IDENTIFICATION AND CHARACTERIZATION OF CAUSATIVE AGENTS OF BROWN LEAF SPOT OF CASSAVA AND PHENOTYPIC RESPONSE OF ELITE CASSAVA GENOTYPES TO INFECTION BY THE PATHOGENS

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DECLARATION

This thesis is my original work and has not been presented for an award of a degree in any other institution or university.

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DEDICATION

This work is dedicated to the Kenya Agricultural and Livestock Research Organization (KALRO) and particularly the Virus Resistant Cassava for Africa (VIRCA) Plus project for the research idea on cassava brown leaf spot disease.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BLS	Brown Leaf Spot
BRI	Biotechnology Research Institute
CABI	Centre for Agriculture and Bioscience International
CAD	Cassava Anthracnose Disease
CBB	Cassava Bacterial Blight
CBSD	Cassava Brown Streak Disease
CMD	Cassava Mosaic Disease
CRRD	Cassava Root Rot Diseases
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
IFAD	International Fund for Agricultural Development
IITA	International Institute of Tropical Agriculture
ISAAA	International Service for the Acquisition of Agri-biotech Applications
ITS	Internal Transcribed Spacers
KALRO	Kenya Agricultural and Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
NaCl	Sodium Chloride
NASE	Namulonge Selection
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PPD	Post-harvest Physiological Deterioration
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
TME	Tropical *Manihot esculenta*

TE	TRIS EDTA
TRIS HCL	Trisaminomethane hydrochloride
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USDA	United States Department of Agriculture
VIRCA	Virus Resistant Cassava for Africa

ABSTRACT

Cassava is one of the most important food crops globally and is a major staple food for more than 700 million people across the tropical and sub-tropical world. Cassava is adversely affected by diseases (caused by viruses, bacteria, nematodes and fungi), pests and adverse environmental constraints. Current research activities in cassava diseases are focused more on viral and bacterial diseases than on fungal diseases, yet phytopathogenic fungi play an important role in causing devastating disease epidemics leading to significant annual yield losses. One of the most important fungal diseases is the cassava brown leaf spot (BLS) disease. This study aimed at identification and characterization of the causative agents of BLS disease in Kenya and the reaction of cassava genotypes to infection by the pathogens. Experimental materials were sourced from the Kenya Agricultural and Livestock Research Organization (KALRO) Biotechnology Research Center, KALRO Kakamega and from a confined field trial of the Virus Resistant Cassava for Africa (VIRCA) project located at KALRO Kandara in Murang'a County. Fungi were isolated and purified on antibiotic-amended PDA media from the symptomatic leaf samples. Identification of the fungal pathogens was based on cultural and morphological characteristics of pure fungal cultures coupled with molecular characterization of individual pathogens. Results of the study delineated three pathogens from the genera *Colletotrichum*, *Cladosporium* and *Alternaria*, working in synergism to produce brown leaf spot disease as observed in the fields. The three fungal pathogens were used, in combination, to challenge five farmer-preferred cassava genotypes TME 204, TME 14, TME 7, Ebwanatereka 1 and Ebwanatereka 2. Symptoms were observed over a period of 56 days (eight weeks) at intervals of seven days, after which disease progress was determined. Analysis of Variance (ANOVA) was carried out using GenStat software, 15th Edition while molecular data was analyzed using Geneious Prime and MEGA 11 softwares. The cassava plants had varied responses depending on genotype. The highest area under disease progress curve (AUDPC) was 178.5 in TME 204 and lowest at 103.8 in Ebwanatereka 2. With regard to symptom severity, Ebwanatereka 2 exhibited a relatively slow response to infection compared to the other genotypes throughout the assessment period. On the other hand, TME 204 maintained a high infection response therefore indicating high level of susceptibility to cassava brown leaf spot disease. Findings of this study will add to the knowledge gap in the management approaches to cassava brown leaf spot disease. More research should be carried out to identify sources of resistance to the disease.

CHAPTER ONE: INTRODUCTION

1.1 Background to the study

Cassava (*Manihot esculenta*) is a woody shrub that belongs to the family Euphorbiaceaea. It is widely cultivated in the tropics and subtropics as an annual crop and used as a major source of carbohydrates. Cassava produces edible starchy storage roots that are long and tapered and covered with a strong detachable skin that is rough and brown on the outside (Wassie, 2019). In developing countries, particularly in sub-Saharan Africa, cassava plays an essential role as a food security crop owing to its ability to grow well in low rainfall and on poor and marginal soils (Mtunguja *et al*., 2019). Being perennial, the crop can be harvested as need arises and has a wide harvesting window that allows it to act as a reserve for famine and is important in management of labour schedules. It serves either as a subsistence and/or a cash crop thus offering flexibility to resource-poor farmers. Having been described as one of the most drought-tolerant crops, cassava has gained popularity and is therefore cultivated widely in the cassava growing countries and in new production zones (Mtunguja *et al*., 2019). This is because the crop is adaptable to vast environments therefore can mitigate the effect of climate change.

In Kenya, cassava is used to manufacture commodities such as gluten-free flour, feeds for animals, sucrose substitute in beverages and confectionary products. The gluten-free carbohydrates in cassava is important in preventing gluten intolerance as well as food allergies. Due to the high fibre content of the crop, it is helpful in reducing cholesterol level. Cassava is also rich in minerals such as manganese, calcium and iron, which is helpful to expectant women (Ministry of Agriculture, 2019).

Cassava is one of the most important food crops globally and ranks fourth as a food crop in developing countries after rice, wheat and maize (Agricultural Research Council, 2014). It is a major staple food for more than 700 million people across the tropical and sub-tropical world and has gained popularity as a source of carbohydrates as well as income for millions of smallholder farmers in sub-Saharan Africa (Legg *et al*., 2014). For optimum growth and yield, cassava requires humid warm climates with temperatures between 25^{o}C and 29^{o}C and evenly distributed annual rainfall of between 1000-1500mm. Regardless of these ideal requirements, cassava is widely adaptable, growing in a range of soils and rainfall regimes (Tan, 2015).

Despite the vast production and utilization of cassava, there are biotic and abiotic stresses that adversely affect its yield thereby limiting the full realization of its immense potential (Bull *et al*., 2011). Much as cassava can survive harsh environmental conditions, its productivity is severely affected by; terminal drought, extremes of heat, salinity, pH and flooding (Tadele, 2018). Biotic stresses affecting the crop include insect pests the common ones being whiteflies, mealybugs, green- and red-spider mites, scales, shoot flies, fruit flies and cassava horn worm. Cassava diseases are mainly caused by viruses, nematodes, bacteria and fungi. The main viral diseases affecting cassava are cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) while bacterial diseases include cassava bacterial blight (CBB) and bacterial stem rot. Among the major fungal diseases are brown leaf spot (BLS), cassava anthracnose disease (CAD), *phyllosticta* leaf spot, white thread and super-elongation disease (Mwang'ombe *et al*., 2013; Titus and Lawrence, 2015). These challenges pose a threat to food security especially in the tropics and sub-tropics (Bull *et al*., 2011; Campo *et al*., 2011). As a way of reducing yield losses to insects and pests, Howeler *et al*. (2013) suggests the use of resistant cassava varieties, use of control agents and managing levels of crop nutrients in order to reduce insect reproduction. On the other hand, cassava diseases can be cotrolled through the use of clean planting materials, elimination of infected plants and crop rotations in order to suppress pathogens. One of the most important factors in effective disease control is accurate identification of the disease causative agent(s). This study was therefore carried out to identify the causative agents of one of the most important phytopathogenic fungal diseases namely cassava brown leaf spot.

1.2 Problem statement

Current research activities in cassava diseases are focused more on viral and bacterial diseases than on fungal diseases, yet phytopathogenic fungi play an important role in causing devastating disease epidemics leading to significant yield losses. This has made plant pathogenic fungi a serious economic factor and a threat to food security thus attracting the attention of all agricultural stakeholders including farmers, breeders and scientists across the globe (Li *et al*., 2020). Cassava fungal diseases include root rots, foliar diseases and stem necrosis disease. These diseases cause a significant loss of planting materials thus making them unsuitable for planting (Boas *et al.*, 2017). Cassava root rot diseases (CRRD) are caused by a complex of soil-borne fungi including *Fusarium* spp. *Phytophthora* spp., *Pythium* spp., *Neoscytalidium* spp. and

Lasiodiplodia spp. (Boas *et al*., 2017). These fungi at times occur in different compositions of species and in some cases restricted to a geographical region. These root rot diseases are a major constraint responsible for up to 80% of yield losses (Boas *et al*., 2017). Foliar diseases include cassava super-elongation disease, white leaf spot and cassava brown spot diseases while cassava stem diseases include cassava bud necrosis and cassava anthracnose disease (McCallum *et al*., 2017; Legg and Álvarez, 2017). Super-elongation disease, caused by the fungus *Sphaceloma manihoticola* causes more than 80% losses in susceptible cassava genotypes while cassava anthracnose disease (CAD), caused by the fungus *Colletotrichum gloeosporioides* (Kunkeaw *et al*., 2010) causes a viability loss of 50-75% in infected planting materials (Legg and Álvarez, 2017).

One of the most important fungal diseases is the cassava brown leaf spot (BLS). It is characterized by large, brown, necrotic spots appearing on older leaves and the infected leaves have a tendency to drop early (Msikita *et al*., 2000). The disease is spread to new leaves and plants by wind or rain splash. BLS disease epidemics in cassava are reported worldwide majorly on lower canopy of crops that are more than five months old. The disease is favored by high humidity and temperature resulting in about 78% disease incidence in the wet savannahs and 98% in the case of transition forest of West African countries. The importance of cassava brown leaf spot disease may be underestimated due to its being confined to the lower canopy leaves. However, the disease causes defoliation which may have a significant effect on yield especially in areas where cassava is extensively grown for commercial production. The effect of defoliation becomes aggravated when the infection is followed by a period of drought (Hillocks and Wydra, 2002).

Since the disease is usually favored by high temperature and humidity, high rainfall areas are more prone to the disease than areas of relatively low rainfall (Hillocks and Wydra, 2002). In this regard therefore, it is advisable to use cultivars that are less susceptible to the disease. The highly susceptible germplasms are often attacked quite early. In these cultivars, the brown spots can cover a great surface area of the leaves and this could significantly reduce photosynthetic activities, thus reducing yields (Moses *et al.*, 2015). The causative pathogen of cassava brown leaf spot disease has been identified and characterized as reported in studies conducted in various countries but in Kenya little has been done to identify and/or characterize the pathogen.

1.3 Justification of the study

Studies on cassava brown leaf spot (BLS) disease have been carried out in various regions including China (Pei *et al*., 2014), Thailand (To-Anun *et al*., 2011), Asia, North- and Latin America and some parts of Africa (Lozano and Booth, 1974). However, little has been done in Kenya to understand the pathogen(s), yet there is increase in cassava production in the country including areas which were not main production zones. In all the production areas, the disease is rampant and has also been observed in recent outbreaks in cassava field trials in the country (H. Obiero, personal communication, September, 2016). Besides, the farmer-preferred cassava genotypes which are currently in use for consumption and research work are susceptible to BLS disease. All these factors necessitate the need to pay more attention to BLS disease in order to enhance its management. The need to identify the causative agent(s) of cassava brown leaf spot disease cannot be overemphasized. Identification of the causative agent(s) is an important pre-requisite in better management of cassava BLS disease thereby avoiding devastating crop yield losses. In the various regions where the disease has been studied, the causative agent has been reported to be a fungus in the genus *Cercospora*. In Kenya, the causative agent(s) has not been studied comprehensively and may or may not be the same fungus. The aim of this study was therefore to identify the causative agent(s) of cassava brown leaf spot in Kenya and to determine the reaction of popular cassava genotypes to infection by the pathogen(s).

1.4 Objectives

1.4.1 Broad objective

To contribute to better management of cassava brown leaf spot disease through identification of the causative agent and assessment of phenotypic reaction of elite cassava genotypes to the pathogen.

1.4.2 Specific objectives

i. To identify and characterize the causal agent of cassava brown leaf spot in Kenya
ii. To determine the phenotypic response of elite cassava genotypes to infection by cassava leaf spot pathogen(s)

1.5 Hypotheses

i. The causative agent for the cassava leaf spot is a fungal pathogen
ii. The selected cassava germplasm have no significant difference in their response to infection by cassava leaf spot pathogen(s)

CHAPTER TWO: LITERATURE REVIEW

2.1 Cassava production in the world

Cassava is grown in more than 105 countries globally including in Asia, North- and Latin America and Africa (Kim *et al*., 2017). Over the years, Africa has been the leading producer of cassava, followed by Asia then the Americas; from 2010 to 2019 the proportions were 58%, 31% and 11% respectively (FAOSTAT, 2021). Cassava is the third most important source of calories in the tropics, after rice and maize. It is important because its starchy tuberous roots are invaluable sources of cheap calories especially in countries where calorie deficiency is widespread (Bayata, 2019). Although cassava roots are high in caloric value, they are deficient in mineral thus placing the populations that rely on cassava as staple in these deficiencies (Okwuonu *et al*., 2021). A potential solution to this challenge is the advancement in biotechnology that has ushered the development of transgenic cassava bio-fortified with iron and zinc (ISAAA AfriCenter, 2020). Over half a billion people in Africa, Asia and Latin America depend on cassava as a staple food (CIAT, 2019). The crop is mainly grown by resource poor farmers, mostly women, often on marginal land. For these people, cassava is essential not only for food security but also for income generation (Mwang'ombe *et al.*, 2013). Being one of the most important food crops in the world, cassava had an annual global production at around 303 million tons in 2019 (FAOSTAT, 2021). The leading world cassava producers in 2019 are illustrated in Table 1. The statistics on cassava production in Kenya are also included for comparison purpose (FAOSTAT, 2021). Being a climate resilient crop, demand for cassava has grown due to its appeal in food security for growing populations in emerging markets, as well as the growing demand for processed cassava products in the industry (Sanginga *et al*., 2015).

Table 1: Leading world cassava producing countries, quantity and acreage in 2019.

	Country	Quantities (Tons)	Acreage (Ha)
1	Nigeria	59,193,708	7,215,162
2	Democratic Republic of the Congo	40,050,112	4,919,457
3	Thailand	31,079,966	1,386,655
4	Ghana	22,447,635	1,027,755
5	Brazil	17,497,115	1,190,121
6	Indonesia	14,586,693	640,526
7	Cambodia	13,737,921	504,940
8	Viet Nam	10,105,224	519,306
9	Angola	9,000,432	945,328
10	United Republic of Tanzania	8,184,093	990,835
11	Kenya	970,587	69,621

Source: FAOSTAT, 2021

2.2 Economic importance of cassava

Cassava is mainly cultivated in the tropics and subtropics as an annual crop and used as a major source of carbohydrates. In sub-Saharan Africa, cassava is mainly grown as a subsistence crop for food by small-scale farmers who sell the surplus. The crop grows well in poor soils with limited labor requirements, and provides food security in time of conflicts where the invader cannot easily remove or destroy because it conveniently grows underground (Tadesse *et al*., 2013). Besides food, cassava is very versatile in that its derivatives and starch are applicable in many products such as confectionery, sweeteners, plywood glues, textiles, paper and drugs (Sanginga *et al*, 2015). The crop is therefore extensively produced, for instance, in 2019 more than 303 million tons were produced, of which 63% was accounted for by Africa. In the same year Nigeria produced over 59 million tons making it the world largest producer (FAOSTAT, 2021).

