CHAPTER 4

CHARACTERISATION OF SOUTH AFRICAN *RALSTONIA SOLANACEARUM* ISOLATES USING MOLECULAR TECHNIQUES

ABSTRACT

Molecular techniques such as polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis have previously been used to illustrate the heterogenicity of different *R. solanacearum* isolates. It is important to determine differences between isolates in order to optimise breeding programs for resistant lines as well as for other control strategies such as crop rotation. The enterobacterial repetitive intergenic consensus (ERIC) region was used to distinguish between eight biovar 2 and 3 isolates. The ribosomal intergenic spacer analysis (RISA) and restriction fragment length polymorphism (RFLP) with *Sau*3A were used to characterise and evaluate possible variation between 44 *R. solanacearum* isolates collected throughout the potato growing regions of South Africa. For ERIC-PCR, the primers ERIC 1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC-3') were used. For RISA-PCR, primers corresponding to the 16S (5'-TTG TAC ACA CCG CCC GTC A-3') and 23S (5'-GGT ACC TTA GAT GTT TCA GTT C-3') spacer regions were selected. The amplified DNA from the RISA-PCR technique was digested with the enzyme *Sau*3A. These primers and enzyme were selected on a basis of availability. The Dice-coefficient was used to construct a distance matrix and isolates were clustered using the unweighted pair group method of arithmetic averages (UPGMA). The ERIC-PCR could successfully distinguish between biovar 2 and 3 isolates. *Sau*3A digestion of the RISA-PCR products also distinguished between these two groups of isolates. No correlation could be drawn between the different isolates and regions from which it was isolated using the above primer sets and enzyme.

1. Introduction

Ralstonia solanacearum is a very heterogeneous species (Fegan *et al.*, 1998). Difference in host range, geographical distribution, pathogenicity, epidemiological relationships and physiological properties are some of the factors contributing to this heterogenicity (Hayward, 1991). Traditionally, strains have been classified into five races on the basis of difference in host range and six biovars based on biochemical properties (Hayward, 1964; Hayward, 1991; Walker and Stead, 1993; Hayward, 2000; Poussier *et al.*, 2000). These classifying systems focused on physiological, biochemical and pathological properties of the pathogen and did not incorporate molecular differences between isolates. Since proteins produced by different strains of bacteria can provide a useful source of information for identification and characterisation of different strains, scientists began to look at molecular properties to sub-divide isolates (Kersters, 1990). With the development of new molecular techniques such as polymerase chain reaction (PCR) (Seal *et al.*, 1993; Hartung *et al.*, 1998) and restriction fragment length polymorphism (RFLP) (Cook *et al.*, 1989; Poussier *et al.*, 1999), it became clear that there existed differences between isolates that has previously been considered identical.

Cook *et al.* (1989) used RFLP groupings to separate strains into two divisions that were genetically distinct from each other and which share only 13.5% similarities. Division 1 consisted of the metabolically more versatile biovars 3, 4 and 5, while division 2 consisted of the other metabolically less versatile biovars 1, 2 and N2. This separation suggested an evolutionary divergence, with division 1 strains mainly originating from the Old World (Asia) and those in division 2 primarily from the New World (America). Another approach that has been followed, involved the partial sequencing of the *hrpB* and endoglucanase genes (Poussier *et al.*, 1999; Poussier *et al.*, 2000). Accordingly isolates could be subdivided into three clusters. Cluster 1 contained all isolates of biovars 3, 4 and 5 and was equivalent to division 1. Cluster 2 contained isolates of biovars 1, 2 and N2 from Africa, the Antilles, USA and central and South America, and was equivalent to division 2. Cluster 3 contained isolates of biovars1 and N2 from Africa and the islands of Reunion and Madagascar. Cluster 3 has previously been found to fall close within either division 1 or 2 depending on the method employed to measure diversity. These findings again highlighted the diversity within *R. solanacearum* and were just one more way to sub-classify different isolates (Poussier *et al.*, 2000). Seal *et al.* (1999) also determined three subgroups within *R. solanacearum*, using primers that were designed to sequence within the 16 rDNA region. Hartung *et al.* (1998) used highly specific primers in their PCR-based assay and

were able to identify 28 different strains. The primer-pair PS96H and PS96I was used and corresponded to a 148-base pair (bp)-long genomic sequence of *R. solanacearum*. Other scientists were also able to determine the genetic diversity of *R. solanacearum* using PCR techniques (Smith *et al.*, 1995; Boudazin *et al.*, 1999).

