IDENTIFICATION AND CHARACTERIZATION OF FUNGAL PATHOGENS ASSOCIATED WITH CASSAVA BROWN LEAF SPOT DISEASE IN WESTERN KENYA

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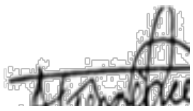
DECLARATION

I hereby declare that this thesis is my original and has not been submitted to the University of Nairobi or any other institution of higher learning for examination, the award of a degree or publication. Where other people's work or my own work has been used, this has been properly acknowledged and referenced in accordance with the University of Nairobi's requirements.



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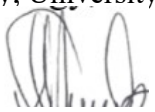

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DEDICATION

To my father Julius Ager Adudu and my late mother Agnes Adhiambo (Nyar Suba) Ager

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LIST OF ABBREVIATIONS AND ACRONYMS

amp	amperes
BIC	Bayesian information criterion
BLASTn	Nucleotide Basic Local Alignment Search Tool
BLS	Brown leaf spot disease
Bp	base pairs
CAD	Cassava anthracnose disease
CBB	Cassava bacterial blight disease
CIAT	Centro Internacional de Agricultura Tropical
CMD	Cassava mosaic virus disease
CONTIG	Contiguous sequence
CTAB	Cetyltrimethylammonium bromide
Cu-DNA	Copper-DNA complex
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organisation
FIESC	Fusarium-incarnatum-equiseti species complex
Freq	Frequency
ITS-1	Internal transcribed spacer-1 sequence
ITS-4	Internal transcribed spacer-4 sequence
kb	Kilobases
MEGA	Molecular Evolutionary Genetics Analysis
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NaCl	Sodium chloride
NJ tree	Neigbor joining phylogenetic tree
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PSI	Pounds per square inch
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation

SE	Standard error of the mean
SDS	Sodium dodecyl sulphate
TAE	Tris-Acetate-EDTA
Tm	Melting temperature
UNITID	University of Nairobi Institute of Tropical and Infectious Diseases
UV	Ultra-violet

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is an important food security crop due to its adaptability to marginal areas. However, brown leaf spot (BLS) is one of the fungal diseases considered to be a major threat in cassava production, causing more than 30% economic damage on susceptible cultivars. To breed cassava for durable resistance, identification and characterization of the fungal pathogens causing BLS is a pre-requisite. Therefore, the aim of this study was to identify and characterize fungal pathogens causing cassava BLS in Western Kenya. Cassava leaf samples with brown leaf spot symptoms were collected from three Counties (Kakamega, Bungoma and Busia) of Western Kenya. The fungal pathogens were isolated by culturing the leaf samples on potato dextrose agar (PDA) amended with the antibiotics. Pathogenicity of the fungal isolates was tested on leaves of susceptible cassava cultivar TMS 60444 using detached leaf assay. A total of 60 fungal isolates were obtained of which thirty five isolates were confirmed to be pathogenic based on detached leaf assay of the susceptible cassava cultivar TMS 60444. Out of the 35 pathogenic isolates, single spore cultures of 13 randomly selected representative isolates were characterized; and they exhibited varied morpho-cultural characteristics including colony colour, colony margin, colony texture, colony diameter and presence of pigments on PDA medium. Based on ITS sequences and phylogenetic analysis, the 13 representative fungal isolates were identified as *Trichothecium roseum* (four isolates), *Fusarium equiseti* (four isolates), *Colletotrichum siamense* (two isolates), *Colletotrichum karstii*, *Diaporthe phaseolorum* (one isolate) and *Bipolaris setariae* (one isolate). This is the first report to identify *T. roseum*, *C. siamense*, *C. karsti* and *D. phaseloreum* as causal agents of cassava BLS. This study reveals that BLS in Western Kenya is caused by a diverse group of fungal pathogens with different morpho-cultural and molecular characteristics. There is need to carry out further studies to investigate the severity of BLS when the cassava host is co-inoculated with the identified fungal pathogens.

CHAPTER ONE
INTRODUCTION

1.1 Background to the study

Many sub-Saharan Countries have resorted to cultivation of cassava (*Manihot esculenta* Crantz) as a food and nutrition security crop due to its ability to tolerate drought, erratic rainfall conditions, low soil fertility and long-term underground storability of the roots (Bryan *et al.*, 2014; FAO, 2021a; Njine, 2010). Importance of cassava as a food crop is emphasised by the fact that it is the third most important food crop after cereals and legumes (Babu *et al.*, 2009), and serves both as a staple and subsidiary food for low income families (CIAT, 2015). Currently, cassava is grown in all countries in Africa with the continent being the largest producer in the world (FAO, 2020). Due to its importance as a food insecurity mitigator, any constraints to cassava production, such as pests and diseases, threaten food security.

Alonso *et al.* (2021) reported that insect pests are a serious threat to cassava production not only due to the direct effect of the pests on cassava itself, but also due to their capacity to as act as vectors to diseases. The most important diseases of cassava are caused by viral pathogens. Cassava mosaic disease causes approximately 30% yield loss, cassava brown streak disease causes an estimated 8% loss while cassava bacterial blight is responsible for up to 92% yield loss (Alonso *et al*., 2021).

Until recently, cassava brown spot (BLS) disease had been neglected because it was deemed to be of less economic importance compared to other foliar diseases (Julião *et al.*, 2020; Powbunthorn *et al.*, 2012). Recent outbreaks of the disease in Brazil have revealed its economic importance (Julião *et al.*, 2020). Pei *et al.* (2014) reported that BLS is the most important fungal disease in China; while Ayesu-Offei and Antwi-Boasiako (1996) reported that cassava BLS affected all cassava cultivars grown in Ghana. As such, the number of studies investigating BLS have been increasing in the past decade. However, most of the studies on cassava diseases simply mention the disease and fail to provide data on its importance, epidemiology, control and most importantly its causative agents (Hillocks, 2002). This is significant since any control method employed to control BLS should take into account its causative agents.

Cassava brown leaf spot disease is believed to be caused by *Passalora henningsii* (Lima *et al.*, 2019; Pei *et al.*, 2014). This pathogen has however undergone numerous taxonomic revisions (Pedro *et al.*, 2000; Phengsintham *et al.*, 2013; Videira *et al.*, 2017). That notwithstanding, BLS shares most of its symptoms with many other fungal diseases, making it difficult to ascertain that BLS is caused by a single fungal pathogenic species. Alonso *et al.* (2021) reported that due to the cryptic nature of most of the cassava diseases, it becomes difficult to diagnose a disease on the basis of symptoms alone. Ng'ang'a *et al.* (2019) reported that symptoms consistent with BLS were caused by a synergistic relationship between *Colletotrichum* spp., *Alternaria* spp. and *Cladosporium* spp. This study utilized samples from a field trial and therefore there may be many fungal pathogens responsible for BLS in cassava.

A handful of methods have been proposed to control BLS. Many studies recommend the use of copper based fungicides, maintaining wide spaces between plants during planting and cultivation of resistant varieties of cassava (Hillocks and Wydra, 2002; Lian and Lan, 1984; Lozano and Booth, 1974; Lozano and Terry, 1976). However, any management strategy is likely to be hindered by inadequate information on the causal agents of cassava BLS since control is often pathogen specific. Such information on the identification and characterization of causal agents of cassava BLS are limited in Kenya.

1.2 Statement of the Problem

Cassava brown leaf spot disease has global incidence occurring in all cassava growing jurisdictions. In Africa, the disease has primarily been reported in West Africa (Ayesu-Offei and Antwi-Boasiako, 1996; Terry and Oyekan, 1976); however, recently a field trial confirmed the disease in Kenya (Ng'ang'a *et al*., 2019). Cassava brown leaf spot disease causes more than 30% economic loss in cassava production (Pei *et al.*, 2014). This is of significance because the government of Kenya is encouraging cultivation of cassava as one of the means to alleviate chronic food insecurity in poverty stricken and semi-arid regions. Such economic loses would adversely affect utilization of cassava as a food and nutrition security crop. There is limited information on BLS infection in cassava in Kenya. Only one report from samples collected from a field trial is available on the causative agent of BLS in Kenya (Ng'ang'a *et al.*, 2019) and therefore identification and characterization of the causal pathogens present in major cassava

growing regions in Kenya are largely unknown. This inadequate knowledge negatively impacts methods of mitigation to control cassava BLS in farmers' fields. The aim of this study was therefore, to identify the causative agents of BLS through morpho-cultural and molecular characterization. This information is important for the development of effective strategies for the management and control of BLS.

1.3 Justification

Cassava is an important root crop cultivated globally in tropical and subtropical regions. The ability to withstand difficult growing conditions with minimum inputs makes it a resilient crop for food and nutrition security. The objective of this study was to identify the causal agents of BLS in western Kenya based on morphology, molecular properties and pathogenicity tests. The accurate identification of the causal agents of BLS is important for correct diagnosis, monitoring of cassava BLS and for effective control of the disease. Knowledge on the variability of the pathotypes is significant in selecting the correct isolates for resistance testing in breeding programmes. The results from this study could be a platform for generating detailed information on the genetic variation of causative agents of cassava BLS in Kenya using molecular approaches. The overall findings of the present study are important in contributing to improving cassava production in Kenya.

Molecular characterization and information on genetic diversity of the pathogen populations are important for developing disease resistant cultivars through breeding or other advanced approaches. This is because in the pathogen population, where random mating occurs, they evolve faster, increasing the probability of overcoming host resistance or developing fungicide resistance as reported by McDonald and Linde (2002).

1.4 Objectives

1.4.1 Main objective

The main objective of this study was to identify and characterize the causal agents of cassava brown leaf spot in Western Kenya using morphological and molecular techniques.

1.4.2 Specific objectives

The specific objectives of the study were:

(i) To isolate and evaluate the pathogenicity of fungal pathogens of cassava infected with BLS from Bungoma, Busia and Kakamega Counties in Western Kenya

(ii) To determine the morpho-cultural and molecular characteristics of the causal agents of cassava BLS

1.5 Hypotheses

The hypotheses tested in this study were:

i. Brown leaf spot disease of cassava in Western Kenya in caused by different fungal pathogens which differ in their pathogenicity.

ii. The causal agents of cassava brown leaf spot disease can be distinguished based on their morpho-cultural and molecular characteristics.

CHAPTER TWO
LITERATURE REVIEW

2.1 Origin of cassava

Cassava (*Manihot esculenta* Crantz) is believed to have originated in South America before being introduced to the other regions of the world (Hillocks, 2002). However, for a majority of the taxonomic and botanical history of the crop, consensus held that cassava had no known ancestry. The discovery of morphologically identical wild population of cassava in the wild in Brazil appears to have been the turning point on this consensus (Allem, 2001). Borrowing from studies from other crops, it had been argued that cassava probably arose from hybridization events between several wild species (Bellotti *et al*., 2001). Recent genetic studies have confirmed the South American origin, but disagree with the hybridization hypothesis (Allem, 2001). Using haplotype data of glyceraldehyde 3-phosphate dehydrogenase (G3pdh) gene, Olsen and Schaal (1999) concluded that cassava most likely originated from a single progenitor and thus hybridization was unlikely. Similarly, Léotard *et al*. (2009) failed to observe evidence for hybridisation thus reinforcing a single domestication event. Moreover, both Olsen and Schaal (1999) and Léotard *et al*. (2009) suggested that the locus of domestication was the southern border of the Amazon river. It is the Portuguese that are credited with introducing cassava in Africa in 1558 by way of West Africa (Guira *et al*., 2017) arriving in East Africa by 1878 (Hillocks, 2002).

2.2 Importance of cassava as a food security crop

Cassava (*Manihot esculenta* Crantz) has emerged as an important food crop due to its ability to tolerate drought, low soil fertility and adapt to changing climatic conditions (Bryan *et al.*, 2014; FAO, 2021a; Njine, 2010). It is the third most important food crop after cereals and legumes worldwide (Babu *et al.*, 2009) serving both as a staple and subsidiary food for low income families (CIAT, 2015). A decline in the cultivation of tubers (cassava being an example) increases the level of food insecurity as reported by Musotsi *et al.* (2008). Due to erratic rainfall patterns, farmers in the Kenyan Lake Victoria Basin have shifted to growing cassava because harvest is guaranteed (Kidasi *et al.*, 2021). This observation is not limited to Kenya; a report by FAO (2017) indicated that cassava is being considered by many governments within Eastern and

Southern Africa as a solution to chronic food insecurity. This has resulted in Africa being the largest cassava producing continent in the world; producing more cassava than the rest of the world combined (FAO, 2021b).

In addition to being a food crop, cassava is important in several industries. In China, cassava is used to produce industrial starch and ethanol (Pei *et al.*, 2014). In several South East Asian countries, cassava is used as a feed and for the production of bio-energy (Chaoping *et al.*, 2015). Cassava leaves are also used as food sources for the eri silkworm that is grown for production of silk (Babu *et al.*, 2009). These uses have the capacity to expand the cassava industry in Africa beyond subsistence farming.

2.3 Nutritional value of cassava

The major importance of cassava as a food and nutrition security crop emanates from the fact that its starchy tuberous roots are a cheap source of calories and can therefore provide daily caloric requirements (Bayata, 2019). Furthermore, the underground storage of the tuber, can ensure food security during periods of famine and war (Bayata, 2019). Cassava tubers are energy dense and compare favourably against other starchy foods like maize and rice.

Cassava has been criticized for being low in lipids it; however it has more lipid content than other tuberous crops like potato and is comparable to rice. Major minerals and vitamins are also contained within cassava tubers (Bayata, 2019). Apart from the roots, the leaves of cassava are also used as food and have a wide range of nutrients. The protein content of cassava leaves, for instance, rivals that of eggs, milk, cheese and soybean. It has been reported that the protein content of cassava leaves is higher than that of other vegetables (Awoyinka *et al*., 1995). Due to the high protein content, cassava leaves have been utilised to enhance breast milk production in lactating women (Aregheore, 2011). Cassava is therefore considered a highly nutritious food and can meet the nutrient requirements of the low-income population in the context of a changing climate (Gleadow *et al*., 2009).

2.4 Constraints to cassava production

Cassava is affected by all major classes of pathogens including bacteria, viruses, nematodes, fungi as well as insects which affect various organs of the plant. This led to the classification of cassava diseases based on the type of pathogen that causes the disease (Lozano and Terry, 1976; Zárate *et al*., 2021), the organ that is affected or a combination of the two (Lin *et al.*, 2019; Lozano and Booth, 1974). Of critical importance among these diseases are the foliar diseases. Leaves are the major photosynthetic organs of the plant, and thus, foliar diseases lead to diminished photosynthetic activity of the leaves. This can lead to reduced photosynthetic surface area of the leaf through chlorosis and necrosis (Lozano and Terry, 1976), dieback and earlier abscission of the leaf (Lian and Lan, 1984). Recent studies on cassava foliar diseases have put significant emphasis on Cassava Bacterial Blight (CBB), Cassava Brown Streak Virus Disease (CBSD) and Cassava Mosaic Virus Disease (CMD). These are regarded as the most destructive and therefore economically important. However, due to the effect of climate change, other foliar diseases are emerging as diseases of concern (Caubel *et al.*, 2017; Garrett *et al.*, 2009; Juroszek *et al.*, 2020). One of the emerging foliar fungal diseases is cassava brown leaf spot.

2.5 Cassava brown leaf spot disease

Cassava Brown Leaf Spot (BLS) has been viewed as being of less economic importance in the past. This has been attributed to it being less destructive compared to other diseases of cassava (Powbunthorn *et al.*, 2012). However, recent reports from Asia and South America have shown significant effects of BLS on cassava production (Julião *et al.*, 2020; Pei *et al.*, 2014). Several authors agree that cassava BLS has global occurrence with its presence in Togo (Verdier *et al.*, 2007), China (Chaoping *et al.*, 2015) and Kenya (Ng'ang'a *et al.*, 2019). Pei *et al.* (2014) asserted that BLS was the most important fungal disease affecting cassava production in China.

Cassava BLS is caused by a fungal pathogen whose taxonomy has undergone tremendous revision over the years. Unfortunately most of these previous names have remained in use. The pathogen, for example, is referred to as *Passalora henningsii* by Pei *et al.* (2014) and *Cercospora henningsii* (Allesch) by Ayesu-Offei and Antwi-Boasiako (1996) and Babu *et al.*, (2009). Hillocks and Wydra (2002) provided ten synonyms for this same pathogen. References for the classification and reclassification of this pathogen into diverse genera is provided by Phengsintham *et al.* (2013). The most recent revision was by Videira *et al.* (2017) who placed

the pathogen into the genus *Clarohilum* with the name *Clarohilum henningsii*. However, since the name *Clarohilum henningsii* is yet to gain universal usage, the name *P. henningsii* will be used within this document. This is mainly due to the fact that most recent papers prefer this name to the plethora of other synonyms. The teleomorphic genus of *P. henningsii* has nonetheless remained *Mycosphaerella* even as the anamorph has undergone revision (Pedro *et al.*, 2000).

2.5.1 Causal agents of cassava brown leaf spot disease

Passalora henningsii as originally described by Braun and Castañeda-Ruiz (1989) was based on the morphologies of conidiophores and conidia. They described the conidiophores as densely fasciculated, sparsely septate, faintly brown and arising from small stromata. The conidia were described as being septate with 1 – 3 septa, clavate and sub-cylindrical and the conidia varied in number (0 – 5). Babu *et al.* (2009) and Pei *et al.* (2014) described that the conidia can either be straight or slightly curved. Phengsintham *et al.* (2013), Videira *et al.* (2017) and Pei *et al.* (2014) observed that conidia form at the apexes of conidiophores. Ayesu-Offei and Antwi-Boasiako (1996) recognised two types of conidia produced by *P. henningsii*; the larger macroconidia (40.5 ± 0.81 × 5.5 ± 0.01 μm) that, in the presence of free water, fragmented into the smaller microconidia (11.2 ± 0.09 × 5.6 ± 0.03 μm). Similar observations are reported by Babu *et al.* (2009) and Pei *et al.* (2014).

To supplement these classical observations, Pei *et al.* (2014) characterised *P. henningsii* on the basis of its biological characteristics. They found that the optimal temperature for mycelial growth and macroconidia germination was 26 – 28 ºC on carrot agar medium, at a pH of 6 and under continuous darkness. The lethal temperature for the macroconidia was 60 ºC for 10 minutes. Ayesu-Offei and Antwi-Boasiako (1996) made additional observation that the number of microconidia and macroconidia produced by lesions was positively correlated with relative humidity and time.

Microconidia are recognised as the infective agent of *P. henningsii.* However, Babu *et al.* (2009) suggested that infection occurred purely through macroconidia. Evidence suggests that even though microconidia and macroconidia can germinate on both leaf surfaces, infection is strictly through the abaxial surface (Ayesu-Offei and Antwi-Boasiako, 1996; Babu *et al.*, 2009; Pei *et*

al., 2014). This can occur either through direct penetration of the abaxial epidermal cells (Babu *et al.*, 2009) or through the abaxial stomatal openings (Lozano and Booth, 1974). Ayesu-Offei and Antwi-Boasiako (1996) also reported formation of appressoria by some microconidia. This has been challenged for example by Thomma *et al.* (2005) who claim that members of *Mycosphaerellaceae* do not develop appressoria. This claim is given credence by Babu *et al.* (2009) who failed to observe appressoria in *P. henningsii* but observed direct infection through germ tubes. Regardless on the means of infection, the consensus is that once the pathogen gains entry into the leaf, it grows in the mesophyll eventually forming stromata. During this time, the leaf remains asymptomatic until the emergence of conidiophores.

