CHAPTER 1

Literature Review

1.1 INTRODUCTION

Ralstonia solanacearum (Smith) (Yabuuchi *et al.*, 1995) is an important soil-borne pathogen with a widespread distribution. It affects crops in the tropics, subtropics and some warm and temperate regions of the world. This pathogen has an unusually broad host range, infecting over 200 species belonging to 50 botanical families (Hayward, 1991; 1994). During the 1990s bacteria wilt was introduced to Western Europe on latent infected seed potatoes (Janse *et al.*, 1998; Hayward, 1991). Bacterial wilt is a devastating disease destroying arable land globally at an astounding speed. The dreaded factor in disease spread is that once the soil has been infected with this pathogen, it is almost impossible to use the area again for any susceptible crops.

The study of bacterial wilt is of economic importance not only in South Africa but also in the rest of the developed and developing world. In the developed world it forms a serious threat to the limited fertile arable land that is available. In the developing countries, the danger lies in the general food shortage that will be aggravated by the decline in crop production due to the presence of bacterial wilt. Furthermore, it has the potential to lead to total crop loss when propagation material is contaminated thus posing a serious threat to the hungry.

Ralstonia solanacearum has been intensively studied both genetically and biochemically, providing a model system for the analysis of bacterial virulence (Salanoubat *et al.*, 2002). Since the lack of success achieved with the implementation of current control measures and the extent of bacterial wilt, the model plant *Arabidopsis thaliana* (L.) Heynh was employed in this study to investigate the plant pathogen interaction (Meinke *et al.* 1998).

This literature review will describe bacterial wilt caused by *R. solanacearum* and the various hosts world-wide. It will describe the importance of the disease and more so, the confinement thereof. It will also give a broad overview of what is known of this bacterial pathogen. The important role of *A. thaliana* in the disease control strategy and how plants defend themselves against pathogen invasion with the focus on bacteria will be

dealt with in this review. Finally, it will highlight the importance of a new pathosystem in Southern Africa.

1.2 THE PATHOGEN

Ralstonia solanacearum previously known as *Pseudomonas solanacearum* (Yabuuchi *et al.*, 1995) is the causal agent of bacterial wilt. It belongs to the Proteobacteria, β subdivision, *Ralstonia* group and the genus *Ralstonia*. It is considered as one of the most important plant pathogenic bacteria due to its broad geographical distribution and great economic losses that occur globally due to its infection and spread (Hayward, 1991). This Gram-negative aerobic bacterium is rod-shaped with polar flagella (Holt, *et al.*, 1994). In South Africa, *R. solanacearum* is considered a prohibited organism (Agricultural Pest Act, 1983 (act 36 of 1983)) and zero tolerance for bacterial wilt in potato seed tubers is applied.

1.2.1 Genome sequence

Ralstonia solanacearum has long been recognized as a model system for investigating the virulence determinants towards the extensive host range on a biochemical as well as a genetic basis (Salanoubat *et al.*, 2002). The complete nucleotide sequence was published by Salanoubat *et al.* (2002) in January 2002 and gave substantial information concerning the evolution of virulence functions. Data revealed the presence of a bipartite genome structure consisting of a 3.7 Mb chromosome and a 2.1 Mb megaplasmid in total and an average high G+C content of 67% (Genin and Boucher, 2002; Salanoubat *et al.*, 2002). The genome has a coding potential for approximately 5120 proteins. The chromosome houses all the mechanisms required for survival, while the megaplasmid carries duplicates of metabolic essential genes as well as all the *hrp* genes that are necessary for plant virulence. This megaplasmid contributes to overall fitness of the bacterium and may provide advantages in adverse environments (Genin and Boucher, 2002).

Alternative Coding Usage Regions (ACURs) are often associated with mobile genetic elements or prophage sequences; acquired through horizontal gene transfer consistent with the inclination that *R. solanacearum* can take up and recombine exogenous DNA through natural transfer (Salanoubat *et al.*, 2002). The presence of transposable elements and the presence of ACURs illustrate the complexity and potential plasticity of the *R. solanacearum* genome. A number of genes with a low G+C content, less than the average of 67%, several virulence genes like effector proteins translocated through the Type III Secretion Pathway (TTSP) and the presence of numerous duplicated ORFs (Open Reading Frames) suggests that ACURs might be virulence islands (PAI). ACURs might, therefore, be involved in the duplication process, consequently contributing to generate genomic variation by catalysing acquisition, loss and generation of genetic material (Genin and Boucher, 2002).

Proof of genetic rearrangement in strain BCC 0300 (GMI 1000) was observed on the megaplasmid where a perfect tandem duplicate of a 31 kb region flanked by insertion sequences was found. Genomic instability is a well-known phenomenon in *R. solanacearum* and this flexibility could be responsible for the genomic diversity of the species, exemplified in terms of host range and the existence of biovars (Genin and Boucher, 2002).

1.2.2 Exopolysaccharide and extracellular enzyme production

A variety of extracellular products that contribute to its ability to colonize host plants and cause disease symptoms are produced by *R. solanacearum*. According to Genin and Boucher (2002), one of the most important extracellular products is an acidic, high molecular mass, extracellular polysaccharide (EPS 1). Studies on EPS 1 suggest that it is the cause of wilting in infected plants, as it blocks the vascular system and thus alters water movement. However, EPS-1 mutants were still pathogenic, poorly colonizing the stem, insinuating that EPS might be responsible for minimizing plant recognition of the bacterial surface structures (Denny *et al.*, 1998; Schell, 2000).

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Ralstonia solanacearum secretes several plant cell wall-degrading enzymes that include an endo-polygalacturonase (PehA), and two exo- polygalacturonases (PehB and PehC), endoglucanase (Egl) and a pectinmethylesterase (Pme). Together the endo-polygalacturonases and exo- polygalacturonases, PehA and PehB, contribute substantially to the virulence of *R. solanacearum*, although each one's individual role in bacterial wilt development might be insignificant (Huang and Allen, 1997; *et al.*, 1998).

1.3 HOSTS

The very extensive host range of *R. solanacearum* includes over 200 plant species in more than 50 plant families. Several species are exclusively susceptible in a specified area such as strawberry, which is only a host in Japan and Taiwan as well as sweet potato bacterial wilt that only occurs in China, but this can change over time. *Eucalyptus* was initially only infected in Brazil and China, but today infection also occurs in Australia, South Africa and Uganda (Hayward, 1991, 1994; Coutinho *et al.*, 2000; Roux *et al.*, 2001). It causes severe loss of monocotyledons such as Moko disease of banana in the tropics, and ginger. Frequently it occurs on solanaceous crops like: tobacco, tomato, potato and eggplant in some warm areas outside the tropics (Agrios, 1997). A few leguminous plants like groundnut and French bean as well as several tree and shrub hosts such as mulberry, olive and cassava were also reported as hosts (Genin and Boucher, 2002; He, *et al.*, 1983). Some ecotypes of *A. thaliana* are also susceptible as shown by Deslandes *et al.* (1998).