The numerous products generated from cassava, such as food, feed, alcohol and starch, are derived from more than one form of cassava ranging from fresh roots and leaves to modified cassava starch. All these products signify potential market development opportunities for

cassava. One of the greatest challenges in cassava marketing is the constraint of short shelf life due to post-harvest physiological deterioration (PPD). PPD decreases the quality of starch thus rendering the roots unpalatable and unmarketable (Zainuddin, *et al*., 2018). In this regard therefore, there is need to process cassava before consumption or selling. Due to this perishability nature of cassava, some market opportunities are close to the centres of production (Titus and Lawrence, 2015).

In countries that produce cassava, urbanization presents prospects for cassava production to a larger consuming population. This implies that markets for fresh cassava can expand if the products are convenient and in a more desirable form. In non-producing countries, the potential for fresh cassava can be realized through innovation and competition (Plucknett *et al*., 2001). Cassava starch is either used directly or as raw material for other products. Qualities of cassava starch include its viscosity, resistance to freezing and to shear stress. The major categories of starch-based products are modified starches for the industry, unmodified starch and sweeteners such as high-fructose syrup and glucose (Kueneman *et al.*, 2010; Tan, 2015).

Another upcoming economic frontier for cassava is in biofuel production (Ziska *et al*., 2009; Jakrawatana *et al*., 2016). However, the development of cassava for this purpose is constrained by its low sugar content compared to crops such as sugar beet, sugar cane and sweet sorghum. Enzymatic processing is therefore required to convert cassava starch to sugar. There are however possibilities of developing high sugar cassava varieties that will allow the enzymatic conversion process be bypassed (Kueneman *et al.*, 2010).

In Kenya, the bulk of cassava produced is for human consumption and surpluses are processed to starch or used for animal feed. Cassava is cultivated virtually throughout the country but mainly in the Western, Coastal and Eastern regions (Githunguri, *et al*., 2017). In Western region of Kenya, cassava roots are peeled, chopped into tiny pieces, dried and milled into flour. At the Coast, apart from the roots, leaves are used as vegetables while in Eastern (Machakos and Kitui Counties), roots are also used as a snack (Githunguri, *et al*., 2017).

2.3 Constraints to cassava production

Despite the vast production and utilization of cassava, there are a number of abiotic and biotic factors that constrain its yield thereby limiting the full realization of its immense potential. These

challenges heavily impact on production, consumption, marketability and economics at local and country levels (Bull *et al*., 2011). Abiotic factors are mainly unfavourable environmental conditions whereas biotic factors comprise pests and diseases.

Abiotic factors include drought, extremes of heat, salinity, pH, flooding, infertile soils, poor management of the crop and planting of sub-optimal materials (USDA, 2003; Bull *et al*., 2011). Despite cassava being able to survive harsh environmental conditions, extremes of these are detrimental. Under high temperature/drought conditions, yield is subsequently decreased following reduced leaf area index (LAI) due to leaf fall and production of smaller and fewer leaves (Okogbenin *et al*., 2013). High levels of salinity affects the growth and nutritional value of cassava. Gleadow *et al.* (2016) indicated that young pre-tuberous cassava plants are less salt tolerant than older plants with storage roots that have already developed. Salinity in cassava significantly reduces leaf area, biomass and photosynthetic rate.

In terms of pH, cassava grows optimally in soil pH ranging between 4.5 - 7.0, below or above which production and yield is compromised (Biratu *et al.*, 2018). Low soil fertility includes low levels of exchangeable potassium since cassava extracts more potassium from the soil than other crops. Other aspects include high levels of unfavorable nutrients (such as aluminium and zinc) and imbalanced nutrient levels in soil (Kintchéa *et al*., 2017). Poor management of cassava include inadequate and late weed control (Albuquerque *et al*., 2014), planting cassava at low density (Silva *et al*., 2013), use of sub-optimal materials such as diseased cuttings or cuttings with destroyed nodes and jagged cuts (Abass *et al*., 2014).

Cassava production has been constrained by numerous pests and diseases which can cause heavy yield losses. Maruthi *et al*. (2018) recorded that cassava is affected by more than 100 mite and insect species and approximately 30 diseases caused by bacteria, viruses, phytoplasmas or fungi.

2.3.1 Arthropod pests infesting cassava

Insects and mites adversely affect cassava yields through feeding (direct and indirect feeding damage) and are important disease vectors. Bellotti *et al*. (1999) described indirect damage through the feeding action on foliage and stem by arthropod pests, which in turn reduces leaf area, leaf life and photosynthetic rate. Prolonged feeding periods (3-6 months) on cell fluids and subsequent reduction of photosynthesis causes severe decrease in root yield. Direct damage

refers to damage to cassava roots directly as exhibited by the burrower bug (*Cyrtomenus bergi*), one of the few pests that cause this kind of damage. Adequate control of these pests in turn controls spread of diseases and ensures yield are maintained at optimum. Whiteflies, mealybugs and green- and red-spider mites are among the common pests while pests such as shoot flies, fruit flies and cassava horn worm are found in Latin America (Howeler, *et al.*, 2013). Others include variegated grasshopper, web mite and cassava scale (Lozano and Booth, 1974). The whitefly, *Bemisia tabaci*, has been recorded as probably the most destructive cassava pest in all areas producing cassava (Howeler *et al.*, 2013). The pest is responsible for transmission of cassava viruses and direct damage through feeding and the sugar exudates predisposes cassava leaves to sooty moulds. Mealybug, *Phenacoccus manihoti*, causes destruction through feeding on cassava and injecting a toxin thereby causing leaf withering (Omongo *et al*., 2012). Parsa *et al.* (2012) records that the pest is native to South America but is now widespread throughout Sub-Saharan Africa since its introduction into Africa in the early 1970s. Cassava mites are also major pests in all regions producing the crop. Green mites were introduced from Latin America and destroyed African cassava production in the early 1970s. Insects cause crop losses, which can be kept at an acceptable minimum through measures such as encouraging biological control agents, use of resistant crop varieties and managing levels of crop nutrient to control insect reproduction. Low-risk selective pesticides can also be used for targeted control where necessary, at right time and quantity (Howeler *et al.*, 2013).

2.3.2 Major diseases affecting cassava

The diseases that are considered of economic value vary to some extent between countries and between continents. For instance, cassava mosaic disease (CMD) occurs wherever the crop is grown in sub-Saharan Africa and the Asian subcontinent, while cassava brown streak disease has been reported in sub-Saharan Africa only (Rey and Vanderschuren, 2017). Cassava bacterial blight occurs in most of the cassava growing areas of South and Central America, the Caribbean, Africa and Asia (CABI, 2021). Root rots are caused by a wide range of fungal pathogens including *Phytophthora* spp., *Fusarium* spp., *Rhizoctonia solani*, *Pythium* spp., *Neoscytalidium* spp., and *Lasiodiplodia* spp. (Boas *et al*., 2017; Bandyopadhyay *et al*., 2006). Symptoms of root rots are observed wherever cassava is grown, especially in cases where cassava crops are left to over mature or fields that are poorly drained (Legg and Alvarez, 2017).

2.3.2.1 Viral diseases

The main viral diseases infecting cassava are cassava brown streak disease (CBSD) and Cassava mosaic disease (CMD). Cassava mosaic disease is endemic in sub-Saharan Africa and symptoms include chlorosis, mottling and mosaic (Alabi *et al*., 2011). The disease has been an important constraint to cassava production in Africa since the 1930s (Legg and Fauquet, 2004). Cassava brown streak disease, a relatively new emergent disease (Taylor *et al*., 2012), causes corky necrosis in roots which render them unfit for consumption. The disease is currently considered a more serious threat to cassava production compared to CMD owing to its rapid spread from East Africa regions to other geographical areas including central Africa (Taylor *et al*., 2012). The agents causing both diseases are transmitted by whiteflies, and subsequent spread through infected stem cuttings (Maruthi *et al*., 2005; Mware *et al*., 2009). Cassava frogskin disease (FSD) has been reported to be caused by a combination of cassava frogskin-associated virus (CsFSaV), cassava polero-like virus (CsPLV), cassava new alphaflexivirus (CsNAV) and cassava torrado-like virus (CsTLV) (de Oliveira *et al*., 2020). Other viral diseases have been described by McCallum *et al*. (2017) and include cassava vein mosaic disease caused by cassava vein mosaic virus and cassava green mottle disease caused by cassava green mottle virus.

2.3.2.2 Bacterial diseases

The most significant bacterial disease affecting cassava production is cassava bacterial blight (CBB) (López and Bernal, 2012). The disease is widespread and is caused by the bacterium *Xanthomonas axonopodis* pv. *manihotis* (López and Bernal, 2012). Where the disease is severe, there could be complete loss of the crop. According to a study by Simiyu *et al*. (2021) there are Kenyan cassava varieties namely, Ebwanatereka 2, Fumbachai, and MM97/0293) that expressed moderate resistance to cassava bacterial blight. Mode of control include cultural practices such as the use of uninfected planting material and resistant germplasm. Bacterial stem rot is another bacterial disease caused by *Erwinia carotovora* var. *carotovora* (Lozano and Bellotti, 1978). It is a relatively new disease restricted to the stem, and the infected plant exhibits dark necrosis, followed by wilting then die-back (FAO, 2013; Hillocks and Wydra, 2002).

2.3.2.3 Fungal diseases

Cassava is affected by a number of fungal diseases which in turn decrease the yield potential of the crop. Legg and Alvarez (2017) categorized fungal diseases of cassava into foliar, stem and root diseases. Foliar diseases of cassava includes cassava super-elongation disease (SED) caused by *Sphaceloma manihoticola* (Alleyne *et al*., 2015). The disease manifests as exaggerated internode lengthening in young stalks and petioles, presence of cankers in leaf blades, stalks and petioles, and deformation of young leaves (Alleyne *et al*., 2015). Cassava anthracnose disease, caused by *Colletotrichum gloeosporioides* is characterized by leaf spots, cankers on branches, stems and fruits, and shoot tip die-back (de Oliveira *et al*., 2020; Pinweha *et al*., 2015). Stem symptoms exhibit as oval pale-brown depressions. Other foliar diseases as desribed by Legg and Alvarez (2017) are cassava brown leaf spot and cassava white leaf spot caused by *Cercospora* spp. Studies on cassava leaf spot diseases carried out in various parts of the world report that fungi of the genus *Cercospora* are the only causative agents of the diseases. Hillocks and Wydra (2002) listed phyllosticta leaf spot caused by *Phoma* sp., and white thread caused by *Fomes lignosus*. Boas *et al*. (2017) described fungal disease of roots as root rots caused by a complex of fungal pathogens. They include dry root rots caused by *Fusarium* spp., soft rots caused by *Phytophthora* spp. and black rots caused by *Neoscytalidium* spp. and *Lasiodiplodia* spp. (Machado *et al*., 2014).

2.4 Cassava brown leaf spot disease (BLS)

The most important fungal disease affecting cassava is the brown leaf spot (BLS) disease as reported by Ayesu-Offei and Antwi-Boasiako (1996) and Robert (2012). The disease causes root yield losses reported globally (Hillocks and Wydra, 2002). Brown leaf spot of cassava is widespread in cassava growing zones. Cassava BLS disease was first reported in East Africa in 1885, later found in India in 1904, and in the Philippines in 1918. It eventually spread to Brazil, Panama, Columbia, Ghana, and other countries by the 1970s (Pei *et al.* (2014).

The disease causative agent(s) exists in diseased leaves of cassava on the plant or leaves on the ground (Legg and Alvarez, 2017). The mode of spread from leaf to leaf or plant to plant is through wind or rain splash. The disease is characterized by leaf yellowing, necrotic lesions leading to drying and senescence (Robert, 2012). Severe defoliation results into reduction on photosynthetic surface translating into remarkable yield losses to the communities depending on cassava for their staple as well as income and/or the ones that utilizes cassava leaves as vegetables.

The most effective management practices of cassava brown leaf spot disease involves planting of disease free cuttings. Cultural practices such as use of clean and disinfected farm tools are encouraged amongst cassava farmers. All crop husks infected with the disease should be completely destroyed in order to minimize spreading of the disease to the next planting. If healthy planting materials are unavailable, chemical control regime could be employed. In this case, the cassava stakes should be dipped in a well constituted fungicide solution before planting (Robert, 2012). Thankappan and Govindaswamy (2021) reported that benomyl benlate or cercobin thiophanate are effective in controlling cassava brown leaf spot disease at the concentration of 0.10% and 0.20%, respectively. From a study conducted by Julião *et al*. (2020), the triazole fungicide (Flutriafol) was also found effective in controlling cassava brown leaf spot at the rate of 0.62 $g.mL^{-1}$.

Literature delineates *Cercospora* as the main cause of cassava brown leaf spot disease (Legg and Alvarez, 2017), following studies by Pei *et al*. (2014) in China, To-Anun *et al*. (2011), in Thailand, and Lozano and Booth (1974) in Asia, North- and Latin America and some parts of

Africa. However, there is little knowledge on the causal agent(s) of the disease in Kenya, yet it is rampant in all cassava production zones and has also been observed in outbreaks in cassava field trials in the country. In addition, the varieties preferred by farmers, which are used for consumption and research work, are susceptible to the disease. In this regard therefore, it is important to study the disease in order to enhance its management thus avoid devastating crop yield losses. Powbunthorn *et al*. (2012) states that cassava brown leaf spot disease causes leaf chlorosis and extensive defoliation resulting in significant yield loss and that infection of a plant by BLS may increase susceptibility to other diseases such as cassava anthracnose disease.

2.4.1 Symptoms and life cycle of cassava brown leaf spot disease

Cassava brown leaf spot occurs on the older, lower leaves and is more manifest 5-6 months after planting (Tsatsia and Jackson, 2012). The disease accelerates senescence and abscission of leaves. Teri *et al*. (1978) describe the onset of symptoms as small circular, greenish-yellow spotty lesions. The circular spots expand 1-8 mm in diameter and sometimes become angular and limited by veins. On the upper surface, spots are brown with dark borders and often surrounded by indistinct yellow halo due to a toxin produced by the advancing mycelium (Figure. 1). Minor veins crossing the spots appear as black necrotic lines. The spots are grey with less distinct borders on the underside. Centres of the spots are usually dry, crack and may fall out leaving shot-holes. As the spots enlarge, the leaves become yellow and fall off.

Severity of the disease is heightened by warm, humid weather in which spores of the fungus are produced on the lower surface. The spores are spread by wind, water-splash and transportation of infected planting material. Generally, older leaves are more susceptible to the disease (Lima *et al*., 2019). However, susceptible varieties are prone to infection and disease manifestation during early growth. The fungus overwinter in soil or plant debris thereby providing inoculum for new infection (Tsatsia and Jackson, 2012).

