BIOASSAY AND RESPONSE OF PYRETHRUM (*Chrysanthemum cinareriifolium*) GENOTYPES TO PATHOGENIC FUNGI IN KENYA

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DECLARATION

Declaration by the Student

This thesis is my original work and has never been presented for the award of an academic degree in any other university and should not be copied, or reproduced in any format without written authority from the author and/or University of Eldoret.

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I dedicate this piece of work to my family who made me believe that words put in writing can stand a test time and change the world.

ABSTRACT

Fungal pathogens are a major production constraint to quantity and quality of pyrethrin in Kenya and other parts of the world. Pyrethrin is a natural insecticide that is cheap, environmentally friendly, acts with celerity, less harmful to both animals and humans and also can be used against a wide range of insects and because of the above importance of pyrethrum and the problem of fungal diseases as a constraint to higher production of the crop, this study was carried out with the following objectives; 1) To assess the morphological diversity and frequency of isolation of the major fungal pathogens affecting pyrethrum production in Kenya, 2) To assess the pathogenicity of identified fungal isolates and response of selected genotypes under greenhouse conditions and 3) To determine the efficacy of selected control agents in management of identified fungal pathogens *in vitro* and therefore, A field visit to major pyrethrum growing zone Nakuru county, was done and infected plant parts with symptoms of fungal infection were sampled and taken to laboratory for analysis. Isolation and identification was done and after identification pathogens with higher isolation frequencies were tested for their pathogenicity and virulence on five pyrethrum genotypes Clone 1 - 4 and P4 in an experiment set in split plot arrangement in CRD with genotypes as main plots and isolates as subplots replicated three times. Data on incidence and severity were scored after inoculation at an interval of 14 days using severity scales. Most virulent pathogens were further selected and taken to the laboratory for testing inhibitory efficacies of selected control agents where a split plot arrangement in CRD was set with isolates as main plots and control agents as sub plots replicated thrice. Agar dilution method and dual culture method were used to test control agents and data on mycelial growth inhibition were scored. Results indicate that there was diversity in fungal pathogens isolated from plants showing symptoms of bud disease, pyrethrum wilt and crown rot disease. There was a significant difference in the pathogenicity and response of selected isolates as $p < 0.05$. All the isolates tested were pathogenic and Clone 4 was more tolerant to most fungi. Botanicals showed promising results as garlic performed well like Carbendazim in the *in vitro* reactions with the test fungi. Selected *Trichoderma* species displayed antifungal properties against all the test fungi. These results indicate that fungal pathogens are still a problem in farmers' fields and available genotypes have varied levels of tolerance which can be exploited in the breeding programs in order to achieve a long-lasting solution to fungal diseases.

***Key words**: Fungi, morphological diversity, Biocontrol, Chrysanthemum cinareriifolium*

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Pyrethrum (*Chrysanthemum cinareriifolium*) is a perennial daisy-like plant that belongs to Genus *Chrysanthemum* of Asteraceae family. Morphologically, pyrethrum is characterized by daisy flowers with white ray florets and yellow disc florets as well as bluish green leaves with lobed appearance that are covered with a wooly material and grows to a height of about 0.8 to 1.0 meters depending on species type (Sun *et al*., 2020). This crop was first identified and grown in Northern Albania and Croatia (Dalmatia) but currently widely distributed in many parts of the world (Bhuiyan *et al*., 2019). Pyrethrum is one of the major cash crops in Kenya alongside coffee, tea and cotton which is mainly grown for the production of an organic pesticide called pyrethrin that contains neurotoxins that acts against most insects both in mammals and crops (Jeran *et al*., 2021; Kaburu *et al*., 2022; Sun *et al*., 2020), However, production trends of pyrethrin in Kenya and other parts of the world have reportedly been affected by biotic factors, abiotic factors as well as socio economic factors (Pethybridge *et al*., 2008).

Pyrethrum flowers are normally harvested and processed to produce pyrethrin, an organic compound made up of chrysanthemic and pyrethric acid which are used in agricultural systems as insecticides as well as insect repellants in non-agricultural systems; for instance, these extracts are used as active ingredients in most insect repellant products such as mosquito coils, hessian strips or ribbons, emulators and vaporizer mats also pyrethrin is a constituent of body lice medicine, and are utilized by humans by applying small amounts of pyrethrin directly to their skin to kill head lice, crab lice and mite lice (scabies)

(Caroline *et al*., 2021). In crop fields, pyrethrum is also planted as companion crop because of its ability to repel insect pests such as spider mites, leafhoppers, cabbage worms, bed bugs and aphids (Liu *et al*., 2021; Lybrand *et al*., 2020). Roots of some varieties of Pyrethrum are used in some countries as a traditional medicine to treat epilepsy, paralysis and rheumatism since these roots contain anacyclin and pellitorine compounds that are useful in management of these diseases (Shahrajabian *et al*., 2021).

Despite the above benefits, the production of pyrethrum has been falling throughout the world due to biotic and abiotic stresses besides the socioeconomic factors. The main biotic agents influencing the yields of pyrethrum are pathogens, insect pests, weeds, nematodes, birds, and mice, whereas the abiotic variables (temperature, humidity, soil properties, and rainfall) define the quality and quantity of pyrethrin (Moslemi, 2017; Suraweera *et al*., 2020).

It has a strong impact of socioeconomic factors on pyrethrum production. As an illustration, small scale farmers in the Kenyan pyrethrum industry have plots that do not exceed one hectare and sell their produce to the Pyrethrum Board of Kenya, who negotiates on their behalf. This means that farmers have a low bargaining power in the global market, which has led to the switching to short-season crops due to low returns in pyrethrum production (Kamau, 2016; Nyoro, 2019). Commercial farms rely heavily on synthetic pesticides in order to contain the losses related to diseases as well as maximize their profitability.

Today, a shift is observed to non-synthetic pesticides which include botanicals, biocontrol agents, and the use of resistant or tolerant variety, better irrigation management, and elimination of alternative hosts as well as extensive spacing within an integrated system. The inefficiencies associated with the use of synthetic pesticides and current management

have led to the need to look into other alternatives, like botanicals and biological agents, that can help in giving the highest production without having to harm biodiversity (Pethybridge, Hay, *et al*., 2008).

Diseases caused by fungal pathogens are a major biotic constraint in most pyrethrum farms in Kenya and they are reported to cause necrosis, chlorosis, damping off, death of flowers and buds, spots on leaves, root rots, blighting of plant parts and wilting of the entire plant resulting in greater losses in pyrethrum yield (Shimira *et al*., 2021). Several pyrethrum diseases such as root rots, tan spots, pink spots, pyrethrum wilt, anthracnose, ray blight and true bud diseases have been reported in Kenya and other pyrethrum producing zones of the world. The high prevalence of major fungal pathogens in many regions could be attributed to favorable environmental conditions for fungus growth and development, air transmission nature of most fungal spores enabling inoculum to persist in the environment for a very long time, ability to be transmitted over long distances and varied modes of reproduction also makes their management to be difficult (Subhash *et al*., 2022). Overreliance on synthetic fungicides which are toxic to both humans and animals, emergence of new pests and diseases due to changes in weather patterns and other inefficiencies of cultural management strategies calls for evaluation of new methods of management of these diseases.

The present study focuses on creating a clear picture and awareness of the major fungal pathogens on pyrethrum fields in Kenya by, describing their diversity that is their morphological characteristics, frequency of isolation, pathogenicity and virulence as well as evaluating selected pyrethrum genotypes for their response to these fungal pathogens and assessing the efficacy of selected botanical extracts and biological agents in

management of the identified fungal pathogens. Addressing the host characteristics, pathogen status and environmentally friendly management strategies will help in boosting pyrethrum production in Kenya (Chappelka & Grulke, 2016).

1.2 Statement of the problem

Fungal pathogens remain to be the major biotic factors hindering the attainment of yield potential of pyrethrum in major growing zones of Kenya and other parts of the world (Liu *et al*., 2023). The majority of the fungi infect plant tissues either passively by entering them through wounds and natural openings or actively by producing enzymes, which break down cell walls (Hamuel, 2015). Transmission of fungal inoculum is through plants to plants, farms to farms and regions to regions through the air, seed and soil in the form of the spores, mycelium and sclerotia. The high humidity, ideal temperatures of around 20 to 30 o C during the criteria of growth and development of the pathogen, and wind that enhances the airborne spread of spores favor fungal diseases (Termorshuizen, 2017).

Farmers in the as well as Kenya areas that produce pyrethrum are losing valuable economic gains of up to 70 per cent due to these diseases during the seed to harvest period and the total production of pyrethrum has declined by as much as 13 per cent due to factors like climate change, under-exploitation of the crop, pest and disease pressure and as well as the decline in market demand due to over reliance on synthetic pesticides (Amwata, 2020). Botanical insecticides, and especially pyrethrin, were used as early as the early 19 th century and by 1928, pyrethrum was actually introduced into Kenya and it was grown in large numbers, with the country producing up to 65 per cent of the global supply between 1960 and 2000. Following this time, the supply of the pyrethrin has shown considerable oscillation owing to biotic, biophysical and socioeconomic factors; currently, the pyrethrin

production businesses are being rejuvenated in Kenya owing to a more widespread movement towards environmental-friendly biopesticides. Continuity of fungal infections is a significant challenge in major pyrethrum manufacturing and research stations in Kenya, and a number of fungal pathogens have been reported as belonging to a causative agent (Grdiša *et al*., 2009; Mulagoli, 2015).

Ray blight was first reported in Australia in the year 1995 to cause a characteristic blighting of the ray florets which resulted in discolored heads that eventually becomes straw colored or withered. In 2012, foliar anthracnose disease that was caused by *Colletotrichum tanaceti* was reported, occurring on the foliage of pyrethrum and appearing as black and water-soaked spots. Authentic bud disease, which is provoked by *Ascochyta* spp., was first introduced in Kenya (1956) in the area of Molo, the disease is characterized by the hanged man syndrome in the bud tissues. The initial outbreak of root rot and the wilting diseases that could be caused by *Rhizoctonia solani* were first reported in India in 1999-2000, highlighting the future occurrence of new phytopathogens. It has been also attributed to the identification of new pests and diseases, the acquisition of resistance, and harmful impacts on biodiversity due to the wide use of synthetic agrochemicals, especially aerosols introduced commercially in 1975 and later spreading in the market (Alam *et al*., 2006; Barimani *et al*., 2013; Javid *et al*., 2013; Sisay *et al*., 2000).

Worldwide commercial cultivation of pyrethrum is practiced as far as Africa, Europe, Australia, and the United States as well as parts of Asia. In Africa, the eastern part is the center of production i.e. Kenya, Tanzania, Uganda and Rwanda which supply a significant percentage of the world market (Isman, 2006). Pyrethrum farming is basically done in high altitude areas in Kenya in the Rift Valley, central, western and coastal provinces. The

leading producer is the Nakuru County where most of the cultivators are small-scale farmers. Loss of Pyrethrum Board in Kenya has led to a sharp reduction in the population of practicing farmers in the country. The attempts to revive the practice of this cash crop have been undermined by population of numerous fungal pathogens in practically all parts of the country (Amwata, 2020). The losses caused by the fungal pathogens are reported to cause huge sums of money every year (Moslemi *et al*., 2016).

Most of the foliar fungal infections produce dark sunken spots that eventually result in massive defoliation, which causes a decrease in the photosynthetically active leaf area and consequently, pyrethrum production (Lelwala *et al*., 2019). Root rot, damping-off, wilting of plants, and eventual death are caused by soil-borne pathogen (Bhuiyan *et al*., 2017). For instance, *Fusarium* spp. which is responsible for Fusarium wilt in pyrethrum causes drooping, wilting, stunting, and reduced tillering. The majority of the tillers wilt in the early stages, killing the entire plant this occurs when underground water and the nutrient flow stops.

1.3 Justification of the study

Diseases caused by fungal pathogens accounts for up to 100% yield losses in pyrethrin content in susceptible varieties (Bhuiyan *et al*., 2019; Pethybridge *et al*., 2007). During these periods between 1964 and the late 1990s, Kenya was the largest producer of pyrethrum which dropped its production to about 2 per cent. Of the world market compared to the 80 per cent. That it had produced.

This has negatively affected the country as exports have declined resulting in low returns, job prospects to most citizens have declined, and the gross domestic product of the country has reduced (Mwega and Ndung'u, 2004). Introduction of low varieties that are prone to

diseases, the appearance of new illnesses and inadequate knowledge about disease-management strategies is seen as the main source of such economic losses. The disease-management strategies of most fungal pathogens have been traditionally cost-effective based on frequent use of fungicides until the emergence of disease-free and resistant planting materials (Sankar *et al*., 2021). This has been achieved through the use of a biotechnological tool which is mutation breeding which has allowed breeding research to be successful in creating high yielding varieties.

Artificial fungicides are costly, have negative impacts on the human body, and a pathogen may develop resistance against such drugs; moreover, lack of appropriate gene modification systems in pyrethrum reduced the achievement of disease-resistant cultivars. As a result, it is necessary to use inexpensive, eco-friendly resources like plant extracts and biocontrol agents (Li *et al*., 2022). Limited data is attributed to the condition of the significant fungal pathogens that attack the pyrethrum grown in Kenya despite the fact that the pathogens have been documented as significant production constraint in most regions where pyrethrum is grown because of the changes in environmental factors. This fact indicates the need to put more efforts in order to learn and control such pathogens. It is also necessary to compare cultivar and line performance to identify their reaction to disease attacks, and draw conclusions which are universal in all cases. Therefore, the current research was aimed at determining the level of incidence, severity, frequency of isolation, pathogenicity, and control of the mentioned fungal pathogens on pyrethrum plants. At the end of the study, pyrethrum genotypes resistant to the wide range of single isolates of fungi were discovered. Moreover, key fungal pathogens that affect pyrethrum in strategic Kenyan areas were morphologies and identified by use of microscopy in order to design

sustainable management mechanisms. The most effective botanical extracts/biocontrol agents were then reported and suggested through in vitro methodology as a cost-effective and efficient way of controlling the identified fungal pathogens in pyrethrum production in Kenya.

1.3 Study objectives

1.3.1 Overall objective

To enhance productivity of pyrethrum through diversity, pathogenicity and *in vitro* efficacy assessments on major fungi affecting production in major growing zones of Kenya.

1.3.2 Specific objectives

1. To assess the morphological diversity and frequency of isolation of the major fungal pathogens affecting pyrethrum production in Kenya
2. To assess the pathogenicity of identified fungal isolates on selected pyrethrum genotypes under greenhouse conditions
3. To determine the *in vitro* inhibitory indices of selected botanical and biocontrol agents for the management of the identified fungal pathogens.

1.3.3 Research questions

Objective one

- Which major fungal pathogens affected pyrethrum production in Kenya?
- What were the morphological characteristics and diversity of fungal pathogens identified?
- What were the isolation frequencies of the identified fungal pathogens?

Objective two

- Which of the identified isolates was more pathogenic and on which pyrethrum genotypes?
- What were the responses of selected pyrethrum genotypes to fungal isolates?
- Which was the most tolerant pyrethrum genotype across all fungal isolates under greenhouse conditions?
- Which was the most susceptible pyrethrum genotype across all fungal isolates under greenhouse conditions?

Objective three

- Which botanical extract showed highest percent inhibition to fungal pathogens *in vitro*?
- Which biological control agent expressed highest percent inhibition to fungal pathogens

  in vitro?
- Which fungal pathogen was resistant to both botanical extracts and biocontrol agents *in vitro*?

CHAPTER TWO

LITERATURE REVIEW

2.1 Botany of Pyrethrum plant

Pyrethrum is scientifically referred to as *Chrysanthemum cinareriifolium* and belongs to Asteraceae family. It is characterized by white ray florets and a yellow disc florets. Pyrethrin are normally highly concentrated in the flower disc floret that is why they are basically cultivated for flower production. These flowers emanate from numerous fairly rigid stems that can grow to about 70-100 centimeters based on genotype. These stems can either be numerous simple or in groups with leaves which are finely cut, multilobed and pubescent (Sun *et al*., 2020b).

2.2 History of pyrethrum production in Kenya

Pyrethrum was originally introduced to Kenya in 1928 from Europe by white settler farmers, and by December of the year 1933, the nation's first commercial harvest was exported to United States, the main market for Kenyan pyrethrum where the dried form of the flower was shipped for processing to final product. In the year 1945, processing industry was built in Nairobi's industrial area by the East Africa Extract Corporation and by 1956, Kenya managed to achieve 90 percent extraction rate and pyrethrum production were at the peak in the year 1993 and following commercialization of synthetic pesticides in American market which were cheaper than botanicals, there was a sharp decline in the international demands (Kuiper, 2019; Njuguna, 2019).

From the reports, Kenya was the world's top producer of pyrethrum for a very long time, providing up to 90% of global pyrethrum needs and other countries that produced

pyrethrum included; Tanzania (7%), Australia (20%), Rwanda (5%), and Uganda (1%) until 1998, when production declined and this position was then replaced by Tasmania, Australia (Ogada *et al*., 2011). Kenyan pyrethrum production stood at 5000 tons per annum of dried flowers in the year 2014 a sharp decrease from a peak of around 18000 tons per annum initially (Dalu *et al*., 2015). Even though Kenya produces the highest-quality pyrethrin in the world, the industry has been plagued by numerous issues such as weather, competition and mismanagement of marketing boards that have contributed to the decline of Kenya's global market share from a peak of 70% to a level of 2% by the year 2018 (Matonda, 2018).

