CHARACTERISATION OF *Phaeoisariopsis griseola,* PHYTOCHEMICAL SCREENING AND FUNGICIDAL ACTIVITY OF SELECTED PLANT EXTRACTS AGAINST *Phaeoisariopsis griseola* OF COMMON BEAN Var. GLP 1127 Mwezi moja

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN PLANT PATHOLOGY

DEPARTMENT OF BOTANY

SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES

MASENO UNIVERSITY

NOVEMBER, 2019

DECLARATION

I, the undersigned, declare that this thesis is my original work and that it has never been presented in any University or institution for academic credit. All sources of information have been acknowledged by means of references and citation.

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ACKNOWLEDGMENTS

This thesis is a product of collaborative efforts of my lecturers at Maseno University, my family and I, and wish to sincerely honour my supervisors: Prof. David Mutisya Musyimi and Dr. Phoebe Anyango Sikuku of the Department of Botany Maseno University for their guidance and support in the course of this research work.

Am greatly indebted to the technical staff of the Department of Botany Maseno University for providing an enabling environment in which I did the laboratory work. The School of Graduate Studies (SGS) library staff is thanked for availing the literature search materials whenever needed.

I also wish to acknowledge with sincere gratitude the contributions of my parents Mr. and Mrs. Obila Johnson for their tireless efforts that has seen me this far. Appreciation to my colleagues John Wekulo, V. Korir, N. Oluoch, A. Nzovu and J. Chesire whose encouragement and company when carrying out the project kept me moving on. Thanks to Mr. William Emitaro of Jaramogi Oginga Odinga University of Science and Technology for assisting with microscopic work.

To my brothers and sisters thank you for your moral support during my postgraduate studies. My deep heart-felt thanks to my lovely wife Lynet Akinyi and son Fidel for their love, support, encouragement, understanding, patience, perseverance and prayers that has seen me through my studies. There are many more people I would wish to remember personally and extend my appreciation to them; all I can do is to thank them and God bless you all.

DEDICATION

To my parents, wife Lynet and son Fidel Meso.

ABSTRACT

Common bean is an important legume and a cheap source of proteins. *Phaeoisariopsis griseola* causing Angular Leaf Spot disease (ALS) is a major constraint for common bean production in Kenya contributing to yield losses estimated at 80%. ALS disease has been difficult to control using cultural practices and resistant varieties which necessitates the need to search for an alternative management option. Synthetic fungicides too have numerous side effects, so the plant-based products are getting more popularity as they are safe to use, easily available and cheap. Most plant extracts possess antifungal activity. *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* exhibit antimicrobial activity though little is known about the success of using these crude extracts to control *Phaeoisariopsis griseola* of common bean. The study was to characterize *Phaeoisariopsis griseola*, phytochemically screen and determine the fungicidal activity of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* extracts against *Phaeoisariopsis griseola* of common bean. The specific objectives were to: morphologically profile *Phaeoisariopsis griseola* from infected common bean, phytochemically screen the plant samples of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* and determine their effects on spore germination using solvent extracts of water, ethanol and methanol, effects on growth, yield and disease severity and incidence. The study was conducted at Maseno University, Department of Botany and greenhouse. Specimens *of Azadirachta indica, Allium sativum* and *Tithonia diversifolia* were collected from Maseno university and its environs. The plants were identified by taxonomists in the Department of Botany. Specimens were then air-dried and ground into fine powder for extraction. Methanol, ethanol and distilled water were used for extraction. *Phaeoisariopsis griseola* were isolated from five diseased common bean plants collected from Ugenya, Bondo and Sabatia sub-counties for morphological profiling and pathogenicity tests done. The pathogen was plated on PDA media and identified based on their cultural and microscopic characters by using taxonomic guides. Fungicidal activity of the extracts was determined using concentrations of 50, 75 and 100% in *vitro.* Synthetic fungicide and water were used as positive and negative controls respectively. In *vivo* evaluations were conducted in the greenhouse using common bean plants Var. GLP 1127 Mwezi moja. Six kilograms of solar sterilized sandy-loam soil was used and two common bean plants Var. GLP 1127 Mwezi moja grown per plastic pot and inoculated with 60ml suspension 2.5 x 10^6 spore/ml of *Phaeoisariopsis griseola* after 21 days. Seven days after disease inoculation, the plants were sprayed by 100% concentration of the extracts with four replications. The plastic pots were arranged in a greenhouse in a completely randomized design. Data was collected on morphological profiling, inhibition of spore germination for the test pathogen, growth, yield and disease index after 7 days until physiological maturity. The data were subjected to Analysis of variance and treatment means separated and compared using Duncan test (P=0.05). Morphological profiling of isolates of *Phaeiosariopsis griseola* from Bondo, Sabatia and Ugenya sub-counties revealed variations in terms of hyphae and spores' characters. Alkaloids, tannins, flavonoids, terpenoids, saponins, cardiac glycosides and sterols were present in nearly all the plants with flavonoids being absent in *Azadirachta indica* and sterols, saponins and alkaloids being absent in *Tithonia diversifolia*. All the plant extracts significantly decreased spore germination of *Phaeiosariopsis griseola* in vitro ($P \leq 0.05$) to a mean of 7.6 for *Allium sativum,* a mean of 7.5 for *Azadirachta indica* and a mean of 7.3 for *Tithonia diversifolia*. The most effective was *Allium sativum* extract. Highest fungicidal effects were observed on plants inoculated with Ugenya isolate followed by Bondo and Sabatia isolates respectively. Moreover, *Allium sativum* and *Azadirachta indica* at 100% concentrations had the highest fungicidal effect while *Tithonia diversifolia* and the other concentrations had moderate to low fungicidal effect proportionate to the concentration percentage. Methanol had the highest extraction potential while water solvent had the least effect. The extracts had higher significant effect on growth index and disease index (P<0. 001). The extracts had no significant effect (P=0.955) on pod weight. From this study plant extracts of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* had higher potential for pathogen control at higher concentrations (100%) hence are recommended as potential botanicals for the control of ALS disease of common bean Var. GLP 1127 Mwezi moja.

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ACRONYMS, ABBREVIATIONS AND SYMBOLS

ALS: Angular leaf spot

^{o}C: Degrees centigrade

Hrs: Hours

Kg: Kilogram

Kg/ha: Kilogram per hectare

IDM: Integrated Disease Management

MT: Metric ton

Mg: Milligram

ML: Millilitre

Min: Minute

Mm: Millimetre

PDA: Potato dextrose agar

PSI: Pressure per Square Inch

±: Plus, or minus

>: Greater than

≤: Equal or less than

Nm: Nanometre

X: Magnification

PHI: Pre harvest interval

SC: Soluble concentrate

MP: Manufacture's prescription

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CHAPTER ONE

INTRODUCTION

1.1. Background to the study

Common bean (*Phaseolus vulgaris*) is among the most important plant cereals in the world (Leitich *et al*., 2016). It can be grouped into dry and green (snap) bean and in Africa majorly produced in climatic regions of temperate and sub-tropical, among other continents (Pamela *et al*., 2014). According to Damiano *et al*. (2014) common bean is consumed throughout the world and comes second in the order of importance. It is the third most important source of calories and fibre in both Eastern and Southern Africa and rich source of protein, zinc and iron (Bode *et al*., 2017; Keller *et al*., 2015; Pamela *et al*., 2014). In 2007, world's production of common bean was estimated at 417,00 Metric tomes (Mt) against a growing demand of more than 500,000 Mt, the deficit largely attributed to extreme biophysical stresses such as climate change, pest and phytopathogenic diseases, and soil fertility (Katungi *et al*., 2011). Phytopathogenic diseases make the largest proportion of the stresses leading to constant production of less than 25% of the potential yield (Ddamulira *et al*., 2014; Mauricio *et al*., 2012).

Fungus *Phaeoisariopsis griseola* is the etiologic agent of angular leaf spot of common bean and very destructive phytopathogen of common bean (Allorent and Savary, 2005). It attacks literally all the aerial plant parts such as stem leaves and pods causing shrivelled pods, sunken seeds and premature defoliation (Allorent and Savary, 2005; Bode *et al*., 2017). Angular leaf spot disease is primarily controlled using synthetic fungicide though effective has numerous shortcomings such as high input cost, human and environmental hazard and development of pathogen resistance towards the fungicide (Ddamulira *et al*., 2014). Moreover, the other effective control is by use of resistant variety which is proving to be expensive to develop and

difficult to maintain due to abundant genetic variability, virulence and pathotype diversity of the pathogen (Sharma and Adikshita, 2017).

The medicinal and antimicrobial activities of extracts from plants are gaining attention of researchers worldwide (Shabana *et al*., 2017). The modern synthetic fungicide has its own advantages and side effects, so the plant-based products are getting more popularity, as they are safe to use, and comparatively easily available and cheap. Many extracts possess antifungal activity (Cherkupally *et al*., 2017). Plant extracts are effective in plant pathogens and are now becoming popular throughout the world (Shabana *et al*., 2017). *Azadirachta indica* (neem) is a medicinal plant of importance with enormous phytochemical compounds such as terpenes and alkaloids on various parts of the plant such as bark, leaves and seeds, and has been reported to have biological properties against pathogenic organisms such as fungi, bacteria, viruses and pests (Pankaj *et al*., 2011; Al-hazmi, 2013). *Azadirachta indica* extracts have activity against phyto-pathogenic bacteria and fungi such as *Xanthomonas vesicatoria* and *Ralstonia solanacearum* (Sarawaneeyaruk *et al*., 2015). Little information has been reported on the use of leaves extracts of *Azadirachta indica* in the management of *Phaeoisariopsis griseola* pathogen of common beans. There is therefore need to determine the potential of neem leaves extracts for fungicidal activity against angular leaf spot disease of common bean. *Allium sativum* commonly known as garlic belongs to the family Alliaceae and among the important earliest known medicinal plants (Stavělíková**,** 2008; Byrappa, 2015). Singh *et al*. (2015) reported that garlic has alliin as its main chemical component which is an alkyl derivative of cysteine alkyl sulfoxide which is responsible for the characteristic odour of garlic thus an important food spice plant. Its usage worldwide has a long history with significant role in disease prevention and control and has been used since long time against human pathogens such as for the treatment of dysentery and worms in infants and adults (Cecilia and Olubunmi, 2014). However, there are less studies regarding the usage of garlic against plant pathogens.

Mexican sunflower (*Tithonia diversifolia*) is a member of Asteraceae family. The plant is usually found growing on the road sides, river banks and uncultivated bare lands and has been used for bruises and wound treatment (Gray *et al*., 2013). It has demonstrated successful antimicrobial activity against food and human pathogens (Rejeki *et al*., 2017). Little information is available on its use for control against plant pathogens. Globally, there is growing demand for natural botanicals among common bean growers for use as bio-fungicides due to the numerous negative effects of the synthetic fungicides on the environment and human health (Cecilia and Olubunmi, 2014). Plant parts of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* have been widely used as medicinal plants and in the control of phyto-pathogens (Rejeki *et al*., 2017). These plants have been reported to have antimicrobial activity against a number of pathogens (Gray *et al*., 2013). Little is known on the use of leaves extracts of neem and Mexican sunflower, and garlic as botanicals in the control of angular leaf spot disease of common beans. This therefore, necessitates the need to search for pathogen management options that are environmentally friendly, harmless to the non-target organisms and human health, and keep the pathogen to levels below economic threshold.

1.2 Statement of the problem

Angular Leaf Spot disease is a major biotic constraint of common bean production in Western Kenya with losses as high as 80% under disease favourable environmental conditions (Leitich *et al*., 2016). *Phaeoisariopsis griseola* the causative agent of angular leaf spot disease is extremely challenging to manage through chemical, cultural techniques or use of resistant varieties. The use of synthetic fungicide in agricultural farming has a number of shortcomings such as being toxic to non-targeted microbes, development of pathogenic resistance, high input costs to the farmers in the third world countries and hazardous to the environment due to residual effect (Sharma and Adikshita, 2017). Angular leaf spot disease thus poses a chronic

threat to food security in Kenya. Lack of elaborate control of angular leaf spot disease has led to yield reduction of 40% -80% (Fikre *et al*., 2011). This calls for the urgent need to identify an eco-friendly, inexpensive and an alternative to the chemical inorganic fungicide that can be used in the control of pathogens. The medicinal and antimicrobial activities of extracts from plants are gaining attention of researchers worldwide. Apart from the use of plant-based products in medicine, the usage of these extracts in plant protection also now becoming popular throughout the world. Little is known about the success of using *Allium sativum, Azadirachta indica* and *Tithonia diversifolia* crude extracts to control *Phaeoisariopsis griseola* of common bean. Synthetic fungicides have been reported to significantly influence common bean growth and yield through disease reduction though limited information is available on the effect of botanicals to influence the herein mentioned parameters.

1.3. Justification

The use of synthetic fungicides in agricultural farming has increased consumer concern and has several drawbacks thus its use is becoming more restrictive due to carcinogenic effects, residual toxicity problems on the environment, development of resistance in pathogenic microbes, toxic to non-target microbes and very expensive for farmers in developing countries (Wang *et al*., 2010). In Kenya, for instance, a bigger percentage of bean production come from medium and small or subsistence farmers who do not apply synthetic fungicides to their crops due to high cost (Leitich *et al*., 2016). The inefficiency of the chemical fungicides in the control of angular leaf spot disease and the high cost for production of a resistant varieties, demands in food production and food security through the use of technology that is not detrimental to humans and the environment is a major challenge to the bean farmers. Due to genetic variability and virulence, the pathogen develops resistance towards the chemical fungicides which in the long-run leads to constant losses that has a significant impact on the production of common

beans over seasons. Taking into account the challenges, it has become important to develop strategies based on rational use of the fungicides or to replace them with alternative products. Plant extracts have shown biological activity that may pave way for an alternative option in the management of angular leaf spot disease due to its fungi-toxic action in the plant. Little information about the fungicidal activity of leaves extracts of *Azadirachta indica* and *Tithonia diversifolia,* and *Allium sativum* against angular leaf spot disease of common bean has been reported. Effective control of angular leaf spot of common bean is deeply anchored on the use of botanicals with fungicidal properties. These extracts have the merits of being readily available in farming localities of the tropics, cheap, eco-compatible, less harmful to non-target organisms and useable in integrated disease management programmes for smallholder, resource-poor farmers. Thus, this study aims to screen the selected plant extracts against *Phaeoisariopsis griseola* pathogens to establish its effectiveness as alternatives to synthetic fungicides in the control of angular leaf spot disease of common beans. This study is vital considering that cultural, chemical and use of resistant varieties control techniques have been employed to get rid of angular leaf spot disease but huge yield losses have continued to occur. This has prompted the need to try out alternative technologies in a bid to develop a suitable control for *Phaeoisariopsis griseola* such as the use of botanicals. The documentation of the effectiveness of the selected plant extracts against angular leaf spot disease of common bean will ultimately provide potential sources of widely sought remedies to the losses incurred in common bean production due to angular leaf spot disease infection. This will lead to isolation of bioactive molecules which will serve as starting materials for laboratory synthesis of drugs as well as models for the production of biologically active compounds (Dhanani *et al*., 2017). This study provides a frame work on the possible plant extracts that can serve as source of constituent for the commercial production of remedies against angular leaf spot disease of common bean.

1.4. Objectives of the study

1.4.1. General objective

The general objective of this study was to characterise *Phaeoisariopsis griseola* from infected common bean, phytochemically screen and determine fungicidal activity of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* plant extracts against *Phaeoisariopsis griseola* causing angular leaf spot disease of common bean Var. GLP 1127 Mwezi moja.