Figure 1: Symptoms of brown leaf spot disease on cassava leaves

Source: Photo taken from KALRO Kandara sampling site (Photo courtesy of Perpetuar W. Ng'ang'a, 2020)

2.4.2 Impact of cassava brown leaf spot disease

Brown leaf spot disease is considered one of the most important fungal diseases of cassava (Powbunthorn *et al*. 2012). Much as the impact induced by BLS is often underrated, infection by the disease brings about leaf chlorosis and extensive defoliation resulting in yield loss of up to 30% (Tsatsia and Jackson, 2012). Additionally, infection of a plant by BLS may increase susceptibility to other diseases (Powbunthorn *et al*., 2012). For instance, there is a significant positive correlation between the occurrence of cassava BLS and cassava anthracnose (Powbunthorn *et al*., 2012). In addition, there is a high likelihood of white leaf spot and root rot infections among the BLS infected plants (Wydra and Verdier, 2002). Powbunthorn *et al*. (2012) observed that BLS is more predominant in humid ecozones while its severity increases with highly branching cassava varieties. In this regard therefore, cassava BLS disease should not be neglected.

Tsatsia and Jackson (2012) reported that yield losses associated with cassava BLS disease are 30% in Africa, 23% in South America, and 17% in India. Nonetheless, serious defoliation necessitating disease control has not been reported in Pacific island countries. The disease is usually observed late in the growth of plants, and mainly on the older leaves.

2.4.3 Identification of cassava brown leaf spot pathogens

The most outstanding methods that have been used in the identification of cassava BLS disease causative agent(s) are Microscopy and use of molecular techniques (Pei *et al*., 2014). For either of these to be employed, the fungus has to first be isolated from lesions of infected leaves. In a study by Ayesu-Offei and Antwi-Boasiako (1996), lesions from infected cassava leaves were surface sterilized using a solution of equal parts of 95% alcohol and 0.001% $HgCl_2$ solution for 2 min and then thoroughly washed in two changes of sterile distilled water. The wet lesions were then placed in sterile Petri dishes lined with wet filter papers and incubated in the dark at room temperature (25-32 °C) to sporulate. In another study by Su *et al.* (2012), sporulation was induced by culturing surface sterilized leaves on PDA media and media made of host tissue.

The role of microscopy is to bring out hyphal and spore morphologies both of which are major characteristics in fungal identification (Su *et al.*, 2012). Microscopy is best utilized together with existing identification keys. Molecular tools, involving DNA and/or RNA extraction, PCR and sequencing techniques enable identification of the fungus at the molecular level. DNA sequencing of the large subunit (LSU) regions and the internal transcribed spacers (ITS) of ribosomal RNA (rRNA), followed by comparative sequence analysis, have been identified as the 'gold standard' for molecular identification (Tsui *et al*., 2011).

2.4.4 Control of cassava brown leaf spot disease

Cassava brown leaf spot management is based on three main approaches namely cultural, chemical control, and cassava genetic resistance (Julião *et al*., 2020). Cultural control measures include reduction of plant density by increasing the spacing between plants to lower the humidity within the plantation. The other aspect is to ensure hygienic agronomic practices are upheld. Genetic resistance control is achieved by use of resistant or tolerant varieties (Onu and Ezeano, 2018). Chemical control of cassava BLS management relies on the use of fungicides such as the trizole group including the flutriafol fungicide.

CHAPTER THREE:MATERIALS AND METHODS

3.1 Description of the study site and experimental materials

The study was conducted at the Kenya Agricultural and Livestock Research Organization (KALRO) Biotechnology Research Institute (BRI), Kabete Centre. Infected leaf samples (Experimental materials) were sourced from BRI, KALRO Kakamega, and from a confined field trial of the Virus Resistant Cassava for Africa (VIRCA) project located at KALRO Kandara (near Thika town), Murang'a County. Infected leaf samples were obtained from different cultivars including those being targeted for improvement by the project and included: TME 204, TME 14, NASE 14, TME 7 and Ebwanateraka, all of which were established at the study sites. These were the cassava cultivars established in the field at the time of sampling.

3.2 Sampling of symptomatic cassava leaves

A total of 80 cassava leaf samples displaying brown spots were collected from the field in the sites listed in Section 3.1. The symptomatic leaves were characterized by brown spots with dark borders on the upper surface and grey with less distinct borders on the underside. The spots were surrounded by indistinct yellow halo and centres were dry. Additional samples were collected from healthy non-symptomatic cassava genotypes established in KALRO-BRI greenhouse at Kabete to serve as negative controls. Samples were separately conserved in khaki envelopes which were appropriately labelled prior to transportation within 24 hours from the field to the laboratory where they were stored at +4 °C before isolation of fungal pathogens.

3.3 Preparation of potato dextrose agar modified with antibiotics

Preparation of the culture medium was done by dissolving 39g of potato dextrose agar (PDA) powder in 1litre distilled water followed by autoclaving at 121 ºC for 20 minutes. To prevent bacterial contamination, 1 mg/ml streptomycin and 0.12 mg/ml neomycin were added to the medium which had been cooled to about 45 °C. Approximately 20 ml of the medium was dispensed under aseptic conditions in a laminar flow cabinet into sterile 9 mm diameter plastic Petri dishes.

3.4 Isolation and purification of fungi

Fungal isolation followed the procedure described by Thilagam *et al*. (2018) with appropriate modifications. The modifications included use of 1.3% (v/v) sodium hydrochloride in place of 0.1% mercuric chloride for surface sterilization, and the use of PDA instead of Rose Bengal media for fungal culturing. Symptomatic leaves were washed thouroughly under running tap water to remove any dirt from the field. They were then sterilized in 70% ethanol for 30 seconds. Small tissue fragments of approximately 5 mm (length) by 3 mm (breadth) were cut using sterile scalpels from the margins of necrotic leaf lesions. The fragments were sterilized in 1.3% (v/v) sodium hypochlorite for 1 minute followed by rinsing three times in sterile distilled water and dried using sterile blotting paper in the lamina flow cabinet. They were then cultured in Petri plates containing antibiotic-amended PDA medium. The plates were incubated at room temperature (23 ± 2 ºC) for seven days. From the colonies that had emerged in the cultured fragments, pure cultures were obtained after sub-culturing each colony on PDA.

3.5 Identification of fungal pathogens

Identification of the pathogens followed both cultural and morphological features (through microscopy) of pure fungal cultures, guided by identification Manuals (Barnett, 1960, Dugan, 2006 and Humber, 1996), studies already conducted in literature and pathology experts in research and academic institutions. Slides for each isolate were prepared and viewed under a light microscope. Using a sterile isolation needle, a small portion of mycelia from each colony was picked and placed on a drop of lactophenol cotton blue dye on a sterilized glass slide. A clean cover slip was then placed gently on top to completely cover the mycelia and dye avoiding any air bubbles. This was repeated for each sample while sterilizing the needle before use. Each slide was viewed first under x10 objective lens, then at x40 for higher magnification. To aid in identification, colony colour, shape, elevation and the surface appearance were used. Microscopic features included type of spores produced, the fruiting body, and the form of hyphae, whether septate or aseptate, branching or non-branching.

Based on the morphological features, identification of the fungal pathogens was guided by Manuals by Barnett (1960), Dugan (2006) and Humber (1996). Guidelines provided in the Manuals aided in narrowing down to possible pathogenic fungi. Three pathogens belonging to

the genera *Colletotrichum*, *Cladosporium* and *Alternaria*, working in synergism, were identified as the causative agents of cassava brown leaf spot. Synergism among the three pathogens was tested by carrying out pathogenicity tests, with single and combined fungal pathogen inoculum as explained in Section 3.6. Morphological identity of the fungi was confirmed by carrying out molecular analysis. Selection of the three pathogens was based on three key factors: (i) culture characteristics delineated through microscopy and guided by identification Manuals (ii) relative incidence of the cultures and (iii) common occurrence of the pathogens in the sampled areas.

3.6 Conduct of pathogenicity test

3.6.1 Growth of experimental plants

Experimental cassava plants were grown and maintained in cleaned, sterilized and fungicide-fumigated greenhouse at the KALRO Biotechnology Research Centre. One susceptible cassava variety, TME 204, was used for pathogenicity tests. The disease-free cassava cuttings containing 3-5 nodes were planted in 12.7cm x 20.3cm polythene planting bags, which were ¾ filled with sterilized forest soil. The soil, enriched with range manure and ¼ inch gravel at the ratio of 50 parts, 20 parts and 8 parts respectively was sourced from Kenya Plant Health Inspectorate Service (KEPHIS). Before planting, the bags were watered to moisten the soil for moisture and ease of planting. The cassava cuttings were planted vertically by pushing into the soil directly by hand, one cutting per bag (Hauser *et al*., 2014). Out of the 100 plants grown, 90 of uniform height and vigor were selected for pathogenicity tests. A complete random design (CRD) was used with three replications with three plants per replicate. To achieve a completely randomized design, random numbers were generated using Microsoft Excel and assigned to each fungal treatment

3.6.2 Inoculum preparation and plant inoculation

Inoculum was prepared following a demonstration film by Kirkhouse Trust (https://www.youtube.com/watch?v=F92GEhJaubc&t=125s) with appropriate modifications. The 14 day old cultures were scraped off the PDA medium while being careful to avoid medium inclusion. Each isolate was then homogenized through blending in 200 ml sterile distilled water followed by straining through a sterile strainer to obtain a spore suspension excluding much of the mycelia. The number of propagules (spores) in the inoculum was ascertained using a

hemocytometer (Abcam, 2015), then adjusted to 1 x 10^6 spores per ml before dispensing in appropriately labelled hand sprayers in readiness for inoculation. The inoculum was prepared as single isolates as well as combinations of the identified fungi, that is, (i) *Colletotrichum* sp. (ii) *Alternaria* sp. (iii) *Cladosporium* sp. (iv) *Colletotrichum* sp. and *Alternaria* sp. (v) *Colletotrichum* sp. and *Cladosporium* sp. (vi) *Alternaria* sp. and *Cladosporium* (vii) All the three species *Colletotrichum, Cladosporium* and *Alternaria* (viii) water control (ix) non-inoculated control.

Each of the four week old cassava plants were spray-inoculated with approximately 10ml of fungal inocula. Each inoculated plant was covered with a clear humidity bag for 48 hours to allow a conducive microenvironment for fungal infection (Figure. 2). Assessment for symptom onset and development was done once a week for eight weeks.

Figure 2: Cassava plants covered in humidity bags after inoculation for fungal infection

3.7 Molecular identification of the fungi causing cassava brown leaf spot disease

Molecular techniques are commonly used in order to overcome taxonomic challenges posed by possible limitation of morphological characteristics or in instances where morphological characteristics are missing, in conflict or ambiguous (Raja *et al*., 2017). In order to confirm identity of the fungi causing BLS at molecular level, molecular diagnostics were carried out. This was achieved through DNA extraction followed by PCR which made use of internal transcribed spacer (ITS) primers of the rRNA region. The ITS region has been used as a universal DNA barcoding marker for fungi (Schoch, 2012; Das and Deb, 2015).

3.7.1 DNA extraction

The rapid DNA extraction protocol was adopted from Cenis (1992) and was used with appropriate modifications. The modifications included: the use of a FastPrep-24™ genogrinder for crushing sample instead of conical grinder, air-drying of DNA pellet instead of vacuum drying and use of molecular grade water in place of TE for pellet resuspension. For each of the one week old fungal cultures, 200 mg of mycelia and spores were scrapped off the PDA medium, put in appropriately labeled self-standing tube containing a ceramic bead. 300 µl of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS (final concentrations) was added. (2%) β-mecaptoethanol was added into extraction buffer prior to use. The mycelia were pulverized at 4.0m/s for 1 min 20 sec using a FastPrep-24™ genogrinder.

Debris were spun down at 13000 rpm for 1 min using Eppendorf 5424 Centrifuge (Germany) and supernatant transferred into well labeled sterile 2 ml Eppendorf tubes. 150 µl sodium acetate pH 5.2 was added followed by incubation at 20 ºC for 10 min and spinning at 13000 rpm for 5 min. The supernatant was transferred into sterile 1.5 ml Eppendorf tube. Equal volume of isopropanol was added, let to stand at room temperature for at least 5min and spun at 13000 rpm for 10 min to pellet the DNA. Supernatant was discarded and pellet washed twice in 500µl of 70% ethanol and air dried for 15 min. Re-suspension of pellet was done in 50µl H_2O. Each sample was then treated with 2µl of 10 mg/ml RNase A and incubated at 37ºC for 30 minutes.

3.7.2 Polymerase chain reaction (PCR) and sequencing

Polymerase chain reaction was set up for a 50 µl reaction volume (Table 2) using internal transcribed primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS4, (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The volumes of PCR components and cycling parameters were adapted from the New England Biolabs OneTaq® Quick-Load® 2X Master Mix with Standard Buffer Manual since this was used as the master mix. Table 2 illustrates the components used for PCR for both primer sets that is ITS 4(R)/1(F), and ITS 4(R)/5(F). Both primer pairs ITS 4(R)/1(F) and ITS 4 (F)/5(R) were expected to amplify about 550 bp. Use of the two primer pairs was opted for in order to amplify different sections of the ITS regions thus maximize the chance of correct identification.

Table 2: Volumes of PCR components

Component	Final Concentration	50 reaction
One-taq Quick load 2X Master Mix with standard buffer	1X	25µl
10µM ITS Primer (F)	0.2 µM	1µl
10µM ITS Primer (R)	0.2 µM	1µl
Template DNA	< 1000 ng	2µl
Nuclease free water		21µl
Total		50µl

For both primer pairs, the PCR cycling conditions were: one cycle of initial denaturation at 94°C for 30 seconds followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 68°C for 1 minute, with a final extension step of 68°C for 5 minutes. The PCR products for both primer sets were between 500bp and 650bp in length and were resolved in 1% agarose gel stained with 1 µg/ml ethidium bromide, under the voltage 80V for 40 minutes.

The PCR products were then purified using QIAquick® PCR purification kit (Cat. No. 28104) from Qiagen. The amplicons were submitted to Molecular and Infectious Diseases Research Laboratory of the University of Nairobi for Sanger sequencing in both forward and reverse directions using the ITS primers.

3.7.3 Sequence analysis

The sequences were downloaded from the server and quality checked by automatic trimming of the ends using Geneious Prime software. The sequences were not edited but used to create consensus sequences using the same software in FASTA format. The FASTA sequences were used to query existing sequences at the National Centre for Biotechnology Information (NCBI) GenBank database. Using selected sequences from the NCBI results, multiple sequence alignment was performed using Geneious Prime 2021.2 in order to generate phylogenetic trees. The alignments were then exported to the software MEGA 11.0.8 and used to generate phylogenetic trees for the pathogens with their near relatives to reflect their evolutionary relationships. This was inferred by the UPGMA (unweighted pair group method with arithmetic mean) method and the branches supported by 1000 bootstrap.

3.8 Confirmation of the causal pathogen of the disease

After the pathogenicity test, the causal pathogens of the disease were confirmed by following the Koch's postulate procedure (LibreTexts, 2021). This is a sequence of observational and experimental steps to isolate and confirm the specific causative agent for a disease. Symptomatic leaves were sampled and taken to the laboratory for fungal re-isolation and later inoculated on healthy cassava plants. The procedure for re-isolation and confirmation of the pathogen was similar to that explained in Sections 3.3 and 3.4.