The method used for conidiophore emergence is still an issue of debate. While Nguyen *et al*. (2016) have agreed that the conidiophores emerge strictly through stomatal openings, Babu *et al.* (2009) and Lozano and Booth (1974) offer the opinion that the emergence is mainly via the rapture of the epidermis.

2.5.2 Symptoms of brown leaf spot disease on cassava

The initial symptoms first appear on the abaxial leaf surface in the form of small, numerous dark green spots. In experimental inoculation studies, this has been observed to occur 9 days after inoculation (Babu *et al.*, 2009). Two days after the initial symptoms, similar spots are observed in the adaxial surface of the leaf (Pei *et al.*, 2014). Within two days of the spots appearing on the adaxial surface, they enlarge and turn brown. The enlarged spots turn greyish and water soaked, the grey colour is due to the presence of conidiophores and conidia in the lesions (Lozano and Terry, 1976). The lesions then become larger, angular and irregular in shape. Some cassava varieties present a dark periphery at the margins of the lesions and/or a halo ring around the lesions. As the disease progresses, the lesions become brittle and break off. This occurs 20 – 30 days after inoculation (Babu *et al.*, 2009). During the course of disease progression, the infected leaves turn yellow, dry and prematurely abscise.

It has been argued in Lozano and Booth (1974) that primary infection is initiated when wind or water transport conidia from old, fallen infected tissue to leaves of new plantings where the

conidia germinate and infect the leaves. Secondary infection occurs when the conidia are transported to new sites of infection by wind or water.

Verdier *et al.* (2007) and Powbunthorn *et al.* (2012) observed that higher incidence for BLS occurred in wet savannah ecological zones. They also observed a positive association between incidence of BLS and abundance of weeds, branching cassava varieties and planting a mixture of cassava varieties. The same authors also observed negative association between BLS and the amount of trees that surrounded the field as well as intercropping. Powbunthorn *et al.* (2012) made similar observations in relation to humid ecozones, branching cassava varieties and number of trees surrounding cassava farms. Increased incidence in the presence of water can be due to increased efficiency of conidial dispersion in water than in wind, especially during splash erosion (Ayesu-Offei and Antwi-Boasiako, 1996). This can also partially explain the phenomenon of preferential infection on old lower leaves, which by virtue of their location, are closer to the ground inoculum than upper younger leaves. Additionally, if one takes into account the earlier cited evidence that macroconidia fragment to microconidia more rapidly in high humidity, the claim can be made that more inoculum will be produced in ecologically wet and/or humid areas than in drier areas. Wind mediated dispersal can be a consequence of the physical dislodging of inocula from infected tissues to healthy leaves. This can also have an indirect effect. When plants are swayed by strong winds, they tend to vibrate and rub on each other. These processes could have the unintended effect of passing inocula to previously healthy plants. This can explain the observation that increase in weeds and planting of branching cassava varieties increases incidence of the disease. Intuitively, these lead to increased surface area that increases probability of inoculation of *P. henningsii*.

Verdier *et al.* (2007) also observed that partial immunity is developed against *P. henningsii* in cassava grown in sandy soil. Given that the nutrient content and water holding capacity of sandy soils is poor, this immunity could be as a result of dilapidated plant health. In fact, it has been observed that members of the genus *Mycosphaerella* prefer to infect well-nourished plants with the effect on weakened plants being significantly limited (Thomma *et al.,* 2005). The combination of chronic water and nutrient deprivation due to competition between weeds and other crops, tend to leave the crops stunted and lowering photosynthetic capacity. It is well

within the boundaries of reason to theorise that similar effects can be achieved in hosts grown in sandy soils.

2.5.3 Management of cassava brown leaf spot disease

A handful of methods have been proposed to control cassava BLS. Many studies recommend the use of copper based fungicides, maintaining wide spaces between plants during planting and cultivating resistant varieties of cassava (Hillocks and Wydra, 2002; Lian and Lan, 1984; Lozano and Booth, 1974; Lozano and Terry, 1976).

Lian and Lan (1984) proposed that the recommended distance for planting was maintaining a distance of 1 × 1 meters between adjacent plants. The rationale of choosing this wide distance was to reduce the relative humidity that would be created if the distance between plants was shorter. This is particularly observed in the plant a few days after planting. The foliar system of the cassava plantings can create a microclimate of low temperature, poor air circulation and high relative humidity near the ground compared to above the plant. These conditions, according to Lozano and Booth (1974) encourage primary infection. It has been suggested that the factor mostly targeted by wider spacing is reduction of relative humidity (Hillocks and Wydra, 2002). Another method recommended to reduce relative humidity is planting during the onset of rain. This is observable during the dry season when the canopy close up creating a dry microclimate that does not favour establishment of the pathogen (Lozano and Terry, 1976). Thus in principle, it controls against secondary infection.

The utility of copper as a control agent depends on copper dissociating to liberate cupric ions which are the active ingredients in copper based fungicides. The cupric ions are taken up by the germinating fungal spores, upon which the copper ions form compounds and complexes with several protein components in the fungi, including imidazoles, phosphates, sulfhydryls and hydroxyls thus disrupting their functions (Husak, 2015). This leads to cell disruption and membrane leakage. Moreover, it has been suggested that cupric ions can lead to DNA damage either via hydroxyl oxidative damage or hydroxyl radical dependent DNA fragmentation. The former DNA damage is attributed to the capacity of copper to form hydroxyl radicals via the Haber-Weiss reaction, the latter on the other hand is due to formation of Cu-DNA complexes

(Husak, 2015). The utility of copper pesticides, though very effective, is not widely used due to the low productivity of cassava (Lian and Lan, 1984).

Since environmental conditions are difficult to control in the field and copper usage is still economically unviable, planting of resistant cassava varieties appears to be the most viable management practice. Several resistant varieties have been developed from naturally occurring resistant parents (Lian and Lan, 1984). Given that resistance is genetically determined, this method of management is the easiest and cheapest to maintain.

2.6 Other fungal pathogens associated with cassava brown leaf spot disease

Recent studies have shown that *Passalora henningsii* may not be the only pathogenic fungus causing cassava BLS. Morphological descriptions consistent with *Colletotrichum* spp. *Alternaria* spp. and *Cladosporium* spp. have showed that these diverse species are causative agents of cassava BLS (Ng'ang'a *et al.*, 2019).

Molecular data has however, only implicated *Passalora henningsii* in cassava BLS. Phylogenetic analysis however clusters *P. henningsii* with other known pathogens that cause leaf spots in other crops such as *Cercospora* spp. and *Pseudoccercospora* spp. (Pei *et al.*, 2014). This has been confirmed by Videira *et al.* (2017) that reclassified *P. henningsii* into the genus *Clarohilum* spp. The absence of molecular data on the possible non-Cercosporoid pathogens limits the extent of pathogenic classification since DNA fingerprinting is currently the gold standard in pathogen identification (Martens *et al.*, 2008) .

CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of cassava leaf samples from the field

Field surveys were carried out in cassava fields of Kakamega, Bungoma and Busia Counties between September and December, 2019. The survey areas were selected based on cassava growing zones described by Jaetzold and Schmidt (1983). In each County, 12 - 15 fields were purposively sampled and in each field 3 – 5 leaf samples were collected from 4 - 5 symptomatic plants. The symptoms observed on cassava plants were necrotic and chlorotic irregularly shaped brown spots on the surface of leaves with clearly defined dark borders; the spots occurred on both the adaxial and abaxial leaf surfaces. The symptomatic cassava plants had symptoms on both the older lower leaves and younger upper leaves (Figure 1).

Sampling procedure involved following the main roads and accessible routes in each surveyed County, and in each available cassava field, stops were made at 5 to 10 km intervals based on vehicle odometers. In cases where there was no suitable field available, the next cassava field was sampled. Symptomatic leaves were detached at the petiole, carefully placed in sample collection paper bags, sealed in the field, correctly labelled and placed in a cooler box. The leaf samples were then transported to the Department of Biochemistry laboratory at the University of Nairobi for fungal isolation within 48 hours post-sampling.

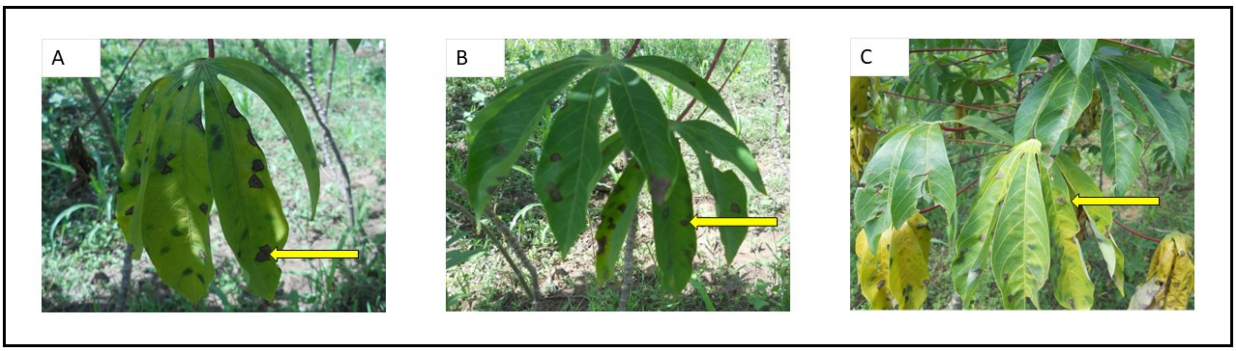

Figure 1: Cassava brown leaf spot disease symptoms observed in the field in Kakamega (A), Bungoma (B), and Busia (C) counties, Kenya

The image shows leaf spots with characteristic brown lesions. The yellow arrows indicate the symptoms on the leaf surface

3.2 Preparation of potato dextrose agar modified with antibiotics

This study used a modified version of the isolation procedure described by Pei *et al.* (2014). Thirty nine grams of potato dextrose agar (PDA) powder was dissolved in 1000 mL of distilled water. This was heated on a hot plate and regularly stirred until it started to boil to ensure even dissolution of the PDA. This solution was then autoclaved for 15 minutes at a temperature of 121 °C and pressure of 15 PSI. After autoclaving, the PDA was allowed to cool to approximately 45 °C then aseptically supplemented with the antibiotics ampicillin and streptomycin each at a concentration of 50 µg/mL in a Class II biosafety cabinet. The PDA modified with antibiotics was then aseptically dispensed in sterile Petri dishes of 90 mm diameter and allowed to cool to room temperature and solidify before use in the culture of sterilized discs of cassava leaf samples.

3.3 Isolation of fungal pathogens from infected cassava leaves

The symptomatic cassava leaves were each washed with running tap water to remove dirt and other debris. The leaves were then washed in 70% ethanol for 30 seconds and rinsed once with sterile distilled water. This was followed by a second wash in 1.3% sodium hypochlorite for 2 minutes followed by 3 washes in sterile distilled water with each wash lasting 2 minutes. The water of the last wash was also plated as controls.

The leaves were then laid on sterile paper towels inside a Class II biosafety cabinet to drain off excess water from their surfaces. Using sterile scalpels, symptomatic portions of the leaves were cut in sizes approximately 1 cm by 0.5 cm. The lesions were cut along their diameters and part of the leaf that displayed no symptom was also cut to make part of the sections. Five such sections were prepared from each leaf from each sampled plant. Sections from the same plant were mixed together by shaking them inside a sterile Petri dish. Five sections were then randomly picked and aseptically placed on the prepared PDA modified with antibiotics plates and labelled. Sections from the same plant were inoculated on the same PDA plate. The plates were then sealed using parafilm and incubated at room temperature (23 ± 2 ºC) under complete darkness for 5 days. For the controls, 100 µL of water from the last wash of each leaf was inoculated on Petri dishes and similarly incubated at room temperature and under complete darkness for 5 days.

After 5 days the fungi that grew from the cultured leaf sections on the PDA modified with antibiotics were aseptically sub-cultured to new PDA plates that were not amended with antibiotics. In a Class II biosafety cabinet, each plate that displayed fungal growth was unsealed and using a sterile inoculating needle, a portion of each morphologically distinct fungal culture was cut from the PDA medium and transferred to another sterile PDA plate and correctly labelled. Each of the plates was then incubated at room temperature (23 ± 2 ºC) under continuous darkness for 10 days to obtain pure cultures. Cultures that did not display any contamination were regarded as pure cultures. Each of the pure cultures obtained after 10 days of incubation was used for detached leaf pathogenicity assay.

3.4 Preparation of spore suspension

The pure fungal isolates obtained were used to prepare spore suspension for detached leaf pathogenicity assays using the modified protocol of Aberkane *et al.* (2002). Each plate was flooded with sterile distilled water using sterile pipettes in a biosafety cabinet. The surface of the fungal mycelia was gently scrapped using sterilized nichrome wire loop to liberate conidia from conidiophores and care was taken to take as little of the media or mycelia as possible. The suspension from individual isolates was transferred to correctly labelled sterile Eppendorf® tubes and mixed vigorously on a vortex mixer for 1 minute. The suspension contained both the spores and hyphal sections. The suspension was strained through a sterile syringe attached to a sterile

filter of pore diameter of 10 µm (Tisch Scientific) to filter out the hyphal fragments retaining only the spore suspension (Aberkane *et al*., 2002). Concentration of the spore suspension was adjusted to 10^6 spores/mL using a haemocytometer.

3.5 *In vitro* detached leaf pathogenicity test

The pathogenicity of all fungal isolates was tested on detached leaves of a healthy susceptible cassava cultivar TMS 60444. Healthy, fully, expanded leaves of cassava cultivar TMS 60444 were detached from the parent plants grown and maintained in a greenhouse within the Faculty of Science and Technology, University of Nairobi. The leaves were sterilized by first washing them with running tap water to remove dirt, followed by washing them with 70% ethanol for 30 seconds, and rinsing once with sterile distilled water. The leaves were then washed with 1.3% sodium hypochlorite for 2 minutes followed by three washing cycles with sterile distilled water. The waters from the last wash of each of the leaves was used for controls. Washing and surface sterilization of the leaf tissues were carried out under aseptic conditions in a Class II biosafety cabinet.

Sterile Petri dishes were lined with sterile paper towels. Six hundred microliters of sterile distilled water was aseptically added to the paper towels using a micropipette. This was to ensure that the relative humidity within the petri dishes exceeded 80%. Leaflets were cut from the previously sterilized leaves then randomly assigned to petri dishes using a random number generator (RANDOM.ORG - True Random Number Service). The leaflets were placed on sterile wire gauzes on top of the paper towels in the petri dishes such that the adaxial leaf surface faced the bottom of the plates. Using sterile inoculating needles, 5 small puncture holes were introduced to each leaf transversely along the leaf's equator. This was to allow infection of leaves by potential fungal pathogens that are unable to penetrate the cuticle during natural infection to overcome this barrier (Imathiu *et al*., 2009; Ntui *et al*., 2010).

Ten microliters of the prepared 10^6 spores/mL spore suspension was inoculated in each of the punctured sites on the leaves. The coverings of the petri dishes were also lined with sterile paper towels and soaked with 400 µL of sterile distilled water. The inoculated leaflets were then covered, sealed using parafilm and incubated at 23 ± 2 ºC for 7 days under a cycle of 12 hours of

daylight and 12 hours of darkness. Controls were similarly treated but the leaves of cassava TMS 60444 were inoculated with water from the last wash. A second set of controls was prepared by inoculating the leaves of cassava TMS 60444 with sterile distilled water.

Any inoculated leaves that showed characteristics that deviated from those displayed by the controls were deemed to have been inoculated with pathogenic fungal isolates. Typical symptoms considered were based on descriptions by Imathiu *et al*. (2009) which included: leaf discolorations, leaf colorations, necrosis and chlorosis on the affected leaves. The percentage area of the symptomatic region was estimated using image J (Schneider *et al*., 2012) and a 1 to 5 scale was developed with 1 being less than or equal to 20%; 2 being more than 20% but less than or equal to 40%; 3 being more than 40% but less than or equal 60%; 4 being more than 60% but less than or equal 80%; and 5 being greater than 80%.

Based on the distinct symptoms displayed on leaves after *in vitro* pathogenicity tests, representative pathogenic isolates of each of the symptom type were randomly selected using a random number generator (RANDOM.ORG - True Random Number Service). These selected pathogenic fungal isolates were inoculated on fresh PDA petri dishes from the previously prepared pure cultures and used for morpho-cultural characterization.

3.6 Preparation of single mycelial cultures of representative pathogenic isolates

The representative isolates that were confirmed to be pathogenic through *in vitro* pathogenicity assays were used to prepare pure cultures for morpho-cultural and molecular identification. A modification of the protocol described by Fei *et al*. (2019) was used. Mycelial suspensions were prepared as described in Section 3.3. Aseptically, in a Class II biosafety cabinet, the suspensions were each serially diluted to 10^{-6}. Using a sterile inoculating loop, a loopful of each of the isolates was streaked on freshly prepared PDA plates, correctly labelled and incubated at 23 ± 2 ºC for 48 hours. After 48 hours, a mycelial plug was cut from the outmost edge of the smallest colony on each plate and transferred to a new PDA plate and correctly labelled. These inoculated plates were incubated at 23 ± 2 ºC for 7 days and maintained at 4 ºC as stock culture for morpho-cultural and molecular characterisation.

3.7 Cultural and morphological identification of fungal pathogens

The pure stock cultures of single mycelium from representative pathogenic isolates were allowed to acclimatize to room temperature by sitting in a Class II biosafety cabinet for 30 minutes. From the stock cultures, mycelial plugs measuring approximately 2 mm by 2 mm were aseptically sub-cultured on fresh PDA plates using sterile inoculating needles and the cultures allowed to grow for 10 days under complete darkness at 23 ± 2 ºC. This was to give ample time for all the pathogenic isolates to sporulate. The pathogenic fungal isolates were characterized based on cultural and morphological characteristics. The cultures were examined for colony colour, colony margin, colony texture, pigmentation on PDA petri dishes and colony diameter (Sompong *et al.*, 2012) based on descriptions by Willey *et al*. (2008). The average colony diameter and the standard error of the mean (SE) were calculated based on three replications for each isolate. Similarly, the mean disease severity score and the SE were calculated based on symptoms of three symptomatic leaves.

Using a sterile inoculating needle, a mycelial mass was detached from the parent culture and transferred to a clean microscope slide. Lactophenol blue dye was added over the mycelial mass on the microscope slide dropwise using a Pasteur pipette until it was flooded. A cover slip was then placed over the mycelial mass and gently pressed to flatten the mycelial mass. Excess lactophenol blue was removed by touching the edge of the coverslip with absorbent paper. However, since some spores may be difficult to visualize in the backdrop of the mycelia, spore prints were also prepared for each of the pathogenic isolates. In this method, 2 – 3 drops of lactophenol blue was placed on a clean microscope slide. Using a pair of forceps, a clean microscope coverslip was placed on the fungal culture and pressed on lightly so that fungal spores could stick on to the coverslip (spore print). The coverslip was then placed on to the microscope slide with the lactophenol blue dye coming into contact with the face containing the spore print. The microscope slide was pressed gently and excess dye was drained by dubbing the edge of the coverslip with absorbent paper. The microscope slides were correctly labelled and viewed under a Leica compound microscope at power 100 objective lens magnification and micrographs taken. Taken together, the cultural and microscopic characteristics were used to assign the pathogenic isolates to known genera or species where possible using a fungal pictorial atlas (Pit and Hocking, 1997).