1.4.2. Specific objectives

1. To profile morphologically isolates of *Phaeoisariopsis griseola* pathogen from infected common bean from Sabatia, Ugenya and Bondo sub-counties.

2. To determine the phytochemical composition of bulbs of *Allium sativum*, and leaves of *Azadirachta indica* and *Tithonia diversifolia.*

3. To determine the effect of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on spore germination of *Phaeoisariopsis griseola* pathogen obtained using solvents extracts of distilled water, methanol and ethanol at 50, 75 and 100% extract concentrations.

4. To determine the effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts on the growth and yield components of common bean Var. GLP 1127 Mwezi moja infected with Ugenya isolate of *Phaeoisariopsis griseola*.

5. To determine the effect of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts on disease incidence and severity of angular leaf spot disease of common bean Var. GLP 1127 Mwezi moja.

1.5. Hypotheses

1. There are morphologically different isolates of *Phaeoisariopsis griseola* pathogen on infected common bean from Sabatia, Ugenya and Bondo sub-counties.

2. There are various phytochemical compounds in *Azadirachta indica, Allium sativum* and *Tithonia diversifolia*.

3. *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts have effects on spore germination of *Phaeoisariopsis griseola* pathogen at different extract concentrations.

4. *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts have significant effect on the growth and yield components of common bean Var. GLP 1127 Mwezi moja.

5. *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts have high significant effect on disease incidence and severity of angular leaf spot disease of common bean Var. GLP 1127 Mwezi moja.

CHAPTER TWO

LITERATURE REVIEW

2.1. Common bean (*Phaseolus vulgaris* L.) botanical description

Common bean is an annual herbaceous plant, erect or climbing with leaves composed of rhomboid or tri-oval shaped leaflets (Katungi *et al*., 2009). Common bean varieties can be indeterminate, determinate and matas low or climbing (Creamer, 2014). The erect plant has stems and fickle tendrils which are made by modification of terminal leaflets while sometimes covered by villi (Damiano *et al*., 2014). It is largely a self-pollinated plant though cross-pollination is possible if the stigma contacts with pollen coated bee when extended (Keller *et al*., 2015). Seeds are non-endospermic and vary greatly in size and colour from the small black wild type to the large white, brown, red, black or mottled seeds of cultivars, which are 7-16 mm long (Katungi *et al*., 2009). Common bean is grown for both food and cash value in monoculture and a number of cropping systems in East Africa (Fikre *et al*., 2011). Over 111,000 years ago, common bean has been reported to have diverged from the Mesoamerica and Andes centres of origin which have led to the gene pools Mesoamerican bean and the Andean bean which can be differentiated on seed size, with Andean bean being large seeded while Mesoamerican is small seeded (Keller *et al*., 2015). Common bean has an extensive root system which is made of taproot, tuberous root and several secondary roots and seeds range in colour from yellow, black, grey, brown, white, red, purple and pinto or fluted (Damiano *et al*., 2014). Chilange *et al*. (2013) stated that common bean is a member of family Fabaceae and an annual, self-pollinating cereal crop (legume) grown almost worldwide under variable climatic conditions from tropical to sub-tropical regions (Damiano *et al*., 2014). Common bean production has been reported to have the potential of reducing food insecurity and poverty levels as the legume serves the primary source of protein and nutrition (Creamer, 2014). Common bean belongs to the genus Phaseolus and a member of the family Fabaceae which is

composed of more than fifty plant species which are all native to America (Damiano *et al*., 2014). According to Damiano *et al*. (2014) the flowers are asymmetric in shape and purple or white in colour while the fruits are variable in colour with 3-12 seeds in the fruit (pod). Common bean thrives well in warm climatic conditions with an average temperature of 16 - 26^{o}C and rainfall of 300-500,000mm throughout the growing season. The production is by means of seeds (Fikre *et al*., 2011). Generally, common bean is considered a short-season crop with most varieties maturing in a range of 65 to 110 days from emergence to physiological maturing (Katungi *et al*., 2009).

2.2. Taxonomy and classification of common bean

Divisions: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Phaseolus*

Specie: *Phaseolus vulgaris* (Damiano *et al*., 2014).

2.3. Common bean varieties in Kenya

The main varieties grown in Kenya are Monei, Amy, Samantha, Teresa, Julia, Vernando, Bronco, Coby, Espadia, Bakara, Rose Coco, Mwitemania, Wairimu, Mwezi Moja, Canadian W, KK 15 (Leitich *et al*., 2016). Mwezi Moja common bean variety was used in the study because it is among the most preferred by farmers, early maturing among other qualities as described in 2.4 (Shisanya, 2004).

2.4. Common bean variety GLP 1127 Mwezi moja

GLP 1127 Mwezi moja common bean variety is an early maturing at about 85 days, vigorously growing and determinate variety (Shisanya, 2004). The seeds are large, light brown, oblong

beige or speckled purple and are well suited for relatively drier, semi-arid or low precipitated areas also performs well in medium rainfall areas during short rain seasons. GLP 1127 Mwezi moja is high yielding though highly susceptible to angular leaf spot disease but symptoms appear during late cycles of the plant life after flowering. The seeds are preferred since they cook fast and tastes well. The market requires freshy, straight, long, rounded in cross-section beans qualities found in Mwezi moja (Leitich *et al*., 2016).

2.5. Ecological requirements for growing common beans

Common beans grow well in temperatures ranging from 15-33 degree centigrade. However, an optimum growing temperature of 20-25 degrees centigrade is essential. Relatively high temperatures affect flowering and pod setting processes (Shisanya, 2004). The crop is very sensitive to frost. Temperatures ranging between 14-32°C though extreme temperatures result to poor flower development and poor pod set (Corte *et al*., 2013). The optimum altitude range between 1,000 – 2,100m above sea level, it however tends to grow and mature faster in low altitude zones and a well distributed medium to high rainfall of about 800-2000mm annually is suitable for the rain fed production. Irrigation should be done if rainfall is inadequate. Excessive rainfall during flowering causes flower abortion and increased disease incidences. Dry weather conditions are needed during harvesting. Common bean crop thrives in a well-drained loam to heavy clay soil which is rich in organic matter, weed free and has an optimum PH of 6.5-7.5. Growth is poor in waterlogged soils (Leitich *et al*., 2016).

2.6. Importance of common bean

In Kenya, the cereal crop is ranked as second most important staple food after maize (Leitich *et al*., 2016). Common bean is consumed daily as part of dietary protein as a vital legume crop for over half a billion people around the world (Corte *et al*., 2013). Almost more than half of

the world population uses this cereal crop for direct consumption which involves Eastern and Southern Africa where it is cultivated in over four million hectors of agricultural land (Leitich *et al*., 2016). Margaret *et al*. (2014) stated that the legume crop is a secure source of food and nutrition mainly in the Sub-Saharan Africa region, and plays a vital role in daily diet as it provides carbohydrates, proteins, vitamins and essential elements to both the urban and rural populations. The crop is estimated to supply more than half of the dietary protein required by households in Africa. This assures the bean producers both food for nutrition and a source of livelihood (Tryphone *et al*., 2016). Beans represent one of the principal crops in East Africa in terms of total area planted and number of farmers involved in production. Common bean production also provides farm households with both source of income and food for nutrition through sales and consumption of part of the produce (Anderson, 2010). Consuming beans also have medicinal benefits as it is recognized that they contribute to treating human ailments like cancer, diabetes, and heart diseases (Bode *et al*., 2017). Evidence points out that poverty levels would have been higher in the absence of development and adoption of improved bean varieties (Leitich *et al*., 2016).

2.7. Common bean production

Common bean yields in Kenya have remained low with an average yield of 585 kg/ha compared to Ethiopia and Rwanda with yields of 1588 kg/ha and 913 kg/ha respectively (Leitich *et al*., 2016). Globally, common bean is cultivated on about 28 million ha, producing on average, approximately 715 kg year-1 ha-1 (Margaret *et al*., 2014). According to Katungi *et al*. (2011) production in 2007 was about 417,000 metric tons while demand was estimated at 500,000 metric ton. The supply deficit is attributed to the severity of biophysical stresses such as climatic variability, insect pests and diseases and declined soil fertility that maintain productivity at less than 25% of potential yield. Plant diseases are the major factor the

contribute to low yields of common bean due to lack of effective control mechanism. Further, Leitich *et al*. (2016) reported that in Kenya, per capita consumption is estimated at 14 kg per year, but can be as high as 66 kg per year in Western Kenya. To counter this production menace alternative control needs to be developed to boost the production to acceptable quantities.

2.8. Constraints of common bean production

According to Tryphone *et al*. (2013) the average yields of common bean has remained low as >500 kg/ha while the potential of current promising released varieties is at 1500 kg/ha.

In Kenya, common bean yield has constantly remained low (Leitich *et al*., 2016). Angular leaf spot disease is basically the most important constraint to common bean production in Africa with annual loss estimated at 374,800MT (Fikre *et al*., 2011). Across farming systems, biotic and abiotic stresses continue to present the major constraints for increased bean production and high yields with bean diseases representing the major constraints to production by reducing yields and seed quality. They can cause yield loss up to 100% of the expected yield, depending on the environment and the varieties used. The major diseases affecting bean production in East Africa including Kenya are Bean Common Mosaic Necrosis Virus, common bacterial blight (*Xanthomonas axonopodis pv. phaseoli*), angular leaf spot (*Phaeoisariopsis griseola*), anthracnose (*Colletotricum lindemuthianum*) and rust (*Uromyces phaseoli*) (Bode *et al*., 2017). Angular leaf spot is the most destructive of the diseases of common bean in Western Kenya (Leitich *et al*., 2016).

2.9. Angular leaf spot disease of common bean

Angular leafspot caused by *P. griseola* attacks only common beans (*Phaseolus vulgaris* L.) and lima beans (*Phaseolus lunatus* L.) (Mongi, 2016). Pathogenic fungi are the main infectious agents in plants, causing alterations during developmental stages including post-harvest.

Angular leaf spot disease of common bean (*Phaseolus vulgaris* L.), caused by the fungus *Phaeoisariopsis griseola* a plant pathogenic fungus (Sartorato, 2002). Angular leaf spot is among the most destructive diseases of common bean particularly in relatively warm, humid, areas with abundant inoculum from infected volunteer plants, infected plant debris, off-season crops and contaminated seeds (Ddamulira *et al*., 2014; Fikre *et al*., 2011). The disease is ranked second among biotic and abiotic factors that constrain bean production in Africa. It is also one of the most widely distributed and damaging diseases of common bean (*Phaseolus vulgaris* L) in tropical and subtropical countries with yield losses on susceptible varieties can be as high as 80%. The infected host exhibits reduced photosynthetic rate due to abnormalities in form and function of chloroplasts of the diseased tissue followed by decline in photophosphorylation, photochemical reaction and carbon dioxide assimilation reducing physiological performance of the canopy (Sharma and Adikshita, 2017; Mongi, 2016). Lack of elaborate control of angular leaf spot disease has led to yield reduction of 40% -80% (Fikre *et al*., 2011).

2.9.1. Taxonomy and diversity of Angular leaf spot (*Phaeoisariopsis griseola*)

Angular leaf spot disease is found in more than sixty countries throughout the world (Sartorato, 2002). In Kenya, forty-four physiological races of *P. griseola* have so far been identified. *Phaeoisariopsis griseola* virulence is assessed based on reaction of isolates on a standard differential set of 12 common bean varieties proposed by CIAT, and divided into two sets of Mesoamerican and Andean, with six varieties each (Leitich *et al*., 2016). The pathogen has been a big challenge to local farmers though little is known of *Phaeoisariopsis griseola* in sub-counties of Sabatia, Bondo and Ugenya and correct pathogenicity test could lead to effective disease management.

2.9.2 Life cycle, epidemiology and dissemination of angular leaf spot disease

Phaeoisariopsis griseola spores germinate on the leaf surface after 3 days of moist conditions, enter the leaf through the stomata and grow inter-cellularly, limited by the leaf veins resulting in an angular lesion shape. Germination of fungal spores is essentially a process during which the normal metabolic and physiological activity is restored after dormancy. which involves spore transformation from a dormant state of low metabolic activity done of high metabolic activities (Singh *et al*., 2014). Infection and sporulation occur in a broad temperature range, from 10 to 33°C (Keller *et al*., 2015). The disease is favoured by intermittent dry-wet and warm-cool weather (Sartorato, 2002). Angular Leaf Spot epidemics are usually observed relatively late in the crop cycle typically about the flowering stage. Lesion multiplication and extension on the foliage lead to defoliation, a prime mechanism leading to reduced physiological performance of the canopy (Allorent and Savary, 2005). According to Allorent and Savary, (2005) spore germination in *P. griseola* is strongly dependent on moisture. Spore germination on the leaf surface only takes place under moist conditions and occurs within three days after spore deposition. *P. griseola* spore germination and hypha-penetration is through the epidermis or stomata. The hemi-biotrophic life cycle of this fungus comprises of intercellular hyphae growth in the plant leaf mesophyll during the biotrophic phase, and sub- sequent hyphae penetration of the host cell causing plasmolysis during the fungus necrotrophic phase on susceptible plant genotypes (Oblessu *et al*., 2015). Little information is available on the effects of plant extracts on fungal spore germination.

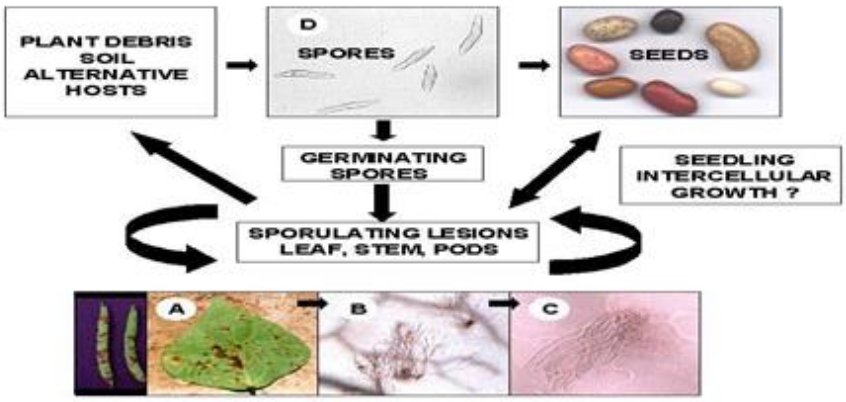


Figure 2.1: Angular leaf spot disease cycle. Courtesy of Allorent and Savary (2005).

2.9.3. Symptomatology of Angular leaf spot disease of common bean

Symptoms induced by *P. griseola* on beans develop on leaves, stems and pods. Lesions can appear on primary leaves within 6 days after inoculation, but usually do not become prevalent until the late flowering or early pod-set stage (Ddamulira *et al*., 2014). The spots are small, angular, dark brown and often so numerous that they give the foliage a checkerboard appearance (Sartorato, 2002). Lesions may increase in size, coalesce and cause necrosis and yellowing. When *P. griseola* attacks a plant, spots originate on the underside of the leaf and are delimited by the veins and veinlets. At first, the lesions are grey, later turn brown and attain an angular shape because of limitation by veins (Allorent and Savary, 2005). Pod lesions are roughly circular and reddish brown with dark brown borders (Sartorato, 2002). Stem lesions are dark brown and elongate (Ddamulira *et al*., 2014). On all lesions, dark stroma appears in abundance. Angular leaf spot disease causes typical symptoms on the leaves with angular shaped lesions as well as lesion multiplication and extension on the foliage lead to defoliation, a prime mechanism leading to reduced physiological performance of plant cover (Allorent and Savary, 2005). Losses can be as high as 70% under disease favourable environmental conditions (Sartorato, 2002). Furthermore, late infection on pods and seeds, also cause scars

that reduce on seed quality and market value (Ddamulira *et al*., 2014). Lesions on other aerial plant parts like stems, petioles, and pods in plates 2.1, 2.2 and 2.3. Angular leaf spot epidemics is majorly observed relatively late in the crop cycle typically the flowering stage (Allorent and Savary, 2005). Effective pathogen identification is anchored on symptomatology by carrying out pathogenicity tests. Pathogenicity test is carried out to establish whether the fungal isolates cause the disease and to determine whether they could induce similar symptoms on inoculation and be re-isolated, thus fulfilling Koch's postulates (Ezeonu *et al*., 2018). There is scanty information on the pathogenicity of the isolates of *P. griseola* fungus.

