# Studies on the interaction between *Arabidopsis thaliana* and African isolates of *Ralstonia solanacearum*

by

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**Courage doesn't always roar.**
**Sometimes courage is the**
**little voice at the end**
**of the day that says:**
**"I'll try again tomorrow"**

Anonymous

**DECLARATION**

I hereby certify that this research, unless specifically indicated to the contrary in the text, is the result of my own investigation and that no part of this thesis has been submitted to any other university.

Weich

Johanna Petronel Weich

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# Summary

*Ralstonia solanacearum* is the causal agent of bacterial wilt resulting in world-wide economic crop losses. The chief objective of this study was to develop a new pathosystem between *R. solanacearum* and *Arabidopsis thaliana*. The results obtained would enable researchers in Africa to limit disease spread due to a better knowledge of the pathogen as well as provide them with a better understanding of the mechanisms involved in plant defence.

The host plant used in the study was *A. thaliana* of which the whole genome has been sequenced. Growth conditions for *A. thaliana* plants in plant growth chambers in South Africa were investigated and subsequently optimized. Various *R. solanacearum* strains were characterized. This was achieved by implementing metabolic profiling and the polymerase chain reaction (PCR) of the hypersensitive response and pathogenicity (hrp) gene region. PCR-RFLP (restriction fragment length polymorphism) together with amplified fragment length polymorphism (AFLP) data grouped the *Eucalyptus* isolates into Biovar 3. This data showed that the PCR-RFLP enabled Biovar classification while the *hrp* PCR method was reliable for diagnosis and enables rapid identification of *R. solanacearum*.

Several ecotypes (Col-5, Nd-1, Kil-O, Be-O, Sf-2, Laer and Cvi) of *A. thaliana* were inoculated and disease development recorded, scoring wilt symptoms on a scale of 0-4. All strains were virulent on at least one ecotype. The Uganda isolate, BCC 0327 (27B), was the most pathogenic. BCC 0302 (CK) from the Congo, revealed a clear differential between the susceptible ecotype, Be-O and resistant ecotype, Kil-O. This was selected for further analysis. Non-virulent strains of *R. solanacearum* were obtained by direct transformation with genomic DNA from a strain carrying the desired knockout insertion (*hrpB* - or *hrcS* -) in the *hrp* gene.

After inoculating the plants with the respective virulent (CK Rif r) and non-virulent (hrcS -) strain, growth of the bacterial populations *in planta* was determined by dilution plating on selective media. A difference of one order of magnitude was present between the resistant and the susceptible ecotypes. Ten days after inoculation Be-O was completely wilted, while no symptoms had developed on Kil-O.

Northern analyses were performed using the Pathogenesis Related (PR)-genes. The data obtained revealed the absence of PR-1, PR-2 and PR-5 expression in Be-O, possibly explaining the rapid onset of disease development. These markers of the salicylic acid pathway were, however, induced in Kil-O conferring the absence of wilt symptoms and thus resistance.

Finally, a new *A. thaliana* - *R. solanacearum* pathosystem was developed, fit for transcriptome analysis. This will aid in the understanding of bacterial wilt, ultimately limiting further disease spread and conservation of vital nutritional food sources in Africa and other developing countries.

# Opsomming

*Ralstonia solanacearum* is die oorsaak van bakteriese verwelk wat wêreldwye ekonomiese verliese veroorsaak. Die hoof doel van hierdie studie was die ontwikkeling en daarstelling van 'n nuwe siektesisteem tussen *R. solanacearum* en *Arabidopsis thaliana*. Die uitkoms bemagtig dan die navorsers in Afrika om verdere verspreiding van die siekte hok te slaan asook die uitbreiding van hul kennis aangaande die weerstands meganismis betrokke by plant beskerming.

*Arabidopsis thaliana* was die gasheerplant wat in hierdie studie gebruik is, en die hele genoom is bekend. Groeitoestande van *A. thaliana* in plantgroeikabinette in Suid-Afrika is ondersoek en geoptimiseer. Verskeie *R. solanacearum* isolate is ook gekarakteriseer deur gebruik te maak van metaboliese profiele en die polimerase ketting reaksie (PKR) van die hipersensitiewe reaksie and patogeniese (*hrp*) geen area. PKR-RFLP (Restriksie fragment lengte polimorfisme) tesame met AFLP (amplifiseerde fragment lengte polimorfisme) data het die *Eucalyptus* isolate gegroepeer in Biovar 3. Hierdie data bewys dan dat die PKR-RFLP tegniek Biovar klassifisering moontlik maak en die *hrp* PKR metode betroubaar is vir diagnostiese gebruik en dus vinning identifisering van *R. solanacearum* bewerkstellig.

Verskeie ekotipes (Col-5, Nd-1, Kil-O, Be-O, Sf-2, Laer en Cvi) van *A. thaliana* is geinokuleer en siekte ontwikkeling is waargeneem, verwelk simptome is geëvalueer op 'n skaal van 0-4. Alle bakteriese isolate was patogenies op ten minste een ekotipe. Die Uganda isolaat BCC 0327 (27B) was die virulentste. BCC 0302 (CK) van die Kongo, het 'n duidelike verskil getoon tussen die vatbare ekotipe, Be-O en die weerstandbiedende ekotipe, Kil-O. Dit is gekies vir verder navorsing. Nie-virulente isolate van *R. solanacearum* is bekom deur middel van direkte transformasie met genomiese DNS van 'n isolaat wat die verlangde uitklop invoeging (*hrpB*$^-$ or *hrcS*$^-$) in die *hrp* geen bevat het.

Nadat die plante met die bepaalde virulente (CK Rif $^{r}$) en nie-virulente (*hrcS* $^{-}$) isolate geinokuleer is, is die groei van die patogeen populasie *in planta* bepaal deur middel van verdunnings uitplating op selektiewe media. 'n Verskil van 1 orde van betekenis is gevind tussen die weerstandbiedende en vatbare ekotipes. Tien dae na inokulasie was Be-O heeltemal verlep, terwyl Kil-O geen simptome getoon het nie.

Northern analises is ook uitgevoer en die Patogeen Verwante (PR)-genes is gebruik. Die data het die afwesigheid and die PR-1, PR-2 en PR-5 uitdrukking in Be-O getoon, dit verklaar moontlik die vinnig aanvang van verwelk en siekte ontwikkeling. Hierdie merkers vir die Salisielsuur padweg is egter geinduseer in Kil-O wat moontlik verantwoordelik is vir die weerstandbiedenheid van die ekotipe.

Uiteindelik is 'n nuwe *A. thaliana* - *R. solanacearum* ontwikkel, geskik vir transkriptoom/microarry analise. Dit sal verder bydra tot die kennis van bakteriese verwelk, met 'n beslissende inperking op die verspreiding van die siekte asook beskerming van noodsaaklike voedsel bronne in Afrika en ander ontwikkelende lande

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# List of Abbreviations

| | |
|---|---|
| aa | Amino acids |
| *Avr* | Avirulence |
| B | Bouchers' liquid media |
| BGT | Bacto-agar Glucose Triphenyltetrazolium chloride |
| bp | Base pairs |
| CFU | Colony Forming Units |
| CTAB | Hexadecyl trimethyl ammonium bromide |
| dATP | Deoxyadenosine triphosphate |
| dCTP | Deoxycytosine triphosphate |
| dGTP | Deoxyguanidine triphosphate |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| dTTP | Deoxythymidine triphosphate |
| EDTA | Ethylenediamine tetraacetic acid |
| EtBr | Ethidium Bromide |
| EtOH | Ethanol |
| HR | Hypersensitive response |
| hr | hour(s) |
| *hrc* | *hrp*-conserved |
| *hrp* | hypersensitive response and pathogenicity |
| kb | Kilobase pairs |
| Mb | Megabase pairs |
| min | Minutes |
| MM | Molecular marker |
| MS | Murashige and Skoog |
| NaAc | Sodium acetate |
| OD | Optical density |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| RE | Restriction Enzyme |
| $Rif^r$ | Rifampicin resistant |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | revolutions per minute |
| s | seconds |
| SDS | Sodium dodecyl sulfate |
| TAE | Tris-acetate ethylenediamine tetraacetic acid |
| TE | Tris-ethylenediamine tetraacetic acid |
| Tris | Tris hydroxy methyl aminoethane |
| TTC | Triphenyltetrazolium chloride |

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# 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).

*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).

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* (hypersensitive response and pathogenicity) 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

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 | Caribbean, Brazil, | 1 | II |
| | other *Musa* spp. | Philippines | | |
| 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

*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 $1 \times 10^4$ 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

(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).

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)

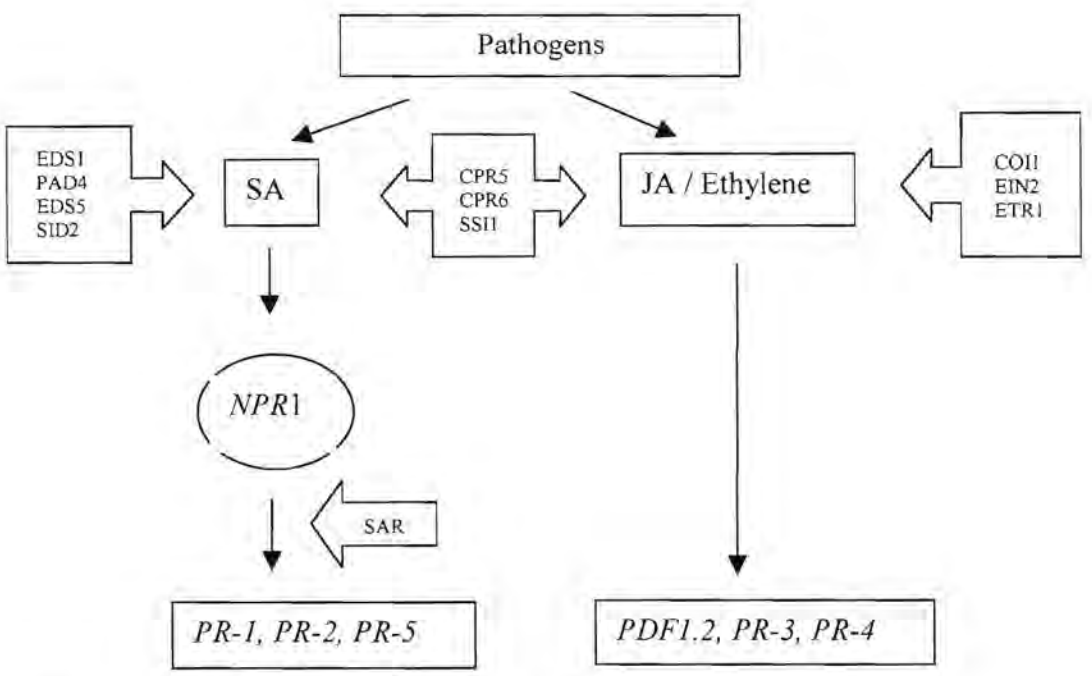



**Figure 1.1. Overview of local and systemic signalling in *A. thaliana* disease resistance.** A number of resistance pathways with different requirements for the signalling for the molecules salicylic acid (SA), jasmonic acid (JA) and ethylene, and led to the induction of sets of defence-related genes, 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.

# CHAPTER 2

# Identification and characterization of African strains of *Ralstonia solanacearum*

## 2.1 INTRODUCTION

Bacterial wilt, caused by *Ralstonia solanacearum*, is an important pathogen of a number of agricultural crops. It has a broad host range, infecting over 200 species belonging to 50 botanical families (Hayward, 1991; Salanoubat *et al.*, 2002). This disease is widely distributed occurring in tropical, subtropical and temperate regions of the world (Hayward, 1991). *R. solanacearum* has been studied on the genetic and biochemical level, and is recognized as a model system for the analysis of bacterial virulence (Salanoubat *et al.*, 2002).

*Ralstonia solanacearum* has been classified into five races, based on host range (Buddenhagen *et al.*, 1962), and into six biovars based on biochemical properties (Hayward, 1990). The pathogen has also been separated into two divisions correlating with the geographical origin based on RFLP (Restriction Fragment Length Polymorphism) analysis by Cook *et al.* (1989), and Cook and Sequeira (1994). Division I, which includes biovars three, four and five, originated in the Old World, and has been named the "Asiaticum" division. Division II, with biovars one, two and N2 (new biovar 2), originated in the New World and has, therefore, been named the "Americanum" division. These divisions were confirmed by sequence analysis of the 16S rRNA gene (Li *et al.* 1993, Taghavi *et al.* 1996) as well as sequencing of the polygalacturonase gene, the endogluconase gene and the 16S-23S rRNA gene inter spacer-region (Seal *et al.*, 1993, 1999). Furthermore, Taghavi *et al.* (1996) revealed a subdivision within Division II, grouping only Indonesian isolates of *R. solanacearum* and the two close relatives: *R. syzygii* and the blood disease bacterium (BDB).

Research performed by Poussier *et al.* (1999, 2000a, 2000b) established that certain "African" isolates from Madagascar, Reunion island, Zimbabwe and Angola belong to Biovar 1 and constitute a distinct phylogenetic group. Data they generated from Amplified Fragment Length Polymorphism (AFLP) and the sequencing of the 16S rRNA and the endoglucanase genes placed the African Biovar 1 isolates under the

"Americanum" division. In contrast data produced by PCR-RFLP of the *hrp* (hypersensitive response and pathogenicity) gene region and the *HrpB* gene sequencing placed this distinct group in the 'Asiaticum' division. What was clear from these studies was that this group, African Biovar 1, is different from the existing divisions. Poussier *et al.* (2000b) suggested that this separation could be ascribed to evolution due to geographical isolation, thus divergence occurred under different natural selection pressures.

The objectives of this study were to confirm the identity of the causal agent of bacterial wilt from *Eucalyptus* plantations in Congo, South Africa and Uganda and from potato fields in South Africa. Phenotypic and molecular techniques were used to characterize these strains. Molecular techniques used included PCR analysis of the 16S rRNA gene and *hrp* genes region, PCR- RFLP and AFLPs.

## 2.2 MATERIALS AND METHODS

### 2.2.1 BACTERIAL STRAINS AND MEDIA

Six strains isolated from various hosts which had been previously characterized as *R. solanacearum* using phenotypic techniques, were included (Boucher *et al.*, 1985; Coutinho *et al.*, 2000; Roux *et al.*, 2001; Van Broekhuizen, 2002) a seventh strain, GMI 1000, was used as a reference strain (Table 2.1). In addition to these strains, 42 strains were isolated from *Eucalyptus* trees in both South Africa and Uganda (Table 2.1, BCC 0307 - BCC 0348). In both countries, the strains were isolated from hosts showing typical symptoms of bacterial wilt.

Strains were routinely grown on solid Bacto-agar glucose triphenyltetrazolium chloride (BGT) media (10 g/l peptone, 1 g/l casmino acid, 1 g/l yeast extract, 15 g/l Bacto-agar, 4 ml of 1.25% triphenyltetrazolium chloride and 25 ml of 20% glucose) (Appendix A, Boucher *et al.*, 1985). They were incubated at 28°C for a minimum period of 24 hr.

When needed, single colonies were transferred into 500 ml liquid B medium (16 g/l peptone, 1 g/l casmino acid, 1 g/l yeast extract) (Appendix A, Boucher *et al.*, 1985) and incubated overnight at 28°C in a rotary shaker. The strains were deposited in the FABI Bacterial Culture Collection, University of Pretoria, Pretoria, South Africa.

**Table 2.1: Isolates of *R. solanacearum* used in this study**

| BCC* number | Sample | Host | Origin | Collector | Reference |
|---|---|---|---|---|---|
| 0300 | GMI 1000` | Tomato | French Guyana | Unknown | Boucher *et al.* (1985) |
| 0301 | K | *Eucalyptus* | South Africa (SA) | J. Roux | Coutinho *et al.* (2000) |
| 0302 | CK | *Eucalyptus* | Congo Kissoko | J. Roux | Roux *et al.* (2001) |
| 0303 | CC | *Eucalyptus* | Congo Civuiti | J. Roux | Roux *et al.* (2001) |
| 0304 | 117 | Potato | SA, Natal, Hlabisha | A.N. Hall | Van Broekhuizen, (2002) |
| 0305 | 92 | Potato | SA, Rooiwal | A.N. Hall | Van Broekhuizen, (2002) |
| 0306 | 62 | Potato | SA, Dendron | A.N. Hall | Van Broekhuizen, (2002) |
| 0327# | 27B | *Eucalyptus* | Uganda | G.Nakabonge | This study |

*FABI Bacterial Culture Collection, University of Pretoria, South Africa

# 0307-0326, 0328-0348 additional strains from wilting *Eucalyptus* found not to be *R. solanacearum*, in this study.

### 2.2.2 PHENOTYPIC METHODS

#### 2.2.2.1 Morphological characteristics

The strains were cultured on BGT media at 28°C. Gram stain, cell morphology and flagellar arrangement were determined (Theron, 1999).

### 2.2.2.2 Biovar differentiation

The method used to differentiate the strains, listed in Table 2.1, into biovars, is based on the utilization of hexose alcohols and disaccharides (Hayward, 1964). The basal medium consisted of the following: 1 g/l peptone, 1 g/l ammonium dihydrogen phosphate ($NH_4H_2PO_4$); 0.2 g/l potassium chloride (KCl), 0.2 g/l magnesium sulphate heptahydrate ($MgSO_4.7xH_20$); 3 g/l agar; 0.08 g/l bromothymol blue and made up to 1 litter with distilled water. The pH was adjusted to 7.0-7.1 (an olivaceous green colour) by drop wise addition of 40% sodium hydroxide (NaOH) solution, and was then autoclaved at 121°C for 30 min. Stock solutions of the various test carbohydrates were prepared (10 % w/v). Solutions of D-glucose, mannitol, sorbitol and dulcitol were sterilized in an autoclave for 30 min at 121°C. Lactose, maltose, and D (+) cellobiose, were filter sterilized into sterile containers. The carbohydrate solution was then added aseptically at a final concentration of 1% to the basal medium and kept at 5°C. The medium was then decanted into 90-mm-diameter Petri dishes under sterile conditions.

The inoculum was prepared as follows: several individual colonies were selected from a 24 hr culture grown on BGT medium (Appendix A, Boucher *et al.* 1985), were mixed with 3 ml of sterile distilled water. A 100 µl of this solution was added to the surface of the agar and the inoculum was spread with a glass hockey stick. The Petri dishes were incubated at 28°C and examined at 3, 7 and 14 days. Petri dishes containing only the basal medium and a verified *Escherichia coli* strain were also inoculated to serve as the controls.

### 2.2.2.3 Physiological tests

Colony morphology on nutrient agar (16 g/l nutrient broth and 20 g/l agar) were determined. Characteristics such as the presence of a diffusible pigment were noted due to the correlation with biovar differentiation. Growth characteristics on BGT media were recorded for all the strains used in this study.

## 2.2.3 MOLECULAR METHODS

### 2.2.3.1 DNA extraction

The total DNA of all strains listed in Tables 2.1 were extracted using a slight modification of the method described by Chen and Kuo (1993). The protocol was as follows: 5 ml B broth was prepared and then inoculated with one individual colony. The overnight culture was decanted into a 1.5 ml Eppendorf tube. Bacterial cells were harvested by centrifugation for 3 min at 12 000 rpm. The cell pellet was resuspended by vigorous pipetting in 200 µl of Lysis buffer (40 mM Tris-acetate, pH 7.8, 20 mM NaAc, 1 mM EDTA, 1% SDS). After resuspension, 66 µl of 5 M NaCl was added to remove protein and cell debris and centrifuged at 12 000 rpm for 10-15 min at 4°C. The supernatant was transferred to a new tube. An equal volume of phenol: chloroform: isoamylalcohol (25: 24:1) was added, and the tube gently shaken until a milky solution formed. The milky solution was centrifuged for 3 min at 12 000 rpm and the supernatant was transferred to a new tube. An equal volume of chloroform was added to the tube and mixed with its contents. The mixture was centrifuged for 3 min at 12 000 rpm. The DNA was precipitated with 400 µl of 100% EtOH overnight at –20°C. Centrifugation was the performed for 15 min at 12 000 rpm, the supernatant discarded and the DNA pellet was carefully retained. The pellet was then washed by the addition of 1 ml of 70% EtOH with subsequent centrifugation for 5 min at 12 000 rpm. The supernatant was discarded, without disturbing the pellet. The pellet was dried in a laminar flow chamber. Excess EtOH was removed by pipetting, and if necessary, with tissue paper. Once the pellet was dried, it was dissolved in 50 µl RNase-water (RNase 20 mg/ml), by vigorous pipetting. The DNA samples were stored at 4°C until required. Quantification of the extracted DNA from the various strains was done on a 1% agarose gel using a range of DNA standards and a 1 kb DNA ladder.

### 2.2.3.2 PCR of the 16S rRNA gene

Amplification of the 16S rRNA genes was performed by PCR as described by Seal *et al.* (1993) using the specific primers OLI1 and Y2 (Appendix B). These primers amplify 287 to 288 bp of *R. solanacearum* and the closely related species, *Pseudomonas syzygii*. The PCR products were visualized by loading 10 µl in a 1% (w/v) agarose/1x TAE (pH 8.0) gel containing 0.1 µg/ml ethidium bromide (EtBr). The gel was run for 1 hr at 80 Volts (V). DNA was visualized under an ultraviolet light and an image was taken with White/Ultraviolet Transilluminator (Ultra Violet Products Ltd., Cambridge, UK).

### 2.2.3.3 PCR-RFLP

Three different primer pairs, RS20 and RS201, RS600 and RS61, RS80 and RS81 (Appendix B), specific for the *hrp* genes of *R. solanacearum*, were used in the PCR reaction (Poussier *et al.* 1999). Primers were designed using the nucleotide sequence of the *hrp* gene region of strain GMI 1000 of *R. solanacearum* (accession no.Z14056 EMBL-Genbank-DDBJ databases). The location of these primers on the *hrp* genes of the reference strain GMI 1000 is illustrated in Fig. 2.1.

The PCR analysis was carried out in a total volume of 20 µl and was conducted in 0.2 ml PCR tubes (ABgene®, Epsom, Surrey, UK) in a GeneAmp® PCR system 2700 (Applied Biosystems, Foster City, California, USA). The reaction mixture contained 1x *Taq* reaction buffer (Gibco BRL Life Technologies, Cergy Pontoise, France), 250 µM of each dNTP's, 1 µM of each primer (Genosys Biotechnologies, Cambridge, England), 2 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Gibco BRL) and 2 µl of genomic DNA (200 - 400 ng) as template. The total reaction volume was made up using sterile distilled water. Negative controls contained all the PCR reagents but no template. A positive control contained 200 ng genomic DNA of the characterized sample GMI 1000.

