Integrated Management of Early Blight of Tomato Caused by *Alternaria solani*

By

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B.Sc. Crop/Soil Science (University of Fort Hare)

Submitted in fulfilment of the

Requirements for the degree of

Master of Science

In the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Sciences (SAEES)

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

Republic of South Africa

January 2016

DISSERTATION SUMMARY

Early blight, caused by *Alternaria solani* (Ellis and Martin) Sorauer, has been reported to reduce tomato crop yields and market value, causing economic losses to tomato growers. There are currently no resistant cultivars available to farmers in the market. Fungicide applications are the standard method for managing the disease on tomatoes. Due to concerns such as cost, exposure risks, chemical residues and other health and environmental hazards, alternative control methods have been sought. Integrated disease management has been proposed as a possible strategy that could aid in reducing fungicide use in crop production. The use of plant resistance activators such as acibenzolar-S-methyl (ASM) along with biological control agents has been reported to have similar efficacy as compared to conventional chemicals in controlling early blight on tomato.

Therefore, this study was aimed at isolating and screening strains of yeasts and bacterial biological control agents (BCAs) against *A. solani* in *in vitro* and *in vivo* bioassays. Subsequently, single and combined applications of potassium silicate (KSil), ASM and BCA treatments were tested for their ability to reduce early blight of tomato under greenhouse and nursery conditions.

Isolation of 171 bacterial and 40 yeast strains were made from tomato leaves. These were screened for their potential to control early blight disease in tomato using a dual culture bioassay on agar plates (bacteria) and their inhibitory effect on spore germination (yeasts). Sixty percent (60%) of the bacterial isolates inhibited mycelial growth of *A. solani,* with zones of inhibition ranging from 6-14 mm on potato dextrose agar (PDA). Thirty-five percent (35%) of the yeast isolates inhibited spore germination of *A. solani* on tryptone soy agar (TSA) with inhibition of conidial germination of between 18 and 82%.

Five bacterial isolates (*Bacillus* spp. XVT8, *Pseudomonas putida* NC13, *Bacillus subtilis* N6/2, *Bacillus subtilis* WESH1 and *Bacillus subtilis* N5) and five yeast isolates (*Meyerozyma guilliermondii* P1-1, *Meyerozyma guilliermondii* C10, *Rodotorula minuta* P1-Orange, *Meyerozyma guilliermondii* Y4 and *Pichia guilliermondii* H5) were further tested under greenhouse conditions against early blight on tomato plants. The greenhouse results showed that *B. subtilis* N6/2, *B. subtilis* N5, *Bacillus* spp. XVT8 and *B. subtilis* WESH1 significantly ($p = 0.001$) reduced early blight severity when compared to the *A. solani* inoculated control,

which showed a final disease severity of 97%. However, the yeast isolates did not control *A. solani* under greenhouse conditions.

None of the liquid potassium silicate concentrations significantly reduced disease severity under greenhouse conditions ($P > 0.05$). The 75% reduced concentration (0.056 g L^{-1}) and the 100% concentration (0.075 g L^{-1}) of ASM significantly ($p = 0.001$) reduced disease severity, by 52% and 68%, respectively, under greenhouse conditions.

When ASM was used in combination with *Bacillus* sp. XVT8+ *Bacillus subtilis* N6/2, a disease reduction of 62 and 66%, respectively, was observed under greenhouse condition, and a disease reduction of 68% and 66%, respectively, was observed under nursery conditions. *Bacillus* sp. XVT8 + *B. subtilis* N6/2 caused a 57% and 46% disease reduction in the greenhouse and nursery trials, respectively. However, none of these treatments was significantly better than *Bacillus* sp. XVT8 applied on its own. When ASM was used in combination with *B. subtilis* N6/2 under greenhouse and nursery conditions it reduced disease levels by 41% and 47%, respectively. When 75% acibenzolar-S-methyl was used alone under greenhouse and nursery conditions it caused disease reductions of 23% and 14%, respectively. Two fungicide treatments, Rovral Flo and Coproxydithane +Petrin reduced disease levels under greenhouse and nursery conditions by 46% and 21%, respectively.

In conclusion, this study demonstrated that the biocontrol agent *Bacillus* sp. XVT8 alone, or in combination with ASM is about three times more effective at controlling early blight disease of tomato under greenhouse and nursery conditions than current fungicide applications. Use of a combination of two biocontrol agents, *Bacillus* sp. XVT8 + *B. subtilis* N6/2, also provided disease control superior to that provided by the fungicides, under both greenhouse and nursery conditions, although the control levels were less than that provided by *Bacillus* sp. XVT8 alone. Only one interation was positive, in the combination treatments (75% ASM+ *B. subtilis* N6/2). All other combinations were antagonistic (biocontrol agents with each other; ASM with *Bacillus* sp. XVT8, or with *Bacillus* sp. XVT8 + *B. subtilis* N6/2).

DECLARATION

I, Ncediwe Pute, declare that:

1. The research reported in this dissertation, except where otherwise indicated, and 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;

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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.

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Prof M.D. Laing (Co-supervisor)

ACKNOWLEDGMENTS

My special and most sincere gratitude goes to the following:

My supervisor, Dr. K.S. Yobo, for his guidance and support during the conduct of the study and final write-up. I sincerely appreciate your permanent availability, support and guidance.

My co-supervisor, Prof. M.D. Laing, for his interest in this study, support, guidance and motivation.

Mrs. S. van der Merwe, for her willingness to help and advice in maintenance of greenhouse and nursery trials.

Nonduduzo Cirha and Jacob Sello Mashilo for assisting me with laboratory experiments and their support throughout the study.

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

National Research Foundation (NRF) for bursaries and the University of KwaZulu-Natal (Competitive Grant Research Funds) for funding this study.

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

My loving family and friends whose support made all the difference and for their enormous support and encouragement.

Postgraduate students in Plant Pathology at UKZN for the love, support, encouragement and friendship.

The Lord God Almighty for making a way and providing people and resources that helped me complete my studies. To Him goes all the Glory.

DEDICATION

This thesis is dedicated to my mother, Nonzima Cynthia Pute, my sisters, Lindiwe James, Nosipho, Usanda, Ncedisa and my loving son, Onalenna Hlelolethu, You guys have shaped the person I have become and taught me that you can achieve anything through hard work and believing in yourself.

DISSERTATION INTRODUCTION

Early blight caused by *Alternaria solani* (Ellis & Martin) Sorauer is a major disease of tomato [*Solanum lycopersicum* (L.) H. Karst.] In severe cases this disease can cause complete defoliation of tomato plants (Peralta et al., 2005). The lack of obvious resistance genes in tomato germplasm has made it difficult to develop resistant cultivars by conventional breeding (Foolad et al., 2000; Chaerani et al., 2007). Early blight control has therefore been based on fungicide applications. However, this strategy is not a good long-term solution because of the cost, exposure risks, fungicide residues and other health and environmental hazards that routine pesticide applications pose (Jagadeesh and Jagadeesh, 2009; Afifi and Zayan, 2010). In an attempt to overcome this problem research efforts have focused on finding sustainable, alternative approaches to controlling the disease. The use of biological control and integrated disease management have been reported to be possible alternatives to controlling plant diseases. However, concerns over the inconsistent performance of biological control agents have been reported (El-Ghaouth et al., 2002; Leverentz et al., 2003). Duffy and Weller (1995) and Varshney and Chaube (2001) suggested that integrating microbial antagonists with other disease control strategies could overcome this shortfall.

The main aim of this research was to develop an integrated disease management approach using a plant defence activator, Acibenzolar-S-methyl (ASM), potassium silicate, and selected bacterial and yeasts biological control agents to control early blight of tomato caused by *A. solani* under greenhouse and nursery conditions.

The specific objectives of this study were as follows:

1. To write a Literature Review on early blight, the causal microorganism, the life cycle, climatic conditions required for infection, symptoms, and economic importance and the available control options;

2. To isolate and screen microorganisms for antagonism against *A. solani in vitro*;

3. To screen *in vivo* the best antagonists against early blight of tomato under greenhouse conditions;

4. To evaluate a range of concentrations of acibenzolar-S-methyl and liquid potassium silicate (KSil) for their ability to control early blight of tomato under greenhouse conditions;

5. Integrate the best control options selected from bacterial and yeast biological control agents with the optimum concentrations of acibenzolar-S-methyl and potassium silicate (KSil) under greenhouse and nursery conditions.

The dissertation consists of five chapters, with each chapter covering a detailed aspect of the research conducted on the integrated control of early blight disease of tomato. With the exception of the literature review, each of the chapters has been written as an independent study, and prepared in the format of a scientific paper. This creates some redundancy in the introductory information, and the references. However, it is the standard dissertation model that has been adopted by University of KwaZulu-Natal.

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

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Tomato [*Solanum lycopersicum* (L.) H. Karst.] is an important vegetable crop that is cultivated worldwide and belongs in the genus *Solanum*, of the family *Solanaceae*, subfamily *Solanoidea* and tribe *Solaneae*. This family includes well known plant species such as potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), sweet pepper (*Capsicum annuum* L.) and eggplant (*Solanum melongena* var. *esculentum*) (Taylor, 1986; Afifi and Zayan, 2010).

In South Africa, tomato is regarded as the second most important horticultural crop following potato. It is cultivated commercially as well by resource poor farmers and home gardeners (Department of Agriculture Forestry and Fisheries, 2013). It is one of the main vegetables used for hawking by small-scale entrepreneurs in the informal sector (Department of Agriculture Forestry and Fisheries, 2010).Its production constitutes 23% of the total vegetable production in South Africa (Department of Agriculture Forestry and Fisheries, 2012).

1.2 Origin and history of tomato

According to Sims (1980), tomato originated from the Andean region which includes Peru, Ecuador, Chile, Colombia and Bolivia. It is reported to have evolved from *Solanum lycopersicum* var. *cerasiforme*, which is known as the cherry form. It is believed that the crop was introduced by the Spanish explorers to Spain and was later taken to Morocco, Turkey and Italy. However, the tomato crop was only accepted as a food crop in the 18th century due to the popular belief that it was poisonous (Harvey et al., 2002).

1.3 Tomato production and constraints in South Africa

In South Africa tomato is grown in both summer and winter in frost-free areas. The major growing areas include Limpopo, the Mpumalanga Lowveld, Middleveld, the Pongola area of KwaZulu-Natal, the southern parts of the Eastern Cape and the Western Cape (Department of

Agriculture Forestry and Fisheries, 2013). Tomato is known to be susceptible to a large number of diseases (Dabbas et al., 2010). These include bacterial wilt, late blight, bacterial speck and spot, as well as early blight disease caused by the fungus *Alternaria solani* Sorauer. These diseases lower production of the crop (Balbi-Peña et al., 2006).

1.4 Taxonomy and morphology of *Alternaria solani*

Alternaria solani is a fungus that causes early blight disease in tomatoes. It is classified under the kingdom Fungi, phylum Ascomycota, class *Dothideomycetes*, subclass *Pleosporomycetidiae*, order *Pleosporales*, family *Pleosporaceae*, genus *Alternaria*, species *solani*, authority Sorauer (Chaerani et al., 2006). It belongs to the fungi imperfecti (deuteromycotina) family in the class *Hyphomycetes* and order *Hyphales*. This family is known to cause a number of diseases on a wide range of agronomic and horticultural plants. This pathogen was first reported in New Jersey by Ellis and Martin in 1882 (cited in Sherf and MacNab, 1986).

According to Agrios (2005), *A. solani* is classified as a deuteromycete due to its lack of known sexual stage. As with other members of this genus, it is identified by transverse and longitudinal septate conidia, with multinucleate cells including dark-collared (melanised) cells. The melanin is reported to act as a protective barrier against adverse environmental conditions. This includes resistance to antagonistic microbes and their metabolites (e.g. hydrolytic enzymes). It also allows the pathogen to survive for several years in the soil. In addition, it increases its resistance to lysis and results in extended survival (Rotem, 1994). It is characterized by the formation of polymorphous conidia either singly or in short or longer chains and provided with cross, longitudinal as well as oblique septa and having longer or short beak.The beaked conidium may possess 9-11 transverse septa as well as vertical septa (Mamgain et al., 2013)

The fungus produces a deeply pigmented gray/black hairy colony with a mycelium that is haploid and septate, becoming darkly pigmented with age. This occurs when the pathogen is cultured on artificial media such as V-8 juice agar. However, its sporulation requires

exposure to light. Thick-walled chlamydospores have also been reported but are not found frequently (Simmons, 2000; 2007).

1.4.1 Symptoms

Early blight symptoms are normally observed on foliage, stems and the fruits (Figure 1A and 1B). They appear as black lesions about 1-2 mm in size (Pscheidt and Stevenson, 1988; Stevenson, 1993). However, under favourable environmental conditions the lesions tend to become enlarged, with a yellow halo. Defoliation may also occur due to the expansion of the lesions, resulting in entire leaves turning chlorotic and necrotic. Other indications such as concentric rings may also be visible on green and ripe tomato fruit, resulting in premature drop of fruits (Jones, 1991).

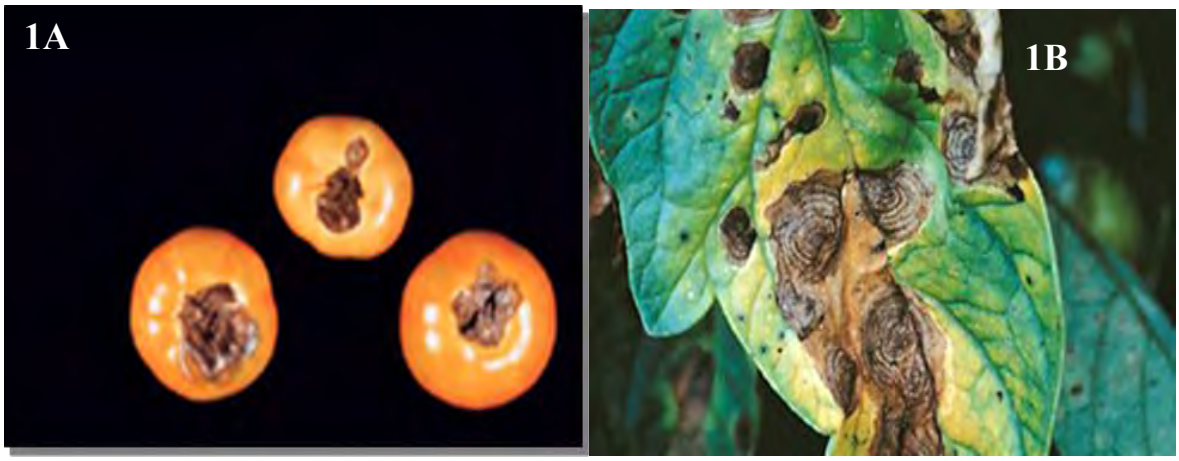

Figure 1.1. Infection of early blight on tomato fruit (A) and foliage (B) (photos courtesy of W.R. Stevenson) (Kemmitt, 2012).http://utahpests.usu.edu/htm/utah-pests-news/up-spring-2012/tomato-diseases-watch

1.4.2 Economic importance and distribution of early blight

Early blight occurs worldwide and is widespread in the tropics, subtropics and temperate zones. According to Hassanein et al. (2010), early blight disease results in high yield losses, resulting in economic losses in every growing season, both directly and indirectly, by fruit infection and by causing sun blotch on defoliated plants. Temperatures ranging from 24-29°C combined with humid environments favour infection (Rotem, 1994).

1.4.3 Alternate hosts

Early blight can affect any plants that belongs to the family *Solanaceae*, whether cultivated or weeds. However, potato (*Solanum tuberosum* L.) are reported to be one of the crops which is mostly affected by early blight disease (Jones, 1991). Jagadeesh and Jagadeesh (2009b) reported that early blight causes major losses in potato fields in most production regions of the world. According to Van der waals et al. (2003) yield losses of from 1% to 60% with the average being 20% have been shown to occur when the disease is not controlled out of the 14 potato growing regions of South Africa.

1.4.4 Disease cycle and epidemiology

The presence of free moisture with optimum temperatures of 28-30°C has been reported to promote conidial germination, which takes place in approximately 40 minutes. However, infection also occurs during alternating wet and dry periods due to renewed growth of desiccated germ tubes when they are re-wetted. The pathogen can penetrate through the leaf epidermis directly or enter through stomata (Kemmitt, 2012). *A. solani* survives between crops cycles in plant debris and on seed (Figure 1.2). In mild climates the pathogen can survive from season to season on volunteer plants of tomato and potato plants such as *S. melongena* (eggplant) as well as other several weedy solanaceous hosts *S. carolinense* L (horse nettle) and *Solanum nigrum* L. (nightshade).

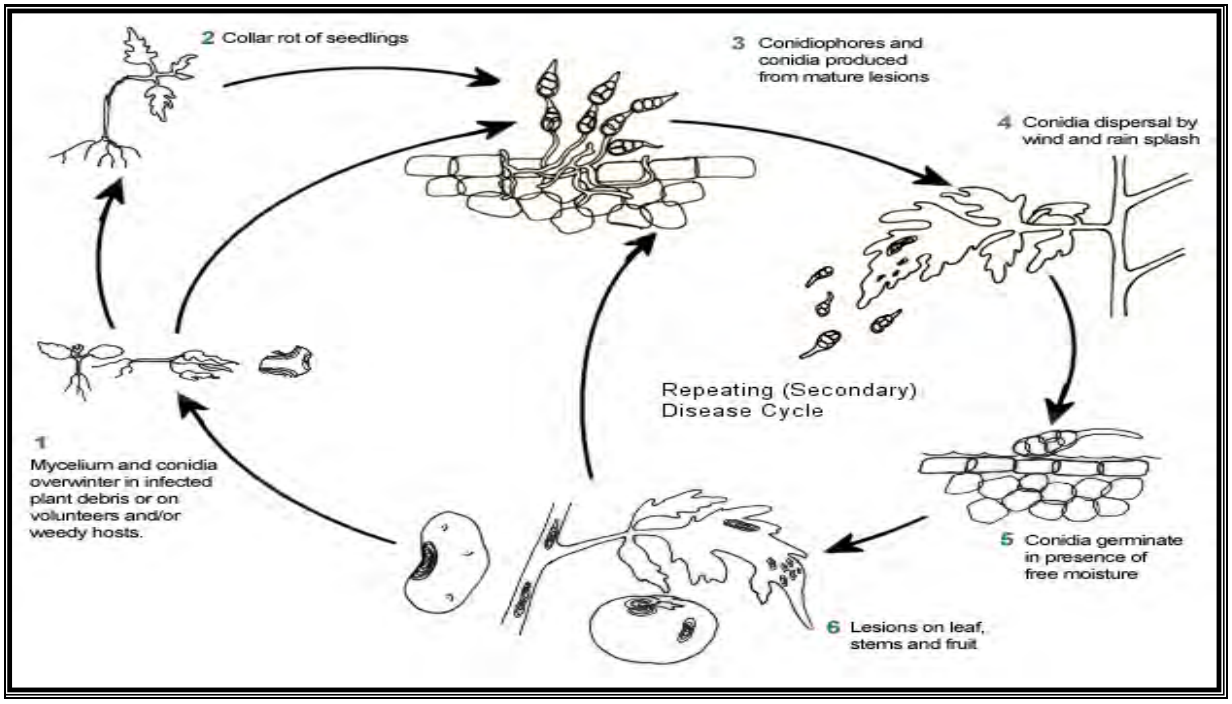

Figure 1.2. Life cycle of early blight in tomato (Kemmitt, 2012).

