Development of an Integrated Management Approach to Controlling Bacterial Speck of Tomato

By

Nonduduzo Charity Ncwane

BSc (Hons), UKZN

Submitted in fulfilment of the

Requirement for the degree of

Master of Science

In the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Sciences

College of Agriculture, Engineering and Sciences

University of KwaZulu-Natal

Pietermaritzburg

Republic of South Africa

December 2015

DISSERTATION SUMMARY

Bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* (Pst) is an economically important bacterial diseases in many tomato growing regions worldwide. The development of bacterial speck epidemics is favoured by cool temperatures, high humidity and prolonged leaf wetness. As a result of infection, dark-brown to black coloured lesions surrounded by halos that eventually lead to premature defoliation are observed. Yield reduction results from the reduced photosynthetic capacity of infected leaves, resulting in flower abortion. Infected tomato fruit become unattractive and unsuitable for sale on the fresh market or for processing.

In this study 250 bacterial and 100 yeast isolates were obtained from diseased and healthy tomato leaf samples. These were screened *in vitro* for activity against *Pst*. Thirty bacterial and 20 yeast isolates demonstrated significant inhibition of *Pst*. During the secondary *in vitro* screening, 10 bacterial and 7 yeast isolates successfully inhibited the growth of Pst on tryptone soy agar (TSA) plates and were selected for further studies under greenhouse conditions. Bacterial Isolates LN17, LN24 and LN10 showed clear zones of inhibition against Pst ranging from 26–29 mm in diameter. During *in vitro* screening, seven yeast isolates were selected, based on their ability to reduce the development of bacterial speck lesions on tomato leaves over a period of 7 d using a detach-leaf technique. Yeast Isolates IB7, Y54 and Y21 moderately suppressed bacterial speck lesions and were rated Class 2 on a five class rating system. Isolate Y25 was the best isolate and was rated Class 1. Scanning electron microscopy revealed that yeast cells colonised the leaf surface.

Ten bacterial and seven yeast isolates selected from *in vitro* screening were further screened under *in vivo* under greenhouse conditions for their ability to control bacterial speck of tomato. Two of the bacterial isolates were identified as *Bacillus cereus*; one as *B. thuringiensis*; 5 as *Bacillus* spp.; and 2 as unidentified *Bacillus* spp. Two of the yeast isolates were identified as *Rhodotorula glutinis;* two as *Rhodotorula mucilaginosa*; and the remaining three isolates as *Cryptococcus magnus*, *C. diffluens* and *Rhodosporidium babjevae,* respectively. Bacterial isolate *Bacillus thuringiensis* LN1 and *Bacillus sp*. LN10 reduced AUDPC units by 25 and 52% and by 51 and 48% in Experiments 1 and 2, respectively compared to the pathogen inoculated control. Yeast isolates *Rhodotorula glutinis* Y25 and *Bacillus cereus* Y14 caused reductions in AUDPC units by 95 and 86% and 42% and 58% in

Experiments 1 and 2, respectively, compared to the pathogen inoculated control. Based on the results from the greenhouse studies, two isolates (one bacterial and one yeast) were selected for further studies under nursery conditions. These two isolates were *Bacillus* sp. Isolate LN10 and *R. glutinis* Isolate Y25.

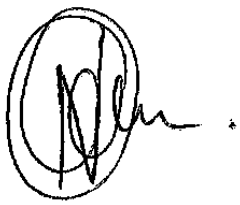
Reduced concentrations of a plant activator, acibenzolar-S-methyl (ASM) and a plant sanitiser didecyl-dimethyl-ammonium chloride (DDAC), were evaluated for their effect on bacterial speck under greenhouse conditions in an effort to use them together with the two selected biological control agents as an integrated strategy to manage bacterial speck. Treatment with 25% of the recommended concentration of acibenzolar-S-methyl caused significant disease suppression (81.2% control) in both Experiments 1 and 2. Treatment with 25% of the recommended concentration of didecyl-dimethyl-ammonium chloride reduced disease severity by 6.3% and 9.3% in Experiments 1 and 2.

The best control strategies were selected for integrated disease management studies under greenhouse and nursery conditions. Two biological control agents, *Bacillus* sp. LN10 and *R. glutinis* Y25, the plant sanitizer DDAC (at 25% of recommended strength) and the plant defence inducer ASM (at 25% of recommended strength) were used. It was found that any combination with 25% ASM caused significant disease reduction. 25 % ASM + *Bacillus sp*. LN10 + *R. glutinis* Y25 had a synergistic effect and gave disease reduction of 99.1% and 92.62% under greenhouse and nursery conditions, respectively, and was more effective than the copper bactericide control. All combinations with 25% DDAC provided no significant disease control. The combination treatment of 25% DDAC + *Bacillus sp*. LN10 + *R. glutinis* Y25 was ineffective under greenhouse and nursery conditions. The combination treatment of the two biological control *Bacillus sp*. LN10 + *R. glutinis* Y25 was ineffective under greenhouse and nursery conditions.

DECLARATION

I, Nonduduzo Charity Ncwane, declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research
2. This dissertation has not been submitted for any degree examination at any other university
3. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted. Then:
    a. Their words have been re-written but the general information attributed to them has been referenced;
    b. Where their exact words have been used, then their writing has been placed inside quotation marks and referenced.
4. 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 dissertation and in the references sections



Signed ----------

Nonduduzo C. Ncwane (Candidate)

Signed ----------

Dr K.S. Yobo (Supervisor)

Signed----------

Prof M.D. Laing (Co-supervisor)

ACKNOWLEDGMENTS

My thanks go to the following persons:

Dr K.S. Yobo, my supervisor, for his unending support, patience, understanding, encouragement, guidance throughout this study, wisdom and assistance in editing this dissertation.

Prof. M.D. Laing, my co-supervisor for his understanding, guidance, wisdom and assistance in editing this dissertation.

Dr M.J. Morris, for his valuable advice used in this study.

Mrs. S. van der Merwe, for all the help in greenhouse trials.

Mr R. Burgdorf, for his willingness to help with the laboratory work, and his advice on research techniques.

Mrs J. Khumalo, Mr J. Madondo, Mrs M. Khambula, Mr S. Mlotshwa, Mr T. Dlamini and Mr V. Dlamini for all their help and technical support in the greenhouses.

To the Plant Pathology RS340 family, thank you very much for assistance, encouragement and prayers.

The Centre for Electron Microscopy (UKZN-PMB) and Disciplines of Horticulture (UKZN-PMB) for allowing me to use some of their equipment.

Many thanks to Sunshine Seedling Services for allowing us to run the nursery trials in their nursery. We appreciate the space, time and the labour that they provided.

My loving family and friends for their patience, support, encouragement and faith in me. I would not have done it without them.

Thanks to the Seedling Growers Association of South Africa for sponsoring this work; National Research Foundation and AgriSeta for bursaries during my study.

DEDICATION

To Thembokwakhe and Balungile Ncwane,

for their support during my study.

May the Almighty God bless you always.

DISSERTATION INTRODUCTION

Bacterial speck disease caused *Pseudomonas syringae* pv. *tomato* (Pst) affects all tomato growing areas worldwide (da Silva *et al*., 2014). Infection of tomatoes by this pathogen results to in dark lesions on the leaves, stems and fruits, resulting into a depreciation of fruit quality and consequently a decrease in the value of the crop (Herman *et al*., 2008). Completely resistant cultivars are not available. Copper based bactericides are not effective enough, and their efficacy is highly dependent on environmental conditions, as well as disease levels (Ekici and Bastas, 2014). Moreover, they cause phytotoxicity and frequent applications of copper bactericides pose risks to human health and the evolution of resistance by the bacterium to copper (Balestra *et al*., 2009). Alternative control strategies include the use of biological control agents, plant defence inducers and plant sanitizers. A combination of these strategies could provide for the sustainable management of bacterial speck of tomato.

The main aim of this study was to develop an integrated disease management strategy using a plant defence activator, a plant sanitiser and novel biological control agents to control bacterial speck of tomato caused by Pst under greenhouse and nursery conditions.

The objectives of this study were:

1. To write a Literature Review on bacterial speck, the causal microorganism, the pathogen life cycle, its epidemiology, symptoms and economic importance, and the available control strategies;

2. To isolate and screen microorganisms for antagonism against bacterial speck of tomato caused by Pst *in vitro*;

3. To screen *in vivo* the best antagonists against Pst under greenhouse conditions;

4. To evaluate a range of concentrations of acibenzolar-S-methyl and didecyl-dimethyl-ammonium chloride for their ability to suppress bacterial speck of tomato under greenhouse conditions;

5. Integrate the best control strategies selected from biological control agents (bacterial and yeast agents) the optimum with concentrations of the plant defence activator (acibenzolar-S-methyl) and the plant sanitizer (didecyl-dimethyl-ammonium chloride) under greenhouse and nursery conditions.

The dissertation is structured in the form of five chapters. Each chapter is focused on a specific objective of the research that was conducted. Apart from Chapter One, the literature review, the other four chapters were independent studies and were written in the form of discrete research chapters, each 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.

This research was undertaken in the Discipline of Plant Pathology, at the University of KwaZulu-Natal, Pietermaritzburg Campus under the supervision of Dr K.S. Yobo and Prof M.D. Laing.

References

Balestra, G.M., Heydari, A., Ceccarelli, D., Ovidi, E., Quattrucci, A., 2009. Antibacterial effect of *Allium sativum* and *Ficus carica* extracts on tomato bacterial pathogens. Crop Protection 28, 807-811.

da Silva, E.O., Martins, S.J., Alves, E., 2014. Essential oils for the control of bacterial speck tomato crop. African Journal of Agricultural Research 9, 2624-2629.

Ekici O., 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.

Herman, M.A.B., Davidson, J.K., Smart, C.D., 2008. Induction of plant defence gene expression by plant activators and *Pseudomonas syringae* pv. *tomato* in greenhouses-grown tomatoes. Phytopathology 98, 1226-1232.

Table of Contents

CHAPTER ONE

Literature Review

1.1. Introduction

Integration of bacterial biological control agents and plant natural defence inducers could be a strategy to combat the severity of bacterial speck disease of tomato (*Solanum lycopersicum* L.). Bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* (*Pst*), and bacterial spot of tomato, caused by X*anthomonas vesicatoria*, are economically important bacterial disease in many tomato-growing regions worldwide (Goode and Sasser, 1980). Bacterial speck and spot often occur together on tomato plants and cause similar symptoms that can be mistaken for each other. They produce leaf lesions surrounded by halos that lead to the defoliation of premature leaves. The yield reduction subsequently, results from the reduced photosynthetic capacity of infected foliage. The pathogen also causes flower abortion and forms lesions on tomato fruit, which makes them unattractive and unsuitable for fresh market or processing.

Development of bacterial spot is favoured by warm and prolonged leaf wetness, while for bacterial speck cool conditions and high humidity are necessary for disease development. Severe losses are experienced when the infections occur on young plants.

Commonly used control measures, largely based on the use of copper bactericides, against the two diseases are not satisfactory because they do not provide sufficient control of the diseases in the field (Marco and Stall, 1983; Vallad *et al*., 2010). Copper based bactericides are inherently inefficient because they can only provide a protectant role, and easily wash off the leaves and fruit of tomato. A further problem is the development of copper resistant strains in Pst populations in many regions of the world (Marco and Stall, 1983; Bender and Cooksey, 1986; Silva and Lopes, 1995; Vallad *et al*., 2010). An increase public concern over pesticide residues found in or on fruit has increased the pressure to look for alternative control methods to manage these two diseases (Pernezny *et al*., 1995; Campbell *et al*., 2005).

1.2. The Crop, Pathogen and Disease

1.2.1. The Crop (Tomato)

Tomato, also known as *Solanum lycopersicum* L. is an edible fruit that belongs to the Family *Solanaceae,* in the Order *Solanales*, and the Genus *Solanum.* The plant is native to South America. In South Africa it is considered as one of the most important vegetables. In the KwaZulu-Natal Province alone, it is the third most important vegetable crop after potatoes (*Solanum tuberosum* L.) and cabbage (*Brassica oleracea* L. var. *capitata* L. f. *alba* DC). Most of the tomato crop is sold fresh, to be consumed as a fresh product or after cooking (DAFF, 2011).

Tomato is an annual plant that is classified as a warm-season crop. The optimum temperature for growth, yield and fruit quality of tomato is 18°Cto 24°C. Extreme temperatures either below 10°C or above 38°C damage both the fruit quality and the plant. Extreme temperatures usually cause the shedding of flowers, resulting in poor fruit set (Naika *et al*., 2005).

Soils for tomato production should be deep, fertile, humus-rich, free-draining and moisture retentive that are free of pathogens. Heavy clay soils are usually not suitable due to their slower drainage that can cause water-logging during prolonged rain or irrigation. Tomato plants prefer a soil pH that is slightly acidic ranging between 6.0 and 6.8.

1.2.2. The causal pathogen (Pseudomonas syringae *pv.* tomato)

According to Bryan (1933), bacterial speck of tomato and its causal agent were originally described and identified in the United State in 1933. It emerged as an economically significant disease in the tomato growing regions of North Carolina. The disease was of minor concern to tomato growers until the last two decades, during which the disease has increased in importance (Jones *et al*., 2003). The disease is now considered as one of the most damaging bacterial disease problem in tomato growing areas worldwide (Goode and Sasser, 1980). This is primarily due to water dissemination which gives the pathogen the ability to move fast through fields during moist conditions, and because it is favoured by cool and wet conditions (Smitley and McCarter, 1982).

P. syringae is a rod-shaped gram-positive bacterium, with multi polar flagella and an aerobic metabolism (Kreig and Holt, 1984). The bacterium is unable to utilise arginine due to the lack of arginine dihydrolase, and has a negative oxidase reaction because of the lack of

cytochrome C oxidase in its respiratory electron transport chain. A distinct *in vitro* characteristic of this bacterium is its yellow fluorescent appearance when cultured on King's B medium due to the siderophore pyoverdin (Cody and Gross, 1987). ***P. syringae*** is a member of the Pseudomonadaceae under the order of Pseudomonadales. Pseudomonadales are classified in the gamma class of Proteobacteria in the bacteria kingdom (Fu, 2008). ***P. syringae*** belongs to the genus *Pseudomonas* based on 16S rRNA analysis and is it named after lilac tree (*Syringa vulgaris*) from which it was first isolated (Anzai *et al*., 2000 and Fu, 2008). *Pseudomonas syringae* pathovar *tomato* DC3000 is an isolate of the pathogen that can cause bacterial speck in tomato and *Arabidopsis thaliana.* The bacterium genome contains circular chromosomes and two plasmids that together encode for open reading frames (ORFs). Its genes are dedicated to regulation which may reflect the need for rapid adaptation to the diverse environment the bacteria typically encounters during its epiphytic growth and subsequent pathogenic stage (Buell *et al*., 2003).

1.2.3. Symptoms of the disease

Lesions of bacterial speck appear as black irregular specks with a maximum diameter of 2mm on the leaves. These lesions are surrounded by the yellow halo (Figure 1a). Speck lesions cause leaf distortion as a result of Pst infection restricting leaf expansion when infection occurs at a very early stage of seedling growth. The lesions are often concentrated near leaf edges, appearing as a burn of the leaf margin resembling bacterial canker symptoms when lesions coalesce (Figure 1b). Only immature fruits are susceptible to infection by *Pst*. Very small black speck lesions develop that are slightly raised and are surrounded by a narrow green to yellow halo, and form a dent on the skin of fruits (Figure 1c).

Bacterial speck and spot diseases often occur together on tomato plants, at any time during the growing season, which is summer in the subtropics. However, bacterial speck is more severe when infection takes place in early spring, which exposes the tomato seedlings to prolonged cool and moist conditions (Schneider and Grogan, 1977). Bacterial spot tends to occur more at a later stage of the growing season when temperatures have risen to between 24^0C and 30^0C (Ji *et al*., 2006). Both diseases may cause significant reductions in tomato yields, especially if the infection occurs early in the season (Yunis *et al*., 1980; Pohronezny and Volin, 1983). When the diseases occur together on a plant more symptoms are observed over the aerial part of the plant and more damage is caused on both the foliage and fruits (Goode and Sasser, 1980; Louws et al., 2001).

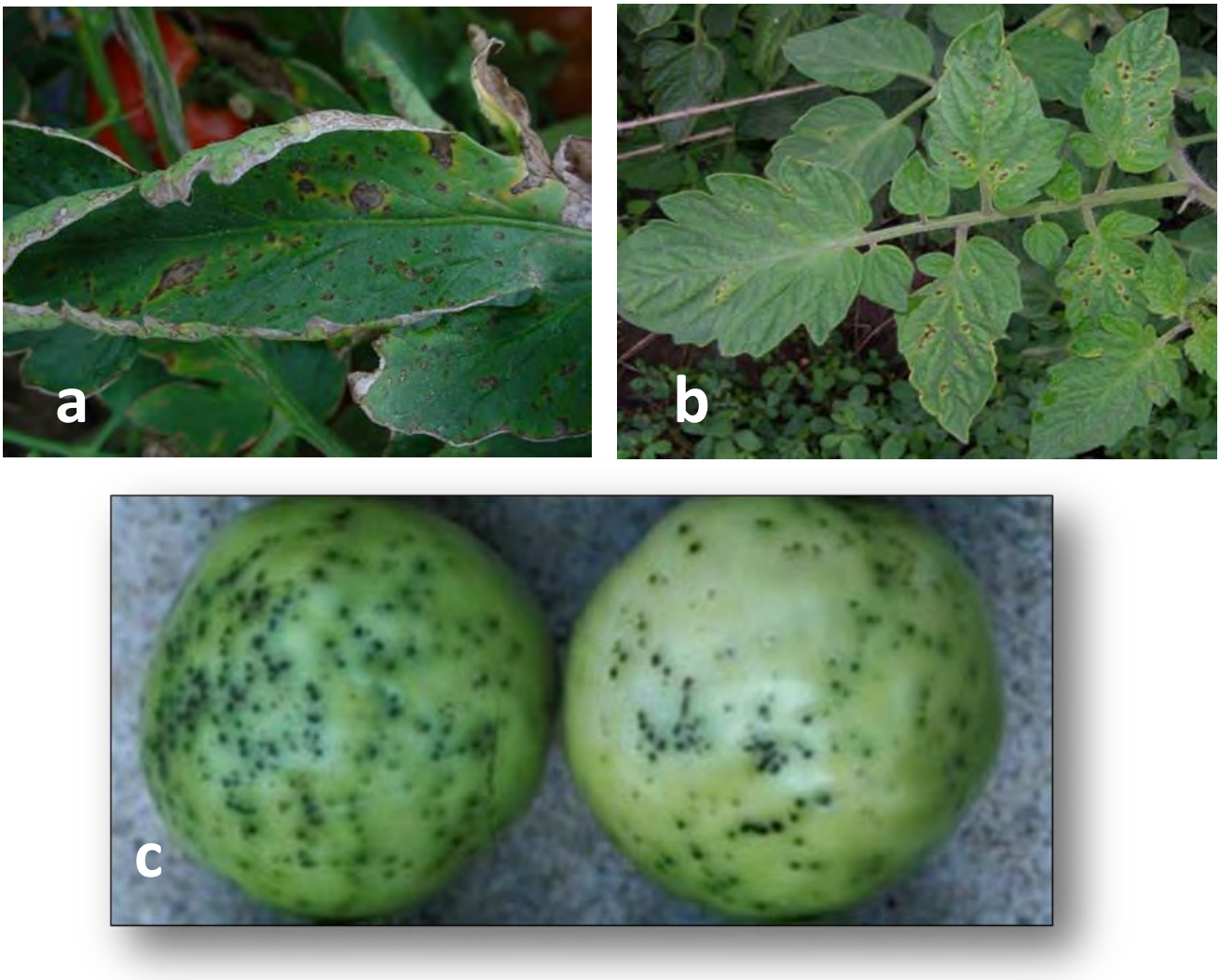


Figure 1: Infection of bacterial speck on tomato leaves and fruits caused by *Pseudomonas syringae* pv *tomato*: (a) Bacterial speck lesions emerging as edge burn lesions at the edge of tomato leaves when lesions coalesce; (b) Symptoms on leaves showing distinct yellow chlorosis around speck lesions (Grabowski, 2014); (c) bacterial speck lesions on the skin of tomato fruits (McCarty, 2014).

1.2.4. Disease Cycle and Epidemiology

Bacterial speck infection and development is favored by temperature ranging from 18ºC - 26ºC, along with high humidity of 80-85%, and regular rain or droplet irrigation. The regular occurrence of cool and moist conditions favour speck development as oppose to warm and moist conditions that favour bacterial spot development (Pernezny and Zhang, 2011). Pathogens multiply in the field after continuous leaf wetness for more than 6 hours, resulting in visible symptoms on the host plant. After 5-6 days, the pathogen is disseminated through wind-driven rain, irrigation droplets and aerosols, and cultural practices such as pruning. The

bacterium enters through natural openings (stomata and hydathodes), wounds created by wind-driven sand, insect punctures, and mechanical means.

P. syringae pv. *tomato* and *X. campestris* pv. *vesicatoria* are able to survive in the soil for a limited period of days to few weeks. However, the usual survival strategy of the *Pst* is in debris, volunteer plants and in seeds. Bacterial speck pathogen survives in these places until they are re-introduced the next season into young seedlings (Figure 2).

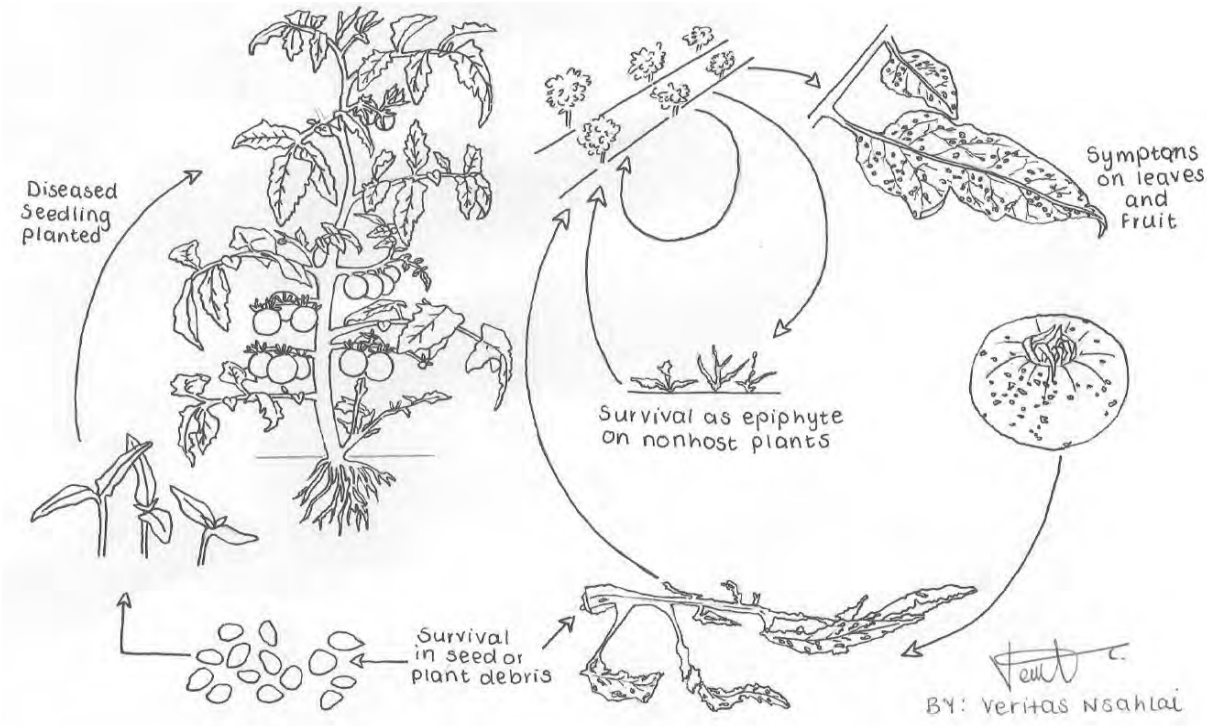


Figure 2: Disease cycle of bacterial speck of tomato. The diagram was adapted from the drawing of bacterial speck disease cycle on pepper plants by Vickie Brewster and redrawn for bacterial speck disease on tomato.

1.3. Control Measures

1.3.1 Chemical control of bacterial speck of tomato

According to Alexander *et al*., (2000), the standard pesticide treatment used for managing bacterial speck disease on tomato is copper combined with mancozeb. This combined standard treatment is usually adequate when environmental conditions favour disease development. However, when environmental conditions favour disease development and inoculum levels are high then this standard treatment become considerably less effective (Conover and Gerhold, 1981; Jardine and Stephens, 1987).

The emergence of copper resistance in both pathogens is as a result of routine sprays with copper-based bactericides, applied to diseased seedling as well as plants, applied on a weekly basis (Marco and Shall, 1983; Cooksey, 1990).

Normally, bacterial speck infection is controlled through the application of fixed copper bactericides (Stall and Thayer, 1962; Marco and Stall, 1983; Cuppels, 1986). However, resistance to copper compounds has been reported in some parts of the world such as Florida, where copper has been used on tomatoes for many years (Cuppels and Elmhirst, 1999). With the presence of copper resistance strains of Pst, copper-based bactericides have become less effective (Alexander *et al*., 1999). Copper resistance has been reported for only a few plant-pathogenic bacteria, primarily in pathovars of *P. syringae* and *X. campestris* (Adaskaveg and Hine, 1985; Bender and Cooksey, 1986; Andersen *et al*., 1991). Previous studies of copper resistance suggest that copper resistant genes cloned from *P. syringae* did not hybridise with copper resistant genes cloned from *X. campestris* in the Southern-blot hybridisation experiments. This suggests that resistance may have evolved independently for each pathogen (Bender *et al*., 1990 and Bender and Cooksey, 1987).

Host resistance has been not proven to be durable (Jones et al., 1998). There are also no commercial cultivars available that are resistant to Pst (Jones *et al*., 1998). As a result of the lack of completely effective bactericides and host resistance, development of alternative strategies for the management of the disease is being investigated.

Alternative strategies include the use of biocontrol agents, such as antagonistic bacteria (EL El-Hendawy *et al.,* 2005; Kavita and Umesha, 2007) and the use of bioactive products, which are referred to as plant activators (Sticher *et al*., 1997; Louws *et al.,* 2001), and the integration of partially effective control measures (Sticher *et al*., 1997; Abd-EL-Ghafar and Mosa, 2001; Anith *et al.,* 2004).

