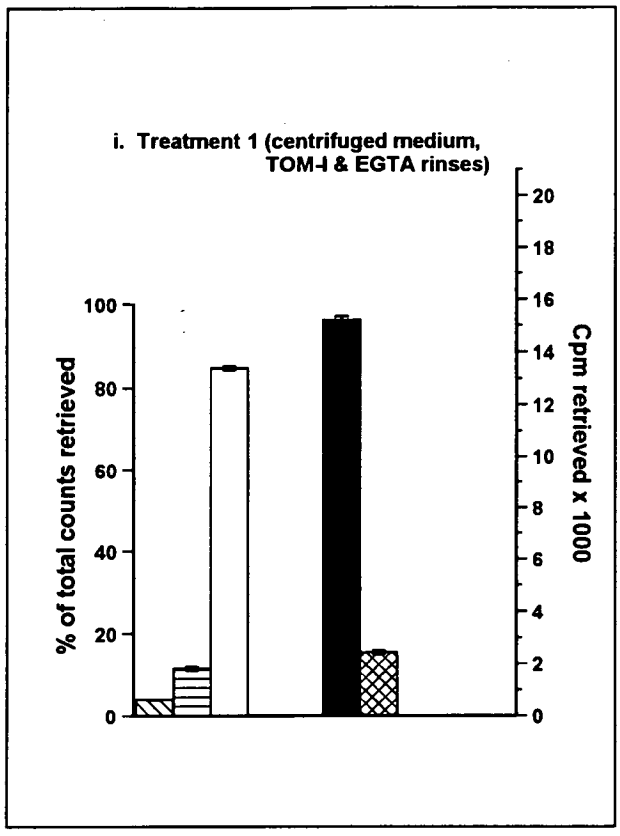


Figure 5.1.7.4.i, Treatment 1:

Distribution of [^{14}C]-pectin eluted from intact cells of *L. esculentum* x *peruvianum*, incubated in centrifuged [^{14}C]-pectic TOM-I medium, in rinses of TOM-I medium and EGTA.

Means were obtained from 3 samples.

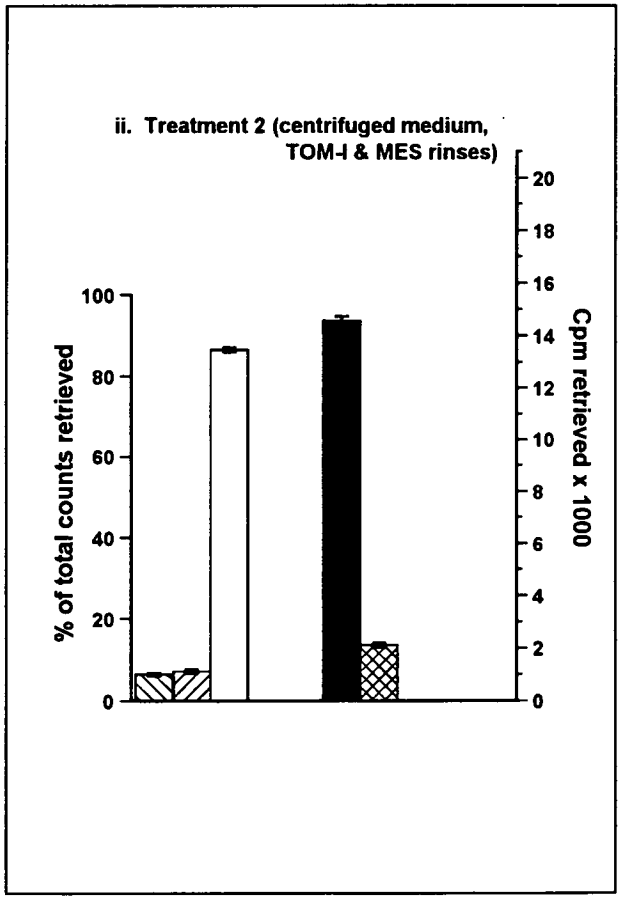


Figure 5.1.7.4.ii, Treatment 2:

Distribution of [^{14}C]-pectin eluted from intact cells of *L. esculentum* x *peruvianum*, incubated in centrifuged [^{14}C]-pectic TOM-I medium, in rinses of TOM-I and MES.

Means were obtained from 3 samples.

KEY

- on filter
- in EGTA rinses
- in MES rinses
- in TOM-I rinses
- total eluted in all rinses
- total associated with cells (filters + EGTA or MES)

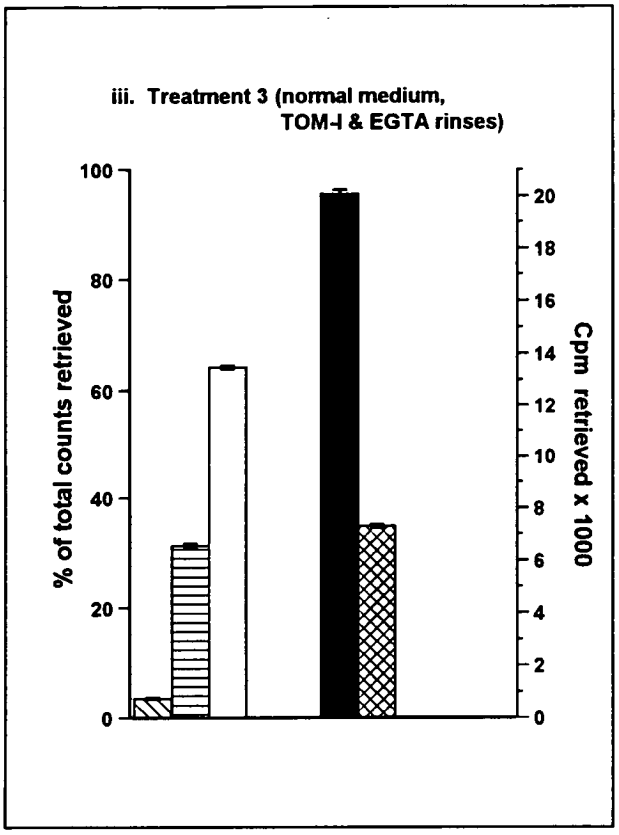


Figure 5.1.7.4.iii, Treatment 3:

Distribution of [^{14}C]-pectin eluted from intact cells of *L. esculentum* x *peruvianum*, incubated in normal [^{14}C]-pectic TOM-I medium, in rinses of TOM-I medium and EGTA.

Means were obtained from 3 samples.

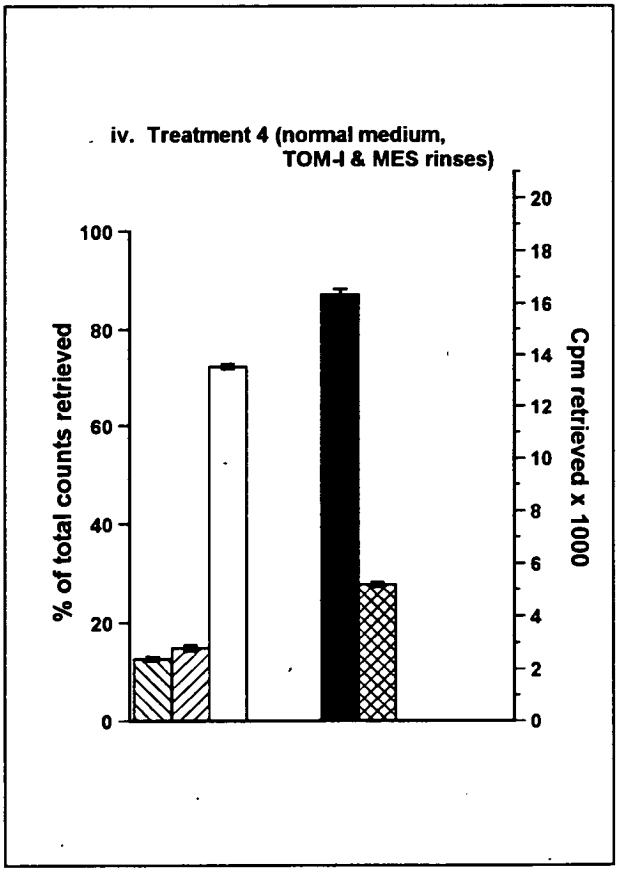
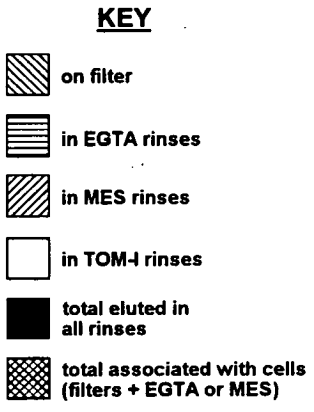


Figure 5.1.7.4.iv, Treatment 4:

Distribution of [^{14}C]-pectin eluted from intact cells of *L. esculentum* x *peruvianum*, supplied with normal [^{14}C]-pectic TOM-I medium, in rinses of TOM-I and MES.

Means were obtained from 3 samples.



those seen in Treatments 1 and 2. In Treatment 3 (Fig. 5.1.7.4.iii), 64% of the [^{14}C]-pectin retrieved was eluted in TOM-I rinses; a further 31% was eluted in EGTA rinses, leaving 3.5% on the filters with the cells. The potential total activity in association with the cells, i.e. EGTA rinses plus counts on the filters, was 35%.

For Treatment 4 (Fig. 5.1.7.4.iv), 72% of the [^{14}C]-pectin retrieved was eluted in TOM-I rinses. Rinses with MES eluted 15%, leaving 13% to filter out with the cells. The potential total activity in association with the cells, i.e. MES rinses plus counts on the filters, was 28%.

The extraction pattern with the EGTA and MES rinses varied from that seen in Treatments 1 and 2 for Treatments 3 and 4; the number of counts extracted in the first rinse of the new washing medium were consistently low, thereafter the counts rose 11-fold for EGTA, with levels remaining at the same level for the third rinse, and five-fold for MES, where levels continued to rise by a factor of 1.5 in the third rinse.

5.1.7.5: Discussion: Elution of pectin with EGTA or MES

The results from Treatment 1, in which cells were incubated in centrifuged [^{14}C]-pectic medium and rinsed with 2 mM EGTA, indicated that a total of 15% of the [^{14}C]-pectin became associated with the cells. EGTA rinses eluted 11% of the [^{14}C]-pectin retrieved, leaving 3.9% on the filters with the cells. Therefore, 75% of the [^{14}C]-pectin which associates with the cells seems to bind ionically to the cell surface and can be eluted once divalent cations have been chelated by EGTA.

3.9% of the [^{14}C]-pectin retrieved, consists of both non-ionically surface-bound [^{14}C]-pectin and internalised [^{14}C]-pectin. This equated to 25% of the [^{14}C]-pectin *associated* with the cells. This 3.9% is less than the 8% which was predicted from the results of the previous two experiments (sects. 5.1.5 and 5.1.4). This may be because deproteinated cell walls do not provide a sufficiently realistic model of the cell walls of living cells, and provide a false figure for the amount of [^{14}C]-pectin which binds ionically to cell walls. The reasons for this are not known, but it is possible the disparity may be due to differences induced during the deproteination process which includes prolonged rinsing in phenol/acetic acid/water in which the acetic acid may extract Ca^{2+} ions from the cell walls and thus reduce the number of potential binding sites for the [^{14}C]-pectin. This comparative paucity of binding sites would lead to an underestimation of the quantity of [^{14}C]-pectin that binds ionically to the cell walls *in vivo*, and a corresponding overestimation of the quantities of actively bound or internalised pectin.

The results from Treatment 2, in which cells were incubated in centrifuged pectic medium and rinsed with MES, indicated that 14% of the [^{14}C]-pectin retrieved was associated with the cells. MES rinses eluted 7.1%, and 6.4% filtered out with the cells. Therefore, 10 mM

MES appears to be considerably less effective at eluting ionically-bound [^{14}C]-pectin from cell surfaces than 2 mM EGTA.

In Treatment 3, in which normal [^{14}C]-pectic TOM-I was used, the results implied that 35% of the [^{14}C]-pectin remained in association with the cells; 31% was apparently externally and ionically bound to the cell walls and was eluted in EGTA rinses, while 3.5% was interpreted as having being internalised or actively bound to the cell walls.

The proportion of [^{14}C]-pectin *associated* with the cells which was elutable in EGTA was greater in Treatment 3 (normal [^{14}C]-pectic medium), 90%, than in Treatment 1 (centrifuged [^{14}C]-pectic medium), 75%. This increase in elutability was presumably due to the increased solubility conferred upon suspended solids of [^{14}C]-pectin in Treatment 3 once extraction of Ca^{2+} ions prevented adoption of the egg-box formation. However the mean number of counts remaining in association with the cells in Treatments 1 and 3 after rinses in EGTA were 614 cpm and 731 cpm respectively; a slight statistical difference exists between these figures, but given the dissimilarities between treatments due to the centrifuged and normal [^{14}C]-pectic incubation media, they were much closer than expected and suggest that the amounts of [^{14}C]-pectin which become enzymically bound to the cell walls, or internalised by cells, remain fairly constant, despite differences in the incubation media.

In Treatment 4, in which cells were incubated in normal [^{14}C]-pectic TOM-I medium and rinsed with MES, 13% of the [^{14}C]-pectin remained associated with the cells, while MES rinses eluted 15% of that retrieved. The ratio of *associated* MES-elutable:non-MES-elutable [^{14}C]-pectin in Treatment 4 was directly comparable with that from Treatment 2.

Comparison of figures obtained with EGTA and MES rinses (not presented here) suggest that either some of the undissolved [^{14}C]-pectin particles failed to enter solution in MES, or that not all the ionically-bound [^{14}C]-pectin was eluted in these rinses. This indicates that 10 mM MES is far less efficient at eluting undissolved and/or ionically-bound [^{14}C]-pectin from the cell walls of suspension-cultured cells of *L. esculentum* x *peruvianum* than 2 mM EGTA. This difference may be attributable to the extraction co-efficient of a 10 mM MES solution, since the percentage of the [^{14}C]-pectin *associated* with the cells which was MES-extractable in Treatments 2 and 4 consistently lay at 53 - 54% despite use of centrifuged and normal [^{14}C]-pectic media. The non-chelating nature of MES may also have contributed to this effect.

However, the data on rinsing patterns suggests that with centrifuged medium there was a limit to the percentage of [^{14}C]-pectin which MES rinses could elute, since the second rinse exhibited the highest concentration of [^{14}C]-pectin of the three applied. With normal [^{14}C]-pectic TOM-I medium, the amounts of [^{14}C]-pectin eluted in MES rinses continued to

increase, suggesting that all undissolved and/or ionically bound [^{14}C]-pectin might have been eluted had enough rinses been applied.

5.1.8: EXPERIMENT TO DETERMINE THE EFFECTS OF PRE-WASHING AND POST-WASHING IN EGTA ON THE ADSORPTION OF [^{14}C]-PECTIN TO CELL WALLS

5.1.8.1: Summary: Cell walls pre- and post-washed with EGTA

An experiment was performed to determine whether the observed low level of ionic binding of [^{14}C]-pectin to deproteinated cell walls of *L. esculentum* x *peruvianum* (sect. 5.1.6), <5%, was due to pre-occupation of the available binding sites by unlabelled pectin. Cell walls were pre-washed in either 2 mM EGTA, to elute pre-bound pectin and free binding sites, or water, prior to incubation in [^{14}C]-pectin TOM-I. Rinses in TOM-I medium were followed by further rinses in either TOM-I medium or 2 mM EGTA.

Cell walls pre-washed in EGTA retained 3.8% of the total [^{14}C]-pectin retrieved when post-washed in TOM-I, and 4.3% when post-washed in EGTA. Cell walls pre-washed in water retained 4.4% of the total [^{14}C]-pectin applied when post-washed in TOM-I, and 4.3% when post-washed in EGTA. Pre-washing with EGTA did not appear to increase the binding capacity of the cell walls for [^{14}C]-pectin, possibly because the [^{14}C]-pectin incubation medium did not contain a high enough concentration of divalent cations to replace those chelated during the pre-wash, or the pre-wash eluted too great a proportion of the surface pectins to which the [^{14}C]-pectin would egg-box.

5.1.8.2: Introduction: Cell walls pre- and post-washed with EGTA

A previous experiment with centrifuged [^{14}C]-pectic TOM-I medium (sect. 5.1.6) indicated that around 3.1% of [^{14}C]-pectin retrieved bound ionically to deproteinated cell walls. However, experiments performed on whole cells with centrifuged pectin (sects. 5.1.5. and 5.1.7) indicated that 11 - 15% remained in association with the cells, most of which was ionically bound to the cell walls and could be eluted in 2 mM EGTA. In order to determine whether the low level of ionic binding observed for deproteinated cell walls was due to a limit in the number of binding sites, or whether a proportion of the available sites had previously been filled by unlabelled pectin the following experiment was performed in which cell walls were pre-washed with EGTA to ensure clearance of binding sites already occupied by unlabelled pectin, and to permit an increased level of [^{14}C]-pectin binding. Cell walls were also post-washed with EGTA after incubation with [^{14}C]-pectin to obtain additional information concerning the amount of ionically-bound pectin.

5.1.8.3: Materials and Methods: Cell walls pre- and post-washed with EGTA

This experiment was performed on deproteinated cell walls of *L. esculentum* x *peruvianum* prepared as described in sections 2.4.1 and 2.7.3. The mass of cell walls used in each sample, 5 mg, was the approximate mass of cell walls present in the 50 mg standard sample of cells. Centrifuged [^{14}C]-pectic TOM-I medium was used throughout, and the tubes were agitated on a rotary tipping table at 18 °C.

Cell walls were pre-washed for 1 h in 3 ml of either 2 mM EGTA or distilled H_2O. All samples were rinsed in 3 x 3 ml of distilled H_2O, and incubated in 0.5 ml centrifuged [^{14}C]-pectic TOM-I medium. After incubation cell walls were rinsed in TOM-I medium (sect. 2.7.2). Three further rinses, 3 x 3 ml, with either TOM-I medium or 2 mM EGTA were applied, then the cell walls were filtered and counted (sect. 2.7.2). A summary of the individual treatments is presented below.

EGTA pre-washed cell walls (Treatments 1 + 2): 1 h wash in 2 mM EGTA,
3 x 3 ml rinses in H_2O,
3 h incubation in [^{14}C] medium,
3 x 3 ml rinses in TOM-I,
Treatment 1: 3 x 3 ml rinses in TOM-I.
Treatment 2: 3 x 3 ml rinses in 2 mM EGTA.

H_2O pre-washed cell walls (Treatments 3 + 4): 1 h wash in distilled H_2O
3 x 3 ml rinses in H_2O,
3 h incubation in [^{14}C] medium,
3 x 3 ml rinses in TOM-I,
Treatment 3: 3 x 3 ml rinses in 2 mM EGTA.
Treatment 4: 3 x 3 ml rinses in TOM-I.

5.1.8.4: Results: Cell walls pre- and post-washed with EGTA

Although percentage values are usually presented with a minimal number of decimal places, in this experiment additional places are detailed to clarify the calculations. For Treatment 1 (Fig. 5.1.8.4.i), in which cell walls were pre-washed in EGTA, TOM-I rinses 1 - 3 eluted 94.5% of the total [^{14}C]-pectin. A further 1.7% was eluted from the cell walls in TOM-I rinses 4 - 6. The filtered cell walls retained an activity of 576 cpm, i.e. 3.8% of the [^{14}C]-pectin retrieved.

For Treatment 2 (Fig. 5.1.8.4.ii), in which cell walls were pre- and post-washed with EGTA, 94% of the [^{14}C]-pectin was eluted in the TOM-I rinses, with a further 3.1% eluted in the EGTA rinses. 2.9% of the [^{14}C]-pectin retrieved remained on the filters (424 cpm) with the

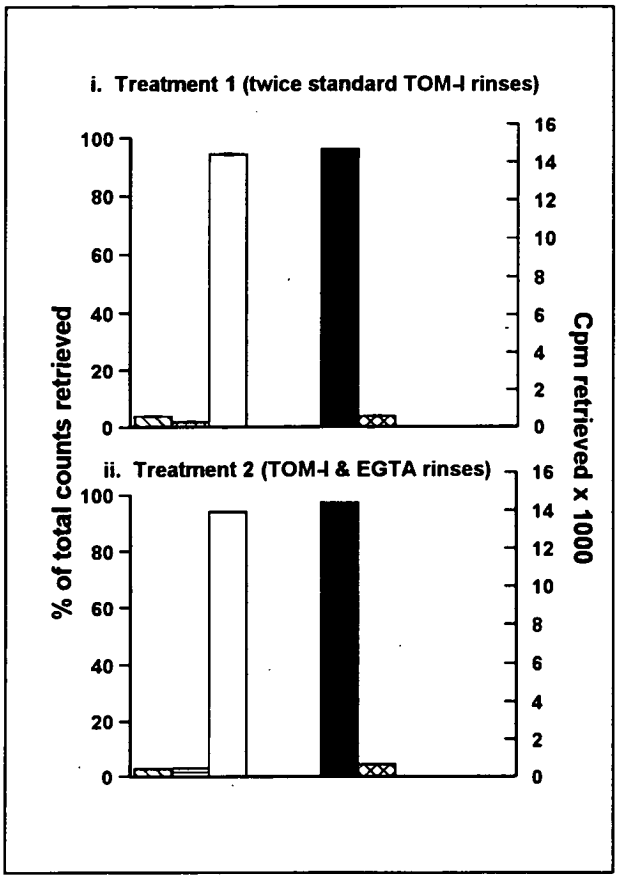


Figure 5.1.8.4.i, Treatment 1:

Distribution of [^{14}C]-pectin eluted from cell walls of *L. esculentum* x *peruvianum*, pre-washed in EGTA, in six rinses of TOM-I medium.

Figure 5.1.8.4.ii, Treatment 2:

Distribution of [^{14}C]-pectin eluted from cell walls of *L. esculentum* x *peruvianum*, pre-washed in EGTA, in rinses of TOM-I and EGTA.

Figure 5.1.8.4.iii, Treatment 3:

Distribution of [^{14}C]-pectin eluted from cell walls of *L. esculentum* x *peruvianum*, pre-washed in water, in six rinses of TOM-I medium.

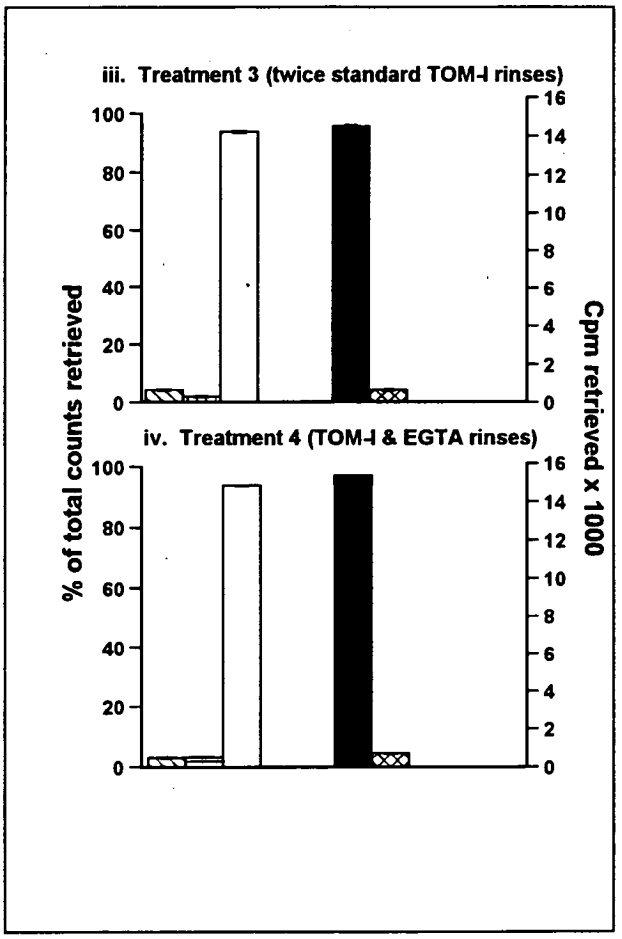
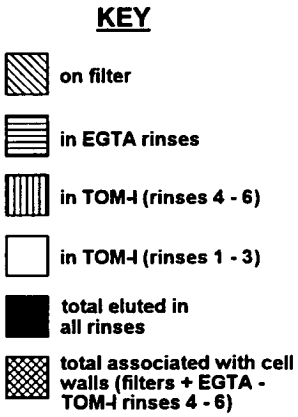


Figure 5.1.8.4.iv, Treatment 4:

Distribution of [^{14}C]-pectin eluted from cell walls of *L. esculentum* x *peruvianum*, pre-washed in water, in rinses of TOM-I and EGTA.

Means were obtained from 3 samples, centrifuged [^{14}C]-pectin TOM-I medium was used throughout.



cell walls. The total activity associated with the cell walls would be ~ 4.3% (filters + EGTA rinses - % eluted by TOM-I rinses 4 - 6 in Treatment 1) of the total [^{14}C]-pectin applied.

