BIO-CONROL OF SELECTED BARLEY FUNGAL PATHOGENS USING *Paenibacillus polymyxa* KaI245 ISOLATED FROM SORGHUM RHIZOSPHERE IN WESTERN KENYA

BY

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DECLARATION

Declaration by the candidate

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or University of Eldoret.

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DEDICATION

To my parents Dr. Charles Chemitei and Mrs. Rosemary Chemitei, my siblings Mr. Kipchirchir Chemitei, Ms. Jepkemoi Rutto, Jepkemoi Terigi and my best friend Mr. Laban Ngobiro.

ABSTRACT

The use of agrochemicals in agriculture has several advantages but has been reported to cause phytotoxicity, hazardous to animals and the environment. In a quest to find alternatives, this study sought to test selected barley pathogens using a sorghum rhizobacterium identified earlier as *Paenibacillus polymyxa* KaI245. The selected barley pathogens were *Drechslera teres* f.sp *teres* (causal agent of barley net blotch) *Rhynchosporium commune* (causal agent of scald) and *Puccinia graminis* f. sp *hordei* (causal agent of barley stem rust). The objectives were; to isolate the fungal pathogens, screen *P. polymyxa* KaI245 for antifungal activity, by both dual culture technique and fungal inhibition by production of volatile organic compounds, enhancement of the bacterium's antifungal activity using organic compounds, characterization of the antifungal compounds produced by the bacterium and greenhouse studies to establish efficacy of bacterial metabolites in controlling barley net-blotch. The fungal colonies were isolated from the symptomatic leaves and identified based on their cultural and morphological characteristics. There was marked variation in the morphology of *D. teres*. Some appeared grey on Potato Dextrose Agar while the majority were black. Conidia were cylindrical in shape having 3 to 6 septa. Some had a conspicuous scar. *R. commune* colonies were initially whitish pink and gradually turned white then grey. It is a slow grower with short and bulbous conidia. *P. graminis* f. sp *hordei* was identified from the brown pustules on the plant stems. Both the urediniospores and the teliospores were examined and identified under the microscope. The growth of mycelia in *D. teres* was inhibited by 47.3% in dual culture with *P. polymyxa* KaI245 while there was no significant inhibition by the bacterium on *R. commune* colonies. *Paenibacillus polymyxa* KaI245 cell-free supernatant obtained from Potato Dextrose Broth inhibited the mycelial growth of *D. teres* by 24.1%, but didn't significantly affect the germination of teliospores, ($P = 0.16$). *P. polymyxa* KaI245 volatile organic compounds inhibited the growth of *R. commune* by 52.9%. These volatiles also had negative effects on the germination of *P. graminis* f. sp *hordei* teliospores. Germination dynamics of volatile exposed teliospores verses non-exposed differed significantly at $P = 0.05$ (chi sq = 27.53, df = 3, $P = 1.4E-11$). Efficacy of *P. polymyxa* KaI245 cell-free culture filtrate was performed in the greenhouse on barley plants infected with *D. teres*. Both the normal and double concentration of the crude bacterial suspension had a mean disease score of 1.8 which translated to 50% disease suppression compared to the positive control. Orius® 430 SC, a commercial synthetic fungicide recorded a mean disease score of 1.0 translating to 72.2% reduction in disease occurrence. Acetone and methanol were used in attempts to enhance the production of antifungal metabolites by the bacterium. Addition of 1% Acetone to the growth medium showed enhancement of the bacterium's antifungal activity, with the inhibition of the fungal mycelium increasing to 27.9% down from 19.7%. Methanol stifled the bacterium's ability to produce antifungal metabolites as was exhibited by reduced inhibition of mycelial growth of *D. teres* (10.3%). Characterization of antifungal metabolites produced by *P. polymyxa* KaI245 using GC-MS revealed 50 different metabolites. Addition of 1% acetone to *P. polymyxa* KaI245 growth medium led to the reduction of antifungal metabolites to 22 although 9 new metabolites were produced. Addition of 1% methanol on the other hand, also resulted in reduced number of antifungal metabolites (35) with 11 new metabolites produced. The bacterium metabolites have the potential to control the selected pathogens of barley as demonstrated in the case of *D. teres*.

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LIST OF ABBREVIATIONS AND ACRONYMS

a.m.u	Atomic mass unit
ANOVA	Analysis Of Variance
DCM	Dichloromethane
E.C	Emulsifiable Concentrate
EABL	East African Breweries Limited
ET	Ethylene
F.A.O	Food And Agriculture Organization of the United Nations
GC/MS	Gas Chromatography Mass Spectrometry
HCN	Hydrogen Cyanide
HPLC	High Performance Liquid Chromatography
ICIPE	International Centre of Insect Physiology & Ecology
I.D	Identity
ISR	Induced Systemic Resistance
JA	Jasmonic Acid (jasmonate)
LB	Luria Bertani
LMWCs	Low Molecular Weight Compounds
Mm	millimeters
Mt	metric tonnes
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
NPK	Nitrogen, Phosphorus Potassium
NPR1	Non-expresser of PR1
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGPR	Plant Growth Promoting Rhizobacteria
PR1	Pathogenesis Related proteins
psi	pounds per square inch
r.p.m	revolutions per minute
RT	Retention Time
SA	Salicylic Acid

SAR	Systemic Acquired Resistance
SAS	Statistical Analysis System
SDW	Sterile Distilled Water
TLC	Thin Layer Chromatography
USDA	United States Department of Agriculture
VAT	Value Added Tax
VOCs	Volatile Organic Compounds
µm	micrometer

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Maize, wheat, rice and sorghum are the main cereals consumed in Kenya in order of quantity (Nzuma and Sarker, 2010; Musyoka *et al*., 2014). Cereals have continued to play a critical role to Kenya's food security. The biggest contribution is particularly by maize (*Zea mays*) which accounts for 56% of land cultivated in Kenya (Kirimi *et al*., 2011). So important is maize that the whole country would whine upon the slightest deficit as was witnessed in most parts of the year 2017. It has been reported that maize contributes more than a third of Kenya's caloric intake (Mohajan, 2014).

Wheat (*Triticum aestivum*), accounts for 17% of Kenya's food consumption (Mohajan, 2014). While the rate of consumption of maize has reduced over the years, the rate of consumption of wheat has been increasing (Nzuma and Sarker, 2010). The production has however not been able to keep up with this consumption rate. Estimates of wheat consumption in Kenya is about 900,000 tonnes per year against 350,000 tonnes produced locally (Onguso & Njau, 2015). Kenya is therefore a net importer of wheat.

Rice (*Oryza sativa*) consumption has gained more prominence compared to wheat and maize (Omondi & Shikuku, 2013). Rice production in Kenya is majorly carried out in irrigation schemes established by the government, namely: Ahero, Mwea, West Kano and Bunyala (Acland, 1971). Kenya has not yet met her own demand and always complemented with imports (Omondi and Shikuku, 2013).

Sorghum (*Sorghum bicolor* L) is the only cereal that is indigenous to Kenya (Kilambya & Witwer, 2013). Its consumption is centered in the areas prone to drought; Kenya's former Eastern, Nyanza, Western and Rift-valley provinces (Nzuma & Sarker, 2010). Current production is expected to increase due to the demand for sorghum in the brewing industry. The East African Breweries Limited (EABL) targeted a 3 fold increase in sorghum industrial use in 2017 (Gwengi, 2016). The company therefore embarked on an aggressive campaign to recruit more farmers to increase sorghum production from 30,000 tons to more than 40,000 tons. The sorghum project came to fruition when the Kenyan president (H.E Uhuru Kenyatta) officiated the opening of senator keg (beer made from sorghum) production facility in Kisumu (western Kenya) in July 2017.

Another cereal crop grown in Kenya is barley. Barley resembles bearded wheat varieties. It is majorly used in malting and does not feature prominently in human nutrition (Viergerer & Tipper, 2014).

1.2 The Origin and Distribution of Barley

Barley (*Hordieum vulgare. L*) is one of the crops that shaped early agriculture (Badr *et al*., 2000). There are suggestions that barley was domesticated more than once (Morrell and Clegg, 2007). Earliest domestication evidence is in the fertile crescent area of Israel and Jordan (Badr *et al*., 2000; Morrell and Clegg, 2007). Other regions thought as barley domestication centers are Ethiopia, Morocco and Egypt (Badr *et al*., 2000). Evidence provided by Blattner *et al.* (2001) however, does not support Morocco as an early region of barley domestication. Currently, it takes the fifth position in terms of production in Kenya and the fourth world-wide (Ullrich, 2010). Barley is grown mainly in the temperate zones with exceptions being East Africa and the Andean zone in South America (Jenkins,

1985). These two regions (East Africa and Andean zone) are found within the tropics. Barley was first used as human food taken raw or roasted, in making breads, porridges and soups (Ullrich, 2010). Overtime however, barley became important in the production of malt, alcohol, animal feeds (Newman and Newman, 2006; Ullrich, 2010) and as food in many developing countries notably Ethiopia, China, India, and Morocco (Newman and Newman, 2006). The decrease of barley's prominence as food can be attributed to the dominance of wheat and rice (Ullrich, 2010). In Kenya, barley is masked by other food grains.

1.3 Barley production and its Importance in Kenya

Barley, a widely adopted cereal crop (Verstegen *et al.*, 2014), was first introduced to Kenya as fodder and first commercialized for malt production in 1929 (Everlyn and Waithaka, 2005). Since then it is mainly grown for malting in beer industries. Currently, the major barley growing counties of Kenya are Narok, Nakuru, Uasin Gishu, Meru, Samburu and Nyandarua in the order of quantity (Ministry of Agriculture, 2015). Some farmers in other regions of Kenya like Trans Nzoia county are now considering a shift from maize to barley production (Dennis, 2015). Reports on cereal production in Kenya, ranks barley a distant fifth despite being the fourth most produced commodity worldwide (Ullrich, 2010). Consequently, Kenya plays an insignificant role on barley production on the global scale. According to the 2015 economic survey, barley production was 77,000 mt, a distant fifth behind maize, wheat, sorghum, and rice (Everlyn, N & Waithaka, M, 2005).

The situation may however change as more farmers are now finding the cultivation of barley more profitable (Lubanga, 2015). In the region (East Africa), barley farmers are

contracted by specific malting industries, where the prizes per bag is set before planting (Pauline, 2016). Further, barley takes four months to mature while wheat takes five months with yields from barley being 1.5 times higher. This is a great advantage that barley cultivation offers since farmers are shielded from any possible losses. The prizes of other cereals are market driven. But even with these advantages, some farmers may opt to switch to wheat farming especially if the wheat prizes are high the previous year (Pauline, 2016). When this happens, the barley shortage may force the local brewing companies to import this raw material (Kavoi *et al*., 2014). As happened in 2008, EABL imported barley so as to meet their then requirement of 100 million kg of barley annually. Currently EABL contracts about 900 farmers to grow barley and about 40,000 farmers to grow sorghum (EABL, 2015). Barley is used to produce lager products while sorghum produces more affordable beer, senator keg. Collectively the contracted farmers pocketed just over 1.5 billion Kenyan shillings in the year 2014 (EABL, 2015). That is a substantive injection into the Economy. Further, the beer industry has been ranked as one of the highest corporate taxpayers (Everlyn and Waithaka, 2005; Mwando *et al.*, 2012). From this perspective therefore, barley and the beer industry should be viewed as very important. Its role in shaping our envisioned future state cannot be over-emphasized.

The occasional shifting of some farmers to wheat production could be a pointer of some underlying challenges in the industry. To initiate and maintain an upward trajectory in barley yield efforts should coalesce towards addressing some of these challenges.

1.4 Constrains of Barley production in Kenya

Barley production in Kenya has been fluctuating (appendix I) since 1960 (USDA, 2016). The lowest yield was recorded in 1961 at 10 Metric Tonnes (MT) while the highest was recorded in 2001 at 113 MT as depicted in Appendix I. These amounts have always not been enough for Kenyan malt industries. The EABL has occasionally imported barley (Kavoi *et al*., 2014) while Keroche (Another significant player in the region) has sometimes sourced its barley from Belgium (Mungai, 2015). Kenya therefore is yet to satisfy her local barley demand. EABL currently contracts about 900 farmers for barley production across the barley growing regions of Kenya (EABL, 2015). To meet the region's (East Africa) demand, the number of farmers should increase. Such prospects however appear bleak as the sector is weighed down with many challenges (Bosibori, 2015). First, attitude change is needed from the Kenyan government. The country, being one that always experiences perennial famine would reasonably resort to direct its efforts to cereals that are mainly used as food. Available literature on grains in Kenya, seem to suggest that barley ranks among the least important crops with more attention focused on corn, wheat, rice, sorghum, and millet. Further, barley is associated with beer which is again saddled with a lot of negativity. While this could partly be driven by ignorance (as in fact more sorghum is used in Kenya's malt industries (EABL, 2015)), it nevertheless underpins the need to diversify the uses of barley in Kenya. As has been mentioned (sec 1.2), barley can be used as fodder as well as human food and it is also found as an ingredient in some non-alcoholic beverages (Appendix II). By embracing these other potential uses, Kenya would reap the full economic and nutritional benefits of barley.

Key to the success of brewing industries is adequate amounts of good quality barley. Consequently, although payments may be agreed upon before plating the grains, profits are always dictated by grain quality (Bosibori, 2015). Grain quality is affected by erratic rain as well as barley diseases. Erratic rains have been blamed on climate change. Due to high production costs, farmers in such cases incur huge loses (Bosibori, 2015; Lusenaka, 2017). These factors would always impede Kenya's prospects of satisfying her barley demand. Finding better ways of managing barley diseases would be the right step in trying to reduce some of these challenges. Another challenge is lodging, where barley slouches and falls due to excess use of fertilizer or heavy rains or winds (Pauline, 2016). This can be particularly concerning in most parts of Kenya since rainfall is projected to increase owing to climate change (Viergerer and Tipper, 2014.). Due to this a lot of grains remain uncollected during harvests by the combined-harvester since they cannot be picked. This translates to losses by the farmers. Although barley is generally expected to be ready for harvest after four months, it may take longer in the cooler growing areas (Kavoi *et al*., 2014). Biomass accumulation is lower in cooler regions. In such cases there is no advantage in growing barley. A practical remedy to such climate induced challenges is to develop more adaptive varieties.

The single most important threat to barley cultivation is diseases caused by the various fungal pathogens. In the USA, fungicide application on barley is estimated to cost 34 US Dollars (about 3400 Ksh) ha^{-1}(Agostinetto *et al*., 2015). Taking into account that disease pressure is higher in the East African region and the fact that the fungicides are not 100 % locally manufactured, the cost here is reasonably higher. Although they affect the folliage,

these pathogens are majorly seed borne (Arabi *et al.*, 2003). Fungal pathogens reduce yield and the quality of barley used to make malt (Arabi *et al*., 2003; McLean *et al.*, 2009). Reduction in quality is due to loss of the protein hordein in infected grains (Mwando *et al*., 2012). Reduction in protein quantity can be a concern in barley intended for human consumption or animal feeds. Poor quality of barley translates to poor quality malt and low-quality beer. This multifaceted effect of barley diseases makes them particularly catastrophic. In some varieties of barley, it has been found that the barley seeds infected especially with *Puccinia graminis* may fail to germinate as a result of the loss of protein in the grain (Mwando *et al*., 2012). This can be particularly concerning since it can be practically difficult for the farmers to discriminate infected seeds. Some of these fungal pathogens include *Puccinia graminis* implicated with stem rust, *Pyrenophora teres* fsp *maculata* causing spot blotch and *P teres* fsp *teres* causal agent for net blotch. *Cochliobolus sativus* also causes spot blotch and can be easily confused with *P. teres* fsp. *maculata* (Arabi *et al*., 2003). The list of fungal pathogens is endless. Currently, these pathogens are managed by fungicides which are always very expensive (Pauline, 2016). This drives up the cost of cultivation and hence low profit margins for the farmers. The main cause of the high prizes of the fungicides is the Kenyan government policy which promotes double taxation (Lusenaka, 2017). For the locally manufactured fungicides, the main ingredients are imported. Tax duties are levied on the individual ingredients as well as the finished product. The result is an exorbitant cost of these farm chemicals.

Alternatively, resistant barley cultivars have been developed to combat some of the pathogens. However, the pathogens can be highly variable with several virulent types,

hence lasting resistance can be difficult to achieve (Arabi *et al*., 2003) . Jalli, (2011) showed that the sexual stage of *Drechslera teres* can create more variants of the pathogen.

1.5 Statement of the Problem

Production of barley in Kenya has had a fluctuating trend since its production was documented as shown in (USDA, 2016). The fluctuation in the production may be attributed to several production constraints, key among them being diseases and insect pests. Some of the serious pathogens of barley include *Blumeria graminis* f.sp *hordei* implicated with powdery mildew, *Rhynchosporium commune* responsible for leaf scald, *Drechslera teres* f. sp. *teres* (Sacc.) Shoemaker causing net-form-net-blotch, and *Cochliobolus sativus* causing spot blotch (Turkington *et al*., 2011).

In the past few years, farmers have been practicing agricultural production intensification for greater yields and profit. Consequently, this has resulted in overdependence on agrochemicals as a relatively reliable method of disease management in order to stabilize economic operations. However, the increasing costs of chemical inputs has several undesirable effects such as development of fungicide resistant strains of the pathogens and their non-target environmental impacts. Most of these pathogens are usually composed of several strains or pathotypes that are capable of evolving rapidly in response environmental changes. It has been shown that crop varieties that were previously known to be resistant may become vulnerable with time (Turkington *et al*., 2011; Mwando *et al*., 2012). Further, the costs of synthetic chemicals have been spiraling up.

In response to these concerns, focus should be directed to the development of alternative methods which are environmentally safe. The current research aims at addressing the above-mentioned concerns.

1.6 Justification of the Study

Our ecosystem is fragile, a fact that we are slowly but surely learning. This recognition has come at a time when feeding humanity has become a real challenge. Our efforts therefore to mitigate this should be coupled with prudent use of essential farm inputs that ensures minimal impact on the environment. Chemical application on crops has been associated with undesirable effects including toxicity to humans and untargeted organisms. With this realization, demand for food that do not contain any toxic substances has been on the rise (Cieślik *et al*., 2014). Further, governments have put forth legislations discouraging the use of some agrochemicals. Consequently, fewer pesticide products are now available to the farmer.

Despite the negative effects associated with chemical control, agrochemicals are still used. At the moment, alternatives are very few. Besides, the levels of control obtained with fungicides are sometimes inadequate (Melo *et al*., 2011). Biological control provides a positive alternative to synthetic chemicals, however it contributes a paltry 1 % of agricultural pesticide sales (O Brien, 2005). A plant growth promoting rhizobacterium, which is a candidate bio-control agent for this study has shown antimicrobial activity against sorghum anthracnose (Makumba, 2016). Its effect on net blotch, rust and scald pathogens of barley is unknown. This study therefore seeks to establish the potential of the bio-control agent against selected barley pathogens and thereby the much-needed alternative.

1.7 Objectives

1.7.1 Broad objective

To establish the effectiveness of *Paenibacillus polymyxa* KaI245 isolate as a bio-control agent of selected barley pathogens.

1.7.2 Specific objectives

1. To establish antagonistic activity of *Paenibacillus polymyxa* KaI245 isolate against important barley fungal pathogens under *in vitro* conditions.
2. To test the efficacy of *Paenibacillus polymyxa* KaI245 culture filtrate against net blotch disease on potted plants in the green house.
3. To conduct chemical studies that will enhance antifungal agents' production from *Paenibacillus polymyxa* KaI245 isolate.
4. To characterize the antifungal compounds produced by *Paenibacillus polymyxa* KaI245 isolate using GC-MS.

1.8 Research Questions

1. Does *Paenibacillus polymyxa* KaI245 isolate exhibit any antagonism towards other cereals' foliar fungal pathogens *in vitro* apart from those of sorghum which it was subjected to?
2. How effective is the culture filtrate of *Paenibacillus polymyxa* KaI245 isolate under greenhouse conditions against barley net blotch?
3. Do varying chemical conditions geared towards enhancement alter the quality and quantity of antifungal agents produced by *Paenibacillus polymyxa* KaI245 isolate?

4. What is the chemical composition of the antifungal agent(s) produced by *Paenibacillus polymyxa* KaI245 isolate?

CHAPTER TWO

LITERATURE REVIEW

2.1 Fungi as plant parasites

Virtually all plant species are susceptible to parasitism by fungi (Sumbali, 2005). A fungal parasite may gain entry to a potential host plant in the following ways: 1) through injuries, 2) direct penetration and 3) through natural openings like stomata. Direct penetration through intact plant surfaces appears to be the most common route of entry (Leonard & Szabo, 2005; Sumbali, 2005).

Based on the modes of nutrition, fungal pathogens can further be grouped to biotrophs, hemibiotrophs, and necrotrophs (Liu *et al*., 2014). Necrotrophs obtain their nutrition from killed cells; during or prior to colonization by deploying cell wall degrading enzymes and phytotoxic compounds (Sumbali, 2005). Biotrophs derive their nutrition from a live host and therefore restricts the amounts of cell wall degrading enzymes released and generally lack toxin production (Mendgen & Hahn, 2002). Further, biotrophs are characterized by the development haustoria. Hemibiotrophs display a biotrophic early phase followed by a late necrotrophic phase (Liu *et al*., 2014). The period of the biotrophic phase varies from pathogen to pathogen.

2.2 Plant-Pathogen interactions

The outcome of infection largely depends on the interaction between the plant and the pathogen. This is a fully known mechanism that involves signal activation, occasionally followed by a quick defense response against a number of plant pathogens (Gururani *et al*., 2012). Because of the discriminate disease pressure exerted by the pathogens, many plants

have developed post invasion resistance under the influence of dominant genes (Chisholm *et al.*, 2006). Pathogens on the other hand, produce an array of effector proteins to counter plants' defenses (Bent & Mackey, 2007). If a pathogen successfully breaches a plant's defense system, a disease would inevitably ensue. As a result, some symptoms become evident on the diseased host which are collectively termed disease syndrome (Sumbali, 2005). These symptoms present an avenue by which a disease is recognized. These symptoms include, damping off, spots, blotch, blight, rust and so on and so forth.

2.3 Diseases of Barley

Numerous diseases of barley have continued to pose great challenge to barley production. Barley is susceptible to all diseases that affect wheat (Acland, 1971). Unlike those of wheat, barley diseases tend to be much more geographically isolated with very few exceptions (Jenkins, 1985). They are most severe when highly susceptible cultivars are planted (Cunfer, 2000). There are however cases whereby some pathogens overcome host resistance in resistant cultivars (Dean *et al*., 2012). Examples that are of worldwide importance are powdery mildew implicated on *Blumeria graminis* (Takamatsu, 2004) various forms of rust diseases caused by species belonging to the genus *Puccinia*, scald caused by *Rhynchosporium commune* (formerly *R. secalis*) (Tekauz, 1991) and net blotch caused by *Drechslera teres* (Jenkins, 1985). This study prioritizes net blotch, scald and stem rust due to their prevalence in the East African region.

2.3.1 Net blotch

Net blotch is widespread in temperate high rainfall regions (Jenkins, 1985). The fungi *Drechslera teres* is the causal agent and has two forms; *Drechslera teres* f. sp *maculata*

and *Drechslera teres* f.sp *teres* (Liu *et al*., 2011). *D. teres* f.sp *teres* inflicts upon the barley leaves symptoms identified by vertical and horizontal crisscrossed brown venation known as net type net blotch (NTNB)(Smedegård-Petersen, 1971). *D. teres* f.sp *maculata*'s symptoms are chlorotic elliptical spots dubbed spot form net blotch (SFNB)(Smedegård-Petersen, 1971). Prevalence of SFNB is as a result of cultivation of vulnerable varieties of barley and retention of stubble (McLean *et al*., 2009).

The symptoms are caused by low molecular weight compounds (LMWCs) and proteinaceous metabolites expressed by *Drechslera teres* (Sarpeleh *et al*., 2007). The proteinaceous metabolites have been implicated with the necrotic spots while the LMWCs induce general chlorosis. Both types may lead to low agricultural output owing to their undesirable impacts on the grain quality (Steffenson *et al*., 1996). *D. teres* also shows great morphological diversity in all the regions that they are present (Steffenson and Webster, 1992; Owino *et al*., 2013). In most developing countries the effects of *D. teres* can be severe.

2.3.1.1 Taxonomy of *Drechslera teres*

D. teres belongs to division Ascomycota; class Dothideomycete; order Pleosporales; family Pleosporaceae; genus *Drechslera* whose formae specialis are *maculata* and *teres* (Liu *et al*., 2011).