1.3.1 Symptom expression

The disease appears as a sudden wilt on solanaceous crops. Infected young plants die rapidly. Agrios (1997) stated that the older plants show wilting, stunting and browning of vascular tissue. A cross-section through the stem usually produces whitish bacterial exudates. Finally the plants wilt completely and die due to destruction of the vascular system by the presence of the bacteria collected in bacterial pockets in the vascular bundle, pith and cortex. Roots also rot and disintegrated.

Latent infection of *R. solanacearum* is well documented. This is when the bacterial pathogen is present at high population densities in the plant but no symptoms are visible. This is a potential source of disease spread since the host is regarded as healthy, and is, therefore, often cultivated further (Denny and Hayward, 2001).

1.3.2 Epidemiology

Ralstonia solanacearum is a soil borne pathogen and has the ability to survive in the soil for extensive periods in the absence of host plants, although this long term survival in soil, is seen by many researchers as a controversial point in the world of bacterial wilt (Sequeira *et al.*, 1993; Nesmith and Jenkins, 1983). Hayward (1991), reported on the survival of the pathogen in the protected niche of a weed's rhizosphere, as well as on the fact that high soil moisture in well drained soils is conducive to bacterial wilt. The population density of biovar 2 in soil declined progressively over time. Although the presence of potato volunteer plants weeds and plant debris aided the long term survival of *R. solanacearum* in soil (Van Elsas, *et al.*, 2000). Survival in soil is dependent on the external temperature. Low circulating temperatures enables the pathogen to survive, while if a high day temperature of 40°C was reached and remained so for more than four hours, the bacterial pathogen population greatly diminished (Hayward, 1991; van Elsas, *et al.*, 2000).

Contamination of irrigation water has been a major source of inoculum, causing new outbreaks on several crops especially in Europe (Elphinstone *et al.*, 1998). In the potato industry surface water contaminants were found to be associated with effluent from potato processing industries and municipal wastewater that handled diseased potatoes (Elphinstone *et al.*, 1998; Janse *et al.*, 1998). Environmental factors including temperature, pH, level of salt and the presence of antagonistic or parasitic organisms play a key role influencing the survival of *R. solanacearum* in aquatic habitats (Van Elsas *et al.*, 2001).

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The pathogen preferably over winters in diseased plants or plant debris, in vegetative propagative organs such as potato tubers or banana rhizomes, on the seeds of some crops like tomato and capsicum, and in the rhizosphere of weed hosts. The weed hosts are usually symptomless carriers of the disease, especially annual weeds, when a non-host crop is propagated in diseased field (Hayward, 1991). Both infected seed tubers and the weed, *Solanum dulcamara* commonly known as 'Bitttersweet' were important sources of inoculum (Janse *et al.*, 1998; Elphinstone *et al.*, 1998; Van Elsas, *et al.*, 2000).

The mode of invasion of the pathogen into the host is through root wounds caused by cultural practices, nematodes, and insects, or at sites of secondary root emergence. Aerial transmission by insects for certain banana strains and the transfer from a diseased tobacco plant to adjacent healthy plants under conditions of high wind and rain has also been documented. The bacteria reach the xylem vessels and spread throughout the plant displaying a strong tissue specific tropism invading and multiplying in the xylem vessels (Salanoubat, 2002). Intercellular spaces of the root cortex and vascular parenchyma are colonized and lead to cell wall disruption, facilitating spread through the vascular system and accumulation of the pathogen in bacterial pockets filled with slimy masses and cell debris (Genin and Boucher, 2002, Agrios, 1997; Vasse *et al.*, 1995; Hayward, 1991).

1.3.2.1 The influence of the environment on epidemiology

A range of biotic and abiotic environmental factors is extremely important in determining the severity of bacterial wilt or if the disease occurs at all (Hayward, 1994). Below is a list of some of the most important factors.

- **Temperature and light**

This is the most important factor affecting the host-pathogen interactions as well as survival in soil. In general, an increase in ambient temperature between 30°C-35°C increases the disease incidence and rate of onset of bacterial wilt on hosts such as tomato as well as *A. thaliana*. This is not true for all strains of *R. solanacearum*. Biovar 3 and 4

increases in virulence with an increase in temperature, while biovar 2 was the most pathogenic at lower ambient temperatures, producing extensive wilt symptoms (Hayward, 1991). Light intensity also seems to play a role in soil survival of the pathogen since potato resistance was more frequently expressed at high light intensities (2000 feet above sea level, 24°C) than at low intensities (1300 feet above sea level, 28°C) (Hayward, 1991).

- **Soil type**

Some soils are conducive to bacterial wilt while other suppresses the disease development. Hayward (1991) also documented that soil type determines the soil moisture, and influences the antagonistic population, which affected the survival of *R. solanacearum* in soil. Nesmith and Jenkins (1983) indicated that suppressive soil factors (possibly linked to biological origin) exist at sites were *R. solanacearum* does not persist from season to season and that conducive soils retain their ability to cause disease after steaming as well as fumigation with methyl bromide.

- **Nematode populations**

Hayward (1991) stated in his review that root infection by nematodes, as expressed by a root galling index, generally correlates with bacterial wilt symptoms as expressed by the percentage of plants wilted. The primary effect is due the increased root wounding, but the pathogen may also modify the plant tissue to such an extent that is becomes favourable for bacterial colonization. Nematode infestation will lead to infection in an otherwise resistant cultivar (Hayward, 1994).

1.3.3 Regulatory systems of *R. solanacearum*: *Hrp* (<u>h</u>ypersensitive <u>r</u>esponse and <u>p</u>athogenicity) genes

The *R. solanacearum hrp* cluster is required for virulence on host species and induction of hypersensitive response in nonhosts (Genin *et al.*, 1992). The *hrpB* gene encodes for components of the type III secretion pathway (TTSP) (Van Gijsegem *et al.*, 1995). Inactivation of one of the more than 20 *hrp* genes causes almost complete loss of ability

to cause disease and multiply in susceptible plants, as well as loss of the ability to cause hypersensitive defence response in resistant plants (Arlat *et al.*, 1992). Type III pathways have a central role in pathogenesis of many bacterial pathogens of plants and animals (Thomas and Finlay, 2003). In *R. solanacearum* Type III secretion requires the production of an Hrp pilus. The *hrpY* gene encodes this structural protein. There is speculation about the possibility of direct protein translocation across the cell wall (Genin and Boucher, 2002).

Three other proteins have been shown to be secreted extracellularly, PopA, PopB and PopC, regulated by the HrpB regulator. Mutants of PopA, PopB and PopC, retain their virulence. This might be due to functional redundancy or that they are required to infect plants that have not yet been tested (Guéneron *et al.*, 2000). TTSP is regulated by at least two environmental factors in *R. solanacearum*. The first is detected when bacteria are grown in 'apoplast-mimicking' minimal medium. The second is a specific inducing signal perceived in the presence of plant cells.

