BIOCHEMICAL ASPECTS OF HOST-PATHOGEN RESISTANCE IN COTTON SUSPENSION CULTURES

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Ac 44	Acala 44
AGPs	arabinogalactan proteins
ANOVA	analysis of variance
Ara	arabinose
BCA	bicinchoninic acid
cDNA	complementary DNA
C. cucumerinum	*Cladosporium cucumerinum*
C. lagenarium	*Colletotrichum lagenarium*
C. lindemuthianum	*Colletotrichum lindemuthianum*
CS	chalcone synthase
4-CL	4-coumarate: CoA ligase
cfus	colony-forming units
DEAE	diethyl amino ethyl
ELISA	enzyme-linked immunosorbent assay
EPS	extracellular polysaccharides
Fuc	fucose
Gal	galactose
GalU/GalA	galaturonic acid
Glc	glucose
HF	hydrogen fluoride
His	histidine

HR	hypersensitive response
HRGP(s)	hydroxyproline-rich glycoprotein(s)
Hyp	hydroxyproline
Im 216	Immune 216
Lys	lysine
Man	mannonse
P. graminis f. sp. *titici*	*Puccinia graminis* f. sp. *tritici*
PAL	phenylalanine ammonia lyase
Pro	proline
Rha	rhamnose
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
Ser	serine
SH	Schenk and Hildebrandt
Thr	threonine
TMS	trimethylsilyl
Tyr	tyrosine
UDP	uracil diphosphate
Val	valine
X. c. malvacearum	*Xanthomonas campestris* pv. *malvacearum*
Xyl	xylose

CHAPTER I

INTRODUCTION

Cell wall modifications appear to be involved in a wide variety of host-pathogen interactions. A number of different conditions affect the expression of cell wall hydroxyproline-rich glycoproteins (HRGPs) or extensins. Their usually low level is strikingly increased in response to wounding, ethylene treatment, elicitor treatment, infection, heat treatment, red light illumination, and under tissue culture conditions (see 34, 225 for pertinent reviews). The accumulation of HRGPs is one of a number of inducible defense mechanisms that many plants exhibit in natural resistance to disease. Other inducible defense mechanisms exhibited by plants include the accumulation of host-synthesized phytoalexins and proteinase inhibitors, deposition of lignin and callose, and increased activity of certain hydrolytic enzymes such as chitinase and β-1,3 glucanases (see 14, 220 for pertinent reviews).

Several research groups have observed that HRGPs accumulated in response to infection and that this accumulation is correlated with disease resistance. For example, Esquerré-Tugayé and Lamport [72] and Esquerré-Tugayé *et. al.* [74] found an approximately 10-fold increase in cell wall HRGP in melon plants infected with the anthracnose fungus, *Colletotrichum lagenarium* . Additionally, artificial enhancement or suppression of HRGP levels in melon plants infected with *C. lagenarium* results in increased or decreased resistance, respectively [71]. Hammerschmidt and coworkers [100] have observed that cell wall hydroxyproline levels, indicative of HRGP levels, increase more rapidly in resistant cultivars than in susceptible cultivars of cucumber plants infected with the fungus, *Cladosporium cucumerinum.* Treatment of melon and soybean hypocotyls with components of fungal cell walls (i.e. elicitors) or plant cell walls (i.e. endogenous elicitors) also stimulates HRGP synthesis [205]. Similarly, elicitor treatment of bean cell cultures results in the accumulation of cell wall hydroxyproline [21]. HRGP mRNAs have also been shown to accumulate in elicitor-treated bean cell suspension

cultures and in race: cultivar-specific interactions between bean hypocotyls and the partially biotrophic fungus, *C. lindemuthianum* [223]. Moreover, HRGP mRNA accumulated earlier in an incompatible interaction (host resistant) than in a compatible interaction (host susceptible) and this accumulation was correlated with the expression of hypersensitive resistance in the former interaction. The enrichment of the cell wall in HRGP seems to be a general response of dicotyledons to infection whereas no significant increases occurred in monocotyledonous host-pathogen interactions [164]. In the 19 dicotyledonous host-pathogen interactions studied [164], melons had the greatest HRGP accumulation (900%) and tobacco the lowest (50%). To date, few studies [164, 168] have been conducted regarding the accumulation of HRGP in the cell walls of a host: pathogen interaction involving a bacterial pathogen.

Primary cell walls of plants contain about 1 to 10% protein [34]. A hydroxyproline-rich glycoprotein, often called extensin, is a major primary cell wall protein. Extensin, like other structural proteins such as collagen and elastin, is a generic term describing a family of related proteins and is involved in extension growth, strengthening of the cell wall, and defense responses. These proteins were originally described by Lamport [148] and given the name "extensin" due to their expected role in extension growth. Extensins are highly processed by co-translational hydroxylation, signal peptide cleavage, post-translational glycosylation, secretion, and the formation of suggested intermolecular cross-links, which produces the insoluble extensin network. In addition to their high hydroxyproline content, extensins have a high level of serine, a highly periodic repeating unit of tetrahydroxyproline-serine, and are rich in basic amino acids. Extensin precursors appear as thin, kinked rods, approximately 80 nm in length organized in a polyproline II helix [105, 236, 250]. Since the structure is linear, extensin may function as a structural polymer and strengthen the cell wall. Secondly, the high level of basic amino acids confer upon it the properties of a polycation and so it may agglutinate negatively charged particles such as the negatively charged glucuronic residues of pectin. Lamport and Epstein [156] have elaborated on this idea by proposing a structural cell wall model in which these glycoproteins are organized around cellulose microfibrils as flexuous rods in a "warp-weft" fashion.

Isolation of HRGPs is a prerequisite to clearly understanding their role in disease resistance. This step however has proved particularly difficult due to the insolubility of HRGP. Direct extraction of extensin precursors from the cell walls of carrot, melon, cucumber, bean , maize, cucumber, and tomato with a salt solution or solubilizing the

HRGPs from a cell homogenate of potato tuber and tobacco using an acidic extraction procedure have been accomplished (see 34, 225, 260 for comprehensive reviews).

Nucleic acid studies have helped to elucidate structural and genetic information regarding extensin. Chen and Varner [38, 39] first characterized and isolated extensin cDNA and an extensin genomic clone from carrot. The carrot genomic clone, pDC5A1, was sequenced and found to contain 25 Ser-(Pro)4 repeat units which are the unhydroxylated precursors of the Ser-(Hyp)4 repeat units in the mature protein [38] . This carrot genomic clone and the later isolated tomato HRGP sequences [223] have been used to follow the induction of HRGP mRNAs in various host-pathogen interactions.

This work reports the first isolation and partial characterization of an insolubilized cell wall HRGP from cotton suspension culture. Several different incompatible interactions (Im 216, AcB_N, OK 1.2 cotton culture lines) and one compatible interaction (Ac 44 cotton culture line) with the bacterial pathogen, *Xanthomonas campestris* pv. *malvacearum* were examined in culture. Additionally, this research attempts to show that cell wall HRGPs play a role in the defense response of cotton to the bacterial pathogen, *X. c. malvacearum*, the causal agent of bacterial blight of cotton.

The upland cotton (*Gossypium hirsutum* L.): *X. c. malvaearum* sytem provides a good model for studying host-pathogen relationships and the mechanism of host-pathogen resistance including the analysis of cotton cell wall HRGPs and their proposed role in disease resistance. The well-defined pathogenic races of *X. c. malvacearum* and known *in vivo* race: cultivar-specific interactions involving this pathogen have been described [24, 25]. The cell lines selected for this research, Ac 44 (completely susceptible to *X. c. malvacearum*), AcB_N, (intermediate resistance to *X. c. malvacearum*), and Im 216 (immune to *X. c. malvacearum*) are suitable for studying the mechanism of resistance *in vitro* to *X. c. malvacearum* . Callus cultures for Ac 44 and Im 216 were established by Ruyack *et. al.* [208] and cultures for AcB_N were newly established during the course of this research. The use of cotton tissue culture allows one to study rapidly *in vitro* host-pathogen interactions and correlate the defense responses directly to the bacterial pathogen, *X. c. malvacearum.*

The results of this research are organized into three major sections. The first section characterizes the growth of the host in the presence of the pathogen and similarly, the pathogen in the presence of the host in both callus and suspension culture.

Additionally, the viability of the host, Ac 44 (completely susceptible to *X. c. malvacearum*), AcB_N, (intermediate resistance to *X. c. malvacearum*), and Im 216 (immune to *X. c. malvacearum*), in the presence or absence of the pathogen is reported. This section also includes a comparative study on the isolation of protoplasts from Ac 44, AcB_N, OK 1.2, Im 216 suspension cultures.

The second section provides details regarding different biochemical parameters measured over time in incompatible and compatible interactions in cotton suspension cultures infected with the bacterial pathogen, *X. c. malvacearum* . These parameters are as follows: sugar profiles/carbohydrate content, amino acid profiles/protein content with special attention drawn to the amino acid, hydroxyproline, and lignin content. Cotton cell walls of various ages were isolated from suspension cultures inoculated and uninoculated with the bacterial pathogen, *X. c. malvacearum.* Important physiological information is obtained from these time courses. Information here relates the differences observed in accumulation or induction of specific sugars, amino acids (especially hydroxyproline), and lignin in response to infection of these different host interactions with *X. c. malvacearum.* Thus, it is possible to show that there are observable differences in the incompatible interaction (host-resistant, Im 216, AcB_N) from that of the compatible interaction (host-susceptible, Ac 44). For instance, the accumulation of hydroxyproline in inoculated resistant cotton cell line is a direct indication of the HRGP involvement in disease resistance to *X. c. malvacearum.*

The third section reports, for the first time, the isolation, purification, and physical characterization of a cotton cell wall bound HRGP from Ac 44 (host-susceptible) and Im 216 (host-resistant) suspension cultures using selective hydrogen fluoride (HF) solvolysis at 0°C. The use of HF solvolysis at 0°C to deglycosylate extensin in tomatoes has been reported [177]. This technique has most often been used to selectively cleave polysaccharides to study their stucture and function (for pertinent reviews see 136, 176). This research attempts to show that HF solvolysis at 0°C is a valuable method for studying and isolating intact, but deglycosylated insoluble HRGPs.

CHAPTER II

LITERATURE REVIEW

Plant Disease Responses in General

General Inducible Defense Mechanisms

Many plants exhibit natural resistance to disease which may involve one of several inducible disease mechanisms. Accumulation of host-synthesized phytoalexin antibiotics, deposition of lignin-like material and callose, accumulation of hydroxyproline-rich glycoproteins (HRGPs), accumulation of proteinase inhibitors, and increases in the activity of certain hydrolytic enzymes such as chitinase and β-1,3 glucanases have been documented in disease resistant species (reviewed in 14, 220). These responses can also be induced by glycan, glycoprotein, or lipid elicitor preparations obtained from fungal cell walls, culture fluids or metabolites such as arachidonic acid and glutathione, and in some cases by structurally unrelated artificial elicitors or mechanical damage [220,19, 55, 63, 64, 66, 68, 261].

Transcriptional Activation of Plant Defense Genes by Fungal and other Elicitors, Wounding, and Infection

Elicitors, wounding, or infection rapidly stimulates the transcription of genes involved in the erection of these plant defenses [37, 54, 64, 106, 161, 233, 261]. Thus, in suspension-cultured bean cells (*Phaseolus vulgaris* L.), fungal elicitor stimulates chitinase and several enzymes of phenyl propanoid biosynthesis (such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CS)) involved in the production of lignin precursors and isoflavanoid-derived phytoalexins, as well as the accumulation of HRGPs [21, 53]. RNA blot hybridization with cloned DNA sequences has shown that elicitor

stimulates accumulation of mRNAs [69, 144, 209, 223]. Increased levels of these mRNAs were also observed in wounded bean hypocotyls and during race- and cultivar-specific interactions following infection with the fungus, *Colletotrichum lindemuthianum*, the causal agent of anthracnose [15, 16, 53, 69]. Finally, Ecker and Davis [69] reported marked accumulation of PAL, 4-coumarate: CoA ligase (4-CL), CS, and HRGP mRNAs in ethylene-treated and wounded carrot roots. These results indicated that at least three distinct signals, ethylene, infection, and wound signals, can affect the expression of plant defense response genes.

Induction of Plant Defense Genes Correlated with the Corresponding Plant Defense Responses

These and similar observation in other systems suggest that specific activation of plant defense genes might underlie expression of the corresponding defense responses [37, 69, 144, 200, 217]. This question was investigated initially by analysis of transcripts completed *in vivo* by isolated nuclei [161]. Elicitor treatment of suspension-cultured *Phaseolus vulgaris* L. cells caused marked transient stimulation of transcription of genes encoding apoproteins of cell wall hydroxyproline-rich glycoproteins (HRGPs) and the phenylpropanoid biosynthetic enzymes, PAL and 4-CL as well as a flavanoid glycoside pathway enzyme, CS; concomitant with the onset of rapid accumulation of the respective mRNAs and hence expression of the phytoalexin (PAL, CS), lignin (PAL), and HRGP defense responses [161]. In related experiments, induction of transcription of PAL, CS, and HRGP genes was also observed in wounded hypocotyls and in infected hypocotyls during race-cultivar specific interactions with the fungus *C. lindemuthianum*, the causal agent of anthracnose [161] and in wounded and ethylene-treated carrot roots [69]. Additionally, transcriptional activation occurred not only in directly infected tissue but also in distant and yet previously uninfected tissue (i.e. induced systemic resistance) indicating there was intercellular transmission of an endogenous signal for defense gene activation [161].

Lignification as a Mechanism of Disease Resistance

What is Lignin

The literature on lignin chemistry is vast, especially that relating to lignins from wood. The work of Sarkanen and Ludwig [214] has been especially beneficial and has

been recently updated by Higuchi [112]and Gross [96]. Lignin is a complex high-molecular weight aromatic polymer whose main building unit is a phenylpropanoid residue (C_6-C_3), almost exclusively derived from *p*-coumaryl, coniferyl, and sinapyl alcohols. Lignin occurs in plant cell walls in close association with cellulose and hemicellulosic polysaccharides. Lignin is most often found in the plant cell walls of xylem tissue, but is also present in the cell walls of pith, root, fruits, buds, bark, and cork. Lignin is found in vascular plants such as lycopods, ferns, gymnosperms and angiosperms, but is absent from non-vascular plants such as fungi and algae. Lignification of the cell wall occurs after the laying down of the polysaccharide components of the wall and towards the end of the growing period of the cell. In general, the lignin laid down first becomes most heavily lignified so that the primary cell wall and middle lamella are more lignified that the secondary cell wall. The purpose of lignification is two-fold: 1) strengthens the cell wall by forming a ramified network through the matrix which anchors the cellulose microfibrils more firmly and 2) lignin protects the microfibrils of the wall from chemical, physical, and biological attack.

Induced Lignification by Fungal Pathogens

Lignification of walls is one of the most common responses of plant tissues to wounding and stress. Rapid lignin deposition may provide a physical and/or chemical barrier to the invading plant pathogen [143, 251]. Lignification has been suggested to be part of the active defence mechanism of several plant species [143] including a number of curcurbits (esp. melons and cucumbers) to the fungal pathogens, *Cladosporium cucumerinum* (causal agent of scab) and *Cladosporium lagenarium* [97, 98, 99, 100, 113, 201]. Cellular lignification has also been found to a factor in the hypersensitive response and disease resistance of wheat to the stem rust fungus, *Puccinia graminis* f. sp. *tritici* [13, 137, 138, 171, 172, 184, 197] and to the appressorial pathogens, *Pyricularia oryze* and *Erysiphe graminis* f. sp. *tritici* [198].

Lignification occurs rapidly at wound margins in wheat leaves following inoculation with non-pathogenic fungi [183, 195] and may be involved in the restriction of such fungi to the wound site [198]. Filamentous pathogenic fungi such as *Puccinia graminis* f. sp. *tritici* have been shown to be potent inducers of this reaction in wheat and appear to be specific for this pathogen [137, 138, 171, 172, 183]. Simply, the growth of the fungus *Puccinia graminis* f. sp. *tritici* is arrested after two or three attempts of penetration and rapid necrosis of host cells occurs which is accompanied by lignification

of the cell contents [171, 172]. In wheat, the reaction of resistant and susceptible plants to *Puccinia graminis* f. sp. *tritici* mainly differs quantitatively in the rate of activation of resistant reactions [171, 172]. A fungal elicitor isolated from the pathogen germ tube walls may represent a postulated race/cultivar unspecific elicitor [171, 172]. It has since been found [137] that Con A-binding glycoproteins in germ tube walls of *Puccinia graminis* f. sp. *tritici* appear to be potent inducers of the hypersensitive lignification response in wheat leaves. This response was preceded by an increase in extractable PAL activity [137] a key enzyme of the phenyl propanoid pathway and lignin biosynthesis. Finally, a range of putative elicitors has been screened for lignification-eliciting activity in wheat . Those elicitors that were found to be most active in inducing lignification were cellulose "Onozuka" R-10, a commercial crude enzyme preparation from *Trichoderma viride*, chitin, chitosan, and ethylene glycol chitin; two β-glucans, pachynan and a cell fraction from *Phytophthora megasperma* f. sp. *glycinea* capable of eliciting phytoalexin accumulation in soybean; a fungal fatty acid, eicosapentaenoic acid, lectin wheat germ agglutinin, and the abiotic treatments, mercuric and cadmium chloride [9].

In cucumbers, muskmelons, and watermelons, induced systemic resistance is associated with an enhanced ability of the host plants to lignify in response to infection by the pathogenic fungi, *Colletotrichum lagenarium* and *Cladosporium cucumerinum* [58, 95, 99, 247]. Induced systemic resistance of cucumber against *C. lagenarium* appears to involve inhibition of penetration by the fungus and is associated with rapid lignification of the epidermis localized around appressoria [99]. This response is different from natural resistance to anthracnose in which the fungus, *C. lagenarium* , is able to successfully penetrate and elicit a hypersensitive-like response [194] similar to the hypersensitive-like response exhibited by wheat to the fungal pathogen, *P. graminis* f. sp. *tritici*. Induced systemic resistance in cucurbits is also associated with enhanced levels of a group of acidic, extracellular peroxidases which accumulate systemically in plant tissues as induced resistance develops [101, 227]. These peroxidases catalyze the final polymerization step of lignin synthesis. Therefore, they may be directly associated with the increased ability of systemically protected tissue to lignify [96]. Finally, enhanced levels of acidic peroxidases have not been documented for the wheat and *P. graminis* f. sp. *tritici* interaction.

Induced Lignification by Bacterial Pathogens

It is important to note that all relevant material related to induced lignification has involved only interactions with fungal pathogens. Little or no work exists that describes natural resistance in plants in response to infection by bacterial pathogens involving induced lignification. Since phytobacterial pathogens are considered passive invaders, there is no physical force, aside from motility, that these pathogens possess to penetrate the host's outer defenses. Thus, the phytobacterial pathogens rely on enzymic or other metabolite activity for successful pathogenesis, including enzymes related to pectin, suberin and cutin, cellulose, hemicellulose, protein, and lipid degradation [93].

General Overview of Plant Cell Wall Components

Plant Cell Wall Components other than Proteins

In general, plant cell walls are comprised of cellulose, hemicelluloses, pectic compounds, lignin, suberin, proteins, and water. The polysaccharide components of the cell wall have been extensively reviewed [167, 179, 188]. Water is a very valuable and extremely important part of the cell wall. Cutin is the functional component of the cuticle deposited on the surfaces and within epidermal walls of aerial parts of plants during extension growth [125]). Cutin is essentially a three dimentional polyester of long chain fatty acids mainly belonging to the C_{16} and C_{18} families, ω-OH fatty acids, ω- and midchain-OH fatty acids, and midchain epoxide fatty acids. Cutin structure, synthesis, and function have been extensively reviewed [139]. Suberin is composed of a phenolic, ligninlike domain, which yields vanillin and *p*-hydroxybenzaldehyde on nitrobenzene oxidation, is attached to unidentified noncellulosic polymers of the wall and is, in turn, polymerized with a hydrophobic polyester domain of C_{14}-C_{30} long-chain fatty acids, fatty alcohols, dicarboxylic acids, and ω-OH fatty acids [8]. Suberin is the functional component of the hydrophobic, water-impermeable, multilamellate walls of cork cells in periderms, and of surfaces of roots and stems, and wounded tissue [191, 192, 193]. It also occurs in the endodermis, hypodermis, and bundle sheath cells of grasses [103]; at the boundary of secretory glands; at the connections between seed coats and vascular tissues; and in certain types of cotton seed hairs [140]. Suberin thus functions to restrict apoplastic movement of solutes and slows or prevents mechanical and microbial attack on walls. Waxes are also hydrophobic compounds found on cuticular surfaces in

characteristic crystalline forms [125]and serve as the major barrier to diffusion of solutes [232] and to microbial penetration. Lignins, described earlier in this chapter. However, lignins are polymers of phenylpropanoid residues that are almost exclusively derived from *p*-coumaryl, coniferyl, and sinapyl alcohols. Their chemistry has been extensively reviewed by Sarkanen and Ludwig [214] and Higuchi [112]. As discussed earlier, lignification of cell walls may make them more resistant to mechanical penetration since lignin confers rigidity to the wall. The structure and function of all components of plant cell wall are thoroughly summarized in a recent review by Bacic and coworkers [8]. In addition, a comprehensive treatise by Goodman and associates [93] discusses the cell wall composition and how it is altered by fungal and bacterial pathogens.

Cell Wall Proteins and Glycoproteins - A General Discussion

Proteins are important components of plant cell walls. Primary walls contain about 1 to 10% protein [34]. Plant walls contain a variety of proteins that have both structural and enzymatic activities. Those that display enzymatic activities are: glycosidase, glycanases, dehydrogenases, and peroxidases which may be important in biosynthesis and modification of wall polymers during growth. Plant cell wall enzymes are extensively reviewed by Bacic and coworkers [8] and Cassab and Varner [34]. Based on published data, it is not possible at this time to distinguish enzymes in the wall from those in the wall space.

Cell walls contain several types of structural proteins as well as various enzymes. Among these is the family of hydroxyproline-rich glycoproteins (HRGPs) or extensins which to date is the best characterized class. We shall consider in detail the HRGP first characterized and named extensin by Lamport [148]. Lamport and Northcote [159] and Dougall and Shimbayashi [65] discovered hydroxyproline (Hyp) as a major amino acid constituent of hydrolysates of cell walls from tissue cultures. There are at least three classes of HRGPs in plants: 1) the cell wall HRGPs or "extensins" (reviewed in 153, 155, 225, 260) and most recently by Cassab and Varner [34]); 2) the arabinogalactan proteins (AGPs) (reviewed in 44, 81, 225, and 3) certain lectins from the Solanaceae family (see 44, 81, 225). These three classes of HRGPs may be readily separated and distinguished from one another by virtue of their unique biochemical and physical properties recently reviewed by Showalter and Varner [225].

Wall proteins, other than extensins, which may also have structural functions have been reported by Brown and Kimmins [27] in bean (*Phaseolus vulgaris*). These proteins are glycosylated, like extensins, but unlike extensin are low in hydroxyproline. Other low hydroxyproline wall proteins have been described in dicotyledon parenchymatous walls by Selvendran *et. al.* [219]. In addition investigators have reported the presence of a glycine-rich protein in petunia [48, 49, 252] and bean [129], a proline-rich protein in carrot [39, 246] and soybean [7]. A 28-kd glycoprotein has been found to accumulate at low water potentials in the cell wall of the growing stems of soybean [23] and is normally associated with early wall growth, in either a catalytic or structural role. In contrast, a 70-kd glycoprotein, mainly extractable from mature cell walls of soybean stems, appeared to decrease at low water potentials [23]. Neither the 28 K nor 70 K protein contained hydroxyproline (Hyp) indicating that they are not related to extensin. Furthermore, a histidine-rich (25 mole%) and tryptophan-rich protein (17 mole%) containing no hydroxyproline was isolated from an arabinogalactan fraction of suspension-cultured sycamore cells (sycamore extracellular polysaccharide) [241]. Yet, a HRGP was isolated from the cell walls of maize cell suspension cultures that was unusually rich in threonine (25 mole%), proline (15 mole%), and hydroxyproline (25 mole%) [131]. In general, monocotyledon cell walls contain low levels of Hyp, with the exception of seed coats and pericarps [22, 249]. In fact, a developmentally regulated HRGP was recently isolated from maize pericarp cell walls [115].

Extensins

Biochemical and Physical Characterization

General Overview of Extensin - 1º and 2º Structure. The following discussion will examine only the cell wall HRGPs or extensins with respect to their biochemical and physical properties and how these properties are related to their proposed biological functions. The cell wall HRGPs or extensins are largely confined to the primary cell walls that are undergoing extension, hence the name "extensin", accorded them by Lamport [148, 149]. Cell wall HRGPs have been extensively reviewed by several different groups [34, 155, 225, 260]. The typical cell walls HRGP or extensin isolated by salt-elution from carrot roots consists primarily (95%) of six amino acids, Hyp, Ser, His, Tyr, Lys, and Val. The abundance of Lys and low content of Asp and Glu contribute to the high isoelectric point and the highly basic nature of this molecule. This glycoprotein consists of 35% protein and 65% carbohydrate. Arabinose represent 97% of the sugar present and

galactose only 3%. The arabinose (ara) is attached via an O-glycosidic linkage to Hyp in short side chains of mainly four (70% of the ara) and three (24% of the ara) residues [149, 150] although some Hyps are not glycosylated. Galactose (gal), on the other hand, in linked to serine in an O-glycosidic linkage (40% of the gal residues) [157]. Extensin has a highly repetitive pentapeptide sequence, Ser-$(Hyp)_4$, characteristic of the backbone of this glycoprotein.

The secondary structure of soluble carrot, tomato, and sycamore-maple extensins has been studied using gel filtration [105, 235, 236], electron microscopy [105, 153, 235, 236, 250], and circular dichroism [250]. Under the electron microscope, extensin appears as thin, kinked rods of approximately 80 nm in length [105, 153, 235, 250]. Consistent with these microscopic observations, circular dichroism analysis indicates that cell wall HRGPs exist in a polyproline II helix, i.e. a left-handed helix with 3 residues per turn and a pitch of 9.36 Å [250]. Moreover, it appears that the carbohydrate moiety of this glycoprotein serves a role in stabilizing this helical conformation, presumably by intramolecular hydrogen bonding, since deglycosylation with anhydrous hydrogen fluoride results in a distortion or unwinding of this helix [105, 235, 250]. Recently, for the first time, Heckman and coworkers [105] have visualized the deglycosylated extensin monomers using transmission electron microscopy as thin, but discernable, extended rods. Furthermore, succinylation also showed that deglycosylated extensin precursors gave anomalously high gel filtration retention times, due to ionic interaction with the column, rather than decreased molecular size [105]. The true molecular weight of the carrot extensin-1 [237, 243] and tobacco HRGP [168] was reported to be ~90 Kd.

Indentification and Isolation of Salt-Extractable Extensins. Chrispeels [40] was the first to identify a salt-extractable hydroxyproline-containing protein in carrot roots, and suggested that it might be the precursor to the covalently bound cell wall extensin [28, 41]. The synthesis and secretion of extensin in carrot roots was shown to be enhanced by slicing and aeration of the tissue [42]. Synthesis of extensin may be involved in structural reformation of the wall, or in disease resistance following wounding [42, 243]. Several laboratories have purified extensin from different plants and tissues such as potato tuber [162], sycamore tissue-culture cells [104], tobacco callus [168], runner bean [180], tomato cell suspension cultures [228], melon callus [166], maize cell cultures [115, 131], and soybean seed coats [32]. The first reported purification of extensin from carrot roots was by Stuart and Varner [243] (a wound induced extensin) and later by Van Holst and

Varner [250] (an extensin enhanced by wounding). A second extensin was isolated from aerated carrot roots named extensin-2 [237].

Hydroxyproline-rich glycoproteins have also been reported in the algae, *Chlamydomonas* and *Volvox*. An extremely hydroxyproline-rich sulfated glycoprotein is expressed under strict developmental control in inverting *Volvox* colonies [216]. Additionally, several cell wall HRGPs have been isolated from *Chlamydomonas reinhardii* [35, 92, 202] as major structural components of the cell walls.

In all these glycoproteins, Hyp is the major amino acid representing approximately 30-45 mole% of the total amino acids (see 34). Other abundant amino acids are Ser, His, Lys, Tyr, Val, and Pro and arabinose and galactose are the only carbohydrates present in the protein. The relative amounts of the oligoarabinose substituents of the Hyp seem to be constant for a given species [158]. For example, in tomato cell suspension cultures, Hyp-tetraarabinosides and triabinosides predominate in both extensin precursors, P1 and P2, isolated [228]. In soybean seed coat extensin, arabinose is mainly bound as Hyp-triarabinosides [32]. In the monocot extensin isolated from maize, 48% of the Hyp is non-glycosylated, while the arabinosylated Hyp occurs as the monosaccharide (15%) and trisaccharide (25%) [131].

Extensin Amino Acid Sequence by Traditional Methods. The complete amino acid sequences of the two different extensin monomers isolated from tomato suspension cultures, P1 and P2, have been reported [229]. From the analysis of tryptic peptide maps, P1 and P2 amino acid sequences were found to be highly repetitive. P1 contains primarily two different peptide blocks: Ser-$(Hyp)_4$-Thr-Hyp-Val-Tyr-Lys and Ser-$(Hyp)_4$ -Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys; and P2 consists of a single-repeating decapeptide, Ser-$(Hyp)_4$ -Val-Tyr-Lys-Tyr-Lys. These sequences of P1 and P2 show two different repeated domains, one of usually glycosylated Ser-$(Hyp)_4$ sequences and the other a nonglycosylated and repeated domain. The significance of these two different domains has been discussed [229]. That is, the glycosylated domain is relatively rigid and the nonglycosylated one flexible so that the proposed structure [70, 229] might allow the weaving of the cellulose microfibrils of the primary wall ("warp") with an extensin network of defined porosity ("weft"), hence the so-called "warp-weft" model. The most recent model of the primary cell wall has been presented by Lamport [154].

Cross-Links. From early studies on the structure of extensin, it was generally thought that the glycoprotein was held in the cell wall by bonding to the polysaccharides. These links were most likely by the carbohydrate side chains of extensin. Later, it was proposed that extensin is slowly insolubulized in the cell wall by a covalent, non-carbohydrate link [51, 228]. One possible covalent link is the isodityrosine discovered in extensin tryptic peptides from cultured tomato cells [151, 157].

Fry [85] isolated isodityrosine, a tyrosine dimer, and showed that the two tyrosine units of extensin molecules were linked by a diphenyl ether bridge. Additionally, Cooper and Varner [51, 52] working with the carrot disc system showed that extensin is secreted from the cytoplasm as a soluble monomer, and that isodityrosine is formed from tyrosine as the glycoprotein is insolubilized in the wall. Wall-bound peroxidase has been implicated in this process ([52, 87]. In contrast to isolating an intermolecular isodityrosine, Epstein and Lamport [70] isolated isodityrosine from two tryptic peptides from cultured-tomato cell walls and showed that the tyrosine dimers were actually intramolecular cross-link forming small closed loops in single extensin molecules, typically found in sequences of Tyr-Lys-Tyr. From these studies, isodityrosine seems to exist in both an inter- and intramolecular form, each having their own function.

Strafstrom and Staehelin [236] have investigated cross-linking patterns in extensin by studying oligomers that can be extracted from carrot cell walls by salt elution. These authors observed that the predominant salt-extractable fraction contained 5.3 half-residues of isodityrosine per molecule and that most of the isodityrosine residues are intramolecular. In general agreement, Fry [86] had calculated that there are about 9 half isodityrosine residues per extensin precursor molecule; however, Stafstrom and Staehelin [236] went on to say that as extensin is rendered insoluble, an additional 2.5 isodityrosine cross-links could be formed intermolecularly, making their total 7.8 half residues of isodityrosine per molecule. Using electron microscopy, they observed that isodityrosine cross-links in extensin oligomers are preferentially formed near the ends of molecules.

A new model for the structure of cell walls is partially related to the structural significance of isodityrosine cross-links to extensin. This model is based on the "warp" (cellulose) and "weft" (extensin) hypothesis proposed by Epstein and Lamport [70]. The role of isodityrosine cross-links may be explained by reviewing the structure of extensin. The positive charges of extensin may interact with the negatively charged uronic acid residues of pectin supporting the idea that extensins are organized around cellulose

microfibrils. Cassab and Varner [34] caution that the insolubilization of extensin may not be the consequence of a covalent link, such as isodityrosine, but of an irreversible change in the conformation of the wall during desiccation as seen in soybean seed development.

Molecular Biology Studies

Not all of the information regarding extensin structure has been determined by traditional protein chemical methods because of the difficulty presented with the presence of many imino acid residues (Hyp) and of many posttranslational modifications. Additionally, the insolubility of extensin has been the major obstacle to its study. A better understanding of the composition, primary sequence, and secondary conformation has only emerged since the discovery of soluble extensin precursors which can be readily eluted from the wall and represent fully-formed extensin molecules which have not yet been cross-linked. Most recently, nucleic acid studies have provided answers to questions concerning the number of different extensin genes and mRNAs as well as the complete primary sequence of extensin elucidated by these techniques.

Chen and Varner [38, 39] broke ground in the area of molecular biology studies related to extensin when they isolated and sequenced a partial cDNA clone for carrot root extensin. This partial cDNA clone [39] isolated from a cDNA library from wounded carrot root mRNA provided the sequence of the carboxyl-terminal end and encoded a peptide containing Ser-$(Pro)_4$ repeats and Tyr-Lys-Tyr-Lys sequences found also in tomato extensin [70, 229]. Later, using extensin cDNA as probes, six different clones from carrot genomic libraries were isolated [38]. The genomic clone, pDC5A1, was characterized and found to contain an open-reading frame possibly encoding extensin and a single intron in the 3'-noncoding region. The derived amino acid sequence contained a putative signal peptide and 25 Ser-$(Pro)_4$ sequences. Two different extensin RNA transcripts were found corresponding to this genomic clone with different 5' start sites. Both of the identified transcripts increased dramatically after wounding, which correlates with the extensin accumulation seen in the cell wall after wounding [38, 42, 243]. In order to confirm that this particular gene encodes the extensin isolated from carrot root it would be necessary to immunoprecipitate the hybrid release RNA translation production with specific antibodies against extensin. Nonetheless, extensin has been immunolocalized in cell walls of carrot roots using antibodies against extensin-1 [238].

Showalter *et. al.* [223, 224] have also isolated and characterized tomato cDNA and genomic clones for extensin using the corresponding carrot extensin clones as probes. The sequence of this clone encodes a polypeptide with numerous Ser-$(Pro)_4$-Val-His and Ser-$(Pro)_4$-Val-Ala repeats. These repeats have not been observed on a protein level. The tomato cDNA sequence; however, shows perfect agreement (excluding the post-translational modifications) with a known tomato cell wall glycopeptide sequence (from extensin) originally characterized by Lamport [151]. The same carrot clones for extensin have been used to isolate petunia cDNA and genomic clones for cell wall HRGPs [225]. These petunia clones encode Ser-$(Pro)_4$-Ser-Pro-Ser-$(Pro)_4$-$(Tyr)_3$-Lys and Ser-$(Pro)_4$-Thr-Pro-Val-Tyr-Lys repeat sequences, nearly identical to those previously elucidated in acid/protease-generated tomato cell wall glycopeptides [151]. Additionally, a tomato HRGP gene sequence has been used to isolate HRGP cDNA clones from bean cell cultures [225] where the presence of numerous Ser-$(Pro)_4$-$(Tyr)_3$-His-Ser-$(Pro)_4$-Lys-His repeat units was observed. A comparison of all know HRGP repeated amino acid sequences to date from either protein or DNA sequence determinations has been presented [225]. The comparison revealed that Ser-$(Hyp/Pro)_4$ repeats were highly conserved but substantial variability in the flanking amino acid sequences was noted.

Biosynthesis and Insolubilization of Extensin

Biosynthesis of Extensin. The general biosynthetic pathway for the cell wall HRGPs is well established (see 60, 203, 211, 225, 260 for reviews), although the subcellular localization of some of these reactions is still controversial. The extensin polypeptide backbone is synthesized on membrane-bound polyribosomes and then undergoes extensive post-translational modifications including hydroxylation of proline residue and glycosylation.

Following transcription, cell wall HRGP mRNA is cytosine-rich and is translated on membrane-bound polyribosomes in the cytoplasm into a proline-rich polypeptide. A putative signal peptide has been identified both at the protein and at the gene level in carrot discs [38] supporting the notion that mRNA is translated on the rough endoplasmic reticulum. Other studies with carrot discs by Weinecke *et. al.* [257] and Robinson and Glas [204] also supported this idea, by isolating organelles and studying kinetics following *in vivo* labeling with [^{14}C] proline they concluded that the major Hyp-containing wound-induced protein(s) of carrot were synthesized first in the endoplasmic reticulum and then transported to the Golgi prior to secretion into the wall.

Since no codon exists for hydroxyproline, proline residues must be hydroxylated post-translationally. Most of the proline residues in the polypeptide are hydroxylated by the action of peptidyl proline hydroxylase [41]. Weinecke *et. al.* [257] found labeled hydroxyproline in protein of the endoplasmic reticulum indicating that some of the plant prolyl hydrolase functions in this organelle. The first characterization of plant prolyl hydrolase [210] indicated that the enzyme was largely soluble, but several other researchers [20, 244, 257] have localized the enzyme in membrane-bound fractions. Later, Sauer and Robinson [215] indicated that the maize prolyl hydrolase is largely localized in the endoplasmic reticulum with small amounts possibly in the Golgi. Cohen *et. al.* [45] confirmed this distribution of prolyl hydrolase after examining the enzyme from rye grass endosperm cells and found it to be localized both in the Golgi and endoplasmic reticulum. The plant prolyl hydrolases studied have several features common to the enzyme involved in collagen biosynthesis namely the following: similar pH optimum, requirements for ferrous ion, inhibition by certain buffers, and requirements for molecular oxygen, ascorbate, and α-keto-glutarate [60, 211]. Tanaka *et. al.* [245] studied the substrate specificity of proline hydroxylase in suspension-cultured *Vinca rosea* and showed that the enzyme recognized the polyproline II helix conformation and hydroxylated tetra-proline sequences.

After hydroxylation is complete, the second type of post-translational modification occurs in the Golgi, namely glycosylation. Only the mechanism of attachment of arabinose to hydroxyproline is known. No information exists concerning the mechanism of attachment of galactose to serine residues in extensin. At least three different arabinosyl transferase activities are responsible for the specific linkages of arabinose residues to hydroxyproline and arabinosylated hydroxyproline [18, 126, 181]. Karr [126] demonstrated the attachment of mono-, di-, tri-, and tetra-arabinosides to protein bound hydroxyproline following incubation of membranes derived from cultured sycamore maple cells with UDP- [^{14}C] Ara. Similarly, arabinosylation of endogenous protein using membrane preparations from cultured cells of potato [181] and bean [18] has been documented.

Following post-translational modifications, a soluble form of extensin is secreted into the wall. Pulse-chase experiments carried out by Chrispeels [40], Brysk and Chrispeels [28] and Cooper and Varner [51] labelled a hydroxyproline-containing glycoprotein in carrot that could be extracted from the cell wall by salt solutions and later

because insolubilized and unextractable. Pope [187]concluded that the wall protein from suspension-cultured sycamore cells is transferred directly from a particulate fraction to the wall, where it is immediately incorporated without passing through a salt-extractable pool. Studies by Stuart and Varner [243] demonstrated a soluble form of extensin that could be salt-extracted from wounded carrot disc walls and was slowly insolubilized in the wall to form insoluble extensin [31, 51, 52]. Because it was later found by Smith *et. al.* [228] that sycamore cells were unsuitable for studying extensin precursors, cultured tomato cells were employed as an alternative and consequently, two distinct salt-extractable extensin precursors were characterized. These soluble forms of extensin closely resemble the bacterial agglutinins isolated from potato [162] and tobacco [168]. Having a net positive charge, such precursors of extensin may play a role in defense by binding invading pathogens [60].

Insolubilization of Extensin. The insolubilization of precursors to form insoluble extensin in the wall is reviewed by Wilson and Fry [260] and Delmer and Stone [60]. Smith [230] using the carrot disc system showed that one HRGP species accounted for most of the protein incorporated into the cell wall and that synthesis in the presence of the proline hydroxylation inhibitor, α,α'-dipyridyl, resulted in a protein which appeared to be a non-hydroxylated, non-glycosylated but still a tightly bound form of the same glycoprotein. This study provided valuable evidence that hydroxylation and arabinosylation are not required for covalent attachment to the wall. However, Stafstrom and Staehelin [235] pointed out that secretion and even cross-linking of unglycosylated extensin does not necessarily mean that the protein may be functioning properly in the wall. They suggested that without carbohydrates, an extended polyprolyl II helix cannot be maintained and thus cannot meet the demands of the "warp and weft" model which requires an elongated rod conformation with accurate spacing of cross-links to provide the necessary strength. However, Heckman *et. al.* [105] found using electron microscopy that deglycosylated extensin monomers were thin, but discernable extended rods suggesting that some degree of structural integrity is maintained even after the carbohydrates are removed.

Cooper and Varner [51] and Fry [85] found that insolubilization of extensin precursors was correlated with another post-translational modification, the formation of isodityrosine cross-links. Wall peroxidases may be responsible for formation of isodityrosine linkages in dicot cell wall glycoproteins. Experiments using wall peroxidase inhibitors such as dithiothreitol, cyanide, and ascorbate indicated that the formation of

isodityrosine linkages in dicot wall glycoproteins and the insolubilization of the soluble extensin precursor was slowed [51, 85]. The potential for a wall-bound peroxidase-ascorbate oxidase system to control the redox potential of the wall and hence the rate of free radical phenolic cross-linking (isodityrosine formation) of wall polymers has been proposed by Cooper and Varner [51, 52]. They found that isolated walls from aerated root slices of carrots have the capacity to insolubilize extensin specifically through the formation of isodityrosine resides. They have reported that cross-linking of extensin *in vitro* is inhibited by acid within the pH range 4 to 7 [52]. This information has caused Cooper and Varner [52] to speculate that the solubility of extensin could be regulated by auxin-induced hydrogen ion (H^+) secretion. They propose two means by which wall pH might regulate extensin solubility: 1) the activity of the enzyme catalyzing the cross-linking would be affected by pH and 2) the structure of extensin would be affected directly since the local environment of the tyrosine side chain could be changed affecting the affinity of the peroxidase cross-linking catalyst or altering the stability of tyrosyl free radical intermediates.

Additionally, Fry [84] has noted that calcium, an inhibitor of cell expansion also promotes the secretion of peroxidase into the wall [242] and thus formation of isodityrosine linkages and insolubilization of extensin. Giberellin, on the other hand, inhibits peroxidase activity thus promoting cell expansion [90]. Finally, Stafstrom and Staehelin [236] suggested that variation of the proportions of the two dimers, inter- and intramolecular isodityrosine links, could also be a means of controlling extensin solubility. Thus, it is quite possible that different peroxidases catalyze formation of the two links, and differential expression of the enzymes could regulate the number of cross-links between different molecules. One last note, cell wall HRGPs are especially stable once they are insolubilized thus making them difficult to solubilize and digest proteolytically.

Roles of Extensin

Structural/Mechanical Function. The distribution of extensin goes beyond the primary wall of plant cells. The Hyp content was determined in several organs and tissues of the soybean plant [31, 32]. It was found that seed coats and root nodules contain the highest ratio of Hyp to dry weight compared to roots, leaves, stems, and flowers. Furthermore, extensin was primarily localized in two of the external layers of the seed coat, palisade epidermal and hour glass cells, and in the cortex of soybean root nodules. Thus, it can be concluded that the distribution of extensin in plant cells is tissue

specific. Additionally, Cassab *et. al.* [32] found that HRGPs were developmentally regulated. Localization of extensin in different plant tissues is necessary in order to assign a possible function to that protein. Specific antibodies to soybean seed coat extensin used for immunocytolocalization revealed marked deposition of the glycoprotein in the walls of both palisade and hour glass cells which changed during different stages of seed development [102].

A new technique called "tissue-printing" on nitrocellulose paper was developed to immunolocalize extensin in different plant tissues as well as plant species [33]. Tissue-printing of developing soybean seeds showed that extensin is mainly localized in the seed coat, hilum, and vascular regions of the seed including sclerenchyma cells. The presence of extensin in sclerenchyma could be related to the mechanical function of this tissue in the plant. Extensin was also immunolocalized in the collenchyma cells of the cortex of soybean root nodules [31]. The presence of extensin in collenchyma cells could also be related to the mechanical function of this tissue in the plant since collenchyma is the first supporting tissue that occurs in a peripheral position in stems, leaves, and floral parts.

Extensin has also been immunolocalized in cell walls of unwounded carrot roots [238]. In carrot phloem parenchyma walls, extensin-1 is distributed uniformly across the wall but is absent from the expanded middle lamella at the intersection of three or more cells, and is reduced in the narrow middle lamella between two cells. This distribution is the same as cellulose. Results from the immunolocalization studies in carrot root suggest that: a) newly synthesized extensin-1 is added to the wall by intercalation of new particles among those existing in the wall (i.e. intussusception), b) extensin cannot cross the middle lamella separating the walls of adjacent cells; and c) incorporation of extensin is a late event in the development of phloem-parenchyma cell walls in carrot, since it is absent in walls of young root tissue. Wounding enhances accumulation of extensin [42, 243], but variability of immunolabeling in carrot roots made it difficult to determine exactly where in the wall newly synthesized extensin becomes incorporated [238].

Role in Plant Growth. The role of extensin in plant growth is not clear. Extensin accumulation in the wall of growing pea epicotyl was coincident with the cessation of cell elongation [212]. Yet, the protein content of the wall increases as well as the content of Hyp and Hyp-arabinosides during elongation of pea stems and these contents changed only slightly once elongation was complete [134]. Thus in elongating pea stems, there is

an inverse relationship between the rate of elongation (extension) and the concentration of HRGPs, so that extensin stiffens the wall during growth, thus reducing the rate of elongation [134].

Role of Extensin in Plant Defense: Response to Stress and Pathogenesis. Several studies suggest a role for extensins in plant defense. A number of observations show extensins accumulate in the cell wall in response to infection, mechanical wounding, ethylene treatment, heat treatment, and red light illumination. These proteins may function in defense simply by forming a more dense, impenetrable cell wall barrier or by providing nucleation sites for the deposition of lignin [258]. Lignin deposition would, of course, provide a protective wall barrier to potential pathogens. In addition, purified HRGPs possess the ability to agglutinate bacteria [162, 168, 250] presumably because of their positively charged nature (polylysine can also agglutinate bacteria). Consequently, these glycoproteins may also function in defense by immobilizing pathogens in the wall.

Immunocytochemical studies of extensin have shown that extensin accumulated in the walls of living, uninfected cells close to sites where fungal and bacterial growth is restricted by plants [165]. Extensins also accumulate in plant papillae which may present a physical barrier to penetration by fungi [165]. Direct evidence for the involvement of papillae as mechanical barriers to penetration has been reported by Israel and coworkers [123]. These researchers demonstrated that the acoustic reflectance (and hence the viscosity, density, and elasticity) of preformed barley papillae (which are resistant to fungal penetration) is greater than that of papillae susceptible to penetration. These observations support the proposed role for extensins in plant disease resistance. Thus, extensin may have a mechanical function in cell extension and a function in disease resistance and stress.

There is an accumulation of cell wall HRGPs in carrot discs [42, 243] and in bean hypocotyls [135] following slicing (mechanical wounding). Similarly wounded tomato stems have been shown to accumulate cell wall HRGPs and HRGP mRNA [224] even though the pattern of RNA accumulation is different for the two plants. Cells in callus or suspension culture show much higher levels of cell wall hydroxyproline than their uncultured counterparts [225], indicating that these tissues behave much like wounded tissue.

Ethylene, a gaseous plant hormone whose level is known to increase following wounding, stimulates and may mediate wound-induced accumulation of cell wall HRGPs [69, 71, 199, 207]. One of the earliest detectable events during plant-pathogen interaction is a rapid increase in ethylene biosynthesis. It has been proposed that ethylene produced in response to biological stress is a signal for plants to activate defense mechanisms against invading pathogens [69]. These researchers found that ethylene markedly increased the levels of HRGP transcripts and the transcripts of three other defense-response genes: PAL, 4-CL, and CS in ethylene-treated carrot roots. However, the accumulation of mRNA for extensin following ethylene treatment was different from that of wounding. Ethylene induced two extensin mRNA (1.8 and 4.0 kb), whereas wounding of carrot root produces the accumulation of an additional extensin mRNA (1.5 kb). These results suggest that the two signal, ethylene and wounding, are distinct [69]. Additionally, Rumeau *et. al.* [207] found that treatment of melon plants with ethylene leads to early induction of the 1.4 and 1.65 kb mRNA species of the five isolated mRNA species (1.4, 1.65, 2.15, 3.2, and 4.5 kb) which hybridize to a genomic clone of HRGP from carrot [38] These results provide further evidence that ethylene can induce the appearance of hybridizable HRGP mRNAs similar to, but more speedily, than infection. Again, this work strengthens the view that ethylene produced during infection is one of the signals responsible for the induction of HRGP mRNAs.

Several groups have observed that HRGPs accumulate in response to infection and that this accumulation is correlated with the expression of disease resistance. Showalter and associates [223] reported the accumulation of HRGP mRNAS in *Colletotrichum lindemuthianum* infected bean hypocotyls at the sites of infection and in tissues distant from the infection sites, a sign of induced systemic resistance, as well as in elicitor-treated bean cells using a genomic clone for carrot extensin as a probe [38]. This same extensin genomic probe has been used to study probable extensin mRNA levels in unwounded and wounded tomato stems and leaf tissue [224]. When tomato stems are wounded, the expression of a new and larger extensin transcript (1.7 kb) occurred, together with the disappearance to two extensin transcripts (both about 1.2 kb) that were present in unwounded stems. The multiple mRNAs present in the same plant may reflect the presence of a multigene family. Treatment of melon and soybean hypocotyls with components of fungal cell walls (i.e. elicitors) also resulted in the stimulation of HRGP synthesis [205]. Similarly, elicitor treatment of bean cell cultures resulted in the accumulation of cell wall hydroxyproline [21], a direct sign of HRGP accumulation.

Extensins accumulate in melon plants infected with the fungus, *Clolletotrichum lagenarium* [71, 73, 164] and in cucumber plants infected by *Cladosporium cucumerinum* [100]. Cell wall hydroxyproline levels, indicative of HRGP levels, increased more rapidly in resistant than in susceptible cultivars of cucumber infected with the fungus, *C. cucumerinum* [100]. Esquerré-Tugayé *et. al.* [71] found an approximately 10-fold increase in cell wall HRGP in melon plants in response to infection with the fungus, *C. lagenarium* . These observations have been extended to many different host-parasite systems and their Hyp/HRGP accumulation appears to correlated with specific defense reactions [100, 164].

Rumeau and associates [206] reported the isolation and *in vitro* translation of cytosine-rich RNA from melon leaves infected by *C. lagenarium.* Data indicate that an increase in translatable cytosine-rich RNA occurred at the onset of *in vivo* HRGP accumulation in infected plants. Among the translation products were two peptides, 56 and 54.5 kd, which appeared to be specifically coded by cytosine-rich RNA. These molecular weights are compatible with that of melon HRGP (55 kd). These data suggest that the two peptides are two HRGP precursors whose synthesis is increased as a result of an increase in translatable cytosine-rich RNA during infection.

Precisely how HRGP accumulation is brought about by wounding, elicitation, and infection is unknown. However some progress has been made toward answering this question. Nuclear run-off experiments, measuring transcription rates in isolated nuclei monitored with [^{32}P] labelled specific transcripts, indicate that HRGP mRNA accumulation is controlled at a transcriptional level at least in wounded bean hypocotyls and elicitor-treated bean cell cultures [161]. Thus, there is a clear indication that ethylene and elicitors, respectively, may be involved at relatively early stages of wound- and infection-regulated HRGP accumulation.

Heat shock also enhanced the accumulation of insoluble extensin found in cell wall of cucumber seedlings [240]. Cucumber seedling, heat-shocked for 40 s at 50°C, acquire disease resistance to the fungal pathogen, *C. cucumerinum.* Heat-shock also causes stimulation of ethylene production. A mechanism for disease resistance induced by heat shock and systemic induced resistance can be proposed from this work. Stermer and Hammerschmidt [240] suggest that an increase in the cross-linking of extensin molecules by the peroxidase isozymes which increase after heat shock could be responsible for the

increased resistance of cell walls from heat-shocked seedlings to digestion by *C. cucumerinum* enzymes and thus provide resistance to fungal attack.

Other roles. A number of other possible functions have been proposed for extensin. There is some indication that cell wall HRGP levels are regulated by phytochrome, since red-light treatment of etiolated pea epicotyls increases the level of wall-bound hydroxyproline [186]. This effect can be reversed by far-red light suggesting that this type of regulation may represent a level of developmental control for the synthesis of HRGPs. Albersheim [2] suggested that extensin could act as a carrier protein, with the function of transporting polysaccharides from the cytoplasm to the wall. Kauss and Glasser [127], in a similar vein, pointed out that extensin might guide the polysaccharides into their correct orientation in the cell wall. An alternative idea involves extensins acting as a primer for the synthesis of the wall polysaccharides, in a similar mechanism to the polymerization of starch and glycogen [27].

In summary, the roles of extensin seem to have in the defense response are: act as nonspecific barriers to pathogen attack, provide matrices for the deposition of lignin, and/or act as nonspecific agglutinins of microbial pathogens. Finally, a few studies to date have shown HRGPs to accumulate in the cell wall in reponse to bacterial infection [164, 168].

Usefulness of Anhydrous Hydrogen Fluoride Solvolysis in Structural Analysis and Isolation of Cell Wall HRGPs

History

The uses of anhydrous hydrogen fluoride (HF) in wood analysis date back to 1869 when Gore [94] determined the formula of this compound as HF and observed that many inorganic and organic materials such as: paper, cotton-wool, calico, gum arabic, India rubber, sealing wax, gelatin and parchment were instantly solubilized by anhydrous HF. Since that time, a limited number of researchers have used anhydrous HF to study the polysaccharides of wood [83] as well as the lignin content of wood [43]. Since 1965, HF has been used to solubilize amino acids and a number of proteins in an attempt to study peptide and protein synthesis (especially the final deprotection step) [213]. Later, Mort and Lamport [177] found that HF could also be used to selectively cleave sugars from

glycoproteins, leaving the protein moiety intact. Since then, this method has been used for deglycosylation of proteins and selective deglycosylation of other polymers.

Most recently, a limited number of researchers have applied anhydrous HF to study structural carbohydrate chemistry in plants. The discovery that HF can selectively solvolyze different glycosidic bonds at different temperatures [177] has proved invaluable in elucidating the complex polysaccharide structures of plant cell walls or other material with an absolute precision not provided by any other known chemical method. That is, by using HF, polysaccharides can be degraded into smaller fragments in high yields which can be used to determine the identity and location of both N - and 0 -acyl substituents on sugars and to aid in the determination of the fundamental structure of the polymer. Two recent reviews summarize the HF methodologies for cell wall analysis [176] and for the structural analysis of polysaccharides [136].

Mechanism of the Reaction between Hydrogen Fluoride and Carbohydrates

The mechanism of action of HF on carbohydrates has been thoroughly discussed by Knirel and associates [136] and summarized briefly by Komalavilas [141]. For example, cellulose, amylose, starch, xylan, and inulin among other materials have all undergone HF solvolysis and the reaction products have been analyzed in detail [59, 178]. These polysaccharides were solubilized in HF at temperatures greater than -20°C and undergo depolymerization to give the corresponding glycosyl fluorides. The resulting glycosyl fluorides were then converted to other products depending on the reaction conditions as shown by scheme presented in Figure 1.

The ability of HF to dissolve polysaccharides is due to the formation of hydrogen bonds between HF and polysaccharides [59] Subsequent depolymerization of the polysaccharide proceeds by way of protonation of the glycosidic oxygen atom and cleavage of the resulting aglycone to give the glycosyl cation. This process is in accordance with the known mechanism of acid hydrolysis of glycosides [182] (refer to Figure 1). Solvolytic cleavage is also promoted through the stabilization of the resulting glycosyl cation by hydrogen fluoride [59]. Additionally, secondary oligomerization can occur by glycosidation of hydroxyl groups of sugars by the glycosyl fluorides when the concentration of sugars is right (see [136] for specific conditions and reaction products). Glycosyl fluorides can also alkylate aromatic compounds with HF acting as a catalyst via

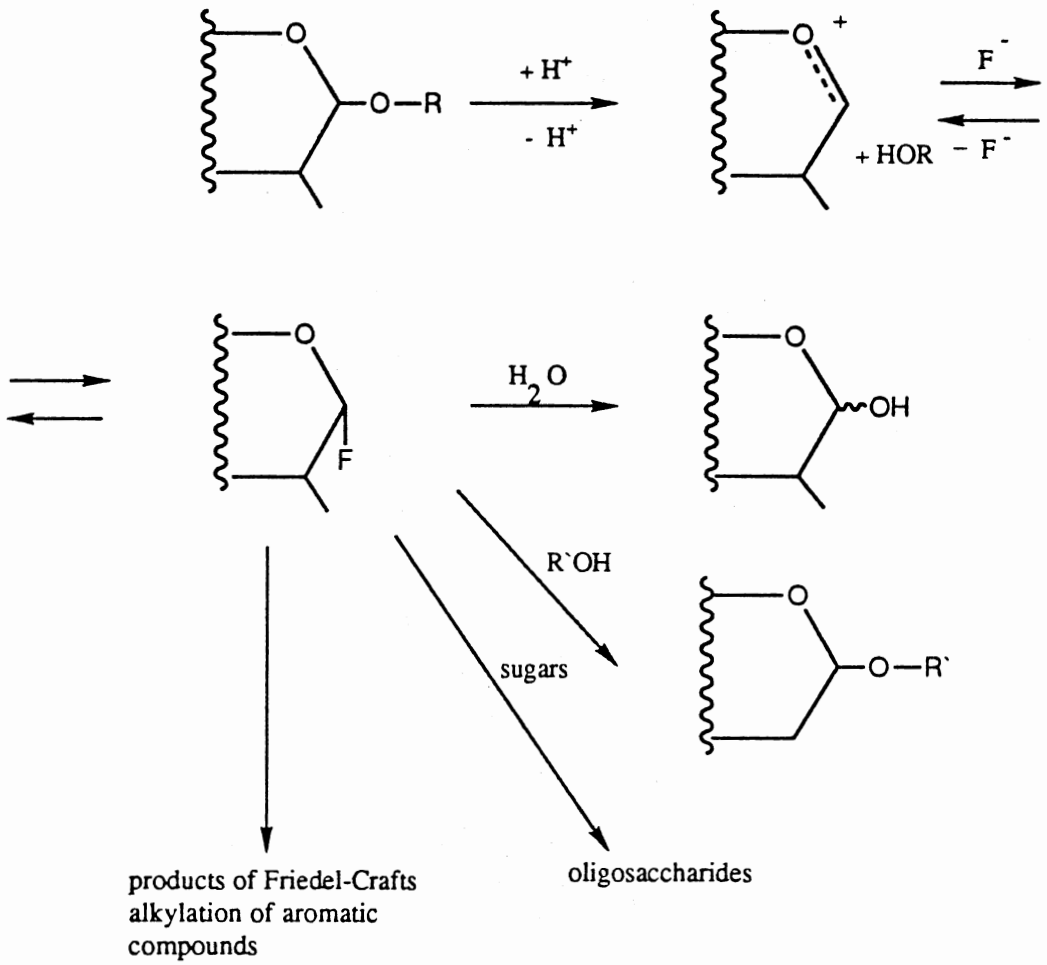


Figure 1. Possible reactions of Glucosyl Fluorides Derived from HF Solvolysis with Various Sugars. (Adapted from P. Komalavilas. 1988. Partial Structural Characterization of Pectin from Cotton Cell Walls Using Selective Hydrogen Fluoride Solvolysis. Ph.D. Thesis. Oklahoma State University. p. 15.)

the Friedel-Crafts mechanism [256]. The stability of glycosyl fluorides varies greatly and is discussed in great detail by Knirel and associates [136]. Hence, selective HF solvolysis at various temperatures has been used extensively in several experiments to study the structure of bacterial and cotton cell wall polysaccharides [141, 142, 145, 146, 174, 175, 176].

Glycoprotein Deglycosylation

In general polysaccharides can be selectively cleaved by HF solvolysis at various temperature (Table 1) [177]. Polysaccharides can be broken into large fragments at low temperatures and then by increasing the temperature broken into smaller fragments [174]. Chemical deglycosylation of glycoproteins and mucoproteins is generally more than 90% complete after 1 h at 0°C for O-glycosidic linkages of neutral sugars while 0-glycosidic linkages of amino sugars require upwards of 3 h at 23°C [176]. That is, for HF solvolysis of the cell wall protein, extensin, all the O-glycosidic linkages of galactose to serine and of arabinose to hydroxyproline are cleaved readily at 0°C within 1 h.

After HF-deglycosylation, extensin monomers gave reproducible tryptic peptide maps and amino acid sequences indicating the highly repetitive nature of the peptides [229]. HF solvolysis for 1 h at 0°C yields no evidence of HF-induced peptide cleavage, but 3 h at 23°C does produce some peptide cleavage [176]. HF degradation does not seem to complicate raising antibodies against the deglycosylated glycoproteins because of the highly repetitive nature of the peptides. Kieliszewski and Lamport [130] showed that antibodies raised against the native and deglycosylated extensin monomers will cross-react in an indirect ELISA. This general method now allows one to quantify the contribution of both glycosylated and non-glycosylated epitopes to the antigenicity of a glycoprotein. However, deglycosylation of extensin monomers leads to a 50% decrease in polyproline II helix content [250]. Yet, the molecule can still be visualized as the typical flexuous rod by electron microscopy [105], even though it is no longer a substrate for the cross-linking enzyme, extensin peroxidase [79].

Thus, HF solvolysis at 0°C can be used to deglycosylate extensin and aid in isolating an intact protein. Mort and Lamport [177] applied anhydrous HF to the problem of studying extensin which is insolubilized in the cell wall (see "Insolubilization of extensin" section presented earlier in this chapter). The premise that these researchers used was that the cell wall protein would be soluble once deglycosylated. However, after

TABLE 1

EXPECTED LABILITY* OF THE GLYCOSIDIC LINKAGES OF VARIOUS SUGAR RESIDUES**

SUGAR RESIDUE	TEMPERATURE OF HYDROGEN FLUORIDE (°C)				
	-70<	-40	-20-23	0	20-25
Pentofuranose	+	+	+	+	+
Pentopyranose	±	±	+	+	+
6-Deoxyhexose	-	±	+	+	+
alpha-Hexose	-	±	+	+	+
beta-Hexose	-	±	±	+	+
2-Amino-2,6-dideoxyhexose	-	-	-	±	+
3-Amino-3,6-dideoxyhexose			+	+	+
4-Amino-4,6-dideoxyhexose			+	+	+
2,4-Diamino-2,4,6-trideoxyhexose					+
2-Amino-2-deoxyhexose	-	-	-	±	±
Uronic acid	-	-	-	±	±
Galactosaminuronic acid	-	-	-	-	±
Mannosaminuronic acid	-	-	-		+
2,3-Diaminuronic acids	-	-	-	-	-
5,7-Diamino-3,5,7,9 tetradeoxynonulosonic acid	-	-	-	-	-

*The labilities indicated are generalizations from the limited number of cases that have been investigated. Branching, anomeric configuration, and some unidentified factors affect the labilities. Key: (+), cleavage; (-), stable; (±), depends on structural peculiarities. **All of the amino sugars were tested in the N-acetylated form. From Y. A. Knirel, E. V. Yinogradov, A. J. Mort. 1989. Application of anhydrous hydrogen fluoride for the structural analysis of polysaccharides. In. Advances in Carbohydrate Chemistry and Biochemistry. vol. 38, in Press.

HF solvolysis the cell wall protein instead of dissolving as expected, remained as an insoluble residue consisting of about 10% of the treated tomato cell wall and an equal proportion of a phenolic component [173, 177]. Lamport [152] suggested that this insoluble residue must contain some other yet unidentified phenolic component acting as a cross-link to prevent solubilization of the cell wall protein. Lamport [153] put forth the idea that isolation of cross-linked peptides and the identification of components involved at the cross-link region would prove that this insoluble residue must contain cross-links and that the protein may be cross-linked to itself independently of any possible links to cell wall polysaccharides. To date, this proof has not been forthcoming.

However, Selvendran *et. al.* [219] reported that extensin from runner beans could be solublized from the cell wall by treatment with warm sodium chlorite and acetic acid, a reagent traditionally used for delignification. Then Mort [173] and O'Neill and Selvendran [180] suggested that the glycoprotein may be held in the wall by phenolic cross-links, which would then account for the effectiveness of the acetic sodium chlorite extraction method. Additionally, the treatment of tryptic peptides from cell walls of tomato and sycamore cell suspension cultures with acetic sodium chlorite [156] led to isodityrosine oxidation lending support to the view that a peroxidase could catalyze the formation of a cross-linked extensin network. Finally, deglycosylation of cell wall glycoproteins using selective HF solvolysis may lead to the eventual isolation of a formerly insoluble, intact HRGP whether by disrupting isodityrosine cross-links or other unknown phenolic cross-links.

The Cotton/ *Xanthomonas campestris* pv. *malvacearum* Host/Pathogen System

General

The upland cotton (*Gossypium hirsutum* L.) and *Xanthomonas campestris* pv. *malvacearum* (*X. c. malvacearum*) constitute a good system in which to study race/cell line specificity and the mechanism of disease resistance. There are eighteen known races that have been distinguished by their differential virulence toward five major resistance genes and a complex of minor genes in cotton [24]). In general, the pattern of the race: cell line specificity suggest that the cotton: *X. c. malvacearum* system obeys the gene-for-gene theory described by Flor [82]. Immunity to bacterial blight of cotton is defined as a response to dilute inocula ($\leq 10^6$ cfu ml^{-1}) of all eighteen known races of *X. c.*

malvacearum where no macroscopic symptoms are produced [116]. Macroscopic symptoms are produced as the typical hypersensitive response (HR), rapid death of individual plant cells at infection sites in the whole plant to the pathogen [133], with inoculum levels of 1 X 10^{6-7} colony-forming units (cfu) per milliliter or higher [116]. Immunity to bacterial blight of cotton was developed by combining several single-gene resistance factors onto a polygenic background described in detail by Brinkerhoff and associates [26]. "Resistance" and "Susceptibility" to bacterial blight have been defined as well by Brinkerhoff and associates (1984) in the field based on a macroscopically visible response seen on the leaves.

