Detection, characterisation and suppression of *Ralstonia solanacearum*

by

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I would like to dedicate this study

to my parents.

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been possible.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The potato (*Solanum tuberosum* L.) is indigenous to the Peruvian and Bolivian Andes mountains in South America, where it was discovered by Spanish explorers in 1532 (Brown, 1993; Steyn, 1999). The potato was shipped to Europe and introduced into Spain in 1573. From Europe it was introduced to North America and the rest of the world, including South Africa (Aartappelreeks, 1974; Brown, 1993; Zuckerman, 1998). Potatoes are currently grown as a major food source in most countries with a temperate climate (Rich, 1983). Globally it is the fourth most important staple food after wheat, rice and maize (Hawkes, 1992).

South Africa is the largest potato producer in Africa, annually producing approximately 1.6 million tons (Potatoes South Africa, 1998/99). The country is divided into 14 production regions, stretching from coastal areas to 2000 m above sea level (Steyn, 1999). Because of the wide climatic spectrum of these regions, potatoes can be produced throughout the year. Tubers are mainly sold on local fresh produce markets and only 6.8% is exported to neighbouring countries such Namibia, Botswana, Mozambique, Angola and Swaziland (Potatoes South Africa, 1998/99). Some of the best known and widely cultivated potatoes in South Africa include cultivars such as Up-to-Date, BP1, Vanderplank and Buffelspoort (Potatoes South Africa, 1998/99). According to Nortje (1999), seed potatoes are produced in all provinces of South Africa with the Western Cape currently producing 35% of all certified tubers. More than 70% of South Africa's potatoes are produced from certified tubers.

One of the most important diseases of potatoes is bacterial wilt. This disease is caused by the bacterium, *Ralstonia solanacearum* (Smith) (Kelman, 1953; Yabuuchi *et al.*, 1995). It was first isolated in 1896 and identified and described by Erwin F. Smith. In 1914, it was reported for the first time on potatoes in South Africa, but it was not until 1978 that research on this disease gained momentum after its presence was established in a number of seed potato crops (Swanepoel and Young, 1988). The pathogen causes typical wilt symptoms in above ground plant parts (Harrison, 1961). The first symptoms usually appear during the warmer spells of the day with a slight drooping in the tip of one or two of the lower leaves. Within a few days infected plants will be completely wilted and eventually die (Kelman, 1953). Tubers may or may not show

external symptoms (Shekhawat *et al.*, 1992). When a diseased tuber is cut in half, distinct brown discoloration and localised decay can be seen in the vascular ring. If slight pressure is applied to the cut tuber, typical greyish-white bacterial slime will ooze from the vascular ring (Shekhawat *et al.*, 1992).

Currently bacterial wilt is not a threat for the South African potato industry and its presence is limited to isolated cases. This is mainly due to the certification scheme implemented on 1 June 1995 (Nortje, 1997). The scheme requires representative samples of all registered seed potatoes to be tested for various diseases. A zero tolerance for *R. solanacearum* is applied to ensure that no infected tubers are sold as seed potatoes.

Ralstonia solanacearum is also the causal agent of bacterial wilt of a number of other plant species (Buddenhagen and Kelman, 1964). Some of the most important agricultural crops affected by this pathogen include: tomato, potato, pepper, tobacco, eggplant, groundnuts and bananas. A number of ornamental plants, woody perennials as well as a very large group of weed species can also be infected (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 2000). The pathogen has a worldwide distribution and has been widely reported in all tropical, subtropical and warm temperate regions around the world (Kelman, 1953; Hayward, 2000).

Since the bacterium can infect various crops without inducing symptoms, tests are also required for detecting latent infection in plant material and in soil samples (Seal and Elphinstone, 1994). Many countries have implemented strict monitoring systems to avoid the introduction of infected material and various methods have been developed for detecting the pathogen *in situ* (Elphinstone *et al.*, 1996). The majority of these methods was primarily developed for detection of *R. solanacearum* in plant material and is subject to various difficulties and shortcomings when used for detection in soil (Jenkins *et al.*, 1967; Seal and Elphinstone, 1994). Most of the problems encountered with detection can be attributed to the heterogeneous nature of soil and the difficulty of adequate soil sampling. Selective media cannot effectively detect pathogen concentrations much lower than 10^3 cfu ml^{-1} of soil sample (Karganilla and Buddenhagen, 1972; Nesmith and Jenkins, 1979; Chen and Echandi, 1981). Using indicator plants to confirm the presence of *R. solanacearum* in suspect fields is effective but time consuming and laborious (Karganilla and Buddenhagen, 1972; Graham and Lloyd, 1978). Serological tests such as enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence antibody staining (IFAS) as well as DNA-based methods such as the polymerase chain reaction (PCR) is highly specific and

sensitive. They are, however rather expensive and not suitable for frequent use in developing countries with limited resources (Seal, 1994; Seal and Elphinstone, 1994).

Once the pathogen has been detected and isolated from soil its identity needs to be confirmed. This can play an important role in the development of control strategies since different strains have different abilities to survive (Elphinstone and Aley, 1993). The information may also be needed 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 deciding which plants to use for crop rotation. Traditionally, a binary system has been used for isolate classification where isolates were differentiated into races or biovars (Buddenhagen *et al.*, 1962; Hayward, 1964; Walker and Stead, 1993; Hayward, 1991). Molecular techniques such as PCR provide a fast alternative to distinguish between different organisms and have previously been used to illustrate the heterogenicity of different *R. solanacearum* isolates (Cook *et al.* 1989; Poussier *et al.* 1999).

Ralstonia solanacearum has an extremely wide host range (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 2000) and is able to survive for prolonged periods in the soil (McCarter, 1976; Graham *et al.*, 1979). Control of this pathogen is therefore difficult and several control methods such as crop rotation and soil amendments should be combined in an integrated control program to combat bacterial wilt. Due to the high costs involved as well as the detrimental effect that the excessive use of chemicals can have on human health and the environment, there has been a global interest in biological control as an alternative method for the control of weeds, insects and plant pathogens (Lydon and Duke, 1989; Lampkin, 1990). Akiew *et al.* (1996) and Kirkegraad *et al.* (1998) illustrated the potential of mustard (*Brassica juncea* L.) and other cruciferous species as biofumigation agents to suppress *R. solanacearum* populations in the soil.

The first objective of this study was to develop an effective detection method for *R. solanacearum* in infested soil. The technique should be easy to apply, affordable, fast and sensitive enough to detect low pathogen concentrations. This technique was compared in terms of sensitivity, affordability and applicability with existing methods. The second objective of this study was to characterise various South African *R. solanacearum* isolates using different molecular techniques. Polymerase chain reactions and restriction fragment length polymorphisms (RFLPs) were used to determine the homogeneity of a collection of *R.*

solanacearum isolates. To conclude this study, different herbal plant species were evaluated as possible biofumigation agents to suppress the pathogen in soil.

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CHAPTER 2

LITERATURE REVIEW

1. Introduction

The potato (*Solanum tuberosum* L.) originated from the Peruvian and Bolivian Andes mountains in South America and wild potato plants can still be found in Central America, Mexico and as far North as Colorado (Aartappelreeks, 1974; Steyn, 1999). It has been cultivated for thousands of years prior to the Spanish conquests of the Americas. Archaeological evidence credits the natives of Peru with cultivating the earliest forms of potatoes approximately 4500 years ago. The importance of the potato in the lives of Andean natives is evident in the religious ceremonies created with the tuber. The Inca people worshiped potato gods and celebrated rituals to ensure the success of their crops (Zuckerman, 1998).

In 1532, Spanish explorers arrived in what is modern day Peru, where amongst others they "discovered" the potato (Brown, 1993). Thirty years later it was shipped to Europe and introduced into Spain (Brown, 1993; Zuckerman, 1998). From Spain it was taken to Italy, then England in 1596 and subsequently Germany in 1601. From Europe, the potato spread to North America and the rest of the world including South Africa where it was initially planted during the late 1600's to provide food for sailors on their way to the Far East (Aartappelreeks, 1974).

Once introduced it became known as the food for the poor and was treated with distrust and fear, that lasted centuries. It was not before the mid 1700s that its potential as a staple food was fully realised (Zuckerman, 1998). Unlike the major cereals such as maize and wheat that are fast approaching the practical limits of their production potential, the yield potential of the potato is still largely under exploited (Zandstra, 1997). Changing consumption patterns in Africa and Asia are resulting in a move away from traditional staple foods such as corn and rice to potato.

2. Potato Production in South Africa

Potatoes are produced throughout the greater part of South Africa with 14 production regions stretching from coastal areas to 2000 m above sea level (Steyn, 1999). In some regions like the Sandveld, potatoes are produced throughout the year. Other regions such as the North Eastern Cape is characterised by a limited production season. Because of South Africa's wide climatic spectrum, potatoes can be produced throughout the year, providing a continuous source of fresh tubers (Potatoes South Africa, 1998/99).

Among the 14 production regions of South Africa, the Northern Province had the highest production of 19.2% during the period of 1998/1999 followed by the eastern Free State (16.3%) and western Free State (14.2%). The total hectares planted during this time reflected the percentage production of the various regions (Potatoes South Africa, 1998/99).

South Africa is the largest potato producer in Africa and approximately 1.6 million tons of potatoes are produced annually (Potatoes South Africa, 1998/99). Worldwide it is the 29th largest producer of potatoes (Encarta, 1999). China is currently the largest producer (42 million tons). It is followed by countries such as Russia (40 million tons), Poland (27 million tons) and the United States of America (22 million tons). Production in South Africa has steadily increased since 1992 from 1.25 million tons to 1.6 million tons in 1999. This increase was mainly due to improved irrigation practices, better farm management and research. Since 1991 the amount of hectares planted decreased from 67 thousand hectares to 59 thousand hectares, indicating more efficient farming systems.

The average market price of potatoes has increased from R7/10kg (1993) to almost R12/10kg in 1999 (Potatoes South Africa, 1998/99). For the 1999 season, the gross value of the potato crop was R1 127 970 m (Matlala, 2001).

Fresh produce markets are the most important distribution channels for potatoes, and of the annual crop only 6.8% is exported. Export has slowly increased from 110 000 to 120 000 tons annually as a result of the free trade agreement between South Africa and some of its neighbouring states. Currently, potatoes are mainly exported to neighbouring countries such Namibia, Botswana, Mozambique, Angola and Swaziland. Small quantities are sporadically exported to other countries in East and Central Africa. In 1998/99 almost 250 tons were exported

to England when the phytosanitary prohibition on South African potatoes was temporarily lifted (Potatoes South Africa, 1998/99).

Each of the different production regions is subject to characteristic climatic conditions and specific diseases, and therefore various cultivars have been identified best suited for each area. Other factors that will influence cultivar selection for production regions are: consumer preferences, planting date, soil type and availability of planting material (Visser, 1999). Although cultivars such as Up-to-Date, BP1, Vanderplank and Buffelspoort are traditionally the best known and widely cultivated seed potatoes in South Africa, various other cultivars are also available and institutes such as the Agricultural Research Council, Roodeplaat, Vegetable and Ornamental Plant Institute are constantly evaluating new cultivars suited for South African conditions.

3. Potato Bacterial Wilt

3.1. Symptom Expression

Potato bacterial wilt is caused by *Ralstonia solanacearum* (Kelman, 1953). The pathogen affects both above and belowground plant parts and damage can occur in two ways; premature wilting of top growth, and rotting of tubers either in the soil or in storage (Shekhawat *et al.*, 1992). According to them the time taken for symptom development depends on various factors such as age of the host plants and environmental factors and will vary from one situation to the next.

3.1.1. Aboveground Symptoms

The first symptoms usually appear during the warmer periods of the day. A slight drooping of the tip of one or two of the lower leaves can be seen midday but, towards the evening, the affected leaves may recover. These symptoms can easily be mistaken for a temporary shortage of soil moisture. However, a few days after the first symptoms have been noticed, wilting will extend to the lower leaves which will be unable to recover (Harrison, 1961). According to Conroy (1969) initially some stems in a potato hill will be wilted while others may appear healthy. Eventually all stems will wilt and die (Fig.1).

In initial stages of wilting, the leaves retain their healthy green colour but, in advanced stages, the foliage turns brown and dies (Conroy, 1969). According to Kelman (1953), a slight yellowing of the lower leaves becomes evident when the first leaflets begin to droop. Wilted leaves may fade to a pale green colour and will finally turn brown. Leaves can also roll upwards and inwards from the margins until they are completely inrolled. This inrolling can be accompanied by the slight yellowing of the foliage (Harrison, 1961).

According to Shekhawat *et al.* (1992) vascular browning is not always present. Harrison (1961) also reported that the vascular tissue appeared normal without any signs of discolouration. He concluded that microscopic examination was required at that point to detect the bacteria.

The presence of the pathogen can easily be demonstrated in the field by placing a cut stem section from the diseased plant in a container of water. Within a few minutes fine milky-white strands that are composed of masses of bacteria, will stream from the margin of the tissue (Kelman, 1953; Shekhawat *et al.*, 1992).

Figure 1. Typical symptoms on aboveground plant parts (Stander L. unpublished data).

3.1.2. Symptoms on Tubers

Tubers may or may not show external symptoms. Where the disease is well advanced, the presence of a greyish brown discolouration is visible through the periderm (Kelman, 1953). According to Harrison (1961), external symptoms vary from small brownish-grey depressions on

the tuber near the point of attachment of the stolon, to large lesions that cover the greater part of the surface of the tuber. In the later stages of disease development bacteria pass through the vascular tissue and will emerge at the eyes as sticky, dirty-white, often bubbly masses to which the soil readily adheres.

Very often when a diseased tuber is cut in half, distinct brown discolouration and localised decay is visible in the vascular ring (Fig.2). If slight pressure is applied to the cut tuber, typical greyish-white bacterial slime will ooze from the vascular ring (Shekhawat *et al.*, 1992). At a later stage the parenchymatous tissue can also be affected where cavities filled with millions of bacteria and decomposed tissue are formed. These cavities constantly expand and ultimately merge. At this stage secondary-rotting organisms may occur, turning large parts of the tuber tissue into a foul-smelling mass (Janse, 1996).

According to Harrison (1961) tuber symptoms do not always correlate with aboveground symptoms. Sometimes tubers from apparently healthy plants show typical vascular discolouration while tubers from wilted plants show no symptoms. Shekhawat *et al.* (1992) suggested that vascular browning in the host might depend on the ability of the invading bacterial strain to produce brown pigments.

Figure 2. Severe symptoms on infected tubers (Stander L. unpublished data).

3.2. Occurrence of Potato Bacterial Wilt

Bacterial wilt of potatoes was first reported in the United States in 1890 (Kelman, 1953). Currently it occurs in most potato-producing regions throughout the world and is especially a problem in tropical and semitropical areas (Martin *et al.*, 1981a). It is a very important limiting factor in potato production and is one of the most serious bacterial diseases occurring on potatoes. In South Africa it was first reported by Doidge in 1914, but it was not until 1978 that research on this disease gained momentum after its presence was established in a number of seed potato crops (Swanepoel and Young, 1988). Although this disease occurs in temperate regions form time to time, it is endemic in the low-altitude, subtropical areas of Mpumalanga and the Northern Province, the costal regions of KwaZulu-Natal and the south western parts of the Western Cape. In other regions such as high-altitude areas of Mpumalanga and the eastern Free State this disease does not occur often but may be a problem from time to time (Aartappelreeks, 1975).

Little information about the occurrence and distribution of this disease in South Africa could be found. According to Cilliers (1992), confirmed cases of bacterial wilt of potatoes drastically increased during 1988 to 1991. However, this data was obtained through routine testing and did not represent the true occurrence and distribution of the disease. It did however raise concern about the increased occurrence of the disease and in 1996 an improved certification scheme for seed potatoes was introduced in order to control further spread of potato bacterial wilt (Nortje, 1997).

4. *Ralstonia solanacearum*

4.1. General Description

Ralstonia solanacearum is a Gram negative, aerobic, chemo-organotrophic rod with rounded ends and does not form spores or capsules (Kelman, 1953; Shekhawat *et al.*, 1992). The bacterial cells are motile but conflicting reports exist as to the number of flagella present on a single cell. According to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) more than one flagellum occur, while authors such as Shekhawat *et al.* (1992) described virulent isolates as non-motile and non-flagellate and avirulent isolates as highly motile with one to four polar flagella.

Depending on the strain, the optimum temperature for growth varies between 27 and 37°C with maximum and minimum temperatures of approximately 39°C and 10 to 15°C respectively (Shekhawat *et al.*, 1992). The bacterium also lacks fluorescent, phenazine and carotenoid pigments.