The difference in the amounts of [^{14}C]-labelled pectin extracted in the first 3 TOM-I rinses of Treatments 1 and 2 were statistically significant, but this must be attributed to biological variation or experimental error since there were no differences in protocol up to this point. EGTA rinses eluted approximately twice as much [^{14}C]-pectin from the cell walls, than TOM-I rinses 4 - 6.

Treatments 3 and 4 showed similar distributions of radioactivity to those exhibited by Treatments 1 and 2, despite the absence of the EGTA pre-wash. In Treatment 3 (Fig. 5.1.8.4.iii) 93.7% of the total [^{14}C]-pectin was eluted in TOM-I rinses 1 - 3, an additional 3.3% was eluted in the EGTA rinses, leaving 3% on the filters with the cell walls. The total [^{14}C]-pectin in association with the cell walls was ~ 4.4% (filters + EGTA rinses - % extracted by TOM-I rinses 4 - 6 in Treatment 4).

The cell walls in Treatment 4 (Fig. 5.1.8.4.iv) were not washed or rinsed in EGTA at any point. The initial TOM-I rinses eluted 93.8% of the total [^{14}C]-pectin applied, while TOM-I rinses 4 - 6 eluted an additional 1.9%, leaving 4.3% on the filters with the cell walls.

There was no notable difference in the amounts of [^{14}C]-labelled pectin extracted in rinses 1 - 3 of TOM-I for Treatments 3 and 4. Again, EGTA rinses extracted approximately twice as much [^{14}C]-pectin as TOM-I rinses 4 - 6.

5.1.8.5: Discussion: Cell walls pre- and post-washed with EGTA

The results from this experiment indicate that pre-washing deproteinated cell walls in 2 mM EGTA to free un-labelled pectin from putative binding sites may have decreased the amount of [^{14}C]-pectin which could bind ionically to the cell walls. This may have been due to an overall reduction in the number of available binding sites after chelation of the divalent cations and elution of the associated unlabelled pectin. The situation might have been exacerbated by an insufficient concentration of appropriate divalent cations in the [^{14}C]-pectin incubation medium to facilitate maximal ionic bonding. Certainly ionic-bonding did not increase after pre-washing with EGTA.

This experiment provided no evidence concerning the pre-occupation of binding sites by unlabelled pectin, however it is possible that pre-occupation is much reduced in deproteinated cell walls in comparison to living cell walls as, in addition to the potential extraction of Ca^{2+} binding sites during the preparation procedures. Redgwell, Melton and Brasch, (1992) suggest that low molecular weight polysaccharides, including pectins to which [^{14}C]-pectin might egg-box, can be leached from cell walls during deproteination washes in phenol/acetic acid/water.

Post-washing with 2 mM EGTA dislodged a greater quantity of [^{14}C]-pectin than further rinses with TOM-I medium, however, it is possible, since counts seem to remain in association with the cell walls on the filters, that rinses with EGTA are not 100% effective in disengaging ionically bound [^{14}C]-pectin from the cell walls. Alternatively, any [^{14}C]-pectin remaining attached to the cell walls may be held there by hydrogen- or covalent-bonds mentioned previously (sect. 5.1.2.2).

Furthermore, a certain number of counts extracted in EGTA may have been dislodged from the walls of the sample tubes to which the [^{14}C]-pectin may have adsorbed (see sect. 3.5.4.b). Release of this small quantity of radioactivity into EGTA rinses could make a proportionally large and important alteration to the counts interpreted here.

5.2: THE FATE OF EXOGENOUSLY APPLIED [^{14}C]-HEMICELLULOSE

5.2.0: GENERAL INTRODUCTION TO HEMICELLULOSE

The [^{14}C]-labelled *L. esculentum* x *peruvianum* hemicellulose preparation dissolved readily in aqueous media, therefore problems similar to those caused by partially dissolved [^{14}C]-pectin in previous experiments (sect. 5.1) were not encountered. The [^{14}C]-hemicellulose preparation was less densely labelled than the [^{14}C]-pectic preparation used in previous experiments, 0.5 ml [^{14}C]-hemicellulosic TOM-I registered approximately 1 400 cpm compared to ~ 16 000 cpm for 0.5 ml centrifuged, and ~ 22 000 cpm for 0.5 ml normal [^{14}C]-pectic TOM-I.

Since hemicellulosic polymers hydrogen-bond to cellulose microfibrils in the cell wall matrix, and the majority of pectic polymers bond ionically through divalent cations, principally Ca^{2+}, to other pectin molecules in the cell wall, it was unlikely that 10 mM MES would detach [^{14}C]-hemicellulose from cell walls to the same extent as it had [^{14}C]-pectin (sect. 5.1.7), especially since effective extraction of hemicellulose from cell walls can generally only be achieved by prolonged treatment with cold alkali (Kato and Matsuda, 1976; Edelmann and Fry, 1992). Therefore, 10 mM MES was held to be a suitable rinsing solution with which to elute [^{14}C]-hemicellulose in the soluble fraction of partitioned cells.

5.2.1: LOCALISATION OF EXOGENOUS [^{14}C]-HEMICELLULOSE APPLIED TO SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC AT pH 5.7

5.2.1.1: **Summary:** Localisation of exogenous hemicellulose at pH 5.7

To gain information about the location of hemicelluloses incubated with suspension-cultured cells in earlier experiments (sect. 4.4), [^{14}C]-labelled polysaccharide binding

assays were performed with [^{14}C]-hemicellulose on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC. Samples of unused [^{14}C]-hemicellulosic media, native media rinses and MES rinses were run on a chromatogram to compare the profiles of the hemicellulose molecules in each.

Results from the assays showed that between 9.3 - 11% of the total [^{14}C]-hemicellulose applied to cells of *L. esculentum* x *peruvianum*, and 14% applied to cells of *L. esculentum* AC became associated with the cells. The percentages, and actual counts, of externally bound [^{14}C]-hemicellulose were very similar for both cell types, 6.8% and 6.3% for *L. esculentum* x *peruvianum* and *L. esculentum* AC respectively, suggesting that external attachment occurred by hydrogen-bonding and was limited by the surface area of the cells. Considerable differences in the amounts and proportions of internalised [^{14}C]-hemicellulose were evident: cells of *L. esculentum* x *peruvianum* internalised 2.6% of the total [^{14}C]-hemicellulose retrieved, while cells of *L. esculentum* AC internalised 8%.

Chromatography indicated that a large proportion of the [^{14}C]-hemicellulose population consisted of highly polymeric material. A considerable proportion of the material internalised by cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC, 53% and 78% accordingly, did appear to be highly polymeric, however, levels of internalised [^{14}C]-hemicellulose were low for both cell types. It was considered possible that the highly polymeric material had been internalised by means of reptation and endocytosis.

5.2.1.2: Introduction: Localisation of exogenous hemicellulose at pH 5.7

A general introduction to the information which it was hoped would be gained from these experiments with [^{14}C]-labelled polysaccharides is given in section 5.0. The results obtained with hemicelluloses in the L-[U-^{14}C] leucine incorporation assays had been extremely consistent, and it was hoped that [^{14}C]-labelled polysaccharide binding assays would provide further information concerning the fate of applied hemicelluloses, in particular, whether any differences existed in the extents to which hemicellulosic oligomers became attached to, and/or internalised by, cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC at pH 5.7 and pH 4.5.

Results from the L-[U-^{14}C] leucine incorporation assays with cells of *L. esculentum* x *peruvianum* had suggested that at pH 5.7 hemicelluloses exerted a slightly inhibitory effect upon the protein metabolism, the effect being more pronounced in cells incubated in ½ d TOM-I medium than in 10 mM MES. Importantly, viability tests showed that incubation with hemicelluloses at pH 5.7 did not have an adverse affect upon the cells.

5.2.1.3: Materials and Methods: Localisation of hemicellulose at pH 5.7

[^{14}C]-labelled polysaccharide binding assays were performed with [^{14}C]-hemicellulose on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC. The [^{14}C]-hemicellulose was extracted from the residue of cell walls of *L. esculentum* x *peruvianum* left after the extraction of [^{14}C]-pectin (sect. 2.4.3). [^{14}C]-hemicellulose was added to fresh native media, TOM-I or SH, at 0.5 mg/ml. Samples of both intact and fractionated cells were taken from cells of *L. esculentum* x *peruvianum*, but fractionated cells only were sampled from *L. esculentum* AC. All incubations and processing were as described for the [^{14}C]-labelled polysaccharide binding assay (sect. 2.7.2).

A 0.5 ml aliquot of the [^{14}C]-hemicellulosic TOM-I medium was scintillation counted to provide an independent assessment of the total number of cpm supplied to the cells, which could then be compared with the number of counts retrieved; in this instance 0.5 ml [^{14}C]-hemicellulosic TOM-I contained 1 409 cpm.

Chromatograms of original incubation media ([^{14}C]-hemicellulosic TOM-I or SH), first rinses in fresh native medium (TOM-I or SH), and first rinses with MES were run as described for experiments in section 5.1.1. As before, samples of initial rinses in TOM-I and MES were taken from the same original sample tube of fractionated cells to provide consistency in the mixture of hemicellulosic compounds originally supplied to the cells.

5.2.1.4: Results: Localisation of exogenous hemicellulose at pH 5.7

5.2.1.4.i: Localisation of exogenously applied [^{14}C]-hemicellulose after 3 h incubation with suspension-cultured cells of *L. esculentum* x *peruvianum* at pH 5.7

5.2.1.4.i.a: [^{14}C]-labelled polysaccharide binding assay

89% and 91% of the [^{14}C]-hemicellulose retrieved was eluted from intact and fractionated cells respectively in the TOM-I rinses (Fig. 5.2.1.4.i.a). With intact cells 11% was retained on the filters. With fractionated cells 2.6% was eluted in post-rupture MES rinses leaving 6.8% on the filters with the solid matter.

The total number of counts retrieved from sample tubes was consistent between tubes of both whole and fractionated cells, and was slightly less than the total of 1 409 cpm measured in 0.5 ml [^{14}C]-hemicellulosic TOM-I medium.

5.2.1.4.i.b: Chromatography

The mobility of polysaccharides in this system is inversely proportional to their degree of polymerisation. The spectrum of differently-sized polymers present in the unused [^{14}C]-hemicellulosic TOM-I medium originally supplied to the cells is shown in Fig. 5.2.1.4.i.b.i. The majority of the radioactivity, 80%, remained at the origin (section 1), which is where the

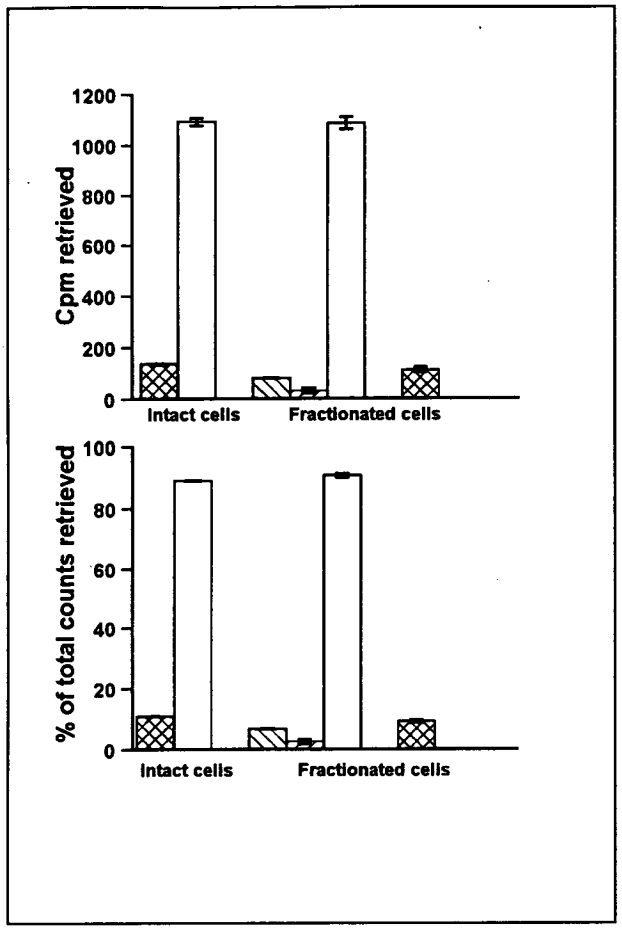


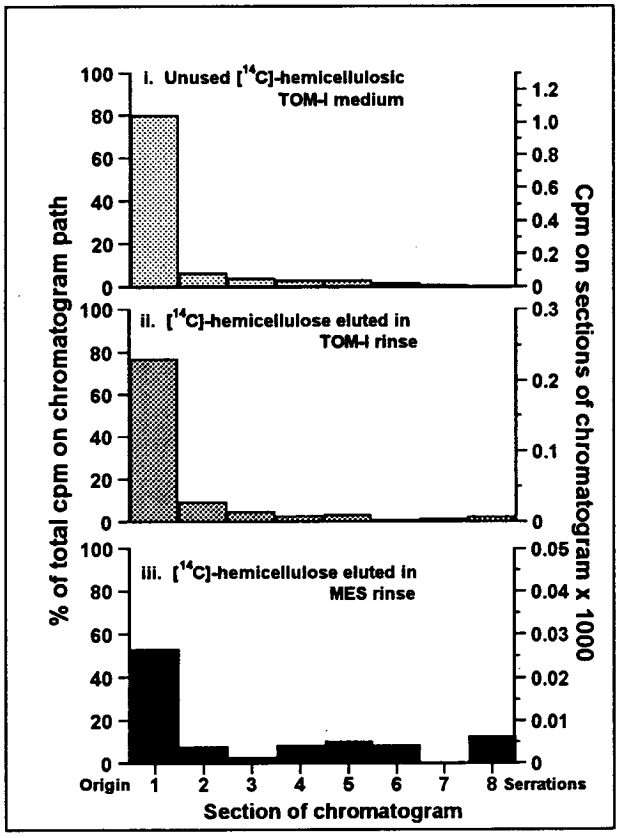
Figure 5.2.1.4.i.a:

Distribution of exogenously applied [^{14}C]-hemicellulose after 3 h incubation at pH 5.7 with cells of *L. esculentum* x *peruvianum*, expressed as total activity (cpm) retrieved, and the corresponding percentage of the total activity.

Means for intact cells were obtained from 2 samples, and those for fractionated cells from 3 samples.

KEY

- total associated with cells
- in TOM-I rinses
- on filter
- in MES rinses



Figures 5.2.1.4.i.b.i, ii, and iii:

Comparison of chromatographic profiles of [^{14}C]-hemicellulosic TOM-I medium before and after incubation with cells of *L. esculentum* x *peruvianum* at pH 5.7.

Measurements shown are for 0.5 ml sample of unused [^{14}C]-hemicellulosic TOM-I medium (Fig. i), and 1 ml samples of [^{14}C]-hemicellulose eluted in TOM-I and MES rinses (Figs. ii and iii).

highly polymeric material would be located. Section 2 exhibited the next largest population of hemicellulosic molecules with 6.2% of the total activity measured. Oligomers which chromatographed to sections 3 - 8 constituted no more than 12% of the total radioactivity in the sample, with no individual section exceeding 3.9% (section 3) of the total radioactivity in the sample.

The hemicellulose extracted from cells in TOM-I rinses showed a highly similar polymer profile to the hemicellulosic medium initially supplied to the cells (Fig. 5.2.1.4.i.b.ii). Again, the highest levels of radioactivity, 77% of the total, were found at the origin, with the next peak of 9%, lying at section 2. The remaining activity located on sections 3 - 8 constituted 14% with the total activity for any section not exceeding 4.6% (section 3).

The amount of [^{14}C]-hemicellulose which might have been internalised by the cells, and released upon rupture, was a low proportion of the original dose supplied to the cells (Fig. 5.2.1.4.i.b.iii). The chromatographic profile of the [^{14}C]-hemicellulose retrieved in MES rinses after cell rupture varied in the ratio of molecules of different mobilities which it contained from the profiles for unused [^{14}C]-hemicellulosic TOM-I medium, and the [^{14}C]-hemicellulose extracted in TOM-I rinses from entire cells.

Again, with the MES rinse, most, 53%, of the hemicellulose population remained at the origin, however this was considerably less than the 80% and 77% recorded previously for samples of unused [^{14}C]-hemicellulosic TOM-I medium and TOM-I rinse. Section 7 maintained a background reading when counted. The radioactivity located on sections 4, 5 and 6 ranged between 7.6 - 9.9%; 2.4% was located on section 2 and 12% on section 8.

5.2.1.4.ii: Localisation of exogenously applied [^{14}C]-hemicellulose after 3 h incubation with suspension-cultured cells of *L. esculentum* AC at pH 5.7

5.2.1.4.ii.a: [^{14}C]-labelled polysaccharide binding assay

A [^{14}C]-labelled polysaccharide binding assay was performed with suspension-cultured cells of *L. esculentum* AC and [^{14}C]-hemicellulose in SH medium at pH 5.7. The sample tubes were rotated at 140 rpm during incubation, and measurements were taken for fractionated cells only.

Most of the [^{14}C]-hemicellulose supplied to the cells, 86%, was eluted in the initial rinses in SH medium (Fig. 5.2.1.4.ii.a). The 14% which remained associated with the cells was divided between the solid fraction retained on the filters, 6.3%, and the soluble cell contents fraction eluted by rinses with MES, 8%.

Between 1 423 - 1 507 cpm were retrieved from samples of *L. esculentum* AC cells in this experiment. This was slightly more than the 1 409 cpm measured in a 0.5 ml aliquot of

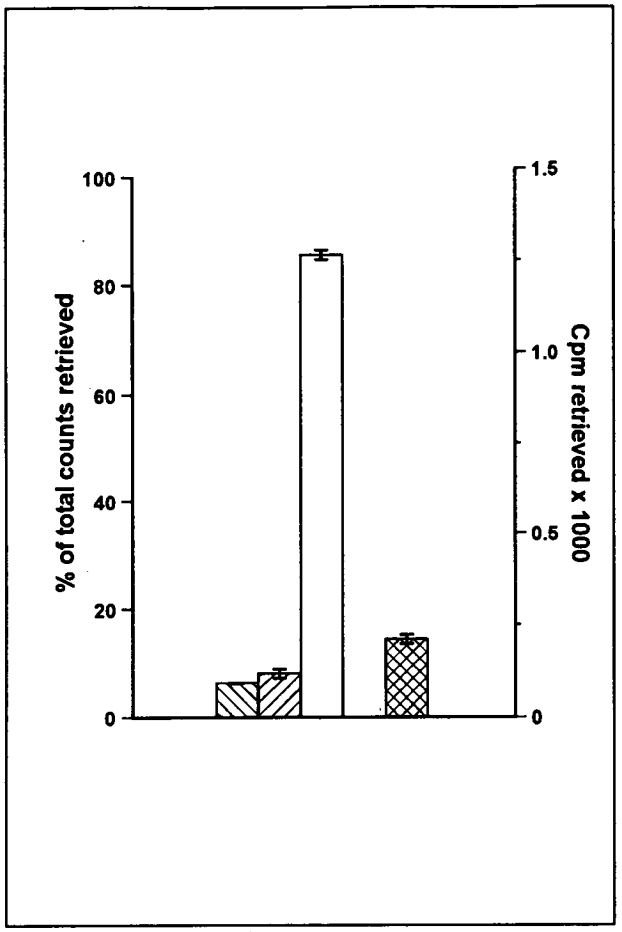


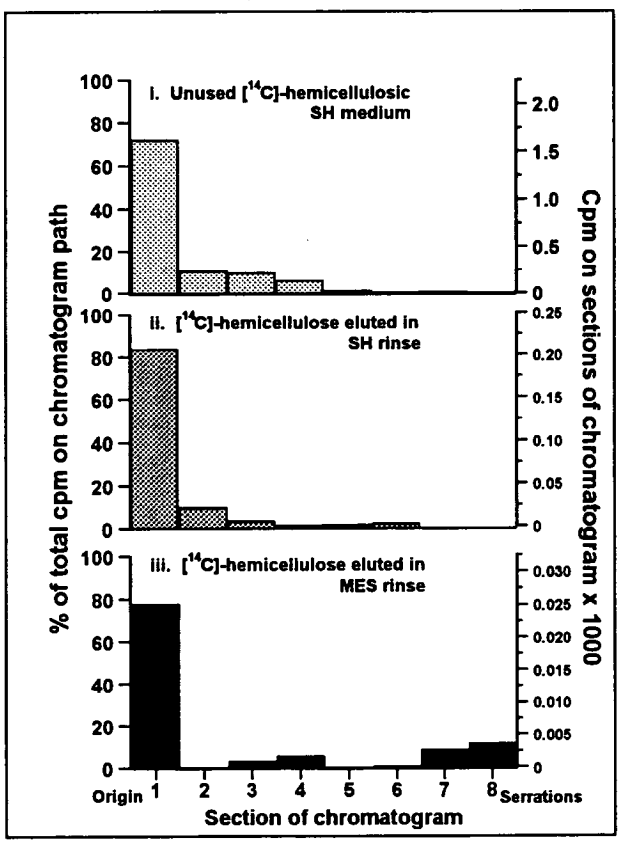
Figure 5.2.1.4.ii.a:

Distribution of exogenously applied [^{14}C]-hemicellulose after 3 h incubation with cells of *L. esculentum* AC at pH 5.7 (fractionated cells only).

Means were obtained from 3 samples.

KEY

- total associated with cells
- in SH rinses
- on filter
- in MES rinses



Figures 5.2.1.4.ii.b.i, ii, and iii:

Comparison of chromatographic profiles of [^{14}C]-hemicellulosic SH medium before and after incubation with cells of *L. esculentum* AC.

Measurements shown are for 0.5 ml sample of unused [^{14}C]-hemicellulosic SH medium (Fig. i), and 1 ml samples of [^{14}C]-hemicellulose eluted in SH and MES rinses (Figs. ii and iii).

unused [^{14}C]-hemicellulosic TOM-I medium, but was sufficiently close to the predicted figure not to cause problems.

5.2.1.4.ii.b: Chromatography

The polymeric profile of the [^{14}C]-hemicellulosic SH medium supplied to the cells is shown in Fig. 5.2.1.4.ii.b.i. Most of the [^{14}C]-hemicellulosic polymers, 72%, remained at the origin. Sections 2, 3 and 4 exhibited the next largest populations of radioactive molecules with 11%, 10% and 6% of the total activity registered respectively. Hemicellulosic oligomers detected on sections 5 - 8 constituted no more than 1.9% of the total radioactivity in the sample, counts ranged between 0 - 24.87 cpm above background levels (0 - 1.2%).

The hemicellulose eluted from cells in SH rinses (Fig. 5.2.1.4.ii.b.ii) showed a profile generally similar to that of the hemicellulosic medium initially supplied to the cells. Most, 84%, of the total [^{14}C]-hemicellulose in the sample was located at the origin, with 9.6% at section 2 and 3.4% at section 3. The remaining 4.7% of the total activity was spread across sections 4 - 8 with measurements for individual sections lying between 0 - 2.1%. A lower proportion of the hemicellulosic molecules which chromatographed to sections 3 and 4 in the unused medium profile were represented on this chromatogram.

A very small amount of [^{14}C]-hemicellulose was eluted with the soluble cell contents in the MES rinses (Fig. 5.2.1.4.ii.b.iii). The profile of this internalised [^{14}C]-hemicellulose exhibited a variety of differences in the hemicellulosic population when compared to the profiles obtained for the unused [^{14}C]-hemicellulosic SH medium and post-incubation SH rinses, although the actual radioactivity levels measured were very low indeed, i.e. 0 - 25.25 cpm, (0 - 78%). The majority of the radioactivity detected remained at the origin, 78% (25.25 cpm). The radioactivity detected on section 2 fell within background levels, as did those on section 5. Section 8 exhibited the next highest levels with 12%, with section 7 accounting for 8.5%. Sections 3 and 4 exhibited 3.1% and 5.4% respectively while the levels on section 6 were very low at 0.77% (0.25 cpm). Therefore, there are peaks present on the chromatogram profile for the MES rinse which represent populations of smaller hemicellulosic molecules for which there are no corresponding peaks in the profiles for the original [^{14}C]-hemicellulosic medium or the SH rinse.

5.2.1.5: Discussion: Localisation of exogenous hemicellulose at pH 5.7

Results from the [^{14}C]-labelled polysaccharide binding assay with cells of *L. esculentum* x *peruvianum* indicated that the level of [^{14}C]-hemicellulose associated with the cells, 9.3%, was comparatively low and at a credible level for a potential oligosaccharin effect to operate. After 3 h incubation 6.8% of the [^{14}C]-hemicellulose retrieved had bound to the solid fraction of the cells, while 2.6% had apparently been internalised.