Studies in the Intact Plant

Work on this host-pathogen system in the intact plant has mainly concerned the possible involvement of phytoalexins in resistance of certain lines of cotton to the bacterial blight pathogen, *X. c. malvacearum*. Bacterial blight-resistant, Im 216, and blight-susceptible, Ac 44, leaf tissue were infected with 1.0 X 10^7 cfu ml^{-1} the pathogen, *X. malvacearum* and examined for signs of disease. The Im 216 leaf tissue showed a typical hypersensitive response characterized by tissue browning and necrosis, and the Ac 44 leaf was eventually destroyed by the pathogen [30]. Ultrastructural studies showed the cytological changes accompanying the rapid collapse of inoculated susceptible cotyledons and that bacterial envelopment accompanied the incompatible response [30]. Additionally, growth studies indicated that colonies of the pathogen develop in leaves from single bacterial cells and that immune cotton leaves produce a small bacteriostatic zone around each bacterial colony [77] characteristic of a hypersensitive response. The growth of the pathogen, *X. c. malvacearum* was studied in susceptible and immune cotton leaves [12, 75]. The bacterial growth was logarithmic in both susceptible and immune cotton leaves but stopped at lower population densities in immune leaves. Growth of bacterial in genetically related cotton lines (AcB_2, AcB_3, Acb_7, OK 1.2) infected with *X. c. malvacearum* was generally similar for the first 24-48 h but differed following this period [12]. These researchers [12, 75] reported that the multiplication of *X. c. malvacearum* stopped abruptly 4 days after inoculation in the immune line, Im 216, which has three major genes and a complex of minor genes for resistance to bacterial blight. In leaves of cotton lines containing resistance genes, B_2, B_3, b_7, B_N in an Ac 44 genetic background, incompatible strains of *X. c. malvacearum* were inhibited 6-8 days after inoculation, whereas compatible strains continued multiplying [222]. Thus it can be concluded from these studies that growth of *X. cmalvacearum* was approximately inversely proportional

to the degree of resistance possessed by the cotton lines, i.e., *X. c. malvacearum* multiplied to the greatest level in leaves of the susceptible line, Ac 44 [76]. Three phytoalexins, low molecular weight antibiotic compounds, were induced in resistant plants upon inoculation of the plants with incompatible races of the blight pathogen, *X. malvacearum* [76, 78]. These phytoalexins were identified as 2,7-dihydroxycadalene, lacinilene c, and lacinilene c 7-methyl ether. Since then, experiments have been conducted to localize these compounds in leaves via fluorescence microscopy [185]. Thus, in the intact cotton plant, there are specific *X. c. malvacearum* -susceptible and -resistant interactions.

Use of Tissue Culture to Study the Cotton/*X. malvacearum* and other Host/Pathogen Systems *In Vitro*

Both callus [117, 189, 221, 231] and suspension cultures [17, 56, 107, 190, 208] of *Gossypium* species have been obtained by a number of laboratories. In general, these studies were undertaken potentially to apply these *in vitro* systems to genetic and crop improvement. In addition, suspension- cultured cells, which do not possess the physiological and morphological complexity of the intact plant, provide a good model for studying host-pathogen relationships and the mechanisms of host-pathogen resistance. The merits of these methods have been examined [62, 121]. Despite misgivings regarding the extrapolation of results to interactions *in vivo* [128], a number of host/pathogen systems have demonstrated that there is a correlation between the response to fungal and bacterial pathogens of intact plants and tissue cultures derived from tobacco [6, 29, 57, 61, 108, 109, 110, 111, 118, 163, 168], lucerne [160], soybean [80, 114] , alfalfa [170], bean [53], potato [122], and cotton [4, 208] plants.

Cotton callus and suspension cultures from cell lines resistant, Im 216 and suceptible, Ac 44 to the bacterial blight pathogen, *X. c. malvacearum* have been established [208]. These two cell lines are good models for studying mechanisms of resistance to *X. c. malvacearum* in cotton culture because of the well-defined pathogenic races of *X. c. malvacearum* and known race: cultivar-specific interactions involving this pathogen [24, 25]. The effect of *X. c. malvacearum* on the growth of the two cotton cell types has been examined in culture [208]. Ac 44 callus had a fresh-weight doubling time of 4 to 5 days, and Im 216 callus had a fresh-weight doubling time of 4 to 9 days; however, in suspension culture the fresh-weight doubling times for Im 216 and Ac 44

were 6 days. Inoculations of Ac 44 and Im 216 callus tissue with *X. c. malvacearum* [208] caused the susceptible line, Ac 44, to brown after 4 days and become completely destroyed after 12 days. The immune line, Im 216, browned after 4 days but continued to grow very slowly even after 4 weeks analogous to growth observed for the uninoculated tissue in the stationary phase of the growth cycle. These results are similar to those which occur in the intact plant [76]. That is, the Im 216 leaf tissue showed a hypersensitive response characterized by tissue browning and necrosis while the Ac 44 leaf tissue was completely destroyed by the pathogen.

CHAPTER III

MATERIALS AND METHODS

Plant Materials

Cotton Lines

The cotton line Acala 44 (Ac 44, blight-susceptible) possesses no genes for resistance to *Xanthomonas campestris* pv. *malvacearum* (Smith 1901) Dye 1978b formerly (E. F. Sm.) Dows (*X. c. malvacearum*) [24] and is completely susceptible to all North American races of this bacterial pathogen. The highly resistant line Immune 216 (Im 216, blight-immune) possesses homozygous resistance to *X. c. malvacearum* including the major resistance genes B_2, B_3, and b_7 in a polygenic background [24, 25, 26]. It is resistant to all known races of *X. c. malvacearum* and shows no macroscopically visible symptoms of natural infection under field conditions [26, 76] The cell lines AcB_N and Acb7 contain the resistance genes, B_N and b_7, respectively, in an Ac 44 genetic background [24]. Cotton lines OK 1.2 and AcIm were derived from segregating generations of a cross between the blight-susceptible line Ac 44 and the blight-immune line Im 216 [185]. Disease severity was graded two weeks after inoculation in the basis of lesion size and was recorded on a scale from 0.0 (highly resistant, no macroscopically visible lesions) to 4.0 (highly susceptible, many macroscopically visible lesions) [26]. Line OK 1.2 had an infection grade of 1.2 [185] and possessed a high level of resistance which appears to be due to the resistance genes B_3 and b_7 [10]. AcIm had an infection grade of 0.0 [185] and possessed an even higher level of resistance probably due to the three major resistance genes B_2, B_3, and b_7, and a complex of minor genes [25, 26].

Plants of Ac 44, Im 216, Acb7, AcB_N, OK 1.2, and AcIm were grown in sterilized six-inch diameter clay pots in Jiffy Mix-Plus (Jiffy Products of American, West

Chicago, IL) in a growth chamber with 12 h, 30°C full-light periods under incandescent plus fluorescent lights, 1 to 2 X 10^4 lux at the level of the plants and 12h, 18°C dark periods. All plants were grown from surface-sterilized, acid-delinted seeds.

Callus cultures

Explant tissue for callus initiation was obtained from 30- to 90-day old plants. Callus cultures were established according to the method of [208]. The original Ac 44 and Im 216 callus cultures were established in 1975 [208]. These were the two major callus cultures used for these studies. The OK 1.2 callus culture was established in 1983 (Earl D. Mitchell, Jr., Dept. of Biochemistry, Oklahoma State University, OK).

Callus cultures from fully-expanded leaves of Ac 44, AcB_N, and Acb7 were established in 1985 and AcIm in 1986 (Janet Rogers, Dept. of Biochemistry, Oklahoma State University, OK) using a modification of the method of Ruyack *et al.* [208]. Leaves were placed in a Mason jar and washed once with distilled water. The leaves were then washed in a 0.1% (v/v) solution of Tween-20 for 5 min, shaken intermittently. The detergent solution was decanted, and the leaves were washed with distilled water. The leaves were sterilized in 5% (v/v) and 0.1% (v/v) Tween-20 for 2 min and then rinsed once with sterile distilled water. The leaves were then washed for 2 min in 70% (v/v) ethanol and rinsed three times with sterile distilled water. On a sterile glass plate, the sterilized leaf was sliced (midrib was removed) into 0.5 to 1.0 cm^2 pieces with a scalpel. Using sterile forceps, the cut explant material was placed in fifty 18 X 150 mm Pyrex culture tubes (Corning Glass works, Corning, NY) containing Shenk and Hildebrandt (SH) solid media [221] with the cut edges flattened into the medium. All sterile operations and transfers were carried out in a laminar flow hood. After 5 to 6 weeks, callus tissue (6 to 10 mm in diameter) from the initial explant was excised and transferred to fresh SH media in 25 X 150 mm Bellco culture tubes (Bellco Glass Inc, Vineland, NJ) for subculture. All subsequent cultures were maintained at 30°C in continuous light and transferred every 3.5 to 6 weeks (usually during the late log phase of callus growth) as needed.

Suspension Cultures

Establishment of Suspension Cultures

In general, cotton suspension cultures were established by transferring either one tube containing 3 to 4 g or two to three tubes containing 7 to 9 g of 3- to 4-week old callus (late log phase) from SH agar medium to 125 ml culture flasks containing 50 ml of liquid SH medium. The flasks were placed on a gyroshaker at 180 rpm in continuous light at 30°C. Alternatively, 10 g of callus was transferred to 500 ml of SH liquid medium (minus agar). All operations were performed using sterile conditions in a laminar flow hood.

Bacterial Culture and Inoculation Method of Callus andSuspension Cultures

Single colonies isolated from *X. c. malvacearum* strain 3631, a spontaneous streptomycin-resistant mutant of the race 3 isolate of [76] maintained frozen at -70°C in 14% (v/v) glycerol in nutrient broth (Difco Laboratories, Detroit, MI) were used. A nutrient broth culture of actively-growing bacteria (middle log phase) was diluted with sterile water saturated with calcium carbonate (0.14 mg ml^{-1}) to make the inoculum 5 X 10^9 colony-forming units (cfu) ml^{-1}. Bacteria in the log phase are actively growing when the absorbance at 600 nm is between 0.10 and 0.30. Colony-forming units are determined using the conversion factor 6.69 X 10^9 cfu ml^{-1} $O.D.^{-1}$ derived from 20 bacterial platings with the aid of a Spiral Plater (Spiral Systems, Inc., Cincinnati, OH).

Cotton suspension cultures containing approximately 20 to 25 g of tissue in 50 ml of SH media were inoculated with 100 µl of the prepared inoculum containing 5 X 10^8 cfu ml^{-1} in culture. The final inoculum density was 1 X 10^7 cfu ml^{-1}. Suspension cultures were inoculated immediately after transfer from callus to SH liquid media. Preparation of the inoculum and all inoculations were performed using sterile conditions in the laminar flow hood. Inoculated suspension cultures were placed on a gyroshaker at 180 rpm in continuous light at 30°C.

Alternatively, cotton suspension cultures containing approximately 3 to 6 g of tissue in 50 ml of SH media were cultured for 9 days (during the log phase of growth).

After 9 days in liquid SH media, the cotton cells were washed once with 3% sorbitol to remove the SH media and filtered through a 30-mesh filter. The cells were then resuspended in 50 ml of 10 mM MES and 3% sorbitol (pH 5.8). The cell-free filtrates were saved. The newly transferred suspension cultures or cell-free filtrates were inoculated with 100 µl of the prepared inoculum containing 5 X 10^8 cfu ml^{-1} in culture. The final inoculum density was 1 X 10^7 cfu ml^{-1}. At appropriate time, 4-ml aliquots were removed and filtered through Miracloth (Calbiochem-Behring Corp., La Jolla, CA) to remove cotton cells. The optical density at 600 nm was taken of the filtered aliquots to determine the bacterial growth. All operations were performed using sterile conditions in a laminar flow hood. Inoculated suspension cultures and cell-free filtrates were placed on a gyroshaker at 180 rpm in continuous light at 30°C as described.

Similarly, cotton callus cultures containing approximately 8 to 9 g of tissue were inoculated with 100 µl of the prepared inoculum containing 5 X 10^8 cfu ml^{-1} in culture. The final inoculum density was 1 X 10^7 cfu ml^{-1} just as for the cotton suspension cultures. Callus cultures were inoculated immediately after subculturing or at a subsequent time following transfer to fresh SH medium.

Viability and Growth Measurements

At appropriate times, usually during the harvest of cotton suspension cells for cell wall preparation, a 2 to 3 ml aliquot was removed from the culture for viability assessment. Average suspension cell viability was determined by staining with 1% (w/v) Evans blue dye, an exclusionary dye. The number of living and dead cells was determined microscopically using a haemocytometer. The percentage of living cells was determined in triplicate or quadruplicate and then averaged.

At appropriate times, the suspension culture cells were collected by filtration in a glass scintered funnel. The fresh weight of the collected cells was obtained before washing them with 100 mM potassium phosphate buffer (pH 7). Alternatively, the suspension cultures were filtered through Miracloth (Calbiochem-Behring Corp., La Jolla, CA) and the cell pad was weighed to determine the fresh weight.

Protoplast Isolation

Protoplasts were prepared from suspension-cultured Ac 44, Im 216, OK 1.2, and AcB_N cotton cells by using the method described in Hussain *et al.* [120]. All procedures were performed under sterile conditions. Callus cells were asceptically transferred to SH medium containing no agar, 8 to 10 d prior to protoplast preparation. Cells were collected by filtration through 11.8/cm mesh filters. An aliquot of the filtered cells was placed in 5 ml 5% (w/v) chromate and shaken at 180 rpm for 24 h and examined microscopically using a haemocytometer to determine the number of cells present. Filtered cells (5.5 to 7.5 g) were transferred to 70 ml of 10 mM MES buffer (pH 5.8) containing 0.6 M sorbitol, 50 units ml^{-1} Cellulysin and 50 units ml^{-1} Macerase (Calbiochem-Behring, San Diego, CA). The flasks were shaken for 3 h at 180 rpm 30°C. The solution was then centrifuged for 5 min at 300*g* and the supernatant removed using suction. The pellet was resuspended in 20 ml 10 mM MES and diluted to 30 ml for counting protoplasts. Yield was calculated as [No. of protoplasts/ mg cells] / [No. of cells/ mg cells].

Preparation of Cell Walls

Source of Cell Walls

Cell walls were obtained from suspension-cultured plant cells and from whole plant tissues. Suspension-cultured plant cells from several cell types of cotton (*Gossypium hirsutum* L.) Ac 44, Acb7, AcB_N, Im 216, and AcIm ranging from fully susceptible- to fully resistant- to the bacterial pathogen, *X. c. malvacearum*, the causal agent of bacterial blight of cotton, were used for cell wall preparation. Cell walls from tomato suspension cultures (*Lycopersicon esculentum* L.), Ac 44 cotton leaves, tobacco (*Nicotiana tabcum* L., cv. Burley White) leaves, and carrot (*Daucus carota* L.) root were obtained from P. Komalavilas, Dept. of Biochemistry, Oklahoma St. University, Stillwater, OK, who had prepared them by the same method employed by this author and described below.

Preparation of Cell Walls from Suspension-Cultured Cells

Suspension cultures were established as described earlier in the Materials and Methods. Cell walls were prepared using a modified procedure described by Komalavilas

and Mort [142]. Cultured cells were collected on a coarse scintered glass funnel during the late log phase of growth, 15-20 days after transfer of callus to suspension media and were washed once with 100 mM potassium phosphate (pH 7) buffer and stored in the freezer at -20°C. The culture fluid may be collected for preparing extracellular polysaccharides (EPS). When enough cells were obtained, the cells were thawed and washed with ten volumes of 100 mM potassium phosphate (pH 7) five times, and with 500 mM potassium phosphate buffer (pH 7) four times to remove extracellular debris. Cells were collected after each wash using centrifugation at 10,000*g* or alternatively, using Nitex-Nylon Mesh HL 3-15 (Tetko, Inc., Precision Woven Screening Media). After initial washing, the cells were resuspended in one volume of 500 mM phosphate buffer (pH 7) and broken using the polytron (Brinkmann Instruments, Inc., Wesbury, NY) at high speed in 4 bursts of 3 min each for a total of 12 min. The polytron was allowed to cool for 2 min between bursts. The sample was kept in ice at 0°C during the entire procedure. Preparations were microscopically examined to indicate that cell rupture was complete. The suspension of broken cells was then centrifuged immediately at 2000*g* for 10 min. The supernatant was decanted and the pellet was washed once, by suspending it in five volumes of the 500 mM phosphate buffer (pH 7), and then centrifuged at 2000*g* for 10 min. The washing was then repeated three times with distilled water. The washed cell walls were resuspended by vigorous stirring in 5 volumes of 1:1 chloroform: methanol (v/v) and placed in a coarse scintered glass funnel. The organic solvent was removed by applying gentle suction to the funnel. This procedure was repeated twice. The cell walls were resuspended in five volumes of acetone and were repeatedly washed with acetone to remove water. The cell walls were then air dried in the exhaust hood for 24 to 28 h and stored in a desiccator.

After air drying, the cell walls used for hydrogen fluoride (HF) solvolysis at 0°C were tested for the presence of starch using iodine. Residual starch was removed by treating the walls with alpha-amylase (Bacillus type II-A, from Sigma Chemical Co.) as described by York *et al.* [262]. The cell walls (10 mg ml^{-1}) were suspended in 100 mM potassium phosphate buffer (pH 7) containing alpha-amylase (50 units ml^{-1}). The suspension was stirred for 48 h at 25°C. The enzyme treated cell walls were centrifuged at 10,000*g* for 10 min. The cell walls were then washed extensively, approximately seven times with distilled water, followed by acetone, air dried, and finally stored in a desiccator. The cell walls prepared from the suspension cells used in the time courses did not have the starch removed.

Cell Wall Composition Analysis

Determination of Sugar Composition

Between 50 and 100 µg of dry whole cell walls or other samples were placed in Teflon-lined, screw-cap glass vials containing 100 nmoles of *myo*-inositol as an internal standard. Methanolysis and derivatization were performed by a modification of the method of Chaplin [36]. One hundred µl of 1.5 M methanolic HCl and 25 µl of methyl acetate were added to the samples and the sealed vials were placed in a heating block at 80°C for 16 h. Caps were tightened after 10 min to prevent leakage. After heating, the vials were removed and cooled to room temperature. Six drops of *t*-butanol were added to each vial and the samples were evaporated under a stream of nitrogen. Samples may be stored at this point prior to trimethylsilylation in a desiccator. Trimethylsilylating reagent was prepared fresh in the exhaust hood by mixing 1 part of Tri-Sil Concentrate (Pierce Chemical Co., Rockford, IL) and 3 parts of dry pyridine. Reagents were mixed using a Teflon-coated 100 µl syringe to keep the Tri-Sil Concentrate and pyridine away from water since they are both hygroscopic. Fifty µl of the reagent was then added to the dried samples, which were mixed and left for 15 min at room temperature to derivatize. The derivatized samples were then evaporated just to dryness under a stream of argon, and redissolved in 100 µl of iso-octane. A 1 µl aliquot was injected into a fused silica capillary column (30 m X 0.25 mm i.d., Durabond-1 liquid phase, J & W Scientific, Inc., Rancho Cordova, CA) installed in a Varian 3300 gas-liquid chromatograph equipped with an on-column injector and helium carrier gas, flow rate = 32.8 cm sec^{-1}. The sample was injected slowly at 105°C, and the temperature was immediately raised to 160°C and held for 4 min before being raised 2°C min^{-1} to 200°C. Peak integration was performed using a Varian 4290 Integrator.

Sugar composition was also determined for whole cell walls treated with hydrogen fluoride (HF) at 0°C for 15 min before methanolysis and derivatization to determine cellulosic glucose.

Determination of Protein Composition and Amino Acid Analysis

Approximately 1 mg dry cell wall samples and other samples were hydrolyzed in 400-600µl of 6 N HCl 24 h *in vacuo* at 110°C in sealed borosilicate tubes. The resulting amino acids were evaporated to dryness and resuspended in 200 µl 0.01 N HCl. The

hydrolyzed samples were then analyzed commercially by a Dionex D-500 amino acid analyzer with a ninhydrin detector [234] by Dr. Ken Jackson, the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, Oklahoma City, OK. Two separate amino acid analyses were performed by this laboratory. One amino acid analysis was used to quantitate all amino acids except hydroxyproline. The second amino acid analysis was used to quantitate hydroxyproline. The two analyses were correlated by equating the glycine from each analysis. The second analysis was needed to separate hydroxyproline and aspartic acid by dropping the analysis temperature from 50°C to 30°C for separation. In all analyses proline, hydroxyproline, and glutamic acid were quantitated by absorbance at 440 nm, while all other amino acids were detected at 570 nm. Data analysis and peak integration were performed using the Dionex D-500 data system and later the Beckman System Gold Chromatography software. Additional computations in this dissertation were performed using the software program Microsoft Excel Version 1.5 [169] and a Macintosh microcomputer.

The percentage of protein in the cell wall samples or other samples was calculated using the following: [Total ng of amino acids determined/ Total ng of sample analyzed] X 100.

Determination of Lignin

Lignin in the cell walls was determined by a modification of the method of Johnson *et al.* [124]. Approximately 1 mg dry cell wall samples were weighed and placed in Teflon-lined screw-cap glass vials. Two hundred µl of 25% (v/v) of fresh acetyl bromide in acetic acid was added to each vial and mixed with the sample. The vials were placed in a 70°C heating block inside the exhaust hood for 30 min. The vials were shaken gently every 10 min to mix the reactants and promote dissolution. After 30 min, the vials were cooled to 15°C in cool water. After cooling, 9 ml of 2 M sodium hydroxide and 50 ml of acetic acid were mixed and 1180 µl of this mixture was added to each sample. The samples were cooled again to 15°C and 20 µl of 7.5 M hydroxylamine hydrochloride was added, mixed well, and the absorbance at 280 nm was read. Ferulic acid was used as a standard and the amount of lignin present was calculated. First, the absorptivity of the Ferulic acid (lignin standard) at this wavelenth was calculated as follows from the amount added: Absorptivity (Ferulic Acid as Lignin Standard) = (Absorbance Ferulic Acid Standard - Absorbance blank) / Concentration of Ferulic Acid as Lignin Standard ($g\ l^{-1}$). The lignin content of an unknown cell wall sample was computed as follows: Lignin % =

[(Absorbance of sample - Absorbance of blank) liters X 100] / [Absorptivity of lignin standard (ferulic acid) X sample weight (g)].

Chemical Analyses

Hydroxyproline Determination

The hydroxyproline content was determined colorimetrically at 560 nm by the method of Drozdz *et al.* [67]. Approximately 1 mg dry cell wall samples or other samples were hydrolyzed prior to hydroxyproline analysis in 6 N HCl at 110°C for 24 h in Teflon-sealed screw-cap glass vials. This simple colorimetric micromethod is based on the oxidation of hydroxyproline with Chloramine T first proposed by Stegemann [239].

Protein Determination

The amount of protein was determined according to the Lowry method (Lowry *et al.*, 1951) or by the Bicinchoninic Acid (BCA) Assay (Pierce Chemical Co., Rockford, IL).

Carbohydrate Determination

The carbohydrate content was determined by the phenol-sulfuric acid method [5]. Portions (usually 50 µl) of the fractions were mixed with 450 µl of water and 12.5 µl of phenol reagent (80% w/v in distilled water) followed by 1.25 ml of concentrated sulfuric acid. Samples were vortex-mixed and the absorption measured at 485 nm after 30 min.

HF Solvolysis at 0°C - Insoluble Cell Wall Protein Analysis

Background Regarding HF Solvolysis and Apparatus

Hydrogen fluoride (HF) is a fuming, volatile liquid at room temperature. The fumes are extremely reactive and are toxic to living entities, corrosive to glass, and many metals. A special apparatus that is closed to the atmosphere and constructed of Teflon with which the HF does not react has been designed [174] to safely handle this material.

Details of the stepwise operation of the apparatus are described by Mort *et al.* [176]. The basic components of the apparatus are shown in Figure 2.

HF Solvolysis of Cotton Cell Walls at 0°C

In a typical experiment (see [176]), 500 mg of dry cell walls were placed in a Teflon reaction vessel along with a 2.5 cm magnetic stirring bar. The whole apparatus was evacuated, and leaks in any part of the system were checked and corrected. HF was transferred from the reservoir to the HF holding vessel. This was done by cooling the reaction vessel with dry ice and acetone and allowing the HF to distill from the HF reservoir. For 500 mg of cell walls, 20 ml of HF was transferred for the solvolysis. Once the HF was transferred to the holding vessel, it was allowed to reach temperature equilibrium (0°C). The reaction vessel containing the cell walls was cooled to 0°C and maintained at 0°C during solvolysis through the use of an ice bath. Once the temperature of HF reached 0°C, it was transferred from the holding vessel to the reaction vessel by using slight nitrogen pressure. The cell walls in the reaction vessel were stirred and the reaction was allowed to continue for 30 min. After 30 min, the reaction was stopped by adding cold (cooled by adding dry ice) ether (300 ml) from the adjacent ether holding vessel. The cooling bath was removed and the quenched mixture was allowed to stir for 30 min and warm to room temperature. After 30 min, the quenched reaction mixture (i.e. HF/ether soution) was filtered using a Teflon filter (50 mm diameter, fine grade, Savillex Corporation).

Filtration of the Quenched Reaction Mixture and Water Extraction

To filter the quenched reaction mixture (HF/ether solution), the reaction vessel was removed and an in-line Teflon filter unit (Savillex Corporation) was attached to it, separating the ether insoluble residue from the HF/ether soluble extract. Nitrogen was used to push the HF/ether soluble portion through the Teflon filter (50 mm diameter, fine grade, Savillex Corporation). The HF/ether souble portion (filtrate) was collected in a Teflon container. The Teflon filter unit was sealed using a Teflon sealing ferrule at the outlet and the whole apparatus was evacuated for 30 to 45 min to dry the remaining ether insoluble material and to remove the ether left behind in the whole HF line. The vacuum was released and the ether insouble residue left on the filter unit was extracted with water three times, resulting in a water soluble extract and a water insoluble residue. The water

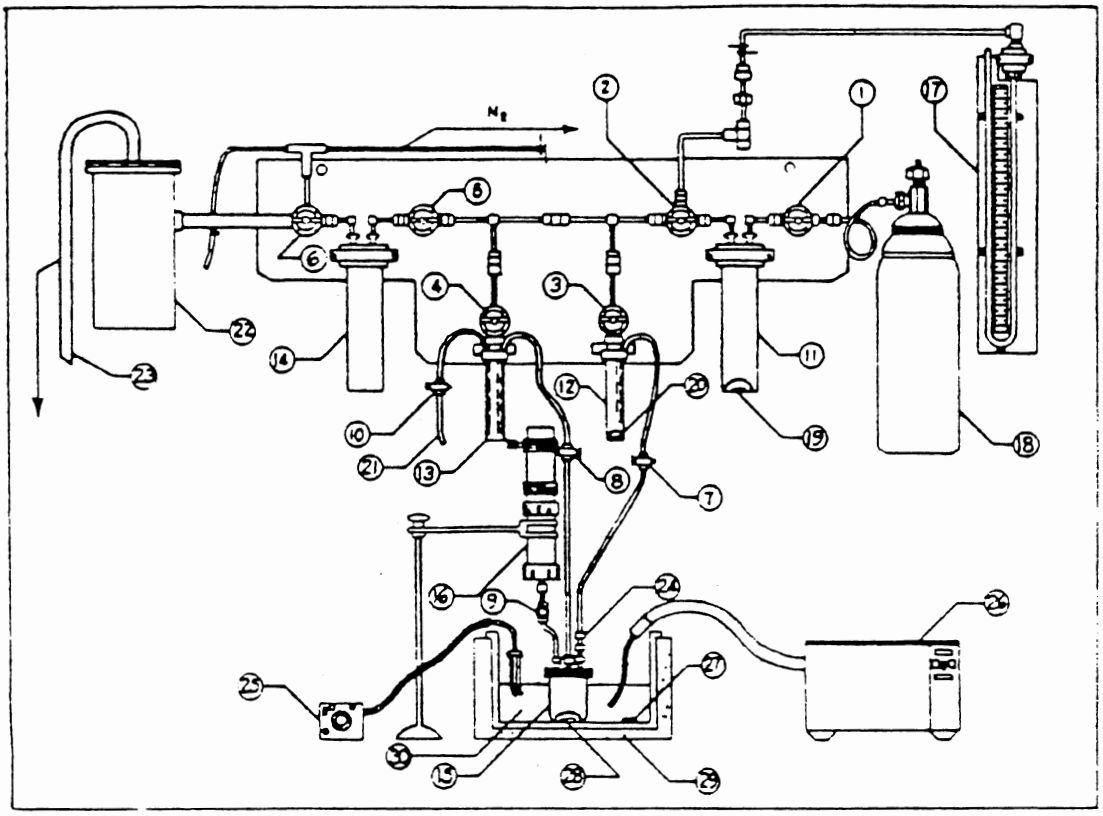


Figure 2. Schematic Representation of the Hydrogen Fluoride Solvolysis Apparatus. 1-8, 10, stopcocks; 9, teflon needle valve; 11-16, Teflon and Kel-F vessel; 17, manometer; 18, hydrogen fluoride tank; 19,20, stirrer bars; 21, exit to the sink for pressure release, if necessary; 22, calcium oxide trap; 23, connection to the vacuum pump; 24, 3 mm to 6 mm adaptor; 25, heater/regulator; 26, immersion cooler; 27, 28, stirrer bars; 29, insulated container; 30, 95% ethanol. (From P. Komalavilas. 1988. Partial Structural Characterization of Pectin from Cotton Cell Walls Using Selective Hydrogen Fluoride Solvolysis. Ph.D. Thesis. Oklahoma State University. p. 26)

soluble extract was dialyzed (3500 MW cutoff) and then lyophilized to obtain the water-soluble oligomers. The water-insoluble residue was lyophilized.

HF/Ether Fraction - Recovery of Sugars

The sugars, mainly monosaccharides and some disaccharides present in the HF/ether soluble fraction (filtrate), were recovered by evaporating the mixture. The filtrate was put in the reaction vessel, but never to more than half of its capacity. The HF/ether complex was evaporated from the reaction vessel and condensed in a cooled collection vessel. The collection vessel was immersed in liquid nitrogen and cooled for 15 min before the vacuum was turned on. The reaction vessel containing the filtrate was immersed in a warm water bath to enhance the removal of the HF/ether complex and stirred on the magnetic stirrer/heater plate. The sugars were retained in the original reaction vessel. When the reaction vessel was dry, the vacuum was released, and the sugars were dissolved in water and lyophilized. The remaining water insoluble material (oil) retained in the reaction vessel was dissolved in ether for N.M.R. Spectroscopy and Mass Spectroscopy. The HF/ether complex in the collection vessel was allowed to come to room temperature and then neutralized by pouring it into excess (~60 g) solid calcium carbonate.

Sodium Chlorite Oxidation of the Water Insoluble Residue

15 or 30 mg samples of Ac 44 HF 0°C water insoluble residue were weighed and placed in 15 ml Corex tubes for sodium chlorite oxidation using a modification of the method presented by Lamport and Epstein [156]. The water insoluble residue was allowed to hydrate in 2.5 ml of fresh 2% (v/v) acetic acid for 15 min in the vacuum oven with no heating. After 15 min, the vacuum was released slowly and the water insoluble residue was microscopically examined to check hydration. After hydration, 2.5 ml of fresh 2% (w/v) sodium chlorite was added to each tube. The resulting 1% (v/v) acetic acid: 1% (w/v) sodium chlorite mixture was placed in a 75°C heating block in the exhaust hood for 30 min. The reaction was stopped by adding excess ascorbic acid. The reaction mixture was filtered using a Teflon filter (50 mm diameter, fine grade, Savillex Corporation) in an inline Teflon filter unit (Savillex Corporation). Nitrogen was used to push the sodium chlorite oxidation soluble extract (filtrate) through the Teflon filter. The remaining sodium chlorite oxdidation residue was washed repeatedly with distilled water and then lyophillized. The sodium chlorite oxidation filtrate was dialyzed (3500 MW

cutoff) for 48 h and then concentrated using a Amicon 10 Concentrator (1000 MW cutoff filter). A portion of the sodium chlorite oxidation filtrate was lyophilized for gel electrophoresis and ELISA.

A time study of sodium chlorite oxidation of 1.00 mg Ac 44 suspension cell walls and 100 µg of Ac 44 HF 0°C water insoluble residue was performed as described above except that the reaction was stopped at 0, 10, 30, and 60 min. Hydroxyproline content for the sodium chlorite extract and the sodium chlorite insoluble residue after hydrolysis were determined using the method of Drozdz *et. al.* [67].

HF Solvolysis at 0°C of Ac 44 and Im 216 Whole Cell Walls for Total Sugar Analysis using Gas-Liquid Chromatography

Duplicate 2 mg samples of Ac 44 and Im 216 cotton suspension cell walls were weighed and placed in small (5 ml) Teflon containers prepared for HF solvolysis. The procedure for HF solvolysis at 0°C was the same as previously described in the Materials and Methods except that approximately 3 ml of HF was transferred to the Teflon sample container and the time of solvolysis was changed to 15 min instead of 30 min. Additionally, the HF was removed by a stream of nitrogen to stop the reaction. The HF-treated whole cell walls were removed and resuspended in 500 µl of *myo*-inositol, the internal standard used for gas chromatographic analysis. One hundred µl of the HF-treated sample containing 100 nmoles of *myo*-inositol was removed and placed in a Teflon-lined, screw-cap glass vial and dried using a stream of nitrogen. Methanolysis and derivatization were performed as previously described in the Materials and Methods. Six hundred µl iso-octane was added to each sample vials after methanolysis and derivatization and a 1 µl aliquot was injected for sugar analysis. The gas-liquid chromatograph column, program, and analysis were identical to that described earlier in the Materials and Methods.

Analytical Methods

Gas-Liquid Chromatography

The trimethylsilyl derivatives of cell wall sugars were separated on a fused silica capillary column (30 m X 0.25 mm i.d., Durabond-1 liquid phase, J & W Scientific, Inc.,

Rancho Cordova, CA) installed in a Varian 3300 gas-liquid chromatograph equipped with an on column injector and helium carrier gas, flow rate 32.8 cm sec^{-1}. One µl aliquots of the samples were injected at 105°C and the temperature was immediately raised to 160°C and held for 4 min, then raised 2°C min^{-1} to 200°C. Peak integration was performed by a Varian 4290 integrator and data analysis by a Macintosh microcomputer using the software Microsoft Excel Version 1.5 [169] .

Gel Filtration Liquid Chromatography

Following HF solvolysis at 0°C, the Im 216 cell wall water-soluble extract was dialyzed (3500 MW cutoff). After dialysis, the Im 216 water-soluble extract was concentrated approximately 10-fold using an Amicon 10 Concentrator (1000 MW cutoff filter). Two ml of the concentrated Im 216 water-soluble extract (total was 6.6 ml) was applied to a Bio-Gel P-2 column (62 X 2.2 cm, Bio-Rad Laboratories, Richmond, CA) and fractions were eluted with 50 mM sodium acetate buffer (pH 5.2). Fractions of 2.5 ml were collected. Sugars in the fractions were detected by the phenol-sulfuric acid test [5]. Protein in the fractions was determined by the Bicinchoninic Acid (BCA) Protein Assay (Pierce Chemical Co., Rockford, IL).

Ion-Exchange Liquid Chromatography

Protein fractions from the Bio-Gel P-2 column were further separated on a column (30 cm X 1 cm) of DEAE-Sephadex anion exchanger (A-25-120 m, Sigma Chemical Co., St. Louis, MO). Samples were applied in 50 mM sodium acetate (pH 5.2) and eluted by a gradient of 50 mM to 1 M sodium acetate buffer (pH 5.2) to remove the negatively-charged sugars. Fractions of 2.5 ml were collected. Sugars in the fractions were determined by the phenol-sulfuric acid method [5]. Protein in the fractions was determined by the BCA Protein Assay (Pierce Chemical Co., Rockford, IL). The protein containing fractions were combined into three fractions, dialyzed (3500 MW cutoff) and lyophillized. Hydroxyproline content was determined after hydrolysis by the method of Drozdz *et. al.* [67] for the combined lyophillized fractions.

High Performance Liquid Chromatography (HPLC): Gel Filtration

The Ac 44 water soluble extract and the sodium chlorite oxidation filtrate were fractionated using a Beckman TSK 2000 SW column (7.5 mm X 30 cm) on a Waters Associates pump system. The elution buffer was 0.3 M sodium acetate buffer (pH 5.2), the flow rate was 0.5 ml min^{-1}, and the chart speed was 0.5 ml min^{-1}. The protein was detected at 280 nm by a Waters Model 440 UV Detector. The molecular weight standards used were salmon testes DNA (Worthington Biochemical Corp.), thyroglobulin (Sigma), bovine serum albumin (Sigma), ovalbumin (Sigma), myoglobin (Sigma), and glycyl-tyrosine (Sigma).

N.M.R.Spectroscopy

Proton (^1H) n.m.r. spectra were recorded with a Varian (Palo Alto, CA) XL-300 N.M.R. spectrometer (300 MHz). ^1H spectra were recorded at 25°C. Approximately 5 mg samples of the Ac 44 and Im 216 ether soluble ether fractions collected after HF solvolysis at 0°C were dried with argon and resuspended in 700 µl of 100% deuterated-chloroform (Aldrich Chemical Co.)

Mass Spectroscopy

Mass spectra of the ether soluble ether fractions isolated from Ac 44 and Im 216 cotton suspension cell walls were recorded using a VG TS-250 mass spectrometer in combination with a Hewlett Packard gas chromatograph by electron-ionization (70 eV).

Scanning Electron Microscopy

Cotton cell walls were dehydrated in two changes of absolute ethanol. The dehydrated walls were dried using a SAMDRI PVT 3 critical point dryer (Tousimis Corp.), and attached to specimen studs, and coated with gold-palladium similar to method described by [32]. The prepared cell walls were viewed using a JOEL JSM 35 scanning electron microscope.

Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described by Laemmli [147]. An acrylamide to bis-acrylamide ratio of 29.2/0.8 was used and the acrylamide concentration in the running and the stacking gel were 12 and 4% respectively. The protein samples were run at 200 volts for 30 min using a Mini-Protean II Dual Slab Cell system (Bio-Rad Laboratories, Richmond, CA). The gels (7.3 X 10.2 cm) were stained using the Bio-Rad silver staining method (Bio-Rad Laboratories, Richmond, CA). The following low molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were run: Phosphorylase B, 97,400 MW, Bovine serum albumin, 66,200 MW, Ovalbumin, 42,669 MW, Carbonic anhydrase, 31,000 MW, Soybean trypsin inhibitor, 21,500 MW, Lysozyme, 14,400 MW.

Immunoblot Analysis

Proteins from the Im 216 and Ac 44 HF 0°C water soluble extracts were electrophoretically transferred at 14 V for 1 h in electroblotting buffer (20 mM Tris and 150 mM glycine solution, pH 8) from 12% SDS-polyacrylamide gels to a nitrocellulose filter using an electroblotting apparatus by the method of Towbin *et al.* [248]. The nitrocellulose filter is placed in Ponceau S solution (0.5% Ponceau S in 1% acetic acid) for 5 min to stain the proteins. After destaining in water for 2 min, the nitrocellulose filter is placed in blocking buffer (5 ml of 0.05% Tween-20) for 1 h to block nonspecific antibody sites on the nitrocellulose. The blots were then incubated with antibodies to deglycosylated tomato extensin precursors dP1 and dP2 for 1 h (description in Kieliszewski and Lamport, [130]) (diluted 1:500) obtained from M. Kieliszewski, Department of Energy Plant Research Laboratory, and Department of Biochemistry, Michigan State University, East Lansing, MI. The nitrocellulose filter is washed with 200 ml of phosphate-buffered saline (PBS) four times to remove nonspecifically bound primary antibody. The nitrocellulose filter is then placed in a diluted solution for 1 h of horseradish peroxidase (HRPO)-anti-immunoglobulin (Ig) conjugate (Cappel) to detect the antibodies. Bound antibodies are then visualized by immunoprecipitation using diaminobenzidine (DAB). The DAB substrate solution (100 ml) consists of 50 mg 3,3'-diaminobenzidine, 2 ml 1% $CoCl_2$ in H_2O, 98 ml PBS, and 0.1 ml 30% H_2O_2, added immediately prior to use. DAB should be handled with care since it is a carcinogen. Western blots were photographed to make a permanent record.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cross-reactivities of antibodies raised against deglycosylated and glycosylated tomato extensin precursors obtained from M. Kieliszewski [130] with the sodium chlorite oxidation filtrate and water-soluble extract of Ac 44 suspension cell walls treated with HF at 0°C and the water-soluble extract of Im 216 suspension cell walls treated with HF at 0°C were determined by indirect ELISA [254]. Each test well of the 96 well Microtest III Tissue Culture Plate (Becton Dickinson & Co., Lincoln Park, NJ) was coated with 2 µg antigen (sodium chlorite filtrate or water soluble extract) in 200 µl Tris buffer (pH 8.8) for 1 h at room temperature then 16 h at 4°C. The plate was washed three times with PBS/Tween (50 mM sodium potassium phosphate (pH 7.4), 150 mM sodium chloride, 0.02% (w/v) potassium chloride, 0.05% (v/v) Tween-20). Remaining protein binding sites were blocked by addition of 200 µl 1% (w/v) bovine serum albumin in PBS-Tween for 30 min at 37°C, followed by washing three times with PBs/Tween. Two hundred µl of the diluted antibody solutions (1:200 or 1:400) were added to each sample well and incubated 2 h at room temperature or alternatively, 1 h at 37 °C. After incubation with the antibody solution, the plate was washed three times with PBS/Tween. Two hundred µl of the conjugate, goat anti-rabbit IgG (Sigma) was added to each well and incubated 2 h at room temperature then the plate was washed three times with PBS/Tween. Two hundred µl of the substrate, *p*-nitrophenylphosphate (1 mg ml^{-1}) in 0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ (pH 10.4) was added to each well and incubated for 30 min at 37°C. The reaction was stopped by the addition of 50 µl of 3 N NaOH to each well, and then the absorbance was determined at 405 nm.

Statistical Analysis

Statistical analysis of the growth, viability, and protoplast data collected was analyzed by N-way analysis of variance (ANOVA) and Student's t-test using the software program Exstatix Version 1.0.1 [132] and the Macintosh microcomputer. ANOVA tests two or more groups to discover if the samples are drawn from the same population. ANOVA analyzes then variances of the groups through the use of the F-ratio. Statistical analysis of variance and least squares means analysis of the biochemical parameter data (amino acid, protein, sugar, and lignin) was done using SAS with the help of Dr. Robert McNew, Department of Statistics, Oklahoma State University. Experimental design was a split-plot, with cell lines as the main plot and presence of the pathogen as the sub-plot.

CHAPTER IV

RESULTS AND DISCUSSION

Growth, Viability, and Protoplast Isolation

Description of Cultures

Initiation of Acb7, AcB_N, AcIm, and "new" Ac 44 occurred easily on SH medium. Friable callus was obtained from all cell lines initiated during this study. Acb7, AcB_N (very dark), AcIm (very dark), and "new" Ac 44 (1985) calli are somewhat brown (the degree of browness varies within each cell line) in color. The visible observation for Ac 44 callus (1985) differs from that of Ruyack *et. al.* (1979) in that Ac 44 callus (1973) is light in color. Note: this line was used predominantly in this research not the 1985 Ac 44 callus. There is no obvious explanation for this difference except that the "new" Ac 44 callus has only been through slightly more than 20 transfers whereas "old" Ac 44 callus has been through hundreds of transfers. Perhaps the production of brown pigments has decreased over time in culture. Suspension cultures were also easily obtained upon transfer of callus from each cell lines studied to liquid SH medium.

Growth of Pathogen in the Presence of the Host

Growth of *X. c. malvacearum* in Ac 44 and Im 216 Suspension Cultures or Cell-Free Filtrates. In order to characterize the cotton: *X. c. malvacearum* host: pathogen interaction in suspension culture, one must first study the growth of each component with respect to the other. The first component of this host: pathogen interaction to be examined is the growth of the bacterial pathogen, *X. c. malvacearum*, stain 3631 in both Ac 44 (susceptible to the pathogen) and Im 216 (immune to the pathogen) suspension cultures. The growth of *X. c. malvacearum* in Ac 44 and Im 216 suspension culture was

determined as a function of its turbidity measured at O.D. 600 nm and is presented in Figures 3 and 4.

The bacteria are considered to be in the log phase of growth when the absorbance at 600 nm is between 0.10 and 0.30. Nutrient borth was used as a blank. Cotton cells in the log phase of growth (9 days after initiating the culture) were filtered and transferred to 10 mM MES and 3% sorbitol (pH 5.8) before inoculation with 1 X 10^7 cfu ml^{-1} (final inoculum density) (see Materials and Methods). Transfer of cotton cells to this media provides no nutritional support for either the bacterial or the cotton cells, but just osmoticum so the cells won't burst. Thus, any growth of the *X. c. malvacearum* in culture will be directly related to its ability to infect the cotton cells and either metabolize degraded cotton cell material to support its own metabolism or use the 10 mM MES and 3% sorbitol solution.

In a previous experiment, *X. c. malvacearum* grew only when host cells were present (data not shown). In this experiment, the cell-free SH filtrate was examined to confirm whether living host cells are necessary for pathogen growth or just some excreted compounds released by the host cells into the medium. That is, can the bacteria utilize compounds in the filtrate to support its own growth? From Figs. 3 and 4, the results do not indicate that the pathogen can grow in the filtrate, but do support the original conclusion that living host cells are necessary for pathogen growth and multiplication. That is, in the inoculated cell-free filtrates of both Ac 44 and Im 216, *X. c. malvacearum* grew poorly, never more than an O.D. 600 nm reading of 0.030. However, the difference in *X. c. malvacearum* growth in the presence of Ac 44 cells vs. Ac 44 cell-free filtrate was quite dramatic (Figure 3). In fact, the one-way ANOVA, O.D. 600 nm vs. host cells treated was found to be significant at the 99.5% level. This difference was not pronounced in *X. c. malvacearum* growth in the presence of Im 216 cells vs. Im 216 cell-free filtrate (Figure 5). The one-way ANOVA, O.D. 600 nm vs. host cells treated was found not to be significant even at the 70% level.

Maximal bacterial growth was observed to occur 6 days after inoculation of Ac 44 suspension cells, O.D. 600 nm = 0.112 (Figure 3). No increase in bacterial growth was seen after 6 days in inoculated Ac 44 suspension culture (Figure 3). Both controls, the uninoculated Ac 44 or Im 216 cell cultures and the *X. c. malvacearum* alone in 10 mM MES and 3% sorbitol did not indicate any growth of the pathogen, O.D. 600 nm <0.010 (see Figs. 3 and 4). However, the uninoculated Ac 44 suspension culture showed some

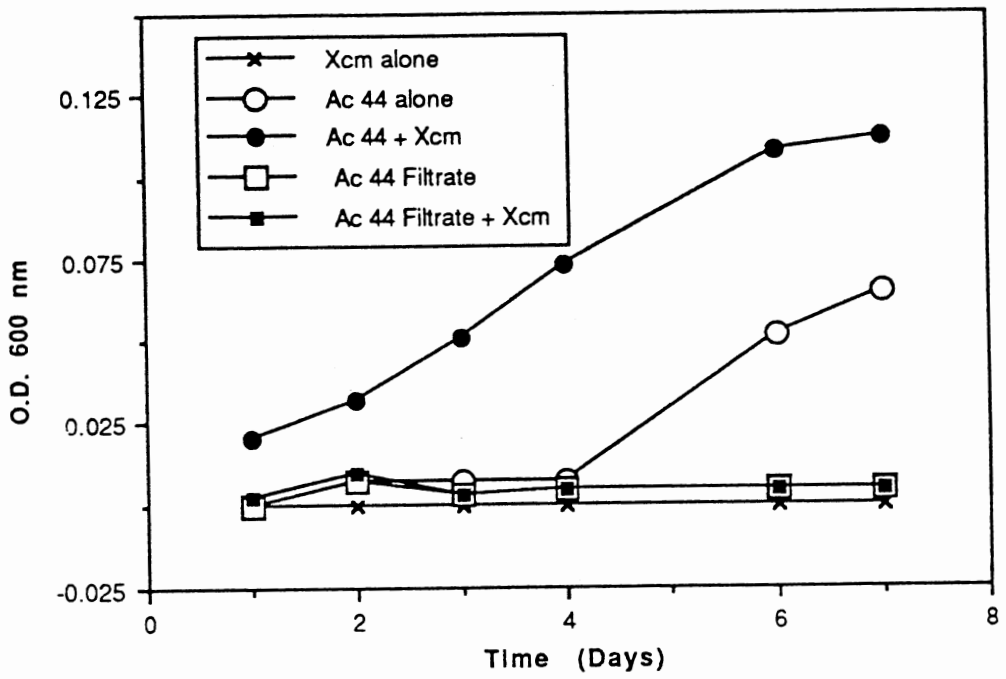


Figure 3. Growth of *X. c. malvacearum* in Ac 44 Suspension Cultures or Ac 44 Cell-Free Filtrates. Ac 44 suspension cultures containing 3 to 6 g of tissue in 50 ml of SH medium (pH 5.8) were filtered and transferred after 9 days to 10 mM MES and 3% sorbitol (pH 5.8). Cell-free SH filtrates and newly transferred suspension cultures were inoculated with *X. c. malvacearum* at a final inoculum density of 1×10^7 cfu ml^{-1}. The optical density at 600 nm was taken of the 4-ml filtered aliquots to determine the bacterial growth.

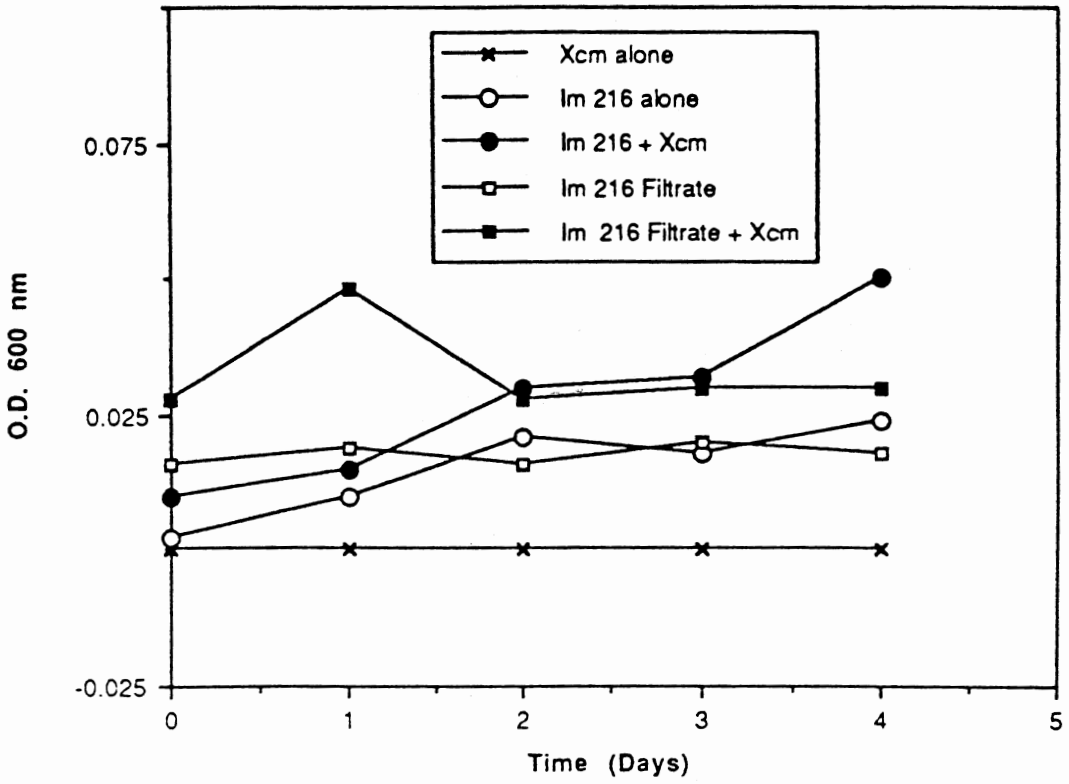


Figure 4. Growth of *X. c. malvacearum* in Im 216 Suspension Cultures or Im 216 Cell-Free Filtrates. Im 216 suspension cultures containing 3 to 6 g of tissue in 50 ml of SH medium (pH 5.8) were filtered and transferred after 9 days to 10 mM MES 3% sorbitol (pH 5.8). Cell-free SH filtrates and newly transferred suspension cultures were inoculated with *X. c. malvacearum* at a final inoculum density of 1 X 10^7 cfu ml^{-1}.The optical density at 600 nm was taken of the 4-ml filtered aliquots to determine the bacterial growth.

possible contamination, the optical density readings began increasing after Day 4. *X. c. malvacearum* was not recovered at the end of the experiment to determine whether or not bacterial growth was actually attributable to *X. c. malvacearum* and not some other bacteria. Yet, these readings were still less than half that of the inoculated Ac 44 culture.

Thus, the results seen in Figs. 3 and 4 necessitate the following observations. First, *X. c. malvacearum* grows best in the presence of live Ac 44 and Im 216 host cells. Second, cell-free filtrates do not support the growth of the pathogen, *X. c. malvacearum*. Third, *X. c. malvacearum* does not grow in 10 mM MES and 3% sorbitol, thus indicating that some nutrient needed to sustain bacterial growth is absent. Four, *X. c. malvacearum* has a different growth behavior in Ac 44 cell culture than in Im 216 cell culture in the presence of live host cells. From the above observations, the following conclusions can be made. First living Ac 44 or Im 216 host cells are required for *X. c. malvacearum* growth *in vitro*. Second, the incompatible interaction involving Im 216 is different from the compatible interaction involving Ac 44. The degree to which these interactions differ cannot be quantitated from this one experiment.

It is possible to suggest that *X. c. malvacearum* growth is inhibited in the presence of Im 216 living host cells to a degree not observed in the presence of Ac 44 living host cells. Specifically, *X. c. malvacearum* growth in the presence of Im 216 cells (Figure 4) is less than half that of similar growth observed in the presence of Ac 44 cells (Figure 3). These results possibly provide information regarding the host responses of Im 216 and Ac 44 suspension cultures to the bacterial pathogen, *X. c. malvacearum*. Although one cannot observe a characteristic hypersensitive response in suspension culture, denoted by rapid cell death of individual plant cells which come into contact with disease resistance of the whole plant to the pathogen [133], it is still possible to clearly note a characteristic "resistant" response *in vitro*, i.e. lack of bacterial growth and bacterial cell death.

From these results, the growth of *X. c. malvacearum* in the presence of cotton suspension cultures was measured using bacterial colony counting procedures and the Spiral Plater (Janet Rogers, Department of Biochemistry, Oklahoma State University, personal communication). Results indicated that there was no growth of *X. c. malvacearum* in the resistant (Im 216) cell-filtrate, that is, the bacterial population remained at the original inoculum density of 1×10^7 cfu ml^{-1} throughout the 15 day length of the experiment. But, growth occurred in the susceptible (Ac 44) cell-filtrate, after 9

days, the bacterial population increased 100-fold, 1 X 10^7 cfu ml^{-1} to 1 X 10^9 cfu ml^{-1} . However in the presence of filtered Ac 44 or Im 216 suspension cells, the bacteria quickly increased from 1 X 10^7 cfu ml^{-1} to 1 X 10^9 cfu ml^{-1} after 3 days and remained at this population density 15 days after inoculation. That, is there was no difference in *X. c. malvacearum* growth in either the susceptible, Ac 44, suspension cultures or the resistant, Im 216, suspension cultures. The maximum population density of *X. c. malvacearum* was 1 X 10^9 cfu ml^{-1} in the presence of live Ac 44 or Im 216 suspension cells. Cell-free filtrates of either Ac 44 or Im 216 stimulate (Ac 44) or maintain (Im 216) *X. c. malvacearum* growth, but do not support bacterial growth as well as the actual host cells in suspension culture. Even though quantitation of bacterial growth using O.D. 600 nm was not as accurate as using the aid of a Spiral Plater to count viable bacteria extracted from the suspension cultures, the simple observation that bacterial grew better in the presence of host cells than in cell-free filtrates (Figures 3 and 4) was comparable to that made later by Janet Rogers (Department of Biochemistry, Oklahoma State University, data unpublished). However, these later experiments performed by Janet Rogers indicated that there was no difference in the growth of *X. c. malvacearum* in the presence of either the susceptible, Ac 44, or the resistant, Im 216, suspension cells in culture.

The *in vitro* host-pathogen interactions can be compared with the *in vivo* host-pathogen interactions even though the pathogen movement between and among host cells is different, there is more uniform moisture, and the production of toxic phytoalexins is significantly lower [4] in suspension culture. The population trends for *X. c. malvacearum* following inoculation into cotyledons of selected cotton cell lines including Ac 44 and Im 216 have been documented [12]. More important, however, is finding that *X. c. malvacearum* growth was logarithmic in susceptible (Ac 44) and immune (Im 216) cotton leaves, but stopped at a lower population density in Im 216 suspension culture cells than in Ac 44 suspension cells. Additionally, in leaves of cotton lines containing resistance genes B_2, B_3, b_7 or B_N in an Ac 44 genetic background, incompatible strains of *X. c. malvacearum* was inhibited 6 to 8 days after inoculation, whereas compatible strains continued multiplying [222].

Growth of Host in Presence of Pathogen

Inoculation of Different Ages of Ac 44 and Im 216 Callus Tissue. Host, Ac 44 and Im 216 (leaf) callus growth was measured as changes in callus height as a function of

time in the presence of the pathogen, *X. c. malvacearum*. Callus cultures of each cell type were inoculated with *X. c. malvacearum*, race 3 as described in the Materials and Methods either immediately after subculturing 26 to 30 day old callus cultures or at subsequent times following transfer to fresh SH solid medium. Appropriate uninoculated control Ac 44 and Im 216 cultures were also monitored. The effects of *X. c. malvacearum* on the growth of different ages of Ac 44 and Im 216 are presented in Figures 5 and 7, respectively. The comparisons of Ac 44 or Im 216 callus growth with time of *X. c. malvacearum* inoculation are presented in Figures 6 and 8, respectively.

The growth curves for each callus cell type, blight-susceptible, Ac 44, and blight-immune, Im 216, are presented in the form of the uninoculated controls (see Figs. 5 and 7). Results indicate that Im 216 callus cells were similar to Ac 44 callus cells in their growth pattern. Ruyack and associates [208]have previously determined that the best environmental conditions for callus proliferation were under continuous light and 30°C. Changes in height of the callus tissue from Ac 44 (susceptible) and Im 216 (resistant) generally followed a sigmoidal curve except that the stationary phase of growth has not yet been fully reached for either cell type at Day 21 following initiation of the culture. Comparison of these data with changes in fresh weight of the Ac 44 and Im 216 callus tissue obtained by subculturing 26 to 30 day old callus cultures [208] showed nearly identical growth patterns. The height doubling time during the log phase was approximately 4 days for both Ac 44 and Im 216 callus. Furthermore, the log phase began at approximately Day 5 or 6 for both Ac 44 and Im 216 and had a duration of at least 16 days. According to Ruyack *et. al.* [208], the stationary phase begins at around 21 days after subculturing 26 to 30 day old Ac 44 and Im 216 callus. Therefore, it is reasonable to expect that since the growth patterns of changes in height and changes in fresh weight are identical, then the length of the log phase and beginning of the stationary phase would also be identical.

As Ruyack *et. al.* [208] noted previously, although the growth curves for the blight-susceptible line, Ac 44 and blight-immune line, Im 216 are similar, there were visible differences in the callus tissues. For example, the Ac 44 tissue was very friable and light, nearly white in color, whereas Im 216 callus was friable and dark, brown in color. These differences in appearance are probably due to phenolics, but no information is available to substantiate this claim at this time. However, as noted earlier, the "new" Ac 44 (1985) callus I initiated 3 years ago is still brown, not white. Perhaps after hundreds of transfers, the ability to produce pigments is altered. Changes in the growth medium

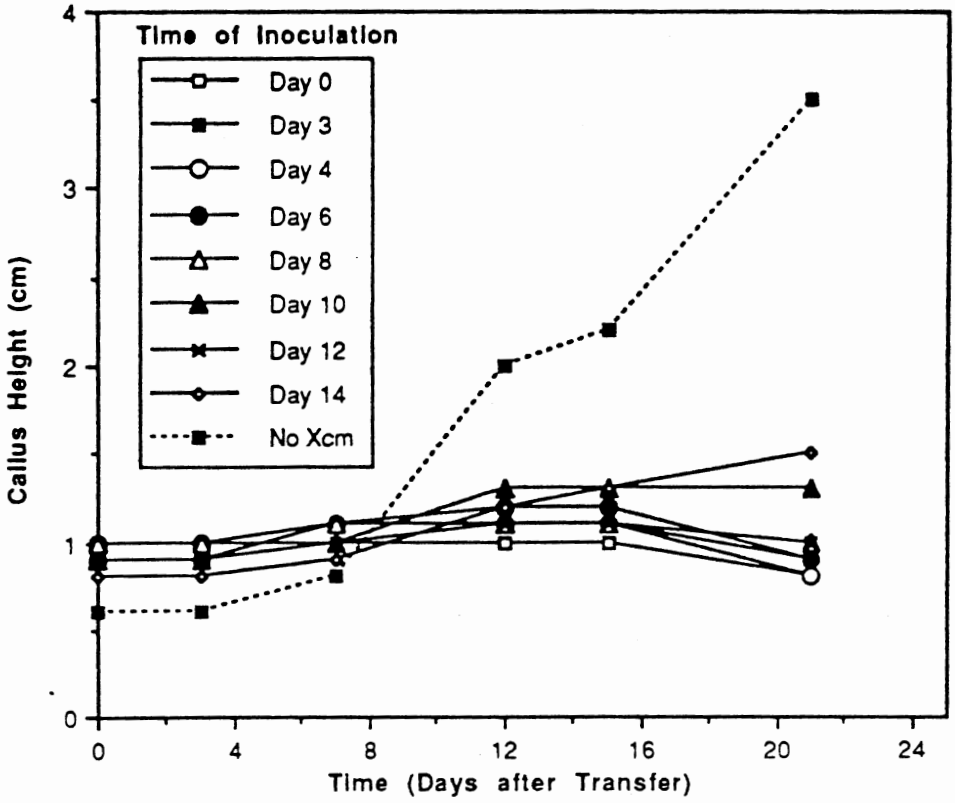


Figure 5. Effect of *X. c. malvacearum* on the growth ages of different of Ac 44 callus. Ac 44 callus cultures containing approximately 8 to 9 g of tissue were inoculated with *X. c. malvacearum* at a final inoculum density of 1 X 10^7 cful ml^{-1} in 50 ml of SH medium (pH 5.8) . Callus cultures were inoculated immediately after transfer (Day 0) to fresh SH or at subsequent times threreafter. The uninoculated Ac 44 callus culture contained no *X. c. malvacearum* . Growth (Height) was measured as function of time with each treatment representing a different date of inoculation.

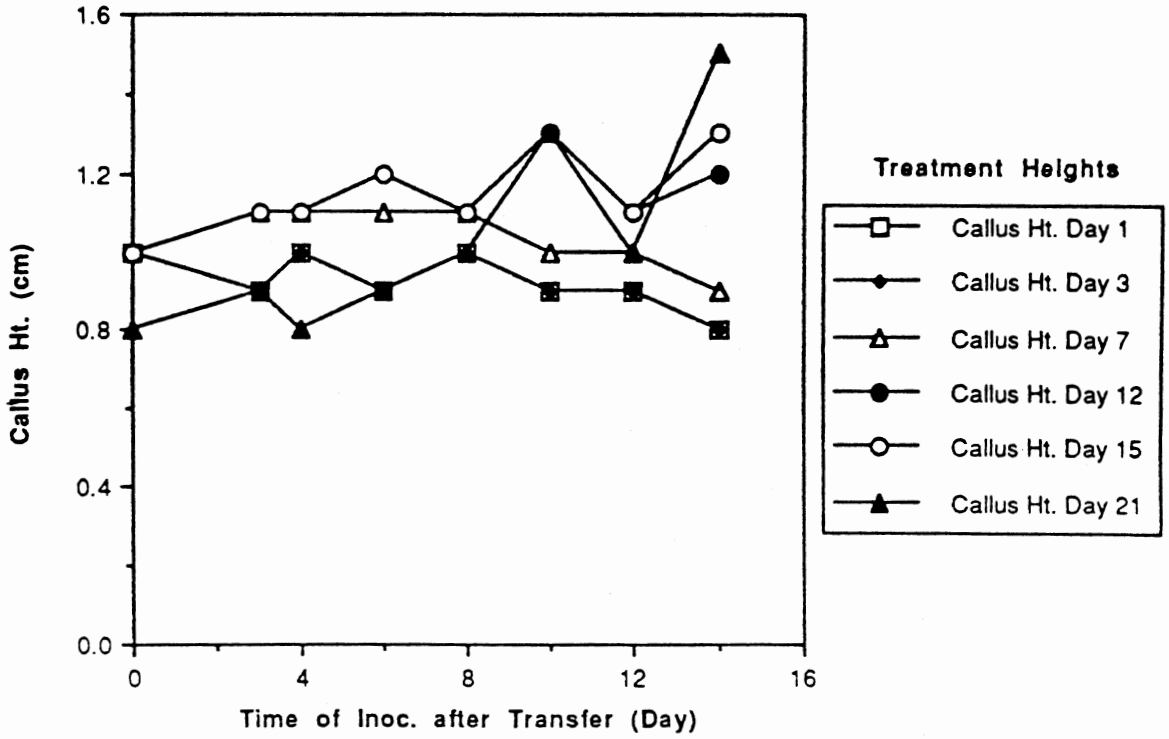


Figure 6. Comparison of Ac 44 Callus Growth with Date of *X. c. malvacearum* Inoculation. Conditions for Ac 44 callus cultures are the same as for Figure 5. Data is presented for only the inoculated Ac 44 callus cultures. Growth (Height) was measured as a function of the date of inoculation with each treatment representing heights at each sampling point.

