UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Mode of action of *Bacillus subtilis* ATCC 55466 as biocontrol agent of postharvest diseases of avocados

By

Wilma Havenga

Submitted to the Faculty of Natural and Biological Sciences
Department of Microbiology and Plant Pathology
University of Pretoria

In partial fulfillment of the requirements for the degree of M.Sc. (Agric)

December 2004

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

I certify that the thesis submitted to the University of Pretoria for the degree of MSc (Agric) has not previously been submitted by me in respect of a degree at any other University

Signed on the 15th of December 2004 at Pretoria.

Wilma Havenga

ACKNOWLEDGEMENTS

Prof. L Korsten, my promotor and mentor, for all her support and advice throughout the years. It was wonderful to be under her tutelage and I will never forget the gift she gave me in inspiring my interest in biocontrol.

Staff members at the Department of Microbiology and Plant Pathology, especially Prof F.C. Wehner, Dr G. Swart, Ms E.S. de Jager, Dr T. Regnier, Dr P. Labuschagne, Ms K. van Dyk, Ms A. Lombard, Mr A. Fourie, Mrs R. Jakobs, Ms K. Joubert, Mrs M. van Eeden and Mr A. Hall for their advice and technical assistance.

Mrs M.F. Smith at the Bioinformatics Agricultural Research Council for statistical analyses.

Prof P.L. Steyn for grammatical care of my thesis.

All the students at the Department of Microbiology and Plant Pathology who made studying at the University truly an adventure.

National Research Foundation for financial support.

All the members and staff at Stimuplant for their support.

Friends and family who never ceased to support and encourage me throughout this venture.

My Dad, Mom and brother who never failed to support me and without their support none of this would have been possible.

Most importantly, Almighty God, who brought me to this path and led me through it, nurturing me and pruning me to grow into what pleases Him. This work is to His honour.

CONTENTS

ACKNOWLEDGEMENTS **2**

CONTENTS **3**

TABLE OF FIGURES **6**

TABLE OF TABLES **8**

CHAPTER 1 .... **9**

GENERAL INTRODUCTION **9**

REFERENCES 11

CHAPTER 2 .... **15**

LITERATURE REVIEW **15**

MODES OF ACTION INVOLVED IN BIOCONTROL OF PLANT PATHOGENS, WITH SPECIAL REFERENCE TO FUNGAL PATHOGENS **15**

1. HISTORY OF BIOCONTROL 15
2. MODES OF ACTION INVOLVED IN BIOCONTROL 16
   2.1. Indirect interactions 17
   2.1.1. Cross-protection or hypo virulence 18
   2.1.2. Systemic acquired resistance 19
   2.1.3. Plant growth stimulation and camouflage 20
   2.2. Direct interactions 22
   2.2.1. Antibiosis 22
   2.2.2. Mycoparasitism and Cell-wall degrading enzymes 26
   2.2.3. Competitive exclusion and physical restriction 30
   2.2.4. Competition for nutrients 31
   2.2.5. Siderophore production 33
3. FACTORS AFFECTING MODES OF ACTION 35
   3.1. Plant host affecting biocontrol modes of action 35
   3.2. Pathogen affecting biocontrol modes of action 38
   3.3. Antagonist affecting biocontrol modes of action 40
4. NEW AND EMERGING TECHNOLOGIES IN THE USE OF BIOCONTROL AGENTS 41
5. CONCLUSION 42
6. REFERENCES 43

CHAPTER 3 .... **54**

COMPARISON OF *BACILLUS SUBTILIS* SUBCULTURES' EFFICACY IN CONTROLLING FUNGAL AVOCADO PATHOGENS *IN VITRO* **54**

1. ABSTRACT 54
2. INTRODUCTION 54

3. Materials and methods 55
3.1. Strains 55
3.2. Isolation and pathogenicity of fungal postharvest avocado pathogens 56
3.3. In vitro evaluation of antagonistic efficacy of Bacillus subtilis subcultures 56
3.4. Genomic fingerprinting of Bacillus subtilis subcultures using RISA primers for polymerase chain reaction 58
3.4.1. DNA extraction 58
3.4.2. Amplification of DNA 59
3.4.3. Separation of DNA bands 59
4. Results 59
4.1. Isolation and pathogenicity of fungal postharvest avocado pathogens 59
4.2. Evaluation of in vitro antagonism of Bacillus subtilis subcultures and reference strains 60
4.3. Genomic fingerprinting of Bacillus subtilis subcultures using the rDNA internal spacer analysis polymerase chain reaction 60
5. Discussion 62
6. References 63

CHAPTER 4 66

MODES OF ACTION INVOLVED IN ANTAGONISM OF *BACILLUS SUBTILIS* AGAINST FUNGAL POSTHARVEST PATHOGENS OF AVOCADO 66

1. Abstract 66
2. Introduction 66
3. Materials and methods 68
3.1. Isolates 68
3.2. Direct interaction between Bacillus subtilis and Colletotrichum gloeosporioides on avocado fruit surfaces using scanning electron microscopy 68
3.3. Diffusible inhibitory metabolites produced by Bacillus subtilis in vitro active against Colletotrichum gloeosporioides 69
3.4. Enzyme activity of Bacillus subtilis and fungal postharvest avocado pathogens in vitro 69
3.4.1. Chitinase activity 70
3.4.2. Extracellular amylases 70
3.4.3. Lipase activity 70
3.4.4. Proteinase activity 71
3.5. Siderophore production by Bacillus subtilis and fungal postharvest avocado pathogens in vitro 71
3.6. Antifungal volatiles produced by Bacillus subtilis active against fungal postharvest avocado pathogens 72
3.6.1. Sealed plate method 72
3.6.2. Aerated plate method 72
4. Results 73
4.1. In vivo studies 73
4.2. Diffusible inhibitory metabolites produced by Bacillus subtilis in vitro active against Colletotrichum gloeosporioides 78
4.3. Enzyme activity of Bacillus subtilis and fungal postharvest avocado pathogens in vitro 78
4.4. Siderophore production by Bacillus subtilis and fungal postharvest avocado pathogens in vitro 78
4.5. Antifungal volatile production in vitro by Bacillus subtilis active against fungal postharvest avocado pathogens 78
4.5.1. Sealed plate method 79
4.5.2. Aerated plate method 80
5. Discussion 81
6. References 83

CHAPTER 5 88

ENVIRONMENTAL CONDITIONS INFLUENCING *IN VITRO* ANTAGONISM OF *BACILLUS SUBTILIS* AGAINST FUNGAL POSTHARVEST AVOCADO PATHOGENS 88

1. Abstract 88
2. Introduction 88
3. Materials and methods 90

3.1. Isolates 90
3.2. Effect of different temperatures on the inhibitory action of Bacillus subtilis on fungal postharvest avocado pathogens in vitro 90
3.3. Effect of nutrients on the inhibitory action of Bacillus subtilis on fungal postharvest avocado pathogens in vitro 91
4. RESULTS 92
4.1. Effect of different temperatures on the inhibiting action of Bacillus subtilis on fungal postharvest avocado pathogens in vitro 92
4.2. Effect of nutrients on the inhibitory action of Bacillus subtilis on fungal postharvest avocado pathogens in vitro 92
5. DISCUSSION 96
6. REFERENCES 98

CHAPTER 6 ..... **112**

PRODUCTION AND PARTIAL CHARACTERISATION OF INHIBITORY METABOLITES PRODUCED BY *BACILLUS SUBTILIS* **112**

1. ABSTRACT 112
2. INTRODUCTION 112
3. MATERIALS AND METHODS 113
3.1. Strains 113
3.2. Optimising antifungal metabolite production by Bacillus subtilis 113
3.3. Antifungal metabolite production and partial characterisation 114
3.3.1. Antifungal metabolite production 114
3.3.2. Extraction of antifungal metabolites 114
3.3.3. Extraction of free acid phenolic compounds 114
3.3.4. Quantification of total phenolic compounds 115
3.3.5. Separation of free acid phenolic compounds by thin layer chromatography 115
3.3.6. Antifungal activity of separated fluorescent spots against Cladosporium cladosporioides 115
3.3.7. High performance liquid chromatography of free acid phenolic compounds 116
4. RESULTS 116
4.1. Optimising antifungal metabolite production by Bacillus subtilis 116
4.2. Quantification of total phenolic compounds 116
4.3. Separation of free acid phenolic compounds by thin layer chromatography 117
4.4. Antifungal activity of separated fluorescent spots against Cladosporium cladosporioides 117
4.5. High performance liquid chromatography of free acid phenolic compounds 119
5. DISCUSSION 119
6. REFERENCES 121

CHAPTER 7 ..... **125**

GENERAL DISCUSSION **125**

REFERENCES 128

SUMMARY **131**

OPSOMMING **133**

TABLE OF FIGURES

Figure 2.1: Modes of action involved in biocontrol and their relation to each other. 18
Figure 2.2: A schematic representation of the factors that influence biocontrol efficacy. 36
Figure 3.1: Dual culture technique: *Bacillus subtilis* inhibiting *Colletotrichum gloeosporioides in vitro* on potato dextrose agar plates. 58
Figure 3.2: Diameter of lesions formed on avocado fruit by fungal postharvest pathogens. 60
Figure 3.3: Banding patterns of DNA products after rDNA internal spacer analysis polymerase chain reaction on selected isolates of *Bacillus subtilis* separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis. 62
Figure 4.1: Scanning electron micrographs of the surface of avocado fruit showing characteristic wax platelets (Photo 1: 4300 x, 2: 3500 x, and 3: 10000 x magnification). 74
Figure 4.2: Scanning electron micrographs of avocado fruit inoculated: 1) with conidia of *Colletotrichum gloeosporioides* alone, 2) and 3) with conidia of *C. gloeosporioides* followed by *Bacillus subtilis* (Photo 1: 1200 x, 2: 7500 x, and 3: 2300 x magnification). 75
Figure 4.3: Scanning electron micrographs of avocado fruit surfaces inoculated with: 1) *Bacillus subtilis*, 2) *B. subtilis* showing attachment structures, and 3) both *B. subtilis* cells and *Colletotrichum gloeosporioides* hyphae (Photo 1: 6000 x, 2: 8500 x, and 3: 5000 x magnification). 76
Figure 4.4: Scanning electron micrographs showing avocado fruit surfaces inoculated with *Colletotrichum gloeosporioides* and *Bacillus subtilis* (Photo 1: 4300 x, 2: 8500 x, and 3: 7000 x magnification). 77
Figure 4.5: Diameter of inhibition zones formed by *Bacillus subtilis* cell-free filtrates on spread plates of *Colletotrichum gloeosporioides*. 78
Figure 4.6: Comparison between the mean percentage inhibition of *Phomopsis perseae* by *Bacillus subtilis* measured on days 1 to 3 after inoculation. 79
Figure 4.7: Comparison between the mean percentage inhibition of *Colletotrichum gloeosporioides* by *Bacillus subtilis* measured on days 1 to 3 after inoculation. 79
Figure 4.8: Comparison between the mean percentage inhibition of *Dothiorella aromatica* by *Bacillus subtilis* measured on days 1 to 3 after inoculation. 80
Figure 4.9: Comparison between the mean percentage inhibition of *Lasiodiplodia theobromae* by *Bacillus subtilis* measured on days 1 to 3 after inoculation. 80
Figure 5.1: Effect of temperature on *Bacillus subtilis* antagonism of *Phomopsis perseae* at respective temperatures. 94
Figure 5.2: Effect of temperature on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides* at respective temperatures. 94
Figure 5.3: Effect of temperature on *Bacillus subtilis* antagonism of *Dothiorella aromatica* at respective temperatures. 95
Figure 5.4: Effect of temperature on *Bacillus subtilis* antagonism of *Lasiodiplodia theobromae* at the respective temperatures. 95
Figure 5.5: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Phomopsis perseae*. 100
Figure 5.6: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Phomopsis perseae*. 101

Figure 5.7: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides*. 102

Figure 5.8: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides*. 103

Figure 5.9: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Dothiorella aromatica*. 104

Figure 5.10: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Dothiorella aromatica*. .. 105

Figure 5.11: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Lasiodiplodia theobromae*. 106

Figure 5.12: Effect of different carbon sources on *Bacillus subtilis* antagonism of against *Lasiodiplodia theobromae*. 107

Figure 6.1: Inhibition zones formed at specific cell concentrations during continuous culturing of *Bacillus subtilis* over a seven day period. 117

Figure 6.2: Free acid fraction of *Bacillus subtilis* growth media and control separated by thin layer chromatography using a silica glass plate, run with acetic acid:methanol:water (8:1:1, v/v/v) and viewed under UV light (254 nm) to separate potential inhibitory phenolic substances. 118

Figure 6.3: Inhibition due to spots of free acid phenolic compounds of *Bacillus subtilis* growth media separated by thin layer chromatography using a silica glass plate run with acetic acid:methanol:water (8:1:1, v/v/v) against *Cladosporium cladosporioides*. 118

Figure 6.4: High performance liquid chromatographs of a) free acid phenolic extract viewed at 430, 325 and 280 nm, and b) control viewed at 360, 320 and 280 nm. 120

TABLE OF TABLES

Table 2.1: Cases where induced resistance was found to be involved in antagonism of pathogenic fungi.... 21

Table 2.2: Antibiotics produced by members of the genus *Bacillus* 27

Table 2.3: Enzymes involved in biocontrol of fungal pathogens.... 29

Table 2.4: Antagonists producing siderophores involved in biocontrol of fungal pathogens.... 34

Table 3.1: *Bacillus subtilis* subcultures and other reference bacteria used for comparison purposes.... 57

Table 3.2: *In vitro* inhibition of avocado fungal pathogens, *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae* by *Bacillus isolates* 61

Table 4.1: Treatment of avocado fruit surfaces prior to scanning electron microscopy viewing 69

Table 4.2: Mean percentage inhibition of fungal pathogens caused by volatile substances produced by *Bacillus subtilis* using the aerated plate method 81

Table 5.1: Nitrogen sources supporting high levels of antagonism against *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae* 99

Table 5.2: Carbon sources supporting high levels of antagonism against *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae*.... 99

Table 6.1: The program followed during separation of samples using high performance liquid chromatography.... 116

Table 6.2: Rf values of fluorescent spots separated using acetic acid : methanol : water as solvent on glass thin layer chromatograph plates 117

Table 6.3: Retention time of the constituents of free acid phenolic extract determined using high performance liquid chromatography.... 119

SUMMARY

Avocados are an economically important crop in South Africa and are mainly exported to Europe. As with any other tropical and subtropical crop, avocados are prone to pre- and postharvest diseases. Until recently, chemical control was the only effective measure to control fungal avocado pathogens. In 1987, a *Bacillus subtilis* isolate was found that showed promise as a biocontrol agent in both pre- and postharvest applications to control postharvest diseases. However, over time variable results has been obtained in semi commercial trials.

From the original *B. subtilis* isolate several subcultures have been made and used over a 15 year period in various experimental trials. The dual culture technique was used to compare the biocontrol activity of the subcultures against postharvest pathogens (*Colletotrichum gloeosporioides*, *Phomopsis perseae*, *Dothiorella aromatica* and *Lasiodiplodia theobromae*). The subcultures differed significantly in their effectiveness and genetic stability. No difference between the subcultures could be found when DNA fingerprinting using RISA PCR was used. The most effective subculture, MI-14, was used in further studies.

The mode of action employed by a biocontrol agent is of utmost importance and can be used to enhance its efficacy. In a previous study it was hypothesized that antibiosis as well as competition for nutrients and space is the modes of action involved in biocontrol of *B. subtilis* against postharvest pathogens of avocado. The direct interaction between *B. subtilis* and *C. gloeosporioides* on avocado fruit were observed using scanning electron microscopy. Cells of *B. subtilis* were observed to colonize the hyphae of *C. gloeosporioides*. In some instances, hyphal walls were lysed in the presence of *B. subtilis* and may be due to the presence of enzymes or antibiotic substances. Conidia of *C. gloeosporioides* did not germinate in the presence of *B. subtilis*. Diffusible inhibitory metabolites active against *C. gloeosporioides* were produced *in vitro* by *B. subtilis*. Inhibitory volatile substances were also produced by *B. subtilis* and were found to be active against *P. perseae*, *D. aromatica* and *L. theobromae* but not *C. gloeosporioides*. Siderophores production as well as chitinase, amylase, lipase and proteinase activity were also observed and may play a role in antagonism.

Antibiotic production by *B. subtilis* is a well-known phenomenon. Most antibiotics are polypeptides and lipopeptides. The involvement of phenolic metabolites in biocontrol by *B. subtilis* is less known. A seven-day-old culture of *B. subtilis* in a minimal medium was analyzed for the presence of free acid phenolic

compounds active against fungi. Free acid phenolic metabolites were found and separated using layer chromatography. TLC plates containing the separated spots were sprayed with *Cladosporium cladosporioides* and plates were observed for inhibition zones. The phenolic substances were present at 7.06 ± 0.95 mg gallic acid ml^{-1}. The phenolic substances fall in the hydroxycinamic acid group due to their fluorescent coloring under UV at 350 nm.

The mode of action involved is also influenced by environmental factors. The effect of temperature and carbon- and nitrogen sources on the *in vitro* inhibitory activity of *B. subtilis* against *C. gloeosporioides*, *P. perseae*, *D. aromatica* and *L. theobromae* were investigated using the dual culture technique. The most effective temperature range for *B. subtilis* was found to be between 20 and 37 °C. At temperatures lower than 15 °C, *B. subtilis* was found to be not very effective, suggesting why postharvest applications followed directly by cold storage do not always work effectively. D-arabinose and D-(+)-mannitol evaluated as carbon source as well as L-glutamic acid, L-glutamine and L-(+)-asparagine used as nitrogen sources support *in vitro* antagonism against the pathogens most effectively. They also do not support the growth of *C. gloeosporioides*, *P. perseae*, *D. aromatica* and *L. theobromae*. These nutrients can potentially be the most effective ones to incorporate in commercial *B. subtilis* formulations.

This study showed the potential role of antagonistic free acid phenolic substances, volatiles and siderophores on inhibition of fungal avocado pathogens. Further studies to confirm their *in situ* activity are required. In conclusion, various factors affect the efficacy of *B. subtilis* against postharvest pathogens of avocado. These factors should be kept in mind when applying the commercial product in order to achieve the best results.

OPSOMMING

Avokados is `n ekonomies belangrike gewas in Suid Afrika en word hoofsaaklik uitgevoer na Europa. Avokados is soos enige tropiese en subtropiese gewas vatbaar vir voor- en na-oes siektes. Tot onlangs was chemiese middels die enigste effektiewe beheermaatreel teen swam avokado patogene. In 1987 is `n *Bacillus subtilis* isolaat gevind wat belowende resultate getoon het as `n biobeheer middel teen avokado na-oes siektes tydens voor- en na-oes toedienings. Varieerende resultate is wel gekry in semi kommersieële proewe.

Verskeie subkulture is van die oorspronklike *B. subtilis* isolaat gemaak en is gebruik in verskeie proewe oor `n tydperk van 15 jaar. Die twee-kultuur tegniek was gebruik om die onderskeie subkulture se biobeheer aktiwiteit teenoor avokado na-oes patogene (*Colletotrichum gloeosporioides*, *Phomopsis perseae*, *Dothiorella aromatica* en *Lasiodiplodia theobromae*) te vergelyk. Die subkulture se effektiwiteit en hul genetiese stabiliteit het betekenisvol van mekaar verskil. DNS profiele van geselekteerde subkulture is deur RISA polimerase ketting reaksie gegenereer en het nie van mekaar verskil nie. Die mees effektiewe subkultuur, MI-14, is in verdere studies gebruik.

Die meganisme van werking wat deur `n biobeheeragent gebruik word, is van kardinale belang en kan gemanipuleer word om sy om sy effektiwiteit te verbeter. In `n vorige studie is dit gestel dat antibiose asook kompetisie vir nutriente en spasie die meganismes van werking is wat deur *B. subtilis* gebruik word in die biobeheer van na-oes avokado patogene. Die direkte interaksie tussen *B. subtilis* en *C. gloeosporioides* op avokado vrugte is deur skandeer elektron mikroskopie ondersoek. Dit is waargeneem dat *B subtilis* die hifes van *C. gloeosporioides* koloniseer. In sommige gevalle waar *B. subtilis* teenwoordig is, is die hifewande geliseer. Dit mag die gevolg wees van ensieme of antibiotiese stowwe. In die teenwoordigheid van *B. subtilis* het *C. gloeosporioides* konidia nie ontkiem nie. Daar is gevind dat *B. subtilis* diffundeerbare inhiberende metaboliete *in vitro* produseer wat aktief is teen *C. gloeosporioides*. Vlugtige inhiberende stowwe is ook deur *B. subtilis* geproduseer en is aktief teen *P. perseae*, *D. aromatica* en *L. theobromae* maar nie teen *C. gloeosporioides* nie. Siderofoor produksie deur *B. subtilis* asook die aktiwiteit van chitinase, amilase, lipase en proteïenase is waargeneem en kan `n rol speel in antagonisme.

Die produksie van antibiotiese metaboliete deur *B. subtilis* in `n bekende verskynsel. Die meeste antibiotiese metaboliete is polipeptiede en lipopeptiede. Die rol van fenoliese metaboliete wat deur *B. subtilis*

geproduseer is in biobeheer, is minder bekend. Sewe-dae-oue fermentasie kulture van *B. subtilis* in `n minimale medium is geanaliseer vir die teenwoordigheid van vrye-suur fenoliese verbindings wat aktief is teen swamme. Vrye-suur fenoliese verbindings is gevind en deur dunlaag chromatografie geskei. Plate wat die geskeide kolle bevat is bedek met *Cladosporium cladosporioides* en is daarna ondersoek vir die vorming van inhibisiesones. Fenoliese verbindings was teenwoordig teen teen `n konsentrasie gelykstaande aan 7.06 ± 0.95 mg gallig suur ml^{-1}. Die fenoliese verbindings wat gevind is word geklassifiseer in die hidroksiesinamiese suur groep na aanleiding van hul fluoresserende kleure onder UV lig by 350 nm.

Omgewingsfaktore beïnvloed ook die meganisme van werking wat gebruik word deur `n biobeheer agent. Die twee-kultuur tegniek is gebruik om die effek van tempratuur asook koolstof- en stikstofbronne op die *in vitro* inhiberende werking van *B. subtilis* teen *C. gloeosporioides*, *P. perseae*, *D. aromatica* en *L. theobromae* te ondersoek. Temprature tussen 20 en 37 °C het die werking van *B. subtilis* die beste ondersteun. By temprature laer as 15 °C is gevind dat *B. subtilis* nie baie effektief was nie, wat kan verduidelik hoekom na-oes toedienings dadelik gevolg deur koue stoor berging nie altyd effektief is nie. Daar was gevind dat *in vitro* antagonisme die beste deur D-arabinose of D-(+)-mannitol as koolstofbron, en L-glutamiensuur, L-glutamien of L-(+)-asparagien as stikstofbron ondersteun was. Hierdie bronne bevorder nie die groei van *C. gloeosporioides*, *P. perseae*, *D. aromatica* of *L. theobromae* nie. Dit kan die beste potensiaal hê om in `n kommersieële *B. subtilis* formulasie gebruik te word.

Hierdie studie toon die moontlike rol van antagonistiese vry-suur fenoliese verbindings, vlugtige stowwe en siderofore op die inhibisie van patogeniese swamme op avokado. Verdere studies om die *in situ* aktiwiteite hiervan te bepaal, is nodig. Verskeie faktore affekteer die werking van *B. subtilis* teen na-oes avokado patogene. Hierdie faktore moet in gedagte gehou word wanneer die produk toegedien word om die beste resultate te behaal.

CHAPTER 1

GENERAL INTRODUCTION

The avocado, *Persea americana* Miller, also known as the "alligator pear", originates from South America. It has been an important constituent of the South Americans' diet for thousands of years (Snowdon, 1990). Currently, avocados are an important fruit crop in many parts of the world, including South Africa. Three main types of avocados can be distinguished, namely the Mexican (subtropical), Guatemalan (semitropical) and "West Indian" (tropical) (Biale & Young, 1971). The most important avocado cultivation regions in South Africa are the Lowveld of the Northern Province and Mpumalanga (Keevy, 1999). Other frost-free parts of the country are also used for cultivation. A number of cultivars, varying in fruit size, shape and colour, are currently cultivated in these regions of which the most important are Fuerte and Hass.

Like all tropical and subtropical fruit, both pre- and postharvest diseases are prevalent on avocados, which can result in major losses. Preharvest diseases include cercospora spot and anthracnose, while stem-end rot and anthracnose are diseases observed postharvestly (Snowdon, 1990; Hartill, 1991). The economic impact of postharvest diseases is difficult to assess, since losses during selective picking, sorting, re-packing and marketing are rarely included (Wilson & Wisniewski, 1989). It is estimated that up to 50 % of the total worldwide production of fruit crops can be wasted as a result of postharvest diseases (Wilson & Wisniewski, 1989).

The most common way to control plant diseases caused by fungi is by means of chemical fungicides. Fungicides are usually not easily biodegradable, since they must persist in the environment for optimal functionality (Campbell, 1989). This causes serious environmental problems due to pesticide build-up in soil or water ecosystems (Campbell, 1989). Pesticides accumulate in predators at the top of the food chain and also adversely affect non-target organisms. During the late 1980's it was estimated that annually 3000 hospitalisations, 200 fatalities and unexpected side effects, occurred annually in the USA alone due to the misuse of pesticides (Campbell, 1989). Incorrect use of fungicides can also lead to build up of pathogen resistance, resulting in reduced effectiveness of the product (Kotzé *et al.*, 1982; Darvas & Kotzé, 1987). Certain pesticides also leave a visible residue on the product that is not allowed for export. Such residues must be manually removed, thereby increasing production cost (Denner & Kotzé, 1986). Finally, small niche industries find it increasingly difficult to manage diseases since relatively few, if any new chemicals are

registered. Major agrochemical companies are reluctant to invest in new products or re-register older products due to perceived lower profit margins (Denner & Kotzé, 1986). Alternative control measures and techniques must be developed to replace chemicals or to minimize their use.

Recently, the use of biological control agents has increased significantly as an alternative to pesticides. Biological control is the use of one organism to control another, especially pests or disease causing organisms (Atlas & Bartha, 1987). The most commonly known definition of biological control in plant pathology, is the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists (Baker & Cook, 1974). Recently, numerous studies were aimed at the use of biological control agents to increase our understanding of the interactions between host, pathogen and antagonist (Andrews, 1992; Fiddaman & Rossall, 1993; Gilbert *et al.*, 1994; Milner *et al.*, 1997; Bellows, 1999; Lindow & Wilson, 1999; van Dijk & Nelson, 2000; Benhamou *et al.,* 2001; Helistö *et al.*, 2001).

In South Africa, biological control of postharvest diseases using natural antagonists has been demonstrated successfully (Korsten *et al.*, 1991). A bacterial antagonist, *Bacillus subtilis*, was isolated and successfully screened *in vitro* and *in vivo* to control postharvest diseases of subtropical crops. The use of *Bacillus* species in the biological control of plant pathogens is well documented (Korsten *et al.*, 1989; Korsten *et al.*, 1991; McKeen *et al.*, 1986). *Bacillus* species are very diverse and commercially useful and occur in almost all environments (Harwood, 1989). The Food and Drug Administration has placed *B. subtilis* under GRAS (Generally Regarded As Safe) status. This is mainly due to the global use of members of this species in several fermentation processes and also due to its general lack of pathogenicity (Harwood, 1989). The efficacy and consistency of *B. subtilis* to control avocado diseases received much attention (Korsten *et al.*, 1988; 1989; 1991; 1993; 1995; van Dyk *et al.*, 1997). However, commercialisation of biocontrol products requires not only proof of its efficacy and consistency, but also its mode of action.

Singular modes of action are rare in nature and often a range of synergistic interactions occurs. Competition for nutrients (Chalutz *et al.*, 1988), competitive colonization (Bhatt & Vaughan, 1962), site exclusion (Janisiewicz, 1988), antibiosis (Pusey & Wilson, 1984; Janisiewicz & Roitman, 1988), induction of host defence mechanisms (Janisiewicz, 1987; Chalutz *et al.*, 1988) and direct interaction with the pathogen (Dubos, 1984; Podile & Prakash, 1996) are some of the more familiar modes of action involved in

antagonism. Several possible modes of action have been postulated for avocado pre- and postharvest diseases, namely competition for nutrients, competitive colonization and antibiosis (Korsten & de Jager, 1995).

The aim of this study was therefore to further investigate the mode of action of the antagonist, *B. subtilis*, and to determine the influence of nutrients and temperature on *in vitro* biocontrol activity. This information is critical for improvement of commercial product formulation. Finally, characterization of antifungal substance/s is required for product registration for commercial use and was therefore also investigated. In this study we hypothesize that antibiosis is the predominant mode of action.

REFERENCES

Atlas, R. M. & Bartha, R. 1987. Microbial Ecology: Fundamentals and Applications. Benjamin Cummings Publishing Company, California.

Andrews, J.H. 1992. Biological Control in the Phyllosphere. Annual Review of Phytopathology 30: 603 – 635.

Bellows, T.S. 1999. Foliar, flower, and fruit pathogens. Pages 841 – 852 In: Handbook of Biological Control: Principles and Applications of Biological Control. Bellows, T.S. & Fisher, T.W. (Eds). Academic Press, San Diego.

Benhamou, N., Bélanger, R.R., Rey, P. & Tirilly, Y. 2001. Oligandrin, the elicitin-like protein produced by the mycoparasite *Pythium oligandrum*, induces systemic resistance to *Fusarium* crown and root rot in tomato plants. Plant Physiology and Biochemistry 39: 681 – 698.

Biale, J.B. & Young, R.E. 1971. The avocado pear. Pages 1 – 63 In: The Biochemistry of Fruits and Their Products. Volume 2. Hulme, A.C. (Ed). Academic Press, London & New York.

Baker, K.F. & Cook, R.J. 1974. Biological Control of Plant Pathogens. San Francisco, Freeman.

Bhatt, D.D. & Vaughan, E.K. 1962. Preliminary investigations on biological control of gray mold (*Botrytis cinerea*) of strawberries. Plant Disease Reporter 46: 342 – 345.

Campbell, R. 1989. Biological Control of Microbial Plant Pathogens. Cambridge University Press, Cambridge.

Chalutz, E., Ben-Arie, R., Droby, S., Cohen, L., Weiss, B. & Wilson, C.L. 1988. Yeasts as biocontrol agents of postharvest diseases of fruits. Phytoparasitica 16: 69.

Darvas, J.M. & Kotzé, J.M. 1987. Fungi associated with pre- and post-harvest diseases of avocado fruit at Westfalia Estate, S.A. Phytophylactica 19: 83 – 85.

Denner, F.D.N. & Kotzé, J.M. 1986. Evaluasie *in vitro* van die effektiwiteit van swamdoders teen *Colletotrichum gloeosporioides* en *Dothiorella aromatica*. South African Avocado Growers' Association Yearbook 8: 48 – 51.

Dubos, B. 1984. Fungal antagonism in aerial agrobiocenoses. Pages 107 – 135 In: Innovative Approaches to Plant Disease Control. Chet, I. (Ed). New York, John Wiley & Sons.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Gilbert, G.S., Handelsman, J. & Parke, J.L. 1994. Root camouflage and disease control. Phytopathology 84: 222 – 225.

Hartill, W.F.T. 1991. Post-harvest diseases of avocado fruits in New Zealand. New Zealand Journal of Crop and Horticultural Science 19: 297 – 304.

Harwood, C.R. 1989. *Bacillus*. Biotechnology Handbooks 2. Plenum Press. New York.

Helistö, P., Aktuganov, G., Galimzianova, N., Melentjev, A. & Korpela, T. 2001. Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components. Journal of Chromatography B 758: 197 – 205.

Janisiewicz, W.J. 1987. Postharvest biological control of blue-mold on apple. Phytopathology 77: 481 – 485.

Janisiewicz, W.J. 1988. Biocontrol of postharvest diseases of apples with antagonist mixtures. Phytopathology 78: 194 – 198.

Janisiewicz, W.J. & Roitman, J. 1988. Biological control of blue-mold and gray-mold on apple and pear with *Pseudomonas cepacia*. Phytopathology 78: 1697 – 1700.

Keevy, C. 1999. Foreword. South African Avocado Growers' Association Yearbook 22: v.

Korsten, L., Bezuidenhout, J.J. & Kotzé, J.M. 1988. Biological control of postharvest diseases of avocado. South African Avocado Growers' Association Yearbook 11: 75 – 78.

Korsten, L., Bezuidenhout, J.J. & Kotzé, J.M. 1989. Biological control of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 12: 10 – 12.

Korsten, L. & de Jager, E.S. 1995. Mode of action of *Bacillus subtilis* for control of avocado post-harvest pathogens. South African Avocado Growers' Association Yearbook 18: 124 – 130.

Korsten, L., de Jager, E.S., de Villiers, E.E., Lourens, A., Kotzé, J.M. & Wehner, F.C. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado post-harvest diseases. Plant Disease 79: 1149 – 1156.

Korsten, L., de Villiers, E.E., de Jager, E.S., Cook, N. & Kotzé, J.M. 1991. Biological control of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 14: 57 – 59.

Korsten, L., de Villiers, E.E., Wehner, F.C. & Kotzé, J.M. 1993. A review of biological control of postharvest diseases of subtropical Fruits. Postharvest Handling of Tropical Fruits. ACIAR Proceedings no. 50: 172 – 185.

Kotzé, J.M., du Toit, F.L. & Durand, B.J. 1982. Pre-harvest chemical control of anthracnose, sooty blotch and cercospora spot of avocados. South African Avocado Growers' Association Yearbook 5: 54 – 55.

Lindow, S.E. & Wilson, M. 1999. Biological control of foliar pathogens and pests with bacterial biocontrol agents. Pages 642 – 650 In: Manual of Industrial Microbiology and Biotechnology. 2nd ed. Demain, A.L. & Davies, J.E. (Eds). ASM Press, Washington, D.C.

McKeen, C.D., Reilly, C.C. & Pusey, P.L. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. Phytopathology 76: 136 – 139.

Milner, J.L., Silo-Suh, L., Goodman, R.M. & Handelsman, J. 1997. Antibiosis and beyond: genetic diversity, microbial communities, and biological control. Pages 107 – 127 In: Ecological Interactions and Biological Control. Andow, D.A., Ragsdale, D.W. & Nyvall, R.F. (Eds). Westview Press, Colorado, USA.

Podile, A.R. & Prakash, A.P. 1996. Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1. Canadian Journal of Microbiology 42: 533 – 538.

Pusey, P.L. & Wilson, C.L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Disease 68: 753 – 756.

Snowdon, A.L. 1990. A Colour Atlas of Post-harvest Diseases and Disorders of Fruits and Vegetables. Wolfe Scientific Ltd, Barcelona, Spain.

Van Dijk, K. & Nelson, E.B. 2000. Fatty acid competition as a mechanism by which *Enterobacter cloacae* suppresses *Pythium ultimum* sporangium germination and damping-off. Applied and Environmental Microbiology 66: 5340 – 5347.

Van Dyk, K., de Villiers, E.E. & Korsten, L. 1997. Alternative control of avocado post-harvest diseases. South African Avocado Growers' Association Yearbook 20: 109 – 112.

Wilson, C.L. & Wisniewski, M.E. 1989. Biological Control of Postharvest Diseases of Fruits and Vegetables: an Emerging Technology. *Annual Review of* Phytopathology 27: 425 – 441.

CHAPTER 2

LITERATURE REVIEW

MODES OF ACTION INVOLVED IN BIOCONTROL OF PLANT PATHOGENS, WITH SPECIAL REFERENCE TO FUNGAL PATHOGENS

"No man is an island, entire of it self; every man is a piece of the continent, a part of the main." – John Donne

No single organism can ever exist on its own. It will always be linked within a matrix of complex interactions with other organisms occurring in the same environment. These interactions can be beneficial, have no effect or can be harmful to one or more of the organisms involved (Gooday, 1988). Managing the interactions between microorganisms is the basis of biocontrol (Bellows, 1999). Understanding the mechanisms through which this can be achieved, is critical to the eventual improvement and wider use of biocontrol (Fravel, 1988). To keep plants healthy, the impact of microorganisms detrimental to the plant must be reduced and the effect of beneficial ones exploited (Milner *et al.*, 1997). Few studies focus on mode of action of biocontrol agents of fungal plant pathogens alone, despite the many articles on biocontrol. This review attempts to alleviate some confusion regarding modes of action.

1. HISTORY OF BIOCONTROL

Fungal pathogens, especially those causing postharvest diseases, are most commonly controlled by means of chemical fungicides (Wilson & Wisniewski, 1989; Roberts, 1994). According to van Driesche & Bellows (1996), the worldwide use of pesticides has increased twelve fold since the 1950's. Essentially, fungicides are toxins and some are carcinogens. To persist in the environment and function optimally, fungicides are formulated not to be easily biodegradable (Campbell, 1989). However, serious environmental problems develop due to the accumulation of pesticide residues in natural resources, affecting non-target organisms (Campbell, 1989). Pesticide residues in food also affect human health. In the USA, up to 3000 hospitalisations and 200 fatalities are due to pesticide misuse annually (Campbell, 1989). Pathogens may build up resistance against fungicides when used incorrectly, leading to less effective control of target plant pathogens (Kotzé *et al.*, 1982; Wilson *et al.*, 1994). New or alternative fungicides must subsequently be developed, which is costly. In some instances, chemical sprays leave residues on fruit surfaces that must be removed manually (Denner & Kotzé, 1986), thereby increasing production cost. It is therefore important to

develop alternative disease control measures to ensure global food security (Skidmore, 1976; Payne & Lynch, 1988; Van Driesche & Bellows, 1996; El-Ghaouth, 1997).

The plant pathologist's definition of biological control is the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists (Baker & Cook, 1974). In the 1920's, the first biological control measures were developed to control insect pests by introducing their natural predators to the environment (Campbell, 1989). At approximately the same time investigations were conducted in the use of non-pathogenic microorganisms to control plant disease (Campbell, 1989). Since then many studies focused on the potential of biological control, which resulted in an increase in knowledge of interactions between host, pathogen and antagonist.

Most reported biocontrol programs focus on control of root pathogens within the rhizosphere environment (Payne & Lynch, 1988). Although many biocontrol agents have been tested *in vitro*, very few agents have been commercialised (Payne & Lynch, 1988; Milner *et al.*, 1997). Biocontrol agents do not provide consistent levels of control and variable results are frequently generated (Wilson *et al.*, 1994; Milner *et al.*, 1997). Environmental parameters influence disease progress. Few biocontrol studies have extended their focus to include the effect of environmental parameters on biocontrol efficacy over time. In addition, the interaction of the antagonist with other microbial inhabitants on the plant surface has not been studied extensively. Biocontrol is often not as effective as chemical control in disease prevention (Dubos, 1984; Wilson *et al.*, 1994). An understanding of the interactions between the environment, host, and pathogen and antagonist populations, may therefore help explain the variation found in effectiveness of biocontrol agents under field conditions.

2. MODES OF ACTION INVOLVED IN BIOCONTROL

Keeping the mode or modes of action of an antagonist in mind is crucial for developing successful biocontrol programs (Milner *et al.*, 1997). Even in successful biocontrol strategies, a more in depth understanding of the mechanism of action may improve the reliability of the agent as well as broadening of its application to other host-pathogen combinations (Milner *et al.*, 1997). It may also help to increase the biocontrol product's activity, optimising the method and timing of its application, and developing more appropriate formulations to enhance efficacy and consistency of its performance. Should important modes of action of biocontrol agents

be identified, it could be used in the isolation of more effective antagonists and ensure improved efficacy (Wilson & Wisniewski, 1989; Milner *et al.*, 1997).

It is relatively easy to study pathogen-antagonist interactions *in vitro*. However, it is much more difficult to prove mechanism of interactions in nature (Mari & Guizzardi, 1998; Calvente *et al.*, 1999). Figure 2.1 gives a brief overview of modes of action involved in biocontrol. The mode of action of the antagonist is not always easy to determine. Thus far, most conclusions regarding modes of action have been based on indirect evidence (Droby & Chalutz, 1994). In the case of *Pseudomonas cepacia*, the antagonist produces an antibiotic, pyrrolnitrin. It is also able to control *Penicillium digitatum* (Pers.: Fr.) Sacc. which is known to be resistant to this antibiotic. Antibiosis can therefore be excluded as the mode of action in this interaction (Mari & Guizzardi, 1998).

Baker's possession principle (Baker, 1987) describes the activity of any antagonistic microbe: "A microorganisms already in a substrate may retain possession against vigorous competitors by: rapidly converting nutrients into its own propagules ("personalized packages") making it unavailable to others; polluting the infection site with antibiotics, phytoalexins, or toxic chemicals; preventing accumulation in the infection site of excess nutrients that attract competitors; or modifying the water potential, rate of oxygen diffusion, pH, or other conditions of the host tissue to limit growth of competitors."

Generally, the modes of action of antagonists are poorly understood. Today it is commonly accepted that a range of different modes of action is involved. These modes of action can be grouped into two categories: those acting indirectly and those that act directly upon the pathogen (Figure 2.1). The type of interaction can be density dependent and can be "regulated" or "switched on" by the specific growth phases in the life cycle of the pathogen or antagonist (Dunny & Leonard, 1997). Microbial interactions should, therefore, be seen over time and space. Modes of action frequently involved in biocontrol systems include antibiosis, induced resistance, competition for space and nutrients, parasitism, and even plant growth promotion (Payne & Lynch, 1988; Milner *et al.*, 1997; Bellows, 1999).

2.1. Indirect interactions

Indirect interactions include cross-protection, hypo virulence and growth stimulation where the antagonist does not act directly on the target pathogen. These interactions act on the host, stimulating its resistance

mechanisms or changing the ecology of the surrounding area to discourage pathogens from germinating and infecting the plant (Payne & Lynch, 1988; Fravel, 1988).