Currently, there is a global concern in the developed countries on the environmental safety due to excessive use of synthetic pesticides that have caused a shift towards demands for pyrethrin, therefore, on its route to recovery, the Kenyan pyrethrum sector is experiencing a transition with great hope that changes in marketing, pest and disease management strategies, breeding for high- yielding pyrethrum varieties as well as technological advancement in production will significantly contribute to the sector in efforts to reclaim its position as an industrial crop of choice for Kenya’s small-scale farmers (Caroline *et al*., 2021).

2.3 Major diseases of pyrethrum

Like any other plant grown in Kenya and other parts of the world, pyrethrum yield is affected by both biotic and a biotic factor as well as socio economic factors. Fungal pathogens are the major biotic factors in almost all parts of the world and are reported to cause root diseases, crown diseases, stem diseases, leaf diseases and also flower diseases which have caused significant losses in quality and quantity of pyrethrin (Scott *et al*., 2011). Since 1928, when the first case of pyrethrum production was introduction in Kenya, several fungal pathogens have been reported to be affecting different parts of the plant (Ogada *et al*., 2011).

Geographical distribution of plant diseases is influenced mainly by environmental factors such as soil characteristics, temperature, relative humidity, rainfall and wind. For instance, different agro ecological zones have varying disease pressures due to differences in agro ecological conditions (Dagar *et al*., 2021). Pyrethrum production in Kenya is highly concentrated in high altitude zones, Nakuru County, a leading producer of pyrethrum and in this region several plant diseases such as pyrethrum wilts, root rots and crown rots, tan spots and bud diseases have been reported to cause yield decline over the years thus the need for continuous crop improvement so that varieties tolerant to these diseases can be developed (Kimani *et al*., 2001).

These fungal pathogens have varied epidemiological characteristics and are also endemic to specific hotspot sites because of different agro ecological conditions of these sites, however due to climate change and other external factors such as extensive use of synthetic fungicides, evolution of fungal pathogens have been reported over the years with new races

emerging and other races disappearing. For instance, a new anthracnose disease was reported in Tasmania (Australia) at around the year 2013 while true and false bud disease of pyrethrum was reported in

Kenya in the year 1946 and it disappeared never to be seen again after a decade (Barimani *et al*., 2013; Matonda, 2018; Ogada *et al*., 2011; Shimira *et al*., 2021).

2.3.1 Pyrethrum wilting

This is a disease of pyrethrum characterized by drying up of parts of the plant or the entire plant and is caused by disruption of water flow from the vascular plant vessels caused by the activity of the pathogens in the vessels or the tracheid by either blocking the vessels or by diverting the plant contents for their growth and development. Wilting is a significant issue in pyrethrum production in Kenya from the reports, huge economic loses has been realized that is up to 50% loses in yield. This wilt disease has been reported to be caused by several pests such as: fungal pathogens (*Fusarium* spp. *Sclerotinia* spp. *Rhizoctonia* spp.) and nematodes (O'Malley *et al*., 2015).

Initial symptoms of wilts due to Fusarium infection include individual branches, especially the lower ones, drying out and losing their foliage that continues until each branch is included, and finally, the entire plant wilts as a result. The major symptoms of *Fusarium* infection include plant wilting, necrosis, and chlorosis of the plant parts as well as damping off of seedlings of seasoned plants. Favorable conditions for the development of this disease are when temperatures are between 28-32 ^{o}C and Relative Humidity of 80-90% and soils with characteristics such as sandy loam soil which are slightly acidic with pH ranges of (5.5 - 6.5) as well as low soil moisture content (Ijaz *et al*., 2020; Pereira *et al*.,

2019; Singh, 2014).

Two *Sclerotinia* species have been found to cause wilting of pyrethrum; these two species are *Sclerotinia minor* and *Sclerotinia sclerotiorum*. These organisms cause crowns rot, head rot and general wilting of plants. The major inoculum is the sclerotia which cause the progression of the crown rot by the growth of the mycelia in soil, and can also grow in stems of plants. The sudden wilting, rot of the roots, leaving of the leaves, and eventual wilting of the plant characterizes the symptoms of this disease. Infection by this pathogen in severe cases is possible only in favorable environmental conditions, namely, temperatures between 15 ^{o}C and 20 ^{o}C that make it possible to be infected, and then hot and humid conditions that contribute to the rapid development of the symptoms of the disease (O'Malley *et al*., 2015).

Rhizoctonia solani fungal pathogen can also cause pyrethrum wilting leading to the development of crown rot, root rot and overall plant wilting. The infection is normally dark brown, necrotic spreads of the roots and on the base of the plant. During later stages, the size of these lesions increases, subsequently making the whole of the plant dry up and wilt. The right environment to this disease is the damp, low drained soils and 15 ^{o}C to 18 ^{o}C temperatures (Cubeta &many years, 1997). Root -knot nematodes (*Meloidogyne hepla*) are a significant limitation to production of pyrethrum, having been reported to make up 95% of the phyto -nematode populations of pyrethrum in Kenya and to result in loss of yield of about 20% to 30%.

They are found in the soil and infect the plant through roots causing root rots, galling of the root system and wounding of the root system providing entry points for secondary pathogens The symptoms include; appearance of knots in the roots, stunting, wilting, and

poor regrowth Lesion nematodes (*Pratylenchus spp*), promote the growth of black lesions, pruning of the root system, yellowing, stuntedness, and eventually withering of the affected plants (Hay *et al*., 2009; Lelwala *et al*., 2019).

2.3.2 Anthracnose disease

The most recent fungal disease that have emerged in many parts of the world is anthracnose disease that is caused by a fungal pathogen *Colletotrichum tanaceti* with the symptoms such as black water-soaked sores that form five days after infection and with time these lesions become large and merge forming holes in the center and could ultimately result in defoliation. The data shows that the pathogen under consideration is a multifaceted foliar disease, as it leads to a decrease in biomass generation due to a decrease in the size of the leaf area. Older foliage also causes a higher duration of symptoms, which can result in up to 67 per cent yield losses in vulnerable cultivars (Huang *et al*., 2022, Lelwala *et al*., 2019, Moslemi, 2017). The inoculum is passed on in form of spores; it was also recorded that seeds can also be a transmission factor. When landed on the epicuticle surface of the prone host, infection is established in 24-48 hrs after inoculation.

The process of the first infection is characterized by the appearance of appressoria on the leaves of pyrethrum and the production of multilobed infection vesicles inside the epidermal cells. This is followed by secondary hyphae that are finer in nature leading to infection and colonization whereby the initial infection presents itself as black necrotic lesions which develop after water-soaked lesions. The best conditions to develop the disease are a climate with high humidity and temperature between 24 ^{o}C and 28 o C with the disease showing symptoms throughout the plant (Lelwala *et al*., 2019; Silva and Michereff, 2013). 243 Foliar diseases Ray blight is one of the floral diseases of pyrethrum

which is introduced by *Stagonosporopsis tanaceti*. Ray blight is the term used to describe the specific ray caused disease of floret, which leads to discolored heads. All plant organs or tissues except the vascular system can become infected by the disease. The initial symptoms are necrotic lesions at the margins of the leaves, which gradually develop to cover all the leaf and result in defoliation and retarded growth of plants. This inoculum may be spread by seed and air spores and mostly occur to the flower and leaves with its symptoms being necrotic lesions (Bhuiyan *et al*., 2017; Javid *et al*., 2013).

Moreover, a similar-symptom disease that is known as True and False bud disease is reported to have been seen in the majority of pyrethrum growing areas of the globe. *Ramularia bellunensis* causes this disease and causes necrotic spots on the leaves, stems and diseased buds are girdled. This was first discovered on bud abnormalities in Kenya in 1946 and the abnormalities were stated to attack flower buds by means of bracts and subsequently assault flower stalk. When this disease continues, the buds begin to dry up and change their color to brown to purple-grey and even the stalk begins to dry up to approximately 25 cm below the dead bracts. The florets and rays being bound on one side are the characteristic symptom of true bud disease that produces the so-called hanged man symptom (Shimira *et al*., 2021).

2.4 Responses of plants to pathogen infection

Fungal pathogens enter plant tissues actively by directly invading cell walls through release of cell wall degrading enzymes or passively through natural openings such as stomata, cuticle, wounds created by other pests or mechanical injuries to the plant and for plants to counter these infection processes, modification of the structure and size of these plant parts either naturally or induced enables plants to resist attack. Some plants develop physical

thick bark that is a mass of dead cells that forms a burrier against pathogens entry, other plants have cellulose cell walls that are heavily packed with pectin forming a strong layer against infection, while others develop thick waxy cuticle that covers their cells from infection (Iqbal *et al*., 2021; Sakamoto, 2021). Crop improvement aims at developing plant varieties with these qualities that can resist the effect of biotic stresses coupled with other agronomic qualities such as yield and quality. There are a few pyrethrum varieties in the market that conforms to these traits thus the need for continued screening to identify resistant varieties for better pyrethrum production.

Environment factors affecting pyrethrum production

These are factors in the environment that plays a key role in the growth and development of most crops and diseases. For instance, certain levels of temperature, moisture and pH may accelerate or suppress disease development; the average annual rainfall demands by the crop are more than 875mm that is rainfall of about 100mm a month is ideal for optimum growth while excess rainfall increases canopy wetness creating a good environment for the development of the disease and also encourages root rots and stunted growth. Kenyan pyrethrum production belt is highly concentrated in regions with this rainfall characteristic; however, pyrethrum crop is also reported to thrive well in the Kisii Highlands in South-West Kenya, which receives between 1800 and 2000 millimeters of annual rainfall. Maximum pyrethrum flush occurs at an average annual rainfall of about 1250 millimeters per annum (Lamichhane *et al*., 2015; Motanya, 2019).

Growth and development of pyrethrum is greatly influenced by temperature. For instance, the quality and quantity of pyrethrin as well as germination of pyrethrum seeds, flowering, elongation of shoot and flower maturation are dependent on prevailing temperatures.

Lower temperatures promote flower initiation and these temperatures should be below 18 ^{o}C for at least six weeks to induce flowering, a process called vernalization and so proper timing of pyrethrum production should be done to ensure maximum yield. Relatively higher temperatures promote flower maturation and since temperatures play a key role in pyrethrum development, exposure to heat stress that is too high or low temperatures causes a reduction in pyrethrin content and quality (Suraweera *et al*., 2015, 2020).

Maximum yield in pyrethrum production can be realized in fertile deep well drained soils with a higher concentration of elemental nutrients such as phosphorus, magnesium and calcium at about pH 5.8. Even though shallow soils can still produce a good harvest when there is a sufficient supply of nutrients and good rainfall and also loamy soil types that are deep and well drained can give good yields. Water logging causes poor re-growths after the first harvest and reduces root expansion that prevents water uptake from the lower soil profiles, as well as soil compaction and cutting height during cut back stage (Atkinson *et al*., 2004).

2.5 Management strategies for diseases in pyrethrum

Crop rotation

This is the process of successively planting several crops on the same piece of land in order to enhance soil health, maximize nutrients, and reduce pest and weed pressure. For instance, in pyrethrum fields infested by nematodes, a four to six year rotational program with crops of Poacea family will help in reducing amount of inoculum in the soil. Most fungal pathogens' inoculum, resides in the soil such as those of *Fusarium* spp. can be managed by rotating pyrethrum plants with non-host plants. This practice of rotating crops helps in breaking the pest cycle by limiting the availability of host plants necessary for

completion of their lifecycle (Marburger, *et al*., 2015).

Intercropping and proper spacing

This is a process of growing crops of different species on the same piece of land at the same time. During the first three months of transplanting and cut back period, pyrethrum plants can be intercropped with most leguminous plants such as garden peas (*Pisium sativum*), pigeon peas (*Cajanus cajan*), Soy beans (*Glycine max*), some species of Common beans (*Phaseolus vulgaris*) and Sunn hemp (*Crotalaria juncea*) (Moorthy, 2018). These cover crops help in fixing nitrogen into the soil, reduces incidence of weeds, covers soil hence conserves soil moisture, increases productivity of land as these crops are utilized as food by farmers and these legumes are also beneficial in management of pests and diseases in that some species of these legumes like common beans suppresses nematode infestation and also reduces damages caused by *Fusarium* wilt pathogens (Moorthy, 2018; J. Mwangi, Waweru, Oeba, & Wepukhulu, 2010).

Pyrethrum must be planted with an interplant distance of 60 cm and an intraplant distance of 30 cm in order to maximize the yield. Where intercropped with other species, proper spatial planning and prudent choice of latter crop companionship are to be employed to ensure that there is ample soil aeration, light penetration and also a canopy which is not inordinately thick so that it covers the pyrethrum. The existence of unfavorable aeration and shading creates an excellent microclimate with which the pests and diseases develop (Moorthy, 2018).

It is crucial to ensure that the agricultural fields are not left with crop residue or volunteer plants where pests and diseases can be found, or to make it difficult to conduct farm management such as weeding, harvesting, planting, and irrigation. Majority of the inoculum related with disease is present in the crop debris hence sanitization measures

should be taken to avoid distribution of the inoculum to new plants. The residues of cut-back pyrethrum must be burned or disposed of in a way that will minimize the incidence of inoculcum in the field (Hay *et al*., 2015). The use of planting materials that are free of pollutants also minimizes the chances of pests and diseases and farmers should buy certified seeds that are registered in Kenya among seed merchants. These seeds are selected from varieties that are resistant or tolerant to pests and diseases to help farmers in managing these constraints.

Physical management strategies

Seed borne pathogens such as *Stagonosporopsis tenaceti* are effectively managed through heat treatment of seeds at temperatures of 50 ^{o}C for 30 minutes to eliminate inoculum that might be found on the seed coat and also steam sterilization of seeds have been reported to reduce inoculum in the seed as well as improving germination of seeds (Bhuiyan, Vaghefi, & Taylor, 2019a). Whereas for soil borne pathogens, such as pseudosclerotia (ray blight) that can survive in the soil for more than six months, dormant and resting spores of *Fusarium* spp. which can survive in the soil for more than five years and nematodes as well can be eradicated through soil solarization by covering soil surface using a clear plastic bag for a period of up to four to six weeks, this will increase soil temperatures to about 40 ^{o}C to 100 ^{o}C or beyond depending on the soil type, time of solarization, season, soil depth and location and these temperatures are beyond tolerable range for pest and thus are lethal for pest survival (Pethybridge, *et al*., 2008a; Rawat, Bisht, & Naithani, 2021; Singh, Pandey, & Sharma, 2023).

Chemical management strategies

This method refers to use of synthetics in management of pests and diseases of pyrethrum

plants. Specific fungicides, nematicides, and insecticides have been proven to work best in management of pests and diseases for instance; site specific foliar sprays with demethylation mode of action have been used successfully against ray blight disease of pyrethrum, nematicides such as Bio-Nematon, soil fumigants such as Ethyl Dibromide and insecticides such as Alpha cypermethrin have also been reported in successful management of pests and diseases (Bhuiyan, *et al*., 2019a).

Breeding for resistance

An innate immunity system exists in plants that enable them to perceive the pathogen presence and be able to defend themselves. These defense mechanisms are either physical, biochemical, physiological or genetic that confers tolerance or resistance to these diseases and in most cases this phenomenon is utilized in integrated pest management strategies by taking advantage of adaptations that many plants has to tolerate plant diseases or any other pest. These adaptations maybe natural or induced (Karban, 2008).

Plant improvement against diseases aims at manipulating the plant characteristics to confer these defense mechanisms. Pyrethrum is a cross pollinated plant and this makes it possible for improvement through conventional means and from the records, efforts to improve pyrethrum through breeding in Kenya dates back to the 1970s when attempts to produce high-yielding varieties coupled with other agronomic characteristics such as resistance to lodging and large flowers with better longevity and quality pyrethrin. Currently, biotechnological tools such as mutagenesis have been utilized in improving pyrethrum plants and from this advances, several clones and varieties have been released into the Kenyan market that includes the following; Clone Ma/71/101, Clone Mo/75/223, P4 and K218 among others (Anderson *et al*., 2021; Gupta *et al*., 2022).

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2.6 Biocontrol agents used in controlling plant diseases.

Natural antagonistic relationships between pathogenic fungi and other organisms have been reported to cause a considerable effect of plant-derived and microbiological agents on controlling fungal diseases. These useful microorganisms are naturally present in the soil and are able to colonize the plant roots and hence help in disease inhibition by inducing systemic resistance and stimulating plant growth. As an example, Trichoderma spp., Beauveria spp., and arbuscular mycorrhizal fungi have been extensively used to the effect in controlling various pathogenic fungi (Guzmán-Guzmán *et al*., 2023; Yao *et al*., 2023.