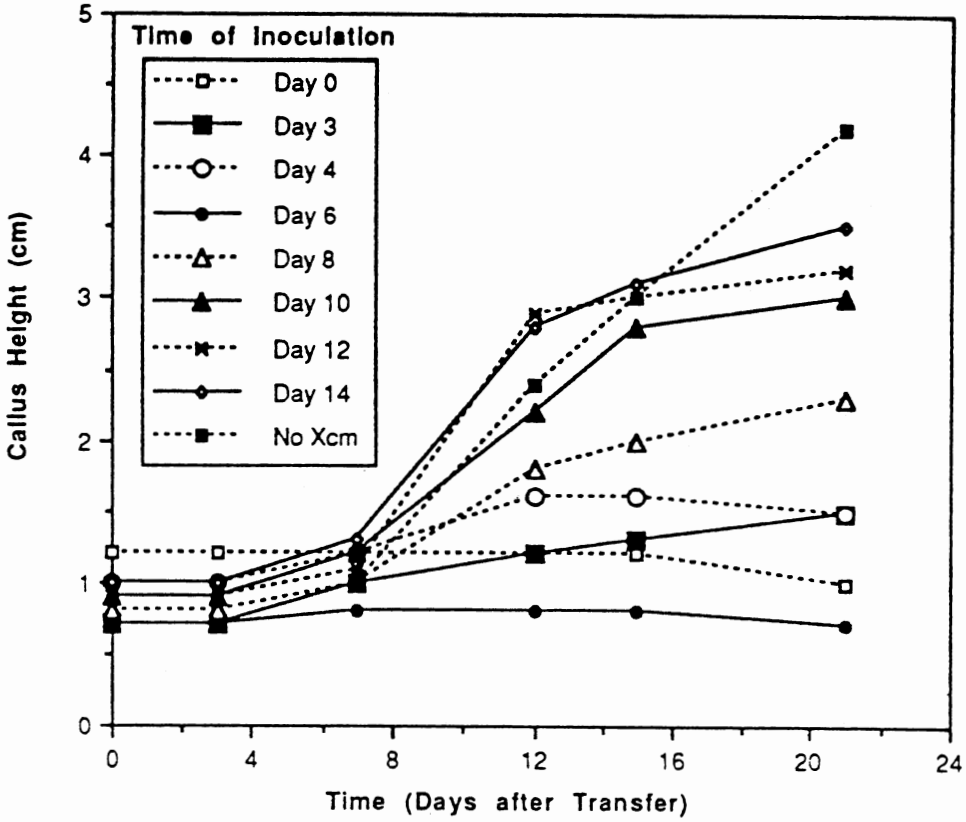


Figure 7. Effect of *X. c. malvacearum* on the growth of different of Im 216 callus. Im 216 callus cultures containing approximately 8 to 9 g of tissue were inoculated with *X. c. malvacearum* at a final inoculum density of 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Callus culture were inoculated immediately after transfer (Day 0) to fresh SH medium or at subsequent times threreafter. The uninoculated Im 216 callus culture contained no *X. c. malvacearum* . Growth (Height) was measured as a function of time with each treatment representing a different date of inoculation.

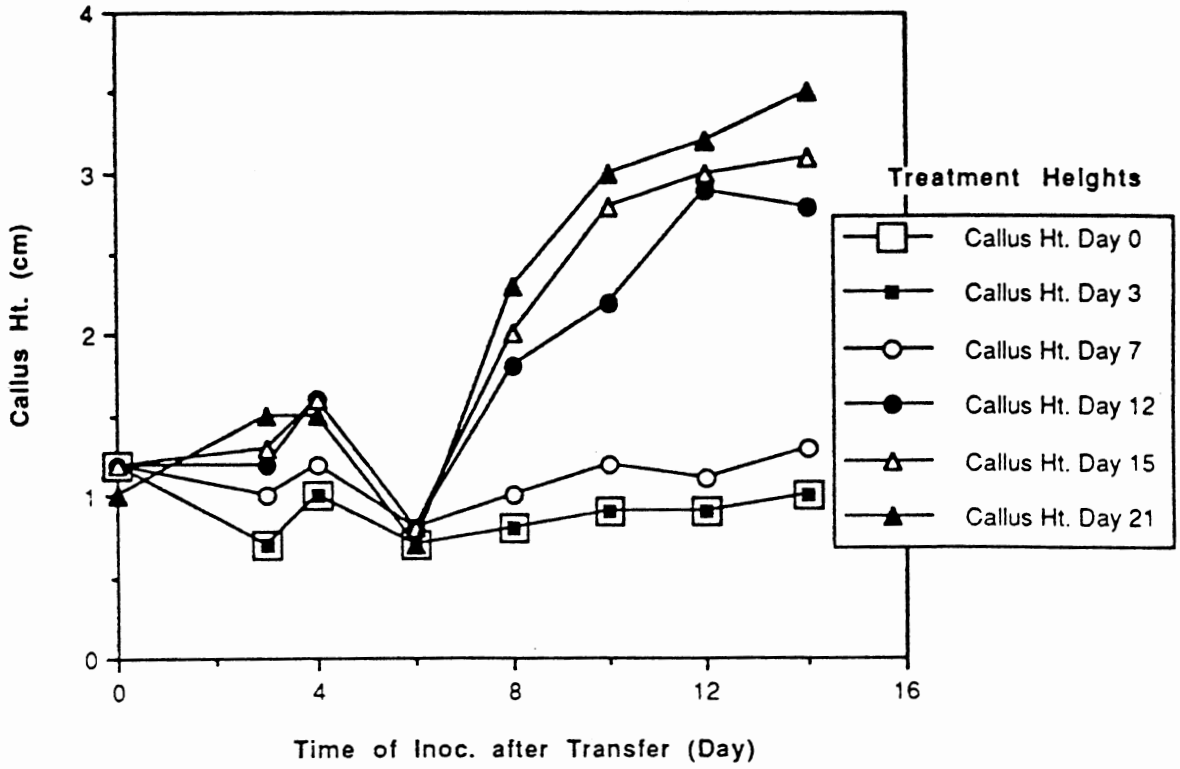


Figure 8. Comparison of Im 216 Callus Growth with Date of *X. c. malvacearum* Inoculation. Conditions for Im 216 callus cultures are the same as Figure 7. Data is presented for only the inoculated Im 216 callus cultures. Growth (Height) was measured as a function of the date of inoculation with each treatment representing heights at each sampling point.

triggered the production of chlorophyll by host tissue and it has been noted that light-examination of the callus tissue showed chloroplasts [208].

The most interesting question concerning callus or suspension cultures of Ac 44 and Im 216 is whether the traits of susceptibility and resistance are exhibited in cell culture. A number of host-pathogen systems have demonstrated that there is a correlation between the response to fungal and bacterial pathogens of intact plants and tissue cultures derived from tobacco [6, 29, 57, 61, 108, 109, 110, 111, 118, 163, 168], lucerne [160], soybean [80, 114] , alfalfa [170], bean [53], potato [122], and cotton [4, 208] plants. One of these studies demonstrated that genes for disease resistance in the intact plant were being expressed in tobacco tissue culture [110]. As in secondary metabolite production, the traits of resistance and susceptibility in tissue culture may depend on the stage of the culture, e.g. log or plateau phase.

Figures 5 and 6 show the growth of Ac 44 callus (changes in height) as a function of time (Figure 5) and time of inoculation (Figure 6). In the one-way ANOVA, the comparison height vs. date of inoculation was significant at the 78% level. In the one-way ANOVA, the comparison height vs. time was significant at the 89% level. In the two-way ANOVA, the comparison height vs. time and date of inoculation was significant at the 90% level with respect to time, but at only the 83% level with respect to date of inoculation.

This information indicates that there was a direct relationship between growth of Ac 44 callus and when the callus was inoculated. Figure 5 presents the changes in callus height over time with treatments being different dates of inoculation. If callus is inoculated immediately after transfer (Day 0), there is no growth as evidenced by no change or a drop in height over the course of 21 days. However, if Ac 44 callus is inoculated 3, 4, 6, 8, 10, 12, and 14 days after transfer, there is still little or no growth of the callus over the course of 21 days, although growth is enhanced when the inoculation time is delayed. Unlike the results in Figure 5, one expected no deviation from the uninoculated callus growth curve until after inoculation. If inoculation of Ac 44 callus occurs 14 days after transfer, there is a positive linear change in growth, but only a 0.60 cm total change in height, a 1.75 fold increase in height over the course of 21 days. The only real growth of callus occurs in the uninoculated control a 7-fold increase in height over the course of 21 days. Figure 6 shows that there is little or no growth in any of the Ac 44 treatments, no matter when the inoculation occurred. The slopes of all the

comparisons of Ac 44 callus growth with time of *X. c. malvacearum* inoculation (Figure 6) were calculated by linear regression to confirm that little or no real growth occurred. These slopes are as follows: Callus Ht. Day 1 = -0.010, Callus Ht. Day 3 = -0.010, Callus Ht. Day 7 = -0.009, Callus Ht. Day 12 = 0.012, Callus Ht. Day 15 = 0.016, Callus Ht. Day 21 = 0.043. Slopes for Days 1, 3, and 7 are negative, thus indicating greater cell death than real growth. Yet, it is not apparent whether the cells just disappear after death or the cell walls still remain. Slopes for Days 12, 15, and 21 are all positive, but very minimally, indicating that growth begins at Day 12 for inoculated cultures independent of when they were inoculated. It is evident, however, that the later the Ac 44 callus was inoculated, the better able the callus was to grow, even if this growth was minimal. Slopes for each day for all cultures showed a strong positive trend in growth. From the two-way ANOVA, the comparison height vs. time and date of inoculation, there is a significant difference at the 83% level for both factors. In other words, there is a positive growth trend observed over time and it is correlated with date of inoculation.

Figures 7 and 8 show the growth of Im 216 callus (changes in height) as a function of time (Figure 7) and time of inoculation (Figure 8). In the one-way ANOVA, the comparison height vs. date of inoculation is significant at the 93% level and the comparison height vs. time is significant at the 99.9% level. In the two-way ANOVA, the comparison height vs. time and date of inoculation is significant at the 99.9% level. These results show that the growth of Im 216 callus is intrinsically linked to both time and date of inoculation. That is, the ability for Im 216 callus growth is dependent on when the callus is inoculated just as was observed for Ac 44 callus growth. It is evident that the later the Im 216 callus is inoculated after transfer, the greater the callus growth. It is obvious that Im 216 callus inoculated >7 days after transfer have growth patterns that are sigmoidal, analogous to the uninoculated control. Callus inoculated < 7 days after transfer did not grow. This is shown by the slopes calculated from the treatment lines drawn in Figure 8. The slopes calculated by linear regression fo the comparisons of Im 216 callus growth with time of *X. c. malvacearum* inoculation are as follows: Callus Ht. Day 1 = -0.005, Callus Ht. Day 3 = -0.005, Callus Ht. Day 7 = 0.008, Callus Ht. Day 12 = 0.138, Callus Ht. Day 15 = 0.163, Callus Ht. Day 21 = 0.204. The one-way ANOVA, height vs. time is significant at the 99.9% level. This trend is clearly seen by the incremental increase in the slopes calculated at each time point. Slopes for Days 1 and 3 are negative, thus indicating greater cell death than real growth. Slopes for Days 7, 12, 15, and 21 are all positive thus indicating that growth begins at Day 7 for inoculated cultures independent of when they were inoculated. It is evident, however, that the later the Im 216 callus was

inoculated, the better able the callus was to grow. Slopes for each day for all cultures showed a strong positive trend in growth. From the two-way ANOVA, the comparison height vs. time and date of inoculation, there is a significant difference at the 99.9% level for both factors. In other words, there is a strong positive growth trend for Im 216 callus observed over time and it is correlated with date of inoculation. This correlation is much stronger than for Ac 44.

It is obvious that inoculated blight-immune Im 216 callus grows better than inoculated blight-susceptible Ac 44 callus (Figs. 5-8). Growth patterns for each cultivar, Im 216 or Ac 44, are the same 0 to 6 days after transfer, but differ dramatically >6 days after transfer. This information correlates well with the fact that both Ac 44 and Im 216 are in the lag phase until 6 days after transfer, and Im 216 is resistant to *X. c. malvacearum.*

Ac 44 and Im 216 callus tested with *X. c. malvacearum* , strain 3631, showed a visually differential response to the pathogen. The Im 216 callus turned brown after inoculation and continued to grow, while Ac 44 callus was destroyed by the pathogen and bacterial slime was produced. Furthermore, Im 216 callus growth in the presence of *X. c. malvacearum* was dramatically different from Ac 44 callus growth. Ac 44, the *X. c. malvacearum* -resistant line growth in the presence of the bacteria was only inhibited if inoculation took place within 6 days after subculturing. Ruyack *et. al.* [208] point out that Im 216 growth (expressed as changes in fresh weight) was depressed when compared to the control, callus turned brown after 4 days, but continued to grow slowly yet even after 4 weeks the Im 216 callus tissue was still visible. However, Ac 44 cells turned brown after 4 days and disintegrated after 12 days following inoculation (Day 0). The results presented in Figs. 5-8 are somewhat similar to that which occurs in the intact leaf when inoculated with *X. c. malvacearum* . The Im 216 leaf tissue shows a hypersensitive response characterized by tissue browning and necrosis, and the Ac 44 leaf is eventually destroyed by the pathogen [12, 30, 77].

Ac 44 and Im 216 callus tissue has now been subcultured through hundreds of transfers over the course of 15 years, and each callus cell type still retains its ability to respond in a "susceptible" (Ac 44) or "resistant" (Im 216) fashion to the pathogen, *X. c. malvacearum.* Another interesting observation is that in order to realistically study cotton/*X. c. malvacearum* interactions, callus should be inoculated within 6 days after transfer to fresh SH medium. Artifacts caused by age of the Im 216 callus occur after 7

days. That is, if 12 or 14 day Im 216 callus cultures were inoculated with the pathogen, the host response is nearly the same as the uninoculated control.

Ac 44 and Im 216 Suspension Culture Growth Curves (Uninoculated). Suspension cultures from leaf callus cells were readily obtained in SH medium. Figure 9 summarizes the growth of Ac 44 and Im 216 cells. Ac 44 suspension cells exhibited a fresh weight doubling time of 2 to 3 days in the log phase and reached stationary-growth phase at 12 days. Im 216 cells in suspension reached stationary-growth phase at 12 days and had a fresh weight doubling time of 2 days. Both of these growth patterns for Ac 44 and Im 216 were sigmoidal just as was observed by Ruyack *et. al.* [208] for increase in Ac 44 and Im 216 dry weight. One obvious difference was the length of the log phase, 7 days for changes in Ac 44 and Im 216 fresh weight and 14 days for increase in Ac 44 and Im 216 dry weight [208].

The Ac 44 and Im 216 growth patterns (changes in fresh weight) were not significantly different in suspension culture. The one-way ANOVA comparisons of fresh weight vs. cotton cell type and fresh weight vs. time were not significantly different even at the 70% level. Visually however, Im 216 suspension cells were noticeably browner than the light colored Ac 44 suspension cells. Thus, it is possible to conclude that uninoculated Ac 44 and Im 216 suspension cultures established by transferring 3 to 4 week old callus do not differ significantly over time with respect to fresh weight even though Im 216 suspension cultures do appear to have a faster growth rate and reach a higher maximal fresh weight than do Ac 44 suspension cultures. In addition, the maximum carrying capacity for each 125 ml culture containing 50 ml SH medium was approximately 30 g of Ac 44 suspension cells and 35 g of Im 216 suspension cells.

Ac 44, AcB_N, Im 216 Suspension Culture Growth Curves Uninoculated and Inoculated with *X. c. malvacearum* . Suspension cultures from leaf callus cells of Ac 44, AcB_N, and Im 216 were readily obtained in SH medium. Figure 10 summarizes the growth of Ac 44, AcB_N, and Im 216 suspension cells over the course of 10 days. The growth patterns obtained in Figure 9 are noticeably different from those in Figure 10. One obvious reason for this difference is that Ac 44 and Im 216 suspension cultures in Figure 10 were initiated with approximately 4 g of callus, whereas Ac 44, AcB_N, and Im 216 suspension cultures in Figure 10 were initiated with 20 to 25 g of callus. Cells from suspension cultures in Figure 10 were collected for cell wall preparation so the host cell density had to be high enough for an adequate yield of cotton cell walls from both

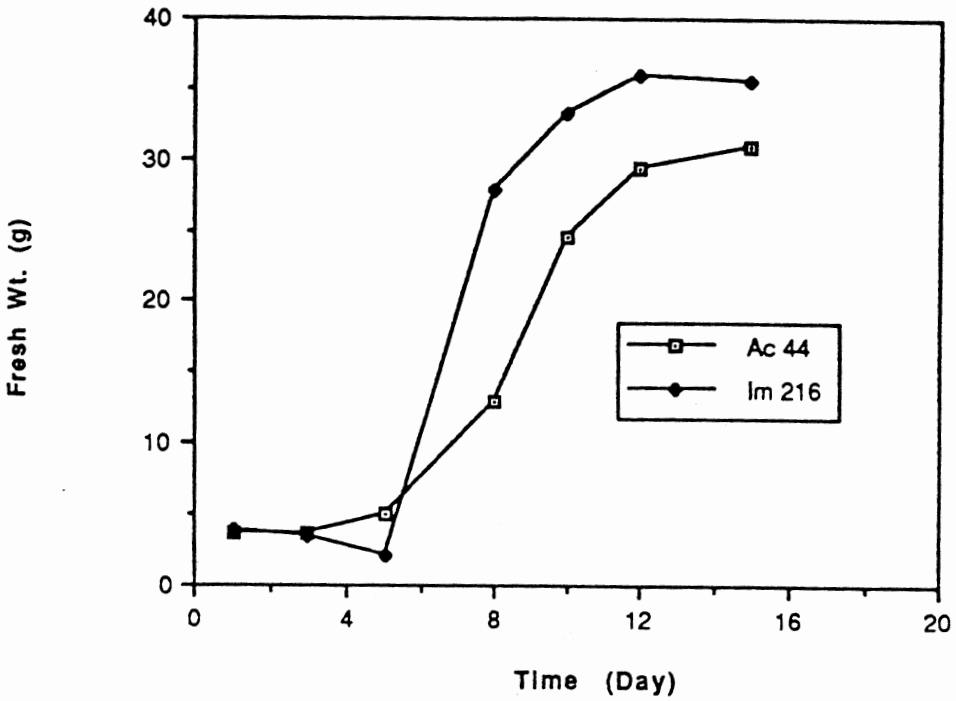


Figure 9. Growth of Ac 44 and Im 216 in Suspension Culture. Ac 44 and Im 216 suspension cultures were established by transferring one tube containing 3 to 4 g of 3 to 4 week old callus (late log phase) from SH agar medium to 125 ml culture flasks containing 50 ml of liquid SH medium. At appropriate times, one flask of the suspension cell was collected by filtration through Miracloth and the cell pad was weighed to determine the fresh weight.

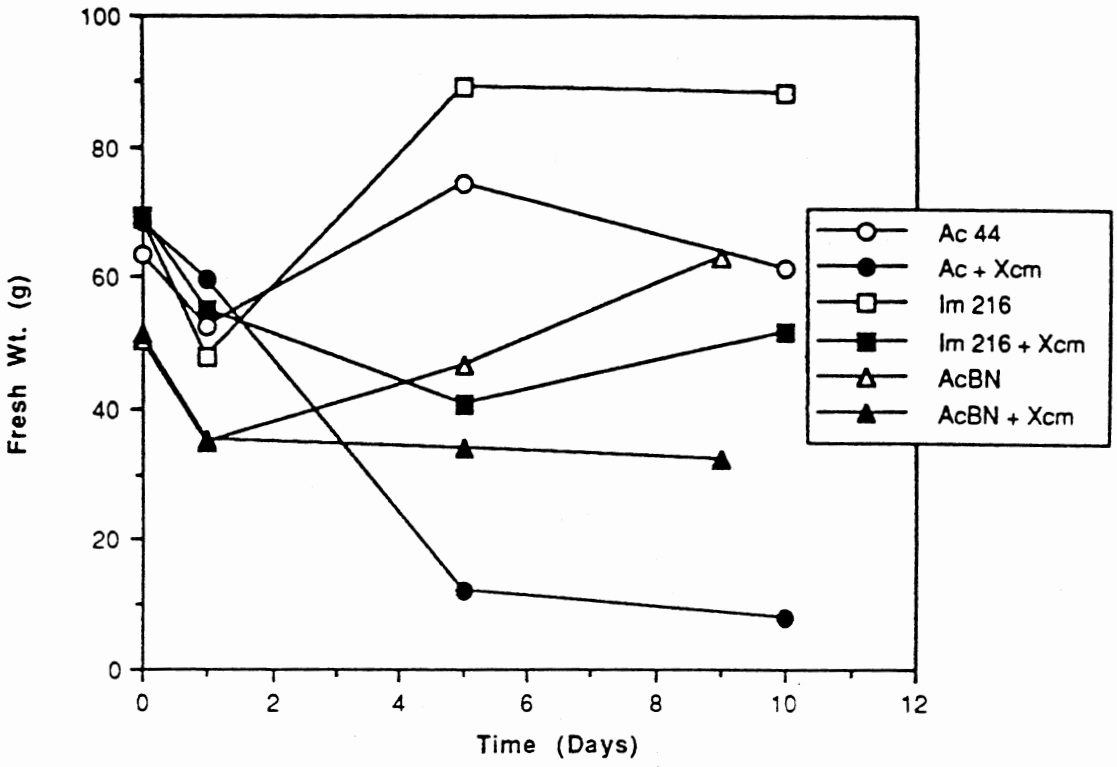


Figure 10. Ac 44, Im 216, and AcB_N Suspension Cell Growth in the Presence and Absence of *X. c. malvacearum* . Ac 44, Im 216, and AcB_N cotton suspension cultures were established by transferring 3 tubes containing 7 to 9 g (total = 20 to 25 g) of 3 to 4 week old callus (late log phase) from SH agar medium to 125 ml culture flasks containing 50 ml of liquid SH medium. Suspension cultures were inoculated with *X. c. malvacearum* at a final inoculum density of 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). At appropriate times, 3 flasks each of uninoculated and inoculated Ac 44, Im 216, and AcB_N suspension cells were collected by a coarse glass scintered funnel and the cell pad was weighed to determine the fresh weight. These cells were used for the viability studies and for cell wall preparation.

inoculated and uninoculated suspension cultures. These cells were also used to study viability. Thus placing 20 to 25 g of Ac 44, AcB_N (assume the maximum carrying capacity is the same as Ac 44 and Im 216), and Im 216 in 50 ml of SH medium, one would only expect about a 30% increase in fresh weight. That is the fresh weight increased from about 60 g (Day 0) to 90 to 100 g after 20 days in uninoculated cultures. This expectation was confirmed after examination of the data presented in Figure 10. The host cell density and size of the inoculum are critical for host cell growth in suspension culture.

All cultures (Ac 44, AcB_N, Im 216) contained 62.0 ± 8.0 g of tissue at Day 0 (time of culture establishment). There is a marked drop in fresh weight 1 day after initiating the culture. This drop varies from cell type to cell type and differs in the presence of the pathogen. The fresh weight drops at Day 1 were calculated as follows: [Day 0 Wt. (g) - Day 1 Wt. (g)]/ Day 1 Wt. (g) X 100. The following percentage fresh weight drops at Day 1 were noted: uninoculated Ac 44, 19.8%; inoculated Ac 44, 13.8%; uninoculated AcB_N, 30.6%; inoculated AcB_N, 32.0%; uninoculated Im 216, 37.5%; inoculated Im 216, 16.6%. These values represent the average of 2 experiments, one of which is exhibited in Figure 10 and the other is not shown. Growth patterns for each experiment are quite similar; thus, it is only necessary to discuss the one represented in Figure 10. The fresh weight drops noted at Day 1 are reversed by Day 5 for all uninoculated suspension cultures, but not for the inoculated suspension cultures. The biochemical reason for these cell weight drops is a change in host environment, i.e. transfer of callus from solid SH medium to liquid SH medium.

From the two-way analysis of variance, comparison of fresh weight vs. presence of pathogen and cell type was significant for both factors at the 91% level. This ANOVA allows the following conclusions to be made: 1) growth is different for each cotton cultivar, Ac 44, AcB_N, and Im 216, 2) growth varies with respect to the presence of the pathogen, *X. c. malvacearum* and 3) growth varies with respect to time. In a simpler sense, uninoculated Ac 44, AcB_N, and Im 216 suspension cultures showed an increase in fresh weight after Day 1, whereas a decrease in fresh weight or no change in fresh weight after Day 1 was observed for the inoculated Ac 44, AcB_N, and Im 216 suspension cultures. The inoculated Ac 44 suspension culture showed a tremendous increase in bacterial slime production after Day 10 which made accurate host fresh weight determinations at points beyond this time virtually impossible. However, inoculated AcB_N and Im 216 suspension cultures had less "slimy", fibrillar material produced by the

bacteria, *X. c. malvacearum.* Additionally, after the initial drop in fresh weight at Day 1 there was no change in fresh weight or in other words, growth was maintained, but not increased throughout the course of studying inoculated Im 216 and AcB_N suspension cultures.

It appears that Ac 44 cultures showed the most marked pathogen effect. Ac 44 is the blight-susceptible cell type and thus susceptibility to *X. c. malvacearum* should result in rapid and continuous Ac 44 cell death just as is shown in Figure 10. In fact at Days 5 and 10, there is a 6.2-fold difference in fresh weight between uninoculated Ac 44 suspension cells and inoculated Ac 44 suspension cells. There are substantial differences in maximum fresh weight reached for uninoculated AcB_N and Im 216 suspension cells and inoculated AcB_N and Im 216 suspension cells, but little overall difference between the calculated differential growths. AcB_N has the resistant gene, B_N, and Im 216 has 3 major resistant genes, B_2, B_3, and b7 in a polygenic background. There was a 1.4-fold fresh weight difference between uninoculated AcB_N suspension cells and AcB_N inoculated suspension cells at Day 5 and a 2.0-fold difference at Day 10. There was a 2.2-fold fresh weight difference between uninoculated Im 216 suspension cells and Im 216 inoculated suspension cells at Day 5 and a 1.7-fold difference at Day 10. In either case, AcB_N and Im 216 inoculated and uninoculated suspension cultures are less affected by *X. c. malvacearum* than Ac 44 inoculated and uninoculated suspension cultures. In addition, Figure 10 shows that inoculated Im 216 cultures have the greatest amount of growth, AcB_N cultures intermediate amount of growth, and Ac 44 culture the least amount of growth.

A case has therefore been made for observable resistance in AcB_N and Im 216 suspension cultures to the pathogen, *X. c. malvacearum* not unlike that seen in intact plants. Based on the ability of Ac 44, AcB_N, and Im 216 to respond to the pathogen, *X. c. malvacearum* seen in Figure 10, it is possible to conclude that susceptibility and resistance are exhibited in callus (Figs. 5-8) and suspension (Figure 10) cell culture. That is, ability for inoculated suspension culture growth is dependent on the cell type and thus the number of resistant genes to *X. c. malvacearum* . Thus, this system, cotton plus *X. c. malvacearum* in suspension culture, would appear to be a useful and valid system for studying the molecular events associated with cotton blight disease resistance.

Viability Studies

Viability of uninoculated and inoculated, Ac 44, AcB_N, and Im 216 suspension cells was determined by Evan's blue staining at various stages of culture growth. A typical view of Evan's blue stained AcB_N suspension cells 3 days after inoculated with *X. c. malvacearum* is shown in Figure 11. Results of the viability studies are shown in Figs. 12-15. This method for staining for dead cells in plant tissues was first described by Gaff and Okag'O-ogola [88]. Evan's blue is excluded by living cells. It is a non-toxic, water-soluble blue pigment and its test properties depend basically on the retention or loss of the semi-permeable properties of the plasmalemma, rather than those of the tonoplast. The viability of the Ac 44, AcB_N, and Im 216 suspension cells was examined over the course of 20 days in the presence and absence of the pathogen, *X. c. malvacearum.* Viability studies are undertaken for 2 main reasons: 1) determining the amount of cell death as a direct result of infection by the pathogen and 2) determining cell death at various stages of normal culture growth.

Figure 12 shows the viability of uninoculated and inoculated Ac 44 suspension cells. The cells were examined on days 0 (inoculation), 1, 3, 5, 7, 10, 12, 13, and 19 after the suspension cultures were established. The cultures were at least 69.1% (inoculated) and 74.7% (uninoculated) alive at Day 0, time of inoculation. Viability gradually decreases from 74.7% to 49.1% after 19 days, or 1.3% decline per day, in uninoculated Ac 44 suspension cultures. The total decline in the percentage of live cells is therefore 25%. This decrease begins to level off at 10 days after inoculation. However, viability dramatically decreased in inoculated Ac 44 suspension cultures from 69.1% to 0% over the course of 19 days. There is a 17-fold decrease in viability over 13 days. This decline is linear with a correlation coefficient of -0.996 through Day 13. That is, viability (cell death) in inoculated Ac 44 suspension cultures is proportional to time after inoculation. There are no detectable live cells after 13 days in inoculated Ac 44 suspension culture.

Figure 13 shows the viability of Im 216 suspension cells. Like Ac 44, the Im 216 cells were examined on days (Day 0 = time of inoculation) 3, 5, 7, 9, 11, 13, and 18 after the suspension cultures were established. The cultures were at least 67.6% (uninoculated) to 68.3% (inoculated) alive at Day 1. Viability gradually decreases from 67.6% to 42.4% after 18 days in uninoculated Im 216 suspension cultures. The total decrease in the percentage of live Im 216 cells is 25.2% virtually identical to that seen in Figure 12

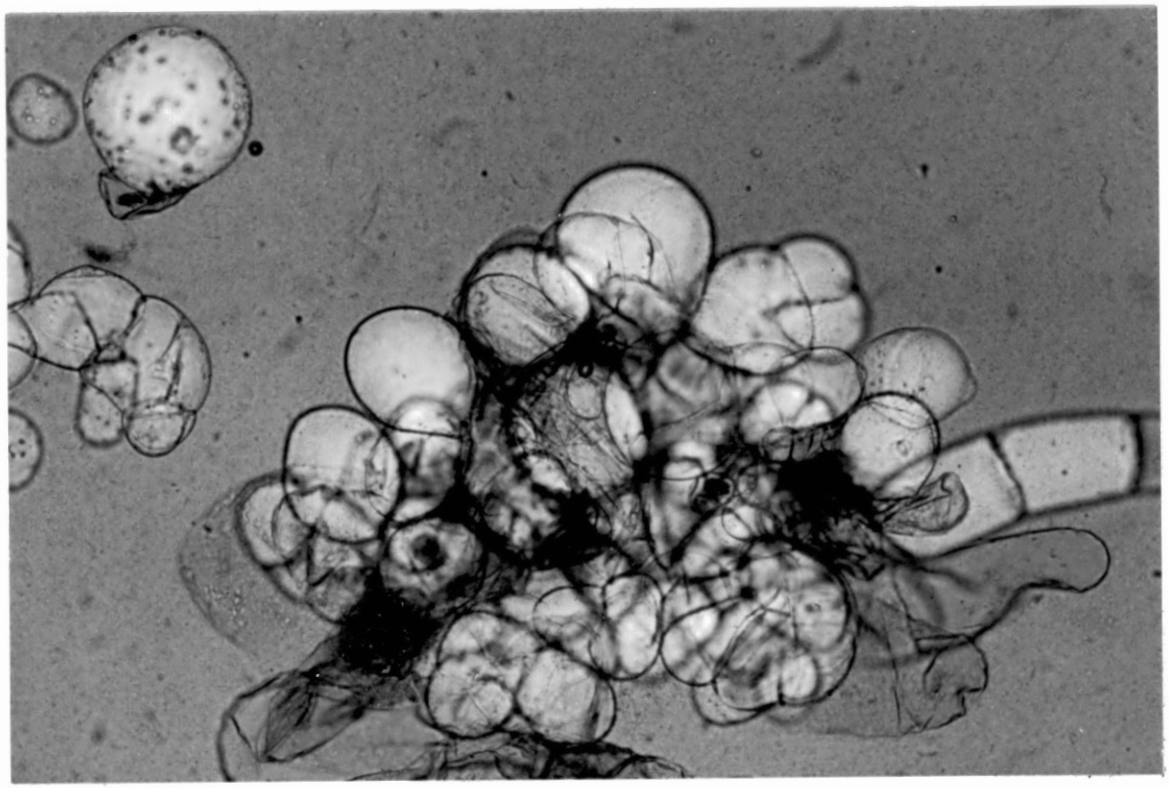


Figure 11. Typical View of Evan's Blue Stained AcB_N Supsension Cells 3 Days after Inoculation with *X. c. malvacearum*.

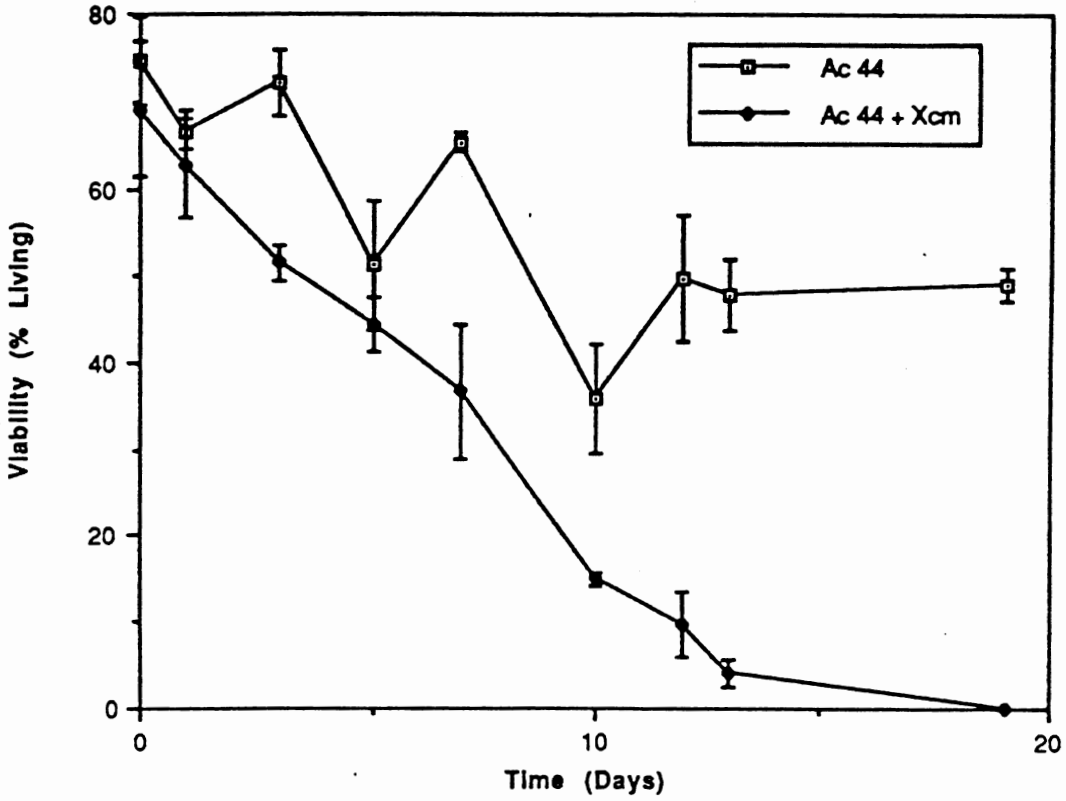


Figure 12. Viability of Uninoculated and Inoculated Ac 44 Suspension Cells. Viability was measured using 1% (w/v) Evan's blue dye] at various stages of uninoculated and inoculated Ac 44 suspension culture growth. Ac 44 suspension cultures were inoculated immediately after callus was transferred to liquid SH medium with *X. c. malvacearum* at a final inoculum density of 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Each time point represents the average cell viability determined from triplicate or quadruplicate determinations ± standard deviation.

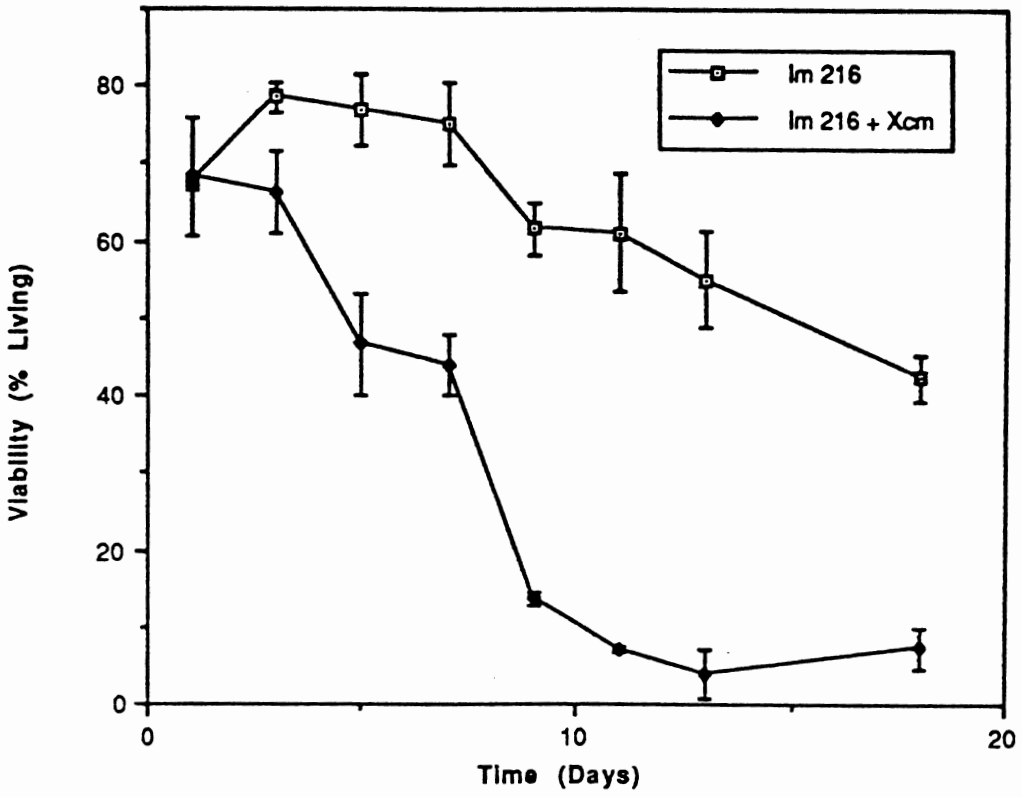


Figure 13. Viability of Uninoculated and Inoculated Im 216 Suspension Cells. Viability was measured using 1% (w/v) Evan's blue dye] at various stages of uninoculated and inoculated Im 216 suspension culture growth. Im 216 suspension cultures were inoculated immediately after callus was transferred to liquid SH medium with *X. c. malvacearum* at a final inoculm density of 1×10^7 cfu ml^{-1} in 50 ml SH medium. Each time point represents the average cell viability determined from triplicate or quadruplicate determinations ± standard deviation.

representing the viability of Ac 44 suspension cells. The decline in viability for uninoculated Im 216 cells is gradual, a loss of only 6.5% after 11 days. However, viability dramatically decreased in inoculated Im 216 suspension cultures from 68.3% to 4.0% after 13 days and then began to increase slightly to 7.3% after 18 days. Im 216 cell viability decreased nearly 9-fold after 18 days, but 17-fold after 13 days. This is the same approximate decline as observed for inoculated Ac 44 suspension cells (Figure 12). This decline in viability of inoculated Im 216 suspension cells is generally linear with a correlation coefficient of -0.964 from Day 3 to Day 13. The viability curve is however sigmoidal, whereas the viability curve for inoculated Ac 44 suspension cells is linear until cell death is complete. Viability (cell death) in inoculated Im 216 suspension culture is proportional to time after inoculation like that of inoculated Ac 44 suspension culture. However, unlike the viability determinations in inoculated Ac 44 suspension culture, there are live Im 216 cells at the end of this study. Thus, one would conclude that Im 216 cells have a greater viability in the presence of the cotton blight pathogen, *X. c. malvacearum* than do Ac 44 cells.

A second viability study of Ac 44 and Im 216 suspension cells inoculated with the pathogen, *X. c. malvacearum* is shown in Figure 14. Both Ac 44 and Im 216 were examined on days 1 (Day 0 = time of inoculation), 5, 10, 15, and 20 after the suspension cultures were established. The cultures were at least 58.2% (Ac 44) and 60.4% (Im 216) alive at the time of inoculation. Both Ac 44 and Im 216 viabilities declined dramatically through Day 10 after inoculation. Im 216 cell viability decreased nearly 14-fold after 15 days while Ac 44 cell viability decreased over 1000-fold after 15 days. All Ac 44 cells were dead at the conclusion of the study (Day 20) while there were still 4.1% Im 216 cells alive. Like the first viability study (Figures 12, and 13) of inoculated Im 216 and Ac 44 suspension cells, the second viability study showed that Im 216 cells reached a leveling off of cell death by Day 15. The second viability study indicates that the rate of Im 216 cell death is less than the of Ac 44 cell death. Again, like the first viability study, this second study strongly suggests that Im 216 cells are more viable in the presence of the cotton blight pathogen, *X. c. malvacearum* than Ac 44 cells.

Figure 15 shows the viability of uninoculated and inoculated AcB_N suspension cells. Like both Ac 44 and Im 216 suspension cells, AcB_N were examined on days 0 (date of inoculation), 1, 3, 6, 7, 9, 15, and 17 days after the cultures were established. The cultures were at least 70.8% (uninoculated) and 70.9% (inoculated) alive at Day 0, date of inoculation. Viability does not decrease in uninoculated AcB_N over the course of

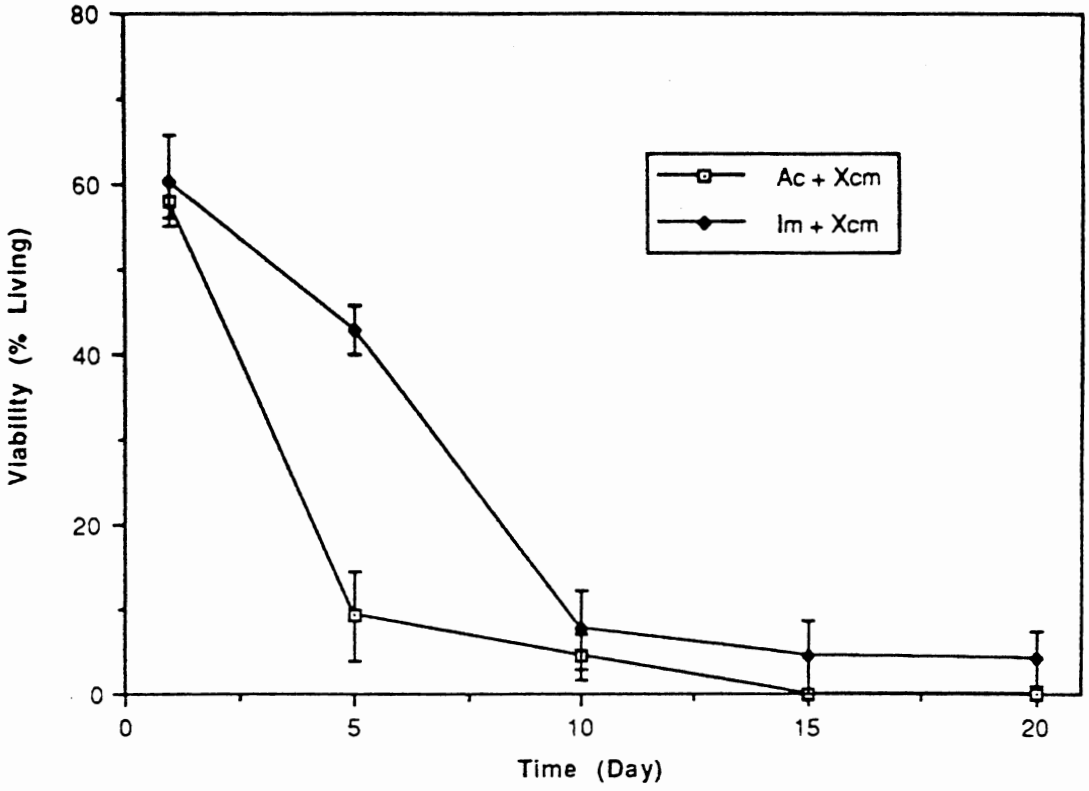


Figure 14. Viability of Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cells (Study 2 - Oct/Nov 1988). Viability was measured using 1% (w/v) Evan's blue dye at various stages of uninoculated and inoculated Im 216 suspension culture growth. Ac 44 and Im 216 suspension cultures were inoculated immediately after callus was transferred to liquid SH medium with *X. c. malvacearum* at a final inoculum density of 1 X 10^7 cfu ml^{-1}. Each time point represents the average cell viability determined from triplicate or quadruplicate determinations ± standard deviation.

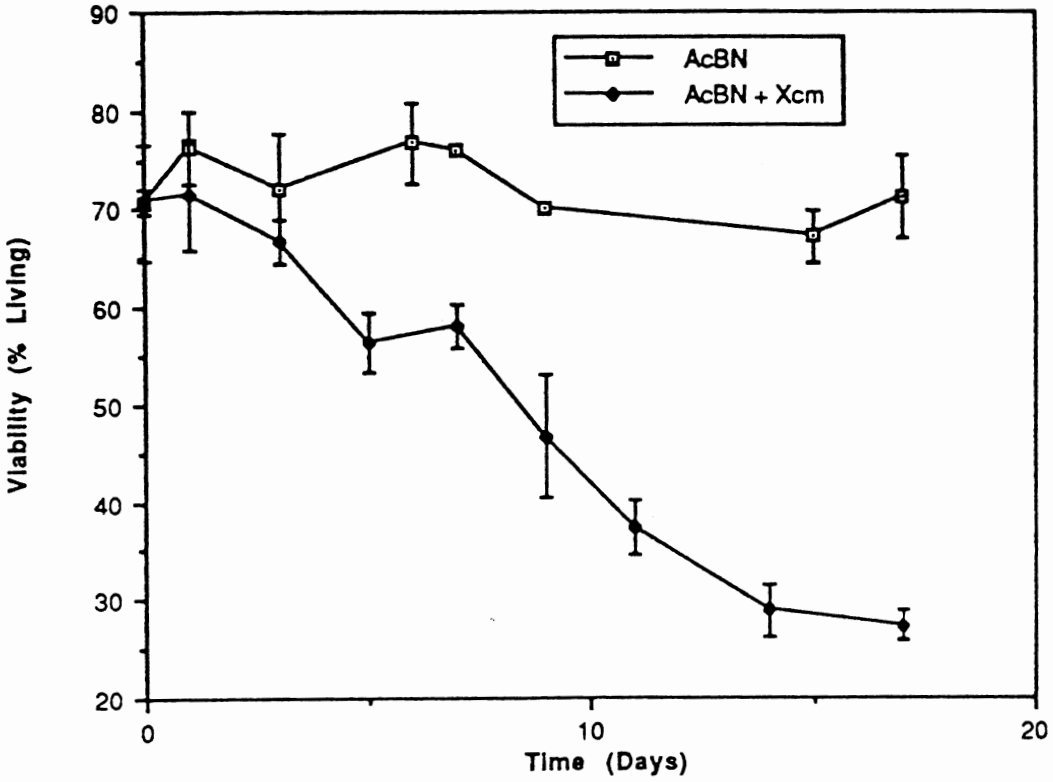


Figure 15. Viability of Uninoculated and Inoculated AcB_N Suspension Cells. Viability was measured using 1% (w/v) Evan's blue dye at various stages of uninoculated and inoculated Im 216 suspension culture growth. AcB_N suspension cultures were inoculated immediately after callus was transferred to liquid SH medium with *X. c. malvacearum* at a final inoculum density of 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Each time point represents the average cell viability determined from triplicate or quadruplicate determinations ± standard deviation.

17 days since the ending percentage of live cells is 71.3%. The standard error of these determinations was only 5%. The finding that there was no decrease in viability for AcB_N suspension cells contrasts with the 25% decrease in viability observed for Ac 44 (Figure 12) and Im 216 (Figure 13) suspension cells. Part of the difference in viability may be due to the fact that the Ac 44 and Im 216 callus cultures were 15 years old at the time of this study and the AcB_N callus culture was only 3 years old at the time of this study. The ability to sustain a high viability may have decreased over the number of years the Ac 44 and Im 216 callus has been in culture.

However, viability dramatically decreased in inoculated AcB_N suspension cultures from 70.9% to 27.4%, a total decrease of 43.5% after 17 days. AcB_N cell viability decreased 2.6-fold after 17 days. The viability curve of AcB_N is similar to that of Im 216 in that cell death levels off after approximately 15 days and the shape of the curve is similarly sigmoidal. Additionally, there is a considerable percentage of live cells remaining in the AcB_N suspension culture 17 days after inoculation. This percentage, 27.4%, is much higher than the 7.3% (1st study, Figure 13) or 4.1% (2nd study, Figure 14) remaining in Im 216 suspension culture. Again, one might expect a higher viability for the AcB_N cells since the callus culture is only 3 years old (~24 transfers) and the Im 216 callus culture is 15 years old and has been through hundreds of transfers. Yet, even after hundreds of transfers for Ac 44 and Im 216 and the limited number of transfers for AcB_N, the suspension cultures behave in a established resistant and susceptible fashion. That is, a percentage of cells of the two resistant cultivars, Im 216 and AcB_N, are able to persist in the presence of the pathogen, whereas the susceptible cultivar, Ac 44, is not. Cell death is complete for Ac 44 in both studies (Figs. 12 and 14) after 15 days.

The two-way analysis of variance, comparison of viability vs. cotton cell type and time, is significant for both factors at the 91% level of significance. The two-way ANOVA, comparison of viability vs. presence of pathogen and time, is significant for both factors at the 99.9% level of significance. However there is a significant interaction effect seen, presence of pathogen X time at the 95% level. The two-way ANOVA, comparison of viability vs. cotton cell type 3.65, and presence of pathogen is significant for both factors at the 96% level. The one-way ANOVA, comparison of viability vs. cotton cell type is significant at the 91% level. The one-way ANOVA, comparison of viability vs. time is significant at the 99.5% level.

From the analyses of variance, several conclusions can be made regarding the data presented in Figs. 12-15. First, viability is different for uninoculated and inoculated suspension cultures, i.e. viability is a function of the presence of the pathogen. Second, viability changes with time. Third, viability is different for Ac 44, AcB_N, and Im 216 suspension cultures. For example, the viability for uninoculated Ac 44 suspension cultures is significantly different from inoculated Ac 44 suspension cultures. The same can be said for the viability of uninoculated and inoculated AcB_N and Im 216 suspension cultures. Thus, if one changes the cell type being measured, the viability is significantly different over the course of time (for the 3 cell types measured) in the absence or presence of the pathogen.

Outside of the statistical studies, the following observations can be made from the results presented in Figs. 12-15. First, viability is greater in the resistant cell typesIm 216 (3 major resistant genes) and AcB_N (1 resistant gene) than in the susceptible cultivar, Ac 44 (no resistant genes) after infection with the pathogen, *X. c. malvacearum* . Second, viability is altered over time in culture. The older tissues, Ac 44 and Im 216, were established as callus more than a decade before the establishment of AcB_N as callus. Third, inoculated suspension cultures have a markedly lower viability than uninoculated suspension cultures. Fourth, differential resistance is expressed by these 3 cell lines, Ac 44, AcB_N, and Im 216 in suspension culture. Each cell type is distinctive in its response to the pathogen, *X. c. malvacearum*, race 3 not unlike the host /pathogen interactions observed in the intact plant [12].

Some analogous viability measurements of host cells have been made in the intact plant. Infiltrated leaf discs of Westburn Immune (WbM (0.0)) have been examined for viability of palisade cells over time (M. Pierce, Oklahoma State University, Dept. of Biochemistry, personal communication). Results indicated that the number of dead cells equaled the initial density of *X. c. malvacearum*, race 3 between 7 days and 10 days after inoculation. The cotton line, WbM (0.0) has the same 3 resistance genes as Im 216, but due to its genetic background, it does not demonstrate as high a resistance to *X. c. malvacearum* [24]. In any event, measurement of cell viability by Evan's Blue dye in WbM (0.0) leaf discs is similar to that measured in suspension culture. By day 10 in inoculated Im 216 suspension cells, viability was less than 10% and at a minimum for the viability curve (see Figure 15). Like in the infiltrated WbM (0.0) leaf disc study, inoculated Im 216 viability at Day 1 is not any different from the uninoculated control, but by Day 2 viability is lower in inoculated plant tissues (suspension cells or leaf discs).

Additionally, there are live cells present at the conclusion of both viability studies in the intact plant and in suspension culture indicating cell delath was not complete. Resistance thus can be expressed in both intact leaf discs and in suspension culture. A second viability study involving inoculated OK 1.2 cotyledons indicated that the first sign of cell death was at 24 h after inoculation (M. Pierce, Oklahoma State University, Department of Biochemistry, personal communication). Examination of Figures 12-15 indicates that cell death begins 1 to 2 days after inoculation in suspension culture. In other studies, mesophyll cells have been examined for yellow-green autofluorescence using fluorescence microscopy for phytoalexins [78, 185]. From these studies, phytoalexins in cotton have been localized at infection sites *in vivo* .

Widholm [259] examined tobacco, rice, soybean, carrot, and tomato cells at various stages of culture growth over the course of 20 days for viability using fluorescein diacetate, and phenosafranin. The cells were examined at days 0 , 2, 4, 8, and 20. The cultures increased rapidly in fresh weight until about day 8 and thereafter the growth rate declined. The cultures were all at least 80% alive at day 8 while day 20 a variable percentage of cells remained alive, tobacco 30-95%, rice 0-50%, soybean 4-30%, carrot 30-40%, and tomato 20-80%. Uninoculated Ac 44, Im 216, and AcB_N suspension cells (Figures, 13, 14, 16) were 45-70% alive after 20 days. Viability measurements in suspension cultures inoculated with plant pathogens are not presently available in other systems. hus, results presented here in Figures 13-16 provide a basis for quantitating cell death as a consequence of inoculating cotton suspension cultures with *X. c. malvacearum*, strain 3631.

Growth of Ac 44, AcB_N, and Im 216 suspension cells in the presence or absence of *X. c. malvacearum* (Figure 11) can be easily compared with the viability of Ac 44, AcB_N, and Im 216 suspension cells in the presence or absence of *X. c. malvacearum* (Figs. 12-15). Increases in fresh weight and maintenance of viability are parallel in the uninoculated suspension cultures of each cultivar. Similarly, the decreases in fresh weight and loss of viability are parallel in the inoculated suspension cultures. That is, cells that are viable are growing by increase in fresh weight evidence and cells that are dying or dead are not growing. The increases seen in fresh weight in uninoculated Ac 44, AcB_N, and Im 216 suspension cells reach a maximum at 10 days after transfer at the described host cell density. Viability at this time is within 10% of Day 0 or Day 1. Just as growth is dramatically different for inoculated and uninoculated suspension cultures of the same cell type so is viability. Additionally, viability and growth are cell type dependent. That is, Ac

44, AcB_N, and Im 216 each had different growth and viability curves. Thus, measuring both growth and viability in suspension culture establishes the cell type specific differences linked to the pathogen, *X. c. malvacearum*. Knowing these differences is necessary for characterizing *in vitro* cotton cell type specific: *X. c. malvacearum* (race specific) differences and using it is a valid host/pathogen system for studying the biochemical and molecular events associated with disease resistance to *X. c. malvacearum*.

Protoplast Isolation

Protoplasts were isolated from four cotton cell types in suspension culture (see Materials and Methods) using an enzymatic digestion mixture consisting of 50 units/ml cellulysin and 50 units/ml macerase. Ac 44 is completely susceptible to the pathogen, *X. c. malvacearum*, whereas, AcB_N has one resistance gene, B_N , OK 1.2 has at least one resistance gene (disease severity = 1.2), and Im 216 has 3 major resistance genes, B_2, B_3, and b_7 in a polygenic background to the pathogen, *X. c. malvacearum*. Protoplast yields of Ac 44, AcB_N, OK 1.2, and Im 216 cotton cell lines are presented in Table 2.

From Table 2, the protoplast yields differ quite dramatically between cotton cell lines. The cotton cell line, Ac 44, has the greatest protoplast yield of 48% which is in good agreement with the protoplast yield obtained by Hussein and coworkers (1987), of 55%. The protoplast yields of AcB_N, OK 1.2, and Im 216 are 6.8%, 2.0%, and 0.97%, respectively. No published data are available for these cell lines. However, a t-test comparing protoplast yields of the resistant cotton cultivars, AcB_N, OK 1.2, and Im 216, to the susceptible cotton cell line, Ac 44, provided significant results. The t-statistic is 2.9808. The difference between Ac 44 and the other 3 cotton cell lines, AcB_N, OK 1.2, and Im 216, was significant (t-value required = 2.951) at the 94% level. The direction of the difference is also statistically significant at the 97% level (t-value required = 2.951). This directionality implies that the protoplast yields are linked to the degree of resistance these cotton cell lines have to *X. c. malvacearum* . That is, the more resistant these cotton cell line to *X. c. malvacearum* , the lower the protoplast yield at this particular enzyme concentration (50 units/ml of both cellulysin and macerase). Differences in protoplast yields within resistant cultivars, AcB_N, Im 216, and OK 1.2, were not significant at ≥90% confidence level. In addition, these results suggest that the cell walls of these 4 cotton cell lines do not have the same sugar composition. Thus, one may speculatively conclude that protoplast yield is directly related to the ability of the enzymatic mixture, cellulysin/ macerase units/ml to digest the cell walls. Therefore, 50 units/ml cellulysin and

TABLE 2

PROTOPLAST YIELDS OF AC 44, IM 216, OK 1.2, AND ACBN COTTON CELL LINES

Cotton Line	Total Fresh Wt. (g) for Cell Count	Fresh Wt. (mg) for Viable Cell Count	No. Protoplasts per ml	No. Protoplasts per mg	No. of Viable Cells per ml	No. of Viable Cells per mg	Protoplast Yield*
Ac 44	5.6	300	530000	1900	240000	4000	48.0 a
AcBN	5.6	100	65000	230	120000	3400	6.8 b
OK 1.2	7.4	100	32000	86	170000	4200	2.0 b
Im 216	6.9	100	20000	58	67000	6000	0.97 b

* = Different letters indicate protoplast yields are significantly different using a t-test at the 94% confidence level.

50 units/ml macerase easily removes the Ac 44 suspension cell wall, but has increasing difficulty associated with resistance to the pathogen, *X. c. malvacearum*, in removing AcB_N, OK 1.2, and Im 216 suspension cell walls.

Time Course Profiles of Several Biochemical Parameters in in Uninoculated and Inoculated Ac 44, AcB_N, and Im 216 Cotton Suspension Cell Walls

Ac 44, AcB_N, and Im 216 Cotton Cell Wall Preparation Summary

Cells walls of Ac 44, AcB_N, Im 216 suspension cells were prepared from inoculated and uninoculated 1, 5, 10, 15, and 20 day old cultures representing the full range of the growth cycle. A summary of the cell wall preparations is shown in Table 3. The other three time courses reflected similar recoveries at each stage of cell wall preparation (data not shown). The suspension cell growth (starting fresh weight) of Ac 44, AcB_N, and Im 216 suspension cells has already been shown in Figure 10.

From the results presented in Table 3, it is apparent that the average percentage of extrapolysaccharides (EPS) in uninoculated suspension cells 40 to 55% lower than that of the inoculated suspension cells. That is, the recovery of washed cells decreases markedly after 10 days in culture due to the presence of the pathogen and the subsequent increase in EPS, irrespective of cultivar. Specifically, there is an approximate 3:1 ratio of average % washed cells recovered to the average % EPS for both uninoculated Ac 44 and Im 216 suspension cells. However, in the inoculated Ac 44 and Im 216 suspension cell, this ratio drops to approximately 1:1. Again, there is a strong suggestion that the presence of the pathogen, *X. c. malvacearum* decreases the collectable Ac 44 and Im 216 cell fresh weight and increases the % EPS. This situation does not change for AcB_N suspension cultures. The ratio of average % washed cells recovered to the average % EPS for uninoculated AcB_N suspension cells is 10:1, while this ratio is only 5:1 for inoculated AcB_N suspension cells.

Thus, it can be concluded first, that the average % washed cells is markedly lower in inoculated suspension cells than in uninoculated suspension cells. Second, the increase in average % EPS is directly related to the presence of pathogen. However, Ac 44 and Im

TABLE 3

AC 44, ACBN, IM 216 COTTON SUSPENSION CELL WALL PREPARATION SUMMARY

Suspension Cell Samples	Starting Cell Fresh Wt. (g)*	Washed Cell Fresh Wt. (g)**	% Washed Cells Recovered	Percentage EPS (%)	Cell Wall Dry Wt. (g)	% (Wall Dry Wt./ Starting Cell Fresh Wt.)	Washed Cell Fr. Wt. (g) / Cell Wall D. Wt. (g)
Ac 44 - Uninoculated							
1 d	52.5	37.0	70.5	29.5	0.27	0.51	137.0
5 d	74.5	64.8	87.0	13.0	0.61	0.82	106.2
10 d	61.5	48.5	78.9	21.1	0.79	1.28	61.4
15 d	64.7	56.8	87.8	12.2	1.00	1.54	56.8
20 d	92.6	56.4	60.9	39.1	0.94	1.02	60.5
Ave. ± S.D.			77.02 ± 10.21	23.0 ± 10.2		1.03 ± 0.36	
Ac 44 - Inoc. with *X. c. malvacearum*							
1 d	59.6	37.3	62.6	37.4	0.28	0.47	133.2
5 d	12.0	7.0	58.3	41.7	0.22	1.83	31.8
10 d	7.8	6.1	78.2	21.8	0.23	2.95	26.5
15 d	27.2	6.0	22.0	78.0	0.23	0.84	26.1
20 d	74.5	15.2	20.4	79.6	0.08	0.11	190.0
Ave. ± S.D.			48.3 ± 23.1	51.8 ± 23.1		1.24 ± 1.03	
Im 216 - Uninoculated							
1 d	48.1	41.5	86.3	13.7	0.44	0.91	94.3
5 d	89.2	64.8	72.6	27.4	1.13	0.27	57.3
10 d	88.3	62.6	70.9	29.1	1.32	1.49	47.4
15 d	89.7	74.4	82.9	17.1	1.41	1.11	52.8
20 d	90.7	62.1	68.5	32.5	1.22	1.34	50.9
Ave. ± S.D.			76.2 ± 7.03	23.8 ± 7.03		1.21 ± 0.20	
Im 216 - Inoc. with *X. c. malvacearum*							
1 d	55.0	39.0	70.9	29.1	0.42	0.76	92.9
5 d	40.8	27.2	66.7	33.3	0.28	0.69	97.1
10 d	51.7	9.5	18.4	81.6	0.34	0.66	27.9
15 d	66.5	18.6	28.0	72.0	0.31	0.47	60.0
20 d	62.9	10.3	16.4	83.6	0.26	0.41	39.6
Ave. ± S.D.			40.1 ± 23.8	59.9 ± 23.8		0.60 ± 0.13	
AcBn - Uninoculated							
1 d	34.8	29.5	84.8	15.2	0.42	1.20	70.2
5 d	46.8	43.4	92.7	7.3	0.89	1.90	48.8
9 d	63.2	60.5	95.7	4.3	1.78	2.82	34.0
Ave. ± S.D.			91.07 ± 4.60	8.93 ± 4.6		1.97 ± 0.66	
AcBn - Inoc. with *X. c. malvacearum*							
1 d	35.2	31.0	88.1	11.9	0.44	1.20	70.4
5 d	34.2	25.2	73.7	26.3	0.44	1.30	57.3
9 d	32.2	28.8	89.4	10.6	0.48	1.50	60.0
Ave. ± S.D.			83.73 ± 7.11	16.27 ± 7.11		1.33 ± 0.12	

*Starting cell fresh weight = the weight of suspension cells from three suspension flasks collected in a coarse-scintered glass funnel.
**Washed cell fresh weight = the weight of the starting suspension cells after washing twice with 0.1 M potassium phosphate buffer, pH 7.0.

216 suspension cultures do not differ in average % washed cells or average % EPS for uninoculated and inoculated cultures, respectively. Third, it appears that the pathogen has a greater affect on the Ac 44 and Im 216 cultures, than on the AcB_N cultures. Analysis of sugars in EPS from Ac 44 and Im 216 cotton suspension culture medium has been performed [141] and results suggest that both Ac 44 and Im 216 are rich in xylose, glucose, and galactose, but not in galacturonic acid indicating that EPS is richer in hemicelluloses than in pectin. Analysis of sugars in EPS from AcB_N cotton suspension culture medium has not been performed. Yet, Ac 44 and Im 216 cell walls differ in the galacturonic acid content. Ac 44 is richer in galacturonic acid than Im 216 cell walls.

In a different vein, examination of the ratio of washed cell fresh weight (g) to cell wall dry weight (g) (=Cell FW / Wall DW) proved to be very interesting. Figure 16 shows the time course of Cell FW / Wall DW for Ac 44 and Im 216 cell wall preparation from uninoculated and inoculated suspension cells. Similarly, Figure 17 shows the time course of Cell FW / Wall DW from AcB_N.

Results in Figures 16 and 17 indicate that the ratio of Cell FW / Wall DW decreases over time for uninoculated and inoculated Ac 44, Im 216, and AcB_N cotton lines. From a N-way analysis of variance (ANOVA), this trend is statistically significant at the 95% confidence level. Significance is checked at the 95% confidence level unless otherwise noted. However, this ratio is not statistically different for the cell types studied nor due to the presence of the pathogen. Yet, the uninoculated Cell FW / Wall DW ratio seems to differ from that of the inoculated Cell FW / Wall DW ratio, but the direction of the difference is not identical for each pair of comparison, i.e., inoculated v. uninoculated Ac 44 or Im 216 or AcB_N. This explains why this difference is not statistically significant after analysis of variance.