With cells of *L. esculentum* AC, 14% of the [^{14}C]-hemicellulose retrieved became associated with the cells; 6.3% bound to the solid fraction of the cells, which correlated well with the level of binding measured for the solid fraction of cells of *L. esculentum* x *peruvianum* and about 8% was internalised

These results are in direct disagreement with those of Smith (1992), who, while establishing the occurrence of endotransglycosylation *in vivo*, observed that negligible amounts of radioactive label became associated with cell wall material, or were taken up by suspension-cultured cells of carrot, rose, chilli pepper, sycamore, tall fescue and maize incubated with [^{3}H]-labelled XG9 xyloglucan oligosaccharides for 6 - 72 h. Smith used much purer polysaccharide preparations than were applied here, and consequently the hydrogen-bonding ability of the xyloglucan was probably extremely limited by the small size of the fragments which would have automatically reduced the levels of externally associated xyloglucan. The hemicellulosic material which appeared to be internalised by suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC may have been a different component of the hemicellulosic fraction, and not xyloglucan. However, cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC may not follow the general pattern observed thus far in other species and may ingest and/or secrete radioactively-labelled hemicellulosic fragments most actively prior to the 6 h starting-point of Smith's investigations.

Approximately equal amounts of [^{14}C]-hemicellulose bonded to the cell walls of both *L. esculentum* x *peruvianum* and *L. esculentum* AC in terms of percentages and actual counts, which suggests that the binding was passive, limited by the surface area available, and therefore occurred to a fairly consistent degree in all samples. Both the actual counts, and percentage of [^{14}C]-hemicellulose internalised by cells of *L. esculentum* AC were significantly greater than the corresponding values in cells of *L. esculentum* x *peruvianum*. This implies that the active uptake of hemicellulosic oligomers occurs more rapidly, or to a greater extent in *L. esculentum* AC than in *L. esculentum* x *peruvianum*.

Paper chromatography of the *L. esculentum* x *peruvianum* and *L. esculentum* AC [^{14}C]-hemicellulosic media and rinses revealed that for all samples the populations of polysaccharides consisted chiefly of large molecules which did not move from the origin. This suggests firstly, that most of the hemicellulose applied to the cells, and most of the hemicellulose which did not associate with the cells, was of high molecular weight. Secondly, that either a considerable proportion of the internalised hemicelluloses consisted of polymers of high molecular weight, or that the MES rinses were contaminated with residual amounts of highly polymeric material after the rinses with native medium.

Generally the levels of hemicellulose internalised by cells of *L. esculentum* x *peruvianum*, extracted by the MES rinses, were low, around 53 cpm. A high proportion, 53%, of the apparently internalised [^{14}C]-hemicellulose remained at the origin, however, the remaining 47% consisted of oligomers which travelled down the chromatogram. A similarly small amount of internalised [^{14}C]-hemicellulosic material was eluted in MES rinses from ruptured cells of *L. esculentum* AC, 107 cpm. This consisted of 78% highly polymeric material, and 22% smaller, more mobile oligosaccharides. The possible internalisation of this small amount of highly polymerised material is interesting, particularly as the means of internalisation of these molecules is currently unknown. While it is debatable whether the larger polysaccharides could be internalised by cells it appears that the smaller, hemicellulosic oligomers, distinguishable by their mobility in the chromatography system, were probably internalised by the cells during the 3 h incubation period. These oligosaccharides may be biologically-active, in which case internalisation may permit external binding-sites to be cleared and the receptors to be resensitised, or, if the receptors are internally located, may permit signal receipt.

The profile of [^{14}C]-hemicellulose molecules extracted in MES rinses from cells of *L. esculentum* AC exhibited a higher representation, percentage-wise, of some smaller molecules than were present in the unused medium. This may indicate selective uptake of oligomers of predetermined sizes by the cells, and might suggest that some are oligosaccharins. However, the low number of counts detected in this sample may not constitute a representative selection of hemicellulosic molecules which become internalised, and thus result in false peaks.

Reptation is a mechanism which has been proposed (Kieliszewski and Lamport, 1994) to explain the extrusion of molecules of extensin, present in gum arabic, too long to pass transversely through pores in the cell wall matrix, from intracellular sites of manufacture through the cell walls to external sites of wounding. It is thought that the molecules migrate endwise through cell wall pores, and that the process is passive, which makes it possible for reptation to operate in both directions (Baron-Epel, Gharyal and Schindler, 1988). In the standard reptation model the polymer chains are thought to move in a curvilinear manner along their own contours through polymer matrices (Russell, Deline, Dozier *et al.*, 1993) and direction of travel does not appear to be absolutely determined. Reversal of the process might provide an explanation for the detection of highly polymeric [^{14}C]-hemicellulosic material in the cell contents. Extensin molecules carry an overall positive charge (Biggs, 1988) while molecules of hemicellulose carry a net negative charge (du Pont and Selvendran, 1987) which might affect the process and direction of movement of the polysaccharides across cell walls (Baron-Epel *et al.*, 1988).

Endocytosis is a mechanism by which substantial polymeric material might be internalised by cells. Although initially thought to be energetically unfeasible in plant cells (Cram, 1980) there is a growing body of evidence that the energy requirements can be met (Saxton and Breidenbach 1988) and that a variety of macro-molecules including heavy metal ions (Hubner, Depter and Robinson, 1985), fluorescent dyes (Opaka, Robinson, Prior *et al.*, 1988) and oligogalacturonic elicitors are ingested in this fashion (Horn *et al.*, 1989).

At present there is no evidence that hemicellulosic material gains entry to cells by endocytosis, however, the molecules are known to possess several features which may qualify them as suitable candidates for endocytotic activity. Cram (1980) stipulated that, if endocytosis were possible, the relevant binding sites would be located on the membrane and would be complex, with internal allosteric sites sensitive to several internal factors. Evidence concerning the structural arrangements of xyloglucan oligomers has shown that biological activity relies upon the presence of specific arrays (McDougall and Fry, 1989a, b; Hinche and Clarke, 1980; and Longman and Callow, 1987), which might fit the requirements stated for endocytotic sequestration. Furthermore Cram noted that endocytosis would cause membrane depolarisation; this phenomenon has been observed in cells exposed to pectic oligomers (Thain *et al.* 1990; Mathieu *et al.* 1991), but no direct evidence has been offered for the ability of hemicellulosic compounds to produce a similar effect. However, potential loss of membrane integrity has been observed in cells of *L. esculentum* AC incubated at low pH in the presence of hemicelluloses (sect. 4.4.3.4.b) and indicates that under certain circumstances these compounds may induce depolarisations to a degree which may be detrimental to the cells.

5.2.2: LOCALISATION OF EXOGENOUS [^{14}C]-HEMICELLULOSE APPLIED TO SUSPENSION-CULTURED CELLS OF *L. ESCULENTUM* X *PERUVIANUM* AND *L. ESCULENTUM* AC AT pH 4.5

5.2.2.1: Summary: Localisation of exogenous hemicellulose at pH 4.5

The distribution and fate of [^{14}C]-hemicellulose applied to suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC at pH 4.5 in 10 mM tartaric acid medium was investigated by means of [^{14}C]-labelled polysaccharide binding assays and chromatography of unused medium and post-incubation rinses.

Cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC exhibited levels of external adsorption of between 6 - 8%, and 5.5% respectively of the [^{14}C]-hemicellulose retrieved, these were consistent with earlier values obtained at pH 5.7 (sect. 5.2.1), reinforcing the perception that external bonding is probably passive and determined by the available surface area. However, at pH 4.5, cells of *L. esculentum* x *peruvianum* internalised only 0.02% of the [^{14}C]-hemicellulose retrieved, while cells of *L. esculentum* AC internalised

6.8%. This suggested that cells of *L. esculentum* x *peruvianum* may exert an active control over hemicellulosic polymer uptake, but that cells of *L. esculentum* AC do not: passive entry of hemicelluloses would result from either the absence of an active control mechanism, or loss of membrane integrity in the cells.

Comparison of chromatography samples indicated that cells of *L. esculentum* x *peruvianum* ingested smaller, more mobile hemicellulosic oligomers, while cells of *L. esculentum* AC ingested a variety of polysaccharides more characteristic of the entire population of hemicellulose molecules present in the original unused [^{14}C]-hemicellulose tartaric acid medium.

5.2.2.2: Introduction: Localisation of exogenous hemicellulose at pH 4.5

L-[U-^{14}C] leucine incorporation assays with hemicellulose had shown that the effects of hemicelluloses upon the protein metabolism of suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC were pH dependant (sects. 4.4.2, 4.4.3 and 4.4.4). Incubation of cells with hemicelluloses at pH 4.5 caused a decrease in overall viability; subsequently a revival in viability was demonstrated in cells of *L. esculentum* AC transferred from *N. physaloides* hemicellulosic medium at pH 4.5 to hemicellulose-free medium at pH 5.7, although this was not paralleled by cells transferred from *L. esculentum* AC hemicellulosic medium, which exhibited signs of membrane damage (sect. 4.4.3.4.b). A revival of protein metabolism was exhibited by cells of *L. esculentum* x *peruvianum* transferred from hemicellulosic media at pH 4.5 to hemicellulose-free media either at pH 4.5 or pH 5.7 (sect. 4.4.4.4.a). Consequently, the location of exogenously applied hemicelluloses after 3 h incubation at pH 4.5 with cells of both types was of great interest.

5.2.2.3: Materials and Methods: Localisation of hemicellulose at pH 4.5

[^{14}C]-labelled polysaccharide binding assays were performed on suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC with [^{14}C]-hemicellulose applied at 0.5 mg/ml in 10 mM tartaric acid (TART) at pH 4.5. Rinses of hemicellulose-free TART were applied in place of the usual rinses in native medium (sect. 2.7.2). Intact and fractionated cells of *L. esculentum* x *peruvianum* were sampled, but only fractionated cells of *L. esculentum* AC were sampled. In the *L. esculentum* AC assay sample tubes were rotated at 140 rpm during the 3 h incubation. After rupturing, cells were rinsed in 10 mM MES and all other procedures were as stated (sect. 2.7.2).

0.5 ml of the [^{14}C]-hemicellulosic TART medium was scintillation counted, and found to contain 2 474 cpm.

1 ml aliquots of original unused [^{14}C] hemicellulosic TART medium, the first TART rinse, and the first MES rinse from a partitioned sample of cells, were loaded onto Whatman 3MM chromatography paper and run in EtOAc/HOAc/H_2O (10:5:6) overnight. Processing and analysis were as previously described (sect. 2.5.1.f). The hemicellulosic samples were run on the same *L. esculentum* x *peruvianum* and *L. esculentum* AC chromatograms as the samples described in sections 5.1.1.4 and 5.2.1.4, therefore direct comparisons may be made between the chromatographic observations and the control track of 10 marker sugars previously referred to (sect. 5.1.1.4). As before, the samples of initial rinses in TART and MES were taken from the same original sample tube of partitioned cells.

5.2.2.4: Results: Localisation of exogenous hemicellulose at pH 4.5

5.2.2.4.i: Localisation of exogenously applied [^{14}C]-hemicellulose after 3 h incubation with suspension-cultured cells of *L. esculentum* x *peruvianum* at pH 4.5

5.2.2.4.i.a: [^{14}C]-labelled polysaccharide binding assay

The rinses in fresh TART (Fig. 5.2.2.4.i.a) eluted 92% of the [^{14}C]-hemicellulose retrieved from intact cells and 94% of that from fractionated cells. 8% of the [^{14}C]-hemicellulose retrieved from intact cells was retained on the filter; while from fractionated cells post-rupture MES rinses eluted 0.02%, leaving 5.9% on the filters with the solid fraction of the cells.

The number of counts retrieved from sample tubes was consistent between tubes of intact and fractionated cells, and was slightly less than the total of 2 474 cpm measured in the 0.5 ml [^{14}C]-hemicellulosic TART medium.

5.2.2.4.i.b: Chromatography

The [^{14}C]-hemicellulosic medium supplied to the cells (Fig. 5.2.2.4.i.b.i) consisted of a spectrum of differently sized polymers. Most of the radioactivity, 87%, remained at the origin. Section 2 exhibited the next largest population of hemicellulosic molecules with 8.5% of the total activity detected. Hemicellulosic oligomers which chromatographed to sections 3 - 8 constituted no more than 4.2% of the total radioactivity in the sample, with no individual section exceeding 1.6% (section 3) of the radioactivity in the sample.

The hemicelluloses extracted from cells in the TART rinses (Fig. 5.2.2.4.i.b.ii) showed a highly similar polymer profile to the hemicellulosic medium initially supplied to the cells. Again, the highest levels of radioactivity, 86% of the total, were found at the origin, with the next peak, 6.7%, lying at section 2. The remaining activity located on sections 3 - 8 constituted 7%, with the total activity for any section not exceeding 2.4% (section 3).

A very small proportion of the original amount of [^{14}C]-hemicellulose supplied to the cells, 52.25 cpm, was eluted in the first MES rinse (Fig. 5.2.2.4.i.b.iii). The profile of [^{14}C]-

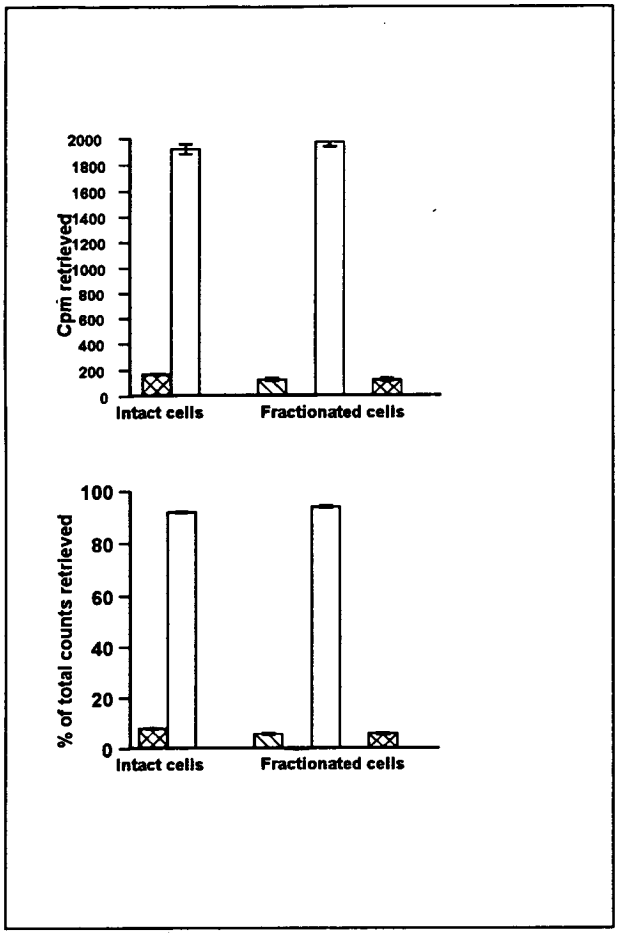


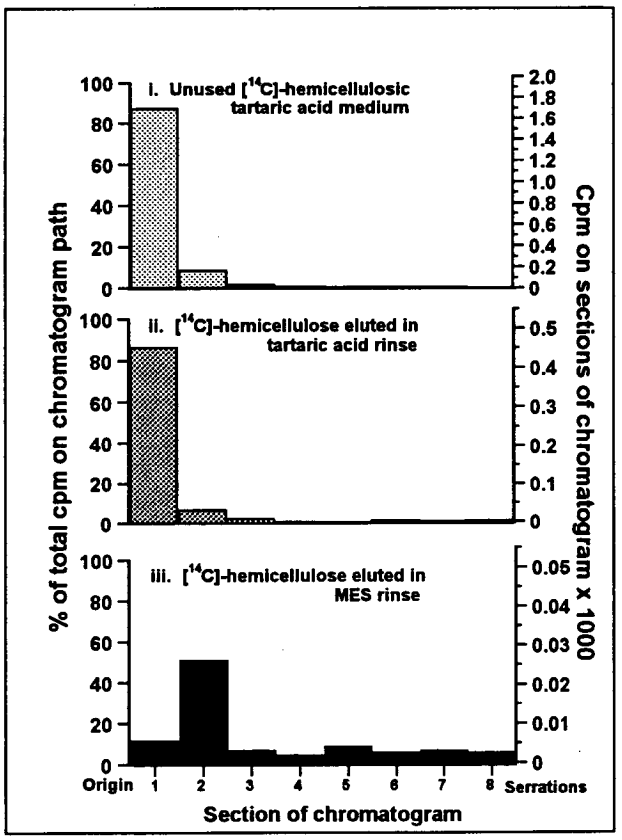
Figure 5.2.2.4.i.a:

Distribution of exogenously applied [^{14}C]-hemicellulose after 3 h incubation at pH 4.5 with cells of *L. esculentum* x *peruvianum*, expressed as total activity (cpm) retrieved, and the corresponding percentage of the total activity.

Means were obtained from 3 samples.

KEY

- total associated with cells: (intact = filter, fractionated = filter + MES)
- in TART rinses
- on filter
- in MES rinses



Figures 5.2.2.4.i.b.i, ii, and iii:

Comparison of composition of [^{14}C]-hemicellulosic TART medium before and after incubation with cells of *L. esculentum* x *peruvianum* at pH 4.5.

Measurements shown are for 0.5 ml sample of unused [^{14}C]-hemicellulosic TART medium (Fig. i), and 1 ml samples of [^{14}C]-hemicellulose eluted in TART and MES rinses (Figs. ii and iii).

hemicellulose extracted in the MES rinse showed that the bulk of the activity chromatographed to section 2 of the chromatogram, 51%, leaving only 12% at the origin. Measurements above background levels were detected on the remaining sections; these ranged between 4.3 - 8.6%. The chromatographic profile of the hemicellulose eluted in the MES rinse varied, with regard to the ratio of molecules of different mobilities it contained, from both the original medium and medium in which intact cells had been incubated.

5.2.2.4.ii: Localisation of exogenously applied [^{14}C]-hemicellulose after 3 h incubation with suspension-cultured cells of *L. esculentum* AC at pH 4.5

5.2.2.4.ii.a: [^{14}C]-labelled polysaccharide binding assay

A [^{14}C]-labelled polysaccharide binding assay was performed as described above (sect. 5.2.2.4.i). The initial rinses in fresh TART (Fig. 5.2.2.4.ii.a) eluted 88% of the total [^{14}C]-hemicellulose applied to the cells. Post-rupture rinses with MES eluted 6.8% of the total [^{14}C]-hemicellulose applied, leaving 5.5% with the solid fraction of the cells on the filters.

5.2.2.4.ii.b: Chromatography

The polymeric profile of the hemicellulosic medium supplied to the cells is shown in Fig. 5.2.2.4.i.b.i. Most of the [^{14}C]-hemicellulose, 87%, remained at the origin. Sections 2, 3 and 4 exhibited the next largest populations of [^{14}C]-labelled molecules with 6.3%, 2.1% and 1.9% of the total activity registered respectively. [^{14}C]-hemicellulosic oligomers detected on sections 5 - 8 constituted no more than 2.2% of the total radioactivity in the sample, counts ranged between 4.75 - 28.8 cpm above background levels, (0.18 - 1.1%).

The hemicellulose eluted from cells in TART rinses showed a profile generally similar to that of the hemicellulosic medium initially supplied to the cells (Fig. 5.2.2.4.ii.b.ii). Most, 85%, of the total [^{14}C]-hemicellulose in the sample was located at the origin, with 6.2% at section 2 and 3.1% at section 3. Sections 4 - 8 contained a total of 5.3% of the total activity, with individual assessments lying between 0.3 - 1.7% (1 - 5.75 cpm). A higher proportion of the hemicellulosic molecules which chromatographed to section 6 in the unused [^{14}C]-hemicellulose TART medium profile were represented on this chromatogram, while a lower proportion of the population at section 7 were detected.

The ratios of the [^{14}C]-hemicellulosic polymers retrieved in MES rinse (Fig. 5.2.2.4.ii.b.iii) varied from the samples of unused medium and post-incubation TART rinse however the actual radioactivity levels measured were very low indeed, i.e. 0 - 14.25 cpm (0 - 69%). Most, 69%, of the [^{14}C]-hemicellulose detected remained at the origin, with 15% at section 2. Sections 3 and 6 registered no counts above the background level. Sections 4, 5, and 7 exhibited 4.8 - 6% of the total radioactivity detected, and section 8 exhibited 16% (3.25 cpm).

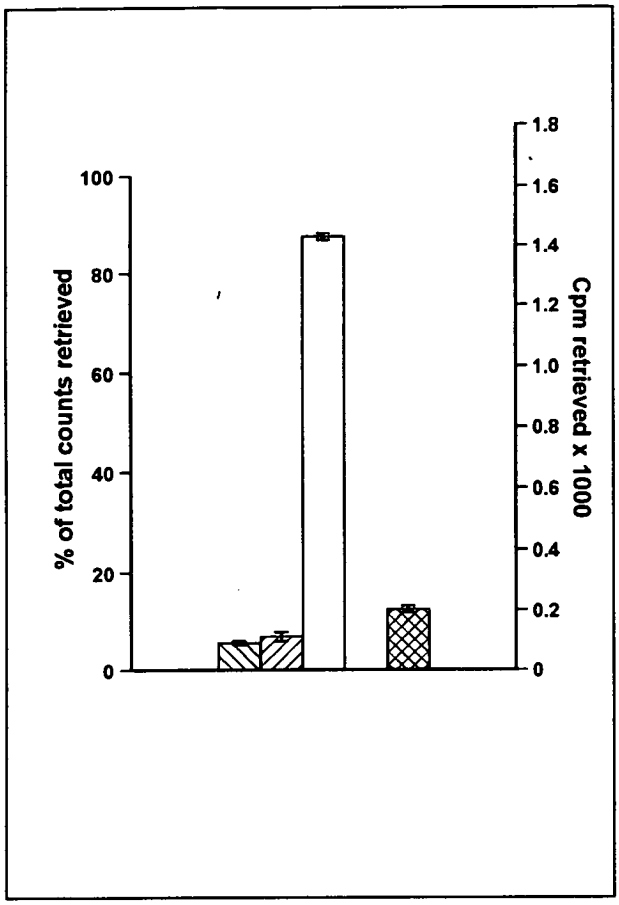


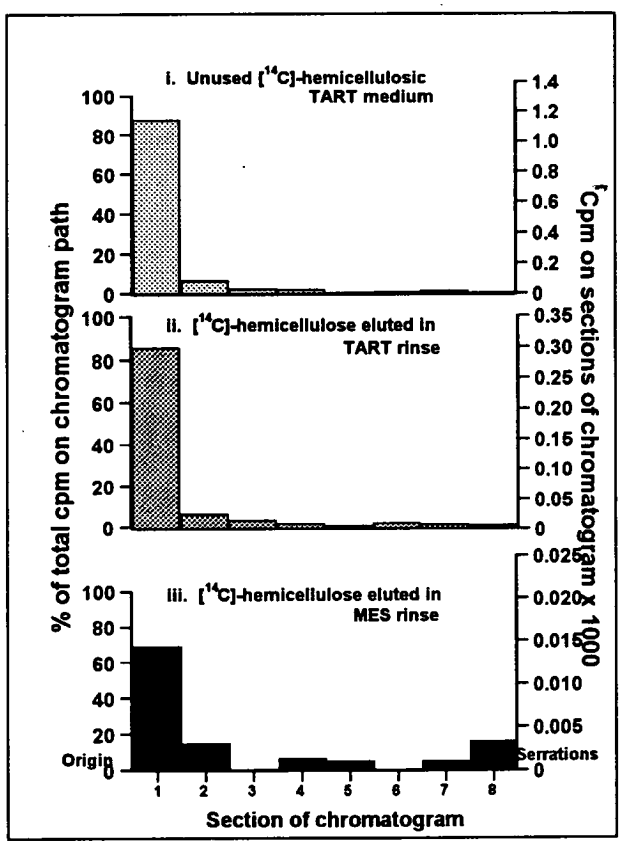
Figure 5.2.2.4.ii.a:

Distribution of exogenously applied [^{14}C]-hemicellulose after 3 h incubation at pH 4.5 with cells of *L. esculentum* AC (fractionated cells only).

Means were obtained from 3 samples.

KEY

- on filter
- in MES rinses
- in TART rinses
- total associated with cells (filter + MES)



Figures 5.2.2.4.ii.b.i, ii, and iii:

Comparison of composition of [^{14}C]-hemicellulosic TART medium before and after incubation with cells of *L. esculentum* AC at pH 4.5.

Measurements shown are for 0.5 ml sample of unused [^{14}C]-hemicellulosic TART medium (Fig. i), and 1 ml samples of [^{14}C]-hemicellulose eluted in TART and MES rinses (Figs. ii and iii).

5.2.2.5: Discussion: Localisation of exogenous hemicellulose at pH 4.5

When cells of *L. esculentum* x *peruvianum* were incubated with [^{14}C]-hemicellulose at pH 4.5, approximately 0.02% of the total [^{14}C]-hemicellulose applied to the cells became internalised. This level is so low that it is possible that internalisation does not occur at all under conditions of low pH, and that the trace of radioactivity detected was due to elution of the residual unassociated [^{14}C]-hemicellulose from the cell debris by the MES rinses.

