CHEMICAL AND MOLECULAR ANALYSIS OF THE CELL WALL COMPOSITION OF TOMATO (*Lycopersicon esculentum*) IN RELATION TO RESISTANCE TO *Ralstonia solanacearum*, CAUSAL AGENT OF BACTERIAL WILT

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ZUSAMMENFASSUNG

Die Bakterielle Welke verursacht durch *Ralstonia solanacearum* gehört zu den wichtigsten bakteriellen Pflanzenkrankheiten weltweit. In der Bekämpfung scheinen integrierte Maßnahmen mit wesentlichem Augenmerk auf der Resistenz von Wirtspflanzen die geeignetsten Mittel. Um die bisher instabile Resistenz der Wirtspflanzen zu erhöhen wurden am Modellsystem Tomate / *R. solanacearum* Untersuchungen zur Rolle der Pflanzenzellwandstruktur in der Interaktion mit dem Pathogen durchgeführt. Extrahierte Pektine einer resistenten Linie wiesen signifikant höhere Methylveresterungsgrade des Homogalakturonans (HG) in der Wurzel (res.: 64,0%, anf.: 7,0%) und im Stängel (res.: 44,0%, anf.: 9,0%) auf als Extrakte einer anfälligen Linie, während in der Monomerenzusammensetzung Unterschiede im Mannoseanteil gefunden wurden. Mittels Immuno-Dot-Blot wurde eine nicht-blockweise Esterverteilung im Pektin aus Stängeln der anfälligen und eine blockweise Verteilung in der resistenten Linie festgestellt. Im Immun-Stängel-Print wurde nach Infektion ein erhöhter Anteil niedrig veresterter HGs mit homogener De-esterifizierung beobachtet, was auf einen spezifischen Abbau nicht-blockweise verteilter Estergruppen durch die bakterielle Pektinmethylesterase hindeutet, und es wurden verstärkt Galaktan- und Arabinanseitenketten des Rhamnogalakturonan I (RGI) und Arabinogalaktanprotein (AGP) in den Xylemwänden der anfälligen Linie nachgewiesen. Der Anstieg im Nachweis homogen de-esterifizierten HGs nach Infektion bestätigte sich in anfälligen nah-isogenen Linien. In immun- histochemischen Untersuchungen wurden in der anfälligen Linie konstitutiv ein erhöhter Anteil homogen de-esterifizierter HGs und geringerer Anteil AGPs in den Gefäßwänden sowie Galaktans des RGI im Xylemparenchym nachgewiesen. Nach Infektion stieg in der anfälligen Linie der Nachweis von niedrig verestertem HG und die homogene Veresterungsstruktur sowie von Galaktan und Arabinan in den Seitenketten des RGI in und um die Gefäße stark an, während sich in der resistenten Linie die Anzahl Gefäße mit erhöhtem Galaktan- und Arabinannachweis signifikant erhöhte, was auf einen Abwehrmechanismus hinweisen könnte. Eine Erhöhung der Basis- Resistenz durch Selektion von Linien mit veränderter Zellwandstruktur könnte möglich sein.

Stichwörter : *Ralstonia solanacearum,* Resistenz, Zellwandpolysaccharide

ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi) is one of the most important and widely distributed plant diseases in the tropics. An integrated approach with emphasis on host plant resistance is the most suitable measure for the control of bacterial wilt. To improve unstable resistance, investigations were conducted in the model system tomato / *R. solanacearum* focussing on the role of plant cell wall structures in interaction with the pathogen. Extracted pectins from the resistant genotype showed significantly higher methyl-esterification of homogalacturonan (HG) in roots (resistant: 64%, susceptible: 7%) and in stems (resistant: 44%, susceptible: 9%) than extracts from the susceptible genotype, while in the monomeric composition differences were observed between the genotypes in the mannose content. In immunodot blot membranes a non-blockwise de-esterification pattern showed in extracts from stems from the susceptible genotype compared to a more blockwise pattern of HG in the resistant genotype. In tissue print assays of stems of the susceptible genotype after infection an increase in low-esterified HG was observed indicating the possible action of pathogen pectinmethylesterase (PME). Also detection of galactan and arabinan side chains of RG I and of arabinogalactan proteins (AGPs) in the xylem cell walls increased in the susceptible genotype. The increase in the homogeneous de-esterification of HG after infection was also confirmed with susceptible, near-isogenic lines. In immuno-histochemical studies the susceptible lines revealed a constitutively higher part of homogeneous de-esterification of HG and a lower part of AGPs in xylem walls, as well as galactan in RG I in the xylem parenchyma. After inoculation an increased labelling of low esterified HG was seen in the susceptible genotype as well as stronger labelling of arabinan and galactan side chains of RG I in and around vessels, while in the resistant genotype after infection labelling of arabinan and galactan side chains of RG I increased significantly, indicating a possible resistance mechanism. Selection of tomato lines with optimal cell wall structure could be a possible venue to increase basic resistance against bacterial wilt.

The biochemical analysis of lipopolysaccharides (LPS) of *R. solanacearum* revealed the typical composition of LPS for *R. solanacearum* strains without major differences among them. Rheological interactions between extracted plant pectins and bacterial LPS were measured *in vitro*. No synergistic effects such as increases in viscosity were recorded in various mixtures of LPS of *R. solanacearum* strain ToUdk2 and pectins from stems of susceptible host plants nor occurred any rheological changes in mixtures with pectin from the resistant plant.

Keywords : *Ralstonia solanacearum*, Resistance, Cell wall polysaccharides

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ABBREVIATIONS

ANOVA	Analysis of variance
APS	Ammoniumpersulphate
AUDPC	Area Under Disease Progress Curve
AVRDC	Asian Vegetable Research and Development Centre
Bv	Biovar
Cfu	Colony forming units
Cm	Centimeter
cv.	Cultivar
Da	Dalton (unit of molecular weight)
Dpi	Days post inoculation
°C	Degree Celsius
DE	Degree of Esterification
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polysaccharide
EPS I	Extracellular polysaccharide I, main virulence factor of *R. solanacearum*
et al.	*et alii* (and others)
FAOSTAT	Food and Agricultural Organization statistic database
Fig.	Figure
G C	Gas Chromatography
g	Gram
h	Hours
HCl	Hydrochloric acid
HR	Hypersensitive response
hrp	Bacterial genes coding for hypersensitive reaction and pathogenicity
hrpB	Regulatory gene of the hrp gene region
HW	Hawaii
KCl	Potassium chloride

kb	Kilobase
kDa	KiloDalton (1,000 Dalton)
K_2O	Potassium
LPS	Lipopolysaccharides
Ltd.	Limited
$MgCl_2$	Magnesium chloride
µl	Microlitre
min	Minute
ml	Millilitre
mM	Millimolar
ND	Not determined
ng	Nanogram
NGA	Nutrient glucose agar
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate- buffered saline
PL	Pectate lyase
P.	Pseudomonas
PC	Phenotype conversion
PehA	Endopolygalacturonase
PehB	Exopolygalacturonase
PehC	Exopolygalacturonase
PglA	Endopolygalacturonase
PglB	Exopolygalacturonase
PglC	Exopolygalacturonase
pH	Potential of hydrogen
PhcA	Central transcriptional regulator of the phenotype conversion system
PIPES	piperazine-N.N-bis(2-ethane sulphonic acid)
Pme	Pectin methylesterase
Po	Potato (*Solanum tuberosum*)

PopA, PopB, PopC	Proteins secreted into the extracellular medium by the *R. solanacearum* Hrp secretion system
PrhA	Plant regulator of hrp genes
PrhI/PrhR	Regulatory components of the plant cell contact-dependent activation of hrp genes
P_2O_5	Phosphorus oxide
PVPP	Polyvinylpolypyrrolidone
pv.	Pathovar
rpm	Rotations per minute
R. solanacearum	*Ralstonia solanacearum*
s	Second
spp.	Species (plural)
SDS	Sodium dodecyl sulphate
Tek	Twenty eight kilodalton protein
TE	Tris EDTA
TEMED	N,N,N,N,-Tetramethyl –Ethylenediamine
TZC	Tetrazolium chloride
UV	Ultraviolet
V	Volt
w/v	Weight per volume

ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi) is one of the most important and widely distributed plant diseases. The following studies were conducted using the model plant tomato to understand the interaction between *R. solanacearum* and its host plant resistance mechanisms at cell wall level. The cell wall pectins of healthy tomato genotypes resistant (H7996) and susceptible (L390) to bacterial wilt were extracted by chelator-soluble extraction from stem and root tissues of 4 week-old plants. The pectins obtained by this extraction procedure were characterized both by composition and degree of methylation. Except for mannose, no significant difference was observed between stems and roots of the genotypes in all the monomers. The degree of methylation (% DM) of homogalacturonans was by a factor of 4 and 6 higher in polysaccharides from stems and roots, respectively, of the resistant genotype H7996 than in extracts from L390. An Immunodot blot assay was conducted for the rapid identification of unbranched homogalacturonan and branched components occurring in extracted pectic polysaccharides using anti-pectin monoclonal antibodies: JIM5 - specific for low ester, JIM7 -high ester, LM7 - non-blockwise de-esterification patterns of homogalacturonan (HGA), LM2 - arabinogalactan protein, LM5 - galactan, LM6 - arabinan epitopes of rhamnogalacturonan I (RG I) respectively. Extracts of both resistant and susceptible tomato genotypes reacted with all the antibodies giving a central dot. No outer ring was observed. Comparing pectic polysaccharides from stems of resistant and susceptible genotypes, the samples from L390 reacted significantly stronger with LM7 than samples from H7996 in both concentrations, indicating a homogeneous de-esterification pattern of the pectic polysaccharides from L390 in comparison to a more blockwise pattern in H7996. Comparing samples from roots, genotype H7996 reacted stronger with antibody LM2 than genotype L390, indicating higher contents of arabinogalactan protein in H7996. It has recently been proposed that some bacterial species, including *R. solanacearum* that are readily culturable in the laboratory, may enter a long-term survival state, when subjected to environmental or laboratory conditions with prolonged starvation or other stress, in which they are not detectable by standard culturability tests. Therefore, the effect of extracted pectic polysaccharides of tomato genotypes on viability and

culturability of *R. solanacearum* was examined. No significant (P=0.05) difference occurred between the numbers of viable and culturable cells from the mixtures with pectic polysaccharides from the genotypes as compared to the control after overnight incubation.

Based on the results obtained in preliminary investigations the research was continued to determine the dynamics of pathogenic processes: interest was concentrated on the vascular colonization by *R. solanacearum* of resistant and susceptible tomato genotypes including near isogenic lines. Changes in the pectic polysaccharides in vascular tissues before and after inoculation with *R solanacearum* were examined. Near isogenic lines and tomato genotypes were compared for resistance to bacterial wilt as measured by disease severity and population density in the mid-stem regions. All symptomless plants were latently infected. Wilt incidence was recorded up to 40 days after inoculation with *R. solanacearum.* On the basis of the area under wilt incidence progress curve (AUDPC), the genotypes were classified into two significantly different groups, i.e. resistant and susceptible genotypes. Two near isogenic lines, NHG 3 and NHG 167, were classified as susceptible, with similar AUDPC as the susceptible standard genotypes L390 and Wva 700. *R. solanacearum* was detected in the mid-stem region of symptomless plants of the resistant genotypes Hawaii 7996, CLN 2123C, CLN1-3-13, CLN4-22-4, CLN1-1-12, CLN1-5-12, BL333, NHG 60, NHG 140, NHG 13 and NHG 162, and of the moderately resistant King Kong 2.

With the antibodies JIM5, JIM7, LM2, LM5, LM6 and LM7 against epitopes present in pectin and AGPs we studied the modification of these wall components during infection by stem tissue prints. The composition and structure of pectins in xylem vessels of tomato in relation to *Rs* was examined in tomato genotypes differing in their resistance level, such as the standard susceptible and resistant genotypes L390 and H7996, respectively, and in near isogenic lines of tomato differing in resistance, obtained from a cross between H7996 and the susceptible Wva700. Increased staining after inoculation occurred with all antibodies except JIM7 in genotype L390 indicating an increase in low esterification and non-blockwise de-esterification pattern of HG and in arabinan and

galactan epitopes of RG I and arabinogalactan proteins. The tissue prints of the susceptible isogenic lines showed a clear difference from the resistant lines after inoculation in binding LM7.

These findings were supported by the immunocytochemical analysis where the density of epitopes was determined and compared in vascular tissues of mid-stem sections from resistant (H7996) and susceptible (L390) tomato genotypes, with and without inoculation with *R. solanacearum*, using the same antibodies. Significant differences were observed between healthy and inoculated H7996 where 70% and 76% of the vessels of inoculated plants showed labeling after staining with LM5 and LM6, respectively, compared to 12% and 8 % of non-inoculated plants. A dramatic increase in the epitopes labeling with a high level of fluorescence in xylem vessels of mid-stems of genotype L390 was observed after inoculation when the reactivities of LM2, LM5, LM6 and LM7 recognizing the arabinogalactan proteins, epitopes of galactan and arabinan side chains of branched regions of pectins and non-blockwise de-esterification pattern of HGA domain of pectic polysaccharides respectively, increased. Labeling with ß–glucosyl Yariv reagent resulted in typical deep red staining of arabinogalactan-proteins.

The immunological results can best be interpreted with a more blockwise distribution of the methyl esters in HG from resistant tomato genotypes and a non-blockwise distribution in the HG from susceptible plants.

Using two independent approaches, chemical and immunological methods, and using resistant and susceptible standard tomato genotypes and near isogenic lines we have found a significant difference in the HG methyl esterification between bacterial wilt resistant and susceptible tomato plants.

LPS was extracted from *R. solanacearum* strains Pss190 and Pss216 (Wang and Lin, 2002) highly and lowly virulent on tomato, respectively, and Pe104 and ToUdk2 from Thailand (Thaveechai, Bangkok), with moderate and high virulence, respectively (Leykun, 2003) and analyzed for fatty acids, phosphates, heptoses, keto-deoxy sugar. The

biochemical analysis revealed the typical composition of LPS in the *R. solanacearum* strains without major differences among them. The rheological interactions between the plant pectins and bacterial LPS were measured. No synergistic effects such as increases in viscosity were recorded in various mixtures of lipo-polysaccharides of *R. solanacearum* strain ToUdk2 and pectins from stems of susceptible host plants.

CHAPTER I

Structural characterization of extracted cell wall polysaccharides from tomato genotypes resistant and susceptible to *Ralstonia solanacearum* and studies of their influence on the physiological state of the pathogen

1.1 INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most cultivated and consumed vegetable worldwide and is grown in various cropping systems and locations. The yearly world production averaged 113,308,298 tons over the last 5 years (FAOSTAT, 2003). Besides the conventional soil system, tomato is grown in greenhouses to satisfy the high world demands (Olympios, 1975). On the market, both field and soil-less grown tomatoes are found. The soil-less system allows production of tomatoes in areas, where suitable soil is not available or where diseases and other conditions make ground production unfeasible (Olympios, 1975; Hochmuth 1999). The greenhouse production requires the simulation of field conditions and adapted varieties. Most of the field varieties do not perform well in the greenhouse environment. Two kinds of tomato varieties, determinate and indeterminate types are cultivated (Rehm and Espig, 1991). The first types are bushy and produce tomato fruits in one harvest, while the indeterminate forms produce year round and are often grown in soil-less culture, which provides plants with nutrients (Hochmuth, 1999). Major advantages of this system are the elimination of need for soil sterilization by chemicals and a precise control of water and nutrients (Olympios, 1975).

Although nutrients, water and climate are major limiting factors, biotic pressure from insects and pathogens can drastically reduce tomato yield. Bacterial wilt is one such major constraint for vegetable production, and especially for tomato in the lowland and highland tropics. Estimates of yield losses due to the disease in tomatoes range from 75 to 100 % (Nirmala *et al*., 2002). The causal agent previously named *Pseudomonas solanacearum,* then reclassified as *Burkholderia solanacearum,* and recently as *Ralstonia*

solanacearum (Yabuuchi *et al*., 1995) is the most destructive pathogen of tomato and its aggressiveness is attributed to its widespread occurrence and high pathogen diversity. *Ralstonia solanacearum* is characterized by the existence of different races, its exceptional ability to survive in the soil and in the roots of non-host plants, and its broad host range. *R. solanacearum* affects over 200 different crops and weed species including economically important host plants such as banana, eggplant, ginger, tobacco, potato (Priou *et al.,* 2002), geraniums (Kim *et al.,* 2002) and tomato (Hayward, 1991). On the basis of host range, *R. solanacearum* strains have been traditionally divided into races, with race 1 affecting Solanaceae, some diploid bananas and other hosts, race 2 affecting triploid bananas and *Heliconia sp*. and race 3 causing damages to potato and tomato. Physiological and genetic characterization resulted in the formation of biovars and divisions (Hayward, 1964, 2001; He *et al.,* 1983, 1986).

R. solanacearum is a soil-borne pathogen and a complex and heterogeneous species. Studies on host range and colony morphology (Kelman, 1953; Buddenhagen *et al*., 1962, 1986), biochemistry (Hayward, 1964, 2001; Harris, 1972), serology (Colleno *et al*., 1976; Schaad *et al*., 1978), membrane proteins (Dristig and Dianese, 1990), and phage susceptibility (Okabe and Goto, 1963) of the bacterium all conclude that the species is composed of a number of distinct strains.

So far, control of bacterial wilt has been ineffective. Breeding for disease resistance has not been very successful because of the extensive variability of bacterial strains and the interactions of a myriad of biotic and abiotic factors. Although intensive efforts have been made to understand the basic mechanism of disease resistance, the fundamental biochemical basis is still unknown.

Resistance against this pathogen is described as quantitative or polygenic on several plants (Wang *et al*., 2000). Natural resistance mechanisms occurring in higher plants can be classified into preformed and induced mechanisms (Schlösser, 1997; Knogge, 1997; Baker *et al*., 1997; Keen, 1999). After the penetration of the invader, induced processes take place. According to the hypothesis of Flor (1971) in host defense, pathogen invasion

is recognized by receptor proteins encoded by plant disease resistance (R) genes, which bind to specific pathogen-derived products of avirulence (Avr) genes. Following recognition of a pathogen, a complex signaling network involving cytosolic Ca^{2+} and H^{+} ions, reactive oxygen species (ROS) (oxidative burst), jasmonate, salicylic acid and ethylene trigger the induction of defense mechanisms (Odjakova and Hadjiivanova, 2001; Brummell and Harpster, 2001). Thus, after the penetration of the pathogen, structural mediations and biochemical responses begin. Structural modifications are accumulation of callose, suberin, lignin, and accumulation of hydroxyproline rich glycoprotein. The biochemical responses are hypersensitive reaction (HR), biosynthesis of phytoalexins and pathogenicity related proteins (PR-proteins) (Knogge, 1997; Odjakova and Hadjiivanova, 2001; Kang and Buchenauer, 2000, 2002, 2003; Graham *et al.,* 2003). Quantitative responses include cell wall modifications in response to pathogen invasion (Prell, 1996; Odjakova and Hadjiivanova, 2001).

Recently, in *R. solanacearum* a number of effector proteins have been identified which specifically interact with plant proteins forming a recognition complex (Boucher and Genin, 2004). *Hrp* genes of *R. solanacearum* code for components of a type III pathway that is typically involved in secretion of proteins required for successful host-pathogen interactions in numerous animal and plant pathogenic bacteria (Gueneron *et al.,* 2000; Genin and Boucher, 2002).

More than 20 *hrp* genes are clustered on the 1.9-Mb megaplasmid spanning 23 kb (Schell, 2000). This gene cluster comprises five transcriptional units which code for a type III secretion apparatus. Presumably, this secretion system serves for transportation of various virulence determinants and avirulence proteins into plant cells by connecting the inner and outer membranes of the bacteria. By providing transfer of these proteins nutrition acquisition and avoidance of defense reactions by the host plant are supported (Schell, 2000).

Genetic analysis of resistance in tomato with molecular markers has led to the observation of an important quantitative trait locus (QTL) on chromosome 6 and showed

that the resistance controlled by this locus could be specific for the type of bacterial strain (Danesh and Young, 1994; Thoquet *et al*., 1996a, b; Mangin *et al*., 1999; Zhang *et al.,* 2002; Lindhout *et al.,* 2003). A molecular linkage map of tomato based on resistance gene analogs (RGA) was constructed where 29 RGAs were located on 9 of the 12 tomato chromosomes (Foolad *et al.,* 2002; Zhang *et al.,* 2002).

Pathogens vary greatly in the way and rate they multiply and spread. Resistance generally affects the multiplication of the pathogen rather than its spread. Bishop and Cooper (1984) observed that various resistance mechanisms probably decrease the extent of xylem colonization although the potential for xylem penetration may be similar in resistant and susceptible cultivars of tomato invaded with fungal pathogens. The formation of tyloses and gels could be induced by wounding. The ontogeny of tyloses and gels and the possible involvement of these structures in resistance to wilt disease has been studied (Wallis and Truter, 1978; Grimault *et al*., 1994). In many host pathogen systems studied, pectic fragments produced during host cell wall degradation can act as endogenous suppressors of the hypersensitive response in the susceptible plants (Moerschbacher *et al.,* 2003) and act as elicitors for the HR in resistant plants (Ridley *et al.,* 2001).

Root infection by *R. solanacearum* occurs through wounds caused by various agents as insects, nematodes, agricultural equipment and natural openings (Kelman and Sequeira, 1965; Schmit, 1978). The pathogen colonizes the exterior of the root and then the intercellular spaces of the cortex, infects the vascular parenchyma, and finally invades the xylem vessel elements (Vasse *et al*., 1995). Electron-dense material on cell walls and pit membranes has been reported during interaction between tomato plants and *R. solanacearum* (Wallis and Truter, 1978; Vasse *et al*., 1995; Rahman *et al.,* 1999; Nakaho *et al.,* 2000). Resistance to wilt in tomato was clearly related to the capacity of the plant to restrict *R. solanacearum* invasiveness in the stem (Bowman and Sequeria, 1982; Grimault and Prior, 1994, 1995; Prior *et al.,* 1996; Leykun, 2003).

Plant cell walls, mainly consisting of polysaccharides including cellulose, hemicellulose and pectin, are an essential barrier for plant pathogens. The cell wall-degrading enzymes secreted by the pathogens during infection and colonization of host plants may play an important role in pathogenesis (Cooper, 1983; Walton, 1994). In many plant- pathogen systems cell wall components have also been suggested to contribute to the susceptible or resistant reaction against pathogens (Kang and Buchenauer, 2000; Perombelon, 2002; Moerschbacher *et al.,* 2003). In potato stem tissues, a higher percentage of methylated and branched pectins has been reported to correlate with resistance against the bacterium *Erwinia carotovora* subsp. *atroseptica* (McMillan *et al*., 1993; Marty *et al*., 1997). A difference in the degree of pectin methylation was also observed between tomato cultivars resistant and susceptible to *Pseudomonas syringae* pv. *tomato* (Venkatesh, 2002).

Growing plants are shaped by an extensible wall that is a complex amalgam of cellulose microfibrils bonded non-covalently to a matrix of hemicelluloses, pectins and structural proteins (Cosgrove, 1997). Jarvis *et al.* (2003) reported that the vascular ring of brassica stems is an interesting, exceptionally flexible model system in which to study how cell wall structure and the mechanical properties of plant tissues are related. Pectins present in the cell walls of plants form a gel phase in which the cellulose-hemicellulose network is embedded. The mechanical characteristics of plant cell walls may depend on detailed structures of their galacturonan components such as esterification patterns, insertion of rhamnose units, presence of neutral side chains, either directly (Yamaoka and Chiba, 1983; Jarvis, 1984) or indirectly, as in the case when free acidic pectin domains are involved in cross-linking and, consequently, in stiffening of the cell wall (Fry, 1986).

Pectic polysaccharides are a major component of the cell wall in vegetable plants and have an important influence on ripeness and plant food texture and are widely used as stabilizing agents in the food industry (Rollin and De Vries, 1990). Pectins are structurally extremely complex. The principal building block of pectins is galacturonic acid (GalA). Besides, they contain large quantities of other sugars, with rhamnose, arabinose, glucose and galactose being the most abundant. The functional properties of

pectins reside primarily in their chemical structure and composition (Fig 1.1). The amount of neutral sugars were shown to determine intercellular attachment (Kikuchi *et al*., 1996). The elucidation of the structure of pectins has been the main centre of research over years (Schols and Voragen, 2003).

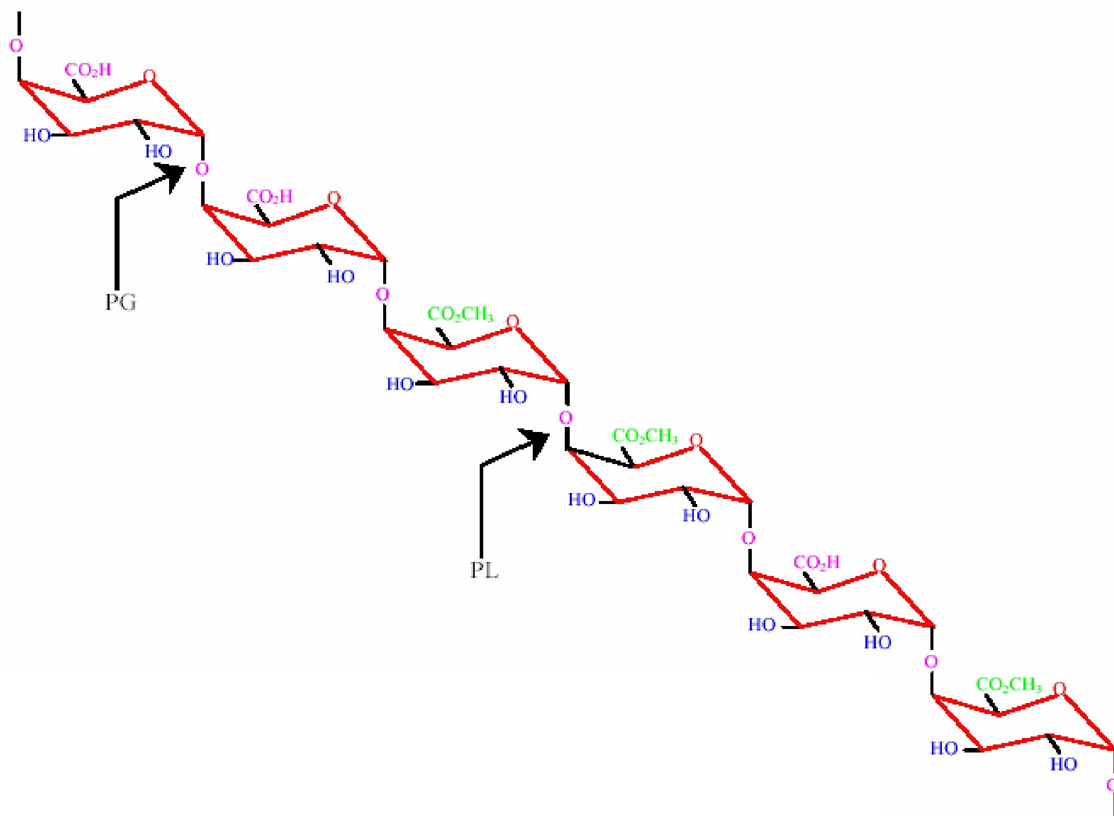


Fig 1.1 Structure of pectin and sites of cleavage of pectin degrading enzymes. PG: polygalacturonase; PL: pectate lyase (Daas *et al.,* 1999)

Four major domains of complex pectic polysaccharides are known: homogalactouronan (HG), rhamnogalacturonans (RG I, RG II) and xylogalacturonan (O'Neill *et al*., 1990; Albersheim *et al*., 1996; Mohnen, 1999; Ridley *et al*., 2001). HG (the smooth region) is a polymer of (1→4)-α linked galacturonic acid (Gal A) which can be methyl-esterified

(C6) and /or acetylated at positions 2/3 and when de-esterified these HG polymers can associate via divalent calcium ions leading to gel formation (Jarvis, 1984). The regions of the pectin molecule that comprise linear chains of α-(1→4)-D-GalA units are referred to as "smooth" regions and the blocks of highly substituted rhamnogalacturonan that interrupt the HG backbone are known as "hairy" regions (De Vries, 1982). RG I (the hairy region) has a backbone of alternating Gal A and rhamnose residues (Lau *et al*., 1987) with 20-80% Rha substituted with (1→4)-ß-D-galactan, (1→5)-α-L-arabinan or arabinogalactan (O'Neill *et al*., 1990; Carpita and Gibeaut, 1993; Schols and Voragen, 1994; Albersheim *et al*., 1996; Mohnen, 1999). RG II consists of a backbone of galacturonosyl residues with complex and diverse side chains (Whit Combe *et al*., 1995), which can be linked by borate di-ester bonds, and appears to be the most conserved of the pectic polysaccharides (Vidal *et al*., 2000).

The degree and distribution of esterified regions differ between plant species and also at different developmental stages of growth during the life cycle of a plant. Liners and Van Cutsem (1992) reported that pectins extracted from tightly attached young calli of carrot have higher amounts of methyl-esterified galacturonic acid residues when compared to that of loosely attached old calli which have a significant amount of non-methyl esterified regions. The degree of methyl esterification (DM) depends on the plant source as well as the cell type and age (Vreeland *et al* 1989; Liner and Van Cutsem, 1992). The degree of methyl-esterification is highly variable in relation to cells, and can greatly influence the pectic network and cell wall properties (Willats *et al*., 2001).

HG is believed to be synthesized in a highly esterified form, but may be subsequently de-esterified by the action of plant or pathogen pectin methylesterase (PMEs) which can remove methyl groups in a blockwise, or random fashion. An abundant class of plasma membrane-associated proteoglycans known as arabinogalactan-proteins (AGPs) are implicated in the control of plant cell proliferation and cell development (Fincher *et al*., 1983; Bacic *et al*., 1986; Pennell *et al*., 1989) (for details see chapter 3).

The availability of pectin-degrading enzymes has led to the emergence of a new approach known as enzymatic fingerprinting to broaden our understanding of the structural diversity of pectins (Limberg *et al.,* 2000). This enzymatic degradation aspect was also used to study pectins from leaves of cultivars of tomato resistant and susceptible to *Pseudomonas syringae* pv. *tomato* (Venkatesh, 2002). The most commonly used enzymes for the purpose are the exo- and endo- polygalacturonases (PG), pectate lyase (PAL), pectin methylesterase (PME), pectin lyase (PL) and ß-galactosidase (De Veau *et al.,* 1993).

Recently, antibodies against structural regions of the pectin molecule have been raised (Knox *et al.,* 1991, 2002, 2003). These antibodies are successfully being used to establish the deposition of the different structural elements of pectins in the plant cell walls. The present studies focus on arabinogalactan-proteins and extracted pectic polysaccharides from genotypes of tomato resistant (H7996) and susceptible (L390) to *R. solanacearum.*

Several virulence factors produced by *R. solanacearum* have been identified, including plant cell-wall degrading enzymes (PG, PMEs) and exo-polysaccharides I (EPS I) (Mc Garvey *et al*., 1999). Plant cell-wall degrading enzymes appear to enhance virulence by promoting invasion and vascular colonization (Allen and Simon, 1997; Kang *et al*., 1994; Mc Garvey, 1997). *R. solanacearum* produces three PGs, called PehA, B and C, which hydrolyze polygalacturonic acid, a long chain polymer of galA residues that is the predominant component of pectins. In addition, *R. solanacearum* produces a PME, which hydrolyzes an ester bond to release methanol from the $–COOCH_3$ groups on the polygalacturonic acid polymer. A substantial proportion of pectin in plants is methylated, but before such pectins can be degraded by PGs, methyl groups must first be removed by PME because PGs cannot attack highly methylated pectins. PGs can degrade pectic polymers in different ways: *endo*- PGs cleave the pectic polymer internally at random, generating a rapid decrease in substrate viscosity, while a second class of PGs remove one (exo-PG) or two (exo-poly-a-D-galacturonosidase) terminal galacturonate residues at a time (Allen *et al.,* 1998).

As in other major groups of Gram-negative, phytopathogenic bacteria, *R. solanacearum hrp* genes have been identified as essential determinants for disease development on compatible hosts and for elicitation of the hypersensitive response (HR) in resistant plants (Boucher *et al*., 1985, 1992, 2002; Lindgren, 1997; Vasse *et al*., 2000). Allen (2001) reported that motility of the bacterium after invasion also contributes to the virulence on tomato. During wilt disease development, when the bacterial density is low and the LysR-type global regulator called *phc*A is not expressed, *R. solanacearum* cells are motile and highly pectolytic. As bacterial populations increase in the host xylem elements, *phcA* is expressed, inducing production of the known virulence factors and reducing motility by repressing *pehSR* expression. *PehSR* is a positive regulator of plant cell wall-degrading polygalacturonases, which are also virulence factors. However, bacterial motility has not been directly measured in the plant during pathogenesis. Numerous Gram-negative bacteria including *R. solanacearum* produce type IV pili (Tfp) important for adhesion, gene transfer and twitching motility (Liu *et al.,* 2001).

An interaction between pectic polysaccharides and a bacterial pathogen at cell walls in the intercellular space was suggested by different authors (Vasse *et al.,* 1995). Since the interaction may be involved in the compatible or incompatible reaction, a reaction of the pathogen to resistance factors of the plant might be possible. *R. solanacearum* was reported to enter the viable but non-culturable (VBNC) state which is explained as the discrepancy between plate counts and total viable counts in a more complex environment, *in planta,* in sterile soil, and in liquid bacterial suspensions, with varying percentages depending on the prevailing environmental or host conditions, presumably induced by shortages of nutrient availability as has been shown to occur with other microbes (Heijnen *et al*., 1995, Van Overbeek *et al*., 1995). A portion of the original cell population that could not be cultured became VBNC and those cells, which could not withstand the stresses, may not survive.