1.5 Control of early blight

Current control strategies for early blight disease are based on fungicide use. However, the commonly used fungicides are not very effective against early blight, and there have been concerns raised about their effects on food safety (Afifi and Zayan, 2010). In addition, limitations such as fungicide resistance, fungicide residues on crops, as well as workers' health and safety concerns have been raised (Jagadeesh and Jagadeesh, 2009). This has led to a search for alternative control measures such biological control, which aims at using beneficial microbes to manage crop diseases (Raupach and Kloepper, 2000; Punja and Utkhede, 2003).

According to Guetsky et al. (2002), the use of biological control methods should create a more sustainable agricultural system and minimize the need for synthetic chemicals in food crop production. The success of biological control in managing various plant disease, using either natural products or antagonistic microorganisms, has been shown in many countries (Papavizas, 1985). They have been shown to be less costly, easily applied and can provide the best control measure under defined conditions. In addition, their application is safe and environmental friendly (Sivan and Chet, 1993). The uses of biological control agents and cultural practise have already been used to manage early blight disease of tomato (Sherf and MacNab, 1986).

1.5.1 Cultural methods

A cultural method is defined as incorporating all aspects of crop husbandry that reduce disease development. These include the use of clean seed, the use of tolerant or resistant varieties, destroying crop debris after harvest, and crop rotation (Westcott, 2001). According to Sherf and MacNab (1986), cultural methods have been successful tools in reducing disease severity caused by various pathogens for centuries. This practise serves as a way of reducing disease below economic threshold levels. Therefore, employing sound cultural practices to control disease in tomato may assist in keeping the crop healthy and keep early blight losses below economic threshold levels (Spletzer and Enyedi, 1999).

(i) **Crop rotation**

Crop rotation is defined as the practice of cultivating different types of crops in the same area in sequential seasons. It is regarded as one of the most important disease management practices and serves as one of the effective strategies in reducing levels of early blight on tomato. A crop rotation including members of different families (non-solanaceous) crops has been found to reduce the initial inoculum of early blight (Madden et al., 1978). This practise is normally conducted by grouping the members of the same family in a crop rotation method based on groups rather than on individual crops. This method helps in removing infected debris which serves as a source of inoculum, thus reducing re-infection of the crop (Sherf and MacNab, 1986).

(ii) Using disease free plants

The use of disease-free certified seed is one way of controlling early blight disease on tomatoes. There are currently no known resistant cultivars available to farmers in the market. However, partial resistance has been observed in wild species of tomato (Afifi and Zayan, 2010). Wild species that were identified as being potential sources of resistant genes include species such as *Solanum habrochaites* (syn. *Lycopersicon hirsutum* L), *Solanum pimpinellifolium L* (syn. *Lycopersicon pimpinellifolium* L), *Solanum peruvianum L* (syn. *Lycopersicon peruvianum L.mill*), and *Solanum chilense (dunal) Reiche* (syn. *Lycopersicon chilense (dunal*) (Foolad et al., 2000; Chaerani et al., 2007).

(iii) Sanitation

According to Tumwine et al. (2002), a broader definition of sanitation refers to the exclusion of inoculum sources in the crop as well as outside the crop. This may be achieved by using various practices such as basic crop hygiene to the elimination of alternate hosts (Hanada, 1988). This kind of practice has been recommended at the field level due to its success in controlling fungal diseases of perennial crops and other diseases such as damping-off diseases (Emebiri and Obiefuna, 1992). However, removing crop residues and preventing weeds is important when controlling early blight disease of tomato. This is due to the fact that the pathogen can survive from season to season as well as in the residues.

1.5.2 Chemical control

Fungicide application has become the standard method for managing early blight disease of tomato (Table 1.1). Fungicide resistance as well as hazardous effect on the environment are some of the limitations associated with the use of chemicals in managing plant diseases (Afifi and Zayan, 2010). There is also an issue of the high cost associated with the use of chemicals as agents of disease control (Yazici et al., 2011). In addition, fungicide use requires application from 1-2 days after transplanting and a 5-7 day spray schedule thereafter, resulting in high input costs (Miller and Miller, 2004). Hazardous effect on the environment has resulted in discontinuation of some of the chemicals used to control early blight. Therefore, this calls for other strategies such as biological control as possible alternative measure to control the disease in tomato production (Urech et al., 1997).

Table 1.1. List of common fungicide used for controlling early blight disease.

Active Ingredient	Type of fungicide	Reference
Chlorothalonil	Protectant	Sahu et al. (2013)
Azoxystrobin	Curative	Bartlett et al. (2002)
Pyraclostrobin	Curative	Kermit (2012)
Trifloxystrobin	Curative	Bartlett et al. (2002)
Copper oxychloride	Protectant	Sahu et al. (2013)
Mancozeb	Protectant	Sarkar and Chowdhary (2004)
Iprodione	Translaminar	Sarkar and Chowdhary (2004)

1.6 Integrated disease management

Integrated control of plant diseases can be defined as combining a number of methods of controlling plant diseases leading to increased disease control, and often in reductions in the number of fungicide applications (El-Khoury and Makkouk, 2010). The various methods include biological, cultural and other control strategies (Andrews, 1983). Combining biological control agents with reduced number of application of fungicides may be an effective way of achieving disease control in plants (Elad et al., 1993; Raupach and Kloepper, 2000, Clarkson et al., 2006). According to Leverentz et al. (2003) using biological control alone may be less effective than commercial fungicides, or it may provide inconsistent control. Lumsden et al. (1995) suggested that using biological control in conjunction with fungicides can reduce the level of chemical and pesticide contaminations in food. However, the use of chemicals with biological control has been reported in only a few systems (Guetsky et al., 2002). Various reasons have been given to explain this problem. According to Harman and Bjorkman (1998), one of the causes of failure in adapting biological control is a lack of knowledge and education among the farmers concerning the product. In addition, Butt and Goettel (2000) noted that market potential is another major factor limiting the success of

biological control agents. These include the absence of strong incentives to develop these agents, as well as the availability of new biodegradable chemical pesticides. This tends to limit the acceptance and transfer of new technology. Bagheri (2010) and Ying and Min (2011) commented that the participation of farmers in the production of biological control agents may play an important role in the adoption of biological control as an alternative disease control management method. The farmers could help in identifying the technical barriers that diminish the efficacy of these products. In addition, farmers will gain an understanding of the biological control product which would help in their adoption of the technology (Valentin, 2002).

1.7 Control of disease using biological control methods

The term "biological control" has been used in various disciplines such as Entomology and Plant Pathology. According to Baker and Cook (1974), biological control can be defined as "the reduction of inoculum or disease producing activity of a pathogen or parasite in its dormant or active state using one or more organism accomplished naturally or by manipulation of the environment, host or antagonist by mass introduction of one or more antagonist". However, in this review the term biological control applies to the use of microbial antagonists to suppress plant diseases.

Previous research has demonstrated that many microorganisms can act as antagonists to plant pathogens (Table 1.2) (Cook, 2000). These organisms can be specific to a pathogen without causing negative effects on beneficial microorganisms (Becker and Schwinn, 1993). Biological control agents may provide a good solution for growers who are working toward an organic status for their crops.

According to El-Khoury and Makkouk (2010), success in the use of antagonistic microorganisms against plant pathogens is well established with the control of crown gall with *Agrobacterium radiobacter* K84 as well as that of seedling blights and damping off disease caused by *Pythium* and *Rhizoctonia* with *Trichoderma harzianum* (Harman and Bjorkman, 1998), *Gliocladium virens* (Lumsden et al., 1996) and *Streptomyces griseus* (Cook et al., 1996).

In order for biological control to be successful and effective, one has to understand the mechanism by which these organisms function. This may result in enhanced control efficacy and reducing the inconsistencies as well as variability in performance (Guetsky et al., 2002).

Table 1.2. Some common biological control agents that have been investigated for control of some common pathogens on various crops.

Organism (BCA)	Target Pathogen	Crop	Disease	Application method	Reference(s)
Trichoderma koningii	*Protomycopsis phaseoli*	Cowpea	leaf smut	Foliar spray	Adejumo et al.,1999
Pseudomonas fluorescens	*Pyricularia oryzae*	Rice	Rice blast	Seed treatment	Vasudevan et al.,2002
Bacillus cereus	*Alternaria alternate*	Tobacco	Brown spot	Foliar application	Fravel and Spur,1977
Aeromonas caviae	*R. solani and Fusarium oxysporum*	Radish	Damping off and Fusarium wilt	Applied on potting sand or soil	De boer et al.,1999
Pseudomonas gladioli b25	*A. Solani*	Tomato	Early blight	Combination treatment	Agrios,1997
Fusarium sambucinum	*R. solani and Fusarium*	Potato	Black scurf and and Fusarium dry rot	Seed treatment	Hijwegen,1992
T. minor	*P. xanthii*	Cucumber	Powdery mildew	Soil and seed treatment	Romero et al.,2003
Bacillus spp	*P. xanthii*	Cucurbit	Powdery mildew	Foliar spray and seed treatment	Gardener and Fravel, 2002
Fusarium oxysporum	*Fusarium oxysporum*	Tomato	Tomato wilt and	Soil incorporation	Gardener and Fravel, 2002
Streptomyces spp	*Alternaria solani* *Clavibacter michiganensis*	Tomato	Early blight and bacterial canker	Seed coating	Rabeendram et al.,1998
Gliocladium virens	*Sclerotinia sclerotiorum*	Cabbage	Sclerotinia Rot	soil inoculation to the root zone	Rabeendram et al.,1998

1.8 Biological control agents used in controlling disease in tomatoes

Biological control agents have been used in controlling various tomato diseases such as bacterial wilt, crown rot and early blight (Table 1.2) (Montealegre, et al., 2005; Yazici et al., 2011; Maji and Chakrabartty, 2014). This includes the use of bacteria, yeast, and filamentous fungi (Caron et al., 1985). These micro-organisms have been reported to be effective as microbial antagonists in controlling plant pathogens (Table 1.2) (Medeiros et al., 2012).

Yazici et al. (2011a) reported that 23 out of 190 bacterial isolate that were screened in a study of bacteria for biological control were effective against early blight. In dual culture agar tests, the inhibitory zones ranged between 9.35 and 31.3mm. The organisms included bacteria such as *Serratia plymuthica* (IK-139) which was highly effective in the *in-vitro* test. Other active bacteria were *Paenibacillus macerans*-GC subgroup A (1.82), *Serratia plymuthica* (1.78), *Bacillus coagulans* (1.75), *Serratia marcescens*-GC subgroup A (1.50), *Bacillus pumilis* –GC subgroup B (1.50) and *Pantoea agglomerans* (1.32). However, there was no evidence on how well the bacteria performed in a greenhouse or field environment. In addition, isolate T39 of *Trichoderma harzianum* (TRICHODEX 20SP, Makhteshim Chemical Works) has also been rported as an effective as a biocontrol agent against tomato diseases in commercial greenhouses (Elad, 1993; Elad and Shtienberg, 2000). In a study conducted by Omar et al. (2005) two bacterial isolates, *Bacillus megaterium* (c96) and *Burkholderia cepacia* (c91), were found to be be antagonistic against *Fusarium oxysporum* f.sp. *radicis-lycopersici*, the causal organism of fusarium crown and root rot of tomato.

Table 1.3. Biological control agents used against tomato pathogens

Organism (BCA)	**Disease**	**Pathogen**	**Reference**
Trichoderma harzianum	Fusarium crown and root rot	*Fusarium oxysporum*	Sundaramoorthy and Balabaskar (2013)
Gliocladium Intraradices	Fusarium crown and root rot	*F. oxysporum*	Datnoff et al. (1995)
Bacillus subtilis	Bacterial wilt	*Rhizoctonia solani*	Chen et al. (2012)
Gliocladium virens	Crown rot	*R. solani*	Lamb and Rosskopf (2001)
Trichoderma spp.	Root and crown rot	*R. solani*	Montealegre et al. (2005)
Pseudomonas spp.	Leaf spot of tomato	*Alternaria solani*	El-Abyad et al. (1993)

Table 1.4. Yeast antagonists that have been tested against different pathogens

Organism	**Crop**	**Pathogen**	**Reference**
Candida guilliermondii (strains 101 , US 7)	tomato	*Botrytis cinerea*	Saligkarias et al. (2002)
Pseudozyma flocculosa	Cucumber	Powdery mildews	Punja and Utkhede (2003)
Candida quercitrusa	Chilli pepper	*Colletotrichum capsici*	Chanchaichaovivat et al. (2007)
Issatchenkia orientalis	Chilli pepper	*C. capsici*	Chanchaichaovivat et al. (2007)
Candida musae	Chilli pepper	*C. capsici*	Chanchaichaovivat et al. (2007)
Pichia guilliermondii,	Chilli pepper	*C. capsici*	Chanchaichaovivat et al. (2007)
Saprophytic yeast	Pistachio nut	*Aspergillus flavus*	Afsah-Hejri (2013)
Pichia guilliermondii	Tomato	*B. cinerea*	Guetsky et al. (2002)

1.9 Systemic resistance in plants

According to Elad et al. (2010) and Ryals and Ward (1994), plants express their own active defence mechanism against biotic stresses. Systemic acquired resistance SAR and induced systemic resistance (ISR) are the two reported forms of induced resistance in which plant defence is preconditioned as a result of prior infection or treatment that causes resistance following a challenge by the pathogen or parasite (Chaudhary et al., 2007). According to Kloepper et al. (1992), "induced systemic resistance (ISR) refers to the systemic protection of the plants following induction with an inducing agent to a single part of the plant". Induction can be as a result of various substances such as microorganisms, metabolic substances of the host plant, or chemical compounds (Achuo et al., 2004). According to Louws et al. (2000), these agents elicit biochemical processes such as the production of phenolics, phytoalexins and enzymes such as glucanases or chitinases which are responsible for rupturing of fungal cell walls (Agrios, 1988).

1.10 Silicon (Si)

Silicon (Si) is regarded as one of the most abundant element in the earths crust as well as in the soil (Datnoff et al., 1997). According to Epstein (1994) , silicon is found in some plant tissue in high concentration and occasionally at levels higher than those of potassium and nitrogen. In agriculture, silicon has been used for centuries for the purpose of controlling disease without a proper understanding of its mechanisms of action (Belanger et al., 1995). Marschner (1995) noted that this element is beneficial in controlling plant diseases and aids in plant growth, including the alleviation of abiotic stresses. It has been reported to reduce mineral toxicity in plants such as those caused by aluminium and manganese, and also preventing zinc and phosphorus deficiency (Marschner, 1995; Tisdale, 1995). In crops such as rice (*Oryza sativa* L.), it was found that using fertilizers that include silicon as one of the elements resulted in higher yields and also reduced rice blast disease severity (Winslow, 1997; Kim et al., 2000).

According to Bocharnikova et al. (2010) and Ma et al. (2001), silicon is taken up by plants from the soil as monosilicic acid (H_4SiO). It accumulates in the epidermal tissues of rice as a polymer of hydrated amorphous silica soon after uptake. Various modes of action of silicon in plants have

been proposed. These include silicon being deposited in plant tissue resulting in the formation of a physical barrier (de Melo et al., 2010). In addition, it reduces the susceptibility of the cell wall to enzymatic degradation by fungi. In a study conducted by Fawe et al., (2001), silicon accumulated close to the pathogens site of entry which led to the conclusion that Si act against the pathogen by increasing the mechanical resistance in the plants. It also serves to enhance phytoalexin production (Cherif et al., 1994).

Silicon has the ability to form complexes with phenolic compounds. The presence of these phenolic compounds in the infection courts acts as a defence mechanism against fungal pathogens and their attack. This resistance mechanism is facilitated by the presence of soluble silicon (Menzies et al., 1991a; Menzies et al., 1991b). According to Inanaga et al. (1995) silicon application can accelerate the production of antifungal compounds after the penetration of pathogens into the epidermal cell.

Silicon can enhance defence responses that are functionally similar to those displayed in systemic acquired resistance. According to Cherif et al. (1994), Si treated plants can significantly increase antioxidant enzymes activities as well as the production of antifungal compounds such as phenolic metabolism products, phytoalexins, and pathogenesis related proteins (Cherif et al., 1994). It has been reported that in the presence of Ca and pectin ions Si accumulates in the epidermal tissues and results in the formation of a cellulose membrane-Si, thus providing protection to the plants. The increase of Si in the sap of the plant results to Si polymerisation which is identified as Si gel hydrated with water molecules (Belanger, 2003; Rodrigues, 2003; Gao et al., 2005).

In studies which were conducted on rice, *Triticum* spp (wheat) and *Cucumis sativus* L. (cucumber), lower disease severity in the Si-treated plants was related to the higher activity of protective enzymes polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) in leaves of the plants (Cai et al., 2008). These enzymes play a significant role in regulating the production and accumulation of antifungal compounds such as phenolic metabolism products (lignin), phytoalexins and pathogenesis-related proteins in plants (Inanaga et al., 1995).

1.11 Use of acibenzolar-S-methyl on plants

Acibenzolar-s- methyl (BION®) is the registered trade mark of a Syngenta product with an active ingredient known as acibenzolar-S-methyl (benzothiadiazole) (ASM) (Novartis, 1997: Csosz et al., 1999). ASM was registered in the United States as a "reduced risk compound' in 1998 as Actigard® (Syngenta Crop Protection Inc., Greensboro, NC) for use on tobacco (Nicotiana tabacum L.), spinach (Spinacea oleracea L.), tomato [Solanum lycopersicum (L.) H. Karst.] cole crops, and leafy vegetables and as BION in Europe (Syngenta Ltd., Basel, Switzerland) on wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) (Tally et al., 1999; Novartis Crop Protection, 1999).