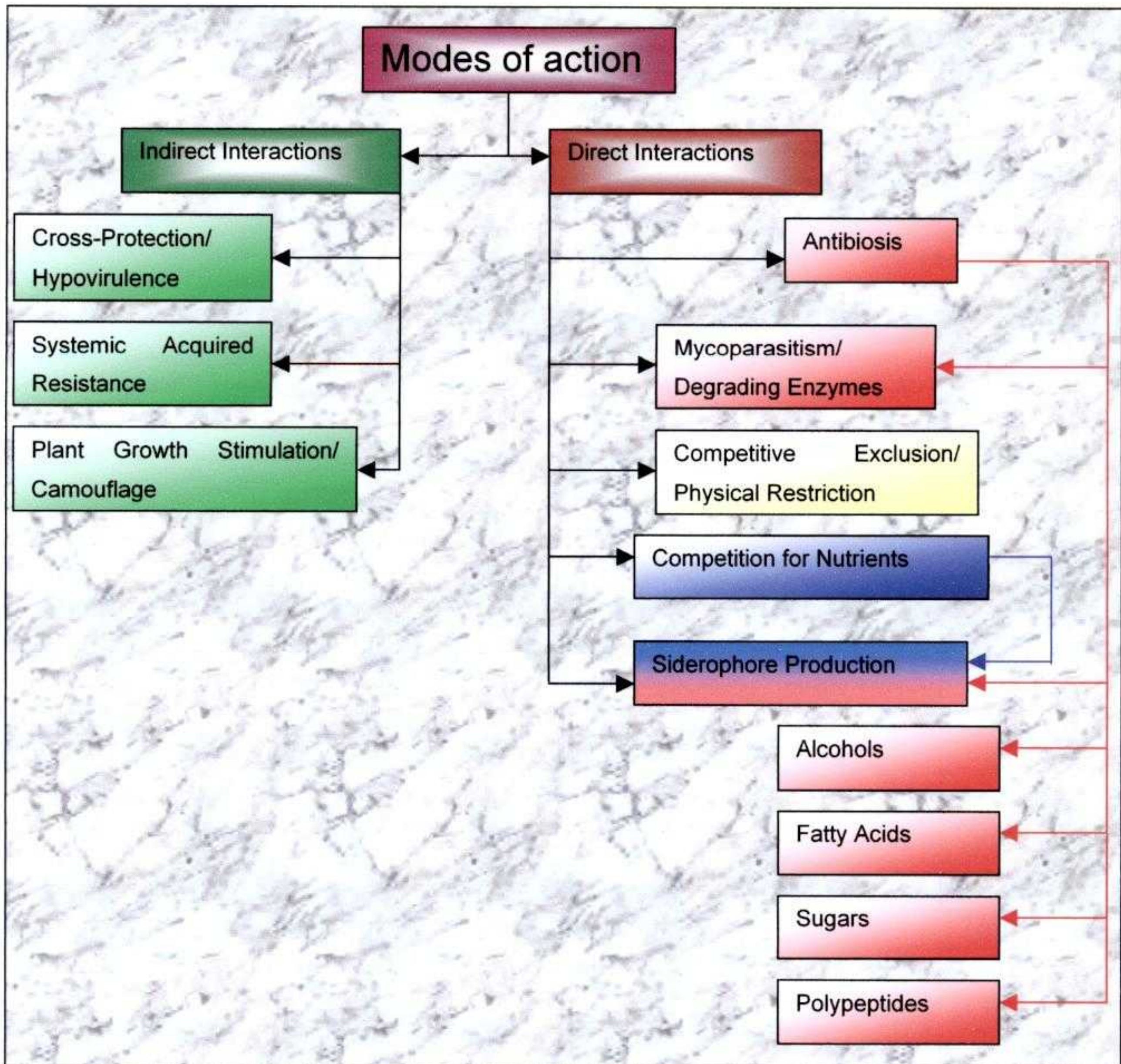


Figure 2.1: Modes of action involved in biocontrol and their relation to each other.

2.1.1. Cross-protection or hypo virulence

Cross-protection occurs when an established virus prevents a related virus from fully expressing its disease-causing ability (Milner *et al.*, 1997; Dodds, 1999; Bellows, 1999). The challenging virus usually fails to accumulate in the host. Since this mode of action occurs only with viruses, it will not be discussed further.

2.1.2. Systemic acquired resistance

Systemic acquired resistance is the development of resistance throughout the whole plant. Plants are challenged by biotic and abiotic elicitors (agents inducing resistance), including non-pathogenic organisms. These induce the plant defence mechanisms and limit or even prevent subsequent infections by pathogens (Van Driesche & Bellows, 1996; Bellows, 1999). Resistance development is seen in areas distant from the original inoculation site and can be very unspecific in its target pathogen (Bellows, 1999).

Elicitors can be grouped into three categories: organic molecules, chemicals and antagonists. Organic elicitors commonly involved in induced resistance include ethylene, chitin, chitosan oligosaccharides and salicylic acid (Wilson *et al.*, 1994; Droby *et al.*, 1996). Chemicals able to induce resistance in plant tissue include benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester, β-aminobutyric acid and 2,6-dichloroisonicotinic acid (Benhamou *et al.*, 2001). Antagonistic organisms are also able to induce resistance, usually by producing a wide range of organic molecules. These molecules include pathogenesis related proteins and jasmonates (Kogel *et al.*, 1995), as well as some of the organic molecules mentioned earlier (Wilson *et al.*, 1994; Droby *et al.*, 1996; Dodds, 1999; Benhamou *et al.*, 2001). Several studies on different fruit crops implicate host exposure to antagonists resulting in induced resistance (Table 2.1). In harvested crops, elicitation can also occur through physical means. Physical treatments that can induce resistance include heat treatment, wounding, gamma radiation and UV-C light (Wilson *et al.*, 1994; Mari & Guizzardi, 1998).

Induction signalling molecules are produced by the plant upon treatment with the elicitor and are used to prime the activation of resistance in the rest of the host (Benhamou *et al.*, 2001). The host act upon the signalling molecules by triggering its gene expression (Benhamou *et al.*, 2001). Defence systems include the lignifications of cell walls through the addition of chemical cross-linkages in cell wall peptides, making it difficult for the pathogen to establish infection through lysis; suberification of tissues, where fatty substance suberin infiltrates cell walls, making them corklike; production of phytoalexins (Paul *et al.*, 1998), chitinases and β-1,3-glucanases (Bellows, 1999). When challenged, a necrotic hypersensitive reaction is elicited in some cases (Benhamou *et al.*, 2001).

Benhamou *et al.* (2001) tested several elicitors against *Fusarium oxysporum* (Schltdl.: Fr.), the casual agent of tomato crown and root rot. The tomato root cells underwent several modifications when treated with the

elicitors. The nature and spectrum of the observed modifications differed depending on the elicitor. This suggests that there may be more than one general pathway followed after elicitation.

Induced resistance is recognized as an important form of resistance in vegetative plant tissues and similar mechanisms may function in harvested fruit. Some reports indicate that certain postharvest biocontrol agents may interact with host tissues, particularly during wounding and thereby enhancing wound healing (Droby & Chalutz, 1994). In studies done by El-Ghaouth (1998), *Candida saitoana* Montrocher also stimulated the production of papillae and other protuberances in the tissue underlying the wounded area. These protuberances might contain phenolic-like substances and could help the tissue to restrict the spread of the invading pathogens.

In many cases a combination of induced resistance and other modes of action may be involved in biocontrol. Weller (1988) used a strain of *Pseudomonas fluorescens* CHAo, to suppress *Thielaviopsis basicola* (Berk. & Broome) Ferraris, causing black root rot of tobacco. In the case of *P. fluorescens*, both the production of antibiotics and siderophores are used in its action. However, it was found that hydrogen cyanide production is also important. Mutant strains of *P. fluorescens* deficient in producing HCN were less suppressive than the normal strain, proving the involvement of induced resistance in its mode of action (Weller, 1988).

2.1.3. Plant growth stimulation and camouflage

A more subtle or indirect mechanism may play a role in keeping plants healthy. Gilbert *et al.* (1994) observed a change in the microbial community surrounding the roots when treating soybeans with *Bacillus cereus* strain UW85. The community changed from one resembling the rhizosphere microbiology to that resembling the soil microbiology. This happened without *B. cereus* UW85 becoming the dominant organism in the community. Coinciding with the population change came a reduction in root disease. These results lead to the development of Gilbert's "camouflage hypothesis". According to this hypothesis, the microbial community changes the root ecology so that it resembles that of the soil, disguising the root (meaning the rhizosphere niche) so that the pathogen will not detect it, or making it less attractive to pathogens thereby protecting it from disease. However, this hypothesis has not been tested or challenged (Gilbert *et al.*, 1994; Milner *et al.*, 1997). According to Milner *et al.* (1997), this may be one mechanism in which antibiotics produced by antagonists work. It may not act directly on the pathogen, but indirectly influencing the microbial community to adapt.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 2.1: Cases where induced resistance was found to be involved in antagonism of pathogenic fungi

Host	Control agent	Disease	Pathogen	Molecules involved	Reference
Apple *Malus sylvestris* Mill.	*Candida saitoana* Montrocher	Postharvest rot	*Botrytis cinerea* (Pers.: Fr.) *Penicillium expansum* Link	Chitinase induction	El-Ghaouth *et al.*, 1998
Apple *Malus sylvestris* Mill. Peach *Prunus persica* Sieb. & Zucc. Citrus	*Pichia guilliermondii* (US-7) Wickerham	Postharvest rot	*Penicillium digitatum* (Pers.: Fr.) Sacc. *Botrytis cinerea* (Pers.: Fr.)	Phytoalexin scoparone, phenylalanine ammonia lyase, peroxidase induction	Wilson *et al.*, 1994
Grapevine *Vitis vinifera* L.	*Bacillus* spp.	Grey mould	*Botrytis cinerea* (Pers.: Fr.)	Phytoalexin induction	Paul *et al.*, 1998
Groundnut *Arachis hypogaea* L.	*Bacillus subtilis* AF1	Crown rot	*Aspergillus niger* Tiegh.	Lipoxygenase	Sailaja *et al.*, 1997
Tobacco *Nicotiana tabacum* L.	*Pseudomonas fluorescens* CHAo	Black root rot	*Thielaviopsis basicola* (Berk. & Broome) Ferraris	Hydrogen cyanide production	Ahl *et al.*, 1986; Stutz *et al.*, 1986
Tomato *Lycopersicon esculentum* Mill.	*Pythium oligandrum* Drechsler	*Fusarium* crown and root rot disease	*Fusarium oxysporum* f.sp. *radicis-lycopersici* Jarvis & Shoemaker	Elicitin: Oligandrin production	Benhamou *et al.*, 2001

117877 26X
b16733812

It is not only root-related pathogens that may be controlled using this approach. Biocontrol in the postharvest arena have also been challenged by indirect manipulation of total populations that already occur on the plant surface. According to Wilson & Wisniewski (1989), there are two basic approaches available in postharvest biocontrol: promoting and managing natural antagonists that already exist on the surface, or by artificial introduction of target antagonists. It has been suggested that certain hosts may control the microbial populations on its surfaces through expression of its own genes (Wilson & Wisniewski, 1989). The host manipulates the microbial community to be more suppressive to diseases. It may become possible in future to modify the host genetics, ensuring that a disease-suppressive microbial population will be sustained.

Some microbial antagonists may play a role in stimulating growth of the host. In the case of *B. cereus* UW85, nodulation of soybeans were increased when treated with the antagonist. This may contribute to plant health (Milner *et al.*, 1997). The possibility of having antagonists that act more indirectly by promoting plant health or altering microbial populations and keeping plants healthy may prove useful in the future.

2.2. Direct interactions

A more direct interaction between the antagonist and pathogen are more commonly found. To date, direct interactions have been more extensively studied compared to indirect interactions. Direct interactions include antibiosis, parasitism, competition for space and nutrients, as well as the production of siderophores and volatiles. However, there is no clear distinction between the direct interactions since siderophore production (molecules produced to sequester iron ions, making them more readily available to the producer organism) can also be seen as competition for nutrients and iron. Also, the production of enzymes, volatiles, and toxic substances by the antagonist can be seen as antibiosis (Fravel, 1988). However, interaction definitions are dependent on the view of the researcher.

2.2.1. Antibiosis

Baker & Cook (1974) defined antibiosis as the inhibition of one organism by a metabolite of another. Fravel (1988) described antibiotics as low molecular weight organic compounds produced by antagonists that are deleterious to the growth of other microbes. Milner *et al.* (1997) saw antibiosis as the production of toxic metabolites like antibiotics, lytic enzymes, and volatile substances. However, antibiosis could result from production of an alcohol or change in pH of the environment or by the production of simple substances not commonly considered to be antibiotics. Thus, metabolites of all types could qualify as products involved in

antibiosis. The use of enzymes and siderophores as biocontrol modes of action are discussed in section 2.2.2 and 2.2.5 respectively.

Of the various forms of antagonism, antibiosis has numerous advantages. Direct physical contact between the antagonistic organism and the target pathogen is not necessary (McKeen *et al.*, 1986). Antibiotic substances may diffuse in water films and water filled pores, or in the case of volatiles, through air filled pores (Fiddaman & Rosall, 1993). The area in which the antibiotic is active will be greater, and its impact will be more rapid and even more effective than other modes of action such as competition or hyper parasitism. Antibiosis may even continue to play a role even after growth ceased from the antagonist. Such reports of antibiotic release from senescent cells within the colony have previously been documented (Baker & Cook, 1974).

In nature, antibiotic production is a common phenomenon and several bacterial antagonists are reported to produce antibiotics that may play a role in protecting crops from pathogen attack. Antibiotics that are produced *in vitro* will not necessarily be produced at the site of action or on the host surface or *in vivo* (Fravel, 1988; Droby & Chalutz, 1994). Some antibiotics are produced only in culture where conditions are optimal (Baker & Cook, 1976; Fravel, 1988). Antibiotics may therefore not always be produced in the phyllosphere. This can be seen where culture filtrates of antagonists are inhibitory to pathogens, while washed cells are not. According to Pfender (1996), this may be due to poor growth of the applied antagonist, or to a lack of antibiotic production under nutritional and physical conditions existing on the phyllosphere. Neighbouring organisms can easily break down antibiotics or it can be inactivated by adsorption to the surface of the host (Blakeman & Brodie, 1976). According to Baker & Cook (1974), antibiosis works best where nutrients are abundant or in excess, such as at specific micro sites i.e. leaking plant parts or organic debris.

Less attention has been given to antibiosis mediated through the production of volatile substances compared to non-volatile antibiotics (Fravel, 1988). Volatile substances produced by antagonists include molecules like ammonia, alkyl pyrones, ethanol, isobutanol, isoamyl alcohol, and isobutyric acid (Fravel, 1988). These molecules can directly inhibit fungi *in vitro*. Fiddaman & Rossall (1993) found that *Bacillus subtilis* produces a volatile substance that is able to inhibit the growth of *Rhizoctonia solani* (J. G. Kühn) and *Pythium ultimum* (Trow).

Milner *et al.* (1997) defined four approaches of determining the role of antibiosis in antagonism. Firstly, mutants unable to produce the antibiotic are tested for activity. If the non-producing strain is unable to give control over the pathogen, antibiosis is involved (Fravel, 1988; Gutterson, 1990; Weller, 1988; O'Sullivan & O'Gara, 1992; Leifert *et al.*, 1995). Secondly, the antibiotic can be purified and tested. If it is effective in the field situation, it is involved in antagonism. However, antibiotics can adsorb onto host tissue and soil particles, inactivating it (Blakeman & Brodie, 1976). The third approach involves the use of a pathogen that is insensitive to the antibiotic. If the antagonist inhibits the pathogen, the antibiotic is not involved and another mechanism may play a role. Finally, genes coding for the antibiotic can be cloned into non-expressing microorganisms. Clones can be evaluated for activity against the pathogen. If it becomes inhibitory, the antibiotic is involved in antagonism.

These strategies were employed to show the involvement of the antibiotic phenazine-1-carboxylic acid in the activity of *P. fluorescens* strain 2-79, against *Gaeumannomyces graminis* var. *tritici* (Sacc.) Oliver & Von Arx, casual agent of take-all of wheat (Weller & Cook, 1983; Bull *et al.*, 1991). The antagonist significantly decreased not only the size, but also the number of lesions, indicating that the antibiotic is a factor in suppression of primary infection by the pathogen. The antibiotic was also isolated from the rhizosphere of healthy roots. A mutant strain, unable to produce phenazine antibiotic was unable to suppress lesion formation, indicating that the antibiotic plays a role in antagonism (Bull *et al.*, 1991). A pyoverdine siderophore and an additional antifungal factor, playing a minor role in disease suppression, were also produced (Hamdan *et al.*, 1991; Slininger & Jackson, 1992; Milner *et al.*, 1997).

Different approaches exist in practice to exploit antibiosis. In the case of Tsuge *et al.* (1996), the antibiotic itself was produced, partially purified and used as a biocontrol agent. Iturin A, produced by *B. subtilis* NB22, was obtained using solid-state fermentation of okara (soy bean curd residue). This approach resulted in a high-efficiency and low production cost of the antibiotic. However, one can reason that following this approach is similar to more traditional chemical control.

An antagonist can produce an array of different antibiotics. Pseudomonads are known to produce a variety of inhibitory compounds that all contribute to disease suppression, though not all work against all pathogens on all hosts (O'Sullivan & O'Gara, 1992; Milner *et al.*, 1997). *P. fluorescens* strain CHAO produces several antifungal compounds, including hydrogen cyanide; antibiotics, including pyoluteorin and 2,4-diacetylphloroglucinol; as well as a siderophore, pyoverdine. Pyrrolnitrin contributes to the suppression of *R.*

solani, while pyoluteorin suppresses *P. ultimum* on cotton seedlings. Tobacco root rot caused by *T. basicola* is suppressed by the production of hydrogen cyanide and 2,4-diacetylphloroglucinol. The antibiotic, 2,4-diacetylphloroglucinol, suppresses take-all of wheat, caused by *G. graminis*. The causal agent of damping-off of cress, *P. ultimum*, is suppressed by pyoluteorin (Milner *et al.*, 1997). *B. subtilis* cell-free filtrates protect fruit from *Monilinia fructicola* (Wint.) Honey (McKeen *et al.*, 1986). The active material were isolated and several iturin peptides were identified, having a low toxicity and lacking allergenic properties, which is active against few bacteria but a wide variety of fungi (Gueldner *et al.*, 1988).

Antibiotics work in different ways. Thus far, the direct effect of a few antibiotics has been determined. By-products of the metabolic activity of *B. cereus* UW85 are antagonistic to oomycete and accumulate in culture supernatants (Milner *et al.*, 1996). Some exhibit their antagonistic activity through the sequestering of calcium and the production of large amounts of ammonium (Milner *et al.*, 1997). This increases the pH of the medium and causes the lysis of oomycete zoospores. However, the increase of the ammonium to calcium ratio does not account for the main ability of *B. cereus* UW85 to suppress disease. Antibiotics, including zwittermicin A and antibiotic B, are also produced. Zwittermicin A is a linear aminopolyol with a broad host range that includes both fungi and bacteria. Its activity includes the reversible inhibition of germ tube elongation of *Phytophthora medicaginis* E.M. Hansen & D.P. Maxwell. Antibiotic B is an aminoglycoside. It has a narrower target range than zwittermicin A, but also shows activity against both bacteria and fungi. These two antibiotics do not account for all antifungal activity of *B. cereus* UW85, suggesting that it produces additional metabolites with antifungal activity (Milner *et al.*, 1997).

With the use of recombinant DNA technology, antibiosis can be readily manipulated and exploited to enhance disease suppression (Spadaro & Gullino, 2004). Antibiotic production regulation and biosynthesis has received a lot of attention recently. New approaches may enhance the amount of antibiotic synthesized or to improve the pattern of antibiotic synthesis. The synthesis of many antibiotics is influenced by specific nutrients and may be applied to enhance the activity of the biocontrol agent (Martin & Demain, 1980; Fravel, 1988; Gutterson, 1990; Thomashow *et al.*, 1990; Clarke *et al.*, 1992; Slininger & Jackson, 1992). Once the genes involved in the production of antibiotics are cloned, they can be placed under the control of a promoter that can be regulated giving control over the amount of antibiotic produced as well as timing of antibiotic production. Multiple copies can be cloned into the biocontrol organism that will also increase the amount of antibiotics produced. However, increasing antibiotic production does not necessarily imply that the biocontrol activity will be enhanced as well. In the case of *P. fluorescent* CHAO, increasing the production of

pyoluteorin, active against *P. ultimum*, resulted in the antagonist becoming phytotoxic to cress and sweet corn (Maurhofer *et al.*, 1992).

There remains some public concern over the use of antibiotic producing antagonists as biocontrol agents of postharvest diseases (Spadaro & Gullino, 2004). Introducing antibiotic producing antagonists into food supplies may have adverse effects on human resistance to antibiotics (Spadaro & Gullino, 2004). Pathogens are also more likely to develop resistance to antibiotics, possibly by only a single mutation, and the antagonist's efficacy is lost. Natamycin is an example of an antibiotic that has been widely used for food preservation to which very little resistance has been found (Droby & Chalutz, 1994). Some reports of members of the genus *Bacillus* producing antibiotics targeting prokaryotic and eucaryotic organisms are summarized in Table 2.2.

2.2.2. Mycoparasitism and Cell-wall degrading enzymes

Parasitism of soil borne and foliar fungal diseases by antagonists is well known (Wilson & Wisniewski, 1994). The term parasitism covers various interactions, including morphological disturbances, overgrowth of one organism by another (especially in the case of fungi), penetration and direct parasitism by production of haustoria, or the lysis of one organism by another (Wilson & Wisniewski, 1994). Necrotrophic interactions occur when the parasite derives nutrients from dead host cells, killed usually by the parasite itself, even though it may not invade the host (Skidmore, 1976).

Several enzymes produced by antagonists are involved in biocontrol, including glucose oxidase, lipase, protease, laminarinase, β-glucosidases, mannanase, xylanase, cellulases, chitinase and chitosanase (Fravel, 1988; Droby *et al.*, 1996; Nielsen & Sørensen, 1997; Frändberg & Schnürer, 1998; Picard *et al.*, 2000). Enzymes involved in biocontrol distort the distinction between parasitism and antibiosis. According to Fravel (1988), antagonists producing cell wall degrading enzymes that may simultaneously parasitise the pathogen and inhibit it through antibiosis. Other enzymes may cause only antibiosis where the antibiotic is an enzymatic end product. In the case of *Talaromyces flavus* (Klöcker) Stolk & Samson TF1, glucose oxidase is produced that catalyses the production of hydrogen peroxide from glucose (Kim *et al.*, 1980). In the case of *Bacillus* spp. X-b, a complex of different enzymes (chitinase, chitosanase, laminarinase, lipase and protease) are produced (Helistö *et al.*, 2001). Several examples of enzymes involved in biocontrol are summarised in Table 2.3.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 2.2: Antibiotics produced by members of the genus *Bacillus*

Antagonist	Type	Structure	Group / antibiotic	Host	Pathogen	Disease	Reference
Bacillus brevis	Peptide antibiotics	Cyclic peptide	Gramicidin S	-	-	-	Katz & Demain, 1977
Bacillus brevis	Peptide antibiotics	Cyclic peptide	Tyrocidine	-	-	-	Katz & Demain, 1977
							Mootz & Marahiel, 1997
Bacillus brevis	Peptide antibiotic	Peptide lactone	Brevistin	-	-	--	Shoji, 1978
Bacillus brevis	Peptide antibiotics	Linear peptide	Edeine	-	-	-	Katz & Demain, 1977
Bacillus cereus	Peptide antibiotic	Linear peptide	Cerexins A to D	-	-	-	Shoji, 1978
Bacillus cereus UW85	3-amino-3-deoxy-D-glucose	-	Kanosamine	Alfalfa *Medicago sativa* L.	*Phytophthora medicaginis* E.M.	Damping-off	Milner *et al.*, 1996
				Tobacco *Nicotiana tabacum* L.	Hansen & D.P. Maxwell		Silo-Suh *et al.*, 1998
					Pythium torulosum Coker & P. Patt		Shang *et al.*, 1999
Bacillus cereus UW85	Aminopolyol	-	Zwittermicin A	Alfalfa *Medicago sativa* L.	*Phytophthora medicaginis* E.M.	Damping-off	Milner *et al.*, 1995
				Tobacco *Nicotiana tabacum* L.	Hansen & D.P. Maxwell		Raffel *et al.*, 1996
					Pythium torulosum Coker & P. Patt		Silo-Suh *et al.*, 1998
							Shang *et al.*, 1999
Bacillus circulans ATCC 21656	Peptide antibiotic	Cyclic peptide	EM-49	-	-	-	Loeffler *et al.*, 1986
Bacillus circulans ATCC 21656	Peptide antibiotic	Cyclic peptide	Octapepin C_1	-	-	-	Shoji, 1978
Bacillus colistinus	Peptide antibiotics		Colistin	-	-	-	Katz & Demain, 1977
Bacillus licheniformis	Peptide antibiotics	Cyclic peptide	Bacitracin A to F	-	-	-	Katz & Demain, 1977
							Podlesek *et al.*, 2000
Bacillus mesentericus	Peptide antibiotic	Peptide lactone	Esperin	-	-	-	Shoji, 1978
Bacillus polymyxa	Peptide antibiotics		Gatavalin	Cauliflower *Brassica oleracea* L. var. *botrytis* L.	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	Cyclic peptides	Polymyxin	-	-	-	Katz & Demain, 1977
							Pichard *et al.*, 1995
							Shoji, 1978
Bacillus polymyxa	Peptide antibiotics	-	Jolipeptin	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	BN 109	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	LI-F	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	Gavaserin	-	*Xanthomonas campestris* pv. *campestris*	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	-	Saltavalin	-	-	-	Pichard *et al.*, 1995
Bacillus polymyxa	Peptide antibiotics	Linear peptides	Tridecaptin A, B, C	-	-	-	Shoji, 1978
Bacillus subtilis	Peptide antibiotics	Cyclic peptide	Mycobacillin	-	-	-	Katz & Demain, 1977
							Shoji, 1978
Bacillus subtilis	Lipopeptide	-	Iturin: Bacillomycin L_c	-	*Ophiostoma ulmi* (Buisman) Nannf.	Dutch Elm Disease	Eshita *et al.*, 1995

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 2.2 (cont.)

Antagonist	Type	Structure	Group / antibiotic	Host	Pathogen	Disease	Reference
Bacillus subtilis	Lipopeptide	-	Surfactin	-	-	-	Jacques *et al.*, 1994 Tsuge *et al.*, 1996
Bacillus subtilis	Lipopeptide	Cyclic peptides	Iturin A, C	Peach *Prunus persica* Sieb. & Zucc.	*Monilinia fructicola* (Wint.) Honey	Brown rot	Gueldner *et al.*, 1988 Jacques *et al.*, 1994
Bacillus subtilis	Lipopeptide	Cyclic peptides	Bacillomycin D, L, F	-	-	-	Jacques *et al.*, 1994 Peypoux *et al.*, 1984
Bacillus subtilis 370	Lipopeptide	-	Mycosubtilin	-	-	-	Peypoux *et al., 1986* Jacques *et al.*, 1994
Bacillus subtilis S499	Lipopeptide	-	Lipobacillin	-	Fungi	-	Jacques *et al.*, 1994
Bacillus subtilis FR-2	Lipopeptide	-	Bacillopeptins A, B, C	Garlic *Allium sativum L.*	*Fusarium oxysporum* (Schltdl.: Fr.)	Basal rot	Kajimura *et al.*, 1995
Bacillus subtilis F-29-3	Peptide antibiotic	Linear peptide	Bacilysin	Mungbean *Phaseolus aureus* Roxb.	*Rhizoctonia solani* (J. G. Kühn)	-	Loeffler *et al.*, 1986
Bacillus subtilis A14	Dipeptide	-	Bacilysin, Anticapsin	-	Bacteria: *Staphylococcus aureus*	-	Sakajoh *et al.*, 1987
Bacillus subtilis 168	Peptide antibiotic	Linear peptide	Bacilysin	-	-	-	Hilton *et al.*, 1988
Bacillus subtilis ATCC 6633		-	Bacilysin	-	Bacteria, fungi	-	Kugler *et al.*, 1990
Bacillus subtilis F-29-3	Lipopeptide	-	Fengymycin / Fengycin	-	-	-	Loeffler *et al.*, 1986 Chen *et al.*, 1995 Lin *et al.*, 1998
Bacillus subtilis YB8	Lipopeptide	-	Plipastatin B1	-	-	-	Tsuge *et al.*, 1996
Bacillus subtilis	Lantibiotic	Peptide lactone	Subtilin	-	Bacteria	-	Shoji, 1978; Klein *et al.*, 1996
Bacillus subtilis ATCC 6633	Phosphono-oligopeptide	-	Rhizocticin A to D	-	Fungi	-	Kugler *et al.*, 1990
Bacillus subtilis ATCC 6633		-	Subsporin A to C	-	Fungi	-	Kugler *et al.*, 1990
Bacillus isolates	Phenolic derivatives	-	Coumarin and isocoumarin: Amicoumacin	-	Bacteria	-	Pinchuk *et al.*, 2002
Bacillus sp. HIL Y-85, 54728	Lantibiotic	-	Mersacidin	-	*Staphylococcus aureus*	-	Bierbaum *et al.*, 1995

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 2.3: Enzymes involved in biocontrol of fungal pathogens

Antagonist	Pathogen	Enzymes	Reference
Aureobasidium pullulans (de Bary) G. Arnaud	*Botrytis cinerea* (Pers.: Fr.) *Penicillium expansum* Link *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. *Aspergillus niger* Tiegh.	Exochitinase β-1,3-glucanase	Castoria *et al.*, 2001
Pichia guilliermondii Wickerham	*Botrytis cinerea* (Pers.: Fr.)	β-1,3-glucanase	Wisniewski *et al.*, 1991
Bacillus subtilis AF1	*Aspergillus niger* Tiegh.	-	Podile & Prakash, 1996
Bacillus sp. X-b	Wood decay fungi	Chitinase Chitosanase Laminarinase Lipase Protease	Helistö *et al.*, 2001
Pythium oligandrum Drechsler	*Phytophthora parasitica* (Breda de Haan) Tucker	Cellulase	Picard *et al.*, 2000
Bacillus pumilus *Bacillus polymyxa*	*Aphanomyces cochleoides* Drechsler *Pythium ultimum* Trow *Rhizoctonia solani* (J. G. Kühn)	Glucanolytic and proteolytic enzymes	Nielsen & Sørensen, 1997
Bacillus pabuli K1 Species of: Pseudomonads, *Corynebacterium-Arthrobacter* group, *Streptomyces*, *Bacillus*	Various fungi *Penicillium roqueforti* Dierckx (J5)	Chitinases	Frändberg & Schnürer, 1994
Pichia anomala Strain K (E.C. Hansen) Kurtzman	*Botrytis cinerea* (Pers.: Fr.)	Exo-β-1,3-glucanase	Jijakli & Leoivre, 1998
Candida saitoana Montrocher	*Botrytis cinerea* (Pers.: Fr.) *Penicillium expansum* Link	Chitinase β-1,3 glucanase	El-Ghaouth *et al.*, 1998
Bacillus cereus UW85	-	-	Gilbert *et al.*, 1994

In the postharvest arena, little is known about biocontrol agents that directly parasitise pathogens (Wilson & Wisniewski, 1994). The yeast, *Pichia guilliermondii* Wickerham, attaches very effectively to the fungal pathogen *Botrytis cinerea* (Pers.: Fr.) hyphae. There is evidence that the antagonist produces hydrolases that may be responsible for the degradation of the fungal cell wall (Droby & Chalutz, 1994; Droby *et al.*, 1996). Cells of *C. saitoana* attach to *B. cinerea* spores and hyphae, preventing the proliferation of the pathogen. In this interaction El-Ghaouth *et al.* (1998) postulated that the yeast might have affected the ability of the fungus to degrade the host tissue. Fungal hyphae were atypical where it was in contact with the yeast, showing alterations ranging from cell wall swelling to degradation of the cytoplasm. These alterations showed similarity to symptoms observed in aged and starved fungal hyphae. Nutritional starvation of the fungi may be caused by the rapid colonization of the yeast. The yeast is also able to produce fungal cell wall degrading enzymes, including chitinase and β-1,3 glucanase. The production of these enzymes may explain some of the observed alterations where the yeast is in contact with the fungal cell wall (El-Ghaouth *et al.*, 1998).

2.2.3. Competitive exclusion and physical restriction

Competition is the interaction between two or more microbial populations that has a simultaneous demand for the same resource, whether it is living space or nutrients (Droby & Chalutz, 1994). If a microbial population already colonized an area, it naturally excludes newcomers. To establish an antagonist before the pathogen arrives is important, especially when the antagonist is not an aggressive colonizer (Morris & Rouse, 1985; Wilson & Wisniewski, 1989; Andrews, 1992). There is no clear distinction between competitive exclusion and competition for nutrients (see section 2.2.4), since the ability to colonize a specific surface goes hand in hand with its ability to take up nutrients thereby depleting the nutrient supply to the surrounding microbes. According to Kinkel *et al.* (1996), competitive ability consists of two distinct parts. Firstly, it describes the ability of a microbe to reduce the relative fitness of co-existing organisms. This can be achieved through predation, antibiosis, parasitism or competition. Secondly, it refers to the ability of the microbe to resist the reduction in its own fitness. It is difficult to directly measure the process of competition. By measuring the reduction in reproductive output, biomass, or vigour of competing microbes, the intensity of competition can be quantified.

Several studies show that exclusion of the pathogen occurs on the host and is an important mode of action in biocontrol. Some *Pseudomonas* species needs to be aggressive colonizers of roots to be successful as biocontrol agents (Bull *et al.*, 1991). In the case of *Cladosporium herbarum* (Pers.: Fr. Link), a saprophytic

fungus, extensive growth on pollen rich rye leaves could prevent the pathogen spores from actually reaching the leaf surface (Skidmore, 1976). When leaf surfaces were inoculated with ice nucleation-deficient strains of *P. syringae* before producing strains were inoculated, the producing strains were excluded (Andrews, 1992). Exclusion may play a role in the suppression of crown gall caused by *Agrobacterium tumefaciens* by *A. radiobacter,* but it has not been proven (Milner *et al.*, 1997).

This mode of action is not usually taken into consideration in the initial selection of antagonists, since screening for it involves more labour-intensive methods. Also, when other more aggressive modes of action, like antibiosis, are involved, the impact of competitive exclusion is seldom directly observed.

2.2.4. Competition for nutrients

Environmental factors severely restrict microbial growth on leaves. The growth of some of the microbes on the plant surface may be limited by the low quantity of nutrients present at any one time (Morris & Rouse, 1985). On newly expanded leaves, the supply of nutrients may be limited. However, as the leaf starts to age, the amount of nutrients on the leaf surface increase. Sometimes water availability will be variable and affect biocontrol (Campbell, 1989; Mercier & Wilson, 1995). The availability of nutrients is not only restricted on leaves, but also on fruit surfaces. Competition for nutrients is described on the phylloplane (Campbell, 1989; Droby & Chalutz, 1994; Bellows, 1999) and various researchers discuss its involvement in biocontrol (see table 2.4). However, indisputable evidence supporting its role in biocontrol is lacking (van Dijk & Nelson, 2000).

The different members of the microbial population that makes up the ecosystem on the plant surface, differs in their ability to grow and take-up nutrients (Droby *et al.*, 1996). According to Droby & Chalutz (1994), bacteria and yeasts are able to take-up nutrients from a dilute solution more rapidly and in greater quantities than the germ tubes of filamentous fungi, due to their large surface-to-volume ratio. Competition can be important at two stages in the pathogen's life cycle (Bellows, 1999). There may be competition during the initial establishment of fresh resource that was not previously colonized by microorganisms. Then, after initial establishment, there can be further competition to secure enough of the limited resources present to permit survival and eventual reproduction (Bellows, 1999).

Most postharvest infections occur through wounds inflicted during harvest and handling (Roberts, 1994). In order to control wound pathogens, the antagonist must be present at the wound site where the antagonism is

to take place (Wilson & Wisniewski, 1989; Roberts, 1994). Wound competence is the ability of the antagonist to grow rapidly at the wound site, be an effective utiliser of nutrients at low concentrations, and survive and develop better than the pathogen on the surface of the fruit and at the infection site under extreme temperature, pH and osmotic conditions. Most postharvest antagonists are able to survive and increase their numbers rapidly in wounds (Droby & Chalutz, 1994).

Competition for nutrients was demonstrated in the interaction of the yeast, *P. guilliermondii*. The antagonist and pathogen were co-cultured on a minimal synthetic medium or in wound leachate solutions (Wisniewski *et al.*, 1991). Spore germination and growth of the pathogen was inhibited when co-cultured with the antagonist. The antagonist concentration determined the inhibition and adding exogenous nutrients reversed the inhibition. This indirect evidence points to the role of nutrient competition in the interaction. There exists a delicate balance at the wound site between the numbers of antagonist and pathogen propagules, affecting the outcome of the interaction and determining whether the wound will become infected or not (Wilson & Wisniewski, 1989). However, Droby & Chalutz (1994) showed that the number of antagonist cells at the wound site does not always determine its efficacy.

To demonstrate nutrient competition as a mechanism of action, it is assumed that the pathogen needs external sources of nutrients for germination and penetration into the host tissue and this assumption is difficult to prove (Droby & Chalutz, 1994). Indirect evidence of the role of nutrient competition can be obtained if the following occurred: 1) spore germination and/or pathogen growth inhibition occurred during co-culturing with the antagonist; 2) pathogen inhibition was dependent on the concentration of the antagonist propagules; and 3) the inhibition could partially or completely be reversed by adding exogenous nutrients (Droby & Chalutz, 1994). Examples of antagonistic interactions where competition for a limited resource is summarised in table 2.4. Siderophore-mediated competition for iron is implicated mostly in rhizosphere systems (see section 2.2.5). Antagonists grow in a microsite, depleting it of certain nutrients. In some cases, fungal spores need exogenous nutrients to germinate (Skidmore, 1976; Andrews, 1992).

Plant-derived long-chain fatty acids are the limiting nutrient in the competition between the antagonist, *Enterobacter cloacae*, and the seed-rotting damping-off fungus *P. ultimum* (Van Dijk & Nelson, 2000). Fatty acids (linolenic acid) are required by the fungus to elicit its germination. Mutants of the antagonist were made with mutations in the fatty acid metabolism (beta-oxidation and fatty acid uptake). These mutants

could not metabolise linoleic acid and they were unable to suppress *Pythium* seed rot. However, when mutants were introduced to complement the mutants, suppression was obtained.

2.2.5. Siderophore production

Iron (Fe^{3+} ions) is limited in soil and on plant surfaces. Siderophores are extracellular, low molecular weight compounds produced by microorganisms and plants to sequester these ions, making them more readily available and giving the produce organism a competitive advantage (Fravel, 1988; Van Driesche & Bellows, 1996). Several antagonistic microbes producing siderophores involved in biocontrol have been described (Table 2.4). Siderophores produced by microbes surrounding the plant surface may enhance plant growth by increasing the availability of iron (Alexander & Zuberer, 1991).

Siderophores are implicated in the inhibition or limitation of growth of some organisms (Bellows, 1999). This is seen in the interaction between some antagonists and pathogens. Some microorganisms produce highly efficient siderophores that out compete those produced by pathogens, limiting their growth (Van Driesche & Bellows, 1996; Bellows, 1999; Calvente *et al.*, 1999). In some cases, siderophores are even implicated in the induction of systemic resistance (Lindow & Wilson, 1999).

Not only Pseudomonads are known to produce siderophores involved in biocontrol. Calvente *et al.* (1999) was able to control blue mould on apple using a combination of a yeast antagonist, *Rhodotorula glutinis* (Fresen.) F.C. Harrison, and its siderophore. Greater control was achieved using the combination rather than the antagonist alone.

The type of fungal pathogen that is to be controlled must be taken into consideration. Some pathogen fungal spores need exogenous sources of nutrients in order to germinate (Blakeman, 1985). Conidia are dormant cells that require a large input of iron to germinate and siderophores may reduce germination through chelating iron, subsequently reduce mycelial growth (Charlang *et al.*, 1981).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 2.4: Antagonists producing siderophores involved in biocontrol of fungal pathogens

Host	Pathogen	Disease	Control agent	Reference
Tobacco *Nicotiana tabacum* L.	*Thielaviopsis basicola* (Berk. & Broome) Ferraris	Black root rot	*Pseudomonas fluorescens*	Ahl *et al.*, 1986
Wheat *Triticum aestivum* L.	*Gaeumannomyces graminis* var. *tritici* (Sacc.) Oliver & Von Arx	Take-all	*Pseudomonas fluorescens*	Weller & Cook, 1983 Pierson & Weller, 1994 Hamdan *et al.*, 1991 Slininger & Jackson, 1992 Milner *et al.*, 1997
Wheat *Triticum aestivum* L.	*Gaeumannomyces graminis* var. *tritici* (Sacc.) Oliver & Von Arx	Take-all	*Pseudomonas putida* B10	Buyer *et al.*, 1989
Cotton *Gossypium hirsutum* L.	*Rhizoctonia solani* (J. G. Kühn)	Damping-off	*Pseudomonas* sp. EM85	Pal *et al.*, 2000
Barley *Hordeum vulgare* L.	-	-	*Pseudomonas* spp.	Buyer *et al.*, 1993

3. FACTORS AFFECTING MODES OF ACTION

Disease suppression by antagonists in the laboratory does not always work as effectively in the field (Milner *et al.*, 1997). One possible explanation is the variable conditions in the field. Physical, chemical and biological environments are continuously changing. The host itself is continuously fluctuating in its surface properties, physically, chemically and biologically. Characteristics of the pathogen will also determine the antagonist's efficacy.

The antagonist population as a biological system fluctuates according to the existing environmental conditions, and in accordance to its population size as well as the presence of other microbial colonizers (Milner *et al.*, 1997). The influence of the host, pathogen and antagonist on biocontrol as well as their interaction with one another over time within an environment will be described in this section (Figure 2.2). The influence of these factors on the efficacy of the biocontrol agent will also be discussed.