2.3.1.2 Morphology of *D. teres*

Between the two forms of the fungus, there exists no clear cut morphological distinction (Campbell *et al*., 1999). As the barley plant senesces, dark, globosely shaped pseudothecia

are brought fourth by the fungus (Liu *et al*., 2011). Ascospores produced are brown and ellipsoidal with transverse septations ranging from three to four and. Conidiophores are lightly swollen at the base and solitary or arise in sets of two or three (Campbell *et al*., 1999). Conidia are cylindrically straight and rounded at the ends, yellowish brown with three to six pseudosepta (Liu *et al*., 2011). In some an inconspicuous scar can be present at one tip (Owino *et al*., 2013).

Other *Drechslera* spp specifically *D. graminea*, has a similar morphology to *D. teres* and also causes some barley foliar diseases (Sivanesan, 1987). Typical symptoms on the barley leaves are however different with *D. graminea* inducing long and extended necrotic stripes (Mathre, 1997). Their differentiation can hence be achieved by disease phenotyping.

2.3.1.3 Life cycle of *D. teres*

Net blotch of barley is stubble-borne disease as the fungus over-seasons by producing pseudothecia (ascocarps) on infected after harvest leftovers (Liu *et al*., 2011). Club shaped asci develops within the mature and fertile Pseudothecia (Mathre, 1997). Each ascus contains eight ascospores. When ascospores mature, they are discharged and spread by wind and goes on to be the primary inoculum (Jordan, 1981). Occasionally, fungal mycelia and conidia carried by seeds released from stubble or an alternate host may serve as a primary inoculum (Mclean *et al*., 2009). Examples of alternative hosts include some wild *Hordeum* species and related species of genera *Avena*, *Bromus* and *Triticum* (Shipton *et al.*, 1973).

Germination of conidia and ascospores on moist leaf surfaces initiates the infection. Germ tubes may sprout from a terminal conidial cell but occasionally from up to four cells at a

time (Caeseele & Grumbles, 1979). Hyphae grow from the germ tubes to different lengths before forming an appresorium for penetration. *D. teres* f. sp. *maculata* has been shown to germinate and grow at a lower rate compared to *D. teres* f. sp. *teres* (Lightfoot & Able, 2010). The fungus infects and feeds necrotrophically by spreading its mycelium intercellularly thereby affecting host cells further from the point of entry. On the other hand, *D. teres* f. sp. *maculata* initially makes a haustorial-like intracellular structures and feeds biotrophically but necrotrophy begins almost immediately. In the entire lifecycle, this is perhaps the most distinguishing step of the *D. teres* forms. *D. teres* f. sp. *teres* can therefore be described as a necrotroph, while *D. teres* f. sp. *maculata* as a hemibiotroph (Liu *et al*., 2011). In successful infection host cells die within 48 hours (Caeseele and Grumbles, 1979).

After the primary colonization, large quantities of conidia are produced to serves as secondary inocula (Liu *et al.,* 2011). This happens throughout a growing season and conidia are scattered by rain and wind to initiate new infections at near or distant barley plantations (Mathre, 1997). As the growing season comes to an end, the fungus further colonizes the dying host, eventually producing pseudothecia which is a protective over-seasoning structure.

2.3.2 Scald

Barley scald, caused by *Rhynchosporium commune* (previously known as *Rhynchosporium secalis* (Arzanlou *et al*., 2015)), is restricted to some environments (Jenkins, 1985). The symptoms can be identified as lesions with dark brown borders. It is frequent in cool and temperate climates having semi humid conditions (Zhan *et al*., 2008). Scald is occasionally severe in Kenya and Turkey (Jenkins, 1985). The disease is economically important due to

reduced yield as well as reduction in quality of barley grains (Arzanlou *et al*., 2015). Its lifecycle is characterized with sporulation and a lengthy symptomless phase, hence classified as a hemi-biotroph (Oliver & Ipicho, 2004). It has been shown however that the fungus secrets some necrosis inducing effectors namely, NIP1, NIP2 and NIP3 (Stefansson *et al.*, 2014). NIP1 has both effector (facilitates infections) and elicitor (trigger resistance by the host) activities (Kirsten *et al*., 2012). This could perhaps be the reason why some scientists would be hesitant to refer *R. commune* as a hemibiotroph and still hold the view that it is a necrotroph. A true necrotroph however has a wide host range but *R. commune* is host-specific on barley. On the other hand *R. commune* has not been shown to form haustoria like other biotrophs (Avrova & Knogge, 2012). A consensus is yet to be reached in this regard.

Although generally considered an imperfect fungi there have been suggestions for the presence of teliomorphic stages by (Linde *et al*., 2003). Stefansson *et al*. (2014) describes the fungus as a haploid ascomycete that causes scald in barley but did not report production of ascospores by the fungus.

2.3.2.1 Life cycle of *R. commune*

Scald fungus has separate generations in a barley growing season *(*Zhan *et al*., 2008). Initial inoculum ranges from conidia on previous crop debris, infected seeds and potentially ascospores (Linde *et al.*, 2003). Secondary dispersal is by splash dispersed conidia, finally overwintering on crop debris, seed and soil (Arzanlou *et al*., 2015). In spite of the fact that existence of a sexual stage has not been proven, a considerably high pathogenic variation exists (Abang *et al*., 2006).

2.3.2.2 Management of Barley Scald

Apart from fungicides, resistant barley cultivars are used to counter the pathogen. Concerns have however been raised due to the rapid evolution of the pathogen in response to host resistance genes (Abang *et al*., 2006). The virulence levels of *R. commune* populations have been shown to change in a short time while the major resistance genes used to control it have proven brief in life span (Barr *et al*., 2004; Stefansson *et al*., 2014). Further, the barley/*R. commune* association resulted from host jump (rather than host tracking) from a different ancestor (Stukenbrock & McDonald, 2008). If this argument holds, then barley may not be a reliable source to look out for resistance genes for lack of extensive co-evolution with the parasite. This could hence further explain why resistance to *R. commune* by resistant barley cultivars is not durable. It also raises questions as to whether it is the inherent fitness of the pathogen or just a very weak host that explains this rapid susceptibility of a previously resistant barley cultivar. Rapid evolution by the pathogen is however difficult to refute since these robust evolution tendencies of *R. commune* has also been witnessed in response to chemical fungicides and climate change (McDonald, 2015). These two (chemical fungicides and climate change) can be described as very recent events hence strengthening the argument that the pathogen has high evolutionary tendencies.

Evolutionary potential of the pathogen reduces when barley stubble is managed (McDonald, 2015) hence suggesting the involvement of sexual cycle.

Due to the complexities presented by *R. commune*, more management measures ought to be put forth. Improvement of seed treatment is such a measure (McDonald, 2015) and is

expected to reduce introduction of *R. commune* to new geographic locations (Arzanlou *et al*., 2015). The bio-product, trichonitrin® from *Trichoderma harzium* strain B1 was shown to be effective in controlling *R. commune* primary infection in seed treatments (Kulichova, 1997). Fröhlich† *et al.* (2012) also showed that commercially available biological agent Proradix® which contains *Pseudomonas* sp. DSMZ 13134 induces resistance of barley towards *R. commune* with enhanced growth yield even under nutrient deprivation. These developments suggest that bio-control of *R. commune* is a promising approach in a bid to control the disease. This study hopes to contribute to this trajectory especially so because of the pathogen's rapid evolution.

2.3.2.3 *R. commune* morphology

Cultures may appear as irregular heaped clumps of mycelium having conidia which may appear beak shaped or short and bulbous (Cromey & Mulholland, 1987).

2.3.3 Rusts

Perhaps the genus that contributes the highest number of pathogens in plants is *Puccinia*. *Puccinia hordei* causes leaf rust in barley (Jenkins, 1985; Polley *et al*., 1993). It is of main concern in the Mediterranean basin but also occurs in Kenya (Jenkins, 1985). It is favoured by warm and moist climate (Woldeab, 2015). It is lethal and susceptible varieties are killed (Jenkins, 1985). Depending on the variety of barley, there can be fast rusting barley and slow rusting barley (Johnson and Wilcoxson, 1978). Fast rusting barley is characterized by short latent period of the rust fungi as well as more urediospores/uredospores production (Johnson and Wilcoxson, 1978).

Puccinia graminis is the causal agent of barley stem rust. *P. graminis* has further been grouped into subspecies, varieties and formae based on spore size and host range (Leonard and Szabo, 2005). It is not important globally but can be severe in the East African highlands (Jenkins, 1985). It is also a pathogen of wheat (*Triticum aestivum*) and oats (*Avena sativa*) and occurs in different races (Fetch Jr, 2009). *Puccinia graminis* is an obligate biotroph and cultured *in vitro* with difficulty (Leonard and Szabo, 2005). It is heteroecious with five distinct spore stages some of which infect an alternate host, the barberry plant. Eradicating the barberry plants has been considered as a possible way of controlling the disease (Dean *et al*., 2012). Sexual reproduction also occurs in the barberry plant and its removal reduces chances of genetic recombination and hence emergence of virulent varieties.

Puccinia striiformis f. sp. *hordei* causes yellow rust or stripe and is one of the most dreaded disease of barley (Jenkins, 1985). There is no known sexual stage for this fungus (Manners, 2016). It has the potential of destroying a barley crop completely (Jenkins, 1985). It completes its lifecycle on alternate host called *Orinthgalem umbellatum* (the garden star-of-Bethlehem) where genetic recombination is thought to occur (Woldeab, 2015).

Because of its severity in East Africa, this study focuses on *P. graminis* the causal agent of barley stem rust.

2.3.3.1 Stem rust

Of all the barley and wheat growing areas in the world, this disease is most severe in East Africa (Acland, 1971). This is so for the following reasons:

- In the temperate wheat growing areas the fungus is checked during winter. There is no winter to check the disease in East Africa.
- The entire East African region experiences different rainfall regimes, and for that reason there must always be a susceptible plantation at some part of the region all year round.
- The conditions mentioned above propagates the emergence of new physiological races and sub-races of the disease. These may gain abilities to attack varieties which had previously proved to be rust resistant. This particular statement, is qualified by the emergence of the more virulent strain *ug*99 in Uganda (Mwando *et al*., 2012).

  Barley and wheat become susceptible to stem rust at the 'boot' stage, that is, before the ear emerges but appears as a bulge inside the uppermost leaf sheath (Acland, 1971). The disease appears as brown pustules on the barley stem. The brown pustules are urediniospores that are so intense that they break the plant cuticle. When the barley plant reaches the end of its lifecycle and begins to senesce the pathogen begins to produce black teliospores.

2.3.3.2 Taxonomy and life cycle of *Puccinia graminis*

Puccinia graminis belongs to kingdom Mycetae; division Amastigomycota; class Basidiomycetes; order Uredinales; family Pucciniaceae and genus *Puccinia.* In the order uredinales, it is the largest genus (Sumbali, 2005).

P. graminis is heteroecious; requiring multiple hosts for a complete life cycle (Leonard & Szabo, 2005). It produces five different spores during the entire life cycle; in the primary host, urediniospores and teliospores, teliospores germinate in the ground to produce basidia and basidiospores, basidiospores infects an alternate plant called the berberry plant

resulting pycnia; flask-shaped structures on the upper leaf surface. Pycniospores form in the pycnium and discharge nectar that attracts insects. Pycniospores, which are male gametes are disseminated among pycnia by insects and rain splash. New growth commences below the berbery leaves where aecia containing aeciospores are produced. It is the aeciospores that eventually serve as primary inoculum to the barley plantation. Upon successful infection, the fungus produces a thick hyphal mass underneath host's epidermis. From the hyphal mat, sporophores containing reddish brown masses of urediniospores that rupture the epidermis are produced. This is the typical disease symptom. The uredinial stage can also survive on barley and wheat debri that may function as a source of primary inoculum the next season (Leonard and Szabo, 2005).

2.4. Plant growth promoting rhizobacteria (PGPR)

The rhizosphere is that region of the soil where the plant roots' influence is exerted. This region has been found to be richer in bacteria than the surrounding soil; a multiple of 10 to 1000 times has been suggested (Lugtenberg and Kamilova, 2009). The bacteria dwelling in this region are the rhizobacteria (Podile and Kishore, 2006). Although focus has always been directed to the beneficial effects of these bacteria it should be emphasized that some may be harmful while others have no effect (Ranjitha, 2015). On the beneficial aspects, these microorganisms promote plant growth by hormonal stimulation, improved nutrient acquisition, and suppression of phytopathogens (Berg, 2009). Members of genera *Azospirillum* and *Rhizobium* are involved in growth enhancement while *Pseudomonas, Bacillus, Stenotrophomonas, Serratia,* and *Streptomyces* influences plant health (Berg, 2009). Plant growth promotion is a direct effect of increased iron uptake by chelating

siderophores, solubilization of inorganic phosphates, production of phytohormones, and volatile compounds affecting the plant's signaling pathways (Podile and Kishore, 2006). Beneficial impact on plant health is due to competition of nutrients and space, antibiosis and promotion of plants' systemic resistance towards a wide spectrum of root and foliar diseases (Podile and Kishore, 2006). Plant Growth Promoting Rhizobacteria can hence be described as a collection of free living bacteria that colonize the rhizosphere with extended benefits to the roots' and the host plant's growth (Lugtenberg and Kamilova, 2009). Microbial inoculants can be developed from the favourable plant-microbe interactions for use in agriculture (Berg, 2009). Plant Growth Promoting Rhizobacteria is therefore set to substitute agricultural synthetic chemicals (Ranjitha, 2015). There market of microbial inoculants is growing worldwide with an estimated yearly growth rate of 10 % (Berg, 2009). It is hoped that the current project is going to make a significant contribution to this growing trend.

This plant-microbe interaction can however be unstable (Ranjitha, 2015). Promising *in vitro* assays cannot guarantee success in field (Zehnder *et al.*, 1997). A further insight to the use of plant growth promoting rhizobacteria is by the exploitation of Type 3 Secretion Systems (T3SSs) (Ranjitha, 2015). T3SSs are conserved membrane-embedded nano-machines utilized by bacterial pathogens to inject virulence effector proteins straight from bacterial cytosol a host cell (Nans *et al.*, 2015). It was previously thought to be present only in pathogenic plant and animal bacteria. The discovery of these systems in the rhizobacteria changed the previous attitude towards it. It is now regarded as a multi-protein nano-machine dedicated in trans-kingdom movement of effector proteins with some influence to

the phenotype of the host plant (Tampakaki, 2014). This realization, coupled with the knowledge of beneficial aspects of rhizobacteria, would contribute to even more efficient strategies to control plant diseases (Ranjitha, 2015). This means that the use of PGPR to combat diseases could be broadened beyond formulations of bio-inoculants in the near future. Further, the knowledge of T3SSs would expand the symbiotic potentials of non-leguminous agricultural plants (Tampakaki, 2014).

2.5 Control of barley diseases

2.5.1 Resistant cultivars

For the control of most fungal barley pathogens, cultivation of resistant cultivars is the most sustainable and environmentally sound approach (Brian and Webster, 1992; McLean *et al*., 2009). Resistance have been found to be controlled by genes that can be inherited (Brian *et al*., 1992). Some type of resistance confers low infection response and is assessed qualitatively while in other types the percentage of leaf tissue affected is reduced and as such assessed quantitatively (Brian *et al.*, 1992). The dynamics of resistance changes with the type of pathogen. Those cultivars, for example, that are resistant to the spot form of *Drechsclera teres* are susceptible to the net form of the disease (Serenius *et al*., 2005; Akhavan *et al*., 2017). Studies of resistant cultivars involves experiments to determine virulence spectrum of the pathogen, with a view to finding resistant cultivars and locating the resistant genes. The genes are then transferred to the susceptible barley cultivars. However, there is always a chance that the resistance can be overcome especially if grown over a wide geographical location (Owino *et al.*, 2014). In sorghum, which is majorly used for food, cultivars with resistance to biotic stresses may compromise the desirable food

qualities. Timu, *et al.*, (2014) showed that sorghum farmers were more likely to cultivate sorghum varieties with desirable marketing attributes. More environmentally friendly ways hence ought to be consistently developed

Induced resistance where plant's physiology is targeted has also been shown as a way to control disease in plants. This type of resistance is inducable in plants through application of a variety of biotic (avirulent or necrotizing pathogens) and abiotic (chemicals such as acibenzolar-s-methyl, ASM) agents with resulting resistance being both long lasting and broad in spectrum (Walters *et al.*, 2010). The inoculation of barley using *Bipolaris maydis* and *Septoria nodorum* both of which are maize (*Zea mays*) pathogens (as mentioned in sec 2.5.3) is a good example to show the use of avirulent pathogens in induction of this type of resistance.

Induced resistance can be divided into systemic acquired resistance (SAR) mediated by salicylic acid (SA) dependent process and induced systemic resistance (ISR) which develops as a result of plant roots' colonization by PGPR and is mediated by ethylene (ET) and jasmonate (JA) sensitive pathway (Walters *et al*., 2010; Yan & Dong, 2014). It is believed that Salicylic Acid induces resistance against biotrophic pathogens (Spanu & Panstruga, 2017) while Jasmonic Acid induces resistance against necrotrophs (Thaler *et al*, 2012; Walters *et al.*, 2013).

Non-expressor of pathogenesis related gene 1 (NPR1) has been revealed as the master controller of SA-mediated responses (Yan & Dong, 2014; Kuai *et al.*, 2015). Salicylic Acid has also been shown to induce copper toxicity tolerance (Kuai *et al*., 2015) and hence strongly suggesting to increase resistance to abiotic stress in plants. However, it has earlier

been shown that induction of SA dependent resistance suppresses JA dependent resistance (Walters *et al*., 2013). This therefore means that resistance against a biotrophic pathogen would compromise resistance against a necrotrophic pathogen.

2.5.2 Use of chemical fungicides

Fungicides are used as crucial component in managing barley fungal pathogens. Fungicides can be used either as seed dressing or foliar application (O Brien, 2005). The fungicides can be classified based on the chemical structure or mode of action and further by means with which it achieves protection to the plant. By structure we may have the azole containing compounds as imidazole or triazole and all interfere with ergosterol biosynthesis (Bossche, 1985). Ergosterol is a primary sterol in yeasts and moulds and is one of the indispensable components of a cell.

Classification by mode of action leads to three groups of fungicides (1) those inhibiting energy production by inhibiting –SH groups, (2) those that inhibit synthesis of proteins, nucleic acids, cell wall and membrane lipids and (3) those which induce indirect effects which change host pathogen interaction.

The third way and perhaps more familiar, fungicides can be either systemic or protectant (Berg, 1986). Systemic fungicides are those that are absorbed by the plant and translocated to other parts where they are toxic to the target fungus (Erwin, 1973). Examples of systemic fungicides include the oxathiins, the pyrimidines and benzimidazoles (Erwin, 1973).

Protectant fungicides are effective against a wide range of pathogens and protect the plant surface where they are applied (Brent and Hollomon, 2007).

Reasonably, protectant fungicides would require multiple applications for the entire period that the plant is vulnerable to infection. Most fungicide formulations have both systemic and protectant activity. Otherwise farmers would most likely prefer to use the systemic fungicides to protectants. All carboxin-related fungicides, with the exception of 'Dexon' and those fungicides that affect cell's energy production are always only protectant while those fungicides that inhibit fungus' biosynthetic pathways are generally systemic (Erwin, 1973; Berg, 1986; Brent and Hollomon, 2007). Because of this quality, the concentration of the fungicide used is low that the slow growing plant is not affected while the vigorously growing fungus is inhibited (Erwin, 1973).

One of the disadvantages of chemical fungicides is the resistance developed by the target pathogens over time (Brent and Hollomon, 2007). Mechanisms of resistance by the fungal pathogens involve, use an alternative pathway, decreased uptake of the toxicant, detoxification or decreased conversions of a non-toxin compound into a toxic compound (Berg, 1986). Based on how the fungicides are resisted they can further be classified into three groups. Those which result from single gene mutations and hence occur more readily are classified as moderate to high resistance, those which result from multiple mutations as moderately resistant while some are low resistant because the ability of mutational modification by the fungus has not yet been demonstrated (Georgopoulos, 1985). Some fungicides have also been shown to have undesirable effects on crop growth (Fromme *et*

al., 2017). Chemical pollution by the chemical fungicides is perhaps what makes them most undesirable (Whipps and Lumsden, 2001). They are non-biodegradable hence accumulate in the ecosystem which may affect human health. They also target other microorganisms some of which might be beneficial to the plants. For these reasons, agrochemicals are continually discouraged (Brent and Hollomon, 2007) while environmentally friendly mechanisms are advocated for, hence the most practical approach is biological control.

The cost of the chemicals have continued to skyrocket globally (Cieślik *et al*., 2014) especially in countries where they are imported (Lusenaka, 2017). In the year 2012 for example, farmers in the Kenya's north rift blamed the high cost of fungicides (some of which cost as high as Ksh 20,000 per 5 litres) for low profit margins they realized (Wambua, 2012). Kenya's problem is particularly compounded by Value Added Tax (VAT) on the imported active ingredients even though the final product remains zero rated. It should be noted that local pesticide manufacturing relies on imported ingredients. The result is that in the recent years locally manufactured pesticide products are becoming more expensive than the imported ones (Lusenaka, 2017). It would be way cheaper if the pesticides were 100% locally manufactured. This project envisions such a possibility.

2.5.3 Biological control

Biological control or simply 'bio-control' can be described as a strategy for reducing the severity of a disease by direct or indirect influence of microorganisms (O Brien, 2005). There is a renewed urge to develop more bio-control agents due to the dangers of agrochemicals. In cases where antagonism is exploited, the microbe is a natural enemy (Lucas and Sarniguet, 1998) where the active secondary metabolites are produced near the

site of action. Reasonably therefore, the use of bio-control agents is currently considered an environmentally friendly option. Bio-control may entail induced resistance to the plant (Jorgensen *et al*., 1996), limit pathogen spread by introducing a competing organism (O Brien, 2005) or using antimicrobial compounds produced by another microorganism (Burkhead *et al*., 1995; O Brien, 2005; Makumba *et al*., 2016). Another mechanism is parasitism (Melo *et al.*, 2011) a phenomenon sometimes called hyper-parasitism.

It is desirable that a candidate bio-control agent should possess as many of these attributes as possible (O Brien, 2005). The exact mechanisms by which antibiotics and enzymes control phytopathogens in the natural environment is yet to be fully understood (Melo *et al*., 2011) but it is believed to initiate plasmolysis of the cells at the hyphal tips or inhibit hyphal growth in some pathogens. Bio-control agents are effective over multiple barley cultivars and pathogens (Jorgensen *et al*., 1996). This offers advantage over the use of resistant cultivars particularly because resistance is always towards one specific pathogen.

Pre-inoculation of barley with *Bipolaris maydis* and *Septoria nodorum* have resulted to increase in disease resistance by barley (Jorgensen *et al*., 1996). The two fungi are pathogens of the common wheat (*Triticum aestivum*) and maize (*Zea mays*). The two that are non-barley pathogens are capable of inducing resistance of barley to *Drechsclera teres* and other pathogens such as *Bipolaris sorokiniana* and *Erysiphe graiminis* f. sp. *hordei* (Jorgensen *et al*., 1996). This approach of biological control can be likened to a two edged sword. As biological organisms, *B. maydis* and *S. nodorum* are pathogens of maize and wheat respectively and their continued use would constitute a risk for the two crops

(Jorgensen *et al*., 1996). They can only be used if the two vulnerable crops do not grow within the vicinity. In Kenya however, maize, wheat and barley are normally found in the same localities, which makes it difficult to implement.

Antimicrobial agents produced by bacteria which directly kills pathogens are called antibiotics (Lugtenberg and Kamilova, 2009). The common source of the antibiotics is from the Plant Growth Promoting Rhizobacteria (PGPR). Examples of antibiotics with bio-control usefulness that have been characterized include, 2,4-diacetylphloroglucinol, phenazines, pyoluteorin, hydrogen cyanide, pyrrolnitrin, and lipopeptides (Haas and Keel, 2003). *Pseudomonas chlororaphis* MA 342; a proven bio-control agent of barley's net-blotch, produces the antifungal compound 2,3-deepoxy-2,3-didehydrorhizoxin (Hökeberg, 1998). Additional means by which *Pseudomonas chlororaphis* MA 342 achieves its suppressive capabilities is by promoting plant's defense system and competing for nutrients and space with the pathogen (Copping, 2004).

Most bio-control antibiotics used so far have broad toxicity and lack antifungal specificity (Haas and Keel, 2003). Future bio-control research should therefore focus on strategies to overcome these challenges.

2.5.3.1 The bio-control agent; *Paenibacillus polymyxa*

This is the candidate bio-control agent used in this study. It was formerly known as *Bacillus polymyxa* (Lal & Tabacchioni, 2009). It is a Gram positive, endospore forming, facultative anaerobic, non-pathogenic bacillus (Lal *et al*., 2012). The bacterium has a wide distribution

ranging from soil, rhizosphere and marine sediments (Lal & Tabacchioni, 2009). It has been shown to be abundant among hydrogen producing facultative anaerobic bacteria in lake Averno sediment in southern Italy (Lal *et al*., 2012). *Paenibacillus polymyxa* KaI245 used in this study was isolated from sorghum rhizosphere in western Kenya (Makumba, 2016).