It triggers induced systemic resistance in plants, and is active against various pathogens such as fungi, bacteria and viruses when used on various plants (Lawton et al., 1996). This is achieved by the activation of various enzymes, lignin and phenolic compounds as well as enhancement of expression in the genes related to resistance in plants (Buzi et al., 2004). It induces host plant resistance and has no direct activity against target pathogens. However, application of this chemical to a range of plants results in a spectrum of responses. It has a long lasting effect on monocotyledonous plants while on dicotyledonous plants its effect has been reported to be minimal. It has been used on various crops to control diseases such as downy mildew (*Plasmopara helianthi)* and *Orobanche cumana* attacking sunflower, fire blight of pear, *Sclerotinia* white mould of soybean, and brown spot and narrow brown leaf spot in rice (Dann et al., 1998; Tosi et al., 1999; Sauerborn et al., 2002).

ASM application showed remarkable protective activity against anthracnose in cucumber. Disease suppression by ASM has been found even when the whole cucumber plants were inoculated with the pathogen 3 hours after treatment with ASM. High levels of control were observed on both the treated leaves (first leaves) and untreated upper leaves. A strong activity against anthracnose was highly reproducible on treated and untreated leaves. However, phytotoxicity of ASM was noted when it was applied at $100gml^{-1}$, resulting in chlorosis, browning and mosaic formations, symptoms that appeared on treated leaves and occasionally on untreated leaves (Malolepsza, 2006).

In another study conducted on the effect of ASM in plants by Dilci et al., (2008), ASM significantly lowered infection caused by both *B. cinerea* and *S. sclerotiorum* on early maturing sunflower variety "Aurasol" (Monsanto), regardless of the application time and dosage applied.

There is much to be done to convince farmers and growers that intergrated disease managent can work and could provide a useful addition to their disease management programmes.however before the strategy can be introduced investigation on how it works has to dertemined.

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CHAPTER TWO

Isolation and *in vitro* screening of biological control agents against *Alternaria solani*

Abstract

In this study 171 bacterial and 40 yeast isolates were screened *in vitro* for antagonistic activity against *Alternaria solani*, the causal agent of early blight disease on tomato. The bacterial and yeast strains were isolated from infected and uninfected tomato leaves, respectively. An *in vitro* bioassay on agar plates was used to screen the bacterial isolates, while inhibitory effects on spore germination of *A. solani* was used to screen the yeast isolates. The results for the *in vitro* bioassay on agar plates showed that 60% of the bacterial isolates inhibited mycelial growth of *A. solani,* with inhibition zones ranging from 6-14 mm. Thirty-five percent (35%) of the yeast isolates inhibited spore germination of *A. solani*. The five most effective bacterial isolates, XVT8, NC13, N6/2, WESH1 and N5, and the five most effective yeast isolates, P1-1, C10, P1-Orange, Y4 and H5, were selected for identification. Based on 16s rRNA sequence, Isolates N5, N6/2 and WESH1 were identified as *Bacillus subtilis* while NC13 was identified as *Pseudomonas putida* strain PYR1 and XVT8 was identified as a *Bacillus* spp. The yeast isolates were sent to Inqaba Biotechnical Industries for genomic identification. Isolates C10, P1-1 and Y4 were identified as *Meyerozyma guilliermondii*, Isolate P1-orange was identified as *Rodotorura minuta* and H5 was identified as *Pichia guilliermondii*. The five best bacterial and five yeast isolates were used for further studies.

2.1 Introduction

Tomato is regarded as one of the world's major vegetable crop and is grown under protected structures as well as under field conditions (Kumra and Phoeling, 2006). According to FAO (2001), it is an important crop for both small and large holder farmers in terms of land occupation, cultivated surface, cash and nutritional value. However, this crop encounters major losses due to numerous diseases affecting it. Early blight, caused by *Alternaria solani* Sorauer, has been reported to be one of the most destructive diseases affecting tomato [*Solanum lycopersicum* (L.) H. Karst.] production (Howell, 2007). Favourable environmental conditions such as high relative humidity, warm temperatures and abundant moisture have been reported to result in rapid disease development causing major yield losses (Agrios, 1988; Rotem, 1994). The fungus causes disease on foliage (leaf blight), stems (collar rot) and fruit, and can result in severe damage during all stages of plant development (Nash and Gardner, 1988).

Fungicides have been the most widely adopted control measure for this disease in most tomato production areas. However, due to environmental concerns, human health issues and the cost of these fungicides, there is a need for alternative methods of control (Herriot et al., 1986). The use of bio-based, eco-friendly, biodegradable plant derived or microbial derived products to control plant pathogens has been suggested as alternatives. According to Jagadeesh and Jagadeesh (2009), biological control agents offer a practical and economical alternative for the management of plant pathogens. Microorganisms such as bacteria and yeasts have been evaluated for their potential as biological control agents in controlling plant diseases. Biological control agents such as *Pseudomonas* spp have been used with success in controlling early blight on tomato (Sharma and Sharma, 2006). Other biological control agents such as *Streptomyces* spp and *Bacillus subtilis* have also been used to control *A. solani* (Mateascu et al., 2002; Sid et al., 2005).

Previous research has shown that yeast isolates can be used as biological control agents against various diseases (Elad et al., 1994). Studies conducted by Cook et al. (1997) and Dik et al. (2001) showed that epiphytic yeast can control *Botrytis cinerea* Pers.:Fr. by colonizing wounds of tomato plants. However, according to Pliego (2011), only few of these organisms actually

work when applied in the field. This is due to a lack of appropriate screening procedures in selecting for the potential organisms under laboratory conditions. According to Krauss (1996), isolating biocontrol agents is a straight forward procedure. However, isolates require proper screening to select the best ones. Field trials are said to be the most realistic approach as far as screening is concerned but are expensive (Pliego et al., 2011). In this study, dual culture plates as well as inhibition of spore germination were used to screen bacteria and yeast species versus *A. solani*. The aim of the study was to isolate potential bacterial and yeast BCA's from the phyllosphere of tomato plants and screen them for antagonistic activities against *A. solani in vitro*.

2.2 Materials and methods

2.2.1 Isolation of *A. solani*

Tomato plants showing early blight disease symptoms were collected from a farm near Pietermaritzburg, KwaZulu-Natal, South Africa. The pathogen was isolated from infected leaf samples. Several small tissue pieces from the infected parts were cut and surface sterilized by soaking in 2% sodium hypochlorite (NaOCl) solution for 5 min. They were then rinsed five times in sterile distilled water. The small tissues were then picked with sterile forceps and wrapped in a sterile paper towel to dry. The small tissues were then plated onto V8 tomato juice agar medium consisting of distilled water (800ml), V8 juice (200ml), $CaCO_3$ (2g) and agar (20g). The plates were incubated at 26 ±2°C and monitored daily for 7 days. Fungal growth suspected to be *A. solani* was aseptically transferred onto fresh V8 tomato juice agar plates and incubated to produce pure *A. solani* cultures. Wet mounts were prepared from pure cultures and viewed under a light microscope for observation of spores and hyphae. Dark coloured, muriform and beaked conidia with septate branched mycelium as well as light brown hyphae which turned darker with age were observed (Ellis and Gibson 1975; Rotem 1994). Cultural characteristics were observed directly by pigmentation on the growing medium and the mycelial growth pattern on PDA plates while sporulation was observed on samples from 10-day-old culture under the microscope. Pure cultures were stored in 30% glycerol at -80^0C for further use.

2.2.2 Pathogenicity test for *A. solani* isolates

A pathogenicity test of the *A. solani* isolate was carried out under greenhouse conditions. Two weeks old tomato seedlings were transplanted into 15 cm diameter pots containing composted pine bark growing medium. Two seedlings were planted per pot. Bacterial cultures were washed by adding sterile distilled water nto the agar plates containing the bacterial culture. The cell concentrations in the suspensions were determined using a Helber counting chamber (Paul Marienfield Superior GmbH & Co, Germany), and adjusted to 10^7 cells ml^{-1} before inoculation onto tomato plants.The seedlings in each pot were inoculated with an *A. solani* suspension using a hand spray at a concentration of 10^4 conidiam l^{-1}. The inoculated plants were covered with clear plastic bags overnight to maintain high relative humidity for fungal infection. Seedlings were monitored for the development of early blight symptoms. Plants with symptoms specific to early blight were sampled and the fungus was re-isolated as previously described in Section 2.1.1.

2.2.3 Isolation of biological control agents (BCAs)

(i) Isolation of bacterial BCAs

Approximately 10g sample of infected and un-infected leaves and stems of tomato plants were colleceted from Hanover farm in Pietermaritzburg. They were washed under running tap water and transferred into 500 ml conical flasks containing 200 ml of sterile distilled water. This was shaken vigorously on a rotary shaker at 180 rpm for 60 min. The leaves were removed and the liquid suspension was used to prepare serial dilutions of 10^{-1} to 10^{-4}. The dilutions were heated in a water bath at 100°C for 15 min. Aliquots of 0.1 ml were plated on tryptone soy agar (TSA) and incubated at 28ºC for 72 h. Individual bacterial colonies were transferred onto clean TSA plates and incubated for 48 h at 28ºC. A total of 171 pure bacterial colonies were selected based on morphological characteristic, sub-cultured and stored in 30% glycerol at -80°C for further use in the study.

(ii) Isolation of yeast BCAs

Approximately 10g sample of un-infected leaves and stems of tomato plants collected from Hanover farm in Pietermaritzburg were washed under running tap water and transferred into 500 ml conical flasks containing 200 ml sterile distilled water. The flask was placed on a rotary shaker at 180 rpm for 60 min to dislodge microorganisms from the leaf and stem surfaces. The leaves and stem sections were removed and the liquid suspension was used to prepare serial dilutions of 10^{-1} to 10^{-4}. One ml of each dilution was plated on nutrient yeast dextrose agar (NYDA) plates consisting of nutrient broth (8 $g.L^{-1}$), yeast extract (5 $g.L^{-1}$), dextrose (10 $g.L^{-1}$), agar (15 $g.L^{-1}$) and chloramphenicol (100 $mg.L^{-1}$). The agar plates were incubated for 48 h at 28ºC. Selected yeast colonies were re-streaked on malt extract agar plates consisting of malt extract (30 $g.L^{-1}$), peptone (3 $g.L^{-1}$), agar (15 $g.L^{-1}$) for single colony isolation. A total of 40 suspected yeast colonies were stored in 30% glycerol at -80°C for subsequent use in the study.

2.2.4 *In vitro* screening of bacterial isolates against *A. solani*

(i) Preliminary screening of bacterial isolates

One hundred and seventy-one bacterial culture plates were each separately washed with sterile distilled water using an L-shape glass rod and the suspensions were separately poured into Erlenmeyer flasks. The concentration of cells was adjusted as required using Helber counting chamber (Paul Marienfield Superior GmbH & Co, Germany). *In vitro* inhibition of mycelial growth of *A. solani* by the bacterial isolates was tested using a dual culture technique as described by Paulitz et al. (1992) and Landa et al. (1997). The experimental design used was a complete randomized design (CRD) with three PDA replicate plates for each isolate. Three 50 µl drops from a 10^8 cfu ml^{-1} bacterial suspension were equidistantly placed on the margins of potato dextrose agar (PDA) plates and incubated at 28°C for 24 h. A 4 mm^2 agar disc cut from fresh V8 agar cultures of *A. solani* was placed at the center of the PDA plates for each bacterial isolate and incubated at 28 ± 1°C for 10 d. The control was treated with distilled water. Data were obtained for the percentage inhibition by using the following formular:

ZOI = (100 x (R1 - R2)/R1 - R1; where R1 is radial growth of the pathogen in Control treatment and R2 is radial growth of the pathogen in dual culture with antagonist) and the width of the zone

of inhibition (ZOI) (measured as the smallest distance between the colonies in the dual culture plate)

(ii) Confirmation of inhibitory activity of selected bacterial isolates

Bacterial isolates selected from the first *in vitro* screening experiment were re-screened for the second time using the dual bioassay technique as described by Paulitz et al. (1992) and Landa et al. (1997). The experimental design used was a complete randomized design (CRD) with three PDA plates for each isolate. Pieces of agar blocks ($4x4m^2$) carrying mycelium of the pathogen grown on V8 media were cut and transferred into the center of each PDA plate. The agar plates were divided into four quadrants and sterile antibiotic disk were placed in each quadrant of the plates and the biocontrol agents were inoculated onto the disk. The plates were incubated at 28°C for 10 d and the inhibition diameters were measured. The above procedure was repeated for 20 bacterial isolates that showed potential to inhibit *A. solani*. The experiment was repeated twice.

2.2.5 *In vitro* screening of yeast isolates against *A. solani*

(i) Preliminary screening of yeast strains

Yeast isolates were screened using a spore germination inhibition test. The experimental design used was a completely randomized design (CRD), with each treatment replicated three times using three Eppendorf tubes for each isolate. Cell suspensions of the yeast isolate were prepared from 2-day-old culture on Malt extract agar plates at a concentration of 10^7 cells ml^{-1}. The plates were washed with sterile distilled water using an L-shaped glass rod and the suspensions were poured into a 50 ml Erlenmeyer flasks. The concentration of cells were adjusted as required using a haemocytometer. A spore suspension of *A. solani* was prepared from 10-day old culture on V8 agar plates at a concentration of 10^4 conidia ml^{-1}. Twenty (20) µl of yeast and 20µl of *A. solani* spore suspension were then added into an Eppendorf tube and mixed. The results were taken by counting the number of *A. solani* germinated (elongated germ tube) after incubation at 28°C, counted every 2 h for 10 h. The experiment was repeated twice and isolates that showed 50% inhibition were selected for secondary screening. The above procedure was repeated for the secondary screening of five selected yeast isolates.

(ii) Confirmation of spore germination inhibition of selected yeast isolates

Twenty yeast isolates showing potential to inhibit the germination of *A. solani* conidia were further screened using the above method described in Section 2.2.5 to confirm their ability to inhibit *A. solani* spore germination.

2.2.6 Identification of bacterial isolates using 16S rRNA sequence analysis

16S rRNA gene fragments from different isolates were amplified according to the method of Garbeva et al. (2003) using BacF, a *Bacillus* specific forward primer, in conjunction with R1378, a universal 16S rRNA reverse primer (Table 2-1) (Heuer et al., 1997). Each 25 µl reaction volume contained (1x) GoTaq® Flexi buffer, 1.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of primer, 1.25 U of GoTaq® DNA polymerase, approximately 50-100 mg template DNA made up to a final volume of 25 µl with nuclease-free water. Control reactions conducted without the DNA template were included in each round of amplifications. Thermal cycling was performed as follows: an initial denaturation, 94°C for 5 min; followed by 30 cycles of denaturation (94°C for 1 min), annealing (65°C for 90 s) and extension (72°C for 2 min); with a final extension of 72°C for 10 min. All samples were kept at 4°C. PCR amplification of the targeted gene fragment (~1300 bp) was confirmed by agarose gel electrophoresis. The resultant amplicons were sent to Inqaba Biotech™ (Hatfield Pretoria, RSA) for sequencing , where they were purified (Wizard PCR Prep Kits, Promega) before being sequenced using the ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA). Both the forward and reverse primers were used and reaction sequences were analysed with an ABI 3130*XL* sequence analyser (Applied Biosystems). The observed results were evaluated by using the standard nucleotide BLAST (BLASTn) from the NCBI web server against previously reported sequences using the GenBank/EMBL/DDBJ database for determination of 100% similarity.

Table 2.1. rRNA primers used for identification of bacterial isolates.

16S rRNA primers	Sequence (5´-3´)	References
P1- BacF	GGGAAACCGGGGCTAATACCGGAT 24	Garbeva et al., (2003)
P2- R1378	CGGTGTGTACAAGGCCCGGGAACG 24	Heuer et al., (1997)

2.2.7 Identification of yeast strains

The selected yeast isolates were sent to Inqaba Biotech for identification at BiotechTM (Hatfield Pretoria, RSA),

2.2.8 Statistical analysis

A general analysis of variance was performed on data collected using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc., 2011). Where ANOVA was significant means were separated using Duncan's multiple range test at a 5% probability level.

2.3 Results

2.3.1 Pathogenicity test for *A. solani* isolates

Three (3) days after inoculation of *A. solani* onto tomato seedlings disease symptoms were observed on all tomato plants. Based on the visual observation of early blight disease symptoms on the leaves, it was therefore concluded that the isolate was pathogenic as it produced typical early blight disease symptoms on tomato plants.

2.3.2 Antifungal bioassay

(i) Preliminary screening of bacterial isolates

A total of 171 bacterial isolates were screened for their ability to suppress *A. solani* in an *in vitro* dual culture bioassay. Of the 171 bacterial isolates, 20 were selected for secondary screening based on their primary screening performance. Sixty percent (60%) of the twenty bacterial isolates inhibited *A. solani* mycelial growth, with inhibition zones ranging from 6-10%. The level of inhibition varied with each bacterial isolate between experiments. Highly significant differences ($P < .001$) were observed among the isolates in the primary and secondary screenings. Isolate N6/2, WESH1 and N6212 caused similar zones of inhibition in primary and secondary screening (Table 2.2). Isolates that performed with consistency in the first and second experiments were selected for further testing under glasshouse conditions (Table 2.2).

Table 2.2. Inhibition of *Alternaria solani* mycelial growth by bacterial isolates on potato dextrose agar

Isolates	1st screening ZOI in mm	2nd screening ZOI in mm
HL22	0.0a	12.0lm
HP22	0.0a	11.3jkl
XVT15	0.0a	9.0de
XVT3	0.0a	0.0a
XVT5	0.0a	9.7efg
NC25	6.0b	11.0ijk
A16	8.3def	10.8ij
HS17	8.3def	10.0fgh
N621	8.7defg	8.7cd
N26	9.3fghi	9.3def
HP16	10.3ijkl	13.0n
N5	10.3ijkl	10.3ghi
N21	10.7jklm	0.0a
N6/2	11.0klmn	11.0ijk
NC13	11.0klmn	10.0fgh
N6212	12.0nopq	12.0lm
XVT8	13.0rstu	10efg
HP28	13.3rstu	12.3mn
XVT13	13.3rstu	9.3def
WESH1	14.0tuvw	14.0o
P-value	0.001	0.001
LSD	1.1	0.8
S.E.D	0.6	0.4
CV %	18.1	18.2

ZOI = zone of inhibition

Means with the same letter in the same column are not significantly different at $P > 0.05$.

Isolates with 0 inhibitions were excluded on the final screening based on their performance.

Isolates which did not show any zones of inhibition zone during the final screening were excluded from the table.

