RAPD Delineation of Native *Bacillus* spp. and Analyses of Their Biocontrol Effect on Tomato Fusarium Wilt

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DECLARATION

I, the undersigned declare that this dissertation for the degree of Master of Science in Biology (Microbial Biotechnology) at the North-West University Mafikeng campus hereby submitted, has not been submitted by me for a degree at this or other university, that it is my own work in design and execution and that all material contained herein has been duly acknowledged.

Signed _____ this day 18/09/2014

DEDICATION

This work is dedicated to:

My husband, Mark Makinde for your love, support on every side, encouragement and belief in me that I can be the best God wants me to be, I love you; and

Our great children, Victoria, God's Delight, God'sBeloved and God'sBeauty for being so supportive even when I had to travel far away. I love you all.

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ABSTRACT

Tomato *(Lycopersicon esculentum* mill) is a very important vegetable crop and easily susceptible to fungal diseases such as Fusarium wilt which is caused by *Fusarium solani*. The use of biocontrol can be used to ameliorate this challenge. Eleven *Bacillus* isolates that were obtained from plant rhizosphere were characterized using Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR). Four out of the eleven were used as antagonists in dual culture of the *Bacillus* and *F. solani in vitro* and *in vivo. In vitro* analysis also included hydrogen cyanide production, phosphate solubilization and Indole acetic acid production while *in vivo* analysis included measurement of growth parameters and using EDX 720 for analysis of mineral/elemental composition of tomato. *In vitro* levels of inhibition of *F. solani* growth revealed that *Bacillus amyloliquefaciens* inhibited the growth of *F. solani* the most by 95.20% and was significantly different (p=0.05) from *Bacillus subtilis, Bacillus pumilus* and *Bacillus cereus* having 82.10%, 70.46% and 55.70% inhibition respectively. They were all significantly different (p=0.05) from the control which had 100% growth. *In vivo* inhibition of *F. solani* growth was measured as percent disease control and disease incidence. Percent disease control *in vivo* was slightly different from *in vitro* and varied from 62.50% in both *B. pumilus* and *B. subtilis*; 75% in *B. amyloliquefaciens* to 81.25% in *B. cereus* which exhibited the highest disease incidence. Disease incidence ranged from 18.75% in *B. cereus, 25%* in *B. amyloliquefaciens* to 37.50% in both *B. pumilus* and *B. subtilis*. This shows that the four *Bacillus* isolates were effective in disease control and reducing disease incidence. Treatment with *B. cereus* enhanced plant growth the most in terms of shoot and root length and root and shoot wet and dry mass while treatment with *B. amyloliquefaciens* enhanced plant growth the most in terms of number of leaves, branches and fruits. The tomatoes from the different plants treated with the antagonists were also analyzed for their mineral composition. The mineral elements common to all treatments are Potassium (K), Calcium (Ca) and Iron (Fe). The analysis showed that in all the treatments except the control, the mineral that was mostly abundant in the tomato was potassium (K). *B. amyloliquefaciens* had the highest percentage of 86.46% with cps/μA of 16.33 while control had the lowest of 22.93% and cps/μA of 8.81. Calcium (Ca) content of *B. cereus* was the highest both in percentage and cps/μA while the control had the lowest concentration of Ca. There was significant difference in the concentration of Iron (Fe) in the treatments compared to the control with the concentration of *B. pumilus* as the highest having 0.88% and 0.70 cps/μA. None of the antagonist produced hydrogen cyanide but all of them showed readings of absorbance with and without tryptophan compared to the control. Treatment with *B.*

amyloliquefaciens and *B. cereus* had the highest readings of absorbance with tryptophan while *B. cereus* had the highest without tryptophan. Only *B. amyloliquefaciens* showed clear zones around the streaked isolates in the test for phosphate solubilization. Three primers, A9B7, OPH 19 and S4 were used to depict the diversity of the eleven *Bacillus* isolates and they produced a total of 76 bands. From the dendrograms drawn, only *B. pumilus* exhibited clear-cut difference compared to the other *Bacillus* isolates. The relatedness of *B. amyloliquefaciens* and *B. subtilis* was emphasized by primer A9B7 even though *B. cereus* was in a different cluster but not distant. Primer OPH 19 revealed that *B. amyloliquefaciens* is closer to *B. cereus* while *B. subtilis* is in a not-distant cluster. In the same vain, primer S4 revealed that *B. amyloliquefaciens* is closely related to *B. pumilus* while *B. cereus* and *B. subtilis* are closely related. It was observed from this study that all randomly selected *Bacillus* isolates used, antagonised the growth of *F. solani in vitro* and *in vivo*. Despite the fact that *B. amyloliquefaciens* antagonised growth by the highest percentage *in vitro*, *B. cereus*, reduced disease incidence the most *in vivo*. This indicated that sometimes there is no correlation between *in vitro* antagonism and *in vivo* disease control. Further research studies can be done to provide information on this non-correlation and also how to clone best expressed potentials from each isolate into one organism.

TABLE OF CONTENTS

DECLARATION i

DEDICATION ii

ACKNOWLEDGEMENTS iii

ABSTRACT v

TABLE OF CONTENTS vii

LIST OF ABBREVIATIONS x

LIST OF FIGURES xi

LIST OF TABLES xii

LIST OF APPENDIX xiiiii

CHAPTER 1 1

INTRODUCTION 1

1.1 GENERAL INTRODUCTION 1

1.2 STATEMENT OF PROBLEM 2

1.3 RESEARCH AIM AND OBJECTIVES: 2

1.3.1 Aim 2

1.3.2 Objectives 2

1.4 SIGNIFICANCE OF THE STUDY 3

1.5 RESEARCH QUESTIONS 3

CHAPTER 2 4

LITERATURE REVIEW 4

2.1 Fate and effects of microorganisms in the biocontrol of diseases of tomato plant 4

2.2 Origin, taxonomy and morphology of Tomato 5

2.3 Effects of microorganisms on tomato pathogens 7

2.4 Adverse effects of using microorganisms as biocontrol agents on non-target organisms 19

2.5 Fate of microbial biocontrol agents in the biocontrol of tomato plant diseases 20
2.6 Fusarium wilt and the fungi pathogens 20
2.7 Cultural, physical and chemical control methods 24
2.8 Biological control and biological control agents (BCAS) 26
2.9 *Bacillus* 27
2.10 Molecular characterization 30
CHAPTER 3 36
MATERIALS AND METHODS 36
3.1 *Bacillus* spp. Inoculum preparation 36
3.2 Preparation of phytopathogenic fungi 36
3.3 Antifungal activities of *Bacillus* isolates 37
3.4 Antagonistic activity of *Bacillus* isolates 38
3.5 Experimental design 39
3.6 Green House experiment 39
3.7 Molecular characterization of *Bacillus* isolates 41
3.8 Statistical Analysis 42
3.9 Data Analysis of RAPD-PCR 43
CHAPTER 4 44
RESULTS 44
4.1 *In vitro* antagonistic effects of *Bacillus* isolates 44
4.2 The Possible mechanism of biocontrol of *Bacillus* isolates 44
4.3 The Biocontrol potential of *Bacillus* isolates on Fusarium wilt of tomato plants in the green house 45
4.4 Growth parameters of tomato plants treated with *Bacillus* isolates 50
4.5 Physical analysis of tomato treated with *Bacillus* isolates 53
4.6 Quantitative result for mineral composition of tomato fruits from each treatment ... 55
4.7 RAPD analysis of *Bacillus* isolates used for biocontrol of tomato Fusarium wilt 57

CHAPTER 5 71

DISCUSSION 71

5.1 *In vitro* antagonistic effects of *Bacillus* isolates on *Fusarium solani* 71

5.2 Mechanism of biocontrol of *Bacillus* isolates against *Fusarium solani* 72

5.3 Biocontrol potentiality of *Bacillus* isolates on Fusarium wilt of tomato plants in the green house 75

5.4 Mineral analysis of tomato using EDX 720 78

5.5 Relationship between growth parameters and disease incidence in biocontrol-treated plants 81

5.6 Molecular characterization and biocontrol of tomato Fusarium wilt 83

CONCLUSION 86

REFERENCES 88

APPENDIX 111

LIST OF ABBREVIATIONS

Abbreviation	Meaning
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
bp	Base pair
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EDS or EDX	Energy-Dispersive X-ray Spectroscopy
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FSP	Inoculation with *F. solani* alone
GRAS	Generally Recognised As Safe
HCN	Hydrogen cyanide production
IAA	Indole Acetic Acid
ISR	Induced Systemic Resistance
keV	kilo electron Volts
LSD	Least Significant Difference
NBNF	No treatment with *Bacillus* isolates and No infection with *F. solani*
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGPR	Plant Growth-Promoting Rhizobacteria
PSM	Phosphate Solubilizing Microorganisms
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SEM	Scanning Electron Microscopy
SEM	Standard Error of Mean
SPSS	Statistical Package for the Social Sciences
SsRNA	Single Stranded Ribonucleic Acid

LIST OF FIGURES

Figure 2.1 Schematic diagram of important mechanisms known for plant growth promotion and biocontrol through rhizobacteria (Source: this study) 15
Figure 2.2 Tomato plant with no infection and no treatment. 23
Figure 2.3 Tomato plant showing signs of yellowing and wilting from the lower leaves. 23
Figure 2.4 Some of the methods used in the cultural control of Fusarium wilt of tomato (Ajilogba and Babalola, 2013) 24
Figure 2.5 Three-fold usefulness of *Bacillus* species. 30
Figure 4.1 Inhibitory effect of the four *Bacillus* isolates against *F. solani in vitro* at *P*=0.05 46
Figure 4.2 Disease incidence (A) and degree of disease control (B) using the different Bacillus isolates in each treatment 48
Figure 4.3 Biocontrol of Fusarium wilt in which the treatments (A, B, C, D) and *F. solani* were simultaneously inoculated into the tomato plant roots 49
Figure 4.4 Dry roots of tomato plants that have been treated with different *Bacillus* isolates. Bac A = *B. amyloliquefaciens,* Bac C = *B. cereus,* Bac P = *B. pumilus,* Bac S = *B. subtilis* 54
Figure 4.5 EDX-Analysis of the different samples of tomato whose plants were treated with *B. cereus* was measured from 0 -40 kilo electron Volts (keV), the peaks of Potassium (K) and Calcium (Ca) are clearly visible for the sample 56
Figure 4.6 Effect of various treatments on the yield of the tomato fruits 58
Figure 4.7 Electrophoretic gel analysis of RAPD polymorphism detected in 11 *Bacillus* isolates using Primer A9B7 66
Figure 4.8 Electrophoretic gel analysis of RAPD polymorphism detected in 11 *Bacillus* isolates using Primer OPH 19 67
Figure 4.9 Electrophoretic gel analysis of RAPD polymorphism detected in 11 *Bacillus* isolates using Primer S4 67
Figure 4.10 Dendrogram from unweighted pair group method with arithmetic averages showing relationships among the 11 *Bacillus* isolates. 68
Figure 4.11 Dendrogram from Unweighted pair group method with arithmetic averages showing relationships among the 11 *Bacillus* isolates. 69
Figure 4.12 Dendrogram from Unweighted pair group method with arithmetic averages showing relationships among the 11 *Bacillus* isolates 70

LIST OF TABLES

Table 2.1 Effect of biocontrol agents on some fungal pathogens of tomato plants 8
Table 2.2 Effect of biocontrol agents on some bacterial disease-causing pathogens of tomato plants..... 11
Table 2.3 Effect of biocontrol agents on viral pathogens of tomato plants..... 12
Table 2.4 Effect of biocontrol agents on nematodes (or Meloidogyne species) that infest tomato 13
Table 2.5 Some antibiotics produced by *Bacillus* as Biological Control Agents..... 31
Table 3.1 Molecular description of *Bacillus* isolates.....37
Table 4.1 Mechanism of inhibition by four *Bacillus* isolates..... 47
Table 4.2 Effect of various treatments on plant growth parameters in tomato plants treated with *Bacillus* isolates but were not infected with *F. solani*..... 52
Table 4.3 Effect of various treatments on plant growth parameters in tomato plants treated with *Bacillus* isolates and infected with *F. solani* 53
Table 4.4 Mineral analysis of tomato fruits from the different treatments in mg/g 57
Table 4.5 Effect of Treatments on tomato fruit color, percent dry weight and moisture content 59
Table 4.6 Number of bands and percentage of DNA polymorphic bands in eleven *Bacillus* isolates amplified with three primers 60
Table 4.7 Number of bands per isolate from each of the primers 61
Table 4.8a Similarity Matrix computed with Jaccard coefficient (using primerA9B7) showing 11 elements with 9 variables and each element has been analyzed..... 62
Table 4.8b Distance matrix based on Jaccard coefficient (using primer A9B7)..... 62
Table 4.8cSimilarity Matrix computed with Jaccard coefficient (using primer OPH 19) showing 11 elements with 12 variables each element have been analyzed..... 64
Table 4.8d Distance Matrix based on Jaccard coefficient (using primer OPH 19)..... 64
Table 4.8e Similarity Matrix computed with Jaccard coefficient (using primer S4) showing 11 elements with 15 variables and each element has been analyzed..... 65
Table 4.8f Distance matrix based on Jaccard coefficient (using primer S4)..... 65

LIST OF APPENDIX

1.0 List of articles already published or accepted 111

1.1. Ajilogba, C. F. & Babalola, O. O. 2013 Integrated management strategies for tomato *Fusarium* Wilt. *Biocontrol Science* 18(3): 117-127. (ISSN 13424815) https://www.jstage.jst.go.jp/article/bio/18/3/18_117/_pdf 111

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1.3. Ajilogba, C. F. & Babalola, O. O. 2014. *Effects and fate of microorganisms in the control of common diseases of tomato* (*in press*) Agriculture and Environmental Pollution: Causes, Consequences and Control. EB/2013/271/ECB ENVBOOKS series, Delhi, India EnvBook series, Delhi India 111

1.4. Ajilogba, C. F. & Babalola O. O. (2013) Biocontrol treatment Of Fusarium wilt In tomato. SASM 2013 (ID. 13, Poster) (The 18th Biennial Conference of the South African Society for Microbiology). 24-27 Nov. Forever Resorts Warmbaths, Bela-Bela. South Africa 111

1.5. Ajilogba, C. F. & Babalola, O. O. (2012) Cultural control of Fusarium wilt of tomato. BioFISA Phase I Programme Closure Conference: Celebrating Four Years of BioFISA, 2-6 September 2012, Irene County Lodge, Centurion, South Africa. 111

2.0. EDX 720 spectrophotometry analysis of the mineral content of each treatment with *Bacillus* isolate 111

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Tomato (*Lycopersicon esculentum* mill) comes second worldwide, behind potato as a vegetable crop. It is estimated that 4.6 million tomatoes is grown in hectares of land annually producing more than 126 million metric tonnes (Panthee and Chen, 2010). It is among the world's most cultivated vegetable crops and known as a healthy food because of its nutritive value and widespread production. As one of the most important nursery-based vegetable crops cultivated for its fleshy fruits, soil-borne pathogens are a major scourge of such crops of the family Solanaceae such as chilli (*Capsicum annuum*), tomato and brinjal (*Solanum melongena*) (Dukare *et al.*, 2011).

In most economically important crops, *Fusarium* is one of the most important genera of fungal plant pathogens (Agrios, 2005; Babalola, 2008; Babalola, 2010a; Babalola, 2010b; Babalola, 2010c; Windels, 2000). *Fusarium solani*, one of the most common *Fusarium* pathogens, is a broad spectrum soil pathogen and the causal agent of root and stem rot diseases of various vegetables (Agrios, 2005; Palmateer *et al.*, 2004). Another devastating disease caused by the *Fusarium* genera is Fusarium wilt (Agrios, 2005; Windels, 2000).

One of the most serious diseases in tomato throughout the world is Fusarium wilt (Charoenporn *et al.*, 2010). Like most vegetable crops, tomato crops depend on fungicides like methyl bromide to combat the various fungal diseases but chemical control of the disease is not satisfactory. Chemical control creates imbalances in the microbial community of the plant which may be unfavourable to the activity of the beneficial organisms. It may also lead to the development of resistant strains of the pathogen (Shanmugam and Kanoujia, 2011). In addition to other potential health, safety and environmental risks, methyl bromide was classified as an ozone-depleting compound and has been banned as at 2004 under the Montreal Protocol (Gullino *et al.*, 2003; Minuto *et al.*, 2006). As a result of this, alternative control measures like biological methods are being considered to suppress or contain this disease.

Biological control, offers a better alternative to the use of chemicals in that it is the use of natural antagonistic organisms to combat pests or suppress plant diseases (Droby *et al.*, 2009; Lugtenberg and Kamilova, 2009). Since Plant Growth-Promoting Rhizobacteria (PGPR) are

known for growth promotion and disease reduction in crops, biocontrol using PGPR represents a potentially attractive alternative disease management method (Jetiyanon and Kloepper, 2002).

The PGPR such as *Bacillus* organisms have received much attention as biocontrol agents. They are able to produce several broad spectrum antibiotics and can withstand adverse conditions due to their ability to form endospores (Cavaglieri *et al.*, 2005). This allows easy formulation and storage of the commercial products obtained from them (Francis *et al.*, 2010; Schallmey *et al.*, 2004). In addition to their antibiotic-producing properties against bacteria, *Bacillus* isolates also exhibit antagonistic behaviour against fungal pathogens (Munibazi and Bullerman, 1998).

1.2 STATEMENT OF PROBLEM

As important as tomato is to health especially in its usefulness as an antioxidant and its effectiveness against common cancers, soil borne pathogens are considered to be the major scourge (Dukare *et al.*, 2011). The pathogenic fungi *(Fusarium oxysporum* or *Fusarium solani*) that cause Fusarium wilt can remain viable in the soil for up to 30 years (Thangavelu *et al.*, 2004). It is destructive to plants and swift. By the time a plant shows any outward sign of infection, it is already too late, the plant wilts and dies.

Every tomato grower is faced with the danger of losses due to Fusarium wilt. Losses in crop production caused by plant pathogens can reach 50% especially on tomato cultivars infected by *Fusarium* organisms in the world and severely limit its production. Treatment with fungicides to control soil-borne fungi has been ineffective in controlling *Fusarium* spp. under conditions conducive for the disease.

1.3 RESEARCH AIM AND OBJECTIVES:

1.3.1 Aim

The purpose of the study was to evaluate the possible biocontrol activities of native *Bacillus* isolates against *Fusarium solani* causing Fusarium wilt of tomato in order to increase tomato production.

1.3.2 Objectives

This present study was therefore designed to:

- identify native *Bacillus* isolates with biocontrol potential for Fusarium wilt of tomato plant *in vitro* and *in vivo*.
- characterize the *Bacillus* isolates having biocontrol potential at the molecular level.
- assay for PGPR activities of the *Bacillus* isolates.

1.4 SIGNIFICANCE OF THE STUDY

From this research, the results from the analyses of the different treatments given to the tomato plants *in vitro* and *in vivo* will add to the body of knowledge on the biocontrol potential of the different *Bacillus* isolates and the disease incidence of wilting by the *F. solani*. Conducting molecular characterization of native *Bacillus* isolates with biocontrol potential for Fusarium wilt of tomato will also provide valuable information to stakeholders for further work that could lead to cloning and formulation to help in the management of Fusarium wilt of tomato caused by *F. solani*.

1.5 RESEARCH QUESTIONS

1. What *Bacillus* isolates are native to Mahikeng?

2. Are they antagonist to the pathogen *F. solani*?

3. How could these native *Bacillus* isolates be characterized at the molecular level?

CHAPTER 2

LITERATURE REVIEW

2.1 Fate and effects of microorganisms in the biocontrol of diseases of tomato plant

Knowledge on the fate and effect of microorganisms as alternative to other ways of controlling plant diseases and pest is essential to the future of biocontrol. Regulatory bodies require concrete information on the possible effects of releasing some microorganisms into the environment. That information can only be generated from research and studies on the fate and effects of microorganisms (Kiewnick, 2007). Some species of microorganisms such as *Saccharomyces cerevisae, Bacillus licheniformis* and *Aspergillus niger* have long been recognized as 'Generally recognised as safe' (GRAS) by regulatory bodies such as the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA), and are being utilized in environmental applications. The safety assessment of these microorganisms is made easy because of previous evaluation; so more attention is concentrated on those microorganisms that could pose greatest risks (Leuschner *et al.*, 2010).

The fate of microorganisms involves four particular areas of microbial ecology which are: community effects, population dynamics, microbial dissemination, and persistence in the environment (Holmberg, 2011). These effects go a long way to affect the strategies that will be used in their formulation into biocontrol products. In a situation where little is known about their interaction with other target and nontarget organisms in the environment, the process towards registration as anti-microbial product is slowed down.

When a microorganism is introduced into a new environment, it is faced with different challenges as well as opportunities. These challenges include its ability to persist, which also varies between strains of the same species; also there is the issue of competition with the resident population in the new community, and the issue of it being predated upon by other pathogens or bacteriophages (Babalola and Glick, 2012b; Xiang *et al.*, 2010). To overcome these challenges, microorganisms may be introduced into sterile soil prior to introduction to the field as it helps them to adapt and deal with the issue of stress in the new environment (Van Dyke and Prosser, 2000).

Monitoring the interaction with and response to both biotic and abiotic influences help to determine the fate of microorganism. This involves following the microorganism overtime and generating data on the microbial population concerning its size, its distribution and

survival times (Whipps, 2001). A microorganism that will be used as a biocontrol agent will be successful if it is able to find its own protective niche within the new environment, colonise it and spread from there to the other parts of the environment (Johansen *et al.*, 2005). Microorganisms have been used in the control of diseases in most solanaceous crops and that includes tomato.

Tomato comes second to potato worldwide, as a vegetable crop. It is known as a healthy food because of its nutritive value which include low sodium, saturated fat and cholesterol value, rich vitamin E (Alpha tocopherol), thiamine, niacin, vitamin B6, folate, magnesium, phosphorus, copper, dietary fiber, vitamin A, vitamin C, vitamin K, potassium and manganese values. The world yearly production was estimated as 145 751 507 Mt in 2010 (FAO, 2012). It is an important cash crop for smallholders and medium-scale commercial farmers (Naika *et al.*, 2005). They contain much vitamin B and C, iron and phosphorus. Yellow tomato contains vitamin A which is important for good eyesight. Red tomato contains lycopene, which is responsible for the red coloration and also an anti-oxidant that may contribute to protection against carcinogenic substances. Lycopene has the ability to neutralize the free radicals that can cause damage to cells (Boyer, 2011). Tomato is an important cash crop for smallholders and medium-scale commercial farmers in Africa (Babalola and Glick, 2012a) but it is prone to infections and diseases by soil –borne pathogens. There are several microorganisms used in the control of tomato disease. Some of these microorganisms are *Bacillus, Trichoderma,* and *Pseudomonas* species.

2.2 Origin, taxonomy and morphology of Tomato

Tomato was native to the South American Andes before Spanish explorers introduced it to the whole world. Scientifically, tomato is known as *Solanum lycopersicon* with synonym as *Lycopersicon esculentum* while botanically, it belongs to the nightshade family or solanaceae which also includes chili peppers, potato and eggplant.

In South Africa, as it is worldwide, tomato is the second most important vegetable crop after potato. It is planted in over 6000 hectares of land in areas like, Mpumalanga, Lowveld and Middleveld, Western Cape, Limpopo, Southern part of the Eastern Cape and the Pongola region of Kwazulu-Natal (DAFF, 2012).

2.2.1 Distribution

Tomato was introduced into Spain by the Spanish explorers and from there it was taken to Europe and Africa. Initially it was thought to be poisonous but was later accepted to be non-poisonous in the 18th century. Tomato is grown in every country in the continent (Philouze and Hedde, 1993) Most of the wild species are found in the Andean mountains of South America while the domesticated one are widely distributed throughout the world (Chetelat *et al.*, 2009). Asia and African account for more than 65% of tomato grown in the world. These countries include China, India, Turkey and Nigeria (Srinivasan, 2010). It is now one of the most widely eaten vegetables worldwide (DAFF, 2012; Hanson *et al.*, 2004). In South Africa, of the total vegetables eaten, tomato constitutes about 24% (DAFF, 2012). Tomato is cultivated on all continents except Antarctica, from sea level to an elevation of 3000 metres above sea level, and in temperate climates they are grown as annuals. Tomato can also be grown as perennials. Sometimes tomato can become weeds when they escape outside the garden and grow where they are not planted.

2.2.2 Planting and harvesting requirement

Tomato can easily be killed by frost and is seen as warm season crop. The plant needs daily optimum temperature of between 20°C and 24°C (Whiting *et al.*, 2012). Temperatures below 12°C lead to poor growth, yield and poor quality of the fruit. During windy dry season, the plant loses many of its flowers while during wet season; it is prone to foliar disease infection. They can grow in quite dry areas, but grow bigger and produce more fruit when they are well-watered (DAFF, 2012). It grows well in a rich well aerated loam soil with nutrients and pH of 6-7. It requires a good amount of nitrogen for growth and better flower and fruit set, phosphorus for vigorous growth and development of root and potassium for the acids and solids of the fruit, firmness and improved taste. It also requires other micronutrients like iron, manganese, calcium, magnesium, boron and molybdenum for its growth and development.

Tomato is a perishable and must be handled with care when they are harvested as they can be harvested manually by picking them in most cases. They can be harvested when they are properly ripe, not fully ripe or not ripe at all. In handling them, care should be taken to avoid injury that could lead to them being infected with microbes which eventually leads to spoilage and low taste quality.

2.2.3 Importance of Tomato in Health

Tomato is so important for its phytochemical properties and it has more health benefitting properties compared to other vegetables. It is one of the vegetables with low calorie; 18 calories per 100 g compared to 902 calories per 100 g in animal fat and 218 calories per 100g in kidney beans. It has no cholesterol and is recommended in cholesterol reduction diet. It is a rich source of many important nutrients, and is quite important to a healthy diet. It is rich in minerals, vitamins, essential amino acids, sugars and dietary fibres. It contains much vitamin B and C, iron and phosphorus. Yellow tomato contains vitamin A which is important for good eyesight. Red tomato contains lycopene which is responsible for the red coloration and also an anti-oxidant that may contribute to protection against carcinogenic substances (Miller *et al.*, 2002). It has the ability to neutralize the free radicals that can cause damage to cells (Boyer, 2011). As a matter of fact, lycopene is thought to be twice as effective as many other kinds of antioxidants, and it has been shown to play a role in the prevention of such common cancers as prostate cancer, lung cancer and breast cancer. In addition, lycopene is thought to help to inhibit the aging process, allowing tomato eaters to remain active longer (Bramley, 2000; Heber and Lu, 2002).

Flavonoids contained in tomato include zeaxanthin, which helps to prevent ultra-violet rays from filtering into elderly people's eyes thereby preventing age related macular disease. It also contains other flavonoid antioxidants such as α and ß-carotenes, xanthins and lutein which enhance good vision and healthy bones and help in preventing oral cavity cancer. It contains reasonable amount of vitamin B complex like folates, thiamine, niacin, riboflavin and other essential minerals like iron, calcium, manganese. 21% of the recommended daily levels per 100 g of vitamin C requirement are found in tomato and it helps to detoxify the blood by removing free radicals (Rudrappa, 2013).

2.3 Effects of microorganisms on tomato pathogens

Various microorganisms have been used to suppress the activities of different tomato pathogens. These soil-borne pathogens attack tomato plants and cause serious diseases which could be fungal (Table 2.1.), bacterial (Table 2.2.), viral (Table 2.3.) and nematicidal (Table 2.4.). These tables reflect some of the tomato diseases, the rhizobacteria that has been used in their biocontrol and their biocontrol mechanisms.

