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.

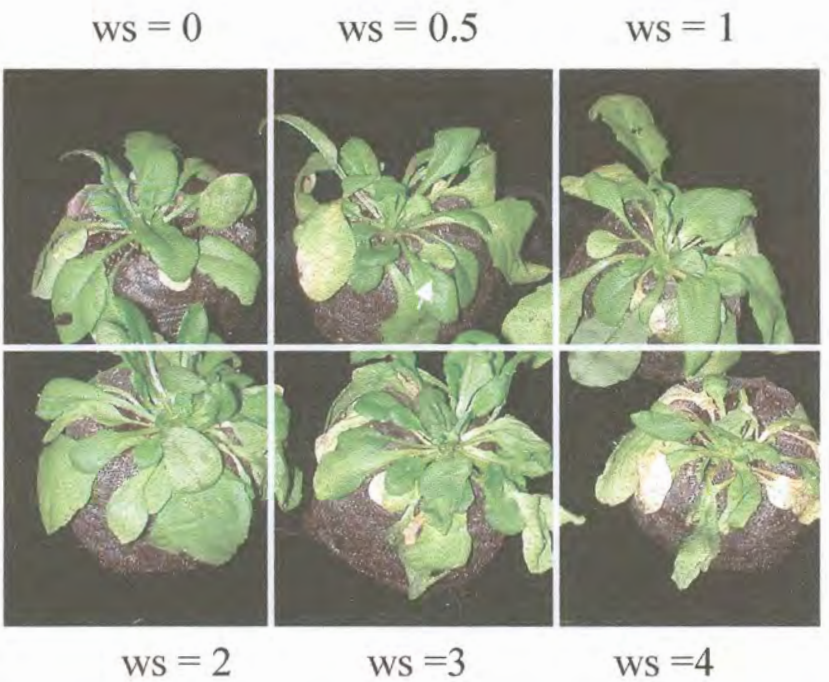

Figure 3.1. Illustrating the different wilt symptoms (ws) on Col-5 inoculated with the control strain, BCC 0300 (GMI 1000). Ws = 0.5: One leaf wilted, shown with the white arrow.

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.