Table 2.3: Confirmation of inhibitory activity of selected bacterial isolates on potato dextrose agar

Isolates	ZOI in mm
HL22	9.7defghi
HP22	8.7cdefg
XVT15	10.7fghi
XVT3	8.0bcde
XVT5	10.0defghi
NC25	11.7hij
A16	8.0bcde
HS17	6.7bcd
N621	8.7cdefg
N26	9.7defghi
HP16	6.2bc
N5	10.3efghi
N21	9.7defghi
N6/2	11.0ghi
NC13	10.0defghi
N6212	12.0ij
XVT8	8.3bcdef
HP28	12.0ij
XVT13	8.3bcdef
WESH1	10.7fghi
P-value	0.0001
LSD	2.3
S.E.D	1.2
CV%	26.6%

ZOI = zone of inhibition

Means with the same letter in the same column are not significantly different at P > 0.05.

Isolates with 0 zone of inhibition –were excluded on the final screening based on their performance.

Isolates which did not show any zone of inhibition during the final screening were excluded on the table.

Isolates NC13, WESH1 (Figure 2-1B) and XVT8, N5 (Figure 2-1C) N6/21(Figure 2-1D) caused inhibition against *A. solani* whereas the Control (figure A) caused no inhibition. However, the greatest inhibition (13 mm) was observed with Isolate N6 2/1 (Figure 2-1D) and the least (7mm) with Isolate N5 in (Figure 2-1C).

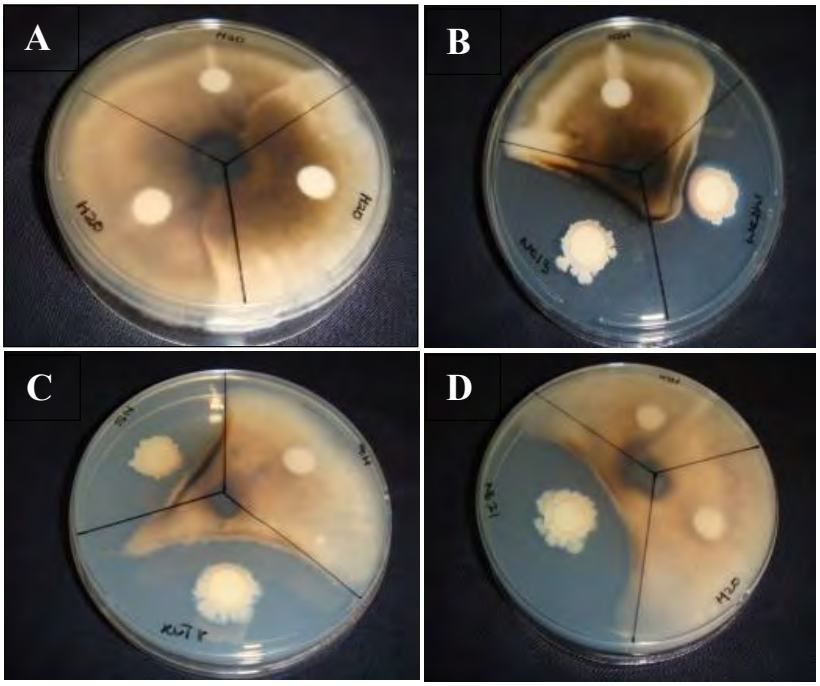

Figure 2.1. *In vitro* antifungal activity of bacterial isolates against *A. solani* on potato dextrose agar. Water control (A); Isolates NC13 and WESH1 (B); Isolates N5, XVT8 and water control (C); Isolates N62/ and two water control (D).

2.3.3 Preliminary screening of yeast

(i) Inhibition of conidial germination by yeast isolates

A total of 40 yeast-like organisms were isolated from tomato plants and screened for antagonistic activities *in vitro* using the conidial germination inhibition test. Isolates varied in their ability to inhibit the conidial germination of *A. solani*. Significant differences ($P < 0.05$) in percentage conidial inhibition were observed among the isolates. Five isolates strongly inhibited germination of *A. solani* conidia compared with the controls. All tested biocontrol agents caused

a difference in reduction of conidia germination of *A. solani* compared to the Control. Isolates Y4, H5 and P1-1 reduced conidial germination below 20% (Figure 2-2).

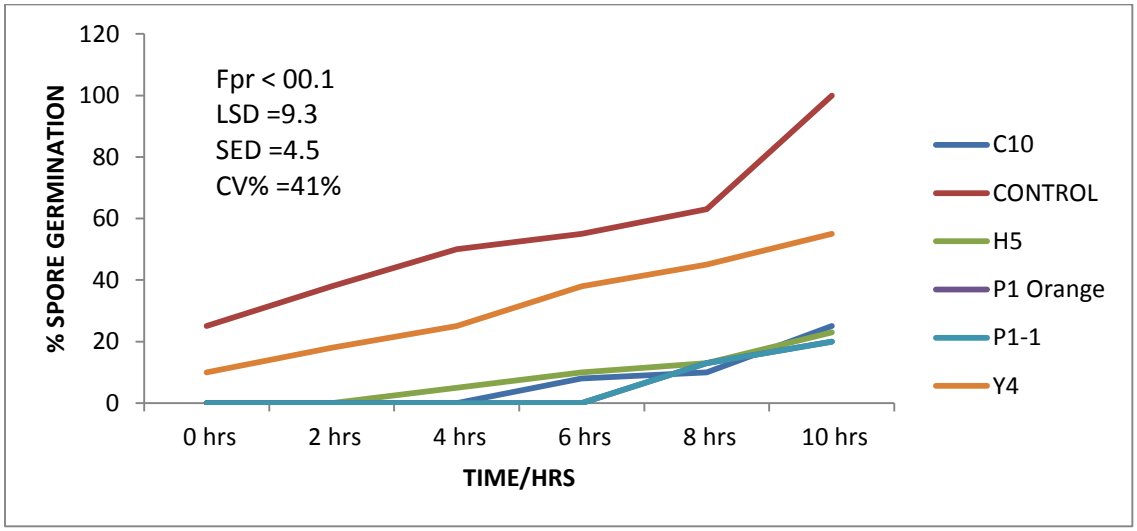

Figure 2.2. Inhibitory effect of five best yeast isolates on spore germination of *A. solani* in *in-vitro* over different time intervals.

(ii) Confirmatory screening for yeast

All five of the yeast isolates showed inhibitory effect against spore germination of *A. solani* with percentage spore germination less than 40%. Conidial germination varied between isolates. Isolate P1-Orange had the highest percentage (30%) of conidial germinated (Figure 2-3) while Isolate Y4 had the lowest level (18%) of conidial germination (Figure 2-3). As expected the control had the highest number of germinated conidial up to 100% at hours.

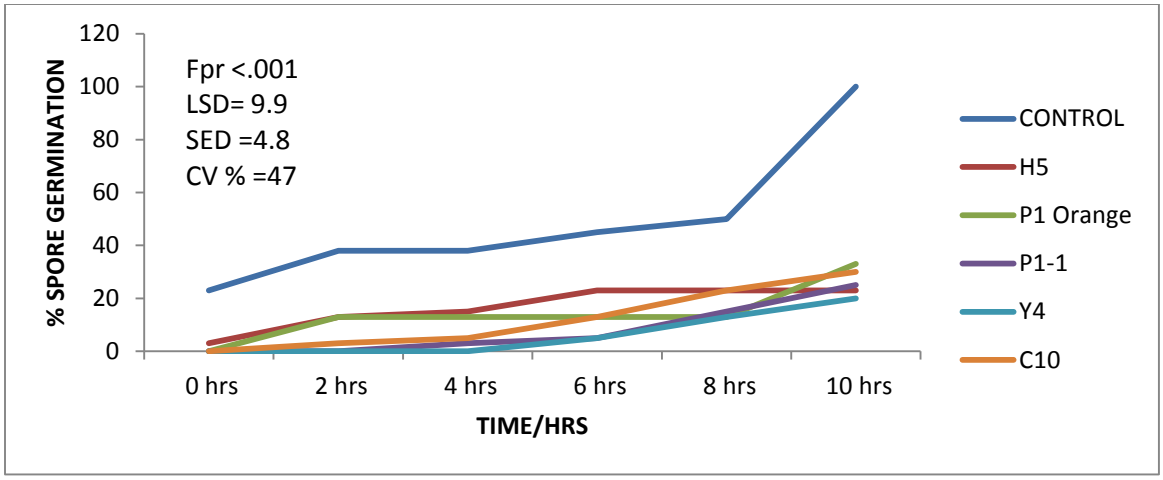

Figure 2.3. Inhibitory effect of five best yeast isolates on spore germination of *A. solani* in-vitro at different time intervals.

2.3.4 Identifications of biological control agents

(i) Identification of bacterial isolates

The PCR amplified products from nine bacterial isolates were sequenced and compared with 16S rRNA sequences in the NCBI database. Based on comparison of the partial sequences by BLAST of the three isolates WESH1, N6/21, N5, N62, HP28 were identified as *Bacillus subtilis* and the HP22 isolate as *Bacillus thuringiensis*, Isolate NC13 was identified as *Pseudomonas putida* while Isolate XVT3 was identified as *Bacillus subtilis* and Isolate XVT8 was identified as a *Bacillus* spp (Table 2.5).

Table 2.4. Identification of biological control agents using 16S rRNA sequence analysis.

S.No.	Isolate name	Identified species	Primer	E-value	% similarity	Accession number
1	WESH1	*Bacillus subtilis*	BacF and R1378	0.0	100%	KJ604990.1
2	N6/21	*Bacillus* spp.	BacF and 16S rRNA	0.0	100%	KJ000215.1
3	XVT8	*Bacillus* ssp.	BacF and 16S rRNA			KJ767389.1
4	HP22	*Bacillus thuringiensis*	BacF and 16S rRNA	0.0	99%	KF973233.1
5	N5	*Bacillus subtilis*	BacF and 16S rRNA	0.0	100%	KF982017.1
6	N62	*Bacillus subtilis*	BacF and 16S rRNA	0.0	100%	KF982026.1
7	HP28	*Bacillus subtilis*	BacF and 16S rRNA	0.0	100%	KF973233.1
8	NC13	*Pseudomonas putida*	BacF and 16S rRNA	0.0	100%	KP192770.1
9	XVT3	*Bacillus subtilis*	BacF and 16S rRNA	0.0	95%	

(ii) Identification of yeast

ITS sequence analysis indicated that Isolates C10, P1-1 and Y4 of the yeast biocontrol agents screened in this study belonged to the species *Meyerozyma guilliermondii*, *Pichia guilliermondii*, *Rodotorura minuta* respectively. Isolate P1-Orange was identified as *Rodotorura minuta* and Isolate H5 was identified as *Pichia guilliermondii* (Table 2-5).

Table 2.5. Blast and identification of yeast isolates.

Isolate name	**Identification species**	**Primer**	**E-value**	**% similarity**	**Accession number**
Y4	*Meyerozyma guilliermondii*	ITS	0.0	100%	JX455762.1
P1 ORANGE	*Rhodotorula minuta*	ITS	0.0	100%	JN837083.1
P1-1	*Meyerozyma guilliermondii*	ITS	0.0	100%	JX455762.1
C10	*Meyerozyma guilliermondii*	ITS	0.0	100%	JX455762.1
H5	*Pichia guilliermondii*	ITS	0.0	99%	HM037942.1

2.4 Discussion

The objective of this research was to isolate antagonistic bacterial and yeasts isolates and to evaluate their efficacy against *A. solani in vitro*. A total of 171 bacterial isolates and 40 yeast isolates were obtained from tomato leaf surface. Only 60% of the bacterial isolates inhibited *A. solani* mycelia growth, with inhibition zone ranging from 6-14mm. Thirty five (35%) of the yeast isolates inhibited spore germination of *A. solani*. Isolates of bacterial and yeast cells from tomato leaves exhibiting a wide range of inhibitory activity against several pathogens have been reported in various studies (Guetsky, 2002).

Dual culture with fungal pathogens on agar plates has often been used as a screening method (Kloepper and Schroth, 1981; Schroth and Hancock, 1982; Yazici, 2011). However, some authors have reported the method as being inappropriate. This is due to its exclusion of host antagonist-pathogen interacting factors as well as lack of selecting biological control agents based on other mechanisms such as root colonisation, induction of systemic resistance as well as niche competition (Lugtenberg et al., 2001; Bakker et al., 2003; Kamilova et al., 2005; Pang et al., 2009). In addition, the use of a spore germination inhibition test has also been reported as an efficient method in screening yeast isolates (Hejri, 2005).

In this study, the greatest inhibition was caused by *Bacillus* sp. N6 2/1(88%) and the least (56%) by *Bacillus subtilis* N5. This suggests that the isolates could be producing antifungal metabolites (AFMs) (Montealegre et al., 2003). Production of zones of inhibition agrees with the report of Basım (1990). It was suggested that *in vitro A. solani* interactions with *B. subtilis* AB-27 and AB-2 strains resulted in production of the targeted zones of inhibition. It has been reported that *B. subtilis* can secrete several antifungal metabolites such as *subtilin, bacitracin, bacillin* and *bacillomycin*, which belong to the iturin family (Alippi and Mónaco, 1994).

The use of *Pseudomonas* species as biological agents to control disease in various crops has been reported in several studies. However, there is no evidence of the organisms being used to control early blight in tomato. In a study conducted by Wei et al. (1991), it was reported that the application of a strain of *Pseudomonas putida* resulted in disease resistance against anthracnose disease of cucumber. In addition, Glandorf et al. (2001) reported that *Pseudomonas putida* supressed Fusarium wilt disease caused by *F. sp. vasinfectum* in cotton cultivar "Rowden" under field conditions. Other *Pseudomonas* species such as *Pseudomonas gladioli* B25 has been reported to control early blight disease by 60.2% using a combination of (seed treatment method + soil application method + nursery bed method + foliar spray method + root dip method) (Jagadeesh and Jagadeesh, 2009). In another study, twenty three bacterial isolates were found to strongly inhibit the growth of *A. solani* by forming inhibition zones larger than 5 mm using a dual culture *in vitro* assay on nutrient agar (NA) medium (Fontenelle et al., 2011). In another study conducted by Chitra et al. (2009) against *R. solani* in a susceptible variety of amaranth, *Pseudomonas sp*. Strain PN026R showed antagonism on NA medium. It was reported that the reduction in disease severity could be due to induction of systemic resistance caused by the organisms.

There is no evidence in the literature reporting on the use of yeast as biological control agents of *A. solani* in tomatoes. Nevertheless, in studies conducted by Elad et al. (1994) and Saligkarias (2002), evidence of significant biocontrol activity by strains of yeasts such as *Rhodotorula rubra* and *Candida pelliculosa* against *Botrytis cinerea* on tomato was observed. Other yeasts isolates have also been found to have efficacy against *B. cinerea*. The ability of yeasts to grow rapidly on the leaf, fruit and flower surfaces as well as in sugar-rich environments has been reported to result in exclusion of other microorganisms by means of competition for space and nutrients (Valdebenito-Sanhueza, 2000). In this study, yeast isolates that exhibited antagonistic properties against *A. solani* were observed. These isolates inhibited spore germination of *A. solani* in spore germination experiments. The isolates were identified as strains of *Rhodotorula minuta* and *Meyerozyma guilliermondii*. *Meyerozyma guilliermondii* (teleomorph) has been reported as biological control of *Botrytis cinerea* and *Penicillium expansum*. However, this organism has only been reported on its anamorphic state (*Candida guilliermondii*). *Meyerozyma guilliermondii* a recently assigned species name for *Candida guilliermondii* is often reported when the organisms is mentioned as biological control (Kurtzman and Suzuki, 2010). *Rhodotorula* strains have been reported to have antifungal activity against important plant pathogens including *B. cinerea* under iron-limited conditions (Calvente et al., 2001). *Rhodotorula minuta* has been identified as a biological control agent effective against *Colletotrichum gloeosporioides* that causes anthracnose of mango (*Mangiferae indica* L.) (Patiño-Vera et al., 2005). Control of grey mould rot using epiphytic yeasts of *Rhodotorula* and *Candida* spp. has been successful for postharvest treatments of strawberry (Helbig, 2002), bean, tomato (Elad et al., 1994; Kalogiannis et al., 2006), citrus fruits, apple, pear, kiwi fruit and table grapes (Lima et al., 1999; El Ghaouth et al., 2000). The results found in this study suggest that the bacterial isolate tested poses a good potential in controlling *Alternaria Solani.*

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CHAPTER THREE

In vivo screening of microbial antagonists against *Alternaria solani* on tomato

Abstract

Five bacterial isolates, (*Bacillus* spp. XVT8, *Pseudomonas putida* NC13, *Bacillus subtilis* N6/2, *Bacillus subtilis* WESH1 and *Bacillus subtilis* N5) and five yeast isolates (*Meyerozyma guilliermondii* P1-1, *Meyerozyma guilliermondii* C10, *Rodotorula minuta* P1-Orange, *Meyerozyma guilliermondii* Y4 and *Pichia guilliermondii* H5) that showed inhibitory effect against *A. solani* during *in vitro* studies were tested against *A. solani* under greenhouse conditions on tomato plants. The biological control agents were applied as foliar spray. The greenhouse results showed that *B. subtilis* N6/2, *B. subtilis* N5, *Bacillus* spp. XVT8 and *B. subtilis* WESH1 significantly (P = 0.001) reduced disease severity of early blight when compared to the *A. solani* inoculated control which showed a final disease severity of 97%. However, the yeast isolates had minimal effect on *A. solani* under greenhouse conditions. The best bacterial and yeast treatments were *Bacillus* spp. XVT8, *B. subtilis* N6/2, *Meyerozyma guilliermondii* C10 and *Pichia guilliermondii* H5 . The study demonstrated that the best two *Bacillus* isolates could be used in an integrated disease management strategy for the control of early blight on tomato.

3.1 Introduction

Various biological control agents have been found following screening of large numbers of microorganisms in both *in vitro* and *in vivo* studies (Berg et al., 2005; Kui et al., 2008). It has been reported that biological control agents found in this way require more extensive laboratory assays and require facilities with controlled environment. For selection of an effective biological control agent, screening should involve a simple and quick *in vitro* or *in vivo* antagonistic assay (Knudsen and Hockenhull, 1997; Kim et al., 2008).

A screening program could be described as the assessment of the potential of biocontrol agents to control diseases in plants. Initial screening is regarded as the most important aspect of the screening procedure. This is because for one to obtain success in developing a commercial biocontrol product, identifying the best isolates has to be done in precisely and conducted in a careful manner (Chiou and Wu, 2003). According to Merriman and Russell (1990) and Folman et al. (2003), failure in performance of many biological control agents lies in the lack of appropriate screening procedures in order to select microorganisms that are consistently effective in controlling the target pathogen. Furthermore, some of the screening procedures do not consider the effect of biotic and abiotic factors that may also influence the efficacy of a biological control agent. There has been an increase in the use of screening procedures that mimic conditions under which the agent will be used. According to Dowling and O'Gara (1994) and Glick (1999), screening systems that mimic field conditions are more likely to result in the selection of effective biocontrol agents. Therefore a combination of *in vitro* and *in vivo* screening procedures can lead to the identification of effective microbial strain that can be integrated into disease management system.

