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

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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 (800ha) and the Eastern Cape (450ha). 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, erythitol, lactose, inositol, fructose and DL-lactate.

Pseudomonas viridiflava CFBP 2107 [T] 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 ≥ 98% sequence similarity to *P. syringae* pv. *tomato*, four strains showed ≥ 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 in1986 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.
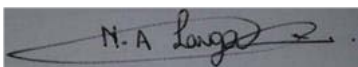
In the evaluation of susceptibility of six commercially available tomato cultivars to *P. syringae* pv. *tomato*, all the six tested tomato cultivars were found to be susceptible to bacterial speck pathogen. Red khaki was found to be the most susceptible cultivar followed by cultivar 9753. Consistency 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: 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:

1. Langa, N.A. *Pseudomonas* species causing foliar diseases of tomato in South Africa. 51st Congress of the South African Society for Plant Pathology (SASPP) at club Mykonos on 21 January 2019. Oral presentation.
2. Langa, N.A. *Pseudomonas* species causing foliar diseases of tomato in South Africa. 6th Annual Agricultural Research Council (ARC) annual postgraduate conference at Roodeplaat, Pretoria on 8 October 2019. Oral presentation.

References

Allen, D.J., Buruchara, R.A., Smithson, J.B. (1998). Diseases of common bean. In the pathology of food and pasture legumes. Allen, D.J and Lenne, J.M (eds). CAB International, Wallingford. pp. 179-235.

Department of Agriculture, Forestry and Fisheries. (2012). Profile of the South African market value chain. Pretoria, South Africa.

Food and Agriculture Organization of the United Nations. (2004). The market for non-traditional agricultural exports. In: FAO commodities and trade technical paper. Hallam, D., Liu, P., Lavers, G., Pilkauskas, P., Rapsomanikis, G., Claro, J (eds). pp. 1-181.

Freeman, B.B. and Reimers, K. (2011). Tomato consumption and health: Emerging benefits. *American journal of lifestyle medicine* **5**: 182-191.

Gilbertson, R.L and Maxwell, D.P. (1992). Common bacterial blight of bean. In plant diseases of international importance. Chaube, S.H and Kumar, J., Mukhopadhyay, A.N. Singh, U.S (eds). Prentice Hall, (vol **2**, pp. 18-39).

He, J., Baldini, R.L., Deziel, E., Saucier, M., Zhang, Q., Liberati, N.T. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harbouring plant and animal virulence genes. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 2530-2535.

Milijasevic, S., Todorovic, B., Rekanovic, E., Potocnik, I., Gavrilovic, V. (2009). Races and hosts of *Pseudomonas syringae* pv. *tomato* in Serbia. *Archives of Biological Sciences* **61**: 93-102.

Miller, J.S. (2002). Phylogenetic relationships and the evolution of gender dimorphism in *Lycium* (*Solanaceae*). *Systematic Botany* **27**: 416-428.

Okabe, N. (1933). Bacterial diseases of plants occurring in Formosa. II. Bacterial leaf spot of tomato. *Society of agricultural tropical Taiwan* **5**: 26-36.

Sundin, G.W., Castiblanco, L.F., Yuan, X., Zeng, Q., Yang, C. (2016). Bacterial disease management: challenges, experience, innovation and future prospects. *Molecular Plant Pathology* **17**: 1506-1518.

Tindall, B.J., Kampfer, P., Euzeby, J.P., Oren, A. (2006). Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. *International Journal of Systematic and Evolutionary Microbiology* **56**: 2715-2720.

Vallad, G.E., Pernezny, K.L., Balogh, B., Wen. A., Figueiredo, J.F.L., Jones, J.B., Momol, T., Muchovej, R.M. Havranek. N., Abdallah, N., Olson, S., Roberts, P.D., (2010). Comparison of kasugamycin to traditional bactericides for the management of bacterial spot on tomato. *Horticultural Science* **45**: 1834-1840.

Varvaro, L. and Guario, A. (1983). Precccupanti manifestazioni di "picchetttatura" e di "maculatura" batterica del pomodoro in Puglia. *L'Infomatore Agrario* **37**: 2741-2747.

Young, J.M, Dye, D.W., Wilkie, J.P. (1978). A proposed nomenclature and classification for plant pathogenic bacteria. *New Zealand agricultural research* **21**: 153-177.

Yunis, H., Bashan, Y., Okon, Y., Henis, Y. (1980). Two sources of resistance to bacterial speck of tomato caused by *Pseudomonas tomato*. *Plant Disease* **64**: 937-939.

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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)

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

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).
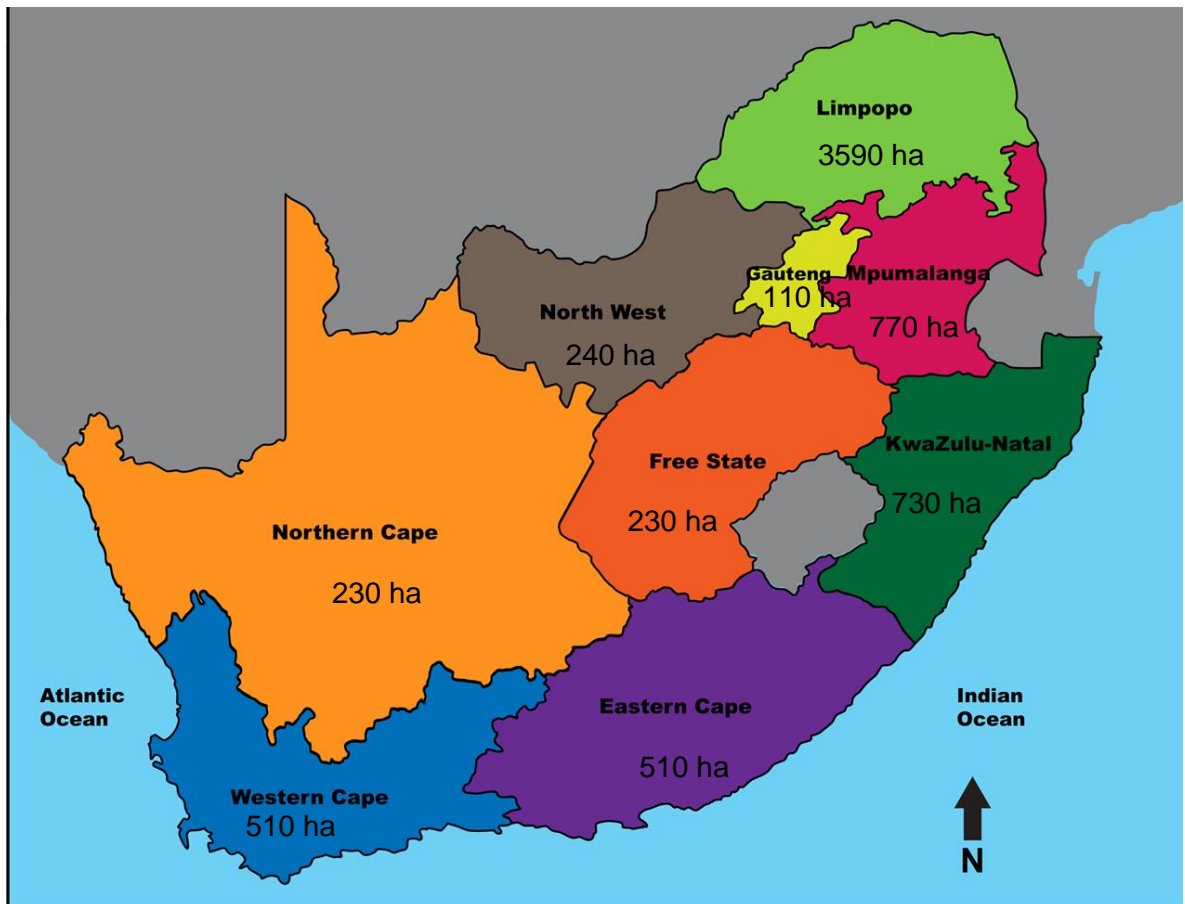
Figure 1.1: Map of South Africa showing area planted under tomatoes per province (http://www.kzntransport.gov.za/public_trans/freight_databank/kzn/industries/Fruit_veg/index_xml.html). Map from https://www.intergate-immigration.com/blog/south-african-provinces/.

1.4 Favorable growth conditions for tomatoes

Crop growth and development are highly dependent on temperature (Lu *et al.*, 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.*, 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.,* 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)

Proximate		
Water	g	94.52
Energy	kcal	18
Protein	g	0.88
Total lipid	g	0.2
Fibers	g	1.2
Sugars	g	2.63
Minerals		
Calcium	mg	10
Magnesium	mg	11
Phosphorus	mg	24
Potassium	mg	237
Sodium	mg	5
Fluoride	µg	2.3
Vitamins		

Vitamin C	mg	13.7
Choline	mg	6.7
Vitamin A	µg	42
α-carotene	µg	449
ß-carotene	µg	101
Lycopene	µg	2573
Lutein-Zeaxanthin	µg	123

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.

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 in1882 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

Kamoun, 2009). 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 solanacearium formerly known as *Pseudomonas solanacearium* 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 at,* 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).

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 (Scheneider 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.
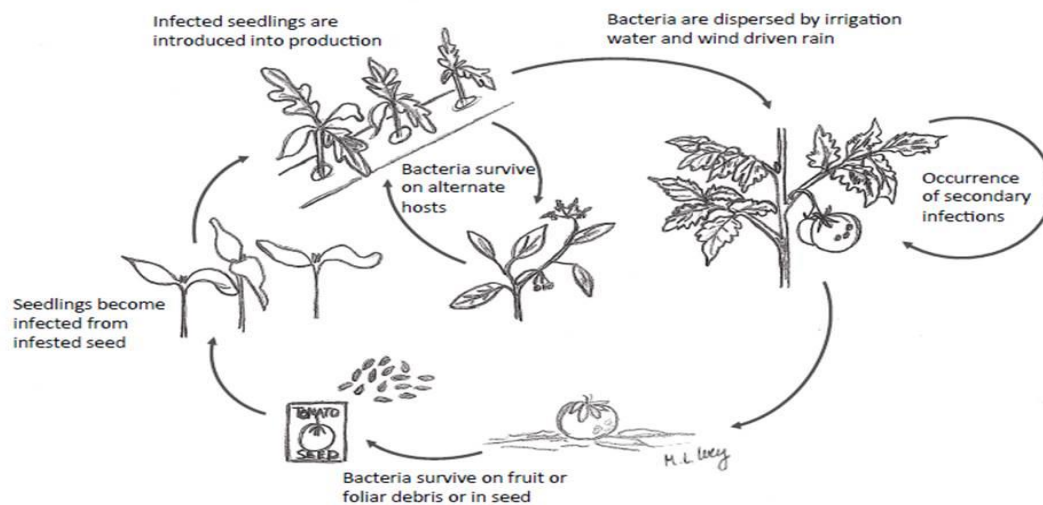
Figure 1.3: Epidemiology of bacterial speck of tomato (Schnieder and Grogan, 1977).

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://tomatodiseasehelp.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. t*omato*, 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 *Pto* because it enables the carriage of virulence proteins into the cytosol of plant hosts.

The *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 *P. syringae* pv. *tomato* strains lack these effectors (Kunkeaw *et al.*, 2010). In the absence of resistance, tomato plants infected with *P. syringae* pv. *tomato* result in bacterial speck of tomato disease. *P. syringae* pv. *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 *P. syringae* pv. *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 (Buonaurio *et al.*, 1996).

1.9 Detection and Characterisation of *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 *et al.*, 1988).

For the accurate identification of *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 *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 *et al.*, 1994).

1.9.1 Isolation and characterisation of *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.

1.9.2 Isolation and characterisation of *Pseudomonas syringae* from seeds

Mohan and Schaad (1987), employed a semiselective agar media for the detection of *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae.* pv. *phaseolicola* in bean seed. Two semiselective media used in the study were KBC which is derived from KB for *P. s.* pv. *syringae* and Modified Sucrose Peptone (MSP) for *P. s.* pv. *phaseolicola.* In the study by Shila *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; Gli*ck 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* genomospecies 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 (*gyrB*), RNA polymerase sigma 70 subunit (*rpo*), RNA polymerase beta subunit (*rpoB*), citrate synthase (*cts*) and glyceraldehyde-3-phosphate dehydrogenase (*gapA*) (Berge *et al.*, 2014; Yamamoto 2010). In the study by Sarkar and Guttman (2004), in a core genome of *P. syringae* a low level of recombination was observed. Kaluzna *et al.*, (2010), observed that MLST presented the highest discrimination among the *P. syringae* strains studied, especially in the case of *P. s. morsprunorum* race 2, as compared to both rep-PCRs (BOX and ERIC). In the study by Gardan *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 *P. syringae* species complex. *P. syringae* pv. *tomato* belongs to phylogroup 1, *P. syringae* pv. *syringae* to phylogroup 2, *P. viridiflava* to phylogroup 7 and *P. cichorii* to phylogroup 11. These phylogroups were obtained from using MLST of four housekeeping genes; *cts, gyrB, rpoD* and *gapA* (Berge *et al.*, 2014).

MLSA is the preferred method for establishing the phylogeny between species and genera (Gomila *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 *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 *et al.*, 2008; Yamamoto *et al.*, 2000; Sarris *et al.*, 2012). The taxonomical revision of *P. putida* strains was based on a MLSA technique with the combined housekeeping genes (16S rRNA, *gyrB*, and *rpoD*); the approach was proven to be reliable for delineating species and greatly facilitated the identification of new strains (Mulet *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 overheard 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 McCarter, 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 McCarter (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 grogram 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 McCarter,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*

al., (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 Syngeta (Kessmann *et al.*, 1996). Acibenzolar-S-methyl (ASM) is an active ingredient of Actigard[TM], 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_2SiO_2) 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

Abbasi, P.A., Soltani, N., Cuppels, D.A. Lazarovits, G. (2002). Reduction of bacterial spot disease severity on tomato and pepper plants with foliar applications of ammonium lignosulfonate or potassium phosphate. *Plant Disease* **86**: 1232-1236.

Abu-Elsamen, F. and Khlaif, H. (1999). Epidemiology and control of bacterial speck of tomato (*Pseudomonas syringae* pv. *tomato*) in the Jordan valley. *Agricultural Sciences* **26**: 205-214.

Abushita, A.A., Hebshi, E.A., Daood, H.G., Biacs, P.G. (1997). Determination of antioxidant vitamins in tomatoes. *Food Chemistry* **60**: 207-212.

Adzitey, F., Huda, N., Rusul, G., Ali., R. (2013). Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. *Biotechnology* **3**: 97-107.

Agrios, G.N. **(1997).** Plant Pathology. 4th edition. Academic press, London. pp. 635.

Agrios, G.N. **(2005).** Environmental effect is on development of the infectious disease. In plant pathology. 5th edition. Elesvier Academic press, Burlington USA. pp. 251-262.

Ajigbola, C.F. and Babalola, O.O. (2013). Integrated management strategies for tomato *Fusarium wilt*. *Biocontrol Sciences* **18**: 117-127.

Alfano, J.R. and Collmer, A. (2004). Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. *Annual Reviews of Phytopathology* **42**: 385-414.

Alvarez, A. M. (2004). Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. *Annual Reviews of Phytopathology* **42**: 339-66.

Anderson, M., Bollinger, D., Hagler, A., Hartwell, H., Rivers, B., Ward, K., Steck, T.R. **(2004).** Viable but nonculturable bacteria are present in mouse and human urine specimens. *Clinical Microbiology* **42**: 753-758.

Anfoka, G.H. (2000). Benzo- (-1,2,3)-thiadiazole-7-carbothioic acid S-Methyl ester induces systematic resistance in tomato (*Lycopersicon esculentum. Mill* cv. *vollendung*) to CMV. *Crop protection* **19**: 401-405.

Arab, L., Steck-Scott, S., Fleishauer, A.T. (2002). Lycopene and the lung. *Experimental Biology and Medicine* **10**: 894-899.

Arie, T., Takahashi, H., Kodama, M., Teraoka, T. (2007). Tomato as a model plant for plant-pathogen interactions. *Plant Biotechnology* **24**: 135-147.

Ayana, G., Fininsa, C., Ahmed, S., Wydra, K. (2011). Effects of soil amendment on bacterial wilt caused by *Ralstonia solanacearium* and tomato yields in Ethiopia. *Plant Protection Research* **51**: 72-73.

Baker, K.F. and Cook, R.J. (1974). Biological control of Plant Pathogens. Freeman, F.W (ed). *American Phytopathological Society*. Minnesota. pp. 433.

Bakir, V., Ozdemir, Z., Yardim, H. (2012). Reaction of some popular hybrid tomato cultivars grown in Aegean region to bacterial speck disease and determination of disease incidence in Sahnalı, Aydın. *Phytopathology.* **41**: 37-42.

Beijerinck, M. (1898). Concerning a contagium vivum fluidum as cause of the spot disease of tobacco leaves. *Phytopathological Classics* **7**: 33-52.

Benhamou, N. and Belanger, R.R. (1998). Benzothiadiazole-mediated induced resistance to *Fusarium oxysporum* f. sp. *radices- lycopersici* in tomato. *Plant physiology 118*: 1203-1212.

Bennie, A.T.P. and Hensley, M. (2000). Maximizing precipitation utilization in dryland agriculture in South Africa-a review. *Hydrology* **241**: 124-139.

Berge, O., Monteil, C. L., Bartoli, C., Chandeysson, C., Guilbaud, C., Sands, D. C., Morris, C.E. (2014). A user's guide to a data base of the diversity of *Pseudomonas syringae* and its application to classifying strains in this phylogenetic complex. *PLoS ONE* **9**: e105547.

Bergeron, M.G. and Ouellette, M. (1998). Preventing antibiotic resistance through rapid genotypic identification of bacteria and of their antibiotic resistance genes in the clinical microbiology laboratory. *Clinical Microbiology* **36**: 2169-217.

Berry, S.Z. and Gould, W.A. (1982). 'Ohio 7870' tomato. *Horticultural science* **17**: 266-267.

Bertolini, E., Olmos, A., Lopez, M.M., Cambra, M. (2003). Multiplex nested reverse transcription–polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in olive trees. *Phytopathology* **93**: 286-292.

Blancard, D. (1997). *A Colour Atlas of Tomato Diseases:* Observations, Identification and Control. John Wiley & Sons, New York. pp. 212.

Bogatzevska, N. (2002). Plant pathogenic bacteria from genus *Pseudomonas* group *syringae* and genus *Xanthomonas* group *vesicatoria* and *axonopodis-* phases of the life cycle (Doctoral dissertation), N. Pushkarov.