Paenibacillus polymyxa has been shown to have great applications in agriculture, industry and medicine (Huang & Yousef, 2012). In agriculture, it has properties including nitrogen fixation, plant growth promotion, and soil phosphorous solubilisation (Lal & Tabacchioni, 2009). The bacterium is also a good bio-control agent for plant parasitic nematodes (Khan *et al*., 2008) and also antagonistic to various phytopathogenic fungi (Kim *et al.*, 2016). Despite these advantages, *P. polymyxa* is the causal agent for potato tuber rot and tomato seedling blight (Caruso *et al.*, 1984). In medical and industrial field, it is known to produce optically active 2,3-butanediol which provides chiral groups in drugs, high value pharmaceutical or for liquid crystals (Celińska & Grajek, 2009). In the current study the ability of the bacterium to control selected barley pathogens by diffusible metabolites and volatiles was assessed.

2.5.3.2 Volatile Organic Compounds (VOCs) as bio-control agents

Microorganisms are known to produce volatile organic compounds (VOCs) some of which can be exploited for biological control. Perhaps the most important property of VOCs is their ability to diffuse and hence inhibit a phytopathogen from a distance (Weisskopf, 2013). Production of the VOCs by microorganisms occur as part of normal metabolism

and mainly functions in communication (Amavizca *et al*., 2017). A classic example of volatile mediated inhibition is the production of a respiratory poison called hydrogen cyanide (HCN), a process called cyanogenesis (Weisskopf, 2013).

Fernando *et al*. (2005) showed that rhizobacteria isolated from canola and soybean plants produce VOCs that inhibit sclerotia and ascospore germination as well as mycelial growth of *Sclerotinia sclerotiorum*. Verspermann *et al*. (2007) also demonstrated the ability of VOCs produced by various bacteria in retarding mycelial growth of different fungal phytopathogens. Apart from the fungal phytopathogens, bacterial VOCs may also inhibit the growth of bacterial phytopathogens. Volatiles from *Bacillus* spp have been shown to have adverse effects on the physiology and ultra-structure of *Ralstonia solanacearum* (Tahir *et al*., 2017). Physiologically, the latter authors showed that VOCs altered the transcriptional expression level of PhcA (a global virulence regulator), type III & IV secretion systems (both mentioned in section 2.4), and extracelluar polysaccharides genes which all make critical contribution to pathogenicity. Further, over-expression of EDSI and NPR1 (both mentioned in sec 2.5.1) suggest the involvement of SA pathway in induction of systemic resistance (Tahir *et al*., 2017).

Generally the beneficial roles of VOCs can be broadly stated as, promoting plant growth, inhibiting the growth of plant pathogens and inducing systemic resistance (Tahir *et al*., 2017). The plethora of antimicrobial activities that may be possessed by a bio-control agent strongly emphasizes the benefits that may be accrued from biological control. On the downside some bacterial volatiles such as ammonia, ethylene and hydrogen cyanide have

been shown to harm plants (Amavizca *et al*., 2017). It is hence important to realize that production of volatiles by a bacterium may not automatically endow plants with health benefits. Further, identification of the active compounds in VOCs-mediated antifungal activity can be a challenging task (Bailly & Weisskopf, 2012). Other challenges as outlined by (Weisskopf, 2013) are; high proportions of unresolved peaks in the GC-MS profiles, test application done once by the researcher while in the field it is slow continuous release by the antagonist, and the knowledge that bio-activity is usually as a result of a mixture of different compounds.

2.6 Improving the antagonistic capabilities of producing bio-control agents

For effective pest control, it may be desirable that the bio-control agents are produced in effective quantities. To achieve this some experiments have been done by optimizing fermentation conditions (Wang *et al*., 2008). Factors altered include medium components, inoculation volume, medium capacity, and fermentation time. For most of these factors, even in optimal conditions, might be costly and high inoculation volumes may be ecologically undesirable (Szekeres *et al*., 2004). Perhaps a better approach is that the antagonists should be improved for increased effectiveness. One such approach is by genetic manipulation of the antagonist with the hope of generating mutants with higher antibiotic production. Most potentially useful bio-control agents are only known to produce asexually and hence limiting the exploitation of breeding to enhance wild-type activity (Melo *et al.,* 2011). Conventional mutagenic techniques therefore comes in handy. Graeme-Cook and Faull (1991) generated mutants of *Trichoderma harzianum* with altered antibiotic production by use of *UV* light mutagenesis. In the experiment the mutants not

only showed elevated levels of antibiotic produced by the wild type but produced two new antifungal compounds. Some of the mutants can however loose the antagonistic activity of the wild type (Ashwini and Srividya, 2014).

The antagonist is not only modified to produce greater yields of antimicrobial agents but to be more tolerant to abiotic stresses. In cases where the antagonist is a mycoparasitic fungi like *Trichoderma spp,* mutant generation would involve developing resistant strains to fungicides. This would help in use of the antagonists together with fungicides for integrated pest management (Melo *et al*., 2011). A mutant antagonist may also be endowed with both the ability to produce greater yields and more tolerance to environmental stress as well. Melo *et al*. (2011) developed mutants of *Coniothyrium minitans* with higher antibiotic yield as well as resistance to fungicide iprodione. Another means by which the production of antimicrobial compounds is manipulated is by use of small quantities of organic compounds (Toghueo *et al.*, 2016). The organic chemicals not only increase the amount of the antimicrobial metabolites produced but also leads to the formation of newer ones. In the current study, attempts to produce better performing strains of *Paenibacillus polymyxa* KaI245, the latter method was employed. The organic chemicals that were used are acetone and methanol. Acetone has been shown to increase the quantity of the antimicrobial agents (Toghueo *et al*., 2016). The table in Appendix III summarizes the various techniques used to stimulate production of secondary metabolites by micro-organisms.

2.7 Methods used in analyzing chemical structures of compounds

2.7.1 Spectrophotometric Techniques

Spectrophotometric techniques are biochemical techniques that exploits the fact that electromagnetic radiation interaction with matter is dependent on the properties of radiation and on the appropriate structural parts of the material(s) involved (Wilson and Walker, 1994). Different parts of matter give rise to radiation and are also affected by radiation in the corresponding region of spectrum. In the attempt to identify the chemical structure of the antifungal agent produced by *Paenibacillus polymyxa* KaI245, mass spectrometry was used.

2.7.1.1 Mass Spectrometry

Mass spectrometry is not dependent on quantum principles like other spectrophotometric techniques (Wilson and Walker, 1994). The term 'spectrometry' in its definition is therefore misleading. Mass spectrometry is used to elucidate molecular weight and molecular formula of an unknown substance. All mass spectrometers are composed of three parts:

- An ionization chamber.
- A mass analyzer.
- A detector.

While using mass spectrometry, molecules are disintegrated to produce fragment ions each of which is represented by a peak in the resultant spectrum. Any material that can be ionized and whose ions can exist in the gas phase can be investigated using mass

spectrometry (Wilson and Walker, 1994). In this study, Gas chromatography was first carried out on the substances and effluents passed through a mass spectrophotometer.

2.7.2 Chromatographic Techniques

These are techniques that are used to separate and identify a wide range of compounds of biochemical interest (Wharton & McCarthy, 1972). In any chromatographic technique substances are separated based on different characteristics inherent to the substances in the mixture. Further, there is always a mobile phase and a stationary phase. In investigating the antifungal agent produced by *Paenibacillus polymyxa* KaI245, gas chromatography was used.

2.7.2.1 Gas Chromatography

Gas chromatography is so called because a carrier gas is used as a mobile phase. The carrier gas is majorly helium however, nitrogen and hydrogen can also be used. Substances are separated based on their volatility and polarity. The stationary phase is a viscous liquid coated on the inner surface of a capillary column or on the surface of solid support in the packed column. A gaseous mixture is passed through either of the columns and separation would depend on the degree of interaction between individual substances and the stationary column. To achieve better resolution while separating, the column length should be increased (Mondello *et al*., 2008). Capillary columns are popularly used but has relatively less sample capacity and hence samples have to be diluted before injection or split inside the gas chromatography inlet (Wharton and McCarthy, 1972). Successful separation is displayed in form of peaks and the time between peaks is called retention time. In this

study, gas chromatography coupled to mass spectrometry was used in characterization of the antifungal agent produced by *Paenibacillus polymyxa* KaI245. Separated molecules were individually passed through mass spectrometer to obtain their molecular masses and chemical structure.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Materials

3.1.1 Diseased Barley Plants

Diseased barley leaves were collected from KARLO-Njoro (0.3˚N, 35.9˚E) in Nakuru county, and Chepkoilel in Uasin Gishu county (0.6˚N, 35.3˚E) barley trial plots. Leaves with characteristic symptoms of diseases of interest were collected from the trial fields. These included leaves with net-form-net-blotch symptoms suspected to be caused caused by *Drechslera teres* f.sp *teres*, scald caused by *Rhynchosporium commune,* and barley stem rust caused by *Puccinia graminis*.

3.1.2. The Bacterial Isolate

A bacterial isolate labelled KaI245 was provided from a previous study conducted by Makumba *et al.* (2016). The isolate had earlier been identified as *Aeromonas hydrophila* using the Potassium hydroxide (KOH) solubility test to establish the Gram stain status and biochemical tests. At a later time however, isolate identification using the 16S rDNA portion, PCR amplification and DNA sequencing revealed that isolate KaI245 was *Paenibacillus polymyxa*. This identity of the KaI245 isolate was adopted for this study.

3.2. Isolation and Identification of Barley Fungal Pathogens

3.2.1 *Drechslera teres* f.sp *teres*

Leaves showing net form net blotch symptoms were cut into convenient sizes (1 cm) and surface sterilised by wetting in 70% ethanol for 10 s and 0.5% sodium hypochlorite solution

for 1 min, then soaked three times for 1 min in Sterile Distilled Water (SDW). They were then dried between sterilised filter papers. Leave fragments were then transferred onto petri dishes containing Potato Dextrose Agar (PDA) amended with streptomycin and incubated for ten days at 28±1˚C in continuous darkness to allow mycelial growth. The mycelia were then transferred onto petri-dishes containing plain PDA to obtain pure colonies. Riddel slides were prepared for microscopy studies. Identification was done using: cultural characteristics of pure cultures and morphological characteristics by microscopy. The fungal cultures were maintained on PDA at 4˚C.

3.2.2 *Rhynchosporium commune*

Rhynchosporium commune is a slow grower and is easily contaminated (Kulichova, 1997). Its isolation was done by using moist chamber technique as described by (Abang *et al*., 2006). Leaves were placed in paper envelopes and allowed to air dry at room temperature for at least 48 h. Dried leaves were wet for 10 seconds in 70% ethanol, surface sterilized for 60 seconds in a 0.5% sodium hypochlorite and rinsed in sterile distilled water for 10 seconds. Wet leaves were pressed dry between sterilised paper towels and placed on a plastic screen. The plastic screen was rested on rubber bands above damp filter paper in a petri dish. Petri dishes were incubated in the dark at 15˚C for 48hours to induce fungal growth and sporulation. Fine needles were used to remove small tufts of mycelia that emerged from the leaf. The mycelial tufts were transferred to petri dishes containing PDA amended with Kanamycin. The petri dishes were incubated at 15˚C for 4 weeks. The mycelia were then transferred to PDA without antibiotics for *in vitro* testing. Identification was also done using cultural and morphological characteristics.

3.2.3 *Puccinia graminis*

Rust spores were collected and maintained as described by Woldeab, (2015) with some adjustments. The spores were collected by tapping barley stalks with rust pustules on to an aluminium foil.

Collected rust spores were inoculated with an atomizer on the seedlings of susceptible barley cultivar (268A) in the greenhouse at the University of Eldoret to produce more pustules. The rust spores were multiplied until sufficient spores were collected by tapping the rusted leaves on a watch glass. The spores (urediniospores) were then transferred to a test tube for storage at -20˚C. Dried barley stalks containing teliospores were obtained as well and kept at -20˚C for *in vitro* antagonism studies. For further characterisation, urediniospores and teliospores were observed under a microscope (Labomed CXR2) and the images photographed using Infinix Note 4 Smartphone.

3.2.4 Pathogenicity test

The fungal isolates were cultured (7-days-old *D. teres* and 21-days-old *R. commune*) and identified based on their colony morphology as well as microscopically by their conidial shape and hyphal characteristics. Further, conidia were inoculated on the susceptible barley cultivars for the expression of symptoms and the same pathogen re-isolated from the lesions. The brown and black pustules of urediniospores and teliospores respectively were scraped onto microscope slides and observed.

3.3 *In vitro* testing of *Paenibacillus polymyxa* KaI245 for antagonistic activity against Barley fungal pathogens

In vitro inhibition of mycelial growth of the isolated fungal phytopathogens by sorghum rhizobacterial isolate labelled KaI245 and identified as *Paenibacillus polymyxa* KaI245 in a previous study by Makumba (2016) was tested using the dual culture technique described by (Paulitz *et al*., 1992) and (Landa *et al*., 1997).

All fungal cultures were maintained on PDA except for *Puccinia graminis*. *P. graminis* urediniospores that were maintained in the laboratory at -20˚C. Those maintained on PDA were kept at 4˚C.

For the assay, *P. polymyxa* KaI245 was streaked onto a PDA plate 2cm from the 2 equidistant edges of the petri-dish, and a 6mm diameter of fresh mycelial plug of *Drechslera teres* f.sp *teres* placed at the center of the petri dish. The plates were incubated at 28˚C for seven days. Readings were taken by measuring the radius of the mycelium towards the antagonist and labelled as ‘r’ and that of the mycelium growing away from the bacterial antagonist and labeled ‘R’. Each experiment was replicated six times. Percent inhibition was calculated using the formula;

$$\frac{R-r}{R} \times 100. \qquad \text{(Makumba, 2016).}$$

Where R: radius of the mycelium away from the bacterial antagonist and

r: radius of the mycelium towards the bacterial antagonist.

The same was done for *R. commune.* However, due to the slow growth rate of the fungus, the bacterium was inoculated 9 days after the fungal growth. Readings were taken after 18

days in the same manner as that of *D. teres* f. sp *teres*. Incubation temperature of *R. commune* and *Paenibacillus polymyxa* KaI245 dual assay was at 20˚C. This temperature was found to be appropriate for both the bacterium and the fungus.

3.4 Barley fungal pathogen inhibition by bacterial volatile organic compounds

Sealed plate method was used for *D teres* f. sp *teres* and *R commune*. *Paenibacillus polymyxa* KaI245 was streaked onto PDA in the bottom of a petri dish. A 6mm diameter mycelial plug was cut from the margin of an actively growing fungal cultures and placed at the center of a second petri-plate containing PDA. The plate with mycelial plug was then inverted over the plate inoculated with the bacterium. The plates were sealed with parafilm and incubated at 28˚C (*D. teres* f. sp. *teres*) and 20˚C (*R. commune*). The diameter of *D. teres* f. sp *teres* was measured daily (every 24hrs) for 7 days and compared to the control. The diameter of *R commune* was measured at every 3 days interval for 18 days and also compared to the control. Controls constituted fungal cultures inverted over non-inoculated PDA. Change in diameters was calculated by subtracting the currently measured diameters from the diameter recorded the previously. Five replicates were set up for each treatment. Percent inhibition of the bacterial volatiles were calculated each time the diameters were measured as follows;

$$\text{Percent inhibition} = \frac{D-d}{D} \times 100.$$ (Makumba, 2016)

Where;

D: is average diameter of the controls at the specific time the diameters were measured and, d: is the average diameter of the fungal colonies exposed to bacterial volatiles at the time of measurement.

A modification of divided plate method (Fernando *et al*., 2005) was used to test the efficacy of bacterial volatiles to control the germination of *P. graminis* teliospores. Filter papers were wetted with Sterile Distilld Water (SDW) and placed in sterile petri dishes. Five dried barley stalks, 5 cm each, containing the black teliospore pustules were then placed on the moist filter papers in the petri-plates. *Paenibacillus polymyxa* KaI245 was then cultured in smaller petri-dishes that were also placed inside the larger petri-plate containing the moist filter papers and barley stalks. The larger cover plate was then inverted and sealed using a parafilm. They were then incubated at 28˚C for 72 h. Five different points of each stalk were scrapped for teliospore observation under a light microscope. 200 slides were prepared for this observation. Germinating and germinated teliospores were counted and compared to the control. Controls had non-inoculated PDA in place of PDA inoculated with the test bacterium. Each treatment was replicated four times.

3.5 Bacterial culture filtrate preparation and testing for its antifungal activity

3.5.1 Antifungal activity against *Drechslera teres* f. sp teres

A single colony of *Paenibacillus polymyxa* KaI245 was cultured in Luria Bertani (LB) broth in a mechanical shaker at 28˚C and 150 r.p.m for 24 h as seed liquid. Seed liquid (1.5 mL) was then inoculated into 200 mL of Potato Dextrose Broth (PDB) medium and

cultured at 28˚C, 120 r.p.m for 7 days (Wang *et al*., 2016). The supernatant was recovered by centrifugation at 4˚C, 13000 rpm for 30 min and carefully pouring out into a clean container without disturbing the pellet.

The supernatant was then used for antifungal assay of *Drechslera teres* f. sp *teres.* The fungal growth medium (PDA) was supplemented with 10% of the supernatant extract (Darna *et al*., 2016).

Fungal mycelium (6mm) were then inoculated on to the centre of the medium. The controls had the fungi inoculated on plain PDA. The plates were then incubated at 28˚C. Colony diameter of the cultured fungi was recorded daily for five days. Changes in diameters were recorded daily and subjected to analysis. The experiment was replicated five times.

3.5.2 Antifungal activity against *Puccinia graminis*

This experiment was done as described by Fernando *et al* (2005) with some modifications. The cell free culture filtrate was tested for its efficacy against the germination of *P. graminis* teliospores. Sterile filter papers (Whatmann No 1) were wetted with 5 mL of the bacterial culture filtrate (cultured in PDB) in petri-dishes. Five 5 cm dried stalks of barley with black teliospore pustules were laid on the filter papers. Controls had the filter paper only wetted with non-inoculated PDB. They were both incubated at 28˚C for 48 h. Afterwards, five random points were scrapped from each of the stalks and observed under the microscope for the germination of the teliospores. A total of 250 microscope slides were prepared and the number of germinating teliospores recorded for further analysis.

3.6. Enhancement of the quality and quantity of antifungal agents produced by *Paenibacillus polymyxa* KaI245

This was done as described by Toghueo *et al.* (2016) with some modifications. Two experimental set-ups were prepared where the bacterium was cultured in PDB, separately supplemented with 1% acetone and 1% methanol and incubated on a mechanical shaker at 28^{o}C, 120 r.p.m. for 7 days. A standard control in which the bacterium was inoculated in PDB was set-up as well. Two other controls had PDB separately supplemented with 1% acetone and 1% methanol dubbed in this study as "acetone-alone-control" and "methanol-alone-control". The purpose of these two controls was to check the antifungal activity of the organic compounds alone against the test fungal pathogen vis á vis the combined antifungal effect of the organic compounds and the antagonistic bacterium. The third uninoculated control, dubbed as "uninoculated-control" had plain PDB. Crude extracts from all the treatments including controls were added (10%) to PDA and *D. teres* f. sp *teres* inoculated as described in section 3.4. The treatments were five in number. Diameters were then measured daily (every 24 h) for seven days. Each treatment was replicated 3 times.

3.7 Production of antifungal agents from *Paenibacillus polymyxa* KaI245 for GC-MS analysis

3.7.1. Batch fermentation

Antifungal agent(s) production by *Paenibacillus polymyxa* KaI245 was carried out in liquid media on shaker flasks. Potato Dextrose Broth: 20.0g of dextrose and 4.0g of infusion from potatoes in 1 litre of distilled water was used as the production medium. The medium was divided into conical flasks each with 200ml and autoclaved. The flasks were then be inoculated with 20% v/v of the rhizobacterial suspension (Wang *et al*., 2008; Farhana *et*

al., 2011) prepared as previously described in section 3.4. The inoculated flasks were incubated at 28^{o}C for 7 days on a rotary mechanical shaker at a revolution speed of 180 r.p.m (Farhana *et al*., 2011; Song *et al*., 2012).

3.7.2. Extraction of antifungal agents

A cell-free filtrate was obtained by centrifuging at 10,000 rpm for 20 min and 4°C (GYROZEN 1730R Micro-centrifuge). Equal volume of ethyl acetate was then added to the supernatant. The mixture was then thoroughly shaken for 2 h in an orbital shaker at 200 r.p.m before putting in a separating funnel and left for 10 minutes for the two layers to separate. The aqueous layer was then run off into a clean beaker and the ethyl acetate layer containing the extracted antibiotics put in a separate beaker. Fresh ethyl acetate was then added to the same aqueous layer and the extraction process repeated 2 more times for complete extraction.The resulting organic phases were mixed and concentrated to dryness under vacuum by using a rotary evaporator (Atta *et al*., 2009). The resulting pellet was re-suspended in dichloromethane (DCM). This was then used for characterisation studies using gas chromatography mass spectrometry (GC-MS).

3.7.3 GC-MS Analysis of antifungal agents

Test antifungal agent samples were analysed as described by (Toghueo *et al.*, 2016). In a screw-capped vial, a sample of approximately 1 mg of dry antibiotic extract sample was dissolved in 0.5 ml of DCM. GC-MS analysis was performed on an Agilent Gas Chromatograph 789A/5975 C mass spectrometer in full scan mode. The conditions were: GC column HP-5 MS low bleed capillary column (30m x 0.25µm film thickness, J & W, Folson, CA, USA), Helium was the carrier gas at a constant flow rate of 1.25 ml/min,

injection volume of 1 µl. The oven temperature program was as follows: 35°C constant for 5 min to 280°C at a rate of 10°C/min for 10.5 min then to 285°C at the rate of 50°C/min for 30min and 70min running time. Ionization was achieved under the electron impact mode (ionization energy of 70 eV). The source and transfer line temperatures were 250°C and 330°C, respectively. Detection was carried out in scan mode: m/z 35 to m/z 700 a.m.u. The detector was switched off in the initial 10 min (solvent delay).

3.7.4 Interpretation of GC-MS spectra

Interpretation of GC-MS mass spectra was conducted using National Institute of Standards and Technology (NIST' 11, 08, 05) and Adams and Chemecol mass spectral databases.

3.8 Greenhouse evaluation of *Paenibacillus polymyxa* KaI245 culture filtrate in controlling net blotch

3.8.1 Pathogenicity test and preparation fungal inoculum

Pathogenicity of *D teres* f. sp *teres* was confirmed under greenhouse conditions. Fungal conidia suspension was prepared by flooding 7-day-old pure plate cultures of all the *D. teres* f. sp *teres* with 20 mL of with sterile distilled water (SDW). Fungal growth (7 days old) was scrapped with the edge of a sterile glass slide (sterilized by dipping in 70% alcohol and flaming) to dislodge the conidia. The conidia suspension was then filtered using sterile cheese cloth (sterilized by autoclaving at 121^{o}C and 1 bar for 15 minutes) to remove mycelia clumps (Morris & Nicholls, 1978; Guarro *et al*., 1998). Conidia counts were done by placing a drop of suspension on an improved Neubauer haemocytometer and examined under a microscope. Total conidia counts were adjusted to 400 conidia/ml (Jorgensen *et al*., 1996) using SDW.

3.8.2. Planting medium and fungal inoculation

The planting medium was prepared from soil, sand and peat in the ratio of 2:1:1 by volume respectively (Samarah, 2005). The soil mixture was sterilized by subjecting it to 121^{o}C and 1 bar for 2 hours in an autoclave and allowed to stand for 7 days to release toxic substances. The soil was then filled (¾-filled) into 20 cm by 10.5 cm diameter plastic pots. Twelve barley seeds of the variety (Nguzo) that is susceptible to net blotch were planted in each of the plastic pots in two rows. These pots were kept in the greenhouse at room temperature (22 ± 5^{o}C) and watered and fertilized (a mixture of 2.5 kg fertilization [N-P-K content 14-5-21%, respectively] with 25 litres of water) regularly (Hyvonen, 2011). After seed germination, thinning was done to leave 6 seedlings per pot.

Fungal inoculation was conducted by spraying 14-day-old plants with a conidial suspension at a concentration of 400 conidia per millilitre using an atomizer, until run off. The plants were then covered using a transparent polythene bag for 72 h to induce high relative humidity conditions of about 100% and 28 ± 5^{o}C for proper germination of conidia and high plant infection. Plants were removed from the polythene chamber after 72 h and placed on greenhouse benches.