Studies have been conducted on the use of *Bacillus* spp. to control foliar disease on tomato [*Solanum lycopersicum* (L.) H. Karst.] plants. According to Sharma and Sharma (2006); Sid et al. (2005) and Mateascu et al. (2002), *Bacillus* spp. have been used to inhibit mycelial growth and spore germination of *A. solani*. Foliar application of *Bacillus coagulans* strain İK-22 and *Bacillus pumilus* significantly reduced disease severity of early blight on tomato (Yazici et al., 2011). However, there is no documented evidence on the use of yeasts to control early blight disease on tomato.

In this chapter, ten microbial antagonists selected from *in vitro* screening studies against *A. solani* were tested under greenhouse conditions for their ability to control early blight disease on tomato plants.

3.2 Materials and methods

3.2.1 Preparation of pathogen inoculum

A. solani was grown on V8 tomato juice agar (V8 tomato juice 200 ml, $CaCO_3$ 3 g, agar 20g in 1 L distilled water). The plates were incubated at 25°C for seven days as described in chapter 2. After 10-14 days, conidia were harvested in sterile distilled water (containing 0.01% of surfactant, Tween-20) by dislodging them off the agar plates with an L-bent glass rod and adjusted to 10^7 conidia.ml^{-1}, the required concentration, using a haemocytometer.

3.2.2 Greenhouse conditions for *in vivo* experiments

All *in vivo* experiments under greenhouse conditions were treated with the same conditions as described in this section.

The greenhouse trial was conducted at the University of KwaZulu-Natal, Pietermaritzburg South Africa. Seeds of the tomato cultivar Rodade were obtained from Starke Ayres (Pty) Ltd, Pietermaritzburg South Africa. The seed were sown in Speedling 128 cavity seedling trays filled with composted pine bark seedling mix (Gromor, Cato Ridge, South Africa). The seeded trays were kept in a greenhouse at a temperature of 26°C during the day and 18°C at night with a relative humidity of 75-85%. The seeds were watered once a day until germination and thereafter two times a day until transplanted using sprinkler irrigation. Irrigation water contained (per litre) NPK Starter Grower fertilizer 2:1:2(43) (1 g) plus trace elements (Ag-Chem Africa (Pty), Pretoria, South Africa). Seedlings were monitored, and at a two leaf stage they were transplanted into 15 cm diameter pots (one plant per pot) filled with composted pine bark (CPB) potting mix media. The seedlings were watered and kept under the same greenhouse conditions as previously described.

3.2.3 Efficacy of bacterial biocontrol agents under greenhouse conditions

Tomato seeds were planted, watered and fertilised as described in Section 3.1.2.

Five bacterial isolates, *Bacillus sp* XVT8, *Pseudomonas putida* NC13, *Bacillus subtilis* N6/2, *Bacillus subtilis* WESH1 and *Bacillus subtilis* N5, were cultured on tryptone soy agar (TSA) medium in 90 mm diameter petri dishes, and incubated at 28°C for 48 h. Bacterial cultures were washed by adding sterile distilled water nto the agar plates containing the bacterial culture. The cell concentrations in the suspensions were determined using a Helber counting

chamber (Paul Marienfield Superior GmbH & Co, Germany) and adjusted to 10^7 cells ml^{-1} before inoculation onto tomato plants. The bacterial suspension was applied as foliar spray using a pressurised 1 L hand spray bottle until run-off and left for 2 h. Plants were then pre-incubated by covering them with transparent plastic bags for 24 h and thereafter sprayed with *A. solani* spore suspension as prepared in Section 3.1.1. The experiment was arranged in a completely randomized design with three replications consisting of single plants per bacterial isolate treatment. A total of six treatments were used: five bacterial treatments and a control (no bacterial BCA treatment, but inoculated control). The plants were assessed for disease severity five days after treatment following a three days interval over a period of three weeks. The experiment was conducted twice.

3.2.4 Efficacy of yeast biocontrol agents under greenhouse conditions

Tomatoes were planted, watered and fertilised as described in Section 3.2.2.

Five yeast isolates, *Meyerozyma guilliermondii* P1-1, *Meyerozyma guilliermondii* C10, *Rodotorula minuta* P1-Orange, *Meyerozyma guilliermondii* Y4 and *Pichia guilliermondii* H5, were grown on malt extract agar (MA) (malt extract 30 g, mycological peptone 5 g and agar 20 g in 1L of distilled water) plates containing 0.1 mg of chloramphenicol and incubated for 48 h. The fresh cultures were used to prepare suspensions at concentrations of 10^4 cells ml^{-1} in sterile distilled water containing 0.01% Tween-80. The cells were dislodged with an L-shaped glass rod and the concentrate was poured into an Erlenmeyer flask. The concentration of cells for each was adjusted as required using a haemocytometer. The suspension was sprayed onto the tomato plants using a 1 L hand spray bottle until run-off and left for 2 h. Plants were then pre-incubated by covering them with transparent plastic bags for 24 h and thereafter sprayed with *A. solani* spore suspension as prepared in Section 3.2.1. The experiment was arranged in a completely randomized design with three replications consisting of single plants per yeast isolate treatment. A total of six treatments were used: five yeast treatments and a control (no yeast BCA treatment, but inoculated control). The plants were assessed for disease severity five days after treatment following a three days interval over a period of three weeks. The experiment was conducted twice.

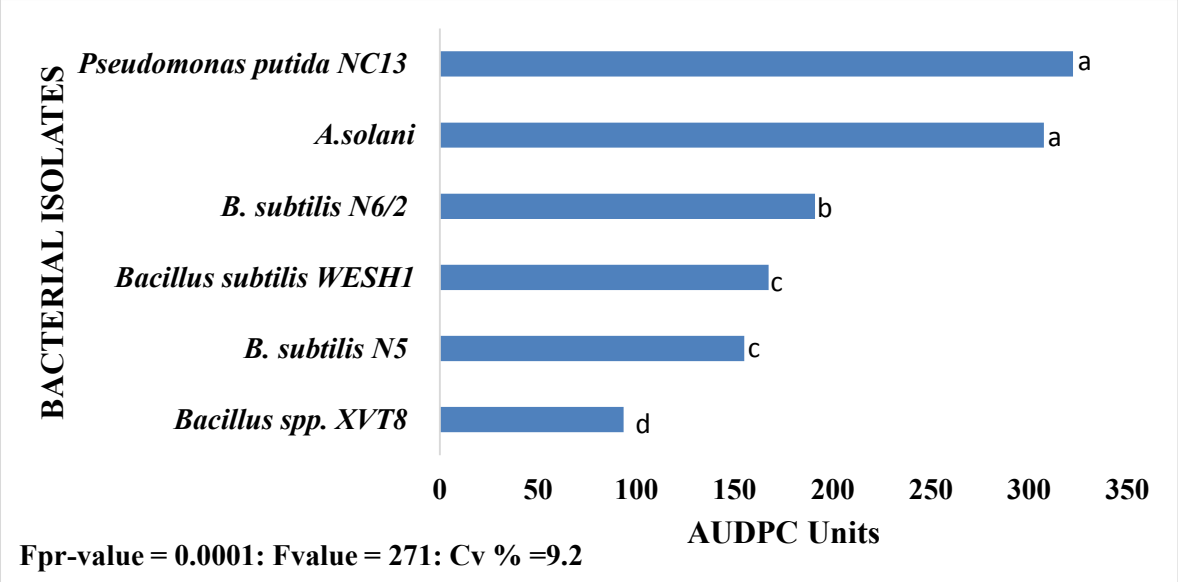
3.2.5 Data analysis

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 = 1 – 3%; 3 = 3 – 6%, 4=6-12, 5=12-25%, 6=25-50%, 7=50-60%, 8=60-70%, 9=70-80%, 10=80-90%, 11=90-100%]. 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.

3.3 Results

3.3.1 Effect of bacterial antagonists on early blight severity

Performance of the five bacterial antagonists against early blight under greenhouse condition is shown in Figure 3.1 and Table 3.1. Highly significant differences (P = 0.001) were observed amongst bacterial antagonists with regards to disease development. In figure 3.1 *Bacillus* spp. XVT8 was strongly antagonistic against the pathogen with the lowest AUDPC score (94), and the highest percent disease reduction (75%) compared to the pathogen inoculated control (308) (Figure 3.1; Table 3.1). Plants treated with *Pseudomonas putida* NC13 developed the highest AUDPC score (323) and showed early blight disease reduction of 11% (Table 3.1). The order of performance in terms of disease reduction when compared to the *A. solani* inoculated control was: *B. subtilis* XVT8 > *B. subtilis* N6/2 >*B. subtilis* WESH1 > *B. subtilis* N5 > *P. putida* NC13 (Figure 3.1 and Table 3.1).



Means followed by the same letter are not significantly different at 5% level of significance according to Duncan's multiple Range Test.

Figure 3.1. Effect of bacterial biological control agents on early blight AUDPC on tomato plants grown under greenhouse conditions.

Analysis of variance of the AUDPC values showed significant differences between treatments (P = 0.001). *Pseudomonas putida* NC13 caused no control against early blight with disease severity by 11% compared to the pathogen inoculated control. *Bacillus subtilis* WESH1 and *B. subtilis* N5 caused moderate levels of disease reduction ranging between 53-54%. *B. subtilis* N6/2 showed a disease control of 63% however, the highest disease reduction was caused by *Bacillus* spp. XVT8 with over 70% reduction in disease severity compared to the pathogen inoculated control in both experiments (Table 3.1).

Table 3.1. The efficacy of five bacterial isolates against early blight disease severity on tomato plants grown under greenhouse conditions

Treatment	Mean foliar disease severity[1,2,3]	% reduction	AUDPC[3,4]
Alternaria solani inoculated Control	97.7a	0.0	307.667a
Pseudomonas putida NC13	87.0b	10.7	322.5a
Bacillus subtilis N5	44.8c	52.9	155c
B. subtilis WESH1	43.8c	53.9	167.5c
Bacillus subtilis N6/2	35.2d	62.5	191b
Bacillus spp XVT8	23.0e	74.7	93.7d
Fpr	0.0001		0.0001
Fvalue	263.7		271
Cv %	7.3		9.2

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

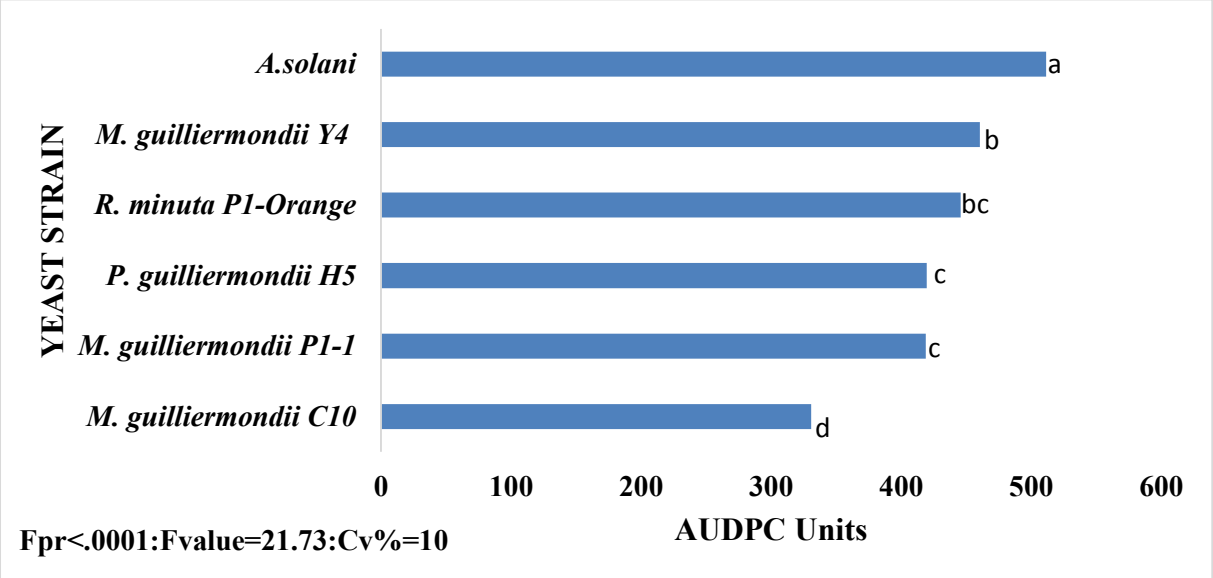
[2]Ratings made on whole plant at three days after inoculation with *Alternaria solani* [1 = 0%; 2 = 1 – 3%; 3 = 3 – 6%, 4=6-12, 5=12-25%, 6=25-50%, 7=50-60%, 8=60-70%, 9=70-80%, 10=80-90%, 11=90-100%].

[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.

3.3.2 Effect of yeast isolates on early blight severity

Analysis of variance of the AUDPC values showed significant differences (P = 0.001 between the treatments. Disease control levels were not significantly different among *Alternaria solani inoculated control* and *Meyerozyma guilliermondii* Y4 (Figure 3.3). There were no significant differences between *Meyerozyma guilliermondii* P1-1 and *Pichia guilliermondii* H5. *Meyerozyma guilliermondii* C10 was the best yeast isolate with an AUDPC value of 331 units.



Means followed by the same letter are not significantly different at 5% level of significance according to Duncan's multiple range test.

Figure 3.2. Effect of yeast biological control agents on early blight AUDPC on tomato plants grown under greenhouse conditions.

There were significant differences among the five yeast isolates (Table 3.2). However, all the yeast isolates tested caused minimal disease reduction of less than 20%. Disease severity ranged from 81-98% which indicates a high disease severity of early blight (Table 3.2). *Meyerozyma guilliermondii* C10 and *Pichia guilliermondii* H5 were the best two isolates.

Table 3.2. The efficacy of five yeast isolates against early blight disease severity on tomato plants grown under greenhouse conditions

Treatment	**Mean foliar disease severity[1,2,3]**	**% reduction**	**AUDPC[3,4]**
Alternaria solani inoculated Control	100a	0.0	511.5a
Meyerozyma guilliermondii Y4	98.0b	2.0	460.7b
Meyerozyma guilliermondii P1-1	86.9c	13.1	419c
Rodotorura minuta P1-Orange	84.1c	15.8	445.8bc
Meyerozyma guilliermondii C10	81.9c	18.0	330.8d
Pichia guilliermondii H5	81.9c	18.0	419.8c
Fpr	0.0001		0.0001
Fvalue	27.04		21.73
Cv	7.07		10

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

[2]Ratings made on whole plant at three days after inoculation with *Alternaria solani* [1 = 0%; 2 = 1 – 3%; 3 = 3 – 6%, 4=6-12, 5=12-25%, 6=25-50%, 7=50-60%, 8=60-70%, 9=70-80%, 10=80-90%, 11=90-100%].

[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.

3.4 Discussion

Screening for potential biocontrol agents based on their antagonistic properties against pathogens is still regarded as a practical approach for identifying effective candidate strains from among abundant plant-associated micro-organisms (Raaijmakers et al., 2002). The aim of the study was to evaluate potential bacterial and yeast biological control agents selected from *in vitro* studies (Chapter Two) for their antagonistic effect against early blight of tomato under greenhouse conditions. According to Fravel (2005) and Mari and Guizzardi (1998), yeast and bacteria were successfully used as biological control agents on fruits and vegetables against various diseases caused by different pathogens.

In this study, five bacterial and five yeast isolates were tested for their inhibitory effect against early blight disease under greenhouse conditions. *Bacillus* spp. XVT8, *B. subtilis* N5, and *B. subtilis* N6/2 showed potential in controlling early blight disease of tomato. However, *Pseudomonas putida* NC13 did not show any ability to reduce early blight severity.The low disease reduction percentage observed corresponds with the AUDPC value which also showed that the treatment were less effective compared to the *Bacillus* spp. XVT8, *B. subtilis* N5, and *B. subtilis* N6/2. AUDPC value of the two effective isolates (*Bacillus* spp. XVT8 and *B. subtilis* N5) and the disease percentage reduction indicated high effectiveness against early blight when compared to the *in vitro* performance. Different strains of *Bacillus* spp. have been reported to aid in promoting plant growth as well as controlling diseases (Kloepper et al.2004). In addition, they are reported to compete with other pathogens for nutrients such as iron and phosphate and also produce secondary metabolites such as antibiotics which suppreses some plant pathogens and others by competing for nutrients such as iron and phosphate (Gardener, 2004). The results observed in the study correlates with those of Adebayo and Ekpo (2005) who reported that *B. subtilis* inhibited the growth of fungal pathogens associated with tomato. It was reported that *B. subtilis* had an effect in inhibiting fungal growth and also promoted growth of tomato. Abdel-Kader (2012) also reported that application of *B. subtilis* showed significant reduction in disease incidence of early blight in tomato when compared with the biological controls which were tested. According to Sharma and Sharma (2006) and Sid et al. (2005), *B. subtilis* was successfully used to inhibit mycelial growth and spore germination of *A. solani*. The *Bacillus* isolates used in this study were selected based on their inhibitory effects on mycelial growth of *A. solani* (Chapter 2). We found this method to be equally effective because two of the *Bacillus* isolates consistently

reduced final disease severity of early blight on tomato under greenhouse conditions in two separate experiments conducted. Two of the bacterial isolates, *B. subtilis* N6/2 and *B. subtilis* XVT8 had the highest reduction of early blight severity on tomato plants under controlled greenhouse conditions. What remains to be seen is if these isolates would perform well under uncontrolled environments, i.e., nursery and field conditions.

The performances of the yeast isolates during greenhouse studies on tomato plants did not correlate with those obtained from *in vitro* studies. High AUDPC as well low disease percentage reduction indicated that all the yeast isolates failed to significantly reduce early blight disease severity under greenhouse conditions. Various observations suggest that competition for nutrients between yeast and plant pathogens is likely to be the main mechanism of action (Janisiewicz et al. 2000). Therefore, it is possible that the failure of the yeast to control the disease could be that the tested isolates were not competitive enough to reduce the effect of *A. solani* under the conducive greenhouse conditions. Moreover, the *A. solani* inoculum used in this study was more than that of the yeast inoculum which means the pathogen could possibly have overwhelmed the competitive abilities of the yeast isolates. Kohl et al. (1995) reported that only strong competitors have the ability to protect necrotic tissue from external colonization by pathogens. Kloepper (1991) recommended the use of rapid pre-screening techniques such as a radicle assay as well as a hypocotyl assay allowing large number of strains to be tested. Cook and Baker (1983) mentioned that the host plant is a participant in virtually any biological control aimed at suppressing the disease producing activities caused by the pathogen. Hence, it is essential that biological control agents selected under laboratory conditions should vigorously be tested on target host-pathogen combinations.