HrpB and the *TTS* genes were induced in response to physical contact of bacteria with plant cells or cell wall fragments. This contact-dependent induction of *hrpB* gene expression requires the outer membrane protein PrhA, but not a functional TTSP. PrhA a ubiquitous and non-diffusible molecule, located in the *A. thaliana* cell wall, it could possibly aid in the translocation of effector proteins at the appropriate time and place, but this still needs to be investigated (Aldon *et al.*, 2000).

1.3.4 Regulation of virulence and phenotypic conversion

The production of *R. solanacearum* virulence determinants is controlled by a complex regulatory network that responds to multiple signals (Schell, 2000). The central player of the regulatory network is PhcA, (putative outer membrane receptor) involved in the Phc cell density-sensing system. PhcA both activates a set of *hrp* genes and virulence genes (EPS biosynthesis, Pme and Egl exoproteins) and represses others (those involved in motility, polygalacturonase and siderophore production, *hrp* genes). The *hrp* and

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virulence genes are expressed differentially during exponential growth in batch cultures (Clough *et al.*, 1996).

An endogenous signal molecule, 3-hydroxypalmitic acid methyl ester (3-OH PAME), controls the levels of active PhcA protein. Only when extracellular 3-OH PAME accumulates above 5 nM (this is at high cell density in the vascular system) repression of PhcA is relieved, resulting in activation/repression of the target genes (Flavier *et al.*, 1997). The above-mentioned model distinguished between early virulence factors, when PhcA is inactive in contrast to late virulence factors during high bacterial populations when PhcA is active.

Spontaneous or induced mutations in *phcA* results in the pleiotrophic morphological changes called 'phenotype conversion' (PC). PC-type mutants correspond to the spontaneous avirulent mutants described as early as 1954, as a change in colony morphology from mucoid to non-mucoid, resulting in reduced virulence. Spontaneous PC-type mutants are most likely the result of distinct mutational events in *phcA*, some of them being reversible in the presence of a susceptible host. This was the first report illustrating the full cycle of phenotypic conversion/reversion in a plant pathogenic bacterium (Poussier *et al.*, 2003). Denny *et al.*, (1994), postulated that *R. solanacearum* shifts between these two dramatically different physiological states, the PC-type being adapted for saprophytic survival while the wild type is specialized for pathogenesis. However, this phenotypic reversion remains controversial and disputed within the scientific community.

1.4 CLASSIFICATION, DIVERSITY AND DETECTION OF *R. SOLANACEARUM*

Ralstonia solanacearum is a heterogeneous species that contains strains that differ in host range, virulence, epidemiological relationships and physiology. A two-fold classification system has been in place, either according to the host range of the strains classifying them

into races (Buddenhagen *et al.*, 1962), or according to the ability of the strains to oxidize various disaccharides and hexose alcohols (Hayward, 1964). This classification (Table 1.1) often gives enough data to predict suitable control measures against the disease outbreak, but requires isolation from the diseased plant, purification of the isolate as well as a two to three week testing period.

Table 1.1: Characteristics of races and their relationship to Biovars and RFLP subdivisions of *R. solanacearum*

Race[&]	Host Range[&]	Geographical Distribution	Biovar[#]	RFLP Division[*]
1	wide	Asia, Australia	3, 4	I
		Americas	1	II & III
2	banana other *Musa* spp.	Caribbean, Brazil, Philippines	1	II
3	primarily potato	Worldwide	2	II
4	ginger	Asia	3, 4	I
5	mulberry	China	5	I

[&]Buddenhagen, 1962; [#]Hayward, 1964; [*]Poussier *et al.*,2000b; Cook & Sequierra, 1994.

Bacterial wilt can be managed most effectively if prophylactic steps are taken such as determination of the presence of the pathogen in soil or planting material prior to crop planting. A technique that is sensitive, specific and quick to detect *R. solanacearum* well below threshold levels is required for control measures. A mere visual inspection is not sufficient due the presence of latent infection on tubers, rhizomes and banana suckers (Seal and Elphinstone, 1994).

Most detection and characterisation methods need a pure culture. Conventional methods for the detection of bacteria were designed to enumerate either the cultural population or the total population. Viable counts are mostly an underestimate due to the fact that all cells are not recoverable. One reason is the viable but non-culturable state that

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R. solanacearum enters (Van Elsas *et al.*, 2001). Another reason is the type of media used for selection. However, all techniques rely heavily on the sampling technique for efficiency. Serial dilution and a high grade of replication are essential to ensure reliability (Seal and Elphinstone, 1994).

Methods that do not need a pure culture are immunological and nucleic acid-based technologies. These depend on cell surface characteristics and either involves direct testing of a macerate or other sample extract to release the nucleic acids from the cells. The following techniques have been used to detect *R. solanacearum*:

(a) Serological detection of *R. solanacearum*

- **Using ELISA**

The Enzyme Linked Immunosorbent Assay (ELISA) does not require sophisticated equipment, is relatively inexpensive, requires a minimum of training and may be readily adapted for use in developing countries in the tropics where the disease is prevalent. An ELISA has been developed that can be use to detect the bacterium in both plant and soil samples in as little as 3 hours and 30 minutes. Monoclonal antibodies are available for *R. solanacearum* and the system had been optimized to detect bacterial levels as low as 1×10^4 CFU/ml (Robinson, 1993).

- **Using Immunostrips**

Immunostrips is developed by Agdia Inc. (Country Road, Elkhart, USA) for the detection of *R. solanacearum* using a monoclonal anti-body. This test is a rapid, specific field diagnostic method of screening crops for *R. solanacearum* and is intended for use with plant samples exhibiting symptoms of *R. solanacearum*. It can also be used to identify bacterial culture examples (www.agdia.com/rs).

(b) Fatty acid profiling

Fatty acid profiling (Stead, 1993) as well as metabolic profiles (Black and Sweetmore, 1993) have sped up the process but also relies on a purified culture. Fatty acid analysis has been used to an extent for both species identification and sub-specific identification of *R. solanacearum*. Intra-specific relationships between *R. solanacearum*, *R. pickettii*, *Pseudomonas syzygii* and the banana blood disease bacterium of the rRNA group II have been studied using fatty acid analysis (Stead, 1993). Results were in agreement with the taxonomic patterns found in DNA-DNA homology studies of these species (Seal and Elphinstone, 1994).

Multivariate analysis of the above-mentioned fatty acid data of 69 *R. solanacearum* species produced four intra-specific clusters. Three of these clusters corresponded with the races 1, 2 and 3 respectively. Biovar 4 strains grouped in the race 1 cluster; the data, however, did not correlate with that obtained before where isolates were grouped into the biovars (Stead, 1993; Seal and Elphinstone, 1994).

