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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.

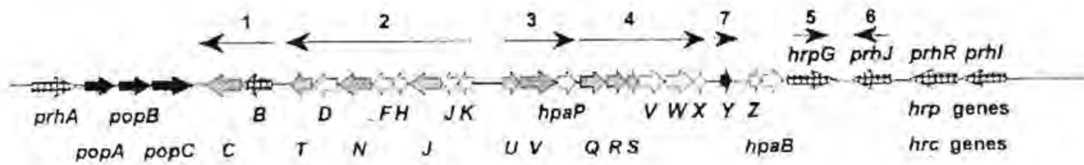


Figure 4.1. Genetic organization of the *R. solanacearum hrp* gene cluster. The thin arrows with numbers indicate the *hrp* transcription units; the thick arrows indicate the different genes. Conserved *hrc* genes are represented by the filled grey arrows, genes encoding secreted proteins by filled black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

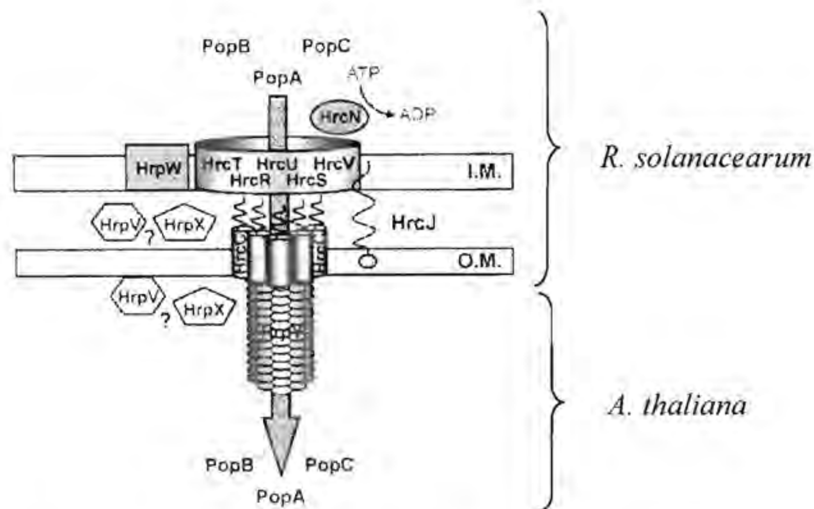


Figure 4.2. Model for the role of the different Hrp proteins in the assembly of *R. solanacearum* type III secretion apparatus. The inner membrane (I.M) and outer membrane (O.M.) of *R. solanacearum* is illustrated (Van Gijsegem *et al*, 2002).

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 (α-^{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 (α-^{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).

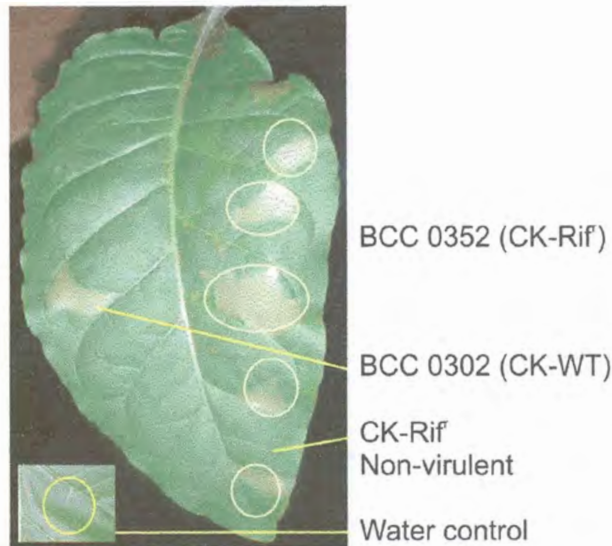


Figure 4.3. The presence and absence of HR respectively, on the nonhost tobacco cv. Bottom Special generated with Rif ^{r} strains. Successful spontaneous mutation of BCC 0302 (CK-wild type) to BCC 0352 (CK Rif ^{r}), encircled. The insert shows a negative control, inoculated with water.

