GENETIC DIVERSITY AND RESISTANCE TO BACTERIAL BLIGHT AMONG KENYAN CASSAVA (*Manihot esculent* Crantz) GERMPLASM

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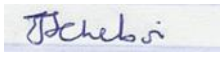
Signature:  Date: 06/08/2023

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DEDICATION

I dedicate this thesis to my late mother, Beatrice Nanjala, and my grandmother, the late Florence Namulanda. To my aunt, Gladys Nakhumwa, for the constant support throughout the challenges of school and life, and my sisters (Mercy Simiyu and Lilian Kaka), for their great sacrifice.

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LIST OF ABBREVIATIONS AND ACRONYMS

AEZ:	Agro-ecological zone
AFLP:	Amplified Fragment Length Polymorphism
ANOVA:	Analysis of variance
AUDPC:	Area under disease progress curve
CBB:	Cassava Bacterial Blight
CBSD:	Cassava Brown Streak Disease
CFU:	Colony Forming Unit
CIA:	Chloroform isoamyl alcohol
CIAT:	International Centre for Tropical Agriculture
CMD:	Cassava Mosaic Disease
CRD:	Completely Randomized Design
CTAB:	Cetyl trimethylammonium bromide
DARwin:	Dissimilarity Analysis and Representation for Windows
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
GxE:	Genotype by Environment
IITA:	International Institute of Tropical Agriculture
KALRO:	Kenya Agricultural and Livestock Research Organization
LSD:	Least Significant Difference
PCA:	Principal component analysis
PCR:	Polymerase chain reaction
PIC:	Polymorphic Information Content
RCBD:	Randomized Complete Block Design
RFLP:	Restriction Fragment Length Polymorphism
SCoT:	Start Codon Targeted
SNP:	Single Nucleotide Polymorphism
SSR:	Simple Sequence Repeats
TrisHCL:	Trisaminomethane hydrochloride
UV:	Ultraviolet radiation
Xam:	Xanthomonas axonopodis pv. manihotis
YPGA:	Yeast Peptone Glucose Agar

GENERAL ABSTRACT

Cassava (*Manihot esculenta* Crantz) has high carbohydrate content and is grown in Africa as an alternative staple crop for food, feed, and bioethanol production. However, the production of cassava encounters limitation due to abiotic and biotic stresses. Of these, the bacterial blight of cassava (CBB) affects most cassava germplasm grown by farmers. Breeding for disease resistance and knowledge of the genetic constitution of the germplasm is key in improving cassava production worldwide. This study aimed to investigate the genetic variation present in the 15 cassava germplasm of Kenya using morphological and molecular markers, while assessing their reactions to CBB. The study involved field and greenhouse experiments. Field trials were carried out at Kakamega and Kiboko research facilities of Kenya Agricultural and Livestock Research Organization (KALRO) using a randomized complete block design (RCBD) with three replications. The cassava plants were spaced 1 m apart within plots, which were spaced 1.5 m apart. Every plot comprised 10 plants, with six plants tagged for data collection. Data for morphological traits were collected at early growth stage, mid-growth stage (reproductive phase), and late growth stage (first branching to maturity). Total DNA was extracted from the 15 cassava varieties using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) protocol. Then, 30 Start Codon Targeted (SCoT) markers were utilized to screen for polymorphism, and seven polymorphic SCoT primers were selected for subsequent analysis. In this study, SCoT markers were preferred because they have a wide genomic coverage. In addition to the field experiments, a greenhouse experiment was established at KALRO Kabete using a completely randomized design (CRD) replicated three times. Differential responses to CBB were observed in both field and greenhouse conditions at 30- and seven-days interval, respectively. The study identified seven clusters based on morphological traits and five clusters based on molecular markers, indicating genetic diversity within the cassava germplasm. Notably, four CBB-resistant varieties independently clustered in sub-cluster A of cluster I using molecular markers, whereas morphological markers did not differentiate between resistant and susceptible varieties. This suggests that morphological traits alone are not indicative of CBB resistance, and resistance may be associated with genetic factors. The identification of CBB-resistant varieties plays a crucial role in developing and deploying improved cassava genotypes, ultimately benefiting farmers and enhancing food security in Africa and beyond.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Cassava (*Manihot esculenta* Crantz), a perennial shrub in the Euphorbiaceae family, originated in South America (Drapal et al., 2019; Olsen & Schaal, 1999). Most small-scale farmers from the tropics majorly grow cassava for its roots with high starch content, making it a crucial food source, providing daily calories to over 800 million people worldwide (Bart & Taylor, 2017; De Souza et al., 2017; Parmar et al., 2017a). Moreover, cassava is a resilient crop, contributing to both food and economic security in marginal areas (Mtunguja et al., 2019; Pushpalatha & Gangadharan, 2020a; Tize et al., 2021a). Previous studies have ranked cassava as the second most important staple food for Africa due to its ability to produce higher carbohydrates per labor unit than most cereals when considering per capita calories consumed (Bayata, 2019; Kacou et al., 2018). Furthermore, several studies have shown that cassava leaves are rich in proteins and essential micronutrients and therefore consumed in some regions (Boukhers et al., 2022; Latif & Müller, 2015; Leguizamón et al., 2021).

Cassava has a cheap cost of production since most farmers prepare and produce their planting materials (Devi & Diarra, 2021; Etany & Walter, 2018). It is cultivated on considerably low inputs and without much technical know-how (Okechukwu et al., 2018). In addition, it is a drought-resilient crop, favoring its production in ecological zones where some crops like maize, wheat, and rice would not survive (Amelework et al., 2021a). Thus, it could bridge the gap in food deficit and provide raw materials for industrial applications. Africa produces about 61% of the world's cassava, while Asia produces 29.5% and America 9.5% (Ferguson et al., 2019). However, despite its potential to promote food and economic security, cassava yields in Africa average 8.8 t/ha, which is significantly lower than both Asia (21.9 t/ha) and South America (12.8 t/ha) (Bennett, 2015). Cassava cultivation in Kenya covers over 90000 ha of land in the western, coastal, and eastern regions and is a low-risk crop that relies on family labor, giving high returns to poor subsistence farmers (Kidasi et al., 2021; Opondo et al., 2020).

Therefore, cassava can improve food security and generate revenue for smallholder farmers in poor marginal lands (Alene et al., 2018). However, its production has remained low mainly due to biotic and abiotic factors.

Due to biotic and abiotic stresses affecting agricultural productivity, food availability and security have remained a significant challenge in developing countries (Bertomeu Pardo & Durán-Romero, 2022). In Kenya, cassava is a vital substitute for staple crop maize because of its exceptional resilience in adverse climatic environments, adaptability to different agro-ecological zones, and high productivity with minimal inputs (Wambua et al., 2020). Recently, cassava has garnered significant attention from governments and industries to address existing food security challenges. This is because of its desirable traits, such as its adaptability to climate change and remarkable productivity even in nutrient-deficient soils. (Pushpalatha & Gangadharan, 2020). In addition to human food, strong demand for cassava has emerged over the years to fulfil industrial needs for producing commodities, including starch, animal feed, and bioethanol (Abass et al., 2018; Chisenga et al., 2019; Devi & Diarra, 2021).

Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD) stand out as the primary viral diseases affecting cassava (Shirima et al., 2022a). On the other hand, Cassava Bacterial Blight (CBB) attributable to *Xanthomonas axonopodis* pv. manihotis (*Xam*) is another significant bacterial disease that impacts cassava productivity worldwide (Zhang et al., 2022a), causing significant yield losses that range from 12% to 100% (Livoi et al., 2021; López & Bernal, 2012a). Previous research has demonstrated that the cassava yield loss caused by *Xam* depends on developmental stage, environmental conditions, and cassava variety (Verdier et al., 2004). Most cassava-growing regions across Africa, Asia, Oceania, and America are affected by CBB (Fanou et al., 2018). Conversely, previous studies have demonstrated that most farmer-preferred cassava cultivars in all cassava-growing regions are susceptible to CBB (Odongo et al., 2019).

Farmers cultivating susceptible varieties manage CBB by rouging deceased plants, planting towards the end of a rainy season, practicing crop rotation, applying quarantine measures, using healthy planting materials, and using chemicals to control insect vectors associated with the bacterium (López & Bernal, 2012b; Toure et al., 2020).

However, these conventional methods are not entirely efficient. Furthermore, chemicals are expensive and unfriendly to the environment (Yu et al., 2012). Therefore, identifying and using varieties resistant to CBB is key to improving cassava production worldwide (Zhang et al., 2022b).

With the goals for locally adapted, drought-resilient, and stress-tolerant crops for sustainable solutions to agriculture and food security in Kenya, the assessment of crop germplasm is a pre-requisite for future breeding work (Andrzejczak & Przysiecka, 2016). Hence, the need to investigate the genetic diversity among Kenyan cassava varieties, identifying varieties to aid in future parental selection for breeding for bacterial blight resistance.

1.2 Statement of the problem

Several studies on cassava genetic diversity have been conducted in Kenya, but there remains a gap in understanding the genetic diversity of specific cassava germplasm in relation to their reactions to Cassava Bacterial Blight (CBB) (López & Bernal, 2012b). CBB poses a significant threat to cassava yield and has contributed to production and consumption deficits in affected regions (Kante et al., 2020).

The infestation of cassava by *Xam* results in leaf wilting and eventually complete defoliation leading to the loss of leafy vegetables in communities where cassava leaves are consumed (Fanou *et al*., 2018). *Xam* is spread from contaminated plant materials (Kerstin Wydra & Verdier, 2002) and is carried from one plant to another by insects or rainwater. The bacteria attach to the underside of the cassava leaves, where they multiply to form micro-colonies providing just enough inoculum to contaminate the lamina tissue through the stomata and/or wounds. Upon entry into the lamina tissue, the bacteria colonize the intracellular spaces in the mesophyll tissues, which rapidly multiply, resulting in lysis of the middle lamella (Boher & Verdier, 1994). The bacteria accumulate in vascular regions of cassava stems, damaging the would-be cleaning planting stakes. CBB is pervasive across all cassava-growing zones of Kenya, although with various incidences and severity depending on the developmental stage of the cassava plants, predisposition of the cassava varieties, climatic conditions, and the inoculant pressure (Fanou *et al*., 2018; Kerstin Wydra & Verdier, 2002).

The effect of CBB on cassava yield is profound, resulting in significant yield losses that range from 12% to 100% (Toure et al., 2020). This reduction in yield directly affects food availability and security (López & Bernal, 2012c) because it is an alternative staple crop for millions of people globally (Wilson et al., 2017a). Farmers have employed several efforts, including proper farm hygiene, planting towards the end of the rainy season, and crop rotation to manage CBB. However, these methods have shown limitations because *Xam* can survive in plant debris and soil (Restrepo et al., 2000), and thus perpetuating the disease.

Additional research has been conducted to identify resistant cassava varieties (Sedano et al., 2017), but the identification and utilization of these varieties remain limited in Kenya (Odongo et al., 2019). The aim of this study was to enhance the knowledge of genetic diversity within cassava germplasm and identify potential resistant varieties to CBB, which can help breeders develop improved cassava genotypes with enhanced disease resistance, ultimately improving cassava production and addressing food security challenges. The findings of this study provide baseline data for the development of improved cassava varieties with enhanced resistance to CBB, ultimately mitigating yield losses and improving cassava production and consumption.

1.3 Justification

Cassava Bacterial Blight (CBB) is a significant bacterial disease that severely affects global cassava production, causing significant yield losses that range from 12% to 100% (Toure et al., 2020). Understanding the genetic diversity of cassava germplasm and its relationship to disease resistance helps breeders identify and select parents that possess inherent resistance (Ogunjobi & Fagade, 2010). This knowledge helps develop effective management strategies and breeding programs aimed at producing CBB-resistant cassava cultivars (Parkes et al., 2013). Genetic diversity studies are vital in determining the ability of a plant to withstand and combat diseases (Karasov et al., 2014). Germplasm with high genetic diversity tend to possess a broader range of resistance genes, enhancing their capacity to withstand pathogen attacks and reduce disease severity (Miedaner, 2016; Perovic et al., 2019).

Therefore, the aim of this study was to identify the presence of diverse genetic traits and their association with CBB resistance (Bohra et al., 2022). Breeders can use this information to select and develop cassava varieties with improved disease resistance, thus reducing yield losses and enhancing cassava production (Narayanan et al., 2021).

Additionally, incorporation of genetic diversity in breeding programs helps breeders avoid over-reliance on single varieties and mitigate the risk of pathogen outbreaks (Nelson et al., 2018a). Furthermore, understanding the genetic basis of disease resistance allows for targeted and efficient breeding efforts (Poland & Rutkoski, 2016;Nelson et al., 2018b), reducing the need for chemical interventions and promoting environmentally friendly and economically viable disease management strategies (Fabre et al., 2012). Therefore, this study provides valuable insights for breeders, farmers, and policymakers to make informed decisions regarding the selection and deployment of CBB-resistant cassava varieties, ultimately leading to improved food security and livelihoods in cassava-dependent communities.

1.4 Objectives

1.4.1 Broad objective

To contribute to cassava improvement through assessment of genetic diversity and screening for resistance to bacterial blight among Kenyan cassava germplasm for increased food production.

1.4.2 Specific objectives

i. To characterize cassava germplasm using morphological and Start Codon Targeted (SCoT) markers.

ii. To identify cassava germplasm with resistance to Cassava Bacterial Blight (CBB) under artificial and natural inoculation conditions.

1.5 Hypotheses

i. No genetic diversity exists among the cassava germplasm
ii. There is no cassava germplasm resistant to Cassava Bacterial Blight (CBB)

CHAPTER TWO

LITERATURE REVIEW

2.1 The origin of cassava and its production

Cassava, a perennial shrub believed to have originated in South and Central America, has a disputed geographical origin (Ferguson et al., 2019b). It was brought to Africa by Portuguese traders during the 16th century, specifically along the coasts of West Africa and nearby islands (Spencer & Ezedinma, 2017). It was then gradually introduced to the rest of the tropical countries, where it was adopted, and is currently grown widely in several parts of the region. Worldwide, Nigeria is the leading cassava grower, producing 59475202 tons, followed by Thailand, which produces 31678017 tons (Arthey et al., 2018; Ikuemonisan et al., 2020; Pipitpukdee et al., 2020).

Today, cassava plays a significant role as food and economic security crop for sub-Saharan Africa, and is an adaptable crop to Africa's diverse environmental conditions ((Mbanjo et al., 2021a; Tize et al., 2021b). Thus, being easily integrated into the farming system of Africa yet full of untapped potential. It is estimated that cassava feeds over 700 million people worldwide (Wilson et al., 2017b). Smallholder farmers contribute about 90% of cassava production in sub-Saharan Africa (Ao et al., 2019; Otekunrin et al., 2019), making it a significant crop in subsistence farming. However, the crop faces significant challenges, including diseases such as CBSD, CBB, and CMD. If not managed well, diseases, such as CBB, constrains cassava production by about 12 to 100% yield loss. *Xam*, which causes CBB, is spread from contaminated plant materials and is carried from one plant to another by insects or rainwater (Dania & Ojeyemi, 2019a). The bacteria attach to the undersurface of the cassava leaves, and they multiply to form microcolonies, providing just enough inoculum to infect the lamina tissue via the stomata and/or laceration (Zárate-Chaves et al., 2021a).

2.2 Importance of cassava

Cassava has unique characteristics such as adaptability in various environments, climate resilience, appreciable production in poor soils, piecemeal harvesting, and cheap propagation materials (Amelework et al., 2021b; Barata et al., 2021; Pushpalatha & Gangadharan, 2020b). As a result, the importance of cassava has increased in tropical and subtropical regions where economic yields of other crops like maize, wheat, and rice, have turned out to be depressed because of climate change and the emergence of new diseases and pests (Amelework et al., 2021b). Therefore, over the years, cassava has shown a great promise as alternative staple food in tropical countries (Awoyale et al., 2021). The significance of cassava is augmented due to its potential industrial application in animal feeds, industrial ethanol, starch, and paper (Abotbina et al., 2022; Chisenga et al., 2019b).

The ever growing world population is not correlated with increased crop yields, and therefore always leading to food shortages associated with increased demand and, consequently, high food prices (Arora, 2019; Nishimoto, 2019). An increase in cassava production provides an excellent promise to meet this continuous demand for food and feed (Amelework et al., 2021c). When consumed as vegetables, cassava leaves offer a diet rich in micronutrients, proteins, and vitamins (Alamu et al., 2021).

2.3 Cassava taxonomy and its morphological features

Cassava (*M. esculenta*, 2n = 36) belongs to *Euphorbiaceae* family and is known to be monoecious (Hu et al., 2021). However, its male and female flowers are separated and mature at different times to escape self-pollination (e Sousa et al., 2021). Cassava is propagated vegetatively using stem cuttings, making it conveniently easy to establish the next cropping cycle (De Oliveira et al., 2020).

Moreover, the crop is exceptionally heterogeneous, and its sexual reproduction leads to unpredictable and diverse genetic diversity (Adjebeng-Danquah et al., 2020a; Baguma et al., 2019; Ferguson et al., 2019c; Ogbonna et al., 2021); hence its propagation from sexual seeds is of great interest to breeders. On the other hand, farmers rarely use cassava sexual seeds for propagation purposes (Ceballos et al., 2004).

Morphological characteristics, including leaf colour, apical leaf colour, petiole colour, and stem exterior colour, are important in distinguishing cassava varieties from one another (Karim et al., 2020a). These morphological characteristics enable farmers differentiate one cassava variety from another. This information is valuable for purposes such as cataloging and identifying different varieties, understanding their genetic diversity, and selecting appropriate varieties based on specific traits like disease resistance or yield potential.

