CHAPTER 2

Identification and characterization of African strains of *Ralstonia solanacearum*

2.1 INTRODUCTION

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

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

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

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

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

2.2 MATERIALS AND METHODS

2.2.1 BACTERIAL STRAINS AND MEDIA

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

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

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

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

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

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

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

2.2.2 PHENOTYPIC METHODS

2.2.2.1 Morphological characteristics

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

2.2.2.2 Biovar differentiation

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

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

2.2.2.3 Physiological tests

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

2.2.3 MOLECULAR METHODS

2.2.3.1 DNA extraction

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

2.2.3.2 PCR of the 16S rRNA gene

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

2.2.3.3 PCR-RFLP

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

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

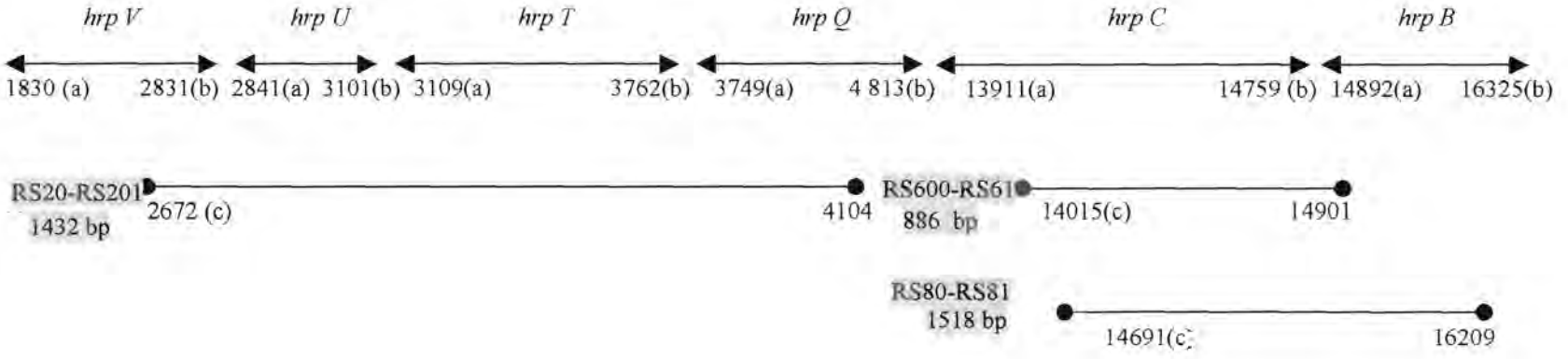

Figure 2.1: Location of the three selective primer pairs (RS20-RS201, RS600-RS61, RS80-RS81), within the *hrp* genes of *R. solanacearum* reference strain GMI 1000 (Poussier *et al.* 1999). Lines with arrowheads indicate the genes of *R. solanacearum* with the various *hrp* clusters given above. Lines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

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

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

2.2.3.4 Amplified Fragment Length Polymorphism (AFLP) analysis

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

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

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

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

2.3 RESULTS

2.3.1. PHENOTYPIC METHODS

2.3.1.1. Morphological characteristics

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

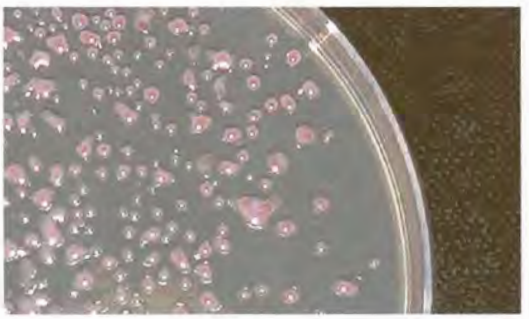

Figure 2.2. Characteristic mucoid colonies of virulent *R. solanacearum* isolates on BGT medium.

2.3.1.2. Biovar differentiation

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

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

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

2.3.2. MOLECULAR METHODS

2.3.2.1. DNA extraction

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

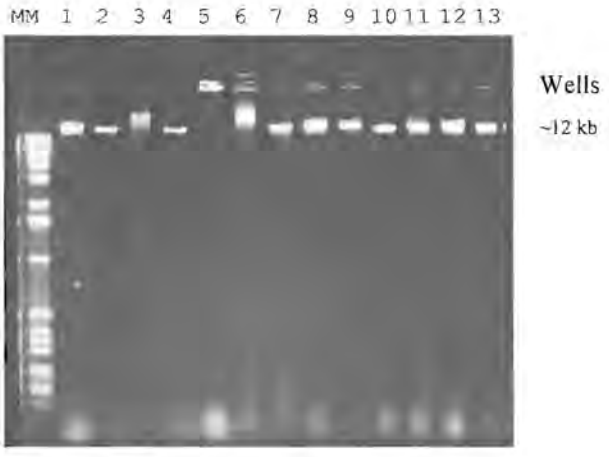

Figure 2.3. Genomic DNA of *R. solanacearum* extracted using the method of Chen and Kuo (1993). MM: 1 kb DNA ladder; lanes 1, 2, 4, 7-13: High molecular weight DNA extracted; lanes 3, 5 and 6: poor quality DNA; lane 5: protein residue in well.