The figures of 5.9% of the total [^{14}C]-hemicellulose retrieved which was associated with the solid fraction of the fractionated cells, and the 8% associated with the intact cells - which, as with the ruptured cells, can be assumed to be almost entirely cell wall bound - agree well with the 6.7% of [^{14}C]-hemicellulose retrieved which was detected on the filters with the solid cellular debris when cells of *L. esculentum* x *peruvianum* were incubated in TOM-I at pH 5.7. Therefore it would appear that conditions of low pH do not radically affect the extent to which [^{14}C]-hemicellulose becomes adsorbed to the cell walls of suspension-cultured cells of *L. esculentum* x *peruvianum*.

The almost complete absence of [^{14}C]-hemicellulose in MES rinses of ruptured cells of *L. esculentum* x *peruvianum* after incubation at pH 4.5 might be due to loss of membrane integrity, since then any internalised [^{14}C]-hemicellulose would have been washed out in the TART rinses. A previous experiment (sect. 4.4.3.4.b) indicated that incubation of cells of *L. esculentum* AC in hemicellulosic TART media at pH 4.5 could induce membrane disruption, visualised by the leaking of fluorescence from cells into the medium during FDA viability tests. However, there was no direct evidence for membrane damage in cells of *L. esculentum* x *peruvianum* exposed to the same conditions (sect. 4.4.3.4.a), so it appears probable that internalisation of hemicellulosic molecules ceases, possibly temporarily, under conditions of low pH. Indeed, the apparent cessation of hemicellulose uptake by cells of *L. esculentum* x *peruvianum* may account, in part, for the lack of adverse effects observed in the earlier experiment (4.4.3.4.b), when cells of *L. esculentum* AC suffered the possible loss of membrane integrity.

The results from section 5.2.2.4.ii.a of these experiments suggest that the distribution and actual amounts of [^{14}C]-hemicellulose which associate with cells of *L. esculentum* AC are independent of pH, since the results from experiment 5.2.1.4.ii.a, in which suspension-cultured cells of *L. esculentum* AC were incubated with [^{14}C]-hemicellulose at pH 5.7 instead of pH 4.5, were very similar. Although the percentages were slightly lower for cells incubated in TART at pH 4.5, there was not as large a difference as might be expected from the differences in the corresponding L-[U-^{14}C] leucine assays at both pHs, or from the difference in patterns of association evident in cells of *L. esculentum* x *peruvianum* at the different pHs. These results infer that, for suspension-cultured cells of *L. esculentum* AC,

the binding of hemicellulosic molecules to cell walls and putative binding sites, and eventual internalisation of the molecules, is not substantially altered by the pH of the incubation medium.

Paper chromatography of samples of unused [^{14}C]-hemicellulosic TART medium and post-incubation TART rinses of cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC revealed that the populations of polysaccharides present consisted chiefly of large molecules which did not move from the origin. Smaller populations of oligomers which travelled some distance from the origin were also present. Comparison of these profiles, especially when expressed as percentages of radioactivity retrieved, showed that the [^{14}C]-hemicellulose eluted in TART rinses was representative of the hemicellulose population illustrated by the [^{14}C]-hemicellulose TART sample, and provided no evidence for preferential adsorption or ingestion of molecules of particular mobilities to and by the cells.

For cells of *L. esculentum* x *peruvianum* most of the potentially internalised [^{14}C]-hemicellulose eluted in the MES rinse chromatographed to section 2 of the chromatogram, 51%, a further 38% chromatographed across sections 3 - 8, leaving only 12% at the origin. Therefore if any internalisation of [^{14}C]-hemicellulose did occur at this low pH, it was chiefly of comparatively mobile oligomers. However, since the counts extracted in the MES rinses were very low, 52.25 cpm, it is not possible to be certain that these [^{14}C] oligomers were ingested by the cells at all.

The profile for the MES rinse from cells of *L. esculentum* AC resembled the profiles from the unused medium and TART rinse samples, and did not show any signs of preferential internalisation of more mobile oligomers. The actual counts retrieved in this rinse were very low, so the accuracy of any comments made concerning the distribution of the potentially ingested hemicellulosic population would be limited. The possibility remains that the activity detected was due to contamination by unassociated hemicellulose which had not been washed out in the previous TART rinses, although this seems unlikely given the information concerning the actual amounts of [^{14}C]-hemicellulose internalised which were obtained from the [^{14}C]-labelled polysaccharide binding assay.

5.2.3: EXPERIMENT TO ASSESS THE ADSORPTION OF [^{14}C] HEMICELLULOSIC POLYMERS TO DEPROTEINATED CELL WALLS

5.2.3.1: **Summary:** Adsorption of hemicellulose to cell walls

A [^{14}C]-labelled polysaccharide binding assay was performed using deproteinated cell walls of *L. esculentum* x *peruvianum* as the substrate to assess the extent of passive hydrogen-bonding between the external surfaces of cells and [^{14}C]-hemicellulose. Previous experiments with live cells had indicated that between 6 - 8% of the total [^{14}C]-

hemicellulose applied adsorbed to the cell walls in this way. This assay showed that 7.1% of the total [^{14}C]-hemicellulose applied became adsorbed to the cell walls, implying that all binding of hemicellulose to external surfaces of cells is of a passive nature, and that in living cells, the binding site for hemicellulosic polymers which become internalised is not in the cell walls, but probably inside the cell or in the plasmalemma.

5.2.3.2: Introduction: Adsorption of hemicellulose to cell walls

Earlier experiments had shown that the percentage of applied [^{14}C]-hemicellulose which became adsorbed to the external surfaces of the cells during [^{14}C]-labelled polysaccharide binding assays was fairly consistent at both pH 5.7 and pH 4.5 for cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC, and lay between 6 - 8% of the total [^{14}C]-hemicellulose retrieved (sects. 5.2.1 and 5.2.2).

Hemicellulose molecules are thought to hydrogen-bond passively to cellulose microfibrils in the cell walls, therefore it is probable that all the [^{14}C]-hemicellulose which adsorbed to the cell walls did so by hydrogen-bonding. The following experiment was performed to quantify this passive adsorption more accurately, by eliminating active bonding and internalisation of [^{14}C]-hemicellulose molecules, which can confuse the issue, by using deproteinated cell walls as the substrate in a [^{14}C]-labelled polysaccharide binding assay.

5.2.3.3: Materials and Methods: Adsorption of hemicellulose to cell walls

A [^{14}C]-labelled polysaccharide binding assay was performed using deproteinated cell walls from suspension-cultured cells of *L. esculentum* x *peruvianum* [5 mg/0.5 ml TOM-l medium], as the substrate (sects. 2.4.1 and 2.7.3). Samples were incubated in [^{14}C]-hemicellulosic TOM-l medium for 3 h on a rotary-tipping table at 18 °C, and rinsed only in 3 x 3 ml TOM-l medium prior to filtering, and counting (sect. 2.7.2).

5.2.3.4: Results: Adsorption of hemicellulose to cell walls

An average of 92 cpm were retained on the filters with the cell walls (Fig. 5.2.3.4), which equated to 7.1% of the [^{14}C]-hemicellulose retrieved. Conversely, an average of 1 209 cpm were eluted in TOM-l rinses; this equated to 93% of the [^{14}C]-hemicellulose retrieved.

5.2.3.5: Discussion: Adsorption of hemicellulose to cell walls

The 7.1% of the [^{14}C]-hemicellulose retrieved which became associated with the cell walls corresponds remarkably well with the predicted figures of 6 - 8% obtained from living cells of *L. esculentum* AC and *L. esculentum* x *peruvianum* in [^{14}C]-labelled polysaccharide binding assays (sects. 5.2.2.4.ii.a and 5.2.2.4.i.a). Therefore deproteinated cell walls of *L. esculentum* x *peruvianum* seem to provide a satisfactory model with which to determine the extent of adsorption of [^{14}C]-labelled hemicellulose to the surface of living cells. This

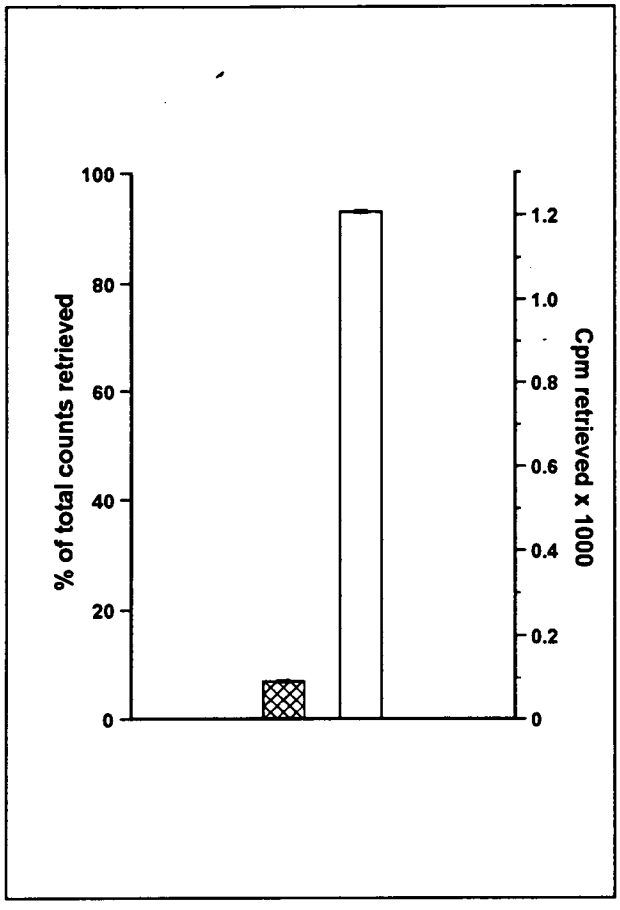


Figure 5.2.3.4:

Distribution of exogenously applied [^{14}C]-hemicellulose after 3 h incubation with deproteinated cell walls from *L. esculentum* x *peruvianum*.

Means were obtained from 3 samples.

may be because the cell wall extraction procedure does not reduce the capacity of the cell walls for hydrogen-bonding. Furthermore, passive hydrogen-bonding seems to account for 100% of the external adsorption of hemicellulose, which implies that there is no active adsorption of hemicellulose molecules to the cell surface, and that molecules which become internalised do not bind to the cell wall prior to ingestion, but possibly to receptors situated internally or in the plasmalemma.

Chapter 6: INVESTIGATION OF APOPLASTIC pH IN THE GRAFT UNION OF COMPATIBLE AND INCOMPATIBLE GRAFTS

"Nature increases plants in several different ways, and it is chiefly Nature's methods which are adopted by gardeners. There are some exceptions. For instance, nothing in Nature is quite like grafting or taking cuttings."

"...root grafting, and the grafting of herbaceous plants, are sometimes done, but only by expert gardeners for special purposes."

Richard Sudell, The New Illustrated Gardening Encyclopædia, circa 1930.

6.1: MEASUREMENT OF pH OF APOPLASTIC FLUID IN THE GRAFT UNION OF HOMO- AND HETEROGRAFTS BETWEEN 1 - 7 DAYS AFTER GRAFTING

6.1.1: **Summary:** pH of apoplastic fluid in the GU

Results from L-[U-^{14}C] leucine incorporation assays indicated that the effects of hemicelluloses upon suspension-cultured cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC were pH-dependant. To assess the range over which hemicellulosic oligomers might operate during grafting *in vivo*, the pH of apoplastic fluid in the GUs of *in vitro* homo- and heterografts of *L. esculentum* and *N. physaloides* was measured 1 - 7 days after grafting.

Across this period the pH of apoplastic fluid in GUs of *L. esculentum* homografts varied by up to 0.56 pH units, while in *N. physaloides* homografts and *N. physaloides/L. esculentum* heterografts it varied by 0.34 units and 0.15 units respectively. For all combinations the lowest pH was recorded four days after grafting: in homografts the pH then increased until day six or seven, while in *N. physaloides/L. esculentum* heterografts a brief rise was followed by a decrease between days five and seven.

The pH minima four days after grafting coincide with the point at which newly generated cells first make contact across the GU and the putative recognition event occurs. These minima may be attributable to the wound-induced presence of acidic pectins in the GU, or to the accumulation of IAA which would be drained as new vascular connections formed thereby leading to a rise in GU pH in compatible grafts, but not in incompatible grafts which fail to establish functional connections.

6.1.2: **Introduction:** pH of apoplastic fluid in the GU

Results from L-[U-^{14}C] leucine incorporation assays with hemicelluloses indicated that the effects of hemicelluloses on suspension-cultured cells of *L. esculentum* x *peruvianum* and

L. esculentum AC were pH-mediated (sects. 4.4.1; 4.4.2; 4.4.3 and 4.4.4). Whether pH fluctuations occur in the GU *in vivo* is of paramount importance in evaluation of the interpretation of particular oligosaccharin signals by suspension-cultured cells. If fluctuations do occur *in vivo* then the pH-related effects of hemicelluloses may be relevant to the grafting process, rather than simply an interesting effect.

No estimations of the pH of apoplastic fluid in entire plants were available, although certain apoplastically active enzymes were known to show performance optima between pH 4 - 5 (Fry, 1988). An experiment was performed with homo- and heterografts of *L. esculentum* AC and *N. physaloides* from 1 - 7 days after grafting to determine the apoplastic pH in the GU.

6.1.3: Materials and Methods: pH of apoplastic fluid in the GU

In vitro homografts of *N. physaloides* and *L. esculentum*, and *N. physaloides/L. esculentum* heterografts were constructed (sects. 2.6.1 and 2.6.2). The pH of each graft in a sample of GUs was assessed on a daily basis for each combination: stock and scion were separated and 5 µl deionised water applied to both exposed GU faces. The 5 µl aliquots were then transferred to the sampling well of a pH Boy-C1 (CamLab) and the pH measured. Mean GU pH values were calculated for each graft combination over a period of 7d after grafting.

6.1.4: Results: pH of apoplastic fluid in the GU

Fluctuations in the pHs of the apoplastic fluid in GUs of all graft combinations were detected in the seven days immediately after grafting. In homografts of *L. esculentum* (Fig. 6.1.4.i) the pH of the GU apoplastic fluid declined steadily from pH 6.4 one day after grafting to pH 5.8 after four days. Between four and six days after grafting the pH rose steadily from pH 5.8 to pH 6.1, and remained steady at pH 6.1 on the seventh day.

The apoplastic fluid in the GU of *N. physaloides* homografts (Fig. 6.1.4.ii) remained at ~ pH 6.05 for two days after grafting. On the third day it rose to pH 6.12, then dropped to pH 5.78 on the fourth day. Between days four to seven it rose again to ~ pH 6.06. The values of GU pH measured one and two days after grafting were similar to the values recorded after six and seven days.

The apoplastic fluid in the GU of *N. physaloides/L. esculentum* heterografts (Fig. 6.1.4.iii) remained between pH 6.03 and pH 6.09 for the first two days after grafting, then fell to pH 5.93 on the third day, and pH 5.85 on the fourth. On the fifth day it rose temporarily to pH 5.95, before falling gradually to pH 5.83 on the seventh day. The differences in pH recorded between days two and three, and four and five after grafting were the largest changes observed between daily assessments.

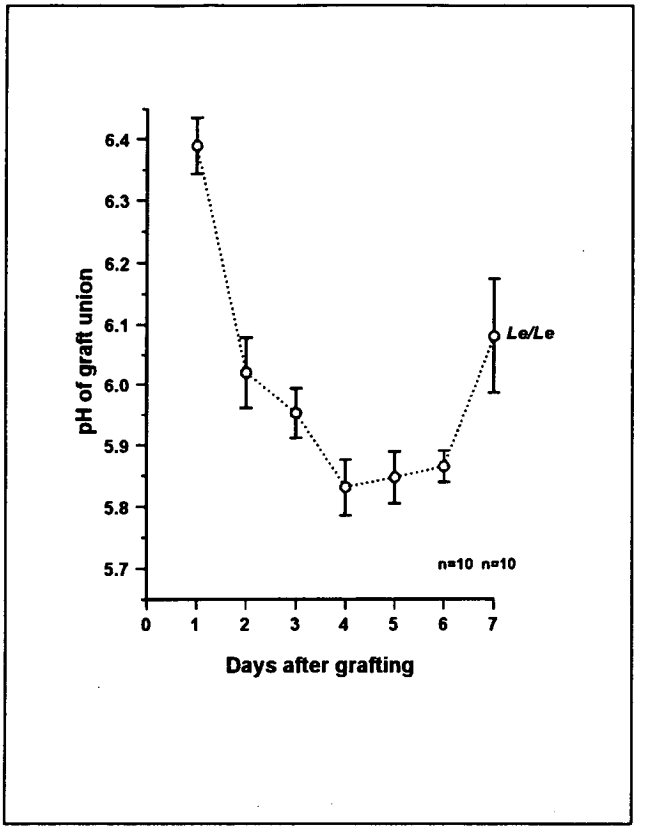


Figure 6.1.4.i:

Mean pH values of apoplastic fluid from the GU of *L. esculentum* homografts between 1 and 7 days after grafting.

Means were obtained from 15 samples unless otherwise specified

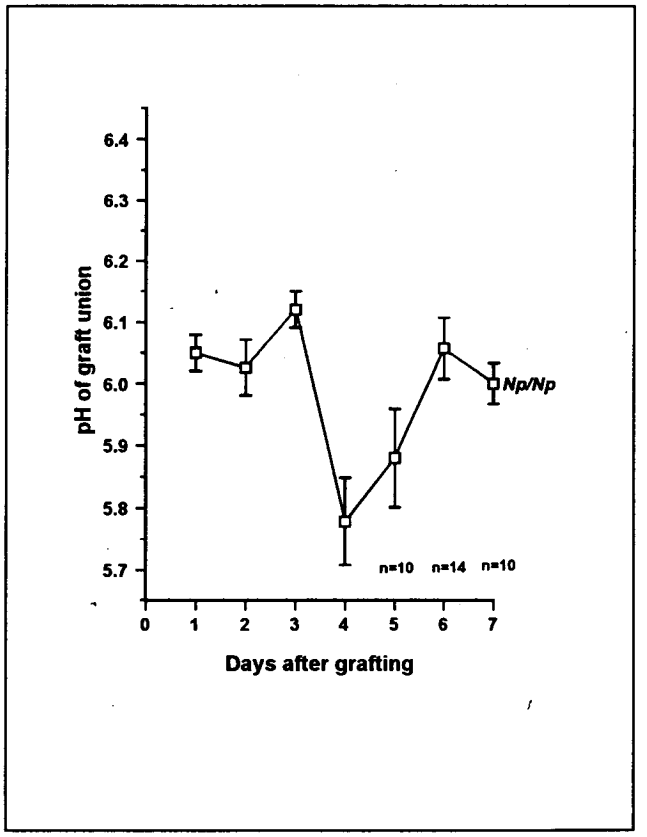


Figure 6.1.4.ii:

Mean pH values of apoplastic fluid from the GU of *N. physaloides* homografts between 1and 7 days after grafting.

Means were obtained from 15 samples unless otherwise specified

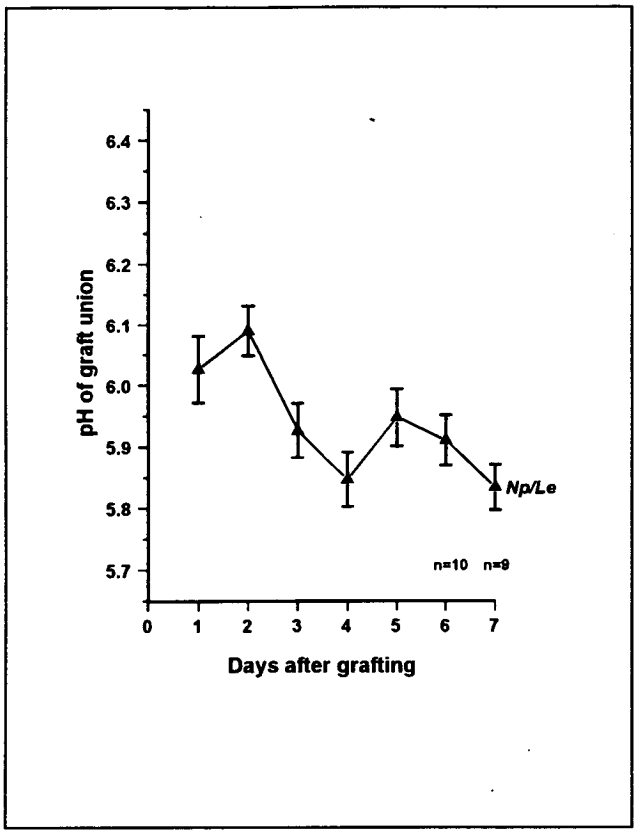


Figure 6.1.4.iii:

Mean pH values of apoplastic fluid from the GU of heterografts of *N. physaloides/L. esculentum* between 1 and 7 days after grafting.

(n = 15 unless otherwise specified)

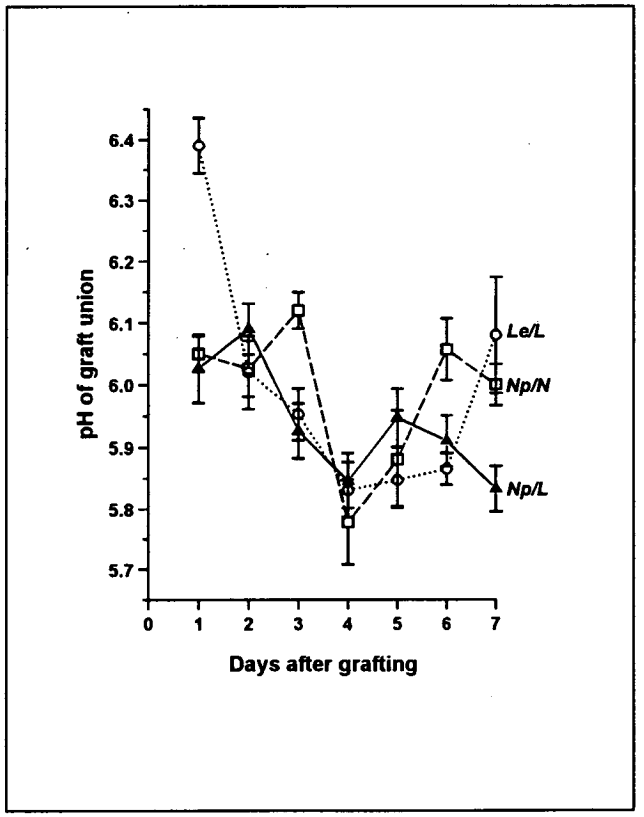


Figure 6.1.4.iv:

Mean pH values of all graft combinations on single axies.

(n values as previously specified)

Direct comparison of Figures 6.1.4.i, ii, and iii can be made by observing Figure 6.1.4.iv, which displays all the data on the same axies. The outstanding feature of this figure is the congregation of minimal pH values, between 5.78 - 5.85 pH units, four days after grafting. The maximum reductions in the pH of GU apoplastic fluid between days one and four were -0.543 pH units for *L. esculentum* homografts, -0.342 units for *N. physaloides* homografts, and -0.244 units for *N. physaloides/L. esculentum* heterografts.

One day after grafting the apoplastic pH of *L. esculentum* homografts was pH 6.39, this was significantly higher than the corresponding values of pH 6.05 and pH 6.026 in *N. physaloides* homografts and *L. esculentum/N. physaloides* heterografts. In *L. esculentum* homografts the most rapid drop in pH (-0.37 units) occurred between days one and two, in *N. physaloides* homografts it occurred between days three and four (-0.342 units), and in *L. esculentum/N. physaloides* heterografts it was more gradual and occurred at a comparatively steady rate between days two and four.

In *L. esculentum* homografts the pH rose steadily after day four. In *N. physaloides* homografts and *N. physaloides/L. esculentum* heterografts the pH both combinations experienced a rise after the day four minima, followed by reductions on days seven and six respectively. The pH of the GU apoplastic fluid of *N. physaloides/L. esculentum* heterografts on day seven was only slightly different from that on day four.

6.1.5: **Discussion:** pH of apoplastic fluid in the GU

In this experiment most of the standard error values were comparatively small which suggests that the measurements reveal actual changes in the pH of GU apoplastic fluid during the preliminary stages of graft development. In all combinations the pH dropped to its lowest value four days after grafting (penultimate low for *L. esculentum/N. physaloides* heterografts), at which time callus cells generated from the opposing graft faces are known to make contact with each other, and the initial putative recognition event is thought to occur. In all combinations the pH increased again after the fourth day.