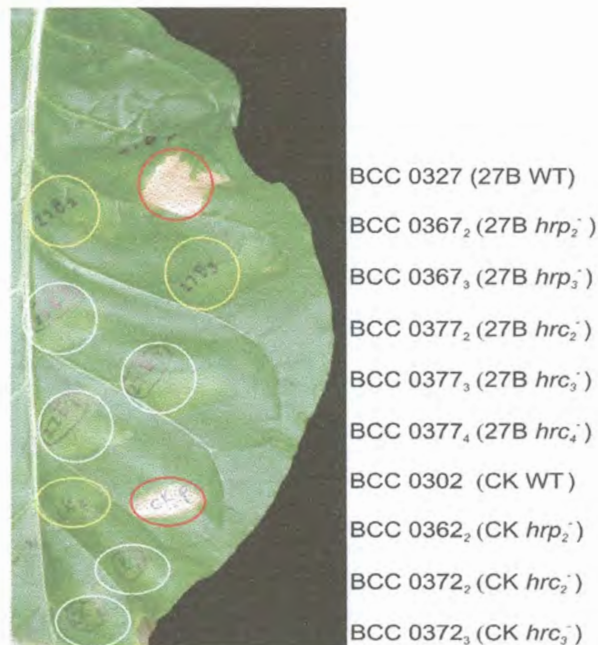


Figure 4.4. The absence of HR when the *hrcS* / *hrpB* gene has been disrupted. Note the characteristic HR present after inoculation with the wild type strain, marked with a red circle. *HrpB*⁻ and *hrcS*⁻ mutants of BCC 0327 (27B) and BCC 0302 (CK) strains marked with yellow and white circles respectively.

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).

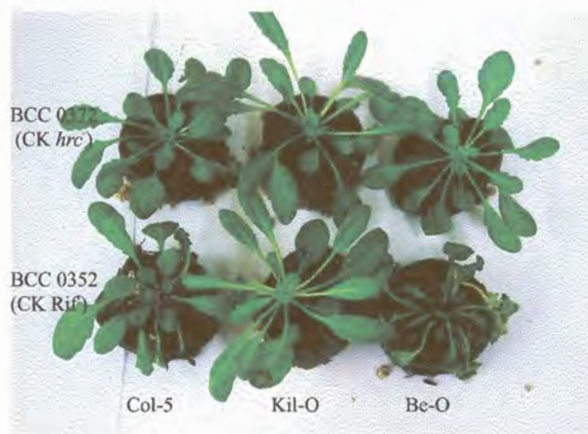


Figure 4.5. Wilt symptoms observed 12 days after inoculation on Be-O and Col-5 inoculated with BCC 0352 (CK Rif ^{r}) and the absence of wilt symptoms after inoculation with BCC 0372 (CK *hrcS*$^-$). No disease symptoms were expressed after inoculation with the *hrcS*-mutant (BCC 0372). Disease development 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.