Table 2.1 Effect of biocontrol agents on some fungal pathogens of tomato plants

Pathogen suppressed	Plant disease	Biocontrol agent	Mechanism	Effect	Reference
Phytophthora infestans	Tomato Late blight	*B. pumilus* SE34	Induced Systemic Resistance (ISR)	Improved plant growth	(Yan *et al.*, 2002)
F. solani	Root rot disease	*B. subtilis, Trichoderma viride*	Mycoparasitism, competition and antibiosis	Biocontrol activity against the diseases and high yield of tomato	(Zaghloul *et al.*, 2007)
Botrytis. cinerea	Grey mold of tomato	*Microdochium. dimerum*		*M. dimerum* is very effective in protecting pruning wounds of tomato plants against *B. cinerea* and reduced disease severity significantly	(Bardin *et al.*, 2008)
F. oxysporum f.sp *radicis-lycopersici* (FORL)	Crown and root rot disease of tomato	*B. subtilis* EU07	Production of antibiotics, fengycin, bacillomycin and iturin	Growth of FORL was efficiently inhibited	(Baysal *et al.*, 2008)

Alternaria solani	Early blight disease of tomato	*Bacillus coagulans, B. pumilus, Serratia plymuthica, S. mercescens, Paenibacillus macerans*	Antifungal metabolites and competition	Reduced disease severity significantly on the field compared to control	(Yazici *et al.*, 2011)
Non-pathogenic strain of *F. oxysporum* Fo47	Tomato wilt	Pathogenic strain of *F. oxysporum* f.sp *lycopersici* Fol8	ISR	Improved growth and reduced infection	(Aime´ *et al.*, 2008)
Rhizoctonia solani	Damping-off of tomato	*B. subtilis* RB14-C	Antibiosis	Promoted plant growth and reduced disease severity	(Szczech and Shoda, 2006)
Pythium ultimium, Macrophomina phaseolina,	Damping-off disease	*Pseudomonas fluorescens*	Antifungal metabolites-antibiosis	Inhibited growth of pathogens	(Goud and Muralikrishnan, 2009)

Pyricularia oryzae					
Pythium aphanidermatum	Damping-off disease of tomato	*Bacillus* spp.	Antifungal metabolites	Inhibited mycelia growth and improved plant growth	(Intana *et al.*, 2008)

Table 2.2 Effect of biocontrol agents on some bacterial disease-causing pathogens of tomato plants

Pathogen suppressed	Plant disease	Biocontrol agent	Mechanism	Effect	Reference
Ralstonia solacearum	Tomato bacterial wilt	*Bacillus* spp.	ISR	Improved plant growth	(Jetiyanon and Kloepper, 2002)
Xanthomonas campestris pv. *vesicatoria* strain 2	Bacterial spot of tomato	*Rahnella aquatilis*	Antibiosis	Reduction in the deleterious effect on tomato seedlings	(El-Hendawy *et al.*, 2005)
Xanthomonas axonopochy	Bacterial spot disease of tomato	*P. fluorescens*	Antibiosis	Promoted plant growth and reduced disease severity	(Abo-Elyousr and El-Hendawy, 2008)

Table 2.3 Effect of biocontrol agents on viral pathogens of tomato plants

Pathogen suppressed	**Plant disease**	**Biocontrol agent**	**Mechanism**	**Effect**	**Reference**
Cucumber mosaic virus (CMV)	Tomato leaf spot	*B. pumilus SE34; B. subtilisIN937b; B. amyloliquefaciensIN937b*	ISR	Improved plant growth	(Zehnder *et al.*, 2000)
Tomato spotted wilt virus (TSWV)	Tomato spotted wilt	*P. fluorescens*	ISR	Significantly reduced incidence of disease	(Kandan *et al.*, 2002)
Tomato yellow leaf curl virus and *Bemisia tabaci*	Tomato yellow leaf curl	*Eretmocerus californicus; Encarsia formosa*	Parasitism	Reduction in the number of *B. tabaci* leading to reduction availability of the virus and so improved tomato production	(Attard, 2002)

Table 2.4 Effect of biocontrol agents on nematodes (or Meloidogyne species) that infest tomato

Pathogen suppressed	Plant disease	Biocontrol agent	Mechanism	Effect	Reference
Meloidogyne javanica	Root nematode	*P. fluorescens* CHAO	ISR-2,4DAPG	Improved plant growth and reduction in galls	(Siddiqui and Shaukat, 2003)
Meloidogyne incognita	Root nematode	*P. fluorescens*	Colonization of roots and direct antagonism	Improved plant growth and reducing galling and nematode multiplication	(Siddiqui, 2004)
M. incognita	Root knot nematodes of tomatoes	*Bacillus thuringiensis*	Competition	Bt7N had 76-84% control of root nematodes	(Mohammed *et al.*, 2008)
M. incognita	Root gall	*P. fluorescens* *P. chlororaphis*	Antagonism	Improved plant growth and reduction in galling	(Jonathan *et al.*, 2000)

2.3.1 Effects of microorganisms on plant

Some microorganisms are very useful for plant growth directly and indirectly. Beneficial effects of microorganisms can be grouped as Plant growth promoting effect and biological control effect (Vassilev *et al.*, 2006; Whipps, 2001). Fig 2.1 shows the mechanism through which these effects are carried out:

2.3.1.1 Mechanisms of Plant growth promoting effect of biocontrol agents

Plant growth promoting consequence of biocontrol agents is a primary effect because of its direct role in promoting plant growth. This effect can be seen in early seedling emergence, helping to colonize roots to ward off pathogens colonising them, promoting production of plant hormones such as gibberellins, auxins and cytokinins, enhancing plant mineral uptake and water utilization. This it does through the following mechanisms;

Phosphate solubilization: this involves the ability of some bacteria to make available to plants soluble phosphorus that has been liberated from insoluble calcium phosphate in the soil. Some microorganisms that liberate and solubilize phosphate include *Agrobacteria, Aspergillus, Bacillus, Enterobacter, Pseudomonas* and *Trichoderma*. Studies have shown that when these microbes are applied around the roots of plants in soils, they promote the growth of such plants and protect the plants from pathogens and their effects (Ouahmane *et al.*, 2007; Rudresh *et al.*, 2005).

Hydrogen cyanide production (HCN): some microorganisms produce Hydrogen cyanide (HCN) which effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations (Pal and Gardener, 2006). Some of the Rhizobacteria involved in the production of HCN are *Bacillus* and *Pseudomonas* and they produce it in the plant root nodules and rhizospheric soils (Ahmad *et al.*, 2008; Charest *et al.*, 2005).

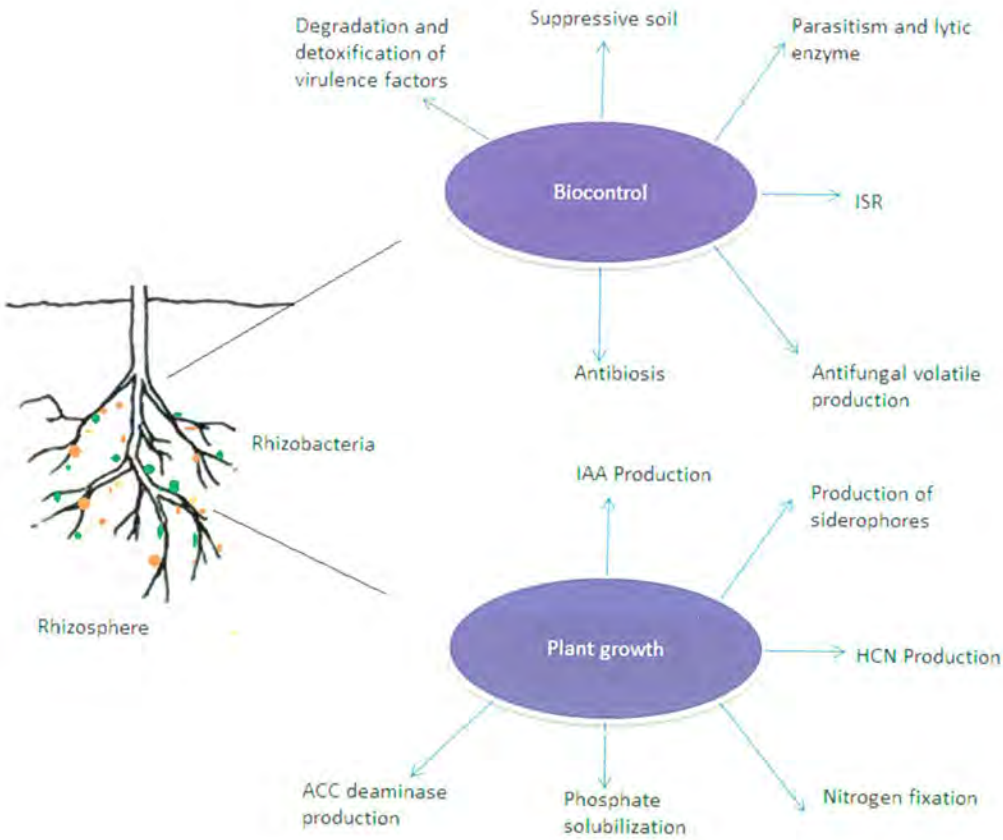

Figure 2.1 Schematic diagram of important mechanisms known for plant growth promotion and biocontrol through rhizobacteria (Source: this study)

Nitrogen fixation: Nitrogen-fixing organisms are generally active in the rhizosphere. They make atmospheric nitrogen available to plants. Some of these nitrogen-fixing microorganisms are *Rhizobium* spp., *Azospirillum* spp., *Azotobacter* spp., *Azolla* spp. and *Cyanobacteria* spp. (Shridhar, 2012). Others that are non-symbiotic nitrogen–fixers are *Bacillus* spp., *Pseudomonas* spp., *Enterobacter* spp. and *Clostridium* spp.

Siderophore production: All living things require iron for growth and when it is insufficient, the issue of competition comes up. Under this condition of insufficient rhizospheric iron, rhizobacteria produce siderophore which is a low-molecular-weight compound to competitively acquire ferric ion (Whipps, 2001). Microorganisms produce and secrete siderophores to sequester iron (Pérez-Miranda *et al.*, 2007). Pathogenic fungi have siderophores with lower affinity so are normally deprived of iron, even though bacteria

siderophores have different ability to sequester iron (Loper and Henkels, 1999). Siderophore solubilises iron in the environment and make it available to the plant. It is a high-affinity chelating agent for ferric iron (Crumbliss and Boukhalfa, 2002) and it helps in inhibiting the growth of phytopathogens (Qing-Ping and Jian-Guo, 2011). *B. subtilis* CAS 15 produced siderophore in pepper plant where it reduced the incidence of Fusarium wilt and promoted growth (Yu *et al.*, 2011). Ability to sequester iron differs from one bacterial siderophore to another but since pathogenic fungi siderophore have a relatively lower affinity for iron they are deprived of this essential element. Sometimes some Rhizobacteria go further to sequester iron from heterologous siderophore of cohabiting microorganisms (Whipps, 2001).

Production of Indole acetic acid (IAA): IAA is one of the most physiologically active auxins which is a plant hormone that regulates the amount, type, and direction of plant growth e.g. *Rhizobium* spp. (Mandal *et al.*, 2007). While working with chickpea, all isolates of *Bacillus* spp., *Pseudomonas* spp. and *Azotobacter* spp. produced IAA while only 85.7% of *Rhizobium* was able to produce it and promoted growth (Joseph *et al.*, 2007).

Production of 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase

Ethylene is one of plant hormones that are gaseous and it helps to regulate a number of developmental processes in plants. ACC is an immediate precursor of ethylene and it can be exuded from plant roots. Ethylene helps to break seed dormancy in many plants but after the seed has germinated, any further increase in the level of ethylene inhibits root elongation (Contesto *et al.*, 2008). Increased growth and yield of plants have been reported with plants that were inoculated with rhizobacteria containing ACC-deaminase (Shaharoona *et al.*, 2007; Zahir *et al.*, 2009).

2.3.1.2 Antagonistic and Biological control effects of some biocontrol agents on different plants

Biological control is the use of microorganisms to suppress or control diseases and infections in plants. These microorganisms might be residents in the soil or introduced into the soil. Some of the Rhizobacteria shown to have biocontrol abilities include; *Pseudomonas* spp., *Bacillus* spp., *Acinetobacter* spp., *Enterobacter* spp., *Micrococcus* spp., (Kumar *et al.*, 2012).

Workers at Montana State university found two strains of *B. pumilus* (strains 203-6 and 203-7) and one of *B. mycoides* (strain Bac J) that reduced the severity of *Cercospora* leaf spot of sugar beet, caused by *Cercospora beticola* sacc (Kloepper *et al.*, 2004). The plant root-

colonizing *B. amyloliquefaciens* strain F2B42 is a naturally occurring isolate and has the ability to stimulate plant growth and suppress plant pathogens (Idris *et al.*, 2007a). Research has it that on several hosts, specific strains of the species *B. amyloliquefaciens*, *Bacillus cereus*, *Bacillus mycoides, Bacillus pasteurii, B. pumilus, Bacillus sphaericus and B. subtilis*, has shown significant reductions in disease incidences and severity (Kloepper *et al.*, 2004).

Bacillus-based biological control agents (BCAs) have great potential in integrated pest management (IPM) systems (plant diseases inclusive). The biocontrol potential of *Bacillus* spp. as important agents to combat root and soil borne pathogen has been reported in many crops including chickpea. Antagonistic and antifungal activities against Fusarium wilt caused by *F. oxysporum* have been shown especially by several *Bacillus* spp. isolated from the rhizosphere of chickpea (Idris *et al.*, 2007a). In terms of plant disease control, biocontrol effects have been observed in sporulating gram positive bacteria like *Bacillus* spp. (Kloepper *et al.*, 2004).

Bacillus spp. was shown to have biocontrol potential against phytopathogenic fungi. For example *B. subtilis* was evaluated for biocontrol activities against *Botrytis cinerea*, a phytopathogenic fungus causing grey mold rot of tomatoes after harvest. The bacterium effectively suppressed the disease at 4°C and 23°C (Siripornvisal, 2010). Antagonistic activity by *B. amyloliquefaciens* LBM 5006 was observed against phytopathogenic fungi, including *Aspergillus* spp., *Bipolaris sorokiniana* and *Fusarium* spp. and pathogenic bacteria (Benitez *et al.*, 2010).

Biological control effects in most cases have secondary effect on the plant in that it helps to suppress the growth of plant pathogens and invariably promotes growth. Some of the mechanisms used to suppress or inhibit the growth of plant pathogens and diseases are;

Detoxification and degradation of virulence factors: Detoxification and degradation of virulence factors is one of the mechanism of biological control for example, albicidin is a toxin produced by *Xanthomonas albilineans* and which can be detoxified by certain biocontrol agents. This involves a protein that is produced and can irreversibly detoxify albicidin as a result of the occurrence of an esterase in *Pantoea dispersa*. This protein can also bind the toxin in both *Klebsiella oxytoca* and *Alcaligenes denitrificans* in an irreversible manner. Also, strains of *Burkholderia cepacia* and *Ralstonia solanacearum* have been observed to hydrolyse a phytotoxin known as fusaric acid, produced by various *Fusarium* species (Compant *et al.*, 2005).

Parasitism and Lytic enzyme production: Parasitism depends on the production of extracellular lytic enzymes and can be observed when pathogenic fungi degrade cell walls. This can be seen in some microorganisms like *Bacillus* degrading chitin because of its ability to produce enzymes that can break it down. Chitin is a major component of the cell wall of fungi and so *Bacillus* can serve as a biocontrol agent against plant fungal by degrading its chitinase cell wall (Kouki *et al.*, 2012).

Antibiosis: Many biological control agents such as *Trichoderma* spp. and *Bacillus* spp. could be effectively used in suppressing diseases caused by *Fusarium* spp., *Drechslera halodes* and *Rhizoctonia solani* as reported by many workers (Abdel-Monaim, 2010; Hashem and Hamada, 2002). A lot of *Bacillus* strains have been considered to be better biocontrol agents compared to fungal and gram negative bacterial biocontrol agents because of their ability to produce spores which invariably enhance their ability to withstand harsh environmental conditions. Also they can produce several broad spectrum antibiotics which can suppress the growth of a wide range of bacterial pathogens (Cui *et al.*, 2012). In addition to their antibiotic properties, *Bacillus* spp. enhance their antagonistic effects against fungal pathogens by competition for basic nutrients and space (Khokhar *et al.*, 2012). Also, some bacterial antagonists produce antifungal antibiotics (Sadfi *et al.*, 2002).

Induced systemic resistance (ISR): When some plants are infected by a disease, they respond with a signal that is salicylic-dependent. This normally led them to express a resistance that is broad-spectrum and long-lasting which is also efficient against bacteria, fungi and viruses. This is because the endogenous levels of salicylic acid (SA) normally increase in the phloem locally and systemically after infection before the occurrence of ISR but in some cases, SA is produced in non-infected parts and this also leads to systemic expression of ISR (Heil and Bostock, 2002). In a natural situation where the pathogen for a particular infection is more than one, the level of the basal resistance from the host is increased as a result of induced resistance (Van Loon and Glick, 2004). In greenhouse or field trials, elicitation of ISR by some strains of *Bacillus* spp. has been demonstrated on *Arabidopsis* spp., bell pepper, cucumber, loblolly pine, muskmelon, sugar beet, watermelon, tobacco, tomato and two tropical crops (green kuang futsoi and long cayenne pepper) (Kloepper *et al.*, 2004).

2.4 Adverse effects of using microorganisms as biocontrol agents on non-target organisms

Some of the adverse effects identified with the use of microorganisms for the biological control of plant diseases and seen as safety issues (hazards) include;

2.4.1 Allergenicity to humans and other animals

Allergenicity is a concern because of direct exposure of workers to the microorganisms but it is not a public health problem because most workers in agricultural settings are exposed to all kinds of allergenic particles. So in using biocontrol agents, allergy is not a new problem but it should be looked into during product formulation and development as safety issues (Hunsberger, 2000).

2.4.2 Displacement of non-target microorganisms

Competitive displacement is defined as the removal of a formerly established species from a habitat as a result of direct or indirect competitive interactions with another species. This can be through competition for carbon or energy; it can also be competition through the activity of PGPR that produce siderophore and make iron unavailable to root pathogens (Reitz and Trumble, 2002).

2.4.3 Pathogenicity to non-target organisms

This is one of the effects of using biocontrol which most people are concerned about. This could mean that the microbial biocontrol agent can also cause disease or is pathogenic to other beneficial organisms. It could also be that apart from helping the plant or pest to be able to induce disease resistant, it could cause disease in other plants or living organisms in that community. This is the most important issue where pathogenicity is concerned. For example, as far as pathogenicity to non-target organism is concerned no harmful effects have been reported on non-target organisms including mammals in using registered strains of *Bacillus* species for biocontrol (Lacey *et al.*, 2001).

2.4.4 Toxigenicity to non-target organisms

Some microorganisms produce antibiotics to help inhibit the growth of pathogens. These antibiotics can become toxic to non-target microorganisms. Even though this potential is there, so far there are no documented examples yet; perhaps because most of these antibiotics are produced in minute quantities and are basically used against the target organisms. For example, the research conducted using wheat take-all (*Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. tritici Walker) showed that *P. fluorescens* strain Q8r1-96 2–79 produced

2,4-diacetylphloroglucinol (2,4-DAPG) of 1850 µg/mL (Okubara and Bonsall, 2008) and which showed no effect on the establishment of mycorrhizal fungi in the rhizosphere (Khan, 2006).

Finally, apart from allergenicity, these other attributes such as displacement of non-target microorganisms, pathogenicity to non-target organisms and toxigenicity to non-target organisms are the same attributes that contribute to the efficacy and usefulness of microbial biocontrol agents.

2.5 Fate of microbial biocontrol agents in the biocontrol of tomato plant diseases

Looking at the various effects of using biocontrol for diseases in tomato plants, the non-use of microbial agents for disease control could be due to the following reasons:

Lack of fundamental information on the various microorganisms to be used as biocontrol is an issue that should be addressed. More research and knowledge into the ecology of these various microorganisms would improve on the technical difficulties experienced so far in the use of microorganisms for biocontrol.

Another important problem hindering the usage of biocontrol for plant disease is the fact that some of the microbial formulations may not be viable in long term plant disease controls (Handelsman, 2002).

Finally, the cost of product development and meeting with the regulatory agencies is high compared to the limitation of the market and its usability by farmers (Handelsman, 2002).

2.6 Fusarium wilt and the fungi pathogens

2.6.1 Origin, taxonomy and morphology

F. solani comprise a complex group of species that are widely distributed worldwide in soils. It causes tuber rot, and stem rots of plants. This complex group of species include at least 50 subspecies lineages (Schollenberger *et al.*, 2005). Taxonomically *F. solani* belongs to the kingdom Fungi, phylum Ascomycota, subphylum Pezizomycota, class Sordariomycetes, subclass Hypocromycetidae, order Hypocreales, family Nectriaceae and genus *Fusarium* (Fry, 2004).

2.6.2 Distribution and environmental conditions

Weather conditions such as temperature and humidity normally affect the distribution of *F. solani* (Kosiak *et al.*, 2004). *Fusarium* spp. are found to be widely distributed in nature in

various environments. Toxic *Fusarium* spp. can cause disease in both plants and animals, including humans. The fungi are mostly present in subtropical regions of the world (McGovern *et al.*, 2001). The optimum temperature for infection is between 27-31°C, but the disease can develop at a temperature that is lower, and soil moisture levels range from 28 to 75% with a pH between 6 and 8 (Glen *et al.*, 2003). However, the highest damage occurs in fields where the moisture content is low.

Fusarium wilt can remain viable in the soil for up to 30 years (Thangavelu *et al.*, 2004). It is destructive to plants and progresses swiftly. By the time a plant shows any outward signs of infection, it is already too late, and the plant wilts and dies.

2.6.3 The disease and the infection behaviour of the pathogens in plant

After conidial germination in the soil, entry of the pathogen into the plant system is through the root tips and is facilitated by the presence of wounds, which can be due to nematode feeding or through vascular wounds which can be sustained by the plant when cuttings are procured for planting or when leaves are detached from the stems.

In the pathosystem of tomato infected by *Fusarium* spp., the pathogen multiplies abundantly through much of the root tissues. It causes severe damage including cell disorganization as a result of *Fusarium* hyphae colonizing the roots and cell wall alterations (M'Piga *et al.*, 1997) which may be due to the production of plant chitinases. In addition, during pathogen ingress in the outer root cortex there is elaboration of callose-enriched wall appositions at sites of attempted fungal penetration. However, penetration of the callus that has grown over the wounds caused by the fungus is impossible. Enzymes may also facilitate *Fusarium* penetration into plant host (Babalola, 2010c). It was earlier proposed that there might be value to transforming biocontrol agents to overproduce hydrolytic enzymes (Babalola, 2007).

Each pathogen that entered the plant system through the root tip then grows intercellularly through the root cortex, and, subsequently rapidly grows into the water-conducting tissue (xylem) (Beckman, 1990). The microconidia produced invade the xylem of the pseudostem where it obstructs the upward movement of water and nutrients. Sieve cells in the stem hinder the conidia from further spread, and, as this happens the spores germinate and grow through the sieve cells where they continue to germinate until the whole xylem system is blocked. As the vessels are plugged and collapse, the water supply to the leaves is blocked, thus causing the wilt symptoms. In most cases, early sign of wilt appears on one-and-a-half month old plants and intensifies gradually. Wilting is seen on the lower leaves first and then extends to

the upper leaves. Leaves, twigs or even the whole plant turns brown and later dies and dries up (Khan and Khan, 2002). Death of the tomato plant is brought about by the failure of the infected xylem to meet the water requirements of the plant (Burgess *et al.*, 2008). As the plant dies, the spores grow into the surrounding tissues where they form chlamydospores that are released back into the soil (Jones, 2000). Chlamydospores can be in the soil until there is enough moisture in the soil for them to germinate and the process is set into motion again. Due to the presence of antagonistic microorganisms, insufficient moisture and alkalinity, germination can be impaired in certain soils.

The disease or infection can be transmitted through infected plant material and through contaminated soil. Other means of spreading the disease is through human movement around the infected field, or the use of irrigation water and implements previously used on an infected crop.

2.6.3.1 Symptoms of Fusarium wilt

Plants affected with Fusarium wilt first develop stunted seedlings and yellowing of the lowest leaves that is often restricted to one side of the plant or a single shoot and later show the defoliation of older leaves as shown in Figure 2.2. and Figure 2.3. The affected leaves wilt and die. Wilting progresses up the stem until the foliage is killed and the stem decays (Lund *et al.*, 1998). For this disease, brown vascular discoloration can be observed in stem tissue cross sections near the soil line (Burgess *et al.*, 2008) even though these stems remain firm and green on the outside. In some cases, affected plants do not die but are often stunted and their yields are quite reduced. In other cases where the inoculum pressure is high, seedlings may damp off as they emerge from the soil.

The general symptoms of many diseases of the root and stems are yellowing, wilting and stunting while a key symptom of pathogens which cause vascular wilt disease, including Fusarium wilt pathogens, is the browning of the internal stem (vascular) tissue (Burgess *et al.*, 2008). Reddish brown discolouration of the xylem vessels is visible inside the stem as lines (if the stem is cut longitudinally) or dots (if it is cut transversely). On the outside of affected stems, white, pink or orange fungal growth can be seen especially in wet conditions.

Figure 2.2 Tomato plant with no infection and no treatment.

Figure 2.3 Tomato plant showing signs of yellowing and wilting from the lower leaves.

2.6.3.2 Isolation of the pathogens from soil and infected plants

Generally, the fungus is isolated from infected plant tissues. These plant tissues are disinfected by soaking each sample in 1% sodium hypochlorite for 2 minutes. The tissues are placed on Potato Dextrose Agar (PDA) and left for incubation at room temperature (25°C) until the growth of colonies becomes visible (5 days). Morphological identification of *Fusarium* spp. is based on characteristics of the macroconidia, phialids, microconidia, chlamydospores, and colony growth traits and could also include using pathogenicity tests (Hibar *et al.*, 2007). Bimonthly infection and re-isolation of the pathogen from host plants is essential to maintain virulence (Babalola, 2007).

2.7 Cultural, physical and chemical control methods

Fusarium wilt is a difficult disease to control (Borrero *et al.*, 2006; Elmer, 2006; L'Haridon *et al.*, 2011). Some of the initial management strategies include cultural control, physical control and chemical control measures.

2.7.1 Cultural control

Cultural control involves practices and farming techniques that will help to increase the quality and quantity of the yield and also reduce the influence of pests and diseases. It involves manipulation of the environment in non-mechanical ways to control plant pests and plant diseases. It includes altering farming practices to make the environment unfavourable for the growth of disease pathogens and pests (Islam, 2001). It is also the purposeful manipulation of a garden or farm in the growing, planting and cultivation of plants to reduce plant disease, pest damage and numbers of pests. It has been shown that the correct implementation of cultural methods to control soilborne pathogens yielded improved soil structure and consequently decreases disease incidence (Neshev, 2008). These methods are mainly preventive and a good knowledge of the nature, behaviour and environmental conditions of the growth of the disease agent is very important in controlling the disease development. Some of the methods used in the cultural control of Fusarium wilt of tomato are shown in Figure 2.4.

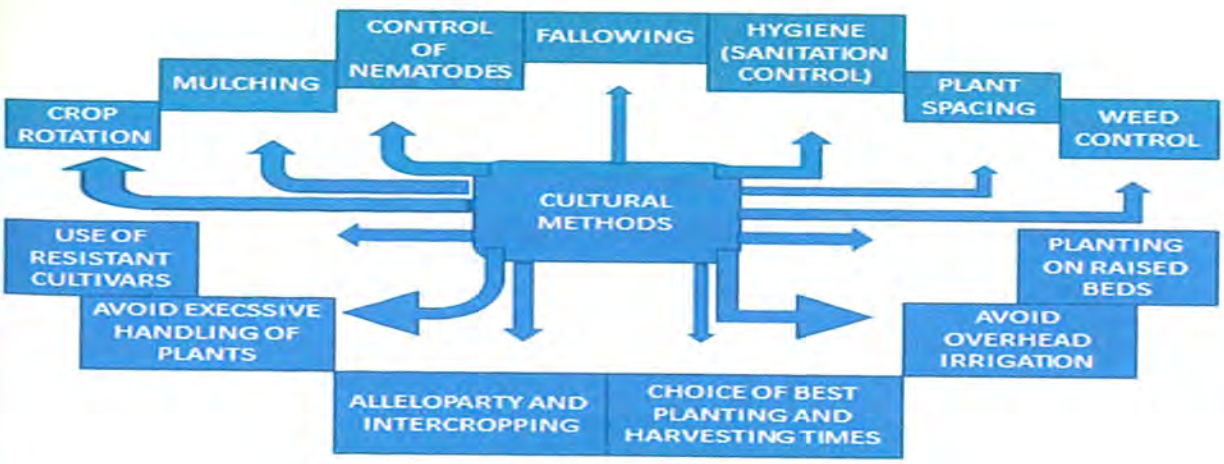

Figure 2.4 Some of the methods used in the cultural control of Fusarium wilt of tomato (Ajilogba and Babalola, 2013).

2.7.2 Physical control

Physical control includes soil solarisation and soil disinfection

2.7.2.1 Soil solarisation

Soil solarisation is done by spreading a clear plastic sheet over the soil for several weeks. This helps to trap solar energy which in turn inhibits soil borne disease pathogens, nematodes, insects and many weed seeds. This is usually done during summer when the air temperature is high and there is intense radiation (Ploeg and Stapleton, 2001).

2.7.2.2 Soil disinfection using heating and steam.

Soil disinfection is usually done as a preplanting method. Hot water can be used and can keep the soil sterilized for up to three years (Tateya, 2001). Steam can also be used especially in greenhouse conditions (Gullino, 2001). Soil disinfection helps to keep the soil sterile and free from disease causing pathogens.

2.7.3. Chemical control

Chemical control is the use of chemicals to control plant diseases (fungicidal, viricidal, bactericidal and nematicidal), pest infestation and weeds. In 2007, Nel *et al.*, (2007) reported that benomyl was partly effective against *F. oxysporum* f.sp *cubense* using the root dip treatment method. This method was applied by using carbendazim on tomato seedlings infected with Fusarium wilt and it led to about 24% increase in yield (Khan and Khan, 2002). Prochloraz has shown to be very effective against Fusarium wilt of tomato and banana (Nel *et al.*, 2007). Fungal diseases may be controlled through the use of fungicides and other agricultural practices; however, new variants of fungi often evolve that are resistant to various fungicides (Sierotzki and Ulrich, 2003). Apart from this, fungicides adversely affect other useful soil microorganisms, human health and also pollute the environment (Gerhardson, 2002). Chemical control measures create imbalances in the microbial community, which may be unfavourable to the activities of the beneficial organisms and may also lead to the development of resistant strains of pathogens. Pollution of the soil by overuse of chemicals has also led to harmful effects on humans such as oncogenesis (Asaka and Shoda, 1996).

Chemical control of the disease is not satisfactory because in most cases the disease is controlled by preplant soil fumigation with methyl bromide. In addition to other potential health hazards, safety and environmental risks, methyl bromide is classified as an ozone-

depleting compound and has been banned from use (Larkin and Fravel, 1998). There are no effective fungicides for the control of Fusarium wilt (Burgess *et al.*, 2008).

The lack of reliable chemical control agents, the development of fungicide resistant pathogens, and the breakdown or circumvention of host resistance by pathogen populations are among the key factors underlying efforts to develop other control measures (Weller *et al.*, 2002). The search for alternative strategies also has been stimulated by public concerns about the adverse effects of soil fumigants such as methyl bromide on the environment and human health (Weller *et al.*, 2002). Since the earliest observations of antagonist disease suppressing soil microorganisms more than 70 years ago, plant pathologists have been fascinated by the idea that such microorganisms could be used as environmentally friendly biocontrol agents both in the field and greenhouses. Thus biological control of Fusarium wilt can offer a potential alternative to chemical fungicides (Alwathnani *et al.*, 2012).

2.8 Biological control and biological control agents (BCAS)

Biological control offers a better alternative to the use of chemicals. It is the use of natural antagonistic organisms to combat pests or suppress plant diseases (Lugtenberg and Kamilova, 2009). Since PGPR (plant growth-promoting rhizobacteria) are known for growth promoting and disease reduction in crops, represents a potentially attractive alternative disease management method (Jetiyanon and Kloepper, 2002).

According to Jacobsen *et al.*, (2004), *Bacillus*-based BCAs have great potential in integrated pest management (IPM) systems (plant diseases included). Multiple *Bacillus* and P*aenibacillus* spp. can promote crop health in a variety of ways. Some populations suppress plant pathogens and pests by producing antibiotic metabolites, while others may directly stimulate nutrient uptake by plants, either through promoting rhizobial and mycorrhizal symbiosis or by fixing atmospheric nitrogen directly (Gardener, 2004). Specific strains of *B. amyloliquefaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides,* and *B. sphaericus* elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts (Kloepper *et al.*, 2004). Elicitation of Induced Systemic Resistance (ISR) by these strains has been demonstrated in greenhouse or field trials on tomato (Choudhary and Johri, 2009; Kloepper *et al.*, 2004).