(c) Metabolic profiles

Metabolic profiles is a term used for a technique that has been commercialised by Biolog Inc. (Hayward, California, USA) and consists of a microtitre plate containing 95 carbon sources and one control well (containing the same reagents as the other 95 wells, but without a carbon source). Cells are cultivated in a nutrient broth to midlogarithmic phase. The actively growing cells are washed. Equal amounts are then pipetted into the wells and incubated at 28°C. If oxidation takes place, the change in redox potential causes the indicator dye tetrazolium to change colour. The pattern produced is species-specific and can be analysed with BIOLOG computer software. Black and Sweetmore (1993) have however developed more representative profiles of *R. solanacearum*. The Biolog Identification System is a useful tool for rapid identification of non-fluorescent plant pathogenic pseudomonads including *R. solanacearum* (Li and Hayward, 1993; Seal and Elphinstone, 1994). However, some researchers found that *R. solanacearum* grows poorly on Biolog medium, giving erratic results (Allen, C., personal communication).

(d) Nucleic acid-based technology, paving the pathway to new discoveries

There are many techniques that can be used. The techniques are based on the same underlying principle: the complementarity of related single-strand molecules of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). Hybridisation is the forming of hydrogen bonds between single-stranded nucleic acid strands, and is highly specific and sensitive due to these high-fidelity hydrogen bonding between complementary nucleic sequences and thus ideal for diagnostic purposes.

- **Polymerase Chain Reaction (PCR)**

This method depends on two oligonucleotide primers, flanking the DNA fragment of interest. Amplification is obtained by repeated temperature cycles, beginning with heat denaturation, followed by annealing of the primers to the complementary sequences, extension from the annealed primers by a thermal stable DNA polymerase (such as *Taq*-polymerase, from *Termus aquaticus*). This leads to an exponential increase of the selected DNA fragment (Seal and Elphinstone, 1994; Gillings and Fahy, 1993).

The sequence of a 292 bp segment of DNA encoding 16S rRNA was determined for 40 *R. solanacearum*, four Blood disease bacterium, two *R. pickettii* and three *Pseudomonas syzygii* strains. Phylogenetic relationships obtained from this PCR analysis confirmed previously obtained results by DNA-DNA/rRNA hybridisations (Seal *et al.*, 1993). Their primer pair, OLI1 and Y2, can detect 1-10 cells of bacteria in this group after 50 rounds of amplification by visualising a 287-288 bp product on agarose gels. Seal, *et al.* (1999) reported the simultaneous detection of species and subdivisions by designing a multiplex PCR where a 288 bp band is produced by all *R. solanacearum* isolates and an additional 409 bp band for Division I strains.

The biggest problem with PCR is inhibition of the reaction due to substances that are naturally in the plant or soil extract subjected to this analysis. Poussier *et al.* (2002) evaluated the different processes and optimised the reaction to obtain reliable PCR detection. They illustrated that the success of PCR depends on the nature of the sample, that the additives such as polyvinylpolypyrrolidone (PVPP), bovine serum albumin

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(BSA) and protein 32 of the T4 phage were very effective in preventing inhibitory effects. The DNA extraction mini kit QIAamp® (Qiagen Ltd., Crawley, West Sussex, UK) appeared to be the most effective tool to guarantee PCR detection of *R. solanacearum* no matter the sample origin. This is the first report of accurate and reliable soil detection *of R. solanacearum* (Poussier *et al.*, 2002).

- **Restriction Fragment Length Polymorphism (RFLP)**

Cook *et al.*, (1989) used RFLP as the basis of a new classification scheme for *R. solanacearum*. Among the 62 strains tested, covering all races and biovars they identified 28 unique multilocus genotypes (MLGs) or better known as RFLP groups, separable into two major divisions. This offered a completely new view of the species complex, reflecting the groupings that had been assigned on the basis of host range and physiological characteristics. The geographical origin was the characteristic that correlated directly to the two divisions, being their respective origin from the Old World, Asia and the New World, the Americas. Division I included biovars three, four and five, originating in Asia, while Division II included biovars one and two, originating from the Americas. This division is hardly surprising, because geographic separate populations are likely to diverge due to the genetic drift and natural selection. Cook and Sequeira (1994) extended the RFLP analysis with an additional 102 strains and found that it correlated with the above-mentioned findings, enabling us to speculate about the origin of the differences between the populations.

- **Phylogeny of biovars based on 16S rRNA sequencing**

Ribosomal RNA (rRNA) represents a small part (about 0.3-0.4%) of the genome and can be divided into three groups: 5S, 16S and 23S according to their sedimentation rates, with the respective chain lengths: 120, 1650 and 3300 nucleotides. The cistrons coding for the rRNA are highly conserved, having evolved less rapidly than the rest of the chromosome – presumably because of the fundamental role of the ribosome. This feature of the rRNA molecule together with the fact that very distantly related taxa have the most highly conserved sequences make it a valuable molecular tool (Logan, 1994). Sequence comparisons of the rRNA revealed 2 clusters (biovar 1 and 2 grouping together in a

cluster and biovar 3 and 4 grouping together in another cluster) in the *Ralstonia* species complex. This was similar to the results obtained by restriction fragment length polymorphism (RFLP) and most likely reflects evolutionary divergence and geographic distribution of the species, the former being primarily of American and the latter of Asian origin (Li *et al.*, 1993; Li and Hayward, 1993; Cook *et al.*, 1989).

- **Genomic fingerprinting**

Genomic fingerprinting is when total DNA is digested with restriction enzymes, either a 4 bp sequence that produces an average fragment size of 256 bp or 6 bp sequence that produces an average fragment size of 4096 bp. Bacterial DNA is digested with enzymes that recognize 4 bp sequences, the digests are resolved on polyacrylamide gels and individual bands visualised under silver staining. Just the high molecular weight fragments are analysed, resulting in high sensitivity. The larger the restriction fragment, the more likely it is to participate in events that might alter the restriction pattern, such as a mutation, deletion and inversion. Genomic fingerprinting enables the scientists to differentiate between strains from all over the world determining the source of inoculum and the means of disease spread (Gillings and Fahy, 1993).

1.5   CONTROL STRATEGIES

Various strategies have been developed to control *R. solanacearum*, but many are limited in general application, due to crop- or site- specificity or lack of funding. Disease control can be achieved by using tolerant cultivars, clean seed and adequate crop rotations. However, no universal solutions are in place. There are only principles that can be applied and adapted in every situation (Hayward, 1991). Adapted preventive measures combined with the use of resistant cultivars are one of the most successful ways to reduce disease incidence (Poussier *et al.*, 2002).

a) Host-plant resistance

The strategy of selection has had some success in the case of tobacco and peanut, but in potato no immunity has been identified while in tomato the resistance in polygenic (Thoquet *et al.*, 1996a; 1996b; Hayward, 1991). In Japan, strawberries are susceptible as seedlings in the nurseries. In the field, however, the mature plants are resistant, this has been attributed to the strong antibacterial substance β-D-glucogallin that is a normal component of healthy tissues. Some resistance has also been achieved by grafting tomato on resistant eggplant rootstocks

b) Cropping systems

In developing countries the use of clean seed and long crop rotations is not feasible in diseased potato fields due to the growing food shortage and the need for staple food. Intercropping with beans, cowpea or corn leads to an overall lower disease incidence on the potato crop. Intercropping reduces soil populations of the pathogen and limits root-to-root transmission (Hayward, 1991). Long term crop rotations with either rye or winter oats was recommended by Pegg and Moffet (1971), in the case of infested ginger fields. Pegg and Moffet (1971), stated that crop rotation and fallowing reduced bacterial populations of the banana strain of *R. solanacearum*. This was likely due to the limited host range of *Helicona* species and banana. Their results suggested that if an organism has such a wide host range as bacterial wilt on ginger (Queensland, Australia), chances of survival are much higher. It is important to rid the fields of potential weed hosts as often as possible to reduce the disease spread (Pegg and Moffet, 1971).