The reasons underlying this general lowering of the GU apoplastic fluid pH in the first four days after grafting are not understood, but might be attributable to the formation of an acidic layer of pectic material across the GU by the wounded surfaces of stock and scion. However, if the changes in GU pH were related to this layer, a further increase in acidity might be expected after day four, when acidic pectic residues are liberated from the layer during thinning, as surfaces of stock and scion make contact. This further reduction in GU pH does not occur, so it is possible that the predominance of polymerised and free pectin in the GU area has little overall effect on the prevailing pH condition.

The *N. physaloides/L. esculentum* heterograft was the only graft combination in which the early pH values, measured up to three days after grafting, were not re-established by the

seventh day; indeed it appears that further decreases in the pH of the apoplastic fluid in the GU might occur after days five and six. It is not known whether the pectic layer is thinned in incompatible grafts, but work by Kollmann *et al.* (1985) suggests that partial thinning may occur in the formation of half plasmodesmata. However, since this combination comes closest to expressing the anticipated drop in apoplastic pH, and yet may not experience thinning of the pectic layer, it is probable that a factor other than the concentration of soluble pectins controls the overall pH of apoplastic fluid. The results obtained in this study do not support an active role for pectin in recognition phenomena in the GU. However, the initial release of pectins into the pectic layer may establish a pH environment in which hemicellulosic molecules can fulfil recognition functions, even if control of the environment lies elsewhere.

During grafting the severance of the vascular conduits must result in the accumulation of certain metabolites in the GU, and efficient transportation of these from the area will not commence until vascular continuity has been re-established. One such metabolite is the auxin IAA (indole-3-acetic acid) which originates in the apex of the plant and travels downwards via the xylem. The overall decrease in GU pH might be ascribed to a general build-up of scion-derived IAA in the GU prior to the reformation of vascular contacts which occurs after contact has been established between stock and scion around day four. If this accounts for the pH changes observed between days one and four, then a gradual increase in GU pH could be expected to accompany the subsequent differentiation of callus cells into xylem wound vessel members (WVMs) which would reconstitute the flow, from the GU region, of accumulated IAA. Such an increase in GU pH was observed in all three graft combinations, and was generally maintained in the two compatible homograft combinations until the end of the monitored period. However in the incompatible heterograft *N. physaloides/L. esculentum* the rise following contact between the stock and scion was short-lived, and the pH dropped on days six and seven, reaching the previous minimum level. This failure to re-establish less acidic pH conditions in the GU is consistent with the inability of this graft combination to form functional conduits across the GU to transport any accumulation of IAA out of the area. A certain amount of IAA may diffuse away once contact between stock and scion occurs, which may account for the brief rise in pH observed on day five, however, this method of dispersal is much less efficient than xylar-transport. It is not known whether the changes in prevailing GU pH actually affect the process of vascular differentiation or whether they constitute a reflection of events within the GU and so only parallel graft development.

This investigation shows clearly that the GU apoplastic pH of these Solanaceous grafts falls to a minimum value of ~ pH 5.8 during the first seven days after grafting, but does not come close to pH 4.5. Consequently it is unlikely that hemicelluloses would operate in the

same manner *in vivo* as they do on suspension-cultured cells in L-[U-^{14}C] leucine incorporation assays at low pHs. Quite what effect hemicelluloses would exert upon cells at these pHs is not known, but could be investigated using both L-[U-^{14}C] leucine incorporation assays and [^{14}C]-labelled polysaccharide binding assays. The actual effect probably lies closer to those observed at pH 5.7 - 6.0 than those observed at pH 4.5, i.e. a moderate inhibition of protein metabolism, which might equate to a cessation of cell division prior to differentiation, resulting in no ill effects such as cell death or plasmolysis. It must be concluded that the hemicellulosic effects detected at pH 4.5 with suspension-cultured cells are not relevant in the recognition phase of these grafts, since the pH of the apoplast, of all combinations, does not fall low enough in the required time-frame. This still leaves very interesting and unexplained apoplastic pH profiles with uniformly clear minima four days after grafting, and cells of *L. esculentum* x *peruvianum* which internalise [^{14}C]-hemicellulose at pH 5.7 but not at pH 4.5.

Chapter 7: DISCUSSION OF POINTS ARISING FROM EXPERIMENTAL WORK

7.1: EVALUATION OF L-[U-^{14}C] LEUCINE INCORPORATION ASSAYS

7.1.1: The L-[U-^{14}C] leucine incorporation assay monitors metabolism

The L-[U-^{14}C] leucine incorporation assay was introduced to monitor protein metabolism in cells to which exogenous cell wall substances had been applied, and thus to identify potential candidates for the compatible "off" signal for cell division which would be accompanied by a corresponding reduction in protein anabolism. The use of suspension cultures enabled much more rapid screening of potential recognition factors in the time available than would have been possible using traditional *in vitro* grafting methods. Those substances which inhibited protein metabolism were considered to be potential candidates for the "off" signal.

Other workers have used analogous assays to provide an index of cell viability (Yamazaki *et al.*, 1983; Bucheli *et al.*, 1990) although, throughout this project, an FDA test was employed to assess viability. However the differences in results from the experiments described in sections 4.4.3 and 4.4.4 show clearly that the FDA viability test and L-[U-^{14}C] leucine incorporation assays produce assessments of separate phenomena, viability and protein metabolism, and are not interchangeable viability tests. Most importantly, the results from these leucine incorporation experiments show that the L-[U-^{14}C] leucine incorporation assay provides a valid assessment of the metabolic activity of cells, rather than a reflection of the viability of the culture as a whole. This is particularly significant if the assay is to be used to gain insight into the reactions of callus cells in the GU of Solanaceous grafts to a range of cell wall-derived components and fragments, and to interpret grafting phenomena. These results suggest that this is a valid and relevant procedure with which to investigate putative recognition events in the GU.

7.1.2: Effect of spent *vs.* fresh medium

In sections 4.3.2 and 4.3.3 the addition of pectic fragments and pectins to cells in 8-d spent media and fresh media (MES or SH), accompanied variations in results. These variable results may highlight a potential danger in this type of assay, namely that when fresh medium is used the instantaneous reactions of cells to the applied exogenous cell wall components may be revealed, which may not be the response evoked during grafting. In searching to identify the "off" signal for cell division in the GU it is important to remember that, *in vivo*, cells which respond to this signal may already have been exposed to a *melange* of cell and cell wall components, released as a consequence of damage inflicted

during graft establishment. Certain of these may have induced particular "wound reactions" including rapid cell division at the cut surfaces, and will probably still be present when the putative "off" signal is delivered. Suspension-cultured cells tested in fresh medium in the absence of this background of endogenous cell debris may react to exogenous pectic molecules as if these are the products of recent wounding, by increasing the rate of cell division, and, accordingly, the rate of protein metabolism measured as L-[U-^{14}C] leucine incorporation. The background of cell wall components, and possible signal interference which accompanies use of spent media, may be a prerequisite for the "off" signal to be perceived as such, rather than as a signal of immediate damage which needs to be repaired.

Similarly, the greater inhibitions observed when hemicelluloses were applied to cells of *L. esculentum* x *peruvianum* in 8-d TOM-l medium rather than MES-based media (sect. 4.4.1) might be attributable to the absence of the background population of polysaccharides, and substantiate the possibility that the absence of such a background population may slow the perception and interpretation of any signals to stop cell division.

7.1.3: The component from deproteinated cell walls which inhibited protein metabolism is of polysaccharide origin

The application of deproteinated cell walls to cells in L-[U-^{14}C] leucine incorporation assays (sect. 4.1) caused considerable inhibition of protein metabolism. The cell wall substance(s) responsible for this inhibition would presumably have been present at fairly low concentrations in the various media, since the alcohol insoluble residue of cell walls supplied to the cells would be largely insoluble in water, and release of fragments due to enzyme activity would probably have been slow and limited. The appearance of inhibition, despite the low final concentration of any soluble cell wall substances in the medium, is consistent with an oligosaccharin effect since oligosaccharins can operate at concentrations as low as 9 nM (McDougall and Fry, 1988). The inhibition of protein metabolism by soluble apoplastic polysaccharides (sect. 4.2) reinforces the probability that the inhibitor of protein metabolism was polysaccharide in nature, although this particular group of polysaccharides might well have been leached from cell walls during the deproteination process (Redgwell *et al.*, 1992). Further work with pectins and hemicelluloses provided evidence that a variety of cell wall polysaccharides can inhibit protein metabolism in cultures of *L. esculentum* x *peruvianum* and *L. esculentum* AC.

7.1.4: Pectin is not the recognition factor

Pectins and pectic fragments have been identified largely as elicitors of defence reactions (Bruce and West, 1982; Nothnagel *et al.*, 1983) and have rarely been implicated in control of growth phenomena (Branca *et al.*, 1988; Tran Thanh Van and Mutaftschiev, 1990) which might include control of cell division. Conversely, hemicelluloses and derivatives have

been reported not to elicit defence reactions (Fry, pers. comm. from Pierpoint, pers. comm.), but oligomers of the hemicellulosic polymer xyloglucan do show anti-auxin activity in the growth of pea internodes (York *et al.*, 1984; McDougall and Fry, 1988), and auxin-mimicking properties in the absence of exogenous auxin (McDougall and Fry, 1990, Fry *et al.*, 1990). Therefore, although pectin was originally suggested as the putative cell wall substance responsible for controlling graft development in the Solanaceous model (Yeoman, 1984; Jeffree *et al.*, 1987), it should not be regarded as the sole candidate for this role, particularly as control of growth, which presumably extends to certain developmental processes, has been shown, here primarily, to be exerted by xyloglucan fragments and not, conclusively, by pectins. Furthermore, the failure to establish a clear pattern of activity for pectins in the L-[U-^{14}C] leucine incorporation assay suggests that either pectin is not active in the recognition process, or may become active after the "off" signal for cell division has been received, but is not the "off" signal *per se*. Since no firm evidence has been generated to confirm the recognition role suggested by Yeoman (1984) and indicated by Jeffree and colleagues (1987) for pectic poly- or oligogalacturonides in this grafting system the hypothesis must be rejected at this stage. However, the possibility that future work may generate the evidence required cannot be excluded either, in which case the complex pectic sidechains of RGII, which may contain structurally specific sequences (sect.4.3.5), should probably receive attention.

7.1.5: The recognition factor may be hemicellulosic

Hemicelluloses have a well-defined role in the control of growth (outlined above sect. 7.1.4), which includes very precise oligosaccharide structure specificity and concentration-related effects. So far no pH effects have been documented with hemicelluloses in these processes. In the course of this study the only applications which produced very consistent and marked reductions in protein metabolism in L-[U-^{14}C] leucine incorporation assays contained hemicelluloses (i.e. hemicelluloses *per se* and cell walls).

Earlier it was stated that the putative recognition factor was probably located in the cell wall, liberated by enzymes, and was likely to be of low mobility on account of its probable size (Baydoun and Fry, 1985). In addition, in order to convey a signal, a suitable candidate molecule should be able to cross the cell wall to potential binding sites on the plasmalemma and subsequently to be cleared from binding sites so as not to saturate them (Horn, Heinstein and Low, 1989). Hemicelluloses fulfil these requirements. Hemicellulose fragments cleaved from cell walls by cellulases (found in the GU of Solanaceous grafts [Jeffree, Gordon and Yeoman, 1989]), are soluble in aqueous media, do cross the cell wall, probably via reptation, and are internalised by cells within three hours (cf. Baron-Epel *et al.*, 1988 with sects. 5.2.1 and 5.2.2). There is also some evidence of selective uptake of hemicellulosic-derived oligomers by cells of *L. esculentum* x *peruvianum*. Evidence to confirm the

mobility/immobility of hemicelluloses in agar would help to align this group of cell wall polysaccharides with Parkinson's (1983) original stipulations that the factor(s) possessed low mobility and high molecular weight. Pectins also fulfil these prerequisites, but, despite extensive testing, could not be confirmed in the proposed role of recognition factor by the present experimental approach.

Furthermore, the extent of effects of hemicelluloses changes at different pHs. This may indicate that multiple points of control exist in the grafting process, which are determined by pH conditions in the GU. The occurrence of pH minima four days after grafting, in all Solanaceous graft combinations tested, appears to be a significant phenomenon, particularly as the initial recognition event is thought to occur at this time, and has not apparently been reported previously. However, it is not known what differences in hemicellulosic activity pH fluctuations of ± 0.56 units would generate.

7.1.6: Nature and cause of plasmolysis at pH 4.5

In sections 4.4.2 and 4.4.3 cells incubated at low pH in 10 mM tartaric acid ± hemicelluloses appeared to become plasmolysed, although the incubation media were not of sufficiently osmolarity to induce plasmolysis. Hanchey and Wheeler (1969) documented the onset of "false plasmolysis" in cells suspended in hypotonic solutions, which was interpreted by Bennet-Clark (1959) as an erratic response to injury, but if so, the type of injury perceived to have been sustained should be considered. Previous work has indicated that hemicellulose-derived poly- and oligosaccharides are not involved in elicitation of defence reactions (Fry, pers. comm. from Pierpoint, pers. comm.), so, although plasmolysed cells occurred more frequently in hemicellulosic media, it is unlikely that the "damage" signal originated from the hemicelluloses, but may have been the result of a secondary effect.

The observations of Horn and colleagues (1991) indicated that cytoplasmic and vacuolar pH changes could be induced in cells, in the absence of elicitors, by adjustments to the pH of the cell-bathing solution. It is thus quite possible that the perceived damage is attributable to the activity of active oxygen species at low pH. Under conditions of normal metabolism, the superoxide radical, O_2^{-1}, is found in many organelles, including the plasma membrane and the cell wall, and can cause serious structural damage to DNA, proteins and membrane lipids (Dhindsa, Plumb-Dhindsa and Thorpe, 1981; Girotti, Thomas and Jordan, 1985). These radicals are detoxified by the enzyme superoxide dismutase (SOD), the activity of which depends upon the availability of metal co-factors and the pH: optimal activity occurs between pH 7.0 - 8.0 (Tzeng, 1989). Below pH 5.6 the scavenging ability of SOD is greatly reduced and Fe is released from the enzyme (Halliwell and Gutteridge, 1984). The increased availability of Fe ions activates the O_2^{-1} radicals, while low pH lengthens their half-lives (Tzeng and DeVay, 1993), thus extending the scope for damage

under the incubation conditions used in these experiments. However, this does not provide an explanation for the higher occurrence of plasmolysed cells in hemicellulosic media.

Some of the plasmolysed cells (sect. 4.4.2.4) exhibited membrane-bound fluorescence which was probably attributable to esterase activity, although previous studies (Holliday, Keen and Long, 1981) have shown that hypersensitive cells of soybean exhibit partial or complete collapse away from the cell wall, and accumulation of autofluorescent phytoalexins when challenged with pathogenic bacteria. If the fluorescence observed here was due to phytoalexin induction this would be the first documented account of hemicelluloses eliciting a defence reaction.

7.2: [^{14}C]-LABELLED POLYSACCHARIDE BINDING ASSAYS

7.2.1: Evaluation of [^{14}C]-labelled polysaccharide binding assays

The [^{14}C]-labelled polysaccharide binding assays (Chapter 5) provided valuable quantitative information concerning the fate of exogenously applied polysaccharides which associate with cells. The final protocol appeared to produce accurate results and to enable complete elution of unassociated polysaccharides (sect. 5.2.2). This assay could be put to good effect in further experiments with more highly purified polysaccharide preparations, in conjunction with complementary L-[U-^{14}C] leucine incorporation assays.

These assays indicated that with cells of *L. esculentum* x *peruvianum* approximately 3.8% of the [^{14}C]-pectin, and about 2.6% (at pH 5.7), or 0.02% (at pH 4.5), of the [^{14}C]-hemicellulose applied to the cells was internalised. Corresponding figures for cells of *L. esculentum* AC indicated that 8.0% of the applied [^{14}C]-hemicellulose was internalised at pH 5.7, and 6.8% at pH 4.5. The mechanisms whereby these polysaccharides traversed the cell walls and plasma membranes are not known, but it is assumed here, in the absence of evidence to the contrary, that trans-wall transport is not an active process.

7.2.2: Trans-wall transport of polysaccharides

Baron-Epel and colleagues (1988) assessed the size of putative trans-wall channels in soybean cells by monitoring the passage of fluorescently-labelled dextrans and proteins of known size through the cell wall into the space between the wall and the plasmolysed protoplast. Their results indicated that polysaccharides of 17.9-kDa, with a Stokes radius of up to 3.3 nm, were not hindered in their movement across the wall and reached equilibrium within 10 - 15 min, while polysaccharides of 41-kDa, with Stokes radius of 4.6 nm, achieved equilibrium after 1 - 2.5 h. The comparatively slow movement of the larger molecules may have reflected a paucity of transport channels of the appropriate size, or have been due to the slow process of reptation, however the time-scale for the achievement of equilibrium fits particularly well with the three hours used in [^{14}C]-labelled polysaccharide binding

assays in this study. Interestingly, the original rates of passage determined by Baron-Epel and colleagues remained unchanged when cells were incubated at pH 3.5 - 4.5. Therefore, it seems likely that internalised [^{14}C]-pectic and hemicellulosic polysaccharides were first delivered to the face of the plasmalemma via passive diffusion and/or active reptation through transport channels in the cell wall.

It is probable that any oligo- or polysaccharide receptor binding sites are located on the external face of the plasmalemma. Most of the published evidence about binding sites concerns elicitors of fungal origin which produce defence reactions and bind to specific receptors, probably proteins or glycoproteins, located in the plasmalemma (Schmidt and Ebel, 1987; Cosio *et al.*, 1988; Cosio *et al.*, 1990; Cheong and Hahn, 1991; Liénart *et al.*, 1991). Horn and colleagues (1989) stated that most elicitors are too large to pass through the plasmalemma, and this would probably hold true for the various polysaccharides which have affected protein metabolism in this study.

7.2.3: Trans-plasmalemma transport of polysaccharides

The results from this study strongly suggest that a significant fraction of exogenously-applied polysaccharides were internalised by the cells. This should probably be interpreted as evidence of clearance of binding sites, which is vital if cells are to remain receptive to environmental stimuli. In view of the size of the molecules, endocytosis is the probable mechanism by which internalisation occurs. Since the rate of polysaccharide passage across cell walls is not affected by pH (Baron-Epel *et al.*, 1988), but the passage of [^{14}C]-hemicellulose across the plasmalemma of cells of *L. esculentum* x *peruvianum* differs with pH (at pH 4.5 uptake ceased almost entirely), there are two possibilities regarding the fate of these hemicellulosic polysaccharides once they have reached the plasmalemma. A fungal hepta-β-glucoside phytoalexin elicitor investigated by Cheong and Hahn (1991), exhibited reduced membrane binding at pHs outside the pH 6.0 - 8.0 range, which suggests that membrane binding of these polysaccharides may be severely limited at pH 4.5, although, Thain *et al.* (1990) obtained almost immediate depolarisation of tomato membranes with oligogalacturonide mixtures at pH 5.5, which implies that membrane binding of pectic fragments can occur at lower pHs. Alternatively, hemicellulose uptake occurs via an active process which requires energy and can be controlled by the cells. However, cells of *L. esculentum* AC appear to be unable to regulate uptake of hemicellulosic oligosaccharides under conditions of low pH, suggesting that accumulation of the polymers in these cells may have occurred passively, possibly as a result of loss of membrane integrity by damage incurred from the activation of superoxide radicals at low pH.

7.3: DETERMINATION OF APOPLASTIC pH OF THE GU

The experiment which determined the apoplastic pH of the GU provided information which will be of great value in future work on recognition phenomena in these Solanaceous species, since it indicates the pH limits within which polysaccharide must exert effects in order to be considered active within the grafting process. The minima which were measured four days after grafting, while probably not constituting recognition events themselves, possibly facilitate, or control, release and/or delivery of the recognition factor. It is particularly significant that the pH minima coincided at this point, and it would be difficult to see how this drop in pH could not play a relevant part in delivery or interpretation of recognition factors. These data also demonstrate that callus cells in the GU would not suffer greatly from membrane damage caused by oxygen radicals, as, at the measured pHs, the combative enzymes would remain operational throughout and production of the radicals would not increase.

7.4: FINAL POINTS FOR CONSIDERATION

7.4.1: Hierarchy of signals in recognition process probable

The pursuit of the identity of the preliminary "off" signal for cell division has been the primary objective of this body of work. However, parallels drawn with the elicitation of phytoalexin production and other defence reactions (Tzeng and DeVay, 1993) indicate that it is probable that the "off" signal will be part of a hierarchy of signals which tap in at different stages in control of graft development to promote or suppress particular metabolic phenomena. Consequently, at this stage it is not possible to say which cell wall components are not involved in the recognition process as a whole, but certainly it is clear that no consistent evidence substantiated the proposed role for pectin as the "off" signal for cell division. This research consistently showed that cell wall derived compounds affect cell suspensions in the manner in which it was anticipated the "off" signal would, but, generally speaking, further examination of hemicellulose activity might be more productive than the continued pursuit of pectin. To this end, use of the L [U-^{14}C] leucine incorporation assay and the polysaccharide binding assay could be productive once more highly purified cell wall preparations are available.

7.4.2: Potential of arabino-galactan proteins as recognition factors

One group of polysaccharide containing compounds which has not yet received widespread consideration for the role of identity-determining messenger molecules in grafting, and should probably receive attention in further studies on this Solanaceous system, are the arabinogalactan proteins (AGPs). These compounds have been discovered in the tissues

of every taxonomic group of angiosperms (Clarke, Anderson and Stone, 1979) and occur in all major plant organs (Fincher, Stone and Clarke, 1983). Although similar in structure to the cell wall polymer extensin, AGPs are not tightly bound to the cell wall matrix and can be extracted with comparative ease (Clarke *et al.*, 1979). AGPs include commercially important gums which are produced in response to wounding and may serve a water-proofing and/or anti-bacterial role while sealing the affected zone (Clarke *et al.*, 1979). There is evidence that, while the structure of these gums is very complex, the terminal disaccharides may be taxonomically distinct (Anderson and Dea, 1969), which implies that a recognition function would be possible. Interestingly it appears that distinguishable differences exist not only at the interspecific level but also, for maize, tobacco, gladiolus and sweet cherry, at the organic level, in that different parts of the same plant can produce distinct groups of AGPs (Van Holst and Clarke, 1986; Raff, Hutchinson, Knox *et al.*, 1979; Khavkin, Misharin, Ivanov *et al.*, 1977; Boutenko and Volodarsky, 1968; Clarke, Harrison, Knox *et al.*, 1977). These "organ-specific antigens" are also expressed by callus cells and collect in culture filtrates, which suggests that they are secreted through the plasma membrane and cell wall (Raff *et al.*, 1979).

Assays suggest that AGPs make up a considerably larger proportion of the plasma membranes in plants than in animals (Komalavilas, Zhu and Nothnagel, 1991) and seem to be localised at the membrane-wall interface in intact cells (Anderson, Clarke, Jermyn *et al.*, 1977) and at the cell surface in protoplasts (Larkin, 1977, 1978). The protein portion of these glycoprotein molecules often consists chiefly of hydrophobic amino acids which, if AGPs were membrane bound, would be embedded within the plasma membrane itself. Thus the carbohydrate moiety would be held on the outer (extra-cellular) face of the cells (Knox and Clarke, 1984). Immediate membrane-membrane contact between plant cells in a grafting scenario is precluded by the existence of the cell walls. However, as already discussed, the porosity of the cell wall is considerable and globular proteins or polysaccharides of considerable size can cross through it readily, providing that factors such as charge and electrostatic forces are favourable.

AGPs have been shown to be secreted in callus (Raff *et al.*, 1979) and by stigma cells (Clarke, Gleeson, Harrison *et al.*, 1979) where they are thought to provide part of an ideal adhesive. As yet there is no direct evidence to show that AGPs are secreted into the adhesive layer which forms between stock and scion, but if such evidence became available then AGPs would be strong contenders for the role of messenger molecules. If, however, AGPs are restricted to the plasmalemma, a receptor role, rather than a signalling role is implied. It remains possible that the putative recognition molecules and their receptors could both be members of this group of compounds.

7.4.3: Auxin inhibition may underlie *N. physaloides/L. esculentum* incompatibility

Experiments performed by Sachs (1969) indicated that the gradients or fluxes resulting from the interruption of auxin transport may guide the orderly differentiation of WVMs in compatible grafts. This certainly appears to be true for *L. esculentum* and *N. physaloides* homografts, but does not explain the disorganised development of xylem WVMs in incompatible *N. physaloides/L. esculentum* heterografts unless the natural dispersal of IAA, when stock and scion come into contact, is prevented by some, as yet, unidentified means. This observation agrees with Parkinson's observations (1983) that when applications of the auxin inhibitor tri-iodo benzoic acid (TIBA) were made to the GU of compatible grafts, a distribution of WVM differentiation highly similar to that seen in the incompatible *N. physaloides/L. esculentum* heterograft was induced. Parkinson (1983) noted that *N. physaloides* exhibits lower levels of acropetal auxin movement than *L. esculentum*, which may contribute to the situation, but is not wholly responsible since incompatibility between these species has been shown to be independent of the species-orientation of stock and scion. Therefore two possibilities exist: either a form of auxin inhibitor is formed when the two species are grafted together, or, in a situation analagous to that reported from pea stem assays (Branca *et al.*, 1988), oligogalactuonides may act as competitive antagonists of IAA in the GU, and may interfere with IAA uptake or dissemination.