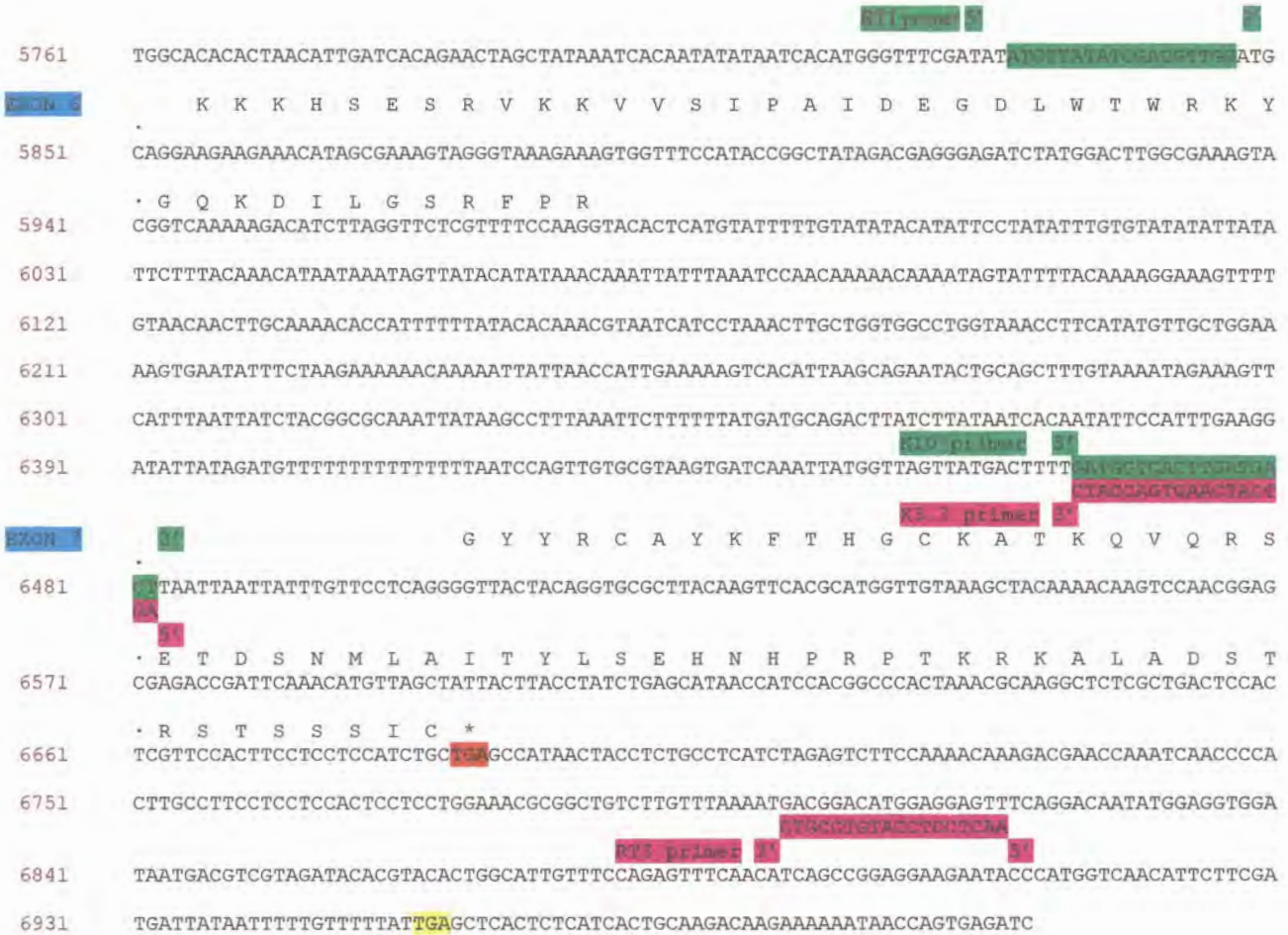

Figure 3.2. Nucleotide sequence of a section of the C-terminal end of the *RRS1* allelic structure of Col-0. Showing the translated exons (start shown in blue) as well as the DNA amplified with the primer pairs: RT1 and RT3 (988 bp), RT1 and K3.3 (650 bp) and K10 and RT3 (353 bp). These are the sizes 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.