Various plant extracts among them the neem, garlic, ginger, Chinese berry, eucalyptus, onion and Aloe vera extracts have been reported to exert antifungal activity over a range of phytopathogens and insect pests and evidence has implicated that they may also alleviate some human pathologies (Vijayan et al., 2023). The botanical pesticides help in eradication of the negative impacts of the synthetic pesticides as they can easily be biodegraded into harmless.

2.7 Gaps and way forward

The high expense, the risks to the environment and human health associated with using synthetic pesticides such as nematicides and fungicides to manage plant diseases, the low economic value of recommended rotational crops, and the lengthy rotational times that slow down production process, the emergence of new pathogenic races that causes the breakdown of resistance in resistant clones, and also limitations of botanical extracts in managing these biotic stresses makes the current management strategies to be less efficient and this calls for more efforts in seeking appropriate alternatives to ensure long term success in supply of pyrethrin.

There is no current documentation on the diversity and status of major fungal pathogens in Kenya as well as sensitivity tests on the major fungal pathogens under different conditions, and since fungal pathogens are a major threat to pyrethrum production because of favorable environmental conditions in almost all agro ecological zones, calls for the need for development of genetically potential varieties to ensure maximum yield. Growing of resistant varieties is a cheaper, easier and environmentally friendly way of comparting pathogen stresses. There is also need to evaluate the locally available botanicals extracted from plant parts as well as biological agents to ensure a long-term success.

This study aims were more concentrated on creating a better understanding of major fungal diseases by determining the diversity and isolation frequencies of major pathogens and testing their virulence and pathogenicity on selected genotypes. Management strategies such as use of botanical extracts and biocontrol were evaluated as well to ensure sustainable management.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Diversity and identification of major fungal pathogens affecting pyrethrum

3.1.1 Study sites, agro ecological conditions and sampling technique

A field visit to two sites in major pyrethrum growing zones of Kenya, Nakuru County was done on November 2022. These sites were Subukia and Molo which lie on different agro ecological zones representing different regions in Kenya. Molo is situated along the Mau forest within the Mau escarpment at coordinates: 0^{o} 15' S and 35^{o} 44' E. this site is a high-altitude zone of between 2,980 to 3,050 meters above sea level whereas annual rainfall ranges between 1,200 to 1,900 millimeters per year in this area while annual temperature ranges between 8 ^{o}C to 23 ^{o}C. Subukia on the other hand lies at a latitude 0^{o} 0' S and longitude 36^{o} 13' E and is located at a low altitude zone of between 2,010 to 2,295 meters above sea level. This region is also characterized by an average annual temperature of between 10 ^{o}C to 26 ^{o}C and rainfall of between 700 to 1,400 millimeters per annum (Kinyori, 2016; Muturi *et al*., 2018).

During the survey in pyrethrum growing zones, each of the zones were considered as clusters based on their variation in weather conditions. In each zone, biased sampling technique was used to maximize on the diversity of the major fungal pathogens affecting pyrethrum. Fresh samples of diseased plant parts (roots, flowers, leaves and stems) showing symptoms were collected and transported in khaki bags to Plant Pathology Laboratory based at the Seed, Crop and Horticultural Sciences in the School of Agriculture and Biotechnology, University of Eldoret, Kenya for morphological diversity assessment

and identification.

3.1.2 Laboratory isolation and identification

Media (Potato Dextrose Agar) preparation

Two hundred grams (200 g) of clean potatoes were pilled, cut into smaller pieces and boiled in 500 ml distilled water for 20 minutes. Filtrate was obtained by sieving using a muslin cloth to remove all potato flesh that might have accompanied the filtrate followed by addition of 20 g of dextrose and 20 g of agar simultaneously while swirling. The resultant mixture was topped up to 1000ml using sterile distilled water and heated while swirling to ensure that dextrose and agar dissolved uniformly. This media was then autoclaved for 15 minutes at 121 psi (pounds per square inch). The sterilized media was then dispensed onto sterile medium sized glass petri dishes and allowed to cool under a laminar flow air system before use (Kipkogei, 2019).

Tissue transfer and culturing of the fungal isolates

Tissue isolation technique was used in this study where, approximately 2 to 5 mm^2 of tissue from the boundary of a lesion of fresh samples was excised and sterilized using 1% sodium hypochlorite solution (NAOCL) for 30 seconds and rinsed thrice using sterile distilled water to remove sterilizing agent from the surface of the tissues. Sterilized tissues were then placed on filter paper to drain excess water followed by plating on solidified PDA The culture plates were then placed inside an incubator (Gallencamp) at 25 ºC - 27 ºC for seven days under an alternating 12 h near-UV light and 12 h dark conditions to allow sporulation (Were, 2018).

3.1.3 Diversity assessment of fungal isolates

Morphological characterization and identification

After seven days, growing fungi were purified unto freshly prepared sterile PDA media and incubated for another seven days. These pure isolates were identified using morphological and microscopic characteristics such as: colony color for both front and reverse side according to pathology charts, as well as growth rates, mycelial growth characteristics while shape and sizes of micro conidia and macro conidia were assessed under a microscope at a total magnification of X1000. After morphological description the fungal isolates were identified using plant pathology manuals, reference books and scientific journals (Kipngeno, 2015).

Frequency of isolation of fungal species

Frequency of isolation refers to number of times every isolate has appeared in every site. Percentage distribution of fungal pathogens causing major fungal diseases were assessed as described by the following formula modified by the author (Benard *et al*., 2013):

$$\textbf{Frequency (\%)} = \frac{\text{No of specific isolates per site}}{\text{Total number of isolates per site}} * 100$$

Results on percentage distribution of isolated fungal species per site and morphological characteristics were presented in form of tables, descriptions graphs and plates.

3.2 Pathogenicity of identified fungal isolates and response of selected genotypes

This study was conducted at the University of Eldoret, school of Agriculture and Biotechnology, department of Seed Crop and Horticultural Sciences under greenhouse conditions and the inoculum of fungal isolates were prepared from the already identified fungi with higher isolation frequencies: (*Fusarium oxysporum, Fusarium solani, Fusarium avenaceum*) which were isolated from wilted pyrethrum plants, *Alternaria alternata* isolated from pyrethrum samples with bud disease symptoms and *Rhizoctonia solani* isolated from rotting roots and crown of pyrethrum plants.

3.2.1 Planting and experimental design in the greenhouse

Two months old pyrethrum plants (P4, Clone1, Clone 2, Clone 3 and Clone 4) were uprooted and split to several clones that were used for this study. Clone1, Clone 2, Clone 3 and Clone 4 are the farmer selected pyrethrum plants that were obtained from the ongoing crop improvement program of Kentegra project in collaboration with the University of Eldoret. P4 variety is a commercial variety that is widely grown by most farmers in Kenya and from field observations; it has been identified to be more susceptible to most fungal pathogens of pyrethrum and was tested against selected genotypes for their response to isolated pathogens.

These clones were potted in black polythene sleeves measuring 35 centimeters diameter and 35 centimeters depth, filled with planting medium to the brim. Planting medium used composed of sterilized forest soil, sand and manure (3:2:1) mixed thoroughly (Lindiro *et al*., 2013). After potting these plants were maintained under greenhouse conditions with regular watering being done to ensure proper plant growth within the analysis period.

Pyrethrum wilt disease was assessed in a split plot arrangement in a completely randomized design (CRD) in the green house with five main plots (four pyrethrum clones; Clone 1 to Clone 4 and one commercial variety; P4) and four sub plots (three fungal isolates identified to cause pyrethrum wilt disease and a control free from fungus) replicated three times. The following (Figure 1) is an experimental layout and a statistical linear model for this arrangement:

REP 1	P2 P4 P1 P3	P3 P2 P1 P4	P1 P3 P2 P4	P2 P4 P1 P3	P2 P3 P1 P4
REP 2	P1 P3 P2 P4	P3 P3 P1 P4	P2 P4 P1 P3	P4 P3 P1 P2	P1 P3 P2 P4
REP 3	P3 P2 P1 P4	P2 P4 P1 P3	P3 P2 P1 P4	P1 P3 P2 P4	P2 P4 P1 P3
KEY	**Clone 3**	**Clone 4**	**Clone 1**	**P4**	**Clone 2**

Figure 1: Randomization plan for the greenhouse experiment: background colours represents the main plots (genotypes), P1 to P4 represents the subplots (treatments) such as Fusarium oxysporum (P1), Fusarium solani (P2), Fusarium graminearium (P3) and a distilled water (control) (P4).

Experimental linear model

Yijkl = $\mu + \alpha i + \varepsilon j\ (i) + \beta k + \alpha\beta ik + \varepsilon ijkl$ where;

Yijkl = Total observation

μ = Overall mean

αi = i^{th} effect of main plot (Genotypes)

εj (i) = j^{th} effect of main plot error

βk = k^{th} effect of sub-plot (Isolates)

αβik = i^{th} and kth interaction effect between Genotypes and Isolates

εijkl = split - plot error

Pyrethrum bud disease and crown rot disease was assessed in a completely randomized design, by applying identified fungus on five selected genotypes in three replications (figure 2). The following is an experimental layout and a statistical linear model for this arrangement that guided analysis:

REP 1	Clone 1	Clone 3	Clone 4	Clone 2	P4
REP 2	Clone 4	Clone 2	Clone 3	P4	Clone 3
REP 3	Clone 2	Clone 3	P4	Clone 4	Clone 1

Figure 2: Randomization plan for screening selected pyrethrum genotypes against *Rhizoctonia solani and Altanaria alternata*

3.2.2 Preparation of inoculum suspension

Pure cultures of identified isolates were sub cultured to ensure significant sporulation on a PDA media under aseptic conditions. The plates were incubated for 10-14 days under 12 hours of light and 12 hours of darkness and temperatures between 18-22 ^{o}C to ensure maximum sporulation (Salman, 2005). After the incubation period, the plates were flooded with 10ml of demineralized water and scrapped with sterile glass rod while gently agitating to ensure that spores were dispersed. The resultant suspension was transferred to 100ml measuring cylinder and sieved using a muslin cloth to discard mycelial mass. Spore concentration was then confirmed by pipetting 1ml of the suspension into a counting slide hemocytometer and viewed through a light microscope.

The concentration of spores per ml of distilled water was adjusted to 2.5 x 10^6 spores/ml

using a Burker Turk Hemocytometer (Counting chamber), a limit that has been reported sufficient to achieve successful infection (Were, 2018). Spore viability was determined through image analysis method by pipetting spores onto freshly prepared PDA and incubating plates for 24 hours under room temperatures. Spores were considered viable if germinating germ tube viewed under the microscope was half the length of the spore after 24 hours of incubation (Paul *et al*., 1993).

3.2.3 Host plant inoculation in the greenhouse

After two months, pyrethrum plants were inoculated with identified isolates in order to assess their response to pathogen infection. Specific isolates were inoculated on specific pyrethrum plant parts on which they were isolated following results from objective one above. For soil borne pathogens that causes crown rots, root rots and wilt disease of pyrethrum, inoculation was done as described by (Morcillo *et al*., 2020) through drenching method where a furrow measuring 10 centimeters deep and 2 centimeters from the plant was prepared around each potted plant and 10ml of the suspension was poured uniformly on the furrow then covered with loose soils.

Foliar diseases inoculation was done by spraying inoculum on the leaves and stems until runoff using a hand sprayer as described by (Were, 2018). Flower buds were inoculated by gently dipping flowers at bud stage on inoculum suspension contained on 25 ml beaker ensuring that all parts of the flower were immersed in the suspension as described by (Huang *et al*., 2022). A control experiment for each replicate was inoculated with sterile distilled water of equal amount as the inoculum. After inoculation these pots were transferred to an inoculation chamber for two days with temperatures maintained at 20 ^{o}C - 25 ^{o}C and humidity of > 70 % to ensure successful infection of the pathogen. Afterwards,

pots were returned to normal greenhouse benches and were assessed for disease development after fourteen days of inoculation. Inoculated pathogens were re isolated following procedures mentioned above to prove the Koch's postulates (Were *et al*., 2016).

3.2.4 Pathogenicity assessment

The pathogenicity of isolates was determined by scoring the incidence and severity of the disease caused by the isolates and was done 14 days after inoculation and at intervals of two weeks thereafter. Days taken for the first symptoms to appear were recorded as well. Disease incidence per treatment was determined according to (Nafula *et al*., 2021) as the number of infected plant parts as part of the total number of plants expressed as a percentage as shown by the formula;

$$\textbf{Disease Incidence (\%)} = \frac{\text{Number of infected pyrethrum parts; leaves/flower/buds/ stems}}{\text{Total number of parts inoculated: leaves/flowers/buds/stems}} \times 100$$

Data collected on incidence of the disease was used to rate the genotype status following the interaction with the disease and was done using a scale of 1-4 adopted from (Manandhar *et al*., 2016). Where scores were awarded based on the disease incidence. DI of 1-10 % scored 1 (Resistant), 11-30% scored 2 (moderately resistant), 31-60 % scored 3 (moderately susceptible), and 51-100 scored 4 (highly susceptible).

Severity of major fungal pathogens on selected pyrethrum plants in the greenhouse was scored using different scales depending on the disease and plant part affected. Wilt disease was scored using a rating scale of 0-6 derived from (Abdulai *et al*., 2020; Barimani *et al*., 2013) and modified. Modification was majorly done so as to accommodated plant stems where; **0**= healthy plants, **1**= yellowing of lower leaves, **2** = Chlorosis and wilting of leaves,

3 = wilting of leaves and yellowing of one stem, **4** = wilting of one stem, yellowing of other stems and wilting of leaves, **5** = wilting of leaves and more than one stem, 6 = completely dead plants.

Destructive sampling was done on genotypes inoculated with crown rot causing fungi, *Rhizoctonia solani*. Two months after inoculation, inoculated genotypes were carefully uprooted, washed in tap water and examined for crown and root discolorations. Crown and root rot disease was scored using a severity scale of 0-2 where **0**= no discoloration, **1**= mild discoloration, **2**= severe discoloration (Moslemi, 2017). Bud disease was scored using a scale of 0-3 where 0= all buds healthy, 1= <10% of buds showing symptoms of the disease, 2= 10-30% of buds infected with bud disease and 3= >30% of buds affected by the disease as described by (Jack, 2013) and modified by the author.

3.2.5 Data analysis and presentation of results

Data on pathogenicity for different fungal isolates on different plant parts were subjected to Analysis of variance (ANOVA) at 5 % level of significance using GenStat Release 16.1, VSN International Ltd. The mean differences were separated using Duncan Multiple Range Test (DMRT). These results were presented in table of means, line graphs with error bars (standard error of means), box-and-whisker plots, figures and plates.

3.3 *In vitro* efficacy of selected biocontrol agents and botanical extracts on pathogenic fungi

This experiment was set at University of Eldoret, Plant Pathology Laboratory where selected control agents were assessed on their inhibitory effects on mycelial fungal growth *in vitro*. Fungal pathogens subjected to control were *Fusarium oxysporum, Fusarium solani, Fusarium graminearium, Alternaria alternata* and *Rhizoctonia solani* that were

found to be pathogenic in the experiment two above. Botanicals, biological agents and synthetic fungicides were tested in this study. The choice of these materials was guided by the principle of their past history on their antimicrobial activities.

Botanicals such as onion bulbs (*Allium cepa*), Aloe leaves (*Aloe vera*), ginger rhizomes (*Zingiber officinale*), leaves of neem (*Azadirachta indica*) and garlic bulbs (*Allium sativum*) were used in this study. Onions bulbs, ginger rhizomes and garlic bulbs were randomly purchased from vendors in Eldoret local market while Neem leaves and Aloe leaves were sourced from University of Eldoret arboretum. Biological agents used in this study included antagonistic fungi such as *Trichoderma* spp. which are commercially available for farmers in most parts of the country. Fungicides selected were a preventive and a curative fungicide such as mancozeb and Carbendazim which are widely used by most farmers to manage fungal pathogens in a wide range of crops and were sourced from commercial shops within Eldoret town (Table 1).

Table 1: Plant extracts, biological control agents and their active ingredients as used in the in vitro efficacy assessment

Control agents	Common name	Scientific name	Active ingredients
Botanicals	Neem leaves Garlic bulbs Ginger rhizomes Aloe leaves Onions bulbs	*Azadirachta indica* *Allium sativum* *Zingiber officinale* *Aloe vera* *Allium cepa*	Nimbidin Allicin Gingerols and shagols High molecular weight compounds Triterpenoids
Biological Agents	Trianum-P Trichotech®	*Trichoderma harzianum* *Trichoderma asperellum*	*Trichoderma harzianum* *Trichoderma asperellum*
Fungicides	Rodazim 50sc	*Carbendazim*	*Carbendazim* 500g/l

3.3.1 Preparation of botanical extracts

Collected plant materials were thoroughly cleaned with tap water and surface sterilized using 1 % sodium hypochlorite for ten minutes then rinsed with sterile distilled water three times to remove traces of sterilizing agent. This was done to eliminate any foreign materials that may have accompanied collected materials. After surface sterilization these materials (ginger, garlic, onions and neem plant parts) were cut into smaller pieces before being air dried in an oven at 60 ^{o}C for 24 hours, there after they were ground into fine powder using an electric grinder at University of Eldoret seed physiology lab 5 according to (Neela *et al*., 2014). Obtained powder was further diluted to form plant crude extracts as follows: 100 grams of each powder were added to 100 grams of distilled water while shaking thoroughly to ensure uniform dilution of 100 % concentration. This resultant mixture was left to stand for 30 minutes on a hot water bath set at 100 ^{o}C and then kept for 24 hours at room temperatures and filtered using a sterile cheese cloth to obtain a pure filtrate for use as the stock solution.