Upon examining Figures 16 and 17, this conflict in direction is presented as follows: the pathogen, *X. c. malvacearum* , in Ac 44 cultures depresses the value of the Cell FW / Wall DW ratio below that of the uninoculated ratio while in AcB_N and Im 216 cultures it elevates the value of the Cell FW / Wall DW ratio above that of the uninoculated ratio. Mere observation of the graphed data suggests that the presence of *X. c. malvacearum* causes the Cell FW / Wall DW ratio to be >20% higher in uninoculated Ac 44 than in inoculated Ac 44 after 10 days. Conversely however, the presence of *X. c. malvacearum* in Im 216 and AcB_N cultures seems to elevate the value of the Cell FW / Wall DW ratio above that of the uninoculated ratio. This divergence in the ratios for

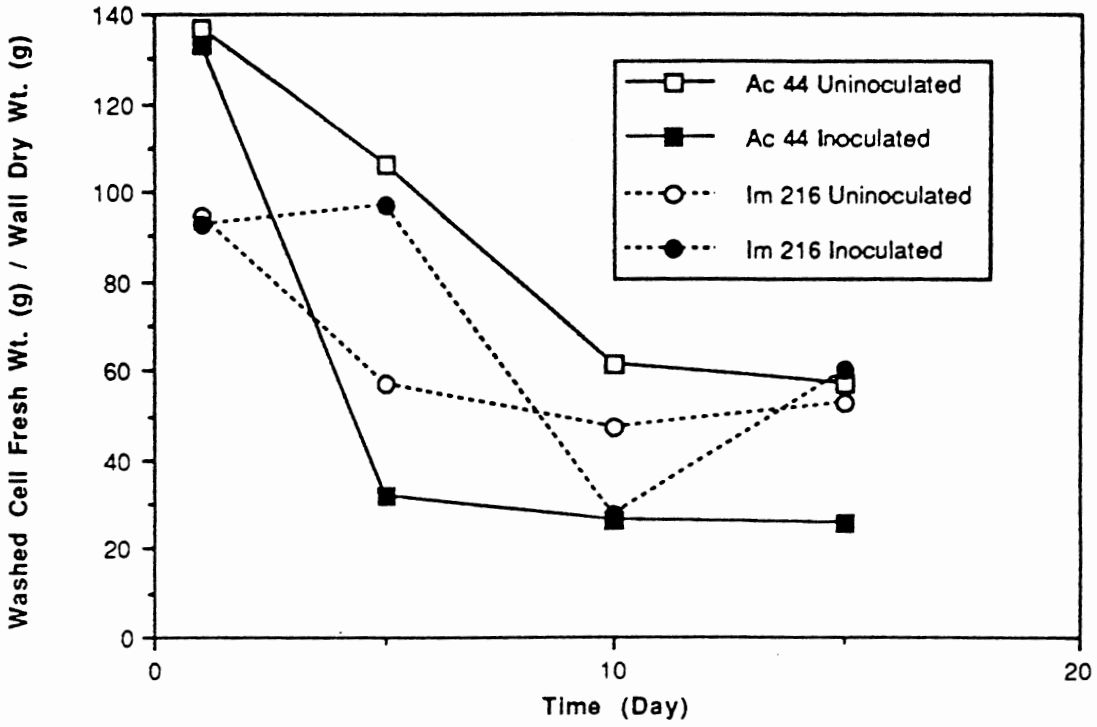


Figure 16. Time course of Washed Suspension Cell Fresh Wt. (g) to Cell Wall Dry Wt. (g) over 15 Days during Ac 44 and Im 21 Cell Wall Preparation from Growing Cells. Ac 44 and Im 216. suspension cultures were inoculated immediately after callus was transferred (~7-9 g of callus per flask) to liquid SH medium with *X. c. malvacearum* at a final inoculum density of 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). At 1, 5, 10, 15, and 20 d, three flasks of cells per treatment were collected by a coarse glass scintered funnel and the cell pad was weighed to determine the fresh weight. The collected cells were washed twice with 0.1 M potassium phosphate, pH 7 and cell walls were prepared as described in the Materials and Methods. The dry weight of the cell walls was determined after drying 48 h. Data represents 1 determination of a single experiment .

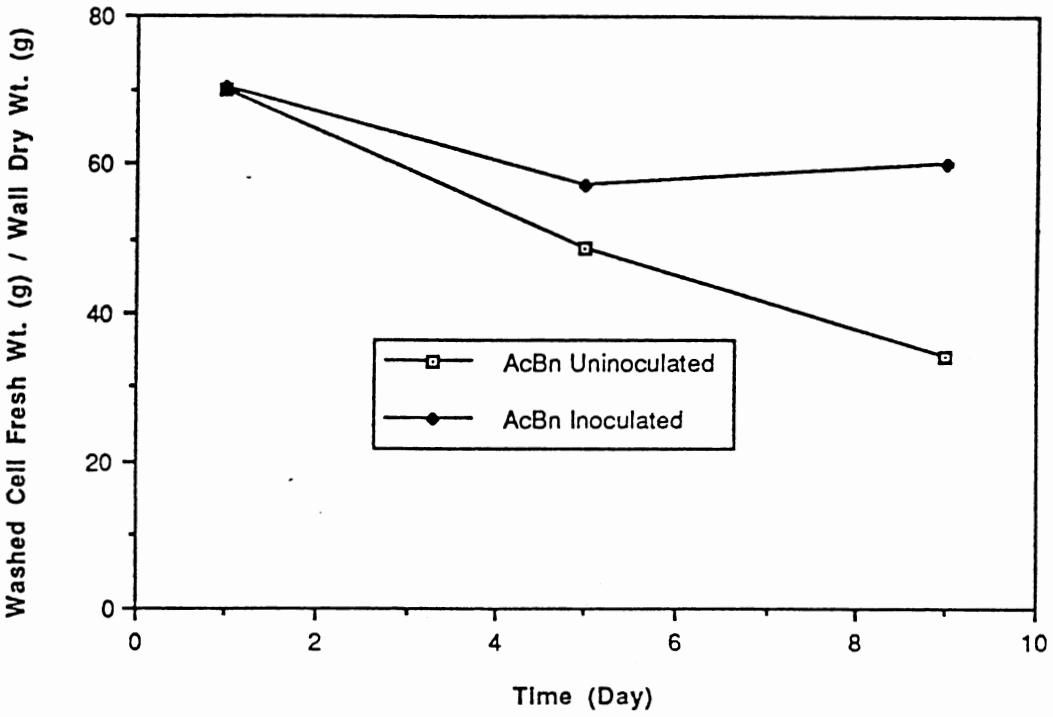


Figure 17. Time course of Washed Suspension Cell Fresh Wt. (g) to Cell Wall Dry Wt. (g) over 9 Days during AcB_N Cell Wall Preparation from Growing Cells. At, 1, 5, 9 d, three flasks of cells were collected. AcB_N suspension cells and cell walls were prepared as described in Figure 16. Data represents 1determination of a single experiment.

uninoculated and inoculated cell lines begins within 5 days after inoculation since the inoculated and uninoculated ratios for each cell line are identical at day 1. This means that either the dry weight recovered for inoculated Ac 44 cell walls is considerably less than that of inoculated AcB_N and Im 216 cell walls or that the washed fresh weight is considerably higher for the inoculated AcB_N and Im 216 cells than the inoculated Ac 44 cells. It appears that washed fresh weight is the major factor affecting the Cell FW / Wall DW ratio after examining Table 3. The washed fresh weight is 3 to 4 times lower for the inoculated Ac 44 cells than the inoculated Im 216 (except for day 10) and AcB_N cells. Thus, it appears that the incompatible interactions differ from the compatible interaction. That is, greater Cell FW / Wall DW ratios are seen in the incompatible interactions, Im 216 and AcB_N, than in the compatible interaction, Ac 44, when compared to their respective control ratios. These observations may have some bearing on resistance to *X. c. malvacearum* and on accumulation of a number of biochemical parameters which will be discussed in this chapter.

Scanning Electron Microscopy of Uninoculated and Inoculated Ac 44 and Im 216 Cell Walls

The superficial ultrastructures of Ac 44 and im 216 cell walls uninoculated and inoculated with the pathogen, *X. c. malvacearum* were examined using scanning electron microscopy (SEM). Figure 18 shows the scanning electron micrographs of 1 day uninoculated and inoculated Ac 44 and Im 216 cell walls.

The inoculated Ac 44 cell walls have a different ultrastructural appearance from that of uninoculated Ac 44 cell walls, as well as from that of uninoculated and inoculated Im 216 cell walls. Even after 1 day, cell walls prepared from inoculated Ac 44 suspension cells are extremely broken and flaky (Figure 19b) while uninoculated Ac 44 and Im 216 suspension cell walls and inoculated Im 216 cell walls are smooth and wavy in appearance, indicating the integrity of the cell walls is maintained (Figures 18a, c, d). Thus, results suggest that the pathogen, *X. c. malvacearum* is responsible for destroying the integrity of the Ac 44 cell walls. Ac 44 is completely susceptible to the pathogen *X. c. malvacearum* and thus would be more likely to be destroyed by cell wall pectolytic and other cell-wall degrading enzymes produced by *X. c. malvacearum*. *X. c. malvacearum* has been reported to produce pectin depolymerizing enzymes [1, 253].

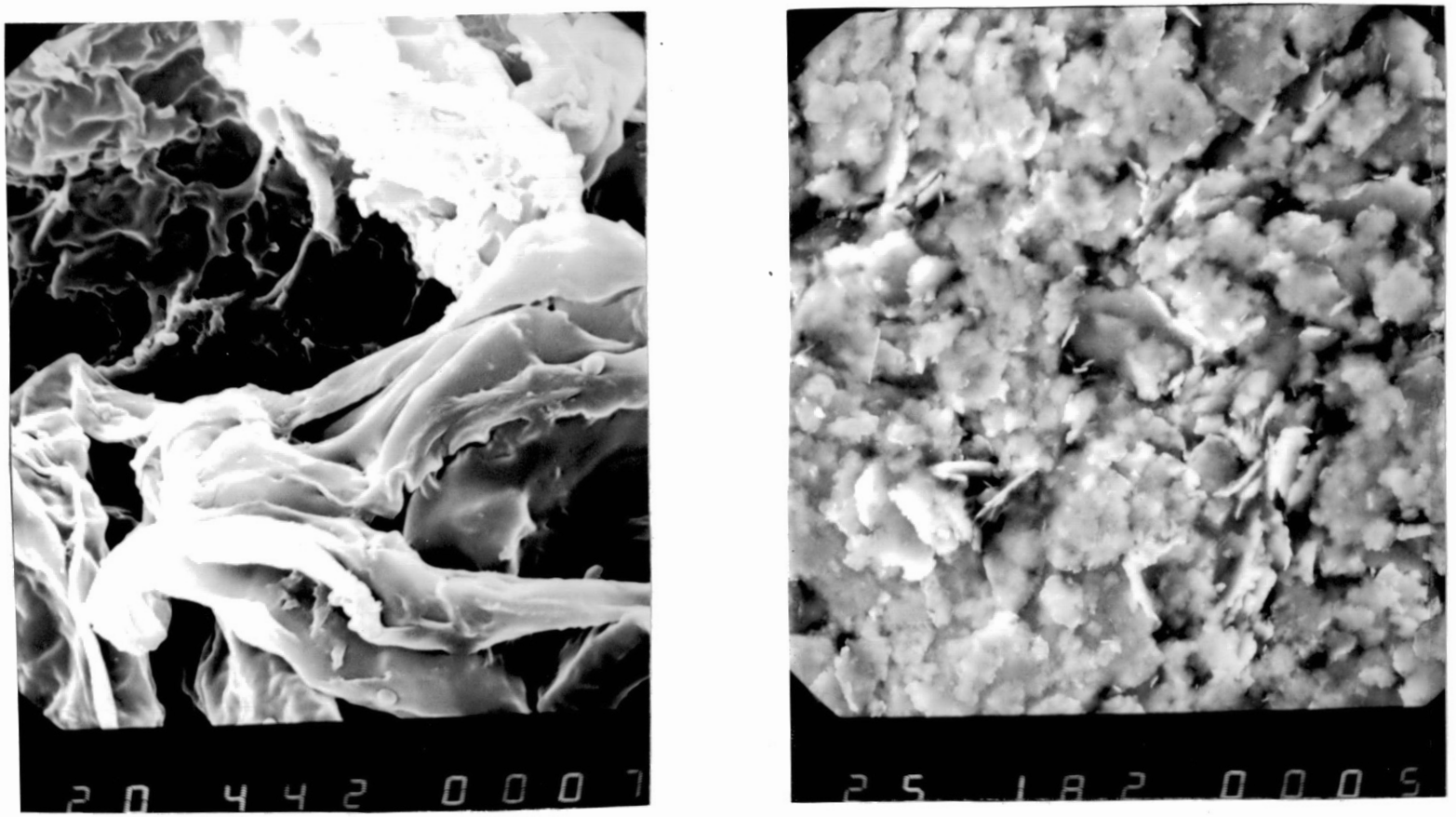


Figure 18a-b. Scanning Electron Microscopy of Uninoculated and Inoculated Ac 44 and Im 216 Cell Walls. Cell walls were prepared from 1 day old suspension cells. Inoculation with *X. c. malvacearum* was at an inoculum density of 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8) as previously described. A. Ac 44 cell walls, 1 d, 4400 X (Left); B. Ac 44 + *X. c. malvacearum* cell walls, 1 d, 1800 X (Right).

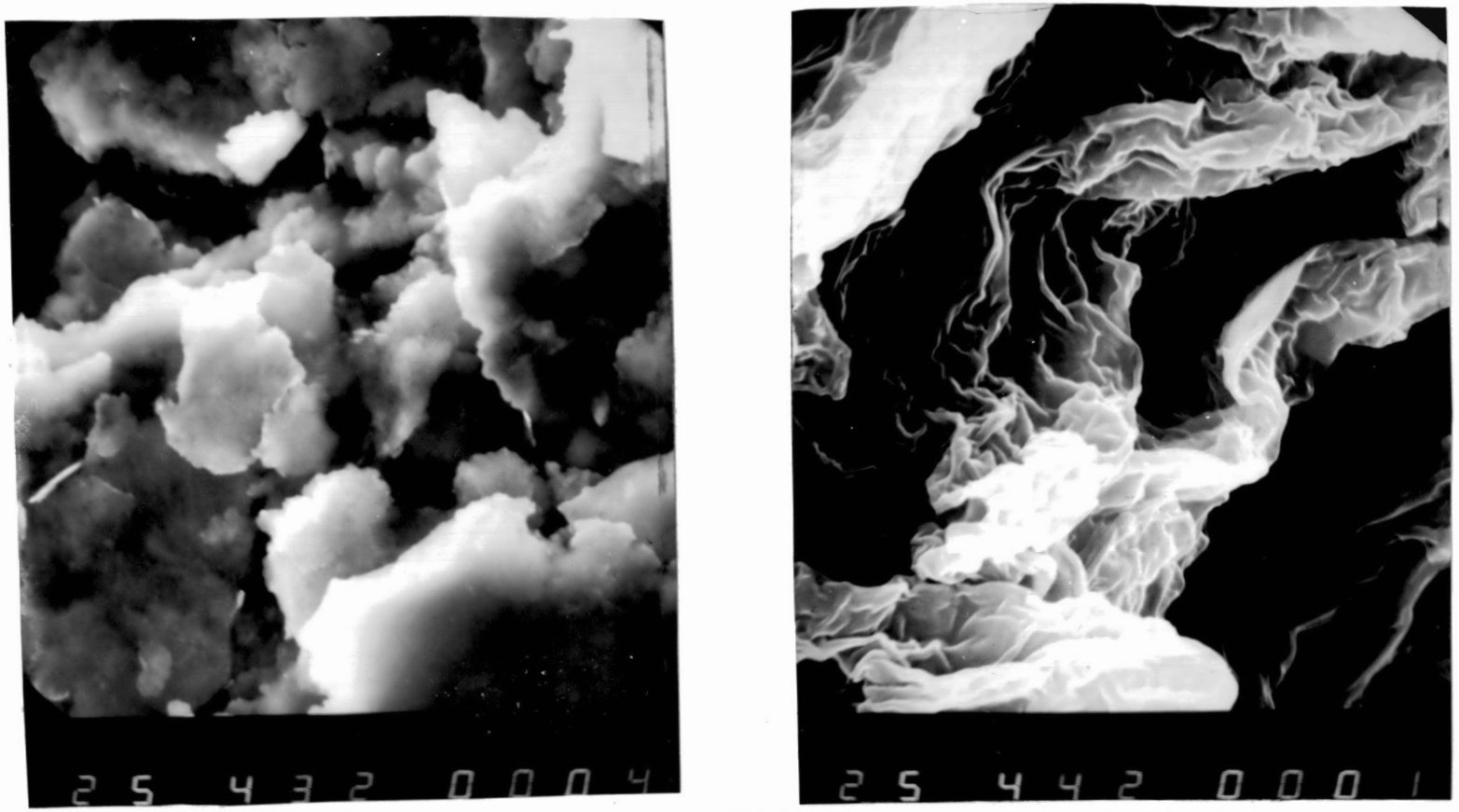


Figure 18c-d. Scanning Electron Microscopy of Uninoculated and Inoculated Ac 44 and Im 216 Cell Walls. Cell walls were prepared from 1 day old suspension cells. Inoculation with *X. c. malvacearum* was at an inoculum density of 1 x 10^7 cfu ml $^{-1}$ in 50 ml SH medium (pH 5.8) as previously described. C. Im 216 cell walls, 1 d, 4300 X (Left); D. Im 216 + *X. c. malvacearum* cell walls, 1 d, 4400 X (Right).

Amino Acid Profiles of Uninoculated and Inoculated Ac 44, AcBN, and Im 216 Cell Walls

The amino acid composition of uninoculated and inoculated Ac 44, AcB_N, and Im 216 cell wall hydrolysates was determined by automated amino acid analysis (see Materials and Methods). Tryptophan and cysteine/cystine were not determined. Amino acids are expressed as total weight percent of the cell walls. Protein is based on the recovery of amino acids as a total weight percent of the cell wall. Data from four different pathogen inoculated time courses are presented in Tables 4-9. Results provide information related to accumulation of specific amino acids in the cell wall with regard to differences in cell type and presence of pathogen.

The amino acids with the highest total weight % in the uninoculated Ac 44, inoculated Im 216, and uninoculated and inoculated AcB_N suspension cell walls were Hyp, Ser, Leu, Val, Lys, Asp, and Glu. In inoculated Ac 44 suspension cell walls all of the amino acids were ≤0.5% (w/w) of the cell wall dry weight. In uninoculated Im 216 suspension cell walls, Hyp was only 0.3-0.4% (w/w) of the cell wall dry weight, but increased dramatically following infection with *X. c. malvacearum* . All other amino acid total weight % (w/w) were generally the same as in the uninoculated Im 216 suspension cell walls. AcB_N cell wall amino acid total weight % (w/w) did not differ significantly in either inoculated or uninoculated cell walls.

Statistical analysis of the amino acid composition and protein content of uninoculated and inoculated Ac 44, AcB_N, and Im 216 cell walls was performed for all four time studies (i.e. replicates). Analysis of variance (split-plot design) indicated that neither the amino acids nor protein varied significantly with time. That is, there was no evidence to support that there was a difference in amino acid or protein accumulation in the total weight % (w/w) of the cell walls of the three cell lines examined. Thus, the least squares means for the amino acid or protein total weight % (w/w) of all time points (1-20 days) for all four time studies, regardless of length of the study, were calculated for each treatment and each cell line. Least squares means attempt to estimate averages as if the data were complete for all reps. Results are shown in Table 10.

All the amino acids and protein least squares means in inoculated Ac 44 cell walls were significantly lower than in the corresponding uninoculated Ac 44 cell walls. However, only the Hyp least squares mean was significantly higher in inoculated Im 216

TABLE 4

AMINO ACID ANALYSIS (TOTAL WEIGHT %) OF AC 44 UNINOCULATED SUSPENSION CELL WALLS

Time	Hyp	Asp*	Thr	Ser	Glu*	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	% Protein
1a	0.45	0.37	0.16	0.25	0.28	0.16	0.17	0.20	0.24	0.03	0.15	0.31	0.13	0.17	0.10	0.32	0.15	3.65
2a	0.57	1.15	0.53	0.66	1.11	0.52	0.53	0.66	0.74	0.15	0.56	1.09	0.40	0.57	0.27	0.89	0.59	10.98
3a	0.48	0.77	0.36	0.48	0.75	0.38	0.38	0.45	0.51	0.10	0.37	0.68	0.25	0.39	0.19	0.64	0.36	7.55
1b	0.73	0.30	0.13	0.19	0.31	0.18	0.17	0.19	0.22	0.02	0.15	0.27	0.17	0.43	0.10	0.30	0.12	3.98
3b	0.67	0.75	0.35	0.39	0.88	0.39	0.38	0.40	0.42	0.08	0.31	0.67	0.21	0.33	0.20	0.58	0.44	7.44
7b	0.32	0.84	0.38	0.39	0.94	0.42	0.36	0.46	0.53	0.07	0.32	0.76	0.08	0.28	0.22	0.65	0.31	7.35
1c	0.34	0.57	0.27	0.38	0.55	0.24	0.28	0.32	0.42	0.05	0.27	0.55	0.23	0.28	0.15	0.47	0.31	5.67
5c	0.82	0.94	0.40	0.49	0.90	0.46	0.43	0.52	0.69	0.16	0.47	0.89	0.34	0.47	0.22	0.75	0.51	9.46
9c	0.51	0.92	0.40	0.48	0.86	0.47	0.43	0.53	0.74	0.13	0.46	0.94	0.30	0.45	0.22	0.72	0.47	9.02
1d	0.74	0.60	0.25	0.43	0.50	0.33	0.27	0.30	0.32	0.08	0.25	0.33	0.17	0.23	0.17	0.53	0.27	5.80
5d	0.57	1.54	0.64	0.87	1.44	0.68	0.66	0.79	0.81	0.25	0.76	0.93	0.40	0.64	0.32	1.13	0.72	13.15
10d	0.63	1.00	0.42	0.64	0.82	0.52	0.40	0.50	0.56	0.15	0.46	0.62	0.29	0.39	0.22	0.78	0.44	8.83
15d	0.52	0.93	0.40	0.60	0.78	0.47	0.38	0.46	0.56	0.07	0.34	0.72	0.24	0.34	0.21	0.71	0.39	8.14
20d	0.68	1.13	0.47	0.73	0.88	0.57	0.46	0.57	0.63	0.15	0.51	0.71	0.35	0.42	0.26	0.87	0.48	9.88

a = Time Study 1 - suspension cultures collected at 1, 2, 3 d
b = Time Study 2 - suspension cultures collected at 1, 3, 7 d
c = Time Study 3 - suspension cultures collected at 1, 5, 9 d
d = Time Study 4 - suspension cultures collected at 1, 5, 10, 15, 20 d
Glu* = Glu + Gln, during acid hydrolysis the Gln is converted to Glu
Asp* = Asp + Asn, during acid hydrolysis the Asn is converted to Asp
Note: Cys and Trp were not determined.

TABLE 5

AMINO ACID ANALYSIS (TOTAL WEIGHT %) OF AC 44 INOCULATED SUSPENSION CELL WALLS

Time	Hyp	Asp*	Thr	Ser	Glu*	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	% Protein
1a	0.34	0.42	0.19	0.28	0.37	0.21	0.22	0.24	0.28	0.04	0.19	0.36	0.16	0.21	0.12	0.35	0.20	4.16
2a	0.44	0.66	0.30	0.41	0.63	0.31	0.32	0.38	0.43	0.09	0.32	0.59	0.26	0.33	0.18	0.55	0.34	6.53
3a	0.36	0.20	0.08	0.18	0.17	0.09	0.13	0.09	0.11	0.01	0.07	0.12	0.09	0.07	0.07	0.19	0.09	2.11
1b	0.55	0.21	0.15	0.15	0.39	0.19	0.18	0.21	0.06	0.04	0.15	0.29	0.10	0.15	0.12	0.27	0.16	3.36
3b	0.72	0.24	0.11	0.15	0.26	0.14	0.14	0.08	0.10	0.02	0.06	0.12	0.08	0.11	0.09	0.25	0.14	2.80
7b	0.28	0.15	0.07	0.08	0.16	0.08	0.08	0.16	0.17	0.03	0.10	0.20	0.05	0.06	0.05	0.15	0.15	2.03
1c	0.50	0.47	0.23	0.33	0.45	0.24	0.26	0.28	0.24	0.07	0.22	0.41	0.21	0.25	0.13	0.40	0.26	4.93
5c	0.39	0.72	0.32	0.44	0.72	0.31	0.39	0.42	0.45	0.11	0.33	0.61	0.29	0.37	0.18	0.54	0.41	7.01
6c	0.37	0.45	0.21	0.30	0.43	0.20	0.24	0.27	0.29	0.05	0.22	0.41	0.20	0.22	0.13	0.39	0.26	4.63
9c	0.34	0.55	0.27	0.37	0.53	0.24	0.29	0.33	0.41	0.05	0.27	0.52	0.20	0.28	0.16	0.46	0.26	5.52
1d	0.61	0.73	0.32	0.48	0.63	0.37	0.33	0.37	0.40	0.11	0.34	0.43	0.21	0.30	0.17	0.60	0.33	6.73
5d	0.15	0.39	0.17	0.22	0.40	0.19	0.19	0.21	0.23	0.04	0.16	0.29	0.12	0.18	0.09	0.29	0.21	3.54
10d	0.30	0.38	0.18	0.27	0.36	0.21	0.20	0.22	0.25	0.01	0.17	0.29	0.09	0.17	0.10	0.36	0.17	3.73
15d	0.38	0.34	0.15	0.24	0.33	0.20	0.17	0.19	0.21	0.04	0.13	0.25	0.10	0.15	0.09	0.30	0.16	3.42
20d	0.24	0.25	0.13	0.12	0.23	0.15	0.12	0.14	0.14	0.03	0.08	0.14	0.09	0.11	0.08	0.24	0.11	2.38

a = Time Study 1 - suspension cultures collected at 1, 2, 3 d
b = Time Study 2 - suspension cultures collected at 1, 3, 7 d
c = Time Study 3 - suspension cultures collected at 1, 5, 6, 9 d
d = Time Study 4 - suspension cultures collected at 1, 5, 10, 15, 20 d
Glu* = Glu + Gln, during acid hydrolysis the Gln is converted to Glu
Asp* = Asp + Asn, during acid hydrolysis the Asn is converted to Asp
Note: Cys and Trp were not determined.

TABLE 6

AMINO ACID ANALYSIS (TOTAL WEIGHT %) OF IM 216 UNINOCULATED SUSPENSION CELL WALLS

Time	Hyp	Asp*	Thr	Ser	Glu*	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	% Protein
1a	0.45	1.50	0.69	0.89	1.45	0.73	0.72	0.82	0.92	0.19	0.66	1.30	0.42	0.69	0.35	1.13	0.76	13.67
2a	0.12	0.41	0.17	0.22	0.37	0.18	0.19	0.21	0.25	0.04	0.17	0.35	0.10	0.18	0.09	0.30	0.20	3.55
3a	0.57	1.11	0.49	0.65	0.98	0.53	0.51	0.58	0.67	0.13	0.46	0.94	0.33	0.47	0.24	0.76	0.55	9.97
1b	0.35	0.78	0.36	0.45	0.75	0.36	0.37	0.43	0.52	0.06	0.41	0.71	0.18	0.31	0.18	0.52	0.43	7.15
3b	0.45	1.00	0.53	0.63	1.04	0.46	0.46	0.53	0.59	0.14	0.46	0.85	0.32	0.47	0.23	0.76	0.62	9.52
7b	0.38	0.85	0.47	0.59	0.85	0.45	0.43	0.51	0.59	0.10	0.40	0.93	0.27	0.40	0.22	0.67	0.54	8.66
1c	0.35	1.27	0.55	0.70	1.24	0.55	0.58	0.68	0.87	0.17	0.57	1.19	0.45	0.59	0.29	0.92	0.66	11.63
5c	0.19	0.76	0.33	0.41	0.79	0.33	0.34	0.41	0.55	0.10	0.36	0.70	0.28	0.37	0.19	0.62	0.47	7.20
9c	0.20	0.74	0.31	0.39	0.75	0.32	0.32	0.40	0.53	0.09	0.34	0.69	0.27	0.35	0.18	0.60	0.43	6.93
1d	0.38	1.25	0.52	0.65	1.05	0.47	0.53	0.62	0.70	0.17	0.49	1.02	0.31	0.52	0.27	0.79	0.56	10.29
5d	0.27	1.05	0.42	0.49	1.05	0.53	0.43	0.53	0.65	0.17	0.45	0.94	0.28	0.50	0.22	0.70	0.51	9.20
10d	0.43	1.46	0.61	0.78	1.30	0.63	0.58	0.71	0.83	0.22	0.57	1.23	0.42	0.61	0.37	0.96	0.79	12.45
15d	0.44	1.66	0.68	0.83	1.43	0.71	0.66	0.80	0.91	0.26	0.69	1.44	0.45	0.73	0.33	1.04	0.81	9.25
20d	0.24	0.39	0.16	0.21	0.36	0.19	0.17	0.21	0.25	0.05	0.16	0.35	0.10	0.17	0.09	0.27	0.18	3.55

a = Time Study 1 - suspension cultures collected at 1, 2, 3 d
b = Time Study 2 - suspension cultures collected at 1, 3, 7 d
c = Time Study 3 - suspension cultures collected at 1, 5, 9 d
d = Time Study 4 - suspension cultures collected at 1, 5, 10, 15, 20 d
Glu* = Glu + Gln, during acid hydrolysis the Gln is converted to Glu
Asp* = Asp + Asn, during acid hydrolysis the Asn is converted to Asp
Note: Cys and Trp were not determined.

TABLE 7

AMINO ACID ANALYSIS (TOTAL WEIGHT %) OF IM 216 INOCULATED SUSPENSION CELL WALLS

Time	Hyp	Asp*	Thr	Ser	Glu*	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	% Protein
1a	1.00	1.50	0.64	0.83	1.28	0.67	0.64	0.75	0.79	0.21	0.60	1.25	0.39	0.69	0.33	0.96	0.64	3.86
2a	0.49	1.20	0.55	0.68	1.11	0.59	0.57	0.65	0.73	0.17	0.52	1.03	0.38	0.56	0.28	0.91	0.63	11.03
3a	0.50	0.85	0.37	0.53	0.74	0.42	0.39	0.44	0.52	0.11	0.34	0.72	0.26	0.35	0.20	0.58	0.41	7.75
1b	0.40	0.73	0.34	0.44	0.73	0.37	0.35	0.40	0.48	0.04	0.32	0.60	0.19	0.29	0.17	0.50	0.40	6.77
3b	0.40	0.89	0.42	0.52	0.90	0.44	0.45	0.49	0.57	0.07	0.47	0.77	0.28	0.40	0.20	0.59	0.54	8.40
7b	0.45	0.58	0.28	0.37	0.56	0.29	0.28	0.32	0.37	0.04	0.24	0.45	0.11	0.21	0.12	0.43	0.27	5.37
1c	0.38	1.00	0.43	0.58	0.99	0.47	0.47	0.54	0.61	0.17	0.45	0.91	0.34	0.47	0.23	0.69	0.57	9.30
5c	0.46	0.70	0.33	0.51	0.72	0.35	0.38	0.41	0.45	0.12	0.33	0.61	0.28	0.35	0.19	0.59	0.45	7.26
6c	0.42	0.89	0.43	0.54	0.90	0.44	0.45	0.51	0.56	0.14	0.39	0.76	0.30	0.44	0.22	0.62	0.50	8.51
9c	0.40	0.65	0.31	0.43	0.65	0.32	0.34	0.37	0.31	0.10	0.28	0.55	0.25	0.33	0.16	0.47	0.36	6.27
1d	1.27	1.90	0.81	1.05	1.62	0.85	0.81	0.95	1.01	0.27	0.76	1.58	0.49	0.88	0.42	1.22	0.81	13.17
5d	1.00	1.19	0.49	0.69	0.93	0.48	0.56	0.65	0.70	0.18	0.50	1.07	0.29	0.57	0.24	0.74	0.47	10.74
10d	1.22	0.89	0.39	0.51	0.69	0.37	0.47	0.51	0.60	0.16	0.37	0.81	0.31	0.46	0.21	0.67	0.40	9.04
15d	1.15	1.20	0.52	0.70	0.24	0.21	0.64	0.69	0.75	0.21	0.53	1.08	0.44	0.63	0.25	0.72	0.57	10.52
20d	0.93	0.78	0.39	0.75	0.82	0.43	0.52	0.50	0.47	0.05	0.21	0.36	0.12	0.19	0.12	0.57	0.33	7.54

a = Time Study 1 - suspension cultures collected at 1, 2, 3 d
b = Time Study 2 - suspension cultures collected at 1, 3, 7 d
c = Time Study 3 - suspension cultures collected at 1, 5, 6, 9 d
d = Time Study 4 - suspension cultures collected at 1, 5, 10, 15, 20 d
Glu* = Glu + Gln, during acid hydrolysis the Gln is converted to Glu
Asp* = Asp + Asn, during acid hydrolysis the Asn is converted to Asp
Note: Cys and Trp were not determined.

TABLE 8

AMINO ACID ANALYSIS (TOTAL WEIGHT %) OF ACBN UNINOCULATED SUSPENSION CELL WALLS

Time	Hyp	Asp*	Thr	Ser	Glu*	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	% Protein
1b	0.40	1.13	0.47	0.64	1.11	0.58	0.55	0.60	0.54	0.12	0.42	0.88	0.24	0.39	0.21	0.79	0.49	9.58
3b	0.38	1.09	0.45	0.61	1.06	0.50	0.50	0.56	0.56	0.63	0.18	0.43	0.93	0.22	0.47	0.81	0.46	9.52
7b	0.50	1.35	0.57	0.79	1.28	0.66	0.67	0.72	0.77	0.14	0.49	1.07	0.34	0.51	0.27	0.96	0.65	11.74
1c	0.61	1.10	0.49	0.55	0.99	0.71	0.59	0.57	0.73	0.12	0.48	0.91	0.32	0.50	0.25	0.72	0.54	10.19
5c	0.33	1.03	0.44	0.50	1.04	0.58	0.54	0.57	0.67	0.13	0.47	0.87	0.24	0.50	0.25	0.75	0.46	9.36
9c	0.35	0.93	0.41	0.46	0.95	0.54	0.47	0.53	0.71	0.12	0.45	0.84	0.26	0.43	0.23	0.72	0.49	8.88

b = Time Study 2 - suspension cultures collected at 1, 3, 7 d
c = Time Study 3 - suspension cultures collected at 1, 5, 9 d
Glu* = Glu + Gln, during acid hydrolysis the Gln is converted to Glu
Asp* = Asp + Asn, during acid hydrolysis the Asn is converted to Asp
Note: Cys and Tryp were not determined.

TABLE 9

AMINO ACID ANALYSIS (TOTAL WEIGHT %) OF ACBN INOCULATED SUSPENSION CELL WALLS

Time	Hyp	Asp*	Thr	Ser	Glu*	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	% Protein
1b	0.51	1.00	0.43	0.57	0.93	0.54	0.48	0.52	0.61	0.16	0.42	0.85	0.28	0.44	0.78	0.75	0.46	9.73
3b	0.43	1.04	0.44	0.58	0.98	0.58	0.51	0.55	0.61	0.10	0.38	0.77	0.29	0.44	0.21	0.70	0.48	9.11
7b	0.33	0.74	0.32	0.42	0.74	0.45	0.35	0.39	0.53	0.12	0.29	0.55	0.19	0.30	0.19	0.60	0.37	6.89
1c	0.45	1.46	0.64	0.71	1.49	0.81	0.76	0.81	1.07	0.19	0.70	1.32	0.45	0.75	0.37	1.07	0.82	13.88
6c	0.59	1.18	0.53	0.60	1.17	0.74	0.63	0.65	0.89	0.16	0.56	1.05	0.39	0.56	0.30	0.97	0.63	11.60
9c	0.50	0.97	0.43	0.51	0.98	0.60	0.54	0.55	0.63	0.14	0.46	0.86	0.32	0.46	0.25	0.79	0.53	9.51

b = Time Study 2 - suspension cultures collected at 1, 3, 7 d
c = Time Study 3 - suspension cultures collected at 1, 6, 9 d
Glu* = Glu + Gln, during acid hydrolysis the Gln is converted to Glu
Asp* = Asp + Asn, during acid hydrolysis the Asn is converted to Asp
Note: Cys and Tryp were not determined.

TABLE 10

LEAST SQUARES MEANS ANALYSIS OF AMINO ACIDS AND PROTEIN

AMINO ACID	PARAMETER	AC 44	ACBN	IM 216
HYP	Uninoculated	0.68 a	0.48 a	0.40 a
	Inoculated	0.48 b	0.57 a	0.69 b
	Difference	0.20 c	-0.09 c,d	-0.29 d
	Average	0.57 e	0.53 e	0.55 e
ASP	Uninoculated	0.94 a	1.24 a	1.25 a
	Inoculated	0.51 b	1.29 a	1.00 a
	Difference	0.43 c	-0.05 d	0.25 c,d
	Average	0.73 e	1.26 f	1.13 f
THR	Uninoculated	0.40 a	0.51 a	0.54 a
	Inoculated	0.23 b	0.54 a	0.44 a
	Difference	0.17 c	-0.03 c	0.10 c
	Average	0.31 d	0.52 e	0.49 e
SER	Uninoculated	0.56 a	0.68 a	0.68 a
	Inoculated	0.32 b	0.69 a	0.61 a
	Difference	0.24 c	-0.01 c	0.07 c
	Average	0.44 d	0.68 e	0.64 e
GLU	Uninoculated	0.88 a	1.14 a	1.16 a
	Inoculated	0.49 b	1.18 a	0.86 b
	Difference	0.39 c	-0.04 c	0.30 c
	Average	0.68 d	1.16 e	1.01 e
PRO	Uninoculated	0.46 a	0.64 a	0.55 a
	Inoculated	0.26 b	0.71 a	0.44 a
	Difference	0.20 c	-0.07 d	0.11 c,d
	Average	0.36 e	0.67 f	0.50 g
GLY	Uninoculated	0.41 a	0.60 a	0.54 a
	Inoculated	0.26 b	0.63 a	0.49 a
	Difference	0.15 c	-0.03 d	0.05 c,d
	Average	0.34 e	0.62 f	0.51 f

TABLE 10 (CONTINUED)

AMINO ACID	PARAMETER	AC 44	ACBN	IM 216
ALA	Uninoculated	0.50 a	0.65 a	0.64 a
	Inoculated	0.29 b	0.68 a	0.55 a
	Difference	0.21 c	-0.03 c	0.09 c
	Average	0.39 d	0.66 e	0.60 e
VAL	Uninoculated	0.58 a	0.71 a	0.75 a
	Inoculated	0.29 b	0.82 a	0.59 b
	Difference	0.29 c	-0.11 d	0.16 c,d
	Average	0.44 e	0.77 f	0.67 f
MET	Uninoculated	0.12 a	0.15 a	0.16 a
	Inoculated	0.06 b	0.18 a	0.13 a
	Difference	0.06 c	-0.03 c	0.03 c
	Average	0.09 d	0.16 e	0.15 e
ILE	Uninoculated	0.41 a	0.48 a	0.52 a
	Inoculated	0.22 b	0.53 a	0.41 a
	Difference	0.19 c	-0.05 d	0.11 c,d
	Average	0.31 e	0.46 f	0.50 f
LEU	Uninoculated	0.73 a	0.95 a	1.08 a
	Inoculated	0.40 b	1.02 a	0.83 b
	Difference	0.33 c	-0.07 c	0.25 c
	Average	0.56 d	0.98 e	0.95 e
TYR	Uninoculated	0.27 a	0.28 a	0.34 a
	Inoculated	0.16 b	0.35 a	0.28 a
	Difference	0.11 c	-0.07 d	0.06 c,d
	Average	0.22 e	0.32 f	0.31 f
PHE	Uninoculated	0.41 a	0.48 a	0.53 a
	Inoculated	0.22 b	0.56 a	0.44 a
	Difference	0.19 c	-0.08 d	0.09 c,d
	Average	0.32 e	0.52 f	0.49 f
HIS	Uninoculated	0.23 a	0.26 a	0.28 a
	Inoculated	0.14 b	0.40 b	0.22 a
	Difference	0.09 c	-0.14 d	0.06 c,e
	Average	0.19 f	0.33 g	0.25 h

TABLE 10 (CONTINUED)

AMINO ACID	PARAMETER	AC 44	ACBN	IM 216
LYS	Uninoculated	0.76 a	0.88 a	0.86 a
	Inoculated	0.44 b	0.96 a	0.69 a
	Difference	0.32 c	-0.08 d	0.17 c,d
	Average	0.60 e	0.92 f	0.78 f
ARG	Uninoculated	0.44 a	0.53 a	0.64 a
	Inoculated	0.26 b	0.61 a	0.49 b
	Difference	0.18 c	-0.08 d	0.15 c,d
	Average	0.35 e	0.57 f	0.57 f
PROTEIN	Uninoculated	7.66 a	9.89 a	9.04 a
	Inoculated	4.10 b	10.50 a	8.27 a
	Difference	3.56 c	-0.61 d	0.77 d
	Average	5.88 e	10.19 f	8.66 f

Note: The letters a and b denote significant differences (t-test) at the 95% confidence level for the two treatments within a single cell line. The letters c, d, e, f, and g denote significant cell line differences (t-test) among treatment differences or among treatment averages at the 95% confidence level. Least squares means were calculated from the total weight % (w/w) data presented in Tables 4-9, for all four time studies. Each time study = one rep.

cell walls than in uninoculated Im 216 cell walls; while, Glu, Val, Leu, and Arg least square means were significantly lower in inoculated Im 216 cell walls than in uninoculated Im 216 cell walls. Furthermore, except for the Hyp least squares mean, all the least squares means of amino acids in uninoculated Im 216 cell walls were higher than in the inoculated Im 216 cell walls. The uninoculated and inoculated AcB_N cell walls did not have statistically significant differences in either the amino acid or protein least squares means, except for His. Yet, the least squares means of all amino acids in inoculated AcB_N cell walls were slightly higher than in the uninoculated AcB_N cell walls.

The least squares means difference between the uninoculated and inoculated treatments for each cell line were compared for statistical significance (Table 10). Least squares means differences in Ac 44, susceptible cell line, and AcB_N, resistant (one resistance gene) cell line, cell walls were statistically significant at ≥92% confidence level for all the amino acids and protein content. In contrast, the two resistant lines, Im 216 and AcB_N, cell walls were not statistically different for any of the amino acids or protein least square mean differences. Ac 44 and Im 216 cell walls were statistically different for only Hyp and protein least squares mean differences.

The least squares mean averages of the two treatments for each cell line were compared across all three cell lines for statistical significance (Table 10). The least squares mean average for Ac 44 cell walls, independent of choice of amino acid or protein content, was always statistically different from the least squares mean averages of AcB_N and Im 216 cell walls. AcB_N and Im 216 cell walls only differed in average His and Pro.

In conclusion, amino acid composition and protein content were different for the resistant cell lines, AcB_N and Im 216, and the susceptible cell line, Ac 44. Ac 44 cell walls, no resistance genes, and AcB_N cell walls, one resistance gene, had the most extreme differences in the amino acid and protein least squares means while Im 216, three resistance genes, was generally neither statistically different from Ac 44 nor AcB_N cell walls (Table 10) except or Hyp and protein. AcB_N is genetically similar to its parent, Ac 44, but has resistant properties somewhat similar to Im 216, placing its least squares means differences intermediate to that of Ac 44 and Im 216. Im 216 and Ac 44 are genetically very different. In addition, it does not appear, in general, that the presence of the pathogen, *X. c. malvacearum*, statistically affects the amino acid or protein least squares means for the resistant cell lines, AcB_N and Im 216, cell walls, but just the

susceptible line, Ac 44, cell walls. The pathogen greatly depresses the total weight % (w/w) of all the amino acids and protein.

Selecting one representative time course for each of the cotton cell types (i.e. 1 rep) simplifies the visual examination of the statistical trends related to the effect of the cell types and presence of pathogen on the total weight percent of each amino acid. Amino acid analysis (total weight %) of cell walls prepared from inoculated and uninoculated Ac 44 and Im 216 suspension cells harvested 1, 5, 10, 15, and 20 days after culture initiation (Uninoculated) or inoculation with the pathogen (Treated) is presented in Figures 19-22 and amino acid analyses from inoculated and uninoculated AcB_N suspension cell harvested, 1, 6, 9 days after inoculation (Treated) or 1, 5, 9 days after culture initiation (Uninoculated) is shown in Figure 23.

Figures 19 and 20 represent the amino acid analysis (total weight %) of uninoculated and inoculated Ac 44 cell walls. Following day 1, all the amino acids are dramatically lower (~40-50 %) including Hyp in the inoculated Ac 44 cell walls than in the corresponding uninoculated Ac 44 cell walls. This would imply that the presence of the pathogen does make a difference in the accumulation of amino acids in the susceptible interaction. This analysis is in agreement with the least squares means analysis done on all four time courses (Table 10). Additionally, in the uninoculated Ac 44 cell wall all the amino acids markedly increased after day 1 except for Hyp, but remained relatively constant from day 5 to day 20.

Figures 21 and 22 represent the amino acid analysis (total weight %) of uninoculated and inoculated Im 216 cell walls (dry weight basis). In contrast to inoculated Ac 44 cell wall hydrolysates, inoculated Im 216 cell wall hydrolysates dramatically increased by >50% in Hyp% over the corresponding uninoculated Im 216 cell wall hydrolysates. In addition, none of the other amino acids (total weight %) changed significantly during infection. In fact, the least squares means analysis (Table 10) showed that all amino acids except for Hyp were lower in the inoculated Im 216 cell walls than in the uninoculated Im 216 cell walls. It does not appear that either the uninoculated Ac 44 or uninoculated Im 216 cell wall hydrolysates have significantly different overall amino acid profiles except for Hyp. Hyp levels are ~50% lower in the uninoculated Im 216 cell wall than in the uninoculated Ac 44 cell walls. Yet, it seems that day 20 amino acid (total weight %) values for the uninoculated Im 216 cell walls are ~50% lower than the corresponding values for the uninoculated Ac 44 cell walls.

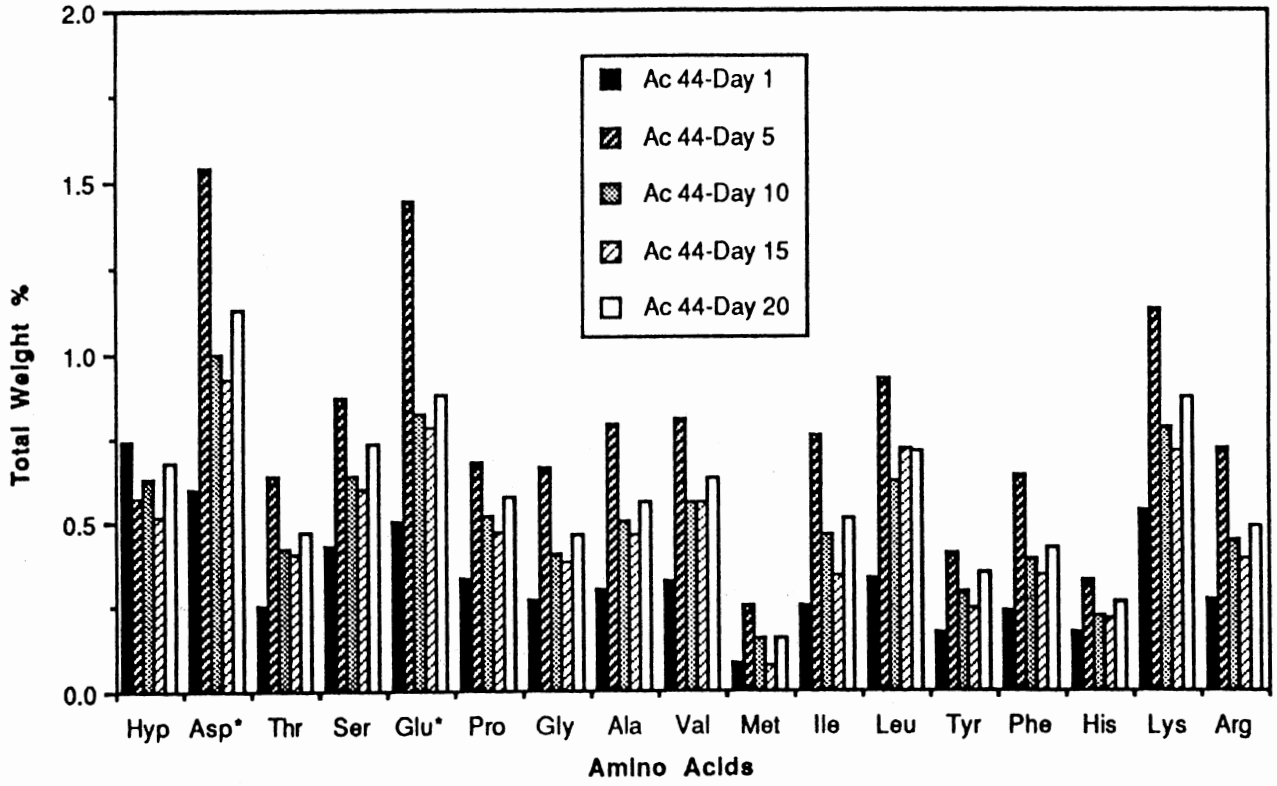


Figure 19. Bar Graph of the Total Weight % of Amino Acids in Uninoculated Ac 44 1, 5, 10, 15, and 20 d Suspension Cell Walls. Cell wall hydrolysates were analyzed for amino acids and converted to total weight % of the cell walls. Asp* and Glu* represent Asp+ Asn and Glu + Gln, repectively. Data is from Table 4 and represents 1 determination of a single experiment.

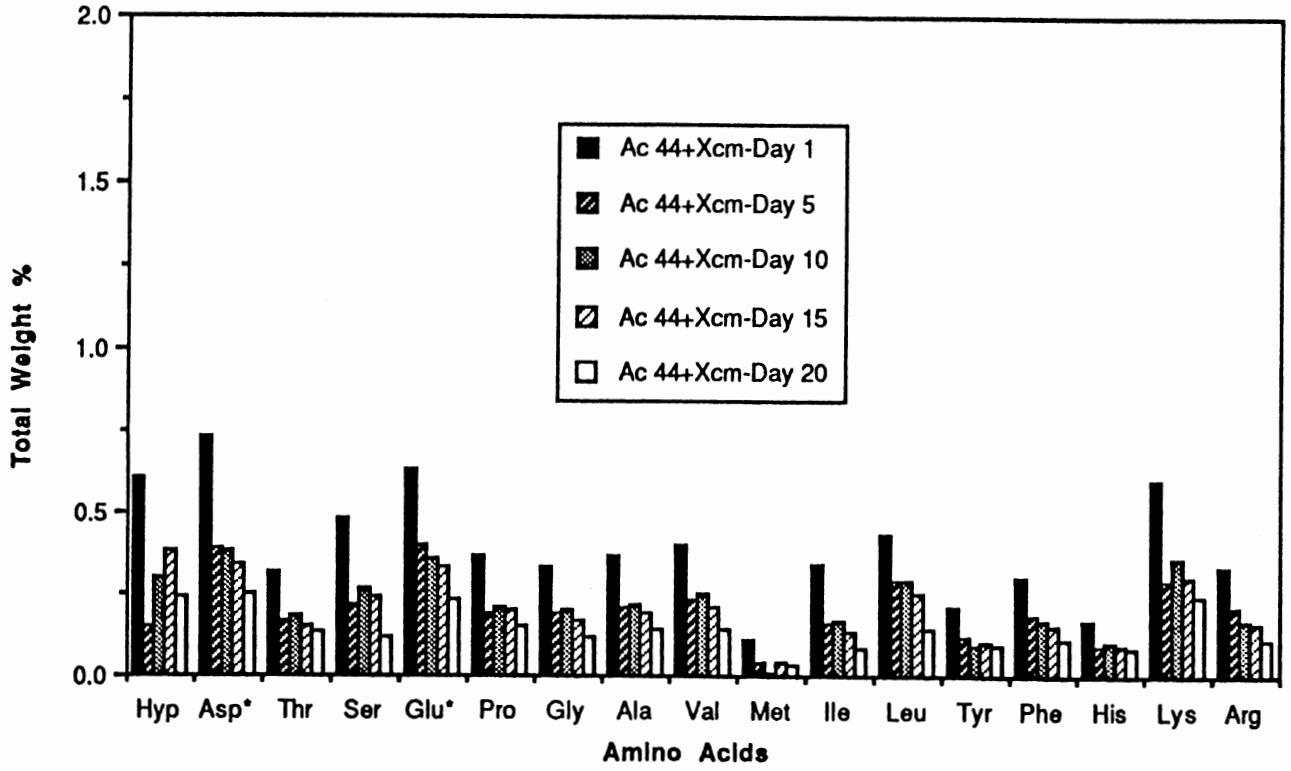


Figure 20. Bar Graph of the Total Weight % of Amino Acids in Inoculated Ac 44 1, 5, 10, 15, and 20 d Suspension Cell Walls. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Cell wall hydrolysates were analyzed for amino acids and converted to total weight % of the cell walls. Asp* and Glu* represent Asp+ Asn and Glu + Gln, repectively. Data is from Table 5 and represents 1 determination of a single experiment.

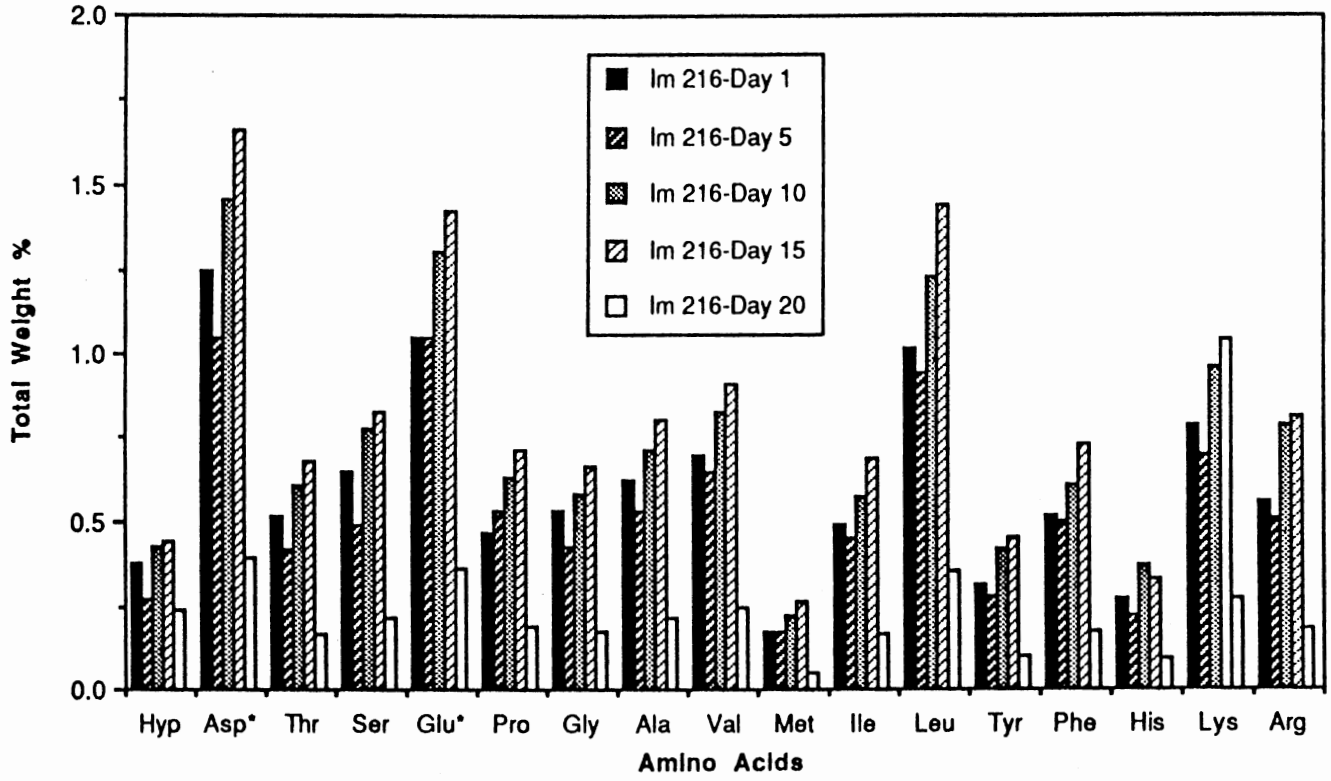


Figure 21. Bar Graph of the Total Weight % of Amino Acids in Uninoculated Im 216 1, 5, 10, 15, and 20 d Suspension Cell Walls. Cell wall hydrolysates were analyzed for amino acids and converted to total weight % of the cell walls. Asp* and Glu* represent Asp+ Asn and Glu + Gln, repectively. Data is from Table 6 and represents 1 determination of a single experiment.

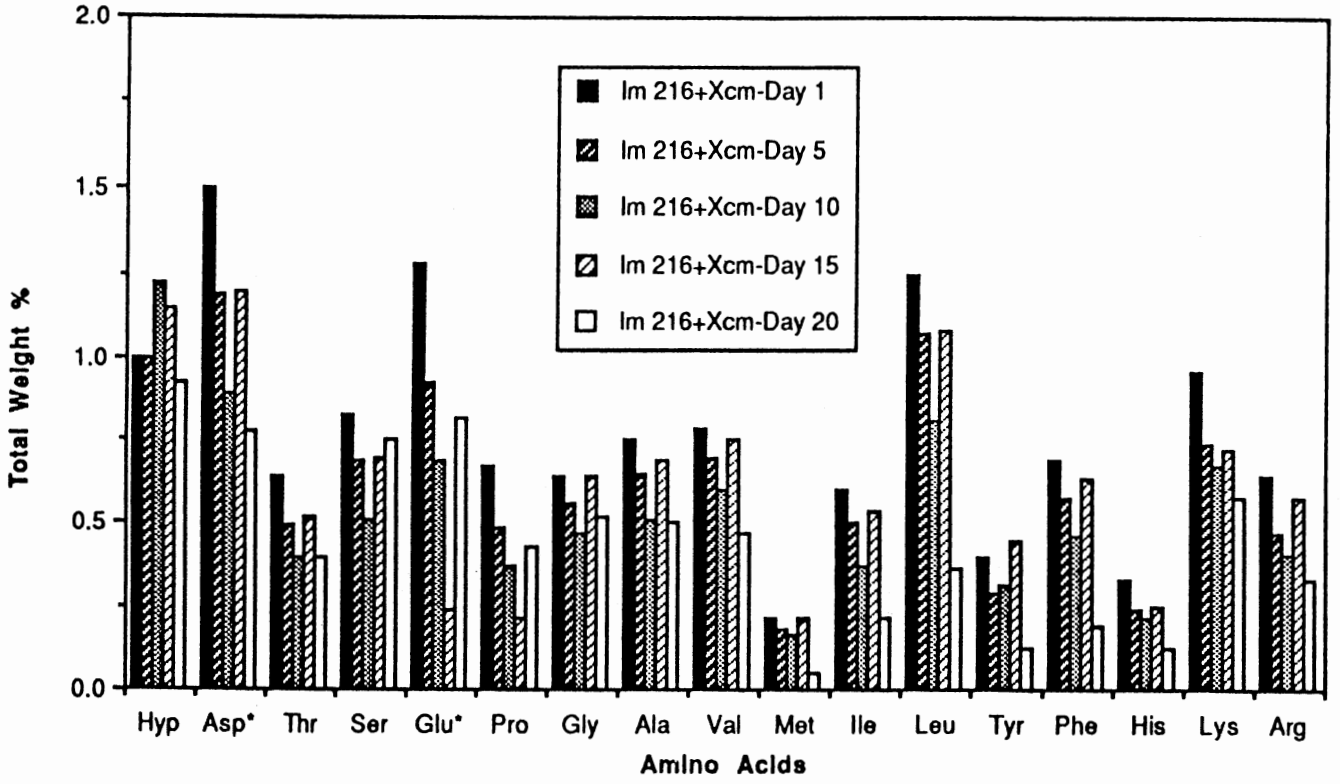


Figure 22. Bar Graph of the Total Weight % of Amino Acids in Inoculated Im 216 1, 5, 10, 15, and 20 d Suspension Cell Walls. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Cell wall hydrolysates were analyzed for amino acids and converted to total weight % of the cell walls. Asp* and Glu* represent Asp+ Asn and Glu + Gln, repectively. Data is from Table 7 and represents 1 determination of a single experiment.

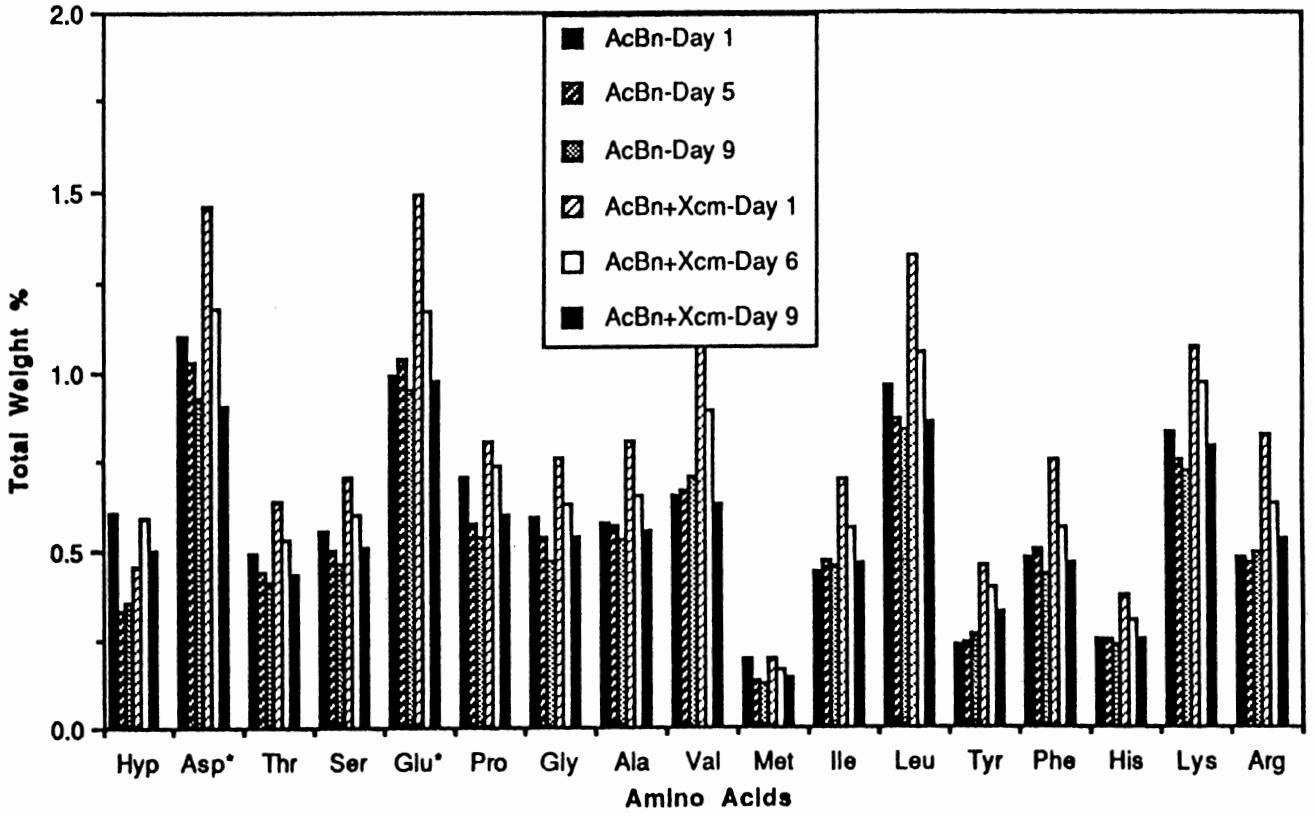


Figure 23. Bar Graph of the Total Weight % of Amino Acids in Uninoculated 1, 5, 9 d and Inoculated 1, 6, 9 d AcB_N Suspension Cell Walls. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Cell wall hydrolysates were analyzed for amino acids and converted to total weight % of the cell walls. Asp* and Glu* represent Asp+ Asn and Glu + Gln, repectively. Data is from Tables 8 and 9 and represents 1 determination of a single experiment.

Thus, the pathogen, *X. c. malvacearum* affects Ac 44 and Im 216 amino acid accumulation. In the compatible interaction, Ac 44, the Hyp level and the other 16 amino acids are depressed by 50% compared to the control values; whereas, in the incompatible interaction, the Hyp level is increased by ~50% and all the other 16 amino acids remained relatively unchanged. The AcB_N inoculated and uninoculated cell wall hydrolysates do not differ in their amino acid profiles except that day 1 inoculated AcB_N cell wall hydrolysate amino acid (total weight %) values are about 0.30% higher than the other days studied. Hyp levels are slightly higher in the inoculated AcB_N cell wall hydrolysates but the difference is not dramatic. These results, like those for Ac 44 and Im 216 cell walls, were in good agreement with the least squares means analysis of AcB_N cell walls (Table 10).

Changes in Hydroxyproline Levels in Uninoculated and Inoculated Ac 44, AcB_N, and Im 216 Suspension Cell Walls. Time course accumulation of Hyp in response to infection by *X. c. malvacearum* in Ac 44, AcB_N, and Im 216 suspension cells is shown in Figures 24 and 25. Data is part of the amino acid profiles presented in Figures 19-23. Special attention is given to the accumulation of Hyp because it has been associated with the time course accumulation of cell wall HRGP [71]. Furthermore, Hyp/HRGP accumulation has been correlated with disease resistance in many different host-parasite systems [71, 100, 164].