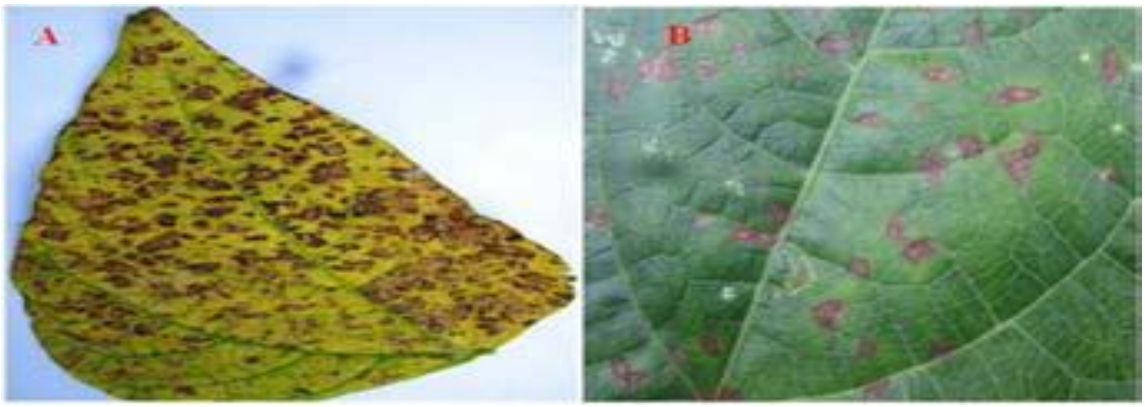


Plate 2.1: Leaf A and B infected by angular leaf spot disease. Courtesy of Allorent and Savary (2005).

Plate 2.2: Angular leaf spot disease causing severe leaf defoliation. Courtesy of Allorent and Savary (2005).

Plate 2.3: Bean pod attacked by angular leaf spot disease. Courtesy of Ddamulira *et al*. (2014).

2.9.4. Effect of angular leaf spot on growth and yield component of common bean

Late infection on pods and seeds causes scars that reduce seed quality and market value (Ddamulira *et al*., 2014). Lack of elaborate control of angular leaf spot disease has led to yield reduction of 40% -80% in have been reported. Angular leaf spot disease is basically the most important constrain to common bean production in Africa with annual loss estimated at 374,800MT (Fikre *et al*., 2011). Across farming systems, biotic and abiotic stresses continue to present the major constraints for increased bean production and high yields with bean diseases representing the major constraints to production by reducing yields and seed quality

(Ddamulira *et al*., 2014). They can cause yield loss up to 100% of the expected yield, depending on the environment and the varieties used. Pathogenic fungi are the main infectious agents in plants, causing alterations during developmental stages including post-harvest. There are very few studies on the effect of angular leaf spot disease on common bean growth and yield component.

2.9.5. Control of angular leaf spot disease

Angular leaf spot disease of common bean is managed through cultural practices such as crop rotation and cultivar mixtures (Sharma and Adikshita, 2017). However, these have limited potential in managing the disease, because land scarcity cannot allow crop rotation to be practiced. Moreover, effective methods of ALS control like use of fungicide are far beyond the means of low resource endowered farmers. This is because of the high cost and long-term consequences fungicide pose to human health and the environment (Ddamulira *et al*., 2014). Angular leaf spot can be more efficiently controlled through fungicide sprays and resistant varieties while the use of genetic resistance is the most appropriate, safe and cost-effective way to control ALS among smallholder farmers (Sartorato, 2002; Ddamulira *et al*., 2014). The use of resistance varieties is highly recommended but according to Sharma and Adikshita (2017) host resistance is difficult to maintain because the abundant virulence and pathotype diversity of *Phaeiosariopsi*s *griseola* renders varieties that are resistant in one location or year susceptible in another. A study by Sharma and Adikshita (2017) reveals that seed treatment and foliar spray with carbendazim and mancozeb either alone or in combination is frequently used for the management of angular leaf spot of beans. But continuous use of these fungicides could result in development of resistance strains and farmers are reporting the ineffectiveness of these fungicides. The use of fungicides in angular leaf spot disease control has been elaborately studies though little has been done on the effect plant extracts for the control of

angular leaf spot disease especially crude extracts of *Azadirachta indica*, *Tithonia diversifolia* and *Allium sativum*.

2.10. Plant extracts

Phytochemical study of plant is important for modern day agriculture but its usefulness cannot be overemphasized if methods are not standardized to obtain comparable and reproducible results. Plants naturally synthesize diverse group of secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs (Ramashish, 2014). In these natural sources, a series of molecules with antifungal activity against different strains of fungus have been found, which have great importance to human and plants (Cherkupally *et al*., 2017). Plants perform as a renewable natural resource of diverse bioactive compounds (Ramadass & Subramanian, 2018). Plant extracts consist of various compounds characteristic to the plant from which they were extracted. Aromatic secondary metabolites synthesized by plants are phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. Botanicals are compounds extracted from plants, which control pathogens on infected crops. Compounds with phenolic structure have high efficiency against plant pathogens because of their antimicrobial activity (Shabana *et al*., 2017). Plants have been proved as useful source of several antifungal molecules that are harmless and caring to the environment (Ramadass & Subramanian, 2018). Obongoya *et al.* (2010) reported that *Azadirachta indica* had reduced disease incidence of *Fusarium oxysporum* of common bean by about 17% while *Tagetes minuta*, *Nicotiana tobacum* and *Inca rosea* had a reduction range of 5.84-9.8%. Analysis of variance at 95% showed that all the parts (leaf, bark and seed) of the test plant (neem) used significantly ($P < 0.05$) inhibited the growth of the fungal organisms against cocoyam rot (Ezeonu *et al*., 2018).The use of botanicals for control of foliar diseases have gained importance due to the recent global awareness negative effect of chemical

fungicides, such as development of resistance, associated resurgence in fungi, accumulation of fungicide residues in food chain, environmental pollution, health risks and high costs (Ramadass & Subramanian, 2018). There are certain advantages in the deployment of botanical pesticides. These are biodegradable, safe to non-target organisms, renewable and suit to sustainability of local ecology and environment (Cherkupally *et al*., 2017). This has awakened new interest in natural products as a source for novel industrial plant protection strategies. It would be advantageous not only to standardize methods of extraction or to test the in vitro antimicrobial efficacy; but the crude extracts or the discovered compounds should be subjected to in vivo testing to evaluate the efficacy in controlling the incidence of disease in crops through pot or field experiments (Ramadass & Subramanian, 2018). It is therefore essential to carry out the complete development of an interesting lead compound into an exploitable product.

2.11. Synthetic chemicals

The adoption of chemicals has had an undeniable benefit for modern agriculture; the yields increase has been significant because of the discovery and application of synthetic measures for pest control (Dayan *et al*., 2009). Due to the continuously growing agronomy sector, there is a growing demand for fungicides and pesticides because of new and existing plant diseases, which could have an adverse impact on yields. Conventional chemicals applied in agriculture for plant disease control such as benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors are related to various problems (Stevic *et al*., 2014); it is already known that chemical fungicides and pesticides have negative effect on the environment and human health. Plant pathogens develop or have already acquired resistance to widely used chemical products. Plant growers feel a responsibility to reduce the application of pesticides as their residues could be found in their produce (Cherkupally *et al*., 2017).

2.12. Neem plant botanical and taxonomic description

Neem is a tropical evergreen tree and a and of two species of Azadirachta genus of Meliaceae family, *Azadirachta indica* A. Juss native to India and *Azadirachta excelsa*. Kack found in Indonesia and Philippines (Pankaj *et al*., 2011; Ravishankar *et al.*, 2018). Native to Indian sub-continent, *Azadirachta indica* is a member of the Mahogany family and has similar properties to its relative, *Melia azedarach* (Pankaj *et al*., 2011). It is a speedy growing tree which can grow up to 30 meters tall with an average girth of 2.5meters (Ravishankar *et al.*, 2018).

The taxonomic positions of neem are as follows:

Order: Rutales

Family: Meliaceae

Genus: *Azadirachta*

Species: *indica*

Latin: *Azadirachta indica* (Pankaj *et al*., 2011).

2.12.1 Uses of neem (*Azadirachta indica*)

In a document by Ahmed (2008) the seeds, bark and leaves contain compounds with proven antiseptic, antiviral, antipyretic, anti-inflammatory, anti-ulcer and antifungal use. *Azadirachta indica* is reported to be a natural source of eco-friendly insecticides, pesticides and agrochemicals. Leaf extracts of *Azadirachta indica* have been reported to be effective in elimination of *Fusarium spp* of African yam bean, brinjal and tomato (Obongoya *et al*., 2010). Alcoholic extracts of *Azadirachta indica* have high inhibitory effect on fungus; the fungus targeted were *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger* and *Aspergillus terreus* (Ravishankar *et al.*, 2018). To date there has been little study of *Azadirachta indica* leaves extracts for fungicidal activity against angular leaf spot disease of common bean.

2.12.2. Phytochemical compounds of *Azadirachta indica* plant

A large number of bioactive secondary metabolites, such as coumarins, alkaloids, limonoids and some essential oils have been isolated from different plant parts of neem plant (Ramashish, 2014). There are around 135 phytocompounds that have been isolated from different parts of neem tree (Ravishankar *et al.*, 2018). These phytocompounds can be divided into two different categories as shown in table 2.1(Ravishankar *et al.*, 2018). Sulphur containing compounds such as trisulphide and tetra-sulphide components of *Azadirachta indica* leaves have antifungal activity against *Trichophyton mentagrophytes* (Pankaj *et al*., 2011). The chemical constituents contain many biologically active compounds that can be extracted from neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, Carotenoids, steroids and ketones (Ahmed, 2008).

Table 2.1: Types of phytocompounds found in the various parts of neem plant

Isoprenoids	**Non- isoprenoids**
Diterpenoids and triterpenoids containing protomeliacins	Proteins (amino acods)
Limonoids (meliantrol)	Carbohydrates(polysaccharides)
Azadirone and its derivatives	Sulphurous compounds
Gedunin and its derivatives	Polyphenolics and their glycosides
Vilasinin type of compounds	Dihydrochalcone, coumarin
C- secomeliacins such as nimbin, salanin and azadirachtin	Tannins and aliphatic compounds.

2.13. Taxonomy and biology of garlic (*Allium sativum*)

It is a member of Liliaceae family having botanical name, garlic (*Allium sativum)* is a common vegetable used widely in almost all parts of the world (Byrappa, 2015; Stavělíková**,** 2008). Originally from Central Asia, garlic is one of the earliest cultivated plants (Bhandari, 2012).

The taxonomic positions of garlic are as follows:

Class: Liliopsida,

Superorder: Liliianae,

Order: Amaryllidales,

Family: Alliaceae,

Genus: *Allium*

Specie: *sativum* (Stavělíková**,** 2008).

There are several varieties of garlic but the most common varieties are white and pink garlic (Cecilia and Olubunmi, 2014). The main chemical constituent of intact garlic is the amino acid "alliin". It is an alkyl derivative of cysteine alkyl sulfoxide, responsible for the typical odour (Singh *et al*., 2015). Allicin is believed to be the natural chemical component that is responsible for antimicrobial effects of garlic (Baljeet *et al*., 2015). Byrappa (2015) stated that, family Liliaceae has more than 500 members which can be distinguished morphologically, by colour and taste though their neutral-ceutical and phytochemical composition are close. For example, extract of *Allium sativum* has antimicrobial properties because of secondary metabolites from amino acids, produced by hydrolysis (Cherkupally *et al*., 2017).

2.13.1 Uses of garlic (*Allium sativum*)

Allium sativum has been used in the treatment of worms and dysentery in children and adults. For cooking it is used for seasoning (Cecilia and Olubunmi, 2014). Allium vegetables, particularly garlic exhibit a broad antibiotic spectrum against both gram-positive and gram-negative bacteria (Stavělíková**,** 2008). The inhibitory effect of garlic is depended on its concentration (Baljeet *et al.,* 2015). Garlic has been used for hundreds of years to treat even fungal, parasitic and viral infections (Lindsey and Staden, 2004). *Allium sativum* is known for having an array of antifungal, antiviral and antibacterial properties (Baljeet *et al.,* 2015). Garlic is also known to be effective against gram-positive and gram-negative such as *Escherichia coli*, *salmonella*, *staphylococcus* and *streptococcus* species (Stavělíková**,** 2008) and against the fungal pathogens such as *Botrytis cinerea*, *Pythium ultimum* and *Rhizoctonia solani* (Lindsey and Staden, 2004).

2.13.2 Phytochemical compounds of garlic

Garlic contains more than 200 chemical compounds with at least 33 sulphur compounds, several enzymes, 17 amino acids and minerals such as selenium (Bhandari, 2012). Garlic's pungent smell and odour and many of its antimicrobial effects are due to the sulphur compounds (Bhandari, 2012). Table 2.2 shows the phytochemical compounds found in garlic.

Table 2.2: Preliminary phytochemical screening of *Allium sativum*

Phytochemical compound	**Observations**
Carbohydrates	+
Proteins	+
Amino acids	+

Volatile oil	+
Saponins	+
Terpenoids	+
Steroids	+
Enzymes	+

Key: + indicates presence - absence

2.14. Taxonomy and biology of Mexican sunflower (*Tithonia diversifolia*)

Family: Asteraceae

Synonym: *Mirasolia diversifolia* Hemsl.

Vernacular/ common names:

English: Mexican sunflower, *Tithonia*, tree marigold.

Kisii: Amaua maroro.

Luo: Maua makech, akech, maua madungo.

Luhya: Maua amalulu (Achieng' *et al*., 2010).

Tithonia diversifolia is a woody herb or succulent shrub, 1.2-3 m tall. Opposite leaves 3-5, attenuate base, acute apex and crenate margin. Leaf size is 5-17 x 5-12 cm, densely pubescent beneath and palmate venation. Occasionally upper leaves are unloaded (Ragasa *et al*., 2014). Flowers are yellow; their ray size is 306 cm x 5-18 mm plate 5. The flower heads are solitary on a peduncle 6-13 cm long as illustrated in plate 5. Each mature stem may bear several flowers at the top of branches. The plant flowers and produces seeds throughout the year. The light weight seeds can be dispersed by wind, water and animals (Anjarwalla *et al*., 2013).

Plate 2.4: *Tithonia diversifolia* plant with yellow flowers (Achieng' *et al*., 2010).

2.14.1 *Uses of Tithonia diversifolia*

Tithonia diversifolia biomass has potential for soil fertility improvement among smallholder farmers (Achieng' *et al.*, 2010). Pesticidal properties of *Tithonia* spp. are well known for sesquiterpene such as lactones and diterpenoids, some of which have biological activities against insects. *Tithonia diversifolia* has been reported to control fungi *phytophthora nicotianae* (Rejeki *et al*., 2017). Chloroform extracts and methanol extracts of *Tithonia diversofolia* have inhibitory effect on salmonella typhi and Staphylococcus (Ogundare, 2007) Most bioassays have been conducted using extracts so are not specific about which compounds are responsible for effects. In Uganda, farmers use it in field and storage pest management although there is no published work to report evidence for these effects and /or its use in the management of angular leaf spot disease.