1.3.2. Biological Control

Biological control agents usually out-compete pathogens through inhibiting the growth of pathogens by several mechanisms such as antibiotic production (Howie and Suslow, 1991), production of secondary metabolites (Dunne *et al*., 1996) or siderophore production (Loper and Buyer, 1991; Meyer, 2007). Biocontrol agents have been reported to play a major role in controlling many plant diseases caused by bacteria, fungi and nematodes (Obradovic *et al.,* 2004; Siddiqui *et al*., 2005; Kavitha and Umesha, 2007).

The increasing occurrence of resistance to copper bactericides among pathovars of Pst has promoted interest in the development of biocontrol agents against foliar bacterial diseases. Biological control of speck can be achieved by using either naturally occurring saprophytic bacteria or non-pathogenic mutant strains of the pathogen (Wilson et al., 1996). The biocontrol agent *P. syringae* Cit7 has been shown to cause a significant reduction in the severity of bacterial speck and spot of tomato under field conditions in various locations in Northern America (Wilson *et al*., 2002, Byrne *et al*., 2005). There is also a promising future for bacteriophages against bacterial speck disease of tomato. Selected bacteriophages have been demonstrated to be able to suppress both bacterial speck and spot under both greenhouses and field conditions (Flaherty *et al*., 2000; Balogh *et al*., 2003; Obradovic *et al*., 2004).

Table 1.1: Some examples of biological control agents used against bacterial speck of tomato in greenhouse and field experiment

Biological Control Agents	Mode of Application	Site of experiment	Comments	Reference
P. syringae Cit7	foliar application	Greenhouse	provided speck suppression by 75% and 56%	Lindow, 1985
P. fluorescens 89B-61	seed application soil drenching	Greenhouse	provided speck suppression by 76.3% and 52%	Ji *et al*., 2006
P. putida B56	foliar application	Field	no significant disease suppression was observed	Wilson *et al*., 2002
P. fluorescens A506	foliar application	Field	speck was significantly reduced by 47.7%	Lindow, *et al*., 1996
B. pumilus SE34	seed application soil drenching	Greenhouse	Speck was reduced by 40.2%	Ji *et. al*., 2006
P.syringae TLP2	foliar application	Greenhouse	provided low disease suppression of 9.8%	Wilson *et al*., 2002
P. fluorescens A506 + *B. pumilus* SE34	foliar (a506) and soil drenching (se34)	Field	not significant suppression of speck observed	Ji *et al*., 2006
P. fluorescens A506 + *P. fluorescens* 89B-61	foliar (a506) and soil drenching (89b-61)	Field	disease suppression was observed however there was no significant difference when compared to each treatment	Ji *et al*., 2006
P. putida B56 + *B. pumilus* SE34	foliar (b56) and soil drenching (se34)	Field	no significant disease suppression	Ji *et al*., 2006
P. putida B56 + P. *fluorescens* 89B-61	foliar (b56) and soil drenching (89b-61)	Field	no significant disease suppression	Ji *et. al*., 2006

1.3.3. Plant Activators

A synthetic compound, acibenzolar-S-methyl (ASM), is an active ingredient of Actigard^{TM}, a product that induces systemic acquired resistance (SAR) and provides for significant suppression of bacterial speck and spot disease in the field (Louws *et al*., 2001; Abbasi *et al*., 2002; Wilson *et al*., 2002). Chemical induction of SAR could be an effective strategy to control this disease. However, application of these chemicals remains to be optimized because phytotoxic effects have been reported (Csinos *et al*., 2001; Louws *et al*., 2001; Romero *et al*., 2001).

ASM is said to stimulate the plant's natural defence mechanisms by inducing SAR in plants to limit pathogenesis of many plant pathogens (Sticher *et. al*., 1997). This ultimately protects the growth of the plant by suppressing pathogen growth. The active ingredient has no direct action against the pathogen and its mode of action is entirely through the activation of the plant's natural defence pathway, which leads to acquire resistance in treated and systemic tissue of the plant (Benhamou and Belanger, 1998).

Systemic Acquired Resistance refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens (Ryals *et al*., 1996). As a result, plants form necrotic lesions from the hypersensitivity response (HR), which is a signal of the activation of SAR pathway (Ward *et al*., 1991; Ryals *et al*., 1996). SAR activation results in the development of a broad-spectrum systemic resistance against multiple pathogens. The activation of SAR is associated with the expression of the set of genes called SAR genes, which encode for SAR marker proteins (Uknes *et al*., 1992). The proteins are associated with SAR activation and many belong to the class of pathogenesis related (PR) proteins, which accumulate in the plant after infection by plant pathogens (Gianinaui *et al*., 1970). These include proteins such as PR-1, β-1,3 glucanase, class II chitinase, hevein-like protein and thaumatin-like proteins (Ward *et al*., 1991).

1.3.4. Induced Systemic Resistance (ISR)

Induced resistance is the physiological state of enhanced defensive capacity elicited by specific environmental stimuli, whereby the plant's physiological defences are potentiated against subsequent abiotic challenge (Choudhary and Johri, 2009). This enhanced state of resistance of the plant is effective against broad range of pathogens or parasites. Unlike SAR,

it does not involve the accumulation of pathogenesis-related proteins or salicylic acid; it relies on a pathway regulated by jasmonate and ethylene (Yan *et al*., 2002).

Induced Systemic Resistance has also been reported to be primed by Plant Growth Promoting Rhizobacteria (PGPR). Plants tend to have the ability to acquire enhanced levels of resistance to pathogens through exposure of biotic stimuli, provided they are PGPRs. The non-pathogenic bacteria in association with plant roots elicit an enhanced state of defence or ISR in plants. This is sometimes referred to as Rhizobacteria-Mediated ISR (Ryu *et al*., 2003; Siddiqui and Saukat, 2004; Meziane *et al*., 2005; Choudhary and Johri, 2009). Several PGPRs that colonise the root system after seed application protect plants against foliar diseases. Some of the PGPRs include *Pseudomonas fluorescens*, *Pseudomonas putida* and *Bacillus pumilus* (Tomma *et al*., 2001). Other PGPR that have been shown to eliciteinduced resistance in tomato plants against other pathogens are listed in Table 1.2.

Table 1.2: PGPR-mediated induction of ISR in different plant species

Bacterial strain	**Plant species**	**Pathogen**	**Elicitors**	**References**
P. putida WCS 358	arabidopsis	*P. syringae*	LPS, Siderophore, Flagella	Meziane *et al*., (2005)
	tomato	*P. syringae*	LPS, Siderophore	Meziane *et al*., (2005)
P. fluorescens CHAO	tomato	*Meloidogyne javanica*	2, 4 DAPG	Siddiqui and Saukat (2004)
P. aeruginosa 7 NSK2	tomato	*Botrytis cinerea*	Pyochelin and Pyocyanin	Audenaert *et al*.(2002)
B. pumilus SE34	arabidopsis	*P. syringae* pv.*maculicola*	SA	Ryu *et al*.(2003)
P. fluorescens 89B-27	tomato, cucumber	*F. oxysporum*	Unknown	Raupach *et al*., 1996
S. marcescens 90-166	tomato,	*C. orbiculare*	Unknown	Raupach *et al*., 1996
	cucumber	*P.* pv. *lacharymans*	Unknown	Raupach *et al*., 1996

1.3.5. Resistant Varieties

The gene-to-gene model proposed by Flor in the 1940's suggested that there is an incompatibility system between plants and pathogens that is controlled by a single dominant gene (Flor, 1971). In this model, the interaction between a pathogen expressing an avirulent (*Avr*) gene product and a host expressing the appropriate resistance (R) gene product results in plant cell death, which stops the spread of the pathogen within the plant (Bent, 1996). Tomato varieties that are resistance to *Pst* strains contain the *avr* gene known as *AvrPto*. The *Pto* gene was originally discovered in *Lycopersicon pimpinellifolium* L., a wild tomato species, and was isolated using Map-based cloning (Martin *et al*., 1993). Mutagenesis of a bacterial speck-resistant tomato line has revealed a second gene *Prf* (Salmeron *et al*., 1994) that is required for *Pto* mediated resistance and fenthion sensitivity, which is a related phenotype mediated by the *Fen* gene (Martin *et al*.,1994).

In 2002, resistance to Race 0 of *Pst* was introduced into many processing tomato varieties and a few fresh-market tomato varieties in North America (Wilson *et al*., 2002). Unfortunately, hybrid varieties heterozygous for *Pto* were not completely resistant to speck. It was also found that *Pto* genes do not confer resistance to Race 1 of *Pst*. Consequently, this variety will not confer resistance where Race 1 is present (Wilson *et al*., 2002). However, in Tanzania two *Pst* resistant cultivars Tonquay and BSS436 have been reported to provide a stable resistance to bacterial speck disease (Shenge et al., 2007). In Turkey there are a number of resistant cultivars that also contain *Pto* gene but still show bacterial speck symptoms. Cultivars such as Prenses and Petrus (from Turkey) showed small speck lesion symptoms and halos, yet they were considered to be resistant when compared to other tomato cultivars without the *Pto* gene. It is possible that the different cultivars have different defence mechanisms (Basim and Turgut, 2013). In Canada resistant cultivars currently available are Ontario 7710, Ontario 7611, Ontario 782 and Farthest North (Pitblado and Kerr, 1980). According to Kozik (2002) resistance of these cultivars against bacterial speck is due to the hybridisation of a sensitive variety A100 with Ontario 7710, a highly resistant cultivar.

1.3.6. The role of Silicon in disease management

Silicon (Si) is a multifunctional element that significantly increases plant tolerance against biotic and abiotic stress (Ma, 2004). This element is reported to play a mechanical role in cell wall re-enforcement sites, such as increasing the thickness of the culm wall and the size of the vascular bundles, thereby enhancing the strength of the stem (Shimoyama,1958). Silicon

alleviates the effect of other abiotic stress such as salt stress, metal toxicity, drought stress, radiation damage, nutrient imbalance, high temperatures and freezing condition (Mitani and Ma, 2005). The beneficial effects are mostly expressed through Si deposition in leaves, stem and hulls (Ma and Tahakashi, 2002; Ma, 2004). As a result, the Si effect in the plant is characterised by the larger deposition effect associated with a greater Si accumulation in the shoots (Mitani and Ma, 2005).

Silicon accumulation varies considerably among plant species. In an experiment by Ma and Tahakashi, (2002), it was observed that Si accumulation in higher plants such as Gramineae and Cyperaceae were high. Plants in the Cucurbitales, Urticales and Commelinaceae showed intermediate Si accumulation. Other plant species mostly dicotyledonous plants had a low Si accumulation (Mitani and Ma, 2005).

Differences in Si accumulation have been attributed to the ability of the roots to absorb Si (Takahashi *et al*., 1990). Silicon is taken up in the form of silicic acid, an uncharged molecule. Three modes of uptake of silicon have been proposed. These are active uptake, passive uptake and rejective uptake. The active mode is where Si uptake is faster than it dissolves in water, resulting in a depletion of Si in the uptake solution. Passive uptake is where the uptake of silicon is similar to the uptake of water, thus no significant changes in the concentration of Si in the uptake solution are observed. In the rejective uptake, plants tend to exclude Si. This is demonstrated by high concentration of Si in the uptake solution (Mitani and Ma, 2005).

Disease suppression by silicon application was first suggested by Isenosuke Onodera, a Japanese plant nutrient chemist who suggested that Si was involved in rice resistance to blast (*Magnoporthe grisea* (T.T. Hebert) M.E. Barr (synonym *Pyricularia grisea* (Cooke) Sacc). He tested blast infected plants and healthy plants obtained from the same field where he discovered that diseased plants always contained less Si than healthy plants. These findings did not necessarily mean that blast infection was reduced by Si accumulation or that plants with less Si content were more susceptible to the disease. His results indicated that there was a relationship between Si content and blast susceptibility (Onodera, 1917 cited in Datnoff and Rodrigues, 2005).

Many significant disease suppression Si experiments have been conducted on monocotyledonous plants. Diseases such as brown spot (*Cochliobolus miyabeanus*); neck blast (*Magnaporthe oryzae*), grain discolouration caused by a disease complex including

Bipolaris oryzae, *Curvularia sp*., *Phoma sp*., *Microdochium sp*., *Nigrospora sp*., and *Fusarium sp*.,; stem rot (*Magnaporthe salvinii* Cattaneo) and root-knot nematodes (*Meloidogyne spp*) were all reduced significantly as a result of Si treatments (Datnoff and Rodrigues, 2005). Though tomato is not considered to be an active absorber of silicon, the little quantity that it absorbs is enough to suppress fusarium crown and root rot (*Fusarium oxysporum* f.sp. *radicis-lycopersici*) of tomato. The increase in the Si content of roots was significantly correlated with the reduction of disease severity of root, crown and stem indicating a silicon-mediated resistance (Huang *et al*., 2011). Another example of tomato disease that was significantly suppressed by silicon is bacterial wilt caused by *Ralstonia solanacearum* on L390 and King Kong2 tomato genotypes. Silicon accumulated in roots, stem and leaves in low concentration, suggesting that silicon is one of the plant activators that can prime for enhanced levels of systemic resistance (Dannon and Wydra, 2004).

1.4. Combination of management strategies

Louws *et al*. (2001) combined the treatments of streptomycin, Clorox, Actigard, Kocide and fungicides both as seed and foliar treatments. It was observed that Kocide application increased fruit infection, while Actigard decreased bacterial speck incidence and severity on the foliage and the fruit of tomato plants, when compared to fungicide alone and fungicide plus Kocide treatment. Clorox seed treatment and streptomycin treatments plus Actigard under field treatment also reduced foliage and fruit symptoms of bacterial speck disease.

Another experiment by Romeo *et al*. (2001) on the combined application of Actigard and copper fungicides, compared with standard treatment of copper plus maneb, on bell pepper plants against bacterial spot in the field was done where by the plant activator application alone or in combination with copper provided disease control that was similar to the standard treatment of copper plus moneb. Weekly application of Actigard alone during the whole crop season reduced fruit yields. While these chemical inducers may provide for foliar disease control, it has unpredictable results on fruit yield.

In another experiment by Louws *et al*., (2001) Actigard reduced bacterial speck disease severity when compared to standard copper bactericide treatments. It reduced the incidence of bacterial speck infection of leaves. However, tomato fruit yield was not affected when compared with the fungicide treatment program that was used in the experiment. It did affect the dry weight of the treated tomato plants when it was compared with other treatments.

In an experiment done by Bashan and de-Bashan (2002) it was observed that inoculation of tomato seed with the plant growth-promoting bacterium (PGPB) *Azospirillum brasilense,* application of streptomycin sulphate, and spraying with copper sprays bactericide as separate treatments against bacterial speck provided no lasting effect on bacterial speck disease severity. However, when the seed inoculation with *A. brasilense* was combined with a single streptomycin foliar treatment and two foliar bactericide application, there was a reduction in disease severity in tomato seedlings. Disease progression was also slowed under favourable conditions. The *A. brasilense* did not induce significant systemic resistance against the bacterial speck pathogen, but levels of salicylic acid were increased. Thus, this suggests that the combination of partially effective disease management treatments with different modes of action could reduce the severity of bacterial speck in tomato.

1.5. References

Abbasi, P.A., Dahmani, A.l., Sahin, J., Hoitink, F., Miller, H.A.J., 2002. Effect of compost amendments on disease severity and yield of tomato in conventional and organic production systems. Plant Disease 86, 156-161.

Abd El-Ghafar, N.Y., Mosa. A.A., 2001. Integration between biological and chemical treatments to control bacterial spot disease of tomato. Egyptian Journal of Phytopathology 29, 33 – 45.

Adaskaveg, J.E., Hine, R.B., 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. Plant Disease 69, 993-996.

Alexander, S. A., Kim, S. H., Waldenmaier, C. M., 1999. First report of copper-tolerant *Pseudomonas syringae* pv. *tomato* in Virginia. Plant Disease 83, 964.

Alexander, S.A., Caldwell, J.S., Hohlt, H.E., Nault, B.A., O'Dell, C.R., Sterrett, S.B., Wilson, H.P., 2000. Virginia Commercial Vegetable Production Recommendations. No. 456-420. VA Coop. Extension Services.

Anith, K.N., Momol, M.T., Kloepper,J.W., Marios, J.J., Olson, S.M., Jones, J.B., 2004. Efficacy of plant growth-promoting rhizobacteria, acibenzolar-S-methyl, and soil amendment for integrated management of bacterial wilt on tomato. Plant Disease 88, 669–673.

Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H., Oyaizu, H., 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. International Journal of Systematic and Evolutionary Microbiology 50, 1563-1589.

Audenaert, K., Pattery, T., Comelis. P., Hotte, M., 2002. Induction of systemic resistance to *Botrytis cinerea* in tomato by *P. aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. Molecular Plant-Microbe Interactions 15, 1147–1150.

Balogh, B., Jones, J.B., Momol, M.T., Olson, S.M., Obradovic, A., King, P., Jackson, L.E., 2003. Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. Plant Disease 87, 949–954.

Bashan, Y., de-Bashan, L.E., 2002. Reduction of bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomato by combined treatments of plant growth-promoting bacterium, *Azospirillum brasilense*, streptomycin sulfate, and chemo-thermal seed treatment. European Journal of Plant Pathology 108, 821–829.

Basim, H., 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., Cooksey, D.A., 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. Journal of Bacteriology 165, 534 - 541.

Bender, C.L., Cooksey. D.A., 1987. Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. Journal of Bacteriology 169, 470 - 474.

Bender, C.L., Malvick, D.K., Conway, K.E., George, S., Pratt, P., 1990. Characterization of pXV1OA, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Applied Environmental Microbiology 56, 176-175.

Bent, A., 1996. Function meets structure in the study of plant disease resistance genes. Plant Cell 8, 1757 - 1771.

Bryan, M. K., 1933. Bacterial speck of tomatoes. Phytopathology 23, 897 - 904.

Buell, R.C., Joardar, V., Lindeberg,M., Selengut, J., Paulsen, I.T., Gwinn, M.L. Dodson, R.J., Deboy, R.T., Durkin, S.A., Kolonay,J.F., Madupu, R., Daugherty, S., Brinkac, L..,

Beanan, M.J., Haft, D.H., Nelson, W.C., Davidsen, T., Zafar, N., Zhou, L.., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., van Aken, S.E., Feldblyum, T.V., Dascenzo, M., Deng, W., Ramos, A.R., James R. Alfano, J.R., Cartinhour, S., Chatterjee, A.K., Delaney, T.P., Lazarowitz,S.G., Martin, G.B., Schneider, D.J., Tang, X., Bender, C.L., White, O., Claire M. Fraser, C.M., Collmer, A., 2003. The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Biological Sciences - Agricultural Sciences 100, 10181 - 10186.

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.

Campbell, H.L., Ji, P., Kloepper, J.W., Jones, J.B., Suslow, T.V., Wilson, M., 2005. Integrated biological control of bacterial speck and spot of tomato under Weld conditions using foliar biological control agents and plant growth-promoting rhizobacteria. Biological Control 36, 358 - 367.

Choudhary, D.K., Johri, B.N., 2009. Interactions of *Bacillus spp*. and plants growth-promoting rhizobacteria, with special reference to induced systemic resistance (ISR). Microbiological Research 164, 493 - 513.

Cody, Y.S., Gross, D.C., 1987. Characterization of pyoverdin (pss), the fluorescent siderophore produced by *Pseudomonas syringae* pv. *syringae*. Applied and Environmental Microbiology 53, 928 - 34.

Conover, R. A., Gerhold, N. R., 1981. Mixtures 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.

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

Csinos, A.S. Pappu, H.R. McPherson, R.M., Stephenson, M.G., 2001. Management of tomato spotted wilt virus in flue-cured tobacco with acibenzolar-*S*-methyl and imidacloprid. Plant Disease 85, 292 - 296.

Cuppels, D.A., 1986. Generation and characterization of Tn5 insertion mutations in *Pseudomonas syringae* pv. *tomato.* Applied and Environmental Microbiology 51, 323 -327.

DAFF, 2011. Eggplant (*Solanum melongena* L.). Pretoria, Republic of South Africa

Dannon, E. A., Wydra, K., 2004. Interaction between silicon amendment, bacterial wilt development and phenotype of *Ralstonia solanacearum* in tomato genotypes. Physiological and Molecular Plant Pathology 64, 233 - 243.

Datnoff, L.E., Rodrigues, F.A., 2005. The role of silicon in suppressing rice disease. APSnet Feature. What pages?

Dunne, C., Delany, I., Fenton, A., Gara, F.O., 1996. Mechanisms involved in biocontrol by microbial inoculants. Agronomy 16, 721 - 729.

El-Hendawy, H.H., Mohamed, E., Osman, N., Sorour, M., 2005. Biological control of bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* by *Rahnella aquatilis.* Microbiological Research 160, 343 - 352.

Flaherty, J.E., Jones, J.B., Harbaugh, B.K., Somodi, G.C., Jackson, L.E., 2000. Control of bacterial spot on tomato in the greenhouse and Weld with H-mutant bacteriophages. Horticultural Science 35, 882 - 884.

Flor, H.H., 1971. Current status of the gene-for-gene concept. Annual Review of Phytopathology 9, 275-296.

Fu, Z., 2008. *Pseudomonas syringae* type III secretion system and effectors. University of Nebraska. ProQuest LLC, Ann Arbor, USA, p 17-18.

Gianinaui, S., Martin, C., Vallbe, J.C., 1970. Hypersensibilite aux virus, temperature et protéines solubles chez le *Nicotiana xanthi* n.c. Apparition de nouvelles macromolécules lors de la repression de la synthese virale. Comptes rendus de l'Académie des Sciences Science and Vie 270, 2382 - 2386.

Goode, M. J., Sasser, M., 1980. Prevention the key to controlling bacterial spot and bacterial speck of tomato. Plant Disease 64, 831 - 834.

Grabowski, M., 2014. Tomato Fruit: Spots on fruit. [http://www.extension.umn.edu/garden/diagnose/plant/vegetable/tomato/fruitspots.html] visited on 15 August 2014.

Howie, W.J., Suslow, T.V., 1991. Role of antibiotic biosynthesis in inhibition of *Pythium ultimum* in the cotton spermosphere by *Pseudomonas fluorescens*. Molecular Plant-Microbe Interactions 4: 393 - 399.

Huang, C.H., Roberts, P. D., Datnoff, L. E., 2011. Silicon suppresses fusarium crown and root rot of tomato. Journal of Phytopathology 159, 546 - 554.

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

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.

Jones, J.B., Momol, T.M., Ivors, K.L., Louws, F.J., 2003. Integrated management of bacterial speck and bacterial spot on tomato. United States Department of Agriculture Cooperative State Research, Education, and Extension Service.

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

Kavitha, R., Umesha, S., 2007. Prevalence of bacterial spot in tomato fields of Karnataka and effect of biological seed treatment on disease incidence. Crop Protection 26, 991- 997.

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

Kreig, N. R., Holt, J. G., 1984. Bergey's Manual of Systematic Biology. Baltimore: Williams and Wilkins, p 141-199.

KZNdea, (Unknown). Tomato. Environmental requirements. [http://www.kzndae.gov.za/Portals/0/Horticulture/Veg%20prod/tomato.pdf]: visited on 14 August 2014.

Loper, J.E., Buyer, J.S., 1991. Siderophores in microbial interactions on plant surfaces. Molecular Plant-Microbe Interactions 4, 5 - 13.

Louws, F.J., Randall-Schadel, B., Lynch, N.P., Shoemaker, P.B., 2001. Integrated Management of Bacterial Speck of Tomato. Department of Plant Pathology, North Carolina State University, Raleigh NC 27695.

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 using a plant activator. Plant Disease 85, 481- 488.

Ma, J.F., Takahashi, E., 2002. Soil fertilizer and plant silicon research in Japan. Amsterdam, Elsevier Science.

Ma. J.F., 2004. Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. Soil Science and Plant Nutrition 50, 11-18.

Marco, G.M., Stall, R.E., 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Disease 67, 779 - 781.

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.

Martin, G.B., Frary, A., Wu, T., Brommonschenkel, S., Chunwongse, J., Earle, E.D., Tanksley, S.D., 1994. A member of the tomato *Pto* gene family confers sensitivity to fenthion resulting in rapid cell death. Plant Cell 6, 1543 - 1552.

McCarty, G., 2014. Wilt, Spot, and Speck…Tis Tomato Disease Season. [http://web.extension.illinois.edu/jsw/eb362/entry_8788] visited on 15 August 2014.

Meyer, J.M., 2007. Siderotyping and bacterial taxonomy: a siderophores bank for a rapid identification at the species level of fluorescent and non-fluorescent *Pseudomonas*. In: Varma, S. and Chincholkar, S. (Eds.), Microbial Siderophores. Springer, New York, pp. 43 - 61.

Meziane, H., Vander, S.I., van Loon, L.C., Hofte, M., Bakker, P.A.H.M., 2005. Determinants of *P. putida* WCS358 involved in induced systemic resistance in plants. Molecular Plant Pathology 6, 177 - 85.

Mitani, N. and Ma, J.F. (2005). Uptake system of silicon in different plant species. Journal of Experimental Botany 56, 1255 - 1261.

Naika, S., De-Jeude, J.L., De-Goffau, M., Hilmi, M., Van-Dam, B., 2005. Cultivation of Tomato: Production Processing and Marketing. Agromisa Foundation and CTA, Wageningen, 92.

National Gardening Association Editors, 2014. Garden Prep for Tomatoes. [http://www.garden.org/foodguide/browse/veggie/tomatoes_getting_started/358]: visited on the 14 August 2014

Obradovic, A., Jones, J.B., Momol, M.T., Balgoh, B., Olson, S.M., 2004. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. Plant Disease 88, 736 - 740.

Onodera, I., 1917. Chemical studies on rice blast. Journal of Science and. Agricultural Society 180, 606 - 617.

Parets, M., Pernezny, K., Roberts, P., 2013. Disease control for Florida Tomatoes. University of Florida, IFAS extension. Gainesville 32611, 35.

Pernezny, K., Kudela, V., Kokoskova, B., Hladka, I., 1995. Bacterial disease 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., Zhang, S., 2011. Bacterial Speck of Tomato. University of Florida, IFAS Extension (UF).