3.8.3. Testing antifungal-agent-culture-filtrate of *Paenibacillus polymyxa* KaI245 in controlling net blotch of Barley

Cell-free antifungal-agent-culture-filtrate from *Paenibacillus polymyxa* KaI245 was used for *in vivo* evaluation in the greenhouse experiments. Treatments for the bio-control experiment were: Plants inoculated with the net blotch pathogen and original concentration of the culture-filtrate, Plants inoculated with the net blotch pathogen and double

concentration (achieved by evaporating to half the original volume on a rotary vacuum evaporator) of the culture-filtrate, Plants inoculated with the net blotch pathogen and Orius® 250 EW (250g of Tebuconazole per litre), a synthetic fungicide at the rate of 0.2% v/v, Plants inoculated with the pathogen alone (+ve Control), and Non-treated plants (–ve control). The experiments were arranged in the greenhouse in a completely randomized design layout achieved using the GLM procedure in SAS-9.1 software in five replications. In each of the barley pots, two leaves were tagged for scoring purposes.

3.9. Disease assessment

The percentage necrotic lesions caused by *Drechslera teres* was scored 7 days post-inoculation (Jorgensen *et al*., 1996). Severity of net blotch on leaves were evaluated using a scale with 21 levels consisting of 5% intervals.

Table 3.1: Severity scale for evaluation of net blotch severity on barley

Scale	Percentage area covered by disease symptom
0	No symptom
1	0-5%
2	5-10%
3	10-15%
4	15-20%
-	-
-	-
-	-
20	95-100%

Based on the disease severity index, % disease suppression of net blotch as described by (Jorgensen *et al*., 1996) was calculated as follows:

%Net blotch Suppression= $\left[\frac{(A-B)}{A}\right]$X100

Where;

A = % disease severity exhibited on the leaves due to *Drechslera teres* f. sp *teres* alone, and

B = % disease severity exhibited on the leaves after inoculation with both the pathogen and the various culture-filtrate concentrations of the best bacterial antagonist and standard control (Orius®).

3.10. Statistical analysis

Each of the experiments was analysed independently. Recorded data was analysed with Statistical Analysis System (SAS®) Software (SAS Institute, 2003). Data obtained from dual culture experiments, inhibition via volatiles and cell-free supernatant was analysed using student's T-test. Treatment effects was tested by Analysis of Variance (ANOVA) and the means compared using the Tukey-Kramer Multiple-Comparison Test at a 5% probability level (Gomez & Gomez, 1984). Chi-square test of independence was used for the analysis of effects of volatiles and cell-free supernatant on the germination of *P. graminis* teliospores. The data obtained from counting germinating teliospores was first grouped to enable analysis by chi-square.

CHAPTER FOUR

RESULTS

4.1 Morphological identification of *Drechsclera teres* f. sp *teres*

The fungus was isolated from the barley leaves displaying the typical net-blotch symptoms (Plate 4.1a). On culturing of *D. teres* on (PDA), there were variations in both colony colour and morphology (Plate 4.1 g & h). Some colonies appeared grey while majority were black; both on the upper and the rear side. Most of the colonies isolated from samples obtained from university of Eldoret barley trial fields were black with a few grey while those isolated from Njoro were mostly grey. The grey ones appeared to be cream on the rear side. There were also cases where colonies differed in colour even after they were sub-cultured from the same parent colony. Some colonies displayed concentric rings (Plate 4.1h). Microscopically, the hyphae ware shown to be septate and branched (Plate 4.1b). Macro-conidia were cylindrical in shape, multicelled and having three to six septa each (Plate 4.1 c & d). Some conidia had conspicuous scars at the terminal end away from the point of attachment to the conidiophore. Immature conidia appeared brown with septa appearing only faintly (Plate 4.1e).

4.2 Identification of *Rhynchosporium commune*

R. commune colonies were initially whitish pink which gradually turned white then grey. The reverse side always appeared brick red (Plate 4.2). Under the microscope, conidia were short and bulbous. For some colonies, orange exudates appeared near the initial point of inoculation. Also notably, was its slow rate of growth which mostly took an average of 21 days to fill the whole petri-plate.

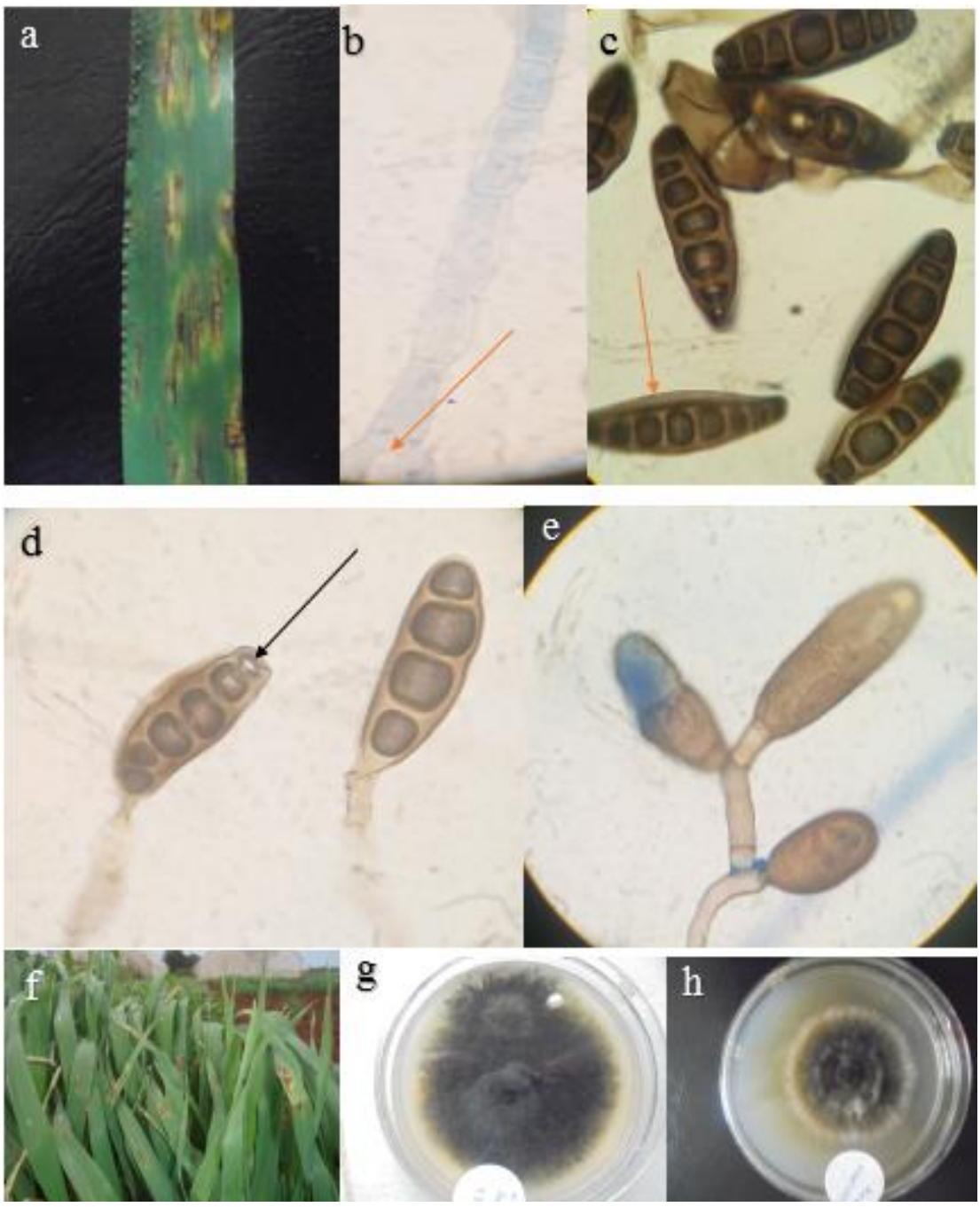

Plate 4.1: ***Drechslera teres.*** **a; Net form net-blotch symptoms on a barley leaf. b; Septate and branched mycelium (pointed by the arrow). c; Conidia with five and six septa at 1000X (pointed by arrow). d; Conidia at 1000X having four and three septa. Both have rounded ends with one displaying a conspicuous scar (black arrow). e; Immature conidia. f; Field barley with net form net blotch infection.g, h;** ***D. teres*** **colonies displaying differences in colour and morphology. g; dark floccose colony with dense sporulation h; grey floccose colony with concentric rings and sparse sporulation. Photos a, f, g, and h were photographed using Sony W810 camera (20 mega-pixels) while micrographs b, c, d, and e, were photographed using infinix Note 4 smartphone camera. All the micrographs were at 1000X magnification.**

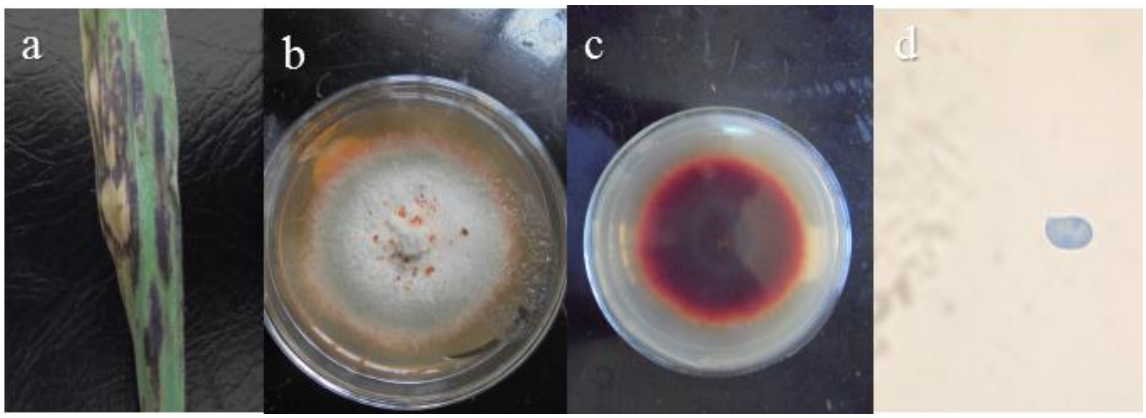

Plate 4.2: *R. commune*. a; Scald symptom on barley leaf. b; Upper view of *R. commune* colony on PDA Note exudates seen near the centre of the petri plate. c; Rear view of *R. commune* colony on PDA. d; Short and bulbous spore.

4.3 Identification of *Puccinia graminis*

Spores of *P. graminis* were obtained from the brown pustules found in an infected barley stem (Plate 4.3a). The pustules were filled with dust-like brown substance. Some pustules had ruptured exposing the dust like substance. The brown 'dust' in the pustules are the urediniodpores. Drying barley stalks (Plate 4.3c) were also obtained from the same fields and teliospores were observed. The urediniospores and teliospores were obtained directly from the infected plant material for observation under the microscope.

The urediniospores appeared orange and circular to ellipsoidal under the microscope (Plate 4.3b). Teliospores were bi-celled, clavate to oblong and yellowish brown. The septum separating the two cells appeared constricted (Plate 4.3d).

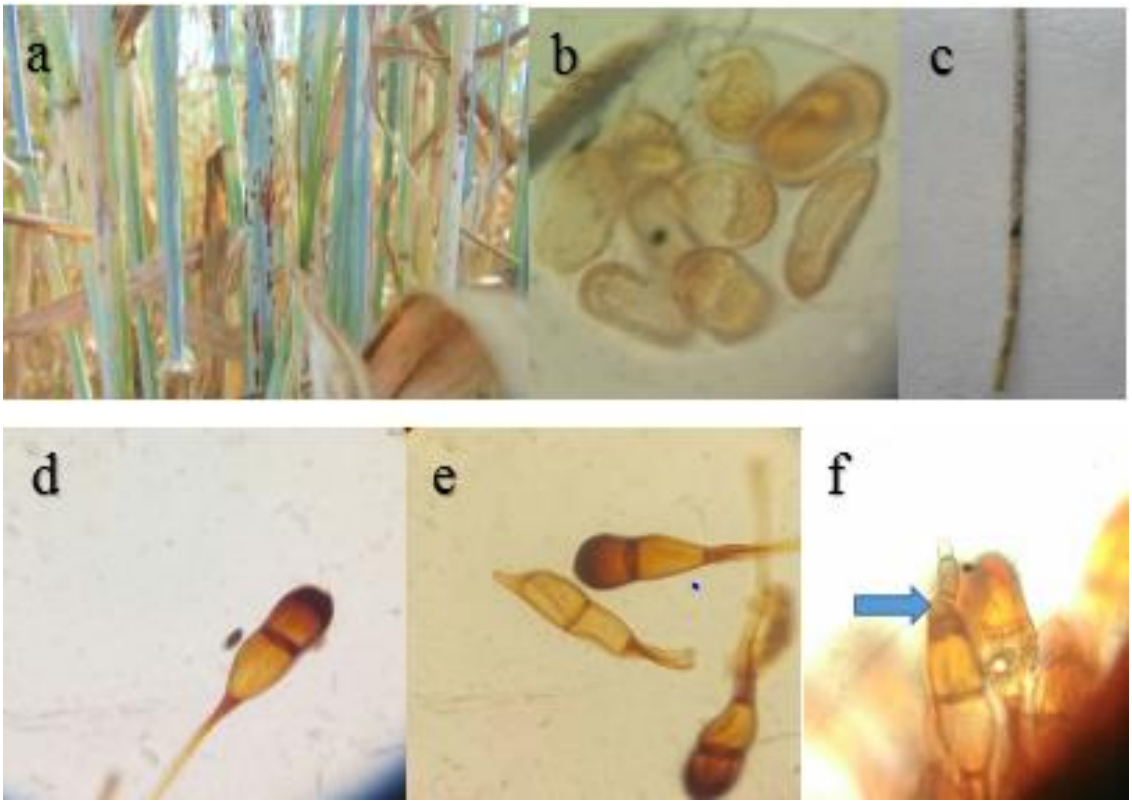

Plate 4.3: ***Puccinia graminis.*** **a; Brown pustules (stem rust) of urediniospores on barley stems. b; Urediniospores. c; Dried barley straw with black pustules of telia. d; A single teliospore. e; A magnification field of view showing a teliospore (in the middle) in the early stage of germination. f; An evident basidium (blue arrow) germinating from a teliospore. Micrographs, b, d, e, and f, were at 1000X magnification. Photographs a and c were recorded using SONY camera while the micrographs were captured using an Infinix Note 4 Smartphone rear camera.**

4.4 *In vitro* antifungal activity of *Paenibacillus polymyxa* KaI245 against Barley fungal pathogens

Effectiveness of *Paenibacillus polymyxa* KaI245's antifungal compounds were tested *in vitro* against *D. teres* f. sp *teres*, *R. commune* and *in vivo* against *P. graminis*. Experiments involved the use of both live cells as well as testing for volatile compounds.

4.4.1 Dual culture Technique

Live bacterial cells were tested against both *D. teres* f sp. *teres* and *R. commune in vitro*. Mycelial growth inhibition was strongest on *D. teres* using the dual culture technique (Plate 4.4A). The strongest percentage inhibition was achieved at 51.00% and the lowest at 42.00%. Average percent inhibition was found to be 47.30%. Further, the difference between 'R' and 'r' was found to be significant t (10) = 13.47 at P = 0.05. Dual plating of *Paenibacillus polymyxa* KaI245 with *R. commune* showed less than full effect in the control of the fungi (Plate 4.4B). The apparent less 'r' compared to 'R' (Appendix VI) can only be attributed to the physical barrier to growth by the bacterial cells rather than to any antagonistic activity. It is also worth mentioning that when the bacteria was cultured at the same time with the fungi *R. commune*, the bacteria always outgrew the fungus. In plate 4.3B below, the bacteria was introduced after 9 days of fungal growth. In all the cases however, the inability of the bacteria to inhibit mycelial growth of *R. commune* was consistent.

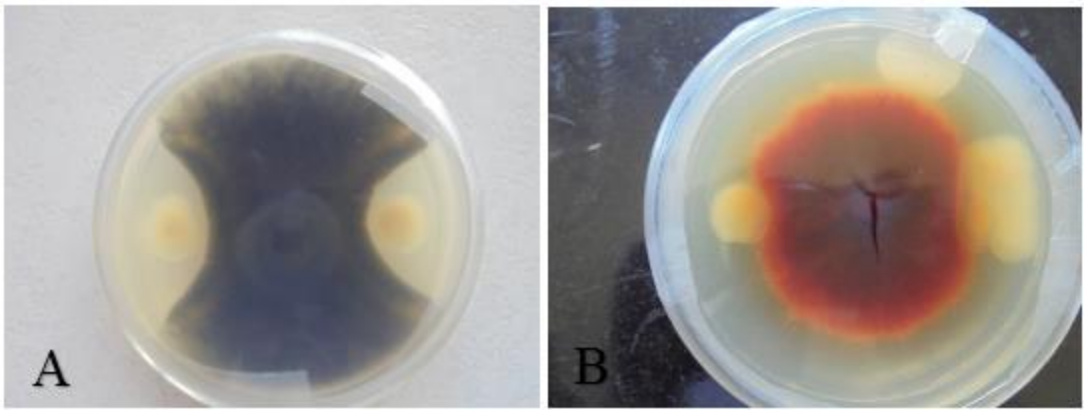

Plate 4.4: Petri plates showing the rear views of barley fungal pathogens isolates co-cultured with the antagonistic bacteria. In 'A', *D teres* inhibition of mycelial growth towards the bacteria is clearly evident. B; The inhibition of *R. commune* mycelia by the bacteria is not effective.

Table 4.1: % mycelia inhibition of *D teres* f sp. *teres* by *Paenibacillus polymyxa* KaI245

Replicate	R	R	% Inhibition
1	35.00	20.00	42.90
2	37.00	18.00	51.40
3	37.00	20.00	46.00
4	33.00	19.00	42.60
5	32.00	16.00	50.00
6	39.00	19.00	51.30
Mean	**35.50**	**18.70**	**47.30**
SD	**2.70**	**1.50**	**4.10**
P **0.000**			

R: radius away from the antagonist.

r: radius towards the antagonist.

SD: Standard Deviation.

The data was recorded on the 7th day of the fungal growth. The highest percentage inhibition was recorded in replicate 2 (51.40%) while the lowest inhibition was recorded in replicate 4 (42.60%).

4.4.2 Fungal inhibition by bacterial volatile organic compounds

Volatile organic compounds produced by *Paenibacillus polymyxa* KaI245 suppressed the growth of *R. commune* by an average of 52.90%. The inhibition rate did not significantly change with time; the lowest was 50.80% (between days 9-12) the highest was 54.10% (between days 3-6) (Table 4.2). After 18 days, *R. commune* colonies grown with the bacteria *P. polymyxa* on the lower plate recorded an average diameter of 25.80mm compared to an average of 56.00mm on the controls. Growth rate was also significantly inhibited. The colonies cultured with bacteria underneath had an average growth rate of 0.80mm/day while the controls had an average of 1.80mm/day. This is shown more clearly by the images depicting smaller colony diameters in Plate 4.5A compared to colonies with relatively larger diameters in Plate 4.5B. The bar graph in Appendix VIII relays information on growth rates. There was no significant difference on the inhibition of growth rate between the days. Despite the live cell cultures having a strong inhibition on *Drechslera teres* as described earlier, the bacterial volatiles did not show any effect on the growth rate of the fungal mycelium (Appendix VI).

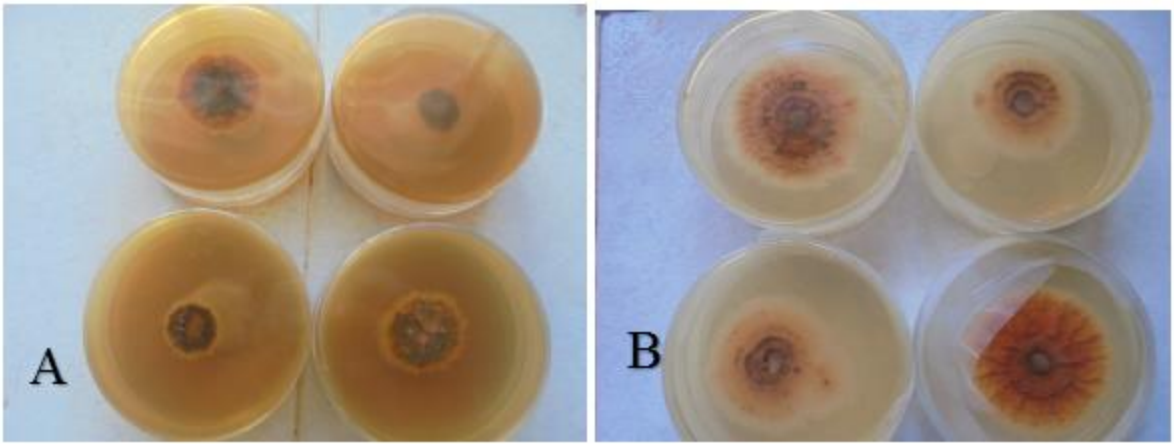

Plate 4.5: Effect of bacterial volatiles (sealed plate method) on the growth of *R. commune*. Both plates A and B represent 18 days old colonies. A: Fungal colony plates inverted on bacterial colony plates. B: View from above shows inverted fungal colony plates on uninoculated/plain PDA plates.

Table 4.2: Inhibition effect of volatile metabolites of *P. polymyxa* KaI245 on *R. commune*

Day	Colony diameters ±SD (mm)		% Inhibition
	Control	Antagonized	
3	29.20±1.5	13.60±1.1	53.4
6	36.60±1.1	16.80±1.3	54.1
9	42.40±2.4	20.20±2.3	52.4
12	48.40±4.3	23.80±4.0	50.8
15	53.20±6.1	25.20±3.1	52.6
18	56.00±9.6	25.80±3.9	53.9
Average			**52.87**
SD			**1.2**
***P* value**			**0.009**

The diameters represents averages from five replicates. The diameters were recorded at 3 days interval for 18 days.

SD: Standard Deviation.

Percentage inhibition was calculated from the average diameters of the antagonized compared to that of control on the same day ((C-A/C)x100) where C = Control diameter and

A = Antagonized diameter. Results show fairly consistent inhibition with the lowest recorded in the 12th day (50.8) and the highest on the 6th day (54.1).

With regard to the effect of volatiles on the germination of *P. graminis* teliospores, a total of 200 slides were prepared and examined. Half of the prepared slides were from the control group (not exposed to the volatiles) while the remaining half represented teliospores from the treated group (exposed to bacterial volatiles). There were 61 glass slides (61%) in the control group where the number of germinating teliospores (Plate 4.6) were between 0 to 5. In the treated group, 88 slides (88%) recorded the number of germinating teliospores in that same range. In the intervals defined by 6 to 15 germinating teliospores, there were 39 slides (39%) in the control group and only 12 (12%) in the treated group. This difference was found to be highly significant at $P = 0.05$ (chi sq value = 27.53, df = 3, P = 1.4E-11). It can further be deduced that there were fewer germinating teliospores in the group that was exposed to *P. polymyxa* KaI245 volatiles compared to the group not exposed to the volatiles (Appendix IXa). The total count of the germinated teliospores was lower by 39% in the group exposed to volatiles as compared to those not exposed. The results therefore strongly suggest that the *P. polymyxa* KaI245 volatiles have negative effects on the germination of *P. graminis* telia.

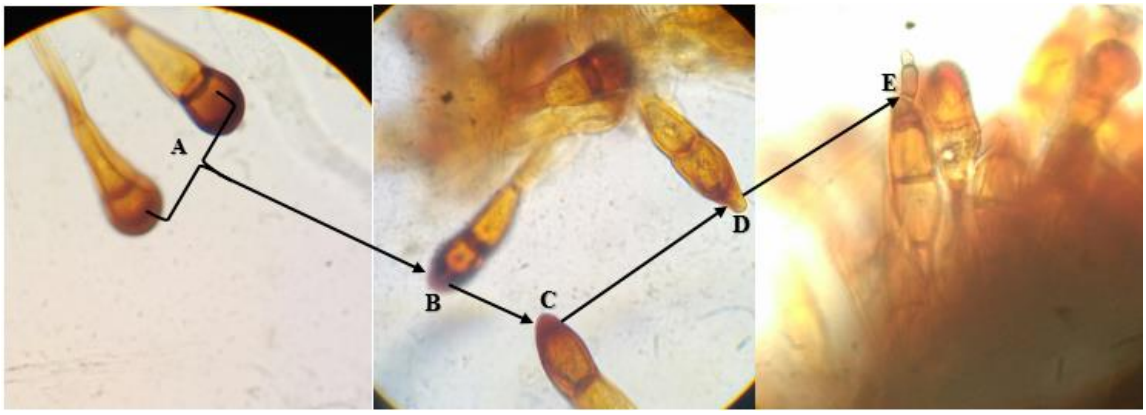

Plate 4.6: Reconstruction of different stages of germination of teliospores. A; teliospores as they appear naturally (non-germinating). B, C, D; teliospores at different germinating stages as evidenced by the shapes of their tips. E; an emerging basidium. B, C, D, & E were all counted as germinating teliopores in all the experiments. All the micrographs are at 1000X magnification.