2.14.2. Phytochemical compounds of *Tithonia diversifolia*

Phytochemical analyses of *Tithonia diversifolia* established that flavonoids and tannins are in small concentrations though alkaloids are found in higher concentrations (Ogundare, 2007; Rejeki *et al*., 2017). These bioactive compounds are usually found in high amounts in storage organs of the plants such as stems, bark, root and leaves (Ogundare, 2007). According to finding of Onaran *et al*. (2016) the plant extracts have showed a different level of antifungal activities in a dose depend manner. Substantial use of chemical pesticides induces problems of health and environmental hazards in agricultural system. So, for plants natural products of antimicrobial activity are best bio-rational alternatives today (Das *et al*., 2010). *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* extracts have been found to contain alkaloids, tannins and flavonoids which are bioactive compound that have antimicrobial activity (Rejeki *et al*., 2017).

2.15. Solvent extraction

First steps in the process of screening medicinal plants for antifungal activity is extraction. Extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular mass (Sasidharan *et al*., 2012). Extraction is an important step in the itinerary of phyto-chemical processing for the discovery of bioactive constituents from plant materials (Dhanani *et al*., 2017).most extraction methods involve collection and authentication of plant material, drying, size reduction, extraction, filtration, concentration, drying and reconstruction (Gupta *et al*., 2012) Selection of a suitable extraction technique is also important for the standardization of herbal products as it is utilized in the removal of desirable soluble constituents, leaving out those not required with the aid of the solvents (Dhanani *et al*., 2017). Differences in the structure of phenolic compounds also

determine their solubility in solvents of different polarity. Therefore, type of extraction solvent as well as the isolation procedures may have a significant impact on the yield of extraction polyphenols from plants material. According to Dhanani *et al*. (2017) selection of suitable extraction process and optimization of various parameters are critical for up-scaling purposes. Extraction efficiency of various techniques are aimed at achieving high efficacy and efficiency (Gupta *et al*., 2012). Various extraction techniques most commonly used include conventional techniques such as maceration, percolation, infusion, decoction and hot continuous extraction. Recently, alternative methods like ultrasound assisted solvent extraction (UASE), microwave assisted solvent extraction (MASE) and supercritical fluid extractions (SFE) have gained increasing interest during the last three decades (Dhanani *et al*., 2017). currently, the open microwave assisted extraction has been reported to be the simplest, convenient, and most rapid technique for extraction of thermolabile phytoconstituents (Gupta *et al*., 2012).

2.16. Choice of solvents

According to Das *et al*. (2010) successful determination of biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate. Gupta *et al*. (2012) indicated that water, ethanol, methanol, chloroform, methylene dichloride and acetone have been used to isolate antimicrobial compounds from plants which is also shared by Das *et al*. (2010) reporting that Since nearly all of the identified antimicrobial compounds from plants are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction. Thus, the most commonly used solvents for preliminary investigations of antimicrobial activity in plants are methanol, ethanol and water. A correct choice of solvent is

fundamental for obtaining an optimal extraction process. When selecting a solvent consideration should be given to the interaction of the solvent with the matrix and the analyte solubility in the solvent. Preferably the solvent should have a high selectivity towards the analyte of interest and exclude unwanted matrix components. Thus, the solvent used for the extraction of bioactive compounds must be critically chosen because it will influence the quantity and quality of the final extract (Syukriah*et al*., 2014). According to Anwar and Przybylski, (2012) polar solvents such as methanol and ethanol, either in their aqueous mixtures, are mostly recommended for the extraction of phenolics from a plant matrix. However, according to Sultana *et al.* (2009) aqueous methanol was found to be more effective in recovering highest amounts of phenolic compounds from rice bran and *Moringa oleifera* leaves.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was undertaken at Maseno University at the Botany laboratory and in the green house. Maseno University is situated in Kisumu County in the western part of Kenya with geographical co-ordinates as 0^0 00′ 17″South 34^0 36′ 02″ East and an altitude of 1,503 meters or 4,934 feet above sea level (KNBS, 2013). The climate of Maseno region is tropical with an average temperature of 20.6^{0}C and an annual rainfall of 1820mm.

3.2 Collection and preservation of *Allium sativum*, *Azadirachta indica* and *Tithonia diversifolia*

Fresh and disease-free *Allium sativum* was purchased from Kibuye market in Kisumu county and disease free and healthy *Azadirachta indica* and *Tithonia diversifolia* leaves were collected from the Maseno University botanic garden and washed under running tap water to eliminate dust and other foreign particles according to a method by Ezeonu *et al*. (2018). Taxonomic identification and authentication of plant specimens was performed by taxonomist at the Department of Botany–Herbarium, Maseno University, Kenya. *Allium sativum* bulbs were separated, washed and sliced to await the drying process according to a modified method of Baljeet *et al*. (2015). The bulbs of *Allium sativum*, leaves of both *Azadirachta indica* and *Tithonia diversifolia* were used in the experiment due to the fact that they contain high levels of active ingredient needed for pathogen control (Baljeet *et al*., 2015; Obongoya *et al*., 2010). Voucher specimens were labelled and brought to the laboratory then stored in the refrigerator at 4°C till use.

3.3 Collection of *Phaeoisariopsis griseola* fungus from Sabatia, Ugenya and Bondo regions

Common bean plants were sampled randomly from the farmers fields based on the typical symptoms of angular leaf spot disease of common bean. The aerial parts of the plant especially the leaves were checked for small, dark brown and numerous spots with angular shape limited by the veins. The diseased leaves were collected from three bean growing ecological zones of upper midland zone 1of Sabatia sub-county in Vihiga county (0^0 31′ 00″North 34^0 34′ 30″ East), lower midland zone 3 of Ugenya sub-county in Siaya county (0^0 10′ 56.2944″North 34^0 17′ 47.9688″ East and low midland zone 4 of Bondo sub-county in Siaya county, 0^0 09′ 73″South 34^0 27′ 64″ East. The sampled diseased plant parts were then zip locked in airtight polyethene bags and placed in ice cooler boxes before they were transported by road to the laboratory. Little information is available on the isolates of *Phaeoisariopsis griseola* pathogen in the selected zones (Leitich *et al*., 2016). The collected leaf samples were washed using tap running water and placed in 2% sodium hypochlorite for 2 minutes then blotted by arranging in between absorbent newspapers and pressed carefully to absorb the moisture and distribute pressure evenly across the samples to keep them intact to avoid any breakage (Leitich *et al*., 2016). The samples were stored at 4 ^{o}C to await isolation.

3.4. Sterilization of materials

All glass wares used in this study were washed with detergent, rinsed and sterilized in a dry, ventilated oven at 160 °C for 2 h. All media were sterilized by autoclaving at a temperature of 121 °C and 15 psi for 20 min. The scalpel and inoculating needle were sterilized by dipping them into 70% ethanol and passing them over a Bunsen burner flame until red hot according to a method by Ezeonu *et al*. (2018).

3.5. Preparation of culture medium

Throughout the *in vitro* experiment, the assayed culture medium employed was LAB M Potato Dextrose Agar (PDA). This medium was used for the growth and maintenance of the fungal isolates. The preparation of Potato Dextrose Agar (PDA) was done according to the manufacturer recipe. The medium was sterilized by autoclaving at 121 ℃ and 15 PSI for 20 min for complete dissolution and homogeneity (Leitich *et al*., 2016). Thereafter, it was allowed to cool to temperature of between 42 and 45 ℃. One capsule of chloramphenicol was added to every 500 ml of sterile cooled PDA so as to prevent bacteria growth. Approximately 15 ml of the cooled amended PDA was poured into each sterile petri dish of 8.6 cm (86 mm) diameter to solidify. The petri dishes that contained the medium were incubated for 24 h at ambient temperature (28 ℃) to check for sterility before use as described by Ezeonu *et al*. (2018). The pathogenicity test was carried out to establish which of the fungal isolates caused the angular leaf spot and to determine whether they could induce similar symptoms on inoculation and be re-isolated, thus fulfilling Koch's postulates (Ezeonu *et al*., 2018).

3.6 Isolation of *Phaeoisariopsis griseola* and inoculum preparation

Diseased leaves obtained from the different localities received similar treatments separately where they were rinsed in three changes of sterile distilled water and small sections of the common bean leaf tissue showing small, angular and dark brown spots on the leaves adjoining healthy tissue were cut using sterilized scalpel and whose surface was sterilized with 70% ethanol (Leitich *et al*., 2016). The cut portions were plated out on solidified PDA. Three cut portions were placed per plate with equal distance between them. Four replicate plates for each of the cut portions were made for each of the isolates. The plates were incubated at 24ºC for 7 days in a non-illuminated incubator for germinate and sub-cultured immediately until a pure

culture of the isolates were obtained and stored in McCartney bottles for further use. Sub culturing of the isolates was made to obtain pure culture. The colonies growing on the plates were identified macroscopically and microscopically (Leitich *et al*., 2016). Direct observation of culture under the light microscope (low power) by careful preparation of slides, staining with cotton blue-in-lactophenol was done according to Ezeonu *et al*. (2018). Identification was done using manuals and guides according to Alexopoulos (1962). Spores from the plates were obtained by adding sterile water and transferred onto potato dextrose agar to obtain cultures of the fungus. Inoculating plates were incubated at 24°C for 14 days.

3.7 Morphological profiling of *Phaeiosariopsis griseola* isolates from Sabatia, Ugenya and Bondo regions

3.7.1 Cultural characteristics

The fungal suspensions of the three samples were separately isolated and purified on Potato Dextrose Agar to obtain pure culture then inoculated on PDA media to study the culture appearance, consistency and elevation. Cultural characteristics (surface and reverse) were assessed after 14 days on PDA at 25 °C in the dark according to Leitich *et al*. (2016).

3.7.2 Lactophenol blue staining (mycelial morphology)

Mycelial morphology was monitored by light microscopy. Thin smears for the three sample plates were prepared separately, heat fixed then flooded with lactophenol blue reagent for one minute. The stain was washed off with tap water and the slides allowed to air dry before being examined microscopically under x400 magnification to reveal microscopic characteristics of the fungus. The hyphae could be either septate or aseptate. Colony colours (surface and reverse) were assessed after 14 days on PDA at 25 °C in the dark (Crous *et al*., 2006).

3.8. Pathogenicity test

Considering the importance of correct identification of plant pathogens, pathogenicity test was carried out to establish whether the fungal isolates caused angular leaf spot disease and to determine whether they could induce similar symptoms on inoculation and be re-isolated, thus fulfilling Koch's postulates (Ezeonu *et al*., 2018). Sabatia, Bondo and Ugenya isolates of *Phaeiosariopsis griseola* causing angular leaf spot disease in common bean was identified following pathogenicity approach. Virulence analysis of Sabatia, Bondo and Ugenya isolates was carried out on common bean. Common bean seeds were planted in ten plastic pots containing 6kg of solarised soil and watered twice daily until after the tri-foliate leaf was fully formed. The fungal inoculum of 1ml from PDA media was serially diluted and about 5 millilitres of 10^{-4} suspension of the fungus inoculum was forced into the underside of three different leaves per pot using hypodermic syringe without a needle. Five millilitres of sterile water were also infiltrated in some common bean plants as controls and left for 48hrs to test for the virulence of the fungus organism according to Emitaro *et al*. (2017). Plants were placed in the greenhouse and observed daily. The inoculated and the uninoculated plants were covered in plastic bags to maintain humidity at its maximum (Narasimha and Srinivas, 2012). Each experiment was repeated twice, with two replications of 10 plants per treatment. Observations were made from one week after inoculation. The pathogens were re-isolated as previously described and their cultural and morphological characteristics were compared with those of the original isolates.

3.9. Phytochemical screening of *Allium sativum, Azadirachta indica* and *Tithonia diversifolia*

The screening was carried out in the Botany laboratory of Maseno university, Kenya. The plant parts were collected from the Botanical Garden of Maseno University and Kibuye market,

identified taxonomically with taxonomists at the Department of Botany Maseno university. The plant parts were thoroughly washed then dried under shade until physiological dryness and then pulverised using the lab mill. The final product was then kept inside tight paper bags to await phytochemical screening. *Allium sativum, Azadirachta indica* and *Tithonia diversifolia* were evaluated for the presence of alkaloids, flavonoids, sterols, cardiac glycosides, saponins, tannins and terpenoids using standard procedures. The phytochemical tests were conducted using standard procedures to identify the constituents as described by Mibei *et al*. (2012); Parithra *et al*. (2017).

3.9.1. Test for tannins

Ferric chloride's Test

Half a gram of dried powder of the selected plant parts were added to 20ml of water in a test tube and boiled for 2 min then filtered using Whatman filter paper to obtain filtrate. A drop of 0.1% ferric chloride was added to the filtrate and observations were made.

3.9.2. Test for saponins

Froth test

Two grams of powdered plant parts was mixed with 20ml of distilled water in a boiling tube then place in a hot water bath for 5 min. Filtrate was obtained by using Whatman filter paper. Ten millilitres of the filtrate were mixed with distilled water and then observations were made.

3.9.3. Test for flavonoids

Alkaline reagent test

A boiling tube of 5g of powdered plant samples with 10mls of ethyl acetate was heated on steam bath for 3 minutes then filtered. 4mls of the filtrate was shaken with 1ml of dilute ammonium solution and observations were made.

3.9.4. Test for terpenoids

Salkowski's test

Five grams of powdered plant samples was placed in test tubes, and 2mls of chloroform added then 3mls of 0.1M sulphuric acid according to and observations were made.

3.9.5. Test for sterols

Salkowski's test

Five grams of powdered plant samples was be added to 10mls of 80% ethanol and boiled in a water bath for two minutes. Filtrate was obtained then concentrated to crystals. Zero point five grams (0.5g) of the ethanoic extract was added to 2mls of acetic anhydride with 2mls of 0.1M sulphuric acid in test tubes and then observations were made.

3.9.6. Test for cardiac glycosides

Kellar-Kiliani test

Five grams of powdered samples was added to 10mls of water in test tubes the heated in water bath for 2 min and then filtered to obtain an extract. Five millilitres of each extract were added to 2mls glacial acetic acid containing one drop of 0.1% ferric chloride solution. Concentrated hydrochloric acid was then added and then observations were made.

3.9.7. Test for alkaloids

Mayer's Test

Two grams of dried plant powder was mixed with 40ml of 0.1M hydrochloric acid in a boiling tube then heat in water bath for 10 minutes. It was cooled then filtered. To the portion of the filtrate a few drops of mayor's reagent were added and observations were made.