2.8.1 Biocontrol by PGPR

PGPR may be defined as free-living rhizosphere-occupying bacteria that enhance plant growth and can also be classified as biocontrol agents, biofertilizers or biopesticides

depending on their activities/abilities (Labuschagne *et al.*, 2010). They can also be defined as root-colonizing bacteria (Rhizobacteria) that exert beneficial effects on plant growth and development, for example, in seedling emergence, colonizing roots, stimulating overall plant growth, mineral nutrition, and water utilization, as well as disease suppression (Choudhary and Johri, 2009). As they control plant pathogens, and promote plant growth, they are also involved with rhizomediation by degrading xenobiotic compounds and enhancing the efficiency of fertilizers (Kloepper *et al.*, 2004). Some of the reasons for the sustained interest in biocontrol by means of PGPR include the following:

- huge crop losses sustained due to diseases including soilborne diseases.
- increasing production costs, especially fertilizer costs
- the global trend toward the use of more environmentally friendly production methods (Labuschagne *et al.*, 2010).

2.8.2 *Bacillus* as PGPR

PGPR such as *Bacillus* spp. have received much attention as biocontrol agents. They are able to produce several broad spectrum antibiotics and have a longer shelf life as a result of their ability to form endospores (Cavaglieri *et al.*, 2005). This allows them to resist adverse environmental conditions and permit the easy formulation and storage of the commercial products (Francis *et al.*, 2010; Schallmey *et al.*, 2004). In addition to their antibiotic properties, *Bacillus* spp. exhibit antagonistic behaviour against fungal pathogens by competition or exploitation, which leads to predation and direct parasitism.

2.9 *Bacillus*

Bacilli are Gram-positive, rod-shaped bacteria and a member of the faranack Firmicutes. They can be facultative anaerobes or obligate aerobes. They can be free-living and pathogenic and test positive for the enzyme catalase. Under stressful environmental conditions, the cells produce oval endospores that can remain dormant over a long period of time under stressful and harsh environmental conditions until favourable conditions are restored. These endospores may remain viable for a long period of time and are resistant to sunlight, heat and chemicals.

2.9.1 Origin, taxonomy and morphology of *Bacillus*

Ferdinand Cohn, a contemporary of Robert Koch in 1872, renamed the bacterium *Bacillus subtilis*. This organism is described as Gram-positive, and it can grow in the presence of

oxygen, forms endospores in its resting phase and represents what has become a large and diverse genus of bacteria named *Bacillus,* in the family Bacillaceae. It was in 2004 that the genus *Bacillus* was split into several families and genera of endospore-forming bacteria, based on Single Stranded Ribonucleic Acid (ssRNA) analysis.

The taxonomic hierarchy of *Bacillus* according to Bergey's Manual 2004 is kingdom: Bacteria; phylum: Firmicutes; class: Bacilli; order: Bacillales; family: Acyclobacillaceae (genus: Acyclobacillus); family: Bacillaceae (genus: Bacillus, Geobacillus); family: Paenibacillaceae (genus: *Paenibacillus, Brevibacillus*); family: Planococcaceae (genus: Sporosarcina).

2.9.2 *Bacillus* distribution and environmental conditions

Bacillus spp. are widely distributed in nature, primarily in soil (Babalola and Akindolire, 2011), from which they invade dust particles. Some are in water while others are in the air. They are so numerous and include:

B. alcalophilus, B. amyloliquefaciens, B. anthracis, B. cereus, B. circulans, B. coagulans, B. firmus, B. laterosporus, B. licheniformis, B. megaterium, B. mycoides, B. polymyxa, B. pseudoanthracis, B. pumilus, B. sphaericus, B. stearothermophilus, B. subtilis, B. thuringiensis, B. vulgatis and *B. weihenstephanensis.*

In extremely harsh conditions, *B. subtilis* has the ability to form tough protective endospores. This ability to withstand harsh conditions makes *B. subtilis* a perfect candidate for probiotics. This is because they are able to balance the microbial community of the human guts which might have been depleted of some of its natural micro flora as a result of illness and breakdown in the immune system (Green *et al.*, 1999). *B. cereus* is mesophilic, and is capable of adapting to wide and varying environmental conditions, widely distributed in nature and commonly found in nature as a saprophyte. It grows optimally at temperatures between 20°C and 40°C (Vilain *et al.*, 2006). Since it is a soil bacterium, it can easily enter different food plants or food made from animals (eggs, meat, and dairy products) as they feed on the plants. It causes about 25% of food-borne diseases which occur when food has been left without refrigeration before being served, and toxins secreted like emetic toxins and enterotoxins into the food will lead to food poisoning (USFDA, 2007).

2.9.3 *Bacillus'* industrial and health significance

Some *Bacillus* spp. are pathogenic to plants, animals, humans and other organisms while some are very important and useful industrially and medicinally.

For example, some of the species secrete large amount of enzymes that are important like the enzyme Barnase secreted by *B. amyloliquefaciens*. Barnase, a ribonuclease is important as it helps to kill plant cells that have been infected by pathogenic fungi in order to stop the spread of the infection. *B. amyloliquefaciens* also is the natural source of amylase which is used in starch hydrolysis (Deb *et al., 2013*). Subtilisin, a protease widely used in commercial products, for example, in synthetic organic chemistry research, laundry and dishwashing, skin care ointments, detergents, food processing, cosmetics and contact lens cleaners, can also be secreted by *B. amyloliquefaciens*. Also the BamH1 restriction enzyme used in Deoxyribonucleic Acid (DNA) research is secreted by *B. amyloliquefaciens* (Leoenen *et al.*, 2014).

Useful antibiotics are also produced by several *Bacillus* spp. like bacitracin produced by *B. subtilis* and polymyxin produced by *B. polymyxa*. Part of the genome of *B. thuringiensis* incorporated into corn and cotton crop resulted in genetically modified plants that are resistant to some insect pests (Bt cotton).

2.9.4 *Bacillus* as biopesticides, biofertilizers and bioinoculants

Biopesticides are formulations containing microorganisms' mixtures that can safely control insect pests. For example *B. thuringiensis* has been formulated into microbial target-specific and environmentally friendly products that help to control insects and pests (Poopathi and Abidha, 2009). *B. subtilis* QST 713 has also been formulated into biopesticides (USEPA, 2006).

Biofertilizers are selected strains of beneficial microbes cultured in the laboratory and packaged in such a way that they can be applied for seed treatment (Muraleedharan *et al.*, 2010). They are preparations containing cells of microorganisms which may be nitrogen fixers, phosphorus solubilizers, sulphur oxidizers or organic matter decomposers

Bioinoculants are microbial inoculants or soil inoculants, they are microbes or beneficial endophytes that have been prepared into microbial products to promote plant health. Their mechanism involves the microbes forming symbiotic relationship with the crop or plant in question and both of them benefitting from the association. Applied microbial inoculants help

to improve plant nutrition, and promote plant growth by stimulating the production of plant hormone (Sullivan, 2001). This three-fold usefulness of *Bacillus* is illustrated below:

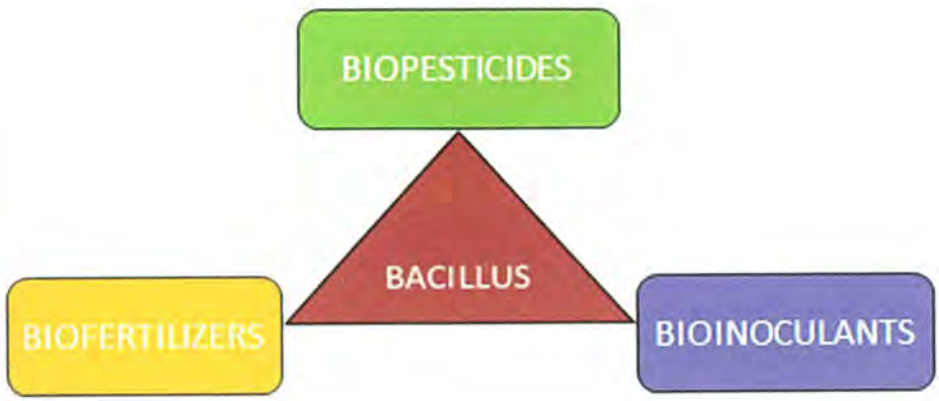

Figure 2.5 Three-fold usefulness of *Bacillus* species.

2.10 Molecular characterization

In the past decade different technologies and techniques have been developed to identify organisms without going through the process of culture based identification. In this case samples can be tested directly, identified and characterised at the molecular level. This is because, chromosomal DNA that is easily reproducible from bacteria that can be used for epidemiological, population genetics and ecological studies can easily be characterised and classified using genetic fingerprinting techniques (Forbes *et al.*, 1991). These techniques help to analyse multiple samples whose genetic diversity can be compared and their individual behaviour studied over time (Toro and Caballero, 2005).

Table 2.5 Some antibiotics produced by *Bacillus* as Biological Control Agents

Bacillus	**Plant pathogen**	**Disease in plant**	**Metabolites produced**	**References**
B. subtilis RB14	*R. solani*	Damping off of tomato	Iturin A and Surfactin	(Asaka and Shoda, 1996)
B. subtilis	*F. oxysporum* f.sp ciceris	Fusarium wilt of chickpea	Subtilin	(Karimi *et al.*, 2012)
B. subtilis AU195	*Aspergillus flavus*	Aflatoxin contamination	Bacillomycin D	(Moyne *et al.*, 2001)
B. amyloliquefaciens FZB42	*F. oxysporum*	Wilt	Bacillomycin and fengycin	(Koumoutsi *et al.*, 2004)
B. subtilis QST713	*B. cinerea* and *R. solani*	Damping off	Iturin A	(Kloepper *et al.*, 2004)
B. subtilis BBG 100	*P. aphanidermatum*	Damping off	Mycosubtilin	(Leclere *et al.*, 2005)
B. cereus UW85	*Phytophtora medicagins*	Damping off	Zwittermycin A, Kanosamine	(Milner *et al.*, 1996 ; Silo-Suh *et al.*, 1994)

Molecular characterization refers to the process by which organisms are identified and differentiated at the level of molecules or genetic components. It is also being able to detect variation in sequences, DNA and genes as a result of their differences or the different factors that have modified them. Small units of RNAs and DNAs that are amplified from nucleic acids of soil are made possible by using molecular techniques. These techniques help to give new insight into how diverse the phylogeny of microbial communities in soils is (Hill *et al.*, 2000). New tools like molecular markers can be effectively applied and targeted genes for

biocontrol can be discovered (Murthy *et al.*, 2006). Some of these molecular techniques are listed below;

2.10.1 Molecular Techniques

These include; the blotting techniques (Western blot, Southern blot, Northern blot), Gel Electrophoresis and Polymerase Chain Reaction (PCR). PCR-based technologies like restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and denaturing gradient gel electrophoresis (DGGE) and an ever increasing number of diagnostic microarrays (Brunner *et al.*, 2007). The following were used in the course of this research;

2.10.1.1 Gel electrophoresis

Gel electrophoresis is a molecular technique used to separate DNA, RNA or proteins. An electrical current is used to separate molecules like DNA, RNA, Proteins and PCR products on agarose gel. PCR products are electrically charged and will usually move towards the opposite charge when a current is applied to the gel. Molecules travel at different speed on the gel depending on their sizes. The larger molecules will travel slowly and will be seen closest to the wells of the gel while smaller molecules will travel faster and reach the far end of the gel.

At the end of the run, products are distributed variously on the gel. In a situation where the gel had been stained with ethidium bromide, the molecules will be seen to the unaided eyes as short blue bands. These can be viewed under the UV transilluminator. The gel can be used in different ways which include the DNA fingerprinting pattern. In their combination with blotting techniques, gels can be used to show whether specific DNA, RNA or protein molecules are present or absent. The dendrogram drawn from the computation of the presence or absence of bands as 1 and 0 respectively shows the relatedness and or diversity among the species.

2.10.1.2 Polymerase Chain Reaction (PCR)

PCR is a biochemical technique used in amplifying DNA using an enzyme called DNA polymerase. PCR plays important roles in fingerprinting, research, paternity testing, diagnosis, mutation, forensics, detection of disease and cloning of genes (Babalola, 2003). The PCR techniques used was the randomly amplified polymorphic DNA PCR.

2.2.1.3 Random Amplified Polymorphic DNA (RAPD) PCR

RAPD is a molecular marker technology based on selective or differential PCR amplification of a small sample of DNA a million fold from short oligonucleotide sequences. This then produces a large amount of DNA products. This molecular marker is based on the PCR amplification of random locations in the genome of the organism in such a way that in order to prime the amplification of the genomic DNA, a single oligonucleotide is used. The primers can anneal on different location in the genome. They must bind to repeated sequences that are inverted in order for the products to be amplified and the sequences must be between 150-4000 base pairs apart. The amount of amplification products is determined by the complementarity of the number and orientation of the sequences to the primer in the genome of the organism. It is commonly used in genetic diversity studies (Kumar and Gurusubramanian, 2011).

Agarose gel electrophoresis is used to separate the amplified PCR products into bands of different patterns which correspond to different molecular weight DNA. Each of these banding patterns are referred to as finger prints of that individual or population and can be used to differentiate it from other genetic types and invariably characterise and classify the organism (Ruiz *et al.*, 2000).

Fingerprints are band patterns of RAPD-PCR and are used to characterize and show the relatedness within a particular bacterial strain or among a bacterial species (Babalola *et al.*, 2002; Welsh and McClelland, 1990). Differentiation between strains and species of *Bacillus* isolates have been carried out using RAPD-PCR fingerprinting oligonucleotide primers with arbitrary sequences (Daffonchio *et al.*, 1998; Daffonchio *et al.*, 1999).

2.10.2 Effects of using RAPD-PCR to test for genetic diversity on various organisms

Some of the effects of using RAPD-PCR to test for genetic diversity on various organisms as carried out by other researchers are listed below;

Baysal *et al.*, (2008) performed RAPD-PCR using primers OPA01, OPA09, OPA 11, OPE02, OPE04, OPF06, and OPH 09 to determine the similarity or difference between *Bacillus subtilis* EUO7 and commercially made QST713 used in the biocontrol of tomato root rot caused by *F. oxysporum* f.sp *radicis-lycopersici* (FORL). It was observed that the difference between the two organisms could be as a result of the ability of EU07 to produce antifungal metabolites against FORL.

Using primers OPA 09, OPA 10 and OPA16, Babalola *et al.*, (2002) showed similarity between *E. sakazakii* and *K. oxytoca* compared to *Pseudomonas* spp. using RAPD-PCR. *Pseudomonas* spp., *Enterobacter sakazakii* and *Klebsiella oxytoca* had been used as biocontrol agents against *Striga hermonthica* (Del.) Benth. causing *Striga*-infestation of maize and sorghum.

RAPD-PCR was used in identifying six different *Fusarium* spp. from plants viz *F. solani, Fusarium semitectum, Fusarium graminearum, Fusarium moniliforme, Fusarium culmorum, and F. oxysporum* having two formae speciales *i.e. F. oxysporum* f.sp *lycopersici* and *F. oxysporum* f.sp *vasinfectum.* Eight primers were used out of which 6 differentiated some and not all the *Fusarium* spp. This is because there was still similarity between 2 or 3 of the *Fusarium* spp. But 2 of the primers differentiated all the *Fusarium* species including the 2 formae speciales and did not show 100% similarity among any of them (El-Fadly *et al.*, 2008).

2.10.3 Importance and advantages of RAPD -PCR

The RAPD technique is simple and it can be applied to a diversity of fields. It requires very small amount of DNA to generate large numbers of genetic markers. It does not require any other form of characterisation like sequencing, cloning etc. before it can be carried out. It does not require previous knowledge of the DNA sequence of the targeted gene. Because the primer will bind randomly on the sequence, this technique is important in comparing the DNA of biological system (plants, animals, microorganisms) in which only few DNA's have been compared. It is quick, simple and efficient. The procedure is not laborious and not time consuming. It is less expensive because the unit cost per assay is low compared to other types of marker technologies. It produces large amounts of fragments (De Vicente and Fulton, 2004). It requires universal primer and it can used to in genomic analysis of different range of species of organisms (Diaz-Guera *et al.,* 2000). It does not require prior knowledge of the sequence of the DNA and it allows multiple loci to be investigated in a single PCR reaction (Bhattacharya and Ranade, 2001).

2.10.4 Reasons for using RAPD-PCR in this study

Since all the rhizobacteria used in this study are *Bacillus*, RAPD-PCR will help to show the similarities and differences between the different *Bacillus* isolates (Irshad and Nawad, 2012). This similarities and differences will help us to understand the variation in their biocontrol

abilities and this could lead to more research where specific genes are isolated and probably cloned as the case may be.

2.10.5 Limitations of RAPD-PCR

One of the prominent draw-backs of this technique is that sometimes band repeatability is low and this makes reproductivity a challenge; and also there might be issues with pseudo bands. Problem of pseudo bands could be resolved by stabilizing amplification protocol and making sure there are no impurities in extracted DNAs (Irshad and Nawab, 2012)

2.10.6 Derivation of dendrograms from the gel pictures

In other to derive dendrograms from gel pictures, a scoring matrix will be created for the gel. Then the bands will be named by their primers and isolate number e.g. OPH1 means primer OPH was used and 1 is isolate 1. On the columns, presence of bands is represented as 1 while absence of band is represented as 0.
Once this is done, the site below is opened and value in column 1 is put in a1, column 2 in a2 and this is done until the entire column values are exhausted. When this is submitted, the similarity matrix is automatically constructed and phenogram is selected which draws the dendrograms.
http://genomes.urv.cat/UPGMA/index.php?entrada=Example2

CHAPTER 3

MATERIALS AND METHODS

3.1 *Bacillus* spp. Inoculum preparation

The following typed cultures and locally isolated organisms (LIO) from the culture collection of the Microbial Biotechnology Research Group of the Biological Sciences department of the North-West University Mafikeng Campus, South Africa were used for this study: *B. subtilis* (ATCC 11774), *B. cereus* (ATCC 11778), *B. amyloliquefaciens* (LIOBac179), *B. pumilus* (LIOBac269), *B. thuringiensis* (LIOBacT51), *B. subtilis* (LIOBacS35), *B. subtilis* (LIOBacS12), *Brevibacillus* (LIOBacBb40), *B. mojavensis* (LIOBacM5) *B. methylotrophicus* (LIOBacM8) and *Bacillus 282* (LIOBac282). The inoculum preparation was carried out according to Cavaglieri et al. (2005). Two loopfuls of each of the bacteria from 3-day old cultures on tryptic soy agar (TSA) were transferred separately to 50 ml tryptic soy broth (TSB) medium and incubated overnight at 28±2°C. Viability was confirmed by standard plate count method using trytone soy broth plus 2% agar (TSBA). These inocula were prepared in order to use them *in vitro* for antifungal activities of the *Bacillus* isolates and also their biocontrol activities *in vivo.*

The inocula for use in the greenhouse were prepared from a 24 h culture of each of the *Bacillus* isolate incubated at 28±2°C. Ten-fold serial dilution was carried out and a concentration of 5×10^7 was achieved. Five ml of *Bacillus* suspension containing 5×10^7 cfu/ml was used as inoculant in the greenhouse.

3.2 Preparation of phytopathogenic fungi

Fusarium solani ATCC 36031 (Davies Diagnostic, South Africa) was used for the research. Fungal strains, and inocula were prepared by culturing them on Potato dextrose agar (PDA) for 10 days in Petridishes. The microconidial suspension of *F. solani* was prepared by pouring 1 ml of sterile water in each Petridish in order to loosen the spores from the medium. The inoculum was then scrapped with the aid of a sterile spatula from the surface of the Petridishes in other to be sure of the viability of the cells and 1 ml was made up to 20 ml in sterile bottles. The bottles were properly shaken in the rotary shaker to dislodge the spores from the mycelia of the fungi to get a concentration of 10^6 spore concentration. The spore concentration was adjusted to the required concentration of 10^9 spores/ml by taking 1ml of the spore suspension and diluting with 9ml sterile distilled water and then injected into sterile soils in the greenhouse (Adebayo and Ekpo, 2005).

Table 3.1 Molecular description of *Bacillus* isolates

Organism Legend	Description	Isolate accession/assigned accession number
Bac A	*B. amyloliquefaciens*	KF433086
Bac C	*B. cereus*	KF433085
Bac P	*B. pumilus*	KF433087
Bac S	*B. subtilis*	KF433084
Bac 282	*B. subtilis*	KF433088
Bac Bb	*Brevibacillus* sp	JX971521
Bac M5	*B. mojavensis*	JX971525
Bac M8	*B. methylotrophicus*	JX971526
Bac S12	*B. subtilis*	JX971518
Bac S35	*B. safensis*	KC113510
Bac T51	*B. cereus*	KC113513

Bac A = *B. amyloliquefaciens,* Bac C = *B. cereus,* Bac P = *B. pumilus,* Bac S = *B. subtilis,* Bac 282 = *B.,* Bac Bb = *Brevibacillus* spp., Bac M5 = *B. mojavensis,* Bac M8 = *B. methylotrophicus,* Bac S12 = *B. subtilis,* Bac S35 = *B. safensis,* Bac T51 = *B. cereus.*

3.3 Antifungal activities of *Bacillus* isolates

Based on antifungal activities, the *Bacillus* isolates were tested for the following:

3.3.1 Detection of Hydrogen cyanide (HCN) production

Production of HCN was detected according to the method of Lorck (1948). Freshly grown cells were spread on a tryptone soy agar (30 g) to which 4.5 g/l glycine had been added and a sterilized filter paper saturated with 1% solution of picric acid and 2% sodium carbonate was placed in the upper lid of the Petridish, which was then sealed with parafilm and incubated at

30°C for 4 days, a change in colour of the filter paper from yellow to reddish brown was an index of cyanogenic activity while no colour change represent no cyanogenic activity.

3.3.2 Determination of Indole acetic acid production (IAA)

Eight grammes of nutrient broth (Merck) was suspended in 500 ml of distilled water; freshly grown cultures were inoculated into 10 ml nutrient broth to which tryptophan had been added (1 mg and 3 mg tryptophan, in order to ascertain the effect of tryptophan concentration on the production of IAA) in each test tube and incubated at 30°C for 48 h. A 4 ml culture was removed from each test tube and centrifuged at 10,000 rpm for 15 min. An aliquot of 1 ml of supernatant was transferred into a fresh tube to which 50 µl of 10 mM orthophosphoric acid and a 2 ml of Salkowski reagent comprising of (1 ml of 0 .5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated at room temperature for 25 minutes. The development of a pink colour indicated the presence of indole acetic acid (Brick *et al.*, 1991). The absorbance of the pink solution from each isolate was measured and recorded at 530 nm using spectrophotometer (Thermo Spectronic, Merck, SA).

3.3.3 Phosphate solubilisation

Phosphate solubilization is a complex phenomenon for selectively screening the bacteria which have the ability to release inorganic phosphate from tricalcium phosphate (TCP). In order to examine the *Bacillus* isolates for their ability to solubilize phosphate, a standard agar medium (pH 6-7) i.e. Pikovskaya's medium; a selective medium for phosphate solubilizing microorganisms (PSM) containing 5 g of TCP as a sole source of phosphorus was prepared and used. The addition of TCP was to enhance formation of halo zones. The medium was autoclaved at 121°C for 15 min and poured into Petridishes. Isolates were streaked on the Petridishes and incubated for 3 days at 27°C. *Bacillus* isolates that were able to solubilize phosphate developed clear zones around colonies.

3.4 Antagonistic activity of *Bacillus* isolates

The test was carried out to see the effect of the *Bacillus* isolates on the fungi mycelial growth *in vitro*. Fungal mycelial growth inhibition tests were performed by plate assay. A loop of *Bacillus* culture was streaked over the surface of a PDA plate (Biolab) and other loopful containing mycelia of *F. solani* was point inoculated. Petridishes were then incubated at 25°C for 10 days and examined for inhibition of fungus mycelium growth by the *Bacillus* isolates. A reduction of mycelial growth around the *Bacillus* isolate compared to the control indicated positive response. The diameters of mycelia growth were measured after 10 days (Yuan *et*

al., 2012). Each experiment was repeated four times. The results were expressed as the mean values with standard error deviation in inhibition distance between the growths of the corresponding *Fusarium* isolate and the presence of the *Bacillus* isolate tested. Percentage mycelial inhibition was calculated as follows:

% inhibition = 1-(fungal growth/control growth) × 100

Control experiment with only growth of *Bacillus* isolates and fungi in two separate Petridishes were observed.

3.5 Experimental design

The experiment was carried out in a completely randomized block design, with 4 main blocks:

- Tomato planted without *F. solani* and *Bacillus* isolates.
- Tomato planted with only the different *Bacillus* isolates.
- Tomato planted with both *Bacillus* isolates and *F. solani.*
- Tomato planted with only *F. solani.*

3.6 Green House experiment

Two-week old tomato seedlings were transplanted into 24cm-diameter pots. Each pot contained sterile vermiculite, peat, and perlite in the ratio 3:4:1. At 5 weeks, the different treatments with *Bacillus* isolates were applied as outlined in the experimental design using 4 trials (each trial comprised of 120 plants) for each of the *Bacillus* isolates, a negative control with no *F. solani* and a positive control with inoculation of *F. solani.* Plants were watered daily in the green house at 25°C at 60-90% relative humidity for 10 weeks. At the end of 10 weeks, samples were harvested to assess the effect of the various *Bacillus* isolates on the different growth parameters. Significant difference was assessed from the mean of each of the different treatments. The experiment was repeated twice.

The growth parameters assessed include: length of shoot, length of leaves, number of branches, fresh and dry mass of shoot and root length and fresh and dry mass of tomato fruits. Four randomly selected seedlings were used to determine each parameter per treatment.

3.6.1 Disease scoring and data recording

Disease incidence was recorded by counting the number of infected plants and dividing it with the total number of plants assessed in each treatment. The result obtained was converted to percentage using the formula:

Disease incidence = (Number of diseased plants /number of plants assessed) x 100 (Haruna *et al.*, 2012).

Percent disease control was obtained by the formula below

$$\frac{\text{Number of diseased plants in control} - \text{Number of disease plant in treatment}}{\text{Number of plants in control}} \times 100$$

3.6.2 Determination of fresh and dry weight of plant shoots and roots

The fresh weight of seedlings of different treatments was determined. This was after recording the symptom development and percentage of infection. They were removed, washed with sterile distilled water, blotted with tissue paper, and weighed. Seedlings were then dried at 28°C for 3 weeks until the dry weights were stable and the dry weights taken.

3.6.3 Determination of fresh and dry weight of Tomato fruits

The samples were harvested at the end of 10 weeks to analyse the quality and mineral composition of the fruits. Freshly plucked tomatoes from each treatment were weighed and the weight recorded. The colours were also noted. Tomato fruits were sliced transversely with sterile scalpel and dried at 28°C for 3 weeks until the dry weights were stable and hence recorded.

3.6.4 Determination of mineral composition of tomato fruits

Harvested tomato fruits were washed and blotted with tissue paper. Each was sliced using sterile scalpel. Each tomato sample was kept on sterile plates and air dried in the oven for 5 weeks and pounded separately in a clean sterile mortar. The pounded samples were taken into the Energy-dispersive X-ray spectroscopy (EDS, EDX, or XEDS) to determine their mineral composition. The EDX 720 is an analytical tool for the chemical characterization of a sample. The data generated by EDX analysis consist of spectra showing peaks corresponding to the mineral elements making up the true composition of the sample being analyzed. It measures a range of atomic elements from sodium (Na) (11) to Uranium (U) (92) (Na-U).

3.7 Molecular characterization of *Bacillus* isolates

3.7.1 DNA extraction from bacterial isolates

Genomic DNA of all isolates were extracted using ZR soil Microbe DNA MiniPrep™ (Zymo Research, USA) extraction kit. It was used to obtain genomic DNA from all *Bacillus* isolates. Bacterial cultures were grown in 10 ml of Luria Bertani broth (Merck) at 37°C for 24 h and then centrifuged at 10,000 rpm (Universal Z300K model centrifuge; HERMLE Labortechnik, Germany) for 5 minutes. The bacterial pellets were resuspended in 200 µl of distilled water and transferred to ZR Bashing Bead™ lysis tube and 750 µl lysis solutions were added to the tube. The bashing bead was secured in a bead beater fitted with a 2-ml tube holder FastPrep® 24 and processed at a maximum speed for 5 minutes. The ZR BashingBead™ lysis tube was centrifuged in a microcentrifuge at 10,000×g for 1 minute, 400 µl of supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and was centrifuged at 7,000×g for 1 minute and 1,200 µl of Soil DNA Binding Buffer was added to the filtrate in the collection tube. Eight hundred micro litres of the mixture of the binding buffer and filtrate was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000×g for 1 minute. Two hundred micro litres of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000×g for 1 minute. Five hundred microliters Soil DNA Wash Buffer was added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000×g for 1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml micro-centrifuge tube and 100 µl DNA Elution Buffer was added directly to the column matrix. The tube was centrifuged at 10,000×g for 30 seconds to elute the DNA.

3.7.2 RAPD-PCR analysis and Agarose gel electrophoresis

Three primers were used in this project: OPH-19 (5'-CTGACCAGCC-3'), S4 (5'-GTCGCCGTCA-3') and A9B7 (5'-GGTGACGCAGGGGTAACGCC-3') (Inqaba biotechnology SA). DNA amplifications were carried out in a total volume of 25 µl (1.5 mM, PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM (each) dNTP, 50 ng primer, 5 U Taq DNA polymerase (Fermentas), 20 mM Tris–HCl, pH 8.4 containing 50 mM KCl), 50 ng template DNA, ddH_2O according to Zhao, (2006). The cycling conditions for RAPD carried out using a C1000 Touch™ thermal cycler (Bio-Rad, USA) were as follows: an initial denaturing at 94°C for 1 minute, 40 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, and 72 °C for 2 minutes. A final extension of 10 minutes at 72°C was included.

3.7.3 Agarose Gel Electrophoresis Procedure

One times Tris-Acetate EDTA (1XTAE) buffer was prepared by adding 4900ml of distilled water to 100ml of 50X TAE (375ml of Tris-Cl, 28.55ml of acetic acid, 50 ml of EDTA and 46.45 distilled water) and filled in the electrophoresis tank. 1.5% agarose gel (4.5g in 300 ml of 1XTAE buffer) was prepared by melting in microwave until boiling. Once agarose was dissolved, it was allowed to cool to ~45°C before pouring into the casting mold or tray. Before pouring the agarose gel, combs of desired sizes were inserted into the tray in such a way that no bubbles were caught under the teeth. After the gel had set, the combs were gently removed. The gel was placed in the electrophoresis tank to which 150 µl ethidium bromide has been added to 1.5 l buffer to cover it to a depth of about 1 cm.