Bohs, L. (2005). Major clades in *Solanum* based on ndhF sequences. In: Keating, R.C., Hollowell, V.C., Croat, T.B (eds) A Festschrift for William G. D'Arcy: the legacy of a taxonomist. Monographs in systematic botany from the Missouri botanical garden. Missouri Botanical Garden Press, St. Louis. (vol. **104**, pp. 27-49).

Bonn, W.G. and Lesage, S. (1984). Control of bacterial speck by copper and etheleynebisdithiocarbamate fungicides: their efficacy and residues on leaves. *Environmental Science and Health* **19**: 20-38.

Bower, C.K. and Daeschel, M.A. (1999). Resistance responses of microorganisms in food environments. *International journal of Food Microbiology* **50**: 33-44.

Bull, C.T., De Boer, S.H., Denny, T.P., Firrao, G., Fischer-Le Saux, M., Saddler, G.S., Scortichini, M., Stead, D.E., Takikawa, Y. (2010). Comprehensive list of names of plant pathogenic bacteria, 1980-2007. *Plant Pathology* **92**: 551-592.

Buonaurio, R., Scarponi, L., Ferrara, M., Sidoti, P., Bertona, A. (2002). Induction of systemic acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease. *Plant pathology* **108**: 41-49.

Buonaurio, R., Stravato, V.M., Cappelli, C. (1996). Occurrence of *Pseudomonas syringae* pv. *tomato* race 1 in Italy on *Pto* gene bearing tomato plants. *Phytopathology* **144**: 9-10.

Braun-Kiewnick, A. and Sands, D.C. (2001). *Pseudomonas*. 3rd edition. In: Laboratory Guide for Identification of *Plant Pathogenic* Bacteria. Jones, N.W., Chun, W (eds). APS Press, St. Paul, MN, USA. pp. 84-120.

Brisset, M.N., Cesbron, S., Thomson, S.V., Paulin, J.P. (2000). Acibenzolar-S-methyl induce the accumulation of defence- related enzymes in apple and protection from fire blight*. Plant Pathology* **106**: 529-536.

Brooks, D. M., Hernandez-Guzman, G., Kloek, A.P., Alarcon-Chaidez, F., Sreedharan, A., Rangaswamy, V., Penaloza-Vazquez, A., Bender, C.L., Kunkel, B. N. **(2004).** Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. *tomato* strain DC3000. *Molecular Plant-Microbe Interactions* **17**: 162-174.

Bryan, M. K. (1933). Bacterial speck of tomatoes. *Phytopathology* **23**: 897-904.

Byrne, J.M., Dianese, A.C., Ji, P., Campbell, H.L., Cuppels, D.A., Louws, F.J., Miller, S.A., Jones, J.B., Wilson, M. (2005). Biological control of bacterial spot of tomato under Weld conditions at several locations in North America. *Biological control* **32**: 408-418.

CAB International. (2005). Protection Compendium CD-ROM. CAB International, Wallingford, UK.

CABI. (2012). *Pseudomonas syringae* pv. *tomato* (Distribution map). Kenya. Kengap horticulture.http://www.plantwise.org/KnowledgeBank/Datasheet.aspx?dsid=45020. (Accessed: 02/11/18).

Cai, R., Lewis, J., Yan, S., Liu, H., Clarke, C.R., Campanile, F., Almeida, N.F., Studholme, D.J., Lindeberg, M., Schneider, D., Zaccardelli, M., Setnbal, J.C.,

Morales-Lizzano, N.P., Bernal, A., Coaker, G., Baker, C., Bender, C.L., Leman, S., Vinatzer, B.A. (2011). The plant pathogen *Pseudomonas syringae* pv. *tomato* is genetically monomorphic and under strong selection to evade tomato immunity. *PLoS Pathogens* **7**: e1002130.

Chamberlain, J.S. and Chamberlain, J.R. (1994). Optimization of multiplex PCRs. In: Mullis, K.B., Ferre, F., Gibbs, R.A (eds). The Polymerase Chain Reaction. Birkhäuser, Boston, MS, USA, pp. 38-46.

Chambers, S.C. and Merriman, P.R. (1975). Perennation and control of *Pseudomonas syringae* pv. *tomato* in Victoria. *Agricultural Research* **26**: 657-663.

Christie, J.R. and Perry, V.G. (1951). A root disease of plants caused by a nematode of *Trichodorus*. *Science* **113**: 491-493.

Clare, B. G. (1993). *Agrobacterium*: Biological plant disease control. In: Advanced Engineered Pesticides. Kim, L (ed). Marcel Dekker, New York, pp. 129-146.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *General Virology* **34**: 475-483.

Cole, D.L. (1999). The efficacy of acibenzolar-S-methyl, an inducer of SAR, against bacterial and fungal diseases of tobacco. *Crop protection* **18**: 267-273.

Conlin, K. C. and McCarter, S. M. (1983). Effectiveness of selected chemicals in inhibiting *Pseudomonas syringae* pv. *tomato* in vitro and in controlling bacterial speck. *Plant Disease* **67**: 639-644.

Conover, R.A. and Gerhold, N.R. (1981). Mixture of copper and maneb or mancozeb for control of bacterial spot of tomato and their compatibility for control of fungus diseases. *Proceedings of the Florida State Horticultural Society* **94**: 154-156.

Cook, C., Reason, C. J. C., Hewitson, B. C. (2004). Wet and dry spells within particular wet and dry summers in the South African summer rainfall region. *Climate Research* **26**: 17-31.

Cooksey, D.A. (1988). Reduction of infection by *Pseudomonas syringae* pv. *tomato* using a non-pathogenic, copper-resistant strain combined with a copper bactericide. *Phytopathology* **78**: 601-603.

Cooksey, D.A. (1990). Genetics of bactericide resistance in plant pathogenic bacteria. *Annual Review of Phytopathology* **28**: 201-219

Crow, W.T. and Han, H. 2005. Sting nematode. Plant Health Instructor, doi:10.1094/PHI-I-2005-1208-01. The American Phytopathological Society Press, St. Paul, MN http://www.apsnet.org/edcenter/intropp/lessons/Nematodes/Pages/StingNematode.aspx. Accessed February 11, 2018.

Datnoff L.E., Elmer W.H., Huber D.M. (2007). Mineral Nutrition and Plant Disease. The American Phytopathological Society Press, St. Paul, MN, USA. pp. 278.

De Boer, S.H., Ward, L.X., Chittaranjan, S. (1995). Attenuation of PCR Inhibition in the Presence of Plant Compounds by Addition of BLOTTO. *Nucleic Acid Research* **23**: 2567-2568.

De Boer, S.H., Wieczorek, A., Kummer, A. (1988). An ELISA test for bacterial ring rot of potato with a new monoclonal antibody. *Plant Disease* **72**: 874-878.

Delahaut, K. and Stevenson, W. (2004). Tomato and pepper disorders: bacterial spot and speck. Cooperative extension publication A2604, University of Wisconsin-Madison p2.

De Leon, L., Rodríguez, A., Lopez, M.M., and Siverio, F. (2008). Evaluation of the efficacy of immunomagnetic separation for the detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. *Applied Microbiol*ogy **104**: 776-786.

Diogo, R.V.C. and Wydra, K. (2007). Silicon-induced basal resistance in tomato against *Ralstonia solanacearum* is related to modification of pectic cell wall polysaccharide structure. *Physiological and Molecular Plant Pathology* **70**: 120-129.

Department of Agriculture, Forestry and Fisheries. (2011). A profile of the South African tomato value chain, Department of Agriculture, Forestry and Fisheries, Pretoria, South Africa.

Department of Water Affairs and Forestry. (2004). National Water Resource Strategy, Pretoria.

Doidge, E.M. (1920). A tomato canker. *Agricultural Journal of South Africa* **1**: 718-721.

Doolittle, S.P. (1916). A new infectious mosaic disease of cucumber. *Phytopathology 6:* 145-147.

Drancourt, M., Bollet, C., Carlioz, A. (2000). 16s ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Clinical Microbiology* **38**: 3623-3630.

Edwardson, J.R. and Christie, R.G. (1991). *Cucumoviruses*. In: CRC Handbook of Viruses Infecting Legumes. CRC Press, Boca Raton, pp. 293-319.

Ellis, J.B. and Martin, G.B. (1882). *Macrosporium solani* E&M. *American Naturalist* **16**: 1003.

Elnifro, E.M., Ashshi, A.M., Cooper, R.J., Klapper, P.E. (2000). Multiplex PCR: optimization and application in diagnostic virology. *Clinical Microbiology Reviews* **13**: 550-570.

Engvall, E. and Perlmann, P. (1971). Enzyme Linked Immunosorbent Assay (ELISA): Quantitative Assay of Immunoglobulin G. *Immunochemistry* **8**: 871-874.

Epstein, E. (1994). The anomaly of silicon in plant biology. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 11-17.

Epstein, E. (1999). Silicon. *Annual Reviews of Plant Physiology and Plant Molecular Biology* **50**: 641-664.

FAOSTAT. (2014). Available from: http://faostat.fao.org. (Accessed:17/09/18).

FAOSTAT. (2016). Available from: http://faostat.fao.org. (Accessed:12/10/18).

Fatmi, M. and Schaad, N.W. (2002). Survival of *Clavibacter michiganensis* subsp. *michiganensis* in infected tomato stems under natural field conditions in California, Ohio and Morocco. *Plant Pathol*ogy **51**: 149-154.

Fauteux, F., Remus-Borel, W., Menzies, J.G., Belanger, R.R*. (2005).** Silicon and plant disease resistance against pathogenic fungi. *FEMS Microbiology Letters* **249**: 1-6.

Flor, H. (1956). The complementary genic systems in flax and flax rust. *Advanced Genetics* **8**: 29–54.

Fourie, H., McDonald A. H., Loots G.C. (2001). Plant-parasitic nematodes in field crops in South Africa. 6. Soybean. *Nematology* **3**: 447-454.

Freeman, B.B. and Reimers, K. (2011). Tomato consumption and health: Emerging benefits. *American journal of lifestyle medicine* **5**: 182-191.

Friedman, M. (2013). Anticarcinogenic, cardioprotective, and other health benefits of tomato compounds lycopene, a-tomatine, and tomatidine in pure form and in fresh and processed tomatoes. *Agricultural Food Chemistry* **61**: 9534-9550.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M.G., Meior, B., Dincher, S., Staub, T., Uknes, S., Metroaux, J.P., Kessmann, H., Ryals, J.A. (1996). Benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant diseases* **10**: 61-70.

Frusciante, L., Carli, P., Ercolano, M.R., Pernice, R., Matteo, A.D., Fogliano, V., Pellegrini, N. (2007). Antioxidant nutritional quality of tomato. *Molecular Nutrition and Food Research* **51**: 609-617

Galan, J.E. and Collmer. A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**: 1322-1328.

Garmyn, M., Ribaya-Mercado, J.D., Russel, R.M., Bhawan, J., Gilchrest, B.A. (1995). *Skin lycopene is destroyed preferentially over beta-carotene during irradiation in humans. Experimental Dermatology* (Vol **4**, Pp: 104-11).

Gevers, D., Cohan, F.M., Lawrence, J.G. (2005). Opinion: re-evaluating prokaryotic species. *Nature Reviews Microbiology* **3**: 733-739.

Ghorbani, R.S.J., Wilcockson, C.G., Leifert, C. (2004). Potato late blight management in organic agriculture. *Pest management* **9**: 11-15.

Glaeser, S.P. and Kampfer, P. (2015). Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. *Systemic and applied microbiology* **38**: 237-245.

Glick, D.L., Coffey, C.M., Sulzinski, M.A. (2002). Simultaneous PCR detection of the two major bacterial pathogens of geranium. *Phytopathology* **150**: 54-59.

Gomila, M., Pena, A., Mulet, M., Lalucat, J., García-Valdés, E. (2015). Phylogenomics and systematics in *Pseudomonas. Front Microbiology* **6**: 214.

Goode, M.J. and Sasser, M. (1980). Prevention the key to controlling bacterial spot and bacterial speck of tomato. *Plant Dis*ease **64**: 831-834.

Goszczynska, T. and Serfontein, J.J. (1998). Milk–Tween agar, a semi-selective medium for isolation and differentiation of *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli. Journal of Microbiological Methods* **32**: 65-72.

Goszczynska, T., Serfontein, J.J., Serfontein, S. (2000). Introduction to practical phytobacteriology. SAFRINET, South Africa. pp. 1-83.

Gudmestad, N.C., Baer, D., Kurowski, C.J. (1991). Validating immunoassay test performance in the detection of *Corynebacterium sepenodicum* during the growing season. *Phytopathology* **81**: 475-480.

Grogan, R.G., Kimble, K.A., Schneider, R.W., Ioamou, N. (1974). Investigations of fungal and bacterial diseases of processing tomatoes. Progress report for Processing Tomato Advisory 155. Board. Department of Plant Pathology, University of California, Davis, California, USA.

Hammond-Kosack, K.E. and Parker, J.E. (2003). Deciphering plant- pathogen communication: fresh perspectives for molecular resistance breeding. *Current Opinion in Biotechnology* **14**: 177-193.

Harris, K.A and Hartley, J.C. (2003). Development of broad-range 16s rDNA PCR for use in the routine diagnostic clinical microbiology service. *Medical Microbiology* **52**: 685-690.

Hartman, G.L. and Huang, Y.H. (1995). Characteristics *of Phytophthora infestans* isolates and development of late blight on tomato in Taiwan. *Plant Disease* **79**: 849-852.

Hass, J.H., More, L.W., Ream, W., Manulis, S. (1995). Universal PCR Primes for Detection of Phytopathogenic *Agrobacterium* Strains. *Environmental Microbiology* **61**: 2879-2884.

Henis, Y. and Bashan, Y. (1985). Epiphytic Survival of Bacterial Leaf Pathogens. In: Microbiology of the Phyllosphere*.* Fokema, N.N. and Den Heuvel, J.V (eds). Cambridge University Press, United Kingdom. pp. 252-268.

Hildebrand, D.C., Schroth, M.N, Sands, D.C. (1988). *Pseudomonas* in: Laboratory guide for identification of plant pathogenic bacteria. Schaad, N.W. (ed): APS Press, St. Paul, MN, USA. pp. 1-398.

Horemans, B., Smolders E., Springael D. (2013). Carbon source utilization profiles suggest additional metabolic interactions in a synergistic linuron-degrading bacterial consortium. *FEMS Microbiology Ecology* **84**: 24-34.

Huang, Q. and Lakshman, D. K. (2010). Effect of clove oil on plant pathogenic bacteria and bacterial wilt of tomato and geranium. *Plant Pathology* **92**: 701-707.

Hulbert, S.H., Webb, C.A., Smith, S.M., Sun, Q. (2001). Resistance gene complexes: evolution and utilization. *Annual Review of Phytopathology* **39**: 285-312.

Ijaz, A., Khan, I., Zareen, S., Khan, M.I., Khan, R. and Haroon, M. (2017). Yield and yield Attributes of tomato (*Lycopersicon esculentum Mill*) cultivars influenced by weed management techniques. *Pakistan Weed Science Research* **23**: 431-438.

Intergate immigration. https://www.intergate-immigration.com/blog/south-african-provinces/ (Accessed 19 August 2019)

Islam, Z. (2001). Control of rice insect pests. Atkinson, A.D (ed). International Rice Research Institute, Philippines. pp. 16-56.

Jagger, I.C. (1916). Experiment with the *cucumber mosaic virus. Phytopathology* **6**: 148-151.

Ji, P., Campbell, H.L., Kloepper, J.W., Sunslow, T.V., Wilson, M. (2006). Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria*. Biological Control* **36**: 358-367.

Jin, Q., Thilmony, R., Zwiesler-Vollick, J., He, S.Y. (2003). Type III protein secretion in *Pseudomonas syringae. Microbes and infection* **5**: 301-310.

Jones, G.D. (1998). The Epidemiology of Plant Diseases. 3rd edition. Kluwer Academic Publishes, London. pp. 371-388.

Jones, J.B. (1991). Bacterial Speck in Compendium of Tomato Diseases. Jones, J.B., Jones, J.P., Stall, R.E., Zitter, T.A (eds). APS Press, St. Paul, MN. pp. 26-27.

Jones, J.B., Bouzar, H., Stall, R.E., Almira, E.C., Roberts, P.D., Bowen, B.W., Sudberry, J., Strickler, P.M., Chun, J. (2000). Systematic analysis of *xanthomonads* (*Xanthomonas* spp.) associated with pepper and tomato lesions. *International Journal of Systematic and Evolutionary Microbiology* **50**: 1211-1219

Jones, J.B., Jones, J.P., Stall, R.E., Zitter, T.A. (1991). *Diseases caused by bacteria.* In Compendium of tomato diseases. The American Phytopathological Society, St. Paul, MN. pp. 73.

Jones, J.P. and Jones, J.B. (1988). Field control of target spot and bacterial speck of tomato. *Proceedings of the Florida State Horticultural Society* **101**:358-361.

Jones, J.B., Stall, R.E., Bouzar, H. (1998). Diversity among *Xanthomonas* pathogenic on pepper and tomato. *Annual Review of Phytopathology* **36**41 - 58.

Jones, J., Zitter, T., Momol, T., Miller, S. (2014). Compendium of tomato diseases and pests, second edition. American Phytopathological Society, St. Paul, MN. pp. 68-168.

Kado, C. I. (2010). Plant Bacteriology. American Phytopathological Society, St. Paul, Minnesota. pp. 72.

Kalloo, G. (1991). Genetic improvement of tomato. In: Monographs on theoretical and applied Genetics. Springer -verlag, Germany. pp. 15-17.

Kaluzna, M., Ferrante, P., Sobiczewski, P., Scortichini, M. (2010). Characterization and genetic diversity of *Pseudomonas syringae* from stone fruits and hazelnut using repetitive-PCR and MLST. *Plant Pathology* **92**: 781-787.

Karjalainen, R., Kangasneim, A., Hamalainen, J., Tegel, J. (1995). Evaluation of PCR Methods for the Detection of Bacterial Ring Rot Infection in Potato. *EPPO Bulletin* **25**:169-175.

Karssen, G. (2002). The plant parasitic nematode genus *Meloidogyne Goldi,* 1892 (Tylenchide) in Europe. Brill, Netherlands. pp. 59-64.

Keinath, A. (2012). Controlling bacterial spot on tomato and pepper. Clemson Cooperative Extension, Illinois. Pp. 91.

Kessmann, H., Oostendorp, M., Staub, T., Goerlach, J., Friedrich, L., Lawton, K., Ryals, J. (1996). *Mode of action of a new plant activator.* Proceedings of the Brighton protection conference: p12.

King, E.O., Ward, M.K., Raney, D.E. (1954). Two simple media for the identification of pyocyanin and fluorescein. *Journal of Laboratory Clinical Medicine* **44**: 301-307.