3.10. Preparation of crude extracts from *Allium sativum*, *Azadirachta indica* and *Tithonia diversifolia*

Ten kilograms each of fresh *Allium sativum* bulbs, leaves of *Azadirachta indica* and *Tithonia diversifolia* were washed thoroughly under running tap water and soaked in 2% solution of sodium hypochlorite for 5 minutes then rinsed thoroughly with sterilized distilled water (Narayana *et al*., 2018). Garlic cloves were slice cut in thin slices to fasten the drying process and all air-dried at room temperature until complete dryness was achieved. The dried *Allium sativum*, *Azadirachta indica* and *Tithonia diversifolia* were ground into fine powder using a laboratory blending machine. Crystal dried garlic slices were ground using mill grinder at the Maseno university farm and their fine powder stored in brown airtight paper bags to prevent moisture from getting in at room temperature to await extraction. Two hundred grams of *Allium sativum*, *Azadirachta indica* and *Tithonia diversifolia* were weighed separately using a top loading balance and each transferred into nine 500ml conical flasks. Five hundred millilitres of solvents of methanol, ethanol and distilled water was added and left at room temperature in the dark for 7 days for the extraction of the bioactive compounds according to the method of Byrappa (2015). The macerated plant tissues were separated from the aqueous solution by filtering using a muslin cloth then re-filtered using Whatman filter paper No. 1. The filtrate was then sterilized by serially filtering through a 0.45 micrometre-pore-size filter and then a 0.22 micrometre-pore-size filter and the filtrate evaporated to dryness to obtain crystals by placing them in water bath at 35^{o}C overnight and then freeze-dried into fine powder for long term storage and kept at 4^{o}C in the dark (Baba and Malik, 2015). When needed the extract was reconstituted by dissolving in water at 5g extract in 10ml water to make the stock solution and stored in the dark at 4^{o}C. One hundred percent of the extract concentration was achieved by measuring 10lms of the stock solution using a measuring cylinder, 75% extract concentration was obtained by measuring 7.5mls of the stock solution plus 2.5mls of water, 50% extract

concentration was achieved by measuring 5mls of the stock solution and 5mls of water, and 0% was achieved by measuring 10mls of sterile water which was used in the experiment as negative control and kept at 4^{o}C until they were required for use according to Narayana *et al*. (2018).

3.11. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on spore germination obtained different solvent extracts of distilled water, methanol and ethanol at 50, 75 and 100% concentrations

Antifungal activity of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* extracts was determined using the paper disc diffusion method of Esmaeili *et al*. (2013). Ten millilitres of Potato Dextrose Agar were poured per petri dish of the 144-petri dish. The fungal growth was adjusted to 0.1 of dilution potato dextrose broth. 5ml of autoclaved 0.01% Tween-20 was added to scintillation vails containing the fungus and vortex thoroughly for 1minute. Wide-bore pipette tip was then used to dilute the inoculum by transferring two separate 0.1ml aliquots of spore suspension into 2ml Eppendorf tubes containing 1.8ml of 0.01% Tween-20 after which each aliquot was enumerated. Zero point one millilitres (0.1mls) of the inoculum suspension contained approximately 10^{8} fungus/ml which were poured over the agar in the petri plates and dispersed using sterile cotton swab. Sterile filter paper discs of 6mm diameter in dimension were soaked for 30 seconds in 10ml of plant extracts in sterile petri dishes at concentrations of 50, 75 and 100%, and immediately introduced into the centre using sterile mounting needle and forceps. Sterile water was used as negative control while synthetic fungicide (AMISTAR TOP) was used as the positive control. Inhibition zones were measured using ruler (mm) to determine the inhibition effect of the extract on the spore germination after incubation at 27^{o}C for 72 hours. All the treatments were replicated four times.

3.12 Determination for the effects of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* extracts on growth component of common bean Var. GLP 1127 Mwezi moja

In vivo evaluations were conducted at Maseno University greenhouse to evaluate the fungicidal effects of methanolic extracts of Azadirachta *indica, Tithonia diversifolia* and *Allium sativum* on angular leaf spot disease of common bean. Certified seeds of GLP 1127 "Mwezi moja" were purchased from certified Simlaw seed dealer in Kisumu which were planted in plastic pots of diameter 20cm containing 6kg of solar sterilised sandy-loam soil collected in Maseno University farm with four holes drilled at the bottom to allow for effective drainage. The pots were arranged on greenhouse floor at 60cm distance from each other horizontally and 30cm from each other under uncontrolled environmental conditions and watered overhead twice a day using micro sprinklers to ensure good germination and growth. Soil was watered and four seeds of GLP 1127 Mwezi moja sowed per pot of which two were thinned immediately after the tri-foliar stage of which two well grown bean plants were during data collection. A basal dose of triple superphosphate granules fertilizer was applied during sowing at the rate of 4g/hole to all the experimental plots. Twenty-eight days after sowing, the selection and tagging were done then all plants inoculated with 60ml suspension 2.5 x 10^6 spore/ml of *Phaeoisariopsis griseola* which was obtained by scraping cultured plates in about 5ml of autoclaved 0.01% tween-20 solution to liberate spores. Mycelium were removed by gravity filtering through at least 4 layers of autoclaved muslin cloth. Spore concentration was calculated with hemocytometer and adjusted to 60ml suspension 2.5 x 10^6 spore/ml and foliar sprayed using a hand-held sprayer. 7days after disease inoculation, 100% concentrated methanolic extracts of *Tithonia diversifolia*, *Azadirachta indica* and *Allium sativum* were sprayed on the common bean, some plants were sprayed with sterile water was as negative control while others were sprayed with synthetic fungicide (AMISTAR TOP) as positive control (Obongoya *et al*., 2010). The experiment was laid out in a complete randomized design

with four replicates. Data was then collected after every seven days till physiological maturity. Methanolic extract at 100% concentration was used for all the greenhouse test since it had shown the best results during the *in vitro* experiment.

3.12.1 Growth

Data on growth was collected for quantitative indicator of plant growth. Components of growth were plant height and stem diameter.

3.12.1.1 Plant height

Plant height (H) was measured using a meter ruler in centimetres from the soil surface at the base of the stem to the furthest point vertically. Data on plant height was collected from two sampled plants every seven days from the 20^{th} day after sowing until physiological maturity.

3.12.1.2 Plant stem diameter

Plant stem diameter was measured using a Vernier calliper in centimetres. Measurement was taken at the top just below the first branch(W1) and slightly above the ground(W2). Two plants were tagged and the points marked with permanent ink. Data was collected every 7 days from the 20^{th} day after sowing until physiological maturity.

Data on plant height and plant stem diameter were used to calculate growth index as a measure of growth in centimetres. The growth index was calculated as follows:

GI=(H+(W1+W2)/2)2

Where; GI = growth index

H = plant height (cm)

W1 = top stem diameter measurement (cm)

W2 = bottom stem diameter measurement (cm), according to method by Irmak *et al*. (2004).

3.13. Determination of the effect of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* extracts on yield component of common bean Var. GLP 1127 Mwezi moja

Yield component involved taking weights of common bean pods in grams and number of seeds per treatment harvested at physiological maturity (Muthomi *et al*., 2017). The pods from two common bean plants per pot were harvested and weighed using a top pan weighing balance and readings recorded in grams according to a method by Wahome *et al*. (2011). Whole plant fresh weight was obtained by uprooting two plants from the pot after watering the soil thoroughly. The remaining soil made into water-soil consistency then sieved to obtain parts of the roots that might have remained in the soil then data on whole plant fresh weight collected in grams using a top pan weighing balance (Muthomi *et al*., 2017). The plants were then dried in the oven at 33^{O}C for three days to obtain data on whole plant dry weight in grams. The data was collected and mean computed for the fresh weight and dry weight in grams (Muthomi *et al*., 2017).

3.14. Assessment of the effect of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* extracts on disease index of common bean Var. GLP 1127 Mwezi moja

Disease index was obtained by collecting data on disease incidence and disease severity. Two plants per pot were tagged for disease assessment. These were done by scoring three trifoliate leaves sampled at the bottom, middle and top of each plant (Wahome *et al*., 2011). The total disease index was computed using score of incidence and severity. Percentage disease indices was calculated using the formula according to Muthomi *et al*. (2017).

$$\text{Disease index (\%)} = \frac{\text{Incidence score + severity score}}{\text{Maximum incidence +maximum severity}} \times 100$$

3.14.1 Assessment of the effect of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* extracts on disease incidence on common bean

The assessment of disease incidence from all the treatments involved the counting of all diseased plants that showed symptoms of angular leaf spot infection. Data on disease incidence commenced 7 days from pathogen inoculation and collected after every 7 days until physiological maturity and the percentage of the disease incidence calculated according to the formula by Muthomi *et al*. (2017).

Percentage disease incidence = (number of infected plants/total number of plants) x 100

Scoring was done based on a rating scale, where;

Low incidence = 1-20%

Moderate incidence = 21-49%

High incidence = 50-100%

3.14.2 Assessment of the effect of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* extracts on disease severity on common bean

Disease severity is the measure of impact of the disease on a plant and involved determination of the percentage of leaf area showing disease symptoms against the whole leaf area in a plant per treatment. Disease severity was assessed at flowering, pod formation, grain filling and physiological maturity stage (Muthomi *et al*., 2017). Data on disease severity commenced 7days from pathogen inoculation and was collected until physiological maturity using a standard scale of 0-5 scale according to Fikre *et al*. (2011).

To determine disease severity a scale of 0-5 was applied.

where;

0 = no disease,

1 = <20% of the leaf area infected,

2 = 21-40% of the leaf area infected,

3 = 41-60% of the leaf area infected,

4 = 61-80% of the leaf area infected

and 5 = 81-100% of the leaf area infected

3.15. Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) helps in identifying the exact extract concentration required to inhibit fungal spore germination under controlled conditions. MIC of the extracts was determined by using broth dilution. The minimum inhibitory concentration was defined as the lowest concentration able to completely inhibit any visible microorganism growth after overnight incubation with media (Yahaya *et al*., 2017). Serial dilution of the extracts in liquid medium were prepared. One millilitre of the prepared broth was dispensed into the test tubes labelled A to D using sterile syringe and needle. A sock solution of 100mg/ml of the extracts was prepared. Then, 1.0ml of the solution was dispensed into test tube A. Subsequently, from tube A, solution was serially transferred until test tube D of 1.0ml of the solution were discarded from it. An overnight culture of the test isolate was prepared in sterile PDA agar. 1ml inoculum was transferred into each test tube from A to D. the final concentration of the extract in each of the test tubes numbered after dilution 100, 50, 25 and 2.5 mg/ml was incubated at 37^{o}C for

24hrs and examined for spore germination. To measure the MIC values, various concentrations of the stock at 12.5, 25, 50 and 100 mg/ml were assayed against the fungus.

3.16. Disposal of experimental materials

Experimental materials used in characterization and fungicidal activity tests were autoclaved to kill the fungus before being washed in detergent. Infected plant materials from the greenhouse were uprooted and burnt to eradicate and prevent spread of the fungus.

3.17. Data analysis

Statistical analysis of the data was conducted using R version 3.6.1 package. Data on inhibition effects of the plant extracts on in vitro spore germination of *Phaeiosariopsis griseola*, effect of *Phaeiosariopsis griseola* on common bean: plant growth, yield component and disease index in the greenhouse were analysed using analysis of variance (ANOVA). Treatment means were separated and compared at P=0.05.

CHAPTER FOUR

RESULTS

4.1. Morphological profiling of *Phaeiosariopsis griseola* isolates from Sabatia, Ugenya and Bondo regions

Morphological characterisation was based on the cultural characteristics and mycelia morphology. Bondo, Sabatia and Ugenya isolates had similar cultural characteristics of flat compact colonies, white at the start then become black homogenously and a grey underside. Microscopically, the pathogen isolated from Sabatia sub-county was observed to be aseptate and hyphae with erect, simple and thick walled conidiophore bearing a head and black in colour while its spores were elliptical in shape, double-walled, unicellular, grey in colour and non-attached as illustrated in plate 4.2c and 4.2d, and table 4.1. Ugenya isolate was aseptate and several had fragmented hyphae with thin walled conidiophores bearing heads split into several loose conidial columns and black in colour while spores were found to be circular, unicellular, double-walled, grey and no attachment as shown in plates 4.3e and 4.3f, and in table 4.1. *Phaeiosariopsis griseola* isolate from Bondo sub-county was observed to be septate and hyphae with erect, simple and thick-walled conidiophore bearing a head and black in colour while spores appeared lemon shaped, grey, unicellular, double walled and non-attached as illustrated in plates 4.1a and 4.1b and table 4.1.

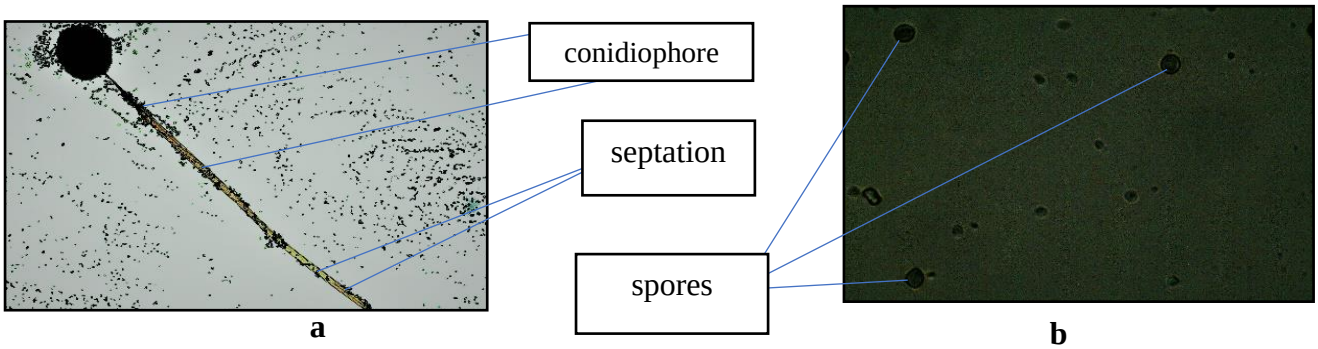


Plate 4.1: Bondo isolate hyphae (a) and Bondo isolate spores (b) mg. x400 showing morphological characteristics.