PCR samples were prepared by mixing 10 µl of the PCR reaction mixture with 10 µl of 1X loading dye in a 1:1 ratio on sterile parafilm. Twenty micro litre samples were loaded into each well in the gel with a sterile micropipette and taking care not to cross-contaminate the wells. Six micro litres of molecular marker (1 kilo base (kb) DNA ladder (Fermentas)) was loaded in the first well of each comb.

The voltage was set to 60 V and top cover was attached, making sure that the polarity of the preparation was placed correctly (wells are placed closest to the negative pole, DNA will migrate to the positive pole).

After about 1h when the loading dye had migrated to the mid-point of the gel, the power was turned off and the gel removed. DNA fragments were visualised by removing the gel slab from the tray and placing it on a UV transilluminator. The outcome of running the gel was recorded/ captured using Chemidoc™ MP imaging system (Bio-RAD USA).

3.8 Statistical Analysis

Analysis of variance (ANOVA) was performed to find out if there was any difference between groups on specific treatments and the degree of significance of the differences among the variables (treatments) was determined using mean values considering the standard error of mean (SEM). Differences were calculated with Least Significant Difference (LSD) analysis (P=0.05) with the Statistical Package for the Social Sciences (SPSS) 11.0 between treatments. Duncan's LSD test at p=0.05 was used to compare the Means. Data are reported in the text, tables and figures as mean values for each treatment per test or for all tests ± standard error of the mean (SEM), which is a statistical parameter measuring the accuracy

with which a sample represents a population. The smaller the standard error, the more representative the sample will be of the overall population.

3.9 Data Analysis of RAPD-PCR

RAPD produces a large number of DNA bands of various sizes from each of the different samples which were prepared. To analyse RAPD data, the total number of unique bands (thus if several lanes share a band, that band is only counted once toward the total) for each primer used were counted. Polymorphic bands were evaluated as being either present (1) or absent (0). The score band data (present or absent) were analysed according to Sokal and Sneath, (1963). A similarity index was used to compare genetic differences.

Similarity index= a/a+b where: a = number of bands showing homology between two samples, b = number of bands showing no homology between two samples. The genetic distance between two genotypes was calculated by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Version 1.8) software (Rohlf, 1990).

CHAPTER 4

RESULTS

Eleven native *Bacillus* species were characterised using RAPD after which *in vitro* analysis to show their biocontrol potentials were carried out. Based on Literature review and random selection, 4 out of the 11 were used as antagonists against *F. solani* in the greenhouse. Results of tests carried out to investigate the mechanisms of biocontrol and plant growth promoting potential of the selected antagonists *in vitro* and *in vivo* are presented below.

4.1 *In vitro* antagonistic effects of *Bacillus* isolates

In vitro analysis revealed that the four *Bacillus* isolates *viz B. amyloliquefaciens, B. cereus, B. pumilus and B. subtilis* inhibited the growth of *F. solani* significantly. The results obtained are presented in Figure 4.1.

The outcomes showed that *B. amyloliquefaciens* inhibited the mycelia growth of *F. solani* the most by 95.20% with only 2.50 mm growth in diameter of *F. solani* while *B. cereus* had the highest growth of *F. solani* mycelia with 23.07 mm growth in diameter and inhibition of 55.70%. The other *Bacillus* isolates also reduced the mycelia growth of *F. solani* significantly as shown on Figure 4.1.

4.2 The Possible mechanism of biocontrol of *Bacillus* isolates

The mechanism of antagonism of the four *Bacillus* isolates was investigated and the results are in Table 4.1.

4.2.1 Phosphate solubilization

Bacillus isolates were examined for their ability to solubilize phosphate as an indirect mechanism of biocontrol by modifying the environmental condition. Only *B. amyloliquefaciens* showed clear zones around the streaked isolate. All the others were negative without any clear zones (Table 4.1).

4.2.2 HCN production

None of these isolates produced HCN (Table 4.1).

4.2.3 IAA production

Production of IAA by all the *Bacillus* isolates was detected by the production of pink colour. Production of IAA was not dependent on the presence of tryptophan even though highest concentration was read from *Bacillus* isolates to which tryptophan had been added. This also

means that there is some correlation between the amount of tryptophan and amount of IAA produced. All the *Bacillus* isolates produced indole acetic acid when grown in media containing tryptophan which was apparent by the production of pink colour (Table 4.1.). Using the spectrophotometer (Thermo Spectronic, Merck, SA), absorbance at 530 nm revealed that *B. amyloliquefaciens* and *B. cereus* had the highest absorbance of 0.29 nm from the test tube having 3 mg tryptophan while *B. subtilis* had the least absorbance of 0.26 nm from the 3 mg test tube. All the *Bacillus* isolates inoculated with or without tryptophan showed different levels of absorbance but the levels of absorbance gradually increased from isolates inoculated without tryptophan to isolates inoculated with 3 mg tryptophan. Increase in the absorbance from zero tryptophan to 1 mg tryptophan was 3% while that of 1 mg tryptophan to 3 mg tryptophan was 13.95% in *B. amyloliquefaciens*. Absorbance values for *B. cereus*, increased by 14.51% (for zero tryptophan to 1 mg tryptophan) while the values increased by 3.52% in samples with tryptophan from 1 mg to 3 mg tryptophan. In *B. pumilus*, the increase from zero tryptophan to 1 mg tryptophan was 3.83% while that of 1 mg tryptophan to 3mg tryptophan was 4.79%. Absorbance level in *B. subtilis*, increased from zero tryptophan to 1 mg with 9.29% while from 1 mg to 3mg with 6.47%. This shows that the highest increase in the level of absorbance based on the increase in tryptophan was exhibited by *B. amyloliquefaciens* while *B. cereus* exhibited the lowest.

4.3 The Biocontrol potential of *Bacillus* isolates on Fusarium wilt of tomato plants in the green house

As a result of the *in vitro* performance of the four *Bacillus* isolates in antagonising the growth of *F. solani*, greenhouse experiments were carried out to analyse their biocontrol activities. Greenhouse experiments were carried out by using completely randomized block design, with 4 main blocks:

- Tomato planted without *F. solani* and *Bacillus* isolates.
- Tomato planted with only the different *Bacillus* isolates.
- Tomato planted with both *Bacillus* isolates and *F. solani*.
- Tomato planted with only *F. solani*

The results obtained are presented in Table 4.3 and Table 4.4 and Figure 4.2a and b, Figure 4.3a, b, c, d and Figure 4.4.

4.3.1 Degree of Disease control using *Bacillus isolates* as treatments

Degree of disease control was measured as a percentage of infected plants or uninfected plants as a fraction of all the plants. Treatments of tomato using the *Bacillus* isolates in the greenhouse were significantly different and varied from 62.50% in both *B. pumilus* and *B. subtilis* to 81.20% in *B. cereus*. The use of *B. cereus* showed the highest disease control while treatment with *B. amyloliquefaciens* was 75% (Figure 4.2b). As such, treatment with *B. cereus* was significantly higher in terms of disease control (Figure 4.3d).

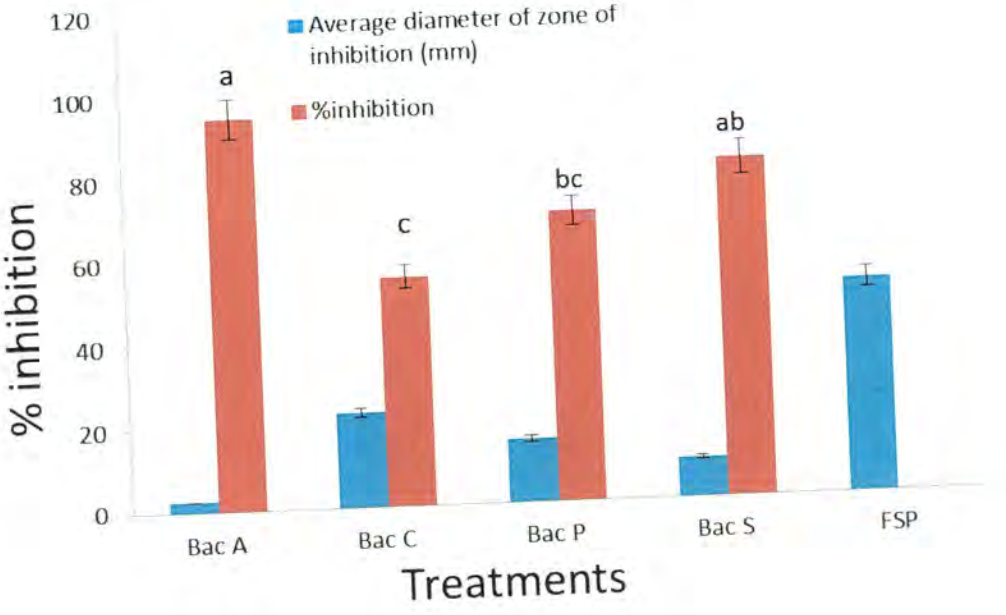

Figure 4.1 Inhibitory effect of the four *Bacillus* isolates against *F. solani in vitro* at P=0.05. Bac A = *B. amyloliquefaciens*, Bac C = *B. cereus*, Bac P = *B. pumilus*, Bac S = *B. subtilis*. Percent inhibition value for Bac A, Bac C, Bac P and Bac S is 95.20, 55.70, 70.46 and 82.10 respectively while FSP is the growth of *F. solani* alone in the Petridish. Each value is average of four replicates.

Table 4.1 Mechanism of inhibition by four *Bacillus* isolates.

Antifungal metabolite production					
Treatments	Phosphate solubilization	HCN	IAA		
			No tryptophan	1mg tryptophan	3mg tryptophan
Bac A	+	-	0.249	0.258	0.294
Bac C	-	-	0.248	0.284	0.294
Bac P	-	-	0.261	0.271	0.284
Bac S	-	-	0.226	0.247	0.263
Control (Sterile distilled water)	-	-	0.064	-	-

All isolates were negative to HCN production and all were positive to the utilization of tryptophan which is a precursor of IAA while only Bac A solubilized phosphate. Bac A = *B. amyloliquefaciens,* Bac C = *B. cereus,* Bac P = *B. pumilus,* Bac S = *B. subtilis.*

4.3.2 Degree of disease incidence in tomato plants after treatment with *Bacillus* isolates

The incidence of disease also followed the pattern of percent disease control with the lowest disease incidence in *B. cereus* and the highest in both *B. pumilus* and *B. subtilis*. Treatment with *B. amyloliquefaciens* was not significantly different from *B. pumilus* and *B. subtilis*. Only treatment with *B. cereus* was significantly different from all the other treatments and the control. In general, the four *Bacillus* isolates were effective in disease control and reducing disease incidence. Degree of disease incidence was 100% in the control which was inoculated with only *F. solani* but no incidence of disease was associated with the plants that were not inoculated with *F. solani* or treated with any of the *Bacillus* isolate (Figure 4.2a and Figure 4.3).

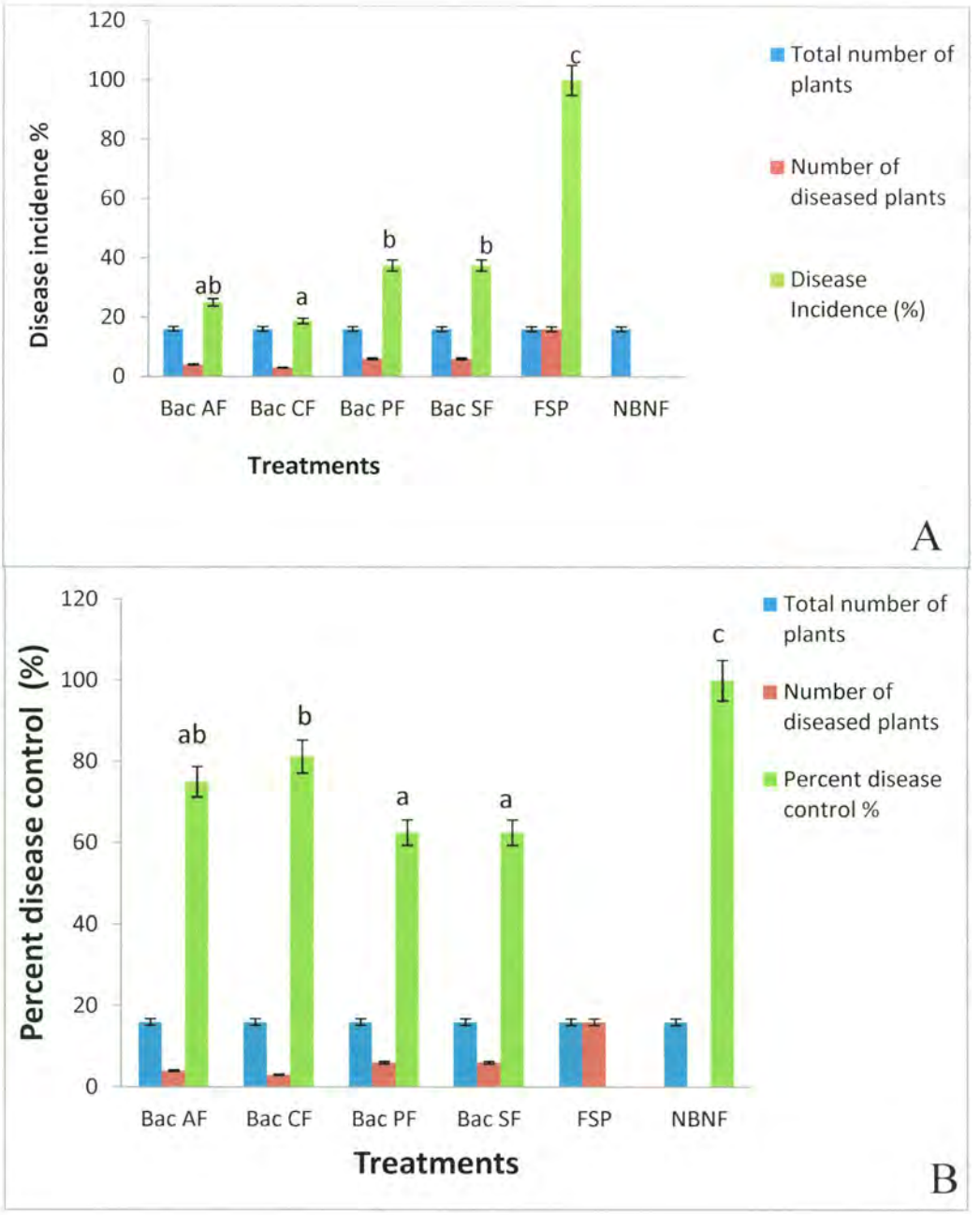

Figure 4.2 Disease incidence (A) and degree of disease control (B) using the different Bacillus isolates in each treatment. Disease incidence (A) was 100% in FSP, 37.50% in Bac P and Bac S, 25% in Bac A and 18.75% in Bac C. Percent disease control (B) was 0% in FSP, 62.50% in Bac P and Bac S, 75% in Bac A and 81.25% in Bac C. Values are mean of four replicates at P= 0.05 by Duncan's Least Significant Difference (LSD). Treatment of *F. solani* with Bac A (BAC AF), *F. solani* with Bac C (BAC CF), F. solani with Bac P (Bac PF), *F. solani* with Bac S (BAC SF), only infection with *F. solani* (FSP) and No treatment with Bacillus isolates and no infection with *F. solani* (NBNF).

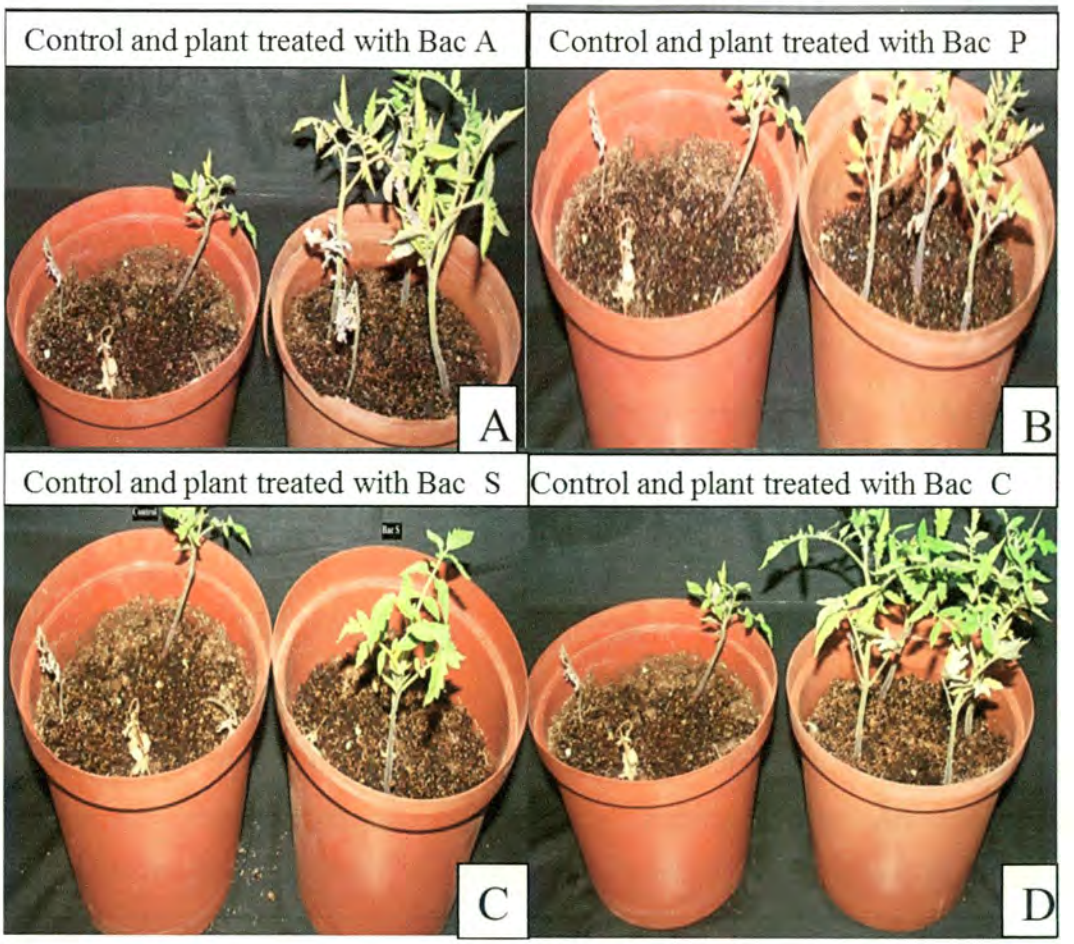

Figure 4.3 Biocontrol of Fusarium wilt in which the treatments (A, B, C, D) and *F. solani* were simultaneously inoculated into the tomato plant roots. Each treatment is compared with the control where there is no treatment. Bac A = *B. amyloliquefaciens,* Bac C = *B. cereus,* Bac P = *B. pumilus,* Bac S = *B. subtilis*. In 'A' above, out of the four plants, 1 was dead, 1 was not fully wilted while the remaining 2 plants were still green. In 'B', one of the four plants was dead while in 'C', 2 out of the 4 plants were dead and in 'D', the four plants were all green compared to the control which had all dead plants except 1

4.4 Growth parameters of tomato plants treated with *Bacillus* isolates

Plant growth parameters that were evaluated from the tomato plants treated with the four *Bacillus* isolates included; the shoot and root length, the number of leaves, the number of branches, the number of fruits, fresh mass of shoot and root length, dry mass of root and shoot length, fresh mass of fruit and dry mass of fruits

4.4.1 Shoot length of the tomato plants treated with *Bacillus* isolates

The shoot length of the plants not infected with FSP but treated with Bac C was 42 mm long and this was longest of the samples. Using Duncan's LSD in which values with different letters are significantly different, this value was significantly different from the control which was 32 mm long and also from Bac P which was 36 mm long and Bac S which was 35 mm long but not significantly different from Bac A which was 38 mm long. While the plants that were infected with FSP and those treated with *Bacillus* isolates also had Bac A and Bac C having the longest shoot length of 38 mm and were significantly different from the control which had shoot length of 24 mm and Bac P and Bac S which had shoot length of 27 mm and 29 mm respectively (Table 4.2 and 4.3).

4.4.2 Root length of the tomato plants treated with *Bacillus* isolates

For plants not infected with FSP but treated with *Bacillus* isolates, the root length of plants treated with Bac C was the longest with 34 mm and was significantly different from the Bac A which had root length of 29 mm, Bac P and Bac S which had 23.25 mm and 23.5 mm respectively. Root lengths of plants treated with Bac C and Bac A were significantly different from the other treatments and from the control with root length of 21.50 mm. Plants infected with FSP and treated with Bac C also had the longest root length of 31 mm and was significantly different from the control having 16.75 mm. Bac A having root length of 29.50 mm was not significantly different from Bac C but both of them were significantly different from Bac P and Bac S having root length of 18.50 mm each (Table 4.2 and 4.3).

4.1.4.3 The number of branches of tomato plants treated with *Bacillus* isolates

Tomato plants treated with Bac C having 22 branches displayed the highest number of branches for plants that were not infected with FSP but treated with Bacillus isolates. It was not significantly different from Bac S having 19 branches but was significantly different from the control with only 16 branches. Bac A and Bac P both having 16 branches each were not significantly different from the control. All the plants that were infected with FSP and treated with Bacillus isolates with the exception of treatment with Bac P having 5 branches were

significantly different from the control (6 branches) with Bac S having the highest number of 15 branches then Bac A with 11 branches and Bac C having 8 branches (Table 4.2 and 4.3).

4.4.3 The number of leaves on tomato plants treated with *Bacillus* isolates

The number of leaves was not necessarily determined by the number of branches. This is seen in treatment with Bac A without infection of FSP having 112 leaves as the highest number of leaves but with 16 branches compared to Bac C with the highest number of branches but 105 leaves. Both of them are significantly different from the control with 74 leaves and Bac P with 80 leaves but not significantly different from Bac S with 97 leaves. For treatments with *Bacillus* where plants had been infected/ inoculated with FSP, plants treated with Bac A had the highest number of 77 leaves and was significantly different from the control having 45 leaves. All the other treatments were significantly different from one another and from the control. Bac C had 56 leaves and quite smaller compared to Bac A and Bac S having 65 leaves (Table 4.2 and 4.3).

4.4.4 The number of fruits of tomato on plants treated with *Bacillus* isolates

The number of fruits from plants treated with Bac A (6.5) and Bac C (6.25) were significantly different from the number of fruits from the control having 3.5 fruits. They were not significantly different from plants treated with Bac P and Bac S having 5 and 4.5 fruit respectively per plant (Table 4.2 and 4.3).

4.4.5 Fresh mass of shoot of tomato plants treated with *Bacillus* isolates

The fresh mass of shoots of plants treated with Bac C was 5.28 g and significantly higher than the control having 1.56 g and the other treatments, Bac P with 2.11 g, Bac S with 2.37 g and Bac A with 2.85 g. There was no significant difference between Bac A, Bac P and Bac S but there was significant difference between Bac A and control (Table 4.3).

4.4.6 Fresh mass of root of tomato plants treated with *Bacillus* isolates

There was significant difference between the root fresh mass of plants infected with FSP and treated with Bac C having 1.45 g and the control having 1.26 g. There was no significant difference between the other treatments and the control (Table 4.3).

4.4.7 Dry mass of shoot of tomato plants treated with *Bacillus* isolates

The dry mass of shoots treated with Bac C with 0.94 g was significantly higher than the other treatments and significantly different from them. The shoot dry mass of plants treated with Bac A of 0.52 g was also significantly higher that of the control which was 0.14 g, Bac P

which was 0.24 g and Bac S which was 0.31 g. Treatments with Bac P and Bac S were not significantly different from each other and the control (Table 4.3).

4.4.8 Dry mass of root of tomato plants treated with *Bacillus* isolates

Treatment with Bac C had the highest root dry mass of 0.27 g and was significantly different from all the other treatments and the control. The control had the smallest root dry mass with 0.05 g and all the other treatments, Bac A with 0.11 g, Bac P with 0.09 g and Bac S with 0.07 g were all significantly higher than the control (Table 4.3).

4.4.9 Relationship between shoot and root length of tomato plants treated with *Bacillus* isolates

The result revealed that the longer the shoot, the longer the roots (Figure 4.4 and Figure 4.5). The root and shoot of the plants treated with Bac C were not significantly different from that of Bac A but were longer than that of the control and treatments with Bac P and Bac S.

Table 4.2 Effect of various treatments on plant growth parameters in tomato plants treated with *Bacillus* isolates but were not infected with *F. solani*

Treatments	Shoot length (mm)	Root length (mm)	Number of branches	Number of leaves	Number of fruits
Bac A	38±1.58bc	29±0.70b	16±0.91a	112±6.58b	6.5±0.64b
Bac C	42±2.12c	34.75±1.70c	22±0.91b	105±5.09b	6.25±0.85b
Bac P	36±1.47ab	23.25±2.35a	16±0.91a	80±5.98a	5±1.08ab
Bac S	35±1.97ab	23.5±2.39a	19±2.16ab	97±3.36b	4.5±0.64ab
Control	32±0.70a	21.5±1.32a	16±1.82a	74±2.08a	3.5±0.28a

Values are mean of 4 replicates ± SE. Each replicate had a total of 120 plants. Values with different letters are significantly different at P= 0.05 by Duncan's LSD. Bac A = *B. amyloliquefaciens,* Bac C = *B. cereus,* Bac P = *B. pumilus,* Bac S = *B. subtilis.*

Table 4.3 Effect of various treatments on plant growth parameters in tomato plants treated with *Bacillus* isolates and infected with *F. solani*

ıts	Shoot length (mm)	Root length (mm)	Number of branches	Number of leaves	Shoot fresh mass (g)	Shoot dry mass (g)	Root fresh mass (g)	Root dry mass (g)
	38±1.22b	29.5±1.04b	11±0.81c	77±4.70d	2.85±0.38b	0.52±0.03b	1.29±0.01a	0.11±0.00d
	38±1.47b	31±1.22b	8±0.40b	56±3.2c	5.28±0.15c	0.94±0.01c	1.45±0.04b	0.27±0.01e
	27±0.70a	18.5±1.65a	5±0.00a	26±3.65a	2.11±0.10ab	0.24±0.07a	1.28±0.00a	0.09±0.00c
	29±2.27a	18.5±1.25a	15±0.81d	65±4.02c	2.37±0.46ab	0.31±0.11a	1.26±0.33a	0.07±0.00b
	24±0.85a	16.75±1.10a	6±0.00a	45±0.91b	1.56±0.21a	0.14±0.00a	1.26±0.01a	0.05±0.00a

Values are mean of 4 replicates ± SE. Values with different letters are significantly different at P= 0.05 by Duncan's LSD. Treatment of *F. solani* with Bac A (BAC AF), *F. solani* with Bac C (BAC CF), *F. solani* with Bac P (Bac PF), *F. solani* with Bac S (BAC SF), only infection with *F. solani* (FSP) and No treatment with *Bacillus* isolates and no infection with *F. solani* (NBNF).

4.5 Physical analysis of tomato treated with *Bacillus* isolates

Fruits from the different samples showed variation in their physical and chemical/mineral composition. Physical analysis of the tomato fruits such as the color of the tomato fruits ranged from light orange to orange to light red and to red. The red color shows the presence of lycopene while in yellow fruits, lycopene is in smaller amount but there is the presence of vitamin A (Table 4.5 and Figure 4.6). Tomatoes from plants treated with *B. amyloliquefaciens* were significantly bigger with dry weight of 12.61% of the wet weight compared to the other treatments and the control whose value was 7.47%. Plants treated with *B. cereus* had tomatoes whose percent dry weight was 9.95% of the wet weight and significantly different from and bigger than those of *B. pumilus* and *B. subtilis* whose percent dry weight were 9.36% and 9.28% respectively of the wet weights (Table 4.5).