Kirk, W., Wharton, P., Hammerschmidt, R., Abu-el Samen, F., Douches, D. (2013). Late Blight. Michigan State University Extension Bulletin E-2945. East Lansing. pp. 23.

Koike, S.T., Cladders, P., Paulus, A.O. (2007). Vegetable diseases, a color handbook. Academic press, USA. pp. 448

Kokoskova, B., Pouvoca, D., Pavela, R. (2011). Effectiveness of plant essential oils against *Erwinia amylovora, Pseudomonas syringae* pv. *syringae* and associated saprophytic bacteria on / in host plants. *Plant Pathology* **93**: 133-139.

Koller, W. **(1998).** Chemical approaches to managing plant pathogens. In: Handbook of integrated pest management. Ruberson, J.R (ed). Dekker, New York. pp. 337-376.

Konstantinidis, K.T. and Tiedje, J.M. (2005). Towards a genome-based taxonomy for prokaryotes. *Bacteriology* **187**: 6258-6260.

Koonin, E.V, Dolja, V.V, Morris, T.J. (1993). Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Critical Reviews in Biochemistry and Molecular Biology* **28**: 375-430.

Kunkeaw, S.S., Tan, G. Coaker. (2010). Molecular and evolutionary analyses of *Pseudomonas syringae* pv. *tomato* race 1. Molecular *Plant-Microbe Interactions* **23**: 415-424.

Knapp, S. 2002. *Solanum* section Geminata. *Flora Neotropica* **84**: 1-405.

KwaZulu-Natal freight transport data bank. http://www.kzntransport.gov.za/public_trans/freight_databank/kzn/industries/Fruit_veg/index_xml.html (Accessed 19 August 2019).

Lamichhane, J.R., Balestra, G.M., Leonardo, V. (2010). Response of commonly cultivated tomato cultivars in Nepal to bacterial speck. *Phytopathology Mediterranea* **49**: 406-413.

Lamour, K. and Kamoun, S. (2009). *Oomycetes genetics and genomics: Diversity interactions and research tools*. Wiley Blackwell, New Jersey. pp. 76-90.

Latorre, B.A., and Jones, A.L. (1979). *Pseudomonas morsprunorum*, the Cause of Bacterial Canker of Sour Cherry in Michigan and its Epiphytic Association with *Pseudomonas syringae*. *Phytopathology* **69**: 335-339.

Lawton, M.B. and MacNeill, B.H. (1986). Occurrence of race 1 of *Pseudomonas syringae* pv. *tomato* on field tomato in southwestern Ontario. *Canadian Journal of Plant Pathology* **8**: 85-88.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K, Delaney, T., Kessmann, H., Staub, T., Ryals, J. (1996). Benzothiadiazole induces disease resistance in

Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant cell* **10**: 71-82.

LeBoeuf, J., Cuppels, D., Dick, J., Loewen, S., Celetti, M. (2005). Bacterial diseases of tomato: bacterial spot, bacterial speck, bacterial canker. OMAFRA Fact Sheet, ISSN 1198-712X.

Lelliott, R.A., Billing, E., Hayward, A.C. (1966). A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Applied Bacteriol*ogy **29**: 470-489.

Lelliott, R. A. and Stead, D.E. (1987). *Methods for the Diagnosis of Bacterial Diseases of Plants*. In: Methods in plant pathology. Blackwell Scientific Publications. Oxford. pp. 33-37.

Lin, C.P., Chen, T.A., Wells, J.M., Van der Zweet, T. (1987). Identification and detection of Erwinia amylovora with monoclonal antibodies. *Phytopathology* **77**: 376-380.

Lindow, S.E., McGourty, G., Elkins, R. (1996). Interactions of antibiotics with *Pseudomonas fluorescens* A506 in the control of fire blight and frost injury to pear. *Phytopathology* **86**: 841-848.

Locke, S. B. (1949). Resistance to early blight and septoria leaf spot in the genus *Lycopersicon. Phytopathology* **89**: 829-836.

Louws, F.J., Fulbright, D.W., Stephens, C.T. and de Bruijn, F.J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied Environmental Microbiology* **60**: 2286-2295.

Louws, F.J., Wilson, M., Campbell, H.L., Cuppels, D.A., Jones, J.B., Shoemaker, P.B., Sahin F., and Miller, S.A. (2001). Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Disease* **85**: 481-48.

Lozano, J.C. and D.W. Wholey. (1974). The production of a bacterial-free planting stock of cassava. *World Crops* **26**: 114-117.

Lu, D., Sun, X., Yan, F., Wang, X., Xu, R., Lu, W. (2013). Effects of high temperature during grain filling under control conditions on the physicochemical properties of waxy maize flour. *Carbohydrate Polymers* **98**: 302-310.

Ma, J. F. and Yamaji, N. (2006). Silicon uptake and accumulation in higher plants. *Trends Plant Science* **11**: 392-397.

Maboko, M.M., du Plooy, C.P., Bertling, I. (2009). Comparative performance of tomato on soilless vs in-soil production systems. *Acta Horticulture* **843**: 319-326.

Macnab, A.A., Sherf, A.F., Springer, J.K. (1983). Identifying diseases of vegetables. The Pennsylvania State University, USA. pp. 20-31.

Mafham, P.J., Boddy, L., Randerson, P.F. (2002). Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles–a critique. *FEMS Microbiology Ecology* **42**: 1-14.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences* **95**: 3140-3145.

Malherbe, S. and Marais, D. (2015). Economics yield and ecology: a case study from the South African tomato industry. *Outlook on Agriculture* **44**: 37-47.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S.V., Machado, M.A., Toth, I., Salmond, G., Foster, G.D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*. **13**: 614-629.

Marques, A.S.A., Corbiere, R., Gardan, L., Tourte, C., Manceau, C., Taylor, J.D., Samson, R. (2000). Multiphasic approach for the identification of the different classification levels of *Pseudomonas savastanoi* pv. *phaseolicola*. *Plant Pathology* **106**: 715-734.

Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P., Willems, A. (2008). Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former

Sinorhizobium). *International Journal of Systematic and Evolutionary Microbiology* **58**: 200-214.

Martin, G. B. (2012). *Suppression and activation of the plant immune system by Pseudomonas syringae effectors AvrPto and AvrPtoB*. In: Effectors in Plant-Microbe Interactions. Martin, F. and Kamoun, S (eds). Wiley-Blackwell, UK. pp. 123-154

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R. (1992). A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae. Nucleic Acids Research.* **20**: 3479-3483.

Massee, G. (1895). The "Sleepy disease" of tomatoes. Garden chronicles series 3. **17**: 707-708.

McLeod, A., Masimba, T., Jensen, T., Serfontein, K., Coertze, S. (2017). Evaluating spray programs for managing copper resistant *Pseudomonas syringae* pv. *tomato* populations on tomato in the Limpopo region of South Africa. *Crop Protection* **102**: 32-42.

Menzel, W., Jelkman, W., Maiss, E. (2002). Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. *Virologic Methods* **99**: 81-92.

Migula, W. (1894). Uber ein neus system der bakterein Karlsruhe. *Bacteriology* **1**: 235-328.

Min, S.C., Dong, S.P., Yeo, H.Y., Seong, H. K., Myung, Y.S., Chang, W.C., Young, S.K. **(2012).** Genetic Differentiation of *Pseudomonas syringae* pv. *tomato* from other *P. syringae* Pathovars using REP-PCR and URP-PCR. *Plant Pathology* **28**: 60-67.

Mohammed, M., Ebrahim, I.D., Houshing, N.R., Ahmad, T. (2013). Growth and yield of tomato (*lycopersicum esculentum* Mill) as influenced by different organic fertilizers. *Agronomy and plant production* **4**: 734-738.

Mohan, S.K. and Schaad, N.W. (1987). An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola* in contaminated bean seed. *Phytopathology* **77**:1390-1395.

Mondal, K. and Mani, C. (2009). ERIC-PCR-Generated Genomic Fingerprints and their Relationship with Pathogenic Variability of *Xanthomonas campestris* pv. *punicae*, the Incitant of Bacterial Blight of Pomegranate. *Current Microbiology* **59**: 616-620.

Moore, E.R.B., Tindall, B.J., Martins Dos Santos, V.A.P., Pieper, D.H., Ramos, J.L., Palleroni, N.J. (2006). Nonmedical *Pseudomonas.* in: The Prokaryotes *Proteobacteria*: Gamma subclass. Springer, New York. (vol. **6**, pp. 21-28).

Moyer, J.W. (2000). Tospoviruses. 2nd edition. In: Encyclopaedia of Microbiology. Hull, R (ed.). Academic Press, London. (vol. **4**, pp. 592-597).

Mui-Yun, W. (2003). Soil borne plant pathogen class project. pp. 728.

Mulet, M., Gomila, M., Lemaitre, B., Lalucat, J., García-Valdes, E. (2012). Taxonomic characterization of *Pseudomonas* strain L48 and formal proposal of *Pseudomonas entomophila* sp. nov. Syst. *Applied Microbiology* **35**: 145-149

Mullis, K.B. (1990). Target amplification for DNA analysis by the polymerase chain reaction. *Annales de biologie clinique* **48**: 579-582.

Mumford, R., Boonham, N., Tomlinson, J., Barker, I. (2006). Advances in molecular phytodiagnostics-new solutions for old problems. *Plant Pathology* **116**: 1-19.

Naika, S., Juede, J., Goffau, M., Hilmi, M., Dam, V. (2005). Cultivation of tomato production, processing and marketing, Agromisa/ CTA. Revised edition, Agrodok -series No 17.

Nowicki, M., Foolad, M.R., Nowakowska, M., and Kozik, E.U. (2012). Potato and tomato late blight caused by *Phytophthora infestans*: An overview of pathology and resistance breeding. *Plant Disease* **96**: 4-17.

Obeng-Ofori, D., Danguah, E.Y., Ofosu-Anim, J. (2007). *Vegetable and spice crop production in West Africa*. City printers limited, Ghana. pp. 53-65.

Okabe, N. (1933). Bacterial diseases of plants occurring in Formosa. II. Bacterial leaf spot of tomato. *Society of Agricultural Tropical Taiwan* **5**: 26-36.

Ozdemir, Z. (2009). Development of a Multiplex PCR assay for the simultaneous detection of *Clavibacter michiganensis* subsp. *michiganensis. Pseudomonas syringae* pv. *tomato* and *Xanthomonas axonopodis* pv. *vesicatoria* using pure cultures. *Plant Pathology.* 91: 495-497.

Palukaitis, P., Roossinck, M., Dietzgen, R.G. Francki, R.I.B. (1992). *Cucumber mosaic virus. Advances in Virus Research* **41**: 281-348.

Palukaitis, P. and Zaitlin, M. (1986). *Tobacco mosaic virus infectivity and replication.* In: The plant viruses. Van Regenmortel, M. H. V., Fraenkel-Conrat, H (eds). Plenum, New York. (vol. **2**, pp. 105-131).

Panagopoulos, C.G. (2000). *Diseases of vegetable crop.* In vegetable diseases. Stamoulis, Athens. pp. 15-189.

Parte, A.C. (2014). LPSN-list of prokaryotic names withstanding in nomenclature. *Nucleic Acids Research* **42**: 613-616.

Pecnold, P.C. and Grogan, R.G. (1973). Deoxyribonucleic acid homology groups among phytopathogenic *Pseudomonas* species. *International Journal of Systematic and Evolutionary Microbiol*ogy **23**: 111-121.

Pedley, K. and Martin, G. (2003). Molecular basis of *Pto* mediated resistance to bacterial speck disease in tomato. *Annual Reviews of Phytopathology* **41**: 215-43.

Peralta, I.E. and Spooner, D.M. (2001). Granule-bound starch synthase (GBSSI) gene phylogeny of wild tomatoes (*Solanum* L. section *Lycopersicon* (Mill) Wettst. Subsection *Lycopersicon. Botany* **88**: 1888-1902.

Pernezny, K., Kudela, V., Kokoskova, B., Hladka, I. (1995). Bacterial diseases of tomato in the Czech and Slovak Republics and lack of streptomycin resistance among copper-tolerant bacterial strains. *Crop Protection* **14**:267-270.

Pernezny, K., Roberts, P.D, Murphy, J.F., Goldberg, N.P. (2003). Compendium of pepper diseases. APS press, USA. pp. 63.

Perveen, R., Suleria, H.A.R., Anjum, F.M., Butt, M.S., Pasha, I., Ahmad, S. (2015). Tomato (*Solanum lycopersicum* L.) carotenoids and lycopenes chemistry; metabolism, absorption, nutrition, and allied health claims-A comprehensive review. *Critical Reviews in Food Science and Nutrition* **55**: 919-929.

Pitblado, R.E., MacNeill, B.H., Kerr, E.A. (1984). Chromosomal identity and linkage relationships of *Pto*, a gene for resistance to *Pseudomonas syringae* pv. *tomato* in tomato. *Plant Pathology* **6**: 48-53.

Pohronezny, K., Leben, C., Larses, P.O. (1977). Systemic invasion of cucumber by *Pseudomonas lachrymans*. *Phytopathology* **67**:730-734.

Pohronezny, K., Moss, M.A., Dankers, W., Schenk, J. (1990). Dispersal and management of *Xanthomonas campestris* pv. *vesicatoria* during thinning of direct-seeded tomato. *Plant Disease* **74**: 800-805.

Pohronezny, K.L., Stall, R. E., Canteros, B. I., Kegley, M., Datnoff, L.E., Subramanya, R. **(1992).** Sudden shift in the prevalent race of *Xanthomonas campestris* pv. *vesicatoria* in pepper fields in southern Florida. *Plant Disease* **76**: 118-120.

Pohronezny, K.L. and Volin, R.B. (1983). *Compendium of tomato diseases*. In: Corky root rot. Jones, J.B., Jones, J.P., Stall, R.E., Zitter, T.A. (eds). The American Phytopathological society, Minnesota. pp.12-13.

Postharvest innovation programme. (nd). http://postharvestinnovation.org.za/commodities/tomatoes/ (accessed: 23 June 2019).

Preston, G.M. (2000). *Psedomonas syringae* pv. *tomato*: The right pathogen, of the right plant, at the right time. *Molecular Plant Pathology* **1**: 263-275.

Rao, A.V. and Agarwal, S. (2000). Role of antioxidant lycopene in cancer and heart disease. *Journal of the American College of Nutrition* **19**: 563-569.

Rice, R.P., Rice, L.W., Tindal, H.D. (1987). Fruit and vegetable production in Africa. Macmillan publishers, U.K. pp. 371.

Robertson, L.D. and Labate, J.A. (2007). Genetic resources of tomato (*Lycopersicon esculentum Mill.)* and wild relatives. In: Genetic improvement of *solanaceous* crops. Razdan, M.K., Matoo, A.K (eds). Science Publishers, USA. pp. 15-18.

Rodrigues, F.A., and Datnof, L.E. (2015). Silicon and Plant Diseases. Springer, Berlin. pp 7-51.

Romero, A.M., Kousik, C.S., Ritchie, D.F. (2001). Resistance to bacterial spot in bell pepper induced by acibenzolar-*S*-methyl. *Plant Disease* **85**: 189-194

Rowell, J.B. (1953). Leaf blight of tomato and potato plants. *Agricola* **320**: 1-29

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., Hunt, M.D. **(1996).** Systemic acquired resistance. *Plant cell* **8**: 1809-1819.

Saha, S., Pfeufer, E., Bessin, R., Wright, S., Strang, J. (2016). Vegetable production guide for commercial growers, 2016-17. University of Kentucky, Cooperative Extension Service. ID-36.

Santos, S.R. and Ochman, H. (2004). Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology* **6**: 754-759.

Sasser, J.N. (1980). Root knot nematodes: A global menace to crop production. *Plant Disease* **64**: 36-41.

Sarkar, S.F. and Guttman, D.S. (2004). Evolution of the Core Genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology* **70**: 1999-2012.

Sarris, P.F., Trantas, E.A., Mpalantinaki, E., Ververdis, F., Goumas, D.E. (2012). *Pseudomonas viridiflava*, a multi host plant pathogen with genetic variation at the molecular level. *PLoS ONE* **7**: e36090.

Savant, N. K., Snyder G.H., Datnoff, L.E. (1997). Silicon management and sustainable rice production. *Advanced Agronomy* **58**: 151-199.

Scheneider, R.W., Hall, D.H., Grogan, R.G. (1975). Effect of bacterial speck on tomato yield and maturity. (Abstr.) Proc. AM. *Phytopathology Society* **2**: 118.

Scheneider, R.W. and Grogan, R.G. (1977). Bacterial speck of tomato: sources of inoculum and establishment of a resident population. *Phytopathology* **67**: 388-394.

Sessa, G. and Martin, G.B. (2000). Signal recognition and transduction mediated by the tomato *Pto* kinase: a paradigm of innate immunity in plants. *Microbes and Infection* **2**:1591-1597.

Shaw, J.G. (1999). Tobacco mosaic virus and the study of early events in virus infections*. Biological Sciences* **354**: 603-611.

Shenge, K.C., Stephan, D., Mabagala, R.B., Mortensen, C.N., Wydra, K. (2008). Molecular characterization of *Pseudomonas syringae* pv. *tomato* isolates from Tanzania. *Phytoparasitica* **36**: 338-351.

Shi, J. and Le Maguer, M. (2000). Lycopene in tomatoes: chemical and physical properties affected by food processing. *Food Science and Nutrition* **40**: 1-42.

Shila, S.J., Islam, M.R., Ahmed, N.N., Dastogeer, K.M.G, Meah, M.B. (2013). Detection of *Pseudomonas Syringae* pv*. lachrymans* Associated with the Seeds of Cucurbits. *Universal Journal of Agricultural Research* **1**: 1-8.

Smith, E. (1910). A new tomato disease of economic importance. *Science* **31**: 794-796.

Smyth, D.S. and Robinson, D.A. (2009). Integrative and sequence characteristics of a novel genetic element, ICE6013, in *Staphylococcus aureus. Journal of Bacteriology,* **191**: 5964-5975.

Snyder, W.C. and Hansen, H.N. (1940). The species concept in *Fusarium. Botany* **27**: 64-67

Spiers, A.J., Buckling, A., Rainey, P.B. (2000). The causes of *Pseudomonas* diversity. *Microbiology* **146**: 2345-2350.

Spooner, D.M., Peralta, I.E., Knapp, S. (2005). Comparison of AFLPs to other markers for phylogenetic inference in wild tomatoes [*Solanum* L. section *Lycopersicon* (Mill.) Wettst. subsection *Lycopersicon*]. *Taxonomy* **54**: 43-61.

Sreedharan, A., Penaloza-Vazquez, A., Escober, M.C., Bender, C.L., Rayas-Duarte, P. (2009). Simple and rapid capillary zone electrophoresis method for the detection of coronamic acid, a precursor to the *Pseudomonas syringae* phytotoxin coronatine. *Agricultural and food chemistry* **57**: 10518-10523.