2.4 Challenges to cassava production

Cassava has a great promise of feeding the ever-growing population across the tropical and subtropical countries of South America, Asia, and Africa. However, the crop faces several constraints that limit its potential yields (Narayanan et al., 2021b). Its production is constrained by existing biotic and abiotic factors (Reichert et al., 2021). Abiotic stresses, including climate variability, soil fertility, and resource limitations, influences the growth and productivity of cassava (Adejuwon & Agundiminegha, 2019; Oluwasanya et al., 2021; Pacheco et al., 2020). For instance, climate variability, such as drought, floods, and temperature fluctuations, disrupt plant growth and development (Cammarano, 2022; Hirpo, 2019; Raza et al., 2019). Drought conditions cause water stress, hindering root development and leading to reduced yields (Kim et al., 2020; Santos et al., 2020). On the other hand, excessive rainfall and flooding promotes root rot and nutrient leaching, negatively affecting cassava plants (Kerddee et al., 2021; Pipitpukdee et al., 2020b). Furthermore, poor soil fertility, nutrient deficiencies, and soil acidity pose challenges to cassava growth and nutrient uptake, further impacting yields.

Among the biotic stresses, CBB, CMD, and CBSD significantly reduces cassava yields (Ano et al., 2021; Ferris et al., 2020; Muhindo et al., 2020). CMD, caused by whitefly-transmitted viruses, leads to stunted growth, leaf deformations, and reduced plant vigor (Al Basir et al., 2021; Shirima et al., 2022b; Time et al., 2020). On the other hand, CBSD causes necrotic lesions and rotting in storage roots, leading to poor quality and yield losses (Maruthi, 2020). CBB is widespread across all cassava growing regions, and the disease is particularly severe in young plants during humid seasons.

Xam infects cassava leaves, resulting in leaf wilting, necrotic lesions, and stem rot (Dania & Ojeyemi, 2019a). It is a gram-negative bacterium, belonging to the gamma proteobacteria (Haq et al., 2021a). It causes a severe disease known as Cassava Bacterial Blight (CBB) when conditions are favorable during wet seasons (Dania & Ojeyemi, 2019b). CBB has historically caused serious starvation and malnutrition in African countries, including Kenya (Zandjanakou et al., 2001). The colonization process of *Xam* begins in the intracellular spaces of mesophyll tissues within the lamina tissue of the crop. The bacteria rapidly multiply and lyse the middle lamella, causing further damage to the plant (CABI, 2023). The accumulation of bacteria in the vascular regions of cassava stems can also harm the planting stakes, affecting the cleanliness of the crop (CABI, 2023b). CBB is widespread across all areas of Kenya under cassava cultivation (Odongo et al., 2019b). However, the incidence and severity of CBB vary depending on several factors. These factors include the developmental stage of the cassava plants, the predisposition of cassava varieties, climatic conditions, and the pressure exerted by the pathogen (Fanou et al., 2018).

CBB can cause 100% loss if it attacks young cassava plants and is not managed effectively (Fanou et al., 2018). The disease originated in Brazil and was first described by Bondar in (1912). In Kenya, CBB exists in all regions where cassava is grown (Odongo et al., 2019) and its severe outbreaks have been associated with environmental fluctuations (Toure et al., 2020). *Xam* infects cassava by multiplying around the stomata on the undersurface of the leaf and entering the vascular tissue, where it rapidly multiplies, colonizes, and lyses the host tissues (Gonzalez, 2022). However, the severity of the disease varies depending on the pathogenicity of *Xam*, the *Xam* population, and the genetics of the cassava plant varieties (Dania & Ojeyemi, 2019). The symptoms of CBB include angular leaf spotting, blight, and wilting. The stems may produce gum exudation, and necrosis can be observed in the stem and root vascular. The plant may also experience leaf loss and die-back (Fanou et al., 2018).

Fig. 2.1: Symptoms for cassava bacterial blight disease in the field. Photo taken by Elphas Simiyu on 23rd May 2021 at Kakamega field trial, showing symptoms of cassava bacterial blight disease.
Key: A = blight, B = angular leaf spot, C = leaf necrosis, D = wilting dieback and necrosis

2.5 Cassava Bacterial Blight Disease

Cassava Bacterial Blight disease (CBB) primarily affects the leaves, stems, and tuberous roots of cassava plants (CABI, 2023) and it manifests as water-soaked lesions on the leaves, which later turn brown and necrotic (Kante et al., 2020b). These lesions often coalesce, resulting in extensive defoliation (Zárate-Chaves et al., 2021b). Stem symptoms include wilting, rotting, and dieback (CABI, 2023), while infected tuberous roots develop brown necrotic lesions, rendering them unfit for consumption or propagation (Fanou et al., 2018). The pathogen enters the host through natural openings or wounds and spreads systemically through the vascular system of the plant (Banito et al., 2022).CBB significantly affects cassava production, causing yield losses of up to 100% in severe cases (Fanou et al., 2018). The disease reduces the photosynthetic capacity of infected plants due to defoliation, leading to reduced carbohydrate production and stunted growth (Parmar et al., 2017b). Moreover, plants infected with CBB have smaller tuberous roots with reduced starch content, affecting both food security and income generation for farmers (Fanou et al., 2018). The disease can cause complete crop failure if left unmanaged, posing a major threat to cassava-dependent communities (Narayanan et al., 2021b).

Management options for CBB include pathogen detection and diagnosis, sanitation and cultural practices, chemical control, and host resistance (Anuj et al., 2022). Early detection of CBB is crucial for effective disease management (López & Bernal, 2012b), and diagnostic tools, including polymerase chain reaction (PCR) and immunological assays, have been developed to detect the presence of *Xam* in infected plant tissues (Catara et al., 2021; Haq et al., 2021b). These tools aid in accurate and rapid diagnosis, enabling timely intervention.

On the other hand, good agricultural practices, including the removal and destruction of infected plant material, reduces the spread of CBB (Pérez et al., 2022). The agricultural practices includes proper field hygiene, avoiding mechanical damage to plants, and using clean planting materials from disease-free sources (Yaméogo et al., 2022; Frimpong et al., 2020). In addition, copper-based bactericides, such as copper oxychloride, have been used to manage CBB (López & Bernal, 2012b). These treatments can suppress the disease, particularly when combined with other management practices. However, the effectiveness of these management options depends on several factors, including farmers' knowledge and adoption of recommended practices, availability and affordability of inputs (such as resistant cultivars or pesticides), and the overall agroecological context (Constantine et al., 2020). Training and putting in place extension programs that provide farmers with knowledge about CBB management practices and promote their adoption are crucial for improving the effectiveness of these options (López & Bernal, 2012b).

Given the challenges and limitations of traditional management options, resistance breeding remains a crucial long-term strategy for managing CBB (Alonso et al., 2022; Zárate-Chaves et al., 2021c) since it provides a sustainable and environmentally friendly approach, reducing the reliance on chemical control and promoting resilience in cassava cultivation systems (Nelson et al., 2018b; Parry et al., 2020). There is a need for continuous research and breeding efforts to improve the resistance levels of cassava cultivars and address emerging challenges posed by CBB (Mbaringong et al., 2017).

Researchers worldwide have used cassava and its wild relative (*Manihot glaziovii*) to develop several hybrids using conventional breeding techniques (Amelework & Bairu, 2022; Wolfe et al., 2019). Cassava breeders take advantage of the resilient traits available in the wild relatives to build more productive varieties under biotic and abiotic stresses (Bredeson et al., 2016). Breeding for cassava genotypes resistant to biotic factors in Africa, including Kenya, Tanzania, Ghana, and Nigeria, started in the 1920s and 1930s (Okogbenin et al., 2013). However, cassava breeding programs have experienced challenges, including the low fertility and asynchronous flowering (Parmar et al., 2017). This hurdle affects the use of conventional breeding techniques in developing resistant cultivars for diseases such as CBB. The International Institute of Tropical Agriculture (IITA) has, however, since 1972, made promising progress in the conventional breeding of cassava against CBB. They sourced resistance to CBB from IITA, International Centre for Tropical Agriculture (CIAT), and East Africa germplasm and developed a promising clone 58308 (Hahn, 1978). Worldwide, surveillance and monitoring programs have been established to track the spread and incidence of CBB in different countries (Rache et al., 2023a). These programs involve close collaboration between national agricultural research institutions, extension services, and international organizations.

Breeding for resistance has been a key focus in managing CBB in Africa (Zárate-Chaves et al., 2021c). Through partnerships with international research institutions and national breeding programs, identification of utilization of germplasm with natural resistance to CBB have been carried out successfully (Sedano et al., 2017b; Sedano et al., 2017; Zhang et al., 2022c). Breeding programs have employed marker-assisted selection techniques to accelerate the breeding process, enabling the identification of resistant traits at early stages (Pathania et al., 2017). In addition, participatory approaches involving farmers have been implemented to ensure the suitability of developed cultivars to local agroecological conditions and the preferences of farmers growing cassava (Almekinders et al., 2006; Weltzien & Christinck, 2017). Encouraging farmers to participate in on-farm trials and demonstrations allows for the assessment of resistant cultivars and other management techniques, thereby facilitating their adoption (Worku et al., 2020).

Conventional breeding approaches have been employed in the search for cassava genotypes resistant to CBB (Parkes et al., 2013b). Researchers have employed techniques such as recurrent selection, hybridization, and backcrossing to introduce and accumulate resistance genes in commercial cassava varieties (Maurya et al., 2021; Mukiibi et al., 2019). Mating designs, such as controlled crosses between resistant and susceptible parents, have been employed to create segregating populations that can be screened for resistance (Bartlett et al., 2020). Advanced breeding methods, including multi-location trials and progeny testing, have been conducted to select and advance the most promising genotypes (Varshney et al., 2014).

Key findings from conventional breeding efforts include the identification of cassava genotypes with varying levels of resistance to CBB (Elegba et al., 2020; Jiwuba et al., 2020). Through rigorous screening and evaluation, researchers have identified sources of natural resistance and successfully introgressed these resistance traits into commercial varieties (Wang et al., 2022). This has promoted the development of improved cassava cultivars that exhibit enhanced resistance to CBB (Zhang et al., 2022d).

However, several challenges have been encountered in combating CBB through conventional breeding. One major challenge is the complexity of the CBB resistance trait, which is controlled by multiple genes (López & Bernal, 2012b). Identifying and introgressing all the necessary resistance genes into a single cultivar remains a difficult task. Furthermore, the genetic variability of the pathogen and its ability to overcome host resistance mechanisms pose ongoing challenges (Ferguson et al., 2019d). The development of durable resistance that can withstand pathogen evolution and adaptation requires continuous breeding efforts and monitoring of pathogen populations (Mundt, 2014). To address some of these challenges, advanced molecular breeding methods have been employed in CBB resistance breeding programs (Pathania et al., 2017). These methods involve the use of molecular markers associated with CBB resistance to assist in the selection of resistant genotypes at early stages of the breeding process. Marker-assisted selection (MAS) techniques have helped expedite the breeding process, improve selection efficiency, and increase the chances of developing CBB-resistant cassava cultivars (Nelson et al., 2018b).

Key findings from research using advanced molecular breeding methods include the identification and validation of molecular markers associated with CBB resistance (Sedano et al., 2017b). These markers have been used to screen large populations of cassava plants, enabling breeders to select individuals with the desired resistance traits. MAS has shown promising results in improving the efficiency and precision of breeding for CBB resistance (Ceballos et al., 2015). However, it should be observed that there are still some gaps in the literature regarding the use of advanced molecular breeding methods to manage CBB.

While significant progress has been made, there is a need for further research to fully understand the genetic basis of CBB resistance in cassava and to identify additional molecular markers associated with resistance (Zhang et al., 2022d). Additionally, studies assessing the durability and long-term effectiveness of CBB resistance conferred through molecular breeding approaches are necessary (Teixeira et al., 2021). Furthermore, to manage diseases such as CBB effectively, it is crucial to assess genetic diversity within cassava germplasm (Mukhopadhyay & Bhattacharjee, 2016). This information aids in selecting candidate parents for breeding programs and optimizing conservation and utilization strategies (Salgotra & Chauhan, 2023a).

2.6 Molecular markers used for genetic diversity

Several molecular markers have been utilized to investigate genetic diversity studies in cassava, including restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP), and start codon targeted (SCoT) polymorphism (Adjebeng-Danquah et al., 2020b; Ferguson et al., 2019e; Naa et al., 2020; Ocampo et al., 2022). The choice of markers relies on diverse factors, including their frequency in the genome, reproducibility, mode of inheritance, polymorphism level, ease of use, and availability (Ramesh et al., 2020). For example, AFLPs, which are dominant markers, have limitations in population genetic analyses of within-breed diversity and inbreeding. On the other hand, co-dominant markers like RFLPs, SNPs, and SSRs provide valuable information on between-breed and within-breed genetic diversity. SNPs, while they have low information content, can detect genetic variations that may impact protein structure or regulation (Joshi, 2020; Safder et al., 2021).

Previous studies have employed various genotyping platforms and assays to assess the genetic diversity. One commonly used approach is Restriction Fragment Length Polymorphism (RFLP) analysis (Harshitha & Sandal, 2022). This technique involves digesting the DNA of different cassava genotypes using specific restriction enzymes that cleave the DNA at predetermined recognition sites. Then, the resulting fragments are separated using agarose gel electrophoresis, and the fragment patterns are analysed. Variations in the lengths of the fragments indicate genetic differences between the genotypes under study. (Ovalle et al., 2020).

Another widely utilized method for evaluating cassava diversity is Random Amplified Polymorphic DNA (RAPD) analysis (Sheeja et al., 2021). RAPD employs short, arbitrary primers to target and replicate random regions of the cassava genome. The amplified fragments are visualized using agarose gel electrophoresis, and the presence/absence of bands in the resulting profiles indicates genetic variation among the tested genotypes (Semagn et al., 2006). RAPD offers a relatively quick and cost-effective approach for evaluating genetic variation in cassava populations (Adu et al., 2021).

SSRs, alternatively known as microsatellites, have also been widely utilized in cassava diversity studies (Beovides et al., 2015; John et al., 2014). SSR markers target specific repetitive DNA sequences with varying numbers of repeated units (Song et al., 2004). The analysis of the lengths of the repeated sequences in different genotypes promotes the determination of genetic variation and relatedness among cassava varieties. SSR markers are highly informative due to their high degree of polymorphism and codominant nature (Zhou et al., 2019), making them valuable tools for studying cassava genetic diversity. Other genotyping platforms and assays that have been employed in molecular characterization studies of cassava include AFLP, SNP genotyping, and DNA sequencing techniques (Bicko et al., 2021; Lokko et al., 2005; Pootakham et al., 2014). These methodologies provide researchers with detailed information about the genetic composition, variability, and relationships among cassava genotypes.

SCoT markers, on the other hand, offer insights into genetic diversity at the gene level, are highly reproducible, and are cost-effective as they only require agarose gel electrophoresis for resolution (Huded et al., 2020; Igwe et al., 2022). SCoT markers have been used in diversity studies for several reasons. Firstly, they have been employed to evaluate the genetic variation within germplasm collections (Owiti et al., 2023). They can detect polymorphisms at the gene level, providing insights into the genetic variation present among different cassava varieties. Additionally, SCoT markers have the potential to identify novel alleles or unique genetic variants within germplasm collections (Tahir et al., 2023). These markers can uncover previously unknown genetic diversity, providing valuable knowledge for expanding the genetic base within breeding populations and introducing new traits into cassava cultivars. Research on using SCoT markers in other crops has yielded various findings. For instance, these markers have been employed for evaluating the genetic diversity and population structure of *Pistacia* species of Iran germplasm collections from different regions (Zarei & Erfani-Moghadam, 2021).

SCoT markers were selected as the chosen marker platform for this study based on several considerations. SCoT markers are a type of PCR-based molecular marker, which specifically targets the start codon region of genes (Alsamman et al., 2017). They offer a simple and cost-effective approach for evaluating genetic diversity in cassava populations (Huang et al., 2014). One of the primary reasons for choosing SCoT markers is their simplicity and ease of use. SCoT markers employ a single primer that targets the conserved start codon sequence, simplifying the experimental design and reducing the associated costs (Kamińska et al., 2020). In addition to their simplicity, SCoT markers have been shown to exhibit high levels of polymorphism (Bhattacharyya et al., 2013). Therefore, it is possible that these markers can detect a broad spectrum of genetic variations in diverse cassava germplasm. The ability to capture such variations is crucial for accurately assessing the genetic diversity of cassava populations

CHAPTER THREE

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF KENYAN CASSAVA GERMPLASM

3.1 Abstract

The selection of appropriate cassava parents is critical for national breeding programs to enhance genetic variability and develop superior recombinant genotypes. This study aimed to determine suitable cassava parents by evaluating 15 Kenyan cassava germplasm for genetic diversity using morphological features and molecular markers. The research was conducted at the Kakamega and Kiboko research stations of the Kenya Agricultural and Livestock Research Organization. A randomized complete block design with three replications was employed, and a 1 m spacing between plants and 1.5 m between plots was observed. Six plants per variety were tagged for data collection. Morphological data were collected at early growth stage, mid-growth stage, and late growth stage from the tagged plants. For molecular analysis, DNA extraction was performed using leaves sampled from the field experiments. Three leaves per tagged plant were randomly collected and then transported to the laboratory for subsequent DNA extraction. Later, DNA extraction was performed following a modified Cetyl Trimethyl Ammonium Bromide (CTAB) protocol, and PCR was performed using 30 Start Codon Targeted (SCoT) primers. Among the primers used, seven exhibited polymorphism. Cluster analysis based on morphological features identified seven clusters, while SCoT marker analysis revealed five main clusters, including sub-clusters A and B within cluster I. The study found that environmental factors did not significantly influence the assessed morphological traits. Notably, SCoT markers demonstrated their potential to generate genetic polymorphism in cassava, thereby offering a valuable tool for genetic diversity studies. These findings provide valuable insights for breeders in national breeding programs, aiding in the selection of appropriate cassava parents to maximize genetic variability and develop superior recombinant genotypes.