2.3.2.2. PCR of the 16S rRNA gene

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

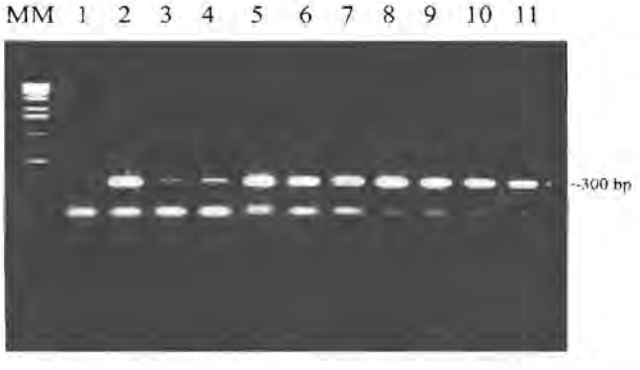

Figure 2.4. PCR amplification of the 16S rRNA region of the bacterial isolates. MM: 1 kb DNA marker; lane 1: negative control with primer dimers present. Lane 2: positive control, lane 3 and 4: Ugandan *Eucalyptus* isolates, BCC 0310 and 0311 (GU 68 and GU 69), weak amplification; lane 5: BCC 0300 (GMI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

2.3.2.3. PCR-RFLP

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

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

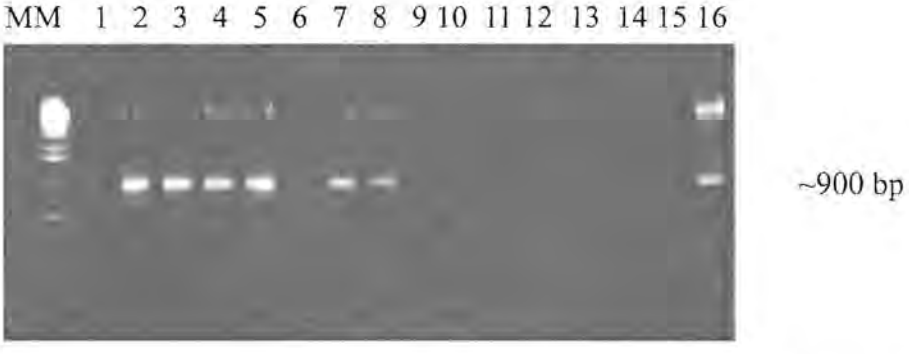

Figure 2.5. Selective amplification of the *hrpC/B* gene fragment of *R. solanacearum* using primers RS600 and RS61. MM: 1 kb molecular marker; lane 1: negative control with sterile distilled water as template; lane 2: positive control, BCC 0300 (GMI 1000). Lane 3-5: BCC 0302 (CK), BCC 0303 (CC) and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