3.8 Molecular identification and characterization of fungal pathogens

3.8.1 Isolation of fungal genomic DNA

The pure stock cultures of single mycelium from representative pathogenic isolates were allowed to acclimatize to room temperature by sitting in a Class II biosafety cabinet for 30 minutes. From the stock cultures, mycelial plugs measuring approximately 2 mm by 2 mm were aseptically sub-cultured on fresh PDA plates using sterile inoculating needles and the cultures allowed to grow for 10 days under complete darkness at 23 ± 2 ºC. It is these cultures that were used for molecular characterisation.

After the 10 days of incubation, genomic DNA was extracted using a modification of the protocol described by Sangpueak *et al.* (2017). From each pathogenic isolate cultured, mycelia biomass approximately measuring 1cm by 1cm was aseptically cut. Care was taken to avoid taking PDA media. The mycelia were then aseptically transferred to a sterile microfuge tube. Eight hundred microliters of extraction buffer was added to each of the microfuge tubes. Using sterile microfuge pestles, the mycelia biomass was macerated. This was followed by homogenization using a vortex mixer for 1 minute. Five hundred and thirty seven microliters of the mixture was aseptically transferred to a sterile microfuge tube. To this, 3 µL of proteinase K and 60 µL of 10% SDS solution were added. The solution was again homogenized using a vortex mixer for 1 minute. This was followed by incubation in a water bath at 65 °C for thirty minutes. A hundred microliters of 5M NaCl solution was then added and mixed using a vortex mixer for 1 minute. Eighty microliters of CTAB-NaCl solution was then added and the mixture homogenized for 1 minute on a vortex mixer. This was followed by incubation in a water bath for 10 minutes at 65 °C. To this, 700 µL of chloroform-isoamyl-alcohol was added and centrifuged at 12000 rpm (Eppendorf MiniSpin® Plus centrifuge) for 15 minutes. The aqueous phase was transferred to a new sterile microfuge tube and care was taken not to transfer the interphase layer. The step proceeding from the addition of 700 µL of chloroform-isoamyl alcohol was repeated thrice until the interphase layer completely disappeared. The final aqueous phase was transferred to a sterile microfuge tube and to it 120 µL (0.6 of the sample volume) of absolute isopropanol at -20 °C was added and the mixture gently mixed by inverting the microfuge tube several times. The mixture was incubated at -20 °C for 1 hour to precipitate genomic DNA.

After this incubation, the microfuge tube with its contents was centrifuged at 14000 rpm (Eppendorf MiniSpin® Plus centrifuge) for 15 minutes to concentrate the precipitated DNA in a pellet at the bottom of the microfuge tube. The supernatant was discarded and the pellet dried by opening the microfuge tube and inverting it over a sterile paper towel for 10 minutes in a Class II biosafety cabinet. After this, 100 µL of 70% ethanol was added to the microfuge tube and centrifuged at 14000 rpm (Eppendorf MiniSpin® Plus centrifuge) for 15 minutes to wash the DNA pellet. The ethanol was removed using a sterile micropipette and the pellet dried by inverting the open microfuge tube over a sterile paper towel for 10 minutes inside a Class II biosafety cabinet. The steps of washing the DNA pellet and drying it was done twice. To the dried DNA pellet in the microfuge tube, 49.5 µL of sterile distilled water was added and left over night at 4 °C to dissolve the DNA. To the dissolved DNA, 0.5 µl of 1 mg/mL RNase A was added and incubated for 30 minutes in a water bath maintained at 37 °C to allow the RNase A hydrolyse any RNA present.

The integrity of the isolated DNA was confirmed by gel electrophoresis. Briefly, 0.8 g of agarose was added to 100 mL of 1× TAE buffer and heated in a microwave oven until boiling to ensure complete dissolving of the agarose. The agarose solution was allowed to cool to about 55 ºC then 1 µL of ethidium bromide solution was added and lightly swirled for homogenization. This was cast into an agarose gel and transfer to an electrophoresis tank filled with 1× TAE buffer. Ten microliters of each of the isolated DNA solution was carefully mixed with a DNA loading dye and loaded into separate wells in the agarose gel. One microliter of 1 kb GeneRuler ladder (Thermo Scientific batch number SM1333) was also loaded to the gel. The gel was allowed to run for 45 minutes at 90 volts and 300 amperes. The gel was then viewed in a UV transilluminator and photos taken by UVP Photodoc-It™.

3.8.2 Polymerase chain reaction and gel electrophoresis

The extracted genomic DNA was used for PCR amplification using universal internal transcribed spacer region (ITS) primers. The primer pair ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') was used for the PCR amplification as described by White *et al.* (1990). The master mix for the PCR reaction was prepared using GoTaq® G2 Hot Start Green Master Mix (catalogue number M742A) for 50 µl reactions as per the manufacturer's

recommendations. The PCR was carried out in Bio-Rad MJ Mini™ Gradient thermocycler (catalogue #PTC-1148) with the following conditions: initial denaturation was set at 94 ºC for 5 minutes, denaturation at 94ºC for 40 seconds. This was followed by annealing at 54 ºC for 40 seconds, then extension at 72 ºC for 40 seconds. The denaturation, annealing and extension cycle was repeated thirty five times. This was followed by a final extension step at 72 ºC for 5 minutes.

To assess whether the PCR was successful, the contents of the PCR tubes were visualized through gel electrophoresis. A 1.2% agarose gel was prepared by adding 1.2 grams of agarose in 100 mL of 1× TAE buffer then boiling in a microwave oven until all the agarose had dissolved. The agarose was allowed to cool to about 55 ºC then 1 µL of ethidium bromide solution was added. The contents were swirled to homogenize the ethidium bromide – agarose solution. The content was then carefully poured into a plastic casting mould. After the agarose gel has been cast, it was submerged into an electrophoresis tank filled with 1× TAE buffer. Five microliters of the contents of each PCR tube was transferred to the wells in the agarose gel. One microliter of 1 kb GeneRuler ladder (Thermo Scientific batch number SM1333) was also loaded to the gel. The electrophoresis was run using Bio-Rad PowerPac™ at 100 volts and current of 300 amperes for one and a half hours. The gel was then viewed in a UV transilluminator and photographs taken by UVP Photodoc-It™.

3.8.3 Sequence analysis and phylogenetic analysis

Cleaning and sequencing of the PCR amplicons was outsourced to the Institute of Tropical and Infectious Diseases (UNITID), University of Nairobi. The sequencing was done both in the 5' to 3' and the 3' to 5' directions using Sanger sequencing. The resultant sequence chromatograms were transferred to the BioEdit software version 7.2.5 for visualization and manual editing to obtain nucleotide sequences (Hall, 1999). Within BioEdit, the sequences were visualised and regions with poor quality sequences occurring at the beginning and at the end of the sequences were manually trimmed. Using the chromatograms as a guide, ambiguous nucleotides were manually edited to the correct nucleotides and those that could not be assigned a nucleotide were left with their assigned ambiguous nucleotide letter. Since sequencing was done in both the forward and reverse directions, each sample had two sequences. For each sample, its two edited sequences were used within BioEdit to obtain a single contiguous (contig) sequence using the

contig assembly program (Huang, 1992). The contig siquences were deposited in Genbank under accession numbers provided in the appendices section.

Each of the resultant contig sequences was then forwarded to Molecular Evolutionary Genetics Analysis (MEGA) software version 11.0.10 (Tamura *et al.*, 2021). For each sequence, a multiple sequence alignment was built within MEGA11. For this, a nucleotide Basic Local Alignment Search Tool (BLASTn) search was done using the web-extension found within the MEGA 11 environment with the following parameters: Nucleotide Collection (nr/nt) was chosen as the database to query, the query was optimized for highly similar sequences and the maximum number of target sequences was set to 5000 sequences. All other parameters within BLASTn were left in their default settings and values. From the results of the BLAST search, selected sequences were added to MEGA 11 for multiple sequence alignment. For a sequence to be included, it had to have an expected value of 0.0, a percentage identity above 90% and the accession length had to be within ± 100 bp of the query sequence and not more than 40 sequences were selected for each alignment.

Once an adequate number of sequences had been added, the sequences were aligned using the MUSCLE algorithm built in MEGA 11 (Edgar, 2004). The alignment was visually inspected for any sequences that were either too long or deviated too much from the other sequences. Such sequences were deleted and the alignment redone, this process was repeated until the resultant alignment was deemed to be satisfactory.

These alignments were used for phylogenetic analysis to create phylogenetic trees. Each alignment was individually assessed within MEGA 11 to find the best nucleotide substitution model to use in the construction of a phylogenetic tree the model selection algorithm was left with the default settings (Masatoshi and Sudhir, 2000). After the algorithm was allowed to run and the results displayed, the model appearing in the results with the least Bayesian information criterion (BIC) value was selected as the most appropriate substitution model for constructing a phylogenetic tree for that specific alignment. After the best model was determined, the aligned sequences were used to construct a phylogenetic tree by the neighbour joining method with 1000 bootstrap replications selected as the test of phylogeny (Saitou and Nei, 1987). All other settings and values were left in their default except for the model parameters which were replaced with

values provided by the substitution model. The resulting tree was exported as a Newick file to the FigTree software version 1.4.4 for visualization and editing.

3.9 Data analysis

All the statistical analyses was carried out using the R statistical software (R-Team, 2022) implemented within RStudio environment (Posit-Team, 2022). Chi-squared tests were carried out to detect statistical differences in the number of pathogenic isolates between the different counties of western Kenya. Graphical representations of these differences were created using the ggplot2 package implemented in RStudio (Wickham, 2016).

CHAPTER FOUR

RESULTS

4.1 Fungal pathogens isolated from cassava leaf samples

A total of 60 fungal isolates were obtained from 29 cassava leaf samples. The samples with their respective fungal isolates obtained are presented in Table 1A and 1B.

Table 1A: Details of fungal pathogens isolated from cassava leaves showing symptoms of brown leaf spot disease in Kakamega County

Leaf sample	County	Location	Village	Number of fungal isolates obtained	ID of fungal isolates	ID of pathogenic fungal isolates
S1	Kakamega	Mweere	Makunga	2	KKG-01A, KKG-1B	KKG-01A
S2	Kakamega	Mweere	Makunga	3	KKG-02A, KKG-02B, KKG-02C	KKG-02A, KKG-02B, KKG-02C
S3	Kakamega	Mweere	Makunga	3	KKG-03A, KKG-03B, KKG-03C	0
S4	Kakamega	Mweere	Makunga	5	KKG-04A, KKG-04B, KKG-04C, KKG-04D, KKG-04E	KKG-04A, KKG-04C, KKG-04D, KKG-04E
S5	Kakamega	Mweere	Makunga	1	KKG-05	0
S6	Kakamega	Mweere	Makunga	3	KKG-06A, KKG-06B, KKG-06C	KKG-06A, KKG-06B, KKG-06C
S7	Kakamega	Mweere	Makunga	2	KKG-07A, KKG-07B	0
S8	Kakamega	Khayimba	Ikoli	1	KKG-08	KKG-08
S9	Kakamega	Khayimba	Ikoli	3	KKG-09A, KKG-09B, KKG-09C	KKG-09B, KKG-09C
S10	Kakamega	Khayimba	Ikoli	10	KKG-10A, KKG-10B, KKG-10C, KKG-10D, KKG-10E, KKG-10F, KKG-10G, KKG-10H, KKG-10I, KKG-10J	KKG-10J, KKG-10H
S11	Kakamega	Khayimba	Ikoli	5	KKG-11A, KKG-11B, KKG-11C, KKG-11D, KKG-11E	KKG-11A, KKG-11B, KKG-11C, KKG-11D, KKG-11E

Key for fungal isolate: The first three letters indicate the county of origin, with 'KKG' representing Kakamega, 'BSI' representing Busia, and 'BGM' representing Bungoma, following this, a number is used to show the plant number from which the pathogen was isolated and lastly, a letter is used to indicate the number of fungal isolates from that specific plant. For example: KKG-11A' tells us that the pathogen was isolated from the 11th plant in Kakamega, and it's the first fungal isolate from that plant

Table 1B: Details of fungal pathogens isolated from cassava leaves showing symptoms of brown leaf spot disease in Busia and Bungoma Counties

Leaf sample	County	Location	Village	Number of fungal isolates obtained	ID of fungal isolates	ID of pathogenic fungal isolates
S12	Busia	Teso South	Otimong'	2	BSI-12A, BSI-12B	BSI-12A, BSI-12B
S13	Busia	Teso South	Otimong'	1	BSI-13	0
S14	Busia	Teso South	Otimong'	1	BSI-14	0
S15	Busia	Teso South	Otimong'	1	BSI-15	BSI-15
S16	Busia	Teso South	Otimong'	2	BSI-16A, BSI-16B	0
S17	Busia	Teso South	Ojude	1	BSI-17	BSI-17
S18	Busia	Alupe	Alupe	1	BSI-18	BSI-18
S19	Busia	Alupe	Alupe	0	0	0
S20	Busia	Teso South	Buteba	0	0	0
S21	Busia	Teso South	Adukumut	2	BSI-21A, BSI-21B	BSI-21B
S22	Busia	Teso South	Adukumut	0	0	0
S23	Busia	Teso South	Adukumut	2	BSI-23A, BSI-23B	BSI-23A, BSI-23B
S24	Bungoma	Bumula	Mateka	3	BGM-24A, BGM-24B, BGM-24C	BGM-24A, BGM-24C
S25	Bungoma	Bumula	Mateka	2	BGM-25A, BGM-25B	BGM-25A, BGM-25B
S26	Bungoma	Bumula	Bunambobi	2	BGM-26A, BGM-26B	BGM-26A, BGM-26B
S27	Bungoma	Bumula	Bunambobi	1	BGM-27	BGM-27
S28	Bungoma	Bumula	Bunambobi	0	0	0
S29	Bungoma	Bumula	Bunambobi	1	BGM-29	BGM-29

Key for fungal isolate: The first three letters indicate the county of origin, with 'KKG' representing Kakamega, 'BSI' representing Busia, and 'BGM' representing Bungoma, following this, a number is used to show the plant number from which the pathogen was isolated and lastly, a letter is used to indicate the number of fungal isolates from that specific plant. For example: KKG-11A' tells us that the pathogen was isolated from the 11th plant in Kakamega, and it's the first fungal isolate from that plant

The highest number of fungal isolates was obtained from leaf samples collected from Kakamega County followed by Busia and Bungoma Counties with 38, 13 and 8 fungal isolates, respectively (Figure 2).

4.2 Pathogenicity of the fungal isolates

Out of the 60 fungal isolates, 58.3% (n = 35) were confirmed to be pathogenic using *in vitro* pathogenicity assays. Kakamega County had the highest number of pathogenic isolates followed by Busia and Bungoma Counties with 57.1% (n = 20), 22.9% (n = 8) and 20% (n = 7) isolates, respectively (Figure 2). The differences were statistically significant (χ^2 =8.9714, p-value = 0.01127). The pathogenic fungal isolates produced four major distinct symptom types on the detached leaf assays with disease severity ranging from 1 to 5. The observed symptoms on the inoculated detached leaves included brown colorations on the leaves, localised chlorosis of the leaves, darkening of major leaf veins and brown rings around the points of inoculation (Figure 3). Table 2A and Table 2B represent the symptoms that were displayed by the pathogenic fungal isolates and their disease severity scores. Out of the 35 pathogenic isolates, 13 were randomly selected for subsequent morphological and genetic characterization based on the distinct symptoms on leaves based on *in vitro* pathogenicity tests.

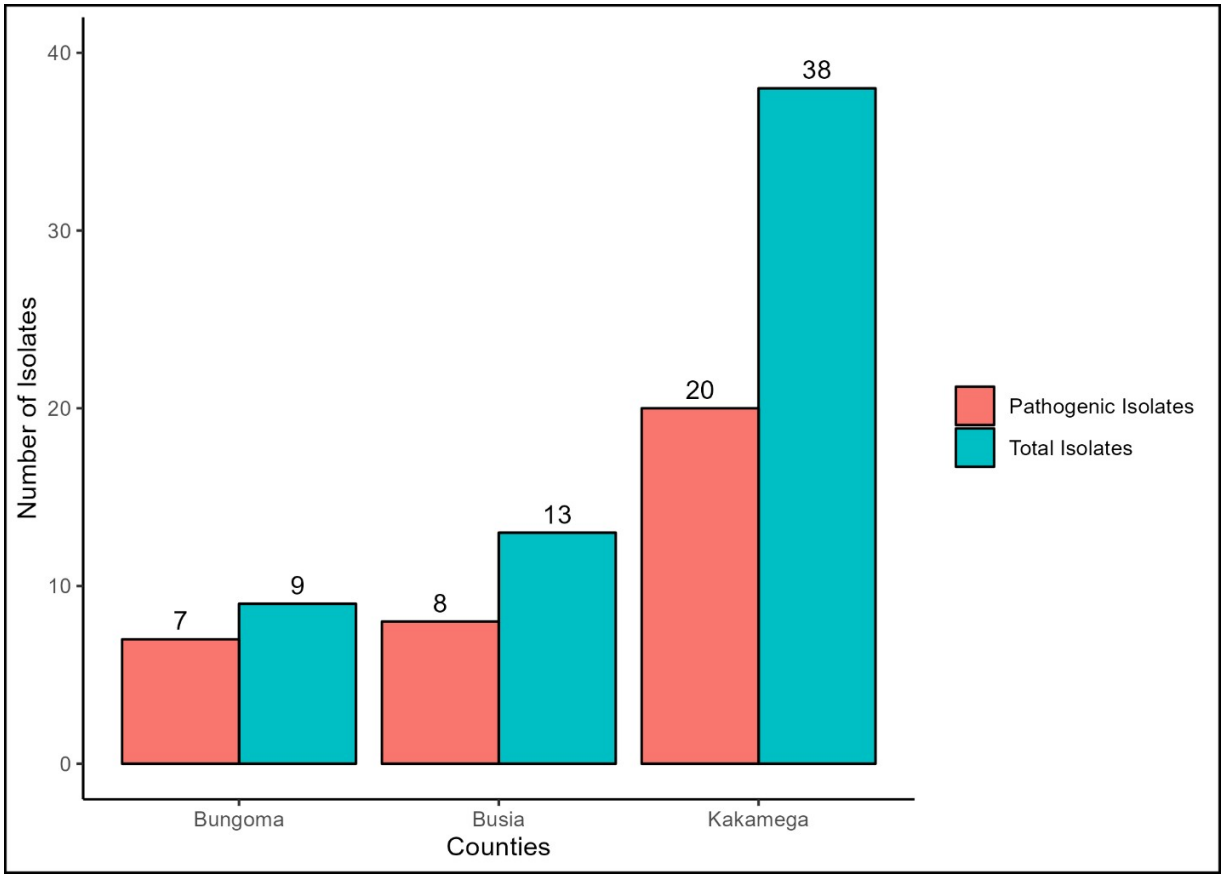

Figure 2: Number of fungal isolates and pathogenic isolates associated with cassava brown leaf spot disease from symptomatic cassava leaf samples collected from Kakamega, Bungoma, and Busia counties in Kenya

Table 2A: Disease severity scores and symptoms displayed from the detached leaf pathogenicity assays