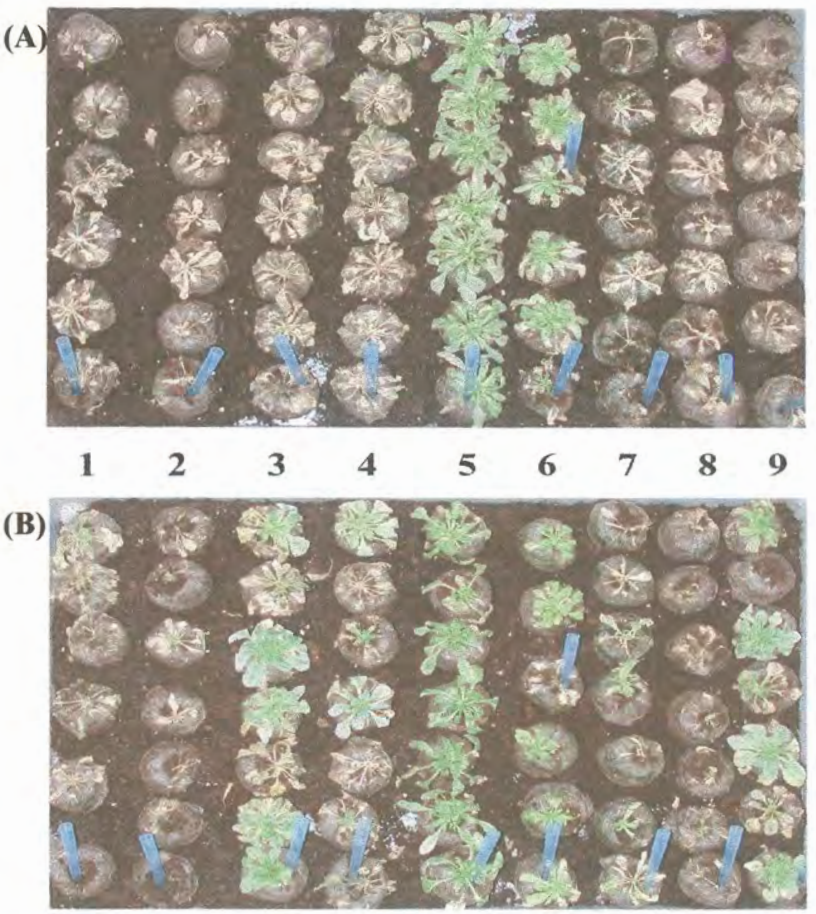

Figure 3.3. Disease symptoms after 18 days in the various *A. thaliana* ecotypes upon inoculation with the control strain, (A) BCC 0300 (GMI 1000), and strain (B) BCC 0302 (CK). Lanes 1 and 4: ecotype Be-O; lanes 2 and 8: ecotype Laer; lanes 3 and 9: ecotype Col-5; lane 5: ecotype Nd-1; lane 6: top blue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

