Characterisation of fluorescent *Pseudomonas* species causing foliar diseases of tomato in South Africa

By

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DISSERTATION SUMMARY

Tomato (Solanum lycopersicum L.) is an important vegetable crop worldwide because of its nutritional benefits. In South Africa, tomatoes are produced in all provinces with Limpopo having the largest production (3600 ha) followed by Mpumalanga (800 ha) and the Eastern Cape (450 ha). Annually, the production of tomato in South Africa is around 600 000 tons. Tomatoes are grown by small and large-scale farmers for domestic and export purposes. The Department of Agriculture, Forestry and Fisheries reported that over 200 000 people are employed for cultivation and processing of tomatoes. Despite its importance, tomato is a host to about 130 plant pathogens, 60 of which cause serious losses and limit its production.

A substantial number of bacteria that causes leaf spots on fruits and vegetable crops and stem necrosis are caused by fluorescent Pseudomonads. These bacteria produce different symptoms depending on the colonized host. In tomato crops, Pseudomonas syringae pv. tomato and Pseudomonas syringae pv. syringae have been reported to cause substantial yield losses of approximately 75% under favourable weather conditions. Closely related Pseudomonas species P. viridiflava and P. cichorii, have also been identified as pathogens of tomato. The objective of this study was to characterise fluorescent Pseudomonas species from diseased tomato isolated between 1991 and 2015 from different provinces of South Africa by using morphological and molecular methods. The study focused more on bacterial speck of tomato, caused by Pseudomonas syringae pv. tomato. The disease is the most prevalent and devastating disease of tomato in South Africa.

Forty-four strains of fluorescent Pseudomonas were obtained from the Plant Pathogenic and Plant Protecting Bacteria collection at the Agricultural Research Council, Plant Health and Protection, Roodeplaat, Pretoria. Type strains of P. syringae pv. syringae, P. syringae pv. tomato, P. viridiflava and P. chicorii were used as reference strains. King’s B medium was used to evaluate colony morphology. The isolates were Gram stained and LOPAT tested. They were evaluated for their ability to utilise eleven different carbon sources, namely
glucose, sucrose, sorbitol, mannitol, erythritol, lactose, inositol, fructose, D (-) tartrate, L (+) tartrate and DL-lactate. Pathogenicity tests were conducted by spraying four weeks old tomato seedlings (cv. Red khaki) with $10^7$ cfu ml$^{-1}$ suspensions of bacterial pathogens. Sprayed plants were kept in a glasshouse with 26°C/20°C day/night temperatures and 65-75% relative humidity and examined daily for development of disease symptoms. To assess genetic diversity among the strains, the genomic DNA extracted from the strains were subjected to rep-PCR fingerprinting using BOX A1R and ERIC 2 primers and Multi Locus Sequence Analysis (MLSA) using two housekeeping genes (cts and gyrB). Partial sequences of the two housekeeping genes were generated for pathogenic strains. The cts and gyrB nucleotide sequences obtained in this study were aligned with sequences of nine *Pseudomonas* reference strains from the Plant Associated and Environmental Microbes Database using the MAFFT 7 online alignment tool. Phylogenetic trees were constructed using MEGA v 5.2 software.

All strains were Gram-negative rods. Thirty-seven strains belonged to the LOPAT group I (*P. syringae*), five to LOPAT II (*P. viridiflava*) and two to LOPAT III (*P. cichorii*). LOPAT I and LOPAT II isolates induced bacterial speck-like symptoms in tomato seedlings seven days after inoculation. *P. syringae* produced water soaked, dark brown spots surrounded by yellow halos. Colonies of *P. syringae* were 3-4 mm in diameter, smooth, round, slightly raised, mucoid and creamy white. Thirty-four strains produced a fluorescent pigment on King’s B media, except for 10 strains (BD 0001, BD 0002, BD 0070, BD 0071, BD 0278, BD 0774, BD 0775, BD 0779, BD 1354 and BD 1355). *Pseudomonas cichorii* and *P. viridiflava* strains produced non-mucoid and creamy colonies fluorescent on KB medium. Thirty strains received as *P. syringae pv. tomato* and the type strain of this pathovar, CFBP 2212 PT, utilised glucose, sucrose, sorbitol, mannitol, lactose, inositol, fructose and D-tartrate as single carbon sources. They did not grow on erythitol, L-tartrate and DL-lactate. *P. syringae pv. syringae* strains (BD 0002, BD 0022, BD 0278, BD 0279, BD 0280, BD 0771 and BD 0774) did not utilize D (-) tartrate and L (+) tartrate just like the type strain of *P. syringae pv. syringae*, CFBP 1392 PT. They grew on a minimal medium containing glucose, sucrose, sorbitol, mannitol, erythritol, lactose, inositol, fructose and DL-lactate.
Pseudomonas viridiflava CFBP 2107 and BD 0146, BD 0156, BD 0223, BD 0224 and BD 0231 did not grow on lactose, inositol and L-tartrate. P. cichorii strains did not utilise sucrose, sorbitol, erythritol, inositol, D(-) tartrate and DL-Lactate. Physiological and nutrient-based tests cannot differentiate P. syringae to the pathovar level. It was therefore necessary to use molecular techniques to identify those strains.

GyrB and cts sequences of 34 strains displayed a high degree of similarity with previously determined sequences belonging to the genus P. syringae. Twenty-four strains showed \( \geq 98\% \) sequence similarity to P. syringae pv. tomato, four strains showed \( \geq 99\% \) to P. syringae pv. syringae and four strains (BD 0002, BD 0022, BD 0771 and BD 0774) clustered with P. syringae pv. syringae however in the concatenated tree clustered with the type strains of P. syringae pv. papulans CFBP 5076\(^{PT}\) and P. s pv dysoxyli LMG 5062 \(^{PT}\). In the concatenated tree, 24 strains clustered with the type strain of P. syringae pv. tomato CFBP 2212\(^{PT}\). These strains originated from four provinces namely North-West, Mpumalanga, Gauteng and Limpopo. Four isolates clustered with the CFBP 1392\(^{PT}\), the type strain of P. syringae pv. syringae. These isolates originated from Mpumalanga. Four strains from Gauteng BD 0002, BD 0022, BD 0771 and 0774 formed a clade with P. syringae pv. papulans LMG 5076\(^{PT}\) and P. s pv dysoxyli LMG 5062 \(^{PT}\). BD 0231 from Limpopo as well as BD 0223 and BD 0224 from Gauteng were P. viridiflava.

Surprisingly, four isolates from Gauteng were found to belong in phylogroup 2a and clustered with P. syringae pv. papulans LMG 5076\(^{PT}\) and P. s pv dysoxyli. Pseudomonas syringae pv. papulans is the causal agent of blister spot of apples and P. s pv dysoxyli is a bacterial disease of dysoxylum spectabile. Both the diseases and pathogens have not been reported in South Africa. However, in 1986, Mansvelt and Hattingh reported a similar disease, bacterial blister bark and blight of fruit spurs of apple. The causal agent was identified as P. syringae pv. syringae. The authors used only physiological, morphological and biochemical method for the identification. In Chapter 2 of this dissertation, strains BD 0002, BD 0022, BD 0771 and BD 0774 were not distinguishable from those of P. syringae pv. syringae by single carbon sources utilisation and colony morphology. In 1986 gene sequencing and MLSA analyses were not commonly
available. It is possible that the disease described in 1986 was caused by *P. syringae* pv. *papulans*. *P. syringae* pv. *syringae* and pv. *papulans* are placed in the phylogroup 2. They induced similar symptoms in pathogenicity tests when inoculated into tomato seedlings. Despite the above speculations, this is the first report of bacterial speck of tomato caused by *P. syringae* pv. *papulans* and *P. s pv dysoxyli*. The reason for a bacterial pathogen of apple and *dysoxylum spectabile* to infect tomato is unclear and should be investigated further. The rep-PCR fingerprints amplified with BOX A1R and ERIC 2 primers ranged from 250 to 3500bp and 250 to 6000 bp pairs respectively. The obtained fingerprints showed genetic similarity within *Pseudomonas syringe* strains isolated from South African tomatoes. Four strains identified as *P. syringae* pv. *papulans* and *P. s pv dysoxyli*; BD 0002, BD 0022, BD 0771 and BD 0774 all isolated from Gauteng failed to produce bands for both primers. The dendrogram of the combined fingerprints of both BOX A1R and ERIC 2 divided *Pseudomonas syringae* into two groups. The majority of strains grouped with the type strain of *P. syringae* pv. *tomato* just like in the concatenated phylogenetic tree. A genetic similarity of 50% was observed among all the *P. syringae* strains.

In the evaluation of susceptibility of six commercially available tomato cultivars to *P. syringae* pv. *tomato*, all the six tested tomato cultivars were found to be susceptible to bacterial speck pathogen. Red khaki was found to be the most susceptible cultivar followed by cultivar 9753. Consistency of the reaction of cultivars to *P. syringae* pv. *tomato* in three independent experiments was observed. The highest number of speck lesions of (59.4 lesions) was noted in the second experiment at the concentration of 10^8 cfu ml^{-1} on Red khaki and the lowest number of speck lesions (14.0 lesions) was recorded in experiment 1, on cultivar 8863 at the concentration of 10^4 cfu ml^{-1}. The highest percentage disease index (PDI), 68.9% was observed on cultivar Red khaki at the highest concentration 10^8 cfu ml^{-1} whilst the lowest PDI 22.2% was observed on three cultivars (9771, 9751 and 9753). The PDI for these three cultivars were not significantly different from each other at the concentration of 10^4 cfu ml^{-1} in all three independent experiments. An increase in the number of lesions and the PDI was observed as the bacterial concentration changed from 10^4 to 10^8 cfu ml^{-1}.
In possible future studies, polyphasic analyses of fluorescent *Pseudomonas* isolated from tomato plants displaying bacterial speck symptoms from a much broader area could give a more detailed and comprehensive understanding of the disease. This could lead to the generation of more disease data and a better species and pathovar representation of speck causing bacterial pathogens in South Africa. Evasion of bacterial pathogens in quarantine is one of the major sources of introducing new bacterial pathogens into new fields, glasshouses and areas where they have not occurred before. To overcome this problem, future research need to be conducted to develop protocols for the detection of bacterial pathogen populations below $10^1$ cfu g$^{-1}$ of seeds produced to ensure sustainable disease-free seed production practices.
DECLARATION

I, Nokubonga Angel Langa, declare that:

i. The research reported in this thesis except otherwise indicated is my original work;

ii. This dissertation has not been submitted for any degree or examination at any other university

iii. This dissertation does not contain other person’s data, pictures or graphs or other information, unless specifically acknowledged as being sourced from other persons

iv. This dissertation does not contain other person’s writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, their words have been re-written, but the general information attributed to them has been referenced.

v. This dissertation does not contain text, graphics or tables copied and pasted from the Internet unless specifically acknowledged and the source being detailed in the thesis and in the References section.

Signed………………………
Date: ……………………
Nokubonga Angel Langa (Candidate)

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Date: 31-07-2020
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Signed….. ……. ……. 
Date: 31-07-2020
Dr T Gosczynska (Co-supervisor)
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DEDICATION

To my late father; Mzikayise Aaron Langa, thank you for being the best dad anyone could’ve hoped for.

To My mom; Martha Langa and brothers; Bongumusa Langa, Nhlanhla Langa and sister; Nompumelelo Langa.

Thank you for the support throughout this journey.
DISsertation Introduction

Bacterial pathogens pose a serious threat to food security since these pathogens hinder crop production and can cause substantial yield losses. Although bacterial disease may occur sporadically, they may cause yield losses of up to 100% on susceptible crops (Sundin et al., 2016). Some of the most important bacterial diseases are those caused by the genus *Pseudomonas*, particularly fluorescent *Pseudomonads*. This genus is widespread and can be found in different environments including soil, water and in plants (He et al., 2004). Approximately 202 bacterial species have been assigned to the *Pseudomonas* genus based on different classification methods (Tindall et al., 2006). Within the genus, *Pseudomonas syringae* comprises of approximately 64 pathovars and has a broad host range.

One crop that is mostly affected by this genus is tomato (*Solanum lycopersicum* L.) which is an important vegetable crop. The vegetable is grown worldwide and has numerous nutritional benefits (Freeman and Reimers, 2011). Some of the most important nutritional benefits provided by tomatoes are lycopene, vitamins, potassium phosphates, calcium and magnesium (Miller, 2002). Confirming its significance, tomato is the most important traded vegetable and accounts for 22% of all world trade by value in vegetable (FAO, 2014). In 2004, South Africa ranked 45th largest exporter of vegetables in the world, however it is not a major exporter of tomatoes. In South Africa, tomatoes are produced in all provinces, with Limpopo being the major productions area, accounting for 3590 hectares (ha) of area planted under tomatoes (DAFF, 2012).

Bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* (Okabe, 1933; Young et al., 1978), is an economically important disease of tomatoes worldwide. It reduces both fruit quality and yield in fresh market and processed tomatoes (Varvaro and Guario, 1983). The disease is seed borne. Cool temperatures and high rainfall are ideal for the disease progression (Yunis et al., 1980). The symptoms on leaves are brown to black spots approximately 2 mm in diameter and are surrounded by a chlorotic yellow halo. As the disease progresses, spots may coalesce (Milijasevic et al., 2009). On fruits it causes dark and small specks which are rarely larger than 1 mm in size. These symptoms are
often confused with those caused by *P. syringae pv. syringae*, the causal agent of leaf spot of tomato.

Bacterial speck of tomato has been reported to cause considerable yield losses; in severe cases it has caused up to 100% yield loss. Just like other bacterial diseases, bacterial speck control relies mostly on an integrated approach, which includes good cultural practices, chemical spray applications and genetic resistance. Planting of pathogen free seeds is an important control strategy. However, this method does not guarantee disease control (Allen *et al.* 1998; Gilbertson and Maxwell, 1992). The use of copper bactericides, against bacterial speck is not adequate, because they do not provide sufficient control of the diseases in the field and also that most strains have developed resistance (Vallad *et al.*, 2010). The use of resistant cultivars remains one of the most economical and effective strategy of controlling the disease (Blancard, 1997).

The aims of this study were to characterise a collection of South African fluorescent *Pseudomonas* isolated from diseased tomatoes between 1991 and 2015 using a polyphasic approach based on morphological, biochemical and molecular methods as well as to evaluate susceptibility of locally and commercially available tomato cultivars to *P. syringae pv. tomato*.

**Research objectives**

The specific objectives of this study include:

1. To characterise fluorescent *Pseudomonas* species by using morphological and biochemical methods;
2. To perform Rep-PCR genomic fingerprints analysis of fluorescent *Pseudomonas* species isolated from tomato in South Africa;
3. To identify South African fluorescent *Pseudomonas* to pathovar level using the multilocus sequence analyses of two housekeeping genes, DNA gyrase Subunit B (*gyrB*) and citrate synthase (*cts*);
4. To evaluate susceptibility of six commercial tomato cultivars to *P. syringae pv. tomato* under greenhouse conditions.
The dissertation has been written in the form of four chapters. Each chapter is focused on a specific objective of the research that was conducted. With an exception of Chapter One, “literature review”, the other three chapters were independent studies and were written in the form of research chapters. Each chapter is following the format of a stand-alone research paper. This format is the standard dissertation model that has been adopted by the University of KwaZulu-Natal because it facilitates the publishing of research out of the dissertation far more readily than the older monograph form of dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information between chapters.

List of conferences or workshop participation emanating from this work:


References


# Table of Contents

DISSENYATION SUMMARY ............................................................................................................. I

DECLARATION........................................................................................................................................ VI

ACKNOWLEDGEMENTS .................................................................................................................... VII

DEDICATION ......................................................................................................................................... VIII

DISSENYATION INTRODUCTION .................................................................................................... IX

Chapter 1 .............................................................................................................................................. 1

Literature Review ............................................................................................................................... 1

1.1 Introduction .................................................................................................................................... 1

1.2 The crop (Tomato) ......................................................................................................................... 2

1.3 Tomato production ......................................................................................................................... 2

1.3.1 Tomato production worldwide ............................................................................................... 2

1.3.2 Tomato production in South Africa ....................................................................................... 3

1.4 Favorable growth conditions for tomatoes .................................................................................. 4

1.5 Nutritional and health benefits of tomatoes ................................................................................ 5

1.6 Diseases of tomato ....................................................................................................................... 7

1.6.1 Major viral diseases of tomato ............................................................................................... 7

1.6.1.1 Cucumber mosaic virus (CMV) .......................................................................................... 7

1.6.1.2 Tobacco mosaic virus (TMV) ............................................................................................. 7

1.6.1.3 Tomato spotted wilt virus (TSWV) .................................................................................... 7

1.6.2 Major fungal pathogens ............................................................................................................ 8

1.6.2.1 Early blight .......................................................................................................................... 8

1.6.2.2 Late blight ........................................................................................................................... 8

1.6.2.3 Fusarium wilt ...................................................................................................................... 9

1.6.3 Major nematode diseases ......................................................................................................... 10

1.6.3.1 Root-knot nematode ........................................................................................................... 10

1.6.3.2 Sting nematodes ................................................................................................................. 10

1.6.3.3 Stubby root nematode ......................................................................................................... 10

1.6.4 Major bacterial diseases of tomato ......................................................................................... 11

1.6.4.1 Bacterial canker .................................................................................................................. 11

1.6.4.2 Bacterial wilt ....................................................................................................................... 11

1.6.4.3 Bacterial spot ....................................................................................................................... 12

1.7 Genus Pseudomonas ..................................................................................................................... 12

1.8 Bacterial speck of tomato ............................................................................................................. 14

1.8.1 Distribution and economic importance .................................................................................... 14
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1 Rep-PCR fingerprinting</td>
<td>101</td>
</tr>
<tr>
<td>3.3.2 Multilocus Sequence Analysis (MLSA)</td>
<td>104</td>
</tr>
<tr>
<td>3.4 Discussion</td>
<td>107</td>
</tr>
<tr>
<td>3.5 References</td>
<td>110</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>116</td>
</tr>
<tr>
<td>Evaluation of susceptibility of six commercially available tomato cultivars in South Africa to <em>Psuedomonas syringae pv. tomato</em> under greenhouse conditions</td>
<td>116</td>
</tr>
<tr>
<td>Abstract</td>
<td>116</td>
</tr>
<tr>
<td>4.1 Introduction</td>
<td>117</td>
</tr>
<tr>
<td>4.2 Materials and Methods</td>
<td>118</td>
</tr>
<tr>
<td>4.2.1 Bacterial isolate</td>
<td>118</td>
</tr>
<tr>
<td>4.2.2 Tomato seedlings and growth conditions</td>
<td>119</td>
</tr>
<tr>
<td>4.2.3 Inoculation of seedlings with the pathogen</td>
<td>119</td>
</tr>
<tr>
<td>4.2.4 Evaluation of disease severity</td>
<td>120</td>
</tr>
<tr>
<td>4.2.5 Statistical analysis</td>
<td>120</td>
</tr>
<tr>
<td>4.3.3 Re-isolation of bacteria from diseased leaves of tomato seedlings</td>
<td>120</td>
</tr>
<tr>
<td>4.3 Results</td>
<td>121</td>
</tr>
<tr>
<td>4.3.1 Response of cultivars to <em>Psuedomonas syringae pv. tomato</em> isolate under greenhouse conditions</td>
<td>121</td>
</tr>
<tr>
<td>4.4 Discussion</td>
<td>124</td>
</tr>
<tr>
<td>4.5 References</td>
<td>127</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>131</td>
</tr>
<tr>
<td>Thesis Overview of the Major Research Findings and their Implications</td>
<td>131</td>
</tr>
</tbody>
</table>
Chapter 1

Literature Review

1.1 Introduction

Tomato (*Solanum Lycopersicum* L.) is one of the most frequently consumed vegetable crops worldwide (Ijaz, 2017). In South Africa tomatoes are grown in all provinces, Limpopo province is the highest producer with 3590 ha (DAFF, 2011). Tomato is greenhouse and field grown since it can adapt to a range of environmental conditions, with the production of 600 000 tons in 2006 recorded in South Africa (http://postharvestinnovation.org.za/commodities/tomatoes/).