ID of Pathogenic Isolate	Severity score	Symptoms
KKG-04E	2 ± 0.58	Displayed dark brown coloration in the middle third of the leaflet about 2mm from the point of inoculation, localized darkened veins and mid rib near the point if inoculation. Throughout the leaflet, areas of faint chlorosis were also visible.
KKG-06C	5 ± 0	
KKG-09B	3.75±0.63	
KKG-11A	3.5 ± 0.65	
KKG-11B	3 ± 0.41	
KKG-11C	3 ± 0.41	
KKG-11D	3.75±0.48	
KKG-11E	3.5 ±0.96	
BGM-29	5 ± 0.0	
BSI-11A	5 ± 0.6	
BSI-12A	1 ± 0	Leaves displayed irregular chlorotic regions that ran parallel on either side of the midrib, dark diffuse rings around the points of inoculation
BSI-018	5 ± 0	
KKG-06A	3.75±0.5	
KKG-06B	4±0.41	Darkened leaf veins near the point of inoculation.
BGM-26B	5 ± 0	
BGM-24C	2.75±0.63	
BGM-027	3±0.91	
KKG-04A	2 ± 0.41	

Table 2B: Disease severity scores and symptoms displayed from the detached leaf pathogenicity assays

ID of Pathogenic Isolate	Severity score	Symptoms
BSI-21B	5 ± 0	The leaves presented uneven chlorosis throughout the leaves. There was darkening of the major leaf veins with the midrib displaying the most darkening and in some leaves, the region surrounding the midrib also displayed darkening
BSI-015	2 ± 0.58	
BSI-23A	3 ± 0.41	
KKG-04D	3 ± 0.41	
KKG-01A	2.75±0.48	
BSI-017	3.25±0.48	
BSI-23B	3±0.71	
BGM-24A	2.5±0.5	
BGM-26A	4 ± 0.41	The leaves presented uneven chlorosis throughout the leaves. There was darkening of the major leaf veins with the midrib displaying the most darkening and in some leaves, the region surrounding the midrib also displayed darkening
KKG-10H	2±0.71	
KKG-10J	1 ± 0	
KKG-04C	1 ± 0	Displayed faint brown rings around the point of inoculation
KKG-9C	2 ± 0.58	
KKG-02B	2.75±0.25	
KKG-02C	1.25±0.25	
KKG-2A	1 ± 0	
KKG-08	1 ± 0	

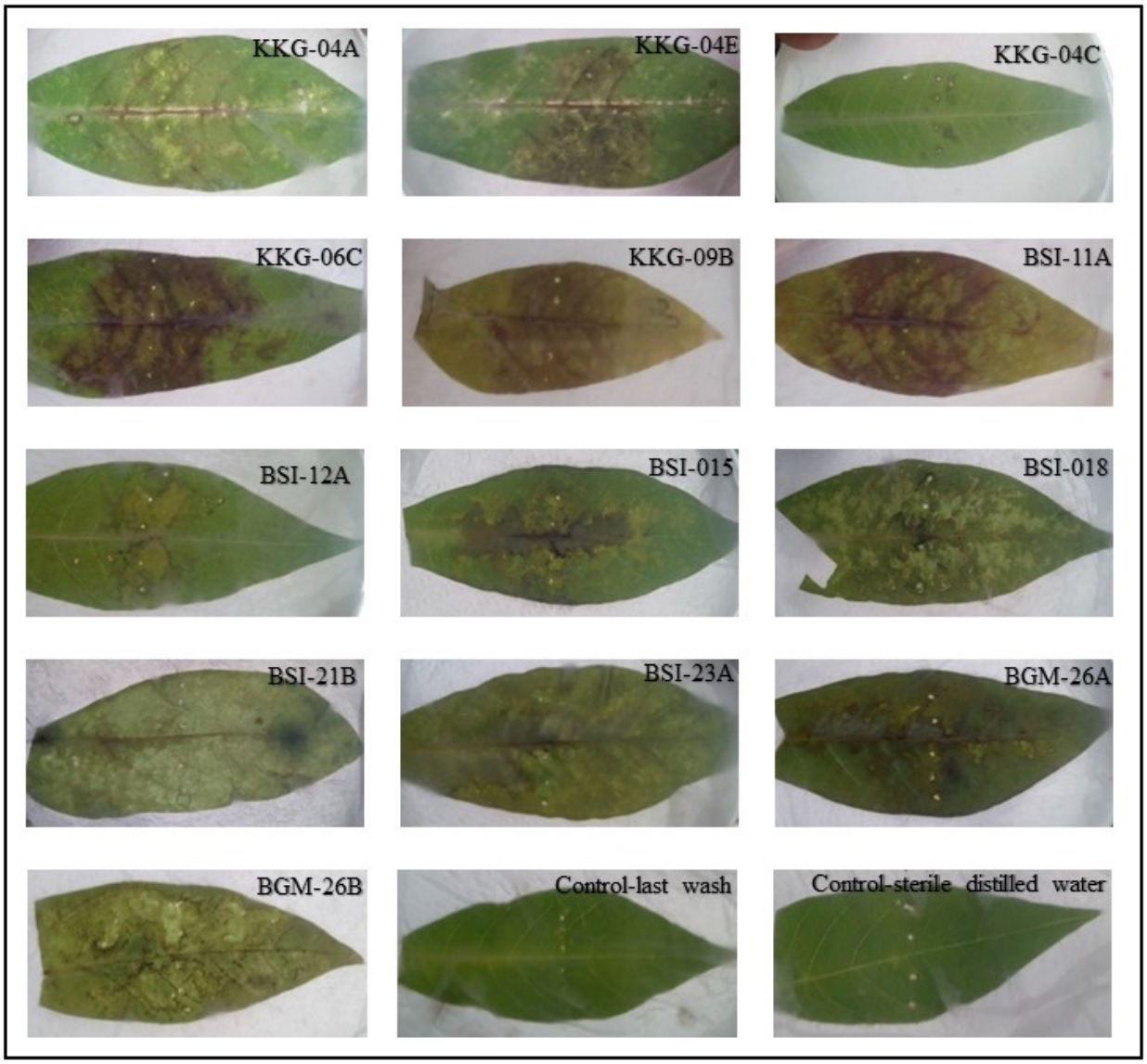

Figure 3**:** Symptoms on cassava leaves caused by representative pathogenic fungal isolates from the detached leaf pathogenicity assay after 7 days of incubation

The leaves were inoculated with fungal pathogens *in vitro*, and the resulting disease symptoms are visible in the images. In addition to the infected leaves, two control leaves were included: one inoculated with sterile distilled water and the other with distilled water from the last wash. The comparison between the control leaves and the infected leaves allows for a clear visualization of the symptoms associated with cassava BLS

4.3 Morpho-cultural characterization of representative pathogenic isolates

The 13 different pathogenic fungal isolates causing cassava BLS displayed varied morpho-cultural characteristics (Tables 3A, 3B and 3C). Isolates KKG-04A, KKG-04E, KKG-09B and BSI-12A showed similar morpho-cultural characteristics (Figure 4). The colonies of these isolates on PDA were orange-pink in colour, flat and granular in texture and the reverse plates displayed darker shades of orange-pink. For isolates KKG-04E and BSI-12A, concentric rings were observed both on the colony surface and in the reverse side of the plate. The conidiophores were simple in structure and bore a mass of conidia at the apex. The conidia were formed, with one conidium below the preceding and offset from the hyphal axis and produced characteristic zigzag chains. The conidia were loosely attached to the conidiophore. The conidia were pyriform in shape and each had one transverse septum. The walls of the conidia were thin and smooth. The isolates reported in Figure 4 were identified as *Trichothecium* spp.

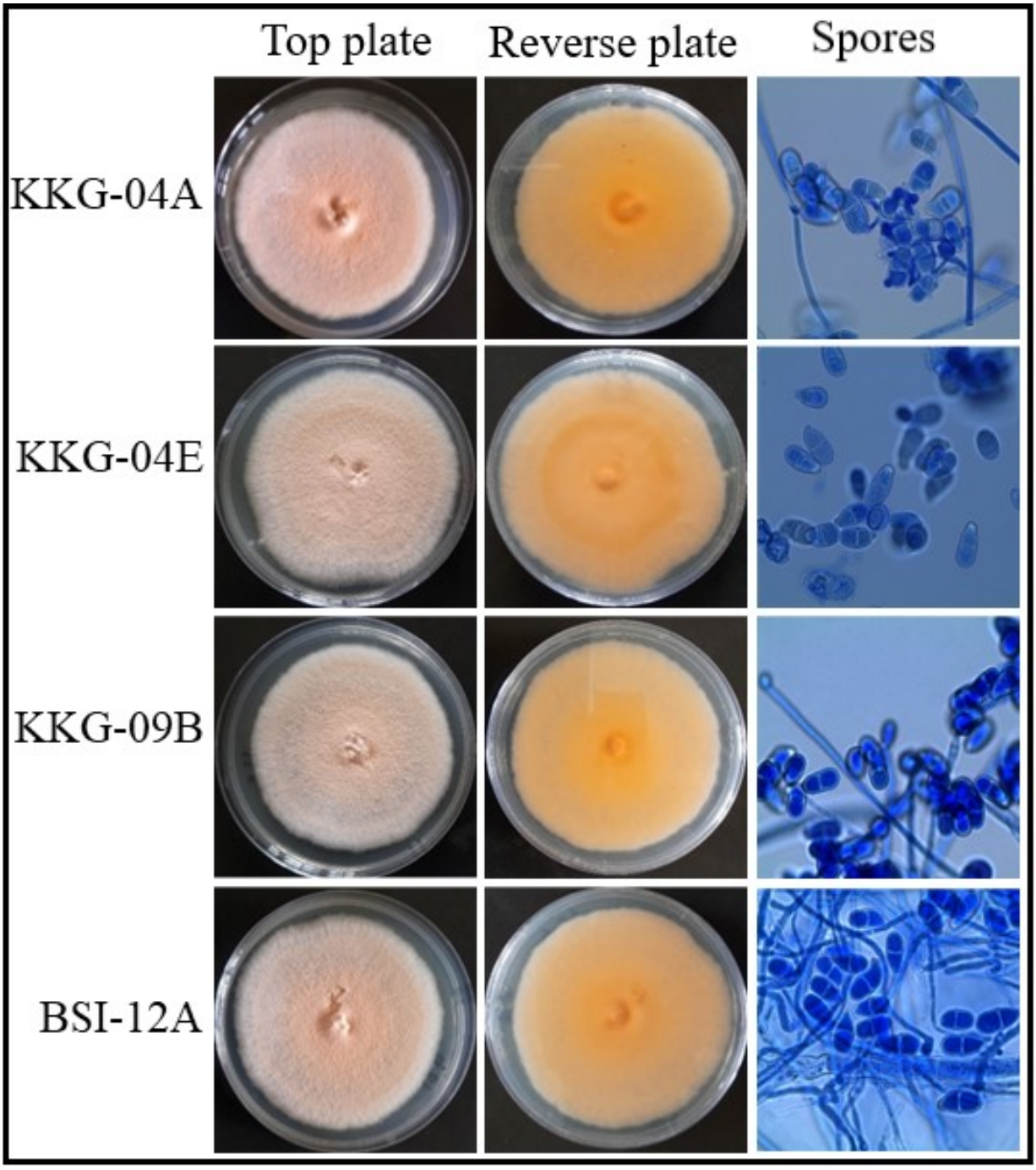

Figure 4: Morpho-cultural characteristics of isolates KKG-04A, KKG-04E, KKG-09B and BSI-12A from cassava leaves showing symptoms of brown leaf spot disease

These are *Trichothecium* spp. isolates associated with cassava brown leaf spot disease. Each row from left to right: Views of the top and bottom of representative isolates grown on potato dextrose agar; and spores as observed with X100 objective lens magnification.

Isolates BSI-018, BSI-23A, BGM-26A and BGM-26B showed similar morpho-cultural characteristics (Figure 5). The mycelial network of the fungal cultures grew extensively on PDA plates, with the hyphae elongating towards the lid of the Petri dish particularly for mature mycelia located at the centre of the culture. The mycelia were white in colour and grew more densely close to the centre of the colony displaying umbonate elevation. This was observed for isolates BSI-018, BGM-26A and BGM-26B with the exception of BSI-23A which presented raised elevation accompanied by significant invaginations into the PDA media. The colonies for isolates BSI-23A and BGM-26B were irregular in shape while BGM-26A and BSI-018 had a circular shape. The margins of the colonies for isolates BSI-018, BGM-26A and BGM-26B were undulate except for isolate BSI-23A which displayed lobate margins with the lobes appearing between successive invaginations. The reverse plate revealed that the colonies for isolates BSI-018, BGM-26A and BGM-26B were a dark shade of cream except for isolate BSI-23A that displayed a light shade of cream. Moreover, the reverse plate view showed that the colonies for isolates BSI-018, BGM-26A and BGM-26B slightly invaginated into the growth media. Macroconidia were observed in the colonies for all the four isolates. The macroconidia were slightly curved in a crescent shape with tapering ends. The macroconidia had at least three septa per conidia; while the microconidia had only one transverse septum (Figure 5). The isolates reported in Figure 5 were identified as *Fusarium* spp.

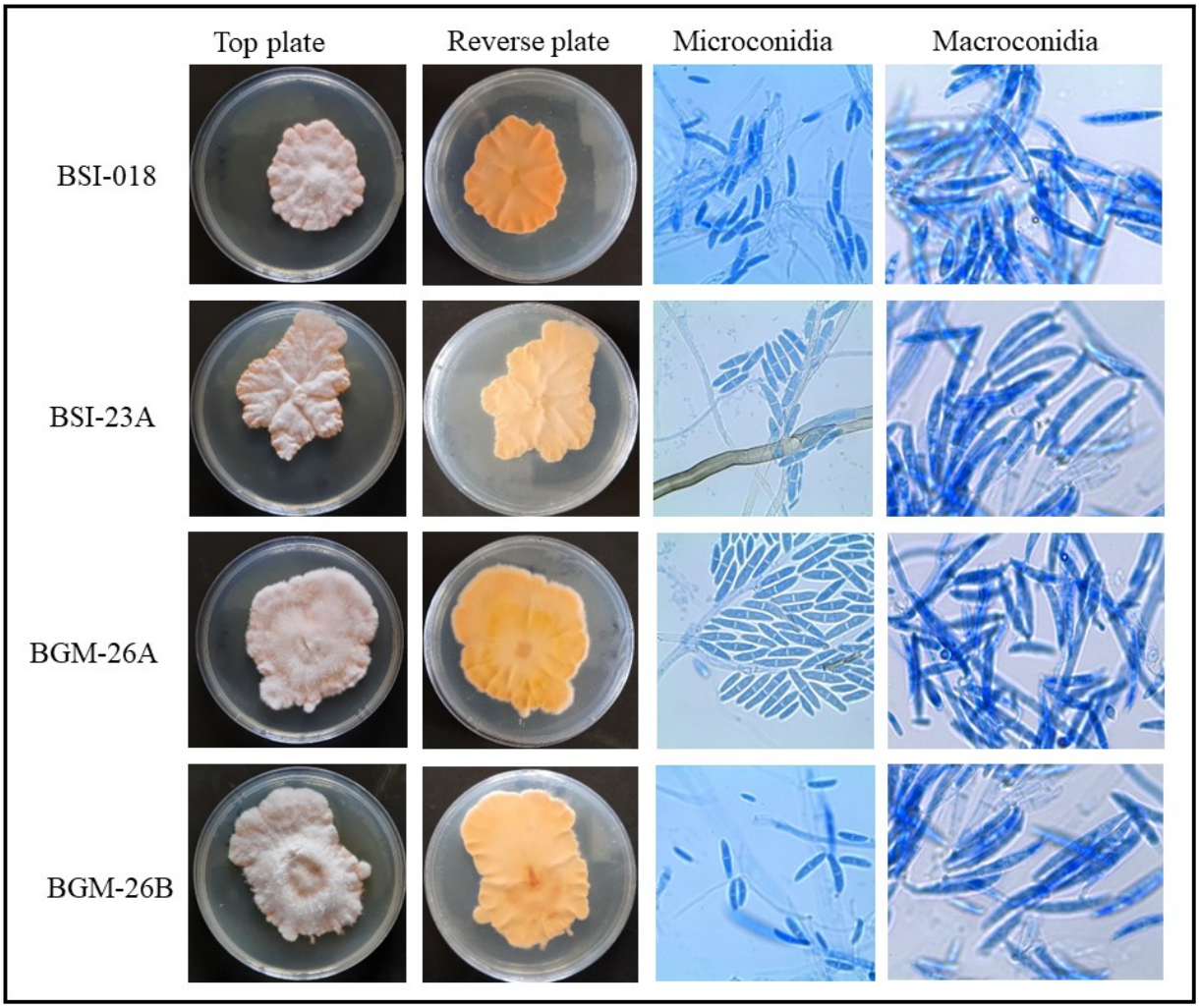

Figure 5: Morpho-cultural characteristics of isolates BSI-018, BSI-23A, BGM-26A and BGM-26B from cassava leaves showing symptoms of brown leaf spot disease

These are *Fusarium* spp. isolates. associated with cassava brown leaf spot disease. Each row from left to right: Views of the top and bottom of representative isolates grown on potato dextrose agar; and microconidia and macroconidia as observed with X100 objective lens magnification

Three isolates namely BSI-015, KKG-06C and BSI-015 displayed similar morpho-cultural characteristics. The isolates produced white and floccose mycelia (Figure 6). Isolate BSI-015 had entire margins and displayed raised elevation with slight invaginations into the PDA media. The reverse plate view revealed concentric rings with dark brown rings near the centre of the culture. Isolate KKG-06C had undulate margins, flat elevation and slight invaginations into the culture media. Isolate KKG-04C has filamentous margin with an orange-cream reverse plate. The conidia of isolates BSI-015, KKG-06C and BSI-015 were hyaline, formed singly and were non-septate. Additionally the conidia were fusiform to cylindrical with rounded ends (Figure 6). The isolates reported in Figure 6 were identified as *Colletotrichum* spp.