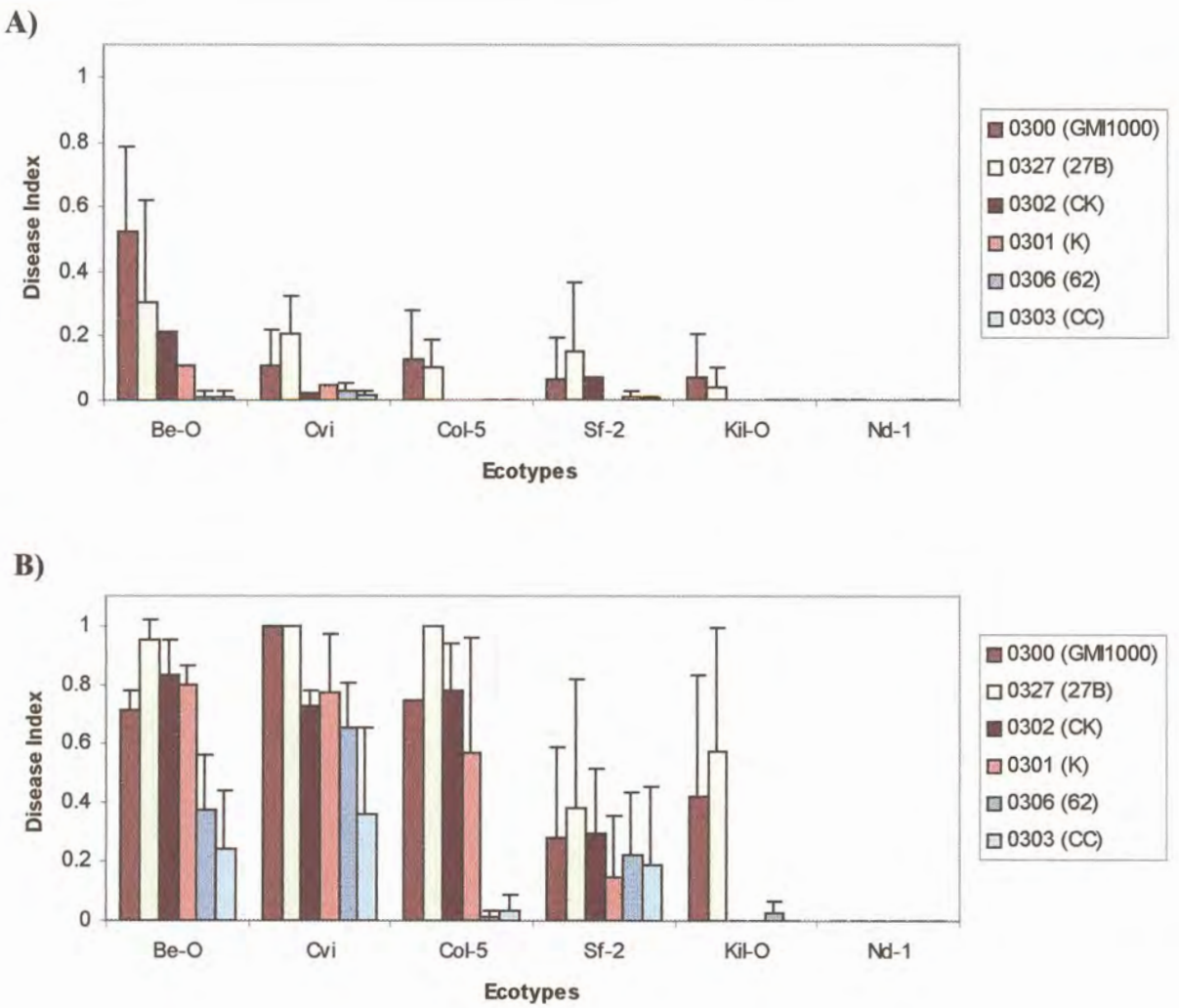

Figure 3.4. Disease symptoms in various *A. thaliana* ecotypes after root inoculation with different strains of *R. solanacearum*. Data presented here are the mean disease indexes at 6 and 18 days (A and B, respectively) of three experiments, with at least six plants per strain per ecotype. Standard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