Once detected and isolated, the pathogen should be thoroughly characterised. This characterisation is not only of academic importance, but is also important to determine the type of pathogen strain present in a certain production area. Gillings and Fahy (1993) suggested that the diversity between isolates poses a number of problems, particularly in the production of resistant cultivars, where strain diversity may explain the failure of many attempts to breed universally resistant lines. The difficulty of developing effective control strategies for bacterial wilt is compounded by a lack of basic knowledge about the ecology and evolution of the pathogen (Cook and Sequeira, 1994). Sufficient knowledge regarding strain differences plays an important role in the development of new control strategies. Different strains have different abilities to survive in soil (Elphinstone and Aley, 1993). It is therefore important to know how strains differ from each other, especially where bare fallow practices are considered as control measure. This information may also be required where crop rotation is used as control measure against bacterial wilt. Since different strains of the pathogen can infect different host plants (Walker and Stead, 1993; Hayward, 2000), knowing what strain is present can play an important role in considering the best plants to use for crop rotation.

The objective of this study was therefore to characterise and evaluate possible variation between different *R. solanacearum* isolates, using different molecular techniques. Isolates were collected from various potato production regions throughout South Africa and have previously been characterised primarily according to their physiological, biochemical and pathological properties (Pers. Comm. A.E. Swanepoel, Vegetable and Ornamental Plant Research Institute, Roodeplaat; Pers. Comm. A.N. Hall, Department of Microbiology and Plant Pathology, University of Pretoria). The enterobacterial repetitive intergenic consensus (ERIC) and ribosomal intergenic spacer analysis (RISA) PCRs as well as RFLP with the enzyme *Sau*3A were used to determine the homogeneity of the collection of *R. solanacearum* isolates.

2. Materials and Methods

2.1. Bacterial Strains

Stock cultures (Appendix 1) were maintained as described in Chapter 3. To prepare cultures for DNA extraction, isolates were streaked and incubated according to methods described in Chapter 3.

2.2. DNA Extraction

Template DNA was extracted from *Ralstonia* isolates (Appendix 1) by a modification of the rapid lyses method described by Sambrook *et al.* (1989). After incubation, a single colony was transferred to standard 1 (STD1) nutrient broth (Biolab) and incubated for 18 hours at 27°C. One millilitre broth was centrifuged (14 000 x g, 10 min), the supernatant was removed and the pellet was washed twice in 1ml sterile physiological buffered saline solution by centrifugation as described above. The pellet was re-suspended in 100µl sterile milli Q water and heated for 10 min at 95°C. The cell lysate was immediately placed on ice until required.

2.3. PCR Amplification and Restriction Fragment Length Polymorphism Analysis

2.3.1. Enterobacterial Repetitive Intergenic Consensus

PCR assays were carried out using oligonucleotide primers ERIC 1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') (MWG-Biotec) (Louws *et al.*, 1994). Amplification was performed on four biovar 2 isolates (SAP111, SAP92, SAP7 and Z23) and four biovar 3 isolates (SAP117, SAP93, SAP6 and NB346) in a total volume of 50µl containing 20mM Tris-HCL (pH 8.4), 50mM $MgCl_2$ 0.1% Triton X-100 (Promega), 200µM each dATP, dCTP, dGTP and dTTP and 0.5U *Taq* polymerase (TaKaRa). Template DNA was added and the tubes placed in a Perkin Elmer (Gene Amp PCR System 2400). PCR conditions were as follows: an initial denaturation step at 95°C for 7 min, followed

by 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 3 min and a final extension step of 72°C for 16 min.

2.3.2. Ribosomal Intergenic Spacer Analysis

The RISA-PCR was performed on 44 *R. solanacearum* isolates (Appendix 1). Primers corresponding to the 16S (5'-TTG TAC ACA CCG CCC GTC A-3') and 23S (5'-GGT ACC TTA GAT GTT TCA GTT C-3') (MWG-Biotec) spacer regions were used for RISA (Fisher and Triplett, 1999). Amplification was performed in a total volume of 50µl containing the same reactants described in 2.3.1. PCR conditions were as follows: an initial denaturation step at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min and a final extension step of 72°C for 10 min.

2.3.3. Restriction Fragment Length Polymorphism Analysis

The amplified DNA from the RISA-PCR techniques was digested with the enzyme *Sau*3A (Promega). Digestions were prepared using 0.4µl enzyme, 2.5µl of the accompanying enzyme buffer (Buffer B), 2.1µl sterile milli Q water and 20µl PCR reaction sample. The mixtures were incubated at 37°C for 2 hours before samples were electrophoresed.