Aloe vera gel which is a liquid fraction obtained from aloe leaves were extracted manually from smooth colorless parenchyma tissue obtained by carefully pilling the outer most cortex cells of the aloe leaf. Obtained aloe tissue was blended in a food blender to obtain a filtrate and sieved using a white man filter paper number 1 to separate the gel from the flesh and this gel was used while still fresh in a food poisoning technique (Karunarathna *et al*., 2021). *Trichoderma* spp. obtained from a commercial shop were cultured in a freshly prepared PDA media and incubated for five days before being used as an antagonist in a dual culture method (Admasu *et al.*, 2023; Suleiman & Emua, 2009).

3.3.2 Experimental design and linear statistical model

An experiment was set in a split plot arrangement in a completely randomized design (CRD) in the soil microbiology laboratory with five main plots that comprised of *Fusarium oxysporum, Fusarium solani, Fusarium graminearium, Alternaria alternata and Rhizoctonia solani* (test fungi) and seven sub plots (botanicals) each replicated three times. Synthetic fungicides were used as a positive check while a negative check comprised of test fungi without any control agent (Figure 3). A similar experiment to test the efficacy of biological agents in split plot design with fungal pathogens as the main plots and two treatments (biological agents) as sub plots replicated three times. Botanicals were tested using food poison technique and biological agents were tested using dual culture technique. The following are layouts of this experiment and a statistical linear model that guided analysis of the data collected.

MAIN PLOTS (Fungal isolates)	SUB PLOTS (botanical extracts and biocontrol agents)						
Fusarium oxysporum	T2	T4	T1	T5	T3	T7	T6
	T1	T7	T2	T6	T4	T5	T3
	T5	T3	T7	T4	T6	T1	T2
Fusarium solani	T1	T7	T2	T6	T4	T5	T3
	T5	T3	T7	T4	T6	T1	T2
	T2	T4	T1	T5	T3	T7	T6
Fusarium avenaceum	T5	T3	T7	T4	T6	T1	T2
	T2	T4	T1	T5	T3	T7	T6
	T1	T7	T2	T6	T4	T5	T3
Alternaria alternata	T4	T6	T1	T2	T5	T3	T7
	T5	T3	T7	T6	T2	T4	T1
	T6	T4	T5	T3	T1	T7	T2
Rhizoctonia solani	T5	T3	T1	T7	T2	T6	T4
	T1	T2	T5	T3	T7	T4	T6
	T7	T6	T2	T4	T1	T5	T3

KEY: **T1** - Neem
T2 - Garlic
T3 - Ginger
T4 - Onions
T5 - Aloe
T6 - Carbendazim (positive check)
T7 - Control (negative check)

Figure 3: Randomization plan for in vitro inhibitory assessment for botanical extracts against major fungal pathogens of pyrethrum

MAIN PLOTS (Fungal isolates)	SUB PLOTS biocontrol agents		
Fusarium oxysporum	T2	T1	T3
	T1	T3	T2
	T3	T2	T1
Fusarium solani	T1	T3	T2
	T2	T1	T3
	T3	T2	T1
Fusarium avenaceum	T1	T2	T2
	T3	T1	T3
	T2	T3	T1
Alternaria alternata	T2	T1	T3
	T3	T2	T1
	T1	T3	T2
Rhizoctonia solani	T2	T2	T1
	T1	T3	T3
	T3	T1	T2

KE **T6** - *Trichoderma harzianum*
T7 - *Trichoderma asperellum*
T9 - Control (negative check)

Figure 4*:* Randomization plan for in vitro inhibitory assessment of biological agents against major fungal pathogens of pyrethrum

Statistical linear model for *in vitro* studies

$Yijkl = \mu + \alpha i + \varepsilon j\ (i) + \beta k + \alpha\beta ik + \varepsilon ijkl$ where;

Yijkl = Total observation

μ = Overall mean

αi = i^{th} effect of main plot (fungal species)

εj (i) = j^{th} effect of main plot error

βk = k^{th} effect of sub-plot (control agents)

αβik = i^{th} and kth interaction effect between fungal species and control agents

εijkl = split - plot error

3.3.3 In vitro inhibitory assessment of fungal isolates against control agents

Efficacy assessment of botanical extracts

Botanicals were assessed using the agar dilution technique as described by (Banito *et al*., 2022) where control agents (botanicals and synthetic fungicides) were added to molten sterile PDA medium maintained at 40 ^{o}C at a given concentration. Concentrations of botanical extracts were determined based on previous reports on mycelial growth inhibition on different fungi invitro (Karunarathna *et al*., 2021) while those of synthetic fungicides were based on manufacturers recommended concentrations.

Two perpendicular lines were drawn at the bottom of each sterilized petri dish to facilitate accurate measuring of mycelial growth patterns during incubation period. 10ml of the control agent (botanical extracts) was added to 100 ml of sterilized potato dextrose agar medium (PDA) and dispensed equally onto five sterile petri dishes. In each treatment, petri dishes containing potato dextrose agar medium (PDA) only served as a negative control while Petri dishes amended with synthetic fungicides served as positive control. Seven day old mycelial plugs were carefully cut to 5mm pieces from the actively growing zone using sterile cork borer and placed at the point of intersection of the drawn lines on each petri dish and incubated, thereafter mycelium growth characteristics were measured at an interval of 72 hours for 9 days (Akaeze, & Aduramigba-Modupe 2017; Song *et al*., 2004).

3.3.4 Efficacy assessments of biocontrol agents

Dual culture technique was used in the testing of the level of inhibition by *Trichoderma harzianum* and *Trichoderma asperellum* on the growth of the fungal pathogens isolated from pyrethrum tissues. In this method, both the test fungi and the control agent were

cultured at equidistant positions towards periphery of petri dishes containing solidified PDA medium and the control contained test fungi without a control agent. Five-millimeter mycelial plugs for both the test fungi and the control agents which were cut using a sterile cork borer were used for this study (Maurya *et al*., 2014) at an interval of 24 hours for three days.

3.3.5 Data collection and analysis

After seven days of incubation, mycelial growth characteristics were recorded that is mycelial growth rate. Inhibition by the control agent (synthetics and botanicals), was recorded by measuring mycelial radial length following treatment with a control agent compared to mycelial radial length of control experiment. Plates of mycelium growth partens were taken during the assessment period. Mycelial growth inhibition was calculated by taking the measurements of the radius of the test fungi under control experiment (R1) and radius of the test fungi under treatment with control agent (R2) and recorded and the percentage inhibition was given by the formula shown below:

$$\mathbf{I}\ (\%) = \mathbf{\{(R1 - R2) / R1\}} \times 100$$

Visual observations were presented in form of plates taken from front and reverse sides of the colony growths as well as the effects of control agents on the mycelial growth characteristics. Also, data on percent inhibition of the control agents on test fungi were subjected to Analysis of variance (ANOVA) at 5 % level of significance using GenStat Release 16.1, VSN International Ltd. The mean differences were separated using Duncan Multiple Range Test (DMRT). These results were presented in table of means, line graphs with error bars (standard error of means), box-and-whisker plots, figures and plates.

CHAPTER FOUR

RESULTS

4.1 Symptomatology, diversity and frequency of isolation of major fungal pathogens of pyrethrum in Kenya

Different plant parts such as crown, flowers, leaves and stems were found to show symptoms of fungal disease infection which were common across the zones and were identified as bud disease, pyrethrum wilt and crown rot diseases. A total of 10 isolates were identified from the zones sampled and distinct morphological differences were observed physically on plates and at microscopic level in terms of spore shape and size as well as mycelial characteristics. *Fusarium oxysporum, Fusarium solani, Fusarium graminearium* and *Fusarium avenaceum* were found to be the major pathogens causing pyrethrum wilt disease. Also, *Sclerotinia minor*, *Alternaria solani* and *Rhizoctonia solani* were isolated from samples with crown rot symptoms while *Phoma* spp., *Alternaria alternata* and *Alternaria tenuissima* were found in tissues showing symptoms of bud disease. These identified fungi were found to have different isolation frequencies, however there was no variation based on sites sampled.

4.1.1 Symptomatology and diversity of fungi causing pyrethrum diseases

Fusarium wilt disease

Pyrethrum wilt disease was diagnosed as described by previous researchers (Kimani *et al*., 2001; Kinyua, 1996; Nattrass, 1950; Njoya-Kimani, 2001) based on the symptoms such as chlorotic leaves on some plants, beginning with the lower leaves and progressing to the leaf petioles as the mid veins gradually turn yellow, upon successful infection, affected pyrethrum display drooping of leaves as the disease progresses then wilting and eventually death of the whole plant Plate 1 (a). Brown discoloration on stems as a sign of developing vascular colonization was observed as shown in plate 1(b) as well as pale bleached lesions on stems that turn yellow and eventually dry as shown in plate 1 (c).

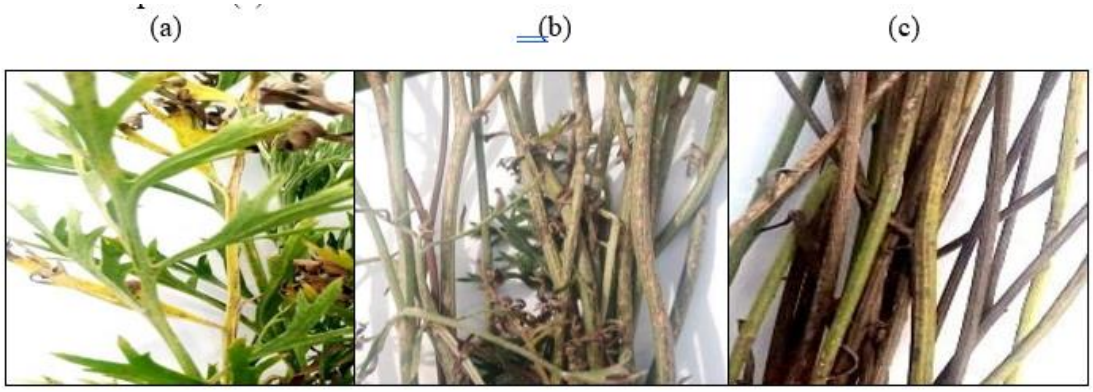

Plate 1: **Pyrethrum plant parts showing symptoms of wilt disease: (a) yellowing of leaves and Leaf petioles, (b) brown discoloration on stems, (c) yellowing and drying of stems (Source : Author, 2022)**

From the wilted parts plate 1, four fungal species were identified belonging genus *Fusarium*: *Fusarium oxysporum*, *Fusarium solani*, *Fusarium avenaceum* and *Fusarium graminearium* (Plate 2). These pathogens were isolated from different plant parts that included leaves, stems, flower stalk and even flower heads that were showing symptoms of wilt disease. Macroscopic as well as morphological characteristics were used in

identifying these pathogens (Arie, 2019; Machado *et al.*, 2015; Moslemi *et al*., 2017c; O'Malley, 2012).

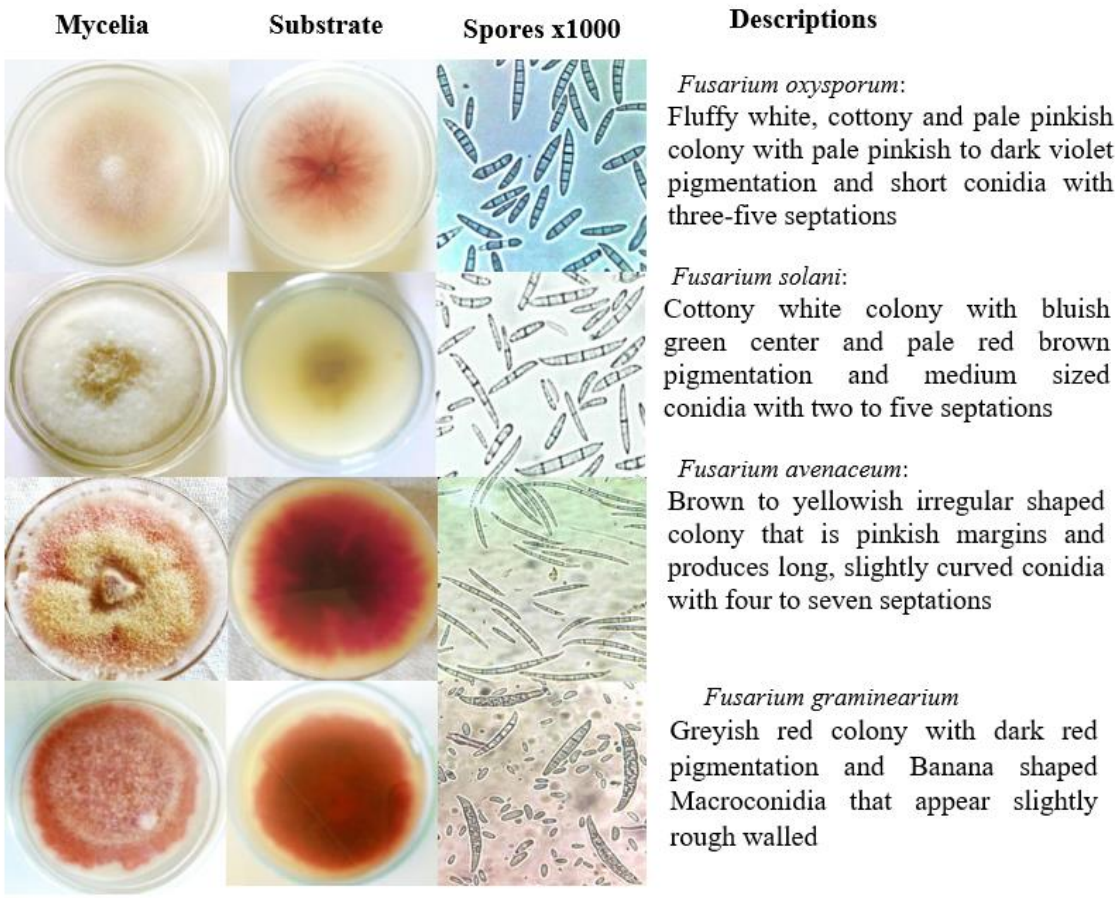

Plate 2: *Fusarium* species isolated from samples with pyrethrum wilt disease (*Fusarium oxysporum*), (*Fusarium solani*), (*Fusarium avenaceum*) *and* (*Fusarium graminearium*). Source Author 2022

Crown rot disease

This disease is characterized by dark, brown necrotic lesions on roots and the other basal parts of the plant such as the crown (Plate 3). These lesions enlarge as the disease progresses to the advanced stages resulting to wilting of the entire plant and in some cases; the crown part of the plant starts rotting and lower leaf petioles starts detaching themselves from the mother plant. (Cubeta & Vilgalys, 1997)

Plate 3: Upper parts of pyrethrum roots showing symptoms of crown rot disease. Source: Author 2022

From infected plant parts (Plate 4), two major fungal isolates were isolated: *Rhizoctonia solani* and *Sclerotinia minor*. These species belong to two different genera and were identified based on their macro and micro characteristics as described by (Al-Fadhal *et al*., 2019; Moni *et al*., 2016; Moslemi *et al*., 2016). Other species identified with lower frequency of occurrences were: *Fusarium oxysporum and Fusarium solani* that were identified (Plate3).