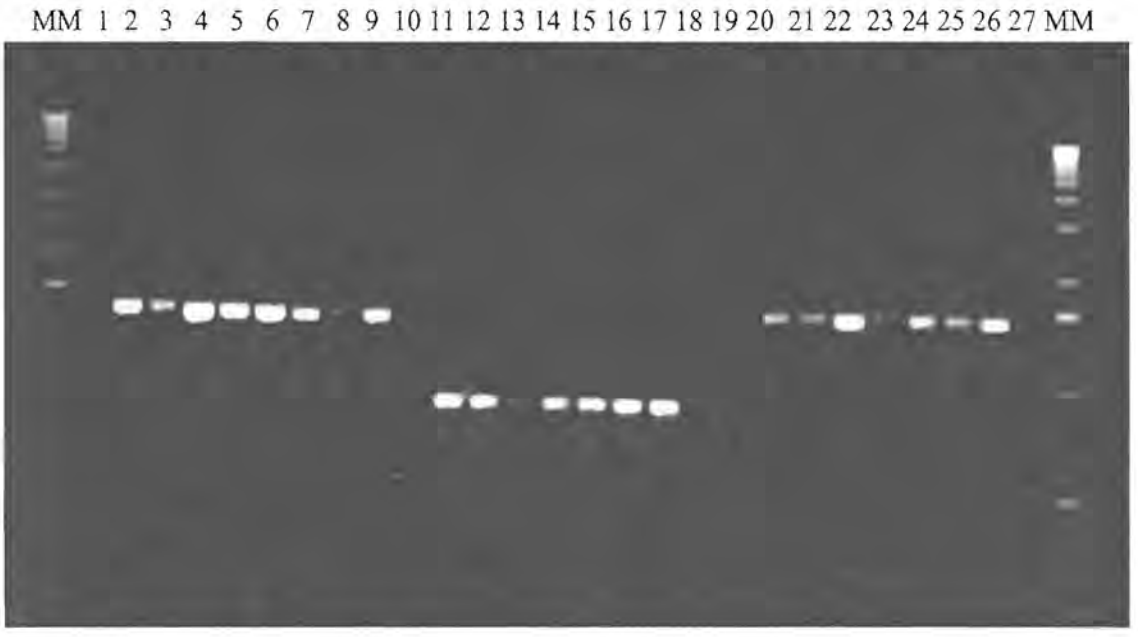

Figure 2.6. Three PCR fragments of the *hrp* cluster amplified from *R. solanacearum* The three primer pairs were RS20-RS201 (fragment 1, ~1500 base pairs (bp), lanes 1-9), RS600-RS61(fragment 2, ~900 bp, lanes 10-18) and RS80-RS81(fragment 3, ~1400 bp, lanes 19-27) (Poussier *et al.*, 1999). MM: 1 kb DNA ladder; lanes 1, 10 and 19: negative control containing sdH_2O; lanes 2, 11 and 20: positive control with reference strain GMI 1000; lanes 3,12 and 21: Ugandan isolate, BCC 0327 (27B); lanes 4,13 and 22: SA Eucalyptus strain, BCC 0301 (K); lanes 5, 14 and 23: Congolese strain, BCC 0302 (CK); lanes 6, 15 and 24: Congolese strain, BCC 0303, (CC); lanes 7, 16 and 25: potato isolate, BCC 0306 (62); lanes 8, 17 and 26: potato isolate, BCC 0305 (92); lanes 9, 18 and 27: potato isolate, BCC 0304 (117).