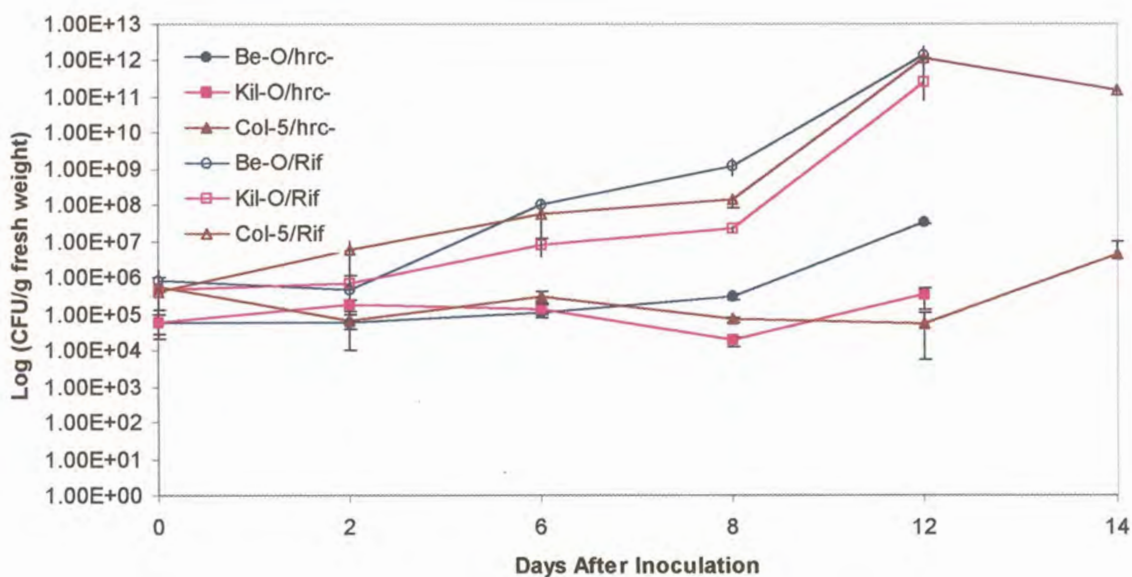
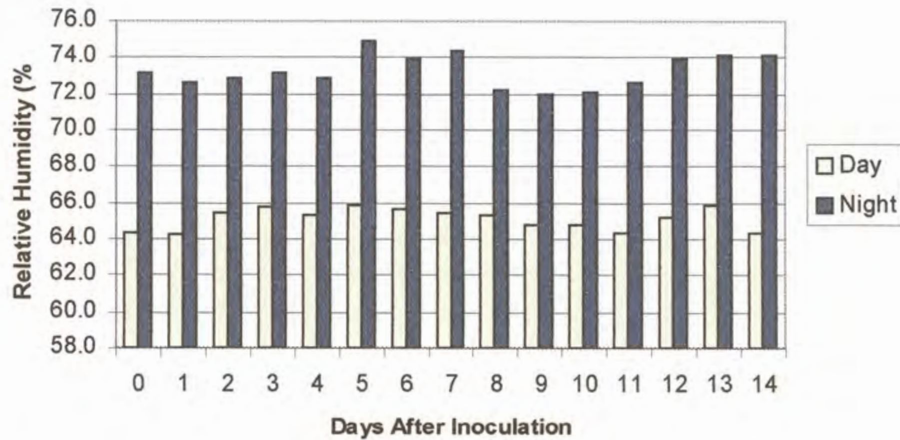


Figure 4.6. Internal bacterial growth curves of BCC 0352 (CK Rif ^{r}) and BCC 0372 (*hrcS*$^-$) strains of *R. solanacearum* in leaves of *A. thaliana* Be-O, Col-5 and Kil-O plants after root inoculation. For each time point, at least triplicate assays were performed on three plants for each *A. thaliana*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

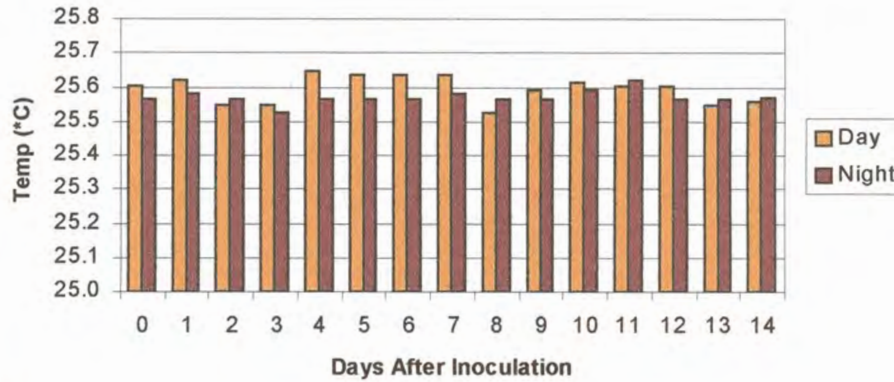
The CK *hrcS⁻* bacteria (BCC 0372) did not cause disease in any of the ecotypes and the amount of CFU varied around 10^5 reaching a maximum at 10^6 for ecotype Col-5 inoculated with strain *hrcS⁻* and 10^7 for ecotype Be-O. All the ecotypes remained healthy and underwent normal development after inoculation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