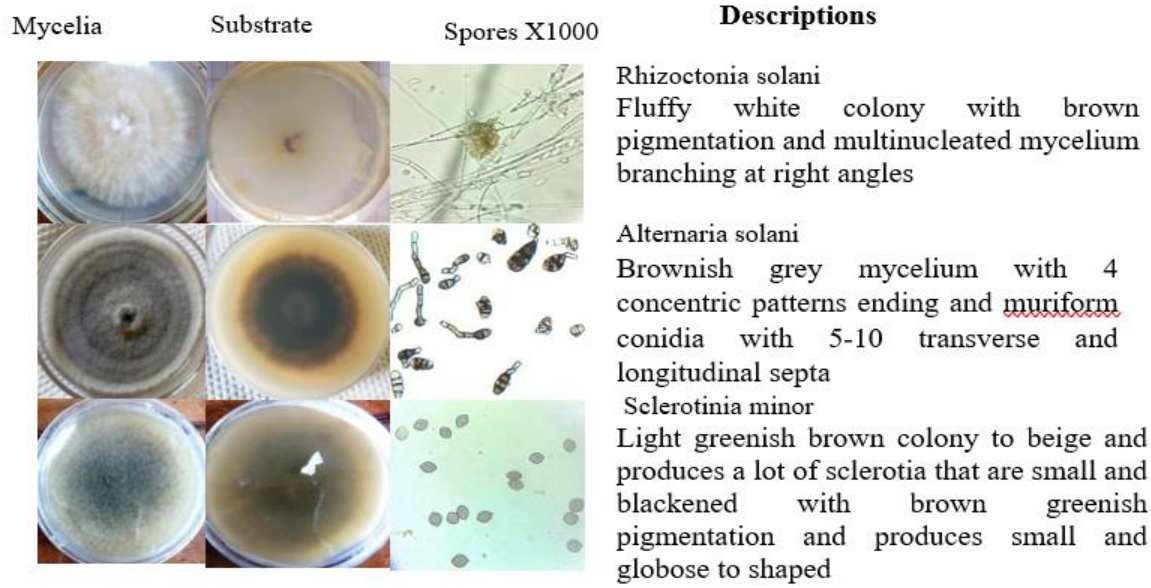

Plate 4: Fungal isolates from crown disease. Source: Author 2022

Bud disease

This disease was identified following the ray florets' blighting symptoms, which cause the heads to turn straw-colored or wilted. The most recognizable sign of bud disease was the emergence of flower buds with a "shepherd's crook," symptom which is brought about by infection and necrosis on one side of the higher flower stem (2 to 3 cm below the flower bud) (Bhuiyan *et al*., 2019) (Plate 5)

Plate 5: Flowers with symptoms of bud disease: Source: Author 2022

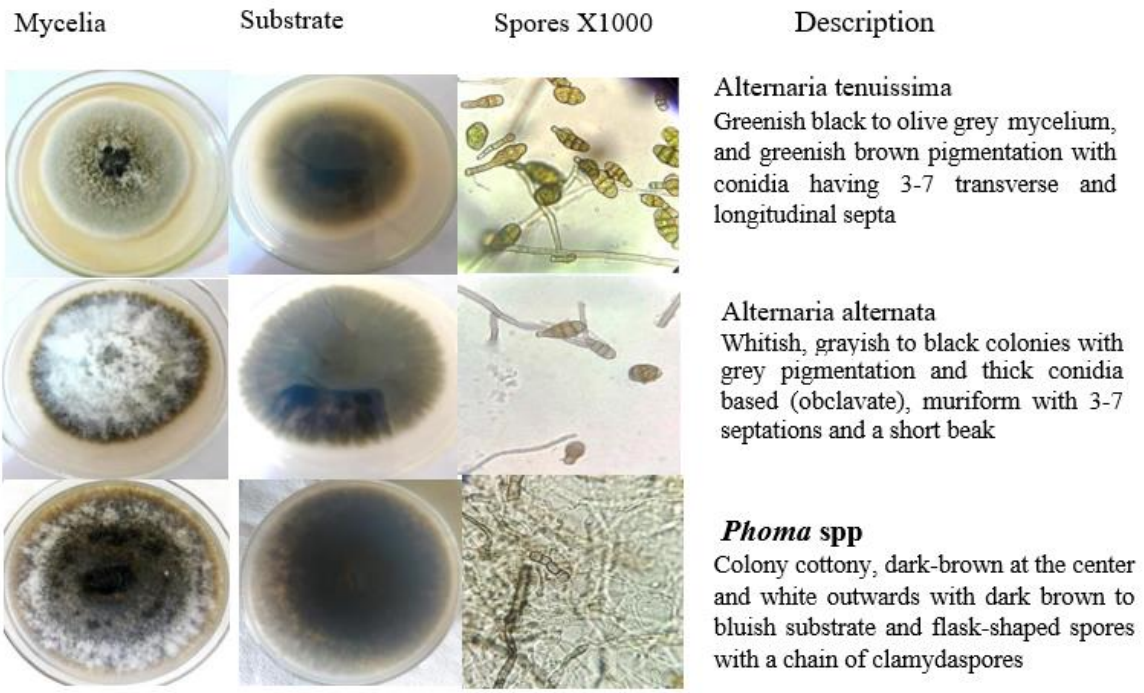

Plate 6: fungal isolates from bud diseased plants. Source: Author 2022

4.1.2 Frequency of isolation of identified fungi associated with pyrethrum diseases

The genus *Fusarium* was the most diverse genus with four species isolated; *F. oxysporum*, *F. Solani*, *F. avenaceum* and *F. graminearium* followed by genus *Alternaria* with three species isolated; *A. solani*, *A. alternata* and *A. tenuissima*, while *Phoma* spp., *Rhizoctonia solani* and *Sclerotinia minor* were single species isolated from specific genera. Seven species were isolated from Subukia while ten species were isolated from Molo site with *F. graminearium, S. minor* and *A. solani* confined to Molo site only. A total of sixty isolates were obtained from Subukia site samples while eighty species were isolated from Molo site samples. With respect to specific pathogens, *F. oxysporum* recorded a highest isolation frequency followed by *F. solani* while *Phoma* spp. and *F. graminearium* had the lowest isolation frequencies (figure 5).

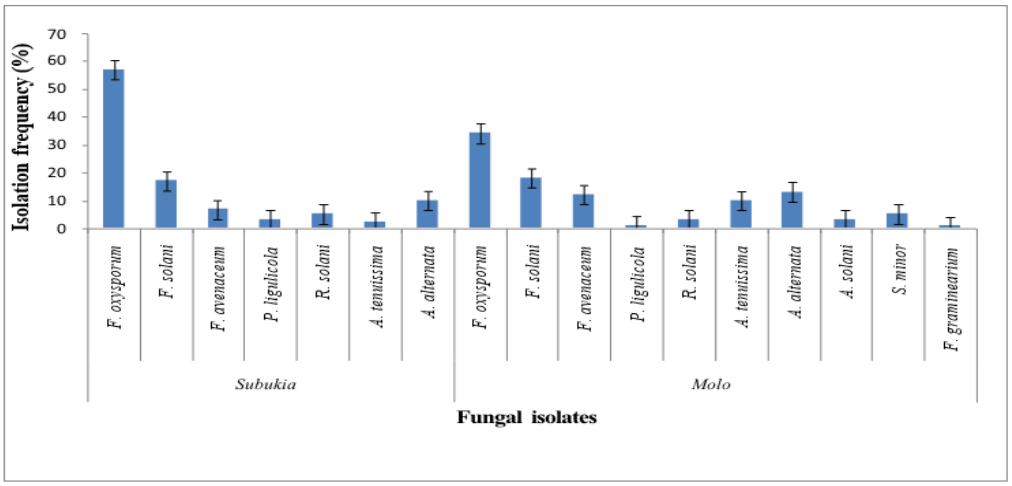

Figure 5: Isolation frequencies of fungal species isolated from Subukia. Error bars represent standard errors

4.2 Pathogenicity of major fungal pathogens affecting pyrethrum genotypes in Kenya

Different pyrethrum genotypes showed different levels of disease severities against different pathotypes of the genus *Fusarium.*

4.2.1 Response of pyrethrum genotypes to wilt disease (Fusarium species)

Three isolates identified as *Fusarium oxysporum*, *Fusarium solani* and *Fusarium avenaceum* that were found to cause pyrethrum wilts and with a higher isolation frequency in experiment one above was inoculated to potted selected pyrethrum genotypes namely; Clone 1, Clone 2, Clone 3, Clone 4 and P4. This experiment was monitored for a period of 126 days after inoculation with assessments being done at an interval of two weeks.

Time taken for the first symptoms to appear was different from genotype to genotype as some such as Clone 1, Clone 2 and P4 were more susceptible to the disease and symptoms were evident fourteen days after inoculation. Clone 3 was resistant to all the isolates inoculated and took forty-two days for the initial symptoms to appear, while Clone 4

appeared to be the most tolerant since it took seventy-two days for its resistance genes to be broken by the invading fungus.

Significant differences were expressed by all the genotypes in terms of their responses to pyrethrum wilt disease severity $p < 0.05$ under equal inoculum pressures. Additionally, disease severity increased progressively with time across all the genotypes with the highest disease severity being recorded on the last day (3.8) while the lowest being recorded on the first day (0.8). This observation was consistent across all the species of *Fusarium* inoculated which was either tolerance or susceptibility to the disease.

Clone 4 recorded the lowest mean severity score (1.1) while P4 recorded the highest mean severity score (3.8). In terms of ranking of the overall responses of genotypes; Clone 4 had the lowest severity followed by Clone 3 then Clone 1 and finally Clone 2 while P4 was the lowest in the rank with a highest severity score. This was assessed on a severity scale of 0 – 6 (Table 2).

Table 2: Response of selected pyrethrum genotypes to different species of Fusarium at an interval of two weeks after inoculation. The assessment was based on a severity scale of 0-6

Genotype	Fungal species	DAYS AFTER INOCULATION									Means		DMRT
		14	28	42	56	70	84	98	112	126	Species	Genotype	
CLONE 4	*F. oxysporum*	0.0	0.0	0.0	0.0	1.4	2.0	2.2	2.5	2.7	1.2		
	F. solani	0.0	0.0	0.0	0.0	1.2	1.4	2.1	2.3	2.6	1.1	1.1	a
	F. avenaceum	0.0	0.0	0.0	0.0	1.1	1.2	1.8	2.1	2.3	0.9		
CLONE 3	*F. oxysporum*	0.0	0.0	0.5	1.4	2.3	2.3	2.4	2.7	2.7	1.6		
	F. solani	0.0	0.0	0.3	1.1	2.1	2.2	2.4	2.5	2.6	1.5	1.5	b
	F. avenaceum	0.0	0.0	0.1	1.1	1.9	2.0	2.3	2.5	2.5	1.4		
CLONE 1	*F. oxysporum*	0.5	1.3	2.5	2.5	2.7	3.3	3.7	2.5	4.2	2.6		
	F. solani	0.5	1.4	2.4	2.6	2.7	3.1	3.4	4.0	4.1	2.7	2.5	c
	F. avenaceum	0.8	0.7	1.6	2.2	2.3	2.5	2.5	3.3	3.3	2.1		
CLONE 2	*F. oxysporum*	1.2	2.3	2.4	2.6	2.8	3.1	3.3	5.0	5.0	3.1		
	F. solani	1.4	2.0	2.4	2.5	2.6	2.7	3.2	3.8	5.0	2.8	2.8	d
	F. avenaceum	1.2	2.0	2.1	2.3	2.5	2.6	2.8	3.6	3.8	2.5		
P 4	*F. oxysporum*	2.0	3.0	3.5	3.6	3.5	4.5	5.1	5.6	5.6	4.0		
	F. solani	2.1	2.5	2.9	3.0	3.6	4.1	4.8	5.3	5.3	3.7	3.7	e
	F. avenaceum	1.9	2.0	2.2	2.3	2.8	3.3	5.0	5.4	5.4	3.4		
Means (Days)		0.8	1.2	1.5	1.8	2.4	2.7	3.1	3.5	3.8	2.3		
DMRT		a	b	c	d	e	f	g	h	i			

Statistics	Genotype (G)	DAI (D)	Species (I)	G x D	D x I	G x I	G x D x I
Probability	< .001	< .001	< .001	< .001	< .001	< .001	< .001
S.E	0.02	0.03	0.02	0.07	0.06	0.04	0.13
S.E.D	0.03	0.05	0.02	0.1	0.08	0.05	0.18
%CV	9.7						

Irrespective of the *Fusarium* species, the severity rating on a scale of 0-4 categorized P4 to be the most susceptible while Clone 4 and Clone 3 were the most tolerant respectively. Grouping was done based on their mean disease severities following inoculation of different species of *Fusarium*. These results point out that selected genotypes were genetically different as could not exhibit similar responses (Figure 6).

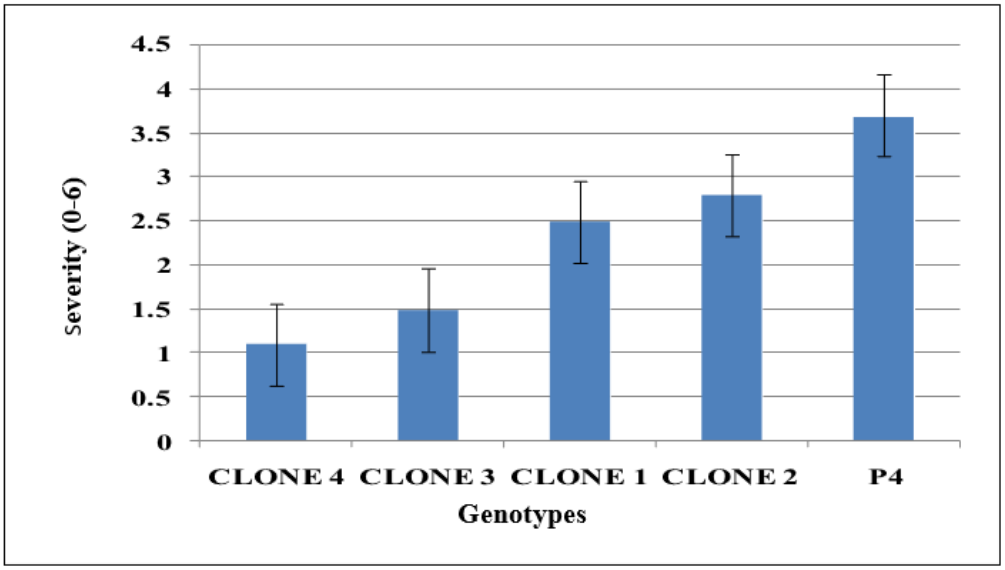

Figure 6: Response of selected genotypes to Fusarium wilt disease. Error bars represent standard error

Disease severity progressed with time as minor symptoms were recorded on the initial stages of genotype development that is fourteen days after inoculation while major severity was recorded on the final day (126 days). Some genotypes such as P4, Clone 2 and Clone 3 showed symptoms of wilt disease fourteen days after inoculation that were yellowing of lower leaves and in other genotypes yellowing and wilting of some of the leaves. Other genotypes exhibited similar symptoms after forty-two days for Clone 3 and after fifty-six days for Clone 4. These results were consistent to all the species of *Fusarium* inoculated. Additionally, some genotypes attained maximum disease severity earlier compared to other genotypes to an extent of completely drying up for the case of P4 with reference to *Fusarium oxysporum*. Additionally, pathogenicity levels of different species of Fusarium increased progressively with time and this was consistent to all the species (Plate 7).

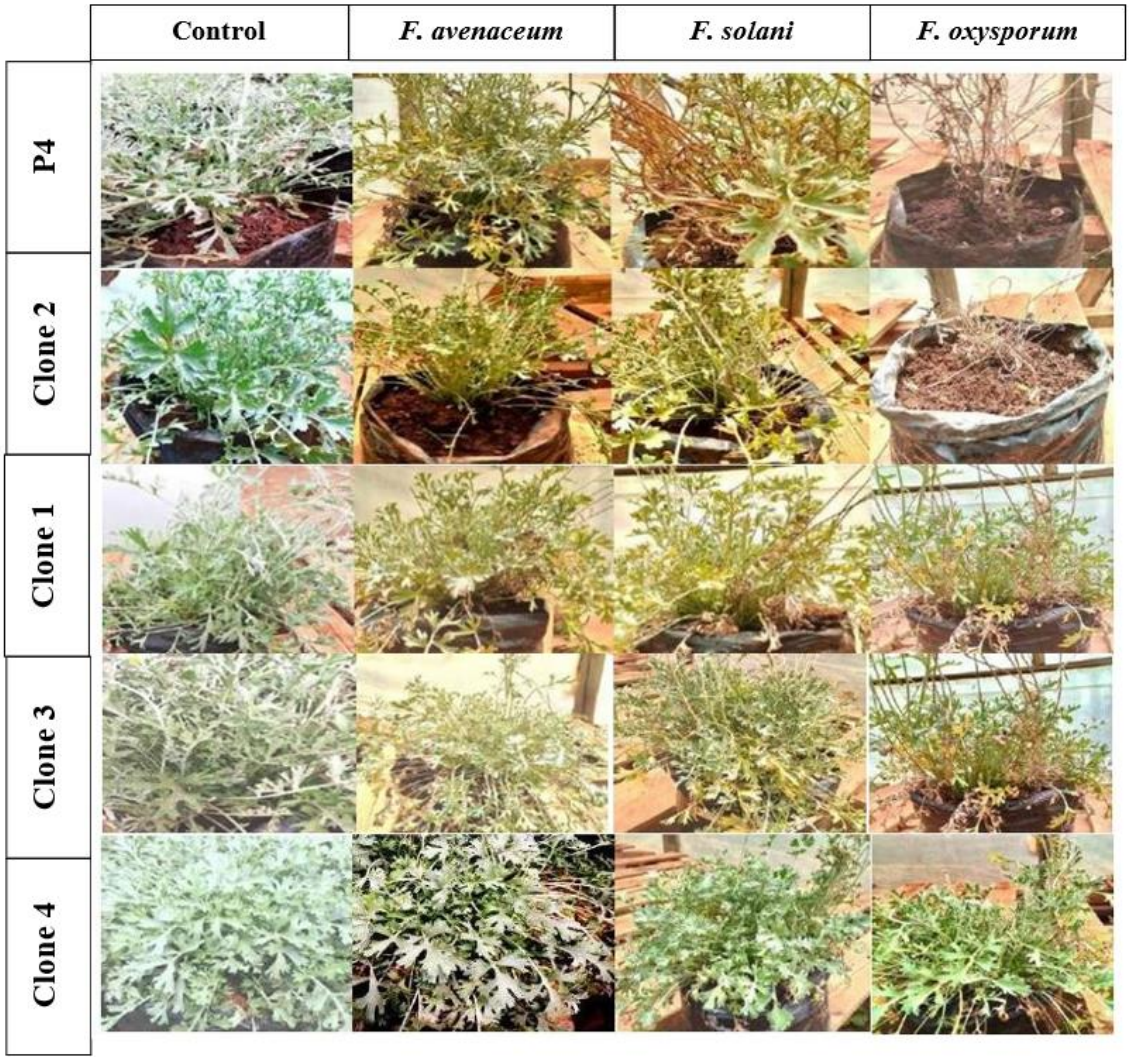

Plate 7: Response of different pyrethrum genotypes to infection by different species of *Fusarium* causing pyrethrum wilt disease under greenhouse conditions. Source: Author 2022

Overall mean severity was highest on the final day and lowest at the initial stages. This implies that tissue colonization increases with time as the pathogen develops and multiplies with time coming in contact with more tissues (Figure 5).