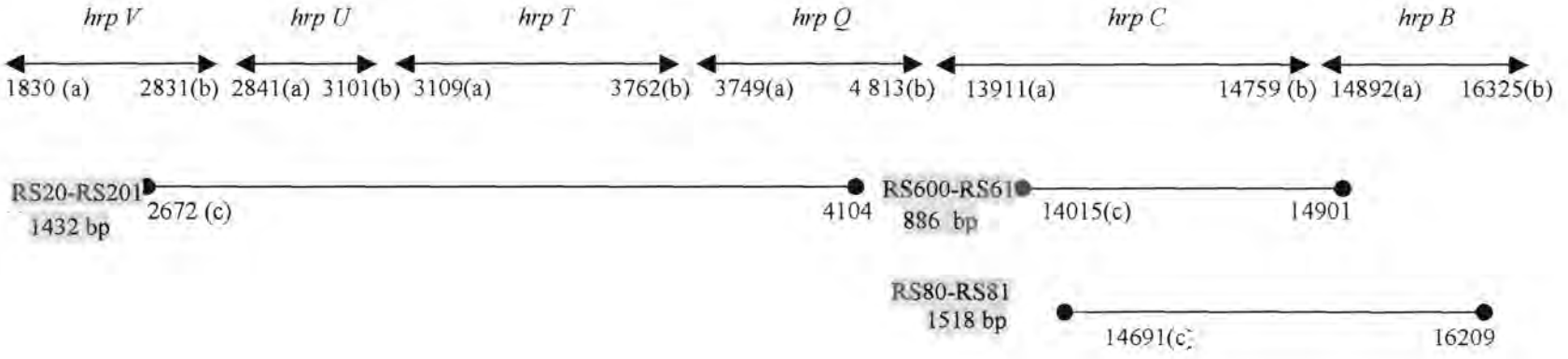



**Figure 2.1: Location of the three selective primer pairs (RS20-RS201, RS600-RS61, RS80-RS81), within the *hrp* genes of *R. solanacearum* reference strain GMI 1000 (Poussier *et al.* 1999).** Lines with arrowheads indicate the genes of *R. solanacearum* with the various *hrp* clusters given above. Lines with circles illustrate the PCR product obtained with the specific primers, to the left of the line and the expected size of the PCR product in highlighted in gray. (a) Nucleotide number of the base at the 5' end of the DNA sequence (accession no. Z14056 EMBL-Genbank-DDBJ databases). (b) Number of the base at the 3' end of the DNA sequence. (c) Number of the base at the 5' end of the primer.

The PCR cycling conditions used included an initial denaturation of 96°C for 2 min. This was followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 68°C for 20 s and elongation at 72°C for 30 s. A final elongation at 72°C for 10 min, then a 4°C hold for 30 s and a final hold at 15°C were included. The PCR products were analysed as described above.

For RFLP, digestions of the PCR products were performed as follows: Fragment 1 (RS20-RS201), with restriction enzymes (RE) *Ava*I and *Pvu*II; Fragment 2 (RS600-RS61) with RE *Hae*II and Fragment 3 (RS80-RS81) with RE *BssH*II. Digestions were carried out in 1.5 ml Eppendorf tubes containing 2 µl specified buffer, 1 U of the enzyme, 12 µl of the PCR product and sterile distilled water to give a final volume of 20 µl and incubated at 37°C for 3 hr. Roche (Roche Molecular Biochemicals, Mannheim, Germany) supplied all enzymes. Five µl of each sample, mixed with loading buffer was loaded onto a 1.5% agarose gel. The gel was run for 2 hr and 30 min at 80 V and then stained with EtBr for 30 min. The gel was visualized under an ultraviolet light. Each PCR-RFLP run was repeated at least twice to confirm reproducibility.

#### 2.2.3.4 Amplified Fragment Length Polymorphism (AFLP) analysis

The QIAamp Dneasy Tissue Kit® (Qiagen Ltd., Crawley, West Sussex, UK) was used to extract DNA from the selected strains according to the manufacturer's protocol. The AFLP protocol as described by Poussier *et al.* (2002) was used. An amount of 200 ng per sample of genomic DNA was digested for 2 hr at 37°C in a final volume of 50 µl, containing 5 U *Msp*I, 5 U *Sac*I (Amersham Biosiences UK Limited, Little Chalfont, Bucks, UK), 0.125 µl BSA (10 µg/ul ) and 2.5 µl 10x 'One Phor All' buffer (Amersham). Ligation was performed by adding 50 pmol double stranded *Msp*I-adapter (5'-GACGATGAGTCCTGAA-3', 5'-CGTTCAGGACTCATC-3'), 5 pmol double stranded *Sac*I adapter (5'-CTCGTAGACTGCGTACAAGCT-3', 5'-TGTACGCAGTCTAC-3') (Genset, Paris, France), 1 µl ATP (10 mM), 1 U T4 DNA ligase (Appligene, Illkirch, Graffenstaden, France) and 2.5 µl 10x 'One Phor All' buffer (Amersham Biosciences) to

the DNA, and incubated for 3 hr at 20°C. The resulting digested, ligated DNA was diluted 8-fold and then stored at 4°C until needed.

Selective amplifications were done with two primers (*Msp*I and *Sac*I, Genset) complementary to the adapter sequences, and the *Msp*I and *Sac*I restriction sites respectively, with additional selective nucleotides at their 3' ends (cytosine for the *Sac*I primer and cytosine plus guanine for the *Msp*I primer). The *Sac*I primer was labelled with $\gamma$-$^{33}$ P [10 µCi (370 kBq) per DNA amplification; (NEN Life Science Products, Boston, USA)] and T4 polynucleotide kinase (Gibco-BRL).

DNA amplifications were carried out in a 50 µl reaction mixture. The template, 5 µl of DNA (dilute, ligated, digested), was added to 45 µl mixture [1x PCR buffer (Gibco-BRL), 1.5 µM labelled *Sac*I primer, 1.2 µM unlabeled *Msp*I primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 3 U *Taq* polymerase (Gibco-BRL)]. Amplifications were performed with a thermocycler (Mastercycler gradient Eppendorf) by using the following reaction cycle: 30 cycles of (94°C for 30 s; 56°C for 1 min; 72°C for 1 min).
PCR products were separated by electrophoresis using 5% polyacryalamide gels and fingerprint patterns were visualized as described by Vos *et al.* (1995). An image was fixed on high performance autoradiogram film. This fixed image was scanned and the digital gel images were imported into the scoring program AFLP-Quantar™ software program (version 1.05, KeyGene products B.V. Wageningen, The Nederlands). Binary data (presence/absence of bands) were obtained and imported into TM4 software and analysed with MEV (Saeed *et al.*, 2003) to generate a hierarchical cluster.

## 2.3 RESULTS

### 2.3.1. PHENOTYPIC METHODS

### 2.3.1.1. Morphological characteristics

All the strains were Gram-negative, rod-shaped cells and had polar flagella. Two strains, BCC 0305 and 0306 (92 and 62) produced a brown pigment on nutrient agar. Eight strains, BCC 0300, 0301, 0302, 0303, 0304, 0305, 0306 and 0327 (GMI 1000, K, CK, CC, 117, 92, 62 and 27B), had colonies that were fluidal and were more than 5 mm in diameter on BGT media (Fig. 2.2). The colonies of the remaining strains (data not shown) varied from tenacious, minute to viscid and <5 mm on this medium.

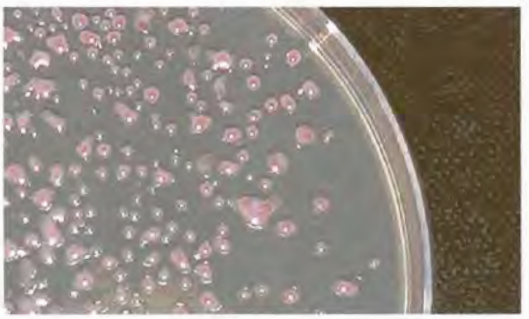


**Figure 2.2. Characteristic mucoid colonies of virulent *R. solanacearum* isolates on BGT medium.**

### 2.3.1.2. Biovar differentiation

The results (Table 2.2) show that strains BCC 0305 (92) and BCC 0306 (62) were unable to utilize or oxidize the hexose alcohols (mannitol, sorbitol and dulcitol) and therefore belong to biovar 2. While BCC 0300-0304 and 0327 (GMI 1000, K, CK, CC, 117 and 27B) were able to utilize the above mentioned hexose alcohols and therefore belong to biovar 3. The other strains were not tested on their ability to metabolise the hexose alcohols and disaccharides because their phenotype weren't characteristic of *R. solanacearum*.

**Table 2.2. Differentiation of *Ralstonia solanacearum* isolates into Biovars based on the ability to metabolise hexose alcohols and disaccharides**

| Isolates | BCC GMI | 0300 1000 | 0301 K | 0302 CK | 0303 CC | 0304 117 | 0305 92 | 0306 62 | 0327 27B | *E.coli* |
|---|---|---|---|---|---|---|---|---|---|---|
| **Oxidation/utilization of hexose alcohols** | | | | | | | | | | |
| **Mannitol** | | + | + | + | + | + | - | - | + | + |
| **Sorbitol** | | + | + | + | + | + | - | - | + | + |
| **Dulcitol** | | + | + | + | + | + | - | - | + | + |
| **Glucose** | | + | + | + | + | + | + | + | + | + |
| **Oxidation of Disaccharides** | | | | | | | | | | |
| **Lactose** | | + | + | + | + | + | + | + | + | + |
| **Maltose** | | + | + | + | + | + | + | + | + | + |
| **Biovar** | | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | |

## 2.3.2. MOLECULAR METHODS

### 2.3.2.1. DNA extraction

High molecular weight DNA (>>12 kb) was obtained from all the strains listed in Table 2.1 (Figure 2.3).

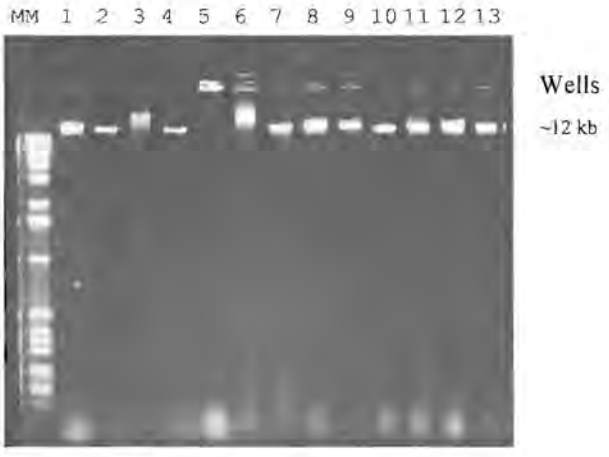



**Figure 2.3. Genomic DNA of *R. solanacearum* extracted using the method of Chen and Kuo (1993).** MM: 1 kb DNA ladder; lanes 1, 2, 4, 7-13: High molecular weight DNA extracted; lanes 3, 5 and 6: poor quality DNA; lane 5: protein residue in well.

### 2.3.2.2. PCR of the 16S rRNA gene

Figure 2.4 shows that a fragment of approximately 290 base pairs of the 16S rRNA was amplified from the seven verified strains of *R. solanacearum.* Twenty-seven of the 42 bacterial isolates referred to in Table 2.1 (BCC 0308-0319, 0321, 0323, 0327 (27B), 0332, 0333, 0338-0348) (Fig. 2.4), revealed the expected PCR product (results not shown with exception of BCC 0310 and 0311).

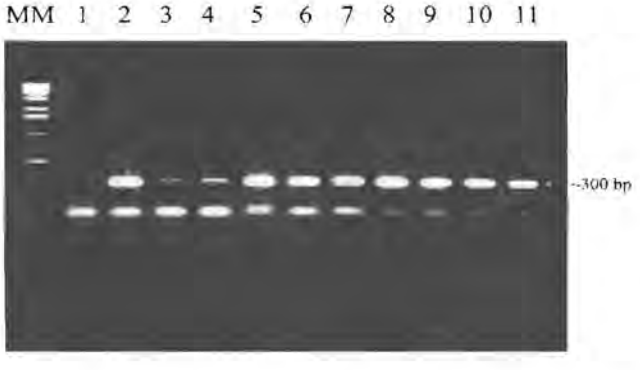



**Figure 2.4. PCR amplification of the 16S rRNA region of the bacterial isolates.** MM: 1 kb DNA marker; lane 1: negative control with primer dimers present. Lane 2: positive control, lane 3 and 4: Ugandan *Eucalyptus* isolates, BCC 0310 and 0311 (GU 68 and GU 69), weak amplification; lane 5: BCC 0300 (GMI 1000); lanes 6 and 7: Congolese *Eucalyptus* isolates, BCC 0302 and 0303 (CK and CC); lane 8: BCC 0301 (K); lanes 9-11: SA Potato isolates, BCC 0304-0306 (117, 92 and 62). The ~300 bp product indicates the PCR fragment.

### 2.3.2.3. PCR-RFLP

One primer pair (RS600 and RS61) was randomly chosen to screen the 27 strains that contained the 16S rRNA amplicon. Only BCC 0327 (27B) from the 27 strains and the seven verified *R. solanacearum* strains had the expected 900 bp product (Fig. 2.5). Based on these results, these eight strains were chosen used for further testing.

DNA from these strains was amplified with the three different primers specific to the *hrp* genes of *R. solanacearum*. Band density was variable depending on a combination of the primer pair and the bacterial DNA used. The primers, RS20 and RS201, produced faint bands with strain BCC 0305 (92), while the primers, RS600 and RS61, gave a poor product with strain BCC 0301 (K) (Fig. 2.6, lanes 8 and 13 respectively). This influenced the RFLP results (Fig. 2.7). It is difficult to explain the link between these two variables, especially taking into account that the same amount of template DNA was used for each strain in every PCR reaction. All the samples were amplified in the same run and prepared from the same master mix.

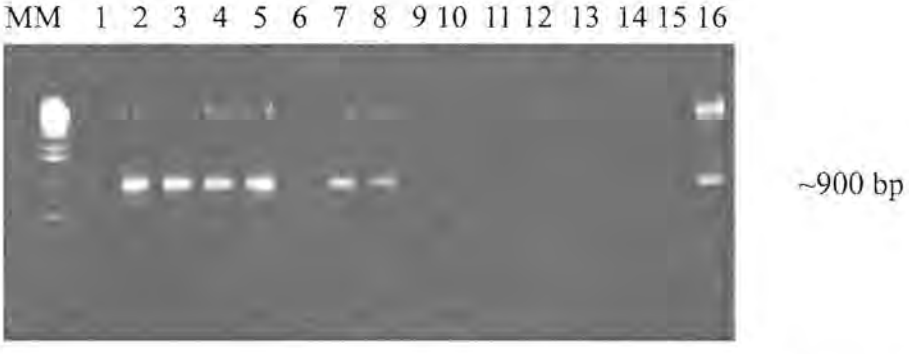



**Figure 2.5. Selective amplification of the *hrpC/B* gene fragment of *R. solanacearum* using primers RS600 and RS61**. MM: 1 kb molecular marker; lane 1: negative control with sterile distilled water as template; lane 2: positive control, BCC 0300 (GMI 1000). Lane 3-5: BCC 0302 (CK), BCC 0303 (CC) and BCC 0305 (92); lane 6: BCC 0301, (K), weak amplification, lane 7: BCC 0306 (62), lane 8: BCC 0327 (27B), lanes 9-15: BCC 0310 (GU 68), BCC 0311 (GU 69), BCC 0339 (3), BCC 0340 (4), BCC 0341 (5), BCC 0344 (9) and BCC 0347 (13): no amplification; lane 16: BCC 0327 (27B)

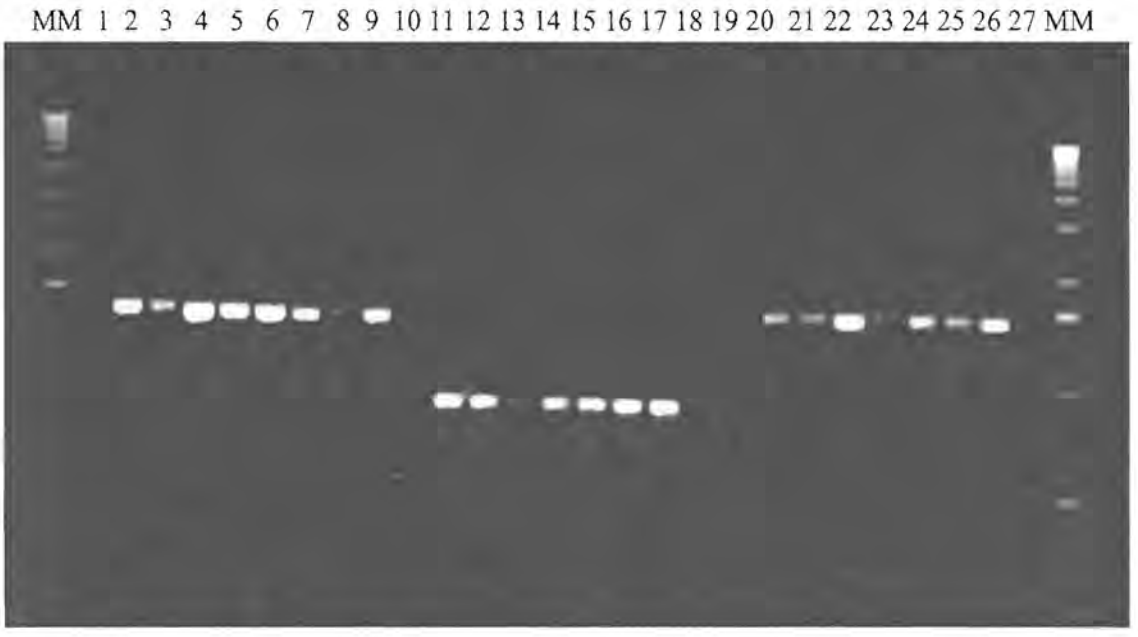



**Figure 2.6. Three PCR fragments of the *hrp* cluster amplified from *R. solanacearum*** The three primer pairs were RS20-RS201 (fragment 1, ~1500 base pairs (bp), lanes 1-9), RS600-RS61(fragment 2, ~900 bp, lanes 10-18) and RS80-RS81(fragment 3, ~1400 bp, lanes 19-27) (Poussier *et al.*, 1999). MM: 1 kb DNA ladder; lanes 1, 10 and 19: negative control containing sd$H_2O$; lanes 2, 11 and 20: positive control with reference strain GMI 1000; lanes 3,12 and 21: Ugandan isolate, BCC 0327 (27B); lanes 4,13 and 22: SA Eucalyptus strain, BCC 0301 (K); lanes 5, 14 and 23: Congolese strain, BCC 0302 (CK); lanes 6, 15 and 24: Congolese strain, BCC 0303, (CC); lanes 7, 16 and 25: potato isolate, BCC 0306 (62); lanes 8, 17 and 26: potato isolate, BCC 0305 (92); lanes 9, 18 and 27: potato isolate, BCC 0304 (117).

After amplification the PCR products, subjected to enzyme digestion, had two to four bands. Different PCR-RFLP patterns were found; *Ava*I had two patterns (AAv2 and AAv3), *Pvu*II had three patterns (APv1, APv2 and APv3), *Hae*II had two patterns (BHa2 and BHa3) and *BssH*II had two patterns (CBs2 and CBs3) (Fig2.7).

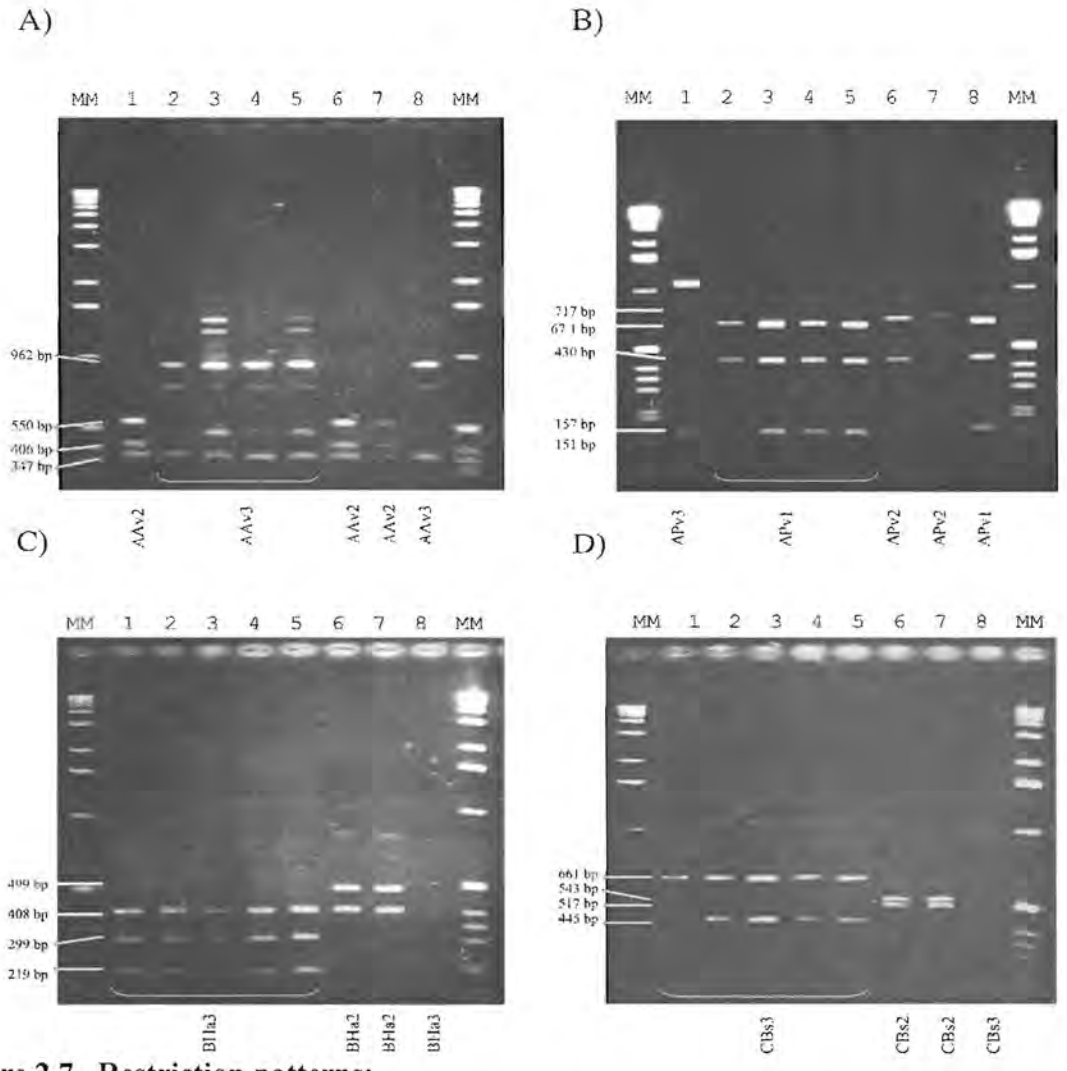



**Figure 2.7. Restriction patterns:**

**A) Fragment 1 digested with *Ava*I** (AAv), **B) Fragment 1 digested with *Pvu*II** (APv), **C) Fragment 2 digested with *Hae*II** (BHa) **and D) Fragment 3 digested with *BssH*II** (CBs). MM: 1 kb DNA Molecular marker; lane 1, BCC 0300 (GMI 1000); lane 2: BCC 0327 (27B); lane 3: BCC 0301 (K); lane 4: BCC 0302 (CK); lane 5: BCC 0303 (CC); lane 6: BCC 0304 (117); lane 7: BCC 0306 (62) and lane 8: BCC 0305 (92). **A)** Lanes 3 and 5: top two bands were partial digest products. Lane 7: Faint bands, digest pattern, AAv2. **B**) Lane 7: Faint bands, digest pattern, APv2. **C)** Lane 8: Faint bands, digest pattern, BHa3. **D)** Lane 8: Faint bands, digest pattern, CBs3. The size of the bands in base pairs was estimated from the sequence of the *hrp* gene region of strain GMI 1000 (Poussier *et al.* 1999).

PCR-RFLP results were summarized in Table 2.3. Here a clear distinction can be made between biovars 2 and 3. It is interesting is that biovar 3 split into two groups differentiating between BCC 0300(GMI 1000), the reference strain and the other biovar isolates.