Tomatoes provide a range of nutrients such as vitamins, minerals, folic acid carotenoids, for example lycopene and antioxidant. Some of these nutrients have been reported to prevent human cardiovascular diseases and cancer (Friedman, 2013; Perveen *et al.*, 2015). Plant pathogens decrease fruit quality and reduce yield (Arie *et al.*, 2007). Jones *et al.*, (1991) reported that tomatoes are prone to bacteria, viruses, fungi and nematodes and these pathogens affect the production of tomatoes.

*Pseudomonas syringae* pv. *tomato* (Okabe, 1933; Young *et al.*, 1978) causes bacterial speck of tomato. The disease occurs worldwide where tomatoes are grown and has been reported to cause severe reduction in fruit quality and yield. It is more prevalent in cool and moist weather conditions (24-26°C) (Bogatzevska, 2002; Cai *et al.*, 2011). The disease causes spots or specks surrounded by a chlorotic yellow halo, as the disease progresses, the lesions may coalesce (Louws *et al.* 2001). The disease may also cause flower abortion, necrosis on stem and on fruits making fruits unmarketable. Bacterial speck is present in South Africa; however, it has been poorly studied (CAB International, 2005). Detection and characterisation of this pathogen is important since it can easily be confused with other pathogens. For instance; biochemically and physiologically, it is hard to distinguish it from *Pseudomonas syringae* pv. *syringae* and its symptoms in the field may often be confused with those of bacterial spot which is caused by *Xanthomonas campestris*.
In the characterization of plant pathogenic bacteria, a number of molecular methods have been used to differentiate and classify bacterial strains below the species level. The methods include Repetitive extragenic palindromic (REP)-PCR (Versalovic, 1991), 16S rDNA restriction analysis (Vaneechottte et al., 1993) and DNA-DNA hybridization (Pecnold and Grogan 1973). These methods have allowed for the delineation of phylogenetic groups, or phylogroups, within the species complex. It is crucial to incorporate the physiological or biochemical methods with molecular methods.

1.2 The crop (Tomato)
Tomato (Solanum lycopersicum, L.) is a perennial plant which can also be grown as an annual plant. Tomato belongs to the family Solanaceae and genus Solanum (Bohs, 2005; Peralta and Spooner, 2001; Spooner et al., 2005). Tomato is the third most economically important crop family after grasses and legumes and the most valuable in terms of vegetable crops (Van der Hoeven et al., 2002). Tomatoes can be eaten raw, added to stews and canned. The family Solanaceae consist of more than 3000 species of economic importance such as; tobacco, pepper, eggplants and potatoes. The species belonging to the family Solanaceae occupy diverse environments (Knapp, 2002).

1.3 Tomato production
1.3.1 Tomato production worldwide
Tomato is an important vegetable crop worldwide and produces high yields (Srinivasan, 2010). Tomato is grown globally and is an important vegetable crop after potatoes and ranks first as a processing crop (FAOSTAT, 2014; Mohammed et al., 2013). Most of the world’s tomato production is concentrated in temperate zones with long summers and winter rainfall. From the year 2001 to 2011 the global tomato production has grown by 47%, Asia showing the strongest regional growth (FAOSTAT, 2014). In 2001, the world tomato production was approximately 105 million tons of fresh fruit from an estimated 3.9 million ha (Naika et al., 2005). According to Robertson and Labate (2007) in 2013, tomato production was estimated to be around 161.8 million tonnes in the world.
According to FAOSTAT (2016); China is the leading producer. China produces about one quarter of the world’s tomato (Table 1.1).

Table 1.1: The list of top 5 most tomato producing countries (FAOSTAT, 2016)

<table>
<thead>
<tr>
<th>Country</th>
<th>Tomato production (metric tonnes)</th>
<th>% of the world total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. China</td>
<td>50552200 m/t</td>
<td>6.82%</td>
</tr>
<tr>
<td>2. India</td>
<td>18227000 m/t</td>
<td>2.46%</td>
</tr>
<tr>
<td>3. United states of America</td>
<td>12574550 m/t</td>
<td>1.69%</td>
</tr>
<tr>
<td>4. Turkey</td>
<td>11820000 m/t</td>
<td>1.59%</td>
</tr>
<tr>
<td>5. Egypt</td>
<td>8533803 m/t</td>
<td>1.15%</td>
</tr>
</tbody>
</table>

1.3.2 Tomato production in South Africa
Tomatoes are grown in all provinces in South Africa (Fig. 1.1). The Limpopo province is the main production area with 3590 hectares. The province accounts for more than 75% of the area planted under tomatoes in the country (DAFF, 2011). The other main leading tomato producing provinces are KwaZulu-Natal, Mpumalanga and Eastern cape.

Annually, SA produces 600 000 tons of tomatoes and ranks 35th in the world. (http://postharvestinnovation.org.za/commodities/tomatoes; Malherbe and Marais, 2015). In Limpopo province, 4523 ha of tomatoes generated 630 million of revenue in 2007 (Statistics South Africa, 2007). Most of the tomatoes are produced in an open field, a small amount is grown under greenhouse protection (Maboko et al., 2009). Almost all open field vegetable production is seasonal (Tsutomu et al., 2007).
Figure 1.1: Map of South Africa showing area planted under tomatoes per province ([http://www.kzntransport.gov.za/public_trans/freight_databank/kzn/industries/Fruit_veg/index_xml.html](http://www.kzntransport.gov.za/public_trans/freight_databank/kzn/industries/Fruit_veg/index_xml.html)). Map from [https://www.intergate-immigration.com/blog/south-african-provinces/](https://www.intergate-immigration.com/blog/south-african-provinces/).

1.4 Favorable growth conditions for tomatoes

Crop growth and development are highly dependent on temperature ([Lu et al.](http://www.kzntransport.gov.za/public_trans/freight_databank/kzn/industries/Fruit_veg/index_xml.html), 2013). Tomato is a warm seasonal crop and grows well in temperatures ranging from 20 to 24°C which is optimum for growth, produce and quality ([Rice et al.](http://www.kzntransport.gov.za/public_trans/freight_databank/kzn/industries/Fruit_veg/index_xml.html), 1987). During flowering and fruit setting tomatoes require adequate moisture content. Well drained soils that are well supplied with organic matter and with pH 6 are beneficial for the growth of tomatoes ([Obeng-Ofori et al.](http://www.kzntransport.gov.za/public_trans/freight_databank/kzn/industries/Fruit_veg/index_xml.html), 2007). Supplying the soil with organic matter containing nitrogen, phosphorous and potassium improves tomato productivity. Tomatoes are extremely sensitive to frost and do
not thrive in cold and extremely hot temperatures. Humidity that is ideal for tomato production ranges from 40 to 70%. High humidity or too low humidity hinders the release of the pollen and its ability to stick to the stigma.

1.5 Nutritional and health benefits of tomatoes

Table 1.2: Nutritional value of 100g of red fresh tomato (USDA, 2000)

<table>
<thead>
<tr>
<th>Component</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>g</td>
<td>94.52</td>
</tr>
<tr>
<td>Energy</td>
<td>kcal</td>
<td>18</td>
</tr>
<tr>
<td>Protein</td>
<td>g</td>
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<td>Sodium</td>
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</tr>
<tr>
<td>Fluoride</td>
<td>µg</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Vitamins
Tomatoes have a high nutritional value and, therefore, represent a major source of vitamins and minerals (Abushita et al., 1997). According to (USDA, 2000); tomatoes are a great source of vitamin A and C, carotenoids, fiber, potassium, and lycopene (Table 1.2). The antioxidant, lycopene is responsible for the red pigment of mature tomato fruit and makes up 90% of the total carotenoid content of tomato (Shi and Le Maguer, 2000). When consumed, this carotenoid functions in searching free radicals, protecting vital biomolecules and modulating cellular signalling or metabolic pathways (Abushita et al., 1997; Frusciante et al., 2007; Rao and Agarwal, 2000). Numerous researches have investigated the link between the intake of tomatoes and disease risk reduction (Freeman and Reimers, 2011). Studies by Arab et al. (2002) and Garmyn et al. (1995) reported that lycopene plays a role in the prevention of skin and lung cancers respectively. According to Toor and Savage (2005), reduction of some cardiovascular diseases in humans has been linked to consumption of tomatoes.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Unit</th>
<th>Value</th>
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</thead>
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</tr>
<tr>
<td>Choline</td>
<td>mg</td>
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<tr>
<td>Vitamin A</td>
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<tr>
<td>Lutein-Zeaxanthin</td>
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<td>123</td>
</tr>
</tbody>
</table>

Table 1.2: Nutritional Value of Tomatoes

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1.6 Diseases of tomato

1.6.1 Major viral diseases of tomato

1.6.1.1 Cucumber mosaic virus (CMV)

_Cucumber mosaic virus_ (CMV) is an important virus worldwide belonging to the _Cucumovirus_ genus and _Bromoviridae_ family. The disease was first described in 1916 infecting cucumber and melon fields of the USA (Doolittle, 1916; Jagger, 1916). CMV has a wide host range, infecting over 1200 plant species of more than 100 families (Edwardson and Christie, 1991). CMV is distributed worldwide in both tropical and temperate regions (Palukaitis et al., 1992). CMV affects many important vegetables and ornamentals. Observed symptoms on tomatoes are slight yellowing, stunting, short internodes and plants may be extremely distorted (MacNab et al., 1983). The disease overwinters in perennial weeds and is transmitted to healthy plants by a variety of aphid vectors in a non-persistent manner and by mechanical transmission.

1.6.1.2 Tobacco mosaic virus (TMV)

_Tobacco mosaic virus_ (TMV) belongs to the genus _Tobamovirus_ (Koonin et al., 1993) and was the first infectious agent identified as a virus and is extensively studied (Beijerinck, 1898). TMV is an economically important viral disease that infect tobacco (_Nicotiana tabacum L._) and other solanaceous crops worldwide. The disease has a broad host range and infects approximately 199 different species from 30 families (Zaitlin, 2000). TMV is a devastating virus, which unlike most viruses does not die when the host plant dies and can withstand high temperatures. The disease is transmitted mechanically from plant to plant via wounds caused by contaminated hands, clothes and tools. Infected plants exhibit mosaic, mottling, curling, yellowing, and stunting (Shaw, 1999). TMV has no known vectors (Palukaitis and Zaitlin, 1986; Shaw, 1999).

1.6.1.3 Tomato spotted wilt virus (TSWV)

_Tomato spotted wilt virus_ (TSWV) belongs to the genus _Orthotospovirus_ in the family _Tospoviridae_. The virus was first discovered in 1915. TSWV has a broad host range and infects over 900 plant species including several crops and weeds.
In nature, TSWV is transmitted by several species of thrips but mainly by the western flower thrips (*Frankliniella occidentalis*) and it can be mechanically transmitted. Once the thrips have acquired the virus, the thrips remain infective for the rest of their lives about (30 to 45 days).

TSWV produces foliar symptoms, but the symptoms differs depending on various factors such as; the genotype, aggressiveness of isolate, host species and environmental factors (Moyer, 2000). The observed symptoms are purple to brown spots on the leaves, stunting, rings on the stem and tip dieback. Concentric rings are observed on green fruits and on red-ripe fruits striking brown rings develop.

1.6.2 Major fungal pathogens

1.6.2.1 Early blight

*Alternaria solani*, the causal agent of early blight of tomato was first discovered in 1882 in New Jersey, USA and was referred to as *Macrosporium solani* (Ellis and Martin, 1882). The disease is predominant in tropical and temperate zones where potatoes and tomatoes are grown. The initial symptoms of the disease are brown to dark leathery oval or angular spots on leaves approximately 0.3 or 0.4 cm in diameter with a thin chlorotic zone around the spot which later fades into the normal green colour (Locke, 1949; Walker, 1952). In tomato, the disease affects the older leaves first and the disease progresses upwards causing the leaves to dry up and drop down. As the disease progresses, it weakens the plant and the susceptibility to infection is increased since there’s an imbalance between the nutrient demand in the fruits and nutrient supply from the leaves (Rowell, 1953). Wet, humid weather favours disease development and the fungus spores are spread mainly by wind.

1.6.2.2 Late blight

*Phytophthora infestans* (Mont.) de Bary is the causal agent of late blight of tomato, an important fungal disease of tomato and potato (*Solanum tuberosum*. L). Late blight causes significant yield losses (Jones *et al.*, 1998). The Irish potato famine in the 1840’s was due to this disease (Ghorbani *et al.*, 2004; Lamour and
Temperature ranging from 16-22°C plays a major role in disseminating the disease and it is more prevalent in high tropical rainfall regions (Hartman and Huang, 1995). A relative humidity of approximately 100% is essential for the sporulation of the causal agent. Yield loss of up to 100% has been observed on unprotected tomato fields (Nowicki et al. 2012). Symptoms expressed, depend on the aggressiveness of the strain and prevailing environmental conditions. The initial symptoms are small, light to dark green and circular water-soaked lesions surrounded by a yellow halo of chlorotic tissue (Kirk et al., 2013).

In the field, the first symptoms that are observed are small, light to dark green and circular to irregularly shaped water-soaked lesions (Kirk et al. 2013). Lesions are often surrounded by a yellow green halo of chlorotic tissue. On tomato fruit, symptoms begin as dark greasy spots and as the disease progresses the spots may cover the entire fruit. White mycelium may be observed on fruit under favourable conditions of late blight sporulation (Stevenson, 1991).

1.6.2.3 Fusarium wilt

Fusarium oxysporum sp. lycopersici causes Fusarium wilt, a soil borne plant pathogen infecting tomato. The disease was first described by G.E. Massee in England in 1895. The disease is of worldwide importance and is predominant in most tomato growing countries. Worldwide, there’s over 100 Fusarium vascular wilt diseases that have been reported. Symptoms begin as slight vein clearing, yellowing, wilting, stunting, leaf death and lack of fruit production also occurs (Snyder and Hansen, 1940). Dark brown lines may be observed running lengthwise if the main stem of the plant is cut, this symptom is a characteristic of the disease and generally can be used for its identification (Mui-Yun, 2003). White or pink growth can be observed outside the affected stem particularly in wet environments (Ajigbola and Babalola, 2013). Using disease free seeds and transplants is required, and hot water treatment should precede planting (Agrios, 2005).
1.6.3 Major nematode diseases

1.6.3.1 Root-knot nematode

Root-knot nematodes (RKN) plant parasites belong to the genus *Meloidogyne* and it is the most damaging nematode genus globally (Sasser, 1980). The genus contains more than 90 species, with some species having several races (Karssen, 2002). The name stems from the fact that the nematode infestation causes galls or root-knot symptoms. In South Africa, root-knot nematodes are the most common and most destructive nematode species and *Meloidogyne javanica* Chitwood is the most economically important species (Fourie *et al*, 2001). Other economically important species that are most widespread are: *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. chitwoodi* and *M. enterolobii*.

1.6.3.2 Sting nematodes

Sting nematodes *Belonolaimus longicaudatus*, are some of the major and most destructive plant parasitic nematodes. Sting nematodes are known to cause the most damage in a variety of crops and is also known as the largest of the nematodes measuring over 3mm in length (Crow and Han, 2005). The observed symptoms caused by sting nematodes are necrotic lesions in the root’s cortical tissues. Root systems of infected plants is greatly reduced, the main roots lose a high percentage of lateral feeder roots and the roots are short, stubby roots, and shrunken lesions particularly at the tips (Crow and Han, 2005).

1.6.3.3 Stubby root nematode

Stubby root nematode belongs in the *Trichodoridae* and *Paratrichodorus* genera an economically important group of nematodes. The common name stem from the fact that the nematode feeds on roots causing “stubby” appearing root system. A characteristic that can be used to differentiate this nematode from others is a solid stylet that is used when feeding. This nematode is an external feeder and causes problems by feeding at the root tips. When the root tips are attacked, they stop growing and frequently appear short, stubby and swollen. The plant is usually stunted, wilt easily, have little to no ability to withstand water
shortage and may be yellowish due to nutrient deficiency caused by a poor root system (Christie and Perry, 1951).

1.6.4 Major bacterial diseases of tomato

1.6.4.1 Bacterial canker

*Clavibacter michiganensis* subsp. *michiganensis* is a bacterium that causes bacterial canker. The disease was originally described in Michigan, United States (Smith, 1910). Tomatoes are the main host of economic importance infected by bacterial canker.

Bacteria can occur on the seed coat as well as within the embryo. Seeds aid as a primary source of inoculum of the bacterium (De Leon *et al*., 2008; Fatmi and Schaad, 2002; Tancos *et al*., 2015). The bacterium enters leaves through hydathodes. The first observed symptom on leaves is wilting which sometimes leads to plant death. Other symptoms that may be observed are leaf chlorosis, vascular discoloration, marginal necrosis, curling and systemic wilting of the plant. Lesions on fruits are creamy, white spots with tan or brown centres on fruits which are referred to as Bird eye spot. Splashing rain and human activity spread the pathogen between the plantings (Jones *et al*., 1991).

1.6.4.2 Bacterial wilt

*Ralstonia solanacearum* formerly known as *Pseudomonas solanacearum* is the causal agents of bacterial wilt on tomato. The primary source of inoculum of the bacterium is soil. The wilting disease affect plants of *Solanaceae* family such as tomato, pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.). Warm temperatures above 24°C, tropical and subtropical climates with high rainfall are conducive for the prevalence of the pathogen (Panagopoulos, 2000). During warm temperatures or hot weather, the younger leaves wilts and in the evening the plants may recover temporarily due to cool weather. The following days the plants wilt permanently. A quick and easy test to identify the bacterium is done, where a lower part of the infected stem is suspended into a glass of water, after
3-5 minutes a milky white stream flows from the infected stem (Koike et al., 2007; Pernezny et al., 2003).

1.6.4.3 Bacterial spot
Four species of *Xanthomonas* namely; *Xanthomonas campestris* pv. *vesicatoria* which is divided into group A and B, *Xanthomonas axonopodis* pv. *vesicatoria* which is in group A, *Xanthomonas perforans* and *Xanthomonas gardeni* causes bacterial spot (Jones et al., 2000; Vauterin et al., 1995). The groups are based on phenotypic and genetic differences. The bacterium was first discovered in South Africa 1914 by Ethel Doidge (Doidge, 1920).

The disease is easily disseminated under favourable conditions, it prefers warmer temperatures around 24 to 30°C. Symptoms caused by the bacterium on leaves are small water-soaked spots which are about a ¼ inch encircled by a yellow halo and the symptoms of bacterial spot are hard to differentiate from those of bacterial speck. On fruits, it causes lesions that are slightly raised referred to as scabby black specks and may cause the fruit to be unmarketable. During favourable weather conditions yield losses of over 66% have been reported (Goode and Sasser, 1980; Louws et al., 2001; Pohronezny et al., 1992;).

Wind driven rain plays a major role in dispersing the pathogen.

1.7 Genus *Pseudomonas*
The genus *Pseudomonas* was described by Migula in 1894. It contains diverse and ecologically important group of bacteria that occur in various environments (Moore et al., 2006; Spiers et al., 2000). The genus *Pseudomonas* has gone through many taxonomic revisions and currently contains approximately 200 species. These bacteria are Gram-negative, aerobic, motile by one or several polar flagella, non-spore-forming straight or slightly curved rods (Parte, 2014). Important diseases on a broad range of hosts are caused by plant pathogenic *Pseudomonads*, and the crops exhibit different symptoms such as spots, soft rot, cankers, blight, and galls (Huang and Lakshman, 2010; Kokoskova et al., 2011).
*P. syringae* is the most important species with over 50 described pathovars (Bull *et al*., 2010). Pathovars of *P. syringae* cause several diseases on a range of economically important crops and threaten the global crop production. *P. syringae* enters the host tissue through wounds and stomata. Virulence on different hosts is maximized by pathogenicity effectors and toxins such as coronatine, syringomycin, phaseolotoxin and tabtoxin. *P. syringae* produces toxins that lack host specificity and cause symptoms on various crops which cannot be infected by the toxin-producing pathogen. Cool temperatures, leaf wetness and cultural practices plays a major role in disseminating the pathogen between host plants (Uppalapati *et al*., 2007). *P. syringae* pathogenicity is reliant on a Type III secretion system (TTSS) which acts as a specialized injection apparatus that delivers virulence proteins. The TTSS secretes effector proteins, known as type III effectors (TTE’s) that alter host cellular processes and promote disease development (Galan and Collmer, 1999; Jin *et al*., 2003).