c) Soil amendment

Hayward (1991) reports on S-H mixture developed in Taiwan that offers broad-spectrum activity against soil borne disease including bacterial wilt. A further study investigated the importance of urea and mineral ash also showed favourable results, as did the addition of sea-shell grit (42% Calcium oxide). More research is needed in this field.

d) Disease avoidance

Losses can be limited by manipulating the date of planting to accommodate seasons that are less favourable for development of bacterial wilt. In winter- and spring-production regions, early planting and harvesting can reduce bacterial wilt and tuber rot. This is only possible for production of merchandise crops because the plant material will carry a latent infection and can not be used as seed or propagation material (Hayward, 1991).

e) Integrated control

Hayward (1991) reported on the integrated approach to bacterial wilt of potato in Japan. The combination of soil fumigation with chloropicin, using a tolerant cultivar and late planting to avoid high temperatures during the planting season, achieved reduced disease incidence. Bacterial wilt posed a serious threat in Burundi, Central Africa. The measures taken to reduce bacterial wilt included crop rotation with cereals, fallowing during the dry season, and herbicide treatment of soil with the main focus on the continuous supply of clean seed. Integrated control contributed to dramatic reduction on the incidence of bacterial wilt, thus illustrating the effectiveness (Berrios and Rubirigi, 1993).

f) Biological control

Biological control is mainly achieved by using antagonistic rhizobacteria and avirulent mutants of *R. solanacearum*. Mienie (1998) investigated the value of bacterial antagonists, such as *Sporosarcina ureae*, *Stenotrophomonas maltophila* and *Pseudomonas resinovorans* isolated from maize roots. These isolates displayed antagonism to *R. solanacearum in vitro*. After greenhouse trials, *P. resinovorans* seemed to be the most promising antagonist with 50% survival over a period of 49 days. According to Hayward (1991) the mechanisms include resistance as well as active colonization of the rhizosphere with antagonistic bacteria of bacteriophage-producing strains of *R. solanacearum*, or protection by protective exclusion. Avirulent strains of *R. solanacearum* were produced by Tn5 mutagenesis and screened on the basis of active penetration in the absence of wilt symptoms. None of these approaches has reached the level of commercial application and more work is needed (Hayward, 1991).

1.6   THE IMPORTANCE OF *ARABIDOPSIS THALIANA* IN PHYTOPATHOLOGY

Arabidopsis thaliana (L.) Heynh is a small plant in the mustard family (Brassicaceae, Caparales) with a broad natural distribution throughout Europe, Asia and North America. Commonly it is referred to as thale cress or mouse-eared cress, naturally found in open-free draining ground, such as sandy or gravely soils (Anderson and Wilson, 2000). During the late 1800's it was rated as an inconspicuous weed (The *Arabidopsis* Genome Initiative, 2000). The adult plants reach a height of 15 to 20 cm with the rosette varying between 2-5 cm and minute flowers, 3 mm long and 1 mm wide. A hundred years later it was the first plant for which the complete genome was sequenced (The *Arabidopsis* Genome Initiative, 2000).

Several plants have been recognized as model genetic systems such as maize, tomato, pea, rice, barley, petunia and snapdragon, but research biologists failed to reach consensus on which species was the most suitable for studying processes common to all plants. This resulting in limited knowledge of our understanding of fundamental aspects of plant growth and development such as flowering, root growth, hormone action and responses to environmental signals (Meinke *et al.*, 1989). The *A. thaliana* genetic system is significantly more tractable than those of the other plant species, which where hampered by long generation times, and large, polyploid, or repetitive genomes. *A. thaliana* exhibits all of the major kinds of defence responses described in other plants. Furthermore, a large number of virulent and avirulent bacterial, fungal and viral pathogens of *A. thaliana* have been collected (Glazebrook *et al.*, 1997).

In the early eighties plant biologists started searching for another model organism suitable for detailed analysis, using the combined molecular tools of genetics and molecular biology. Petunia and tomato received a lot of interest but the focus started to move to *A. thaliana* when a detailed genetic map was released together with publications

outlining the value of *A. thaliana* for research in plant physiology and biochemistry (Meinke *et al.*, 1989).

Anderson and Wilson (2002), summarised the attributes that contributed to the global success of *A. thaliana* as the lab-rat for plant biologists. The plant, today known as a model dicotyledon, is an ideal genetic tool due to its small diploid genome that has little repetitive DNA. *A. thaliana* can complete its entire life cycle in six weeks, a single self-pollinated plant producing thousands of seeds that can easily be transformed by *Agrobacterium tumefaciens*. Furthermore it has a small genome consisting out of five chromosomes of which the complete sequence is known (The *Arabidopsis* Genome Initiative, 2000). As many as 142 ecotypes exist in the native population and are available for research (Mitchell-Olds, 2001).

Mutants defective in almost every aspect of plant growth and development have been identified and studied by the various research groups over the world (Meinke *et al.*, 1989). Novel insights into events subsequent to pathogen recognition in *A. thaliana* have been obtained from mutants altered in defence (Buell, 1998). Several mutant groups in *A. thaliana* exist today: lesion mimic mutants, phytoalexin mutants as well as enhanced susceptibility and resistance mutants, respectively. With the variety of mutants available it is possible to determine which defence pathways are activated during pathogen attack, and what leads to the subsequent resistance or susceptibility. As research progresses, the different mutants will be linked to specific genes finally leading to a better understanding of the various genes involved in plant response pathways (Glazebrook *et al.*, 1997).

1.6.1 Understanding plant-pathogen interactions

The host plant can be resistant or susceptible to an avirulent or virulent pathogen, respectively. The interaction between a susceptible host and a virulent pathogen is termed compatible whereas the interaction between a resistant host and an avirulent pathogen is termed incompatible. Tolerance is defined as the overall ability for a plant to

withstand the development of the pathogen without major yield losses (Prior *et al.*, 1994). The other factor is the environment, which can influence both the growth and resistance of a host plant, the rate of multiplication of the pathogen population, the pathogen virulence as well as pathogen dispersal by wind, water, insect vectors, and so on. All together they form and are referred to as 'the disease triangle', if any one of the factors is absent, no disease can develop (Agrios, 1997; Buell, 1998).

Buell (1998) states that plants are under a constant assault of microorganisms attempting to obtain access to the reservoir of nutrients within the plant. As disease seems the exception rather than the rule, the question arises: How do plants protect themselves? In the following paragraphs this will be briefly discussed.

