# 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.