Chérif et al. (2002) and Fravel (2005) explained that antifungal activities *in vitro* do not always correlate with disease reduction *in vivo*. Ran et al. (2005) also observed the lack of correlation between *in vitro* and *in vivo* effectiveness of biological control agents. However, some authors have reported a positive correlation between *in vitro* and *in vivo* studies (Askew and Laing, 1994; Glick, 1995; De Boer et al., 1999; Zhang et al., 1999; Khalid et al., 2004). The results obtained in the current study suggest that the bacterial isolates selected during *in vitro* studies have potential as biocontrol agents against *A. solani* on tomato. However, further nursery/field studies need to be conducted to ascertain the real potential of these bacteria biological control agents under field conditions.

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CHAPTER FOUR

The effect of potassium silicate and a plant defence activator, acibenzolar-s-methyl, on early blight of tomato under greenhouse conditions

Abstract

Different concentrations of potassium silicate (KSil) and acibenzolar-S-methyl (ASM) were evaluated for their efficacy in controlling early blight disease of tomato under greenhouse conditions. Four concentrations (50, 100, 150 and 200 ppm) of a liquid formulation of potassium silicate (KSil) and four reduced acibenzolar-S-methyl (ASM) concentrations (25, 50, 75 and 100 percent) were used in the study. None of the potassium silicate concentrations significantly reduced disease severity of early blight under greenhouse condition ($P > 0.05$). The 75 and 100 percent concentrations of ASM significantly ($P = 0.001$) reduced disease severity of early blight by 52 and 68% respectively. Analysis of the distribution of silicon as a results of potassium silicate applications in the tomato plant showed that there was minimal uptake of potassium silicate and hence accumulation of silicon in the tomato plants. There were no significant differences in silicon accumulation observed between the different potassium silicate treatments in the leaves as well as the stems ($P > 0.05$).

4.1 Introduction

At present, application of selected fungicides continues to be the primary means of control of early blight disease of tomato. However, these chemicals have been reported to be hazardous on the environment as well as human health (Yazici et al. 2011). Alternative control strategies are therefore being sought as a possible solution to the problem (Abdel-Kader. 2012). Induced resistance has been used successfully in plant disease control since 1950. This strategy has been reported to have several benefits such as increasing the ability of susceptible plants to withstand pathogens in a non-genetic way (Kuć, 2001). Various types of inducers including microorganisms (fungi, bacteria), chemicals, metabolic substances of the host plant, plant extracts as well as ultraviolet light have been reported to induce resistance (Métraux et al., 1991; Kessmann et al., 1994; Achuo et al., 2004; Obradovic and Jones,

2005). Silicon and acibenzolar-S-methyl (ASM) have been reported to be among these chemical compounds which have been used as disease resistance inducers in plants.

Csosze et al. (1999) reported that ASM is a member of a novel class of inducers of systemic acquired resistance which activates gene expression and disease resistance in plants. According to Louws (2000), the compound has no direct microbial activity, however, "it assists the plants to obtain pre-infection biochemical processes that confer resistance to the same spectrum of pathogens as a biological elicitor". ASM has demonstrated an ability to manage a number of plant diseases including bacterial spot on tomato [*Solanum lycopersicum* (L) H. Karst.] as well as tomato spotted wilt (Csinos et al. 2001; Huang, 2012). Even though ASM has shown efficacy in disease suppression, negative effects have been reported, such as reduced plant growth and yield (Csinos et al. 2001; Louws et al. 2001). According to Louws et al. (2001), many growers are reluctant to adopt ASM due to documented concerns over reduced or delayed yields as well as whether the achieved control justifies the additional cost of ASM compared with the use of fungicides. Nevertheless, even though high application rates of ASM have been studied, the use of low application rates has often been overlooked.

Fertilisation with plant-available forms of silicon has been shown to reduce a number of diseases in various crops such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), sugarcane (*Saccharum* sp. L.), maize (*Zea mays* L.), and cucumber (*Cucumis sativa* L.), as well as citrus. Even though mechanisms in which silicon compounds function is not clear it has been shown to be efficient in reducing levels of plant diseases (Epstein. 1994; Liang and Ding, 2002). Some authors agree that silicon acts as a physical barrier in cell walls, preventing the penetration of fungal hyphae into host tissues (Ma and Yamaji, 2006). However, others believe that the disease reduction effects provided by silicon are related to the priming of plant defense reactions (Rodrigues et al. 2003; 2004; Yang et al. 2003). Ma and Yamaji (2006) reported that for a plant to benefit from silicon, it has to be able to acquire the element in high concentrations, whether it is a monocotyledon or a dicotyledon. Tomato is regarded as a non-accumulator because it has a rejective mode of uptake which tends to minimise silicon uptake (Mitani and Ma, 2005). The objective of this study was to evaluate range of reduced concentrations of ASM and potassium silicate (KSil) for their efficacy in suppressing early blight of tomato under greenhouse conditions.

4.2 Materials and methods

4.2.1 Preparation of pathogen inoculum

A cultures of A. Solani was grown on V8 tomato juice agar [V8 tomato juice 200 ml, $CaCO_3$ 3 g, agar 20g in 1 L distilled water]. The plates were incubated at 25°C for seven days as described in (Chapter 2). After 10-14 days, conidia were harvested in sterile distilled water (containing 0.01% of a surfactant, Tween-20) by dislodging them off the agar plates with an L-bent glass rod, and then adjusting their concentration to 10^7 conidia ml^{-1}using a haemocytometer.

4.2.2 Greenhouse conditions for *in vivo* experiments

The greenhouse trial was conducted at the University of KwaZulu-Natal, Pietermaritzburg South Africa. Seeds of tomato (*Solanum lycopersicum* (L.) H. Karst.) of the cultivar Rodade were obtained from Starke Ayres (Pty) Ltd, Pietermaritzburg, South Africa. The seed were sown in Speedling 128 cavity seedling trays filled with composted pine bark seedling mix (Gromor, Cato Ridge, South Africa). The seeded trays were kept in a greenhouse at a temperature of 26°C during the day and 18°C at night with a relative humidity of 75-85%. The seeds were watered once a day until germination and thereafter two times a day until transplanted using sprinkler irrigation. Irrigation water contains (per litre) NPK Starter Grower fertilizer 2:1:2(43) (1 g) plus trace elements (Ag-Chem Africa (Pty), Pretoria, South Africa. Seedlings were monitored, and at a two leaf stage they were transplanted into 15 cm diameter pots filled with a composed pine bark (CPB) potting mix media. The seedlings were watered and kept under the same greenhouse conditions as previously described.

4.2.3 Evaluating the effect of different concentrations of potassium silicate on early blight disease under greenhouse condition

Tomato plants (1 plant/pot) were grown in 150 mm diameter pots containing composted pine bark in a greenhouse maintained at 25 – 28ºC with a relative humidity (RH) of 75%. There were four treatment concentrations of KSil including an untreated, inoculated Control. These were 0 (Control), 50, 100, 150, and 200ppm. Potassium chloride (KCl) salts was also used as a positive control to quantify the effect of the potassium. A liquid formulation of dissolved potassium silicate Agrisil K50 (PQ Corporation, South Africa) with a concentration of 20.5% silicon was used as the silicon source. The treated plants in pots were arranged in a completely randomised design with three replicates per treatment concentration. Plants were

then pre-incubated by covering them with transparent plastic bags for 24h and thereafter sprayed with an *A. solani* spore suspension and again covered with a transparent plastic bag to increase humidity. The plants were fertilized weekly with 150 ml per pot of limestone ammonium nitrate (LAN) to ensure that nutrients were not a limiting factor for growth. The plants were assessed for disease severity three days after treatment following a three days interval over a period of three weeks. The experiment was conducted twice.

4.2.4 Effect of acibenzolar-S-methyl (ASM) on early blight under greenhouse conditions

Four concentrations of ASM were evaluated in this study. These were: 25%=0.02 $g.L^{-1}$, 50%=0.03 $g.L^{-1}$, 75%=0.04 $g.L^{-1}$ and 100%=0.075 $g.L^{-1}$, where 100%=0.075 $g.L^{-1}$ is the recommended concentration. 500ml of treatments were sprayed onto five weeks old tomato plants until run-off and left for 24 h. Plants were then pre-conditioned by covering them with transparent plastic bags for 24 h and then spraying them with an *A. solani* 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 three replications consisting of single plants per ASM treatment. A total of five treatments were used: four different ASM treatments and a pathogen-inoculated control. ASM treatments were applied every 14 days. The plants were assessed for disease severity three days after treatments were applied following a three days interval over a period of three weeks. The experiment was conducted twice.

4.2.5 Uptake and accumulation of potassium silicate in tomato plants

The experiment was set up in the same way as described under Section 4.2.3 above with the exception that the tomato plants were not treated with the pathogen, *A. solani*. There were four KSil treatments (50, 100, 150, and 200 $g.L^{-1}$) and a KCl control. Leaf samples (three leaves per replicate) were taken from each treatment each week for a period of six weeks. The samples were frozen at -80 ^{0}C until used for analysis to determine the silicon accumulation levels. The experiment was done twice.

(i) Setting up a standard curve

Quantities of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 ml of Si standard solution was separately transferred into a 50 ml volumetric flask. Thirty (30) ml of acetic acid (20%) and 10 ml of ammonium molybdate solution (54 $g.L^{-1}$, pH 7.0) was added and the volumetric flasks were shaken up to mix the content thoroughly. After 5 min, 5 ml of 20% tartaric acid

and 1 ml reducing solution was added. The content solution was adjusted to 50 ml with 20% acetic acid. The reducing solution was made by mixing Solution A (2 g of Na_2SO_3 and 0.4 g of 1-amino-2-naphthol-4-sulfonic acid in 25 ml of ddH_2O) and Solution B (25 g of $NaHSO_3$ in 200 ml of ddH_2O). This was adjusted to 250 ml with ddH_2O and stored in a tightly stoppered plastic bottle in the dark. The absorbance was measured at 650 nm after 30 min using a CT-8 Series Double Beam UV/VIS Spectrophotometer E-Chrom Tech (Chrom Tech-CT, Taiwan).

(ii) Pre-treatment of leaf samples

A 100 mg leaf sample previously dried at 70ºC for one week and ground powder using a mortar and pestle. The sample powder was transferred into a 100 ml polyethylene tube. A 3 ml solution of 50% NaOH was added and the tube was covered with a loose-fitting plastic cap. It was gently vortexed, then autoclaved at 121℃ for 20 min. The solution was transferred to volumetric flask and adjusted to 50 ml with ddH_2O. This procedure was repeated for all the leaf samples from the various KSil treatments.

(iii) Sample determination

One millilitre (1 ml) of a pre-treated leaf sample solution was transferred to a 50 ml volumetric flask. A 30 ml solution of 20% acetic acid and 10 ml of ammonium molybdate solution (54 $g.L^{-1}$, pH 7.0) were added and the volumetric flask was shaken for the content to mix thoroughly. After 5 min, 5 ml of 20% tartaric acid and 1 ml of the reducing solution were added and adjusted to 50 ml with 20% acetic acid. The mixture was allowed to stand for 30 min and the absorbance was measured at 650 nm using a CT-8 Series Double Beam UV/VIS Spectrophotometer E-Chrom Tech (Chrom Tech-CT, Taiwan).

4.2.6 Scanning electron microscopy analysis of tomato plant leaves treated with potassium silicate

Fresh leaf samples which were stored at -80 ^{0}C were used for silicon microanalysis using scanning electron microscopy (SEM) using Energy Dispersive X-ray (EDX). Samples were prepared for SEM by primary and secondary fixation. The fixation protocol began by immersing two pieces of 0.5 mm of excised epidermis in 3% glutaraldehyde. They were then treated twice with sodium cacodylate buffer for 5 min. For secondary fixation, the samples were fixed in 20% osmium tetroxide. Samples were rinsed in sodium cacodylate buffer for another 5min. They were then dehydrated in graded ethanol series (30%, 50%, 70%, 90% and

100%) for 10 min. The samples were transferred to critical point drying (CPD) machine basket in 100% ethanol (Quorum K850 critical point dryer from Quorum Technologies). During CPD, the ethanol was replaced with liquid CO_2. The CO_2 was heated and pressurized to its critical point at which the liquid converts to gas without damaging effect of the surface tension on the sample. The samples were then mounted on aluminium stubs with double sided sticky tape and sputter coated with gold (Eiko IB.3 ion coater, Tokyo, Japan). The dried samples were then viewed using SEM.

4.2.7 Statistical analysis

Disease ratings of percentage foliar infection consisted of an estimate of percent area infected (% LAI) using a Horsfall-Barratt Scale (1945) where [1 = 0%; 2 = 1 – 3%; 3 = 3 – 6%, 4=6-12, 5=12-25%, 6=25-50%, 7=50-60%, 8=60-70%, 9=70-80%, 10=80-90%, 11=90-100%]. The estimates were used to calculate the Area Under the Disease Progress Curve (AUDPC) for each treatment (Shanner and Finney, 1977). The AUDPC values, the final disease severity values (arcsine transformed) and the silicon quantities in leaf samples 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 a 5% probability level.

4.3 Results

4.3.1 Efficacy of different potassium silicate concentrations on disease severity of early blight

Significant differences at (P = 0.0001) were observed among the AUDPC values resulting from the four different concentration of KSil. The KCl treated control and 100ppm were not significantly different from water treated control, however they were significantly different compared with other treatments (Figure 4.1).

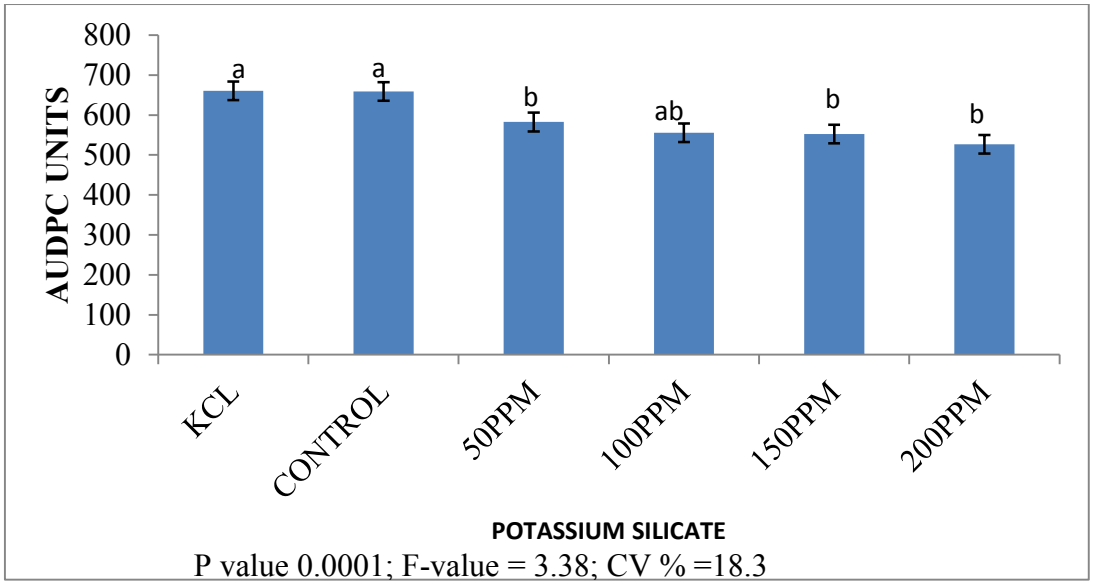

Figure 4.1. Effect of different liquid potassium silicate concentrations on severity of early blight under greenhouse conditions.

Significant differences (P = 0.0089) were observed between the different KSil treatments with regards to disease severity and AUDPC units. Plant treated with KSil showed minimal disease control. None of the plants treated with KSil concentrations reduced disease severity over 12% relative to the water control (Table 4.1).

Table 4.1. Evaluation of the efficacy of several potassium silicate concentrations for the control of early blight disease on tomato under greenhouse condition

Treatment	Foliar disease severity[1,2,3]	% Early blight reduction	AUDPC[3,4]
KCl	89b	- 0.3	661a
0 (Water Control)	88.7a	0	659a
50 ppm	84.9b	4.3	583b
100 ppm	84.7b	4.5	555ab
150 ppm	82.9c	6.5	552b
200 ppm	78.4a	11.6	527b
F – ratio	8.66		3.38
P – value	0.0089		0.0001
CV%	5.5		18

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

[2]Ratings made on whole plant at three days after inoculation with *Alternaria solani* [1 = 0%; 2 = 1 – 3%; 3 = 3 – 6%, 4=6-12, 5=12-25%, 6=25-50%, 7=50-60%, 8=60-70%, 9=70-80%, 10=80-90%, 11=90-100%].

[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.

4.3.2 Efficacy of various ASM concentrations on disease severity of early blight

There were significant differences (P = 0.001) in the AUDPC values after treatment with the four ASM concentrations, and the pathogen inoculated control. ASM at a 75% concentration (0.056 g l^{-1}) and at 100% (0.075 g l^{-1}) were the most effective in reducing the levels of early blight, with AUDPC units below 150. All the ASM treatments were significantly better than the pathogen inoculated control (Figure 4-2).

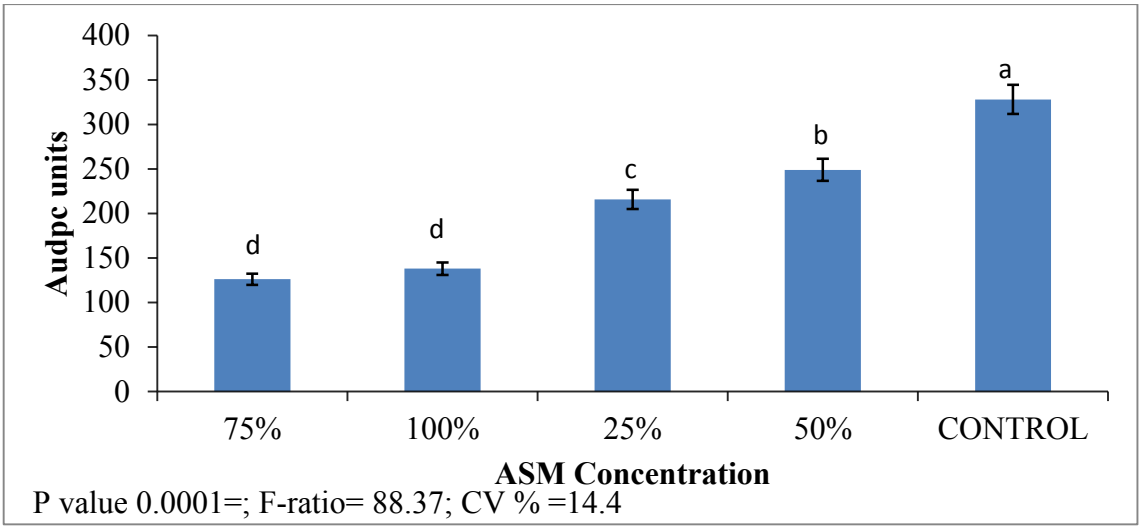

Figure 4.2. Effect of different concentrations of acibenzolar-S-methyl on disease AUDPC of early blight under greenhouse conditions.