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

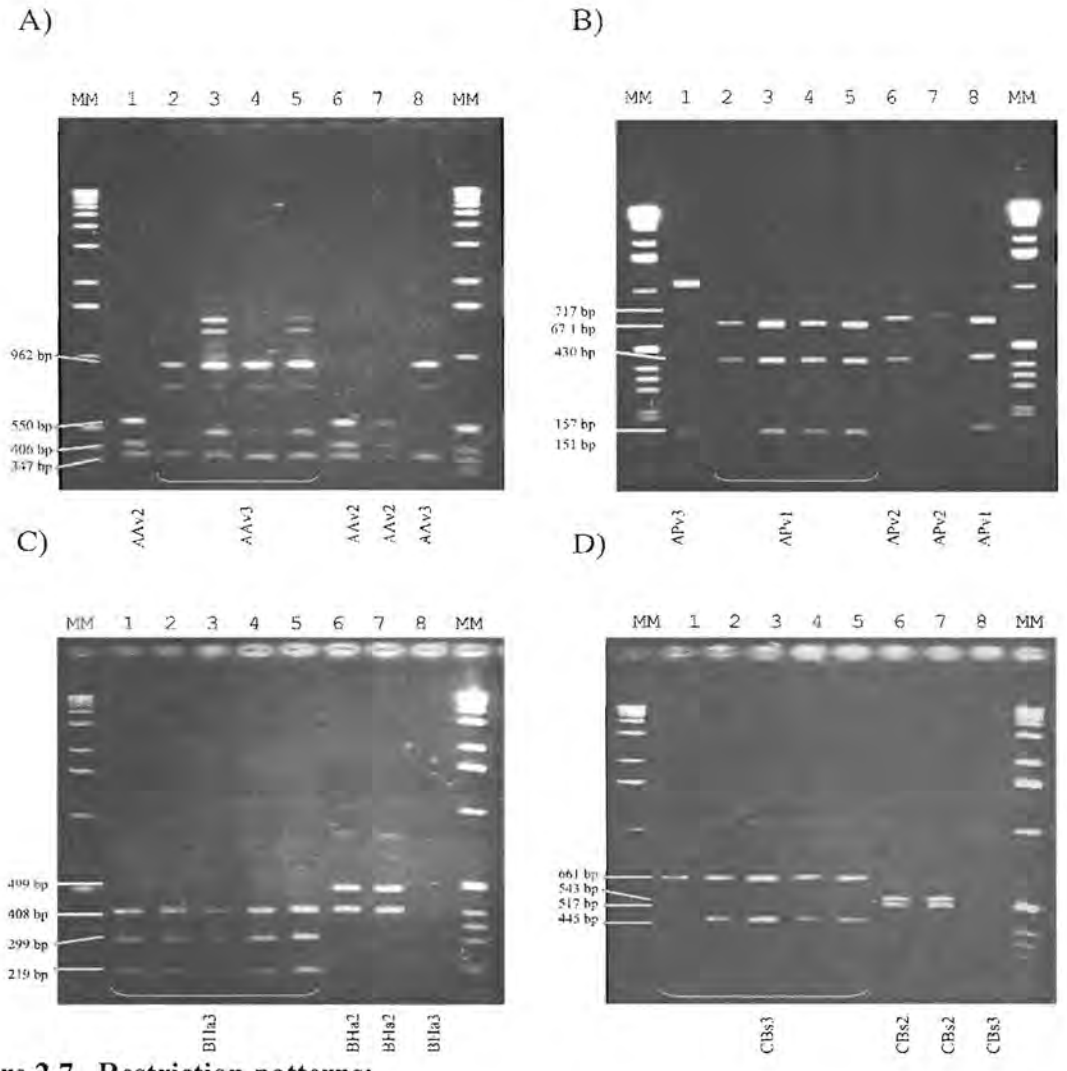

Figure 2.7. Restriction patterns:

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

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

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

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

2.3.2.4. AFLP analysis

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

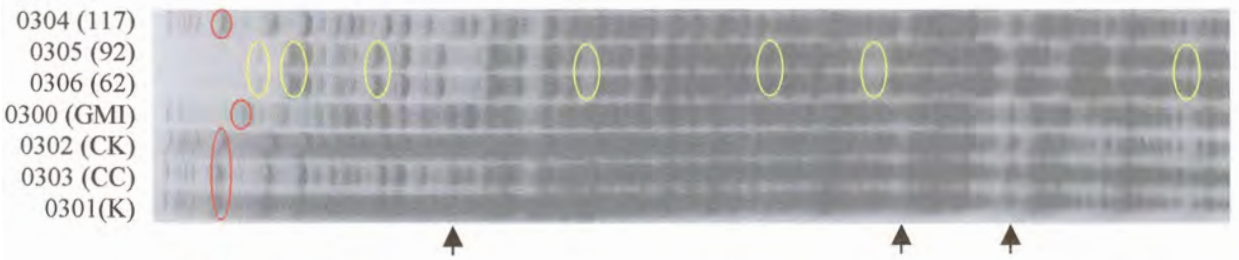

Figure 2.8. AFLP data viewed as an image on high performance autoradiogram film. A clear distinction can be made between biovar 3 (BCC 0300 (GMI 1000), BCC 0302 (CK), BCC 0303 (CC) and BCC 0304 (117)) and biovar 2 (BCC 0306 (62) and BCC 0305 (92)) isolates. Yellow ovals show the similarities between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

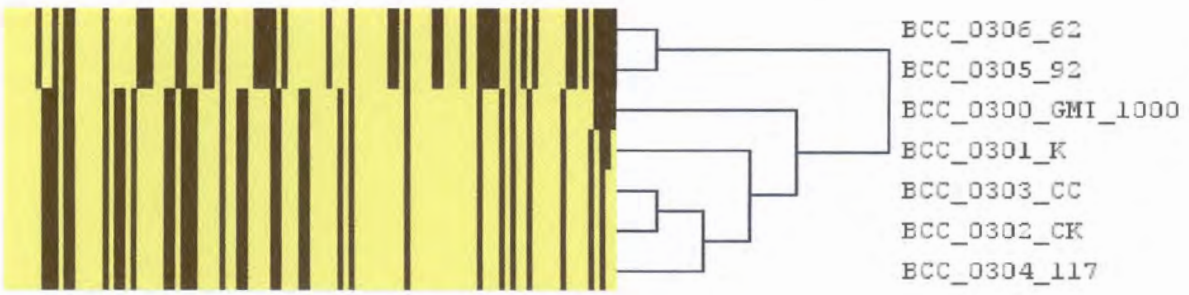

Figure 2.9. AFLP data viewed as a dendogram.

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

2.4. DISCUSSION

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

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

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

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

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

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

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

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

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