One representative time study, as previously described, is presented. In uninoculated Ac 44 suspension cultures, the Hyp level varies very little over the course of 20 days (Figure 24). There is a net decrease in Hyp% from day 1 to day 5 from 0.74% (w/w) to 0.57% (w/w), respectively. This net drop in Hyp % is reversed by day 10 since the Hyp% is 0.63% (w/w) and by day 20 the Hyp% is 0.68% (w/w), nearly the same as day 1. The average Hyp% (averaged over 20 days) for the uninoculated Ac 44 suspension cell walls is 0.63 ± 0.08% (w/w) of the cell wall dry weight. In the inoculated Ac 44 suspension cell walls, the level of Hyp drops dramatically from 0.61% (w/w) at day 1 to 0.15% (w/w) at day 5, a net decrease of 75%. This Hyp level increases ~50% at day 10 to 0.30% (w/w), but is still 50% below the starting value and is maintained at approximately this Hyp level through day 20. The average inoculated Ac 44 Hyp% (averaged over 20 days) is 0.34 ± 0.16% (w/w) of the cell wall dry weight. While the uninoculated Ac 44 cell wall Hyp% changes very little, the inoculated Hyp% decreases markedly and does not every significantly increase after the initial 75% drop. In other

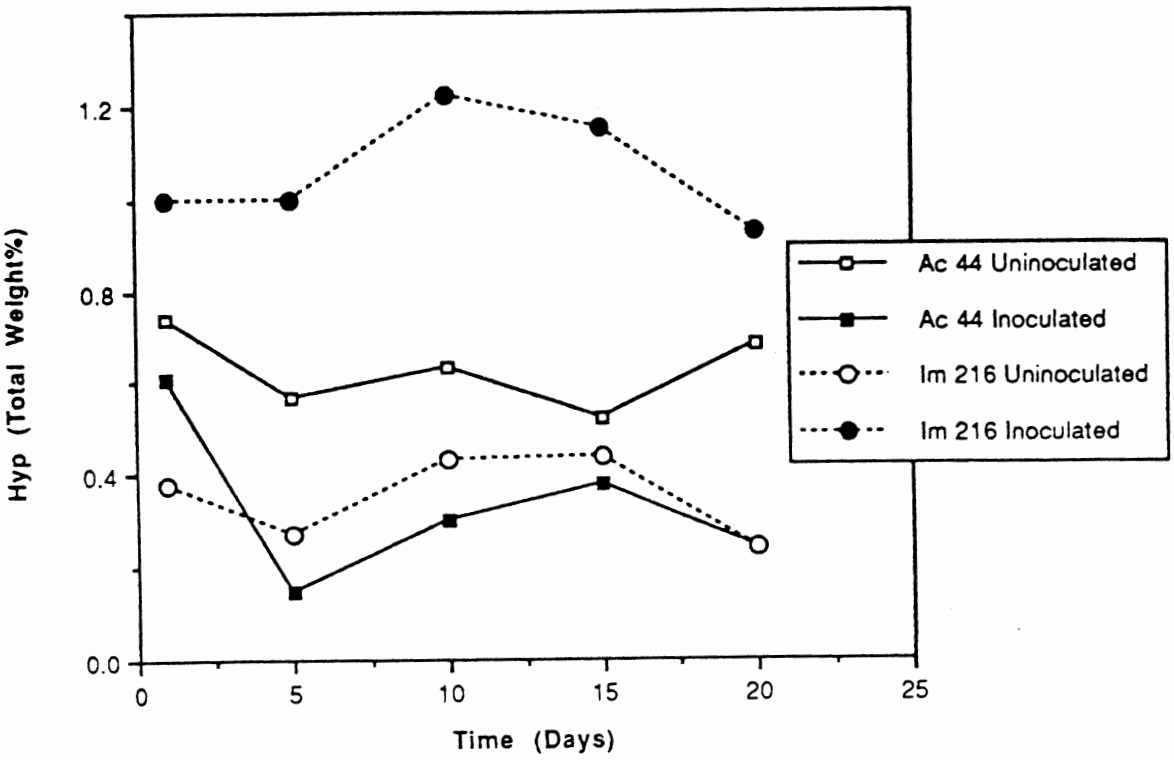


Figure 24. Time Course Accumulation of Hydroxyproline (Total Weight %) in Response to Infection by *X. c. malvacearum* in Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Hyp was determined from amino acid analysis of cell wall hydrolysates. Hyp % represents the Hyp calculated as total weight % of the cell walls. Data is from Figures 19-22 and represents 1 determination of a single experiment.

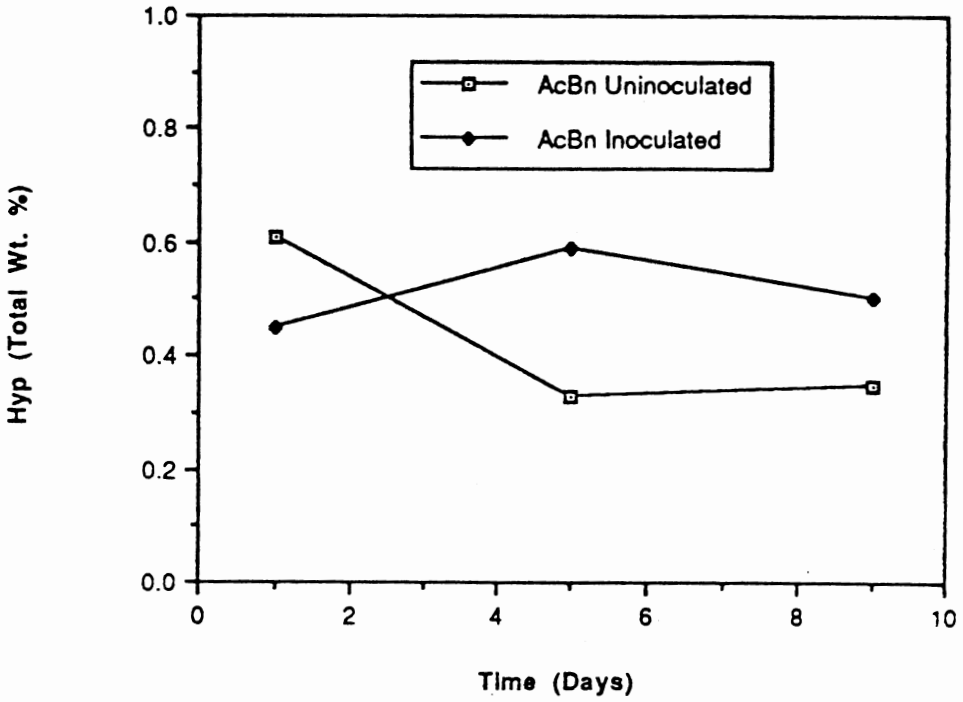


Figure 25. Time Course Accumulation of Hydroxyproline (Total Weight %) in Response to Infection by *X. c. malvacearum* in AcB_N Suspension Cell Walls. Cell walls were prepared from 1, 5, 9 d old suspension cultures as previously described. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Hyp was determined from amino acid analysis of cell wall hydrolysates. Hyp % represents the Hyp calculated as total weight % of the cell walls. Data is from Figure 23 and represents 1 determination of a single experiment.

words, the average Hyp% for the uninoculated Ac 44 cell walls is always 1.8-fold higher than in the inoculated Ac 44 cell walls. In fact, the least squares means of uninoculated and inoculated Ac 44 cell wall Hyp (total weight%, w/w)were statistically different for all four time studies (Table 10). Thus, the presence of the pathogen, *X. c. malvacearum* dramatically affects the Hyp content in the cell wall, by immediately lowering it at day 5 after infection and keeping the Hyp% at ~50% below the starting value.

In uninoculated Im 216 cell walls, the Hyp% does not change dramatically over the course of 20 days (Figure 24), the average Hyp% (averaged over 20 days) is 0.35 ± 0.08% (w/w) of the cell wall dry weight. However, the Hyp level does drop overall from its starting value of 0.38% (w/w) at day 1 to 0.27% (w/w) at day 5, a net decrease of 29% or a 1.4-fold total drop, but increases to 0.24% (w/w) by day 20. Yet, in inoculated Im 216 cell walls (see Figure 25), the Hyp% values are all significantly higher than their respective uninoculated counterparts. That is, the average inoculated Hyp% (averaged over 20 days) is 1.11 ± 0.13% (w/w) which is 68.5% higher than the average uninoculated Hyp% (0.35 ± 0.0.08% (w/w)) in Im 216 cell walls. In other words, there is an average 3.2-fold difference between the average uninoculated Hyp% values and the average inoculated Hyp% values in Im 216 cell walls. Again, just as in the inoculated Ac 44 cell walls, the presence of *X. c. malvacearum* dramatically affects the Hyp content in the cell walls. The Hyp levels in inoculated Im 216 cell walls are significantly higher than in the uninoculated Im 216 cell walls. This difference was substantiated by the statistical analysis of the Hyp least squares means for the inoculated and uninoculated Im 216 cell walls (Table 10). This observation is inverse to that seen in uninoculated and inoculated Ac 44 cell walls, i.e., Hyp% (w/w) is lower in inoculated cell walls than in uninoculated cell walls.

Results for uninoculated and inoculated AcB_N cell walls (Figure 25) were the same as for Im 216 cell walls. That is, the Hyp level initially increased 31% or 1.4-fold in inoculated AcB_N cell walls by day 5 (0.65% (w/w)) over the level at day 1 (0.45% (w/w)) while the Hyp level initially decreased 54% or 1.8-fold in the uninoculated AcB_N cell walls from 0.61% (w/w) at day 1 to 0.33% (w/w) at day 5. The average Hyp% (averaged over 20 days) in uninoculated AcB_N cell walls is 0.43 ± 0.13% (w/w) while the average Hyp% in inoculated AcB_N cell walls is 0.51 ±0.06% (w/w) of the cell wall dry weight. However, the least squares means of uninoculated and inoculated AcB_N cell wall Hyp (total weight %, w/w) were not statistically different for all four time studies (Table 10).

From Figures 24 and 25, and the statistical analyses of all four time studies (Table 10), it is thus possible to conclude the following: the susceptible line, Ac 44 responds in an opposite direction to the pathogen, *X. c. malvacearum* than do the resistant lines, Im 216 and AcB_N. That is, there is a significant linkage between cell type and pathogen to the Hyp level. It is important to glean from these results that the Hyp% (w/w) in inoculated treatments of Ac 44 and Im 216 were significantly different from their respective uninoculated treatments. Hyp% (w/w) in inoculated AcB_N cell walls was not statistically different from the uninoculated AcB_N cell walls. Inoculated Ac 44 cell walls showed a net decrease in Hyp% (w/w), as compared to the control, while both Im 216 and AcB_N cell walls showed a net increase in Hyp% (w/w) (Table 10). Thus, the presence of some resistance genes does seems to alter the behavior of the suspension cells to synthesis and accumulation of Hyp in the presence of the pathogen. Therefore, it can be concluded that the ability to synthesize Hyp in inoculated cell walls may be related to the presence of resistance genes.

Furthermore, the change in Hyp, either net increase or net decrease at day 5 in Ac 44, AcB_N, and Im 216 cell walls is correlated with growth of the suspension cells (see Figure 10). Like Hyp%, at day 5, the fresh weight had increased or decreased with respect to fresh weight at day 1. In uninoculated suspension cells for all three cultivars, Ac 44, AcB_N, and Im 216, the fresh weight increased ~20%, ~40%,~15%, respectively, from day 1. On the other hand, fresh weight for inoculated suspension cells for all three cell types decreased ~55%, ~45%, ~30%, respectively, from day 1. Figure 10 showed that inoculated Im 216 cultures had the greatest amount of growth, AcB_N cultures intermediate amount of growth, and Ac 44 cultures had the least amount of growth. Similarly, average Hyp% was greatest in inoculated Im 216 cell walls, intermediate in inoculated AcB_N cell walls, and lowest in inoculated Ac 44 cell walls.

Since the data presented in Figures 24 and 25 is in the form of Hyp total weight % of the cell walls, then recovery of cell walls (Table 3, Figures 16 and 17) and growth of suspension cultures (Figures 9 and 10) are not directly related to the ability of inoculated or uninoculated suspension cultures to synthesize or accumulate Hyp/HRGP in the cell walls. What is important is that 5 days after culture initiation, the log phase of growth begins (see Figure 19) and there is an associated increase in metabolic activity. In addition, bacterial (*X. c. malvacearum*) growth is a maximum at this time (see Figures 3-4).

Studies at the genetic level would clarify biochemical events associated with synthesis and accumulation of Hyp/HRGP and whether the same or different genes are expressed or whether they are differentially expressed in uninoculated and inoculated cotton suspension cells. Additionally, these studies would provide the basis for analyzing cell type differences observed in Hyp/HRGP accumulation and whether these differences are related to the presence or absence or one or more resistant genes to *X. c. malvacearum.* Several unsuccessful attempts have been made during the course of this research using a carrot genomic clone, pDC5A1, for a HRGP gene [38] to identify cotton HRGP gene(s). This work can be continued in the future so that one might be able to correlate the accumulation of cotton HRGP with an accumulation of cotton HRGP mRNA.

Several conclusions from the time course accumulation of Hyp (total weight %) in Ac 44, AcB_N, and Im 216 suspension cell walls can be made. Data collected indicate that the Hyp accumulation in inoculated cotton suspension cell walls is based on differences in resistant (Im 216 or AcB_N) and susceptible (Ac 44) cell lines. The relationship of resistance to production of HRGP, indicated by the Hyp accumulation in the incompatible interactions, is suggested by these studies. However, mechanisms for triggering synthesis of HRGP are not known for cotton. The susceptible line, Ac 44, HRGP may be destroyed by proteases possessed by *X. c. malvacearum* , since the Hyp level drops so dramatically at day 5 after inoculation and is consistently lower than the control. To date, there have been 2 extracellular proteases (P1 and P2) isolated from *X. c. malvacearum*, race 3 [91]. It is thought that P1 has a probable role in pathogenicity toward cotton. It has been shown that P1 cleaves N-terminally to aspartic acid and cysteic acid. Additionally, Hyp% is much higher in the inoculated resistant lines, Im 216 and AcB_N, than in inoculated Ac 44 cell walls.

Previous work on melon infected by the fungal pathogen, *Colletotrichum lagenarium* demonstrated that the level of HRGP is greatly increased in the cell walls of diseased plants [71]. Further work with this host/pathogen system provided the amount of Hyp in the cell walls of different host species, both susceptible and resistant to different species of *Colletotrichum* ([164]. Additionally, these authors studied the amount of Hyp in the cell walls of a number of other host/pathogen systems. These researchers found that the cell walls of healthy control plants usually contained low amounts of Hyp and proteins; whereas, inoculation produced a general increase in cell wall Hyp and, to a lesser extent in cell wall proteins, in all plant materials. Thus, taxonomically unrelated plant species such as melons, beans, alfalfa, all showed a general increase in cell wall Hyp in response to

species of *Colletotrichum* or other pathogens such as *Pseudomonas phaseolicola* (bacterium) and *Uromyces appendiculatus* (fungi). The accumulation of HRGP is a general response of dicotyledons to challenge by both compatible and incompatible pathogens.

The work in this dissertation has indicated that there is an accumulation of HRGP (proportional to Hyp) in resistant cultivars, Im 216 and AcB_N, in response to infection by the bacterial pathogen, *X. c. malvacearum*. Maximal accumulation occurred 5 to 10 days after challenge with the pathogen (see Figures 24 and 25). The extent of Hyp/HRGP accumulation (as compared to the control) varied in the three host-pathogen interactions studied. The immune cultivar, Im 216, had the highest Hyp accumulation, 72% at day 5, the intermediate resistant cultivar, AcB_N, had a Hyp accumulation of 10% at day 5, and the susceptible cultivar, Ac 44, had a Hyp accumulation of -73% at day 5. The Hyp/HRGP accumulation in Im 216 and AcB_N suspension cell walls is of the same order of magnitude as values reported for the cucumber-*Cladosporium cucumerinum* system [100] in which all resistant cucumber varieties exhibited an average 61.4% increase in Hyp content 48 H after inoculation, while susceptible cell cultivars showed only an average 7.9% increase in HRGP content. Additionally, Stermer and Hammerschmidt [240] found that 3 to 4 days after inoculation, there was a rapid rise in Hyp content of cell walls from both inoculated and heat-shocked, inoculated cucumber seedlings. Finally, the time course appearance of HRGP mRNAs in infected melon leaves [207] is consistent with the same time course accumulation of cell wall HRGP which starts 3 to 4 days after inoculation [71].

A number of reports have suggested that HRGPs might be involved in the defense reactions in melons [71], in tobacco [168], in potato [162], and in cucumber [100]. Hyp and the corresponding HRGPs increased in all infected resistant cultivars, but the extent varied according to the host studied. In the present work, it is reasonable to conclude that cotton HRGP is present and accumulates only in the incompatible interactions, AcB_N and Im 216, studied in suspension culture. The compatible interaction, Ac 44, showed a diminished Hyp level and therefore lower HRGP than the uninoculated suspension cell walls. Thus, it is possible that cotton HRGP is involved in resistance to the blight pathogen, *X. c. malvacearum*. The HRGP role in disease resistance has been reported to be due to its linear structure [250] and high level of basic amino acids [162, 164, 168] which confer the functions of strengthening the cell wall and agglutination of negatively charged particles or cells such as bacteria.

There has been previous work that suggests that HRGP accumulation is similar to a phytoalexin response. First in the cotton (leaves)-*X. c. malvacearum* system , higher amounts of phytoalexins were found in all 17 of the incompatible interactions than in the 13 compatible interactions in the cotton (leaves) [222]. Additionally, in cucumber-*Cladosporium cucumerinum* system, the resistant cultivars exhibited an earlier and higher increase in phytoalexins than susceptible ones [100]. Second, HRGP synthesis can be elicited by elicitors of both fungal and plant origin which are known elicitors of phytoalexins [205]. Third, a molecular analysis of HRGP accumulation in *Phaseolus vulgaris* in response to *Colletotrichum lindemuthianum* has shown that the mRNAs hybridizing to a carrot genomic clone of HRGP are increased earlier in the incompatible interaction than in the compatible interaction and these increases are magnified by an elicitor preparation from this fungal pathogen [223]. The correlation between HRGP and phytoalexins has not been made in cotton suspension culture or the intact cotton plant and should be investigated in the future to contribute toward a complete understanding of the mechanism of disease resistance to *X. c. malvacearum*.

Changes in Protein Levels in Uninoculated and Inoculated Ac 44, AcB_N, and Im 216 Suspension Cell Walls. Cell wall protein for all four time courses is shown in Tables 4-9 and the statistical analysis of the least squares means is shown in Table 10. The time course changes from one representative time course of 1, 5, 10, 15, and 20 day Ac 44 and Im 216 cell wall protein and 1, 5/6, and 9 day AcB_N cell wall protein in the presence or absence of *X. c. malvacearum* are expressed in Figures 26 and 27. Statistical analysis of the results indicated that both cell type and presence of pathogen significantly affected the cell wall protein (%).

In uninoculated Ac 44 suspension cell walls (see Figure 26), the average protein % (averaged over 20 days) is $9.16 \pm 2.40\%$ (w/w) while the inoculated Ac 44 cell walls have an average protein % of $3.96 \pm 1.46\%$ (w/w). Cell wall protein immediately decreases after inoculation by 39% from day 1 (6.73% (w/w)) to day 5 (3.54% (w/w)) and decreases by an additional 20% from day 5 to day 20 (2.38% (w/w). On the other hand, cell wall protein has a marked increase from day 1 (5.80% (w/w)) to day 5 (13.15% (w/w)) in uninoculated Ac 44 cell walls and then levels off to between 8.83% (w/w) and 9.88% (w/w) protein. The uninoculated Ac 44 average cell wall protein % is 2.3-fold higher than the inoculated Ac 44 average cell wall protein %. This difference was statistically significant for all four time studies (Table 10).

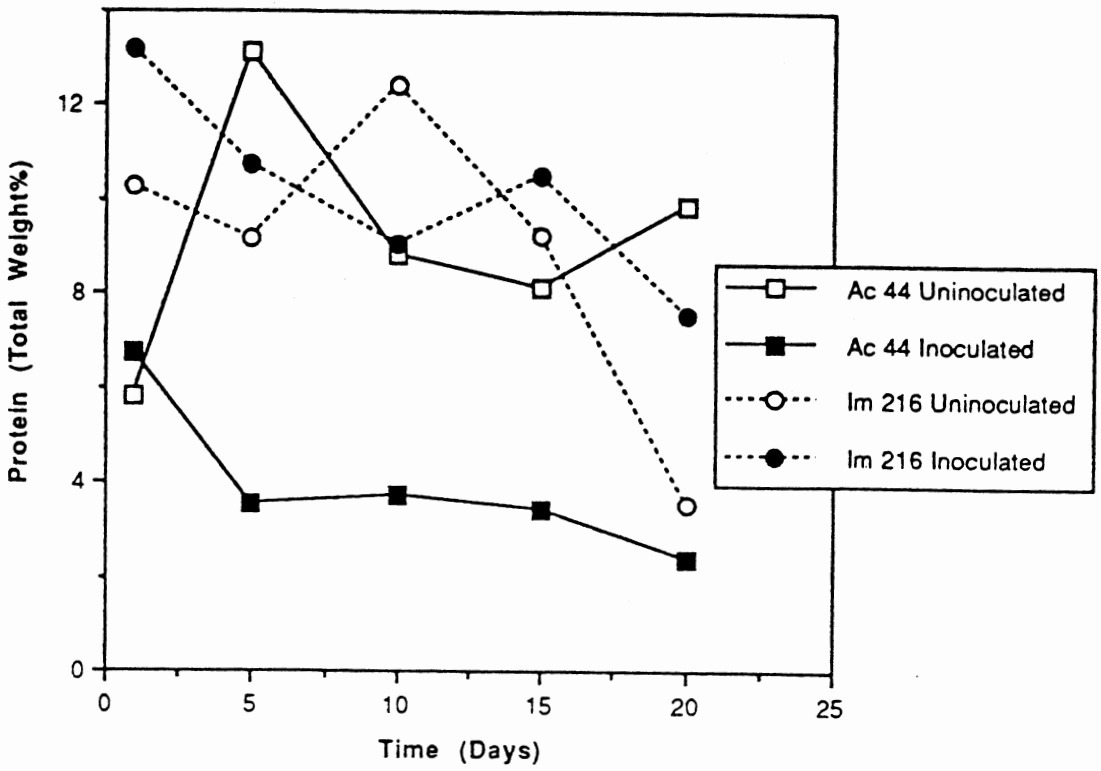


Figure 26. Time Course Accumulation of Protein (Total Weight %) in Response to Infection by *X. c. malvacearum* in Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Protein was determined from amino acid analysis of cell wall hydrolysates. Protein % represents the protein calculated as total weight % of the cell walls. Data is from Tables 4-7 and represents 1 determination of a single experiment.

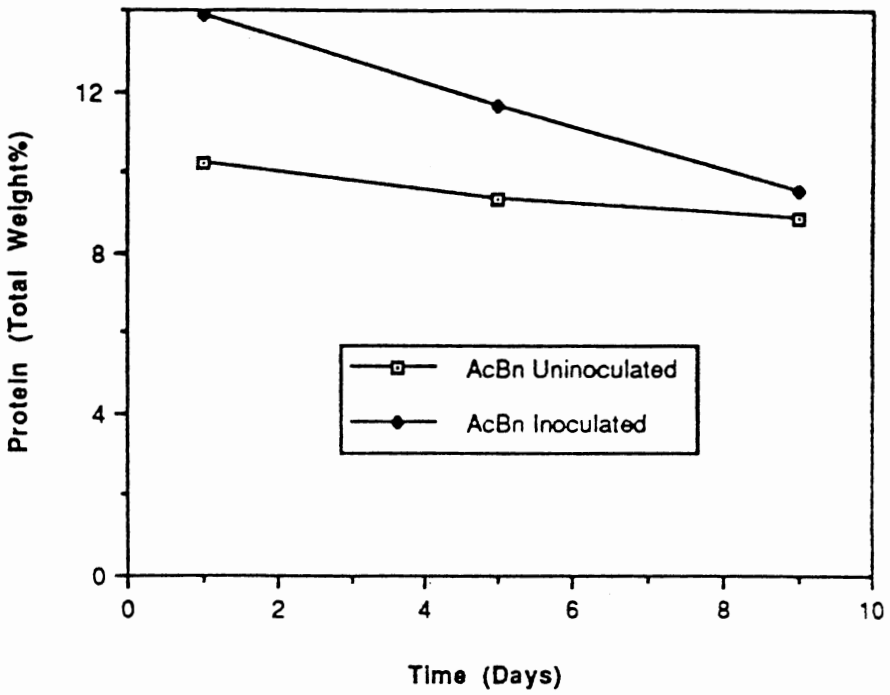


Figure 27. Time Course Accumulation of Protein (Total Weight %) in Response to Infection by *X. c. malvacearum* in AcB_N Suspension Cell Walls. Cell walls were prepared from 1, 5, 9 d old suspension cultures as previously described. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Protein was determined from amino acid analysis of cell wall hydrolysates. Protein % represents the Hyp calculated as total weight % of the cell walls. Data is from Tables 8-9 and represents 1 determination of a single experiment.

In uninoculated Im 216 cell walls (Figure 26), the average protein % (averaged over 20 days) is 8.95 ± 2.94% (w/w) while in the inoculated Im 216 cell walls, the average protein % is about 12% higher, 10.20 ± 1.88% (w/w). That is, there is an average 1.14-fold difference between the uninoculated and inoculated protein cell wall content. This difference is fairly small. Statistical analysis of the protein least squares means for all four time studies showed this difference was not significant (Table 10).

In uninoculated AcB_N cell walls (Figure 27), the average protein % (averaged over 20 days) is 9.48 ± 0.54% (w/w) while in the inoculated Im 216 cell walls, the average protein % is about 19% higher, 11.66 ± 1.79% (w/w). That is, there is an average 1.22-fold difference between the uninoculated and inoculated protein cell wall content. Again, like inoculated and uninoculated Im 216 cell walls, the protein difference is very small in AcB_N cell walls whether inoculated or not. Statistical analysis of the protein least squares means for all four time studies showed this difference was not significant (Table 10). In general, inoculation with *X. c. malvacearum* produced a general increase in cell wall Hyp, but to a lesser extent in cell wall proteins in the resistant cultivars, Im 216 and AcB_N, but just the opposite was observed in the susceptible cultivar, Ac 44. Mazau and Esquerré-Tugayé [164] found the same trend in all 16 different host-pathogen system studies except that an increase in accumulation of Hyp and cell wall protein also occurs in susceptible plants.

The uninoculated cell walls have nearly the same average cell wall protein % over the course of 20 days for this one time study. Uninoculated Ac 44 cell walls have a cell wall protein % average of 9.16 ± 2.40% (w/w), uninoculated Im 216 cell walls have an average of 8.95 ± 2.94% (w/w), and AcB_N cell walls have an average of 9.48 ± 0.54% (w/w). Statistical analysis of all four time studies indicated that the uninoculated protein least squares mean for Ac 44 cell walls was different from that of AcB_N cell walls, but not from that of Im 216 cell walls. Th uninoculated protein least squares mean of AcB_N cell walls was not statistically different from that of Im 216 cell walls. Yet, the resistant cultivars, Im 216 and AcB_N responded quite differently to the pathogen, *X. c. malvacearum* than did the susceptible cultivar, Ac 44. This difference was statistically significant for all four time studies (Table 10). This difference was also consistent with results reported earlier for growth (Figure 11), viability (Figures 12-15), cell wall recovery (Table 3, Figures 16 and 17), amino acid profiles (Figures 19-23), accumulation of Hyp (Figures 24 and 25), and now protein (Figures 26 and 27). The accumulation of

Hyp (Figures 24 and 25) follows the same pattern as the protein profiles (Figures 26 and 27) and the time course of the ratio of washed cell fresh weight to cell wall dry weight (Figures 16 and 17). In every case, the presence of the pathogen coupled to the cell line chosen significantly affected the dependent variable measured at ≥90% confidence level from the various analyses of variance of the results. Therefore, one would conclude first that the biochemical parameters of Hyp% and protein% in the suspension cell walls, and the biological parameters of growth (suspension cell fresh weight), viability, and cell wall recovery are different for uninoculated and inoculated suspension cultures/cell walls, i. e., these parameters are a function of the presence of the pathogen. Second, these parameters differ from cell type to cell type chosen as the host. Thus, the measured parameter differs within a cell line (inoculated v. uninoculated) and between cell lines (Ac 44 v. AcB_N v. Im 216).

The information gained by this research helps define resistant properties in cotton suspension cultures. A set of biochemical characteristics is being measured *in vitro* for resistant cell types in an incompatible interaction and for a susceptible cell type in a compatible interaction for the cotton suspension culture-*X. c. malvacearum* host-pathogen system. These data may be related to the defense response. For example, since Hyp accumulates in the resistant cell lines, AcB_N and Im 216, and does not in the susceptible cell line, Ac 44, then one must infer than HRGP is synthesized or HRGP genes are being induced or expressed differentially in the resistant cell types but not in the susceptible cultivar. As mentioned earlier, nucleic acid studies would provide the definitive information to directly correlate increases in Hyp/HRGP to the induction of particular genes or determine whether the genes are just differentially expressed in resistant, AcB_N and Im 216, cell lines, but not in the susceptible cell line, Ac 44. Therefore, the Hyp/HRGP level is linked to resistance of AcB_N and Im 216 to the pathogen, *X. c. malvacearum* . The capacity in which cotton HRGP functions in resistance to *X. c. malvacearum* is not known. Since the protein profiles mirror the Hyp profiles, one might conclude that new protein is being synthesized in the resistant cell lines and that it could be HRGP. Additionally, the dramatic decrease in Hyp and protein in inoculated Ac 44 cell walls may signify a change in gene expression of HRGP or other proteins elicited by the pathogen. Perhaps, *X. c. malvacearum* produces a protease or other enzymes that partially destroy intact HRGP or prevent synthesis of new HRGP or other proteins in the susceptible cuell line, Ac 44, but not in the resistant cell lines, AcB_N and Im 216. This hypothesis is strengthened by the fact that the *X. c. malvacearum* extracellular protease, P1, may have a role in pathogenicity toward cotton [91]. In any case, the nature of

resistance in the cotton-*X. c. malvacearum* system is very poorly understood. Therefore, the results presented in this dissertation provide new insight into the mechanisms of resistance in cotton through the use of plant tissue culture.

Carbohydrate Profiles of Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls

Ac 44 and Im 216 cell walls from 1, 5, 10, 15, and 20 day uninoculated and inoculated with *X. c. malvacearum* , were analyzed for carbohydrates using gas-liquid chromatography. A representative gas chromatogram of the trimethylsilyl (TMS) derivatives of sugars from uninoculated (15-20 day old) cotton suspension cell walls is shown in Figure 28. The TMS-derivatives of sugars in the cell walls, whether uninoculated or inoculated, Ac 44 or Im 216, have the same standard sugar profile with nearly identical retention times. The sugars are identical, and only the quantitative analysis of the sugars between cell types and in the presence of the pathogen differs. The time course accumulation of overall sugar total weight percent in uninoculated and inoculated Ac 44 and Im 216 cell walls is presented in Figure 29. Both Ac 44 and Im 216 cell walls have arabinose (ara), rhamnose (rha), fucose (fuc), xylose (xyl), galacturonic acid (galU), mannose (man), galactose (gal), and glucose (glc). The quantities of each sugar are determined by comparison with known quantities of sugar standards and an internal standard, *myo*-inositol. The time course profiles of each of the eight individual sugars, expressed as mole percent and total weight percent of the cell wall, is examined in uninoculated and inoculated 1, 5, 10, 15, and 20 day Ac 44 and Im 216 cell walls. Results are shown in Figures 30-37. Plain methanolysis does not account for cellulosic glucose. Thus, the glucose quantities in Figures 30-37 represent only non-cellulosic glucose and are a fraction of the total glucose in the cell wall. Hydrogen fluoride treatment of cell walls at 0° C breaks the glycosidic bonds of cellulose, resulting in the glucose monomers.

Analysis of variance of time course accumulation of the total sugar content (total weight %) and for each individual sugar (total weight %) in uninoculated and inoculated Ac 44 and Im 216 cell walls was conducted for the results shown in Figures 29-37. Sugar content (total weight %) in the cell wall changes very little over the course of 20 days within any treatment tested. These changes in sugar content were not significant at the 95% confidence level. Least squares means were calculated for total sugar and Ara, Rha,

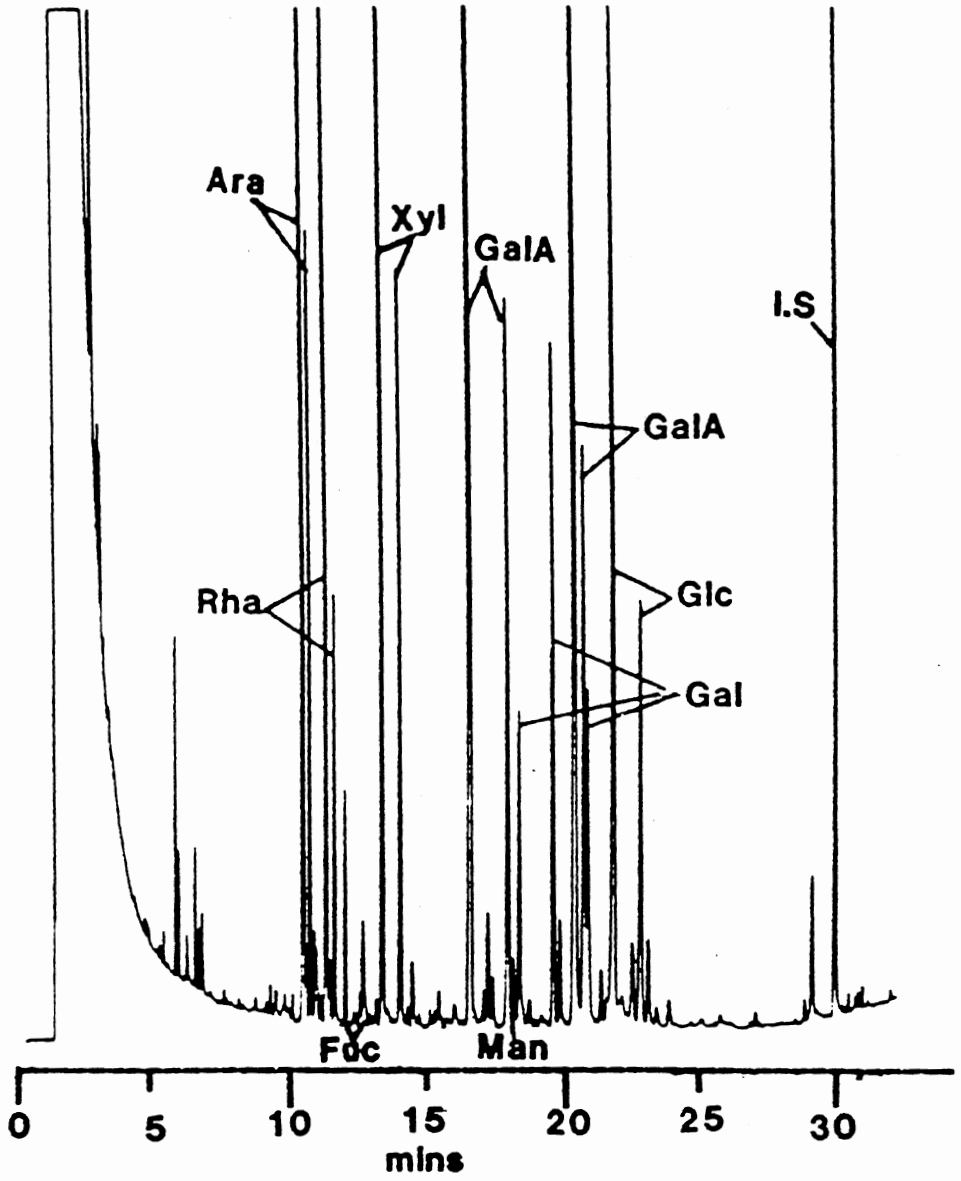


Figure 28. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides from Uninoculated 15-20 d old Ac 44 Suspension Cell Walls. Peaks are identified as follows: Ara, arabinose; Rha, rhamnose; Fuc, fucose, Xyl, xylose; GalU, galacturonic acid; Man, mannose; Gal, galactose; Glc, glucose; IS, *myo* - inositol, the internal standard. [Adapted from P. Komalavilas. 1988. Partial Structural Characterization of Pectin from Cotton Cell Walls Using Selective Hydrogen Fluoride Solvolysis. Ph.D. Thesis. Oklahoma State University. p. 3)

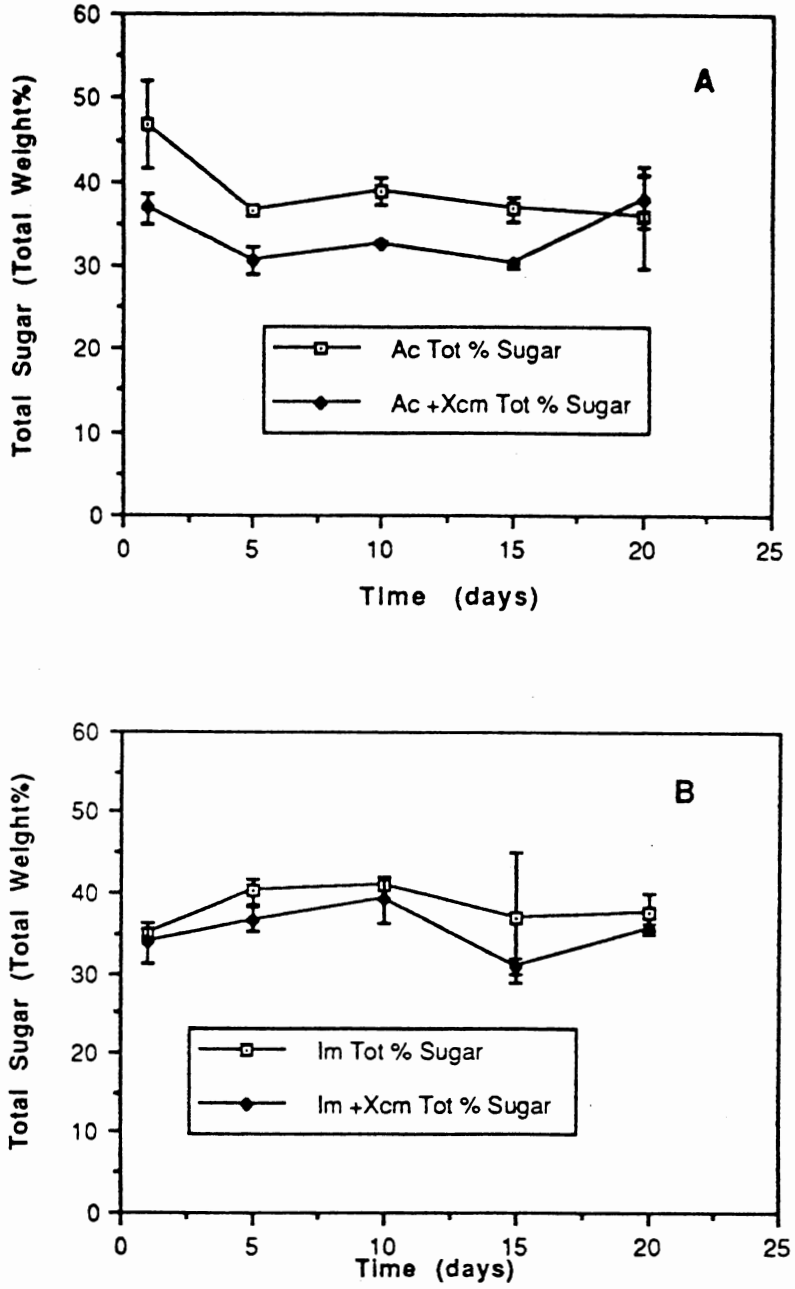


Figure 29. Time Course Accumulation of Overall Sugar Total Weight Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Ac 44 total sugar content, B. Im 216 total sugar content.

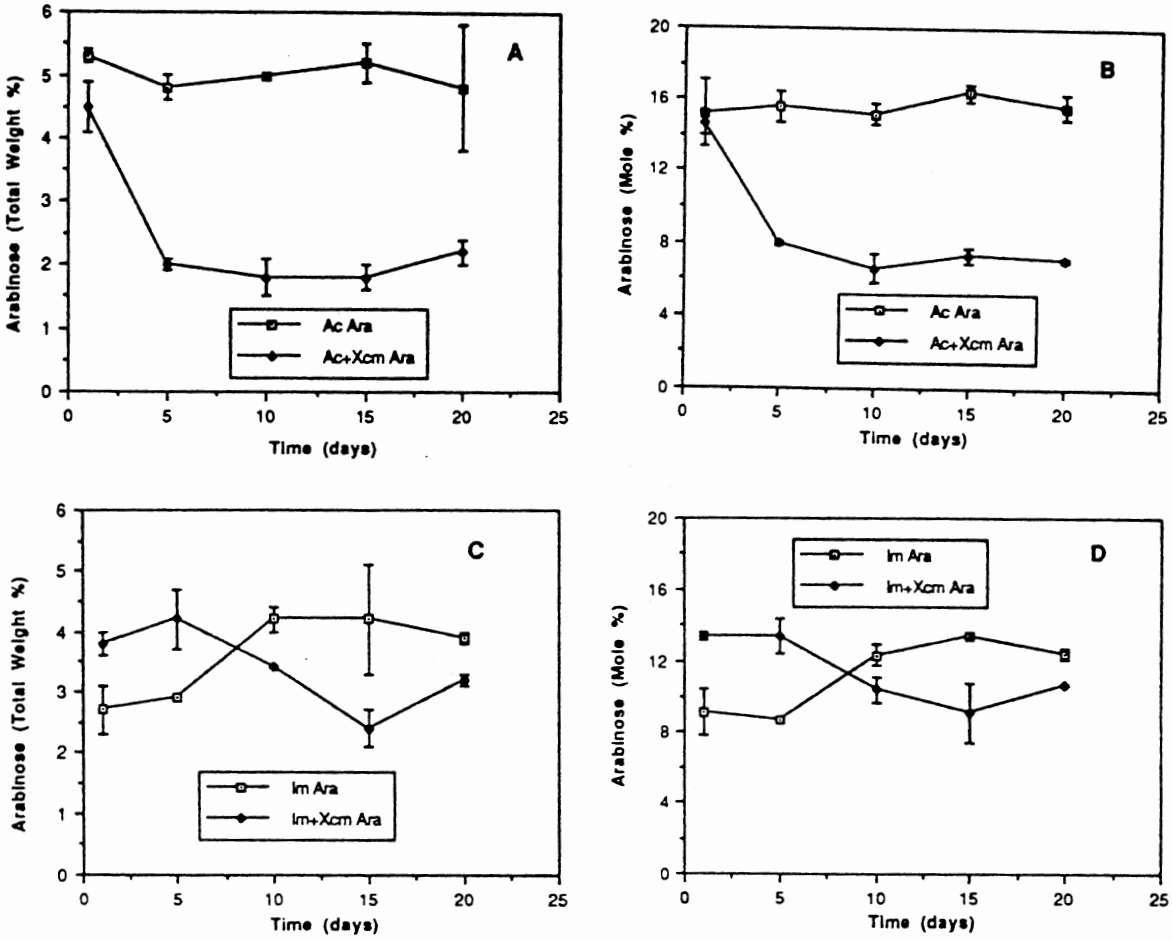


Figure 30. Time Course Accumulation in Arabinose Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1 $X10^7$ cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of Ara in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of Ara in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of Ara in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of Ara in Uninoculated and Inoculated Im 216 Cell Walls.

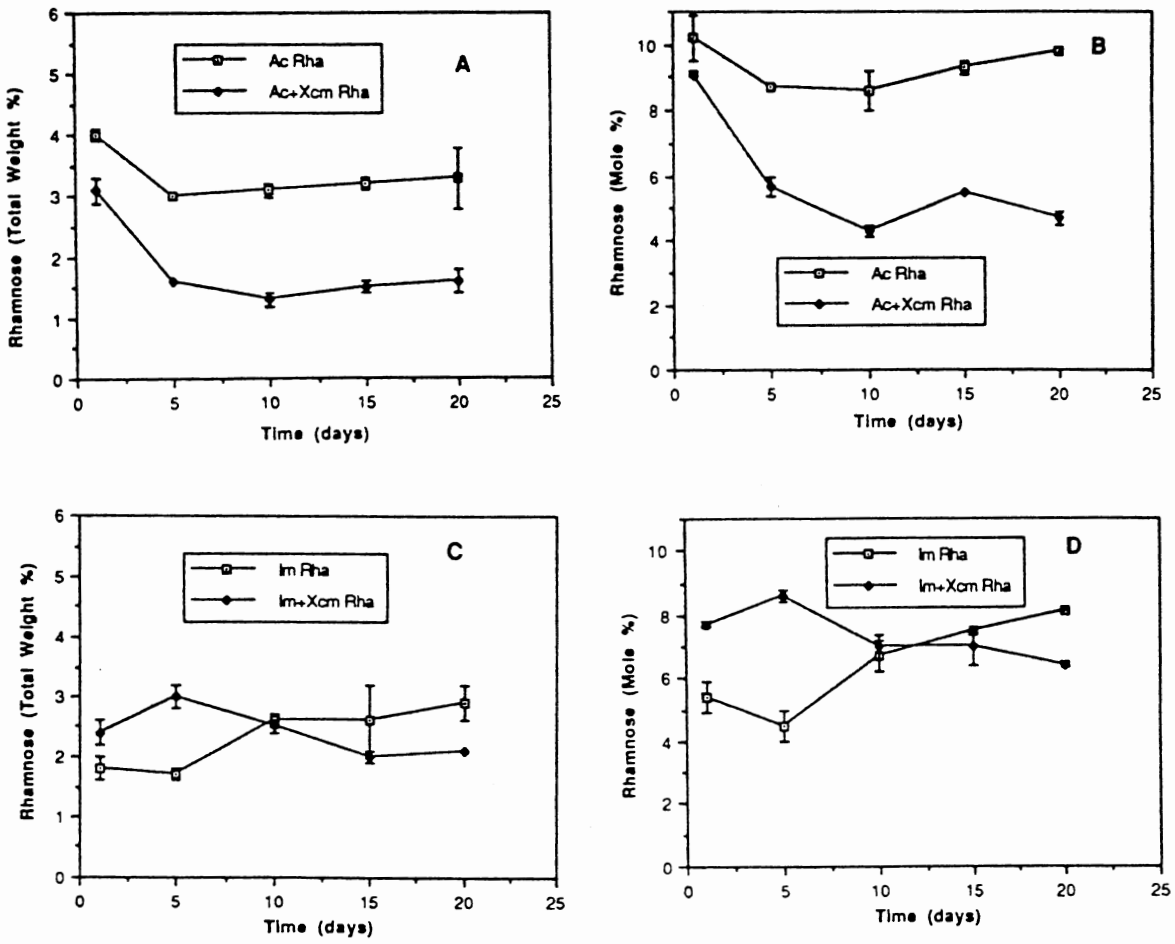


Figure 31. Time Course Accumulation in Rhamnose Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of Rha in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of Rha in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of Rha in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of Rha in Uninoculated and Inoculated Im 216 Cell Walls.

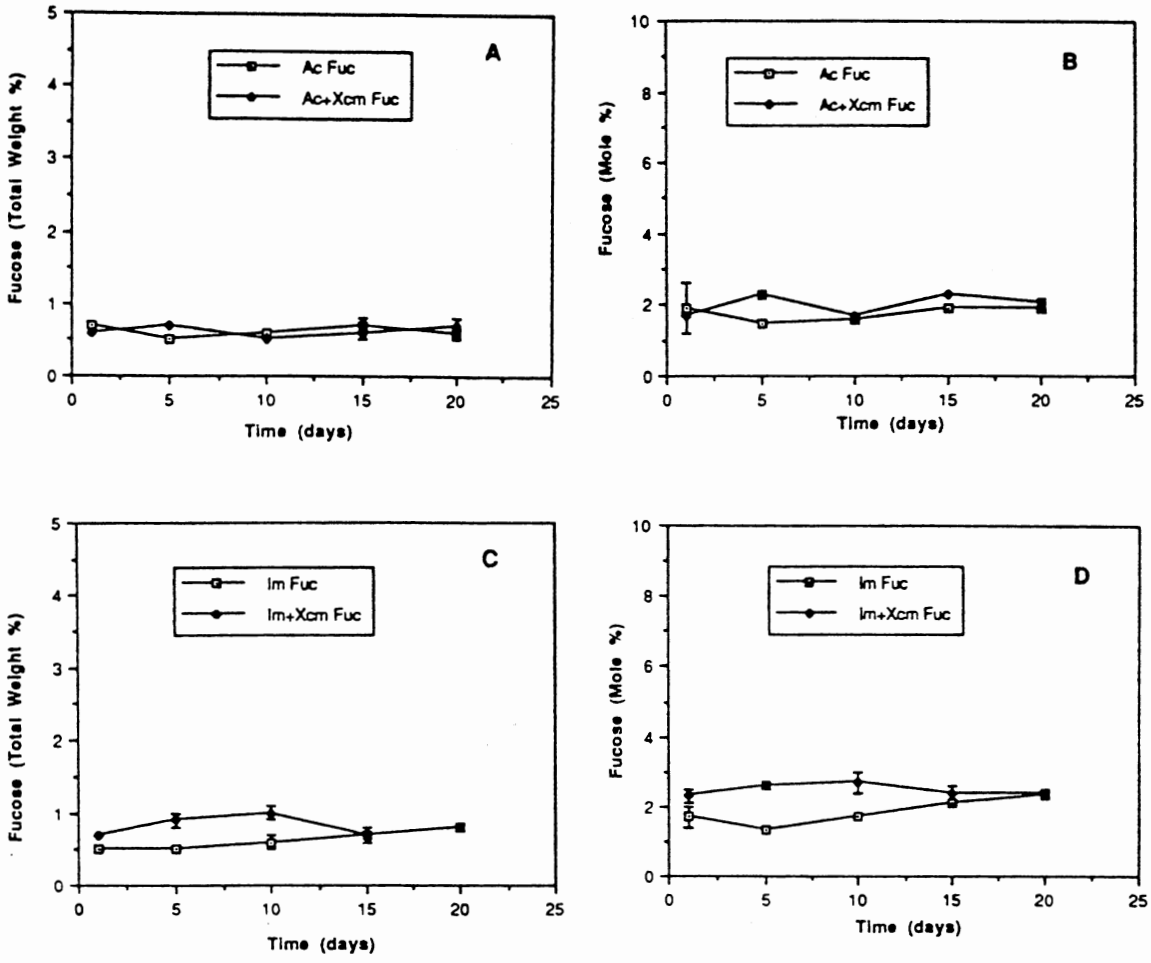


Figure 32. Time Course Accumulation in Fucose Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of Fuc in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of Fuc in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of Fuc in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of Fuc in Uninoculated and Inoculated Im 216 Cell Walls.

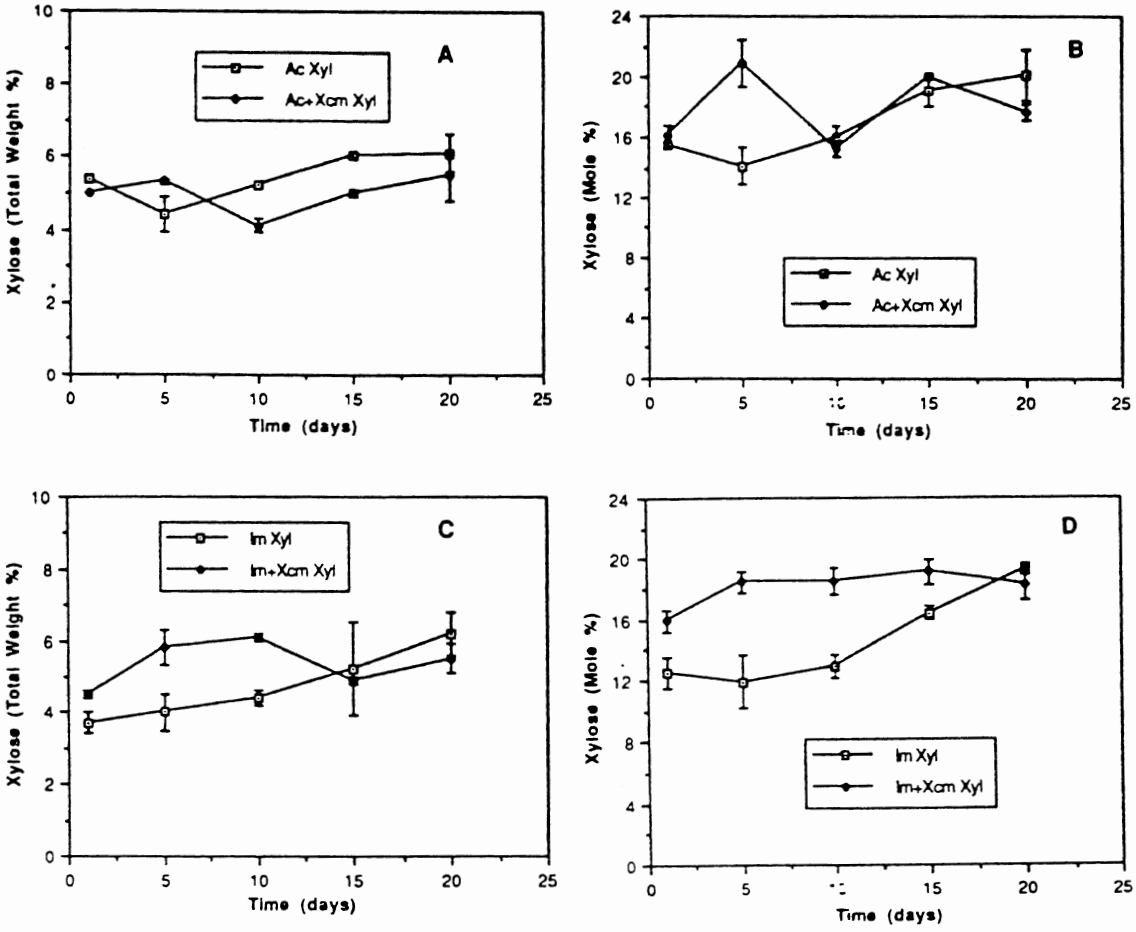


Figure 33. Time Course Accumulation in Xylose Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1 X 10^7 cfu ml^{-1}in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of Xyl in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of Xyl in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of Xyl in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of Xyl in Uninoculated and Inoculated Im 216 Cell Walls.

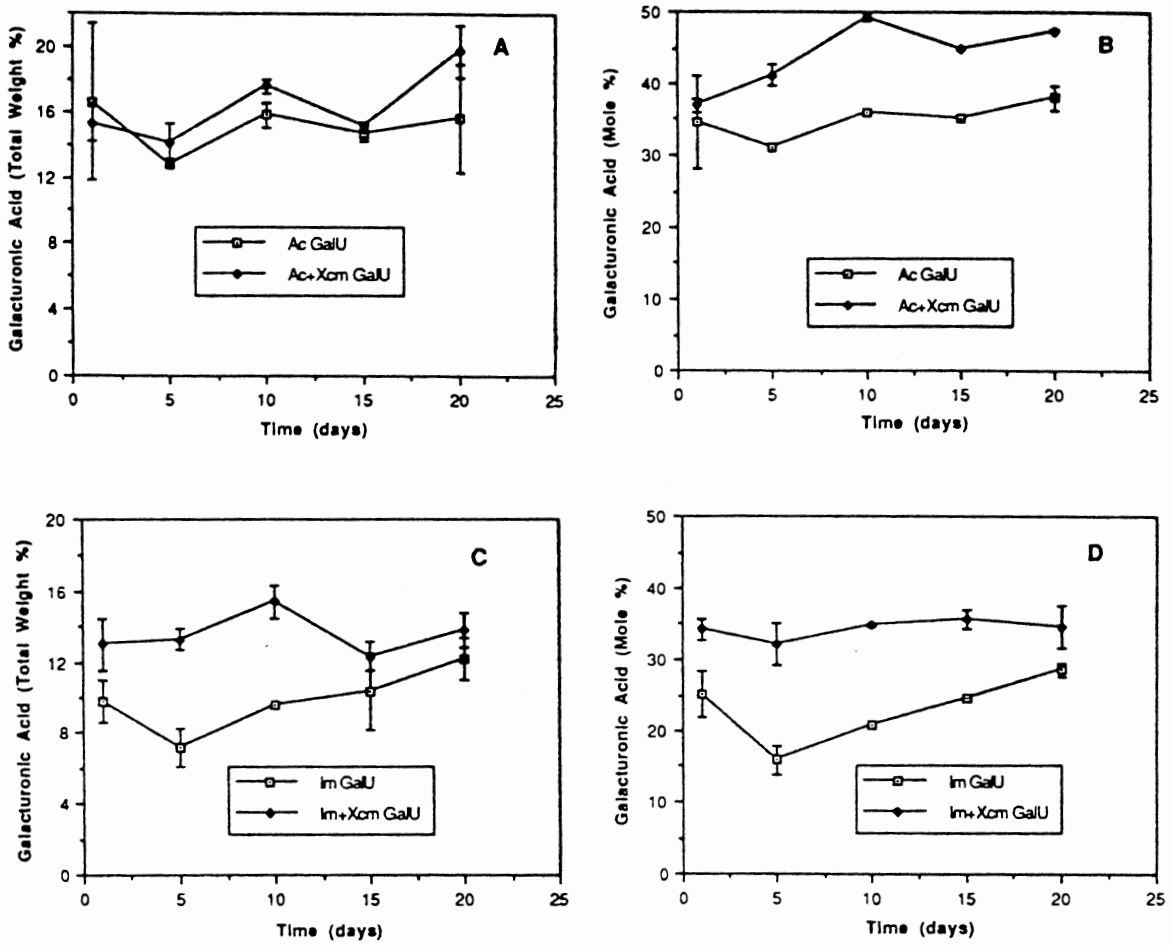


Figure 34. Time Course Accumulation in Galacturonic Acid Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of GalU in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of GalU in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of GalU in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of GalU in Uninoculated and Inoculated Im 216 Cell Walls.

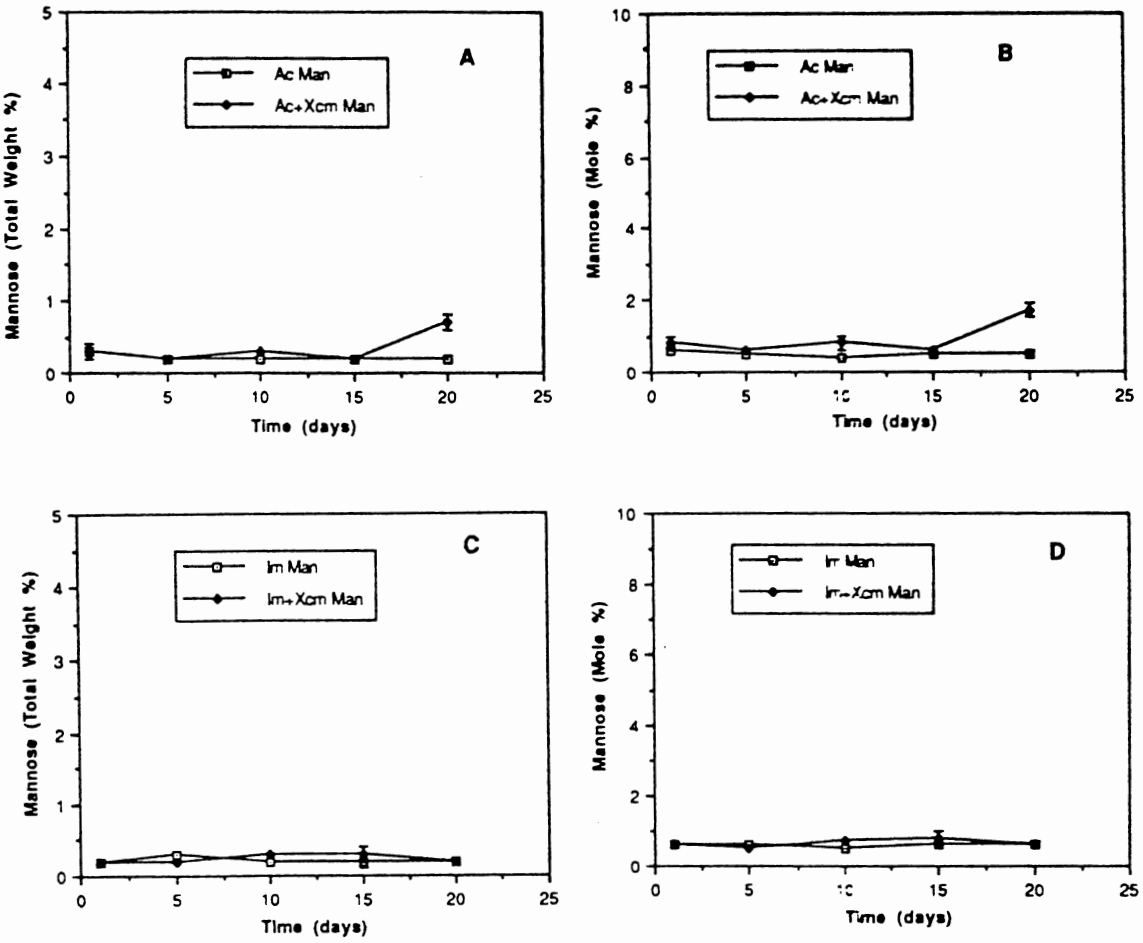


Figure 35. Time Course Accumulation in Mannose Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of Man in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of Man in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of Man in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of Man in Uninoculated and Inoculated Im 216 Cell Walls.

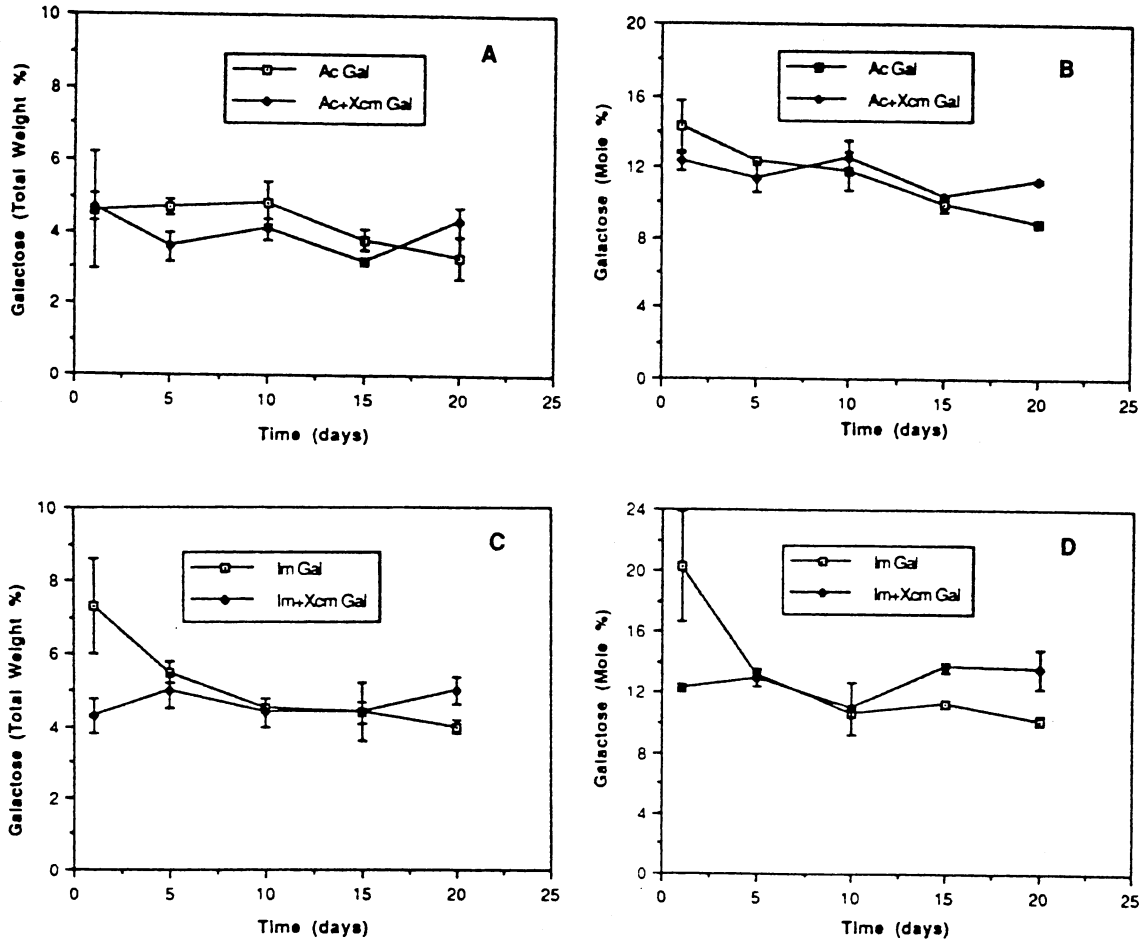


Figure 36. Time Course Accumulation in Galactose Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of Gal in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of Gal in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of Gal in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of Gal in Uninoculated and Inoculated Im 216 Cell Walls.