**Table 2.3. Classification of the PCR-RFLP patterns identified between the eight strains of *R.solanacearum* into Biovars**

| | ***Ava I*** | ***Pvu II*** | ***Hae II*** | ***BssH II*** | **Strains BCC** | **Biovar** |
|---|---|---|---|---|---|---|
| **Group I** | AAv2 | APv3 | BHa3 | CBs3 | 0300 (GMI 1000) | 3 |
| **Group II** | AAv3 | APv1 | BHa3 | CBs3 | 0327 (27B), 0301 (K), 0302 (CK), 0303 (CC), 0304 (117) | 3 |
| **Group III** | AAv2 | APv2 | BHa2 | CBs2 | 0306 (62), 0305 (92) | 2 |

### 2.3.2.4. AFLP analysis

Visual examination of the fixed image produced by the AFLP analysis enabled us to compare the different isolates. It was possible to distinguish between biovars biovar 3 (BCC 0300 (GMI 1000), BCC 0302 (CK), BCC 0303 (CC) and BCC 0304 (117)) and biovar 2 (BCC 0306 (62) and BCC 0305 (92)) isolates (Fig. 2.8).

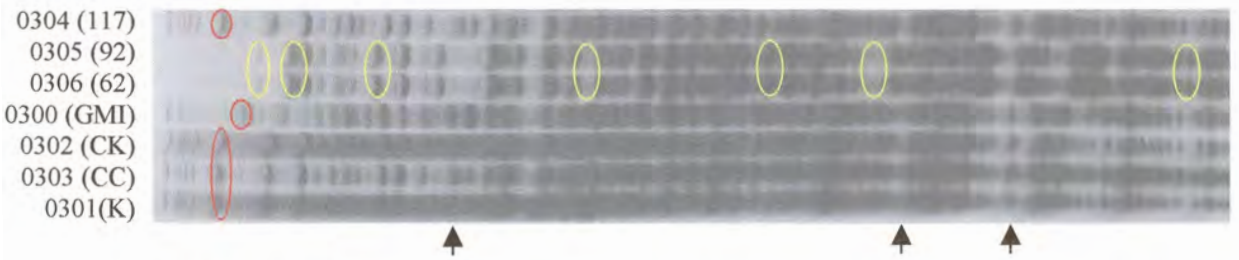



**Figure 2.8. AFLP data viewed as an image on high performance autoradiogram film.** A clear distinction can be made between biovar 3 (BCC 0300 (GMI 1000), BCC 0302 (CK), BCC 0303 (CC) and BCC 0304 (117)) and biovar 2 (BCC 0306 (62) and BCC 0305 (92)) isolates. Yellow ovals show the similarities between the two, biovar 2 strains, BCC 0306 (62) and BCC 0303 (92). Red ovals show one of the differences between the strain GMI 1000 and the other biovar 3 strains. Black arrows show the similarity between the four, biovar 3 strains.

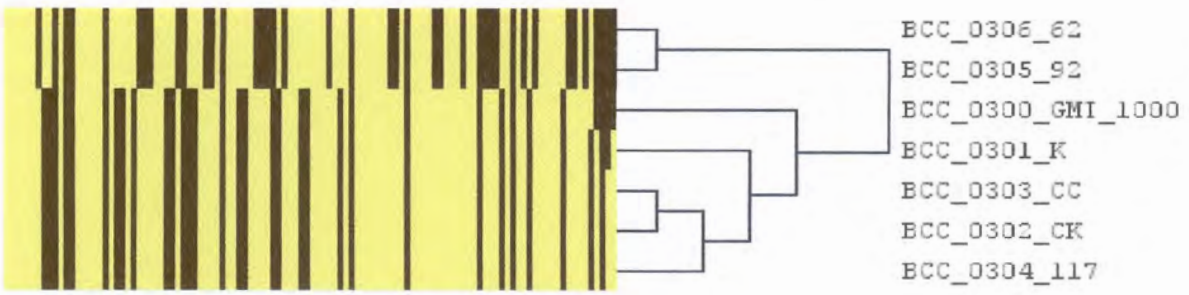



**Figure 2.9. AFLP data viewed as a dendogram.**

The hierarchical cluster analysis (Fig. 2.9) revealed that the biovar 2 isolates (BCC 0306 (62) and BCC 0305 (92)) grouped closely together. The same was true for the Congolese isolates, BCC 0302 (CK) and BCC 0303 (CC). From the data in Fig. 2.9 the Congolese isolates are grouped closer to the biovar 3, potato isolate (BCC 0304 (117)) than to the South African *Eucalyptus* isolate (BCC 0301 (K)), and the last isolate to fall in the cluster was BCC 0300 (GMI 1000). This data confirmed the PCR-RFLP results and thus verifies the PCR-RFLP method as reliable.

## 2.4. DISCUSSION

The hypothesis of this chapter was that the Bacterial Wilt of *Eucalyptus* in the Congo and Uganda was caused by the world-renowned wilt pathogen, *R. solanacearum*. A secondary objective was to develop fast and reliable identification of *R. solanacearum* in order to employ management strategies as soon as possible after an outbreak occurs.

Forty-nine isolates of what was believed to be *R. solanacearum* were used in this study. The samples originated from French Guyana, Uganda, Congo and South Africa. The objective of this study was to identify which *Eucalyptus* bacterial wilt samples were indeed *R. solanacearum* and to fully characterize them. The strain, BCC 0300 (GMI 1000), was used as positive control and reference strain due to the fact that it had been used in previous publications (Poussier *et al.*, 1999, 2000a).

Hayward established the traditional approach for classifying *R. solanacearum* into biovars in 1964 (Hayward, 1964; 1991; 1994). This is based on the utilization of single alcohols and carbohydrates. Several carbon sources can be used but differentiation into biovars can be determined by utilization of these hexose alcohols: mannitol, sorbitol and dulcitol plus oxidation of lactose, maltose and D (+) cellobiose. The identity of 41 strains could not be established on the basis of phenotypic characterization (Hayward, 1964), used in this study. The unknown strains should have been discarded at this stage, but due to my limited experience in the field of bacterial morphology, they were submitted to molecular tests.

OLI1 and Y2 primer-pairs (Seal *et al.*, 1993) from the 16S rRNA area, known to amplify *R. solanacearum* and nearby family members such as *R. syzygii* and the Blood Disease bacterium were used. Samples that yielded a bright broad band most likely had the highest homology to the primer pair used, while amplification was also obtained with notably less product, seemingly lower homology. At this stage we are trying to sequence these low homology strains to verify if these might be another bacterial pathogen of *Eucalyptus*.

The 34 isolates (seven of known and 27 of unknown identity) were also subjected to the three *hrp*-primer pairs test designed by Poussier *et al.* (1999), see Fig. 2.4. These primer pairs could amplify only eight isolates, confirming that these eight isolates were definitely *R. solanacearum*. The isolates were the following: South African potato isolates: BCC 0304 (117), BCC 0305 (92), BCC 0306 (62); SA *Eucalyptus* isolate: BCC 0301 (K); Congolese *Eucalyptus* isolates: BCC 0302 (CK) and BCC 0303 (CC); Ugandan *Eucalyptus* isolate: BCC 0327 (27B) and the reference strain from tomato: BCC 0300 (GMI 1000) (Table 2.1). This result supports the hypothesis that *R. solanacearum* as present in Uganda and Congo. Varying band density was observed between the amplified DNA isolates. Determining the reason behind this was difficult, since the same sample did not yield a poor DNA product during the different amplifications. All samples were amplified in the same run, prepared from the same DNA aliquot and master mix. Southern hybridisation studies of the digested DNA hybridised with part of the *hrp*-gene isolated from GMI 1000, would complement these results showing the presence of the *hrp* gene due higher sensitivity.

Restriction Enzyme Digestion, first described by Poussier *et al.* (1999) was used for further confirmation of the eight isolates used in this study. Together with the PCR amplification the technique is known as PCR-RFLP. Poussier *et al.* (1999) assessed the genetic diversity among a worldwide collection consisting of 120 strains of *R. solanacearum,* using RFLP of the *hrp* gene region. Fifteen different profiles were identified and grouped the strains into eight clusters. These clusters could be linked to biovars. This enabled comparison of the results described in this study. The two potato isolates grouped in biovar 2, while one potato isolate, the South African *Eucalyptus* isolate, two Congo *Eucalyptus* isolates and one Ugandan *Eucalyptus* isolate grouped under biovar 3. The control, BCC 0300 (GMI 1000), from French Guyana also grouped in biovar 3 but under a different RFLP group (Table 2.3). None of the African isolates (BCC 0301-0306 and 0327) studied here grouped in the 'Africanum' division suggested by Poussier *et al.*, (2000a; 2000b).

Clear differences could be distinguished between the biovar 2 and biovar 3 isolates after the AFLP analysis (Fig. 2.8) with respect to presence or absence of bands. This AFLP-autoradiogram data were analysed further and the resulting dendogram, Fig. 2.9, revealed the close grouping of the biovar 2 and biovar 3 groups, respectively. The differences in the biovar 3 cluster were due to three polymorphisms that were present and, were not considered further in this chapter.

Since 1964 many molecular methods involving PCR (Fegan *et al.*, 1998, Seal *et al.*, 1993) have been investigated, aiming to rapidly identify *R. solanacearum* as the cause of bacterial wilt but none could distinguish between the biovars with such ease and clarity as the methods described here. However multiplex, real-time, fluorogenic PCR assay offered potential advantages in the routine indexing of potato tubers and other plant material for *R. solanacearum* (Weller *et al.*, 2000). Four primer pairs have been published to amplify *R. solanacearum* but were not able to differentiate it from closely related family (Denny and Hayward, 2001). Although these four primer pairs have the ability to detect *R. solanacearum* under high dilutions, they have not been exhaustively tested for sensitivity and specificity, so should be used with caution (Denny and Hayward, 2001).

The molecular method presented here, PCR-RFLP, is much less time-consuming and producing reliable and accurate data 72 hr after the primary isolation from the field. Furthermore the method is easy and reliable. Cost is low, after initial set-up of the Thermocycler and primer pairs, the maintenance of the system is limited to disposables such as PCR reagents. This diagnostic technique will enable researchers to identify the pathogen rapidly. Management strategies can then be implemented within one week after a new outbreak occurs.

# CHAPTER 3

# The susceptibility and resistance of different ecotypes of *Arabidopsis thaliana* to African *Eucalyptus* strains of *Ralstonia solanacearum*

## 3.1. INTRODUCTION

*Arabidopsis thaliana* has been widely used as a model plant for studying plant-pathogen interactions as well as stress responses to name only a few. It has a small diploid genome with little repetitive DNA. *A. thaliana* are self-fertile plants that reproduce very efficiently in just six to eight weeks bringing forth thousands of seeds per plant (Anderson and Wilson, 2000). Another advantage is the natural genetic diversity within *A. thaliana* with the different ecotypes that exist. This has enabled scientists to exploit the genetic variability.

Control of the pathogen *R. solanacearum,* which is a serious threat not only to forestry in Southern Africa, but also to the potato industry in South Africa, is limited to agricultural practices. No universal solution exists, only principles that can be applied and adapted in particular situations (Hayward, 1991). Hayward (1991) reviewed the situation of bacterial wilt and defined the following control strategies: cropping systems, soil amendment, disease avoidance, integrated control and biological control. The most effective strategy remains breeding for host-plant resistance, but until 1998 simple inherited resistance had not been clearly demonstrated (Deslandes *et al.,* 1998). The genetic basis of resistance to *R. solanacearum* has been studied extensively in tomato, and revealed complex mechanisms of polygenic resistance governed by several loci (Thoquet *et al.,* 1996a; 1996b).

The nature of the pathogen, the limitations of available control strategies for the disease as well as the economical and social importance of the crops affected by *R. solanacearum* gave reason to use *A. thaliana* as the model plant. Deslandes *et al.,* (1998) developed a new pathosystem between *A. thaliana* and *R. solanacearum.* Recently the genomes of this soil-borne bacterium (Salanoubat *et al.*, 2002), and the plant (The *Arabidopsis* Genome Initiative, 2000) have been sequenced. This has paved the way for a better understanding of resistance and susceptibility.

Hammond-Kosack and Jones (1996) described resistance (or pathogen failure) as the result of one of the following reasons. (1) The plant is a non-host, i.e. it cannot support pathogen invasion and growth, (2) preformed structural barriers and toxic compounds are present that limits pathogen

growth or, (3) the plant recognizes the pathogen and forms defence mechanisms that localizes the pathogen and restricts growth (Heath, 2000).

Compatibility (susceptibility) is described as where the plant does not recognize the pathogen, preformed barriers as well as activated responses are ineffective and the pathogen invades the plant leading to either latent infection or disease, and thus plant death. 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).

Various genes that affect regulation of defence and thus resistance in *A. thaliana* have been disclosed. RRS1 (recognition of *R. solanacearum*) was identified by Deslandes *et al.* in 1998 and characterised in 2002, this was specific for the ecotype, Nederzenz-1 (Nd-1) and confers resistance to several strains of *R. solanacearum*. In the latter paper they illustrated that the dominant (RRS1-S) and the recessive (RRS1-R) alleles from susceptible and resistant accessions encode highly similar predicted proteins. Sequence analysis of the RRS1 genes present in two homozygous intragenic recombinant lines revealed that several domains of the RRS1-R are essential for its resistance function (Deslandes *et al.*, 2002).

PopP2 was identified as the corresponding Avr protein from *R. solanacearum* to RRS1-R (Deslandes *et al.*, 2003). These two proteins interacted in yeast, and targeted the nucleus, but only when both were complete (i.e. no deletions or only some part of the respective proteins) and were present at the same time. Nd- 1 (RRS1-R) plants are resistant to GMI 1000 and no wilting symptoms develop after inoculation. However, upon inoculation with a mutated Δ*popP2* bacterial strain, symptoms developed similar to those in a susceptible interaction. Bacterial complementation with the cloned *popP2* gene reversed this virulence observed in Nd-1 plants, confirming the role in resistance.

The presence of PopP2 was investigated (Lavie *et al.*, 2004). They were able to verify that popP2 was present in most 'Asiaticum' (Biovars 3,4 and 5) and 'African' (Biovar 1) isolates but were generally absent from the 'Americanum' (other Biovar 1 and 2) isolates. They did however establish that these genes were not essential for virulence since a mutant strain derived from BCC

0300 (GMI 1000) strain with disabled PopP2 and PopP3 genes was still able to cause disease on tomato and *A. thaliana* (Lavie *et al.*, 2004).

The aim of this chapter was to identify a resistant *A. thaliana* ecotype as well as a susceptible ecotype to at least one of the African isolates of *R. solanacearum.*

The virulence of seven isolates, respectively from the Congo, Uganda and South Africa was tested against eight *A. thaliana* ecotypes. The strain BCC 0300 (GMI 1000) from French Guyana was used as a positive control (Table 3.1). Subsequently, a DNA fragment of the RRS1 locus of *A. thaliana* ecotypes used in the virulence studies was sequenced. The presence of the PopP2 gene was also investigated by PCR analysis. These molecular characterisations of the host and the pathogen were done in an attempt to select a pathosystem that is different from the previously *A. thaliana* ecotype Nederzenz-1 (Nd-1) and *R. solanacearum* (BCC 0300 (GMI 1000)), pathosystem (Deslandes *et al.*, 1998; Lavie *et al.*, 2004).

## 3.2. MATERIALS AND METHODS

### 3.2.1. Bacterial isolates

The *R. solanacearum* isolates selected were: BCC 0300-0306 and 0327 (GMI 1000, K, CK, CC, 117, 92, 62 and 27B).

### 3.2.2. Plant materials

#### 3.2.2.1. *Arabidopsis thaliana* ecotypes

Ecotypes presented in this study are presented in Table 3.1

**Table 3.1. Phenotypic description of Ecotypes used in this study**

| Name code | Name | Origin | Leaf hairiness | Leaf posture | Rosette size and compactness | Leaf margin |
|---|---|---|---|---|---|---|
| Col-5* | Columbia | USA | Glabrous or smooth | Half upright | Medium, compact rosette | Slightly serrated |
| Nd-1* | Nedersenz | Germany | Weakly hairy | Incompletely flat | Medium, very loose rosette | Sinuate |
| Kil-0* | Killean | UK | Hairy | Upright | Large, loose rosette | Slightly serrated |
| Be-O* | Bensheim | Germany | Weakly hairy | Upright | Very large, loose rosette | Dentate |
| Sf-2* | Not available | Spain | Hairy | Strongly upright | Very large, loose rosette | Serrate |
| Laer** (Ler-O) | Landsberg erecta | Germany | Hairy | Not available | Not available | Smooth |
| Cvi * | Cape Verde Islands | Cape Verde Islands | Hairy | Strongly upright | Medium, very loose rosette | Smooth |

*http://www.shingen.nig.ac.jp
** http://www.arabidopsis.org

### 3.2.2.2. Seed preparation

Preparation of the seeds was done one calendar month in advance of the planned inoculation. MS medium (Murashige and Skoog, 1962) was poured in 90-mm Petri-dishes under sterile conditions. Seeds were soaked for three hr to overnight in 1 ml of the fungicide Benlate® (2,5 gram/L) and afterwards rinsed with sterile distilled water. 1 ml of Jik® (2,4%) was added to the seeds for 15 min. The seeds were washed three times with sterile distilled water. 300 µl of the seed suspension was pipetted onto the MS plate with 700 µl of sterile distilled water. The plate was then shaken in such a manner that seeds spread over the whole surface. Finally, the plate was turned at an angle of 45° to allow excess water to collect that was removed with a pipette. Seedlings were grown for one week at 20°C in a growth chamber under constant fluorescent light.

### 3.2.2.3. Plant growth conditions

The seedlings were carefully removed from the agar with metal forceps and transferred to Jiffy pots (Jiffy France, Lyon, France). The trays were then covered with cling wrap. After 24 hr, slits were cut in the cling wrap. After 48 hr the cling wrap was removed completely. Plants were watered well with distilled water on a regular basis to keep Jiffy pots moist. Care was taken not to water the seedlings from the top. Plants were allowed to grow for three weeks in short day conditions (10 hr/14 hr, day/night regime) under constant light at 500 $\mu E/s/m^2$.

## 3.2.3. Plant inoculation

### 3.2.3.1. Inoculum

Inoculum was prepared under sterile conditions. Four individual colonies were transferred to 500 ml of B broth. The flasks were then incubated on a shaker at 100 rpm for 20 hr. Just before inoculation, the concentrations of the bacterial suspensions for each isolate was determined with a spectrophotometer (Spectronic®, 20 Genesys™, Rochester, USA), with a fixed wave-length of 600 nm and using a ten fold dilution. The dilution was prepared as follows: 10 µl of bacterial broth in 90 µl of sterile broth was used. The absorbance was converted to colony forming units (CFU): 1 $OD_{600}$ = 1 $x10^9$ CFU /µl. This was then diluted to a give a final concentration of 1 $x10^9$ CFU /µl. Two litre inoculum suspensions were used per tray (100 jiffy pots per tray).

### 3.2.3.2. Preparation of the plants

If any flowers were present, they were removed. Root wounding was induced by removing the bottom third of the Jiffy pot, approximately 2 cm, with scissors. Contamination with the different strains was limited by placing plants with exposed roots in separate trays without drainage holes. The inoculum was added to the tray in such a manner that the Jiffy pots were almost completely

covered. After 20 min the plants were transferred to a tray covered with about 3 cm of soil (Soil for balcony plants and Geranium base dressing, Hawitaflor, Vechta, Germany). The excess bacterial suspension was autoclaved and discarded. The plants were then transferred to a growth chamber at 25°C (16 hr/8 hr, day/night, constant light 500 µE/s/$m^2$).

### 3.2.4. Rating of disease progress on *A. thaliana*

After inoculation the plants were evaluated daily for wilt symptom development. The plants were rated on a scale from zero (no disease) up to 4 (100% wilted/dead plants), (see Table 3.2, Fig. 3.1). Subsequently the data was used to calculate the Disease Index using the formula:

$$\mathbf{DI = [\sum( n_i \times v_i) / (V \times N)]}$$

Where DI = Disease Index; $n_i$ = number of plants with respective disease rating; $v_i$ = disease rating (0, 1, 2, 3 or 4); V = the highest disease rating (4); and N = the number of plants observed. Published by Winstead and Kelman, (1952) used with slight modification as described here.

**Table 3.2: Rating scale of wilted *A. thaliana* plants inoculated with *R. solanacearum***

| Scale | Description |
|---|---|
| 0 | No Disease |
| 0.5 | First leaf wilting |
| 1 | 2 or more leaves wilted, less than 25% of the leaves wilted |
| 2 | Less than 50% of the leaves wilted |
| 3 | More than 50% to 75% of the leaves wilted |
| 4 | 76-100% wilt, the plant yellows and dies |

### 3.2.5. Experimental planning

During the preliminary experiments, the control strain BCC 0300 (GMI 1000), was inoculated on Col-5, the susceptible ecotype, and Nd-1, the resistant ecotype. Disease development was classic, with wilting of Col-5 within a week and no symptom development on Nd-1 (Deslandes *et al.*, 1998).

The first trial (April 2003) included ecotypes: Col-5, Be-O, Kil-O, Sf-2, Nd-1 and Cvi, the second and third trials (May 2003) included the same ecotypes than above as well as the ecotype Laer. During November 2003 a final repeat was carried out including the most resistant ecotypes, Sf-2 and Kil-O and the most susceptible ecotypes, Be-O and Col-5. In each trial at least six plants per ecotype was inoculated with each isolate. Two to three replicates were included in each trail. Ecotypes that were screened with the various *R. solanacearum* isolates are presented in Table 3.1. BCC 0300 (GMI 1000) was used as the control strain throughout the study to determine if environmental factors were optimal for disease development.

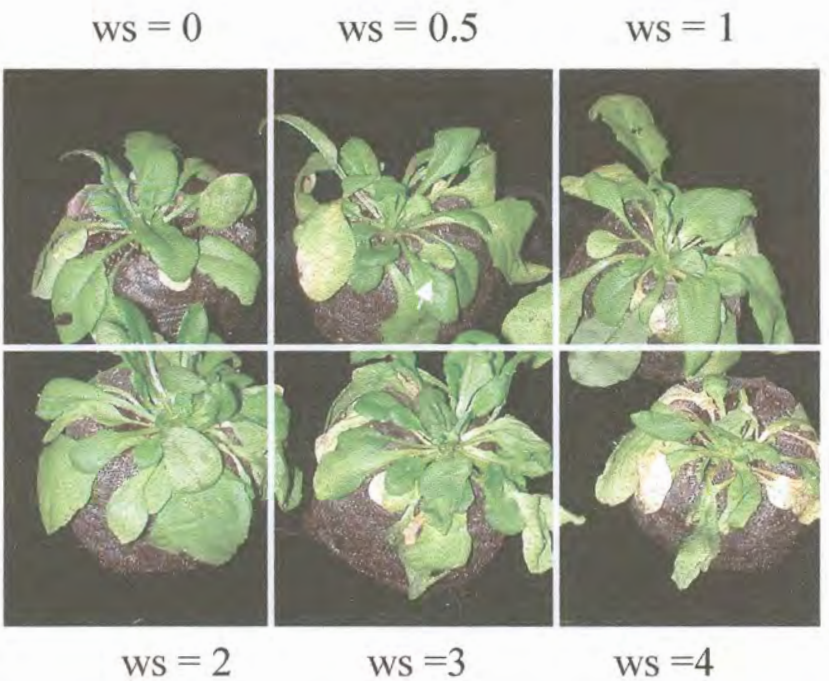



**Figure 3.1. Illustrating the different wilt symptoms (ws) on Col-5 inoculated with the control strain, BCC 0300 (GMI 1000).** Ws = 0.5: One leaf wilted, shown with the white arrow.