4.2. Taxonomy

The causal agent of bacterial wilt was first isolated in 1896 and identified and described as *Bacillus solanacearum* by Erwin F. Smith (Kelman, 1953). In 1914, the name was changed to *Pseudomonas solanacearum*. For almost 80 years the pathogen was grouped within the genus *Pseudomonas*. By 1973 this genus was divided into five homology groups. Based on rRNA-DNA hybridisation, *P. solanacearum* was placed in homology group II (Hayward, 2000).

In 1992, a new genus *Burkholderia* was proposed based on 16S rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid composition and phenotypic characteristics (Yabuuchi *et al.*, 1992). Seven species from the original homology group II were transferred to this new genus, including *P. solanacearum*. In 1993, the new genus *Burkholderia*, was validated and *P. solanacearum* became known as *Burkholderia solanacearum* (Hayward, 2000).

In 1995, a new genus derived from *Burkholderia* was proposed by Yabuuchi *et al.* (1995). According to them, *B. picketti* and *B. solanacearum* showed similarities that were different from the remaining five *Burkholderia* species. Based on the results of phenotypic characterisation, cellular lipid and fatty acid analysis, phylogenic analysis of 16S rDNA nucleotide sequences and rRNA-DNA hybridisation three species (*B. picketti*, *B. solanacearum* and *Alcaligenes eutrophus*) were transferred to the new genus, *Ralstonia*. This proposal was validated in 1996 and *B. solanacearum* changed to *Ralstonia solanacearum* (Hayward, 2000).

Difference in host range, geographical distribution, pathogenicity, epidemiological relationships and physiological properties made *R. solanacearum* a very heterogeneous species (Hayward, 1991). Infra subspecific classification was therefore needed in order to define and categorise this diversity especially in targeted plant breeding, epidemiological investigations and quarantine (Gillings and Fahy, 1993a).

A binary system has been used since the early 1960's (Hayward, 1991). This system reflected two different approaches to subspecific classification. The one classified strains into different races according to host affinity, while the other used selected biochemical properties as the basis for separation into biovars. These were informal groupings at the infra subspecific level and were not governed by the Code of Nomenclature of Bacteria.

In 1962, Buddenhagen *et al.* proposed the race classification system. Since then five races have been distinguished based primarily on difference in host range and pathogenicity (Table 1) and, to a lesser extent differences in colony form and pigmentation (Buddenhagen *et al.*, 1962; Walker and Stead, 1993; Hayward, 2000).

Using differences in carbon source utilisation and nitrate metabolism, strains were alternatively divided into five biovars (Table 2). This system has been used since 1964 (Hayward, 1964; Hayward, 1991; Hayward, 2000) and according to Hayward (1994b), was specially designed in the context of epidemiology rather than taxonomy.

It was however found that biovar 2 isolates were not as uniform in phenotype as was previously thought. According to Gillings and Fahy (1993a), biovar 2 strains collected in the Amazon basin had different phenotypic properties both in terms of pathogenicity on various *Solanum* species and in metabolic activity compared to those isolated in the Andean highlands. The second metabolically more versatile phenotype had been designated biovar N2 (Hayward *et al.*, 1990; Gillings and Fahy, 1993b).

There was however, little apparent correlation between these two systems (Sequeira, 1993). According to him, the only real comparison could be drawn between race 3 (the potato race) and biovar 2, although all biovar 2 strains did not necessarily belong to race 3. This lack of concordance was due to the fact that the race system was based on ecological categories while the biovar system was based on classical phenotypic characters (Gillings and Fahy, 1993a).

In 1989, 28 restriction fragment length polymorphism (RFLP) groups were identified, using a selection of DNA probes that specified virulence or the hypersensitive response (Gillings and Fahy, 1993a). According to Hayward (2000), the number of RFLP groups has increased to 46 and will continue to grow as more isolates are examined.

According to Sequeira (1993), the RFLP groups previously described by Cook *et al.* in 1989 could be separated into two divisions that were genetically distinct from each other, sharing 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. Within each division, the coefficients of similarity were very high (78% for division 1 and 62% for division 2).

Another approach that has been followed, involves the partial sequencing of the *hrpB* and endoglucanase genes. Using these techniques, scientists could subdivide the isolates 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).

Table 1 Classification of races according to primary hosts affected (Walker and Stead, 1993; Hayward, 2000)

Race	Host(s) primarily affected
1	Solanaceous hosts, diploid bananas and a variety of non-solanaceous hosts
2	Triploid banana and *Heliconia* spp.
3	Primarily potato, to a lesser extent tomato and a few weed hosts such as black nightshade
4	Mainly ginger
5	Mulberry

Table 2 Comparison between different biovars of *Ralstonia solanacearum* (Hayward, 1994b)

Property	Biovar 1	Biovar 2	Biovar 3	Biovar 4	Biovar 5
Utilisation of: mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-
Trehalose	+	-[1]	+[2]	+[2]	+
Oxidation of: lactose	-	+	+	-	+
Maltose	-	+	+	-	+
cellobiose	-	+	+	-	+
Gas from nitrate (de-nitrification)	-[1]	-[1]	+	+	+

[1] Uncommon isolates give a positive reaction.
[2] Uncommon isolates give a negative reaction.

4.3. Worldwide Distribution

Ralstonia solanacearum has been reported on every major continent and islands from the warm temperate to tropical regions of the world. Few other diseases of plants are comparable in terms of its geographical distribution (Kelman *et al.*, 1994). Most records of bacterial wilt were within latitudes 40°N and 40°S with high summer temperatures and high rainfall (Hayward, 2000).

However, it is not clear where the disease originated from and if it was from one continent or if it evolved separately in many locations (Buddenhagen and Kelman, 1964).

It is presumed that the disease originated from South America. However, authors such as Hayward (1991) and Sequeira (1993) postulated that there might have been separate evolutionary origins since there are marked differences in the geographical distribution of different biovars. In general, division 2 (i.e. biovars 1, 2 and N2) has evolved primarily in the Americas and division 1 (i.e. biovars 3, 4 and 5) in Asia (Sequeira, 1993). Biovar 1 is the only biovar that has been reported from Florida to North Carolina. In Asia biovar 3 is predominant, with biovar 1 being absent from most parts. Biovars 2, 3 and 4 occur in Australia, China (together with biovar 5),

Indonesia, Papua New Guinea and Sri Lanka. While biovars 1 to 4 have been found in the Philippines (Hayward, 1991).

In future, genomic fingerprinting could be used to shed more light on the evolution and population structure of *R. solanacearum* (Gillings and Fahy, 1993a). Data obtained through techniques such as RFLP analysis might help to clarify the origins and natural hosts of particular strains. For example, Poussier *et al.* (2000) used partial sequencing of the *hrpB* and endoglucanase genes to divide isolates into 3 clusters. Clusters 1 and 2 corresponded to the previously defined divisions 1 and 2 but cluster 3 included biovar 1 and N2 isolates which originates from Africa. These isolates were clearly different from the other strains suggesting a separate evolution (Poussier *et al.*, 2000).

4.4. Host Range and Specificity

With the exception of *Agrobacterium tumefaciens*, the causal agent of crown gall, no other bacterial pathogen attacks as many different plant species as does *R. solanacearum*. Several hundred species, representing more than 50 plant families, have been identified as hosts of this pathogen with the number of new host species still increasing. Some of the most important agricultural crops affected by *R. solanacearum* include tomato, potato, pepper, tobacco, eggplant, groundnuts and bananas. A number of ornamental plants, woody perennials as well as a very large group of weed species can also be infected (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 2000).

Where hosts are concerned, it is important to realise that various factors such as temperature, soil type and rainfall may coincide to influence susceptibility of the host and symptom development (Hayward, 1991). According to him, another reason for the erratic occurrence of bacterial wilt on certain hosts in specific areas could also be due to strain differences in the pathogen. For example, *R. solanacearum* affects *Eucalyptus* only in Brazil and China but not in Australia. Similarly, bacterial wilt of cassava is confined to Indonesia although the host is widely cultivated in other countries where the pathogen is endemic. According to Kelman (1953), hosts do not necessarily show typical wilt symptoms and latent infections are not an uncommon phenomenon.

Apart from potatoes, other Solanaceous crops of economic importance include tomato, eggplant, tobacco and pepper (Hayward, 2000). According to Hayward (1994a), bananas, plantains and ginger are other economically important hosts. Some ornamental plants include bird-of-paradise, geranium and anthurium (Hayward, 1994a) and chrysanthemum (Shekhawat *et al.*, 1992). Leguminous hosts include groundnuts as well as cowpea and some beans. Trees and shrubs can also be affected and perennial fruit and nut crops include custard apple, cashew, cassava and mulberry, with *Eucalyptus* spp. representing woody perennial hosts. Other plants affected are strawberry, sweet potato, papaya, radish and cucumber (Hayward, 1994a). Various weed species can also be infected. Common South African weed species are blackjack, the common thorn apple and black nightshade (Swanepoel and Young, 1988; Swanepoel, 1992).

4.5. Epidemiology

4.5.1. Survival

There are conflicting results regarding the ability of *R. solanacearum* to survive in soil and its persistence in the field cannot be predicted (McCarter, 1976). Survival of the pathogen in soil can be affected by many factors such as initial inoculum concentration, whether the land is left fallow or cropped with a non-susceptible host or the physical, chemical and biological properties of the soil (Moffett *et al.*, 1983). The organism can survive in some soils for long periods even in the absence of host plants, whereas in other soils its population declines rapidly in spite of cultivation with susceptible crops. Race 1 of *R. solanacearum* has been shown to survive in bare-fallowed field plots for at least four years and race 3 for one to two years (Graham *et al.*, 1979). According to Lloyd (1978) there are a number of "sheltered sites" where the bacterium might survive in infected soil between successive plantings of a susceptible crop. These sites are alternative weed hosts, infected host debris, self-sown potato tubers and deeper soil layers.

There have been numerous reports of crop plants and weed species that act as alternative hosts for the pathogen. These plants can be infected and not express any symptoms (Shekhawat *et al.*, 1992). However, since plant debris rapidly decomposes in warm, moist soils, it would provide only temporary sheltered sites for the pathogen. Infected tubers on the other hand can provide shelter for a longer period (Graham *et al.*, 1979). According to Granada and Sequeira (1983) the pathogen also survives by colonising the rhizospheres of non-host plants.

Graham and Lloyd (1979) reported that the pathogen could be detected at all soil depths between 15 and 75 cm but not in the 0 to 15 cm zone, probably due to desiccation during dry weather prior to soil sampling. This could explain why various soil disinfectants performed poorly in the control of bacterial wilt. Martin *et al.* (1981b) were able to detect the pathogen in the first 30 cm of the topsoil. They stated that the vertical distribution of *R. solanacearum* is influenced by various factors such as soil type and rainfall and varies from one site to the next.

4.5.2. Source of Inoculum and Dispersal

Ralstonia solanacearum is regarded as a soilborne pathogen with primarily two sources of inoculum, namely infested soil and infected tubers (Shekhawat *et al.*, 1992). Infected seed tubers are the most effective inoculum source for short and long distance dispersal. Developing countries often lack seed certification programs and farmers are forced to retain tubers from plants grown in infested soils in order to have sufficient seed for the next crop. This practice will promote the problem and spread the disease even further (Ciampi *et al.*, 1980).

Tubers can carry the pathogen in three different ways: in vascular tissues, on the tuber surface and in lenticels (Shekhawat *et al.*, 1992). It was found that during storage the bacterial population decreased rapidly on the tuber surface to non-detectable levels, but survived in vascular tissues for at least eight months at 10 to 15°C and at room temperature. Only when temperatures dropped to 1.6°C, did the pathogen loose its virulence and viability in tubers and only if it was stored for long periods (Nielsen, 1963). Sunaina *et al.* (1989) could still detect the pathogen in lenticels and vascular tissue after 240 days of storage at 4°C.

Infected plant material decaying in soil releases masses of bacterial cells in a slime layer. These slime masses adhere to soil particles and form pellets, enhancing soil survival (Shekhawat *et al.*, 1992).

According to Shekhawat *et al.* (1992), secondary spreading of the disease from infected plants takes place by root contact, irrigation water, farm implements and people and animals moving through infected fields. Kelman and Sequeira (1965) concluded that infection by *R. solanacearum* can occur in roots without any external wounding of the root system if the number of cells in the inoculum is relatively high. Under high soil moisture levels, the pathogen emerges

or is released from the roots of infected plants and spread to adjacent roots of healthy plants, particularly when the roots are in close proximity. Where tubers are cut for planting, cutting knives can also spread the pathogen to healthy tubers (Shekhawat *et al.*, 1992). It was suggested that the pathogen could have an epiphytic phase in its life cycle and together with infected alternative host debris such as weed species can provide alternative inoculum sources (Buddenhagen and Kelman, 1964; Hayward and Moffett, 1978; Graham *et al.*, 1979).

4.5.3. Conditions Favourable for Disease Development

Ralstonia solanacearum predominates in tropical and subtropical regions where warm, humid conditions enhance disease development. The optimum temperature for development of bacterial wilt of potato is between 25 and 36°C (Kelman, 1953). According to Thurston (1963) infection occurred at a soil temperature of 12.8°C but symptoms did not develop unless the soil temperature remained at 21.1°C or higher for several days. Lower-temperature strains of *R. solanacearum* exist and are capable of causing disease under cooler conditions. Although disease incidence is usually lower under cooler conditions, significant losses may still occur (Elphinstone, 1996).

High soil moisture levels favour disease development and the pathogen cannot withstand desiccation. Temperature and soil moisture have a synergistic effect and wilt incidence will decline when soil moisture levels drop to 8 to 10% of water holding capacity. Wilt incidence will decline when the maximum/minimum temperature falls below 20/15°C (Shekhawat *et al.*, 1992).

4.5.4. Mechanism of Infection and Colonisation

According to Kelman (1953), *R. solanacearum* is a typical wound pathogen on roots. Under field conditions the pathogen can also infect plants by penetrating the natural gaps between primary and secondary roots (Kang *et al.*, 1994). Wounding can occur during cultural practices or through nematodes (Shekhawat *et al.*, 1992). Moffett *et al.* (1981) demonstrated that the organism entered certain hosts through stomata but, to a lesser extent.

After entry, the pathogen rapidly colonises the intercellular spaces of epidermal tissue, damaging the middle lamellae of cells and degrading the cell walls. This result in the destruction of xylem

tissues and production of tyloses that block the vascular system and cause the plants to wilt (Kang *et al.*, 1994).

In 1978, Wallis and Truter conducted a detailed study on the histopathology of tomato plants infected with *R. solanacearum*. According to them the initial colonisation of host tissue did not occur in the xylem vessels of the roots as was first expected, but in small diameter cells adjacent to the large xylem vessels. Twenty-four hours after inoculation, stimulation of tyloses formation was noted in invaded as well as non-invaded cells. According to Agrios (1997), tyloses form in xylem vessels of most plants under various conditions of stress and during invasion by most of the xylem-invading pathogens. These structures are overgrowths of the protoplast of adjacent living parenchymatous cells that protrude into xylem vessels through pits, inhibiting further spread of the pathogen. Increased production of indole acetic acid and other growth substances are believed to be responsible for their formation (Sequeira and Kelman, 1962; Sequeira, 1965).

During their study, Wallis and Truter (1978) noted that bacteria migrated from the infected cells through a narrow, slightly elongated neck and enters the lumina of the tyloses. During the next 24 hours most tyloses matured and collapsed, possibly due to extracellular enzyme activity of the pathogen, releasing the bacteria into the xylem vessels. After their release, the bacterial concentration increased steadily and it was noted that the bacterial cells were often imbedded in a fine granular matter. This granular matter increased until many xylem vessels were filled, plugging the vessels and preventing normal water uptake and resulting in rapid wilting of the host plant.

According to Schell (1996), *R. solanacearum* disrupts cell walls to facilitate spreading through the vascular system into the rest of the host. One of the possible enzymes released is cellulase and according to Ofuya and Wood (1981), it reaches its maximum activity at the advanced stages of infection.

Techniques such as immunofluorescence (IF) microscopy using specific antibodies can be used to visualise the infection process of bacterial cells (McGarvey *et al.*, 1999).

4.5.5. Influence of Environmental factors on the Virulence of *Ralstonia solanacearum*

Ralstonia solanacearum has many specialised genes relating to interactions with its host and it has been postulated that host-pathogen interactions are strongly regulated by the environment (Denny *et al.*, 1998). The pathogen has presumably evolved to recognise selected environmental signals that are important for its survival or successful host-colonisation. It reacts upon these signals to regulate its virulence. Exactly what these environmental signals are and how they interact, are still not clear.

a) Phenotypic Conversion

One of the characteristics of *R. solanacearum* is the rapid loss of pathogenicity once it is maintained in culture. The pathogen therefore has to be inoculated into host plants and re-isolated to maintain virulence (Kelman, 1953). When the pathogen undergoes a change in colony morphology from mucoid to non-mucoid, it shows a greatly reduced capacity to cause wilt in plants. This phenomenon where virulence is lost is also referred to as phenotypic conversion (PC) and is thought to be regulated by the pathogens environment. According to Denny *et al.* (1994) these PC-type strains still retain pathogenicity because they are able to infect plants and cause stunting and proliferation of adventitious roots and should not be called avirulent.