3.9 Reaction of elite cassava genotypes to infection by fungi causing cassava brown leaf spot disease

After identification of cassava brown leaf spot causative pathogens, inoculum was prepared in order to challenge elite cassava genotypes and assess their phenotypic response to the disease. This experiment was conducted in Biotechnology Research Centre greenhouses. Inoculum was prepared following a similar procedure as described in Section 3.6.2. Five cassava genotypes were selected namely TME 204, TME 14, TME 7, Ebwanateraka 1 and 2. These are farmer preferred varieties and have gained popularity as genotypes of choice for research work. Much as the TME series cultivars are known to be susceptible to BLS, continuous research is important to check their susceptibility over time. In addition, other cultivars can be checked against these susceptible genotypes in order to map them in the susceptibility-resistant continuum.

For each variety, disease-free cassava cuttings containing 3-5 nodes were planted in 12.7 cm x 20.3 cm polythene planting bags, ¾ filled with sterilized soil. The soil, enriched with range manure and ¼ inch gravel at the ratio of 50 parts, 20 parts and 8 parts respectively was sourced from Kenya Plant Health Inspectorate Service (KEPHIS). Before planting, the bags were watered to moisten the soil and ease of planting. The cassava cuttings were planted vertically by pushing into the soil directly by hand, one cutting per bag (Hauser *et al*., 2014). Out of the 35 plants established for each variety, 27 of uniform height and vigor were selected for inoculation. A complete random design (CRD) was used for the experiment and consisted of three replicates with three plants per replication. The bags were watered on need basis.

The nine plants were spray-inoculated with fungal isolate combination (Section 3.6.2) 4 weeks after planting. This set up was repeated for the control experiments which were established in a separate greenhouse to avoid any chances of contamination. The two greenhouses were similar in terms of physical location and growth conditions of temperature and relative humidity were maintained in the same manner. The inoculated plants were covered with a clear humidity bag for 48 hours to allow a conducive microenvironment for fungal infection. Observation for disease symptom development was done daily and the first severity score recorded seven days post planting. A subjective scoring scale was used and the range was between 1 and 6 (Inglis, 1988) where 1=no disease, 2=1-10% leaflet area with lesions, 3=11-25% leaflet area with lesions, 4=26-50% leaflet area with lesions and limited chlorosis, 5=51-75% leaflet area with

lesions and extensive chlorosis, 6=76% and above of leaflet area with lesions and defoliation. Scores were taken weekly for a period of eight weeks (56 days), after which data analysis was done to ascertain how varied the genotypes were in their response to infection with the pathogens. This experiment was carried out in two greenhouse cycles, that is, between March – May and between June – August 2020.

3.9.1 Experimental design

The experiment was performed in the KALRO Biotechnology Research Centre greenhouses where plants were established and maintained. The plants were spray-inoculated with the fungal inoculum. Other plants were sprayed with distilled water while others remained non-inoculated. These acted as the experimental control and were set up in a separate greenhouse to avoid chances of contamination especially when watering the plants. There was no resistant control since this is a novel study with little that has been done in Kenya to investigate BLS in cassava. There are no documented cassava varieties in Kenya. A completely randomized design was adopted for the experimental and control treatments. To achieve randomization, random numbers were generated using Microsoft Excel application, random numbers assigned to the varieties and experiment set. Disease symptoms were assessed each week for eight weeks.

3.9.2 Rating of cassava brown leaf spot disease severity on elite cassava genotypes

Severity score data for individual plants was used to assess how the cassava genotypes responded to infection by the pathogens and disease progression over time. This assessment would be used to determine if the genotypes had any significant difference in their response to the disease over the assessment period.

3.9.3 Data analysis

Disease incidence and severity data were analyzed using Genstat statistical program, 15th Edition. Analysis of Variance (ANOVA) was used to test for significant differences in disease expression among the genotypes and means separated using Fischer's Unprotected least significant difference at P = 0.05. Area under the disease progress curve (AUDPC) score was computed using disease severity data in order to compare different cassava genotypes against time of infection through the season. The AUDPC was calculated using the midpoint rule method (Campbell and Madden, 1990) using the formula:

$AUDPC = \Sigma_{i=1}^{n-1} [(t_{i+1} - t_i)(y_i + y_{i+1})/2]$

Where “t” is time in days of each reading, “y” is the percentage of affected foliage at each reading and “n” is the number of readings.

CHAPTER FOUR: RESULTS

4.1 Symptoms of cassava brown leaf spot disease under field conditions

Cassava brown leaf spot disease displayed as brown spots with dark borders on the upper surface and grey with less distinct borders on the underside. There was an indistinct yellow halo surrounding the spots and the centres were dry (Figure. 3). There were no variations in disease severity and how symptoms presented among the sampled genotypes at the time of sampling. The symptoms were similar to those observed during the pathogenicity tests, and the severity progression varied among the test genotypes during the assessment period. The disease is most frequent during hot and humid seasons.

Figure 3: Symptoms of cassava brown leaf spot disease under field conditions

4.2 Pathogen isolation, purification and identification

The identified pathogenic fungi from cassava leaves were *Colletotrichum* sp., *Alternaria* sp. and *Cladosporium* sp. The three pathogens were common in all the sampled areas and their cumulative relative incidences were 41%, 24% and 18%, respectively as shown in Figure 4. The percentage incidences were calculated from each of the three sampled areas, that is; 54%, 37% and 33% in KALRO BRI, KALRO Kakamega and KALRO Kandara respectively for *Colletotrichum*; in the same respective order, *Alternaria* incidences were 21%, 24% and 28% while *Cladosporium* incidences were 16%, 20% and 19%. One isolate of each pathogen was obtained. Other isolated fungi (16%) were either non-pathogenic, saprophytic or secondary pathogens and thus they were not used for inoculation. Cultural and morphological features of the three pathogens as recorded in Table 3 showed that all the three fungi had septate hyphae.

Colletotrichum sp. had white cottony colonies which formed as clusters upon maturity while the spores were borne on dark clusters of fruiting bodies. *Alternaria* sp. had gray, woolly colonies with muriform conidia while *Cladosporium* sp. had colonies that were olive-green in colour and velvety to suede-like in texture, and the micro-and macroconidia had tapering ends.

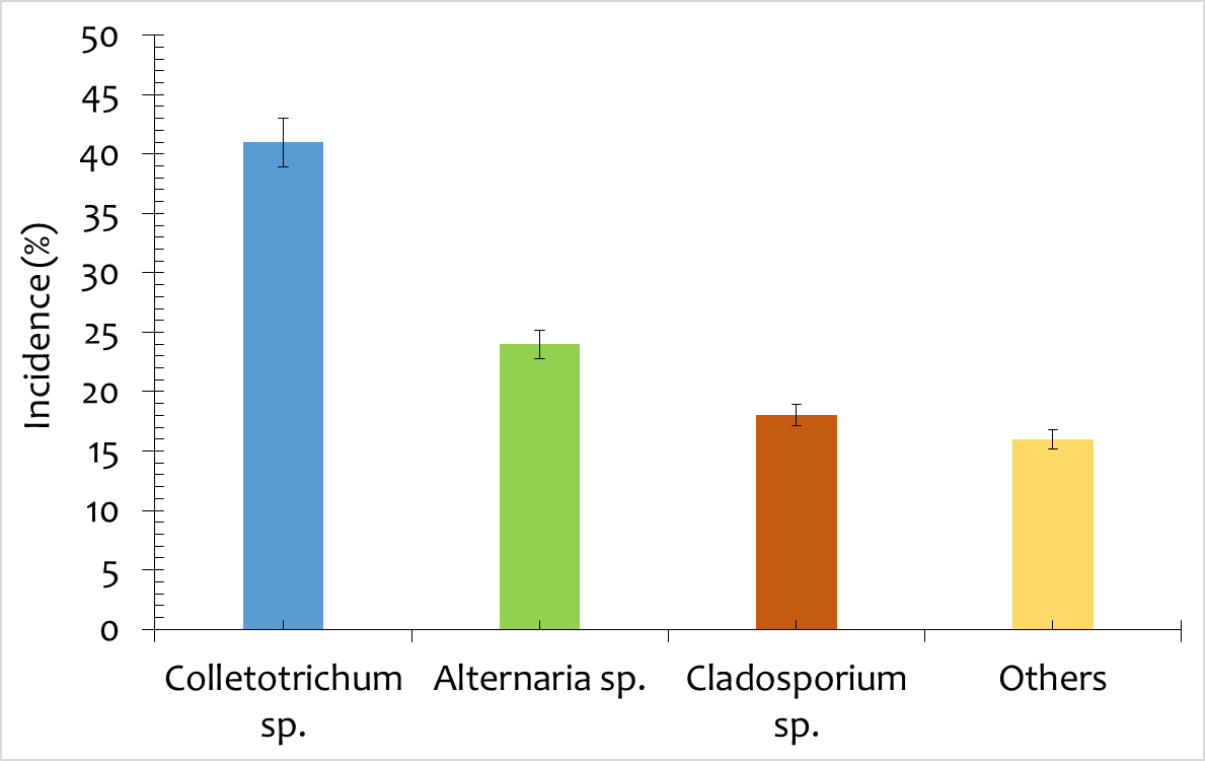

Figure 4: Incidence of fungal pathogens associated with cassava brown spot disease

Table 3: Cultural and morphological characteristics of fungal pathogens isolated from infected cassava leaves

Fungi	**Cultural characteristics on PDA**	**Microscopic characteristics**	
		Spores	**Hyphae**
Colletotrichum sp.	White colonies; white cottony clusters upon maturity	Borne on dark clusters of fruiting bodies	Septate
Cladosporium sp.	Colonies olive-green and velvety to suede-like in texture.	Micro-and macroconidia with tapering ends	Septate
Alternaria sp.	Greyish, woolly colonies	Muriform conidia	Septate

4.3 Growth and morphological characteristics of the pathogens on solid medium

The three fungal pathogens associated with cassava brown spot disease were characterized on basis of colony colour, fruiting bodies and conidia production on PDA medium. Growth rate of the fungal pathogens was not determined because focus was more on identification of BLS fungal agents and how selected cassava genotypes respond to infection by the fungi.

Colletotrichum: Colonies were white in colour and upon maturity they appeared as white cottony clusters throughout the media. Spores were borne on dark clusters of fruiting bodies more evident on the back side of the culture as shown in the Figure 5. Conidiophores, which emanated from a rather weak stroma, each bore conidia which appeared crescent shaped upon their release.

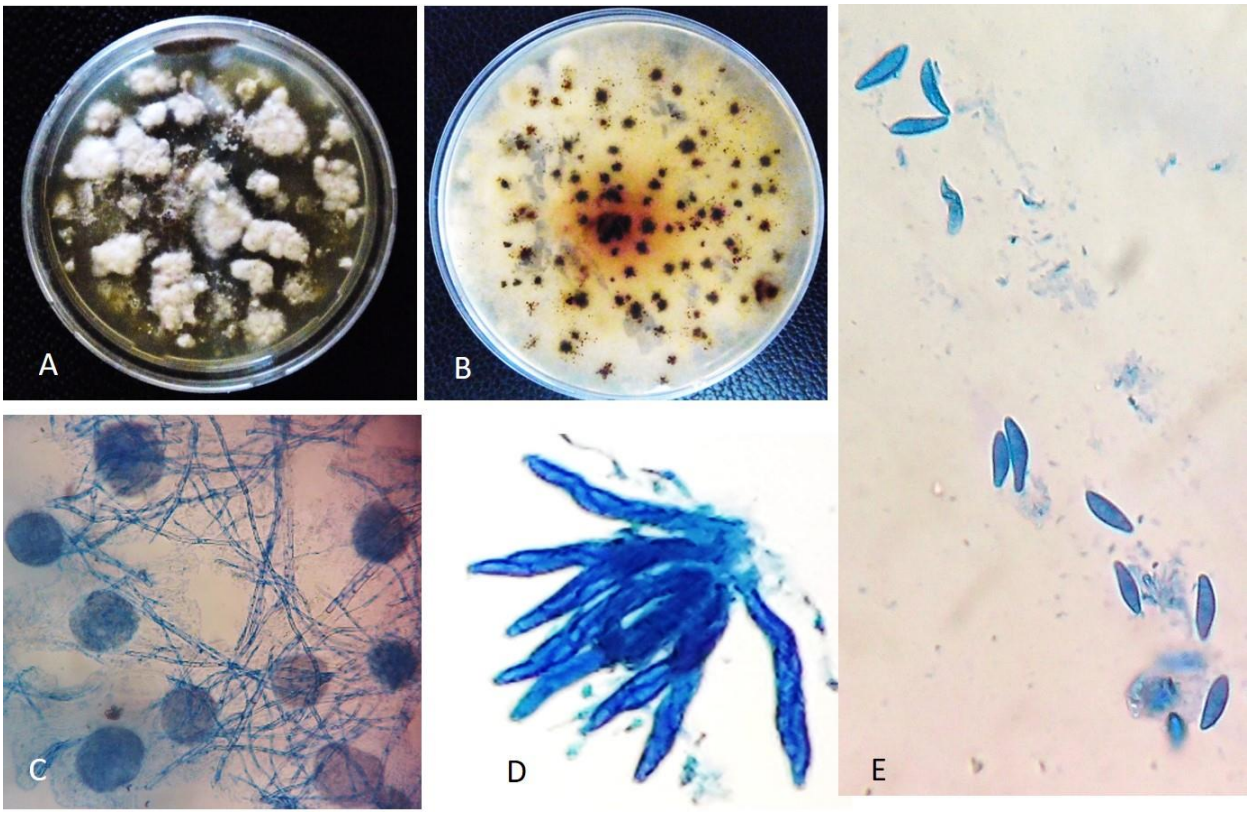

Figure 5: Cultural and morphological characteristics of *Colletotrichum* sp.: (A and B) front and back sides of culture on PDA medium; (C) fruiting bodies as seen under light microscope; (D) conidiophores bearing conidia; (E) released crescent-shaped conidia

Cladosporium: The colonies in this study grew rather moderately and it matured to produce large amounts of conidia. The colony was olivaceous green and velvety to suede-like in texture. *Cladosporium* produced erect, septate hyphae. Conidiophores were also septate and showed tree-like branching (Figure 6).