3.1. Plant host affecting biocontrol modes of action

The plant host has a dual role in biocontrol. Through its own resistance mechanisms it reduces the pathogen's activities (Clarke, 1996; Droby *et al.*, 1996). It also provides the meeting ground for the pathogen and antagonists in which they will interact. The host affects the environment in which the interaction between the pathogen and the antagonist will occur by its excretion of exudates, ion and water uptake, gaseous exchange, as well as the UV light and temperature on its surface (Baker & Cook, 1974; Campbell, 1989; Leibinger *et al.*, 1997; Bellows, 1999). Environmental factors also drive the dynamic nature of microbial communities on plant surfaces and influence plant growth and development as well as all plant-microbe and even microbe-microbe interactions (Spurr, 1994). Microbial ecosystems are extremely complex and the interactions on the plant surface are affected by both physical and chemical (or nutritional) variables (Morris & Rouse, 1985).

In the preharvest scenario, the strategy of disease control is to ultimately keep the plant as healthy as possible and keep the inoculum levels of the pathogen as low as possible (Arul, 1994; Ippolito & Nigro, 2000). The major factors causing decay of fresh produce in the postharvest scenario are fungal infection, and/or physiological processes such as senescence (Arul, 1994; Roberts, 1994). Factors that accelerate senescence and favour microbial growth can promote postharvest decay. These include physiological and mechanical injuries and exposure to undesirable storage conditions (Arul, 1994). Any treatment that slows

the rate of senescence and inhibits microbial growth is a key factor in controlling disease in the postharvest arena (Roberts, 1994).

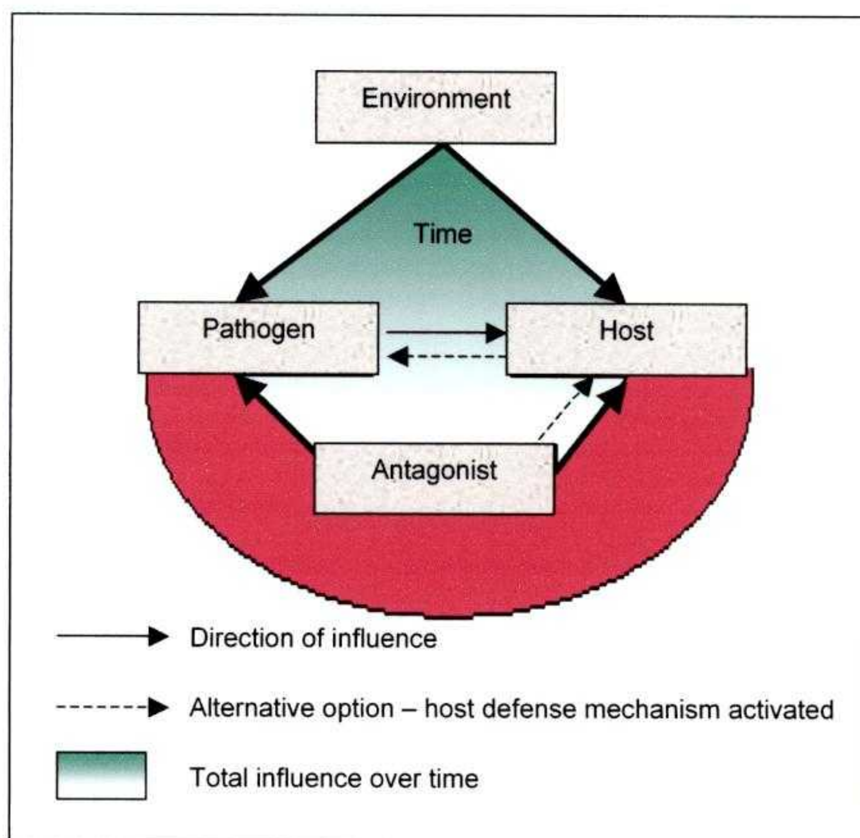


Figure 2.2: A schematic representation of the factors that influence biocontrol efficacy.

Aerial plant surfaces are hostile environments to the colonizing microorganisms (Campbell, 1989; Andrews, 1992). Microbial growth is restricted on plant surfaces due to the impact of environmental factors. Nutrient levels are low (Derridji, 1996), and microclimate variables exist, including surface moisture, temperature and irradiation, which may impact on the microbial community growth (Van Driesche & Bellows, 1996). Colonization, as well as growth and effectiveness of antagonism are affected (Romantschuk *et al.*, 1996).

Only a thin layer of air, a few millimetres thick, separates the leaf surface from the macro environment. The leaf itself influences the temperature, moisture and air currents within this layer (Andrews, 1996). This is a dynamic environment due to fluctuating environmental factors, including temperature, relative humidity, dew, rain, wind and UV-radiation, which may be cyclic or noncyclic in nature. Factors such as the position in the plant canopy, shape, size and surface topography of the leaf and fruit also influences the environment (Andrews, 1992). In the phyllosphere, leaf age and position influences the size of colonizing microbial populations (Jacques, 1996). On senescent and necrotic leaves, microbes are exposed to greater

environmental extremes, including humidity and temperature fluctuations, than exists on similarly exposed living leaves (Pfender, 1996). In the postharvest scenario, the environment is more easily manipulated. Temperature, humidity and gas composition can easily be controlled under storage conditions (Pusey, 1994).

Physical characteristics of plant surfaces vary among plant species, cultivars of the same species, different growth stages as well as distribution within the same plant (Andrews, 1996). The physical surface of a plant is made up of epidermal, guard and special functional cells such as trichomes, or leaf hairs, which varies considerably in size and shape, structure and density (Spurr, 1994). The phylloplane is a rough environment, giving the impression of hills, peaks, valleys and craters. Leaf veins, epidermal cells and cuticles may vary in shape and size, while trichomes, stomata and hydatodes may be present or absent and also varies in shape and size (Andrews 1992). Leaf surfaces may contain wax, embedded within and upon the cuticle, which is made up of long chain hydroxycarbons, alkyl esters, free primary alcohols and fatty acids. These waxes vary in thickness, composition, distribution and resistance to abrasion. Young leaves can regenerate wax (Andrews, 1992). However, this ability declines with age resulting in retention of water films and leaching of nutrients (Andrews, 1992).

The chemical composition of plant surfaces varies greatly (Derridji, 1996). The phylloplane is not an energy-rich environment, although there may be temporary patches of excess carbon compounds. Nutrients on the phylloplane originate endogenously or exogenously and include diverse carbohydrates, amino acids, organic acids, sugar alcohols, mineral trace elements, vitamins, hormones as well as antimicrobial compounds such as phenols and terpenoids (Andrews, 1992; Derridji, 1996). In the phylloplane, nutrients are important since they directly provide a growth substrate for microbes and indirectly induce the synthesis of secondary metabolites such as antibiotics and siderophores. Exogenous sources of nutrients include soil particles, dust, ions and solutes in rainwater, aphid honeydew, pollen, dead microbes, as well as bird and insect excrements (Spurr, 1994). Endogenous nutrients are removed from leaves by wounding, the leaching action of rain, dew and fog, or active exudation through guttation by hydatodes or even cuticles (Derridji, 1996; Jacques, 1996; Schönherr & Baur, 1996). Exudate concentrations vary both quantitatively and qualitatively with host, leaf position, leaf surface, age of plant, light temperature, fertility, pH, leaching medium and leaf injury. Nutrients are frequently limiting on the phylloplane. One nutrient affects the utilization of another, while some nutrients may be toxic to some microbial species (Andrews, 1992). In senescent and necrotic tissue, the distinction between the surface and the interior of the plant becomes difficult (Pfender, 1996),

making more nutrients available as the leaf dies. However, some nutrients are exported to other parts of the plant.

The biological composition on the plant surface is consists of bacteria, yeasts and fungi. These microbes succeed one another over time, so that the composition of the interacting community on the plant surface is not constant (Andrews, 1996). Microbial population size and composition vary under the influence of biotic and abiotic factors that affect population density and influence the host (Jacques, 1996). There are two different types of surface inhabitants: endophytes (parasitising the internal plant tissues and using nutrients from the plant to grow) and epiphytes (growing on the plant surface and utilizing available nutrients) (Spurr, 1994). Bacteria are usually the first colonists of the phylloplane, followed by yeasts and then filamentous fungi (Andrews, 1996). Sources of inoculum include soil, seed, air, and buds as well as over wintering shoots. Air carried spores are probably the primary source of filamentous fungi.

The available inoculum, the environment and host phenology directs the sequential microbial colonization of the phylloplane (Blakeman, 1985). Local effects like the degree of insect infestation, existing weather conditions and cropping practices alter this pattern. Factors that may influence colonization are births, immigration, emigration and growth rate of colonists, as well as the position of the leaf in the tree canopy (Andrews, 1996; Lindow, 1996). The preferred colonization sites on the leaf are along the veins and in grooves above the anticlinal wall of epidermal cells. This may be because of the localized concentrations of nutrients, protection from erosion, trapping and deposition of the colonists, as well as the retention of water films (Andrews, 1992).

The plant host has genes that, when expressed, affect the microbial community on its surface and the area surrounding it (Milner *et al.*, 1997). Gilbert *et al.* (1994) found that in certain cases the rhizosphere communities surrounding disease-resistant cultivars showed more similarities to the microbial communities in the surrounding soil than those of susceptible cultivars (see section 2.1.3). This may play a role in protecting the host from pathogen attack.

3.2. Pathogen affecting biocontrol modes of action

The pathogen remains one of the most important considerations in the selection of biocontrol agents, since each pathogen differ in their interaction with the host. This is due to genetic diversity and variable levels of ecological fitness (Blakeman, 1985). Successful plant surface colonizing fungi has to locate nutrients and

convert it into a viable reproductive or migratory form without yielding to competition with neighbours, unfavourable conditions or unfavourable host responses (Rayner, 1996). Pathogens also react differently to antagonists, since pathogens can vary in terms of virulence, they may also differ in their susceptibility to antagonist action. Blakeman (1985) describes three different types of pathogens: unspecialised necrotrophes, specialized necrotrophes and biotrophic pathogens. For biocontrol to be successful, the weak link in the pathogen's life cycle should be identified as the "the window of opportunity". The target biocontrol agent's mode of action should fit into the window of opportunity, disrupting the disease cycle.

Unspecialised necrotrophic pathogens include *B. cinerea*, *Cladosporium*, *Alternaria*, *Cochliobolus* and *Septoria* species (Blakeman, 1985; Andrews, 1992). They grow saprophytically on the plant surface before formation of an infection structure (Blakeman, 1985; Andrews, 1992). Nutrients from the spore itself, nutrients leaked from the plant surface and other nutrient sources like pollen and aphid honeydew, act to sustain the growth of this group of pathogens. The window of opportunity that is applicable in this circumstance is to obstruct the uptake of nutrients required for growth (Andrews, 1992). The saprophytic phase will be controlled when the antagonist competes for nutrients, preventing its establishment. When antagonistic organisms are present in high enough numbers in the area surrounding the pathogen spores, loss of endogenous nutrients from the spore may occur, reducing or preventing germination (Blakeman, 1985). The production of enzymes or antibiotics by the antagonist may also be effective against these pathogens (Blakeman, 1985).

The saprophytic phase of the specialized necrotrophic pathogens on the plant surface prior to penetration may be totally absent or very limited. They require less exogenous nutrients than unspecialised necrotrophic pathogens (Blakeman, 1985). Pathogens belonging to this group include some species of *Colletotrichum*, causing anthracnose. They form well-developed appressoria directly from the spore, from either no or a very short germ tube. Excess nutrients encourage the pathogen to grow saprophytically, suppressing the normal pathogenic behaviour (Blakeman, 1985). The weak link in the disease cycle of specialized necrotrophic pathogens is therefore the formation of appressoria (Blakeman, 1985). By adding competing bacteria to the spores, the extension of the germ tube is reduced and the number of appressoria formed increases since nutrient depravation enhances appressoria development (Koomen & Jeffries, 1993). The possibility exists that siderophores may directly assist in controlling fungi (Fravel, 1988). Iron may be fixed at a site within the conidium of the pathogen, inducing germination (Blakeman, 1985). Should germination take place in unsuitable conditions, pathogenesis may be reduced.

No saprophytic phase occur before biotrophic spores germinate and penetrate the host, even though long germ tubes can be produced (Blakeman, 1985; Andrews, 1992). Endogenous nutrients inside the spore entirely support growth of biotrophic pathogens. This means that competition for nutrients from epiphytes and biocontrol agents will not be effective against biotrophic pathogens. In the case of biotrophic pathogens, it was found that germ tubes and appressoria are sites of nutrient leakage. Yeasts and yeast-like fungi were found to stimulate germination of uredospores and growth of germ tubes of some rust fungi, benefiting the pathogen (Blakeman, 1985). These pathogens are difficult to control using biocontrol. Direct acting modes of action, like antibiosis and direct parasitism, are the best modes of action to combat biotrophic pathogens (Blakeman, 1985; Roberts, 1994).

The way a pathogen reacts to competition can be important at two stages in the pathogen's life cycle. Competition may occur during the initial establishment of new niche's that was not previously colonized by microorganisms. Then, after initial establishment, there can be further competition to secure enough of the limited resources present to permit survival and eventual reproduction (Bellows, 1999).

Microorganisms show traits that classify them as either adept at the initial colonizing phase, or as able to withstand and prevent subsequent phases of competition. A feature of r-strategists (ruderal species) is a highly reproductive capacity (Atlas & Bartha, 1987). They produce large amounts of reproductive bodies, like spores, increasing the chance that some may find newly available resources. They are efficient at the dispersal of these reproductive bodies, which is mostly resistant to harmful environmental conditions, and establish themselves in disturbed habitats or available resources. In contrast to the r-strategists, K-strategists flourish in more stable environments (Atlas & Bartha, 1987). They are able to cope with competition for space and limited resources. Plant pathogens are spread across this r – K range of characteristics. These traits will determine which mode of action will be the most effective against the pathogen (Bellows, 1999).

3.3. Antagonist affecting biocontrol modes of action

As with pathogens, biocontrol agents are also spread across the r – K range of evolutionary adaptive strategies (Andrews, 1992). An antagonist will be most effective when it is set in its optimal relationship (Janisiewicz & Korsten, 2002). Some pathogens infect hosts when some kind of disturbance occurs, such as wounding or when there is a change in the microbial ecology on the host surface (Blakeman, 1985). Biocontrol agents showing r-strategist characteristics will be able to colonize available resources, reducing

the available amount of nutrients, and have some survival mechanism, such as spores, which will persist on the host (Bellows, 1999). These agents will be in place before the pathogen infection cycle can begin. In cases where the pathogen has already infected the host, K-strategist biocontrol agents will be the best option, as a more competitive species will be required to be effective (Bellows, 1999).

The antagonists timing of application is also of importance (Andrews, 1992). Most antagonistic organisms currently used for biological control are most effective when they are applied prior to infection by the pathogen and do not appear to control previously established infections (Ikediugwu *et al.,* 1994; El-Ghaouth, 1997; El-Ghaouth *et al*., 1998). Smilanick *et al.* (1993) tested two yeasts and two *Pseudomonas* species against *M. fructicola*, causal agent of brown rot of stone fruit. When the two yeasts were applied prior to pathogen inoculation, they were able to protect fruit from infection. However, when applied after inoculation by the pathogen, they were unable to control decay. On the other hand, the two bacteria were able to protect wounds up to 12 hours after inoculation with the pathogen (Smilanick *et al*., 1993). It seems that it depends on the mode of action employed by the antagonist will be able to control established infections or not.

Since the action of the antagonist is under genetic control, its ability to take-up nutrients, produce antibiotics, siderophores and enzymes can be self-regulated (growth phase, nutrient status). It can also be triggered by reigning environmental conditions (exogenous nutrients, temperature, humidity). In the case of spore-forming bacteria, like *Bacillus* species, antibiotics are produced when sporulation is initiated, or when the stationary phase of growth is reached (Nakano & Zuber, 1990). Genes involved in Fengycin synthesis, an antibiotic produced by *B. subtilis*, is initiated during two different stages of cell growth. The promoter for the genes, are active during the log phase, and again during the early stationary phase (Lin *et al*., 1999). Cell density, or quorum sensing, may also affect the production of antibiotics. Phenazine, an antibiotic produced by *Pseudomonas aureofaciens* is regulated by cell density (Pierson *et al*., 1994). Nutrients may also affect the production of antibiotics. Milner *et al.* (1995) found temperature and the nutrient base for growth affected the accumulation of the antibiotic zwittermicin A, produced by *B. cereus* UW85.

4. NEW AND EMERGING TECHNOLOGIES IN THE USE OF BIOCONTROL AGENTS

Changing the environment surrounding the biocontrol agent, host and pathogen has received recent attention in an attempt to enhance biocontrol efficacy (Gilbert *et al*., 1994). Manipulation of the biocontrol agent or the plant host may lead to improved disease control and it is possible not only to add resistance

genes to plants but also to induce changes in the plant environment to promote colonization by antagonists (Andrews, 1996). According to Morris & Rouse (1985), an alternative to inoculate the plant surface with large quantities of biocontrol microbes is to alter the ecosystem to favour the increase of the indigenous antagonists. This can be accomplished by adding certain nutrients to the plant surface. This strategy, however, requires a comprehensive understanding of the ecosystem.

By combining biocontrol agents a mixture of different biocontrol systems, the genetic diversity of the biocontrol systems pool increases (Pierson & Weller, 1994; Milner *et al.*, 1997). The resulting treatments may utilize a wide range of modes of action under a broader range of environmental conditions, and may even persist longer in the environment. The combination may also suppress a wider range of pathogens (Pierson & Weller, 1994). Two *Pseudomonas* isolates were tested against take-all of wheat, both in combination and on their own. The mixture was up to 30 % more effective in the field than using either of the isolates alone. They postulated that the combined treatment might have enhanced root colonization or increased the complexity of the protective barrier to the take-all fungus (Weller & Cook, 1983; Pierson & Weller, 1994).

Biocontrol agents may never give us the same level of control as that of chemicals. However, it may help reduce the amount of chemicals used on a crop, thereby decreasing the cost and improving the impact on the environment (Dubos, 1984; El-Ghaouth, 1997). By combining hot water spray and fruit brushing, the incidence of postharvest disease caused by *Alternaria alternata* (Fr.: Fr. Keissl.) improved on mango (Prusky *et al.*, 1999). Using alternative control measures such as these together with biocontrol agents might improve the total control achieved (Pusey *et al.*, 1986). Janisiewicz *et al.* (1998) evaluated calcium treatment and biocontrol agents, either alone or in combination, to control postharvest decay of apples. Each of the treatments gave some control, but not as high as combining the calcium treatment with the biocontrol. Similar results have been found on citrus (Obagwu & Korsten, 2003).

5. CONCLUSION

Biocontrol is currently one of the few alternatives for using chemicals to control fungal diseases. However, the success of biocontrol agents has been inconsistent (Andrews, 1996). This fact has lead to the study of the interactions between the host, pathogen, and biocontrol agent, as well as factors that influence them. We now know more about how biocontrol systems work compared to a few years ago. With a better understanding of biocontrol systems, future biocontrol products can be selected and screened more

successfully. By deciding on a biocontrol agent with specific modes of action, it may be possible to select an agent that is suitable for a specific pathogen-host-environment combination.

To ensure the future success of a biocontrol agent, it is crucial to keep the interaction between the biocontrol agent, host, pathogen and environment in mind when doing laboratory studies. According to Upper & Hirano (1996), it is important to make sure that the behaviour of a system in the greenhouse or growth chamber mimics the in the field scenario before starting extensive and exhaustive laboratory studies, particularly in the case of plant–microbe interactions.

Focusing on altering the application of biocontrol agents, combined treatments as well as genetic enhancement is no longer futuristic, and has received much attention recently. It will be possible that in time, and with a more comprehensive understanding of biocontrol systems and factors, biocontrol may still provide a practical, holistic and economically viable solution for consistent disease control.

6. REFERENCES

Ahl, P., Voisard, C. & Défago, G. 1986. Iron bound-siderophores, cyanic acid, and antibiotics involved in suppression of *Thielaviopsis basicola* by a *Pseudomonas fluorescens* strains. Journal of Phytopathology 116: 121 – 34.

Alexander, D.B. & Zuberer, D.A. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biology and Fertility of Soils 12: 39 – 45.

Andrews, J.H. 1992. Biological control in the phyllosphere. Annual Review of Phytopathology 30: 603 – 635.

Andrews, J.H. 1996. Phyllosphere ecology – past, present, and future. Pages 285 – 294 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Arul, J. 1994. Emerging technologies for the control of postharvest diseases of fresh fruits and vegetables. Pages 1 - 10 In: Biological Control of Postharvest Diseases – Theory and Practice. Wilson, C. L. & Wisniewski, M. E. (Eds). CRC Press, Boca Raton.

Atlas, R.M. & Bartha, R. 1987. Microbial Ecology: Fundamentals and Applications. Benjamin Cummings Publishing Company, California.

Baker, K.F. 1987. Evolving concepts of biological control of plant pathogens. Annual Review of Phytopathology 25: 67 – 85.

Baker, K.F. & Cook, R.J. 1974. Biological Control of Plant Pathogens. San Francisco, Freeman.

Bellows, T.S. 1999. Foliar, flower, and fruit pathogens. Pages 841 – 852 In: Handbook of Biological Control: Principles and Applications of Biological Control. Bellows, T.S. & Fisher, T.W. (Eds). Academic Press, San Diego.

Benhamou, N., Bélanger, R.R., Rey, P. & Tirilly, Y. 2001. Oligandrin, the elicitin-like protein produced by the mycoparasite *Pythium oligandrum*, induces systemic resistance to *Fusarium* crown and root rot in tomato plants. Plant Physiology and Biochemistry 39: 681 – 698.

Bierbaum, G., Brötz, H., Koller, K.P. & Sahl, H.G. 1995. Cloning, sequencing and production of the lantibiotic mersacidin. FEMS Microbiology Letters 127: 121 – 126.

Blakeman, J.P. 1985. Ecological succession of leaf surface micro organisms in relation to biological control. Pages 6 – 30 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Brodie, I.D.S. & Blakeman, J.P. 1976. Competition for exogenous substrates *in vitro* by leaf surface micro-organisms and germination of conidia of *Botrytis cinerea*. Physiological Plant Pathology 9: 227 – 239.

Bull, C.T., Weller, D.M. & Thomashow, L.S. 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. Phytopathology 81: 954 – 959.

Buyer, J.S., Sikora, L.J. & Chaney, R.L. 1989. A new growth medium for the study of siderophore-mediated interactions. Biology and Fertility of Soil: 8: 97 – 101.

Buyer, J.S., Kratzke, M.G. &Sikora, L.J. 1993. A method for detection of Pseudobactin, the siderophore produced by a plant-growth-promoting *Pseudomonas* strain, in the barley rhizosphere. Applied and Environmental Microbiology: 59: 677 – 681.

Calvente, V., Benuzzi, D. & de Tosetti, M.I.S. 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. International Biodeterioration & Biodegradation 43: 167 – 172.

Campbell, R. 1989. Biological Control of Microbial Plant Pathogens. Cambridge University Press, Cambridge.

Castoria, R., De Curtis, F., Lima, G., Caputo, L., Pacifico, S. & De Cicco, V. 2001. *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. Postharvest Biology and Technology 22: 7 – 17.

Charlang, G., Bradford, N., Horowitz, N. & Horowitz, R. 1981. Cellular and extracellular siderophores of *Aspergillus nidulans* and *Penicillium chrysogenum*. Molecular and Cellular Biology 1: 94 – 100.

Chen, C.L., Chang, L.K., Chang, Y.S., Liu, S.T. & Tschen, J.S.M. 1995. Transposon mutagenesis and cloning of the genes encoding the enzymes of fengycin biosynthesis in *Bacillus subtilis*. Molecular and General Genetics 248: 121 – 125.

Clarke, D.D. 1996. Coevolution between plants and pathogens of their aerial tissues. Pages 91 – 101 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Clarke, G.R.G., Leigh, J.A. & Douglas, C.J. 1992. Molecular signals in the interactions between plants and microbes. Cell 71: 191 – 199.

Denner, F.D.N. & Kotzé, J.M. 1986. Evaluasie *in vitro* van die effektiwiteit van swamdoders teen *Colletotrichum gloeosporioides* en *Dothiorella aromatica*. South African Avocado Growers' Association Yearbook 8: 48 - 51.

Derridji, S. 1996. Nutrients on the leaf surface. Pages 25 – 42 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Dodds, J.A. 1999. Cross-protection and systemic acquired resistance for control of plant diseases. Pages 549 – 556 In: Handbook of Biological Control: Principles and Applications of Biological Control. Bellows, T.S. & Fisher, T.W. (Eds). Academic Press, San Diego.

Droby, S. & Chalutz, E. 1994. Mode of action of biocontrol agents of postharvest diseases. Pages 63 – 75 In: Biological Control of Postharvest Diseases – Theory and Practice. Wilson, C.L. & Wisniewski, M.E. (Eds). CRC Press, Boca Raton.

Droby, S., Chalutz, E., Wisniewski, M.E. & Wilson, C.L. 1996. Host response to introduction of antagonistic yeasts used for control of postharvest decay. Pages 73 - 89 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Dubos, B. 1984. Biocontrol of *Botrytis cinerea* on grapevines by an antagonistic strain of *Trichoderma harzianum*. Pages 370 – 373 In: Current Perspectives in Microbial Ecology. Klug, M.J. & Reddy, C.A. (Eds). American Society for Microbiology, Washington, D.C.

Dunny, G.M. & Leonard, B.A.B. 1997. Cell-cell communication in gram-positive bacteria. Annual Review of Microbiology 51: 527 – 564.

El-Ghaouth, A. 1997. Biologically-based alternatives to synthetic fungicides for the control of postharvest diseases. Journal of Industrial Microbiology & Biotechnology: 19: 160 – 162.

El-Ghaouth, A., Wilson, C.L. & Wisniewski, M. 1998. Ultrastructural and cytochemical aspects of the biological control of *Botrytis cinerea* by *Candida saitoana* in apple fruit. Phytopathology 88: 282 – 291.

Eshita, S.M., Roberto, N.H., Beale, J.M., Mamiya, B.M. & Workman, R.F. 1995. Bacillomycin L_c, a new antibiotic of the iturin group: Isolations, structures, and antifungal activities of the congeners. The Journal of Antibiotics 48: 1240 – 1247.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Frändberg, E. & Schnürer, J. 1994. Chitinolytic properties of *Bacillus pabuli* K1. Journal of Applied Bacteriology 76: 361 – 367.

Frändberg, E. & Schnürer, J. 1998. Antifungal activity of chitinolytic bacteria isolated from airtight stored cereal grain. Canadian Journal of Microbiology 44: 121 – 127.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant diseases. Annual Review of Phytopathology 26: 75 – 91.

Gilbert, G.S., Handelsman, J. & Parke, J.L. 1994. Root camouflage and disease control. Phytopathology 84: 222 – 225.

Gooday, G.W. 1988. Principles of microbial behaviour in ecosystems. Pages 7 – 32 In: Micro-organisms in Action: Concepts and Applications in Microbial Ecology. Lynch, J.M. & Hobbie, J.E. (Eds). Blackwell Scientific Publication, Oxford.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. & Cutler, H.G. 1988. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. Journal of Agricultural and Food Chemistry 36: 366 – 370.

Gutterson, N. 1990. Microbial fungicides: recent approaches to elucidating mechanisms. Critical Review of Biotechnology 10: 69 – 91.

Hamdan, H., Weller, D.M. & Thomashow, L.S. 1991. Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79 and M4-80R. Applied and Environmental Microbiology 57: 3270 – 3277.

Helistö, P., Aktuganov, G., Galimzianova, N., Melentjev, A. & Korpela, T. 2001. Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components. Journal of Chromatography B 758: 197 – 205.

Hilton, M.D., Alaeddinoglu, N.G. & Demain, A.L. 1988. Synthesis of bacilysin by *Bacillus subtilis* branches from prephenate of the aromatic amino acid pathway. Journal of Bacteriology 170: 482 – 484.

Ikediugwu, F.E.O., Emoghene, A.O. & Ajiodo, P.O. 1994. Biological control of the shoot diseases *Amaranthus hybridus* caused by *Choanephora cucurbitarum* with *Bacillus subtilis*. Journal of Horticultural Science 69: 351 – 356.

Ippolito, A. & Nigro, F. 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruit and vegetables. Crop Protection 19: 715 – 723.

Jacques, P., Hbid, C., Vanhentenryck, F., Destain, J., Baré, G., Razafindralambo, H., Paquot, M. & Thonart, P. 1994. Quantitative and qualitative study of the production of broad-spectrum antifungal lipopeptides from *Bacillus subtilis* S499. Pages 1067 – 1070 In: ECB6: Proceedings of the 6th European Congress on Biotechnology. Alberghina, L., Frontali, L. & Sensi, P. (Eds). Elsevier Science, B. V.

Jacques, M.A. 1996. The effect of leaf age and position on the dynamics of microbial populations on aerial plant surfaces. Pages 233 – 248 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Janisiewicz, W.J., Conway, W.S., Glenn, D.M. & Sams, C.E. 1998. Integrating biological control and calcium treatment for controlling postharvest decay of apples. HortScience 33: 105 – 109.

Janisiewicz, W.J. & Korsten, L. 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40: 411–441.

Jijakli, M.H. & Lepoivre, P. 1998. Characterization of an exo-β-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. Phytopathology 88: 335 – 343.

Kajimura, Y., Sugiyama, M. & Kaneda, M. 1995. Bacillopeptins, new cyclic Lipopeptide antibiotics from *Bacillus subtilis* FR-2. The Journal of Antibiotics 48: 1095 – 1103.

Katz, E. & Demain, A.L. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible functions. Bacteriological Reviews 41: 449 – 474.

Kim, K.K., Fravel, D.R. & Papavizas, G.C. 1980. Identification of a metabolite produced by *Talaromyces flavus* as glucose oxidase and its role in the biocontrol of *Verticillium dahliae*. Phytopathology 78: 488 – 492.

Kinkel, L.L., Newton, M.R. & Leonard, K.J. 1996. Quantifying microbial competition on leaves. Pages 265 – 276 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Klein, C., Kaletta, C. & Entian, K. –D. 1993. Biosynthesis of the lantibiotic subtilin is regulated by a Histidine kinase/response regulator system. Applied and Environmental Microbiology 59: 296 – 303.

Kogel, K. -H.; Ortel, B.; Jarosch, B.; Atzorn, R.; Schiffer, R. & Wasternack, C. 1995. Resistance in barley against the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*) is not associated with enhanced levels of endogenous jasmonates. European Journal of Plant Pathology 101: 319 – 332.

Koomen, I. & Jeffries, P. 1993. Effects of antagonistic microorganisms on the post-harvest development of *Colletotrichum gloeosporioides* on mango. Plant Pathology 42: 230 – 237.

Kotzé, J.M., Du Toit, F.L. & Durand, B.J. 1982. Pre-harvest Chemical Control of Anthracnose, Sooty Blotch and Cercospora Spot of Avocados. South African Avocado Growers' Association Yearbook 5: 54 - 55.

Kugler, M., Loeffler, W., Rapp, C., Kern, A. & Jung, G. 1990. Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: Biological properties. Archives of Microbiology 153: 276 – 281.

Leibinger, W., Breuker, B., Hahn, M. & Mendgen, K. 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. Phytopathology 87: 1103 – 1110.

Leifert, C., Li, H., Chidburdee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. & Harbour, A. 1995. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology 78: 97 – 108.

Lin, G.H., Chen, C.L., Tschen, J.S.M., Tsay, S.S., Chang, Y.S. & Liu, S.T. 1998. Molecular cloning and characterization of fengycin synthetase gene *fenB* from *Bacillus subtilis*. Journal of Bacteriology 180: 1338 – 1341.

Lin, T.P., Chen, C.L., Chang, L.C., Tschen, J.S.M. & Liu, S.T. 1999. Functional and transcriptional analyses of a fengycin synthetase gene, *fenC*, from *Bacillus subtilis*. Journal of Bacteriology 181: 5060 – 5067.

Lindow, S.E. 1996. Role of immigration and other processes in determining epiphytic bacterial populations. Pages 155 – 168 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Lindow, S.E. & Wilson, M. 1999. Biological control of foliar pathogens and pests with bacterial biocontrol agents. Pages 642 - 650 In: Manual of Industrial Microbiology and Biotechnology. 2nd ed. Demain, A.L. & Davies, J.E. (Eds). ASM Press, Washington, D.C.

Loeffler, W., Tschen, S. -M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T. –F. & Wu, T. –G. 1986. Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3: A comparison with activities of other *Bacillus* antibiotics. Journal of Phytopathology 115: 204 – 213.

Mari, M. & Guizzardi, M. 1998. The postharvest phase: Emerging technologies for the control of fungal diseases. Phytoparasitica 26: 59 – 66.

Martin, J.F. & Demain, A.L. 1980. Control of antibiotic biosynthesis. Microbiological Reviews 44: 230 – 251.

Maurhofer, M., Keel, C., Schnider, U., Voisard, C., Haas, D. & Defago, G. 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHAO on its disease suppressive capacity. Phytopathology 82: 190 – 195.

McKeen, C.D., Reilly, C.C. & Pusey, P.L. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. Phytopathology 76: 136 – 139.

Mercier, J. & Wilson, C.L. 1995. Effect of wound moisture on the biocontrol by *Candida oleophila* of gray mold rot (*Botrytis cinerea*) of apple. Postharvest Biology and Technology 6: 9 – 15.

Milner, J.L., Raffel, S., Lethbridge, B.J. & Handelsman, J. 1995. Culture conditions that influence the accumulation of zwittermicin A. Applied Microbiology and Biotechnology 43: 685 – 691.

Milner, J.L., Silo-Suh, L., Lee, J.C., He, H., Clardy, J. & Handelsman, J. 1996. Production of kanosamine by *Bacillus cereus* UW85. Applied and Environmental Microbiology 62: 3061 – 3065.

Milner, J.L., Silo-Suh, L., Goodman, R.M. & Handelsman, J. 1997. Antibiosis and beyond: genetic diversity, microbial communities, and biological control. Pages 107 - 127 In: Ecological Interactions and Biological Control. Andow, D.A., Ragsdale, D.W. & Nyvall, R.F. (Eds). Westview Press, Colorado, USA. / Oxford.

Morris, C.E. & Rouse, D.I. 1985. Role of nutrients in regulating epiphytic bacterial populations. Pages 63 – 82 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Mootz, H.D. & Marahiel, M.A. 1997. The tyrocidine biosynthesis operon of *Bacillus brevis*: Complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. Journal of Bacteriology 179: 6843 – 6850.

Nakano, M.M. & Zuber, P. 1990. Molecular biology of antibiotic production in *Bacillus*. Critical Reviews in Biotechnology 10: 223 – 240.

Nielsen, P. & Sørensen, J. 1997. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiology Letters 22: 183 – 192.

Obagwu, J. & Korsten, L. 2003. Integrated control of citrus green and blue molds using *Bacillus subtilis* in combination with sodium bicarbonate or hot water. Postharvest Biology and Technology 28:187-194.

O'Sullivan, D.J. & O'Gara, F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiological Reviews 56: 662 – 676.

Pal, K.K., Tilak, K.V., Saxena, A.K., Dey, R. & Singh, C.S. 2000. Antifungal characteristics of a fluorescent *Pseudomonas* strain involved in the biological control of *Rhizoctonia solani*. Microbiological Research 155: 233 – 242.

Paul, B., Chereyathmanjiyil, A., Masih, I., Chapuis, L. & Benoît. 1998. Biological control of *Botrytis cinerea* causing grey mould disease of grapevine and elicitation of stilbene phytoalexin (resveratrol) by a soil bacterium. FEMS Microbiology Letters 165: 65 – 70.

Payne, C.C. & Lynch, J.M. 1988. Biological control. Pages 261 – 287 In: Micro-organisms in Action: Concepts and Applications in Microbial Ecology. Lynch, J.M. & Hobbie, J.E. (Eds). Blackwell Scientific Publication, Oxford.

Peypoux, F., Pommier, M.T., Das, B.C., Besson, F., Delcambe, L. & Michel, G. 1984. Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. The Journal of Antibiotics XXXVII: 1600 – 1604.

Peypoux, F., Pommier, M.T., Marjon, D., Ptak, M., Das, B.C. & Michel, G. 1986. Revised structure of Mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. The Journal of Antibiotics XXXIX: 636 – 641.

Pfender, W.F. 1996. Microbial interactions preventing fungal growth on senescent and necrotic aerial plant surfaces. Pages 125 – 138 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Picard, K., Tirilly, Y. & Benhamou, N. 2000. Cytological effects of cellulases in the parasitism of *Phytophthora parasitica* by *Pythium oligandrum*. Applied and Environmental Microbiology 66: 4305 – 4314.

Pierson, E.A. & Weller, D.M. 1994. Use of mixtures of fluorescent Pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology 84: 940 – 947.

Pierson, L.S. III, Keppenne, V.D. & Wood, D.W. 1994. Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. Journal of Bacteriology 176: 3966 – 3974.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. & Urdaci, M.C. 2002. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Research in Microbiology 153: 269 – 276.

Podile, A.R. & Prakash, A.P. 1996. Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF1. Canadian Journal of Microbiology 42: 533 – 538.

Podlesek, Z., Comino, A., Herzog-Velikoja, B. & Grabnar, M. 2000. The role of the bacitracin ABC transporter in bacitracin resistance and collateral detergent sensitivity. FEMS Microbiology Letters 188: 103 – 106.

Prusky, D., Fuchs, Y., Kobiler, I., Roth, I., Weksler, A., Shalom, Y., Fallik, E., Sauberman, G., Pesis, E., Akerman, M., Ykutiely, O., Weisblum, A., Regev, R. & Artes, L. 1999. Effect of hot water brushing, prochloraz treatment and waxing on the incidence of black spot decay caused by *Alternaria alternata* in mango fruits. Postharvest Biology and Technology 15: 165 – 174.

Pusey, P.L. 1994. Enhancement of biocontrol agents for postharvest diseases and their integration with other control strategies. Pages 77 – 88 In: Biological Control of Postharvest Diseases – Theory and Practice. Wilson, C.L. & Wisniewski, M.E. (Eds). CRC Press, Boca Raton.

Pusey, P.L., Wilson, C.L., Hotchkiss, M.W. & Franklin, J.D. 1986. Compatibility of *Bacillus subtilis* for postharvest control of peach brown rot with commercial fruit waxes, Dichloran, and cold-storage conditions. Plant Disease 70: 587 – 590.

Raffel, S.J., Stabb, E.V., Milner, J.L. & Handelsman, J. 1996. Genotypic and phenotypic analysis of zwittermicin A-producing strains of *Bacillus cereus*. Microbiology 142: 3425 – 3436.

Rayner, A.D.M. 1996. Antagonism and synergism in the plant surface colonization strategies of fungi. Pages 139 – 154 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Roberts, R.G. 1994. Integrating biological control into postharvest disease management strategies. HortScience 29: 758 – 762.

Romantschuk, M., Roine E., Björklöf, K., Ojanen, T., Nurmiaho-Lassila, E-. L. & Haahtela, K. 1996. Microbial attachment to plant aerial surfaces. Pages 43 – 57 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Sailaja, P.R., Podile, A.R. & Reddanna, P. 1997. Biocontrol strain of *Bacillus subtilis* AF1 rapidly induces lipoxygenase in groundnut (*Arachis hypogaea* L.) compared to crown rot pathogen *Aspergillus niger*. European Journal of Plant Pathology 104: 125 – 132.

Sakajoh, M., Solomon, N.A. & Demain, A.L. 1987. Cell-free synthesis of the dipeptide antibiotic bacilysin. Journal of Industrial Microbiology 2: 201 – 208.

Schönherr, J. & Baur, P. 1996. Cuticle permeability studies: A model for estimating leaching of plant metabolites to leaf surfaces. Pages 1 – 23 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Shang, H., Chen, J., Handelsman, J. & Goodman, R.M. 1999. Behaviour of *Pythium torulosum* zoospores during their interaction with tobacco roots and *Bacillus cereus*. Current Microbiology 38: 199 – 204.

Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. Advances in Applied Microbiology 24: 187 – 214.

Silo-Suh, L.A. Stabb, E.V., Raffel, S.J. & Handelsman, J. 1998. Target range of zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. Current Microbiology 37: 6 – 11.

Skidmore, A.M. 1976. Interactions in relation to biological control of plant pathogens. Pages 507 – 528 In: Microbiology of Aerial Plant Surfaces. Dickinson, C.H. & Preece, T.F. (Eds). Academic Press, London.

Slininger, P.J. & Jackson, M.A. 1992. Nutritional factors regulating growth and accumulation of phenazine 1-carboxylic acid by *Pseudomonas fluorescens* 2-79. Applied Microbiology and Biotechnology 37: 388 – 392.

Smilanick, J.L., Denis-Arrue, R., Bosch, J.R., Gonzalez, A.R., Henson, D. & Janisiewicz, W.J. 1993. Control of postharvest brown rot of nectarines and peaches by *Pseudomonas* species. Crop Protection 12: 513 – 520.

Spadaro, D. & Gullino, M.L. 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. International Journal of Food Microbiology 91: 185 – 194.

Spurr, H.W. 1994. The microbial ecology of fruit and vegetable surfaces: its relationship to postharvest biocontrol. Pages 11 – 23 In: Biological Control of Postharvest Diseases – Theory and Practice. Wilson, C.L. & Wisniewski, M.E. (Eds). CRC Press, Boca Raton.

Stutz, E.W., Défago, G. & Kern, H. 1986. Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76: 181 – 185.

Thomashow, L.S., Weller, D.M., Bonsall, R.F. & Pierson, L.S. III. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Applied and Environmental Microbiology 56: 908 – 912.

Tsuge, K., Ano, T. & Shoda, M. 1996. Isolation of a gene essential for biosynthesis of the Lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Archives of Microbiology 165: 243 – 251.