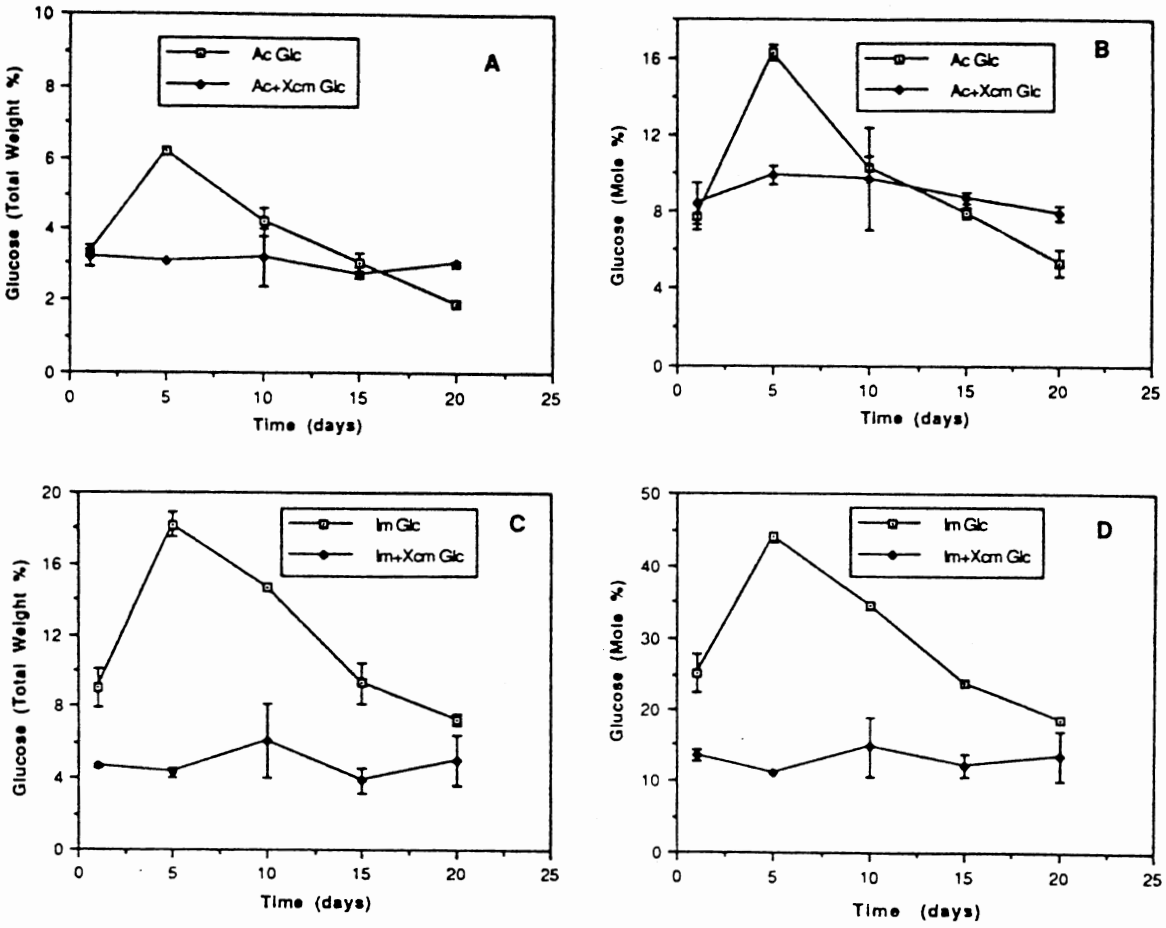


Figure 37. Time Course Accumulation in Glucose Total Weight Percent and Mole Percent in Uninoculated and Inoculated Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described. Suspension cells were inoculated with 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Sugar content is expressed as total weight % in the cell wall from duplicate determinations ± standard deviation. A. Total Weight % of Glc in Uninoculated and Inoculated Ac 44 Cell Walls. B. Total Mole % of Glc in Uninoculated and Inoculated Ac 44 Cell Walls. C. Total Weight % of Glc in Uninoculated and Inoculated Im 216 Cell Walls. D. Total Mole % of Glc in Uninoculated and Inoculated Im 216 Cell Walls.

Fuc, Xyl, GalU, Man, Gal, and Glc total weight % (days = reps). Results are presented in Table 11.

The average sugar total weight % for the entire time course for uninoculated Ac 44 cell walls is 39.0 ± 4.1% (w/w), in inoculated Ac 44 cell walls is 33.7 ± 3.1% (w/w), in uninoculated Im 216 cell walls is 38.1 ± 2.2% (w/w), and in inoculated Im 216 cell walls is 35.2 ± 2.8% (w/w). Komalavilas [141] reported that the Ac 44 cell wall sugar content for uninoculated 15-20 day cell walls was 48% and the Im 216 cell wall sugar content for uninoculated 15-20 day cell walls was 53%, values which are ~10-15% higher than reported for this work. Analysis of variance indicated that Im 216 sugar content was not significantly different from Ac 44 sugar content, i.e. no cell line difference (see Table 11). The least squares mean average for Ac 44 cell wall was not statistically different from that of Im 216 cell walls (Table 11). But, inoculated cell walls have a statistically significantly different sugar content (least squares means) than uninoculated cell walls (Table 11). From Table 11, the inoculated total sugar least squares mean difference of Im 216 cell walls was not statistically different from the total sugar least squares mean difference of Ac 44 cell walls. This means that the pathogen lowered the sugar content in both Ac 44 and Im 216 cell walls by approximately the same amount. The general conclusion for the total sugar profile is that the pathogen, *X. c. malvacearum* lowered the sugar content of the cell wall, but that sugar content did not differ between cell lines.

Each of the following eight sugars (mole% and total weight%): Ara (Figure 30), Rha (Figure 31), Fuc (Figure 32), Xyl (Figure 33), GalU (Figure 34), Man (Figure 35), Gal (Figure 36), Glc (Figure 37) were examined separately with respect to cell line and inoculation over the course of 20 days. Analysis of variance of the data for all eight sugars was performed with respect to the dependent variable (mole% and total weight%) and the independent variables (time, cell line, and inoculation). For each sugar examined in Ac 44 and Im 216 suspension cell walls, sugar content (total weight %) and sugar composition (mole %) does not significantly change over the course of 20 days (no correlation with time). Least squares means were calculated for all sugars (total weight%) in Ac 44 and Im 216 cell walls (Table 11). All comparisons were examined for significance at the 95% confidence level. Ac 44 and Im 216 cell walls differed significantly in the total weight % of GalU, Gal, and Glc, but do not differ in the total weight % of Ara, Rha, Xyl, and Man as shown by the least squares mean averages in Table 11. Similarly, Ac 44 and Im 216 cell walls differed significantly in the mole% of only GalU and Glc, but not in Ara, Rha, Fuc, Xyl, Man, and Gal (least squares means not

TABLE 11

LEAST SQUARES MEANS ANALYSIS OF SUGARS

SUGAR	PARAMETER	AC 44	IM 216
ARA	Uninoculated	5.02 a	3.58 a
	Inoculated	2.46 b	3.40 a
	Difference	2.56 c	0.18 d
	Average	3.74 e	3.49 e
RHA	Uninoculated	3.32 a	2.32 a
	Inoculated	1.82 b	2.40 a
	Difference	1.50 c	-0.08 d
	Average	2.57 e	2.36 e
FUC	Uninoculated	0.62 a	0.62 a
	Inoculated	0.62 a	0.82 b
	Difference	0.00 c	-0.20 c
	Average	0.62 d	0.72 d
XYL	Uninoculated	5.42 a	4.70 a
	Inoculated	2.98 a	5.36 a
	Difference	0.44 c	-0.66 c
	Average	5.20 d	5.03 d
GALU	Uninoculated	15.10 a	9.82 a
	Inoculated	16.36 a	13.56 b
	Difference	-1.26 c	-3.74 d
	Average	15.73 e	11.69 f
MAN	Uninoculated	0.22 a	0.22 a
	Inoculated	0.34 a	0.24 a
	Difference	-0.12 c	-0.02 c
	Average	0.28 d	0.23 d
GAL	Uninoculated	4.24 a	5.14 a
	Inoculated	3.98 a	4.62 a
	Difference	0.26 c	0.52 c
	Average	4.11 d	4.88 e

TABLE 11 (CONTINUED)

SUGAR	PARAMETER	AC 44	IM 216
GLC	Uninoculated	3.72 a	11.70 a
	Inoculated	3.04 a	4.80 b
	Difference	0.68 c	6.90 d
	Average	3.38 e	8.25 f
TOTAL SUGAR	Uninoculated	39.02 a	38.10 a
	Inoculated	33.66 b	35.24 a
	Difference	5.36 c	2.86 c
	Average	36.34 d	36.67 d

Note: The letters a and b denote significant differences (t-test) at the 95% confidence level for the two treatments within a single cell line. The letters c, d, e, and f denote significant cell line differences (t-test) among treatment differences or among treatment averages at the 95% confidence level. Least squares means were calculated from the sugar total weight % (w/w) data for all four time studies. Each time study = one rep.

shown). There were cell line differences in sugar accumulation and sugar composition. Only the inoculated Ara and Rha least squares means in Ac 44 cell walls differed significantly from their uninoculated counterparts (Table 11). On the otherhand, only the Fuc, GalU, and Glc least squares means differed significantly from their uninoculated counterparts in Im 216 cell walls (Table 11). In addition, specific sugar differences were observed with respect to inoculation. The pathogen, *X. c. malvacearum*, affected Ac 44 and Im 216 cell wall sugars unequally. That is, the least squares mean differences for Ac 44 and Im 216 cell walls presented in Table 11 were significantly different for Ara, Rha, GalU, and Glc.

Correlation of all sugar total weight% and mole% with all variables has been made. There seems to be a strong correlation between Rha and Ara, 0.96. there is a fairly significant negative correlation between GalU and Glc, -0.86. These correlations are easily verified by looking at Figures, 30, 31, 32, 33 and 37. The Ara (Figure 30) and Rha (Figure 31) sugar profiles (mole% and total weight%) are clearly parallel to each other. Each change in magnitude of the Ara total weight % or mole % is mirrored by the exact same change in Rha. On the other hand, an increase in Glc (Figure 37) (total weight% or mole%) is accompanied by a decrease in GalU (Figure 34). The inoculated GalU (total weight% and mole%) is 4% higher than the uninoculated GalU, but the inoculated Glc (total weight% and mole%) is conversely at least 4% lower than the uninoculated Glc.

Examination of each sugar profile (total weight% and mole%) (Figures 231-38) provides useful information in characterizing the cell walls of the susceptible cell line, Ac 44 and the immune cell line, Im 216. Describing the carbohydrate composition of the cell wall will help relate structural differences in these two cell types to resistance to *X. c. malvacearum* . The mole% and total weight% profiles of each sugar for either Ac 44 or Im 216 cell walls do not differ qualitatively, but do differ quantitatively. Thus, observations made for Ara mole%, for example, could be made for Ara total weight%.

The Fuc and Man levels (Figures 32 and 35) (total weight% or mole%) do not change at all during the time course for either inoculated or uninoculated Ac 44 or Im 216 cell walls. There is not a cell line difference for the least squares mean averages for Fuc and Man in Ac 44 and Im 216 cell walls (Table 11). Fuc accumulation (total weight%) is approximately 0.50-0.60% of the Ac 44 or Im 216 cell wall dry weight and only 2.0% of the cell wall total sugar composition (mole%). Man accumulation (total weight %) is approximately 0.20-0.30% of the Ac 44 or Im 216 cell wall dry weight and only 0.50-

0.60% of the total sugar composition (mole%). The inoculated Im 216 Fuc least squares mean is significantly different from its uninoculated counterpart (Table 11).

Ara and Rha have similar profile patterns and they are positively correlated (see Figures 30 and 31, respectively). This is probably due to the fact that there are side chains of arabinose on the rhamnogalacturonan I and II in the cell wall, therefore, quantities of both Ara and Rha are directly related to each other. Both Ara and Rha total weight% and mole% in uninoculated Ac 44 cell walls remain essentially constant throughout the 20 day time course. Uninoculated Ac 44 cell walls had an average Ara and Rha total weight % of $5.0 \pm 0.2\%$ and $3.3 \pm 0.4\%$ calculated over 20 days, respectively and an average Ara and Rha mole% of $15.6 \pm 0.9\%$ and $9.3 \pm 0.6\%$. Ara and Rha values for uninoculated Ac 44 cell walls differ by about 1.6-fold where Ara is higher than Rha. Yet, the inoculated Ac 44 cell walls, Ara and Rha (total weight% and mole%) drop dramatically from day 1 to day 5. Ara total weight% falls from 4.5 ± 2.0 (66% decrease) and Ara mole% drops from 14.6% to 8.0% (45% decrease). After day 5, Ara (total weight% or mole%) is maintained at this lower level throughout the course of the experiment. Rha total weight% drops from 3.1% to 1.6% (49% decrease) and Rha mole% falls from 9.1% to 5.7% (47% decrease). After day 5, Rha (total weight% and mole%) is maintained at this lower level throughout the course of the experiment. There was no significant difference in the Rha and Ara least squares means for inoculated uninoculated Ac 44 cell walls (Table 11). Day 5 differences in sugar content between inoculated and uninoculated cell walls would seem to correlate with the differences noted earlier in Hyp% and suspension cell fresh weight (growth). Changes in Rha and Ara content indicate changes in the composition of the rhamnogalacturonan I region of Ac 44 and Im 216 cell wall pectin.

The uninoculated and inoculated Im 216 cell wall Ara and Rha (total weight% and mole%) profiles cross between day 5 and day 10 after inoculation. Initially (day 1), the inoculated Ara and Rha levels are higher than the uninoculated Ara and Rha levels, but fall during the course of the experiment as the uninoculated Ara and Rha levels increase. The pattern thus resembles two S-shaped curves with one starting high and ending low (inoculated) and the other starting low and ending high (uninoculated). The uninoculated Im 216 cell wall Ara mole% begins at 9.1% (day 1) and ends at 12.4% (day 20) while Rha mole% begins at 5.4% (day 1) and ends at 9.1% (day 20). On the other hand, inoculated Im 216 cell walls Ara mole% begins at 13.4% (day 1) and ends at 10.6% (day 20) while Rha mole% begins at 7.7% (day 1) and ends at 6.4% (day 20). A similar situation exists for Im 216 cell wall Ara and Rha total weight %. In uninoculated Im 216 cell walls, the

Ara total weight% begins at 2.7% (day 1) and ends at 3.9% (day 20) while Rha total weight% begins at 1.8% (day 1) and ends at 2.9% (day 20). Then in inoculated Im 216 cell walls, the Ara total weight% begins at 3.8% (day 1) and ends at 3.2% (day 20) while Rha total weight% begins at 2.4% (day 1) and ends at 2.1% (day 20). In any event, the necessary conclusions to be made from these data are that Ac 44 and Im 216 Ara and Rha content (total weight% and mole%), respectively, are not significantly different (Table 11). However, the inoculated and uninoculated Im 216 cell walls do have significantly different Ara and Rha content after examining the least squares means (Table 11) even though uninoculated and inoculated Ac 44 cell walls are not significantly different in Ara and Rha content. It is important to remember that the Hyp-arabinosides are typical of cell wall HRGP and a large percentage of the cotton cell wall Ara could be glycosidically bound to HRGP [149, 150]. In addition, changes in Rha and Ara reflect alterations in the composition of rhamnogalacturonan I, a particular potion of the cell wall pectin. These differences are indicative of the differences between resistance and susceptibility that may be related to the structure of the cell walls. Since GalU content is very different, then the pectin content may play a role in resistance to *X. c. malvacearum*.

Xyl content , total weight% or mole%, (Figure 33) does not significantly differ in Ac 44 and Im 216 cell walls. The average Xyl mole% calculated over 20 days is statistically different in inoculated Ac 44 ($18.0 \pm 2.2\%$) and Im 216 cell wall ($18.1 \pm 1.1\%$) from their respective uninoculated controls ($17.0 \pm 2.3\%$ and $14.6 \pm 2.8\%$). Yet, Xyl total weight% is not statistically different in inoculated Ac 44 and Im 216 cell walls from their respective uninoculated controls. The average Xyl total weight% in uninoculated Ac 44 cell walls is $5.4 \pm 0.6\%$ and in inoculated Ac 44 cell walls is $5.0 \pm 0.5\%$. The average Xyl total weight% in uninoculated Im 216 cell walls is $4.7 \pm 0.9\%$ and in inoculated Im 216 cell walls is $5.4 \pm 0.6\%$. The Xyl mole% in inoculated Ac 44 and Im 216 cell walls is 1.0% and 3.0%, respectively, higher than in uninoculated Ac 44 and Im 216 cell walls. Thus, Xyl content, least squares mean averages, are not statistically different in the blight-susceptible Ac 44 cell walls and the blight-immune Im 216 cell walls (Table 11). Xylose differences in Ac 44 and Im 216 cell wall could be linked to the structure of arabinoxylan, a hemicellulose or xyloglucan, a β 1-4 linked polymer of glucose with xylosyl sidechains.

Gal and Xyl, total weight% and mole% profiles are quite similar. Gal content (Figure 36) does significantly differ in Ac 44 and Im 216 cell walls shown by the least squares mean averages in Table 11. The average Gal total weight% calculated over 20

days in uninoculated Ac 44 and Im 216 cell walls is 4.2 ± 0.6% and 5.1 ± 1.2%, respectively. The average Gal total weight% in inoculated Ac 44 and Im 216 cell walls is 4.0 ± 0.5% and 4.6 ± 0.3%, respectively. It is obvious from Figure 36 that Gal total weight% and mole% do not change during the 20 days of this time course except for minor fluctuations. The average Gal mole% in uninoculated Ac 44 and Im 216 cell walls is 11.4 ± 1.9% and 13.1 ± 3.7%, respectively. While in inoculated Ac 44 and Im 216, the average Gal mole% is 11.6 ± 0.7% and 12.7 ± 1.0%, respectively. While the least squares mean averages were different for Ac 44 and Im 216 cell walls, there was no significant difference due to the presence of the pathogen, *X. c. malvacearum* (Table 11). It is important to remember that the serine of HRGP are glycosylated with one galactose molecule [157]. Thus, a percentage of Ac 44 and Im 216 cell wall Gal is probably bound to the Ser of cotton HRGP. In addition, galactose is part of the variable side-chains of rhamnogalacturonan I. Difference in Ac 44 and Im 216 cell wall Gal could reflect differences in the number of Gal residues on the variable side-chains of rhamnogalacturonan I or even rhamnogalacturonan II.

The key sugars in this study are Glc (Figure 34) and GalU (Figure 37). These two sugars are negatively correlated. That is, the pathogen causes the Glc content to decrease while it causes the GalU content to increase. Together they compose 50% of the total sugars in the cell wall. The average GalU mole% for uninoculated Ac 44 and Im 216 cell walls calculated over 20 days is 34.9 ± 2.3% and 23.0 ± 4.3%, respectively. The average GalU total weight% for uninoculated Ac 44 and Im 216 cell walls is 15.1 ± 1.3% and 9.8 ± 1.6%, respectively. The average GalU mole% for inoculated Ac 44 and Im 216 cell walls is 43.9 ± 4.4% and 34.3 ± 1.1%, respectively. The average GalU total weight% for uninoculated Ac 44 and Im 216 cell walls is 16.4 ± 2.0% and 13.6 ± 1.0%, respectively. The average Glc mole% for uninoculated Ac 44 and Im 216 cell walls is 9.5 ± 3.0% and 29.2 ± 9.0%, respectively. The average Glc total weight% for uninoculated Ac 44 and Im 216 cell walls is 3.7 ± 1.4% and 11.7 ± 4.1%, respectively. The average Glc mole% for inoculated Ac 44 and Im 216 cell walls is 8.9 ± 0.8% and 13.1 ± 1.2%, respectively. The average Glc total weight% for inoculated Ac 44 and Im 216 cell walls is 3.0 ± 0.2% and 4.8 ± 0.8%, respectively.

There is a dramatic 45-50% increase in Glc mole% and total weight% in both uninoculated Ac 44 and Im 216 cell walls at day 5. By day 10, however, this burst of Glc mole% and total weight% is diminished to nearly the same level as day 1 and remains fairly constant through day 20. The inoculated Glc mole% and total weight% changes

very little throughout the length of the time course for either Ac 44 or Im 216 cell walls, but the inoculated Im 216 Glc mole% and total weight% is 50% lower than the uninoculated Im 216 cell walls. This dramatic drop in Glc content was significant after examining the least squares means (Table 11). It is important to note that the cell wall inoculated and uninoculated Im 216 cell wall Glc mole% and total weight% are significantly greater than that of inoculated and uninoculated Ac 44 cell walls. That is, the Glc least squares mean averages for Im 216 cell walls was significantly different from the Glc least squares mean averages for Ac 44 cell walls (Table 11). The Im 216 Glc least squares mean average is more than twice that of the Ac 44 Glc least squares mean average. That is, the pathogen does not equally affect the Glc total weight% (significant interaction effect). In fact, the uninoculated Im 216 cell wall Glc mole% and total weight% are approximately three times higher than the uninoculated Ac 44 cell wall Glc mole%. In general, the inoculated Ac 44 and Im 216 cell wall Glc total weight% and mole% is lower than that of the uninoculated Ac 44 and Im 216 cell walls. This difference was significant for only inoculated and uninoculated Im 216 cell walls (Table 11). Presumably, since only non-cellulosic glucose is determined from these analyses, then differences in Ac 44 and Im 216 cell wall glucose content would be related to the structure of glucomannan, a hemicellulose or xyloglucan, a β 1-4 linked polymer of glucose with xylosyl sidechains.

GalU is the major sugar component of the cell wall. The highest GalU mole%, 49.1%, was observed in 10 day inoculated Ac 44 cell walls. Inoculation causes a 26% real increase in the average GalU mole% for Ac 44 cell walls and 50% for Im 216 cell walls over the course of 20 days. The increase in GalU mole% in inoculated Ac 44 and Im 216 cell walls is gradual, but >day 5 the GalU mole% is substantially greater than the uninoculated cell walls. This increase in inoculated GalU content was significant for Im 216 cell walls but not for Ac 44 cell walls (Table 11). It is interesting to note that while the Im 216 cell wall Glc content (both total weight% and mole%) is higher than the Ac 44 cell wall Glc content, the Im 216 cell wall GalU content is lower than the Ac 44 cell wall GalU content whether inoculated or inoculated. This is shown by comparing the least squares mean averages calculated for GalU and Glc for both Ac 44 and Im 216 cell walls (Table 11). Thus, Ac 44 cell walls have higher amounts of pectin than Im 216 cell walls. Furthermore, inoculation causes an increase in GalU in both Ac 44 and Im 216 cell walls and thus an increase in pectin. This increase is greatest in inoculated Im 216 cell walls. This was shown by the fact that the GalU least squares mean difference was three times greater in Im 216 cell walls than in Ac 44 cell walls (another significant interaction effect) (Table 11).

It has been noted previously [196] that cell wall alteration can contribute to disease resistance by forming a barrier that is more resistant to attack by the pathogens cell wall degrading enzymes including various pectin-degrading enzymes. Pectinases are the key enzymes involved in the process of tissue maceration and cell death in plant cell walls and are produced by many plant pathogen, most notably in the soft-rot diseases caused by *Erwinia* sp. [11, 46, 47, 89]. *X. c. malvacearum* was reported to produce pectin depolymerizing enzymes only under appropriate inducing conditions [1]. It is not certain whether *X. c. malvacearum* pectinase activity is present in inoculated Ac 44 and Im 216 suspension cultures, since the cell wall GalU increased after inoculation in both cultures. A logical experiment would be to measure pectinase activity in inoculated Ac 44 and Im 216 suspension cultures and relate enzyme activity to cell death and thus susceptibility and resistance to the bacterial pathogen, *X. c. malvacearum* . Additionally, pectinase activity could be measured in the cell walls isolated from uninoculated and inoculated cotton suspension cells and related to changes in GalU. With this information in mind, differences in the GalU content of the Ac 44 and Im 216 cell walls can presumably be linked to structural differences in the cotton cell wall in resistant and susceptible lines. These structural differences can then be related to susceptibility and resistance to *X. c. malvacearum* and cell wall destruction or maintenance. Therefore, the GalU and Glc content differences observed in the susceptible Ac 44 cell walls and the resistant Im 216 cell walls might explain why it is relatively easy to produce protoplasts from Ac 44, but not from Im 216. Protoplast yields (see Table II) appear to be inversely proportional to the number of resistance genes in each cell line. These data further strengthen the hypothesis that cell wall alterations are important in cotton resistance to *X. c. malvacearum* . In any event, the observed differences in sugar composition of Ac 44 and Im 216 cell walls enhance the understanding of the host response to *X . malvacearum* and the biochemical differences in a compatible host and a incompatible host.

In addition, it is necessary to study the glycosylation pattern of Hyp in the cell walls of uninoculated and inoculated Ac 44 and Im 216 suspension cells to determine if an increase in Hyp corresponds to an increase in HRGP. This is done by determining the Hyp-arabinosides. Thus, the presence of Man, Ara, Gal, Xyl, Glc, GalU, Fuc, and Rha in the cell walls of inoculated and uninoculated Ac 44 and Im 216 cell walls does not identify whether or not any of these sugars is attached to Hyp.

Lignin Profiles of Uninoculated and Inoculated Ac 44, AcB_N, and Im 216 Suspension Cell Walls

Lignin deposition was determined by colorimetric microanalysis [124] in cotton cell walls (dry weight basis) of cultured cells from, AcB_N and Im 216, and the susceptible variety, Ac 44 for several different time periods. One representative time course of lignin percent of 1, 5, 10, 15, and 20 day inoculated and uninoculated Ac 44 and Im 216 cell walls is shown in Figure 38 and for 1, 5/6, and 9 day inoculated and uninoculated AcB_N cell walls is shown in Figure 39.

Statistical analysis of the lignin content of uninoculated and inoculated Ac 44, AcB_N, and Im 216 cell walls was performed for all four time studies (i.e. replicates). Analysis of variance (split-plot design) indicated that the lignin content did not vary significantly with time for the three cell lines examined. Thus, the least squares means for the lignin percent (w/w) of all time points (1-20 days) for all four time studies, regardless of length of the study were calculated for each treatment and each cell line. Results are shown in Table 12.

The lignin least squares means for inoculated AcB_N and Im 216 cell walls were greater than the lignin least squares means for their uninoculated counterparts (Table 12). However, only the lignin least squares mean of inoculated Im 216 cell walls was significantly greater than the uninoculated Im 216 cell walls. The lignin least squares mean for inoculated and inoculated AcB_N and Ac 44 cell walls were not statistically different.

The lignin least squares mean difference between the uninoculated and inoculated treatments for each cell line were compared for statistical significance (Table 12). The lignin least squares mean difference for Im 216, three resistance genes, was statistically different from AcB_N, one resistance gene, and Ac 44, no resistance genes. The lignin least squares mean average for Ac 44, AcB_N, and Im 216 cell walls were all statistically different (Table 12). Thus, Ac 44, AcB_N, and Im 216 cell walls had characteristically different lignin content . AcB_N, had the greatest lignin percent (largest least squares mean), Im 216 had the intermediate lignin percent, and Ac 44, the susceptible line, had the lowest lignin percent (Figures 38-39, Table 12). The pathogen, *X. c. malvacearum* significantly triggered an increase in lignin in Im 216 cell walls, but only slightly caused

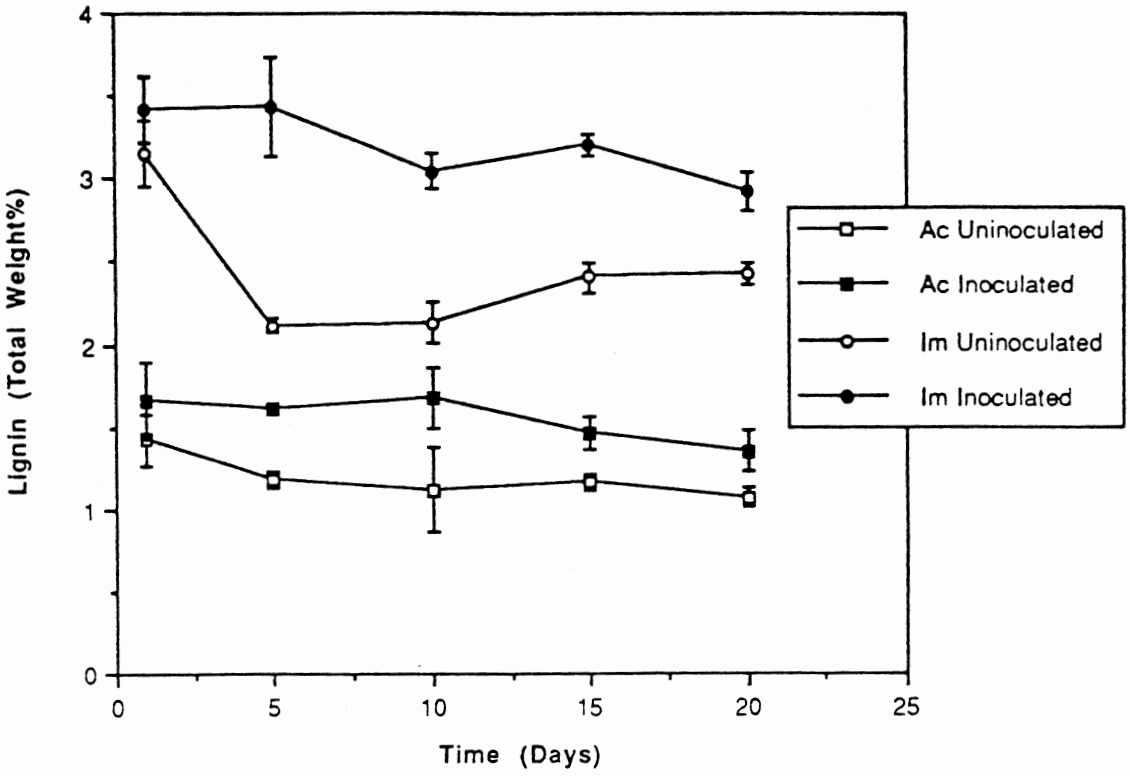


Figure 38. Time Course Accumulation of Lignin (Total Weight %) in Response to Infection by *X. c. malvacearum* in Ac 44 and Im 216 Suspension Cell Walls. Cell walls were prepared from 1, 5, 10, 15, and 20 d old suspension cultures as previously described Suspension cells were inoculated with 1×10^7 cfu ml^{-1} in 50 ml SH medium (pH 5.8). Lignin was determined spectrophotmetrically (see Materials and Methods) and expressed as total weight % of the cell walls. Data represent 2 reps with duplicate determinations ± standard deviation.

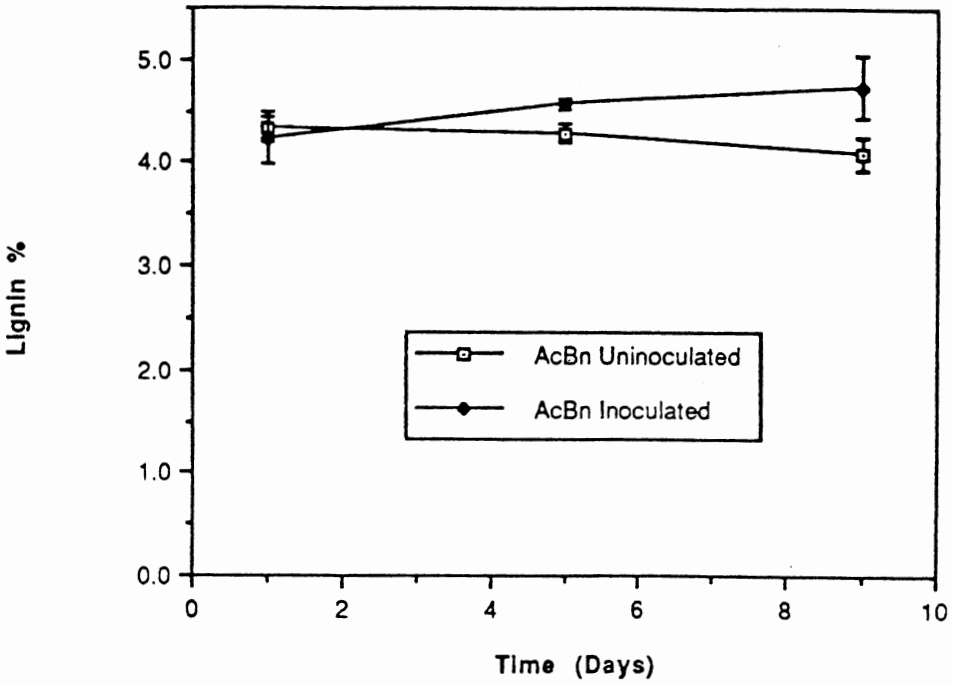


Figure 39. Time Course Accumulation of Lignin (Total Weight %) in Response to Infection by *X. c. malvacearum* in AcB_N Suspension Cell Walls. Cell walls were prepared from 1, 5, and 9 d old suspension cultures as previously described Suspension cells were inoculated with 1×10^7 cfu ml^{-1}. Lignin was determined spectrophotmetrically (see Materials and Methods) and expressed as total weight % of the cell walls. Data represent 2 reps with duplicate determinations ± standard deviation.

TABLE 12

LEAST SQUARES MEANS ANALYSIS OF LIGNIN

PARAMETER	AC 44	ACBN	IM 216
Uninoculated	1.42 a	3.75 a	2.42 a
Inoculated	1.39 a	3.97 a	3.44 b
Difference	-0.03 c	0.22 c	1.02 d
Average	1.40 e	3.86 f	2.93 g

Note: The letters a and b denote significant differences (t-test) at the 95% confidence level for the two treatments within a single cell line. The letters c, d, e, f, and g denote significant cell line differences (t-test) among treatment differences or among treatment averages at the 95% confidence level. Least squares means were calculated from the total lignin % (w/w) data for all four time studies. Each time study = one rep.

an increase in AcB_N cell walls, but did not cause a change in lignin in Ac 44 cell walls (Table 12).

For the representative lignin study shown in Figures 38 and 39, the average lignin content calculated over 10 days was highest in uninoculated AcB_N cell walls, 4.24 ± 0.11% and 4.51 ± 0.20% in inoculated AcB_N cell walls. Im 216 had an intermediate lignin content of 2.44 ± 0.38% (calculated over 20 days) in uninoculated cell walls and 3.2% ± 0.20% in inoculated cell walls. Ac 44 had the lowest lignin content of 1.19 ± 0.12% (calculated over 20 days) in uninoculated cell walls and 1.55 ± 0.13% in inoculated cell walls. These averages are very similar to the least squares means calculated from all four time studies (Table 12). In all three cell lines, the inoculated cell wall lignin was generally higher than the corresponding uninoculated cell wall lignin ≥day 5, this elevation was statistically significant for Im 216 cell walls. The two resistant cell lines, AcB_N and Im 216, had elevated cell wall lignin content (see least squares mean averages Table 12), two to four times higher, compared to the susceptible cell line, Ac 44.

It appears that there is a 1.5-fold drop in lignin% in uninoculated Im 216 cell walls, but not in inoculated Im 216 cell walls. By day 5, in all treatments examined, *X. c. malvacearum* caused a marked increase in cell wall lignin over the uninoculated treatment. At day 10, this increase in lignin% is 45 to 48% greater than the uninoculated Ac 44 and Im 216 cell wall lignin%, but only 15% greater than the uninoculated AcB_N cell wall lignin%. After the initial decrease or increase in lignin content observed by day 5, the lignin% for all treatments is maintained at a rather constant level.

The lignin enhancement in inoculated AcB_N and Im 216 cell walls parallels the time course of Hyp enhancement (Figures 24 and 25). Inoculated Ac 44 cell walls have no accumulation of Hyp (Figure 26) even though there is an enhancement of cell wall lignin. Perhaps, there is a correlation between lignin accumulation and Hyp accumulation in the cotton suspension cell wall. Localization of the enhanced Hyp with lignified cell walls would prove such an association exists and whether it is connected with the resistance of cotton to *X. c. malvacearum* . The cotton suspension culture-*X. c. malvacearum* system would thus provide a viable new system to study the suggested interactions between lignin synthesis, cell wall bound phenols and cell wall HRGP in incompatible and compatible interactions. Greater lignification in the cell walls of AcB_N and Im 216 than in the cell walls of Ac 44 provides strong evidence for the role lignin has in host defense to *X. c. malvacearum* . Rapid lignin deposition may provide a physical

and/or chemical barrier to the invading pathogen [251]. Since peroxidases catalyze the final polymerization step of lignin synthesis, it would be interesting to isolate AcB_N and Im 216 peroxidases in suspension culture and relate peroxidase activity to accumulation of lignin in the cell wall. Furthermore, the bacterial pathogen, *X. c. malvacearum* may induce greater activity of cotton peroxidases and thus cause an increase in lignin deposition. These experiments need to be completed before more definitive mechanisms can be developed.

Previous work has related lignin deposition as an early event in the resistance of cucumber to the fungal pathogen, *Cladosporium cucumerinum* (Hammerschmidt et al, 1984) and in several other cucurbits [143]. Cellular lignification has been shown to occur rapidly at wound margins of wheat leaves following inoculation with pathogenic and non-pathogenic fungi and may be involved in the restriction of the fungi to the the wound site [137, 138, 171, 172, 183, 195, 198]. The work in this dissertation provides relevant information regarding induced lignification in an interaction involving the bacterial pathogen, *X. c. malvacearum* in cell walls isolated from cotton suspension cultures. Past work has concentrated exclusively on interaction involving fungal pathogen, since lignin provides a physical barrier to further haustorial penetration. In addition, lignin is measured here quantitatively using a colorimetric micromethod described originally by Johnson *et. al.* [124] instead of the *in situ* tissue staining method using phloroglucinol HCl.

Preliminary Compositional Analysis of Ac 44 Cotton Leaf, Ac 44 Cotton Suspension, Im 216 Cotton Suspension, AcB_N Cotton Suspension, AcIm Cotton Suspension, Tomato Suspension, Tobacco Callus, and Carrot Root Cell Walls

Introduction

Preliminary compositional analysis of Ac 44 cotton suspension, Im 216 cotton suspension, AcB_N cotton suspension, AcIm cotton suspension suspension, tomato suspension, tobacco callus, and carrot root cell walls was performed in order to provide information on the amount and the chemical nature of the biochemical polymers in these plant cell walls. The chemical composition of the four cotton cell line suspension cell

walls prior to HF treatment at 0°C was established. HF solvolysis of Ac 44 and Im 216 cotton suspension cell walls at 0°C is discussed later in this chapter. In addition, the composition of cotton suspension cell walls can be compared to the tomato suspension, tobacco callus, carrot root, and cotton leaf cell walls.

The carbohydrates of Ac 44 cotton suspension, Im 216 cotton suspension, Ac 44 cotton leaf, tomato suspension, tobacco leaf and carrot root cell walls have been previously determined [141]. All walls tested were rich in galacturonic acid (GalA), an indication of high amounts of pectin in the cell walls. Sugars (includes only non-cellulosic glucose) accounted for 48% (w/w) of the Ac 4 cotton suspension cell walls, 53% (w/w) of the Im 216 cotton suspension cell walls, 30% (w/w) of the Ac 44 cotton leaf cell walls, 52% (w/w) of the tomato suspension cell walls, 33% (w/w) of the tobacco leaf cell walls, and 38% of the carrot root cell walls.

Amino Acid Analysis and Protein Content

Protein contents of Ac 44 cotton suspension, Im 216 cotton suspension, AcB_N cotton suspension, AcIm cotton suspension suspension, tomato suspension, tobacco callus, and carrot root cell walls were determined by automated amino acid analysis using a Dionex D-500 amino acid analyzer with a ninhydrin detector. The mole percent of amino acids and the protein percent (w/w) in the different cell walls is presented in Table 13. (Note: These analyses differ from those in Tables 4-9.) Amino acid analyses showed that Ac 44 cotton leaf cell walls were especially rich in Gly (11.00 mole%), and Leu (11.18 mole%) with lesser amounts of Asp (9.32 mole%), Glu (9.93 mole%), Ala (9.43 mole%), but practically no Hyp (0.25 mole%). This composition is in direct contrast to that of Ac 44 cotton suspension cell walls, which were rich in Hyp (11.96 mole%), Leu (9.02 mole%), Asp (8.74 mole%), Ser (7.56 mole%), Gly (7.44 mole%), Ala (8.74 mole%), Lys (7.35 mole%), and Val (7.10 mole%). The ~50-fold difference in the Hyp mole% in Im 216 or Ac 44 cotton suspension cell walls from the Hyp mole% in the Ac 44 leaf cell walls is another example where Hyp/HRGP levels are strikingly increased under tissue culture conditions [148]. The Hyp content of cells in culture is usually much greater than the Hyp content in leaf tissue.

The amount of protein in Ac 44, Im 216, AcB_N, and AcIm cotton suspension cell walls was found to 4.68%, 2.93%, 7.87%, and 12.96% (w/w) of the cell walls, respectively. Ac 44 leaf cell wall protein was 12.51% (w/w) of the dry weight of the cell

TABLE 13

AMINO ACID COMPOSITION OF VARIOUS PLANT CELL WALLS

Amino Acid Mole %	Ac 44 Leaf	Ac 44 Suspension	Im 216 Suspension	AcBn Suspension	AcIm Suspension	Tomato Suspension	Tobacco Callus	Carrot Root
Hyp	0.25	11.96	18.33	3.69	7.13	28.90	24.53	10.44
Asp*	9.32	8.74	9.48	11.70	12.94	7.51	6.32	9.77
Thr	5.02	4.23	3.82	5.16	5.22	3.99	4.99	4.74
Ser	5.29	7.56	8.59	8.04	9.11	7.83	6.37	6.09
Glu*	9.93	6.10	6.09	9.92	9.40	5.03	4.17	7.45
Pro	6.32	5.54	4.66	6.42	6.84	3.99	7.88	4.26
Gly	11.00	7.44	6.95	10.20	9.92	5.59	8.05	11.12
Ala	9.43	7.37	7.10	9.36	8.94	4.95	5.12	7.25
Val	7.25	7.10	5.43	6.78	6.70	7.60	8.47	7.74
Met	1.55	0.99	1.18	0.97	0.88	0.49	0.71	1.06
Ile	5.27	4.34	3.36	3.66	3.11	3.32	2.79	4.64
Leu	11.18	9.02	7.56	7.39	5.70	5.12	5.06	8.70
Tyr	2.80	2.90	3.51	1.10	0.84	2.97	2.35	2.42
Phe	5.12	3.69	2.82	2.44	2.49	2.33	2.05	3.48
His	1.89	2.38	2.24	2.21	2.00	2.18	3.12	2.32
Lys	3.96	7.35	5.60	7.93	6.06	5.88	6.44	5.32
Arg	4.42	3.29	3.28	3.04	2.73	2.33	1.56	3.19
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
% Protein	**12.51**	**4.68**	**2.93**	**7.87**	**12.69**	**4.44**	**12.10**	**1.21**

Asp* = denotes Asp + Asn, during hydrolysis Asn is converted to Asp
Glu* = denotes Glu + Gln, during hydrolysis Gln is converted to Glu
Note: Cys and Trp were not determined. Data values are for one batch of cell walls.

wall. Tomato suspension cell walls, tobacco callus cell walls, and carrot root cell walls had protein contents of 4.44, 12.10, 1.21% (w/w), respectively. Ac 44 leaf cell walls (12.51%), Ac Im suspension cell walls (12.69%), and tobacco callus cell walls (12.10%) have a greater percentage of protein in their cell walls (w/w) than do Ac 44 suspension cell walls (4.68%), Im 216 suspension cell walls (2.93%), AcB_N suspension cell walls (7.87%), tomato suspension cell walls (4.44%), and carrot root cell walls (1.21%).

The amino acid mole% profiles of the other three cotton suspension cell walls, AcB_N, Im 216, and AcIm, are similar to that of Ac 44 except that the Hyp mole% is variable. The Hyp mole% in Im 216 suspension cell walls is 18.33 %, in AcB_N Hyp mole% is only 3.69%, and in AcIm suspension cell walls, the Hyp mole% is 7.13%. It appears that AcB_N and AcIm grow more slowly in both callus and suspension culture and thus may not have synthesized the same amount of Hyp-rich protein in the cell walls. In addition, these two cultivars, AcB_N and AcIm, have undergone far fewer transfer (<30) than either Im 216 or Ac 44 (>300). These four cell types differ in their resistance to the bacterial pathogen, *X. c. malvacearum*. AcIm and Im 216 each have the three resistant genes B_2, B_3, and b_7 in a polygenic background (the genetic background is different, see the Materials and Methods), while AcB_N had one resistant gene, B_N, and Ac 44 has no resistant genes. AcIm and AcB_N suspension cell walls have strikingly higher Asp, Gly, and Ala (mole%) than do either Ac 44 or Im 216 suspension cell walls.

The Hyp mole% is highest in tomato suspension and tobacco callus cell walls, 28.90% and 24.33%, respectively, while carrot root cell walls have 10.44%. Carrot root cell walls also have a high mole% (content) of Gly (11.12%), Leu (8.70%), Val (7.74%), Ala (7.25%), Glu (7.45%), and Asp (9.77%). Tomato suspension and tobacco callus have abundant Hyp, Asp, Ser, Gly, and Val. The amino acid compositions of the purified HRGPs isolated from carrot root [243], tomato suspension [228], and tobacco callus [168] have been reported. These HRGPs represent only soluble precursors of the mature insoluble cell wall bound HRGP. In studying cotton cell wall protein insolubilized in the cell walls, it is necessary to first study the amino acid composition of all the cell wall hydrolysates. Thus, these data can be compared to tomato suspension, carrot root, and tobacco callus cell wall hydrolysates directly during the preliminary analysis of the cotton suspension cell walls. Later, when the cotton HRGP is isolated its amino acid composition can be compared to that of the purified tomato, tobacco, and carrot HRGPs.

The Hyp and Ser occur in a 4:1 ratio for tomato suspension and tobacco callus cell walls, but 2:1 for Ac 44 and Im 216 cotton suspension and carrot root cell walls (Table 13). A 4:1 ratio is found for the recurrent pentapeptide sequenced by Lamport for a salt-elutable tomato suspension HRGP [72, 151] and the Hyp-rich domain of potato lectin [3]. Since amino acid analyses were conducted on intact cotton suspension cell walls, the results in this study are not directly comparable to amino acid compositions reported for a maize HRGP [131], tomato extensin precursor P1 and P2 [228], a melon HRGP [166], bacterial agglutinin from potato [162], and a tobacco HRGP [168]. However, the amino acid profile of intact melon cell walls [166] shows similar Hyp, Ser, and Gly (mole%) to that of Ac 44 and Im 216 suspension cell walls. The prominant amino acids (mole%) in melon cell walls are Hyp (18.8%), Ser (9.4%), Pro (6,8%), Gly (7.7%), Val (8.7%), and Lys (11.0%). In Im 216 suspension cell walls, the major amino acid component (mole%) are Hyp (18.33%), Asp (9.48%), Ser (8.59%), Gly (6.95%), Ala (7.10%), Leu (7.56%), Glu (6.09%), Lys (5.60%), and Val (5.43%). Thus, preliminary analysis of cultured cotton cell walls strongly suggests that HRGP is present and it is in substantially higher amounts than in Ac 44 leaf cell walls (if present at all).

Hydroxyproline Content

Hydroxyproline content (% Hyp) was determined on a total weight percent basis of the cell wall dry weight by two different analytical methods. Results are shown for the Hyp content of Ac 44 leaf, Ac 44 suspension, Im 216 suspension, AcB_N suspension, AcIm suspension, carrot root, tomato suspension, and tobacco callus cell walls in Table 11. Statistical analysis of the results in Table 14 by the Student's t-test and one-way ANOVA indicated that there is no significant difference between the two analytical methods in calculating the % Hyp in all of the cell walls tested. Additionally, the % Hyp in each of the plant cell walls examined is significantly different from each other at the 95% confidence level. The accumulation of Hyp (w/w) is least in Ac 44 leaf, $\leq$0.10%, and carrot root cell walls, ~0.10%. These two tissues represent the cell walls derived from non-cultured sources and an expected, the Hyp level is at least 3-fold lower than in the cultured cell walls with the lowest % Hyp. Of the four cotton suspension cell walls AcB_N, with one resistance gene, has the least accumulation of Hyp, ~0.34% (w/w) while AcIm (3 resistance genes) suspension cell walls have the greatest accumulation of Hyp, ~0.87% (w/w). It is interesting to note that the cotton cell typeswith no resistance genes to *X. c. malvacearum* , Ac 44, has nearly the same % Hyp (w/w) , ~0.76% as the cotton cell

TABLE 14

COMPARISON OF HYDROXYPROLINE CONTENT IN VARIOUS PLANT CELL WALLS DETERMINED BY TWO DIFFERENT METHODS

	% Hyp	
Wall Sample	Colorimetric Determination*a	Amino Acid Analysis*a
Ac 44 Leaf	0.10 ± 0.0070	0.03
Ac 44 Suspension	0.91 ± 0.12	0.62 ± 0.14**
Im 216 Suspension	0.46 ± 0.066	0.49 ± 0.12**
AcBn Suspension	0.36 ± 0.062	0.31
AcIm Suspension	0.74 ± 0.0050	0.97
Carrot Root	0.072 ± 0.029	0.13
Tomato Suspension	0.93 ± 0.054	1.32
Tobacco Callus	2.22 ± 0.430	3.11

* = % Hyp expressed as the average of 4 determinations ± S.D.
** = % Hyp expressed as the average of 4 different cell wal batches ± S.D.
a = no statistically significant difference (t-test) in % Hyp determined by the two methods at the 95% level of significance.

line with 3 resistance genes to *X. c. malvacearum*, AcIm, ~0.86%. Furthermore, Ac 44 suspension cell walls have approximately twice the % Hyp (w/w) as the other resistant cell line with 3 resistant genes to *X. c. malvacearum*, Im 216 (see Table 14).

Tomato suspension cell walls have ~1% Hyp (w/w) while tobacco callus cell walls have ~2-3% Hyp (w/w). Tobacco callus cell walls accumulate at least twice as much Hyp as any of the other plant cell walls tested. tobacco callus cell walls, therefore are a very good source for extracting HRGP. In fact, a HRGP has been isolated from tobacco callus tissue cultures by an acidic-ethanol extraction procedure [168]. In addition, Hyp-rich (2-3%) melon callus tissue has been used as a source for isolating two HRGPs also using an acidic-ethanol extraction procedure [166]. Stuart and Varner [243] have isolated a salt-extractable HRGP from aerated carrot root discs which was composed of 50% Hyp. Apparently, the HRGP is not bound tightly to the cell wall since in carrot root cell walls (see Table 14), the Hyp-content is only 0.03-0.10% (w/w) of the cell wall and can be easily eluted using salt solutions. Additionally, a salt-extractable maize HRGP has been isolated from suspension culture whose cell walls had only ~0.15% Hyp on a dry weight basis [131]. Precedent has been set and therefore it should be possible to isolate a cotton HRGP from suspension cultures of any of the four cell types since the lowest % Hyp (w/w) was 0.4%.

Lignin Content

The lignin content of Ac 44 leaf, Ac 44 suspension, Im 216 suspension, AcB_N suspension, AcIm suspension, carrot root, tomato suspension, tobacco callus cell walls and the Ac 44 HF 0°C water insoluble residue was determined using a colorimetric method using acetyl bromide (see Materials and Methods, Johnson *et. al.* [124]) and results are shown in Table 15. Lignin content is expressed as a percent of the dry weight of the cell walls using ferulic acid as a standard. The most lignified cell walls examined were from AcIm suspension cultures, 4.58 ± 0.22%, while the least lignified cell walls examined were from Ac 44 suspension cultures, 0.98 ± 0.034%, a 4.7-fold difference. When Ac 44 suspension cell walls were treated with HF solvolysis at 0 °C, there is a 3-fold increase in lignin content (%). The lignin content may have increased on a percent basis due to the nearly complete deglycosylation of cell wall protein and because liquid-phase HF solvolysis is a useful technique for determining the lignin composition of the cell walls [176]. Thus, HF solvolysis of the cell walls at 0° C would leave the residual lignin shell in the water insoluble residue. The Ac 44 leaf cell walls, AcB_N suspension cell walls, and

TABLE 15

LIGNIN ANALYSIS OF VARIOUS PLANT CELL WALLS

Sample	Lignin %**
Cotton Samples	
Ac 44 Leaf	4.04 ± 0.45
Ac 44 suspension	0.98 ± 0.034
AcBn suspension	4.10 ± 0.13
Im 216 suspension	2.35 ± 0.21
AcIm suspension	4.58 ± 0.22
Ac 44 HF 0°C Water Insoluble Residue*	3.10 ± 0.11
Non-Cotton Samples	
Carrot root	0.97 ± 0.10
Tobacco callus	3.82 ± 0.11
Tomato suspension	2.86 ± 0.41
Wood chips	11.10 ± 2.38

*= HF solvolysis of Ac 44 cell walls is discussed in the next section of this chapter.

** = Lignin % expressed as average of 2 determinations ± S.D.

tobacco callus cell walls all have ~4% (w/w) lignin, while Im 216 suspension cell walls have ~2.4% (w/w) lignin and tomato suspension cell walls have ~2.9% lignin. Carrot root cell walls lignin is essentially the same as for Ac 44 suspension cell walls, 0.97 ± 0.10% and 0.98 ± 0.034% (w/w), respectively.

The lignin contents of this study are generally in good agreement with results reported previously [141] for Ac 44 suspension cell walls (~1%), Im 216 suspension cell walls (~1%), and Ac 44 leaf cell walls (~5%). It seems apparent that there is a significant difference in the cell wall lignin content between the blight-susceptible cotton cell line, Ac 44 and the blight-resistant cotton cell lines, AcIm, AcB_N, and Im 216. The lignin content of all 3 resistant cell typesis 2 to 4 times greater than in the susceptible cultivar. This difference has been shown to be statistically significant (see Figures 38 and 39). Thus, increased lignification in the cell walls of resistant cell lines may prevent pathogen from spreading to uninfected plant cells and causing plant disease as well as explain the increased difficulty in isolating protoplasts from these cell lines (Table 2).

Hydrogen Fluoride Solvolysis of Ac 44 and Im 216 Cotton Suspension Cell Walls at 0°C - Characterization of the Fractions and Recovery of Hydroxyproline-Rich Proteins

Introduction

Relatively little information is available concerning the cell wall proteins except for the structural glycoprotein, extensin. Extensins are Hyp-rich cell walls glycoprotein (HRGPs) implicated in a structural/mechanical function in strengthening the plant cell walls [31, 32, 33, 34, 40, 238, 243], control of plant growth [134, 212], and in disease resistance [71]. Work related to studying extensin has been reviewed extensively in chapter 2 of this dissertation. Past isolations of cell walls HRGPs (extensin) have concentrated on the soluble precursors since extensin is highly insolubilized by extensive phenolic cross-links in the cell wall [152]. Even reagents such as anhydrous HF failed to solubilize extensin [177]. Recent data supports this hypothesis that extensin is insolubilized by extensive phenolic cross-links: first, isolation of tomato extensin tryptides contained the cross-linked amino acid isodityrosine [70], and second, monomeric tomato extensin precursors were extracted from cell walls of rapidly growing intact cells

[228], and from an isolated carrot cell wall fraction where a time-dependent insolubilization of extensin precursor occurred [52]. Assembly of extensin thus occurs *in muro* . Preliminary attempts to directly extract these glycoproteins from intact cotton (Ac 44, AcB_N, Im 216) suspension cell walls with salt solutions failed to solubilize significant amounts of Hyp-rich protein. This same failure was observed by Mazau and associates [166] during salt-extraction of HRGPs from intact melon cell walls. These researchers chose an acidic extraction procedure which led to the recovery of a HRGP from a melon callus homogenate of 0.4 mg of bound Hyp per gram (dry weight) of melon callus.

This section describes the use of anhydrous HF solvolysis of cotton suspension cell walls at 0°C to purify cell wall proteins after deglycosylation. A description of the information obtained from the products of HF solvolysis of cell walls from Ac 44 and Im 216 suspension cultures at 0°C is given here. Fractions of Ac 44 and Im 216 suspension cell walls treated by HF solvolysis at 0°C are further purified and characterized to isolate Hyp-containing cell wall protein, possibly HRGP.

Solvolysis of Ac 44 Cotton Suspension Cell Walls at 0°C

Fractionation and Recovery of Hydroxyproline. Ac 44 suspension cell walls (500 mg) were treated by HF solvolysis at 0°C for 30 min. The reaction was stopped by the addition of ether. The procedure of the fractionation of Ac 44 cell walls is shown in scheme I (Figure 40). Summary of the fractionation of Ac 44 cell walls after HF solvolysis at 0°C and recovery of the Hyp in each fraction by two different analytical methods is shown in Table 16. Analysis of 3 separate cell wall HF treatments indicated that 55.9 ± 13.4% of the original weight of the cell walls was recovered in the HF/ether soluble fraction and 44.7 ± 14.3% in the ether residue. The ether residue was suspended in distilled water and extracted three times with water. After water extraction, about 19.7 ± 5.8% of the original weight of the walls became soluble in water and about 10.4 ± 1.5% of the original weight of the walls was recovered as the insoluble residue. Only an average of 53.8% of the weight of the ether insoluble residue was recovered after water extraction. HF solvolysis at 0°C removes nearly all sugars from the cell walls including those in cellulose. This temperature then is of particular interest in this study because HF solvolysis at 0°C deglycosylates cell wall proteins potentially making them easier to isolate, purify, and characterize. In this study, the cell wall protein of particular interest is HRGP, since it has well-established roles in maintaining the structural integrity of the cell

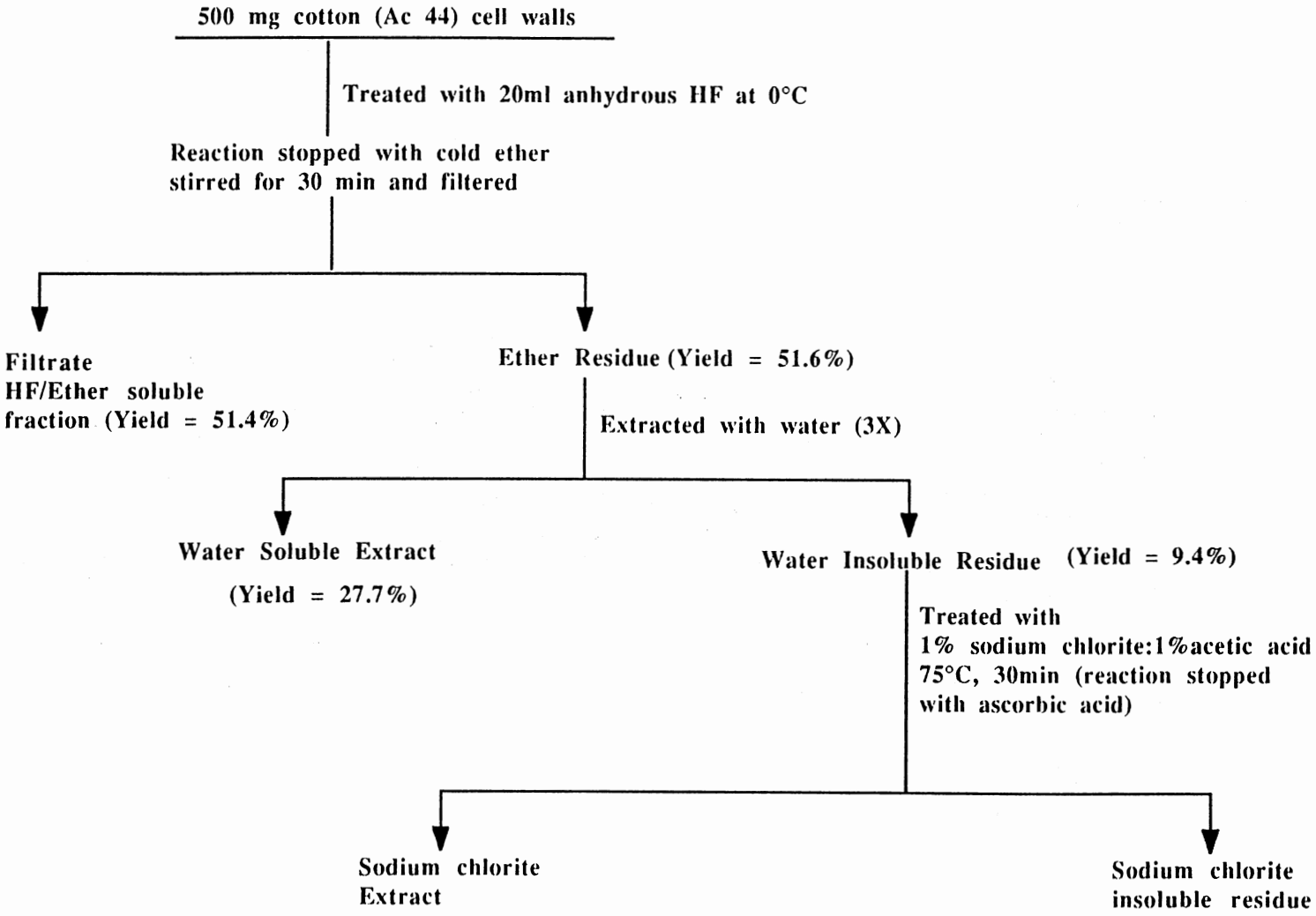


Figure 40. Scheme 1 for HF Solvolysis and Fractionation of Ac 44 Cell Walls at 0ºC

TABLE 16

FRACTIONATION OF AC 44 CELL WALLS USING HF SOLVOLYSIS AT 0°C AND RECOVERY OF HYDROXY-PROLINE BY TWO DIFFERENT METHODS

Fractions from Ac 44 cell walls after HF solvolysis at 0° C	Treatment Number	Fraction Weight (mg)	% Recovery of starting material	% Hyp in each fraction*	Weight (mg) Hyp in each fraction*	% Hyp Recovery of starting material*
Untreated Ac 44 Cell Walls (Starting Material)	1	500.0	100.0	0.478 ± 0.056 (0.539 ± 0.041)	2.39 (2.70)	100.0 (100.0)
Ether Insoluble Residue	1	258.0	51.6	1.132 ± 0.068	2.92	122.2
	2	210.8	42.0			
	3	370.0	74.0			
	Ave. ± S.D.	279.6 ± 66.8	55.9 ± 13.4			
HF/Ether Soluble Extract (Water Soluble Portion)	1	256.9	51.4	0.224 ± 0.100 (0.000)	0.58 (0.00)	24.3 (0.0)
	2	289.0	57.8			
	3	124.2	24.8	(0.000)	(0.00)	(0.0)
	Ave. ± S.D.	223.4 ± 71.3	44.7 ± 14.3			
Water Insoluble Residue	1	46.8	9.4	2.876 ± 0.073 (4.486)	1.34 (2.27)	56.1 (84.1)
	2	62.5	12.5	3.722 ± 0.113 (3.904)	2.33 (2.44)	97.5 (90.4)
	3	46.4	9.3	(2.828)	(1.31)	(48.5)
	Ave. ± S.D.	51.9 ± 7.5	10.4 ± 1.5	3.299 ± 0.0.423	1.84 ± 0.50	76.8 ± 20.8
	(Ave. ± S.D.	(51.9 ± 7.5)	(10.4 ± 1.5)	(3.739 ± 0.687)	(2.01 ± 0.50)	(74.3 ± 18.5)
Water Soluble Extract	1	138.4	27.7	0.267 ± 0.031 (0.0054)	0.37 (0.0075)	15.5 (0.3)
	2	70.2	14	0.612 0.0142 (0.0143)	0.43 (0.010)	18.0 (0.4)
	3	87.3	17.5	(0.0109)	(0.010)	(0.4)
	Ave. ± S.D.	98.6 ± 29.0	19.7 ± 5.8	0.440 ± 0.172	0.40 ± 0.03	16.8 ± 1.3
	(Ave. ± S.D.	98.6 ± 29.0	(19.7 ± 5.8)	(0.0102 ± 0.0037)	(0.009 ± 0.001)	(0.37 ± 0.05)

* = % Hyp determined by a colorimetric method (Data expressed as averages ± S.D. for 4 determinations (see Materials and Methods) and by amino acid analysis (values are in parentheses).

Note: Fraction wt. and % Recovery of starting material, averages ± S.D. for 3 cell wall batches.

wall and plant defense to pathogen attack (see literature review in chapter 2 for further details).

Therefore, each of the isolated fractions was tested for Hyp in an attempt to identify Hyp-rich fractions for further purification (see Table 16). From the colorimetric analysis of Hyp (see Materials and Methods), the starting Ac 44 cell wall material has 0.478 ± 0.056% (w/w) Hyp on a cell wall dry weight basis. The amino analysis of the the starting Ac 44 cell walls indicated there was 0.539 ± 0.041 Hyp % (w/w). These two different analyses of the Ac 44 cell walls differ by about 10% in their estimation of Hyp and thus seem quite comparable. 24.3% (w/w) of the Hyp is found in the ether soluble fraction using the colorimetric analysis, but 0% of the Hyp is recovered using amino acid analysis. Perhaps, there is some compound in the HF/ether soluble fraction reacting with the Chloramine T to give a false report of Hyp. In contrast, the ether insoluble residue shows a 122.2% recovery of Hyp (colorimetric analysis), clearly in excess of the Hyp % (w/w) in the starting Ac 44 cell walls indicating some inaccuracy in the colorimetric determination of Hyp. Generally speaking the amino acid analysis is thought to more accurate than the colorimetric analysis of Hyp, although the colorimetric assay has a sensitivity in the μg range.

In any event, the ether insoluble residue is extracted with water and the majority of the Hyp is recovered in the water insoluble residue, 76.8 ± 20.8% (w/w) (colorimetric analysis) or 74.3 ± 18.5% (w/w) (amino acid analysis), of the starting cell wall Hyp. The Hyp content is 3.299 ± 0.423% (w/w) of the water insoluble residue. This Hyp enrichment is >6-fold from the starting cell wall material. Only 16.8 ± 1.3% (w/w) of the Hyp is recovered in the water soluble extract determined by the colorimetric analysis or 0.37 ± 0.05% determined by amino acid analysis. Clearly, the target fraction for isolating Hyp-rich protein, perhaps HRGP, is the water insoluble residue. Yet, the water insoluble residue by definition is not soluble in water and thus, the omnipresent problem of purifying intact cell wall HRGP is still not resolved. Even after deglycosylation of Ac 44 cell walls using HF solvolysis at 0°C, the cell wall protein is still insoluble. This fact has been noted previously in the unsuccessful attempt to solubilize tomato suspension cell glycoprotein using HF solvolysis at 0°C [173, 177].

Use of Sodium Chlorite Oxidation to Solubilize Ac 44 Cell Walls and Ac HF 0°C Water Insoluble Residue Hyp-rich Proteins. As just mentioned, Ac 44 cotton suspension cell wall protein was not solubilized by HF solvolysis at 0°C. One of the reasons for this

is the extensive cross-linking of the cell wall protein via phenolic compounds to form an insoluble network. This hypothesis has been supported by several pieces of evidence: 1) the identification of a tyrosine derivative called isodityrosine [85] in tryptic peptides of extensin [70] and 2) the release of soluble extensin glycopeptides after acid chlorite oxidation of cell walls [173, 180], and 3) extraction of monomeric extensin precursors from tomato cell walls of rapidly growing intact cells [228] and from monomeric extensin precursors from the isolated carrot cell wall fraction where a time-dependent insolubilization of extensin precursor occurred [52]. In addition, it has been proposed that peroxidases could catalyze the formation of a cross-linked extensin network *in muro* [153, 155].