Chapter 8: FUTURE WORK

8.1: DETERMINATION OF CELL SENSITIVITY TO POLYSACCHARIDE EFFECTS

8.1.1: pH range of effects of potential recognition factors

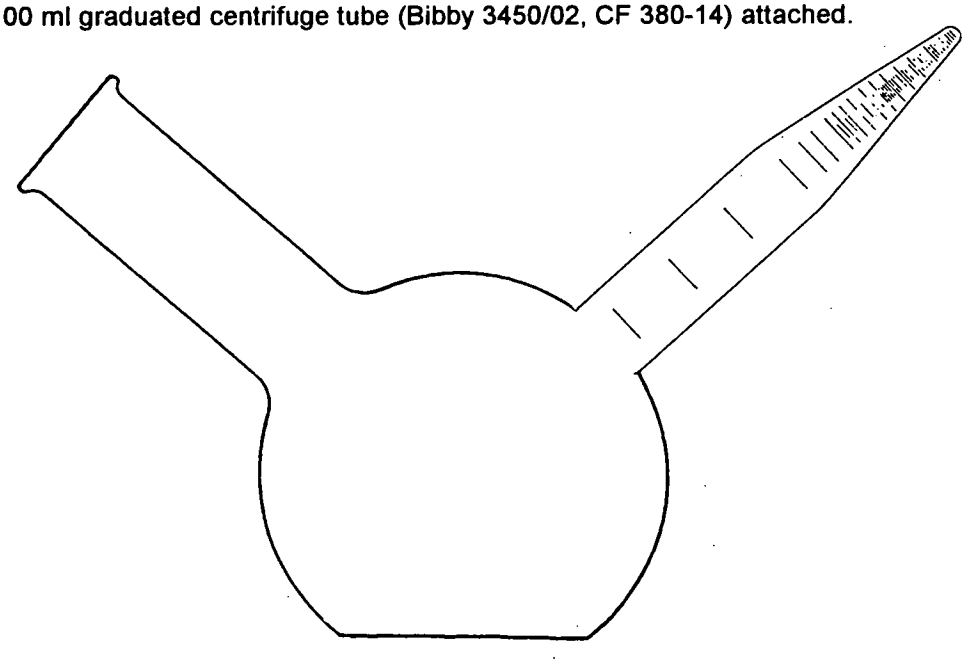
Given the differences in the extent of inhibitive effects caused by hemicelluloses at different pHs (sects. 4.4.1 and 4.4.2), prospective recognition factors, should be screened in a series of L-[U-^{14}C] leucine incorporation assays across the pH range 4.5 - 6.5 to establish patterns of activity both within, and outside, the range of pH observed in the GU, i.e. ~ pH 5.8 - 6.4 (Chapter 6). Better characterisation of size and structure of hemicellulosic and/or pectic polysaccharides responsible for effects already documented would permit particularly promising candidates to be assayed directly by insertion into the GU of *in vitro* grafts.

8.1.2: Influence of background polysaccharide population on signal interpretation

The effect of oligosaccharin signals on suspension-cultured cells may depend upon the mixture of oligo- and polysaccharides present in the incubation medium. Comparison of cell metabolism activities in spent and fresh media ± test polysaccharides would provide a preliminary survey of the situation. Tests to rule out synergism could be performed by the addition of a known population of polysaccharide molecules to the medium together with the test substance and ommitting each polysaccharide in turn.

8.1.3: Long-term effects of potential "off" signals for cell division

The "bird" flask illustrated below would permit a more direct assessment of the effects of polysaccharide fractions on cell division in suspension-cultures. This flask consists of a standard round-bottomed flask (Bibby 1100/18, FK 120-35), one side of which is flattened, with a 100 ml graduated centrifuge tube (Bibby 3450/02, CF 380-14) attached.



Test substances could be added to the medium in a sterile form. Flasks could then be inoculated with normal suspension-cultures, and the volume of cells assessed for the life of the culture (up to 3 weeks). Inversion of the flask, causing cells to sink into the centrifuge tube, would permit the settled cell volume (scv) to be read off. Comparison of scv in flasks ± test substances could provide a series of non-intrusive data from sister cultures, and back-up conclusions drawn from L-[U-^{14}C] leucine incorporation assays regarding the cessation of cell division. Simultaneous cell counts from a parallel set of inoculated flasks could provide complementary data about the size of cells and state of culture division activity (sect. 3.2.4.a.iii and 3.2.4.b.i).

8.1.4: Nature and extent of membrane damage/plasmolysis at pH 4.5

The application of vital dyes to cells of *L. esculentum* AC before and after transfer from hemicellulosic tartaric acid media to hemicellulose-free media at pH 4.5 or pH 5.7 could indicate the extent to which membrane integrity is lost during incubation ± hemicellulose at low pH. A series of patch-clamping experiments could measure electrolyte leakage from the cells and clarify the hypersensitive-like defence responses induced by hemicelluloses at low pH by characterising the metabolic behaviour of "plasmolysed" cells produced by these conditions (sect. 7.1.6).

8.2: DETERMINATION OF FATE OF EXOGENOUSLY APPLIED POLYSACCHARIDES

8.2.1: Characterisation of polysaccharides binding to cells

A series of [^{14}C]-polysaccharide binding assays using characterised fractions of [^{14}C]-labelled polysaccharide would provide more detailed information regarding the size of polysaccharides involved in binding, and particularly those that are internalised.

8.2.2: Detection of structural alterations in [^{14}C]-labelled polysaccharides after incubation

Evidence of structural alterations in particular oligomers after incubation or internalisation by the cells could be revealed by submitting samples of unused and used media and cell rinses to 2-dimensional paper chromatography. Structural alterations should affect the chromatographic mobility of the molecules, and produce different patterns between samples. The use of thin-layer chromatography would permit the retrieval of substances exhibiting altered mobilities for further analysis.

8.2.3: Nature of mechanism for internalisation of [^{14}C]-hemicellulose in cells of *L. esculentum* x *peruvianum*

Cells of *L. esculentum* x *peruvianum* internalise [^{14}C]-hemicellulose at pH 5.7, but not at pH 4.5. This may be due to inhibition of an active internalisation process, or because the

pH lies outside the binding range for the polysaccharides (sect. 7.2.3). The existence of an active internalisation process could be investigated by performing a [^{14}C]-labelled polysaccharide binding assay at 4 °C and pH 5.7 with cells incubated with [^{14}C]-hemicellulose. Cessation of internalisation at 4 °C would indicate the existence of an active pathway. This could be substantiated by incubating membranes from *L. esculentum* x *peruvianum* with [^{14}C]-hemicellulose across a range of pHs, and measuring the radioactivity which became associated with them. Complete absence of binding would indicate that binding and internalisation require energy. However, if binding occurred at moderate pHs, but ceased at pH 4.5, the lack of internalisation observed in cells of *L. esculentum* x *peruvianum* at pH 4.5 was more probably due to pH than the cessation of an active system.

8.3: EVALUATION OF RESULTS WITH EXPERIMENTS ON *IN VITRO* GRAFTS

8.3.1: Characterisation of [^{14}C]-labelled polysaccharides that bind to callus cells in the GU

Application of [^{14}C]-labelled polymers of pectin or hemicellulose to the GU of *in vitro* grafts would permit an assessment of polysaccharide binding *in vivo*. After incubation, the grafts could be separated, excess [^{14}C]-polysaccharide gently rinsed away, callus cells on either side of the GU excised. Techniques used in [^{14}C]-labelled polysaccharide binding assays could be applied to the cells to determine adsorbtion and internalisation of polysaccharide.

8.3.2: Suitability of *L. esculentum* x *peruvianum* cells as model for *L. esculentum* AC in L-[U-^{14}C] leucine incorporation assays

An *in vitro* grafting experiment could evaluate the extent to which suspension-cultured cells of *L. esculentum* x *peruvianum*, which were 75% *L. peruvianum* genetically, provided a suitable substitute for *L. esculentum* AC until it became available in suspension-culture. If *L. peruvianum* formed compatible grafts with *L. esculentum* cv. Ailsa Craig, and incompatible ones with *N. physaloides*, it would imply that use of the suspension-culture of *L. esculentum* x *peruvianum* had been justified.

Seeds of *L. peruvianum* are available, upon request, from the Tomato Genetics Stocks Center, UC, Davis, California, USA, and can be germinated either after passage through a Galapagos giant tortoise, or after 25 minutes in a 1:4 (v/v) solution of commercial sodium hypochlorite (10 - 14% free chlorine) followed by thorough rinsing. The first internodes (Parkinson and Yeoman, 1982), are approximately 9 - 20 mm long. Tissue survives normal sterilisation, grafting manipulations, and incubation of grafts. However, when submitted to the staining and clearing processes prior to microscope examination; stock and scion tend to separate, thus making interpretation of events in the GU difficult.

A grafting experiment has been performed with *L. peruvianum*, but due to the low number of *L. peruvianum*-containing grafts which survived the fixing process intact, and the uncharacteristic failure of the *L. esculentum* material to graft to itself, no conclusions could be drawn about the behaviour of *L. peruvianum* within this Solanaceous model system.

8.3.3: Characterisation of contents of apoplastic fluid from the GU

The samples of GU apoplastic fluid (Chapter 6) were pooled, according to graft type and number of days after grafting, and frozen. These could be blotted on nylon membranes (Jeffree, 1993) and a profile of polysaccharides present in the apoplast during the first seven days after grafting obtained. Any molecules identified could be tested in L-[U-^{14}C] leucine incorporation assays and/or [^{14}C]-polysaccharide binding assays.

Chapter 9: CONCLUSIONS

Since the principles and phenomena discovered in scientific research become better characterised and understood with subsequent research into an area, the reader should note that conclusions presented here outline the current state of understanding of the situation which emerged from this program of research, and should not be interpreted as a definitive statement of final understanding.

The primary objective of this research was to identify the nature of the compound responsible for the "off" signal for cell division in compatible grafts. Although cell wall-derived polysaccharides did produce effects in the L-[U-^{14}C] leucine incorporation assay (Chs. 3 and 4), which were consistent with a slowing or cessation of cell division, it has not been possible, in the time available, to identify this compound. Neither has it been possible to test the relevance of results obtained from the L-[U-^{14}C] leucine incorporation assay in the GU of real grafts. The following are presented as realistic conclusions drawn from this body of information, on the understanding that they are open to future enquiry to clarify mode of operation, relevance to grafting and any other points of discussion.

Conclusions:

1. The L-[U-^{14}C] leucine incorporation assay model system, designed to enable rapid screening of potential candidates for the "off" signal, provides a powerful tool with which to investigate all manner of metabolic effects of oligosaccharides or other biologically-active compounds on this plant material.
2. The L-[U-^{14}C] leucine incorporation assay assesses current metabolic activity in cells and should not be employed as a viability test.
3. The use of suspension-cultured cells of *L. esculentum* x *peruvianum* in preliminary assays, prior to generation of cultures of *L. esculentum* AC, was justified as this material generally produced results similar to those obtained at a later date with cells of *L. esculentum* AC.
4. A component of cell walls slows L-[U-^{14}C] leucine incorporation in cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC.
5. Repeatable effect of pectins and pectic fragments could not be established with the L-[U-^{14}C] leucine incorporation assay. The basis of the variability detected was not determined.

6. The pectic preparations were not toxic to cells of *L. esculentum* x *peruvianum* or *L. esculentum* AC.

7. At pH 4.5, hemicellulosic preparations were somewhat toxic to cells of *L. esculentum* x *peruvianum* and *L. esculentum* AC.

8. The effect of hemicelluloses in the L-[U-^{14}C] leucine incorporation assay was pH-dependent.

9. Incubation of *L. esculentum* x *peruvianum* and *L. esculentum* AC cells at pH 4.5 induced false plasmolysis, which was exacerbated in the presence of hemicelluloses.

10. Membranes are not the active signalling molecules in this system, but may be the site of receptors.

11. Experiments with radioactively-labelled cell wall polysaccharides showed that a percentage of pectin applied to cells of *L. esculentum* x *peruvianum*, <15%, became associated with the cells. 75% of the *associated* pectin bound to the outside of the cells, while the remaining 25% was internalised.

12. The quantity of [^{14}C]-hemicellulose which became externally bound to cells was probably determined by the surface area available, and did not exceed 8% of the polysaccharide applied.

13. Cells of *L. esculentum* AC internalised 8% of [^{14}C]-hemicellulose applied at pH 5.7, and 6.8% at pH 4.5.

14. Cells of *L. esculentum* x *peruvianum* incorporated 2.6% of [^{14}C]-hemicellulose applied at pH 5.7, but only 0.02% at pH 4.5.

15. Changes of up to 0.56 units in the prevailing apoplastic pH of the GU area occur in the first seven days after grafting, with the lowest pH detected on the fourth day, in all combinations tested.

In summary, the observations reported here provide some evidence that components of the normal internodal cell walls have effects on cell metabolism which may be consistent with the hypothesis of Yeoman (1984) that cell wall fragments could be involved in cell signalling leading to graft incompatibility or compatibility. However, the results provide little support for the suggestion of Jeffree, Yeoman, Parkinson *et al.* (1987) that a pectic fragment might be the signalling molecule. The activity of hemicellulose fractions of the cell walls in slowing protein synthesis and the relationship of this effect with pH are significant findings in that few oligosaccharin effects involving hemicelluloses are documented. The role of hemicelluloses in cell signalling, and their possible involvement in graft compatibility requires further investigation.

REFERENCES

ALBERSHEIM, P. (1976). The primary cell wall. In: *Plant Biochemistry*, (Eds. J. Bonner and J.E. Varner), pp 226-274. Academic Press, New York.

ALBERSHEIM, P., DARVILL, A.G., McNEIL, M., VALENT, B.S., SHARP, J.K., NOTHNAGEL, E.A., DAVIS, K.R. and YAMAZAKI, N. (1983). Oligosaccharins: naturally-occurring carbohydrates with biological regulatory functions. In: *Structure and Function of Plant Genomes*. NATO Advanced Study Institute on the Structure and Function of the Plant Genome, Lake Garda, Italy.(Eds. O. Ciferri and L. Dure) pp 293-312. Plenum, New York.

ALBERSHEIM, P., DARVILL, A.G., SHARP, J.K., DAVIS, K.R. and DOARES, S.H. (1986). Studies on the role of carbohydrates in host-microbe interactions. *Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions*. NATO Series, **Vol. H4**; (Ed. Ben Lutenberg), pp. 297-309. Springer-Verlag, Heidelberg.

ALDINGTON, S. and FRY, S.C. (1993). Oligosaccharins. *Advances in Botanical Research*, **19**, 1-101.

ALDINGTON, S. and FRY, S.C. (1994). Rhamnogalacturonan-II - a biologically active fragment. *Journal of Experimental Botany*, **45**, 287-293.

ANDERSON, R.L., CLARKE, A.E., JERMYN, M.A., KNOX, R.B. and STONE, B.A. (1977). A carbohydrate-binding arabinogalactan-protein from liquid suspension cultures of endosperm from *Lolium multiflorum*. *Australian Journal of Plant Physiology*, **4**, 143-158.

ANDERSON, D.M.W. and DEA, I.C.M. (1969). Chemotaxonomic aspects of the chemistry of *Acacia* gum exudates. (Review). *Phytochemistry*, **8**, 167-176.

APOSTOL, I., HEINSTEIN, P.F. and LOW, P.S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Role in defense and signal transduction. *Plant Physiology*, **90**, 109-116.

ATSATT, P.R., HEARN, T.F., NELSON, R.L. and HEINEMAN, R.T. (1978). Chemical induction and repression of haustoria in *Orthocarpus purpurascens* (Scrophulariaceae). *Annals of Botany*, **42**, 117-1184.

AUGUR, C., YU, L., SAKAI, K., OGAWA, T., SINAY, P., DARVILL, A.G. and ALBERSHEIM, P. (1992). Further studies of the ability of xyloglucan oligosaccharides to inhibit auxin-stimulated growth. *Plant Physiology*, **99**, 180-185.

AYERS, A.R., EBEL, J., FINELLI, F., BERGER, N. and ALBERSHEIM, P. (1976a). Host-pathogen interactions. IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. *Plant Physiology*, **57**, 751-759.

AYERS, A.R., EBEL, J., VALENT, B. and ALBERSHEIM, P. (1976b). Host-pathogen interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var *sojae*. *Plant Physiology*, **57**, 760-765.

AYERS, A.R., VALENT, B., EBEL, J. and ALBERSHEIM, P. (1976c). Host-pathogen interactions. XI. Composition and structure of wall-released elicitor fractions. *Plant Physiology*, **57**, 766-774.

BAILEY, J.A. (1983). Biological perspectives of host-pathogen interactions. In: *The Dynamics of Host Defence*. (Eds. J.A. Bailey and B.J. Deverall), p1. Academic Press, Sydney.

BARON-EPEL, O., GHARYAL, P.K. and SCHINDLER, M. (1988). Pectins as mediators of wall porosity in soybean cells. *Planta*, **175**, 389-395.

BASHAM, H.G. and BATEMAN, D.F. (1975). Killing of plant cells by pectic enzymes: the lack of direct injurious interaction between pectic enzymes or their soluble reaction products and plant cells. *Phytopathology*, **65**, 141-153.

BAUER, W.D., TALMADGE, K.W., KEEGSTRA, K. and ALBERSHEIM, P. (1973). The structure of plant cell walls. II. The hemicellulose of suspension-cultured sycamore cells. *Plant Physiology*, **51**, 174-184.

BAYDOUN, E.A.-H. and FRY. S.C. (1985). The immobility of pectic substances in injured tomato leaves and its bearing on the identity of the wound hormone. *Planta*, **165**, 269-276.

BENHAMOU, N., MAZAU, D. and ESQUERRÉ-TUGAYÉ, M.T. (1990). Immunocytochemical localization of hydroxyproline-rich glycoproteins in tomato root cells infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*: study of a compatible interaction. *Phytopathology*, **80**, 163-173.

BENNET-CLARK, T.A., (1959). Water relations of cells. In: *Plant physiology*, (Ed. F.C. Steward) Vol **2**, pp 105-191. Academic Press, New York.

BIGGS, K.J. (1988). Tyrosine cross-links in plant cell wall glycoprotein. Ph.D. Thesis, University of Edinburgh.

BISHOP, P.D., MAKUS, D.J., PEARCE, D.J. and RYAN, C.A. (1981). Proteinase inhibiting-inducing factor activity in tomato leaves resides in oligosaccharides enzymically released from cell walls. *Proceedings of the National Academy of Sciences, U.S.A.*, **78**, 3536-3540.

BISHOP, P.D., PEARCE, G., BRYANT, J.E. and RYAN, C.A. (1984). Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves. Identity and activity of poly and oligogalacturonide fragments. *Journal of Biological Chemistry*, **259**, 13172-13177.

BOHOOL, B.B. and SCHMIDT, E.L. (1974). Lectins: A possible basis for specificity in the rhizobium-legume root nodule symbiosis. *Science*, **185**, 269-271.

BOIS, F. (1992). The influence of some natural cell-wall derived precursors on organogenesis and differentiation of wild strawberry (*Fragaria vesca* L.) callus cultures. *Plant Cell, Tissue and Organ Culture*, **28**, 91-96.

BOLLER, T. and VOGELI, U. (1984). Vacuolar localization of ethylene-induced chitinase in bean leaves. *Plant Physiology*, **74**, 442-444.

BORMANN, F.H. (1966). The structure, function and ecological significance of root grafts on *Pinus strobus* L. *Ecological Monographs*, **36**, 1-26.

BOUTENKO, R.G. and VOLODARSKY, A.D. (1968). Analyse immunochimique de la differenciation cellulaire dans les cultures de tissus de Tabac. *Physiologie Vegetale*, **6**, 299-309.

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

BRANCA, C., DE LORENZO, G. and CERVONE, F. (1988). Competitive inhibition of the auxin-induced elongation by α-D-oligogalacturonides in pea stem segments. *Physiologia Plantarum*, **72**, 499-504.

BRIDGE, M. and KLARMAN, W.L. (1970). Ultraviolet induction of an antifungal chemical in soybeans. *Phytopathology*, **60**, 1013.

BROWN, I., MANSFIELD, J., IRLAM, I., CONRADS-STRAUCH, J. and BONAS, U. (1993). Ultrastructure of interactions between *Xanthomonas campestris* pv. vesicatoria and pepper, including immunocytochemical localization of extracellular polysaccharide and the AvrBs3 protein. *Molecular Plant-Microbe Interactions*, **6**, 376-386.

BROWN, R. and RICKLESS, P. (1949). A new method for the study of cell division and cell extension with primary observations on the effect of temperature and nutrients. *Proceedings of the Royal Society of London Series B: Biological Sciences*, **136**, 110-125.

BRUCE, R.J. and WEST, C.A. (1982). Elicitation of casbene synthetase activity in castor bean. The role of pectic fragments of the plant cell wall in elicitation by a fungal endopolygalacturonase. *Plant Physiology*, **69**, 1181-1188.

BRUCE, R.J. and WEST, C.A. (1989). Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiology*, **91**, 889-897.

BUCHELI, P., DOARES, S.H., ALBERSHEIM, P., and DARVILL, A. (1990). Host-pathogen interactions XXXVI. Partial purification and characterization of heat labile-molecules secreted by the rice blast pathogen that solubilize plant cell wall fragments that kill plant cells. *Physiological Molecular Plant Pathology*, **36**, 159-173.

BYRNE, H., CHRISTOU, N.V., VERMA, D.P.S. and MACLACHLAN, G.A. (1975). Purification and characterization of two cellulases from auxin-treated pea epicotyls. *Journal of Biological Chemistry*, **250**, 1012-1018.

CERVONE, F., DE LORENZO, G., DEGRÀ, L. and SALVI, G. (1987). Elicitation of necrosis in *Vigna unguiculata* Walp. by homogeneous *Aspergillus niger* endopolygalacturonase and by α-D-galacturonate oligomers. *Plant Physiology*, **85**, 626-630.

CHEONG, J.-J. and HAHN, M.G. (1991). A specific, high-affinity binding site for the hepta-β-glucoside elicitor exists in soybean membranes. *The Plant Cell*, **3**, 137-147.

CHRISPEELS, M.J. (1969). Synthesis and secretion of hydroxyproline-containing macromolecules in carrots. I. Kinetic analysis. *Plant Physiology*, **44**, 1187-1195.

CLARKE, A.E., ANDERSON, R.L. and STONE, B.A. (1979). Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry*, **18**, 521-540.

CLARKE, A.E., GLEESON, P., HARRISON, S. and KNOX, R.B. (1979). Pollen-stigma interactions: Identification and characterization of surface components with recognition potential. *Proceedings of the National Academy of Sciences, U.S.A.*, **76**, 3358-3362.

CLARKE, A.E., HARRISON, S., KNOX, R.B., RAFF, J., SMITH, P. and MARCHALONIS, J.J. (1977). Common antigens and male-female recognition in plants. *Nature*, **265**, 161-163.

CLARKE, A.E. and KNOX, R.B. (1978). Cell recognition in flowering plants. *The Quarterly Review of Biology*, **53**, 3-28.

CLELAND, R.E. (1981). Wall extensibility: hormones and wall extension. In: *Plant Carbohydrates. II. Extracellular carbohydrates. Encyclopedia of Plant Physiology*, New Series, Volume **13B**. (Eds. W. Tanner and F.A. Loewus), pp255-276, Springer, Berlin.

COPES, D. (1969). Graft union formation in Douglas-fir. *American Journal of Botany*, **56**, 285-289.

COSIO, E.G., FREY, T., VERDUYN, R., VAN BOOM, J. and EBEL, J. (1990). High-affinity binding of a synthetic heptaglucoside and fungal glucan phytoalexin elicitors to soybean membranes. *FEBS Letters*, **271**, 223-226.

COSIO, E.G., PÖPPERL, H., SCHMIDT, W.E. and EBEL, J. (1988). High-affinity binding of fungal β-glucan fragments to soybean (*Glycine max* L.) microsomal fractions and protoplasts. *European Journal of Biochemistry*, **175**, 309-315.

COUTTS, M.P. (1983). Root architecture and tree stability. *Plant and Soil*, **71**, 171-188.

CRAM, W.J. (1980). Pinocytosis in plants. *New Phytologist*, **84**, 1-17.

DARVILL, A.G., McNEIL, M. and ALBERSHEIM, P. (1978). Structure of plant cell walls VIII. A new pectic polysaccharide. *Plant Physiology*, **62**, 418-422.