Phenotypic conversion-types are easily recognised on TZC medium (Denny *et al.*, 1993). The colonies are round, dark red and butyrous, which contrast sharply with the typical irregularly round, mucoid, pink or white, fluidal virulent colonies. True PC-type strains are also markedly more motile. According to Denny *et al.* (1993), these changes in colony type and subsequent reduced virulence of the pathogen are mainly due to the loss of one or more components of extracellular polysaccharides (EPS) and the reduction in extracellular endoglucanase activity.

These types routinely appear when the pathogen is cultured for five to seven days in glucose containing broth with an organic nitrogen source without shaking (Denny *et al.*, 1993). They also arise during prolonged culture on agar plates and in wilted plants, which suggests that there may be natural conditions that favour their appearance or multiplication. The large number of PC-type mutants that arise under certain conditions may be because they have a selective advantage over the wild type. According to Denny *et al.* (1994) the oxygen limitation in unshaken broth cultures

appeared to select PC-mutants due to their higher motility and positive aerotaxis while a high-salt mineral medium allowed PC cells to grow better than the wild type.

Denny *et al.* (1994), hypothesised that *R. solanacearum* normally exist as the PC-type in soil but can switch to the wild type when a suitable host is found. According to them this shift in phenotype occurs when some endogenous inducer (EI) exceeds a critical concentration. Exactly what this inducer is and how it regulates PC is still not clear but according to Schell (1996), 3-OH PAME (3-hydroxypalmitic acid methyl ester) might be one example of such an inducer. In soil, the bacteria survive as microcolonies with relatively low cell densities and slow rates of multiplication that might prohibit sufficient 3-OH PAME levels to accumulate, thereby favouring the PC-type. These PC-type cells can conserve energy and cellular resources because of their reduced production of extracellular polysaccharides and proteins, enhancing survival. When the pathogen encounters a host plant, it begins to multiply and later reaches a sufficient cell density to produce high enough levels of 3-OH PAME to switch on virulence gene expression (Fig.3).

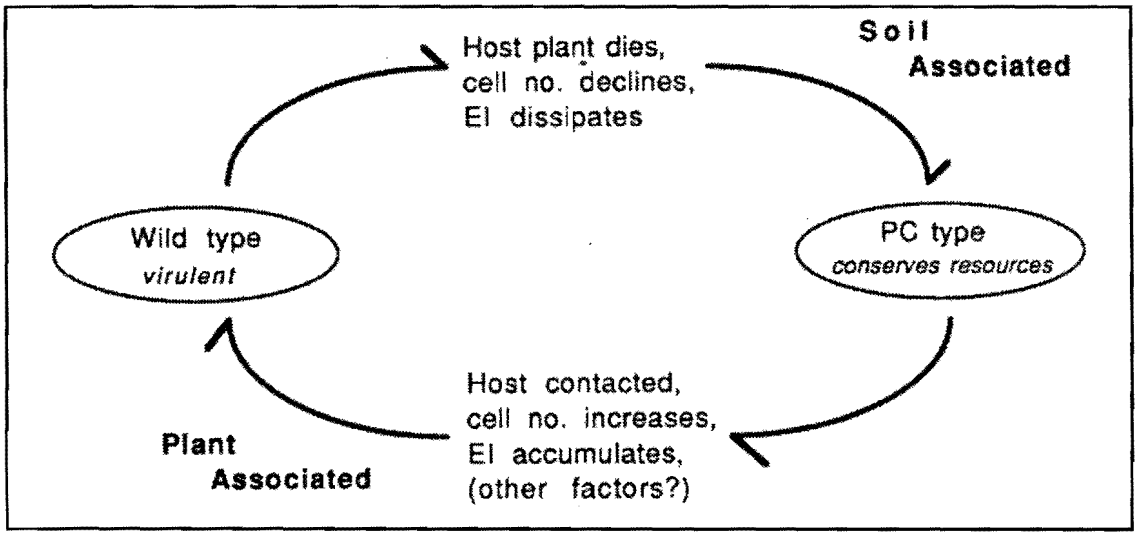

Figure 3. Postulated switching between the wild type and phenotypic conversion-type that might occur when bacteria move between soil and host plants. EI = endogenous inducer (Denny et al., 1994).

b) Virulence Factors

In order to understand how the control of virulence genes functions, it is necessary to understand the biochemistry and possible physiological functions of the pathogen's individual virulence factors. According to Denny *et al.* (1993), the extracellular polysaccharide (EPS I) is the primary virulence factor. Extracellular proteins (EXPs) such as cell wall-degrading enzymes; endoglucanase and *endo*-polygalacturonase enzymes are relatively minor virulence factors (Denny *et al.*, 1993; Denny *et al.*, 1998).

Despite many years of investigation, the exact role of extracellular polysaccharides (EPS) in the pathogenesis of plant-pathogenic bacteria remains unclear (Kao *et al.*, 1992). According to them the slime is thought to play a role in virulence by occluding the vascular system of the plant and reducing water transport and by protecting the bacteria from plant defence compounds.

The extracellular polysaccharide, EPS I, is the major known virulence factor of *R. solanacearum* and is released extracellularly in large quantities (Schell, 1996). It is an unusual nitrogen-rich polysaccharide and is composed of a trimeric repeating unit of N-acetyl-galactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2,4,6-trideoxy-D-glucose (McGarvey *et al*, 1998). Quantification of EPS I in infected plants, using an enzyme-linked immunosorbent assay (ELISA), revealed that the bacterial populations in susceptible tomato cultivars produced greater amounts of EPS I per plant than those in resistant cultivars (McGarvey *et al.*, 1999). According to Huang *et al.* (1995), the production of EPS I requires the 18-kb *eps* gene cluster, which encodes several membrane-associated and soluble polypeptides involved in its biosynthesis and export. The *eps* gene cluster contains at least 12 genes that are probably transcribed as an operon from a single environmentally-regulated promoter (Schell, 1996). According to him, extracellular polysaccharide synthesis also involves portions of the *ops* gene cluster, which may encode synthesis of a common sugar precursor.

The pathogen also produces many EXPs that are likely or proven virulence factors. Some of these EXPs are plant cell wall-degrading enzymes such as endoglucanase (Egl), polygalacturonases (PglA and PglB) and pectin methylesterase (Pme) (Denny *et al.*, 1998). According to them, extracellular proteins as a whole have a major role in pathogenesis. However, once inside the host, *R. solanacearum* does not necessarily require these individual enzymes for

wilting. It is more likely that they function in root invasion, spreading into the vascular system or acceleration of disease development (Huang *et al.*, 1995; Schell, 1996).

c) Regulation of Virulence

According to Denny *et al.* (1993), the virulence of *R. solanacearum* is a trait regulated by a sophisticated, multi-component network that is sensitive to the environment. According to Schell (1996), the pathogen must cope with two very different environments, namely soil and plant. Denny *et al.* (1994), hypothesised that the pathogen normally exist as the PC type in soil but when it encounters a suitable host plant, it begins to multiply and later reaches a sufficient cell density to start producing extracellular virulence factors (Fig.3).

The production of virulence factors is controlled by a complex network that contains at least 12 regulatory genes (Schell, 1996). This network coordinates the expression of the different virulence genes in simultaneous response to multiple cues encountered during the various stages of pathogenesis and during soil survival, working together primarily to direct the syntheses of EPS I. Exactly how this network is regulated is still not known but is projected to function as illustrated in Fig.4.

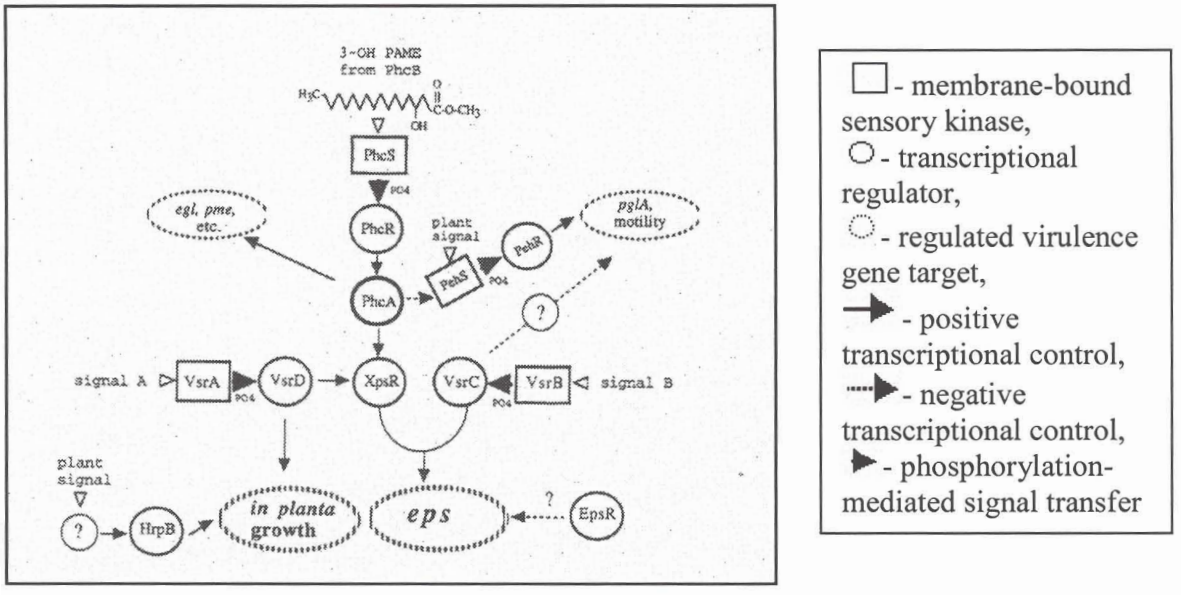

Figure 4. Schematic presentation of the organisation and operation of the virulence gene regulatory network (Schell, 1996).

4.6. Detection and Isolation

Various semi selective media can be used for the detection of *R. solanacearum*. In 1954, Kelman developed a semi selective medium containing tetrazolium chloride. On this medium, virulent colonies are fluidal, slightly raised, slimy and appear creamy-white with or without pink centres. These centres are typically comma-shaped. Avirulent colonies on the other hand are round, butyrous in texture and dark red (Kelman, 1954; Shekhawat *et al.*, 1992). Kelman's TZC medium is still used for soil and tissue isolations with moderate success. Other well-known media were developed by Graham and Lloyd (1979) and Granada and Sequeira (1983). These were modified versions of Kelman's TZC medium, containing antibiotics such as penicillin, chloramphenicol, polymixin B sulphate and bacitracin as well as crystal violet. The antibiotics and crystal violet reduced background population, resulting in more selective media. Karganilla and Buddenhagen (1972) and Chen and Echandi (1981) also modified TZC medium, adding various antibacterial as well as antifungal substances, resulting in extremely expensive media. A major limitation of all these media was that low population densities of the pathogen were not detectable (Nesmith and Jenkins, 1979). Especially in soil samples, the concentration of pathogen cells must exceed 10^4 cfu ml^{-1} soil sample before it can be detected (Karganilla and Buddenhagen, 1972; Nesmith and Jenkins, 1979; Chen and Echandi, 1981). According to Karganilla and Buddenhagen (1972), it appeared that the performance of a selective medium depended on the types of pathogen strains present in a region as well as on the soil itself.

Indicator plants can be used to detect the presence of the pathogen in soil (Graham and Lloyd, 1978). The indicator plant species should show disease symptoms within a short period of time following infection. Tomato and potato seedlings have been used as indicator plants. It takes two to three weeks for symptom development and slightly lower concentrations (10^4 cfu ml^{-1} soil sample) could be detected (Karganilla and Buddenhagen, 1972; Graham and Lloyd, 1978).

Detection methods based on immunological and nucleic acid based technologies have also been developed for the isolation of *R. solanacearum*. Although these techniques are generally more expensive, they are more accurate, -rapid and -sensitive than traditional plating techniques (Seal and Elphinstone, 1994). Serological detection techniques include ELISA and IF antibody staining. These techniques are commonly used to monitor the presence of *R. solanacearum* in host plants. Various ELISA kits have been developed for the detection of *R. solanacearum* primarily in tissue samples. Bellstedt and van der Merwe (1989) described two ELISA methods

for the detection of *R. solanacearum* biovar 2 and 3 in South Africa. These systems were based on a biotin/avidin system. In this system the high affinity of avidin for biotin was utilised. Where the two systems were combined, all strains of *R. solanacearum* from various localities in South Africa could be detected. Although ELISA techniques are useful for rapid and repetitive diagnostic tests, improvements are still required to extract the bacteria that adhere to soil particles and to inhibit non-specific reactions between antibodies and soil or plant material (Seal and Elphinstone, 1994).

Although DNA-based methods offer highly specific and sensitive means to detect the pathogen, they are more expensive than serological techniques (Seal, 1994). The polymerase chain reaction (PCR), one of the most well known DNA-based methods for the detection of *R. solanacearum*, is not dependent on the use of purified DNA (Gillings and Fahy, 1993a). Various primers have been designed to detect *R. solanacearum*. These primers were based on the published DNA sequence of the gene for endopolygalacturonase (*peh* A). The DNA fragments produced by the various PCR techniques can be further classified using RFLP techniques. Restriction fragment length polymorphism analysis is especially useful in distinguishing among different biovars or races of *R. solanacearum* (Seal, 1994). Although RFLP analysis is very discriminatory, it is time-consuming and not suitable for rapid screening of isolates (Seal and Elphinstone, 1994).

According to Black *et al.* (1998), the BACTID kit and Biolog system can be used to detect or confirm *R. solanacearum.* The BACTID kit is used to eliminate saprobic bacteria and to achieve preliminary identification (usually to the genus level) with as few tests as possible using media in micro-centrifuge tubes. The system includes software for identifying bacteria from test results. The system can partly differentiate between biovars of *R. solanacearum*. The high costs however, makes it too expensive for routine laboratory analysis especially in less-developed countries. The limited shelf life of plates is a further drawback.

4.7. Control

Due to its extremely wide host range and its ability to survive for prolonged periods in the soil, control of *R. solanacearum* is complicated and no single control measure can be used on its own to eradicate this pathogen. Bacterial wilt is particularly a problem because there is no chemical registered for the use on potatoes although various chemicals such as Bavistin and Biltox have

been tested (Shekhawat *et al.*, 1992). An integrated control program should therefore be implemented where a variety of control methods be combined and applied during different stages of cultivation in order to exclude the pathogen from all phases of potato production. The primary objective of such an integrated control strategy would be to limit the survival and multiplication of the pathogen as well as alternative hosts and interacting nematodes by means of cost-effective programs. The efficiency of a particular system would be location-specific since it must be adapted to suit certain climatic conditions, soil type, pathogen strain, farming system as well as the socio-economic situation of a specific area (Elphinstone and Aley, 1993; Mienie, 1997).

In developing countries many of the available control strategies that have been developed against *R. solanacearum* are not cost-effective and cannot be applied (Hayward, 1991). According to him, the disease can only be contained in these countries, unlike in developed countries where the use of resistant cultivars, pathogen-free seed, and adequate crop rotations are used to fight the battle against bacterial wilt. Various cultural practices can however be manipulated with little extra costs to inhibit growth and development of the pathogen without affecting growth of the crop. Some of these practices include: control before planting (i.e. field selection and preparation), control during planting and crop development and control during harvesting and thereafter.

4.7.1. Field Selection and Preparation

a) Field Selection

The field where the potato crop is planted must be bacterial wilt free. Various techniques such as PCR and ELISA are available to detect the pathogen in soil. Apart from high costs these techniques are impeded by inadequate soil sampling and are usually not an economic option for farmers. Another more affordable method for the detection of the pathogen in soil is to plant susceptible indicator plants and to evaluate them for symptom development over a period of time (Graham and Lloyd, 1978).

According to Kelman (1953), losses can also be minimised to some extend if farmers select well-drained sites or avoid low places in fields where the disease has been detected before. Farmers should also avoid fields where tomato, eggplant or tobacco has been grown in the past three years (Ali, 1995).

b) Crop Rotation, Bare Fallow Practices and Intercropping

Crop rotation with non-host crops has widely been investigated as a control measure where the disease is known to be present. According to Hartman and Elphinstone (1994), rotation with maize, rice and finger millet can reduce the incidence of wilt to some extent. Various other crops such as sorghum, wheat, cowpea, onion, garlic and carrot have also been used in different combinations to suppress pathogen levels within the soil (Shekhawat *et al.*, 1992). Scientists still do not agree on how long crop rotation programs should be. According to Shekhawat *et al.* (1992) two to three years of rotation should be enough while others such as Mienie (1997) suggested longer periods of four to five years.