Pitblado, R.E., Kerr, E.A., 1980. Resistance to bacterial speck (*Pseudomonas tomato*) in tomato. Acta Horticulturae. 100, 379 - 382.

Pohronezny, K., Volin, R.B., 1983. The effect of bacterial spot on yield and quality of fresh market tomatoes. Horticultural Science 18, 69 - 70.

Raupach, G.S., Liu, L., Murphy, J.F., Tuzun, S., Kloepper, J.W., 1996. Induced systemic resistance in cucumber and tomato against cucumber mosaic (cucumovirus) using plant growth promoting rhizobacteria (PGPR). Plant Disease 80, 891 - 894.

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.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., Hunt, M.D., 1996. Systemic Acquired Resistance. The Plant Cell 8, 1809 - 1819.

Ryu C.M., Hu, C.H., Reddy, M.S., Kloepper, J.W., 2003. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae.* New Phytology, 160, 413 - 420.

Salmeron, J.M., Barker, S.J., Carland, F.M., Mehta, A.Y., Staskawicz, B.J. 1994. Tomato mutants altered in bacterial disease resistance provide evidence for a new locus controlling pathogen recognition. Plant Cell 6, 511 - 520.

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

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. Journal of Plant Protection Research 47,103 - 111.

Shimoyama, S., 1958. Effect of calcium silicate application to rice plants on the alleviation of lodging and damage from strong gales. Studies in the improvement of ultimate yields of crops by the application of silicate materials. Japanese Association for the Advancement of Science, 57 - 99.

Siddiqui, A., Haas, D., Heeb, S., 2005. Extracellular Protease of Pseudomonas fluorescens CHA0, a biocontrol factor with activity against the root-knot nematode Meloidogyne incognita. Applied and Environmental Microbiology 71, 5646 - 5649.

Siddiqui, A., Saukat, S.S., 2004. Systemic resistance in tomato induced by biocontrol bacteria against the root-knot nematode, *Meloidogyne javanica* is independent of SA production. Phytopathology 152, 48 - 54.

Silva, V.L., Lopes, C.A., 1995. *Pseudomonas syringae* pv. *tomato* resistant to copper in copper-sprayed tomato Welds. *Fitopatologia Brasileira* 20, 85 - 89.

Smitley, D.R., 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.

Stall, R.E., Thayer, P.L., 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. Plant Disease 46, 389 - 392.

Sticher, L., Mauchmani, B., Metraux, J.P., 1997. Systemic acquired resistance. Annual Review of Phytopathology 35, 235 - 270.

Takahashi, E., Ma, J.F., Miyake, Y., 1990. The possibility of silicon as an essential element for higher plants. Comments on Agricultural and Food Chemistry 2, 99 - 122.

Thomma, B.P.H.J., Tierens, K.F.M., PenninckxI, A.M.A., Mauch- Mani, B., Broekaert, W.F., Cammue, B.P.A., 2001. Different micro-organisms differentially induce *Arabidopsis* disease response pathways. Plant Physiology and Biochemistry 39, 673 - 80.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., Ryals, J., 1992. Acquired resistance in *Arabidopsis*. Plant Cell 4, 645 - 656.

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.

Voloukis, A.E., Bender, C.L., Cooksey, D.A., 1993. Similarities between copper resistance gene from *Xanthomonas campestris* and *Pseudomonas syringae*. *American Society of Microbiology* 59, 1627-1634.

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

Wilson, M, Campbell, H.L. Jones, J.B. Suslow. T.V., Cuppels. D.A., 1996. Biological control of bacterial speck of tomato. Phytopathology 86, 549.

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.

Yan, Z., Reddy, M.S., Ryu, C.M., McInroy, J.A., Wilson. M., Kloepper, J.W., 2002. Induced systemic resistance against tomato late blight elicited by plant growth promoting rhizobacteria. Phytopathology 92, 1329 - 33.

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

CHAPTER TWO

Isolation, *in vitro* screening and identification of bacterial and yeast isolates as potential biological control agents against bacterial speck on tomato

Abstract

Two hundred and fifty bacterial isolates, and one hundred yeast isolates were screened *in vitro* for antagonistic activity against *Pseudomonas syringae* pv. *tomato*, the causal agent of bacterial speck on tomato. These isolates were obtained from tomato leaf samples collected from several seedling nurseries and tomato fields around Pietermaritzburg, South Africa. For bacterial isolates *in vitro* dual-test screening was done on Tryptone Soy Agar and incubated at 28^0C for 7 days in which the zones of inhibition created by each of the isolates was used as the selection criteria for the best potential isolates. The 10 best bacterial isolates created significant zones of inhibition, ranging from 17 to 29 mm in diameter. These were selected for identification and for further studies. Seven yeasts isolates were selected for identification and for further studies based on their *in vitro* activity on detached leaf bioassay against the bacterial speck pathogen. All the selected bacterial isolates were identified as *Bacillus* spp. Four of the selected seven yeasts isolates belong to the genus *Rhodoturula*, two isolates were *Cryptococcus* species and one isolate belonging to the genus *Rhodosporidium.*

2.1 Introduction

Tomato (*Lycopersicon esculentum* Mill) is the second most important vegetable crop after potato (*Solanum tuberosum* L.) in South Africa. Tomato is cultivated both commercially by subsistence farmers, small scale farmers and home gardeners. China is reported to be the largest producer of tomatoes in the world, followed by the USA, Turkey, India and Egypt (DAFF, 2013). These countries produce most of the world's tomato. Globally commercial sectors contribute 95% of the total production and the emerging sector only produces 5% (DAFF, 2013).

Bacterial speck and spot of tomato are economical important bacterial diseases in most tomato growing regions worldwide (Goode and Sasser, 1980). Bacterial speck of tomato is caused by *Pseudomonas syringae* pv. *tomato* (Pst) (Jones *et al*., 1991). The main source of infection of this disease is the use of contaminated seeds which serves as a source of

inoculum. The disease results in leaf distortion which restricts leaf expansion (Good and Sasser., 1980; Liete *et al*., 1995). The disease causes necrotic zones surrounded by chlorotic halos in the leaves, especially if infection occurs at an early stage of seedling growth (McCarter *et al*., 1983; Sijam *et al*., 1991). As a result, yield is significantly reduced due to reduced photosynthetic capacity of infected foliage, and also due to flower abortion and spotted fruits that are rejected. The lesions are often concentrated near the leaf edge, causing margin burn resembling symptoms of bacterial canker. The lesions on infected immature fruit are slightly raised, black and are surrounded by a narrow green to yellow halo. These usually form a dent on infected fruit (Louws *et al*., 2001).

Traditionally, agrochemicals are the most effective and immediate solution to most plant disease problems but their persistent residues may cause health problems (Howarth, 1991). Biological control using microorganisms to suppress plant diseases offers an alternative to the use of synthetic chemicals. Several members of *Bacillus spp*. have been used as biological control agents against several soilborne and foliar pathogens (Thomashow and Bakker, 2015). These organisms are known to produce various antimicrobial substances such as bacteriocins and siderophores, which act against plant pathogens (Foldes *et al*., 2000; Peralta *et al.,* 2005; Pathma *et al*., 2011). Yeasts have been used as biological control agents against *Phytophthora infestans* (Mont.) de Bary on tomato; *Colletotrichum acutatum* J.H. Simmonds (1968); and *Penicillium expansum* Link ex. Thom of apple (*Malus domestica* Borkh., 1803) in a postharvest study (Xu *et al*., 2011). The mechanisms of action by yeast include competition for food substrates and space (Ippolito *et al*., 2000 and Jijakli *et al*., 2001). A common approach in screening for potential biological control agent is to perform *in vitro* dual-test bioassays using an antibiotic disc for bacterial strains (Taechowisan *et al*., 2009) and detached-leaf assays for yeast strains (Pacheco *et al*., 2012).

In vitro screening provides a rapid pre-screening technique that allows for large number of microbial isolates to be tested. However, there has been both positive and negative feedback on this technique in selecting for the most effective and aggressive isolates for greenhouse and field studies. Some isolates that were initially considered to be effective have been found to be ineffective when tested under greenhouse conditions (Zengeni and Giller, 2007).

Biological control agents against bacterial speck disease have been reported by other researchers. Ji *et al*., (2006) reported that *Pseudomonas syringae* Strain Cit7 effectively suppressed bacterial speck severity under greenhouse conditions whilst *Pseudomonas*

fluorescens Strain 89B-61 reduced the severity of bacterial speck in the field. To the best of our knowledge there has been no report on yeast biological control agents being tested against bacterial speck of tomato. The aim of this chapter was to isolate potential bacterial and yeast strains from tomato leaves and to screen them *in vitro* for their activity against Pst.

2.2. Materials and Methods

2.2.1. Source of bacterial speck pathogen

The Pst isolate used in this study was provided by Mr Kobus Serfontein (QMS AgriScience (Pty) Ltd, Letsitele, Limpopo, South Africa). The isolate was grown on tryptone soy agar (TSA) and stored in 30% glycerol at -80^{0}C.

2.2.2. Isolation of yeast biocontrol agents

Approximately twenty-five diseased and healthy tomato leaf samples were collected from seedling nurseries and tomato fields around Pietermaritzburg, South Africa. The samples were washed under running tap water for 10 min. The clean leaves were cut into small pieces and a 1.0 g sample was weighed out and immersed in 9.0 ml of sterile distilled water in 50 ml Erlenmeyer flasks. The flasks were placed into a 28°C water bath shaker for 1 h. The suspensions were serially diluted to 10^3. One ml (1 ml) aliquots of 10^{-1}-10^{-3} dilutions were plated on malt extract agar (MEA) supplemented with chloramphenicol (added to slow down the growth of bacterial colonies) (Spurr and Welty, 1975; Larran *et al*., 2001). Presumptive yeast isolates were seen as budding cells under the light microscope after 48 h of incubation at 28^{0}C. Maximum of four single yeast colonies per plate were sub-cultured on TSA and stored at -80°C in 30% glycerol (v/v) (Obradovic *et al*., 2005).

2.2.3. Isolation of bacterial biocontrol agents

Diseased and healthy tomato leaf samples were collected from commercial and subsistence farmers' fields in and around Pietermaritzburg, South Africa. The samples were washed under running tap water for 10 min. The clean leaves were cut into small pieces and a 1.0g sample was weighed out and immersed in 9.0ml of distilled water in 50 ml Erlenmeyer flasks. The flasks were heated in a rotary water bath at 70^{0}C for 20 min to kill all vegetative bacterial cells. The suspensions were serially diluted to 10^3. One ml (1 m*l*) aliquots of 10^1 and 10^3 dilutions were plated on tryptone soy agar (TSA). Plates were incubated at 28^{0}C for 48 h. Maximum of four single bacterial colonies were selected per plate and streaked on TSA. The

TSA plates were incubated at 28^0C for 48 h to obtain pure cultures. Pure cultures were stored at -80^0C in 30% glycerol (v/v) (Obradovic *et al*., 2005).

2.2.4. In vitro screening of bacterial isolates

Suspensions of bacterial isolates and the bacterial speck pathogen was aseptically prepared by picking a colony with the tip of a sterile toothpick into an Eppendorf tube containing 1 ml of sterile distilled water. Approximately 0.5 µl of the pathogen suspension was spread over the surface of TSA plate using a bent glass rod and allowed to dry for 15 min under a laminar flow bench. Three sterile antibiotic paper disks, size 9 mm diameter were placed onto the media surface in a triangular shape at equal distance from each other. Accordingly, 0.1 µl of the different bacterial suspensions were introduced onto the sterile paper disks. Suspension of each bacterial isolate was separately introduced onto a sterile antibiotic paper disc. Hence there were three bacterial isolates per TSA plate. A total of 250 bacterial isolates were tested against the bacterial speck pathogen. The plates were incubated at 28°C for a period of 7 d. On the 7^{th} day, each plate was assessed for antagonistic activity by measuring the diameter of zone of inhibition created by each bacterial isolate. There were three replicate per isolate and the experiment was repeated once. Bacterial isolates with a mean diameter of zone of inhibition greater than 20 mm were selected for secondary screening using the protocol under Section 2.2.4. This was done to confirm the performance of the isolates that were selected for further studies.

The data collected was analysed using analysis of variance (ANOVA) with GenStat (VSN International Ltd, Version 14.1 Edition). Means of zones of inhibition were separated using Fishers least significant difference test at the 5% probability level.

2.2.5 In vitro screening of yeast isolates

The detach-leaf assay method was used for *in vitro* yeast bioassays. Leaves of 3 - 4 week old tomato seedlings (cv. Rodade) were washed under running tap water for 10 min. The leaves were surface sterilised by submerging the leaves in 70% ethanol for 2 min, then rinsed with sterile distilled water (Larran *et al*., 2001). The leaves were then transferred into a 1% sodium hypochlorite solution for a period of 5 min (Abdul-Baki, 1974; Larran *et al*., 2001), then thoroughly rinsed with sterile distilled water. The surface of the leaves was air-dried under the laminar flow in petri dishes until they were completely dry.

Yeast suspensions were made in Eppendorf tubes using the same technique as previously reported under Section 2.2.4. The leaves were placed on water agar (WA) in a 90 mm petri dish on the abaxial surface. A drop of the yeast suspension was placed onto each leaf then gently spread using a bent glass rod. Plates with the inoculated leaves were incubated at 28°C for 24 h. The pathogen was subsequently introduced using the same technique as with the introduction of yeast isolates. The plates were left on a laboratory bench at ambient temperature until disease rating was done on Day 7. The leaf area occupied by speck lesions was estimated using a rating scale described by Irzhansky and Cohen (2006) after modification for bacterial speck disease where Class 0 = no visible symptoms; Class1 = 10% of the total leaf area has speck lesions; Class 2 = 25% of the total leaf area has speck lesions; Class 3 = 50% of the total leaf area has speck lesions; Class 4 = 75% total leaf has speck lesions and Class 5 = 100% of the leaf area has speck lesions. Best isolates were selected based on the class rating.

2.2.6. Electron microscopy analysis of yeast treated tomato leaves

Before pathogen inoculation, six tomato leaf samples per yeast isolate treatment were used for preparation of conventional scanning electron microscopy (SEM) through 1 h fixation in 3% buffered glutaraldehyde, and then washed twice (5 min per each wash) in sodium cacodylate buffer. The leaf samples were dehydrated in graded ethanol [10%, 30%, 50%, 70% and 90% (v/v)] for 10 min respectively. Samples were finally immersed twice for 10 min each time in 100% ethanol. The dehydrated leaf samples were dried using a critical point dryer (Model Quorum K850, Quorum Technologies Ltd, East Sunsex, United Kindom). Each dried tomato sample was mounted onto SEM stubs and sputter coated with gold-palladium (Eiko IB3, Tokyo, Japan) for conventional high vacuum imaging. Samples were then viewed using a Zeiss Evo LS IS VP (Thornwood, New York, USA) scanning electron microscopy.

2.2.7. Molecular identification of selected bacterial isolates from secondary in vitro screening

Bacterial isolates were grown on 10% TSA and incubated at 28^0C for 24 h. Using Milli-Q purified water, 1 m*l* of each bacterial isolate suspensions was made in a 2 m*l* Eppendorf tube. Suspensions were all vortexed and heated at 95°C on a heat block for a period of 15 min.

25 µ*l* reaction volume were made containing the following:

- 1X GoTaq® Flexi buffer
- 1.75 mM $MgCl_2$
- 0.2 mM of each dNTP
- 0.2 µM of Primer containing 16SRNA
    - 16S rRNA gene fragment was used, *Bacillus* specific BacF Forward Primer (Garbeva *et al*., 2003) and Universal 16S rRNA reverse primer
- 1.25 µ*l* of GoTaq® DNA Polymerase
- 2 µ*l* of Template, DNA
- Then Nuclease-free water was used to make-up the final volume of 25 µ*l*.

Control reactions without DNA template were included in each round of amplification. Thermal cycling on the reaction and control reactions was performed as follows:

- Initial denaturation at 94°C for 5 min
- 30 cycles of denaturation at 94°C for 1 min
- Annealing at 65°C for 90 sec
- Extension at 72°C for 2 min
- Final Extension at 72°C for 10 min
- Samples were kept at 4°C overnight.

PCR amplification of the targeted gene fragment (1300bp) was confirmed by agarose gel electrophoresis.

1 % agarose was made by mixing:

- 100 m*l* of TAE Buffer
- 1 g of Agarose gel
- 10 µ*l* Nucleic Acid Gel (Syber Safe)

The mixture was heated for 30 sec in a microwave until it dissolved. The gel was run in a TAE Buffer. Preparations on Parafilm for loading sample into the 1% agarose gel well were done by mixing 2µl of the loading dye, 3µl of the sample, and base pair ladder. This was done for every sample. The electrophoresis was run at 80V for the first 5 min then 100V for 45 min. DNA extractions products were sent to Inqaba Biotechnology Industries (Pty) Ltd (Hatfield, Pretoria, South Africa) for sequences and identification to species level.

2.2.8. Molecular identification of selected yeast isolates from isolate from in vitro *bioassays*

Selected yeast isolated were subcultured on 10% TSA plates and incubated at 28^0C for 24 h. The isolates were then sent to Inqaba Biotechnology Industries (Pty) Ltd (Hatfield, Pretoria, South Africa) for identification to species level.

2.3. Results

2.3.1. In vitro screening of bacterial isolates

Results of the primary and secondary tests *in vitro* screening are presented in Table 2.1. Out of the 250 bacterial isolates that were screened, 31 isolates created zones of inhibition after a period of 7 d post inoculation. During the secondary *in vitro* screening 13 isolates (LN10, LN129, LN33, SC16, LN5, LN17, LN18, LN11, LN35, LN1, LN24, LN16 and LN35) were confirmed for antagonistic activity against Pst with an average diameter of zone of inhibition ranging from 17-29 mm. Significant (P = 0.001) zones of inhibition were observed with Isolates LN10, LN11, LN24 LN17, LN16, LN33, LN1, LN5 and LN35 (Table 2.1).The rest of the isolates that were not inhibitory to the test pathogen were not included in Table 2.1.

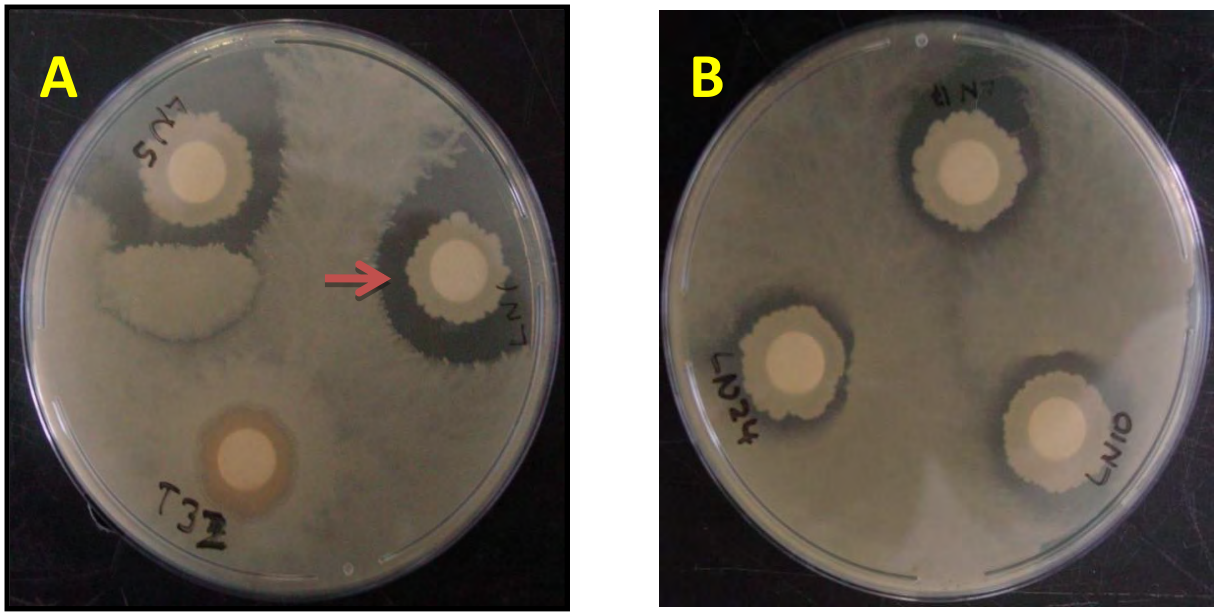


Fig. 2.1: Bacterial isolates LN1, LN5 (Fig.2.1A); LN24, LN10, and LN17 (Fig.2.1B) illustrating antagonist activity against Pst. Isolate T32 (Fig. 2.1A) showed zero antagonistic activity against the bacterial speck pathogen.

Table 2.1: Primary and secondary *in vitro* screening results of bacterial isolates that showed activity against *Pseudomonas syringae* pv. *tomato* on TSA plates.

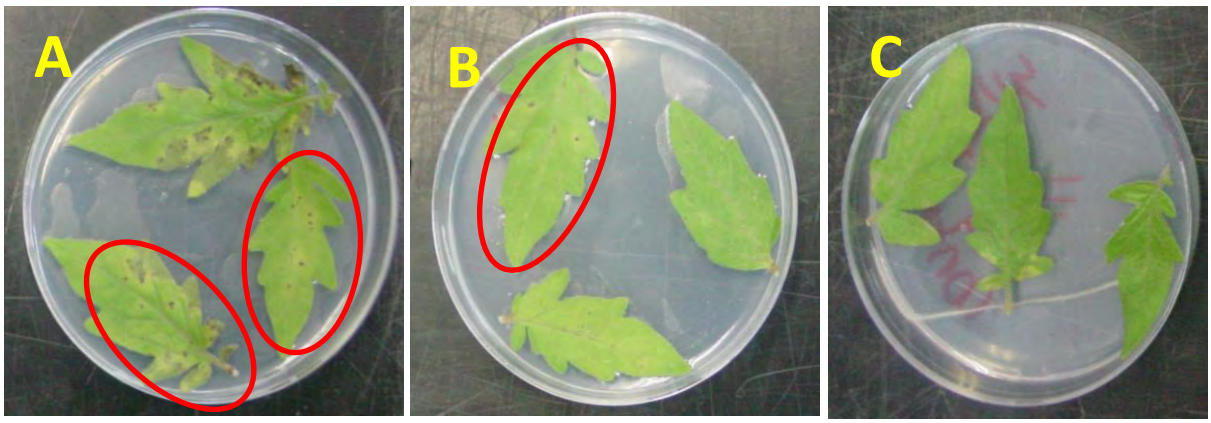
Treatments	Source of the sample	Diameter of the Zone of Inhibition (mm)	
		Primary screening	Secondary screening
LN8	Scottville	9a	-
LN7	Scottville	10ab	-
LN2	Scottville	12.67bc	-
NC13	Camperdown	13.67cd	-
LN129	Scottville	13.67cd	19ab
LN79	Scottville	13.67cd	-
LN90	Scottville	13.67bcd	-
LN62	Scottville	16cde	-
LN63	Scottville	16cde	-
LN61	Scottville	17def	-
LN46	Scottville	17.67ef	-
NC273	Camperdown	18ef	-
NC092	Camperdown	18.33efg	18a
NC093	Camperdown	18.33efg	-
LN11	Scottville	19efg	28.67f
LN112	Scottville	19efg	-
NC094	Camperdown	19efg	-
HPL08	Glenwood	19.33efg	-
LN10	Scottville	20fgh	29f
LN52	Scottville	20fgh	-
LN33	Scottville	22ghi	25de
SC16	Ukulinga	23hij	17.33a
LN5	Scottville	24ijk	21.17bc
LN17	Scottville	24ijk	26.67ef
LN18	Scottville	25ijk	20abc
LN51	Scottville	25ijk	-
LN35	Scottville	25.33ijk	20abc
LN1	Scottville	26jk	22.33cd
LN24	Scottville	26jk	27ef
LN16	Scottville	26.67jk	25.33e
LN25	Scottville	27k	-
cv%		10.4	7.4
lsd		3.281	2.011
sed		1.64	0.98
P-value		10.4	<.001

"-" = Isolates were not selected for secondary screening

** Rest of the 250 bacterial isolates were not included in Table 2.1 because they showed zero activity against the bacterial speck pathogen

2.3.2. In vitro screening of yeast isolates

The results of the detach-leaf assay are presented in Table 2.2. Five yeast isolates; Y1, Y14, Y93, Y94 and IB1 scored a rating of 3. About 50% of the leaf area had speck lesions after a period of 7 d post inoculation with the speck pathogen. Isolates IB7, Y54, and Y21 were rated Class 2 with 25% of the leaf area covered with speck lesions, while Y25 was rated Class 1 with significantly fewer symptoms (Fig 2.2B) visible on the leaf surface.



Pathogen inoculated control | Y25 isolate | Uninoculated control

Fig. 2.2: *In vitro* screening of yeast Isolate Y25, compared to pathogen inoculated control (*Pseudomonas syringae* pv. *tomato*) and uninoculated control 14 d post inoculation using the detach-leaf assay method.

The pathogen inoculated tomato leaves (Fig. 2.2A) developed more speck lesions covering the leaf surface; while leaves treated with the yeast isolate Y25 (Fig. 2.2B) developed ca. 5% of leaf area covered with bacterial speck lesions on one leaf with the rest of the leaves showing no symptoms. The leaves of the uninoculated Control (Fig. 2.2C) developed no bacterial speck lesions.

Table 2.2. *In vitro* screening of yeast isolates against the bacterial speck pathogen in the detach-leaf assay experiment, 14 d after pathogen inoculation.

Yeast Isolate	Class of Rating Index	Comments
Uninoculated control	0	No speck lesions
Pathogen inoculated control	3	Irregular and small circular speck lesions on the leaf edges
Y1	3	Irregular speck lesions on the edges of the leaf
Y14	3	Irregular speck lesions surrounded by halo
Y21	2	Small circular lesions surrounded by halo
Y25	1	Circular lesions surrounded by halo. Only ca. 5% of the leaf area with lesions.
Y93	3	Irregular speck lesions surrounded by halo
Y54	2	Small circular lesions surrounded by halo
Y94	3	Irregular speck lesions on the leaf
IB7	2	Small circular lesions surrounded by halo
IB1	3	Irregular speck lesions surrounded by halo

2.3.3. Electron microscope viewing of yeast treated leaves

Six leaf samples treated with Isolate Y25 were observed under electron microscope for the presence of yeast cells. Electron microscopy images confirmed the presence of yeast cells on the leaf surface (Fig. 2.3). The cells were observed after 48 h post inoculation. This was performed to ensure that we observe the ability of yeast cell of adhering on the surface of the tomato leaves. The experiment was repeated twice.