This review focuses on *P. syringae pv. tomato*; which is a bacterium classified in phylum *Proteobacteria*, class *Gammaproteobacteria*, and family *Pseudomonadaceae* (Kado, 2010). *P. syringae pv. tomato* DC3000 plant pathogen infects *Arabidopsis thaliana* and *Nicotiana benthamiana* and is also an important pathogen of tomato (Mansfield *et al*., 2012). The bacterium is reported to be of economic importance in tomato, although it has been isolated from numerous other crops and weed species as an epiphyte (Preston, 2000). *P. syringae pv. tomato* produces fluorescent pigment on King’s B under ultraviolet light and therefore belongs in the fluorescent group of phytopathogenic *Pseudomonads* (King *et al*, 1954). After incubation at about 26°C for 48 hours, colonies are examined for fluorescence under ultraviolet light. On general medium, the pathogen is circular, smooth and creamy white. Accurate identification of pathogen is essential, and the identification methods should be reliable.
1.8 Bacterial speck of tomato

1.8.1 Distribution and economic importance

Bacterial speck of tomato is a devastating plant pathogen worldwide (Fig. 1.2) and has been reported to reduce both fruit quality and yield in fresh market and processed tomatoes (Varvaro and Guario, 1983). Bacterial multiplication and an increase of epiphytic population is favoured when environmental conditions are cool. Fruits infected with bacterial speck produces small dark spots, and the fruits may be regarded as of poor quality by consumers since appearance plays a major role (Bryan, 1933). From the time of infection and environmental conditions plays a pivotal role in the yield loss.

Grogan et al (1974) observed significant yield reductions in all plants that were inoculated with *P. syringae pv. tomato*; total yields were reduced by an average of 15% and total ripe fruit yield was reduced by 59%. In Israel, yield reduction of approximately 75% in plants infected at an early stage of growth have been reported, this may be due to that at an early stage of growth plants are more sensitive or prone to the disease (Yunis et al., 1980). Three-leaf stage plants are more susceptible to the disease than matured plants. Yield reduction of 13% was observed on older plants whilst infections in younger seedlings resulted to a greater reduction (Scheneider et al., 1975).

![Distribution map of bacterial speck of tomato (P. syringae pv. tomato) around the world, CABI (2012).](image)

Figure 1.2: Distribution map of bacterial speck of tomato (*P. syringae pv. tomato*) around the world, CABI (2012).
1.8.2 Epidemiology

Bacterial speck is a seed borne pathogen. Seeds contaminated with *P. syringae* pv. *tomato* are a primary source of inoculum (Chambers and Merriman, 1975; Henis and Bashan, 1985). Upstone (1971) suggested that infected debris was the source of infection in the field. The pathogen can be found in soil for a limited period, on plant debris, weeds, non-host plants, or as an epiphyte on symptomless tomato transplants for longer periods of time (Schneider and Grogan, 1977). Cool temperatures ranging from 18 to 24°C and relative humidity of about 80 to 100% favours the disease development, these factors play a crucial role in disseminating the pathogen. For the dissemination of the bacterium; wind carrying rain droplets, water for irrigation and handling of plants whilst still wet are also contributing factors to disseminating the disease (Fig.1.3) (Pohronezny *et al*., 1990).

It has been indicated by many studies that favourable temperatures and leaf wetness can cause plants that have low level or intensity of the bacterial population to exhibit symptoms within as few as 3-5 days. The bacterium penetrates through the stomata, wounds and hydathodes and the possibility of secondary infection is highly likely (Jones, 1991). Bacterial speck of tomato may often be confused with bacterial spot and the diseases may often occur simultaneously in mixed infections (Delahaut and Stevenson, 2004). The diagnosis is best accomplished by vigilant inspection of fruit symptom since foliage symptoms are much more difficult to differentiate from bacterial spot. For the development of control measures of the bacterium, a better understanding of bacterial speck of tomato epidemiology is essential.
1.8.3 Symptoms
On leaves, symptoms of bacterial speck consist of small black spots approximately 2mm in diameter that can frequently be noticeable even on the basement of leaves (Fig.1.4). As the spots gets older, a yellow halo develops around the spots. The chlorotic yellow halo is generated by the phytotoxin, coronatine (Young et al. 1986). The phytotoxin plays a vital role in the virulence of the pathogenic bacteria *P. syringae pv. tomato*. The toxin is not-host specific; other several *Pseudomonas syringae* can produce it and is a secondary metabolite. Members of *P. syringae* produces the coronatine phytotoxin which induces chlorosis on several hosts (Brooks et al., 2004).

Spots are very small and do not infiltrate very deeply into the fruit and appears to be flat or sunken, can be raised and vary in colour from brown to black. On green fruits, darker green haloes are observed whilst on ripe fruits the spots have slight yellow haloes. It is difficult to distinguish leaf symptoms of bacterial speck from other diseases of tomatoes. The disease causes defoliation in severe cases. The disease reduces photosynthetic capacity of the infected plant, causes flower abortion, and spots on the fruit. Therefore, making the fruits unfit for fresh market.
or for processing. Early in the season, the disease is much more prevalent and may cause reduction in tomato yield significantly (Pohronezny and Volin, 1983; Yunis et al., 1980).

Figure 1.4: Tomato plant exhibiting symptoms of bacterial speck on leaves and fruits; small black spots, with a yellow chlorotic halo around the spots and on fruits it causes small raised, black lesions (Lamichhane et al., 2010; https://tomatodiseasethelp.com/bacterial-speck).

1.8.4 Bacterial speck of tomato races

*P. syringae* pv. *tomato* has two races (0 and 1) that have been described worldwide. Race 1 was first detected in Canada and California in 1993 (Lawton and MacNeil, 1986). The occurrence of new races of the pathogen is problematic in breeding tomato cultivars for resistance. The races differ in terms of the expression of avirulence factors. In the races of *P. syringae* pv. *tomato*, resistance is conferred by the *Pto* gene which carries the avirulence gene, *avrPto* and *AvrPtoB*. The *Pto* gene has been introduced into numerous processing tomatoes and a few fresh markets tomato cultivars in North America (Wilson et al., 2002). Due to the increase in selection pressure caused by growing tomato cultivars resistant to race 0, has led to the development of the new race 1 even on tomato hybrids heterozygous for the *Pto* gene (Buonaurio et al., 1996). TTSS is required as an effector for the successful expansion of *P. syringae* pv. *tomato*
in the host (Alfano and Collmer, 2004). The system is an important key for the virulence factor of \textit{Pto} because it enables the carriage of virulence proteins into the cytosol of plant hosts.

The \textit{Pto} gene encodes a protein kinase which is responsible for activating the immune system of the host, thus inhibit bacterial multiplication and preventing disease development (Martin, 2012). Race 1 of \textit{P. syringae} pv. \textit{tomato} strains lack these effectors (Kunkeaw \textit{et al.}, 2010). In the absence of resistance, tomato plants infected with \textit{P. syringae} pv. \textit{tomato} result in bacterial speck of tomato disease. \textit{P. syringae} pv. \textit{tomato} is identified based on the absence or presence of typical disease symptoms on tomato cultivars. According to Pedley and Martin (2003), no cultivated tomato cultivars are resistant to race 1 although some processing tomato cultivars can be resistant to strains of \textit{P. syringae} pv. \textit{tomato} race 0. The development of bacterial speck of tomato symptoms indicates a compatible reaction between the host and the pathogen. Therefore, if symptoms are observed on the host it indicates the existence of race 1 of the pathogen (Buonauroi \textit{et al.}, 1996).

1.9 Detection and Characterisation of \textit{Pseudomonas syringae}

It is important to accurately detect and identify plant pathogens in order to prevent a disease dissemination by applying correct disease management measures. Identification of a pathogen is the first step in successful plant disease management. Diagnosis of unknown pathogens requires symptom observation in the field, pathogen isolation from infected tissues, characterization, pathogenicity tests and confirmation of Koch’s postulate (Alvarez, 2004). According to Agrios (2005), special manuals are used as a reference. When a pathogen is found on an infected plant, a pathogen is known to cause such a disease and the diagnostician is confident that no other causal agents are involved, then the diagnosis of the disease may be considered complete.

Identification and classification of plant pathogenic bacteria has mainly been based on phenotypic and biochemical methods. These methods have been of great value however, these techniques are laborious and time-consuming (Hildebrand \textit{et al.}, 1988).
For the accurate identification of \textit{P. syringae pv. tomato}, the pathogen should be isolated, purified and characterized by a series of biochemical, physiological and pathogenicity tests (Braun- Kiewnick and Sands, 2001; Goszczynska \textit{et al}., 2000). Phenotyping and genotyping are two approaches used to identify phytopathogenic bacteria. Phenotyping techniques include colony morphology, carbon source utilization, pathogenicity test and serological tests. Generally, these methods are time consuming and require simpler equipment than those of genotyping. The advances of using genomic techniques for characterization of bacteria over the past decade have significantly simplified and improved pathogen detection and identification. For characterisation of organisms into species, subspecies and pathovar level, DNA fingerprinting has been broadly explored (Louws \textit{et al}., 1994).

\subsection*{1.9.1 Isolation and characterisation of \textit{Pseudomonas syringae} from plant tissues}

The isolation of the pathogen from diseased tissues is done by excising a portion of the infected leaves exhibiting speck symptoms typically from the margins of lesions. The excised leaves are thoroughly rinsed under running tap water and placed into drops of sterile distilled water in a sterile Petri dish. The suspension is left to stand in the laminar flow for a few minutes. A loopfull of the suspension is streaked on the surface of dried King’s B medium (King’s 1954). The inoculated plates are incubated at 26°C until bacterial growth starts to develop and are frequently checked for the detection of fluorescent colonies under ultraviolet light at approximately 367nm.

\subsection*{1.9.2 Isolation and characterisation of \textit{Pseudomonas syringae} from seeds}

Mohan and Schaad (1987), employed a semiselective agar media for the detection of \textit{Pseudomonas syringae pv. syringae} and \textit{Pseudomonas syringae} pv. \textit{phaseolicola} in bean seed. Two semiselective media used in the study were KBC which is derived from KB for \textit{P. s. pv. syringae} and Modified Sucrose Peptone (MSP) for \textit{P. s. pv. phaseolicola}. In the study by Shila \textit{et al}., (2013), external surfaces of seed samples for each cucurbit species were sterilized with
10% Clorox for 1 min. Then the samples were washed with sterile water and the excess water was removed using blotter paper. Seeds were later on placed on the Nutrient Agar (NA) plates. Plates were incubated in an inoculation chamber at 27°C for 2 days to allow for the growth of bacteria associated with the seeds. Pure bacterial culture colonies are obtained by streaking a loopfull of the suspension on semiselective media and incubated until colonies develop (Schaad et al., 2013)

1.10 Detection methods commonly used for the identification of Pseudomonas

1.10.1 Selective or differential media
Several techniques have been developed to identify *P. syringae pv. tomato* but isolation on media remains the primary identification method. There are different types of media used to study microbes such as selective, minimal, differential, general and nutrient media. The growth media can be solid, semi-solid and liquid (broth). Selective and differential media have been widely used for the detection of phytopathogenic bacteria (Sequeira, 1983). The use of pure colony for re-inoculation of susceptible host plants to carry out Koch’s postulate remains an easiest and most accurate method for the demonstration that the observed bacterium is undeniably the pathogen. Semi selective media facilitate the growth and identification of target bacteria since non-target bacteria are inhibited. Numerous selective media have been developed to facilitate the isolation of *P. syringae pv. tomato*. (King’s et al., 1954; Pohronezny et al., 1977). In the study by Goszczynska and Serfontein (1998) *P. syringae pv. tomato* strain was found to grow on Milk tween (MT) medium, which is a semi selective medium.

1.10.2 Determinative tests
The *Pseudomonas* genus is heterogenous and is divided into two groups; fluorescent and non-fluorescent, based on the fluorescent pigments production on Iron- deficient media (King’s et al., 1954). The determinative tests of Levan production, Oxidase activity, Potato soft rot, Arginine dihydrolase activity and Tobacco hypersensitive response (LOPAT) are used to classify *P. syringae* from
other species of fluorescent *Pseudomonads*. Furthermore, the tests divides species into five groups (Lelliott *et al.*, 1966; Lelliott and Stead, 1987). The five groups are distinguished as follows; Group I (LOPAT ++++), Group II (LOPAT +---), Group III (LOPAT --++), Group IV (LOPAT +++++) and Group V (LOPAT +++.). *P. syringae* belongs in the LOPAT Group I. The shortcoming of LOPAT tests is; it does not distinguish pathovars within *P. syringae*.

### 1.10.3 Pathogenicity confirmation

Diagnosis and identification of phytopathogenic bacteria primarily depends on disease symptoms, large bacterial population present in the infected area and the absence of other pathogens (Agrios, 2005). According to Goszczynska *et al.*, (2000), it is imperative to determine pathogenicity and implement Koch’s postulate in the identification of phytopathogenic bacteria. Once the pathogenicity of a bacterial isolate has been established, several characterisation techniques can be used. Hypersensitive reaction on tobacco leaves indicates the pathogenic nature of the tested bacterium, but it is not a substitute for pathogenicity test on susceptible host plants (Latorre and Jones, 1979). In the study done by Wreikat *et al.*, (2006), four weeks old tomato seedlings were rubbed with a water suspension of carborundum, then the leaves were sprayed with a *P. syringae* pv. *tomato* bacterial suspension of $10^7$cfu/ml.

### 1.10.4 BIOLOG

According to Mafham *et al.*, (2002) primarily, the GN2 microplate was initially created for the quick identification of Gram-negative bacteria, but it can also be used for evaluating functional variety of natural microbial communities. The Biolog GN microplates contain a redox tetrazolium dye, which changes color as a result of cellular respiration providing a metabolic fingerprint. The 96 well GN microplate comprises of 95 substrate containing wells and one without a carbon source which serves as a negative control. Shenge *et al.*, (2008) stated that the Biolog technique correctly identified all the *P. syringae* pv. *tomato* isolates to the species level, but it was vague for the differentiation of the pathovar. Only 41% of the isolates were correctly identified to the pathovar level. In the study by
Horemans et al., (2013) it was reported that, the assay is a great tool for exploring interactions between bacterial strains.

1.10.5 Enzyme-linked immunosorbent assay (ELISA)

Engvall and Perlmann (1971) originally described the ELISA method. The method uses antibodies and is one of the most commonly used serological diagnostic procedure (Clark and Adams, 1977). The ELISA assay is typically performed in 96-well polystyrene plates, which binds antibodies and proteins. There are different types of ELISA’s namely direct, indirect, sandwich and competitive and all are modified from the basic technique. Two antibodies are used by this assay namely; monoclonal and polyclonal antibodies (De Boer et al., 1988; Westra et al., 1994). The monoclonal antibody is sensitive and specific, but its limitations are it is expensive, can be overly specific and cannot detect all the strains in the population of the target bacterium (Lin et al., 1987). For bacteria, the sensitivity is about $10^5$ to $10^6$ cfu/ml, lower concentration can make the targeted organism difficult to detect and the sensitivity varies depending on the organism and sample freshness (Gudmestad et al., 1991). The ELISA method have been extensively used to detect the presence of the phytotoxin, coronatine produced by *P. syringae* (Sreedharan et al., 2009; Zhao et al., 2001). Furthermore, the assay is cheap, simple and suitable for processing numerous samples. Many ELISA kits have been developed for the identification of bacteria and have been used in numerous protocols (Nolasco et al. 2002).

1.10.6 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was first discovered by Mullis (1983). The assay is a powerful tool that it has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. The assay is extensively used to detect and identify plant pathogens (Mumford et al. 2006). The technique makes numerous copies of a particular region of DNA and uses a single primer set (targeting a specific gene) to detect an organism. Specific primer set for specific species can detect the targeted organism in the presence of other organisms. Specific primers play a major role in confirming the
absence or presence of target microorganism or specific features such as virulence factors (Anderson et al., 2004; Bergeron and Ouellette 1998;). Zaccardelli et al., (2005), the results showed that the PCR protocol was suitable for the specific detection of *P. syringae* pv. *tomato* in pure culture and in symptomatic and asymptomatic plant materials. According to (De Boer et al., 1995; Hass et al., 1995; Karjalainen et al., 1995) numerous plant pathogenic bacteria such as *Pseudomonas*, *Xanthomonas*, *Clavibacter*, *Agrobacterium* and *Erwina* has been detected by the use of PCR.

### 1.10.7 Multiplex-PCR

Multiplex PCR is a modification of normal PCR that uses multiple primers within a single PCR mixture to detect, identify and/or differentiate bacteria; therefore, it is not labour intensive (Chamberlain and Chamberlain, 1994). The technique amplifies more than one target sequence in a reaction to produce amplicons of varying sizes specific for different DNA sequences (Adzitey et al., 2013). One of the downfalls of using this type of PCR is using multiple primers on multiple templates may cause inefficient binding of some primers to their templates (Elnifro et al., 2000). Moreover, multiplex PCR assay have been largely applied for the detection of numerous pathogens including *P. syringae* (Bertolini et al., 2003; Glick et al., 2002; Menzel et al., 2002; Ozdemir, 2009).

### 1.10.8 Repetitive extragenic palindromic PCR (REP-PCR)

Repetitive extragenic palindromic PCR (REP-PCR) has been recognized as one of the most effective technique for bacterial strain typing. The method is based on PCR amplification of regions between short interspersed repetitive elements that are dispersed throughout the genome of prokaryotes and eukaryotes (Versalovic et al., 1991). The technique uses three specific primers; Repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus sequence (ERIC) and a subunit of the Box element (Box) (Martin et al., 1992). Several studies have shown the differential potential of rep-PCR for different *P. syringae* pathovars (Kaluzna et al., 2010; Louws et al., 1994; Vicente and
Roberts, 2007). The distribution of the repetitive patterns of the sequences differs from one bacterium to another.

Marques et al., (2000) reported that BOX-PCR independent of other rep-PCR primers, outlined *P. syringae* genospecies whilst Mondal and Mani (2009), stated that ERIC primer is the most effective method in determining the genetic diversity among a population of many bacterial plant pathogenic genera, including *Xanthomonas* and *Pseudomonas*. In a study by Min et al., (2012), genetic differentiation of *P. syringae pv. tomato* was checked against other *Pseudomonas syringae* pathovars such as *maculicola, glycinea, mori, mellea, pisi, tabaci* and *panici*. Genomic fingerprinting was carried out according to the methods of Rep-PCR with BOX, ERIC, and REP primers of Louws et al. (1994) and different primers generated different band sizes of DNA fingerprints.

1.10.9 16S ribosomal RNA

16S ribosomal RNA sequences have been lengthily used in the classification and identification of bacteria. The gene is a vital gene and is found in all organisms, it is a common target for sequencing studies because of the large database used for comparison (Santos and Ochman, 2004). Numerous species have been renamed and reclassified based on the use of 16S rRNA (Woo et al., 2008). Using 16S rRNA followed by DNA sequencing allows the amplification and identification of bacterial DNA (Harris and Hartley, 2003; Woo et al., 2008). After sequencing, the sequences are compared with known nucleotide sequences on numerous databases such as Genbank in order to identify the bacteria (Drancourt et al., 2000; Harris and Hartley, 2003). Recent studies have shown that the use of *gyrB* is the best alternative to using 16S rRNA gene for phylogenetic studies of *Pseudomonas* species (Yamamoto et al. 2000).