Due to the insolubility of Ac 44 cell wall proteins in the water insoluble residue after HF solvolysis at 0°C, a mild sodium chlorite/acetic acid procedure was used to isolate the Hyp-rich protein. This method has been used successfully to solubilize cell wall glycoprotein by several groups of researchers [173, 177, 180, 218, 219]. Sodium chlorite oxidation is a selective way to destroy phenols and thus break the indicated network of cross-linked glycoproteins in the cell walls and solubilize the glycoproteins. Sodium chlorite oxidation has been most often used to delignify wood [214], Previously, O'Neill and Selvendran [180] have used sodium chlorite/acetic acid for isolating HRGP from depectinated parenchyma cell wall material of runner beans.

A time course of sodium chlorite oxidation of Ac 44 cell walls and Ac HF 0°C water insoluble residue is presented in Figure 41. Sodium chlorite oxidation differs in Ac 44 cell walls (glycosylated) and Ac HF 0°C water insoluble residue (deglycosylated). From Table 13, there is a higher Hyp% in the Ac 44 HF 0°C water insoluble residue than in Ac 44 cell walls indicating that perhaps the water insoluble residue is more accessible to sodium chlorite oxidation thus releasing more Hyp. From the analysis of variance, this difference is statistically significant at the 95% confidence level. From the results, Hyp is released into the sodium chlorite extract after 10 min of treatment with sodium chlorite/acetic acid. There is an increase in % Hyp (w/w) from 0.95% to 1.1% after 10 min in the Ac 44 HF 0°C water insoluble residue sodium chlorite extract. This is accompanied by a rapid decrease in % Hyp in the sodium chlorite residue of the Ac 44 HF 0°C water insoluble residue from 0 min to 10 min of 2.6% to 0.91% (w/w). This indicates that a Hyp-rich protein is being solubilized during sodium chlorite oxidation and phenolic crosslinks are being broken either between protein-glycoprotein or glycoprotein-

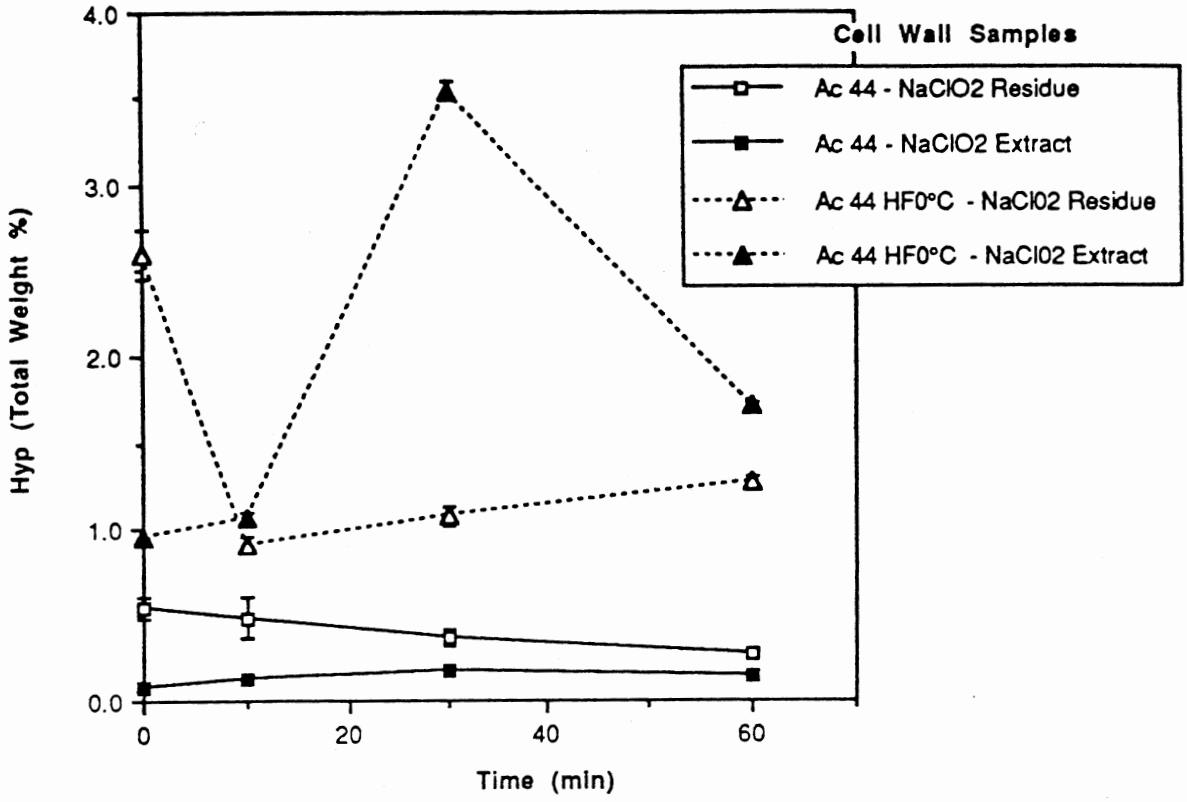


Figure 41. Time Course of Sodium Chlorite Oxidation of Ac 44 Suspension Cell Walls and Ac 44 HF 0°C Water Insouble Residue. 1.00 mg of Ac 44 cell walls and 100 µg of Ac 44 HF 0°C water insoluble residue were treated with 1% sodium chlorite: 1% acetic acid solution for 0, 10, 30, and 60 min. Sodium chlorite oxidation was measured as a function of hydroxyproline (total weight %) (w/w) of the cell wall dry weight present in each fraction. Duplicate determinations of Hyp were performed after acid hydrolysis.

polysaccharides. This result reinforces the hypothesis that HRGP is insolubilized *in muro* by extensive phenolic cross linkages.

Maximal solubilization of the Hyp-rich cell wall protein is seen after 30 min in the sodium chlorite extracts of both Ac 44 cell walls and Ac HF 0°C water insoluble residue. At this time, the percent of Hyp-rich material is 3.5% (w/w) in the sodium chlorite extract of Ac HF 0°C water insoluble residue and only 0.2% (w/w) in the sodium chlorite extract of Ac 44 cell walls. At 0 min, the percent of Hyp-rich cell wall protein in the sodium chlorite extracts was 0.95% (w/w) in the Ac 44 HF 0°C water insoluble residue and 0.083% in the Ac 44 cell walls. The increase in % Hyp in sodium chlorite extracts is matched with a corresponding decrease in % Hyp in the sodium chlorite residues. At 0 min, the sodium chlorite residue of Ac 44 cell walls had 0.55% (w/w) Hyp and after 30 min only 0.27% (w/w) Hyp. At 0 min, the sodium chlorite residue of Ac 44 HF 0°C water insoluble residue had 2.6% (w/w) Hyp and after 30 min only 1.3% Hyp. Thus, there is a net increase in Hyp in the sodium chlorite extracts of 50% after 30 min and a net decrease of 50% in Hyp in the sodium chlorite residues. Therefore, sodium chlorite oxidation solubilizes 50% of the cotton HRGP or deglycosylated HRP. Since there is a greater percentage of Hyp in the sodium chlorite extract of Ac 44 HF 0°C water insoluble residue, this material was used in further isolation of a cotton HRP.

For quantitative isolation of a cotton cell wall HRP, 15.0 mg or 30.0 mg of Ac 44 HF 0°C water insoluble residue was treated with 1% sodium chlorite: 1% acetic acid at 75°C for 30 min. The general scheme for sodium chlorite oxidation of Ac 44 HF 0°C water insoluble residue and subsequent biochemical analyses of the sodium chlorite soluble extract and sodium chlorite insoluble residue is shown in Figure 42. Summary of the fractionation of Ac 44 HF 0°C water insoluble residue after sodium chlorite oxidation and recovery of Hyp in each fraction by two different methods is presented in Table 17. Analysis of two different sodium chlorite treatments indicated that there was some variability in the recovery of the two fractions: the sodium chlorite soluble extract and the sodium chlorite residue and the recovery of Hyp within the two fractions. Recovery of the sodium chlorite extract is 23.3% (w/w) and 51.7% (w/w) of the starting weight compared to the recovery of the sodium chlorite residue is 36.0% (w/w) and 19.0% (w/w) of the starting weight, respectively, for the two treatments. The second treatment seems more reliable and agrees well with the time course of sodium chlorite oxidation shown in Figure 42. A control sample of Ac HF 0°C water insoluble residue was treated with 2% acetic acid at 75°C, for 30 min. Recovery was not comparable to the sodium chlorite treatment.

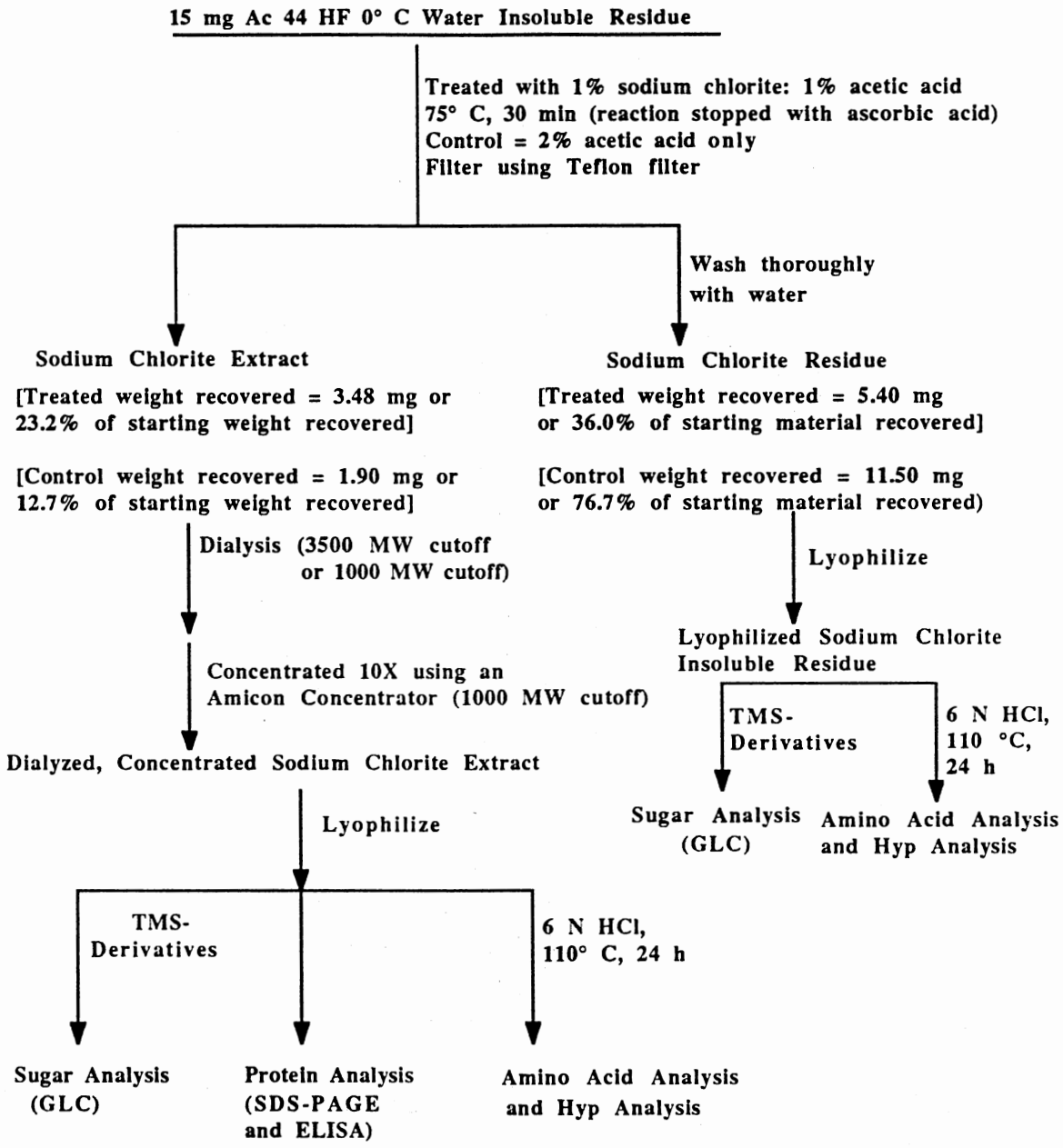


Figure 42. Scheme II for Sodium Chlorite Oxidation of Ac 44 HF 0° Water Insoluble Residue

TABLE 17

FRACTIONATION OF AC 44 HF 0°C WATER INSOLUBLE RESIDUE AFTER SODIUM CHLORITE OXIDATION AND RECOVERY OF HYDROXYPROLINE BY TWO DIFFERENT METHODS

Fractions from Ac 44 cell walls after HF solvolysis at 0° C	Treatment Number	Fraction Weight (mg)	% Recovery of starting material	% Hyp in each fraction*	Weight (mg) Hyp in each fraction*	% Hyp Recovery of starting material*
Ac 44 HF 0°C Water Insoluble Residue (Starting Material)	1	15.0	100.0	2.876 ± 0.073 (4.486)	0.431 (0.729)	100.00 (100.00)
	2	30.0	100.0	2.876 ± 0.073 (4.486)	0.863 (1.346)	100.00 (100.00)
Sodium Chlorite Extract						
Control (2% Acetic Acid)	1	1.9	12.7	0.315 ± 0.0246	0.006	1.39
Treated (1% Sodium Chlorite: 1% Acetic Acid)	1	3.5	23.3	3.608 ± 0.506 (0.254)	0.126 (0.00889)	29.23 (1.22)
	2	15.5	51.7	(7.167)	(1.111)	(82.54)
Sodium Chlorite Residue						
Control (2% Acetic Acid)	1	11.5	76.7	2.240 ± 0.530	0.258	59.86
Treated (1% Sodium Chlorite: 1% Acetic Acid)	1	5.4	36.0	1.906 ± 0.322 (1.363)	0.103 (0.0736)	23.90 (10.10)
	2	5.7	19.0	(2.159)	(0.1231)	(9.14)
Eluate from Amicon Filter for concentration of Sodium Chlorite Extract						
Control (2% Acetic Acid)	1	1.9	12.7	0.031 ± 0 .001	0.000589	0.14
Treated (1% Sodium Chlorite: 1% Acetic Acid)	1	3.5	23.3	0.054 ± 0.002	0.00188	0.44

* = % Hyp determined by a colorimetric method (see Materials and Methods), data expressed as averages ± S.D. for 4 determinations and by amino acid analysis (values are in parentheses).

Approximatley 12.7% of the starting material was recovered in the extract and 76.7% was recovered in the residue. This of course means that 2% acetic acid does not solubilize the HRP of Ac 44 HF 0°C water insoluble residue, but the 1% sodium chlorite: 1% acetic acid does solubilize the Hyp-rich cell wall protein. Lastly, during concentration of the sodium chlorite extract only 0.44% of the Hyp (w/w) was lost.

Using the colorimetric determination of Hyp, recovery of Hyp in the sodium chlorite extract is 82.5% (w/w) of the starting material for the second treatment, but only 29.2% (w/w) for the first treatment. The amino acid analysis determination of Hyp does not agree well with the colorimetric determination of Hyp for the first treatment. Recovery of Hyp in the sodium chlorite residue is 9.2% (w/w) of the starting material for the second treatment and 23.9% (w/w) for the first treatment. Only 1.4% (w/w) of the starting material Hyp was recovered in the control 2% acetic acid extract, but 59.9% (w/w) of the Hyp was recovered in the control 2% acetic acid residue. It is obvious that a significant proportion of Hyp is solubilized by sodium chlorite oxidation after 30 min at 75°C and the % Hyp is more than triple that in the sodium chlorite residue, 7.2% (w/w) to 2.2% (w/w) (amino acid analysis) (see Table 17).

The premise that sodium chlorite would solubilize Ac 44 (cotton) Hyp-rich cell wall protein is proven to be correct by the results presented in Figures 41 and Table 17. In fact, 50% (w/w) (Figure 41) to 82% (w/w) (Table 14) of the Hyp was released and recovered in the sodium chlorite extract. O'Neill and Selvendran (1980) found that 80% (w/w) of the Hyp was released at 70°C after two 15 min treatments with 0.12% (v/v) acetic acid and 0.3% sodium chlorite.

Analysis of the Ac 44 HF 0°C Water Soluble Extract and Sodium Chlorite Extract of the Ac 44 HF 0°C Water Insoluble Residue by High Performance Liquid Chromatography: Gel Exclusion Chromatography. High performance liquid chromatography (HPLC): gel exclusion chromatography was used to determine the molecular weights of putative proteins in the Ac HF 0°C water soluble extract (water soluble extract) and the sodium chlorite soluble extract of Ac 44 HF 0°C water insoluble residue (sodium chlorite extract). The HF solvolysis scheme for Ac 44 cell walls at 0°C is shown in Figure 40 and for the sodium chlorite fractionation scheme refer to Figure 42. A mixture of protein standards of known molecular weights were resolved by gel exclusion HPLC. These standards were as follows in order of decreasing MW: salmon testes DNA ($>10^6$), thyroglobulin (6.7×10^5), bovine serum albumin (6.9×10^4), ovalbumin ($4.4 \times$

10^4), myoglobulin (1.72 X 10^4), glycyl tyrosine (2.38 X 10^2). A molecular weight (MW) standard curve was constructed from their elution pattern and the MWs of the Ac 44 HF 0°C water soluble extract and sodium chlorite extract protein peaks (UV-absorbing) were resolved from their elution time.

The HPLC trace of the water soluble extract on a TSK SW 2000 gel filtration column is shown in Figure 43. It showed that there are 3 major UV-absorbing peaks of low MWs. Peak 1 had a MW of ~6500 d, Peak 2 has a MW of ~5100 d, and Peak 3 has a MW of ~673 d. There is a heterogeneous mixture of higher MW proteins with MWs of ≤1,72 X 10^4 d, but are not well-defined and are in low concentrations. These proteins are not resolved by HPLC even at an attenuation of 0.02 full-scale. The protein concentration determined by the Lowry method of the water soluble extracts is very low, 0.093 μg μl^{-1}, while the protein concentration determined from the UV-absorbance at 280 nm is 0.676 μg μl^{-1}, a 7.3-fold difference in the 2 assays. These determinations indicate that the HPLC gel exclusion peaks in Figure 43 may not be protein, but just UV-absorbing low molecular weight material, even at an attenuation of 0.02 full-scale. The HPLC gel filtration analysis does not adequately resolve heterogeneous mixture of proteins that are in the Ac 44 HF 0°C water soluble extract at low concentrations.

The HPLC trace of the sodium chlorite extract on a TSK SW 2000 gel filtration column is shown in Figure 44. It showed that there is a heterogeneous mixture of proteins beginning with MWs in the range of thyroglobulin, 6.7 X 10^5 d. These proteins are not resolved by HPLC gel filtration even at an attenuation of 0.02 full-scale because they may be a very low concentrations. There are possibly two peaks corresponding to low molecular weight UV-absorbing material, probably not protein, in the MW range of 5000-7000 d. These same low MW UV-absorbing material peaks were observed in the Ac 44 HF 0°C water soluble extract (see Figure 43). The protein concentration of the sodium chlorite soluble extract of the Ac 44 HF 0°C water insoluble residue is 0.64 μg μl^{-1} from the Lowry protein determination and 0.668 μg μl^{-1} from the UV absorbance at 280 nm. These two protein measurements are in good agreement with each other and suggest that the material examined in the sodium chlorite extract is protein, at least for those peaks in a MW range >7000 d. Again, the HPLC gel filtration analysis of the sodium chlorite extract doe not adequately resolve any proteins of interest. Potential HRPs (deglycosylated) would be in the range of at least 55,000 d [166].

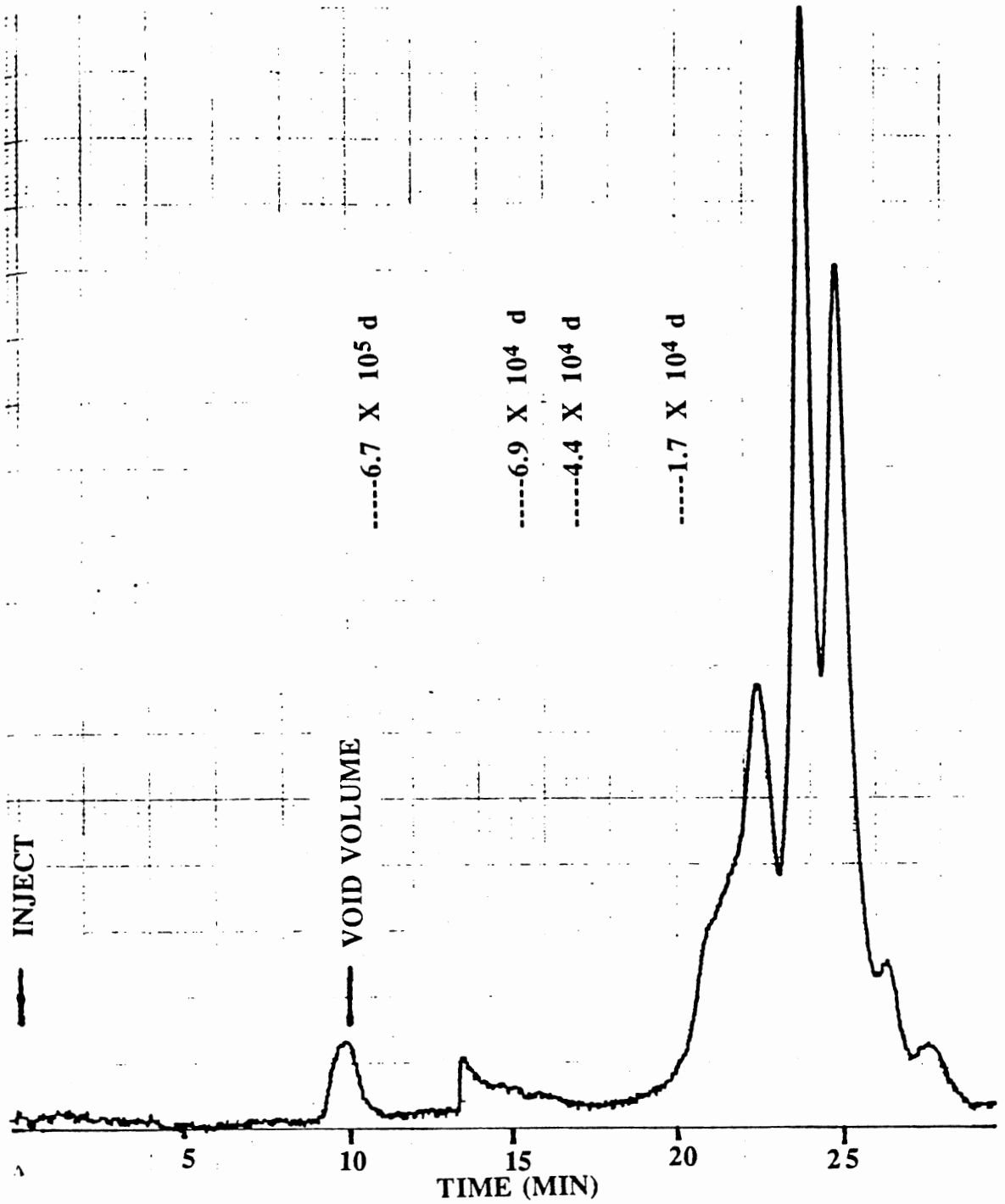


Figure 43. HPLC Chromatogram of the Ac 44 HF 0°C Water Soluble Extract on a TSK SW 2000 Gel Filtration Column (7 5 mm X 30 cm). The elution buffer was 0.3 M sodium acetate, pH 5.2 and the flow rate was 0.5 ml/min. Detection was by UV absorbance at 280 nm. The sample was dissolved in the elution buffer, filtered using a 0.45 µ filter, and 50 µl were injected.

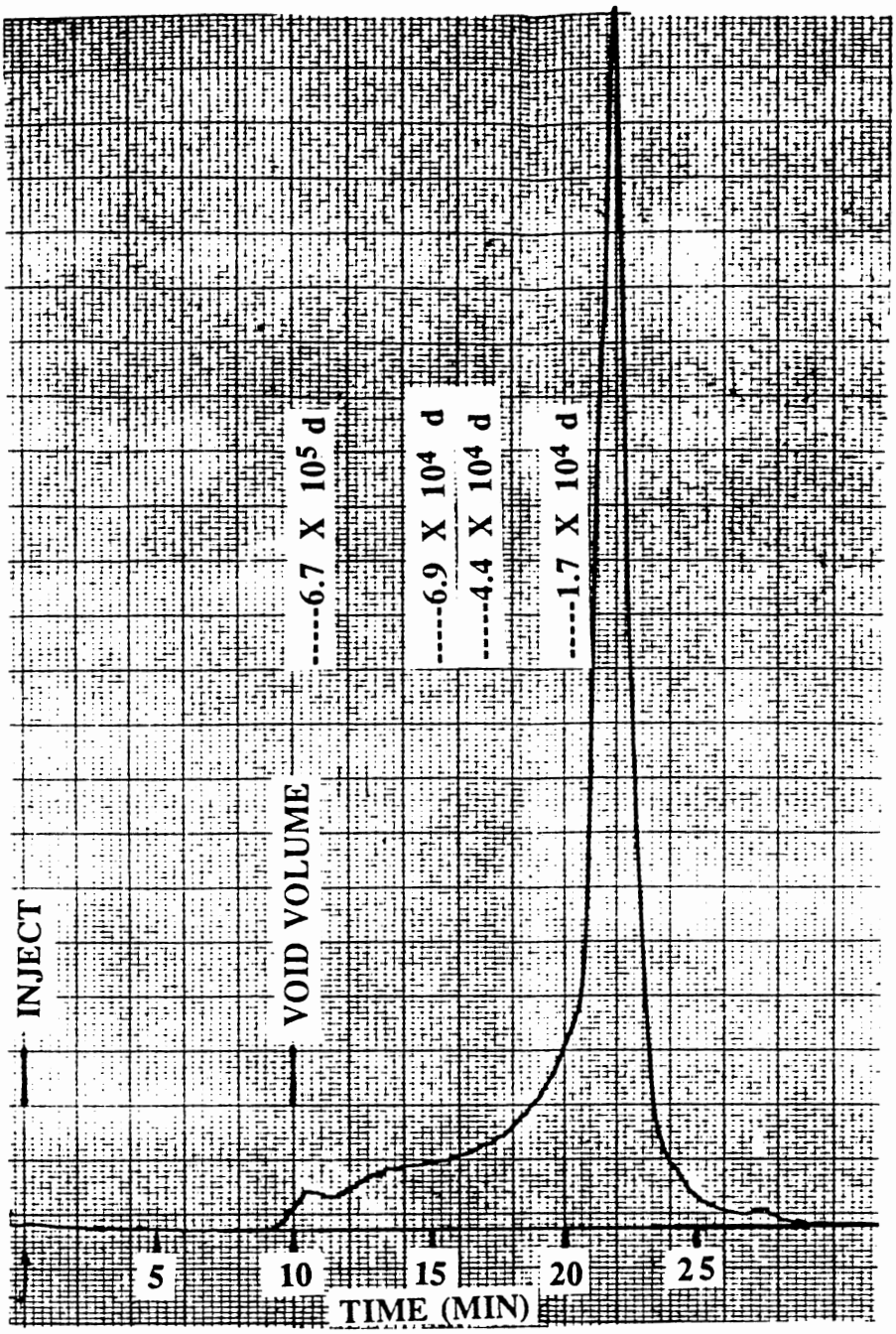


Figure 44. HPLC Chromatogram of the Sodium Chlorite Extract of Ac 44 HF 0°C Water Insoluble Residue on a TSK SW 2000 Gel Filtration Column (7.5 mm X 30 cm). The elution buffer was 0.3 M sodium acetate, pH 5.2 and the flow rate was 0.5 ml/min. Detection was by UV absorbance at 280 nm. The sample was dissolved in the elution buffer, filtered using a 0.45 μ filter, and 50 μl were injected.

Amino Acid Composition of Different Fractions of Ac 44 Cell Walls Following HF Solvolysis at 0°C and Sodium Chlorite Oxidation. The amino acid compositions of the different fractions of Ac 44 cell walls following HF solvolysis at 0°C and sodium chlorite oxidation were determined by amino acid analysis and results are given in Table 18. Ac 44 suspension cell walls are rich in Hyp (16.33 ± 3.20 mole%), Asp (8.53 ± 0.50 mole%), Ser (7.88 ± 0.79 mole%), Gly (7.06 ± 0.27 mole%), Leu (8.03 ± 0.73 mole%), Lys (7.37 ± 0.13 mole%), Ala (5.80 ± 0.40 mole%), and Val (6.66 ± 0.31 mole%). These 8 amino acids compose nearly 70% of the total protein recovered after amino acid analysis in the Ac 44 cell walls. The recovered protein is 3.36 ± 0.93% (w/w) of the Ac 44 cell walls.

The usefulness of HF solvolysis at 0°C to deglycosylate cell wall proteins and isolate HRGP is dependent on avoiding destruction of the Hyp and therefore the Hyp-rich protein during the fractionation. Amino acid analysis shows that little protein is extracted by ether, 0.014 ±0.002% (w/w) and more importantly, Hyp is not in this fraction. The water insoluble residue had a Hyp mole % of 15.68 ± 1.78% and then is treated with sodium chlorite to solubilize the Hyp-rich protein. The water soluble extract has a Hyp mole% of 1.63 ± 0.24%. The water insoluble residue contains 22.49 ± 1.69% (w/w) protein while the water soluble extract contains 0.60 ± 0.25% (w/w) protein. Thus, most of the protein and especially the Hyp-rich protein is recovered in the Ac 44 HF 0°C water insoluble residue. The overall amino acid composition of the intact Ac 44 suspension cell walls and Ac 44 HF 0°C water insoluble residue are nearly identical. This indicates that the protein material in both have the same relative composition. It is especially interesting to note that the Hyp mole% of the Ac 44 suspension cell walls and Ac 44 HF 0°C water insoluble residue are virtually equal, 16.33 ± 3.20% and 15.68 ± 1.78%, respectively. There is far less Hyp in the water soluble extract, but greater amounts of Ser, Gly, and Lys than in the water insoluble residue and Ac 44 suspension cell walls.

During sodium chlorite/acetic acid treatment free ε-amino groups of lysine undergo oxidation, yielding α-aminoadipic acid (O'Neill and Selvendran, 1980) which co-chromatographs with lysine. About 30% of the lysine is oxidized after 30 min, hence the Lys from the amino acid analysis contains both Lys and α-aminoadipic acid. For the sodium chlorite soluble extract, the protein content was calculated to be 14.76 ± 6.66% (w/w) from the recovery of the amino acids. Amino acid composition of the water insoluble residue and the sodium chlorite extract is shown in Table 18. The mole% of Ser and Hyp increase substantially in the sodium chlorite extract. The two sodium chlorite

TABLE 18

AMINO ACID COMPOSITION OF AC 44 CELL WALLS BEFORE AND AFTER HF SOLVOLYSIS AT 0°C

		FRACTIONS AFTER HF SOLVOLYSIS AT 0°C OF AC 44 SUSPENSION CELL WALLS				
Amino Acid Mole %	Ac 44 Suspension	Water Insol. Residue	Water Soluble Extract	Sodium Chlorite Ox. Residue	Sodium Chlorite Ox. Extract	Ether Extract-Water Soluble Portion
Hyp	16.33 ± 3.20	15.68 ± 1.78	1.63 ± 0.24	12.30 ± 3.21	17.36 ± 14.44 (31.80*)	0.00 ± 0.00
Asp*	8.53 ± 0.50	8.33 ± 0.46	9.33 ± 2.42	9.32 ± 1.15	10.79 ± 1.42 (9.39)	6.86 ± 6.86
Thr	4.13 ± 0.21	4.19 ± 0.26	4.65 ± 0.59	3.94 ± 0.56	4.09 ± 0.91 (3.18)	4.52 ± 0.69
Ser	7.88 ± 0.79	9.98 ± 0.92	16.32 ± 3.95	7.74 ± 0.52	12.10 ± 1.48 (10.61)	16.10 ± 3.24
Glu*	5.73 ± 0.34	5.83 ± 0.87	9.32 ± 2.38	7.10 ± 0.69	7.50 ± 1.72 (5.78)	21.79 ± 3.48
Pro	5.20 ± 0.32	5.72 ± 0.86	4.76 ± 2.15	6.05 ± 0.37	6.91 ± 0.53 (6.38)	0.00 ± 0.00
Gly	7.06 ± 0.27	7.64 ± 0.16	14.44 ± 1.86	8.96 ± 1.06	8.04 ± 0.82 (7.21)	14.55 ± 14.55
Ala	6.80 ± 0.40	7.20 ± 0.12	8.37 ± 0.33	7.88 ± 0.20	6.06 ± 1.32 (4.71)	17.54 ± 17.54
Val	6.66 ± 0.31	6.62 ± 0.20	5.02 ± 0.59	6.94 ± 0.44	5.05 ± 1.50 (3.55)	0.00 ± 0.00
Met	0.90 ± 0.15	0.31 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 (0.00)	0.76 ± 0.76
Ile	3.88 ± 0.49	3.41 ± 0.28	2.63 ± 0.78	4.61 ± 0.00	2.54 ± 0.92 (1.61)	0.00 ± 0.00
Leu	8.03 ± 0.73	7.47 ± 0.58	5.48 ± 2.01	10.03 ± 0.47	4.97 ± 1.49 (3.48)	0.00 ± 0.00
Tyr	2.71 ± 0.32	1.58 ± 0.14	1.41 ± 0.65	0.12 ± 0.05	0.58 ± 0.16 (0.41)	0.00 ± 0.00
Phe	3.28 ± 0.38	2.63 ± 0.15	1.82 ± 0.53	3.70 ± 0.01	1.72 ± 0.70 (1.01)	0.00 ± 0.00
His	2.32 ± 0.05	2.33 ± 0.13	1.94 ± 0.07	1.94 ± 0.18	2.18 ± 0.19 (1.99)	0.00 ± 0.00
Lys	7.37 ± 0.13	8.25 ± 0.53	10.26 ± 3.07	6.40 ± 0.88	7.37 ± 1.00 (6.37)	17.88 ± 9.70
Arg	3.17 ± 0.11	2.80 ± 0.15	2.62 ± 0.89	2.97 ± 0.57	2.70 ± 0.26 (2.51)	0.00 ± 0.00
Total	100.00	100.00	100.00	100.00	100.00 (100.00)	100.00
% Protein	**3.36 ± 0.93**	**22.49 ± 1.69**	**0.60 ± 0.25**	**15.31 ± 6.99**	**14.76 ± 6.66 (21.42)**	**0.014 ± 0.002**

Asp* = denotes Asp + Asn, during hydrolysis Asn is converted to Asp.
Glu* = denotes Glu + Gln, during hydrolysis Gln is converted to Glu
Note: Cys and Trp were not determined. Data values are presented as the Average ± S.D. for 2-3 reps (batches).
**Disparate values for Hyp mole% from the 2 treatments 2.92% and 31.80%, thus used only the second treatment.

extracts had extremely disparate values for the Hyp mole%, 2.92% and 31.80%, respectively. The first treatment underestimated the amount of Hyp. Since the analysis of Hyp during amino acid analysis is done separately and correlated with the other amino acids (see Materials and Methods), I must conclude that this Hyp value is wrong and makes the other amino acid compositions artificially high.

In the second treatment, 31.80 mole% of Hyp was observed in the sodium chlorite extract. The relative molar ratio of Hyp (31.80%) to Ser (12.10%) for the sodium chlorite soluble glycoprotein (deglycosylated by HF solvolysis at 0°C) from cotton suspension cell walls is nearly 2.63:1 which is in excellent agreement with results reported for the chlorite-soluble glycoprotein from runner beans, (35.3%/13.9% = 2.54:1) [180] and from the chlorite-soluble glycoprotein from tomato suspension culture cells (37.5%/12.6% = 2.98:1) [177]. The amino acids, Hyp and Ser constitute 42.4% of the total in the Ac 44 HF 0°C sodium chlorite extract. As noted earlier, the HRGP is tenaciously bound to the cell wall as an insoluble complex [104, 218, 219]. Therefore, sodium chlorite extraction of the Ac 44 HF 0°C water insoluble residue releases the insoluble HRGP (mostly deglycosylated HRP after HF solvolysis at 0°C) with minimal modification immediately ready for further purification. O'Neill and Selvendran [180] proposed from the known action of sodium chlorite/acetic acid on phenolic or polyphenolic material that the HRGP is held in the cell wall by phenolic cross-links. Yet, these phenolic cross-links cross-links may not involve the formation of isodityrosine residues as has been reported previously [70, 85] since there is a reduced proportion of Tyr, 0.41 mole% in the Ac 44 HF 0°C sodium chlorite extract versus an average of 9.8 mole% reported in carrot extensin-1. Reduced Tyr, 3.9 mole% was also observed for carrot extensin-2 [237]. A noteworthy feature of the chlorite-soluble glycoprotein from cotton suspension cell walls is its similarity in amino acid composition with material solubilized by chlorite treatment of runner bean cell walls [180] and suspension-cultured tomato cells [173]. Thus, the chlorite treatment may act in a specific way to solubilize the HRGPs. Further fractionation of the chlorite-soluble glycoprotein from runner beans by ion-exchange chromatography removed the uronic acids and enabled the isolation of 3 distinct groups of HRGPs [180]. Three distinct groups of HRGPs were also observed from the incorporation of radioactive amino acids into wall glycoproteins and kinetic studies of the rate of radioactive labelling [187]. Further fractionation of the sodium chlorite extract of HF-treated Ac 44 cotton suspension cell walls is indicated from these results in order to isolate cotton HRGP(s).

Determination of the function(s) of the cell-wall glycoprotein(s) depends, in part, on their association with other cell-wall polymers. Evidence from this research (sodium chlorite oxidation treatment) suggests that the cotton suspension cell wall HRGP is cross-linked within the cell wall by phenolic cross-links. These phenolic cross-links could be between polysaccharides and the HRGP or between the HRGP and other cell wall proteins. No direct covalent linkage of glycoproteins to polysaccharides has been demonstrated. Thus, if phenolic cross-links exist between the glycoprotein and polysaccharides (making the HRGP insoluble) then sodium chlorite/acetic acid treatment would disrupt them rendering the glycoprotein in soluble form.

Sugar Composition of Different Fractions of Ac 44 Cell Walls following HF Solvolysis at 0°C and Sodium Chlorite Oxidation. The sugar compositions of each of the different fractions isolated after HF solvolysis at 0°C and sodium chlorite oxidation was determined by gas chromatographic analysis of the TMS-derivatives. Results are shown in Table 19. HF solvolysis at 0°C deglycosylates cell wall proteins by removing almost all sugars from the walls including those in cellulose. Thus, the glucose in all fractions treated with HF solvolysis at 0°C represents both cellulosic and non-cellulosic glucose. Plain methanolysis of walls gives only the non-cellulosic glucose composition. Therefore, without any HF treatment, 36.8 ± 1.4% (w/w) of the Ac 44 suspension cell walls was recovered as sugars. The untreated Ac 44 cell walls have 5 times the molar percent of GalA (35%) as Glc (7%). After HF treatment, 56.4 ± 9.99% (2 reps with 2 determinations each) (w/w) of the Ac 44 suspension cell walls was recovered as sugars. Treatment of the Ac 44 cell walls with HF at 0°C increases the relative mole percent of glucose 5-fold. This indicates that 80% of all the Glc in the cell wall is part of cellulose. Glucose is 45.1 ± 1.7% (w/w) of the total cell wall sugar. After HF treatment, GalA mole% decreases to 13%, while the Glc mole% increases to 40%. In addition, the relative molar contributions of GalA and Glc together as a function of the total sugar content increases by 10%. Besides the Glc and GalA compositions changing after HF treatment at 0°C, the other relative mole percents do not change significantly.

At least 86.4 ± 11.8% (w/w) of the ether soluble fraction (water soluble portion) is accounted for by sugars. This fraction is especially rich in glucose,~48 mole% of the total sugar content. Recovery of sugars in the HF/ether fraction is expected since solvolysis at 0°C cleaves nearly all possible sugar linkages (see Table 1). The sugar composition of total cell wall material treated with HF at 0°C except that the relative mole percent of GalA drops by 50% from 19.1% to 8.9% and the relative mole percents of Xyl and Glc each

TABLE 19

MOLE PERCENT OF SUGARS IN DIFFERENT FRACTIONS OF AC 44 CELL-WALL MATERIAL AFTER HF-SOLVOLYSIS AT 0°C

Material	Cell Batch	Percent of original cell wall weight recovered	Apparent percent sugar in sample	Ara	Rha	Fuc	Xyl	GalA	Man	Gal	Glc
Whole cell walls (15 d)	1	100.0	36.8 ± 1.4	16.4 ± 0.5	9.2 ± 0.2	1.9 ± 0.1	19.0 ± 1.0	35.0 ± 0.0	0.5 ± 0.1	9.9 ± 0.4	7.9 + 0.3
Cell wall after HF 0°C*	1	100.0	66.3 ± 1.2	12.4 ± 0.6	7.3 ± 0.2	1.3 ± 0.1	14.2 ± 0.6	17.9 ± 1.3	0.4 ± 0.0	7.2 ± 0.2	39.4 ± 1.7
	2	100.0	46.6 ± 0.8	9.4 ± 0.0	7.4 ± 0.0	1.2 ± 0.0	12.8 ± 0.0	20.2 ± 0.4	0.6 ± 0.0	6.4 ± 0.4	41.9 ± 0.2
Ether-fraction water soluble portion	1	58.0	98.0 ± 3.0	12.6 ± 0.2	8.4 ± 0.2	1.6 ± 0.0	15.7 ± 0.5	10.2 ± 0.2	0.4 ± 0.0	6.8 ± 0.1	44.2 ± 1.0
	2	24.8	75.0 ± 2.2	10.0 ± 0.6	12.0 ± 3.4	1.4 ± 0.0	16.4 ± 0.8	7.6 ± 0.4	0.4 ± 0.0	5.6 ± 0.3	46.6 ± 1.4
Water soluble extract	1	27.7	72.6 ± 7.4	5.9 ± 0.1	4.4 ± 0.1	0.8 ± 0.0	6.3 ± 0.2	45.2 ± 0.2	0.5 ± 0.1	4.9 ± 0.1	32.0 ± 0.4
	2	14.0	67.9 ± 4.3	3.4 ± 0.4	2.2 ± 0.0	0.5 ± 0.1	4.4 ± 0.0	71.5 ± 0.0	Trace	2.6 ± 0.2	15.4 ± 0.7
	3	17.5	62.0 ± 20.8	7.0 ± 0.0	5.1 ± 0.0	0.8 ± 0.0	8.1 ± 0.3	50.8 ± 0.4	0.4 ± 0.2	5.1 ± 0.2	22.6 ± 0.0
Residue after water extraction	1@	12.5	45.1 ± 5.9	1.8 ± 0.0	0.7 ± 0.0	0.2 ± 0.0	2.6 ± 0.0	87.2 ± 0.4	0.4 ± 0.0	1.1 ± 0.0	5.9 ± 0.3
	2	9.3	46.4 ± 7.2	3.2 ± 0.4	1.5 ± 0.2	0.4 ± 0.0	4.1 ± 0.4	80.0 ± 1.2	0.2 ± 0.2	1.6 ± 0.3	9.1 ± 0.3
Sodium chlorite extract	1**	6.5	27.0 ± 3.0	3.8 ± 0.8	1.4 ± 0.2	0.6 ± 0.4	2.7 ± 0.6	82.7 ± 0.4	0.2 ± 0.0	1.0 ± 0.2	7.3 ± 1.0
	2***	2.1		1.3 ± 0.0	0.8 ± 0.0	---	4.4 ± 0.2	83.6 ± 2.4	---	Trace	10.0 ± 2.6
Residue after sodium chorite extraction	1**	3.3	43.6 ± 1.2	0.8 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	1.0 ± 0.0	93.0 ± 0.4	0.2 ± 0.0	1.2 ± 0.8	3.4 ± 0.4
	2***	2.4	50.9 ± 0.9	1.3 ± 0.0	0.4 ± 0.0	Trace	2.2 ± 0.2	89.6 ± 0.5	0.2 ± 0.2	1.0 ± 0.4	5.2 ± 0.9

Sugar compositions of each fraction were determined after methanolysis and trimethyl-silylation (see Materials and Methods).
All the samples marked with an * were given a 15-min treatment in HF at 0°C to solubilize cellulose and any other sugar polymers resistant to methanolysis. Cell walls were all prepared from suspension cells harvested in late log phase 15-20 days after the culture was initiated). The starting material for the sodium chlorite treated fractions marked ** and *** were from two different preparations of the Ac 44 HF 0°C water insoluble residue by HF solvolysis at 0°C.
All fractions were derived from the same batch of Ac 44 whole cell walls except for the fractions marked by an @.
Data is expressed as the average ± S.D. for 2 determinations/rep.

increased by 5%. A significant portion of the Ara, Rha, Fuc, Xyl, Man, Gal, and even Glc (non-cellulosic) have been solvolytically cleaved at 0°C. These sugar monomers are found in the HF/ether soluble fraction. Thus, the polymers like arabinogalactan, arabinan, galactan, xyloglucan are broken down into their respective monomers. Those sugars that remain after HF solvolysis at 0°C are either water soluble and are recovered in the water soluble extract or are water insoluble or unable to be solvolytically cleaved at 0°C and are recovered in the water insoluble residue. Approximately 67.4 ± 13.7% (w/w) of the water soluble extract is accounted for by sugars, while 45.8 ± 6.6% (w/w) of the water insoluble residue is accounted for by sugars.

The major sugar components of both the water insoluble residue and water soluble extract are GalA and Glc. GalA and Glc account for 80% of all the sugars left in the water soluble extract and 92% of all the sugars left in the water insoluble residue. Thus, uronic acid linkages and certain glucosidic linkages are not labile to HF solvolysis at 0°C. Therefore, it is possible to conclude that the GalA-rich portion of pectin (homogalacturonan) was not broken down. The sugar compositions of each of the fractions analyzed (excluding sodium chlorite fractions) after HF solvolysis at 0°C are quite similar to those obtained by Komalavilas [141].

The sodium chlorite extract and sodium chlorite insoluble residue sugar compositions are quite similar to the sugar composition of the water insoluble residue. This is expected since sodium chlorite/acetic acid treatment oxidizes phenolic cross-links, but does not affect the polysaccharides themselves nor the glycoprotein. Thus, the sodium chlorite soluble extract is about 27.0 ± 3.0% (w/w) sugar and the sodium chlorite insoluble residue is 47.2 ± 3.8% (w/w) sugar. Like the water insoluble residue and the water soluble extract, the 2 major sugar components of the sodium chlorite extract and sodium chlorite insoluble residue are GalA and Glc. These two sugars account for 96% and 92%, respectively, of all the sugar in these fractions.

Therefore, when isolating a HRGP from the Ac 44 HF 0°C water soluble extract, water insoluble residue or sodium chlorite extract, it is important to remember that ~40.0% of the fraction weight is GalA. The presence of GalA may affect the migration of the protein(s) during SDS- polyacrylamide gel electrophoresis (SDS-PAGE). Even if there is incomplete solubilization of the proteins by SDS, the presence of GalA would give the proteins negative charges and allow migration in SDS-PAGE, although resolution of the

proteins may inadequate and the apparent MW may differ if the proteins were devoid of GalA.

Solvolysis of Im 216 Cotton Suspension Cell Walls at 0°C

Fractionation and Recovery of Hydroxyproline. Im 216 suspension cell walls (500 mg) were treated like Ac 44 suspension cell walls with HF solvolysis at 0°C for 30 min. The reaction was stopped by the addition of ether. The procedure for fractionation of the Im 216 cell walls is shown in scheme III (Figure 45). Summary of the fractionation of Im 216 cell walls after HF solvolysis at 0°C and recovery of Hyp in each fraction by two different analytical methods is shown in Table 20.

Analysis of the results in Table 20 indicates that 70.5% (w/w) of the original weight of the Im 216 suspension cell walls was recovered in the HF/ether soluble fraction. This value seems to be quite high since 6.8% (w/w) of the original weight of the walls was recovered in the water insoluble residue and 31.5% (w/w) of the weight of the walls was recovered in the water soluble extract. Results from the fractionation of the Ac 44 suspension cell wall (Table 13) indicate that ~56% (w/w) of the original weight of the Ac 44 suspension cell walls was recovered in the HF/ether soluble fraction. More of the Im 216 suspension cell walls were recovered after HF solvolysis at 0°C in the water soluble extract and water insoluble residue combined, 38.3% (w/w) than in the Ac 44 HF 0°C water soluble extract and water insoluble residue combined, 30.1% (w/w).

As noted earlier during the discussion of the solvolysis of Ac 44 cotton suspension, HF solvolysis at 0°C nearly completely deglycosylates cell wall proteins which makes them easier to isolate, purify, and characterize. The cell wall protein of interest is a HRGP. Therefore, each of the isolated fractions were tested for Hyp in an attempt to identify Hyp-rich fractions for further purification of Im 216 HRGP (see Table 20).

The starting Im 216 cell wall material has 0.455 ± 0.66% (w/w) Hyp based on the colorimetric analysis and 0.555% (w/w) Hyp based on the amino acid anlaysis. The Im 216 cell walls and the Ac 44 cell walls have virtually the same % Hyp (w/w) (see Tables 16 and 20). The colorimetric and amino acid analyses are generally in agreement, ~18% difference, in their respective determination of % Hyp (w/w) in the cell wall and thus seem comparable. Approximately 37.3% of the Hyp is recovered in the HF/ether soluble

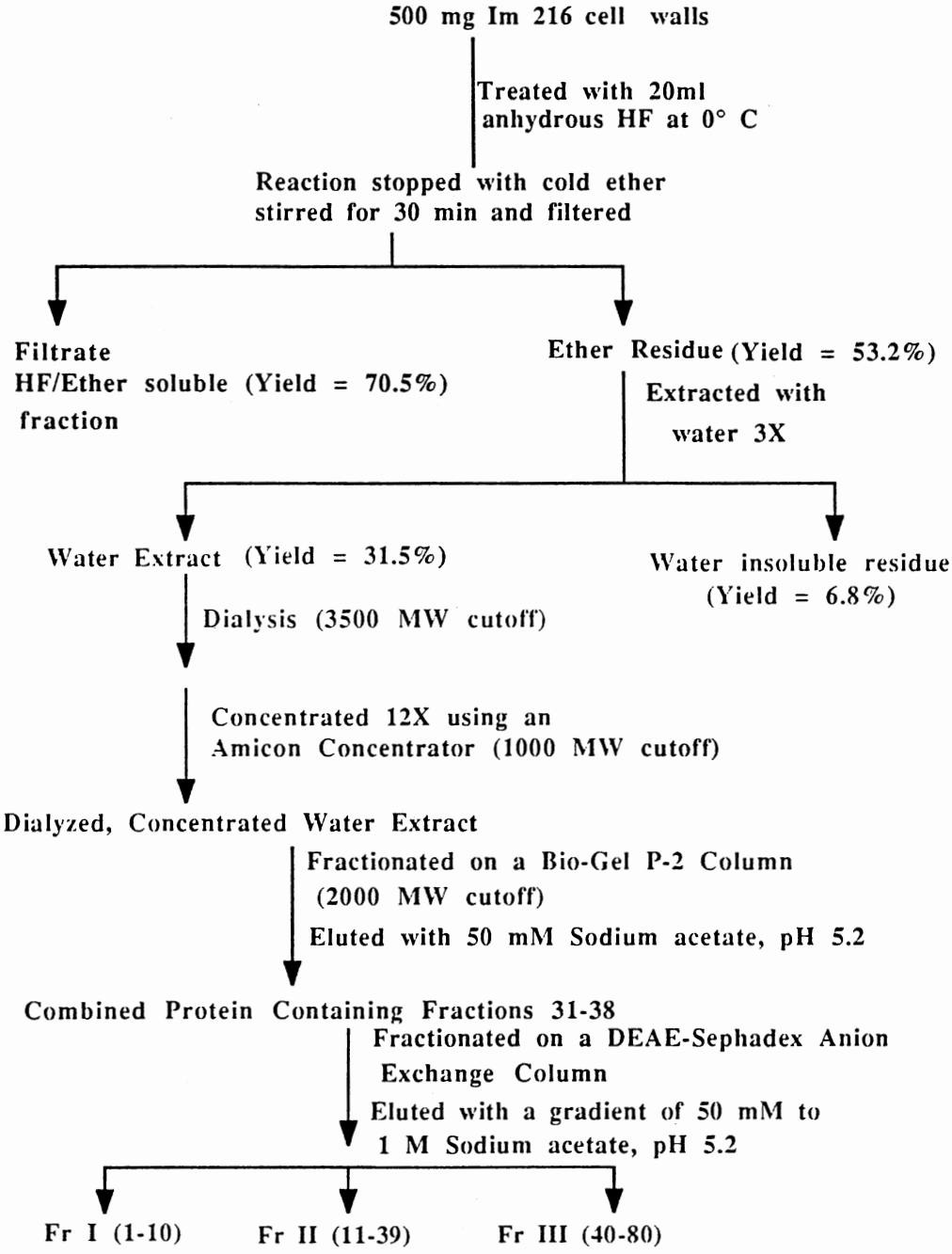


Figure 45. Scheme III for HF Solvolysis and Fractionation of Im 216 Cell Walls at 0° C

TABLE 20

FRACTIONATION OF IM 216 CELL WALLS USING HF SOLVOLYSIS AT 0°C AND RECOVERY OF HYDROXY-PROLINE TWO DIFFERENT METHODS

Fractions from Im 216 cell walls after HF solvolysis at 0° C	Fraction Weight (mg)	% Recovery of starting material	% Hyp in each fraction*	Weight (mg) Hyp in each fraction*	% Hyp Recovery of starting material*
Untreated Im 216 Cell Walls (Starting Material)	500.0	100.0	0.455 ± 0.066 (0.555)	2.28 (2.78)	100.0 (100.0)
HF/Ether Soluble (Water Soluble Portion)	352.2	70.5	0.242 ± 0.0796 (0.000)	0.85 (0.00)	37.3 (0.0)
Water Insoluble Residue	34.0	6.8	4.430 ± 0.842 (2.750)	1.51 (0.94)	66.2 (33.6)
Water Soluble Extract	157.5	31.5	0.317 ± 0.070	0.499	21.9
Water Soluble Extract - Dialyzed and Concentrated	41.3	8.3	0.294 ± 0.0658 (0.0450)	0.121 (0.0071)	5.3 (2.6)
Combined P2 Column Fractions of Dialyzed and Concentrated Water Extract	5.1	1.0			
DEAE Sephadex Fractions - Dialyzed of Combined P2 Fractions					
I (1-10)	1.6**	<1.0**	0.104 ± 0.005	0.0017	0.074
II (11-39)	4.7**		0.100 ± 0.0338	0.0047	0.21
III (40-80)	11.4**		0.079 ± 0.029	0.0090	0.39

* = % Hyp determined by a colorimetric method (see Materials and Methods), data expressed as averages ± S.D. for 4 determinations and by amino acid analysis (values are in parentheses).

** = Weight recovered is misleading, that is the total weight recovered from the DEAE Sephadex column must be < 5.1 mg if the weight of the combined P2 column fractions is calculated correctly to be 5.1 mg.

fraction using the colorimetric analysis to determine % Hyp, but no Hyp is recovered in the HF/ether soluble fraction using the amino acid analysis to determine % Hyp. Perhaps there is some compound in the HF/ether soluble fraction reacting with the chloramine T to give false values of Hyp.

Approximately 66.2% (w/w) of the Hyp according to the colorimetric assay is recovered in the water insoluble residue. The amino acid anlaysis gives a recovery of 33.6% (w/w). Approximately 21.9% (w/w) of the Hyp determined by colorimetric analysis is recovered in the water soluble extract. The amino acid anlaysis gives a recovery of 2.6%. The Hyp content of the water insoluble residue is 4.430 ± 0.842% (w/w) (colorimetric analysis), this value marks a Hyp-enrichment of nearly 10-fold from the Hyp% 0.455 ± 0.066% (w/w) of the starting material. The target fraction for isolating Hyp-rich proteins is the water insoluble residue just as was seen for the Ac 44 suspension cell walls after HF solvolysis at 0°C. Again, the water insoluble residue by definition is insoluble in water and thus makes isolation of a HRP (mostly deglycosylated after HF solvolysis at 0°C) very difficult if not impossible.

Analysis of the Im 216 HF 0°C Water Soluble Extract. This time a slightly different approach was used to isolate Hyp-rich cell wall proteins. Instead of treating the water insoluble residue with sodium chlorite/acetic acid to solubilize the cell wall HRGP or HRP (deglycosylated), the Im HF 0°C water soluble extract was dialyzed, concentrated, and put through a Bio-gel P2 gel filtration column and a DEAE-Sephadex (anion-exchange) column in order to purify the Hyp-rich protein containing fractions. A side note, this experimental attempt at isolating an Im 216 cotton suspension cell wall HRGP/HRP was done prior to sodium chlorite/acetic acid solubilization of Ac 44 suspension cell walls or Ac 44 HF 0°C water insoluble residue. As a reminder, the water soluble extract after HF solvolysis at 0°C of Im 216 suspension cell walls was found to be about 31.5% (w/w) of the total cell walls. The total weight percent of sugar in this fraction was 47.04 ± 9.25% (w/w).

Fractionation of the Im 216 HF 0°C water soluble extract (2.0 ml of the 6.6 ml concentrated extract) on a Bio-gel P2 column is shown in Figure 46. Fractions 31-38 were dialyzed and lyophillized and the weight recovered (5.1 mg) was 1.0% (w/w) of the starting Im 216 suspension cell walls. The protein in Fractions 31-38 was determined to be 500 μg. Thus, there is ~10% (w/w) protein in the combined P2 fractions.

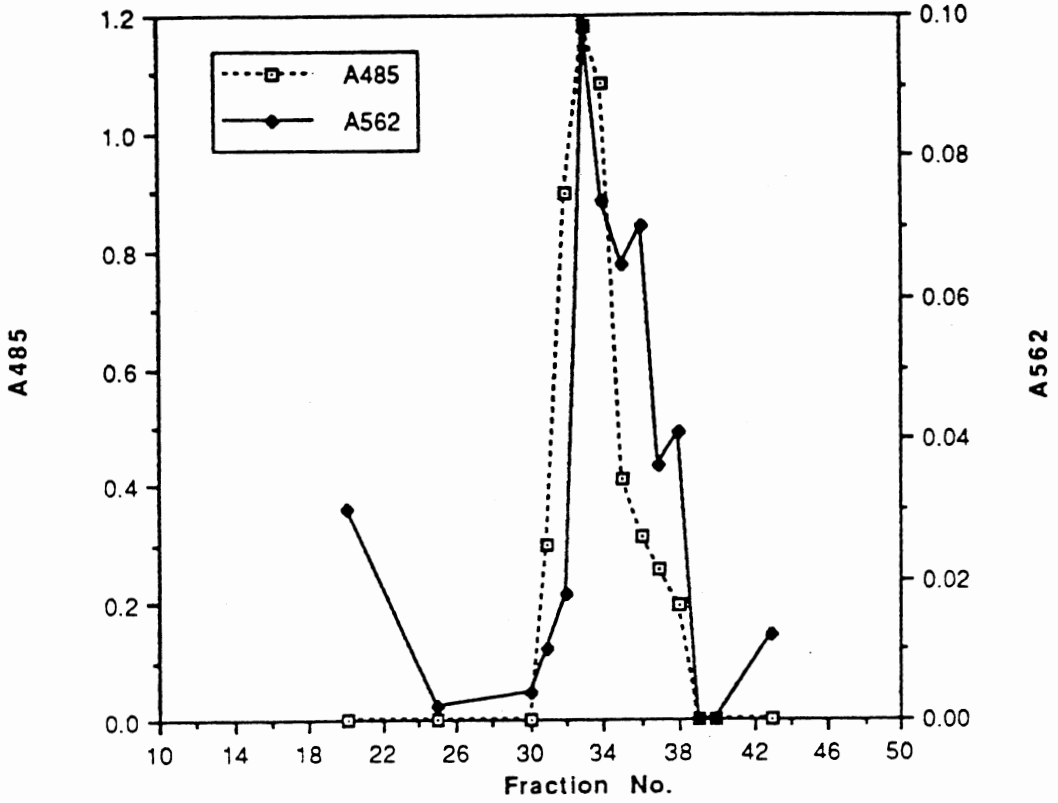


Figure 46. Chromatography on a Bio-gel P2 (62 cm X 2.5 cm) Gel Filtration Column of the Water Extract from Im 216 Cotton Suspension Cell Walls after HF Solvolysis at 0°C in 0.05 M Sodium Acetate, pH 5.2. Two ml of the dialyzed and concentrated Im 216 HF 0°C water soluble extract was applied to the column. 2.5 ml fraction were collected. 75 µl of the fractions were tested for sugar by the phenol sulfuric acid test (A485). One hundred µl of the fractions were tested for protein by the bicinchoninic acid (BCA) assay (A562). Protein-containing fractions 31-38 were pooled, dialyzed, and lyophilized (5.1 mg).

The combined P2 fractions containing ~500 μg protein were further purified using a DEAE-Sepahdex (anion-exchange) column (Figure 47). Protein was eluted using a gradient of 0.05 M to 1.0 M sodium acetate, pH 5.2. Fractions (2.5 ml) were collected and analyzed for protein and sugar. Fraction I (Tubes 1-10) contained neutral sugars, Fraction II contained the major protein peaks and the acidic sugars, polygalacturonic acid, and Fraction III contained miscellaneous protein and sugars eluted at high ionic strength (1.0 M sodium acetate, pH 5.2). Each of the combined DEAE-Sepahdex fractions was dialyzed and lyophillized. Lyophillized combined DEAE-Sepahdex fractions I, II, and III were analyzed for Hyp using the colorimetric method of Drozdz *et. al.* [67]. Results are presented in Table 20. Each of the three fractions contained ~0.100% (w/w) Hyp. Fraction III had the greatest % Hyp recovered, but it was only 0.39% of the total Hyp in the starting material. DEAE-Sephadex combined Fractions I, II, and II were electrophoresed by SDS-PAGE, but no proteins were discernable.

Amino Acid Composition of Different Fractions of Im 216 Cell Walls following HF Solvolysis at 0°C. The amino acid composition of the different fractions of Im 216 cell walls following HF solvolysis at 0°C was determined by amino acid anlaysis and results are given in Table 21. Im 216 suspension cell walls are rich in Hyp (15.30 ± 5.32 mole%), Asp (9.09 ± 0.64 mole%), Ser (7.72 ± 0.62 mole%), Gly (7.54 ± 0.55 mole%), Ala (6.93 ± 0.39 mole%), Leu (8.24 ± 1.05 mole%), Lys (6.36 ± 0.54 mole%), Glu (6.43 ± 0.79 mole%), and Val (6.17 ± 0.54 mole%). These 9 amino acids compose nearly 74% of the total protein recovered after amino acid analysis in the Im 216 suspension cell walls. The recovered protein was 3.62 ± 0.77% (w/w) of the Im 216 suspension cell walls. The amino acid composition of Im 216 suspension cell walls (Table 21) is clearly analogous to the amino acid composition of the Ac 44 suspension cell walls (Table 18).

The usefulness of HF solvolysis at 0°C to deglycosylate cell wall proteins is extremely important, but for isolating HRGP/HRP it is essential not to destroy Hyp during the fractionation. Amino acid analysis (Table 21) shows essentially no protein in the Im 216 HF 0°C ether fraction (water soluble portion), 0.005% (w/w) of the Im 216 suspension cell walls. More importantly, there is no Hyp in this fraction, all the Hyp-rich protein is in either the Im 216 HF 0°C water insoluble residue or water soluble extract. Note: the Im 216 cell walls used for the HF solvolysis at 0°C had 18.33 mole% Hyp. The Im 216 HF 0°C water insoluble residue had a Hyp mole% of 8.13% while the Im 216 HF 0°C water soluble extract had a Hyp mole% of 10.18%. However, the overall amino

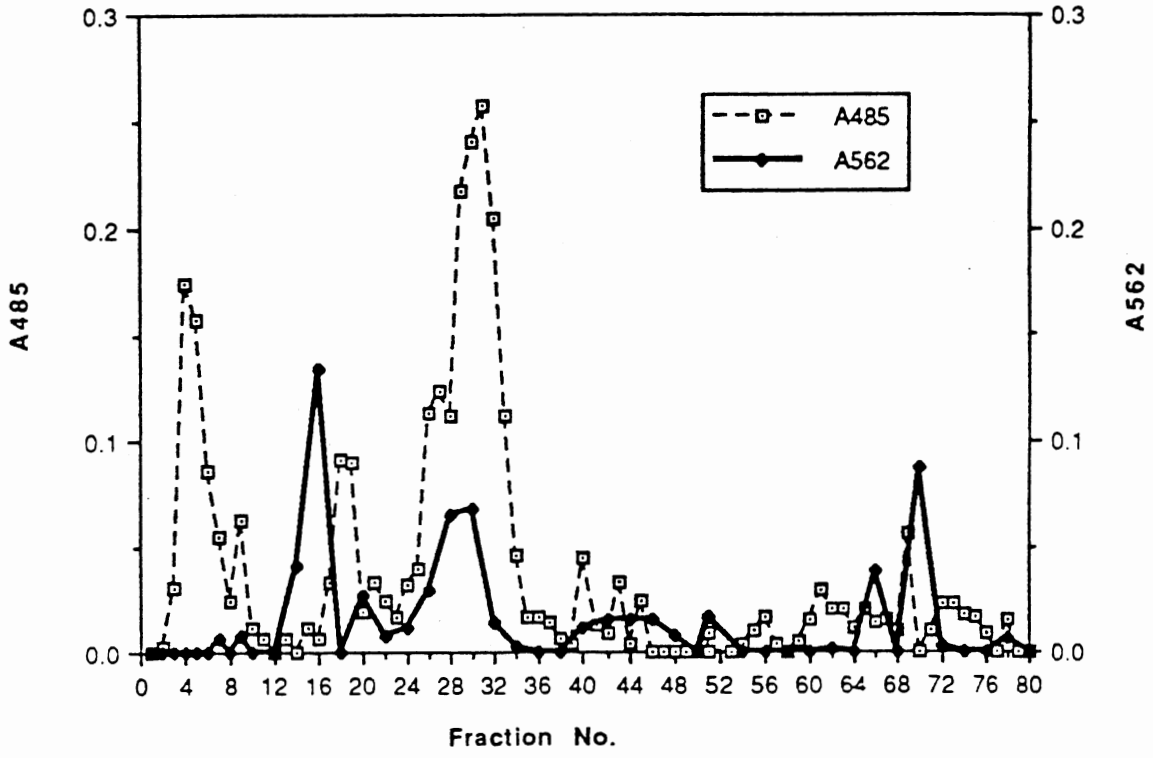


Figure 47. Chromatography on a DEAE-Sephadex (Anion-Exchange) Column (30 cm X 1 cm) of the Combined Protein Fractions 31-38 of the Bio-gel P2 Column of the Water Extract from Im 216 Cotton Suspension Cell Walls after HF Solvolysis at 0°C using a Gradient of 0.05 M to 1.00 M Sodium Acetate, pH 5.2. The lyophillized combined protein Bio-gel P2 fraction, 5.1mg, was dissolved in 1.0 ml of 0.05 M sodium acetate, pH 5.2 and applied to the column. Fractions of 2.5 ml were collected. Seventy-five µl of the fractions were tested for sugar by the phenol sulfuric acid test (A485). One hundred µl of the fractions were tested for protein by the BCA assay (A562). Tubes 1-10 were pooled as Fraction I (neutral sugars), Tubes 11-39 were pooled as Fraction II (major protein peaks and acidic sugars), and Tubes 39-80 were pooled as Fraction III (miscellaneous protein and sugar at very low levels). Fractions I, II, and III were dialyzed and lyophilized. Quadruplicate determinations of Hyp were performed on each lyophilized fraction after acid hydrolysis.