3.2 Introduction

Cassava (*Manihot esculenta* Crantz) holds significant agricultural importance, mainly in the tropics, for its adaptability to poor marginal soil, harsh climatic conditions, and high production per unit of land and labor (Udoro et al., 2021). Moreover, cassava has a flexible harvesting time all year round since its edible storage roots can stay below ground for more than 24 months without going bad (Enesi et al., 2022; Frimpong et al., 2020b). Cassava has a promising potential to bridge the food security gap in sub-Saharan countries (Olarinde et al., 2020) reducing cases of famine in the regions where cassava is grown and consumed. However, cassava production is significantly low in Kenya because of biotic factors (Paul et al., 2022).

It is critical to accurately characterize Kenyan cassava germplasm; thus, identifying genetic variability for selection of potential candidate parents in breeding against biotic stresses (Ferguson et al., 2019f; Perez-Fons et al., 2020). Thus, assessing the genetic diversity of cassava germplasm is an important step in the development of improved cassava varieties that exhibit enhanced resistance to biotic factors (Manze et al., 2021). Genetic diversity pertains the presence of different varieties and variability of genetic traits within a population or group of individuals. Understanding the genetic diversity of cassava germplasm helps breeders gain valuable insights into the genetic composition and potential traits of different cassava varieties (Swarup et al., 2021a).

To explore the genetic diversity of cassava germplasm, several approaches can be employed. One approach is the characterization of agronomic, morphological, and yield traits (Diniz & de Oliveira, 2019; Kamanda et al., 2020; Karim et al., 2020b). This involves studying the observable characteristics and performance of cassava plants, such as plant height, leaf shape, tuber yield, disease resistance, and other agronomic traits. The evaluation of these traits helps breeders assess the genetic variations and select parental candidates that possess desirable traits for further breeding.

Molecular characterization techniques are another important approach in assessing the genetic diversity of cassava germplasm. Researchers use different molecular markers, such as DNA-based markers, to identify specific regions of the genome that exhibit genetic variations among different cassava genotypes (Amiteye, 2021; Kesawat & Das Kumar, 2009). These markers provide insights into the genetic diversity and relatedness of cassava varieties, enabling breeders to make informed when selecting candidate parents. The employment of these diverse approaches helps breeders to comprehensively understand the genetic diversity within available cassava germplasm (Ferguson et al., 2019f).

This knowledge allows breeders to select suitable parental candidates that possess the desired characteristics, such as resistance to biotic factors. The incorporation of genetic diversity into breeding programs promotes development of improved cassava varieties that exhibit enhanced resistance, better productivity, and adaptability to various environmental conditions (Palanivel & Shah, 2021; Salgotra & Chauhan, 2023b). Previous assessments of genetic diversity in cassava varieties have explored various approaches to managing the challenge of disease resistance (Nybom & Lācis, 2021). Some studies have identified and used resistant sources available in the germplasm, but the emergence of new strains of pathogens has rendered the existing germplasm insufficient in conferring resistance to these strains (Rache et al., 2023b; Velásquez et al., 2018). Therefore, the identification and incorporation of new germplasm that exhibits diverse genetic traits and resistance mechanisms are necessary to address this ongoing challenge (Zhang et al., 2017). The incorporation of diverse genetic material in breeding programs not only improves the resistance to CBB but also enhances the overall genetic resilience of cassava crops, making them better equipped to withstand future challenges and environmental changes (Govindaraj et al., 2015; Swarup et al., 2021b).

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3.3 Materials and methods

3.3.1 Plant materials

Fifteen (15) cassava germplasm (Table 3.1) were collected from KALRO research stations of Kakamega, Embu, and Mtwapa. The germplasm included both the local commercial varieties and landraces to ensure a comprehensive representation of the cassava germplasm in the study. This approach allowed for the assessment of genetic diversity across genotypes that are relevant to the local farming communities. The cassava germplasm were then planted in experimental field trials established at the Kakamega and Kiboko research stations of KALRO in the 2020/2021 cropping season

Table 3.1: List of 15 cassava germplasm, source of material, and description of the germplasm used for the establishment of experimental field trials for characterization using morphological features and SCoT-PCR tools

Germplasm	Source of material	Description
NASE 14	KALRO-Kakamega	Commercial genotype
Migyera	KALRO-Alupe	Landrace
Fumbachai	KALRO-Alupe	Landrace
Ebwanatereka 2	KALRO-Kakamega	Commercial genotype
Kisimbani	KALRO-Mtwapa	Commercial genotype
Mariakani	KALRO-Mtwapa	Commercial genotype
KME 1	KALRO-Embu	Commercial genotype
KME 2	KALRO-Embu	Commercial genotype
Karembo	KALRO-Mtwapa	Commercial genotype
Ex- Ndoro	KALRO-Embu	Commercial genotype
Tajirika	KALRO-Mtwapa	Commercial genotype
MM96/9308	KALRO-Kakamega	Commercial genotype
MM97/0293	KALRO-Kakamega	Commercial genotype
MM96/2480	KALRO-Kakamega	Commercial genotype
KCA 2	KALRO-Embu	Commercial genotype

3.3.2 Site Descriptions

The germplasm were planted in experimental field trials conducted at two different KALRO research stations: Kakamega and Kiboko. The selection of these research stations was based on their representation of different agro-ecological zones, allowing for a broader assessment of the genetic diversity across varying environments. The Kakamega research station, located at coordinates approximately 0.2733°S latitude and 34.7278°E longitude, provided a suitable site for evaluating cassava germplasm in the Western region of Kenya. This region is characterized by a tropical rainforest climate, with abundant rainfall and relatively high humidity. The annual rainfall in this region ranges from 1500 to 2000 millimetres or even more.

The plentiful rainfall contributes to the lush vegetation and fertile soil, making it suitable for a various crops, including cassava. The average annual temperatures at Kakamega research station are approximately 20°C to 28°C (68°F to 82°F), providing a favourable growing environment for various plant species.

On the other hand, the Kiboko research station, with its coordinates approximately 2.4686°S latitude and 37.8409°E longitude, represented a different agro ecological zone in the Eastern region of Kenya. Kiboko receives low rainfall, from 500 to 800 millimetres, annually. The semi-arid conditions of the region create challenges for rain-dependent crops but make it a suitable location to study drought-tolerant crops like cassava. The average annual temperatures at Kiboko research station are approximately 25°C to 30°C (77°F to 86°F), indicating hotter and drier conditions compared to Kakamega..

3.3.3 Experimental Layout and Design

The experimental design for this study involved the replication of the experiments three times using a randomized complete block design (RCBD). The purpose of this design was to minimize potential sources of variability and ensure the robustness of the results. Cassava cuttings, approximately 30 cm in length, were used for planting in the experimental units. Each experimental unit had dimensions of 1 x 10 m. The spacing between individual cassava plants within a plot was maintained at 1 m, allowing sufficient room for their growth and development.

To facilitate the organization and separation of the plots, alleyways measuring 1.5 m were established between the plots. These alleyways served as physical dividers between adjacent plots and helped prevent interference or cross-contamination between the cassava plants. The randomized complete block design was implemented by dividing the research area into blocks, with each block representing a distinct replication of the experiment. The allocation of the different cassava genotypes within each block was randomized to minimize any potential bias or systematic effects.

During the planting process, care was taken to ensure uniformity and accuracy. The cassava cuttings were planted at the predetermined spacing within the experimental units, maintaining consistent distances between plants to avoid overcrowding or uneven competition for resources. Throughout the experimental period, standard agricultural practices were employed to ensure optimal plant growth and minimize confounding factors. This included appropriate soil preparation and adequate weed management.

3.3.4 Morphological data collection

3.3.4.1 Field experiment

Eighteen morphological features (Table 3.2) were analysed according to Fukuda et al., (2010). Data was collected on morphological traits, including leaf colour, colour of stem exterior, and petiole colour, from the 15 Kenyan cassava germplasm. The plants were sampled and tagged for data collection by randomly selecting six representative plants per variety. This sampling approach was chosen to ensure a sufficient representation of each variety while minimizing the workload and time required for data collection. The choice of these specific time points for data collection aligns with the typical growth and development stages of cassava and allows for a comprehensive evaluation of its agronomic and morphological characteristics throughout its growth cycle.

The assessment of agronomic and morphological traits of cassava was performed at three different stages: three (early growth stage), six (mid-growth stage), and nine months after planting (late growth stage). At the early growth stage, data were collected to assess the initial growth and emergence phase of the cassava plants. Traits such as apical leaf colour and pubescence on apical leaves were recorded. These traits provide insights into the early morphological characteristics and leaf development of the plants.

During the mid-growth stage, the reproductive phase of the cassava plants begins. Data collection focused on traits related to flowering, reproductive performance, and potential yield. Traits such as leaf lobe margin, leaf vein colour, petiole colour, orientation of the petioles, prominence of foliar scars, colour of stem cortex, and colour of stem epidermis were assessed. These traits are indicative of the reproductive capacity of the plants and can provide valuable information about their yield potential.

In the late growth stage, data collection included traits that reflect the plant's maturity and overall architecture. Traits such as colour of stem exterior, distance between leaf scars, growth habit of stem, colour of end branches of the adult plant, length of stipules, and stipule margin were assessed. These traits contribute to understanding the plant's structural characteristics, branching patterns, and maturity, which can impact its productivity and suitability for different agricultural purposes.

Table 3.2: List of morphological features used to assess the diversity of 15 cassava germplasm.

No.	Trait descriptor	Score code	Sampling time
1	Apical leaf colour	3 = Light green, 5 = Dark green, 7 = Purplish green, 9 = Purple	3 MAP
2	Pubescence on apical leaves	0 = Absent, 1 = Present	3 MAP
3	Central leaf shape	1 = Ovoid, 2 = Elliptic-lanceolate, 3 = Obovate-lanceolate, 4 = Oblong-lanceolate, 5 = Lanceolate, 6 = Straight or linear, 7 = Pandurate, 8 = Linear-piramidal, 9 = Linear pandurate, 10 = Linear-hostatilobalate	6 MAP
4	Leaf colour	3 = Light green, 5 = Dark green, 7 = Purple green, 9 = Purple	6 MAP
5	Leaf lobe number	Count	6 MAP
6	Leaf lobe margin	3 = Smooth, 7 = Winding	6 MAP
7	Leaf vein colour	3 = Green, 5 = Reddish-green in less than half of the lobe, 7 = Reddish-green in more than half of the lobe, 9 = All red	6 MAP
8	Petiole colour	1 = Yellowish-green, 2 = Green, 3 = Reddish-green, 5 = Greenish-red, 7 = Red, 9 = Purple	6 MAP
9	Petiole orientation	1 = Inclined upwards, 3 = Horizontal, 5 = Inclined downwards, 7 = Irregular	6 MAP
10	Prominence of foliar scars	3 = Semi-prominent, 5 = prominent	9 MAP
11	Colour of stem cortex	1 = Orange, 2 = Light green, 3 = Dark green	9 MAP
12	Colour of stem epidermis	1 = Cream, 2 = Light brown, 3 = Dark brown, 4 = Orange	9 MAP
13	Colour of stem exterior	3 = Orange, 4 = Greeny-yellowish, 5 = Golden, 6 = Light brown, 7 = Silver, 8 = Grey, 9 = Dark brown	9 MAP
14	Distance between leaf scars	3 = Short, 5 = Medium, 7 = Long	9 MAP
15	Growth habit of stem	1 = Straight, 2 = Zig-zag	9 MAP
16	Colour of end branches of adult plant	3 = Green, 5 = Green-purple, 7 = Purple	9 MAP
17	Length of stipules	3 = Short, 5 = Long	9 MAP
18	Stipule margin	1 = Entire, 2 = Split or Forked	9 MAP

Key: MAP = Months after planting

3.3.5 Morphological data analysis

Morphological features of the 15 cassava varieties were analysed using the multivariate analysis technique GenStat 15th Edition software version. A cluster analysis was conducted using the GenStat 15th Edition software. This involved assessing the morphological traits of the cassava varieties to identify similarities and dissimilarities. The cluster analysis generated a dendrogram to visually represent the grouping or clustering of varieties based on their morphological characteristics. Moreover, Principal Component Analysis (PCA) was carried out, rather than performing an Analysis of Variance (ANOVA), to generate Eigenvalues, and each trait contribution's relevance to variation was accounted for by each principal component (PC). The decision to use PCA was based on the nature of the data and the research objective.

ANOVA is typically applied to test for significant differences between groups or treatments based on mean values. It is commonly used for analysing quantitative data with categorical independent variables. However, in the case of morphological traits, which often exhibit complex patterns of variation and are difficult to categorize into distinct groups, ANOVA may not be the most appropriate analysis method. PCA, on the other hand, is a dimensionality reduction technique that allows for the exploration of patterns and relationships in multivariate data. It helps to identify the key sources of variation and the underlying structure within the dataset. Therefore, in this study, PCA was selected as the data analysis technique for the morphological traits due to its ability to capture the complex relationships and patterns in the data, providing a comprehensive understanding of the trait variability among the cassava varieties.

3.3.6 Molecular Characterization

3.3.6.1 Sample collection

At three months after planting, at least three leaves per variety were collected from the field trials and pressed between two sheets of flipcharts in the order in which they were collected, ensuring that the leaves did not overlap. The flipcharts containing the leaves samples were placed between two corrugated cardboards. The corrugated cardboards were put between two herbarium frames and tightly tied using a sisal twine roll. This was followed by transportation to the laboratory at KALRO, Biotechnology Research Institute-Kabete centre, to extract genomic DNA to perform the SCoT-PCR analyses.

3.3.6.2 DNA Extraction

The extraction of DNA was performed using a modified CTAB method described by Turaki et al., (2017). About 100 mg of dried leaf tissues were put in a 2 ml microfuge tube after which it was filled with 750 µl of warm (65˚C) CTAB extraction buffer (100mM Tris-HCl pH 8.0, 2% CTAB, 20mM EDTA pH 8.0, 1.4M NaCl, and 5% β-Mercaptoethanol). The tissues were ground using a sterile ceramic bead on the fast-prep machine at 4m/s for 60 seconds. This step was repeated for complete homogenization of the tissues. Tubes containing homogenate were incubated at 65°C for 30 minutes in a water bath with inversions after every 10 minutes to mix the content and then incubated on the bench for 10 minutes. The microfuge tubes were spun at 14000 rpm for 1 minute, after which the supernatant was transferred to sterile 1.5 ml eppendorf tubes, and an equal volume of chloroform isoamyl alcohol (24:1) was added and mixed gently by inverting. Then, centrifugation was performed at 14000 rpm for 10 minutes. A total of 500 µl clear aqueous solution was pipetted into a fresh sterile 1.5 ml tube, after which ice-cold isopropanol (500 µl) was added, gently inverting to mix well before incubating for 30 minutes at -20°c. The eppendorf tubes were centrifuged at 14000 rpm for 10 minutes, then discarded the supernatant. Next, the pellet was washed using 600 µL 70% ethanol, followed by a 5 min centrifugation at 14,000 rpm before discarding the supernatant. The process was repeated twice, followed by air-drying the pellet at room temperature for 30 minutes to remove the residual ethanol. Later, the DNA was resuspended in nuclease-free water (100 µl).

3.3.6.3 DNA Quality and Quantity

DNA quality and quantity were assessed for each extracted sample. To determine DNA quality, a small portion of the extracted DNA (5 µl) was resolved on a 0.8% w/v agarose gel stained with a fluorescent dye (1% Ethidium bromide). Agarose gel electrophoresis was performed by loading the DNA samples onto the gel. The gel was then subjected to electrophoresis at a voltage of 75 V for 45 min, allowing the DNA fragments to migrate through the gel matrix.

After electrophoresis, the gel was visualized under ultraviolet (UV) light. The DNA bands were examined for their integrity, clarity, and absence of degradation. However, the DNA ladder was not used in the assessment of the quality of DNA. This is because the inclusion of a DNA ladder was not necessary as it would not provide additional information relevant to the evaluation of DNA integrity.

To determine the quantity of extracted DNA, a NanoDrop2000 Spectrophotometer was used. A total of 1 µl volume of each sample with extracted DNA was pipetted onto the sample pedestal of the spectrophotometer. The instrument measured the absorbance of the DNA sample at specific wavelengths, typically 260 nm, to estimate the concentration of DNA in the sample. The spectrophotometer also provided information on the purity of the DNA by measuring the absorbance ratio at different wavelengths (260 nm/280 nm). This ratio can indicate the presence of contaminants or impurities in the DNA sample.

3.3.6.4 Marker Analysis

A total of 30 Start Codon Targeted (SCoT) primers specific for SCoT markers (Table. 3.3) were used. The extracted DNA from the 15 cassava varieties was amplified using 2 µL template DNA, BioLabs 2X oneTaq master mix, 10mM forward primer, 10mM reverse primer, and PCR grade water. The conditions for PCR were: 94°C (3 minutes) and 35 cycles of 94°C (30 seconds), 52°C (20 seconds), 72 °C (30 seconds), and 72 °C (5 minutes) final extension.

Agarose gel electrophoresis analysis was performed to investigate the polymorphism of Start Codon Targeted (SCoT) markers in the 15 cassava germplasm. A 2% agarose gel was utilized, and the electrophoresis was conducted at a voltage of 85 V. The gel was labeled based on the arrangement of the cassava germplasm as presented in Table 3.7. PCR results (Fig. 3.7) were visualized under a gel documentation system (Uvitec Gel Documentation | Thermo Fisher Scientific - AU) after gel electrophoresis on 1% w/v agarose gel.

Table 3.3: Selection of Highly Polymorphic Start Codon Targeted (SCoT) Primers for Molecular Characterization of 15 Kenyan Cassava Germplasm. A total of 30 SCoT primers were used in the screening process.