2.4. Gel Electrophoresis and Data Analysis

PCR products were electrophoresed onto 1.4 % agarose gels in 1 x Tris-Borate EDTA buffer (TBE) (Maniatis *et al.*, 1982) at 100 V for two hours. Gels were stained with 10µl ethidium bromide (Merck) and viewed under UV light. Sizes of products were confirmed by comparison with a molecular mass marker (100 bp ladder, Promega).

The RFLP gels were photographed and the photographs were scanned and analysed with the Gel compar 4.0. computer programme (Applied Maths Kortrijk, Belgium). The Dice-coefficient was used to construct a distance matrix. A tolerance of 0.8% in band position was allowed. The unweighted pair group method of arithmetic averages (UPGMA) was used to cluster the isolates.

3. Results

3.1. PCR Amplification

3.1.1. Enterobacterial Repetitive Intergenic Consensus

DNA was successfully extracted from the four isolates representing biovar 3 (SAP117, SAP93, SAP6 and NB346). These isolates produced a single DNA fragment of approximately 650 bp. No DNA fragments were obtained from the four isolates representing biovar 2 (SAP111, SAP92, SAP7 and Z23) (Fig.1).

3.1.2. Ribosomal Intergenic Spacer- and Restriction Fragment Length Polymorphism Analysis

DNA was successfully extracted from the 44 isolates studied. A single DNA fragment of approximately 900 bp was obtained from each isolate (results not shown). *Sau*3A digestion of the RISA-PCR products resulted in DNA fragments ranging from approximately 100 to 450 bp in size (Fig. 2.1-2.3). A level of genetic variation was observed in the *R. solanacearum* population tested in this study. The dendogram constructed from this data showed two distinct groups (Fig. 3). The first group comprised all four biovar 3 isolates. All biovar 2 isolated grouped in the second group. No correlation could be draw between the different isolates and the regions from where it was collected.

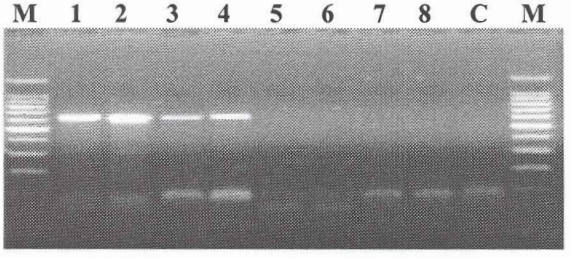

Figure 1. Agarose gel electrophoresis of ERIC-PCR products from DNA of *Ralstonia solanacearum* isolates. M = 100 bp DNA molecular weight marker. Lanes 1-4 represent biovar 3 isolates (SAP117, SAP93, SAP6 and NB346). Lanes 5-8 represent biovar 2 isolates (SAP111, SAP92, SAP7 and Z23). C = water control.

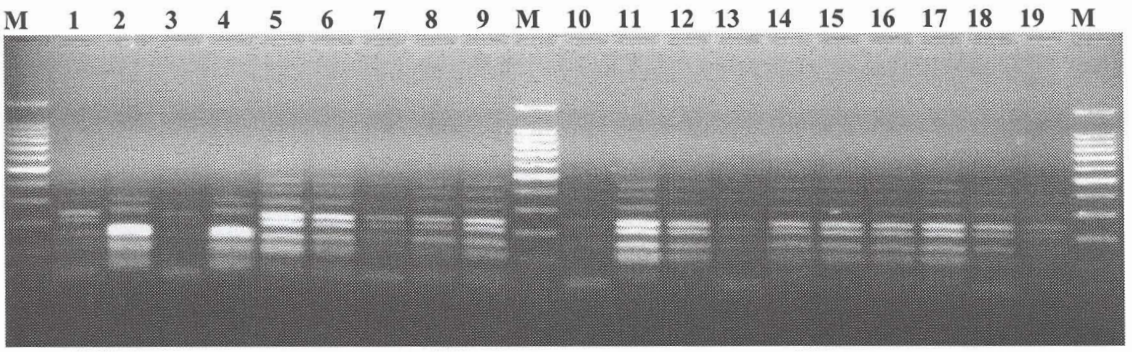

Figure 2.1. Restriction enzyme (*Sau*3A) digestion patterns of *Ralstonia solanacearum* RISA-PCR products. M = 100 bp DNA molecular weight marker. Lane 1: SAP1, Lane 2: SAP6, Lane 3: SAP111, Lane 4: SAP93, Lane 5: SAP23, Lane 6: SAP3, Lane 7: SAP46, Lane 8: SAP62, Lane 9: SAP19, Lane 10: SAP2, Lane 11: SAP22, Lane 12: SAP60, Lane 13: SAP7, Lane 14: SAP45, Lane 15: SAP79, Lane 16: SAP16, Lane 17: SAP64, Lane 18: SAP92, Lane 19: SAP66.