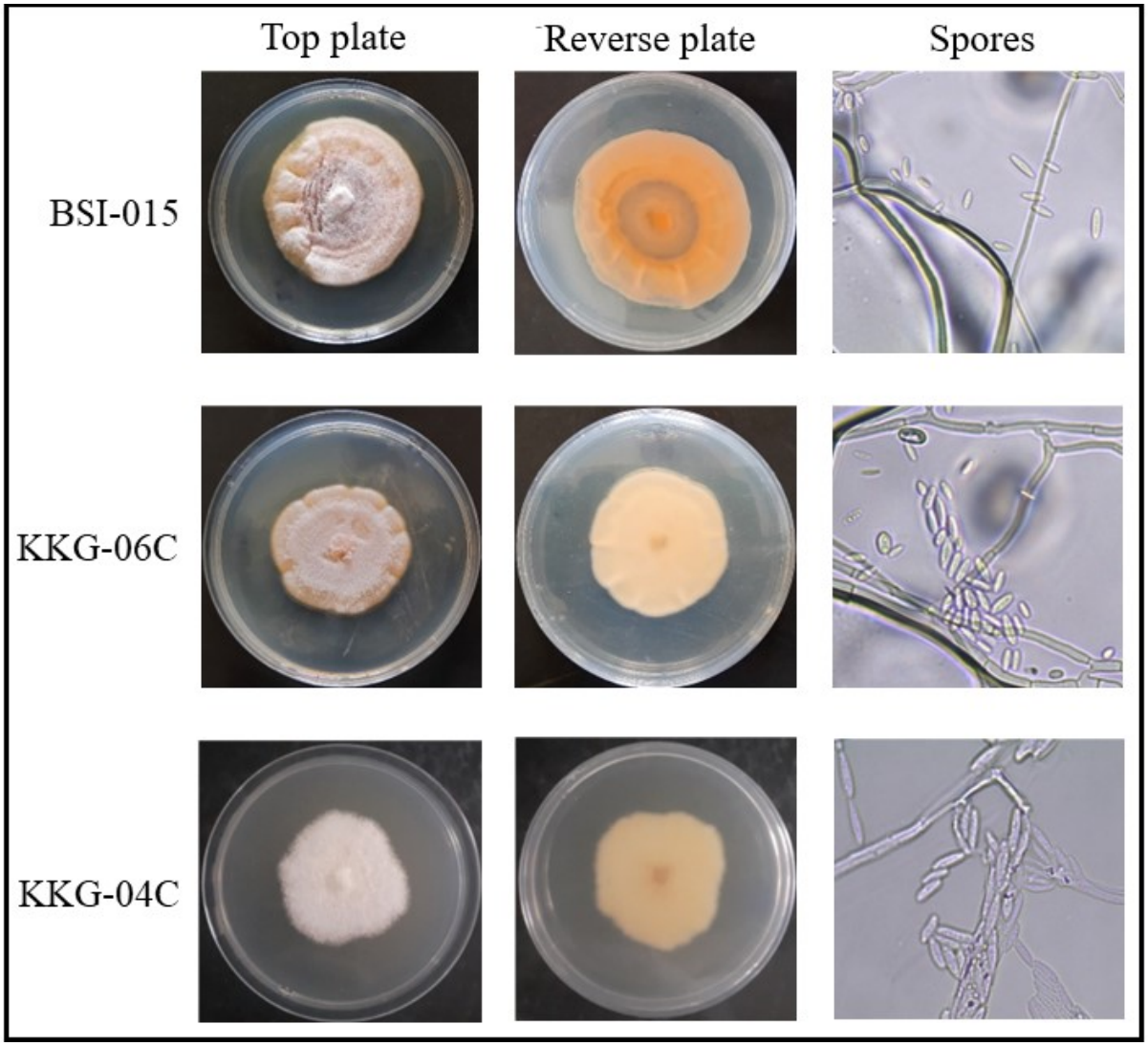

Figure 6: Morpho-cultural characteristics of isolates BSI-015, KKG-06C and KKG-04C associated with cassava brown leaf spot disease

These are *Colletotrichum* spp. isolates associated with cassava brown leaf spot disease. Each row from left to right: Views of the top and bottom of representative isolates grown on potato dextrose agar; and spores as observed with X100 objective lens magnification.

Isolate BSI-11A had an umbonate elevation and a central region of tuft white mycelia of higher density than the outer regions (Figure 7). The growth rate was rapid for this isolate. The underside of the plate was tan in colour with the darkest portion being at the central region near the high mycelia island. The conidia were ellipsoidal or irregular in shape with most of these having one septum (Figure 7). The isolate reported in Figure 7 was identified as *Diaporthe* spp.

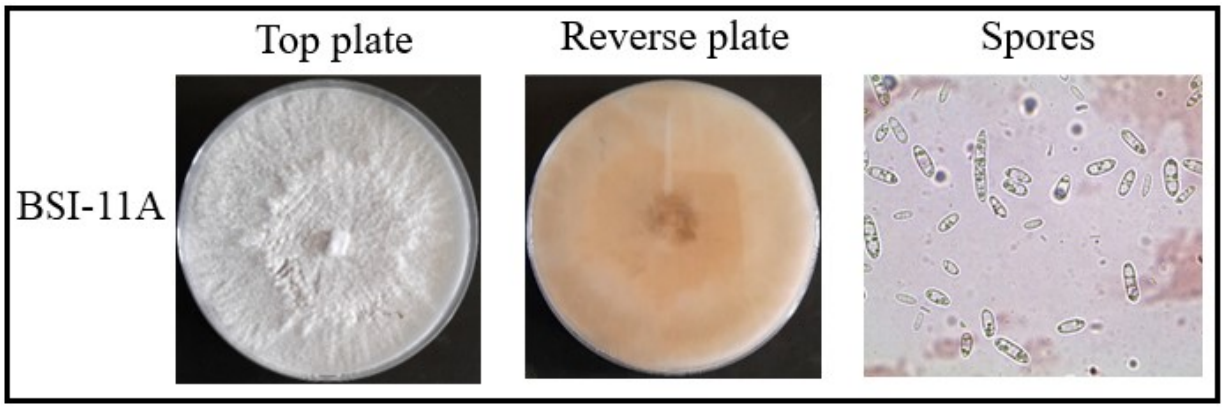

Figure 7: Morpho-cultural characteristics of isolate BSI-11A isolated from cassava leaves showing symptoms of cassava brown leaf spot disease

This is an isolate of *Diaporthe* spp. associated with cassava brown leaf spot disease. The row from left to right: Views of the top and bottom of representative isolates grown on potato dextrose agar; and spores as observed with X100 objective lens magnification

Isolate BSI-21B showed the following characteristics: the top plate view revealed that the isolate was roughly circular in shape on the PDA plate, however, its margins though filamentous, were slightly undulate. The isolate had a raised elevation but it was crateriform with the outer regions having younger mycelia being more raised and numerous. The outer mycelia were white in colour and gradually changed into grey towards the centre of the colony. Concentric rings were observed from the top plate view. The reverse plate view revealed the same margin patterns and the concentric rings were brown. The conidia were fusiod in shape and a slightly protruding hilum where the conidia attached on the conidiophore. The conidia occurred in whorl at the tips of the conidiophore. The conidia had between 4 and 7 cells each but the majority had 7 cells. The conidia were dark brown in colour and were numerous (Figure 8). The isolate reported in Figure 8 was identified as *Bipolaris* spp.

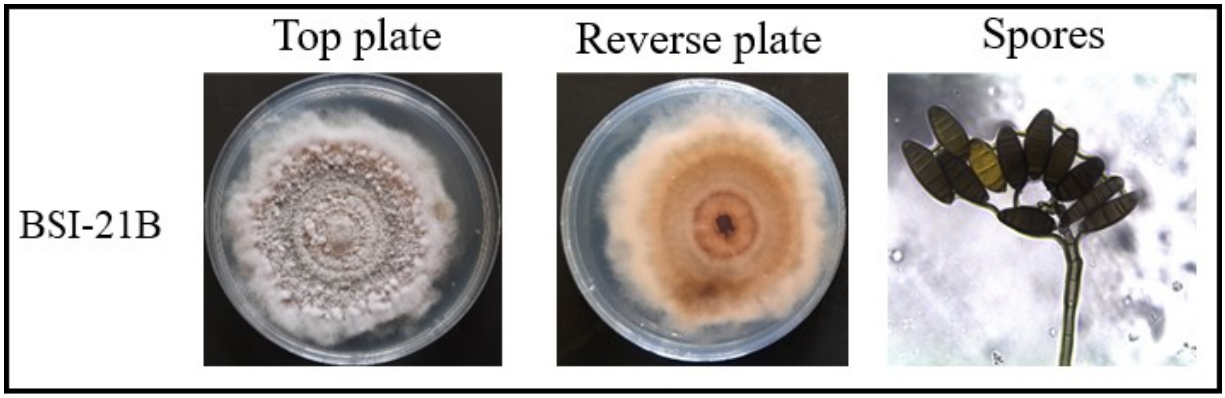

Figure 8: Distinct morpho-cultural traits exhibited by isolate BSI-21B isolated from cassava leaves in western Kenya that showed symptoms of cassava brown leaf spot disease

This is an isolate of *Bipolaris* spp. associated with cassava brown leaf spot disease. The row from left to right: Views of the top and bottom of representative isolates grown on potato dextrose agar; and spores as observed with X100 objective lens magnification.

Table 3A:Morpho-cultural characteristics of pathogenic fungal isolates from cassava leaves sampled from western Kenya showing symptoms of brown leaf spot disease

Isolate	Origin (County)	Morpho-cultural characteristics			
		Surface	Underside	Diameter (mm)	Conidia
KKG-04A	Kakamega	Homogeneously orange-pink in colour, flat, granular in texture, concentric rings barely visible	Light orange in color, a single concentric ring near the center of the culture	71 ± 1.2	Loosely attached to conidiophores, pyriform in shape, single transverse septum, smooth walled.
KKG-04E	Kakamega	Homogeneously orange-pink in colour, flat, granular in texture, concentric rings clearly visible	Light orange in color, several single concentric rings of deep orange color	75 ± 1.4	Loosely attached to conidiophores, appear at the apex of conidiophores pyriform in shape, single transverse septum, smooth walled
BSI-12A	Busia	Homogeneously orange-pink in colour, flat, granular in texture, concentric rings clearly visible	Light orange in color, a single concentric ring near the center of the culture, intense orange color near the center of culture.	69 ± 0.4	Loosely attached to conidiophores, pyriform in shape, single transverse septum, smooth walled.
KKG-09B	Kakamega	Homogeneously orange-pink in colour, flat, granilar in texture, concentric rings bearly visible	Light orange in color, several single ring of deep orange color near center of culture	72 ± 1.1	Loosely attached to conidiophores, pyriform in shape, single transverse septum, smooth walled.
BSI-018	Busia	Mycelia were tall regularly reaching the covering of the Petri dish, white in colour, grew more densely close to the centre of the colony, undulate margins	Dark shade of cream in colour, slight invaginations into the PDA	47 ± 2.4	Microconidia were rare, conidia were spindle-like in chape and slightly crescent in shape, multi-septate
BSI-23A	Busia	Moderately aerial, white in colour, raised elevation, lobate margins, irregular in shape, visible invaginations	Light shade of cream in colour, deep invaginations into the PDA between lobes	62 ± 0.8	Microconidia were rare, conidia were spindle-like in shape and slightly crescent, one transverse septum

The values include colony diameters ± standard errors of the mean

Table 3B: Morpho-cultural characteristics of pathogenic fungal isolates from cassava leaves sampled from western Kenya showing symptoms of brown leaf spot disease

Isolate	Origin (County)	Morpho-cultural characteristics			
		Surface	Underside	Diameter(mm)	Conidia
BGM-26B	Bungoma	Mycelia were tall regularly reaching the covering of the Petri dish, white in colour, grew more densely close to the centre of the colony, undulate margins	Dark shade of cream in colour, slight invaginations into the PDA	62 ± 1.8	Microconidia were rare, conidia were spindle-like in shape and slightly crescent, one transverse septum
BSI-015	Busia	Mycelia that was white and floccose, slight invaginations, margin was entire, concentric rings were visible	Brown near the center, light cream near the margins, skight invaginations concentric rings of brown and orange colours.	58 ± 0.4	Hyaline, cylindrical in shape with rounded ends, conidia appeared singly.
KKG-06C	Kakamega	Mycelia that was white and floccose, slight invaginations near the margins, margin was entire, concentric rings were visible	Evenly light cream in colour, shallow invagination.	59 ± 0.4	Hyaline, cylindrical in shape with rounded ends, conidia appeared singly.
KKG-04C	Kakamega	Mycelia that was white and floccose, slight invaginations near the margins, margin was entire, concentric rings were visible	Evenly light cream in colour.	43 ± 1.5	Hyaline, cylindrical in shape with rounded ends, conidia appeared singly
BSI-11A	Busia	Umbonate elevation, grew rapid growth	Tan in colour, darkest portion being at the central region.	90 ± 0.0	Ellipsoidal, or irregular in shape, single transverse septum

The values include colony diameters ± standard errors of the mean

Table 3C: Morpho-cultural characteristics of pathogenic fungal isolates from cassava leaves sampled from western Kenya showing symptoms of brown leaf spot disease

Isolate	Origin (County)	Morpho-cultural characteristics			
		Surface	Underside	Diameter (mm)	Conidia
BGM-26A	Bungoma	Mycelia were tall regularly reaching the covering of the Petri dish, white in colour, grew more densely close to the centre of the colony, undulate margins	dark shade of cream in colour, slight invaginations into the PDA, orange concentric rings	65 ± 3.7	Microconidia were rare, conidia were spindle-like in shape and slightly crescent, one transverse septum.
BSI-21B	Busia	Margins though filamentous and slightly undulate, crateriform elevation, grey-white in colour, concentric rings visible, granular near the centre	Concentric rings were coloured in different shades of brown	75 ± 0.4	Conidia were fusiod, slightly protruding hilum, conidia occurred in whorls at tip of conidiophores, 3 – 7 cells, numerous

The values include colony diameters ± standard errors of the mean

4.4 Molecular characteristics of fungal isolates

4.4.1 Genomic DNA isolation and PCR amplification

Fungal genomic DNA was successfully extracted from the pathogenic isolates and confirmed by agarose gel electrophoresis which showed high molecular weight DNA bands. The fungal genomic DNA of all the 13 pathogenic isolates was successfully amplified and produced the expected amplicons of ≈600 bp (Figure 9).

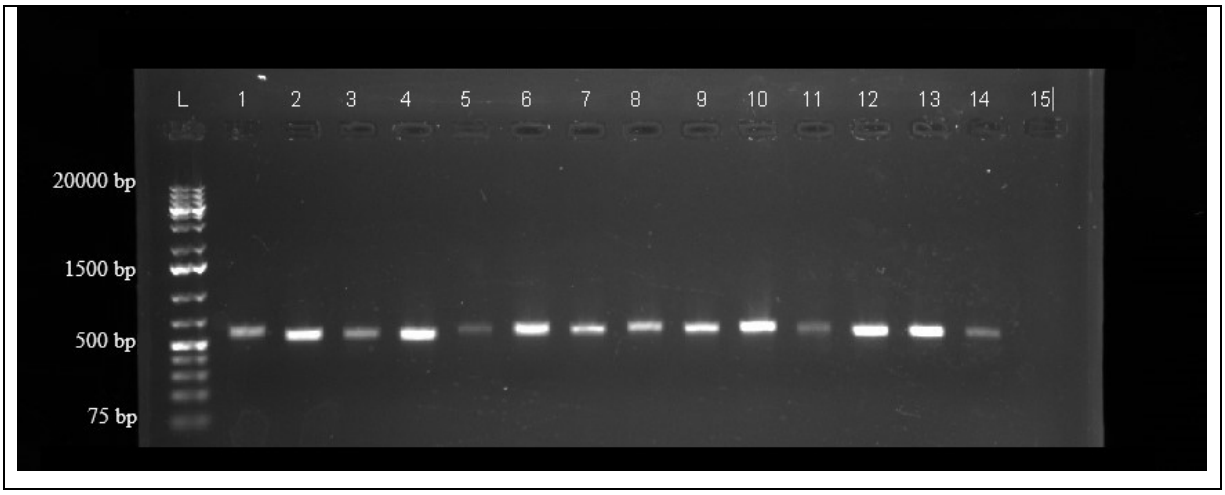

Figure 9: Image of agarose gel electrophoresis showing PCR amplification of fungal genomic DNA using ITS1/4

Amplicons of ≈ 600 bp for all the pathogenic isolates are amplified. 1 = KKG-04A, 2 = KKG-04C, 3 = KKG-04E, 4 = KKG-6C, 5 = KKG-9B, 6 = BSI-11A, 7 = BSI-12A, 8 = BSI-015, 9 = BSI-018, 10 = BSI-21B, 11 = BSI-23A, 12 = BGM-26A, 13 = BGM-26B, 14 = positive control. 15 = negative control

4.4.2 Nucleotide composition

The nucleotide composition of the contig sequences revealed varying sequence lengths which ranged from 511 to 649 bp, with an average of 598.3 bp and a standard error (SE) of 11.4 bp. The nucleotide base with the highest frequency was cytosine (C) at 27.2%, followed by adenine (A), guanine (G) and thymine (T) at 25.3%, 25.1% and 22.4% respectively. The pyrimidine bases (cytosine and thymine) displayed greater deviations in their means than the purine bases (adenine and guanine) (Table 4).

Table 4: Nucleotide composition of the analysed pathogenic isolates showing the length of the isolate sequence, the nucleotide frequencies and the percentage proportion of each nucleotide

Isolate	Length (bp)	Adenine		Cytosine		Guanine		Thymine	
		Freq	%	Freq	%	Freq	%	Freq	%
KKG-04A	644	156	24.2	195	30.3	173	26.9	120	18.6
KKG-04C	599	143	23.9	156	26.0	151	25.2	149	24.9
KKG-04E	511	116	22.7	170	33.3	136	26.6	89	17.4
KKG-06C	609	146	24.0	157	25.8	156	25.6	150	24.6
KKG-09B	649	158	24.3	195	30.0	173	26.7	123	19.0
BSI-11A	612	156	25.5	152	24.8	159	26.0	145	23.7
BSI-12A	636	154	24.2	194	30.5	168	26.4	120	18.9
BSI-015	626	153	24.4	168	26.8	161	25.7	144	23.0
BSI-018	581	159	27.4	151	26.0	136	23.4	135	23.2
BSI-21B	615	159	25.9	136	22.1	141	22.9	179	29.1
BSI-23A	533	146	27.4	141	26.5	126	23.6	120	22.5
BGM-26A	580	160	27.6	149	25.7	135	23.3	136	23.4
BGM-26B	583	161	27.6	149	25.6	137	23.5	136	23.3
Mean	598.3	151.3	25.3	162.5	27.2	150.2	25.1	134.3	22.40
SE	11.4	3.4	0.5	5.7	0.8	4.4	0.4	5.9	0.9

Freq = nucleotide frequency; SE = standard error of the mean

4.4.3 Identification of fungal pathogens based on BLAST analysis

BLASTn analysis was used in the identification of the fungal pathogens in cassava BLS. Based on BLASTn analysis, the cleaned sequences for the isolates KKG-04A, KKG-04E, KKG-09B and BSI-12A were identified as *Trichothecium roseum*. The four isolates had percentage identities of more than 99.5% with known sequences of *T. roseum* in GenBank database at an expected E value of 0.00 and query coverage of more than 99%. Isolates KKG-04A, KKG-09B and BSI-12A had high homology to a sequence of *T. roseum* with the accession number of MW478332 while isolate KKG-04E was homologous to *T. roseum* sequence of accession number MN882763 (Table 5).

BLASTn analysis identified isolates BSI-018, BSI-23A, BGM-26A and BGM-26B as *Fusarium equiseti.* Each of the isolates showed high similarity to known sequences of *F. equiseti* in GenBank database. Isolate BSI-018 had a percentage identity of 100% with a query cover of 100% to *F. equiseti* of accession number MH578585 isolated from diseased *Citrus reticulate*. Isolate BSI-23A had a percentage identity of 99.44% with a query coverage of 99% to a known member of *F. equiseti* with the accession number MN202708 that was identified as an endophyte of crucifer. Isolate BGM-26A had a percentage identity of 100% and a query cover of 100% with *F. equiseti* accession number MT560323 that was isolated from potato rot. Isolate BGM-26B showed similarity with *F. equiseti* of accession number MT560323 at 99.83% with a query cover of 100% (Table 5).

BLASTn analysis for isolates KKG-04C, KKG-06C and BSI-015 classified these isolates to the genus *Colletotrichum.* Isolates KKG-04C had close homology to a known sequence of *Colletotrichum siamense* of accession numbers MW578479 with query coverage and percentage identity of 100%. Isolate KKG-06C had a percentage identity of 99.67% at 100% query coverage to KU642471. The BLASTn analysis revealed that isolate BSI-015 was more closely related to *Colletotrichum* sp. of accession number MF076603 isolated as an endophyte of *Aristolochia triangularis* with a percentage identity of 99.68% and query cover of 99% (Table 5).