The VBNC state could be a long-term survival mechanism induced by an oligotrophic environment, and cells can remain as VBNC cells for more than a year (Mc Dougald *et al.,* 1998). It might be possible that in the infection process, the pathogenic bacteria

become VBNC in response to a signal such as decreased nutrient availability, resistance responses of host plants or due to the wilting process prior to the return of the bacteria to the soil. However, this response might differ from plant to plant according to their resistance level. The VBNC state of bacteria has significance for epidemiology and ecology of bacterial pathogens in natural environments and constitutes an adaptive strategy of non-spore-forming bacteria allowing survival under adverse conditions (Roszak and Colwell, 1987). VBNC cells of pathogenic bacteria may therefore provide a potential reservoir for infection, which evades detection by most standard methods (Xu *et al.,* 1982; Roszak *et al.,* 1984; Oliver, 1993; Oliver and Bockian, 1995; Colwell *et al.,* 1996, Rahman *et al.,* 1996; Whitesides and Oliver, 1997; Mc Dougald *et al.,* 1998).

Many gram-negative organisms such as soil microbes including *R. solanacearum, Pseudomonas fluorescens* and *Salmonella enterica serovar typhimurium* (Binnerup *et al*. 1993; Turpin at al., 1993; Van Overbeek *et al*. 1995; Pernezny *et al*., 1997; Van Dyke *et al*., 1998; Alexander *et al*., 1999; Ghezzi *et al*., 1999) have been reported to respond to changes in environmental conditions by entering into the VBNC state. Processes of injury (DNA damage) may cause VBNC phenomena, as observed in cold-stressed *Vibrio vulnificus* (Oliver, 2000), however, its role attributed to “programmed cell death” or “cellular suicide” (Bloomfield *et al.,* 1998; Hochman, 1997; Nystroem, 1998) was not well investigated.

Moreover a VBNC state of *R. solanacearum* was recently demonstrated to retain its pathogenicity (Steck *et al.,* 2001). The ability of VBNC cells to resuscitate has raised some controversy, however, it has recently been reported that “VBNC” cells of *R. solanacearum* can be resuscitated (Steck *et al.,* 2001), suggesting that natural conditions may be able to “trigger” growth of VBNC cells. Two other reports (Oliver, 1995; Oliver and Bockian, 1995) showed growth (resuscitation) of VBNC cells of *V. vulnificus,* an organism which has become the paradigm of the VBNC hypothesis in environmental chambers and in a mouse model, and also, more recently, in the laboratory under certain conditions (Whitesides and Oliver, 1997). For conventional microbiology, viability and culturability are equivalent. Recovery of culturable cells from a population of non-

culturable cells (resuscitation) is ultimately required and is the keystone for the confirmation of the VBNC hypothesis (Steck *et al.,* 2001). However, reports demonstrated that the presence of a low level of residual culturable cells within the population of VBNC cells are able to grow and multiply in response to changes which are suggested to trigger resuscitation of VBNC cells such as host organisms or added nutrients in laboratory samples. Hence, investigations on other resuscitation methods are required to support the VBNC state of bacteria. Studying the VBNC state of *R. solanacearum* in plant tissue and the percentages of cells entering into this state in relation to the level of host resistance and through the progress of infection may further elucidate the interaction of the pathogen with its host plant.

We analyzed the extracted pectic polysaccharides from healthy resistant (H7996) and healthy susceptible (L390) cultivars of tomato using the recently developed technique of immuno-profiling in which immuno-reactive components occurring in the preparation of pectic polysaccharides can be resolved on the basis of their differing mobilities on nitrocellulose membranes and their reaction to highly specific antibodies (Willats and Knox, 1999). We also investigated water-soluble AGP using the monoclonal antibody LM2 (Smallwood *et al*., 1996; Serpe *et al.,* 2002) by immuno-localization studies. Specific antibody probes to defined oligosaccharide components of the pectic side chains have currently been developed and have been used to gain insight into the function of these side chains during cell development and cell expansion in a range of plant systems: JIM5 recognizes a low-ester epitope of HG, JIM7 recognizes a high-ester epitope of HG, LM5 an epitope of (1→4)-ß-galactan of RG I, LM6 an epitope of (1→5)-α- arabinan of RG I, and LM7 the non-blockwise de-esterification pattern of HG. In our present study we also investigated the influence of extracted pectic polysaccharides from both resistant and susceptible tomato cultivars on the VBNC *state* of the bacteria.

1.2 MATERIALS AND METHODS

1.2.1 Plant material

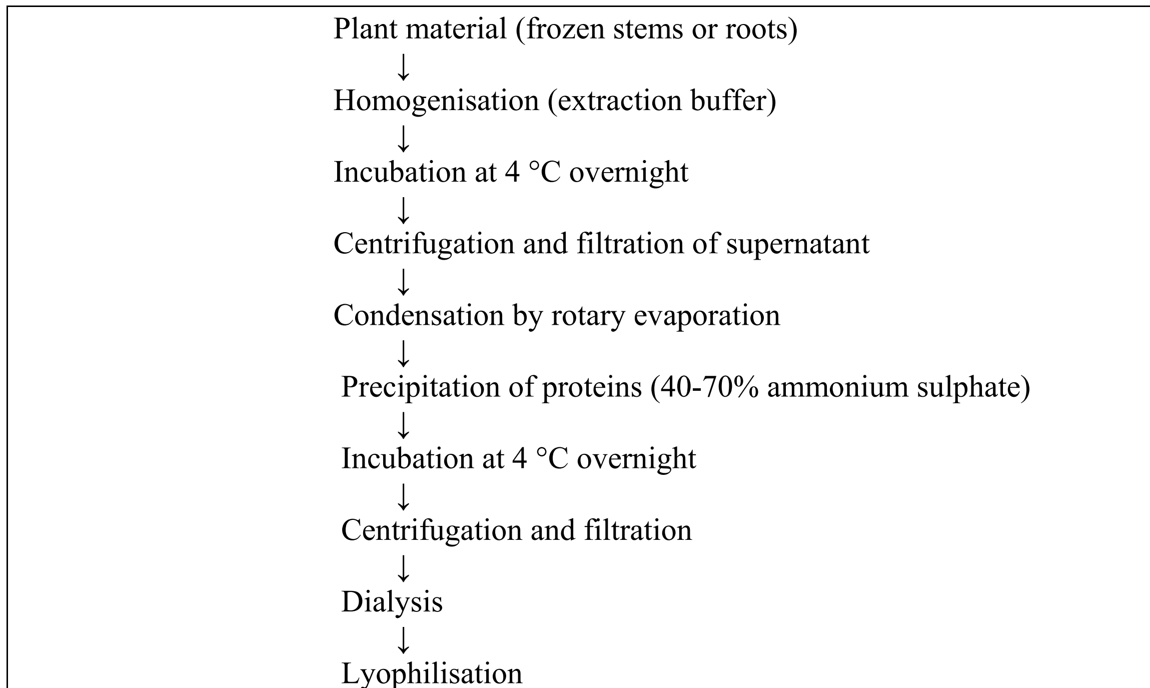
Tomato genotypes Hawaii 7996 and L390, resistant and susceptible to bacterial wilt (Jaunet and Wang, 1999) were received from the Asian Vegetable Research and Development Centre (AVRDC), Taiwan. Genotypes King Kong and King Kong-2 with moderate resistance level were obtained from Asian Vegetable Research and Development Centre (AVRDC), Taiwan. Seeds were sown in a greenhouse (20°C day/night temperature, 14h of light per day / 30K Lux, and 70% relative humidity) and transplanted to individual pots with 330g of soil (Fruhstorfer Erde, Type P, with 150mg/l N, 150mg/l P_2O_5, and 250mg/l K_2O). During the experiment plants were kept in a climate chamber with 30/27 °C day/night temperature, 85% relative humidity, 14 hours light, and 30K Lux. After 4 weeks the stems and roots were harvested and kept frozen for further trials.

1.2.2 Extraction of pectic polysaccharides

Pectic polysaccharides were extracted from stem and root tissues of 4 week-old plants of the four tomato genotypes following the method of Sonnenberg (1994).

About 100-200 g of stem material was harvested and immediately frozen at -20 °C. The root system was thoroughly washed to remove all traces of substrate and also frozen. The frozen stem or root material was homogenized with 100 mM NaH_2PO_4 buffer (pH 5.5) (Sigma) containing 4% (w/v) polyvinylpolypyrrolidone (PVPP) (Sigma), 20 mM ethylenediamine tetraacetic acid (EDTA) (Sigma) and 2% (w/v) sodium ascorbate (Sigma) in a mixer-blender at its maximum speed for few minutes till the material was homogenized. After keeping the homogenate overnight at 4 °C, it was centrifuged at 9,000 x g (Sorvall RC-5B refrigerated centrifuge) for 30 min at 4 °C. The supernatant was filtered through cheese cloth and condensed to one fifth of the initial volume using a

rotary evaporator (RV 05 rotary evaporator, Janke and Kunkel GmbH, u. Co KG, IKA WERK Staufen im Breisgau, Germany), which was held at a temperature below 35 °C.



Scheme for the extraction of pectins from plant stems and roots (Sonnenberg, 1994)

Proteins were precipitated from the supernatant by slowly adding ammonium sulphate to a saturation of 40-70% and the suspension was kept stirring overnight at 4°C. The suspension was centrifuged at 9,000 x g for 30 min to remove the proteins. The supernatant containing pectic polysaccharides was filtered through cheese cloth and filter paper (Schleicher and Schüll, Germany, with a pore size 320 mm) and dialyzed against demineralised water at 4 °C for 72 h and water being changed after every two hours. After reducing the volume by further condensation by a rotary evaporator, the solution containing the crude pectic polysaccharides was lyophilized. These pectin extractions were repeated three times from each genotype being grown and harvested thrice producing pectic polysaccharides in triplicate.

1.2.3 Acid hydrolysis of pectic polysaccharides

Monosaccharide compositions from the above extracted pectic polysaccharides produced in triplicate from each genotype were analyzed again in triplicate by capillary gas–liquid chromatography of the trimethyl-silylated methyl-glycosides using the method of Chaplin (1982) with inositol as the internal standard. Briefly, carefully dried samples (approx. 100 µg) were methanolyzed (200 µL 1.5 M methanolic HCl, 50 µL methyl acetate, 16 h, 80 °C) and derivatized using a commercial silylating agent (Silyl 2110, Chromatographie Service, Langerwehe, Germany) containing hexamethyldisilizane, trimethylchlorosilane and pyridine (2:1:10). Prior to injection, samples were dried under a gentle stream of nitrogen and dissolved in 30 µL isooctane. One µL samples were split-injected (1:10, injection port temperature 230 °C) into the gas–liquid chromatograph (Hewlett-Packard model HP 5840A GC with a flame ionization detector maintained at 260 °C) equipped with an OV-1 column (25 m × 0.32 mm i.d.; 0.2 µm film thickness; Macherey-Nagel, Düren, Germany). Carrier gas was nitrogen at 1 ml/min. The temperature was held at 140 °C for 4 min, then increased at 2 °C/min to 200 °C, followed by a 6 °C/min rise to 250 °C. Pectins extracted in triplicate from each genotype were used and the trial was repeated twice.

1.2.4 Quantitative determination of uronic acids

Uronic acids were quantified following the method described by Blumenkrantz and Hansen (1973). Extracted pectic polysaccharides were dissolved at 10 mg/ml of water. To 200 µL of the solution, 1.2 ml of H_2SO_4 was added. The tubes were cooled in crushed ice. The mixture was shaken in a vortex mixer and the tubes heated in a water bath at 100°C for 5 min. After cooling in a water ice-bath, 20 µL of m-hydroxy-diphenyl reagent (Sigma) was added. The tubes were shaken, and within 5 min absorbance measurements were made at 520 nm in a Beckman DU spectrophotometer. Because carbohydrates produce a pinkish chromogen with sulphuric acid at 100 °C, a blank sample was run without the addition of the reagent, which was replaced by 20 µL of 0.5% NaOH. The

absorbance of the blank sample was subtracted from the total absorbance. The determination of uronic acids from each extracted sample was performed in triplicate.

1.2.5 Total protein determination

The total protein content was determined using the Bradford Reagent (Sigma) assay (Bradford, 1976). The samples were prepared at a concentration of 1mg/ml. One hundred microliter of the sample were mixed with 900 µL Bradford reagent [0.1 g Coomassie brilliant blue G-250 (Sigma) in 50 ml of EtOH (95%), added to 100ml phosphoric acid (85%) diluted to 1000 ml with water and filtered]. Samples were measured at 595 nm against a BSA standard.

1.2.6 Determination of degree of methyl esterification (DM) of pectic polysaccharides

The degree of methylation of the extracted pectic polysaccharides was determined following the method of Wojciechowski (1996). Five milligram of the samples were weighed and added to 400 µL of water and 1.25 ml of KOH (200 mM). All steps were carried out at 4 °C. The tubes were closed air-tight and shaken at 80 rpm at 4 °C for 5 h. After centrifuging the samples at 7,000 x g for 10 min at 4°C, the supernatant was filtered with Millipore filtration unit using filters with a pore size of 0.45 µm (Pall Life Sciences, Centrifugal Devices Nanosep, MF GHP).

Seven hundred fifty microliter of filtrate were mixed with 750 µL potassium-phosphate buffer (pH 4.0). Five microliter alcohol oxidase from Pitchia Pastoris, Sigma (400 u/ml) was added and incubated at 25°C in a water bath for 15 min. To this 20 µL of Fluoral-P reagent (Sigma) (15mg/ml) was added and vortexed. After measuring the volume of the samples, they were incubated at 60 °C in a water bath for 15 min. The tubes were cooled in ice for 5-10 min and measured again with the fluorometer at Ex - 405 nm and Em - 503 nm. The standard curve was prepared using methanol. The determination of degree of esterification of each sample was performed in triplicate.

1.2.7 Immuno-dot Assay

Immunodot assays were carried out as described in Willats *et al.* (1999, 2000). Extracted pectic polysaccharides were dissolved in water at a concentration of 10 mg/ml and applied as 1-µL aliquots to a nitrocellulose membrane (Scheicher and Schüll, Dassel, Germany) in a 5- or 10-fold dilution series.

Following the application of samples, membranes were left to air-dry for 30 min. All subsequent treatments were conducted at room temperature. Membranes were blocked with phosphate buffered saline (PBS) (NaCl = 135mM, KCl = 3mM, $Na_2HPO_4.2H_2O$ = 10mM, KH_2PO_4 = 2mM) containing 5% skim milk powder (MPBS, pH 7.2) for 1 h prior to incubation in primary antibodies (hybridoma supernatants of JIM5, JIM7, LM2, LM5, LM6, LM7, diluted 1/10 in MPBS, received from P. Knox, University of Leeds, UK) for 1.5 h. After washing extensively under running tap water and for 10 min in PBS containing 0.1% (v/v) Tween 20 (PBST), membranes were incubated for 1.5 h in the secondary antibody (anti-rat horseradish peroxidase conjugate, Sigma) diluted 1/1000 in MPBS. Membranes were again washed as described prior to development in substrate solution [25ml deionized water, 5ml methanol containing 10mg/ml 4-chloro-1-naphthol, 30 µL 6% (v/v) H_2O_2]. Citrus pectin (63-66% DM) and apple pectins (70-75% DM) (Sigma (Poole, Dorset, UK) were used as controls. The results were obtained based on visual evaluation by keeping the membranes on an illuminating table, giving color grade values from 0-4 for controls and 0-7 for samples. Depending on the intensity of the dots obtained we assigned the lower values for samples and the higher values up to grade 7 for controls. The samples showed the presence of a central dot and were differentiated from the controls which formed the central dot and an outer ring. The immunodot assay was repeated three times from pectins that were extracted from each genotype in triplicate.

1.2.8 Quantification of VBNC bacterial cells

Ralstonia solanacearum strain ToUdk2 was grown at 30°C for 48h on NGA medium (0.3 % beef extract, 0.5 % Bacto peptone, 0.25 % D-glucose, 1.5 % agar). Bacterial colonies

were harvested with sterile water and the suspension adjusted to an optical density of 0.06 at 600nm. Cells were concentrated by filtration on a 0.2 µm pore-size isopore membrane, black polycarbonate filter (Millipore, USA.).

To determine the number of VBNC cells after incubation of *R. solanacearum* with pectic polysaccharides, extracted pectic material of the tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt respectively, were dissolved in water at a concentration of 10 mg/ml and mixed 1 ml with 1ml of the inoculum suspension of a 48 h old culture of *R. solanacearum* strain To-Udk2 of OD 0.06 at 600 nm. After overnight incubation at room temperature the suspension was assayed for viable bacteria by the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes Inc., Eugene, Oreg. USA). This assay uses two fluorescent nucleic acid stains, Component-A, nucleic acid stain, 3.34 mM and Component-B, propidium iodide, 20 mM (Molecular Probes, Eugene, Pregon, USA), which differ in their ability to penetrate cell membranes. Component-A can enter cells with and without an intact cell membrane. Component-B can only enter cells with a compromised membrane. As a result, cells with intact membranes (i.e. viable bacteria) stain fluorescent green, and bacteria with damaged membranes (i.e. dead bacteria) stain fluorescent red.

From the same samples, an aliquot was streaked on NGA. Live bacterial suspensions as control as well as the incubated mixture of pectin from both genotypes H7996 and L390, and bacterial suspension were diluted tenfold up to 10^{-7}. One hundred microliter of the dilutions were plated in duplicates on NGA agar medium and colonies were counted after incubation at 30°C for 48 hours. The concentration of VBNC cells is calculated by subtracting the concentration of culturable cells from the concentration of viable cells.

One milliliter of each dilution of the bacterial suspension of the mixture with pectin was stained by adding 1 µL of Component A and 2 µL of Component B, and incubating at room temperature for 30 min in the dark. The cells were re-collected on a 0.2-µm-pore-size filter and counted.

At least 100 grids per sample over at least ten fields of vision under the microscope (Zeiss fluorescence microscope with a broad range filter for green and red fluorescence (Olympus B x 60 Epifluorescence) at a magnification of 250 x were scored in one replication of the tested sample. The average number of green cells was calculated per one milliliter of inoculum suspension to determine the viability of bacterial cells.

1.2.9 Statistical Methods

Data were processed using analysis of variance in SAS (the SAS System for Windows V8, Release 8.02 TS Level 02M0; 1999-2001. Institute INC., Cary, USA). For all analyses a significance level of P = 0.05 or lower was used. The bacterial counts on media, were expressed as colony forming units per gram of fresh matter or per milliliter of bacterial suspensions, and analyzed using parametric analysis procedures in SAS.

1.3 RESULTS

1.3.1 Carbohydrate composition of pectic polysaccharides from tomato stems and roots

Pectic polysaccharides from stems and roots of tomato genotypes H7996 (resistant) and L390 (susceptible) to *R. solanacearum* were obtained by chelator-soluble extraction.

Monomeric composition of pectic polysaccharides from stems of genotypes H7996 and L390 was generally similar, with galactose (38-39 %) in highest concentration, followed by arabinose, rhamnose, xylose and glucose in pectins from genotype H7996, and followed by glucose, arabinose and lower contents of rhamnose and xylose in genotype L390 (Table 1.3.1). The total carbohydrate composition made up 15-20 % of the extracted stem material. Mannose was only found in pectins from stems of genotype L390. Total uronic acids made up 1-2 %, and protein 10-20 % of the extracted stem material.

Table 1.3.1 Carbohydrate composition of pectic polysaccharides from stems and roots of healthy tomato genotypes H7996 and L390, resistant and susceptible to *R. solanacearum*, respectively. Neutral monosaccharides were obtained by GC (Chaplin, 1982), total content of proteins (Bradford, 1976), total content of uronic acids (Blumenkrantz and Hansen, 1973) and degree of methylation (DM) (Wojciechowski, 1996) were determined by spectrophotometric methods. Content is expressed as % weight with standard error values of pectins from three repeated trials and pectin extractions. Tukey test at P = 0.05.

sample	[% WT]		composition			Monomeric	Total protein	Uronic acids	% DM
	rha	ara	xyl	man	glc	gal			
H7996-stems	19.8±14.0a	23.8±15.5a	9.7±4.9a	0±0**a**	8.5±5.9a	37.9±19.5a	19.8±7.7a	1.6±0.4a	44.0±5.0**a**
L390-stems	6.9±1.2a	13.9±3.3a	5.6±1.5a	5.0±1.0**b**	29.1±15.7a	39.3±14.1a	10.5±3.0a	1.3±0.2a	9.0±3.8**b**
H7996-roots	4.4±3.6a	6.1±5.0a	25.3±6.9a	0±0**a**	11.8±1.9a	51.9±0.5a	7.8±1.9a	0.2±0.03a	64.0±0**a**
L390-roots	9.7±1.3a	19.0±0.6a	3.2±0.3a	3.2±0.3**b**	7.4±0.1a	57.4±1.9a	2.7±0.08a	1.2±0.3a	7.0±0.9**b**

In pectins from roots, galactose content was predominant with more than 50% of the total % weight, followed by xylose, glucose, arabinose and rhamnose in genotype H7996, and by arabinose, rhamnose, glucose, and xylose, in genotype L390. Root pectin of the latter genotype contained additionally 3% mannose. Except for mannose, no significant difference was observed between stems and roots of the genotypes in all the monomers. Comparing genotypes, only the pectin of roots of genotype L390 contained mannose.

The degree of methylation (% DM) of homogalacturonans was by a factor of 4 and 6 higher in polysaccharides from stems and roots, respectively, of the resistant genotype H7996 than in extracts from L390.

1.3.2 Characterization of extracted pectic polysaccharides by immunodot assay

Pectic polysaccharides from genotypes H7996 and L390 were characterized for unbranched homogalacturonan and branched components using monoclonal anti-pectin antibodies recognizing different epitopes (Table 1.3.2).

Table 1.3.2 Characterization of EDTA-extracted pectic polysaccharides from stems and roots of healthy tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively, and the moderately resistant genotypes King Kong and King Kong 2 by immunodot assay with six antibodies specific for different pectic epitopes.

Quantities of 100 µg and 50 µg pectin were applied to the nitrocellulose membrane. Apple and citrus pectins (10 µg and 5 µg, respectively) were used as controls. For visual grade evaluation a scale of 0-4 for samples and 0-7 for controls was used. Antibody specificities: JIM5 - specific for low ester, JIM7 -high ester, LM7 - non-blockwise de-esterification patterns of homogalacturonan (HGA), LM2 - arabinogalactan protein, LM5 - galactan, LM6 - arabinan epitopes of rhamnogalacturonan I (RG I).

Samples	µg	JIM5[1]	JIM7	LM2	LM5	LM6	LM7
L390-stems	**100**	1.2 ± 0.4[2] a A	1.2 ± 0.6 a A	1.4 ± 0.8 a A	1.2 ± 0.2 a A	1.6 ± 0.5 a A	2.1 ± 0.05 **a** A
	50	1.0 ± 0.5 a A	0.7 ± 0.4 a A	1.5 ± 0.7 a A	0.7 ± 0.1 a A	1.1 ± 0.4 a A	1.5 ± 0 **a** A
H7996-stems	**100**	1.0 ± 0.6 a A	0.9 ± 0.1 a **A**	0.8 ± 0.3 a **A**	1.3 ± 1.1 a A	0.9 ± 0.6 a **A**	0.8 ± 0.3 **b A**
	50	0.3 ± 0.3 a **A**	0.7 ± 0.2 a **A**	0.6 ± 0.4 a **A**	0.7 ± 0.6 a A	0.7 ± 0.6 a A	0.2 ± 0.1 **b A**
H7996-roots	**100**	3.5 ± 0.5 a A[3]	4 ± 0 a **B**	4 ± 0 **a B**	3.2 ± 0.2 a A	3.7 ± 0.2 a **B**	4 ± 0 a **B**
	50	3.2 ± 0.7 a **B**	4 ± 0 a **B**	4 ± 0 **a B**	3 ± 0 a A	2.7 ± 0.2 a A	3 ± 0 a **B**
L390-roots	**100**	1.7 ± 1.5 a A	1.7 ± 1.2 a A	1.4 ± 0.6 **b** A	1.5 ± 0.5 a A	2 ± 1 a A	1.8 ± 1.1 a A
	50	1.2 ± 0.7 a A	1.6 ± 1.4 a A	1.4 ± 0.6 **b** A	1.2 ± 0.7 a A	1.8 ± 1.1 a A	1.3 ± 0.6 a A
King Kong-stems	**100**	1[4]	1.5	0.25	2.5	1.25	0.66
	50	0.5	1	0.33	2	0.75	0.5
King Kong2-stems	**100**	4	3	3	4	3	4
	50	3	2	2	2	2	3
Citrus pectin[5]	**10**	7	7	0.5	5.5	5	0
	5	7	7	0.5	5.5	4.5	0
Apple pectin[6]	**10**	6	7	0.5	5.5	4.5	0
	5	6	6	0.5	5.5	4.5	0

[1] Antibodies with specificity to pectic epitopes
[2] Color grade values with standard errors for three repeated trials with pectin from three extractions
[3] Tukey test at P = 0.05; small letters: comparison between genotypes; capital letters: comparison between stem and root extracts
[4] No triplicate extraction done, therefore no SE values , [5] Degree of esterification: 63-66 % , [6] Degree of esterification: 70-75 %

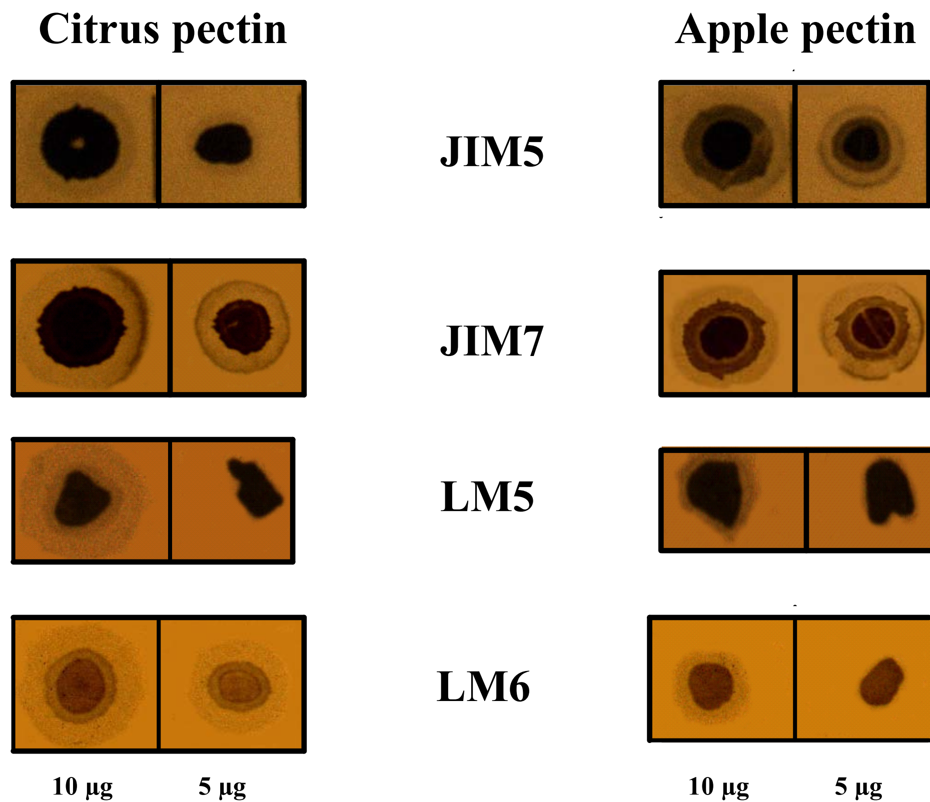


Fig.1. Immunodot-binding assay of commercial samples of pectic polysaccharides. Samples of citrus pectin (CP) and apple pectin (AP) were dissolved in demineralized water and applied at 1- µl volumes (10mg/ml) to a dry nitrocellulose membrane.
After drying the nitrocellulose was blocked and probed with the anti-low-ester pectin (JIM5), anti-high-ester pectin (JIM7), anti-(1→4)-ß-D-galactan (LM5) and anti-(1→5)-α-L-arabinan (LM6) rat monoclonal antibodies. Antibody binding was detected with anti-rat IgG linked to horse-radish peroxidase as a dark bluish coloration.

Probing commercial samples of citrus and apple pectin with anti-low-ester antibody JIM5, at least two components were detected, an outer ring and a central dot (Fig.1). In case of citrus pectin, JIM5 binding was most intense in the central dot, whereas apple pectin produced a dot and an intense outer ring. Both commercial pectins did not show any specificity for the LM7 epitope which recognizes the non-blockwise de-esterification patterns of HGA (photo not shown), but reacted intensely with a central dot with LM5 and LM6, which bind to the epitopes (1→4)-ß-D-galactan, and (1→5)-α-L-arabinan, respectively (Fig. 1, Table 1.3.2). A generally slight reaction occurred for controls with LM2 which is specific for arabinogalactan-protein except a strong reaction with pectin from stems of genotype KK2 (photo not shown).

Extracts of both resistant and susceptible tomato genotypes reacted with all the antibodies giving a central dot (Table 1.3.2.). No outer ring was observed. Comparing pectic polysaccharides from stems of resistant and susceptible genotypes, the samples from L390 reacted significantly stronger with LM7 than samples from H7996 in both concentrations (Fig. 2), indicating a homogeneous de-esterification pattern of the pectic polysaccharides from L390 in comparison to a more blockwise pattern in H7996. No significant difference between genotypes was found in epitope-binding specificities of JIM5, JIM7, LM2, LM5 and LM6. Antibody reactions indicated the presence of branched pectic polyssaccharides with low quantities of galactan and arabinan epitopes in stems of both genotypes. Generally, samples from genotype L390 reacted slightly stronger with the antibodies than samples from H7996.

Comparing samples from roots, genotype H7996 reacted stronger with antibody LM2 than genotype L390 (Fig. 3), indicating higher contents of arabinogalactan protein in H7996. No significant difference between root extracts of the two genotypes was found when probing with antibodies JIM5, JIM7, LM5, LM6 and LM7.

Comparing pectic polysaccharides from stems and roots, extracts from roots of genotype H7996 showed significantly stronger reactions with antibodies JIM5, JIM7, LM2, LM7

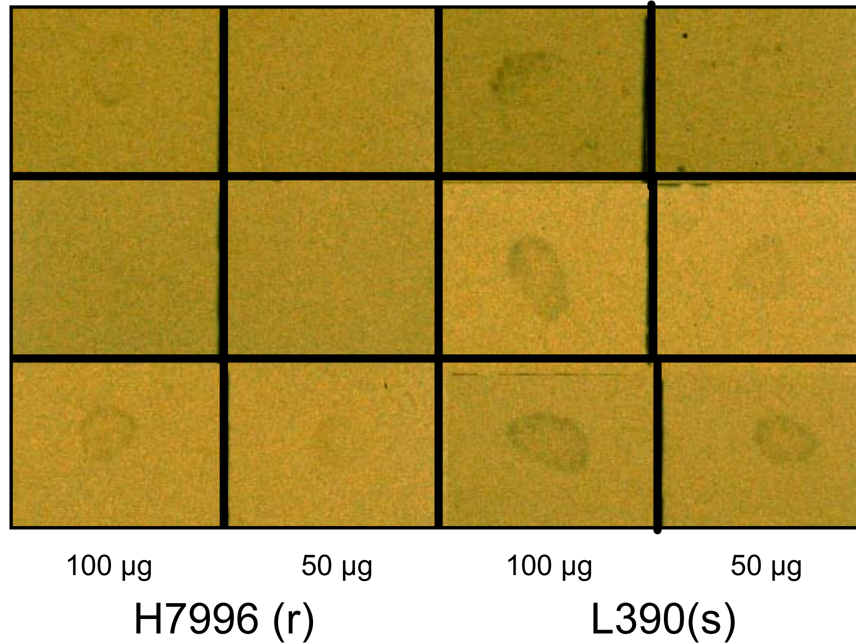


Fig. 2. Pectins from healthy stems of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively were probed on nitrocellulose membrane at 100 µg/ml and 50 µg/ml by immuno dot blot assay. The two genotypes showed differences in labeling with antibody LM7 which recognizes the non-blockwise de-esterification pattern of HG.
Results from three different sets of extracted pectins are shown (upper, middle, lower rows). The trial was repeated thrice.

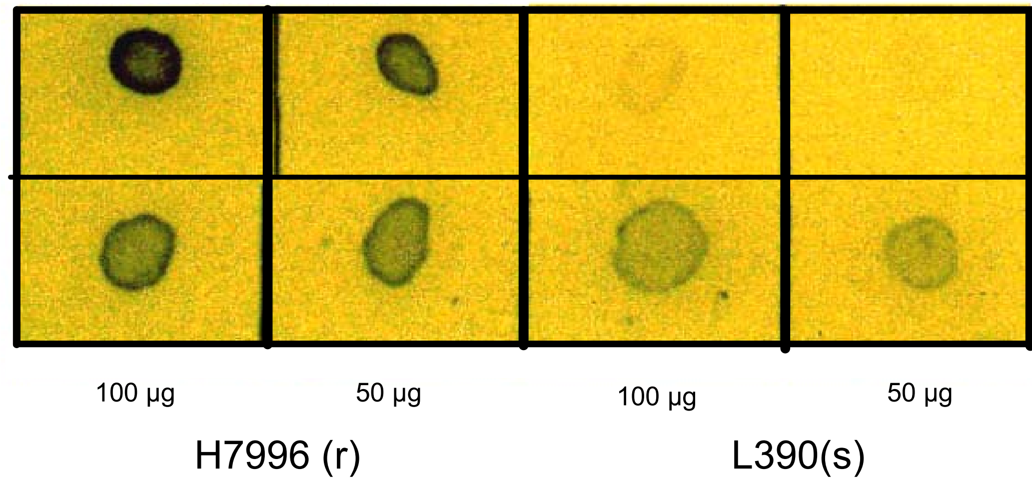


Fig. 3. Dot blots of pectic polysaccharides (50 µg/ml, 100 µg/ml) obtained from roots of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively, stained for arabinogalactan proteins with antibody LM2. Results from two sets of extracted polysaccharides (upper and lower rows); the trial was repeated three times.

and less with LM6 than extracts from stems, indicating a high esterification in a non-blockwise pattern of HG, presence of arabinogalactan protein and RG I with arabinan side chains, and small quantities of a pectin with low esterified HG. The root polysaccharides from genotype L390 seem to be less branched than from H7996. No differences between extracts from stems and roots were observed for genotype L390.