DARVILL, A.G., ALBERSHEIM, P., McNEIL, M., LAU, J.M., YORK, W.S., STEVENSTON, T.T., THOMAS, J., DOARES, S., GOLLIN, D.J., CHELF, P. and DAVIS, K. (1985). Structure and function of plant cell wall polysaccharides. *Journal of Cell Science Supplement*, **2**, 203-217.

DAVIES, W.P. and LEWIS, B.G. (1981). Development of pectic projections on the surface of wound callus cells of *Daucus carota* L. *Annals of Botany*, **47**, 409-414.

DAVIS, K.R., DARVILL, A.G., ALBERSHEIM, P. and DELL, A. (1986a). Host-pathogen interactions. XXIX. Oligogalacturonides released from sodium polypectate by endopolygalacturonic acid lyase are elicitors of phytoalexins in soybean. *Plant Physiology*, **80**, 568-577.

DAVIS, K.R., DARVILL, A.G., ALBERSHEIM, P. and DELL, A. (1986b). Host-pathogen interactions. XXX. Characterization of elicitors of phytoalexin accumulation in soybean released from soybean cell walls by endopolygalacturonic acid lyase. *Zeitschrift fur Naturforschung Section C - Biosciences*, **41**, 39-48.

DAVIS, K.R., DARVILL, A.G. and ALBERSHEIM, P. (1986c). Host-pathogen interactions. XXXI. Several biotic and abiotic elicitors act synergistically in the induction of phytoalexin accumulation in soybean. *Plant Molecular Biology*, **6**, 23-32.

DAVIS, K.R., LYON, G.D., DARVILL, A.G. and ALBERSHEIM, P. (1982). A polygalacturonic acid lyase isolated from *Erwinia caratovora* is an elicitor of phytoalexins in soybeans. *Plant Physiology*, **69**, S-142.

DE WIT, P.J.G.M. and KODDE, E. (1981). Further characterization and cultivar-specificity of glycoprotein elicitors from culture filtrates and cell walls of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Physiological Plant Pathology*, **18**, 297-314.

DHINDSA, R.S., PLUMB-DHINDSA, P. and THORPE, T.A. (1981). Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *Journal of Experimental Botany*, **32**, 93-101.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. and SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, **28**, 350-356.

DU PONT, M.S. and SELVENDRAN, R.R. (1987). Hemicellulosic polymers from the cell walls of beeswing wheat bran. I. Polymers solubilised by alkali at 2 °C. *Carbohydrate Research*, **163**, 99-113.

EBEL, J., AYERS, A.R. and ALBERSHEIM, P. (1976). Host-pathogen interactions. XII. Response of suspension-cultured soybean cells to the elicitor isolated from *Phytophthora megasperma* var. *sojae*, a fungal pathogen of soybeans. *Plant Physiology*, **57**, 775-779.

EBERHARD, S., DOUBRAVA, N., MARFÀ, V., MOHNEN, D., SOUTHWICK, A., DARVILL, A. and ALBERSHEIM, P. (1989). Pectic cell wall fragments regulate tobacco thin-cell-layer explant morphogenesis. *The Plant Cell*, **1**, 747-755.

EDELMANN, H.G. AND FRY, S.C. (1992). Factors that affect the extraction of xyloglucan from the primary cell walls of suspension-cultured rose cells. *Carbohydrate Research*, **228**, 423-431.

EIS, S. (1972). Root grafts and their silvicultural implications. *Canadian Journal of Forest Research*, **2**, 111-120.

ESQUERRÉ-TUGAYÉ, M.T., LAFITTE, C., MAZAU, D., TOPPAN, A. and TOUZÉ, A. (1979). Cell surfaces in plant-microorganism interactions. II. Evidence for the accumulation of hydroxyproline-rich glycoproteins in the cell wall of diseased plants as a defense mechanism. *Plant Physiology*, **64**, 320-326.

ESQUERRÉ-TUGAYÉ, M.T. and LAMPORT, D.T.A. (1979). Cell surfaces in plant-microorganism interactions. I. A structural investigation of cell wall hydroxyproline-rich glycoproteins which accumulate in fungus-infected plants. *Plant Physiology*, **64**, 314-319.

ESQUERRÉ-TUGAYÉ, M.T. and MAZAU, D. (1974). Effect of a fungal disease on extensin, the plant cell wall glycoprotein. *Journal of Experimental Botany*, **25**, 509-513.

FARMER, E.E., PEARCE, G. and RYAN, C.A. (1989). *In vitro* phosphorylation of plant plasma membrane proteins in response to the proteinase inhibitor inducing factor. *Proceedings of the National Academy of Sciences, USA*, **86**, 1539-1542.

FELIX, G., GROSSKOPF, D.G., REGENASS, M. and BOLLER, T. (1991). Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells. *Proceedings of the National Academy of Sciences, U.S.A.*, **88**, 8831-8834.

FINCHER, G.B., STONE, B.A. and CLARKE, A.E. (1983). Arabinoglactan-proteins: Structure, biosynthesis and function. *Annual Review of Plant Physiology*, **34**, 47-70.

FOSKET, D.E. and TORREY, J. (1969). Hormonal control of cell proliferation and xylem differentiation in cultured tissues of *Glycine max* var. Biloxy. *Plant Physiology*, **44**, 871-880.

FRY, S.C. (1982a). Phenolic components of the primary cell wall: feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. *Biochemical Journal*, **203**, 439-504.

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

FRY, S.C. (1986). *In-vivo* formation of xyloglucan nonasaccharide: A possible biologically active cell wall fragment. *Planta*, **169**, 443-453.

FRY, S.C. (1988). The growing plant cell wall: chemical and metabolic analysis. Ed. Wilkins, M., Longman Scientific and Technical, Harlow, Essex.

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

FRY, S.C. (1989b). Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. *Physiologia Plantarum*, **75**, 532-536.

FRY, S.C., DARVILL, A.G. and ALBERSHEIM, P. (1983). Amino acid transport and protein synthesis: possible primary targets of biologically active cell wall fragments. *Interactions between nitrogen and growth regulators in plant development.* Monograph 9, (Ed. M.B. Jackson) British Plant Growth Regulator Group, Wantage, Oxfordshire, pp. 33-43.

FRY, S.C., McDOUGALL, G.J., LORENCES, E.P., BIGGS, K.J. and SMITH, R.C. (1990). Oligosaccharins from xyloglucan and cellulose: modulators of the action of auxin and H^+ on plant growth. In: *Hormone Perception and Signal Transduction in Animals and Plants.* Symposium XLIV Society for Experimental Biology. (Eds. J. Roberts, C. Kirk and M. Venis), pp 285-298.

FUSHTEY, S.G. (1957). Studies in the physiology of parasitism. XXIV. Further experiments on the killing of plant cells by fungal and bacterial extracts. *Annals of Botany*, **21**, 273-286.

GARNER, R.J. (1967). *The grafter's handbook.* (3rd edition) Shenval Press Ltd., London and Harlow.

GÄUMANN, E. (1950). Principles of Plant Infection. Crosby Lockwood and Son Ltd., London.

GIEBEL, J. and STOBIECKA, M. (1974). Role of amino acids in plant tissue response to *Heterodera rostochiensis*. I. Protein, proline and hydroxyproline content in the roots of susceptible and resistant solanacous plants. *Nematologica*, **20**, 407.

GILROY, S., FRICKER, M.D., READ, N.D. and TREWAVAS, A.J. (1991). Role of calcium in signal transduction of *Commelina* guard cells. *Plant Cell*, **3**, 333-344.

GIROTTI, A.W., THOMAS, J.P. and JORDAN, J.E. (1985). Lipid photooxidation in erythrocyte ghosts: sensitization of the membranes towards ascorbate and superoxide induced peroxidation and lysis. *Archives of Biochemistry and Biophysics*, **236**, 138-251.

GLAZENER, J.A., ORLANDI, E.W., HARMON, G.L. and BAKER, J.C. (1991). An improved method for monitoring active oxygen in bacteria-treated suspension cultures using luminol-dependent chemiluminesence. *Physiological Molecular Plant Pathology*, **39**, 123-133.

GOLLIN, D.J., DARVILL, A.G. and ALBERSHEIM, P. (1984). Plant cell wall fragments inhibit flowering and promote vegetative growth in *Lemna gibba* G3. *Biology of the Cell*, **51**, 275-280.

GRAHAM, B.F. and BORMANN, F.H. (1966). Natural root grafts. *Botanical Review*, **32**, 255-292.

GREEN, T.R. and RYAN, C.A. (1972). Wound-induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. *Science*, **175**, 776-777.

GREEN, T.R. and RYAN, C.A. (1973). Wound-induced proteinase inhibitor in tomato leaves. Some effects of light and temperature on the wound response. *Plant Physiology*, **51**, 19-21.

GROSSKOPF, D.G., FELIX, G. and BOLLER, T. (1991). A yeast-derived glycopeptide elicitor and chitosan or digitonin differentially induce ethylene biosynthesis, phenylalanine ammonia-lyase and callose formation in suspension-cultured tomato cells. *Journal of Plant Physiology*, **138**, 741-746.

GUR, A. and BLUM, A. (1973). The role of cyanogenic glycoside in incompatibility between peach scions and almond rootstocks. *Horticultural Research*, **13**, 1-10.

GUR, A., SAMISH, R.M. and LIFSHITZ, E. (1968). The role of cyanogenic glycoside of the quince in the incompatibility between pear cultivars and quince rootstocks. *Horticultural Research*, **8**, 113-134.

HADWIGER, L.A. and SCHWOCHAU, M.E. (1971). Ultraviolet light-induced formation of pisatin and phenylalanine ammonia lyase. *Plant Physiology*, **47**, 588-590.

HAHN, M.G., DARVILL, A.G. and ALBERSHEIM, P. (1981). Host-pathogen interactions. XIX. The endogenous elicitor, a fragment of plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. *Plant Physiology*, **68**, 1161-1169.

HALLIWELL, B and GUTTERIDGE, J.M.C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*, **319**, 1-14.

HAMBLIN, J. and KENT, S.P. (1973). Possible role of phytohaemagglutinins in *Phaseolus vulgaris* L. *Nature, New Biology*, London, **245**, 28-30.

HAMMERSCHMIDT, R., LAMPORT, D.T.A. and MULDOON, E.P. (1984). Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerinum*. *Physiological Plant Pathology*, **24**, 43-47.

HANCHEY, P., WHEELER, H. (1969). Pathological changes in ultra-structure: false plasmolysis. *Canadian Journal of Botany*, **47**, 675-678.

HARTMANN, H.T. and KESTER, D.E. (1975). *Plant Propagation: Principles and Practices.* (3rd edition) Englewood Cliffs, New Jersey; Prentice-Hall, Inc..

HAYASHI, T., KATO, Y. AND MATSUDA, K. (1980). Xyloglucan from suspension-cultured soybean cells. *Plant and Cell Physiology*, **21**, 1405-1418.

HAYASHI, T. and MACLACHLAN, G. (1984a). Pea xyloglucan and cellulose. I. Macromolecular organization. *Plant Physiology*, **75**, 596-604.

HAYASHI, T. and MACLACHLAN, G. (1984b). Pea xyloglucan and cellulose. III. Metabolism during lateral expansion of pea epicotyl cells. *Plant Physiology*, **76**, 739-742.

HAYASHI, T., MARSDEN, M.P.F. and DELMER, D.P. (1987). Pea xyloglucan and cellulose. V. Xyloglucan-cellulose interactions *in vitro* and *in vivo*. *Plant Physiology*, **83**, 384-389.

HESLOP-HARRISON, J. (1975). Incompatibility and the pollen-stigma interaction. *Annual Review of Plant Physiology*, **26**, 403-425.

HESLOP-HARRISON, J. and HESLOP-HARRISON, Y. (1970). Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. *Stain Technology*, **45**, 115-120.

HINCHE, J. M. and CLARKE, A.E. (1980). Adhesion of fungal zoospores to root surfaces is mediated by carbohydrate determinants of the root slime. *Physiological Plant Pathology*, **16**, 303-307.

HITCHCOCK, C. and NICHOLS, B.W. (1971). Biological degredation of plant fatty acids. In: *Plant Lipid Biochemistry*, (Eds. J.F.Sutcliffe and P.Mahlberg) pp 201-235. Academic Press, London.

HOLDEN, M.A. (1985). Studies on epidermal grafting in the Solanaceae. Ph.D. Thesis, University of Edinburgh.

HOLDEN, M.A., JEFFREE, C.E. and YEOMAN, M.M. (1987). Development of "epidermal" grafts on cultured internodes of *Datura stramonium*. *New Phytologist*, **106**, 497-503.

HOLLIDAY, M.J., KEEN, N.T. and LONG, M. (1981). Cell death patterns and accumulation of fluorescent material in the hypersensitive response of soybean leaves to *Pseudomonas syringae* pv. *glycinea*. *Physiological Plant Pathology*,**18**, 270-287.

HOPPER, D.G., VENERE, R.J., BRINKERHOFF, L.A. and GHOLSON, R.K. (1975). Necrosis induction in cotton. *Phytopathology*, **65**, 206-213.

HORN, M.A., HEINSTEIN, P.F. and LOW, P.S. (1989). Receptor-mediated endocytosis in plant cells. *The Plant Cell*, **1**, 1003-1009.

HORN, M.A., MEADOWS, R.P., APOSTOL, I., JONES, C.R., GORENSTEIN, D.G., HEINSTEIN, P.F. and LOW, P.S. (1991). Effect of elicitation and changes in extracellular pH on the cytoplasmic and vacuolar pH of suspension-cultured soybean cells. *Plant Physiology*, **98**, 680-686.

HOSSAIN, M.A. (1986). Studies on grafting in the Leguminosae using *in vitro* techniques. Ph.D. Thesis, University of Edinburgh.

HUBNER, R., DEPTA, H., and ROBINSON, D.G. (1985). Endocytosis in maize root cap cells. Evidence obtained using heavy metal salt solutions. *Protoplasma*, **129**, 214-222.

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

JEFFREE, C.E. (1993). Immobilization of pectin, xyloglucan and other soluble plant polysaccharides on blotting membranes. *New Phytologist*, **125**, 695-706.

JEFFREE, C.E., GORDON, F. and YEOMAN, M.M. (1989). Pectinaceous beads and pectinase on callus cell surfaces in graft unions and in culture. In: NATO ASI Series, Vol. **H35**, *Cell Separation in Plants*, (Eds. D.J. Osborne and M.B. Jackson), pp 287-299. Springer-Verlag, Berlin, Heidelberg.

JEFFREE, C.E. and YEOMAN, M.M. (1981). A study of the intracellular and intercellular distribution of the *Datura stramonium* lectin using an immunofluorescent technique. *New Phytologist*, **87**, 463-471.

JEFFREE, C.E. and YEOMAN, M.M. (1983). Development of intercellular connections between opposing cells in a graft union. *New Phytologist*, **93**, 491-509.

JEFFREE, C.E., YEOMAN, M.M., PARKINSON, M. and HOLDEN, M.A. (1987). The chemical basis of cell to cell contact and its possible role in differentiation. *British Plant Growth Regulator Group*, Monograph **16**. "Advances in the Chemical Manipulation of Plant Tissue Cultures." Eds. M.B. Jackson, S.H. Mantell and J. Blake.

JIN, D.F. and WEST, C.A. (1984). Characteristics of galacturonic acid oligomers as elicitors of casbene synthetase activity in castor bean seedlings. *Plant Physiology*, **74**, 989-992.

JOSELEAU, J.-P. and CHAMBAT, G. (1984). Structure of the primary cell walls of suspension-cultured *Rosa glauca* cells. II. Multiple forms of xyloglucans. *Plant Physiology*, **74**, 694-700.

KATO, Y. AND MATSUDA, K. (1976). Presence of a xyloglucan in the cell wall of *Phaseolus aureus* hypocotyls. *Plant and Cell Physiology*, **17**, 1185-1198.

KATO, Y. and MATSUDA, K. (1980a). Structure of oligosaccharides obtained by controlled degredation of mung bean xyloglucan with acid and *Aspergillus oryzae* enzyme preparation. *Agricultural and Biological Chemistry*, **44**, 1751-1758.

KATO, Y. and MATSUDA, K. (1980b). Structure of oligosaccharides obtained by hydrolysis of mung bean xyloglucan with *Trichoderma viride* cellulase. *Agricultural and Biological Chemistry*, **44**, 1759-1766.

KATO, Y. and MATSUDA, K. (1985). Xyloglucan in cell walls of suspension-cultured rice cells. *Plant and Cell Physiology*, **26**, 437-445.

KEEGSTRA, K., 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**, 188-196.

KEENAN, P., BRYAN, I.B. and FRIEND, J. (1985). The elicitation of the hypersensitive response of potato tuber tissue by a component of the culture filtrate of *Phytophthora infestans*. *Physiological Plant Pathology*, **26**, 343-355.

KEPPLER, L.D. and NOVACKY, A. (1986). Involvement of membrane lipid peroxidation in the development of a bacterially induced hypersensitive reaction. *Phytopathology*, **76**, 104-108.

KHAVKIN, E.E., MISHARIN, F.I., IVANOV, E.N. and DANOVITCH, K.N. (1977). Embryonal antigens in maize caryopses: The temporal order of antigen accumulation during embryogenesis. *Planta*, **135**, 225-231.

KIELISZEWSKI, M.J. and LAMPORT, D.T.A. (1994). Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *The Plant Journal*, **5**, 157-172.

KLEMENT, Z. (1982). Hypersensitivity. In: *Phytopathogenic Prokaryotes* Vol. **2**, (Eds. M.S. Mount and G.H. Lacey) pp 149-177. Academic Press, New York.

KNOX, R.B. and CLARKE, A.E. (1984). Cell recognition in plants. In: *Developmental Control in Animals and Plants* (2nd edition) (Eds. C.F. Graham and P.F. Waring), pp 191-217. Blackwell Scientific Publications, Oxford.

KOHLE, H., YOUNG, D.H. and KAUSS, H. (1984). Physiological changes in suspension-cultured soybean cells elicited by treatment with chitosan. *Plant Science Letters*, **33**, 221-230.

KOLLMANN, R. and GLOCKMANN, C. (1985). Studies on graft unions. I. Plasmodesmata between cells of plants belonging to different unrelated taxa. *Protoplasma*, **124**, 224-235.

KOLLMANN, R., YANG, S., and GLOCKMANN, C. (1985) Studies on graft unions. II. Continuous and half plasmodesmata in different regions of the graft interface. *Protoplasma*, **126**, 19-29.

KOMALAVILAS, P., ZHU, J-K. and NOTHNAGEL, E.A. (1991). Arabinogalactan-proteins from the suspension culture medium and plasma membrane of rose cells. *Journal of Biological Chemistry*, **266**, 15956-15965.

KURANTZ, M.J. and ZACHARIUS, R.M. (1981). Hypersensitive response in potato tuber: elicitation by combination of non-eliciting components from *Phytophthora infestans*. *Physiological Plant Pathology*, **18**, 67-77.

KUROSAKI, F., TSURUSAWA, Y. and NISHI, A. (1987). The elicitation of phytoalexins by Ca^{2+} and cyclic AMP in carrot cells. *Phytochemistry*, **26**, 1919-1923.

KUNTZ, J.E. and RIKER, A.J. (1956). Oak wilt. Agricultural Experimental Station of the University of Wisconsin Bulletin, p.19.

LAMB, C.J., LAWTON, M.A., DRON, M. and DIXON, R.A. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. *Cell*, **56**, 215-224.

LAMPORT, D.T.A. (1965). The protein component of primary cell walls. *Advances in Botanical Research*, **2**, 151-218.

LAMPORT, D.T.A. (1967). Hydroxyproline-O-glycosidic linkage of the plant cell wall glycoprotein extensin. *Nature*, **216**, 1322-1324.

LAMPORT, D.T.A. (1969). The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymic degredation of primary cell walls. *Biochemistry*, **8**, 1155-1163.

LAMPORT, D.T.A. (1973). The glycopeptide linkages of extensin: O-D-galactosyl serine and O-L-arabinosyl hydroxyproline. In: *Biogenesis of Plant Cell Wall Polysaccharides* (Ed. F. Leowus), pp 149-164. Academic Press, New York.

LAMPORT, D.T.A. (1978). Cell wall carbohydrates in relation to structure and function. In: *Frontiers of Plant Tissue Culture, Proceedings of 4th International Congress - Plant Tissue and Cell Culture*, (Ed. T.A. Thorpe) pp 235-244. International Association Plant Tissue Culture, University of Calgary, Canada.

LAMPORT, D.T.A. (1986). The primary cell wall: a new model. In: *Cellulose: structure, modification and hydrolysis*. (Eds. R.A. Yound and R.M. Rowell), pp 77-90. Wiley, New York.

LAMPORT, D.T.A., KATONA, L. and ROERIG, S. (1973). Galctosylserine in extensin. *Biochemical Journal*, **133**, 125-131.

LARKIN, P. (1977). Plant protoplast agglutination and membrane-bound β-lectins. *Journal of Cell Science*, **26**, 31-46.

LARKIN, P. (1978). Plant protoplast agglutination by artificial carbohydrate antigens. *Journal of Cell Science*, **30**, 283-292.

LAU, J.M., McNEIL, M., DARVILL, A.G., and ALBERSHEIM, P. (1985). Structure of the backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary cell wall of plants. *Carbohydrate Research*, **137**, 111-125.

LEE, S.-C. and WEST, C.A. (1981). Polygalacturonase from *Rhizopus stolonifer*, an elicitor of casbene synthetase activity in castor bean (*Ricinus communis* L.) seedlings. *Plant Physiology*, **67**, 633-639.

LIÉNART, Y., GAUTIER, C. and DRIGUEZ, H. (1991). Silica-bound *N*-acetylglucosaminyl residues elicit laminarinase activity in *Rubus* protoplasts. *Plant Science*, **77**, 41-45.

LINDSAY, D.W., YEOMAN, M.M. and BROWN, R. (1974). An analysis of the development of the graft union in *Lycopersicon esculentum*. *Annals of Botany*, **38**, No. 156, 639-646.

LIPETZ, J. (1970). Wound-healing in higher plants. *International Review of Cytology*, **27**, 1-28.

LOEHLE, C. and JONES, R.H. (1990). Adaptive significance of root grafting in trees. *Functional Ecology*, **4**, 268-271.

LONGMAN, D. and CALLOW, J.A. (1987). Specific saccharide residues are involved in the recognition of plant root surfaces by zoospores of *Pythium aphanidermatum*. *Physiological Molecular Plant Pathology*, **30**, 139-150.

LOW, P.S. and HEINSTEIN, P.F. (1986). Elicitor stimulation of the defense response in cultured plant cells monitored by fluorescent dyes. *Archives of Biochemistry and Biophysics*, **249**, 472-479.

LYON, F.M. and WOOD, R.K.S. (1975). The hypersensitive reaction and other responses of bean leaves to bacteria. *Annals of Botany*, **40**, 470-491.

LYON, G.D. and ALBERSHEIM, P. (1982). Host-pathogen interactions. XXI. Extraction of a heat-labile elicitor of phytoalexin accumulation from frozen soybean stems. *Plant Physiology*, **70**, 406-409.

MANSFIELD, J.W. (1986). Recognition, elicitors and the hypersensitive reaction. In: *Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions. NATO ASI Series* Vol. **H4** (Ed. B. Lutenberg), pp 433-437. Springer-Verlag, Berlin, Heidelberg.

MANSFIELD, J.W. and BROWN, I.R. (1986). The biology of interactions between plants and bacteria. In: *Biology and Molecular Biology of Plant-Pathogen Interactions*. (Ed. J.A. Bailey), pp 71-98. Springer-Verlag, Berlin, Heidelberg.

MARFÀ, V., GOLLIN, D.J., EBERHARD, S., MOHNEN, D., DARVILL, A. and ALBERSHEIM, P. (1991). Oligogalacturonides are able to induce flowers to form on tobacco explants. *The Plant Journal*, **1**, 217-225.

MASUTA, C., VAN DEN BULCKE, M., BAUW, G., VAN MONTAGU, M. and CAPLAN, A.B. (1991). Differential effects of elicitors on the viability of rice suspension cells. *Plant Physiology*, **97**, 619-629.

MATHIEU, Y., KURKDJIAN, A., XIA, H., GUERN, J., KOLLER, A., SPIRO, M.D., O'NEILL, M., ALBERSHEIM, P. and DARVILL, A. (1991).Membrane responses induced by oligogalacturonides in suspension-cultured tobacco cells. *The Plant Journal*, **1**, 333-343.

McDOUGALL, G.J. and FRY, S.C. (1988). Inhibition of auxin-stimulated growth of pea stem segments by a specific nonasaccharide of xyloglucan. *Planta*, **175**, 412-416.

McDOUGALL, G.J. and FRY, S.C. (1989a). Anti-auxin activity of xyloglucan oligosaccharides: The rôle of groups other than the terminal α-L-fucose residue. *Journal of Experimental Botany*, **40**, 233-238.