Specifically, the morphological expression corresponds to the severity scores with respect

to different species of *Fusarium* against the pyrethrum genotypes. For instance, *Fusarium oxysporum* (Plate 8) caused total drying of the whole plant (P4 and Clone 2) by day 126 while Clone 1, Clone 3 and Clone 4 proved tolerant to this species of *Fusarium.* However, and *Fusarium avenaceum* (Plate 10) did not lead to total drying of all clones as well as P4 at the end of 126 days post-inoculation.

Different isolates exhibited varied levels of pathogenicity which were significantly different as $p < 0.05$ as shown in appendix iii below. *Fusarium oxysporum* was more pathogenic followed by *Fusarium solani* and the least pathogenic was *Fusarium avenaceum figure 7.*

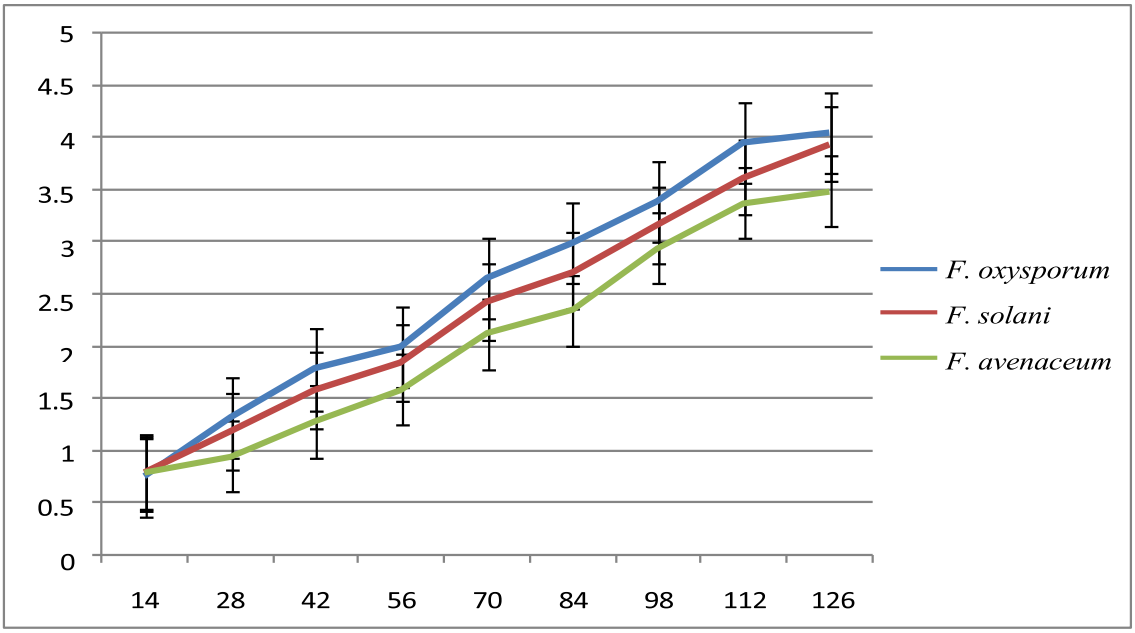

Figure 7: Pyrethrum wilt disease progress from 14th to 126th day after inoculation (Scale 0-6)

4.2.2 Response of pyrethrum clones to bud disease

There was a significant difference between the pyrethrum genotypes in their response to bud disease $p < 0.05$ (Appendix ii). Clone 4 exhibited more tolerant qualities since less buds were affected by bud disease at the time of assessment, followed by Clone 1; However, Clone 2, Clone 3 and P4 appeared to be more susceptible to bud disease since it had highest severity as shown in figure 3 and plate 7 below (severity scale 1-3).

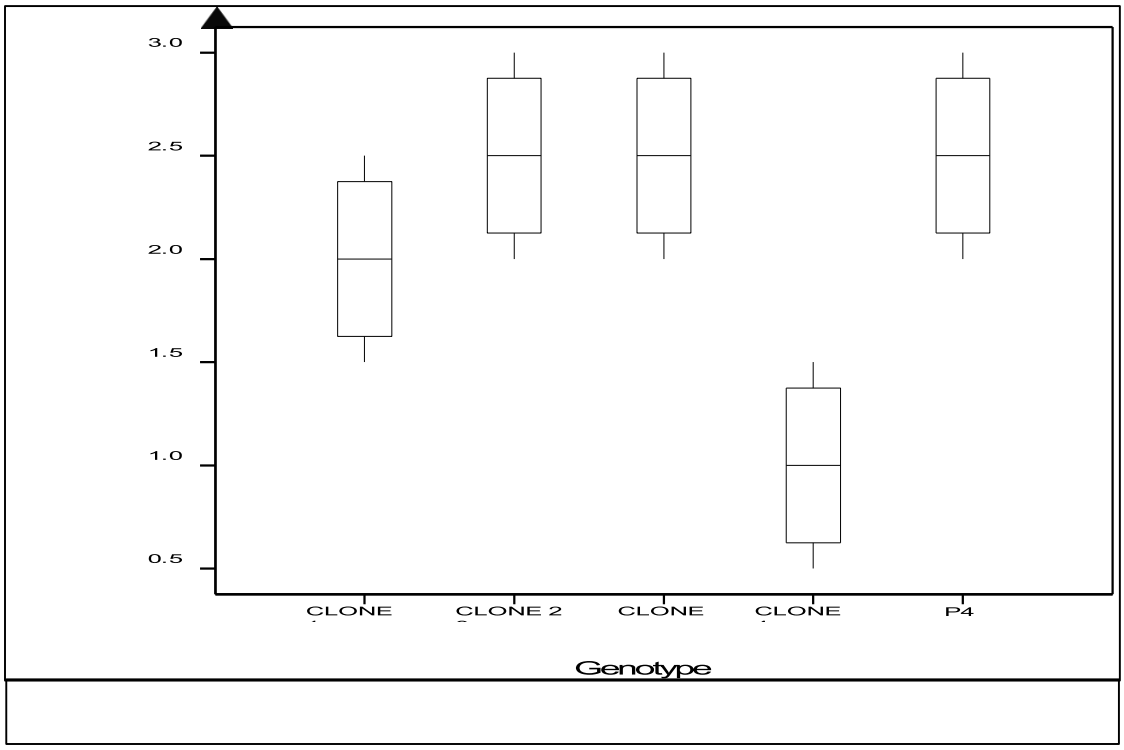

Figure 8: Severity responses and distribution of pyrethrum clones to bud disease

In ascending order to susceptibility of pyrethrum clones to bud disease, Clone 4 was the most tolerant followed by Clone 1, Clone 2 and Clone 3 respectively. In contrast, P4 showed highest susceptibility despite being the currently grown variety for commercial purposes (Plate 11).

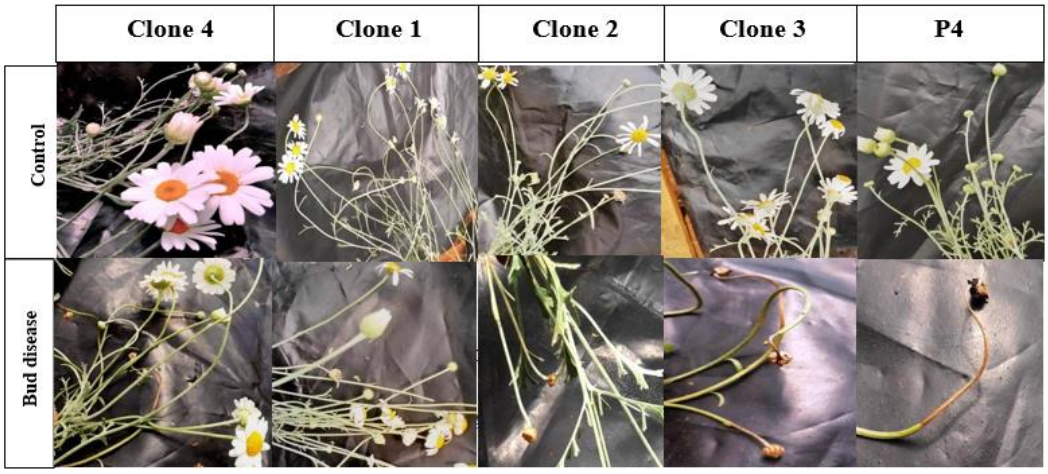

Plate 8: Response of different pyrethrum genotypes to infection by *Alternaria alternata* causing bud disease under greenhouse conditions. Source: Author 2022

4.2.3 Response to crown rot disease

Selected genotypes differed significantly in their response to crown rot disease $p<0.05$ (Appendix iii). P4 exhibited severe crown discoloration followed closely by Clone 2 whereas Clone 1 and Clone 3 did not differ significantly and with minor crown discoloration. However, Clone 4 proved tolerant to crown rot disease with approximately sixty days after inoculation as displayed in the box-and-whisker plot on a 0-2 severity scale (Figure 9).

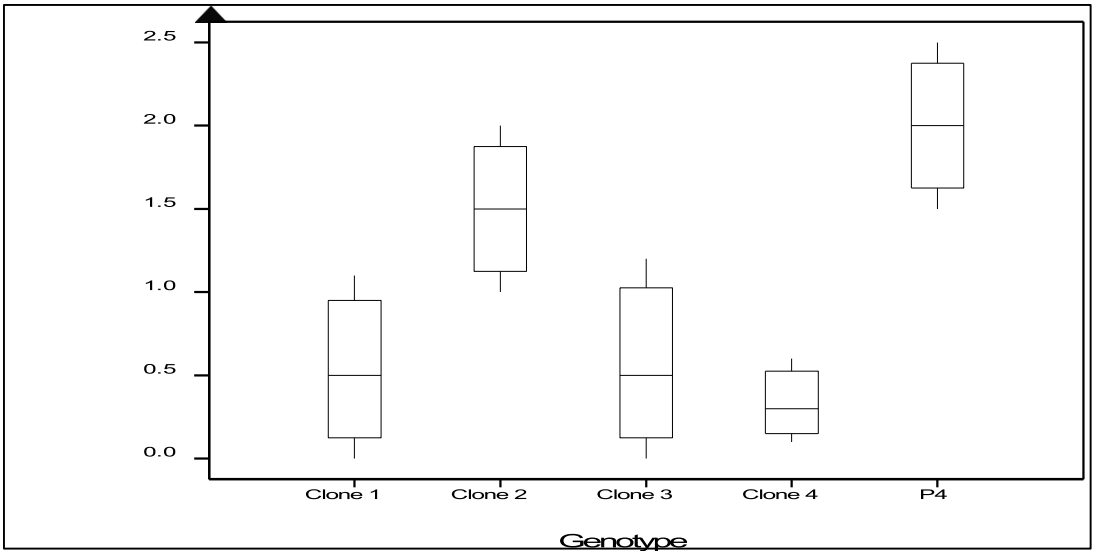

Figure 9: Mean differences and distribution of pyrethrum Clones in their response to crown rot disease

In terms of phenotypic expressions, P4 was the most susceptible to crown rot disease while clone 4 proved tolerant 60 days post-inoculation (Plate 9).

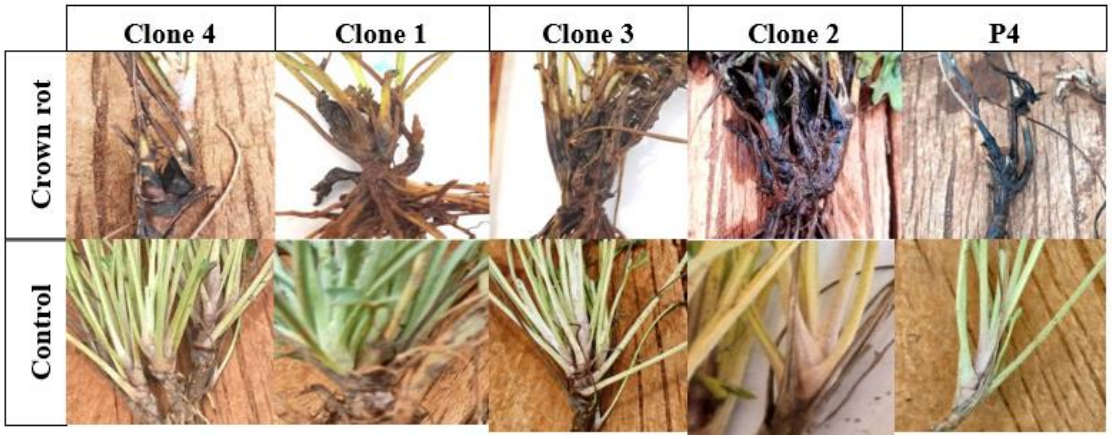

Plate 9: Response of different pyrethrum genotypes to infection by *Rhizoctonia solani* (crown rot disease) under greenhouse conditions. (Source: Author, 2023)

4.3 *In vitro* reactions of botanical extracts and biocontrol agents on pathogenic fungi

4.3.1 Effects of botanical extracts on mycelial growth of different fungi

Varied levels of inhibition of test fungi (*Fusarium oxysporum, Fusarium solani, Fusarium avenaceum, Alternaria alternata and Rhizoctonia solani*) by selected control agents T1 (*Allium sativum*), T2 (*Zingiber officinale*), T3 (*Azadirachta indica*), T4 (*Aloe vera*), T5 (*Allium cepa*), T6 (negative control), and T7 (positive control) was noticed throughout the inhibition period. Maximum inhibition of mycelial radial growth was recorded in T7 in all test fungi treated with a synthetic fungicide except for *Alternaria alternata* in which the maximum inhibition was achieved with garlic while zero inhibition was recorded among all test fungi in T6 that were incubated with no control agent, these results were consistent in all the fungi subjected to this control agent, however, there was a great variation in the fungicidal levels of treatment in the fungi tested.

There was a progressive growth of the mycelium radial length from third day to ninth day in all the treatments tested. Maximum radial length was attained by most controls within day nine and this is when the inhibition rates of the control agents were assessed. Maximum inhibition was evident on test fungi treated with garlic and a positive control Carbendazim as there was a no growth during the inhibition period. This observation Trans versed all test fungi except for *Alternaria alternata* where, maximum inhibition was achieved with garlic alone during day 3, day 6 and also day 9 (Figure 10).

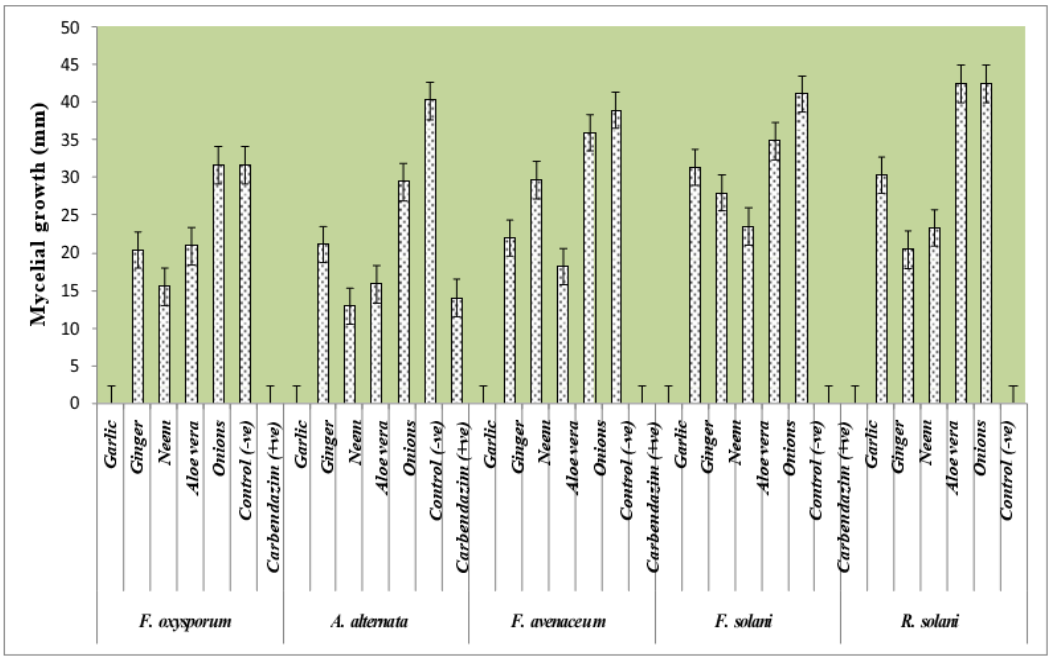

Figure 10: Inhibitory effects of plant extracts on mycelial growth of *Fusarium oxysporum, Alternaria alternata, Fusarium avenaceum, Fusarium solani, and Rhizoctonia solani*. Error bars represents standard errors.