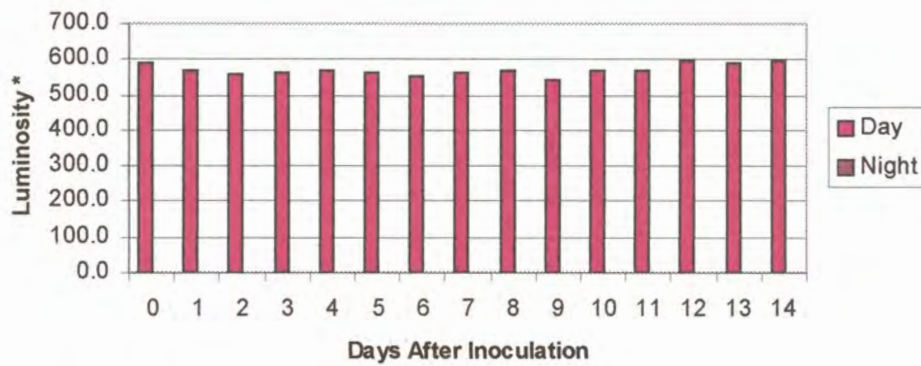


Figure 4.7. The changes in humidity (A), temperature (B) and light (C) during the 14 days after inoculation. **A)** Average relative humidity during the day was approximately 65%, and during the night, 73%. **B)** Average day/night temperatures remained stable at 25.5°C while **(C)** the plants were 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).

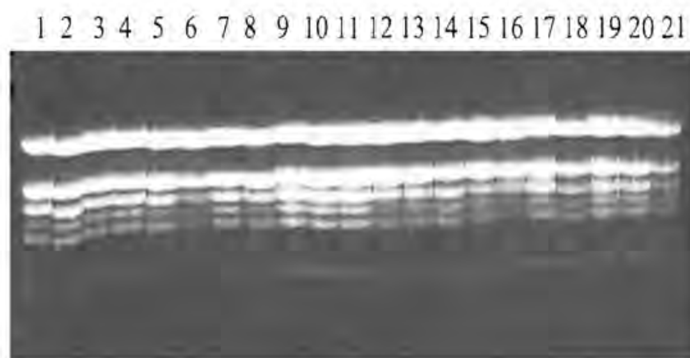


Figure 4.8. Formaldehyde-agarose electrophoresis of total RNA isolated from *A. thaliana* ecotypes. Equal loading of the RNA was checked by illumination of the gel under an UV-light achieved by adding EtBr to the loading buffer.

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

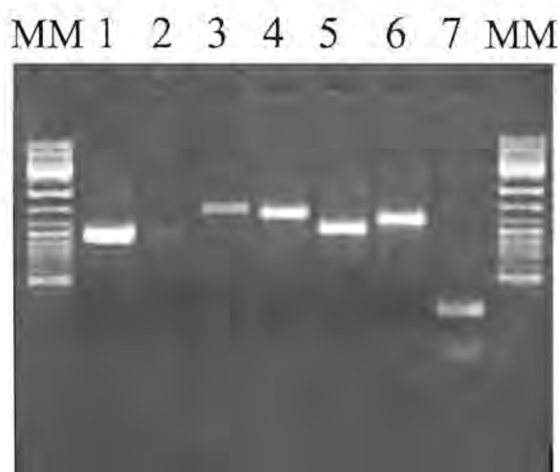


Figure 4.9. Agarose gel electrophoresis of the PCR products of the probes prior to probe labelling. MM: Molecular marker; lanes 1 and 2: PR-1A and PR-1B; lane 3: PR-2; lane 4: PR-3; lane 5: PR-4, lane 6: PR-5 and lane 7: 16S rRNA *R. solanacearum*.