Primer No.	Primer	Sequence
1	SCoT 6	5' - CAACAATGGCTACCACGC - 3'
2	SCoT 90	5' - CCATGGCTACCACCGGCA - 3'
3	SCoT 27	5' - ACCATGGCTACCACCGTC - 3'
4	SCoT 36	5' - GCAACAATGGCTACCACC - 3'
5	SCoT 8	5' - CAACAATGGCTACCACGT - 3'
6	SCoT 43	5' - CAATGGCTACCACCGCAG - 3'
7	SCoT 11	5' - AAGCAATGGCTACCACCA - 3'
8	SCoT 28	5' - CCATGGCTACCACCGCCA - 3'
9	SCoT 48	5' - ACAATGGCTACCACTGGC - 3'
10	SCoT 21	5' - ACGACATGGCGACCCACA - 3'
11	SCoT 7	5' - CAACAATGGCTACCACGG - 3'
12	SCoT 18	5' - ACCATGGCTACCACCGCC - 3'
13	SCoT 5	5' - CAACAATGGCTA CCACGA - 3'
14	SCoT 13	5' - ACGACATGGCGACCATCG - 3'
15	SCoT 19	5' - ACCATGGCTACCACCGGC - 3'
16	SCoT 25	5' - ACCATGGCTACCACCGGG - 3'
17	SCoT 93	5' - ACCATGGCTACCAGCGCA - 3'
18	SCoT 12	5' - ACGACATGGCGACCAACG - 3'
19	SCoT 20	5' - ACCATGGCTACCACCGCG - 3'
20	SCoT 30	5' - CCATGGCTACCACCGGCG - 3'
21	SCoT 23	5' - CACCATGGCTACCACCAG - 3'
22	SCoT 55	5' - ACAATGGCTACCACTACC - 3'
23	SCoT 29	5' - CCATGGCTACCACCGGCC - 3'
24	SCoT 10	5' - CAACAATGGCTACCAGCC - 3'
25	SCoT 59	5' - ACAATGGCTACCACCATC - 3'
26	SCoT 60	5' - ACAATGGCTACCACCACA - 3'
27	SCoT 84	5' - ACGACATGGCGACCACGT - 3'
28	SCoT 31	5' - CCATGGCTACCACCGCCT - 3'
29	SCoT 52	5' - ACAATGGCTACCACTGCA - 3'
30	SCoT 4	5' - CAACAATGGCTACCACCT - 3'

From the PCR results, gel pictures of the seven highly polymorphic SCoT marker primers (Table 3.4) were selected for allele scoring to characterize the 15 cassava varieties. The amplified DNA fragments obtained from the PCR reactions were subsequently used for downstream analysis, such as genetic diversity assessment using SCoT markers. Bands of DNA at different fragment sizes based on the DNA molecular size marker were recorded in an excel workbook as 1 for presence and 0 for absence, and the data were used to analyze the genetic relationships and diversity among the cassava varieties.

Table 3.4: List of seven SCOT markers used to characterize the 15 cassava germplasm

Primer No.	Primer	Sequence
1	SCoT 11	5' - AAGCAATGGCTACCACCA - 3'
2	SCoT 13	5' - ACGACATGGCGACCATCG - 3'
3	SCoT 19	5' - ACCATGGCTACCACCGGC - 3'
4	SCoT 28	5' - CCATGGCTACCACCGCCA - 3'
5	SCoT 43	5' - CAATGGCTACCACCGCAG - 3'
6	SCoT 48	5' - ACAATGGCTACCACTGGC - 3'
7	SCoT 90	5' - CCATGGCTACCACCGGCA - 3'

The data obtained from the PCR gel picture was processed and converted into binary matrices, where the presence of a DNA fragment was assigned a value of 1, and its absence was assigned a value of 0. This binary representation allowed for the analysis of genetic variation among the 15 cassava germplasm. The frequency of the alleles across the markers was determined, and the polymorphism information content (PIC) was calculated. PIC provides a measure of the informativeness of a marker in distinguishing between different genotypes. The PIC values were calculated and recorded in Table 3.8.

Following the scoring of bands (presence/absence) across the 15 germplasm, the data was compiled and organized in an Excel workbook. The Excel workbook contained information about the presence or absence of specific DNA fragments for each germplasm sample. The data generated in the Excel workbook was further analysed using the genetic analysis software package Dissimilarity Analysis and Representation for Windows (DARwin). This is a widely used software tool for analysing genetic diversity and conducting dissimilarity analyses. It provides various statistical methods and visualization tools to explore the genetic relationships among samples.

In DARwin, the binary data matrix was imported, and dissimilarity analysis was performed to calculate the genetic distances between pairs of cassava germplasm. Based on these genetic distances, a dendrogram or other graphical representations were generated to visualize the genetic relationships among the 15 cassava germplasm. The use of DARwin allowed for the exploration of genetic diversity, identification of genetic clusters, and assessment of the genetic relatedness among the cassava genotypes based on the SCoT marker data. This analysis provided valuable information about the genetic structure and diversity within the set of cassava germplasm studied.

3.4 Results

3.4.1 Morphological characterization

3.4.1.1 Frequency distribution of the 15 cassava varieties according to morphological traits

The distribution of the morphological features in the 15 cassava varieties is presented in Figs. 3.1, 3.2, 3.3, and 3.4. The 15 cassava varieties varied in their morphological features. Overall, 80% of the 15 germplasm exhibited a dark-green leaf colour, while about 20% of the germplasm had a light green leaf colour (Fig. 3.1a). All the germplasm under this study had a smooth lobe margin. The central leaflet of the 15 varieties consisted of 40% elliptic-lanceolate, 53.3% lanceolate, and 6.7% straight or linear (Fig. 3.1b).

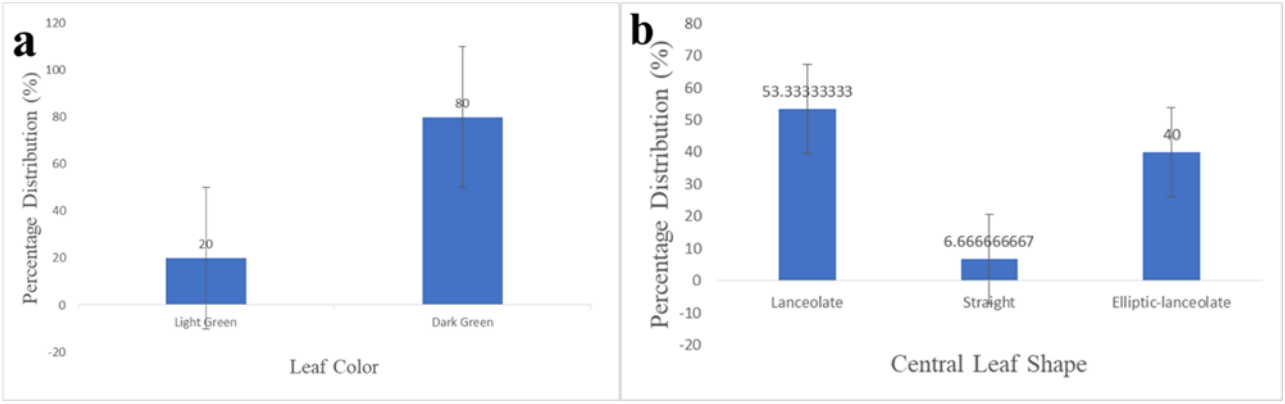

Fig 3.1: Percentage distribution of (a) leaf colour, and (b) central leaf shape among 15 Kenyan cassava germplasm.

The apical leaf colour of 86.7% of cassava germplasm was purplish-green, and the apical leaf colour of 13.3% was purple (Fig. 3.2a). Furthermore, the apical leaves of 60% cassava germplasm were pubescent (Fig. 3.2b).

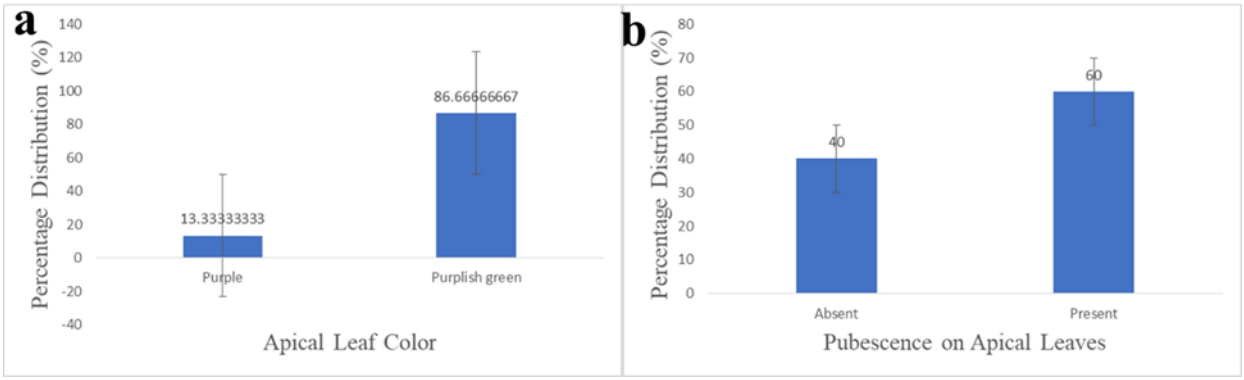

Fig. 3.2: Percentage distribution of (a) Apical leaf colour; (b) pubescence on apical leaves among 15 Kenyan cassava germplasm.

In this study, 13.33% of the germplasm had five leaf lobe numbers, while 87.67% had seven leaf lobe numbers (Fig. 3.3a). The leaf vein of 33.3% of the germplasm was green, while 20% of the germplasm showed a reddish-green leaf vein colour in more than half of the lobe, and 46.7% had reddish-green in less than half of the lobe (Fig. 3.3b).

Regarding petiole colour 13.3% of the germplasm were yellowish-green, 13.3% reddish-green, 26.7% greenish-red, 13.3% red, and 33.3% red were purple (Fig. 3.3c). For petiole orientation, about 66.7%, 6.7%, and 26.7% of the cassava germplasm had a horizontal, upward inclination, and an irregular petiole orientation, respectively (Fig. 3.3d).

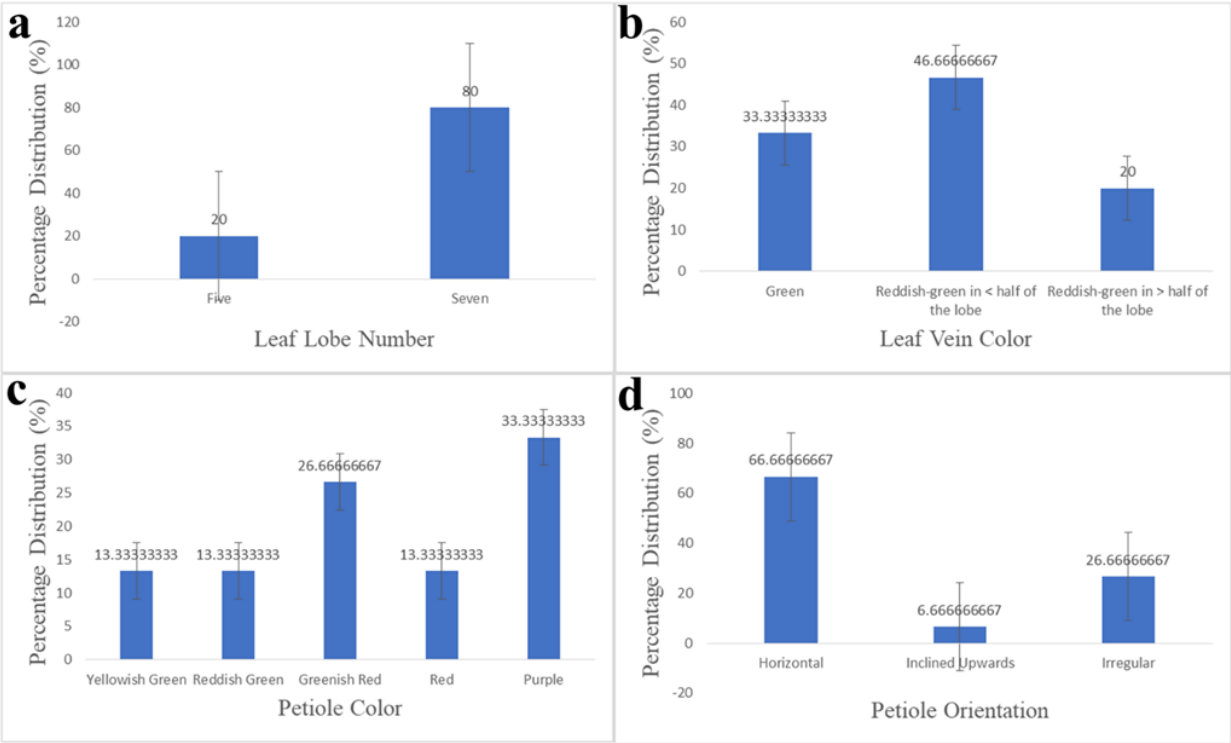

Fig. 3.3: Percentage distribution of (a) leaf lobe number; (b) leaf vein colour; (c) petiole colour; and (d) petiole orientation among 15 Kenyan cassava germplasm.

On the other hand, the stem cortex of 66.7% of the germplasm was dark-green (Fig. 3.4a) and light green in 33.3% of the germplasm. About 40% of the germplasm had light green stem epidermis, while 60% were dark green (Fig. 3.4b). The stem exterior for 66.7% of the germplasm was silver, 6.7% light brown, and 26.7% dark brown. The adult plants of the germplasm had either green-purple (93.3%) or purple (6.7%) branches (Fig. 3.4c).

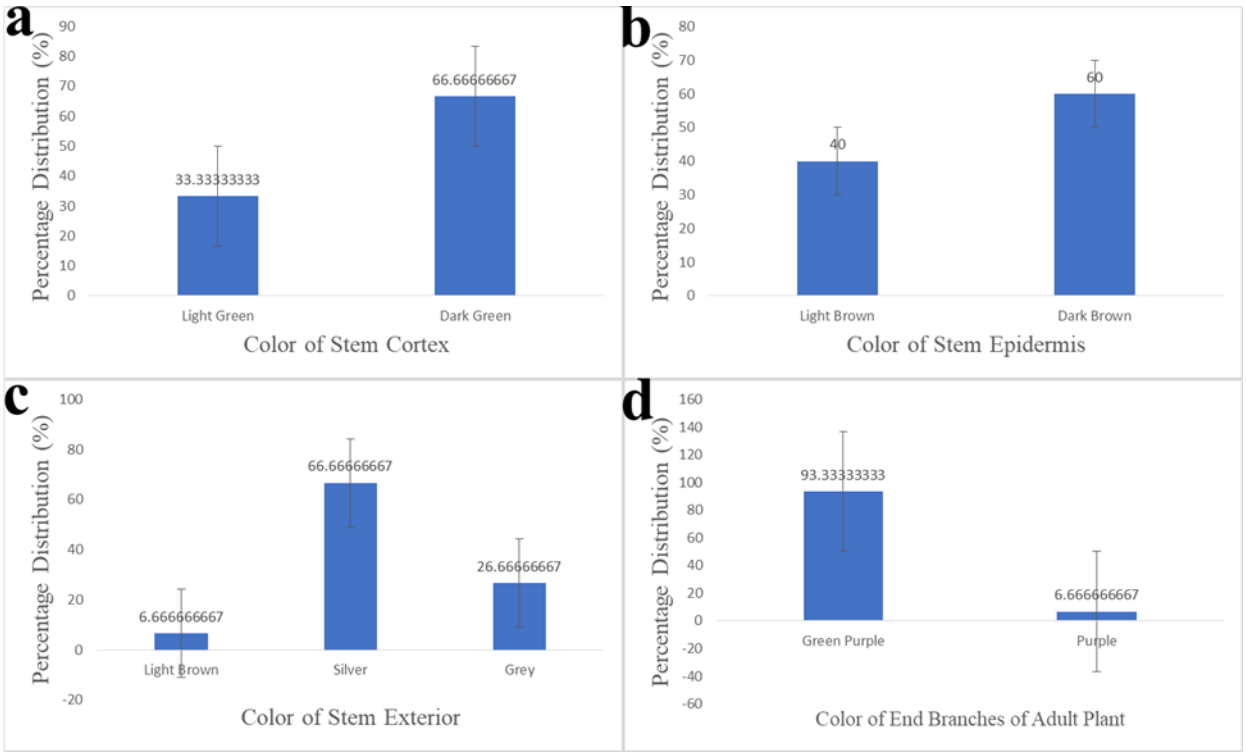

Fig. 3.4: Percentage distribution of (a) colour of stem cortex; (b) colour of stem epidermis; (c) colour of stem exterior; and (d) colour of end branches of adult plant among 15 Kenyan cassava germplasm.

3.4.1.2 Principal component analysis of morphological characters

The principal component analysis revealed 55.04% of total variation among the 15 cassava varieties (Table 3.5). The contribution of the first three components in Principal Component Analysis (PCA) was as follows: 55.04% of the variance was accounted for by the Principal Component 1 (PC1), 15.89% by the Principal Component 2 (PC2), and 9.58% by the Principal Component 3 (PC3). With PC1 explaining 55.04% of the total variance, it captured the most significant amount of information from the dataset. PC1 was significant among seven varieties, including Ebwanatereka 2, Ex – ndoro, Fumbachai, KME 1, KME2, Mariakani, and NASE 14, as evidenced by their positive score of the component (Table 3.5). In contrast, Karembo, KCA 2, Kisimbani, Migyera, MM96/2480, MM96/9308, MM97/0293, and Tajirika had negative scores of the component, suggesting that the component is not significant to these varieties.