Treatment effects were significantly different in their levels of inhibition among all the test fungi after the incubation period as $p<0.05$ (Appendix 4). Significant mycelial growth inhibition was expressed by selected control agents with garlic and Carbendazim (100%) showing full inhibition followed by neem (81%), Aloe (54%), Ginger (37) while least inhibition was recorded in treatment with onions (20%) for *Fusarium oxysporum* figure 13 (i). Garlic completely inhibited mycelial growth of *Alternaria alternata* (100%). Some botanicals such as Neem (72%), Aloe (65%) performed better than the positive check Carbendazim (65%) while ginger (19.67%) and onions (6%) showed the least inhibition as shown in figure 13 (ii) below.

Positive control (Carbendazim) and garlic had similar levels of inhibition in the case of *Fusarium avenaceum, Fusarium solani* and *Rhizoctonia solani* (100%) while neem, Aloe,

Ginger and onions followed in the rank respectively as shown in figure 13(iii), (iv) and (v) below.

In terms of the overall performance of the selected botanical extracts, varied levels of inhibition that were significantly different $p<0.05$ were recorded on different test fungi. Garlic showed promising results with a mean of 100% inhibition which was followed closely by the positive check Carbendazim, Neem, Aloe vera, Ginger and onions followed respectively but with significant differences. Minimum inhibition was recorded in test fungi treated with onions extracts across all the test fungi. *Rhizoctonia solani* stood out to be more inhibited by the plant extracts (70%). *Alternaria alternata* and *Fusarium avenaceum* were the least inhibited by the plant extracts (57%) while *Fusarium oxysporum* and *Fusarium solani* were moderately inhibited (62%) and (63%) respectively (Figure 11, Plate 10).

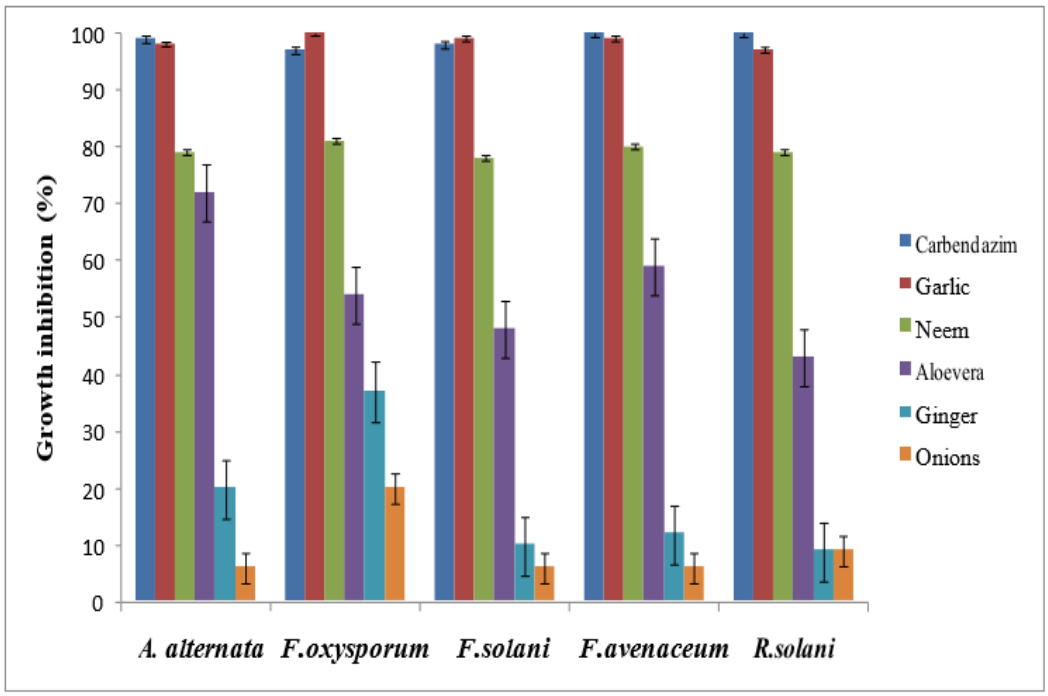

Figure 11: Mycelial growth inhibition (%) by plant extracts against *Alternaria, Fusarium* and *Rhizoctonia* species *in vitro*. Error bars represent standard error

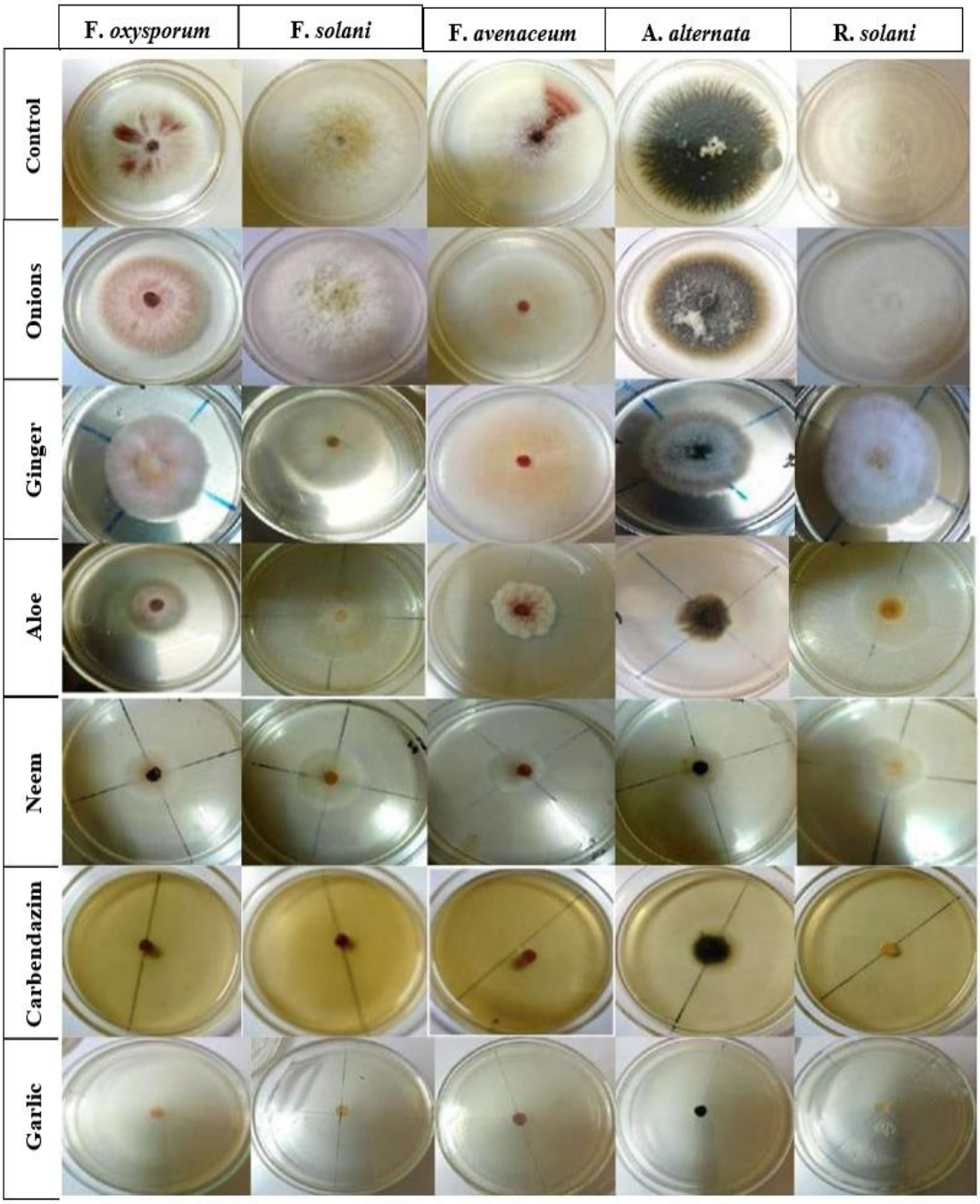

Plate 10: Inhibitory effects of selected botanical extracts on pyrethrum fungal pathogens under invitro conditions within nine days of incubation.
(Source: Author, 2023)

4.3.2 Effects of *Trichoderma harzianum* and *Trichoderma asperellum* on mycelial growth

Two biocontrol agents *Trichoderma harzianum* and *Trichoderma asperellum* were tested in this study against the five test fungi. It was observed that there was a significant difference between the two fungi in the mycelial growth inhibition of the test fungi $p < 0.05$ (Appendix IV). *Fusarium solani* (81%) was more inhibited by *Trichoderma asperellum* followed closely by *Fusarium oxysporum* (70%), *Rhizoctonia solani* (58%), *Fusarium avenaceum* (55%) while *Alternaria alternata* (45%) was least controlled. There was no significant difference in the response of *Fusarium avenaceum* and *Rhizoctonia solani* as displayed in Figure 12.

Trichoderma harzianum inhibited mycelial growth of the fungi at varied levels. There was a greater inhibition in mycelial growth of *Fusarium solani* (87%), followed by *Fusarium oxysporum* (81%), *Rhizoctonia solani* (64%), *Fusarium avenaceum* (57%) and *Alternaria alternata* (57%) (Figure 12).

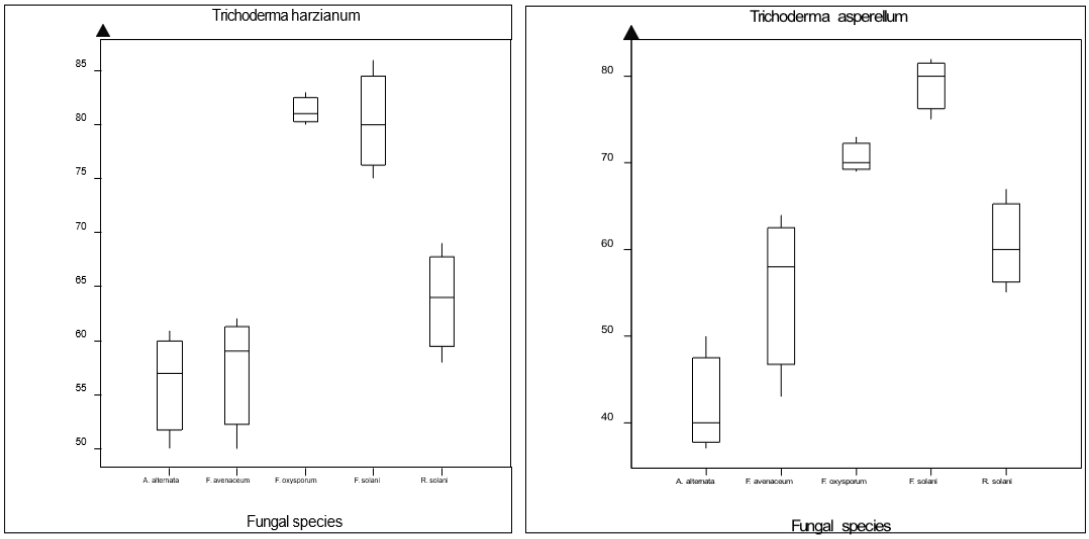

Figure 12 Inhibitory effects of biocontrol agents on mycelial radial length of major pyrethrum disease causing pathogens under in vitro conditions

Relative to control experiment, the biocontrol agents namely *Trichoderma asperellum* and *Trichoderma harzianum* slowed down the growth rate of *Fusarium*, *Alternaria* and *Rhizoctonia* species *in vitro*. This resulted into distinct inhibition zones separating the biocontrol agents and the pathogenic fungal species six days post-incubation. In addition to this, there was no overlap in all interactions between the biocontrol agents and the pathogenic fungal species (Plate 11)

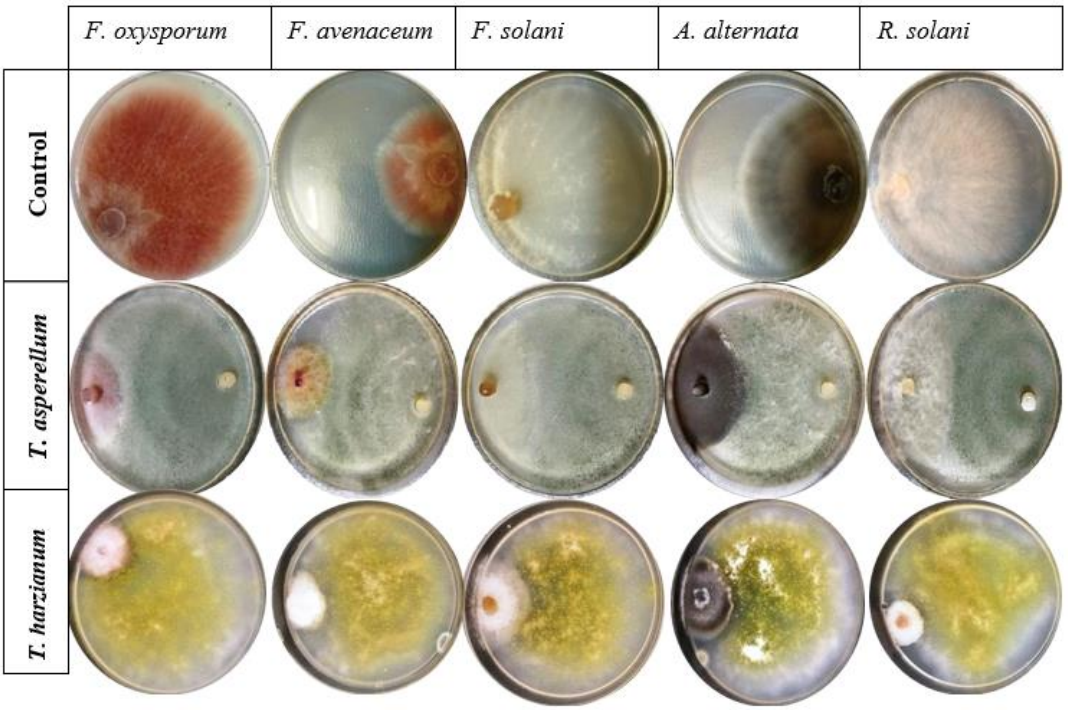

Plate 11: Inhibitory effects of selected species of Trichoderma on fungal pathogens of pyrethrum under invitro conditions during six days of incubation. (Source: Author, 2023)

CHAPTER FIVE

DISCUSSION

5.1 Symptomatology, diversity and frequency of isolation of major fungal pathogens

Pyrethrum wilts, crown rot and bud disease were the major fungal diseases identified in Subukia and Molo regions where pyrethrum is grown. Pyrethrum is grown in the Subukia and Molo regions, which were found to be major areas of concern. Pathogens known to cause wilting can have invaded the plant tissues passively or actively through direct penetration in susceptible cultivars in the establishment of hyphae in the presence of the hot and damp climate, which is endemic in these areas. After successful colonization, the hyphae entered epidermal cells and spread in vascular tissues, thus hampering water and nutrient flow via xylem vessels and causing wilting of plants (Moslemi, 2017). Moreover, the root systems that are believed to be infected by the crown rot fungi are through root pores which are used to colonize the crown.

The action of the enzymes that follow the secretion breaks down the cell walls, making the tissues soft and rottable by adverse environmental factors that eventually lead to the death of the organisms in harsh environments (Kader, 2002). Additionally, there was a bud disease, which was characterised by shepherd crook deformities in flower buds, which were caused by infection two or three centimetres below the floral apex on one side of the stem. This disease resulted in permanently dry flower heads probably because of fungal obstruction of vascular conduits (xylem and phloem) to inhibit the movement of photosynthates, water and other nutrients to the apex (O'Malley, 2012; Pethybridge, Hay, *et al*., 2008).

The morphological variations of the isolated species of fungi could be caused by induced

variation within the strains of pathogen, which were already in the environment, adaptations to changing abiotic conditions, and an increased ability to overcome resistance in newly introduced cultivars, as well as morphologically diversified reproductive, survival, and dispersal mechanisms (Bahram and Netherway, 2022). *Sclerotinia minor* and *Fusarium graminearium* were confined to Molo site only and this could be due to their ability to adapt to environmental conditions of this site hence environmental specificity.

Differences in isolation frequencies were recorded among the different species and this could indicate the existence in variability in terms of virulence of these fungi, specificity to different environmental conditions and also susceptibility of the host (Singh Saharan *et al*., 2023). *Fusarium oxysporum* had the highest frequency of isolation while *Phoma ligulicola* had the lowest isolation frequency. This could mean that *F*. *oxysporum* was the most virulent pathogen and the most adapted to prevailing environmental conditions present at the pyrethrum growing zones under study (Liu *et al*., 2021; Moslemi *et al*., 2016; Motanya, 2019; Nyoro, 2019).