Table 4.3: Effect of *P. polymyxa* KaI245 volatiles on germination of *P graminis* telia

Class (Nos. of germinated teliospores)	Control set-ups		Treated set-ups		Totals
	No. of glass slides	%	No. of glass slides	%	
0	6	6	8	8	14
1−5	55	55	80	80	135
6−10	30	30	7	7	37
11−15	9	9	5	5	14
Totals	**100**		**100**		**200**
***P* = 0.000**					

Class: Grouped number of teliospores (grouping was done to validate analysis by chi-square).

No. of slides: The frequency with which the numbers of germinating teliospores in the corresponding groups appeared.

The inhibition of germination of teliospores is clearly seen in the 6-10 category; 30% of slides in the control group vis á vis 7% of the slides exposed to volatiles.

4.5 Antifungal activity of the cell free *P. polymyxa* culture filtrate

The cell free supernatant was tested to show its efficacy in inhibiting *D. teres* f. sp *teres* and *P. graminis in vitro*. The mycelial growth of *D. teres* f. sp *teres* was inhibited when the culture filtrate of *P. polymyxa* KaI245 was added to the fungal cultivation medium (PDA). The media was supplemented with 10% of *P. polymyxa* KaI245 culture filtrate. The diameter of the fungal mycelium was inhibited by an average of approximately 24% compared to the control (Plate 4.7). This inhibition however, was not enough for the rate of growth of treated means (m = 6.52, sd = 3.2, n = 5) to be significantly different t (8) = -1.11, P = 0.15 to the controls (m = 9.2, sd = 4.3, n = 5) at P = 0.05 (Table 4.4). With regard to the use of disks, less consistent results were obtained with some showing less prominent zones of inhibition while no mycelial inhibition was shown in some (Plate 4.8).

The cell free supernatant was also used to moisten filter papers upon which barley stalks, containing black teliospore pustules were placed and incubated for 48 h. Filter papers moistened using sterile distilled water (SDW) were used as controls. A total of 250 glass slides were prepared for observation, 125 for the controls and the rest for the treated ones (Table 4.5). In the control group, 56% of the glass slides had the number of germinating teliospores being at most 5 while in the treated group, 69.6% fell in that same category. Moreover, 30.4% of glass slides in the treated group had germinating teliospores ranging from 6 to 15 while 44% of slides in the control group were in the same category. Generally, there were fewer germinating teliospores in the treated group compared to the controls. These differences were however not found to be significant (chi-sq = 5.1, P = 0.16, df = 3)

at P = 0.05. The cell free supernatant exerts negligible inhibition on the germination of teliospores.

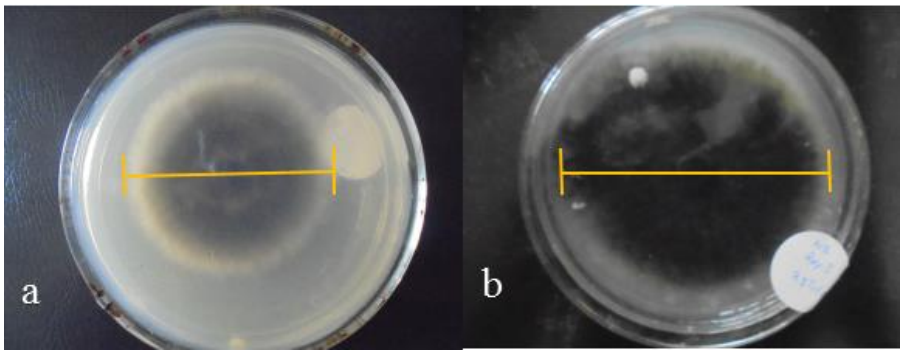

Plate 4.7 Pictures depicting the effect of *Paenibacillus polymyxa* cell free supernatant on the *D. teres* colony diameter. Both 'a' and 'b' represent 7 days old colonies. The colony in 'a' grows on medium amended with 10% *P. polymyxa* cell free supernatant while that in 'b' grows on plain PDA. Slight difference in diameter (24.1% inhibition) can be noted.

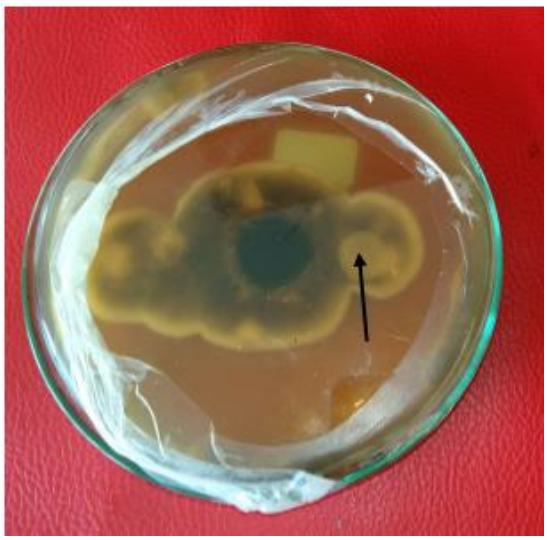

Plate 4.8: Evidence for the inability of the bacterial cell free supernatant to inhibit the growth of *D. teres* mycelium using paper disk. The arrow points to the paper disk.

Table 4.4: Mycelia inhibition of *D. teres* f sp. *teres* in media amended with *Paenibacillus polymyxa* KaI245 cell free culture

	Colony diameters±sd (mm)		
Day	**Control**	**Amended media**	**% Inhibition**
1	19.25±1.5	14.60±1.8	24.15
2	34.75±3.2	26.20±2.2	24.60
3	38.75±3.3	29.60±2.6	23.61
4	48.50±2.6	37.00±3.6	23.71
5	55.00±3.6	41.60±4.6	24.36
Mean			**24.09**
SD			**0.45**
***P* value**		**0.15**	

The *P* value was obtained from comparison of the rates of growth (daily change in diameter). The growth rates were significantly different at $P \leq 0.05$. Diameters represents means from five replicates.

Table 4.5: Effect of cell free *P. polymyxa* culture filtrate on the germination of *P. graminis* teliospores.

Class (Nos. of germinated teliospores)	Control set-ups		Treated set-ups		Totals
	No. of glass slides	%	No. of glass slides	%	
0	8	6.4	9	7.2	17
1−5	62	49.6	78	62.4	140
6−10	49	39.2	33	26.4	82
11−15	6	4.8	5	4	11
Totals	**125**	**100**	**125**		**250**
***P* = 0.16**					

Class: **Represents grouped numbers of germinating telia.**

No of slides: **Frequency of slides where the corresponding numbers (class) of germinating telia were observed.**

Control: **Barley stalks placed on filter papers moistened with sterile distilled water.**

Treated: **Barley stalks placed on filter papers moistened with cell free supernatant.**

The cell-free supernatant was not effective in inhibiting germination of *P. graminis* teliospores.

4.6 Compositional analysis of volatile organic compounds (antifungal agents) produced by *P. polymyxa* KaI245 using GC-MS techniques

Compositional analysis of the bacterial volatile organic compounds (antifungal agents) was done using GC-MS and interpretation done using National Institute of Standards and Technology (NIST' 11, 08, 05) and Adams and Chemecol mass spectral databases.

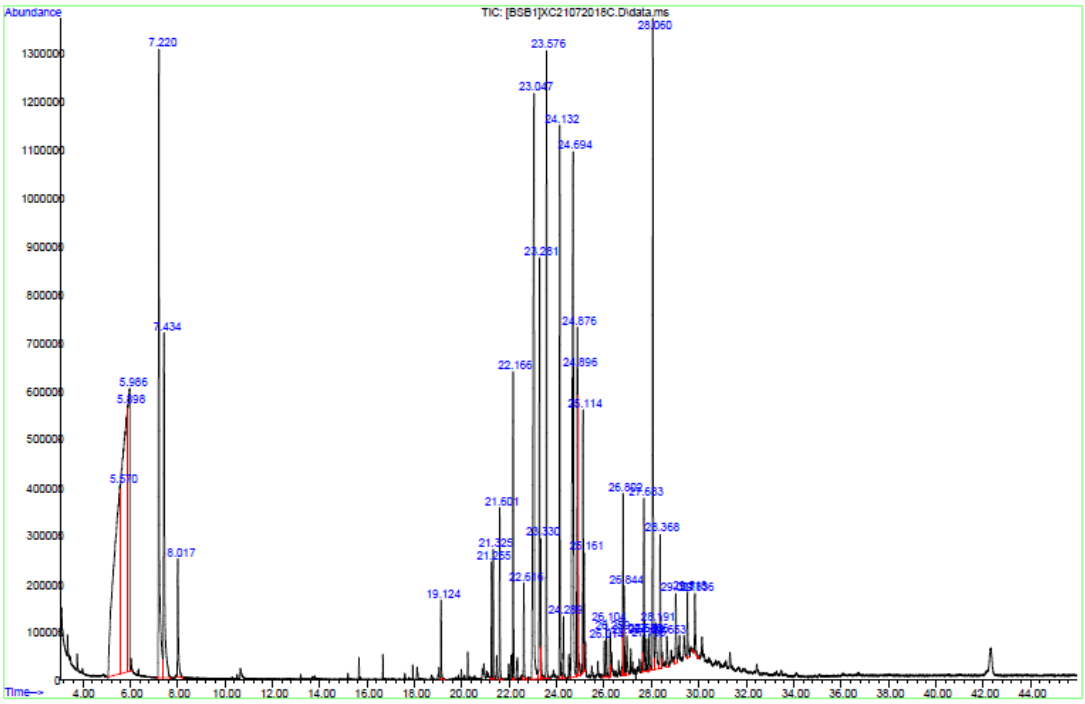

Fig 4.1. GC-MS mass spectra of bacterial volatile organic compounds of ethyl acetate extracts from *P. polymyxa* KaI245 cultures. This gave a total of 50 metabolites.

Figure 4.1 shows the volatile metabolite profile obtained by GC-MS analysis of the ethyl acetate extracts of *P. polymyxa* KaI245 cultures at 28˚C at the speed of 180 r.p.m for 7 days. There were a total of 50 volatile organic metabolites of which butanediol<2,3> (RT ≈ 5.97) was the most abundant at 21.20% (Appendix X). 6-octadecenoic acid (RT ≈ 24.69) and Hexadecanoic acid (RT ≈ 23.04) were the second (8.25%) and third (7.66%) most

abundant metabolites respectively. Of the detected metabolites, 58% (29/50) had relative abundance of less than 1% with Decane (RT ≈ 10.46) at 0.03% being the least recorded.

4.7 Greenhouse evaluation of *Paenibacillus polymyxa* KaI245 culture filtrate in controlling net blotch

The crude bacterial extract was assessed for its efficacy in controlling the pathogen *D. teres* f sp. *teres* in the greenhouse. A susceptible barley cultivar, Nguzo, was used for this experiment. Foliar applications of both the double concentration of the extract and the normal concentration had a mean disease score of 1.8 with no significant difference between them at $P \leq 0.05$ (Table 4.6). They both represented 50% reduction in disease score as compared to the control. The crude extract was however not equally as effective as the chemical fungicide Orius® 250 EW (25%w/w tebuconazole) at a rate of 0.2% v/v. The foliar spray of the chemical fungicide recorded a mean disease score of 1.0 representing 72.2% disease reduction.

The positive control had a mean disease score of 3.6 and was significantly different from the rest. The maximum disease score of 5 was recorded in the untreated control compared to 3 and 2 for the crude bacterial extracts and chemical fungicide respectively. A minimum disease score of 0 was recorded for the chemical fungicide while a minimum of 1 and 2 was recorded for bacterial extracts and the untreated control respectively. These are shown in Plate 4.9. Except for the non-inoculated plants (E_1 and E_2), each pair of leaves in the lower row represent a maximum and a minimum score for each treatment.

Table 4.6: Mean disease score and percent reduction of infection of net blotch on the leaf of Nguzo Barley variety

Treatment	Mean disease score ±SD	% Reduction of disease score
D. teres alone (+ve control)	3.6±0.7[a]	–
D. teres + Orius® 250 EW	1.0±0.8[b]	72.2
D. teres + Double Conc culture filtrate	1.8±0.8[c]	50
D. teres + Normal Conc culture filtrate	1.8±1.1[c]	50
***P* value**		**0.00428**

Means followed by the same letter in each column are not significantly different at *P* = 0.05. The means were obtained from five replicates. From the table it can be seen that Orius® was the most superior in controlling net-blotch of barley *in vivo*.

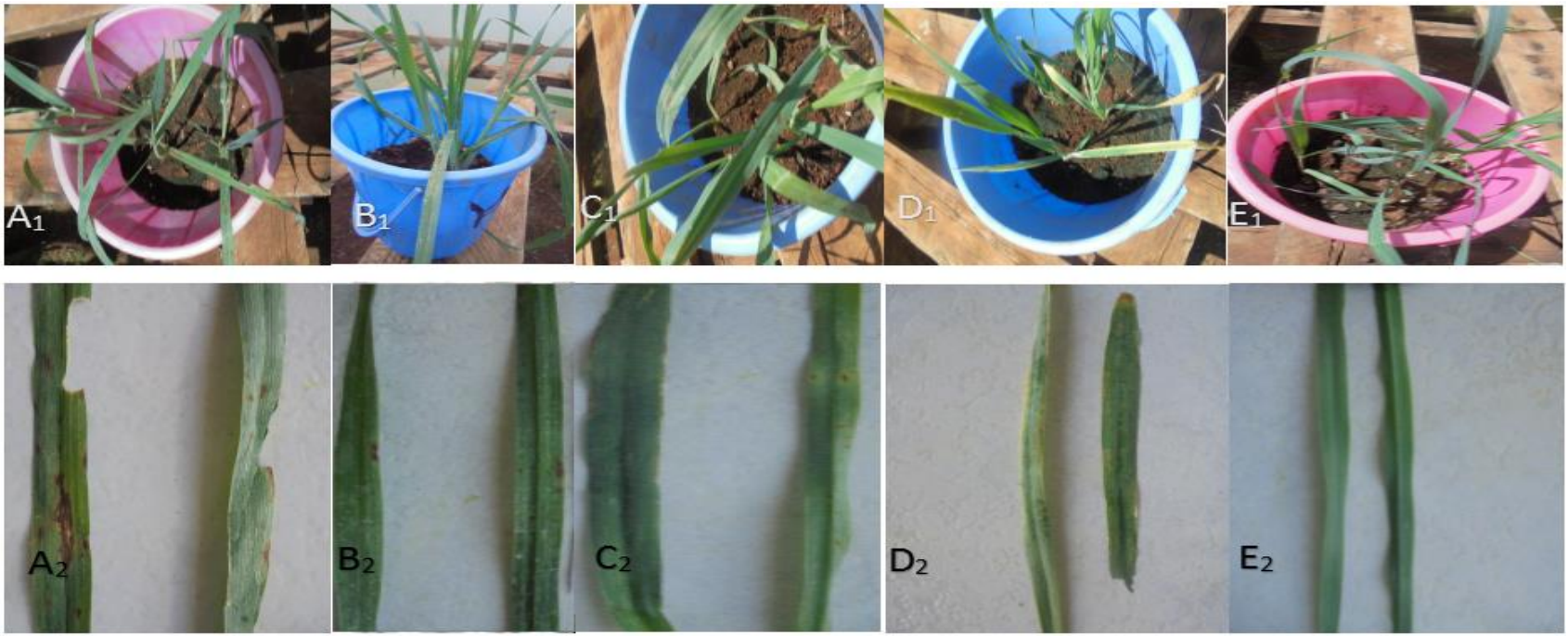

Plate 4.9: Different reactions by barley to *D teres* fsp *teres*. A_1; Plants inoculated with *D. teres* f. sp *teres* alone, B_1; Plants inoculated with *D. teres* f. sp *teres* and the normal concentration of the crude bacterial extract. C_1; Plants inoculated with *D teres* fsp *teres* and double concentration of the crude bacterial extract. D_1; plant inoculated with *D. teres* f. sp *teres* and Orius® 250 EW (a chemical fungicide). E_1; Non-inoculated plants. The images in the lower row provides detailed evidence of the lesions (or lack of it) of the corresponding plants above them.

4.8 Enhancement of the production of high quality and quantity of antifungal agent(s) by *Paenibacillus polymyxa* KaI245

Organic compounds, viz: 1% acetone and 1% methanol were added to the antifungal agent production growing medium (PDB) which had been inoculated with the antagonistic *Paenibacillus polymyxa* KaI245. The cell-free supernatant was then tested against *D. teres*, whose results indicated that acetone had an enhancing effect in production of antifungal agents by the bacterium. The KaI245 inoculated-acetone-cell-free-supernatant impeded mycelial growth by 27.9% while the KaI245 inoculated (standard) control supernatant obtained without the addition of any organic compound save the bacterium alone impeded the mycelial growth by 19.7%. Despite this numerical difference, means from the two treatments were not significantly different at $P = 0.05$ (Table 4.7). Further, the "acetone-alone-control" supernatant did inhibit the fungal mycelium by 13.6%. This inhibition differed significantly from the "uninoculated-control". The "methanol-alone-control" supernatant on the other hand stifled the bacterium's ability to produce antifungal agents that would impede the growth of the fungi. Fungal growth % inhibition was reduced to 10.3% down from 19.7% achieved by the KaI245-inoculated control supernatant. Further, the mean growth rate of the fungal colonies growing in 'methanol' cell free supernatant amended media did not significantly differ from the ones growing on plain media.

Table 4.7: Effects of acetone and methanol on the ability of *P. polymyxa* KaI245 to impede the growth of *Drechslera teres* f sp. *teres*

Day	KaI245+Ace	KaI245+Met	KaI245 only	PDB+Ace	PDB+Met	Plain PDA
1.	5.67±1.5	7.6±1.1	7.6±0.6	8.3±2.1	7.6±2.3	8.6±2.1
2.	8.7±1.2	10.3±0.6	8.6±1.2	9.3±0.6	9.6±0.6	9.3±2.9
3.	7±2	7.6±1.2	7.6±0.6	8.3±0.6	7.3±0.6	9.6±1.5
4.	5.3±0.6	9±1	7.6±0.6	7.3±0.6	8.3±2.1	9.6±0.6
5.	8±1	7±0	8±1	8.3±1.2	9±1	10.3±1.2
6.	6.7±1.5	9.7±0.6	6.3±0.6	8±0	10.3±0.6	9.6±0.6
Mean	6.89^{a}	8.57^{bcd}	7.67^{ab}	8.25^{bc}	8.72^{cd}	9.55^{d}
% inh	27.9	10.3	19.7	13.6	8.7	--

Change in mycelial diameter in PDA amended with various inhibitory agents (change in diameter was obtained by subtracting consecutive recorded diameters). PDB; Potato Dextrose Broth, Ace; acetone, Met; methanol. PDA; Potato Dextrose Agar. Percent inhibition was calculated relative to the diameter obtained from plain PDA. Means followed by the same letters did not differ significantly at *P* = 0.05.

4.9 GC-MS compositional analysis of altered volatile compounds production by *Paenibacillus polymyxa* KaI245 due to addition of organic compounds

The GC-MS chromatograms in figures 4.2 and 4.3 shows variation in the production of volatile organic compounds due to exposure of the antifungal producing bacterium to methanol and acetone respectively. In cultures where methanol was added, there was a reduction in the number of volatiles metabolites to 35 down from 50 recorded with untreated cultures (Appendices X and XI). The most abundant metabolites (cultures amended with 1% methanol) were octadecanoic acid (RT ≈ 24.91), hexadecanoic acid (RT ≈ 23.10) and butanediol<2,3> (RT ≈ 5.42) with 16.93%, 13.36% and 10.84% relative abundance respectively. Despite the reduction in the total number of metabolites produced there were 17 new metabolites (highlighted in Appendix XI) when methanol was added. Of the detected metabolites 48.57% (17/35) had relative abundance of less than 1% with alpha-Pinene having the lowest abundance of 0.03%.

In cultures amended with 1% acetone, a total of 21 metabolites were detected (Appendix XII). The most abundant metabolites were ethyl oleate (RT ≈ 24.90), hexadecanoic acid (RT ≈ 22.98) and octadec-9-enoic acid (RT ≈ 24.64) with 13.15%, 11.27% and 10.76% relative abundance respectively. Of the 21 metabolites detected, 9 of them were new (highlighted in Appendix XII). Only 2 of the 21 metabolites had relative abundances of less than 1%; both of which are phenol, 2,5-bis(1,1 –dimethyl)- at 0.60% (RT ≈ 18.15) and formic acid, dodecyl ester at 0.53% (RT ≈ 19.08). Octadecane (C18) (RT ≈ 21.33) having a relative abundance of 2.24% was unique to acetone exposed medium.

Some metabolites were detected in at least two categories while others were detected in all the three. They differed majorly in respective relative abundance. The untreated had 6 metabolites with the highest abundance methane-treated had 5 while acetone-treated had 8. This information is summarized in table 4.8.

Table 4.8. Volatile metabolites identified by GC-MS in the dichloromethane suspended ethyl acetate extracts of *P. polymyxa* KaI245 cultured without and in the presence of acetone and methanol

Chemical name	Abundance of compounds identified in at least two of the extracts (%)		
	Untreated.	**Acetone**	**Methanol**
Butanediol<2,3>	21.20	7.34	10.84
Ethylbenzene	3.51	1.52	0.91
p-Xylene	2.46	----	0.28
Dodecane	0.34	----	0.26
Tetradecane (C14)	0.86	1.11	0.75
Pentadecane	0.96	----	0.95
Hexadecane<1>	0.8	1.8	0.77
Hexadecane (16)	1.64	2.37	1.09
Heptadecane (C17)	0.89	1.16	1.31
Isopropyl-tetradecanoate	1.85	3.06	1.74
*****	0.95	1.12	1.15
Nonadecane (C19)	1.59	2.8	----
Hexadecanoic acid	7.66	11.27	13.36
Ethyl-hexanoate	4.53	2.8	----
Isopropyl-hexadecanoate	3.74	7.8	5.27
Oleic acid	----	6.57	6.44
Hexadecanol	3.57	----	0.25
Octadecanoic acid	6.03	----	16.93
Eicosene<1>	3.54	7.09	4.64
28-Nor-17,beta(H)-Hopane	2.32	----	2.68

The chemicals have been arranged in the order of their retention times (RT). Chemicals were identified through comparison of their spectra with those of known components of the NIST library; Relative abundance of the extract components was automatically generated from electronic integration of individual pictures of the chromatogram relative to the total area.

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***** 7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin.**

The underlined values represent the highest for the corresponding metabolite.