Table 3.5: Principal component scores and the percentage variation among the germplasm

No.	Germplasm	PC 1
1	Ebwanatereka 2	3.5666
2	Ex- Ndoro	3.4979
3	Fumbachai	2.1621
4	Karembo	-3.7441
5	KCA 2	-0.2108
6	Kisimbani	-1.1238
7	KME 1	0.7495
8	KME 2	2.8466
9	Mariakani	4.0847
10	Migyera	-1.661
11	MM96/2480	-3.5461
12	MM96/9308	-5.3704
13	MM97/0293	-4.9791
14	NASE 14	4.7661
15	Tajirika	-1.0381
	Percentage Variation	**55.04**

Based on its high latent vector (loadings), the most significant trait was petiole colour (latent vector = 0.83923), followed by leaf vein colour (latent vector = 0.38183) and petiole orientation (latent vector = 0.29989) (Table. 3.6). Central leaf shape, apical leaf colour, the colour of end branches of adult plant, leaf lobe number, length of stipules, and pubescence on apical leaves were also positively associated with the principal component. In this study, evidenced by their high latent vector (loadings), three morphological features were the most significant in separating the different germplasm: petiole colour, petiole orientation, and leaf vein colour (Table 3.6). In addition, morphological features, including central leaf shape, apical leaf colour, colour of the end branches of the adult plant, leaf lobe number, length of stipules, and prominence of foliar scars positively contributed to the differentiation of the germplasm.

The cassava germplasm had varied petiole colour, including yellowish-green, reddish-green, greenish-red, red, and purple. The most consistent colour of the petioles was purple and greenish red. Germplasm that exhibited the purple colour of petioles were Ebwanatereka 2, Ex-ndoro, KME 2, Mariakani, and NASE 14. The greenish red petiole colour was observed in KCA 2, Kisimbani, Migyera, and Tajirika. Fumbachai and KME 1 were observed to have red petiole colour while MM96/9308 and MM97/0293 had yellowish green petiole colour.

A high number of the germplasm had a horizontal petiole orientation, including Ebwanatereka 2, Ex-ndoro, Karembo, Kisimbani, KME 1, Migyera, MM96/2480, MM96/9308, MM97/0293, and Tajirika. Fumbachai, KCA 2, Mariakani, and NASE 14 had an irregular petiole orientation. However, the petiole orientation of KME 2 was inclined upwards. Moreover, most cassava germplasm had either the lanceolate or elliptic-lanceolate central shape of the leaflet. However, Fumbachai had a straight central shape of the leaflet, distinguishing it from the rest.

Table 3.6: Latent vector (loadings) of the principal component analysis for the cassava germplasm

No.	Morphological trait descriptor	PC 1
1	Central leaf shape	0.07798
2	Apical leaf colour	0.093
3	Colour of end branches of adult plant	0.0501
4	Colour of stem cortex	-0.01479
5	Colour of stem epidermis	-0.03624
6	Colour of stem exterior	-0.03126
7	Distance between leaf scars	0
8	Flowering	-0.02113
9	Growth habit of stem	0
10	Leaf colour	-0.1523
11	Leaf lobe margin	0
12	Leaf lobe number	0.05926
13	Leaf vein colour	0.38183
14	Length of stipules	0.10473
15	Petiole orientation	0.29989
16	Prominence of foliar scars	0.04349
17	Pubescence on apical leaves	-0.01364
18	Stipule margin	-0.0046
19	Petiole colour	0.83923

3.4.1.3 Cluster analysis based on 18 morphological traits

The cluster analysis using morphological traits classified the 15 cassava varieties into seven classes (Fig. 3.5). These classes reflect different genetic relationships and similarities among the varieties. Cluster I comprised Ebwanatereka 2, Migyera, NASE 14, Kisimbani, MM97/0293, KME 2, Karembo, and MM96/9308. Ebwanatereka 2 and NASE 14 of cluster I recorded a 95% genetic relatedness, suggesting the most similarity between the two varieties.

Tajirika and MM96/2480 clustered together in Group IV, revealing genetic similarity between the two varieties. Clusters II, III, V, VI, and VII were genetically distinct, with Ex- Ndoro, Fumbachai, KCA 2, KME 1, and Mariakani, respectively. These clusters indicate unique genetic characteristics and differences compared to the other cassava varieties.

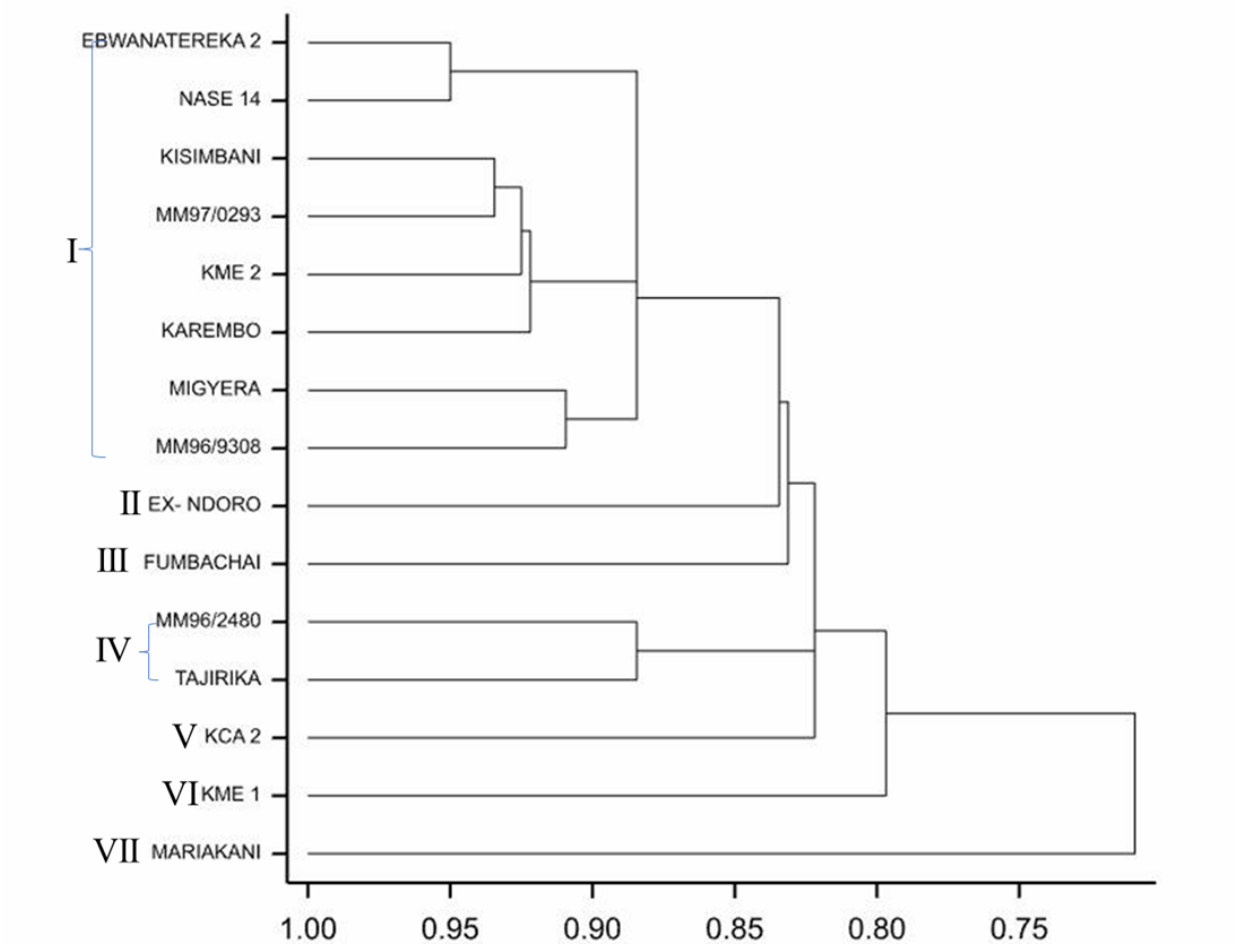

Fig. 3.5: Dendrogram based on morphological features showing relationships among 15 Kenyan cassava germplasm

3.4.2 Molecular characterization

3.4.2.1 DNA quality

The extraction of DNA from the young leaves of 15 cassava varieties was successful, namely Ebwanatereka 2, Ex-Ndoro, Fumbachai, Karembo, KCA 2, Kisimbani, KME 1, KME 2, Mariakani, Migyera, MM96/2480, MM96/9308, MM97/0293, NASE 14, and Tajirika. Subsequent analysis using agarose gel electrophoresis revealed clear bands across all the studied varieties. (Fig. 3.6).

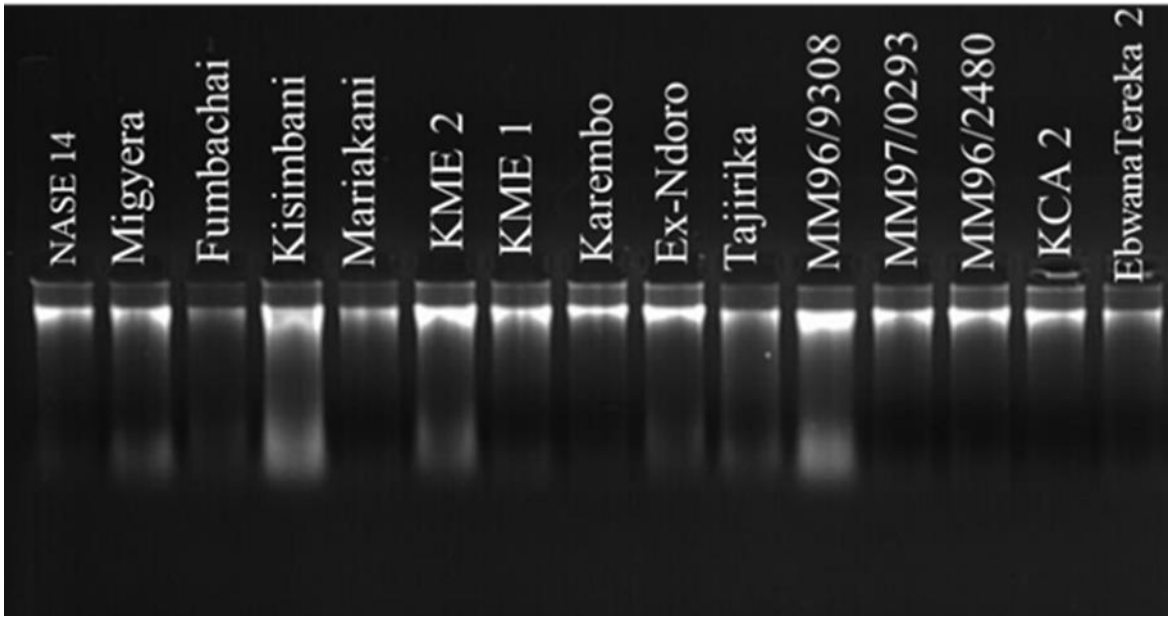

Fig. 3.6: Gel picture showing DNA extracted from 15 cassava germplasm

3.4.2.2 DNA quantity

The varieties differed in the quantity of extracted DNA based on Nanodrop readings (Table 3.7). For example, MM97/0293 yielded the highest extractable DNA (4289 ng/µl), while Fumbachai yielded the lowest extractable DNA (107.9 ng/µl). Meanwhile, MM96/2480, NASE 14, Karembo, Tajirika, and MM96/9308 also had very high DNA quantities of 4262, 4235, 3725, 3561, and 1011 ng/µl, respectively. Mariakani, Kisimbani, KME 2, and KME 1 yielded 614.6, 719.7, 745.9, and 752 ng/µl, respectively. On the other hand, satisfactory amounts of extractable DNA of 119.7 (KCA 2), 353.5 (Ex-ndoro), and 355.6 ng/µl were obtained from KCA 2 (Migyera).

Table 3.7: Results of extracted DNA quantified using a NanoDrop2000 Spectrophotometer

Sample ID	Genotype	DNA	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	Nase 14	4235	ng/µl	84.706	40.194	2.11	1.94
2	Migyera	355.6	ng/µl	7.112	3.183	2.23	0.83
3	Fumbachai	107.9	ng/µl	2.158	1.287	1.68	0.41
4	Kisimbani	719.7	ng/µl	14.393	6.775	2.12	1.1
5	Mariakani	614.6	ng/µl	12.293	5.694	2.16	1.1
6	KME 2	745.9	ng/µl	14.917	6.885	2.17	1.2
7	KME 1	752	ng/µl	15.041	6.926	2.17	0.81
8	Karembo	3725	ng/µl	74.508	34.064	2.19	1.01
9	Ex-ndoro	353.5	ng/µl	7.07	3.181	2.22	0.6
10	Tajirika	3561	ng/µl	71.212	33.618	2.12	2.3
11	MM96/9308	1011	ng/µl	20.216	9.585	2.11	1.77
12	MM97/0293	4289	ng/µl	85.78	42.191	2.03	1.38
13	MM96/2480	4262	ng/µl	85.235	41.081	2.07	2.1
14	KCA 2	119.7	ng/µl	2.393	1.095	2.19	2.61
15	Ebwanatereka 2	235.6	ng/µl	4.712	2.235	2.11	2.9

3.4.2.3 Polymerase Chain Reaction (PCR)

Fig. 3.7 displays the results of the agarose gel electrophoresis, revealing highly polymorphic SCoT markers across the studied cassava germplasm. The distinct bands observed in the gel indicate genetic variations among the genotypes, reflecting the presence of different DNA fragments amplified by the SCoT markers. The labeling of the gel corresponds to the arrangement of the cassava genotypes as provided in Table 3.7, enabling easy identification and comparison of the amplified DNA fragments among the different genotypes. The successful visualization of polymorphic SCoT markers in the agarose gel demonstrates the suitability of this marker system for evaluating genetic variation in the cassava germplasm. These results provide a foundation for further genetic analyses, such as marker-assisted breeding and diversity studies, contributing to the understanding and utilization of the cassava genotypes examined. A total of 30 SCoT markers were evaluated to investigate the presence of genetic variations in the 15 cassava germplasm. Among these markers, several exhibited varying patterns of amplification and polymorphism across the studied genotypes. The monomorphic markers, which showed identical banding patterns across all genotypes, were SCoT 5, 6, 7, 8, 21, 25, 31, 60, 18, 27, 29, 30, 36, 59, 84, and 20. These markers failed to capture genetic variations among the cassava genotypes and, therefore, were considered non-informative for the diversity analysis.

SCoT 52 amplified only one sample (Mariakani), indicating a unique genetic profile specific to that particular genotype. On the other hand, SCoT 93 displayed faint bands that were difficult to score accurately due to their low intensity. SCoT 4, 12, 55, 23, and 10 did not amplify any fragments across the entire set of cassava genotypes, suggesting the absence of specific DNA sequences targeted by these markers. In contrast, SCoT 11, 13, 19, 28, 43, 48, and 90 exhibited polymorphism among the 15 cassava genotypes. These markers generated diverse banding patterns, reflecting significant genetic variations among the studied genotypes.

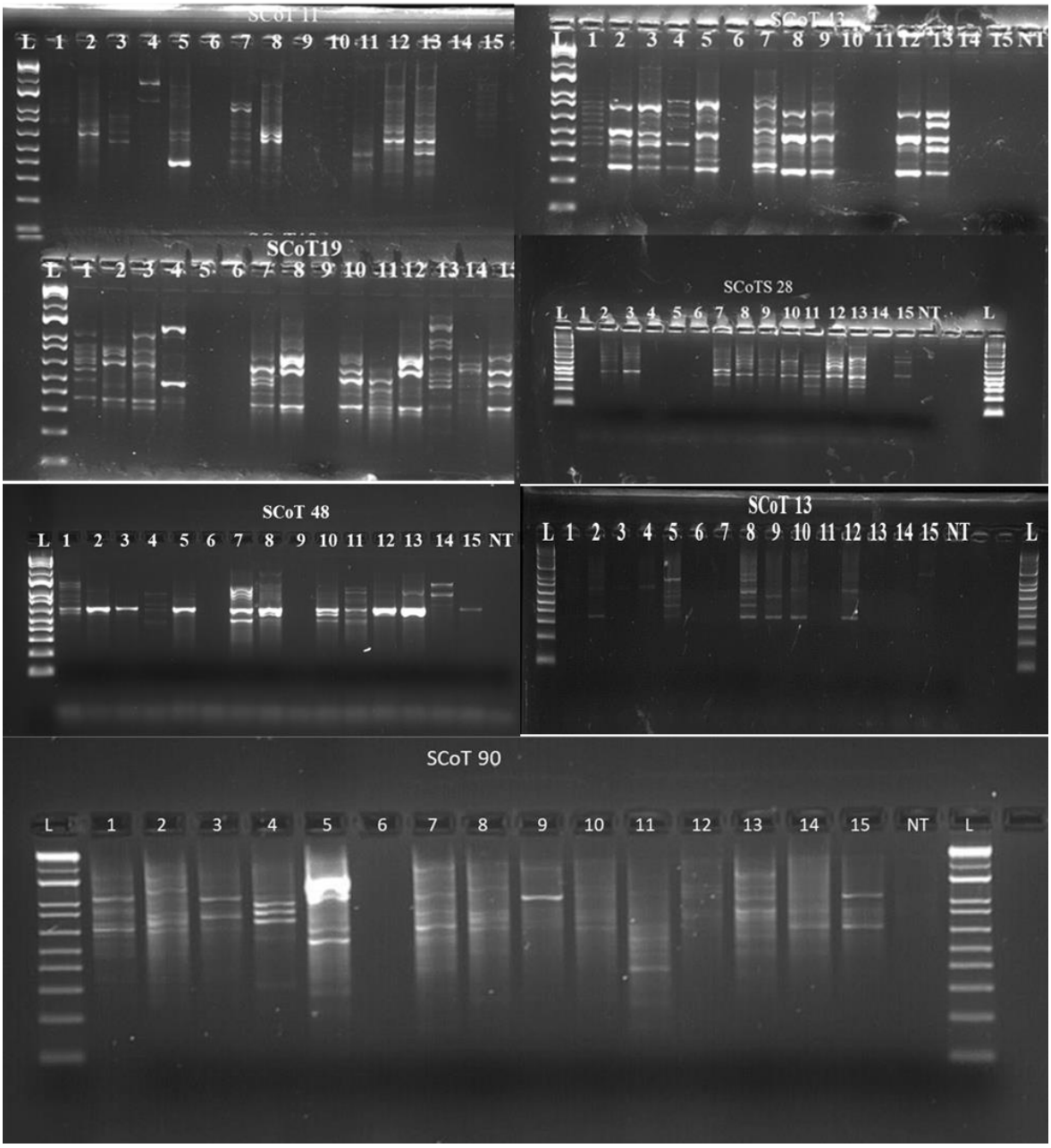

Fig. 3.7: Agarose gel electrophoresis image displaying highly polymorphic Start Codon Targeted (SCoT) markers in the 15 cassava germplasm

3.4.2.4 Cluster analysis based on SCoT markers

All seven SCoT markers revealed 310 DNA fragments. Among the 310, 215 were polymorphic (Table 3.8). The mean polymorphic fragments were 30.71. More polymorphic fragments (55) across the 15 cassava varieties were detected using SCoT 19. In addition, SCoT 11 had the highest polymorphism percentage (83.7%), while SCoT 28 had the lowest polymorphism percentage (40.6%). The average polymorphism percentage across germplasm was 67.4%. Based on the PIC values, the marker SCoT 11 was the most discriminative marker with a PIC percentage value higher than 0.5. Thus, SCoT 11 detected the highest genetic variation among the 15 cassava varieties, with a value of 0.54. Meanwhile, ScoT 19, 48, and 43 had 0.42, 0.35, and 0.32 PIC, respectively. Alternately, ScoT 90, 13, and 28 had low PIC (0.30, 0.16, and 0.07, respectively).