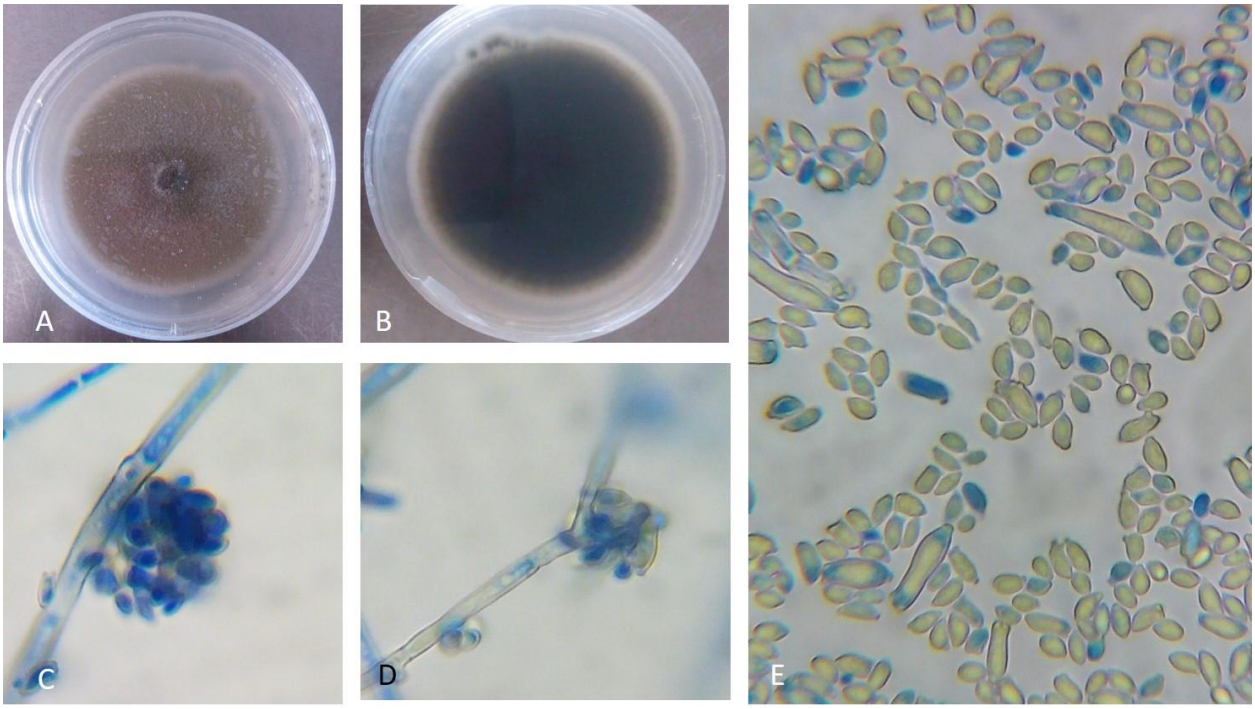

Figure 6: Cultural and morphological characteristics of *Cladosporium* sp.: (A and B) front and back side of culture on PDA medium, respectively; (C and D) Conidiophores bearing conidia (E) Micro- and macroconidia upon release from conidiophores

Alternaria: Colonies grew rapidly, were woolly and were covered with grayish, short hyphae. The back side of the culture was typically black due to production of pigment (Figure 7). The fungus possessed hyphae and conidiophores that were septate with muriform conidia (transverse and longitudinal septation). The conidia were produced singly or in acropetal chains at the apex of the conidiophores.

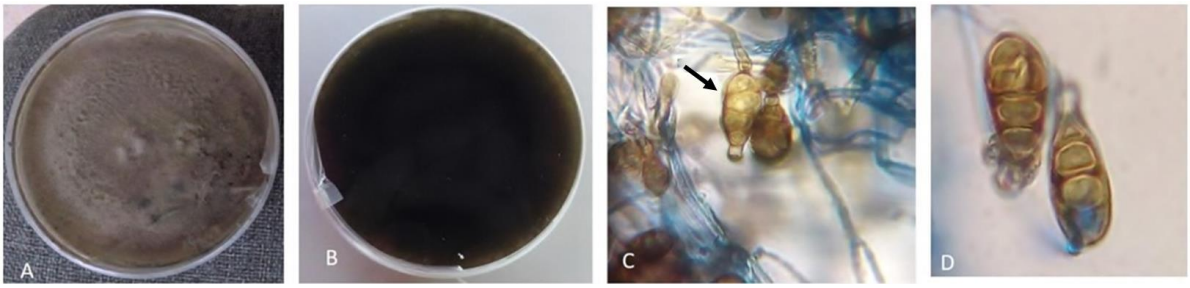

Figure 7: Cultural and morphological characteristics of *Alternaria* sp.: (A and B) front and back side of culture on PDA medium, respectively; (C) spores borne at the conidiophore terminus (arrow); (D) mature spores

4.4 Molecular identification and phylogenetic analysis of fungal pathogens

Identification of the isolated fungal pathogens based on morphological features was complemented by use of the internal transcribed spacer (ITS) sequences. The ITS, a conserved rDNA sequence, has been extensively used to identify and characterize fungal isolates. This has enabled performance of phylogenetic analysis (Toledo *et al*., 2013). Sequencing of the ITS and phylogenetic analysis were in accordance with results of morphological observations thus confirming the three pathogens, *Colletotrichum* sp., *Cladosporium* sp. and *Alternaria* sp.

4.4.1. PCR amplification using universal primers

After polymerase chain reaction with ITS 1/4 and 4/5 primer combination, the result is as shown in Figure 8. The expected band size was between 500 bp and 600 bp and was confirmed by use of a size marker (1 Kb Plus DNA Ladder –Invitrogen™).

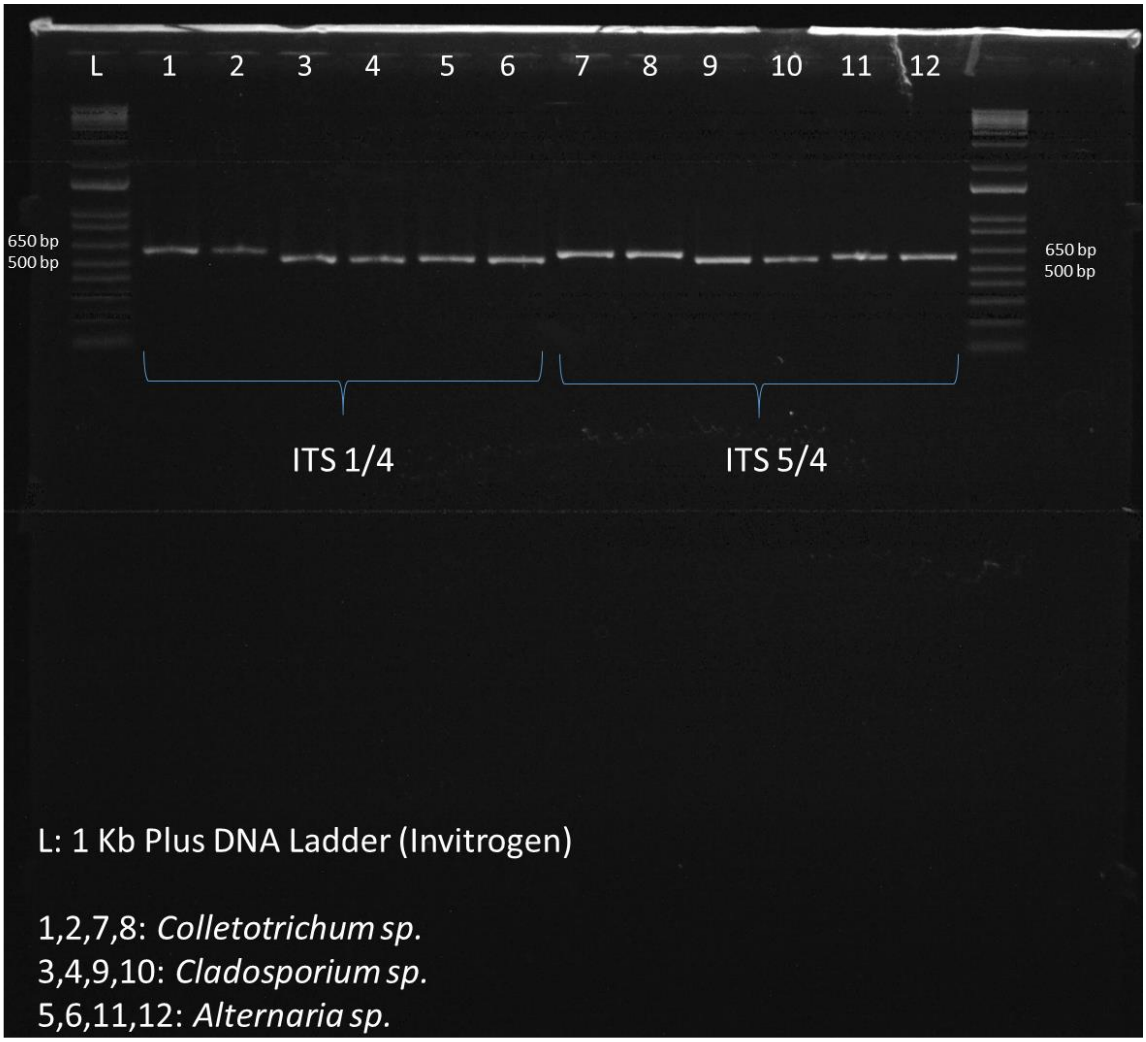

Figure 8: Purified PCR products for *Colletotrichum* sp., *Cladosporium* sp. and *Alternaria* sp.

4.4.2. Phylogenetic analysis

The rDNA ITS 1/4 and 4/5 sequences of the *Colletotrichum*, *Cladosporium* and *Alternaria* isolates and those of ten related species selected from BLAST results for each isolate, and the outgroup *Puccinia graminis* were used to compile a data matrix for the unweighted pair group method with arithmetic mean (UPGMA) analysis. Table 4 shows the alignment results.

Table 4: Comparison of cassava brown leaf spot fungal pathogen sequences with sequences deposited in GeneBank sequences, showing sequences with best match, query coverage and similarity percentages for both ITS 1/4 and ITS 4/5

Isolate code	Pathogen	Sequence with best match	Query coverage Percentage (%)	Percentage similarity (%)
S5	*Alternaria* sp.	MN909169 *Alternaria brassicae*	100	99.81
S11	*Alternaria* sp.	MK078593 *Alternaria alternata*	99	99.65
S3	*Cladosporium* sp.	MG873077 *Cladosporium tenuissimum*	99	99.81
S9	*Cladosporium* sp.	MH884146 *Cladosporium sp.*	100	99.82
S1	*Colletotrichum* sp.	MH051303 *Colletotrichum karstii*	99	99.44
S7	*Colletotrichum* sp.	LC488858 *Colletotrichum karstii*	99	99.17

The phylogenetic trees that resulted from the analysis were rooted with the outgroup *Puccinia graminis* and are shown in Figure 9 to Figure 14. The two primer sets ITS 1/4 and ITS 4/5 were used for each sample (pathogen) to eventually give two phylogenetic trees per pathogen.

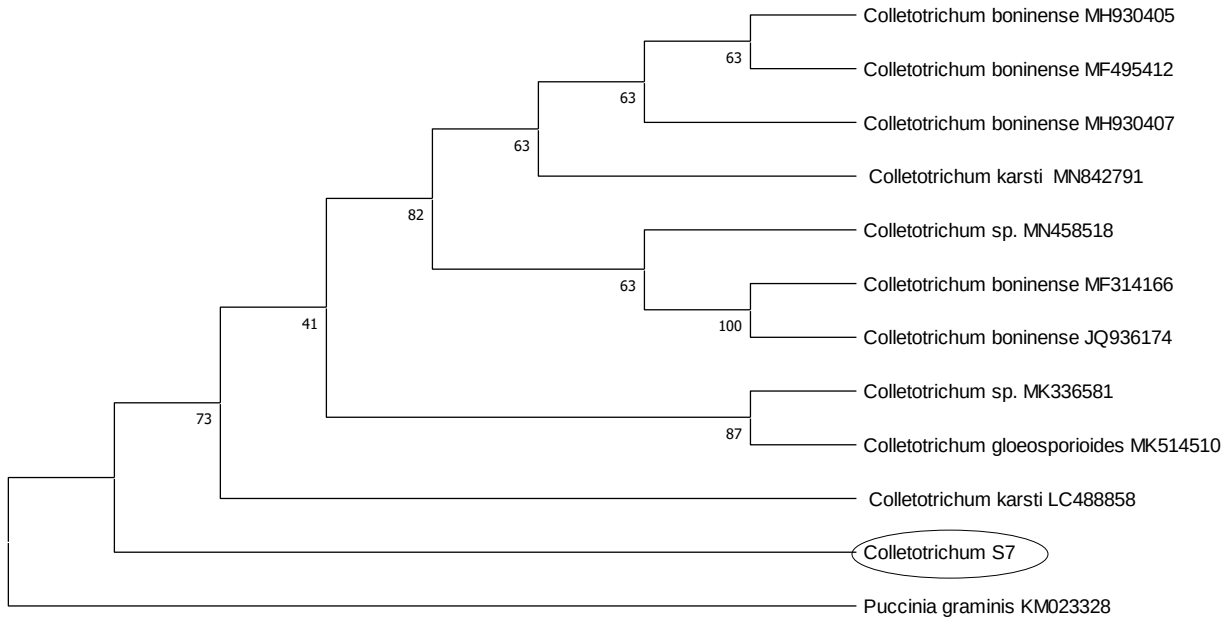

Figure 9: Phylogenetic tree of *Colletotrichum* sp. (labelled *Colletotrichum* S7) isolated from cassava leaves and its near relatives. The sequence was obtained through amplification with ITS primers 4 and 5. The UPGMA method was used to infer the evolutionary history and the tree branches supported by bootstrap percentages. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

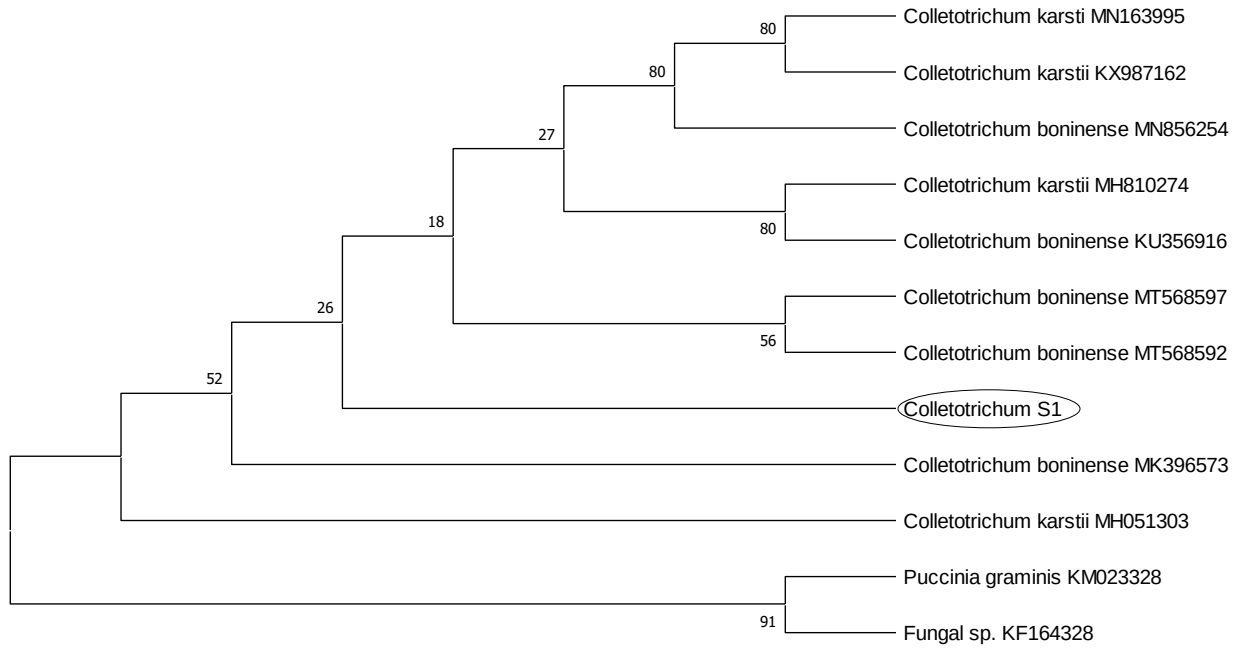

Figure 10: Phylogenetic tree of *Colletotrichum* sp. (labelled *Colletotrichum* S1) isolated from cassava leaves and its near relatives. The sequence was obtained through amplification with ITS primers 1 and 4. The UPGMA method was used to infer the evolutionary history and the tree branches supported by bootstrap percentages. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