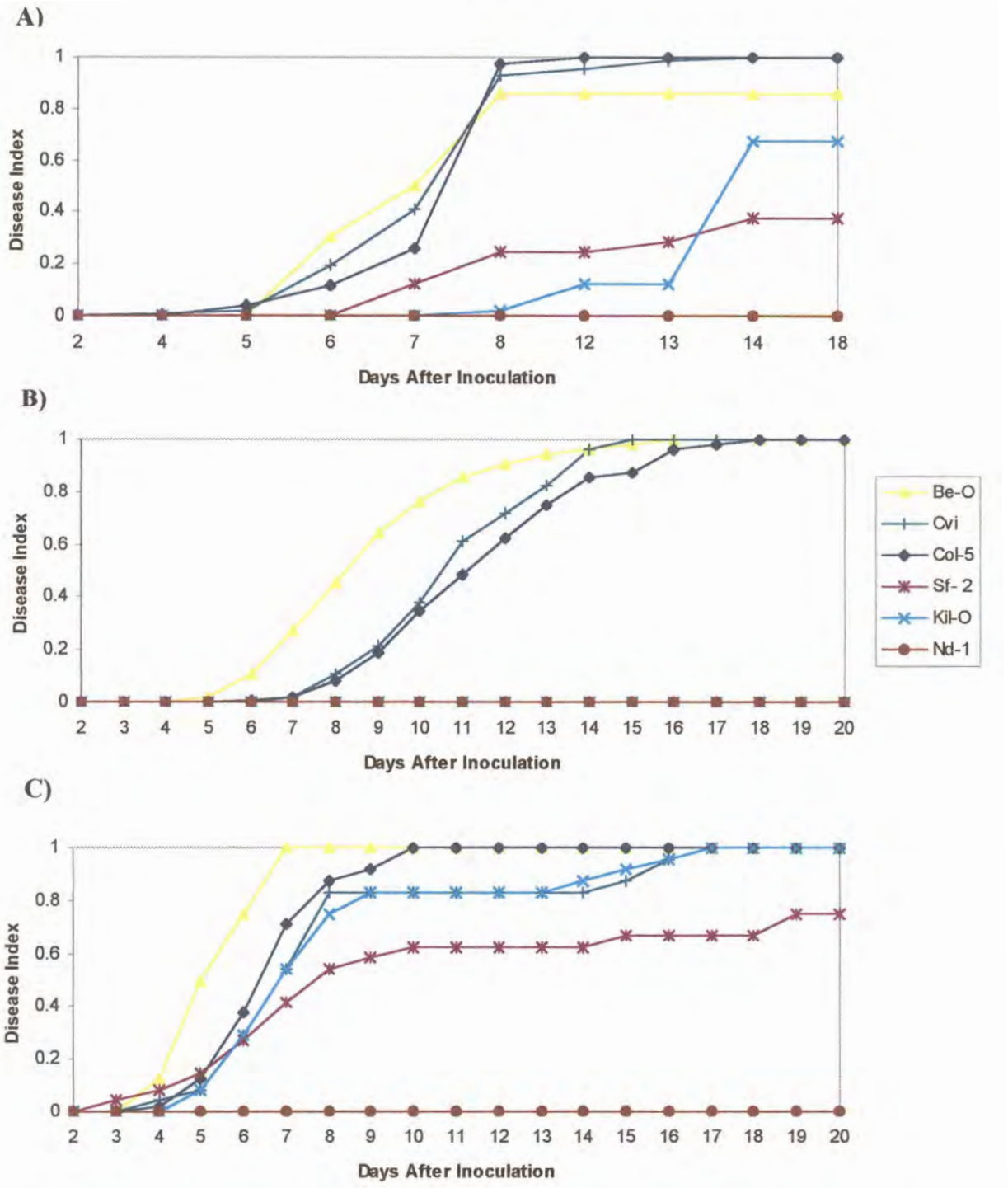

Figure 3.5. Progression of disease symptoms in the different *A. thaliana* ecotypes over 18 days after root inoculation by the *R. solanacearum* control strain, BCC 0300 (GMI 1000) during the three replicate experiments (A, B and C). Data points were generated from at least six plants. During experiment 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.