Upper, C.D. & Hirano, S.S. 1996. Predicting behaviour of phyllosphere bacteria in the growth chamber from field studies. Pages 277 – 284 In: Aerial Plant Surface Microbiology. Morris, C.E., Nicot, P.C. & Nguyen-The, C. (Eds). Plenum Press, New York.

Van Dijk, K. & Nelson, E.B. 2000. Fatty acid competition as a mechanism by which *Enterobacter cloacae* suppresses *Pythium ultimum* sporangium germination and damping-off. Applied and Environmental Microbiology 66: 5340 – 5347.

Van Driesche, R.G. & Bellows, T.S. Jr. 1996. Biological Control. Chapman & Hall, New York.

Weller, D.M. 1988. Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology 26: 379 – 407.

Weller, D.M. & Cook, R.J. 1983. Suppression of take-all of wheat by seed treatments with fluorescent Pseudomonads. Phytopathology 73: 463 – 469.

Wilson, C.L., El-Ghaouth, A., Chalutz, E., Droby, S., Stevens, C., Lu, J.Y., Khan, V. & Arul, J. 1994. Potential of induced resistance to control postharvest diseases of fruits and vegetables. Plant Disease 78: 837 – 844.

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

Wisniewski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C. & Chalutz, E. 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I. Characterization of attachment to *Botrytis cinerea*. Physiological and Molecular Plant Pathology 39: 245 – 258.

CHAPTER 3

COMPARISON OF *BACILLUS SUBTILIS* SUBCULTURES' EFFICACY IN CONTROLLING FUNGAL AVOCADO PATHOGENS *IN VITRO*

1. ABSTRACT

With the global move away from pesticides, biocontrol has become a viable alternative. Postharvest diseases, anthracnose and stem-end rot of avocado are difficult to control. Previously, *Bacillus subtilis* was isolated and proved effective as biocontrol agent of avocado pre- and postharvest diseases. However, during semi-commercial trials levels of control were not constant. The *in vitro* efficacy of the subcultures used in these studies was therefore compared to determine possible reasons for product performance variability. The dual culture technique was used and all subcultures and reference isolates were tested against four important fungal pathogens (*Phomopsis perseae, Colletotrichum gloeosporioides, Dothiorella aromatica* and *Lasiodiplodia theobromae*) causing postharvest diseases of avocado. The efficacy of the subcultures differed significantly. Subculture MI-14 was the most effective. RISA PCR was used to ascertain if the subcultures were still closely related, even though their efficacies differed. All subcultures showed the same DNA fingerprint, differing from that of other *B. subtilis* isolates included as reference strains.

2. INTRODUCTION

Pre- and postharvest fruit diseases are of major concern for avocado growers. Postharvest diseases are difficult to control since there is a wide spectrum associated fungal pathogens involved in disease development, especially in the case of stem-end rot (SE) (Darvas & Kotzé, 1987; Korsten *et al.,* 1995). Control of anthracnose caused by *Colletotrichum gloeosporioides* Penzig. is also challenging since the pathogen makes use of latent infections. As alternative to chemical control, biocontrol has received increasing attention and have been used either on its own or as part of an integrated disease management system (Roberts, 1994).

In previous studies, *Bacillus subtilis* was isolated and proved effective against various postharvest diseases of avocado (Korsten, 1993). The antagonist was evaluated successfully on a semi-commercial scale in field and packhouse trials (Korsten *et al.*, 1989; 1991; 1992; 1993; 1994; 1997; van Dyk *et al.*, 1997). However, control was not always consistent when evaluated over 15 years. Initially, stock cultures were made of the

original isolate. Subcultures were used in subsequent studies. The subcultures of *B. subtilis* used in most of these studies were incorporated over time into the bacterial culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

The main objective of this study was to compare the *in vitro* efficacy of the abovementioned subcultures. The dual culture technique (Skidmore, 1976), initially used to select *B. subtilis* amongst other avocado epiphytes, was used to determine if all subcultures are still efficacious. DNA fingerprinting, using the RISA (rDNA internal spacer analysis) PCR (Polymerase Chain Reaction), was employed to ascertain if the subcultures originated from the same isolate are still closely related. The RISA PCR targets the spacer regions between the 23S and 16S ribosomal genes (Campbell *et al.*, 1993; Jensen *et al.*, 1993; Farber, 1996). The spacer region is highly conserved and the length as well as the sequence of these regions can be used to compare related bacteria, even though the RISA PCR is usually employed in the analysis of bacterial communities (García-Martínez *et al.*, 1999; Ranjard *et al.*, 2000).

3. MATERIALS AND METHODS

3.1. Strains

Bacillus subtilis (B246), isolated in 1984 by Korsten (1993) from avocado (*Persea americana* Mill.) leaves in Tzaneen, South Africa, was used throughout this study. Subcultures for use were stored in 30 % glycerol with quarter strength Ringer's (Merck, Johannesburg, S.A.) solution at –70 ºC, and grown on standard 1 nutrient agar (STD1) (Biolab, Merck) for use. Freshly streaked cultures on STD1 were incubated at 28 ºC for 24 h prior to use. Consecutive subculturing of isolate was recorded over time and summarized in Table 3.1. The identity of all strains was verified using the API system (API 50 CH for Gram-positive bacteria (BioMérieux)).

The following fungal pathogens were used in this study: *Colletotrichum gloeosporioides* (Dr. G. Swart, Department of Microbiology and Plant Pathology, University of Pretoria, S.A.), *Dothiorella aromatica* (Sacc.) Petrak & Sydow and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (both isolated by M. Schoeman, Department of Microbiology and Plant Pathology). *Phomopsis perseae* Zerova was isolated from SE lesions on avocado fruit as described in the following section. All fungi were maintained on potato dextrose agar (PDA) (Biolab, Merck) slants as well as in sterile water at 25 ºC. An agar disk containing fungus was placed on PDA and incubated at room temperature for three days at near UV light, the purity thereof checked and subsequently used throughout this study.

3.2. Isolation and pathogenicity of fungal postharvest avocado pathogens

Fuerte cultivar avocados were collected from the Pretoria fresh produce markets and were ripened at room temperature. All fruit showing typical SE symptoms were used for isolation. Fruit were surface sterilized by wiping with 70 % ethanol. Small sections of skin and pulp surrounding the diseased lesions (2 x 2 mm) were aseptically cut and placed on PDA plates. The plates were incubated at 25 °C for three to six days. Fungi growing from the samples were isolated, purity ensured and identified. Prof. F.C. Wehner confirmed the isolates' identity (Department of Microbiology and Plant Pathology). Pathogenicity was confirmed using the fruit inoculation method described below.

Unripe Fuerte avocados were surface sterilized as described and three holes were made with a five mm diameter cork borer on the equatorial region of the fruit. Three fruit were used per isolate. Agar disks were punched from the periphery of the actively growing fungal pathogen cultures using a five mm diameter cork borer and were placed in the holes with the side containing fungal growth inserted first. Holes were sealed with the removed avocado plugs and covered with Parafilm. The negative control consisted of agar disks containing no fungal growth. Fruit were incubated at 25 °C to ripen and observed daily for the development of decay symptoms. As soon as typical black necrotic lesions developed, fungi were re-isolated and identity confirmed (as described previously).

3.3. In vitro *evaluation of antagonistic efficacy of* Bacillus subtilis *subcultures*

The dual culture method of Skidmore (1976) was modified and used in this study. Fungal isolates were cultured on PDA for seven days at 25 °C and used in this assay. Disks were aseptically punched from the periphery of the actively growing fungal culture using a cork borer with a four mm diameter. Disks were placed in the center of a round 90 mm Petridish containing PDA, with the fungal growth in contact with the PDA plate. Plates were incubated at 25 °C until the fungal colony reached a five mm diameter. Bacterial cultures were streaked on STD1 and incubated for 24 h at 25 °C. Bacteria were subsequently streak inoculated (two cm long streaks) 30 to 35 mm from the center of the fungal disk on three equidistant places on the Petridish. Plates containing only the test fungal culture, without any bacterial streaks, served as controls. Figure 3.1 depicts what the test plates looked like.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 3.1: *Bacillus subtilis* subcultures and other reference bacteria used for comparison purposes

Culture	*MPUP Code	°Reference Code	Known history	Reference
Bacillus cereus	2	DSMZ 9	Reference strain	None
Bacillus licheniformis	214	5A1	Reference strain	None
Bacillus licheniformis	494	B40	Reference strain	None
Bacillus licheniformis	-	PS-Mango	Commercial isolate	None
Bacillus licheniformis	-	MAL	Trial mango antagonist	None
Bacillus licheniformis	-	MAH	Trial mango antagonist	None
Bacillus spp.	19	Natto	Reference strain	None
Bacillus subtilis	16	Avo 13	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	17	Avo 32	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	18	Avo 66	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	22	Avo 225	Isolated by L. Korsten	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	23	ATCC 663	Reference strain	None
Bacillus subtilis	24	ATCC 6051	Reference strain	None
Bacillus subtilis	25	ATCC 11774	Reference strain	None
Bacillus subtilis	27	A6	Unknown	Korsten *et al.*, 1988; Korsten *et al.*, 1989
Bacillus polymyxa	32	DSMZ 36	Reference strain	None
Bacillus subtilis	35	A6 (Daleen)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	44	A6 (87)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	45	A6 (91)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	46	A6 (Lise)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	47	A6 (Lise - 1)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	48	A6 (89 - 1)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	49	A6 (Lise - lig)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	50	A6 (Lise - wit)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	51	A6 (89 - lig)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	52	A6 (89 - wit)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	79	A6 (88)	Unknown	Korsten *et al.*, 1989; Korsten, 1993
Bacillus subtilis	80	ATCC 55466	Deposited at ATCC	None
Bacillus subtilis	-	A6 (Estelle)	Unknown	Towsen *et al.*, 1995, Korsten *et al.*, 1998
Bacillus subtilis	-	A6 (Erika)	Unknown	Korsten *et al.*, 1991; Korsten *et al.*, 1992; Korsten *et al.*, 1993; Korsten *et al.*, 1994; Van Dyk *et al.*, 1997
Bacillus subtilis	-	PS-S	Commercial isolate	None
Bacillus subtilis	-	PS-B	Commercial isolate	None
Bacillus subtilis	-	MI-14	Subculture used 1999	Havenga *et al.*, 1999; Korsten & de Jager, 1995

* MPUP code as assigned to isolates in the culture collection at the University of Pretoria
°Reference code: DSMZ (German Collection of Microorganisms and Cell Cultures), ATCC (American Type Culture Collection), A6 (original biocontrol bacterium), other codes are the researcher's own codes
No MPUP code assigned

The antagonists were also streak inoculated on PDA plates without fungal cultures as an additional control. Duplicate plates were used and the experiment was repeated three times. Plates were maintained at 25 °C. Growth of the fungus, measured from the centre of the fungal disk to the periphery of the culture (in the direction of the bacterium), as well as the distance to the nearest edge of the bacterium's growth were measured 3, 6, 10 and 14 days after inoculation with the antagonist. The percentage growth inhibition was determined by the following formula: $(K_r - r1) / K_r \times 100 = \%$ Growth Inhibition. K_r represents the radius of the control fungal pathogen's growth and r1 represents the radius of the pathogen's growth towards the bacterial antagonist as measured from the centre of inoculation. The means of the percentage inhibition for each bacterium were calculated for each fungus. The most resistant or most susceptible groups of treatments, with a probability of 95% for the correct decision, were selected according to the Multiple t-distribution test procedure of Gupta & Panchapakesan (1979). Data were analysed using the statistical program GenStat (2000).

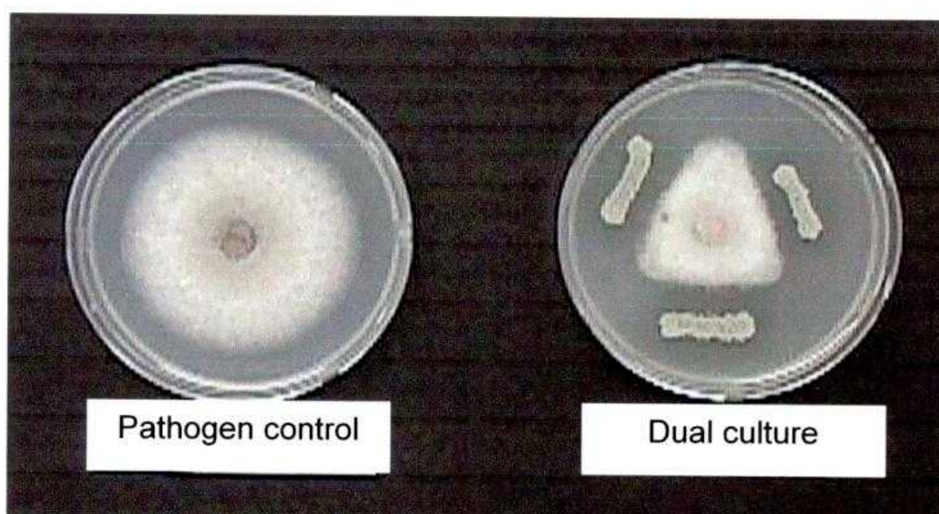


Figure 3.1: Dual culture technique: *Bacillus subtilis* inhibiting *Colletotrichum gloeosporioides in vitro* on potato dextrose agar plates.

3.4. *Genomic fingerprinting of* Bacillus subtilis *subcultures using RISA primers for polymerase chain reaction*

3.4.1. DNA extraction

Template DNA was extracted from bacterial isolates (2, 23, 25, 35, 79, 80, A6 (Estelle), A6 (Erika), PS-S, MI-14) using a modification of the rapid lysis method (Maniatis *et al.*, 1982). Single colonies were transferred to Luria Bertani broth (LB) (Biolab, Merck) and shake incubated (70 rpm) for 24 h at 37 °C. One ml of the

suspension was centrifuged (14 000 x g) for 10 min. The supernatant was removed and the pellet was washed twice by resuspending the pellet in one ml quarter strength Ringer's solution and centrifuged (14 000 x g) for 10 min. The pellet was resuspended in 100 μl sterile milli Q water and heated for 10 min at 95 °C. Cell lysate was immediately placed on ice.

3.4.2. Amplification of DNA

RISA primers (MWG Biotech Germany), 16S rDNA (5'-TTG TAC ACA CCG CCC GTC A-3') and 23S rDNA (5'-GGT ACC TTA GAT GTT TCA GTT C-3') were used for the amplification of DNA (McManus & Jones, 1996). A reaction mixture containing 1 x reaction buffer (100 mM NaCl; 50 mM Tris-HCl, pH 8.0; 0.1 mM DDT; 50 % glycerol and 1 % Triton X-100) (Promega, Johannesburg), 50 pmoles of each of the oligonucleotide primers, 0.625 mM of each of the dNTPs, 2.5 mM $MgCl_2$, milli Q water and 0.2 U of *Taq* DNA polymerase (Promega) were set up to which 1.5 μl of cell lysate was added. A Perkin Elmer 2400 was used for thermal cycling. The following cycle was selected: initial denaturation for seven min AT 95 °C followed by thirty cycles of denaturation for one min at 94 °C, annealing for one min at 52 °C and extension for three min at 72 °C. The reaction was completed with a final 10 min extension at 72 °C. As positive control, a pure *Pseudomonas aeruginosa* DNA sample (B. Steyn, Department of Microbiology and Plant Pathology, University of Pretoria) was included. Water alone was used as negative control.

3.4.3. Separation of DNA bands

Ten μl of the amplification products were separated on a 1.2 % agarose (Sigma) gel in Tris-Borate buffer (TBE) (45 mM Tris-borate, 1 mM $Na_2EDTA.2H_2O$) and visualized by ethidium bromide staining (Maniatis *et al.*, 1982) and transillumination. The Vilber Lourmat camera system was used to photograph the DNA gels. A 1 kbp standard ladder was included for band size reference.

4. RESULTS

4.1. Isolation and pathogenicity of fungal postharvest avocado pathogens

Pathogenic efficacy of the isolated fungi, *P. perseae*, *C. gloeosporioides*, *D. aromatica* and *L. theobromae* is summarised in Figure 3.2. Of the four fungi evaluated for pathogenicity, *D. aromatica* and *L. theobromae* caused the greatest lesion formation followed by *C. gloeosporioides* and *P. perseae*. All test fungi were re-isolated from lesions produced and Koch's postulate could therefore be confirmed.

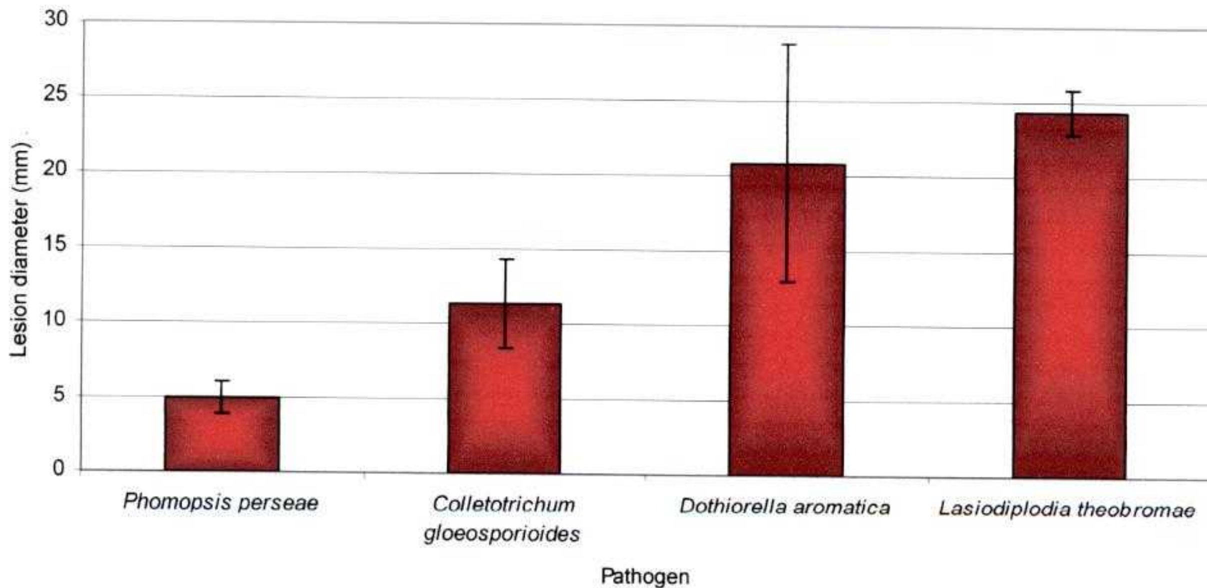


Figure 3.2: Diameter of lesions formed on avocado fruit by fungal postharvest pathogens.

4.2. Evaluation of in vitro *antagonism of* Bacillus subtilis *subcultures and reference strains*

The inhibitory efficacy of subcultures and reference strains against fungal avocado pathogens is summarised in Table 3.2. The antagonists, listed from the best (indicated in clear blocks) to the least effective antagonist (grey blocks) and the two blocks significantly less effective by a probability of $P < 0.05$ according to the Multiple t-distribution test. The most effective antagonists against all four pathogens *in vitro* are 16, 22, MI-14, MAH, 214, MAL, PS-M and A6 Erika, of which only MI-14 and A6 Erika are subcultures of the initial biocontrol organism. Antagonists MAH, MAL, PS-M and 214 are all *B. licheniformis* isolates. The commercial isolates (PS-S and PS-B), however, is still efficacious, but not to the same extent as subculture MI-14. Antagonist A6 Erika is the most effective and A6 Estelle the least effective compared to the rest of the subisolates.

4.3. Genomic fingerprinting of Bacillus subtilis *subcultures using the rDNA internal spacer analysis polymerase chain reaction*

Figure 3.3 depicts the fingerprint of the antagonists. Isolates MI-14, PS-S, A6 (Erika), A6 (Estelle), 80, 79 and 35 all have the same characteristic set of DNA bands. Isolate 80 contained less DNA and the band is not as bright as the rest. The band pattern of isolates 2, 23 and 25 are unique and are not subcultures of the initial biocontrol organism.

Table 3.2: *In vitro* inhibition of avocado fungal pathogens, *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae* by *Bacillus isolates*

Phomopsis perseae		*Colletotrichum gloeosporioides*		*Dothiorella aromatica*		*Lasiodiplodia theobromae*	
Antagonist	Mean % Inhibition	Antagonist	Mean % Inhibition	Antagonist	Mean % Inhibition	Antagonist	Mean % Inhibition
16	61.1	16	56.57	PS-M	54.45	16	41.38
MAH	61.1	MAH	56.2	MAH	49.78	MAH	40.8
214	60.92	PS-M	54.22	22	48.07	214	38.2
PS-M	60.67	214	54.15	16	40.72	PS-M	37.02
18	58.57	18	51.72	18	39.73	18	31.25
MI-14	57.32	22	49.82	214	38.95	22	30.7
24	56.22	MI-14	48.82	MI-14	35.92	48	24.67
22	55.83	MAL	46.98	24	34.3	45	24.05
80	53.47	24	44.52	MAL	34.1	A6 Erika	22.43
MAL	53.12	A6 Erika	42.02	PS-B	32.63	MAL	22.22
A6 Erika	52.82	45	40	PS-S	32.3	MI-14	20.92
PS-S	52.53	48	38.93	23	32.15	17	19.95
PS-B	51.95	23	37.76	A6 Erika	32.15	24	19.8
17	51.92	80	37.57	48	31.95	79	18.18
45	50.88	35	35.58	35	30.92	PS-B	17.23
44	49.7	79	35.53	79	30.62	80	16.73
35	49.23	51	35.28	17	30.5	PS-S	16.47
79	48.68	PS-S	35.1	45	30.35	23	16.1
52	48.57	44	34.55	80	28.6	35	15.97
48	48.05	52	33.93	44	28.4	44	15.77
51	45.43	PS-B	33.82	19	16.65	51	15.22
23	44.2	17	32.85	51	14.17	52	14.93
19	29.83	19	26.65	52	12.22	19	0
49	28.25	25	22.92	25	1.73	25	0
27	27.35	A6 Estelle	22.38	A6 Estelle	1.67	27	0
47	25.33	49	21.3	47	0.97	46	0
46	23.63	27	20.4	46	0.42	47	0
A6 Estelle	22.6	46	17.65	27	0	49	0
50	19.32	50	16.77	49	0	494	0
25	13.48	47	16.32	494	0	50	0
494	10.17	494	10.87	50	0	A6 Estelle	0
$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$	
SEM = 4.06		SEM = 2.73		SEM = 5.53		SEM = 5.11	

SEM = Standard error of means

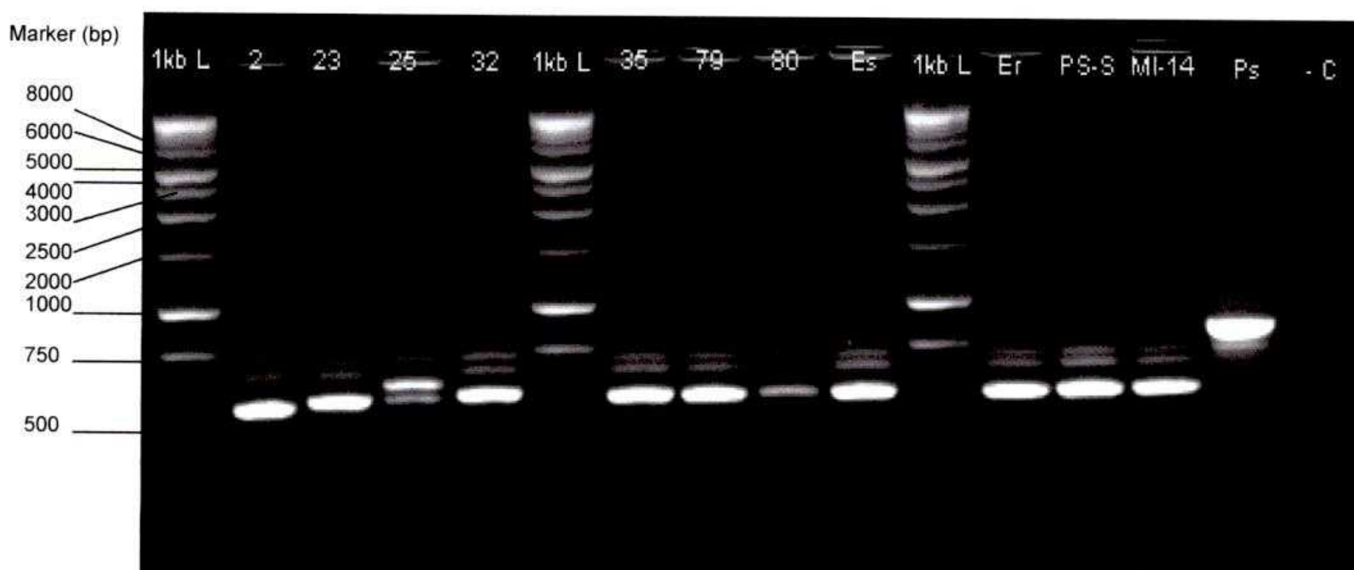


Figure 3.3: Banding patterns of DNA products after rDNA internal spacer analysis polymerase chain reaction on selected isolates of *Bacillus subtilis* separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Isolates 35, 79, 80, Es, Er, PS-S and MI-14 are all subcultures of *B. subtilis* biocontrol agent of avocado postharvest diseases. Isolate 2 (*Bacillus cereus*), 23 (*Bacillus subtilis* ATCC 663) and 25 (*B. subtilis* ATCC 6051) are reference cultures used. Isolate Ps is pure DNA from *Pseudomonas aeruginosa* as positive control and –C is water used as a negative control.

5. DISCUSSION

In this study significant differences were found in the *in vitro* activity of *B. subtilis* subcultures against postharvest fungal avocado pathogens. The subcultures used in various studies over a 15 year period differed in terms of their biocontrol effectivity *in vitro*. Theoretically, the activity of the subcultures should not differ, but some were found to be less effective. Isolate A6 Erika performed well against *P. perseae* and *L. theobromae*. Korsten *et al.* (1997) found this subculture to be effective against preharvest diseases of avocado. Black spot, caused by *Pseudocercospora purpurea* Cooke, and sooty blotch, caused by a specie of *Akaropeltopsis* that was controlled effectively for three consecutive years with and without combining it with the commercial fungicide, copper oxychloride. The commercial isolates (PS-S and PS-B), however, is efficacious, but not to the same extent as subculture MI-14, used in the mode of action studies (Korsten & de Jager, 1995; Havenga *et al.*, 1999).

The variability in the effectiveness of *B. subtilis* subcultures to inhibit fungal pathogens of avocado may be ascribed to phenotypic variation. Variation in efficacy under commercial and semi-commercial conditions was observed (Korsten *et al.*, 1993; Korsten *et al.*, 1998) and subculturing in order to obtain pure cultures may contribute thereto. Reinheimer *et al.* (1995) found variants in a community of *Lactobacillus helveticus*

ATCC 15807 that are able to ferment mannose and fructose. However, these variants make up a very small percentage of the total population. This implies that during subculturing, selection might take place if care is not taken to preserve stock cultures from the initial isolate. Rainey & Rainey (2003) found that individuals of *P. fluorescens* making up a population work together for the benefit of the group, even though it may be costly to the individual itself. They state that some phenotypic characteristics are sacrificed for the benefit of the population. Specific traits may be lost while others are selected for during continuous subculturing.

Since the subcultures differed significantly in their *in vitro* efficacy, contamination was suspected. All subcultures showed identical DNA banding patterns or fingerprints using RISA primers in PCR. The observed banding pattern of the subcultures differed from those of the reference strains *B. subtilis* ATCC 663 and *B. subtilis* ATCC 11774. Thus, the possibility of contamination is ruled out as a possible reason for the observed variability in product performance.

This study highlights the importance of managing stock cultures, subcultures and culture purity to ensure consistent results. The subculture MI-14 was used further throughout this study. Of all the subcultures, it showed the greatest antagonistic activity against all fungal pathogens tested. No differences were observed between the subcultures using RISA PCR. Sequencing of the subcultures may further support these findings and should be considered for future studies.

6. REFERENCES

Campbell, K.D., East, A.K., Thompson, D.E. & Collins, M.D. 1993. Studies on the large subunit ribosomal RNA genes and intergenic spacer regions of non-proteolytic *Clostridium botulinum* types B, E and F. Research in Microbiology 144: 171 – 180.

Darvas, J.M. & Kotzé, J.M. 1987. Fungi associated with pre- and postharvest diseases of avocado fruit at Westfalia estate, South Africa. Phytophylactica 19: 83 – 85.

Farber, J.M. 1996. An introduction to the hows and whys of molecular typing. Journal of Food Protection 59: 1091 – 1101.

García-Martínez, J., Acinas, S.G., Antón, A.I. & Rodríguea-Valera, F. 1999. Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity. Journal of Microbiological Methods 36: 55 – 64.

GenStat for Windows. 2000. Release 4.2. Fifth Edition. Oxford: VSN International.

Gupta, S.S. & Panchapakesan, S. 1979. Multiple Decision Procedures: Theory and Methodology of Selecting and Ranking Populations. John Wiley & Sons, New York.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Jensen, M.A., Webster, J.A. & Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Applied and Environmental Microbiology 59: 945 – 952.

Korsten, L. 1993. Biological Control of Avocado Fruit Diseases. Ph.D. thesis, University of Pretoria, Pretoria.

Korsten, L., Bezuidenhout, J.J. & Kotzé, J.M. 1988. Biological control of postharvest diseases of avocado. South African Avocado Growers' Association Yearbook 11: 75 – 78.

Korsten, L., Bezuidenhout, J.J. & Kotzé, J.M. 1989. Biocontrol of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 12: 10 – 12.

Korsten, L., de Villiers, E.E., de Jager, E.S., Cook, N. & Kotzé, J.M. 1991. Biological control of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 14: 57 – 59.

Korsten, L., Lonsdale, J.H., de Villiers, E.E. & Kotzé, J.M. 1992. Effect of *Bacillus subtilis* and fungicide sprays for control of pre-harvest diseases of avocado. South African Avocado Growers' Association Yearbook 15: 9 – 11.

Korsten, L., de Villiers, E.E., Rowell, A. & Kotzé, J.M. 1993. Postharvest biological control of avocado fruit diseases. South African Avocado Growers' Association Yearbook 16: 65 – 69.

Korsten, L., de Villiers, E.E., Duvenhage, J.A. & Kotzé, J.M. 1994. Control of avocado pre-harvest diseases with *Bacillus subtilis* and fungicide sprays. South African Avocado Growers' Association Yearbook 17: 32 – 34.

Korsten, L. & de Jager, E.E. 1995. Mode of action of *Bacillus subtilis* for control of avocado post-harvest pathogens. South African Avocado Growers' Association Yearbook 18: 124 – 130.

Korsten, L., de Jager, E.S., de Villiers, E.E., Lourens, A., Kotzé, J. M. & Wehner, F. C. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado postharvest diseases. Plant Disease 79: 1149 – 1156.

Korsten, L., de Villiers, E.E., Wehner, F.C. & Kotzé, J.M. 1997. Field sprays of *Bacillus subtilis* and fungicides for control of preharvest fruit diseases of avocado in South Africa. Plant Disease 81: 455 – 459.

Korsten, L, Towsen, E. & Claasens, V. 1998. Evaluation of Avogreen as post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. South African Avocado Growers' Association Yearbook 21: 83 – 87.

McManus, P.S. & Jones, A.L. 1996. Genetic fingerprinting of *Erwinia amylovora* by repetitive sequence-PCR and PCR-ribotyping. Acta Horticulture 411: 281 – 283.

Maniatis, T., Fritsch, E.F. & Sambrook, J. 1982. Molecular Cloning: a Laboratory Manual. Cold Spring Harbour Laboratory. Cold Spring Harbour, New York.

Ranjard, L., Nazaret, S., Gourbière, F., Thioulouse, J., Linet, P. & Richaume, A. 2000. A soil microscale study to reveal the heterogeneity of Hg(II) impact on indigenous bacteria by quantification of adapted phenotypes and analysis of community DNA fingerprints. FEMS Microbiology Ecology 31: 107 – 115.

Rainey, P.B. & Rainey, K. 2003. Evolution of cooperation and conflict in experimental bacterial populations. Nature 425: 72 – 74.

Reinheimer, J.A., Morelli, L., Bottazzi, V. & Suárez, V. 1995. Phenotypic variability among cells of *Lactobacillus helveticus* ATCC 15807. International Diary Journal 5: 97 – 103.

Roberts, R.G. 1994. Integrating biological control into postharvest disease management strategies. HortScience 29: 758 – 762.

Skidmore, A.M. 1976. Interactions in relation to biological control of plant pathogens. Pages: 507 – 528 In: Microbiology of Aerial Plant Surfaces. Dickinson, C.H. & Preece, T.F. (Eds). Academic Press, London.

Skidmore, A.M. & Dickinson, C.H. 1976. Colony interactions and hyphal interference between *Septoria nodurum* and phylloplane fungi. Transactions of the British Mycological Society 66: 57 – 64.

Towsen, E., van Wyngaardt, S., Verschoor, J.A. & Korsten, L. 1995. The importance of monitoring antagonists survival for efficient biocontrol. South African Avocado Growers' Association Yearbook 18: 121 – 123.

Van Dyk, K., de Villiers, E.E. & Korsten, L. 1997. Alternative control of avocado post-harvest diseases. South African Avocado Growers' Association Yearbook 20: 109 – 112.

CHAPTER 4

MODES OF ACTION INVOLVED IN ANTAGONISM OF *BACILLUS SUBTILIS* AGAINST FUNGAL POSTHARVEST PATHOGENS OF AVOCADO

1. ABSTRACT

Postharvest diseases are of economic importance to the avocado industry. The most effective control of postharvest fungal pathogens causing these diseases is through the use of fungicides. However, due to the global movement away from the excessive use of chemicals, research into alternative control measures, such as biocontrol have become important. In biocontrol, two basic strategies can be followed, one a preventative and the other a curative approach. *Bacillus subtilis*, previously described as an effective antagonist against postharvest pathogens of avocado, was further evaluated *in vitro* and *in vivo* to determine its mode of action. Scanning electron microscopy studies aimed at elucidating the interactions between *B. subtilis* and *Colletotrichum gloeosporioides* indicated that the antagonist can prevent conidia of *C. gloeosporioides* from germinating. The antagonist also attached to the hyphae of *C. gloeosporioides* and caused extensive lysis. Inhibitory substances were produced *in vitro* by *B. subtilis* that may represent antibiotics, enzymes or siderophores. *In vitro* tests showed that *B. subtilis* expressed chitinase, extracellular amylase, lipase and proteinase activity. Siderophores are also produced by *B. subtilis*. Volatiles produced by *B. subtilis* inhibited *Phomopsis perseae*, *Dothiorella aromatica* and *Lasiodiplodia theobromae in vitro*. However, *C. gloeosporioides* showed no sensitivity towards these volatiles.

2. INTRODUCTION

All subtropical fruit are prone to fungal attack either during fruit development or after harvesting. Postharvest diseases, such as stem-end rot (SE) and anthracnose, cause major economic losses in avocado production. Several fungal pathogens have been associated with SE, and include *Dothiorella aromatica* (Sacc.) Petrak & Sydow (Darvas & Kotzé, 1987; Korsten *et al.,* 1995), *Thyronectria pseudotrichia* (Schw.) Seeler, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Colletotrichum gloeosporioides* Penzig., *Phomopsis perseae* Zerova, *Pestalotiopsis versicolor* (Speg.) Steyaert, and *Fusarium* species. However, infection patterns of the pathogens differ and therefore require different disease control strategies. Most SE pathogens may enter fruit through freshly cut stem-end lesions during harvesting, or postharvestly due to handling and bruising (Darvas *et al.*, 1987; Koomen & Jeffries, 1993). The postharvest pathogen, *C.*

gloeosporioides, typically infect preharvestly and either cause lesions or remain latent until fruit ripen, resulting in postharvest infections (Koomen & Jeffries, 1993).

The wide spectrum of fungi associated with SE and their infection patterns make control of this disease difficult. Biological control strategies can either focus on curative or preventative approaches depending on the target pathogen. Keeping the diversity in fungal pathogen infection mechanisms in mind, it could be important to select both approaches in order to achieve total control of avocado diseases. Latent infections, employed by *C. gloeosporioides,* are especially problematic since the pathogen has already attached itself to the fruit surface and developed appressoria. Infection is only seen when fruit soften and ripen, decreasing the amount of inhibitory substances in the avocado skin (Prusky & Plumbley, 1992).

To develop a successful control strategy that will target all types of infection patterns, the specific mode of action of an antagonist has to be taken into consideration (Andrews, 1992; Droby & Chalutz, 1994; Guetsky *et al.*, 2002). Knowledge of the mode of action may also help in optimising the method and timing of application, as well as enhancing the activity of antagonists by modifying its formulation (Wilson & Wisniewski, 1989). In addition, information regarding the antagonist and its mode of action is necessary for product registration.

Several different modes of action can be involved in antagonism between pathogens and antagonists. These include antibiosis (Baker *et al.,* 1983; Fravel, 1988; Asaka & Shoda, 1996; Bull *et al.*, 1998), competition for space and nutrients (Wisniewski *et al.*, 1989; Dik, 1991; Calvente *et al.*, 1999), induced resistance in the host (Wilson *et al.*, 1994; Arras, 1996; Elad, 2000) and direct interactions between the pathogen and antagonist (Droby & Chalutz, 1994; Nielsen & Sørensen, 1997). The modes of action involved in *Bacillus subtilis* antagonism against *C. gloeosporioides* were previously investigated (Korsten & de Jager, 1995; Havenga *et al.*, 1999). Three modes of action were found to be involved, namely competitive colonization, competition for nutrients and antibiosis.

In this study the modes of action of *B. subtilis* against a spectrum of SE pathogens were investigated. In all assays, *C. gloeosporioides* were included for comparative purposes. Interactions between *B. subtilis* and *C. gloeosporioides* were viewed using Scanning Electron Microscopy (SEM). The possibility that antibiotic substances, enzymes and volatiles are involved was also evaluated.

3. MATERIALS AND METHODS

3.1. Isolates

Subculture MI-14 of *B. subtilis* (Chapter 3) was used throughout this study. Stock cultures of antagonist, *B. subtilis* were stored in 30 % glycerol with Ringer's solution (Merck, Johannesburg) at –70 °C. Cultures were maintained on standard 1 nutrient agar (STD1) (Biolab, Merck) and plates were incubated at 28 °C for 24 h before use.

Fungal pathogens isolated and identified in Chapter 3 were used throughout this study (*C. gloeosporioides, P. perseae, D. aromatica* and *L. theobromae*). All fungi were maintained on both potato dextrose agar (PDA) (Biolab) slants and mycelium-containing agar plugs in sterile water, kept at room temperature. An agar disk (5 mm) containing the fungus was placed on PDA and incubated at 25 – 28 °C for three days prior to use.

3.2. Direct interaction between Bacillus subtilis *and* Colletotrichum gloeosporioides *on avocado fruit surfaces using scanning electron microscopy*

Ripe Fuerte cultivar avocado fruit were obtained from Westfalia Estate for use in *in vivo* antagonism studies. The *B. subtilis* isolate was streaked onto STD1 and incubated for 24 h at 25 °C. Cells were suspended in sterile quarter strength Ringer's using a sterile glass rod. Cell counts were determined with a Petroff-Hausser counting chamber and adjusted to 10^7 cells/ml. Fungal spores of *C. gloeosporioides* were harvested from seven-day-old cultures and spore counts were made using a Haemacytometer. Spore suspensions were adjusted to contain 10^4 spores/ml.

The surfaces of ten avocado fruit were wiped with 70 % ethanol. Fruit were left for five min to air dry before three 25 mm^2 areas were marked with a water insoluble marker. The protocol described in Table 4.1 was followed. For each treatment, three samples of inoculated avocado areas were aseptically removed 6, 24 and 72 h after application and prepared for viewing using SEM. Sterile distilled water, as well as *B. subtilis* and *C. gloeosporioides* alone served as controls.

Excised avocado skin samples were placed in 0.075 M Phosphate buffer (0.15 M Na_2HPO_4. $2H_2O$ (Saarchem, Merck); 0.15 M $NaH_2PO_4.2H_2O$ (Saarchem) containing 2.5 % Gluteraldehyde (Dawson *et al.*, 1969) for 24 h. Samples were washed three times in 0.075 M Phosphate buffer. Samples were dehydrated through a series of ethanol concentrations (1 x 50 %, 1 x 70 %, 1 x 90 % and 3 x 100 %) for 15 min each.

Samples were dried in a Hitachi HCP-2 critical point dryer and mounted on aluminium stubs. Specimen stubs were coated with gold palladium in an Eiko IB-3 ion coater. Stubs were viewed in a Hitachi 840 JEOL SEM operating at 5 kV. All samples were observed and photos were taken of possible interactions between *C. gloeosporioides* and *B. subtilis*, as well as the control treatments.

Table 4.1: Treatment of avocado fruit surfaces prior to scanning electron microscopy viewing

Treatment number	Treatment	Time interval between applications			
		0 h	4 h	24 h	48 h
1	Sterile distilled water (100 μl)	+	-	-	-
2	*B. subtilis* alone (50 μl of 10^7 cells/ml + 50 μl sterile water)	+	-	-	-
3	Fungal suspension alone (50 μl of 10^4 spores/ml + 50 μl sterile water)	+	-	-	-
4	*B. subtilis* (50 μl of 10^7 cells/ml). Application of fungal test culture (50 μl of 10^4 spores/ml) after time intervals	+	+	+	+
5	Fungal test culture (50 μl of 10^4 spores/ml). Application of *B. subtilis* (50 μl of 10^7 cells/ml) after time intervals	-	+	+	+

+ indicates application of treatment

- indicates no application

3.3. *Diffusible inhibitory metabolites produced by* Bacillus subtilis in vitro *active against* Colletotrichum gloeosporioides

Antibiotic production medium (Chapter 6) was inoculated with *B. subtilis*. The inoculated liquid culture medium was shake incubated at 25 °C for seven days and two ml of the suspension was daily filtered through a 0.22 μm pore sized acetate filter (Millipore, Separation Scientific, Johannesburg). Spore suspensions of *C. gloeosporioides* were made by pipetting sterile water onto PDA plates and removing mycelia and spores with a sterile streaking rod. Spore suspensions were spread plated over PDA. Wells were made in the agar with a sterile five mm diameter cork borer and 0.25 ml of the bacterial liquid culture filtrate was pipetted into the wells. The absence or presence of inhibition zones were noted and were measured when formed. A filtrate of sterile antibiotic production medium was used as a negative control. The averages and standard deviation between formed inhibition zone diameters were calculated.