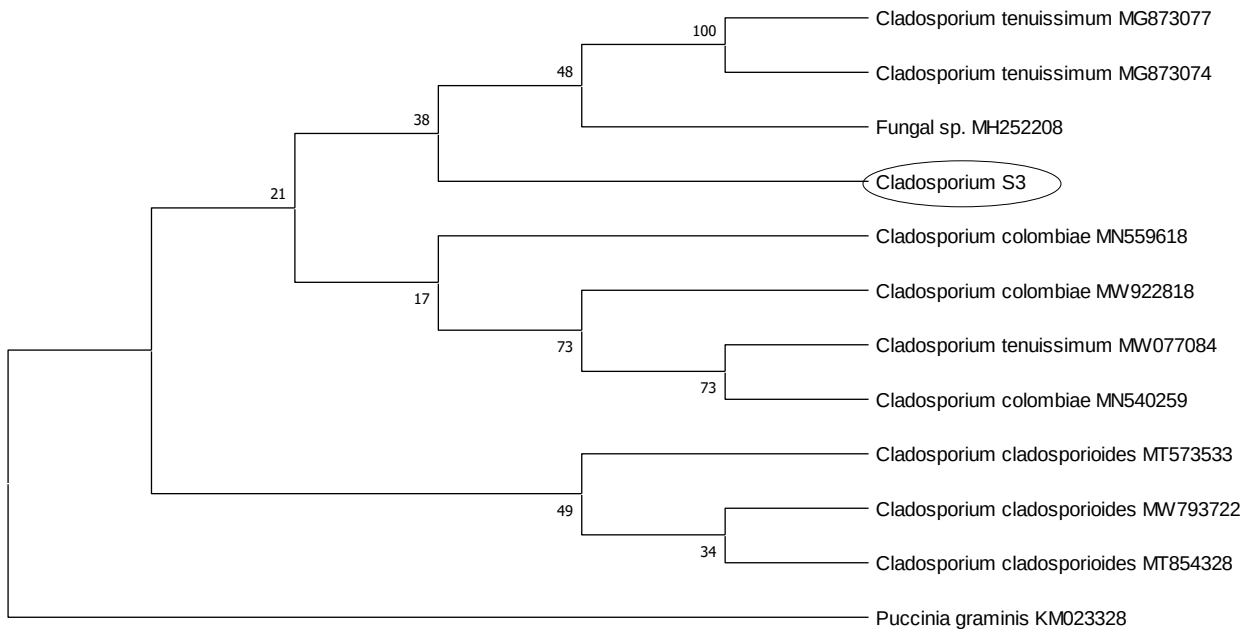

Figure 11: Phylogenetic tree of *Cladosporium* sp. (labelled *Cladosporium* S3) isolated from cassava leaves and its near relatives. The sequence was obtained through amplification with ITS primers 1 and 4. The UPGMA method was used to infer the evolutionary history and the tree branches supported by bootstrap percentages. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

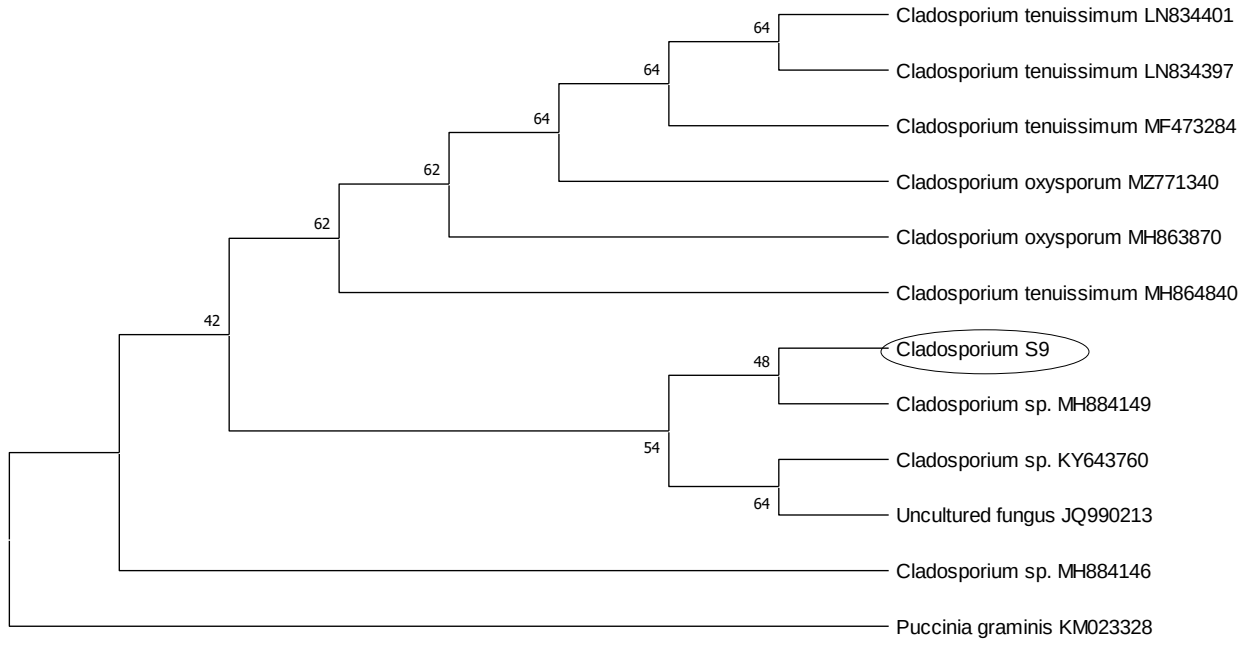

Figure 12: Phylogenetic tree of *Cladosporium* sp. (labelled *Cladosporium* S9) isolated from cassava leaves and its near relatives. The sequence was obtained through amplification with ITS primers 4 and 5. The UPGMA method was used to infer the evolutionary history and the tree branches supported by bootstrap percentages. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

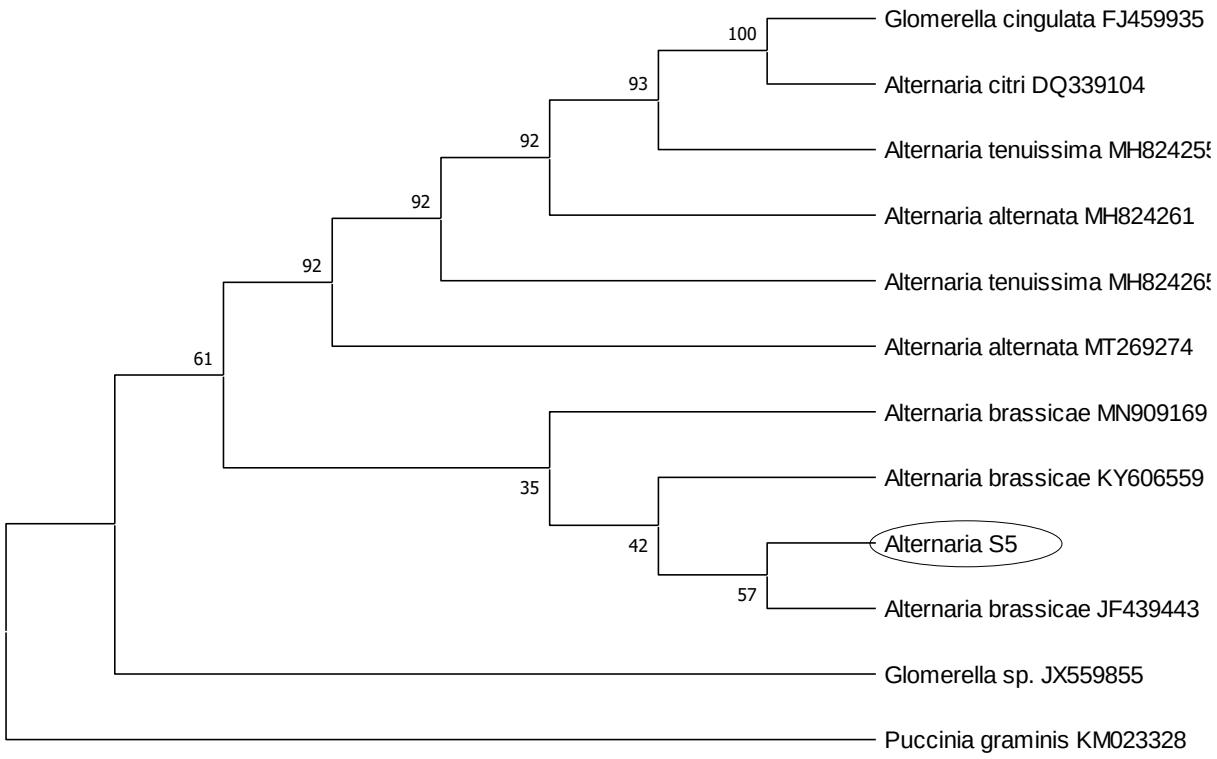

Figure 13: Phylogenetic tree of *Alternaria* sp. (labelled *Alternaria* S5) isolated from cassava leaves and its near relatives. The sequence was obtained through amplification with ITS primers 1 and 4. The UPGMA method was used to infer the evolutionary history and the tree branches supported by bootstrap percentages. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

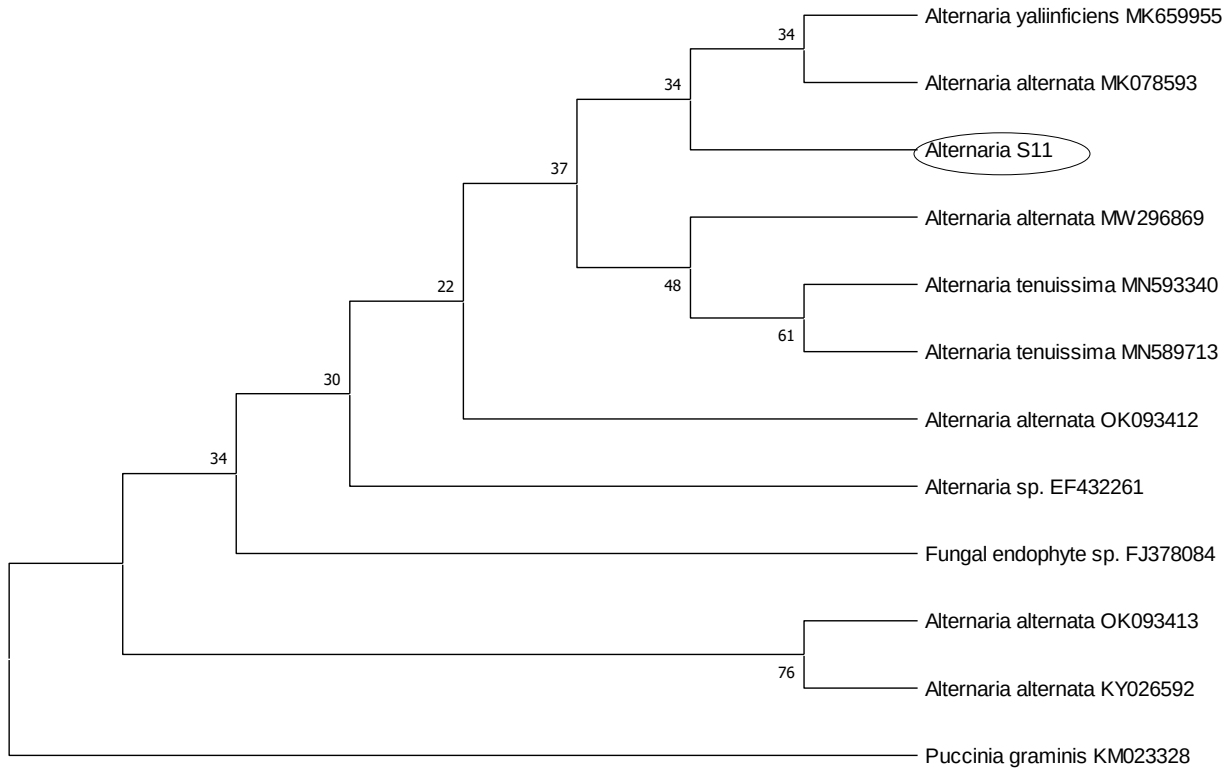

Figure 14: Phylogenetic tree of *Alternaria* sp. (labelled *Alternaria* S11) isolated from cassava leaves and its near relatives. The sequence was obtained through amplification with ITS primers 4 and 5. The UPGMA method was used to infer the evolutionary history and the tree branches supported by bootstrap percentages. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

4.5 Pathogenicity tests

The onset of disease symptoms manifested as small circular chlorotic (greenish-yellow) spotty lesions on the leaf surface (Figure 15). These chlorotic lesions were observed on all inoculated leaves.

The foliar disease symptoms exhibited by the cassava plants indicated that cassava brown leaf spot disease resulted from the combination of the three fungal pathogens belonging to the genera *Colletotrichum*, *Cladosporium* and *Alternaria*. For all the pathogen-inoculated plants, symptom development began as small circular, greenish yellow, chlorotic spots. As time progressed, the spots became more distinct and they differed with the type of fungal inoculum. It was observed that mixed infection produced more severe symptoms compared to infection with single isolates and that a combination of the three pathogens produced symptoms typical of cassava brown leaf spot disease as observed in the fields. All inoculated plants had started to show symptoms one week post inoculation.

Inoculation with *Colletotrichum* sp produced tiny round spots which would later coalesce especially at the leaf edges to produce blight-like symptoms (Figure 16) while *Cladosporium* sp produced tiny, sunken plain brown spots which were not typical of cassava brown leaf spot symptoms (Figure 17). *Alternaria* produced brown spots that were not typical of BLS disease (Figure 18). Inoculation with two pathogen combinations, that is, *Colletotrichum* and *Cladosporium*, *Colletotrichum* and *Alternaria*, and *Cladosporium* and *Alternaria* (Figures 19-21) produced brown blight-like leaf symptoms which were not typical of cassava BLS symptoms. It was therefore concluded that a combination of the three fungal pathogens (Figure 21), in synergism, were the causative agents of cassava brown leaf spot disease. This combination of pathogens was then used to challenge selected elite cassava genotypes in order to assess their phenotypic response to the disease, and if the responses were varied depending on genotype.