Crop rotation can be combined with bare fallow practices (Jackson and Gonzals, 1979). During the bare fallow period all plants (i.e. weeds and volunteer potato plants) should be eradicated. The failure to eliminate all alternative hosts in crop rotation has been considered as one of the major factors that accounts for the insufficient control of bacterial wilt. The level of disease control with the use of crop rotation and bare fallow methods was however very variable. It appeared to be dependent on several factors such as the ability of the pathogen to survive in the absence of a host, climatic conditions and soil type (Akiew *et al.*, 1993).
Intercropping can be used to reduce the incidence of bacterial wilt as well as root-to-root transmission of the pathogen. In Burundi, intercropping with beans has been used successfully while corn and cowpea proved to be the most effective intercrops in the Philippines (Hayward, 1991). Autrique and Potts (1987) reported that intercropping potato with maize or haricot beans markedly reduced the incidence and rate of disease development in the potato crop.

c) Soil Preparation

Infected soil can be treated to some extend prior to planting. Since *R. solanacearum* is sensitive to desiccation, soil should be deep ploughed after the last potato harvest and left exposed preferably to high temperatures (35 to 40°C). Deep ploughing during a rotation system disturbs the soilborne inoculum of the pathogen, resulting in a decrease in inoculum levels and infection potential (Shekhawat and Chakrabarti, 1995; Mienie and Theron, 1999) and one or two deep ploughings of infested soil reduced wilt incidence by about 70% (Shekhawat *et al.*, 1992).

Soil amendments can be used with some degree of suppression although most have only been tested in pot culture experiments and are not yet commercially applicable (Hartman and Elphinstone, 1994). Chellemi *et al.* (1992), had some success in greenhouse experiments where they used composted organic amendments such as mushroom compost, poultry broiler litter and yard waste in different combinations to suppress bacterial wilt. Mixtures containing urea and calcium (S-H mixtures) and even seashell grit have also been used with various degrees of success and large nitrogen inputs, particularly sewage sludges, also helped to suppress the pathogen (Hartman and Elphinstone, 1994). Soil can also be disinfected with chemicals such as stable bleaching powder. However, most of such chemicals proved to be detrimental to the crop (Shekhawat *et al.*, 1992).

Steam was used for soil disinfection where temperatures were maintained at 85°C for 15 minutes but this method was not practical over large areas and only proved to be effective in seedbeds or greenhouses (Shekhawat *et al.*, 1992). According to Hartman and Elphinstone (1994) fumigants like chloropicrin have been tested for control of bacterial wilt on tomatoes but were not economically feasible over large areas. Although crop losses could be reduced, complete control or eradication could not be obtained.

Soil solarisation has been evaluated as a possible control measure but only a slight reduction in population levels could be obtained. Saumtally *et al.* (1993) suggested that higher temperatures might prove to be more effective and different combinations of transparent and dark plastic should be investigated.

The burning of vegetation can reduce the bacterial wilt incidence by spreading straw in furrows and set alight. However, this practice is dangerous and not often used (Shekhawat *et al.*, 1992).

4.7.2. Planting and Crop Development

a) Pathogen-free Propagative Material

It is crucial to use pathogen-free propagative material to minimise the concentration of initial pathogen inoculum. *R. solanacearum* can easily be disseminated through infected propagative material such as potato tubers (Hayward, 2000). A major problem is the ability of the pathogen to infect tubers without symptom expression. This latent infection of tubers is particularly

problematic in developing countries where certified seed is not readily available (Ciampi *et al.*, 1980). If there is no proper seed certification program, farmers have no alternative but to use potentially infected tubers for their next crop.

South Africa has a very effective seed certification program. According to Nortje (1999) seed potatoes are produced in all the provinces of South Africa with the Western Cape currently producing 35% of all certified tubers. More than 70% of South Africa's potatoes are produced from certified tubers.

The South African seed certification program functions according to the Plant Improvement Act (Act 53 of 1976). It was primarily implemented to reduce the incidence of tuber-carried pathogens and to provide farmers with high quality seed potatoes. This program is based on a true generation concept and was implemented on 1 June 1995. According to this concept, seed potatoes should be phased out of the program within eight generations to limit pathogen population build-up. Selected material from the National Cultivar Collection is multiplied *in vitro* and certified as generation 0 (G0). The first field multiplication is certified as G1 with subsequent generations certified up to G8. After each field multiplication seed potatoes advance to the next generation until it is phased out after eight generations. With each successive generation the probability that the tubers are infected with one or more pathogens increases. Each generation has three quality classes built in to compensate for diseases. If a seed crop exceeds a given diseases index, it is graded in a lower class. The third class is automatically phased out, despite the generation (Nortje, 1997). The latter class is considered as standard grade seed and is only suitable for the production of table potatoes.

A zero tolerance for *R. solanacearum* is maintained (i.e. if this organism is detected, farmers are immediately notified and their crop rejected). This ensures that no infected tubers can be sold as seed potatoes. However, once rejected as seed potatoes, farmers are still allowed to sell tubers as table potatoes. If these tubers should wrongfully be used as seed potatoes, the danger of spreading the disease still exists.

b) Resistant Cultivars

The use of resistant potato cultivars plays an important role in the integrated control of bacterial wilt. Unfortunately the complexities of host-pathogen-environment interaction make breeding for

resistance extremely difficult (Tung *et al.*, 1990) and no immunity has as yet been identified in potato (Hayward, 1991).

The effect of temperature on resistance to wilt caused by different strains and races of *R. solanacearum* have been investigated (Tung *et al.*, 1990; Tung and Schmiediche, 1995). Resistance was found to be temperature sensitive as well as strain specific. A wide range of genes for resistance needs to be incorporated into a genotype to provide adequate resistance. This should reduce strain specificity and improve the stability of resistance under changing environmental conditions (Tung *et al.*, 1990).

Since the early 1970's, *Solanum phureja* Juz. & Buk., a diploid cultivated species, has been used extensively in breeding programs by authorities such as the International Potato Centre in Peru, as the major source of resistance to bacterial wilt. It has, however, not been successful in all environments (Hayward, 1991). Its resistance is temperature sensitive and it is therefore best suited for higher elevations or cooler climates (Laferriere *et al.*, 1998). Resistant genes from other diploid potato species have been identified and used in breeding programs. Some of these species are: *S. chacoense* Bitt., *S. ciliatum* Lam., *S. jamesii* Torr., *S. multidissectum* Hawkes, *S. pinnatisectum* Dun., *S. raphanifolium* Hawkes, *S. sisymbrifolium* Lam., *S. sparsipilum* Bitt. and *S. stenotomum* Juz. & Buk. (Madalageri and Patil, 1995). Other *Solanum* spp. evaluated were: *S. acaule* Bitt., *S. berthaultii* Hawkes, *S. blanco-galgosi* Ochoa, *S. boliviense* Dun., *S. brachycarpum* Ochoa, *S. chomatophilum* Bitt., *S. demissum* Lindl., *S. polytrichon* Rydb., *S. stoloniferum* Schlechtd and *S. sucrense* Hawkes (Hartman and Elphinstone, 1994). According to Laferriere *et al.* (1998) another source of resistant genes was *S. commersonii* Dun., a diploid wild potato species from south-eastern South America. Attempts to incorporate this species (as with many other) into potato breeding programs were however thwarted by its sexual incompatibility with tetraploid *S. tuberosum*. To bypass this obstacle, protoplasts from the two species were electro fused where after the fused protoplasts multiplied to form callus tissue. This tissue differentiated to give rise to shoots that were used in further breeding programs. The stability of resistance of these plants under different field conditions and temperature regimes is still unknown and has to be evaluated.

According to French and De Lindo (1982), resistance to bacterial wilt in potato is a partially dominant character and is more of a polygenic type. Tung and Schmiediche (1995) agreed on the dominant character of resistance and suggested that only a few genes control it although the

number of genes involved is still unknown. Tung *et al.* (1990) and Tung and Schmiediche (1995) suggested that the genes for resistance in the host evolved independently from the pathogen and that a gene-for-gene relationship does not seem to be applicable to bacterial wilt.

Whether or not resistance to *R. solanacearum* is controlled by minor or major genes is not considered to be a point of great concern in practical breeding (Tung *et al.*, 1990). According to them, the inheritance and expression of resistance seem to be complex and new methods other than conventional breeding techniques should be evaluated to increase and stabilise resistance. Genetic engineering techniques are therefore being used to strengthen the basic composition of the host plant through the incorporation of suitable genes from other sources. Antimicrobial genes coding for lytic enzymes such as cecropins isolated from the lepidopteran *Hyalophora* were used for the control of bacterial diseases of plants and showed great potential against *R. solanacearum* (Montanelli *et al.*, 1995). According to Hayward (1991), lysozyme and other potent antibacterial proteins derived from insects can also be introduced into potatoes to increase resistance.

c) Time or Season of Planting

The pathogen can also be escaped by delaying the date of planting until temperatures are lower in summer- and autumn-production regions. In winter- and spring-production regions, early planting and harvesting can reduce bacterial wilt and tuber rot. Short duration early maturing potato varieties would be ideal for this practice since the time for crop development is reduced to escape the disease (Shekhawat *et al.*, 1992). However, this only applies for the production of table potatoes since the danger of latent infection still exists (Mienie and Theron, 1999). Seed carrying the latent pathogen can enhance disease epidemics when planted under conditions favourable for disease development (Hayward, 1991). In Brazil, changing of planting dates to avoid the rainy season was also suggested as a mean to decrease losses in potato crops (Kelman, 1953).

d) General Cultural Practices

In an attempt to reduce cost of planting material, farmers in developing countries often use tuber cuttings as seed (He, 1995). Cutting of seed tubers increases the risk of bacterial infection. During the cutting process the pathogen can be spread from a diseased tuber to healthy ones by

contaminated blades. When whole tubers are used the incidence of bacterial wilt is lower (Shekhawat *et al.*, 1992). According to them when cut tubers were used, disease incidence increased by more than 2.5 times and yield decreased by more than 40%.

Before any seed lot is handled, all containers, tools and implements should be thoroughly washed and disinfected. Labourers should also be informed about the impact of the disease and be issued with detergents for personal hygiene to prevent spread of the disease by their boots and hands (Mienie, 1998). All implements used in infected fields should be cleaned to get rid of excess soil and properly disinfected before being used in other fields. Sodium hypochlorite (3.5 to 5%) can be used for general disinfection and 4% formalin and steam (Conroy, 1969) or 0.5% carbolic acid (Jeyes Fluid) (Mienie and Theron, 1999) is suitable to clean and disinfect machinery. Movement through infected fields should also be limited since soil attached to vehicles, shoes and animal hooves can spread the pathogen to adjacent fields (Swanepoel and Bosch, 1988).

Due to the wounding caused by nematodes (*Meloidogyne* spp.), *R. solanacearum* would be able to infect tubers easier where nematodes are present (Elphinstone and Aley, 1993). Controlling nematodes will result in a decrease in the number of infection loci and control the spread and development of bacterial wilt (Kelman, 1953). According to Akiew *et al.* (1993) effective control methods include nematode-resistant varieties, chemical soil treatment, soil fumigation and rotation with crops that are resistant to nematodes.

Removing and destroying wilted plants limits the spread and development of bacterial wilt since these plants act as inoculum sources. Potato plants should be carefully monitored throughout the season and wilted plants should be removed and burned immediately (Mienie, 1997).

Where infection is very high, irrigation should be temporarily suspended to avoid further spread to adjacent fields. Farmers should also avoid the use of irrigation water that might be contaminated by *R. solanacearum* (i.e. water from irrigation dams near infested fields) (Swanepoel and Bosch, 1988).

Since root injuries facilitate infection, injury to the roots should be avoided during cultivation. Where intercropping is applied, care should also be taken not to damage the roots of potato plants (Kelman, 1953). Post-emergence earthing-up of potatoes is a common practice. It should be carefully applied without injuring potato plant parts. General post-emergence tillage should also

be restricted to an absolute minimum, limiting damage (Shekhawat *et al.*, 1992). According to Chakrabarti *et al.* (1995) earthing-up should be applied immediately after planting, when damage to plant parts should be minimal.

e) Chemical Control

Currently there is no chemical registered in South Africa for the control of potato bacterial wilt. Some of the chemicals that have been tested elsewhere against *R. solanacearum* include Bavistin, Emisan 6, plantomycin and Biltox (Shekhawat *et al.*, 1992). Most of these have been tested on other hosts such as tobacco, jute and ginger and have not been tested on potatoes. According to Hartman and Elphinstone (1994), an experimental bactericide, Terlai, showed potential in reducing wilt in pot culture experiments. Where it was applied in conjunction with *Pseudomonas fluorescence* it reduced wilt significantly (Machmud and Machmud, 1994).

Studies on the use of antibiotics as control agents have been performed with varying success. An antibiotic C-6, similar to erythromycin, was found to effectively inhibit the pathogen in diseased potato tubers. Pre-treatment of seed tubers with 500 to 1000 ppm of the antibiotic followed by two foliar sprays at the same concentration effectively controlled the disease and also increased the yield per unit area by approximately three times (Shekhawat *et al.*, 1992).

f) Biological Control

The primary agents used to control bacterial wilt biologically include antagonistic rhizobacteria and avirulent mutants of *R. solanacearum*. These organisms are invasive but non-wilt inducing and compete for vascular colonisation of xylem vessels and induce host resistance (Hayward, 2000). Various actinomycetes and bacteria such as *Pseudomonas fluorescens* and *Bacillus polymyxa* have been reported to be active control agents. These organisms have been found to delay the development and reduce the incidence of bacterial wilt (Shekhawat *et al.*, 1992). *Pseudomonas cepacia*, commonly isolated from maize roots, showed antagonistic potential in culture and pot trials (Hartman and Elphinstone, 1994). Other organisms such as *B. mesentericus*, *B. megaterium*, *B. subtilis*, *B. mycoides* and even *Erwinia* spp. have been reported potential biocontrol agents for *R. solanacearum* (Hartman *et al.*, 1993).

According to Hayward (1991) the mechanisms involved in biocontrol include induced resistance, or competitive exclusion. The active colonisation of the rhizosphere with antagonistic soil bacteria or bacteriocin- and bacteriophage-producing strains of *R. solanacearum* can protect the plant from virulent infections. The induction of host plant resistance as the result of artificial inoculations with heat-killed virulent strains, avirulent mutants or incompatible strains of *R. solanacearum* in roots, stems and leaves of host plants has also been reported (Trigalet *et al.*, 1994). According to them, the factors involved in the resistance response of the host plant are very complex and although biocontrol seems promising under controlled conditions, it has not proven effective in natural environments.

In an attempt to limit the factors that influence biocontrol efficacy, endophytic antagonists derived from the wild-type pathogen have been evaluated (Trigalet *et al.*, 1994). Once established within the plant it provides continuous protection. Avirulent mutants of *R. solanacearum* are deficient in production of exopolysaccharides and appeared to be the most likely candidates for endophytic biocontrol agents. Although these organisms had the ability to multiply within susceptible host tissue upon inoculation by the stem puncture technique, their systemic spreading was limited and their populations declined after a while (Trigalet *et al.*, 1994).

According to Hayward (1991), few of the tested approaches to biological control of bacterial wilt have reached a stage of commercial application and much more research is needed. Biocontrol strategies developed in laboratories often failed under natural conditions either since root colonisation of the biocontrol agent was too poor or too dependent on environmental greenhouse conditions (Trigalet *et al.*, 1994). Mienie (1998) evaluated *Pseudomonas resinovorans* as potential control agent. Results indicated that treatment with the antagonist significantly reduced the severity of bacterial wilt. *P. resinovorans* is currently being registered as biocontrol agent for potato bacterial wilt in South Africa. Although not yet registered, it has already been widely distributed and used amongst local potato farmers (Pers. Comm. N.J.J. Mienie, Potatoes South Africa, Seekoegat).

4.7.3. Harvesting and Storage

a) Sanitation Practices

According to Mienie (1998), all storage and seed handling equipment should be properly cleaned and disinfected after harvest to prevent survival and build up of inoculum on surfaces or in potato debris. All tubers, vines, soil, old boxes and bags should be removed and destroyed (Chakrabarti *et al.*, 1995). Storage bins, walls and floors should be washed with hot soapy water, thoroughly rinsed and disinfected.

b) Time of Harvest

Kelman (1953) suggested that the harvest date of potatoes grown in infested soil or in seasons when bacterial wilt is severe should be advanced. Trials conducted in India indicated that early harvesting reduced bacterial wilt and tuber rot (Shekhawat *et al.*, 1992). This is not always applicable because harvesting tubers when they are not fully developed may present marketing problems.