1.6.2 How do plants protect themselves?

Generally plants have two main magazines of weapons to protect themselves, firstly structural barriers, and secondly biochemical reactions. Physical obstacles inhibit the pathogen from gaining entrance and spreading throughout the plant, while the biochemical reactions take place in the cells and tissues of the plants and produce substances which are either toxic to the pathogen or create conditions that inhibit pathogen growth in the plant (Agrios, 1997).

Pre-existing defence structures such as thick cuticle and often a wax layer that is present on the plant surface as well as chemical defences including leaf exudates that are toxic to fungi, as well as phenolic compounds and tannins. Plant surface cells also contain several hydrolytic enzymes such as glucanases and chitinases. Resistance also occurs due to several factors such as lack of recognition between the host and pathogen, the absence of host receptors to detect fungal toxins, causing subsequent disease, or an insufficient supply of or the total absence of essential substances in the case of an obligate parasite (Agrios, 1997).

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The following induced structural defences, cytoplasmic defence in the case of slow growing weakly pathogenic fungi, cell wall defence structures such as formation of cork layers, abscission layers, tyloses and gums. They are formed during infection. Hypersensitive response follows, starting the cascade of induced biochemical defences.

The most common new cell functions and compounds include a burst of oxidative reactions (Bolwell, 1999), increased ion movement, disruption of cell walls and cellular compartmentalization, cross-linking of phenolics with cell wall components and strengthening of the cell wall as well as peroxidase production. Production of anti-microbial substances such as phytoalexins and the induction and subsequent accumulation of pathogenesis related (PR) proteins also fall under these cell functions (Agrios, 1997; Bol *et al.*, 1990).

1.6.3 What is resistance?

Hammond-Kosack and Jones (1996) described resistance (or pathogen failure) as the result of one of the following reasons. Firstly the plant is a non-host, i.e. it cannot support pathogen invasion and growth, secondly preformed structural barriers and toxic compounds are present that limit pathogen growth, or thirdly the plant recognizes the pathogen and forms defence mechanisms that localize the pathogen and restrict growth. The latter was confirmed by Heath (2000). This resistance can be termed as the state of the host when no disease develops during a plant-pathogen interaction (Glazebrook *et al.*, 1997)

Arabidopsis thaliana, like other flowering plants, exhibits specificity in resistance to plant pathogens. Most phytopathogenic organisms, unlike *R. solanacearum*, are able to infect and cause disease on only one or a small number of host species. This specificity suggests that initiation of a pathogenic interaction is dependent on the ability of the pathogen to recognize, invade and grow in suitable host plants. Then there also exists the potential for the host plant to detect the presence of the pathogen and rapidly induce

expression of a complex series of defence responses, which limit growth and spread of the pathogen within the plant labelled as resistant (Kunkel, 1996).

1.6.4 The costs of resistance

Resistance indeed is costly, so why do resistant polymorphisms persist in plant populations (Purrington, 2000)? A popular view among plant scientists is that plant populations and their pathogens are in equilibrium, with the frequency of resistant alleles highest in those populations in which the virulence of the pest is the highest or in which the pest is common. In the review by Purrington (2000), he lists the following costs of resistance to insects. Firstly that pollinators visit plants with extensive chemical defences less frequently, this could lead to reduced pollination and seed production. Secondly, that resistance to one type of herbivore leads to greater susceptibility of a more specialized pest. Thus trade off exists between resistance and tolerance, due to the fact that highly resistant plants might be less able to tolerate herbivory when it is occurs. Thirdly, herbivore damage in itself may cause a further fitness reduction due the subsequent reduced regrowth potential

Inducible defences are more likely to be more cost effective compared to constitutive expression. But inducible systems depend on maintaining wound-detection pathways, defence precursors and storage vesicles. All require allocation of both energy and resources away from growth and reproduction. It is not straightforward to measure the fitness costs. The only way would be to compare the fitness of uninduced plants that possess the above components (i.e. resistant genotypes) to the fitness of plants that lack all the components (i.e. susceptible genotypes) (Purrington, 2000). Heil (2002) stated that integrative measures of fitness must be applied under natural conditions so that the researchers can fully understand the costs and benefits of induced resistance.

If resistance is indeed costly, a breeder's most effective strategy may not be to select for excellence of resistance, if that implies sacrificing yield or quality, but to select for

moderate resistance. The entire genotype of a cultivar is more important and valuable than a single gene. Brown (2002) concluded this review by stating that there is an urgent need to study disease response as part of the biology of the whole plant and to stop focussing on one fragment.

1.6.5 Gene-for-gene interactions

The gene-for-gene hypothesis was based on correlated genetic studies of both host and pathogen. For each gene that conditions resistance in the host, there is a corresponding gene that conditions virulence in the pathogen (Flor, 1971). Single race-specific resistance (R) genes have been highly effective in blocking diseases in many crop species. However, this type of resistance often becomes ineffective as members of the pathogen population emerge that avoid plant recognition, requiring the introduction of new resistant traits (Quirino and Bent, 2003).

Today, two models exist for interpreting this interaction, the first being the receptor-ligand model in which products (ligands) of resistance genes are receptors that specifically detect the pathogen avirulence genes either directly as a protein product or indirectly as an enzyme product. The resistant receptor has a two-fold function, primarily to recognize the pathogen and secondarily to signal down stream resistance response genes. These genes inhibit the pathogen integration, conferring resistance (Ellis *et al.*, 2000; Glazebrook *et al.*, 1997). Although most genes encode Leucine Rich Repeat (LRR)-containing proteins (Bent, 1996; Hammond-Kosack and Jones, 1997) implying protein-protein interactions or ligand binding this has only been illustrated in two cases. These are the rice blast resistance protein Pi-ta and Avr-Pita from *Magnaporthe grisea* and RRS1-R/PopP2 of *A. thaliana* and *R. solanacearum* (Jia *et al.*, 2000; Deslandes *et al.*, 2003).

Many other research groups have characterized LRR-R proteins but have failed to show direct R-Avr interactions, noting other interactions. The responsiveness of tomato to

Pseudomonas syringae avrPto gene product requires two R proteins, one being a NB-LLR protein (Prf), and the other being a Pto kinase (Pto). Pto physically interacts with AvrPto, while no evidence can be found for Prf interaction (Tang *et al.*, 1996; Quirino and Bent, 2003). These contradicting results lead to the formulation of the 'guard hypothesis' (second model).

In the place of strong and direct LRR/Avr protein-protein interactions postulated above in the receptor-ligand model, *R*-encoded LRR proteins may commonly detect physical alterations of a plant-protein that are caused by the pathogen Avr protein. The term 'guard' acknowledges the concept that Avr proteins are often virulence factors that attack host cellular machinery, with the R protein altering the cell to this attack (guarding) and calling forth a broad-scale counterattack. This hypothesis is strongly supported by the results obtained by Mackey *et al.*, (2002) where they identified RIN4 (RPM1-interacting protein) as an *A. thaliana* protein that physically interacted with *P. syringae* AvrB in a yeast to hybrid screen. RIN4 functions as a negative regulator of plant defence responses leading to disease in susceptible plants. In resistant plants RIN4 is the direct binding target of AvrRpm1 and AvrB, these pathogen effector proteins induce phosphorylation of RIN4, this is perceived by RPM1 and leads to defence activation (Mackey *et al.*, 2002).