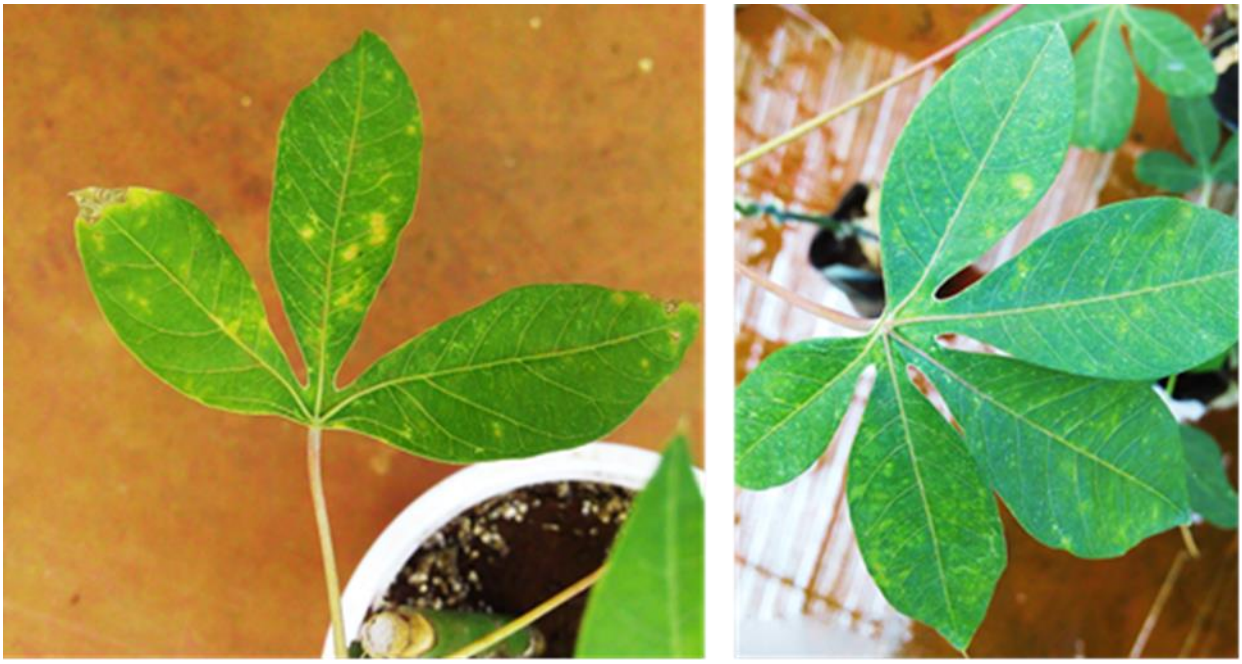

Figure 15: Cassava leaves displaying chlorotic spots on surface at the beginning of symptom development at 7 days post-inoculation upon combined inoculation with *Colletotrichum*, *Cladosporium* and *Alternaria* species

Figure 16: Foliar disease symptoms on cassava plants inoculated with an isolate of *Colletotrichum* sp. at 14 days post-inoculation

Figure 17: Foliar disease symptoms on cassava plants inoculated with an isolate of *Cladosporium* sp.; at 21 days post-inoculation

Figure 18: Foliar disease symptoms on cassava plants inoculated with an isolate of *Alternaria* sp.; at 14 days post-inoculation

Figure 19: Foliar disease symptoms on cassava plants inoculated with a combination of *Colletotrichum* and *Cladosporium* spp. isolates; at 14 days post-inoculation

Figure 20: Foliar disease symptoms on cassava plants inoculated with a combination of *Colletotrichum* and *Alternaria* spp. isolates; at 14 days post-inoculation

Figure 21: Foliar disease symptoms on cassava plants inoculated with a combination of *Cladosporium* and *Alternaria* spp. isolates; at 14 days post-inoculation

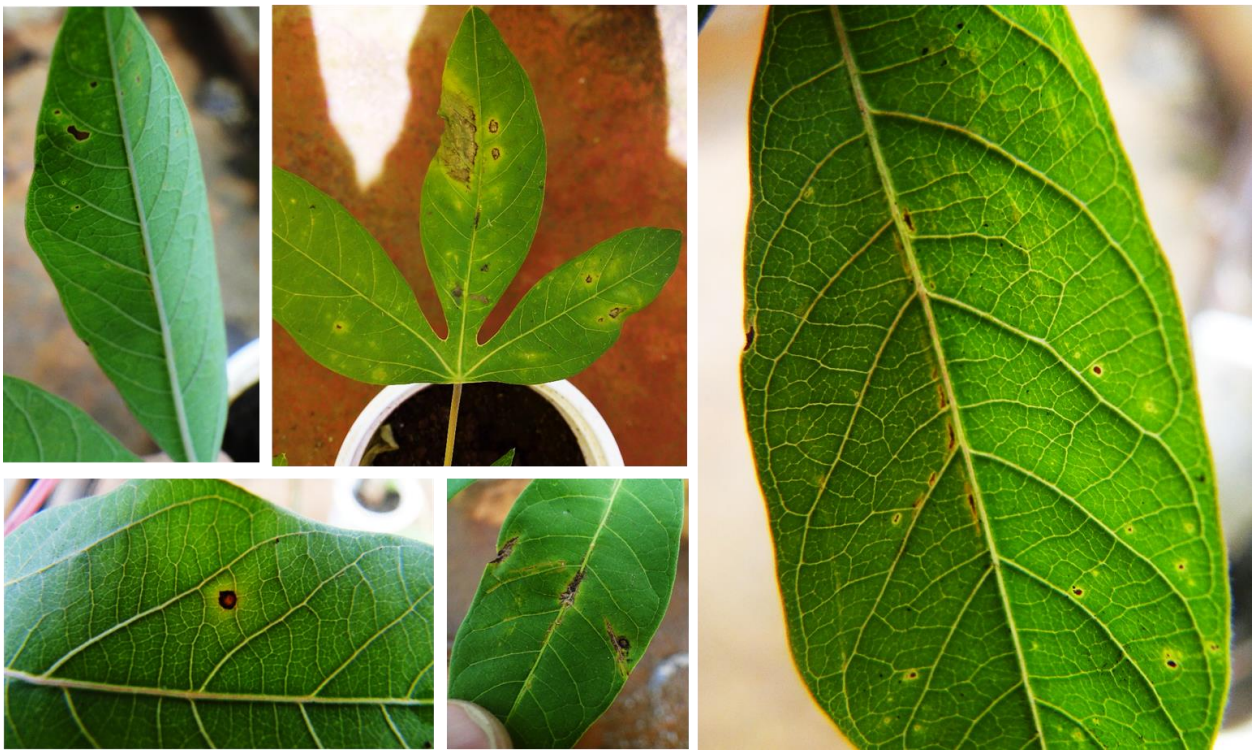

Figure 22: Foliar disease symptoms on cassava plants inoculated with a combination of *Colletotrichum*, *Cladosporium* and *Alternaria* spp. isolates; at 14 days post-inoculation

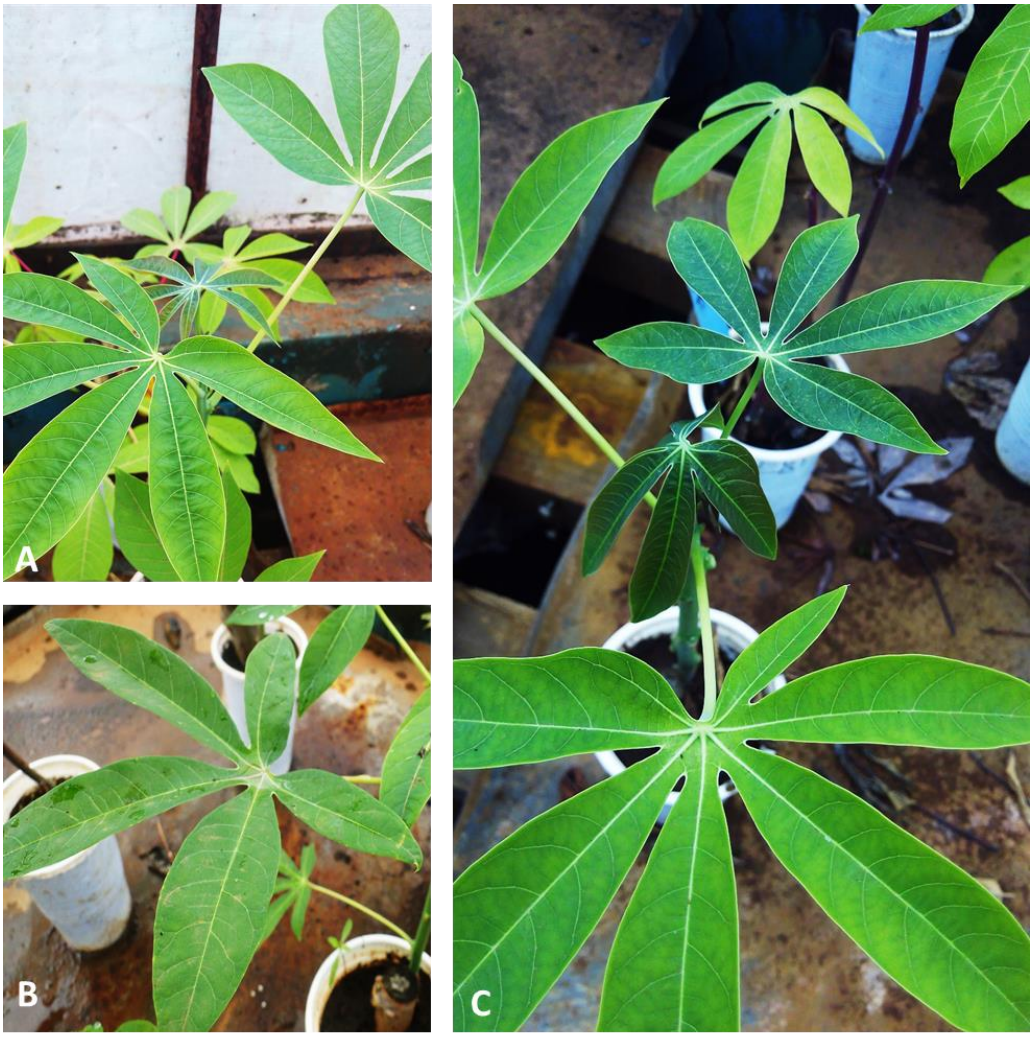

Figure 23: Experimental negative controls: (A and B) cassava plants sprayed with distilled water; (C) un-inoculated cassava plants

4.6 Phenotypic symptom assessment of elite cassava genotypes

This experiment was carried out in two greenhouse cycles, that is, between March – May and between June – August 2020 and the two experiments generally exhibited similar trends as far as symptom development was concerned.

4.6.1 Symptom development and disease incidence

Symptom development started within the first seven days post inoculation and an average of 84% of the plants exhibited the disease symptoms on their leaves (Figure 24). The incidence

progressed steadily to 87% to 89% to 89% to 98% in day 14, day 21, day 28 and day 35, respectively (Figure 24). From day 42 to day 56 post inoculation, all plants (100%) in all the genotypes had disease symptoms. 100% disease incidence for genotypes Ebwanatereka 1 and Ebwanatereka 2 was attained at day 35 and day 42, respectively. TME 204, TME 14 and TME 7 had 100% disease incidence from day 7 post inoculation and this explains the superimposition of the three disease progress curves into one in Figure 24. All plants in these genotypes exhibited leaf spots (Figure 24).

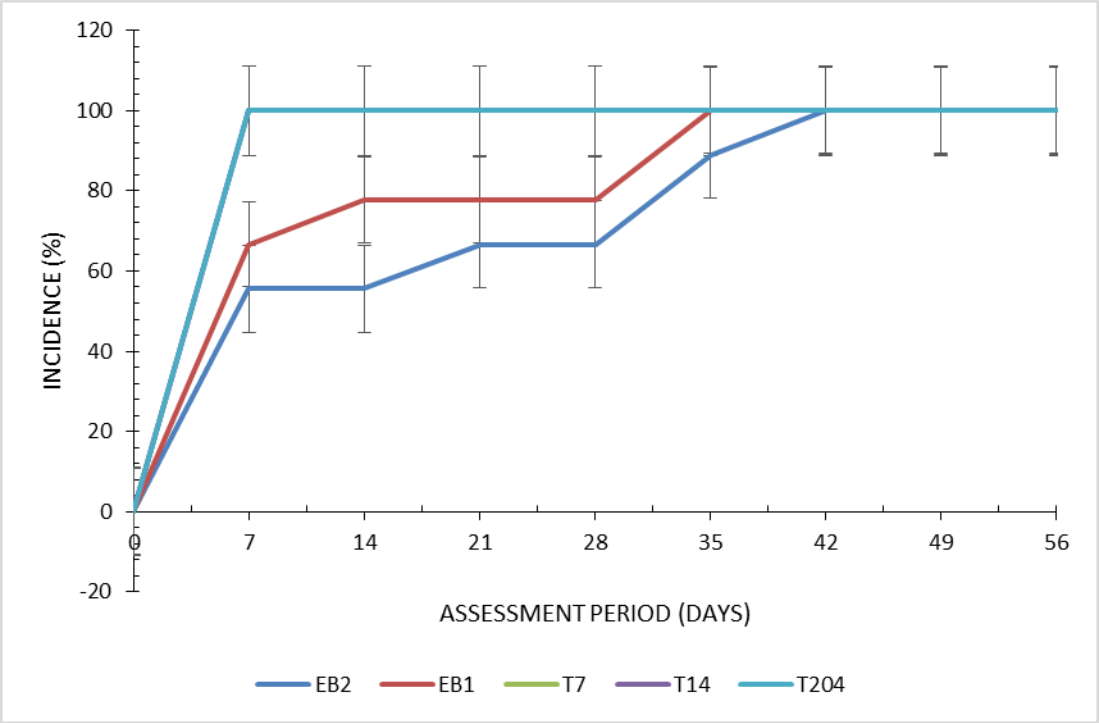

Figure 24: Percentage disease incidence over time for cassava genotypes TME 204, TME 14, TME 7, Ebwanatereka 1 and 2

4.6.2 Brown leaf spot disease severity and area under disease progress curves

As brown leaf spot disease incidence increased among the genotypes, disease severity on individual plants also increased (Figure 25). At 7 day post inoculation (dpi) 84% of all the plants were infected and exhibited the brown leaf spot symptoms with a disease severity score of 2 representing 1-10% of leaflet area expressing lesions. All the plants had leaf spot symptoms by day 42 with a severity score of between 2 and 5. During the assessment period, there was varied disease severity progression among the genotypes as indicated by the varying severity score as

shown in Figure 25. Severity progression for the TME 204 was relatively fast compared to the other genotypes, followed by TME 14 then TME 7, then Ebwanatereka 1 (EB1) and lastly Ebwanatereka 2 (EB2), which showed a relatively slow disease progression. At the beginning of the assessment period (7 dpi), significant difference (P=0.05) was observed between two genotype groups, that is, (EB1, EB2) and (TME 7, TME 14, TME 204) at 1.56, 1.67, 2, 2 and 2, respectively (Figure 25). Towards the end of the assessment period (42-56 dpi), there were significant differences (P=0.05) between EB2, EB1 and TME 7 but there was no significant difference between TME 14 and TME 204. There were significant differences (P=0.05) among EB2, EB1 and TME 7 from 14 dpi to 56 dpi; severity score at 56 dpi was 3.0, 3.3 and 3.8, respectively and 4.1 for both TME 14 and TME 204. In terms of area under disease progress curve (AUDPC), the highest value recorded was 178.5 in TME 204 and lowest at 103.8 in Ebwanatereka 2. The other AUDPC values were 169.9, 157.1 and 127.9 for TME 14, T7 E and Ebwanatereka 1, respectively (Figure 26).