Extracts from stems of the moderately resistant genotype King Kong2 reacted strongly with all the antibodies, while King Kong extracts showed overall less intense reactions.

1.3.3 Effect of extracted pectic polysaccharides on viability and culturability of *R. solanacearum*

EDTA-extracted pectic polysaccharides from stems of tomato genotypes H7996 and L390 were mixed 1:1 (v/v) with a suspension of *R. solanacearum* (OD = 0.06 at 600 nm corresponding to 7.8×10^7 CFU/ml) and assayed for viable and culturable forms of bacteria after overnight incubation.

Table 1.3.3 Number of viable and culturable cells of *R. solanacearum* (cfu/ml) after mixture with pectins (10 mg/ml) extracted from tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively.
Values represent cell counts of three repeated trials with standard errors (P=0.05 with Tukey test)

Cell counts	**Control** [1]	**H 7996** [2]	**L 390**
viable [3]	$3.02x10^7 \pm 5.06x10^6$ a	$3.3x10^7 \pm 8.4x10^6$ a	$1.4x10^7 \pm 3.7x10^5$ a
culturable [4]	$5.5x10^7 \pm 2.6x10^7$ a	$5.2x10^7 \pm 1.6x10^7$ a	$5.4x10^7 \pm 3.6x10^7$ a

[1] Control: Sterile water
[2] Extracted pectins dissolved in water, 10 mg/ ml
[3] Detection of viable cells by fluorescence staining with the LIVE/DEAD *Bac*Light Viability Kit, observed under a fluorescence microscope at a magnification of 250x.
[4] Aliquots of serial dilutions were plated on NGA medium and bacterial colonies counted after 48 h incubation at 30 °C.

No significant (P=0.05) difference occurred between the numbers of viable and culturable cells from the mixtures with pectic polysaccharides from the genotypes as compared to the control after overnight incubation (Table. 1.3.3).

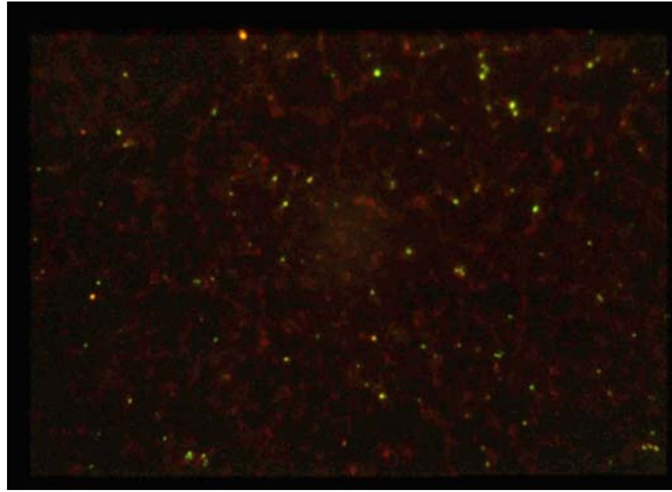


Fig. 4. Viable *R. solanacearum* cells (green fluorescent) in mixture with pectins from tomato stem tissue, stained by the LIVE/DEAD *Bac*Light Viability Kit, collected on the 0.2 µm pore-size filter, observed under fluorescence microscope at a magnification of 250x.

1.4 DISCUSSION

The primary cell wall is composed of a rigid cellulose-hemicellulose network that is embedded in a hydrated matrix of pectic polysaccharides and structural glycoproteins (Carpita and Gibeaut, 1993; Willats *et al.,* 2001).

Many extractants have been used to solubilize pectins from plant material such as water-soluble solids (WSS), chelating-agent-soluble solids (ChSS), dilute-alkali (50mM NaOH)-soluble solids (DASS) and concentrated-alkali (4M KOH)-soluble solids (CASS) as described in Orfila *et al.* (2002). For instance, chelating agents such as EDTA as used in the present studies capture calcium ions, resulting in solubilization of HG-HG interactions of the calcium-pectate gel. However, besides HG, also RG I, arabinan, and galactan are solubilized, which implies that these polysaccharides are interconnected (De Vries *et al*., 1982). The bulk of these structurally highly complex chelator-soluble polymers probably originate from the middle lamella.

The components from the supernatant of the tissue macerate after ammonium sulphate fractionation are rich in galactose, arabinose and xylose, but also contain rhamnose and glucose. These presumably represent arabinogalactan and xylogalacturonans which are present in the side chains of pectic polysaccharides and components of the cell walls (Mc Cartney *et al.,* 2000; Vierhuis *et al.,* 2001). Schols *et al.,* (1995) reported the presence of xylogalacturonan in pea, which is a type of HG substituted at C-2 or C-3 positions with α-D-xylose, and does not appear to be as widespread as the other pectic domains. The abundance of arabinose is probably due to the presence of a neutral arabinan, as also reported in pea stems by Pauly *et al.* (2000). Glucose is only a minor component of the polysaccharides in the extract. Majewska-Sawka *et al.* (2002) reported the presence of xyloglucan (RG I) bearing terminal α-(1→2)-fucosyl residues in the guard cells in sugar beet leaves (*Beta vulgaris L.*).

The most important constituent sugars in the extracted pectic fractions as detected by gas chromatography are galactose, glucose and arabinose, while only low quantities of uronic

acids were detected by photometric determination. This may be due to incomplete hydrolysis during analysis (Orfila *et al.* 2002) possibly caused by the presence of methyl- or acetyl- esterified HG, or branched RG I domains covalently bound to HG.

Also Goldberg *et al.* (1989) reported that the use of the chelating agent EDTA for extracting pectins from the cell walls led to the removal of only negligible amounts of uronic acids. Additionally, the low content of uronic acids in the pectic polymers is typical for pectins derived from primary cell walls (Ryden and Selvendran, 1990). The range of pectic polysasccharides obtained indicates the presence of highly branched pectins derived from the primary cell wall. The total uronic acids could derive from galacturonan or alternating RG I and RG II type side chains (Jarvis *et al*. 1988), or partly from the hemicellulosic fraction. This was found in extracts from some species where glucuronic or 4-O-methyl glucuronic acid was attached to xylose. Therefore, we did not use the uronic acid content as an indicator of pectin content.

The degree of methylation was observed to be higher in tomato genotype H7996 resistant to *R. solanacearum* than in the susceptible genotype. It is suggested that the high degree of pectin methylation could be one of the factors contributing to the resistance of genotype H7996 to *R. solanacearum*. Also in other host-pathogen systems such as potato and *Erwinia carotovora* subsp. *atroseptica* and tomato and *Pseudomonas syringae* pv. *tomato* pectins of the resistant genotype were higher methyl-esterified. It is suggested that these genotypes are less easily degraded by pectinolytic enzymes, which cannot act on branched or highly methylated galacturonans (McMillan *et al.,* 1993; Venkatesh, 2002).

Populations of pectic polysaccharides with varying compositions are solubilized to different degrees by all these above described extractants (Willats *et al*., 2001; Dinu, 2001). The presence of arabinan- and galactan-containing pectic polymers has also been reported in ChSS extracts from tomato fruit (Gross, 1984; Seymour *et al*., 1990). Another part of pectic polysaccharides which are not extracted by our method may be constituents of the non-EDTA soluble fraction. Also Knox *et al.* (2002) suggested the existence of a population of pectic polysaccharides that are tightly associated with the cellulose-

hemicellulose network. The covalent association of pectic polysaccharides to xyloglucan has been reported in *Rosa glauca* cell suspension cell walls (Thompson and Fry, 2000) and the presence of cellulose-associated xyloglucan has been reported in pea stems (Pauly *et al.,* 2000).

^{13}C-NMR on tomato cell wall material indicated a population of pectic polysaccharides that is tightly associated with cellulose microfibrils (Fenwick *et al.,* 1996). Thus, a cellulose-associated cell wall micro-domain, which may be subjected to structural modulation during growth and might also be altered after invasion of a pathogen may play a role in the determination of cell wall properties. Extensive studies of the pectic polysaccharides in the pea testa also identified xylose-rich pectins and xylogalacturonan domains (Renard *et al*., 1997; Huisman *et al.,* 1998; Le Goff *et al*., 2001) representing cell wall polymers that appear to be highly cross-linked. Nevertheless, according to our hypothesis, these less soluble or water and EDTA- unsoluble polysaccharides would not come into contact with the pathogen in the intercellular space during the first phases of infection. Thus, the non-EDTA soluble fraction is not the goal of our characterization studies.

The pectic material is supposed to be covalently bound, if not to the cellulose then to other components such as phenolic material and proteins, so as to be enmeshed with the cellulose fibres. Moerschbacher *et al.* (2003) also reported that the pectic fractions extracted by HF-solvolysis are usually contaminated with considerable amounts of hemicelluloses. The contents of higher amounts of xylose and glucose in the roots of the resistant genotype (H7996) than in the roots from the susceptible (L390) could be explained by the presence of xyloglucan. Usually pectic polymers contain very low quantity mannose so the difference observed between the resistant and susceptible genotypes in mannose content where susceptible genotype L390 had higher amount of mannose was not important and could be indicating the presence of mannan as the chelator used for extraction can also solubilize some nonpectic polymers. Hence it would

be interesting to study the role of these mannose containing nonpectic polymers in resistance reactions.

The immunodot assays indicated that the chelating agent fractions contained galactan and arabinan epitopes, providing evidence that branched RG I domains with arabinan and galactan side chains are present in the chelator-soluble fractions of root and stem cell walls of tomato genotypes. Also Jones *et al.* (1997) and Willats *et al.* (1998) found RG I with arabinan and galactan side chains in EDTA-extracts. Therefore, the treatment with EDTA was especially effective for extracting low methylated pectic acids.

The monoclonal antibodies with defined epitopes are powerful tools for the dissection of the molecular structure of complex pectic polysaccharides. Both the degree and pattern of de-esterification influence the capacity of the antibodies JIM5, JIM7, LM2, LM5, LM6, and LM7 to bind to HG domains (Willats *et al.,* 2001). The extracted pectic polysaccharides reacted with all the antibodies producing a central dot. Our pectin samples showed low to high degrees of methyl esterification being higher in the resistant genotype, and typical reactions of highly branched polysaccharides because of the detection of arabinan and galactan epitopes, while differences occurred in the non-blockwise de-esterification pattern, with a regular de-esterification in pectin from genotype L390 and a more blockwise pattern in genotype H7996. HG which is synthesized in esterified form is subsequently de-esterified by the action of plant or pathogen PME which is acting in blockwise or non-blockwise pattern, respectively. In our study it was observed that in the susceptible genotype which differed from resistant genotype H7996 in non-blockwise pattern and this difference must be due to the action of the plant PME. It has been observed that some plant PMEs may have a non-blockwise action pattern as reported in pea stems (Willats *et al.,* 2001). More Interestingly, inhibitor proteins of plant PMEs have been identified that modifies the resulting distribution of methyl esters within the HG polymers, possibly shifting from a blockwise towards a more random pattern. It is tempting to speculate that the direct or indirect product of the Sr5-gene might possess such a PME modulating activity that would lead to loss of processivity of the enzyme (Camardella *et al.,* 2000; Moerschbacher, 2003). PMEs may

have diverse and complex roles during growth and development. In the interaction between tomato and *R. solanacearum* the following scenario may occur: the four pectinolytic enzymes comprising one pectin methylesterase (PME) and three polygalacturonases produced by *R. solanacearum* can degrade pectin into oligomers and supply the bacteria with monogalacturonic acid as a growth substrate (Tans-Kersten *et al.*, 1998). First, PME demethylates pectin and thereby facilitates following degradation of pectin by endopolygalacturonase PehA (also designated PglA) and exopolygalacturonase PehB (PglB) and PehC (PglC). Nonetheless, the true biological function of PME still has to be revealed, since Tans-Kersten *et al*. (1998) found that strains deficient in PME which also exhibited no detectable polygalacturonase activity on highly methylated pectin, showed no differences in virulence to the wild-type strains.

PehA is known to degrade polygalacturonate internally which results in trimers and larger galacturate oligomers. PehB, however, releases digalacturonic acid and PehC produces monogalacturonic acid from the same substrate (Tans-Kersten *et al*., 1998). Huang and Allen (1997) reported that strains lacking exopolygalacturonase (PehB) were less virulent than strains lacking endopolygalacturonase (PehA). It was suggested that these two enzymes may work additively or synergistically, since in an experiment a *pehA pehB* double mutant exhibited a much lower virulence. These results were even more pronounced when soil inoculation was used rather than petiole inoculation, leading to the hypothesis that both PehA and PehB are involved in promoting ingress of *R. solanacearum* from the wounds into the vascular system, supplementary to their nutritional role (Huang and Allen, 1997).

JIM5- and JIM7-recognized epitopes are produced by a wide range of degrees and distribution of methyl esterification. The optimal binding requirements of JIM5 are not fully defined. JIM5 has the capacity to bind to a wide range of HGA epitopes with varying degrees (up to 40%) and patterns of methyl-esterification (Willats *et al*., 2000). Therefore, discrete microdomains of the cell wall matrix of our tomato pectic polysaccharides are likely to contain HG with a mixture of HG methyl-ester distribution patterns resulting in a complex combination of physical properties. *In vitro*, analysis of

calcium-mediated pectic gels indicated that strength, water holding capacity, and porosity of gels was significantly influenced by both the pattern as well as degree of methyl-esterification of HG domains. HG is usually highly methyl-esterified prior to insertion into cell walls (Mohnen, 1999; Zhang *et al*., 1992). It is therefore likely that the activity of PMEs from plant origin, but also, in case of infection, deriving from the pathogen results in varying de-esterification action patterns and is an important mechanism for modifying matrix properties *in planta*. Thus, differences in the esterification degrees and patterns were found after infection of tomato with *R. solanacearum* in a susceptible genotype by immuno-histological studies (chapter III).

Our observation of dots without rings from all our samples stained with the antibodies indicates the presence of highly branched pectic polysaccharides. When a sample of pectic polysaccharides is applied to nitrocellulose for staining, unbranched HG-rich components will migrate to produce an outer ring that is separate from less mobile, branched components forming a central dot (Willats and Knox, 1999). Thus, these observations confirm the GC- results where high quantities of neutral sugars were found. Also Round *et al.* (1997) found by Atomic force microscopy that pectins from tomato fruits revealed to possess long branches, the existence of which was not known previously. The neutral components of pectin are known to be rich in galactose and arabinose, and occur as side chains of RG I. They have been described as branched or hairy regions, probably occurring in covalent association with pectins (Willats *et al*., 1999). The elevated levels of arabinose in our pectins may therefore be due to branched arabinans not recognized by LM6. Knox *et al.* (1999) reported that galactan which is known to be involved in cell elongation is attached to the acidic pectic domain and also indicated that it was separate from a distinct HG-rich component.

The immunoprofiling technique is complementary to histochemical immunolocalization studies in that it provides biochemical information on epitope occurrence. For both JIM5 and JIM7 the degree rather than the pattern of methyl esterification is the most important factor influencing binding.

Staining occurred with antibodies LM5 and LM6, specific for galactan and arabinan across genotypes and origin of pectin. These are the first defined epitopes occurring in the side chains of pectic polysaccharides to be immunolocalized in the developing plants. The side chains of RG I domains may affect the accessibility by enzymes with wall modifying properties to the sites of action within the cell wall matrix. Thus, it may be speculated that these parts cannot easily be degraded by enzymes of a pathogen. Additionally, a high content of branched RG I makes pectin a less suitable gelling agent (Ryden and Selvendran, 1990), which would create more unfavourable conditions for a bacterial pathogen (chapter III). Nevertheless, we could not detect differences in arabinan and galactan epitopes in pectins from the susceptible and the resistant genotype by the immuno-dot-blot method.

The binding of LM7 specific for the non-blockwise de-esterification domain of HG was observed in extracts from the susceptible genotype, but significantly less in the resistant genotype. The non-blockwise de-esterification pattern makes HG more suitable for calcium-pectate gels. These gels are favourable for pathogen survival in case of infection (Rudolph, 2001), and thus may constitute a factor supporting the survival and multiplication of *R. solanacearum* in the susceptible genotype in contrast to the resistant genotype.

Therefore, the compositional analysis and characterization of pectic polysaccharides from tomato stems and roots of both resistant and susceptible genotypes showed that the pectins of tomato stems are a heterogeneous group of polysaccharides and that the difference in pectin composition e.g. the esterification pattern of HG could be related to the resistance of genotypes. King Kong 2 reacted strongly with all the antibodies indicates that the pectins must be branched and were recognized by these antibodies used whereas it was not the case with genotype King Kong.

Our results showed that the extracted pectic polysaccharides from both resistant and susceptible genotypes had no influence on *R. solanacearum* to enter VBNC. Because there are few studies on the VBNC condition in plants and soil, especially using

R. solanacearum, it is not known which soil, plant or bacterial parameters influence entry of cells into the VBNC state. In former studies, indications were found that the number of VBNC cells was higher in a resistant genotype than in a susceptible one in the early phase of infection (Leykun, 2003). It is possible that conditions known to influence the survival of bacteria such as various host resistance responses, moisture, temperature, pH, O_2 availability, nutrient availability, soil texture and physiological status of the introduced bacteria could trigger cell-entry into the VBNC state.

Nevertheless, we could not find that pectic polysaccharides are involved in triggering the VBNC state of *R. solanacearum in vitro*. This does not exclude a possible interaction of pectic polysaccharides with the pathogen in *planta*, causing a change in the bacterial condition. If the VBNC condition is involved in the etiology of bacterial plant-pathogenic diseases, either via bacterial survival or in the infection process itself (Steck *et al*., 2001), then it could be expected that the appearance of VBNC cells might differ as the host genotypes differ in resistance.

1.5 SUMMARY

Pectic polysaccharides were extracted from stem and root tissues of 4 week-old plants of tomato genotypes H7996 and L390, resistant, susceptible, respectively, and King Kong and King Kong 2, moderately resistant to bacterial wilt, by EDTA-extraction.

Monomeric composition of pectic polysaccharides from stems of genotypes H7996 and L390 was generally similar, with galactose (38-39 %) in highest concentration, followed by arabinose, rhamnose, xylose and glucose in pectins from genotype H7996, and followed by glucose, arabinose and lower contents of rhamnose and xylose in genotype L390.

In pectins from roots, galactose content was predominant with more than 50% of the total % weight, followed by xylose, glucose, arabinose and rhamnose in genotype H7996, and by arabinose, rhamnose, glucose, and xylose, in genotype L390. Except for mannose, no significant difference was observed between stems and roots of genotypes in all the monomers.

A comparison of the degree of pectin esterification between the susceptible and resistant genotypes revealed significant differences. The pectins from the resistant genotype H7996 was more esterified than those from the susceptible genotype L390.

Pectic polysaccharides from genotypes H7996 and L390 were characterized for unbranched homogalacturonan and branched components using monoclonal anti-pectin antibodies recognizing different epitopes. Extracts of both resistant and susceptible tomato genotypes reacted with all the antibodies giving a central dot. No outer ring was observed.

Comparing pectic polysaccharides from stems of resistant and susceptible genotypes, the samples from L390 reacted significantly stronger with LM7 than samples from H7996 in both concentrations, indicating a homogeneous de-esterification pattern of the pectic

polysaccharides from L390 in comparison to a more blockwise pattern in H7996. No significant difference between genotypes was found in epitope-binding specificities of JIM5, JIM7, LM2, LM5 and LM6.

Comparing samples from roots, genotype H7996 reacted stronger with antibody LM2 than genotype L390, indicating higher contents of arabinogalactan protein in H7996. No significant difference between root extracts of the two genotypes was found when probing with antibodies JIM5, JIM7, LM5, LM6, LM7.

Extracts from stems of the moderately resistant genotype King Kong2 reacted strongly with all the antibodies, while King Kong extracts showed less intense reactions.

EDTA-extracted pectic polysaccharides from stems of tomato genotypes H7996 and L390 were assayed for viable and culturable forms of bacteria after overnight incubation. No significant difference occurred between the numbers of viable and culturable cells from the mixtures with pectic polysaccharides from the genotypes as compared to the control indicating that pectins did not influence *R. solanacearum* to enter into VBNC state.

To further verify these results and our hypothesis we tested fresh stem material for its polysaccharide composition by tissue prints and immunocytochemical studies by using the same antibodies to confirm the dynamic nature of the pectic network which is reflected in the resistance mechanisms.

CHAPTER II

Structural characterization by tissue prints of pectic polysaccharides in xylem vessels of tomato in relation to infection by *Ralstonia solanacearum*

2.1 INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* ranks as one of the most important if not the most important disease of bacterial origin in tropical and subtropical regions of the world (Buddenhagen and Kelman, 1964; Persley, 1986; Kleinhempel, 1989; Hayward, 1991, 2001; Chellemi *et al.,* 1998). Among bacterial diseases, there is no equally harmful organism concerning the actual number of plants killed each year in major crops such as banana, groundnut, tobacco and tomato (Kelman, 1998). An important host of *R. solanacearum* is tomato (*Lycopersicon esculentum* Mill), an important and widely distributed vegetable grown throughout the tropics and subtropics in the open field or in plastic houses. Both production systems are subjected to high losses when infected by *R. solanacearum*, which may constitute a major limitation of production.

Since *R. solanacearum* can survive in soil (Granada and Sequeria, 1983; Hara and Ono, 1985), has a wide host range of over 200 plant species (French, 1986; Hayward, 2000) and easily multiplies in or around host plants (Kelman and Sequeria, 1965), control of this pathogen is difficult. Many authors have stressed the complexity of the epidemiology of bacterial wilt and involvement of many interacting factors (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 1991).

R. solanacearum (formerly *Pseudomonas solanacearum* and, more recently, *Burkholderia solanacearum*) (Yabuuchi *et al*., 1992; Yabuuchi *et al.*, 1995) is classified as a member of the ß-subdivision of the class Proteobacteria. Its genetic information is organized in a 5.8-megabase (Mb) genome that is separated into two replicons: a 3.7-Mb chromosome and a 2.1-Mb megaplasmid (Salanoubat *et al*., 2002). The soilborne

pathogen, a Gram-negative, aerobic, motile rod that naturally infects roots (Hayward, 2001; Genin and Boucher, 2002), causes bacterial wilt.

R. solanacearum is a heterogeneous species, referred to as 'species complex' including strains differing largely in host range, geographical distribution, pathogenicity, epidemiological relationships and physiological properties (Buddenhagen and Kelman, 1964; Palleroni and Doudoroff, 1971; Hayward, 1991; Seal and Elphinstone, 1994). According to French (1986) the insufficient taxonomy of *R. solanacearum* handicaps resistance breeding for crops which cannot be accomplished without an understanding of the pathogen and its diversity. Therefore, it is of paramount importance to generate a stable taxonomy and nomenclature which characterizes subspecific groups of the pathogen corresponding to epidemiology, pathogenicity and host range in order to help plant breeders and plant pathologists, and to enhance the capability to predict the properties of *R. solanacearum* strains (Fegan and Prior, 2004). To describe this intraspecific variability various classification systems are used. Thus, the pathogen is separated into six biovars based on utilization of three disaccharides and three hexose alcohols (Hayward, 1961, 1964; He *et al.,* 1983, 1986; Hayward *et al.*, 1992) and into five races mainly based on host range (Buddenhagen *et al.,* 1962; Quinon *et al.,* 1964; Lum, 1973; He *et al.,* 1983; Buddenhagen, 1986).

The pathogen (Fig. 2.1) causes substantial yield losses worldwide. Losses of about 75% of potato due to bacterial wilt have been described, whereas in tomato, being one of the most susceptible crops, *R. solanacearum* can result in total destruction of the harvest (Persley *et al.,* 1986; Hayward, 2000; Elphinstone, 2004). In Hawaii, up to 50% of the ginger harvest has been destroyed due to *R. solanacearum* in 1998 and 1999 (Yu *et al.,* 2003; Alvarez *et al.,* 2004).

Fig. 2.1 *R. solanacearum* on tetrazolium chloride agar (strain UQRS585).

Losses are rising because agriculture is extending into areas where susceptible crops have not been cultivated before (Persley *et al.*, 1986). In countries, where *R. solanacearum* is considered a quarantine pathogen, significant economic losses can be caused by destruction of entire infected crops, supplementary eradication strategies and restriction of further cultivation on contaminated land (Elphinstone, 2004). In addition to significant yield losses, bacterial wilt is also responsible for indirect damage which is difficult to assess. This includes interference with land usage and disposal of susceptible crops. The presence of *R. solanacearum* in fields discourages the planting of many vegetables on family farms and home gardens which leads to a considerable reduction in food source (Kelman, 1998; Hayward, 2000).

Breeding for resistant cultivars is one of the main approaches to control the disease, but even though this has led to good levels of site-specific resistance, breakdown of resistance has been repeatedly established in tomato cultivars grown in the heat stress of the lowland humid tropics (Hanson *et al.,* 1996; Hayward, 2000). Moreover, bacterial wilt is still a limiting factor in tropical and subtropical agriculture, because new and more virulent strains are continuously reported (Cook *et al.*, 1989). Especially when vegetables

are bred for the international trade, the complete absence of *R. solanacearum* is most important, since dissemination of strains on vegetative propagating material is a serious problem (Hayward, 2000).

The great economic losses can be attributed to the broad geographical distribution and extensive host range of the pathogen. Every year continuous cropping of susceptible cultivars increase the severity of the problem (Persley *et al.*, 1986). Furthermore, due to the lack of reliable means of control, it is almost impossible to restrict the disease, once it appears (Genin and Boucher, 2002).

The disease affects not only solanaceous plants such as tomato, potato, tobacco and eggplant, as suggested by SMITH in 1896, but also monocotyledons, trees and shrubs (Hayward, 1991; Genin and Boucher, 2002). However, most of the susceptible species belong to the Solanaceae, while the Asteraceae (Compositae) and three families of leguminous plants form the second and third largest number of host plants (Hayward, 1994a). Monocotyledons are less frequently attacked by *R. solanacearum*, except for banana (*Musa* spp.), ginger and their relatives, representing important host species in some areas (Hayward, 2000). Numerous economically important crops as well as many weed hosts have been reported to be attacked by bacterial wilt, some of which may be symptomless carriers of the disease (Horita and Tsuchiya, 2001; Daughtrey, 2003). Alternative plants such as weed hosts or presumed non-host plants enable the pathogen to survive without a susceptible crop for prolonged periods, hence complicating the disease control by rotations (Persley, 1986).

On certain hosts, various incongruities concerning the distribution of the disease have been established. In some areas, where bacterial wilt is endemic certain hosts are attacked, but not in other places where the disease also is prevalent. For instance, the disease has been reported to affect cashew (*Anacardium occidentale*) in Indonesia, but not in Brazil though grown in locations where the disease is indigenous on solanaceous hosts and environmental conditions are favourable to the disease (Hayward, 2000). The reasons for these differences have not been investigated yet. However, Hayward (1991,

2000) suggested this might be attributed to distinct environmental aspects, such as temperature, rainfall, or advantageous biotic and abiotic soil factors. Another explanation could be that particular strains pathogenic for certain hosts may have evolved only in some areas and cannot be found anywhere else (Hayward, 1991, 2000).

The difficulty of developing effective control strategies for bacterial wilt is compounded by a lack of basic knowledge about the ecology and evolution of *P. solanacearum.* It is likely that the difficulty in developing stable, improved resistant cultivars is due, in large part, to the apparent variability of the pathogen (Elphinstone, 1996, 2004), and to the continued appearance of new, more virulent strains. Control of bacterial wilt will be more effective if resistant cultivars could be associated with the crop management measures, such as crop rotation, soil amendment and proper irrigation (Lemaga *et al.,* 2002).

Host resistance is defined as the ability of the host to hinder the growth and/or development of the pathogen, whereas tolerance is the ability of the host to endure the presence of the pathogen (Parlevliet, 1979), the latter can be expressed by less severe (reduced) disease symptoms and/or less damage. Accordingly, when *R. solanacearum* infects or colonizes plant genotypes without causing wilt symptoms, these genotypes may be classified as resistant or tolerant to the disease. But when yield losses or other undesirable influence in the symptomless plants are considered, the above definitions for resistance or tolerance must be modified. Resistance to bacterial wilt has been defined in the past as a high percentage of plant survival under certain infection pressure. However, latent colonization of *R. solanacearum* without obvious wilting symptoms has been reported in some resistant cultivars of tomato (Hayward, 1991; Grimault and Prior, 1993, 1994, 1995; Prior *et al.,* 1996). The ability of *R. solanacearum* to infect and colonize tomato and many other crops and weeds without causing symptoms has resulted in its widespread dispersal and subsequent establishment in different environments (Hayward, 1991). This is particularly true in temperate climates where temperatures are usually below optimum for the pathogen multiplication, resulting in latent infection rather than development of the disease. Late season outbreaks may also result in a greater proportion of the infection remaining latent (Ciampi *et al.,* 1980). It can be carried over wide

distances in vegetative propagating material and is distributed in latently infected seedlings, where the organism remains viable and pathogenic and causes disease outbreaks under favourable conditions (Hayward, 1964). Resistant plants are often partially colonized by the pathogen and show reduced damage by the disease (Prior *et al*., 1996).

Two types of plant resistance responses can be distinguished, non-host and host or race/cultivar specific resistance (Vanderplank, 1968 and 1982; Prell, 1996). In both cases, the biochemical processes involved in pathogen resistance are similar. Thus, after the penetration of the pathogen or pest, structural mediations and biochemical responses begin. Preformed resistance factors usually include preformed structural, morphological and chemical factors such as leaf position or colour, trichomes, dynamic of stomatal thickness and opening, quality of cuticle (thickness, quantity and quality of waxes), and cell wall characteristics (thickness and composition) acting as barriers and providing resistance against potential invaders, while chemical compounds such as phytoanticipins may be directly toxic or indirectly after transformation (Schlösser, 1997; Knogge, 1997; Baker *et al*., 1997; Keen, 1999). These include phenols, phenolic glycosides, saponins (steroids and triterpenoides), glycosinolates and cyanogenic glycosides present in high concentration in particular tissues. On the other hand, after the penetration of the invader, induced processes take place. A complex signaling network involving cytosolic Ca^{2+} and H^{+} ions, reactive oxygen intermediates (oxidative burst), jasmonate, salicylic acid and ethylene triggers the induction of defence mechanisms (Odjakova and Hadjiivanova, 2001). In tomato, several QTL controlling resistance have been found, but in different studies, markers spanning a large region of chromosome 6 showed strong association with the resistance (Mangin *et al.,* 1999; Lindhout, *et al.,* 2003). Phenotypic evaluation of wilt resistance in cultivar H7996 has been previously conducted by inoculating *R. solanacearum* onto wounded roots and monitoring wilt symptom development and colonization (Grimault and Prior, 1994; Grimault *et al.,* 1994; Grimault and Prior, 1993). Current quarantine tests include visual inspection of the incidence of disease symptoms in the field, greenhouse or storage samples. However, symptomless genotypes and vegetative propagation materials may carry and protect virulent pathogen populations.

Reactions involved in resistance of tomato to *R. solanacearum* were observed, such as tyloses, gums, cell wall breakdown, and modifications to the primary cell wall. Plant cells are encapsulated within a complex, fibrous wall with properties crucial to the form and function of the plants. The cell wall acts as an exoskeleton to give the plant cell its shape and to allow its high turgor pressures. As the skin of the plant cell, the wall participates in adhesion, cell-cell signaling, defense, and numerous growth and differentiation processes. The wall of enlarging plant cells is composed of approximately 30% cellulose, 30% hemicellulose, and 35% pectin, with perhaps 1-5% structural protein on a dry weight basis. Substantial deviations from these values maybe found, notably in the grasses, where, for example, walls of growing maize coleoptiles consist of 55% hemicellulose, 25% cellulose, and only 10% pectin (Crosgrove, 1997). Pectin forms a gel phase in which the cellulose-hemicellulose network is embedded.

Pectins are the most soluble of the wall polysaccharides. Like the hemicelluloses, pectins also constitute a heterogeneous group of polysaccharides, characteristically containing acidic sugars such as galacturonic acid. Some pectins have a relatively simple primary structure such as homogalacturonan (HGA), a linear polymer of (1→4) α galacturonic acid. Rhamnogalactouronan I (RG I) has repeating subunits of (1 →2) α-L-rhamnosyl-(1→ 2)-α-D-galacturonyl disaccharides, with the long side chains of arabinan and arabinogalactan. The size of RG I is reported to range from 500-2000 KDa (Talbott and Ray, 1992a). Many of the acidic residues in pectins are esterified with methyl, acetyl, and other unidentified groups (Kim and Carpita, 1992; McCann *et al,* 1994). Recently borate di-esters of rhamnogalacturonan II were identified (Kobayashi *et al*, 1996; O' Neill *et al,* 1996; Ishii and Matsunaga, 1997) indicating that such borate esters likely affect cell wall mechanics (Findeklee and Goldbach, 1996).

In addition to the major polysaccharides, growing plant cell wall also contain structural proteins (Showalter, 1993). Wounding, pathogen attack, and treatment with elicitors increase expression of many of these proteins. Numerous enzymes may be found associated with the cell walls (Fry, 1995). Some can modify the major polysaccharides of the plant wall, e.g. pectinases, pectin methyl esterases, endoglucanases etc.

Arabinogalactan proteins (AGPs) form a very large and diverse group of macromolecules in plants and can be subdivided most readily into extracellular proteoglycans composed of a hydroxyproline polypeptide backbone to which branched 1,3:1,6 galactan chains are attached by O-glycosidic bonds. The galactan is substituted by arabinose residues and minor amounts of glucose, uronic acids, xylose and rhamnose and membrane-associated glycoproteins (Fincher *et al.,* 1983; Bacic *et al.,* 1986; Pennell *et al.,* 1989). Water-soluble AGPs are antigenic and capable of generating monoclonal antibodies with reactivities inhabitable by L-arabinose, D-galactose and associated dissacharides (Anderson *et al.,* 1984).