3.4. *Enzyme activity of* Bacillus subtilis *and fungal postharvest avocado pathogens* in vitro

The production of chitinase, amylase, lipase and proteinase were investigated *in vitro* on selective media. Plates were either streak inoculated with *B. subtilis* or inoculated with a four mm diameter agar plug

containing *C. gloeosporioides*, *P. perseae*, *D. aromatica*, or *L. theobromae*. Three plates were streak inoculated on four equidistant spots with *B. subtilis* culture. For each test fungal culture, three plates were inoculated with a five mm diameter agar plug taken from the actively growing periphery of the fungus on PDA. Petridishes were inoculated with four plugs equidistant from each other. Plates were incubated at 25 °C for up to seven days and monitored daily.

3.4.1. Chitinase activity

The medium of Frändberg & Schnürer (1994) was modified to contain 4 % chitin instead of 1.5 % w/v. The medium contained the following: 4 % w/v colloidal chitin from crab shell; 8.6 mM K_2HPO_4; 11.0 mM KH_2PO_4; 2.8 mM $MgSO_4.7H_2O$; 8.6 mM NaCl; 6.7 mM KCl (all from Saarchem); 0.9 mM $CaCl_2.2H_2O$ (Fluka, Sigma-Aldrich); 0.05 % w/v yeast extract (Biolab); and 2 % w/v bacteriological agar (Biolab). The pH was adjusted to 6.6. The medium was autoclaved for 15 minutes at 121 °C. The plates were inoculated as previously described, incubated at 25 °C and the presence or absence of growth on the minimal medium was noted after 7 days. Results were compared qualitatively in relation to the presence or absence of growth.

3.4.2. Extracellular amylases

To test for extracellular amylase activity, Petridishes containing starch medium (Skinner & Lovelock, 1979) was prepared. The medium contained: 50 ml Czapec solution A (94.1 mM $NaNO_3$; 26.8 mM KCl; 0.2 mM $MgSO_4.7H_2O$); 50 ml Czapec solution C (23 mM K_2HPO_4 (all from Saarchem)); 1 ml zinc solution (3.5 mM $ZnSO_4.7H_2O$ (AnalaR, British Drug Houses (BDH)); 1 ml copper solution (2 mM $CuSO_4.5H_2O$ (Pro Analysi, Merck)); 50 ml starch solution (20 % w/v starch (Biolab) in distilled water; heated slowly to 70 – 80 °C and slowly added to the rest of the media); 1.2 % w/v bacteriological agar (Biolab); and 850 ml distilled water. The medium was sterilized for 30 min at 121 °C. Plates were inoculated as previously described, incubated at 25 °C for three days and then covered with Gram's iodine (Sigma). The presence or absence of clear zones in the agar surrounding bacterial and fungal growth was noted. Results were compared qualitatively in relation to the presence or absence of clear zones.

3.4.3. Lipase activity

To test for lipase activity, Petridishes containing Tween-80 medium (Skinner & Lovelock, 1979) was prepared. The medium contained: 1 % w/v peptone (Biolab); 8.6 mM NaCl (Saarchem); 0.7 mM $CaCl_2.2H_2O$ (Fluka); 0.05 mM bromocresol purple (Pro Analysi); and 1.5 % w/v biological agar (Biolab). The pH was adjusted to 5.4. A 10 % v/v tween-80 stock solution was made by adding tween-80 (Sigma) to distilled water

that was heated to 65 ± 5 °C. Both medium and tween-80 stock solution was autoclaved for 10 minutes at 121 °C. The final medium contained 10 ml of the tween-80 stock solution and 90 ml of the medium before plates were poured. Plates were inoculated as previously described, incubated at 25 °C for four days and monitored for change in colour of the medium from yellow to purple-blue. Results were compared qualitatively in relation to whether the medium colour changed or not.

3.4.4. Proteinase activity

To test for proteinase activity, Petridishes containing casein hydrolysis medium (Skinner & Lovelock, 1979) was prepared. The medium contained: 7.3 mM KH_2PO_4; 6.7 mM KCl; 0.8 mM $MgSO_4.7H_2O$ (all from Saarchem); 0.7 mM $CaCl_2.2H_2O$ (Fluka); 1 % w/v glucose (Sigma); 2.5 % v/v skim milk; and 1.2 % w/v bacteriological agar (Biolab). The pH was adjusted to 5.4 and the medium was autoclaved for 30 min at 121 °C. The plates were inoculated as previously described, incubated at 25 °C and monitored for four days for the formation of clear zones surrounding growth. The presence or absence of clear zones in the agar was noted. Results were compared qualitatively in relation to the presence or absence of clear zones.

3.5. Siderophore production by Bacillus subtilis *and fungal postharvest avocado pathogens* in vitro

The methods of Schwyn & Neilands (1987), Buyer *et al.* (1989), and Alexander & Zuberer (1991) were combined to evaluate the production of siderophores. In brief, 2 mM chrome azurol S (CAS) (Sigma) was slowly added to an iron solution (1 mM $FeCl_3.6H_2O$ (AnalaR) in 10 mM HCl (Sigma)) in a 5:1 v/v ratio to make solution A. Solution B consisted of 5 mM hexadecyltrimethylammonium bromide (HDTMA) (Fluka) dissolved over low heat distilled water. Solution A was slowly added to solution B in a ratio of 3:2 v/v and autoclaved (CAS stock solution). The following was autoclaved for 15 min at 121 °C and cooled to 50 °C to form the RSM stock solution: 6.35 mM $Ca(NO_3)_2.4H_2O$ (Saarchem); 2 mM $MgSO_4.7H_2O$ (Saarchem); 0.2 M N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) (Fluka); 0.1 M NaOH (Merck); 30 % w/v bacteriological agar (Biolab). The following were each autoclaved before adding to the RSM stock solution: 1 M KH_2PO_4 (Saarchem) at 1:0.002 v/v; 10 % casaminoacid solution (Difco) at 1:0.17 v/v; and 30 % sucrose (Saarchem) at 1:0.07 v/v. The following was filter sterilized before adding to RSM stock solution: 0.007 M $ZnSO_4.7H_2O$ (AnalaR) at 1:0.002 v/v; 0.009 M $MnSO_4.H_2O$ (Saarchem) at 1:0.002 v/v; 0.02 % w/v thiamine HCl (Sigma); and 0.001 % w/v biotin (Fluka). The CAS stock solution was slowly added to RSM stock solution, to keep foam from forming, at 1:9 v/v and dispensed into Petridishes. Plates were either streak inoculated with *B. subtilis* or inoculated with a four mm diameter agar plug containing *C. gloeosporioides*, *P. perseae*, *D. aromatica* or *L. theobromae*. Three replicate plates were inoculated for each organism and the experiment

was repeated four times. Plates were incubated at 25 °C for four days and monitored for the formation of yellow to orange zones surrounding the growth on the blue plates. The presence or absence of yellow or orange zones was noted. Results were compared qualitatively in relation to whether medium colour changed or not.

3.6. Antifungal volatiles produced by Bacillus subtilis *active against fungal postharvest avocado pathogens*

The sealed plate (3.6.1) and the aerated plate (3.6.2) methods of Fiddaman & Rossall (1993) were followed. The radial growth of the fungal pathogens were measured and compared to the untreated control by determining the percentage inhibition (Chapter 3). The means of percentage inhibition for each fungus were calculated. Data was analysed using the statistical program GenStat (2000). Weighted analysis of variance was used to test for differences between the main effects for both pathogens and mediums, as well as the pathogen-by-medium interaction. Fisher's protected t-test least significant difference was used to test for differences between means per day (Snedecor & Cochran, 1980).

3.6.1. Sealed plate method

The method of Fiddaman & Rossall (1993) was followed. The antagonist was shake incubated at 25 °C overnight in Nutrient Broth (NB) (Biolab) and 100 μl was spread plated over Nutrient Agar (NA) (Biolab) or STD1. Plates were incubated at 25 °C for 24 h. Fungal pathogens were grown on PDA for four to seven days. Five mm diameter plugs, obtained from the periphery of actively growing cultures, were used to centrally inoculate PDA plates. The treatment consisted of fungal pathogen inoculated plates inverted and placed over the antagonistic bacterial cultures, so that the antagonist could not contaminate the fungal cultures. The plates were sealed together with Parafilm and incubated at 25 °C. Control treatment consisted of two identical pathogen-containing plates sealed together. Three replicates were made from each fungus and the experiment was repeated four times. The experiment was designed to determine if the antagonist could produce antifungal volatiles. Measurements of fungal radial growth were taken over a three-day period incubated at 25 °C. Data was statistically analysed as previously described in 3.6.

3.6.2. Aerated plate method

As in the method of Fiddaman & Rossall (1993), an agar strip, 5 mm wide, was removed from the centre of PDA containing plates, creating a division. The antagonist was shake incubated at 25 °C overnight in NB

and 50 µl was spread plated over one of the PDA containing sides. Petridishes were incubated at 25 ºC for 24 h. The other PDA side was inoculated with a 5 mm diameter plug obtained from the periphery of actively growing fungal cultures on PDA. Plates were incubated at 25 ºC for four days. Plates inoculated with the fungus alone served as control. Three replicates were made from each of the fungi and the experiment was repeated four times. The pathogen's colony diameter was measured daily for four days. Data was statistically analysed as previously described in 3.6.

4. RESULTS

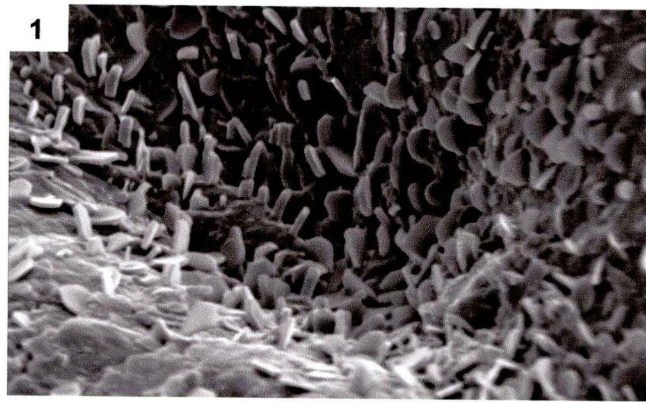
4.1. In vivo *studies*

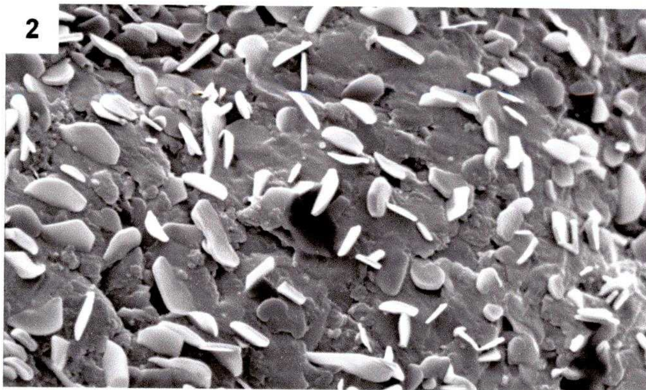
Scanning electron micrographs (Figure 4.1) show the avocado fruit surface being characteristically corrugated providing multiple microbial sites to colonise. The avocado surface has distinct protruding wax platelets.

Germinating conidium of *C. gloeosporioides* was observed attaching to the fruit surface, forming an attachment sheath (Figure 4.2). Cells of *B. subtilis* were typically found to be at least five times shorter than *C. gloeosporioides* spores (Figure 4.2). Inoculation of the avocado fruit surface with *B. subtilis* followed by fungal conidia, showed inhibition of germination of *C. gloeosporioides* spores (Figure 4.2).

In Figure 4.3 *B. subtilis* cells were observed actively dividing. Extracellular slime and micro colonies were observed (Figure 4.3). In addition to attaching to the avocado fruit surface, *B. subtilis* also colonized *C. gloeosporioides* hyphae (Figure 4.3).

Conidia of *C. gloeosporioides* inoculated onto avocado fruit germinated, forming germtubes and hyphae. When *B. subtilis* was inoculated 24 hours later, it colonized the hyphal surface. In certain cases hyphal lysis was observed (Figure 4.4). Colonization of *C. gloeosporioides* by *B. subtilis* did not always coincide with damage to hyphae (Figure 4.4).





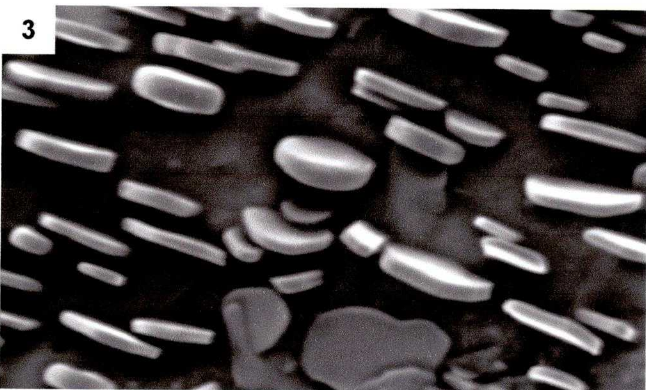


Figure 4.1: Scanning electron micrographs of the surface of avocado fruit showing characteristic wax platelets (Photo 1: 4300 x, 2: 3500 x, and 3: 10000 x magnification).

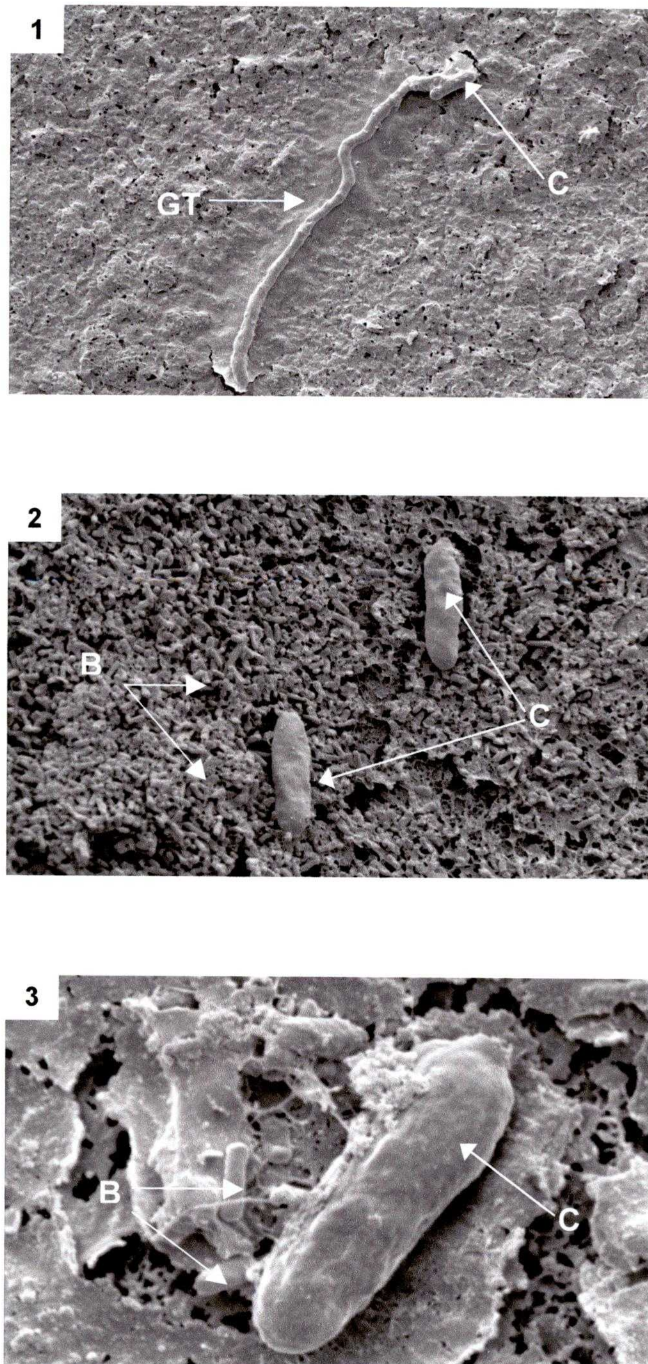


Figure 4.2: Scanning electron micrographs of avocado fruit inoculated: 1) with conidia of *Colletotrichum gloeosporioides* alone, 2) and 3) with conidia of *C. gloeosporioides* followed by *Bacillus subtilis* (Photo 1: 1200 x, 2: 7500 x, and 3: 2300 x magnification).

Abbreviations: C = conidium of *C. gloeosporioides*, B = *B. subtilis* cells, GT = germ tube of *C. gloeosporioides*

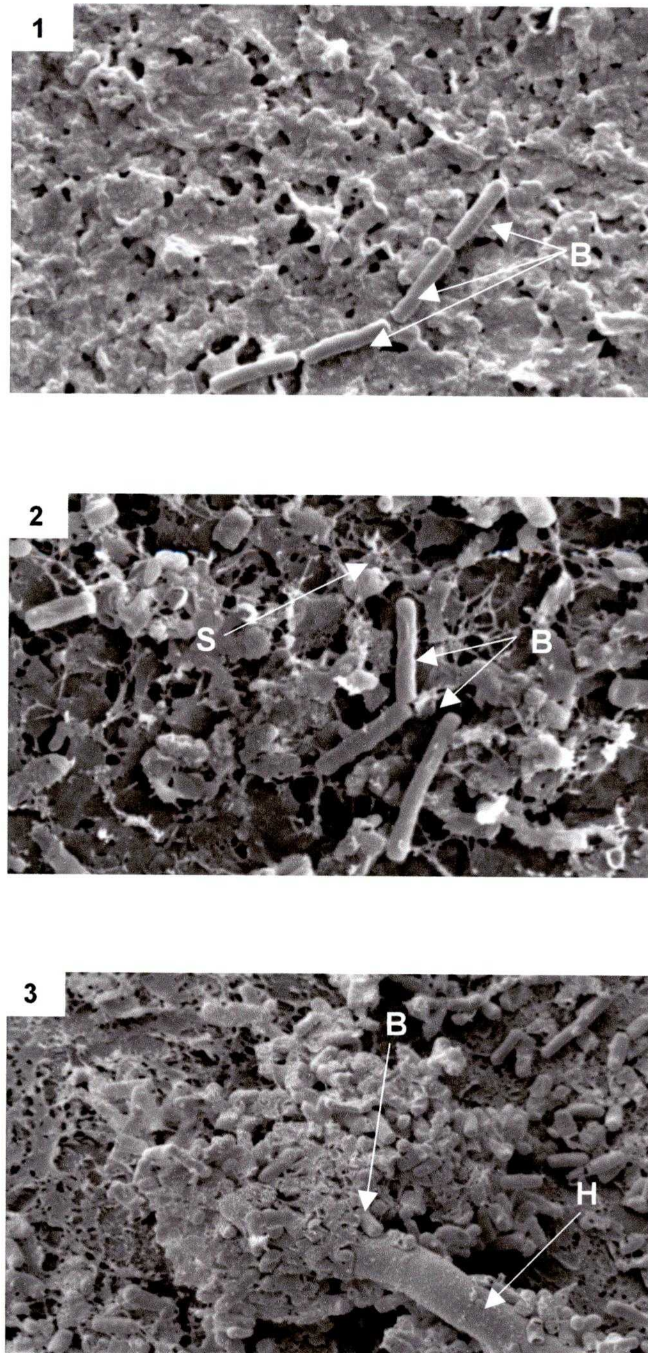


Figure 4.3: Scanning electron micrographs of avocado fruit surfaces inoculated with: 1) *Bacillus subtilis*, 2) *B. subtilis* showing attachment structures, and 3) both *B. subtilis* cells and *Colletotrichum gloeosporioides* hyphae (Photo 1: 6000 x, 2: 8500 x, and 3: 5000 x magnification).

Abbreviations: B = *B. subtilis* cells, H =*C. gloeosporioides* hyphae, S = slime formed by *B. subtilis*

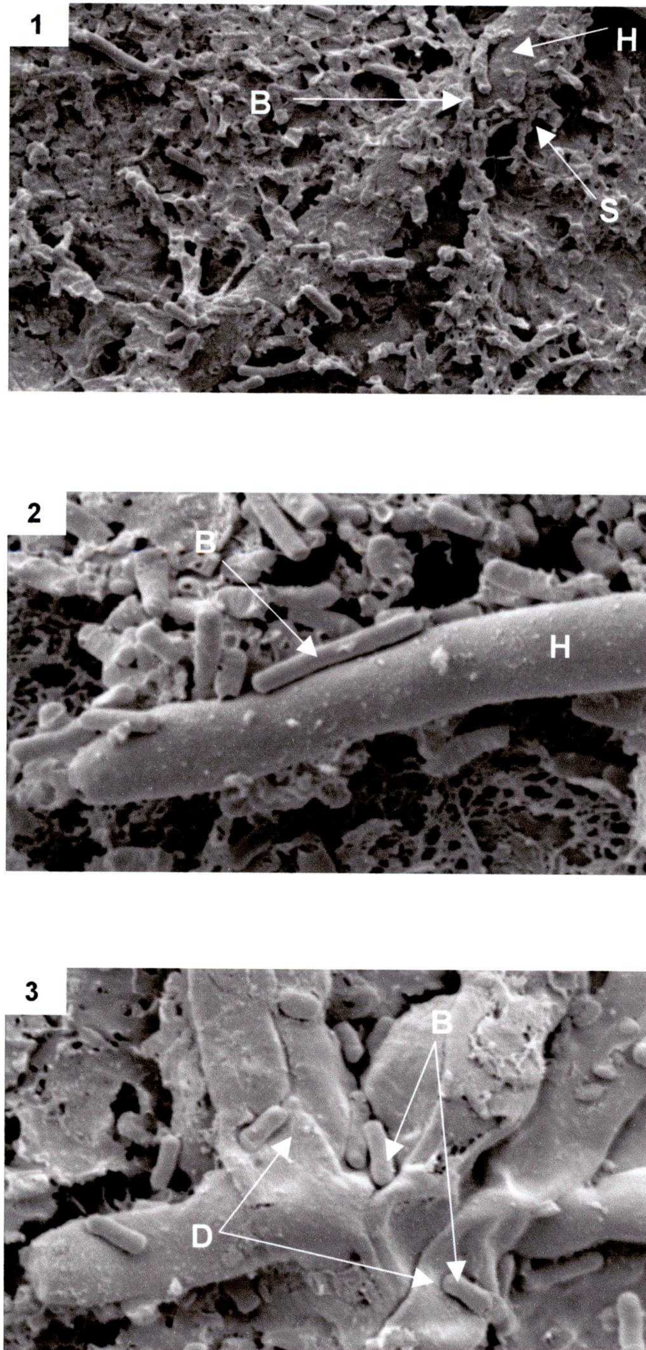


Figure 4.4: Scanning electron micrographs showing avocado fruit surfaces inoculated with *Colletotrichum gloeosporioides* and *Bacillus subtilis* (Photo 1: 4300 x, 2: 8500 x, and 3: 7000 x magnification). Abbreviations: B = *B. subtilis* cells, D = Damage caused by *B. subtilis* to fungal hypha, H =*C. gloeosporioides* hyphae, S = slime formed by *B. subtilis*

4.2. Diffusible inhibitory metabolites produced by Bacillus subtilis in vitro *active against* Colletotrichum gloeosporioides

Extracellular inhibitory substances were produced by *B. subtilis* from day two and consistently increased over time (Figure 4.5).

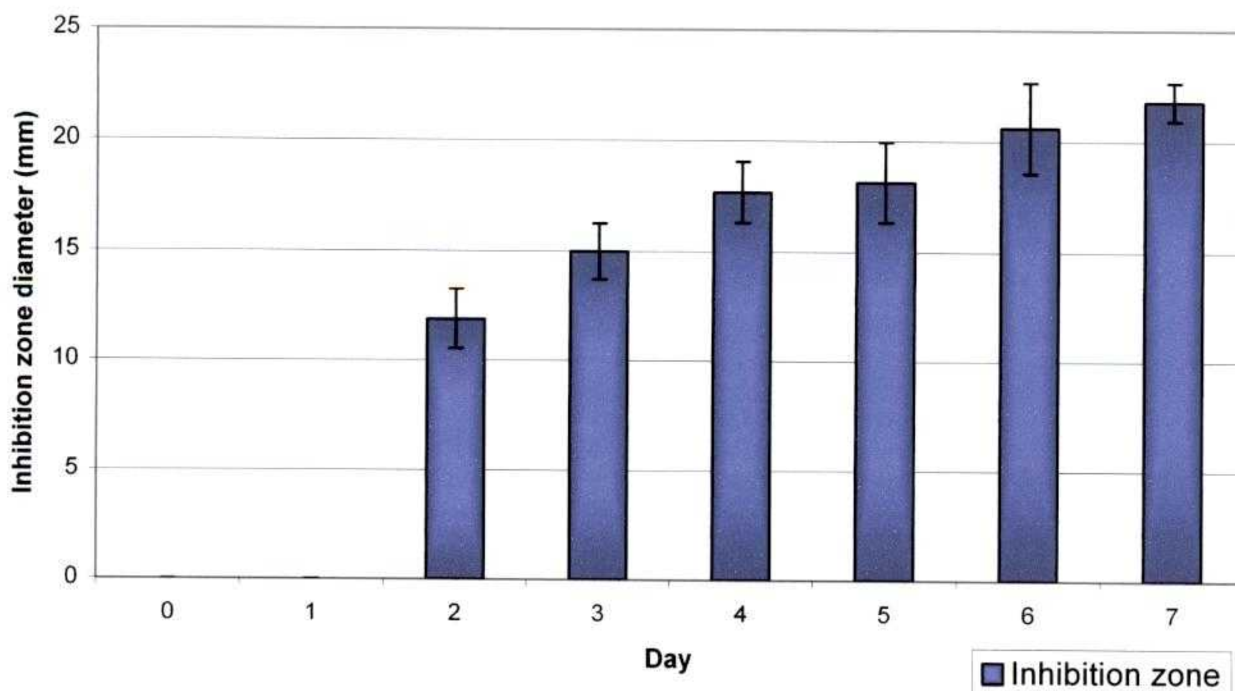


Figure 4.5: Diameter of inhibition zones formed by *Bacillus subtilis* cell-free filtrates on spread plates of *Colletotrichum gloeosporioides*.

4.3. Enzyme activity of Bacillus subtilis *and fungal postharvest avocado pathogens* in vitro

Qualitatively, all fungal pathogens tested, as well as *B. subtilis*, were observed to break down chitin, starch, lipids and casein.

4.4. Siderophore production by Bacillus subtilis *and fungal postharvest avocado pathogens* in vitro

Siderophore production was observed on all tested organisms, except *P. perseae*.

4.5. Antifungal volatile production in vitro *by* Bacillus subtilis *active against fungal postharvest avocado pathogens*

For both the sealed and aerated plate method, the data was acceptably normally distributed, but the treatment variances were excessively heterogeneous and significance was obtained at the 5 % level of significance.

4.5.1. Sealed plate method

Significant radial mycelial growth inhibition was observed for *P. perseae* (Figure 4.6), *D. aromatica* (Figure 4.8) and *L. theobromae* (Figure 4.9). A volatile substance was produced by *B. subtilis* that is able to decrease the growth rate of the fungi, when compared to the control. Although the volatile substance inhibited radial growth of *P. perseae* and *L.* theobromae, it affected *C. gloeosporioides* (Figure 4.7) less. The highest percentage fungal pathogen inhibition was higher on STD1 when compared to NA.

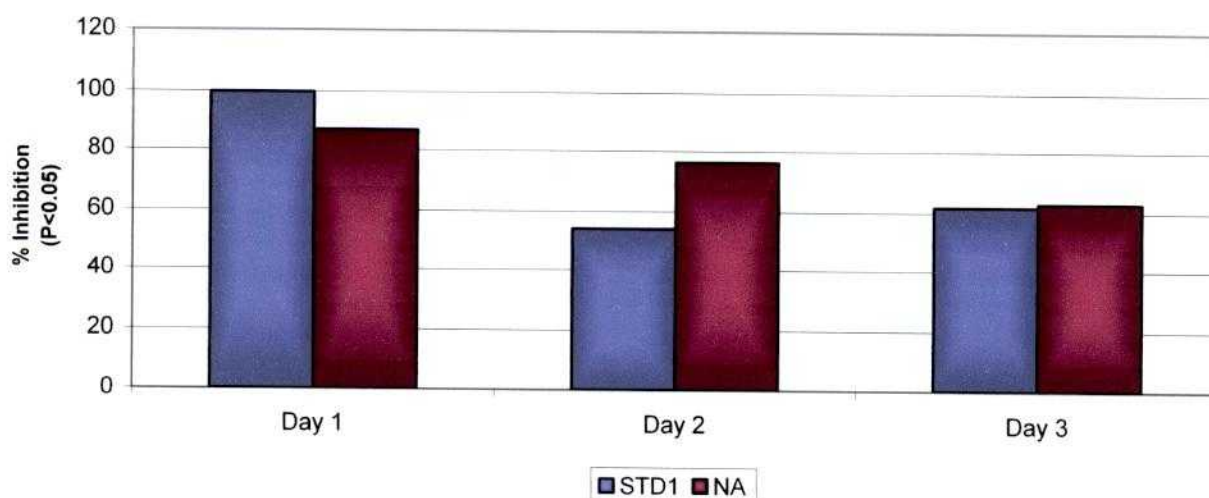


Figure 4.6: Comparison between the mean percentage inhibition of *Phomopsis perseae* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.

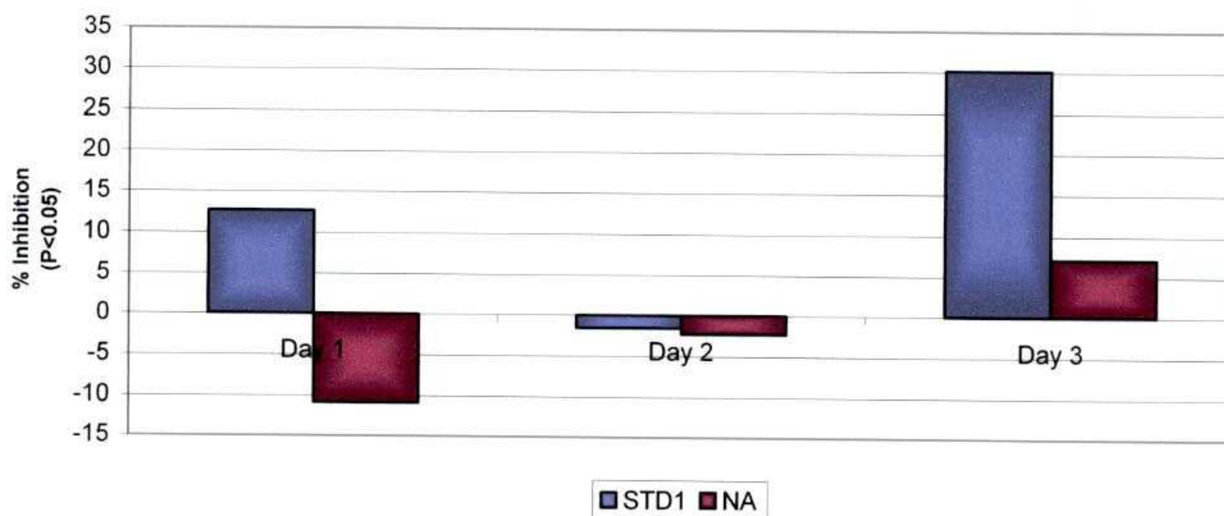


Figure 4.7: Comparison between the mean percentage inhibition of *Colletotrichum gloeosporioides* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.

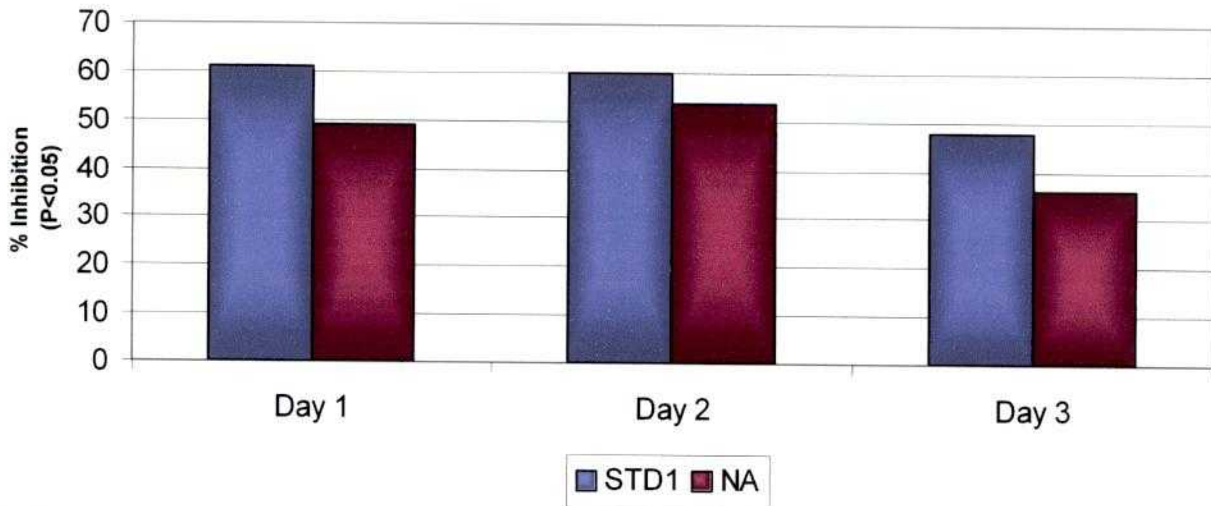


Figure 4.8: Comparison between the mean percentage inhibition of *Dothiorella aromatica* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.

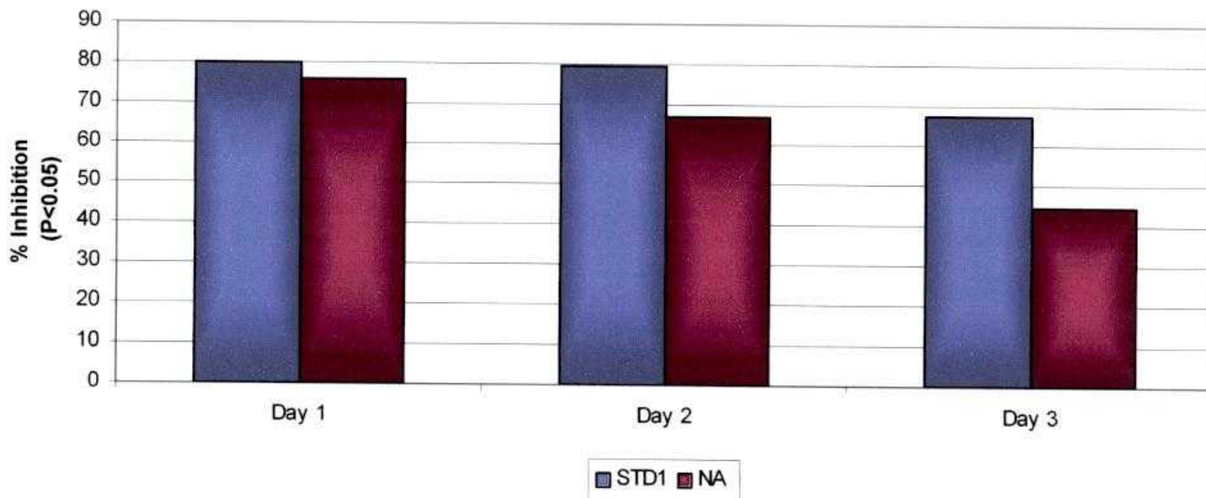


Figure 4.9: Comparison between the mean percentage inhibition of *Lasiodiplodia theobromae* by *Bacillus subtilis* measured on days 1 to 3 after inoculation.

4.5.2. Aerated plate method

The radial mycelial growth of all fungi was inhibited to some extent, as seen in Table 4.2. This implied that *B. subtilis* produced some volatile substance that was able to inhibit the growth of these pathogens. However, the extent to which the pathogens were inhibited was much lower than that observed when using the sealed plate method. There were no significant differences when the percentage inhibition of the four pathogens was compared (Table 4.2). After two days, the small area available for growth of *L. theobromae* was overgrown and no further measurements could be taken.

Table 4.2: Mean percentage inhibition of fungal pathogens caused by volatile substances produced by *Bacillus subtilis* using the aerated plate method

Pathogen	Day 1		Day 2		Day 3		Day 4	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Phomopsis perseae	-5.94	32.8	-8.905	12.103	2.647	5.344	1.496	3.841
Colletotrichum gloeosporioides	13.50	2.3	-0.689	0.961	-0.700	0.676	3.760	0.256
Dothiorella aromatica	12.02	9.5	-16.847	7.093	-15.942	13.401	-8.225	4.777
Lasiodiplodia theobromae	0.57	10.6	2.820	5.379	-	-	-	-
Fprob	P = 0.633		P = 0.154		P = 0.454		P = 0.072	
% CV	50.5		5.45		9.72		78.38	

SEM = Standard error of means

- Indicates no measurements were taken on these days

Data analysed using weighted ANOVA.

5. DISCUSSION

This study was the first to show the activity of volatile substances produced by *B. subtilis* that is inhibitory against fungal pathogens of avocado fruit. The production of antifungal volatile substances by *B. subtilis* is not unusual. Knox *et al.* (2000) observed *B. subtilis* producing antifungal volatiles active against *Fusarium oxysporum* (Schltdl.: Fr.). The fungal pathogens differed in their sensitivity to the volatile substance, with *P. perseae*, *L. theobromae* and *D. aromatica* being more sensitive. However, *C. gloeosporioides* was not affected by the volatile substance, indicating that the volatile might not play a role in *in vivo* antagonism against *C. gloeosporioides*. Therefore, other modes of action must be involved in the interaction between *C. gloeosporioides* and *B. subtilis*. This observation corresponds to that reported by Fiddaman & Rossall (1993). They found that various fungi react differently to volatiles produced by *B. subtilis* NCIMB 12376. They also stated that this variation in activity might reflect differences in sites of action of the volatiles, or even differences in the ability of the fungi to detoxify these metabolites. The volatiles produced by *B. subtilis* NCIMB 12376 were tentatively identified as alcohols, aldehydes, ketones and esters. In the current study, two different agar media were used to test for the production of antifungal volatiles, STD1 and NA. A greater inhibitory activity was found with STD1 medium, especially against *L. theobromae* and *D. aromatica*. The carbon sources and peptone levels in STD1 are known to be higher than that of NA. Fiddaman & Rossall (1993; 1994) found that by increasing the D-glucose levels in the medium, a significant increase in the volatile activity could be observed. Furthermore, volatile production increased with the addition of complex carbon sources, like starch, even though the same inhibitory levels yielded by D-glucose was not reached. Knox *et al.* (2000) found that with the interaction between *B. subtilis* and *F. oxysporum*, volatile production

increased with higher nitrate levels in the media. Similarly, peptone was able to increase volatile production in *Streptomyces* (Fiddaman & Rossall, 1994). However, Fiddaman & Rossall (1994) found that there was not necessarily a correlation between the ability of a medium to support growth and its ability to inhibit the fungal pathogens. They state that some nutrients may yield higher volatile production, without increasing bacterial growth and that a range of different volatiles may act synergistically. They also found the inhibitory effect of the sealed plate being more effective than the aerated plate method (Fiddaman & Rossall, 1993).

In vivo inhibition of *C. gloeosporioides* germination by *B subtilis* was observed when conidia were applied to avocado fruit after *B. subtilis*. This indicates competitive colonisation or competition for nutrients, as suggested by Korsten & de Jager (1995). When bacteria colonise fruit surfaces they utilise nutrients from the surrounding area (Brodie & Blakeman, 1976). This could imply that only limited nutrients would be available for other microbes. However, *C. gloeosporioides* is a specialised necrotrophic pathogen that does not require exogenous nutrients for germination and appressoria formation (Blakeman, 1985). The involvement of nutrient competition can thus be excluded, leaving only direct inhibitory interaction by enzymes or antibiotics produced by the biocontrol agent. Upadhyay & Jayaswal (1992) also observed this phenomenon in the interactions between *Pseudomonas cepacia* and phytopathogenic fungi.

The antagonist, *B. subtilis*, produced a substance effective against *C. gloeosporioides in vitro*. The cell-free filtrate contained diffusible substances, which may be enzymes or antibiotics. In this study, the amount of inhibitory substance/s produced by *B. subtilis* in liquid culture increased over time. The stationary phase is usually the signal for *B. subtilis* to switch on its survival genes (Msadek, 1999). In this study, single cells that colonised the hyphal surface of the pathogen did not cause damage to the fungal mycelium. However, when *B. subtilis* was present in high numbers, hyphal lysis was observed. In this case cell density was related to hyphae damage. This concept is known as quorum sensing or cell density-dependent regulation, which is a type of gene regulatory system. It has been found to play a role in antibiotic production (Dunny & Leonard, 1997; Msadek, 1999). With *B. subtilis*, a small regulator molecule (usually a small peptide or modified peptide) is produced at the basal level (Dunny & Leonard, 1997). When the bacterial cell concentration reaches a certain level, these molecules attain a certain concentration, which result in a signal to switch on selected genes (Dunny & Leonard, 1997). Quorum sensing may thus impact on the efficiency of *B. subtilis* as a biocontrol agent.