Table 3.8: Summary information on total bands (DNA fragments), total polymorphic bands (polymorphic fragments), percentage of polymorphic bands, and polymorphism information content (PIC) obtained using seven SCoT markers in 15 cassava germplasm

Primer	Total bands	Polymorphic bands	Percentage of polymorphic bands	PIC
SCoT 11	43	36	83.7	0.54
SCoT 13	18	12	67.7	0.16
SCoT 19	75	55	73.3	0.42
SCoT 28	32	13	40.6	0.07
SCoT 43	68	48	70.6	0.32
SCoT 48	42	30	71.4	0.35
SCoT 90	32	21	65.6	0.30
Total	**310**	**215**		
Average	44.30	30.71	67.4	0.31

Variations within the 15 cassava varieties were established using the ScoT primer markers analysis. The Start Codon Targeted (SCoT) markers identified and classified cassava germplasm into five main clusters (Fig. 3.8). Consistent with the morphological markers, Ebwanatereka 2, NASE 14, Migyera, Karembo, MM97/0293, and MM96/9308 grouped in one cluster. Also, KCA 2, KME 2, and Kisimbani were observed to fall in independent clusters. Using the SCoT marker primers, Ex- ndoro and Mariakani grouped in cluster III (Fig 3.8). Cluster I had sub-clusters A and B.

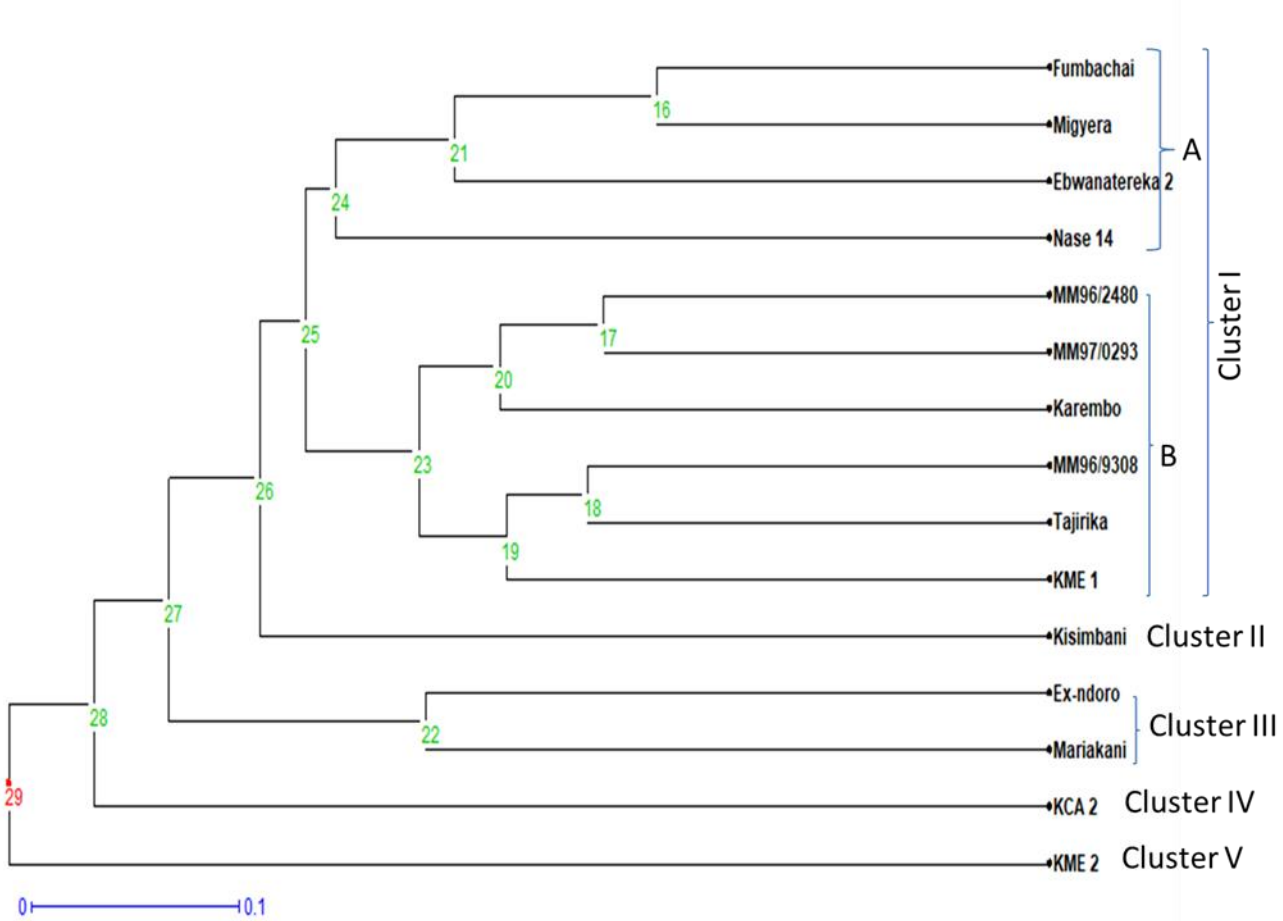

Fig. 3.8: Hierarchical clustering of 15 Kenyan cassava germplasm based on seven highly polymorphic SCoT marker primers

3.5 Discussion

Morphological traits, such as petiole colour, petiole orientation, and leaf vein colour, are significantly important in the early identification, characterization, and selection of cassava germplasm with desirable characteristics (Agre et al., 2015). These traits provide valuable information for researchers and breeders in their efforts to improve cassava varieties. One of the key advantages of morphological traits is their ability to serve as visual markers for the identification and differentiation of cassava genotypes (Okogbenin et al., 2013). Petiole colour, for instance, exhibits variation from green to purple, which can be easily observed and recorded. This variation enables quick assessment and classification of different genotypes based on their petiole colour, facilitating the early identification and characterization of cassava germplasm.

Similarly, petiole orientation is another important morphological trait that contributes to the overall plant architecture and growth habit (Adu et al., 2020). It can range from erect to spreading, and this variation provides additional information for researchers and breeders. The examination of petiole orientation provides insights into the growth patterns and vigor of different cassava genotypes, aiding in their characterization and selection for specific breeding goals (Diaguna et al., 2022; Ha et al., 2016).

Scientific evidence from various studies supports the significance of morphological traits in cassava improvement efforts. For instance, studies conducted by Jansson et al., (2009) and Pujol et al., (2005) have highlighted the importance of using morphological traits, including petiole colour, petiole orientation, and leaf vein colour, to identify, characterize, and classify cassava germplasm. In the study, the analysis of morphological features revealed significant differences among the 15 cassava varieties. This variation in leaf attributes, including petiole colour, leaf colour, leaf vein colour, and leaf shape (e.g., lanceolate central leaf), provides a basis for distinguishing and identifying different cassava genotypes (Tiago et al., 2020).

The identification of distinct leaf attributes is particularly important for breeding programs as it allows researchers to select elite genotypes with specific traits of interest. For example, the presence of purplish-green apical leaf colour, dark green leaf colour, reddish-green leaf vein, and lanceolate central leaf can be indicative of certain desired characteristics, such as higher nutritional content or better adaptation to specific environmental conditions (Fongod et al., 2012). Petiole colour, petiole orientation, and leaf vein colour can be linked to breeding efficiency. Petiole colour can be an indicator of disease resistance or tolerance, as certain colours may be associated with resistance to specific pathogens (Kawuki et al., 2011). Petiole orientation can influence the plant's ability to capture sunlight efficiently, affecting photosynthesis and overall growth (Yang et al., 2022). Leaf vein colour may be associated with nutrient uptake and transport efficiency, contributing to the plant's overall health and productivity (Hu et al., 2021).

The use of Principal Component Analysis (PCA) in discriminating cassava germplasm based on morphological traits has proven to be effective in this study. PCA allowed for the reduction of several correlated variables into a smaller number of independent components, revealing three significant morphological features: petiole colour, petiole orientation, and leaf vein colour. These features were found to be responsible for the phenotypic differences among the 15 cassava germplasm analyzed. The study also highlighted the practicality of these traits in identifying appropriate varieties and assessing genetic diversity in cassava. The morphological characteristics, including petiole colour, leaf vein colour, petiole orientation, length of stipules, and colour of the apical leaves, positively contributed to the PCA and aided in the discrimination of cassava germplasm. The results of the Principal Component Analysis indicated that 55.04% of the total variability in the morphological analysis could be explained by the identified features. This finding underscores the usefulness of Fukuda et al.'s morphological descriptor (2010) in identifying variability within cassava germplasm. The effectiveness of PCA in discriminating cassava germplasm using morphological traits has been supported by related studies in the field. For example, research by (Asare et al., 2011) demonstrated the utility of PCA in characterizing the diversity of cassava landraces based on morphological traits. Similarly, a study by Bakare et al., (2022) employed PCA to identify key morphological traits linked to root yield and quality in cassava.

Herein, the utilization of SCoT markers provided valuable insights into the genetic diversity of the cassava germplasm. Analysis based on SCoT markers revealed a significant number of polymorphic fragments, indicating a wide range of genetic variations among the 15 cassava varieties. This demonstrates that SCoT markers are effective in detecting genetic diversity at the molecular level. The mean polymorphic fragments per marker, with an average of 30.71, further supports the reliability and robustness of SCoT markers in capturing genetic variations. The polymorphism percentage across the germplasm was calculated to be 67.4%. This relatively high value indicates a considerable level of genetic diversity within the studied cassava varieties.

The ability of SCoT markers to identify and classify this genetic diversity makes them a valuable tool for genetic variation studies. Moreover, the use of SCoT markers allows for the detection of genetic variations that may not be visible through traditional morphological markers, providing a more comprehensive understanding into the genetic landscape of cassava germplasm (Ferguson et al., 2012).

PIC values calculated for each SCoT marker offer insights into their discriminatory power. SCoT 11 displayed the highest PIC value of 0.54, indicating that it can detect a broad spectrum of genetic variations and that it has potential as a highly informative marker. On the other hand, SCoT 90, 13, and 28 had lower PIC values, suggesting limited discriminatory power. These results emphasize the importance of marker selection in genetic studies, as markers with higher PIC values can provide more detailed information about genetic diversity and assist in identifying varieties with unique genetic characteristics (Kumar et al., 2020).

The cluster analysis according to SCoT marker data resulted in the classification of the 15 cassava varieties into five distinct clusters. This clustering pattern reflects the genetic relatedness and differentiation among the varieties. Clusters I, II, III, IV, and V encompassed different varieties, indicating varying degrees of genetic similarity or divergence. The clustering information obtained from SCoT markers can be utilized for germplasm conservation efforts, breeding programs, and the identification of suitable parental combinations for trait improvement (Salgotra & Chauhan, 2023).

The cluster analysis based on morphological characteristics varied from that based on genetic markers, consistent with previous studies (Bellemou et al., 2020), suggesting an inadequate correlation between them, thus a bottleneck for the exclusive use of genetic markers in diversity studies. The combination of morphological traits and SCoT markers provided complementary information, allowing for a comprehensive assessment of the genetic variation and differentiation. These findings have important implications for cassava breeding programs, germplasm conservation, and the selection of parental combinations for trait improvement.

3.6 Conclusions

The insights gained from this study can guide future research and breeding efforts aimed at harnessing the genetic potential of cassava. The identified traits that contribute significantly to the genetic variation, such as petiole colour, petiole orientation, and leaf vein colour, can be targeted for further investigation and application in breeding programs. Additionally, the genetic relationships among the varieties and the clustering pattern observed can provide information on the development of breeding strategies that maximizes the genetic diversity and enhance desirable traits in future cassava cultivars.

CHAPTER FOUR

IDENTIFICATION OF CASSAVA GERMPLASM WITH RESISTANCE TO CASSAVA BACTERIAL BLIGHT

4.1 Abstract

Bacterial blight of cassava (CBB), caused by *Xanthomonas axonopodis* pv. manihotis (*Xam*), mainly threatens cassava production worldwide, leading to significant yield reduction. Effective management of CBB requires breeders to develop and deploy resistant cassava varieties. This study aimed to assess the resistance of 15 cassava germplasm to CBB in different agro-ecological environments and evaluate the implications for CBB management. Field trials were carried out in Kakamega and Kiboko research stations of Kenya Agricultural and Livestock Research Organization (KALRO), using a Randomized Complete Block Design (RCBD) with three replications. Additionally, a greenhouse trial was established under a completely randomized design (CRD). The evaluation of CBB symptoms was carried out at 7-day intervals in the greenhouse and 30-day intervals in the field. The scoring of CBB symptoms followed a scale ranging from 1 (no symptoms) to 5 (plant death). The 7-day interval in the greenhouse experiment allowed for the early detection of disease symptoms and provided a rapid assessment of the response of cassava germplasm to CBB under controlled conditions. On the other hand, the 30-day interval in field experiments allowed for longer observation periods to capture the progression and development of CBB symptoms in natural field conditions. The results demonstrated significant variation ($P < 0.001$) in the response of the cassava germplasm to CBB under natural infectivity and artificial inoculation. At the Kiboko trial site, all 15 cassava varieties exhibited tolerance to natural *Xam* infection. In the Kakamega field trial, Ebwanatereka 2, KME 2, Migyera, Karembo, Fumbachai, KME 1, Tajirika, Kisimbani, MM97/0293, NASE 14, Mariakani, and Ex-ndoro showed tolerance to CBB. However, under artificial inoculation, some initially tolerant varieties showed susceptibility to CBB. The identification of tolerant cassava genotypes, including NASE 14, Migyera, Ebwanatereka 2, Fumbachai, and MM97/0293, provides potential candidates for breeding programs aimed at developing CBB-resistant cassava cultivars. These tolerant genotypes can be integrated into breeding efforts to enhance the resistance of future cassava varieties to CBB.

4.2. Introduction

Cassava (*Manihot esculenta* Crantz) is a staple food crop, which plays a crucial role in food security and generation of income for millions of people in tropical regions (De Souza et al., 2017). However, its production is severely constrained by various diseases, among which bacterial blight (CBB), attributable to *Xanthomonas axonopodis* pv. manihotis (*Xam*), poses a significant challenge (Mbaringong et al., 2017). CBB is prevalent in Africa, Latin America, and Asia, where cassava is extensively cultivated (McCallum et al., 2017). Its symptoms include the development of angular leaf spots, wilting, plant dieback, and eventually, complete plant death (Fanou et al., 2018).

CBB not only causes direct yield losses but also affects the quality of cassava roots, rendering them unsuitable for consumption or processing (Gbadegesin, 2013). Yield losses attributed to CBB can vary depending on different cultivars, different environmental conditions, or both. According to Toure et al. (2020), CBB can lead to 100% yield loss under certain circumstances. Different cassava varieties exhibit varying levels of susceptibility to CBB. Some varieties may possess inherent resistance or tolerance mechanisms that allow them to withstand the disease's impact, resulting in lower yield losses (Sundin et al., 2016). On the other hand, environmental conditions are vital in determining CBB severity and its subsequent impact on yields (Toure et al., 2020). Factors such as temperature, humidity, and rainfall patterns can create favorable conditions for the development and spread of the CBB pathogen (Fanou et al., 2018). For example, high humidity and prolonged leaf wetness promote the growth and dissemination of *Xam* (Gbadegesin, 2013; Sundin et al., 2016). In such conditions, the disease can rapidly progress, leading to higher yield losses compared to periods with less favorable environmental conditions.

The severity of CBB is influenced by a combination of factors, including the virulence of the pathogen, susceptibility of cassava varieties, and prevailing environmental conditions (Fanou et al., 2018). *Xam* is known for its high aggressiveness and ability to rapidly spread within and between fields (Daniel et al., 2019). Cassava varieties exhibit varying levels of resistance to CBB, with some being highly susceptible while others show moderate to high tolerance or resistance (Mbaringong et al., 2017; Teixeira et al., 2021b).