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CHAPTER 3

DEVELOPMENT OF AN ALTERNATIVE DETECTION METHOD FOR *RALSTONIA SOLANACEARUM* IN SOIL

ABSTRACT

Various methods have been developed for the detection of *Ralstonia solanacearum*. The majority of these were developed to detect *R. solanacearum* in plant material. Applying these methods for detection of the pathogen in soil, presented its own set of problems. An alternative method was therefore developed in this study to detect *R. solanacearum* in soil samples. The technique was based on using a catch crop or indicator plant system. Tissue culture potato plants were used to "entrap" the pathogen from artificially infected soil suspensions. Initially different enrichment broths were used for pathogen enrichment. Buffelspoort cultivar tissue culture potato plants were placed in artificially inoculated soil suspensions, incubated and tissue isolations prepared. The sensitivity of the trapping technique was evaluated using different pathogen concentrations. The efficacy of the trapping technique was compared with selective media and the commercial ELISA kit currently used in South Africa for the isolation of *R. solanacearum*. Although pathogen cell growth was enhanced in sterile conditions, most of the enrichment broths were unsuccessful in terms of enrichment when tested in a soil system. This was mainly due to the proliferation of various soil organisms, causing plants to die before tissue isolations could be made. It was therefore decided to exclude the enrichment step and only use sterile distilled water. Using the trapping technique, the pathogen was successfully re-isolated from concentrations up to 10^1 cfu ml^{-1} in soil suspensions. The technique appears to be practical, not needing specialised equipment and presents an alternative approach to detect *R. solanacearum* in soil samples. However, soil samples taken from a potato field with a well-documented history of bacterial wilt tested negative. None of the other methods used could detect the pathogen, emphasising the difficulties relating to soil testing.

1. Introduction

Throughout the years, various techniques have been developed for the detection of *Ralstonia solanacearum*. Since the pathogen has the ability to spread latently through infected plant parts, many countries have implemented strict quarantine regulations and detection systems to prevent further introduction of the pathogen into new fields (Elphinstone *et al.*, 1996). The majority of detection techniques were primarily developed for the detection of the pathogen in infected plant material. It is however also important to be able to detect the pathogen in infected soil (Nesmith and Jenkins, 1979). Although many of the techniques were originally developed for plant tissue detection it can also be used for soil detection. However, this approach is subject to various difficulties and shortcomings such as low recovery efficacy. Most of the problems encountered can be attributed to the heterogeneous nature of soil and the difficulty ensuring adequate sampling (Jenkins *et al.*, 1967).

One of the techniques most often used for the detection of *R. solanacearum* in soil, is to plant indicator plants (Jenkins *et al.*, 1967; Graham and Lloyd, 1978). These plants show disease symptoms within a short period of time following infection. Tomato and potato seedlings have commonly been used as indicator plants (Graham and Lloyd, 1978). After plant emergence, it took approximately two to three weeks for symptom development and concentrations of 10^4 colony forming units (cfu) ml^{-1} soil sample were detected (Jenkins *et al.*, 1967). According to the authors this method was slow, laborious and provided inconsistent results.

Various different selective and semi-selective media have also been developed. Most of these were based on Kelman's tetrazolium chloride medium (TZC) (Kelman, 1954). One of these media was developed by Graham and Lloyd (1979) and modified by Engelbrecht (1994). This modified version of the traditional TZC medium contains antibiotics such as penicillin G potassium salt, bacitracin, polymixin B sulphate and chloramphenicol. Although there are numerous variations, none of these media gained wide acceptance (Granada and Sequeira, 1983). Most of the media allows growth of too many background bacteria, are appropriate only for certain strains of the pathogen and are difficult and expensive to prepare. Another problem is that the media can only detect the pathogen effectively in concentrations from 10^4 cfu ml^{-1} soil sample (Jenkins *et al.*, 1967; Nesmith and Jenkins, 1979).

Serological techniques can also be used to detect the pathogen. Jenkins *et al.* (1967) prepared antiserum in rabbits against *R. solanacearum* and was able to detect pathogen concentrations of 10^4 cfu ml^{-1} per soil sample within three days. Although they were able to detect the pathogen, their technique did not distinguish between live and dead bacterial cells. Other immunological techniques have been developed which include immunofluorescent (IF) antibody staining and enzyme linked immunosorbent assays (ELISA) (Seal, 1998). According to Elphinstone *et al.* (1998) the IF technique can detect pathogen concentrations of 10^4 cfu ml^{-1} per sample. The ELISA kit developed by Prof. D.U. Bellstedt, Department of Biochemistry, University of Stellenbosch and used by Potatoes South Africa in their certification program, effectively detects pathogen concentrations of 10^3 cfu ml^{-1} of sample (Bellstedt and van der Merwe, 1989). Although the use of these techniques can result in detection of lower concentrations than selective media, they are expensive and not suitable for frequent use in developing countries where resources are limited (Seal, 1994; Seal and Elphinstone, 1994).

The ideal diagnostic test required for the detection of *R. solanacearum* should be rapid, specific and able to detect low bacterial concentrations in a soil sample. It should also be easy to apply, affordable and not dependable on specialised equipment. The primary objective of this study was therefore to develop an alternative method for the detection of *R. solanacearum* in soil samples. The efficacy and affordability of this technique were evaluated and compared to selective media and ELISA techniques currently used in South Africa.

2. Materials and Methods

2.1. Plant Material

Tissue culture potato plantlets, cultivar Buffelspoort, were obtained from the Department of Plant Production and Soil Science, University of Pretoria. The plantlets were multiplied and maintained on medium as described by Joerdens-Roettger (1987). Plants were kept in a growth room at 18 to 24°C with a 12 h light/dark cycle for four to maximum six weeks depending on root development.

2.2. Cultures

A.E. Swanepoel of the Vegetable and Ornamental Plant Research Institute, Roodeplaat and Mr. A.N. Hall of the Department of Microbiology and Plant Pathology, University of Pretoria, supplied the original collection of R. solanacearum isolates (Appendix 1). Stock cultures were stored in McCartney bottles at room temperature in sterile distilled water. For this study the South African isolate 111 (biovar 2) was randomly selected. To prepare fresh inoculum, stock suspensions were streaked out on Kelman's TZC agar medium containing 2,3,5-triphenyltetrazolium chloride and incubated at 28°C for 72 h (Kelman, 1954; Appendix 2). After incubation, virulent colonies (irregularly round, white fluidal colonies with pink, typically comma-shaped centres) were picked up with an inoculation loop and suspended in sterile distilled water. The viable total concentration was determined using the spread plate technique. Dilution series were prepared, plated out on TZC medium, incubated for 72 h at 28°C, colonies counted and concentrations adjusted using sterile distilled water. This set of procedures was used throughout all experiments where fresh inoculum was required.

2.3. Suspension Preparation, Inoculation and Evaluation

2.3.1. Evaluation of Pathogen Growth in Different Enrichment Broths

Different enrichment broths were prepared based on Kelman's TZC medium (Kelman, 1954) and those described by Graham and Lloyd (1979) and Granada and Sequeira (1983). The medium of Graham and Lloyd (1979) was modified by replacing 0.00005% (w/v) actidione with 0.01% (w/v) polymixin B sulphate and was labelled SMSA (abbreviation derived from "selective media from South Africa") (Engelbrecht, 1994; Appendix 2). The medium described by Granada and Sequeira (1983) was modified by using 0.00005% (w/v) penicillin G potassium salt, 0.0025% (w/v) bacitracin, 0.0005% (w/v) chloramphenicol and 0.01% (w/v) polymixin B sulphate together with 0.0005% (w/v) crystal violet and was labelled modified TZC (M-TZC) (Appendix 2). The agar component of the media was omitted and either sterile distilled water or sterile standard nutrient solution (Hydro Grow, Ocean Agriculture, Johannesburg) was used to prepare the different enrichment broths. The enrichment broths were autoclaved and allowed to cool down. Sterile tissue culture test tubes, containing 9 ml of each solution were inoculated with 1 ml

inoculum (1 x 10^6 cfu ml^{-1}) and incubated for two weeks at room temperature. Each treatment (solution) was represented by fifteen replicates. Every three days, three replicates were removed from each treatment and used to calculate the pathogen concentration. Dilution series were prepared from each replicate, plated out on TZC medium and concentrations calculated using the spread plate technique. After a set of tubes was plated out, it was discarded and not used for further evaluation. After two weeks the average cell concentration per treatment was calculated and expressed as $\log_{10}$. The inoculated sterile water treatment acted as control.

2.3.2. Evaluation of Host-Pathogen Interactions in Different Enrichment Broths

Tissue culture plants were removed from their tubes and rinsed in sterile distilled water to dislodge remaining pieces of medium. A few roots were cut to facilitate pathogen entry and plants were placed into tissue culture test tubes containing 9 ml of one of the enrichment broths and inoculated with 1 ml inoculum (2 x 10^6 cfu ml^{-1}). These test tubes were incubated at 20 ± 2°C with a 12 h light/dark cycle for three weeks. Each treatment consisted of twelve replicates. Every week, four plants were removed and surfaced sterilised, using a 1% sodium hypochlorite solution. After 30 sec, plants were rinsed three times in sterile distilled water and placed in sterile Petri dishes containing 9 ml sterile Ringers solution. Plants were cut into small pieces, allowed to diffuse and after 15 min a dilution series was prepared from each Petri dish. Samples were plated out on TZC medium, incubated at 28°C for 72 h and evaluated for presence of *R. solanacearum* colonies. Each treatment also included three control plants, placed in the different enrichment broths without adding the pathogen. The experiment was repeated to confirm results.

2.3.3. Evaluation of Host-Pathogen Interactions in Different Enrichment Broths Containing Soil

The procedures described in 2.3.2 were repeated but this time soil was added to the different enrichment broths. Soil solutions were prepared by adding 100 g of red, clay-loam soil, collected at the University of Pretoria's experimental farm (previously tested negative for *R. solanacearum* by L. Stander), to 900 ml of each growth medium described under 2.3.1. The solutions were agitated vigorously on a mechanical shaker for 30 min, allowed to settle for 2 min and filtered through a Whatman no.1 filter to remove insoluble material. The prepared soil solutions were then used with tissue culture plants as described in 2.3.2.

2.4. Comparison Between the Trapping Technique and Procedures Used in South Africa for the Detection of *R. solanacearum* in Soil Samples

2.4.1. Lowest Pathogen Concentration Detectable

Soil suspensions were prepared by adding 100 g pathogen free soil to 900 ml sterile distilled water (Refer to 2.3.3). Inoculum was prepared and the concentration calculated at 3 x 10^6 cfu ml^{-1} using the spread plate technique. A dilution series for dilution factors 10^{-1} to 10^5 was prepared using sterile distilled water. Each treatment-concentration tested consisted of five replicates.

Three different approaches were selected to determine the detectibility of *R. solanacearum* at various concentrations. The different techniques were repeated to validate results.

a) Trapping Technique

The procedures described in 2.3.2 and 2.3.3 were repeated, except that only sterile distilled water was used to prepare soil solutions and four to five plants were placed together in sterile tissue culture bottles. Each bottle contained 45 ml soil solution and 5 ml inoculum from each concentration. Final concentrations were between 3 x 10^5 and 3 x 10^{-1} cfu ml^{-1} and each concentration was represented by four replicates (bottles). Due to the large number of individual plants, plants from each replicate were pooled together and tissue isolations made after seven days incubation.

b) Selective Media

Standard TZC, M-TZC and SMSA agar media were prepared (Appendix 2). Dilution series were prepared from each inoculum concentration and 100µl plated out in duplicate on the different media and incubated at 28°C. After 72 h cultures were evaluated for presence of *R. solanacearum* colonies.

c) ELISA

Aliquots from each inoculum concentration were sent to Potatoes South Africa, Seekoegat-laboratory for analyses, using the commercial ELISA kit to detect *R. solanacearum* (Bellstedt and van der Merwe, 1989). A multi scan spectrophotometer was used to analyse the samples at 405 nm. The cut-off value for positive results was an absorbance of 0.15 and lower readings were regarded as negative results (Pers. Comm. N.J.J. Mienie, Potatoes South Africa, Seekoegat).

2.4.2. Economic Comparison

Costs of the chemicals required for the different techniques were obtained and the average cost per treatment calculated in order to give a relative indication of the affordability of each technique. Quotes were obtained from Sigma-Aldrich, Merck Laboratory Supplies and Labretoria. Costs of glassware, general appliances and specialised equipment such as the multi scan spectrophotometer were not taken into account.

2.4.3. Detection from Soil Samples

a) Soil Sampling

Soil samples were taken from a potato field (20 m x 3 m) with a well-documented history of bacterial wilt from the experimental farm at the University of Pretoria (Stander, 2001). Soil sampling was done based on the method described by Pradhanang (1999). A soil auger was used and 14 samples were taken randomly. The top 10 cm soil was discarded and the 10 cm below that was collected and mixed together. A 4 mm mesh screen was used to remove large soil particles and insoluble material. Five, 100 g sub-samples were taken from the sieved soil and dissolved in 900 ml sterile distilled water. The samples were agitated for 30 min on a mechanical shaker and allowed to settle for 2 min and filtered through a Whatman no.1 filter to remove insoluble material.

b) Comparison Between Different Techniques

The techniques described in 2.4.1 were repeated, using suspensions prepared from the soil samples collected in 2.4.3a. Five replicates were made from each sub-sample resulting in 25 isolations per treatment.

3. Results

3.1. Suspension Preparation, Inoculation and Evaluation

3.1.1. Evaluation of Pathogen Growth in Different Enrichment Broths

Ralstonia solanacearum could effectively grow in all media tested for enrichment (Fig.1). Inoculation in the two TZC solutions, both the SMSA solutions and the M-TZC solution prepared with sterile distilled water, resulted in an increase in pathogen concentrations between 10^{10} and 10^{13} cfu ml^{-1} after two weeks. The cell concentrations in the distilled water, nutrient solution and the M-TZC solution prepared with distilled water, remained in the range of 10^{6} to 10^{7} cfu ml^{-1}.

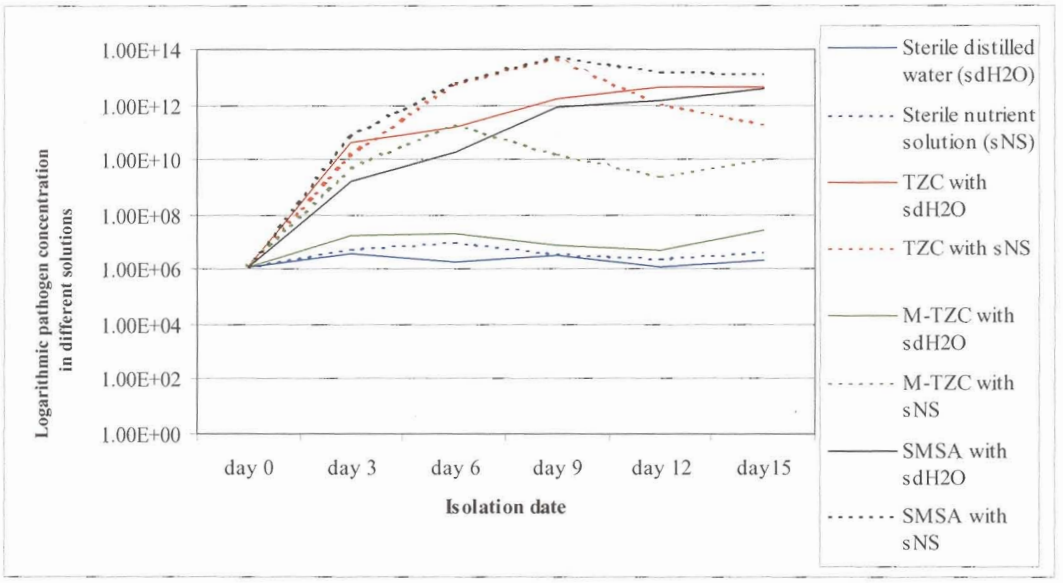

Figure 1. Evaluation of *Ralstonia solanacearum* growth in different enrichment broths expressed as Log_{10} cfu ml^{-1}

3.1.2. Evaluation of Host-Pathogen Interactions in Different Enrichment Broths and Different Enrichment Broths Containing Soil

Positive isolations were made from all plants maintained in distilled water, nutrient solution and TZC solution prepared with distilled water (Table 1). These plants did not disintegrate and only started to turn yellow after three weeks. The majority of plants in the TZC solution prepared with sterile nutrient solution, both the SMSA solutions and the M-TZC solutions prepared with sterile distilled water and sterile nutrient solution disintegrated after two to three weeks and it was difficult to make tissue isolations. None of the plants showed typical wilt symptoms, but the majority of leaves turned light yellow after a week or two and older leaves started to drop, especially in the modified TZC, M-TZC and SMSA solutions.