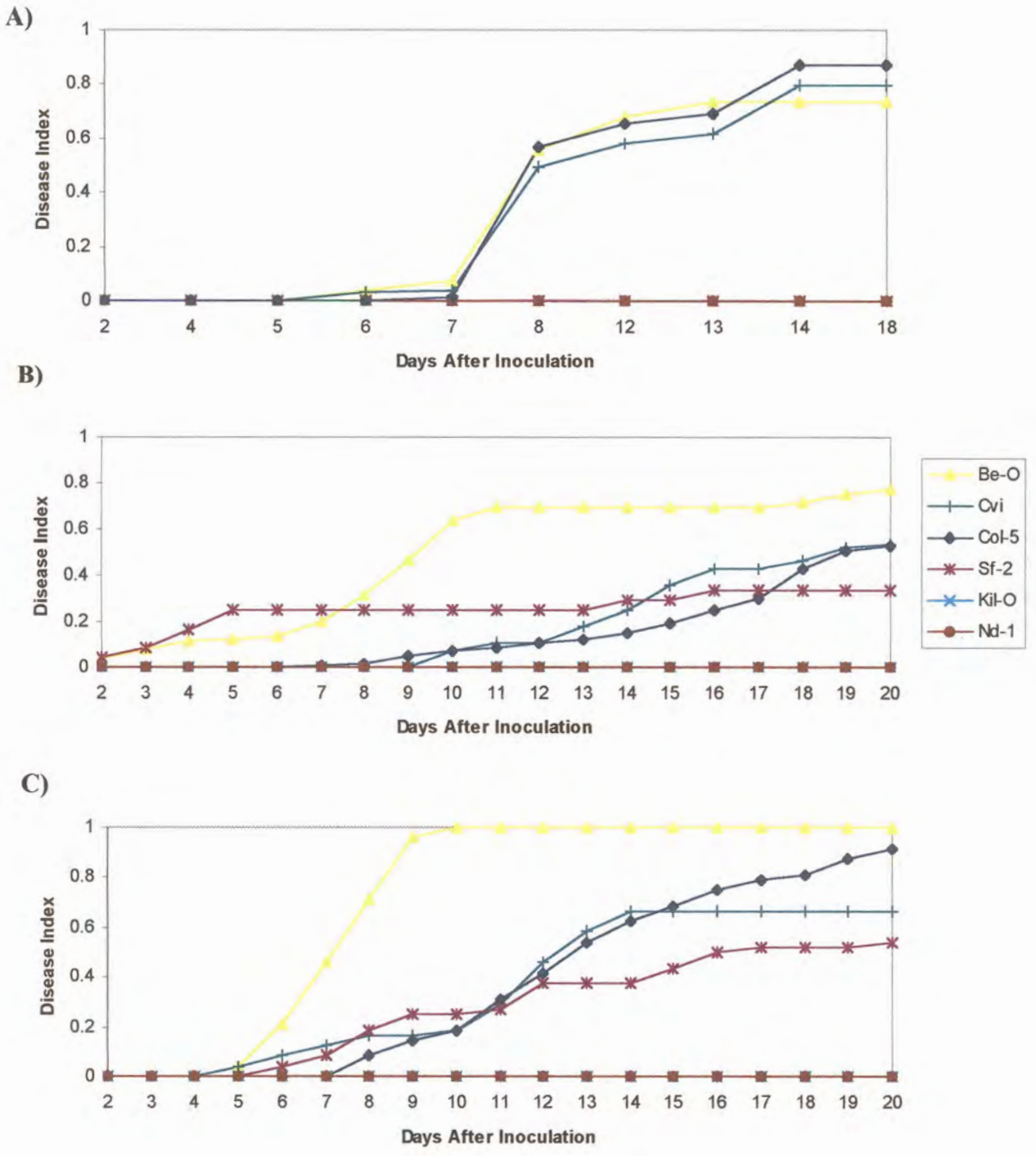

Figure 3.6. Progression of disease symptoms in the different *A. thaliana* ecotypes over 18 days after root inoculation with the *R. solanacearum* isolate BCC 0302 (CK) during the three experiments (A, B and C). Data points were generated from at least six plants.

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.

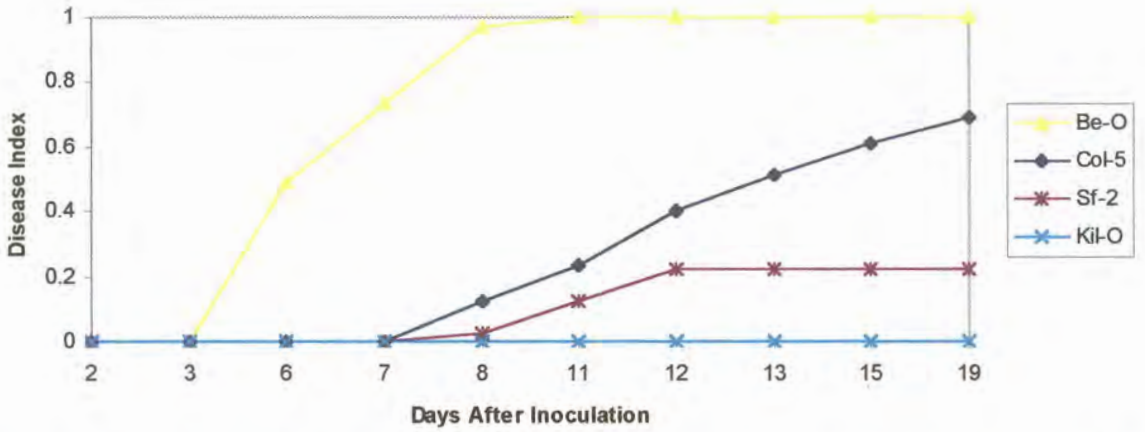

Figure 3.7. A comparison between the most susceptible ecotypes, Be-O and Col-5, with the more resistant ecotypes, Kil-O and Sf-2, over 19 days after inoculated with isolate BCC 0302 (CK). The values of 18 plants were pooled for each data point.