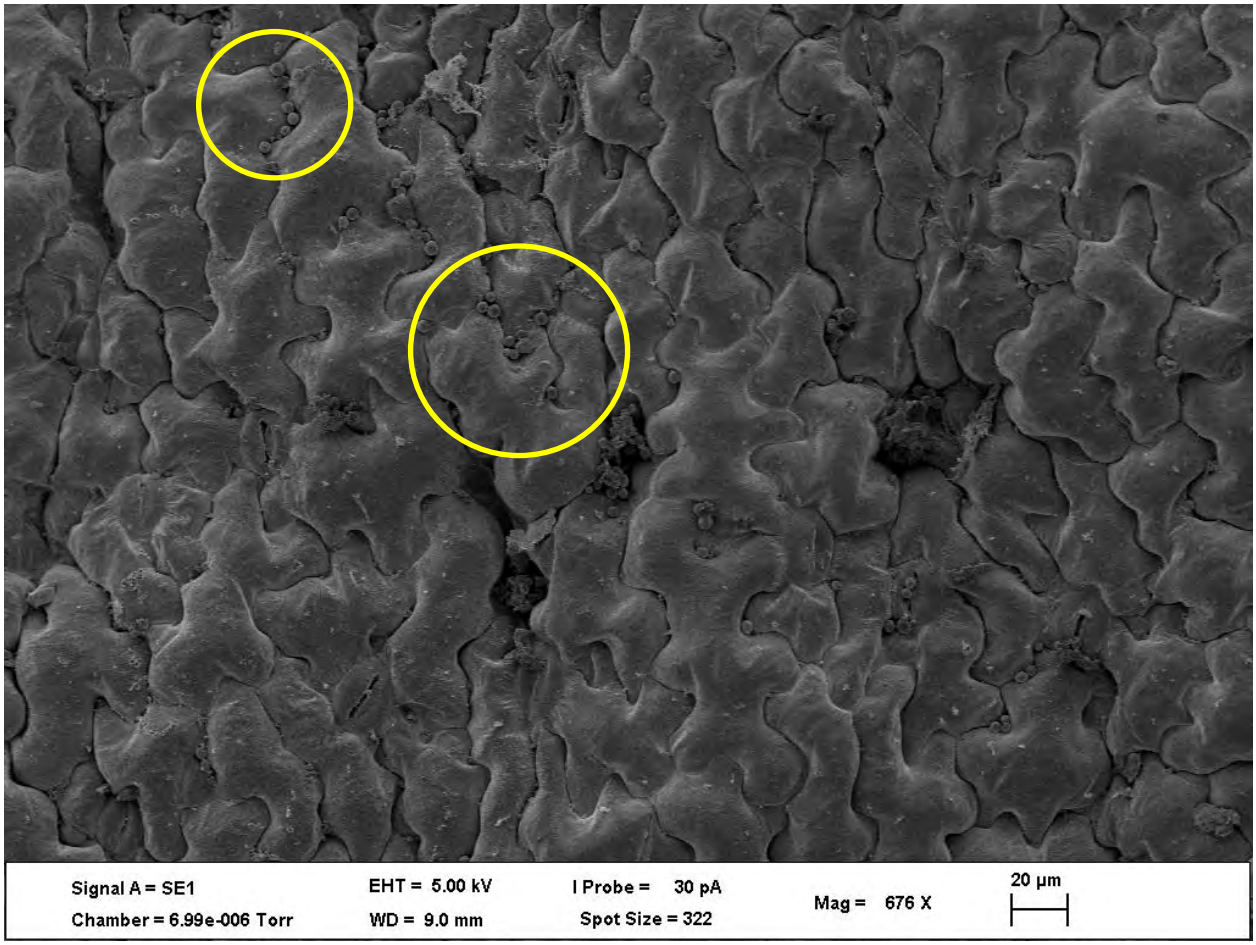
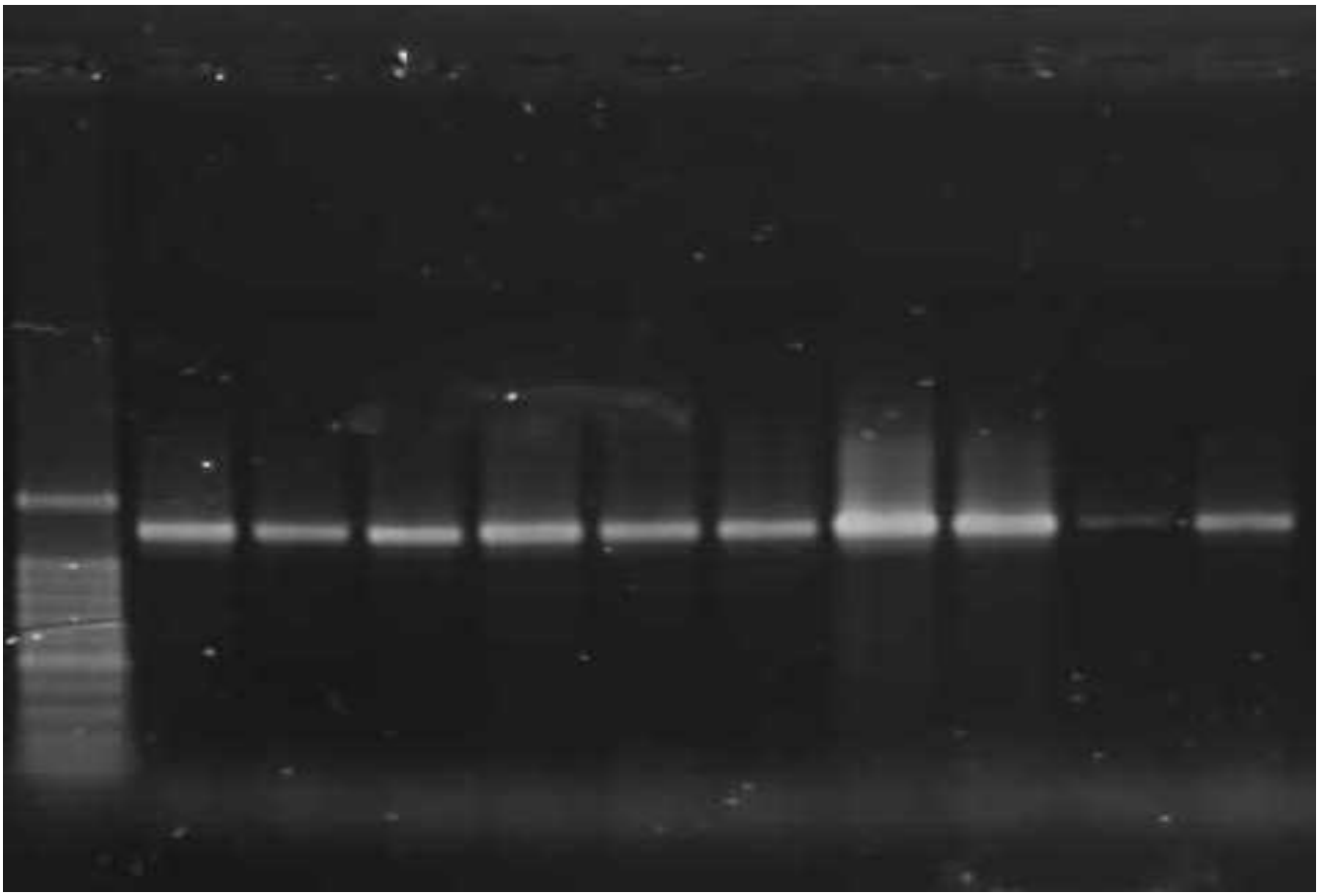


Fig 2.3: Yeast cells present on tomato leaf surface 48 h after treatment before introduction of the bacterial speck pathogen on the leaf.

2.3.4. Molecular identification of selected isolates from in vitro *screening*

The PCR amplification bands of the best ten bacterial isolates are shown in Fig. 2.4. Bacterial isolates LN10, LN16, LN17, LN18 were identified as *Bacillus* sp*;* while LN1 was *Bacillus thuringiensis*, LN5 and LN24 were identified as *Bacillus cereus* (Table 2.3). For the yeast isolates Y1 was identified as *Cryptococcus magnus*, Y14 *Cryptococcus diffluens*, Y21 *Rhodotorula mucilaginosa*, Y25 and Y54 *Rhodorula glutinis*, Y93 *Rhodotorula babjevae* and Y94 as *Rhodosporidium babjevae* (Table 2.3).

MM LN1 LN10 LN5 LN18 LN16 LN17 LN35 LN24 LN33 LN11



Key: MM = molecular marker; LN = bacterial isolates

Fig 2.4: PCR amplification bands for ten unknown bacterial isolates.

The molecular bands confirmed the presence of genetic material that matched the genus *Bacillus*. DNA material for all the 10 bacterial isolate were estimated to be 800bp when compared with the levels of the molecular marker in Lane 1. The size of the molecular marker in Lane 1 is between 100 to 1500 bp.

Table 2.3: Identity of bacterial and yeast isolates that showed activity against *Pseudomonas syringae* pv. *tomato* during *in vitro* studies

Isolate Name	Identified Species	Primer	E-value	% Similarity	Accession Number
Y1	*Cryptococcus magnus*	ITS1	0.0	100	HG532072.1
Y14	*Cryptococcus diffluens*	ITS1	0.0	99	KC152904.1
Y21	*Rhodotorula mucilaginosa*	ITS1	0.0	100	KJ182676.1
Y25	*Rhodotorula glutinis*	ITS1	0.0	100	JQ686831.1
Y54	*Rhodotorula glutinis*	ITS1	0.0	100	EF194846.1
Y93	*Rhodotorula mucilaginosa*	ITS1	0.0	100	KJ183057.1
Y94	*Rhodosporidium mabjevae*	ITS1	0.0	100	JX188219.1
LN1	*Bacillus thuringiensis*	BacF & 16S rRNA	0.0	99	KF921618.1
LN5	*Bacillus cereus*	BacF &16S rRNA	0.0	100	JX130375.1
LN10	*Bacillus* sp	BacF & 16S rRNA	0.0	100	KF848991.1
LN16	*Bacillus* sp	BacF & 16S rRNA	0.0	100	HM566575.1
LN17	*Bacillus* sp	BacF & 16S rRNA	0.0	100	KF848991.1
LN18	*Bacillus* sp	BacF & 16S rRNA	0.0	100	KF848991.1
LN24	*Bacillus cereus*	BacF & 16S rRNA	0.0	100	KJ534497.1
LN33	*Bacillus* sp	BacF & 16S rRNA	0.0	100	KF848987.1
LN35	*Bacillus* sp	Unidentified	Unidentified	Unidentified	Unidentified
LN11	*Bacillus* sp	Unidentified	Unidentified	Unidentified	Unidentified

2.4. Discussion

Results of the *in vitro* screening showed that out of the 250 bacterial isolate screened, only 10 isolates demonstrated significant activity against Pst. Isolate LN10 created the largest zones of inhibition (29 mm) and was selected for further studies under greenhouse conditions. Five other bacterial isolates LN1, LN5, LN24, LN16 and LN17 were also selected for further studies due to the consistency in their performances during the primary and secondary screening.

The zones of inhibition created by the selected bacterial isolates suggest that they were antagonistic to Pst. The bacterial isolates selected for further studies were identified as *Bacillus* spp. Isolates of *Bacillus* spp. has been used as biocontrol agents against several plant diseases (Cawoy *et al*., 2011). These include post-bloom fruit drop of citrus (*Rutaceae* L.) (Sonoda *et al*., 1996); gray mould of strawberries (*Fragaria* x *ananassa*, Duchesne) (Swadling and Jeffries, 1996), yam leaf spot (Michereff *et al*., 1994) and early leaf spot of peanut (*Arachis hypogaea* L.) (Kokalis-Burelle *et al*., 1992). Isolates of *Bacillus* spp. have also been shown to produce a range of antibiotics against different bacterial and fungal pathogens (Abou-Zeid *et al*., 2009; Chen *et al*., 2012). In this study, the bacterial isolates which were identified as *Bacillus* spp. did produce inhibitory substances/compounds by creating zones of inhibition during the *in vitro* primary and secondary screening. Tolba and Soliman (2013) also recommended that *Bacillus* spp. be considered as candidates for the biological control of bacterial plant pathogens.

The *in vitro* screening technique used was effective in selecting isolates with biocontrol potential, although it had some challenges. A similar experiment was carried out on *Bacillus* sp. on *Alternaria alternata* (Fr.) Keiss (1912), where an agar diffusion method was described by Pajand and Paul (2000). The two methods differ through creation of wells in the agar using cork-borer in the agar diffusion technique, whereas in the antibiotic disc bioassay, sterile discs were placed on top of the agar and the potential biocontrol isolate was introduced (Palaksha *et al*., 2010). We chose the antibiotic disc bioassay because it reduced the risk of contamination into the agar surface considering the large number of isolates that were to be screened in the experiment.

The *in vitro* detached-leaf assay screening method was used for screening the yeast isolates whereby 20 out of 100 yeast isolates reduced bacterial speck lesions on the detached tomato leaves. Out of the 20 yeast isolates seven caused significant reduction in the development of

bacterial speck lesions. These isolates were rated between Classes 1-3. The detached-leaf assay was an effective method for screening the yeast isolates against the bacterial speck pathogen. Another screening method to identify biocontrol strains of yeasts that has been reported in the literature is the whole-plant assay. Whole-plant assay is a much more complex screening method in comparison to the detached-leaf assay because it requires so much space, so many plants, and would be difficult to perform sterilization procedures prior to the commencement of the experiment.

For many years *Bacillus* sp. has been used to suppress diseases in different of crops. The commercialized *Bacillus* product, Quantum-4000 (Gustafson Inc. USA) is being used on peanuts and has been available since 1983 (Turner and Bockman, 1991). *Bacillus* strain L324-9 was used to control take-all disease caused by *G. graminis* var. *tritici;* as well as *Bacillus subtillis* strain RB14 which suppressed damping-off of tomato (*Lycopersicon esculentum* L.) caused by *Rhizoctonia solani* Kühn (Asaka and Shoda, 1996). Wilson *et al*. (2002) showed that *Bacillus pumilus* SE34 controlled bacterial speck on tomato plants. The collective disease suppression effect given by *Bacillus* spp as biological control agents on different plant diseases and crops show the possibility that they could have against bacterial speck disease on tomato.

In conclusion, both screening methods allowed the selection of bacterial and yeast isolates as potential biological control agents against the bacterial speck pathogen through the creation of zones of inhibition on agar plates, and the reduction in development of bacterial speck on detached-leaves, respectively. *Bacillus* spp which have been used as biological control agents against many diseases both under greenhouse and field conditions were prominent in the isolates obtained from tomato leaves. It is our hope that the performance of these isolates under laboratory would be replicated under greenhouse conditions and in the nursery.

2.5. Reference

Abdalla, S.A., Algam, S.A.A., Ibrahim, E.A., EL-Naim, A.M. 2014. *In vitro* screening of *Bacillus* isolates for biological control of early blight disease of tomato in Shambat soil. World Journal of Agricultural Research 2, 47-50.

Abdul-Baki, A.A. 1974. Hypochlorite and tissue sterilization. Journal of Plant Biology 115, 373 - 376.

Abou-Zeid, A.M., Altalhi, A.D., Abd El-Fattah, R.E., 2009. Bacterial control of pathogenic fungi isolated from some wild plants in Taif Governorate, Saudi Arabia. Mycopath 7, 11 - 20.

Akhtar, K.P., Saleem, M.Y., Asghar, M., Ali, S., Sarwar, N., Elahi, M.T., 2012. Resistance of *Solanum* species to *Phytophthora Infestans* evaluated in the detached-leaf and whole-plant assays. Pakistan Journal of Botany 44, 1141 - 1146.

Anderson, M., Bollinger, D., Hagler, A., Hartwell, H., Rivers, B., Ward, K., Steck, T.R., 2004. Viable but non-culturable bacteria are present in mouse and human urine specimens. Journal of Clinical Microbiology 42, 753 - 758.

Asaka, O. and Shoda, M. 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Applied Environmental Microbiology 62, 4081-4085.

Barghouthi, S.A., 2011. A universal method for the identification of bacteria based. Indian Journal of Microbiology 51, 430 - 444.

Cawoy, H., Bettiol, W., Fickers, P., Ongena M., 2011. *Bacillus*-based biological control diseases. Pesticides Use and Management.

Chen, N., Jin, M., Qu, H.M., Chen, Z.Q., Chen, Z.L., Qui, Z.G., Wang, X.W., Li, J.W., 2012. Isolation and characterization of *Bacillus* sp. producing broad spectrum antibiotics against human and plant pathogenic fungi. Journal of Microbiology Biotechnology 22, 256 - 263.

Daff, 2010. A profile of the South African tomato market value chain. [http://www.nda.agric.za/docs/amcp/tomatomvcp2010-11.pdf], accessed on the 20th August 2014.

Daff, 2013. A profile of the South African tomato market value chain. [http://www.nda.agric.za/doaDev/sideMenu/Marketing/Annual%20Publications/Commodity%20Profiles/field%20crops/Tomato%20Market%20Value%20Chain%20Profile%202013.pdf], Accessed on the 20th August 2014.

Davis, R. M., Lanini, W.T., Hamilton, G., Spreen, T. H., Osteen. C., 1998. The Importance of Pesticides and Other Pest Management Practices in US Tomato Production. USDA, Document No: 1-CA-98.

Foldes, T., Ba-Ânhegyi, I., Herpai, Z., Varga, L., Szigeti, J., 2000. Isolation of *Bacillus* strains from the rhizosphere of cereals and in vitro screening for antagonism against

phytopathogenic, food-borne pathogenic and spoilage micro-organisms. Journal of Applied Microbiology 89, 840 - 846.

Garbeva, P., van Veen, J.A., Elsas, J.D. (2003). Predominant *Bacillus* spp. in Agricultural Soil under Different Management Regimes Detected via PCR-DCGE. Microbial Ecology 45, 302-316.

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

Howarth, F.G., 1991. Environmental impact of classical biological control. Annual Review of Entomology 36, 485 - 509.

Inacio, J., Flores, O., Martins, I.S., 2008. Efficient identification of clinically relevant *Candida* yeast species by use of an assay combining pan fungal loop-mediated isothermal DNA amplification with hybridization to species-specific oligonucleotide probes. Journal of Clinical Microbiology 46, 713 - 720.

Ippolito, A., El-Ghaouth, A., Wilson, C.L., Wisniewski, M.A., 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biology and Technology 19, 265 - 272.

Irzhansky, I., Cohen, Y., 2006. Inheritance of resistance against *Phytophthora infestans* in *Lycopersicon pimpinellifolium* L3707. Euphytica 149, 309 - 316.

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.

Jijakli, M.H., Grevesse, C., Lepoivre, P., 2001. Modes of action of biocontrol agents of postharvest diseases: challenges and difficulties. Bulletin-Organisation Interrnationale de Lutte Biologique et Integree, Section Regionale Ouest Palearctique 24, 317 - 318.

Jones, J.B., Jones, J.P., Stall, R. E., Zitter, T. A., 1991. Compendium of Tomato Diseases. American Phytopathological Society, St. Paul, MN.

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

Kalogiannis, S., Tjamos, S.E., Tjamos, E.C., Stergiou, A., Antoniou,P.P., Ziogas B.N. (2006). Selection and evaluation of phyllosphere yeasts as biocontrol agents against grey mould of tomato. European Journal of Plant Pathology 116, 69 - 76.

Kokalis-Burelle, N., Backman, P.A., Rodriguez-Kabana, R., Ploper, L.D., 1992. Potential for biological control of early leaf spot of peanut using *Bacillus cereus* and chitin as foliar amendments. Biological Control 2, 321 - 328.

Larran, S., Monaco, C., Alippi, H.E., 2001. Endophytic fungi in leaves of *Lycopersicon esculentum Mill.* World of Journal of Microbiology and Biotechnology 17, 181 - 184.

Leite, R.P., Jones, J.B., Somodi, G.C., Minsavage, G.V., Stall, R.E., 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed by DNA amplification. Plant Diseases 79, 917 - 922.

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 using a plant activator. Plant Disease 85, 481- 488.

Lund, M., Nordentoft, S., Pedersen, K., Madsen, M., 2004. Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR. Journal of Clinical Microbiology 42, 5125 - 5132.

Mareckova, M.S., Cermak, L., Novotna, J., Plhackova, K., Forstova, J., Kopecky, J., 2008. Innovative methods for soil DNA purification tested in soils with widely differing characteristics. Applied Environmental Microbiology 74, 2902 - 2907.

McCarter, S.M., Jones, J.B., Gitaitis, R.D., Smitley, D.R., 1983. Survival of *Pseudomonas syringae* pv. *tomato* in association with tomato seed, soil, host tissue, and epiphytic weed hosts in Georgia. Phytopathology 73, 1393 - 1398.

Michereff, S.J., Silveira, N.S.S., Reis, A., Mariano, R.L.R., 1994. Epiphytic bacteria antagonistic to *Curvularia* leaf spot of yam. Microbiology Ecology 28, 101 - 110.

Mitterer, G., Huber, M., Leidinger, E., Kirisits, C., Lubitz, W., Mueller, M.W., Schmidt, W.M., 2004. Microarray-based identification of bacteria in clinical samples by solid-phase PCR amplification of 23S ribosomal DNA sequences. Journal of Clinical Microbiology 42, 1048 - 1057.

Neu, H.C., Gootz, T.D., 1996. Antimicrobial chemotherapy. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; Chapter11. Available from: [http://www.ncbi.nlm.nih.gov/books/NBK7986/]

Nilsson, H., Taneera, J., Castedal, M., Glatz, E., Olsson, R., Wadstrom, T., 2000. Identification of *Helicobacter pylori* and other *Helicobacter* species by PCR, hybridization and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. Journal of Clinical Microbiology 38, 1072 - 1076.

Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., Guven, K., Iriarte, F. B., 2005. Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. Plant Disease 89, 712 - 716.

Ott, S.J., Musfeldt, M., Ullmann, U., Hampe, J., Schreiber, S., 2004. Quantification of intestinal bacterial populations by real-time PCR with a universal primer set and minor groove binder probes: a global approach to the enteric flora. Journal of Clinical Microbiology 422, 2566 - 2572.

Owen, R.H., Dymock, D., Booth, V., Weightman, A.J., Wade, W.G., 1999. Detection of un-culturable bacteria in periodontal health and disease by PCR. Journal of Clinical Microbiology 37, 1469 - 1473.

Pacheco, C.A., Martelli, I.B., Polydoro, D.A., Schinor, E.H., Pio, M.R., Kupper, K.C., Azevedo, F.A., 2012. Resistance and susceptibility of mandarins and their hybrids to *Alternaria alternata*. Science and Agriculture (69)6, 386 - 389.

Palaksha, M.N., Ahmed, M., Das, S. 2010. Antibacterial activities of garlic extract on streptomycin-resistant *Staphylococcus aureus* and *Escherichia coli* solely and in synergism with streptomycin. Biology 1, 12-15.

Pathma, J., Rahul, G.R., Kamaraj, K.R., Subashri, R., Sakthivel, N., 2011. Secondary metabolite production by bacterial antagonists. Journal of Biological Control 25, 175.

Peng, X., Luo, W., Zhang, J., Wang, S., Lin, S., 2002. Rapid detection of *Shigella* species in environmental sewage by an immune capture PCR with universal primers. Applied Environmental Microbiology 68, 2580 - 2583.

Peralta, I.E., Knapp, S., Spooner, D.M., 2005. New species of wild tomatoes (*Solanum* section *Lycopersicon: Solanaceae*) from Northern Peru. Systematica Botanica 30, 424 - 434.

Perry, D., Slade, H., 1961. Transformation of streptococci to streptomycin resistance. Journal of Bacteriology 83, 443 - 449.

Pitblado, R.E., MacNeill. B.H., 1983. Genetic basis of resistance to *Pseudomonas syringae* pv. *tomato* in field tomatoes. Canadian Journal of Plant Pathology 5, 251 - 255.

Richards, G.P., Watson, M.A., Fankhauser, R.L., Monroe, S.S., 2004. Geno group I and II noroviruses detected in stool samples by real-time reverse transcription-PCR using highly degenerate universal primers. Applied Environmental Microbiology 70, 7179 - 7184.

Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A Review, Biological Control 50, 205 - 221.

Shrestha, A., Kim, B.S., Park. D.H., 2014. Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper. Biocontrol Science and Technology 24, 763 - 779.

Sigal, N., Senez, J.C., Le Gall, J., Sebald, M., 1963. Base composition of the deoxyribonucleic acid of sulfate-reducing bacteria. Journal of Bacteriology 85, 1315 - 1318.

Sijam, K., Chang, C. J., Gitaitis, R. D., 1991. An agar medium for the isolation and identification of *Xanthomonas campestris* pv. *vesicatoria* from seed. Phytopathology 81, 831 - 834.

Sonoda, R.M., Guo, Z.T., Nemec, S., 1996. Effect of spray applications of *Bacillus* subtilis on postbloom fruit drop of citrus. Phytopathology 86, 52 - 53.

Spilker, T., Coenye, T., Vandamme, P., Li-Puma, J.J., 2004. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. Journal of Clinical Microbiology 42, 2074 - 2079.

Spurr Jr, H.W., Welty, R.E., 1975. Characterisation of endophytic fungi in healthy leaves of *Nicotiana* spp. Phytopathology 65, 417 - 422.

Swadling, I., Jeffries, R.P., 1996. Isolation of microbial antagonists for biocontrol of gray mould disease of strawberries. Biocontrol Science and Technology, 6.

Taechowisan, T., Chuaychot, N., Chanaphat, S., Wanbanjob, A., Tantiwachwutikul, P., (2009). Antagonistic effects of *Streptomyces sp*. SRM1 on *Colletotrichum musae.* Biotechnology 8, 86 - 92.

Thomashow, L., Bakker P.A.H.M., 2015. Microbial Control of root pathogenic fungi and oomycetes. Principles of Plant Microbe Interactions, 165 - 173.

Tolba, I.H., Soliman, M. A., 2013. Efficacy of native antagonistic bacterial isolates in biological control of crown gall disease in Egypt. Annals of Agricultural Science 58, 43 - 49.