Various bacterial avirulence genes have been cloned. In general no common features are recognized in the predicted gene products. The function of the bacterial avirulence gene product is not known, but it is widely speculated that it assists the pathogen in colonizing and gaining nutrition from the host. A reduction of virulence has been observed when avirulence genes are mutated (Ellis *et al.*, 2000).

Five distinct classes of host resistance proteins are known; three contain leucine-rich repeats (LRRs). The most abundant class is nucleotide-binding-site-leucine-rich-repeat (NBS-LRR) with a nucleotide binding site (NBS) in the N-terminal portion and several imperfect leucine-rich repeats (LRR) in the C-terminal portion. LRR proteins are involved in protein-protein interactions; these proteins are the common structural denominator of all these proteins. The N-terminal domain also called the TIR domain

due to sequence and structural similarity to the cytoplasmic domains of the Toll and interleukin-1 receptor can also affect specificity (Ellis *et al.*, 2000).

Two different strategies were used to identify *R*-genes, using the virulent and avirulent bacterial, fungal and viral pathogens that have been collected. One strategy was identification of a virulent pathogen isolate on one *A. thaliana* ecotype and then screening the other ecotypes for resistance. The resistant ecotype was then crossed with the susceptible ecotype and the F2 progeny were tested to determine whether resistance segregated as a single gene. Many single loci, more than 40 genes that govern resistance to oomycetes, viruses and bacteria have been identified in this way (Glazebrook *et al.*, 1997). In cases of tolerance, the pathogen grows to the same extent in tolerant and susceptible ecotypes, but the tolerant ecotype shows no symptoms (Glazebrook *et al.*, 1997).

A different strategy was followed for some of the bacterial pathogens. Cloned avirulence (*avr*) genes were used to convert virulent strains into isogenic avirulent strains, which were used to identify *R* genes. An approach like this ensures that the observed difference in the host is the effect of a single *avr-R* gene interaction, and is not affected by a difference in the pathogen strains other than the presence or absence of the *avr*-gene itself (Glazebrook *et al.*, 1997).

A mutagenesis approach has been used to identify *R* genes corresponding to particular *avr*-genes. This strategy was based on the fact that a single *avr*-gene was responsible for the avirulent pathogen, thus that host resistance was due to a single *R*-gene and a mutation of this gene would eliminate resistance. In the absence of the isogenic strains, this approach would be very risky leading to a low success rate (Glazebrook *et al.*, 1997).

1.6.6 Hypersensitive Response (HR)

Hypersensitive response is the incompatible response between a pathogen and a host that is characterized by the appearance of a necrotic lesion at the site of pathogen penetration, thus limiting disease spread and activating defence genes (Heath, 2000). It is also referred to as gene for gene cell death since HR is often the outcome of R-Avr protein recognition. Agrios (1997) defined HR as the death of host cells within hours of pathogen contact, thought to limit the obligate biotrophic pathogen's access to water and nutrients. In interactions with hemibiotrophic and necrotrophic pathogens, the role of HR is less clear since these pathogens can obtain nutrients from dead tissue. However, HR can be phenotypically diverse, ranging from death of a single cell to a spreading chlorosis and a secondary necrotic region in the surrounding uninfected tissue. This is attenuated by relative humidity (Hammond-Kosack and Jones, 1996; O'Donnell et al., 2003).

1.6.7 Signal transduction pathways

There are at least two signal transduction pathways leading to disease resistance in A. thaliana (Murray et al., 2002; Feys and Parker, 2002). The first is systemic acquired resistance (SAR) a form of strong disease resistance in plants and the second pathway is regulated by jasmonic acid and ethylene (Fig. 1.1). SAR is produced in plants after hypersensitive response or gene-for-gene programmed cell death and causes a signal to be transmitted throughout the plant conferring resistance to a broad spectrum of pathogens. Salicylic acid (SA) plays a key role in the establishment of SAR: resistant tissue contains elevated levels of SA; treatment of plants with SA induces defence gene expression and resistance; and SA is required in the responding tissue for defence gene expression and subsequent resistance (Glazebrook et al., 1997; Agrios, 1997; Uknes et al., 1992). SAR can also be induced by the synthetic immunomodulator such as 2,6-dichloroisonicotinic acid (INA) as well as benzothiadizole (BTH). Conclusive proof that SA is required for SAR came from the analysis of transgenic plants expressing the bacterial salicylate hydroxylase gene, nahG, responsible for converting SA to the inactive

form, catechol. These *nahG* mutants were unable to activate the SAR pathway (Ryals *et al.*, 1996). SAR has been closely correlated with a set of nine gene families, five of these families encode for pathogenesis-related (PR) proteins, a set of extracellular polypeptides long associated with pathogen infection (Bol *et al.*, 1990).

The plant defensin gene *PDF1.2* in *A. thaliana* has been shown to be blocked by the ethylene response mutant, *ein2-1* as well as the jasmonate response mutant, *coi1-1* (Fig. 1.1). Penninckx *et al.* (1998) presented the genetic evidence for the strict requirement of both the jasmonate and ethylene signalling pathways for the induction of the pathogen responsive gene, *PDF1.2* in *A. thaliana*. This synergistic effect has been confirmed by the constitutive JA mutant, *cev1*, clearly showing the inhibitory effect of JA on plant growth as well as the induction of plant defence responses (Ellis *et al.*, 2002). Viljayan *et al.* (1998), illustrated that jasmonate is essential for plant defence against the oomycete pathogen, *Pythium*. Insect herbivory induced JA in the *npr1* mutant as well as in wild-type plants, research found that SA was able to reduced this enhanced resistance (Fig. 1.1) (Stotz *et al.*, 2002), thus illustrating the interaction of JA and SA. Finally, Shirano *et al.* (2002) suggested that SA not only functions as a critical downstream signal but also up regulates the expression of certain *R* genes.

Bleecker and Kende (2000) stated the ethylene synthesis is associated with plant stress. However, ethylene seems to mediate defence response to some pathogens and to suppress them in others. Ethylene insensitivity, like which is exhibited in the tomato mutant, *never-ripe*, conferred tolerance to bacterial leaf pathogens, caused reductions in vascular wilt symptoms, not imparting reduced resistance. During a compatible interaction selective pressure on the pathogen population is minimal, thus tolerance might be a more durable way of disease control (Lund *et al.*, 1998).