### 3.2.6. Molecular analysis between the ecotypes

Plants that were not subjected to the virulence tests were used for DNA extraction.

**3.2.6.1.** Extraction of genomic DNA from *A. thaliana*

A modification of Doyle and Doyle's (1987) CTAB isolation procedure was used for genomic DNA from *A. thaliana*. CTAB extraction buffer consisted of 100 mM Tris-HCL, pH 8, 1.4 M NaCl, 20 mM EDTA, and 2% (w/v) CTAB (hexadecyltrimethylammonium bromide) and was autoclaved. Afterwards 0.5 ml of β-mercapto-ethanol was added to 100 ml buffer. Fresh leaf tissue was ground in liquid nitrogen, transferred to a 1.5 ml Eppendorf tube and 800 µl of extraction buffer was added. The mixture was incubated at 65°C for 30 min and tubes were inverted every 10 min. Phenol-chloroform was then added (500 µl) and mixed well. Centrifugation was done at 12 000 rpm for 10 min at 25°C, the aqueous top phase transferred to a new tube and 500 µl chloroform added, mixed well while incubating at room temperature for 15 min. Centrifugation was done at 12 000 rpm for 10 min at 25°C, the aqueous top phase transferred to a new tube and 0.8 times the volume of isopropanol was added. It was thoroughly mixed and immediately centrifuged for 1 min at high speed. The supernatant was discarded carefully and the pellet washed twice with 70% ethanol. Hereafter the pellet was dried and suspended in 60 µl of RNase treated TE (pH 8). DNA samples were visualized on a 1% agarose gel. The quality and concentration were verified using standards as controls.

**3.2.6.2.** Sequence analysis of the C-terminal region of the RRS1 locus in different *A. thaliana* ecotypes

Two forward primers, RT1 and K10 and two reverse primers RT3 and K3.3, Appendix B, were used in different combinations to amplify the C-terminal end of the RRS1 allelic structure. This was done to determine if the stop codon was present in the susceptible allele or absent in the resistant allele. Combinations used were RT1 and RT3, to obtain the full-length product, RT1 and K3.3, to amplify the 3' end of the RRS1 allelic structure and K10 and RT3 to amplify the 5' end of RRS1 (Fig. 3.2). The last two products were purified and subjected to sequencing.

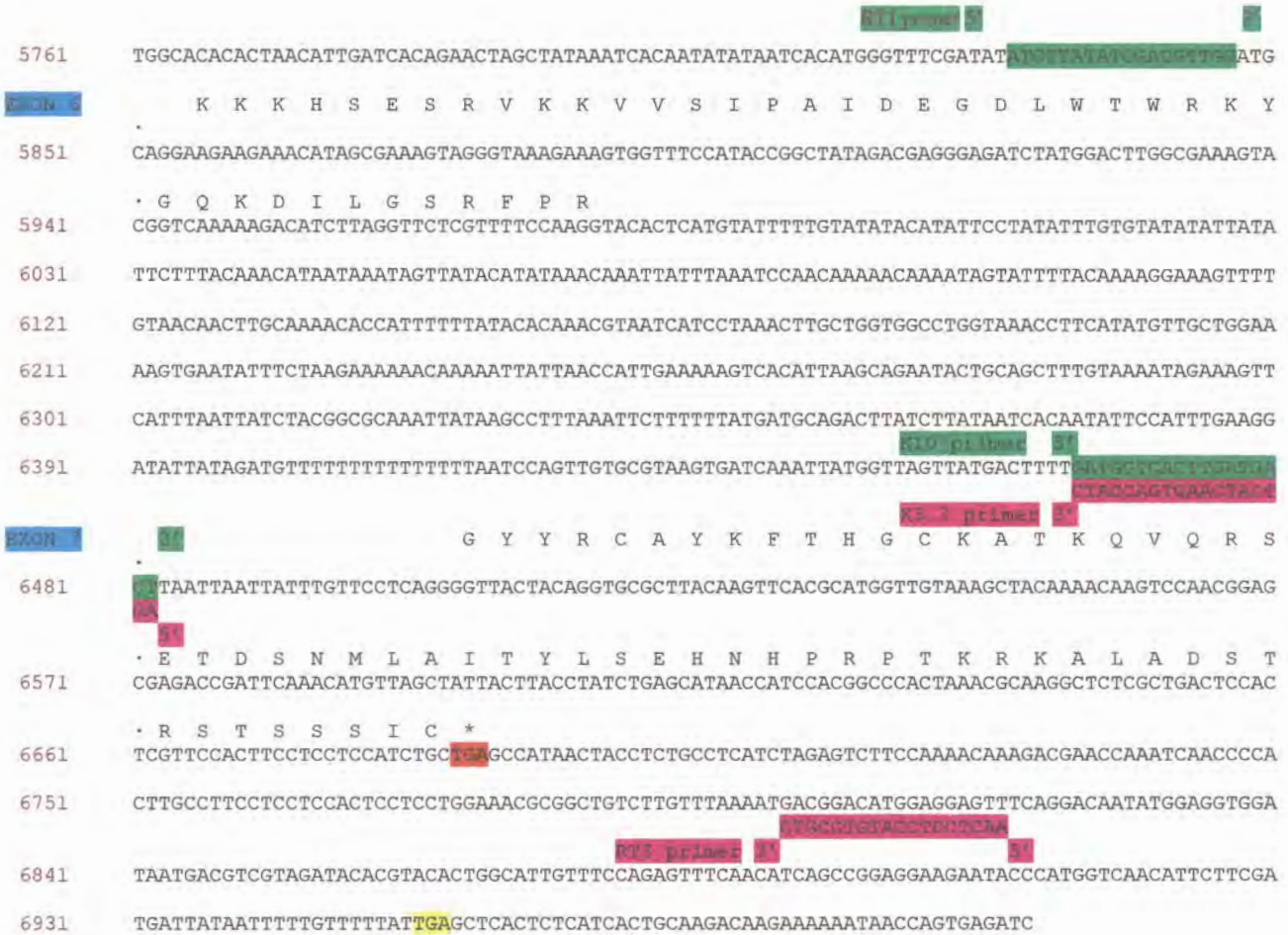



**Figure 3.2. Nucleotide sequence of a section of the C-terminal end of the *RRS1* allelic structure of Col-0.** Showing the translated exons (start shown in blue) as well as the DNA amplified with the primer pairs: RT1 and RT3 (988 bp), RT1 and K3.3 (650 bp) and K10 and RT3 (353 bp). These are the sizes of the amplified products expected for Col-0 (Col-5). The stop codon investigated in this study is shown in red. Stop codon in yellow is where other ecotypes like Kil-O, will terminate translation. Numbers to the left of the nucleotide sequence is the positions on the cosmid clone available in Genbank; accession number AB020744, on chromosome 5. The forward primers are shown in green and the reverse primers in pink.

The PCR was carried out in a total volume of 50 µl and was conducted in 0.2 ml PCR tubes (ABgene®) in a GeneAmp® PCR system 2700 (Applied Biosystems). The reaction mixture contained 1x *Taq* reaction buffer (Invitrogen), 200 µM of each dNTP's, 1 µM of each primer, 1.5 mM $MgCl_2$, 0.5 U of *Taq* DNA polymerase (Invitrogen) and 3 µl of genomic DNA (60 ng) as template. The reaction volume was made up with sterile distilled water. Negative controls contained all the PCR reagents excluding the template.

The PCR cycling conditions used included an initial denaturation of 95°C for 1 min and 5 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 2 min and elongation at 72°C for 1 min. Another 24 cycles followed with denaturation at 94°C for 1 min, annealing at 54°C for 1 min and 30 s, and elongation at 72°C for 1 min. A final elongation at 72°C for 10 min, then a 4°C hold for 30 s and a final hold at 15°C were included. PCR products were visualized by loading 10 µl on a 1% (w/v) agarose/1x TAE (pH 8.0) gel containing about 0.1 µg/ml EtBr. The gel was run for one hr at 80 V and the PCR product visualized.

The remaining 40 µl was subjected to purification through an S400 microspin column (Amersham Biosciences) and sequencing performed at LIPM, CNRS-INRA, Castanet-Tolosan, France using primer RT1 and K10 respectively (Appendix B).

### 3.2.7. Molecular analysis of the *Pop* P2 gene in *R. solanacearum*

#### 3.2.7.1. *Pop* P2 PCR

The presence of the *Pop* P2 gene in the eight *R. solanacearum* isolates was investigated by PCR analysis. DNA was extracted as described (Chapter 2) and was subjected to PCR-amplification. Primers used were Pop P2.1 forward and Pop P2.7 reverse (Appendix B) (a gift from S. Gunnac, LIPM, CNRS-INRA, Castanet-Tolosan, France). The total reaction volume was 50 µl and the PCR was conducted in 0.2 ml tubes. The reaction mixture contained 1x Buffer 3 (with 1.5 mM $MgCl_2$) (Roche) 200 µM of each dNTP's, 0.5 µM of each primer, 0,875 U of *Taq* + *Pwo* DNA polymerase (Expand High Fidelity PCR system, Roche) and 5 µl of genomic DNA (200 - 400 ng) as template. The reaction volume was made up with sterile distilled water. Negative controls contained all the

PCR reagents but no genomic DNA. PCR cycling conditions were as follows: an initial denaturation of 95°C for 2 min. This was followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s and elongation at 72°C for 30 s. A final elongation at 72°C for 10 min, then a 4°C hold for 30 s and a final hold at 15°C were included. PCR products were visualized as described previously.

## 3.3. RESULTS

### 3.3.1. Screening of the ecotypes against the strains

Plants in trays (Fig. 3.3.) were screened on a daily basis, means were determined and data are presented in Figs. 3.4 – 3.7. From the first histogram as illustrated in Fig. 3.4 A, it is clear that Be-O and Cvi already developed disease as soon as 6 days after inoculation with all six strains of *R. solanacearum*, while Nd-1 developed no disease. The water control also showed no disease symptoms (data not shown). Be-O, Cvi, Col-5, Sf-2 and Kil-O showed some response at this stage to BCC 0300 and BCC 0327 (GMI 1000 and 27B). The second histogram, Fig. 3.4 B, represented the mean disease index 18 days after inoculation with the various *R. solanacearum* strains. Be-O, Cvi, Col-5 and Sf-2 were susceptible to most of the isolates, while Nd-1 was resistant to all the isolates and the water control also developed no disease. Ecotype Kil-O showed resistance to three of the eucalyptus isolates.

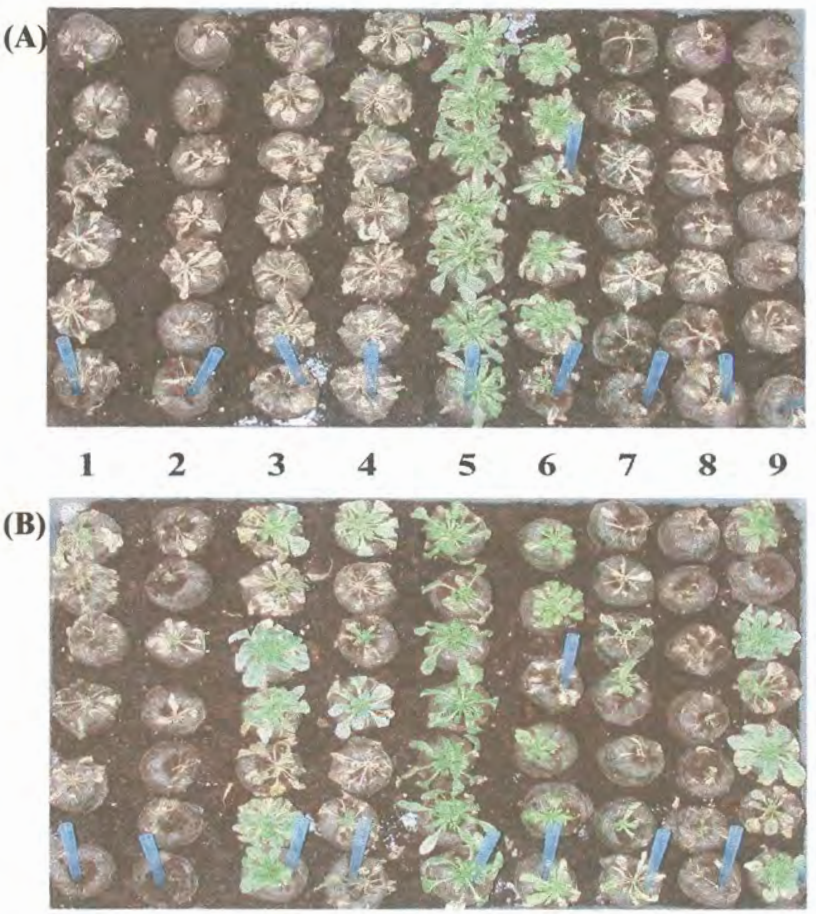



**Figure 3.3. Disease symptoms after 18 days in the various *A. thaliana* ecotypes upon inoculation with the control strain, (A) BCC 0300 (GMI 1000), and strain (B) BCC 0302 (CK).** Lanes 1 and 4: ecotype Be-O; lanes 2 and 8: ecotype Laer; lanes 3 and 9: ecotype Col-5; lane 5: ecotype Nd-1; lane 6: top blue marker: Sf-2 and bottom blue marker: Kil-O; lane 7: ecotype Cvi.

According to Figure 3.4 A and B, disease responses can be classified into four groups: a highly susceptible ecotype (Be-O) with first wilt between 3-5 days and total wilt after 10 days; Susceptible ecotypes (Col-5 and Cvi) with first wilt between 4-6 days and total wilt after two weeks; A intermediate ecotype (Sf-2) where first wilt developed after 6 days and 12 days later the mean disease index was below 0.6; Resistant ecotypes, where no wilting occurred 18 days after inoculation, or the mean disease index was below 0.3. The last group included ecotype, Nd-1 and depending on the isolate used Kil-O (resistant to isolates: BCC 0301-0303 (K, CK and CC)).

Fig 3.4 A and B also revealed that all the *R. solanacearum* isolates used were pathogenic since they caused wilt of the two most susceptible ecotypes, Be-O and Cvi. The virulence of the strains could be ranked from the most virulent (DI=1) to the least virulent (DI=0.3): BCC 0300 (GMI 1000) > 0327 (27B) > 0302 (CK) > 0301 (K) > 0306 (62) > 0303 (CC).

The *Arabidopsis – Ralstonia* pathosystem showed classical disease progress curves in all trials conducted (Fig. 3.5 – 3.7). An initial lag phase was seen followed by exponential increase of disease over time. The disease progress curves of the different ecotypes infected with isolates BCC 0300 (GMI 1000) and BCC 0302 (CK) are presented in Fig. 3.5 and Fig. 3.6 respectively. The disease progress curves of three trials for BCC 0300 (GMI 1000) (Fig. 3.5) and BCC 0302 (CK) (Fig. 3.6) revealed that wilt symptom development and progress differed between the respective trials. However, the ranking of the ecotypes according to resistance and susceptibility, remained more or less the same between the trials.

However the ecotypes Kil-O, varied between wilt symptom development varied between susceptible and resistant responses between the trials. For example ecotype Kil-O was resistant against GMI 1000 in one trial (Fig. 3.5 A) but susceptible in another trial (Fig. 3.5 B). This trend of variation in the susceptibility of ecotype Kil-O was also seen for isolates BCC 0327 (27B), BCC 0303 (CC) and BCC 0302 (CK) (data not shown). The isolates BCC 0306 (62) and BCC 0303 (CC) exhibited relative low virulence to *A. thaliana*. However, upon testing isolate BCC 0306 (62) on egg plant, the strain behaved aggressively (data not shown).

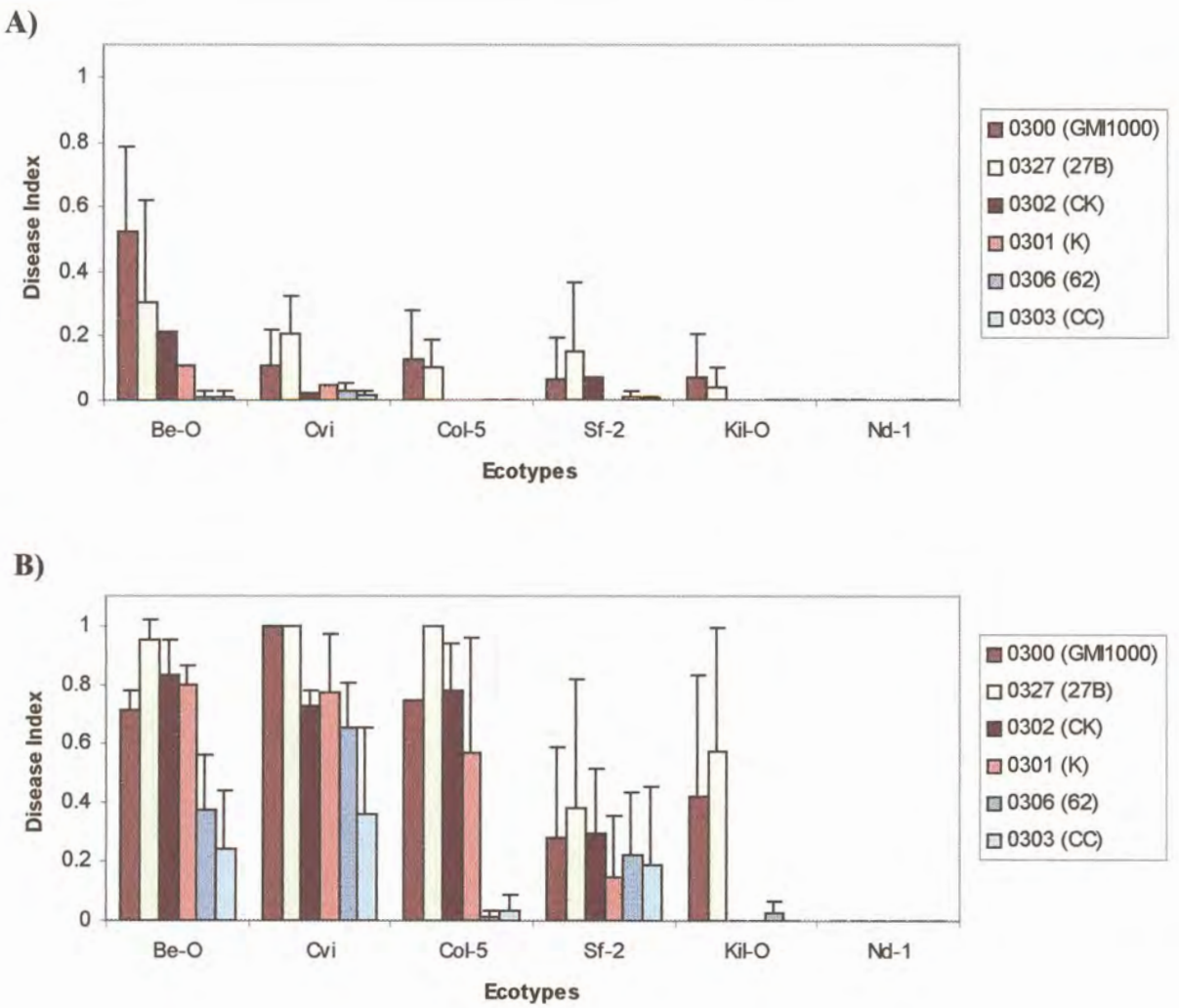



**Figure 3.4. Disease symptoms in various *A. thaliana* ecotypes after root inoculation with different strains of *R. solanacearum*.** Data presented here are the mean disease indexes at 6 and 18 days (A and B, respectively) of three experiments, with at least six plants per strain per ecotype. Standard deviation was calculated between the experiments. The negative control was water inoculated plants and no disease was present (DI=0).

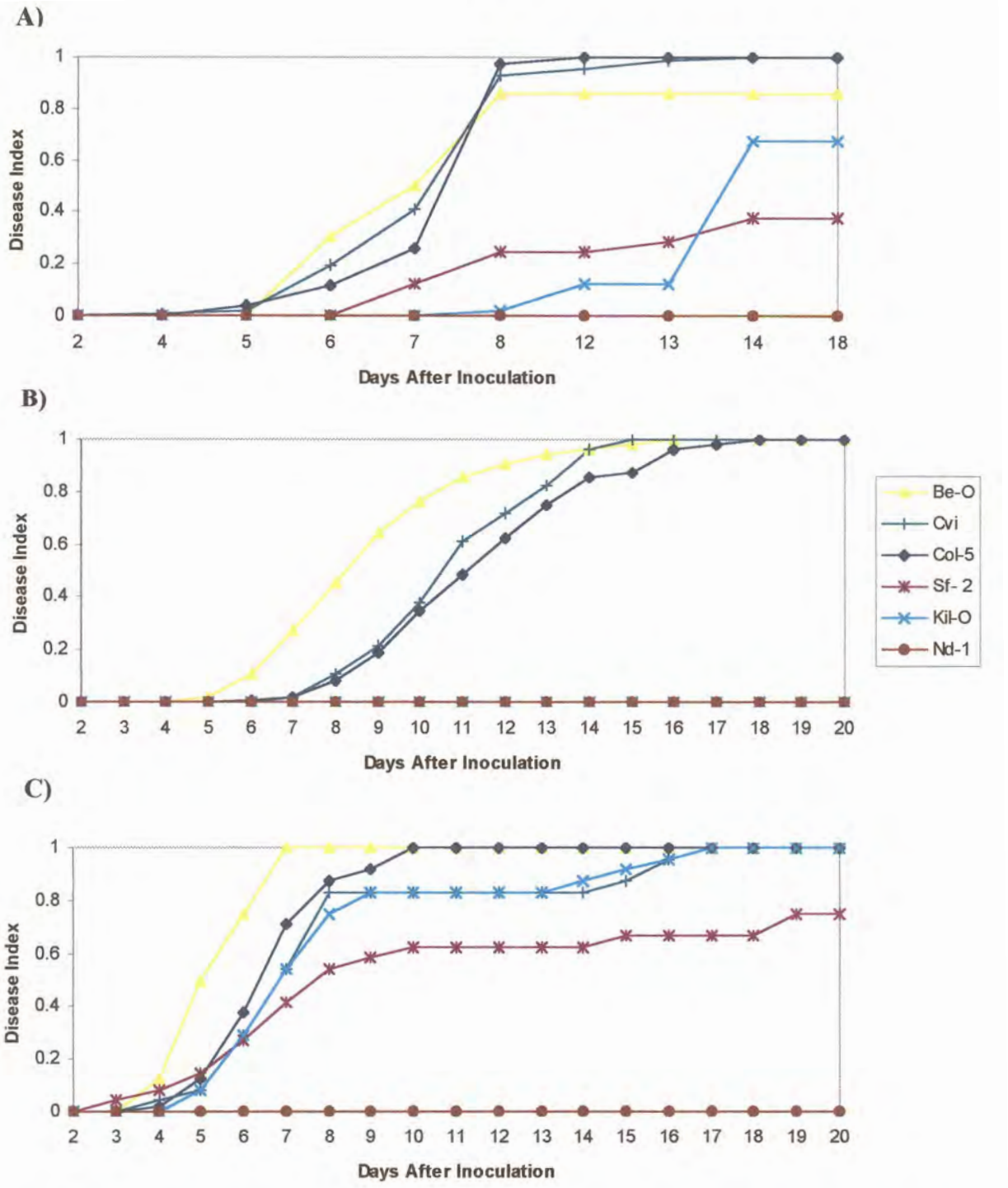



**Figure 3.5. Progression of disease symptoms in the different *A. thaliana* ecotypes over 18 days after root inoculation by the *R. solanacearum* control strain, BCC 0300 (GMI 1000) during the three replicate experiments (A, B and C).** Data points were generated from at least six plants. During experiment 3, C, disease occurred after 4 days and overall symptoms appeared to be more severe, disease occurring on ecotypes Kil-O and Sf-2 as well.