5.2 Pathogenicity of major fungal pathogens affecting pyrethrum genotypes

Diverse symptoms were observed on five different pyrethrum genotypes screened. The common symptoms were wilting, crown rot and bud diseases which were expressed fourteen (14), sixty (60) and ten (10) days respectively after inoculation thus confirming the pathogenicity of the isolated fungal pathogens. They exhibited similar symptoms of the respective diseases as those that had initially been identified in the field; however, the severity levels varied significantly from one genotype to the other. For all the fungal isolates tested under greenhouse conditions, morphologically identical isolates to those initially identified were re-isolated from the pyrethrum tissues post-inoculation thus

fulfilling the requirements of the Koch's postulates as described by (Bhunjun *et al*., 2021). All the isolates tested were pathogenic and were able to infect and cause diseases on the genotypes tested at varied severities thus corresponds to the findings by the earlier researchers (Moslemi *et al.*, 2017b; Pethybridge *et al.*, 2004; Pethybridge, Jones, *et al.*, 2008; Pethybridge & Wilson, 1998) that identified pathogenic fungal species that were infectious to the pyrethrum plants under pot and field experiments.

In the genus *Fusarium*, *Fusarium oxysporum* was extremely virulent and caused the most severe wilt disease of all the genotypes, and *Fusarium avenaceum* had relatively low virulence between all clones of pyrethrum. Nonetheless, Fusarium solani was not highly aggressive in character where decreased levels of virulence and severity were recorded against the pyrethrum clones placed under conditions of control. This may be attributed to diversification in the genetic structure of the species at the species level hence distinguishing the level of pathogenicity in the conditions of the host pathogen between favorable conditions and adverse conditions, where the disease may thrive. There could also be that these three species of the genus *Fusarium* possessed different capacities in regards to potential sporulation post-infection, type and amounts of toxins and their capacity to surmount host resistance mechanisms at morphological, physiological and genetic levels (Sakr, 2022).

Considering the number of days following inoculation, there was the expression of progressive disease severity in all of the fungal pathogens with time can be explained by the fact that once the pathogens had successfully inoculated, the pathogens kept reproducing and colonizing more cells and tissues in the host plant, thus, causing death and damage to more tissues as the days went provide conditions to do so. There might also have

been secondary infections by other pathogens resulting in injury of the tissue over time at the accelerated rate of tissue damage due to oxidative mechanisms, with increasing severities as the process continued (Simko and Piepho, 2012).

Differences in genetic compositions of genotypes tested could have led to different responses to pathogen infections under greenhouse conditions. For instance, Clone 4 expressed higher tolerance to most pathogenic fungi while P4 (commercially grown variety) was more susceptible to pathogenic fungi. This could be due to varying response mechanisms by these two genotypes through activated stress signal pathways leading to synthesis of pathogenesis related proteins that have antifungal activities and induces systemic resistance in the host plant. Therefore, it is possible that these Clone 4 and P4 synthesized varying levels of such proteins hence varying levels of resistance. In terms of virulence, *Fusarium* wilt disease differed from one genotype to the other and this could be due to the varying response channels where some of the genotypes may have synthesized antagonistic antifungal compounds with strong bonds that took time to be broken (Shoresh *et al*., 2010; Were, 2018).

5.3 *In vitro* inhibitory reaction of botanical extracts and biocontrol agents against pathogenic fungi

Selected plant extracts and biocontrol agents expressed varied antifungal potentials against major fungal pathogens of pyrethrum under *in vitro* conditions. The radial growth of the test isolates was inhibited by botanical extracts. Namely, the highest percentage inhibition was obtained with garlic extract, which was used in combination with the positive control Carbendazim, whereas the lowest percentage inhibition was recorded with onion extract at the same concentration of 50 0. The observations align with the results of Kipkogei *et al*. (2019),

who stated that extracts of ginger and turmeric reduced the growth of Alternaria alternata, whereas extracts of neem and other plants inhibited the growth of the papaya anthracnose in the form of the mycelium in the in-vitro conditions (Kugui, 2021). More literature has shown that aqueous plant extracts are able to inhibit mycelial growth of the fungal pathogens within a range of 50% to 100% (Ogbebor *et al*., 2007), which also agrees with our data whereby the use of garlic showed complete inhibition at 50% concentration against the major isolates. Allicin, which is a sulphur containing compound, is abundant in garlic extracts and can be found in the extracts of garlic in concentrations that are about three times more than onion. The compounds have the antibacterial and antifungal activity based on the penetration of the fungal cells, spores, and cytoplasm, which involves the alteration of the essential protein thiols and consequently disrupts the electron transport chain used to produce respiration (Benkeblia, 2004; Yasmeen *et al*., 2020).

High molecular weight compounds contained in aloe vera extracts inhibits mycelial growth of the fungi, the results of this study are in line with those of (Sitara *et al*., 2011), who stated that constituents of aloe vera have toxic effects on mycelial growth of most fungi.

It has been demonstrated that Nimbidin compounds that are found in Azadirachta indica can cause cell wall degradation in a wide range of microorganisms, thus making the mycelial growth of the fungi tested inhibited (Rodrigues *et al*., 2019). On the other hand, gingerols and shogaols formed as a result of ginger extracts help in the antifungal effect of the extract through the inhibition of germination of spores, which in turn inhibit the growth and development of fungi (Choudhary *et al*., 2022). Trichoderma spp had strong microlaisis effects on the growth of the mycelia of the major pathogenic fungi of pyrethrum; the intensity of the antifungal effect was however variable among the pathogens

studied in the context of biocontrol. The differences can be related to the different sets of bioactive metabolites produced and the different mechanisms the two Trichoderma species used in antagonizing the pathogens. In this perspective, Trichoderma harzianum proved to be more effective in inhibiting the development of pathogenic fungus in terms of their mycelia in comparison to Trichoderma asperellum which showed relatively lower inhibitory activities in the culture media. Additionally, the identified hostility can also be due to the release of secret of antibiotic substances or parasitic relationships with fungal hyphae, which leads to their disintegration, penetration, and lysis, as it has been previously described (Amin *et al*., 2010; Barari, 2016; Bastakoti *et al*., 2017). The use of aqueous extracts of several plant species as a natural source of fungicidal-like properties has been emphasized by their antifungal properties.

This study demonstrates the possibility of extracts of some plants to be used as fungicides. Numerous researchers have also noted that the antifungal properties of various plant species and emphasized the significance of plants as a potential source of natural fungicides. This would lead to conservation of environment by reducing overreliance on synthetic fungicides that are hazardous to the ecosystem as well as reduction on the cost of production since plant extracts are relatively cheaper and can be locally available in the common farmer settings.

CHAPTER SIX

CONCLUSIONS, RECOMMENDATIONS AND WAY FORWARD

6.1 Conclusions

Fusarium, Rhizoctonia and *Alternaria* species are the major pathogenic fungi affecting pyrethrum growth, yield and flower quality Molo and Subukia zones of production in Kenya. These fungi differed in their cultural and morphological characteristics such as mycelial growth, mycelial color, substrate color as well as micro characteristics and hence their diversities.

Clone 4 was the most tolerant while P4 was the most susceptible genotypes to wilt, crown and bud diseases under greenhouse conditions. The genotype responses also varied from one pathogenic fungus to the other within the same genus of *Fusarium* and between genera.

Among the plant extracts, garlic extracts was the most efficacious with 100% inhibition against *Fusarium*, *Alternaria* and *Rhizoctonia* species *in vitro*. However, *Trichoderma harzianum* ranked second best after garlic in terms of inhibition of mycelial growth of the three genera.

6.2 Recommendations

Sensitization and increased awareness on the identified fungal pathogens among the key stakeholders along the pyrethrum value chain.

The inclusion of Clone 4 in breeding programs aiming at conferring tolerance towards *Fusarium* species, *Alternaria alternata* and *Rhizoctonia solani* as the major pathogenic fungi.

Garlic extract and *Trichoderma harzianum* are recommended for use as best non-chemical approach in the management of *Fusarium*, *Alternaria* and *Rhizoctonia* species affecting pyrethrum performance in Kenya.

6.3 Way forward

Establish genetic variability among the isolated pathogenic fungi using molecular techniques to ascertain the validity of the observed morphological characteristics.

Determine and map the exact genes responsible for the observed tolerance and susceptibility responses among the screened pyrethrum clones with respect to different pathogenic fungi.

Profile the active biochemical compounds with antifungal properties in garlic, document their mode of actions, perform efficacious assessment under natural environmental conditions and formulate a bio fungicide for use in the management of major fungal pathogens.

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APPENDICES

Appendix I: Anova tables for the response of different pyrethrum genotypes to pyrethrum wilt disease caused by Fusarium spp.

Analysis of Variance **Variate: Severity** **Source of Variation**	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Replication stratum	2	0.0	0.0	0.5	
Genotypes	4	364.0	91.0	3448.8	<.001
Residual	8	0.2	0.0	0.7	
Isolates	2	12.7	6.3	170.6	<.001
Genotypes.Isolates	8	3.5	0.4	11.8	<.001
Residual	20	0.7	0.0	0.7	
Days	8	411.0	51.4	1023.7	<.001
Genotypes.Days	32	37.1	1.2	23.1	<.001
Isolates.Days	16	2.9	0.2	3.7	<.001
Genotypes.Isolates.Days	64	13.2	0.2	4.1	<.001
Residual	240	12.0	0.1		
Total	404	857.4			

Appendix II: Analysis of Variance table showing the response of different pyrethrum genotypes to bud disease

Variate: Severity **Source of variation**	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Genotypes	4	4.951	1.238	5.140	0.016
Residual	10	2.407	0.241		
Total	**14**	**7.357**			

Appendix III: Analysis of Variance table showing the different responses of Pyrethrum genotypes to crown rot disease

Variate: Severity **Source of variation**	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Genotypes	4	6.607	1.652	9.460	0.002
Residual	10	1.747	0.175		
Total	**14**	**8.353**			

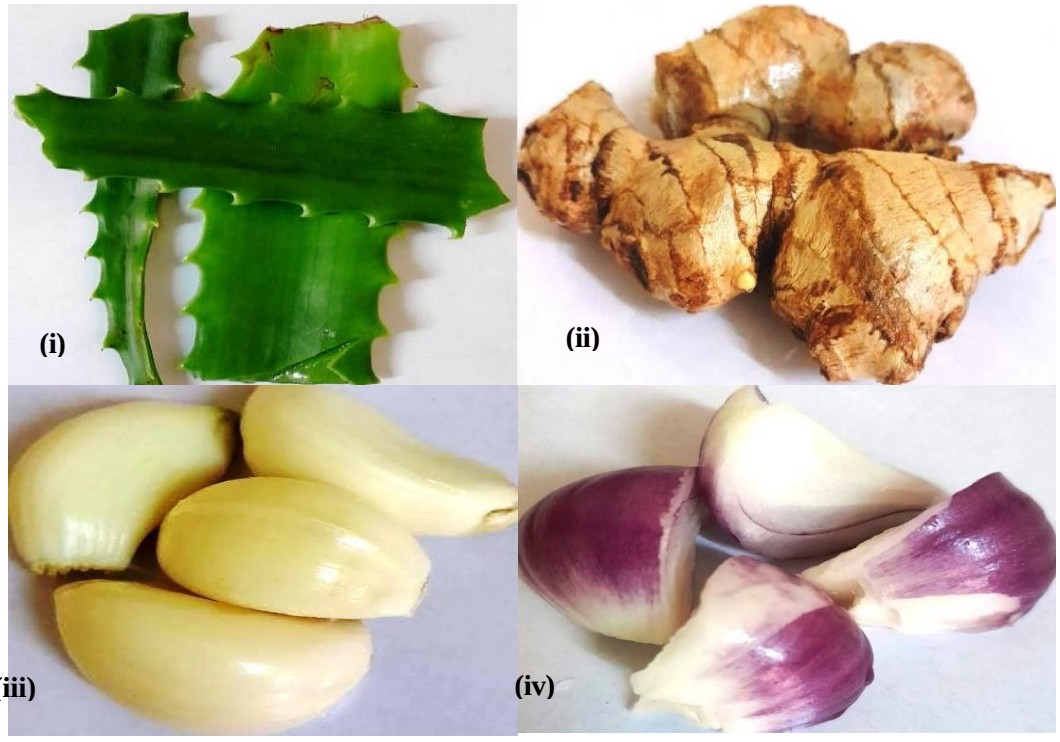
Appendix IV: Anova tables for inhibitory effects of selected botanical agents

Analysis of variance **Variate: %_inhibition** **Source of Variation**	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
REP stratum	2	34.343	17.171	2.25	
Fungal species	4	2439.714	609.929	79.88	<.001
TREATMENT	6	116192.5	19365.41	3234.41	<.001
ISOLATE.TREATMENT	24	8106.019	337.751	56.41	<.001
Residual	60	359.238	5.987		
Total	**104**	**127192.9**			

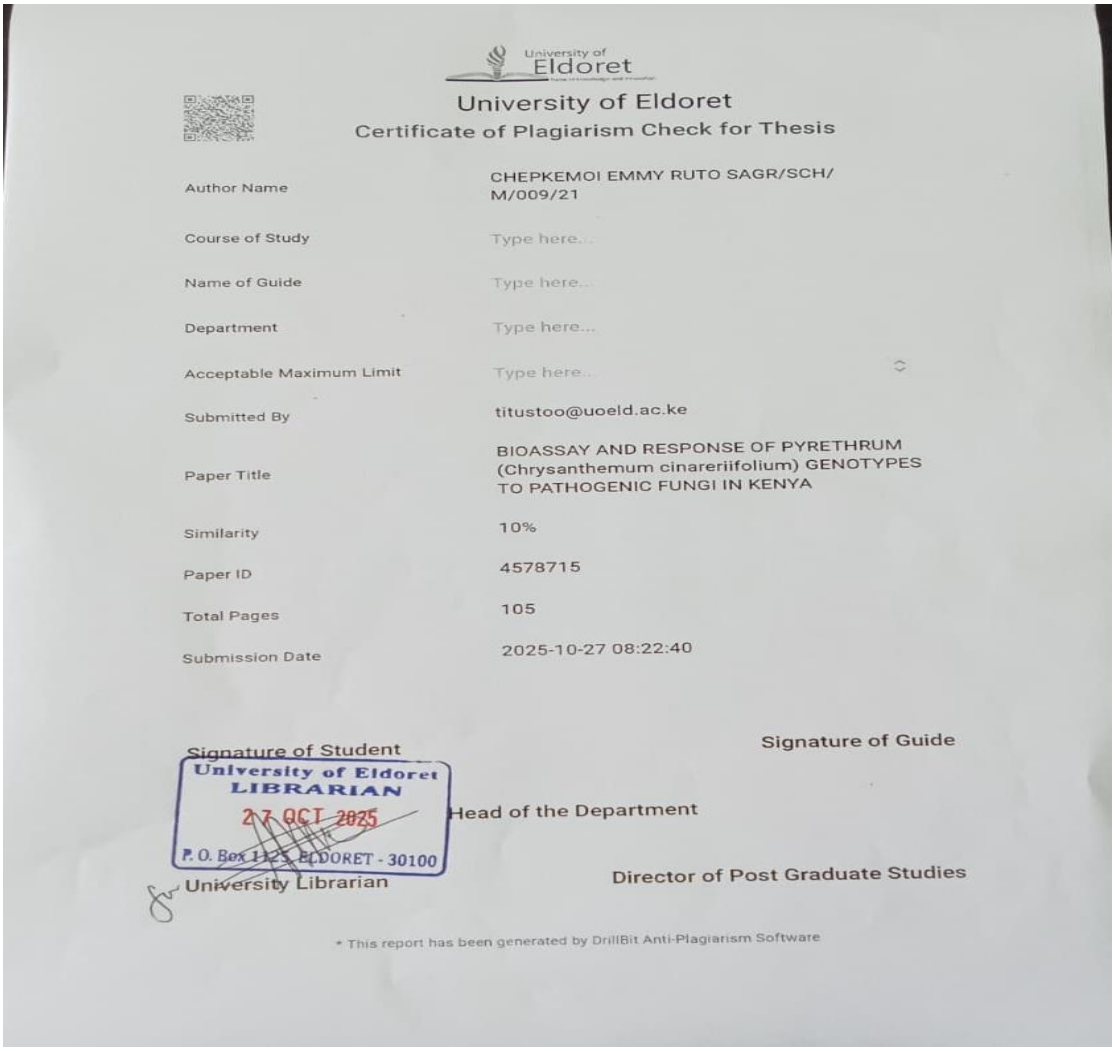
Appendix V: Anova tables for inhibitory effects of selected biocontrol agents

Analysis of variance Variate: %_inhibition Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
REP stratum	2	40.267	20.133	0.34	
Isolate	4	4554.533	1138.633	19.13	<.001
Treatment	1	425.633	425.633	83.46	<.001
Isolate.Treatment	4	105.867	26.467	5.19	0.016
Residual	10	51	5.1		
Total	**104**	**127192.9**			

Appendix VI: A sample of selected botanicals used in the inhibitory tests: Source: Municipal market Eldoret.



Appendix VII: Plagiarism check report



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