1.10.10 Multilocus sequence typing (MLST) and Multilocus sequence analysis (MLSA)

Phylogenetic analysis using MLST has become an integral tool in bacterial evolution analysis studies. The MLST technique differentiate bacterial isolates below species level by using several gene regions (Maiden et al., 1998).
According to Szabo (2014), MLST is a highly discriminatory technique which characterizes bacterial isolates since it uses approximately 400-500 bp fragments from 7 housekeeping genes to investigate the genetic diversity among bacterial isolates. Some of the housekeeping genes used in this method are; DNA gyrase beta subunit (\textit{gyrB}), RNA polymerase sigma 70 subunit (\textit{rpo}), RNA polymerase beta subunit (\textit{rpoB}), citrate synthase (\textit{cts}) and glyceraldehyde-3-phosphate dehydrogenase (\textit{gapA}) (Berge \textit{et al.}, 2014; Yamamoto 2010). In the study by Sarkar and Guttman (2004), in a core genome of \textit{P. syringae} a low level of recombination was observed. Kaluzna \textit{et al.}, (2010), observed that MLST presented the highest discrimination among the \textit{P. syringae} strains studied, especially in the case of \textit{P. s. morsprunorum} race 2, as compared to both rep-PCRs (BOX and ERIC). In the study by Gardan \textit{et al.}, (1999), nine genomospecies were identified by DNA-DNA hybridization and were later reflected to phylogroups based on housekeeping genes. To date, thirteen phylogroups have been identified within the \textit{P. syringae} species complex. \textit{P. syringae} pv. \textit{tomato} belongs to phylogroup 1, \textit{P. syringae} pv. \textit{syringae} to phylogroup 2, \textit{P. viridiflava} to phylogroup 7 and \textit{P. cichorii} to phylogroup 11. These phylogroups were obtained from using MLST of four housekeeping genes; \textit{cts}, \textit{gyrB}, \textit{rpoD} and \textit{gapA} (Berge \textit{et al.}, 2014).

MLSA is the preferred method for establishing the phylogeny between species and genera (Gomila \textit{et al.}, 2015). The MLSA approach is a robust technique used to determine whether a particular isolate belongs to a previously described species, or whether it represents a new species. The MLSA method has been extensively used to classify a diverse and previously undescribed group of prokaryotes (Gevers \textit{et al.}, 2005). Several studies have shown how powerful and reliable this method is for identifying new species within a genus (Konstantinidis and Tiedje, 2005; Martens \textit{et al.}, 2008; Yamamoto \textit{et al.}, 2000; Sarris \textit{et al.}, 2012). The taxonomical revision of \textit{P. putida} strains was based on a MLSA technique with the combined housekeeping genes (16S rRNA, \textit{gyrB}, and \textit{rpoD}); the approach was proven to be reliable for delineating species and greatly facilitated the identification of new strains (Mulet \textit{et al.}, 2012).
1.11 Control of plant diseases caused by bacteria

1.11.1 Correct farming practices

Cultural control comprises of farming practices that help to increase the quality and quantity of the yield and reduce the influence of pests and diseases. The environment is manipulated in non-mechanical ways to control pests and diseases by making the environment unfavourable for the growth of diseases and pests (Islam, 2001). Cultural practice does not provide sufficient control of the disease and have not generally been significantly implemented by commercial growers (Conover and Gerhold, 1981; Lawton and MacNeil, 1986). For effective control of bacterial pathogens, the life cycle of the pathogen and its disease cycle must be fully understood (Lozano and Wholey, 1974). The use of disease-free seeds and transplants should always be the starting point in preventing the pathogen. Seeds that are infested with the bacteria can be treated with hot water at the temperature of 50°C for approximately 25 minutes.

The shortcoming of using hot water treatment is that, it can reduce seed germination. Seeds, tools and greenhouse surfaces can be disinfected by using chlorine or hydrochloric acid (LeBeouf et al., 2005; Saha et al., 2016). During the production of transplants, sanitary measures play a major role in the production of healthy transplants. Good sanitary measures may include; removal of weeds, sterilization of tools, removing infected material from the greenhouse and controlling the relative humidity. It is very important to minimise contact between seedlings, therefore spacing between plants is crucial and minimal handling of seedlings in greenhouses is advised. Bacterial speck of tomato is disseminated by wind-driven rain, therefore it’s important to minimise leaf wetness and substitute overhead irrigation with furrow irrigation. Handling of plants whilst wet should be minimized and plants should be allowed to dry before transporting to the field (LeBoeuf et al., 2005). Symptomatic seedling trays close or opposite to the trays which contain infected plants should be immediately removed. Rotation with non-solanaceous crops is vital, a minimum crop rotation of 2 years is recommended for bacterial speck (Jones et al., 2014).
1.11.2 Chemical control

Copper has been extensively used in the agricultural sector since early 1800’s for combating bacterial diseases. Many studies have demonstrated the efficacy of copper compounds (Conlin and McCarther, 1983; Cooksey, 1988;) this control method is inefficient (Bashan, 1997). This is mainly due to pathogen having developed resistance to copper compounds (Pernezny et al., 1995). Applying copper bactericides frequently may result in the emergence of copper resistance of bacterial speck pathogen (Cooksey, 1990). Despite the development of copper resistant bacterial strains, copper compounds are routinely used as a standard treatment to manage foliar diseases. It has been reported that the efficacy of copper products increases if mixed with the fungicide, mancozeb. Copper and mancozeb are protectant products, so treatments should be applied before symptoms appear and should be applied on frequently to slow disease progress (Agrios, 1997; Jones et al., 2014; Keinath, 2012; LeBoeuf et al., 2005). McLeod et al., (2017) noted that copper+ mancozeb were ineffective in managing bacterial speck of tomato in the Limpopo province, South Africa.

In the study by Jones and Jones (1988), it was reported that copper containing chemicals applied alone or in combination with other fungicides, resulted in excellent control of bacterial speck of tomato. In another experiment, frequency of application was evaluated, and it was noted that copper sprays applied twice a week provided better disease control than once weekly applications. Conlin and McCarther (1983), reported that both streptomycin and copper compounds provided sufficient control of bacterial speck of tomato since lesions on foliage and fruit was greatly reduced and this spray program was to be included in the tomato transplant spray program. However, disease control did not result in increased yields. Streptomycin, an antibiotic and copper are regarded as the most effective and commonly used agents for control of bacterial speck with tomatoes (Conlin and McCarther, 1983; Bonn and Lesage, 1984). Streptomycin lost its effectiveness due to the emergence of resistant strains in the 1960s (Thayer and Stall, 1961). The use of antibiotics has been reported to be ineffective and most countries have banned its use in controlling foliar diseases. Long term use of these biocides has induced undesirable pathogen resistance (Bower and Daeschel, 1999; Louws et al., 2001). In a study done by Louws et
(2001) it was observed that copper resistant *P. syringae pv. tomato* strains were prevalent in all fields consistent with many grower observations that copper sprays were not effective to limit bacterial speck incidence. It was further demonstrated that copper applications can increase bacterial speck incidence. Based on the findings, farmers will need to rely on other methods to reduce or eliminate bacterial speck.

According to Koller (1998), copper ions are not degraded in soil and build-up to high levels in areas with a history of intensive copper application. The use of copper has many shortcomings such as toxicity, environmental impact and reduced copper sensitivity among strains. The residues of these agents in soil and food are harmful to the environment and human health. High levels of copper in the soil may cause plant stress and reduce soil fertility and that may lead to having adverse effects on crop yield and quality (Dumestre *et al.* 1993).

### 1.11.3 Biological control

Chemical use is associated with many disadvantages hence many strategies are being employed to substitute the use of chemicals. One of the major problems with using chemicals as a mode of controlling diseases, is the development of resistance of pathogen strains to chemicals. The increasing incidences of resistance to copper bactericides by many pathogens has promoted interest in the development of biocontrol agents against foliar bacterial diseases. Biological control offers a striking alternative to chemical use. The strategy reduces inoculum density or virulence (Baker and Cook, 1974). This method is environmentally friendly and has been reported to control several bacterial diseases such as crown gall caused by *Agrobacterium tumefaciens* (Clare, 1993) and fire blight of pear caused by *Erwinia amylovora*, (Lindow *et al.*, 1996).

Foliar biological control agents such as *P. syringae* strain Cit7; *Pseudomonas Xuorescens* strain A506 and *P. putida* strain B56 are frequently used (Wilson *et al.*, 2002). In the study by Wilson *et al.*, (2002) it was observed that while these foliar bacterial strains have been shown to provide protection against both bacterial speck and bacterial spot of tomato, only a moderate level of disease control was achieved (Byrne *et al.*, 2005; Wilson *et al.*, 2002). In the study by Ji
et al., (2006), the results obtained indicated that the foliar biological control agent *P. syringae* strain Cit7 was the most effective of the three-biological control agent, providing significant suppression of bacterial speck and bacterial spot. When PGPR strain *P. fluorescens* 89B-61 was applied as a seed treatment and soil drench. It significantly reduced foliar severity of bacterial speck. Combined use of foliar biological control agent Cit7 and 89B-61 provided significant control of both bacterial speck and spot.

1.11.4 Activation of natural plant defences

Systemic Acquired Resistance is a signal transduction pathway that plays an important role in the ability of plants defending themselves against pathogens (Ryals *et al*., 1996). As a result, plants develop necrotic lesions from the hypersensitive response (HR), which is a signal of the activation of SAR pathway (Ryals *et al*., 1996; Ward *et al*., 1991). Plants such as tobacco (*Nicotiana tabacum* L), cucumber (*Cucumis savitis* L.) and *Arabidopsis thaliana* function as model plants to elucidate the biochemical, genetic and molecular mechanisms of SAR (Sticher *et al*., 1997). This strategy of activation of natural plant defences through SAR has been widely used to control many bacterial and fungal diseases (Louws *et al*., 2001), as molecules that activates systemic resistance are elicited, thus protecting tissues against subsequent attack from a wide range of pathogens (Hammond-Kosack and Parker, 2003).

SAR is an environmentally friendly strategy used for controlling plant diseases. SAR is biologically induced however some chemicals can trigger it including salicylic acid and its synthetic analogues such as acibenzolar-S-methyl (ASM) which was developed by Syngenta (Kessmann *et al*., 1996). Acibenzolar-S-methyl (ASM) is an active ingredient of Actigard™, which is a product that induces SAR and has proven useful to limit field incidence of bacterial speck and spot in tomato production in the field (Abbasi *et al*., 2002; Louws *et al*., 2001; Wilson *et al*., 2002). It has been shown to slow the development and spread of spot and speck in the field when applied on a 7 to 14-day schedule, beginning 1 week after transplanting (Saha *et al*., 2016). ASM has no antimicrobial activity and has been reported to protect monocotyledonous and dicotyledonous plant species against many diseases, those caused by bacteria are included (Buonaurio *et al*., 2002;
Brisset et al., 2000; Cole, 1999; Friedrich et al., 1996; Lawton et al., 1996; Romero et al., 2001; Werner et al., 2002).

In a study done by Louws et al., (2001) it was noted that, the integrated use of seed treatments, transplant treatments, and field use of Actigard reduced losses due to bacterial speck. Induced resistance is a transient phenomenon more especially in open field conditions, more applications of ASM are needed to increase the efficacy and duration of the crop protection. Buonaurio et al., (2002) reported that, ASM alone or in combination with copper hydroxide slightly increased marketable yield furthermore, some parameters of fruit quality such as Brix values, pH and colour were not affected in ASM treated plants. According to Anfoka (2000), ASM may be able to control the infections of other important diseases such as *Clavibacter michiganensis* subsp. *michiganensis* and *Fusarium oxysporum* sp. *radices* (Benhamou and Belanger, 1998; Werner et al., 2002).

1.11.5 Silicon

In the earth’s crust, silicon (Si) is the second most abundant element after oxygen comprising up to 70% of soil mass (Epstein, 1994; Ma and Yamaji, 2006; Savant et al., 1997). Initially silicon was not recognized as an essential nutrient, but it plays a major role in plant growth and production. Several studies have shown the effectiveness of Si in controlling several fungal and bacterial diseases (Epstein, 1999; Fauteux et al., 2005; Rodrigues and Datnof, 2015). Si accumulation varies considerably among plant species, it was reported that dicots are poor accumulators however positive results against biotic and abiotic stress following application have been observed. According to Datnofft et al. (2007), Si application has many beneficial properties including enhanced yield, growth, plant production, structure design (height, stature, root penetration into the soil, photosynthetic capacity, resistance to environment, and tolerance to frost).

In the study by Diogo and Wydra (2007), tomato genotypes were treated with potassium silicate solution (K<sub>2</sub>SiO<sub>2</sub>) substrate against bacterial wilt which is caused by *R. solanacearum*, and it was observed that the disease incidence was reduced by 38.1% and 100% respectively in moderate resistant tomato and the
resistant genotype grown under growth chamber condition. A reduction of 57.8% of wilt severity was obtained in the moderately resistant tomato cv. King Kong 2 when it was treated with silicon fertilizer (Ayana et al., 2011). In the study by Xue et al., (2010), it was reported that Si could control bacterial blight caused by Xanthomonas oryzae pv. oryzae in rice (Oryza sativa L.) grown hydroponically.

1.11.6 Resistant varieties
According to the ‘Gene-for-gene’ hypothesis (Flor, 1956), for each resistance gene in the host there is a corresponding avirulence gene in the pathogen conferring resistance and vice versa. Many studies have reported that the development of resistant cultivars is one of the most effective and environmentally friendly strategies for controlling bacterial speck (Blancard 1997; Hulbert et al. 2001; Yu et al.,1995). Using chemicals for controlling bacterial speck remains expensive, therefore the use of resistant cultivars serves as a better alternative for controlling the disease. Pedley and Martin, (2003), reported that some processing tomato cultivars are resistant to race 0 strains of P. syringae pv. tomato but there are no cultivated cultivars that are resistant to race 1. Resistance to race 0 strains of P. syringae pv. tomato is controlled by a single resistance gene, Pto. The Pto gene, recognizes either of two pathogen effectors namely AvrPto or AvrPtoB. The Pto gene was originally discovered in Lycopersicon pimpinellifolium L., a wild tomato species, and was isolated using Map-based cloning (Pitblado et al., 1984).

The interaction between wild tomatoes (Solanum Lycopersicon) and P. syringae pv. tomato has been reviewed broadly and extensively characterized at the molecular level, therefore is ideal for evolutionary studies (Sessa and Martin, 2000). Bakir et al., (2012) tested the reaction of commonly grown hybrid cultivars in the Aegean region against bacterial speck of tomato and disease symptoms were observed in all cultivars seven days after inoculations. In another study of Turgut and Basim (2013), cultivars grown in the field and greenhouses in Turkey were also tested against bacterial speck and it was noted that 7 cultivars were resistant to the disease out of 93 cultivars tested. Many cultivars have been bred
for resistance to bacterial speck of tomato such as; Ontario 7710, Tosporodo, Ontario 7611, Ontario 782, Ohio 7870 (Berry and Gould, 1982).

1.12 References


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Chapter 2

Physiological and biochemical characterization of fluorescent *Pseudomonas* species causing foliar diseases of tomato in South Africa

Abstract

*Pseudomonas syringae pv. tomato* is a causal agent of bacterial speck of tomato, an economically important foliar disease of tomato. Heavy economic losses are experienced by the tomato seed and plantlets industry under favorable conditions. In this study, 44 strains of *Pseudomonas spp.* isolated predominantly from tomatoes exhibiting speck-like symptoms in different provinces of South Africa between 1991 and 2015 were characterized using physiological and biochemical techniques. All the tested strains were Gram negative and most strains produced a fluorescent blue pigment on King’s B medium. Thirty-seven strains belonged to LOPAT group I (*P. syringae*), five to LOPAT group II (*P. viridiflava*) and two to LOPAT group III (*P. cichorii*). On Tryptic soy agar (TSA) medium, strains of *P. syringae* produced smooth, mucoid and creamy white colonies whilst those of *P. viridiflava* and *P. cichorii* were distinguished by slightly yellowish colonies. Most strains of *P. syringae* induced bacterial speck-like symptoms in inoculated tomato seedlings cv. Red Khaki in a greenhouse-conducted pathogenicity test. Brown to black lesions were surrounded by a chlorotic yellow. Symptoms of *P. viridiflava* included wilting, yellowing and stem necrosis. LOPAT group III strains were non-pathogenic. A 689-bp fragment was amplified in all strains when COR1/2 primers were used. Physiological and nutrient-based tests cannot differentiate *P. syringae* to the pathovar level. It is necessary to use molecular techniques to identify those strains.

Keywords: *Pseudomonas*, Bacterial speck, Coronatine, Pathogenicity
2.1 Introduction

In South Africa, tomato (Solanum lycopersicum L.) is the second most important vegetable crop after potatoes (Solanum tuberosum L.). Tomatoes are grown by small- and large-scale farmers for domestic and export purposes (DAFF, 2016). Bacterial speck of tomato is caused by Pseudomonas syringae pv. tomato (Okabe 1933; Young et al., 1978). The disease is one of the most important foliar disease in different tomato production areas and can cause substantial yield losses when conditions are favourable (Blancard, 1997; Devash et al., 1980; Okabe 1933; Young et al., 197; Yu et al., 1995). The disease may occur anytime during the growing season but is more severe under low temperatures (18-24°C) and high humidity (Jones, 1991). The bacterium enters the host through stomata and bases of leaf trichomes and multiplies in the intercellular spaces (Bashan et al., 1981, Preston, 2000). Pseudomonas syringae pv. tomato is disseminated via infested seed and has the ability to survive in infected seed or transplants, plant debris or remain as an epiphyte on weeds (Jones et al., 2014; Louws et al., 2001; Wilson et al., 2002). The disease hampers the tomato production worldwide (Shenge et al., 2007) and both Department of Forestry and Fisheries (DAFF) and the Agricultural Research Council (ARC) have listed it as present in South Africa (DAFF 2016).

Bacterial speck symptoms may appear on all aerial plant parts, including leaves, fruits, stems and flowers. Small, round dark water-soaked spots with or without a yellow halo are the first symptoms (Jones, 1991). The yellow halo is due to the phytotoxin coronatine, which plays a role in virulence of this bacterium. As the disease progresses, the spots coalesce forming necrotic lesions. On fruits small, brown to black, slightly raised spots (1-3mm in diameter) are formed. On stems elongated, black lesions may appear on stems. Bacterial speck symptoms on tomato may often be confused with those of bacterial spot, caused by Xanthomonas species (Cuppels et al., 2006).

Pseudomonas syringae is a Gram-negative bacterium that causes a wide range of diseases in several plant species (Alfano and Collmer, 1996; Hirano and Upper, 2000). P. syringae is genetically diverse and is divided into more than 64 pathovars and 9 genomospecies, based on the host range and pathogenicity
Pseudomonas syringae is a Gram-negative rod belonging to the Gammaproteobacteria. These bacteria are usually fluorescent on iron deficient media such as King’s B (King et al., 1954), produce levan formations on sucrose-rich media, are oxidase and arginine dihydrolase negative, do not rot potato and induce a hypersensitivity reaction in tobacco (Nicotiana tabacum L.) (Palleroni, 1984), placing them in the LOPAT group I of Lelliott and Stead (1987). Although a large number of plant species can be infected by P. syringae, each strain demonstrates a certain degree of host specificity, for example P. syringae pv. tomato prefers to attack tomatoes (Bull and Koike, 2015).

The purpose of this study was to characterise South African strains of fluorescent Pseudomonas isolated from diseased tomatoes between 1991 and 2015 by using morphological, phenotypic and biochemical methods. Strains were obtained from the South African National Culture Collection of Plant Pathogenic and Plant Protecting Bacteria (PPPPP) at the Agricultural Research Council, Plant Health and Protection (ARC-PHP).