When soil was added to each solution, positive isolations of *R. solanacearum* could only be made from plantlets maintained in distilled water and nutrient solution. One to three positive isolations could be made from plants in the TZC solution prepared with distilled water and both of the SMSA solutions but no positive isolations could be made from plants in the TZC solution prepared with nutrient solution and the M-TZC solutions. Disintegration of plants in the modified enrichment broths started after one or two weeks and it was difficult to make tissue isolations (Table 2).

3.2. Comparison Between the Trapping Technique and Procedures Used in South Africa for the Detection of *R. solanacearum* in Soil Samples

3.2.1. Lowest Pathogen Concentration Detectable and Economic Comparison Between Different Techniques

Results of the lowest concentration detectable as well as the time needed for results to develop are presented in Table 3 and the general costs involved are presented in Table 4. The trapping technique could detect the lowest pathogen concentration (10^1 cfu ml^{-1}) but needed ten days to produce results. It was also more expensive than the selective media, but was more affordable than the ELISA. Although the other techniques only needed three days to produce results, it

could only detect the pathogen from concentrations higher than 10^3 (M-TZC and SMSA) and 10^4 cfu ml^{-1} (TZC).

3.2.2. Detection from Soil Samples

Although the trapping technique, selective media and the ELISA kit were used for the detection of *R. solanacearum*, no positive detection could be made.

Table 1 Comparison of different enrichment broths to enable successful entrapment of *Ralstonia solanacearum* in potato tissue culture plantlets

Enrichment broth	Tissue isolations[a]											
	Week 1				Week 2				Week 3			
	1	2	3	4	1	2	3	4	1	2	3	4
Sterile distilled water (sdH_2O)	+	+	+	+	+	+	+	+	+	+	+	+
Sterile nutrient solution (sNS)	+	+	+	+	+	+	+	+	+	+	+	+
TZC prepared with sdH_2O	+	+	+	+	+	+	+	+	+	+	+	+
TZC prepared with sNS	+	+	+	+	+	-	-	-	+	+	-	-
M-TZC prepared with sdH_2O	+	+	+	-	+	-	-	-	+	-	-	-
M-TZC prepared with sNS	+	+	+	-	+	-	-	-	+	-	-	-
SMSA prepared with sdH_2O	+	+	+	-	+	+	-	-	+	-	-	-
SMSA prepared with sNS	+	+	+	+	+	-	-	-	+	-	-	-

[a] Positive detection of *R. solanacearum* indicated with (+) and absence with (-)

Table 2 Comparison of different enrichment broths added to soil solutions to enable successful entrapment of *Ralstonia solanacearum* in potato tissue culture plantlets

Enrichment broth	Tissue isolations[a]											
	Week 1				Week 2				Week 3			
	1	2	3	4	1	2	3	4	1	2	3	4
Sterile distilled water (sdH_2O)	-	+	+	+	+	+	+	+	+	+	+	+
Sterile nutrient solution (sNS)	-	+	+	+	-	+	+	+	+	+	+	+
TZC prepared with sdH_2O		-	-	-	-	-	-	-	-	-	-	+
TZC prepared with sNS		-	-	-	-	-	-	-	-	-	-	-
M-TZC prepared with sdH_2O	-	-	-	-	-	-	-	-	-	-	-	-
M-TZC prepared with sNS		-	-	-	-	-	-	-	-	-	-	-
SMSA prepared with sdH_2O	-	-	-	+	-	-	-	-	-	-	-	+
SMSA prepared with sNS	-	-	+	+	-	-	-	+	-	-	-	-

[a] Positive detection of *R. solanacearum* indicated with (+) and absence with (-)

Table 3 Comparison of different isolation techniques to evaluate the successful detection of *Ralstonia solanacearum* concentrations in soil solutions and the rapidity of each technique

Technique	Inoculum concentration (cfu ml^{-1})[a]							Days required for results
	10^5	10^4	10^3	10^2	10^1	10^0	10^{-1}	
Tissue culture plants	+	+	+	+	+	-	-	10
TZC medium	+	+	-	-	-	-	-	3
M-TZC medium	+	+	+	-	-	-	-	3
SMSA medium	+	+	+	-	-	-	-	3
ELISA	+	+	-	-	-	-	-	3

[a] Positive detection of *R. solanacearum* indicated with (+) and absence with (-)

Table 4 Comparison of general costs involved with the different techniques used for the detection of *Ralstonia solanacearum* in soil solutions

Technique	Cost (Rand) in 2001	General remarks
Tissue culture plants	R 352.94	Cost include purchase price of 100 Buffelspoort tissue culture plants, one 24 cm, no.1 Whatman filter paper and 1 *l* TZC medium
TZC medium	R 51.82	Costs calculated for 1 *l* medium
M-TZC medium	R 106. 82	Costs calculated for 1 *l* medium
SMSA medium	R106.79	Costs calculated for 1 *l* medium
ELISA	R 750.00	Purchase price of one ELISA kit. A multi scan spectrophotometer is also needed

4. Discussion

Initially modified enrichment broths, based on commercial selective media were used to enrich *R. solanacearum* concentrations. Although these enrichment broths were able to increase pathogen concentrations under sterile conditions, it was unsuccessful when soil was added to the system. This was most likely due to the proliferation of other soil microorganisms. These organisms infected the plants and caused them to disintegrate before successful tissue isolations could be made. It was therefore decided to only use sterile distilled water for the preparation of soil suspensions. Since this solution was unable to increase pathogen concentrations, only concentrations from 10^1 cfu ml^{-1} and higher could be detected. With the selective media we were able to detect concentrations of 10^3 cfu ml^{-1} (M-TZC and SMSA) and 10^4 cfu ml^{-1} (TZC) while the ELISA proved effective from 10^4 cfu ml^{-1}. Although the selective media and ELISA methods produced results within three days, the ELISA technique was expensive and required specialised equipment such as the multi scan spectrophotometer. The selective media were the most affordable and although less sensitive for the detection of low pathogen concentrations, could be the best method to use in developing countries with limited resources. The trapping technique could effectively be used as an alternative method for the detection of *R. solanacearum* in artificially inoculated soil samples but should not replace the traditional selective media. Another possible application of the tissue culture technique could be the revival of old cultures. One of the characteristics of *R. solanacearum* is the rapid loss of pathogenicity once it is maintained in culture (Kelman, 1953). Previously scientists had to stem-inoculate mature susceptible host

plants with cell suspensions and re-isolate the pathogen after symptom development to maintain virulence (Kelman and Sequeira, 1965). The tissue culture plants were able to take up the pathogen within a week and based on colony morphology, virulent colonies could successfully be re-isolated (Kelman, 1953). One of the disadvantages of this technique is that it requires a continuous supply of tissue culture potato plants. Where plants are not readily available or facilities do not permit own *in vitro* multiplication of potato plants, scientists will have to use traditional methods for detection and revival of old cultures.

Ralstonia solanacearum could not be detected in the soil samples from an infected potato field. This could probably be due to incorrect sampling since Jenkins *et al.* (1967), Elphinstone (1993) and Pradhanang (1999) reported similar problems with soil sampling. According to them, it was difficult to obtain adequate dispersal of soil and bacteria. Smaller samples (2 g) should have been taken and more sub-samples prepared (Pradhanang, 1999). Bacteria do not to exist as individual cells and colonies are enveloped in mucilage (Jenkins *et al.*, 1967). According to Lloyd (1978) these enveloped colonies could act as a sheltered site where the bacteria survive between successive plantings of a susceptible crop. Since a 4 mm mesh screen was used to sieve the soil samples, many of the aggregates could have been removed, resulting in a lower pathogen concentration. Graham and Lloyd (1979) reported that the pathogen could be detected at all soil depths between 15 and 75 cm but not in the 0 to 15 cm zone, most probably due to desiccation during dry weather prior to soil sampling. This could explain why *R. solanacearum* was not detected. Since the soil was extremely dry and was undisturbed for six months since the last potato crop, the pathogen could have been present in deeper soil layers and samples should have been taken deeper. According to Elphinstone (1993) sampling depths should be between 20 and 30 cm. Before collecting soil samples, field plots should be ploughed to distribute any inoculum present and samples taken from a depth of 25 cm (Pradhanang, 1999). Due to practical limitations, this could not be done at the time of sampling.

Although many different methods exist for the detection of *R. solanacearum*, and the trapping technique could possibly be used as an alternative method for the detection of the pathogen in artificially inoculated soil suspensions, soil sampling remains a problem. Another way to overcome the difficulty of soil sampling is to use indicator plants in suspected fields (Jenkins *et al.*, 1967; Graham and Lloyd, 1978). Although this method takes longer to obtain results, it is cheaper and easier to apply and could possibly be used to give an indication of the presence of *R. solanacearum* in a field.

This study showed the successful development of an alternative approach to isolate *R. solanacearum* from artificially inoculated soil samples. The trapping technique proved highly sensitive but not rapid enough for commercial use and cannot replace selective media and the ELISA technique.

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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.

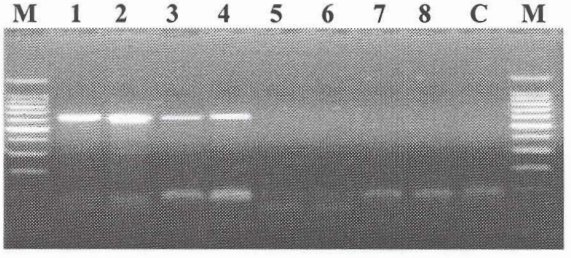

Figure 1. Agarose gel electrophoresis of ERIC-PCR products from DNA of *Ralstonia solanacearum* isolates. M = 100 bp DNA molecular weight marker. Lanes 1-4 represent biovar 3 isolates (SAP117, SAP93, SAP6 and NB346). Lanes 5-8 represent biovar 2 isolates (SAP111, SAP92, SAP7 and Z23). C = water control.

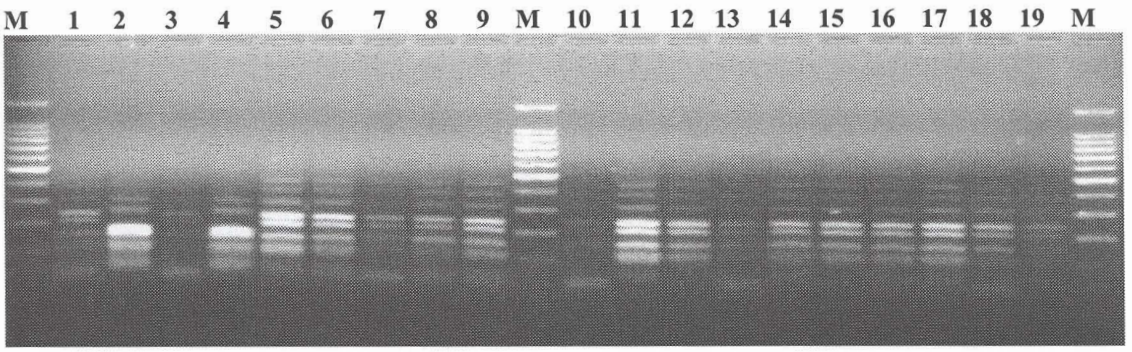

Figure 2.1. Restriction enzyme (*Sau*3A) digestion patterns of *Ralstonia solanacearum* RISA-PCR products. M = 100 bp DNA molecular weight marker. Lane 1: SAP1, Lane 2: SAP6, Lane 3: SAP111, Lane 4: SAP93, Lane 5: SAP23, Lane 6: SAP3, Lane 7: SAP46, Lane 8: SAP62, Lane 9: SAP19, Lane 10: SAP2, Lane 11: 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.

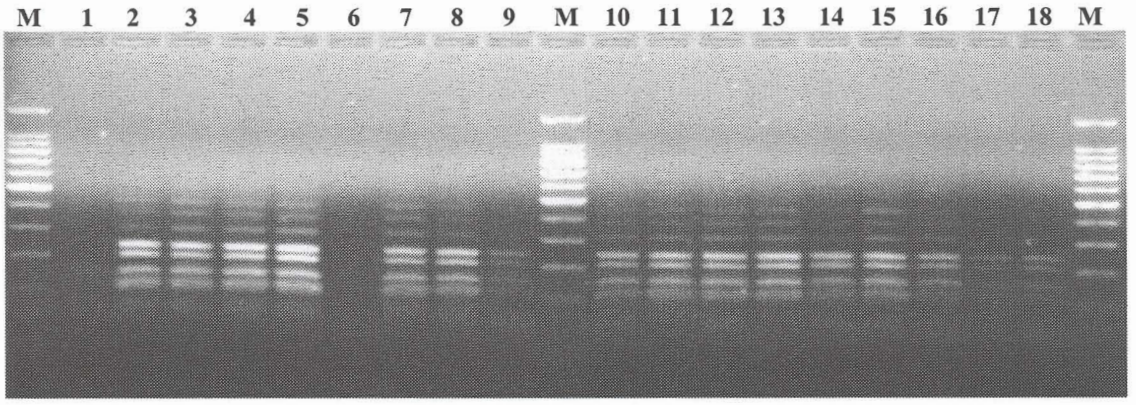

Figure 2.2. Restriction enzyme (*Sau*3*A*) digestion patterns of *Ralstonia solanacearum* RISA-PCR products. M = 100 bp DNA molecular weight marker. Lane 1: SAP117, Lane 2: SAP27, Lane 3: SAP26, Lane 4: SAP76, Lane 5: SAP17, Lane 6: SAP25 Lane 7: SA1, Lane 8: SA3, Lane 9: SA20, Lane 10: SA2, Lane 11: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

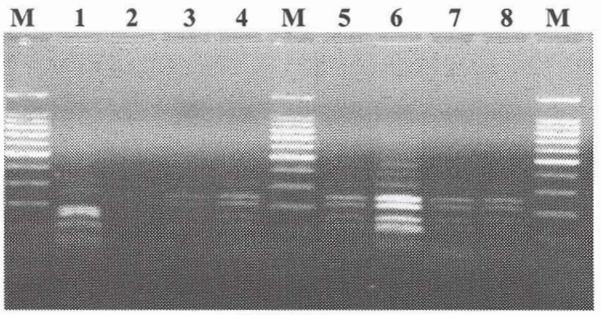

Figure 2.3. Restriction enzyme (*Sau*3A) digestion patterns of *Ralstonia solanacearum* RISA-PCR products. M = 100 bp DNA molecular weight marker. Lane 1: NB346, Lane 2: NB980221 (not enough PCR product obtained, excluded from further comparison), Lane 3: NK15, Lane 4: D109, Lane 5: PB1-2, Lane 6: TB40,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

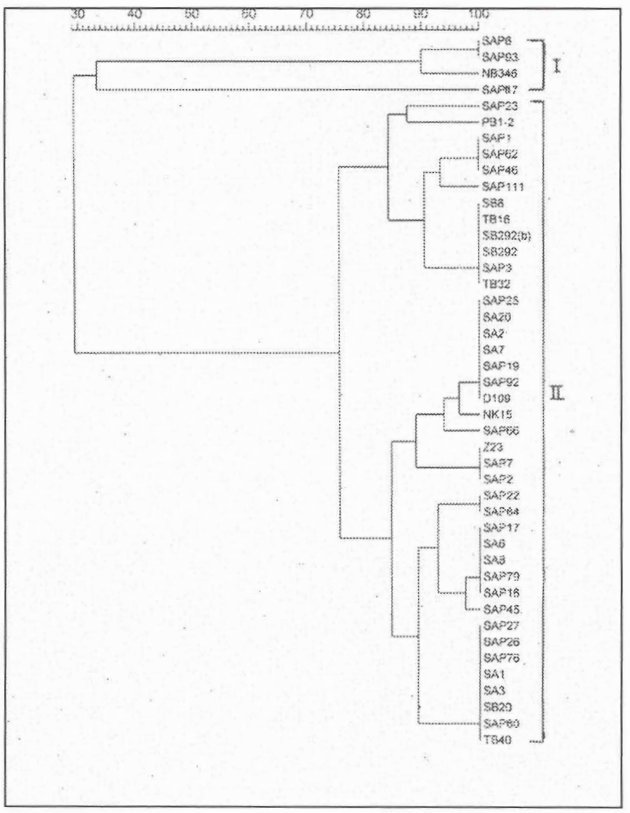

Figure 3. Dendogram showing the grouping of representative RFLP band profiles of *Ralstonia solanacearum* isolates using the unweighted pair group of arithmetic averages method.

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.