Significant differences (P = 0.001) were observed among the ASM treatments (P = 0.0001). The 75% concentration (0.056 g l^{-1}) and 100% (0.075 g l^{-1}) treatments provided equivalent control levels that were not significantly different, but were significantly more effective than the treatments of 25% (0.018 g l^{-1}) and 50% (0.038 g l^{-1}). (Table 4.2).

Table 4.2. Evaluating the efficacy of acibenzolar-S-methyl on early blight severity under greenhouse conditions

Treatment	Foliar disease severity[1,2,3]	% Early blight reduction	AUDPC[3,4]
Control	80a	0	328a
25% (0.018 g l^{-1})	40b	48	216c
50% (0.038 g l^{-1})	40b	48	249b
75% (0.056 g l^{-1})	39c	67	126d
100% (0.075 g l^{-1})	32b	57	138d
P – value	0.0001		0.0001
F– ratio	163.49		88.3
CV%	7.9		14.4

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

[2]Ratings made on whole plant at three days after inoculation with *Alternaria solani* [1 = 0%; 2 = 1 – 3%; 3 = 3 – 6%, 4=6-12, 5=12-25%, 6=25-50%, 7=50-60%, 8=60-70%, 9=70-80%, 10=80-90%, 11=90-100%].

[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.

4.3.3 Uptake and accumulation of silicon in tomato plants

(i) Standard curve

Linear regression equation for the determination of silicon content was acquired as y = 0.0243x – 0.0016, R^2 = 0.887 is shown in Figure 4.3, where y = OD value and x is the time in weeks for setting up the standard curve (Figure 4.3).

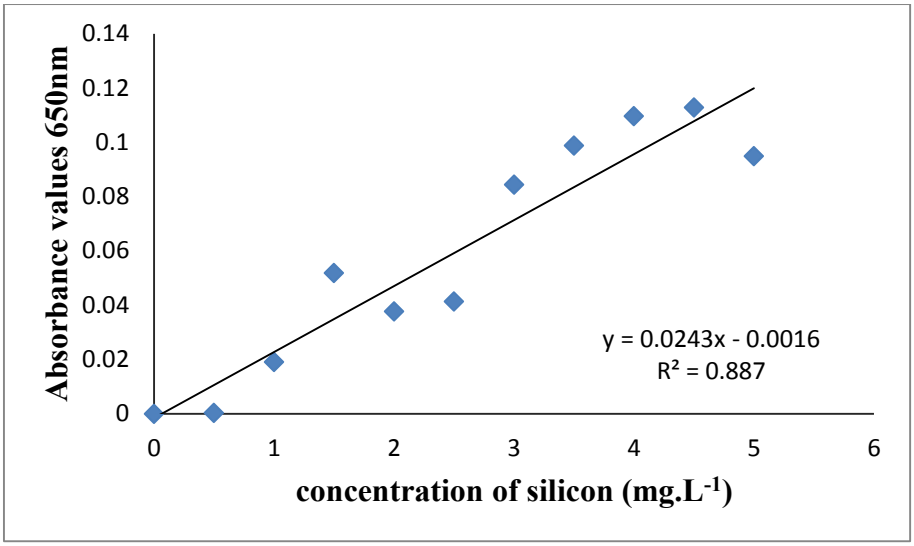

Figure 4.3. A standard curve for the determination of the silicon content in tomato leaves

(ii) Silicon accumulation in tomato plants

Accumulation of silicon in stems and leaves was evaluated after six weeks. The results indicate that there no significant difference across the silicon treatments at P = 0.005. Silicon accumulation in stems was significantly higher than in the leaves with the highest recorded level of 0.93 mg g^{-1} of leaf sample (Table 4.3). The results showed that stems accumulated more silicon, even in silicon-deprived control plants. The ANOVA showed that there was no difference between any of the treatments and the control in Week2, Week3, Week4 and Week6 (Figure 4.5). Significant differences were observed on week5.

Table 4.3. Levels of silicon uptake and accumulation in leaves and stems after six weeks of growth under greenhouse conditions

	Time/weeks					
Treatment Concentration (ppm)	**WK2**	**WK3**	**WK4**	**WK5**	**WK6**	**STEMS**
KCL	0.057a	0.059a	0.063a	0.065ab	0.068a	0.093a
50	0.058 a	0.063b	0.064a	0.064a	0.069a	0.091a
100	0.059a	0.062b	0.063a	0.066b	0.069 a	0.093a
150	0.063b	0.063b	0.064a	0.066b	0.070b	0.092a
200	0.057a	0.061b	0.064a	0.065ab	0.069a	0.092a
F-ratio	0.11	0.53	0.19	0.14	0.51	0.79
P-value	0.37	0.71	0.94	0.10	0.73	0.42
CV (%)	13.07	3.33	10.7	4.3	4.3	3.0

Means with the same letter are not significantly different (P = 0.05) according to Duncan's multiple range test.

4.3.4 Silicon levels in leaves using Energy Dispersive X-ray (EDX)

Minimal absorption of silicon over a period of six weeks was observed in tomato leaves using EDX on a scanning electron microscope (Table 4.4). Silicon mapping showed that leaves accumulated more silicon, even in the control plants that were not provided with additional silicon. There were no significant differences between the treatments during week except for treatment 150ppm which had a slightly higher silicon absorption compare to other treatments. Week 1. Even though the level of silicon absorption was minimal across all treatments a gradual increase in silicon uptake was observed with increase in the concentration of silicon. Each treatment showed a slight increase absorption of the element over time.

Table 4.4. Evaluation of levels of silicon in tomato plants treated with potassium silicate, with scanning electron microscopy energy dispersive X-ray (SEMEDX)

	Time/weeks					
Treatment Concentration	**WK2**	**WK3**	**WK4**	**WK5**	**WK6**	**STEM**
50	0.01a	0.03a	0.04b	0.06a	0.06a	0.13a
100	0.03a	0.04a	0.05ab	0.08a	0.12b	0.19a
150	0.04a	0.05a	0.07a	0.08a	0.13b	0.19a
200	0.04a	0.08a	0.08a	0.09a	0.13b	0.19a
F-ratio	1.50	3.46	4.95	2.03	0.02	3.36
P-value	0.28	0.07	0.03	0.18	5.62	0.07
CV (%)	57	41	21	21	23	16

Means with the same letter are not significantly different (P =0.05) according to Duncan's multiple range test.

EDX showed that the levels of silicon absorbed by tomato were much lower than most (Figure 4.4) other elements that are present within tomato leaf samples. Silicon levels were observed to be higher in the stems than in the leaves.

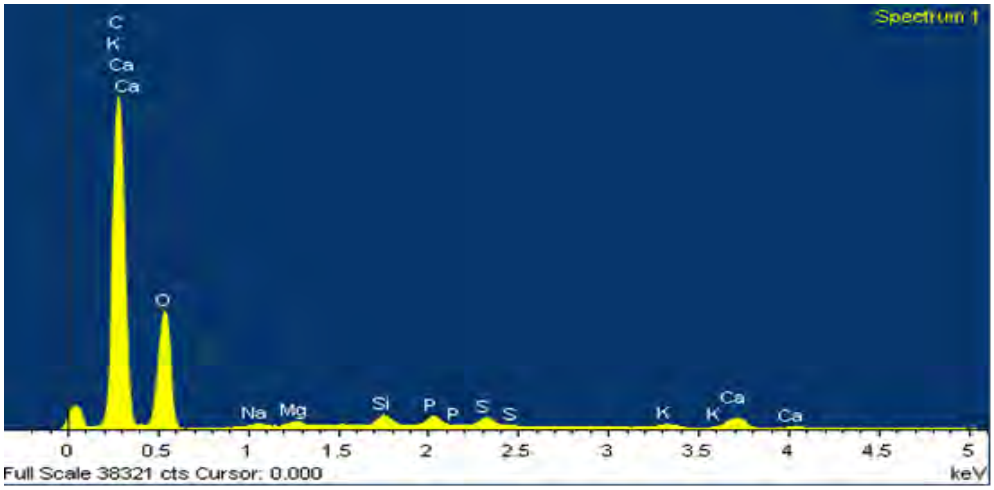

Figure 4.4. Silicon concentration peak amongst other elements found in a sample of tomato leaf using energy dispersive X-ray.

4.4 Discussion

Sticher et al. (1997) reported that identifying a suitable disease resistance inducer may help in protecting plant against various diseases which are caused by pathogens such as fungi, viruses and bacteria. It can also be seen as a way of adding or contributing to sustainable agriculture (Edreva and Kostoff, 2004). In this study, four treatments of potassium silicate and four treatments of ASM were tested for their efficacy in controlling early blight of tomato under greenhouse conditions. The results demonstrated that none of the potassium silicate treatments were effective in controlling the disease. However, all of the ASM treatments provided moderate control of 48 to 67% reduction in disease severity.

According to Ma and Takahashi (2002) and Lana et al. (2003), plants possess different abilities when it comes to silicon accumulation. Monocotyledons are classified as silicon accumulators, accumulating more Si in their shoots compared to other species, and especially when compared with silicon non-accumulators (some dicotyledons). Ma and Yamaji (2006) suggested that xylem loading (e.g. the transport of silicon from cortical cells to the xylem) is the most important determinant for a high level of silicon to accumulate in monocotyledons. Therefore, the much lower accumulation of silicon in tomato observed in the study might be explained by a lower density of the transporter to transport silicon from the external solution

into the cortical cells as well as the absence of transporter to transport Si from cortical cells to the xylem.

ASM has been reported to activate resistance in many crops against a broad spectrum of diseases when applied in relatively small amounts. These include fungi, bacteria and viruses. It has been reported that in monocotyledonous plants the activated resistance by ASM typically is long lasting, while the lasting effect is less pronounced in dicotyledonous plants. Out of the four ASM concentrations (25% (0.018 g l^{-1}), 50% (0.038 g l^{-1}), 75% (0.056 g l^{-1}) and 100% (0.075 g l^{-1})) two concentrations (75% (0.056 g l^{-1}) and 100% (0.075 g l^{-1}) resulted in a control effect above 50% against early blight disease. The results obtained in the study are in agreement with the one reported by Fritz (2005) where significant differences were observed among the treatments. The result obtained in the study agree with the results found by Madhusudhan et al. (2008) where ASM resulted in 66 and 68% reduction of local lesions in tomato and bell pepper as a result of TMV infection.

The SEM-EDX analysis was mainly used to observe the silicon levels in plant samples and the elemental distribution in the tomato plant tissues, while it is also a method for the rapid determination of the silicon content in plant tissues, as described by Wei-min et al. (2005). Hence, only the results of the rapid determination of the silicon content analyses were considered for analysis.The microanalysis using scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) performed on the plant samples also showed that silicon was absorbed in small amount. This explains failure of the potassium silicate applications to control early blight disease.

The 75% (0.056 g l^{-1}) and 100% (0.075 g l^{-1}) concentrations of ASM showed potential to control early blight in tomato. The effect of low concentrations of ASM in early blight control is important as it could be integrated with other disease management strategies to effectively manage the pathogen.

4.5 References

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CHAPTER FIVE

Integrated management of early blight of tomato using biological control agents and plant defence activator under greenhouse and nursery conditions

Abstract

A plant defence activator, acibenzolar-S-methyl (ASM), two biological control agents, *Bacillus subtilis* N6/2 and *Bacillus* sp. XVT8, as well as combinations thereof were evaluated for their efficacy in controlling early blight of tomato under greenhouse and nursery conditions. The treatments were applied as foliar sprays. Individual applications of 75% (0.056 g l^{-1}) concentration of acibenzolar-S-methyl, Rovral Flo and Coproxydithane+Petrin treatment failed to control early blight disease in both greenhouse and nursery conditions. *Bacillus* sp. XVT8 and *B. subtilis* N6/2 treatments significantly (P = 0.0001) reduced early blight disease severity in both greenhouse and nursery trials. A single application of *Bacillus* sp. XVT8 resulted in a significant reduction (P = 0.0001) in the levels of early blight disease in both greenhouse and nursery trials. However, a single treatment of *B. subtilis* N6/2 failed to control early blight under nursery conditions. The combination treatment of 75% acibenzolar-S-methyl + *Bacillus* sp. XVT8 + *B. subtilis* N6/2 showed disease suppression of 62% and 66% in the greenhouse and nursery trials, respectively. Combination treatments with 75% ASM and *Bacillus* sp. XVT8 reduced disease severity under greenhouse and nursery conditions by 66% and 68%, respectively. The combination of *Bacillus* sp. XVT8 + *B. subtilis* N6/2 caused a 57% and 46% disease reduction in the greenhouse nursery conditions, respectively. The combined treatment of 75% ASM + *B. subtilis* N6/2 failed to control early blight disease in both the greenhouse and nursery experiments.

5.1 Introduction

Tomato (*Solanum lycopersicum* (L.) H. Karst.) is an important vegetable because of its health benefits and phytochemical properties. Even though the crop is important for its nutritional value and as an important cash crop for smallholders and medium-scale commercial farmers in Africa, diseases such as early blight still remain a challenge for its production (Babalola

and Glick, 2012). Early blight has been reported to reduce the quantity and quality of tomato fruit produced by tomato crops globally (Tewari and Vishunavat, 2012). Even though the application of fungicides has been adopted as a standard practice to control early blight, various concerns such as the harmful effects of fungicides to human health and the environment have been raised (Janisiewicz and Korsten, 2002). This in turn has driven a search for alternative disease control measures (Nordlund, 1996; Glick and Bashan, 1997; El-Khoury and Makkouk, 2010).

The use of microbial antagonists such as bacteria has been suggested as one of the strategies that could play a role in controlling plant diseases (Lima et al., 1997; Harman et al., 2004). However, biological control has sometimes been reported to be less effective than commercial fungicides (El-Ghaouth et al., 2002; Leverentz et al., 2003). To overcome this shortfall, the use of microbial antagonists combined with commercial fungicides has been suggested. Moreover, application of mixtures of microbial antagonists could also serve as a way of reducing the inconsistency in performance that is normally observed when a single biological control agent is used (Droby et al., 1998; Janisiewicz, 1988; Duffy and Weller, 1995; Varshney and Chaube, 2001). In addition, it has been suggested that combining two or more antagonists could more closely mimic natural conditions on the phylloplane, and might broaden the spectrum of biocontrol activity. This in turn would enhance the efficacy and reliability of biocontrol, and allow the combination of various control measures without the need for genetic engineering (de Medeiros et al. 2012). However, the combination of different treatments increases the complexity and costs associated with such treatments. In some cases, combinations of biological control agents may be antagonistic (Yobo, 2005).

Chemically induced systemic acquired resistance (SAR) has also been suggested as one of the strategies that could be used to control disease in plants, indirectly. A synthetic compound, acibenzolar-S-methyl (ASM), has been reported to induce SAR and to provide significant suppression of various tomato diseases (Louws et al., 2001; Abbasi et al., 2002; Wilson et al., 2002). According to Batista et al. (2006), adopting integrated disease management practices, as well as implementing management strategies to reduce fungicide use in crop production, could minimise losses in tomato production. Integration of biological control agents with ASM has been used successfully to control bacterial spot of tomato under greenhouse experiments (Obradovic et al., 2005). In a study conducted by Fritz (2005), a strong, direct inhibitory effect of ASM *in vitro* on *Alternaria* isolates was documented. Fifty

three percent (53%) reduction of mycelial discs on potato dextrose agar was observed when compared to the control. The objective of this study was to investigate individual and combined effect of a plant defence activator, ASM, and two biological control agents for the management of early blight under greenhouse and nursery conditions.

5.2 Materials and methods

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 greenhouse studies (Chapter Three). The tested isolates were *B. subtilis* N6/2 and *Bacillus* sp. XVT8.

A plant defence activator, ASM, was used in the study. The concentration used was selected based on the studies carried out under greenhouse conditions on early blight 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%.

The greenhouse trial was conducted at the University of KwaZulu-Natal, Pietermaritzburg South Africa. Seeds of tomato (*Solanum lycopersicum* (L.) H. Karst.) (Cultivar Rodade) were obtained from Starke Ayres (Pty) Ltd, Pietermaritzburg South Africa. The seed were sown in Speedling 128 cavity seedling trays filled with composted pine bark (CPB) seedling mix (Gromor, Cato Ridge, South Africa). The seeded trays were kept in a greenhouse at a temperature of 26°C during the day and 18°C at night with a relative humidity of 75-85%. The seeds were watered once a day using sprinkler irrigation until germination and thereafter twice a day until transplanted. Irrigation water contains (per litre) NPK Starter Grower fertilizer 2:1:2(43) (1 g) plus trace elements (Ag-Chem Africa (Pty), Pretoria, South Africa). Seedlings were monitored, and at a two leaf stage they were transplanted into 15 cm diameter pots filled with composed pine bark potting mix media. The seedlings were watered and kept under the same greenhouse conditions as previously indicated above.

5.2.3 Inoculum preparation of *A solani* for the greenhouse and nursery experiments

A culture of *A. solani* was grown on V8 tomato juice agar [V8 tomato juice 200 ml, $CaCO_3$ 3 g, agar 20g in 1 L distilled water]. The plates were incubated at 25°C for seven days as described in Chapter Two. After 10-14 days, conidia were harvested in sterile distilled water (containing 0.01% of surfactant, Tween-20) by dislodging them off the agar plates with an L-bent glass rod and adjusted to 10^7 conidia ml^{-1} using a haemocytometer.

5.2.4 Treatment preparation for greenhouse and nursery experiments

(i) Inoculum preparation of biological control agents

Bacterial isolates *Bacillus* sp. XVT8 and *B. subtilis* N6/2, were cultured in tryptone soy agar (TSA) medium in 9 mm petri plates. The plates were incubated at 28°C for 48 h (Chapter Two). Bacterial cultures were washed by adding sterile distilled water nto the agar plates containing the bacterial culture. The conidial concentration in the suspension was measured using a Helber counting chamber (Paul Marienfield Superior GmbH & Co, Germany), and it was adjusted to10^7 conidia ml^{-1} before inoculation. The bacterial suspension was applied as foliar sprays using 1 L hand spray bottles.