The production of chitinase, amylase, lipase and proteinase was observed in this study. Enzymes reported to be produced by *B. subtilis* and implicated in biocontrol include chitinase (Frändberg & Schnürer, 1998; Helistö *et al.*, 2001), chitosanase, laminarinase, lipase and protease (Helistö *et al.*, 2001), as well as glucanolytic and proteolytic enzymes (Nielsen & Sørensen, 1997). These enzymes may cause damage to *C. gloeosporioides* hyphae where *B. subtilis* attached itself directly onto the fungal cell wall. Keeping in mind that effective biocontrol is not necessarily ensured by the potential of an organism to produce cell wall degrading enzymes (Elad, 2000), further studies should be done to determine their role in *in vivo* biocontrol of postharvest pathogens by *B. subtilis.*

In this study it was found that *B. subtilis* produces siderophores. *Bacillus* species are known to produce siderophores of which at least two have been identified, namely 2,3-dihydroxybenzoate (Rowland & Taber, 1996; Bsat & Helmann, 1999) and itoic acid (Ito, 1993). Lindow & Wilson (1999) described siderophores that may play a dual role in antagonism, both competing for nutrients and inducing resistance in the host. However, the involvement of siderophores in *in vivo* antagonism against fungal postharvest avocado pathogens has yet to be proven.

This study shed some light on the different types of modes of action that may be involved in the control of fungal avocado postharvest pathogens by *B. subtilis.* Results indicate that antibiosis is involved through the production of inhibitory volatile substances and soluble cell-free filtrates, which may contain antibiotics, enzymes or siderophores. The direct interaction studies using SEM also indicates that competitive colonization is a potential mode of action. Further studies should focus on identifying the siderophore, volatile substance or antifungal component involved in antagonism since there is evidence that more than one of these substances may be involved. Determining which nutrients and temperatures could enhance antagonism will also be useful when designing better formulations and applications for the commercial biocontrol product.

6. REFERENCES

Alexander, D.B. & Zuberer, D.A. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biology and Fertility of Soils 12: 39 – 45.

Andrews, J.H. 1992. Biological control in the phyllosphere. Annual Review of Phytopathology 30: 603 – 635.

Arras, G. 1996. Mode of action of an isolate of *Candida famata* in biological control of *Penicillium digitatum* in orange fruits. Postharvest Biology and Technology 8: 191 – 198.

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

Baker, C.J., Stavely, J.R., Thomas, C.A., Sasser, M. & MacFall, J.S. 1983. Inhibitory effect of *Bacillus subtilis* on *Uromyces phaseoli* and on development of rust pustules on bean leaves. Phytopathology 73: 1148 – 1152.

Blakeman, J.P. 1985. Ecological succession of leaf surface micro organisms in relation to biological control. Pages 6 – 30 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Brodie, I.D.S. & Blakeman, J.P. 1976. Competition for exogenous substrates *in vitro* by leaf surface micro-organisms and germination of conidia of *Botrytis cinerea*. Physiological Plant Pathology 9: 227 – 239.

Bsat, N. & Helmann, J.D. 1999. Interaction of *Bacillus subtilis* Fur (ferric uptake repressor) with the dhb operator *in vitro* and *in vivo*. Journal of Bacteriology 181: 4299 – 4307.

Bull, C.T., Wadsworth, M.L., Sorensen, K.N., Takemoto, J.N., Austin, R.K. & Smilanick, J.L. 1998. Biological Control 12: 89 – 95.

Buyer, J.S., Sikora, L.J. & Chaney, R.L. 1989. A new growth medium for the study of siderophore-mediated interactions. Biology and Fertility of Soils 8: 97 – 101.

Calvente, V., Benuzzi, D. & De Tosetti, M.I.S. 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. International Biodeterioration & Biodegradation 43: 167 – 172.

Darvas, J.M. & Kotzé, J.M. 1987. Fungi associated with pre- and postharvest diseases of avocado fruit at Westfalia estate, South Africa. Phytophylactica 19: 83 – 85.

Darvas, J.M., Kotzé, J.M. & Wehner, F.C. 1987. Pathogenicity of fungi causing pre- and postharvest diseases of avocado fruit. Phytophylactica 19: 489 – 493.

Dawson, R.M.C., Elliot, W.H. & Jones, K.M. 1969. Data for Biochemical Research. 2nd Edition. Claredon press, Oxford.

Dik, A.J. 1991. Interactions among fungicides, pathogens, yeasts, and nutrients in the phyllosphere. Pages 412 – 429 In: Microbial Ecology of Leaves. Andrews, J.H. & Hirano, S.S. (Eds). Springer-Verlag, New York.

Droby, S. & Chalutz, E. 1994. Mode of action of biocontrol agents of postharvest diseases. Pages 63 – 75 In: Biological Control of Postharvest Diseases – Theory and Practice. Wilson, C. L. & Wisniewski, M. E. (Eds). CRC Press, Boca Raton.

Dunny, G.M. & Leonard, B.A.B. 1997. Cell-cell communication in Gram-positive bacteria. Annual Review of Microbiology 51: 527 – 564.

Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Protection 19: 709 – 714.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Fiddaman, P.J. & Rossall, S. 1994. Effect of substrate on the production of antifungal volatiles from *Bacillus subtilis*. Journal of Bacteriology 76: 395 – 405.

Frändberg, E. & Schnürer, J. 1994. Chitinolytic properties of *Bacillus pabuli* K1. Journal of Applied Bacteriology 76: 361 – 367.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant diseases. Annual Review of Phytopathology 26: 75 – 91.

GenStat for Windows. 2000. Release 4.2. Fifth Edition. Oxford: VSN International.

Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E. & Dinoor, A. 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology 92: 976 – 985.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Helistö, P., Aktuganov, G., Galimzianova, N., Melentjev, A. & Korpela, T. 2001. Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components. Journal of Chromatography B 758: 197 – 205.

Ito, T. 1993. Enzymatic determination of itoic acid, a *Bacillus subtilis* siderophore, and 2,3-dihydroxybenzoic acid. Applied and Environmental Microbiology 59: 2343 – 2345.

Knox, O.G.G., Killham, K. & Leifert, C. 2000. Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*. Applied Soil Ecology 15: 227 – 231.

Koomen, I. & Jeffries, P. 1993. Effects of antagonistic microorganisms on the post-harvest development of *Colletotrichum gloeosporioides* on mango. Plant Pathology 42: 230 – 237.

Korsten, L. & de Jager, E.S. 1995. Mode of action of *Bacillus subtilis* for control of avocado post-harvest pathogens. South African Avocado Growers' Association Yearbook 18: 124 – 130.

Korsten, L., de Jager, E.S., de Villiers, E.E., Lourens, A., Kotzé, J. M. & Wehner, F. C. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado postharvest diseases. Plant Disease 79: 1149 – 1156.

Lindow, S.E. & Wilson, M. 1999. Biological control of foliar pathogens and pests with bacterial biocontrol agents. Pages 642 - 650 In: Manual of Industrial Microbiology and Biotechnology. 2nd ed. Demain, A.L. & Davies, J.E. (Eds). ASM Press, Washington, D.C.

Msadek, T. 1999. When the going gets tough: survival strategies and environmental signalling networks in *Bacillus subtilis*. Trends in Microbiology 7: 201 – 207.

Nielsen, P. & Sørensen, J. 1997. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiology Ecology 22: 183 – 192.

Prusky, D. & Plumbley, R.A. 1992. Quiescent infections of *Colletotrichum* in tropical and subtropical fruits. Pages 289 – 307 In: *Colletotrichum*: Biology, Pathology and Control. Bailey, J.A. & Jeger, M.J. (Eds). CAB International, Wallingford.

Rowland, B.M. & Taber, H.W. 1996. Duplicate isochorismate synthase genes of *Bacillus subtilis*: regulation and involvement in the biosyntheses of menaquinone and 2,3-dihydroxybenzoate. Journal of Bacteriology 178: 854 – 861.

Schwyn, B. & Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160: 47 – 56.

Skinner, F.A. & Lovelock, D.W. 1979. Identification Methods for Microbiologists, Academic Press, London.

Snedecor, G.W. & Cochran, W.G. 1980. Statistical Methods. 7th Edition. Iowa State University Press. Ames, Iowa.

Upadhyay, R.S. & Jayaswal, R.K. 1992. *Pseudomonas cepacia* causes mycelial deformities and inhibition of conidiation in phytopathogenic fungi. Current Microbiology 24: 181 – 187.

Wilson, C.L., El Ghaouth, A., Chalutz, E., Droby, S., Stevens, C., Lu, J.Y., Khan, V. & Arul, J. 1994. Potential of induced resistance to control postharvest diseases of fruits and vegetables. Plant Disease 78: 837 – 844.

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

Wisniewski, M., Wilson, C. & Hershberger, W. 1989. Characterization of inhibition of *Rhizopus stolonifer* germination and growth by *Enterobacter cloacae*. Canadian Journal of Botany 67: 2317 – 2323.

CHAPTER 5

ENVIRONMENTAL CONDITIONS INFLUENCING *IN VITRO* ANTAGONISM OF *BACILLUS SUBTILIS* AGAINST FUNGAL POSTHARVEST AVOCADO PATHOGENS

1. ABSTRACT

Environmental conditions affect the efficacy of biocontrol agents. Understanding and exploiting conditions required for optimal functioning is essential for the successful implementation of biocontrol systems. The effect of different temperatures on *in vitro* antagonism of *Bacillus subtilis* on avocado postharvest pathogens *Phomopsis perseae*, *Dothiorella aromatica*, *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides* were investigated. The *in vitro* effect of 19 carbon sources, 18 amino acids and ammonium chloride on antagonism was also studied. Low temperatures did not support antagonism of *B. subtilis*. However, temperatures higher than 15 °C increased antagonistic efficacy. Nitrogen sources that increased antagonism without supporting the growth of the tested pathogens included L-glutamic acid, L-glutamine and L-(+)-asparagine, while carbon sources were D-arabinose, D-(+)-mannitol and citrate.

2. INTRODUCTION

A thorough understanding of the impact of environmental conditions on the antagonist is required in order to establish effective preharvest biocontrol systems. Preharvestly low nutrient availability, temperature fluctuations, high levels of UV radiation and dry conditions are the norm (Mari & Guizzardi, 1998; Spadaro & Gullino, 2004). In contrast, biocontrol agents applied postharvestly have an advantage since harvested fruit are kept in areas where the atmosphere is controlled and temperature and humidity are more constant (Wilson & Pusey, 1985; Ippolito & Nigro, 2000). It is well known that environmental conditions affect the survival and efficacy of bacterial biocontrol agents (Morris & Rouse, 1985; Gueldner *et al.*, 1988; Hase *et al.*, 1999; Duffy & Défago, 1999; Knox *et al.*, 2000). Most importantly, the mode of action employed by the antagonist can be triggered, enhanced or even inactivated by environmental conditions.

According to Morris & Rouse (1985), and Hase *et al.* (1999), a microbial community size is limited by the availability of at least one growth-limiting variable. Some nutrients may stimulate infection by the pathogen (Dik, 1991). On the other hand, the normal pathogenic behaviour of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara was found to be suppressed and it developed more saprophytically by adding supplementary nutrients (Blakeman, 1985). Appressoria development was also enhanced by depriving the

pathogen of nutrients (Blakeman & Brodie, 1977; Blakeman, 1985). The involvement of nutrient competition in biocontrol is often cited as a potential mode of action (Brodie & Blakeman, 1976; Blakeman & Brodie, 1977; Dik, 1991; Janisiewicz & Bors, 1995), but is difficult to prove. The competing microorganisms must both require a specific nutrient, which must not be available in abundance (Brodie & Blakeman, 1976; Blakeman & Brodie, 1977). Alternatively, if the growth-limiting nutrient of the biocontrol agent and the pathogen differ, the addition of the nutrient favoured by the biocontrol agent may increase its growth and subsequently its population size. Specific nutrients may also enhance the production of antifungal volatiles (Fiddaman & Rossall, 1994) and antibiotics (Milner *et al.*, 1995). Thus, nutrients incorporated into a biocontrol product can affect its subsequent effectiveness.

Commercial low temperature storage may reduce the effectiveness of the antagonist (Mari & Guizzardi, 1998). On the other hand, antagonists can be selected that are effective at these temperatures (Wilson & Pusey, 1985). The mode of action involved in biocontrol may also be enhanced or inactivated by the reigning temperature. Ohno *et al.* (1995) found temperature enhances the antibiotic production of iturin A and surfactin, with each antibiotic preferentially produced at a specific temperature.

In a previous study, the commercial product, Avogreen® (Stimuplant CC, Pretoria, South Africa), with *B. subtilis* as the active ingredient, performed well on a semi-commercial scale (van Dyk *et al.*, 1997). However, in one postharvest trial, the incidence of decay increased when compared with the untreated control (Korsten *et al.*, 1998). The ineffectiveness of Avogreen® in that case was ascribed to product formulation which might have contained nutrients that could stimulate pathogen growth (Korsten *et al.*, 1998). A subsequent study on the role of various nutrients in the *in vitro* interaction between *B. subtilis* and the anthracnose pathogen, *Colletotrichum gloeosporioides* Penzig., showed that some nutrients enhance the antagonist efficacy (Havenga *et al.*, 1999). The nutrients that enhanced the antagonist activity were subsequently incorporated into a new powder and liquid formulation (Personal communication, Prof P.L. Steyn, Stimuplant CC, 2003). Previously, Korsten & Cook (1996) found that the best growth temperature for *B. subtilis*, biocontrol agent of avocado fruit disease, was between 30 and 37 ºC. However, after treatment of avocado fruit in the packinghouse, fruit are immediately placed in cold storage at 5.5 to 7 ºC (Personal communication, Derek Donkin, South African Avocado Growers' Association, South Africa, 2004). This may also have contributed to the lowered efficacy of Avogreen® in the trial.

In this study, the effect of specific nutrients and temperatures on *in vitro* antagonism of *B. subtilis* against stem-end rot (*Phomopsis perseae* Zerova, *Dothiorella aromatica* (Sacc.) Petrak & Sydow and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.) and anthracnose (*C. gloeosporioides*) pathogens were investigated.

3. MATERIALS AND METHODS

3.1. Isolates

Bacillus subtilis subculture MI-14 (Chapter 3) was used throughout this study. Stock cultures of the antagonist were stored in 30 % glycerol with Ringer's (Merck, Johannesburg, S.A.) solution at –70 °C. Cultures were maintained on standard 1 nutrient agar (STD1) (Biolab, Merck) and plates were incubated at 25 – 28 °C for 24 h before use.

Fungal pathogens isolated and identified in Chapter 3 (*C. gloeosporioides*, *P. perseae*, *D. aromatica* and *L. theobromae*) were used throughout this study. All fungi were maintained on potato dextrose agar (PDA) (Biolab, Merck) slants as well as keeping mycelium-containing plugs in sterile water at room temperature. An agar disk (5 mm) containing the fungus was placed on PDA and incubated at 25 – 28 °C for three days prior to use.

3.2. Effect of different temperatures on the in vitro *inhibitory action of* Bacillus subtilis *on avocado fungal postharvest pathogens*

The dual culture technique described in Chapter 3 was used to compare the effect of temperature on *in vitro* activity of *B. subtilis* on avocado postharvest pathogens. The antagonist-pathogen combinations were placed on PDA and incubated at 4, 10, 15, 20, 25, 30, 37 and 42 °C. Duplicate plates were used and the experiment was repeated three times. Petridishes containing either *B. subtilis* or one of the test fungi incubated at the specified temperatures served as controls. Radial growth of the pathogens was measured after seven days' incubation at the specified temperature and recorded. The percentage inhibition was determined as described in Chapter 3. Data was analysed using the statistical program GenStat (2000). Fisher`s protected t-test least significant difference was used to test for differences between means per day. Significance was obtained at the 5 % level of significance (Snedecor & Cochran, 1980).

3.3. Effect of nutrients on the in vitro *inhibitory action of* Bacillus subtilis *on avocado fungal postharvest pathogens*

The minimum salt-based medium of Janisiewicz *et al.* (1992) was used. The medium consisted of the following: 13 mM $Na_2HPO_4.7H_2O$ (Sigma, Johannesburg, S.A.), 20.4 mM KH_2PO_4 (Saarchem, Merck), 2 % v/v Hutner's vitamin free mineral base, 1.5 % w/v bacteriological agar (Biolab, Merck) in double distilled water. The pH of the medium was adjusted to 6.8 before autoclaving. Hutner's vitamin-free mineral base consisted of the following: 52.3 mM nitrilotriacetic acid (dissolved and neutralized with KOH to pH 7), 0.0075 mM $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.4 mM $FeSO_4.7H_2O$ (all from Sigma), 58.6 mM $MgSO_4.7H_2O$ (Saarchem), 22.7 mM $CaCl_2.2H_2O$ (Fluka, Sigma-Aldrich, Johannesburg, S.A.), and 5 % v/v Metals 44. The mineral base was supplemented with 0.000001 % w/v D-(+)-biotin (Fluka), 0.0001 % w/v nicotinic acid, and 0.00005 % w/v thiamine hydrochloride (both from Sigma). The pH of the Hutner's mineral base was adjusted to 6.6 – 6.8. Metals 44 consisted of the following: 8.6 mM ethylenediaminetetraacetic acid, 1.8 mM $FeSO_4.7H_2O$, 0.09 mM $Co(NO_3)_2.6H_2O$ and 0.05 mM $Na_2B_4O_7.10H_2O$ (all from Sigma), as well as 3.8 mM $ZnSO_4.7H_2O$ (AnalaR, British Drug Houses (BDH)), 0.9 mM $MnSO_4.H_2O$ (Saarchem) and 0.2mM $CuSO_4.5H_2O$ (Pro Analysi, Merck). Precipitation was prevented by adding of a 6 N H_2SO_4 (uniLAB, Saarchem) solution. All nitrogen and carbon sources were filter sterilized and added to the medium after autoclaving. Where the effect of different carbon sources was investigated, glucose was replaced by the carbon source to be tested, added at a concentration of 10 % w/v. The following carbon sources were used: acetate, benzoate, citrate, D-gluconic acid, D-(+)-glucose, glycerol, D-(-)-lyxose, L-(-)-malic acid, D-(-)-mannitol, pectin, peptone, pyruvate, L-(-)-rhamnose, D-(-)-sorbitol, starch, D-(+)-trehalose, L-(+)-xylose (all from Sigma), and D- and L-arabinose (Merck). Ammonium chloride (NH_4Cl (Sigma)) was replaced with the following amino acids as nitrogen source at a concentration of 1 % w/v: L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cystein, L-isoleucine, L-leucine, L-methionine, L-serine, L-tyrosine, L-valine (all from Merck), L-glutamine, L-glutamic acid, L-phenylalanine, L-proline (all from Sigma), glycine (Saarchem), L-histidine (BDH), and L-lysine (Fluka). The dual culture technique described in Chapter 3 was used. The antagonist-pathogen combinations were placed on the various nutrient combinations. Duplicate plates were used and the experiment was repeated three times. Plates were maintained at 25 °C. Petridishes containing either *B. subtilis* or the fungus on its own were also incubated on the nutrient combination plates and served as controls. Radial growth of the pathogens was measured after seven days and recorded. Percentage inhibition was determined as described in Chapter 3 and statistically analysed as described for 3.2.

4. RESULTS

4.1. Effect of different temperatures on the in vitro *inhibitory activity of* Bacillus subtilis *on avocado fungal postharvest pathogens*

The *in vitro* activity of *B. subtilis* at 10, 15, 20, 25 and 30 °C is depicted in Figure 5.1 to 5.4. No pathogen growth was observed after seven days at 37 and 42 °C. The optimal radial growth temperature of *P. perseae* and *D. aromatica* was 25 °C, while the radial growth of *C. gloeosporioides* was best at 30 °C. The radial growth of *L. theobromae* was supported equally at 15, 20, 25 and 30 °C. However, Petridishes allowed only for measurements up to 40 mm and *L. theobromae* grew faster than the other fungi. In the experiment conducted at 4 °C, no bacterial growth was observed after seven days and growth was restricted at 10 °C. The best inhibition against *P. perseae* was observed at 25 °C. Inhibition of *C. gloeosporioides* was best at 30 °C. There was no significant difference between inhibition at 15, 20, 25 and 30 °C against *D. aromatica*. Antagonism against *L. theobromae* was greatest at 15 and 20 °C after seven days' incubation. In general, 15 °C supported the best inhibition of all pathogens.

4.2. Effect of nutrients on the in vitro *inhibitory activity of* Bacillus subtilis *on avocado fungal postharvest pathogens*

Antagonism of *B. subtilis* against *P. perseae* was most effective when evaluated on the following amino acid base mediums: L-arginine, L-(+)-asparagine, L-cysteine, L-glutamic acid, L-glutamine, L-methionine and L-serine (Table 5.1; Figure 5.5). However, of these, L-methionine supported the growth of *P. perseae* most effectively. Growth of *P. perseae* was the least on L-aspartic acid and L-(+)-glutamic acid. The amino acids L-isoleucine and L-lysine did not support antagonism at all. Of the carbon sources tested, acetate, D-(-)- and L-(-)-arabinose, citrate, glycerol, peptone, pectin and D-(+)-trehalose supported antagonism most effectively (Table 5.2; Figure 5.6). However, pathogen growth was supported by L-(-)-arabinose, glycerol, peptone and D-(+)-trehalose, while the least growth was observed on D-(-)-arabinose, citrate, D-(+)-glucose and D-(+)-mannitol. Antagonism was not supported by benzoate, D-gluconic acid, pyruvate, L-(-)-malic acid, L-(-)-rhamnose and L-(+)-xylose.

All the amino acids evaluated supported antagonism of *B. subtilis* against *C. gloeosporioides*, except for L-cysteine, L-isoleucine and L-lysine (Figure 5.7; Table 5.1). The inorganic nitrogen source, NH_4Cl, supported antagonism most effectively. Amino acids that did not support radial growth of *C. gloeosporioides* represented L-alanine, L-(+)-asparagine, L-aspartic acid, L-arginine, L-cysteine, L-glutamic acid, L-

glutamine, L-isoleucine, L-lysine and L-methionine. Antagonism was most effective on D-(+)-glucose and D-(+)-trehalose carbon base medium and to a lesser extent L-(-)-arabinose, glycerol, D-(+)-mannitol, pectin, peptone, pyruvate, D-(-)-sorbitol and starch (Figure 5.8; Table 5.2). Antagonism was not evident on citrate-amended medium. Peptone and pectin supported the growth of the pathogen most effectively, while acetate, D-(-)- and L-(-)-arabinose, benzoate, citrate, D-gluconic acid, L-(-)-malic acid, D-(+)-mannitol, pyruvate, D-(-)-sorbitol, starch and D-(+)-trehalose did not.

The amino acid bases that supported *in vitro* antagonism of *D. aromatica* by *B. subtilis* most effectively was NH_4Cl, L-alanine, L-arginine, L-(+)-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid and L-proline (Figure 5.9; Table 5.1). However, radial growth of *D. aromatica* was supported by all amino acids, with L-aspartic acid, L-cysteine, glycine and L-lysine being the most effective. The highest percentage inhibition was measured on D-(+)-glucose, D-(+)-mannitol, pectin, peptone, pyruvate, D-(-)-sorbitol, starch and D-(+)-trehalose as carbon source (Figure 5.10; Table 5.2). Of these, peptone, pyruvate and D-(+)-mannitol did not support radial growth of the pathogen effectively.

The most effective nitrogen sources for supporting antagonism of *B. subtilis* against the four pathogens are listed in Table 5.1. For each pathogen, the sources supporting the growth of the other three pathogens were marked. The remaining sources are those that do not support growth of any of the pathogens. The nitrogen sources that support antagonism of all four pathogens without enhancing growth of any of the pathogens tested, are L-(+)-asparagine, L-glutamic acid and L-glutamine. Table 5.2 lists all carbon sources that supported high levels of inhibitory activity of *B. subtilis* against the four pathogens tested. Antagonism against both *P. perseae* and *L. theobromae* were effectively supported by D-(-)-arabinose and citrate while they were not as conductive to antagonism against *C. gloeosporioides* or *D. aromatica*. However, neither carbon source enhanced the growth of any of the pathogens tested. D-(+)-mannitol supports inhibition of *C. gloeosporioides*, *D. aromatica* and *L. theobromae* effectively, and *P. perseae* to a lesser extent. However, it does not support the growth of any of the pathogens tested.

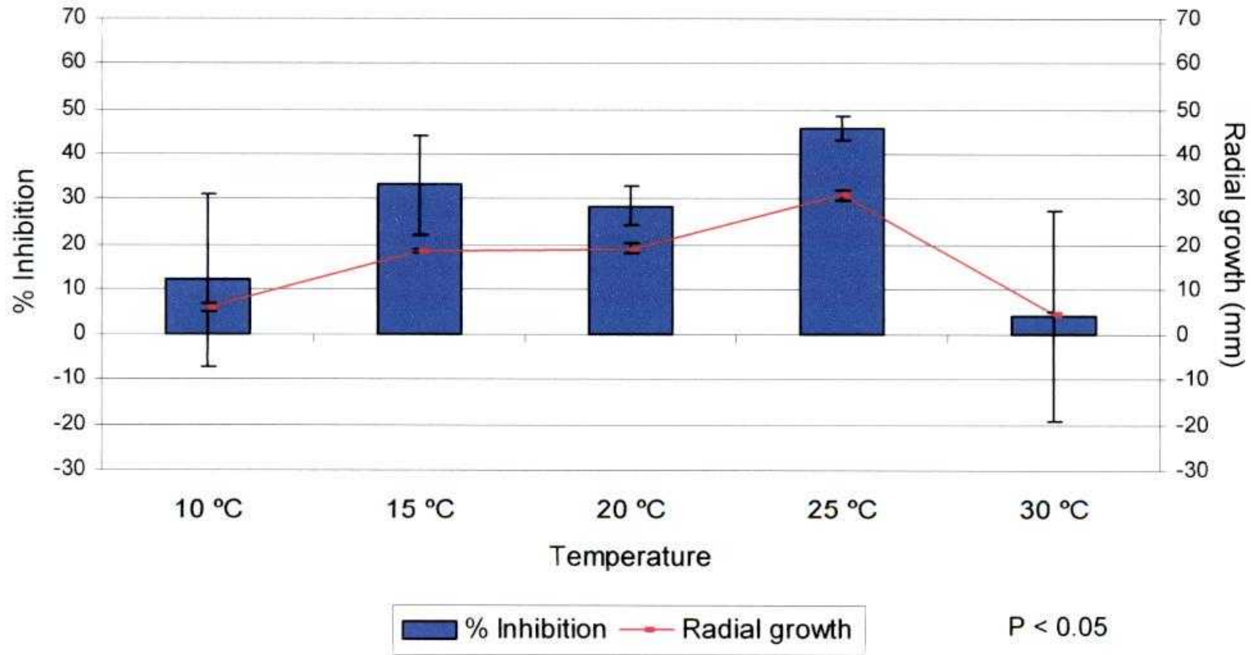


Figure 5.1: Effect of temperature on *Bacillus subtilis* antagonism of *Phomopsis perseae* at respective temperatures.

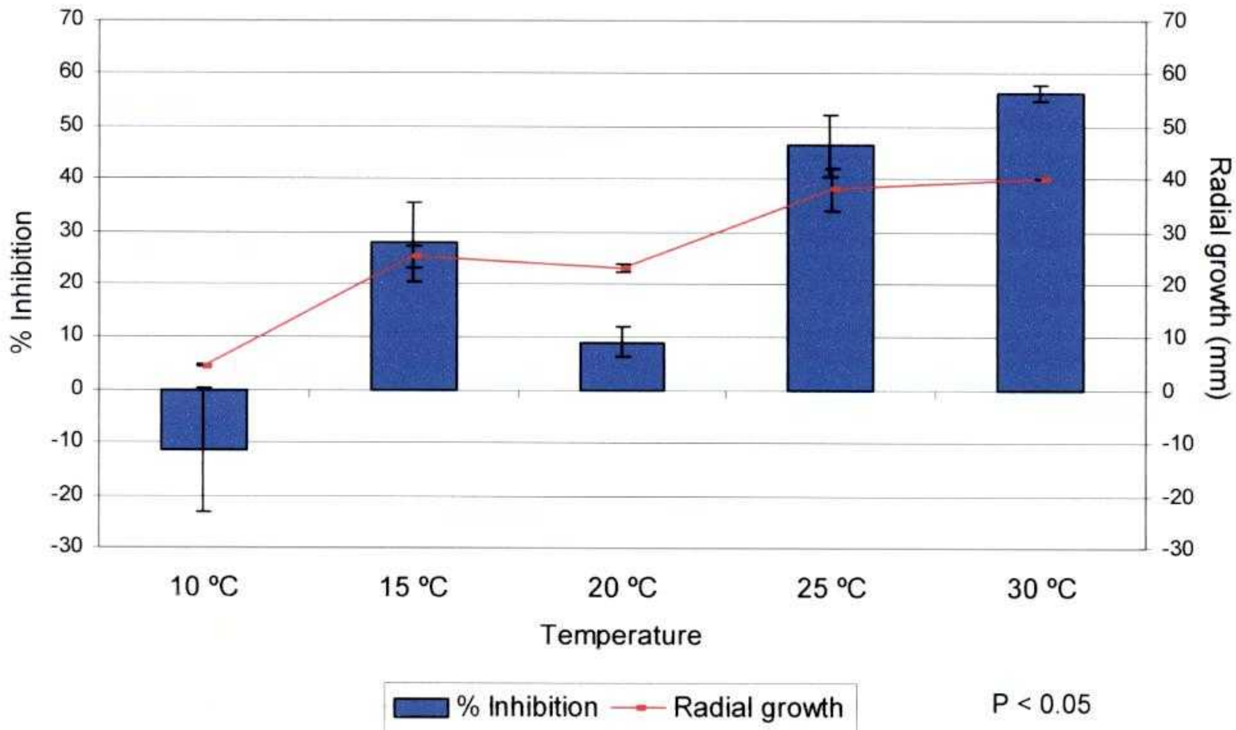


Figure 5.2: Effect of temperature on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides* at respective temperatures.

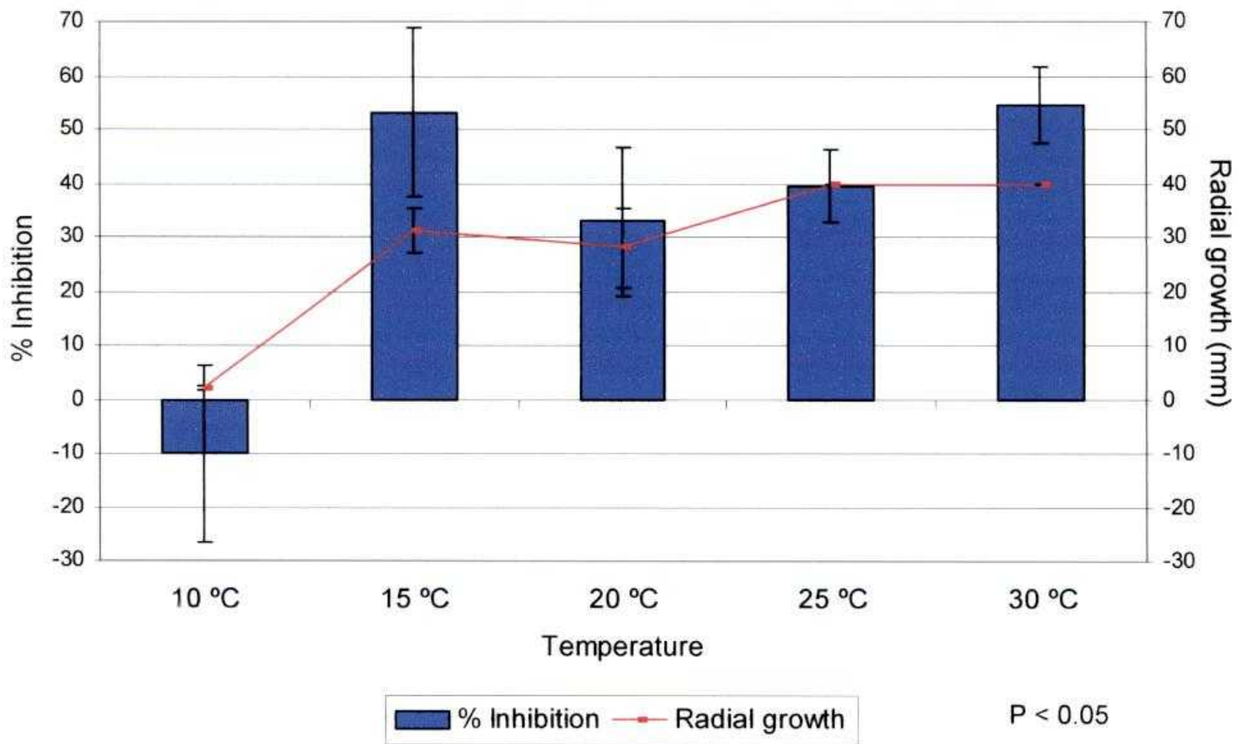


Figure 5.3: Effect of temperature on *Bacillus subtilis* antagonism of *Dothiorella aromatica* at respective temperatures.

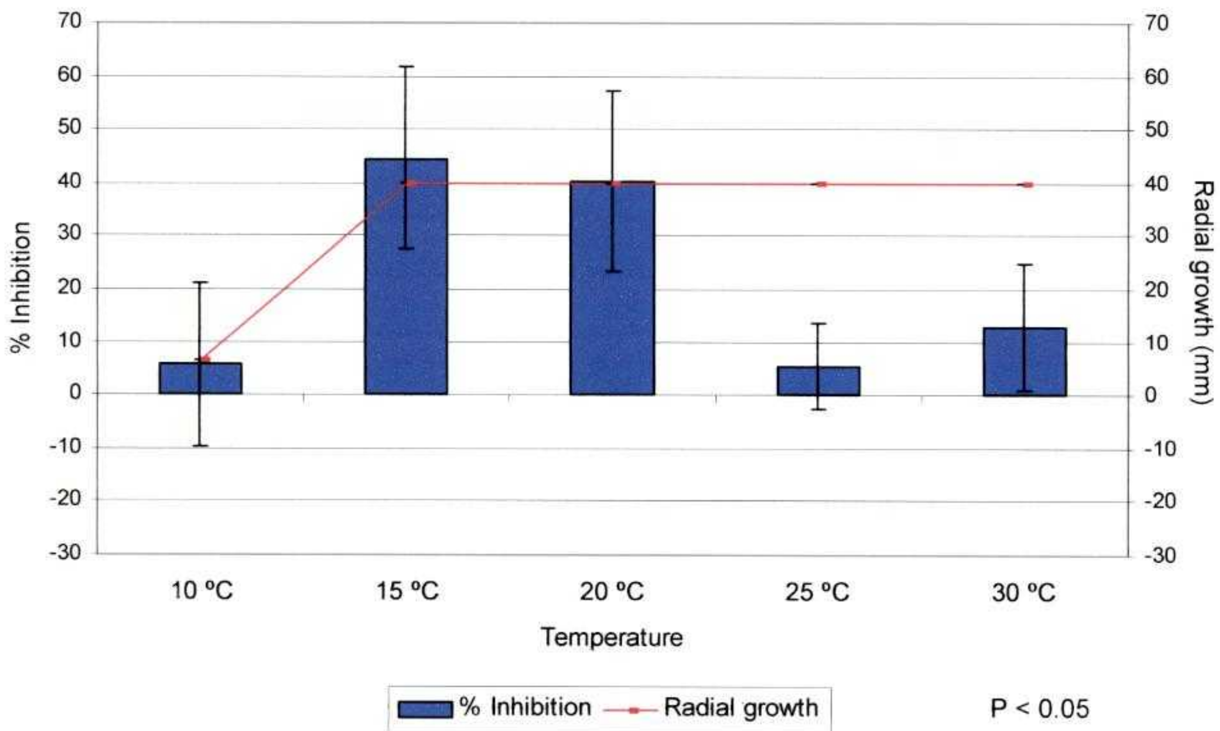


Figure 5.4: Effect of temperature on *Bacillus subtilis* antagonism of *Lasiodiplodia theobromae* at the respective temperatures.

5. DISCUSSION

In this study, prevailing temperatures and available nutrients impacted on the effectiveness of antagonism between *B. subtilis* and the fungal avocado pathogens causing stem-end rot and anthracnose. Temperatures negatively affected *in vitro* antagonism of *B. subtilis* against a range of fungal avocado pathogens. Most of the fungal pathogens tested, were able to grow at temperatures of 10 to 15 ºC. Of the pathogens evaluated, *P. perseae* grew the slowest and the least uniform, making radial growth measurement difficult and resulting in increased variability. The least variability was observed on day seven. Since *L. theobromae* was fast growing, less significant differences were observed. At 10 ºC, little or no antagonistic activity was observed between *B. subtilis* and the avocado pathogens. The antagonist was found to be unable to effectively inhibit radial growth of the various fungal pathogens tested at low temperatures. However, at higher temperatures, inhibition increased. In a previous study, higher culturing temperatures (30 and 37 ºC) increased the growth and subsequent yield of cell mass of *B. subtilis* (Korsten & Cook, 1996).

The previously reported variability in efficiency of *B. subtilis* in postharvest studies may be attributed to temperature under commercial conditions. In this study, the antagonist worked most effectively at 30 to 37 ºC *in vitro*. Picked avocado fruit take around two hours to reach the packinghouse (Personal communication, Derek Donkin, South African Avocado Growers' Association, South Africa, 2004). After fruit pass through the packinghouse, they are stored at between 5.5 to 7 ºC for up to 48 h (Personal communication, Derek Donkin, South African Avocado Growers' Association, 2004). Fruit are containerized or placed in a cool truck for transport to the ports for export, or local markets, and are kept at between 5.5 and 7.5 ºC, depending on the moisture content of the fruit (Personal communication, Derek Donkin, South African Avocado Growers' Association, 2004). From packing to arrival at the European market can take on average 27 d (Personal communication, Derek Donkin, South African Avocado Growers' Association, 2004). The temperatures at which fruit are maintained after packing is not optimal for the *in vitro* activity of *B. subtilis* and can contribute to its variable effectiveness. Upadhyay *et al.* (1991) found that by increasing the temperature from 18 to 37 ºC antagonism of *Pseudomonas cepacia* against *Trichoderma viride* Pers could be improved. Postharvest heat treatment of oranges was found to enhance the efficacy of *Pseudomonas glathei* against *P. digitatum* (Pers.) Sacc. (Huang *et al.*, 1995). After treatment with the antagonist, the fruit were stored at 30 ºC for 24 hours, stimulating the multiplication of the antagonist and delaying pathogen spore germination. Incubation of fruit at 20 to 30 ºC might also increase the efficacy of *B. subtilis* against postharvest diseases on avocado. However, in this study the pathogens were found to grow well at these temperatures and this might neutralize the possible advantage in increased antagonism. The effect of temperature on the shelf life of the

fruit must also be taken into consideration. By choosing antagonists that are active at the prevailing conditions when infection occurs, can enhance biocontrol efficacy (Wilson & Pusey, 1985; Mari & Guizzardi, 1998). Postharvest diseases of apple, caused by *Penicillium expansum* Link., were effectively controlled at 5, 10 and 20 °C (Sholberg *et al.*, 1995). These *B. subtilis* isolates originated from apples stored for six to seven months at 1 °C. Leibinger *et al.* (1997) found that *B. subtilis* applied preharvestly survived well in the field, but population size decreased after storage at 2 °C. Temperature was also found to affect the modes of action involved in biocontrol (Gupta & Utkhede, 1986; Fiddaman & Rossall, 1993; Ohno *et al.*, 1995; Graumann *et al.*, 1997).

In the current study, various nutrients were found to enhance the *in vitro* antagonistic efficacy against avocado stem-end rot and anthracnose pathogens. In *in vitro* studies, medium with citric acid, D-(+)-galactose, pyruvate and benzoate incorporated into it was found to provide a basis for sustained inhibition by *B. subtilis* against *C. gloeosporioides* (Havenga *et al.*, 1999). The formation, size and sustainability of inhibition zones were used as criteria to study the impact of nutrient sources on biocontrol, and not percentage inhibition of the pathogen radial growth as was used in other studies (Skidmore & Dickinson, 1976; Whipps, 1987; Upadhyay & Rai, 1987). In this study, the enhancing effect of pyruvate and the amino acids L-(+)-asparagine and L-aspartic acid on *B. subtilis* antagonism against *C. gloeosporioides*, as well as the low effectiveness of starch to increase antagonism was confirmed (Havenga *et al.*, 1999). Various studies showed the effective enhancement of biocontrol by adding nutrients (Dik, 1991; Upadhyay *et al.*, 1991; Janisiewicz *et al.*, 1992; Janisiewicz & Bors, 1995; Manjula & Podile, 2001). The mode of action of a biocontrol agent can also be affected by nutrients (Wisniewski *et al.*, 1991; Slininger & Jackson, 1992; Krebs *et al.*, 1993; Fiddaman & Rossall, 1994; Milner *et al.*, 1995; Duffy & Défago, 1999).

Nutrients incorporated into a commercial product should enhance the antagonist activity without supporting pathogen growth (Janisiewicz *et al.*, 1992; Havenga *et al.*, 1999). From the current study it was found that the best amino acids to incorporate into a commercial formulation against the tested pathogens would be L-glutamic acid, L-glutamine and L-(+)-asparagine. Ammonium chloride, L-cysteine and L-arginine also supported antagonism without increasing the growth of most of the tested fungal pathogens. It was further found that carbon sources most effective for enhancing antagonistic potential of the biocontrol product are D-arabinose and D-(+)-mannitol. Citrate is also a good alternative, but it did not sustain antagonism against *C. gloeosporioides*. However, in the previous study (Havenga *et al.*, 1999) it did support the production of an inhibition zone.

In the initial Avogreen® formulation, a rich growth medium was used in the fermentation process to obtain high total counts. However, it was modified after Korsten *et al.* (1998) found that the incidence of postharvest decay increased when Avogreen® was used in postharvest dips compared with the control treatment. Currently, the liquid and powder Avogreen® formulations consists of a minimal medium containing tri-ammonium citrate and L-aspartic acid as carbon and nitrogen sources respectively (Personal communication, Prof P.L. Steyn, Stimuplant CC, Pretoria, South Africa). The use of specific nutrients in the formulation of a commercial product is supported by the results obtained in this study. Fuchs *et al.* (2000) found that the medium in which an antagonist is grown affect its subsequent activity. A less-rich growth media helped *Pseudomonas* sp. Pf153 to protect cucumber roots to a greater extent than when grown on a rich media. A formulation of *P. fluorescens* F113 controlled damping-off of sugarbeet, but was less-effective when nutrients were added (Moënne-Loccoz *et al.*, 1999). These findings support the use of a minimal medium. Both the powder and liquid formulations have a shelf life of at least one year (Studies done by W. Havenga at Stimuplant CC, Pretoria, South Africa). The consistent efficacy of the product under various preharvest commercial conditions is currently still under investigation but preliminary results indicate the product is effective (Madel van Eeden, personal communication, University of Pretoria).