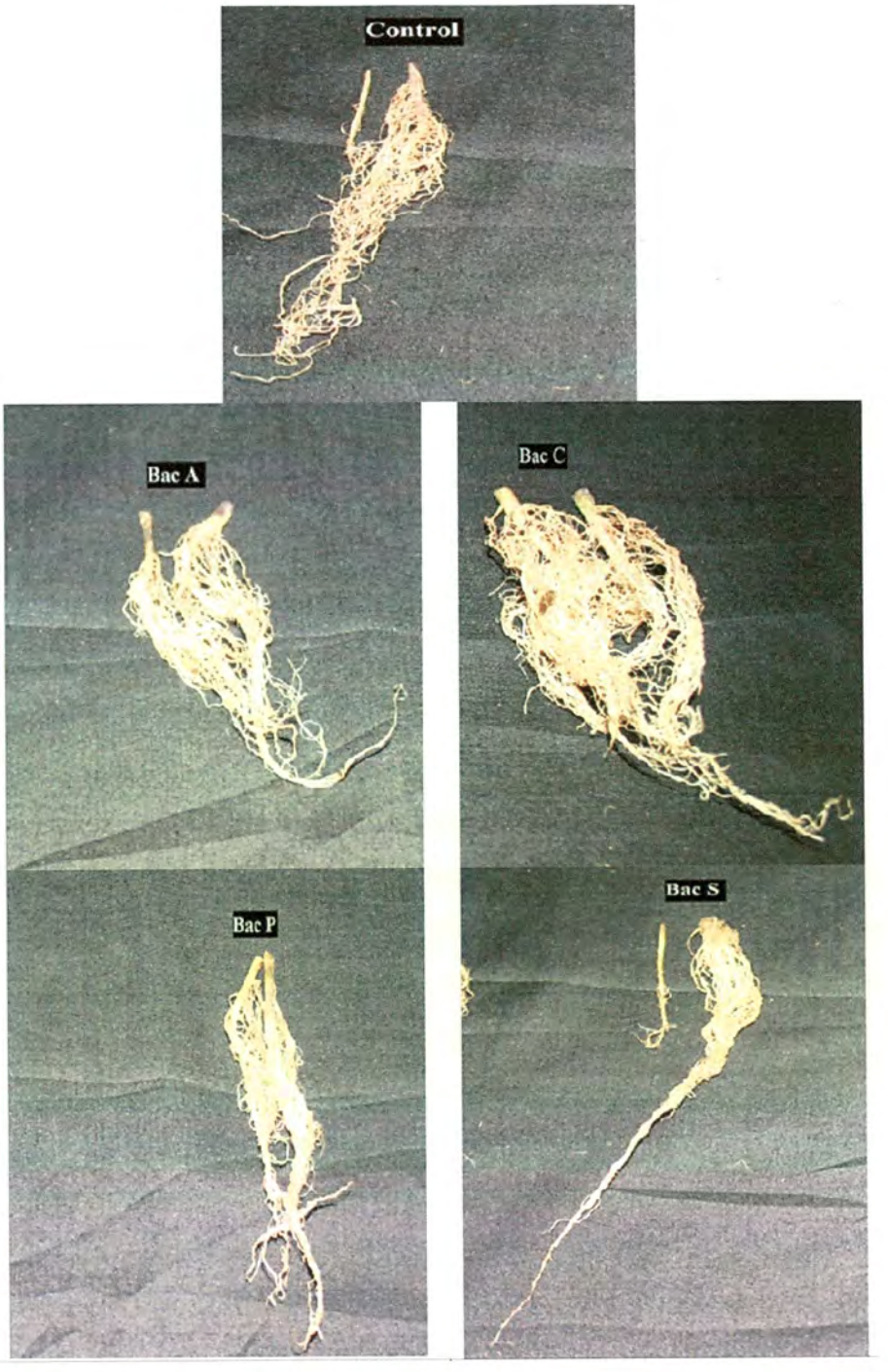

Figure 4.4 Dry roots of tomato plants that have been treated with different *Bacillus* isolates. Bac A = *B. amyloliquefaciens,* Bac C = *B. cereus,* Bac P = *B. pumilus,* Bac S = *B. subtilis*

4.6 Quantitative result for mineral composition of tomato fruits from each treatment

The mineral content of the tomato fruits from each treatment was analysed by using the EDX 720. It uses the Scanning Electron Microscopy (SEM) technique. The beam from the electron using uniform energy activates the atoms in the sample which automatically send out X-rays of specific energies for each element. The radiation from this X-rays is shown as spectra with different peaks which give information about the elemental composition of the sample. For a particular energy (wavelength) of fluorescent light emitted by an element, the number of photons per unit time (generally referred to as peak intensity or count rate) is related to the amount of that analyte in the sample. Therefore, by determining the energy of the X-ray peaks in a sample's spectrum, and by calculating the count rate of the various elemental peaks, the elemental composition and concentration of elements of the samples is established. The minerals of interest in the tomato samples analysed included Potassium (K), Calcium (Ca), Sulphur (S), Iron (Fe), Zinc (Zn), Manganese (Mn), Bromine (Br), Rubidium (Rb), Aluminium (Al), Sulphur (S) and Cobalt (Co). This data is presented in Figure 4.5 and appendix 2.0. The analysis shows that in all the treatments except the control that was not infected and not treated, the mineral that was mostly abundant in the fruits is K. Plants treated with Bac A had tomatoes with the highest percentage of K which is 86.46% of the dry weight with cps/μA of 16.33 while plants not treated nor infected (NBNF) had the lowest at 22.93% of the dry weight and cps/μA of 8.81. Apart from plants treated with Bac A, the composition of K in all the other treatment is not significantly different. The negative control had a high percent of K but the cps/μA which was 2.47 is significantly different from the others and the lowest. This emphasis was also seen in the graphical analysis showing that its peak for K is the lowest (Figure 4.5). Each treatment has a table showing qualitative and quantitative analysis and a graph of spectra showing the peaks of elemental composition. Using the analysis of plants treated with Bac C as an example, on the table with qualitative analysis, list of elements from Na to U present in the sample was outlined as K, S, Rh, Ca, Mn, Fe, Co and Cu. Their intensity which corresponds to their concentration is given as counts per seconds per micro Ampere (cps/μA). The quantitative analyses were given in % with K having 79.584% and its intensity was 13.485. On the graph showing the peaks, the K peak was the highest while the Co peak was the lowest.

The fruits from all the treatments had four basic minerals common to them and they are K, Ca, Fe, and S except for NBNF whose composition did not include S. Ca content of Bac C was the highest both in percentage and cps/μA. Treatment with Bac P had 13.70 and 1.27 in

percent and cps/μA respectively while that of FSP and NBNF had the lowest concentration for Ca. There was significant difference in the concentration of Fe in the treatments compared to the control NBNF with the concentration of Bac P as the highest having 0.88 and 0.70 in percentage and cps/μA.

These results were converted to mg/g based on the fact that the EDX 720 result reported the concentration of mineral based on intensity. So with the knowledge of the dry matter of each treatment, the percent of each treatment was calculated from the dry weight and then converted to mg/g. These results were in agreement with the use of the raw percentage and cps/μA for the concentration of potassium but were different for the other minerals (Table 4.4 and Appendix 2.0). This shows that the conversion helped to reveal even the smallest details in terms of similarity or difference that is significant.

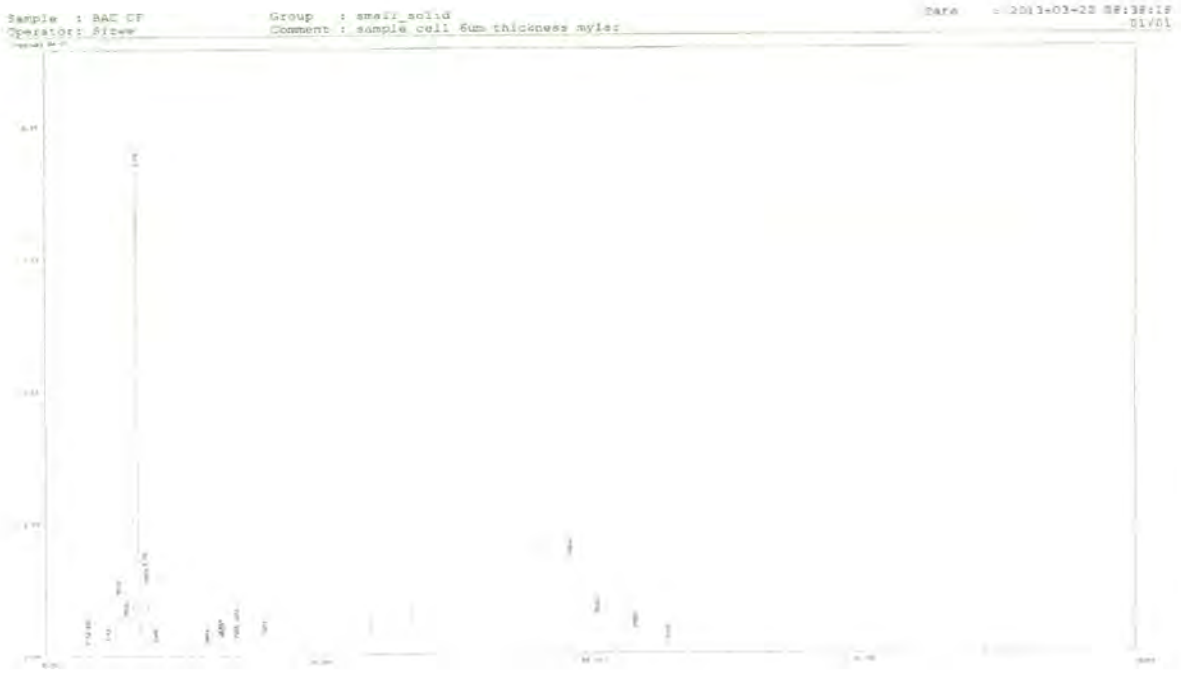

Figure 4.5 EDX-Analysis of the different samples of tomato whose plants were treated with *B. cereus* was measured from 0 -40 kilo electron Volts (keV), the peaks of Potassium (K) and Calcium (Ca) are clearly visible for the sample

Table 4.4 Mineral analysis of tomato fruits from the different treatments in mg/g

Treatments	K (mg/g)	Ca (mg/g)	S (mg/g)	Fe (mg/g)	Cu (mg/g)	Rb (mg/g)	Mn (mg/g)
Bac AF	3.88±0.08	0.44±0.08	0.10±0.03	0.02±0.00	0.06±0.0	0.01±0.00	-
Bac CF	1.00±0.07	0.21±0.07	0.03±0.02	-	-	-	-
Bac PF	2.75±0.08	0.46±0.08	0.11±0.03	0.03±0.00	-	0.01±0.00	0.01±0.00
Bac SF	3.53±0.11	0.73±0.11	0.10±0.055	0.02±0.00	-	-	-
FSP	1.80±0.08	0.18±0.09	0.10±0.04	0.01±0.06	-		-

Four minerals were common to all samples i.e. K, Ca, S and Fe. Values are mean of 4 replicates ± SE. Values with different letters are significantly different at P= 0.05 by LSD. Treatment of *F. solani* with Bac A (BAC AF), *F. solani* with Bac C (BAC CF), *F. solani* with Bac P (Bac PF), *F. solani* with Bac S (BAC SF), only infection with *F. solani* (FSP) and no treatment with *Bacillus* isolates and no infection with *F. solani* (NBNF).

4.7 RAPD analysis of *Bacillus* isolates used for biocontrol of tomato Fusarium wilt

Out of 17 different oligonucleotide primers used only three of them showed clear and sharp bands for the 11 *Bacillus* isolates. Primers S4 and OPH-19 are decamers while primer A9B7 is a 20-mer primer. The total number of bands or fragments scored from the three primers was 76 and they were used for the estimation of genetic diversity from the eleven *Bacillus* isolates (Table 4.6 and 4.7). Out of the 76 bands, the finger prints generated are shown on Figures 4.7, 4.8 and 4.9.

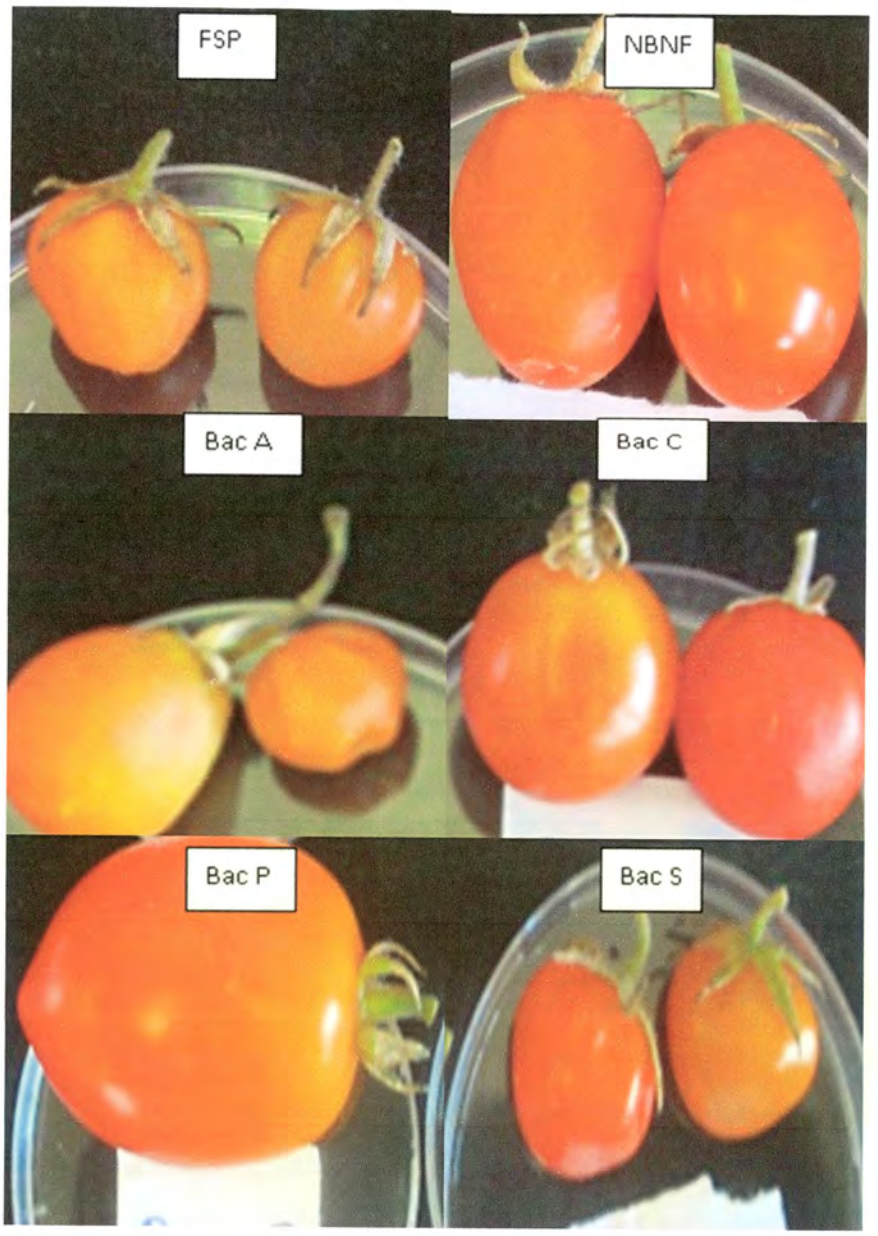

Figure 4.6 Effect of various treatments on the yield of the tomato fruits. The different *Bacillus* isolates had effect on the colour of the tomato ranging from orange to red. Bac A = *B. amyloliquefaciens,* Bac C = *B. cereus,* Bac P = *B. pumilus,* Bac S = *B. subtilis,* FSP = Plants only infected with *F. solani,* and NBNF = No treatment with *Bacillus* isolates and no infection with *F. solani*

Table 4.5 Effect of Treatments on tomato fruit color, percent dry weight and moisture content

Treatment	Treatments	Description of fruit	% dry weight	% moisture content
Bac A + *F. solani*	Bac AF	Orange	12.61±1.42 a	87.39±1.42a
Bac C + *F. solani*	Bac CF	Light orange	9.95±1.42a b	90.06±1.42a b
Bac P+ *F. solani*	Bac PF	Light red	9.36±1.42b	90.06±1.42b
Bac S + *F. solani*	Bac SF	Light red	9.28±1.42b	90.72±1.42b
Negative control (NBNF)	FSP	Red	7.47±1.42b	92.53±1.42b
Positive control (*F. solani*)		Light orange		

Tomato plants treated with the four *Bacillus* isolates have variation in colour of their fruits. Tomato colour ranged from orange to red based on the different *Bacillus* isolates used for the treatment. Percent dry weight and wet weight are mean values of 4 replicates ± SE. Values with different letters are significantly different at $P= 0.05$ by LSD. Treatment of *F. solani* with Bac A (BAC AF), *F. solani* with Bac C (BAC CF), *F. solani* with Bac P (Bac PF), *F. solani* with Bac S (BAC SF), only infection with *F. solani* (FSP) and *no* treatment with *Bacillus* isolates and no infection with *F. solani* (NBNF).

4.7.1 Molecular characterization using RAPD-PCR Primer A9B7

A 20-mer primer of arbitrary sequence 5'-GGTGACGCAGGGGTAACGCC-3' was used. Products of the RAPD-PCR yielded varying sizes and they were separated by gel electrophoresis. Each lane with the exception of lane 3 had a minimum of 1 and a maximum of 5 visible bands which ranged in size from 75 to 600 base pair (bp) (Figure 4.7). Isolates Bac 282, Bac M8, Bac S35 and Bac T51 showed minimum polymorphism. In the region of 100 bp, most isolates had bands. Thirty-three bands were produced from this primer having 90.90% polymorphism (Table 4.6 and Table 4.7).

The dendrogram obtained from the RAPD marker showed 6 classes of clusters which showed relatedness (Figure 4.10). Cluster analysis revealed that out of the eleven *Bacillus* isolates, ten of them paired in twos with each other, isolate from lane 1 paired with isolate from lane 4, isolate from lane 2 paired with isolate from lane 6, isolate from lane 5 paired with isolate from lane 10, while isolates from lane 7 and 8 also formed a pair. Isolates from 9 and 11 showed close similarity and also formed a pair. Isolate from lane 3 did not cluster with the others which show it is either from the same strain or a different species. This is also in agreement with the result from the similarity matrix computed with Jaccard coefficient. Basically, the distance matrix showed that isolate from lane 3 was distant from the other *Bacillus* isolates (Table 4.8a and Table 4.8b). This shows that the similarity between isolate 9 and isolate 11 was the closest followed by similarity between isolate 7 and isolate 8, Isolate 7 and isolate 9 and between isolate 7 and isolate 11.

Table 4.6 Number of bands and percentage of DNA polymorphic bands in eleven *Bacillus* isolates amplified with three primers

Primer	Primer sequence	Number of fragments scored	Percentage of polymorphic loci (%)
S4	5'-GTCGCCGTCA-3'	27	92.59
OPH-19	5'-CTGACCAGCC-3'	16	87.50
A9B7	5'-GGTGACGCAG GGGTAACGCC-3'	33	90.90

Table 4.7 Number of bands per isolate from each of the primers

Isolates	Primer A (A9B7)	Primer S4	Primer OPH (OPH 19)	Total
	Number of fragments scored			
Bac A	5	2	4	11
Bac C	3	3	2	8
Bac P	0	3	1	4
Bac S	3	3	0	6
Bac 282	3	0	2	5
Bac Bb	3	3	2	8
Bac M5	4	2	2	8
Bac M8	3	3	1	7
Bac S12	3	2	0	5
Bac S35	3	3	2	8
Bac T51	3	3	0	6
Total	**33**	**27**	**16**	**76**

Table 4.8a Similarity Matrix computed with Jaccard coefficient (using primerA9B7) showing 11 elements with 9 variables and each element has been analyzed

	A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**
A1	1	0.167	0.000	0.333	0.143	0.333	0.286	0.333	0.143	0.143	0.143
A2		1	0.000	0.000	0.000	0.500	0.400	0.200	0.200	0.200	0.200
A3			1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A4				1	0.200	0.167	0.167	0.200	0.200	0.200	0.200
A5					1	0.167	0.400	0.500	0.500	0.500	0.500
A6						1	0.600	0.400	0.400	0.400	0.400
A7							1	0.750	0.750	0.400	0.750
A8								1	0.500	0.200	0.500
A9									1	0.500	1.000
A10										1	0.500
A11											1

Table 4.8b Distance matrix based on Jaccard coefficient (using primer A9B7)

	A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**
A1	0	0.833	1.000	0.667	0.857	0.667	0.714	0.667	0.857	0.857	0.857
A2		0	1.000	1.000	1.000	0.500	0.600	0.800	0.800	0.800	0.800
A3			0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A4				0	0.800	0.833	0.833	0.800	0.800	0.800	0.800
A5					0	0.833	0.600	0.500	0.500	0.500	0.500
A6						0	0.400	0.600	0.600	0.600	0.600
A7							0	0.250	0.250	0.600	0.250
A8								0	0.500	0.800	0.500
A9									0	0.500	0.000
A10										0	0.500
A11											0

4.7.2 Molecular characterization using RAPD-PCR Primer OPH 19

OPH 19 is a decamer primer with arbitrary sequence of 5'-CTGACCAGCC-3'. Products of the RAPD-PCR separated on gel electrophoresis revealed that lane 4, lane 9 and lane 11 produced no bands. Even though some of the bands were not sharp, the minimum number of bands produced was 1 while the maximum was 3. The band sizes ranged from 750 to 4000 bp (Figure 4.8). Isolates Bac M5 and Bac M8 showed minimum polymorphism. There were 16 bands in all with the regions of 1000 and 1500 bp having the most bands and 87.5% polymorphism (Table 4.6 and Table 4.7).

The dendrogram clearly depicts 5 major clusters (Figure 4.11). Only isolate 3 and isolate 8 formed individual clusters. Isolates 1, 2 and 6 formed a cluster; isolates 5, 10 and 7 formed another cluster while isolates 4, 9 and 11 also formed a different cluster. The similarity matrix revealed the relatedness among isolates 1, 2and 6 as 0.500, isolates 5, 10 as 1.000 and with isolate 7 as 0.333. While the similarity between isolates 4, 9 and 11 was 1.000 (Table 4.8c and 4.8d).

4.7.3 Molecular characterization using RAPD-PCR Primer S4

The primer S4 is a decamer with the arbitrary sequence of 5'-GTCGCCGTCA-3'. Gel electrophoresis revealed that the products separated for all the isolates except for isolate 5 which produced no bands. Bands produced per isolate ranged from 1 to 3 bands and the band sizes ranged from 300 to 2000 bp (Figure 4.9). Isolates that showed minimum polymorphism were Bac A, Bac M5 and Bac S12. There were 27 bands in all with 92.59% polymorphism (Table 4.6 and Table 4.7) and the region around 1000 bp having the highest number of bands.

Similarity and Distance matrix based on Jaccard coefficient showed that isolates 1, 7 and 3 are closely related while isolates 6 and 11 are also closely related. But most of the other isolates were distant in their relationship with one another (Table 4.8e and Table 4.8f)

The dendrogram revealed that there were 2 basic clusters while the other five groups had only one isolates each (Figure 4.12). Primer did not display very sharp reproducible bands but it also reveals that though the isolates were from the same root, they are different strains.

Primers A9B7, OPH19, S4 were able to complement one another in revealing the relationship between the eleven isolates. This is seen in isolate Bac P which was not shown to produce any bands using primer A9B7 but produced sharp bands using primers S4 and OPH 19

Table 4.8c Similarity Matrix computed with Jaccard coefficient (using primer OPH 19) showing 11 elements with 12 variables each element have been analyzed

	OPH 1	**OPH 2**	**OPH 3**	**OPH 4**	**OPH 5**	**OPH6**	**OPH 7**	**OPH 8**	**OPH 9**	**OPH 10**	**OPH 11**
)H1	1	0.500	0.250	0.000	0.000	0.500	0.200	0.000	0.000	0.000	0.000
)H2		1	0.000	0.000	0.000	0.333	0.000	0.000	0.000	0.000	0.000
)H3			1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
)H4				1	0.000	0.000	0.000	0.000	1.000	0.000	1.000
)H5					1	0.000	0.333	0.000	0.000	1.000	0.000
)H6						1	0.333	0.000	0.000	0.000	0.000
)H7							1	0.000	0.000	0.333	0.000
)H8								1	0.000	0.000	0.000
)H9									1	0.000	1.000
)H10										1	0.000
)H11											1

Table 4.8d Distance Matrix based on Jaccard coefficient (using primer OPH 19)

	OPH 1	**OPH 2**	**OPH 3**	**OPH 4**	**OPH5**	**OPH 6**	**OPH 7**	**OPH 8**	**OPH 9**	**OPH 10**	**OPH 11**
)H1	0	0.500	0.750	1.000	1.000	0.500	0.800	1.000	1.000	1.000	1.000
)H2		0	1.000	1.000	1.000	0.667	1.000	1.000	1.000	1.000	1.000
)H3			0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
)H4				0	1.000	1.000	1.000	1.000	0.000	1.000	0.000
)H5					0	1.000	0.667	1.000	1.000	0.000	1.000
)H6						0	0.667	1.000	1.000	1.000	1.000
)H7							0	1.000	1.000	0.667	1.000
)H8								0	1.000	1.000	1.000
)H9									0	1.000	0.000
)H10										0	1.000
)H11											0

Table 4.8e Similarity Matrix computed with Jaccard coefficient (using primer S4) showing 11 elements with 15 variables and each element has been analyzed

	S41	**S42**	**S43**	**S44**	**S45**	**S46**	**S47**	**S48**	**S49**	**S410**	**S411**
S41	1	0.250	0.667	0.000	0.000	0.250	1.000	0.250	0.333	0.000	0.250
S42		1	0.200	0.000	0.000	0.000	0.250	0.000	0.250	0.000	0.000
S43			1	0.000	0.000	0.500	0.667	0.200	0.250	0.200	0.500
S44				1	0.000	0.200	0.000	0.000	0.000	0.000	0.000
S45					1	0.000	0.000	0.000	0.000	0.000	0.000
S46						1	0.250	0.200	0.250	0.200	0.500
S47							1	0.250	0.333	0.000	0.250
S48								1	0.250	0.200	0.200
S49									1	0.000	0.250
S410										1	0.200
S411											1

Table 4.8f Distance matrix based on Jaccard coefficient (using primer S4)

	S41	**S42**	**S43**	**S44**	**S45**	**S46**	**S47**	**S48**	**S49**	**S410**	**S411**
S41	0	0.750	0.333	1.000	1.000	0.750	0.000	0.750	0.667	1.000	0.750
S42		0	0.800	1.000	1.000	1.000	0.750	1.000	0.750	1.000	1.000
S43			0	1.000	1.000	0.500	0.333	0.800	0.750	0.800	0.500
S44				0	1.000	0.800	1.000	1.000	1.000	1.000	1.000
S45					0	1.000	1.000	1.000	1.000	1.000	1.000
S46						0	0.750	0.800	0.750	0.800	0.500
S47							0	0.750	0.667	1.000	0.750
S48								0	0.750	0.800	0.800
S49									0	1.000	0.750
S410										0	0.800
S411											0

(Figure 4.7, 4.8, 4.9). Isolates Bac S and Bac T51 did not produce any bands with primer OPH 19 but had visible bands on primers A9B7 and S4. RAPD-PCR using primer S4 did not produce bands on isolate Bac 282 but was different on primer A9B7 and OPH 19 with visible bands. Based on the sizes of the bands primer A9B7 was able to amplify band sizes with lower base pair (75-600 bp) compared to primer S4 whose sizes ranged from 300 to 2000 bp and primer OPH 19 that was able to amplify very high band sizes (750-4000 bp).

Figure 4.7 Electrophoretic gel analysis of RAPD polymorphism detected in 11 *Bacillus* isolates using Primer A9B7. M represent a 1 kb marker, 1-11 represent different isolates; (1) *B. amyloliquefaciens*; (2) *B. cereus* (3) *B. pumilus*; (4) *B. subtilis*; (5) *B. subtilis*; (6) *B. brevibacillus*; (7) *B. mojavensis* (8) *B. methylotrophicus*; (9) *B. subtilis*; (10) *B. safensis*; (11) *B. cereus*.

Figure 4.8 Electrophoretic gel analysis of RAPD polymorphism detected in 11 *Bacillus* isolates using Primer OPH 19. M represent a 1 kb marker, 1-11 represent different isolates; (1) *B. amyloliquefaciens*; (2) *B. cereus* (3) *B. pumilus*; (4) *B. subtilis*; (5) *B. subtilis*; (6) *B. brevibacillus*; (7) *B. mojavensis* (8) *B. methylotrophicus*; (9) *B. subtilis*; (10) *B. safensis*; (11) *B. cereus*.

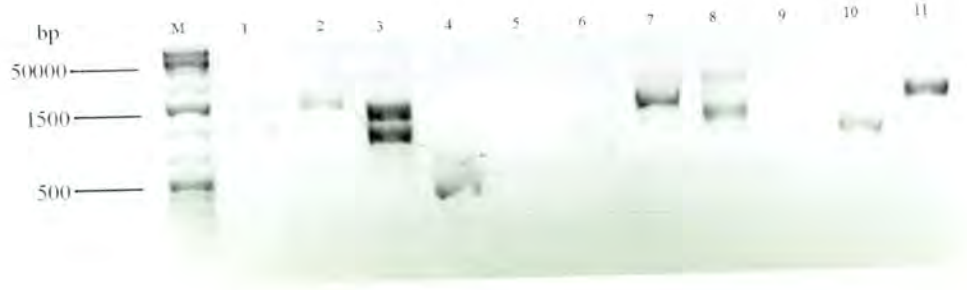

Figure 4.9 Electrophoretic gel analysis of RAPD polymorphism detected in 11 *Bacillus* isolates using Primer S4. M represent a 1 kb marker, 1-11 represent different isolates; (1) *B. amyloliquefaciens*; (2) *B. cereus* (3) *B. pumilus*; (4) *B. subtilis*; (5) *B. subtilis*; (6) *B. brevibacillus*; (7) *B. mojavensis* (8) *B. methylotrophicus*; (9) *B. subtilis*; (10) *B. safensis*; (11) *B. cereus*.

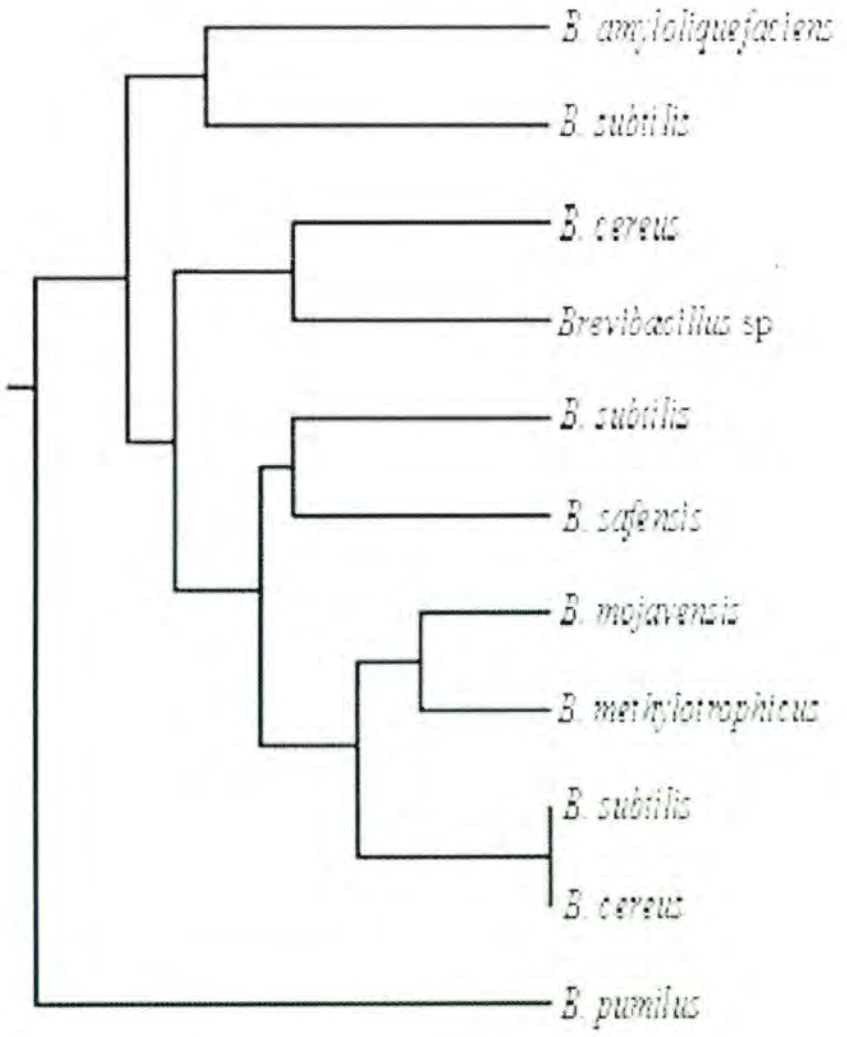

Figure 4.10 Dendrogram from unweighted pair group method with arithmetic averages showing relationships among the 11 *Bacillus* isolates. Genetic distances were obtained by RAPD analysis with a twenty-mer primer A9B7. Distance and similarity matrix were based on Jaccard coefficient (Table 4.8a and b).