2.2 Materials and Methods

2.2.1 Bacterial strains

Forty-four strains used in this study are listed in Table 2.1. Strains were obtained from the Plant Pathogenic and Plant Protecting Bacteria (PPPPP) National Collection at the Agricultural Research Council, Plant Health and Protection (ARC-PHP) in Pretoria, South Africa. The strains were isolated from diseased tomato plants exhibiting speck-like symptoms in different provinces of South Africa between 1991 and 2015. Out of the forty-four strains; thirty were obtained as Pseudomonas syringae, pv. tomato, seven as P. syringae pv. syringae, five P. viridiflava and two strains were P. cichorii. Reference strains of P. syringae pv. tomato, P. syringae pv. syringae, P. viridiflava and P. cichorii were obtained from the Française de Bactéries Phytopathogènes (CFBP), France (Table 2.2).

Stock cultures of all isolates were stored in nutrient glycerol yeast extract broth (0.8g nutrient broth, 15ml glycerol, 0.2g yeast extract, 0.5g glucose in 100ml distilled water) at -80°C. The growing cultures were recovered on King’s B
medium (King et al., 1954). The medium contained 20 g proteose peptone No. 3; 1.5 g K$_2$HPO$_4$; 1.5 g MgSO$_4$. 7H$_2$O; 15 ml glycerol and 15.0 g agar in 1 litre distilled water. Inoculated plates were incubated at 28°C for 48 hours. Cultures were routinely checked for purity and colony characteristics.

Table 2.1: Bacterial strains used in the study obtained from the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) National Collection at the Agricultural Research Council, Plant Health and Protection (ARC-PHP) in Pretoria, South Africa

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Stored as</th>
<th>Host</th>
<th>Plant part isolated from</th>
<th>Province isolated from</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD 0001</td>
<td><em>P. syringae</em> pv. <em>tomato</em></td>
<td>Tomato</td>
<td>Fruit</td>
<td>Gauteng</td>
<td>1991</td>
</tr>
<tr>
<td>BD 0002</td>
<td><em>P. syringae</em> pv. <em>syringae</em></td>
<td>Tomato</td>
<td>Fruit</td>
<td>Gauteng</td>
<td>1994</td>
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<td>BD 0022</td>
<td><em>P. syringae</em> pv. <em>syringae</em></td>
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<td>Leaf</td>
<td>Gauteng</td>
<td>1995</td>
</tr>
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<td>BD 0028</td>
<td><em>P. syringae</em> pv. <em>tomato</em></td>
<td>Tomato</td>
<td>Seed</td>
<td>Gauteng</td>
<td>1995</td>
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<td>North-West</td>
<td>1996</td>
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<td><em>P. syringae</em> pv. <em>tomato</em></td>
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<td>Stem</td>
<td>North-West</td>
<td>1996</td>
</tr>
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<td><em>P. syringae</em> pv. <em>tomato</em></td>
<td>Tomato</td>
<td>Fruit</td>
<td>North-West</td>
<td>1996</td>
</tr>
<tr>
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<td><em>P. syringae</em> pv. <em>tomato</em></td>
<td>Tomato</td>
<td>Leaf</td>
<td>Limpopo</td>
<td>1997</td>
</tr>
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<td><em>P. syringae</em> pv. <em>tomato</em></td>
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<td>Stem</td>
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<td>1998</td>
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<td>Stem</td>
<td>Limpopo</td>
<td>1998</td>
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<td>Strain number</td>
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<td>Plant part isolated from</td>
<td>Province isolated from</td>
<td>Year of isolation</td>
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Table 2.2: Reference strains used in the study obtained from the Collection Francaise de Bacteries Phytopathogenes (CFBP), France.

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<td>CFBP 2101</td>
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<td>P. cichorii</td>
<td>2007</td>
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</table>

2.2.2 Morphological tests

2.2.2.1 Colony morphology on different growth media

Colony morphology was assessed on two media. King’s B (KB) was described by King et al. (1954) for the non-selective isolation and pigment production of *Pseudomonas* species. Tryptone soy agar (TSA) is a non-selective growth medium. Bacterial strains were streak-plated on KB and TSA (Difco™, Le Pont de Claix, France). The inoculated plates were incubated at 28°C for 48–72 hours. The colony morphology was evaluated visually on both media. Plates of KB were observed under a long wave (350 nm) ultraviolet light for presence of fluorescent pigment.
2.2.2.2 Gram staining and KOH solubility test

The Gram staining procedure (Gram, 1884) and the KOH solubility test were done as described by Goszczynska et al. (2000). To determine the KOH solubility, a drop of 3% aqueous solution of potassium hydroxide (3% aq., w/v) was placed on a microscope slide. A single colony was removed from the TSA medium using a cool, sterile inoculation loop and mixed with the 3% KOH solution on the microscope slide until an even suspension was obtained. The loop was lifted from the slide and if a mucoid thread was observed it was considered a Gram-negative bacterium. When a watery suspension was produced it was considered a Gram-positive bacterium.

2.2.3 LOPAT

The LOPAT test is used to distinguish pathogenic from non-pathogenic fluorescent Pseudomonads and further divides strains into five groups (I-V) (Lelliott and Stead,1987; Schaad et al., 2001). The LOPAT test consists of levan production, oxidase reaction, potato soft rot test, arginine dihydrolase and tobacco hypersensitive reaction. A 24-48 hours old pure cultures of bacterial isolates listed in Table 2.1 were used in the LOPAT tests.

(i) Levan production

The levan test shows the ability of a bacterial isolate to produce polysaccharides on a sucrose-rich medium. Colony characteristics were determined on nutrient agar (3g Beef extract (Difco), 5 g peptone (Difco) 15 g agar in 1 L distilled water) supplemented with 5% sucrose Nutrient Sucrose Agar (NSA) (Lelliot et al., 1966). Bacterial strains were streaked onto plates containing NSA using a cotton swab and incubated for 3-5 days at 28°C. Levan was produced when colonies were convex, domed, white and mucoid (Lelliot and Stead, 1987).

(ii) Oxidase reaction

The test for oxidase reaction was done according to the method of Kovacs (1956). A 1% aqueous solution of NNN'N'- tetramethyl-p-phenylene-diamine-
Dihydrochloride was prepared in a sterile petri dish. Cotton swab was dipped into the solution and then used to pick the bacterial growth from the TSA medium plates. The production of a purple colour within 10 seconds was recorded as a positive reaction, within 10-60 seconds, as delayed positive and the absence of coloration as a negative reaction.

(iii) Potato soft rot
Fresh potato tubers were washed, peeled, alcohol flamed, and sliced into approximately 7mm- 1 cm width (one slice for each strain and one slice for control). The slices were dipped in alcohol and flamed, placed in 90 mm Petri-dishes, then sterile distilled water was added to a depth of half the slice of the potato. A well in the centre of each slice of potato was made with a sterile blade. The well was spot inoculated with a loopful of a 24-hour-old bacterial culture. Positive results were indicated by rotting beyond the point of inoculation, while lack of rotting indicated negative results as described by Goszczynska et al. (2000).

(iv) Arginine dihydrolase
Tubes containing arginine medium (1 g peptone, 5 g NaCl, 0.3 g K₂HPO₄, 10 g L-(+) arginine HCl, 0.01 phenol red, 15 g agar in 1 L distilled water) were stab-inoculated with a 24 hr-old bacterial culture grown on the TSA medium and covered with sterile mineral oil. The arginine-dihydrolase enzyme activation needs culture medium acidification, for which the bacterium must first use the glucose present in the medium causing a pH drop (indicated by a medium colour change from yellow to pink). Color changes were recorded after incubation at 26°C for 24-48 hrs. The color change from yellowish orange to pink/red was considered positive (Schaad et al., 2001).

(v) Tobacco Hypersensitive Response (HR) Test
Hypersensitivity test was conducted in tobacco (Nicotiana tabacum cv. Samsun) leaves according to the method of Klement (1963). A 24-hr-old bacterial culture
from TSA plate was suspended in sterile distilled water making the concentration of approximately $10^6$-$10^7$ CFU/ml. The concentration was confirmed by spread plating ten-fold serial dilutions of a suspension on TSA plates and counting the number of colonies after incubation at 28°C for four days. The suspension was infiltrated into the lower surface of a mature tobacco leaf, forcing the suspension into the leaf. Distilled water was used as a negative control. Plants were kept in a greenhouse at 24°C/20°C day/night temperatures. Positive reaction was observed when the infiltrated area became dry and necrotic after 24 hrs and negative reaction was observed when the infiltrated area was not dry and necrotic after 24 hrs. A hypersensitivity reaction is triggered in a tobacco plant when inoculated with pathogenic bacteria that are not pathogens of tobacco (Klement and Goodman, 1967). This reaction was used as diagnostic tool for identification of fluorescent pathogenic *Pseudomonas* (Lozano and Sequeira, 1970).

2.2.4 Utilization of single carbon sources
Bacterial strains listed in Table 2.1 were evaluated for their ability to utilise eleven different carbon sources, namely glucose, sucrose, sorbitol, mannitol, erythritol, lactose, inositol, fructose, D (-) tartrate, L (+) tartrate and DL-lactate. The minimal medium was prepared by adding NH$_4$H$_2$PO$_4$ (0.5 g), KCl (0.1 g), MgSO$_4$.7H$_2$O (0.1 g) to 500 ml distilled water. The indicator, 0.5 ml bromothymol blue (1.6 % aq.) was added, stirred for 10 min and the pH adjusted to 7.2. Bacteriological agar (12.0 g) was then added to the medium. The medium was autoclaved at 121°C for 15 min and allowed to cool down to 50 °C after autoclaving. Individual carbohydrates were prepared separately by dissolving 0.5 g of a carbohydrate in 5ml of distilled water. Each carbohydrate solution was filter sterilised and mixed with the cooled medium (Goszczynska et al., 2000). The carbohydrate utilisation test was performed by streak inoculating a loopful of 24 hr old bacterial culture grown on TSA plates. The inoculated media plates were incubated for 72 hrs at 28°C. Carbohydrate utilisation was indicated by growth of the inoculated bacterium on the medium containing the single carbon source.
2.2.5 Pathogenicity Test

Four (4) wk old tomato seedlings (cv. Red khaki) were dusted with an abrasive substance, carborundum. Carborundum was used to create wounds in leaves, allowing the bacterial suspension to enter the tissues. The plants were inoculated by spraying both surfaces of a leaf with $10^7$ cfu/ml bacterial suspension. Concentration of the bacterial suspension was confirmed by preparing serial ten-fold dilutions and spread-plating 0.1 ml of each dilution on King’s B medium plates and incubated at 28°C for 2 days before counting the number of colonies. A hand-held sprayer was used to spray the plants until suspension ran off. After inoculation, plants were covered with polyethylene plastic bags for 24 hr to contain the moisture. Plants were kept in the glasshouse with 24°C/20°C day/night temperatures and 65-75% relative humidity. Control plants were sprayed in the same manner with sterile distilled water. Disease development on tomato leaves was assessed seven days after inoculation. Re-isolations of bacteria on TSA and King’s B media were made from the plants. The identity of isolated bacteria was confirmed by observing colony morphology, fluorescence under UV light, oxidase and KOH solubility tests as well as utilisation of single carbon sources thus fulfilling the Koch’s postulates (Agrios, 2005).

2.2.6 DNA extraction

Genomic DNA extraction of bacterial strains listed in Table 2.1 was conducted by using the cetyltrimethylammonium bromide (CTAB) method as described by Wilson (1989). Bacterial growth of each strain grown on TSA medium for 48 hrs was suspended in 1 ml of distilled water in an Eppendorf tube and centrifuged for 3 min at 13 000 rpm/min. Supernatant was removed, and the pellet was re-suspended in 570 µl of Sodium Chloride-Tris-EDTA (STE) buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, pH 8.0). Cells were lysed by incubation with sodium dodecyl sulfate. Cell debris and polysaccharides were precipitated with CTAB/NaCl. Chloroform/isoamyl alcohol was used to extract DNA. The DNA was precipitated with isopropanol and washed with 70% ethanol. DNA was air dried in a laminar for approximately 20 min and dissolved in 100 µl of sterile, nuclease free water (Qiagen). Purified DNA was quantified using a NanoDrop and was
adjusted to the concentration of 20 to 25 ng µl⁻¹. The DNA was stored at -20 °C until further analysis.

2.2.7 Polymerase Chain Reaction
Primers specific for the detection of coronatine-producing isolates of fluorescent *Pseudomonas* were used, Primer pair: COR1, 5′ GGA CTC AGC AGT ATC ATC TCG GGA CG 3′ and COR2, 5′ TGC AGG GTC TTG GGG AGC ACG 3′ (Cuppels *et al.*, 2006). These primers were originally developed for the specific detection of coronatine-producing isolates of fluorescent *Pseudomonas*. Coronatine is a non-host-specific phytotoxin produced by several members of the *Pseudomonas syringae* group of pathovars (Bender *et al.*, 1999). Specific primers used for the detection of *Pseudomonas syringae pv. tomato* were not able to amplify any PCR products.

PCR amplification was performed in 0.2 ml thin-walled PCR tubes in the 2720 thermal cycler (Applied Biosystems, Singapore). The method of Cuppels *et al.* (2006) was followed with amendments. Amplification of DNA was performed in a total volume of 20 µl. All reactions contained, 11.4 µl of nuclease free water, 4 µl 5X Green Go Taq Flexi buffer, 1.2 µl 25 Mm MgCl₂, 0.2 µl Go Taq DNA polymerase 500 u 5u/µl, 0.2 Mm each dNTP, 0.4 µl of the forward and reverse primer and 2 µl of DNA template. The reference strains of *P. syringae pv. tomato* (CFBP 2212), *P. syringae pv. syringae* (CFBP 1392), *P. viridiflava* (CFBP 2107) and *P. cichorii* (CFBP 2101) were used as positive controls. A negative control contained sterile nuclease-free water in place of a template DNA.

The amplification conditions were initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 94°C for 59 sec, annealing at 65°C for 45 sec, extension at 72°C for 3.5 min and the final extension at 72°C for 3 min. After amplification, the expected PCR products were stored at 4°C until electrophoresis. Electrophoresis of PCR products was performed in 1.5% agarose gel stained with 10 µl of ethidium bromide. The gel was run at 100 V for 45 min in 1×TBE buffer. Gels were observed and photographed using the BIO RAD molecular imager® Gel Doc™ XR+ with image lab™, software.
2.3 Results

2.3.1 Colony morphology on different growth media

On the TSA medium, colonies of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* were identical. The colonies were round, circular, 3-4 mm in diameter, smooth, mucoid and creamy white. Colonies of *P. viridiflava* and *P. cichorii* were similar to those of *P. syringae*. The only difference was in colour, as colonies were slightly yellowish (Fig. 2.1).

![Image of colony morphology on TSA medium](image)

Figure 2.1: Colony morphology of *Pseudomonas* strains on TSA medium; A = *P. syringae* pv. *tomato* (BD 0151); B = *P. syringae* pv. *syringae* (BD 0280), C = *P. viridiflava* (BD 0223) and D = *P. cichorii* (BD 0229).
Thirty-four isolates used in the study produced a bluish fluorescent pigment on the KB medium. However, BD 0001, BD 0002, BD 0070, BD 0071, BD 0278, BD 0774, BD 0775, BD 0779, BD 1354 and BD 1355 did not produce this fluorescent pigment. Colonies of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* were identical on KB, the colonies were 3-4 mm in diameter, smooth, round, slightly raised, mucoid and creamy white. *P. cichorii* and *P. viridiflava* strains produced non-mucoid, creamy colonies (Fig. 2.2).

![Fluorescence on KB under UV light. *P. syringae* pv. *tomato* isolates; A = (BD 1357) fluorescent and B = *P. syringae* pv. *syringae* (BD 0278) non-fluorescent under UV light.](image)

2.3.2 Gram staining and KOH solubility test

All strains listed in Table 2.1 and Table 2.2 were Gram-negative and rod-shaped (Fig 2.3). In the KOH solubility test all isolates produces a mucoid thread when the loop was slightly raised from the glass slide containing bacterial solutions.
Figure 2.3: Gram staining (A) and KOH (B) reactions of BD 0165 (*P. syringae* pv. *tomato* isolate). All strains used in the study were Gram-negative rods and KOH positive.

### 2.3.3 LOPAT tests

Based on the results obtained from the LOPAT tests, all *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* belonged to LOPAT Group Ia (Fig. 2.4.1), *P. viridiflava* to the LOPAT Group II (Fig. 2.4.2) and *P. cichorii* to the LOPAT Group III (Fig. 2.4.3; Table 2.3)

Figure 2.4.1: LOPAT tests results of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* belonging to the Lopat group Ia. A-Levan production (positive), B- Oxidase reaction (negative), C -Potato soft rot (negative), D- Arginine dihydrolase (negative) and E- Tobacco hypersensitive reaction (positive).
Figure 2.4.2: LOPAT tests results of *P. viridiflava* belonging to the LOPAT group II. A-Levan production (negative), B-Oxidase reaction (negative), C-Potato soft rot (positive), D-Arginine dihydrolase (negative) and E-Tobacco hypersensitive reaction (positive).

Figure 2.4.3: LOPAT tests results of *P. cichorii* belonging to the LOPAT group III. A-Levan production (negative), B-Oxidase reaction (positive), C-Potato soft rot (negative), D-Arginine dihydrolase (negative) and E-Tobacco hypersensitive reaction (positive).
(i) Levan production
Levan is produced through the action of the enzyme sucrase. The enzyme is mostly produced by fluorescent *Pseudomonads* that utilise sucrose as a sole carbon source. On NSA, *P. syringae* pv. *tomato* strains and *P. syringae* pv. *syringae* produced colonies that were white, convex, mucoid and domed whilst *P. viridiflava* and *P. cichorii* isolates did not produce any levan type colonies.

(ii) Oxidase reaction
*P. syringae* pv. *tomato*, *P. syringae* pv. *syringae* and *P. viridiflava* strains were oxidase negative. Two strains of *P. cichorii*, BD 0229 and BD 0245 were oxidase positive (Table 2.3).

(iii) Potato soft rot
Potato slices inoculated with strains of *P. cichorii*, *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* showed no signs of rot (Figure 2.4.1 and Figure 2.4.3). Five strains of *P. viridiflava* induced rot in potato tuber slices (Figure 2.4.2).

(iv) Arginine dihydrolase
All strains used in this study (Table 2.1) were arginine dihydrolase negative.

(v) Tobacco hypersensitive reaction
All strains used in this study produced a necrotic lesion on the infiltrated leaf area (Table 2.3).
Table 2.3: Results of the KOH solubility, LOPATs, pathogenicity test, fluorescence on KB and PCR.

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(+) = positive; (-) = negative. LOPAT tests included Levan production (L), Oxidase reaction (O), Potato soft rot (P), Arginine dihydrolase (A) and Tobacco hypersensitivity response (T). Fluorescence was evaluated on KB medium. Pathogenicity test was done on tomato plants (cv. Red Khaki). PCR was performed with primers COR1, 5’ GGA CTC AGC AGT ATC ATC TCG GGA CG 3’; COR2, 5’ TGC AGG GTC TTG GGG AGC ACG 3’ (Cuppels et al., 2006) designed for the detection of strains producing a phytotoxin, coronatine.
2.3.4 Utilisation of single carbon sources

The results of utilisation of single carbon sources are presented in Table 2.4. *P. syringae* pv. *tomato* strains and the type strain of this pathovar, CFBP 2212, utilised glucose, sucrose, sorbitol, mannitol, lactose, inositol, fructose and D-tartrate as single carbon sources. The strains did not utilise erythitol, L-tartrate and DL-lactate. Seven strains of *P. syringae* pv. *syringae* did not utilise D-tartrate and L-tartrate just like the type strain of *P. syringae* pv. *syringae*, CFBP 1392. The strains grew on a minimal medium containing glucose, sucrose, sorbitol, mannitol, erythitol, lactose, inositol and DL-lactate. *Pseudomonas viridiflava* CFBP 2107 and BD 146, BD 156, BD 223 BD 224 and BD 231 did not grow on lactose, inositol and L-tartrate. *P. cichorii* strains did not utilise sucrose, erythritol, D (-) tartrate and DL-Lactate.