Turner, J.T. and Backman, P.A. (1991). Factors relating to peanut yield increases after seed treatment with Bacillus subtilis. Plant Disease 75, 347-353.

USDA. (2010). U.S. Tomato Statistics (92010). Pages U.S. and state tomato data include acreage, yield, production, price and value broken out by fresh and processing. Economic Research Service http://usda.mannlib.cornell.edu.

van der Lelie, D., Lesaulnier, C., McCorkle, S., Geets, J., Taghavi, S., Dunn, J., 2006. Use of single-point genome signature tags as a universal tagging method for microbial genome surveys. Applied Environmental Microbiology 72, 2092–2101.

Wilson, C.L., Wisniewski, M.E., 1989. Biological control of postharvest diseases of fruit and vegetables: an emerging technology. Annual Review of Phytopathology 27, 425 - 441.

Wisniewski, M.E., Wilson, C.L., 1992. Biological control of postharvest diseases of fruit and vegetables: recent advances. Horticultural Science 27, 94 - 98.

Xu, M.X., Jeffries, P., Pautasso, M., Jeger, M.J., 2011. Combined use of biocontrol agents to manage plant diseases in theory and practice. Phytopathology 101, 1024 - 1031.

Yasuoka, M.O., (2002). A multiplex polymerase chain reaction– based diagnostic method for bacterial vaginosis. Obstetrics and Gynecology 100, 759 - 764.

Zengeni, R., Giller, K.E., 2007. Effectiveness of indigenous soybean rhizobial isolates to fix nitrogen under field conditions of Zimbabwe. Symbiosis 43, 129 - 135.

CHAPTER THREE

In vivo screening of bacterial and yeast antagonists against *Pseudomonas syringae* pv. *Tomato* and disease suppression on tomato plants

Abstract

Ten bacterial and seven yeast isolates previously selected from *in vitro* studies were evaluated against *Pseudomonas syringae* pv. *tomato* under greenhouse conditions on tomato plants. Two bacterial isolates *Bacillus thuringiensis* LN1 and *Bacillus* sp. LN10 reduced bacterial speck severity, reducing AUDPC units by 25% and 52% in Experiment 1, respectively, and by 51% and 48%, respectively, in Experiment 2 compared to the inoculated Control. Among the yeast isolates, *Cryptococcus diffluens* Y14 and *Rhodotorula glutinis* Y25 consistently reduced bacterial speck severity, reducing AUDPC units by 42% and 95%, respectively, in Experiment 1, and by 58% and 86%, respectively, in Experiment 2. *Bacillus* sp. LN10 and the yeast isolate *Rhodotorula glutinis* Y25 were the most effective biocontrol isolates that successfully suppressed bacterial speck disease of tomato. *Bacillus* sp. LN10 and *Rhodotorula glutinis* Y25 were therefore selected for further studies.

3.1. Introduction

Biological control is a term used to describe the application of microbial antagonists to suppress diseases of plants. The organism that is used to suppress the disease causing pathogenic microorganism is referred to as the biological control agent (BCA) (Pal and Gardener, 2006). These BCAs are reported to be specific in their activity against plant pathogens, and are less toxic and more flexible than chemical pesticides (Koberl *et al*., 2013). Microbial antagonists such as yeast, bacteria and fungi are promising disease management tools and are continuously being investigated by many researchers (Droby, 2006; Korsten, 2006; Sharma *et al*., 2009).

Both natural and artificial microbial antagonists can be used as biological control agents (Sharma *et al*, 2009). Natural microbial antagonists involve the use of microorganism that already exists on the surface of the leaf or fruit that is to be protected (Janisiewicz, 1987; Sabiczewski *et al*., 1996). Artificial microbial organisms are used when an organism from another ecosystem is used to protect a plant against infection (Sharma *et al*., 2009). Although much research has been done on the use of microbial antagonists to manage crop pests and

diseases, the mechanisms by which they control the pathogens' activity has not yet been fully understood in many cases (Sharma *et al*., 2009). Understanding the mode of action helps in developing additional means and procedures to improve their performance and would also assist in selecting the more effective and desirable strains of antagonists (Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992). However, there are several modes of actions that have been suggested to explain biological control activity of microbial antagonists. They include competition for nutrients and space (Ippolito *et al*., 2000; Jijakli *et al*., 2001); production of antibiotics; direct parasitism and induction of resistance in the host plants (Janisiewicz *et al*., 2000; Barkai-Golan, 2001; El-Ghaouth *et al*., 2004).

Due to the concurrent infection of tomato by bacterial speck and spot diseases in many cases, similar strategies to control the two diseases need to be developed. Unfortunately current methods of managing the diseases are unsatisfactory because they do not provide sufficient control of either of the diseases (Marco and Stall, 1983; Vallad *et al*., 2010; Araujo *et al*., 2012). This failure to control bacterial speck satisfactorily is as a result of the inherent inefficiencies of copper as a bactericide, combined with bacterial resistance development to copper based bactericides (Marco and Stall, 1983; Turgut and Basim, 2013). However, tomato cultivars have been developed with resistance to bacterial speck disease (Jones and Jones, 1989; Silva and Lopes, 1995; Turgut and Basim, 2013). In Tanzania, the tomato cultivars Tonquay and BSS436 were reported to be tolerant to bacterial speck (Shenge *et al*., 2007). In Turkey, the speck-resistant tomato cultivars Prenses and Petru were released. However, they are not fully resistant to the disease (Turgut and Basim, 2013). In a study by Turgut and Basim (2013), they showed that the tomato cultivar Ontario 7710 is still resistant against bacterial speck disease. They also found that Atalay, Party, Piccadilly, Tuty, Yeniceri, Petru, and Prenses are cultivars that showed resistance against bacterial speck infection during their study in Turkey.

Biological control of bacterial speck pathogen has been successfully studied in most part of the world. According to Wilson *et al*. (2002), a collection of non-pathogenic bacteria from tomato leaves were screened *in vivo* under greenhouse conditions for their ability to reduce the severity of bacterial speck disease. Bacterial isolate *P. syringae* Cit 7 provided disease reduction of bacterial speck disease by 78%. Generally, *in vivo* screening of potential antagonists selected from *in vitro* screening studies is to confirm the efficacy of the antagonist isolates. In many cases, the biological control agent does not replicate their *in vitro* performance under field conditions. For example, bacterial isolates *P. syringae* strains TLP2

and *P. fluorescens* A506 were not effective in suppressing bacterial speck under greenhouse conditions although they performed very well under *in vitro* conditions (Wilson *et al*., 2002).

Bacillus spp are among the potential antagonistic bacterial isolates that were selected in Chapter Two against bacterial speck of tomato. *Bacillus* sp. is a gram positive singly flagellated rod-shaped and spore formers microorganism that lives under aerobic or facultative anaerobic (Priest, 1993). This microorganism is mostly found in the soil, water and plant body (Gardner, 2004). Most *Bacillus* spp survive as saphrophytes in soil where form inactive spores. *Bacillus* species have several advantages over other bacteria because of its long life shelf which results from their ability to form endospores and the broad spectrum activity of their antibiotics (Cavaglier *et al*., 2005). *Bacillus* species has been used in the agricultural sector for plant growth promotion where it fixes nitrogen and eventually increasing the growth of plants such as sugar beet; and also serves as a biological control agent where it controls roots and shoot diseases of plants (Beneduzi *et al*., 2008).

In this chapter ten bacterial and seven yeast isolates selected from *in vitro* screening studies for antagonistic activity against Pst were tested on tomato plants under greenhouse conditions for their ability to suppress bacterial speck infection.

3.2. Materials and methods

3.2.1. Production of seedlings and greenhouse conditions under in vivo *studies*

All *in vivo* studies under greenhouse conditions were subjected to the temperature of 28°C during the day and 20°C in the night with a relative humidity of 80-95%.

Tomato (*Solanum lycopersicum* L.) seeds (cultivar Rodade) were obtained from McDonalds Seeds (Pty.) Ltd Pietermaritzburg, Republic of South Africa. The seeds were planted in seedling trays filled with commercially prepared composted pine bark (CPB) growing medium (Gromor (Pty) Ltd, Cato Ridge, South Africa). Seedling trays were placed in a greenhouse, and were irrigated once a day until germination and thereafter two times a day until transplanted. Four weeks after germination the seedlings were transplanted into 15 cm diameter pots filled with a composted pine bark potting mix (Gromor), one seedling per pot. The pots with seedlings were placed in a greenhouse for one week to allow them adapt to the new medium. Plants were irrigated two times a day with water containing NPK Starter Grower Fertilizer 2:1:2 (43) plus trace element (Ag-Chem Africa (Pty) Ltd, Pretoria, South Africa).

3.2.2. Biological control efficacy under greenhouse conditions

a. Bacterial isolates

Ten bacterial isolates were streaked onto fresh tryptone soy agar (TSA) plates and incubated at 28^0C for 72 h. Cells of bacterial isolates were harvested by washing the surface of the plates with distilled water using a bent glass rod to dislodge the bacteria from the surface of the agar. Bacterial suspensions were adjusted to $1x10^6$ cells.ml^{-1} using a haemocytometer. The suspensions were sprayed onto the seedlings until run-off and left for 48 h. Plants were then pre-incubated by covering the pots with a transparent plastic bags for 24 h and thereafter sprayed with a Pst suspension prepared in the same manner as the bacterial isolates. The experiment was arranged in a completely randomized design with four replications consisting of single plants per bacterial isolate treatment. A total of 11 treatments were used: 10 bacterial treatments and an untreated, inoculated Control (no bacterial BCA treatment, but pathogen-challenged control). The plants were assessed for disease severity five days after *Pst* treatment over a period of four weeks. The experiment was done twice.

b. Yeast isolates

Suspensions of seven yeast isolates were prepared as described in Section 3.2.2 (a). The suspensions were sprayed onto seedlings in a greenhouse until run-off and then left for 48 h. The plants were further treated in the same manner as described in Section 3.2.2(a). The experiment was arranged in a completely randomised design with four replication consisting of a single plant per yeast isolate. A total of 8 treatments for each experiment were used: seven yeast treatments and an untreated, pathogen inoculated Control (no bacterial BCA treatment, but pathogen-challenged disease control). The plants were assessed for disease severity five days after *Pst* treatment over four weeks. The experiment was done twice.

3.2.3. Disease rating

Disease rating was done according to a Horsfall-Barratt rating scale where 1 = 0%; 2 = 0 – 3%; 3 = 3 – 6%; 4 = 12 – 25%; 5 = 25 – 50%; 6 = 50 – 75%; 7 = 75 – 88%; 8 = 88 – 94%; 9 = 94 – 97%; 10 = 97 – 100% (Horsfall and Barratt, 1945). The plants were assessed for disease severity five days after *Pst* inoculation. Disease severity was rated two times each week for four weeks. All disease severity values were analysed using AUDPC for rate of disease development (Shaner and Finney, 1977) before statistical analysis.

3.2.4. Data analysis

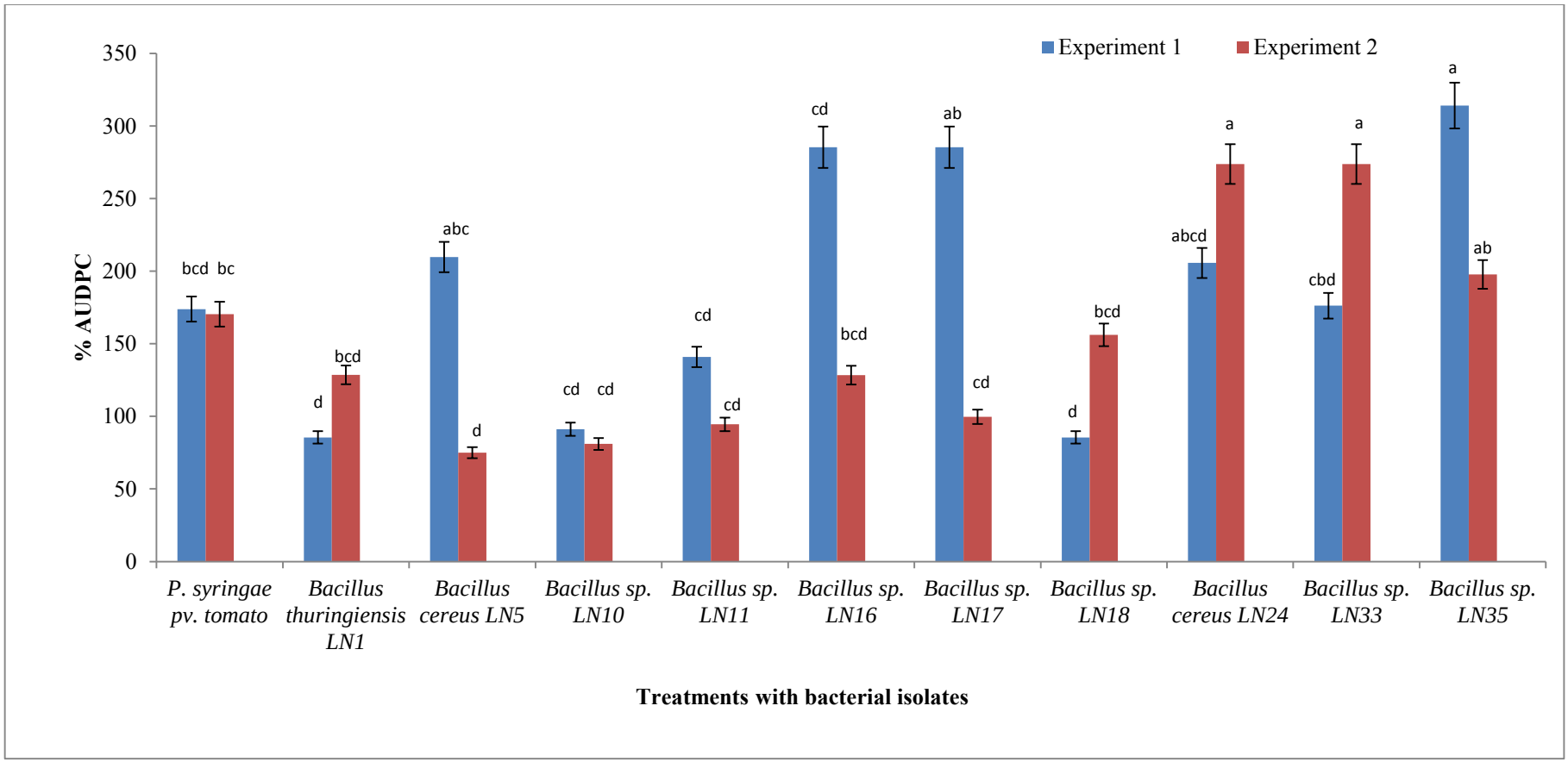
The AUDPC values obtained were subjected to analysis of variance (ANOVA) using GenStat (VSN International Ltd, Version 14.1 Edition). Treatment means were separated using Duncan's multiple range test at the 5% probability level.

3.3. Results

3.3.1. Efficacy of bacterial isolates in reducing bacterial speck severity

The AUDPC graph in (Fig 3.1) illustrate that the best treatments in Experiment 1 are two bacterial isolates *B. thuringiensis* LN1 and *Bacillus* sp. LN10 with reduced AUDPC units by 25% and 52%, and by 51% and 48% in Experiments 1 and 2, respectively, when compared to the inoculated Control treatment (Table 3.1). *Bacillus* sp. LN10 was the only bacterial isolate that showed consistency in both experiments in suppressing bacterial speck development (Table 3.1).

Bacillus thuringiensis LN1 and *Bacillus* sp. LN10 provided significant disease suppression when compared to the inoculated Control in both Experiment 1 and 2 (Table 3.1).



Experiment 1: P-value = 0.0006; F-value = 4.4; CV% = 41.51; Experiment 2: P-value = 0.0001; F-value =6.67; CV% = 35.94

Fig. 3.1: The effect of bacterial biocontrol agents on the development of bacterial speck of tomato under greenhouse conditions.

Table 3.1: Summary of the efficacy of ten bacterial isolates against bacterial speck disease severity on tomato plants grown under greenhouse conditions.

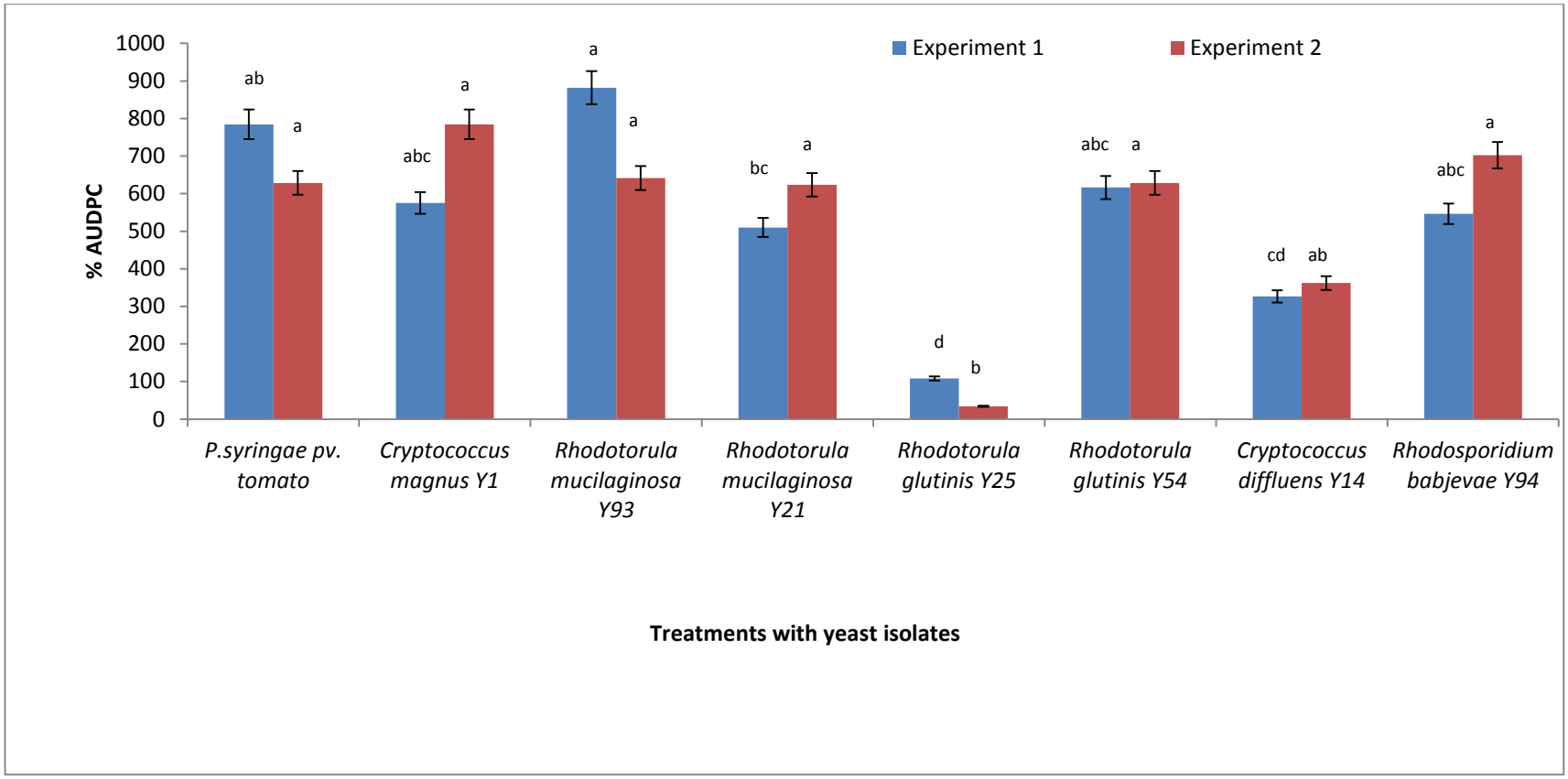
Treatment	Experiment I		Experiment II	
	AUDPC[1,2]	**% AUDPC reduction**	**AUDPC[1,2]**	**% AUDPC Reduction**
P. syringae pv. *tomato* Control	170.44 bc	0	173.94 bcd	0
Bacillus thuringiensis LN1	128.63 bcd	24.53	85.50 d	50.86
Bacillus cereus LN5	75.00 d	56.09	209.75 abc	-20.59
Bacillus sp. LN10	81.00 cd	52.48	91.19 cd	47.57
Bacillus sp. LN11	94.50 cd	44.56	140.94 cd	18.97
Bacillus sp. LN16	128.38 bcd	24.53	285.50 cd	-64.14
Bacillus sp. LN17	99.75 cd	41.48	285.50 ab	-64.14
Bacillus sp. LN18	156.19 bcd	8.36	85.50 d	50.86
Bacillus cereus LN24	273.75 a	-60.61	205.69 abcd	-18.25
Bacillus sp. LN33	273.75 a	-60.61	176.25 cbd	-1.33
Bacillus sp. LN35	197.75 ab	-16.02	314.13 a	-80.85
F – ratio	6.67		4.40	
P – value	0.0001		0.0006	
CV%	35.94		41.51	

[1]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)

[2]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates. % AUDPC = Control-X/Control *100, where X = *P. syringae* pv. *tomato* control.

3.3.2. Efficacy of yeast isolates in reducing bacterial speck severity

The AUDPC graph shows that isolate *Rhodotorula glutinis* - Y25 to be the best control treatment in both Experiment 1 and 2, whilst isolate *Rhodotorula mucilaginosa* Y93 showed minor ability to suppress bacterial speck disease (Fig, 3.2). In Experiment 1, two yeast isolates, *Rhodotorula glutinis* Y25 and *Cryptococcus diffluens* Y14, reduced AUDPC units by 95% and 42% when compared to *P. syringae* pv. *tomato* inoculated control (Table 3.2). In Experiment 2 the two isolates reduced AUDPC units by 86% and 58%, respectively (Table 3.2). These two yeast isolates were the most consistent isolates in reducing bacterial speck severity in the two experiments. In both experiments *Rhodotorula glutinis* Y25 provided significant disease reduction (Table 3.2) when compared to the inoculated control.



Experiment 1: P-value = 0.0302; F-value = 2.75; CV% = 52.77; Experiment 2: P-value = 0.0018; F-value = 4.77; CV% = 41.07

Fig. 3.2: The effect of seven yeast isolates on the development of bacterial speck disease of tomato under greenhouse conditions.

Table 3.2: Summary of the efficacy of seven yeast isolates against bacterial speck severity on tomato plants grown under greenhouse conditions

Treatment	Experiment I		Experiment II	
	AUDPC[3]	%AUDPC reduction	AUDPC[1,2]	% AUDPC Reduction
P. syringae pv. *tomato* Control	628.5 a	0	784.5 ab	0
Cryptococcus magnus Y1	784.5 a	-24.82	575.4 abc	26.65
Cryptococcus diffluens Y14	362.1 ab	42.39	326.6 cd	58.37
Rhodotorula mucilaginosa Y21	623.5 a	0.80	510.1 bc	34.98
Rhodotorula glutinis Y25	33.8 b	94.67	108.2 d	86.21
Rhodotorula glutinis Y54	628.5 a	0.0	616.6 abc	21.40
Rhodotorula mucilaginosa Y93	641.3 a	-2.04	881.9 a	-12.42
Rhodosporidium babjevae Y94	702.4 a	-11.76	546.4 abc	30.35
F – ratio	2.75		4.77	
P – value	0.03		0.002	
CV%	52.7		41.1	

[1]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)

[2]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates. % AUDPC = Control-X/Control *100, where X = *P. syringae* pv. *tomato* control.

3.4. Discussion

In this study, two bacterial isolates (*B. thuringiensis* LN1 and *Bacillus* sp. LN10) and two yeast isolates (*Rhodotorula glutinis* Y25 and *Cryptococcus diffluens* Y14) consistently suppressed bacterial speck disease in two separate greenhouse experiments. Of the bacterial isolates that showed potential against the bacterial speck pathogen during the *in vivo* screening in the greenhouse, *Bacillus* sp. LN10 was the most consistent in suppression of bacterial speck disease. *Rhodotorula glutinis* Y25 was the only yeast isolate that consistently suppressed bacterial speck on tomato plants.

A number of studies have been conducted on the ability of biological control agents to suppress bacterial speck and spot diseases of tomato. In a study by Ji *et al.* (2006), biological controls agents *B. pumilus* SE34 and *P. fluorescens* 89B-61 were applied using seed and root application while *P. syringae* Cit 7 was applied using foliar applications, with disease reductions ranging between 72-74% and 12-56% in Experiments 1 and 2 respectively. Moreover, foliar, seed and root application methods have all been reported to provide consistent and significant disease suppression of bacterial speck disease of tomato under field conditions (Wilson *et al*., 2002; Byrne *et al*., 2005). In this study, the foliar application method was used because isolates were obtained from tomato leaves and stems.

In this study it was observed that foliar application of both *Bacillus* sp. LN10 and *Rhodotorula glutinis* Y25 significantly suppressed the severity of bacterial speck on tomato plants. The possible mechanism of both bacterial and yeast isolates could be their ability to form biofilm on the surface of the leaf (Verstrepen and Klis, 2006), whereby group of cells stick to each other and adheres on a leaf surface using extracellular polymeric substance to adhere to the leaf surface (Ramey *et al*., 2004). Hence the cells are able fend off competition from the pathogen. When yeast cells are introduced onto the leaf surfaces, they ferment all exuded sugar present, then convert sugar to ethanol and carbon dioxide, and then begin adhering to each other (Verstrepen, *et al*., 2003). Biofilm populations protect the plant from infection by utilising all nutrients and waste products found on the leaf surface, through diffusion gradient. This eliminates competition by other microorganisms that arrive on the leaf (Sauer *et al*., 2002).

Many microorganisms have been screened against bacterial speck of tomato including *Bacillus* species and *Pseudomonas* species. In an experiment done by Wilson *et al*., (2002), biocontrol isolates *P. syringae* Strain TLP2 and Cit7, *P. fluorescens* Strain A506, and the mutant Pst DC3000 hrp were screened for their ability to reduce bacterial speck disease severity. Bacterial isolate *P. syringae* Cit 7 was the most effective, providing a disease reduction of 78% under greenhouses condition. In another similar experiment to screen bacterial isolates against bacterial speck and spot diseases, *Bacillus pumilus* SE34 provided significant suppression of bacterial speck under field conditions (Ji *et al*., 2006). To the best of our knowledge there have been no reported studies on the screening of yeasts as potential biological control agents against Pst. However, yeast biocontrol agents have been used against pathogens on a number of other crops.