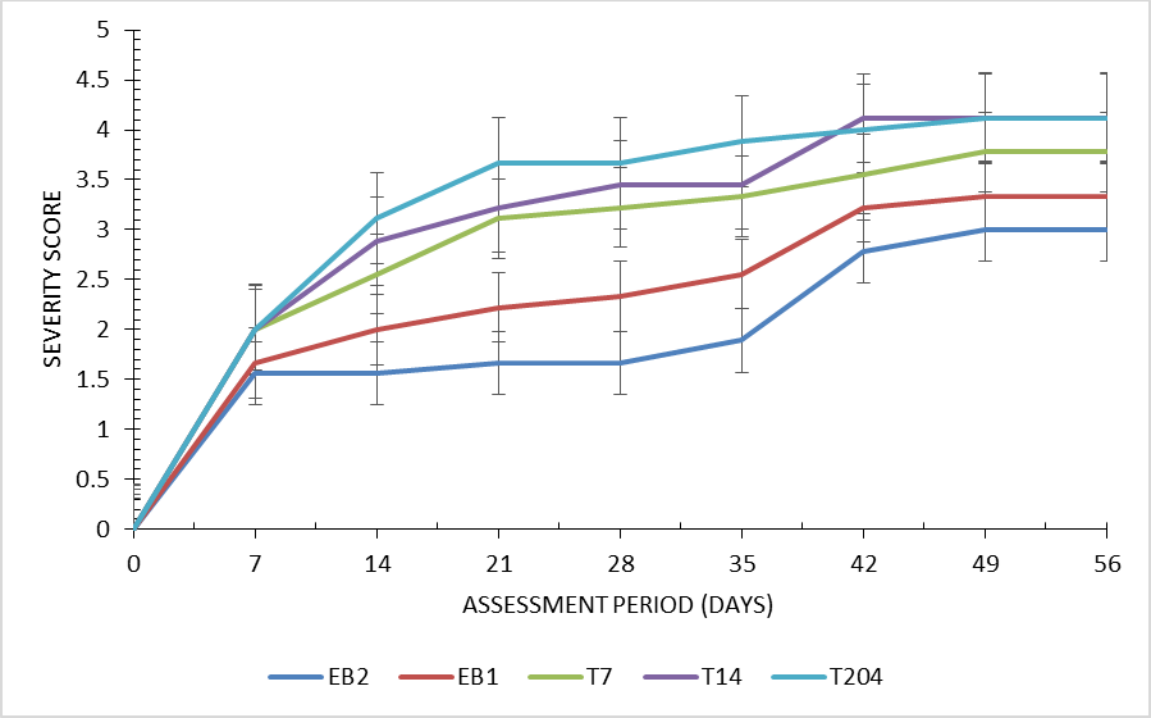

Figure 25: Disease severity scores over time for five farmer preferred cassava genotypes. Error bars indicate standard error of the mean for each of the assessment periods

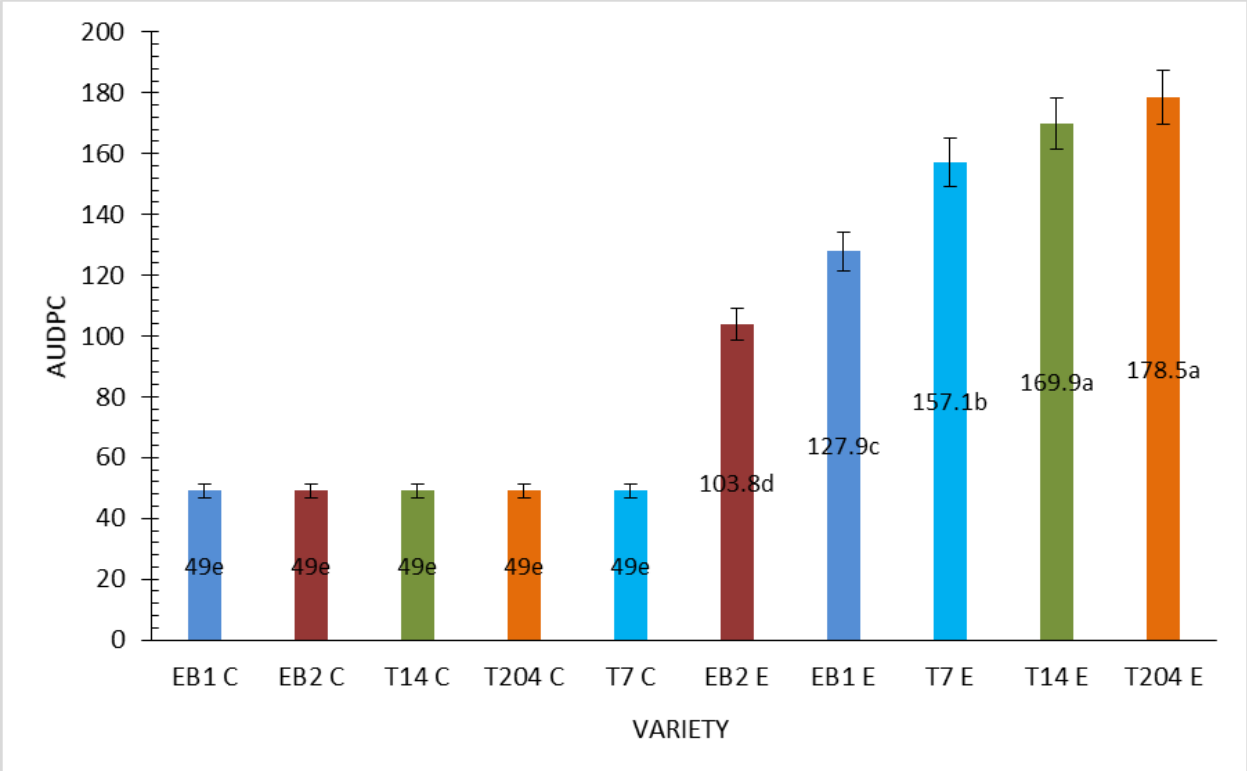

Figure 26: Mean AUDPC scores of control (C) and fungal (E) treatments calculated from disease severity rating on TME 204, TME 14, TME 7, Ebwanatereka 1 and 2 cassava genotypes.

Error bars indicate standard error of the means

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

This study aimed at identifying the causative agents of brown leaf spot disease of cassava and the phenotypic response of elite cassava genotypes to infection by the pathogen(s). Recognition of fungal disease symptoms and accurate identification of the causative agents is imperative in development of effective management practices and may also help in the control and prevention of disease spread (Jain *et al*., 2019).

Morphological features through microscopy and molecular diagnostics through ITS region amplification identified the causative agents of the disease to be *Colletotrichum* sp., *Alternaria,* sp. and *Cladosporium* acting in synergism to produce brown leaf spots on the cassava leaves. Peng *et al*. (2021) confirmed that some diseases are not as a result of infection by one pathogen, but rather a synergy of multiple pathogens. The three pathogens produce phytotoxins as explained by Jayawardena *et al*. (2016) for *Colletotrichum*, Meena *et al*. (2017) for *Alternaria* and Alwatban *et al*. (2014) for *Cladosporium*, and the toxins play an important role in plant colonization, and the indistinct yellow halo produced around the brown spot is due to the toxins produced by the advancing mycelia. The probable mechanism of interaction among the three BLS pathogens could be explained by Chatterjee *et al*. (2016) after studying three fungal species with different life styles. In the study, there were new metabolites not present in single infections but present in synergy.

Through the pathogenicity tests carried out in this study, it has been shown that each of these pathogens causes leaf spots, whether as single isolates or in combinations only that typical BLS symptoms were observed when the three pathogens were combined. The three pathogens have been shown to cause leaf spots in various plants including cassava (*Manihot esculenta*). *Colletotrichum* produced tiny round spots which would later coalesce especially at the leaf edges to produce blight-like symptoms as attested to by Silva *et al*. (2017). *Colletotrichum* is one of the most important genera of phytopathogenic fungi in agriculture which is commonly associated with leaf spots. The genus is a major agent of crop losses globally due to its destructive nature. It is nearly universally pathogenic, occurring on a wide range of host plants (Silva *et al*., 2017). In almost all major families of dicots, most monocot families, some gymnosperms and ferns. In cassava, *Colletotrichum* has been implicated as the main cause of brown leaf spot disease, the

ultimate case of which is yellowing and defoliation thus affecting tuber production and leaf yield. The fungus is widespread in most cassava growing regions as well as across ecozones (To-Anun *et al*., 2011). *Colletotrichum* species, as described by Cannon *et al*. (2012), cause devastating diseases in a wide range of economically important crops such as coffee, maize, sorghum, sugar cane, strawberries, mango, avocado and many other crops. *Colletotrichum* species use a wide range of approaches to colonize and obtain nutrients from their hosts (Baroncelli *et al*., 2017). One of the infection mechanisms is the development of an appressorium from the germinating spore on the host plant surface, followed by turgor driven penetration of the cuticle and epidermal cells by infective hyphae. Establishment within plant tissues is aided by the production of host-induced virulence effectors by the fungus (Cannon *et al*., 2012). The genus *Colletotrichum* contains approximately 189 species categorized into 11 major species complexes, also known as phylogenetic lineages (Baroncelli *et al.,* 2017). The highest prevalence of this pathogen in this study underpins its importance in causing leaf spots which in turn reduces the photosynthetic surface thereby causing significant yield losses in the affected crops.

Symptoms produced by *Cladosporium* sp exhibited as tiny, sunken plain brown spots which were not typical of cassava brown leaf spot symptoms. Thomma *et al*. (2005) confirms that Cladosporium sp. has been implicated as an important pathogen causing leaf spots in plants including spinach, pecan nuts, cucumber and peach. It can also exist as a saprophyte, living on decaying plant tissue. Its conidiophores are usually tall and upright with branching at the apex. Conidia are produced singly or in chains. Studies conducted on *Cladosporium* were limited on the role of *Cladosporium* in cassava brown leaf spot disease but its implication in other plant leaf spots and high prevalence in this study made it deserve a deeper investigation. Inoculation of cassava leaves with *Cladosporium* yielded tiny, sunken spots on the upper side of leaf which were rough to touch on the leaf underside. The scenario was similar to spots caused by the fungus on cucumber plant (Thomma *et al*., 2005). Bensch *et al.* (2012) describes *Cladosporium* species to be cosmopolitan in distribution and common on all types of plant, fungi, debris, soil, textiles and other organic materials. The authors further stated that *Cladosporium* comprises one of the largest and most heterogenous hyphomycetes genera. The species colonize as secondary pathogens on lesions caused by phytopathogenic fungi. Bensch *et al.* (2012) adds that other *Cladosporium* species are phytopathogens, causing leaf spots and other lesions, or can be

hyperparasites on other fungi. These facts could possibly explain the relatively high prevalence of *Cladosporium* sp. observed in this study.

Symptoms produced by *Alternaria* produced brown spots that were not typical of BLS disease. There were no detailed studies on the role of *Alternaria* sp. in cassava brown leaf spot disease reported in literature. Nonetheless, its prevalence through all the isolation procedures and sampled areas warrants its inclusion in this study. Besides, the typical BLS symptoms produced upon combined inoculation with the three pathogens indicated that *Alternaria* played a key role in BLS disease development. *Alternaria* species have been shown to be important pathogens of a wide variety of crops such as potato and tomato where they cause severe early blight (Stammler *et al*., 2014). Pathogenicity of the *Alternaria* species is dependent on susceptibility or resistance of host plant as well as the quantitative production of host-specific and non-host specific toxins (HSTs and nHSTs) (Meena *et al*., 2017).

Morphological identification of the three fungal pathogens was complemented by molecular identification through amplification of the ribosomal internal transcribed spacer (ITS) DNA region by use of ITS 1, 4 and 5 primers (Schoch *et al*., 2012). The ITS has been universally accepted as the official barcoding marker for fungi. It is a conserved ribosomal DNA sequence that has been used widely both singly and in combination with other universal sequences to identify and characterize as well as carry out phylogenetic analysis of fungal isolates (Schoch *et al*., 2012). Each of the isolated fungus was subjected to PCR amplification with the ITS primer combination ITS 1/4 and 4/5 with ITS 4 being the reverse primer. The sequences obtained proved to be highly homologous to those of species of *Colletotrichum, Cladosporium* and *Alternaria*, thus morphological identification was duly confirmed through molecular analysis. Due to homology of each of the BLS pathogens with a number of their relative species in the GenBank, this study identified pathogens to the genus level.

Symptom development upon inoculation of healthy cassava plants with the fungal pathogens is a visible effect of disease on the plants. The symptoms observed in this study began as chlorotic patches which later became necrotic lesions on the leaf surface. Upon inoculation with the identified brown leaf spot disease pathogens, the different cassava genotypes expressed varied phenotypic response over the assessment period. Infection and severity progression for the

genotype TME 204 was relatively fast compared to the other genotypes, while genotype Ebwanatereka 2 (EB2) showed the slowest disease progression over the assessment period. Severity progression of the other genotypes was in between the two genotypes. This indicated that TME 204 was highly susceptible to BLS disease, followed by TME 14, then TME 7, while Ebwanatereka 2 was resistant to the disease, followed by Ebwanatereka 2. The slow progress of disease in Ebwanatereka 1 and Ebwanatereka 2 could indicate possible resistance mechanisms in these genotypes. The high disease incidence among the genotypes TME 204, TME 14 and TME 7 indicate that they were susceptible to cassava brown leaf spot disease.

5.2 Conclusion

i. The present study has delineated three pathogens belonging to the genera *Colletotrichum, Cladosporium* and *Alternaria*, acting in synergism to produce BLS disease symptoms.
ii. Phenotypic responses upon challenging selected genotypes with the identified pathogens showed that there were significant differences among the genotypes, with TME 204 exhibiting fast infection and rapid severity progression, while EB 2 slow disease severity progression.

5.3 Recommendations

The findings of this study could lead to further research studies in areas that will enable better understanding of cassava brown leaf spot causative pathogens in Kenya, and how to better manage them to avoid devastating crop yield losses. The following recommendations are deduced from this study:

i. Results of this study provide a foundation for investigation of sources of disease resistance in resistant genotypes Ebwanatereka 1 and 2.
ii. Further research should be carried out to investigate the mechanism of the synergism among the three pathogens.
iii. Other molecular methods such as DNA sequencing of the ribosomal large subunit (LSU), coupled with ITS analyses followed by comparative sequence analysis, can be used to identify the pathogens to the species level.
iv. Further studies should be conducted to test reaction of other cassava genotypes and elite lines to the cassava BLS disease causal agents, and in other agro-ecological zones.

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