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).

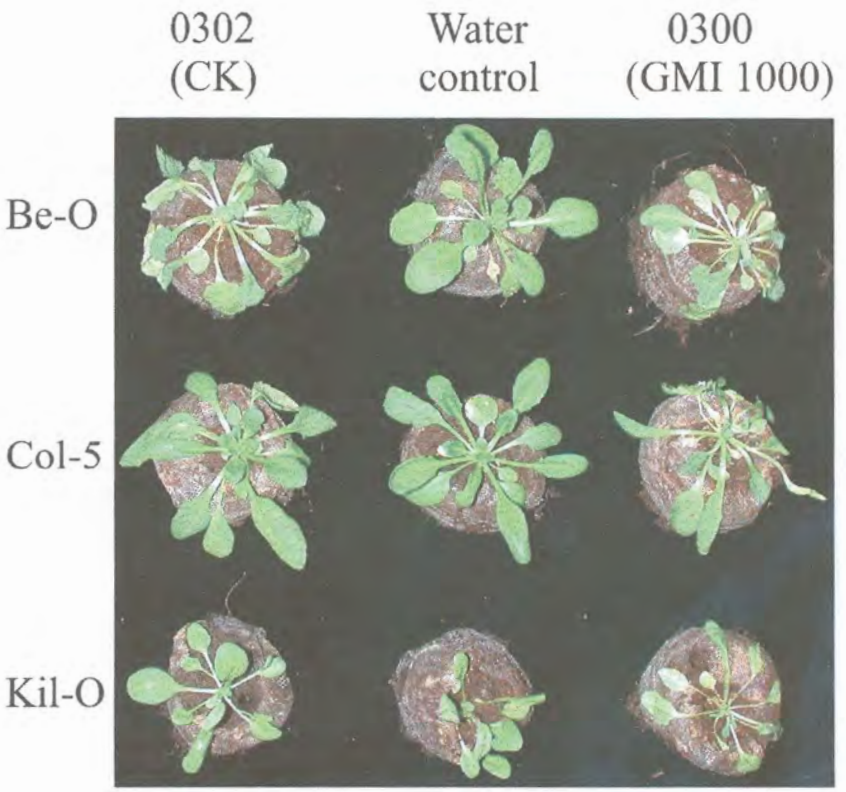

Figure 3.8: Illustration of wilt symptoms of selected *A. thaliana* ecotypes Be-O, Col-5 and Kil-O, one week after inoculation with *R. solanacearum* strains BCC 0302 (CK) and BCC 0300 (GMI 1000) compared to the water control.

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.

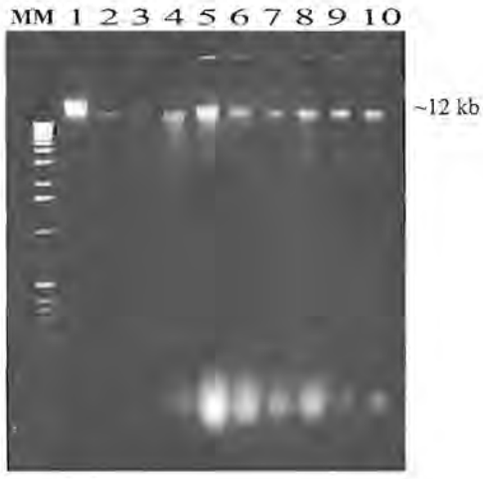

Figure 3.9. DNA extracted from selected *A. thaliana* ecotypes. MM: 1 kb molecular marker; lane 1: 500 ng DNA Standard; lane 2: 50 ng DNA Standard; lanes 3 and 4: ecotype Cvi; lanes 5 and 6: ecotype Kil-O; lanes 7 and 8: ecotype Col-5; lanes 9 and 10: ecotype Be-O.