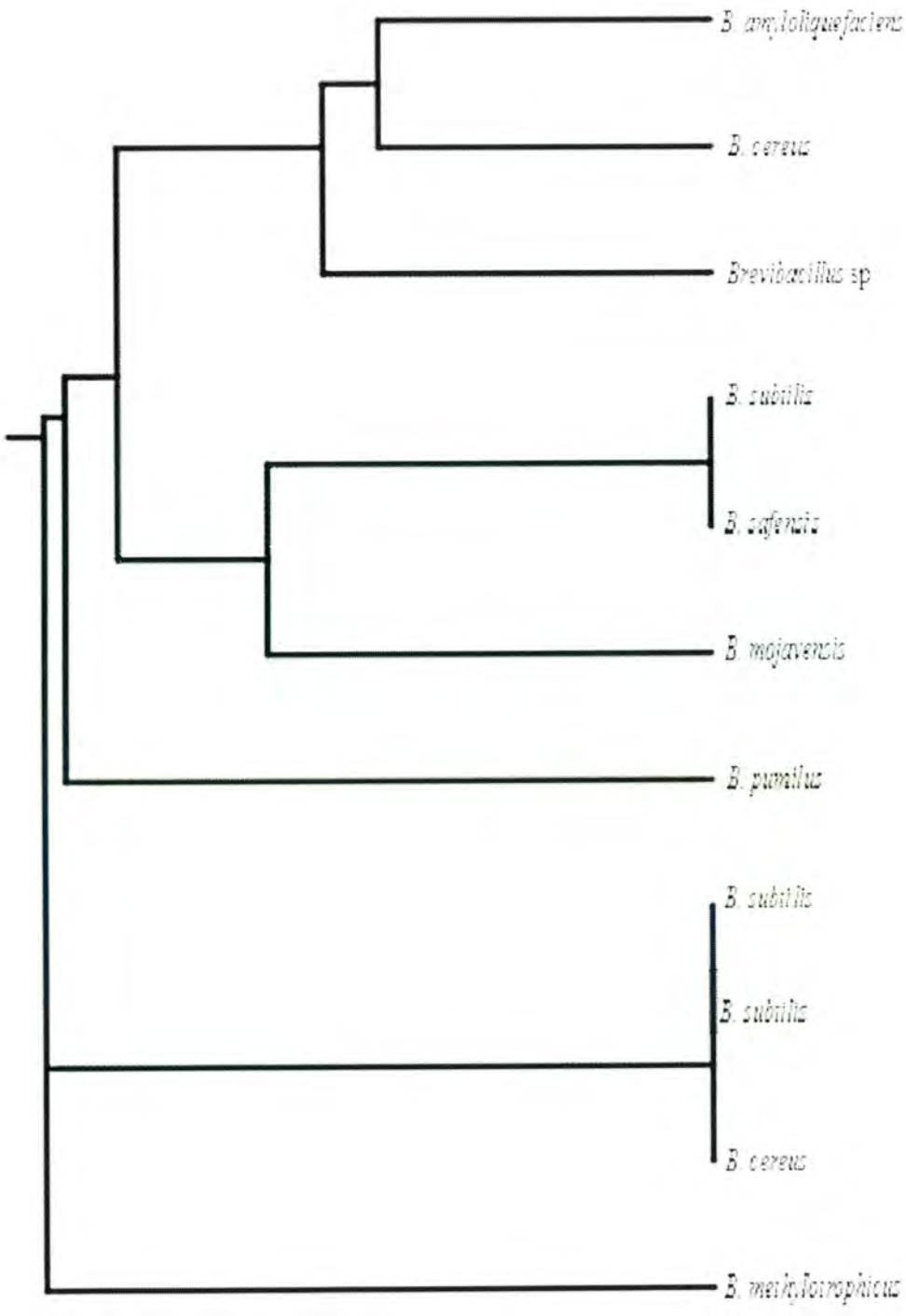

Figure 4.11 Dendrogram from Unweighted pair group method with arithmetic averages showing relationships among the 11 *Bacillus* isolates. Genetic distances were obtained by RAPD analysis with a decamer primer OPH19. Distance and similarity matrix were based on Jaccard coefficient (Table 4.8c and d).

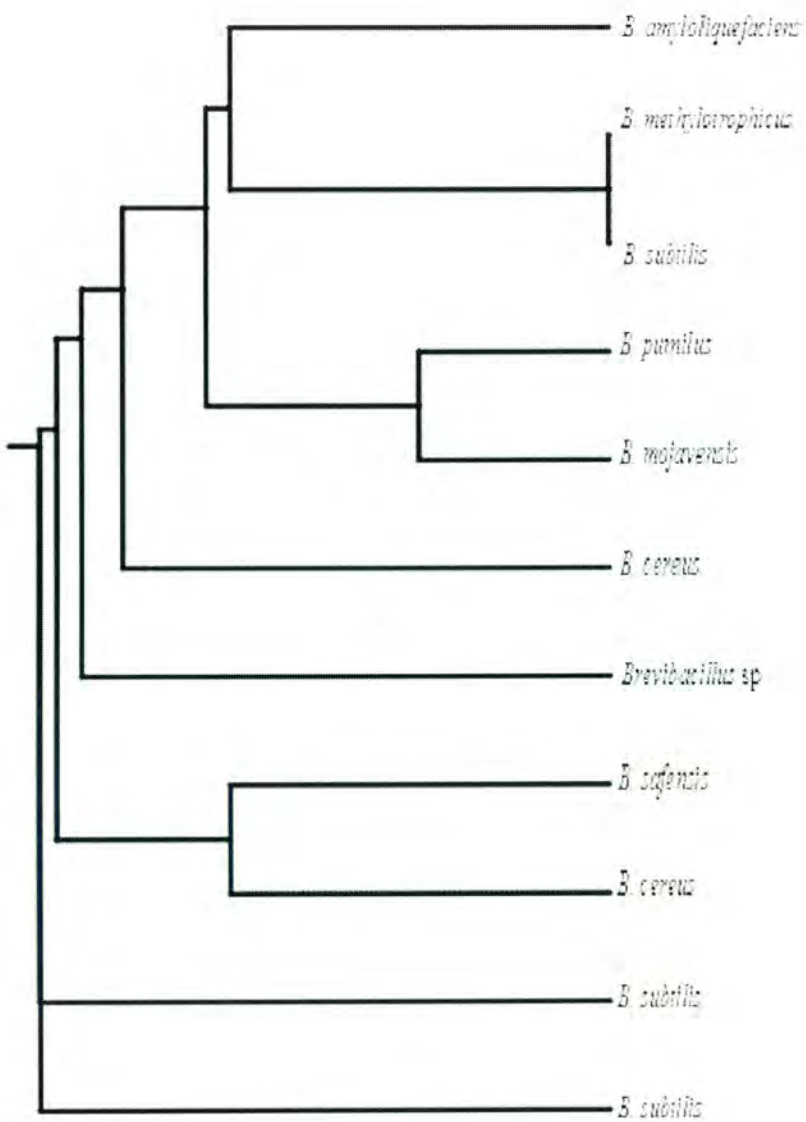

Figure 4.12 Dendrogram from Unweighted pair group method with arithmetic averages showing relationships among the 11 *Bacillus* isolates. Genetic distances were obtained by RAPD analysis with a decamer primer S4. Distance and similarity matrix were based on Jaccard coefficient (Table 4.8e and f).

CHAPTER 5

DISCUSSION

Rhizobacteria from rhizospheric soils are very important because they can naturally inhibit and antagonise soil-borne plant pathogens (Weller *et al.*, 2002). Some of these microorganisms can colonize the rhizosphere or the soil around the roots of plants effectively and efficiently. They are referred to as Plant growth-promoting rhizobacteria (PGPR). They are involved in growth promotion of plants and also suppressing plant pathogens in biocontrol through different mechanisms (Nihorimbere *et al.*, 2011). This was apparent in all the eleven *Bacillus* isolates used for the project as they were all rhizospheric organisms of plants in Mafikeng, North West province of South Africa.

Fusarium solani, is an economically important and significant disease of tomato plants, with no substantial chemical methods of control. This is because it is also a soil-borne pathogen and can resist unfavourable environmental conditions over a long period of time. PGPR have been reported to be both effective and efficient against nematode, viral, bacterial and fungal diseases (Harish *et al.*, 2009; Kloepper *et al.*, 2004). The uses of *Bacillus* isolates to control soil borne pathogens have been well documented (Jacobsen *et al.*, 2004). It is against this background that this study was carried out. This present study was conducted to evaluate the effectiveness of *Bacillus* isolates against *F. solani* and to determine the genetic diversity and/or relatedness of the *Bacillus* isolates that are able to suppress or control the activities of *Fusarium* pathogen.

5.1 *In vitro* antagonistic effects of *Bacillus* isolates on *Fusarium solani*

Four out of the eleven *Bacillus* isolates obtained from the rhizosphere were evaluated for their PGPR potential antagonism against *F. solani in vitro*. The result revealed that all the *Bacillus* isolates suppressed the mycelial growth of *F. solani* in varying degrees ranging from 55.70% by *B. cereus* (Bac C) to 95.20% by *B. amyloliquefaciens* (Bac A). This inhibitory activity of Bac A has been reported to be as a result of antifungal compounds or metabolites released into the PDA medium (Dihazi *et al.*, 2012). Also it has been reported that *B. amyloliquefaciens* strains inhibited the growth of a variety of fungal pathogens because of their ability to produce a vast array of antibiotics such as zwittermicin, bacillomycin, fengycin, bacilysin and difficidin (Athukorala *et al.*, 2009; Chen *et al.*, 2009). *B. subtilis* also inhibited the growth of *F. solani* by 82.10% *in vitro*. This result is in agreement with the findings of Adebayo and Ekpo (2005), that *B. subtilis* also inhibited fungal growth and

promoted that of tomato plant in greenhouse trial. *B. subtilis* has been shown to exhibit broad spectrum antimicrobial activities over diverse fungal and bacteria pathogen (Grover *et al.*, 2009). Over 70% of mycelial growth of *F. solani in vitro* was inhibited by *B. pumilus* (Bac P). This may be as a result of production of antibiotic, competition with pathogen for nutrients and direct antagonism (Akhtar *et al.*, 2010). *Bacillus* isolates are known to reduce wilting index in *F. udum*, increase plant growth and cause rapid colonization of tomato tissue in order to induce systemic resistance against *F. oxysporum* (Kloepper *et al.*, 2004).

5.2 Mechanism of biocontrol of *Bacillus* isolates against *Fusarium solani*

5.2.1 Phosphate solubilization

B. amyloliquefaciens was the only isolate that solubilized phosphate which could be the reason why it exhibited 92.50% growth inhibition of *F. solani in vitro*. It was also reflected in the biocontrol activity *in vivo* in which case the disease control by *B. amyloliquefaciens* was 75%. This result is similar to the findings of Calvo *et al.* (2010), that the main strain isolated from potato crop rhizosphere that solubilizes tricalcium phosphate (58%) belonged to *B. amyloliquefaciens* isolates and they also had *in vitro* antagonism against *Rhizoctonia solani* and *Fusarium solani*. *B. amyloliquefaciens* sks-bnj-1 (AY 932823) possesses multiple plant growth-promoting traits which included production of indole-3-acetic acid (IAA), solubilization of zinc, production of ACC deaminase, solubilization of phosphate, and production of phytases, HCN and cellulases. It also improved the growth of soybean by improving nutrient assimilation, rhizosphere properties and yield (nutrient content of soybean) compared to unioculated control (Sharma *et al.*, 2013). *B. amyloliquefaciens* AM1 and D29 inhibited the growth of *R. solanacearum* T-91 and produced IAA, siderophore and solubilize phosphate (Almoneafy *et al.*, 2012). Even though *B. subtilis, B. pumilus* and *B. cereus* did not solubilize phosphate in this study, the findings from Maheswar and Sathiyavani (2012) however indicated that *B. subtilis* and *B. cereus* isolated from groundnut rhizosphere were able to solubilize phosphate. *B. subtilis* inhibited the growth of *F. oxysporum* (25-34%) *in vitro* and *Botryodiphodia theobromae* isolated from post-harvest rotten yam tuber (100%) and it was able to solubilise phosphate and promote elongation of root in seedlings (70-74%) of *Cicer arietinum* compared to the control (Swain and Ray, 2009). *B. subtilis* strain D16 inhibited the growth of *R. solanacearum,* produced IAA and siderophore but did not solubilize phosphate which is similar to the result of this research (Almoneafy *et al.*, 2012).

Other *Bacillus* isolates that solubilise phosphate include *B. thuringiensis, B. sphaericus* and *B. megaterium* (Akgül and Mirik, 2008). Phosphorus is a very important macronutrient needed for plant growth and development. Microorganisms help to convert insoluble phosphorus in the soil to soluble ones that are accessible by plants for growth and increased yield (Saharan and Nehra, 2011). This helps to increase the uptake of phosphorus by plants (Chen *et al.*, 2006 ; Igual *et al.*, 2001), they are therefore quite important in the biotechnological aspect of agriculture as per meeting the phosphorus needs of plants

5.2.2 HCN production

Positive colour change of filter paper to reddish brown indicated the production of HCN. None of the four *Bacillus* isolates produced HCN which is in accordance to Singh *et al.* (2008) report where *Bacillus* isolates were all negative for HCN. Cyanide is a toxin and dreaded chemical produced by many rhizobacteria. Some bacteria synthesis it, others excrete it and yet others metabolize it in other to avoid predation and competition (Zeller *et al.*, 2007).

B. amyloliquefaciens sks-bnj-1 (AY 932823) produced HCN among other Plant growth promoting characteristics; improved plant growth and increased soybean yield (Sharma *et al.*, 2013). Reports have shown that HCN influences plant growth indirectly especially isolates from rhizosphere of chickpea, rice and mangrove (Joseph *et al.*, 2007; Samuel and Muthukkaruppan, 2011; Shobha and Kumudin, 2012).

5.2.3 IAA production

All the *Bacillus* isolates produced indole acetic acid from tryptophan which could enhance plant growth. Production of IAA was evidenced by pink colour produced by all isolates in different concentrations. *B. amyloliquefaciens* produced as much IAA as *B. cereus* but more than the other isolates even in the absence of tryptophan and this is in agreement with most findings as been cited in literature (Bottini *et al.*, 2004). For example, *B. amyloliquefaciens* KPS46 produced auxins, which is the commonest form of IAA and supported growth of soybean (Buensanteai *et al.*, 2008). Calvo *et al.,* (2010) reported that most of the strains isolated from the rhizosphere of potato crop were from the *B. amyloliquefaciens* strain and they produced IAA, others are *B. amyloliquefaciens* AM1 and D29 and *B. subtilis* strain D16 (Almoneafy *et al.*, 2012). The ability of *B. amyloliquefaciens* FZB24 to enhance growth and control plant disease could be as a result of production of plant hormones such as indole-3-acetic acid (IAA) (Bloemberg and Lugtenberg, 2001). Our findings revealed that, *B. cereus*

also produced IAA with or without tryptophan which is in agreement with the findings of Jetiyanon *et al.*, (2008) where *B. cereus* RS87 significantly promoted growth of root length, plant height and seedling emergence as compared to the control and produced IAA. *B. subtilis* B1, B6, B28 and B99 significantly promoted growth and biocontrol activity against *F. oxysporum* f.sp *ciceris* in chickpea compared to untreated control (15.8-44.8%). They were observed to produce IAA, HCN and antifungal volatiles among others (Karimi *et al.*, 2012). *B. subtilis* WR-W2 and *B. amyloliquefaciens* MR-A1 produced different concentration of IAA with *B. subtilis* producing more compared to *B. amyloliquefaciens*. Even in the absence of tryptophan, the result of this study indicated production of IAA, unlike the result from Mishra and Kumar, (2012), where there was no production of IAA in the absence of tryptophan. Sometimes, auxins are produced when there is a precursor such as L-tryptophan, which helps to increase the production of IAA in *Bacillus amyloliquefaciens* FZB42 (Idris *et al.*, 2007b).

All *Bacillus* isolates from this study produced IAA, which is in agreement with Joseph *et al.*, (2007) report on chickpea. Production of IAA in plants helps to increase root dry weight and thereby increase the plants ability to take up N, P, K compared to the control which is non-inoculated (Etesami *et al.*, 2009). This is because IAA is quite important in elongation of root and root is important in helping to transport nutrients to plants parts (Saharan and Nehra, 2011). IAA helps to stimulate plant growth and increased the uptake of N, P, K, Ca and Mg in sweet potato cultivar (Farzana and Radizah, 2005). It caused increase in vegetables especially cucumber, pepper and tomato (Kidoglu *et al.*, 2007) and also responsible for early growth promotion in soybean (*Glycine max* L) and corn (*Zea mays* L) (Cassa´na *et al.*, 2009). The response of plant to different concentration of auxins (Sarwar and Frankenberger, 1994) is different and the difference can depend on the type of microorganism (Ahmad *et al.*, 2005). Even though some microorganisms produce high concentration of auxins i.e. IAA and this helps to increase plant growth and yield in wheat crop (Khalid *et al.*, 2004), while others producing low concentration of IAA also improve plant growth (Tsavkelova *et al.*, 2007), in otherwords IAA is not needed in large quantity. This is in agreement with our findings where all *Bacillus* isolates produced IAA with or without tryptophan. They were able to inhibit growth of *F. solani in vitro* and *in vivo* and were also able to promote growth and control disease.

5.3 Biocontrol potentiality of *Bacillus* isolates on Fusarium wilt of tomato plants in the green house

5.3.1 Percent Disease control and Disease incidence of treatment tomato plants

Various *Bacillus* spp. have long being used for pest and plant disease control (Nagorska *et al.*, 2007; Ongena and Jacques, 2008). In this study, *B. amyloliquefaciens* was able to reduce disease incidence markedly. This ability to inhibit the growth of *F. solani in vitro* was not related to the biocontrol effect in the greenhouse. This result is similar to the observation of Wulff *et al.*, (2002) in which *B. amyloliquefaciens'* ability to inhibit the growth of *X. campestris* pv *campestris* was not related to the biocontrol effect in the greenhouse. Similar observations were earlier described by Schreider *et al.*, (1988) and Utkhede and Gaunce, (1983). This might be because the quantity and quality of antibiotics produced *in vivo* is not the same as those produced *in vitro*. Also it could be that the population of *Bacillus* spp. produced *in planta* might not be located close enough to the pathogenic cell to cause any kind of inhibition or death (Schreider *et al.*, 1988). It could also be the composition of the type of agar used and the systemic application of the *Bacillus* spp. either before or after inoculation with the fungal pathogen which may affect antibiosis *in vitro* (Bell *et al.*, 1995). The inhibition of the growth of *F. oxysporum* f. sp *albedinis* (Foa), causal agent of Bayond disease (a very destructive date palm diseases in Morocco and Algeria) by *B. amyloliquefaciens* could be as a result of the production of a variety of antibiotics such as zwittermicin A, difficidin, fengycin, bacillomycin and bacilysin (Athukorala *et al.*, 2009; Chen *et al.*, 2009).

In this study *B. subtilis* reduced disease incidence by 62.50%, which was similar to the result of Chen *et al.*, (2012b). The ability of *B. subtilis* to have above 50% biocontrol efficacy over *Ralstonia solanacearum* under greenhouse conditions was as a result of production of biofilm (Chen *et al.*, 2012a). Biofilm helps to create a hostile environment for the fungal pathogen (Zhang *et al.*, 2011). Its production could be as a result of the production of surfactin, which can act as a biocontrol agent and also help to stimulate the production of biofilm. From this study, biocontrol activities of *B. subtilis* may be due to production of IAA and some other metabolites which further research will highlight. According to Montealegre *et al.*, (2005), biocontrol activities of *B. subtilis* may also be due to its ability to produce other antifungal metabolites like subtilin, bacitracin and bacillin which can have antagonistic effects on fungal pathogens.

Inhibition of growth of *F. solani* by *B. subtilis* in this study was higher compared to the result of 34.40% disease reduction reported by Morsy *et al.*, (2009) and 53.30% reported by Abdel-Monaim, (2011). Disease reduction of Fusarium wilts of pepper caused by *F. oxysporum* schl. f.sp *capsici* ranged from 56.90% to 44.40% depending on the application of *B. subtilis* CAS 15 before and after inoculation with *F. oxysporum*. The result also showed that *B. subtilis* CAS 15 may suppress Fusarium wilt by induced systemic resistance (Yu *et al.*, 2011). In order to manage growth of *F. solani* in causing damping off and wilt disease in chickpea, *B. subtilis* inhibited the growth *in vitro* and percent inhibition ranged from 53.5% to 28.40% compared to 82.10% inhibition *in vitro* in this study and 62.50% disease reduction *in vivo*. But according to Baysal *et al.*, (2008) the case was different in the percent reduction of crown and root rot of tomato by *B. subtilis* EU07 which was 75% while percent inhibition was 64%. This was due to production of antibiotics by *B. subtilis* EU07 evidenced by the amplification of Fen D, Bmy A, and Itu C genes for antibiotics fengycin D, Bacillomycin A and iturin C. *B. subtilis* is known to produce a wide range of antifungal agents such as rhizocticins, alboleutin, iturins, bacitracin, fengycin, botrycidin and clorotetain that are effective as biocontrol agents against wide array of fungi which includes *Penicillium, Aspergillus* and *Fusarium* spp. (Baysal *et al.*, 2008). Though in this study, randomly amplified bands indicated that some genes were present but further research using specific primers can help to target specific genes that can be targeted, quantified and cloned.

B. pumilus inhibited the growth of *F. solani in vitro* by 70.46% and reduced disease incidence by 62.50%. Like the other treatments, *B. pumilus* showed that inhibition of *F. solani in vitro* was not related to biocontrol effect *in vivo*. The percentage of disease reduction is higher compared to the result reported by Wulff *et al.*, (2002) who noted that the percent reduction of black rot incidence in cabbage ranged from 17.20% to 55.10%. This was due to the production of secondary metabolites such as amphomycin, acivicin, arthrobactin, valinomycin, stenothricin, colistin and enterochelin. Inoculation with *B. pumilus* significantly increased shoot dry weight of plants coinoculated with *F. oxysporum* as compared to those inoculated with *F. oxysporum* alone. This could be attributed to the antagonistic effects of *Bacillus* spp. on pathogens (Akhtar *et al.*, 2010; Bapat and Shah, 2000; Chan *et al.*, 2003). It was observed that *B. pumilus* N43 inhibited the growth of *R. solani* Q1 in dual culture. This could be as a result of the production of antibiotics as seen in induced hyphal deformation, leaking of the cytoplasm and increase in the vacuoles of the cytoplasm as shown in the micrograph (Huang *et al.*, 2012). Similar results were observed by Montealegre *et al.*, (2003) while using *Bacillus* spp. to treat *R. solani.*

According to Wulff *et al.*, (2002), some *Bacillus* spp. ability to inhibit growth of pathogen *in vitro* is generally not related to their ability *in vivo*. This is also the case with the percent disease reduction using *B. cereus* which inhibited growth *in vivo* by 81.25% while it inhibited growth of *F. solani in vitro* by 55.7%. *In vitro* percent inhibition of the growth of *F. solani* in chickpea by *B. cereus* ranged from 29.40% to 12.40% (Abdel-Monaim, 2011). This is lower compared to the finding of this study.

B. cereus 28-9 was able to inhibit the conidial growth of *B. elliptica* B061, the causal agent of Lily leaf blight *in vitro* as clear zones in the Petridish (Huang *et al.*, 2005). *B. cereus* UW85 suppressed damping-off of alfalfa caused by *Phytophthora medicaginis*. It produced 2 antibiotics zwittermicin A and kanosamine which enhanced the suppression of the pathogen (Handelsman *et al.*, 1990).

B. cereus A47-2 and A47-3 were able to control take-all disease on wheat seedlings caused by *Gaeumannomyces graminis* var. *tritici*, and *Rhizoctonia* root rot caused by *R. solani* AG-8. They increased plant growth and reduced disease incidence consistently with a range from 39 to 35% (Ryder *et al.*, 1999).

5.3.2 Dry matter content of tomatoes from different treatment

The dry matter of tomatoes in this study ranged from 12% to 61% in treatment with *B. amyloliquefaciens* to 7.46% in the control. This is slightly higher compared to those reported by Majkowska-Godomska *et al.* (2008) and Turhan and Sentz (2009) (7%-3.83%) but similar to those reported by Babu *et al.*, (2012) where dry matter content ranged from 10.23% to 2.89%. In order to improve the quality of tomato both processed and fresh, the dry content matter must be at least 5% (DePascale *et al.*, 2001). In tomatoes, 8% of the dry matter content makes up the mineral composition especially K and P. These minerals have a way of affecting the taste of tomatoes by affecting the buffering capacity, titratable acidity and pH of the tomatoes (Petro-Turza, 1987; Yilmaz, 2001). The high dry matter could be as a result of the interaction between the microorganisms since all of the treatments produced higher percent of dry matter including the plants that were infected and not treated (control). The nutritional quality of harvested crops can be linked to the biological activities of microorganisms in the soil and the soil organic matter. This is because microorganisms are responsible for production of vital biochemical compounds that are the basis of the nutritional values in crops (Marler and Wallin, 2006). When there are increased activities of microorganisms in the soil, soil fertility is increased which leads to higher levels of dry

matter in harvested crops and invariably increase in mineral composition leading to production of healthy nutrient-rich foods.

5.3.3 Moisture content of tomato from different treatment

Moisture content ranged from 92.53% to 87.38% in the control experiment treated with *B. amyloliquefaciens*. This result is similar to the moisture content range of tomato fruits planted using organic or conventional system with range from 94.80% to 92.00% (Pieper and Barrett, 2009). They also observed that moisture content of tomatoes from conventional system was more compared to organic system. This can be important in this study as those plants treated with *Bacillus* spp. had less moisture content compared to the control. It is also similar to those reported by Gupta *et al.*, (2011) where moisture content ranged from 94.45% to 92.24%.

5.4 Mineral analysis of tomato using EDX 720

Mineral content of tomato is affected by various factors which include maturity level of the plant at the time of harvest, soil type, types of organic and inorganic matter in the soil. Tomatoes were analyzed to know the effect of the various treatments on the mineral content. A total of eleven different mineral elements were detected. The spectrometers of the EDX 720 enable non-destructive, rapid analysis of solids and liquids at the ppm level without much pre-treatment. This is quite important as other analysis can be carried out on the samples after they have been used for analysis in EDX 720.

EDX 720 allowed the detection of various minerals in the tomato samples. The mineral elements detected were potassium, calcium and sulphur (macro elements) manganese, zinc, copper and iron (micro elements), cobalt, bromine and rubidium and aluminium (trace elements).

5.4.1 Potassium content

The concentration of K in the treatments ranged from 3.88 mg/g to 1.00 mg/g. Treatment with *B. amyloliquefaciens* had the highest concentration of K with 3.88 mg/g while treatment with *B. cereus* had the least with 1.00 mg/g. treatment with *B. pumilus*, *B. subtilis* and *F. solani* had 2.75 mg/g, 3.53 mg/g and 1.80 mg/g respectively. These results are similar to the concentration range from 4.29 to 0.28 g/kg reported by Adotey *et al.*, (2009), 1.55g/kg reported by Zaichick, (2002), 4114.77 to 2391.08 mg/kg reported by Shar *et al.*, (2012) and 1.56 g/kg in medicinal plants reported by Serfor-Armah *et al.*, (2001). But it is lower than the concentration that ranged from 7.31 to 3.26 g/kg reported by Quartey *et al.*, (2012). Fresh

tomatoes are rich in K which is an important cell and body fluid component that helps in controlling blood pressure and as a result the heart beat (Freebern, 2012). Potassium also helps to transmit nerve impulses. It is also important in coagulating ATP with Sodium. Deficiency of K in the human body can lead to muscular weakness and paralysis, cardiac and mental disorder and nerve irritability (Adotey *et al.*, 2009). In plants, it helps to control opening and closing of the stomata and involved in enzyme activation. Deficiency leads to growth reduction, browning of leaves and reduced fertility (Morgan and Connolly, 2013)

5.4.2 Calcium content

Calcium content of tomato from the various treatments ranged from 0.18 mg/g to 0.73 mg/g with treatment with *B. subtilis* having the highest value and the control having the lowest. *B. cereus*, *B. amyloliquefaciens* and *B. pumilus* had concentration of 0.21 mg/g, 0.44 mg/g and 0.46 mg/g respectively. This is similar to the range of 76.41 to 77.75 mg/100g and 2.47 to 3.83 g/kg reported by Olaniyi *et al.*, (2010). Calcium is an essential component that is needed for the formation of fibrinogen which is very important and needed for the clotting of blood. It is also an important component of teeth and bones. Deficiency can lead to osteoporosis and rickets in children (Adotey *et al.*, 2009). Calcium can be provided by vegetables even though in low quantity but can add up to complement the daily intake needed by the body for proper functioning. Calcium is important as it helps to determine the rigidity of the structure of the cellwall (Hepler, 2005).

5.4.3 Sulphur content

The concentration of sulphur in the various treatments ranged from 0.11 mg/g in the treatment with *B. pumilus* to 0.03 mg/g in the treatment with *B. cereus*. Treatments with *B. amyloliquefaciens*, *B. subtilis* and control had 0.10 mg/g, 0.10 mg/g and 0.10 mg/g of sulphur respectively. It is important and used for production of amino acids which is an important and fundamental building block. It is important in the skin, hair and nails. As a component of insulin, it helps in reducing blood sugar. It is also important in the digestion and absorption of fat. In plants it is present as sulphate as is important for the deep rich green color and it is very important in protein synthesis (Sahota, 2005)

5.4.4 Iron content

The iron content ranged from 0.00 mg/g in treatments with *B. cereus* to 0.030 mg/g in treatment with *B. pumilus*. Treatments with *B. amyloliquefaciens*, *B. subtilis* and control had 0.02 mg/g, 0.02 mg/g and 0.01 mg/g of Fe respectively. Christian and West, (1998) had

similar result with millet (range from 39.75 mg/kg to 50.37 mg/kg). The result from this study was lower than the range of 11.61 to 12.29 mg/100g reported by Gupta *et al.*, (2011). Fe is needed in small quantity to help prevent chlorosis in plants (Morgan and Connolly, 2013).