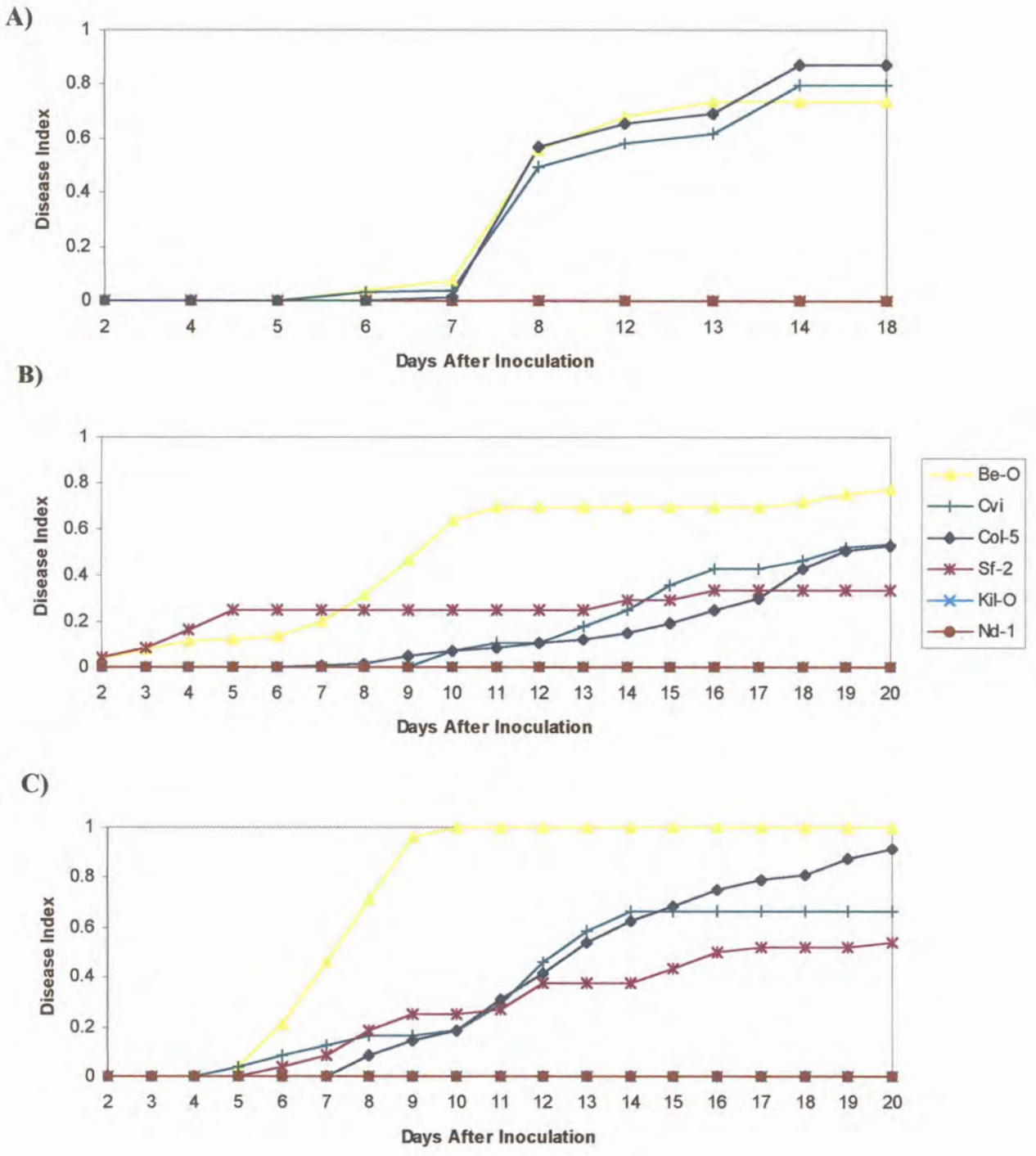



**Figure 3.6. Progression of disease symptoms in the different *A. thaliana* ecotypes over 18 days after root inoculation with the *R. solanacearum* isolate BCC 0302 (CK) during the three experiments (A, B and C).** Data points were generated from at least six plants.

A final experiment (experiment 4) was carried out on the seemingly most susceptible and the most resistant ecotypes that could be used in a potential southern African pathosystem, to confirm results obtained until now. Fig. 3.7 depicts the most susceptible ecotype (Be-O) against the more tolerant ecotypes (Col-5 and Sf-2) and the relative resistant ecotype (Kil-O). Showing the disease development over time it is clear that wilt symptoms appear first in Be-O and follows an exponential increase. Col-5 and Sf-2 reveals an initial lag phase. Sf-2 reveals a reduced exponential increase and reaching a plane, while the exponential increase continues for Col-5.

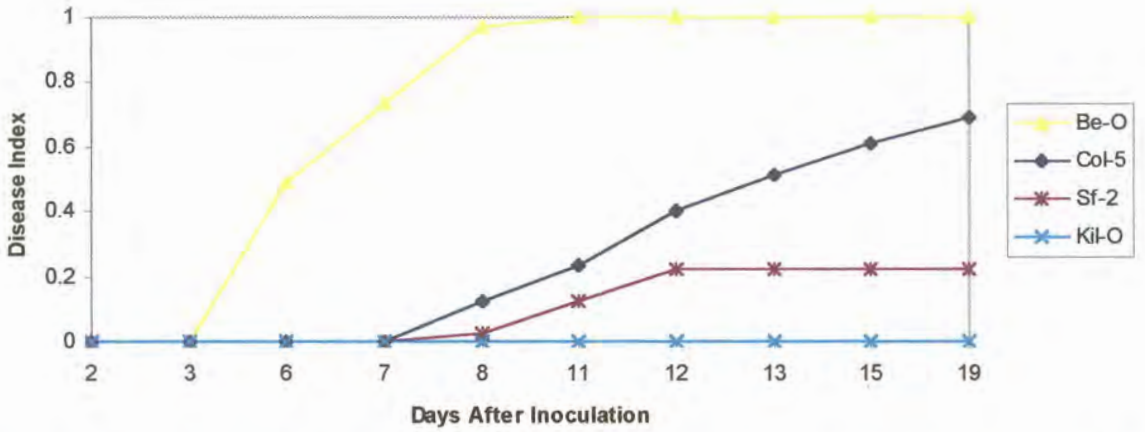



**Figure 3.7. A comparison between the most susceptible ecotypes, Be-O and Col-5, with the more resistant ecotypes, Kil-O and Sf-2, over 19 days after inoculated with isolate BCC 0302 (CK).** The values of 18 plants were pooled for each data point.

The wilt phenotypes that were converted to disease index and then depicted in the various graphs used to illustrate disease development are shown in Fig. 3.8. The control strain BCC 0300 (GMI 1000) is much more virulent the African strain BCC 0302 (CK). The strain BCC 0300 (GMI 1000) induced serious wilt (ws=4, DI=1) on all three ecotypes screened here, while the water control, middle lane, remained healthy (DI=0). Isolate BCC 0302 (CK), however, did produce wilt symptoms (ws) on ecotypes Be-O (ws=4, DI=1) and Col-5 (ws=1, DI=0.25) while ecotype Kil-O remained healthy (DI=0).

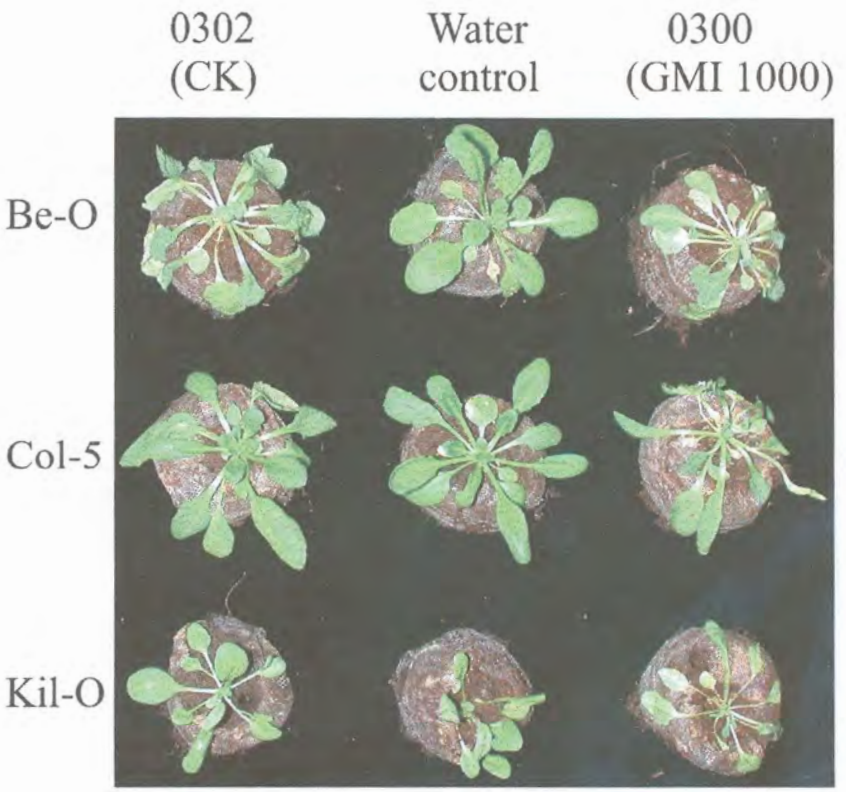



**Figure 3.8: Illustration of wilt symptoms of selected *A. thaliana* ecotypes Be-O, Col-5 and Kil-O, one week after inoculation with *R. solanacearum* strains BCC 0302 (CK) and BCC 0300 (GMI 1000) compared to the water control.**

### 3.3.2. Molecular analysis between the ecotypes

**3.3.2.1.** Extraction of genomic DNA from *A. thaliana*

Electrophoresis (Fig. 3.9) indicated that genomic DNA was successfully extracted from ecotypes Cvi, Kil-0, Col-5 and Be-O. High molecular weight DNA (>>12 kb) was obtained, with little protein residue visible in the wells.

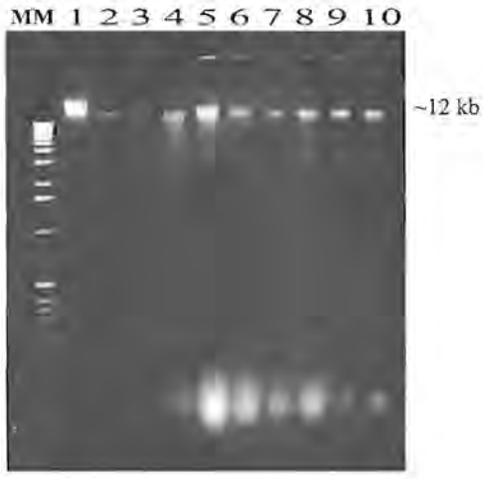



**Figure 3.9. DNA extracted from selected *A. thaliana* ecotypes.** MM: 1 kb molecular marker; lane 1: 500 ng DNA Standard; lane 2: 50 ng DNA Standard; lanes 3 and 4: ecotype Cvi; lanes 5 and 6: ecotype Kil-O; lanes 7 and 8: ecotype Col-5; lanes 9 and 10: ecotype Be-O.

**3.3.2.2.** Sequence analysis of the C-terminal region of the RRS1 locus in different *A. thaliana* ecotypes

The expected band sizes for Col-5 were as follows: primer pair 1 (RT1 and RT3) = 988 bp; primer pair 2 (RT1 and K3.3) = 651 bp; primer pair 3 (K10 and RT3) = 353 bp (Fig. 3.10). Ecotype Cvi was not amplified with primer pairs 1 or 2, but did produce a faint band with primer pair 3. Primer pair 2 yielded two bands where only the top band, was expected. This PCR was repeated and only the top band (651 bp) was obtained (data not shown). The PCR products obtained with primer pair 3, K10 and RT3, were submitted for sequencing using K10 to amplify the sequencing reaction. The results for the sequencing data whether the stop codon is present or absent are presented in Table 3.3.

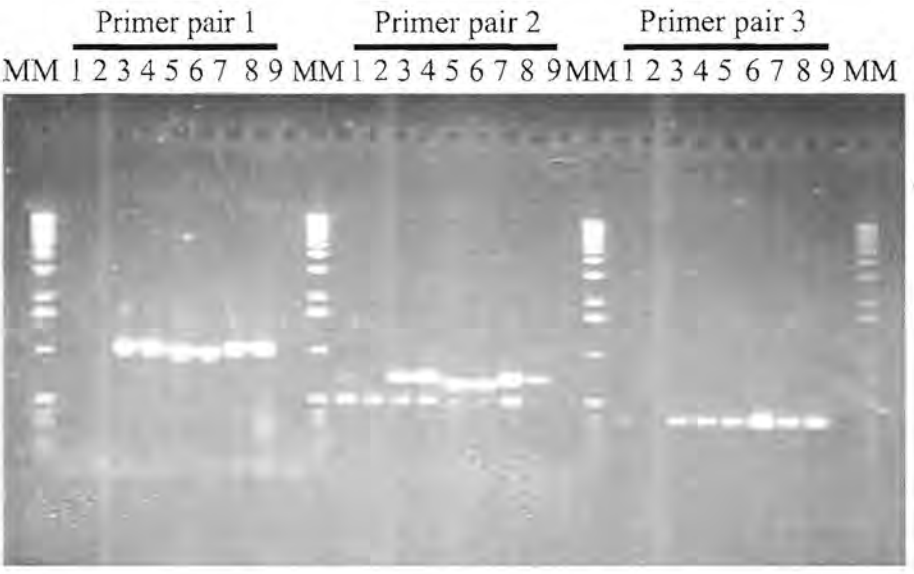



**Figure 3.10.** ***A. thaliana*** **DNA amplified with RRS1 primers.** Primer pair 1 = RT1 & RT3; primer pair 2 = RT1 & K3.3 and primer pair 3 = K10 & RT3. The PCR products were separated on a 1 % agarose gel, MM = 1 kb. Lanes 1 and 2: ecotype Cvi; lanes 3 and 4: ecotype Kil-O; lanes 5 and 6: ecotype Col-5; lanes 7 and 8: ecotype Be-O and line 9: Negative control without template DNA.

**Table 3.3: Presence of stop codon in the RRS1 gene of the *A. thaliana* ecotypes**

| Ecotype | Stop codon* | Reference |
|---|---|---|
| Col-5 | Yes | Deslandes *et al.*, 2002 |
| Nd-1 | No | Deslandes *et al.*, 2002 |
| Sf-2 | No | Olivier (personal communication, LIPM, CNRS-INRA, Castanet-Tolosan, France) |
| Cvi | Unknown | PCR product too faint to sequence |
| Kil-0 | No | This study |
| Be-O | No | This study |

* Presence of the stop codon as found in the RRS1 allele of ecotype Columbia (Col-5)

### 3.3.3. Molecular analysis of the Pop P2 gene in *R. solanacearum*

#### 3.3.3.1. Pop P2 PCR

The presence of the Pop P2 gene sequence was investigated by PCR analysis. Amplification revealed the absence of the gene sequence in the two Biovar 2 isolates (Fig 3.11, lanes 8 and 9) and the presence of the 850 bp gene product in the Biovar 3 isolates (Fig 3.11, lanes 2-5). In BCC 0327 (27B) a main amplification product, 510 bp, was obtained that was smaller than the expected gene size. Exactly the same DNA samples were amplified with the *hrp*-primers in Chapter 2, confirming the DNA quality.

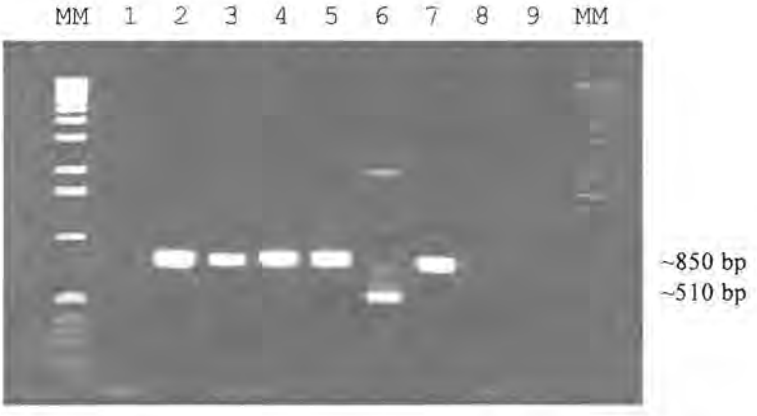



**Figure 3.11. PCR amplification of the *PopP2* gene of *R. solanacearum* using primer pair, Pop P2-1 and Pop P2-7.** MM: 1KB DNA Molecular marker; lane 1: Negative control, no DNA template; lane 2: BCC 0301 (K), lane 3: BCC 0302 (CK), lane 4: BCC 0303 (CC), lane 5: BCC 0300 (GMI 1000), lane 6:BCC 0327 (27B), lane 7: BCC 0304 (117), lane 8: BCC 0306 (62) and lane 9: BCC 0305 (92).

## 3.4. DISCUSSION

The aim of this chapter namely to identify two *A. thaliana* ecotypes with a differential expression i.e. resistant and susceptible phenotype to the pathogen *R. solanacearum* recovered from *Eucalyptus* spp. in southern Africa was fulfilled. The ecotypes identified were Be-O (susceptible) and Kil-0 (resistant) when inoculated with BCC 0302 (CK). The stop codon that was found in the susceptible ecotype Col-5 in a related study (Deslandes *et al.* 1998), was not present in the susceptible ecotypes Be-0 and Cvi, or in the resistant ecotype Kil-0. The PopP2 gene is present in the biovar 3 isolates, was detected in the PCR tests on African *R. solanacearum* biovar 3 isolates with exception of isolate BCC 0327 (27B)

It is important to note that RRS1 (*A. thaliana*) - POP P2 (*R. solanacearum*) is one of the few characterised gene for gene interactions in plants. Wanting to ensure that we were working on a different pathosystem than the French lab (Deslandes *et al.*, 1998) the RRS1 and POP P2 screening was carried out. However resistance in our system could be polygenic as is known for the *R. solanacearum* – Tomato pathosystem (Thoquet *et al.*, 1996a, 1996b). To investigate this the next step could be performing genetic crosses to determine if a single gene or multiple loci govern resistance. This supports our choice of the plant *A. thaliana* as it offers a diverse array of genetic tools that would make the study of quantitative trait loci easier.

Despite the high fluctuations in wilting, the trend in disease development remained the same and response could be classified into four groups, ranging from highly susceptible to resistant ecotypes (see Results, 3.3.1). A relatively resistant ecotype and a highly susceptible ecotype were needed for a pathosystem. Ecotype Be-O presented wilting symptoms in most cases, even when inoculated with the less virulent isolates like BCC 0301, 0303 and 0306 (K, CC and 62), while ecotype Kil-O displayed resistance to isolates BCC 0301-0303 (K, CK and CC). Isolate BCC 0302 (CK) was the most virulent in this group and was selected for our studies. Isolate BCC 0302 (CK) did however present intermediate virulence when compared to all strains tested with the control strain, BCC 0300 (GMI 1000), and isolate BCC 0327 (27B), which were the most virulent. However, this

Congolese *Eucalyptus* pathogen was strongly aggressive to ecotype Be-O and no symptoms developed in ecotype Kil-O, thus forming the ideal basis for a new African pathosystem.

High fluctuations were common between the different experiments carried out from March 2003 until November 2003. The main reason seemingly being different environmental conditions at the time of inoculation, as well as the physical state of the plants on the day of inoculation. Although conditions, in the growth chamber as well as the disease chamber were set at fixed temperatures, light conditions and humidity, conditions varied due to the external environment. For example extreme heat during the European summer caused the chambers to run at an overall higher temperature in spite of attempts to keep it stable, while a lot of rain leads to an increased humidity. Diseases such as aphids and powdery mildew occurred more frequently during the warmer season and plants had to be treated accordingly. A waiting period of at least one-week had to follow treatment, but this still affected plant response to *R. solanacearum,* resulting in a slightly higher disease tolerance. Another influence is the number of plants present in the chamber during the growth-phase: more plants lead to an overall lower disease threshold, due to a larger source of potential inoculum. Variation is commonly associated with the life of a biological organism and the absence of hereof would be unexpected.

The variation could be due to the high variability of *R. solanacearum*. Sequeira (1994) stated that *R. solanacearum* is a highly variable organism and obtaining a stable resistance in the host plants, potato and tomato, is very difficult. Even the control strain, BCC 0300 (GMI 1000) obtained from the Boucher-lab (LIPM, CNRS-INRA, Castanet-Tolosan, France) has a reputation for its lack of stability in culture. Extensive reviews have dealt with this pathogen, concerning resistance to *R. solanacearum*: all stated high variability in host-pathogen interactions as a challenge (Prior *et al.*, 1994).

*Lycopersicon esculentum,* tomato (cv Marion and cv. Bonnie Best) is also used as a host for *R. solanacearum*, plants were inoculated via the petioles or by soil drench (Saile *et al.*, 1998, Tans Kersten *et al.*, 1997). The latter inoculation method was investigated on *A. thaliana* but this lead to even higher variability in disease incidence, suggesting that *A. thaliana* might be a poor host for *R. solanacearum*. However *A. thaliana* offers various genetic tools, the complete genome sequence

is known as well as its small size enables us to perform high throughput experiments in a limited space. These positives and the fact that the French lab (Deslandes *et al.*, 1998) also used *A. thaliana* as the host for *R. solanacearum* motivated us to use *A. thaliana*.

The resistant ecotype Kil-0 does not have the stop-codon that is associated with susceptibility in ecotype Col-5. These sequences of Kil-0 are similar to the resistant ecotype, Nd-1 (Table 3.3). This similarity can not be explained by contamination of Kil-O seed with Nd-1 since it was confirmed by four different seed stocks, generated at different times (data not show). Previous data as well as studies by J. Olivier (LIPM, CNRS-INRA, Castanet-Tolosan, France) validated susceptibility of Kil-O to BCC 0300 (GMI 1000). The absence of the stop codon in Sf-2, Kil-O and Be-O suggests that these ecotypes could contain the RRS1-R allele. However, full-length sequencing of the RRS1-R alleles would be required to confirm the results, since it is known that this is not the only difference between the susceptible ecotype, Col-5 and the resistant ecotype, Nd-1. Furthermore the absence of a stop codon in both Kil-O and Be-O, suggested that the particular R-gene was not involved in the resistant/susceptible phenotype of these ecotypes. The size difference seen in the PCR products (Fig 3.10) in lanes 5 and 6 after amplification of the first part of the RRS1 sequences (primer pairs 1 and 2) can be most likely explained by the presence of a deletion in the intron area between exon 6 and exon 7 in ecotype Col-5 (Fig. 3.10).

Deslandes et al., 2002, compared the amino acid sequences of RRS1-R and RRS1-S. All together 21-aa differences were found. Analysis of the derived amino acid sequence revealed the presence of several domains associated with resistance proteins. These motifs included the Toll/Interleukin Receptor (TIR), the NBS (nucleotide binding site, domain), an NLS site, and six imperfect 23- to 25-aa coding for LLR's (leucine rich repeats). This also was the first report of an R protein with a group III conserved WRKY domain (Figure 3 in Deslandes *et al.*, 2002). The most interesting difference was the presence of a stop codon in RRS1-S (shown in red in Fig. 3.9), 90 aa, before the C-terminal end of the RRS1-R allele. During structure-function analysis these 90 aa appeared to be vital since an exact copy of RRS1-R without the 90 aa was susceptible to *R. solanacearum* (Deslandes *et al.*, 2002).