Table 2.4: Utilisation of sole carbon sources by *Pseudomonas* strains used in the study.

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(+)=positive; (-)=negative
3.5 Pathogenicity Test

Thirty-seven bacterial strains isolated from diseased tomatoes in South Africa induced disease symptoms in tomato plants cv. Red Khaki (Table 2.3). The symptoms were observed seven days after inoculation. Plants sprayed with bacterial suspensions of *P. syringae* pv. *tomato* and *P. syringae pv. syringae* developed water soaked, dark brown to black spots on leaves. Spots were surrounded by yellow halos (Fig. 2.5 B and C).

*P. viridiflava* type strain CFBB 2107 and three *P. viridiflava* isolates from this study induced yellowing and wilting of leaves and stem necrosis (Fig. 2.5 D). Two isolates of *P. viridiflava*, BD 0146 and BD 0156, *P. chicorii* CFBP 2101, BD 0229 and BD 0245, one strain received as *P. syringae* pv. *tomato* and one strain of *P. syringae pv. syringae* were non-pathogenic. Tomato seedlings sprayed with sterile distilled water did not develop any symptoms (Fig 2.5 A).
Figure 2.5: Symptoms exhibited by tomato plants sprayed with: (A) sterile distilled water-no symptoms, (B) *P. syringae* pv. *tomato* strain BD 1357- necrotic spots surrounded by wide yellow halos (C) *P. syringae* pv. *syringae* strain BD 0280- necrotic spots surrounded by narrow yellow halos and (D) *P. viridiflava* strain BD 0223- leaf wilt and yellowing and stem necrosis.
2.3.6 Polymerase Chain Reaction (PCR)

The PCR results are shown in Table 2.3 and Figure 2.6. Purified DNA was amplified using coronatine primer set COR1/2 (Cuppels et al., 2016). Originally, these primers were developed for the specific detection of coronatine-producing isolates of fluorescent Pseudomonas; however, they amplified the DNA at the annealing temperature of 65°C. All the strains including strains of \textit{P. viridiflava} (CFBP 2107 and BD 0231) and \textit{P. cichorii} (CFBP 2101 and BD 0229) were able to synthesize the toxin coronatine and produced the expected 689-bp PCR product (Fig. 2.6).

![PCR products amplified from various Pseudomonas syringae isolates using primers COR1, 5’ GGA CTC AGC AGT ATC ATC TCG GGA CG 3’; COR2, 5’ TGC AGG GTC TTG GGG AGC ACG 3’ (Cuppels et al., 2006). Lane 1 is a negative control. Lane 2 is the \textit{P. syringae pv. tomato} type strain (CFBP 2212), lane 3 and 4 are \textit{P. syringae pv. tomato} strains (BD 0151 and BD 0165), lane 5 is a \textit{P. syringae pv. syringae} type strain (CFBP 1392), Lane 6 and 7 are \textit{P. syringae pv. syringae} strains (BD 0279 and BD 0280), lane 8 is a \textit{P. viridiflava} type strain (CFBP 2107), lane 9 is a \textit{P. viridiflava} strain (BD 0231), lane 10 is a \textit{P. cichorii} type strain (CFBP 2101) and lane 12 is a \textit{P. cichorii} strain (BD 0229).]
2.4 Discussion

In this study, 44 strains of fluorescent *Pseudomonas* isolated from diseased tomatoes in South Africa between 1991 and 2015 were characterised by using morphological, physiological and biochemical methods. The strains were compared with the type strains of *P. syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *P. viridiflava* and *P. cicorii*. All strains were Gram negative rods and the majority produced a diffusible fluorescent pigment on KB medium. Nine isolates of *P. syringae* pv. *tomato* failed to fluoresce on KB media. KB media is commonly used to detect fluorescent *Pseudomonas* species. However, some strains fail to produce a fluorescent pigment when cultured on this medium (Reyels *et al.*, 1981). Several studies have attempted to enhance the fluorescent production by developing new media (Dulla *et al.*, 2010). The LOPAT tests (Lelliot *et al.*, 1966) were very useful in the preliminary identification of tomato isolates. Based on Lopat tests, strains were found to belong to three groups. All *P. syringae* isolates were in the Lopat Group Ia, *P. viridiflava* was in the Lopat Group II and *P. cicorii* was in the Lopat Group III. The Lopat Group I of Lelliot *et al.* (1966) was divided into two subgroups by Sands *et al.* (1970). Subgroup Ia contained strains that were Levan and tobacco HR positive, oxidase reaction, arginine dihydrolase activity and potato soft rot negative. Strains that were Levan, oxidase, arginine dihydrolase and potato soft rot negative, and tobacco HR positive were placed in the subgroup Ib. Misaghi and Grogan (1969) used the LOPAT tests to identify 26 isolates of *P. syringae* pv. *lachrymans* from cucumbers (*Cucumis sativus*. L.). LOPAT is still used for preliminary identification of fluorescent *Pseudomonas* (Goszczynska *et al.*, 2000).

All strains of *P. syringae* pv. *tomato* and the type strain of this pathovar, CFBP 2212, utilised glucose, sucrose, sorbitol, mannitol, lactose, inositol, fructose and D-tartrate as single carbon sources. The strains did not grow on erythritol, L-tartrate and DL-lactate. The inability of *P. syringae* pv. *tomato* to utilise erythritol is one of the tests distinguishing it from *P. syringae* pv. *syringae* (Goszczynska *et al.*, 2000). *P. chicorii* strains differed from all other isolates in this study by not utilising sorbitol and growth on a minimal medium containing L-tartrate. *P. viridiflava* strains did not utilise lactose but all other isolates utilized it. Jones *et al.* (1986) suggested that sucrose, erythritol and DL-lactate are the most
important carbon sources for distinguishing *P. syringae* pv. *tomato*, *P. syringae* pv. *syringae* and *P. viridiflava*. In the study done by Serfontein and Hattingh (1990), out of 45 South African strains, one strain of *P. syringae* pv. *tomato* failed to utilize D (-) tartrate, however the strain was pathogenic on tomato. According to Goszczynska et al. (2000), utilisation of single carbon sources is useful in the identification of bacteria, especially fluorescent *Pseudomonas*. Peix et al. (2009) reported phenotypic characteristics such as carbon sources utilisation, production of antibiotics, cell shape, extracellular enzymes, antibiotic resistance and the type of flagellum are suitable to differentiate pathovars in the *Pseudomonas* genus.

In the identification of bacteria, the development of a disease symptom is one of the first indications of a potential infection (Kritzman, 1991). A slight difference in symptoms was observed between *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae*, but was barely noticeable. Spots induced by strains of *P. syringae* pv. *syringae* were slightly smaller and the yellow hallows were narrower. In the study done by Milijasevic et al. (2009), *P. syringae* pv. *tomato* strains induced small (2-mm) dark brown spots on stab-inoculated immature tomato fruits whilst *P. syringae* pv. *syringae* Ks-101 strain developed large, black sunken spots, twice in size compared to those inoculated with *P. syringae* pv. *tomato*. In our study, the tomato plants did not produce any fruits so the lesions on fruits were not observed. Three strains of *P. viridiflava* caused yellowing of lower leaves, wilting, and stem necrosis. Some strains of *P. viridiflava* and *P. cichorii* were non-pathogenic on tomato, both these *Pseudomonas* species have been reported as opportunistic pathogens and sometimes can be secondary invaders (Goumas et al., 1999). In the study by Jones et al. (1984), based on the inoculation results, *P. viridiflava* strains were reported to be weak pathogens of tomato. Jones et al. (1986), reported that *P. cichorii* is dependent on moisture and temperature ranging from 20-32°C for infection and lesion development. Furthermore, pathogenicity can be lost by consecutive sub-culturing and also environmental conditions, cultivar and the bacterial strain also have an effect on pathogenicity.

PCR with primers COR1 and COR2 (Cuppels et al., 2006) designed to detect the coronatine-producing *Pseudomonas syringae* yielded the amplicons of approximately 689 bp from all tested *P. syringae* pv. *tomato* and *P. syringae* pv.
syringae isolates. Interestingly, Strains of *P. viridiflava* and *P. cichorii* also produced an amplicon of 689 bp. Coronatine has been widely studied and is known to be produced by five *Pseudomonas syringae* pathovars namely *P. syringae* pv. *tomato*, *P. maculicola*, *P. morsprunorum*, *P. atropurpurea*, and *P. glycinea* (Shim *et al.*, 2003). Shim *et al.* (2003) found that *Pseudomonas syringae* pv. *actinidiae* strains and *Pseudomonas syringae* pv. *glycinea* strains produced coronatine. Although coronatine genes were detected in the *Pseudomonas syringae* pv. *actinidiae* strains, this does not indicate that the coronatine phytotoxin is synthesized by the strains. Most phytotoxins produced by *Pseudomonas syringae* are not host-specific and may cause symptoms on many plants that cannot be infected by the toxin-producing pathogen.

In South Korea, *P. cichorii* JBC1 was reported to be the causal agent of leaf spot on soybeans (*Glycine max* L.) (Yu and Lee, 2012), and is highly virulent. Ramkumar *et al.* (2015) found that the *P. cichorii* strain (JBC1) codes for phytotoxin coronatine which promotes *P. syringae* virulence (Zheng *et al.*, 2012). Primers specific for the detection of *P. syringae* pv. *tomato* published by Bereswill *et al.* (1994) were also used in this study but did not amplify the expected product even from the DNA of the type strain of *P. syringae* pv. *tomato*.

Based on morphological, phenotypic and biochemical test results, South African *Pseudomonas* strains isolated from tomatoes belonged to three species; *P. syringae*, *P. viridiflava* and *P. cichorii*. Physiological and nutrient-based tests, however, cannot differentiate *P. syringae* to the pathovar level (Little *et al.*, 1998). It is necessary to use molecular techniques to identify strains further.

2.5 References


Chapter 3

Molecular characterisation of fluorescent *Pseudomonas* species causing foliar diseases on tomato using REP-PCR fingerprinting and multilocus sequence analysis (MLSA)

Abstract

Fluorescent *Pseudomonads* cause a wide range of diseases in various agricultural crops. Despite being present in South Africa these bacteria remain poorly studied. This study aimed at molecular characterisation of 34 strains of fluorescent *Pseudomonas* isolated from diseased tomatoes in South Africa between 1991 and 2015. MLSA analyses of two housekeeping genes, *gyrB* and *cts*, and rep-PCR fingerprints obtained with BOXA1R and ERIC 2 primers showed that isolates from tomatoes belong to three species, *Pseudomonas syringae*, *P. viridiflava* and *P. cichorii*. within *P. syringae*, strains were found to belong to 2 phylogroups namely, phylogroup 1 and 2 (a and b). *Pseudomonas viridiflava* was found to belong to phylogroup 7 and *P. cichorii* in phylogroup 11. This is the first report of *P. syringae* pv. *papulans* and *P. syringae* pv. *dysoxyli* inducing a disease on tomatoes. The research findings in this study may help in epidemiological studies of bacterial speck and in breeding for resistance programmes.

Keywords: *Pseudomonas syringae*, Phylogenetic group, MLSA, rep-PCR
3.1 Introduction

*Pseudomonas syringae* is a common foliar bacterium which causes a wide range of important diseases on numerous hosts and is commonly found in diverse environments. Approximately, 200 crop species of economic importance are infected by *P. syringae* (Zembek *et al.*, 2018). *P. syringae* taxonomy and its pathovars have been extensively studied and greatly debated in the last 40 years (Young *et al.*, 1992). The bacterium is divided into more than 64 pathovars, based on the plant host from which they were originally isolated (Gardan *et al.*, 1999, Gomila *et al.*, 2017; Young, 2010). *Pseudomonas* pathovars cause a variety of symptoms such as water-soaking, hypertrophic growth, spots, specks, cankers, chlorosis, necrosis, soft rots, yellowing and blights (Murillo and Sesma, 2001; Kokoskova *et al.*, 2011).

*P. syringae* pv. *tomato* is a seedborne pathogen responsible for bacterial speck of tomato worldwide. It can survive on weed hosts, host debris and in soil (Chambers and Merriman, 1975; Scheneider and Grogan, 1977). The disease is favoured by cool temperatures and is disseminated by wind driven rain, overhead irrigation and handling of wet plants. Primarily, brown to black spots may be observed on leaves and after some time the spots are surrounded by a yellow halo. As the disease progresses the lesions may expand and coalesce. The disease causes yield reduction since it renders the fruits to be unmarketable and often lead to plant death when the conditions are conducive. Apart from *P. syringae* pv. *tomato*, *P. syringae* pv. *syringae* had been reported as the causal agent of bacterial speck outbreaks on tomato (Gitaitis *et al.*, 1985). Closely related *Pseudomonas* species *P. cichorii*, and *P. viridiflava* have also been identified as pathogens of tomato (Alivizatos, 1986; Malathrakis and Goumas, 1897; Mirik *et al.*, 2011; Wilkie and Dye, 1974). The symptoms vary depending on the host and environmental conditions.

Generally, *P. syringae* pathovars have been identified based on the classical bacteriological LOPAT tests (levan production, Oxidase reduction, Potato soft rot, Arginine dihydrolase and tobacco hypersensitive reaction) and pathogenicity test (Lelliot *et al.*, 1996; Stead, 1992). The LOPAT tests further divides fluorescent *Pseudomonas* strains into five groups, however it is impossible to correctly identify each of the pathovars by means of these biochemical tests.
Traditional methods of identifying bacteria such as biochemical tests, microscope and serology remains the first approach of identifying bacteria. The development of genomic methods for characterisation of bacteria over the years has greatly simplified and improved pathogen detection and identification (Varadi et al., 2017).

In the last two decades, molecular methods such as DNA-DNA hybridization, multi locus sequence typing (MLST), multi locus sequence analysis (MLSA) and 16S rRNA gene sequencing, have been a reliable aid to the identification of diverse bacteria (Cho and Tiedje, 2001). Garden et al. (1999) conducted the DNA-DNA hybridization research and determined that Pseudomonads contained nine genomospecies. Multi locus sequence analysis (MLSA) of four housekeeping genes done by Mulet et al. (2010), divided the genus Pseudomonas into two lineages namely Pseudomonas fluorescens and Pseudomonas aeruginosa. Berge et al. (2014) showed that based on MLST, the P. syringae species complex is divided into 13 phylogroups, with P. syringae pv. tomato belonging to phylogroup 1, P. syringae pv. syringae to phylogroup 2, P. viridiflava to phylogroup 7 and P. cichorii to phylogroup 11. Phylogroup 2 contains numerous pathovars such as P. syringae pv. syringae, P. syringae pv. dysoxyli and P. syringae pv. papulans, P. syringae pv. lapsa, P. syringae pv. aptata etc (Berge et al., 2014; Bull et al., 2011). This phylogroup is the most ubiquitous of P. syringae and is found in all habitats and has three subgroups 2a, 2b and 2c (Berge et al., 2014).

The present research was initiated to characterise a collection of fluorescent Pseudomonas strains from South African tomatoes by molecular methods. The methods included MLSA analyses of two housekeeping genes, DNA gyrase subunit B (gyrB) and citrate synthase (cts) and the rep-PCR genomic fingerprinting. As far as we know, this study is the first comprehensive molecular study of fluorescent Pseudomonas species infecting tomato, characterised using molecular techniques in South Africa.
3.2 Materials and Methods

3.2.1 Bacterial strains

The bacterial strains used in this study are listed in Table 3.1. Strains were obtained from the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) National Collection at the Agricultural Research Council, Plant Health and Protection (ARC-PHP) in Pretoria, South Africa. Four strains BD 1146 (CFBP 2212T), BD 1130 (CFBP 1392PT), BD 1149 (CFBP 2107T) and BD 1152 (CFBP 2101T) were obtained from the Collection Francaise de Bacteries Phytopathogenes (CFBP), France. Strains from PPPPB culture collection were isolated from diseased tomato plants exhibiting speck-like symptoms in five provinces of South Africa between 1991 and 2015. These strains have been characterised in the previous chapter by using physiological, morphological and biochemical methods.

Strains were stored in nutrient glycerol yeast extract broth (0.8 g nutrient broth, 15 ml glycerol, 0.2 g yeast extract, 0.5 g glucose in 100 ml distilled water) at -80°C. The growing cultures were recovered on King’s B medium (King et al., 1954). Inoculated plates were incubated at 28°C for 48 hr. Cultures were routinely checked for purity and colony characteristics.
Table 3.1 Forty-four strains used in the study for gene sequencing and rep-PCR fingerprinting

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<th>Gene</th>
<th>rep-PCR primer</th>
<th>Identity</th>
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<td>rep-PCR primer BOX&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> (+) gyrB sequences obtained, no accession numbers from Genbank yet; (-) gyrB not sequenced

<sup>b</sup> (+) cts sequences obtained, no accession numbers from Genbank yet; (-) cts not sequenced
Genomic fingerprints obtained using BOX A1R primer; (-) No Box A1R fingerprints
Genomic fingerprints obtained using ERIC 2 primer; (-) No ERIC 2 primer fingerprints

3.2.2 DNA extraction
Genomic DNA was extracted using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, USA) following manufacturer’s instructions. The Elution solution (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) was directly pipetted onto the centre of the column and was centrifuged for 1 min at ≥ 6500 × g in order to dissolve the DNA. Purified DNA was quantified using a Dyna Quant 200 fluorometer (Nano drop, Hoefer, San Francisco, CA, USA). The DNA was stored at -20°C until further analysis.

3.2.3 Repetitive sequence-based polymerase chain reaction (REP-PCR)
Rep-PCR was carried out using BOX A1R primer (5’-CTACGGCAAGGCGACGCTGACG-3’) and ERIC 2 primer (5’-AAGTAAGTGACTGGGGTGAGCG-3’) independently (Louws et al., 1994). Primers were synthesized by Inqaba Biotechnologies (Pretoria, South Africa). The PCR reaction contained 11.7 µl of nuclease free water, 2 µl 5X Green Go Taq Flexi buffer, 2.8 µl 25Mm MgCl2, 0.2 µl Go Taq DNA polymerase 500u 5u/µl, 0.2Mm each dNTP, 0.5 µl DMSO, 0.4 µl of each primer and 2 µl of DNA template in a total volume of 20 µl. PCR amplifications were performed in 2720 thermal cycler (Applied Biosystems, Singapore) by using the method of Cho et al. (2012) with modifications. The following parameters were used for amplifications: 1 cycle at 94°C for 3 min, then the next 30 cycles consisting of 1 min at 94°C, 65.1°C at 1 min and 3 min for 72°C and 1 cycle at 72°C for 10 min.

The amplified PCR products and Gene Ruler 1 kb marker (Thermoscientific) were analysed by gel electrophoresis on a gel containing 1% agarose in 1×TBE buffer. The gels were stained with ethidium bromide for 20 min in the dark and destained under running water, then viewed and photographed with BIO RAD molecular imager® Gel Doc™ XR+ with image lab™ software.
The resulting fingerprints were analysed using the BioNumerics V 2.0 software package (Applied Maths, Ghent, Belgium). The similarity among digitized profiles were calculated using the Pearson correlation, and an average linkage (UPGMA) dendrogram was derived from the profiles. Experiments were repeated at least three times to confirm the reproducibility of banding patterns.

3.2.4 Amplification and sequencing of gyrB and cts genes
Primers used for PCR amplification and sequencing of cts and gyrB genes were developed by Sarkar and Guttman (2004) and Yamamoto et al. (2000) respectively. The primers used were cts-Fp AGTTGATCAGGGGCWGCC, cts-Rp TGATCGGTGATCTCGCAGGG, gyrB M13-R CAGGAAACAGCTATGACC and M13(-21) TGATAAAACGACGGCCAGT. Amplification of DNA was done in 50 µl reaction volume containing PCR buffer (10 mM Tris-HCl, pH 9.0 at 25°C; 50 mM KCl, 0.1% Triton X-100); 1.5 mM MgCl₂; 150 µM dNTPs; 1.0 µM each primer; Taq polymerase (Promega Corp., Madison, WI) 1 unit per reaction volume and 25-50 ng DNA template µl⁻¹.