BLASTn analysis for isolate BSI-11A suggested that it belonged to *Diaporthe phaseloreum* at 99.83% identity with the closest homology to a sequence of accession number KT964567 with query coverage of 98% (Table 5). BLASTn analysis showed that Isolate BSI-21 had a query cover of 100% and percentage identity of 99.84% to a known sequence of *Bipolaris* spp. at an E-value of zero. The BLASTn analysis could not classify this sequence down to the species level (Table 5).

Table 5: Parameters and fungal pathogens identification based on BLASTn analysis comparing sequences from this study to homologous sequences from GenBank and the presumptive species the pathogen belongs to

Isolate	Accession (This study)	Species Name	Max Score	Total Score	Query Cover (%)	E value	Per. ident	Acc. Len	Accession (Reference)
KKG-04A	OP002076	*T. roseum*	1175	1175	100	0	99.5	1520	MW478332
KKG-04E	OP002077	*T. roseum*	942	942	99	0	100	613	MN882763
KKG-09B	OP002078	*T. roseum*	1195	1195	99	0	100	1520	MW478332
BSI-12A	OP002079	*T. roseum*	1175	1175	100	0	100	1520	MW478332
BSI-018	OP002080	*F. equiseti*	1066	1066	100	0	100	586	MH578585
BSI-23A	OP002081	*F. equiseti*	966	966	99	0	99.4	542	MN202708
BGM-26A	OP002082	*F. equiseti*	1072	1072	100	0	100	585	MT560323
BGM-26B	OP002083	*F. equiseti*	1070	1070	100	0	99.8	585	MT560323
KKG-04C	OP002084	*C. siamense*	1107	1107	100	0	100	612	MW578479
KKG-06C	OP002085	*C. siamense*	1114	1114	100	0	99.7	612	KU642471
BSI-015	OP002086	*Colletotrichum* spp.	1142	1142	99	0	99.7	975	MF076603
BSI-11A	OP002087	*D. phaseolorum*	1109	1109	98	0	99.8	603	KT964567
BSI-21B	OP002088	*Bipolaris* spp.	1131	1131	100	0	99.8	620	MG182688

Max Score = Maximum alignment score between query sequence and subject sequence

E value = Expected value

Per. indent = Percentage identity

Acc. Len = Accession length of homologous GenBank sequences

4.4.3 Phylogenetic analysis of representative pathogenic isolates

The phylogenetic analysis placed isolate KKG-04A in a monophyletic clade with *T. roseum* isolate of accession number MK522521. Isolate KKG-04E formed a monophyletic clade with a *T. roseum* strain of accession number KP317992 isolated from diseased beans. Isolate KKG-09B and isolate BSI-12A were clustered in a monophyletic clade that included *T. roseum* isolates from the GenBank database with strong bootstrap support value of 73.25%. This *T. roseum* clade

had bootstrap support of 99.98% (Figure 10). The *T. roseum* clade was paraphyletic to a clade that had sequences of *Hypocreales* spp. supported by a bootstrap value of 99.4%. These two clades formed a monophyletic clades that had close homology to a clade that had *T. crotocinigenum* sequences supported by a bootstrap support of 94.9%. The most basal clade was populated with sequences of *T. ovalisporum* with bootstrap support values of 99.93% (Figure 10)

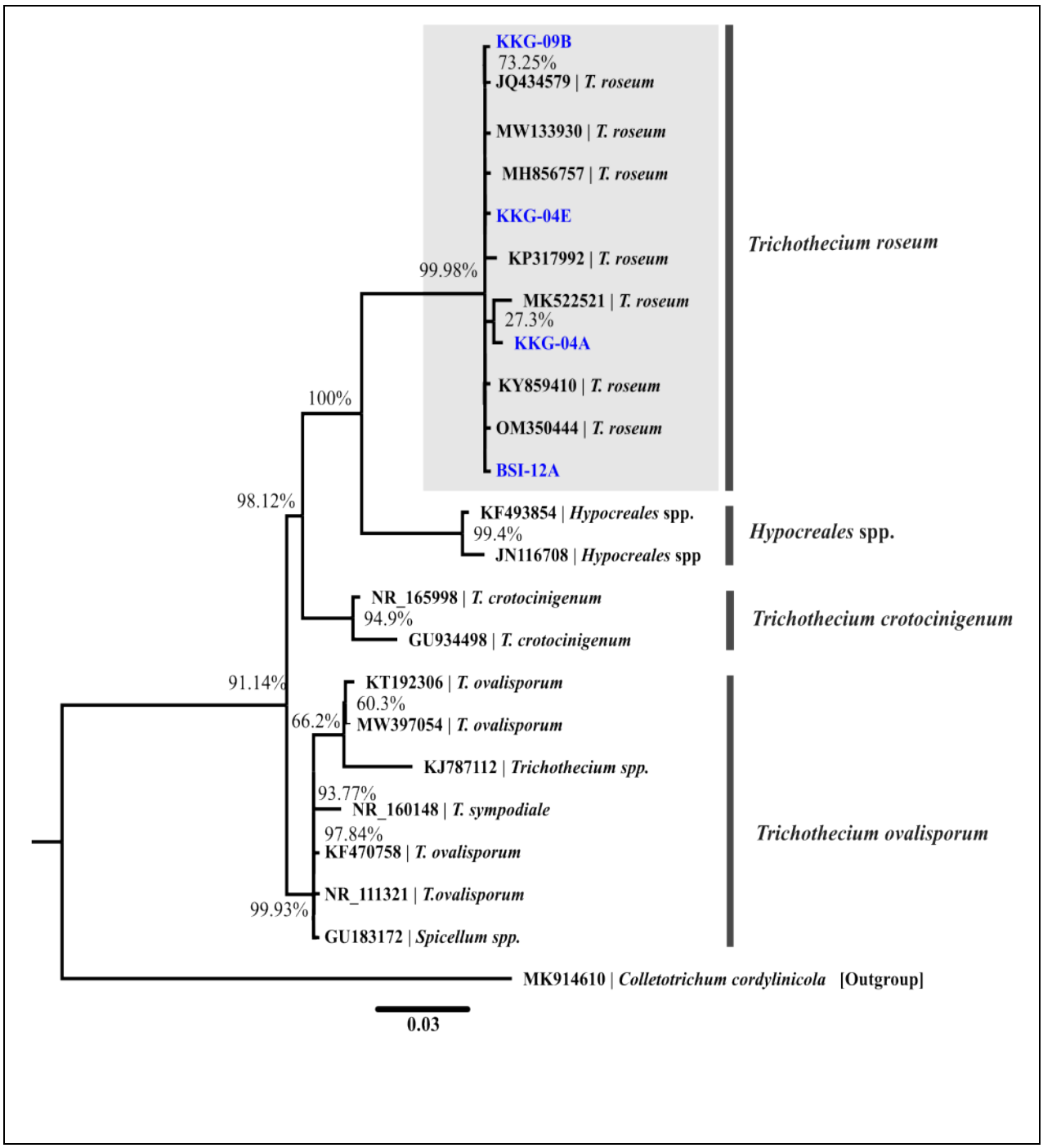

Figure 10: A Neighbour Joining tree for *Trichothecium roseum* isolates from the current study

The tree is drawn to scale with the scale bar representing the number of nucleotide substitutions per site. The values at the nodes are bootstrap support values; the isolates from this study are presented in blue.

The phylogenetic analysis placed isolates BSI-018, BSI-23A, BGM-26A and BGM-26B in monophyletic clade with known sequences of *Fusarium equiseti* with bootstrap support of 90.03% (Figure 11). Isolate BSI-23A formed a monophyletic clade with two *F. equiseti* reference sequences: MN202708, isolated as an endophyte of crucifer and MT560375, a causative agent of potato rot. This clade was supported by 79.78% bootstrap replications. Isolate BSI-018 clustered in a monophyletic clade with MH578585, a pathogen of *Citrus reticulata*, with bootstrap support of 78.0%. Isolates BGM-26A and BGM-26B formed a monophyletic clade with *F. equiseti* of accession number MT560337 (a causative agent of potato rot) with a bootstrap value of 82.15%. Overall, the clade that clustered *F. equiseti* isolates had high bootstrap support of 90.03% (Figure 11). The clustering of isolates BSI-018, BSI-23A, BGM-26A and BGM-26B with known isolates of *F. equiseti* confirmed the identity of these isolates as *Fusarium equiseti*.

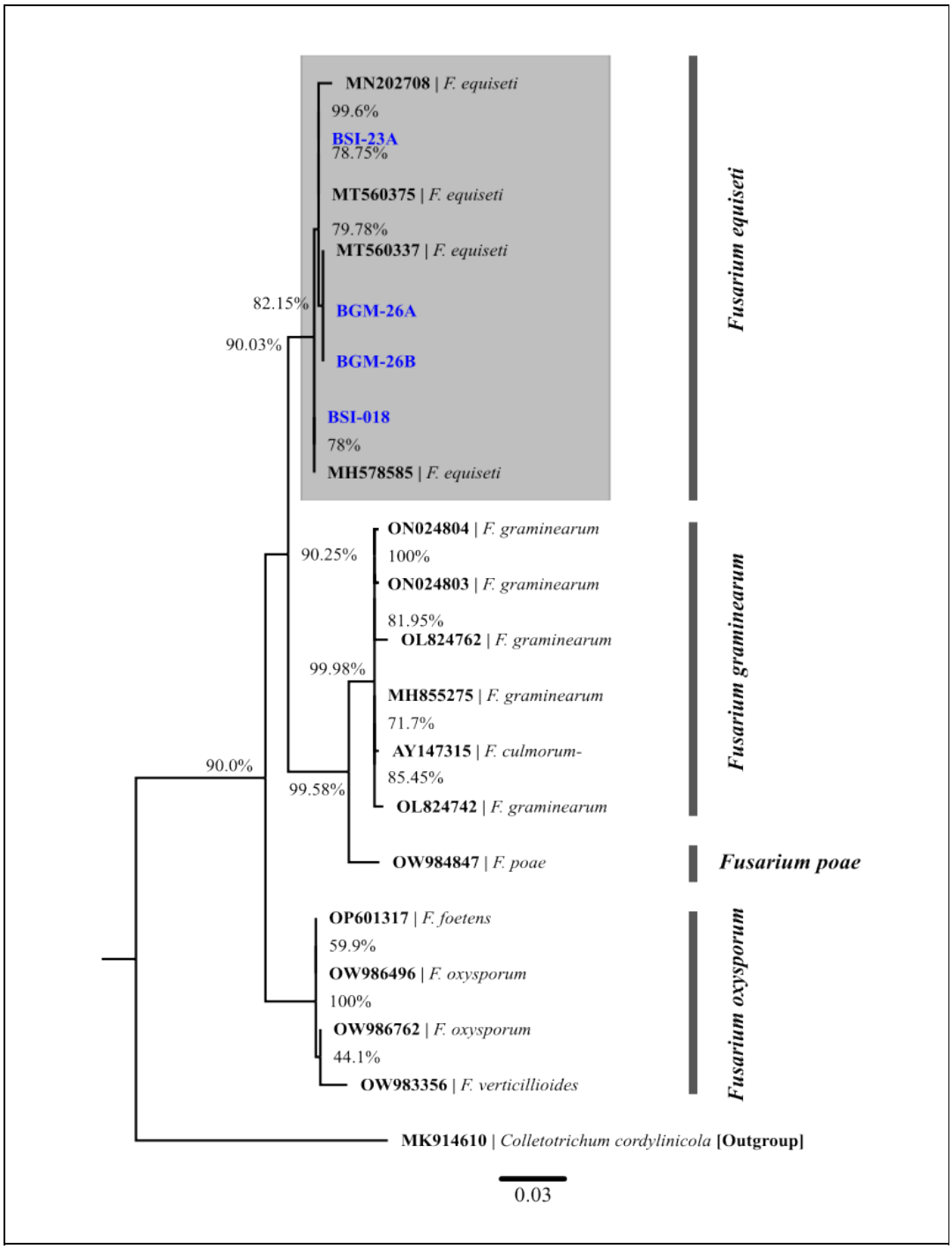

Figure 11: A Neighbour Joining tree for *Fusarium equiseti* isolates from the current study. The tree is drawn to scale with the scale bar representing the number of nucleotide substitutions per site. The values at the nodes are bootstrap support values; the isolates from this study are presented in blue

Phylogenetic analysis revealed that isolates KKG-04C, KKG-06C and BSI-015 belonged to the genus *Colletotrichum.* The inferred phylogenetic tree bifurcated into two major clusters that corresponded with *Colletotrichum gloeosporioides* species complex (CGSC) and *Colletotrichum boninense* species complex (CBSC) (Figure 12). Isolate KKG-04C was clustered within a clade that also contained isolate KKG-06C and two known isolates of *C. siamense* of accession numbers MW578479 and KU64248 implicated as the cause of anthracnose. This monophyletic clade of *C. siamense* isolates was supported by a bootstrap value of 77.17% and occurred within the CGSC which was strongly supported by 99.74% of bootstrap replications. Isolate BSI-015 clustered with *C. karstii* isolates of accession numbers OL824836 identified as a phytopathogen to *Juglans mandshurica* and KX578794 to form a monophyletic clade with bootstrap support of 57.85% and occurred within CBSC (Figure 12). The clustering of the isolates to known members of *Colletotrichum* spp. identified isolates KKG-04C and KKG-06C as *Colletotrichum siamense* and isolate BSI-015 as *Colletotrichum karstii*.

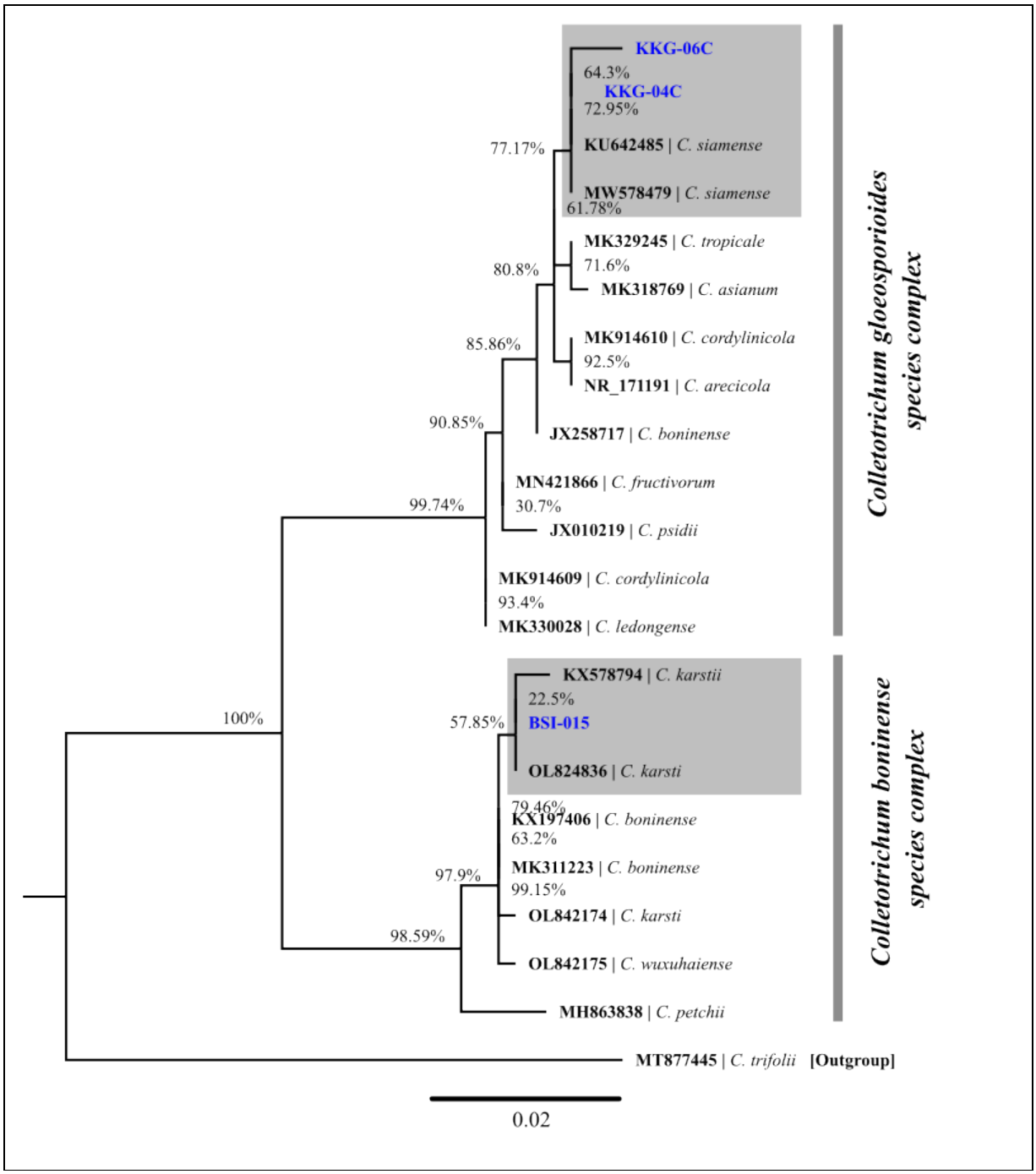

Figure 12: A Neighbour Joining tree for *Colletotrichum siamene* and *Colletotrichum karstii*. isolates from the current study

The tree is drawn to scale with the scale bar representing the number of nucleotide substitutions per site. The values at the nodes are bootstrap support values; the isolates from this study are presented in blue

Phylogenetic analysis placed Isolate BSI-11A in a monophyletic clade with *Diaporthe phaseloreum* of accession number AF001020 isolated from soybean. This monophyletic clade was supported by a bootstrap value of 36.5%, this clade clustered within a clade of known sequences of *D. phaseolorum* supported by a bootstrap value of 95.49% (Figure 13). This clade was a sister clade to a cluster that had *Phomopsis* spp. supported by a bootstrap value of 99.8%. The phylogenetic tree bifurcated into two more monophyletic clusters consisting of members of *Phomopsis* spp. and other *Diaporthe* spp. (Figure 13).