A study by Lavie *et al.*, 2004, confirmed that PopP2 was usually absent from the "Americanum" isolates, biovar 2 but present in the "African", biovar 1 as well as in the "Asiaticum", biovar 3, isolates. PopP2 PCR analysis was carried out to ascertain if it explained the varying virulence of the isolates. The primers used to amplify part of the gene were located at the beginning of the promotor and 880 bp downstream from the promotor, amplifying about 850 bp out of the total 1525 bp of the full length pop P2 gene. Data generated during this study confirmed the absence of the *PopP2* gene in the two Biovar 2 isolates analysed, and the presence in biovar 3 isolates. However, amplification of biovar 3 isolate, 27B, produced a smaller band than expected as well as several additional bands. Sequencing of these products should be carried out to determine their identity. A possibility could be that 27B has the PopP2 gene with an internal deletion. Isolate BCC 0302 (CK) has the *PopP2* gene, but if it is expressed is not yet known. It does not trigger resistance in Be-O, although the absence of the stop codon in the RRS1 allele suggests a functional RRS1-R gene.

Isolates BCC 0301 (K) and BCC 0327 (27B) will be used in future studies in the lab employing the relevant *hrp*$^-$ and Rif$^r$ strains, however the *A. thaliana – R. solanacearum* pathosystem with ecotypes Be-O and Kil-O, and isolate BCC 0302 (CK) will be further investigated in Chapter 4.

# CHAPTER 4

# Characterisation of a new pathosystem between *Arabidopsis thaliana* and an African isolate of *Ralstonia solanacearum* from *Eucalyptus*

## 4.1. INTRODUCTION

*Ralstonia solanacearum*, the cause of bacterial wilt, is one of two bacterial pathogens known to infect *Eucalyptus* in South Africa. *R. solanacearum* was first reported to infect *Eucalyptus* spp. in South Africa in 2000 (Coutinho *et al.*, 2000). Plantation forestry covers more than 1.5 million ha in South Africa and about half of this is *Eucalyptus* spp. (Department of Water Affairs and Forestry, 1995). High yielding clonal hybrids which are costly to maintain are mostly used (Roux *et al.*, 2000). *R. solanacearum* has the potential to destroy these plantations therefore, the rapid identification and subsequent disease management is vital. One of the most convenient, inexpensive and environmentally sound ways to control plant disease is to utilize disease resistant varieties (Agrios, 1997).

Resistance is either the presence of preformed barriers and chemical compounds, or due to activation of defence mechanisms as a result of pathogen recognition (Hammond-Kosack and Jones, 1996). Hypersensitive response (HR) and systemic acquired resistance (SAR) have received the most attention among the inducible responses. HR 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). Agrios (1997) defined HR as the death of host cells within hrs of pathogen contact, but 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 aggravated by relative humidity (Hammond-Kosack and Jones, 1996; O'Donnell *et al.*, 2003). *R. solanacearum* is known to form a HR when infiltrated into the tobacco leaves (Boucher *et al.*, 1985). This was exploited in this study to test the rifampicin resistant ($Rif^r$) and hypersensitive response and pathogenicity defective (*hrp*$^-$) strains according to their ability to form a HR on a susceptible tobacco cultivar. The antibiotic rifampicin is a bactericidal agent that inhibits protein synthesis, due to its binding to the β subunit of RNA polymerase, thus blocking the pathway of elongation (Sambrook *et al.*, 1989). *R. solanacearum* becomes resistant

when for example a point mutation occurs altering the RNA polymerase in such a way that the rifampicin can no longer bind enabling the bacteria to grow on Rif$^r$ plates. These mutants are spontaneous mutants. Often Rif $^r$ are also defective in their exopolysaccharide production, producing red flat colonies with clear halos, thus wild-type like strains that gave rise to pink mucoid colonies, with white halos were selected (Boucher *et al.*, 1985). *Hrp* genes were originally defined in *Pseudomonas syringae* pv. *phaseolica*, and play a key role because they control both the ability to cause disease on susceptible host plants as well as the ability to induce the local defence reaction termed HR in resistant plants (Van Gijsegem *et al.*, 1995). The *hrp* genes have been identified in representatives of all major groups of Gram-negative plant-pathogenic bacteria except *Agrobacterium tumefaciens*, and in each case are organized in a gene cluster spanning 23 to 40 kb of DNA (Van Gijsegem *et al.*, 1995). In all Type III Secretion Pathways nine proteins are conserved, and eight of these have homologues that are involved in the biogenesis of the type III related flagellar complex. In plant pathogens, the corresponding genes have been renamed *hrc* (for *hrp* conserved) followed by the letter corresponding to the Ysc homologue (which explain why *hrpU* was renamed *hrcS)* (Van Gijsegem *et al.*, 2002).

Boucher *et al.* (1985) was the first to apply induced mutagenesis to *R. solanacearum* in order to increase the number of avirulent mutants to further facilitate genetic and physical analysis of the pathogen. Following Tn5 mutagenesis, 8250 clones were screened and 12 avirulent mutants with a wild-type phenotype were obtained. Arlat *et al.*, (1992) illustrated that the *hrp*-gene cluster is associated in six transcriptional units, by using the transposon Tn5-B20. GMI 1402, *hrcS* mutant was generated during this above-mentioned study. HrpB and HrpG are encoded by the first gene in transcription unit 1 and unit 5 respectively. In order to illustrate the regulatory role for HrpB in *hrp* gene expression, a *hrpB* mutation was constructed by inserting Ω interposon at the unique *Bgl*II site within the *hrpB* DNA sequence. This insertion abolished the functional role of *hrpB* as shown by the inability to cause HR on tobacco (Genin *et al.*, 1992). The location of the respective regulators of the *hrp* and *hrc* gene clusters including the transcription units 1 – 7 are illustrated in Fig 4.1. Fig. 4.2 displays the role of *hrcS* located in the inner

membrane of *R. solanacearum*, illustrating that the disruption of this core gene disrupts the translocation of PopA, PopB and PopC.

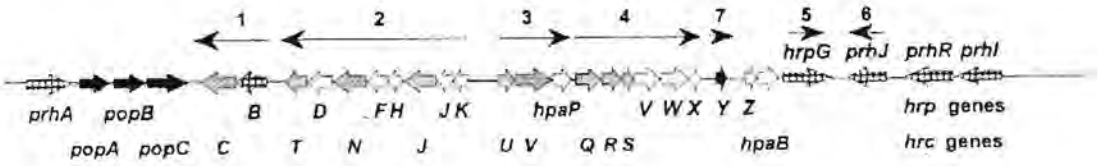



**Figure 4.1. Genetic organization of the *R. solanacearum hrp* gene cluster.** The thin arrows with numbers indicate the *hrp* transcription units; the thick arrows indicate the different genes. Conserved *hrc* genes are represented by the filled grey arrows, genes encoding secreted proteins by filled black arrows, and regulatory genes by hatched white arrows. (Van Gijsegem *et al*, 2002)

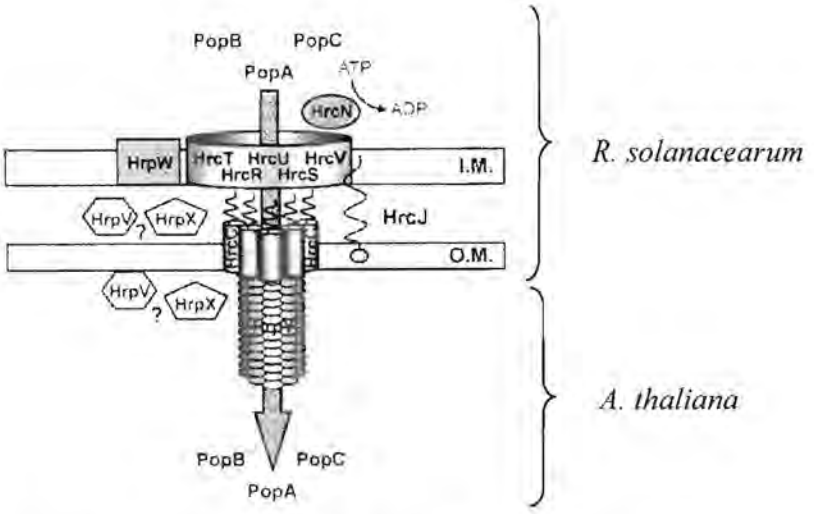



**Figure 4.2. Model for the role of the different Hrp proteins in the assembly of *R. solanacearum* type III secretion apparatus.** The inner membrane (I.M) and outer membrane (O.M.) of *R. solanacearum* is illustrated (Van Gijsegem *et al*, 2002).

SAR is a systemic resistance response that is induced after the formation of a necrotic lesion, either as part of HR or as a disease symptom (Ryals *et al.*, 1996). SAR is associated with the expression of three pathogenesis-related genes: PR-1, PR-2 and PR-5, Table 4.2 (Uknes *et al.*, 1992). Pathogen attack elicits jasmonic acid (JA) or ethylene production activating PR-3, PR-4 (Table 4.2) and PDF 1.2 (Feys and Parker, 2000). In potato HR and PR-gene expression have been correlated with resistance to *Phytophthora infestans* (Smart *et al.*, 2003). Data until now has revealed that each plant species has the ability to respond in unique ways to many different pathogens, while the molecular mechanisms responsible for resistance remain ambiguous in most plant-pathogen interactions (Smart *et al.*, 2003).

The objective of this chapter was to characterize the new pathosystem arising between an African isolate of *R. solanacearum*, BCC 0302 (CK) from *Eucalyptus* and two susceptible ecotypes, Be-O and Col-5 and a resistant ecotype, Kil-O. The significance of the apparent healthy phenotype of inoculated plants was investigated in order to determine if this could be labelled as tolerance or resistance. Growth curve analysis was performed to quantify the bacterial growth *in planta* using Rif$^r$ strains as well as *hrp*$^-$ strains. Development of these genetic tools is described in this Chapter. Furthermore, northern analysis was carried out to estimate the amount of RNA's transcribed by the selected *A. thaliana* ecotypes during disease development. The probes used in this study (Table 4.2) were chosen to evaluate the expression of salicylic acid (SA), jasmonic acid (JA) and ethylene pathways during disease development in a susceptible ecotype, and in a seemingly resistant ecotype. With this investigation we hoped to clarify the plant-pathogen interaction between the selected ecotypes, supporting the data obtained from the bacterial growth curves.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Bacterial growth curves

#### 4.2.1.1. Preparing rifampicin resistant (Rif $^{r}$) mutants

Selected strains, BCC 0301 (K), BCC 0302 (CK) and BCC 0327 (27B), stored at –70°C were revived by streaking the inoculum on BGT medium (Appendix A). Two individual colonies were selected 48 hrs after incubation at 28°C and transferred to 5 ml of B broth (Appendix A), supplemented with glucose (0.5%). After overnight incubation at 28°C, it was centrifuged for one min at 3000 rpm, discarding the supernatant. This was repeated until all the broth had been centrifuged. The resulting pellet was then spread on BGT medium containing rifampicin (50µg/ml) and glucose (0.5%). The plates were incubated at 28°C for two to three days. Single wild-type like, mucoid colonies (see Chapter 2, Fig. 2.2), were selected and overnight cultures were prepared.

After 24 hrs incubation on a rotational shaker at 28°C, the cells where harvested by centrifugation of the broth. The pellet was then resuspended in 3 ml of sterile distilled water. This was then diluted ten fold and approximately 250 µl infiltrated into a fully expanded tobacco leaf, *Nicotiana tabacum* cv. Bottom special, as described by Galiana *et al.*, (1997). Every isolate was infiltrated twice on each leaf with the respective concentrations and on three different leaves per plant. The overnight culture was stored at room temperature to prepare glycerol stocks if the Rif $^{r}$ strain was virulent.

Plants were observed on a daily basis for HR. This was done for 7 consecutive days. Water was infiltrated to illustrate a negative control. Rif $^{r}$ mutants that produced a HR were selected (Fig. 4.3). Cultures stored overnight that produced a HR were streaked out on BGT medium (Appendix A) with the appropriate antibiotics to determine the stability of the mutant. Glycerol stocks were now prepared from the overnight culture to preserve the transformed strain.

### 4.2.1.2. Preparing hrp⁻ mutants

Total genomic DNA was extracted (3.2.3.1) from the two *hrp*⁻ *R. solanacearum* strains GMI 1525 (Genin *et al.*, 1992) and GMI 1402 (Arlat, *et al.*, 1992). They are derivatives of BCC 0300 (GMI 1000). The disruption is in the *hrp*B gene for GMI 1525 and in the *hrc*S (*hrp*-conserved) gene for GMI 1402. These strains were kindly provided by Dr. Stéphane Genin (LIPM, CNRS-INRA, Castanet-Tolosan, France). GMI 1525 is spectinomycin resistant and was grown on BGT medium with 0.5 % glucose and a final antibiotic concentration of 40 µg/ml. The strain GMI 1402 is kanamycin resistant and used at a final concentration of 50 µg/ml. DNA extractions were performed as described previously and the subsequent DNA concentration was checked on a gel (Chapter 3).

The receiver strains were BCC 0301 (K), BCC 0302 (CK), BCC 0306 (62) and BCC 0327 (27B), were prepared by growing them for three days in minimal media (one-quarter strength of M63 (Maniatis *et al.*, 1982), with a final concentration of 2% glycerol), on a rotary shaker at 30°C. A final optical density (OD) of about one was needed. This bacterial growth solution was placed on a nitrocellulose filter on B medium without glucose (Appendix A), with total genomic DNA of GMI 1525 or GMI 1402, of a concentration of 0.3 µg/µl.

Incubation was carried out at 30°C for two to three days. The bacterial growth was collected by quick centrifugation of the nitrocellulose filter using 1 ml of sterile distilled water to collect the bacterial growth. The filter was removed and the remaining suspension mixed by vortexing. 100 µl of this suspension was streaked onto a selection plate of BGT medium (Appendix A) and the antibiotic (spectinomycin or kanamycin) at the above-mentioned concentration. Incubation followed at 30°C for two to three days. Transformed colonies were tested as described above. The *hrp*-mutants that were no longer capable of inducing a HR were chosen (Fig. 4.4).

#### 4.2.1.3 Inoculations and dilution plating

Inoculation of *A. thaliana* was performed as described in 3.2.3. The ecotypes were Be-O, Kil-O and Col-5. Two hundred plants were inoculated for each ecotype and four groups of three plants selected per day for the bacterial growth analysis carried out on the same day. The aerial parts of each group of *A. thaliana* plants inoculated with either the BCC 0352 (CK Rif$^r$) strain or the BCC 0372 *(CK hrc$^-$)* strain of *R. solanacearum* were pooled separately. Afterwards they were weighed and sterilized in 250 ml of 70% ethanol for 3 min, washed three times in sterile distilled water and ground with a mortar and pestle after addition of sterile distilled water (3 ml per gram of fresh weight). Serial dilutions of the ground material were performed with sterile distilled water. Serial dilutions were increased as the disease development progressed. The bacteria were spread on solid BGT medium (Appendix A) containing the relevant antibiotics (BCC 0352 (CK Rif$^r$) strain: rifampicin, 50 µg/ml and selected BCC 0372 (CK *hrc$^-$*) strain: kanamycin, 50 µg/ml) and grown at 28°C for two days. At least triplicate assays were performed for each time point, bacterial strain and *A. thaliana* ecotype. Environmental factors were closely monitored using a HOBO® data logger (Onset computer corporation, Bourne, USA).

### 4.2.2. Northern analysis

#### 4.2.2.1. Plant material harvested for Northern analysis

Plants were grown and inoculated as described in Chapter 3. The wild-type strain BCC 0302 (CK) was inoculated on the susceptible ecotypes, Be-O and Col-5 and on the apparent resistant ecotype Kil-O. Plants were harvested at 0, 4, 7 and 14 days after inoculation. Wilt symptoms were recorded as described in Table 3.2 (p.60). T=0, was taken before inoculation. In a second trail only ecotypes Kil-0 and Beo were inoculated, plants were harvested at 0, 2, 4, 7 and 14 days after inoculation.

#### 4.2.2.2. RNA isolation

The hot phenol method (Perry and Francki, 1992) was used with modifications as described by Gu *et al.* (2000). Total rosettes were collected, frozen in liquid nitrogen and stored at -80°C until needed. 50 ml tubes were prepared with 4 ml extraction buffer (100 mM LiCl, 100 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS and 6 ml of water-saturated phenol, incubated at 80°C. Frozen leaf tissue was removed from -80°C, ground in liquid nitrogen with a mortar and a pestle, and transferred to the pre-prepared 50 ml tubes. Samples were immediately thoroughly vortexed for 30 s to optimize the extraction of RNA, 4 ml of chloroform was added and each sample was vortexed for 10 s. Samples were centrifuged at 4°C for 15 min at a speed of 10 000 rpm. The supernatant was transferred to clean 50 ml tubes. One volume of 4 M LiCl was added to each 50 ml tube. Samples were then incubated for one hrs at –80°C. Frozen samples were incubated at room temperature for 10 min and then centrifuged for 30 min at 11 500 rpm (10°C), afterwards carefully discarding the supernatant. The pellet was then washed in 4 ml of 70% ethanol, centrifuged for 10 min at 11 500 rpm (10°C), the ethanol was finally discarded. The pellet was left to air dry. The dry pellet was resuspended in 500 µl DEPC-water (distilled water treated with diethyl pyrocarbonate), and transferred to clean RNase free 2 ml microfuge tubes. Phenol (500 µl) was added to the microfuge tubes, briefly vortexed, and centrifuged at 12 000 rpm for 15 min, where after the aqueous phase was transferred to new 2 ml microfuge tubes, and 500 µl of chloroform was added and briefly vortexed. After being centrifuged at 12 000 rpm for 15 min the aqueous phase (supernatant) was transferred to a new 2 ml microfuge tube and 2 volumes of ethanol and 0.1 volume of 3M NaOAc, pH 5.2 added. The 2 ml microfuge tubes were then incubated at –20°C overnight, after which it was centrifuged for 30 min at 12 000 rpm (4°C) in order to form the pellet. The pellet was washed with 500 µl of 70% ethanol and subsequently centrifuged for 10 min. The pellet was dried and resuspended in 100 µl DEPC-water, and stored at –70°C. The RNA concentration was determined with a spectrophotometer (GeneQuant pro, RNA/DNA calculator, Amersham Biosciences).

#### 4.2.2.3. RNA evaluation

An RNA denaturing gel was run to visualize the RNA stained with EtBr and then Northern blotted. A 500 ml stock solution of 10 X Running buffer (MOPS), (DEPC-water, 200 mM 3-[N-Morpholino] propanesulfonic acid (MOPS), pH 7, 50 mM Sodium Acetate (NaAc), 100 mM EDTA, pH 8 with NaOH) was prepared. Using the stock solution a 1.2% agarose formaldehyde gel [1.2 g agarose, 97 ml DEPC-water, 10 ml of 10x MOPS buffer, 3 ml formaldehyde (37% (12.3M) solution)] was prepared.

20 µg of total RNA was mixed with 5x RNA loading buffer [a few crystals bromophenol blue, 8 µl 500 mM EDTA, pH 8, 72 µl 37% (12.3M) formaldehyde, 200 µl of 100% glycerol, 308.4 µl formamide, 400 µl 10x MOPS buffer, 11.6 µl DEPC-water] and 1:10 dilution EtBr (50 µg/ml). These samples were separated on the 1.2% agarose formaldehyde gel for 2 hrs. Equal loading of gels was verified by visualization under UV-light. Gels were then transferred overnight to a Hybond-N membrane (Amersham Biosciences) by upward capillary blotting (Ausubel *et al.*, 1997) (using a 10x SSC blotting solution (175g NaCl, 88g Sodium Citrate, pH 7). The RNA-containing side of the membrane was cross-linked with an UVI-tec Cross-linker CL-508 (St John's Innovation Center, Cambridge, UK) at 0.167 Joules. Membranes were stored at 4°C until needed.

#### 4.2.2.4. Probe generation by PCR and purification

PR 1-5 genes in *E. coli* were kindly provided by the lab of Dr. Yves Marco, LIPM, CNRS-INRA, Castanet-Tolosan, France. Plasmid DNA's were extracted using the QIAprep® Spin Miniprep Kit (Qiagen) according to the protocol used by the manufacturer. The plasmids were amplified with the universal primer pair M13 forward and M13 reverse (Appendix B). Part of the 16S rRNA of *R. solanacearum* was also included for probing and was amplified as described in Chapter 3.

For the PR 1- 5 probes PCR was carried out in a total volume of 40 µl and was conducted in 0.2 ml PCR tubes (ABgene®) in a GeneAmp® PCR system 2700, Applied Biosystems). The reaction mixture contained 1x *Taq* reaction buffer (Invitrogen) and 200 µM of each dNTP's. 0.4 µM of each universal M13 forward and reverse primers (Appendix B), 1.5 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Invitrogen) and 1 µl of plasmid DNA (1 µl plasmid diluted with 200 µl water) as template. The reaction volume was made up with sterile distilled water.

PCR cycling conditions used included an initial denaturation of 96°C for 2 min. This was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 67°C for 30 s and elongation at 72°C for 1 min. A final elongation at 72°C for 10 min, then a 4°C hold for 30 s and a final hold at 15°C were included. Full-length PCR products were generated and visualized by loading 10 µl on a 1% (w/v) agarose/1x TAE (pH 8.0) gel containing about 0.1 µg/ml EtBr. The gel was run for one hr at 80 V. DNA was visualized under an ultraviolet light (White/Ultraviolet Transilluminator, Ultra Violet Products Ltd.).

The products were cleaned up with QIAquick®PCR Clean-up Kit (Qiagen) according to the manufacturer's instructions. The products were then sequenced using the BigDye® Terminator v.1.1 Cycle sequencing Kit (Applied Biosystems) and an ABI PRISM® Genetic analyzer (Applied Biosystems) and results were subjected to a BLAST N search, confirming the identity (Table 4.2). The following volumes of the above-mentioned PCR products determined by gel analysis (Figure 4.9) were labeled for probes: PR-1: 2 µl, PR-2: 5 µl, PR-3 to PR-5: 3 µl and for the *R. solanacearum* probe: 3 µl to obtain an equal concentration of each probe.

#### 4.2.2.5. Probe labelling

Labelling of the probes was preformed by random priming of ($\alpha$-$^{32}$P) deoxyadenosine 5' triphosphate (dATP) (Amersham Biosciences) using HexaLabel Plus ™ DNA Labelling Kit (Fermentas Inc., Hanover, USA) according to the manufacturer's instructions with

slight modification as presented here. The following components were placed in a reaction tube: about 100 ng template DNA, hexanucleotide in a 5x reaction buffer and DEPC-water to a volume of 40 µl. Denaturing was carried out at 97°C, followed by brief centrifugation and incubated on ice thereafter. The following components were added: 200 µM of each dNTP including ($\alpha$-$^{32}$P) dATP (4 µCi), 1x reaction buffer and 10U of Exo (-) Klenow enzyme. Incubation was carried out at 37°C for 1 hr. Afterwards unincorporated nucleotides were removed by using the PCR Clean-up kit (Qiagen). Boiling for 10 min denatured the eluted probes.