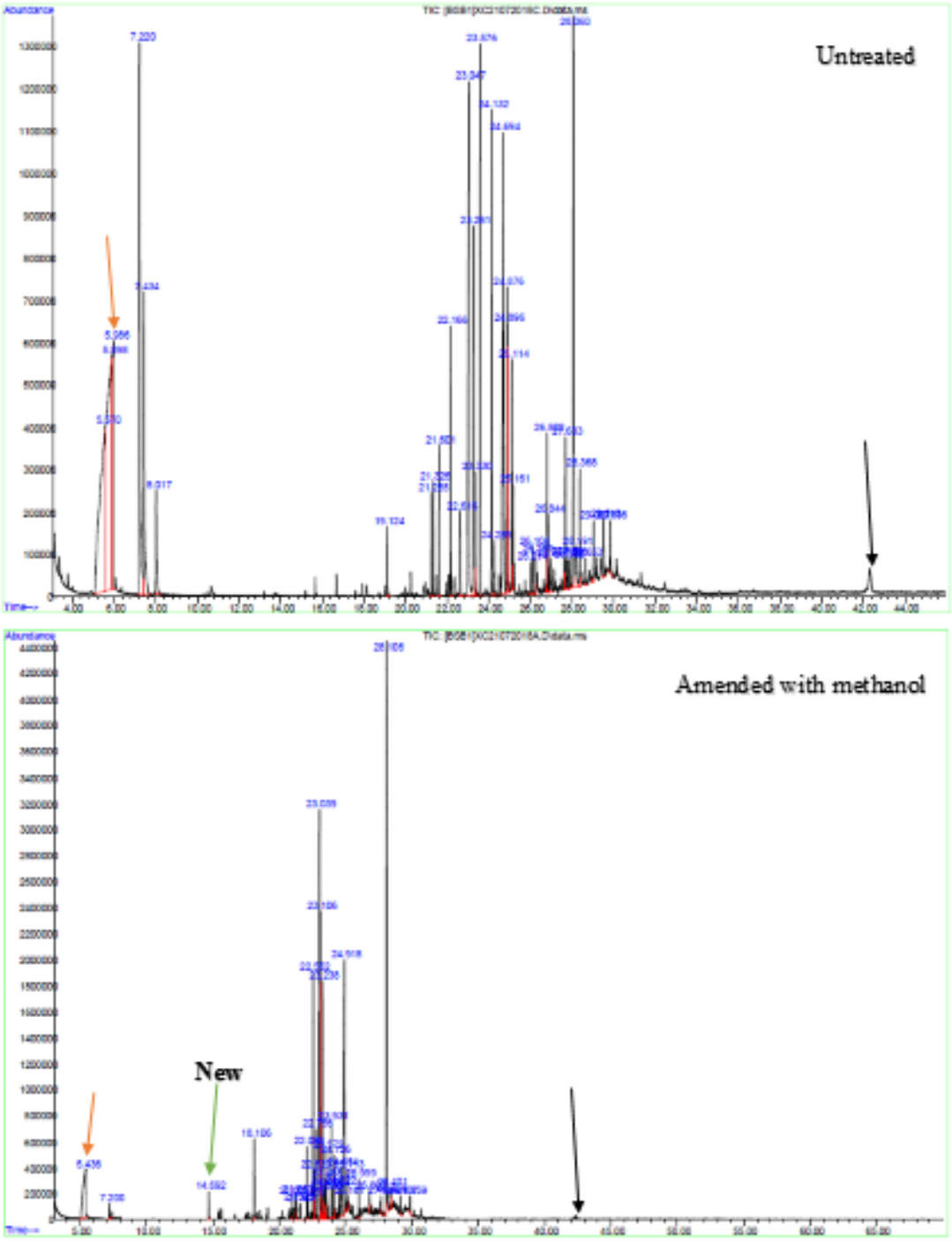

Figure 4.2. GC-MS chromatograms of ethyl acetate extracts from untreated and methanol-amended *P. polymyxa* KaI245 cultures. The chromatograms are at different scales. Points of similarities and differences can be noted. The untreated sample; 50 metabolites. Sample amended with methanol; 35 metabolites with 17 new ones.

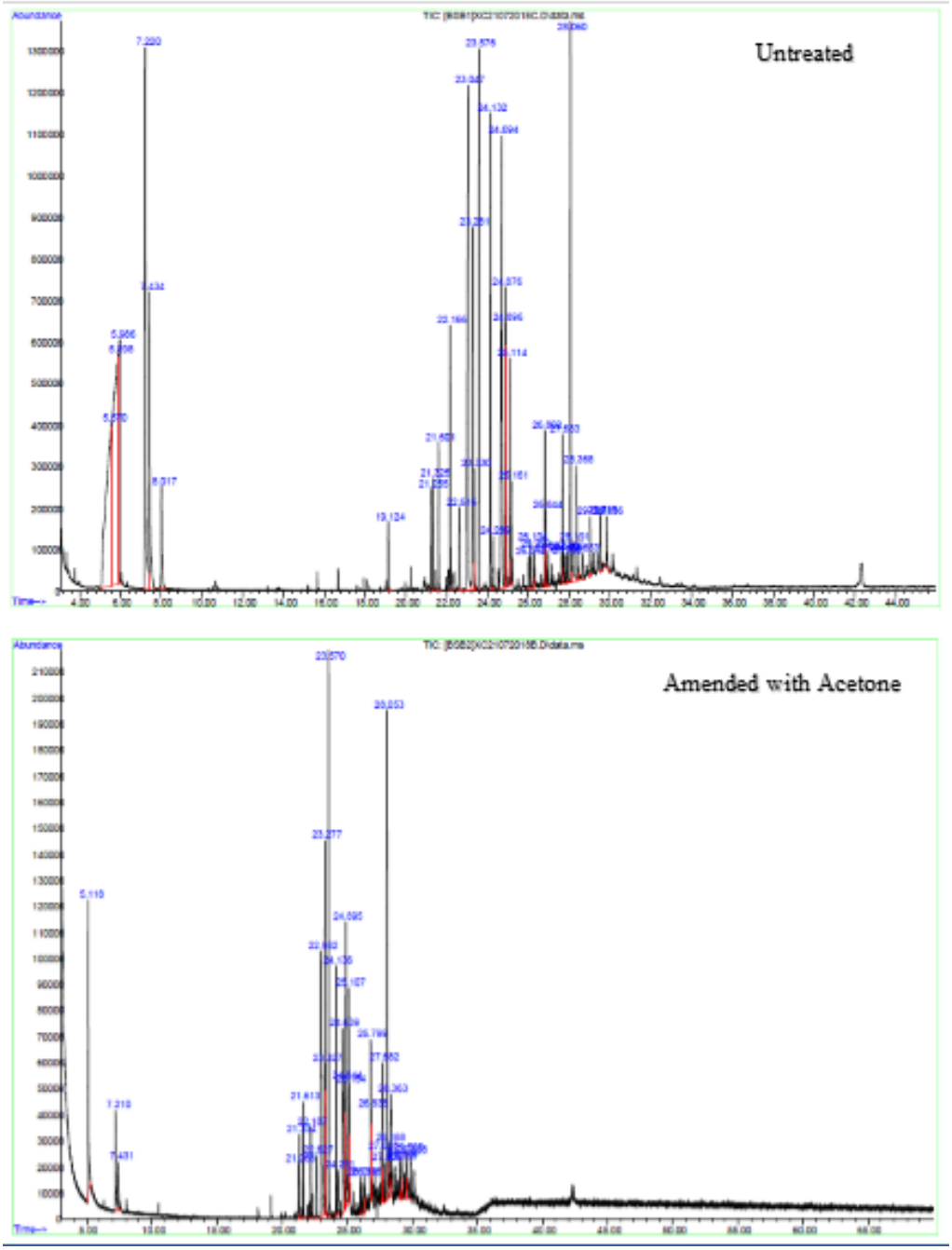

Figure 4.3. GC-MS chromatograms of ethyl acetate extracts from untreated and acetone-amended *P. polymyxa* KaI245 cultures. The chromatograms are at different time scales. Points of similarities and differences can be noted. Untreated sample: 50 metabolites. Sample treated with acetone: 21 metabolites with 9 new ones.

CHAPTER FIVE

DISCUSSION

5.1 Isolation and identification of barley fungal pathogens

D. teres f. sp *teres* was isolated from the leaves presenting crisscrossed necrotic lesions, characteristic for net-form net blotch. The isolates of *D. teres* showed much variation in colony characteristics. This variation was independent of the locality of the fungus as variation occurred both within and between the two localities (Njoro and Chepkoilel) which were the sources of the diseased plant materials. The color of the colonies ranged from dark to grey with some colonies having cream concentric rings. This phenomenon was also observed and described in greater detail by Owino *et al*. (2013) who also showed that the conidia from all their isolates, morphological groups notwithstanding, had three septa (4 segments) while some conidia displayed conspicuous scars at their tips. The latter observation can also be reported from the current study. However, conidia with three through to six septa were observed. This result has therefore enriched our knowledge of morphological diversity of the net blotch fungi—*D. teres*. This is particularly significant because the isolates used in the above cited study were also obtained from the same localities as the ones used in this study. The conidia with those different septations were obtained from the same colony. Liu *et al*. (2011) did report that the number of septa in conidia ranges from three to five. In some cases, the colony morphology differed among isolates sub-cultured from the same parent colony. This is a case that was also reported by (Louw *et al.*, 1996).

The brown border present in the scald disease symptoms in barley, represents the advancing front of the *R. commune* fungus. The cream patch is the dried part due to

depletion of nutrients by the fungus.' The leaves were obtained from Chepkoilel, in Uasin Gishu County, during a cool and wet season. Similar lesions have been described extensively by several authors with the similar symptoms shown by (Avrova & Knogge, 2012). The pink greyish colonies obtained in this study were isolated from the barley leaf showing those symptoms. Salamati & Tronsmo (1997) also described these colonies as pink although they conceded that variation may exist. A very slow growth rate of the isolate characteristic to *R. commune* was noted. Although the spores have been defined as beak shaped, appearance of short and bulbous spores is also a possibility (Cromey & Mulholland, 1987) as was the case in this study.

Barley stem rust symptoms characterized by brown pustules caused by *Puccinia graminis* as shown in this study have been extensively described by several authors. Similar results were shown by Dean *et al*. (2012). Microscopic observation revealed orange and circular to ellipsoidal urediniospores as well as teliospores born on pedicels which were bicelled, clavate to oblong and yellowish brown. Similar microscopic examinations were found by Leonard & Szabo, (2005). Further, similar microscopic observation of teliospores were reported by Dheepa *et al.* (2016). In that case however, the pathogen in question was *Puccinia horiana*, the causative agent of chrysanthemum white rust. There exist extensive morphological similarities among different species of genus *Puccinia*.

5.2 Antagonistic activity of *Paenibacillus polymyxa* KaI245 against barley fungal pathogens

5.2.1 Dual Assay

In the present investigation, *Paenibacillus polymyxa* KaI245 strongly impeded growth of *Drechslera teres* f sp. *teres* as evidenced by prominent zones of antibiosis due to diffusible

metabolites. The live bacterial cells inhibited the growth of the fungus by approximately 47%. It can therefore be deduced that the bacteria acts through production of substances that may be toxic to the fungi. Microbes acting through such mechanism of action have a wide action spectrum and is more effective than any other mode of action (Leelasuphakul *et al.*, 2008). This is also true for this bacterium since it was also shown to inhibit growth of *Colletotrichum sublenolium*, a pathogen of sorghum (Makumba, 2016). Further, it was shown by Lee *et al*. (2013) that *Paenibacillus polymyxa* strain E681 produces fusaricidin with capability to control *Phytophthora capsici* the causative agent of phytophthora blight in red-pepper. Ligon *et al*. (2000) showed that the bacterium *Pseudomonas fluorescens* BL915 produces pyrrolnitrin, hexyl-5-propylresorcinol and HCN and enzymes such as chitinase and gelatinase. Like the bacteria used in the current research, *P. fluorescens* BL915 produced zones of antibiosis against *Rhizoctonia solani*.

The same results could however not be replicated with *R. commune.* The apparent zones of antibiosis can only be attributed to the physical presence of the bacterium that only prevents the growth of the fungal mycelium beyond the point of bacterial inoculation. Such a phenomenon can happen with any bacteria regardless of whether or not it produces antifungal metabolites. This variation in response by different fungi to a bacterial inhibitor has been shown to be common. Ashwini and Srividya (2014) showed that *Bacillus subtilis* inhibited different fungal pathogens to varying degrees even after it was shown to produce different mycolytic enzymes. *Paenibacillus polymyxa* strain A21 for example, has been shown to produce β-1, 3-1, 4-glucanase (Li *et al*., 2015). Despite these evidences of enzyme production, *Paenibacillus polymyxa* strain GBR1 presented considerable variation

in its antifungal activity in dual culture; it was strongly antagonistic to *Colletotrichum gloeosporioides, C. acutatum* and *C. destructans* while being moderately antagonistic to *Alternaria mari* and *Botrytis cinerea* (Kim *et al.*, 2016). This is an indication that fungi have different tolerance levels to bacterial antifungal systems.

As is suggested in the preceding section, perhaps different fungi stimulate the production of antifungal metabolites to varying degrees. Suresh and Nelson (2016) reported that antibiotic production by bacteria varies depending on the competing organism. *Rhynchosporium commune* used in this experiment may not have stimulated the bacterium to release adequate antifungal metabolites against it.

5.2.2 Bioactivity of *Paenibacillus polymyxa* KaI245 Volatile Organic Compounds

Despite the bacterial cells producing strong antifungal diffusible metabolites in dual assay, there was no effect of bacterial volatile metabolites on the growth of *D. teres*. The volatiles strongly inhibited *R. commune* colonies though the bacterium did not show any antagonism in dual plate assay. The rate of growth of *R. commune* was inhibited by approximately 53%. It has been shown that susceptibility to bacterial volatile compounds varies greatly among different targets (Hunziker *et al*., 2015). They further showed that the fast growing *Rhizoctonia solani* was little affected even by a strongly inhibiting strain of *Pseudomonas* strain SO4. Growth rate may therefore be suggested as one of the factors affecting the sensitivity of a fungus to volatile compounds. This is reasonably so since between the antagonist and the target there is no point of contact. In the current study, *D. teres* had a significantly higher growth rate as compared to *R. commune*. The greater susceptibility of

R. commune to the bacterial volatiles serves to reinforce the line of thought that a fungus with a slower growth is more sensitive to gaseous substances.

Moreover, an interesting observation is that the same bacterium that was inhibitory to *D. teres* via diffusible metabolites was not inhibitory via volatile emission and the events were vice versa for *R. commune*. We therefore speculate that the most effective bacteria in dual assay may not necessarily be the most efficient volatiles producer and vice versa. The observation is critical since it informs on future such studies. Many scientists, as was done by Vinodkumar *et al*. (2017), are always tempted to choose only the most effective antagonists in dual assay for volatile production studies. This study affirms that the degree of effectiveness of dual plate assay in antagonism should not always be the basis upon which a bacterium is assessed for volatile mediated control.

Volatiles mediated control of *P. graminis* teliospores was also assessed. The number of germinating teliospores that were not exposed to the bacterial volatiles were more by 39% compared to the telia exposed to bacterial volatiles. Analysis by chi-square suggested a strong relationship between volatile emissions and inhibition of teliospore germination. The number of germinating teliospores in the treated group was majorly found in the lower rank with fewer numbers. Here again the volatiles seem to be inhibiting the germination of teliospores while the cell free supernatant doesn't. Most experiments with *P. graminis* are done *in vivo* due to the difficulty to grow the fungus *in vitro*. However, the results here mimics that obtained with *R. commune*.

In all the antagonistic studies done in this study, a general trend was observed. *D. teres* which is inhibited by diffusible metabolites in the dual culture assay is not inhibited by volatiles and vice versa. For pathogens that are closely related as is the case with *D. teres* and *R. commune* (both are ascomycetes), it is quite intriguing. The range of activity of most chemical fungicides is normally among closely related pathogens. The mode of action of such a bacterium is most likely the production of mycolytic enzymes like cellulases. Closely related fungi should be having about the same structure and hence expected to be equally vulnerable to stresses like antagonism. As shown by Zhan *et al*. (2008) and Stefansson *et al*. (2014), what was observed in this study could be resistance by *R. commune*. However, the antagonist in question is a sorghum rhizobacterium. That is to say, between the two organisms (*R. commune* and *P. polymyxa* KaI245 the sorghum rhizobacterium), there may be no sufficient co-evolution to back up that argument. Alternatively, it could be that with *R. commune*, the bacterium produces insufficient antimicrobial metabolites. Most researchers suggest that the latter is more likely.

A clear-cut difference between net-form-blotch-pathogen and the other two is that it is a necrotroph while *R. commune* and *P. graminis* fall in hemibiotroph and obligate biotroph categories respectively. Necrotrophs are known to be robust in the production of phytotoxic metabolites. The net-blotch pathogen has been shown to produce phytotoxic proteinaceous metabolites in culture (Sarpeleh *et al*., 2009). Perhaps this could trigger the bacterium to produce more antifungal compounds and hence appear more antagonistic towards *D. teres*. This judgment can also be backed further since, despite optimizing the culture conditions, the bacterial culture filtrate supernatant consistently failed to match the inhibition achieved

in dual culture. The supernatant was obtained from a ‘bacterium-only’ broth. We speculate that the bacterium failed to produce enough antimicrobials due to lack of an effective stimulant. Indeed, as highlighted in Appendix III, co-inoculation of a bacterial antagonist with another organism enhances the production of secondary metabolites (Combès *et al.*, 2012; Marmann, *et al.*, 2014).

Because these are biological systems, results reveal more than just the apparent need for competition for production of more antimicrobials. Synergism has also been shown as a factor in altering the antifungal activity of a bacterium. Li *et al*. (2015) used *Paenibacillus polymyxa* A21 to improve the antagonistic activity of *Streptomyces lydicus* A01 against *Botrytis cinerea*, the causal agent of gray mold disease in berries.

The phytopathogens, depending on their modes of nutrition (whether necrotroph, biotroth or hemibiotroph) produce different metabolites and probably differently alter the production of antimicrobials of a given antagonist. This could inform on future developments that the range of activity of a given bio-control agent could be biased against a group of phytopathogens based on their mode of nutrition. The bacterium used in this study, *Paenibacillus polymyxa,* was shown to be effective against *Colletotrichum sublenolium,* a necrotroph, by (Makumba *et al*., 2016). Further, it has been shown that *P. polymyxa* E681 controls phytophthora blight caused by *Phytophthora capsici* a necrotrophic oomycete (Lee *et al*., 2013). In the current study, the result was only exhibited on another necrotroph that is *D. teres*.

5.2.3 Cell-free supernatant

The cell-free culture filtrate supernatant of *Paenibacillus polymyxa* KaI245 was not equally effective as the live cells. Amendment of fungal growth medium with 10% cell-free supernatant retarded the growth of *D. teres* by about 24%. Inhibition of about 47% was achieved while using live cells. Such disparity has been shown to occur in other bacterial antifungal systems (Darna *et al*., 2016). It has been hypothesized that for some bacterial antifungal systems, defence mechanisms are activated when the microoganisms are in close proximity (Lacroix, 2011). This experiment could lend some credence to that line of thought.

The production of antifungal metabolites by *P. fluorescens* BL915 mentioned in sec 5.2.1 above, was found to be regulated by a two component regulatory system activated upon the receipt of an environmental or internal physiological signal (Hill *et al*., 1994). In this study, the bacterium was first cultured in broth. *D. teres*, that was co-cultured with the bacterium in the dual assay was missing. We can therefore suggest that *D. teres* stimulates the bacterium to produce more antifungal agents translating to greater inhibition in dual assay.

It can also be argued that, amending the media is a one-off application of the antifungal agents while in dual culture there is a continous constant release of the same by the bacterium. This argument is borrowed from Weisskopf, (2013) where the challenges of antifungal volatiles are addresed. There are other experiments however, where both the live cells and the supernatant (10%) have not shown much discrepancy between their antifungal

activities (Petatán-Sagahón *et al*., 2011; Darna *et al*., 2016). These facts make it difficult to further the latter argument.

The effect of cell-free supernatant on germination of teliospores was also checked. The cell-free supernatant was used to moisten the filter papers upon which barley stalks containing black pustules of teliospores were placed and incubated. In all the glass slides counted (250), the number of germinating teliospores in samples treated with bacterial supernatant was lower by 20%. This was however a negligible reduction as was also shown by Dheepa *et al*. (2016). Teliospore germination of a related pathogen, *Puccinia horiana*, was inhibited by liquid-based formulations of *Bacillus* spp. Liquid based formulation of KaI245 used in this study did not show significant antifungal activity against *P. graminis*. The weak antifungal activity displayed here may also be accounted for as has been explained in the case of *D. teres*. The effect of live cells in this case could not be inferred to due to the difficulty of cultivating the fungus *in vitro*. Due to the failure of the live bacterial cells to control *R. commune in vitro*, this fungus was not selected for this experiment.

5.3 Greenhouse studies

In the present study, efficiency of *P. polymyxa* KaI245 cell-free culture-filtrate supernatant in suppressing net blotch infection on barley plants in the greenhouse was found to be significant compared to the positive control (infected but not treated). This effectiveness is an indication that metabolically active cells may not be needed for *in vivo* antagonism of *D. teres*. Such cases can be advantageous as the shortcomings of using live cells may be by-passed (Darna *et al*., 2016). It was not however as superior to tebuconazole (20% w/w),

a standard fungicide that was used in this experiment. Different concentrations of the bio-fungicide can be further explored.

5.4 GC-MS compositional analysis of *Paenibacillus polymyxa* KaI245 culture filtrate

The volatile organic compounds produced by *P. polymyxa* KaI245 were analysed by GC-MS and 50 compounds were detected. Majority of the compounds included alkanes, alkenes, esters, fatty acids, and alcohols. The most abundant volatile metabolite was found to be 2,3-butanediol at 21.20%. These results corroborate many studies where *P. polymyxa* has been shown to be the most promising non-pathogenic producer of 2,3-butanediol (Ma *et al*., 2011). It can be produced industrially by hydrolysis of 2,3-butene oxide (Gräfje *et al*., 2000). In *Paenibacillus polymyxa*, it is produced by a process known as butanediol fermentation (De Mas *et al.*, 1988). The key enzyme involved in its production was found to be 2,3-butanediol dehydrogenase belonging to the medium chain dehydrogenase/reductase superfamily (Yu *et al.*, 2011).

It has been well established that 2,3-butanediol is a plant growth enhancement agent (Amavizca *et al*., 2017) as well as an inducer of increased stress resistance in plants (Bitas *et al*., 2013). In-deed *Paenibacillus polymyxa* GBRI was shown to stimulate the growth of tobacco seedlings (Kim *et al*., 2016). This is a characteristic also shared by acetoin which has been shown to induce plant growth promotion, particularly leaf surface area, and systemic resistance (Saïd *et al*., 2005). Relative abundance of acetoin was however very low at 0.05%. Hexadecanoic acid (also known as palmitic acid) has been touted as the most common saturated fatty acids found in plants, animals and microorganisms (Hussain *et al*., 2017).

Phenolic compounds have been shown to be toxic to cells (Breinig *et al.,* 2000) including some plants at different stages of growth especially during seed germination (Massalha *et al*., 2017). It should however be noted that the phenolic compound detected from this bacterium (RT ≈ 18.11) had a very low relative abundance (0.47%). This phenomenon could explain the observation by Kim *et al.* (2016) where tobacco seedlings further away from *Paenibacillus polymyxa* in a petri dish had vigourous growth while those growing near the point of inoculation were suppresed.

In an experiment by Yuan *et al*. (2012) all benzene componds produced by the bacterium *Bacillus amyloliquefaciens* NJN-6 showed antifungal activity towards *Fusarium oxysporum. D. teres* used in this experiment is of the same feeding characteristics as *F. oxysporum* as well as taxonomic classification. Benzene compounds produced by *P. polymyxa* KaI245 in this study is hence expected to exert the same antifungal activity to *D. teres.* These compounds (benzene containing compounds produced by *P. polymyxa* kaI245) were, ethylbenzene (RT ≈ 7.22), benzene, 1,4-dichloro- (RT ≈ 10.66), and benzyl benzoate (RT ≈ 21.10). However due to low benzene content, toluene, ethyl benzene, propylbenzene, and isopropyl benzene are less effective in inhibiting the growth of fungi (Yuan *et al*., 2012).

Most of the alcohols produced by this bacterium must be derived from fatty acid synthesis intermediates (Schulz & Dickschat, 2007). Alcohols have been shown to be weakly active against a basidiomycete wood decaying fungi (Humphris *et al.,* 2001). This explains the

antifungal activity of the bacterium's volatiles towards *P. graminis* in the petri dish experiment.

5.5 Enhancement of *Paenibacillus polymyxa* KaI245 for the production of high quality and quantity of antifungal agents

The bacterium was cultured in potato dextrose broth separately supplemented with 1 % methanol and 1 % acetone. The quantity of metabolites produced (in terms of different metabolites produced), reduced when the growth medium was treated with acetone and methanol. The most notable was the reduction of 2,3-butanediol in both cases. There were however new secondary metabolites produced by the bacterium. It has been shown that culturing microbes with small organic molecules stimulates the silent genes to produce new secondary metabolites (Pettit, 2011).

However, this stimulation of new secondary products must find meaning within certain contexts. In this case, any alteration of the secondary metabolites of the bacterial isolate KaI245 will be meaningful if it translates to more inhibition of the test fungi. Greater inhibition was achieved when the bacterium medium was supplemented with acetone. Toghueo *et al*. (2016) showed that addition of 1 % acetone to culture of *Aspergillus niger* lead to the production of a new metabolite. There were 9 new metabolites in this case. Addition of methanol on the other hand negated the activity of the bacterial extracts despite presence of 11 new metabolites. The alteration of the production of the volatiles by methanol does not prove beneficial in enhancing antimicrobial activities of the bacterium. Since acetone had a positive impact on the antagonistic capabilities of the bacterium, special emphasis has been directed to some of the metabolites produced. 2,3-Butanediol (RT = 5.12) was also produced in all the different treatments. This is consistent with *P.*

polymyxa as it has been referred to as an unmatched producer of 2,3-butanediol (Jiang *et al*., 2015). In agriculture the compound has been shown to enhance the growth of plants (Amavizca *et al*., 2017)**,** induces increased stress resistance in crops (Bitas *et al.*, 2013) and regulates auxin homeostasis and cell expansion in Arabidopsis **(**Zhang *et al*., 2007). Ethyl benzene (RT = 1.52) has been shown to be weakly antagonistic to *Fusarium oxysporum* (Yuan *et al.*, 2012). Hexadecenoic acid (RT = 22.98) is known for its active antibacterial activity (Yff *et al*., 2002) indicating that the inhibitory activity of *Paenibacillus polymyxa* extends to some bacteria. Indeed, *P. polymyxa* strain CR1 is potent against *Pseudomonas syringae* DC300, *Xanthomonas campestris* 93-1 and *Bacillus cereus* (Weselowski *et al.*, 2016). Hexadecane (RT = 19.15) has antifungal activities against *Alternaria brassicae* and *A. cinerea* (Geng *et al*., 2016) and is 100% effective against *Fusarium oxysporum* f. sp *niveum* (Raza *et al.*, 2015). These four metabolites were also produced by the bacterium cultured in plain PDB. They all endow the bacterium with the ability to improve plant growth via various mechanisms. The use such a bacterium as a bio-fungicide would make more economical sense given that it also enhances the plant growth (Vanitha & Ramjegathesh, 2014). This is the reason the bacterium has received much attention over the years (Weselowski *et al.*, 2016).