Antibodies to defined pectic antigens and epitopes are important probes for the study of function and organization of plant cell walls (Freshour *et al.,* 1996; Knox, 1999, 2002, 2003; Mc Cabe *et al*., 1997; Willats *et al.,* 1999, 2001, 2003). HGA derived oligogalacturonides generated by pectinolytic cleavage are involved in signaling processes during development and in defense responses to plant pathogens (Huxham *et al.,* 1999; Knox *et al.,* 1999; Samaj *et al.,* 1999; Brent *et al.,* 2001; Moerschbacher, 2003). Tomato pectins are revealed to possess long branches, the existence of which was not known previously (Round *et al.,* 1997).

Pectins are subject to a number of modifications that alter their conformation and linkage in the wall and this could explain the correlation of pectins with onset of resistance mechanisms (McMillan *et al.,* 1993; Venkatesh, 2002). Electronmicroscopic studies on *R. solanacearum* development in stems showed that the limitation of bacterial spread associated with the resistance of tomato to bacterial wilt was mainly attributed to an induced, non-specific, physical barrier. These studies indicated that the resistance does not arise from an inability of the bacteria to invade the roots, but rather from a limitation of their spread from the collar to the mid-stem. Electron-dense materials accumulated in or around pit cavities in parenchyma cells next to vessels with bacteria, and in vessels with bacteria (Nakaho *et al.,* 2000). Plugging the vessels by bacterial mass (Vasse *et al.,* 1995) and complete occlusion of vessels by bacteria, gum and tyloses (Wallis and Truter, 1978) have been considered to be the cause of wilting. The bacteria enter the roots at sites

of secondary root emergence (Kelman and Sequeria, 1965; Schmit, 1978) or at root tips (Vasse *et al.,* 1995) and progress to the xylem, then spreading systemically in the plant. Large numbers of bacteria secreting EPS I and proteins involved in the pathogenesis causing impaired water transport and lead to disease symptoms in the host, finally ending in wilting and death (Buddenhagen and Kelman, 1964). Certain cell wall degrading enzymes secreted by *R. solanacearum* could also be playing an important role in wilting mechanisms (Mc Garvey *et al.,* 1999). Hrp genes, encoding the type III secretion machinery, have been shown to be key determinants for pathogenicity in the vascular bacterium *R. solanacearum* (Vasse *et al.,* 2000 a&b). Plant resistance to pathogens in various interactions is sometimes associated with a hypersensitive response (HR) (Carney and Denny, 1990; Arlat *et al.,* 1994; Genin and Boucher, 2002), a phenomenon often controlled by single dominant loci, and some of the genes controlling this type of response have been cloned and characterized (Baker *et al.,* 1997; Gebhardt, 1997; Hammond-Kosack and Jones, 1997).

With the antibodies against epitopes present in pectin, we studied the modification of these wall components during infection using tissue printing. The imprint is formed by soluble molecules released at the surface that bind irreversibly to the membrane. The print, which is remarkably faithful to the original anatomy, can then be probed with a variety of localizing reagents to probes. This study compares infection of resistant and susceptible cultivars in relation to modification in pectic epitopes of tomto stem cell walls which could be involved in resistance mechanisms.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Seven tomato genotypes with high resistance level to bacterial wilt and two susceptible genotypes were received from the Asian Vegetable Research and Development Centre (AVRDC), Taiwan: Hawaii 7996, CLN2123C, CLN1-3-13, CLN4-22-4, CLN1-1-12, CLN1-5-12, BL333, and Wva 700 and L390 respectively. Genotype King Kong-2 [moderately resistant (Leykun, 2003; Dannon, 2003, Dannon and Wydra 2004)] was obtained from Taiwan. Additionally six near isogenic lines deriving from Wva 700 and H7996 were used: NHG 3 and NHG 167 (susceptible), NHG 13, NHG 60, NHG 162, and NHG 140 (resistant). Seeds were sown in a greenhouse (20°C day/night temperature, 14h of light per day / 30K lux, and 70% relative humidity) and transplanted after four weeks to individual pots with 330g of soil (Fruhstorfer Erde, Type P, with 150mg/l N, 150mg/l P_2O_5, and 250mg/l K_2O).

2.2.2 Reaction of tomato genotypes to bacterial wilt

The reaction of the above mentioned tomato genotypes and near isogenic lines with the fluidal and highly virulent *R. solanacearum* isolate To-Udk2 obtained from Thailand was determined as described by Winstead and Kelman (1952) by inoculating the plants: Hawaii 7996, CLN2123C, CLN1-3-13, CLN4-22-4, CLN1-1-12, CLN1-5-12, BL333, and Wva 700 and L390 and the near isogenic lines of tomato: NHG 3 and NHG 167 (susceptible), NHG 13, NHG 60, NHG 162, and NHG 140 (resistant) (AVRDC, 2001) differing in their susceptibility to *R. solanacearum*. Bacterial inoculum was produced by streaking a single colony on NGA agar medium (0.3 % beef extract, 0.5 % Bacto peptone, 0.25 % D-glucose, 1.5 % agar) and incubating at 30 °C for 48 h. Cells were harvested from agar plates by flooding with sterile, distilled water and an optical density of 0.06 at 600 nm wavelength (Spectrotonic 20 Bausch and Lomb), corresponding to about 7.8×10^7 colony-forming units per milliliter (CFU/ml) was adjusted.

Ten four-week old plants per genotype were inoculated by soil drenching with 33 ml of bacterial suspension per pot, corresponding to about 10^7 CFU/g of soil, around the base of the plants directly after transplanting. After inoculation plants were kept in a climate chamber with 30/27 °C day/night temperature, 85% relative humidity, 14 hours light, and 30K Lux. Pots were watered after inoculation up to the soil field capacity without producing a surplus.

Symptom development was evaluated daily in 5 severity classes (Fig. 2.2.2) with class 0 = no wilt symptom, class 1 = one leaf wilted, class 2 = two or more leaves wilted, class 3 = all leaves except the tip wilted, class 4 = whole plant wilted, and class 5 = death (collapse) of the plant (Winstead and Kelman, 1952).

The mean wilt disease severity at the evaluation dates of 5 to 40 days after inoculation of each genotype was calculated and used to determine the area under disease progress curve (AUDPC) of genotypes using evaluation dates according to the following formula. The data on incidence was used only for results. Wilt incidence is the proportion of dead plants at the evaluation date out of the total number of plants in the treatment. The area under disease progress curve (AUDPC) was calculated on the basis of disease severity and of wilt incidence using the following equation (Shaner and Finney, 1977; Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum [(x_i + x_{i-1})/2](t_i - t_{i-1})$$

x_i and x_{i-1} is wilt incidence at time t_i and t_{i-1}, respectively,
t_i and t_{i-1} are consecutive evaluation dates.
and $t_i - t_{i-1}$ was equal to 1.

Fig.2.2.2 Illustrated key for symptom assessment of bacterial wilt, where 0 = no wilt, 1 = single leaf wilted, 2 = two or more leaves wilted, 3 = all leaves except the tip wilted, 4 = all leaves wilted, and 5 = collapse (death) of the plant.

2.2.3 Quantification of latent infections in stems

Bacterial numbers were quantified in stems of all the genotypes and near isogenic lines, which had been evaluated for symptom development as described by Li and Jan (1984). The two susceptible reference genotypes L390 and Wva 700 showed first symptoms 5 days after inoculation and were severely attacked 10 days after inoculation with no survival of the plants. Three symptomless plants per resistant genotype were randomly harvested four weeks after inoculation. The mid-stem pieces (5-10 g) were surface-sterilized by submerging the sample in 70% alcohol for less than one minute, rinsed in sterile water, and macerated by adding about 20 ml sterile, distilled water. The macerate was filtered through cheesecloth and centrifuged for 10 minutes (7000 x g) (Sorvall RC-5B refrigerated centrifuge). The pellet was re-suspended in 1 ml sterile, distilled water. All suspensions were serially, tenfold diluted and 100 µl from at least four dilution levels were plated in duplicates on Triphenyl Tetrazolium Chloride medium (TTC): 20g Bacto peptone, 5g Glucose, 1g Casamino acids, 15g Bacto agar and 1000 ml H_2O (Kelman, 1954). After autoclaving, 10 ml of filter-sterilized solution of 0.5% (w/v) of 2, 3, 5-

Triphenyl Tetrazolium Chloride (SERVA, Germany) were added to the medium before pouring into Petri dishes. Typical bacterial colonies were counted after 48 hours of incubation at 30°C and calculated as colony forming units per gram of fresh matter (CFU/g). Identity of colonies was identified by NCM-ELISA and PCR (Leykun 2003).

2.2.4 Tissue printing

Tissue printing was carried out by pressing the cut surface of mid-stems from both healthy and inoculated genotypes as well as from near isogenic lines of tomato 5 days after inoculation firmly and evenly onto a nitrocellulose membrane (ELISA Kit, Biorad, Germany) for approximately 15-20 sec as described by Jones *et al.* (1997) and McCartney *et al.* (2000). The tissue printing of all genotypes and near isogenic lines was repeated three times with newly grown plants for each trial and in each trial prints were made in duplicate for each antibody to test the repeatability of the method, with 2 healthy and 2 inoculated plants per antibody per print which were picked randomly from each healthy and inoculated genotypes and isogenic lines. Each stem material was then tested for all the six antibodies JIM5, JIM7, LM2, LM5, LM6, and LM7.

After the prints had dried, the nitrocellulose membrane was blocked by incubation with phosphate buffered saline (PBS) containing 5% milk powder (MPBS, pH 7.2) for 1 h prior to incubation in primary antibodies (hybridoma supernatants of JIM5, JIM7, LM2, LM5, LM6, LM7, received from P. Knox, University of Leeds, UK) diluted 1/10 in MPBS for 1.5 h. After washing extensively under running tap water and for 10 min in PBS containing 0.1% (v/v) Tween 20 (PBST), membranes were incubated in the secondary antibody (anti-rat horseradish peroxidase conjugate, Sigma) diluted 1/1000 in MPBS for 1.5 h. Membranes were again washed as described above and developed in substrate solution [25 ml deionized water, 5 ml methanol containing 10 mg/ml 4-chloro-1-naphthol, 30 µl 6% (v/v) H_2O_2] until a clear colour reaction developed. All steps were performed at room temperature. The trial was repeated three times as said above and results were obtained based on visual evaluation of the color intensity in increasing catergories from -, (+), +, (++), ++, (+++), +++ of the membranes on an illuminating

table. Data of the repetitions were compared and their repeatability confirmed, and representative results are given.

2.2.5 Statistical Methods

Data were processed using analysis of variance in SAS (the SAS System for Windows V8, Release 8.02 TS Level 02M0; 1999-2001. Institute INC., Cary, USA). For all analyses a significance level of P = 0.05 or lower was used. The bacterial counts on media, expressed as colony forming units per gram of fresh matter or per milliliter of bacterial suspensions and/or stem macerates, were log transformed and analyzed using parametric analysis procedures in SAS. A lack of growth on plates of all replications is plotted on the log scale as one, which gives zero CFU g^{-1}.

Tukey's Studentized Range (TSR) test (P = 0.05) was used within parametric analysis of variance (ANOVA) as incorporated in SAS version 8.02 (SAS Inc., Cary, USA) to compare AUDPC data of respective resistant genotypes based on bacterial numbers in stems and the area under wilt disease progress curve.

2.3 RESULTS

2.3.1 Symptom development in tomato genotypes

Wilt incidence was recorded up to 40 days after inoculation of tomato genotypes inoculated with *R. solanacearum.* On the basis of the area under wilt incidence progress curve (AUDPC), the genotypes were classified into two significantly different groups, i.e. resistant and susceptible genotypes.

Two near isogenic lines NHG 3 and NHG 167 were classified as susceptible, with similar AUDPC as the susceptible standard genotypes L390 and Wva 700. First symptoms appeared 5 days after inoculation, and plants were severely attacked 10 days after inoculation, resulting in plant death.

Table 2.3.1 Symptom development expressed as area under wilt incidence progress curve of the near isogenic tomato lines NHG3 and NHG167 and the susceptible standard genotypes Wva700 and L390 after inoculation with *R. solanacearum* strain ToUdk2 (10^7 CFU/g of soil).

Genotype	AUDPC of wilt incidence
L390	98.1 ± 4.6 a
Wva	99.5 ± 3.3 a
NHG 3	102.2 ± 3.7 a
NHG 167	100.7 ± 3.5 a

Genotypes Hawaii 7996, CLN 2123C, CLN 1-3-13, CLN 4-22-4, CLN 1-1-12, CLN 1-5-12, BL333 and the lines NHG 60, NHG 140, NHG 13 and NHG 162 did not show any wilt symptoms and were grouped as resistant. King Kong 2 was identified as moderately resistant in former trials. (see section 2.2.1)

2.3.2 Latent bacterial multiplication

R. solanacearum was detected in the mid-stem region of symptomless plants of the resistant genotypes Hawaii 7996, CLN 2123C, CLN1-3-13, CLN4-22-4, CLN1-1-12, CLN1-5-12, BL333, NHG 60, NHG 140, NHG 13 and NHG 162 and of the moderately resistant King Kong 2 with bacterial numbers of 885 to 2.9 x10^7 CFU/g stem. Significant differences in bacterial density between genotypes occurred with significantly higher bacterial numbers in genotypes NHG 60, NHG 140, and King Kong 2 than in CLN 1-3-13 and CLN 2123C, and in CLN 4-22-4 than in CLN 2123C ($P = 0.0001$) (Fig. 2.3.2).

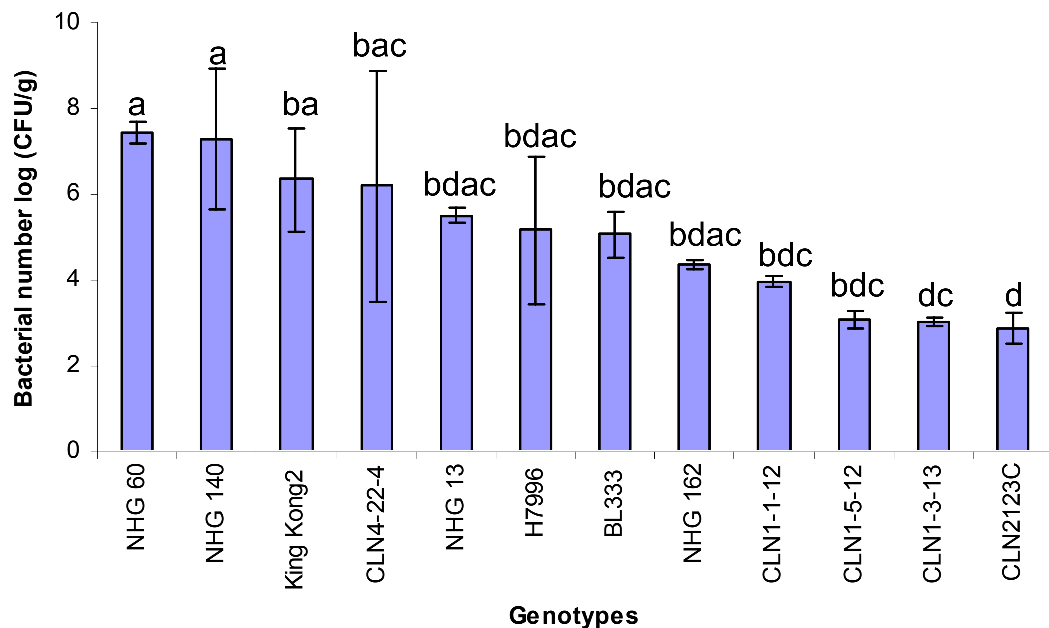


Fig 2.3.2 Bacterial numbers in the mid-stems of asymptomatic plants of 12 genotypes of tomato at 4 weeks after inoculation with *R. solanacearum* To-Udk2. Data are means of 4 plants ± SE. Letters indicate significant differences among genotypes.

Among the resistant genotypes, CLN 2123C showed the lowest bacterial density in the stem with about 3 CFU/g stem. No significant differences were observed among the resistant isogenic lines where the bacterial numbers in these isogenic lines ranged from 2.3 x 10^4 to 2.9 x 10^7 CFU/g stem with the lowest in NHG 162 being 2.3 x 10^4 CFU/g stem.

2.3.3 Characterization of pectic polysaccharides by immunochemical stem tissue printing

The resistant tomato genotypes CLN 2123C, CLN 1-3-13, H7996, CLN 4-22-4, CLN 1-1-12, CLN 1-5-12, BL333, NHG 60, NHG 13, NHG 140, NHG 162 and moderately resistant King Kong 2 and the susceptible genotypes L390, Wva 700, NHG 3 and NHG 167 were characterized for the composition of the pectic polysaccharides of stem sections by immunological staining with the monoclonal anti-HG-antibodies JIM5, JIM7 and LM7, and antibodies LM5 to (1→4)-ß-galactan and LM6 to (1→5)-α-arabinan epitopes, occurring in side chains of RG I, and LM2 specific for arabinogalactan protein with and without inoculation with *R. solanacearum* strain ToUdk2.

Table 2.3.3.1 Characterization of pectic polysaccharides in xylem vessels of resistant and susceptible healthy and inoculated tomato genotypes by reaction with antibodies specific to low ester (JIM5), high ester (JIM7) or non-blockwise de-esterification patterns of HG (LM7), arabinan (LM6), galactan (LM5) and arabinogalactan protein (LM2) on nitrocellulose membrane after stem tissue print[1]. Resistance reaction to *Rs* from symptom observations.

Genotypes	Reaction to *Rs*[2]	JIM5- low ester		JIM7- high ester		LM7- n-block		LM6-arabinan		LM5-galactan		LM2-AGP	
		H	I	H	I	H	I	H	I	H	I	H	I
CLN 2123C	R	+[3]	++	+++	+++	-[4]	-	+	+	-	-	+	(++)
CLN 1-3-13	R	+	++	+++	+++	-	-	+	+	-	++	+	++
H7996	R	+	++	+++	+++	-	-	+	+	+	+	+	+
CLN 4-22-4	R	+	+	+++	+++	-	-	+	+	+	+	+	+
CLN 1-1-12	R	+	++	+++	+++	-	(+)	+	+	+	+	+	+
CLN 1-5-12	R	+	(++)	+++	+++	-	(+)	+	+	+	+	+	+
King Kong 2	MR	+	+	+++	+++	-	-	+	(++)	-	+	++	++
BL333	R	+	+	++	++	-	-	+	+	+	+	+	+
L-390	S	+	++	+++	+++	-	++	+	++	+	++	+	++
Wva	S	+	++	+++	+++	-	(+)	+	+	+	+	+	+

[1] Trial repeated three times, table shows representative results
[2] Reaction to *Ralstonia solanacearum*: R= resistant, MR= moderately resistant, S=susceptible
[3] Color intensity was measured from -, (+), +, (++), ++, (+++), +++ and H = healthy genotype and I = inoculated genotype, 5dpi
[4] (-) no staining was found

Among healthy resistant and susceptible genotypes no clear differences in cell wall composition were observed. After inoculation all genotypes except King Kong 2, BL333 and CLN 4-22-4 showed stronger labeling with JIM5 indicating an increase in low-esterified pectin epitopes. No difference in the binding specificity was observed between inoculated and non-inoculated plants after labeling with JIM7. Increased staining after inoculation occurred with all antibodies except JIM7 in genotype L390 indicating an increase in low esterification and non-blockwise de-esterification pattern of HG (Fig. 2.3.3.1.) and in arabinan (LM6) (Fig. 2.3.3.2 a) and galactan (LM5) (Fig. not shown) epitopes of RG I and arabinogalactan proteins (LM2) (Fig. 2.3.3.2 b). In genotype CLN1-3-13 galactan and AGP, and in genotype KK2 galactan labeling increased after inoculation.

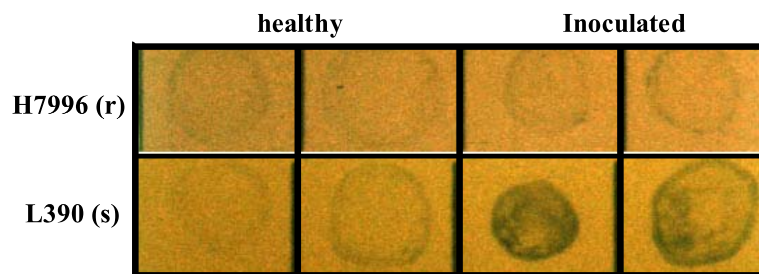


Fig. 2.3.3.1 Stem tissue prints of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, in healthy and inoculated treatments after staining with LM7 specific for non-blockwise de-esterification of homogalacturonan.
The susceptible genotype showed intense labeling with LM7 after inoculation indicating the increase in non-blockwise de-esterification of homogalacturonan, whereas the resistant genotypes showed less or no labeling before and after inoculation.

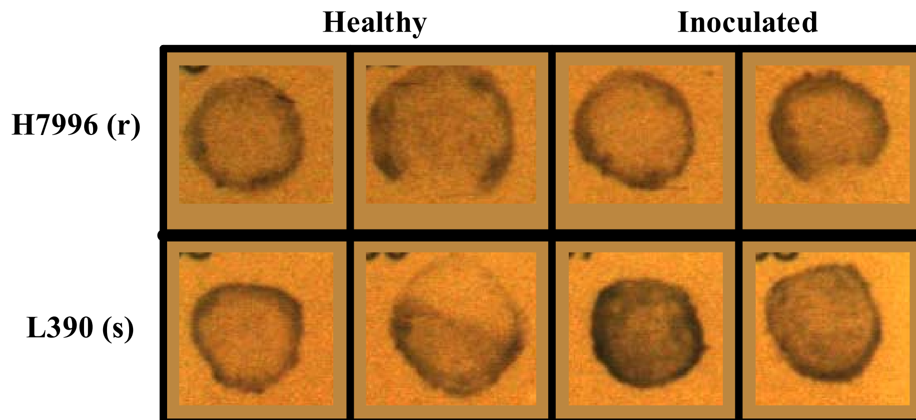


Fig. 2.3.3.2a Stem tissue prints of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, in healthy and inoculated treatments after staining with LM6 specific for arabinan side chain of RG I.
The susceptible genotype showed intense labeling with LM6 after inoculation indicating the increase in arabinan side chain of homogalacturonan, whereas the resistant genotypes showed less labeling before and after inoculation.

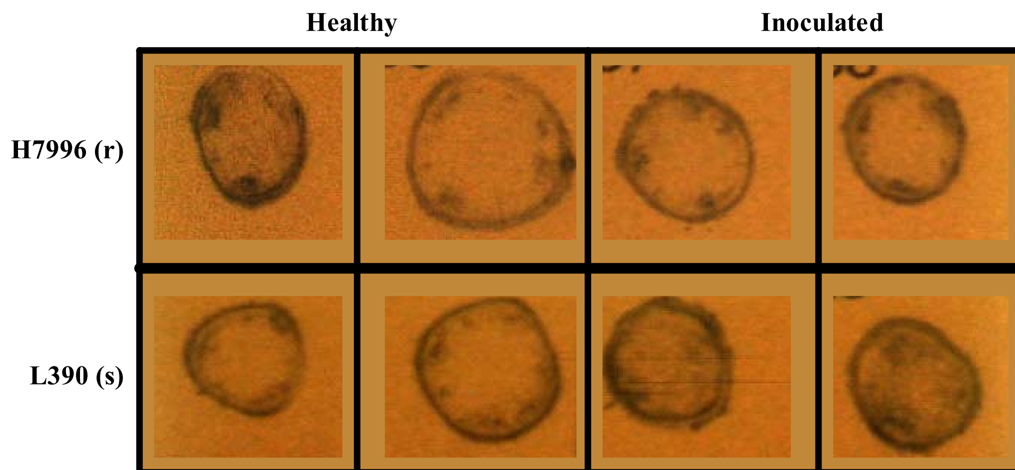


Fig. 2.3.3.2b Stem tissue prints of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, in healthy and inoculated treatments after staining with LM2 specific for arabinogalactan protein.
The susceptible genotype showed intense labeling with LM2 after inoculation indicating the increase in arabinogalactan proteins, whereas the resistant genotypes showed less labeling before and after inoculation.

Table 2.3.3.2 Characterization of pectic polysaccharides in xylem vessels by tissue prints[1] in the healthy and inoculated near isogenic lines of tomato: NHG 60, NHG 13, NHG 140, NHG 162, NHG 3, and NHG 167 differing in susceptibility to *R. solanacearum*. The susceptible isogenic lines NHG 3 and NHG 167 showed intense labeling after inoculation. Resistance reaction to *Rs* from symptom observations.

Isogenic Lines	Reaction to *Rs*[2]	JIM5- low ester		JIM7- high ester		LM7- n-block		LM6- arabinan		LM5- galactan		LM2- AGP	
		H	I	H	I	H	I	H	I	H	I	H	I
NHG 60	R	++	++[3]	+++	(+++)	-[4]	-	+	++	+	(++)	++	++
NHG 13	R	(++)	++	(+++)	+++	-	(+)	(+)	++	+	(+++)	+	++
NHG 140	R	++	++	(+++)	+++	-	(+)	+	+	+	+	+	+
NHG 162	R	+	+	+++	+++	-	-	+	++	(+)	(+)	+	+
NHG 3	S	+	+++	++	++	-	+	+	+	+	++	+	++
NHG 167	S	(++)	+++	++	++	(+)	++	+	(++)	+	(++)	+	(+++)
L390	S	+	++	+++	(+++)	-	++	+	++	+	++	+	++

[1] Trial repeated three times, table show representative, repeatable results
[2] Reaction to *Ralstonia solanacearum*: R= resistant, S= susceptible
[3] Color intensity was measured from -, (+), +, (++), ++, (+++), +++ and H = healthy genotype and I = inoculated genotype, 5dpi
[4] (-) no staining was found

Comparing healthy plants of resistant and susceptible isogenic lines, tissue prints of the susceptible lines: NHG 3 and NHG 167 showed less intense labeling for HG with high degree of esterification (JIM7) than the resistant lines: NHG 60, NHG 140, NHG 13 and NHG 162.

NHG 60 and NHG 13 (resistant) as well as NHG 3 and NHG 167 (susceptible) isogenic lines showed increases in arabinan (LM6), galactan (LM5), and arabinogalactan protein epitopes in the side chains of RG I after inoculation. Susceptible lines NHG 3 and NHG167 showed intense labeling with JIM5 and LM7 after inoculation, indicating an increase in low esterification patterns and in the non- blockwise de-esterification pattern of HG, respectively (Fig 2.3.3.2 c), while no differences were observed between treatments for the prints of the other genotypes stained with JIM5 and with any genotype stained with JIM7. The tissue prints of the susceptible isogenic lines showed a clear difference from the resistant lines after inoculation in binding LM7.

NHG 60 showed the highest number of bacteria in latently infected plants and increased labeling for galactan and arabinan epitopes (Table 2.3.3.2), whereas NHG 140 also showed high bacterial populations but no change in labeling with LM5 and LM6 after inoculation, indicating no relation between bacterial numbers and cell wall epitopes.

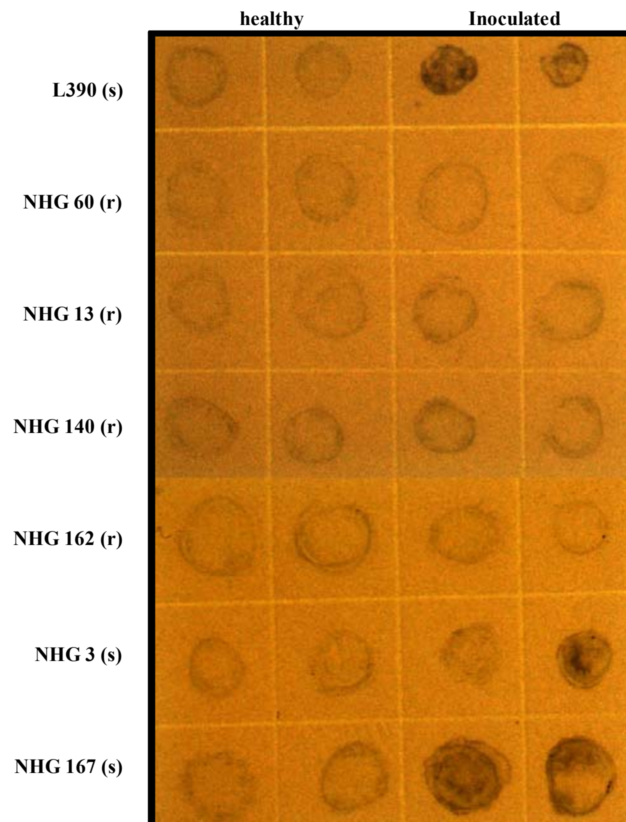


Fig 2.3.3.2c Characterization of pectic polysaccharides in xylem vessels in tomato isogenic lines by tissue printing before and after inoculation with *R. solanacearum*. Susceptible genotypes: NHG167, NHG3 and L390 showed intense labeling with LM7 after treatment indicating an increase in the epitope of non-blockwise de-esterification pattern of homogalacturonan whereas no change was observed in the resistant lines.

2.4 DISCUSSION

Genotypes were grouped in resistant and susceptible after inoculation with *R. solanacearum* strain To-udk2. The resistant, completely asymptomatic genotypes were used for the subsequent study on latent infection. Susceptible genotypes severely wilted and 'resistant' genotypes showed variation in bacterial density in stems. Several solanaceous species, such as tobacco (*Nicotiana tabacum* L.), potato (*Solanum tuberosum* L.) and also tomato, are known to be symptomless carriers of the pathogen and latent infection appears to be a common trait in bacterial wilt pathogenesis (Prior *et al.,* 1994).

Li and Jan, 1984; Liao *et al.,* (1998) found that latent infection in some resistant groundnut cultivars affected root proliferation and tolerance to drought, which may explain the low productivity of the resistant cultivars in infested areas. Different reaction types of plants to latent infections were related to resistance levels in some genotypes, and, thus, latent infection studies may provide information to improve breeding efforts.

All resistant genotypes exhibited high populations of bacteria in their stems, regardless of their resistance level. Effective resistance mechanisms in the stem could be the reason for the low bacterial concentration in the stem of genotype CLN2123C, different to other resistant genotypes used in our study. On the other hand, an additional restriction of bacterial invasion and /or multiplication, at the root level might play a role as resistance mechanism (Dannon and Wydra, 2004). Grimault *et al.* (1995) and Vasse *et al.* (2002) reported a decrease of bacterial density in mid-stems compared to the collar. Grimault *et al.* (1994) found a significant correlation between the bacterial population at mid-stems level and the degree of resistance. Investigating the resistance mechanisms of tomato to *R. solanacearum*, Chellemi *et al.* (1998) found that the amino acids and organic acids present in the xylem fluid of tomato plants may be determinants for resistance against bacterial wilt disease. Phenols and ascorbic acid present in high amount in roots and stems may also play a role in resistance of tomato to *R. solanacearum* (Kumar *et al.*, 2002). Interestingly, Rajan and Bose (2002) reported the presence of proteins bands PPO1 (polyphenol Oxidase), PPO 12 and PPO9 in roots of resistant cultivars, while

bands PPO2, PPO4, PPO5 and PPO7 were found in the moderately resistant ones. However, these bands were not reported to relate to resistance at roots level.

Genotypes CLN 2123C and CLN 1-3-13 with high resistance to bacterial wilt, combined with low pathogen colonization were identified. Thus, it is suggested to include the quantification of the colonization of the mid part of stems of asymptomatic plants as a complementary criterion for evaluating tomato germplasm for resistance to wilt, in addition to wilt severity.

Tissue prints of susceptible lines after inoculation, showed an increased labelling with LM 5, LM 6 and LM 2 indicating an increase in various pectic polysaccharide epitopes: galactan and arabinan and arabinogalactan protein, except no increased labeling for arabinan epitope in NHG3, and also no change in Wva700. Comparing the resistant genotypes galactan and AGP increased in some resistant genotypes after inoculation with *Ralstonia solanacearum*, and arabinan increased in three of ten resistant genotypes, whereas no change was observed in H7996 in labeling with LM6, LM5 and LM2 after inoculation. The increased de-esterification after inoculation may be due to the activity of pectin methylesterase of *Rs*, an enzyme which was shown to be involved in the pathogenesis of bacterial wilt (Allen *et al.,* 1998).

The difference between resistant and susceptible genotypes in non-blockwise de-esterification pattern of HG could be due to the action of the cell wall degrading enzyme PME of the pathogen, thus increasing the capacity to form gels and these gels which act as reservoir of water and nutrients for the bacteria can promote pathogen growth in susceptible genotype whereas in the resistant genotype the bacterial movement is restricted due to the formation of the cell wall thickening which is a common feature of resistance mechanism and at the same time there is deposition of new wall material with different composition which can then strengthen the wall and hence can provide resistance to pectic enzymes. JIM5 labeling was seen to increase for all genotypes except the resistant isogenic lines and King Kong 2, BL333 and CLN4-22-4, after inoculation indicating increased low esterification (low DE) pattern of HG which could result from

degradation of pectic polysaccharides in the cell walls in the susceptible genotype *R. solanacearum* and could be the onset of the resistance mechanisms in the resistant genotypes, and in many host pathogen systems studied, pectic fragments produced during host cell wall degradation can act as endogenous suppressors of the hypersensitive response in the susceptible plants (Moerschbacher *et al.,* 2003) and act as elicitors for the HR in resistant plants (Ridley *et al.,* 2001), whereas no change was observed in labeling with JIM7 before or after inoculation. All the resistant genotypes were observed to be latently infected showing presence of bacteria after quantification indicating the bacteria present in mid stems has a role in degrading the pectic polysaccharides. Thus, the degradation of plant wall material and, at the same time, deposition of new wall material could indicate a resistance mechanism in the resistant genotype (Rahman *et al.,* 1999).