Therefore, through their ability to reduce the severity of bacterial speck on tomato seedling, isolates *Rhodotorula glutinis* Y25 and *Bacillus* sp. LN10 were selected for further testing as part of an integrated strategy to manage bacterial speck of tomato under both greenhouse and nursery conditions.

3.5. References

Abdalla, S.A., Algam, S.A.A., Ibrahim, A.E., El Naim, M. A., 2014. *In vitro* screening of *Bacillus* isolates for biological control of early blight disease of tomato in Shambat soil. World Journal of Agricultural Research 2, 47-50.

Araujo, E.R., Pereira, R.C., Ferreira, M.A.S.V., Quezado-Duval, A.M., Caf'e-Filho, A.C., 2012. Sensitivity of *Xanthomonads* causing tomato bacterial spot to copper and streptomycin and *in vivo* infra-specific competitive ability in *Xanthomonas perforans* resistant and sensitive to copper. Journal of Plant Pathology 94, 79 – 87.

Barkai-Golan, R., 2001. Postharvest Diseases of Fruit and Vegetables: Development and Control. Elsevier Sciences, Amsterdam, The Netherlands.

Beneduzi, A., Perez, D., Vorgas, K.L., Bodanese-zanettini, H. M., Pasaglia, L.M.P., 2008. Evalution of genetic diversity and plant growth promoting activities of nitrogen fixing bacteria isolates from rice fields in south Brazil. Applied Soil Ecology 39, 311-320.

Bogino, P.C., Oliva, M., Giordano, W., Sorroche, F.G., 2013. The role of bacterial biofilms and surface components in plant-bacterial associations. International Journal of Molecular Science 14, 15838-15842.

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.

Cavaglieri, L. Orlando, J. Rodriquez, M.I., Chutze, S., Etcheverry, M., 2004. Biocontrol bacillus subtillis against Fusarium Verticillioides in vitro and at the Maize root level. Research in Microbiology 156, 748-754.

Coates, L., Johnson, G., 1997. Postharvest diseases of fruits and vegetables. Plant Pathogens and Plant Diseases 33, 534 – 548.

Droby, S., 2006. Improving quality and safety of fresh fruit and vegetables after harvest by the use of biocontrol agents and natural materials. Acta Horticulturae 709, 45-41.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? Postharvest Biology and Technology 52, 137-145.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M.E., 2004. Biologically based alternatives to synthetic fungicides for the postharvest diseases of fruit and vegetables. Diseases of Fruit and Vegetables 2, 511–535.

Gardener, B.B.M., 2004. Ecology of Bacillus and Paenibacillus spp. Agricultural systems symposium. The nature of microbes: Bacillus spp. Phytopathology 94, 1252-1258.

Ippolito, A., El-Ghaouth, A., Wilson, C.L., Wisniewski, M.A., 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biology and Technology 19, 265–272.

Janisiewicz, W.J., Tworkoski, T.J., Sharer, C., 2000. Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients. Phytopathology 90, 1196 - 1200.

Ji, P., Campbell, H.L., Kloepper, J.W., Jones, J.B., Suslow, T.V., Wilson, M., 2006. Integrated biological control of bacterial speck and spot of tomato under field conditions using folia biological control and growth-promoting rhizobacteria. Biological Control 36, 358 – 367.

Jijakli, M.H., Grevesse, C., Lepoivre, P., 2001. Modes of action of biocontrol agents of postharvest diseases: challenges and difficulties. Bulletin-OILB/SROP 24, 317 – 318.

Jones, J.P., Jones, J.B., 1980. Field control of target and bacterial speck of tomato. Proceedings of the Florida State Horticultural Society 101, 358-361.

Koberl, M., Schmidt, R., Ramadan, B., Berg, G., 2013. The microbiome of medicinal plants: diversisty and importance for plant growth, quality and health. Front Microbiology 4, 1-9.

Korsten, L., 2006. Advances in control of postharvest diseases in tropical fresh produce. International Journal of Post-Harvest Technology Innovation 1, 48-61.

Lindow, S.E., Leveau, J.H., 2002. Phyllosphere microbiology. Current Opinion Biotechnology 13, 238-243.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S.V., Machado, M.A., 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular Plant Pathology 13, 618-629.

Pal, K. K., Gardener, B. M., 2006. Biological control of plant pathogens. The plant health Instructor. DOI: 10.1094/PHI-A-2006-1117-02.

Priest, F.G., 1993. Systematics and ecology of bacillus. In bacill subtillis and other gram positive bacteria. Biochemistry, physiology and molecular genetics. Senenshein, A.L. Hoch, J. And Cosick, R. (Editors). American Society of Microbiology, Washington DC, USA, pp 3-16.

Ramey, B.E., Koutsoudis, M., Bodman, S.B., Fuqua, C., 2004. Biofilm formation in plant-microbe associations. Current Opinions in Microbiology 7, 602 - 609.

Rudappa, T., Biedrzycki, M.L., Bias, H.P., 2008. Causes and consequences of plant-associated Biofilms. FEMS Microbiology Ecology 64, 153-166.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., Davies, D.G., 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of Bacteriology 184, 1140 - 1154.

Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biological Control 50, 205 - 221.

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

Sabiczewski, P., Bryk, H., Berezynski, S., 1996. Evaluation of epiphytic bacteria isolated from apple leaves in the control of postharvest apple diseases. Journal of Fruit and Ornamental Research 4, 35-45.

Stall R.E., Thayer P.L., 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. Plant Disease Reporter 46, 389 - 392.

Stoodley P., Sauer K., Davies D. G., Costerton, J.W., 2002 Biofilms as complex differentiated communities. Annual Review of Microbiology 56, 187 - 209.

Sutherland, I.W., 2001. Biofilms exopolysaccharides: A strong and sticky framework. Microbiology 147, 3 - 9.

Turgut, A; 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.

Turkel, S., Korukluoglu, M., Yavuz, M., 2014. Biocontrol activity of the local strain of *Metschnikowia pulcherrima* on different postharvest pathogens. Biotechnology Research International, ID 397167: 6.

Verstrepen, K.J., Derdelinckx, G. Verachtert, H. Delvaux, F.R., 2003. Yeast flocculation: what brewers should know. Applied Microbiology and Biotechnology 61, 197 - 205.

Verstrepen, K.J., Klis, F.M., 2006. Flocculation, adhesion and biofilm formation in yeasts. Molecular Microbiology 60, 5 - 15.

Wilson, C.L., Wisniewski, M.E., 1989. Biological control of postharvest diseases of fruit and vegetables: an emerging technology. Annual Review of Phytopathology 27, 425 – 441.

Wilson, M. 1997. Biocontrol of aerial plant diseases in agriculture and horticulture: current approaches and future prospects. Journal of Industrial Microbiology and Biotechnology 19, 188 – 191.

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

Wisniewski, M.E., Wilson, C.L., 1992. Biological control of postharvest diseases of fruit and vegetables: Recent Advances. Horticultural Science 27, 94 – 98.

Zhang, H., Ge, L., Chen, K., Zhao, L., Zhang, X., 2014. Enhanced biocontrol activity of *Rhodotorula mucilaginosa* cultured in media containing chitosan against postharvest diseases in strawberries: Possible mechanisms underlying the effect. Journal of Agriculture, Food and Chemistry 62, 4214 - 4224.

CHAPTER FOUR

The effect of acibenzolar-S-methyl and didecyl-dimethyl-ammonium chloride on bacterial speck of tomato under greenhouse conditions

Abstract

A range of concentrations of acibenzolar-S-methyl and didecyl-dimethyl-ammonium chloride were evaluated for their ability to control bacterial speck caused by *Pseudomonas syringae* pv. *tomato* under greenhouse conditions in two separate experiments. The disease rating was at an interval of twice per week over a period of four weeks and the experiment was repeated twice. Reduced concentrations [25% (0.018 g l^{-1})] of acibenzolar-S-methyl reduced bacterial speck severity by 81.2% in Experiment 1 and 2, respectively. Increasing the concentrations from 25 – 100 percent of the recommended dose (0.018 – 0.075 gl^{-1}) did not result in a significant increase in disease reduction among the treatments ($P < 0.05$) in both Experiment 1 and 2. However, all four concentrations of acibenzolar-S-methyl significantly reduced the severity of bacterial speck relative to the inoculated Control ($P < 0.05$). For didecyl-dimethyl-ammonium chloride, the use of a reduced concentration [25% (0.19 ml l^{-1})] resulted in a reduction in the severity of bacterial speck by 11% and 15% in both Experiment 1 and 2. Increasing the concentrations from 25% to 100% of the recommended dose (0.19-0.075 ml l^{-1}) only increased disease reduction from 6% to 17% in Experiment 1. In Experiment 2 disease severity actually increased from by 5% as the concentrations increased from 25% to 100% of the recommended dose (0.19-0.75 ml l^{-1}). These were significantly different ($P < 0.05$) from the inoculated Control. The reduced concentrations (25%) for both acibenzolar-S-methyl and didecyl-dimethyl-ammonium chloride control were therefore selected for integrated control experiments in Chapter Five.

4.1. Introduction

Systemic acquired resistance (SAR) is an integrated set of resistance events that are activated by an initial infection by a pathogen, with the objective of limiting the spread of the invading pathogenic microorganism (Choudhary *et al*., 2015). Recognition by plants of a pathogenic attack leads the plant to respond through activating several local responses in the cells surrounding the infection site. These localized responses include the hypersensitive response,

deposition of cellulose, physical thickening of the cell by lignification, synthesis of various antibiotics and proteins. SAR is also associated with an increased level of salicylic acid (SA), both locally and systemically, and the induction of specific set of genes that encode for pathogenesis-related (PR) proteins (Pieterse and Wees, 2014).

Acibenzolar-S-methyl (ASM), with the trade name Bion® in South Africa (Syngenta SA, Ltd, Johannesburg, South Africa), and Actigard® in the United States of America (Syngenta Crop Protection, Greensboro, USA), is a plant activator that induces SAR, and thereby provides protection against a broad spectrum of plant pathogens, including bacterial speck of tomato (Tally *et al*., 1999). Research on ASM has demonstrated that it is effective in controlling a number of plant diseases such as bacterial wilt of Solanaceae (Pradhanang *et al*., 2005), powdery mildew (Gorlach *et al*., 1996), root knot nematodes (Molinari and Baser, 2010) and tomato spotted wilt virus (Csinos *et al*., 2001). Although ASM provides significant suppression of bacterial speck and spot diseases of tomato in the fields, it has been demonstrated to have a harmful impact on plant growth and yield in some cases (Csinos *et al*., 2001; Louws *et al*., 2001; Romero *et al*., 2001). These effects may include severe phytotoxicity, reduced early plant growth and possibly death of plants (Csinos, 2001).

Didecyl-dimethyl-ammonium chloride (DDAC), with the trade name Sporekill®, is an agricultural disinfectant or plant sanitizer that is alleged to be active against some pathogenic microorganisms on plants. Didecyl-dimethyl-ammonium chloride is reported to have high efficacy against *Fusarium oxysporum* f.sp. *cubense (Foc)* that cause fusarium wilt of banana in South Africa (Nel *et al*., 2007). This disinfectant is also effective for cleaning contaminated tools that have been exposed to plant diseases (Nel *et al*., 2007). In South Africa, didecyl-dimethyl-ammonium chloride is also commonly used in the post-harvest sector including for the control of post-harvest diseases of mango fruits (Fourie and Serfontein, 2011).

The main aim of this study was to evaluate the effect of a range of concentrations of ASM and DDAC to control bacterial speck disease on tomato plants.

4.2. Materials and methods

4.2.1. Production of seedlings and greenhouse conditions on in vivo studies

All *in vivo* studies under greenhouse conditions were subjected to mean temperatures of 28°C during the day and 20°C in the night, with a relative humidity of 75-90%.

Tomato (*Solanum lycopersicum* L.) seeds (cultivar Rodade) were obtained from McDonalds Seeds (Pty.) Ltd, Pietermaritzburg, South Africa. The seeds were planted in seedling trays filled with commercially prepared composted pine bark (CPB) growing medium (Gromor, Cato Ridge, South Africa). Seedling trays were placed in a greenhouse, and were irrigated once a day with tap water until germination and thereafter two times a day until transplanted. Four weeks after germination the seedlings were transplanted into 15 cm diameter pots filled with a composted pine bark potting mix (Gromor, Cato Ridge, South Africa), with one seedling per pot. The pots with seedlings were placed in a greenhouse for one week to allow them adapt to the new medium. Plants were irrigated twice a day with a nutrient solution (per litre) containing NPK Starter Grower Fertilizer 2:1:2 (43) (1 g) plus trace element (Agri-Chem Africa (Pty) Ltd, Pretoria, South Africa).

4.2.2. Inoculum preparation

Pseudomonas syringae pv. *tomato* was grown on fresh tryptone soy agar (TSA) plates and incubated at 28^0C for 72 h. Cells of the pathogen were harvested by washing the surface of the plates with sterile distilled water using a bent glass rod to dislodge the bacteria from the surface of the agar. Pathogen suspension was adjusted to $1x10^6$ ml^{-1} using a haemocytometer.

4.2.3. Evaluation of reduced concentrations of acibenzolar-S-methyl under greenhouse conditions

Four concentration of ASM were evaluated in this study. These were: 25% X, 50% X, 75% X, and X, where X ($0.018gl^{-1}$) as the recommended concentration. The treatments were sprayed onto five weeks old tomato plants until run-off and left for 24 h. Plants were then preconditioned by covering them with a transparent plastic bag for 24 h and then sprayed with a suspension of Pst until run-off. The treated plants were covered again with transparent plastics bags for another 24 h. The experiment was arranged in a completely randomized design with six replications consisting of single plants per ASM treatment. A total of five treatments were used: four

different ASM treatments and an inoculated control. ASM treatments were applied every 14 days. The plants were assessed for disease severity five days after *Pst* treatments were applied at an interval of two times a week over a period of four weeks. The experiment was done twice.

4.2.4. Evaluation of didecyl-dimethyl-ammonium-chloride under greenhouse conditions

Four concentration of DDAC were evaluated in this study. These were: 25% X, 50% X, 75% X, and X, where X (0.19 ml l^{-1}) as the recommended concentration. The treatments were sprayed onto five weeks old tomato plants until run-off and left for 24 h. Plants were then preconditioned by covering them with a transparent plastic bags for 24 h, before spraying them with a Pst suspension until run-off . The treated plants were covered again with transparent plastics bags for another 24 h. The experiment was arranged in a completely randomized design with six replications consisting of single plants per DDAC treatment. A total of five treatments were used: four different DDAC concentration treatments and an inoculated Control. The DDAC treatments were applied every 21 days. The plants were assessed for disease severity five days after *Pst* inoculation. Disease severity was rated two times each week for four weeks. The experiment was done twice.

4.2.5. Data analysis

Disease ratings of percentage foliar infection consisted of an estimate of percent area infected (% LAI) using the Horsfall-Barratt Scale (1945) where 1 = 0%; 2 = 0 – 3%; 3 = 3 – 6%; 4 = 12 – 25%; 5 = 25 – 50%; 6 = 50 – 75%; 7 = 75 – 88%; 8 = 88 – 94%; 9 = 94 – 97%; 10 = 97 – 100%. The estimates were used to calculate the Area Under the Disease Progress Curve (AUDPC) for each treatment (Shanner and Finney, 1977). The AUDPC values and the final disease severity values (arcsine transformed) were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc, 2011). Treatment means were separated using Duncan multiple range test at the 5% probability level.

4.3. Results

4.3.1. Disease control by a range of concentrations of acibenzolar-S-methyl under greenhouse conditions

It was observed that the inoculated Control plants developed more disease compared to all the plants treated with ASM at all concentrations, in both experiments. All ASM treatments caused significant disease suppression (P = 0.0001). The 25% concentration (0.018 g l^{-1}) and the 50% concentration (0.038 g l^{-1}) caused an average of 81.2% disease reduction. (Table 4.1; Fig 4.1).

At a 50% concentration (0.038 g l^{-1}), disease reduction was 81.2% in Experiment 1 and 88.73% in Experiment 2. At a 75% concentration (0.056 g l^{-1}) disease reduction was 88.73% and 83.9% in Experiment 1 and 2, respectively. At the recommended concentration, 100 % (0.075 g l^{-1}) gave the lowest level of disease reduction (78.2%) in Experiment 1 and 88.73% in Experiment 2 (Table 4.1; Fig 4.1). The reduced concentrations were as effective as the recommended concentration (Table 4.1).

4.3.2. Different dose response of didecyl-dimethyl-ammonium chloride under greenhouse conditions

The effects of treatments of tomato plants with reduced concentrations of DDAC are shown in Table 4.2 and Fig 4.2. A reduced concentration (25% - 0.19 ml l^{-1}) was the most effective in reducing disease severity after four weeks relative to the inoculated Control (Fig 4.2). There was no significant difference in the level of disease control provided by the different reduced concentration treatments. Treatment with the 25% concentration caused 6.3% and 9.8% reductions of bacterial speck in Experiments 1, and 1 (Table 4.2).

At 50% concentration (0.38 ml l^{-1}) disease reduction by DDAC was 7.61% in Experiment 1 and only 2.50 % in Experiment 2. The 75% concentration of DDAC (0.56 ml l^{-1}) gave a disease reduction of 9.75% in Experiment 1 and only 2.50% in Experiment 2. The 100% concentration (0.75 ml l^{-1}) gave a disease reduction of 17.26% in Experiment 1, but only 2.50 % in Experiment 2. Among the four treatments with DDAC none effectively reduced disease severity on the seedlings after four weeks.

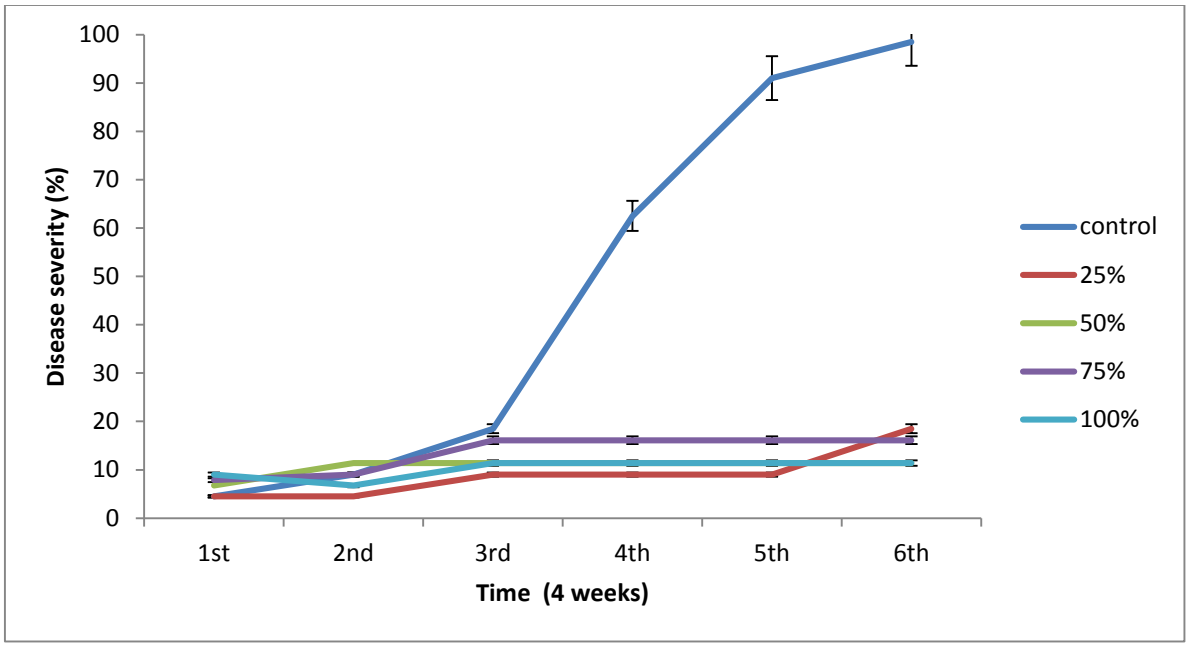


Figure 4.1**:** Effect of four different acibenzolar-S-methyl treatment concentrations on bacterial speck severity under greenhouse conditions.

Table 4.1: A summary of the efficacy of various concentrations (0.019, 0.038, 0.056 and 0.075 g l^{-1}) of acibenzolar-S-methyl treatments application on tomato plants against bacterial speck under greenhouse conditions.

Treatment	Experiment 1			Experiment 2		
	Foliar disease severity[1,2,3]	**% Reduction in speck severity**	**AUDPC[3,4]**	**Foliar disease severity[1,2,3]**	**% Reduction in speck severity**	**AUDPC[3,4]**
Pathogen inoculated control	98.5 a	-	855.25 a	98.5 a	-	839.5 a
25% (0.018 g l^{-1})	18.5 b	81.22	176.5 c	18.5 b	81.22	156.25 c
50% (0.038 g l^{-1})	18.5 b	81.22	176.5 c	11.10 c	88.73	195.5 bc
75% (0.056 g l^{-1})	11.10 b	88.73	191.69 c	15.86 bc	83.9	248.81 b
100% (0.075 g l^{-1})	21.48 b	78.2	399.5 b	11.10 c	88.73	187.19 bc
F – ratio	90.27		23.46	311.53		132.59
P – value	0.0001		0.0001	0.0001		0.0001
CV%	15.30		33.58	9.09		15.44
Contrast						
Control vs 25%	<0.0001		<0.0001	<0.0001		<0.0001
Control vs 50%	<0.0001		<0.0001	<0.0001		<0.0001
Control vs 75%	<0.0001		<0.0001	<0.0001		<0.0001
100% vs 25%	0.5935		0.0197	0.0125		0.3976
100% vs 50%	0.5939		0.0197	1.0000		0.8182
100% vs 75%	0.0551		0.0280	0.0879		0.1033
100% vs Control	<0.0001		<0.0001	<0.0001		<0.0001
25% vs 50%	1.0000		1.0000	0.0152		0.2867
25% vs 75%	0.1456		0.8613	0.3757		0.0199
50% vs 75%	0.1456		0.8613	0.0879		0.1542

[1]Visual ratings of foliar disease severity (0 – 100) using Horsfall-Barratt scale. Numbers are arcsine transformed.

[2]Ratings made on whole plant at four weeks after inoculation with *Pseudomonas syringae* pv. *tomato* [1 = 0%; 2 = 0 – 3%; 3 = 3 – 6%; 4 = 12 – 25%; 5 = 25 – 50%; 6 = 50 – 75%; 7 = 75 – 88%; 8 = 88 – 94%; 9 = 94 – 97%; 10 = 97 – 100%]

[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan multiple range test (DMRT)

[4]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates

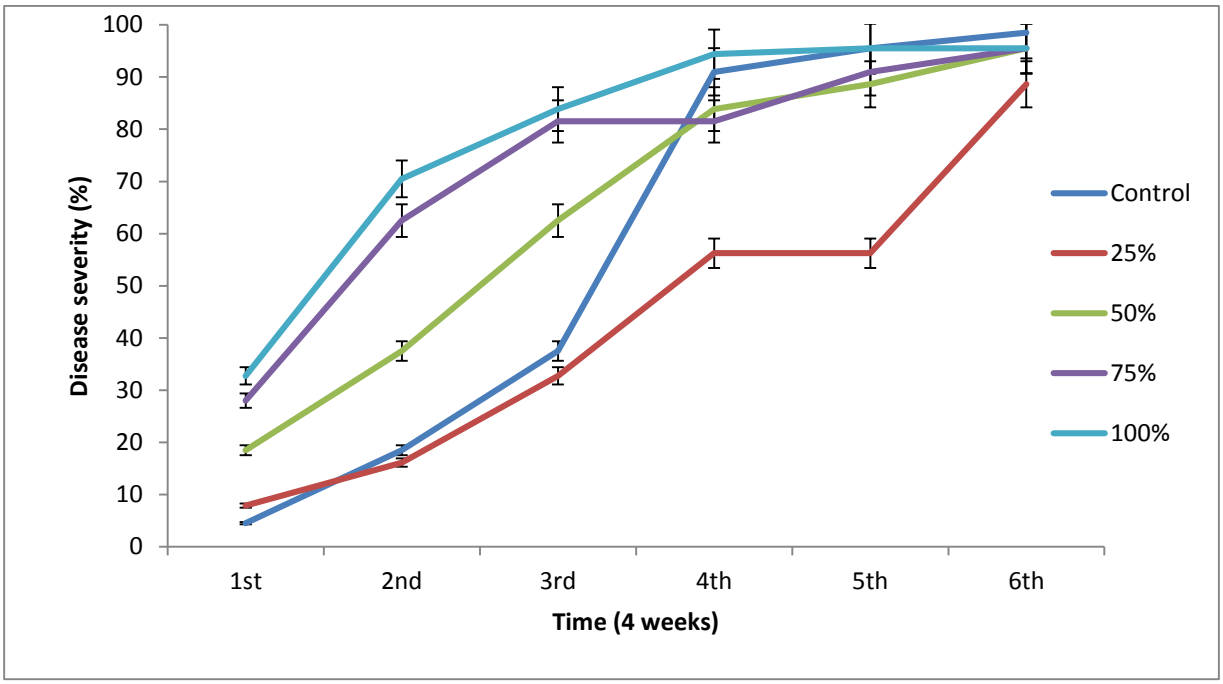


Figure 4.2: Effect of four different of didecyl-dimethyl-ammonium chloride treatment concentration on bacterial speck severity under greenhouse conditions.

Table 4.2: Summary of the efficacy of different concentrations (0.19, 0.38, 0,56 and 0.75 ml l^{-1}) of didecyl-dimethyl-ammonium chloride treatments application on tomato plants against bacterial speck disease under greenhouse conditions.