Efforts to manage CBB and mitigate its impact on cassava production have been undertaken (Daniel et al., 2019; Sedano et al., 2017). Traditional control methods, such as cultural practices and chemical treatments, have been employed to reduce disease incidence (Fanou et al., 2018). However, these approaches are not entirely effective, and their sustainability and environmental impact are questionable. Breeding for CBB resistance has emerged as a promising strategy to combat the disease (Hahn, 1978). Cassava breeding programs worldwide have made significant progress in developing CBB-resistant varieties (Zhang et al., 2022). The incorporation of genes are responsible for resistance in the wild relatives of cassava has proven successful in enhancing CBB resistance in cultivated varieties (Zhang et al., 2017). Marker-assisted selection (MAS) has been utilized to expedite the breeding process and facilitate the identification of resistant genotypes (Pathania et al., 2017). However, the idea of developing commercially viable, high-yielding, and locally adapted genotypes that are resistant to CBB remains a priority (Thiele et al., 2021).

The purpose of this study was to assess the resistance of 15 cassava germplasm to CBB and evaluate their performance under different agro-ecological environments. Through the identification of resistant genotypes, the study aimed to contribute to the development of CBB-resistant cultivars, which can effectively manage the disease and reduce yield losses. Understanding the role of environmental conditions and cassava variety types in predisposing cassava plants to CBB is crucial for effective disease management (Graziosi et al., 2016). Environmental factors, such as high humidity and prolonged leaf wetness, promotes a conducive environment for the pathogen to grow and spread (Velásquez et al., 2018). Additionally, certain cassava varieties may lack the necessary genetic traits that confer resistance or tolerance to CBB, promoting their susceptiblity to the disease (Mbaringong et al., 2017). Therefore, by investigating the interaction between environmental conditions, cassava varieties, and CBB incidence, we can gain insights into the mechanisms that contribute to the susceptibility or resistance of cassava plants to CBB (Sedano et al., 2017).

4.3 Materials and Methods

4.3.1 Field Experiments

4.3.1.1 Germplasm

A collection of 15 Kenyan cassava germplasm was chosen for this study, comprising a combination of commercial genotypes and landraces. This selection aimed to ensure the representation of diverse genetic backgrounds and variations in traits associated with cassava bacterial blight resistance. The inclusion of both commercial genotypes and landraces provided a comprehensive evaluation of resistance, considering the different breeding histories and adaptability of these varieties.

The chosen commercial genotypes included NASE 14, Ebwanatereka 2, Kisimbani, Mariakani, KME 1, KME 2, Karembo, Ex- Ndoro, Tajirika, MM96/9308, MM97/0293, MM96/2480, and KCA 2. These genotypes are widely cultivated in various regions of Kenya, making them important representatives of the country's cassava production. Their inclusion allowed for an assessment of resistance in commonly grown commercial varieties and provided insights into their potential for disease management.

Additionally, two landraces, Migyera and Fumbachai, were included in the study. Landraces possess unique genetic traits that have been developed through generations of cultivation and adaptation to specific local conditions. Including these landraces provided a broader representation of the genetic diversity in the Kenyan cassava germplasm and allowed for evaluation of traditional varieties' resistance to cassava bacterial blight

4.3.1.2 Site Description

Two field experiments were established at KALRO in Kakamega (Kakamega County) and Kiboko stations (Makueni County). The Kakamega trial site (approximately 0.2733°S latitude and 34.7278°E longitude) was previously cultivated with sweet potatoes. The annual rainfall in the Kakamega region ranges from 1500 to 2000 millimetres or even more, while the average annual temperatures are approximately 20°C to 28°C (68°F to 82°F).

On the other hand, the Kiboko trial site (approximately 2.4686°S latitude and 37.8409°E longitude) had no immediate previous crop. In Kiboko, the annual rainfall varies between 500 to 800 millimetres and the average annual temperatures of approximately 25°C to 30°C (77°F to 86°F).

4.3.1.3 Field Layout and Design

The field trials were established using RCBD, with three replications, to ensure reliable results. Each cassava variety was planted using healthy stem cuttings measuring approximately 30 cm in length. The experimental units were arranged in plots measuring 1 x 10 m, with a 1 m spacing between plants. Alleyways measuring 1.5 m in width were used to separate plots, facilitating convenient access and minimizing any potential inter plot interference.

4.3.1.4 Field Data collection

Disease symptom data were collected as part of the field evaluation process to assess the presence and severity of cassava bacterial blight. The data collection began three months after planting, allowing sufficient time for disease establishment and symptom development. At 30-day intervals, observations were made on a total of six selected cassava plants per variety. These plants were previously tagged to ensure accurate identification and consistent monitoring throughout the study.

To quantify the severity of the disease symptoms, a subjective scoring system was employed. The scoring scale ranged from 1 to 5, enabling the classification of symptom severity in a qualitative manner. A score of 1 indicated the absence of any visible symptoms, signifying healthy plants. On the other hand, a score of 5 denoted complete plant death, reflecting severe infection and the plant's inability to recover.

Intermediate scores were assigned based on the progression and intensity of symptoms observed. The specific scoring criteria utilized were as follows: a score of 2 represented angular leaf spotting, which involved the presence of angular-shaped spots or lesions on the leaves. A score of 3 indicated leaf wilting, characterized by significant drooping or wilting of the foliage, suggesting further progression of the disease.

For plants assigned a score of 4, noticeable plant dieback was observed, with sections of the plant displaying necrosis or decay. This score reflected advanced disease progression and substantial damage to the plant. The systematic data collection approach employed in this study ensured a comprehensive evaluation of cassava bacterial blight symptoms over time. By starting the data collection three months after planting, it allowed for the differentiation between disease-related symptoms and initial establishment issues or non-disease-related stressors. The subjective scoring system provided a means to compare and quantify the severity of symptoms across the different cassava varieties under evaluation.

4.3.1.5 Field Data Analysis

The first step in the data analysis was the calculation of the Area Under the Disease Progress Curve (AUDPC) for each cassava variety. AUDPC provides a measure of disease severity over time. For each of the six tagged plants per variety, the first two infection scores were averaged, and this average was multiplied by the time interval (30 days) between the two readings to obtain the trapezoid area. This process was repeated for the second, third, fourth, and fifth infection readings, and the trapezoid areas were summed to calculate the cumulative AUDPC for each plant (Campbell & Madden, 1990). The formula used to calculate AUDPC is a shown below:

$$\mathbf{AUDPC} = \sum_{i=1}^{n-1} 0.5\left(x_{i+1} + x_i\right)\left(t_{i+1} - t_i\right)$$

Where n = total number of observations, x_i = an assessment of disease at i^{th} observation, t_i = time in days at i^{th} observation.

Next, the data collected from the six tagged plants per variety at each month was subjected to General Analysis of Variance (ANOVA) (in Randomized blocks) Fisher's unprotected least significant difference test in Gen-stat 15^{th} edition. The LSD was tested at P = 0.05. The Fisher's unprotected least significant difference test was performed as a post-hoc test to determine significant differences in means.

The means generated by ANOVA were recorded for further analysis and interpretation. To determine the overall mean disease data per variety, a calculation was performed by combining the means generated through the ANOVA for the six successive time points of data collection per variety. This calculation was aimed at obtaining a representative average of the disease progression across the entire duration of the study.

First, the means from the six time points were summed for each variety, resulting in a cumulative value representing the total disease severity recorded over the entire data collection period. To obtain the overall mean, the cumulative value was divided by the number of time points (six) in the data collection. This division ensured that the resulting value represented an average disease severity across all time intervals.

4.3.2 Greenhouse Experiment

The greenhouse experiment was conducted at KALRO Kabete. This was to evaluate the responses of the 15 cassava germplasm varieties to Cassava Bacterial Blight (CBB) under artificial inoculation with Xam. Each cassava variety was planted using healthy stem cuttings measuring approximately 30 cm in length. The greenhouse trial was established using a Completely Randomized Design (CRD), replicated three times, which ensured that the treatments were randomly assigned to minimize bias. The trial consisted of a total of 45 plants per replication, with three plants representing each of the 15 cassava germplasm varieties.

To facilitate optimal growth and development, the cassava plants were cultivated in polyethylene nursery bags. These bags had dimensions of five inches in height and eight inches in diameter, providing sufficient space for root development. The bags were filled with sterilized forest soil to a depth of three inches. This soil preparation ensured a clean and suitable substrate for the cassava plants, minimizing the interference of external factors that could affect their growth and disease reaction. The treatments (different cassava germplasm varieties) were randomly assigned to the replications, ensuring that each variety had an equal opportunity to express its reaction to CBB.

4.3.2.1 Inoculum sourcing

To obtain the inoculum for the greenhouse experiment, leaves infected with *Xam* were collected from cassava fields in Kakamega. The decision to use this location (Kakamega) was because of its known CBB-prevalence status and thus served as suitable sources for obtaining diseased plant material. During the sampling process, care was taken to select leaves displaying typical symptoms of CBB infection. Only leaves with visible disease lesions and characteristic angular leaf spots were collected to ensure the presence of *Xam* and maintain the integrity of the inoculum.

To preserve the infected leaves for further use, a method utilizing silica gel was employed. The sampled leaves were carefully wrapped in a paper towel to prevent direct contact with the silica gel. These wrapped leaves were then placed in 1 kg khaki bags, and silica gel was added to the bags. Silica gel, known for its moisture-absorbing properties, effectively dried and preserved the plant materials. The leaves were then transported to KALRO Kabete labs for inoculum preparation.

4.3.2.2 Preparation of the pathogen

About 1 g of the sampled cassava leaves infected with CBB was then cut using a scalpel blade and ground in 10 ml sterile distilled water. One millilitre of the homogenate containing the bacteria was streaked on a petri dish filled with approximately 12 ml of semi-selective yeast peptone glucose agar (YPGA). This was followed by incubation at 28ºC for 48 hours (Fig. 4.1). YPGA medium was chosen for its ability to promote the growth of *Xam*, the causal agent of CBB, while inhibiting the growth of other microorganisms.

4.3.2.3 Confirmation of the pathogen

To confirm the presence of *Xam*, the extracted bacterial content obtained from the sampled cassava leaves was inoculated onto a susceptible cassava germplasm check variety (Tajirika) to observe CBB symptoms on the cassava variety. This inoculation process was conducted in a controlled greenhouse environment. First, the extracted bacterial content obtained from the sampled cassava leaves was prepared for inoculation. The starting concentration of the bacteria in the suspension was determined to be 5×10^7 colony forming units per milliliter (CFU/ml), using serial dilutions.

To achieve the desired concentration for successful inoculation, the bacterial suspension was adjusted using a dilution factor of 35. This adjustment involved diluting the initial suspension with a sterile water to attain a final concentration of 1.43 x 10^6 CFU/ml. This process ensured that the bacterial suspension was at an appropriate density for effective inoculation onto the susceptible cassava germplasm check variety.

4.3.2.4 Inoculation procedures in the greenhouse experiment

The greenhouse experiment was inoculated with the extracted *Xam*. The bacterium was extracted from cassava leaves and isolated according to the method in section 4.3.2.2 of this study, yielding both white and yellow mucoid bacteria on YPGA media in Petri dishes. The white and yellow bacteria were differentiated based on their ability to produce carotenoid-like yellow pigments (Xanthomonadins) and mucoid xantham gum.

Based on suggestions by Mora et al., (2019), the procedure for inoculating cassava plants in the glasshouse followed a method involving stem puncturing and the placement of a bacterial suspension using a 10 µl pipette tip. First, a bacterial suspension of *Xam* was prepared as described in section 4.3.2.3 in this study. This likely involved growing the bacteria in a suitable culture medium, such as nutrient agar or broth, and adjusting the concentration to the desired level for inoculation. Cassava plants (8 weeks old) were inoculated using an adjusted final concentration of 1.43 x 10^6 CFU/ml of the bacteria.

The actual inoculation procedure involved puncturing the stems of the cassava plants at a specific location. This puncture point served as an entryway for the bacterial suspension. Careful attention was given to ensure the accuracy and precision of the puncturing process. Using a 10 µl pipette tip, the bacterial suspension was then delivered into the puncture wound. This precise volume of the suspension was placed within the stem, allowing the pathogen to infiltrate the plant tissues. After the inoculation, the puncture wound was left to heal naturally.

To create suitable conditions for the establishment of CBB in the greenhouse, specific measures were taken to regulate temperature and humidity. These controlled greenhouse conditions aimed to replicate the natural environment conducive to CBB development and ensure reproducibility of the experiment.

Proper watering techniques were employed to maintain the necessary temperature range. Early in the morning, the plants were watered by spraying, allowing the water to evaporate gradually throughout the day. This gradual evaporation helped in maintaining an optimal humidity level within the glasshouse. The evaporation process was facilitated using the natural forces of the sun, contributing to the regulation of temperature and humidity. To prevent heat loss and maintain consistent environmental conditions, all greenhouse windows and doors were covered. This covering effectively prevented the escape of heat, enabling the glasshouse to retain a controlled and stable temperature.

4.3.2.5 Greenhouse data collection

The timing of data collection was planned to capture the progression of disease symptoms. Data collection began seven days after inoculation, which allowed sufficient time for the disease to manifest in the cassava plants. Subsequently, data was collected from three plants per variety in every replication at six consecutive time points, with a consistent interval of seven days between each measurement. This time frame enabled the observation and documentation of changes in disease severity over the course of the experiment.

A subjective scale of disease severity was used to evaluate and quantify the symptoms exhibited by the cassava plants in the greenhouse experiment. This qualitative measure provided a framework for categorizing and assessing the extent of disease progression observed in the plants. The scale consisted of five distinct categories, each representing a specific level of disease severity as described in section 4.3.1.4 of this study.

4.3.2.6 Greenhouse data analysis

This study used collected data on disease severity recorded at the six time points to generate the Area Under the Disease Progress Curve (AUDPC). The computation of AUDPC for all three plants per variety per replication followed the formula specified in Section 4.3.1.5 of this study. To analyze the data collected at each time point, a General analysis of variance (no blocking) was carried out using Fisher's unprotected least significant difference (LSD) test in Gen-stat 15th edition. The LSD was determined at $P = 0.05$.

Furthermore, the means generated by ANOVA for each variety were recorded for further analysis and interpretation. General Analysis of Variance (no blocking) was performed on the AUDPC values and the Fisher's unprotected LSD test was used at a significance level of P = 0.05 to compare the AUDPC values among different varieties.

4.3.3 General Statistical analysis

To examine the relationship between the reaction of the 15 cassava varieties to CBB in the greenhouse experiment and CBB-prevalent Kakamega region, a statistical analysis using the Spearman's rank correlation coefficient was conducted. The purpose of this analysis was to establish whether there was a correlation between the reaction of different cassava varieties to CBB in the controlled greenhouse environment and their performance in the CBB-prevalent region of Kakamega. The statistical analysis was performed using the Gen-stat 15th edition software, which provided the necessary tools to perform the Spearman's rank correlation coefficient. The statistical significance was evaluated at P = 0.05.

In this study, the evaluation of genotype resistance or susceptibility to CBB involved a multi-step methodology. Firstly, the AUDPC was calculated for each genotype. Subsequently, the AUDPC values were converted into a disease rating scale represented as a percentage. This conversion process allowed the translation of the cumulative disease progression data into a more easily interpretable and standardized format. Genotypes with a percentage falling below 25% were classified as "highly resistant," while those between 25% and 37.5% were categorized as "resistant", 37.5% and 50% as "moderately resistant", 50% and 62.5% as "moderately susceptible", 62.5 and 75 as "susceptible”, and above 75% as “highly susceptible".

4.4 Results

4.4.1 Isolation and identification of the two Xanthomonas strains

The white and yellow strains were successfully isolated on yeast peptone glucose agar (YPGA), as shown in Fig. 4.1. The white strains formed white stripes on the agar with droplets of white exudates on the stripes (Fig. 4.1b). At the same time, the yellow strains were mucoid, convex, and yellow stripes on YPGA medium (Fig. 4.1 a and b).

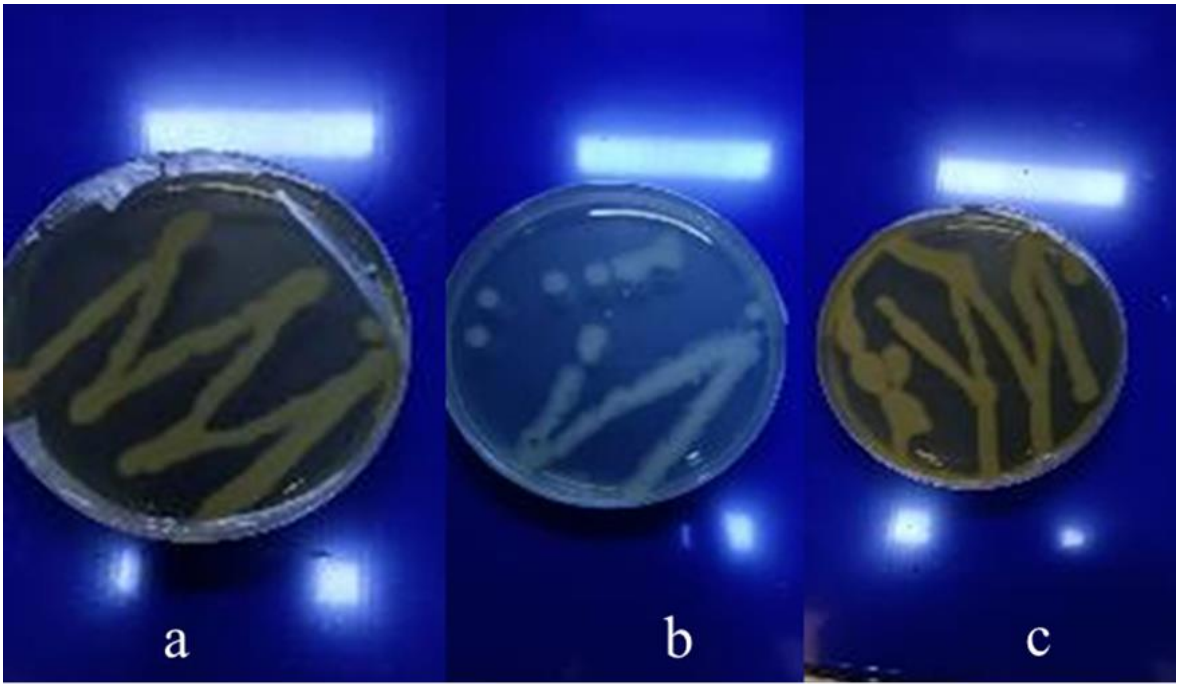

Fig. 4.1: *Xanthomonas axonopodis* pv. manihotis on YPGA after 48 hours of incubation at 28°C

4.4.2 Reaction of the 15 Kenyan cassava germplasm to bacterial infection in the greenhouse

The test cassava varieties in the greenhouse, which were inoculated with *Xam*, showed varying symptoms, such as chlorosis of the leaves, wilting and defoliation, as well as plant dieback. For MM96/2480, plant wilting was observed seven days post-inoculation (7 dpi) (Fig.4.2a). Moreover, MM96/2480 showed about 90% of plant defoliation and dieback due to *Xam* infection at 28 days post-inoculation (28 dpi) (Fig. 4.2b). Ebwanatereka 2 exhibited tolerance to *Xam* infection at seven and 28 days after inoculation (Fig. 4.4).