In conclusion, this study highlighted the importance of temperatures and nutrients on the efficacy of *B. subtilis*. Antagonists must be able to not only survive, but also flourish in the environment where they are applied. The antagonist, *B. subtilis* is most effective at temperatures of 15 °C or higher. In order for antagonists to be successful, the formulation in which it is applied must be optimised. The biocontrol agent formulation must be active against all tested pathogens, as well as being affordable and practical. Since there is no single carbon and nitrogen source that will ensure optimal antagonist efficacy, while maintaining minimal growth of all the pathogens, a compromise must be made in the choice of ingredients. A minimal medium with specific carbon and nitrogen sources that will not support the growth of the target pathogens, but provide the required nutrition for optimal antagonist activity would be the preferred choice. Future studies should focus on the efficacy of the formulated product Avogreen® under commercial conditions both in pre- and postharvest applications.

6. REFERENCES

Blakeman, J.P. 1985. Ecological succession of leaf surface micro organisms in relation to biological control. Pages 6 – 30 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 5.1: Nitrogen sources supporting high levels of antagonism against *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae*

Phomopsis perseae	*Colletotrichum gloeosporioides*	*Dothiorella aromatica*	*Lasiodiplodia theobromae*
L-arginine	NH_4Cl	NH_4Cl	NH_4Cl
L-cysteine[D]	L-valine[PC]	L-glutamic acid°	L-aspartic acid[D]
L-(+)-asparagine°	L-tyrosine[C]	L-alanine	L-cysteine[D]
L-glutamic acid°	L-histidine[C]	L-(+)-asparagine°	L-glutamic acid°
L-glutamine°	L-arginine	L-arginine	L-lysine[DL]
L-serine[C]	L-glutamic acid°	L-proline[PC]	L-valine[PC]
	L-(+)-asparagine°	L-cysteine[D]	L-glutamine°
	L-glutamine°	L-aspartic acid[D]	L-(+)-asparagine°
	L-alanine	L-lysine[DL]	L-proline[PC]
	L-proline[PC]	Glycine[CD]	L-histidine[C]
	L-serine[C]	L-glutamine°	L-isoleucine
	L-aspartic acid[D]		
	Glycine[CD]		
	L-phenylalanine[PC]		

[P] - Stimulated *P. perseae* growth
[C] - Stimulated *C. gloeosporioides* growth
[D] - Decreased *D. aromatica* growth
[L] - Decreased *L. theobromae* growth
° - Source does not support pathogen growth and supports antagonism against all four pathogens tested

Table 5.2: Carbon sources supporting high levels of antagonism against *Phomopsis perseae*, *Colletotrichum gloeosporioides*, *Dothiorella aromatica* and *Lasiodiplodia theobromae*

Phomopsis perseae	*Colletotrichum gloeosporioides*	*Dothiorella aromatica*	*Lasiodiplodia theobromae*
L-(-)-arabinose[P]	D-(+)-glucose[CDL]	D-(+)-trehalose[PDL]	D-(+)-mannitol°
Acetate[DL]	D-(+)-trehalose[PDL]	D-(+)-glucose[CDL]	D-gluconic acid[L]
Glycerol[PC]	Glycerol[PC]	D-(-)-sorbitol[PDL]	D-(+)-trehalose[PDL]
D-(+)-trehalose[PDL]	L-(-)-arabinose[P]	D-(+)-mannitol°	L-(-)-arabinose[P]
Peptone[PCL]	Pectin[CDL]	Pyruvate[L]	Peptone[PCL]
Pectin[CDL]	Pyruvate[L]	Starch[DL]	Citrate°
D-(-)-arabinose°	D-(+)-mannitol°	Peptone[PCL]	D-(-)-sorbitol[PDL]
Citrate°	Peptone[PCL]	Pectin[CDL]	Pectin[CDL]
D-(+)-glucose[CDL]	D-(-)-sorbitol[PDL]	D-gluconic acid[L]	Glycerol[PC]
D-(-)-sorbitol[PDL]	Starch[DL]	L-(-)-arabinose[P]	Starch[DL]
			D-(+)-glucose[CDL]
			Pyruvate[L]
			Acetate[DL]
			D-(-)-arabinose°

[P] - Stimulated *P. perseae* growth
[C] - Stimulated *C. gloeosporioides* growth
[D] - Stimulated *D. aromatica* growth
[L] - Stimulated *L. theobromae* growth
° - Source does not support pathogen growth, supporting high levels of antagonism against some pathogens

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

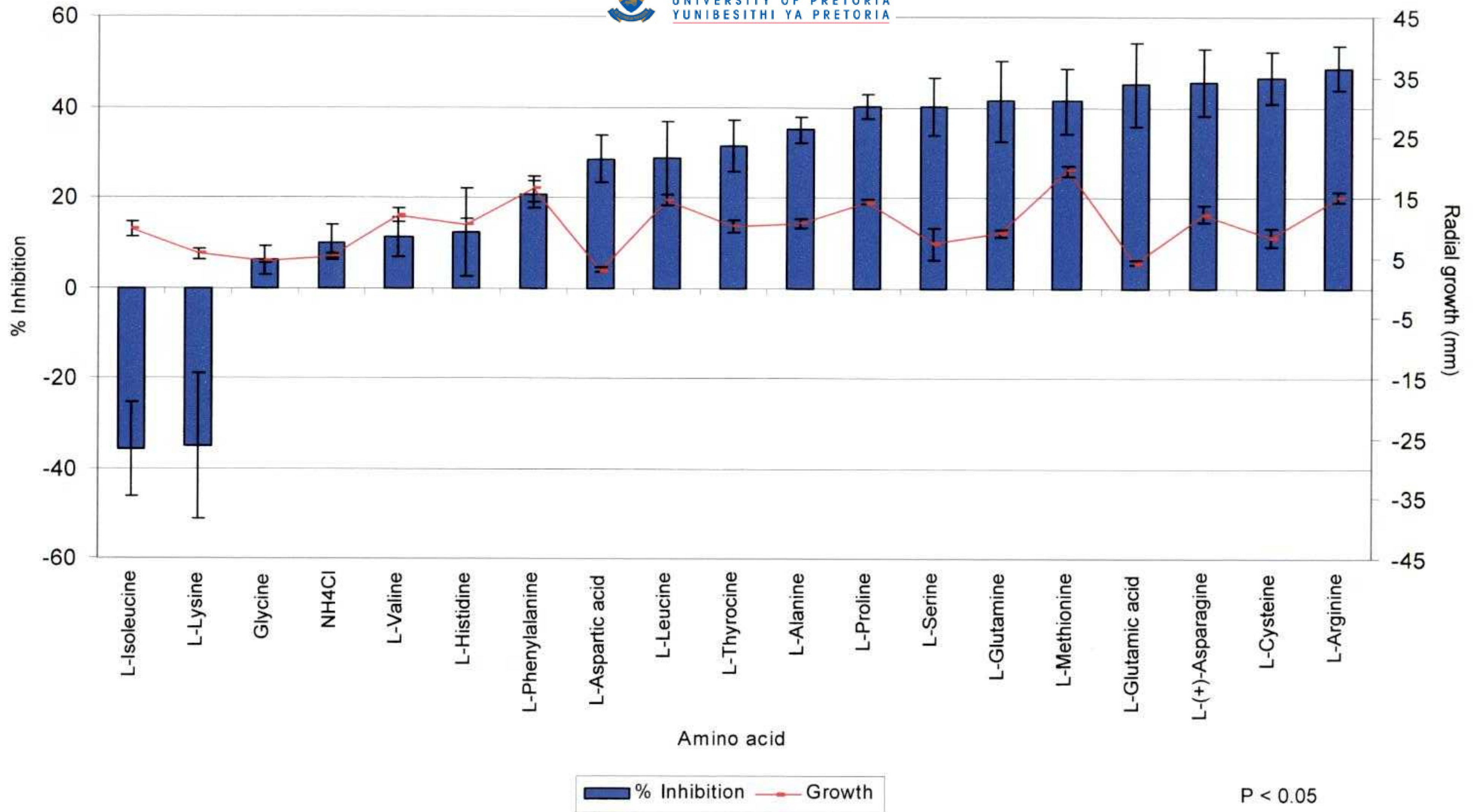


Figure 5.5: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Phomopsis perseae*.

% Inhibition

60
40
20
0
-20
-40
-60

Radial growth (mm)

45
35
25
15
5
-5
-15
-25
-35
-45

Benzoate
L-(+)-Xylose
L-(-)-Malic acid
Pyruvate
L-(-)-Rhamnose
D-Gluconic acid
Starch
D-(+)-Mannitol
D-(-)-Lyxose
D-(-)-Sorbitol
D-(+)-Glucose
Citrate
D-(-)-Arabinose
Pectin
Peptone
D-(+)-Trehalose
Glycerol
Acetate
L-(-)-Arabinose

Carbon source

% Inhibition
Growth

P < 0.05

Figure 5.6: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Phomopsis perseae*.

% Inhibition: 60, 40, 20, 0, -20, -40, -60

Radial growth (mm): 45, 35, 25, 15, 5, -5, -15, -25, -35, -45

L-Cysteine, L-Lysine, L-Isoleucine, L-Leucine, L-Methionine, L-Phenylalanine, Glycine, L-Aspartic acid, L-Serine, L-Proline, L-Alanine, L-Glutamine, L-(+)-Asparagine, L-Glutamic acid, L-Arginine, L-Histidine, L-Thyrocine, L-Valine, NH4Cl

Amino acid

% Inhibition — Growth

P < 0.05

Figure 5.7: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides*.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

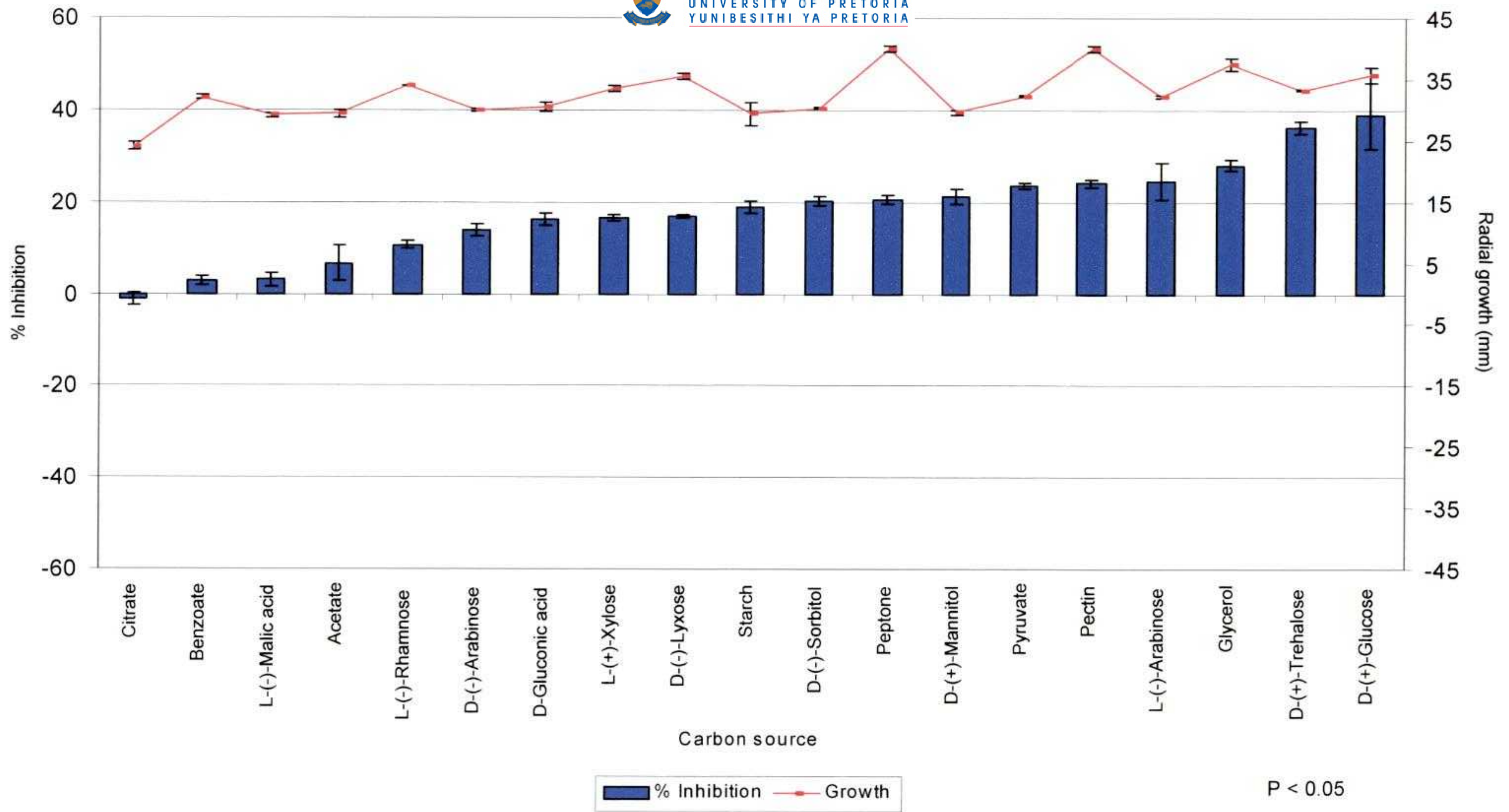


Figure 5.8: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Colletotrichum gloeosporioides*.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

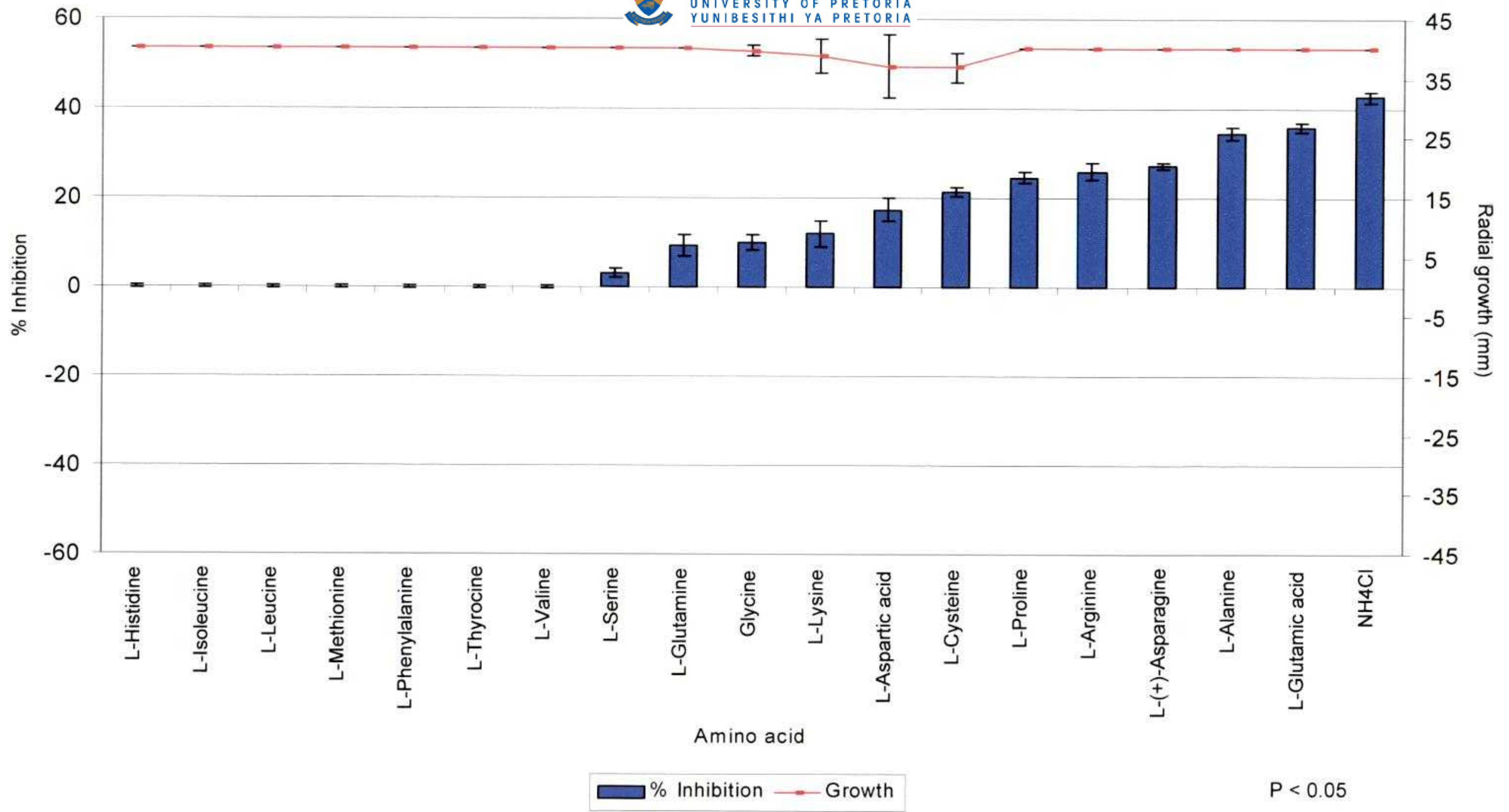


Figure 5.9: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Dothiorella aromatica*.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

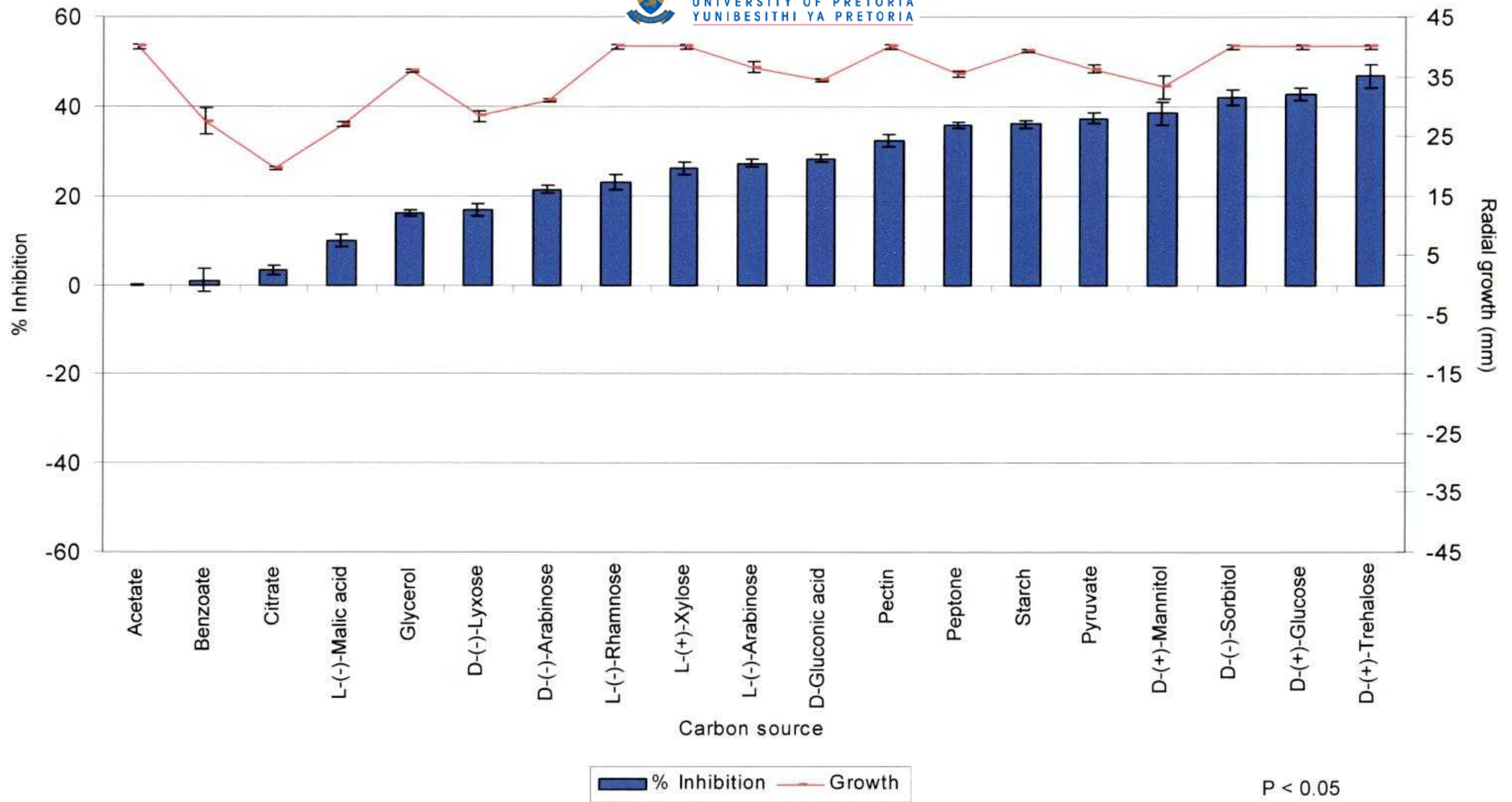


Figure 5.10: Effect of different carbon sources on *Bacillus subtilis* antagonism of *Dothiorella aromatica*.

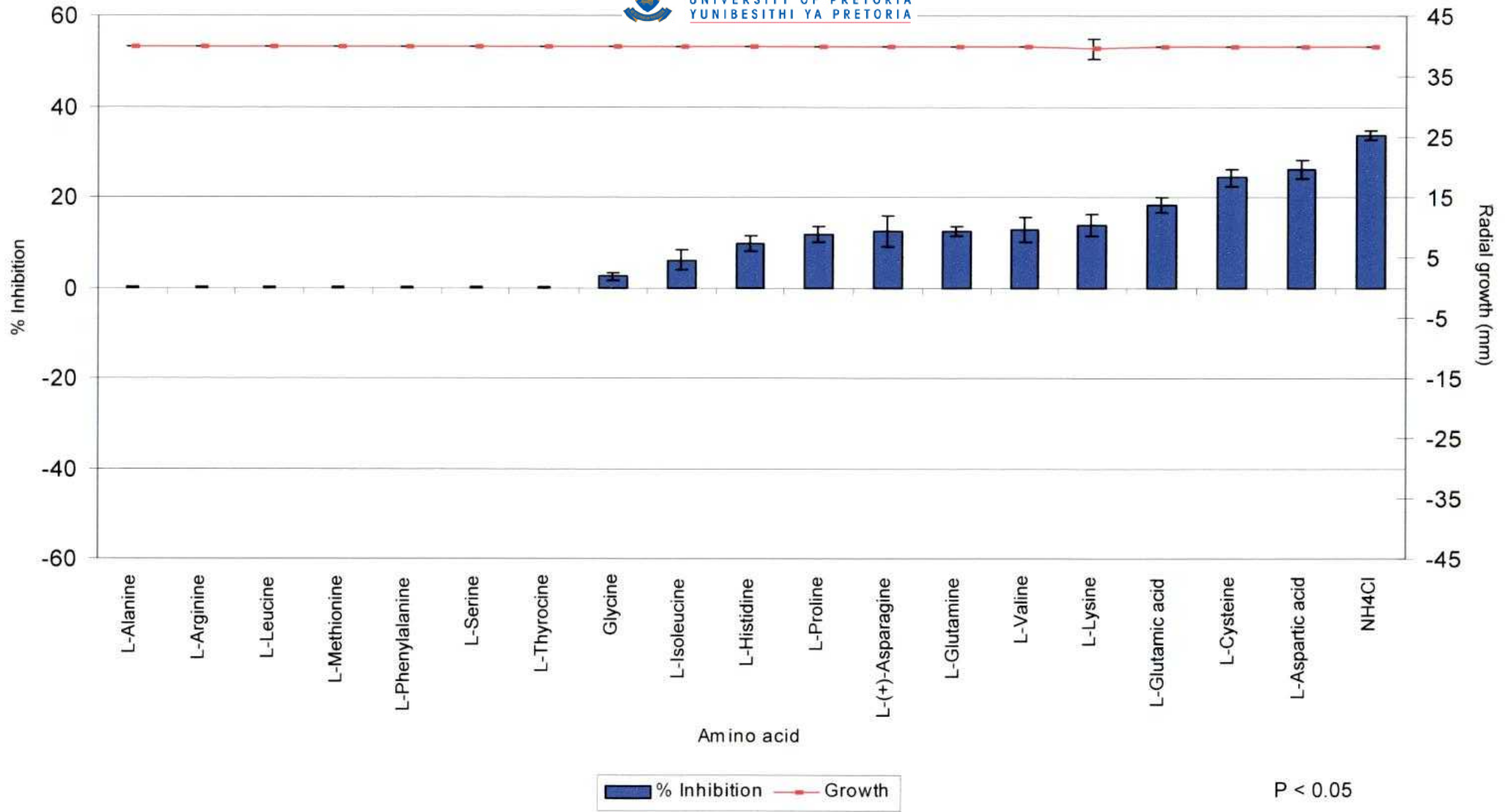


Figure 5.11: Effect of different amino acids and ammonium chloride on *Bacillus subtilis* antagonism of *Lasiodiplodia theobromae*.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

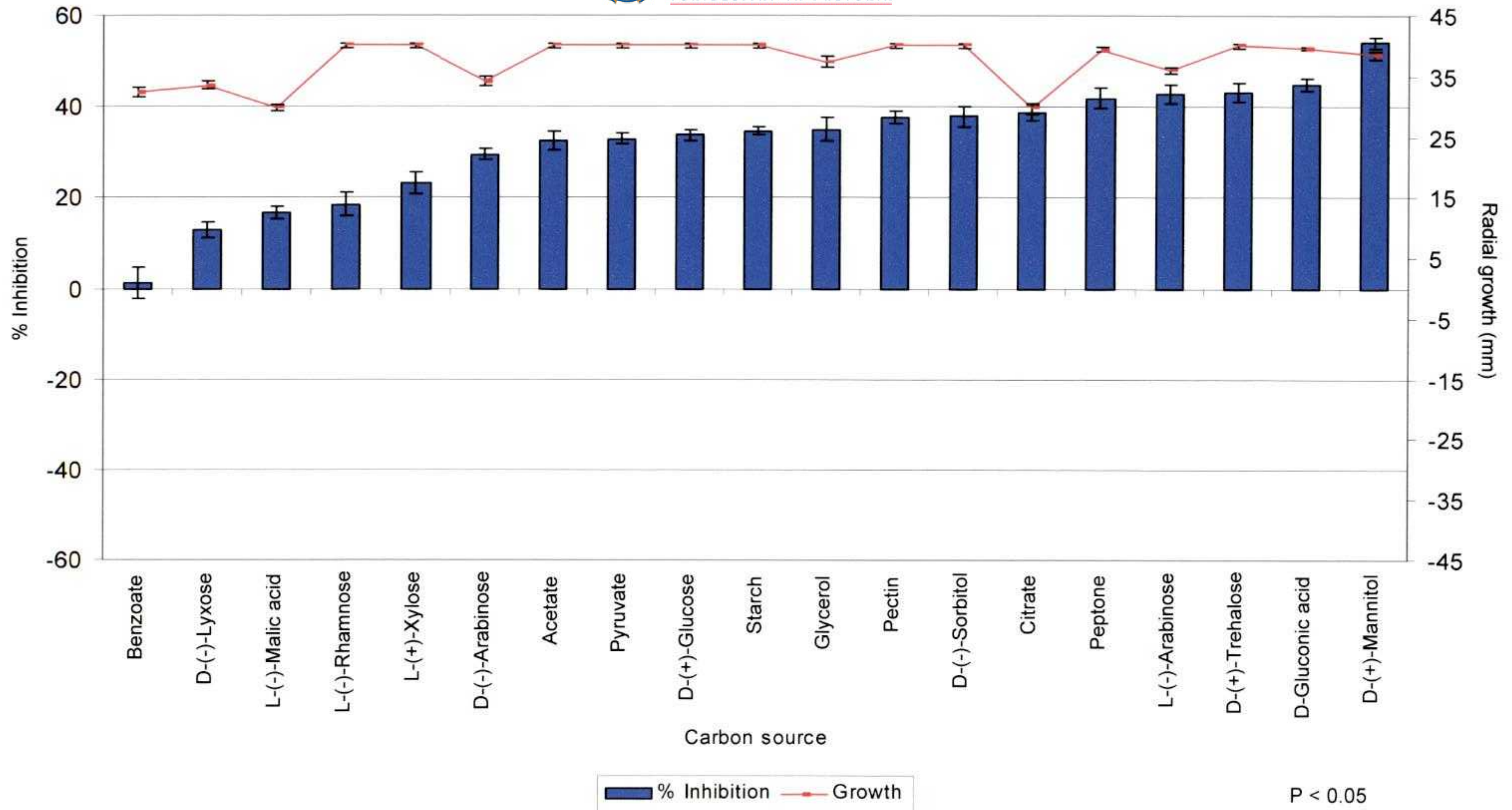


Figure 5.12: Effect of different carbon sources on *Bacillus subtilis* antagonism of against *Lasiodiplodia theobromae*.

Blakeman, J.P. & Brodie, I.D.S. 1977. Competition for nutrients between the epiphytic micro organisms and germination of spores of plant pathogens on beetroot leaves. Physiological Plant Pathology 10: 29 – 42.

Brodie, I.D.S. & Blakeman, J.P. 1976. Competition for exogenous substrates *in vitro* by leaf surface micro-organisms and germination of conidia of *Botrytis cinerea*. Physiological Plant Pathology 9: 227 – 239.

Dik, A.J. 1991. Interactions among fungicides, pathogens, yeasts, and nutrients in the phyllosphere. Pages 412 – 429 In: Microbial Ecology of Leaves. Andrews, J.H. & Hirano, S.S. (Eds). Springer-Verlag, New York.

Duffy, B.K. & Défago, G. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Applied and Environmental Microbiology 65: 2429 – 2438.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Fiddaman, P.J. & Rossall, S. 1994. Effect of substrate on the production of antifungal volatiles form *Bacillus subtilis*. Journal of Applied Bacteriology 76: 395 – 405.

Fuchs, J.-G., Moënne-Loccoz, Y. & Défago, G. 2000. The laboratory medium used to grow biocontrol *Pseudomonas* sp. Pf153 influences its subsequent ability to protect cucumber from black root rot. Soil Biology and Biochemistry 32: 421 – 424.

GenStat for Windows. 2000. Release 4.2. Fifth Edition. Oxford: VSN International.

Graumann, P., Wendrich, T.M., Webber, M.H.W., Schröder, K. & Marahiel, M.A. 1997. A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. Molecular Microbiology 25: 741 – 756.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. & Cutler, H.G. 1988. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. Journal of Agricultural and Food Chemistry 36: 366 – 370.

Gupta, V.K. & Utkhede, R.S. 1986. Factors affecting the production of antifungal compounds by *Enterobacter aerogenes* and *Bacillus subtilis*, antagonists of *Phytophthora cactorum*. Journal of Phytopathology 117: 9 – 16.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Hase, C., Mascher, F., Moënne-Loccoz, Y. & Défago, G. 1999. Nutrient deprivation and the subsequent survival of biocontrol *Pseudomonas fluorescens* CHA0 in soil. Soil Biology and Biochemistry 31: 1181 – 1188.

Huang, Y., Deverall, B.J. & Morris, S.C. 1995. Postharvest control of green mould on oranges by a strain of *Pseudomonas glathei* and enhancement of its biocontrol by heat treatment. Postharvest Biology and Technology 5: 129 – 137.

Ippolito, A. & Nigro, F. 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruit and vegetables. Crop Protection 19: 715 – 723.

Janisiewicz, W.J. & Bors, B. 1995. Development of a microbial community of bacterial and yeast antagonists to control wound-invading postharvest pathogens of fruits. Applied and Environmental Microbiology 61: 3261 – 3267.

Janisiewicz, W.J., Usall, J. & Bors, B. 1992. Nutritional enhancement of biocontrol of blue mold on apples. Phytopathology 82: 1364 – 1370.

Knox, O.G.G., Killham, K. & Leifert, C. 2000. Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*. Applied Soil Ecology 15: 227 – 231.

Korsten, L. & Cook, N. 1996. Optimizing culturing conditions for *Bacillus subtilis*. South African Avocado Growers' Association Yearbook 19: 54 – 58.

Korsten, L., Towsen, E. & Claasens, V. 1998. Evaluation of Avogreen as post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. South African Avocado Growers' Association Yearbook 21: 83 – 87.

Krebs, B., Junge, H., Ockhardt, A., Höding, B., Heubner, D. & Erben, U. 1993. *Bacillus subtilis* – an effective biocontrol agent. Pesticide Science 37: 427 – 433.

Leibinger, W., Breuker, B., Hahn, M. & Mendgen, K. 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. Phytopathology 87: 1103 – 1110.

Manjula, K. & Podile, A.R. 2001. Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. Canadian Journal of Microbiology 47: 618 – 625.

Mari, M. & Guizzardi, M. 1998. The postharvest phase: emerging technologies for the control of fungal diseases. Phytoparasitica 26: 59 – 66.

Milner, J.L., Raffel, S.J., Lethbridge, B.J. & Handelsman, J. 1995. Culture conditions that influence accumulation of zwittermicin A by *Bacillus cereus* UW85. Applied Microbiology and Biotechnology: 43: 685 – 691.

Moënne-Loccoz, Y., Naughton, M., Higgins, P., Powell, J., O'Connor, B. & O'Gara, F. 1999. Effect of inoculum preparation and formulation on survival and biocontrol efficacy of *Pseudomonas fluorescens* F113. Journal of Applied Microbiology 86: 108 – 116.

Morris, C.E. & Rouse, D.I. 1985. Role of nutrients in regulating epiphytic bacterial populations. Pages 63 – 82 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Ohno, A., Ano, T. & Shoda, M. 1995. Effect of temperature on production of lipopeptide antibiotics, Iturin A and Surfactin by a dual producer, *Bacillus subtilis* RB14, in solid-state fermentation. Journal of Fermentation and Bioengineering 80: 517 – 519.

Sholberg, P.L.., Marchi, A. & Bechard, J. 1995. Biocontrol of postharvest diseases of apple using *Bacillus* spp. isolated from stored apples. Canadian Journal of Microbiology 41: 247 – 252.

Skidmore, A.M. & Dickinson, C.H. 1976. Colony interaction and hyphal interference between *Septoria nodorum* and phylloplane fungi. Transactions of the British Mycological Society 66: 57 – 64.

Slininger, P.J. & Jackson, M.A. 1992. Nutritional factors regulating growth and accumulation of phenazine 1-carboxylic acid by *Pseudomonas fluorescens* 2-79. Applied Microbiology and Biotechnology 37: 388 – 92.

Snedecor, G.W. & Cochran, W.G. 1980. Statistical Methods. 7th Edition. Iowa State University Press. Ames, Iowa.

Spadaro, D. & Gullino, M.L. 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. International Journal of Food Microbiology 91: 185 – 194.

Upadhyay, R.S. & Rai, B. 1987. Studies on antagonism between *Fusarium udum* Butler and root region microflora of pigeon pea. Plant and Soil 101: 79 – 93.

Upadhyay, R.S., Visintin, L. & Jayaswal, R.K. 1991. Environmental factors affecting the antagonism of *Pseudomonas cepacia* against *Trichoderma viride*. Canadian Journal of Microbiology 37: 880 – 884.

Van Dyk, K., de Villiers, E.E. & Korsten, L. 1997. Alternative control of avocado post-harvest diseases. South African Avocado Growers' Association Yearbook 20: 109 – 112.

Whipps, J.M. 1987. Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. The New Phytologist 107: 127 – 142.

Wilson, C.L. & Pusey, P.L. 1985. Potential for biological control of postharvest plant diseases. Plant Disease 69: 375 – 378.

Wisniewski, M.E., Biles, C., Droby, S., McLaughlin, R., Wilson, C.L. & Chlautz, E. 1991. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*: I. Characterization of attachment to *Botrytis cinerea*. Physiological and Molecular Plant Pathology 39: 245 – 258.

CHAPTER 6

PRODUCTION AND PARTIAL CHARACTERISATION OF INHIBITORY METABOLITES PRODUCED BY *BACILLUS SUBTILIS*

1. ABSTRACT

The mode of action of *Bacillus subtilis* as biocontrol agent is often ascribed to the production of inhibitory secondary metabolites. Some of these metabolites reportedly have a wide spectrum of antifungal activity. Fungal pathogens causing postharvest avocado diseases are effectively controlled under commercial conditions using a strain of *B. subtilis*. Inhibitory substances produced *in vitro* pointed to antibiosis as one of the modes of action involved in the interaction. The *in vitro* production of inhibitory metabolites by *B. subtilis* was evaluated over time to determine the optimal time for extracting large quantities of the metabolite. A high concentration of inhibitory substances was present in the cell free filtrate of *B. subtilis* growth media by day seven. The possibility that the inhibitory substance may be a phenolic compound was investigated. Antifungal compounds, inhibitory to *Cladosporium cladosporioides*, were found in the free acid fraction of *B. subtilis* growth medium. The fraction was analysed using thin layer chromatography and high performance liquid chromatography and results indicate that the phenolic compounds produced belong to the hydroxycinamic family of compounds.

2. INTRODUCTION

Using *Bacillus subtilis* as a biocontrol agent against fungal plant pathogens has been investigated by numerous researchers (Loeffler *et al.*, 1986; Pusey *et al.*, 1986; Kugler *et al.*, 1990; Fiddaman & Rossall, 1993; Korsten *et al.*, 1993; Ikediugwo *et al.*, 1994; Podile & Prakash, 1996; Sailaja *et al.*, 1997). Antibiosis is commonly employed in biocontrol by *B. subtilis* (McKeen *et al.*, 1986; Gueldner *et al.*, 1988; Leifert *et al.*, 1995). In previous studies, *B. subtilis* proved to be an effective biocontrol agent against avocado postharvest diseases (Korsten *et al.*, 1991; Korsten, 1993). A commercial product, Avogreen®, containing *B. subtilis*, was formulated and registered (Korsten *et al.*, 1998). Studies on the mode of action involved in the antagonism of *B. subtilis* against postharvest fungal pathogens of avocado provide evidence that one or more antifungal metabolites are involved (Korsten & de Jager, 1995; Havenga *et al.*, 1999).

The production of antibiotic metabolites by the specie *B. subtilis* is well documented (Chapter 2) and it is also common to find more than one antibiotic metabolite produced. Cell-free filtrates of *B. subtilis* were reportedly

used to protect fruit from *Monilinia fructicola* (Wint.) Honey (McKeen *et al.*, 1986). The inhibitory substances were isolated and several iturin peptides were identified, active against a wide variety of fungi (Gueldner *et al.*, 1988). Most research has focused on peptide and lipopeptide antibiotics (Katz & Demain, 1977; Shoji, 1978; Peypoux *et al.*, 1984; Loeffler *et al.*, 1986; Sakajoh *et al.*, 1987; Jacques *et al.*, 1994; Chen *et al.*, 1995; Klein *et al.*, 1996; Tsuge *et al.*, 1996; Lin *et al.*, 1998; Pinchuk *et al.*, 2002). Less information is available concerning non-peptide antibiotics, including phenolic compounds (Pinchuk *et al.*, 2002).

The aim of this study was to determine what inhibitory substances are produced by *B. subtilis* and if phenolic compounds are partly responsible for antagonism.

3. MATERIALS AND METHODS

3.1. Strains

Bacillus subtilis subculture MI-14 (Chapter 3) was used throughout this study. The antagonist was stored in 30 % glycerol with Ringer's (Merck, Johannesburg, S.A.) solution at –70 ºC. Cultures were maintained on standard 1 nutrient agar (STD1) (Biolab, Merck) and plates were incubated at 28 ºC for 24 h before use.

Colletotrichum gloeosporioides Penzig. (Chapter 3) and *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (M. Muller, Department of Microbiology and Plant Pathology, University of Pretoria) was maintained on potato dextrose agar (PDA) (Biolab, Merck) slants as well as in sterile water at room temperature. An agar disk containing mycelia and spores of the fungus was placed on PDA and incubated at room temperature for three days prior to use. Fungal spore suspensions were made by pipetting sterile water onto PDA plates and removing mycelia and spores with a sterile streaking rod (Chapter 4).

All solvents used in this study were supplied by uniLAB (Saarchem, Merck, Johannesburg, S.A.).

3.2. Optimising antifungal metabolite production by Bacillus subtilis

Antibiotic production medium (APM) (McKeen *et al.*, 1986) was inoculated with *B. subtilis*. Antibiotic production media contained the following: 2 % (m/w) D-(+)-glucose and 0.5 % (m/w) DL-glutamic acid (both from Sigma, Johannesburg, S.A.); as well as 4.13 mM $MgSO_4.7H_2O$; 5.74 mM K_2HPO_4; 6.7 mM KCl (all from Saarchem, Merck); and 1 ml trace element solution (2.95 mM $MnSO_4.H_2O$ (Saarchem); 0.64 mM $CuSO_4.5H_2O$ (Pro Analysi, Merck); 0.05 mM $FeSO_4.7H_2O$ (Sigma) in 100 ml distilled water) per litre distilled

water. The pH was adjusted to 6.0 – 6.2. Erlenmeyer flasks (250 ml) containing 100 ml APM were autoclaved at 121 °C for 15 min. Flasks were inoculated with a single colony of bacteria growing on STD1 and shake incubated (70 rpm) at 25 °C for seven days. Absorbency measurements were taken **(240 nm)** and viable counts were made daily for seven days. In addition, two ml of the culture suspension was filtered through a 0.22 μm pore sized acetate filter (Millipore, Separation Scientific, Johannesburg). Spore suspensions were made as described above and were spread plated over PDA. Holes were made in the agar with a five mm diameter cork borer and 0.25 ml of the filtrate was pipetted into the hole. Three holes were made per Petridish, serving as one replicate. The absence or presence of inhibition zones were noted and were measured when formed. A filtrate of sterile antibiotic production medium was used as a negative control. The experiment was done in duplicate. Data was statistically analysed using Microsoft® Excel 2000.