Treatment	Experiment 1			Experiment 2		
	Foliar disease severity[1,2,3]	**% reduction in speck severity**	**AUDPC[3,4]**	**Foliar disease severity[1,2,3]**	**% reduction in speck severity**	**AUDPC[3,4]**
Pathogen inoculated control	98.5 a	-	872.75 b	98.5 a	-	1054.8 b
25% (0.19 ml l^{-1})	92.26 b	6.34	861.5 b	88.9 c	9.75	812.06 c
50% (0.38 ml l^{-1})	91 b	7.61	1139.5 a	96.04 b	2.50	1198.4 b
75% (0.56 ml l^{-1})	88.9 b	9.75	1245 a	96.04 b	2.50	1388 a
100% (0.75 ml l^{-1})	81.5 c	17.26	1216.5 a	96.04 b	2.50	1461.4 a
F – ratio	38.95		12.34	24.40		24.53
P – value	0.0001		0.0001	0.0001		0.0001
CV%	2.93		9.96	2.32		8.93
Contrast						
Control vs 25%	0.0001		0.8830	0.0001		0.0054
Control vs 50%	0.0001		0.0029	0.0009		0.0737
Control vs 75%	0.0001		0.0002	0.0009		0.0005
100% vs 25%	0.0001		0.0003	0.0001		0.0001
100% vs 50%	0.0001		0.3216	1.0000		0.0031
100% vs 75%	0.0012		0.7097	1.0000		0.3416
100% vs Control	0.0001		0.0004	0.0009		0.0001
25% vs 50%	0.4025		0.0021	0.0001		0.0001
25% vs 75%	0.0450		0.0001	0.0001		0.0001
50% vs 75%	0.2048		0.1806	1.0000		0.0227

[1]Visual ratings of foliar disease severity (0 – 100) using Horsfall-Barratt scale. Numbers are arcsine transformed.

[2]Ratings made on whole plant at four weeks after inoculation with *Pseudomonas syringae* pv. *tomato* [1 = 0%; 2 = 0 – 3%; 3 = 3 – 6%; 4 = 12 – 25%; 5 = 25 – 50%; 6 = 50 – 75%; 7 = 75 – 88%; 8 = 88 – 94%; 9 = 94 – 97%; 10 = 97 – 100%, 11 = 100%]

[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan multiple range test (DMRT)

[4]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment data.

4.4. Discussion

Management of bacterial speck disease of tomato continues to be a challenge for tomato growers worldwide. Diligent application of effective preventative programs targeting the various sources of *Pst* and the implementation of control measures before symptoms could provide effective management of bacterial speck of tomato plants (McGrath and Smart, 2009; Rhoade, 2015). Fortunately, the products ASM (Obradovic *et al*., 2005; Haung *et al.,* 2012) and DDAC (Serfontein, 2014) provide relatively effective products for the management of bacterial speck disease of tomato.

In this study, our overall results consistently found that applications of ASM significantly reduced bacterial speck disease severity and AUDPC units. All four of the ASM concentrations reduced AUDPC and the final bacterial speck severity levels ranging by 78 to 88% in the two experiments.

Graves and Alexander (2002) found that ASM (10g a.i. ha^{-1}) applied at 7-10 days intervals significantly controlled bacterial speck on tomato under field conditions Our results suggest that the rate recommended by the manufacturer may not be necessary to manage bacterial speck because a concentration as low as 2.343g a.i.ha^{-1} was enough to control the disease, and it provided the highest level of control. Therefore, this concentration of 25% of the recommended dose (0.019 g l^{-1} or 2.343 a.i. ha^{-1}) was chosen the optimum dose of ASM to be included in the integrated management study in Chapter Five.

The 25 % concentration of DDAC significantly reduced bacterial speck disease when compared with inoculated Control. However, none of the DDAC concentrations effectively controlled bacterial speck, (2.5 – 17 percent reduction in disease levels) in the two experiments conducted.

DDAC is best described as a quaternary ammonium compound that is a disinfectant that has been adapted for use on a variety of plants both pre- and post-harvest (Serfontein, 2014). In our study the tomato plants were first treated with the four concentrations of DDAC before inoculating with Pst. The treatments were done in this order because DDAC has no curative action or residual presence, and works by killing the target pathogens on the surface of plant leaves though disrupting the bacterial cell membranes, which causes cell leakage and death (Serfontein, 2014, Yoshimatsu and Hiyama, 2007). Our aim was to observe the effect of DDAC on the severity of Pst infection after the plant leaves were surface sterilised. High

levels of disease severity compared to the control with no DDAC were observed, which indicates that DDAC successfully sanitized the leaf surface and eliminating the microflora population on the leaf surface. This therefore creates less competition on the leaf surface for the pathogen. According to Serfontein (2014) tomato plants treated with DDAC had almost zero *Pst* cells on the leaf in two experiments. This is because in his experiments the DDAC treatments were applied shortly after inoculation of tomato plant with *Pst*. As a result, the DDAC treatments killed all the *Pst* cells. The two contrasting results reflect different experimental conditions, and the question is which of these reflects the reality of the epidemiology of bacterial speck in nurseries and farms on tomato crops.

In conclusion, a reduced concentration of 25% of the recommended concentration of ASM and DDAC was the most effective dose for these two compounds. This concentration was therefore chosen for further testing as part of an integrated strategy to manage bacterial speck of tomato under both greenhouse and nursery conditions, presented in Chapter Five.

4.5 References

Choudhary, D.K., Kasotia, A., Jain, S. Vaishnav, A., Kumari, S. Sharma, K. P., Varma,A., 2015. Bacterial-mediated tolerance and resistance to plants under abiotic and biotic stresses. Journal of Plant Growth Regulation , DOI: 10.1007/s00344-015-9521-x

Csinos, A. S., Pappu, H. R., McPherson, R. M., Stephenson, M. G., 2001. Management of tomato spotted wilt virus in flue-cured tobacco with acibenzolar-S-methyl and imidacloprid. Plant Disease 85, 292-29.

Fourie, A., Serfontein, J.J., 2011. The effect of post-harvest, packhouse treatment of Sporekill as part of a mango packline on lenticel damage. Mango Grower Association Research Journal 31, 45-50.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.-H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H., Ryals, J. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8, 629- 643.

Graves, A.S., Alexander, S.A., 2002. Managing bacterial speck and spot of tomato with acibenzolar-S-methyl in Virginia. Plant Health Progress 10.1094/PHP-2002-0220-01-RS.

Horsfall, J. G., Barratt, R. W., 1945. An improved grading system for measuring plant disease. Phytopathology 35, 665.

Huang, C.-H., Vallad, G. E., Zhang, S., Wen, A., Balogh, B., Figueiredo, J. F. L., Behlau, F., Jones, J. B., Momol, M. T., Olson, S. M., 2012. Effect of application frequency and reduced rates of acibenzolar-S-methyl on the field efficacy of induced resistance against bacterial spot on tomato. Plant Disease 96, 221-227.

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 using a plant activator. Plant Disease 85, 481-488.

McGrath, M.T., Smart, C., 2009 Unknown. Managing bacterial diseases of tomato in the field. [http://vegetablemdonline.ppath.cornell.edu/NewsArticles/Tom_Bacter_06.html]: Accessed on 18th August 2015.

Molinari, S., Baser, N. 2010. Induction of resistance to root-knot nematodes by SAR elicitors in tomato. Crop Protection 29, 1354-1362.

Nel, B., Steinberg, C., Labuschagne, N., Viljoen, A. 2007. Evaluation of fungicides and sterilants for potential application in the management of Fusarium wilt of banana. Crop Protection 26,697-705.

Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., Guven, K., Iriarte, F. B. 2005. Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. Plant Disease. 89, 712-716.

Pieterse, C.M.J., Wees, S.C.M., 2014. Induced disease resistance. In, Principles of Plant-Microbe Interactions. B. Lugtenberg, Ed. Springer, Heidelberg, Germany.

Pradhanang, P. M., Ji, P., Momol, M. T., Olson, S. M., Mayfield, J. L., Jones, J. B., 2005. Application of acibenzolar-S-methyl enhances host resistance in tomato against *Ralstonia solanacearum*. Plant Disease 89, 989-993.

Rhoades, H. 2015. Bacterial speck identification and tips for control for bacterial speck on tomato plants. [http://www.gardeningknowhow.com/edible/vegetables/tomato/tomato-bacterial-speck.htm]: Accessed on 18th August 2015.

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.

SAS Institute Inc., 2011. SAS® 9.3 System Opinion: Reference. Second Edition. Cary, NC. USA.

Serfontein, K., 2014. Sporekill still relevant after two decades. [https://agrosifu.files.wordpress.com/2014/11/hygrotech__spring-forum_2014_pg-28-29-1.pdf]: Accessed on 18th August 2015.

Shaner, G., Finney, R.E., 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67, 1051 – 1056.

Tally, A., Oostendorp, M., Lawton, K., Staub, T., Bassi, B. 1999. Commercial development of elicitors of induced resistance to pathogens. In: Induced Plant Defenses against Pathogens and Herbivores. Biochemistry, Ecology, and Agriculture. A. A. Agrawal, S. Tuzun, and E. Bent, Eds. American Phytopathological Society, St. Paul, MN, 357-369

Yoshimatsu, T., Hiyama, K., 2007. Mechanism of the action of dodecyl-dimethyl-ammonium chloride (DDAC) against *Escherichia coli* and morphological changes of the cells. Biocontrol Science 12, 93 -99.

CHAPTER FIVE

Integration of biological control agents, a plant defence activator and a plant sanitizer against bacterial speck of tomato under greenhouse and nursery conditions

Abstract

A plant defence activator, acibenzolar-S-methyl (ASM), a plant sanitizer, didecyl-dimethyl-ammonium chloride (DDAC), and two biological control agents, *Bacillus* sp. LN10 and *Rhodotorula glutinis* Y25, and their combinations were evaluated against bacterial speck of tomato under greenhouse and nursery conditions. All treatments were applied as foliar sprays. Individual application of a 25% dose of ASM was the only treatment that significantly (P = 0.0001) reduced bacterial speck severity under both greenhouse and nursery conditions. All combination treatments that included 25% ASM with either 25 % DDAC, *Bacillus* sp. LN10 or *R. glutinis* Y25 resulted in a significant reduction of bacterial speck severity under both greenhouse and nursery conditions. The combination treatment of 25% ASM + *Bacillus* sp. LN10 + *R. glutinis* Y25 resulted in disease suppression of 99.0% and 92.62% in the greenhouse and nursery, respectively. Without 25% acibenzolar-S-methyl, the various combination treatments such as 25% DDAC + *R. glutinis* Y25 + *Bacillus* sp. LN10 were not effective in either the greenhouse or the nursery. The chemical control, using a standard copper bactericide, did not deliver a significant reduction of bacterial speck in the greenhouse or the nursery.

5.1. Introduction

Bacterial speck is an economically important bacterial disease in many tomato-growing regions worldwide, and is caused by the pathogen *Pseudomonas syringae* pv *tomato* (Pst) (Ji *et al*., 2006). Early infection of tomato plants, which is favoured by cool and humid conditions, results in fast disease progression, which leads to a loss of photosynthetic capacity in infected foliage (Wilson *et al*., 2002, Ji *et al*, 2006). The consequence of this is a reduction in fruit yield and quality with reduced market value. The largest economic losses resulting from bacterial speck occur in the tomato seed and seedling industry as a result of the

rejection of seed or seedlings that are contaminated or infected by the pathogen, Pst (Turgut and Basim, 2013).

Cultural practices such as crop rotation, sanitation programs and weed control do not provide sufficient control of bacterial speck and therefore they have not been implemented by most commercial growers. The most common practice to control bacterial speck is the use of copper based bactericides. However, these products are inherently ineffective because they are non-persistent, contact bactericides that are easily washed off tomato leaves. They have become less effective because of the development of copper resistance in the pathogen population in many tomato-growing regions (Obradovic *et al*., 2005). The use of copper bactericides also poses risk to human health due to their mammalian toxicity (Balestra *et al*., 2009). Frequent applications cause phytotoxicity in tomato crops. They also result in high levels of copper residues because not many growers respect the post chemical application waiting period before harvest (Granata *et al*., 2011; da Silva *et al*., 2014). Therefore, there is an urgent need to develop effective, alternative strategies to control bacterial speck disease of tomato. These methods include the use of biological control, plant resistance inducers (e.g. acibenzolar-S-methyl) and plant sanitizers (e.g. didecyl dimethyl ammonium chloride).

Biological control may provide a control tool to complement or replace the traditional chemical approach to managing bacterial diseases. Bacterial biological control agents have been studied as a possible alternative/strategy for managing bacterial speck of tomato. Examples of such bacterial agents include isolates of the genera *Bacillus* and *Pseudomonas* (Wilson *et al*., 2002, Ji *et al*., 2006). Even though there have been no studies on the use of yeasts to control bacterial speck of tomato, yeast isolates have been shown to control *Botrytis cinerea* on grapes (*Vitis vinifera* L.) (Per.:Fr) and *Penicillium* spp. infections of strawberry [*Fragaria* × *ananassa* (Weston) Duchesne ex Rozier (pro sp.)] (Coates and Johnson, 1997; Wilson, 1997; Zhang *et al*., 2014). In biological control the mechanisms of control include competition for nutrients and space (Ippolito *et al*., 2000; Jijakli *et al*., 2001), production of antibiotics, direct parasitism and the induction of systemic resistance in the plants (Janisiewicz *et al*., 2000; Barkai-Golan, 2001; El-Ghaouth *et al*., 2004). Thus far, there is evidence that biological control agents could reduce bacterial speck severity when used alone. For example, a non-pathogenic strain of *Pseudomonas syringae* Cit7 significantly reduced bacterial speck disease by 78% and 28% under greenhouse and field conditions, respectively (Wilson *et al*., 2002). Although this biological control agent could not be combined with copper bactericides due to its sensitivity to copper, it was suggested that there

is a potential for integration with plant defence activators which are not active against bacteria (Wilson *et al*., 2002).

Acibenzolar-S-methyl (ASM) is a plant defence activator that provides protection to many crops against a broad spectrum of plant pathogens, including bacterial speck of tomato (Tally *et al.,* 1999). Important diseases of tomato such as bacterial wilt [*Ralstonia solanacearum* (race 1)], bacterial speck (*P. syringae* pv. *tomato*) and bacterial spot (*Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas vesicatoria*) and tomato spotted wilt (Csinos *et al*., 2001) have been effectively controlled by ASM under greenhouses and field conditions (Anita *et al.,* 2004; Ji *et al.,* 2006). ASM is a chemical compound that triggers systemic acquired resistance (SAR) in treated plants. SAR is a natural defence mechanism in plants that is activated by pathogens to protect the plants against a broad range of pathogenic infections (Kuc, 2001; Oostendrop *et al*., 2001; Anith *et al*., 2004). The remarkable value of ASM is that of being able to contribute to an enhanced suppression of various diseases when it is integrated with other control strategies such as biological control agents or copper based bactericides (Anith *et al*., 2004; da Silva et al., 2014).

Didecyl-dimethyl-ammonium chloride (DDAC) is a quaternary ammonium compound (QAC) disinfectant that is commonly used to manage post-harvest diseases of mango fruits. According to Serfontein (2014), there are a number of studies that are in progress on DDAC worldwide. He reported that DDAC was found to be as effective as copper for the control of leaf curl (*Taphrina deformans* (Berk.) Tul.) of peaches [*Prunus persica* (L.) Batsch] when applied during conditions that our favourable for infection. This agricultural disinfectant is reported to give a better control of diseases when combined with copper based agrochemicals (Serfontein, 2014).

Integration of biocontrol agents with the conventional disease management practices present new opportunities in plant protection and could provide for more efficient disease management practices (Obradovic, *et al*., 2005). Integration of different disease control practices is important in order to achieve satisfactory disease suppression (Ji *et al*., 2006, Obradovic *et al*., 2005). This concept has lead researchers to improve the level of disease control provided by individual biocontrol agents through using mixtures of biological control agents. A number of biocontrol mechanisms may function in a mixed population of biological control agents (Elad *et al*. 1993; Guetsky *et al*., 2001; Xu *et al*., 2010). A combination of two biological control agents may provide increased (Le Floch *et al*., 2009; Yobo *et al*., 2010),

reduced (Bora *et al*., 2004; Elliott *et al*., 2009) or similar (Janousek et *al*., 2009; Dooley and Beckstead 2010) levels of biocontrol when compared to individual applications. Therefore, the combination of two or more biological control agents will not always result in increased efficacy when compared to the efficacy of the individual agents.

The aim of this chapter was to determine whether the control of bacterial speck of tomato could be enhanced through combined use of selected biological control agents, a plant defence activator and a plant sanitizer, under both greenhouse and nursery conditions.

5.2. Method and material

5.2.1. Biological control agents, plant defence activator and plant sanitizer

The biological control agents used in this study were selected based on their performance during previous greenhouse studies (Chapter Three). These were *Bacillus* sp. LN10 and the yeast isolate *Rhodotorula glutinis* Y25.

A plant defence activator, acibenzolar-S-methyl (ASM), and a plant sanitizer, didecyl-dimethyl-ammonium chloride (DDAC), were used in this study. The concentrations used for each was selected based on the prior studies carried out under greenhouse conditions on bacterial speck of tomato (Chapter Four).

5.2.2. Production of seedlings and greenhouse conditions

All greenhouse bioassays were treated under the same conditions as described in this section. Greenhouse experiments were subjected to the temperature of 25˚C during the day and 20˚C at the night, with a relative humidity of 75-90%.

Tomato (*Solanum lycopersicum* L.) seeds (cultivar Rodade) were obtained from McDonalds Seed (Pty.) Ltd, Pietermaritzburg, South Africa. The seeds were planted in seedling trays filled with composted pine bark (CPB) medium. (Gromor, Cato Ridge South Africa). Seedling trays were placed in a greenhouse and were irrigated once a day with tap water until germination and thereafter twice a day until transplanted. Four weeks after germination the seedlings were then transplanted into 15 cm diameter pots filled with potting mix growth medium (Gromor, Cato Ridge, South Africa), one seedling per pot. The pots with seedlings were placed in a greenhouse for one week to allow them adapt to the new medium. Plants were irrigated twice a day with nutrient solution containing NPK Starter Grower Fertilizer 2:1:2 (43) (1 g) plus trace elements [Agri-Chem Africa (Pty) Ltd., Pretoria, South Africa].

5.2.3. Inoculum preparations of Pseudomonas syringae *pv.* tomato *for greenhouse and nursery experiments*

A pathogenic strain of *Pst*, previously isolated, was grown on fresh tryptone soy agar (TSA) plates and incubated at 28°C for 72 h. Cells of the pathogen were harvested by washing the surface of the plates with sterile distilled water using a bent glass rod to dislodge the bacteria from the surface of the agar. The cell concentration was adjusted to $1x10^6$ ml^{-1} using a haemocytometer.

5.2.4. Treatments preparations for greenhouse and nursery experiments

a. Inoculum preparation of biological control agents

Rhodotorula glutinis Y25 and *Bacillus sp.* LN10 were aseptically-cultured on tryptone soy agar (TSA) plates for 48 h at 28 °C. Cells of the biological control agents were harvested by washing the surface of the plates with sterile distilled water using a bent glass rod to dislodge the microflora from the surface of the agar. Bacterial and yeast treatment suspensions were each adjusted to 1 x 10^6 ml^{-1} using a haemocytometer. Biological control agents were applied once weekly for both greenhouse and nursery trials.

b. Plant defence activator - Acibenzolar-S-methyl (ASM)

A 25% reduced concentration treatment of a full strength (0.0075 g L^{-1}) ASM was prepared by measuring out 0.019 g and dissolving it into 1000 ml of tap water in a 2 L spray bottle. The mixture was shaken to form a homogenous solution. Spray treatment of the tomato plants was repeated every 14 d for both the greenhouse and nursery trials.

c. Plant sanitizer – Didecyl-dimethyl-ammonium chloride (DDAC)

A 25% reduced concentration treatment of full strength (0.75 ml^{-1}) DDAC was prepared by measuring out 0.19 ml and mixed it into 1000 ml of tap water in a 2 L garden spray bottle. The mixture was shaken to form a homogenous solution. Spray treatments with DDAC were repeated every 21 days for both the greenhouse and nursery trials

d. Copper oxychloride

Copper oxychloride (Farmers Agricare, Pietermaritzburg) was used as a bactericide control. A full strength (1.5 g L^{-1}) suspension was prepared by mixing 1.5 g of the bactericide into 1000 ml of tap water in a 2 L garden spray bottle. The mixture was shaken well to form a homogenous mixture. Copper oxychloride sprays were applied once during the greenhouse trials and weekly during the nursery trial.

5.2.5. Efficacy of treatment combinations under greenhouse conditions

The tomato seedlings to be used in the greenhouse were prepared and fertilized, as described in Section 5.2.2. There were a total of 17 treatments. Among the treatments were combinations of two, three and four treatments. The treatments were as follows: (1) 25% DDAC; (2) 25% ASM; (3) *Bacillus* sp. LN10; (4) *R. glutinis* Y25; (5) 25% DDAC + 25% ASM; (6) 25% DDAC + *Bacillus* sp. LN10; (7) 25% DDAC + *R. glutinis* Y25; (8) *R. glutinis* Y25 + 25% ASM; (9) *Bacillus* sp. LN10 + 25% ASM; (10) *Bacillus* sp. LN10 + *R. glutinis* Y25; (11) 25% DDAC + 25% ASM + *R. glutinis* Y25; (12) 25% DDAC + 25% ASM + *Bacillus* sp. LN10; (13) 25% DDAC + *R. glutinis* Y25 + *Bacillus* sp. LN10; (14) 25% ASM + *Bacillus* sp. LN10 + *R. glutinis* Y25; (15) 25% DDAC + 25% ASM + *Bacillus* sp. LN10 + *R. glutinis* Y25; (16) *P. syringae* pv. *tomato* inoculation only; and (17) copper hydroxide.

The treatments were applied in the following order with a waiting period of 24 h between treatment applications: 25 % DDAC followed by 25 % ASM then *Bacillus* sp. LN10 and finally *R. glutinis* Y25. Plants were then preconditioned by covering them with transparent plastic bags for 24 h and then sprayed with Pst suspensions until run-off. The treated plants were covered again with transparent plastic bags for another 24 h. The experiment was arranged in a randomized complete blocks design with six replicates. The plants were assessed for disease severity five days after treatment with Pst under greenhouse conditions. The experiment was done two times.

5.2.6. Efficacy of treatment combinations under nursery conditions

Nursery trials were conducted at Sunshine Seedling Services (Old Wartburg Rd, Pietermaritzburg, South Africa). The trials were conducted in an unprotected tunnel, with approximately 10-20% shading on the roof. Five to six week old tomato seedlings (cv Rodade), provided by Sunshine Seedling Services, were transplanted into 20 cm diameter pots (one seedling per pot) filled with growing medium comprising of a mixture of vermiculite and coco peat. The plants were irrigated once a day for a period of 20 minutes. The irrigation water contained NPK fertiliser 3:1:3 [38] 0.75 g L^{-1}; $CaNO_3$ 0.25 g L^{-1}; Microplex at 1 g per 1000 L (Sunshine Seedling Services, Pietermaritzburg, South Africa). The number of treatments and treatment combinations, number of replicates per treatment, the experimental design and treatment application were the same as detailed under Section 5.2.5 except that the copper oxychloride application was done weekly for the copper bactericide control. The plants were assessed for disease severity five days after *Pst*

inoculation. Disease severity was rated two times each week for four weeks. The experiment was done two times.

5.2.7 Disease scoring and data analysis

Disease ratings of percentage foliar infection consisted of an estimate of percent leaf area infected (% LAI) using the Horsfall-Barratt Scale (1945) where 1=0%; 2=0–3%; 3 = 3 – 6%; 4 = 12 – 25%; 5 = 25 – 50%; 6 = 50 – 75%; 7 = 75 – 88%; 8 = 88 – 94%; 9 = 94 – 97%; 10 = 97 – 100%. The estimates were used to calculate the Area Under the Disease Progress Curve (AUDPC) for each treatment (Shanner and Finney, 1977). The AUDPC values and the final disease severity values (arcsine transformed) were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc, 2011). Treatment means were separated using Duncan multiple range test at 5% probability level.

The benefits of using treatment combinations were compared with separate/individual application of each treatment were estimated. When two or more treatments were applied together, their effects could be classified as % Reduction in speck severity antagonistic, additive or synergistic. Antagonistic effects means the efficacy of the combination(s) is lower than the sum of the individual components' efficacies. Additive effects means that the efficacy of the combination(s) is equal to the sum of the separate efficacies, and synergistic effects means the efficacy of the combination(s) is greater than the sum of the separate efficacies. The expected disease control levels were calculated according to Abbott's formulas (Levy *et al*., 1986) as follows:

$$\boldsymbol{E_{(exp)} = a + b - (a \times b)/100; \text{ and } SF = E_{(obs)}/E_{(exp)}}$$

where a = control efficacy of treatment "a" when applied alone; b = control efficacy of treatment "b" when applied alone; $E_{(exp)}$ = expected control efficacy by the combination(s); $E_{(obs)}$ = observed control efficacy by the combination(s); and SF = the synergy factor achieved by the combination(s). When SF = 1, the interaction between the treatments is additive; when SF < 1, the interaction is antagonistic; and when SF > 1, the interaction is synergistic (Levy *et al*., 1986).

5.3. Results

5.3.1. Efficacy of individual and combination treatments in reducing bacterial speck severity under greenhouses conditions

Individual treatment applications of *Bacillus* sp. LN10, *R. glutinis* Y25 and 25% DDAC did not provide any significant control of bacterial speck disease of tomato, with disease reduction of 12.5, 8.85 and 5.27%, respectively. In contrast, the 25% ASM treatment resulted in significant suppression of bacterial speck by 98.97% when compared to the inoculated Control (Table 5.1).

The combination treatment of *Bacillus* sp. LN10 and *R. glutinis* Y25 was ineffective against bacterial speck. The combination treatments of 25% ASM + *Bacillus* sp. LN10, and 25% ASM + *R. glutinis* Y25 provided disease reductions of 97.9% and 95.45%, respectively. The three-way combination of 25% ASM + *R. glutinis* Y25 + *Bacillus* sp. LN10 caused a disease reduction of 99.1%. However, all combinations of 25% DDAC + any biological control agent were ineffective in controlling the disease (Table 5.1).

Most of the combination treatments were antagonistic. Only two were synergistic and one was additive (Table 5.2). The copper hydroxide Control treatment did not provide a significant disease reduction compared to the pathogen inoculated Control tomato plants (Table 5.1).

Table 5.1: Efficacy of individual and combined applications of two biological control agents, didecyl-dimethyl-ammonium chloride, acibenzolar-S-methyl and their combination treatments against bacteria speck of tomato under greenhouse conditions.