4.2.3.3. Northern hybridisation

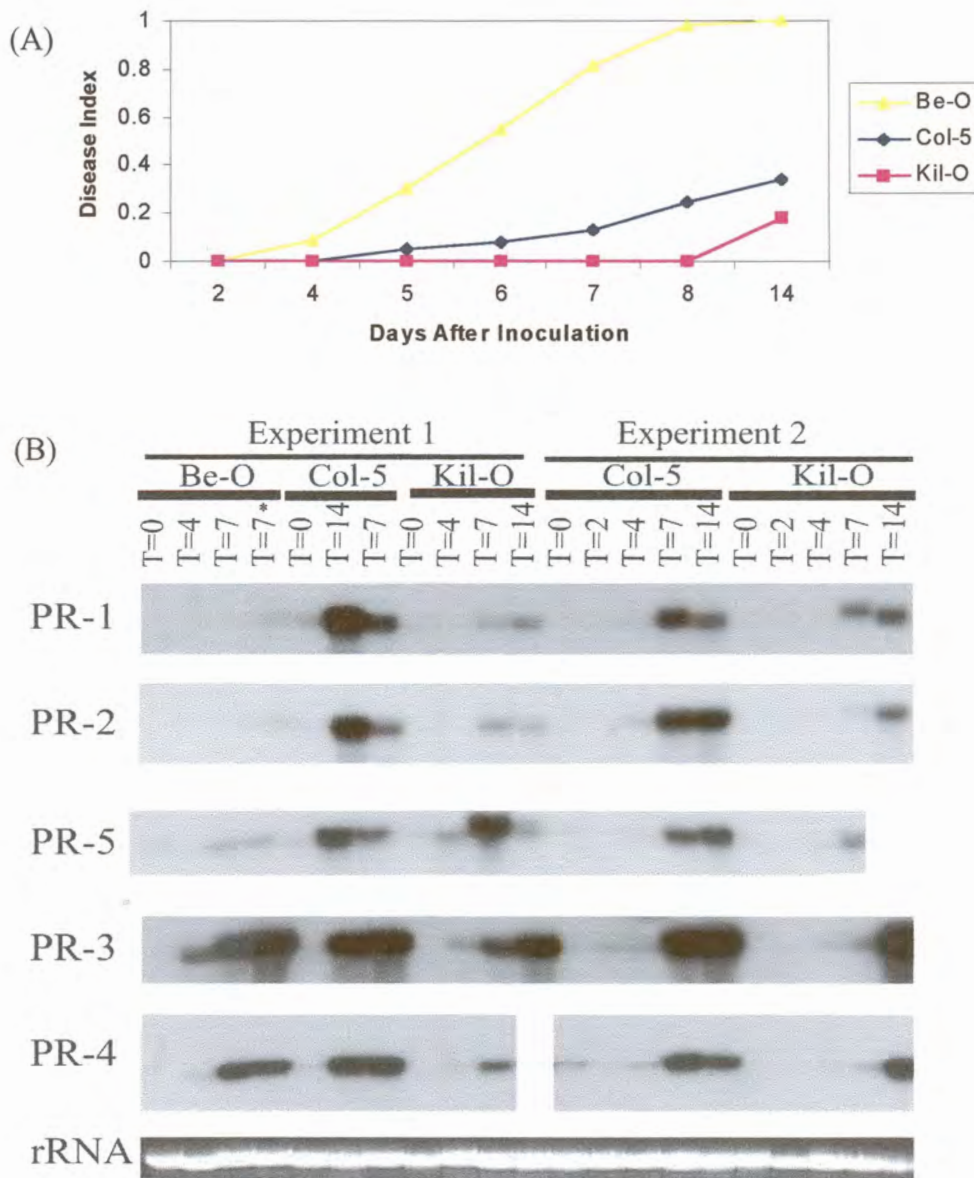


Figure 4.10. (A) Progressio•n of disease symptoms and (B) northern analysis of PR proteins in three *A. thaliana* ecotypes inoculated with *R. solanacearum* strain BCC 0302 (CK). Data points were generated from readings taken from at least 63 plants per ecotype per day (a). Probes used for each blot 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),

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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

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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.