(ii) Plant defence activator - Acibenzolar-S-Methyl (ASM)

A 75% concentration treatment of a full strength (0.075 g l^{-1}) ASM was prepared by measuring 0.056 g and dissolved in 1000 ml of tap water in a Aqua Systems 2L Garden Pressure Sprayer. The mixture was shaken to form a homogenous solution. ASM treatments were repeated every 14 d for both the greenhouse and nursery trials.

5.2.5 Testing treatment combinations under greenhouse conditions

Tomato seedlings used for greenhouse studies were prepared and fertilized as described in Section 5.2.2. A total of 9 treatments were evaluated in this study. Among the treatments were combinations of two and three treatments. The treatments consisted of (1) Fungicide (Rovral Flo), (2) *B. subtilis* N6/2, (3) *Bacillus* sp. XVT8, (4) 75% ASM (0.056 g l^{-1}), (5) *B. subtilis* N6/2 + *Bacillus* sp. XVT8, (6) *B. subtilis* N6/2 + 75% ASM, (7) *Bacillus* sp. XVT8 + 75% ASM, (8) *B. subtilis* N6/2 + *Bacillus* sp. XVT8 + 75% ASM, (9) *A. solani* inoculate control. The treatments were applied following a waiting period of 24 h between treatment applications: *B. subtilis* N6/2 followed by *Bacillus* sp. XVT8 and 75% ASM.

The plants were arranged in a randomized complete block design (RCBD) with six replicates per treatment. Plants were fertilized weekly with 150 ml per pot of limestone ammonium nitrate to ensure that nutrients were not a limiting factor for growth. The pathogen was inoculated by spraying 10^7 ml^{-1} of a conidial suspension on leaf surfaces.

Before and after inoculation each plant was sprayed with distilled water to create high humidity conditions and covered with a plastic bag for 24 h. The plants were then assessed for disease severity five days after treatment with *A. solani* inoculum following a three days interval over a period of four weeks. The experiment was conducted twice and the data was pooled for statistical analysis.

5.2.6. Testing 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 drip irrigated once a day for a period of 20 min. 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 plants were then assessed for disease severity five days after treatment with *A. solani* inoculum over a period of four weeks following an interval of three days. The trial was repeated once and the data pooled for statistical analysis.

In the nursery experiment, most experimental techniques used were as described in Section 5.2.5, such as the preparation and application of pathogen inoculum. The same treatments and treatment combinations were used except for the Fungicide Control which consisted of a weekly treatment with Coproxydithane and Petrin, which is the standard fungicide used at the nursery where the experiment was conducted.

5.2.7 Disease scoring and data analysis

Disease ratings of percentage foliar infection consisted of an estimate of percentage leaf area infected (%LAI) using a Horsfall-Barratt Scale (1945) where [1 = 0%; 2 = 1 – 3%; 3 = 3 – 6%, 4=6-12, 5=12-25%, 6=25-50%, 7=50-60%, 8=60-70%, 9=70-80%, 10=80-90%, 11=90-

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's multiple range test at the 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 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 was calculated according to Abbott's formulas (Levy *et al*., 1986) as follows:

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

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 early blight severity under greenhouses conditions

The levels of disease control provided by the treatments with single biological control agents, *Bacillus spp.* XVT8 and *B. subtilis* N6/2, and the fungicide control treatment, Rovral Flo, were not significantly different (63.3%, 62.9% and 53.5%, respectively), and fell into the best class of treatments. Treatment with the biocontrol agent *Bacillus spp.* XVT8 alone resulted in the lowest overall AUDPC values. The treatment combination of *Bacillus* sp. XVT8 + *B.*

subtilis N6/2 caused a disease reduction of 53.4% which was almost exactly the same as the commercial fungicide treatment (53.5%), and was not significantly more effective than either of the biocontrol agents by themselves.

Treatment with 75% ASM alone failed to control early blight disease under greenhouse conditions with a disease reduction of only 14%. However, *B. subtilis* N6/2 + 75% ASM provided moderate control of early blight disease, with a disease reduction of 41%, which is close to the level of control provided by the fungicide (53.6%), but was less than the biocontrol agent by itself (62.9%). However, the combination of the two biological control agents and ASM (*B. subtilis* N6/2 + *Bacillus* sp. XVT8 + 75% ASM) provided for disease reduction of 62.4%, and *Bacillus* sp. XVT8 + 75% ASM provided a disease reduction 65.9%, the best treatment overall, although it was not significantly more effective than the biocontrol agent by itself (Table 5.1).

Table 5.1: Efficacy of individual and combined applications of two biological control agents, ASM and their combination treatments against early blight of tomato under greenhouse conditions.

Treatments	Final disease severity	% Early blight reduction	AUDPC
A. solani	86.27a	0	766.5a
Rovral Flo	40.17d	53.5	292.88c
Bacillus sp XVT8	31.65d	63.3	121.5d
B. subtilis N6/2	31.98d	62.9	247.25c
75% ASM	74.53b	13.6	222.17cd
Bacillus sp. XVT8 + *B. subtilis* N6/2	40.16d	53.4	428.17b
B. subtilis N6/2 + 75% ASM	50.68c	41.3	270c
Bacillus sp. XVT8 +75% ASM	29.61d	65.7	398.92b
B. subtilis N6/2+ *Bacillus* sp. XVT8+75% ASM	32.44d	62.4	126.25d
F-value	37.68		33.31
P-value	0.0001		0.0001
CV%	16.47		37.14

Means with the same letter are not significantly different (P =0.05) according to Duncan's multiple range test.

ASM = acibenzolar –S- methyl

The failure of the combination treatments to have higher levels of activity than the biocontrol agents by themselves is reflected in the analysis in Table 5.2, which shows that all the combination treatments resulted in antagonistic effects between the treatments (Table 5.2).

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

Treatment	Efficacy Observed	Efficacy expected	Synergy factor	Interactive effect
Bacillus sp. XVT8 + *B. subtilis*	65.67	79.26	0.5	antagonistic
N6/2 + ASM	41.26	59.26	0.5	antagonistic
Bacillus sp. XVT8 +ASM	13.60	59.97	0.9	antagonistic
N6/2+ *Bacillus* sp. XVT8+75% ASM	65.67	-227.24	-0.2	antagonistic

ASM= acibenzolar-S-methyl

5.3.2 Integrated control of early blight of tomato under nursery conditions

Significant differences were observed among the single treatments (p = 0.0001) (Table 5.3). *Bacillus subtilis* N6/2 and 75% ASM treatments applied alone performed poorly, and reduced early blight disease under nursery conditions by only 25% and 24%, respectively. However, *Bacillus* sp. XVT8 applied alone caused a disease reduction of 65%. This was better than its performance during the greenhouse experiment. The standard fungicide treatment of Coproxydithane + Petrin also provided for poor levels of control of early blight disease with a 23% disease reduction (Table 5.3).

When 75% ASM was applied alone it provided a poor level of disease control of 23.9%. When 75% ASM was used in combination with *Bacillus* sp. XVT8, the best overall control level was achieved of 69.9% disease reduction, although this was not significantly different to the biocontrol agent by itself. When 75% ASM was used in combination with *Bacillus subtilis* N6/2 a moderate level of disease control was provided, of 48.3%, which was significantly better than either of the treatments by themselves (23.9% and 25.2%, respectively). When 75% ASM was used in combination with both of the biological control agents, *Bacillus* sp. XVT8 + *B. subtilis* N6/2 a disease reduction of 66.9% was observed. This was not significantly better than performance of *Bacillus sp*.XVT8 by itself (Table 5.3).

The AUDPC values show that the best treatment was achieved with a combination of 75% ASM with both of the biological control agents. However, this was not significantly better than the biocontrol agent *Bacillus* sp. XVT8 treatment on its own. The least effective

treatment was the fungicide treatment, which fell into the same class as the untreated, inoculated Control. The biocontrol agent *Bacillus subtilis* N6/2 on its own performed poorly, with a high AUDPC value.

Table 5.3: Efficacy of individual and combined applications of two biological control agents, ASM and their combination treatments against early blight of tomato under nursery conditions

Treatment	Final disease severity	% Early blight reduction	AUDPC
A. solani	97.95a	0	552.5a
Coproxydithane +Petrin	77.00b	21.3	507.17a
Bacillus sp. XVT8	33.43d	65.8	218.25de
B. subtilis N6/2	73.27b	25.2	391bc
75% ASM	74.53b	23.9	289cd
Bacillus sp. XVT8 + *B. subtilis* N6/2	40.49cd	58.7	298.58cd
B. subtilis N6/2+75 % ASM	50.68c	48.3	460ab
Bacillus sp. XVT8+75% ASM	29.53d	69.9	291cd
B. subtilis N6/2 + *Bacillus* sp. XVT8 + 75% ASM	32.44d	66.9	157.75e
F-value	40.29		11.82
P-value	0.0001		0.0001
CV%	18.0		38.37

Means with the same letter are not significantly different (P =0.05) according to Duncan's multiple range test

ASM= acibenzolar-S-methyl

The combination of the two biological control agents, and the combinations of 75% ASM with one or two biological control agents were mostly antagonistic. The one exception was the combination of *B. subtilis* N6/2 with 75% ASM in the nursery trial that resulted in a strong additive effect (Table 5.4).

Table 5.4: Calculation of the nature of the interactions of treatment combinations for the control of early blight on tomato under nursery conditions using Abbot's formula (Levy *et al*., 1986)

Treatment	Efficacy Observed	Efficacy expected	Synergy Factor	Interactive effect
Bacillus sp. XVT8 + *B. subtilis* N6/2	58.67	73.27	0.7	Antagonistic
B. subtilis N6/2 + ASM	48.26	42.31	1.14	Synergistic
Bacillus sp. XVT8 + 75% ASM	69.85	72.82	0.96	Antagonistic
B. subtilis N6/2 + *Bacillus* sp. XVT8 + 75% ASM	66.88	-297.7	- 0.2	Antagonistic

ASM = acibenzolar-S-methyl

5.4 Discussion

Two biological control agents and a 75% concentration of ASM were tested either singly or in combinations for their efficacy in controlling early blight disease under greenhouse and nursery conditions. Overall, *Bacillus* sp. XVT8, *Bacillus* sp. XVT8 + 75% ASM, and *Bacillus* sp. N6/2 + *Bacillus* sp. XVT8 +75% ASM were consistent in controlling early blight in both greenhouse and nursery conditions. The treatment combination of *Bacillus* sp. XVT8 + *B. subtilis* N6/2 was antagonistic under both greenhouse and nursery conditions. The 75% ASM treatment was consistently ineffective against early blight and provided relatively poor control. It was not additive or synergistic with either biocontrol agent in the greenhouse trial, and was additive in action with *Bacillus* sp. N6/2 in the nursery trial. It did not improve the performance of the biocontrol agent *Bacillus* sp. XVT8 significantly in either experiment. Acoording to Jacobsen et al. (2004) *Bacillus* based biocontrol play a vital role in controlling disease in plants.This is due to their production of wide spectrum of antibiotics as well as their ability to form endospore and this is evident on the results obtained from the study.The results obtained in the study correlate with the study where *Bacillus* sp. was used as biological control agent against Fusarium wilt of tomato under greenhouse condition a disease reduction of 62.5-81.2 % was achieved (Ajilogba et al.,2013) . I another study the use of *bacillus sp* was found to have a better performance in controlling powdery mildew in

cucumber and gray mold in tomato with results that were as effeicient as the results obtained from using fungicide (Kim et al., 2013).

Previous reports on the effect of ASM on early blight disease severity under greenhouse and nursery conditions have not been documented due to early blight being controlled using commercial fungicide alone. However, it has been reported that when ASM was used in controlling bacterial speck and spot under greenhouse condition it did not have any significant impact on the population of bacterial speck and spot (Louws et al., 2001). Saad et al. (2015) and Soleimani and Kirk (2012) reported that ASM did not provide any control against of *A. solani* when tested *in vitro*. This correlates with the results observed in the study in which ASM alone failed to control early blight and its contribution towards the performance of the treatment combinations was trivial

This study contradicts various studies where it was observed that combining different biological control agents resulted in better disease control (Duffy et al., 1996; Raupach and Kloepper, 1998; Mishra et al., 2011). In this study the combination of the two biological control agents was consistently antagonistic when compared to the *Bacillus* spp. XVT8 by itself. Given the complexities and costs involved in applying multiple biocontrol agents, or biocontrol agents combined with ASM, this is the best outcome, especially as it was substantially more effective than the standard fungicide.

In conclusion ASM did not control early blight disease when used as a single treatment as well as in combination with the biological control agents. In addition biological control used in the study showed to be less effective in controlling early blight disease under greenhouse and nursery conditions when used in combination. However even though the combined biological control were not effective one single treatment of biological control agents showed to be more effective compared to the current fungicide program used for controlling early blight disease.

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DISSERTATION OVERVIEW

The search for alternatives to agrochemicals has attracted the attention of many researchers globally in the last few decades. This interest has been prompted by the necessity to increase crop production to meet the demands for food (Ray et al., 2013). Plant diseases alone have been shown to drastically reduce crop yields thereby necessitating the need to find strategies to control them (Agrios, 2005).

Integrating biological control agents (BCA) with other means of control such as plant defence activators has been reported to increase reliability and consistencies of BCAs. This may be used as an alternative strategy that can be practiced on a large scale. Moreover, combinations of BCAs with plant defence activators has been shown to be effective and may offer a better option to control plant disease on a large scale (El Ghaouth et al., 2000; Usall et al., 2001; Arras et al., 2002 Droby et al., 2003; Spadaro et al., 2004; Lima et al., 2005). On the other hand, this would increase the costs of disease control.

Therefore, this study was aimed at isolating potential bacterial and yeast BCAs and screening them *in vitro* and *in vivo* against early blight of tomato caused by *A. solani*. A range of concentrations of acibenzolar-S-methyl and potassium silicate were evaluated for their ability to control early blight of tomato under greenhouse conditions. The best treatments were combined and tested in an effort to formulate an integrated approach to manage early blight under greenhouse and nursery conditions.

In this study, the following outcomes were achieved:

- In an *in vitro* dual-culture assay 10 bacterial isolates strongly inhibited the growth of *A. solani*.
- *Bacillus subtilis* N6/2, *B. subtilis* N5, *Bacillus* sp. XVT8 and *B. subtilis* WESH1 significantly ($p = 0.001$) reduced disease severity of early blight in an initial trial.
- None of the liquid potassium silicate concentrations significantly reduced disease severity of early blight under greenhouse condition. Little potassium silicate was taken up and translocated into the leaves by tomato.
- Individual applications of 75% (0.056 g L^{-1}) ASM and both the fungicide treatments Rovral Flo (100mL/100) Land Coproxydithane +Petrin failed to control early blight disease under both greenhouse and nursery conditions.

- Biocontrol treatments with *Bacillus* sp. XVT8 and *B. subtilis* N6/2 alone significantly (P = 0.0001) reduced early blight disease severity under both greenhouse and nursery conditions.
- A single treatment of *Bacillus* sp. XVT8 provided better and more consistent control of early blight disease than a single treatment of *B. subtilis* N6/2 under greenhouse and nursery conditions
- Combination of 75% reduced concentration of ASM and *Bacillus* sp .XVT8 controlled early blight in the greenhouse and nursery by 66% and 68% respectively compared to the pathogen inoculated control treatment. However, these levels of control were not significantly better than the biocontrol agent by itself.
- The combination treatment of 75% ASM + *Bacillus* sp. XVT8 + *B. subtilis* N6/2 resulted in disease suppression of 62% and 66% in the greenhouse and nursery, respectively. Neither of these results were significantly better than *Bacillus* sp. XVT8 by itself.

***In vitro* bioassays**

Sixty percent (60%) of the bacterial isolates inhibited mycelial growth of *A. solani*, with inhibition zones ranging from 6-14 mm. Thirty-five percent (35%) of the yeast isolates inhibited spore germination of *A. solani*.

The isolates screened were identified isolates N5, N6/2 and WESH1 were identified as *Bacillus subtilis* while NC13 was identified as *Pseudomonas putida* strain PYR1 and XVT8 was identified as a *Bacillus* sp using 16S rRNA sequencing,. The yeast isolates C10, P1-1 and Y4 were identified as *Meyerozyma guilliermondii*, Isolate P1-Orange was identified as *Rodotorula minuta* and H5 was identified as *Pichia guilliermondii*.

Greenhouse trials

The study demonstrated that the best two *Bacillus* isolates could be used in an integrated disease management strategy for the control of early blight on tomato. The performance of the yeast isolates during greenhouse studies on tomato plants did not correlate well with those obtained in the *in vitro* studies. None of the yeast isolates reduced early blight disease severity under greenhouse conditions. It is clear that the *in vitro* test used here did not replicate conditions in the greenhouse, on the leaf of tomato, where the competition with *A.*

solani takes place. More work is needed to discover a better primary screening method to identify competitive yeast strains to control foliar diseases of crops. Use of detached tomato leaves in the laboratory may be an option.

The 75% reduced concentration (0.056 g L^{-1}) and the full strength (0.075 g L^{-1}) ASM showed potential in controlling early blight of tomato.

The microanalysis using scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) performed on the plant samples treated with potassium silicate showed that silicon was absorbed in minute amount which explains why KSil had no effect on the disease, and there was no difference in the levels of silicon taken up as a result of differences in concentrations of KSil that were applied.

Nursery trial

ASM on its own provided little control of early blight, a 23.9% reduction. When 75% ASM was used in combination with XVT8 *Bacillus sp.* + *Bacillus subtilis* N6/2, disease reductions of 66% and 68% respectively were observed. However, a disease reduction of 47% was observed when 75% ASM was combined with *B. subtilis* N6/2. A combination of two biological control agents *Bacillus sp.* XVT8 + *B. subtilis* N6/2 resulted in a significant disease reduction of 57%. Coproxydithane +Petrin fungicide resulted in minimal disease reduction of 21%. These results are in agreement with the studies conducted by various works where it was observed that combining different biological agents results a better and consistent disease control (Duffy et al., 1996; Raupach and Kloepper, 1998; Mishra et al., 2011).

Overall conclusion

These results indicate that biological control agents alone, or in combination with ASM, can be used successfully in a sustainable disease management program**.**

Proposed future research priorities

- Selecting more efficient biological control agents suitable for integrated application with ASM.

- To evaluate the efficacy of all the combination treatment that showed potential in controlling early disease under fields conditions.
- Use of participatory approach to evaluate the efficacy of the treatment combination under farmer's conditions.
- An evaluation of the financial aspects of applying a range of control measures to a tomato crop for the control of early blight.

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