Srinivasan, R. (2010). Safer tomato production methods. A field guide for soil fertility and pest management. *AVRDC- The world vegetable centre Shanhua Taiwan AVRDC* **97**:10-740.

Statistics South Africa. (2007). Census of commercial agriculture, 2007, Limpopo, Report No. 11-02-10 (2007), Statistics South Africa, Pretoria, South Africa.

Stevenson, W.R. (1991). Diseases caused by fungi: late blight. In: Compendium of tomato diseases. Jones, J.B., Jones, J.P., Stall, R.E., Zitter, T.A (eds). APS Press, St. Paul, MN. pp. 17-18.

Sticher, L., Mauchmani, B., Metraux, J.P. (1997). Systemic acquired resistance. Annual Reviews of *Phytopathology* **35**: 235-270.

Szabo, J. (2014). Molecular Methods in Epidemiology of *Methicillin Resistant Staphylococcus aureus* (MRSA): Advantages, disadvantages of different techniques. *Diagnostic Microbiology and Infectious Disease* **3**: 147.

Tancos, M.A., Lange, H.W., Smart, C.D. (2015). Characterizing the genetic diversity of the *Clavibacter michiganensis* subsp. *michiganensis* population in New York. *Phytopathology* **105**: 169-179.

Thayer, P.L. and Stall, R.E. (1961). A survey of *Xanthomonas vesicatoria* resistance to streptomycin. *Proceedings of the Florida Horticultural Society* **75**: 163-165.

Tomato disease help: treatments, control and prevention. https://tomatodiseasehelp.com/bacterial-speck (Accessed 13 August 2019).

Turgut, A. and Basim, H. (2013). Sensitivity of tomato (*Solanum lycopersicum*) cultivars from Turkey to bacterial speck (*Pseudomonas syringae* pv. *tomato*). *African Journal of Biotechnology* **12**: 1793-1801.

Toor, R.K. and Savage, G.P. (2005). Antioxidant activity in different fractions of tomatoes. *Food Research International* **38**:487-494.

Tsutomu, A., Hideki, T., Motoichiro, K., Tohru, T. (2007). Tomato as a model plant for plant-pathogen interactions. *Plant Biotechnology* **24:** 135-147.

United States Department of Agriculture, Agricultural Research Service. (2000). USDA Nutrient Database for Standard Reference, Release 13. The Service, Washington, D.C

Uppalapati, S.R., Ishiga, Y., Wangdi, T., Kunkel, B. N., Anand, A., Mysore, K. S., and Bender, C.L. (2007). The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. *Molecular Plant-Microbes Interactions* **20**: 955-965.

Upstone, M.E. (1971). Control of bacterial diseases of tomatoes in Jersey. Proc. 6th Br. Insecticide. Fungicide. Conf. pp. 211-214.

Vauterin, L., Hoste, B., Kersters, K., Swings, J. (1995). Reclassification of *Xanthomonas*. *Systemic Bacteriology* **45**: 472-489.

Vaneechoutte, M., De Beenhouwer, H., Claeys, G., Verschraegen, G., De Rouck, A., Paepe, N., Elaichouni, A., Portaels, F. (1993). Identification of Mycobacterium species by using amplified ribosomal DNA restriction analysis. *Clinical Microbiology* **31**: 2061-2065.

Van der Hoeven, R.S., Ronning, C., Giovannoni, J.J., Martin, G., Tanksley, S.D. (2002). Deductions about the number, organization, and evolution of genes in the tomato genome based on analysis of a large expressed sequence tag collection and selective genomic sequencing. *The Plant Cell* **14**:1441-1456.

Varvaro, L. and Guario, A. (1983). Preoccupanti manifestazioni di picchiettatura e di macchiettatura batterica del pomodoro in Puglia. *Informatore Agrario* **37**:27471–27473.

Versalovic, J., Koeuth, T., Lupski, J.R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19:** 6823-6831.

Vicente, J. and Roberts, S. (2007). Discrimination of *Pseudomonas syringae* isolates from sweet and wild cherry using rep-PCR. *Plant Pathology* **117**: 383-392.

Walker, J.C. (1952). Diseases of Vegetable Crops. McGraw Hill Book Company, New York. pp. 529.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P., Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* **3**: 1085-1094

Westra, A.A.G., Slack, S.A, Drennan, J.L. (1994). Comparison of some diagnostic assays for bacterial ring rot of potato: a case study. *American Journal of Potato Research* **71**: 557-565.

Werner, N.A., Fulbright, D.W., Podolsky, R., Bell, J., Hausbeck, M.K. (2002). Limiting populations and spread of *Clavibacter michiganensis* subsp*. michiganensis* on seedling tomatoes in the greenhouse. *Plant Disease* **86**: 535-542.

Wilson, M., Campbell, H.L., Ji, P., Jones, J.B., Cuppels, D.A. (2002). Biological control of bacterial speck of tomato under Weld conditions at several locations in North America. *Phytopathology* **92**: 1284-1292.

Woo, P.C.Y., Lau, S.K.P., Teng, J.L.L., Tse, H., Yuen, K.Y. (2008). Then and now: use of 16s rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical Microbiology and Infection* **14**: 908-934.

Wreikat, B.I., Al-Banna, L.S., Khlaif, H.M. (2006). Detection and identification of bacterial speck of tomato (*Pseudomonas syringae* pv. *tomato*) by Polymerase Chain Reaction. *Agricultural Sciences* **2**: 45-56.

Xue, G., Sun, W., Song, A., Li, Z., Fan, F., Liang, Y. (2010). Influence of silicon on rice growth, resistance to bacterial blight and activity of pathogenesis-related proteins. *Scientia Agricola* **43**: 690-697.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R.W., Vivian, A., Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiology* **146:** 2385-2394.

Young, J.M, Dye, D.W., Wilkie, J.P. (1978). Genus VII *Pseudomonas* Migula 1894. In: A proposed nomenclature and classification for plant pathogenic bacteria. Young, J.M., Dye, D.W., Bradbury, J. F., Panagopoulos., C.G., Robbs., C.F (eds). *New Zealand agricultural research* (vol. **21**, pp. 153-177).

Young, J.M., Dye, D.W., Wilkie, J.P. (1986). Bacterial Speck. In: Vegetable Diseases and Their Control. Sherf, A.F., MacNab, A.A., (eds). John Wiley & Sons Inc, New York. pp. 610-614.

Yu, Z.H., Wang, J.F., Stall, R.E., Yallejos, C. C. (1995). Genomic localization of tomato genes that control a hypersensitive reaction to *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye. *Genetics* **141**: 675-682.

Yunis, H., Bashan, Y., Okon, Y., Henis, Y. (1980). Two sources of resistance to bacterial speck of tomato caused by *Pseudomonas tomato*. *Plant Disease* **64**: 937-939.

Zhao, Y.F., Jones, W. T., Sutherland, P., Palmer, D. A., Mitchell, R.E., Reynolds, P.H.S., Bender, C.L. (2001). Detection of the phytotoxin coronatine by ELISA and localization in infected plant tissue. *Physiological and Molecular Plant Pathology.* **58**: 247-258.

Zaitlin, M. (2000). *Tobacco mosaic virus. AAB descriptions of plant viruses* on CD-ROM and online**,** No. 370**.** Adams, M.J., Antoniw, J.F., Barker, H., Jones, A.T., Murant, A.F., Robinson, D (eds). Wellesbourne, United Kingdom.

Zaccardelli, M., Spasiano, C., Bazzi, C., Merighi, M. (2005). Identification and *in planta* detection of *Pseudomonas syringae* pv. *tomato* using PCR amplification of *hrpZpst. Plant Pathology* **111**: 85-91.

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

(Gardan *et al.*, 1999; Young *et al.*, 1996; Young, 2010; Thakur *et al.*, 2016). *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 hypersensivity 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 (PPPPB) 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 (PPPPB) 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 -80ºC. 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_2HPO_4; 1.5 g $MgSO_4$. $7H_2O$; 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

Strain number	Stored as	Host	Plant part isolated from	Province isolated from	Year of isolation
BD 0001	*P. syringae* pv. *tomato*	Tomato	Fruit	Gauteng	1991
BD 0002	*P. syringae* pv. *syringae*	Tomato	Fruit	Gauteng	1994
BD 0022	*P. syringae* pv. *syringae*	Tomato	Leaf	Gauteng	1995
BD 0028	*P. syringae* pv. *tomato*	Tomato	Seed	Gauteng	1995
BD 0034	*P. syringae* pv. *tomato*	Tomato	Stem	North-West	1996
BD 0035	*P. syringae* pv. *tomato*	Tomato	Stem	North-West	1996
BD 0070	*P. syringae* pv. *tomato*	Tomato	Fruit	North-West	1996
BD 0071	*P. syringae* pv. *tomato*	Tomato	Leaf	Limpopo	1997
BD 0085	*P. syringae* pv. *tomato*	Tomato	Stem	North-West	1998
BD 0091	*P. syringae* pv. *tomato*	Tomato	Stem	Limpopo	1998

Strain number	Stored as	Host	Plant part isolated from	Province isolated from	Year of isolation
BD 0151	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	1999
BD 0159	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	1999
BD 0164	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	1999
BD 0165	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	1999
BD 0269	*P. syringae* pv. *tomato*	Tomato	Stem	Mpumalanga	2002
BD 0278	*P. syringae* pv. *syringae*	Tomato	Leaf	Mpumalanga	2002
BD 0279	*P. syringae* pv. *syringae*	Tomato	Leaf	Mpumalanga	2002
BD 0280	*P. syringae* pv. *syringae*	Tomato	Stem	Mpumalanga	2002
BD 0283	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	2002
BD 0284	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2002
BD 0285	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	2002
BD 0774	*P. syringae* pv. *syringae*	Tomato	Stem	Gauteng	2007
BD 0775	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2007
BD 0778	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2007
BD 0779	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2007

Strain number	Stored as	Host	Plant part isolated from	Province isolated from	Year of isolation
BD 0780	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2007
BD 1354	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2015
BD 1355	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2015
BD 1357	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2015
BD 1358	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2015
BD 1359	*P. syringae* pv. *tomato*	Tomato	Stem	Gauteng	2015
BD 1361	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	2015
BD 1366	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	2015
BD 1367	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	2015
BD 1368	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	2015
BD 0770	*P. syringae* pv. *tomato*	Tomato	Leaf	Gauteng	2007
BD 0771	*P. syringae* pv. *syringae*	Tomato	Stem	Gauteng	2007
BD 0146	*P. viridiflava*	Tomato	Stem	Gauteng	1999
BD 0156	*P. viridiflava*	Tomato	Stem	Gauteng	1999
BD 0223	*P. viridiflava*	Tomato	Stem	Gauteng	2000
BD 0224	*P. viridiflava*	Tomato	Stem	Gauteng	2000
BD 0231	*P. viridiflava*	Tomato	Leaf	Limpopo	2000
BD 0229	*P. cichorii*	Tomato	Stem	Eastern cape	2000

Strain number	Stored as	Host	Plant part isolated from	Province isolated from	Year of isolation
BD 0245	*P. cichorii*	Tomato	Leaf	Gauteng	2001

Table 2.2: Reference strains used in the study obtained from the Collection Francaise de Bacteries Phytopathogenes (CFBP), France.

Strain number	Isolated from	Scientific name	Year obtained
CFBP 2212	Tomato	*P. syringae* pv. *tomato*	2007
CFBP 2107	Tomato	*P. viridiflava*	2007
CFBP 1392	Onion	*P. syringae pv. syringae*	2007
CFBP 2101	Tomato	*P. cichorii*	2007

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 K_2HPO_4, 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, erythitol, lactose, inositol, fructose, D (-) tartrate, L (+) tartrate and DL-lactate. The minimal medium was prepared by adding $NH_4H_2PO_4$ (0.5 g), KCl (0.1 g), $MgSO_4.7H_2O$ (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 ⁰C 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 $MgCl_2$, 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).
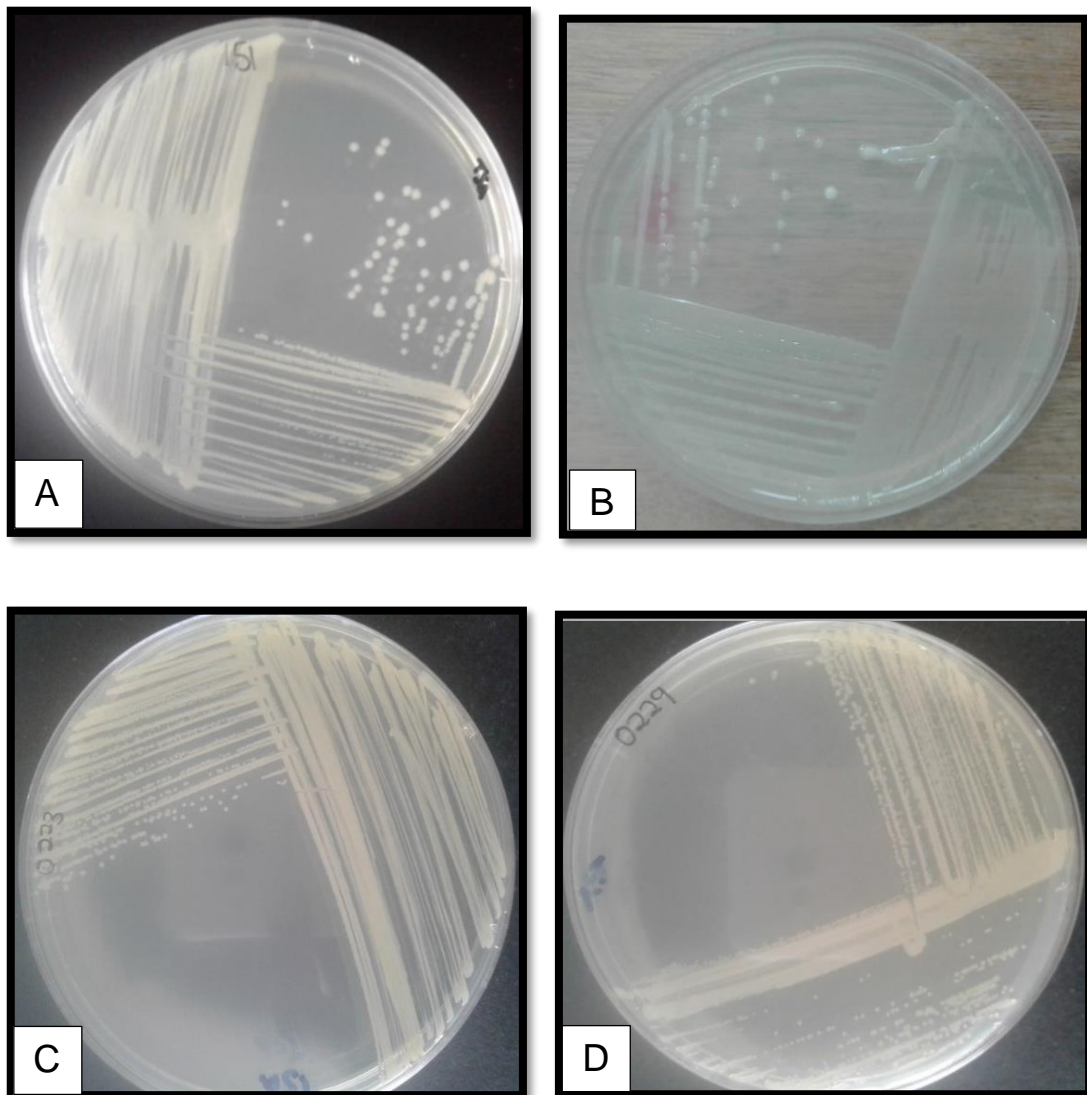
Figure 2.1: Colony morphology of *Pseudomonas* strains on TSA medium; A = *P. syringae* pv. *tomato* (BD 0151); B = *P. syringae.* pv. *syringae* (BD 0280), C = *P. viridiflava* (BD 0223) and D= *P. cichorii* (BD 0229).

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).
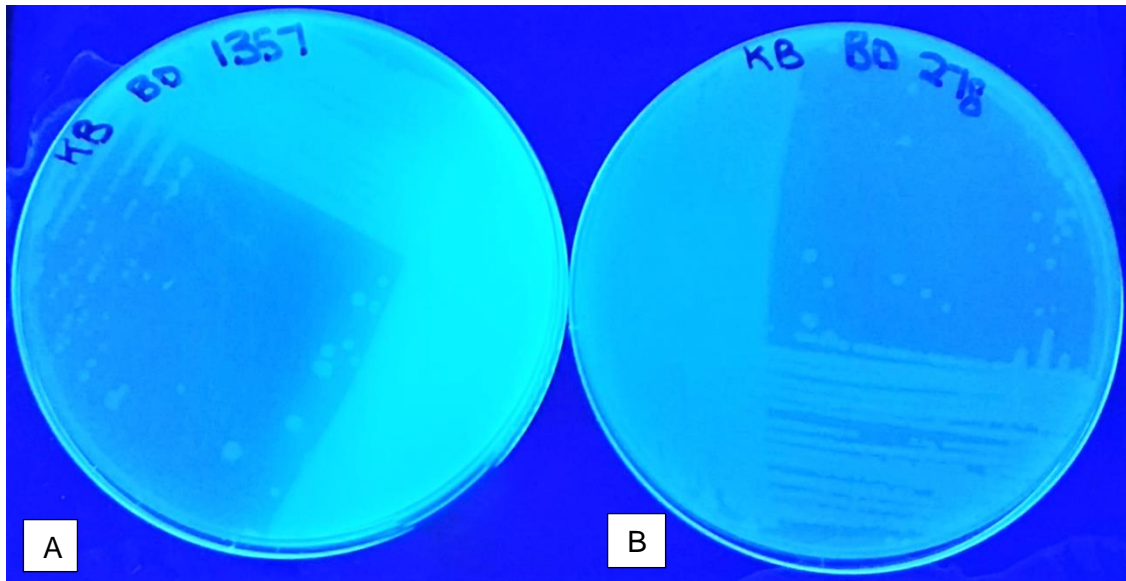
Figure 2.2: Fluorescence on KB under UV light. *P. syringae* pv. *tomato* isolates; A = (BD 1357) fluorescent and B = *P. syringae* pv. *syringae* (BD 0278) non-fluorescent under UV light.