5.4.5 Copper content

The copper content of treated tomatoes ranged from 0.009 mg/g in treatment with *B. subtilis* to 0.005 mg/g in the *B. cereus*. The control had no copper content while *B. amyloliquefaciens* and *B. pumilus* had 0.006 mg/g and 0.007 mg/g respectively. This result shows that these *Bacillus* isolates can increase the Cu content of tomatoes since the control which was not treated with any *Bacillus* isolate was deficient in it. It is important in the synthesis and stability of chlorophyll and several other enzymatic reactions involved in photosynthesis. Deficiency in plants leads to necrosis of the apical meristem and subsequent growth reduction (Uchida, 2000).

5.4.6 Rubidium content

The rubidium content of tomatoes whose plants were treated is quite small and limited to only two of the treatments i.e. *B. amyloliquefaciens* having concentration of 0.01 mg/g and *B. pumilus* having concentration of 0.01 mg/g and the control having the least concentration of 0.001 mg/g. Rubidium behaves like potassium and it is important in helping patients suffering from depression as a result of dialysis by supplementing their rubidium level (Canavese *et al.*, 2001). In lower organisms rubidium can replace potassium. In plants, addition of rubidium to medium solution increased leaf size and over growth of sugar beet (El-Sheikh and Ulrich, 1970). The presence of Rubidium in Plants treated with *Bacillus* isolates is worthy of note. As such, the relationship between the amount of inoculum of *Bacillus* isolates and the concentration of rubidium can be observed. So far sources of rubidium have not been researched extensively, therefore the use of *Bacillus* treated tomato could also be considered a potent source.

5.4.7 Zinc content

The concentration of Zinc in tomatoes whose plants were treated ranged from 0.006 mg/g in *B. subtilis* to 0.001mg/g in the control. *B. amyloliquefaciens* and *B. pumilus* had 0.004 mg/g and 0.005 mg/g. It is important in several enzymes like carbonic anhydrase. It is also important in the normal metabolism of vitamin A. Its deficiency can lead to night blindness because of its interaction with vitamin A deficiency. This is because zinc is required to

convert retinol to retinal which helps to prevent night blindness (Christian and West, 1998). Even though the control contained Zn, *Bacillus* treated plants had higher concentration. This result reveals that intake of tomato from *Bacillus* treated plant has advantage over those that have not been treated. Also, the intake of *Bacillus*-treated tomato as a source of Zn can also be researched into. Zn is quite important as a component of protein and deficiency in tomato plants was observed to affect stem elongation (Broadley et al., 2007).

5.4.8 Manganese content

Manganese content of tomatoes was only available to plants that have been treated with *B. cereus* and *B. pumilus* and they are 0.002 mg/g and 0.12 mg/g respectively. It is important in urea formation and energy metabolism, and it is also a key component of the antioxidant enzyme superoxide dismutase (Holley *et al.*, 2011). It is important in the oxidation of indole acetic acid in plants and it is a constituent of pyruvate carboxylase (Uchida, 2000).

5.4.9 Cobalt content

Cobalt was only found in tomatoes whose plants had been treated with *B. cereus* and the concentration was 0.0005 mg/g. This is needed in ultra-trace amount and it is important in the synthesis of vitamin B_{12} (Pilon-Smit *et al.*, 2009). Gad, (2006) observed that the addition of 8ppm of Cobalt to soil led to increased growth of pea plants (*Pisum sativum L.*), number and weight of nodules and seed yield.

From these findings, it is observed that tomato treated with the *Bacillus* isolates contained more mineral content and can be said to be more nutritious compared to the control. These mineral elements are important to health even though some of them were in small quantity and others are only needed in small quantity.

5.5 Relationship between growth parameters and disease incidence in biocontrol-treated plants

In this study, the various treatments reduced disease incidence and promoted growth parameters compared to the control in the greenhouse. They were all effective in promoting tomato growth which led to increase in the shoot and root dry weight compared to the control. This is because where *Bacillus* spp. or and their by-products are applied to plants, the outcome is disease control (Gardener, 2004). Disease incidence was highest in treatment with *B. subtilis* and *B. pumilus* and disease control was least in both treatments. Plant growth-promoting activities in terms of root and shoot length, number of leaves and branches, number of fruits in both treatments were also the least. Disease incidence was least in

treatment with *B. cereus* while disease control and plant growth promoting activities were highest. In treatment with *B. amyloliquefaciens*, disease incidence was higher than treatment with *B. cereus* but lower than treatment with *B. subtilis* and *B. pumilus*. Plant growth-promoting activities of *B. amyloliquefaciens* were lower than treatment with *B. cereus* but higher than treatments with *B. subtilis* and *B. pumilus*. The dry weight of treatment with *B. amyloliquefaciens* was the highest compared with the other treatments and the control. This shows that the higher the plant growth promoting activities of the treatments the lower the disease incidence and higher disease control. Also, the result of disease reduction in the green house did not correlate with the result of antifungal activities *in vitro*. These results are similar to the examples indicated below.

According to Singh *et al.*, (2008), chir-pine seeds treated with *B. subtilis* BN1 demonstrated early seed emergence, viability and increased biomass. In comparison to uninoculated seeds and seeds infested with *M. phaseolina*, disease severity was significantly reduced. *B sphaericus* and *B. brevis*-2 increased plant length significantly while *B. megaterium, B. polymyxa, B. sphaericus*, *B. brevis* -1 and *B. thuringiensis* increased the number of pods significantly by 30-54%. Pod weight was increased by 25% while seed yield by 35% in plants treated with *B. thuringiensis* (de Freitas *et al.*, 1997).

Shoot and root length were enhanced as well as increase in fresh biomass and total dry matter using rhizospheric *Bacillus* spp. for the biocontrol of anthracnose caused by *Colletotrichum acutatum* on pepper. AB05 (*B. amyloliquefaciens*) and AB12 (*B. subtilis*) inhibited the growth of *C. acutatum* by 60% and induced increase in weight of pepper fruit. In the greenhouse disease was more than 30%. These rhizobacteria solubilized phosphate and produced phytohormone IAA which are factors regarded as systemic acquired resistance induced in different and diverse plants making such isolates to be considered as potential biocontrol agents (Lamsal *et al.*, 2012).

Reports have shown that two strains of *B. pumilus* (203-6 and 203-7) and one of *B. mycoides* (Strain Bac J) were able to significantly reduce the severity of *Cercospora* leaf spot of sugar beet which is caused by *Cercospora beticola* Sacc. by eliciting ISR (Bargabus *et al.*, 2002; Bargabus *et al.*, 2004; Kloepper *et al.*, 2004). Also, growth of banana plantlets increased and Fusarium wilt of banana caused by *F. oxysporum cubense* was controlled as a result of treatment with *B. pumilus* ENF24 (Figueiredo *et al.*, 2010). *B. cereus* was effective in suppressing alfalfa diseases, thereby enhancing the emergence of seedling and increasing

nodulation in common beans (Camacho *et al.*, 2001; Figueiredo *et al.*, 2007). *B. megaterium* has been found to increase growth parameters in the root which include the length of the root and the dry matter content of the root (Kaymak *et al.*, 2008).

Findings from this study showed that the result of the greenhouse was quite different from the result *in vivo* which means that antifungal activities *in vitro* does not always correlate with disease reduction *in vivo.* This is in agreement with Schmledeknecht *et al.*, (2001) who reported that *B. subtilis* FZB24 and FZB37 inhibited mycelial growth of *F. oxysporum, R. solani* and Sclerotinia *sclerotiosum in vitro*. Incidence of *F. oxysporum* disease was significantly reduced by up to 50% while plant height, root and shoot fresh weight increased significantly compared to the control. According to Chérif *et al.*, (2002), the reason for this is because bacteria that antagonise soil-borne pathogen *in vitro* are not necessarily the most effective *in vivo* and vice-versa.

Colonization of root was not inspected in this research but from the morphology of the root samples, those treated with *Bacillus* isolates had more root hairs compared to the uninoculated control.

5.6 Molecular characterization and biocontrol of tomato Fusarium wilt

From the results of the 3 primers, the high similarity matrix among the *Bacillus* isolates was not unexpected because they are of the same species while their diversity was also not unexpected because of the differences in their genetic level. It was also observed that even in isolation of the same species polymorphism exist but not as high because of the fact that they are from the same species.

In this study, out of 10-mer primers, S4 and OPH 19, OPH 19 produced the lowest number of bands that is 16. The dendrogram also showed that the *Bacillus* isolate Bac C (OPH 2) that had the best growth is distant from Bac P (OPH 3) and Bac S (OPH 4) but closest to Bac A (OPH 1) and Bac Bb (OPH 6). This relatedness is also observed in their biocontrol activities where Bac C had the highest disease control of 81.20% *in vivo* which was quite close to disease control of 75% by Bac A even though Bac C only produced IAA while Bac A produced both IAA and solubilized phosphate. While using primer A9B7, even though Bac A was closer to Bac S than Bac P, the effect can also be likened to the effect from the use of primer OPH 19. This also means that the relationship between primer OPH 19 and biocontrol activities of the *Bacillus* isolates is the same with primer A9B7. Primer S4 showed the

diversity among all isolates. This is reflected in their different mechanism of biocontrol and confirms the variations in their different growth parameters.

In this study, all the primers were quite informative since they all have high level of polymorphism 90.90% for primer A9B7, 92.59% for primer S4 and 87.5% for primer OPH 19. Baysal *et al.*, (2008) while using primer OPH 19 in characterizing 2 strains of *Bacillus subtilis*, EU 07 and QST 713 had 6 observed bands which included 2 polymorphic bands which is 33.3% polymorphism. This is also in agreement with Kozyrenko *et al.*, (2001), who observed that the banding patterns were marked differently from one another with each primer. Also, the common amplicons in the agarose profile differ only in the intensity of their bands in the RAPD spectra.

Using primer A9B7, the number of bands from this study is in agreement with the observation recorded by El-Mokadem and Hadia (2008), whose level of polymorphism was higher. In their study, Primer A9B7 produced bands ranging from 2-6 bands with only 1 common band in 5 isolates including the control showing 77.27% polymorphism.

According to Abdel Ghany and Zaki, (2003) while using primer A9B7, RAPD products from four *Gossypium barbadense* cultivars generated bands between 300 and 1500bp each cultivar having a minimum of 2 bands and a maximum of 4 bands which is also similar in terms of number of bands observed in this study with minimum of 1 and a maximum of 5 visible bands which ranged in size from 75 to 600 bp. Even though *G. Barbadense* is an angiosperm, this result shows the ability of the primer to bind randomly to genes on its genomes.

Using primer A9B7 was quite informative and was able to distinguish six isolates of rhizobacteria from rhizosphere of water hyacinth out of which one of the isolates produced no bands and number of bands ranged between 2 and 4 bands among the other five (Abou-Shanab *et al.*, 2007), which is also quite similar to the result of this study.

Based on the different primers used, the 11 *Bacillus* isolates of this study were grouped into 2 basic clusters using primer A9B7 viz; cluster with only isolate 3(Bac P) and the cluster including the 5 sub clusters. Primer OPH 19 was able to group the 11 isolates into 5 basic clusters and in this case only isolate 3 and isolate 8 had individual clusters. Primer S4 showed greater diversity among the *Bacillus* isolate by grouping them into 8 clusters. The following isolates, 2, 4, 5, 8, 9 and10 formed individual clusters, isolates while the others formed 2 main clusters.

This diversity experienced based on each of the three primers used might account for the differences in the biocontrol activities of the different *Bacillus* isolates *in vitro* and *in vivo.* Using the example of isolate 1 (Bac A) and isolate 2 (Bac C), primers A9B7 and S4 help to show that they are diverse and distant but a converging point from primer OPH 19 helps to reveal their similarity in being involved in biocontrol but at different levels. Bac A had highest percentage of inhibition *in vitro* while Bac C had highest percentage of disease reduction *in vivo*. This similarity and diversity can also be emphasized with isolate 3 (Bac P) and isolate 4 (Bac S), which both had similarity in the percentage of disease reduction *in vivo* but zone of inhibition varied slightly between them.

CONCLUSION

Biocontrol in this study was brought about by the *Bacillus* isolates, their by-products and metabolites produced in the form of antibiotics. The result of this research showed that the four *Bacillus* isolates can be used for biocontrol of Fusarium wilt of tomato. It also emphasized the fact that sometimes there is no correlation between *in vitro* antagonism and *in vivo* diseases control. This could help to open more research prospects with a view to understanding the interactions for the latter and how to harness the resulting opportunities.

The purpose of the study was to analyse the biocontrol activities of native *Bacillus* isolates against Fusarium wilt pathogen of tomato in order to eradicate the disease. This was carried out as seen in the *in vitro* and *in vivo*. *In vitro* biocontrol activities were seen in the antifungal activities of the various *Bacillus* isolates as they had varying degrees of *Fusarium* growth inhibition. This was also observed in the various mechanisms of the biocontrol activities which included phosphate solubilization ability, production of indole acetic acid as seen in the utilization of tryptophan and hydrogen cyanide production. *In vivo* biocontrol activities were seen in the ability of the *Bacillus* isolates to reduce disease incidence and promote growth as observed in the various growth parameters

The objectives of this research as outlined were also achieved in the molecular characterization of the native *Bacillus* isolates as this provided information on relatedness of the various *Bacillus* isolates. Since RAPD analysis amplifies genes randomly, some of the genes that aided the biocontrol abilities of the *Bacillus* isolates were amplified, but only further research work on targeted genes will be able to elucidate this. This also put into perspective the issues of particular and specific genes in each *Bacillus* isolate of interest that can also be cloned in further research studies.

Also, this present results shows that tomato plants treated with *Bacillus* isolates contain vital micro and macro nutrients compared to untreated tomatoes and those that are infected with Fusarium wilt. This variation in the nutritive values of the different tomatoes could be as a result of the different treatments on the plants and the microbial interactions that took place. All the tomatoes are good sources of qualitative mineral elements even those infected with *Fusarium.* This means that those infected with *Fusarium* also had vital mineral elements. Even though treatment with *B. cereus* supported growth and controlled Fusarium wilt, treated plants contained small quantity of mineral elements compared to the others. Therefore research studies should be carried out to understand this and how to improve the various

treatments so that they will not just increasing vegetative growth but can also be involved in reproductive aspect of the plants, the mineral composition inclusive. The similarity and diversity of the *Bacillus* isolates as seen in the RAPD-PCR put into perspective the variation in the biocontrol activities of the isolates *in vitro* and *in vivo*. Extension of this research work can be carried out on the formulation of these *Bacillus* isolates into microbial products that can be easily employed as biocontrol agents that will eventually lead to eradicating the problem of Fusarium wilt.

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APPENDIX

1.0 List of articles already published or accepted

1.1. Ajilogba, C. F. & Babalola, O. O. 2013 Integrated management strategies for tomato *Fusarium* Wilt. *Biocontrol Science* 18(3): 117-127. (ISSN 13424815) https://www.jstage.jst.go.jp/article/bio/18/3/18_117/_pdf

1.2. Ajilogba C. F., Babalola O. O. and Ahmad F. (2013) Antagonistic effects of *Bacillus* species in biocontrol of tomato Fusarium wilt. Studies on Ethno Medicine 7(3): 205-216. http://www.krepublishers.com/02-Journals/S-EM/EM-07-0-000-13-Web/S-EM-07-0-000-2013-Contents/EM-07-0-000-13-Contents.htm

1.3. Ajilogba, C. F. & Babalola, O. O. 2014. *Effects and fate of microorganisms in the control of common diseases of tomato* (*in press*) Agriculture and Environmental Pollution: Causes, Consequences and Control. EB/2013/271/ECB ENVBOOKS series, Delhi, India EnvBook series, Delhi India

1.4. Ajilogba, C. F. & Babalola O. O. (2013) Biocontrol treatment Of Fusarium wilt In tomato. SASM 2013 (ID. 13, Poster) (The 18th Biennial Conference of the South African Society for Microbiology). 24-27 Nov. Forever Resorts Warmbaths, Bela-Bela. South Africa

1.5. Ajilogba, C. F. & Babalola, O. O. (2012) Cultural control of Fusarium wilt of tomato. BioFISA Phase I Programme Closure Conference: Celebrating Four Years of BioFISA, 2-6 September 2012, Irene County Lodge, Centurion, South Africa

2.0. EDX 720 spectrophotometry analysis of the mineral content of each treatment with *Bacillus* isolate

Biocontrol Science, 2013, Vol. 18, No. 3, 117—127

Review

Integrated Management Strategies for Tomato *Fusarium* Wilt

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Received 20 December, 2012/Accepted 30 January, 2013

***Fusarium* wilt is caused by the fungal pathogens, *Fusarium oxysporum* or *Fusarium solani*. It is a devastating disease that affects many important food and vegetable crops and a major source of loss to farmers worldwide. Initial strategies developed to combat this devastating plant disease include the use of cultural, physical and chemical control. None of these strategies have been able to give the best results of completely ameliorating the situation except for the cultural method which is mainly preventive. A good knowledge of the nature, behaviour and environmental conditions of growth of the disease agent is very important to controlling the disease development in that case. Biological control has been shown to be an environmentally friendly alternative. It makes use of rhizospheric and endophytic microorganisms that can survive and compete favourably well with the *Fusarium* wilt pathogen. They include plant growth-promoting rhizobacteria (PGPR) such as *Bacillus* spp. and *Pseudomonas* spp.. For PGPR to control or inhibit the growth of the *Fusarium* wilt pathogen, they make use of mechanisms such as indole acetic acid production, siderophore production, phosphate solublilization, systemic resistance induction and antifungal volatile production among others.**

Key words : Biological control / Disease development / Pathogen / Plant growth-promoting rhizobacteria.

INTRODUCTION

Fusarium is one of the more troublesome genera of fungal plant pathogens, causing devastating diseases like *Fusarium* wilt and *Fusarium* root/stem rot in numerous economically important crops. It is one of the most serious diseases affecting tomato plants throughout the world, especially in upland areas (Charoenporn et al., 2010). *Fusarium* wilt is caused by *Fusarium oxysporum* Schlecht or *Fusarium solani*. Formae speciales of *F. oxysporum* can usually only cause *Fusarium* vascular wilt on one plant host species. The predominant hosts for *F. solani* are vegetable crops but some strains may be infectious to man. There are more than 100 *Fusarium* vascular wilt diseases worldwide (Burgess et al., 2008). Apart from causing diseases, they colonize outer cells of roots as harmless endophytes after the pathogen has killed the root tissues and others live as saprophytes in soil (Burgess et al., 2008). Some strains of *F.oxysporum* are not pathogenic and may even antagonize the growth of pathogenic strains and can be used as biological control agents (Fravel et al., 2003). To provide an environmentally friendly *Fusarium* disease control system, the use of antagonistic microorganisms represents an alternative disease management strategy (Lugtenberg and Kamilova, 2009).

This review will summarize the knowledge available for the use of native *Bacillus* spp. with biocontrol potential to combat *Fusarium* wilt of tomatoes. The origin, taxonomy and morphology of the *Fusarium* wilt pathogen will be introduced together with the pathogen and the disease. The initial management strategies will be reviewed and then the use of biological control will be discussed.

FUSARIUM WILT AND THE FUNGAL PATHOGENS

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Ethno Med, 7(3): 205-216 (2013)

Antagonistic Effects of *Bacillus* Species in Biocontrol of Tomato *Fusarium* Wilt

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KEYWORDS *Bacillus* spp. Biological Control. *Fusarium solani*. Inhibition. *L. esculentum*

ABSTRACT *Fusarium solani* is the causative organism of *Fusarium* wilt of tomato (*Lycopersicon esculentum* Mill). Four *Bacillus* spp identified as *B. amyloliquefaciens, B. cereus, B. pumilus* and *B. subtilis* were tested for biocontrol activities *in vitro* as zone of inhibition and *in vivo* as percent disease control and disease incidence. The result from *in vitro* analysis showed that *B. amyloliquefaciens* inhibited the growth of *F. solani* the most by 95.2% while *B. cereus* had the highest growth of *F. solani* and inhibition of 55.7%. *B pumilus* and *B. subtilis* showed inhibition of 70.46% and 82.1% respectively and were significantly lower ($p=0.05$) than the control with 100% *F. solani* growth. *In vivo, B. cereus* had the least disease incidence and highest percent disease control (18.75% and 81.2%). This was significantly different from the control (100% and 0%), *B. amyloliquefaciens* (25% and 75%), *B. pumilus* (37.5% and 62.5%) and *B. subtilis* (37.5% and 62.5%). Also, growth parameters like shoot and root length from treatment with *B. cereus* (38 mm ±1.47 and 31 mm ±1.22) were significantly longer ($p=0.05$) compared to the control. This result shows that these four *Bacillus* spp are very effective biocontrol agents and should be harnessed for further biocontrol applications.

INTRODUCTION

Tomato is one of the most important vegetables because of its health benefits and phytochemical properties. Because of its low calorie and absence of cholesterol, it is one of the recommendations of diets needing low cholesterol. They are quite rich in many important nutrients and vitamins which include phosphorus and potassium and also vitamins B and C. They are also very important against common cancers like breast and prostate cancer.

As important as tomato is nutritionally and in being an important cash crop for smallholders and medium-scale commercial farmers in Africa, soil-borne pathogens inflicts a lot of diseases and infections on it (Babalola and Glick 2012). Such diseases include Bacterial wilt, root knot nematodes disease, early blight, late blight and Fusarium wilt. Fusarium wilt is a devastating disease of tomato and causes a lot of loss to farmers worldwide. symptoms begins as gradual yellowing and wilting of the lower leaves (Khan and Khan 2002) which is brought about by the growth of the microconidia inter-cellularly in the xylem of the stem and root. As a result of the failure of the infected xylem of the plant to meet the water requirement of the plant, death of the tomato plant is inevitable (Burgess et al. 2008). Spores from the conidia are released into surrounding tissues as the plant dies. They later form chlamydospores that fall back into the soils (Jones 2000). These spores can remain in the soil for as long as 30 years until favourable conditions are available and they can re-infect plants (Thangavelu et al. 2004).

Several microorganisms are being used in the control of tomato pests and diseases. Listed among tomato pest control agents are *F. compactum* and *F. arthrosporioides* (Babalola 2010a,b,c). Included in tomato disease control agents are *Trichoderma, Pseudomonas* and *Bacillus* species. Bacillus-based biocontrol agents are quite important in the management of pests and plant diseases (Jacobsen et al. 2004). Varieties of *Bacillus* and *Paenibacillus* help to promote the health of crops and control diseases by producing antibiotic metabolites, suppressing plant pathogens, others antagonise plant pathogens by competing for nutrients like iron and phosphate, others indirectly fix nitrogen which they make available to the plants and help stimulate plant nutrient uptake (Gardener 2004). This research seeks to elucidate the biocontrol abilities of these four *Bacillus* spp in order to make use of these abilities for further biocontrol interventions.

MATERIAL AND METHODS

Bacillus spp Inoculum Preparation

The following typed cultures and locally isolated organisms (LIO) from the culture collec-

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Fate and effects of microorganisms in the control of common diseases of tomato

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Abstract

Tomato (*Lycopersicon esculentum* mill) is a very important vegetable and fruit crop. It is nutritious and contains vitamin A, B and the antioxidant lycopene that is useful in the prevention and treatment of common cancers. Tomatoes are easily susceptible to fungal, bacterial, viral, and nematodal diseases and infections. Such diseases include wilts, root rots, canker, spots and specks, tomato spotted wilt virus, tomato bushy stunt virus and root knot. These diseases lead to stunted growth in tomato seedlings, wilting and death of the tomato plants. This in turn leads to little or no yield from the tomato to the farmer. The effects microorganisms on tomatoes can both be beneficial and adverse. The beneficial effects include promoting plant growth and biocontrol while the adverse effects include non-target microorganisms being displaced from their habitats, the microorganisms being pathogenic to non-target organisms, the microorganisms being toxic to non-target organisms and humans/other animals beings allergic to the microorganisms. The fate of these microorganisms can be determined by monitoring them and generating data about their microbial ecology over time to ascertain how they will fare if they are formulated as this helps to determine their future. This review seeks to outline the importance of tomato plant, the various diseases affecting it, and the fate and the effect of using microorganisms to stop these infections.

Keywords

Biological control, diseases, effects, fates, microorganisms, tomato

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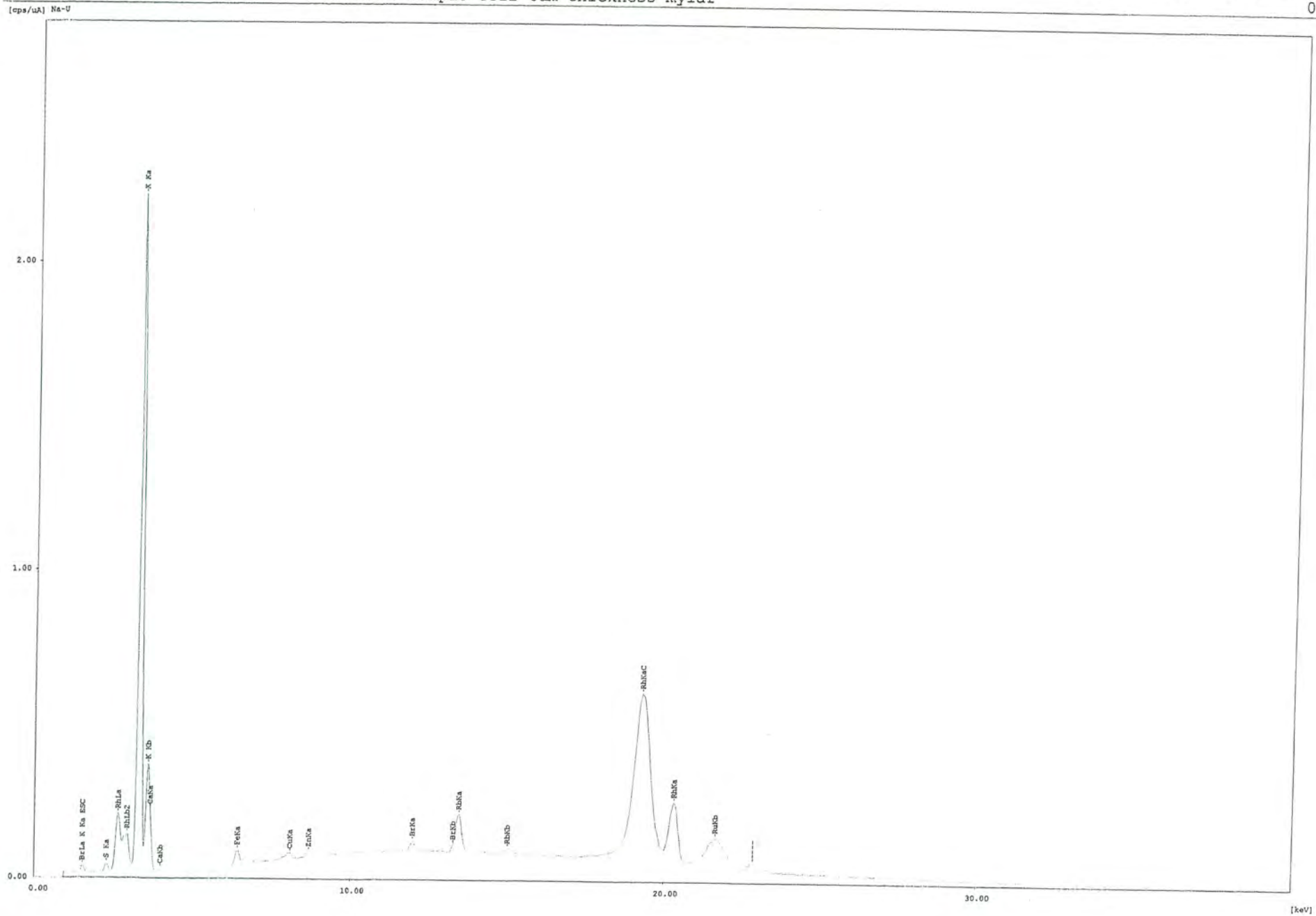

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Measurement Condition

Instrument: EDX-720 Atmosphere: Air Collimator: 10(mm) Spin: Off

Analyte	TG	kV	uA	FI	Acq.(keV)	Anal.(keV)	Time(sec)	DT(%)
Na-U	Rh	50	155-Auto	----	0 - 40	0.00-40.00	Live- 100	41

Qualitative Result

Element: Br, K , S , Rh, Ca, Fe, Cu, Zn, Rb, Ru

Peak List

Channel	Line	keV	Net Int.(cps/uA)	
Na-U	BrLa	1.54	0.0079	
	K KaESC	1.54	0.1331	
	S Ka	2.30	0.1929	qf
	RhLa	2.66	1.3112	
	RhLb2	2.94	0.8762	
	K Ka	3.32	16.3320	qf
	K Kb	3.60	2.6641	
	CaKa	3.68	0.8686	qf
	CaKb	4.02	0.1216	
	FeKa	6.42	0.4894	QF
	CuKa	8.08	0.2271	QF
	ZnKa	8.68	0.2143	QF
	BrKa	11.98	0.3535	QF
	BrKb	13.28	0.0612	
	RbKa	13.46	1.3840	QF
	RbKb	15.00	0.2513	
	RhKaC	19.28	6.9199	
	RhKa	20.30	2.4850	
	RuKb	21.62	1.2439	
	----	22.86	0.3421	

Quantitative Result

Analyte	Result	(Std.Dev.)	Proc.-Calc.	Line	Int.(cps/uA)
====[No. 1 Layer]====<	Layer1		>==========		
	6.000 um	(------)	Fix	--------	--------
C10H8O4	100.000 %	(------)	Fix	--------	--------
====[No. 2 Layer]====<	Base		>==========		
K	86.484 %	(0.168)	Quan-FP	K Ka	16.3320
Ca	9.857 %	(0.160)	Quan-FP	CaKa	0.8686
S	2.386 %	(0.064)	Quan-FP	S Ka	0.1929
Fe	0.625 %	(0.011)	Quan-FP	FeKa	0.4894
Rb	0.307 %	(0.003)	Quan-FP	RbKa	1.3840
Cu	0.137 %	(0.006)	Quan-FP	CuKa	0.2271
Zn	0.108 %	(0.005)	Quan-FP	ZnKa	0.2143
Br	0.096 %	(0.003)	Quan-FP	BrKa	0.3535

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Comment : sample cell 6um thickness mylar