#### 4.2.2.6. Northern hybridisation

Membranes were prehybridised for 1 hr at 65°C with 20 µl of the Puregene hyb-9 hybridisation solution (Genta Systems, Plymouth, MN, U.S.A.), supplemented with with 0.1 mg/ml of heat denatured salmon sperm DNA (Sigma, Missouri, USA). Denatured probes were separately added and hybridised overnight at 65°C with the same buffer. Membranes were subjected to a threefold wash at high stringency: firstly, at 65°C in 2x SSC/0.1% SDS (1x SSC was 0.15M NaCl, and 0.015M sodium citrate) for 20 min; secondly, in 1x SSC/0.1% SDS for 10 min, and the final wash was for 10 min in 0.1x SSC/0.1% SDS. Radioactivity was monitored with a Geiger Counter.

Hybridisation with the *R. solanacearum* ribosomal probe was carried out as mentioned above except that after the overnight Hybridisation at 65°C, the membrane was washed for one hr in 1x SSC/0.1% SDS.

#### 4.2.2.7 Autoradiography

Blots were sealed with cling wrap as well as a plastic bag then exposed to the Molecular Imager (Bio-Rad Laboratories, Hercules, USA) for 3 hrs and subsequently visualized. Thereafter the membranes were exposed to high performance autoradiogram film, Hyperfilm™ (Amersham Biosciences) in a Hypercassette (Amersham Biosciences) at -80°C for 48 hrs. The film was then processed by 5 min incubation in a developer solution

(PolyconA, variable contrast x-ray developer (Axim, Midrand, SA)), 1 min in a fix solution (Prefix high speed x-ray fixer (Axim, Midrand, SA)), and thereafter washed with water and allowed to dry.

## 4.3 RESULTS

### 4.3.1. Bacterial growth curves

#### 4.3.1.1. Rifampicin resistant (Rif $^{r}$) strains

Rif $^{r}$ strains were generated to enable us to cleanly re-isolate the bacterial strains from *A. thaliana* and quantify the bacterial counts by dilution plating on the selective media. The following Rif $^{r}$ strains were generated: BCC 0351 (K), BCC 0352 (CK) and BCC 0357 (27B) (Table 4.1.) and their virulence confirmed by their ability to cause HR on the non-host tobacco (Fig. 4.3). BCC 0350 (GMI 1000) has the ability to grow on media containing 50 µg/ml rifampicin.

#### 4.3.1.2 *HrpB*$^{-}$ / *HrcS*$^{-}$ mutants

*HrpB*$^{-}$ / *HrcS*$^{-}$ mutants were generated to serve as a negative control during the dilution plating since the amount of bacteria *in planta* should stay the same. This also provided a valuable tool as a negative control in future micro-array analysis. Eight *HrpB*$^{-}$ / *HrcS*$^{-}$ mutants were generated (Table 4.1). Their lack of virulence was confirmed by the absence to cause HR on the non-host tobacco (Fig. 4.4).

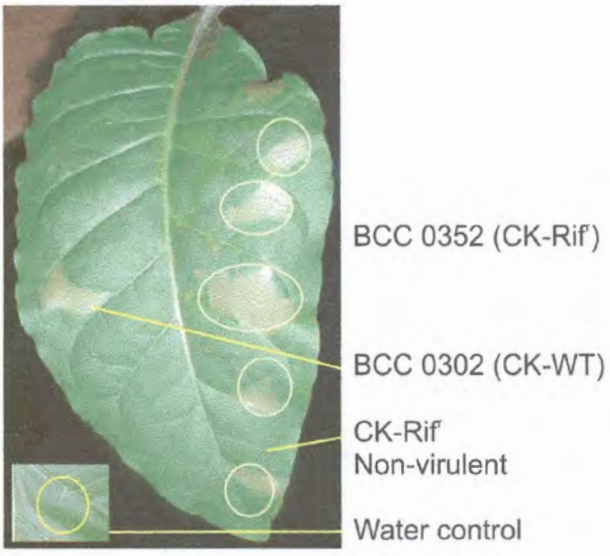



**Figure 4.3. The presence and absence of HR respectively, on the nonhost tobacco cv. Bottom Special generated with Rif $^{r}$ strains.** Successful spontaneous mutation of BCC 0302 (CK-wild type) to BCC 0352 (CK Rif $^{r}$), encircled. The insert shows a negative control, inoculated with water.

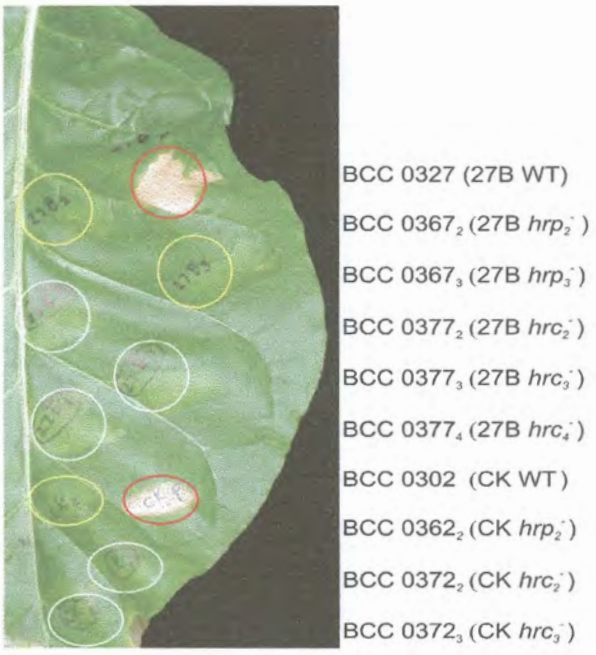



**Figure 4.4. The absence of HR when the *hrcS* / *hrpB* gene has been disrupted.** Note the characteristic HR present after inoculation with the wild type strain, marked with a red circle. *HrpB*$^-$ and *hrcS*$^-$ mutants of BCC 0327 (27B) and BCC 0302 (CK) strains marked with yellow and white circles respectively.

**Table 4.1: Isolates of *R. solanacearum* produced in this study**

| Wild-type | | Rif$^r$ | *HrpB*$^-$ | *HrcS*$^-$ |
|---|---|---|---|---|
| **BCC number** | **Strain** | **BCC number** | **BCC number** | **BCC number** |
| 0301 | K | 0351 | 0361 | 0371 |
| 0302 | CK | 0352 | 0362 | 0372 |
| 0306 | 62 | X | 0366 | 0376 |
| 0327 | 27B | 0357 | 0367 | 0377 |

X – Not obtained

#### 4.3.1.3 *A. thaliana* analysis

The derivatives, BCC 0362 and BCC 0372 of strain BCC 0302 (CK) (Table 4.1) were inoculated into the two susceptible ecotypes Be-O and Col-5 as well as the resistant ecotype Kil-O, caused wilting symptoms on the susceptible ecotypes, Be-O and Col-5. The Kil-O ecotype did not wilt.

Disease symptoms were evaluated and plant material harvested 0, 2, 6, 8 and 12 days after inoculation. Twelve days after inoculation ecotype Be-O showed extensive wilting, while Col-5 displayed wilt symptom 1, and Kil-O showed no symptoms (Fig. 4.5). Dilution plating followed by colony counts that were log transformed, pooled and presented as a growth curve (Fig. 4.6).

The data (Fig. 4.6) revealed that the bacteria multiplied in an almost linear fashion in the aerial parts of all the ecotypes inoculated with strain BCC 0352 (CK Rif$^r$), reaching very high bacterial concentrations ($10^{12}$ CFU bacteria per g fresh weight) 12 days after root inoculation. Surprisingly, the bacterial density in the seemingly resistant ecotype Kil-O where little wilt symptoms (2/60 plants completely wilted) were present 12 days after inoculation (Fig. 4.5), was only one order of magnitude lower ($10^{11}$ CFU bacteria per g fresh weight).

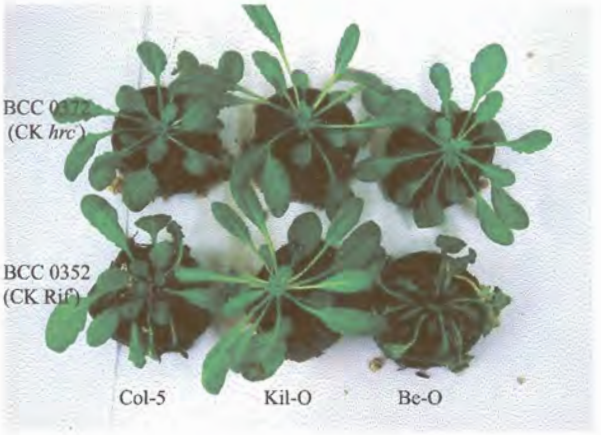



**Figure 4.5. Wilt symptoms observed 12 days after inoculation on Be-O and Col-5 inoculated with BCC 0352 (CK Rif $^{r}$) and the absence of wilt symptoms after inoculation with BCC 0372 (CK *hrcS*).** No disease symptoms were expressed after inoculation with the *hrcS*-mutant (BCC 0372). Disease development was present in the most susceptible ecotype, Be-O, where it progressed to wilt symptom (ws) (Chapter 3) = 4 while Col-5 expressed a ws = 2, and the resistant ecotype, Kil-O showed no disease development. Hereafter, these plants were subjected to analysis to determine the amount of bacteria present on day twelve.

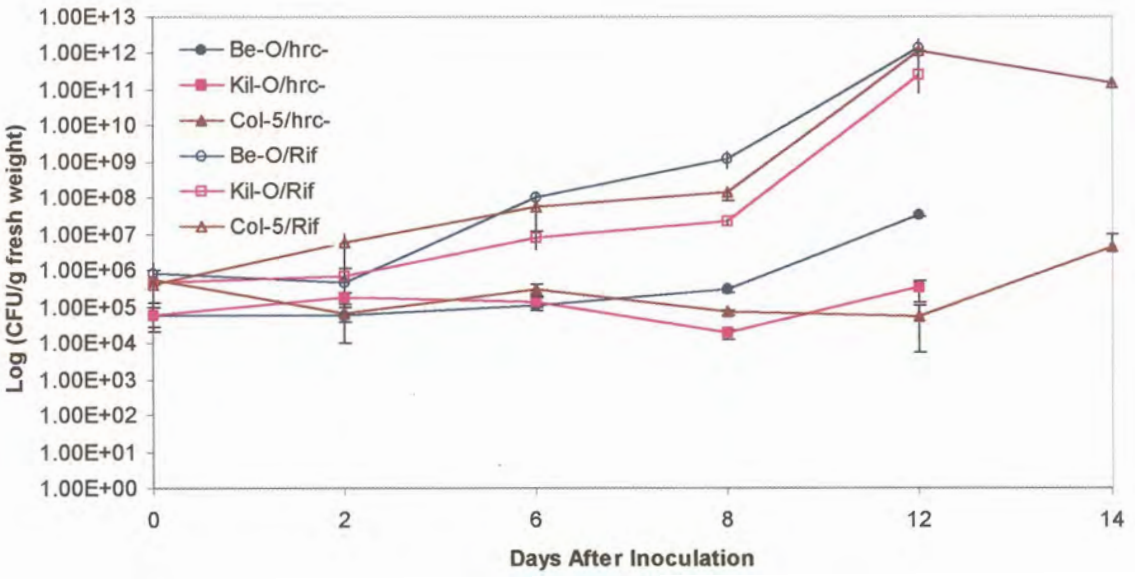
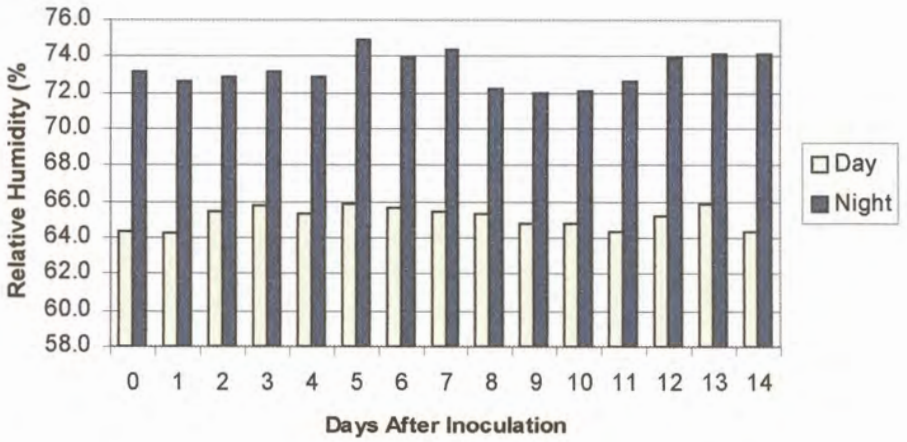



**Figure 4.6. Internal bacterial growth curves of BCC 0352 (CK Rif $^{r}$) and BCC 0372 (*hrcS* $^{-}$) strains of *R. solanacearum* in leaves of *A. thaliana* Be-O, Col-5 and Kil-O plants after root inoculation.** For each time point, at least triplicate assays were performed on three plants for each *A. thaliana* accession and bacterial strain.

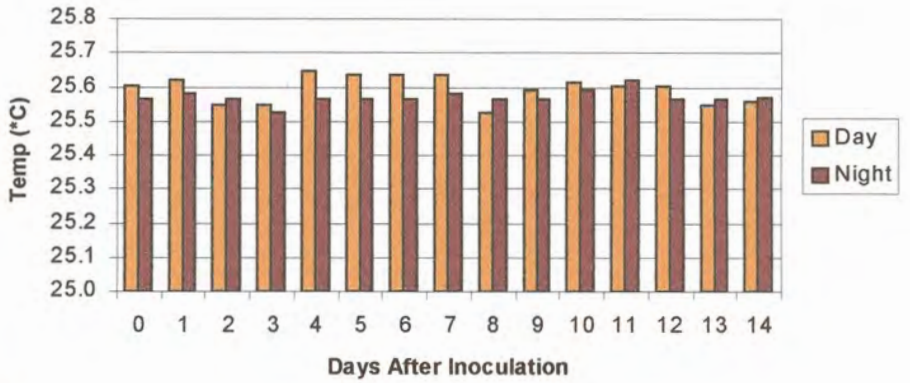
The CK *hrcS*⁻ bacteria (BCC 0372) did not cause disease in any of the ecotypes and the amount of CFU varied around $10^5$ reaching a maximum at $10^6$ for ecotype Col-5 inoculated with strain *hrcS*⁻ and $10^7$ for ecotype Be-O. All the ecotypes remained healthy and underwent normal development after inoculation with the *hrcS*⁻ CK (BCC 0372) bacteria.

Environmental factors were closely monitored to determine that they where constant and furthermore to detect if the results were influenced in any way. Conditions were more stable than expected in middle summer, January, South Africa. The results as depicted in the bar graphs (Fig. 4.7 A-C) are averages taken every 15 min for the duration of the bacterial numeration experiment. Data was pooled to give the respective day/night readings. Humidity was relatively high, during the day an average of 65% was measured and at night 74%. The day/night temperatures were very stable averaging out at 25.5°C, while the light condition was just as constant with a steady reading of 550 $\mu E/s/m^2$ during the day.

A)




B)




C)

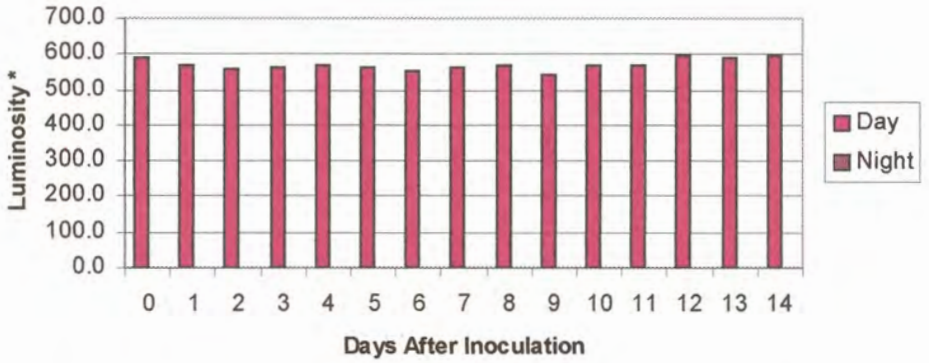



**Figure 4.7. The changes in humidity (A), temperature (B) and light (C) during the 14 days after inoculation.** **A)** Average relative humidity during the day was approximately 65%, and during the night, 73%. **B)** Average day/night temperatures remained stable at 25.5°C while **(C)** the plants were grown under constant light at about 550 µE/s/m$^2$ (*) during the day.

### 4.3.2. Northern analysis of the *A. thaliana* ecotypes

#### 4.3.2.1. RNA evaluation

RNA formaldehyde gel analysis revealed that the hot phenol method yielded high quality total RNA and concentrations varied between 1 and 6 µg/µl. The samples from the two experiments were loaded on the same RNA formaldehyde gel with a final concentration of 20 µg/µl (Fig. 4.8).

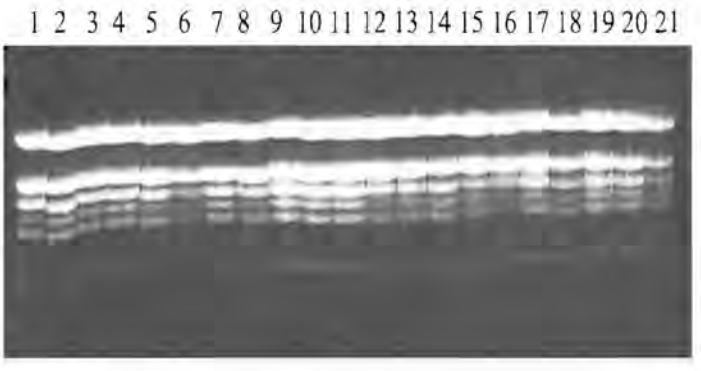



**Figure 4.8. Formaldehyde-agarose electrophoresis of total RNA isolated from *A. thaliana* ecotypes.** Equal loading of the RNA was checked by illumination of the gel under an UV-light achieved by adding EtBr to the loading buffer.

#### 4.3.2.2. Probe generation and probe labelling

Plasmid DNA's were extracted and amplified with the universal primer pair M13 forward and M13 reverse (Appendix B, Fig. 4.8). Table 4.2 shows that the probes were almost totally homogenous to the GENBANK accessions.

**Table 4.2.** Characteristics of Northern probes

| Probe | Genbank accession number | DNA sequence homology | Description | TAIR accession number |
|---|---|---|---|---|
| PR-1 | AT2G14610.1 | 97% | Pathogenesis-Related Protein 1 | Locus 206 4294 |
| PR-2 | AT3G57260.1 | 96% | Beta 1-3 Glucanase | Locus 208 2543 |
| PR-3 | AT3G12500.1 | 97% | Basic endochitinase | Locus 209 2502 |
| PR-4 | AT3G0470.1 | 90% | Hevein-related Protein Precursor | Locus 208 4918 |
| PR-5 | AT1G75040.1 | 98% | Thaumatin-like Protein 5 | Locus 203 7235 |

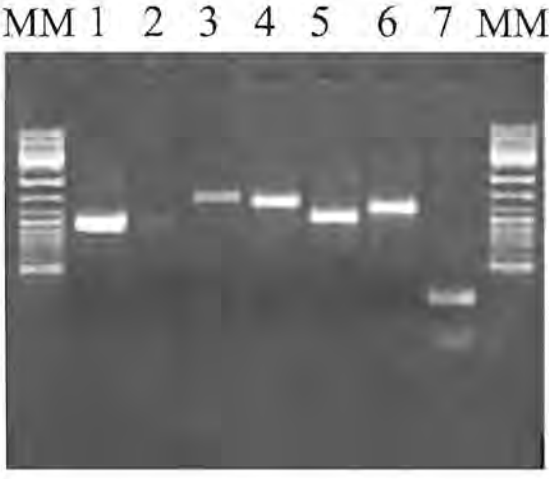



**Figure 4.9. Agarose gel electrophoresis of the PCR products of the probes prior to probe labelling.** MM: Molecular marker; lanes 1 and 2: PR-1A and PR-1B; lane 3: PR-2; lane 4: PR-3; lane 5: PR-4, lane 6: PR-5 and lane 7: 16S rRNA *R. solanacearum*.

### 4.2.3.3. Northern hybridisation

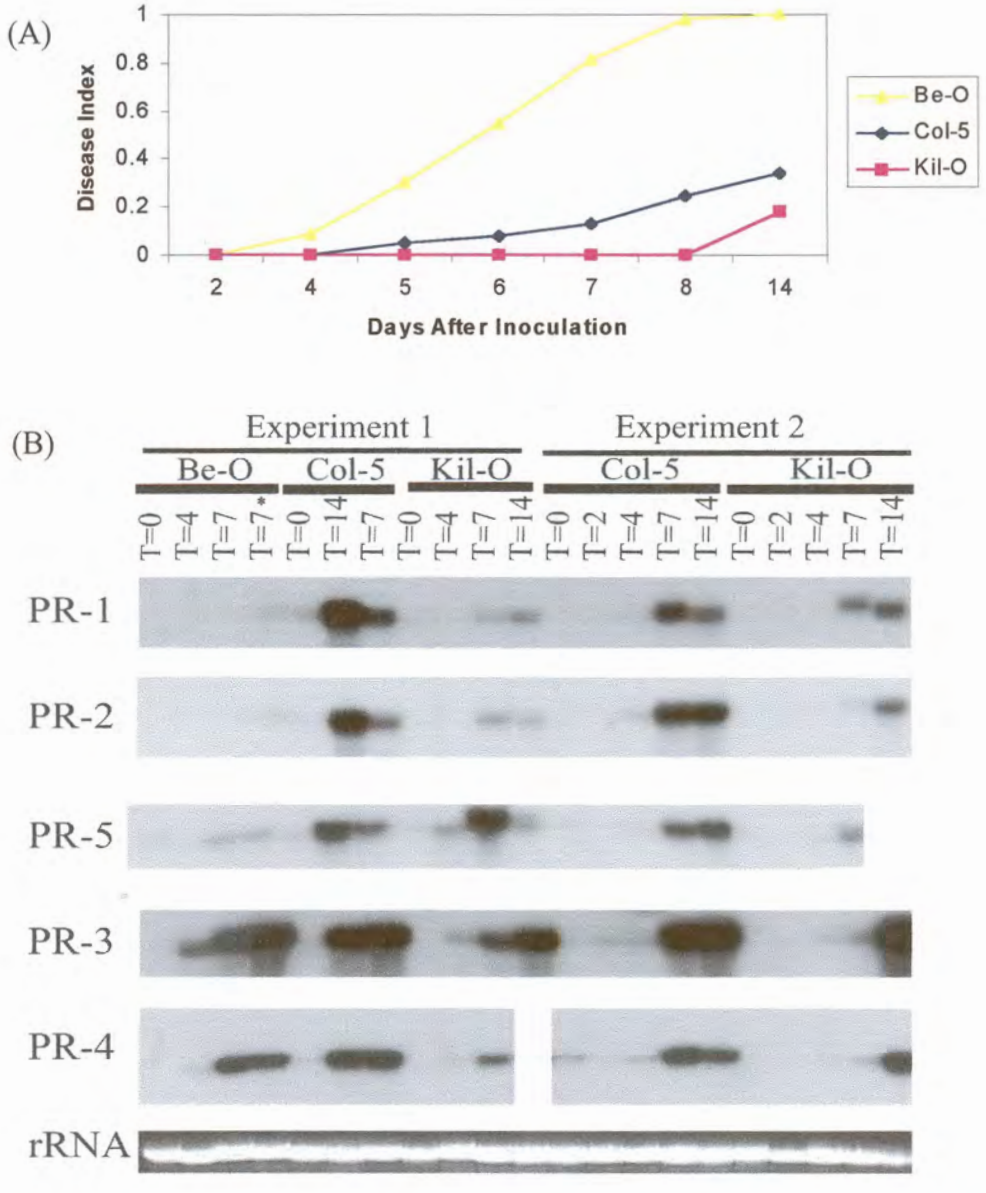



**Figure 4.10. (A) Progression of disease symptoms and (B) northern analysis of PR proteins in three *A. thaliana* ecotypes inoculated with *R. solanacearum* strain BCC 0302 (CK).** Data points were generated from readings taken from at least 63 plants per ecotype per day (a). Probes used for each blot are identified to the left. Numbers at the top refer to the days after inoculation when the plant material was harvested for the RNA analysis. Gaps are present when a narrower gel system was used due to a time constraint, consequently, all the samples could not be loaded.