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.
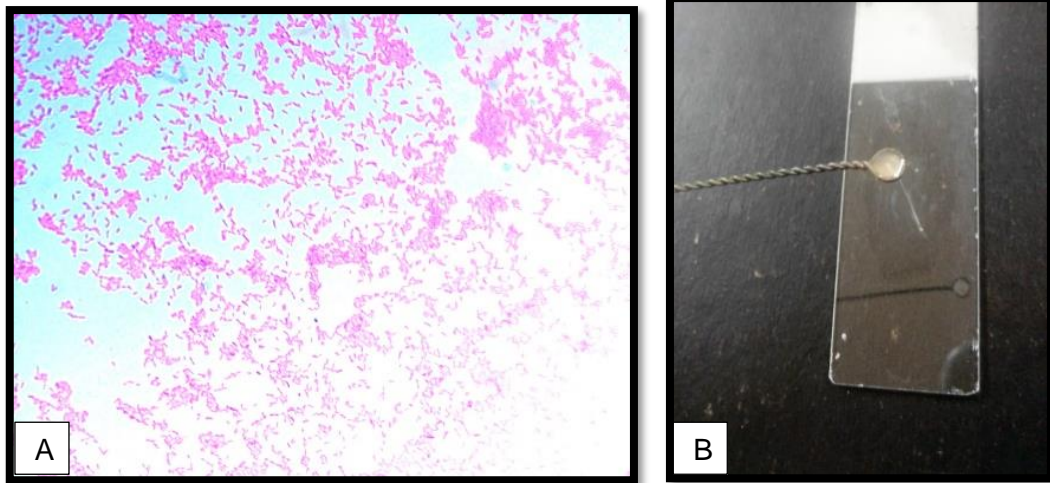
Figure 2.3: Gram staining (A) and KOH (B) reactions of BD 0165 (*P. syringae* pv. *tomato* isolate). All strains used in the study were Gram-negative rods and KOH positive.

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)
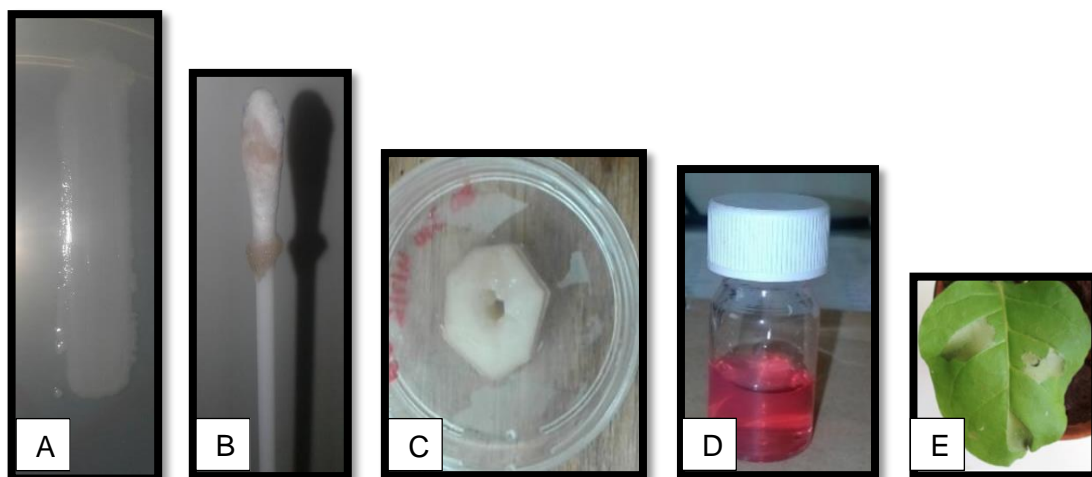
Figure 2.4.1: LOPAT tests results of *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* belonging to the Lopat group Ia. A-Levan production (positive), B-Oxidase reaction (negative), C -Potato soft rot (negative), D- Arginine dihydrolase (negative) and E- Tobacco hypersensitive reaction (positive).
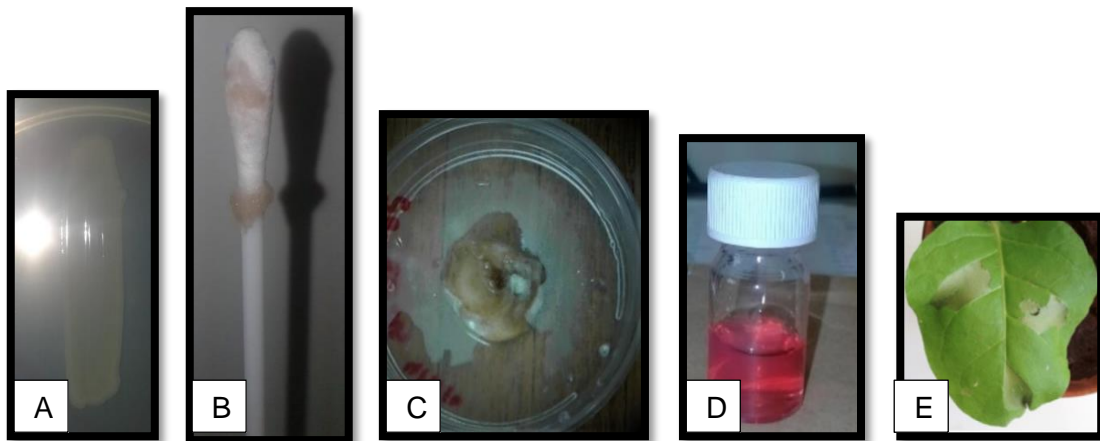
Figure 2.4.2: LOPAT tests results of *P. viridiflava* belonging to the LOPAT group II. A-Levan production (negative), B-Oxidase reaction (negative), C-Potato soft rot (positive), D-Arginine dihydrolase (negative) and E-Tobacco hypersensitive reaction (positive).
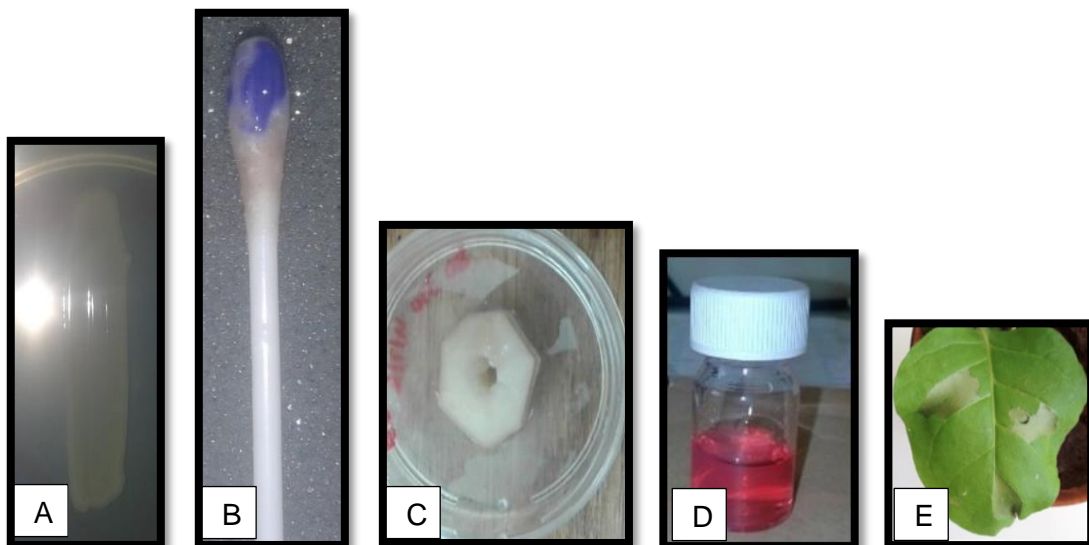
Figure 2.4.3: LOPAT tests results of *P. cichorii* belonging to the LOPAT group III. A-Levan production (negative), B-Oxidase reaction (positive), C-Potato soft rot (negative), D-Arginine dihydrolase (negative) and E-Tobacco hypersensitive reaction (positive).

(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.

Strain ID	KOH solubility	LOPAT tests					LOPAT group	Pathogenicity test	Fluorescence on KB	PCR
		L	0	P	A	T				
CFBP 1212	+	+	-	-	-	+	Ia	+	+	+
BD 0001	+	+	-	-	-	+	Ia	+	-	+
BD 0028	+	+	-	-	-	+	Ia	+	+	+
BD 0034	+	+	-	-	-	+	Ia	+	+	+
BD 0035	+	+	-	-	-	+	Ia	+	+	+
BD 0070	+	+	-	-	-	+	Ia	+	-	+
BD 0071	+	+	-	-	-	+	Ia	+	-	+
BD 0085	+	+	-	-	-	+	Ia	-	+	+
BD 0091	+	+	-	-	-	+	Ia	+	+	+
BD 0151	+	+	-	-	-	+	Ia	+	+	+
BD 0159	+	+	-	-	-	+	Ia	+	+	+
BD 0164	+	+	-	-	-	+	Ia	+	+	+
BD 0165	+	+	-	-	-	+	Ia	+	+	+
BD 0269	+	+	-	-	-	+	Ia	+	+	+
BD 0283	+	+	-	-	-	+	Ia	+	+	+
BD 0284	+	+	-	-	-	+	Ia	+	+	+
BD 0285	+	+	-	-	-	+	Ia	+	+	+

Strain ID	KOH solubility	LOPAT tests					LOPAT group	Pathogenicity test	Fluorescence on KB	PCR
		L	0	P	A	T				
BD 0775	+	+	-	-	-	+	la	+	-	+
BD 0778	+	+	-	-	-	+	la	+	+	+
BD 0779	+	+	-	-	-	+	la	+	-	+
BD 0780	+	+	-	-	-	+	la	+	+	+
BD 1354	+	+	-	-	-	+	la	+	-	+
BD 1355	+	+	-	-	-	+	la	+	-	+
BD 1357	+	+	-	-	-	+	la	+	+	+
BD 1358	+	+	-	-	-	+	la	+	+	+
BD 1359	+	+	-	-	-	+	la	+	+	+
BD 1361	+	+	-	-	-	+	la	+	+	+
BD 1366	+	+	-	-	-	+	la	+	+	+
BD 1367	+	+	-	-	-	+	la	+	+	+
BD 1368	+	+	-	-	-	+	la	+	+	+
BD 0770	+	+	-	-	-	+	la	+	+	+
CFBP 1392	+	+	-	-	-	+	la	+	+	+
BD 0002	+	+	-	-	-	+	la	-	-	+
BD 0022	+	+	-	-	-	+	la	+	+	+
BD 0771	+	+	-	-	-	+	la	+	+	+

Strain ID	KOH solubility	LOPAT tests					LOPAT group	Pathogenicity test	Fluorescence on KB	PCR
		L	0	P	A	T				
BD 0774	+	+	-	-	-	+	Ia	+	-	+
BD 0278	+	+	-	-	-	+	Ia	+	-	+
BD 0279	+	+	-	-	-	+	Ia	+	+	+
BD 0280	+	-	-	-	-	+	Ia	+	+	+
CFBP 2107	+	-	-	+	-	+	II	+	+	+
BD 0146	+	-	-	+	-	+	II	-	+	+
BD 0156	+	-	-	+	-	+	II	-	+	+
BD 0223	+	-	-	+	-	+	II	+	+	+
BD 0224	+	-	-	+	-	+	II	+	+	+
BD 0231	+	-	-	+	-	+	II	+	+	+
CFBP 2101	+	-	+	-	-	+	III	-	+	+
BD 0229	+	-	+	-	-	+	III	-	+	+
BD 0245	+	-	+	-	-	+	III	-	+	+

(+) = positive; (-) = negative. LOPAT tests included Levan production (L), 0xidase 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* strain*s* 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, erythitol, D (-) tartrate and DL-Lactate.

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

Strain	Glucose	Sucrose	Sorbitol	Mannitol	Erythrotol	Lactose	Inositol	Fructose	D (-) tartrate	L (+) tartrate	DL-lactate
CFBP 2212	+	+	+	+	-	+	+	+	+	-	-
BD 0001	+	+	+	+	-	+	+	+	+	-	-
BD 0028	+	+	+	+	-	+	+	+	+	-	-
BD 0034	+	+	+	+	-	+	+	+	+	-	-
BD 0035	+	+	+	+	-	+	+	+	+	-	-
BD 0070	+	+	+	+	-	+	+	+	+	-	-
BD 0071	+	+	+	+	-	+	+	+	+	-	-

Strain	Glucose	Sucrose	Sorbitol	Mannitol	Erythrotol	Lactose	Inositol	Fructose	D (-) tartrate	L (+) tartrate	DL-lactate
BD 0085	+	+	+	+	-	+	+	+	+	-	-
BD 0091	+	+	+	+	-	+	+	+	+	-	-
BD 0151	+	+	+	+	-	+	+	+	+	-	-
BD 0159	+	+	+	+	-	+	+	+	+	-	-
BD 0164	+	+	+	+	-	+	+	+	+	-	-
BD 0165	+	+	+	+	-	+	+	+	+	-	-
BD 0269	+	+	+	+	-	+	+	+	+	-	-
BD 0283	+	+	+	+	-	+	+	+	+	-	-
BD 0284	+	+	+	+	-	+	+	+	+	-	-
BD 0285	+	+	+	+	-	+	+	+	+	-	-
BD 0770	+	+	+	+	-	+	+	+	+	-	-
BD 0771	+	+	+	+	-	+	+	+	+	-	-
BD 0775	+	+	+	+	-	+	+	+	+	-	-
BD 0778	+	+	+	+	-	+	+	+	+	-	-
BD 0779	+	+	+	+	-	+	+	+	+	-	-

Strain	Glucose	Sucrose	Sorbitol	Mannitol	Erythrotol	Lactose	Inositol	Fructose	D (-) tartrate	L (+) tartrate	DL-lactate
BD 0780	+	+	+	+	-	+	+	+	+	-	-
BD 1354	+	+	+	+	-	+	+	+	+	-	-
BD 1355	+	+	+	+	-	+	+	+	+	-	-
BD 1357	+	+	+	+	-	+	+	+	+	-	-
BD 1358	+	+	+	+	-	+	+	+	+	-	-
BD 1359	+	+	+	+	-	+	+	+	+	-	-
BD 1361	+	+	+	+	-	+	+	+	+	-	-
BD 1366	+	+	+	+	-	+	+	+	+	-	-
BD 1367	+	+	+	+	-	+	+	+	+	-	-
BD 1368	+	+	+	+	-	+	+	+	+	-	-
CFBP 1392	+	+	+	+	+	+	+	+	-	-	+
BD 0002	+	+	+	+	+	+	+	+	-	-	+
BD 0022	+	+	+	+	+	+	+	+	-	-	+
BD 0278	+	+	+	+	+	+	+	+	-	-	+
BD 0279	+	+	+	+	+	+	+	+	-	-	+
BD 0280	+	+	+	+	+	+	+	+	-	-	+
BD 0771	+	+	+	+	+	+	+	+	-	-	+

Strain	Glucose	Sucrose	Sorbitol	Mannitol	Erythrotol	Lactose	Inositol	Fructose	D (-) tartrate	L (+) tartrate	DL-lactate
BD 0774	+	+	+	+	+	+	+	+	-	-	+
CFBP 2107	+	-	+	+	+	-	-	+	+	-	+
BD 0146	+	-	+	+	+	-	-	+	+	-	+
BD 0156	+	-	+	+	+	-	-	+	+	-	+
BD 0223	+	-	+	+	+	-	-	+	+	-	+
BD 0224	+	-	+	+	+	-	-	+	+	-	+
BD 0231	+	-	+	+	+	-	-	+	+	-	+
CFBP 2101	+	-	-	+	-	+	-	+	-	+	-
BD 0229	+	-	-	+	-	+	-	+	-	+	-
BD 0245	+	-	-	+	-	+	-	+	-	+	-

(+) = 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).
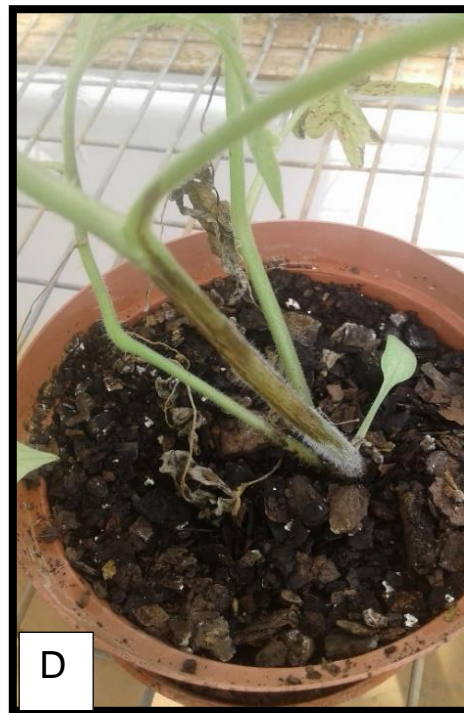
Figure 2.5: Symptoms exhibited by tomato plants sprayed with: (A) sterile distilled water-no symptoms, (B) *P. syringae* pv. *tomato* strain BD 1357- necrotic spots surrounded by wide yellow halos(C) *P. syringae* pv. *syringae* strain BD 0280- necrotic spots surrounded by narrow yellow halos and (D) *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 *P. viridiflava* (CFBP 2107 and BD 0231) and *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).
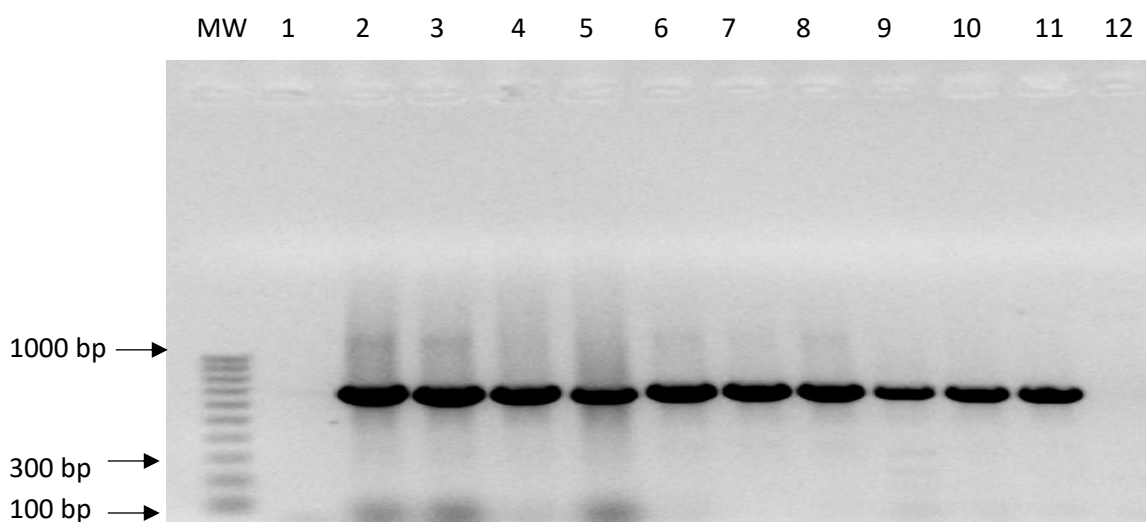
Figure 2.6: PCR products amplified from various *Pseudomonas syringae* isolates using primers COR1, 5′ GGA CTC AGC AGT ATC ATC TCG GGA CG 3′; COR2, 5′ TGC AGG GTC TTG GGG AGC ACG 3′ (Cuppels *et al.*, 2006). Lane 1 is a negative control. Lane 2 is the *P. syringae* pv. *tomato* type strain (CFBP 2212), lane 3 and 4 are *P. syringae* pv. *tomato* strains (BD 0151 and BD 0165), lane 5 is a *P. syringae* pv. *syringae* type strain (CFBP 1392), Lane 6 and 7 are *P. syringae* pv. *syringae* strains (BD 0279 and BD 0280), lane 8 is a *P. viridiflava* type strain (CFBP 2107), lane 9 is a *P. viridiflava* strain (BD 0231), lane 10 is a *P. cichorii* type strain (CFBP 2101) and lane 12 is a *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. cichorii*. 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. cichorii* 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 erythitol, L-tartrate and DL-lactate. The inability of *P. syringae* pv. *tomato* to utilise erythitol 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

Agrios, G.N. (**2005).** Plant Pathology. 5th edition. Academic Press, USA. Pp. 37-41.