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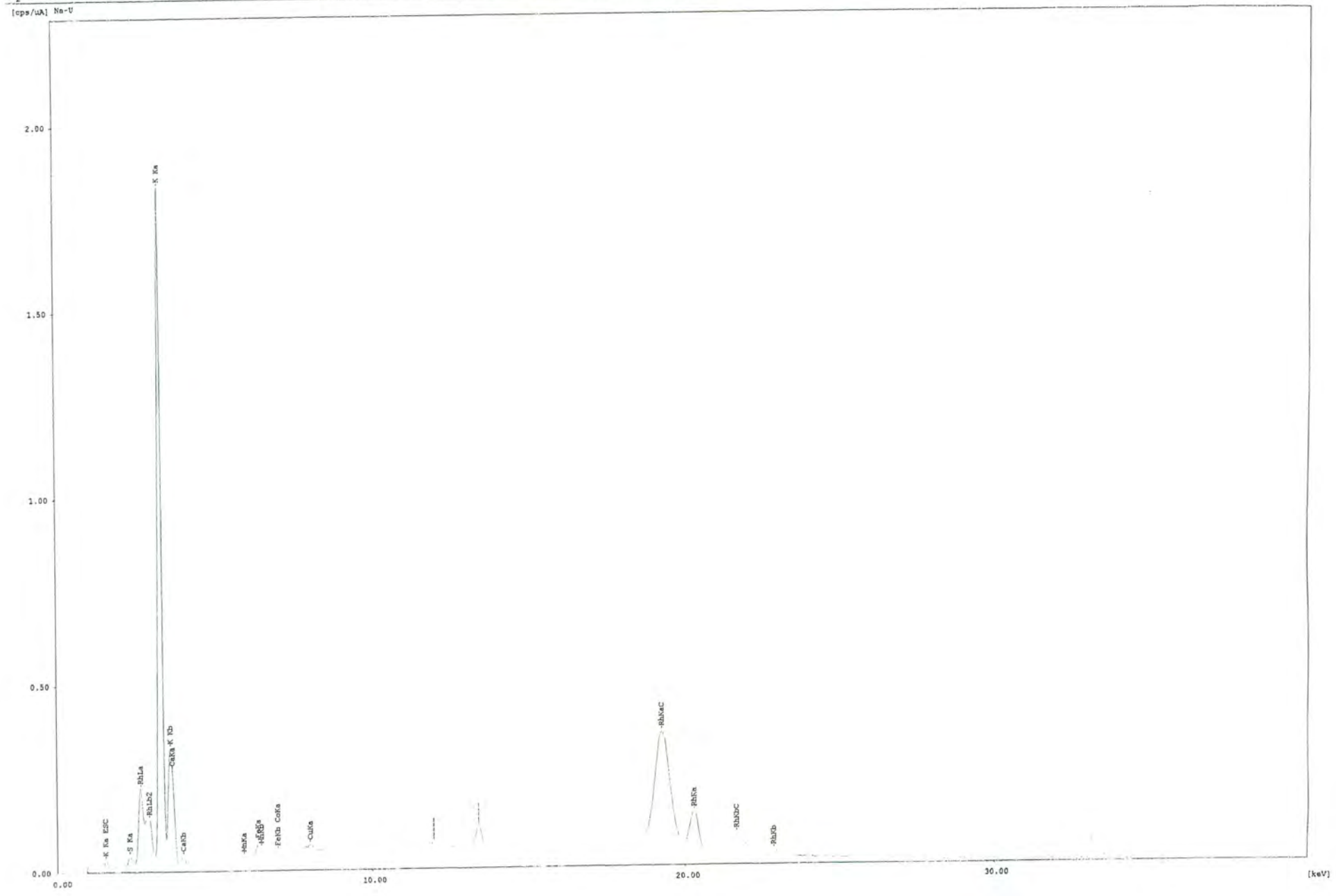

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Group : small_solid
Date : 2013-03-20 08:38:18

Measurement Condition

Instrument: EDX-720 Atmosphere: Air Collimator: 10(mm) Spin: Off

Analyte	TG kV	uA	FI	Acq.(keV)	Anal.(keV)	Time(sec)	DT(%)
Na-U	Rh 50	225-Auto	----	0 - 40	0.00-40.00	Live- 100	40

Qualitative Result

Element: K , S , Rh, Ca, Mn, Fe, Co, Cu

Peak List

Channel	Line	keV	Net Int.(cps/uA)	
Na-U	K KaESC	1.54	0.1129	
	S Ka	2.30	0.1948	qf
	RhLa	2.66	1.4257	
	RhLb2	2.94	0.8544	
	K Ka	3.32	13.4853	qf
	K Kb	3.62	2.3560	
	CaKa	3.68	1.4046	qf
	CaKb	4.02	0.1966	
	MnKa	5.94	0.0780	QF
	FeKa	6.40	0.3685	QF
	MnKb	6.50	0.0117	
	FeKb	7.02	0.0553	
	CoKa	7.02	0.0384	QF
	CuKa	8.04	0.2047	QF
	----	11.98	0.1656	
	----	13.44	0.6913	
	RhKaC	19.28	4.0921	
	RhKa	20.28	1.3488	
	RhKbC	21.64	0.7242	
	RhKb	22.84	0.2121	

Quantitative Result

Analyte	Result	(Std.Dev.)	Proc.-Calc.	Line	Int.(cps/uA)
====[No. 1 Layer]====< Layer1		>==========			
	6.000 um	(------)	Fix	--------	--------
C10H8O4	100.000 %	(------)	Fix	--------	--------
====[No. 2 Layer]====< Base		>==========			
K	79.584 %	(0.142)	Quan-FP	K Ka	13.4853
Ca	16.851 %	(0.142)	Quan-FP	CaKa	1.4046
S	2.692 %	(0.057)	Quan-FP	S Ka	0.1948
Fe	0.533 %	(0.010)	Quan-FP	FeKa	0.3685
Mn	0.154 %	(0.011)	Quan-FP	MnKa	0.0780
Cu	0.140 %	(0.005)	Quan-FP	CuKa	0.2047
Co	0.045 %	(0.008)	Quan-FP	CoKa	0.0384

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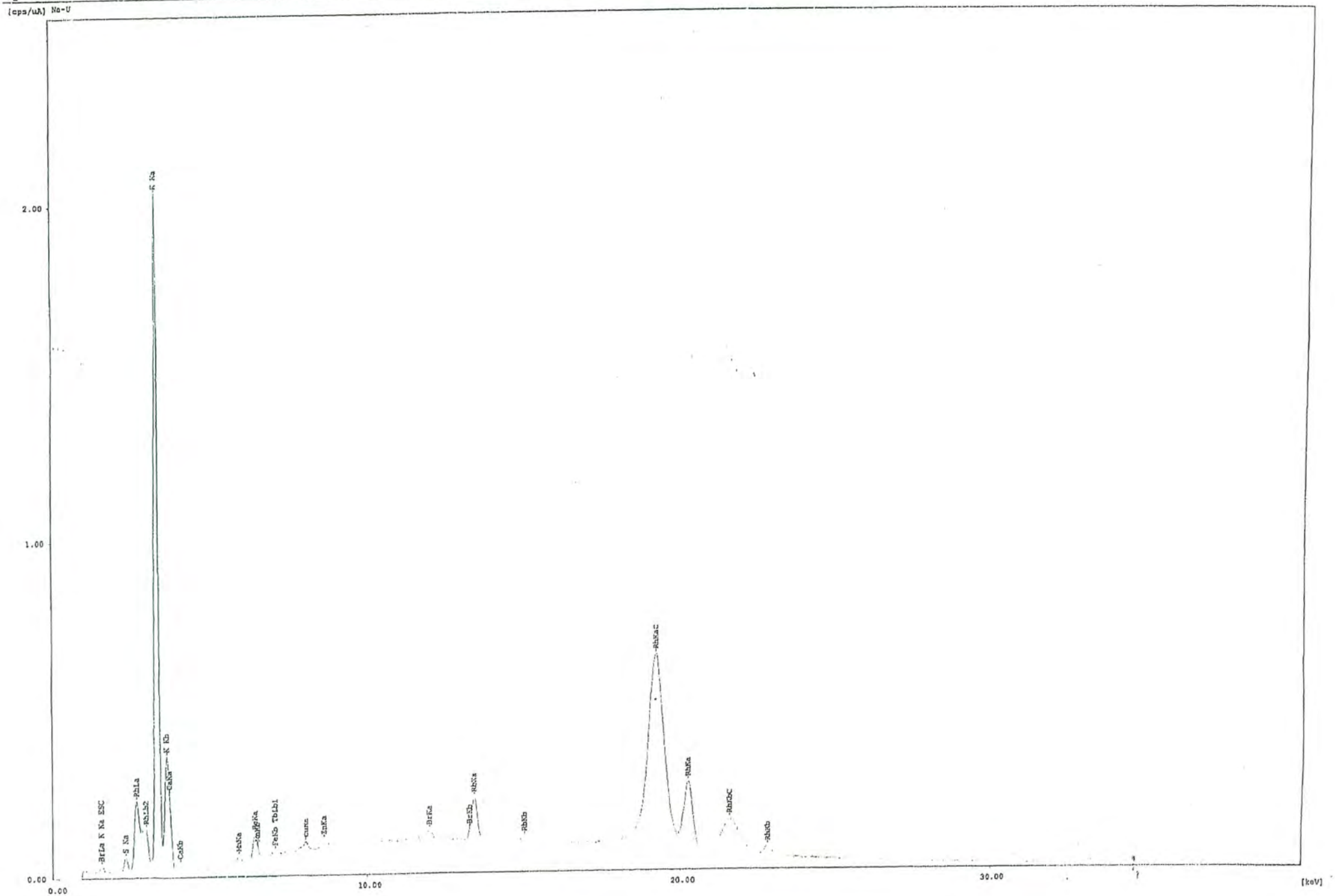

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Date : 2013-03-20 09:07:51

Measurement Condition

Instrument: EDX-720 Atmosphere: Air Collimator: 10(mm) Spin: Off

Analyte	TG kV	uA	FI	Acq.(keV)	Anal.(keV)	Time(sec)	DT(%)
Na-U	Rh 50	141-Auto	----	0 - 40	0.00-40.00	Live- 100	39

Qualitative Result

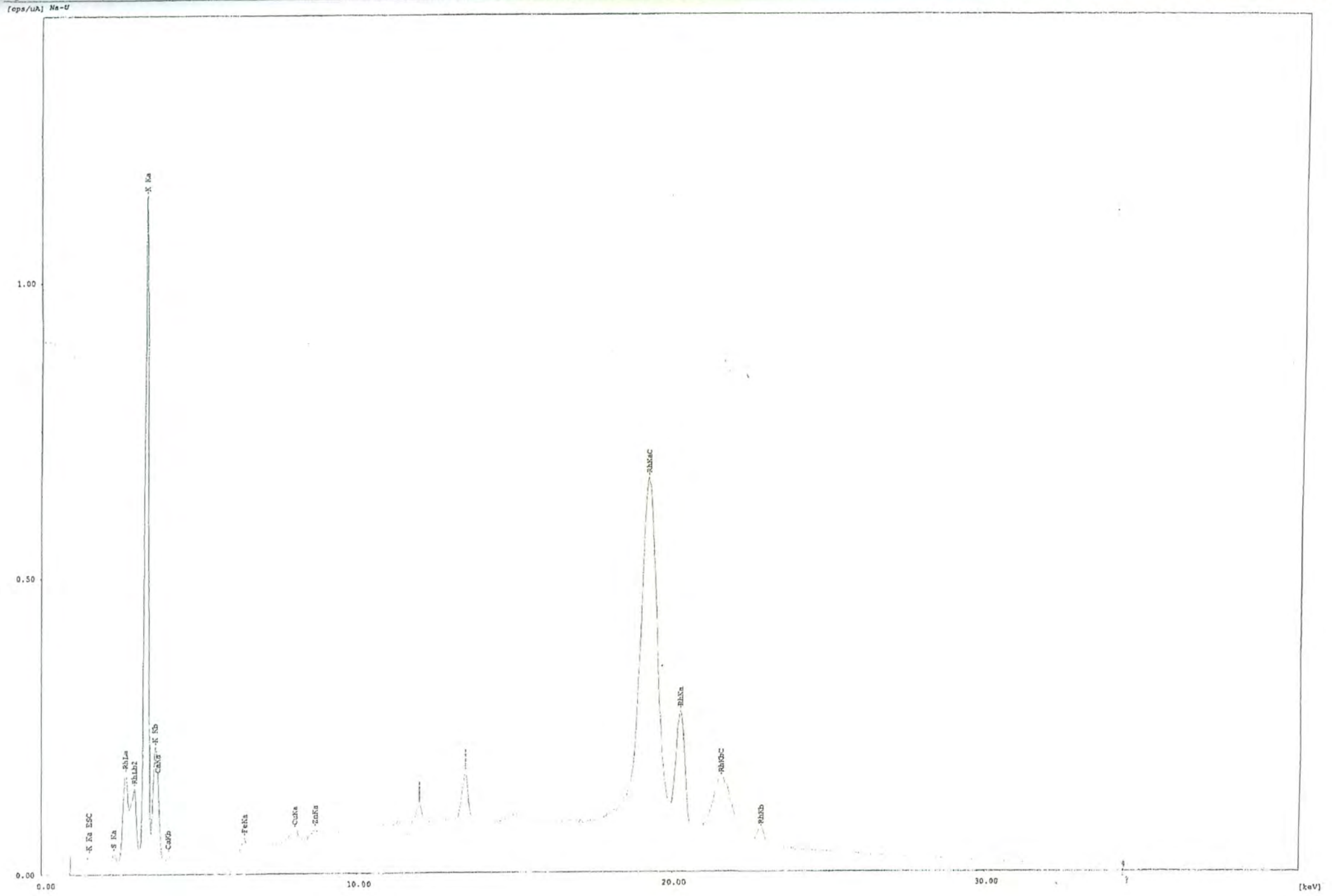
Element: Br, K , S , Rh, Ca, Mn, Fe, Tb, Cu, Zn, Rb

Peak List

Channel	Line	keV	Net Int.(cps/uA)	
Na-U	BrLa	1.54	0.0079	
	K KaESC	1.54	0.1209	
	S Ka	2.30	0.2778	qf
	RhLa	2.64	1.4379	
	RhLb2	2.94	0.8795	
	K Ka	3.30	14.9291	qf
	K Kb	3.60	2.5693	
	CaKa	3.68	1.2760	qf
	CaKb	4.02	0.1786	
	MnKa	5.90	0.2208	QF
	FeKa	6.42	0.7033	QF
	MnKb	6.50	0.0331	
	FeKb	7.06	0.1055	
	TbLb1	7.06	0.0606	
	CuKa	8.06	0.3291	QF
	ZnKa	8.64	0.3019	QF
	BrKa	11.96	0.3963	QF
	BrKb	13.28	0.0634	
	RbKa	13.42	1.4603	QF
	RbKb	15.02	0.2574	
	RhKaC	19.26	7.3008	
	RhKa	20.26	2.5175	
	RhKbC	21.56	1.3065	
	RhKb	22.82	0.3721	

Quantitative Result

Analyte	Result	(Std.Dev.)	Proc.-Calc.	Line	Int.(cps/uA)
====[No. 1 Layer]====<	Layer1				
	6.000 um		(------) Fix	--------	--------
C10H8O4	100.000 %		(------) Fix	--------	--------
====[No. 2 Layer]====<	Base				
K	80.729 %	(0.174)	Quan-FP	K Ka	14.9291
Ca	13.705 %	(0.161)	Quan-FP	CaKa	1.2760
S	3.518 %	(0.077)	Quan-FP	S Ka	0.2778
Fe	0.886 %	(0.013)	Quan-FP	FeKa	0.7033
Mn	0.380 %	(0.013)	Quan-FP	MnKa	0.2208
Rb	0.324 %	(0.004)	Quan-FP	RbKa	1.4603
Cu	0.198 %	(0.007)	Quan-FP	CuKa	0.3291
Zn	0.152 %	(0.006)	Quan-FP	ZnKa	0.3019
Br	0.108 %	(0.004)	Quan-FP	BrKa	0.3963


[cps/uA] Na-U
1.00
0.50
0.00
0.00
10.00
20.00
30.00
[keV]
K Ka
K Ka ESC
S Ka
RhLa
RhLb2
CaKa
K Kb
CaKb
FeKa
CuKa
ZnKa
RhKaC
RhKa
RhKbC
RhKb

Sample : BAC SF
Operator: Sizwe
Comment : sample cell 6um thickness mylar
Group : small_solid
Date : 2013-03-20 08:53:43

Measurement Condition

Instrument: EDX-720 Atmosphere: Air Collimator: 10(mm) Spin: Off

Analyte	TG kV	uA	FI	Acq.(keV)	Anal.(keV)	Time(sec)	DT(%)
Na-U	Rh 50	155-Auto	----	0 - 40	0.00-40.00	Live- 100	40

Qualitative Result

Element: K , S , Rh, Ca, Fe, Cu, Zn

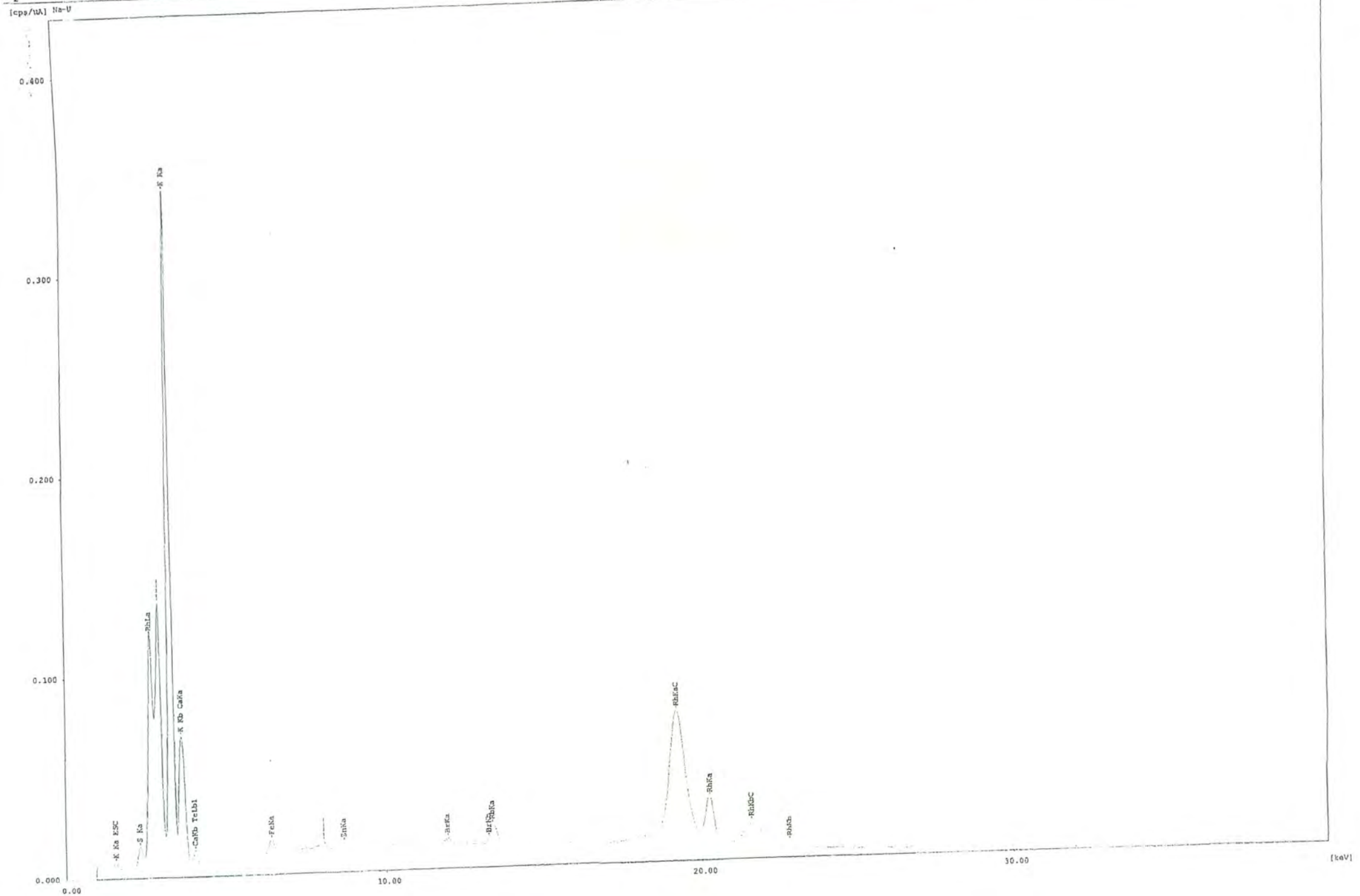
Peak List

Channel	Line	keV	Net Int.(cps/uA)	
Na-U	K KaESC	1.54	0.0737	
	S Ka	2.30	0.1076	qf
	RhLa	2.66	1.0346	
	RhLb2	2.94	0.8768	
	K Ka	3.32	8.3521	qf
	K Kb	3.60	1.4718	
	CaKa	3.68	0.8440	qf
	CaKb	4.02	0.1182	
	FeKa	6.44	0.2209	QF
	CuKa	8.04	0.1937	QF
	ZnKa	8.68	0.1467	QF
	----	11.98	0.3553	
	----	13.44	1.0002	
	RhKaC	19.26	7.4896	
	RhKa	20.28	2.5212	
	RhKbC	21.56	1.3202	
	RhKb	22.84	0.3558	

Quantitative Result

Analyte	Result	(Std.Dev.)	Proc.-Calc.	Line	Int.(cps/uA)
====[No. 1 Layer]====<	Layer1				
	6.000 um	(------)	Fix		
C10H8O4	100.000 %	(------)	Fix		
====[No. 2 Layer]====<	Base				
K	80.095 %	(0.222)	Quan-FP	K Ka	8.3521
Ca	16.604 %	(0.224)	Quan-FP	CaKa	0.8440
S	2.426 %	(0.109)	Quan-FP	S Ka	0.1076
Fe	0.522 %	(0.018)	Quan-FP	FeKa	0.2209
Cu	0.216 %	(0.011)	Quan-FP	CuKa	0.1937
Zn	0.137 %	(0.009)	Quan-FP	ZnKa	0.1467

Sample : FSP
Operator: Sizwe
Group : small_solid
Comment : sample cell 6um thickness mylar



Sample : FSP
Operator: Sizwe
Comment : sample cell 6um thickness mylar
Group : small_solid
Date : 2013-03-20 09:24:03

Measurement Condition

Instrument: EDX-720 Atmosphere: Air Collimator: 10(mm) Spin: Off

Analyte	TG	kV	uA	FI	Acq.(keV)	Anal.(keV)	Time(sec)	DT(%)
Na-U	Rh	50	990-Auto	----	0 - 40	0.00-40.00	Live- 100	41

Qualitative Result

Element: K , S , Rh, Ca, Te, Fe, Zn, Br, Rb

Peak List

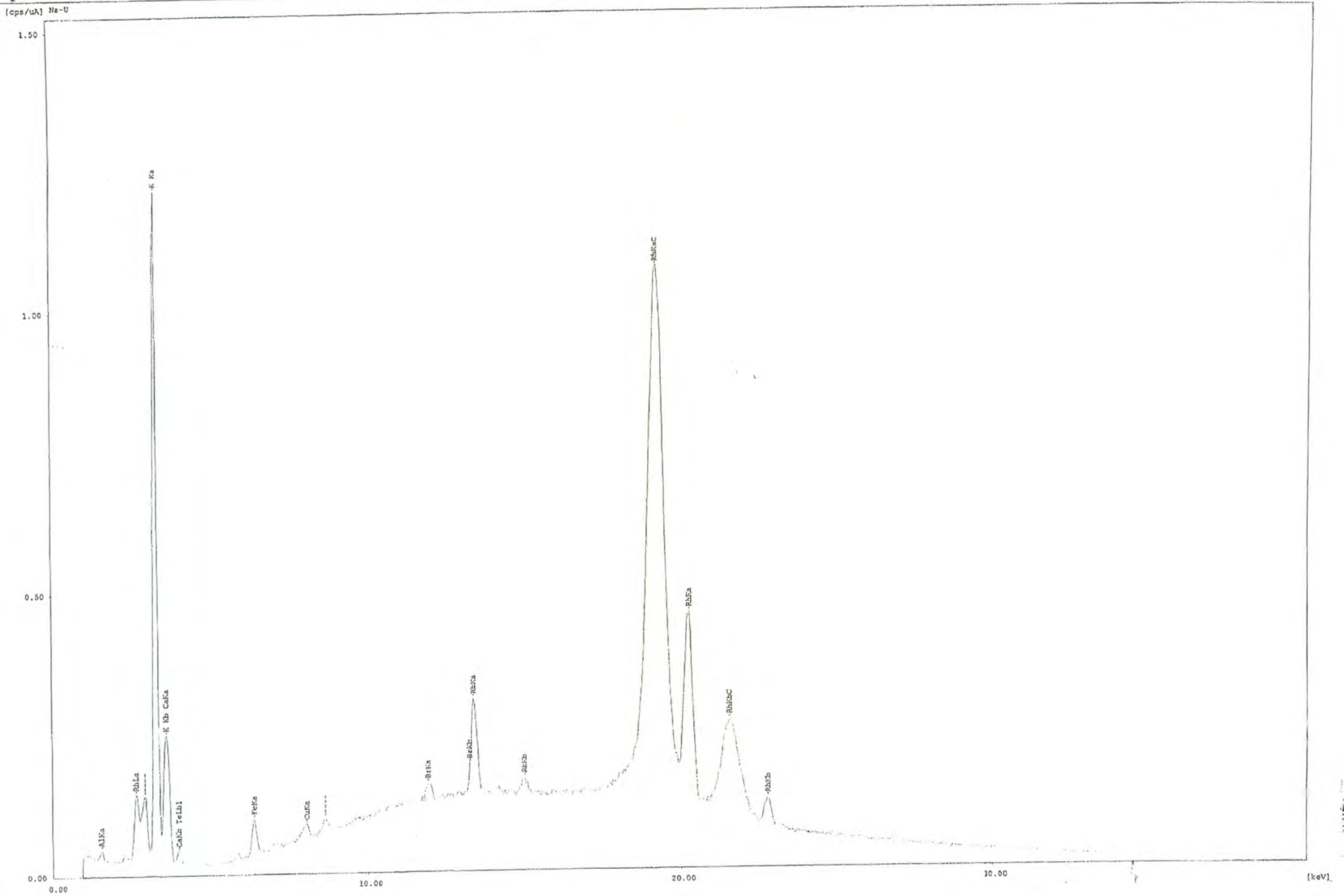
Channel	Line	keV	Net Int.(cps/uA)	
Na-U	K KaESC	1.56	0.0199	
	S Ka	2.30	0.0647	qf
	RhLa	2.66	0.7903	
	----	2.94	0.9246	
	K Ka	3.30	2.4774	qf
	K Kb	3.62	0.3468	
	CaKa	3.62	0.1252	qf
	CaKb	4.02	0.0175	
	TeLb1	4.02	0.0308	
	FeKa	6.42	0.0732	QF
	----	8.06	0.0356	
	ZnKa	8.68	0.0264	QF
	BrKa	11.96	0.0367	QF
	BrKb	13.28	0.0055	
	RbKa	13.40	0.1154	QF
	RhKaC	19.28	0.8385	
	RhKa	20.28	0.2923	
	RhKbC	21.58	0.1588	
	RhKb	22.82	0.0428	

Quantitative Result

Analyte	Result	(Std.Dev.)	Proc.-Calc.	Line	Int.(cps/uA)
====[No. 1 Layer]====<	Layer1				
	6.000 um	(------)	Fix	--------	--------
C10H8O4	100.000 %	(------)	Fix	--------	--------
====[No. 2 Layer]====<	Base				
C	85.416 %	(0.176)	Quan-FP	K Ka	2.4774
Ca	8.742 %	(0.193)	Quan-FP	CaKa	0.1252
S	4.977 %	(0.095)	Quan-FP	S Ka	0.0647
Fe	0.570 %	(0.013)	Quan-FP	FeKa	0.0732
Rb	0.154 %	(0.003)	Quan-FP	RbKa	0.1154
Zn	0.081 %	(0.005)	Quan-FP	ZnKa	0.0264
Br	0.060 %	(0.003)	Quan-FP	BrKa	0.0367

Operator: Sizwe

Comment : sample cell 6um thickness mylar



Sample : NBNF
Operator: Sizwe
Comment : sample cell 6um thickness mylar
Group : small_solid
Date : 2013-03-20 09:14:43

Measurement Condition

Instrument: EDX-720 Atmosphere: Air Collimator: 10(mm) Spin: Off

Analyte	TG	kV	uA	FI	Acq.(keV)	Anal.(keV)	Time(sec)	DT(%)
Na-U	Rh	50	100-Auto	----	0 - 40	0.00-40.00	Live- 100	39

Qualitative Result

Element: Al, Rh, K , Ca, Te, Fe, Cu, Br, Rb

Peak List

Channel	Line	keV	Net Int.(cps/uA)	
Na-U	AlKa	1.56	0.1032	qf
	RhLa	2.66	0.8054	
	----	2.94	0.8119	
	K Ka	3.30	8.8120	qf
	K Kb	3.64	1.2337	
	CaKa	3.64	0.4718	qf
	CaKb	4.02	0.0660	
	TeLb1	4.02	0.1000	
	FeKa	6.42	0.5368	QF
	CuKa	8.08	0.2772	QF
	----	8.66	0.2600	
	BrKa	11.94	0.3822	QF
	BrKb	13.28	0.0611	
	RbKa	13.42	1.9526	QF
	RbKb	15.02	0.3512	
	RhKaC	19.24	11.9728	
	RhKa	20.28	4.3184	
	RhKbC	21.56	2.2409	
	RhKb	22.82	0.6587	

Quantitative Result

Analyte	Result	(Std.Dev.)	Proc.-Calc.	Line	Int.(cps/uA)
====[No. 1 Layer]====<	Layer1		>==========		
	6.000 um	(------)	Fix	--------	--------
C10H8O4	100.000 %	(------)	Fix	--------	--------
====[No. 2 Layer]====<	Base		>==========		
Al	75.964 %	(5.070)	Quan-FP	AlKa	0.1032
K	22.937 %	(0.077)	Quan-FP	K Ka	8.8120
Ca	0.896 %	(0.031)	Quan-FP	CaKa	0.4718
Fe	0.103 %	(0.002)	Quan-FP	FeKa	0.5368
Rb	0.060 %	(0.001)	Quan-FP	RbKa	1.9526
Cu	0.024 %	(0.001)	Quan-FP	CuKa	0.2772
Br	0.015 %	(0.001)	Quan-FP	BrKa	0.3822