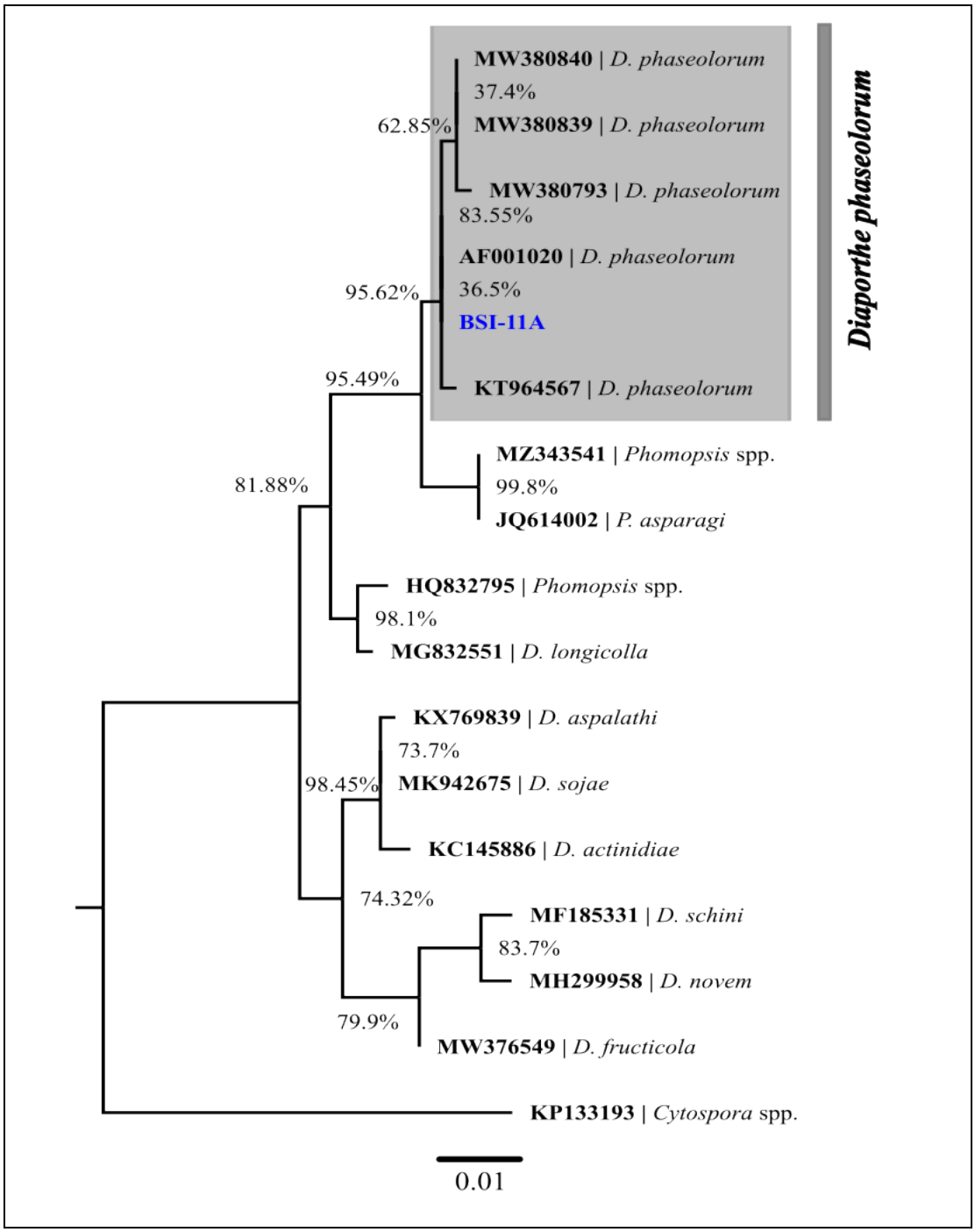

Figure 13: A Neighbour Joining tree for *Diaporthe phaseolorum* isolate from the current study The tree is drawn to scale with the scale bar representing the number of nucleotide substitutions per site. The values at the nodes are bootstrap support values; the isolate from this study is presented in blue

The phylogenetic tree inferred for isolate BSI-21B formed two clusters, one cluster contained *Bipolaris* spp. isolates and the other cluster contained isolates belonging to *Curvularia* spp. and *Setosphaeria prolata* (Figure 14). Phylogenetic analysis placed isolate BSI-21B in a cluster with known *Bipolaris* spp. isolates. Isolate BSI-21B clustered with three *Bipolaris setariae* isolates of accession numbers MT750303 (causing leaf spot of millet), MT755708 (causing leaf spot of millet) and MT750304 (causing leaf spot of millet) with a bootstrap support of 86.5%. The clustering of isolate BSI-21B in a monophyletic clade with *Bipolaris setariae* reference sequences confirms the identity of this isolate as *Bipolaris setariae* (Figure 14).

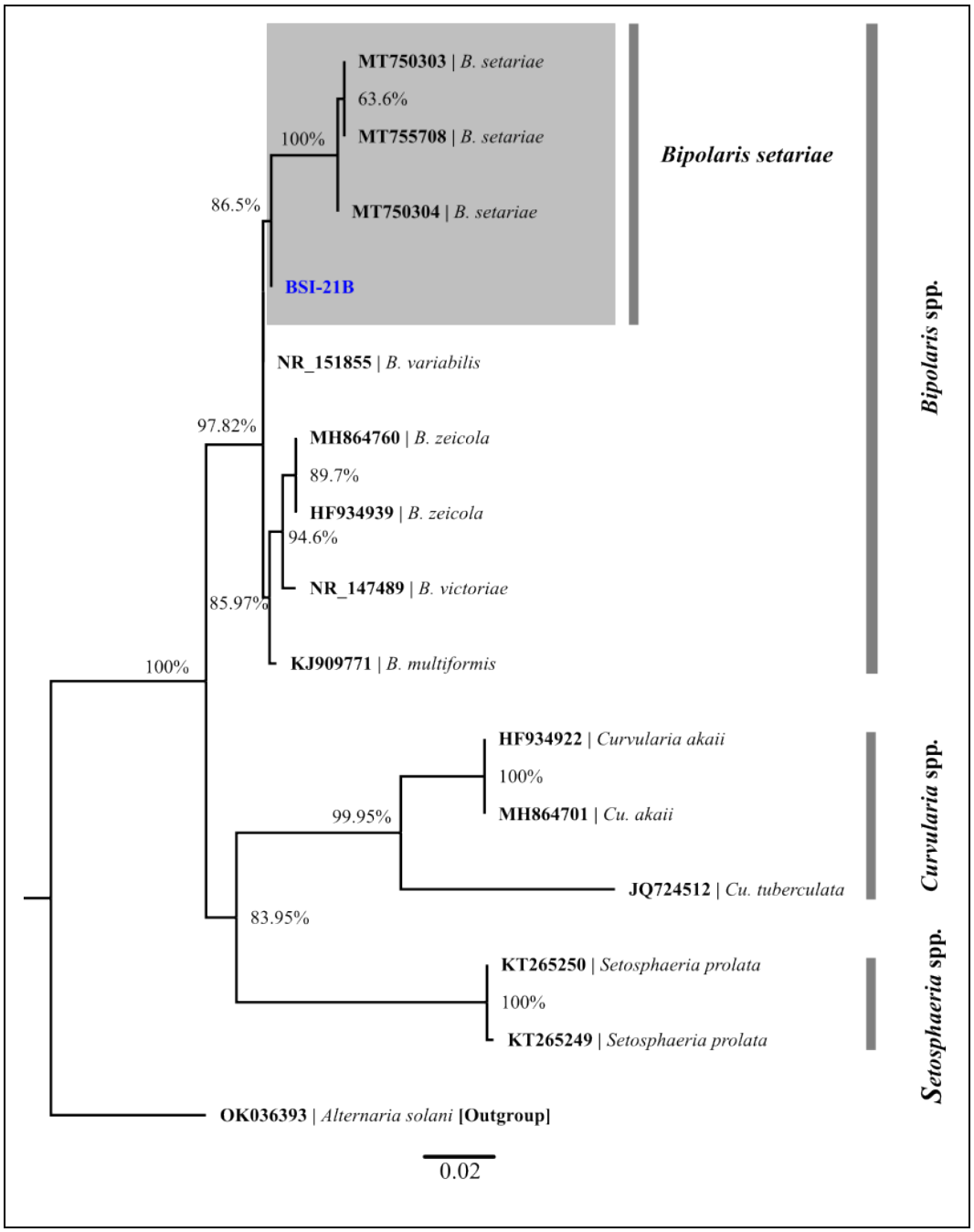

Figure 14: A Neighbour Joining tree for *Bipolaris setariae* isolate from the current study

The tree is drawn to scale with the scale bar representing the number of nucleotide substitutions per site. The values at the nodes are bootstrap support values; the isolate from this study is presented in blue

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

This study is the first to elucidate the aetiology of cassava BLS from different cassava growing Counties in Western Kenya. A study by Ng'ang'a *et al.* (2019) in Kenya on cassava BLS reported the occurrence of several fungal pathogens from samples collected from a field trial at Kenya Agricultural and Livestock Research Organization (KALRO) and therefore there is lack of information on causal agents of cassava BLS and their diversity in farmers' fields in Western Kenya.

The current study confirmed the presence of cassava BLS in cassava fields in three Counties (Kakamega, Bungoma and Busia) of Western Kenya, which are the major cassava growing regions in Kenya (Kidasi *et al.*, 2021). A total of 60 fungal isolates were obtained from cassava leaf samples collected from the three Counties and 58.3% of the isolates were pathogenic. Of the pathogenic isolates, 57.1%, 22.9% and 20.0% were isolated from samples collected from Kakamega, Busia and Bungoma Counties, respectively, indicating that BLS is present in all cassava growing regions in Western Kenya. This confirms reports from previous studies that cassava BLS occurs in all cassava growing regions worldwide (Chaoping *et al.*, 2015; Verdier *et al.*, 2007).

The pathogenicity tests revealed four different types of symptoms presented by the different pathogenic isolates that included chlorosis and browning of the leaf lamella and veins. This suggested an underlying variability of pathotypes and the different severity scores point to different level of aggressiveness between pathotypes (Sakr, 2020, 2018). Therefore, further studies need to be performed to confirm the virulence and/or aggressiveness of the isolated fungal pathogens causing cassava BLS.

5.1.1 Morpho-cultural characteristics of causal agents of cassava brown leaf spot disease

The fungal isolates obtained were placed into different groups based on the morpho-cultural characteristics. Four isolates namely BSI-018, BSI-23A, BGM-26A and BGM-26B produced white, dense, aerial mycelia with abundant microconidia with a single septum. The observed

characteristics are typical of members within the *Fusarium incarnatum-equiseti* species complex (Crous *et al.*, 2021; Summerell, 2019; Wang *et al.*, 2019). However, the presence of a single septum and the rarity of microconidia and phialides in BSI-018, BSI-23A, BGM-26A and BGM-26B supported the phenotypic identification of the isolates as *Fusarium equiseti* (Pit and Hocking, 1997).

The morpho-cultural characteristics of isolates KKG-04A, KKG-04E, KKG-09B and BSI-12A suggested that they were members of the same taxon. All these isolates displayed colonies that were flat, granular and orange pink in color with the granulose morphology arising from heavy conidiation. The conidia were pyriform in shape and each, had one transverse septum and formed at the apex of conidiophores in characteristic zigzag chains. The chains were loosely attached and the conidia easily broke off during slide preparations. The observed morpho-cultural characteristics in this study fits previous description for *Trichothecium roseum* as described by Firouzianbandpey *et al.* (2021), Kwon *et al.* (2010) and Pit and Hocking (1997).

Three isolates BSI-015, KKG-06C and BSI-015 produced mycelia that were white and floccose in nature. The conidia were hyaline, formed singly and were non-septate. Additionally the conidia were fusiform to cylindrical with rounded ends. Based on these characteristics, these isolates were identified to belong to the genus *Colletotrichum* spp. (Pit and Hocking, 1997). However, morpho-cultural traits alone could not be used for taxonomic classification of *Colletotrichum* spp. to the species level. Other authors have experienced such drawbacks; Weir *et al.* (2012) argued that some species within the *Colletotrichum gloeosporioides* species complex cannot be distinguished using phenotypic keys. Furthermore, most of the morpho-cultural traits suggested for species discrimination of *Colletotrichum* spp. by Oliveira *et al.* (2018) and Yang *et al.* (2009) largely overlap between species.

Isolate BSI-11A had a centrally elevated region of tuft white mycelia of higher density than the outer regions and the conidia were ellipsoidal or irregular in shape. The occurrence of white tuft mycelia has previously been associated with *Diaporthe* spp. (Sun *et al.*, 2012). However, it has also been reported that growth conditions can affect the morphological presentation of *Diaporthe* spp. (Mena *et al.*, 2020). The morpho-cultural characteristics observed for this isolate were in

agreement with previous studies that used the same characteristics to identify *Diaporthe* spp. (Costamilan *et al.*, 2008; Gao *et al.*, 2014), and therefore, BSI-11A was classified as *Diaporthe* spp. Morpho-cultural characterization could not delineate BSI-11A to species level due to morphological conservation and phenotypic plasticity resulting in different members of *Diaporthe* spp. having overlapping traits (Guo *et al.*, 2020; Hilário *et al.*, 2021; Mostert *et al.*, 2001).

Isolate BSI-21B presented with mycelia that was white but gradually turned light grey with age and the outer younger mycelia were fluffy and abundant. The light grey regions also had abundant conidiation and the conidia were brown, fusiform occurring in clusters at the tip of conidiophores, with 4-7 cells per conidium. Similar morphological traits have been recorded for members of *Bipolaris* spp. (Pit and Hocking, 1997; Sun *et al.*, 2020). However, *Bipolaris* spp. is a morphologically conserved genus with different species sharing the same morpho-cultural characteristics (Jayawardena *et al.*, 2021). This makes taxonomic classification based only on morphology difficult, thus, without other specific, distinguishing features, isolate BSI-21B was designated as *Bipolaris* spp. based on morpho-cultural traits.

It was not possible to identify all the fungal isolates to species level using only morphological characteristics, thus molecular tools were implemented to assist in the identification of the *Trichothecium*, *Fusarium*, *Colletotrichum*, *Diaporthe* and *Bipolaris* species.

5.1.2 Molecular characteristics of causal agents of cassava brown leaf spot disease

In the present study, the genomic DNA of the selected 13 pathogenic fungal isolates were successfully amplified using ITS1 and ITS4 primers with PCR products of ≈ 600 bp. This confirms the universality of ITS markers in amplification of fungal pathogens (Belisário *et al.*, 2018; Chen *et al.*, 2020; Hami *et al.*, 2021).

Phylogenetic and BLASTn analysis of the pathogenic fungal isolates classified isolates KKG-04A, KKG-04E, KKG-09B and BSI-12A as *Trichothecium roseum*, and isolates BSI-018, BSI-23A, BGM-26A and BGM-26B as *Fusarium equiseti*. Unlike BLASTn analysis, phylogenetic analysis was able to delineate all isolates down to the species level with isolates KKG-04C, and

KKG-06C identified as *Colletotrichum siamense*, BSI-015 as *Colletotrichum karstii,* BSI-11A as *Diaporthe phaseolorum* and BSI-21B as *Bipolaris setariae.* This study supports the use of molecular techniques in studies of fungal diversity and species identification (Kapli *et al.*, 2020; Martens *et al.*, 2008).

In this study, four isolates namely KKG-04A, KKG-04E, KKG-09B and BSI-12A, were identified as *T. roseum*. Based on the *in vitro* pathogenicity tests, all the isolates were confirmed to be pathogenic. This is the first report that has associated *T. roseum* to cassava BLS. Previous studies on the pathogenicity of *T. roseum* have concentrated on infection of fruits and vegetables mainly as a post-harvest pathogen (Zhang *et al.*, 2020). Studies emanating from Asia implicate *T. roseum* as the causal agent of pink mold rot of oranges (Kwon *et al.*, 2013a), pears (Kwon *et al.*, 2013b), strawberries (Kwon *et al.*, 2010) and tomatoes (Han *et al.*, 2012). Furthermore, several virulent factors have been identified in *T. roseum* (Dai *et al.*, 2019; Oh *et al.*, 2014; Soukupová *et al.*, 2003; Žabka *et al.*, 2006) confirming that *T roseum* is a pathogenic fungus with a broad range of plant hosts. In *Pyracantha coccinea, T. roseum* has been shown to cause die back disease (Firouzianbandpey *et al.*, 2021); and it was determined as the causal agent for a new leaf spot disease of mango trees (ShiLan *et al.*, 2011). In addition to plants hosts, *T. roseum* also affects round worms' eggs and embryos by retarding development of the eggs and delaying embryogenesis (Blaszkowska *et al.*, 2013). The current study has expanded the host range of *T. roseum* to include cassava.

Molecular identification confirmed four isolates BSI-018, BSI-23A, BGM-26A and BGM-26B as members of *Fusarium equiseti*. Each of the isolates was confirmed to be pathogenic in the detached leaf pathogenicity assay. This is the first study that has confirmed the association of *F. equiseti* with cassava BLS. This fungal pathogen has been reported to have broad host range. Ismail *et al.* (2021) found that *F. equiseti* infected the leaves of *Cucumis melo* and presented with brown necrotic spots with chlorotic halos. *Lagenaria siceraria*, a member of the *Cucurbitaceae* family, was also found to be infected with *F. equiseti* and presented similar symptoms (Aslam *et al.*, 2021). Hami *et al.* (2021) suggested that *F. equiseti* was more virulent than other Fusaroid species such as *F. solani* and *F. oxysporum* in causing chilli wilt. Since this is the first report of *F. equiseti* causing brown leaf spot disease in cassava, further studies are required to evaluate the

virulence of the *F. equiseti* isolates in cassava; as well as establish the virulence factors associated with the pathogen on the crop.

The isolates KKG-06C and KKG-04C were identified as *Colletotrichum siamense* while BSI-015 was identified as *Colletotrichum karstii* and revealed high similarity to the reference sequences in the GenBank database. *Colletotrichum siamense* and *C. karstii* have been reported to belong to two different species complexes, that is, *C. gloeosporioides* species complex and *C. boninense* species complex, respectively (Schena *et al.*, 2014). This is the first study reporting occurrence of *C. siamense* and *C. karstii* in cassava infected with BLS. The results obtained in this study contradicts the findings of Liu *et al.* (2019), who reported *C. siamense* and *C. karstii* to be the causal agents of cassava anthracnose disease (CAD). However, since cassava anthracnose sometimes displays similar symptoms to BLS, there is need for further studies to determine the fungal disease caused by *C. siamense* and *C. karstii* in cassava. In a previous study in Kenya from a field trial, Ng'ang'a *et al.* (2019) showed that *Colletotrichum* spp. was the causal agent of BLS, although the study did not delimit the actual species that caused the disease.

This study determined that the isolate BSI-11A belonged to *Diaporthe phaseolorum* based on both morpho-cultural and molecular data. The phytopathology status of this isolate was tested using the detached leaf pathogenicity assay and it confirmed that this is the first report to associate *D. phaseolorum* with cassava BLS. In a previous study, *D. phaseolorum* was implicated in causing root rot disease of cassava in Brazil (Boas *et al.*, 2017). Additionally, there is precedence that members of the genus *Diaporthe* spp. are able to infect cassava leaves and cause leaf spots in cassava; for instance in Rwanda, *Diaporthe manihotia* has been reported to be the causal agent of cassava leaf spot disease (Gao *et al.*, 2014). Furthermore, the infection of other organs of the plant by *Diaporthe* spp. can present symptoms on the leaves of the plant as is the case of soybean stem canker disease caused by *D. phaseolorum* (Mena *et al.*, 2020).

The isolate BSI-21B was confirmed to be *Bipolaris setariae* based on the ITS sequences. This indicates that *B. setariae* is one of the causal agents of cassava BLS which was in agreement with a previous study by Manamgoda *et al.* (2014). Members of the genus *Bipolaris* have been reported to cause brown spot symptoms when they infect other crop plants including cereals and

grasses (Marin-Felix *et al.*, 2017). *Bipolaris oryzae* has been shown to be a cause for devastating diseases in rice and led to the Bengal famine in India in 1943 (Bhunjun *et al.*, 2020; Manamgoda *et al.*, 2014). Therefore, identification of *B. setariae* as one of the causal agents of cassava leaf spot disease is of great concern in cassava production. Studies in China also reported that *B. setariae* can infect cassava leaves and cause leaf spot disease (Chunfang *et al.*, 2012). In addition, 50% of cassava plants in a plantation in China was reported to be infected by *B. setariae* (Shi *et al.*, 2010). This shows that *B. setariae* as one of the causal agent of cassava BLS has worldwide distribution.