McDOUGALL, G.J. and FRY, S.C. (1989b). Structure-activity relationships for xyloglucan oligosaccharides with anti-auxin activity. *Plant Physiology*, **89**, 883-887.

McDOUGALL, G.J. and FRY, S.C. (1990). Xyloglucan oligosaccharides promote growth and activate cellulase: Evidence for a rôle of cellulase in cell expansion. *Plant Physiology*, **93**, 1042-1048.

McFARLAND, D. and RYAN, C.A. (1974). Proteinase inhibitor-inducing factor in plant leaves. A phylogenetic survey. *Plant Physiology*, **54**, 706-708.

McNEIL, M., DARVILL, A.G. and ALBERSHEIM, P. (1980). Structure of plant cell walls. X. Rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. *Plant Physiology*, **66**, 1128-1134.

McNEIL, M., DARVILL, A.G. and ALBERSHEIM, P. (1982). Structure of plant cell walls. XII. Identification of seven differently linked glycosyl residues attached to O-4 of the 2,4-linked L-rhamnosyl residues of rhamnogalacturonan I. *Plant Physiology,* **70**, 1586-1591.

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

MELTON, L.D., McNEIL, M., DARVILL, A.G., ALBERSHEIM, P. and DELL, A. (1986). Structural characterization of oligosaccharides isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydrate Research*, **146**, 279-305.

MESSIAEN, J., READ, N.D., VAN CUTSEM, P. and TREWAVAS, A.J. (1993). Cell wall oligogalacturonides increase cytosolic free calcium in plant cells. *Journal of Cell Science*, **104**, 365-371.

MIEDZYBRODZKA, M.B.W. (1981). Studies on the development of the graft union in *Lycopersicon esculentum* Mill. Ph.D. Thesis, University of Edinburgh.

MILLER, P.W. (1965). The etiology of blackline in grafted Persian walnuts. *Plant Disease Reporter*, **49**, 954.

MILLNER, M.E. (1932). Natural grafting in *Hedera helix*. *New Phytologist*, **32**, 2-25.

MOERSCHBACHER, B., KOGEL, K.H., NOLL, U. and REISENER, H.J. (1986). An elicitor of the hypersensitive lignificaiton response in wheat leaves isolated from the rust fungus *Puccinia graminis* f.sp. *tritici*. I. Partial purification and characterization. *Zeitschrift fur Naturforschung*, **41c**, 830-838.

MOORE, P.J., DARVILL, A.G., ALBERSHEIM, P. and STAEHELIN, L.A. (1986). Immunogold localization of xyloglucan and rhamnogalacturonan I in the cell walls of suspension-cultured sycamore cells. *Plant Physiology*, **82**, 787-794.

MOORE, P.J. and STAEHELIN, L.A. (1988). Immunogold localization of the cell-wall-matrix polysaccharides rhamnoglacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways. *Planta*, **174**, 433-445.

MOORE, R. (1982). Studies of vegetative compatibility-incompatibility in higher plants. V. A morphometric analysis of the development of a compatible and an incompatible graft. *Canadian Journal of Botany*, **60**, 2780-2787.

MOORE, R. (1984). A model for graft compatibility-incompatibility in higher plants. *American Journal of Botany*, **71**, 752-758.

MOORE, R. and WALKER, D.B. (1981a). Studies of vegetative compatibility-incompatibility in higher plants. I. A structural study of a compatible autograft in *Sedum telephoides* (Crassulaceae). *American Journal of Botany*, **68**, 820-830.

MOORE, R. and WALKER, D.B. (1981b). Studies of vegetative compatibility-incompatibility in higher plants. II. A structural study of an incompatible heterograft between *Sedum telephoides* (Crassulaceae) and *Solanum pennellii* (Solanaceae). *American Journal of Botany*, **68**, 831-842.

MUZIC, T.J. (1958). Role of parenchyma cells in graft union in *Vanilla* orchid. *Science*, **127**, 82.

MUZIC T.J. and LaRUE, C.D. (1954). Further studies on the grafting of monocotyledonous plants. *American Journal of Botany*, **41**, 448-55.

NARI, J., NOAT, G., RICARD, J., FRANCHINI, E. and MOUSTACAS, A-M. (1983). Catalytic properties and tentative function of a cell wall β-glucosyltransferase from soybean cells cultured in vitro. *Plant Science Letters*, **28**, 313-320.

NEILSON-JONES, W. (1969). In: *Plant Chimeras*. (Ed. W. Neilson-Jones) pp 1-13, Camelot Press Ltd., London and Southampton.

NISHITANI, K. and MASUDA, Y. (1982). Acid pH-induced structural changes in cell wall xyloglucans in *Vigna angularis* epicotyl segments. *Plant Science Letters*, **28**, 87-94.

NISHITANI, K. and MASUDA, Y. (1983). Auxin-induced changes in the cell wall xyloglucans: Effects of auxin on the two different subfractions of xyloglucans in the epicotyl cell wall of *Vigna angularis*. *Plant and Cell Physiology*, **24**, 345-355.

NOTHNAGEL, E.A., McNEIL, M., ALBERSHEIM, P. and DELL, A. (1983). Host-pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. *Plant Physiology*, **71**, 916-926.

OPARKA, K.J., ROBINSON, D., PRIOR, D.A.M., DERRICK, P. and WRIGHT, K.M. (1988). Uptake of Lucifer Yellow CH into intact barley roots: Evidence for fluid-phase endocytosis. *Planta*, **176**, 541-547.

PARKINSON, M. (1983). Studies on cellular interactions in cultured grafts of the Solanaceae. Ph.D. Thesis, University of Edinburgh.

PARKINSON, M., JEFFREE, C.E. and YEOMAN, M.M. (1987). Incompatibility in cultured explant-grafts between members of the Solanaceae. *New Phytologist*, **107**, 489-498.

PARKINSON, M. and YEOMAN, M.M. (1982). Graft formation in cultured explanted internodes. *New Phytologist*, 91, 711-719.

PAUL, THE APOSTLE (C.57). Romans, Ch.11^{w11-25}.

PEARCE, R.B. and RIDE, J.P. (1978). Elicitors of the lignification response of wheat. *Annals of Applied Biology*, **89**, 306. (Abstract).

PEARCE, R.B. and RIDE, J.P. (1982). Chitin and related compounds as elicitors of the lignification response in wounded wheat leaves. *Physiological Plant Pathology*, **20**, 119-123.

PIEHL, M.A. (1962). The parasitic behaviour of *Dasistoma macrophylla*. *Rhodor*, **64**, 331-336.

POWELL, D.A., MORRIS, E.R., GIDLEY, M.J. and REES, D.A. (1982). Conformations and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gels. *Journal of Molecular Biology*, **155**, 517-531.

RAFF, J.W., HUTCHINSON, J.F., KNOX, R.B., and CLARKE, A.E. (1979). Cell recognition: Antigenic determinants of plant organs and their cultured callus cells. *Differentiation*, **12**, 179-186.

RAMASARMA, T. (1982). Generation of H_2O_2 in biomembranes. *Biochimica Biophysica Acta*, **694**, 69-93.

REDGWELL, R.J., MELTON, L.D. and BRASCH, D.J. (1992). Cell wall dissolution in ripening kiwifruit (*Actinidia deliciosa*). Solubilisation of the pectic polymers. *Plant Physiology*, **98**, 71-81.

RIDE, J. P. (1975). Lignification in wounded wheat leaves in response to fungi and its possible rôle in resistance. *Physiological Plant Pathology*, **5**, 125-134.

RIDE, J. P. (1978). The role of cell wall alterations in resistance to fungi. *Annals of Applied Biology*, **89**, 302-306.

ROBERTSEN, B. (1986). Elicitors of the production of lignin-like compounds in cucumber hypocotyls. *Physiological Molecular Plant Pathology*, **28**, 137-148.

ROBY, D., TOPPAN, A. and ESQUERRÉ-TUGAYÉ, M.T. (1985). Cell surfaces in plant-microorganism interacations. V. Elicitors of fungal and of plant origin trigger the synthesis

of ethylene and of cell wall hydroxyproline-rich glycoprotein in plants. *Plant Physiology*, **77**, 700-704.

RUMEAU, D., MAZAU, D. and ESQUERRÉ-TUGAYÉ, M.T. (1986). Hydroxyproline-rich glycoproteins (HRGPs) in infected plants: Signaling, accumulation and gene expression. In: *Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions. NATO ASI Series* Vol **H4**(Ed. B. Lutenberg), pp 377-384. Springer-Verlag, Berlin, Heidelberg.

RUSSELL, T.P., DELINE, V.R., DOZIER, W.D., FELCHER, G.P., AGRAWAL, G., WOOL, R.P. and MAYS, J.W. (1993). Direct observation of reptation at polymer interfaces. *Nature*, **365**, 235-237.

RYAN, C.A. (1968). Synthesis of chymotrypsin inhibitor I protein in potato leaflets induced by detachment. *Plant Physiology*, **43**, 1859-1865.

RYAN, C.A. (1974). Assay and biochemical properties of the proteinase inhibitor-inducing factor, a wound hormone. *Plant Physiology*, **54**, 328-332.

RYAN, C.A., BISHOP, P., PEARCE, G., DARVILL, A.G., McNEIL, M. and ALBERSHEIM, P. (1981). A sycamore cell wall polysaccharide and a chemically related tomato leaf polysaccharide possess similar proteinase inhibitor-inducing activities. *Plant Physiology*, **68**, 616-618.

RYAN, C.A. and HUISMAN, W. (1970). The regulation of synthesis and storage of chymotrypsin inhibitor I in leaves of potato and tomato plants. *Plant Physiology*, **45**, 484-489.

SACHS, T. (1969). Polarity and the induction of organised vascular tissues. *Annals of Botany*, **33**, 263-275.

SACHS, T. (1991). *Pattern formation in plant tissues*. Cambridge University Press.

SACHS, T. and COHEN, D. (1982). Circular vessels and the control of vascular differentation in plants. *Differentiation*, **21**, 22-26.

SALZWEDEL, J.L., DAUB, M.E. and HUANG, J.-S. (1989). Effects of singlet oxygen quenchers and pH on the bacterially-induced hypersensitive reaction in tobacco suspension cell cultures. *Plant Physiology*, **90**, 25-28.

SARKANEN, K.V. and LUDWIG, C.H. (1971). Definition and nomenclature. In: *Lignins: Occurrence, Formation, Structure and Reactions* (Eds. K.V. Sarkanen and C.H. Ludwig), Chapter 1, pp 1-18. Wiley-Interscience.

SAXTON, M.J. and BREIDENBACH, R.W. (1988). Receptor-mediated endocytosis in plants is energetically possible. *Plant Physiology*, **86**, 993-995.

SCHENK, R.U. and HILDEBRANDT, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany*, **50**, 199-204.

SCHMIDT, W.E. and EBEL, J. (1987). Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions from soybean *Glycine max*. *Proceedings of the National Academy of Sciences, U.S.A.*, **84**, 4117-4121.

SCHOFIELD, A.D. (1988). Cellular interactions between host and parasite. Ph.D. Thesis, University of Edinburgh.

SCHOTTENS-TOMA, I.M.J. and DE WIT, P.J.G.M. (1988). Purification and primary structure of a necrosis-inducing peptide from the apoplastic fluids of tomato infected with *Cladosporium fulvum* (syn. *Fulvia fulva*). *Physiological Molecular Plant Pathology*, **33**, 59-67.

SERR, E.F. and FORDE, H.I. (1959). Blackline, a delayed failure at the union of *Juglans regia* trees propagated on other *Juglans* species. *Proceedings of the American Society of Horticultural Science*, **50**, 149-150.

SHACKLOCK, P.S., READ, N.D. and TREWAVAS, A.J. (1992). Cytosolic free calcium mediates red light-induced photomorphogenesis. *Nature*, **358**, 753-755.

SLUSARENKO, A.J., LONGLAND, A. and FRIEND, J. (1986). Expression of plant genes in the hypersensitive reaction of French bean (*Phaseolus vulgaris*) to the plant pathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola*. In: *Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions. NATO ASI Series* Vol. **H4**,(Ed. B. Lutenberg), pp 367-376. Springer-Verlag, Berlin, Heidelberg.

SMITH, R.C. (1992). Xyloglucan endotransglycosylation in the apoplast of plant cell suspension cultures. Ph.D. Thesis, University of Edinburgh.

SPELLMAN, M.W., McNEIL, M., DARVILL, A.G. and ALBERSHEIM, P. (1983). Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamogalacturonan II. *Carbohydrate Research*, **122**, 131-153.

STAKMAN, E.C. (1915). Relations between *Puccinia graminis* and plants highly resistant to its attack. *Journal of Agricultural Research*, **4**, 193-200.

STERMER, B.A. and HAMMERSCHMIDT, R. (1984). Disease resistance induced by heat shock: association with ethylene production and cell wall HRGP accumulation. In: *13th Annual UCLA Symposia "Cellular and Molecular Biology of Plant Stress", Journal of Cell Biochemistry Supplement* **8B**, Abstract p 257. Alan R. Liss Inc., New York.

STODDARD, F.L. and McCULLY, M.E. (1980). Effects of excision of stock and scion organs on the formation of the graft union in *Coleus*: A histological study. *Botanical Gazette*, **141**, 401-412.

TAKEBE, I., OTSUKI, Y. and AKIO, S. (1968). Isolation of tobacco mesophyll cells in intact and active state. *Plant and Cell Physiology*, **9**, 115-124.

TALMADGE, K.W., 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 detailed analysis of the pectic polysaccharides. *Plant Physiology*, **51**, 158-173.

THAIN, J.F., DOHERTY, H.M., BOWLES, D.J., and WILDON, D.C. (1990). Oligosaccharides that induce proteinase inhibitor activity in tomato plants cause depolarisation of tomato leaf cells. *Plant, Cell and Environment*, **13**, 569-574.

THOMAS, J.R., McNEIL, M., DARVILL, A.G. and ALBERSHEIM, P. (1987). Structure of plant cell walls. XIX. Isolation and characterization of wall polysaccharides from suspension-cultured Douglas fir cells. *Plant Physiology*, **83**, 659-671.

TIEDEMANN, R. (1989). Graft union development and symplastic phloem contact in the heterograft *Cucumis sativus* on *Cucurbita ficifolia*. *Journal of Plant Physiology*, **134**, 427-440.

TOPPAN, A., ROBY, D. and ESQUERRÉ-TUGAYÉ, M.T. (1982). Cell surfaces in plant-microorganism interactions. III. *In vivo* effect of ethylene on hydroxyproline-rich glycoprotein accumulation in the cell wall of diseased plants. *Plant Physiology*, **70**, 82-86.

TORREY J.G. and FOSKET, D.E. (1970). Cell division in relation to cytodifferentiation in cultured pea root segments. *American Journal of Botany*, **54**, 1072-1080.

TRAN THANH VAN, K. and MUTAFTSCHIEV, S. (1990). Signals influencing cell elongation, cell enlargement, cell division and morphogenesis. In: *Proceedings of the VIIth International Congress on Plant Tissue and Cell Culture* (Eds. H.J.J. Nijkamp, L.H.W. van der Plas and J. van Aartrijk) pp 514-519. Kluwer, Dordrecht.

TRAN THANH VAN, K., TOUBART, P., COUSSON, A., DARVILL, A.G., GOLLIN, D.J., CHELF, P. and ALBERSHEIM, P. (1985). Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins. *Nature*, **314**, 615-617.

TSIVION, Y. (1978). Physiological concepts of the association between parasitic angiosperms and their hosts - a review. *Israel Journal of Botany*, **27**, 103-121.

TZENG, D.D.-S. (1989). Hydroxyl radical and singlet oxygen are the key role factors involved in the photodynamic biocidal activity of methionine-riboflavin mixture. *Botanical Bulletin of Academia Sinica*, **30**, 39-48.

TZENG, D.D. and DEVAY, J.E. (1993). Role of oxygen radicals in plant disease development. In: *Advances in Plant Pathology*, Vol.**10**, (Eds. J.H. Andrews and I.C. Tommerup), pp 1-34. Academic Press, London.

VANCE, C.P., KIRK, T.K. and SHERWOOD, R.T. (1980). Lignification as a mechanism of disease resistance. *Annual Review of Phytopathology*, **18**, 259-288.

VAN HOLST, G-J. and CLARKE, A.E. (1986). Organ-specific arabinogalactan-proteins of *Lycopersicon peruvianum* (Mill.) demonstrated by crossed electrophoresis. *Plant Physiology*, **80**, 786-789.

VAN HUYSTEE, R.B. (1987). Some molecular aspects of plant peroxidase biosynthetic studies. *Annual Review of Plant Physiology*, **38**, 205-219.

WALKER-SIMMONS, M., HADWIGER, L. and RYAN, C.A. (1983). Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proeinase inhibitors in tomato leaves. *Biochemical and Biophysical Research Communications*, **110**, 194-199.

WALKER-SIMMONS, M. and RYAN, C.A. (1984). Proteinase inhibitor synthesis in tomato leaves. Induction by chitosan oligomers and chemically modified chitosan and chitin. *Plant Physiology*, **76**, 787-790.

WARDROP, A.B. (1971).Occurrence and formation in plants. In: Lignins: Occurrence, Formation, Structure and Reactions. (Eds. K.V.Sarkanen and C.H.Ludwig), Chapter 2, pp 19-41. Wiley-Interscience.

WARREN, S.D., BLACK, H.L., EASTMOND, D.A. and WHALEY, W.H. (1988). Structural function of butresses of *Tachigalia versicolor*. *Ecology*, **69**, 532-536.

WEINSTEIN, L.I., HAHN, M.G. and ALBERSHEIM, P. (1981). Host-pathogen interactions. XVIII. Isolation and biological activity of glycinol, a pterocarpan phytoalexin. *Plant Physiology*, **68**, 358-363.

WILSON, L.B. and FRY, J.C. (1986). Extensin - a major cell wall glycoprotein. *Plant, Cell and Environment*, **9**, 239-260.

WOLFF, S.P., GARNER, A. and DEAN, R.T. (1986). Free radicals, lipids and protein degredation. *Trends in Biochemical Sciences*, **11**, 27-31.

WOLPERT, J.S. and ALBERSHEIM, P. (1976). Host-symbiont interactions. I. The lectins of legumes interact with the O-antigen-containing lipopolysaccharides of their symbiont *Rhizobia*. Biochemical and Biophysical Research Communications, **70**, 729-737.

YAMAOKA, T. and CHIBA, N. (1983). Changes in the coagulating ability of pectin during the growth of soybean hypocotyls. *Plant Cell Physiology*, **24**, 1281-1290.

YAMAZAKI, N., FRY, S.C., DARVILL, A.G. and ALBERSHEIM, P. (1983). Host-pathogen interactions. XXIV. Fragments isolated from suspension-cultured sycamore cell walls inhibit the ability of the cells to incorporate [^{14}C] leucine into proteins. *Plant Physiology*, **72**, 864-869.

YEOMAN, M.M. (1984). Cellular recognition systems in grafting. In: *Encyclopedia of Plant Physiology New Series*, Vol. **17**, *Cellular Interactions*, pp 453-472 (Eds. H.F.Linskens and J.Heslop-Harrison), Springer-Verlag, Berlin.

YEOMAN, M.M. and BROWN, R. (1976). Implications of the formation of the graft union for organisation in the intact plant. *Annals of Botany*, **40**, 1265-1276.

YEOMAN, M.M., KILPATRICK, D.C., MIEDZYBRODZKA, M.B., and GOULD, A.R. (1978). Cellular interactions during graft formation, a recognition phenomenon? *Society for Experimental Biology Symposium* **XXXII**, *Cell-Cell- Recognition*, (Ed. A.S.G. Curtis) pp 139-159. Cambridge University Press, Cambridge.

YORK, W.S., DARVILL, A.G. and ALBERSHEIM, P. (1984). Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. *Plant Physiology*, **75**, 295-297.

ZUROSKE, G., MAKUS, D.J., and RYAN, C.A. (1980). The direction and rate of transport of the proteinase inhibitor inducing factor out of wounded tomato leaves. *Plant Physiology*, **65**, Supplement 6, 150.

APPENDIX I: SUPPLIERS' NAMES AND ADDRESSES

A & J BEVERIDGE LTD	5 Bonnington Road Lane, Edinburgh, Scotland. EH6 5BP
AGAR SCIENTIFIC LTD	66a Cambridge Road, Stansted, Essex, England. CM24 8DA
ALDRICH CHEMICAL CO LTD	Gillingham, Dorset, England.
AMERSHAM INTERNATIONAL PLC	Amersham Place, Little Chalfont, Buckinghamshire,England. HP7 9NA
BDH CHEMICALS LTD	Poole, England.
BECKMAN INSTRUMENTS (UK) LTD	Progress Road, Sands Industrial Estate, High Wycombe, Buckinghamshire, England. HP12 4JL
J BIBBY SCIENCE PRODUCTS LTD	Stone, Staffordshire, England. ST15 0SA
BIO-RAD LABORATORIES LTD	Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, England. HP2 7TD
CAMLAB LTD	Nuffield Road, Cambridge, England. CB4 1TH
DuPONT (UK) LTD	Wedgwood Way, Stevenage, Hertfordshire, England. GG1 4QN
EVANS ELECTROSELENIUM LTD	Halstead, Essex, England.
FISONS SCIENTIFIC EQUIPMENT	Bishop Meadow Road, Loughborough, Leicestershire, England. LE1 0RG
FLOW LABORATORIES LTD	c/o ICN Biomedicals Ltd, Unit 18, Thame Park Business Centre, Wenman Road, Thame, Oxfordshire, England. OX9 3XA
GENEVAC SALES DEVELOPMENT LTD	The Sovereign Centre, Farthing Road, Sproughton,Ipswich, England. IP1 5AP
GREINER LABORTECHNIK LTD	Station Road, Cam, Dursley, Gloucestershire, England. GL11 5NS
IMPERIAL LABORATORIES	West Portway, Andover, Hampshire, England. SP10 3LF
LSL, LABORATORY SALES (UK) LTD	Unit 20-21, Transpennine Trading Estate, Rochdale, West Yorkshire, England. OL11 2PX
LUCKHAM LTD	Victoria Gardens, Burgess Hill, Sussex, England. RH15 9QN

MILLIPORE (UK) LTD	The Boulevard, Ascot Road, Croxley Green, Watfordex Hertfordshire,England. WD1 8YW
MSE	Bishop Meadow Road, Loughborough, Leicestershire England. LE11 0RG
SAVANT	c/o International Equipment Co Ltd, Lawrence Way, Brewers Hill Road, Dunstable, Bedfordshire, England. LU6 1BD
SCIENTIFIC INSTRUMENT CENTRE	London, England.
SIGMA CHEMICALS LTD	Fancy Road, Poole, Dorset, England. BH17 7NH
STERILIN LTD	Lampton Road, Hounslow, Middlesex, England. TW3 4EE
UNIPATH LTD	Basingstoke, Hampshire, England.
WHATMAN LABSALES LTD	St. Leonard's Road, 20/20 Maidstone, Kent, England. ME16 0LS
ZINSSER ANALYTIC (UK) LTD	Howarth Road, Maidenhead, Berkshire, England. SL6 1AP

APPENDIX II: COMPOSITION OF MEDIA

The composition of Murashige and Skoog basal salt mixture (Sigma cat. no. M5524)

	mgL^{-1}
NH_4NO_3	1650.00
KNO_3	1900.00
$CaCl_2.2H_2O$	440.00
$MgS0_4.7H_2O$	370.00
KH_2PO_4	170.00
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.4H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2.EDTA$	37.30
$FeSO_4.7H_2O$	27.80

Composition of Schenk and Hildebrandt medium (1972) (Imperial Laboratories product no. 9-650-50):

	mgL^{-1}
$CaCl_2.2H_20$	200.00
$CoCl_2.6H_2O$	0.10
$CuSO_4.5H_2O$	0.20
$FeSO_4.7H_20$	15.00
H_3BO_3	5.00
KI	1.00
KNO_3	2500.00
$MnS0_4.4H_2O$	13.20
$MgS0_4.7H_2O$	400.00
Na_2EDTA	20.00
$Na_2MoO_4.2H_2O$	0.10
$NH_4H_2PO_4$	300.00
$ZnSO_4.7H_2O$	1.00
i-Inositol	1000.00
Nicotinic Acid	5.00
Pyridoxine HCl	0.50
Thiamine HCl	5.00

Composition of Flow Labs Murashige and Skoog medium without sucrose, IAA, kinetin and agar, (cat. no. 26-100-24):

	mgL^{-1}
$CaCl_2.2H_2O$	440.00
$CoCl_2.6H_2O$	0.025
$CuS0_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KH_2PO_4	170.00
KI	0.83
KNO_3	1900.00
$MgSO_4.7H_2O$	370.00
$MnSO_4.4H_2O$	22.30
$NaMoO_4.2H_2O$	0.25
$NH4NO_3$	1650.00
$ZmSO_4.7H_2O$	8.60
Myo-inositol	100.00
Nicotinic acid	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine	2.00