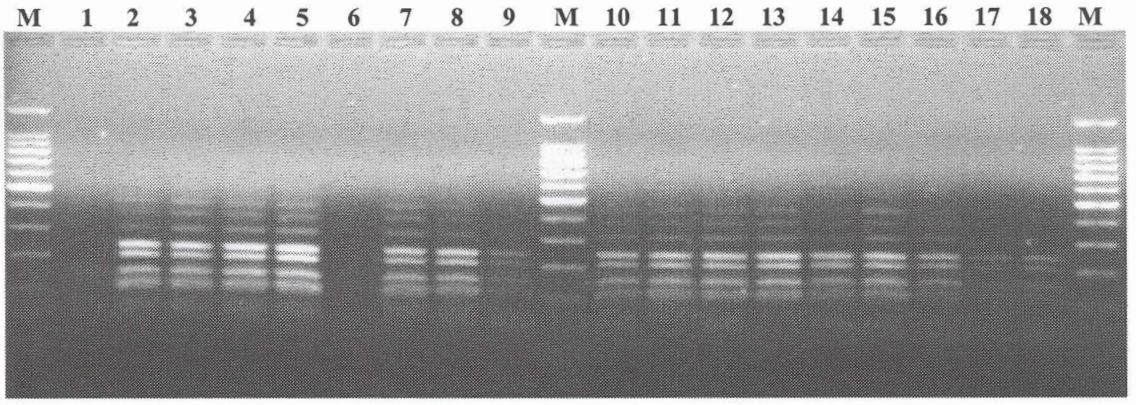

Figure 2.2. Restriction enzyme (*Sau*3*A*) digestion patterns of *Ralstonia solanacearum* RISA-PCR products. M = 100 bp DNA molecular weight marker. Lane 1: SAP117, Lane 2: SAP27, Lane 3: SAP26, Lane 4: SAP76, Lane 5: SAP17, Lane 6: SAP25 Lane 7: SA1, Lane 8: SA3, Lane 9: SA20, Lane 10: SA2, Lane 11: SA6, Lane 12: SA7, Lane 13: SA8, Lane 14: SB8, Lane 15: SB292(b), Lane 16: SB292, Lane 17: SB20, Lane 18: Z23.

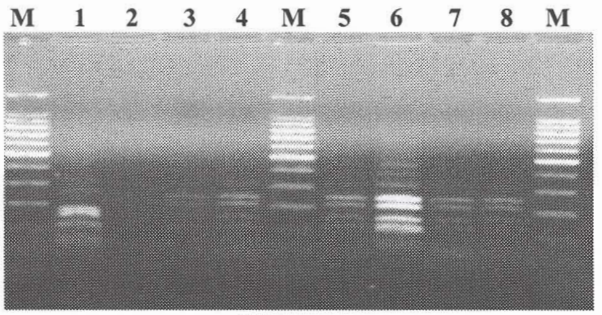

Figure 2.3. Restriction enzyme (*Sau*3A) digestion patterns of *Ralstonia solanacearum* RISA-PCR products. M = 100 bp DNA molecular weight marker. Lane 1: NB346, Lane 2: NB980221 (not enough PCR product obtained, excluded from further comparison), Lane 3: NK15, Lane 4: D109, Lane 5: PB1-2, Lane 6: TB40, Lane 7: TB32, Lane 8: TB16.