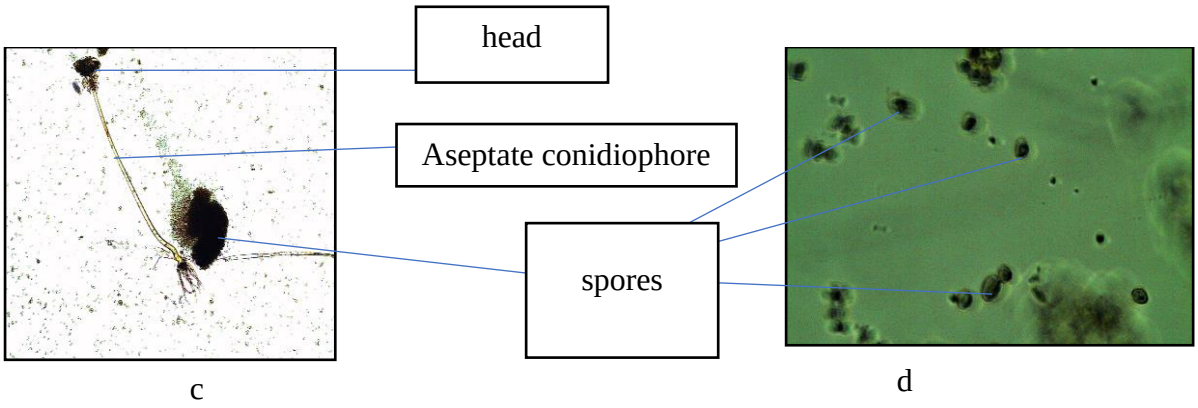


Plate 4.2: Sabatia isolate hyphae (c) and Sabatia isolate spores (d) mg. x400

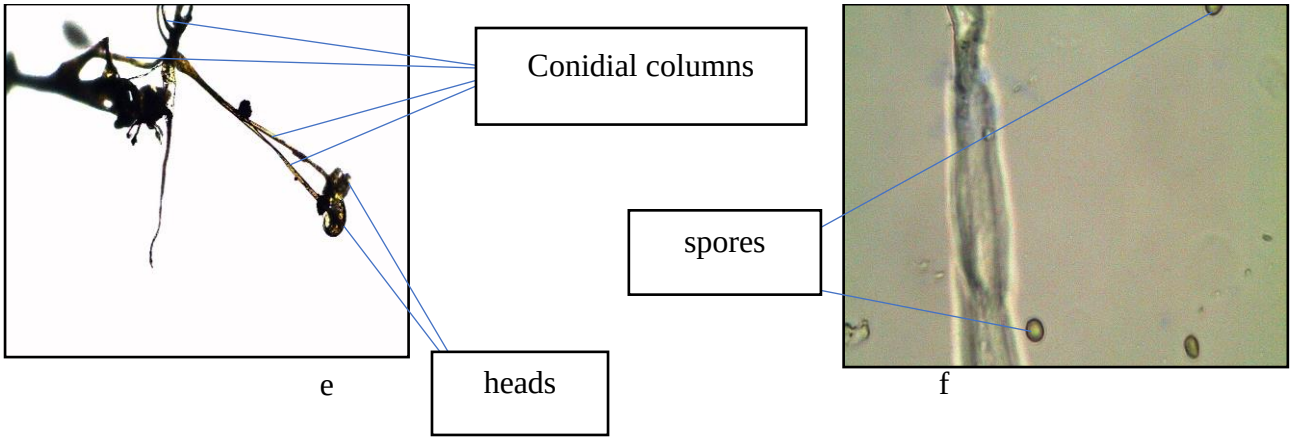


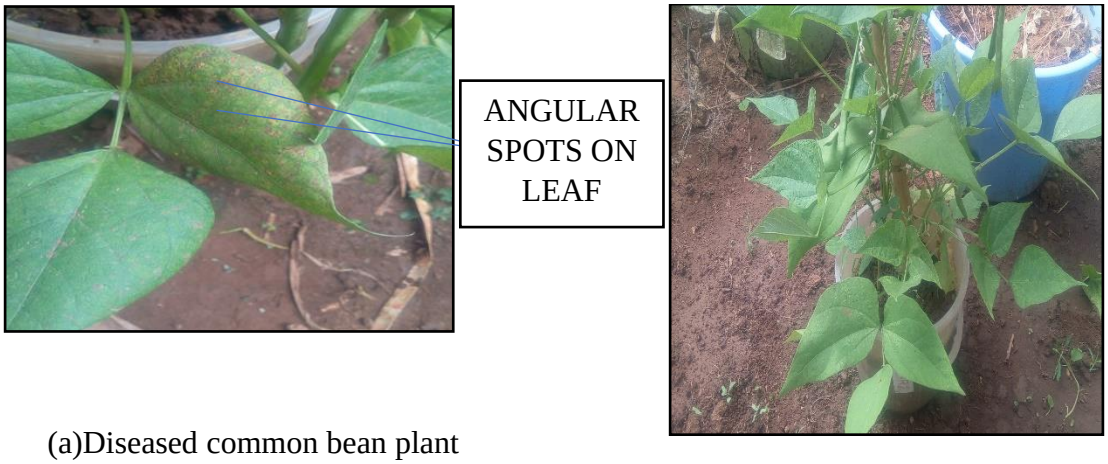
Plate 4.3: Ugenya isolate hyphae (e) and Ugenya isolate spores (f) mg. x400

Table 4.1. Morphological characterisation of isolates of *Phaeiosariopsis griseola*

Hyphae characteristics	**Sabatia isolate**	**Ugenya isolate**	**Bondo isolate**
Septation	aseptate	aseptate	Septate
Colour	Black	Black	Black
Spore characteristics			
Spore shape	elliptical	circular	lemon-shaped
Number of cells	unicellular	unicellular	Unicellular
Spore colour	Grey	Grey	Grey
Wall characteristics	double	Double	Double

4.2. Test of pathogenicity of the isolates

Phaeiosariopsis griseola was isolated from Sabatia, Bondo and Ugenya common bean plants and its pathogenicity was confirmed by verification of Koch's postulations. Common bean plants inoculated with Sabatia, Bondo and Ugenya isolates showed typical angular leaf spot symptoms of small, angular, dark brown and numerous spots on the leaves, the interaction was considered pathogenic as shown in plate 4.4 (a). At first, the lesions were grey, later turn brown and attain an angular shape because of limitation by veins. On all lesions, dark stroma appeared in abundance. Angular leaf spot disease causes typical symptoms on the leaves with angular shaped lesions as well as lesion multiplication and extension on the foliage (Allorent and Savary, 2005; Bode *et al*., 2017). The uninoculated common bean plants showed no symptoms of angular leaf spot disease hence served as control as shown in plate 4.4 (b). There was indeed high virulence of the Bondo, Sabatia and Ugenya isolates and thus great pathogenicity of fungi examined. Data document that Sabatia isolate was highly pathogenic and caused the highest disease severity. Ugenya isolate exhibited the lowest disease severity on common bean plants followed by Bondo isolate. On the basis of this result, Sabatia isolate was used in subsequent experiments.



(a)Diseased common bean plant

(b)Healthy common bean plant

Plate 4.4. (a)Symptoms of angular leaf spot disease on diseased common bean plant and (b)a healthy plant. Photos courtesy of Simon Meso.

4.3. Phytochemical screening of *Azadirachta indica, Tithonia diversifolia* and *Allium sativum*

4.3.1 Test for tannins

The test for tannins in *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* there was change in colour to brownish green of black which indicated a positive test as presented in table 4.2.

4.3.2 Test for saponins

There was formation of emulsion which indicated the presence of saponins in *Azadirachta indica* and *Allium sativum* unlike for Tithonia *diversifolia* where no formation of emulsion was observed which indicated the absence of saponins as presented in table 4.2.

4.3.3Test for flavonoids

There was no colour change which indicated a negative test for flavonoids in *Azadirachta indica* unlike for *Tithonia diversifolia* and *Allium sativum* where there was colour change of yellow colouration which indicated a positive test as shown in table 4.2.

4.3.4 Test for terpenoids

There was the formation of an interface of reddish-brown colouration that indicated the presence of terpenoids in *Azadirachta indica, Tithonia diversifolia* and *Allium sativum* as presented in table 4.2.

4.3.5 Test for sterols

There was a colour change from violet to blue which indicated a positive test for sterols in *Azadirachta indica* and *Allium sativum* while the colour remained violet for *Tithonia diversifolia* which indicated the absence of sterols as shown in table 4.2.

4.3.6 Test for cardiac glycosides

A violet ring appeared below the acetic acid layer and a greenish ring also formed gradually throughout the thin layer. This indicated the presence of cardiac glycosides in *Azadirachta indica, Tithonia diversifolia* and *Allium sativum as* presented in table 4.2. and appendix 5.

4.3.7 Test for alkaloids

The formation of a slightly turbid of heavy precipitate indicated the presence of alkaloids in *Azadirachta indica* and *Allium sativum* unlike *Tithonia diversifolia* where no formation of turbidity was observed which indicated the absence of alkaloids as shown in table 4.2.

Table 4.2. Qualitative phytochemical screening of *Azadirachta indica, Tithonia diversifolia,* and *Allium sativum* as suggested above

	Plant material		
Phytochemical	*Azadirachta indica*	*Tithonia diversifolia*	*Allium sativum*
Tannins	+	+	+
Saponins	+	_	+
Flavonoids	_	+	+
Terpenoids	+	+	+

Sterols	+	_	+
Cardiac glycosides	+	+	+
Alkaloids	+	-	+

Key= + present; - absent

4.4.0 Effect of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on spore germination obtained using different solvent extracts of distilled water, methanol and ethanol

The effect of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on spore germination is presented in table 4.3. Three types of concentration 50%, 75% and 100 % of the extracts were used to test the spore germinate of *Phaeiosariopsis griseola* obtained from methanol, ethanol and water as solvents. *Allium sativum* methanolic extract had the highest effect on spore germination at 100% concentration followed by *Tithonia diversifolia* ethanoic extract at 100% concentration, both methanolic and ethanoic extracts of *Azadirachta indica* at 100% concentration also showed high efficacy on fungus spore germination inhibition compared to synthetic fungicide (AMISTAR TOP) and distilled water (control) as presented in table 4.3. All the extracts at 50%, 75% and 100% concentrations performed better than the synthetic fungicide (AMISTAR TOP). Sterile water (control) had no effect on fungus spore germination. All the plant extracts at 100% concentration showed the highest activity on spore germination followed with 75% while 50% extract concentration had the least fungicidal activity. Methanolic extracts of all the plants showed more effectiveness than ethanoic extract. Water extracts had the least activity. The findings showed that all the treatments differed significantly compared to the controls on spore germination *in vitro,* the most effective was methanolic *Allium sativum* extract at 100% concentration as shown in table 4.3.

Table 4.3. Effect of *Azadirachta indica*, *Allium sativum* and *Tithonia diversifolia* crude extracts on spore germination using different solvent extracts of distilled water, methanol and ethanol at different extract concentration of 50%, 75% and 100%, sterile water and synthetic fungicide (AMISTAR TOP)

Plant extract	Concentration (%)	Water	Ethanol	Methanol
		Inhibition zones (mm)		
Allium sativum	50	6.63±0.03d	9.09±0.04cd	9.03±0.04c
	75	14.34±0.05c	14.89±0.05c	20.70±0.07b
	100	16.77±0.06ab	20.95±0.06ab	26.10±0.11a
Azadirachta indica	50	7.97±0.0cd	6.79±0.03cd	7.30±0.04cd
	75	16.08±0.0b	14.89±0.05b	19.70±0.06b
	100	18.51±0.0ab	22.28±0.12a	22.95±0.12a
Tithonia diversifolia	50	8.74±0.04cd	10.59±0.05c	11.18±0.05c
	75	17.00±0.06b	16.21±0.06b	21.01±0.06b
	100	16.77±0.06ab	21.29±0.10ab	24.62±0.09a
Control (sterile water)	0%	6.0±0e	6.0±0e	6.0±0e
Fungicide (AMISTAR TOP)	MP	6.4±0.07d	6.4±0.07d	6.4±0.07d

Legend: Means followed by different letters down the columns differ significantly at P=0.05 by Duncan test. ± standard error. Each value is an average of four replicates.

4.4.1 Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic extracts at 100% concentration on growth of *Phaeiosariopsis griseola* fungal isolates obtained from Sabatia, Ugenya and Bondo sub-counties

The highest fungal growth reduction (inhibition) was observed on fungal petri plates cultured with pathogen isolated from Ugenya sub-county followed by Bondo isolate and Sabatia isolate which was least inhibited by the methanolic plant extracts at 100% concentration respectively. Variability was also highest in plants inoculated with Ugenya pathogen isolate as shown in table 4.4. Sabatia, Bondo and Ugenya isolates showed significant differences in their response to methanolic extracts of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* P=00.5.

Table 4.4. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentration on growth of *Phaeiosariopsis griseola* fungus obtained from Sabatia, Ugenya and Bondo sub-counties

Pathogen isolates	**Inhibition zone(mm)**
Ugenya	7.00±0.06a
Bondo	6.79±0.05b
Sabatia	6.56±0.03c
LSD	6.78

Legend: Means followed by different letters down the column are statistically different at P=0.05 by Duncan test. ± standard error.

4.4.2 The effects of extraction potential of methanol, ethanol and water solvents on the fungicidal activity of plant extracts on Ugenya isolate of *Phaeiosariopsis griseola* of common bean

The highest extraction potential was observed when methanol solvent, which was followed by ethanol while water solvent had the least extraction potential as shown in table 4.5. Interaction effect between extracts and pathogen, and solvent all had a significant effect on spore germination. Methanol, ethanol and sterile water differed significantly on their extraction potential as presented in table 4.5.

Table 4.5. The effects of extraction potential of methanol, ethanol and water solvents on the fungicidal activity of plant extracts on Ugenya isolate of *Phaeiosariopsis griseola*

Solvents	**Inhibition zone(mm)**
Methanol	7.03±0.06a
Ethanol	6.79±0.05b
Sterile water	6.63±0.04c
LSD	6.82

Legend: Means followed by different letters down the column are statistically different at p=0.05 by Duncan test. ± standard error.

4.5.0 Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on the growth and yield components of common bean Var. GLP 1127 Mwezi moja infected with Ugenya isolate *Phaeoisariopsis griseola*

Methanolic plant extracts of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* all had significant effects on growth index P<0.001. Synthetic fungicide (AMISTAR TOP) had

the highest significant effect followed by *Allium sativum* had higher significant effect, then *Tithonia diversifolia* and lastly *Azadirachta indica* on growth index compared to the negative control (sterile water). Sterile water (control) had the least effect on growth index. Thus, Synthetic fungicide (AMISTAR TOP) differed significantly with *Allium sativum, Azadirachta indica* and *Tithonia diversifolia* compared to the negative control. *Allium sativum, Azadirachta indica* and *Tithonia diversifolia* differed insignificantly compared to the negative control as presented in table 4.6.

Table 4.6. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on growth components of common bean Var. GLP 1127 Mwezi moja infected with Ugenya *Phaeoisariopsis griseola* isolate

Plant extracts	**Growth index(cm)**
Azadirachta indica	36.74±1.12ab
Tithonia diversifolia	34.63±1.82ab
Allium sativum	33.48±1.22b
Fungicide (AMISTAR TOP)	28.8c
Control (sterile water)	39.88±0.45a
LSD	34.84

Legend: Means followed by different letters down the column are statistically different at P=0.05 by Duncan test**.** ± standard error.

4.6.0 Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on pod weight of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Methanolic extracts of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* had no significant effect (P=0.96) on pod weight as shown in table 4.7. *Allium sativum* performed better than *Tithonia diversifolia* and *Azadirachta indica.* The effect of the synthetic fungicide (AMISTAR TOP), and *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* extracts all differed insignificant compared to the negative control.

Table 4.7. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on pod weight of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Plant extracts	**Pod weight (g)**
Allium sativum	15.31±2.13a
Azadirachta indica	14.43±2.76a
Tithonia diversifolia	14.09±1.83a
Fungicide (AMISTAR TOP)	14.95±1.2a
Control (sterile water)	12.05±2.81a
LSD	14.41

Legend: Means followed by different letters down the column are statistically different at p=0.05 by Duncan test**.** ± standard error.

4.6.1 Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on number of seeds of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Allium sativum extract had highest significant effect (*P*=0.03) on number of seeds followed by synthetic fungicide (AMISTAR TOP). *Azadirachta indica* and *Tithonia diversifolia* extracts had least significant effect on number of seeds compared to sterile water (control). The effect of *Allium sativum* and synthetic fungicide (AMISTAR TOP) on seed number did not differ significantly while the effect of their effects differed significantly with those of *Azadirachta indica* and *Tithonia diversifolia* compared to the negative control (sterile water). The effects of *Azadirachta indica* and *Tithonia diversifolia* on seed number differed insignificantly as shown in table 4.8.

Table 4.8. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on number of seeds of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Plant extract	**Number of seeds**
Allium sativum	36.25±4.17a
Azadirachta indica	27.0±2.83b
Tithonia diversifolia	26.08±2.79b
Fungicide (AMISTAR TOP)	34.5±3.12a
Control (sterile water)	21.25±3.73c
LSD	29.43

Legend: Means followed by different letters down the column are statistically different at p=0.05 by Duncan test**.** ± standard error.

4.6.2 Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on whole plant fresh weight of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Synthetic fungicide (AMISTAR TOP) had highest effect on whole plant fresh weight followed by Azadirachta *indica, Allium sativum* and *Tithonia diversifolia* extracts respectively compared to sterile water (negative control). All the methanolic plant extracts and the synthetic fungicide (AMISTAR TOP) differed insignificantly compared to the negative control on their effects on whole plant fresh weight as shown in table 4.9.

Table 4.9. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* metanolic crude extracts at 100% concentrations on whole plant fresh weight of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Plant extract	**Whole plant Fresh weight(g) (shoot plus roots)**
Azadirachta indica	52.00±4.42a
Allium sativum	48.01±5.61a
Tithonia diversifolia	39.63±4.65ab
Fungicide (AMISTAR TOP)	58.03±7.82a
Control (sterile water)	27.78±2.91c
LSD	45.88

Legend: Means followed by different letter down the column are statistically different at P=0.05 by Duncan test**.** ± standard error.