In the resistant genotypes as well as in the resistant isogenic lines, there was no relation observed between bacterial numbers and cell wall epitopes. Two of the resistant genotypes CLN 2123C and CLN 1-3-13 with lower number of bacteria in the mid-stems showed increased labeling for AGP after inoculation and increased binding for galactan epitope with the latter genotype. More intense binding of LM5 and LM6 may be because of epitope accessibility at lower DE is increased by conformational changes in pectin structure (Willats *et al*., 2000). The high labeling of the pectic galactan and arabinan epitopes may also be related to the presence of the low methyl-esterified HG in the cell walls of the susceptible genotype after inoculation. LM5 and LM6 epitopes are present at cell corners and these are the first defined epitopes to be attacked by the pathogen. The de-esterification by pathogen PME makes the galactan and arabinan side chains more accessible to the LM5 and LM6 antibodies and thus, indicating the degradation of pectic polymers as the presence of pathogen causes damage in those tissues, hence the pathogen degenerates the pit membrane and can move from vessel to vessel thus causing wilting of the whole plant could be speculated as in susceptible genotypes (Nakaho *et al.,* 2000). The increased labeling of arabinan and galactan epitopes on the other hand in resistant isogenic lines (NHG 60 and NHG 13) after inoculation was due to increased branching of RG I making the epiotpes less degradable by pathogen PME.

The increased labeling with LM7 which is specific for non-blockwise distribution of methyl ester groups, indicates a change in physico-chemical properties of the cell wall, since these HG can form calcium-mediated gels with distinct properties in terms of porosity and elasticity (Willats *et al*., 2001). This observation also indicates the possible action of pathogen PME, resulting in non-blockwise de-esterification, and, thereby, making the pectin more suitable for gel formation.

Thus, resistance of wilt in tomato was related to the capacity of the plant to restrict *R. solanacearum* multiplication in the stem, similar to other vascular bacterial diseases (Cho *et al.,* 1973), inhibiting bacterial growth, or limiting the effects of bacterial virulence factors. The changes on the cell wall level that were observed after infection in the susceptible genotypes were generally not observed in the same intensity in the resistant genotypes. Therefore it is suggested, that pectic polysaccharides are involved in the host pathogen interactions and might play a role as resistance mechanisms. Further investigations were conducted to identify and confirm the role of these cell wall components in host pathogen interactions. However, these results contribute to the understanding of resistance mechanisms of tomato to bacterial wilt caused by *R. solanacearum* at molecular level.

2.5 SUMMARY

On the basis of the area under disease progress curve (AUDPC), the genotypes were classified into two significantly different groups, i.e. resistant and susceptible genotypes. The two near isogenic lines NHG 3 and NHG 167 were classified as susceptible, with similar AUDPC as the susceptible standard genotypes L390 and Wva 700. The near isogenic lines NHG 13, NHG 60, NHG 162 and NHG 140 were grouped as resistant genotypes which did not show any wilt symptoms. Significant differences in bacterial density between genotypes occurred in the mid-stem region of symptomless plants of the resistant genotypes, with genotype **CLN 2123C** showing the lowest bacterial density with 2.9 CFU/g. No significant differences were observed among the resistant isogenic lines.

All genotypes and isogenic lines were characterized for the composition of the pectic polysaccharides of stem sections by immunological staining of tissue prints with monoclonal antibodies. Among healthy resistant and susceptible genotypes no clear differences were observed. After inoculation increased staining occurred in genotype L390 with all antibodies except Jim 7 indicating an increase in low esterification and non-blockwise distribution of esterification of HG and an increase in arabinan and galactan epitopes of RG I and arabinogalactan proteins. All genotypes except KK2 and BL333 showed stronger labeling with JIM5. No difference in the binding specificity was observed between inoculated and non-inoculated plants after labeling with JIM7.

Some resistant as well as susceptible isogenic lines showed increases in arabinan (LM6), galactan (LM5), and arabinogalactan protein epitopes in the side chains of RG I after inoculation. Susceptible lines NHG 3 and NHG 167 showed intense labeling with JIM5 and LM7 after inoculation indicating the low esterification pattern and non blockwise de-esterification of HG in these susceptible lines. The tissue prints of the susceptible isogenic lines showed a clear difference from the resistant lines after inoculation in binding LM7.

CHAPTER III

Immunocytochemical differences in methyl-ester distribution and side chain composition of pectic polysaccharides from tomato in response to *Ralstonia solanacearum*

3.1 INTRODUCTION

Tomatoes are one of the most widely distributed vegetables grown throughout the tropics and subtropics. They are used as a fresh vegetable and can also be processed and canned as a paste, juice sauce, powder or as a whole. World production has increased approximately 10% since 1985 (FAOSTAT, 2003) reflecting a substantial increase in dietary use of the tomato. Nutritionally, tomato is a significant dietary source of vitamin A and C.

One of the major constraints in production of tomato is damage caused by pathogens (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 1991). Bacterial wilt is one of the most devastating plant diseases and affects more than 200 plant species, the most susceptible commercial crops being potato, eggplant, pepper, banana, groundnut and tomatoes (Hayward, 1991, Elphinstone, 2004). *Ralstonia* (formerly *Pseudomonas*) *solanacearum* (Smith) (Yabuuchi *et al*., 1995), causal agent of the disease, is widely distributed in tropical, subtropical, and some warm temperate regions of the world (Kelman, 1953; Hayward, 1991; Chellemi *et al*., 1998). *Ralstonia solanacearum* which is a soil-borne bacterium, enters host plant roots through wounds or at lateral root emergence points, colonizes the root cortex, and subsequently invades the developing xylem vessels (Vasse *et al*., 1995; Wallis and Truter, 1978). Once established in the xylem, the pathogen spreads rapidly throughout the plant, inducing yellowing, stunting, wilting, necrosis, and finally plant death. Hikichi *et al.* (1997) observed that *R. solanacearum* invading and colonizing the roots spreads into the collars and multiplies in xylem vessels of stems, the intercellular spaces and the inner spaces between the

epidermis and the cortex, and that bacterial multiplication in stem tissues correlated with susceptibility and resistance of tomatoes to bacterial wilt.

R. solanacearum is a Gram-negative bacterium that exists as an array of biotypes with varying cultural, physiological and pathogenic traits (Buddenhagen and Kelman, 1964; Fraser *et al.,* 2001). Strains of *R. solanacearum* differ in host range, geographical distribution, pathogenicity and physiological properties (Hayward, 1964, 2001; Harris, 1972; He *et al*., 1983, 1986; Seal and Elphinstone, 1994).

More recently, *R. solanacearum*, especially the biovar 3, race 1 phenotype which is the most persistent and potentially the most destructive, has increased in importance as a quarantine organism and became subject of investigations in cool temperate climates, following reports of potato brown rot disease (race 3) outbreaks in some European countries (Elphinstone, 1996, 2004; Janse, 1996; Stead *et al.,* 1996). Hence, because of its extensive host range, worldwide distribution and destructive economic impact, bacterial wilt can be ranked as the most important among the phytopathogenic bacterial diseases (Kelman, 1998).

Because of the economic importance of this disease, research has been carried out on this disease from different aspects such as disease epidemiology, host plant resistance and other means of disease control (Sutton, 1982; Snijders, 1990; Snijders and Krechting, 1992; Parry *et al*., 1995; McMullen *et al*., 1997). Studies of wilt in potatoes (Priou *et al.,* 2002) suggested the use of bacterial wilt-free seed potatoes of a less susceptible variety under farmer cultural practices. Also the use of improved cultural practices with the existing farmer varieties has the potential to significantly reduce wilt and increase yield. Crop rotation, another important component of integrated disease management, significantly reduced wilt and increased yields even under serious bacterial wilt infestation in potato (Lemaga *et al.,* 2002).

Use of resistant cultivars remains the key strategy to control bacterial wilt, especially caused by the broad host range race 1 strains of *R. solanacearum* (Hartman and

Elphinstone, 1994), since it is the simplest and most effective way among possible control options. Breeding for resistance in tomato has generally resulted in good levels of site-specific resistance. However, breakdown of resistance has been frequently reported in tomato cultivars grown away from the original breeding areas (Grimault and Prior, 1993; Hanson and Wang, 1996).

Even though bacterial wilt is a disease of major international importance, very little is known about the cellular mechanisms or genetic underlying host plant resistance. Therefore, understanding the mechanism of host resistance is essential for the long-term management of bacterial wilt. In their association with pests or pathogens, plants evolved an impressive array of defensive mechanisms to avoid pest damage or diseases (Baker *et al.*, 1997; Knogge, 1997; Schlösser, 1997; Keen, 1999; Nandakumar *et al.*, 2000). Many resistance mechanisms in plants in response to pathogen invasion were described, such as the modification of the cell wall in response to infection by vascular pathogens including the formation of wall appositions (Beckman and Talboys, 1981), papilla formation (Inoue *et al.*, 1994), callous deposition (Beckman *et al.*, 1989), production of gels or gums (Van der Molen *et al.*, 1977), formation of tyloses (Beckman *et al.*, 1972; Grimault *et al.*, 1994), and lignification (Kang and Buchenauer, 2000). Besides these morphological defense responses, numerous biochemical alterations may also be involved in resistance mechanisms including the synthesis and accumulation of pathogenesis-related (PR) proteins (Aist, 1976; Smart, 1991; Benhamou *et al.,* 1989, 1990; Kang and Buchenauer, 2000, 2002, 2003).

Resistance in tomato is known to be temperature-sensitive and strain-specific (Krausz and Thurston, 1975; Mew *et al.,* 1977; Martin and Nydegger, 1982). Temperature is the most important factor affecting the host- pathogen interactions as well as survival in soils. In general, increase in ambient temperature to 30-35°C increases the incidence and the rate of onset of bacterial wilt on hosts such as tomato. Plants that are resistant at moderate temperature may become susceptible at a higher temperature (Hayward, 1991). Also, high soil moisture accumulations resulting from either a high water table or heavy rainfall usually favors development of bacterial wilt.

The primary cell wall of dicotyledonous plants principally consists of the cellulose and hemicellulose polysaccharides network, embedded in a pectic matrix that is structurally complex and heterogeneous (Willats *et al.,* 2002; Ridley, 2001). The pectic polysaccharides also form the middle lamella, and are involved in cell adhesion (Jarvis, 2003). The cell wall provides different mechanical properties at different times during cell development. The cell wall assembly has a major influence on the mechanical properties of cells and tissues, although as yet there is a relatively poor understanding of the effect of specific molecular interactions on wall behavior. Zivanovits *et al.* (2004) suggested that the mechanical properties of the pectin network of the plant cell wall may be modulated through the control of the response of the pectic network to the osmotic stress of cell contents and the composition of the apoplastic sap. The plant cell wall is, thus, a complex matrix with physical properties conferred by the interaction of constituent structural polymers (Redgwell and Selvendran, 1986; McCann and Roberts, 1991). Pectic polysaccharides are probably the most complex class of cell wall polysaccharides (O'Neill *et al*., 1990) (as discussed in chapter 1). The structure and material properties of pectins is characterized by cross-linkages with divalent cations and with possible esterification to other cell wall polymers (Schols and Voragen, 2003). The complex structural composition of pectins reflects perhaps their functional diversity. It is not surprising, therefore, that pectins play multifaceted roles during the life cycle of a plant. Interestingly, the unique gelling property of pectins is also one of the factors that can determine the fate of a host-pathogen interaction. For instance, pectins may act as defense barriers against the invasion of microorganisms. The resistance of primary cell walls to enzyme digestion has been suggested to be the result of the formation of calcium bridges between pectin chains (Ferguson, 1984). On the other hand, Rao *et al*. (1982) indicated that pectins of plant cell walls play a critical role in adherence of bacterial cells to the host surface. However, the elucidation of the role of the structural composition of pectins in host–pathogen interactions is a topic that has received scant attention, and this aspect is the focus of the present investigation. Specific AGPs or AGP epitopes appear to be associated with differentiation events during the life cycle of the plant. AGPs are also significant components of a number of plant gums or exudates and confer special properties on these plant products (Showalter, 2001). AGPs are proteoglycans with poly-

and oligosaccharide units covalently attached to their protein moiety (Van Holst and Klis, 1981) and are known to be present in higher plants and in their exudates. One of the most well characterized AGPs to date is LeAGP-1, a major AGP in tomato. LeAGP-1 has four distinct regions: N-terminal signal sequence for secretion, a central hydroxyproline/ proline-rich region interrupted by a short lysine-rich basic region, and a hydrophobic C-terminal sequence identified as a GPI-anchor (glycosylphosphatidylinositol-anchor) addition sequence. LeAGP-1 influences stem elongation, lateral branching, fruit production and seed development. Alterations of plant cell wall components as pectins and AGPs may contribute to induced resistance mechanisms as defense barriers against the invasion of pathogens. Lignification and the production of other structural barriers in cell walls, eg. formation of calcium bridges between pectic chains, were observed in many plant species following attempted infection by pathogenic organisms. (Ferguson, 1984).

Ralstonia solanacearum has a lower rate of multiplication in resistant plants. Pre-existing morphological barriers as well as induced mechanical barriers at the penetration site in response to pathogen attack may hamper the spread of the pathogen (Rahman *et al.,* 1999). Bacterial wilt resistance is not associated with resistance to bacterial root invasion but with the capability of the plant to limit *R. solanacearum* colonization in tomato stems (Grimault *et al.,* 1993, 1994). Similar bacterial numbers in roots of tomato varieties with different degree of resistance were also observed by Prior *et al.* (1994), and Dannon, (2004) who concluded that resistance did not result from a limitation of bacterial penetration in roots, but resistance mechanisms were localized in the mid-stem. Similarly, Grimault *et al.* (1995) and Vasse *et al.* (2002) reported a decrease of bacterial density in mid-stems compared to the collar. Wilting of the plants is therefore the most obvious symptom that reflects the invasion of the pathogen and is possibly a result of restricted water movement due to the formation of slime or electron dense material (Vasse *et al*., 1995), that surrounds the bacterial masses in the stem xylem vessels.

The complexity of tomato - *R. solanacearum* interactions, are the result of subtle combinations between genetic sensor-regulator systems governing *R. solanacearum*

pathogenicity (Schell, 1996) and strong host-genotype-environment interactions. Although the resistance in tomato genotype Hawaii 7996 appeared to be monogenic and dominant in a Mendelian genetic study (Grimault and Prior, 1995), quantitative genetic studies indicate that it is under control of 6 quantitative resistance loci (QRL) (Thoquet, 1996; Zhang *et al.,* 2002; Lindhout *et al.,* 2003). Resistant plants are often partially colonized by the pathogen and show partial tolerance of the disease (Prior *et al.,* 1996). Continuous distribution of the pathogen appears to be necessary for colonization of the stems and development of the disease symptoms, as also reported by Rodriguez *et al.,* (2003) in tomato cultivars, resistant and susceptible to *Fusarium oxysporum f. sp. lycopersici* races 0 and 1. However, the mechanism by which colonization is restricted remains unknown. A role of cell wall polysaccharides has been suggested. Thus, pectin esterification was observed to be higher in potato cultivars resistant to *Erwinia carotovora* subsp. *atroseptica* (McMillan *et al*., 1993) and tomato cultivars differing in resistance to *Pseudomonas syringae* pv. tomato (Venkatesh, 2002), and the degree of methyl esterification, which was seen to be higher in the resistant genotypes was related to resistance mechanisms indicating the role of pectins in response to pathogen invasion. In this study the possible resistance mechanisms involving the role of pectic polysaccharides will be elucidated.

Resistance responses of soybean against fungal pathogens may manifest in the expression of pathogenicity related proteins (PRPs) or pathogen inducible genes (Graham et al., 2003). *R. solanacearum* contains a set of conserved pathogenicity genes, the *hrp* (hypersensitive response and pathogenicity) gene cluster, which codes for a type of bacterial protein secretion system, known as the Type III secretion system (Vasse *et al.,* 2000a). So far, PopA, PoPB & PoPC were secreted via the *R. solanacearum* Hrp pathway (Arlat *et al.,* 1994, Genin & Boucher, 2002). Together with P*opA,* P*opB* and P*opC* form an operon that is controlled by the *hrpB* regulatory gene. PopA is responsible for a hypersensitive-like reaction when *R. solanacearum* cells are infiltrated into plant tissue at high concentration (Genin and Boucher, 2002). PopB and PopC both exhibit characteristics indicating that they might be aimed at eukaryotic cells. PopB possesses a functional bipartite signal, which is hypothesized to be carried into the plant cell, where it

is targeted at the nucleus. PopC, however, displays 22 tandem leucin-rich repeats (LRR), which correspond to the eukaryotic cytoplasmic LRR consensus, implying that PopC might be transported into the plant cell cytoplasm (Gueneron *et al*., 2000). Moreover, PopC is homologous to plant LRR proteins serving for development and resistance. Thus, Gueneron *et al*. (2000) propose that PopC could imitate plant LRR proteins and thereby alters a specific signal transduction cascade or else PopC could prevent identification of the pathogen by influencing effectors of *R. solanacearum*. Though, in experiments *popABC* mutants are reported to be as virulent as wild-type strains. Investigations have also been undertaken to analyze the involvement of bacterial surface appendages in the type III-related interactions with host cells. Two roles have been proposed for these type III-dependent appendages: either in the attachment to eukaryotic cells and/or in the delivery of proteins into host cells. Vasse *et al.* (2000a) and Blocker *et al.* (2003) identified a type III-associated Hrp-pilus in *R. solanacearum* which is involved in delivering the Pop A proteins into the host cell which are assumed to form pores in the eukaryotic cell membrane.

Based on results from microscopic observations, Vasse *et al.,* (2000b) suggested that *hrp* mutants, such as hrpB mutant and hrcV mutant of *R. solanacearum* strain GMI1000 showed reduced infection, colonization, and multiplication ability in the vascular system of tomato roots. The mutants also induce a defense reaction similar to a vascular HR at one protoxylem pole of invaded tomato plants. Vasse *et al.* (1998) also suggested that these mutants which compete with the pathogenic strain for space within xylem vessels and induce local, non-specific resistance may lead to significant protection against tomato bacterial wilt. Hikichi *et al.,* (2003) suggested that the *hrp* mutants, which are deficient in type III secretion machinery, lose their ability to colonize and multiply in host tobacco plants immediately after invasion, resulting in a loss of their ability to induce host responses and the subsequent provocation of disease.

When the plants and pathogens interact, plants use multiple mechanisms to accumulate bioactive levels of oligosaccharins which then acts as the signal molecules to elicit the

defense responses and on the other hand the pathogens use corresponding mechanisms to prevent signal molecules from accumulating (Ridley *et al.,* 2001).

Growth of *R. solanacearum* in host tissues was associated with pronounced alterations including the disintegration of host cell walls, which indicates the production of cell-wall-degrading enzymes (PG, PMEs) during infection and spread in the host tissues. An extracellular enzyme consortium breaks down plant cell walls and facilitates bacterial invasion and spread by digesting cortical cell walls, the pectic gels surrounding lateral root emergence points, and the pit membranes that separate adjacent xylem vessels (Allen *et al.,* 1998).

We have characterized the interactions between wall-matrix polysaccharides by examining the cell-tissue, and species-dependent expression of cell wall epitopes using well characterized monoclonal antibodies. The distribution of pectic epitopes in the primary cell walls of higher plants is rather well-documented (Bush and McCann, 1999; Bush *et al*., 2001; reviewed in Willats *et al*., 2001, 2002, 2003).

To elucidate the role of pectins and AGPs, which are important components of the extracellular matrix (Clarke *et al*., 1978) in cell wall architecture in the interaction between *R. solanacearum* and tomato genotypes, we have established the use of monoclonal antibody probes (JIM5, JIM7, LM5, LM6, LM7, and LM2) for specific pectic epitopes [low ester and high esterification pattern of homogalacturonan (HG), galactan, arabinan side chains of rhamnogalacturonan I (RG I), non-blockwise pattern of de-esterification of HG and arabinogalactan protein (AGP)] respectively.

The monoclonal antibody LM2 detecting specific patterns of expression of AGPs, both temporal and spatial (Knox *et al*., 1989, 1991; Stacey *et al*., 1990; Pennell *et al*., 1991, 1992; Li *et al*., 1992) was used in this study. Immunolocalization of AGP has also been performed using other monoclonal antibodies (Serpe *et al.,* 2002).

The artificial AGP carbohydrate antigen (ß–glycosyl Yariv reagent) binds to AGP and forms a red precipitate (Yariv *et al*., 1962, 1967; Jermyn and Yeow, 1975; Clarke *et al*., 1975; Fincher *et al*., 1983). AGPs are preferentially associated with the vascular bundles and epidermis (Bacic *et al*., 1986; Pennell *et al*., 1989). The protein portion represents up to 7% of the molecule and is rich in hydroxyproline, alanine and serine. The carbohydrate portion consists of arabinose (36%) and galactose (64%) with 1,3:1,6-galactan substituted by arabinofuranosyl residues (Keegstra *et al*., 1973; Fincher *et al*., 1983).

Therefore, one of the most powerful ways to study pectin in its physiological and developmental contexts is by the use of anti-pectin antibody probes based analyses of HGA, RG I, RG II (Knox *et al.,* 2003). The extensive structural and conformational variety and the dynamic nature of the pectic network presumably reflects the range of properties it provides to the cell wall matrix, in terms of mechanics, ionic and hydration conditions, signals, potential for molecular interactions and capacity to be degraded by plant and microbial pectinases, and thus, its possible role in host-pathogen interactions. The present paper therefore reports characteristics of these pectins and AGPs from the mid-stem sections of both resistant and susceptible tomato genotypes being recognized by specific monoclonal antibodies before and after infection with *R. solanacearum*.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

The tomato genotypes H7996 with high resistance level and L390, susceptible to bacterial wilt were received from the Asian Vegetable Research and Development Center (AVRDC), Taiwan. Seeds were sown in a greenhouse (20°C day/night temperature, 14h of light per day / 30K Lux, and 70% relative humidity) and transplanted after four weeks to individual pots with 330g of soil (Fruhstorfer Erde, Type P, with 150mg/l N, 150mg/l P_2O_5, and 250mg/l K_2O). During the experiments plants were kept in a climate chamber with 30/27 °C day/night temperature, 85% relative humidity, 14 hours light, and 30K Lux.

3.2.2 Inoculum preparation

Ralstonia solanacearum isolate To-Udk2 from Thailand was used for inoculating the plants. Bacterial inoculum was produced by streaking a single colony on NGA (0.3 % beef extract, 0.5 % Bacto peptone, 0.25 % D-glucose, 1.5 % agar) agar medium and incubating at 30 °C for 48 h. Cells were harvested from agar plates by flooding with sterile, distilled water, and an optical density of 0.06 at 600 nm wavelength (Spectrotonic 20 Bausch and Lomb), corresponding to about 7.8×10^7 colony-forming units per milliliter (CFU/ml) was adjusted.

3.2.3 Inoculation of tomato plants

Four week old plants were inoculated by soil drenching with 33 ml of bacterial suspension per pot, corresponding to about 10^7 CFU/g of soil, around the base of the plants. Pots were watered after inoculation up to the soil field capacity but without producing a surplus, and kept in a climate chamber. Both healthy as well as inoculated plants were harvested at 5 dpi for the study by immuno-fluorescence microscopy and histochemical detection of AGPs.

3.2.4 Immuno-histochemical preparations and microscopy

Sections of both healthy and inoculated mid-stems from tomato genotypes H7996 and L390 were cut in slices of about <0.5 mm and collected in a fixative (4% paraformaldehyde in 50 mM PIPES buffer, pH 6.9) by free-hand sectioning. The sections were treated with PBS (phosphate buffer saline) with 5% skim-milk powder for 1 h at room temperature for blocking. Incubation with the primary antibodies (JIM5, JIM7, LM7, LM5, LM6 or LM2 received from P. Knox, University of Leeds, UK) (Table 3.2.4) was performed over night at 4°C or for 2 h at room temperature, at 1:10 dilution in PBS with 5 % skim-milk powder. The sections were washed with PBS-0.1% Tween 20 (v/v) 3-5 times for 5 minutes each and followed by dH_2O. Incubation with the secondary antibody anti-rat IgG FITC (Sigma) at 1:50 concentration in PBS-5 % milk powder was then performed over night at 4°C or for 2 h at room temperature. The sections were washed again with PBS-0.1% Tween 20, 3-5 times for 5 min each followed by 3-5 times washing with dH_2O. Finally, the sections were mounted in Citifluor (AF1) antifade (Plano, Wetzlar, Germany) on glass slides. Specimens were observed under a photomicroscope (Zeiss fluorescence microscope) equipped with epifluorescence illumination with a filter system appropriate for fluoroscein fluorescence, 520-560 nm. Fluorescein has λ_{Ex}=495 nm and λ_{Em}=518 nm. Increasing color intensity evaluated as -, F (few, for single vessels), ±, (+), +, ++, (+++), +++. The immuno-histochemical trial was repeated three times with newly grown plants of both healthy and *Ralstonia solanacearum* strain ToUdk2 inoculated resistant (H7996) and susceptible (L390) tomato genotypes. 3 healthy and 3 inoculated plants were picked randomly out of set of 5 plants and sections were made for both genotypes. On each slide 5-6 mid stem sections were observed and evaluated as per the grades described and slides were made in duplicate. Control samples were taken from both genotypes and treated same way as described above except for the step with the primary antibodies was omitted. The labeled vessels in case of healthy and inoculated resistant tomato genotype (H7996) were counted in treatments with LM5 and LM6 and Data were processed using analysis of variance in SAS (the SAS System for Windows V8, Release 8.02 TS Level 02M0; 1999-2001.

Institute INC., Cary, USA). For all analyses a significance level of P = 0.05 or lower was used.

Table 3.2.4 Antibodies used for structural analysis of pectic polysaccharides

Pectin domain	1° Ab	Epitope recognized
HG	JIM 5	low methyl esterification grade 31-40%
HG	JIM 7	'high' methyl esterification grade 15-80%
HG	LM 7	non-blockwise de-esterification
RG-I	LM 5	(1→4)-ß-D-galactan
RG-I	LM 6	(1→5)-α-L-arabinan
AGP	LM 2	arabinogalactanprotein

HG = homogalacturonan, RG = rhamnogalacturonan, AGP = arabinogalactan protein

3.2.5 Histochemical detection of Arabinogalactan protein (AGPs)

AGPs in tomato stem tissues were stained as described by Majewska-Sawka *et al.* (2002). The hand-cut sections of <0.5 mm of both healthy and inoculated tomato mid-stems from genotypes H7996 and L390 were taken from plants 5 days after inoculation with *R.*

solanacearum strain ToUdk2. Stem sections were stained with 300 µM solutions of ß-glucosyl Yariv phenylglycoside (ß-D-Glc)$_3$ [Australia Biosupplies, Parkville, Australia] in 1% NaCl (Clarke *et al.,* 1975), which specifically binds and precipitates AGPs. As a control, sections from the same sample were stained with 300µM solutions of α-galactosyl Yariv phenylglycoside which does not bind AGPs. After staining for 72 h at 4 °C, the sections were washed overnight in 1% NaCl to remove excess unbound reagents, then briefly rinsed with distilled water, air-dried, and observed under the Zeiss microscope.

3.3 RESULTS

3.3.1 Cytochemical localization of pectic polysaccharides

The structural composition of pectic cell wall polysaccharides of mid-stem sections of healthy and *R. solanacearum*-inoculated tomato genotypes H7996 (resistant) and L390 (susceptible) was established by immuno-labeling with the antibodies JIM5, JIM7 and LM7, binding to low methyl-esterified, highly methyl-esterified and non-blockwise patterns of de-esterification of homogalacturonan (HG), respectively, and LM5, LM6 and LM2, specific for galactan and arabinan side chains of rhamnogalacturonan (RG I) and arabinogalactan protein, respectively, and subsequent microscopic observations. Horizontal stem sections were compared for labeling of epidermis, chlorenchyma, angular collenchyma, cortical fibres, phloem, cambium, xylem parenchyma, metaxylem, protoxylem, mark parenchyma and inner parenchyma.

Comparing stem sections of healthy plants of genotypes H7996 (H) and L390 (L), a stronger labeling of the galactan side chains of RG I (LM5) (Fig. 1 a-d) and of AGPs (LM2) (Fig. 1 e, f) was observed in the resistant genotype in the xylem parenchyma (Fig. 1c, d) and in walls of single vessels (Fig. 1 f), respectively, compared to sections of genotype L390 (Fig. 1 a, c, and e, respectively) (see also Tab. 3.3.1.). On the contrary, sections of genotype L390 reacted stronger with antibody LM6 in single xylem (Metaxylem) vessels and slightly stronger around the vessels (Fig. 1 g,), and stronger with antibody LM7 around xylem vessels (Fig. 1 i) compared to H7996 (Fig. 1h and j, respectively). Antibodies JIM5 and JIM7 stained only few vessels in both genotypes while no or only low labeling occurred in other tissues of healthy plants (Tab. 3.3.1.). No labeling or no differences between genotypes were observed in the other evaluated stem tissue components. Significant differences were observed between healthy and inoculated H7996 where 70 % and 76 % of the vessels showed more labeling after inoculation in treatment with LM5 and LM6 respectively, in contrast to only 12 % and 8 % of the vessels being brighter in healthy H7996 in treatments with LM 5 and LM6 respectively,

indicating an increase in galactan and arabinan epitopes, in the inoculated resistant genotype.

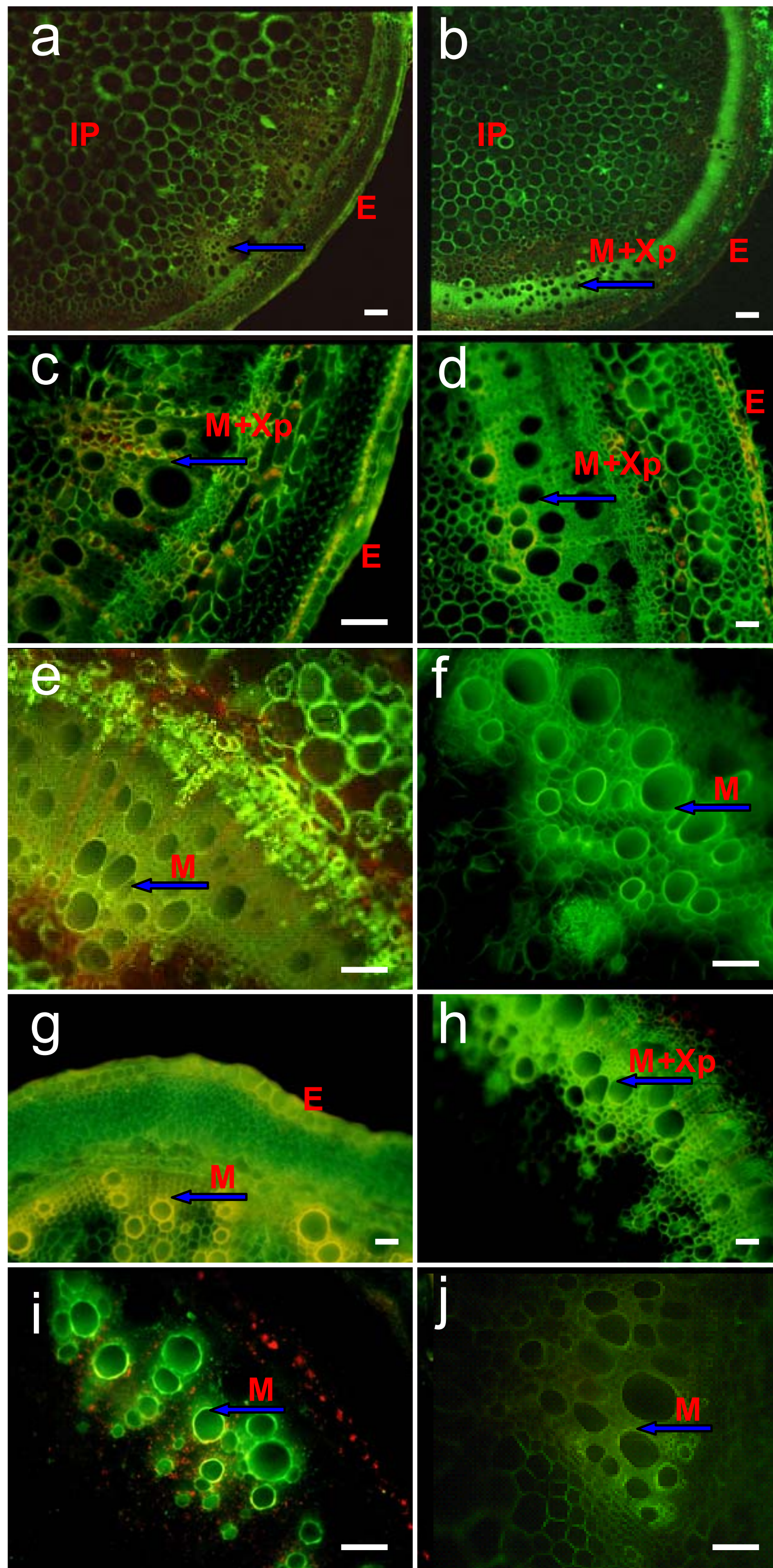

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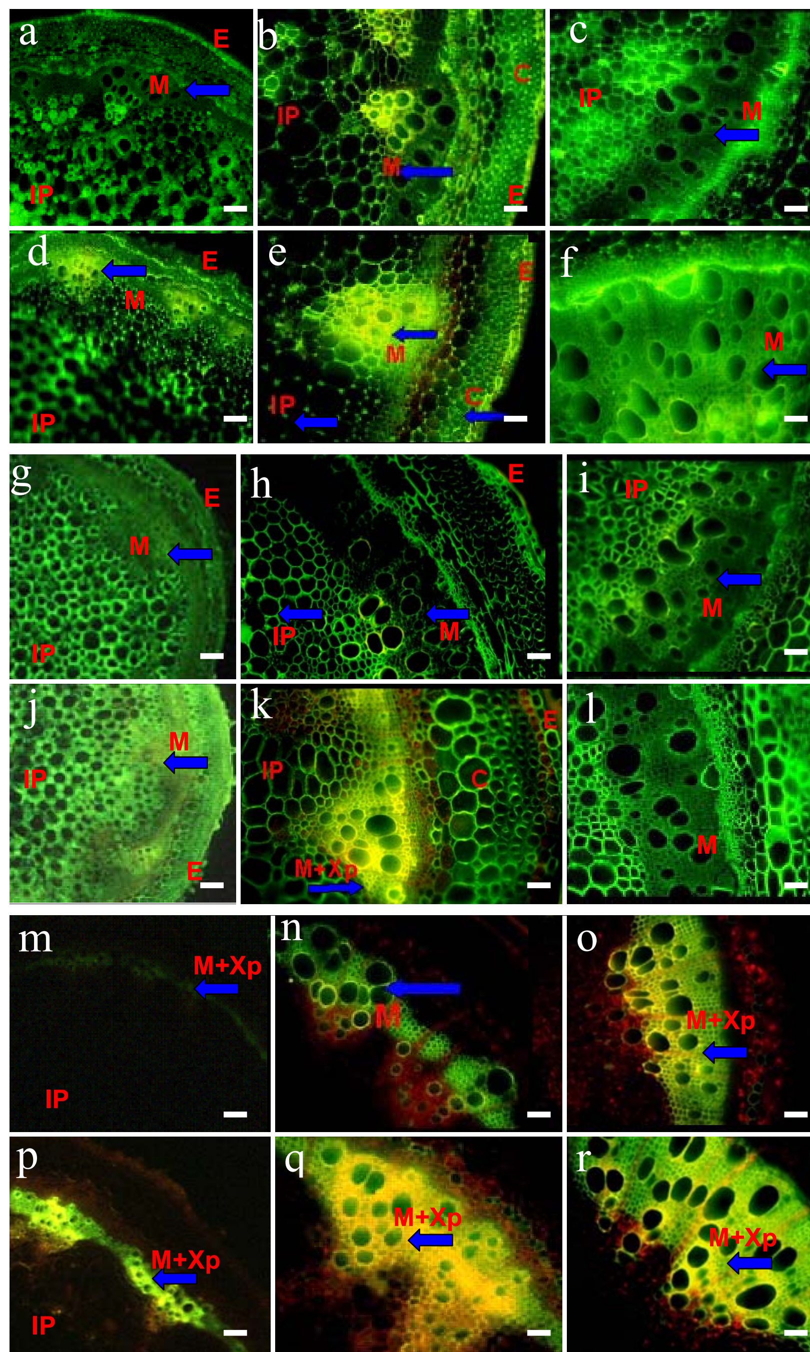
Fig. 1. Pectin epitopes in stem tissues of healthy tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively. a, c: immunofluorescent labelling by LM5 specific for galactan in L390 was observed in the inner parenchyma (IP) and cortex (C), but less in the metaxylem (M) and xylem parenchyma (Xp). b, d: in H7996 a strong labelling by LM5 occurred in the xylem parenchyma (Xp); a, b = 2.5 x objectives, c, d = 10 x objectives; e: reaction of LM2 specific for arabinogalactan protein with xylem parenchyma of L390. f, in H7996 LM2 showed stronger signals in metaxylem (single) vessels than in L390. g: in L390 labelling by LM6 specific for arabinan was detected in epidermis, cortex and metaxylem (single vessel), but no signal was seen in the inner parenchyma (not shown). h: in H7996 signals, which were less intense than in L390, were detected in the metaxylem (single) vessels indicating more of the arabinan epitope in side chains of RG I. i, L390 showed stronger labelling in the tissue around xylem vessels by LM7 which recognizes the non-blockwise de-esterification pattern of HG, compared to the resistant genotype H7996 (j). Bars = (a, b =200µm). (c, d, e, f, g, h, I, j = 100 µm). The sections were mounted in Citifluor (AF1) antifade (Plano, Wetzlar, Germany) on glass slides. Specimens were observed under a photomicroscope (Zeiss fluorescence microscope) equipped with epifluorescence illumination with a filter system appropriate for fluoroscein fluorescence.