The following are some of the metabolites unique to the bacterium cultured in acetone amended medium. Phenol (2,5-bis(1,1-dimethylethyl) (RT = 18.15) is known to inhibit cellular activities and also has negative effects on plant seed germination (Breinig *et al*., 2000). This implies that the use of this metabolite should only be beyond the stage of seed germination. Oleic acid (RT = 24.29) has significant reduction effect in mycelial growth of *Pythium ultimatum*, an oomycete (Walters *et al*., 2013). This indicates that addition of

organic solvents may help to broaden the antimicrobial activity of the bacterium. Medically, it can be used as a delivery material for antifungal agents particularly for cutaneous diseases (Verma *et al*., 2014). Hydrazides of octadic-9-enoic acid (RT = 24.64) are used as cheap starting material in the synthesis of biologically active 5-(alkenyl)-2-amino-1,3,4-oxadiazole with known antibacterial activity (Banday, 2010). The production of these two metabolites (oleic acid and 5-(alkenyl)-2-amino-1,3,4-oxidiazole) makes the significance of the bacterium to industries extend beyond the traditional 2,3-butanediol. This is imperative from industrial point of view. 1-Nonadecane (26.80), has antimicrobial activity and cytotoxic as well (Arora & Kumar, 2018).

The fact that 2,3-butanediol has been shown to enhance plant growth further merits the exploitation of this biocontrol agent from *P. polymyxa.* KaI245. Its use as a fungicide would make more economical sense given that it also enhances plant's growth (Vanitha & Ramjegathesh, 2014). Further, it has been shown that when *P. polymyxa* is cultured under anaerobic conditions, it has the ability to produce large amounts of R-isomer of 2,3-butanediol (Ma *et al*., 2011). This is very important from the industrial perspective.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. Variation in the septation of conidia of *Dreschlera teres* has been shown to be richer than earlier thought. Conidial septation varied from three to six among the *D. teres* isolates identified and used in this study.
2. *In vitro* antagonism studies of diffusible metabolites via dual culture technique confirmed antifungal activity of the sorghum rhizobacterium against *D. teres*. The average percentage inhibition was 47.3%. It was also shown that the same was not effective against *R. commune*.
3. Amendment of the fungal growth medium with 10% bacterial supernatant resulted in a reduction of *D. teres* growth rate by about 24%. This reduction was however not found to be significant. However, a seven-day old bacterial supernatant showed only negligible inhibition of germination of *P. graminis* teliospores.
4. The bacterial volatiles did not show any effect on the growth of *D. teres* but showed strong retardation on the growth of *R. commune* and germination of *P. graminis* teliospores.
5. Green house experiments showed that the crude *Paenibacillus polymyxa* KaI245 cell free filtrate reduced the mean disease score of net blotch symptoms by 50%. This value was achieved both by the double and normal concentration of the bacterial supernatant. This was however no match to tebuconazole (20%w/w) [Orius®250 EW], a synthetic fungicide, which recorded a reduction in disease score of 72.2%. However, unlike the synthetic fungicide, the bacterium has shown broader spectrum of action and can be used on other cereals as well.

6. Addition of 1% acetone to potato dextrose broth inoculated with the bacterium KaI245 enhanced the performance of the supernatant towards *D. teres* while methanol reduced the antifungal activity of the supernatant.
7. There was total of 50 different volatile organic compounds produced by the *Paenibacillus polymyxa* KaI245. On addition of 1% acetone to the growth medium the number reduced to 22 but with 9 new metabolites while with 1% methanol it reduced to 35 with 11 new metabolites.

6.2 Recommendations

1. Further research should be carried out to shed some light on the reasons why most antagonistic bacteria do not exert their antimicrobial activity uniformly against related pathogens. This information would be crucial in the development of better ways to enhance the antimicrobial activity of any antagonistic bacteria.
2. In evaluating a microbe's inhibitory potential, production of volatile organic compounds should be assessed independently from the production of diffusible metabolites. Microbial ability or inability to show antimicrobial activity in dual culture cannot be the basis upon which production of antagonistic volatiles is predicted. It was shown in this study that this aspect could vary greatly with respect to the test phytopathogen.
3. Greater focus should coalesce around use of volatiles in controlling phytopathogens. More research should be done to overcome the challenges mentioned in section 2.5.3.2.
4. Additional characterization of non-volatile compounds using HPLC is needed in-order to gain more insights on the antimicrobial nature of *Paenibacillus polymyxa* KaI245.

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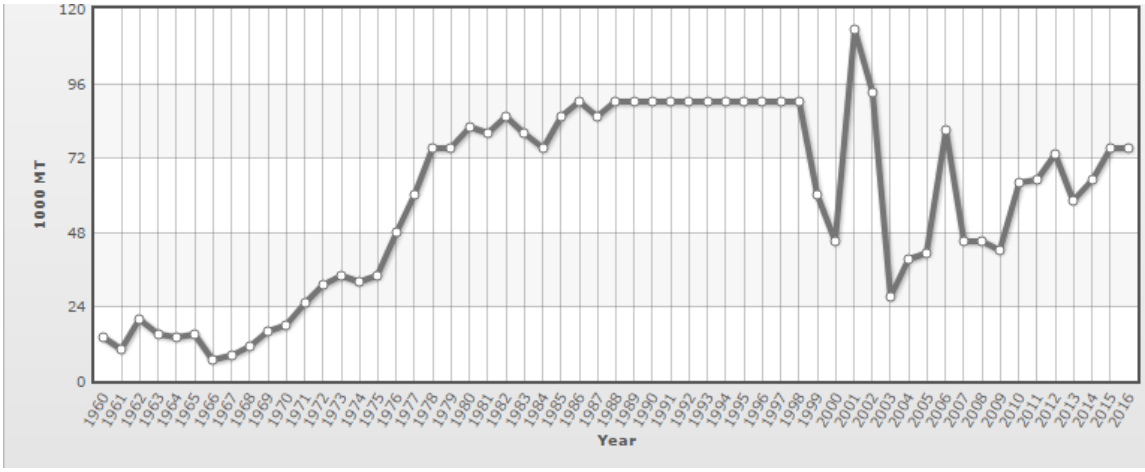
APPENDICES

Appendix I: **Barley production trend from 1960 to 2016 as provided by(USDA, 2016). Source**; *https://www.indexmundi.com/agriculture/?country=ke&commodity=barley&graph=production*



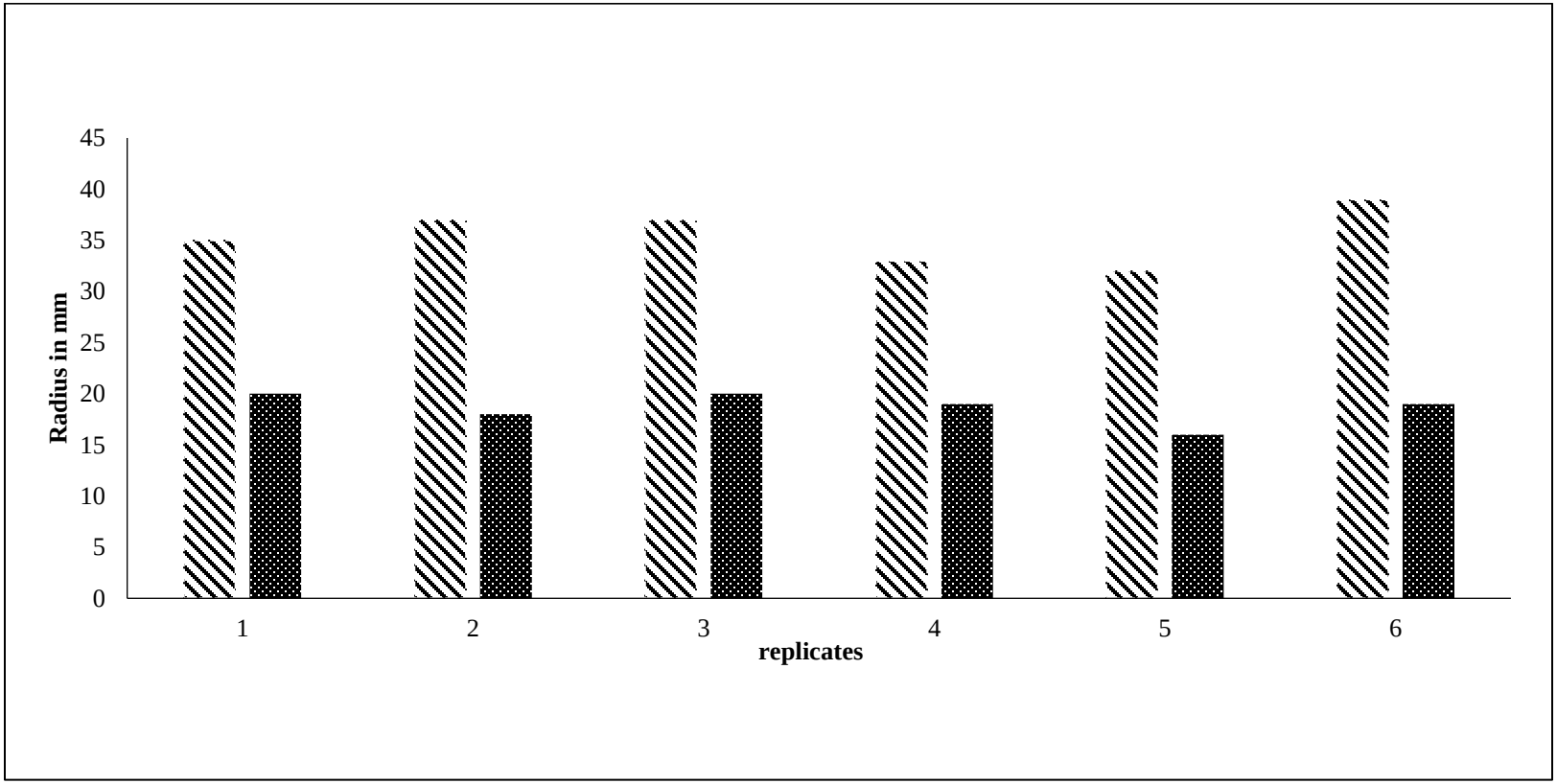
Appendix II: **Milo; a popular beverage which contains barley (shown by the blue arrow) Source; (Author, 2017)**

Appendix III: Summary of various strategies used to enhance the production of antimicrobial agents from micro-organisms

	Strategy.	Source(s).
1.	Varying medium conditions; For example, inoculation volume, Medium capacity, rotary speed, Fermentation time and temperature	 (Bode *et al.*, 2002) (Y.-H. Wang *et al*., 2008) (Song et al., 2012)
2.	Replacement of native gene promoter regions with constitutive and inducible promoters for over-expression of transcription factors.	(Brakhage & Schroeckh, 2011)
3.	Co-inoculation with one or more micro-organisms. Competition induced metabolite An endophytic fungus *Paraconiothyrium variable* inhibited *Fusarium oxysporum*.	(Marmann *et al.*, 2014) (Combès *et al*., 2012)
4.	Fermentation in the presence of non-ionic adsorption resins. Media treatment with amberlite XAD7 and growth in vermiculite had divergent Patterns of antibiosis relative To non-treated controls.	 (de la Cruz *et al*., 2012)
5.	Addition of small molecule elicitors such epigenetic modifiers. Examples are DNA methyltransferases (5-azacytidine) and histone deactylase Inhibitors (suberoylanilide hydroxamate)	 (Williams *et al*., 2008)
6.	Addition of organic solvents to growth medium. Culturing of *Aspergillus niger* in Potato Dextrose Broth supplemented with 1% acetone, 1%	(Pettit, 2011)

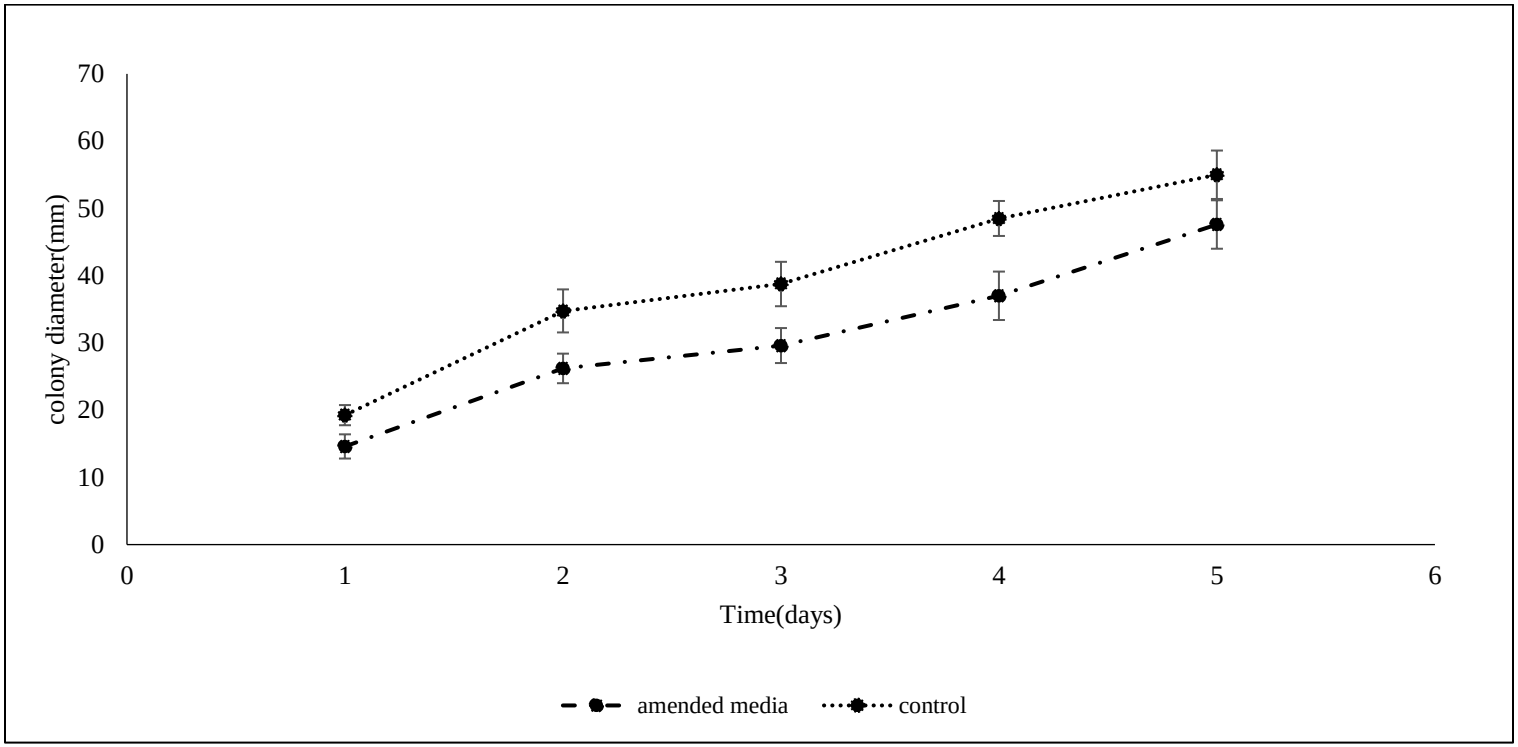
DMSO, and 1% ethanol increased the
production of secondary metabolites
in addition to production of newer ones. (Toghueo *et al*., 2016)

Appendix IV: Effects of diffusible bacterial antifungal metabolites on the growth of *D teres* f sp. *teres*



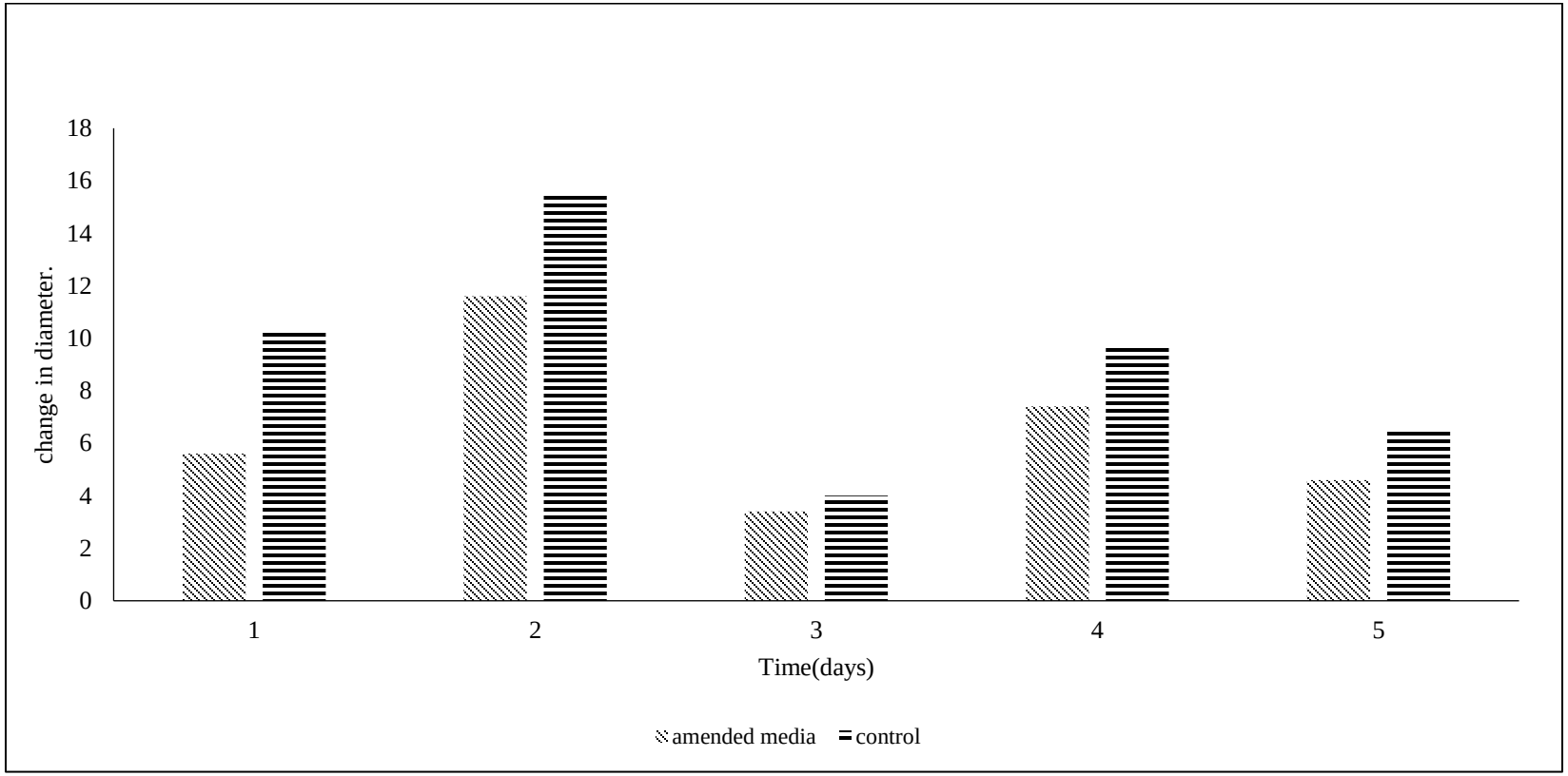
R r. R: Radius of the mycelium growing away from the bacterium, r: Radius of the mycelium growing towards the bacterium. The inhibition from all the replicates is consistent.

<u>Appendix Va:</u> Effect of bacterial supernatant extract on the growth rate of *D. teres* f sp *teres*



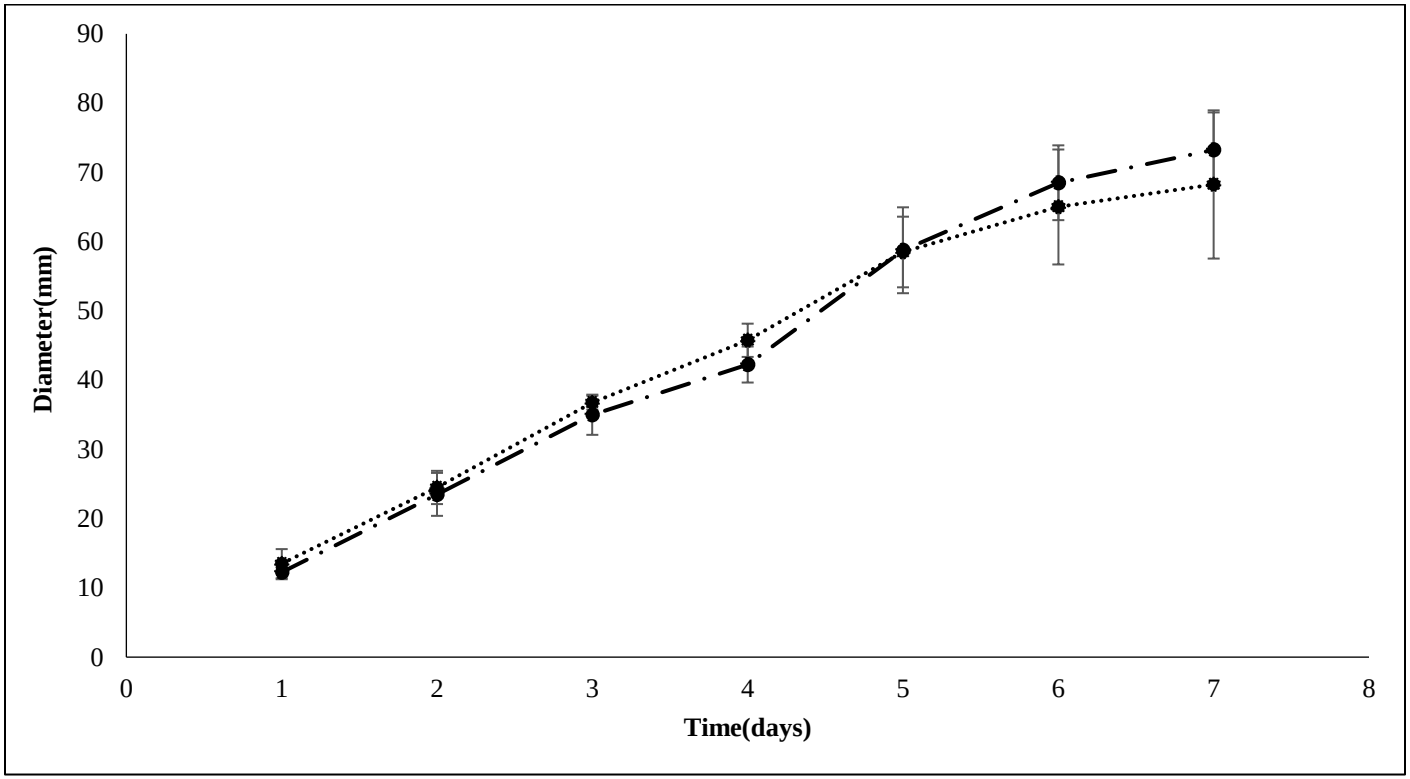
Real inhibition occurred only in the first day. Fairly parallel line graphs show the same growth rate of the fungi growing in amended media and non-amended media. The error bars represent standard deviations.

<u>Appendix Vb:</u> Effect of cell-free supernatant extract on growth rate (change in colony diameter) of *D teres* f. sp *teres*



Differences in the change in colony diameter. It can be seen for example that change in colony diameter of the control in the first day is smaller than the colony that received the supernatant treatment on the second day. Such inconsistencies are what made it lack significant statistical difference.