3.3. Antifungal metabolite production and partial characterisation

3.3.1. Antifungal metabolite production

Flasks containing sterile APM were inoculated with a single colony of bacteria growing on STD1 and shake incubated (70 rpm) at 25 °C for seven days.

3.3.2. Extraction of antifungal metabolites

On day seven, production medium was centrifuged (14 000 x g) for 10 min to remove bacterial cells. The supernatant was filtered through a 0.22 μm pore sized acetate filter into Eppendorf tubes (crude cell-free extract) and stored in the dark at 4 °C until needed.

3.3.3. Extraction of free acid phenolic compounds

The pH of the crude cell-free extract was lowered to ± 2.6 by adding tetrafluroacetic acid (TFA) at a final concentration of 20 % (v/v) and mixed well. A 1:1 (v:v) solution was made with diethylether. The solution was mixed and allowed to stand for five minutes to allow separation of the different phases. The supernatant was removed with a micropipette and placed in a new Eppendorf tube. This procedure was repeated four times. The supernatants were combined and diethyl ether allowed to evaporate completely. The resulting precipitate was dissolved in 100 μl methanol and stored at 4 °C. An extraction of sterile APM was also done and served as the negative control.

3.3.4. Quantification of total phenolic compounds

Total soluble phenolics were quantified using the Folin-Ciocalteu's reaction (Swain & Hillis, 1959; Harborne, 1984). The volumes used were scaled down in order to use 96-well microtiter plates (Lasec, Johannesburg). Microtiter plate wells were inoculated with 370 µl distilled water. Samples to be analysed were added to the well at 5 µl per well, followed by 50 µl of 20% (w/v) Na_2CO_3 (Sigma) solution. Folin Ciocalteu's Phenol reagent (Sigma) was added as a colorimetric indicator to each well at 25 µl per well. Water served as negative control. The solutions in each well were mixed thoroughly with a micropipette and the plate was incubated at 37 °C for 30 minutes. Three wells were used per sample and the analysis was done in triplicate. Absorbency was measured with a Multiskan Ascent VI. 24 354-00973 (version 1.3.1). Data were calculated as equivalent gallic acid in mg / ml extract from the standard curve using an equation: $y = 1.3527x + 0.0109$ ($R^2 = 0.9989$) (Dr. T. Regnier, 2000, personal communication).

3.3.5. Separation of free acid phenolic compounds by thin layer chromatography

Thin layer chromatography (TLC) was used to evaluate possible antifungal compounds produced by *B. subtilis*. Crude extract, consisting of cell-free filtrates of *B. subtilis* growth medium at day seven, free acid extract and the growth medium used as control were analysed. For optimal separation of phenolic compounds, various solvents were tested, namely benzene:acetic acid:water (6:7:5, v/v/v) (BAW), toluene:acetic acid (4:1, v/v), acetic acid:methanol:water (8:1:1, v/v/v) (AMW) and water alone. Pre-coated glass plates (Silica Gel 60 F-254) as well as aluminum pre-coated TLC plates (SIL G-100UV_{254}) (both from Merck) were tested and the glass plates proved to be the better basis. All assays were run in triplicate and spots and bands were visualized with a CAMAT 50 Hz UV lamp (254 and 366 nm).

3.3.6. Antifungal activity of separated fluorescent spots against Cladosporium cladosporioides

Three volumes, 10, 20 and 30 µl, of the crude extract as well as the free acid extracts were spotted onto the TLC plates and ran with AMW. The plates were left under an extractor fan overnight to remove all traces of volatile solvents. The method described by Homans & Fuchs (1970) was used to detect antifungal activity. Plates were sprayed with a nutrient broth containing *C. cladosporioides* at 10^6 spores per ml. The broth consisted of two solutions, A and B, mixed together in a ration of 1:6 A:B (v/v). Solution A consisted of a 30 % (w/v) glucose (Sigma) solution. Solution B contained 0.04 M KH_2PO_4 (Saarchem), 0.02 M Na_2HPO_4 (Sigma), 0.04 M KNO_3 (Sigma), 4.1 mM $MgSO_4.7H_2O$ (Saarchem) and 0.02 M NaCl (Saarchem). Plates

were incubated in a moisture chamber at 25 °C for 48 to 96 h and observed for the formation of clear inhibition zones.

3.3.7. High performance liquid chromatography of free acid phenolic compounds

Dr. T. Regnier (Department of Microbiology and Plant Pathology, University of Pretoria) performed the high performance liquid chromatography (HPLC) analysis. The sample (10 μl) was injected into a HPLC column. The chromatographic system consisted of Varian 9012 high pressure pumps (three phases), a manual injector, an integrated system controller, a MALsil C18, 5 micron, reverse-phase analytical column (250 x 4.6 mm, five μm particle size), and a system spectra 6000 LP UV diode array detector with an attached analysis computer and data storage system (OS/2 WARP, Thermo Separation Products). The mobile phase, consisting of water and acetonitrile, as well as the flow rate used at specific times are summarized in table 6.1.

Table 6.1: The program followed during separation of samples using high performance liquid chromatography

Time: Minutes	% Acetonitrile	Flow rate
0	10	1.5
10	30	1.6
13	50	1.9
15	55	1.9
17	10	1.6

4. RESULTS

4.1. Optimising antifungal metabolite production by Bacillus subtilis

Antifungal substance concentration increased with the cell concentration in the seven day incubation period (Figure 6.1). After day five, the inhibition zone formed started to level off and it was decided that further analysis should focus on the antifungal metabolites present on day seven.

4.2. Quantification of total phenolic compounds

Phenolic compounds were present in the free acid extract at a concentration equivalent to 7.06 ± 0.95 mg gallic acid ml^{-1}. The growth medium was used as control and contained a total of phenolic compounds equivalent to 0.04 ± 0.01 mg gallic acid ml^{-1}.

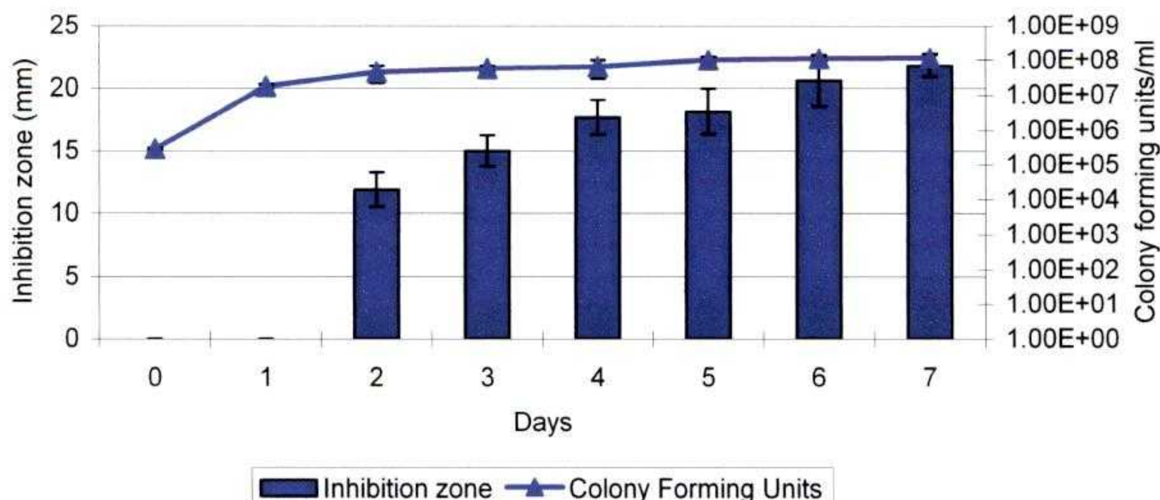


Figure 6.1: Inhibition zones formed at specific cell concentrations during continuous culturing of *Bacillus subtilis* over a seven day period.

4.3. Separation of free acid phenolic compounds by thin layer chromatography

Both water and BAW did not result in any separation of bands. The best solvent proved to be AMW. The free acid fraction was separated on pre-coated silica glass plate using AMW as solvent (Figure 6.2). Four spots were observed under UV illumination at 254 nm. No spots were noticed in the control sample. The Rf values of each of the spots are summarised in Table 6.2. As the concentration of the applied sample increased, the spot migrated further up the plate and in the 30 µl sample, spots c and d migrated together.

Table 6.2: Rf values of fluorescent spots separated using acetic acid : methanol : water as solvent on glass thin layer chromatograph plates

Observed fluorescent spot	Rf Value on silica plates using AMW	Colour at 254 nm UV illumination
a	0.22	Blue
b	0.31	Pink-red
c	0.59	Blue
d	0.64	Blue

Rf = mobility relative to front

4.4. Antifungal activity of separated fluorescent spots against Cladosporium cladosporioides

Growth of C. cladosporioides was inhibited by spots originating in the free acid fraction at the three concentrations tested. The inhibition zone corresponded to spots numbered a and b in Figure 6.3. As the applied concentration increased, the inhibitory activity also increased. Spot c and d combined also formed an inhibition zone.

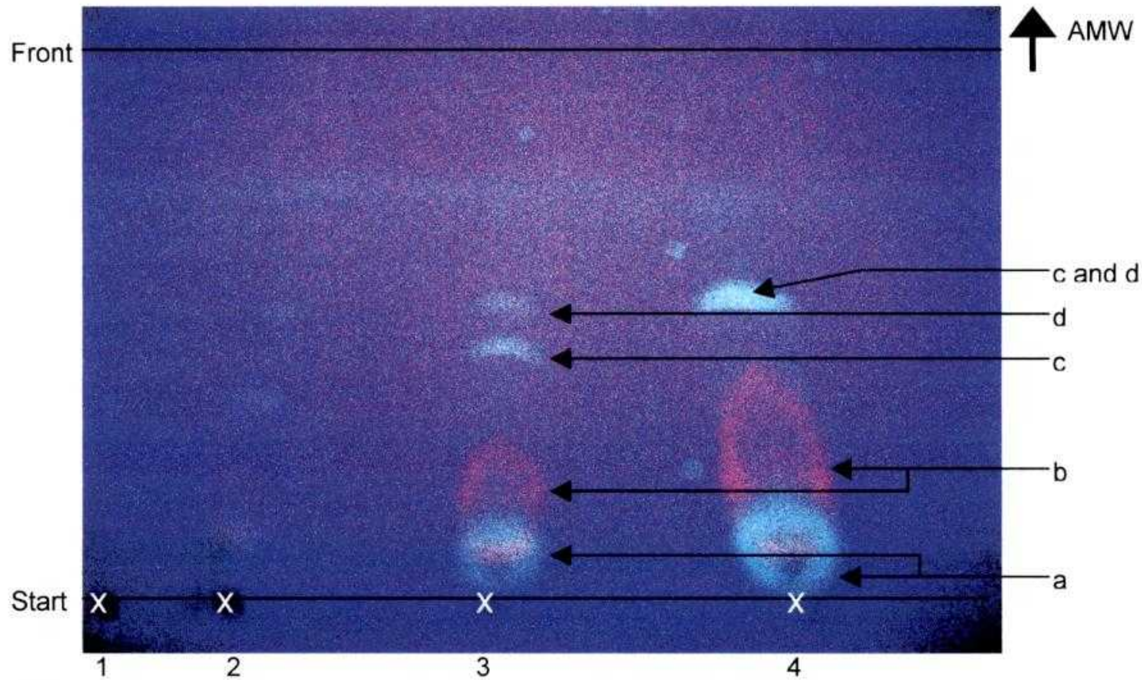


Figure 6.2: Free acid fraction of *Bacillus subtilis* growth media and control separated by thin layer chromatography using a silica glass plate, run with acetic acid:methanol:water (8:1:1, v/v/v) and viewed under UV light (254 nm) to separate potential inhibitory phenolic substances.
(No. 1 = control at 20 µl, no. 2 = 10 µl free acid extract, no. 3 = 20 µl free acid extract and no. 4 = 30 µl free acid extract)

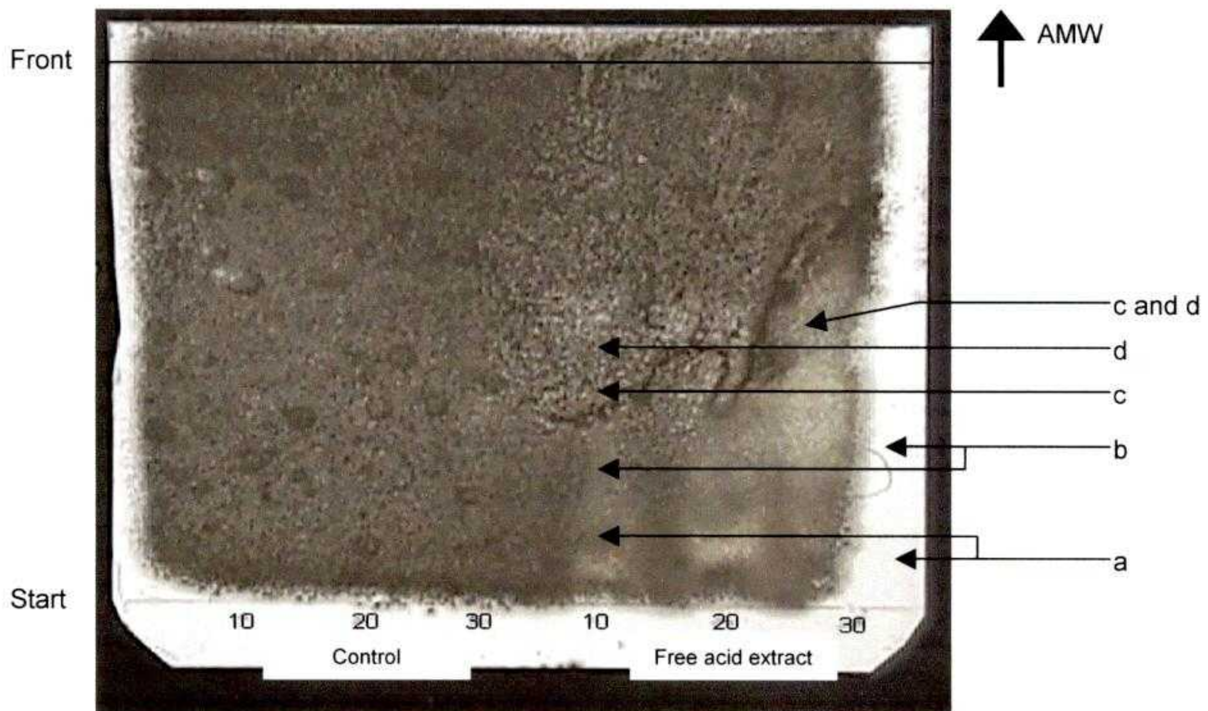


Figure 6.3: Inhibition due to spots of free acid phenolic compounds of *Bacillus subtilis* growth media separated by thin layer chromatography using a silica glass plate run with acetic acid:methanol:water (8:1:1, v/v/v) against *Cladosporium cladosporioides*.

4.5. High performance liquid chromatography of free acid phenolic compounds

In the HPLC analysis, eight peaks were observed at 325 nm while 13 peaks were evident at 280 nm (Figure 6.4). Of these 21 peaks, ten peaks coincided (1 and 2, 8 and 9, 11 and 12, 13 and 14, as well as 16 and 17). No peaks were observed at 430 nm. Table 6.3 summarises the time each of these peaks were observed. The percentage area provides an idea of how prominent a peak was and was calculated as the area covered by a peak compared to the whole area covered at a specific absorbency. The largest peaks were 2, 3, 6, 7, 11, 13 and 17.

Table 6.3: Retention time of the constituents of free acid phenolic extract determined using high performance liquid chromatography

Peak	RT (min)	Maximum Absorbency (nm)	% Area occupied	Resolved
1	2.306	280	6.00 + 3.38	No
2	2.376	325	18.10	Yes
3	3.046	280	49.77	Yes
4	3.058	325	4.44	Yes
5	4.140	325	5.15	Yes
6	5.296	280	18.59	Yes
7	5.373	325	11.18	Yes
8	7.213	280	1.45	Yes
9	7.264	325	8.65	Yes
10	8.450	280	1.51	Yes
11	8.929	325	12.18	Yes
12	8.935	280	0.95	Yes
13	9.661	325	21.68	Yes
14	9.668	280	3.41	Yes
15	12.050	280	0.60	Yes
16	14.132	280	1.01	Yes
17	14.134	325	18.61	Yes
18	15.147	280	3.58	No
19	15.573	280	3.80	No
20	15.973	280	3.51	No
21	16.547	280	2.47	No

RT = Retention time

5. DISCUSSION

A cell-free culture medium of *B. subtilis* produced a visible inhibition zone on PDA against *C. gloeosporioides* after day three of fermenting using the agar plate well method. The observed inhibition zone might have been due to the production of one or more inhibitory substances. It has previously been shown that antifungal metabolites were produced by *B. subtilis* (Chapter 4). Antibiotics reported to be produced by *B. subtilis* are most commonly peptides and lipopeptides (Peypoux *et al.*, 1984; Loeffler *et al.*, 1986; Peypoux *et al.*, 1986; Gueldner *et al*, 1988, Jacques *et al.*, 1994; Chen *et al.*, 1995; Eshita *et al.*, 1995; Kajimura *et al.*, 1995; Tsuge *et al.*, 1996; Lin *et al.*, 1998). However, Pinchuk *et al.* (2002) showed that phenolic compounds such as amicoumacin are also produced *B. subtilis*.

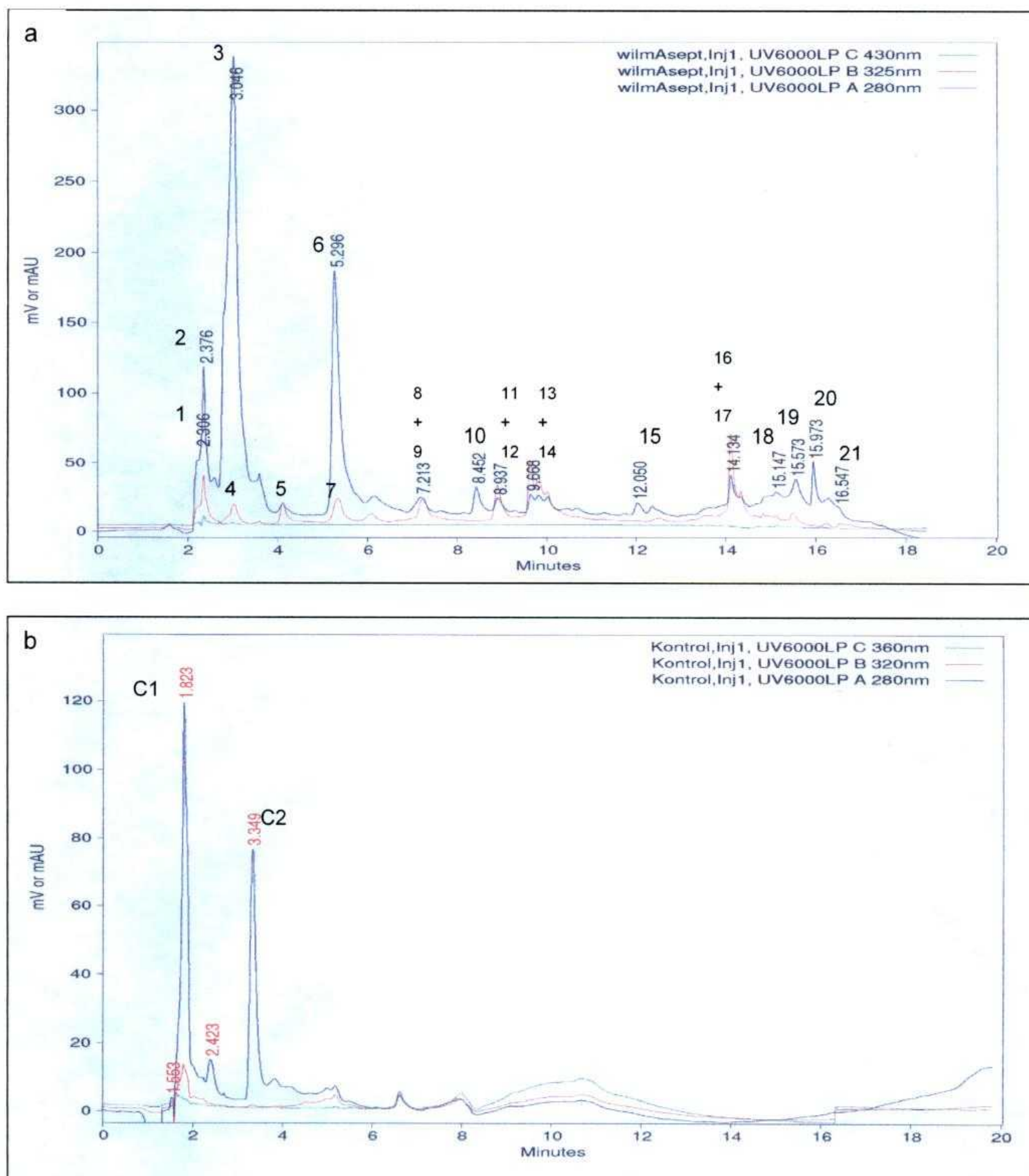


Figure 6.4: High performance liquid chromatographs of a) free acid phenolic extract viewed at 430, 325 and 280 nm, and b) control viewed at 360, 320 and 280 nm.

This study proved that *B. subtilis* secreted free acids into the growth medium. Pinchuk *et al.* (2002) also found free acids excreted by a *B. subtilis* isolate. In this study, analysis using TLC showed that four different compounds (a, b, c and d) were produced and that a and b were inhibitory to *C. cladosporioides,* a standard test organism for antifungal metabolites on TLC plates. Compounds c and d alone did not affect the fungal

growth, but the combination of c and d at the higher concentration did. Substance a was active at a concentration equivalent to 141.2 μg gallic acid ml^{-1} and b at a concentration equivalent to 211.8 μg gallic acid ml^{-1}.

Identification of the family of molecules produced by *B. subtilis* is possible if the visible spectral range and observed colours during UV illumination are kept in mind. The pink to mauve fluorescence under UV is usually associated with the coumaric group of compounds that include ferulic, sinapic, caffeic and ρ-coumaric acid (Harborne, 1984). Spot b presents this type of fluorescence and could then be one compound of the coumaric family. The HPLC analysis confirmed the production of at least eight compounds. Peak 17 (RT = 14 min), which represents a maximum absorption at 320 nm, could be ferulic acid as the coinjection with a standard of ferulic acid resulted in only one higher peak at the same time. Ferulic acid is mostly produced by plant roots and decomposing plant residues and plays a role in the protection of the plant cell wall (Caspersen *et al.*, 2000). Ferulic, ρ-coumaric, o-coumaric, caffeic, coumarin and umbelliferone are known to be germination inhibitors of wheat rust uredospores (Towers & Yamamoto, 1985). In contrast to coumaric acids, cinnamic acids are known to give two spots when chromatographed in aqueous solvents. They separate into the *cis*- and *trans*-isomers, explaining the formation of spots c and d (Harborne, 1984; Towers & Yamamoto, 1985). Moreover, another *B. subtilis* isolate was found that produced amicoumacin, a member of the isocoumarin or coumaric acid group (Pinchuk *et al.*, 2002).

Future studies should focus on the further identification of the inhibitory phenolic substances as well as their effect on fungal postharvest avocado pathogens. Since a variety of antifungal metabolites may be produced, the presence of antifungal peptide and lipopeptides should also be investigated. Even though the production of these antifungal metabolites is observed *in vitro*, their role in the *in vivo* inhibitory activity of *B. subtilis* against postharvest pathogens of avocado needs to be investigated.

6. REFERENCES

Caspersen, S., Alsanius, B.W., Sundin, P. & Jensén, P. 2000. Bacterial amelioration of ferulic acid toxicity to hydroponically grown lettuce (*Lactuca sativa* L.). Soil Biology and Biochemistry 32: 1063 – 1070.

Chen, C.L., Chang, L.K., Chang, Y.S., Liu, S.T. & Tschen, J.S.M. 1995. Transposon mutagenesis and cloning of the genes encoding the enzymes of fengycin biosynthesis in *Bacillus subtilis*. Molecular and General Genetics 248: 121 – 125.

Eshita, S.M., Roberto, N.H., Beale, J.M., Mamiya, B.M. & Workman, R.F. 1995. Bacillomycin L_c, a new antibiotic of the iturin group: Isolations, structures, and antifungal activities of the congeners. The Journal of Antibiotics 48: 1240 – 1247.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. & Cutler, H.G. 1988. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. Journal of Agricultural and Food Chemistry 36: 366 – 370.

Harborne, J.B. 1984. Phytochemical methods: a guide to modern techniques of plant analysis. Chapman & Hall, New York.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Homans, A.L. & Fuchs, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. Journal of Chromatography 51: 327 – 329.

Ikediugwu, F.E.O., Emoghene, A.O. & Ajiodo, P.O. 1994. Biological control of the shoot diseases *Amaranthus hybridus* caused by *Choanephora cucurbitarum* with *Bacillus subtilis*. Journal of Horticultural Science 69: 351 – 356.

Jacques, P., Hbid, C., Vanhentenryck, F., Destain, J., Baré, G., Razafindralambo, H., Paquot, M. & Thonart, P. 1994. Quantitative and qualitative study of the production of broad-spectrum antifungal lipopeptides from *Bacillus subtilis* S499. Pages 1067 – 1070 In: ECB6: Proceedings of the 6th European Congress on Biotechnology. Alberghina, L., Frontali, L. & Sensi, P. (Eds). Elsevier Science, B. V.

Kajimura, Y., Sugiyama, M. & Kaneda, M. 1995. Bacillopeptins, new cyclic Lipopeptide antibiotics from *Bacillus subtilis* FR-2. The Journal of Antibiotics 48: 1095 – 1103.

Katz, E. & Demain, A.L. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible functions. Bacteriological Reviews 41: 449 – 474.

Klein, C., Kaletta, C. & Entian, K. –D. 1993. Biosynthesis of the lantibiotic subtilin is regulated by a Histidine kinase/response regulator system. Applied and Environmental Microbiology 59: 296 – 303.

Korsten, L. 1993. Biological Control of Avocado Fruit Diseases. Ph.D. thesis, University of Pretoria, Pretoria.

Korsten, L. & de Jager, E.E. 1995. Mode of action of *Bacillus subtilis* for control of avocado post-harvest pathogens. South African Avocado Growers' Association Yearbook 18: 124 – 130.

Korsten, L., de Villiers, E.E., de Jager, E.S., Cook, N. & Kotzé, J.M. 1991. Biological control of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 14: 57 – 59.

Korsten, L., de Villiers, E.E., Rowell, A. & Kotzé, J.M. 1993. Postharvest biological control of avocado fruit diseases. South African Avocado Growers' Association Yearbook 16: 65 – 69.

Korsten, L, Towsen, E. & Claasens, V. 1998. Evaluation of Avogreen as post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. South African Avocado Growers' Association Yearbook 21: 83 – 87.

Kugler, M., Loeffler, W., Rapp, C., Kern, A. & Jung, G. 1990. Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: Biological properties. Archives of Microbiology 153: 276 – 281.

Leifert, C., Li, H., Chidburdee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. & Harbour, A. 1995. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology 78: 97 – 108.

Lin, G.H., Chen, C.L., Tschen, J.S.M., Tsay, S.S., Chang, Y.S. & Liu, S.T. 1998. Molecular cloning and characterization of fengycin synthetase gene *fenB* from *Bacillus subtilis*. Journal of Bacteriology 180: 1338 – 1341.

Loeffler, W., Tschen, S. -M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T. –F. & Wu, T. –G. 1986. Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3: A comparison with activities of other *Bacillus* antibiotics. Journal of Phytopathology 115: 204 – 213.

McKeen, C.D., Reilly, C.C. & Pusey, P.L. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis.* Phytopathology 76: 136 - 139.

Peypoux, F., Pommier, M.T., Das, B.C., Besson, F., Delcambe, L. & Michel, G. 1984. Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. The Journal of Antibiotics XXXVII: 1600 – 1604.

Peypoux, F., Pommier, M.T., Marjon, D., Ptak, M., Das, B.C. & Michel, G. 1986. Revised structure of Mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. The Journal of Antibiotics XXXIX: 636 – 641.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. & Urdaci, M.C. 2002. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Research in Microbiology 153: 269 – 276.

Podile, A.R. & Prakash, A.P. 1996. Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF1. Canadian Journal of Microbiology 42: 533 – 538.

Pusey, P.L., Wilson, C.L., Hotchkiss, M.W. & Franklin, J.D. 1986. Compatibility of *Bacillus subtilis* for postharvest control of peach brown rot with commercial fruit waxes, Dichloran, and cold-storage conditions. Plant Disease 70: 587 – 590.

Sailaja, P.R., Podile, A.R. & Reddanna, P. 1997. Biocontrol strain of *Bacillus subtilis* AF1 rapidly induces lipoxygenase in groundnut (*Arachis hypogaea* L.) compared to crown rot pathogen *Aspergillus niger*. European Journal of Plant Pathology 104: 125 – 132.

Sakajoh, M., Solomon, N.A. & Demain, A.L. 1987. Cell-free synthesis of the dipeptide antibiotic bacilysin. Journal of Industrial Microbiology 2: 201 – 208.

Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. Advances in Applied Microbiology 24: 187 – 214.

Swain, T & Hillis, W.E. 1959. The phenolic constituent of *Prunus domestica* I. The quantitative analysis of phenolic constituents. Journal of Science of Food and Agriculture 10: 63 – 68.

Towers, G.H.N & Yamamoto, E. 1985. Interactions of cinnamic acid and its derivatives with light. Pages 271 – 287 In: Annual proceedings of the phytochemical society of Europe: the biochemistry of plant phenolics. Van Sumere, C.F. & Lea, P.J. (Eds). Clarendon Press, Oxford.

Tsuge, K., Ano, T. & Shoda, M. 1996. Isolation of a gene essential for biosynthesis of the lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Archives of Microbiology 165: 243 – 251.

CHAPTER 7

GENERAL DISCUSSION

Avocado, *Persea americana* Mill., is an economically important crop in South Africa (Keevy, 1999). Major losses occur due to pre- and postharvest diseases. Postharvest diseases include stem-end rot (SE), caused by *Dothiorella aromatica* (Sacc.) Petrak & Sydow (Darvas & Kotzé, 1987; Korsten *et al.,* 1995), *Thyronectria pseudotrichia* (Schw.) Seeler, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Colletotrichum gloeosporioides* Penzig., *Phomopsis perseae* Zerova, *Pestalotiopsis versicolor* (Speg.) Steyaert, and *Fusarium* species. Anthracnose is caused by *C. gloeosporioides*. The pathogens causing avocado diseases are mainly controlled using chemical pesticides. With the worldwide movement away from excessive use of chemicals, a need for alternative control strategies evolved in the avocado industry. Biocontrol has been investigated as an alternative approach, especially when combined within an integrated pest management system (Roberts, 1994). In 1987, a *Bacillus subtilis* isolate was found on avocado leaves that showed promise as a biocontrol agent for control of avocado fungal diseases (Korsten, 1993). The isolate was evaluated and found effective in laboratory and semi-commercial trials, both in the field and in the packhouse (Korsten *et al.*, 1989; Korsten *et al.*, 1993; Korsten *et al.*, 1994; van Dyk *et al.*, 1997). Disease control using the biocontrol agent was often comparable to that achieved with commercially used chemicals, but occasionally results were found to be variable.

The main aim of this thesis was to investigate the modes of action involved in the antagonism of *B. subtilis* against postharvest pathogens of avocado. Previous studies showed that various modes of action might be involved (Korsten & de Jager, 1995; Havenga *et al.*, 1999). Since there is a wide range of pathogens involved in pre- and postharvest diseases of avocado, various infection patterns can be used, making effective control difficult. The biocontrol agent needs to act in both a preventative and curative way. The modes of action of *B. subtilis* against fungal postharvest avocado pathogens found in this study may play a role in both.

For biocontrol to be most effective, the biocontrol agent needs to be present before the pathogen arrives, when using the preventative approach. The antagonist needs to out compete the pathogen for space and nutrients. *In vivo* studies on avocado fruit using scanning electron microscopy (SEM) showed that where *B. subtilis* was present before the pathogen, conidia of *C. gloeosporioides* were unable to germinate.

Competition for nutrients was proposed as one of the main modes of action. However, *C. gloeosporioides* is a specialized necrotrophic pathogen and does not require any additional nutrients for germination (Blakeman, 1985). In *in vitro* studies, it was found that *B. subtilis* produces inhibitory substances. These substances may prevent conidia of *C. gloeosporioides* from germinating. It was also found that *B. subtilis* produces antifungal volatiles that may play a further role in the inhibitory activity. This finding is in accordance with Fiddaman & Rossall (1993) who evaluated volatile substances produced by *B. subtilis* that inhibits the growth of *Rhizoctonia solani* (J. G. Kühn) and *Pythium ultimum* (Trow). However, if the pathogen has already established an infection, the curative approach is the only alternative to ensure control.

Enzyme activity of *B. subtilis* was investigated in this study and chitinase, amylase, protease and lipase activity was found. Various lytic enzymes are reported to be produced by *B. subtilis* and are implicated in biocontrol, including chitinase (Frändberg & Schnürer, 1998; Helistö *et al.*, 2001), chitosanase, laminarinase, lipase and protease (Helistö *et al.*, 2001), as well as glucanolytic and proteolytic enzymes (Nielsen & Sørensen, 1997). These enzymes may cause the damage to *C. gloeosporioides* hyphae where *B. subtilis* attached itself directly onto the fungal cell wall which was observed during direct interaction studies on avocado fruit observed using SEM. In this study, cell-free inhibitory metabolites as well as inhibitory volatile substances were found to be produced by *B. subtilis* and may also act directly on a cellular level against the pathogen.

Since *B. subtilis* is a well-known producer of antibiotic substances, antibiotic production by the biocontrol agent was investigated. Most antibiotic substances produced by *B. subtilis* are peptides and lipopeptides (Katz & Demain, 1977; Shoji, 1978; Peypoux *et al.*, 1984; Loeffler *et al.*, 1986; Sakajoh *et al.*, 1987; Chen *et al.*, 1995; Tsuge *et al.*, 1996). Few studies focused on phenolic compounds utilised by biocontrol agents (Pinchuk *et al.*, 2002). In this study, a combination of thin layer chromatography (TLC) and high performance liquid chromatography combined with UV illumination was used to study free acid phenolic compounds produced by *B. subtilis*. Four fluorescent spots were observed on silica coated glass plates using acetic acid:methanol:water (8:1:1, v/v/v) as solvent. Two of these spots were inhibitory against *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (a standard test organism for antifungal metabolites on TLC plates). The free acid phenolic compounds produced by *B. subtilis* belong to the hydroxycinamic acids group. Further characterisation of these spots is of importance and should be considered in future studies.

The antagonistic efficacy of *B. subtilis* was evaluated *in vitro* using the dual culture technique, initially used to select the isolate (Korsten *et al.*, 1989). A high variability was found in the antagonistic activity with the subcultures evaluated. However, this effect can be ascribed to phenotypic variability in the culture (Reinheimer *et al.*, 1995) or even consecutive subculturing. Genetic comparisons between the different subcultures were done using DNA fingerprinting with RISA PCR. All of the representative subcultures showed identical banding patterns and differed from other *B. subtilis* reference strains. Even though phenotypic variability was found, environmental conditions may also play a role in the consistent performance of the biocontrol agent.

By providing optimal conditions for antagonist performance, more consistent disease control results can be ensured. The commercial formulation of the biocontrol agent can play a role in product performance and efficacy (Korsten *et al.*, 1998). Various nutrients were tested *in vitro* to determine their effect on antagonism against four postharvest fungal pathogens. The effect of these nutrients on the pathogens was also investigated. It was recommend that only nutrients that enhance antagonism, while not supporting the growth of the pathogen should be incorporated into the commercial formulation. The nutrients identified were L-glutamic acid, L-glutamine and L-(+)-asparagine as nitrogen source as well as D-arabinose and D-(+)-mannitol as carbon source. The efficacy of the new formulation is currently being tested in the field and in packhouse trials under commercial conditions.

In this study it was found that the environmental conditions also affect the biocontrol agent's effectiveness. In previous packhouse trials, *B. subtilis* was applied in the wax after the fruit were washed and was followed by storage in cold chambers (Korsten *et al.*, 1998). However, significant control was not achieved (Korsten *et al.*, 1998). The effect of various temperatures on *in vitro* antagonism of *B. subtilis* against postharvest fungal pathogens indicated that *B. subtilis* did not grow well at 10 ºC, which is the commercially used temperature for export. In a study by Huang *et al.* (1995), *Pseudomonas glathei* was applied to oranges. After application the fruit were stored at 30 ºC for 24 h prior to cold storage and an increase in efficacy of the biocontrol agent was found. A similar approach, taking into consideration the conditions necessary for fruit quality and shelf life, should perhaps be considered in future using *B. subtilis*. Temperature also affects the mode of action utilised by a biocontrol agent and can be used to enhance the efficacy.

In conclusion, *B. subtilis* is an effective biocontrol agent of avocado fungal pathogens. Results from this study provide some evidence of the mode of action employed by *B. subtilis*. This information can be used to

improve future biocontrol product formulation and application. By understanding the factors affecting the antagonist's efficacy a more stringent application program can be devised to support more consistent performance. This study confirms the involvement of antibiosis in the form of cell-free secondary metabolites, siderophores and volatiles, as well as direct interaction through lytic enzymes as modes of action used by *B. subtilis* against fungal postharvest avocado pathogens. Since *B. subtilis* employs such a wide range of modes of action, working directly and indirectly, few if any fungal pathogens will be able to overcome its control.

REFERENCES

Blakeman, J.P. 1985. Ecological succession of leaf surface micro organisms in relation to biological control. Pages 6 – 30 In: Biological Control on the Phylloplane. Windels, C.E. & Lindow, S.E. (Eds). The American Phytopathological Society, St. Paul, Minnesota, USA.

Chen, C.L., Chang, L.K., Chang, Y.S., Liu, S.T. & Tschen, J.S.M. 1995. Transposon mutagenesis and cloning of the genes encoding the enzymes of fengycin biosynthesis in *Bacillus subtilis*. Molecular and General Genetics 248: 121 – 125.

Darvas, J.M. & Kotzé, J.M. 1987. Fungi associated with pre-and post-harvest diseases of avocado fruit at Westfalia Estate, S.A. Phytophylactica 19: 83 – 85.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Frändberg, E. & Schnürer, J. 1994. Chitinolytic properties of *Bacillus pabuli* K1. Journal of Applied Bacteriology 76: 361 – 367.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Helistö, P., Aktuganov, G., Galimzianova, N., Melentjev, A. & Korpela, T. 2001. Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components. Journal of Chromatography B 758: 197 – 205.

Huang, Y., Deverall, B.J. & Morris, S.C. 1995. Postharvest control of green mould on oranges by a strain of *Pseudomonas glathei* and enhancement of its biocontrol by heat treatment. Postharvest Biology and Technology 5: 129 – 137.

Katz, E. & Demain, A.L. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible functions. Bacteriological Reviews 41: 449 – 474.

Keevy, C. 1999. Foreword. South African Avocado Growers' Association Yearbook 22: v.

Korsten, L. 1993. Biological Control of Avocado Fruit Diseases. Ph.D. thesis, University of Pretoria, Pretoria.

Korsten, L. & de Jager, E.S. 1995. Mode of action of *Bacillus subtilis* for control of avocado post-harvest pathogens. South African Avocado Growers' Association Yearbook 18: 124 – 130.

Korsten, L., Bezuidenhout, J.J. & Kotzé, J.M. 1989. Biocontrol of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 12: 10 – 12.

Korsten, L., de Villiers, E.E., Rowell, A. & Kotzé, J.M. 1993. Postharvest biological control of avocado fruit diseases. South African Avocado Growers' Association Yearbook 16: 65 – 69.

Korsten, L., de Villiers, E.E., Duvenhage, J.A. & Kotzé, J.M. 1994. Control of avocado pre-harvest diseases with *Bacillus subtilis* and fungicide sprays. 17: 32 – 34.

Korsten, L, Towsen, E. & Claasens, V. 1998. Evaluation of Avogreen as post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. South African Avocado Growers' Association Yearbook 21: 83 – 87.

Loeffler, W., Tschen, S. -M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T. –F. & Wu, T. –G. 1986. Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3: A comparison with activities of other *Bacillus* antibiotics. Journal of Phytopathology 115: 204 – 213.

Nielsen, P. & Sørensen, J. 1997. Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiology Letters 22: 183 – 192.

Peypoux, F., Pommier, M.T., Das, B.C., Besson, F., Delcambe, L. & Michel, G. 1984. Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. The Journal of Antibiotics XXXVII: 1600 – 1604.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. & Urdaci, M.C. 2002. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Research in Microbiology 153: 269 – 276.

Reinheimer, J.A., Morelli, L., Bottazzi, V. & Suárez, V. 1995. Phenotypic variability among cells of *Lactobacillus helveticus* ATCC 15807. International Diary Journal 5: 97 – 103.

Roberts, R.G. 1994. Integrating biological control into postharvest disease management strategies. HortScience 29: 758 – 762.

Sakajoh, M., Solomon, N.A. & Demain, A.L. 1987. Cell-free synthesis of the dipeptide antibiotic bacilysin. Journal of Industrial Microbiology 2: 201 – 208.

Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. Advances in Applied Microbiology 24: 187 – 214.

Tsuge, K., Ano, T. & Shoda, M. 1996. Isolation of a gene essential for biosynthesis of the Lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Archives of Microbiology 165: 243 – 251.

van Dyk, K., de Villiers, E.E. & Korsten, L. 1997. Alternative control of avocado post-harvest diseases. South African Avocado Growers' Association Yearbook 20: 109 – 112.