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CHAPTER 5

SUPPRESSION OF *RALSTONIA SOLANACEARUM* IN SOIL USING HERBAL PLANT MATERIAL

ABSTRACT

Due to its extremely wide host range and its ability to survive for prolonged periods in the soil, control of *R. solanacearum* is complicated. No single method exists for the control of this pathogen and different options should be considered in an integrated program to effectively control bacterial wilt. Due to the detrimental effect of pesticides on human health and the environment, agricultural industries have been forced to consider alternative disease control strategies that is safer and more in line with natural farming systems. One such option is the use of natural plants or microbes to prevent disease development. One component of biological control is the incorporation of plant material into soil, providing a biofumigation effect to control soilborne pathogens. Mustard (*Brassica juncea* L.) and other cruciferous species have previously been shown to be effective in reducing *R. solanacearum* populations in soil. In this study, thirteen herbal plant species were evaluated as biofumigation agents to suppress *R. solanacearum* in soil. The capability of these species acting as host plants for *R. solanacearum* (biovar 2 and 3) was also determined. Crude extracts were prepared from non-host species and tested *in vitro* for possible inhibition of the pathogen. Plant material from non-host species was incorporated into artificially inoculated soil and a glasshouse pot trail was conducted to determine whether or not *R. solanacearum* could be suppressed in soil. Marjoram, nasturtium and parsley acted as hosts for *R. solanacearum* (biovar 2), while chive, coriander, marjoram, mustard, nasturtium and parsley were hosts for *R. solanacearum* (biovar 3). Crude extracts from non-host species were unable to suppress the pathogen *in vitro*. Difficulties were experienced with soil inoculation and biofumigation could not be applied successfully.

1. Introduction

Once *Ralstonia solanacearum* has been detected and identified in a field, various control measures can be implemented to exclude the pathogen from further spread and infection to subsequent or alternate crops. Some of these methods include; crop rotation and intercropping with non-host plant species (Hayward, 1991; Hartman and Elphinstone, 1994), soil amendments with organic and inorganic material (Chellemi *et al.*, 1992; Hartman and Elphinstone, 1994), using resistant potato cultivars (Tung *et al.*, 1990), chemical control (Shekhawat *et al.*, 1992) and biological control (Hartman *et al.*, 1993).

Due to its wide host range (Kelman, 1953; Buddenhagen and Kelman, 1964; Hayward, 2000) and ability to survive for prolonged periods in the soil (McCarter, 1976; Graham *et al.*, 1979), control of *R. solanacearum* is complicated and several methods should be combined in an integrated program to effectively manage the disease. The primary objective of an integrated control strategy would be to limit the survival and multiplication of the pathogen and eliminate alternative hosts and interacting nematodes by means of cost-effective programs (Elphinstone and Aley, 1993). The effectiveness of any integrated approach will depend on factors such as climatic conditions, soil type, pathogen strain present, farming system as well as socio-economic situation of a specific area.

Interest in the field of biological control as an alternative method for the control of weeds, insects and plant pathogens has grown due to the global move towards more environmental friendly and natural farming systems (Lydon and Duke, 1989; Lampkin, 1990). Traditionally biological control has been defined as the total or partial destruction of plant pathogen populations by other microorganisms (Agrios, 1997). However, plant material or extracts can also be used for the suppression of pathogens and are also classified as biological control (Lampkin, 1990). Plant material has been used successfully as green manure by incorporating it into the soil for use as a biofumigant to control soilborne diseases (Akiew *et al.*, 1996). Interest in the use of biofumigation has particularly increased recently due to the prohibition of certain synthetic soil fumigants such as methyl bromide (Kirkegraad *et al.*, 1998). According to Kirkegraad and Sarwar (1998), biofumigation refers to the suppression of soilborne pests and pathogens by biocidal compounds released in soil when certain plant material is broken down. During the decomposition of plant residues, substances such as glucosinolates can be hydrolysed to release a range of volatile compounds known as isothiocyanates and other secondary compounds such as

oxazolidinethiones, nitriles and thiocyanates (Akiew *et al.*, 1996; Kirkegraad and Sarwar, 1998). The type of isothiocyanate released is specific to the type of glucosinolate present in the tissue. Isothiocyanates are the same class of chemical compounds produced by the decomposition of metham sodium (vapam), a commercial soil fumigant (Akiew *et al.*, 1996). Since some of the substances released by biofumigation have broad biocidal activity, including insecticidal, nematicidal, fungicidal, antibiotic and phytotoxic effects, it can be used as part of an integrated control program to suppress soilborne pathogens (Kirkegraad and Sarwar, 1998).

Akiew *et al.* (1996) incorporated mustard (*Brassica juncea* L.) into *R. solanacearum* infested soil and were able to suppress the pathogen population to an undetectable level after a four to five week incubation period. Kirkegraad and Sarwar (1998) were also able to successfully suppress pathogen populations with the incorporation of cruciferous green manures.

The medicinal and antimicrobial properties of herbal plant species have been recognised since before 2000 B.C. (Craker *et al*, 1986; Roberts, 1999). Various studies have since been conducted on the medicinal use and antimicrobial properties of herbs. Thanassoulopous *et al.* (1997) used essential oils from different herbs for biological control of *Botrytis cinerea.* It was therefore decided to conclude this study by evaluating various readily available, fast growing herbal plant species as green manure for biofumigation properties to suppress *R. solanacearum* in artificially inoculated soils.

2. Materials and Methods

2.1. Cultures

For the first part of this study the South African isolates, 111 (biovar 2) and 117 (biovar 3) were randomly selected (Appendix 1). For the subsequent biofumigation studies only isolate 111 (biovar 2) was used. To prepare fresh inoculum the techniques described in Chapter 3 were used.

2.2. Plant Material

Thirteen herbal species were selected for this study: *Ocimum basilicum* L. (basil), *Borago officinalis* L. (borage), *Apium graveolens* L. (celery), *Matricaria recutita* L. (chamomile), *Foeniculum vulgare* Mill. (fennel), *Melissa officinalis* L. (lemon balm), *Anethum graveolens* Linn. (dill), *Allium tuberosum* Rottler (chive), *Coriandrum sativum* L. (coriander), *Origanum majorana* L. (marjoram), *Brassica alba* L. (mustard), *Tropaeolum majus* L. (nasturtium) and *Pertoselinum crispum* L. (parsley). These species were selected according to availability, rapid germination and the production of large amounts of plant material within a relatively short period. Commercial seed was bought from a local nursery and germinated in seedling trays containing 2:1 sand-peat mixture. Seedling trays were placed in a dark growth cabinet at 20°C until seedlings were 2 to 3 cm long. Trays were then moved to a glasshouse where seedlings were hardened off for three to four weeks at 25/20°C day/night temperatures.

2.3. Host Determination

After four weeks, plants were transplanted in 2 *l* pots containing 2 kg of a sterile 1:1 clay-bark mixture (v/v). Sterile vermiculite was soaked in inoculum of either isolate 111 (biovar 2) or 117 (biovar 3) at 1 x 10^6 cfu ml^{-1} for 2 h (1:2 m/v) where after 40 g vermiculite was added to each pot and mixed with the top third of the soil. Pots were thoroughly watered and plants evaluated for symptom development each week for eight consecutive weeks. Each treatment (herbal plant species) consisted of six pots inoculated with isolate 111 (biovar 2) and six pots inoculated with isolate 117 (biovar 3).

Each plant was tested individually for the presence of the pathogen according to the technique described by Swanepoel (1992). Plants were removed from the pots and the roots and bottom 10 cm of the stems washed in tap water to remove excess soil. This material was surface sterilised in 1% sodium hypochlorite for five minutes and then rinsed three times in sterile distilled water. Prepared plant material was placed into sterile Petri dishes containing 9 ml sterile Ringers solution and cut into fine pieces with a sterile scalpel. After 30 minutes diffusion time a dilution series were prepared, plated out on TZC medium (Kelman, 1954), incubated and evaluated for the presence of *R. solanacearum* according to the methods described in Chapter 3.

2.4. *In Vitro* Tests with Crude Extracts

The basil, borage, celery, chamomile, fennel, lemon balm and dill were selected for *in vitro* testing of crude extracts from the original 13 species based on their non-host characteristics. Crude extracts were prepared according to the method described by Terblanche and de Villiers (1998). Plant material (roots, stems and leaves) was removed, surface sterilised in a 1% sodium hypochlorite solution for five minutes and then rinsed three times in sterile distilled water. Crude extracts were prepared by homogenising plant material in sterile distilled water (1:1 v/v). Seeded TZC agar plates were prepared by pouring an additional thin layer of cool TZC medium, to which *R. solanacearum* inoculum (1 x 10^4 cfu ml^{-1}) was added, to standard TZC agar plates (Stander, 2001). Four wells were punched in each agar plate using a sterile cork bore and each well was filled with 100µl suspension. Treatments (suspensions) included a 1% sodium hypochlorite solution and sterile water that acted as controls, crude extracts and sterile extracts prepared from the selected herbal plant species. Crude extracts were sterilised with the aid of a Millipore filter. Each treatment was repeated three times. Plates were incubated at 28°C and formation of inhibition zones evaluated and measured after 72 h.

2.5. Soil Suppression (a)

Seed from the seven species selected in 2.4. was sown and seedlings raised according to the method described in 2.2. After four weeks, six plants from each species were transplanted in 2 *l* pots containing 2 kg sterile 1:1 clay-bark mixture (v/v) and kept in a glasshouse for six to eight weeks. Plants from each species were removed, rinsed and the whole plant cut into 1 to 2 cm pieces.

Class 1, BP1 potato tubers (generation 1) were taken from cold storage 48 h prior to planting and 50 to 69 g tubers selected. A 2:1 clay-bark soil mixture (v/v) was steam sterilised, added to 1 part non-sterilised bark, thoroughly mixed and 2 kg mixture weighed off into 2 *l* pots. Each treatment was divided into two parts and consisted of five replicates. For part 1, vermiculite was soaked in biovar 2 inoculum (refer to 2.3) and together with 20 g herbal material, were added to each pot and thoroughly mixed. For part 2, the vermiculite was omitted and only 20 g of cut plant material was added to each pot and thoroughly mixed. Control treatments were also included where herbal plant material was omitted. Tubers were planted 10 cm deep, pots were placed in a

glasshouse and watered regularly. Glasshouse temperatures ranged between 10 to 12°C min and 20 to 22°C max (winter temperatures). After emergence, potato plants were weekly evaluated for typical wilt symptoms.

After six weeks, plants were removed and tissue isolations made on TZC medium from the bottom 10 cm of stems to determine the presence of *R. solanacearum* (refer to 2.3). Tubers were harvested, weighed and cut open to evaluate possible ring symptoms in the vascular ring (Harrison, 1961). Developing tubers smaller than 1 cm were discarded. The average amount of tubers per plant, average mass of tubers per plant and average mass per tuber were statistically analysed. Variance of analysis to separate means were carried out followed by Duncan's multiple range test used at the 5% significance level to determine the possible effect of incorporated plant material on tuber production (SAS Institute, 1999).

2.6. Stem Inoculation

To regain possible loss of virulence, *R. solanacearum* isolate 111 (biovar 2) was inoculated into potato plants and re-isolated according to the technique described by Kelman and Sequeira (1965). Mature potato plants were inoculated by piercing the stem with a sterile needle through a drop of bacterial suspension (10^7 cfu ml^{-1}) placed on a leaf axil at the third node below the stem tip. After inoculation plants were transferred to a glasshouse and temperatures maintained between 25 and 30°C. After first symptoms of wilt appeared, plants were removed, tissue isolations prepared and the pathogen re-isolated on TZC medium.

2.7. Soil Suppression (b)

Soil inoculation was repeated but instead of soaked vermiculite, 50 ml isolate 111 inoculum (1 x 10^5 cfu ml^{-1}) was added to each pot after tubers were planted (Martin and Nydegger, 1982). Isolate 111 inoculum (1 x 10^6 cfu ml^{-1}) was again added to each pot after a week. Pots were placed over drip trays and watered daily. Glasshouse temperatures ranged between 18 to 20°C min and 30 to 35°C max (summer temperatures). After emergence, potato plants were weekly evaluated for typical wilt symptoms. After six weeks, plants were removed, tissue isolations made and plates evaluated for positive *R. solanacearum* growth as described in 2.5.

3. Results

3.1. Host Determination

None of the 13 selected herbal plant species developed typical wilt symptoms but the pathogen was re-isolated from several species (Table 1).

Table 1 Host determination of the different herbal plant species (results from individual replicates not included)

Herbal specie	Host determination	
	Biovar 2	Biovar 3
Ocimum basilicum L. (basil)	-	-
Borago officinalis L. (borage)	-	-
Apium graveolens L. (celery)	-	-
Matricaria recutita L. (chamomile)	-	-
Foeniculum vulgare Mill. (fennel)	-	-
Melissa officinalis L. (lemon balm)	-	-
Anethum graveolens Linn. (dill)	-	-
Allium tuberosum Rottler (chive)	-	+
Coriandrum sativum L. (coriander)	-	+
Origanum majorana L. (marjoram)	+	+
Brassica alba L. (mustard)	-	+
Tropaeolum majus L. (nasturtium)	+	+
Pertoselinum crispum L. (parsley)	+	+

3.2. *In Vitro* Tests with Crude Extracts

After incubation, *R. solanacearum* colonies developed evenly throughout the seeded TZC plates and clear zones could be measured around all wells inoculated with the 1% sodium hypochlorite solution. Although some degree of suppression was noted around wells inoculated with non-filtered crude extracts, no clear inhibition zones developed. Bacterial growth (not *R. solanacearum*) developed in and around these wells and suppression was possibly as result of these endophytes and not because of antibacterial properties of the plant extracts. No inhibition zones developed around wells inoculated with filter-sterilised crude extracts or sterile distilled water.

3.3. Soil Suppression (a)

No wilt symptoms developed during the six-week period after emergence. All tissue isolations tested negative for the presence of *R. solanacearum* and no internal symptoms could be seen within the vascular rings of cut tubers. There was no significant difference in the average amount of tubers produced per treatment, the average mass of tubers produced per treatment or the average mass per tuber of the different treatments

3.4. Stem Inoculation and Soil Suppression (b)

First wilt symptoms appeared after twelve days and *R. solanacearum* was successfully re-isolated from stem inoculated potato plants and used in the second soil inoculation trial.

No wilt symptoms developed during the six-week period after emergence. All tissue isolations tested negative for the presence of *R. solanacearum* and no internal symptoms could be seen within the vascular rings of cut tubers. Due to a very low percentage emergence (28%) no statistical analysis could be performed on the tuber production between the different treatments.

4. Discussion

Although only 13 herbal species were tested in this study, the general ability of the pathogen to survive in alternative hosts was confirmed. Biovar 3 was able to infect more herbal species than biovar 2. This coincided with previous observations that biovar 3 has a wider host range than biovar 2 (Swanepoel, 1992). Strains of *R. solanacearum* were differentiated into 5 races according to host range (Buddenhagen *et al.*, 1962; Walker and Stead, 1993). South African biovar strains could be designated to race 1 (Swanepoel and Young, 1988) that affected a wide range of host plants including solanaceous hosts, diploid bananas and a variety of non-solanaceous plants (Buddenhagen *et al.*, 1962). Biovar 2 could be designated as race 3 (Buddenhagen and Kelman, 1964). This race is restricted to potato, to a lesser extent tomato and a few solanaceous weed species (Buddenhagen *et al.*, 1962). *Origanum majorana* L. (marjoram), *Tropaeolum majus* L. (nasturtium) and *Pertoselinum crispum* L. (parsley) belonged to the

families *Labiatae*, *Tropaeolaceae* and *Umbelliferae* respectively. These results did not coincide with previous findings that biovar 2 (race 3) only infected solanaceous plants (Buddenhagen *et al.*, 1962) and should be further investigated.

No wilt symptoms developed during the six-week period after emergence, all tissue isolations tested negative for the presence of *R. solanacearum* and no internal symptoms could be seen within the vascular rings of cut tubers. From these results it could be speculated that the pathogen was unable to successfully infect the potato plants. This can be attributed to various factors such as low inoculum concentration, low soil temperatures or low soil water content (Kelman and Sequeira, 1965; Ciampi *et al.*, 1980; Chen and Echandi, 1984). Although *R. solanacearum* has the ability to survive for periods of several years under field conditions (McCarter, 1976; Graham *et al.*, 1979) its viability in soil under controlled conditions is known to be poor (Granada and Sequeira, 1983; Shekhawat and Perombelon, 1991). This was confirmed by our findings.