Amplifications were done according to Morris et al. (2008) for both genes, with the initial denaturation of 30 s at 94 °C, followed by 40 cycles of denaturation of 30 s at 94 °C, annealing of 63°C at 90 s, extension 1 min at 72 °C and final extension of 10 min at 72 °C. PCR amplifications were carried out in the ABI Applied Biosystems 2720 thermal cycler (Singapore). Amplified products were electrophoresed in 1 % agarose for 45 min at 80 V and purified by using ExoSAP PCR cleanup reagent (Affymetrix, Danta Clara, CA, USA), as per the manufacturer’s instructions. PCR products were sequenced by Inqaba Biotechnology (Pretoria, South Africa).

3.2.5 Phylogenetic analysis
The obtained sequences of gyrB and cts genes were analysed for homology using Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI). Sequence alignment was carried out using
Multiple Alignment using Fast Fourier Transform (MAFFT) V 7 (Katoh et al., 2017). After alignment, sequences were trimmed in BioEdit Sequence Alignment Editor (Hall, 1999). DNA Sequence Polymorphism Analysis of Large Datasets (DnaSP) V 6.12.03 was used to concatenate sequences of both genes (Rozas et al., 2017). Phylogenetic trees for individual genes and concatenated sequences were constructed on Mega V 5.2 (Tamura et al., 2011) using the maximum likelihood method (Felsenstein, 1981). Evolutionary distances were calculated using Kimura’s two-parameter model (Kimura, 1980). The bootstrap test (Felsenstein, 1985) with 1000 bootstrap replicates was estimated and shown next to the branches of the phylogenetic tree. Sequences of type and pathotype strains were obtained from the GenBank database according to Hwang et al. (2005) scheme for multilocus sequence analysis. *Pseudomonas graminis* LMG 21661 strain, was used as an outgroup.

### 3.3 Results

#### 3.3.1 Rep-PCR fingerprinting

The rep-PCR fragments amplified with the BOX A1R and ERIC 2 primers, ranged in size from approximately 250 to 3500 bp for BOX A1R and from 250 to 6000 bp for ERIC 2 primer. ERIC 2 primer yielded a higher number of bands compared to BOX A1R primer (Fig. 3.1a and Fig. 3.1b). Four strains, BD 0002, BD 0022, BD 0771 and BD 0774, all from Gauteng, did not produce bands for both primers and were not included in the analyses. The fingerprint pattern showed a high degree of genetic similarity among the strains. Notable differences were observed between the major groups, within each group of strains fingerprints were similar. A 50% similarity was observed among all the *P. syringae* strains. Twenty-four strains of *P. syringae pv. tomato* showed 85% similarity amongst each other. Three strains of *P. syringae pv. tomato* BD 0035 (North West), BD 0269 (Limpopo) and BD 0091 (Limpopo), were less similar to other *P. syringae* pv. *tomato* strains and each other. These three strains were isolated from tomato stems. Three strains of *P. viridiflava* BD 0146, BD 0156 and BD 223 grouped with *P. viridiflava* showing 60% similarity. A similarity of 55% was observed among *P. cichorii* strains when grouped with their type strain CFBP T 2101 (Fig. 3.2).
Figure 3.1a: Agarose gel electrophoresis of BOX A1R PCR fingerprinting patterns of *Pseudomonas* isolates. The sizes of GeneRuler 1 kb DNA ladder (Thermoscientific) are indicated in base pairs. Fingerprinting patterns of *P. viridiflava* isolates (BD 1149, BD 0223), *P. syringae* pv. *tomato* isolates (BD 0282, BD 1358, BD 1146, BD 0164, BD 0195, BD 0775, BD 0286, BD 0151, BD 0284), *P. syringae* pv. *syringae* isolates (BD 1130, BD 0280), *P. cichorii* isolates (BD 1152, BD 0229) are presented.
Figure 3.1b: Agarose gel electrophoresis of ERIC 2 PCR fingerprinting patterns of *Pseudomonas* isolates. The sizes of GeneRuler 1 kb DNA ladder (Thermoscientific) are indicated in base pairs. Fingerprinting patterns of *P. viridiflava* isolates (BD 1149, BD 0223), *P. syringae* pv. *tomato* isolates (BD 0164, BD 0775, BD 1146, BD 0034, BD 0282, BD 1358, BD 0195, BD 0286, BD 0195), *P. syringae* pv. *syringae* isolates (BD 1130, BD 0280), *P. cichorii* isolates (BD 1152, BD 0229) are presented.
Figure 3.2: Dendrogram based on BOX A1R and ERIC 2 fingerprints (rep-PCR) of fluorescent Pseudomonads isolated from diseases tomato. Fingerprints similarity was calculated using Pearson’s curve-based correlation coefficient using UPGMA clustering method. PSS- P. syringae pv. syringae, PST- P. syringae pv. tomato, PV- P. viridiflava and PC- P. cichorii.
3.3.2 Multilocus Sequence Analysis (MLSA)

Partial *gyrB* and *cts* sequences were obtained for 38 strains. *GyrB* and *cts* sequences of 34 strains displayed a high degree of similarity with previously determined sequences belonging to the genus *P. syringae*. Twenty-four strains showed ≥ 98% sequence similarity to *P. syringae* pv. *tomato*, three strains (BD 0278, BD 0279 and BD 0280) showed ≥ 99 to *P. syringae* pv. *syringae* and four strains (BD 0002, BD 0022, BD 0771 and BD 0774) blasted with *P. syringae* pv. *syringae* however in the concatenated tree clustered with the type strain of *P. syringae* pv. *papulans* CFBP 5076<sup>T</sup>.

Twenty *gyrB* sequences and one *cts* sequence were submitted to Genbank. Accession numbers are listed in Table 3.1. The length of *gyrB* sequences ranged from 507 bp to 840 bp and 584 bp to 980 bp for *cts*. GyrB and cts nucleotide sequences were used to construct Maximum Likelihood trees for individual genes before concatenation. These trees showed similar topologies (data not shown).

In the concatenated tree (Fig. 3.3) 24 strains clustered with the type strain of *P. syringae* pv. *tomato* CFBP 2212<sup>PT</sup>. These strains originated from four provinces namely North-West, Mpumalanga, Gauteng and Limpopo. Four isolates clustered with the CFBP 1392<sup>PT</sup>, the type strain of *P. syringae* pv. *syringae*. These isolates originated from Mpumalanga. Four strains from Gauteng BD 0002, BD 0022, BD 0771 and 0774 formed a clade with *P. syringae* pv. *papulans* LMG 5076<sup>PT</sup>. BD 0231 from Limpopo as well as BD 0223 and BD 0224 from Gauteng were *P. viridiflava* (Figure 3.3).
Figure 3.3: Maximum likelihood phylogenetic tree of *Pseudomonas* isolates from diseased tomato and reference strains based on the concatenated sequences of *gyrB* and *cts*. The scale bar indicates similarity distances given as percentage values and bootstrap values of 1000 replicates were applied. Bootstrap values higher than 65% are shown. *P. graminis* served as an outgroup.


PG- Phylogroup
3.4 Discussion

An accurate identification of pathovars of *P. syringae* commonly found in diverse environments is crucial in order to understand the genetic polymorphism of isolates and can be used to devise suitable agricultural management practices (Cepni and Gurel, 2012). Traditionally, bacteria are identified using phenotypic and biochemical methods, however these methods are not sufficient for pathovar designation. In this study, MLSA based on *cts* and *gyrB* genes and rep-PCR fingerprinting using BOX A1R and ERIC 2 primers successfully identified *Pseudomonas* strains to species levels. Several studies have shown that rep-PCR fingerprinting and using partial sequences of genes are suitable methods when identifying and classifying *P. syringae* strains (Berge et al., 2014; Bull et al., 2011; Gardan et al., 1999; Hwang et al., 2005; Louws et al., 1994; Maiden et al., 1998; Marques et al., 2008; Mulet et al., 2010; Sarkar and Guttman 2004; Yamamoto et al., 2000).

In this study, the partial sequences of two genes, *cts* and *gyrB* were generated for 34 strains of *Pseudomonas* species isolated from diseased tomato in South Africa between 1991 and 2015. These strains were isolated from tomato plants showing the bacterial speck-like symptoms. Phylogenetic analyses revealed that 24 of those strains were *P. syringae pv. tomato*. Both sequences of the *cts* and *gyrB* genes were 98 to 100% homologous to that of *P. syringae pv. tomato* and all clustered with the type strain of that pathovar in the concatenated phylogenetic. These bacteria were isolated from diseased tomatoes collected from Gauteng, Limpopo, North West and Mpumalanga. Previously, all isolates listed in Table 3.1 were subjected to PCR with primers specific for the detection of coronatine-producing isolates of fluorescent *Pseudomonas, COR1 and COR2* (Cuppels et al., 2006). All strains, including type strains of *P. syringae pv. tomato, P. syringae pv. syringae, P. viridiflava and P. cichorii* produced the 689 bp band. Primers COR1 and COR2 were not useful for identification of fluorescent *Pseudomonas* isolates from tomato to the species level.

MLSA analysis of two housekeeping genes, *gyrB* and *cts*, revealed that most isolates are *P. syringae pv. tomato*. Three strains, all from Mpumalanga were *P. syringae* pv. *syringae*. Rep-PCR fingerprints dendrogram confirmed the
phylogenetic analysis. Single carbon source utilisation pattern was also able to distinguish these two pathovars. *P. syringae* pv. *tomato* isolates did not utilise erythitol, while *P. syringae* pv. *syringae* isolates did utilize this carbon source. Four strains from Gauteng grouped with both *P. syringae* pv. *papulans* and *P. syringae* dysoxyli. Additional genes will need to be used to distinguish between these closely related pathovars therefore, the identity of the four strains is currently unclear. The use of the two housekeeping genes; gyrB and cts was not enough to discriminate the strains since the four strains clustered closely with *P. syringae* pv. *papulans* and *P. syringae* pv. dysoxyli. *Pseudomonas syringae* pv. *papulans* is the causal agent of blister spot of apples and *P. syringae* pv. dysoxyli is a bacterial disease of *dysoxylum spectabile* (Humm, 1946; Rose, 1916).

Both *Pseudomonas syringae* pv. *papulans* and *P. syringae* pv. dysoxyli have not been reported in South Africa. However, in 1986, Mansvelt and Hattingh reported a similar disease, bacterial blister bark and blight of fruit spurs of apple. The causal agent was identified as *P. syringae* pv. *syringae*. The authors used only physiological, morphological and biochemical methods. In Chapter 2 of this dissertation, strains BD 0002, BD 0022, BD 0771 and BD 0774 were not distinguishable from those of *P. syringae* pv. *syringae* by single carbon sources utilisation and colony morphology. In 1986 gene sequencing and MLSA analyses were not commonly available. It is possible that the disease described by Mansvelt and Hattingh (1986) was caused by *P. syringae* pv. *papulans*. *P. syringae* pv. *papulans* and *P. syringae* pv. dysoxyli are placed in the phylogroup 2 or genomospecies 1 (Berge *et al*., 2014; Gardan *et al*., 1999). These isolates induced similar symptoms similar to those of *P. syringae* pv. *syringae* in pathogenicity tests when inoculated into tomato seedlings. Marcelletti and Scortichini (2014) suggested that genetically related host plants are infected by closely related pathogenic microorganisms. Apples (*Malus domestica* B.) and tomatoes represent two genetically distinctive plant groups. It is not common that they are infected by the same pathovar. Reason for a bacterial pathogen of apple infecting tomatoes is unclear and should be investigated further. Three strains identified as *P. viridiflava*; BD 0231, BD 0223 and BD 0224 caused the bacterial speck like symptoms in pathogenicity tests. These strains induced yellowing, wilting and stem necrosis on inoculated tomatoes cv. Red Khaki.
The Rep-PCR fingerprints obtained in this study using BOXA1R and ERIC2 primers showed genetic similarity within *Pseudomonas syringae* strains isolated from South African tomatoes. The dendrogram with combined fingerprints of BOX A1R and ERIC 2 divided *Pseudomonas syringae* into two groups. The majority of strains grouped with the type strain of *P. syringae* pv. *tomato* just like in the concatenated phylogenetic tree. Fingerprints produced by *P. syringae* pv. *tomato* isolates were almost identical, some strains having or missing one or two bands. These results were in line with those of Louws et al. (1994), who reported that isolates of the same pathovar have almost identical REP, BOX and ERIC fingerprints. Three strains formed a clade with *P. syringae* pv. *syringae* CFBP 1392PT. These were the same strains that were identified as *P. syringae* pv. *syringae* by MLSA. Four strains clustered with *P. syringae* pv. *papulans* and *P. syringae* pv. *dysoxyli* by MLSA did not produce bands in rep-PCR.

Rep-PCR genomic fingerprints are used to assess genetic diversity of bacterial strains, not to identify isolates to the species level. Borges et al. (2003) studied the genetic diversity of *Escherichia coli* isolated from polluted waters using rep-PCR. Ninety-eight strains were used in the study. Majority of the strains formed 28 clusters with a 70% similarity cut-off. However, some strains of *E. coli* produced fingerprints that were under 50% similar to the main clusters. The similarity between the more distant isolates was only 37%. Scortichini et al. (2003) observed diversity among isolates from the same host plant as well as among isolates from the same site, isolated at the same time. All isolates in this study were from one host, tomato. However, correlations and similarities in fingerprints for isolates from the same province or collected in the same year were not found. Nonetheless repetitive-DNA markers have been used with success in the identification of a large number of Gram-negative bacteria worldwide (Marques et al., 2008; Pour and Taghavi 2011; Rombouts et al., 2015; Trantas et al., 2013).

The research in study revealed that the strains that causes leaf spots of tomato in South Africa used in this study belonged to four phylogroups. Strains belonged to phylogroup 1, 2 (a and b), 7 and 11. However, more strains must be isolated from the bacterial speck-affected tomatoes countrywide and identified using
sequences of more than two housekeeping genes. Moloto et al. (2016) and Trantas et al. (2013) used the gyrB and cts sequences in studies determining the phylogroups and pathovars within the Pseudomonas syringae genus.

3.5 References


Chapter 4

Susceptibility of six commercially available tomato cultivars in South Africa to *Pseudomonas syringae pv. tomato* under greenhouse conditions

Abstract

Bacterial speck of tomato caused by *Pseudomonas syringae pv. tomato* is an economically important disease of tomato worldwide. The commonly used control measures for the disease are not effective; however, the use of resistant cultivars remain one of the most promising control strategies in managing the disease. Six commonly cultivated tomato (*Solanum lycopersicum* L.) cultivars in South Africa were tested for susceptibility to *Pseudomonas syringae pv. tomato* under greenhouse conditions. A virulent strain of *P. syringae pv. tomato* (BD 0165) with disease concentrations of $10^4$ and $10^8$ cfu ml$^{-1}$ were sprayed inoculated on 4-wk old seedlings grown in a glasshouse at 24/20°C (day and night temperatures) respectively. Control plants were sprayed with sterile distilled water and the experiment was repeated three times. Symptoms were observed 7 days post inoculation, and final lesions on leaves were counted 21 days post inoculation. A modified Chambers and Merriman rating scale (0-9) was used to evaluate the disease severity of the six cultivars. The higher concentration of $10^8$ cfu ml$^{-1}$ caused a high number of lesions and percentage disease index (PDI) compared to the lower concentration of $10^4$ cfu ml$^{-1}$. Red khaki was the most susceptible cultivar with the highest PDI of 68.9% at the concentration of $10^8$ cfu ml$^{-1}$ in all the three independent experiments. Cultivar 8863 was the least susceptible cultivar throughout the three independent experiments. This cultivar had the lowest number of lesions of 14.0 in experiment 1 at a concentration of $10^4$ cfu ml$^{-1}$ and the PDI of 22.2% throughout the three independent experiments at $10^4$ cfu ml$^{-1}$. In this study, none of the cultivars were classified to be resistant to bacterial speck of tomato, however disease severity varied among the cultivars used. Cultivar Red Khaki was found to be the most susceptible cultivar regardless of the inoculum concentration that was used.

Keywords: Cultivar, susceptibility, percentage disease index, concentration
4.1 Introduction

Tomato (*Solanum lycopersicium* L.) is an important vegetable crop and is produced in all provinces in South Africa. Bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* (Okabe) Young, Dye and Wilkie is an important disease in many tomato growing areas worldwide and is favoured by cool, moist environmental conditions (CAI *et al.*, 2011; Bogatsevska, 1988). The disease causes severe damages of tomatoes both in the greenhouse and the field worldwide (Bashan *et al.*, 1978, Devash *et al.*, 1980, Smitley and Mc Carter, 1982). Chambers and Merriman (1975) reported that bacterial speck of tomato is a seed-borne pathogen. According to Young *et al.* (1986), the disease affects the first flowers which prevents further flowering and can cause substantial yield losses. The disease affects many parts of the plant such as flowers, buds, stems, petioles and sometimes may lead to the death of the plant (Louws *et al.*, 2001; Preston, 2000). Bacterial speck has been reported to cause yield losses of about 75% in plants at an early stage of growth and about 5% in plants infected later in the season (Yunis *et al.*, 1980a). The phytotoxin, coronatine which is produced by the pathogen causes yellow chlorotic halo around the specks on the leaves (Young *et al.*, 1986). On fruits it causes black specks therefore makes the fruit to be unmarketable.

Numerous control measures have been implemented to eliminate or control bacterial speck on tomatoes. While many studies have demonstrated the efficacy of copper compounds and streptomycin sprays against bacterial speck of tomato (Conlin and McCarter, 1983; Cooksey, 1988; Jardine and Stephens, 1987), these control methods are not efficient (Bashan, 1997; Da Silva and Lopes, 1995, Pernezny *et al.*, 1995). This is mainly because the pathogen strains have developed resistance to copper compounds (Cooksey, 1990; Cooksey and Azad, 1992; Pernezny *et al.*, 1995), which were the most common antibacterial agents used in disease prevention programs (Yunis *et al.*, 1980b). Using chemicals is expensive especially for small holder farmers so the use of resistant cultivars may serve as a better alternative for control. Much weight has been placed on developing cultivars that are resistant to bacterial speck of tomato. According to Basim and Turgut (2013); Blancard (1997); Lamichhane *et al.* (2010); Yu *et al.*
Resistance in the host plant or the use of resistant cultivars is the most effective strategy for managing bacterial speck of tomato.

The occurrence of new races is a cause for concern when it comes to breeding for resistant cultivars against bacterial speck. Two races of the pathogen have been described in the world; race 0 and 1 (Lawton and MacNeil, 1986; Bogatsevska, 1989). The incompletely dominant, resistant gene *Pto* (Kozik, 2002) is responsible for resistance against bacterial speck of tomato. The gene was originally discovered in *Lycopersicon pimpinellifolium* L., a wild tomato species and was isolated using Map-based cloning (Martin *et al*., 1993). Since its discovery, the gene has been introgressed into many tomato cultivars by backcrossing. The pathogen population structure has gradually shifted from race 0 to race 1 due the wide use of tomato cultivars carrying the *Pto* gene for resistance to race 0 (Kunkeaw *et al*., 2010; Thapa *et al*., 2015). In recent years some tomato cultivars and wild species which possess resistance to the disease have been found (Shenge *et al*., 2007; Turgut and Basim, 2013). The aim of the present study was to evaluate the susceptibility of six commercial tomato cultivars to bacterial speck of tomato caused by *P. syringae* pv. *tomato* using two inoculum concentrations (10^4 cfu ml^{-1} and 10^8 cfu ml^{-1}) under greenhouse conditions.