Table 3.3.1 Characterization of pectic polysaccharides from mid-stem sections of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, before and five days after inoculation with *R. solanacearum* strain To-Udk2. Antibody specificities: JIM5 - specific for low ester, JIM7 - high ester, LM7 - non-blockwise de-esterification patterns of HGA, LM2 - arabinogalactan protein, LM5 - galactan, LM6 - arabinan epitopes, respectively of RGI. Increasing color intensity evaluated as -, F (few, for single vessels), (+), +, ++, +++. H= healthy H7996, Hi= inoculated H7996, L= healthy L390, Li= inoculated L390. Trial was repeated three times with newly grown plants.

Antibodies	Xylem parenchyma				Single vessel				Around vessels			
	H	Hi	L	Li	H	Hi	L	Li	H	Hi	L	Li
JIM5	-	-	-	-	F	F	F	F	-	-	-	+++
JIM7	-	-	-	±	F	F	F	F	-	-	-	+++
LM7	++	(+++)	++	++	++	(+++)	++	+++	-	-	+	+++
LM2	++	++	++	++	++	++	F	+++	-	-	-	+++
LM5	++	++	+	+	±	++	(+)	+++	-	-	-	+++
LM6	+	+	+	++	+	+++	++	+++	-	-	±	+++

Strong differences between sections of healthy and inoculated plants of the susceptible genotype were observed around vessels after staining with all the antibodies (Tab. 3.3.1., Fig. 2a, b). Labeling with LM6 after inoculation in L390 showed intense fluorescence localized mainly in around vessels and also in the xylem parenchyma and single vessels, indicating arabinan side chains of RG I in branched regions of the pectic polysaccharides. After inoculation, a dramatic increase in the epitopes labeling and a high level of fluorescence in single vessels of mid-stems of genotype L390 was observed in labeling with LM2, LM5, LM6 and LM7 recognizing the arabinogalactan proteins, epitopes of

galactan and arabinan side chains of branched regions of pectins and non-blockwise de-esterification pattern of HGA domain of pectic polysaccharides respectively.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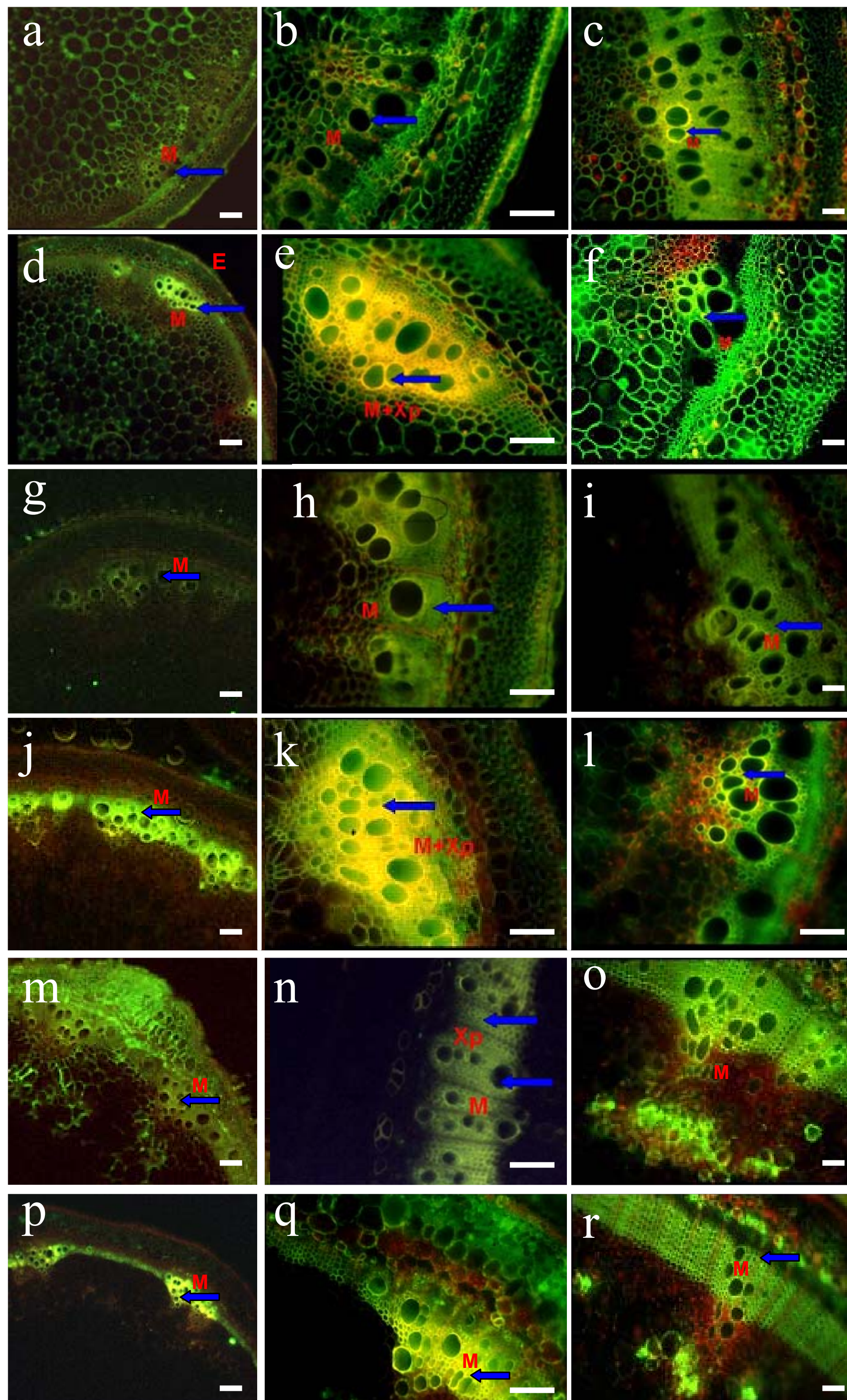
M+Xp

Fig. 2A. Pectin epitopes in stem tissues of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively. A (a, b), labelling by JIM5 in L390 before inoculation was in similar pattern as seen in the resistant genotype H7996 (c). A significant difference was seen between the genotypes after inoculation where L390 showed a more intense labelling around the vessels (d, e) compared to H7996 in (f). No significant difference in labeling with Jim 7 was seen between H7996 (i) and L390 (g, h) before inoculation, whereas after inoculation much stronger signals were detected around vessels and, less, in the xylem parenchyma of L390 (j, k) compared to H7996 (l). (m, n), labeling by LM7 in L390 before inoculation was stronger around vessels than H7996 in (o). (p, q), after inoculation L390 showed stronger labelling in single vessels and around vessels with LM7 which recognizes the non-blockwise de-esterification pattern of HG than H7996 (r). Bars = (a, d, g, j, m, p =200µm). (b, e, h, k, n, q, c, f, i, l, o, r =100 µm)

Labeling of single vessels increased more than threefold and was generally intense in reactivities of LM2 and LM5 antibodies after infection in the inoculated susceptible genotype (L390) (Tab. 3.3.1.). After inoculation, labeling of single vessels with antibodies LM2 (Fig. 2B p&q), LM5 (Fig. 2B d&e), LM6 (Fig. 2B j&k) and LM7 (Fig. 2A p&q) and, stronger, around vessels with all the antibodies, increased in the susceptible genotype indicating an increase in AGPs, galactan and arabinan side chains of RG I and the non-blockwise deesterification pattern of homogalacturonan (HG), respectively. Different than in comparison of genotypes, a reaction was observed around vessels with JIM5 (Fig. 2 d&e) and JIM7 (Fig. 2 j&k) after inoculation in the susceptible genotype. The JIM 5 binding polymers appeared as irregular aggregates in inner parenchyma and some vessels of the susceptible genotype after infection (Fig. 3 a&b). These pectins are distributed in the middle lamella or in the intercellular spaces. The resistant genotype (H7996) showed no reaction after inoculation around vessels and in the xylem parenchyma, but the labeling in single vessels showed a significant increase in the



a
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Fig. 2B. Pectin epitopes in stem tissues of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively. (a, b), labeling by LM5 in L390 before inoculation was similar in single vessels and around vessels but less labeling was observed in xylem parenchyma as compared to H7996 (c). After inoculation L390 showed intense labeling of the single (metaxylem) vessels and around vessels (d, e); as compared to H7996 which showed increased labeling of only single vessels after inoculation (f). Significant difference was seen in single vessels and around vessels between H7996 (i) and L390 (g, h) after labeling with LM6 in healthy plants being stronger in L390 than H7996, whereas stronger signals were detected in the single (metaxylem) vessels and, around vessels but less, in the xylem parenchyma of L390 (j, k) after inoculation compared to inoculated H7996 (l), which showed increased labeling of only single vessels after inoculation. A similar intensity of labeling by LM2 was observed in the xylem parenchyma and around vessels of L390 (m, n) and H7996 (o) before inoculation but significant difference was observed where H7996 showed more labeling of single vessels than L390 before inoculation. After inoculation L390 (p, q) showed stronger labeling for LM2 in single vessels and around vessels than H7996 (r). Bars = (a, d, g, j, m, p =200µm). (b, c, e, f, h, I, k, l, n, o, q, r = 100 µm).

binding of LM6 (specific for arabinan) (Fig. 2B i&l) and LM5 (specific for galactan) (Fig. 2B c&f) epitopes of branched RG I.

An increase in fluorescence of degenerating cells (around vessels) in the transmitting tissue of inoculated L390 occurred with both MAbs, JIM 5 and JIM 7. Both these antibodies revealed the homogeneous presence of HG epitopes in epidermal and parenchymal cell walls throughout the tomato stems. The controls, where primary antibodies were omitted, showed a lack of fluorescence in all the tissues except a low autofluorescence in the xylem vessels (Fig. 2C).

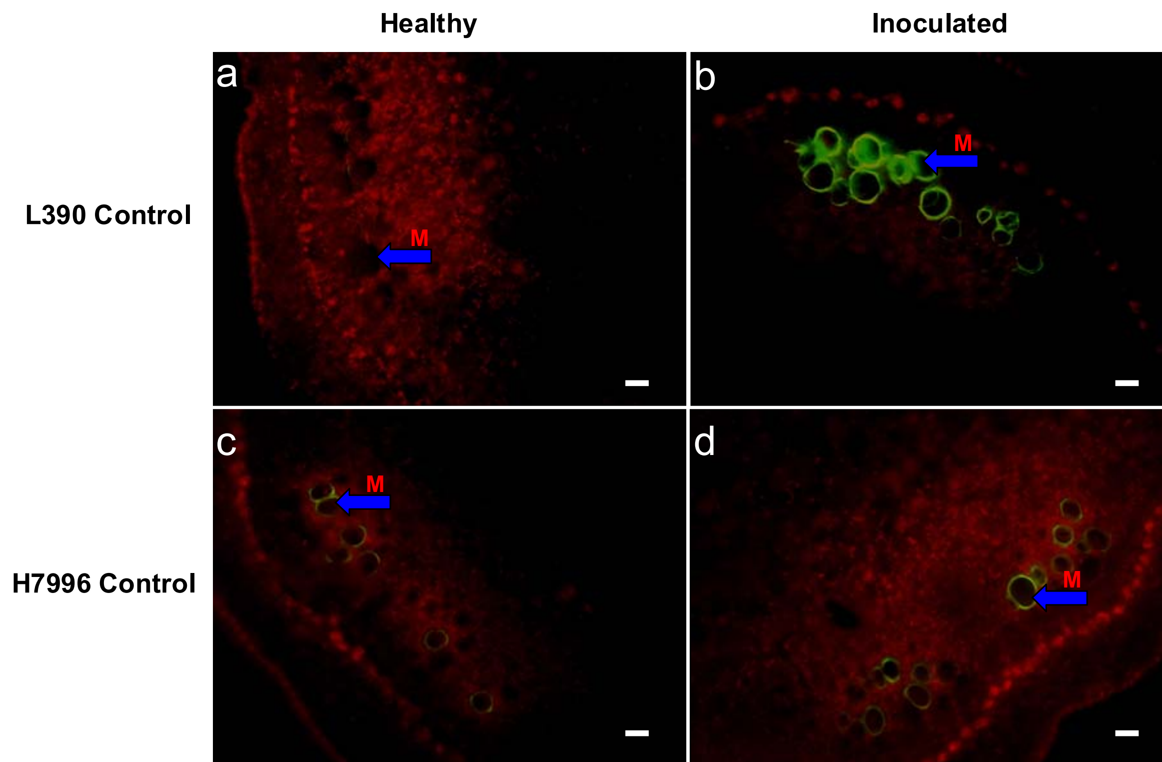


Fig. 2C. Control samples where primary antibody was omitted or stem tissues of 4-week old tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively. (a), L390 control before inoculation showed no labeling of vessels. (b) A significant difference was seen between the healthy and inoculated L390 where single vessels showed autofluorescence labeling. (c), H7996 control before inoculation showed no labeling of vessels. A significant difference was observed between genotypes after inoculation where L390 showed a more intense labelling of the metaxylem vessels (M) (single vessels) compared to H7996 in (d). Bars = 100µm (a, b,c,d).

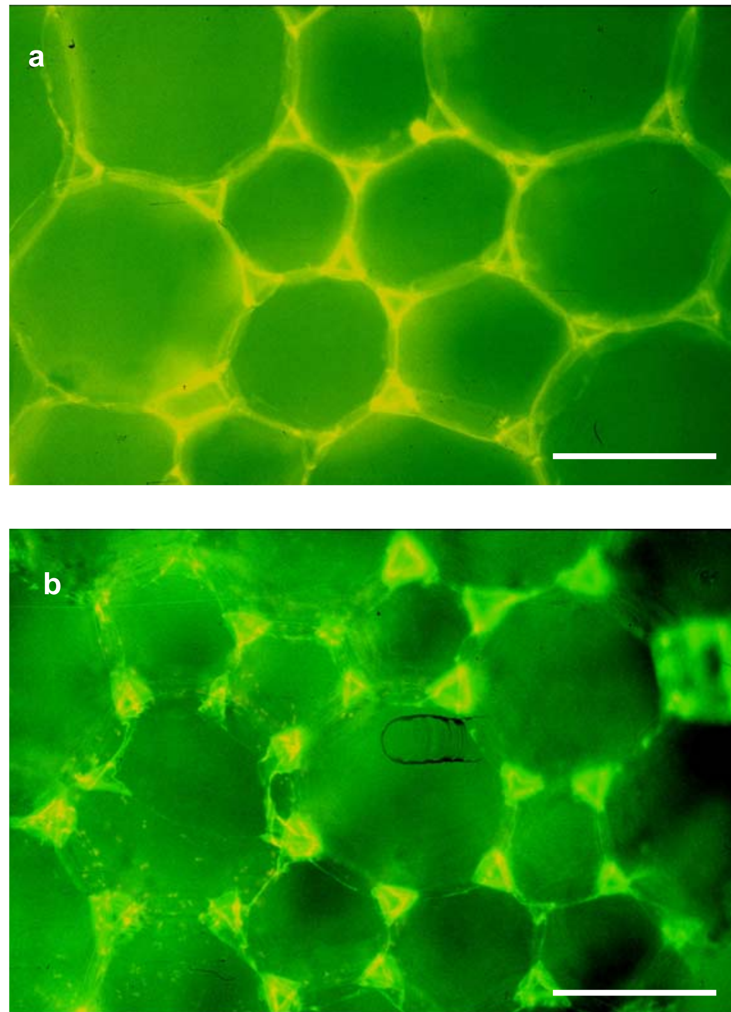


Fig. 3. (a), Demonstration of lowly esterified homogalacturonans with the antibody JIM5 in healthy plants of tomato genotype L390, susceptible to bacterial wilt. Labeling was recognized in the inner parenchyma. (b), Irregular aggregates in cell corners were observed after inoculation with *R. solanacearum*. Bars = 200 µm (a, b).

3.3.2 Histochemical localization of Arabinogalactan-Protein

Labeling with ß–glucosyl Yariv reagent resulted in typical deep red staining of arabinogalactan-proteins. The outer epidermal wall showed a clearly defined red zone beneath the cuticle and a similar red zone at the inner surface, while staining of the inner epidermal wall was much less intense. Control reactions with α–galactosyl Yariv reagent resulted in a complete lack of staining (Fig. 4b). The vesicles stained with ß–glucosyl derivative were evident around the periphery of the cells. The staining patterns of both healthy and inoculated resistant and susceptible genotypes were similar. Both immunochemical and histochemical methods revealed the expression of these proteoglycans in the xylem.

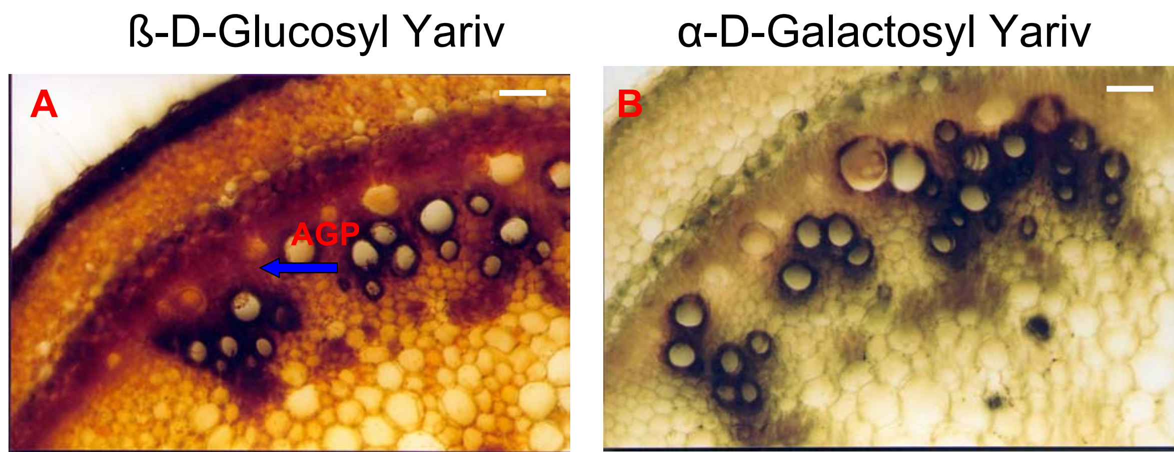


Fig. 4. Demonstration of arabinogalactan-proteins stained with ß-glucosyl Yariv reagent in stem tissues of healthy plants of tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, in the xylem parenchyma and around xylem vessels and in the cuticle layer. Weak staining was observed in the inner parenchyma and cortex (A), compared to a control sample treated with $(\alpha\text{-D-Gal})_3$ Yariv reagent, which shows no staining (B). Bars = 100 µm (A, B).

3.4 DISCUSSION

Immunocytochemical investigations revealed differences between tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively, in structure and composition of their pectic cell wall components and arabinogalactan proteins as observed by staining with monoclonal antibodies specific for epitopes of branched pectins.

The pectic polysaccharides form a hydrated cross-linked three-dimensional network in the matrix of primary plant cell walls and are some of the most structurally complex macromolecules that exist in nature. Comparing the healthy resistant and susceptible genotypes H7996 and L390, respectively, the labeling of arabinogalactan protein and galactan epitopes, presumably side chains of RG I, was higher in H7996 genotype than in L390. These polymers could contribute to a higher resistance in genotype H7996, since arabinogalactan protein has been reported to be involved in the resistance reactions (Mc Cann and Roberts, 1996).

An important component of the extracellular matrix and the plasma membrane are arabinogalactan-proteins (Fincher *et al*., 1983; Samson *et al*., 1983 and 1984), and play a role as messengers in cell-cell interactions during differentiation. Receptors for AGPs are present on the cell surface, making the high availability of AGPs possible (Kreuger and Van Holst, 1993). This high reactivity could explain the strong labeling of AGP epitopes in the susceptible genotype after inoculation and interaction with the pathogen. Pathogens tend to attack cell corners, and the complex mixture of pectins and arabinogalactan proteins (AGPs) found there may have a role in enmeshing the invading organism as well as in signaling to elicit through the release of small pectic fragments defense mechanisms (Mc Cann and Roberts, 1996).

Also Grimault *et al.* (1994) and Vasse *et al.* (1995) observed in their histochemical studies deposition of electron-dense material, they supposed gums, at the cell wall, where preferentially the bacteria were distributed, being involved in the resistance reaction.

Thus, the degradation of plant wall material and, at the same time, deposition of new wall material could indicate a resistance mechanism in the resistant genotype. The arabinan and galactan epitopes are the first defined epitopes occurring in the side chains of pectic polysaccharides to be immunolocalized in developing systems (Willats *et al.,* 1999)**,** and the observations reported here may indicate differences between genotypes H7996 and L390 in expression patterns of RG I pectic domains during plant development.

After inoculation with *R. solanacearum,* dramatic cell wall changes were observed in the susceptible genotype (L390), as indicated by increased labeling with all the antibodies. Specificity of JIM5 makes it a highly selective probe for low methyl-esterification regions of HG and are thought to be a major factor in the cross-linking of HG chains by calcium ions and thereby gel formation by HG domains. This gel could form favorable conditions for the pathogen, and play an important role in the compatible interaction, resulting in wilting and finally death of the plant. It is assumed that the antibody JIM5 reacts only with partly de-esterified pectins, and that optimal binding requires the presence of groups that are methyl-esterified in a range of 31% to slightly over 40%. As for JIM7, the pattern of esterification does not significantly influence binding capacity (Willats *et al*., 2000). The increased de-esterification after inoculation may be due to the activity of pectin methylesterase of *R. solanacearum*, an enzyme which was shown to be involved in the pathogenesis of bacterial wilt (Allen *et al.,* 1998). After infection many resistance mechanisms are started. This fluorescence – in the highly susceptible genotype L390 should be partly due to pectins and AGPs, although these polymers are abundant in xylem tissues, immunolabelling of these pectic polymers and HRGPs might not be the only reason for the observed fluorescence. Also lignification may contribute to a yellowish-green shine of xylem vessels by autofluorescence. Due to the characteristic of the tissue print to display a colour reaction in the presence of pectic and AGPs epitopes because lignin compounds as well as phenolics are not transferred to the nitrocellulose membrane, this assay can confirm the participation of these pectins and AGPs to fluorescence observed in microscopical studies. Also the strong autofluorescence in xylem tissues that were observed in *R. solanacearum* controls of both genotypes might be due to such an increase of phenolics as an host plant response to pathogen infection.

Thus, the strong fluorescence of xylem vessels might be caused both by phenolic compounds and lignification, additional to the increase in pectic epitopes and AGPs as confirmed by tissue prints.

Also the increased labeling with LM7 which is specific for non-blockwise distribution of methyl ester groups, indicates a change in physico-chemical properties of the cell wall, since these HG can form calcium-mediated gels with distinct properties in terms of porosity and elasticity (Willats *et al*., 2001). This observation also indicates the possible action of pathogen PME, resulting in non-blockwise de-esterification, and, thereby, making the pectin more suitable for gel formation. The action of the pectic enzymes of *Rs* has been obscure to date, and these observations might elucidate the highly specific molecular interaction between these enzymes and plant cell wall structures, in order to create a suitable environment for pathogen multiplication.

In plant development, the highly methyl-esterified HG is first inserted into cell walls and can be viewed as a raw material from which finely tuned functionalities can be created by the action of plant-owned and pathogen-derived pectin methyl-esterases. Enzymatic cell wall degradation by pathogens was supposed to create the gels typically found in the vessels of wilting plants (Beckman, 1987). In addition, these enzymes may also release nutrients that enable rapid bacterial multiplication. Also Cooper (1983) and Walton (1994) suggested that the cell wall degrading enzymes secreted by fungal pathogens during infection and colonization of host plants may play an important role in pathogenesis.

The complexity of HG or other domains of pectic polysaccharides is due to both biosynthesis and, most notably, their modification in the cell wall during growth. However, the sheer extent to which pectins are modified and the functional possibilities created indicates that these polymers have a high potential to play specific biological roles. The high level of pectin in single vessels and around vessels with non-blockwise de-esterification of methyl-ester groups in the inoculated susceptible genotype L390 indicates a direct role in maintaining cell wall to cell wall links through calcium-mediated

cross-linking. Additionally, the LM7-binding pectin may play a defensive role at points of intercellular attachment (Willats *et al.,* 2001).

The high labeling of the pectic galactan and arabinan epitopes may also be related to the presence of the low methyl-esterified HG in the cell walls of the susceptible genotype after inoculation. The de-esterification by pathogen PME could make the galactan and arabinan side chains more accessible to the LM5 and LM6 antibodies. LM5 and LM6 epitopes are present at cell corners and these are the first defined epitopes to be attacked by the pathogen. Thus, the presence of the pathogen could cause damage in those tissues and possibly release pectic fragments by cell wall degrading enzymes such as PME and endo, exo PGs at the penetration site, where the pathogen degenerates the pit membrane and moves from vessel to vessel and these fragments generated can then act as suppressors of resistance response in the susceptible genotype (Nakaho *et al.,* 2000).

Hence our results show for the first time that *R. solanacearum* is capable of degrading pectin compounds of the plant cell wall. Pectin degradation has also been reported during root decay of rubber trees by *Rigidoporus lignosus* by Nicole and Benhamou (1993). The porosity of the cell wall matrix may be altered in these regions, which could determine the capacity of enzymes to reach the sites of action in the pit fields (Orfila, 2000). HG is usually synthesized in a largely methyl-esterified form in the Golgi apparatus and could be de-esterified in the cell wall by the action of pectin methyl esterases of *R. solanacearum* followed by degradation by PGs may produce HG fragments which could act as signal molecules determining resistance or susceptibility of the host plant, as shown for the near isogenic wheat lines resistant and susceptible to wheat stem rust fungus (Moerschbacher *et al.,* 2003).

AGPs increased in the susceptible genotype after inoculation, as shown by immunolocalization studies with antibody LM2 and the presence of these AGPs was also confirmed by histochemical staining with the synthetic phenyl glycosides, Yariv reagent, that have the ability to bind to AGPs and to block their function within living cells. The staining of AGPs by Yariv is simply through the precipitation and aggregation of soluble

arabinogalactan-protein molecules. Due to the nature of AGPs it is very likely that the sugar moiety is involved in binding the antibody but a contribution of the protein moiety, even though it is relatively small, cannot be ruled out. The sugar side chains are probably the most variable part of the molecule, generating many epitopes and differences in size and charge (Kreuger and Holst, 1995). Some AGPs can interact with pectin, most likely through ionic interactions like negative charged ions bonded by Calcium bridges.

AGPs are presumably involved in molecular interactions and cellular signaling at the cell surface. Certain AGPs are associated with xylem development. More specifically, such AGPs are associated with, and hypothesized to function in, secondary cell wall thickening and can identify cells committed to programmed cell death (PCD) of xylem cells in order to allow water transport (Gao and Showalter, 2000; Showalter, 2001; Schopfer *et al.*, 1995). It has been found that Yariv reagent effectively blocks water uptake which might be due to its binding to AGPs, and thus, inhibiting AGPs may contribute to wilting of the plant (Ding and Zhu, 1997). It should be noted that several investigators have found that AGPs often copurify with pectin (Serpe and Nothnagel, 1994, 1995; O'Neill and Selvendran, 1985; Carpita *et al*, 1989; Shea *et al.*, 1989). Since cell wall thickening is the common feature of plant resistance mechanism, and it was reported that for example tomatoes, affected by vascular wilt disorders, frequently respond by secretion of normal or modified wall components as a vascular coating onto xylem vessel walls it could be likely that the AGPs which are associated with the cell wall thickening might be a part of pathogen defence by physical barriers. It has been shown that LeAGP-1 is associated with secondary cell wall thickening of differentiating metaxylem in tomato roots and stems (Gao & Showalter, 2000). Therefore, LeAGP-1 might serve as a marker for wall thickening and lignification in cellular differentiation processes.

AGPs might contribute to cell wall thickening by association with other cell surface molecules or with one another. For example LeAGP-1 was reported to interact with pectin by clusters of basic amino acid residues or by Ca^{2+} mediated binding. However, it remains doubtful whether these bindings are strong enough to paly a role in xylem cell

wall strengthening and therefore in induced resistance (Showalter, 2001). Association of AGPs among themselves are thought to involve ionic interactions and plant analogs of Yariv agents such as flavonols glycosides or oxidative crosslinking (Showalter, 2001). The latter is associated with the oxidative burst, a rapid, transient production of huge amounts of reactive oxygen species (ROS) by plant cells, as a defense strategy against pathogens. It might be assumed that also AGPs located in resistant tomato genotype H7996 were crosslinked by these mechanisms and therefore strengthened cell walls, thus slowing the ingress of *R. solanacearum* into the xylem tissues. This could suggest the role of AGPs as resistant factor as they were already abundant in the midstem tissues of resistant tomato genotype and might be increasingly produced by susceptible genotype as a reaction to pathogen infection.

In the resistant genotype after inoculation the epitopes of arabinan and galactan side chains of branched pectins showed significantly increased labeling. The greater binding of LM5 and LM6 may be because of epitope accessibility at lower DE is increased by conformational changes in pectin structure (Willats *et al*., 2000). The increased labeling for arabinan and galactan side chains may be due to their higher accessibility after a possible deesterification after pathogen action on the cell wall. This could explain that the restricted movement of the pathogen inspite cell wall degrading enzymes causing release of pectic fragments such as degraded RG I that might form an electron-dense material possibly interacting with bacterial polymers surrounding the bacterial masses which could then result in thickening of pit membranes and hence bacteria cannot move from vessel to vessel indicating a resistance mechanism activated at the penetration site as was reported by Nakaho *et al.,* (2000). It could be speculated, that the resistant plant newly synthesizes pectic polymers with changed composition in reaction to an infection. Bacterial lipopolysaccharides and pectic polysaccharides from the host were reported to form precipitates in an incompatible interaction between *Pseudomonas syringae* pv. *phaseolicola* and pectins from bush bean, but not in the compatible interaction (Wydra, 1991).

The pathogen infection caused pronounced alterations of the host cell walls and middle lamella matrices. The pectin matrix of the plant is found throughout the primary cell wall, but is most concentrated in the middle lamella between plant cells (Carpita and Gibeaut, 1993). Therefore, pectic molecules probably are the first polysaccharides to be degraded by the pathogen during infection. Hence our results indicate that *R. solanacearum* is capable of degenerating cell walls in a tomato genotype susceptible to bacterial wilt by the production of enzymes and degrading the pectic components in a non-blockwise pattern and thus differentiating between the resistant and susceptible genotypes used in this study at the pectin level.

3.5 SUMMARY

The structural composition of pectic cell wall polysaccharides of healthy and *R. solanacearum* inoculated mid-stem sections of tomato genotypes H7996 (resistant) and L390 (susceptible) was established by immuno-labeling with the antibodies JIM5, JIM7 and LM7, binding to low methyl-esterified, highly methyl-esterified and non-blockwise patterns of de-esterification of homogalacturonan (HG), respectively, and LM5, LM6 and LM2, specific for galactan and arabinan side chains of rhamnogalacturonan (RG I) and arabinogalactan protein, respectively, and subsequent microscopic observations.