Alfano, J.R and Collmer, A. (1996). Bacterial pathogens in plants: life up against the wall. *Plant Cell* **8**: 1683-1698.

Bashan, Y., Sharon, E., Okon, Y., Henis, Y. (1981). Scanning electron and light microscopy of infection and symptom development in tomato leaves infected with *Pseudomonas tomato. Physiological Plant Pathology* **19**:139-144.

Bereswill, S., Bugert, P., Voelksch, B., Ullrich, M., Bender, C. L., Geider, K. (1994). Identification and relatedness of coronatine producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. *Applied Environmental Microbiology* **60**: 2924-2930.

Bull, C.T., and Koike, S.T. (2015). Practical benefits of knowing the enemy: modern molecular tools for diagnosing the etiology of bacterial diseases and understanding the taxonomy and diversity of plant-pathogenic bacteria. *Annual Reviews of Phytopathology* **53**: 157-180

Blancard, D. (1997). A colour atlas of tomato diseases: observations, identification and control. John Wiley & Sons, New York. pp. 212.

Cuppels, D.A., Louws, F.J., Ainsworth, T. (2006). Development and evaluation of PCR based diagnostic assays for the bacterial speck and bacterial spot pathogens of tomato. *Plant Disease* **90**: 451-458.

Department of Agriculture, Forestry and Fisheries. (2017). A profile of the South African tomato market value chain. Pretoria.

Devash Y., Okon, Y., Henis, Y. (1980). Survival of *Pseudomonas tomato* in soil and seeds. *Phytopathology* **99**:175-185.

Dulla, G.F.J., Kraslleva, K.V., Lindow, S.E. (2010). Interference of quorum sensing in *Pseudomonas syringae* by bacterial epiphytes that limit iron availability. *Environmental Microbiology* **12**: 1762–74.

Gardan, L., Shafik, H., Belouin, S., Brosch, R., Grimont, F., Grimont, P.A.D. (1999). DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *International Journal Systematic Bacteriology* **49**: 469-478.

Goszczynska, T., Serfontein, J.J. and Serfontein, S. (2000). Introduction to practical phytobacteriology. SAFRINET, South Africa. pp.1-83.

Gram, H. C. **(1884).** Über die isolierte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. Abstr. *Fortschritte der Medizin 2*: 185-189.

Hirano, S.S., and Upper, C.D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*-a pathogen, ice nucleus and epiphyte. *Microbiology and Molecular Biology Reviews* **64**: 624-53.

Jones, J.B. (1991). Bacterial Speck. In: Compendium of tomato diseases. Paul, M.N. J.B. Jones, J.B., Jones, J.P., Stall, R.E., Zitter, T.A (eds). APS Press, Saint Paul. pp. 26- 27.

Jones, J.B., Gitaitis, R.D., McCarter, S.M. (1986). Fluorescence on single-carbon sources for separation of *Pseudomonas syringae* pv. *syringae, P. syringae* pv. *tomato,* and *P. viridiflava* on tomato transplants. *Plant Disease* **70**: 151-153.

Jones, J.B., Jones, J.P., McCarter, S.M., and Stall, R.E. 1984. *Pseudomonas viridiflava:* causal agent of bacterial leaf blight of tomato. *Plant disease* **68**: 341-342.

Jones, J.B., Raju, B.C., and Engelhard, A.W. (1984). Effects of temperature and leaf wetness on development of bacterial spot of geraniums and chrysanthemums incited by *Pseudomonas cichorii. Plant Disease* **68**: 248-251

Jones, J., Zitter, T., Momol, T., Miller, S. (2014). Compendium of tomato diseases and pests. 2nd edition. APS Press, Saint. Paul, MN. pp. 168.

King, E.O., Ward, M.K., Raney, D.E. (1954). Two simple media for the identification of pyocyanin and fluorescein. *Journal of Laboratory Clinical Medicine* **44**: 301-307.

Klement, Z. (1963). Rapid detection of pathogenicity of phytopathogenic *Pseudomonads. Nature* **199**: 299-300.

Klement, Z., and Goodman, R.N. (1967). The hypersensitive reaction to infection by bacterial plant pathogens. *Phytopathology* **5**: 17-44.

Kovacs, N. (1956). Identification of *Pseudomonas pyocyana* by the oxidase reaction. *Nature* **178**: 703.

Kritzman, G. (1991). A method for detection of seed-borne bacterial diseases in tomato seeds. *Phytoparasitica* **19**: 133-141.

Lelliott, R.A., Billing, E., Hayward, A.C. (1966). A determinative scheme for the fluorescent plant pathogenic *pseudomonads. Applied Bacteriology* **29**: 470-489.

Lelliott, R. A., and Stead, D.E. (1987). Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell Scientific Publications, United Kingdom. pp. 169-199.

Little E.L., Bostock R.M., Kirkpatrick B.C. (1998). Genetic characterisation of *Pseudomonas syringae* pv. *syringae* strains from stone fruits in California. *Applied and Environmental Microbiology* **64**: 3818-3823.

Lozano, J.C., and Sequeira, L. (1970). Differentiation of races of *Pseudomonas solanacearum* by a leaf infiltration technique. *Phytopathology* **60**: 833-838

Louws, F.J., Wilson, M., Campbell, H.L., Cuppels, D.A., Jones, J.B., Shoemaker, P.B., Sahin, F., and S.A. Miller. (2001). Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Disease* **85**: 481-488.

Milijasevic, S., Todorovic, B., Rekanovic, E., Potocnik, I., Gavrilovic, V. (2009). Races and hosts of *Pseudomonas syringae* pv. *tomato* in Serbia. *Biological Science* **61**: 93-103.

Misaghi, I., and Grogan, R.G. (1969). Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent *Pseudomonads. Phytopathology* **59**: 1436-1450.

Palleroni, N.J. (1984). Genus *Pseudomonas.* In Bergey's Manual of Systematic Bacteriology. Krieg, N.R. and Holt, J.G. (eds). Williams & Wilkins, Baltimore, MD, USA. (vol. **1**, pp. 141-199).

Peix, A., Ramirez-Bahena, M. H., Velazquez, E. (2009). Historical evolution and status of the taxonomy of genus *Pseudomonas. Infection, Genetics and Evolution* **9**: 1132-1147.

Preston, G.M. (2000). *Psedomonas syringae* pv. *tomato*: The right pathogen, of the right plant, at the right time. *Molecular Plant Pathology* **1**: 263-275.

Ramkumar, G., Lee, S.W., Weon, H.Y., Kim, B.Y., Lee, Y.H. (2015). First report on the whole genome sequence of *Pseudomonas cichorii* strain JBC1 and comparison with other *Pseudomonas species. Plant Pathology* **64**: 63-70.

Reyes, E.A., Bale, M.J., Cannon, W.H., Masten, J.M. (1981). Identification of Pseudomonas aeruginosa by pyocyanin production on tech agar. *Journal of Clinical Microbiology* **13**: 456–8

Sands, D.C., Schroth, M.N., Hilderbrand, D.C. (1970). Taxonomy of phytopathogenic *Pseudomonads. Bacteriology* **101**: 9-23.

Schaad, N. W., Jones, JB., Chun, W. (2001). Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd edition. APS Press, St. Paul, Minnesota. pp. 398.

Serfontein, J.J. and Hattingh, M.J. (1990). Characterization of *Pseudomonas syringae* pv. *tomato* by numerical analysis of phenotypic features and total soluble proteins. *Phytopathology* **128**: 220-234.

Shenge, K.C., Mabagala, R.B., Mortensen, C.N., Stephan, D., Wydra, K. (2007). First report of bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* in Tanzania. *Plant Disease* **91**: 462.

Shim, H.H., Koh, Y.J., Hur, J.S., Jung, J.S. (2003). Identification and characterization of coronatine-producing *Pseudomonas syringae* pv. *actinidiae. Biotechnology* **13**: 110-118.

Thakur, S., Weir, B.S., Guttman, D.S. (2016). Phytopathogen genome announcement: draft genome sequences of 62 *Pseudomonas syringae* type and pathotype strains. *Molecular plant microbe interaction.* **29**:243-246.

Wilson, K. (1989). Preparation of genomic DNA from bacteria. In *Current protocols in* molecular biology. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K (eds). John Wiley & Sons, New York. pp. 241-245.

Wilson, M., Campbell, P., Jones, J., Cuppels, D. (2002). Biological control of bacterial speck of tomato under field conditions at several locations in North America. *Phytopathology* **92**: 1284-1292.

Young, J.M. (2010). Taxonomy of *Pseudomonas syringae. Plant Pathology* **92**: 1

Young J.M., Saddler G.S., Takikawa Y., DeBoer S.H., Vauterin L., Gardan, L., Gvozdyak R.I., Stead, D.E. (1996). Names of plant pathogenic bacteria 1864-1995. *Reviews of Plant Pathology* **75**: 721-763.

Yu, S.M., and Lee, Y.H. (2012). First report of *Pseudomonas cichorii* associated with leaf spot on soybean in South Korea. *Plant Disease* **96**: 142.

Yu, Z.H., Wang, J.F., Stall, R.E., Vallejos, C.C. (1995). Genomic localization of tomato genes that control a hypersensitive reaction to *Xanthomonas campestris pv. vesicatoria* (Doidge) Dye. *Genetics* **141**: 675-682.

Zheng, X.Y., Spivey, N.W., Zeng, W. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signalling cascade that inhibits salicylic acid accumulation. *Cell Host and Microbe* **11**: 587-96.

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 Arica 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

(Bradbury, 1986; Gardan *et al.,* 1991; Young and Triggs, 1994). 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 2212^{PT}), BD 1130 (CFBP 1392^{PT}), BD 1149 (CFBP 2107^{T}) and BD 1152 (CFBP 2101^{T}) 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

Strain number	Geographical origin -Province	Gene		rep-PCR primer		Identity
		gyrB[a]	*Cts*[b]	BOX[c]	ERIC[d]	
BD 0002	Gauteng	+	+	-	-	*P. syringae* pv. *papulans*
BD 0022	Gauteng	MK737966	+	-	-	*P. syringae* pv. *papulans*
BD 0034	North-West	MK737967	+	+	+	*P. syringae* pv. *tomato*
BD 0035	North-West	MK770404	+	+	+	*P. syringae* pv. *tomato*
BD 0071	Limpopo	MK770405	+	-	-	*P. syringae* pv. *tomato*
BD 0091	Limpopo	MK770407	+	+	+	*P. syringae* pv. *tomato*
BD 0146	Gauteng	-	-	+	+	*P. viridiflava*
BD 0151	Gauteng	MK770408	+	+	+	*P. syringae* pv. *tomato*
BD 0156	Gauteng	-	-	+	+	*P. viridiflava*
BD 0159	Gauteng	+	+	+	+	*P. syringae* pv. *tomato*
BD 0164	Gauteng	MK614767	MK614768	+	+	*P. syringae* pv. *tomato*
BD 0165	Gauteng	MK862143	+	+	+	*P. syringae* pv. *tomato*
BD 0193	Gauteng	MK862144	+	+	+	*P. syringae* pv. *tomato*
BD 0195	Gauteng	MK862145	+	+	+	*P. syringae* pv. *tomato*
BD 0223	Gauteng	+	+	+	+	*P. viridiflava*
BD 0224	Gauteng	+	+	+	+	*P. viridiflava*
BD 0229	Eastern cape	-	-	+	+	*P. cichorii*

Strain number	Geographical origin -Province	Gene		rep-PCR primer		Identity
		gyrB[a]	*Cts*[b]	BOX[c]	ERIC[d]	
BD 0231	Limpopo	+	+	+	+	*P. viridiflava*
BD 0245	Gauteng	-	-	+	+	*P. cichorii*
BD 0269	Mpumalanga	MK862146	+	+	+	*P. syringae* pv. *tomato*
BD 0278	Mpumalanga	MK862147	+	+	+	*P. syringae* pv. *syringae*
BD 0279	Mpumalanga	MK862148	+	+	+	*P. syringae* pv. *syringae*
BD 0280	Mpumalanga	+	+	+	+	*P. syringae* pv. *syringae*
BD 0282	Gauteng	MK862149	+	+	+	*P. syringae* pv. *tomato*
BD 0283	Gauteng	MK862150	+	+	+	*P. syringae* pv. *tomato*
BD 0284	Gauteng	-	-	+	+	*P. syringae* pv. *tomato*
BD 0285	Gauteng	MK862151	+	+	+	*P. syringae* pv. *tomato*
BD 0286	Gauteng	MK862152	+	+	+	*P. syringae* pv. *tomato*
BD 0770	Gauteng	+	+	+	+	*P. syringae* pv. *tomato*
BD 0771	Gauteng	+	+	-	-	*P. syringae* pv. *papulans*
BD 0774	Gauteng	+	+	-	-	*P. syringae* pv. *papulans*
BD 0775	Gauteng	MK862153	+	+	+	*P. syringae* pv. *tomato*
BD 0778	Gauteng	MK862154	+	+	+	*P. syringae* pv. *tomato*
BD 1355	Gauteng	MK862155	+	+	+	*P. syringae* pv. *tomato*
BD 1357	Gauteng	+	+	+	+	*P. syringae* pv. *tomato*

Strain number	Geographical origin -Province	Gene		rep-PCR primer		Identity
		gyrB[a]	*Cts*[b]	BOX[c]	ERIC[d]	
BD 1358	Gauteng	+	+	+	+	*P. syringae* pv. *tomato*
BD 1359	Gauteng	+	+	+	+	*P. syringae* pv. *tomato*
BD 1361	Gauteng	+	+	+	+	*P. syringae* pv. *tomato*
BD 1360	Gauteng	-	-	+	+	*P. syringae* pv. *tomato*
BD 1368	Gauteng	+	+	+	+	*P. syringae* pv. *tomato*
BD 1130 **CFBP 1392**	United Kingdom	+	+	+	+	*P. syringae pv. syringae*
BD 1146 **CFBP 2212**	United Kingdom	+	+	+	+	*P. syringae pv. tomato*
BD 1149 **CFBP 2107**	Switzerland	+	+	+	+	*P. viridiflava*
BD 1152 **CFBP 2101**	United Kingdom	+	+	+	+	*P. cichorii*

[a] (+) *gyrB* sequences obtained, no accession numbers from Genbank yet; (-) *gyrB* not sequenced