Bipolaris setariae belongs to a complex called *Curvularia-Drechslera-Bipolaris* species complex (CDBC), which includes *Curvularia* spp. *Drechslera* spp. and *Bipolaris* spp. One of the members in the complex known as *Curvularia lunata* has been reported to affect 9, 13 and 38% of the cassava fields in Benin, Ghana and Nigeria, respectively, causing cassava stem blight with incidences of up to 80% (Msikita *et al.*, 2007); showing that CDBC as causal agents of cassava diseases are widely spread in Africa. Members of CDBC are known phytopathogens in many crop plants (Manamgoda *et al.*, 2014).

5.2 Conclusion

Six different fungal pathogens namely *Trichothecium roseum*, *Fusarium equiseti*, *Colletotrichum karstii*, *Colletotrichum siamense*, *Bipolaris setariae* and *Diaporthe phaseolorum* were isolated from cassava leaves showing symptoms of cassava brown leaf spot disease in western Kenya. This is the first study that has implicated *T. roseum*, *C. karstii*, *C. siamense* and *D. phaseolorum* as causative agents of BLS. This study therefore expands the number of fungal pathogens that are responsible for causing cassava BLS. Earlier studies on BLS within Africa have indicated that the disease is caused by members of *Passalora* spp. and *Cercospora* spp. It seems that the disease is caused by a myriad of fungal pathogens. The results showed that the fungal pathogens causing cassava BLS in western Kenya are morphologically and genetically variable. A high level of genetic variation is one of the fungal mechanisms that could overcome host resistance to avoid host recognition. Furthermore, based on the distribution of fungal isolates, the current study determined that Kakamega County had the highest number of pathogenic isolates,

followed by Busia and Bungoma Counties. This suggests that the severity of cassava BLS may be higher in Kakamega County compared to Busia and Bungoma counties.

5.3 Recommendations

Based on the findings of this study, the following recommendations are drawn:

i. There is need to investigate the effect of co-inoculation of more than one fungal pathogen causing cassava BLS since synergistic effects are known to affect disease expression.

ii. There is need to determine whether virulence of the identified fungal pathogens is correlated with morphological and genetic variations. In particular, how the genetic factors affect virulence and phenotypic expressions of the disease in cassava.

iii. There is need to identify and characterize fungal pathogens associated with cassava BLS from other cassava growing regions.

iv. There is need to screen available cultivars for tolerance/resistance to cassava BLS.

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APPENDICES

Appendix I: Nucleotide sequences for representative pathogenic isolates from cassava leaves collected from western Kenya

Isolate	Contig sequence	GenBank Accession Number
KKG-04A	GTCATTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGG TGAACCAGCGGAGGGATCATTATAGAGTTAACAAAACAACT CCCAACCCTTTGTGAACCTTACCTACCGTTGCTTCGGCGGAC CGCCCCGGGCGCTGCGTGCCCCGGACCCAAGGCGCCCGCCG GGGACCACACGAACCCTGTTTAACAAACATGTGTATCCTCTG AGCGAGCCGAAAGGCAACAAAACAAATCAAAACTTTCAACA ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC GAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGG CATGCCTGTCCGAGCGTCATTTCAACCCTCGGGCCCCCCCCT TTTCCCCTCGCGGGGGAGGGGGCGGGCCCGGCGTTGGGGCC CAGGCGTCCTCCAAGGGCGCCTGTCCCCGAAACCCAGTGGC GGCCTCGCCGCTGCCTCCTCCGCGTAGTAGCACAAACCTCGC GGGCGGAAGGCGGCGCGGCCACGCCGTAAAACCCCAAACTT TTACCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGA ACTTAAGCATATCATAGCGGAGGA	OP002076

Isolate	Contig sequence	GenBank Accession Number
KKG-04E	AGTTAACAAAACAACTCCCAACCCTTTGTGAACCTTACCTAC CGTTGCTTCGGCGGACCGCCCCGGGCGCTGCGTGCCCCGGA CCCAAGGCGCCCGCCGGGGACCACACGAACCCTGTTTAACA AACATGTGTATCCTCTGAGCGAGCCGAAAGGCAACAAAACA AATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG CCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAAC CCTCGGGCCCCCCCCTTTTCCCCTCGCGGGGGAGGGGGCGG GCCCGGCGTTGGGGCCCAGGCGTCCTCCAAGGGCGCCTGTC CCCGAAACCCAGTGGCGGCCTCGCCGCTGCCTCCTCCGCGTA GTAGCACAAACCTCGCGGGCGGAAGGCGGCGCGGCCACGCC GTAAAACCCCAAAA	OP002077
KKG-09B	TTGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTT GGTGAACCAGCGGAGGGATCATTATAGAGTTAACAAAACAA CTCCCAACCCTTTGTGAACCTTACCTACCGTTGCTTCGGCGG ACCGCCCCGGGCGCTGCGTGCCCCGGACCCAAGGCGCCCGC CGGGGACCACACGAACCCTGTTTAACAAACATGTGTATCCTC TGAGCGAGCCGAAAGGCAACAAAACAAATCAAAACTTTCAA CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA TCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG GGCATGCCTGTCCGAGCGTCATTTCAACCCTCGGGCCCCCCC CTTTTCCCCTCGCGGGGGAGGGGGCGGGCCCGGCGTTGGGG CCCAGGCGTCCTCCAAGGGCGCCTGTCCCCGAAACCCAGTG GCGGCCTCGCCGCTGCCTCCTCCGCGTAGTAGCACAAACCTC GCGGGCGGAAGGCGGCGCGGCCACGCCGTAAAACCCCAAA CTTTTACCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCT GAACTTAAGCATATCAATAAGCGGAGGA	OP002078

Isolate	Contig sequence	GenBank Accession Number
BSI-12A	GTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTG GTGAACCAGCGGAGGGATCATTATAGAGTTAACAAAACAAC TCCCAACCCTTTGTGAACCTTACCTACCGTTGCTTCGGCGGA CCGCCCCGGGCGCTGCGTGCCCCGGACCCAAGGCGCCCGCC GGGGACCACACGAACCCTGTTTAACAAACATGTGTATCCTCT GAGCGAGCCGAAAGGCAACAAAACAAATCAAAACTTTCAAC AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG GCATGCCTGTCCGAGCGTCATTTCAACCCTCGGGCCCCCCCC TTTTCCCCTCGCGGGGGAGGGGGCGGGCCCGGCGTTGGGGC CCAGGCGTCCTCCAAGGGCGCCTGTCCCCGAAACCCAGTGG CGGCCTCGCCGCTGCCTCCTCCGCGTAGTAGCACAAACCTCG CGGGCGGAAGGCGGCGCGGCCACGCCGTAAAACCCCAAACT TTTACCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTG AACTTAAGCATATCAA	OP002079
BSI-018	TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCG TTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTC CCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATC AGCCCGCGCCCCGTAAAACGGGACGGCCCGCCCGAGGACCC TAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAA TAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC GATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC CGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA ACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGC GTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCG TAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACG CCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTA GGAATACCCGCTGAACTTAAGCATATCAATAAGGCGGAG	OP002080

Isolate	Contig sequence	GenBank Accession Number
BSI-23A	GAGGAAGTAAAAAGTCGTAACAAGGTCTCGTTGGTGAACCA GCGGAGGGACATTACCGAGTTTACAACTCCCAAACCCCTGT GAACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCC GTAAAAAGGGACGGCCCGCCCGAGGACCCCTAAACTCTGTT TTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAA CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAAC GCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT GAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT CTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGC TCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAA TCGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCAT ACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACC CCAACTTCTGAATGTGACCTCGGATCAGGTAGGA	OP002081
BGM-26A	TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCG TTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTC CCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATC AGCCCGCGCCCTGTAAAAAGGGACGGCCCGCCCGAGGACCC TAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAA TAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC GATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC CGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA ACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGC GTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCG TAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACG CCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTA GGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAG	OP002082

Isolate	Contig sequence	GenBank Accession Number
BGM-26B	TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCG TTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTC CCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATC AGCCCGCGCCCTGTAAAAAGGGACGGCCCGCCCGAGGACCC TAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAA TAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC GATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC CGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA ACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGC GTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCG TAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACG CCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTA GGAATACCCGCTGAACTTAAGCATATCAATAAGGCGGAGGA	OP002083
KKG-04C	TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCG TTGGTGAACCAGCGGAGGGATCATTACTGAGTTTACGCTCTA TAACCCTTTGTGAACATACCTATAACTGTTGCTTCGGCGGGT AGGGTCTCCGTGACCCTCCCGGCCTCCCGCCCCCGGGCGGGT CGGCGCCCGCCGGAGGATAACCAAACTCTGATTTAACGACG TTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACA ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC GAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGG CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTG GTGTTGGGGCCCTACAGCTGATGTAGGCCCTCAAAGGTAGT GGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTTACGT CTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCC AATTTTCCAAAGGTTGACCTCGGATCAGGTAGGAATACCCG CTGAACTTAAGCATATCA	OP002084

Isolate	Contig sequence	GenBank Accession Number
KKG-06C	TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCG TTGGTGAACCAGCGGAGGGATCATTACTGAGTTTACGCTCTA TAACCCTTTGTGAACATACCTATAACTGTTGCTTCGGCGGGT AGGGTCTCCGTGACCCTCCCGGCCTCCCGCCCCCGGGCGGGT CGGCGCCCGCCGGAGGATAACCAAACTCTGATTTAACGACG TTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACA ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC GAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGG CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTG GTGTTGGGGCCCTACAGCTGATGTAGGCCCTCAAAGGTAGT GGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTTACGT CTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCC AATTTTCCAAAGGTTGACCTCGGATCAGGTAGGAATACCCG CTGAACTTAAGCATATCATAAGGCGGAG	OP002085
BSI-015	TTGGTCATTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGT TGGTGAACCAGCGGAGGGATCATTACTGAGTTACCGCTCTAT AACCCTTTGTGAACATACCTACAACTGTTGCTTCGGCGGGTA GGCCGTCCCCTGAAAAGGACGCCTCCCGGCCCGGACCGGAC CCCCCGCGGGACCGGACCCGGCGCCCGCCGGAGGATAACCA AACTCTATTGTAACGACGTTTCTTCTGAGTGGCATAAGCAAA ATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATC GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC CGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA ACCCTCAAGCTCTGCTTGGTGTTGGGGCTCTACGGTCGACGT AGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTT GCGTAGTAACATTTCGTCTCGCACTGGGATCCGGAGGGACTC TTGCCGTAAAACCCCCCAATTTTCCAAAGGTTGACCTCGGAT CAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAGGCG G	OP002086

Isolate	Contig sequence	GenBank Accession Number
BSI-11A	GTCTTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGG TGAACCAGCGGAGGGATCATTGCTGGAACGCGCTTCGGCGC ACCCAGAAACCCTTTGTGAACTTATACCTATTTGTTGCCTCG GCGTAGGCCGGCCTCTTCACTGAGGCCCCCTGGAGACAGGG AGCAGCCCGCCGGCGGCCAACTAAACTCTTGTTTCTATAGTG AATCTCTGAGTAAAAAACATAAATGAATCAAAACTTTCAAC AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGG GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTT GGTGATGGGGCACTGCCTTCTAGCGAGGGCAGGCCCTGAAA TCTAGTGGCGAGCTCGCTAGGACCCCGAGCGTAGTAGTTAT ATCTCGTTCTGGAAGGCCCTGGCGGTGCACTGCCGTTAAACC CCCAACTTCTGAAAATTTGACCTCGGATCAGGTAGGAATACC CGCTGAACTTAAGCATATCAATAAGCGGAGGA	OP002087
BSI-21B	TGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGT AGGTGAACCTGCGGAGGGATCATTACACAACAAAATATGAA GGTCTGGCTTCGCGGCCGGCTGAAATATTTTTTTCACCCATG TCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCAC CAGGACCAAACCATAAACCTTTTTTTTTATGCAGTTGCAATC AGCGTCAGTAAAAACAATGTAATTAATTACAACTTTCAACA ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATC GAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGG CATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTTTGCTTG GTGTTGGGCGTTTTTGTCTCCCTCTTTCTGGGAGACTCGCCTT AAAACGATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCA CATAATTTGCGCTTTGTATCAGGAGAAAAGGACGGTAATCC ATCAAGACTCTACATTTTTAACTTTTGACCTCGGATCAGGTA GGGATACCCGCTGAACTTAAGCATATCAATAA	OP002088

Appendix II: Sequences of fungal isolates used in the phylogenetic analysis

Strain/Isolate	Accession number	Species	Host/Source	Location
KKG-09B	**OP002078**	***T. roseum***	**Cassava**	**Kenya**
LCP 47.624	JQ434579	*T. roseum*	Cheese	France
GZCC19-0523	MW133930	*T. roseum*	Water	China
CBS 566.50	MH856757	*T. roseum*	-	Canada
KKG-04E	**OP002077**	***T. roseum***	**Cassava**	**Kenya**
B-1	KP317992	*T. roseum*	Beans	-
MTP1529	MK522521	*T. roseum*	-	-
KKG-04A	**OP002076**	***T. roseum***	**Cassava**	**Kenya**
52_1B12	KY859410	*T. roseum*	Refrigerator	Turkey
HM1	OM350444	*T. roseum*	-	-
BSI-12A	**OP002079**	***T. roseum***	**Cassava**	**Kenya**
ENT4	KF493854	*Hypocreales* spp.	Red sandalwood	-
PSU-ES199	JN116708	*Hypocreales* spp.	Sea grass	-
CBS 129.64	NR_165998	*T. crotocinigenum*	-	Hungary
OTU270	GU934498	*T. crotocinigenum*	Willows	-
40a2	KT192306	*T. ovalisporum*	*Populus cathayana*	-
Morchella sextelata	MW397054	*T. ovalisporum*	-	-
B-6	KJ787112	*Trichothecium* spp.	*Crocus sativus*	China
CBS 227.76	NR_160148	*T. sympodiale*	-	France
CGMCC 3.17049	KF470758	*T. ovalisporum*	*Cordyceps militaris*	China
DAOM:186447	NR_111321	*T. ovalisporum*	*Megachile rotundata*	Canada
HF8	GU183172	*Tricothecium spp.*	Rice	China
HNBL 53	MK914610	*C. cordylinicola*	Arecanut palm	China
OTU49	MN202708	*F. equiseti*	Crucifer	China
BSI-23A	**OP002081**	***F. equiseti***	**Cassava**	**Kenya**

N-32-1	MT560375	*F. equiseti*	Potato	China
N-11-2	MT560337	*F. equiseti*	Potato	China
BGM-26A	**OP002082**	***F. equiseti***	**Cassava**	**Kenya**
BGM-26B	**OP002083**	***F. equiseti***	**Cassava**	**Kenya**
BSI-018	**OP002080**	***F. equiseti***	**Cassava**	**Kenya**
G388	MH578585	*F. equiseti*	*Citrus reticulata*	Pakistan
NFG44	ON024804	*F. graminearum*	Wheat	India
NFG43	ON024803	*F. graminearum*	Wheat	India
NFG26	OL824762	*F. graminearum*	Wheat	India
CBS 186.32	MH855275	*F. graminearum*	-	-
FC12	AY147315	*F. cullmorum*	-	Germany
NFG6	OL824742	*F. graminearum*	Wheat	India
IHEM:13813	OW984847	*F. poae*	Grains	Belgium
30,2-C	OP601317	*F. foetens*	Bulding	Costa Rica
IHEM:22249	OW986496	*F. oxysporum*	Human nail	France
IHEM:23153	OW986762	*F. oxysporum*	Human blood	Belgium
IHEM:04197	OW983356	*F. verticillioides*	Human peritoneal liquid	Italy
BTMH5	MT750303	*B. setariae*	Millet	India
BTMH9	MT755708	*B. setariae*	Millet	India
BTMH6	MT750304	*B. setariae*	Millet	India
BSI-21B	**OP002088**	***B. setariae***	**Cassava**	**Kenya**
CBS 127716	NR_151855	*B variabilis*	Pennisetum	Argentina
CBS 127731	MH864760	*B. zeicola*	-	-
CBS 317.64	HF934939	*B. zeicola*	Buffalo grass	-
CBS 327.64	NR_147489	*B. victoriae*	Avena sativa	USA
CBS480.74	KJ909771	*B. multiformis*	*Tribulus terrestris*	South Africa
CBS 127730	HF934922	*Cu. akaii*	Buffalo grass	-
CBS 127727	MH864701	*Cu. akaii*	-	-
DBOF152	JQ724512	*Cu. tuberculata*	Marine sediment	India
CBS 128059	KT265250	*Exserohilum*	-	

		prolatum		
		Exserohilum		
CBS 128058	KT265249	*prolatum*	-	
AMaNaL	OK036393	*Alternaria solani*	Tomato	India
101A	MW380840	*D. phaseolorum*	Cassava	Colombia
19A	MW380839	*D. phaseolorum*	Cassava	Colombia
90A1n	MW380793	*D. phaseolorum*	Cassava	Colombia
pssp5	AF001020	*D. phaseolorum*	Soybean	-
BSI-11A	**OP002087**	***D. phaseolorum***	**Cassava**	**Kenya**
WAA02	KT964567	*D. phaseolorum*	Grass	Malaysia
I7T2	MZ343541	*Phomopsis* spp.	-	-
HB6	JQ614002	*P. asparagi*	Aspragus	-
LH20	HQ832795	*Phomopsis* spp.	*Camellia sinensis*	China
TS-131	MG832551	*D. longicolla*	Kiwi fruit	China
Isolate 508	KX769839	*D. aspalathi*	Soybean	Brazil
17-DIA-122	MK942675	*D. sojae*	Soybean	USA
ICMP:13683	KC145886	*D. actinidiae*	*Actinidia deliciosa*	New Zealand
L5N71	MF185331	*D. schini*	*Solanum quitoense*	Ecuador
MLT16	MH299958	*D. novem*	Soybean	Canada
Yeh 0068	MW376549	*D. fructicola*	*Ipomoea pescaprae*	Taiwan
3.3.2	KP133193	*Cytospora* spp.	Inga spp.	Ecuador
KKG-06C	**OP002085**	***C. siamense***	**Cassava**	**Kenya**
KKG-04C	**OP002084**	***C. siamense***	**Cassava**	**Kenya**
Cg130	KU642485	*C. siamense*	-	-
ZJ5	MW578479	*C. siamense*	-	-
C-141	MK329245	*C. tropicale*	Mango	Taiwan
C-1076	MK318769	*C. asianum*	Mango	Taiwan
HNBL 5	NR_171191	*C. arecicola*	arecanut palm	China
M2P5E1	JX258717	*C. boninense*	Soybean	-
Isolate 15	MN421866	*C. fructivorum*	*Nectandra lineatifolia*	Ecuador
CBS 145.29	JX010219	*C. psidii*	*Psidium spp.*	Italy

HNBL 9	MK914609	*C. cordylinicola*	Arecanut palm	China
EIPP 58	MK330028	*C. ledongense*	Coffee	China
AGMy0178	KX578794	*C. karstii*	*Citrus sinensis*	South Africa
BSI-015	**OP002086**	***C. karstii***	**Cassava**	**Kenya**
CDH057	OL824836	*C. karstii*	*Juglans mandshurica*	South Korea
F212102	KX197406	*C. boninense*	*Hylocereus undatus*	Taiwan
Isolate 37	MK311223	*C. boninense*	Satsuma orange	China
YMF 1.04944	OL842174	*C. karstii*	Aquatic plants	China
F34	OL842175	*C. wuxuhaiense*	Aquatic plants	China
CBS 125957	MH863838	*C. petchii*	-	Netherlands
XJLKMH2WJ	MT877445	*C. trifolii*	Alfalfa	China

The entries in bold were obtained from the current study, '-' = data is absent from GenBank database