Treatment	**Final disease severity**	**% Reduction**	**AUDPC**
P. syringae pv. *tomato* inoculated Control	65.32 bcd	0	459.33 e
Copper hydroxide sprayed Control	61.88 cd	5.27	431,31 e
25% DDAC	58.89 d	9.84	665.90 d
25% ASM	0.67 e	98.97	72.54 f
Bacillus sp.LN10	57.12 d	12.55	429.73 e
R. glutinis Y25	59.51 d	8.85	384.83 e
25% DDAC + 25% ASM	0.88 e	98.65	0.00 f
25% DDAC + *Bacillus* sp. LN10	74.72 abcd	-14.39	841.52 b
25% DDAC +*R. glutinis* Y25	79.60 ab	-21.86	722.69 cd
25% DDAC+ 25% ASM +*R. glutinis* Y25	1.61 e	97.54	5.56 f
25% DDAC + 25% ASM +*Bacillus* sp. LN10	2.03 e	96.89	35.44 f
25 % DDAC+ *Bacillus* sp. LN10 +*R. glutinis* Y25	85.42 a	-30.77	965.88 a
25 % DDAC+ 25 5 ASM + *Bacillus sp*. LN10 + *R. glutinis* Y25	84.62 a	-29.55	684.92 d
25% ASM + *R. glutinis* Y25	2.97 e	95.45	16.06 f
25% ASM+ *Bacillus* sp. LN10	1.37 e	97.9	29.56 f
25% ASM+*Bacillus* sp. LN10 + *R. glutinis* Y25	0.59 e	99.1	24.50 f
Bacillus sp. LN10 + *R. glutinis* Y25	77.58 abc	-18.77	809.90 bc
F-value	59.75		117.95
P-value	0.0001		0.0001
CV%	22.86		20.20

Key: DDAC = didecyl-dimethyl-ammonium chloride; ASM = acibenzolar-S-methyl

Table 5.2: Calculation of benefits of treatment combinations for the control of bacterial speck on tomato under greenhouse conditions using Abbots formula (Levy *et al*., 1986)

Treatment	**Efficacy Observed**	**Efficacy Expected**	**Synergy Factor**	**Interactive Effect**
25 % DDAC + 25 % ASM	98.65	99.07	1.00	Additive
25 % DDAC + *Bacillus* sp. LN10	-14.39	21.16	-0.68	Antagonistic
25 % DDAC +*R. glutinis* Y25	-21.86	31.47	-0.69	Antagonistic
25 % DDAC+25 % ASM +*R. glutinis* Y25	97.54	31.47	3.10	Synergistic
25% DDAC +25 % ASM +*Bacillus* sp. LN10	96.89	-0.86	-112.66	Antagonistic
25% DDAC+ *Bacillus* sp. LN10 +*R. glutinis* Y25	-30.77	20.31	-1.51	Antagonistic
25% DDAC+ ASM + *Bacillus sp*. LN10 + *R. glutinis* Y25	-29.55	-951.44	0.03	Antagonistic
ASM + *R. glutinis* Y25	95.45	99.06	0.96	Antagonistic
ASM+ *Bacillus* sp. LN10	97.9	99.10	0.99	Antagonistic
ASM+*Bacillus* sp. LN10 + *R. glutinis* Y25	99.1	10.45	9.49	Synergistic
Bacillus sp. LN10 + *R. glutinis* Y25	-18.77	20.29	-0.93	Antagonistic

Key: DDAC = didecyl-dimethyl-ammonium chloride; ASM = acibenzolar-S-methyl

5.3.2. Efficacy of individual and combination treatments in reducing the severity of bacterial speck under nursery conditions

Application of individual treatments of *Bacillus* sp. LN10, *R. glutinis* Y25 and 25% DDAC cause no significant reduction in disease levels of bacterial speck, with disease reduction levels of 1.6, 13.8 and 29.46%, respectively. However, 25% ASM alone caused significant suppression of bacterial speck with reduction in disease severity of 90.51% (Table 5.3).

The combination treatment of *Bacillus* sp. LN10 + *R. glutinis* Y25 was ineffective against bacterial speck. However, combinations of 25% ASM + *R. glutinis* Y25, 25% ASM + *Bacillus* sp. LN10, 25% ASM + *R. glutinis* Y25 + *Bacillus* sp. LN10, and 25% ASM + 25 % DDAC significantly suppressed bacterial speck with disease reductions of 93.11%, 89.27%, 92.62% and 89.36%, respectively, compared to the pathogen inoculated Control (Table 5.3). However, this was not significantly different to the ASM treatment on its own. Hence the other treatments provided little or no control relative to ASM.

None of the combination treatments of 25% DDAC with any of the biological control agents provided significant disease control. Examples of such combinations include 25% DDAC + *Bacillus* sp.LN10, and 25% DDAC + *R. glutinis*Y25, which provided 20% and 0% disease suppression, respectively (Table 5.3).

Combination treatments once again resulted in a range of interactions, most of which were antagonistic. Only three were additive and none was synergistic (Table 5.4). The copper hydroxide bactericide treatment provided no significant control of bacterial speck.

Table 5.3: Efficacy of individual and combined treatments with two biological control agents, didecyl-dimethyl-ammonium chloride, acibenzolar-S-methyl and their combination treatments against bacteria speck of tomato under nursery conditions.

Treatment	**Final disease severity**	**Disease control %**	**AUDPC**
P. syringae pv. *tomato* inoculated Control	65.32 abc	0	459.33 ab
Copper oxychloride spray Control	61.88 bcd	5,27	431.31 ab
25 % DDAC	46.08 e	29.46	292.73 c
25 % ASM	6.20 g	90.51	73.50 d
Bacillus sp.LN10	64.22 abc	1.68	500.08 a
R. glutinis Y25	56.30 cd	13.81	386.38 b
25 % DDAC + 25 %ASM	6.95 gf	89.36	77.73 d
25 % DDAC + *Bacillus* sp. LN10	52.11 de	20.22	443.83 ab
25 % DDAC + *R. glutinis* Y25	65.32 abc	0	492.79 a
25 % DDAC + 25 % ASM + *R. glutinis* Y25	6.10 g	90.66	60.85 d
25 % DDAC + 25 % ASM + *Bacillus* sp. LN10	12.29 f	81.19	129.73 c
25 % DDAC + *Bacillus* sp. LN10 + *R. glutinis* Y25	67.31 ab	-3.05	413.02 b
25 % DDAC + 25 % ASM + *Bacillus sp*. LN10 + *R. glutinis* Y25	4.82 g	92.62	62.13 d
25 % ASM + *R. glutinis* Y25	4.50 g	93,11	55.19 d
25 % ASM + *Bacillus* sp. LN10	7.01 fg	89.27	59.58 d
25 % ASM + *Bacillus* sp. LN10 + *R. glutinis* Y25	4.82g	92.62	48.13 d
Bacillus sp. LN10 + *R. glutinis* Y25	72.23a	-10.57	450.73 ab
F-value	99.97		60.06
P-value	0.0001		0.0001
CV%	13.86		23.13

Key: DDAC = didecyl-dimethyl-ammonium chloride; ASM = acibenzolar-S-methyl

Table 5.4: Calculation of the benefits of treatment combinations for the control of bacterial speck on tomato under nursery conditions using Abbotts formula (Levy *et al*., 1986)

Treatment	**Efficacy Observed**	**Efficacy Expected**	**Synergy Factor**	**Interaction Effect**
25% DDAC + 25 %ASM	89.36	93.31	0.96	Antagonistic
25 % DDAC + *Bacillus* sp. LN10	20.22	30.65	0.66	Antagonistic
25% DDAC +*R. glutinis* Y25	0	39.20	0	Antagonistic
25% DDAC+ 25% ASM +*R. glutinis* Y25	90.66	-234.45	-0.39	Antagonistic
25% DDAC + 25% ASM +*Bacillus* sp. LN10	81.19	76.85	1.06	Additive
25% DDAC+ *Bacillus* sp. LN10 +*R. glutinis* Y25	-3.05	38.12	-0.08	Antagonistic
25% DDAC+ 25% ASM + *Bacillus sp*. LN10 + *R. glutinis* Y25	92.62	-483.17	-0.19	Antagonistic
25 % ASM + *R. glutinis* Y25	93,11	91.82	1.01	Additive
25% ASM+ *Bacillus* sp. LN10	89.27	91.27	0.98	Antagonistic
25% ASM+ *Bacillus* sp. LN10 + *R. glutinis* Y25	92.62	85.00	1.09	Additive
Bacillus sp. LN10 + *R. glutinis* Y25	-10.57	15.26	-0.69	Antagonistic

Key: DDAC = didecyl-dimethyl-ammonium chloride; ASM = acibenzolar-S-methyl

5.4. Discussion

Inconsistency in the poor efficacy of copper based bactericides that are routinely used against bacterial speck disease of tomato has led to the search for alternative control methods such as biological control to manage the disease (Wilson *et al*., 2002). It is concerning that the copper bactericide used in these experiments was completely ineffective. This is the standard treatment across South Africa that nurseryman and tomato farmers use on a routine basis. Yet it was entirely ineffective in both the greenhouse and the nursery experiments.

DDAC has been promoted heavily in South Africa as a plant sanitiser, with recommendations that it can be used to control bacterial speck and spot on tomato, especially in the nursery industry. In the nursery experiment here, it only provided 29.46% control of bacterial speck. This is inadequate for a commercial nursery or a tomato farmer, but relative to the complete failure of the copper bactericide, it is not surprising that it has been adopted by nurseryman.

It has been observed in a number of studies that individual application of biological control agents can significantly suppress Pst *in vitro* but that their performances are not always consistent *in vivo*. This suggests that combinations of different control approaches may be needed to achieve satisfactory disease suppression in practice (Ji *et al*., 2006; Wilson *et al*., 2002). In an attempt to develop an integrated approach to the control of bacterial speck disease of tomato, combination treatments were formulated, based on prior studies documented in Chapters Two, Three and Four. The best treatments identified previously were two foliar biological control agents, *Bacillus* sp. LN10 and *R. glutinis* Y25, and 25% concentrations of DDAC and ASM. These treatments were evaluated alone or in combinations against bacterial speck of tomato under both greenhouse and nursery conditions.

The plant defence activator, ASM, at a 25% concentration significantly suppressed bacterial speck severity when applied alone or in combination with the other three treatments, or when combined with the two biocontrol agents. The 25% DDAC did not provide for significant suppression of bacterial speck when applied alone or in combination with either biological control agents, *Bacillus* sp. LN10 or *R. glutinis* Y25. Combinations and individual treatments with either *Bacillus* sp. LN10 or *R. glutinis* Y25 also did not provide for any significant disease suppression of bacterial speck of tomato. The copper based bactericide applied as the standard treatment control was also ineffective in controlling bacterial speck disease on

tomato. The levels of disease control provided by the various treatments and their combinations were similar for both greenhouse and nursery experiments.

In a similar study done by Obradovic *et al*. (2005) they found that the plant defence inducer (Actigard a.i. acibenzolar-S-methyl) effectively induced a high level of plant resistance against bacterial spot disease (caused by *X. vesicatoria*), with the result that it completely prevented the development of the bacterial spot symptoms under greenhouse conditions. Obradovic et al (2005) found that a combination treatment of a bacteriophage and ASM resulted in the elimination of hypersensitive reaction lesions. This may have occurred because the phage application decreased the pathogen population on the leaf surface, allowing the induced defence mechanism to develop within the tomato plant. In another study it was shown that application of ASM either singly or in combination has the benefit of minimising the risk of yield reduction (Huang *et al* 2012). Thus, these publications were in line with our findings that bacterial speck on tomato was significantly reduced under both greenhouse and nursery conditions with the combination of ASM and biological control agents, although the ASM provided the bulk of the disease control.

No study was found on the combination of a plant sanitizer and a plant defence activator. In our experiment the combination successfully suppressed bacterial speck consistently under greenhouse and nursery conditions, although the ASM provided the bulk of the disease control. This was expected because the different modes of action of these two agrochemicals should complement each other. The plant sanitizer disrupts bacterial cell membranes, and causing cellular leakage and death of the cells of Pst (Serfontein, 2014). In contrast, ASM triggers the production of plant defence compounds (Huang *et al*., 2012). Combinations of the plant sanitizer and the biological control agents *Bacillus* sp. LN10 and *R. glutinis* Y25 were ineffective. We suggest that since the treatments were applied prior to inoculation of the pathogen, the plant sanitizer could have killed the cells of both biological control agents, thus leaving the leaf surface with no competitive antagonist to compete against Pst when it was introduced. This is a basic problem with plant sanitisers, in that they cannot be combined with biological control agents without killing the biological control agents.

Individual and combination application of biological control agents *Bacillus* sp. LN10 and *R. glutinis* Y25 did not provide satisfactory control of bacterial speck of tomato under either greenhouse and nursery conditions. These results were not consistent with their performance in prior research, as documented in Chapter Three where both isolates were effective under

greenhouse conditions. Ji *et al*. (2006) showed that the combination of biological control agents, *P. syringae* Cit7 and *P. fluorescens* 89B-61 against bacterial speck resulted in significantly greater suppression than when applied individually under greenhouse conditions but did not enhance suppression under field conditions. This confirmed that combinations of biological control agents are not always synergistic (Ji *et al*., 2006). The result of the two biological control agents' combination treatment in our experiment showed antagonistic effect, but the individual applications under both greenhouse and nursery conditions were inadequate anyway.

In summary the 25% ASM treatment, applied either alone or in combination with any of the other treatments significantly suppressed bacterial speck disease of tomato. Neither of the biological control agents was effective in practice, either in the greenhouse or in the nursery. Hence, there is still a need to find novel biological control agents that will be effective under field conditions. Given the failure of both the *Bacillus* and yeast isolates in this study, perhaps the best option is bacteriophages, which can be combined with ASM to maximise disease control.

5.5. Reference

Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M., Jones, J. B., 2004. Efficacy of plant growth–promoting rhizobacteria, acibenzolar-*S*-methyl, and soil amendment for integrated management of bacterial wilt on tomato. Plant Disease 88, 669-673.

Balestra, G.M., Heydari, A., Ceccarelli, D., Ovidi, E., Quattrucci, A., 2009. Antibacterial effect of *Allium sativum* and *Ficus carica* extracts on tomato bacterial pathogens. Crop Protection. 28, 807–811.

Barkai-Golan, R., 2001. Postharvest Diseases of Fruit and Vegetables: Development and Control. Elsevier Sciences, Amsterdam, The Netherlands.

Bora, T., Ozaktan, H., Gore, E., and Asian, E., 2004. Biological control of *Fusarium oxysporum* f. sp. *melonis* by wettable powder formulation of the two strains of *Pseudomonas putida*. Journal of Phytopathology, 152: 471-475.

Coates, L., Johnson, G., 1997. Postharvest diseases of fruits and vegetables. Plant Pathogens and Plant Diseases 33, 534 – 548.

Csinos, A. S., Pappu, H. R., McPherson, R. M., Stephenson, M. G., 2001. Management of Tomato spotted wilt virus in flue-cured tobacco with acibenzolar-S-methyl and imidacloprid. Plant Disease 85, 292-29.

da Silva, E.O., Martins, S.J., Alves, E. 2014. Essential oils for the control of bacterial speck in tomato crop. African Journal of Agriculture Research 9, 2624-2629.

Dooley, S.R., Beckstead, J., 2010.Characterizing the interaction between a fungal seed pathogen and a deleterious Rhizobacterium for biological control of cheatgrass. Biological Control 53, 197-203.

Elad, Y., Zimand, G., Zaqs, Y., Zuriel, S., Chet, I. 1993. Use of *Trichoderma harzianum* in combination or alternation with fungicides to control cucumber gray mold (*Botrytis cinerea*) under commercial greenhouse conditions. Plant Pathology 42, 324-332.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M.E., 2004. Biologically based alternatives to synthetic fungicides for the postharvest diseases of fruit and vegetables. Diseases of Fruit and Vegetables 2, 511–535.

Elliott, M., Shamoun, S.F., Sumampong, G., James, D., Masri, S., and Varga, A., 2009. Evaluation of several commercial biocontrol products on European and North American populations of *Phytophthora ramorum*. Biocontrol Science of Technology19, 1007-1021.

Granata, T., Alfa, M., Giuffrida, D., Rando, R., Dugo, G. (2011). Contamination of the food products by lead, cadmium and copper in the area at risk of Gela (Sicily). Epidemiology Prevenzione. 35, 94-100.

Guesky, R., Shtienberg, D., Elad, Y., Dinoor, A. 2001. Combining biocontrol agents to reduce the variability of biological control. Phytopathology, 91, 621-627.

Huang, C.H., Vallad, G. E., Zhang, S., Wen, A., Balogh, B., Figueiredo, J. F. L., Behlau, F., Jones, J. B., Momol, M. T., Olson, S. M., 2012. Effect of application frequency and reduced rates of acibenzolar-S-methyl on the field efficacy of induced resistance against bacterial spot on tomato. Plant Disease 96, 221-227.

Ippolito, A., El-Ghaouth, A., Wilson, C.L., Wisniewski, M.A., 2000. Control of postharvest decay of apple fruit by *Aureobasidium* pullulans and induction of defense responses. Postharvest Biology and Technology 19, 265–272.

Janisiewicz, W.J., Tworkoski, T.J., Sharer, C., 2000. Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients. Phytopathology 90, 1196 - 1200.

Janousek, C.N., Lorber, J.D., and Gubler, W.D., 2009. Combination and rotation of bacterial antagonists to biocontrol powdery mildew on pumpkin. Journal of Plant Disease Protection, 116, 260-262.

Ji, P., Campbell, H.L., Kloepper, J.W., Jones, J.B., Suslow, T.V., Wilson, M., 2006. Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control and growth-promoting rhizobacteria. Biological control 36, 358 – 367.

Jijakli, M.H., Grevesse, C., Lepoivre, P., 2001. Modes of action of biocontrol agents of postharvest diseases: challenges and difficulties. Bulletin-OILB/SROP 24, 317 – 318.

Kuc, J. 2001. Concepts and directions of induced systemic resistance in plants and its application. European Journal of. Plant Pathology 107, 7-12.

Le Floch, G., Vallance, J., Benhamou, N., Rey, P., 2009. Combining the oomycete *Pythium oligandrum* with two other antagonistic fungi: Root relationships and tomato grey mold biocontrol. Biological Control 50, 288-298.

Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., Guven, K., Iriarte, F. B., 2005. Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. Plant Disease 89, 712-716.

Oostendrop, M., Kunz, W., Dietrich, B., Staub, T. 2001. Induced disease resistance in plants by chemicals. European Journal of Plant Pathology 107, 19-28.

Serfontein, K., 2014. Sporekill still relevant after two decades. [https://agrosifu.files.wordpress.com/2014/11/hygrotech__spring-forum_2014_pg-28-29-1.pdf]: Accessed on 18th August 2015.

Tally, A., Oostendorp, M., Lawton, K., Staub, T., Bassi, B. 1999. Commercial development of elicitors of induced resistance to pathogens. In: Induced Plant Defenses against Pathogens and Herbivores. Biochemistry, Ecology, and Agriculture. A. A.

Agrawal, S. Tuzun, and E. Bent, Eds. American Phytopathological Society, St. Paul, MN, 357-369

Turgut, A; 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.

Wilson, M. 1997. Biocontrol of aerial plant diseases in agriculture and horticulture: current approaches and future prospects. Journal of Industrial Microbiology and Biotechnology 19, 188 – 191.

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

Xu, X.M., Robinson, J.D., Jeger, M., Jeffries, P., 2010. Using combinations of biocontrol agents to control *Botrytis cinerea* on strawberry leaves under fluctuating temperatures. Biocontrol Science of Technology 20, 359-373.

Yobo, K.S., Laing, M.D., Hunter, C.H., 2010. Application of selected biological control of damping-off caused by *Rhizoctonia solani*. African Journal of Biotechnology 9, 1789-1796.

Zhang, H., Ge, L., Chen, K., Zhao, L., Zhang, X., 2014. Enhanced biocontrol activity of *Rhodotorula mucilaginosa* cultured in media containing chitosan against postharvest diseases in strawberries: Possible mechanisms underlying the effect. Journal of Agriculture, Food and Chemistry 62, 4214 - 4224.

DISSERTATION OVERVIEW

The bacterial speck disease of tomato caused by *Pseudomonas syringae* pv. *tomato* (Pst) is a devastating disease of tomato (*Solanum lycopersicum* (L.) H. Karst worldwide (Shenge *et al*., 2008; Cai *et al*., 2011; da Silva *et al*., 2014). There are a number of control strategies that have been used to manage bacterial speck on tomato plants. These control strategies include the use of partially resistant cultivars (Milijasevic *et al*., 2009) and the use of copper-based bactericides (Balestra *et al*., 2009). However, these control methods are only partly effective, and significant yield losses still occur. Therefore there is a research gap to find other alternative methods to manage the disease.

In this study, we aimed to isolate potential biological control agents from the surface of tomato fruits and leaves. Both *in vitro* and *in vivo* screening of the potential biological control agents were done against Pst. Reduced concentrations of a plant defence activator, acibenzolar-S-methyl (ASM), and a plant sanitiser didecyl-dimethyl-ammonium chloride (DDAC) were evaluated for their ability to suppress bacterial speck of tomato under greenhouse and nursery conditions. The best treatments were combined and evaluated in an effort to formulate an integrated approach to manage bacterial speck disease of tomato under greenhouses and nursery conditions.

In this study, the following outcomes were achieved:

- Seventeen potential biological control agents, ten bacterial and seven yeast isolates, all obtained from the phyllosphere of tomato plants, inhibited the growth of Pst in *in vitro* studies.

- *Bacillus* sp. LN10 and *Rhodotorula glutinis* Y25 were the most effective biological control agents that were selected for subsequent integrated control studies out of the 350 potential biological control agents that were screened.

- Individual applications *Bacillus* sp. LN10, *R. glutinis* Y25 and a 25% reduced concentration of DDAC provided no significant suppression of bacterial speck. In contrast, a 25% reduced concentration of ASM was highly effective in suppressing bacterial speck disease of tomato under greenhouses and nursery conditions.

- Combination treatments of 25% reduced ASM + *Bacillus* sp. LN10 + *R. glutinis* Y25 significantly suppressed bacterial speck of tomato when compared to the inoculated Control,

under both greenhouse and nursery conditions. None of these treatments were significantly more effective than ASM on its own.

- None of the combination treatments that contained 25% DDAC were effective against bacterial speck when compared to inoculated Control, under greenhouses and nursery conditions.

***In vitro* screening**

In this study, *Bacillus* and yeast isolates from the phyllosphere of tomato plants provided significant inhibition of Pst during *in vitro* studies. The levels of antagonism differed between isolates. Out of 350 isolates, only two were selected for further studies under nursery conditions. This shows that large numbers of isolates must be tested by stringent screening to be able to select appropriate isolates to be used under field conditions

Greenhouse and nursery trials

The correlation between *in vitro* and *in vivo* performance of biological control agents has been found to be inconsistent by other researchers (Palazzini *et al*., 2007). In this study we found that some biological control agents that had provided for significant control of Pst under *in vitro* conditions performed poorly during *in vivo* screening under greenhouse conditions. Two isolates, *Bacillus* sp. LN10 and *R. glutinis* Y25, were not consistent in their performance, providing significant suppression of bacterial speck during *in vivo* screening under greenhouses conditions.

A range of concentrations of ASM and DDAC were evaluated for their ability to reduce bacterial speck These experiments showed that a 25% reduced concentrations of ASM could significantly reduce levels of bacterial speck of tomato. The recommended full strength concentration was not more effective than the 25% concentration. This outcome would reduce the risk of phytotoxicity, an issue that has been reported in some trials.

All combination treatments containing 25% ASM successfully reduced bacterial speck severity and were better than other combinations. The copper bactericide used as a standard treatment by nurseries was completely ineffective, which confirms the critical need to develop better control measures against bacterial speck and spot.

Overall conclusion

This study was apparently the first to test yeast isolates against bacterial speck disease of tomato. The successful screening of yeast isolates that were antagonistic against bacterial speck *in vitro* gives hope that yeasts could be effective antagonists against bacterial speck. Combinations of biological control agents and a reduced concentration of ASM could provide an alternative management option to control bacterial speck of tomato.

Future studies

Throughout this study no yeast study done on control of bacterial speck of tomato was found. In this study yeasts were identified as potential biological control agents to control bacterial speck disease of tomato. Further research may discover a more effective yeast isolate. There is a need for studies to elucidate the mode of action of the bacterial and yeast isolates in order to fully understand their biocontrol efficacy, and therefore, how to use them appropriately under the right conditions.

All treatments of the plant sanitizer, DDAC, were applied after the biological control agents. This was the wrong order of treatment because the DDAC would have eliminated the biological control agents from the leaf surfaces as the mechanism as a sanitiser is to rupture cell wall of any microorganism present on the leaf surface. The order needs to be reversed, whereby the DDAC is applied first, allowed to dry, and then the biological control agent is applied. This combination may be additive or synergistic, because the tomato leaves would be left clear of any competition for the beneficial biological control agents, which could then colonise the leaves fully.

Reference

Balestra, G.M., Heydari, A., Ceccarelli, D., Ovidi, E., Quattrucci, A., 2009. Antibacterial effect of *Allium sativum* and *Ficus carica* extracts on tomato bacterial pathogens. Crop Protection. 28, 807–811.

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., Setubal, J.C., Morales-Lizcano, 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 Pathog 7: e1002130. http://dx.doi.10.1371/journal.ppat.1002130

da Silva, E.O., Martins, S.J., Alves, E. 2014. Essential oils for the control of bacterial speck in tomato crop. African Journal of Agriculture Research 9, 2624-2629.

Milijasevic, S., Todorovic, B., Potočnik, I., Rekanivic, E., Stepanovic, M., 2009. Effects of copper-based compounds, antibiotics and a plant activator on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganensis* in greenhouse tomato seedlings. Pesticide and Phytomedicine 24, 19–27.

Shenge, K.C., Stephan, D., Mabagala, R.B., Mortensen, C.N., Wydra, K., 2008. Molecular Characterization of *Pseudomonas syringae* pv. *tomato* Isolates from Tanzania. Phytopathology 36, 338-351.

Palazzini, J.M., Ramirez, M.L., Alberione, E.J., Torres, A.M., Chulze, S.N., 2009. Osmotic stress adaptation, compatible solutes accumulation and biocontrol efficacy of two potential biocontrol agents of Fusarium head blight in wheat. Biological Control 51, 370-376.