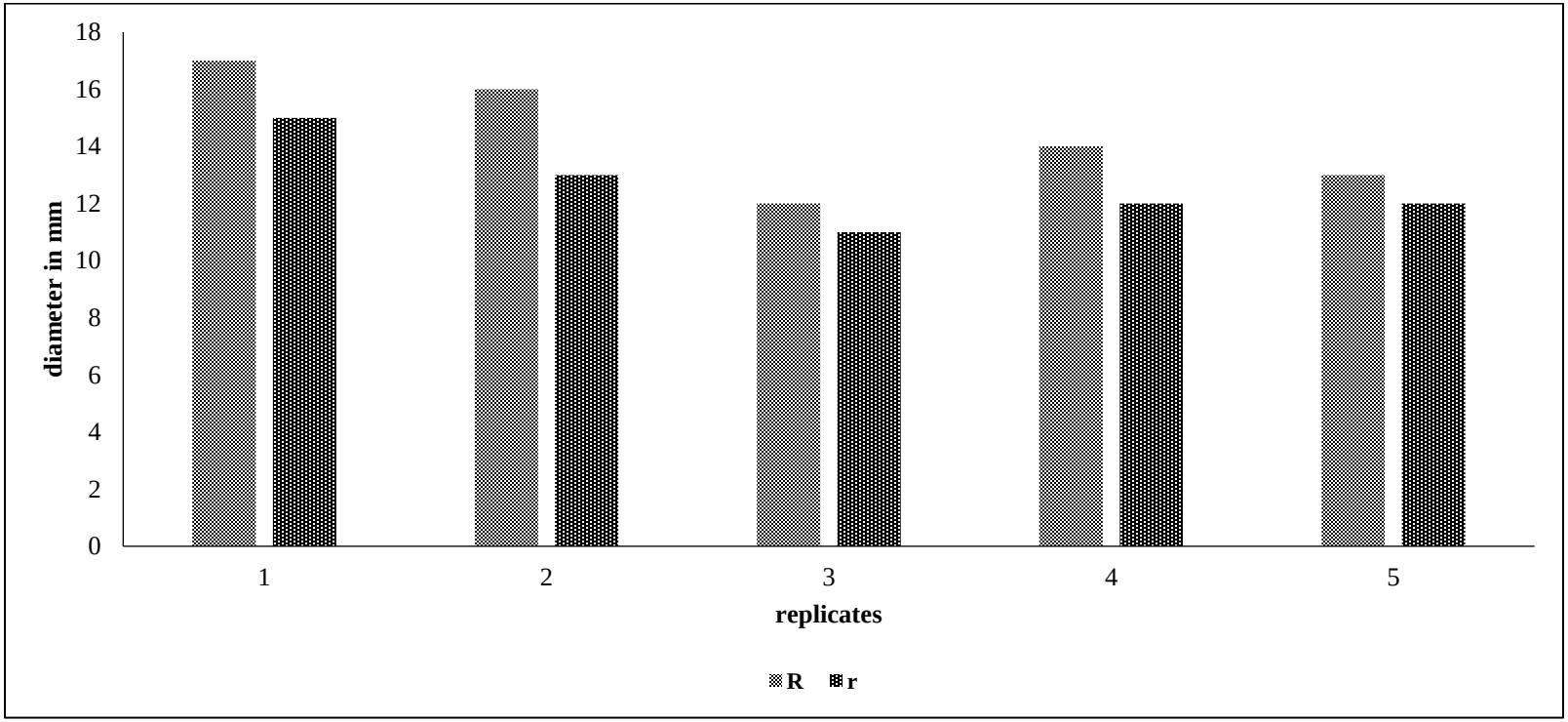
<u>Appendix VI:</u> Ineffectivity of *P. polymyxa* volatiles in controling the growth of *D teres* f. sp *teres in vitro*



…….. Control. – · – · – · – · *P. teres + P. polymyxa*

Line graphs of the *D. teres* colonies exposed to antifungal metabolites and those not exposed to antifungal metabolites fairly superimpose. Volatile organic compounds from *P. polymyxa* does not have any effect on the mycelial growth of *D. teres*.

<u>Appendix VII:</u> The less than full control of *R commune* by diffusible metabolites of *P. polymyxa* KaI245 *in vitro*

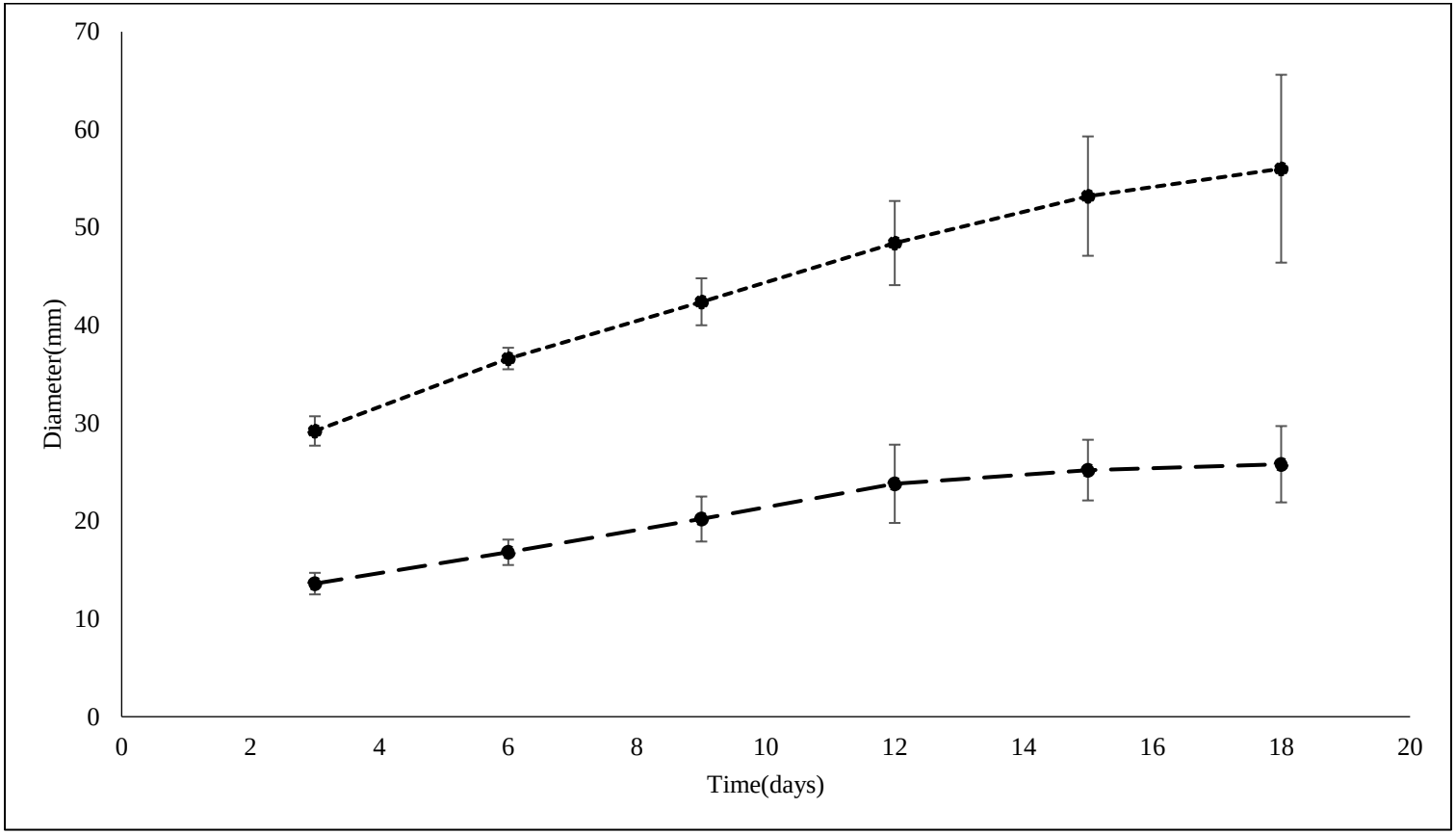


R; Growth away from the bacteria

r; Growth towards the bacteria

There is a consistent negligible difference in the two sets of radiuses. Further, controls in one replicate records an unprecedented lower value than those colonies that received what was supposed to be inhibitory treatment. For that reason, there wasn't a statistical difference between these two categories.

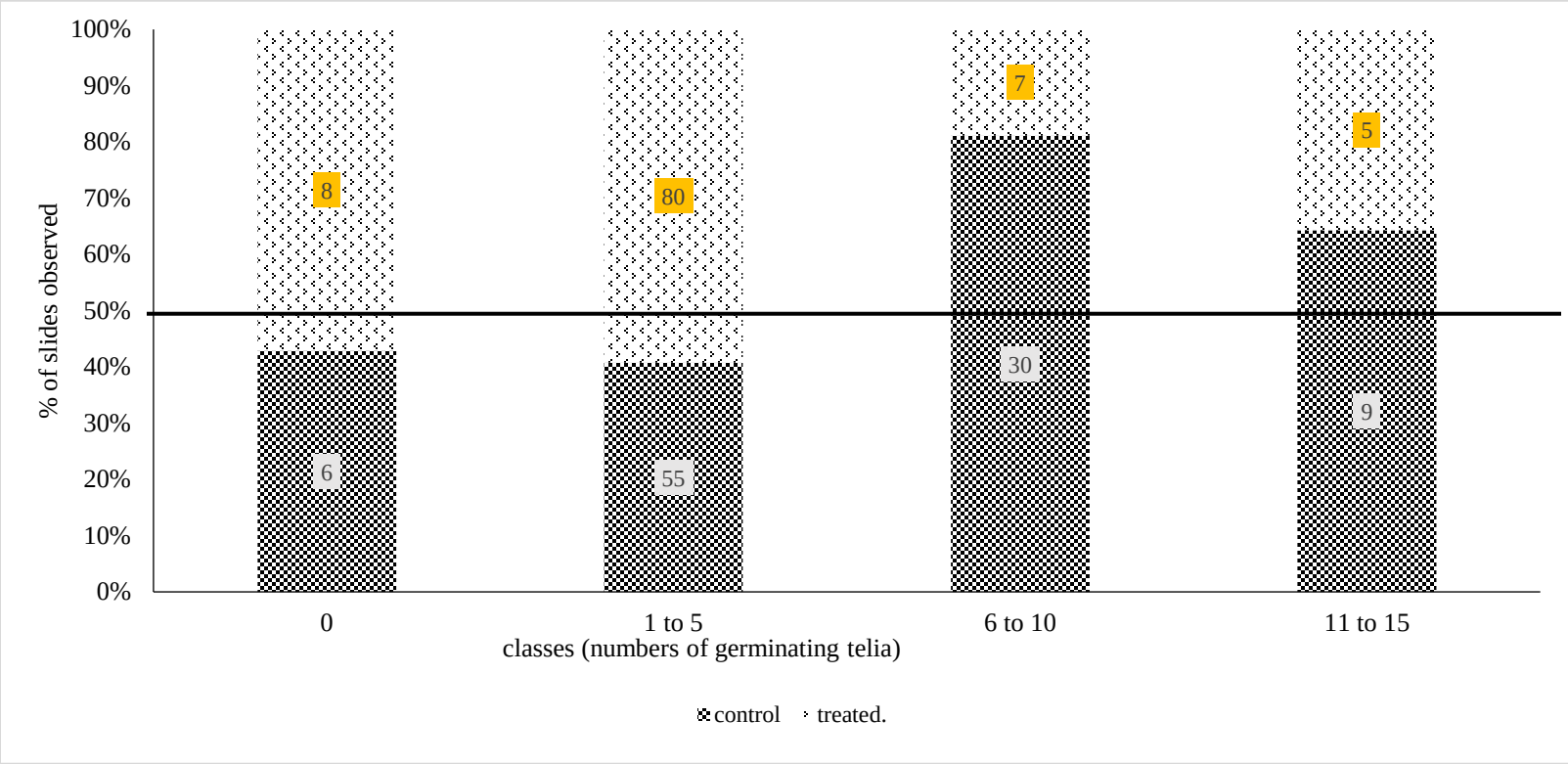
<u>Appendix VIII:</u> Inhibition of *R. commune* growth rate by *P. polymyxa* KaI245 volatiles



------- **Control.** – – – – – ***R. commune* + *P. polymyxa***

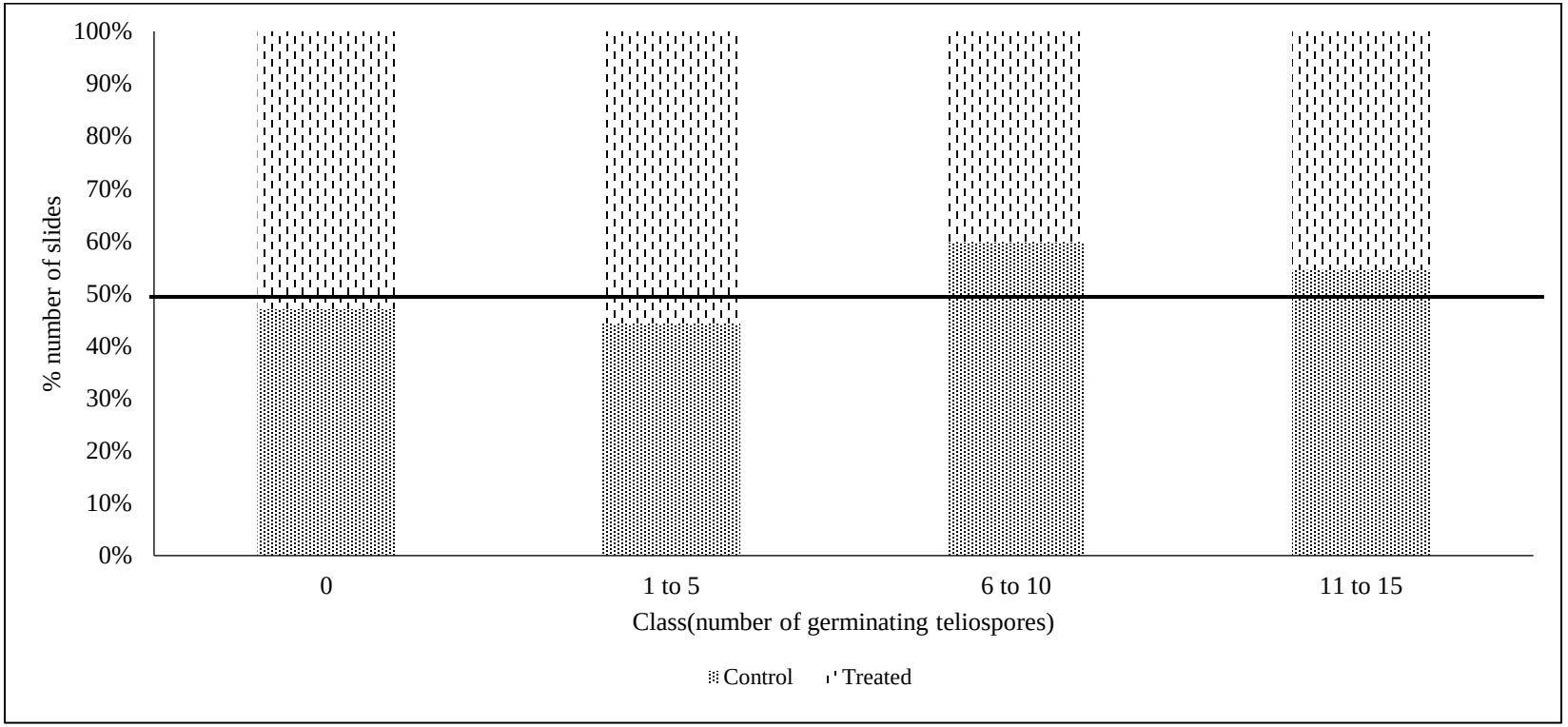
Clear difference in the mycelial growth of *R. commune* colonies exposed to *P. polymyxa* KaI245 volatiles and non-exposed colonies. *Paenibacillus polymyxa* KaI245 strongly inhibits the mycelial growth of *R. commune*.

- **<u>Appendix IXa:</u> Relative numbers of germinating teliospores among the volatile exposed and non-exposed samples**



The numbers of the germinating teliospores in each of the category occupies different proportions of the columns. The effect of the bacterial volatiles can be evidenced by assessment of the attainment or lack of 50% parity. There are major shifts in the 6-10 and 11-15 categories. *Paenibacillus polymyxa* KaI245 deterred the germination of *P. graminis* teliospores.

Appendix IXb: Relative percentages of germinating teliospores among the ones treated with *P. polymyxa* KaI245 cell-free supernatant and untreated controls



The numbers of the germinating teliospores in each of the category occupies different proportions of the columns. The effect of the bacterial volatiles can be evidenced by assessment of the attainment or lack of 50% parity. There are no major shifts in all the categories. *Paenibacillus polymyxa* KaI245 cell-free supernatant didn't deter the germination of *P. graminis* teliospores.

Appendix X: Volatile organic compounds obtained by GC-MS from ethyl acetate extracts of *P. polymyxa* KaI245 cultured in PDB

RT	Library/ID	Peak area	% abudance
3.37	Acetoin	502044	0.05
5.97	Butanediol<2,3->	228489744	21.20
6.36	Cyclohexane, ethyl-	2876885	0.27
7.22	Ethylbenzene	37776579	3.51
7.43	p-Xylene	26463039	2.46
8.02	o-Xylene	9373446	0.87
10.31	Mesitylene	419675	0.04
10.46	Decane<n->	270213	0.03
10.66	Benzene, 1,4-dichloro-	1787870	0.17
10.99	Limonene	1195775	0.11
12.28	Undecane<n->	1088958	0.10
13.79	2-Carene	2705121	0.25
13.87	Dodecane	3631409	0.34
15.20	Isobornyl acetate	2227922	0.21
15.33	Tridecane	6281566	0.58
15.66	Nonane, 2,2,4,4,6,8,8-heptamethyl-	5240934	0.49
16.57	Tetradecene<1->	2130787	0.20
16.67	Tetradecane (C14)	9300357	0.86
16.86	Aromadendrene<allo->	1645082	0.15
17.59	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	5763560	0.53
17.76	Ionone<methyl-gamma->	2303746	0.21
17.93	Pentadecane	10298124	0.96
18.11	Phenol, 2,4-bis(1,1-dimethylethyl)-	5026263	0.47
18.72	Dodecanoic acid	4907221	0.46
19.04	Hexadecene<1->	8639962	0.80
19.13	Hexadecane (C16)	17718712	1.64
19.86	Octane, 1,1'-oxybis-	5477193	0.51
20.25	Heptadecane (C17)	9635512	0.89
20.87	Cinnamaldehyde<2-hexyl-(Z)->	8475802	0.79
20.94	Tetradecanoic acid	7869640	0.73
21.10	Benzyl Benzoate	6756105	0.63
21.47	Salicylate<2-ethylhexyl->	11934644	1.11
21.60	Isopropyl tetradecanoate	19885238	1.85
21.98	7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	10284629	0.95
22.35	Nonadecane (C19)	17157081	1.59
22.61	Methyl hexadecanoate	17100917	1.59
23.04	Hexadecanoic acid	82567177	7.66
23.28	Ethyl hexadecanoate	48837994	4.53

23.57	Isopropyl hexadecanoate	40283372	3.74
24.14	Hexadecanol<n->	38425044	3.57
24.29	6-Octadecenoic acid, methyl ester, (Z)-	21344617	1.98
24.52	Methyl octadecenoate	9280628	0.86
24.69	6-Octadecenoic acid	88873059	8.25
24.88	Octadecanoic acid	64935205	6.03
25.11	Eicosene<1->	38105379	3.54
26.01	Docosane	24004353	2.23
26.26	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	12973990	1.20
26.32	Tetracosane	28374420	2.63
31.31	Myristyl myristate	42031128	3.90
32.45	28-Nor-17.beta.(H)-hopane	24960297	2.32

RT: Retention time in minutes

Appendix XI: Volatile organic compounds obtained by GC-MS from ethyl acetate extracts of *P. polymyxa* KaI245 cultured in PDB treated with methanol

RT	Library/ID	Peak area	%abudance
5.42	Butanediol<2,3->	65966224	10.84
5.59	2,3-Butanediol, [R-(R*,R*)]-	3480445	0.57
7.20	Ethylbenzene	5514347	0.91
7.42	Benzene, 1,3-dimethyl-	4542510	0.75
8.01	p-Xylene	1681461	0.28
8.98	.alpha.-Pinene	160369	0.03
13.87	Dodecane	1602809	0.26
14.54	Dodecane, 4,6-dimethyl-	1070156	0.18
14.69	Ageratochromene<6-demethoxy->	7846586	1.29
15.33	Tridecane	1858353	0.31
15.54	4-Isopropyl-1,3-cyclohexanedione	4965856	0.82
16.58	Hexadecanol<n->	1507053	0.25
16.67	Tetradecane (C14)	4574185	0.75
17.35	Pentadecane, 2,6,10-trimethyl-	3479116	0.57
17.58	Decane, 2,3,5,8-tetramethyl-	5334572	0.88
17.70	Tridecane, 5-propyl-	3804753	0.63
17.93	Pentadecane	5798393	0.95
18.11	Phenol, 2,5-bis(1,1-dimethylethyl)-	18918349	3.11
18.57	Cyclohexane, 1,2,4-trimethyl-	4881175	0.80
19.04	Hexadecene<1->	4702092	0.77
19.12	Hexadecane (C16)	6654948	1.09
20.25	Heptadecane (C17)	7963502	1.31
21.25	Octadecene<1->	10890362	1.79
21.60	Isopropyl tetradecanoate	10616781	1.74
21.98	7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	6980096	1.15
22.34	Nonadecane (C19)	12260887	2.01
23.10	Hexadecanoic acid	81348884	13.36
23.57	Isopropyl hexadecanoate	32103887	5.27
24.66	Oleic Acid	39171305	6.44
24.91	Octadecanoic acid	103079662	16.93
25.11	Eicosene<1->	28254175	4.64
28.40	Hexacosane	37670075	6.19
28.82	2,6,10,14-Tetramethyl-7-(3-methylpent-4-enylidene) pentadecane	37881766	6.22
29.42	Pyrimidine-2,4,6(1H,3H,5H)-trione, 5-[3-[2-(4-tert-butylphenoxy)ethoxy]benzylidene]-	25813910	4.24
32.44	28-Nor-17.beta.(H)-hopane	16300236	2.68

Shaded metabolites represent the new ones due to methanol treatment.
RT: Retention Time (minutes). ID: Identity. R: Carbon chain

Appendix XII: Volatile organic compounds obtained by GC-MS from ethyl acetate extracts of *P. polymyxa* KaI245 cultured in PDB treated with acetone

RT	Library/ID	Peak area	%abundance
5.12	Butanediol<2,3->	7500046	7.34
7.21	Ethylbenzene	1555698	1.52
7.43	Benzene, 1,3-dimethyl-	1174821	1.15
16.74	Tetradecane (C14)	1133430	1.11
18.15	Phenol, 2,5-bis(1,1-dimethylethyl)-	610939	0.60
19.08	Formic acid, dodecyl ester	536669	0.53
19.15	Hexadecane (C16)	2425408	2.37
20.27	Heptadecane (C17)	1187958	1.16
21.27	Hexadecene<1->	1843365	1.80
21.33	Octadecane (C18)	2292764	2.24
21.61	Isopropyl tetradecanoate	3128967	3.06
21.99	7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	1145891	1.12
22.63	Methyl hexadecanoate	2860834	2.80
22.98	Hexadecanoic acid	11511673	11.27
23.57	Isopropyl hexadecanoate	7963696	7.80
24.29	Oleic Acid	6710317	6.57
24.53	Methyl stearate	1526168	1.49
24.64	Octadec-9-enoic acid	10993779	10.76
24.90	Ethyl Oleate	13427155	13.15
25.11	Eicosene<1->	7244984	7.09
26.80	1-Nonadecene	9006069	8.82

Shading reveals the new metabolites produced as a result of acetone treatment.

RT: Retention Time. ID: Identity

Appendix XIII: **Research Permit**

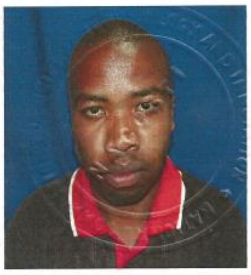
THIS IS TO CERTIFY THAT:
MR. KIPKOGEI CHEMITEI
of UNIVERSITY OF ELDORET, 0-30108 TIMBOROA,has been permitted to conduct research in *Uasin-Gishu County*

on the topic: *BIO-CONTROL OF SELECTED BARLEY PATHOGENS USING RHIZOBACTERIA ISOLATED FROM SORGHUM RHIZOSPHERE IN KENYA.*

for the period ending:
12th April,2019

.....
Applicant's Signature

Permit No : NACOSTI/P/18/79087/21616
Date Of Issue : 12th April,2018
Fee Recieved :Ksh 1000



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Director General
National Commission for Science, Technology & Innovation

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