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.

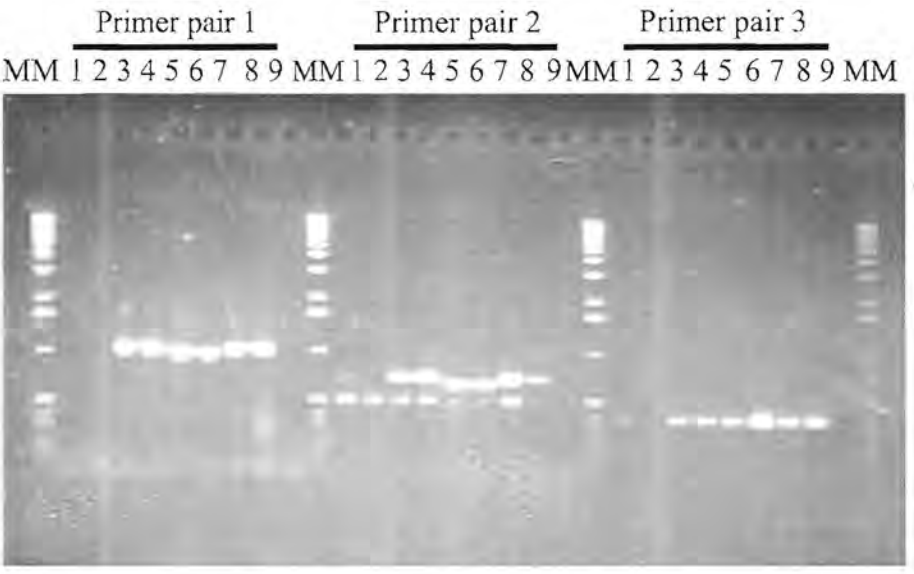

Figure 3.10. ***A. thaliana*** **DNA amplified with RRS1 primers.** Primer pair 1 = RT1 & RT3; primer pair 2 = RT1 & K3.3 and primer pair 3 = K10 & RT3. The PCR products were separated on a 1 % agarose gel, MM = 1 kb. Lanes 1 and 2: ecotype Cvi; lanes 3 and 4: ecotype Kil-O; lanes 5 and 6: ecotype Col-5; 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.

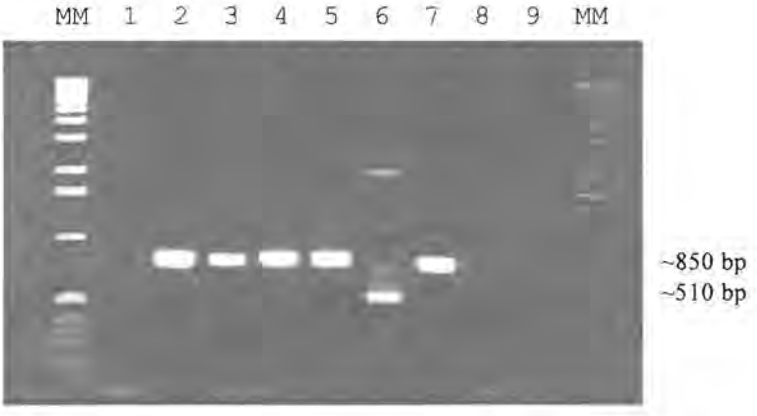

Figure 3.11. PCR amplification of the *PopP2* gene of *R. solanacearum* using primer pair, Pop P2-1 and Pop P2-7. MM: 1KB DNA Molecular marker; lane 1: Negative control, no DNA template; lane 2: BCC 0301 (K), lane 3: BCC 0302 (CK), lane 4: BCC 0303 (CC), lane 5: BCC 0300 (GMI 1000), lane 6:BCC 0327 (27B),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