### 4.2 Materials and Methods

#### 4.2.1 Bacterial isolate

The *Pseudomonas syringae* pv. *tomato* isolate BD 0165 was obtained from Plant Pathogenic and Plant Protecting Bacteria (PPPPB) National Collection at the Agricultural Research Council, Plant Health and Protection (ARC-PHP) in Pretoria, South Africa. The strain was isolated from diseased tomato leaf exhibiting speck-like symptoms in the Gauteng province, South Africa in 1999.

Stock cultures of the bacterial isolate were stored in nutrient glycerol yeast extract broth (0.8 g nutrient broth, 15 ml glycerol, 0.2 g yeast extract, 0.5 g glucose in 100 ml distilled water) at -80°C. The cultures were recovered on Tryptone Glucose extract Agar (TGA) (Difco, Madison) medium (3.0 g beef extract, 5.0 g tryptone, 1.0 g dextrose and 15.0 g agar). The inoculated plates were incubated
at 28°C for 48 hr. The bacterial inoculum was prepared by washing 48-hour-old cultures of bacteria with sterile distilled water. Concentration of bacterial cells in the suspension was adjusted to $10^4$ and $10^8$ cfu ml$^{-1}$ by a serial dilution plating method.

4.2.2 Tomato seedlings and growth conditions
The tomato cultivars used in this study were obtained from a commercial company Sakata Seed Southern Africa (South Africa, Pretoria) producing tomato seeds and seedlings. Six commonly grown cultivars of tomato in South Africa; Red khaki, 9771, 886, 9751, 9752 and 9753 were tested. Tomato seeds were sown in speedling® 60 trays, and after germination the seedlings were transplanted into 10 cm diameter pots containing thoroughly mixed sterile growing medium [ consisting of vermiculite (8 kg), composted pine bark seedling mix (12.5 kg), potting mixture (75kg), agricultural lime (200 g), super phosphate (96 g), limestone ammonium nitrate (LAN) (70g) and potassium phosphate (60 g)]. Plants were kept at the greenhouse at the Agricultural Research Council, Roodeplaat, Pretoria and were arranged in a completely randomized design (RCD) at relative humidity of approximately 70-80% and temperatures of 24°C and 20°C day/night respectively.

4.2.3 Inoculation of seedlings with the pathogen
The *P. syringae* pv. *tomato* BD 0165 isolate was used to inoculate the tomato plants. The inoculum was prepared by mixing the bacterial suspension grown from the TGA medium with sterile distilled water to make up the concentration of $10^4$ and $10^8$ cfu ml$^{-1}$. Inoculation of plants was carried out on 4 wk-old tomato plants. Five replicates (one plant per replicate) were used per cultivar and per concentration. Plants were first dusted with carborundum in order to create wounds, then were later sprayed with the inoculum on the leaves as well as underneath the leaves using a hand-held sprayer until leaf surfaces were uniformly wet. Control plants were sprayed in the same manner with sterile distilled water. Immediately after spraying, plants were covered with clear polyethylene bags for 72 hours to retain the moisture and were kept at 24°C/20°C
day/night in the glasshouse. Bags were removed after 72 hours and plants were observed for disease symptoms. The experiment was repeated three times.

4.2.4 Evaluation of disease severity
Lesions on tomato plants were observed on the 7th day post inoculation (dpi), however lesions were counted on the 21st dpi. Disease ratings were classified using a modified Chambers and Merriman rating scale (1975); where 0= no lesions, 1= 1-10 lesions per plant, 2= 11-20 lesions per plant, 3= 21-30 lesions per plant, 4= 31 to 40 lesions per plant, 5= 41 to 50 lesions per plant, 6= 51-60 lesions per plant, 7= 61-70 lesions per plant, 8=71-80 lesions per plant and 9= more than 80 lesions per plant. The percentage disease index values were calculated using the following formula:

\[ PDI = \frac{\text{Sum of all ratings}}{\text{Total number of observations} \times \text{Maximum rating scale}} \times 100 \]

4.2.5 Statistical analysis
Data from each of the three experiment were subjected to analysis of variance (ANOVA) using SAS software version 9.4 (SAS Institute Inc, 2016). Where ANOVA was significant, treatment means were separated using Duncan’s multiple range test (DMRT) at 5% significant level.

4.3.3 Re-isolation of bacteria from diseased leaves of tomato seedlings
A portion of the infected leaves were excised and thoroughly rinsed with running tap water and was left to air-dry in the laminar flow. Small sections of the leaves were cut and chopped with a sterile scalpel, placed in 100 µl of sterile distilled water and set aside for approximately 10 minutes. A volume of 50 µl was drawn from the extract and was streaked on to King’s B and TGA media. Plates were incubated at 28°C for 48 hr and monitored frequently for colony development.
4.3 Results

4.3.1 Response of cultivars to *Pseudomonas syringae pv. tomato* isolate under greenhouse conditions

Approximately 7 dpi, typical bacterial speck symptoms surrounded by a chlorotic yellow halo (Fig. 4.1 B and C) were observed on all cultivars. Disease symptoms progressed faster on the most susceptible cultivars; (Red khaki and cultivar 9753) and specks symptoms became more visible and distinct over time. Lesions counted on 21 dpi at a concentration of $10^8$ cfu ml$^{-1}$ had a large number of specks on seedlings of tomato plants compared to the lower concentration of $10^4$ cfu ml$^{-1}$ (Fig. 4.1 D).

The least susceptible cultivar, cultivar 8863 sprayed with $10^4$ and $10^8$ cfu ml$^{-1}$ concentration had 14.0 and 20.0 number of lesions. The highest number of lesions, 59.0 was recorded for $10^8$ cfu ml$^{-1}$ for the respective concentrations on Red khaki and the lowest number of lesions 14.0 was recorded for $10^4$ cfu ml$^{-1}$ on cultivar 8863. The most susceptible cultivars formed necrosis on the stems and leaves coalesced severely (Fig. 4.1 D, E, and F). Control plants showed no disease symptoms (Fig. 4.1 A).
Figure 4.1: Symptoms caused by *P. syringae* pv. *tomato* isolate (BD 0165) on tomato seedlings from six cultivars grown under greenhouse conditions. A-tomato plant sprayed with sterile distilled water (control), B-symptoms observed at 7 dpi using $10^4$ cfu ml$^{-1}$, C-specks surrounded by a chlorotic yellow halo, D-lesions observed at 21 dpi at $10^8$ cfu ml$^{-1}$, E-specks coalesced as the disease progressed, F-necrosis on stem and severe coalescing of specks.

All the six tested cultivars were susceptible to bacterial speck of tomato regardless of the concentration that was used. Based on the number of lesions; cultivars Red khaki, 9753 and 9752 were significantly different from the other three cultivars ($P<0.0001$) (Table 4.1) showing high susceptibility to the disease.
Red khaki cultivar was found to be the most susceptible cultivar with PDI values ranging from 44.1% to 68.9% whilst the less susceptible cultivar, 8863 had PDI values ranging from 22.2% to 33.3% (Table 4.2).

Consistency in cultivar responses in the three separate experiments was observed. The cultivars with the high number of lesions, also had the high PDI values. Cultivar Red khaki in experiment 2, had 59.4 lesions (average) and had the highest PDI value of 68.9% (Table 4.1). For Red khaki, in all the three separate experiments the values remained constant for both bacterial concentrations used.

Table 4.1: The effect of two inoculum concentrations (10^4 cfu ml\(^{-1}\) and 10^8 cfu ml\(^{-1}\)) of \textit{P. syringae} pv. \textit{tomato} on six tomato cultivars under greenhouse conditions.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Trial 1</th>
<th></th>
<th>Trial 2</th>
<th></th>
<th>Trial 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td></td>
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<td>10^8 CFU ml(^{-1})</td>
<td>10^4 CFU ml(^{-1})</td>
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<td>10^4 CFU ml(^{-1})</td>
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<td>No. of lesions</td>
<td></td>
</tr>
<tr>
<td>RK</td>
<td>35.8a</td>
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<td>36.6a</td>
<td>59.4a</td>
<td>35.2a</td>
<td>56.8a</td>
</tr>
<tr>
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<td>25.2b</td>
<td>43.0b</td>
<td>25.8b</td>
<td>41.4b</td>
</tr>
<tr>
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<td>37.0c</td>
<td>20.2c</td>
<td>37.4c</td>
<td>21.0c</td>
<td>35.0c</td>
</tr>
<tr>
<td>C9771</td>
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<td>24.0de</td>
<td>16.0d</td>
<td>22.8e</td>
<td>14.4d</td>
<td>25.0de</td>
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<td>26.6d</td>
<td>17.0cd</td>
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<td>16.4d</td>
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</tr>
<tr>
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<td>20.2e</td>
<td>15.8d</td>
<td>20.8e</td>
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<tr>
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<td>86.55</td>
<td>39.18</td>
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<tr>
<td>CV%</td>
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<td>9.86</td>
<td>13.23</td>
<td>10.24</td>
<td>13.26</td>
<td>12.09</td>
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</table>

Means with the same letter(s) are not significantly different at P=0.05 according to Duncan Multiple Range Test (DMRT).
Table 4.2: PDI of six cultivars inoculated with $10^4$ and $10^8$ cfu ml$^{-1}$ *Pseudomonas syringae* pv. *tomato* concentrations under glasshouse conditions.

<table>
<thead>
<tr>
<th>Cultivars</th>
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<th></th>
<th></th>
<th>Trial 2</th>
<th></th>
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<th>Trial 3</th>
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<tr>
<td></td>
<td>$10^4$ CFU ml$^{-1}$</td>
<td>$10^8$ CFU ml$^{-1}$</td>
<td>PDI</td>
<td>$10^4$ CFU ml$^{-1}$</td>
<td>$10^8$ CFU ml$^{-1}$</td>
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<td>$10^8$ CFU ml$^{-1}$</td>
<td>PDI</td>
</tr>
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<td></td>
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<td></td>
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<td>37.8cd</td>
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<tr>
<td>CV%</td>
<td>7.21</td>
<td>10.29</td>
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<td>8.71</td>
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</table>

Means with the same letter(s) are not significantly different at P=0.05 according to Duncan Multiple Range Test (DMRT).

### 4.3.2 Re-isolation of bacteria from diseased leaves of tomato seedlings

The *Pseudomonas syringae* pv. *tomato* colonies re-isolated from diseased leaves were raised, smooth and mucoid on TGA and fluoresced on KB. Plants inoculated with sterile distilled water were asymptomatic and colonies similar to those isolated from the cultivars inoculated with BD 0165 were not isolated on the media.

### 4.4 Discussion

The aim of this study was to evaluate six tomato cultivars against *P. syringae* pv. *tomato* isolate using two inoculum concentrations ($10^4$ and $10^8$ cfu ml$^{-1}$). Based on the obtained results, none of the cultivars were resistant to isolate BD 0165 of *P. syringae* pv. *tomato*. It was noted that the six cultivars responded differently to
the two concentrations of *P. syringae pv. tomato*. The difference in susceptibility levels is caused by differences in the multiplication rate of *P. syringae pv. tomato* in the apoplast of the cultivars (Babelegoto *et al*., 1988). The highest PDI was observed for Red khaki (68.9%) and cultivar 9753 (55.5%); the lowest PDI 22.2% was observed with the lowest concentration of $10^4$ cfu ml$^{-1}$ on three cultivars; 9771, 9751 and 8863. The reactions of the cultivars were consistent in all the three separate experiments, with cultivar Red khaki being highly susceptible and cultivar 8863 being the least susceptible. It was established that as the bacterial load increased, the PDI and the number of lesions also increased. This was in agreement with Kozik and Sobiczewski (2007), where tomato leaves were sprayed using two concentrations of $10^7$ and $10^8$ cfu ml$^{-1}$ and the latter concentration was found to give the most uniform and consistent results.

Necrotic spots generally surrounded by a chlorotic yellow halo caused by the phytotoxin, coronatine (Bender *et al*., 1999) produced by bacterial speck were observed in all the six cultivars. The most susceptible cultivar, Red khaki developed necrosis on the stem and spots coalesced severely. In all the three experiments, symptoms appeared on all six cultivars at 7 pdi. The temperature of the test conditions (24°C) and high relative humidity (70-80%) favoured the multiplication of bacteria and hence the development of symptoms. These results contradicted the results of Lamichhane *et al*. (2010) and Turgut and Basim (2013) where symptoms appeared as early as 2 days post inoculation. However, it should be noted that there are lot of factors that affect symptom development such as the growth stage of the inoculated plants, cultivar, method of inoculation that is used and environmental conditions. Kozik and Sobiczewski (2007) assessed different inoculation methods and it was noted that spray inoculation method produced high disease severity compared to rubbing upper leaf area with cheese cloth dipped in bacterial suspension and spraying detached leaves with bacterial suspension using hand sprayer.

Five cultivars (Red khaki, 9753, 9771, 9751 and 8863) showed constant PDI values in all the three separate experiments at the lower concentration of $10^4$ cfu ml$^{-1}$. The higher inoculum level of $10^8$ cfu ml$^{-1}$ allowed for a better separation of cultivars with different disease levels compared to $10^4$ cfu ml$^{-1}$. In the study by
Scott et al. (2010), it was observed that cultivars considered susceptible were more affected than the cultivars with intermediate resistance, when inoculated with a higher concentration of inoculum. However, when the inoculum concentration was low there were no apparent differences between susceptible and resistant plants. Therefore, in this study, the higher concentration of inoculum allowed a better differentiation of degrees of susceptibility between the cultivars.

Significant bacterial speck of tomato symptoms were observed on tomato plants. The obtained results showed that the presence and PDI of bacterial speck of tomato on the six cultivars was high. Bacterial speck of tomato was reported by Okabe (1933) and Bryan (1933) and has since been an important disease of tomato globally. It has been reported that host resistance is an efficient and effective strategy in managing and controlling bacterial speck of tomato (Hulbert et al., 2001; Blancard, 1997; Yu et al., 1995; Scott et al., 1989). The evaluation of different cultivars of tomatoes against bacterial speck of tomato has been largely reported worldwide (Ekici and Bastas, 2014; Kozik, 2002; Kozik and Sobiczewski, 2007; Shenge et al., 2007) but not much studies have been done in South Africa to date.

The identification of resistance to bacterial speck of tomato in existing commercial cultivars of tomato is a significant contribution of the present work as the cultivars are readily available for use by the tomato growers, without the need of a long breeding process. In addition, constant monitoring of the variability of the pathogen is needed, to prevent the emergence of new pathogen races. This study will contribute to knowledge about the susceptibility of tomato cultivars to bacterial speck disease as a basis for the development of breeding programs to develop resistant lines to \textit{P. syringae pv. tomato}.
4.5 References


Chapter 5

Thesis Overview of the Major Research Findings and their Implications

Introduction

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable after potatoes worldwide and ranks first among the processing crops. Despite its importance, tomato is susceptible to over 200 diseases caused by pathogenic bacteria, fungi, viruses and nematodes. Among these diseases, fluorescent *Pseudomonas* species (*Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas viridiflava* and *Pseudomonas cichorii*) play a major role in causing diseases on tomato; however, remains poorly studied in South Africa. Globally, *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* (*Pss*) have been reported as causal agents of disease outbreaks on tomato. Closely related *Pseudomonas* species, *P. cichorii* and *P. viridiflava* have also been identified as pathogens of tomato.

The aim of the present study was to characterise fluorescent *Pseudomonas* isolates using the following specific objectives: The specific objectives were as follows: (1) To characterise fluorescent *Pseudomonas* species by using morphological and biochemical methods (2) To do Rep-PCR genomic fingerprints analysis of fluorescent *Pseudomonas* species isolated from tomato in South Africa (3) To identify South African fluorescent *Pseudomonas* to pathovar level using the multilocus sequence analyses of two housekeeping genes, *DNA gyrase Subunit B* (*gyrB*) and *citrate synthase* (*cts*) (4) To evaluate the susceptibility of six commercial tomato cultivars to *P. syringae* pv. *tomato* under greenhouse conditions.
Chapter 2: Physiological and biochemical characterisation of fluorescent *Pseudomonas* species causing foliar disease of tomato in South Africa

Major findings:

- Strains were found to belong to three LOPAT groups with LOPAT group 1 consisting of most strains.
- Carbon source utilization was able to distinguish strains of *P. syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *P. viridiflava* and *P. cichorii*.
- Although the studied strains are fluorescent *Pseudomonas*, some strains did not produce the fluorescent pigment on King’s B medium.
- *P. cichorii* strains did not produce any symptoms on cultivar Red Khaki.
- All the tested *Pseudomonas* strains yielded a 689 bp amplicon when COR1 and COR2 primers were used. These primers were designed to detect coronatine-producing *Pseudomonas syringae* isolates.

Implications:

Although bacteria are traditionally identified or characterized by morphology and biochemical methods, these methods do not distinguish bacterial isolates to pathovar level. Both *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* isolates belonged to LOPAT group 1. The carbon source utilization method suggested that this method is able to differentiate between closely related species, *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae*. Strains of *P. cichorii* did not cause any symptoms, this could be due to that this bacterium requires higher temperatures for it to induce symptoms.

Chapter 3: Molecular characterization of fluorescent *Pseudomonas* species causing foliar diseases on tomato using REP-PCR fingerprinting and MLST

Major findings:

- The results from MLSA were in agreement with those of LOPAT characterization and confirmed the strains as *P. syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *P. viridiflava* and *P. cichorii*.
• Isolates belonging in Phylogroup 2, formed two separate clades; 2a and 2b. Strains isolated from Mpumalanga were closely related to the type strain of *P. syringae* pv. *syringae* CFBP 1392<sup>PT</sup>, and strains isolated from Gauteng were closely related to *P. syringae* pv. *papulans* type strain CFBP 5076<sup>PT</sup> and *P. syringae* pv. *dysoxyli* LMG 5062<sup>PT</sup>.

• No shifts by *Pseudomonas* species to tomato in South Africa was observed. Although strains were isolated in different years, they grouped according to their phylogroups in the phylogenetic tree.

• The results of the combined rep-PCR fingerprinting dendrogram were in agreement with those of MLSA.

• Both BOX A1R and ERIC 2 primers were able to identify bacterial strains to species level.

Implications:

The four strains (BD 0002, BD 0022, BD 0771 and BD 0774) earlier identified as *P. syringae* pv. *syringae* using biochemical methods were all isolated from Gauteng, clustered closely with the type strain of *P. syringae* pv. *papulans* LMG 5076<sup>PT</sup> and *P. syringae* pv. *dysoxyli* LMG 5062<sup>PT</sup>. More genes have to be used to distinguish between these pathovars since the use of two genes did not discriminate these pathovars.

These strains did not produce fingerprints with both primers. So, these strains were not included in the rep-PCR dendrogram. These strains had formed a separate clade in MLSA, so it is not clear if they would have formed a separate cluster in rep-PCR fingerprint dendrogram as well.

All the strains were isolated from the same host, tomato. As a result, host specificity was not observed among the tested isolates. In future, isolates should be isolated from different host plants and include geographically distant isolates in order to identify possible host or geographically related genetic polymorphism.
Chapter 4: Evaluation of susceptibility of six commercially available tomato cultivars to *Pseudomonas syringae* pv. *tomato* in South Africa under greenhouse conditions

Major findings:
- A pathogen inoculum as low as $10^4$ cfu ml$^{-1}$ was pathogenic to susceptible cultivars.
- Red khaki was the most susceptible cultivar with the PDI of 68.9% throughout the three independent experiments followed by cultivar 9753 with the PDI of 55.6% in experiment 2.
- No significant difference was observed between 9771, 9751 and 8863 at the concentration of $10^4$ cfu ml$^{-1}$.

Implications:
An inoculum concentration of $10^8$ cfu ml$^{-1}$ caused severe disease symptoms on susceptible cultivars. The disease severity of the six tested cultivars increased as the inoculum concentration increased from $10^4$ to $10^8$ cfu ml$^{-1}$.

The cultivars were screened under greenhouse conditions, only. It would be significant to also check the susceptibility of these cultivars in the field to see if the cultivars response is consistent to that under greenhouse conditions.