Comparing stem sections of healthy plants of genotype H7996 and L390, a stronger labeling of the galactan side chains of RG I by antibody LM5 in the xylem parenchyma and AGPs of single vessels by antibody LM2 was observed in the resistant genotype.

Genotype L390 reacted stronger in single xylem vessels with antibody LM6 and around xylem vessels with antibody LM7, and, slightly, antibody LM6. Antibodies JIM5 and JIM7 stained only few vessels in both genotypes while no labeling occurred in other tissues of healthy plants. No labeling or no differences between genotypes were observed in the other evaluated stem tissue components.

Labeling of single vessels increased more than threefold and was generally intense in reactivities of LM2 and LM5 antibodies after infection in the inoculated susceptible genotype (L390). After inoculation, labeling of single vessels with antibodies LM2, LM5, LM6 and LM7, and, stronger around vessels with all the antibodies, increased in the susceptible genotype indicating an increase in the epitope binding recognizing the AGPs, galactan and arabinan side chains of RG I and non-blockwise deesterification pattern of homogalacturonan (HG), respectively.

Different than in comparison of genotypes, a reaction was observed around vessels with JIM5 and JIM7 after inoculation in the susceptible genotype. The JIM 5 binding

polymers appeared as irregular aggregates in xylem parenchyma and some vessels of the susceptible genotype after infection.

The resistant genotype (H7996) showed no reaction after inoculation around vessels and in the xylem parenchyma, but the labeling in xylem vessels showed a significant increase in the binding of arabinan and galactan epitopes of branched RG I.

The controls, where primary antibodies were omitted, showed a lack of fluorescence in all the tissues but slight autofluorescence was observed around vessesls.

Labeling to detect arabinogalactan-proteins with ß–glucosyl Yariv reagent resulted in typical deep red staining of the outer epidermis and vascular bundle, in particular, the outer epidermal wall and xylem elements. The staining patterns of both healthy and inoculated resistant and susceptible genotypes were similar.

CHAPTER IV

Chemical composition of lipopolysaccharides from *Ralstonia solanacearum* and their interactions with cell wall pectins from tomato stems

4.1 INTRODUCTION

The pseudomonads, like all Gram-negative bacterial cells contain complex polymers called lipopolysaccharides (LPS) as their outermost layer. Besides providing an impermeable barrier against entry of harmful substances, LPS also interact with eukaryotic hosts (Newman and Erbs, 2003). In plants, LPS appear to interact with the cell wall by direct contact of the bacteria or by release of micelles containing LPS of the bacterial cell surface (Rudolph, 2001). Several data indicate that the LPS play a role in pathogenesis in susceptible hosts and in resistance induction in incompatible hosts (Laux *et al.,* 1996; Müller *et al.,* 1996). The effects of LPS on plant cells have recently been reviewed by Dow *et al.* (2000).

The LPS have a "three in one" type of architecture, consisting of Lipid A, an oligosaccharide core region and a chain of repeating sugars or oligosaccharide units called O-chains or O-antigen (Fig 4.1). Gram-negative bacteria that are mutated and lack O-chains are referred to as rough forms because of their appearance on agar plates; they are usually avirulent in nature. The genes for the synthesis of LPS are distributed throughout the bacterial chromosome and have been well characterized in *Ralstonia solanacearum* (Kao and Sequeria, 1991), the causal agent of bacterial wilt in over 200 host species (Yabuuchi *et al*., 1995). Several LPS genes are clustered in loci, the predominant cluster has been shown to be 6.5kb in length.

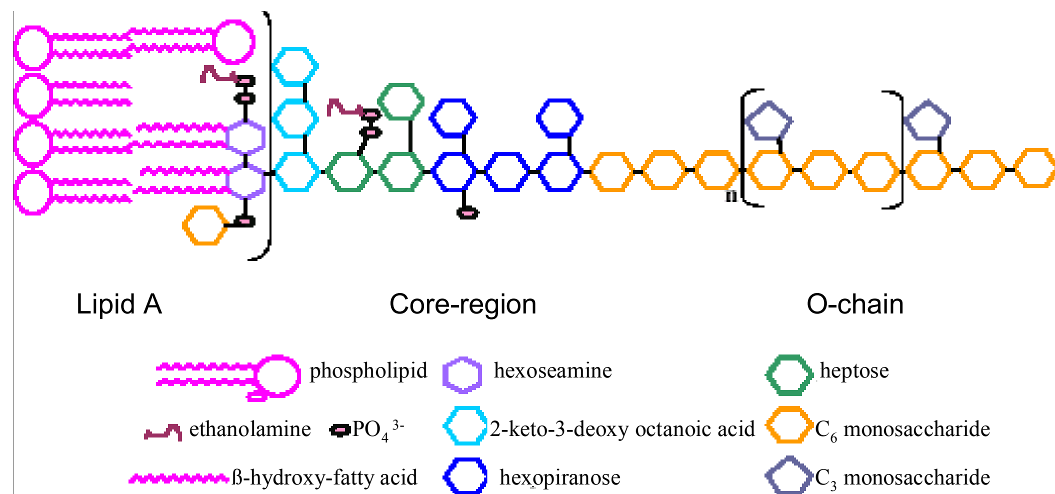


Fig 4.1 General structure of bacterial lipopolysaccharides (Luderitz *et al.,* 1982)

The LPS molecules are anionic due to the presence of phospholipid groups and hence can bind to charged compounds e.g. pectins from hosts to form aggregates. The interaction of bacterial LPS with plant pectins has been studied in several host-pathogen combinations (Wydra, 1991; Grolms, 1996; Liehe, 1998; Laux, 1998; Venkatesh, 2002). The degree of interaction is influenced by various factors such as temperature, pH, and the presence of divalent cations as calcium or magnesium.

In these studies, bacterial LPS and plant pectins mixed *in vitro* exhibited two types of rheological behaviour, synergistic and non-synergistic interaction. A synergistic interaction was almost always synonymous with a compatible combination while no synergistic interaction accompanied an incompatible combination of host and pathogen. A synergistic interaction *in vitro* can be directly related to conducive conditions *in vivo* in

which the bacteria can multiply (Laux *et al.,* 1996; Grolms and Rudolph, 1997). Thus, it seems possible that the bacterial LPS and plant pectins are involved in specific host-pathogen interactions. Graham (1983) suggested that the O-specific sugar moieties of LPS play a vital role in recognition by binding to a specific receptor on the host cell. Results of a study by Cody and Gross (1987) also suggest that these biopolymers indeed play a governing role in early pathogenesis. The host specificity of the *Pseudomonas* pathovars was suggested to be due to the specific structure of the LPS molecule, mainly the O-chain (Rudolph, 2001).

LPS is thought to contribute to the restrictive membrane permeability properties of the outer membrane, allowing bacterial growth and survival in harsh environments which may include niches within eukaryotic hosts (Newman *et al.,* 2000, 2001, 2003). It is not known whether direct contact is involved in LPS-mediated effects, but it has recently been shown that a network of functions required for virulence of *R. solanacearum* is activated by contact with plant cells (Aldon *et al.,* 2000). Moreover, the outermost portion of the LPS molecule, the Oantigen chain, is not the active moiety in triggering plant responses. A significant fraction of LPS may be released from bacteria as micelles or blebs during growth (Beveridge, 1999) and this may be the form in which LPS interacts with eukaryotic cells. The most studied effect of LPS or LPS protein complexes on plant cells is its ability to prevent the hypersensitive response (HR) induced in plants by avirulent bacteria. Newman *et al.* (2001, 2003) studied this effect of LPS by infiltration of heat-killed *R. solanacearum* bacteria into leaves of tobacco which delayed or prevented the appearance of disease symptoms or the HR when the leaves were subsequently inoculated with live bacteria in compatible or incompatible interactions. The activity responsible for the prevention of HR was subsequently shown to reside in the LPS of *R. solanacearum*, specifically in the lipid A- core structure.

In contrast, several reports have described defence-related responses induced by LPS treatment of plants. *R. solanacearum* LPS induced a polypeptide of unknown function and also soluble peroxidase activity (Leach *et al.,* 1983). LPS of *Xanthomonas campestris* induced ß-1, 3-glucanase in *Brassica sp.* (Newman *et al.,* 1995). Other

changes induced by LPS include induction of antimicrobial activity (Rathmell and Sequeira, 1975) and changes in plant cell wall ultrastructure (Graham *et al.,* 1977). In addition to direct effects of LPS on gene expression, LPS pre-treatment of pepper leaves altered patterns of gene expression induced by subsequent challenge with bacteria. Genes encoding the PR-proteins and basic ß-1,3-glucanase were not induced by *Salmonella minnesota* LPS, but *X. campestris* LPS gave weak, transient expression (Newman *et al.,* 2000). However pre-treatment of pepper leaves with LPS from either source caused marked changes in degree of expression following subsequent challenge with *X. c. campestris* and *X. c. vesicatoria* (Newman *et al.,* 2000, 2001). LPS is suggested to have sensitized the leaf tissue so that it reacted more rapidly and strongly to bacterial challenge.

Bacterial wilt caused by *R. solanacearum* which is a highly variable species comprising five biovars and five races (Hayward, 1991). Race 1 strains occur in tropical areas and are highly diverse, as demonstrated by their wide host range including solanaceous crops. A population of *R. solanacearum* race 1 from tomato was analyzed for aggressiveness, idnetifying Taiwanese strains Pss 190 as most virulent on tomato and Pss 216 as the least virulent (Jaunet and Wang, 1999).

Rheology is the science of deformation and flow of matter and involves the study of the manner in which materials respond to applied stress or strain. The word owes its origin to the greek words *'panta rhei'* meaning *'everything flows'*. Rheology is a useful tool to study the physical properties of matter and has immediate applications in various fields such as geology, soil mechanics, polymer industries, bioengineering, cosmetics, pharmaceutics and in food industry.

The ability of a polymer to form a gel depends on the molecular weight of the polymer and the nature of intermolecular interactions. These interactions have been broadly classified as Newtonian or non-Newtonian. By definition, Newtonian interactions have a straight line relation between the shear stress (*the ratio of force to area gives a shear stress across the liquid and is usually expressed in Pascal, N/m2*) and shear rate (*the*

velocity gradient which forms between the two surfaces gives a shear rate) with a zero intercept ($\tau = \eta \times D$, where η = viscosity, τ = torque, D = shear rate). All fluids that do not obey the above rule are known as non-Newtonian fluids ($\eta = \tau/D$). The equations that relate the stress and strain are called rheological equations.

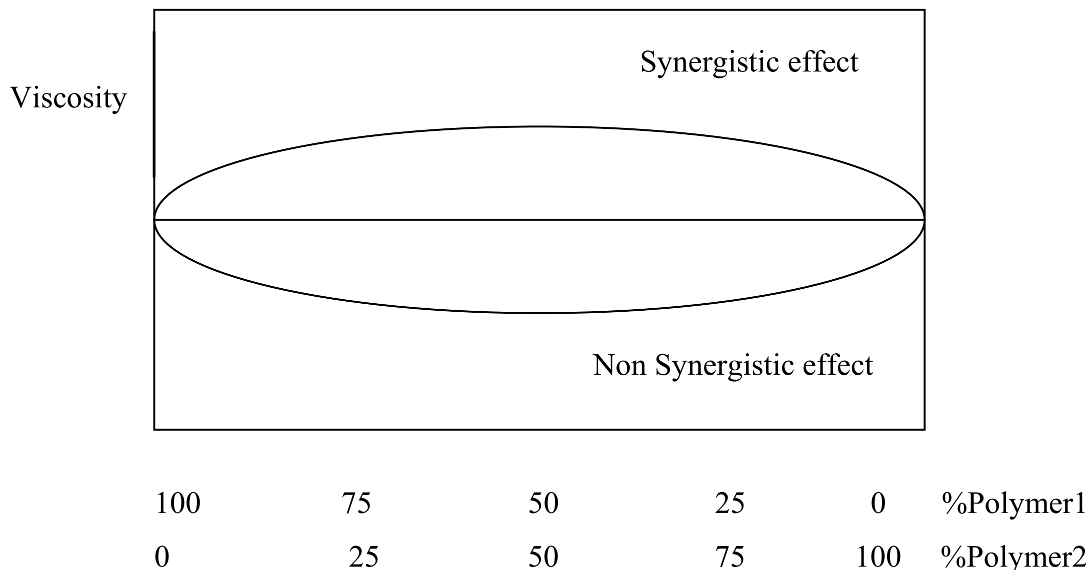


Fig 4.2 Theoretic curve showing mixture of two polymers: synergistic and nonsynergistic effects.

In synergistic interactions, mixing of polymers results in gels characterized by high rigidity and superior to that which would be expected from a linear combination of the rigidities of the gels formed by each individual polymeric component. Also, the addition of a small amount of a non-gelling polymer to a gelling one may induce a strengthening of the resulting gel or even, some polymers that are individually non-gelling can yield gels on mixing. Many mixed systems of polysaccharides show this highly specific non-additive behaviour, which is currently termed synergism (Copetti *et al.,* 1997).

But, such synergistic interactions occur rarely in nature, although several cases have been reported such as oil spill in marine life and plant viral synergism (Vance *et al.,* 1995). Such intermolecular interactions are consequences of chemical, physical or thermodynamic factors and the interaction is a function of the different primary and secondary structures of the component chains in the polymer system. Cairns *et al.* (1997) classified the synergistic interactions between polysaccharides as single polymer network containing a second polymer, interpenetrating networks, phase-separated network, or coupled network, depending on the kind of polymer network that results from the interactions.

In most cases studied, biological polymers such as LPS and plant pectins from different sources show no synergistic interaction or exclusion. Only in very rare cases the interaction between polymers of different origin is synergistic, leading to an increase in viscosity when two polymers are mixed.

Studies by Wydra (1991) revealed an interaction between LPS of *Pseudomonas syringae* pv. *phaseolicola* and bush bean agglutinin, a pectic substance, resulting in an increased viscosity and yield stress when combining components of the compatible interaction *in vitro*. Grolms (1996) and Laux (1998) demonstrated that the bacterial LPS in general and their O-chains in particular bind to pectins from susceptible cultivars in several host-pathogen systems. The molecular interactions of the compatible host-pathogen combination were always accompanied by an increase in viscosity and yield stress characteristic of a synergistic effect. An antagonistic phenomenon observed in incompatible combinations resulted in no synergistic effect or exclusion.

To investigate possible interactions in the host-pathogen system *R. solanacearum* and tomato, in the present studies partially purified LPS preparations were obtained from strains *R. solanacearum* race 1, biovar 3 and partly characterized biochemically. Additionally, the interaction of these LPS-preparations with extracted pectins from stems of tomato was studied.

Thus, whenever a synergistic interaction between bacterial LPS and plant pectins was recorded, the pectins originated from **leaves** of a plant that was susceptible to the bacteria as reported for *P. syringae* pv. *tomato* (Venkatesh, 2002). Pectins in these systems have never been extracted from stems, which are the primary sites of multiplication of *R. solanacearum* in tomato and of expression of resistance against this pathogen (Grimault *et al.,* 1993, 1994). To further verify the hypothesis on involvement of LPS and pectin in the host-pathogen interaction we selected the *R. solanacearum* strain ToUdk2 race 1, biovar 3 from Thailand, characterized its LPS partly by chemical analysis and studied the rheological interactions of the partially purified LPS with pectins from both susceptible and resistant genotypes of tomato.

4.2 MATERIALS AND METHODS

4.2.1 Growth media used in bacterial cultures

The following media were routinely used for the growth and maintenance of the bacterial cultures. The composition of the media is given below.

Nutrient broth

Ingredient	Concentration
Yeast extract	1g
Bacto peptone	10g
Casamino acid	1g
Glucose	10g
dH_2O	ad 1000ml

Nutrient Glucose Agar (NGA)

Ingredient	Concentration
Beef extract	3g
Bacto peptone	5g
D-Glucose	2.5g
Agar	15g
dH_2O	ad 1000ml

4.2.2 Extraction of lipopolyssaccharides from strains of *R. solanacearum* (Westphal and Jann, 1965)

LPS was extracted from *R. solanacearum* strains Pss190 and Pss216 obtained from AVRDC, Taiwan (Wang and Lin, 2002) highly and lowly virulent on tomato, respectively, and Pe104 and ToUdk2 obtained from Thailand (Thaveechai, Kasetsart University, Bangkok), with moderate and high virulence, respectively (Leykun, 2003). Hundred litres of nutrient broth supplemented with 1% (w/v) proteose peptone (see above) were inoculated with 3 L of preculture of strain Pss190 in a 100 L fermenter (Model U 100, Bauner + Diesel GmbH, Germany) maintained at 28 °C under permanent stirring and aerobic conditions. For the production of preculture bacterial cultures were initiated from single colonies grown on NGA agar medium and subsequently transferred to 100 ml of nutrient broth. After 24 h of growth at 28 °C under stirring, 1 ml of the culture was transferred to 3 L of nutrient broth to serve as the preculture. This production was conducted at the Institut für Mikrobiologie und Genetik, Universität Göttingen, laboratory of Prof. W. Liebl. The cells were harvested at mid-logarithmic phase after 30 h incubation at 28 °C (Fig 4.2.2) by centrifugation at 12,000 x g for 15 min. The pellets were suspended by swirling in a solution containing 0.1% (w/v) NaCl and 10mM EDTA, pH 7.0, and centrifuged at 10,000 x g for 20 min at 4 °C. Strain Pss190 was additionally produced in 10 L batch cultures (designated as Pss190-WW) at the Institut für Mikrobiologie, Universität Hannover, laboratory of Prof. Aulich. Strain Pss216 and strains Pe104 and ToUdk2 were produced in a 10 L fermenter and in 1 L batch cultures, respectively. In all extractions, the washing steps were repeated at least five times to remove the adsorbed exopolysaccharides (EPS) from the bacterial pellets. Cultures harvested from the 10 L fermenter and the 1 L batch cultures were centrifuged at 6,000 x g (Sorvall RC-5B refrigerated centrifuge, Sorvall, Germany). Additionally, strain Pss190 produced in 20 L batch culture was washed only with distilled water or with EDTA (see above) to study the influence of differences in washings on LPS composition. Then the washed pellets were lyophilized. From the fermenter cultures and from other batch cultures about 10-40 mg/l pure LPS (see below) were received.

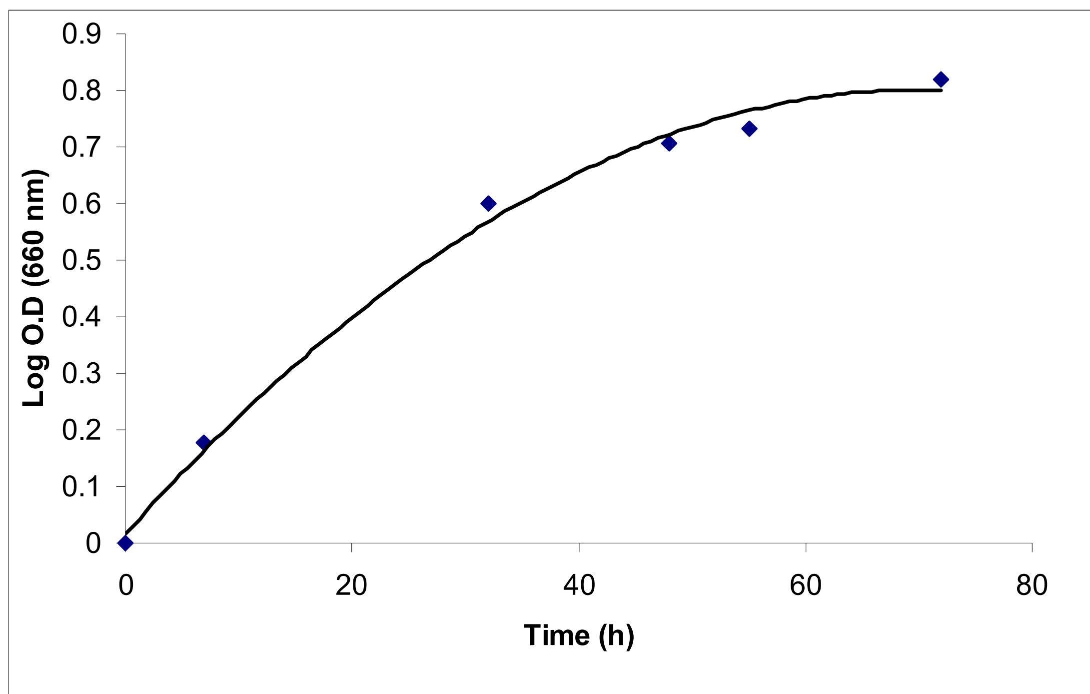
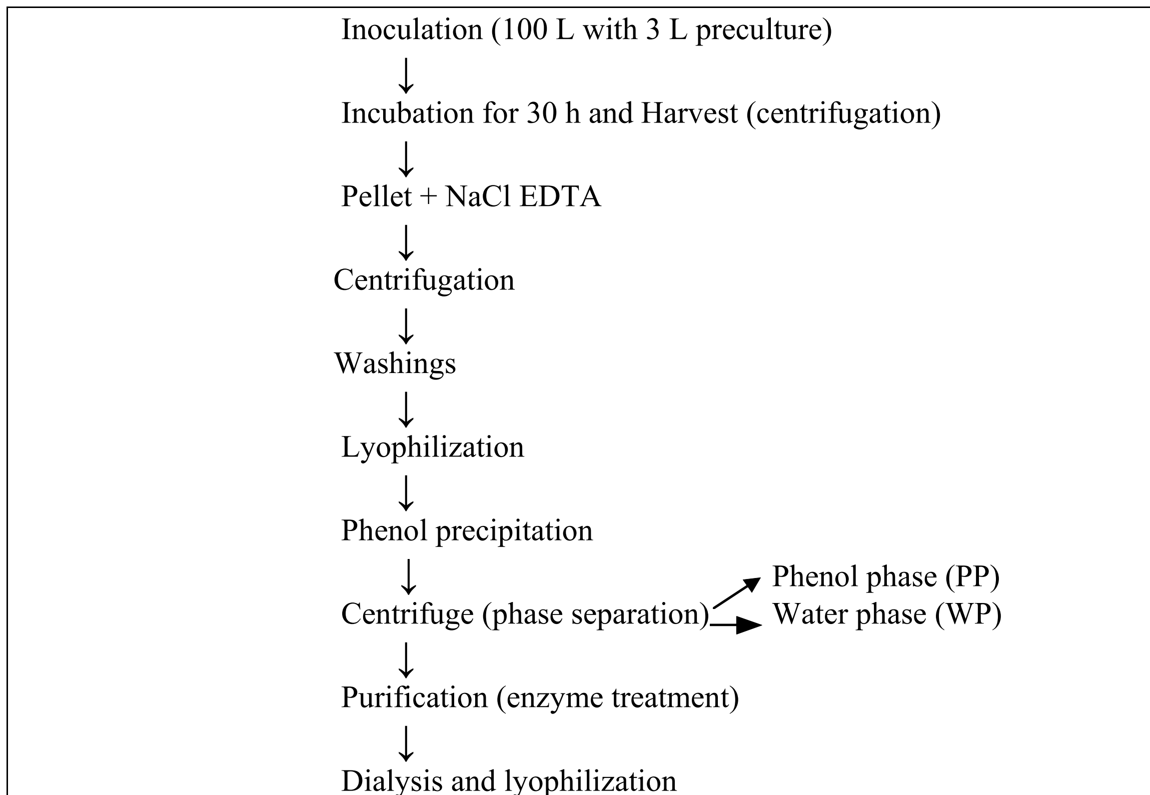


Fig 4.2.2 Growth curve of *R. solanacearum* strain Pss190 in 1 L batch cultures at 28 °C. Bacterial cultures were harvested at the mid-logarhythmic phase after 30 h incubation.

LPS were extracted from the lyophilized bacterial pellets following the method as described by Westphal and Jann (1965). The lyophilized cells were suspended in water (15 ml/g dry weight). The slurry was warmed to 68 °C and mixed with an equal volume of 90 % (v/v) pre-warmed phenol (60 °C). The mixture was incubated at 68 °C in a water bath for 15 min with frequent stirring and cooled to 4 °C before centrifugation at 10,000 x g for 20 min for phase separation (phenol and water phases). The upper aqueous phase containing LPS, polysaccharides, RNA and salts was carefully siphoned off and transferred to a sterile flask, and the lower organic phase containing proteins, lipids, phospholipids and DNA was dialyzed and then lyophilized.



Scheme for the extraction of bacterial lipopolysaccharides (Westphal and Jann, 1965)

Purification of LPS

The lyophilized crude LPS were dissolved in sterile water (5 mg/ml) and clarified by centrifugation at 10,000 x g for 20 min to remove insoluble materials. Protein and DNA contamination in the supernatant were determined spectrophotometrically at 260 nm and 280 nm, respectively. The aqueous phase was then treated with RNAse (Sigma), proteinase K and DNAse (Sigma) (100 µg/ml each) at 37 °C overnight, followed by dialysis against demineralized water with frequent water change for 72 h at 4 °C to remove salts. The dialyzed sample was recovered by lyophilization. The purified LPS were then characterized biochemically.

4.2.3 Biochemical analysis of LPS

The analyses were conducted in collaboration with U. Zähringer, Zentrum für Medizin und Biowissenschaften, Borstel, Germany. Generally all measurements were repeated three times and data given are means of three replicates.

4.2.3.1 Phosphate analysis

Phosphate was determined by the modified method of Bartlett (1959) as described in Gross (1990). LPS solution (2 mg/ml) and the standard NaH_2PO_4 (1 mM) (Sigma) were taken at different volumes (5, 10, 15, 20 µL) and dried overnight. To this 100 µL of the reagent containing 62.7 ml H_2O, 30.6 ml H_2SO_4, and 6.7 ml 70% $HClO_4$ (Sigma) were added and incubated at 100 °C for 1 h and then at 165 °C for 2 h. The samples were cooled down to room temperature and 1 ml of reagent C (see below) was added and the mixture was incubated at 37 °C for 90 min and samples were read at 820 nm.

Reagent A: 1 ml 1 M Na- Acetate solution (Sigma), 1 ml 2.5 % Ammonium-molybdate solution (Sigma), 7 ml H_2O.

Reagent B: 10 % Ascorbic acid (Sigma)

Reagent C: 9 ml of Reagent A and 1 ml of Reagent B.

4.2.3.2 Analysis of KDO

The keto-deoxy sugar 2-keto-3-deoxy-octonate (KDO) was determined as described by Karkhanis *et al.* (1978). The LPS (2-4 mg/ml) were mixed with two volumes of 0.2 N H_2SO_4, heated for 30 min at 100 °C and centrifuged at 10,000 x g for 10 min. Five hundred µL of the supernatant were transferred to a clean test tube into which 250 µL of HIO_4 [0.04M HIO_4 in 0.125%(v/v) H_2SO_4] (Sigma) were mixed. After 20 min of incubation at 25 °C, 250 µL of $NaAsO_2$ (Sigma) were added (2.6% $NaAsO_2$ in 0.5 N

HCl). Immediately after the disappearance of brown color, 500 µL of TBA (0.6% thiobarbituric acid, dissolved in hot water) were mixed and the sample was incubated for 15 min at 100 °C. While hot, 1ml of DMSO (Sigma) was added, and after cooling the optical density (OD) was read at 548 nm in a photometer at Zentrum für Medizin und Biowissenschaften, Borstel, Germany.

4.2.3.3 Analysis of heptoses

Heptoses were determined following the method of Wright and Rebers (1972). To 0.5 ml of LPS (2 mg/ml), 4.5 ml of H_2SO_4 (1 vol. H_2O plus 6 vol. conc. H_2SO_4) were added on an ice bath. The sample was first incubated for 3 to 10 min at 0 °C and again for 3 min at 25 °C. One hundred microliter of freshly prepared L-cysteine-HCl [3% (w/v) in water, were admixed and the sample was heated for 20 min at 100 °C. After incubating for 1 h at 25 °C, the absorbencies at 505 nm and 545 nm were read in a photometer. The difference in the absorptions (505 minus 545 nm) was used to quantify heptoses.

4.2.3.4 Analysis of fatty acids

Fatty acids were analyzed according to the method described by Smith *et al.* (1985). The LPS (2 mg/ml) preparations were hydrolyzed with a 1.5 % solution of acetyl chloride in methanol (100°C, 4 h) in sealed ampoules and analyzed on a GC 3400 gas chromatograph (Varian) combined with an ITD 800 mass spectrometer (Finnigan, Germany) and an IBM PC AT computer; a quartz capillary column packed with OV-1701 (0.25 µm) was used. The temperature program started at 125 °C for 1 min and then increased at 7 °C/min to 275 °C, where it was held for 10 min. Identification of fatty acids was performed using a computer database of standard spectra from the National Bureau of Standards of the United States.

4.2.3.5 Analysis of sugars and amino acids

LPS (2 mg/ml) were hydrolyzed with 2 M 1-2 N trifluoroacetic acid (4 h/100 °C) and the alditol acetates of the carbohydrates were analyzed by gas liquid chromatography (GLC) (Gross, 1990). Quantification of amino compounds was performed on an automated amino acid analyzer Kontron Chromakon 500 equipped with a Kontron Anacomp 220 computer, after acid hydrolysis using 8M HCl at 100 °C at overnight incubation.

4.2.3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

LPS were separated on a 1 mm thick 10% polyacrylamide gel using a minigel apparatus (10 x 10 cm, Biometra Co., Germany).

Buffers, chemicals and reagents used for SDS-PAGE

Acrylamide stock	30% (w/v) acrylamide
	0.8% (w/v) bisacrylamide
3X separating gel buffer	1M Tris- HCl, pH 8.8
	0.3% (w/v) SDS
3X stacking gel buffer	1M Tris-HCl, pH 6.8
	0.3% (w/v) SDS
TEMED	10% (w/v)
APS	10% (w/v)
Denaturing buffer (5X)	62.5 mM Tris-HCl, pH 6.8
	2% (w/v) SDS
	10% (v/v) glycerol
	0.002% (w/v) bromophenolblue
Electrode buffer (10X)	400mM Tris pH 8.3
	600mM glycine
	1% (w/v) SDS

Table 4.2.3.6 Formulation for preparing a 1mm-thick acrylamide gel

Ingredients	Separating gel (10%)	Stacking gel (3%)
Acrylamide stock solution	2.0 ml	250.0 µl
Separating gel buffer	2.0 ml	-
Stacking gel buffer	-	500.0µl
TEMED	100.0 µl	50.0µl
APS	15.0 µl	15.0 µl
Double-distilled water	3.0 ml	1.75 ml

Procedure for preparing the polyacrylamide gel

The glass plates were cleaned with 70% ethanol and air-dried. A polymerization cassette was made using the two glass plates. All the constituents for the separating gel except ammoniumpersulphate (APS) and TEMED were added in a 10ml side arm Erlenmeyer flask. The flask was sealed with a rubber stopper and de-aerated. APS and TEMED were added and the mixture was dispended between the sandwiched glass plates, overlaid with water-saturated iso-butanol or water and allowed to polymerize. A 3% stacking gel was prepared as described above and layered over the polymerized separating gel. A comb was fixed and the gel was allowed to set for at least 1 h.

Preparation of LPS samples

Aqueous suspensions of LPS (0.2-0.4 mg/ml) derived from the strain Pss 190 were mixed with one fifth volume of 5 X denaturing buffer (to a final concentration of 1 X) and incubated at 65 °C for 75 min under stirring.

Electrophoresis conditions

Ten microliters containing 2-4 µg LPS were loaded per well, and the gel was subjected to electrophoresis at 8 V for 30 min (stacking phase) and at 100 V for 1 h for separation.

Staining

Following electrophoresis, the LPS were detected on the gel by silver staining as described by Heukeshoven and Dernick (1988).

Steps	Solutions
1. Fixing (2 h)	30% (v/v) ethanol
	10 % (v/v) acetic acid
2. Incubation (2 h)	30% (v/v) ethanol
	0.5 % (w/v) sodium acetate
	0.5% (v/v) glutaraldehyde
	0.2% (w/v) sodium thiosulphate
3. Washing (H_2O, 3 x 10 min)	double distilled water
4. Staining (45min)	0.1% (w/v) $AgNO_3$
	0.01% (v/v) formaldehyde
5. Developing (3-10 min)	2.5% (w/v) Na_2CO_3
6. Stopping (5-10 min)	500mM EDTA
7. Washing (3 x 10 min)	double distilled water

4.2.4 Rheological interactions between plant pectins/ bacterial LPS

Lipopolysaccharides were extracted from *R. solanacearum* strain ToUdk2 as described under section (4.2.2). Pectins were obtained from stems of susceptible (L390) and resistant (H7996) genotypes of tomato (section 1.2.2). Ten milligrams and 50 mg of pectins and 5 mg of LPS were dissolved each in 1 ml of demineralized water separately. Plastic viscosity and shear stress of the solutions were measured separately and later in mixtures for combinations of different quantities of LPS with pectin of tomato genotype L390 (susceptible) or pectin of tomato genotype H7996 (resistant): 80:20, 60:40, 40:60, 20:80 in a total volume of 500 μL. Pectins (10 mg and 50 mg) extracted from stems in triplicate from each genotype were mixed with LPS (5 mg) and each combination was run on a viscometer thrice.

Equipment used

Rheological experiments were conducted using a rotation viscometer (Brookfield Model DV-III, Karlsruhe, Germany) with a CP 4/40 cone plate according to the manufacturer's instructions. The rheometer was calibrated initially using the Brookfield standard oil for 70 cycles. The viscosity measurements were averaged over 40 seconds for 70 cycles in total. The temperature was maintained by means of a circulating water bath at 21 °C during the measurements.

Mathematical calculations

Plastic viscosity and shear stress were measured and yield stress (τ_0) and consistency index (k) were calculated using Bingham's and Power's equations using the software WinGather V1.1. provided by Brookfield Engineering Laboratories Inc., Middleboro, USA. All rheological data were processed using the Microsoft Xact 6.0 computer program for graphics.