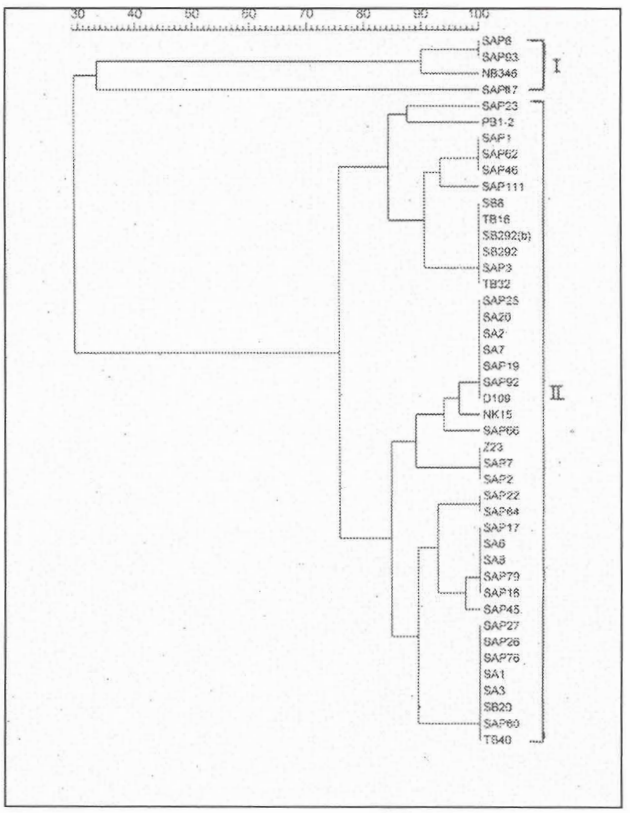

Figure 3. Dendogram showing the grouping of representative RFLP band profiles of *Ralstonia solanacearum* isolates using the unweighted pair group of arithmetic averages method.

4. Discussion

The ERIC- and RISA-PCR techniques were successfully used in this study to distinguish between biovar 2 and biovar 3 isolates. Currently biovars are identified based on differences in carbon source utilisation and nitrate metabolism. This system has been used since 1964 and can distinguish between five biovars (Hayward, 1964; Hayward, 1991; Hayward, 1994; Hayward, 2000). Although various carbon sources can be used, biovars are primarily identified based on their ability to utilise three hexose alcohols (mannitol, sorbitol and dulcitol) and the production of acids from three disaccharides (lactose, maltose and cellobiose) (Hayward, 1994). The preparation of these substrates is laborious and time consuming. Since the ERIC- and RISA-PCR techniques could distinguish between biovar 2 and 3 within a few hours, it can produce results much faster. It should however also be tested against biovars currently not occurring in South Africa (biovars 1, 4 and 5) to conclude whether or not it can replace traditional identification methods.

In this study the ERIC- and RISA-PCR techniques could not be used to draw a correlation between the different isolates and the regions from which it was sampled. The primers and enzyme used were selected on a basis of availability but various other primer sets and enzymes have previously been used to group *R. solanacearum* isolates. Fegan *et al.* (1998) sequenced the 16S-23S rRNA gene intergenic spacer region, polygalacturonase gene and the endoglucanase gene from various *R. solanacearum* isolates to confirm the two divisions previously described. The primers used for sequencing the 16S-23S rRNA gene intergenic spacer region were L1 (5' AGT CGT AAC AAG GTA GCC G) and PS-23Sr (TAC TAC GTC CTT CAT CG). For the polygalacturonase gene the primers PehF (CAG CAG AAC CCG CGC CTG ATC CAG) and PehR (ATC GGA CTT GAT GCG CAG GCC GTT) were used. The primers EndoF (ATG CAT GCC GCT GGT CGC CGC) and EndoR (GCG TTG CCC GGC ACG AAC ACC) were used for the amplification of the endoglucanase gene. Seal *et al.* (1999) used a whole set of different primer pairs (OLI1 + Y2, OLI1 + BV345, DIV1F + DIV1R, DIV2F + DIV2R, DIV2F + ITSR) to distinguish between the same two divisions. Many restriction enzymes exist, with each one recognising a particular and different nucleotide sequence (Farber, 1996). Alvarez *et al.* (1993) used *Bam*HI or *Eco*RI digestion to show the relationships between strains isolated from different hosts. Gillings *et al.* (1993) and Gillings and Fahy (1993) used *Hae*III digestion of an amplified polygalacturonase gene fragment of *R. solanacearum* to divide the isolates into different RFLP

groups. DNA was also digested with a mixture of *Eco*RI and *Hind*III to defined RFLP groups (Tsuchiya and Horita, 1998).

It is therefore clear that one has to be very careful in choosing the correct molecular method and enzyme that could answer specific questions. Although isolates from different areas did not group together in this study, looking at another region within the genome of *R. solanacearum* could have yielded different results. Genotypes recognised by molecular techniques must correspond to a recognised phenotype/ecotype so that these groupings are universally useful for plant pathologists, plant breeders and bacterial taxonomists alike (Fegan, 1998). Although molecular techniques provide a whole new field for the classification of organisms, it should not be considered as the only criteria used for classification. Traditional phenotypical and pathological observations should and will always play a fundamental role in classification.

5. Literature Cited

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