According to Ciampi *et al.* (1980), potatoes grown under cool conditions (12-22°C) produced no visually infected tubers and no stem infection could be detected. Swanepoel (1990) however found that BP1 potato plants could develop symptoms where temperatures were as low as 14 to 16°C. To promote rapid disease development, glasshouse temperatures were maintained at 26 to 34°C during the day and 22 to 28°C during the night (Graham *et al.*, 1979). The best growth of inoculated *R. solanacearum* took place between 30 and 35°C (Shekhawat and Perombelon, 1991) and Gallegly and Walker (1949) also found that bacterial wilt increased with an increase in temperature. When temperatures were higher during our second soil inoculations, symptoms again did not developed and it can therefore be concluded that insufficient infection was not due to unfavourable temperatures maintained in the glasshouse.

Although wounding of roots is not absolutely essential for infection (Chen and Echandi, 1984) cutting the lateral roots with a scalpel along one side of the plant to a depth of approximately 4 cm prior to soil inoculation can enhance infection (Winstead and Kelman, 1952). We decided against root cutting since uniform wounding could not be ensured. However, since no apparent infection occurred in this study, wounding should be considered for future studies.

Kelman and Sequeira (1952) observed that where wounding did not occur, infection could only take place if inoculum concentration were higher than 10^4 to 10^5 cfu ml^{-1} soil sample. They did however not specify whether or not these concentrations were the initial inoculum concentrations

of the final concentrations after soil inoculation. Chen and Echandi (1984) found that there was a direct correlation between the inoculum concentration and the number of cells that adhered to roots. They used inoculum concentrations of 10^6 to 10^7 cfu g^{-1} of soil and concluded that wilt severity increased as inoculum concentration increased. Although inoculum concentrations of 10^5 to 10^6 cfu ml^{-1} inoculum were used in our trial, the dilution factor of the soil was not taken into account and possibly resulted in too low concentrations after soil inoculum for successful infection.

Survival of *R. solanacearum* is most efficient in wet, but well drained soils and survival is affected by soil desiccation and flooding (Kelman, 1953; Buddenhagen and Kelman, 1964). High soil moisture should be maintained for the duration of the trial (Kelman and Sequeira, 1965) but during our first soil inoculations, pots were not watered immediately after inoculation and allowed to dry out between each watering. According to Nesmith and Jenkins, 1979) highest populations of the pathogen were found in saturated (but not flooded) soils. According to them, colony counts declined rapidly in flooded or dry soil. This could explain why infection was again unsuccessful during both the soil inoculations. Soil should not have been allowed to dry out between watering during the first soil inoculations and was probably too wet during the second trial. This wet conditions probably attributed to very poor plant emergence (28%) and most tubers rotted within a week after planting.

According to Kelman (1953), *R. solanacearum* has the ability to loose its virulence under certain conditions, resulting in a reduced ability to cause wilt in host plants. This phenomenon is also referred to as phenotypic conversion (PC) and is thought to be regulated by environmental signals. Exactly what these environmental signals are and how they interact are not yet clear (Denny *et al.*, 1998).

It has been hypothesised that *R. solanacearum* normally exists as the PC-type in soil (Denny *et al.*, 1994). When conditions are suitable, the pathogen will switch to its wild type to cause infection and induce wilt symptoms in the host. Although the environment may play a role in this switching (Denny *et al.*, 1998) pathogen concentration may also play an important role (Schell, 1996). According to him, phenotypic conversion occurs when some endogenous inducer, probably 3-hydroxypalmitic acid methyl ester (3-OH PAME) exceeds a critical concentration. When pathogen concentrations in the soil are too low, sufficient 3-OH PAME cannot accumulate and the pathogen would not be able to cause wilt symptoms in its host. A much longer incubation

period will then be necessary to ensure sufficient cell multiplication and therefore high enough levels of 3-OH PAME to switch on virulence.

This could explain why infection was not successful in this study and should be taken into consideration in future studies.

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CHAPTER 6

GENERAL DISCUSSION

Ralstonia solanacearum is a diverse pathogen, capable of causing significant crop losses throughout the world on a diverse range of crops (Hayward, 2000). On potatoes, the pathogen affects both above and belowground plant parts and damage can occur in two ways; premature wilting of top growth, and rotting of tubers either in the soil or in storage (Shekhawat *et al.*, 1992). Bacterial wilt is currently not a threat for the South African potato industry due to an effective certification program (Nortje, 1997). However, due to its wide host range and ability to survive for long periods in the soil, its presence needs to be continuously monitored to prevent serious future outbreaks. It is therefore important to have techniques available for its detection, both in plant material and in soil. Various techniques such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody staining have been developed for the detection of *R. solanacearum* in plant material (Bellstedt and van der Merwe, 1989; Elphinstone *et al.*, 1998). However, none of these techniques were developed for soil systems. The primary objective of this study was therefore to development an alternative technique for the detection of *R. solanacearum* in soil systems and to compare it with techniques currently used in South Africa.

In South Africa, techniques that are commercially used for the detection of *R. solanacearum* in plant material are ELISA and selective media (Pers. Comm. N.J.J. Mienie, Potatoes South Africa, Seekoegat). These techniques were primarily developed for detection of the pathogen in plant material and not in soil systems. If used for detection of *R. solanacearum* in soil samples, the techniques are not sensitive enough to detect low pathogen concentrations (Graham and Lloyd, 1978; Nesmith and Jenkins, 1979; Seal, 1994; Seal and Elphinstone, 1994). The technique developed in this study was based on using an indicator plant system. Tissue culture potato plants were successfully used to trap the pathogen in artificially inoculated soil suspensions. This trapping technique was found to be sensitive and could detect pathogen concentrations as low as 10^1 cfu ml^{-1} soil suspension within ten days. Although the other techniques only require three days to produce results, it could only detect the pathogen at concentrations higher than 10^3 or 10^4 cfu ml^{-1} depending on the technique used. The trapping technique used in this study proved to be

a successful alternative method for the detection of *R. solanacearum*, was practical and did not require specialised equipment. It was however found to be not rapid enough for commercial use and can therefore not replace the existing ELISA and selective media diagnostic methods.

Once the pathogen has been isolated, identity needs to be confirmed. Traditionally, a binary system has been used for isolate classification (Hayward, 1991). Isolates could either be differentiated into five races according to hosts primarily affected (Buddenhagen *et al.*, 1962; Walker and Stead, 1993) or more commonly into five biovars (Hayward, 1964). Biovars can be distinguished based on the isolates' ability to utilise different carbon sources. Molecular techniques such as polymerase chain reaction (PCR) provide a fast alternative to distinguish between different organisms and it was decided to evaluate the enterobacterial repetitive intergenic consensus (ERIC)-PCRs' ability to distinguish between biovar 2 and 3 isolates. Eight South African isolates were selected and the technique was successfully used to distinguish between these two groups.

Isolates can also be characterised using molecular techniques such as restriction fragment length polymorphism (Gillings and Fahy, 1993). This classification can play an important role in the development of new control strategies since different strains have different abilities to survive (Elphinstone and Aley, 1993). Different strains also have the ability to infect different plant species and care should be taken to choose non-host plants for crop rotation (Walker and Stead, 1993; Hayward, 2000). Integrated control programs should therefore not only be adapted to suit certain climatic conditions, soil type, farming system and the socio economic situation of a specific area, but also the pathogen strain (Elphinstone and Aley, 1993; Mienie, 1997). The ribosomal intergenic spacer analysis-PCR and RFLP with *Sau*3A were used to characterise and evaluate possible variation between 44 *R. solanacearum* isolates collected throughout the potato growing areas of South Africa. These techniques could also distinguish between biovar 2 and 3 isolates but no correlation could be drawn between the different isolates and the regions from which they were isolated.

Once the pathogen has been detected and identity confirmed, a suitable control program could be implemented. Since there is no chemical registered for the control of *R. solanacearum*, herbal plant material was evaluated for its potential use in biofumigation to suppress the pathogen in soil. This biofumigation was based on the method described by Kirkegraad *et al.* (1998) but proved unsuccessful in our study. Although the pathogen could not be suppressed successfully,

new hosts for *R. solanacearum* were determined. Biovar 3 was able to infect marjoram, nasturtium, parsley, coriander, chive and mustard and biovar 2, marjoram, nasturtium and parsley.

For this study it was found that *R. solanacearum* could successfully be detected from artificially inoculated soil systems using the trapping technique. Molecular techniques such as the ERIC-PCR and RFLP with *Sau*3A could be used to distinguish between biovar 2 and 3 isolates, but could not be used to draw a correlation between the different isolates and the regions from which they were isolated. Biofumigation could not be applied successfully.

Future studies should focus on the detection of *R. solanacearum* in naturally infested soil using the trapping technique. The molecular techniques used in this study should be tested against biovars 1, 4 and 5 to determine whether or not they can distinguish between all five *R. solanacearum* biovars. Biofumigation should be re-evaluated under different glasshouse conditions to determine if herbal plant material could be used to suppress *R. solanacearum* in soil.

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SUMMARY

DETECTION, CHARACTERISATION AND SUPPRESSION OF *RALSTONIA SOLANACEARUM*

by

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The primary objective of this study was to develop an alternative method for the detection of *Ralstonia solanacearum* in soil systems. The pathogen could successfully be detected with this trapping technique. The technique was easy to apply and sensitive enough to detect pathogen concentrations of 10^1 cfu ml^{-1} soil suspension. Results could not be obtained fast enough for commercial application and the technique could therefore not replace traditional selective media and the ELISA-technique currently used in South Africa. The second objective of this study was to evaluate different molecular techniques for the identification and characterisation of different *R. solanacearum* isolates. The ERIC-PCR was used on eight biovar 2 and 3 isolates and could successfully distinguish between the two groups. The RISA-PCR and RFLP with *Sau*3A were used to characterise 44 *R. solanacearum* isolates. Although the techniques could distinguish between the two groups of biovars, it could not be used to draw a correlation between the isolates and the different regions from which they were isolated. The last objective of this study was to evaluate 13 herbal species for their potential use as biofumigation agents to suppress *R. solanacearum* in soil. Problems were experienced with soil inoculation and suppression of the pathogen could not be evaluated successfully.

OPSOMMING

OPSPORING, KARAKTERISERING EN ONDERDRUKKING VAN *RALSTONIA SOLANACEARUM*

deur

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Die primêre doel van hierdie studie was om 'n alternatiewe tegniek te ontwikkel vir die opsporing van *Ralstonia solanacearum* in grondmonsters. 'n Lokaastegniek is ontwikkel waarmee die patogeen suksesvol opgespoor kon word. Die tegniek was maklik toepasbaar en sensitief genoeg om patogeenkonsentrasies so laag as 10^1 kolonie-vormende eenhede ml^{-1} grondoplossing mee op te spoor. Dit kon egter nie vinnig genoeg resultate lewer om kommersieël aangewend te word nie en was dus nie geskik om tradisionele selektiewe media en die ELISA-tegniek te vervang wat tans in Suid-Afrika gebruik word nie. Die tweede doel van hierdie studie was om molekulêre tegnieke te ondersoek waarmee *R. solanacearum* isolate uitgeken en gekarakteriseer kon word. Die ERIC-PCR is gebruik om agt biovar 2 en 3 isolate mee te ondersoek en kon suksesvol tussen die twee groepe biovars onderskei. Die RISA-PCR en RFLP met *Sau*3A is gebruik om 44 *R. solanacearum* isolate mee te ondersoek. Hoewel die tegnieke onderskeid kon tref tussen die twee groepe biovars, kon geen korrelasie getref word tussen die isolate en die verskillende streke waaruit dit geïsoleer was nie. Die laaste doel van hierdie studie was om 13 kruiespesies te ondersoek vir moontlike gebruik in bioberoking vir die onderdrukking van *R. solanacearum* in grond. Probleme is ondervind met grondinokulasie en onderdrukking van die patogeen kon nie suksesvol ondersoek word nie.

APPENDIX 1

RALSTONIA SOLANACEARUM ISOLATES ISOLATED THROUGHOUT SOUTH AFRICA FROM INFECTED POTATOES

***Ralstonia solanacearum* isolates collected throughout the potato growing regions of South Africa**

No.	Isolate	Biovar	District	Supplier
1	SAP 1	2	Dendron	A.N. Hall + A.C. Hayward
2	SAP 6	3	Winterton	A.N. Hall + A.C. Hayward
3	SAP 111	2	Zimbabwe	A.N. Hall
4	SAP 117	3	Hlabisa	A.N. Hall
5	SAP 93	3	Ladysmith	A.N. Hall
6	SAP 23	2	Warmbad	A.N. Hall + A.C. Hayward
7	SAP 3	2	Vivo	A.N. Hall + A.C. Hayward
8	SAP 46	2	Badplaas	A.N. Hall
9	SAP 62	2	Rooiwal	A.N. Hall
10	SAP 19	2	Dendron	A.N. Hall + A.C. Hayward
11	SAP 2	2	Dendron	A.N. Hall + A.C. Hayward
12	SAP 22	2	Vivo	A.N. Hall + A.C. Hayward
13	SAP 60	2	Unknown	A.N. Hall
14	SAP 7	2	Winterton	A.N. Hall + A.C. Hayward
15	SAP 45	2	Barkly Wes	A.N. Hall
16	SAP 79	2	Douglas	A.N. Hall
17	SAP 16	2	Letsitele	A.N. Hall + A.C. Hayward
18	SAP 64	2	Unknown	A.N. Hall
19	SAP 92	2	Dendron	A.N. Hall

20	SAP 66	2	Unknown	A.N. Hall
21	SAP 27	2	Pietersburg	A.N. Hall + A.C. Hayward
22	SAP 26	2	Dendron	A.N. Hall + A.C. Hayward
23	SAP 76	2	Plooysburg	A.N. Hall
24	SAP 17	2	Piketberg	A.N. Hall + A.C. Hayward
25	SAP 25	2	Vivo	A.N. Hall + A.C. Hayward
26	SA 1	2	Clanwilliam	A.E. Swanepoel
27	SA 3	2	Worcester	A.E. Swanepoel
28	SA 20	2	Piketberg	A.E. Swanepoel
29	SA 2	2	Worcester	A.E. Swanepoel
30	SA 6	2	Piketberg	A.E. Swanepoel
31	SA 7	2	Piketberg	A.E. Swanepoel
32	SA 8	2	Piketberg	A.E. Swanepoel
33	SB 8	2	Sandveld	A.E. Swanepoel
34	SB 292(b)	?	Unknown	A.E. Swanepoel
35	SB 292	?	Unknown	A.E. Swanepoel
36	SB 20	2	Piketberg	A.E. Swanepoel
37	Z 23	2	Dendron	A.E. Swanepoel
38	NB 346	3	Bergville	A.E. Swanepoel
39	NK 15	2	Itala Valley	A.E. Swanepoel
40	D 109	2	North Cape	A.E. Swanepoel
41	PB 1-2	2	Warmbad	A.E. Swanepoel
42	TB 40	2	Davel (Ermelo)	A.E. Swanepoel
43	TB 32	2	Alberton	A.E. Swanepoel
44	TB 16	2	Middelburg	A.E. Swanepoel

APPENDIX 2

2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE MEDIUM (TZC-1 *l*)

Peptone	10g
Casein hydrolysate	1g
Glycerol	5 ml
Distilled water	975 ml
Agar bacteriological	18g
2,3,5-Triphenyltetrazolium chloride	0.05g in 25 ml distilled water

Peptone, casein and glycerol are added to the distilled water and thoroughly mixed. The agar is added separately to the bottles and autoclaved for 15 min at 121°C. The tetrazolium chloride is autoclaved for 7 min at 121°C and added to the autoclaved peptone mixture while still hot. The mixture is shaken gently and poured into Petri dishes.

MODIFIED TZC MEDIUM CONTAINING CRYSTAL VIOLET (M-TZC-1 *l*)

Peptone	10g
Casein hydrolysate	1g
Glycerol	5 ml
Crystal violet	5 mg (1 ml stock solution: 0.25 g 50 ml^{-1} distilled water)
Distilled water	975 ml
Agar bacteriological	18g
2,3,5-Triphenyltetrazolium chloride	0.05g in 25 ml distilled water

Peptone, casein, glycerol and crystal violet are added to the distilled water and thoroughly mixed. The agar is added separately to the bottles and autoclaved for 15 min at 121°C. The tetrazolium chloride is autoclaved for 7 min at 121°C and added to the autoclaved peptone mixture while still hot.

The following antibiotics are added (using filter sterilization) to the medium just before pouring (Stock solutions can also be prepared and 1 ml added to the medium):

Polymixin B sulphate	100 mg	(1 g in 10 ml distilled water)
Bacitracin	25 mg	(0.25 g in 10 ml distilled water)
Chloramphenicol	5 mg	(0.25 g in 50 ml methanol)
Penicillin G potassium salt	0.5 mg	(0.05 g in 100 ml distilled water)

SELECTIVE MEDIA FROM SOUTH AFRICA (SMSA-1 *l*)

This medium is prepared in the same way as the M-TZC medium except that no crystal violet is added.