Murray *et al.* (2002), concluded that SA appears to be required for biothropic pathogens whereas JA and ethylene are needed against necrothrophic plant pathogens, thus that the type of virulence appeared to determine which defence response are engaged in *A. thaliana*. However, the SA pathway seems to be activated due to defence response while

the JA/ethylene pathway is linked to wounding. Today the scientific world is fully aware of the occurrence of cross talk between these pathways as well as pathways independent of these signalling pathways (Fig. 1.1) (Hunt *et al.*, 1997; Maleck and Dietrich, 1999; Gu *et al.*, 2000)
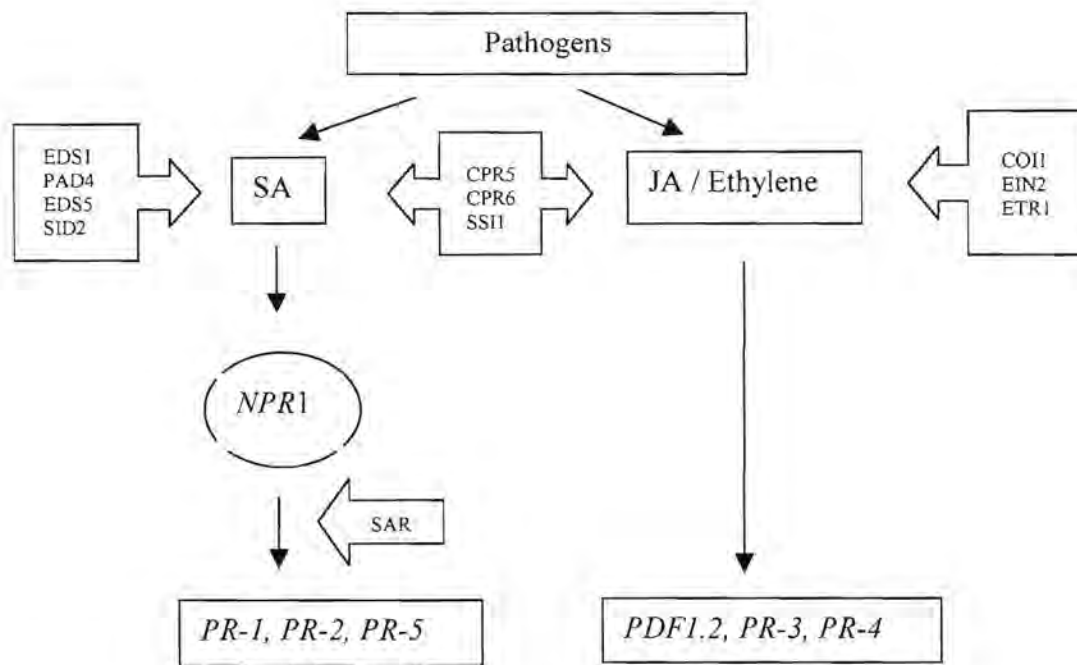

Figure 1.1. Overview of local and systemic signalling in *A. thaliana* disease resistance. A number of resistance pathways with different requirements for the signalling for the molecules salicylic acid (SA), jasmonic acid (JA) and ethylene, and led to the induction of sets of defence-related genes, have been defined in *A. thaliana*. SAR is regulated by *NPR1*. Analysis of several resistance-up regulating mutants (*cpr5, cpr6, ssi1*) also point to cross talk between the SA- and JA/Ethylene-responsive processes. Genes that are known to be required for the different pathways are shown at the correspondent sites (Feys and Parker, 2002).

1.6.8 Pathogenesis-related (PR) proteins

General characteristics associated with PR-genes are the following: Initially they were known to be acidic, but later basic homologues were identified, they are resistant to proteases, located extracellularly although some are localized in the vacuole and can be divided into several groups designated from PR-1 to PR-11 (Bol *et al.*, 1990; Hammersmidt and Nicholson, 2000). Similar patterns of proteins are induced after the incompatible interaction with plants with different types of pathogens and each interaction, in turn, induces a broad range of defence interactions. The expression of a number of PR genes is developmentally regulated in healthy plants. However, the accumulation of PR proteins are generally associated with pathogen infection, wounding and abiotic stress. For example, PR-3, PR-4 and the corresponding mRNAs are present at different levels in the roots, stems and flowers of healthy tobacco plants. After exposure to ethylene the following PR genes were expressed: Bean seedlings accumulated PR-2 and PR-3 while pea accumulated only PR-2. Tobacco and potato seedlings revealed a higher accumulation of PR-3 and PR-4, after exposure to auxin and a phytopathogen respectively. In maize PR-1, PR-2 and PR-5 accumulated after treatment with mercuric chloride, while in barley PR-2 accumulation was only present in resistant plants after pathogen infection and not in the susceptible plants (Bol *et al.*, 1990).

As seen in the few examples mentioned above several lines of evidence have been used to support the role of PR proteins in resistance. These include observations that the PR proteins are induced during the HR and appear systemically in plants expressing SAR. PR-2 (β-1,3-glucanase) and PR-3 (chitinases) proteins exhibit anti-fugal activity *in vitro* and may release elicitors from the cell wall of the pathogen (Fig. 1.1). Transformation of plants with PR-genes has also provided a means to test the role of these proteins in resistance. An example of constitutive expression PR-1 in transgenic tobacco and PR-5 in transgenic potato lead to the increased resistance to oomycete pathogens, while PR-2 and PR-3 transformation of tobacco lead to increased resistance to other fungi. Thus, evidence exists that PR genes are responsible of some plant defence strategies (Fig. 1.1) (Hammersmidt and Nicholson, 2000).

1.7   Aim of this study

The aim of this study was to develop a new African pathosystem between the pathogen *R. solanacearum* and the plant, *A. thaliana*, and the characterization thereof. The objectives of this study were threefold. Firstly, to rapidly identify the cause of bacterial wilt in *Eucalyptus* plantations or potato fields; secondly to screen various ecotypes of *A. thaliana* with the characterized strains for resistance and susceptibility. Thirdly, to establish a stable and reliable pathosystem fit for future transcriptome studies and subsequent evaluation hereof by investigating the bacterial growth curve and northern analysis with defence-related probes.

The hypotheses are that the causative agent of *Eucalyptus* bacterial wilt is *Ralstonia solanacearum,* that this isolate from a woody host will be able to induce wilting in *A. thaliana*, and finally, that an *A. thaliana* ecotype resistant to this African *R. solanacearum* isolate does exist.

The literature review in Chapter 1 focuses on *R. solanacearum*, the spread of the disease and its impact world-wide. The role of *A. thaliana* in finding resistance genes to this pathogen and how *R. solanacearum* mediates disease development in susceptible hosts forms part of this review. A review of the virulence factors of *R. solanacearum*, as well as resistance genes that have been characterized with *Arabidopsis* pathosystem, is discussed. Chapter 2 focuses on the characterization of *R. solanacearum*, while Chapter 3 describes the experiments to test with the *R. solanacearum* strains for virulence on various ecotypes of *A. thaliana*, partially fulfilling the aim of this study. Chapter 4 discusses the characterization of the pathosystem, while Chapter 5 provides a summary of the outcome of the experiments and a discussion of the relevance of results obtained.