TABLE 21

AMINO ACID COMPOSITION OF IM 216 SUSPENSION CELL WALLS BEFORE AND AFTER HF SOLVOLYSIS AT 0°C

Amino Acid Mole %	Im 216 Suspension*	Im 216 HF 0°C Water Insol. Residue	Im 216 HF 0°C Water Sol. Extract	Im 216 HF 0°C Ether Fraction-Water Sol. Portion
Hyp	15.30 ± 5.31	8.13	10.18	0.00
Asp*	9.09 ± 0.64	10.59	11.70	9.38
Thr	4.07 ± 0.29	4.57	5.09	12.95
Ser	7.72 ± 0.62	9.31	11.20	33.58
Glu*	6.43 ± 0.79	8.08	4.58	0.00
Pro	5.35 ± 0.65	5.62	6.62	44.09
Gly	7.54 ± 0.55	7.82	11.70	0.00
Ala	6.93 ± 0.39	7.46	8.40	0.00
Val	6.17 ± 0.54	6.18	6.11	0.00
Met	0.93 ± 0.24	1.03	0.00	0.00
Ile	3.75 ± 0.39	4.09	2.54	0.00
Leu	8.24 ± 1.05	9.04	5.85	0.00
Tyr	3.10 ± 0.29	1.80	1.27	0.00
Phe	3.05 ± 0.27	3.28	1.53	0.00
His	2.33 ± 0.15	2.40	1.27	0.00
Lys	6.36 ± 0.54	7.39	9.67	0.00
Arg	3.69 ± 0.41	3.21	2.29	0.00
Total	100.00	100.00	100.00	100.00
% Protein	**3.62 ± 0.77**	**32.42**	**0.41**	**0.005**

Asp* = denotes Asp + Asn, during hydrolysis Asn is converted to Asp
Glu* = denotes Glu + Gln, during hydrolysis Gln is converted to Glu
Note: Cys and Trp were not determined.
*Data values are presented as the Average ± S. D. of 3 reps (batches).

acid composition of the water insoluble residue is much closer to that of the intact suspension cell walls. The water soluble extract is much richer than the intact Im 216 cell walls in Asp (11.70 mole%), Ser (11.20 mole%), Gly (11.70 mol%), and Lys (9.67 mol%), but poorer in Thr (5.09 mole%), Glu (4.58 mole%), Ile (2.54 mole%), and Met (0.00 mole%).

From these results, both the water soluble extract and water insoluble residue is somewhat Hyp-rich. However, it is much easier to work with the Im 216 HF 0°C water soluble extract because of its solubility. Yet, results from the Hyp analysis (colorimetric method, see Table 17) indicate that two-thirds of the Hyp-rich protein is in the water insoluble residue while only one-third of the Hyp-rich protein is in the water soluble extract. In addition, column chromatography of the Im 216 HF 0°C water soluble extract by Bio-gel P2 gel filtration column (Figure 46) and DEAE-Sepahdex anion exchange column (Figure 47) did not effectively separate a Hyp-rich glycoprotein. Sodium chlorite/acetic acid treatment, discussed earlier in this chapter, seems a viable method to solubilize and isolate HRGP or HRP after HF solvolysis at 0°C from both Ac 44 and Im 216 suspension cell walls (water insoluble residue).

Sugar Composition of Different Fractions of Im 216 Cell Walls following HF Solvolysis at 0°C. The sugar compositions of each of the different fractions isolated after HF solvolysis at 0°C was determined by gas chromatographic analysis of the TMS-derivatives. Results are shown in Table 22. HF solvolysis at 0°C deglycosylates cell wall proteins by removing almost all sugars from the walls including those in cellulose. Thus, the glucose in all fractions treated with HF solvolysis at 0°C represents both cellulosic and non-cellulosic glucose. Plain methanolysis of walls gives only the non-cellulosic glucose content. Therefore, without any HF treatment 36.9 ± 8.1% (w/w) of the Im 216 suspension cell walls was recovered as sugars. After HF treatment, 65.9 ± 7.4% (w/w) (2 reps with 2 determinations each) was recovered as sugars, about 10% more than for Ac 44 suspension cells walls (see Table 19). Treatment of the Im 216 cell walls with HF at 0°C increases the relative mole percent of glucose 2-fold, but more importantly 50% (w/w) of the total sugar composition of the Im 216 cell wall is glucose. Approximately 40% (w/w) of the total sugar composition of the Ac 44 cell wall is glucose (see Table 19). The untreated Im 216 cell walls have equal molar ratios of GalA and Glc, both ~24 mole%, but after HF treatment, the Im 216 cell walls have ~50 mole% Glc and ~14 mole% GalA. After HF treatment, the relative molar contributions of GalA and Glc combined increases by 15%.

TABLE 22

MOLE PERCENT OF SUGARS IN DIFFERENT FRACTIONS OF IM 216 CELL-WALL MATERIAL AFTER HF-SOLVOYSIS AT 0°C

Material	Percent of original cell wall weight recovered	Apparent percent sugar in sample	Ara	Rha	Fuc	Xyl	GalA	Man	Gal	Glc
Whole cell walls (15d)	100.0	36.9 ± 8.1	13.5 ± 0.2	7.6 ±0.0	2.1 ± 0.1	16.4 ±0.4	24.6 ± 0.3	0.6 ±0.0	11.3 ± 0.2	23.9 ±0.2
Cell wall	100.0	59.4 ± 5.0	9.0 ± 0.4	5.1 ± 0.1	1.3 ± 0.0	12.1 ± 0.4	13.6 ± 1.0	0.4 ± 0.0	6.8 ± 0.2	51.6 ± 0.8
after HF 0°C*	100.0	72.3 ± 0.2	8.6 ± 0.8	5.3 ± 0.1	1.4 ± 0.2	13.7 ± 0.5	14.2 ± 1.0	0.4 ± 0.0	6.6 ± 0.2	49.8 ± 0.6
Ether-fraction water soluble portion	46.8	67.3 ± 0.7	8.5 ± 0.1	5.4 ± 0.0	1.2 ± 0.0	12.6 ± 0.2	7.9 ± 0.0	0.3 ± 0.0	6.0 ± 0.0	58.2 ± 0.0
Water soluble extract	31.5	47.0 ± 9.2	3.5 ± 0.1	1.3 ± 0.0	0.4 ± 0.0	4.1 ± 0.3	74.6 ± 0.6	0.3 ± 0.1	3.0 ± 0.0	12.8 ± 0.4
Residue after water extraction	6.8	36.0 ± 3.0	4.6 ± 0.2	1.5 ± 0.0	0.5 ± 0.0	5.8 ± 0.3	71.6 ± 0.7	0.2 ± 0.2	3.3 ± 0.1	12.4 ± 0.6

Sugar compositions of each fraction were determined after methanolysis and trimethyl-silylation (see Materials and Methods).
All the samples marked with an * were given a 15-min treatment in HF at 0°C to solubilize cellulose and any other sugar polymers resistant to methanolysis. Cell walls were all prepared from suspension cells harvested in late log phase 15-20 days after the culture was inititated. All fractions were derived from one batch of Im 216 cell walls.
Note: Data expressed as average ± S.D. of 2 determinations

Approximately 67.3 ± 0.7% (w/w) of the Im 216 HF/ether soluble fraction (water soluble portion) is accounted for by sugars. This fraction is especially glucose rich, 58.2 mole% of the total sugar content. Most of the sugar linkages are labile during HF solvolysis at 0°C (see Table 1). The sugar composition of the Im 216 HF/ether soluble fraction is very similar to the Im 216 HF treated cell walls except for GalA and Gal which are both lower. A significant proportion of the Ara, Rha, Fuc, Xyl, Man, Gal, and even Glc (non-cellulosic) have been solvolytically cleaved at 0°C. These sugar monomers are found in the HF/ether soluble fraction. Thus, the cell wall polymers like arabinogalactan, arabinan, galactan, xyloglucan are broken down into their sugar monomers.

Both the Im 216 water soluble extract and water insoluble residue are rich in GalA, 72-75 mole% of the total sugar content. Approximately 47.05 ± 9.25% (w/w) of the water soluble extract is accounted for by sugars and 36.0 ± 3.0% of the water insoluble residue is accounted for by sugars. A significant portion of the sugars have been solvolytically cleaved at 0°C and those that are soluble in water are in the water soluble extract and those that can't be solvolytically cleaved at 0°C and are not soluble in water are in the water insoluble residue. The major components of both the water insoluble residue and water insoluble residue are GalA and Glc. GalA and Glc account for 87% of all the sugars left in the water soluble extract and 84% of all the sugars left in the water insoluble residue. It seems that certain uronic acid and glucose linkages are not labile to HF solvolytic cleavage at 0°C in Im 216 cell walls. Thus, the galacturonic acid rich portion of pectin (homogalacturonnan) was not broken down into monomers in either Ac 44 cell walls or Im 216 cell walls by HF solvolytic cleavage at 0°C.

Characterization of the Ether Soluble Fraction-Ether Oil of Ac 44 and Im 216 Cell Walls after HF Solvolysis at 0°C by ^{1}H-Nuclear Magnetic Resonance Spectroscopy and Mass Spectroscopy

Structure of the Ether soluble fraction-ether oil of Ac 44 and Im 216 cell walls after HF solvolysis at 0°C was investigated by ^{1}H-n.m.r. spectroscopy and mass spectroscopy. The ^{1}H-n.m.r. spectra of the Ac 44 and Im 216 HF 0°C ether soluble fraction - ether oils are shown in Figures 48 and 49, respectively. Specific structures cannot be attributed to either Ac 44 or Im 216 HF 0°C ether soluble seem to be a complex mixture composed mainly of a backbone of aliphatic hydrocarbons with associated methyl groups. The only

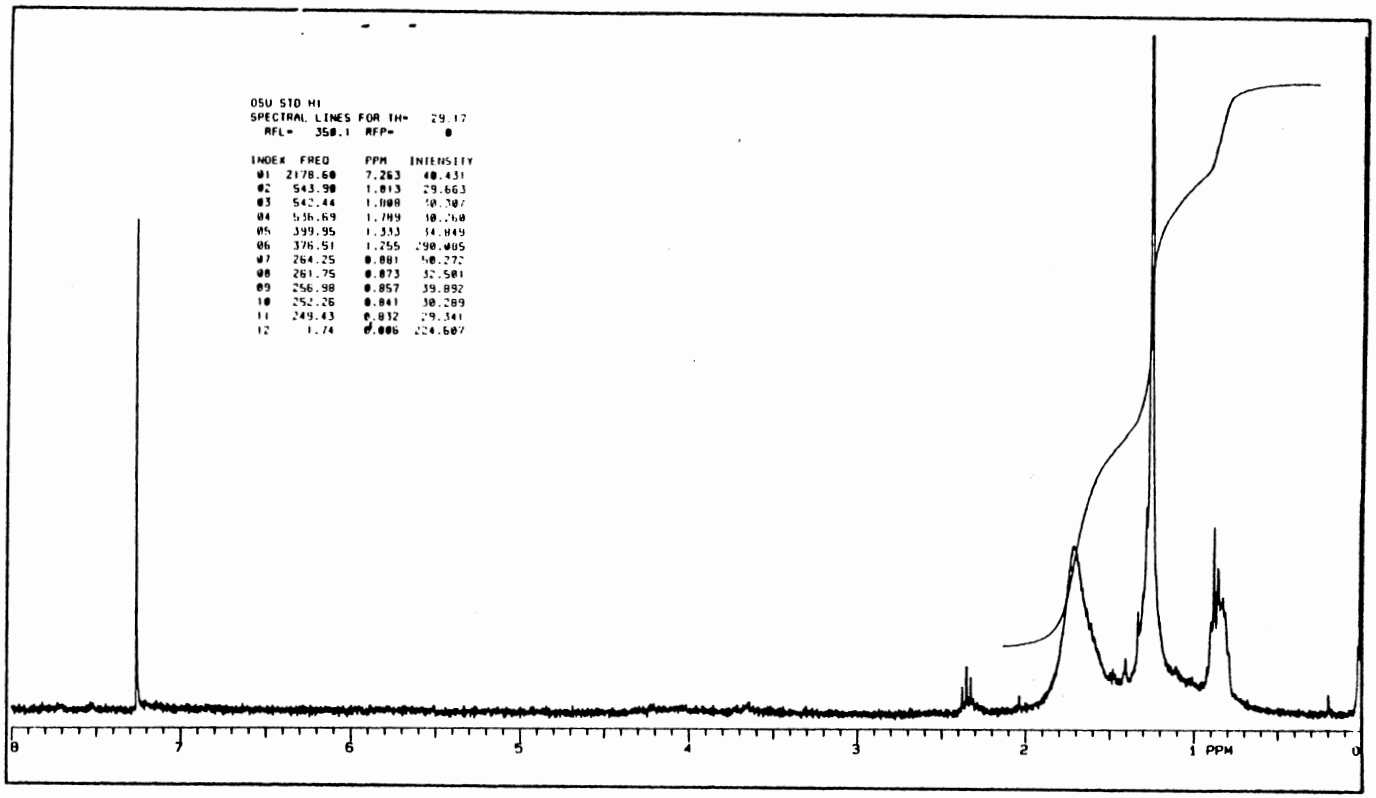


Figure 48. ^{1}H N.M.R. Spectrum of Ac 44 HF 0°C Ether Soluble Fraction - Ether Oil. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in d-chloroform using TSP as an internal standard at 0.00 p.p.m.

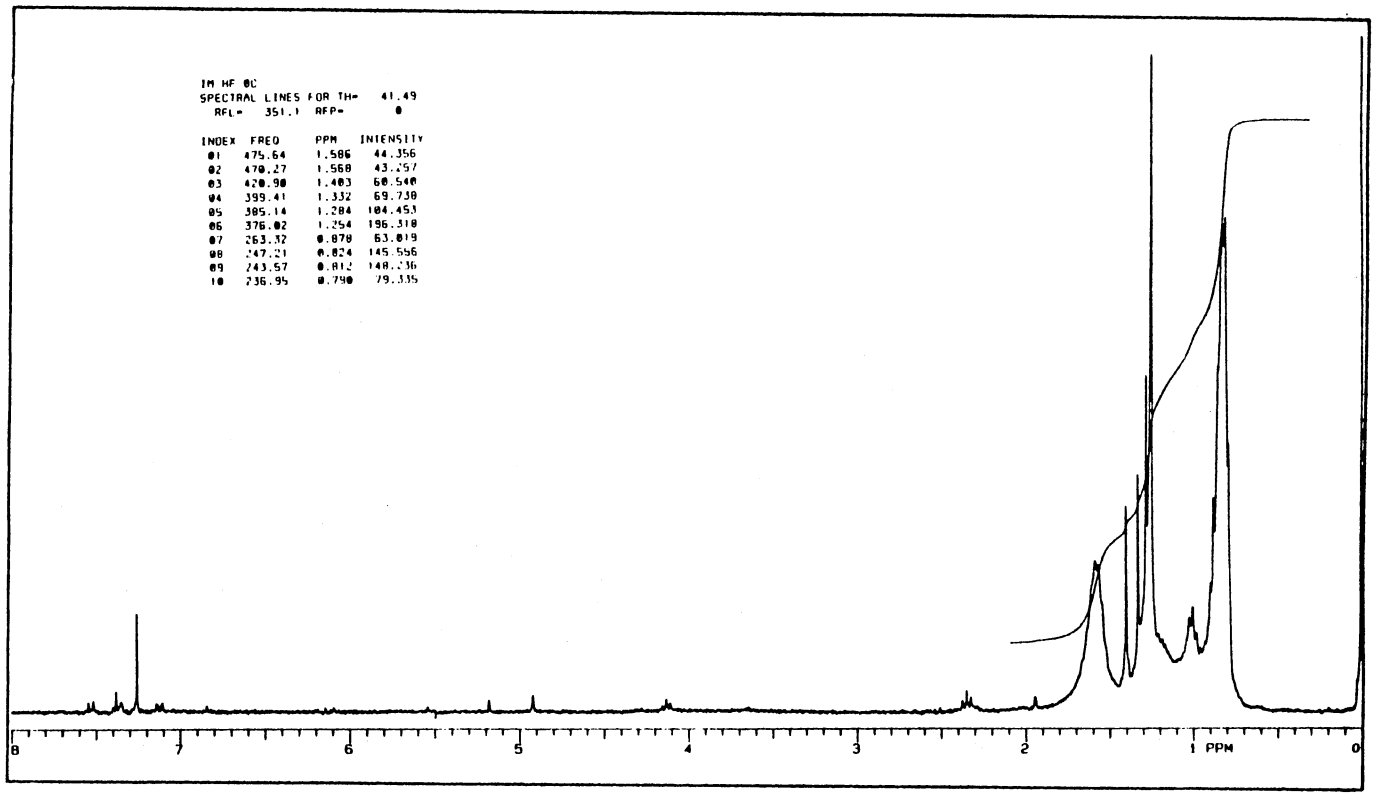


Figure 49. ^{1}H N.M.R. Spectrum of Im 216 HF 0°C Ether Soluble Fraction - Ether Oil. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in d-chloroform using TSP as an internal standard at 0.00 p.p.m.

peaks observed were between 0.80 and 2.0 p.p.m., strongly suggesting the saturated hydrocarbon nature of the ether oil mixture.

The peaks observed in both spectra at 0.83-0.88 p.p.m. are with long chain hydrocarbons. Three different kinds of methyl groups are on the hydrocarbons. The peak at 7.26 p.p.m. observed in Figure 49 and to a lesser degree in Figure 50, is $CHCl_3$ contamination of the $CDCl_3$ solvent. The peaks observed between 1.789 and 1.813 p.p.m. in the ether soluble fraction-ether oil of the HF treated Ac 44 cell walls (Figure 48) are seemingly shifted downfield in the ether soluble fraction - ether oil of HF treated Im 216 cell walls (Figure 49) to between 1.568 and 1.586 p.p.m. The peaks with the greatest intensity observed at 1.255 p.p.m. and 1.33 p.p.m. are common to both Ac 44 and Im 216 ether oils. the Im 216 ether oil has at least two different peaks not observed in the spectrum for the Ac 44 ether oil at 1.284 p.p.m., 1.403 p.p.m. the identity of each ^1H signal in Figures 49 and 50 was not possible from this n.m.r. spectroscopy analysis.

In essence, these spectra indicate the Ac 44 and Im 216 HF 0°C ether soluble fraction - ether oils contain aliphatic methyl groups associated with a hydrocarbon chain such as in lipids. There are no olefenic nor aromatic proteins nor are there ($-CH_2-$) methylene protons associated with aromatic groups. Further elucidation of the composition and structure of the Ac 44 and Im 216 HF 0°C ether soluble fraction - ether oils was carried out using gas-liquid chromatography - mass spectroscopy.

Results from the mass spectroscopy analysis are nearly as conclusive as for ^1H n.m.r. spectroscopy analysis. Only the Im 216 HF 0°C ether soluble fraction - ether oil gave mass spectra of any kind (spectra not shown). No ions, $m/z > 220$ were observed. No molecular ion is observed. Perhaps TMS-derivatization of the Ac 44 and Im 216 ether soluble fraction - ether oils would enable proper identification of the molecular components of this fraction. At best, the mass spectra indicate that the Im 216 HF 0°C ether soluble fraction - ether oil contains small molecular weight saturated hydrocarbons which might be part of a lipid.

Isolation and Characterization of Ac 44 and Im 216 Cotton Suspension Cell Wall Proteins following HF Solvolysis at 0°C

Electrophoresis Analysis

Numerous attempts to characterize Hyp-rich glycoproteins by electrophoresis were unsuccessful due to their very poor migration in a number of different electrophoretic conditions as noted for tomato extensin precursors, P1 and P2 [228] salt-extractable HRGP from aerated carrot discs [243], HRGP from tobacco callus [168], and a Hyp-rich bacterial agglutinin from potato [162]. HRGP may not migrate well because of the combination of high carbohydrate, hydroxyproline, and basic amino acid content [243]. In this system, the galacturonic acid content is very high in both the Ac 44 HF 0°C water soluble extract and water insoluble residue, which contains a high percentage of Hyp. Nevertheless, the true molecular weight of the carrot HRGPs [237, 243] and tobacco HRGP [168] has been reported to be about 90 Kd. To improve electrophoretic characterizations of HRGPs, a number of researchers have deglycosylated the HRGPs from melon [166], tomato [228], carrot [237], and bacterial agglutinin from potato [162] and reported apparent molecular weights for the deglycosylated HRGP of 40-56 Kd based on the migration in SDS-PAGE.

Similarly, deglycosylation of Ac 44 and Im 216 cotton suspension cell wall proteins was performed using HF solvolysis at 0°C, by a procedure originally described by Mort and Lamport [177]. This procedure led to significantly reduced amounts of Ara, Rha, Fuc, Xyl, Man, Gal, and Glc, ~90% in the water insoluble residue recovered after HF solvolysis at 0°C (see Tables 16 and 19). HF solvolysis did not significantly alter the glycoproteins since Im 216 and Ac 44 HF 0°C water insoluble residues do not differ significantly in their amino acid compositions from that of the intact suspension cell walls although the water soluble extracts do differ (see Table 18 and 21). Further treatment of the Ac 44 HF 0°C water insoluble residue with sodium chlorite/acetic acid solubilized the Hyp-rich cell wall protein as evidenced by the amino acid composition of (Table 18) and the release of Hyp (Figure 41 and Table 14) in the chlorite soluble extract in comparison to the amino acid compositions of the chlorite extracted glycoproteins from runner beans [180] and from tomato suspension cells [173].

Results indicate that the Ac 44 HF 0°C water soluble extract and chlorite soluble extract of the water insoluble residue have the same two proteins migrating in 12% SDS-PAGE with apparent molecular weights of 65.6 ± 2.2 Kd and 60.3 ± 1.8 Kd (12 determinations) (Figure 50). The Ac 44 and Im 216 HF 0°C water insoluble residue has one clearly visible protein band with an apparent molecular weight of 64.0 ± 2.2 Kd (4 determinations) (Figure 51), nearly indistinguishable from the higher molecular weight protein seen in the Ac 44 HF 0°C water soluble extract and sodium chlorite extract. The Ac 44 and Im 216 HF 0°C water insoluble residue did not solubilize well in the SDS sample buffer and proteins were not resolved well in this system. In addition, not all of the protein moved into the resolving gel as evidenced by silver staining of the stacking gel. There are also diffuse protein bands seen with apparent molecular weights of ~31Kd and 14-21.5 Kd.

It is evident that HF solvolysis at 0°C causes some of the protein to be solubilized in the water soluble extract and some is insolublized in the water insoluble residues since the protein with the apparent molecular weight of 65.6 Kd is observed in both fractions. However, the lower molecular weight protein, ~60 Kd seems to be absent from the water insoluble residues of either Ac 44 or Im 216 suspension cell walls after HF solvolysis at 0°C although it may be masked by the intensive streaking of stained material in the gel. The proteins seen at 65.6 Kd and 60.3 Kd must be deglycosylated Hyp-rich proteins since sodium chlorite oxidizes the phenolic cross-links liberating the HRGP from the cell wall and from the amino acid composition of the sodium chlorite extract. Following this line of reasoning, the Ac 44 HF 0°C water soluble extract with 2 discernable protein bands at the same apparent molecular weight of 65.6 Kd and 60.3 Kd are the same as seen in the sodium chlorite extract, but are less concentrated.

Thus, from the electrophoresis analysis by 12% SDS-PAGE, there are 2 major proteins isolated from cotton suspension cell walls after HF solvolysis at 0°C with apparent molecular weights of 65.6 Kd and 60.3 Kd. It is not known yet whether these proteins can be positively identified as HRGP (extensin) until after the immunological analyses are complete. These analyses will be discussed next. However, the apparent molecular weights of the potential cotton HRGPs (deglycosylated) especially the lower molecular protein, 60 Kd, are comparable to those obtained after SDS-PAGE by other researchers for deglycosylated HRGPs in melon [166], tomato [228], and potato [162], ~55Kd. Different apparent molecular weight for cotton, melon, tomato and potato HRGP would seem to be due to the degree of glycosylation.

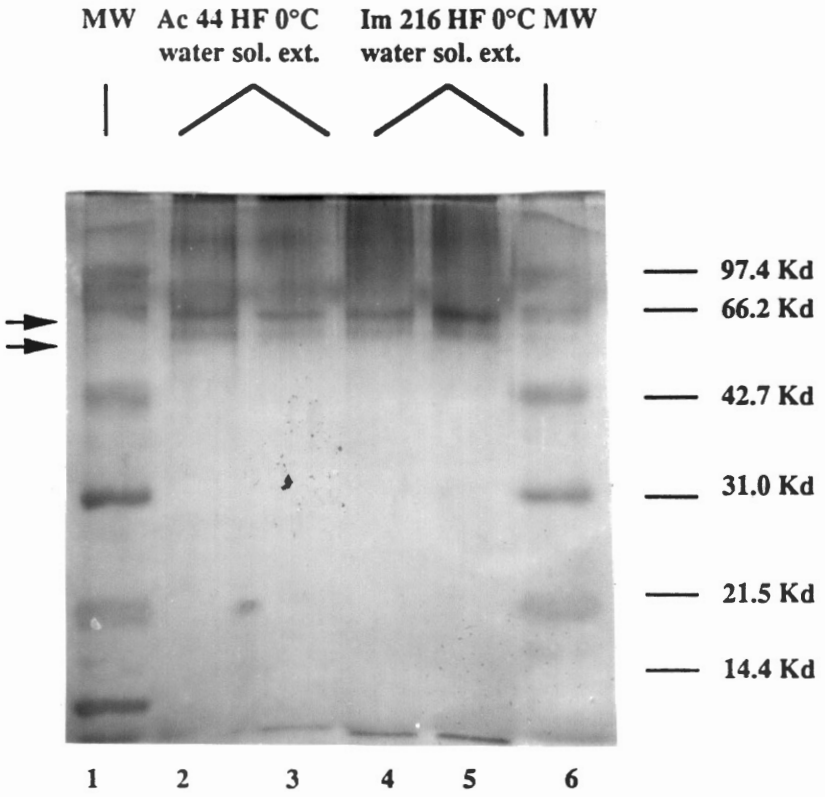


Figure 50. 12% SDS-PAGE of the Water Soluble Extract and the Sodium Chlorite Extract of the Water Insoluble Residue of Ac 44 Suspension Cell Walls after HF Solvolysis at 0ºC. Lanes 1 and 6 contain MW markers as indicated on the far right (Phosphorylase B, 97.4 Kd; BSA, 66.2 Kd; ovalbumin, 42.7 Kd; carbonic anhydrase, 31.0 Kd; soybean trypsin inhibitor, 21.5 Kd; and lysozyme, 14.4 Kd). Lanes 2 and 3 contain 2.5 µg and 5.0 µg protein from Ac 44 HF 0ºC water soluble extract, respectively. Lanes 4 and 5 contain 4.0 µg and 8.0 µg protein from sodium chlorite extract of the Ac 44 HF 0ºC water insoluble residue. At the far left, arrows indicate the two major proteins isolated from the cotton suspension cell walls after HF solvolysis at 0ºC, 65.6 Kd and 60.3 Kd.

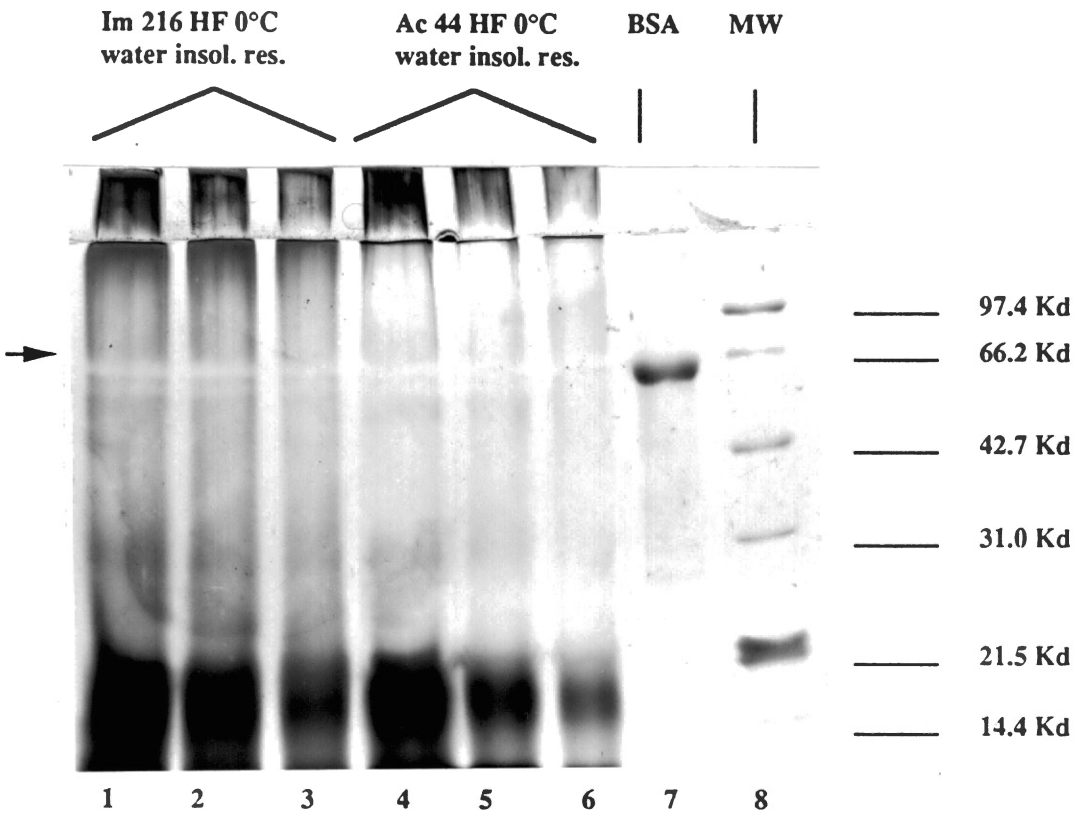


Figure 51. 12% SDS-PAGE of the Water Insoluble Residue of Ac 44 and Im 216 Suspension Cell Walls after HF Solvolysis at 0°C. Lanes 1-3 contain 54 µg, 40 µg, and 27 µg protein from the Im 216 HF 0°C water insoluble residue. Lanes 4-6 contain 38 µg, 28 µg, and 19 µg protein from the Ac 44 HF 0°C water insoluble residue. Lane 5 contains the BSA standard, 66.2 Kd. Lane 6 contains the MW markers as described in Figure 50.

Immunological Analysis

ELISA. Enzyme-line immunosorbent assays (ELISA) are an especially sensitive method used in detecting common antigenic determinants on molecules in solution or on cell surfaces or investigating antibody specificities or affinities. Polyclonal antibodies raised against cell wall fractions from higher plants [119] and from extensin precursors [50, 130] and monoclonals raised against *Chlamydomas* wall HRGPs [226] and *Fucus* cell wall carbohydrates [255] have been employed in immunological approaches to studying the structure of the cell walls. Results are presented here describing the properties and cross-reactivities of Ac 44 and Im 216 HF 0°C water soluble extracts and Ac HF 0°C sodium chlorite extract of the water insoluble residue with polyclonal rabbit antibodies raised against two HF-deglycosylated extensin precursors of tomato, dP1 and dP2. Reactivities were determined by an indirect non-competitive sandwich type of ELISA technique [254]. At the same time, a more definitive identification of a cotton extensin in the fractions can be made from the ELISA.

Figure 52 shows the cross-reactivities of polyclonal antibodies from deglycosylated tomato extensin dP1 and dP2 precursors with HF-deglycosylated Ac 44 and Im 216 cotton suspension cell wall fractions. Results indicate that the antibodies raised against deglycosylated tomato dP1 and dP2 cross-react with proteins seen in the water soluble extracts and sodium chlorite extract. The extent of antibody cross-reactivity depends on the number of common epitopes that exist between the deglycosylated tomato precursors, dP1 and dP2 and the deglycosylated cotton fractions. All three HF-treated (0°C) cotton fractions show substantial cross-reactivity with both of the antibodies raised against deglycosylated tomato dP1 and dP2 extensin precursors even though the antigen concentrations (amount of protein) vary in the wells. From analysis of tryptic peptide maps, P1 contains two different peptide blocks: Ser-$(Hyp)_4$-Thr-Hyp-Val-Tyr-Lys and Ser-$(Hyp)_4$-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys and P2 consists of a single-repeating decapeptide, Ser-$(Hyp)_4$-Val-Tyr-Lys-Tyr-Lys [229]. Figure 52 also shows extensive cross-reactivity of the heterologous reactions anti-dP1/dP2 ($65.7 \pm 1.4\%$) and anti-dP2/dP1 ($71.6 \pm 2.6\%$) indicating great similarity in the nonglycosylated domains of the two tomato precursors. This result has already been discussed [130]. As for the HF-treated cotton cell wall fractions examined, the Ac HF 0°C and Im HF 0°C water soluble extract react well with anti-dP1 and anti-dP2 although the percent cross-reactivity differs between the two cell types because there is a 2.7-fold difference in the amount of protein

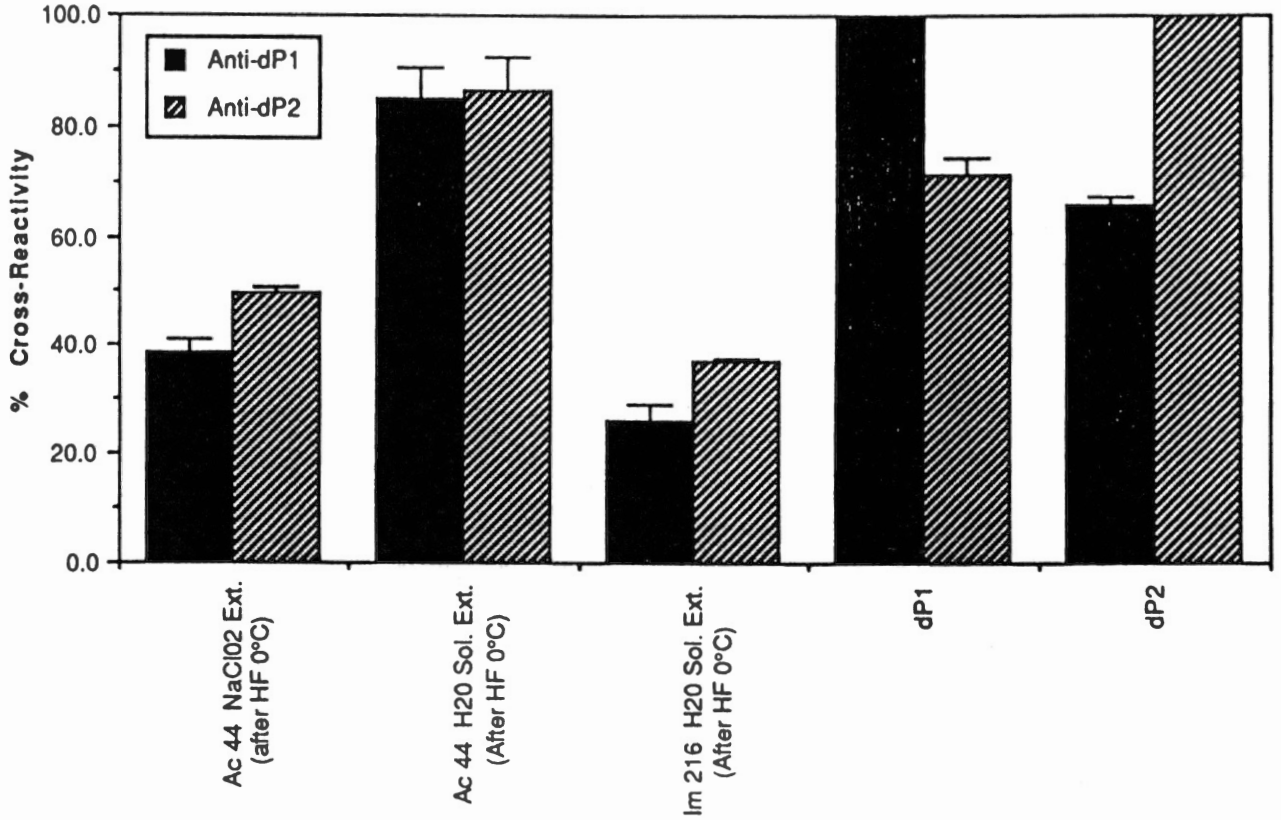
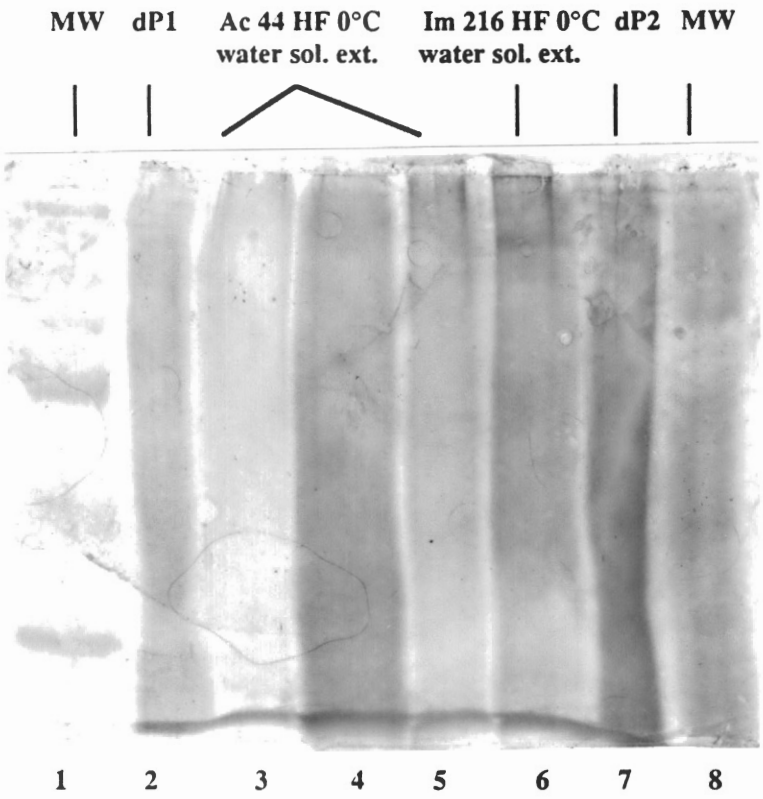


Figure 52. Cross-Reactivity of Ac 44 and Im 216 Water Soluble Extracts and the Ac 44 Sodium Chlorite Extract of the Water Insoluble Residue after HF Solvolysis at 0°C of Ac 44 and Im 216 Cotton Suspension Cell Walls with Polyclonal Antibodies against Tomato Extensin Precursors, dP1 and dP2. ELISA wells were coated as follows: Ac 44 HF 0°C sodium chlorite extract has 0.34 µg protein/well, Ac 44 HF 0°C water soluble extract had 0.088 µg protein/well, Im 216 HF 0°C water soluble extract had 0.033 µg protein/well, deglycosylated tomato extensin precursors, dP1 and dP2 antigens, 0.2 µg/well. Cross-reactivity for a given antibody towards the sample antigens is expressed a percentage of either anti-dP1/dP1 and anti-dP2/dP2.

in the sample wells. The Ac 44 HF 0°C chlorite soluble extract of the water insoluble residue has substantially less cross-reactivity than the two water soluble extracts to the antibodies raised against the two deglycosylated extensin precursors even though there is 4-fold more protein loaded in the wells than in the Ac 44 HF 0°C water soluble extract.

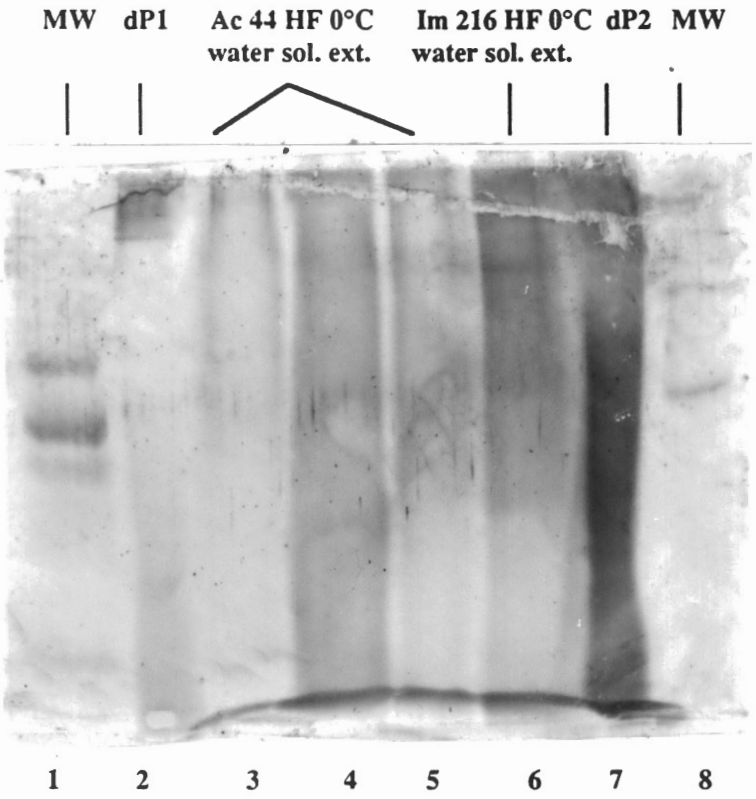
Even considering differences in the amount of protein loaded per sample well, the Ac 44 and Im 216 HF 0°C water soluble extracts and the Ac HF 0°C chlorite soluble extract of the water insoluble residue contain similar epitopes to both deglycosylated tomato extensin precursors. Slightly stronger reactivity, 2-10%, reactivity is shown for the antibody raised against the deglycosylated tomato extensin precursor, dP2 than for dP1. The reactivity of the Ac 44 HF 0°C chlorite soluble extract is only 38.6% and 49.7% for anti-dP1 and anti-dP2, respectively while the reactivity of the Ac 44 HF 0°C water soluble extract is 85.1% and 86.8% for anti-dP1 and anti-dP2, respectively. This difference may be attributed to the fact the sodium chlorite extract represents a more purified form of the cotton HRGP and thus may no longer have the same epitopic similarity with the deglycosylated tomato precursors, dP1 and dP2. The ultimate conclusion from the ELISA is that the HF-treated cotton fractions cross-reacted significantly with polyclonal antibodies raised against deglycosylated tomato extensin precursors, dP1 and dP2, thus confirming some epitopic similarity with the two deglycosylated tomato precursors, dP1 and dP2. Therefore, the deglycosylated cotton HRGP isolated from suspension cultures has a similar primary structure to the deglycosylated tomato extensin precursors, dP1 and dP2 with more structural similarity existing between the deglycosylated cotton HRGP and the deglycosylated tomato extensin precursor dP2. It is thus possible to proclaim these HF-treated cotton fractions to have at least "extensin-like" molecules. The extent of true homology awaits further biochemical analyses: peptide mapping and amino acid sequence determination of the major repeating peptides of these HF-treated cotton fractions to see if these peptides are highly periodic like other extensins.

Western Blot Analysis. Western blot analysis of the reactivities of Ac 44 and Im 216 HF 0°C water soluble extracts with the two polyclonal antibodies raised against the deglycosylated tomato extensin precursors, dP1 and dP2 is shown in Figure 53. There is some cross-reactivity of the Ac 44 and Im 216 HF 0°C water soluble extracts with the antibody to the deglycosylated tomato extensin precursors, dP2, but not to the deglycosylated tomato extensin precursor, dP1. However, there appears to be a certain amount of non-specific binding to both antibodies (see Figure 53). The specificity and accuracy of the Western blot analysis over other immunological analyses including ELISA, would provide definitive evidence that the Ac 44 and Im 216 HF 0°C water soluble extracts contain "extensin-like" molecules and that they are structurally similar to the deglycosylated tomato extensin precursor, dP2 which has the major repeating decapeptide, Ser(Hyp)$_4$-Val-Tyr-Cys-Tyr-Lys [229]. Further Western blot analysis is currently underway in order to determine specifically whether the isolated cotton cell wall proteins after SDS-PAGE (Figures 50 and 51) are "extensin-like" molecules.



A. Anti-dP1

Figure 53a. Western Blot Analysis of the Reactivities of Ac 44 and Im 216 HF 0°C Water Soluble Extracts with the Two Polyclonal Antibodies Raised Against the Deglycosylated Tomato Extensin Precursors, dP1 and dP2. Lanes 1 and 8 contain the MW markers as shown in Figure 50. Lanes 2 and 7 contain 15.6 µg of the dP1 and 12.5 µg dP2 control antigens. Lanes 3-5 contain 3.28 µg, 10.4 µg, 16.2 µg protein from three batches of Ac 44 HF 0ºC water soluble extract. Lane 6 contains 4.72 µg protein from Im 216 HF 0ºC water soluble extract. A. Anti-dP1.



B. Anti-dP2

Figure 53b. Western Blot Analysis of the Reactivities of Ac 44 and Im 216 HF 0°C Water Soluble Extracts with the Two Polyclonal Antibodies Raised Against the Deglycosylated Tomato Extensin Precursors, dP1 and dP2. Description is the same as presented for Figure 53a except the proteins are reacted with the antibody raised against dP2.

CHAPTER V

SUMMARY AND CONCLUSIONS

This research has further established the utility of cotton tissue culture to study *in vitro* host-pathogen interaction in the *Gossypium hirsutum* L. - *X. c. malvacearum* system and correlate the defense responses directly to the bacterial pathogen, *X. c. malvacearum*. The cotton cell lines selected for this research differ in the number of resistance genes to *X. malvacearum*. The main cotton cell lines used were: the susceptible cell line, Ac 44, which possesses no resistance genes to *X. c. malvacearum*; the intermediate resistant line, AcB_N, which possesses 1 resistance gene, B_N, in an Ac 44 genetic background; and the highly resistant line, Im 216, which possesses homozygous resistance to *X. c. malvacearum* including the major resistance genes B_2, B_3, and b_7 in a polygenic background. In general, this research characterizes biochemical host-specific responses to the bacterial pathogen, *X. c. malvacearum*, race 3 in tissue culture. Initially, cotton suspension growth and viability for all three cell lines were characterized in the presence and absence of the pathogen and related to resistance and susceptibility to *X. c. malvacearum*. Growth of the pathogen was also established in the presence of host suspension cells. Next, differences in several biochemical parameters were measured over time in Ac 44, AcB_N, and Im 216 suspension cultures and then related to resistance and susceptibility to *X. c. malvacearum*. Finally, cotton HRGPs were isolated from Ac 44 and Im 216 suspension cell walls following HF solvolysis at 0°C.

In order to characterize the cotton - *X. c. malvacearum* host-pathogen interaction in suspension culture, the growth of each component with respect to the other was examined. From crudely measuring the growth of *X. c. malvacearum* in Ac 44 and Im 216 suspension cultures (Figures 3-4), it was concluded that first, there were significant differences in the ability of the pathogen to grow in these two cotton cell lines in suspension culture. That is, bacterial growth in the incompatible interaction involving Im

216 is different from the compatible interaction involving Ac 44. Second, growth of the pathogen, *X. c. malvacearum* only occurred in the presence of live host cells whether Ac 44 or Im 216, since suspension-cell filtrates do not support growth of the pathogen. A clearly "resistant" response *in vitro* was noted in Im 216 suspension cell cultures.

The growth of the cotton host in the presence of *X. malvacearum* was measured both in callus culture and suspension cell culture (Figures 5-8). There is statistically significant relationship between the growth of Ac 44 and Im 216 callus and when the callus inoculated. That is, the later the Ac 44 and Im 216 callus were inoculated, the better able the callus was able to grow. Finally, the inoculated blight-immune Im 216 callus growth significantly better than the inoculated blight-susceptible Ac 44 callus. Ac 44 and Im 216 callus tissue have now been subcultured through hundreds of transfers over the course of 15 years, and each callus cultivar still retains its ability to respond in a "susceptible" (Ac 44) or "resistant" (Im 216) fashion to the pathogen, *X. c. malvacearum*. Results are clearly similar to those observed in the intact plant.

The growth of Ac 44, AcB_N, and Im 216 cells in suspension was examined in the presence and absence of the pathogen, *X. c. malvacearum* (Figures 9-10). The following conclusions can be made following statistical analysis of the data: 1) growth is significantly different with respect to each cell line, Ac 44, Im 216, or AcB_N, 2) growth varies with respect to the presence of the pathogen, *X. c. malvacearum* and 3) growth varies with respect to time. Ac 44 suspension cell cultures showed the most marked pathogen effect observed in the form of rapid and continuous Ac 44 cell death. A case has therefore been made for observable resistance in AcB_N and Im 216 suspension cultures to the pathogen, *X. c. malvacearum* not unlike that seen in the intact plants. Based on the ability of Ac 44, AcB_N, and Im 216 to respond to the pathogen, *X. c. malvacearum*, it is possible to conclude that suspension culture growth is dependent on the cell types and thus the number of resistance genes to *X. c. malvacearum*.

Viability of uninoculated and inoculated, Ac 44, AcB_N, and Im 216 suspension cells was determined by Evan's blue staining at various stages of suspension culture growth (Figures 11-15). From the statistical analysis of the studies, it was concluded that viability is a function of the presence of the pathogen and a function of the cotton cell line. The viability for uninoculated suspension cultures is significantly higher than in inoculated suspension cultures. Viability is different in all three cotton cell lines, Ac 44, AcB_N, and

Im 216. Viability is greater in the resistant cell lines, Im 216 and AcB_N, than in the susceptible cultivar, Ac 44 after infection with *X. c. malvacearum.*

Protoplast yields differ quite dramatically among cotton cell lines (Table 2). The cotton cell line, Ac 44 (blight-susceptible), has the greatest protoplast yield of 48%. The protoplast yields of the three resistant lines, AcB_N, OK 1.2, and Im 216 were 6.8%, 2.0%, and 0.97%, respectively. These yields differ significantly (t-test) from the protoplast yields of the susceptible line, Ac 44. The cell wall degrading enzymes used removed the susceptible line, Ac 44 suspension cell walls, with relative ease, but had increasingly difficulty in removing the resistant lines' suspension cell walls depending on the number of resistance genes present. Differences in protoplast yields may be due to the differences observed in Ac 44 and Im 216 glucose and galacturonic acid content in the cell walls as well as increased lignification of the cell wall in resistant cell lines than in susceptible cell lines.

Cell walls were prepared from the three cotton suspension cell lines, Ac 44, AcB_N, and Im 216 either inoculated or uninoculated with the blight pathogen, *X. c. malvacearum.* Greater suspension cell wall fresh weight to cell wall dry weight ratios are seen in the incompatible interactions, Im 216 and AcB_N, than in the compatible interaction, Ac 44 (Table 3, Figures 16-17). Examination of the suspension cell walls using scanning electron microscopy indicated that inoculated Ac 44 cell walls have a different ultrastructural appearance than do uninoculated Ac 44 cell walls and also from uninoculated and inoculated Im 216 cell walls. The results suggest that the pathogen, *X. c. malvacearum* is responsible for destruction of the cell wall in the compatible interaction, Ac 44.

Several biochemical parameters were measured over time in Ac 44, AcB_N, and Im 216 cell walls prepared from uninoculated and inoculated suspension cultures. Statistically significant differences in all biochemical parameters measured (see Tables 10-12): Hyp% (Figures 24-25), Protein% (Figures 26-27), Lignin% (Figures 38-39), Total Sugar% (Figure 29), and Individual Sugar% (Figure 30-37) (w/w) of the cell wall dry weight were observed between uninoculated and inoculated Ac 44, AcB_N, and Im 216 suspension cell walls at ≥90% confidence level. The biological parameters of growth, cell viability, and cell wall recovery are also significantly different for uninoculated and inoculated suspension cultures/cell walls. Both the biochemical and biological parameters are a function of the presence of the pathogen and a function of the cell type chosen.

These parameters differ between cell type treatments (Ac 44 v. AcB_N v. Im 216) and within cell type treatments (inoculated v. uninoculated). In addition, only in the incompatible interactions, AcB_N and Im 216 were Hyp (Figures 24-25) and protein (Figures 26-27) accumulated. Lignin was accumulated in both the compatible and incompatible interactions (Figures 38-39). Thus, from the growth studies, and time studies of different biochemical parameters resistant properties in cotton suspension culture were defined. That is, what are the biochemical and growth responses in a compatible interaction and in an incompatible interaction. For example, since Hyp accumulates in the resistant cell lines, AcB_N and Im 216, and does not in the susceptible cell line, Ac 44, then one must conclude that HRGP is under transcriptional or translational control or there are different HRGP stabilities in the resistant cell lines and in the susceptible cell line. Similarly protein profiles mirror the Hyp profiles so that it is possible to conclude new proteins, some of which may be HRGP, are synthesized in the incompatible interaction , but not in the compatible interaction. Accumulation of Hyp in the incompatible interaction is a direct indication of the HRGP involvement in disease resistance to *X. c. malvacearum* in cotton. The dramatic decrease in Hyp and protein in inoculated Ac 44 cell walls may signify enzymatic activity of the pathogen. *X. c. malvacearum* may partially destroy intact HRGP by protease action or prevent synthesis of HRGP or other proteins. From the measurement of several biological and biochemical parameters in Ac 44, AcB_N, and Im 216 suspension cells/ cell walls there is new insight into the mechanisms of host resistance to *X. c. malvacearum* achieved through the use of tissue culture.

Preliminary compositional analysis of Ac 44 cotton leaf, Ac 44 cotton suspension, Im 216 cotton suspension, AcB_N cotton suspension, AcIm cotton suspension, tomato suspension, tobacco callus, and carrot root cell walls were performed in order to provide information on the amount and the chemical nature of the biochemical polymers in the these plant cell walls. Table 13 showed the amount of protein (w/w) in each of these wall samples. The amount of protein in Ac 44, Im 216, AcB_N, and AcIm cotton suspension cell walls was found to be 4.68%, 2.93%, 7.87%, and 12.96% (w/w) of the cell walls, respectively. Tomato suspension cell walls, tobacco callus cell walls, and carrot root cell walls had protein contents of 4.44%, 12.10%, 1.21% (w/w), respectively. Ac 44 leaf cell walls had the highest protein content, 12.51% (w/w). Amino acid analyses (Table 13) showed that Ac 44 cotton leaf cell walls were Hyp-poor and and all the suspension cultured cotton cell lines, Ac 44, AcB_N, AcIm, and Im 216 were Hyp-rich. This difference in Hyp mole% in suspension cell walls from that in the intact plant is not

unusual since cultured cells normally have greater Hyp. Of all the plant cell walls studied, the Hyp mole% is highest in tomato suspension and tobacco callus cell walls, 28.9% and 24.3%, respectively, while carrot root cell walls have 10.4%. The amino acid composition of Ac 44 and Im 216 suspension cell walls are similar. Preliminary amino analysis of cotton suspension cell walls strongly suggest that they are typical of cell walls containing the cell wall protein, HRGP.

Hyp content (% Hyp) for Ac 44 cotton leaf, Ac 44 cotton suspension, Im 216 cotton suspension, AcB_N cotton suspension, AcIm cotton suspension, tomato suspension, tobacco callus, and carrot root cell walls was determined on a total weight percent basis of the cell wall dry weight by two different analytical methods (Table 14). These two analytical methods were not significantly different in determining the %Hyp in each of the cell walls tested. Tobacco callus, tomato suspension cell walls have ~1% and 2.3% Hyp (w/w), respectively. Ac 44 suspension cell walls have a % Hyp of ~0.76% (w/w), AcB_N suspension cell walls have a & Hyp of ~0.34% (w/w), Im 216 suspension cell walls have a % Hyp of 0.50% (w/w), and AcIm suspension cell walls have a % Hyp of ~0.87% (w/w). Ac 44 leaf and carrot cell walls only have ≤0.10% (w/w) Hyp. Precedence indicates that cotton suspension cell walls contain enough Hyp in the cell walls to be considered as a source for isolating HRGP(s).

The lignin content of Ac 44 cotton leaf, Ac 44 cotton suspension, Im 216 cotton suspension, AcB_N cotton suspension, AcIm cotton suspension, Ac 44 HF 0°C water insoluble residue, tomato suspension, tobacco callus, and carrot root cell walls was determined spectrophotometrically (Table 15). The most lignified cell walls examined were from AcIm suspension cultures while the least lignified cell walls examined were from Ac 44 suspension cultures and carrot root cell walls. HF solvolysis at 0°C causes a 3-fold increase in lignin content seen in the Ac 44 HF 0°C water insoluble residue. There is a statistically significant difference in the cell wall lignin content between the blight-susceptible cotton cell line, Ac 44, and the blight-resistant cotton cell lines, AcIm , Im 216, and AcB_N. The lignin content of all 3 resistant cell lines is 2 to 4 times greater than seen in the susceptible cell line. Thus, increased lignification in the cell walls of resistant cell lines may prevent the pathogen from spreading to uninfected plant cells in culture and causing cell death.

The last section of this dissertation described the use of anhydrous HF solvolysis of Im 216 and Ac 44 suspension cell walls at 0°C to purify cell wall proteins after

deglycosylation. Cell walls isolated from Im 216 and Ac 44 suspension cultures were separated into 3 major fractions after HF solvolysis at 0°C, a HF/ether soluble fraction, a water soluble extract and a water insoluble residue. HF solvolysis at 0°C removes nearly all sugars from the cell walls including those in cellulose. The cell wall proteins are deglycosylated by this procedure making them easier to characterize and isolate. The cell wall protein of particular interest in this research is HRGP because of its well established role in resistance to plant disease. Each of the isolated fractions of Ac 44 and Im 216 cell walls following HF solvolysis at 0°C were tested for Hyp to identify Hyp-rich fractions for further purification. The majority of the Hyp is recovered in the water insoluble residue of both Ac 44 (Table 16) and Im 216 (Table 20). There is a typical Hyp-enrichment of 6-10 fold in the HF 0°C water insoluble residues from the Ac 44 and Im 216 starting cell wall material. Lesser amounts of Hyp were recovered in the water soluble extract. Even after deglycosylation of the cell wall proteins using HF solvolysis at 0°C, the cell wall protein remains insoluble in the water insoluble residue. N.M.R. spectroscopy and mass spectroscopy analyses of the Ac 44 (Figure 48) and Im 216 (Figure 49) HF 0°C ether soluble fraction - ether oils indicated the mixture was a plethora of saturated hydrocarbons of unknown identity.

Sodium chlorite oxidation is a selective way to destroy phenols and break the indicated network of cross-linked glycoproteins in the cell walls and solubilize the HRGPs. Following sodium chlorite oxidation of the Ac 44 HF 0°C water insoluble residue, a significant proportion of Hyp (50-82%) is solubilized in the sodium chlorite extract after 30 min at 75°C and the % Hyp (w/w) is more than triple that in the sodium chlorite residue (Table 17).

HPLC gel exclusion chromatography of the Ac 44 HF 0°C water soluble extract and sodium chlorite extract of the water insoluble residue did not adequately separate any proteins in these fractions (Figures 43-44). In addition, chromatography of the Im 216 HF 0°C water soluble extract on a Bio-gel P2 (gel filtration) (Figure 46) and a DEAE-Sepahdex (anion-exchange) (Figure 47) column did not isolate a cotton HRGP.

The amino acid compositions of different fractions of Ac 44 (Table 18) and Im 216 (Table 21) cell walls following HF solvolysis at 0°C were determined by amino acid analysis. Ac 44 and Im 216 suspension cell walls are rich in Hyp, Asp, Ser, Gly, Leu, Lys, Ala, Glu, and Val. These amino acids compose 70-74% of the total protein recovered after amino acid analysis in the Ac 44 and Im 216 suspension cell walls. The

recovered protein is 3.4% of the Ac 44 cell walls and 3.6% of the Im 216 cell walls. Most of the protein and especially the Hyp-rich protein is recovered in the Ac 44 and Im 216 HF 0°C water insoluble residue. The overall amino acid composition of the intact Ac 44 and Im 216 suspension cell walls and Ac 44 and Im 216 HF 0°C water insoluble residues are clearly similar. This indicates that the protein material in both have the same relative composition.

The amino acid composition of the Ac 44 HF 0°C sodium chlorite extract (Table 18) has a relative molar ratio of Hyp (31.80 mole%) to Ser (12.10 mole%) of 2.63:1, a ratio seen for other HRGPs. The cotton suspension cell wall chlorite-extracted glycoprotein has a similar overall amino composition to other chlorite-extracted glycoproteins. The Ac 44 HF 0°C sodium chlorite residue has a similar amino acid composition to the Ac 44 HF 0°C water insoluble residue. Thus, the chlorite treatment of cotton cell walls may act in a specific way to solubilize the HRGP. Further fractionation of the sodium chlorite extract of HF-treated Ac 44 suspension cell walls is indicated in order to isolate cotton HRGP(s). From this research, there is strong evidence that the cotton suspension cell wall HRGP is cross-linked by the cell wall phenolic cross-links.

The sugar compositions of each of the different fractions of Ac 44 (Table 19) and Im 216 (Table 22) suspension cell walls after HF solvolysis at 0°C were determined by gas chromatographic analysis of the TMS-derivatives. HF solvolysis at 0°C removes almost all the sugars of the cell walls including those in cellulose. Without any HF-treatment, 37% of the Ac 44 and Im 216 suspension cell walls were recovered as sugars. After HF treatment, 56% of the Ac 44 suspension cell walls and 66% of the Im 216 suspension cell walls were recovered as sugars. HF-treatment of Ac 44 and Im 216 cell walls increases the relative mole percent of glucose 2 to 5-fold, 45-50 mole%. The Ac 44 and Im 216 HF 0°C HF/ether fraction contains a significant portion of the Ara, Rha, Fuc, Xyl, Man, Gal, and Glc which was removed from the cell walls after HF solvolysis at 0°C. Approximately 86% of the Ac 44 ether soluble fraction is accounted for by sugars and 67% of the Im 216 ether soluble fraction. This fraction is especially rich in glucose, 48 mole% and 58 mole%, respectively for Ac 44 and Im 216. Approximately 67% (w/w) of the Ac 44 HF 0°C water soluble extract is accounted for by sugars, while 46% (w/w) of the water insoluble residue is accounted for by sugars. Similarly, 47% of the Im 216 HF 0°C water soluble extract is accounted for by sugars and 36% fo the water insoluble residue is accounted for by sugars. The major components of both the water insoluble residue and water soluble extract are GalU and Glc which account for ~90 mole% of the

sugars left in these fractions. GalU is 75-96 mole% of the Ac 44 HF 0°C water soluble extract, water insoluble residue, sodium chlorite extract, sodium chlorite residue and the Im 216 HF 0°C water soluble extract and water insoluble residue. The Ac 44 HF 0°C sodium chlorite extract and sodium chlorite residue sugars are almost exclusively GalA and Glc, 92-96 mole% of the total sugar in these fractions. Therefore, it is possible to conclude that the GalU-rich portion of pectin (homogalacturonan) was not broken down by HF solvolysis of Ac 44 and Im 216 suspension cell walls at 0°C.

Electrophoresis analysis of Ac 44 HF 0°C water soluble extract and chlorite soluble extract have the same two proteins migrating in 12% SDS-PAGE with apparent MWs of 65.6 ± 2.2 Kd and 60.3 ± 1.8 KD (Figure 50). The Ac 44 and Im 216 HF 0°C water insoluble residues (Figure 51) had one clearly visible protein band with an apparent MW of 64.0 ± 2.2 Kd, nearly identical to the 65.6 ± 2.2 Kd protein seen in the Ac 44 HF 0°C water soluble extract and sodium chlorite extract. These proteins have apparent MWs similar to that reported for other HRGPs.

Immunological analysis by ELISA (Figure 52) revealed that Ac 44 and Im 216 HF 0°C water soluble extracts and Ac 44 HF 0°C sodium chlorite extract contain similar epitopes to both deglycosylated tomato extensin precursors, dP1 and dP2. Slightly stronger reactivity is shown for the antibody raised against dP2. It can be concluded that the deglycosylated cotton HRGP contained in the HF-treated (0°C) fractions isolated from suspension cultures has a similar primary structure to the deglycosylated tomato extensin precursors, dP1 and dP2. This specificity and accuracy of the Western blot analysis (Figure 53) over other immunological analyses including ELISA, would provide definitive evidence that the Ac 44 and Im 216 HF 0°C water soluble extracts may contain "extensin-like" molecules and that they are structurally similar to the deglycosylated tomato extensin, dP2 which has the major repeating decapeptide, Ser-(Hyp)4-Val-Tyr-Cys-Tyr-Lys. Further Western blot analyses are in progress. The extent of true homology awaits further biochemical analyses: peptide mapping and amino acid sequence determination of the major repeating peptides of these HF-treated cotton fractions to see if these peptides are highly periodic like other extensins.

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