[b] (+) *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 $MgCl_2$, 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 de-stained 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 AGTTGATCATCGAGGGCGCWGCC, *cts*-Rp TGATCGGTTTGATCTCGCACGG, *gyrB* M13-R CAGGAAACAGCTATGACC and M13(-21) TGTAAAACGACGGCCAGT. 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 MgCl$_2$; 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 AB 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.
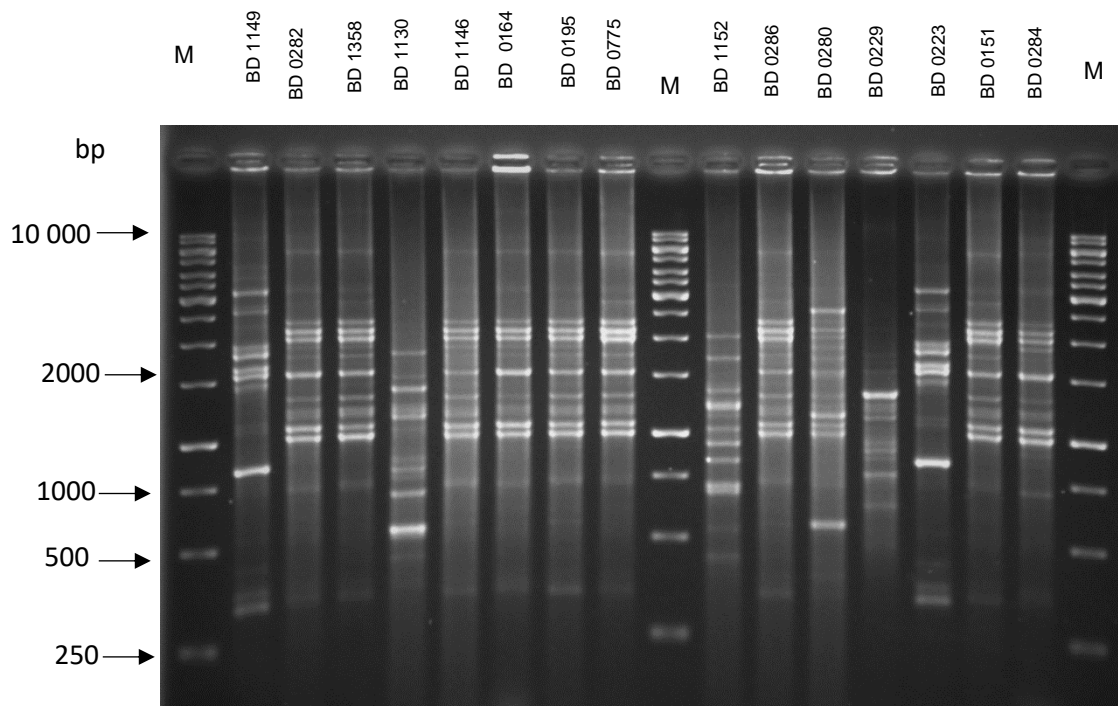
Figure 3.1b: Agarose gel electrophoresis of ERIC 2 PCR fingerprinting patterns of *Pseudomonas* isolates. The sizes of GeneRuler 1 kb DNA ladder (Thermoscientific) are indicated in base pairs. Fingerprinting patterns of P. *viridiflava* isolates (BD 1149, BD 0223), *P. syringae* pv. *tomato* isolates (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.
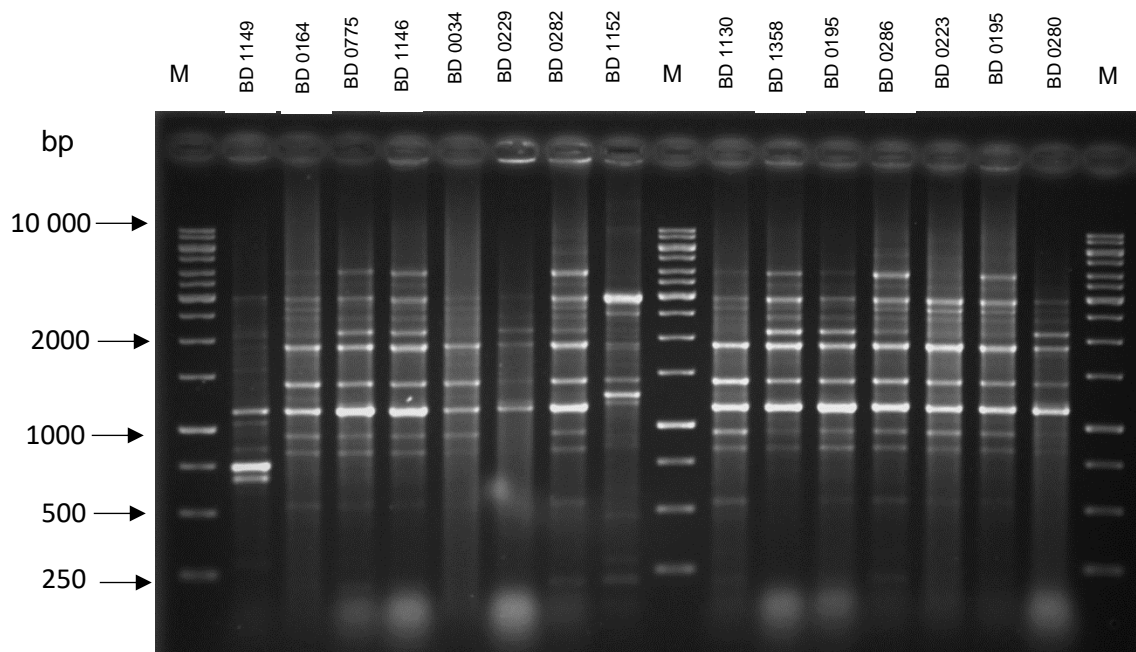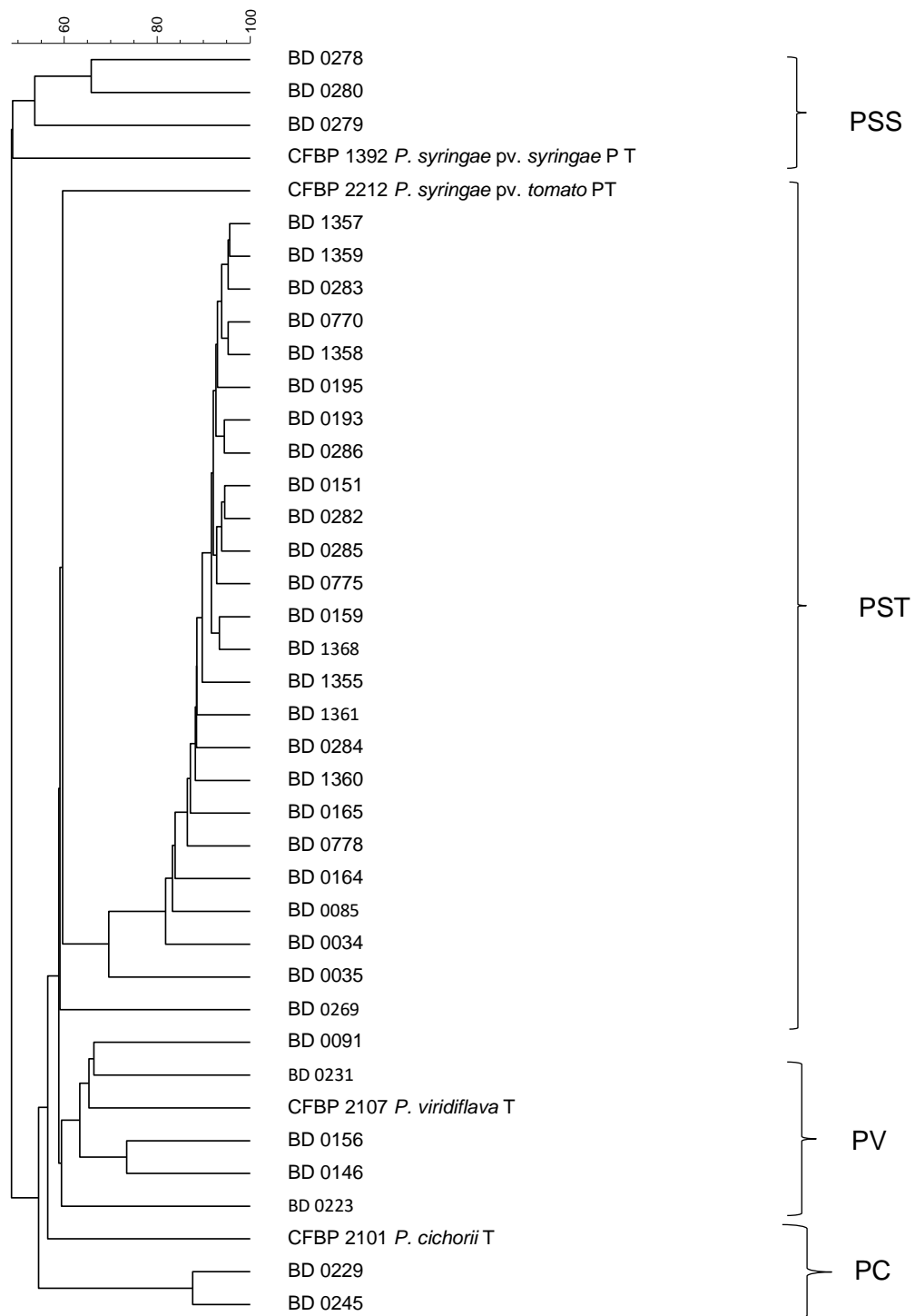
Figure 3.2: Dendrogram based on BOX A1R and ERIC 2 fingerprints (rep-PCR) of fluorescent *Pseudomonads* isolated from diseases tomato. Fingerprints similarity was calculated using Pearson's curve-based correlation coefficient using UPGMA clustering method. PSS- *P. syringae* pv. *syringae*, PST- *P. 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 $\geq$ 98% sequence similarity to *P. syringae* pv. *tomato*, three strains (BD 0278, BD 0279 and BD 0280) showed $\geq$ 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[T].

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 [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]. 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.

PST- *P. syringae* pv. *tomato*, PSS- *P. syringae* pv. *syringae*, PSP- *P. syringae* pv. *papulans,* PSD- *P. syringae* pv. *dysoxyli*, PV- *P. viridiflava* and PC- *P. cichorii.*

PG- Phylogroup
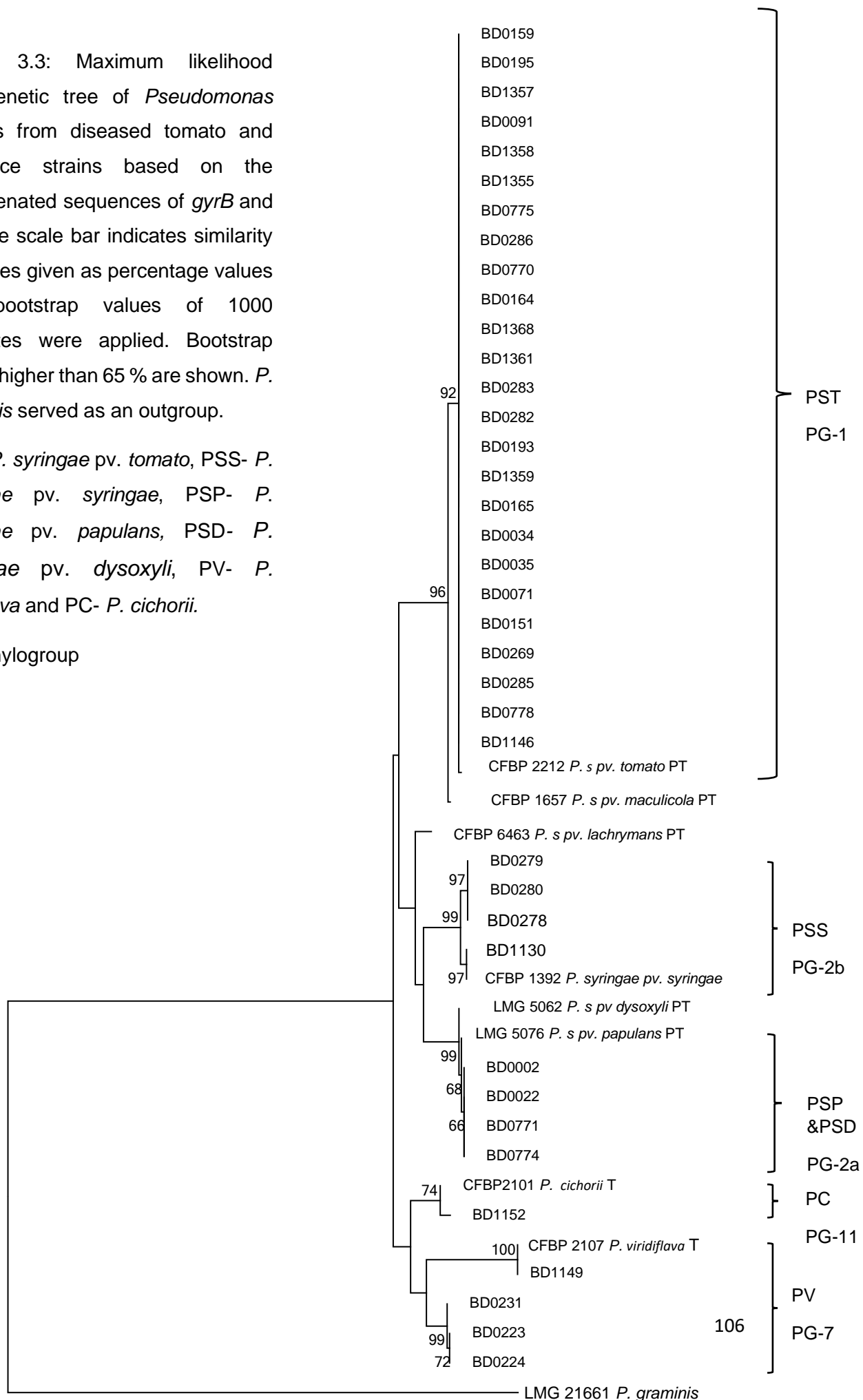
3.4 Discussion

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 Hattings (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 syringe* 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 1392[PT]. 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

Alivizatos, A.S. (1986). Tomato pith necrosis caused by *Pseudomonas viridiflava. Phytopathology* **15**: 41-47.

Berge, O., Monteil, C.L., Bartoli, C., Chandeysson, C., Guilbaud, C., Sands, D.C., Morris, C.E. (2014). User's Guide to a data base of the diversity of *Pseudomonas syringae* and its application to classifying strains in this phylogenetic complex. *PLoS ONE* **9**: 9.

Borges, L.G., Vechia, V.G., Corcao, G. (2003). Characterisation and genetic diversity via rep-PCR of *Escherichia coli* isolates from polluted waters in southern Brazil. *FEMS microbiology ecology.* **45**: 173.180.

Bull, C. T., Clarke, C.R., Cai, R., Vinatzer, B.A., Jardini, T.M., Koike, S.T. (2011). Multilocus sequence typing of *Pseudomonas syringae sensu lato* confirms previously described genomospecies and permits rapid identification of *P. syringae* pv. *coriandricola* and *P. syringae* pv. *apii* causing bacterial leaf spot on parsley. *Phytopathology* **101**: 847-858.

Bradbury, J.F. (1986). Guide to plant pathogenic bacteria. CAB International Mycological Institute. pp.154-177.

Cepni, E. and Gurel, F. (2012). Variation in extragenic repetitive DNA sequences in *Pseudomonas syringae* and potential use of modified REP primers in the identification of closely related isolates. *Genetics and Molecular Biology* **35**: 650-656.

Chambers, S.C. and Merriman, P.R. (1975). Perennation and control of *Pseudomonas syringae* pv. *tomato* in Victoria. *Agricultural Research* **26**: 657-663.

Cho, MS., Park, D.S., Yun, Y.H., Kim, S.H., Shim, M.Y., Choi, C.W., Kim, Y.S. (2012). Genetic Differentiation of *Pseudomonas syringae* Pathovar *tomato* from

Other *P. syringae* Pathovars using REP-PCR and URP-PCR. *Plant Pathology* **28**: 60-67.

Cho, J.C. and Tiedje, J.M. (2001). Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. *Applied and environmental microbiology* **67**: 3677-3682.

Cuppels, D.A., Louws, F.J., Ainsworth, T. (2006). Development and evaluation of PCR based diagnostic assays for the bacterial speck and bacterial spot pathogens of tomato. *Plant Disease* **90**: 451-458.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**: 368-76.

Felsenstein, J. (1985). Phylogenies and the comparative method. *The American Neutralist* **25**: 1-15.

Gardan, L., Cottin, S., Bollet, C., Hunault, G. (1991). Phenotypic heterogeneity of *Pseudomonas syringae*. *Research in Microbiology* **142**: 995-1003.

Gardan, L., Shafik, H., Bedouin, S., Broch, R., Grimont, F., Grimon, P.A.D. (1999). DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. Nov. and *Pseudomonas cannabina* sp. Nov. (ex Sutic and Dowson 1959). *Systematic Bacteriology* **49**: 469-478.

Gitaitis, R.D., Jones, J.B, Jaworski, C.A. Phatak, S.C. (1985). Incidence and development of *Pseudomonas syringae* on tomato transplants in Georgia. *Plant disease* **69**: 32-35.

Gomila, M., Busquets, A., Mullet, M., García-Valdes, E., Lalucat, J. (2017). Clarification of taxonomic status within the *Pseudomonas syringae* species group based on a phylogenomic analysis. *Frontiers in Microbiology* **8**: 2422.

Goumas, D.E., Malathrakis, N.E., Chatzaki, A.K. (1999). Characterization of *Pseudomonas viridiflava* associated with a new symptom on tomato fruit. *European Journal of Plant Pathology* **105**: 927-932.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for window 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Humm, H.J. (1946). Marine agar-digesting bacteria of the South Atlantic coast. *Duke university marine station bulletin.* **3**: 45-75.

Hwang, M.S.H., Morgan, R.L., Sarkar, S.F., Wang, P.W., Guttman, D.S. (2005). Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae. Applied and environmental Microbiology* **71**: 5182-5191.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111-120.

King, E.O., Ward, M.K., Raney, D.E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *Laboratory and Clinical Medicine* **44**: 301-307.

Kokoskova, B., Pouvoca, D., Pavela, R. (2011). Effectiveness of plant essential oils against *Erwinia amylovora, Pseudomonas syringae* pv. *syringae* and associated saprophytic bacteria on/in host plants. *Plant Pathology* **93**: 133-139.

Lelliott, R.A., Billing, E., Hayward, A.C. (1966). A determinative scheme for the fluorescent plant pathogenic *Pseudomonads. Applied Bacteriology* **29**: 470-489.

Louws, F.J., Fulbright, D.W., Stephens, C.T., de Bruijn, F.J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied Environmental Microbiology* **60**: 2286-2295.

Katoh, K., Rozewicki, J., Yamada K.D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics* **20**: 1160-1166.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., and Feavers, I.M. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 3140-3145.

Malathrakis, N.E., and Goumas, D.E. (1987). Bacterial soft rot of tomato in plastic greenhouse in Crete. *Annals of applied Biology* **111**: 115-123.

Mansvelt, E.L. and Hattingh, M.J. (1986). Bacterial blister bark and blight of fruit spurs of apple in South Africa caused by *Pseudomonas syringae* pv. *syringae*. *Plant Disease* **70**: 403-405.

Marcelletti, S. and Scortichini, M. (2014). Definition of plant pathogenic genomospecies of the *Pseudomonas syringae* species complex through multiple comparative approaches. *Phytopathology* **104**: 1274-1282.

Marques, A.B.S., Marchaison, A., Gardan, L., Samson, R. (2008). BOX-PCR based identification of bacterial species belonging to *Pseudomonas syringae- P. viridiflava* group. *Genetics and Molecular Biology* **31**: 106-115,

Mirik, M., Aysan, Y., Sahin, F. (2011). Characterization of *Pseudomonas cichorii* from different hosts in Turkey. *International Journal of Agriculture and Biology* **13**: 203-209.

Moloto, V.M., Goszczynska, T., du Toit, L.J., Coutinho, T.A. (2016). A new pathovar of *Pseudomonas syringae*, pathovar *allii*, isolated from onion plants exhibiting symptoms of blight. *European Journal of Plant Pathology* **147**: 591-603.

Morris, C.E., Sands, D.C., Vinatzer, B.A., Glaux, C., Guilbaud, C., Buffiere, A., Yan, S., Domiguez, H., Thompson, M.B. (2008). The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *International Society for Microbial Ecology* **2**: 321-334.

Mulet, M., Lalucat, J., Garcıa-Valdes, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environmental Microbiology* **12**: 1513-1530.

Murillo, J. and Sesma, A. (2001). The biochemistry and molecular genetics of host rage definition: *Pseudomonas syringae*. *Phytopathology Mediterranea* **40**: 3-26.

Pour, N.G. and Taghavhi, S.M. (2011). Comparison of *P. syringae* pv. *syringae* from different hosts based on pathogenicity and BOX-PCR in Iran. *Journal of Agriculture, Science and Technology* **13**: 431-442.

Rombouts, S., Vaerenbergh, J.V., Volckaert, A., Baeyen, S., De Langhe, T., Declerq, B., Lavigne, R., Maes, M. (2015). Isolation and characterization of

Pseudomonas syringae pv. *porri* from leek in Flanders. *European Journal of Plant Pathology* **144**: 185-198.

Rose, D.J. (1916). Blister spot of apples. *Phytopathology* **6**: 110.

Rozas, J., Feerrer-Mata, A., Sanchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsis, S.E., Sanchez-Gracia, A. 2017. DnaSP v6: DNA Sequence Polymorphism Analysis of Lage Datasets. *Molecular Biology and Evolution* **34**: 3299-3302.