Bingham's equation

$$\tau = \tau_0 + \eta D$$

τ = shear stress, τ_0 = yield stress (shear stress at zero shear rate),

η = plastic viscosity, D = shear rate

Power's equation

$$\tau = k\,D^n$$

τ = shear stress, D = shear rate, k = consistency index, n = flow index

4.3 RESULTS

4.3.1 Biochemical analysis of LPS from *R. solanacearum*

LPS were extracted from strains Pss 190, Pss 216, Pe 104 and Toudk2. LPS extracts from the water phase and the phenol phase from all above *R. solanacearum* strains and additionally the treatments of strain Pss190 water washed (Pss190-WW) and EDTA washed (Pss190-EW) were treated with RNAase, DNAase and Proteinase K to further purify the LPS before their chemical composition was analyzed. The analysis of strains Pss190-EW washed with EDTA solution and Pss190-WW which was washed with distilled water did not show clear differences (table 4.3.1). Similarly, the composition of LPS from the lowly virulent strain Pss 216 and the moderately virulent strain Pe 104 produced in 10 L and 1 L batch cultures, respectively, purified LPS was obtained. The LPS of the Thai strain To-Udk2 was used in our plant inoculation studies (see below). The 1 L batch cultures of To-Udk2 also produced pure LPS which were further characterized biochemically.

The LPS extracts from strains Pss190-EW and Pss190-WW did not show clear differences in the chemical composition (table 4.3.1). In the water phase extracts, which normally contain the major part of the extracted LPS, the total phosphate content which is a part of the Lipid A and the core region, was 124-206 nmol/mg of LPS across strains. About 40-80 nmol/mg of keto-deoxy sugar (KDO) which is typical for the core region, were obtained. The heptose content, another component typical for the core region was determined in both the water and phenol phases with 38-137 nmol/mg of LPS.

The monosaccharides rhamnose, glucose, arabinose are typical components of the O-chain. Among them, rhamnose was the most dominant, followed by glucose, with 904-1299 nmol/mg of rhamnose in the water phase and 149-651 nmol/mg in the phenol phase, and 55-247 nmol/mg LPS of glucose in the phenol phase across treatments and strains.

A dimer of acetylated amino sugars N-acetylglucosamine/mannosamine was generally detected in both phases in high quantity, while ethanolamine phosphate was detected in high quantity in the water phase and lower quantity in the phenol phase and N-acetylgalactosamine was generally not detected or in some cases in small quantities. These amino sugars typical for the LPS were observed in all our *R. solanacearum* strains. The analysis of fatty acids which constitute the Lipid A region of LPS showed the presence of tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), 9-octadecanoic acid (C18:1), and 3-hydroxy-tetradecanoic acid (C3OH-14:0) in water and phenol phases across strains and treatments.

The amino acids alanine and glycine typical for the core region of LPS were present in both the phases at 83-204 nmol/mg, and 39-83 nmol/mg, respectively, in the water phase and 345-611 nmol/mg and 91-187 nmol/mg, respectively, in the phenol phase of LPS in all the strains of *R. solanacearum*.

The biochemical analysis revealed the typical composition of LPS in the *R. solanacearum* strains without major differences among them.

Table 4.3.1 Chemical composition of the lipopolysaccharides from *R. solanacearum* strains Pss190-WW, Pss190-EW, Pss216 and Pe104 obtained from the water phase (WP) and phenol phase (PP) after purification with enzymes (DNAase, RNAase, and Proteinase K). KDO: 3-deoxy-D-manno-octulosonic acid, $PO4^{3-}$: phosphate, C14:0: tetradecanoic acid, C3OH-14:0: 3-hydroxy-tetradecanoic acid, C16:0: hexadecanoic acid, C18:1: 9-octadecanoic acid, GalN: N-acetylgalactosamine, Etn: ethanolamine phosphate, GlcN/ManN: N-acetylglucosamine / N-acetylmannosamine.

nmol/mg	**Pss190-WW**		**Pss190-EW**		**Pss216**		**Pe104**	
	WP	**PP**	**WP**	**PP**	**WP**	**PP**	**WP**	**PP**
KDO	76	-	52	-	40	-	56	-
HEXN	114	-	240	-	190	-	105	-
PHOSPHATES	184	-	206	-	148	-	124	-
NEUTRAL SUGARS								
Rhamnose	960	490	938	149	1299	651	904	365
Arabinose	0	22	0	0	0	27	0	0
Glc	94	86	202	247	95	65	202	55
Heptose	135	137	105	-	118	81	101	38
FATTY ACIDS								
C14:0	35	69	49	-	68	46	37	40
C3OH-14:0	86	176	110	-	157	121	99	103
C16:0	3	21	5	4	5	16	3	12
C18:1	0	11	7	0	6	13	8	9
C2OH-14:0	7	13	9	0	13	9	8	9
AMINO SUGARS								
GalN	0	12	42	47	0	0	0	0
EtN	443	20	199	16	113	21	720	6
GlcN+ManN	403	387	544	202	590	320	535	75

[1] Pss 190-WW = *R. solanacearum* strain Pss190 water washed, Pss 190-EW = EDTA washed
[2] WP = water phase, PP = phenol phase
[3] not determined

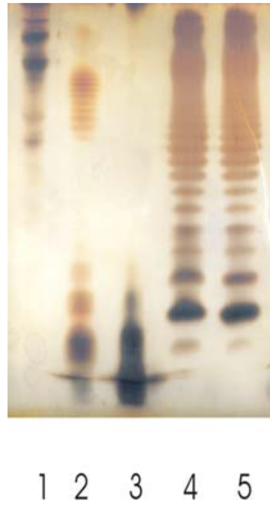


Fig 4.3.3 LPS was extracted by the hot phenol-water method of Westphal and Jann (1965) and resolved on a 10% polyacrylamide gel and visualized by silver staining.
Lane 1: protein standard Serva P4 (2µg), Lane 2: *E. coli* O111 LPS, Sigma (2µg), Lane 3: *E. coli* F515 LPS SB 111,66 (1µg), Lanes 4 & 5: *R. solanacearum* LPS strains Pss190 (3µg).

The LPS extracted from *R. solanacearum* strains Pss190, Pss216 and Pe104 were characterized by SDS-PAGE. After staining with silver a characteristic ladder-like pattern (Fig 4.3.3) was obtained. The ladder-like part represents the O-antigen region. The form of LPS as revealed by gel electrophoresis was the smooth (S) type.

The highest mobility bands of LPS from *Rs* (lanes 4 & 5) differed from those of the LPS standards used (lanes 1, 2 & 3). For all the strains similar ladder-like patterns were observed.

4.3.2 Interaction studies between bacterial LPS and plant pectins

Interactions between bacterial LPS and plant pectins were measured as changes in viscosity and shear stress in a cone plate viscometer for minimal quantities (<500 µl) at the concentration of 10 mg/ml and 50 mg/ml (pectins) and 5 mg/ml LPS.

No synergistic effects indicated by increased viscosity were recorded in various mixtures of lipopolysaccharides of *R. solanacearum* strain ToUdk2 and pectins from stems of susceptible genotype L390 (data not shown), nor were any significant changes in the viscosity measurements observed for any combination of *R. solanacearum* LPS with pectins from the resistant genotype H9776 (Fig 4.3.4). The figures obtained were similar for both resistant and susceptible genotypes hence only resistant H7996 is shown in the below given fig.

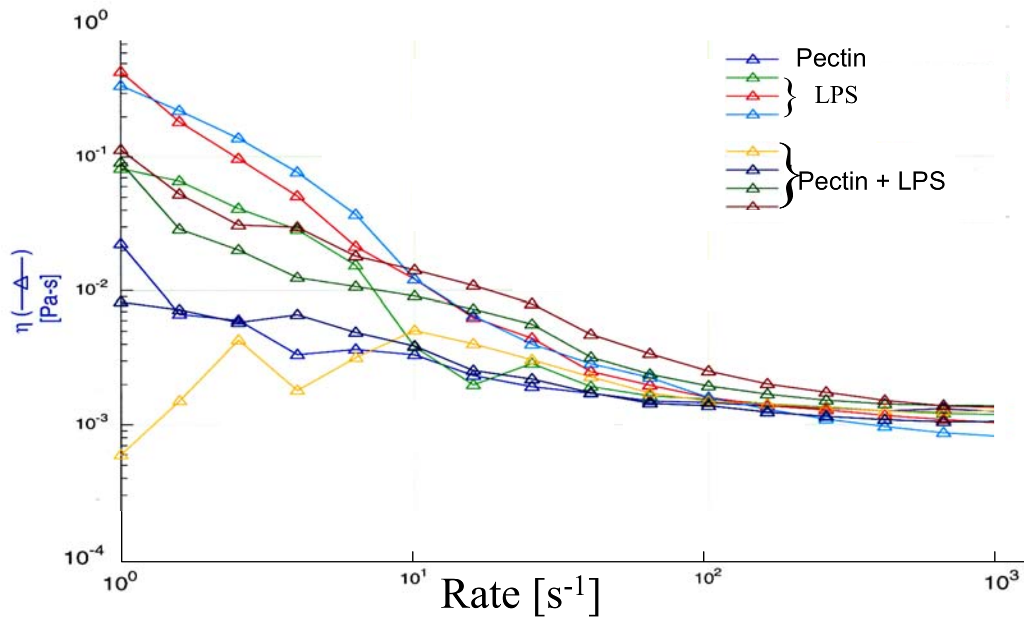


Fig 4.3.4 Rheological properties measured as increase in viscosity in mixtures of LPS from *R. solanacearum* strain ToUdk2 (5 mg/ml) and pectins (50 mg pectin/ml) from resistant (H7996) genotype of tomato measured in a cone-plate rheometer. No synergistic (gel formation) nor inhibitory interaction was observed in mixtures. Blue curve: pectin from L390, Light green, red, light blue curves: LPS (repeated three times), Mixtures: LPS:pectin (L390) yellow curve= 80:20, dark blue= 60:40, green= 40:60, brown= 20:80

4.4 DISCUSSION

The typical components of the LPS core from pseudomonads such as glucose, rhamnose, heptoses, glucosamine, galactosamine, 2-keto 3-deoxy-D manno-octonic acid (KDO), alanine, glycine and phosphate were detected in the LPS from *R. solanacearum* by biochemical analysis. The presence of rhamnose was also observed in the phenol phase indicating that the LPS was also present in the phenol phase (PP). The presence of high amounts of rhamnose is in line with the observations in phytopathogenic strains of *Pseudomonas* pvs. in which the predominant hexose sugar is rhamnose (Basu, 1991; Laux, 1998; Müller, 1998; Janson, 1999; Verbanets *et al.,* 2003). The core also contained rare components such as galactose, ribose, and arabinose as also observed by Varbanets *et al.* (2003) in *R. solanacearum* strains. Galactose was previously revealed in the core oligosaccharides of the LPS from *Salmonella enterica* and *E. coli* strains whereas arabinose was found in the core oligosaccharides from *P. mirabilis* (Holst, 1999). KDO and heptose contents are routinely tested in LPS analysis since these compounds serve as valuable markers for LPS.

The lipid A part of *Ralstonia solanacearum* is highly phosphorylated. Also strains of *P. s.* pv. *tomato* were reported to contain highly phosphorylated lipid A cores (Das *et al,* 1994). A possible role of the degree of phosphorylation of LPS in modulating the function of the outer membrane as a permeability barrier has been discussed (Ray *et al.,* 1994).

The detection of dimers of galactosamine and mannosamine in the water and the phenol phases are suggested to be the result of the incomplete separations of the LPS in the phenol extraction, probably due to incomplete phase separation through contamination of the fractions with extracellular polysaccharides and proteins. The tetradecanoic acid, hexadecanoic and octadecanoic acids of the lipid A fraction was reported to be a distinguishing feature of *R. solanacearum* strains (Varbanets *et al.,* 2003). The profile of 3-hydroxy acids in lipid A fraction of *R. solanacearum* can serve as an additional chemotaxonomic criterion for the elucidation of the phylogenetic relationship between

microorganisms (Stead, 1992). Also in other studies with *Pseudomonas syringae* strains decanoic and dodecanoic acids were observed (Venkatesh, 2002). The component analyses of the hydrophobic lipid A of *Pseudomonas syringae* pv. *apii* showed the presence of the 2-hydroxy-dodecanoic acid, 3-hydroxy-decanoic acid, decanoic acid and 3-hydroxy-dodecanoic acid in the ratio of approximately 2:2:1:3. Various authors suggested, that dodecanoic acid is a distinguishing feature of the lipid A fraction for Pseudomonads, such as *P. s.* pv. *apii, P. s*. pv. *tomato* (Müller, 1998), *P. aeruginosa*, *P. acidovorans, P. syncyanea, P. putida* and *P. aminovorans* (Das *et al.* , 1994, Wilkinson, 1977). In *P. s.* pv. *syringae*, *P. s.* pv. *phaseolicola* and *P. s.* pv. *atrofaciens* C16:O, C18:O, C16:1 and C18:O were detected in addition to the four fatty acids mentioned above (Zdorovenko *et al.* , 2001). Nevertheless, differences among the pseudomonads were also observed. Thus, LPS from *P. s.* pv. *apii* seems to possess a fatty acid profile that is similar to that of *P. s.* pv. *tomato* but different from that of *P. s.* pv. *phaseolicola* or *P. s.* pv. *atrofaciens.* Distinctions in the composition of fatty acids were also observed between individual strains belonging to the species *B. cepacia* and *R. solanacearum* (Stead, 1992). But, in our studies we could not detect differences between strains of *R. solanacearum* using these general analysis methods. In the studies performed by Montrozier and Cerantola, (1997) the isolate of *B. cepacia* elaborates two LPS-related polymers with linear and trisaccharide repeating units. Hence, simultaneous production of more than one Oside chain by Gram-negative bacteria is not unusual. This has been also noted for another *Burkholderia* species, *B. pseudomallei* (Knirel *et al*., 1992; Perry *et al.,* 1995) and a number of other organisms like *P. aeruginosa* and also *R. solanacearum* (Kocharova *et al.,* 1993). Another characteristic is the occurrence of the less usual D-isomer of rhamnose as described for *B. cepacia* by Shashkov *et al.,* (1986) which is also known to occur in other organisms such as *E. coli, R. solanacearum* and several pathovars of *Pseudomonas syringae* (Ovod *et al.,* 1996; Knirel *et al.,* 1994). Nevertheless, the L-isomer is also found in O-antigens of other strains of *B. cepacia*.

Alanine, cysteine and glycine have been reported as typical amino acid components of LPS from the pseudomonads (Das *et al.,* 1994). However, the LPS of *R. solanacearum* showed no presence of cysteine.

LPS from *R. solanacearum* were characterized by SDS-PAGE and visualized by silver staining. The electrophoretic behavior of LPS in the presence of SDS reflects its basic structure. The typical ladder-like pattern was observed indicating a smooth (S) type of LPS which has the O-chain. This pattern is due to the heterogeneity of the length of the antigenic side-chain in the LPS molecules (Goldman and Leive, 1980; Palva and Mäkelä, 1980). Also in other Gram-negative bacteria the ladder-like pattern of the O-chain was observed. Thus, Ovod *et al.* (1997) reported that some strains of *P. syringae* have identical O-chain repeats (e.g. *P. s.* pv. *apii* and *P. s.* pv. *tomato* or *P. s.* pv. *cannabina* and *P. s.* pv. *maculicola*). Also Varbanets *et al.* (2003) reported that the structure of the O-PS varies with the strain, and different types of the O-PS structure may occur in one strain.

In *in vitro* interaction studies mixing bacterial LPS with pectins of stems of the susceptible genotype L390 no significant increase in viscosity (data not shown) was observed, and, also, no synergistic interaction nor an inhibitory effect after mixing LPS with pectin from the resistant genotype H9776 (fig: 4.3.4) could be demonstrated. In other host-pathogen systems, synergistic interactions were observed in mixtures of LPS from *P. s.* pv. *tomato* with pectins from leaves of a tomato cultivar susceptible to *P. s.* pv. *tomato* race 0 and race 1 (Grolms, 1996). Synergistic interactions have also been reported in compatible combinations of LPS from *P. s.* pv. *phaseolicola* and pectins from leaves of its susceptible host, the bush bean cv. Red Kidney (Wydra, 1991; Laux, 1998), *P. s.* pv. *coriandricola* and the susceptible coriander cv. Corry (Liehe, 1998), and, more recently, between LPS from *X. axonopodiss* pv. *manihotis* and pectins from young leaves of the susceptible cassava cv. Ben 86052 (Witt, pers. communication).

Although our rheological experiments revealed no interaction between LPS of *R. solanacearum* with pectins from the susceptible nor the resistant tomato genotypes, former studies suggested an interaction of cell wall components of *R. solanacearum* with tobacco cell walls, inducing disease resistance in tobacco whereby bacterial cells became immobilized at their binding site through an envelopment process which involves an active restructuring of the plant cell wall surface (Goodman *et al.,* 1976; Graham *et al.,*

1977). This binding and encapsulating process has not been observed in a susceptible interaction, when the virulent bacteria remain free and multiply in the intercellular space (Anderson and Jasalavich, 1979; Rudolph and Mendgen, 1985). In light of the fact that bacterial LPS bind chemically to plant pectins via H-bonds or Van-der-Waals forces it has been speculated that the viscous gel that results from a compatible combination serves as a reservoir of water where the bacteria can survive, multiply and further the infection process.

No synergistic interactions were in general apparent in compatible nor incompatible combinations. Our results could not support the hypothesis that bacterial LPS are actively involved in interactions with pectic plant cell wall components, although these observations in the mixture of LPS and pectin could be due to low purity of the pectins and the method adopted for extraction. Pectic polymers require a specific length of the side chain for entanglements, too short or too long side chains will inhibit getting entangled (Schols & Voragen, 2003). Our extracted pectins also had too many branches which could be reason for observing no interaction between the two polymers (pectins and LPS). The impurities like EPS during the LPS extraction could also contribute to the non-entanglements of the bacterial and pectic polysaccharides.

4.5 SUMMARY

LPS were extracted from *R. solanacearum* strains Pss190, Pss216, Pe104 and Toudk2. The biochemical analysis revealed the typical composition of LPS in the *R. solanacearum* strains without major differences among them. Comparing LPS extracts from strains which were EDTA-washed and water-washed before extraction, no clear differences in the chemical composition were detected.

The total phosphate content was 124-206 nmol/mg of LPS across strains, and 40-80 nmol/mg of keto-deoxy sugar (KDO) was determined. The heptose content, was determined in both the water and phenol phases with 38-137 nmol/mg of LPS. Among the monosaccharides, rhamnose was the most dominant, followed by glucose, with 904-1299 nmol/mg in the water phase and 149-651 nmol/mg in the phenol phase, and 55-247 nmol/mg LPS of glucose across treatments and strains. The amino sugars N-acetylglucosamine/mannosamine typical for the LPS were observed in all our *R. solanacearum* strains. The analysis of fatty acids showed the presence of *R. solanacearum*-characteristic tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), 9-octadecanoic acid (C18:1), and 3-hydroxy-tetradecanoic acid (C3OH-14:0).

The amino acids alanine and glycine typical for the core region of LPS were present in both the phases at 83-204 nmol/mg, and 39-83 nmol/mg, respectively, in the water phase and 345-611 nmol/mg, 91-187 nmol/mg, respectively, in the phenol phase of LPS in all the strains of *R. solanacearum*.

The LPS were characterized by SDS-PAGE and visualized by the silver staining method. A characteristic ladder-like pattern indicating the presence of smooth type LPS was observed.

No synergistic nor inhibitory effects were recorded in various mixtures of lipopolysaccharides of *R. solanacearum* strain ToUdk2 and pectins from stems of susceptible and resistant host plants, respectively, and hence no interaction observed.

CONCLUSIONS

The present studies elucidate the role of pectic polysaccharides and AGPs from tomato genotypes in resistance to *R. solanacearum*. The data suggest a relationship between susceptibility or resistance to bacterial wilt and the properties of the pectic fractions and AGPs of stems.

Although useful levels of resistance have been obtained in some crop species such as tobacco and groundnut, breeding of tomato lines with stable resistance to *R. solanacearum* has so far met with only limited success. Genetic analysis resulted in the detection of general and specific quantitative trait loci important for resistance. Genetically characterized individuals are available which can now be used to create further lines that lack or possess known combinations of the different resistance loci for molecular or cytological studies. The increased awareness of the complexity and dynamic nature of the pectic network has been largely due to the development of appropriate tools to determine its structural complexity and to dissect this complexity at the cell biological level. Isolation of the cell wall polysaccharides from tomato stems yields a fraction containing almost all polysaccharides present and few other components. These pectic fractions composed of homogalacturonans (HGs) differing in the degree of methylesterification and of rhamnogalacturonans (RG) I, which are rich in galactose and arabinose, which occur as side chains, and RG II, extracted by the chelating agents are usually contaminated with considerable amounts of hemicelluloses. The neutral components of pectin are known to belong to the most variable biological molecules and were therefore chosen for further studies on their involvement in the resistance reaction.

The use of the chelating agent EDTA for extracting pectins from the cell walls led to the removal of only negligible amounts of uronic acids which form the backbone of the homogalacturonan molecule. The low content of uronic acids in the pectic polymers is typical for pectins derived from primary cell walls. The elevated levels of rhamnose indicate the presence of a rhamnogalacturonan, and high levels of arabinan and galactan in our pectins may derive from side chains of RG I, arabinogalactan proteins or galactans

and arabinans, which have been described as branched or hairy regions, probably occurring in covalent association with pectins (Willats *et al*., 1999). Thus, the range of pectic polysaccharides demonstrated by biochemical and immunochemical analysis of stems of tomato genotypes resistant and susceptible to *R. solanacearum* is typical for the presence of highly branched pectins in the tomato stems.

The degree of methylation of HGs was observed to be higher in tomato genotype H7996 resistant to *R. solanacearum* than in the susceptible genotype. It is suggested that the high degree of pectin methylation could be one of the factors contributing to the resistance of genotype H7996 to *R. solanacearum*. Also in other host-pathogen systems such as potato and *Erwinia carotovora* subsp. *atroseptica* and tomato and *Pseudomonas syringae* pv. *tomato* pectins of the resistant genotype were higher methyl-esterified. It is suggested that these genotypes are less easily degraded by pectinolytic enzymes, which cannot act on branched or highly methylated galacturonans (McMillan *et al.,* 1993; Venkatesh, 2002). In a susceptible genotype differences in the esterification degrees and patterns were found due to pathogen activity after infection of tomato with *R. solanacearum* by immuno-histological studies. But, in case that HGs can be degraded by pathogen enzymes, degradation products such as oligomeres of galacturonic acid are also known to elicit defense responses in resistant genotypes. Thus, the degree of pectin methylation can only be one factor among others that control the resistance of a given genotype; accumulation of hydroxyproline rich glycoproteins and production of phytoalexins, lectins and proteinase inhibitors must also be taken into account.

We believe that immunoprofiling complements the repertoire of techniques currently available for pectin analysis and has considerable potential in applications where the rapid analysis of small amounts of material is required. Furthermore, although the assays we have described have been applied specifically to pectic polysaccharides and AGPs, this type of assay could readily be extended to other polysaccharides or macromolecules with the use of antibodies of appropriate specificities. These antibodies are to complex carbohydrates. Serendipity might play a role in antibody selection and can lead to the isolation of antibodies with the specificities of interest. Antibodies can be used in a range

of approach and techniques that can extend to the localization of these epitopes in the plant material (Schols and Voragen, 2003).

Our result showed the differences between the resistant and the susceptible tomato genotypes, and further investigations with genotypes representing near isogenic lines differing in resistance to bacterial wilt were carried out to confirm these preliminary data. Our results also showed that the extracted pectic polysaccharides from both resistant and susceptible genotypes had no influence on *R. solanacearum* to enter the viable but non-culturable (VBNC) state. This does not exclude a possible interaction of pectic polysaccharides with the pathogen in *planta*, causing a change in the bacterial condition.

Tissue prints also showed an increased labeling after inoculation with *R. solanacearum* with the specific antibodies indicating an increase in the pectic polysaccharide epitopes galactan and arabinan in xylem vessels of the resistant genotype and galactan, arabinan and arabinogalactan protein as well as HG with a more homogeneous degree of esterification in the xylem parenchyma of the susceptible genotype. The increased de-esterification in a homogeneous pattern after inoculation may be due to the activity of pectin methylesterase of *Rs,* an enzyme which was shown to be involved in the pathogenesis of bacterial wilt. Galactans and arabinans are the first defined epitopes occurring in the side chains of pectic polysaccharides to be immunolocalized in the developing plants. The side chains of RG I domains may affect the accessibility by enzymes with wall modifying properties to the sites of action within the cell wall matrix. Thus, it may be speculated that a higher concentration of side chains of RG I as detected for galactan and of arabinan in xylem vessels of the respective resistant isogenic lines cannot easily be degraded by enzymes of the pathogen. Additionally, a high content of branched RG I makes pectin a less suitable gelling agent (Ryden and Selvendran, 1990), which would create more unfavourable conditions for a bacterial pathogen which in a gel is well protected against desiccation and resistance mechanisms of the plant. *Ralstonia* uses pectic enzymes as pathogenicity determinants when infecting tomatoes, although relative virulence is affected by the effect of temperature on enzyme production.

Wilting of plants infected with bacteria has been connected with a blocking of water conductance resulting from mechanical plugging of xylem vessels. For bacterial wilt of tomato, plugging of vessels has been described in connection with bacterial masses, exopolysaccharide (EPS) produced by *R. solanacearum*, or tyloses. Occlusion of vessels by gels is a common phenomenon following infection of both susceptible and resistant genotypes of many species by bacterial pathogens. The present studies showed that *R. solanacearum* infects tomato at the vascular tissues and these tissues are rich in different types of pectins. If pectins with gelling properties are present, occlusion of vessels by interaction of pathogen- and plant derived poylsaccharides might be enhanced and infection facilitated, and thus these pectins might contribute to a fast establishment of the disease in a susceptible gentoype.

The biochemical analysis of lipopolysaccharides of *R. solanacearum* strains revealed the typical composition of LPS in the *R. solanacearum* strains without major differences among them. The rheological interactions between the plant pectins and bacterial LPS were measured. No synergistic effects such as increases in viscosity were recorded in various mixtures of lipopolysaccharides of *R. solanacearum* strain ToUdk2 and pectins from stems of susceptible host plants. Our results could not support the hypothesis that bacterial LPS are actively involved in interactions with pectic plant cell wall components, although these observations in the mixture of LPS and pectin could be due to low purity of the pectins and the method adopted for extraction. LPS impurity with EPS could also account for non-entanglements of the two polymers.

The present study strongly supports the hypothesis that susceptibility was related to cell wall pectin esterification. Higher labeling of homogeneously esterified pectic polysaccharides in host cell walls of the susceptible gentoype compared to a blockwise esterification with lower concentration of methylesters in the resistant genotype may indicate that these pectins exert a number of effects contributing to defense reactions against *R. solanacearum*. They may act along with other defense responses such as deposition of lignin and formation of cell wall appositions.

After inoculation with *R. solanacearum,* dramatic cell wall changes were observed in the susceptible genotype (L390), as indicated by increased labeling with all the antibodies. The present study demonstrates that pectin is degraded during stem colonization of tomato genotypes by *R. solanacearum.* Our results also show that alteration of pectic polysaccharides could result from bacterial pectinases and suggests that degradation of these polymers mostly occurs during xylem colonization in the stems. These observations on the distribution of pectin epitopes in plant tissues at the level of cells, and tissue systems would appear to be novel in terms of the understanding of the biology of pectins. To resolve the roles of pectins will require monitoring the degree of esterification and the distribution of methyl esters in a series of stem cuts at different stages of plant maturity.

Immunocytochemical investigations with monoclonal antibodies specific for epitopes of pectins revealed differences between healthy tomato genotypes H7996 and L390, resistant and susceptible to bacterial wilt, respectively, in structure and composition of their pectic cell wall components and arabinogalactan proteins. In our resistant (H7996) and susceptible (L390) genotypes no significant diference was observed in labeling with JIM5 and JIM7. Specificity of JIM5 makes it a highly selective probe for low methyl-esterification regions of HG, which are thought to be a major factor in the cross-linking of HG chains by calcium ions and thereby gel formation by HG domains (Schols & Voragen, 2003). The immunological results presented a more blockwise distribution of the methyl esters in HG from resistant tomato genotypes and a non-blockwise distribution (LM7) in the HG from susceptible plants. In our tomato genotypes we did not observe any relation between the degree and pattern of methyl esterification of HG, as has also been reported by Willats *et al.,* (2001) where they suggested that LM7 epitope contains both methyl esterified and un-esterified Gal A residues. It has been observed that some plant PMEs may have a non-blockwise action pattern as reported in pea stems (Willats *et al.,* 2001). More Interestingly, inhibitor proteins of plant PMEs have been identified that modifies the resulting distribution of methyl esters within the HG polymers, possibly shifting from a blockwise towards a more random pattern (Moerschbacher, 2003).

Studying the pectic epitopes after inoculation with *R. solanacearum*, the increased labeling with LM7 indicates the possible action of pathogen PME, resulting in non-blockwise de-esterification. The mode of action of the pathogen PME on plant polysaccharides has not been described before. The high level of pectin with non-blockwise de-esterification of methyl-ester groups were observed in single vessels and around vessels in the inoculated susceptible genotype L390. The high labeling of the pectic galactan and arabinan epitopes may also be related to the presence of the low methyl-esterified HG in the cell walls of the susceptible genotype after inoculation. Hence our results show for the first time which pectin compounds of the plant cell wall *R. solanacearum* is capable to degrade. In the resistant genotype after inoculation the epitopes of arabinan and galactan side chains of branched pectins showed significantly increased labeling. The greater binding of LM5 and LM6 may be because of epitope accessibility at lower DE due to pathogen action is increased by conformational changes in pectin structure. Methylation of the carboxyl groups of galacturonic acid and the formation of bridges between rhamnogalacturonan chains offer resistance to certain pectic enzymes as does calcium, and together they confer the gelling property of pectic substances which suggests the restricted movement of bacteria in the resistant genotype by formation of a barrier, and at the same time in susceptible genotype forms favorable conditions by providing the nutrients for the bacteria to grow as the cell wall thickening formed is not strong enough to restrict the bacterial movement.

The inhibition of the bacterial growth maybe simply due to the failure of the resistant plant to provide the host factors (i.e. nutrients) necessary for the multiplication of the pathogen. Susceptible plants that provide these host factors may allow bacteria to grow rapidly and cause disease. Although Pagel and Heitfuss (1989) found that potato tuber rotting susceptibility of six cultivars was inversely related to cell wall pectin esterification, other studies (Weber, 1990) reported the converse. However, as presented also in our study, the ranges of resistance and pectin esterification in the tomato genotypes against *Rs,* used were too narrow to draw firm conclusions.

AGPs might contribute to cell wall thickening by association with other cell surface molecules or with one another. For example LeAGP-1 (Chapter III) was reported to interact with pectin by clusters of basic amino acid residues or by Ca^{2+} mediated binding. However, it remains doubtful whether these bindings are strong enough to paly a role in xylem cell wall strengthening and therefore in induced resistance (Showalter, 2001). Association of AGPs among themselves are thought to involve ionic interactions and plant analogs of Yariv agents such as flavonols glycosides or oxidative crosslinking (Showalter, 2001). The latter is associated with the oxidative burst, a rapid, transient production of huge amounts of reactive oxygen species (ROS) by plant cells, as a defense strategy against pathogens. It might be assumed that also AGPs located in resistant tomato genotype H7996 were crosslinked by these mechanisms and therefore strengthened cell walls, thus slowing the ingress of *R. solanacearum* into the xylem tissues. This could suggest the role of AGPs as resistance factor as they were already abundant in the midstem tissues of resistant tomato genotype and might be increasingly produced by susceptible genotype as a reaction to pathogen infection.

Hence our results suggest that *R. solanacearum* is capable of degenerating cell walls in a tomato genotype susceptible to bacterial wilt by the production of enzymes and degrading the pectic components in a non-blockwise pattern and thus differentiating between the resistant and susceptible genotypes used in this study at the cell wall (pectins & AGPs) level. These studies provide a basis for further studies on the cell wall in relation to resistance mechanisms. The early stages of infection of tomato with *R. solanacearum*, especially through the root system, needs to be examined in detail. The observations reported here demonstrate that modulation of the pattern and degree of methyl-esterification of pectic HG occurs within discrete regions of primary cell walls and, in particular, that a non-blockwise pattern of methyl esters of HG is an abundant feature of HG of susceptible tomato genotypes. The pattern and degree of methyl group distribution significantly affect the mechanical and physiological properties of pectins and are therefore likely to influence the *in vivo* functionalities of pectic HG domains. Understanding the cell biological context of the products of PME action will be crucial for determining the functions of PME multigene family members. A study of the

structure of the pectic polysaccharides carrying these neutral side chains of RG I and the enzymes that are responsible for the synthesis and any modifications of the neutral side chains of RG I will be of considerable interest.

The action of the pectic enzymes of *R. solanacearum* has been obscure to date, and these observations might elucidate the highly specific molecular interaction between these enzymes and plant cell wall structures, in order to create a suitable environment for pathogen multiplication. Enzymatic mechanisms for the de-esterification of pectic polysaccharides are known to occur within the cell but little is known of their precise location, activity or turnover. One possible approach to help resolve pectic polysaccharides metabolism within the wall would be to develop antibodies to plant PME and extend the methods used in the study to simultaneously locate PME distribution and pectic polysaccharides epitopes in the different tissues.

Although recent work has placed some structural features in cell biological context, there is much still to be done in this area, particularly concerning the occurrence and function of micro-domains of the primary cell wall matrix. Since immunolocalization studies with these anti-pectin antibodies and antibody to AGP in tomato stems revealed differences in the distribution of the pectins and AGPs within the cell wall, additional investigations should be carried out with further tomato gentoypes. Nevertheless, it is doubtful that a tomato line exists that carries resistance to all strains of *R. solanacearum,* since tomato breeding programs are usually specific for certain geographic locations and hundreds of different strains of the pathogen are present worldwide. Further research on the resistance mechanisms could provide an insight into the basis of resistance and provide criteria for selection of more resistant genotypes by traditional or molecular plant breeding methods.

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