4.6.3 Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on whole plant dry weight of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Allium sativum had the highest effect on whole plant dry weight followed by synthetic fungicide, then *Azadirachta indica* and lastly *Tithonia diversifolia* methanolic extracts compared to negative control sterile water. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on whole plant dry weight deferred insignificantly while that of synthetic fungicide and the extracts also differed insignificantly (P=0.05) as shown in table 4.10.

Table 4.10. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on whole plant dry weight of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

Plant extract	**Dry weight(g) (shoot plus roots)**
Allium sativum	24.26±2.71a
Azadirachta indica	20.25±1.77a
Tithonia diversifolia	19.83±1.72a
Fungicide (AMISTAR TOP)	23.2±2.58a
Control (sterile water)	19.15±1.99a
LSD	21.40

Legend: Means followed by different letter down the column are statistically different at P=0.05 by Duncan test**.** ± standard error.

4.7.0. Effect of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on disease index of angular leafspot disease in common bean Var. GLP 1127 Mwezi moja

Synthetic fungicide (AMISTAR TOP) had the highest significant effect followed by *Allium sativum*, then *Tithonia diversifolia*, and lastly *Azadirachta indica* on disease index compared the control (sterile water). Synthetic fungicide (AMISTAR TOP), and *Azadirachta indica*, *Allium sativum* and *Tithonia diversifolia* extracts effect on disease index differed significantly(P=0.05) whereas *Allium sativum* and *Tithonia diversifolia* differed significantly with *Azadirachta indica* methanolic extract (P=0.05). All the treatment differed significantly with the control as shown in table 4.11.

Table 4.11. Effect of *Azadirachta indica*, *Allium sativum* and *Tithonia diversifolia* methanolic crude extracts at 100% concentrations on disease index of *Phaeiosariopsis griseola* on common bean Var. GLP 1127 Mwezi moja

Plant extracts	**Disease index (%)**
Allium sativum	46.67±4.49b
Tithonia diversifolia	45±3.59b
Azadirachta indica	35±4.35c
Fungicide (AMISTAR TOP)	70±5.77a
Control	20±0d
LSD	42.73

Legend: Means followed by different letter down the column are statistically different at P=0.05 by Duncan test**.** ± standard error.

4.8.0. Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration of the various methanolic extracts on the fungus, the MIC varies with the extracts used. The MIC values varied from 0.025-0.1mg/ml for the three extracts($P \leq 0.05$). *Allium sativum* extracts had the highest MIC followed by *Azadirachta indica* and the least was *Tithonia diversifolia* extract as shown in table 4.12.

Table 4.12. Minimum inhibitory concentration (MIC) of the various methanolic extracts on *Phaeiosariopsis griseola*

Microorganism	**Methanolic plant extracts**	**Minimum Inhibitory Concentration MIC (mg/ml)**
Phaeiosariopsis griseola	*Allium sativum*	0.025
	Azadirachta indica	0.05
	Tithonia diversifolia	0.1

CHAPTER FIVE

DISCUSSIONS

5.1. Morphological characterisation of isolates of *Phaeiosariopsis griseola* of common bean Var. GLP 1127 Mwezi moja

Cultural characterisation of *P. griseola* revealed similarities among the different isolate with all of the isolates showing compact colonies with a grey underside. Significant variations among Bondo, Sabatia and Ugenya isolates were observed in terms of hyphae and spore microscopically. From the findings of this study the conidial size, septation and number of conidiophores among the isolates of *Phaeiosariopsis griseola* were found to vary considerably but the variations were not significant which is in agreement with the findings of Crous *et al*. (2006). The presence or absence of different strains of *Phaeiosariopsis griseola* is an important area which many studies have tended to overlook. If different strains are present but not identified, confusing and conflicting results can be obtained while dealing with other aspects of the fungus especially during screening and breeding for resistance. These morphological variations may be attributed to a number of factors ranging from environmental, host type among other factors.

5.2 Pathogenicity test of the *Phaeiosariopsis griseola* isolates

Considering the importance of correct identification of plant pathogens, *Phaeiosariopsis griseola* causing angular leaf spot disease in common bean Var. GLP 1127 Mwezi moja was identified following pathogenicity approach. There was positive pathogenicity outcome. The findings concurred with studies by Ddamulira *et al*. (2014) and Sartorato (2002) who obtained similar results when Phaeiosariopsis *griseola* was artificially inoculated with common bean.

These results demonstrated the fact that all the fungi isolates investigated for pathogenicity were indeed pathogenic as presented in plates 4.1, 4.2 and 4.3, and table 4.1.

5.3. Phytochemical analysis of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* plant parts

The present work which was undertaken to screen for phytochemical compounds for controlling fungal diseases in common bean, confirmed the presence of several fungicidal compounds in *Azadirachta indica, Allium sativum* and *Tithonia diversifolia*. The results are in agreement with Ramadass (2018) that reported that plants have several antifungal compounds. Most phytochemicals play an important role in protection against fungal pathogens by affecting pathogens' physiology, morphology and ultrastructure or indirectly by promoting plant systemic resistance (Sales *et al*., 2016). Plant that produces such product, antimicrobial activities should be tested against appropriate microbes to confirm the activity and to ascertain the parameters responsible for the activity and this leads to the results obtained from this research work on phytochemical screening of *Azadirachta indica, Tithonia diversifolia,* and *Allium sativum* extracts. The phytochemical investigation of *Allium sativum* bulbs indicated the presence of alkaloids, flavonoids, saponin, tannins and cardiac glycoside. This is in agreement with the work done by Idowu *et al*. (2008); Hunasagi *et al*. (2018). These classes of compounds especially alkaloids, saponins, tannins and flavonoids are known to have fungicidal activity against several pathogens (Shabana *et al*., 2017). The presence of these phytochemicals confirmed the fungicidal activities of extract of *Allium sativum* bulb. Phytochemical screening of the leaves extract of *T. diversifolia* revealed that among the substances investigated, presence of phenolic compounds was detected (total phenols, tannins and flavonoids) while alkaloids, sterols and saponins were not detected. The presence of some of these secondary metabolites suggest that the plant might be of fungicidal importance which is in agreement with results of

Rasaga *et al*. (2014) differing only in the presence of sterols which may be attributed to the part used to obtain the extract in the study. Rasaga *et al*. (2014) used flowers of *T. diversifolia*. The difference in the phytochemical composition from these two studies could be due to variable distribution of phytochemicals compounds in different parts of *Tithonia diversifolia* plant. Similar results were also reported with the earlier studies of Ogundare (2007) and Rejeki *et al*. (2017). From the test of *Azadirachta indica*, the following biologically active compounds were tested positive; alkaloids, saponins, tannins, terpenoids, cardiac glycosides and sterols which is partially in agreement with the findings of Ahmed (2008) on the absence of flavonoids as shown in Table 4.2. These class of compounds especially terpenoids, alkaloids, saponins and tannins are known to have antimicrobial activity against several pathogens Rejeki *et al*. (2017). The exploitation of plant products for the management of plant diseases have achieved greater significance in recent times due to its readily available nature, antimicrobial activity, easy biodegradability, non-phytotoxicity, besides inducing resistance in host.

5.4. Effect of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on spore germination obtained using different solvent extracts of distilled water, methanol and ethanol

The findings showed the inhibition of spore germination in vitro with all tested extracts with the most effective being methanolic *Allium sativum* extract at 100% concentration. *Allium sativum* is a spice with global recognition. In this study, *Allium sativum* had inhibited spore germination when tested. The inhibitive effect was proportional to the concentration for all the methanolic plant extracts of *Allium sativum*, *Azadirachta indica* and *Tithonia diversifolia*; the higher the concentration of the extracts showed more inhibitive effects. These effects are in accordance with the results of Singh *et al*. (2014) who reported that garlic extract had effective spore germination inhibition on *Alternaria dauci*. The fungicidal action of *Allium sativum* is

due to the compound allicin. It has strong antimicrobial and antifungal activities (Keerio *et al*., 2017). Thus, inhibition of fungi observed in this study may be related to allicin or ajoene which curbs the performance of some enzymes that are important to fungi. The results clearly indicate that the *Allium sativum* methanolic extract showed the highest inhibitory activity. The aqueous extracts of *Allium sativum* showed least fungicidal activity than the ethanolic extract against the test organism, which is in agreement with earlier report by Abdulaziz *et al*. (2018) which reported that methanol had a higher extraction ability than ethanol and water. *Allium sativum* has been reported to have fungicidal activity on *Puccinia tritina* a wheat fungus that causes wheat leaf rust (Shabana *et al*., 2017). Similar results were reported by Keerio *et al*. (2017) with *Allium sativum* being effective in controlling *Fusarium oxysporum* fungus. The fungicidal activity of *Azadirachta indica* leaves extract against fungal plates showed considerably spore germination inhibition activity similar findings were also presented by Ravishankar *et al*. (2018) that alcoholic extracts of *Azadirachta indica* had high inhibitory effect on fungus; the fungus targeted were *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger* and *Aspergillus terreus*. The leaves extract of *Azadirachta indica* and *Tithonia diversifolia* plant has been previously reported to show antifungal activity Parekh & Chanda (2007). Effectiveness of *Azadirachta indica* leaves extract in this experiment also agree with the work of Pankaj *et al*. (2011) which reported its antifungal activity against *Trichophyton mentagrophytes*. The results also in agree with the findings of Keerio *et al*. (2017) in which it was found to have fungicidal activity against *Fusarium oxysporum* fungus. The results also conform to Ezeonu *et al*. (2018) who reported that 5% aqueous leaf extract of neem was shown to cause inhibition in growth of six tested fungal pathogens (*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Candida albicans* and *Microsporum gypseum*) and further confirmed that aqueous neem extracts inhibited *A. niger* more than *C. albicans*, while alcohol neem extract inhibited *C. albicans* better than *A. niger*. The highest

extraction potential effect was observed with methanol solvent while water solvent had the least effect. Similar results were reported by Sultana *et al*. (2009) that aqueous methanol was found to be more effective in recovering highest amounts of phenolic compounds from rice bran and *Moringa oleifera* leaves than ethanol and water. Extracts obtained using methanol as solvent had higher activity followed by ethanol and then water. This shows that the extraction yield increases with increasing polarity of the solvent used in extraction. The results of this study are in agreement with the findings of Quy *et al*. (2014). From the experiment done, about three quarter of the results showed that as the concentrations of the plant extracts increases the inhibition effectiveness of the extracts also increased which is partly in agreement with the findings of Effiong *et al*. (2016). These results were also in agreement with the findings of Onaran *et al*. (2016) that concluded; the plant extracts showed a different level of antifungal activities in a dose depend manner. Among the isolates Ugenya was confirmed to be the most susceptible to the plant extracts this could be attributed to its relatively thin walls, unlike Bondo isolate that had relatively thicker conidial walls that might have prevented rapid influx of the plant extracts into the conidia and therefore resist rapid accumulation of toxic levels, this findings are in agreement with Somai and Belewa (2011) that reported that the conidia of *A. parasiticus* were more resistant to the effects of the plant extracts than were the conidia which indicated that the walls of *A. flavus* conidia are relatively thinner than those of *A. parasiticus* conidia, which could explain the difference observed among the Ugenya, Bondo and Sabatia isolates.

5.5. Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on the growth and yield components of common bean Var. GLP 1127 Mwezi moja infected with *Phaeoisariopsis griseola*

From this study there was significant increase in the growth index and yield components in *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* extract treatments in comparison to the control. All measured parameters gave significant differences from their respective controls. *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* extracts showed a promotive effect on plant height, stem diameter, pod weight, number of seeds, fresh weight and dry weight with increasing time compared to control ones. The promotive effect could be due to triterpene which acts by delaying the transformation of ammonium nitrogen into nitrate nitrogen as reported by Al hazmi (2013). Growth and yield stimulating effect of some medicinal plant extracts *P. pinatta, A. marmelos, A. indica, B. campestris, P. nigrum, E. tirucalli* have been observed (Pattnaik *et al*., 2012). Okunlola and Thomas (2013) also reported *Azadirachta indica* extract had effect on the growth and yield of jute under sole and mixed cropping system. Plant height and plant girth increased in comparison to control. The growth stimulating effect is not exclusively by its adverse effect on pathogen or by an increase in nutrient uptake. However, substances with hormone like properties can stimulate of effect biomass allocation in plants. In addition to hormones, medicinal plant extracts contain saponins and polyphenols which could be the active compounds causing the effect on growth and yield (Anderson, 2010).

5.6. Effect of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on disease index of angular leafspot disease in common bean Var. GLP 1127 Mwezi moja

In this study, evaluation of the fungicidal activity of the extracts of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* showed high efficacy in reducing disease index. Similar effect of these plant extracts effectiveness against *Alternaria solani* have been reported Nashwa and Abo-Elyousr (2012); Yaran *et al*. (2011). This study showed that the crude extracts of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* exhibit fungicidal effect on *Phaeoisariopsis griseola*. From the results *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts reduced disease incidence and severity in comparison to the negative control. The results confirm that *Allium sativum* was the most effective of the plant extracts which is in agreement with the findings of Nashwa and Abo-Elyousr (2012) which reported that most effective treatments with plant extracts were *A. sativum* at 1% and 5% concentration, followed by *D. stramonium* at 1% and 5% concentration against early blight disease caused by *Alternaria solani*. *Allium sativum* was observed have most of the phytochemicals (glycosides and saponins) which have antifungal activity which is in agreement with Ezeonu *et al*. (2018) which observed that saponins exhibit potent antifungal activity and are often present in relatively high levels in healthy plants. Plants with most phenolic compounds have high efficiency against plant pathogens because of their antimicrobial activity as a result have been implicated as determinants of a plant's resistance to fungal attack (Shabana *et al*., 2017). The leaf extracts of *Azadirachta indica* have been reported to substantially reduce the number of infected leaves and number of lesions on foliage, and curtail disease development, which in turn, protected flowers and capsules from infection (Enikuomehin, 2005). Obongoya *et al*. (2010) reported that *Azadirachta indica* had reduced disease incidence of *Fusaruim oxysporum* of common bean by about 17% which was also echoed by Ezeonu *et al*. (2018) that neem had

significantly($P<0.05$) inhibited the growth of the fungal organisms against Cocoyam rot. Similarly, the result of this study also showed a positive effect on reducing *Phaeoisariopsis griseola* pathogen. So, these extracts could be useful in the control of *Phaeoisariopsis griseola* fungus of common bean. The results indicated that various crude extracts had fungicidal activity. This may be attributed to the secondary metabolites of the extracts, including terpenoids, flavonoids and other phenols. These metabolites work antagonistically and as a result the pathogen can never develop resistance (Njue *at al*., 2014). This further strengthens this study on *Phaeoisariopsis griseola* control of common bean by *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* extract. A study was undertaken to evaluate the effectiveness of 33 plant extracts against leaf spot of ground nut. All treatments including *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* gave considerable reduction in disease incidence and severity (Hussain *et al*., 2013). Qualitative screening of phytochemical compounds of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* showed the presence of phenols, flavonoids, tannins, alkaloids and saponins as illustrated in table 4.2. These class of compounds independently or in combination may be responsible for the broad range of fungicidal properties of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia*. These plants contain at least 35 biologically active phenolic compounds which are present predominantly in the seeds, leaves and other parts of the plant Mondall *et al*. (2009); Nahak and Sahu (2010) are the most active fungicidal ingredients for effective control of angular leaf spot disease of common bean. Interestingly the important issue that must be noticed in the present work is the effectiveness of synthetic fungicide which appeared to be most effective in reducing the angular spot disease in common bean under greenhouse and *in vitro* study similar findings were also reported by Ezeonu *et al*. (2018) where ketoconazole which was used as the positive control showed 100% inhibition against the growth of the fungi *A. oryzae* and *A. niger*. However, easy availability of plant species coupled with less phytotoxicity and environmental

hazards make them a potential alternative (Cherkupally *et al*., 2017). Most plants contain substances that can be used for anti-microbial purpose of which are precursors for the synthesis of useful pesticides. Crude extracts of plants have been used as antimicrobial (Shabana *et al*., 2017). The antimicrobial value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the phytopathogens.