Sarkar, S. and Guttman, D. S. (2004). Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied Environmental Microbiology* **70**: 1999-2012.

Schneider, R.W. and Grogan, R.G. (1977). Bacterial speck of tomato: sources of inoculum and establishment of a resident population. *Phytopathology* **67**: 388-394.

Scortichini M., Marchesi U., Dettori M.T., Rossi M.P. (2003). Genetic diversity, presence of the *syrB* gene, host preference and virulence of *Pseudomonas syringae* pv. *syringae* strains from woody and herbaceous host plants. *Plant Pathology* **52**: 277–286.

Stead, D.E. (1992). Techniques for detecting and identifying plant pathogenic bacteria. In: techniques for the rapid detection of plant pathogens. Duncan, J.M. and Torrance, L (eds). Blackwell Scientific Publications, Oxford, UK. pp. 235.

Tamura, K., Peterson, D., Stecher, G., Nei, M., Kumar, S. (2011). MEGA 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Thakur, S., Weir, B.S., Guttman, D.S. (2016). Phytopathogen genome announcement: draft genome sequences of 62 *Pseudomonas syringae* type and pathotype strains. *Molecular plant microbe interaction*. **29**:243-246.

Trantas, E.A., Sarris, P.F., Mpalantinaki, E.E., Pentari, M.G., Ververdis, F.N., Goumas, D.E. (2013). A new genomovar of *Pseudomonas cichorii*, a causal agent of tomato pith necrosis. *European Journal of Plant Pathology* **137**: 477-493

Varadi, L., Luo, J.L., Hibbs, D.E., Perry, J.D., Anderson, R.J., Orenga, S., Groundwater, P.W. (2017). Methods for the detection and identification of pathogenic bacteria: past, present and future. *Chemical Society Reviews* **46**: 4818-4832.

Wilkie, P. and Dye, D.W. (1974). *Pseudomonas cichorii* causing tomato and celery diseases in New Zealand. *New Zealand journal of Agricultural Research* **17**: 123-130.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A. Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyr*B and *rpo*D genes. *Microbiology* **146**: 2385-2394.

Young, J.M. (2010). Taxonomy of *Pseudomonas syringae. Plant Pathology* **92**: 5-14.

Young, J.M. and Triggs, C.M. (1994). Evaluation of determinative tests for pathovars of *Pseudomonas syringae* van Hall 1902. *Applied Bacteriology* **77**: 195-207.

Young, J.M., Takikawa, Y., Gardan, L., Stead, D.E. (1992). Changing concepts in the taxonomy of plant pathogenic bacteria. *Annual Reviews of Phytopathology* **30**: 67-105.

Zembek, P., Danilecka, A., Hoser, R., Eschen-Lippold, L., Benicka, M., Grech-Baran, M., Rymaszewski, W., Barymow-Filoniuk, I., Morgiewicz, K., Kwiatkowski, J., Piechocki, M., Poznanski, J., Lee, J., Hennig, J., Krzymowska., M. (2018). Two Strategies of *Pseudomonas syringae* to Avoid Recognition of the HopQ1 Effector in *Nicotiana* Species. *Front Plant Sciences* **9**: 978.

Chapter 4

Susceptibility of six commercially available tomato cultivars in South Africa to *Psuedomonas syringae pv. tomato* u der 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 lycopersicum* 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.*

(1995). 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:

$$PDI = \frac{\text{Sum of all ratings}}{\text{Total number of observations x Maximum rating scale}} \; x \; 100$$

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 steaked 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).
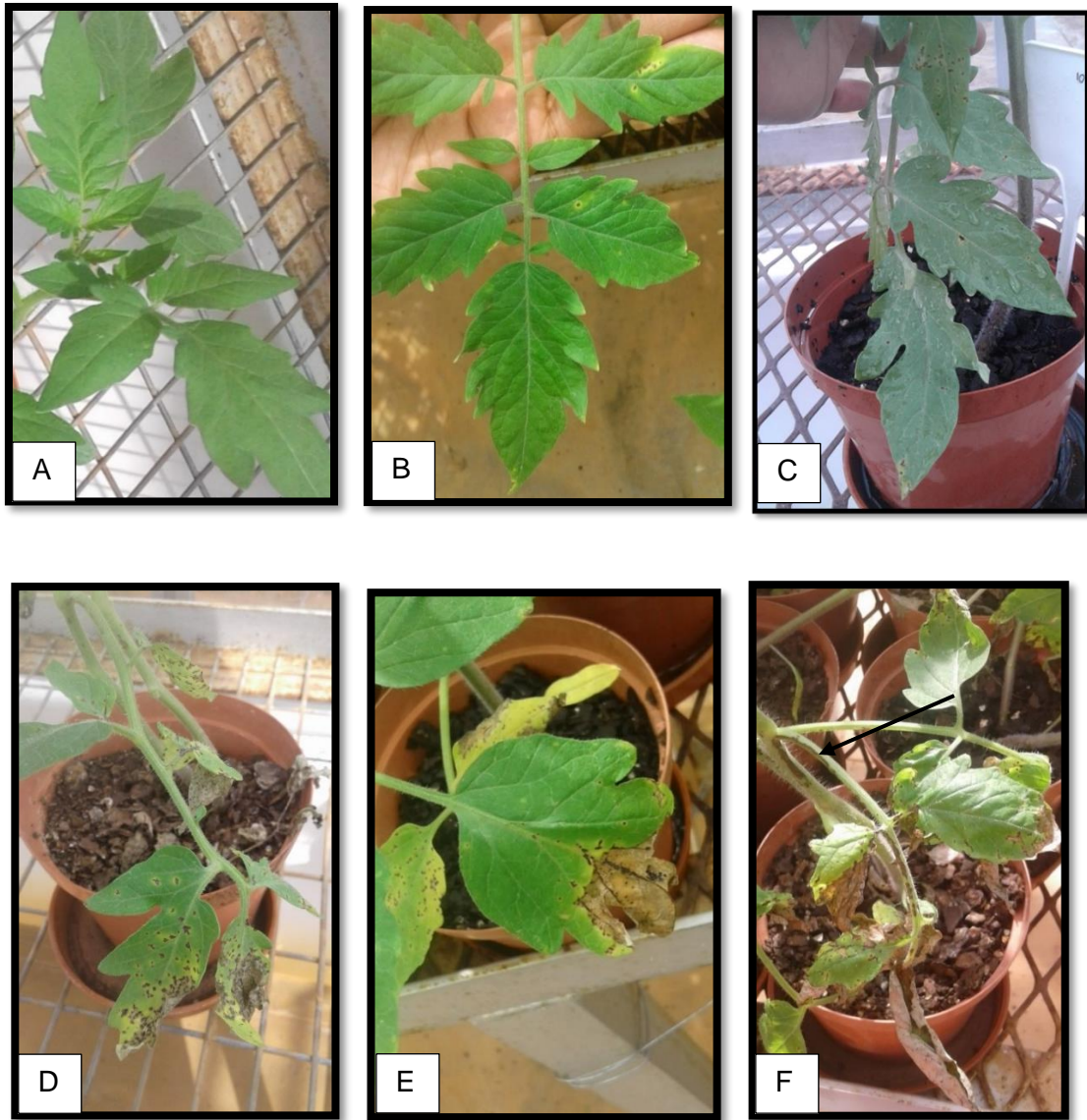
Figure 4.1: Symptoms caused by *P. syringae* pv. *tomato* isolate (BD 0165) on tomato seedlings from six cultivars grown under greenhouse conditions. A-tomato plant sprayed with sterile distilled water (control), B-symptoms observed at 7 dpi using 10^4 cfu ml^{-1}, C-specks surrounded by a chlorotic 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 *P. syringae* pv. *tomato* on six tomato cultivars under greenhouse conditions.

Cultivars	Trial 1		Trial 2		Trial 3	
	Concentrations		Concentrations		Concentrations	
	10^4 CFU ml^{-1}	10^8 CFU ml^{-1}	10^4 CFU ml^{-1}	10^8 CFU ml^{-1}	10^4 CFU ml^{-1}	10^8 CFU ml^{-1}
	No. of lesions		No. of lesions		No. of lesions	
RK	35.8a	58.0a	36.6a	59.4a	35.2a	56.8a
C9753	25.2b	44.6b	25.2b	43.0b	25.8b	41.4b
C9752	19.0c	37.0c	20.2c	37.4c	21.0c	35.0c
C9771	17.2cd	24.0de	16.0d	22.8e	14.4d	25.0de
C9751	15.8cd	26.6d	17.0cd	29.6d	16.4d	29.6cd
C8863	14.0d	20.2e	15,8d	20.8e	14.8d	22.0e
F-value	56.26	86.55	39.18	78.94	41.18	45.54
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
CV%	11.46	9.86	13.23	10.24	13.26	12.09

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.

Cultivars	Trial 1		Trial 2		Trial 3	
	Concentrations		Concentrations		Concentrations	
	10^4 CFU ml^{-1}	10^8 CFU ml^{-1}	10^4 CFU ml^{-1}	10^8 CFU ml^{-1}	10^4 CFU ml^{-1}	10^8 CFU ml^{-1}
	PDI		PDI		PDI	
RK	44.4a	68.9a	44.4a	68.9a	44.4a	68.9a
C9753	33.3b	55.6b	33.3b	53.3b	33.3b	53.3b
C9752	24.4c	44.4c	26.7c	44.4c	26.7c	42.2c
C9771	22.2c	33.3d	22.2d	31.1de	22.2d	31.1d
C9751	22.2c	33.3d	22.2d	37.8cd	22.2d	35.6cd
C8863	22.2c	28.9d	22.2d	28.9e	22.2d	33.3d
F-value	100	58.8	64.67	32.6	64.67	34.39
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
CV%	7.21	10.29	8.71	13.42	8.71	12.61

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 *P. syringae* pv. *tomato.*

4.5 References

Babelegoto, N.M., Varvaro, L., Cirulli, M. (1988). Epiphytic and endophytic multiplication of *Pseudomonas syringae* pv. *tomato* (Okabe) Young *et al.* in susceptible and resistant tomato leaves. *Phytopathologia Mediterranea* **27**: 138-144.

Bashan, Y., Okon, Y., Henis, Y. (1978). Infection studies of *Pseudomonas tomato,* causal agent of bacterial speck of tomato. *Phytoparasitica* **6**: 135-145.

Bashan, Y. (1997). Alternative strategies for controlling plant diseases caused by *Pseudomonas syringae*. In: "*Pseudomonas syringae* pathovars and related pathogens. developments in plant pathology". Rudolph, K., Burr, T.J., Mansfield, J.W., Stead, D., Vivian, A., von Kietzell, J (eds). Kluwer Academic Publishers, Dordrecht, The Netherlands. (vol. **9**, pp 575-583)*.*

Basim, H. and Turgut, A. (2013). Sensitivity of tomato (*Solanum lycopersicum*) cultivars from Turkey to bacterial speck (*Pseudomonas syringae* pv. *tomato*). *African Journal of Biotechnology* **12**: 1793-1801.

Bender, C.L., Alarcon-Chaidez, F., Gross, D.C. (1999). *Pseudomonas syringae* phytotoxins: mode of action, regulation and biosynthesis by peptide and polyketide synthetases. *Microbiology and Molecular Biology Review* **63**: 266-292.

Bogatzevska, N. (1988). *Pseudomonas syringae* pv. *tomato* causal agent of bacterial speck of tomato. *Plant science* **25**: 91-96.

Bogatzevska, N., Sotirova, V., Stamova, L. 1989. Race of *Pseudomonas syringae pv. tomato* (Okabe). *Science* **42**: 129-130.

Blancard, D. (1997). A colour atlas of tomato diseases: Observations, Identification and Control. John Wiley & Sons, New York. pp. 212.

Bryan, M.K. (1933). Bacterial speck of tomatoes. *Phytopathology* **23**:897-904.

Cai, R. M., J. Lewis, S. C. Yan, H. J. Liu, C.R. Clarke, F. Campanile. (2011). The plant pathogen *Pseudomonas syringae* pv. *tomato* is genetically monomorphic and under strong selection to evade tomato immunity. *PLoS Pathol*gy. **7**: E1002130.

Chambers, S.C. and Merriman, P.R. (1975). Perennation and control of *Pseudomonas tomato* in Victoria. *Australian journal of Agricultural research* **26**: 657-663.

Cooksey, D.A. (1988). Reduction of infection by *Pseudomonas syringae* pv. *tomato* using a non-pathogenic, copper-resistant strain combined with a copper bactericide. *Phytopathology* **78**: 601-603.

Cooksey, D.A. (1990). Genetics of bactericide resistance in plant pathogenic bacteria. *Annual   Reviews of Phytopathology* **28**: 201-219

Cooksey, D.A. and Azad, H.R. (1992). Accumulation of copper and other metals of copper-resistant plant-pathogenic and saprophytic *pseudomonads. Applied and Environmental Microbiology* **58**: 274-278.

Da Silva, V.L. and Lopes, C.A. (1995). *Pseudomonas syringae* pv. *tomato* resistant to copper-sprayed tomato fields. *Fitopatologia Brasileira* **20**: 85-89.

Devash, Y., Okon, Y., Henis, Y. (1980). Survival of *Pseudomonas tomato* in soil and seeds *Phytopathologische Zeitschrift* **99**: 175-185.

Ekici, O. and Bastas, K.K. (2014). Determination of the resistance reactions of some tomato cultivars against bacterial speck disease. *Selcuk journal of Agriculture and food sciences* **28**: 42-51.

Hulbert, S.H., Webb, C.A., Smith, S.M., Sun, Q.   (2001). Resistance gene complexes: evolution and utilization. *Annual Review of Phytopathology* **39**: 285-312.

Jardine, D. J. and Stephens, C.T. (1987). Influence of timing of application and chemical on control of bacterial speck of tomato. *Plant Disease* **71**: 405-408.

Kozik, E.U. (2002). Studies on resistance to bacterial speck (*Pseudomonas syringae* pv. *tomato*) in tomato cv. Ontario 7710. *Plant Breeding* **121**: 526-530.

Kozik, E.U. and Sobiczewski, P. (2007). Assessment of inoculation techniques suitability for determination of tomato plants resistance to bacterial speck (*Pseudomonas syringae* pv. tomato). *Phytopathology* **44**: 17-25.

Kunkeaw, S., Tan, S., Coaker, G. (2010). Molecular and evolutionary analyses of *Pseudomonas syringae* pv. *tomato* race 1. *Molecular Plant Microbe Interactions* **23**: 415-424.

Lawton, M.B. and MacNeill, B.H. (1986). Occurrence of race 1 of *Pseudomonas syringae* pv. *tomato* on field tomato in southwestern Ontario. *Canadian journal of Plant Pathology* **8**: 85-88.

Lamichhane, J.R., Balestra, G.M., Varvaro, L. (2010). Response of commonly cultivated tomato cultivars in Nepal to bacterial speck. *Phytopathology Mediterranea* **49**: 406-413.

Louws, F.J., Wilson, M., Campbell, H.L., Cuppels, D.A., Jones, J.B., Shoemaker, P.B., Sahin, F., Miller, S.A. (2001). Field control of bacterial spot and bacterial speck of tomato. *Plant Disease* **85**: 481-488.

Martin, G.B., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **262**: 1432-1436.

Okabe, N. (1933). Bacterial diseases of plants occurring in Formosa. II. Bacterial leaf spot of tomato. *Society of Agricultural Tropical Taiwan* **5**: 26-36.

Pernezny, K., Kudela, V., Kokoskova, B., Hladka, I. (1995). Bacterial diseases of tomato in the Czech and Slovak Republics and lack of streptomycin resistance among copper-tolerant bacterial strains. *Crop Protection* **14**: 267-270.

Preston, G. M. (2000). *Pseudomonas syringae* pv. *tomato*: The right pathogen, of the right plant, at the right time. *Molecular Plant Pathology* **1**: 263-275.

SAS Institute Inc. (2016). SAS® 9.4 System operations: 5th edition Cary, NC, USA.

SAS Institute Inc. (2016). System operations 5th edition, Cary, NC, USA.

Scott, J.W., Jones J.B., Somodi G.C. (1989). Genetic resistance to bacterial spot in tomato. p73–76. In: "Tomato and Pepper Production in The Tropics". Proceedings of the International Symposium on Integrated Management Practices. 21-26 March 1988. AVRDC, Taipei, Taiwan.

Shenge, K.C., Mabagala, R.B., Mortensen, C.N. (2007). Evaluation of locally available tomato varieties and introductions for resistance to bacterial speck and bacterial spot diseases in Tanzania. *Plant Protection* **47**: 103-111.

Smitley, D.R. and McCarter, S.M. (1982). Spread of *Pseudomonas syringae* pv. *tomato* and role of epiphytic populations and environmental conditions in disease development. *Plant Disease* **66**: 713-717.

Thapa, S.P., Miyao, E.M., Davis, R.M., Coaker, G. (2015). Identification of QTLs controlling resistance to *Pseudomonas syringae* pv. *tomato* race 1 strains from the wild tomato, *Solanum habrochaites* LA1777. *Theoretical Applied Genetics* **128**: 681-692.

Turgut, A. and Basim, H. (2013). Sensitivity of tomato (*Solanum lycopersicum* cultivars from Turkey to bacterial speck (*Pseudomonas syringae* pv. *tomato*). *African Journal of Biotechnology* **15**: 1793-1801.

Young, J.M., Dye, D.W., Wilkie, J.P. (1986). Bacterial Speck. In: Vegetable Diseases and their Control. Sherf, A.F. and MacNab, A.A (eds). John Wiley and Sons Inc, New York. pp. 610-614.

Young, J.M., Dye, D.W., Wilkie, J.P. (1986). Bacterial Speck. In: Vegetable Diseases and Their Control. Sherf, A.F., MacNab, A.A., (eds). John Wiley & Sons Inc, New York. pp. 610-614.

Yu, Z.H., Wang, J.F., Stall R.E., Vallejos, C.C. (1995). Genomic localization of tomato genes that control a hypersensitive reaction to *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye. *Genetics.* **141**: 675-682.

Yunis, H., Bashan, Y., Okon, Y., Henis, Y. (1980a). Two sources of resistance to bacterial speck of tomato caused by *Pseudomonas tomato. Plant Disease* **64**: 851-852.

Yunis, H., Bashan, Y., Okon, Y., Henis, Y. (1980b). Weather dependence yield losses and control of bacterial speck of tomato caused by *Pseudomonas tomato. Plant Disease* **64**: 937-939.

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[PT], and strains isolated from Gauteng were closely related to *P. syringae* pv. *papulans* type strain CFBP 5076[PT] and *P. syringae* pv. *dysoxyli* LMG 5062 [PT].

- 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[PT] and *P. syringae* pv. *dysoxyli* LMG 5062 [PT]. 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.