Wilt symptoms on the ecotype Be-O occurred as early as 2 days after inoculation and 6 days later (day 8) the rosettes were completely shrivelled (Fig. 4.10 A). The Northern blot revealed that PR-1 and PR-2 were not present and PR-5 accumulated slightly on day 7 at ws = 2 (7) and ws = 4 (7*). However, PR-3 and PR-4 was strongly expressed when the DI was higher than 0.4.

The first wilt symptoms on ecotype Col-5 occurred 5 days after inoculation, increasing to a maximum DI of 0.4 seven days later (day 14). Delayed disease symptom response being evident when compared to ecotype Be-O (Fig. 4.10 A). However the Northern blot (Fig 4.10 B) revealed sustained induction of all the PR genes in both experiments. An increase in the accumulation of PR-1, PR-2 and PR-5 transcripts could be seen from 7 days to 14 days after inoculation. Accumulation of PR-3 transcripts started as early as two days after inoculation and all the PR genes exhibited very strong accumulation at 7 and 14 days after inoculation.

Ecotype Kil-O did not show any wilt symptoms until two weeks after inoculation when 5 of the 63 plants started to show wilt symptoms. The Northern blot revealed accumulation of PR-1, PR-2 and PR-5 transcripts at 7 days post inoculation with a further increase at day 14. In the first experiment PR-5 transcripts reached the maximum at 7 days after inoculation. Strong accumulation of transcripts PR-3 and PR–4 was displayed, despite the fact that DI was below 0.2.

The *R. solanacearum* ribosomal probe showed an increase in transcript accumulation for Col-5, a slight increase in Be-O and no transcript accumulation for Kil-O (data not shown). Although this result seems promising, experiments should be repeated to arrive to a final conclusion.

## 4.4 DISCUSSION

Virulent strains of *R. solanacearum* causes wilt symptoms in more than 50 plant families (Hayward, 1994). The main objective of the research undertaken in this project was to establish a new pathosystem in southern Africa enabling one to gain a better understanding of disease development and thereby finding strategies to limit disease spread. Determining the bacterial growth populations during disease development is one of the final characterization steps for the pathosystem.

Bacterial growth curves were needed to firstly determine if the plant reduces bacterial growth during a seemingly resistant interaction. Secondly, in the case of a susceptible interaction to determine if wilt occurs due to clogging up of the xylem vessels by the bacteria and extracellular compounds like the acidic extracellular polysaccharides (EPS1) (Schell *et al.*, 1994).

Rif$^{r}$ strains of *R. solanacearum* with the ability to grow on rifampicin media producing mucoid, pathogenic colonies, were generated to investigate the amount of viable cells present at different disease ratings over time. This was more reliable than just counting the wild type cells on BGT media, since the leaf extracts contain fungi and other bacteria that can overgrow the *R. solanacearum* cells. *Hrp*B$^{-}$/*hrc*S$^{-}$ were used as a negative control.

Bacterial multiplication estimated *in planta* confirmed my classification of Be-O and Col-5 as being susceptible to *R. solanacearum* strain BCC 0302 (CK). However, it also revealed that the apparently healthy and thus seemingly resistant ecotype, Kil-O, supported high bacterial populations only one order of magnitude lower than that present in the two susceptible ecotypes (respectively $10^{12}$ and $10^{11}$ CFU bacteria per g fresh weight). This was not as low as expected or as found in trials by Deslandes *et al.* (1998), where the resistant ecotype, Nd-1, showed reduced bacterial multiplication with a

maximum of $10^7$ CFU bacteria per g fresh weight, 10 days after root inoculation. The lack of symptoms in ecotype Kil-O can thus not be ascribed to the fact that bacteria reached low population levels, as was the case with Nd-1 (Deslandes *et al.*, 1998). A possibility might be the physical adaptation of this ecotype, for example, the presence of larger xylem vessels that can not be clogged up as soon as the susceptible ecotypes. This however needs to be confirmed by microscopy.

The inoculated bacterial strains, BCC 0352 (CK Rif$^r$) and BCC 0362 (CK *hrcS*$^-$) were detected in the rosettes as soon as one hr after inoculation at concentrations ranging between $10^5$ and $10^6$ CFU per g fresh weight. Deslandes *et al.* (1998), explained this phenomenon as a passive adsorption and rapid diffusion throughout the plant due to capillary forces exerted by transpiration. The ability to multiply was dependent on the plant genotype, i.e. *A. thaliana* ecotype and the bacterial strain used.

The ecotypes inoculated with the defective BCC 0372 (CK *hrcS*$^-$) strain remained healthy, and plant development was normal. The bacterial growth curves illustrate the inability of the BCC 0372 (CK *hrcS*$^-$) strain to have multiplied *in planta* to higher levels than $10^5$ CFU bacteria per g fresh weight. This was also described by Godiard *et al.* (2003) and also occurred in a Δ*hrp* derivative of GMI 1000 (Deslandes *et al.*, 1998). This confirms that the effective multiplication *in planta* relies on a functional *hrp* gene.

The molecular mechanisms responsible for resistance remain ambiguous in most plant-pathogen interactions (Smart *et al.*, 2003). However, the *A. thaliana – R. solanacearum* pathosystem has been studied intensively, and the first *R-avr* gene pair was identified (Deslandes *et al.*, 1998). This specific RRS1-R-PopP2 interaction did not appear to be responsible for resistance in this study. It is possible that each plant species has the ability to respond in an unique way to different pathogens (Smart *et al.*, 2003).

During my study I also found that the resistance in Kil-O to *R. solanacearum* is temperature sensitive, due to the fact that Kil-O showed little to no resistance during high temperatures (data not shown). This can be attributed to two factors (Hayward, 1991),

one may be that the virulence factors of the pathogen are only expressed during high temperatures. This, however, is not likely because BCC 0302 (CK) caused extensive wilt of Be-O and Col-5 at lower temperatures. Another factor, which is more probable, is that Kil-O is no longer capable of inducing the resistance genes at high temperatures.

This study revealed that the SA responsive genes PR-1, PR-2 and PR-5 were expressed in ecotype Col-5 inoculated with strain BCC 0302 (CK), whereas the corresponding transcripts were present at lower levels in ecotype Kil-O and either not detected or not present in the highly susceptible ecotype Be-O. These PR genes have previously been shown to be induced by exogenous application of SA or the chemical derivatives thereof (2,6-dichloroisonicotinic acid and benzothiadiazole) as well as by the bacterial pathogen *Pseudomonas syringae* pv. tomato (Uknes *et al.*, 1992). SA is a putative endogenous signal for SAR (Uknes *et al.*, 1992). Although PR-1, PR-2 and PR-5 are all induced during SAR, the regulation of the genes can be separated genetically for example, in *eds* mutants after infection by the pathogen PR-1 levels are severely reduced, whereas PR-2 and PR-5 levels match those of the wild type plants (Maleck and Dietrich, 1999).

The lack of the expression of the SA responsive genes PR-1, PR-2 and PR-5 indicates that this defence-related pathway was not activated in Be-O. This possibly explains the rapid onset and development of wilt symptoms in this ecotype. Col-5 however, displayed reduced wilting symptoms compared to Be-O and complete wilt appeared three weeks post inoculation. O'Donnell *et al.*, (2003) stated that susceptible hosts do possess a defence response that limits but does not stop pathogen growth. The increase in expression as seen in Col-5, might be attributed to this basal resistance. A possibility might be that Col-5 does recognize the pathogen to some extent and defence related pathways are up regulated, holding back pathogen progress until the bacterial populations overcome the resistance resulting in disease development.

The *A. thaliana* PR-4 gene is SA-responsive and induced by ethylene and JA (Maleck and Dietrich, 1999). The PR-3 gene, encoding for basic endochitinase, has been shown to be induced by the exogenous application of ethylene (Hirsch *et al.*, 2002). The data

presented in this study revealed simultaneous expression of PR-3 and PR-4 genes in the resistant and susceptible ecotypes starting at 2 days after inoculation for PR-3, and at 4 days after inoculation for PR-4 continuing up to 14 days after inoculation. Although expression did not appear to be constitutive, it might be due to root-wounding induced during inoculation. However, among the plant-pathogenic bacteria, *R. solanacearum* has long been known to produce ethylene gas and this is maximal in the early exponential phase (Weingart *et al.*, 1999), this might explain the increase of these ethylene responsive genes, but the question arises why does expression not decline as disease progressed? A negative control other than the zero time point should be included in future studies, it was omitted in these experiments due to a space limitation for growing the plants. Two options exist, the first is using the *hrpB* ⁻ or *hrcC* ⁻ derivatives of BCC 0302(CK) for inoculation, data generated in this way would differentiate between resistance and the mere recognition of a non-virulent bacterial population, as well as defence response due to wounding. The second option is the use of a water control i.e. plants are cut and soaked in water, this data would show the response of the environment as well as wound response.

The expression levels of the PR-3 and PR-4 genes did not distinguish the susceptible from the resistant ecotypes. Expression can possibly be linked to the amount of bacteria present in the plants. This was suggested by Smart *et al.* (2003). Studying the tomato – *Phytophthora infestans* pathosystem they found that PR-gene expression appeared earlier in the susceptible interaction during which the pathogen multiplied at a higher rate. Thus a greater number of plant cells responded to the presence of the pathogen. Hirsch *et al.* (2002), found that both PR-3 and PR-4 gene expression was present in the susceptible ecotype (Col-0) but absent in the resistant ecotype (Nd-1) after inoculation with the *R. solanacearum* strain BCC 0300 (GMI 1000). In addition, bacterial growth curves showed the high rate of bacterial multiplication in the susceptible ecotype (Col-0) when compared to the resistant ecotype (Nd-1), a difference of 3 orders of magnitude. This matched the PR-3 and PR-4 gene expression levels as presented by Hirsch *et al.* (2000) agreeing with the hypothesis of Smart *et al.* (2003). Data presented here for PR-3 and PR-4 in the ecotype Be-O infected with *R. solanacearum*, BCC 0302 (CK) did not match

the result of Hirsch *et al.* (2000) i.e. up-regulation in the susceptible ecotype. However, there was only a difference of 1 order of magnitude of bacterial multiplication between the susceptible (Be-O) and resistant (Kil-O) ecotypes. This may explain the lack of difference in PR-3 and PR-4 expression if the notion of Smart *et al.* (2003) is correct that expression of these genes correlates with bacterial levels *in planta*.

The resistance observed in Kil-O might either have a simple or complex genetic basis. A complex genetic basis could include preformed resistance barriers, the absence of targets for virulence factors or some yet undiscovered phenomenon controlled by Quantitative Trait Loci (QTL) affected by environmental factors such as heat and drought stress. The way of investigation QTL would be to generate genetic crosses between the parents, to produce F1 and F2 populations. Screening these offspring for resistance would enable us to determine if a single gene on multiple loci controls resistance.

In conclusion, a comparison between the ecotypes Be-O and Kil-O infected with the Congolese *Eucalyptus* strain of *R. solanacearum* BCC 0302 (CK), gives substantial evidence of a new pathosystem. Further research is required in order to determine what is the basis of the healthy Kil-O phenotype, despite the high number of bacteria present in the plants. Transcriptome studies investigating the pathosystem is currently underway and should clarify the responses characterized here, possibly revealing genes more appropriate than the PR genes for the exploration into the mechanisms of resistance.

# CHAPTER 5

# Concluding Discussion

*Ralstonia solanacearum* is a soil-borne pathogen of considerable importance with a global distribution, causing devastating crop losses (Hayward, 1991). *A. thaliana* is a model plant. The overall objective of this study was to develop a new plant-pathosystem, both stable and reliable, as a basis for future transcriptome (microarray) studies.

According to Quirino and Bent (2003), establishing an *A. thaliana-R. solanacearum* pathosystem requires the following criteria: (i) Optimization of the conditions for growth of *A. thaliana.* (ii) Development of a successful pathogen inoculation protocol; (iii) establishment of disease scoring methods; (iv) a source of virulent and avirulent *R. solanacearum* strains; and finally (v) identification of susceptible and resistant ecotypes of *A. thaliana*. Growing *A. thaliana* and development of a pathogen inoculation method leading to disease development proved to be an arduous task. Growth conditions as well as a disease scoring method adopted from Deslandes *et al.* (1998) are described in Chapter 3.

The first aim of this study was to confirm the identity of the causal agent of bacterial wilt occurring in *Eucalyptus* plantations in the Congo, Uganda and South Africa. These isolates provided a promising new source of virulent *R. solanacearum*. Forty-nine isolates causing bacterial wilt of solanaceous crops and *Eucalyptus* were subjected to 16S rRNA and *hrp* (hypersensitive response and pathogenicity) gene PCR analysis. Seven had been characterized previously using metabolic profiling and were confirmed in this study by *hrp* gene PCR analysis. Only one of the remaining 42 isolates, namely BCC 0327 (27B), was positively identified as *R. solanacearum*. This strain was the most pathogenic causing sudden and severe wilt of *A. thaliana.* This correlated well to the renowned strain GMI 1000 (BCC 0300) used as control in this study. Seven ecotypes, namely Col-5, Nd-1, Kil-O, Be-O, Sf-2, Laer and Cvi were screened in this study and only Nd-1 exhibited resistance to this new virulent isolate.

The results from the *hrp* gene PCR products were further analysed using Restriction Fragment Length Polymorphism (RFLP). Amplified Fragment Length Polymorphism (AFLP) analysis was also carried out. The PCR-RFLP data grouped the seven *R.*

*solanacearum* isolates into their respective biovars. An analysis of the AFLP data confirmed this by clustering the two biovars separately. The latter technique revealed the presence of at least three polymorphisms in the biovar 3 group. However, due to sample size this was not enough to define sub-clustering, but it did provide valuable information. A polymorphism detected with both the RFLP and the AFLP analyses distinguished BCC 0300 (GMI 1000) from BCC 0327 (27B). The RFLP and AFLP analyses verified that the PCR analyses were true and reliable with a high level of accuracy. The *hrp* gene PCR based diagnostic technique will now enable researchers to identify the pathogen rapidly. Management strategies can then be implemented within a week after an outbreak occurs, thus fulfilling the first objective of this study.

The second objective was to identify susceptible and resistant ecotypes to at least one *R. solanacearum* strain. Seven natural ecotypes of *A. thaliana* were screened for their response against eight isolates of *R. solanacearum*. Four of these bacterial isolates from the Congo, Uganda and South Africa were from the host tree, *Eucalyptus*. A clear differential response was obtained with the Congolese isolate BCC 0302 (CK), using Be-O as the susceptible ecotype and Kil-O as the resistant ecotype.

The third and final objective of this research study was characterizing the new pathosystem between BCC 0302 (CK) and the selected ecotypes. This included determining the growth rate of the bacterial populations during disease development as well as studying the accumulation of defence-related transcripts. Be-O was confirmed as susceptible supporting high concentrations of bacterial growth *in planta* ($10^{12}$ CFU bacteria per g fresh weight); while that of Kil-O was one order of magnitude lower ($10^{11}$ CFU bacteria per g fresh weight) ten days after root inoculation. At this stage ecotype Be-O exhibited severe wilting symptoms while Kil-O exhibited none. A non-pathogenic *R. solanacearum* derivative of BCC 0302 (CK) was produced by *hrc*S-disruption in the *hrp* gene. This disclosed that the bacteria needed a functional *hrp* gene system to be pathogenic, and that symptoms were due to bacterial multiplication, and not on account of toxic compounds released during colonization of the vascular system. More research is required to determine why Kil-O can maintain such high bacterial populations without

visible disease symptoms. This could be achieved by performing a basic anatomical analysis to establish if it is purely a physical attribute of this ecotype, for example, the presence of larger xylem vessels. Furthermore, a genetic analysis would be advisable to ascertain if the trait responsible for resistance is dominant or recessive and thereafter to establish if it is a monogenic or polygenic trait.

Northern analysis revealed that the salicylic acid (SA) responsive genes represented by the pathogenesis related probes PR-1, PR-2 and PR-5 were either not detected or not present during infection of Be-O, while Kil-O did show accumulation of the transcripts after one week increasing towards the end of the second week. The lack of expression of these genes revealed that these defence-related pathways were not activated in Be-O. This could explain the rapid onset of disease development in this ecotype. PR-3 and PR-4 genes which are markers of the jasmonate / ethylene pathway did not show any differentiation between the accumulation of the transcripts for the susceptible ecotype, Be-O and the resistant ecotype, Kil-O. According to Smart *et al.* (2003) working on the tomato-*Phytophthera infestans* pathosystem, expression might be linked to the number of pathogenic bacteria present in the plant, thus expression occurring earlier in the compatible interaction. Since bacterial populations differed with only one order of magnitude in this study, this could lead to similar expression of the PR-3 and PR-4 genes, responding to the presence of *R. solanacearum* in the vascular system.

The French-South African pathosystem between *A. thaliana* and *R. solanacearum* induces excessive wounding when cutting the roots for inoculation; this however is not entirely true to nature where root wounding occurs on account of nematodes, other insect feeding insects and mammals as well as mechanical wounding during agricultural practices. Introducing the bacteria by soil soaking did not produce homogenous results, (data not shown) and the French lab (Yves Marco, personal communication), confirmed this.

The trials with the new virulent African isolate collected in Uganda, BCC 0327 (27B), appear promising, since it was the only isolate that presented high virulence. It was as

pathogenic as BCC 0300 (GMI 1000), a strain isolated from tomato, of which the whole genome has been sequenced. This was the first step towards an exhaustive functional analysis of virulence determinants in this pathogen. At this stage BCC 0327 (27B) has to be screened against more *A. thaliana* ecotypes to obtain a resistant ecotype other than Nd-1, which is used in the French *A. thaliana-R. solanacearum* (BCC 0300; GMI 1000) (Deslandes *et al.,* 1998) pathosystem. All other molecular tools are in place to exploit this potential promising pathosystem.

The hypothesis of this study was threefold: Firstly that bacterial wilt of *Eucalyptus* was caused by the phytopathogenic bacteria *R. solanacearum,* secondly that this isolate from a woody host will be able to induce wilting to *A. thaliana*, and thirdly that an *A. thaliana* ecotype resistant to this African *R. solanacearum* isolate does exists. The data obtained in this study supported the three-fold hypothesis.

Given that the genome sequences of both host and pathogen are available, it is logical in this post-genomic age to exploit microarray technology (Naidoo, *et al.,* 2002). Microarrays are a type of large-scale reverse Northern blot, which allows researchers to obtain information on gene expression simultaneously for thousands of genes (Harrington, *et al.,* 2000; Wu, *et al.,* 2001). By performing promoter comparisons in sets of co-regulated genes identified in microarray experiments, common elements can be identified and a signalling pathway can be elucidated. Furthermore, microarrays can be used to: identify genes involved in virulence by comparing expression profiles of virulent and avirulent bacterial strains, screen genes induced specifically in the host in response to attack by the pathogen, and identify disease resistance or important downstream effector genes in the host (Naidoo *et al.,* 2002).

As this new African pathosystem has now been established it can be used for transcriptome/microarray analysis in order to investigate the mechanisms of resistance as described above. This will assist us in understanding plant defence mechanisms thereby contributing to containing and preventing the spread of *R. solanacearum* in plantations and into arable land. Novel strategies arising from these studies will prevent the erosion

of basic nutrition sources in Africa and other developing countries by bacterial wilt disease. This will make a significant contribution to feeding and sustaining rural populations.

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# Appendix A

## 1. Bacto-agar Glucose Triphenyltetrazolium chloride (BGT) Media

For one litre

| | |
|---|---|
| Peptone | 10g |
| Casmino acid / Casein hydrolysate | 1g |
| Yeast extract | 1g |
| Agar bacteriological | 15g |
| | |
| 1.25% Triphenyltetrazolium chloride (TTC) | 4ml |
| 20% Glucose | 25ml |

Mix the peptone, casmino acid and yeast extract in 500 ml of water, then add agar. Add distilled water to a final volume of one liter. Autoclave the mixture for 20 minutes at 121°C. Add 4ml of TTC with a concentration of 1.25% and 25ml of 20% Glucose and the selected antibiotic if required, just before pouring.

**1,25% Triphenyltetrazolium chloride (TTC)**

1.25 g TTC dissolved in 100 ml of EtOH, store at 4°C, and cover bottle with foil.

**20% Glucose**

Dissolve 20 g of glucose in 100 ml of sterile distilled water and autoclave. After opening store at 4°C.

2. **B MEDIA** (LIQUID BROTH)

| | |
|---|---|
| Peptone | 10 g |
| Casmino acid / Casein hydrolysate | 1 g |
| Yeast extract | 1 g |

Peptone, casmino acid and yeast extract is added to 500 ml of distilled water and mix thoroughly. Add distilled water to a final volume of one liter. Autoclave the mixture for 20 minutes at 121°C. Add 25 ml of 20% Glucose and antibiotics if required.

**3. Antibiotics added**

| | **Stock solution** | **Final concentration** |
|---|---|---|
| **Rifampicin** | (50 mg in 1 ml of ethanol) | 50 mg |
| **Kanamycin** | (50 mg in 1 ml of distilled water) | 50 mg |
| **Spectinomycin** | (40 mg in 1 ml of distilled water) | 40 mg |

Kanamycin and Spectinomycin were dissolved in the water and filter-sterilized through 0.2 µm sterile filters. Antibiotic stocks were stored at –20°C

# APPENDIX B

Primers used in the study

| Primer | Length (bp) | Sequence 5'-3' | % GC |
|---|---|---|---|
| OLI1 | 21 | GGG GGT AGC TTG CTA CCT GCC | 67 |
| Y2 | 24 | CCC ACT GCT GCC TCC CGT AGG AGT | 67 |
| RS20 | 20 | GCT CGT GGT CCT GCT CGT GT | 65 |
| RS201 | 20 | CTC ACC GCC ACT GCC CAT TA | 60 |
| RS600 | 20 | TTT CTC CAT CCT GCC CGC CA | 60 |
| RS61 | 20 | CCA GGG CGA AGT AGA TGT TT | 50 |
| RS80 | 20 | TTG AAA GAG CAG GTG AAG CA | 45 |
| RS81 | 20 | CGA TGA TGT TGG ACG GAT TG | 50 |
| Pop P2.1 | 27 | CCC AAG CTT TGG TCA GAC GCG GGA AGC | 63 |
| Pop P2.7 | 25 | GAA GAT CTG CCA GCC GAT GTA CGC G | 60 |
| RT1 | 18 | ATG TTA TAT CGA CGT TGG | 38 |
| RT3 | 18 | AAC TCC TCC ATG TCC GTC | 55 |
| K3.3 | 18 | AGT CAT CAA GTG ACC ATC | 44 |
| K10 | 18 | GAT GGT CAC TTG ATG ACT | 44 |
| universalM13 F | 18 | GTT TTC CCA GTC ACG ACG TTG | 79 |
| universalM13 R | 21 | TGA GCG GAT AAC AAT TTC ACA CAG | 48 |