5.7. **Minimum inhibitory concentration of methanolic extracts of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* on *Phaeoisariopsis griseola***

Minimum Inhibitory Concentration (MIC) is the lowest concentration able to completely inhibit any visible microorganism growth after overnight incubation with media (Yahaya *et al*., 2017). The MIC results showed that increasing concentration had an increasing effect in inhibiting the fungus used. Since the MIC values indicated the definite nature of the fungicidal activity of these plants, the inhibition zones values only indicated the extent of effectiveness of the extracts with increasing concentration. High minimum inhibition concentration observed for *Tithonia diversifolia* at 0.1mg/ml may be an indication of the highest concentration of the extract required to inhibit the fungus spore germination. This may also indicate low efficacy or that the organism has higher potential for developing resistance to the bioactive compounds in the plant, is reported to be related to the relatively thick layer in their outer membrane which prevents the entry of the inhibition substances (Yahaya *et al*., 2017). The low MIC value observed for allium sativum is a good indication of high efficacy against the fungus. This also means that lower concentration of the extract is required to inhibit spore germination of the fungus (Yahaya *et al*., 2017). This outcome is remarkable considering that angular leaf spot disease of common bean is extremely destructive and has led to low yield thus affecting the livelihoods of common bean growers. On the other hand, disparity in the MIC may be due to the variable sensitivity to the chemical substances related to different resistant levels (Yahaya

et al., 2017). The fungicidal activity of the plants is attributed to the various chemical constituents in them. Active singly or in combination inhibit spore germination of *P. griseola* by binging with their protein molecules, acting as chelating agents, altering their biochemical systems and preventing utilization of available nutrients to the organisms (Orchue *et al*., 2013).

CHAPTER SIX

CONCLUSION, RECOMMENDATIONS AND SUGGESTIONS FOR FURTHER RESEARCH

6.1 Conclusion

The study confirmed the existence of morphological variations of *Phaeiosariopsis griseola* among the isolates from Bondo, Sabatia and Ugenya. These morphological variations may be attributed to a number of factors ranging from environmental, host type among other factors.

The phytochemical screening confirmed that *Azadirachta indica*, *Allium sativum* and *Tithonia diversifolia* plants are rich in phytochemicals. The findings confirmed the presence of phytochemicals like alkaloids, saponins, tannins, cardiac glycosides, terpenoids, flavonoids and sterols in nearly all the plants.

These plants have confirmed fungicidal activity on spore germination of *Phaeiosariopsis griseola* fungus but there was some degree of variation in their fungicidal activities. This study showed that all the plants contained most of the active phytochemical compounds which justify their fungicidal property and extract effectiveness increased with the concentration. Methanol solvent was confirmed t have the highest extraction potential followed with ethanol and lastly sterile water. This finding confirm that the more polar a solvent is the more its extraction potential and vice versa.

All the various types of plant extracts were found to be effective against *Phaeiosariopsis griseola* fungi-induced in common bean Var. GLP 1127 Mwezi moja examined. Therefore, there is possibility that *Azadirachta indica*, *Allium sativum* and *Tithonia diversifolia* extracts can control *Phaeiosariopsis griseola* of common bean. *Allium sativum* methanolic extracts at high concentrations should be used in controlling and management of angular leaf spot disease of common bean.

There is need to harness the potential of these plant extracts which are eco-friendly and biologically degradable to control *Phaeiosariopsis griseola* pathogen as it would help ameliorate the cost and negative effects of continuous use of synthetic fungicides. Considering the high fungicidal activity of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* extracts under the test conditions, the extracts may be strong candidates for future field tests. The study revealed significant findings that would be beneficial for the common bean producers.

6.2. Recommendation

Effective pathogen management is anchored on pathogen identification and characterization, it is therefore recommended that for proper disease control appropriate tools such as pathogenicity tests should be done for accurate pathogen identification.

The leaves extract of Azadirachta *indica and Tithonia diversifolia* and bulbs of *Allium sativum* had fungicidal activity against *Phaeoisariopsis griseola* and therefore should be used to inhibit its spore germination and growth.

The *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* plants should be used in management and control of angular leaf spot as they were effective in disease management.

Methanolic extract should be encouraged in control of angular leaf spot disease as it was more effective in inhibiting the growth of *Phaeoisariopsis griseola*. Therefore, methanol as extraction solvent should be encouraged for plant extraction because of its high extraction potential compared to ethanol and sterile water.

Allium sativum should be used in the control of angular leaf spot disease of common bean as it was the most effective than *Azadirachta indica and Tithonia diversifolia* in control of disease incidence and severity.

Highest concentrations of about 50% to 100% of the extract should be used when controlling the disease to achieve better results.

6. 3 Suggestions for further research

1. It was not possible to obtain good distinct differences among the isolates of *Phaeiosariopsis griseola* from the different regions it would be prudent for future studies to conduct molecular profiling to give clear variations.

2. Isolates characterized were obtained in Bondo, Sabatia and Ugenya sub counties and its recommended that other isolates should be obtained from other regions of the country to determine whether there are existing variations.

3. Extensive research still needs to be done on phytochemicals of this plant for the development of cost-effective drugs for the future. More so, since many of the existing synthetic drugs cause various side effects, drug development using plant-based compounds could be useful in meeting this demand for newer drugs with minimal side effects.

4. Fungicidal activity of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* was determined using crude extract of the plants and there is need for more studies to be carried out to determine the individual compounds that have fungicidal activity.

5. Parts of the plants used in this study were leaves of *Azadirachta indica and Tithonia diversifolia* and bulbs of *Allium sativum*; therefore, more studies should be carried out using flowers, roots and bark to determine their fungicidal activity.

6. Disease incidence and severity reduction using the extracts were carried out in the greenhouse under controlled environment and therefore we recommend that similar studies be carried out under natural environment to determine their effectiveness.

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APPENDICES

Appendix 1: PDA media preparation

During isolation of fungi, an artificial growth medium Potato Dextrose Agar (PDA) used was prepared according to the manufacturer's specifications by ingredients such as potatoes infusion (200g), dextrose (2g), Agar (15g) and distilled water to make the volume 500ml which were sterilized in an electric operated autoclave at 15 PSI for 15 minutes. Media was allowed to cool then poured into petri plates to cover the bottom according to a method by Ezeonu *et al*. (2018).

Appendix 2: Information on synthetic fungicide (AMISTAR TOP)
(Adopted from tps://www.syngenta.co.ke at 1445hrs on 8th August 2018)

Trade mark name: AMISTAR TOP is a broad-spectrum fungicide a product by Syngenta for control of grey leaf spot, leaf blight and common rust in maize. Rust, angular leaf spot and anthracnose in French and common beans. Ascochyta leafspot and powdery mildew on snow peas and powdery mildew in roses.

Active ingredients: 250g/litre Azoxystrobin

125g/litre Difenoconazole

Formulation: soluble concentrates (SC)

WHO classification

II

Mode of action:

Azoxystrobin inhibits spore germination ta early stages of fungal development, this confers excellent protection against invasion by fungalpathogens.it is also active against post germination stages of the life cycle in a broad range of fungal species.it also confers anti-sporulant activity against a wide range of diseases.

Difenoconazole is taken up by the plant and acts on the fungal pathogen during penetration and haustoria formation. It stops development of the fungi by interfering with the biosynthesis of sterols in cell membranes, although the mode of action permits protective and curative use, it is recommended to apply the product early enough to avoid irreversible crop damage and build-up of the disease.

Rate of application: 0.5L/Ha with a 14-day PHI.

Appendix 3: Analysis of variance (ANOVA) table on the effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on inhibition, growth index and components of yield against *Phaeiosariopsis griseola*

Parameter	**Source**	**DF**	**SS**	**MS**	**F**	**Pr > F**
Inhibition effect by extracts on *in vitro* spore germination of *Phaeiosariopsis griseola*	Model	13	109.59	8.430	128.784	<2e-16
	Error	2	14.97	7.483	114.321	<2e-16
		2	12.03	6.017	91.926	<2e-16
		26	13.57	0.522	7.975	<2e-16
	Corrected total	412	26.97	0.065		
Effect of extracts on growth index	Model	4	313.6	78.40	3.957	0.00989**
	Error	2	23.9	11.97	0.604	0.55239
		4	167.5	41.88	2.114	0.10126
	Corrected total	33	653.8	19.81		

Effect of extract on pod weight	Model	4	34.5	8.63	0.164	0.955
	Error	2	31.0	15.52	0.295	0.746
		4	388.6	97.15	1.849	0.143
	Corrected total	33	1733.9	52.54		
Effect of extract on number of seeds	Model	4	1134	283.47	2.310	0.0785
	Error	2	105	52.53	0.428	0.6554
		4	514	128.40	1.046	0.3984
	Corrected total	33	4050	122.73		
Effect of extract on fresh weight	Model	4	2874	718.6	2.761	0.0439*
	Error	2	1066	532.8	2.047	0.1452
		4	785	196.1	0.753	0.5629
	Corrected total	33	8590	260.3		
Effect of extracts on dry weight	Model	4	176.6	44.14	0.918	0.465
	Error	2	8.3	4.17	0.087	0.917
		4	299.3	74.84	1.556	0.209

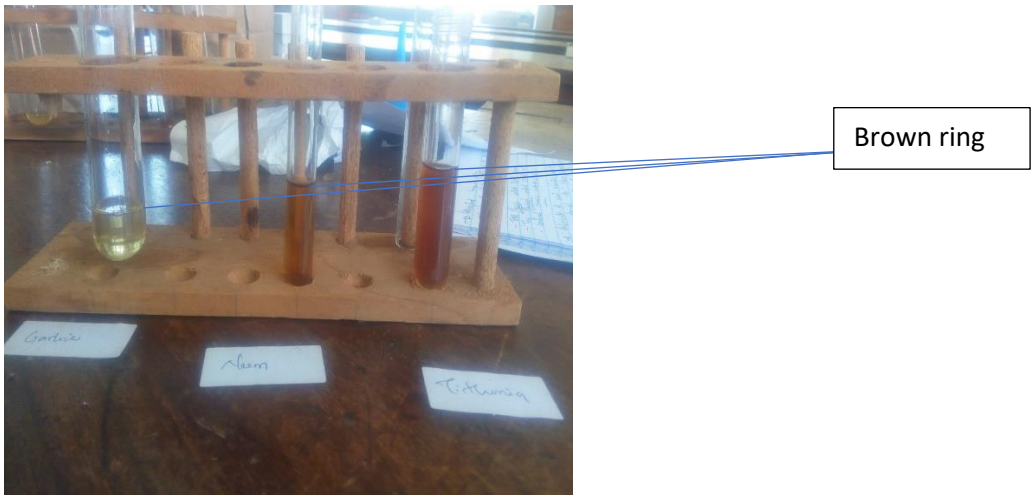
	Corrected total	33	1587.1	48.09		

Appendix 4: Analysis of variance (ANOVA) table on the effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on disease index against *Phaeiosariopsis griseola*

Parameter	**source**	**DF**	**SS**	**MS**	**F**	**Pr > F**
Effects of *Azadirachta indica, Allium sativum* and *Tithonia diversifolia* crude extracts on disease index against *Phaeiosariopsis griseola*	Model	4	**6006**	**1501.5**	**10.772**	**1.O7e-05*** **
	Error	2	**22**	**89**	**1144.4**	**8.210 0.00128****
		4	**378**	**94.4**	**0.678**	**0.61239**
	Corrected total	33	**4600**	**139.4**		

Appendix 5: Garlic drying process

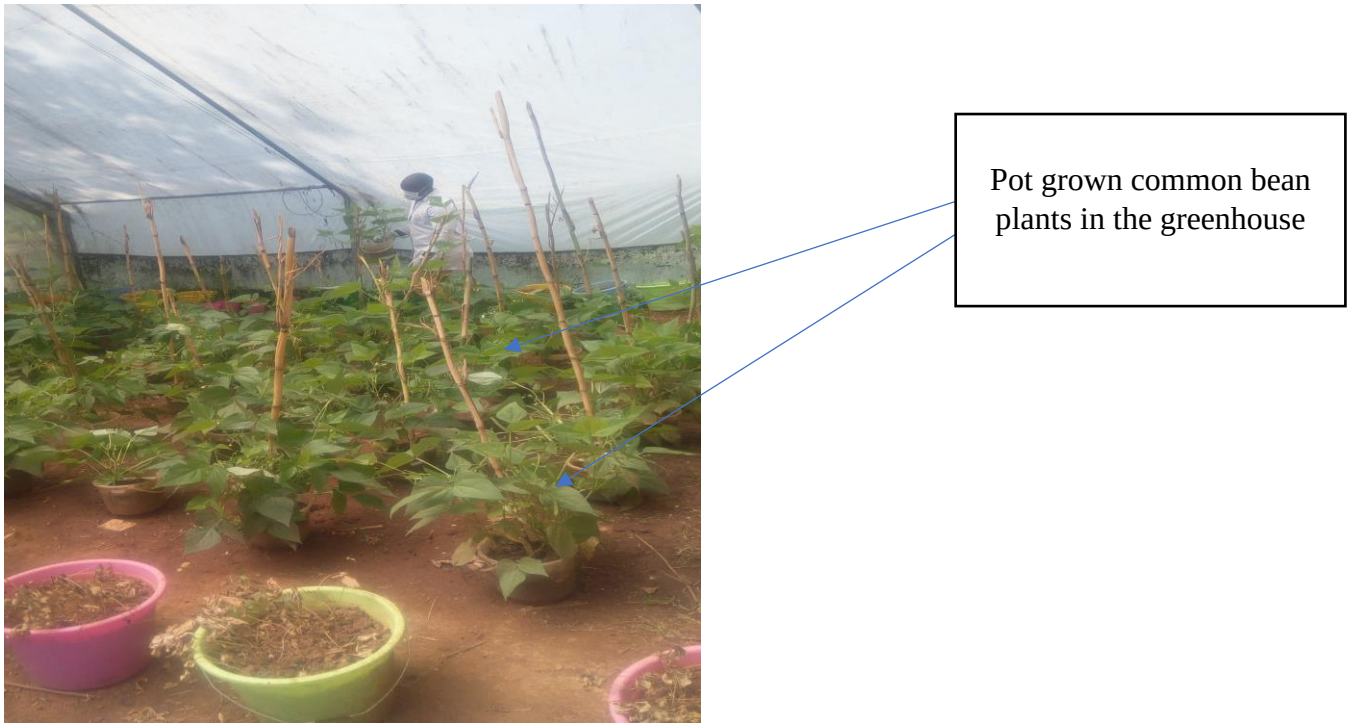
Appendix 6: Phytochemical screening of plant samples: Test for cardiac glycoside that shows interface formation of a brown ring (positive test).



Appendix 7: Grinding of plant material for extraction

Appendix 8: Application of synthetic fungicide using a hand held sprayer in the green house

Appendix 9: Pots containing common bean arranged in a completely randomized design



Appendix 10: Diseased common bean infected with *Phaeiosariopsis griseola* in the green house