Fig. 4.2: Response for MM96/2480 to CBB infection (a) at seven- and (b) 28-days post inoculation, respectively at the Kabete greenhouse

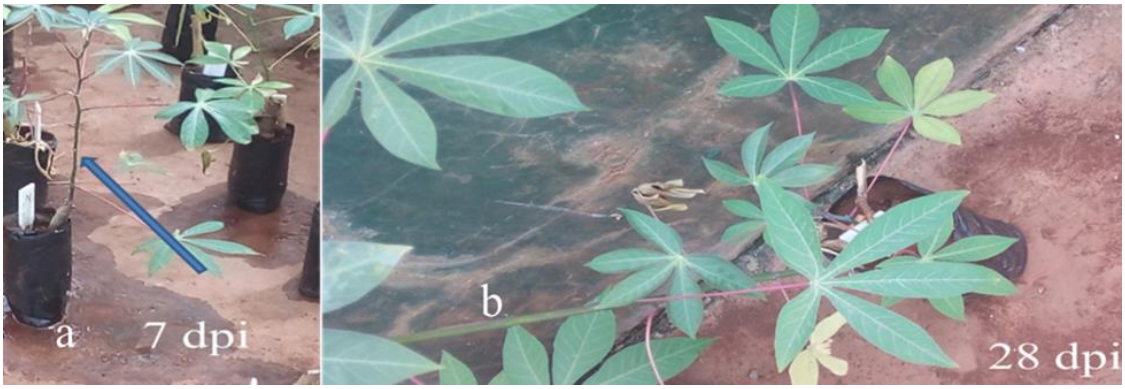

Fig. 4.3: Cassava Bacterial Blight response for Ebwanatereka 2 (a) at seven- and (b) 28-days post inoculation, respectively at the Kabete greenhouse

The analysis of variance (ANOVA) performed on AUDPC values showed significant variations within the 15-cassava germplasm ($p<.001$) in terms of their responses to CBB. Migyera, NASE 14, Fumbachai, Ebwanatereka 2, and MM97/0293 were moderately resistant. However, KCA 2, MM96/9308, Kisimbani, Ex- Ndoro, Mariakani, Karembo, KME 1, and KME 2 were moderately susceptible. At the same time, MM96/2480 and Tajirika were susceptible.

Table 4.1: Greenhouse AUDPC values observed among the 15 Kenyan cassava germplasm

Germplasm	**AUDPC**	**Percentage (%)**	**Interpretation**
MM96/2480	115	66	S
Ebwanatereka2	71.2	41	MR
Ex- Ndoro	94.5	54	MS
Karembo	92.2	53	MS
KME 1	94.5	54	MS
KME 2	96.8	55	MS
MM96/9308	101.5	58	MS
MM97/0293	80.5	46	MR
Tajirika	109.7	63	S
KCA 2	86.3	49	MS
Kisimbani	88.7	51	MS
Mariakani	91	52	MS
Fumbachai	72.3	41	MR
Migyera	68.8	39	MR
NASE 14	67.7	39	MR
Probability	(P<.001)		
CV%	5.8		
LSD	8.657		

Key: MR is Moderately Resistant; MS is Moderately Susceptible; S is Susceptible.

4.4.3 Field evaluation

4.4.3.1 Kakamega Field

Angular leaf spot symptoms (Fig. 4.4) were selectively observed on Ex-ndoro, Kisimbani, KME 1, KME 2, Migyera, MM96/2480, MM96/9308, NASE 14, KCA2, Fumbachai, Karembo, and Ebwanatereka 2 at three and four MAP. However, at three months after planting, no symptoms were observed on Mariakani, Tajirika, and MM97/0293. Therefore, the incidence of angular leaf spotting among the 15 varieties from three to four MAP was 80%. On the other hand, wilting of the leaves of some of the tagged plants was observed on EX-ndoro, Kisimbani, KME 2, Karembo, KME 1, MM96/2480, Tajirika, MM96/9308, Mariakani, and KCA 2 at five months after planting. As a result, the incidence of leaf wilting at five MAP was 66.7%.

ANOVA performed on AUDPC values showed significant variations among the 15 cassava varieties ($p<.001$) in terms of their responses to CBB under natural inoculation in a CBB-prevalent region. Migyera and Ebwanatereka 2 were resistant. Mariakani, Ex-ndoro, KME 2, Karembo, Kisimbani, KME 1, Tajirika, Fumbachai, MM97/0293, and NASE 14 were moderately resistant. At the same time, MM96/2480, and MM96/9308 were moderately susceptible.

Spearman's rank correlation coefficient analysis between the glasshouse and CBB-prevalent Kakamega region revealed a significant ($P<.001$) correlation in the reaction of the cassava germplasms to CBB. The spearman’s rank correlation was 0.739, revealing a positive association.

Fig. 4.4: CBB symptoms at three months after planting in the Kakamega field trial

Table 4.2: Kakamega field AUDPC values observed among the 15 Kenyan cassava germplasm

Germplasm	AUDPC	Percentage (%)	Interpretation
NASE 14	300	40	MR
MM96/9308	375	50	MS
MM96/2480	390	52	MS
Ex- Ndoro	365	49	MR
KCA 2	375	50	MS
Migyera	275	37	R
KME 1	325	43	MR
KME 2	360	48	MR
Ebwanatereka2	260	35	R
Fumbachai	315	42	MR
Karembo	325	43	MR
Kisimbani	325	43	MR
Mariakani	330	44	MR
MM97/0293	300	40	MR
Tajirika	325	43	MR
Probability	(p<.001)		
CV%	11.6		
LSD	25.07		

Key: R is Resistant; MR is Moderately Resistant; MS is Moderately Susceptible

4.4.3.2. Kiboko field

The reaction of the 15 cassava germplasm over time is shown in Table 4.3. Angular necrotic spotting of the leaves was observed on Ebwanatereka2, Ex-ndoro, Karembo, KCA 2, Mariakani, Migyera, MM96/2480, MM96/9308, MM97/0293, and NASE 14 from three to eight months after planting (MAP). At the same time, angular necrotic spotting of the leaves was observed on Kisimbani and KME 1, from four to eight MAP. At four months after planting, Tajirika had angular leaf spots, which disappeared in the subsequent five and six months after planting. The symptoms reappeared at seven and eight months after planting. On the other hand, Fumbachai exhibited angular leaf spots three months after planting, which disappeared. The CBB disease incidence in the Kiboko field was 80% three months after planting. However, no blight, wilting, dieback, or vascular necrosis was observed in the Kiboko field. Based on the AUDPC values, the reaction of the 15 cassava varieties to CBB varied (P<.001). Kisimbani, KME 1, Tajirika, KME 2, Ebwanatereka2, Karembo, Mariakani, MM96/9308, and Fumbachai had high resistance to CBB in the Kiboko field. Nevertheless, Ex- ndoro, KCA 2, Migyera, MM96/2480, MM97/0293, and NASE 14 were resistant.

Table 4.3: Kiboko field AUDPC values observed among the 15 Kenyan cassava germplasm

Germplasm	AUDPC	Percentage (%)	Interpretation
Kisimbani	205	27	HR
KME 1	202.5	27	HR
Tajirika	205	27	HR
KME 2	217.5	29	HR
Ebwanatereka2	165	22	HR
Ex- Ndoro	225	30	R
Karembo	180	24	HR
KCA 2	245	33	R
Mariakani	200	27	HR
Migyera	215	29	R
MM96/2480	240	32	R
MM96/9308	200	27	HR
MM97/0293	235	31	R
NASE 14	235	31	R
Fumbachai	160	21	HR
Probability	(P<.001)		
CV %	18.5		
LSD	25.28		

Key: MAP is months after planting; R is Resistant; HR is Highly Resistant

The performance of the 15-cassava germplasm varied across the two environments (Table 4.4) depending on their resistance level. Ebwanatereka 2 and Migyera were resistant to CBB across the two environments, suggesting the influence of genotype on cassava's resistance to CBB. However, some varieties, such as KCA 2, MM96/9308, and MM96/2480, were resistant to CBB in the Kiboko but exhibited moderate susceptibility in Kakamega, indicating that the environment influences the prevalence of CBB. For example, environments that receive high levels of rainfall throughout the year are more likely to be affected by CBB than environments with a low amount of rain.

Table 4.4: Performance of 15 cassava germplasm on response to CBB in varied environments (Kakamega and Kiboko)

Germplasm	**Kakamega**		**Kiboko**	
	AUDPC	**Interpretation**	**AUDPC**	**Interpretation**
Ebwanatereka2	260	R	165	HR
Ex- Ndoro	365	MR	225	R
Fumbachai	315	MR	160	HR
Karembo	325	MR	180	HR
KCA 2	375	MS	245	R
Kisimbani	325	MR	205	HR
KME 1	325	MR	202.5	HR
KME 2	360	MR	217.5	HR
Mariakani	330	MR	200	HR
Migyera	275	R	215	R
MM96/2480	390	MS	240	R
MM96/9308	375	MS	200	HR
MM97/0293	300	MR	235	R
NASE 14	300	MR	235	R
Tajirika	325	MR	205	HR
Probability	(P<.001)		(P<.001)	
CV %	11.6		18.5	
LSD	25.07		25.28	

NB: Migyera and Ebwanatereka 2 exhibited CBB resistance across the two environments

4.5 Discussion

The findings of this study provided vital information and insights into the response of 15 Kenyan cassava germplasm to bacterial infection caused by *Xam* in both greenhouse and field conditions. The greenhouse experiment allowed close observation of the different cassava genotypes and assessment of their susceptibility to *Xam*. As reported in previous studies, the variation in symptom development and severity among the cassava varieties highlights the complexity of the plant-pathogen interaction (CABI, 2023). The significant wilting, defoliation, and dieback observed in MM96/2480 demonstrated its high susceptibility to *Xam* infection, underscoring the importance of identifying susceptible genotypes to address potential yield losses and disease management. The varied responses observed among the remaining cassava varieties suggests the presence of diverse mechanisms influencing their resistance or susceptibility to *Xam*. Similar findings have been reported in other studies, highlighting the genetic complexity underlying plant-pathogen interactions (Fanou et al., 2018). Understanding the genetic basis of these responses is essential for effective breeding strategies aimed at improving disease resistance in cassava crops.

The outcomes of the two field experiments, conducted in both Kakamega and Kiboko regions, yielded a comparable range of responses in the cassava germplasm to CBB infection. Angular leaf spot symptoms were evident across several varieties, exhibiting varying severities and incidence rates. Our observations corroborate findings from previous studies, indicating that CBB affects cassava germplasm in diverse ways, depending on genetic factors and environmental conditions (Sedano et al., 2017). Notably, some varieties, such as Migyera, Fumbachai, NASE 14, and Ebwanatereka 2, demonstrated resistance to CBB in both the field experiments. This resistance is of utmost significance for breeding programs and disease management, as it offers potential solutions to manage CBB and mitigate its detrimental impact on cassava production. Conversely, other varieties, including MM96/2480 and KCA 2, were susceptible to CBB in the CBB hotspot area of Kakamega. However, it is important to note that the selection of KALRO Kakamega and Kiboko as representative field locations was based on certain considerations.

Kakamega was chosen because it is recognized as a hotspot area for CBB in Kenya (Chege et al., 2017), where the incidence of the disease as well as its severity is relatively high. The experiment in Kakamega aimed to assess the performance of the cassava germplasm under conditions that are more conducive to CBB proliferation. On the other hand, Kiboko was selected as a comparative study location due to its distinct environmental characteristics. Kiboko receives lower rainfall (between 500 and 800 millimetres) compared to Kakamega (from 1,500 to 2,000 millimetres or more) (Lul, 1985), and the drier conditions may not be as favourable for the rapid proliferation and spread of CBB (Dania & Ojeyemi, 2019). While Kakamega and Kiboko were chosen to represent different climatic conditions, it is essential to acknowledge that these two locations alone may not provide a comprehensive understanding of the performance and adaptability of the identified tolerant varieties across various agro ecological zones in Kenya. However, there is need to conduct additional experiments in other regions that encompass diverse environmental factors to ensure the broader applicability of the results.

The results obtained in this study highlight how evaluating cassava germplasm for their resistance or tolerance to *Xam* infection is an important step. Identification of resistant or tolerant varieties is crucial for developing effective strategies for disease management and ensuring sustainable cassava production (McCallum et al., 2017). The results indicate that some varieties, such as Migyera, Ebwanatereka 2, Fumbachai, and NASE 14, hold promise as potential CBB-resistance sources. These varieties may provide important genetic resources for breeding programs aimed at developing improved cassava cultivars with enhanced disease resistance. Notably, the susceptibility or resistance of cassava germplasm to CBB can vary across different regions and environments (Toure et al., 2020). Thus, there is need for further studies to evaluate the performance of these varieties in other locations and under diverse climatic conditions. Additionally, investigating the basis of resistance at molecular level and understanding the mechanisms underlying this resistance involved in the interaction between cassava and the pathogens would provide valuable insights for targeted breeding efforts (Nelson et al., 2018).

4.6 Conclusion

This study revealed a wide range of responses, with some varieties displaying resistance or tolerance to *Xam* infection. These findings play a crucial role in identifying potential sources of resistance and the development of improved cassava cultivars with enhanced disease resistance. The results of the greenhouse experiment demonstrated that MM96/2480 was susceptible to *Xam* infection. In the field experiments conducted in Kakamega and Kiboko, varying degrees of angular leaf spot symptoms were observed, with some varieties showing resistance or tolerance to CBB.

Notably, Migyera, Fumbachai, Ebwanatereka 2, and NASE 14 were tolerant to CBB in both field locations and controlled greenhouse conditions. The correlation analysis between the greenhouse and the CBB hotspot area (Kakamega) field experiment indicated a positive association, suggesting that greenhouse evaluations can provide valuable insights into the field performance of cassava germplasm. These findings emphasize the importance of evaluating cassava germplasm for disease resistance and tolerance and highlight the potential of certain varieties as sources of resistance to CBB. Further research is needed to validate these findings in different regions and environments and to elucidate the genetic basis of resistance. The knowledge gained from this study will inform decisions in developing sustainable strategies for managing diseases, thus improving cassava production in Kenya and other regions facing similar challenges.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

5.1 General discussion

Evaluating cassava germplasm diversity through phenotypic and genotypic characterization is critical in understanding the genetic makeup and potential features of these cassava varieties (Ferguson et al., 2019). Other studies have highlighted the importance of evaluating cassava genotypes for resistance to CBB, a devastating disease which significantly affects cassava production (Mbaringong et al., 2017). This study conducted field experiments in distinct environmental conditions, such as the CBB hotspot in Kakamega and the less favorable Kiboko region, aiming to gain insights into how these germplasm varieties respond to varying disease pressure. Studies have indicated that distinct environmental conditions influences the severity of CBB in different regions (soto Sedano et al., 2017).

Regions with higher rainfall and humidity, such as Kakamega, are more conducive to CBB development. On the other hand, regions like Kiboko, which receive lower rainfall, may experience reduced disease pressure (Wydra et al., 2007). Thus, conducting field experiments in these diverse locations allowed the assessment of the adaptability of the germplasm to varying disease pressures and environmental conditions.

Moreover, greenhouse experiments are crucial for controlled assessments of the reaction of the different cassava varieties to CBB (Ogunjobi & Fagade, 2010). Under greenhouse conditions, disease development is monitored, controlled, and replicated, offering valuable insights into the inherent resistance or susceptibility of the different cassava varieties to the disease.

Herein, the combination of field and greenhouse experiments provided a comprehensive assessment of the performance of the 15 Kenyan cassava germplasm in response to CBB. It allowed for the comparison of the outcomes from different environmental conditions, promoting the understanding of genetic factors attributable to disease resistance and the potential for breeding resistant cassava varieties. The Kakamega experiment provided insights into how genotypes fared in a CBB hotspot, while the Kiboko experiment offered information about their performance under less favorable disease conditions. Additionally, the greenhouse experiment provided a controlled setting for systematically testing the reaction of the genotypes to CBB.

The assessment of phenotypic and genotypic characteristics in cassava germplasm is crucial in understanding the diversity of the crop as well as the potential for improving available germplasm (Fregene et al., 2001). Previous research have emphasized on the importance of phenotypic characterization to identify visible variations in morphological and agronomic traits among cassava varieties (Adu et al., 2020). Phenotypic data can offer valuable insights into how these varieties perform in varied environmental conditions, enabling breeders to select traits of interest for targeted improvement. Moreover, genotypic characterization using molecular markers has been well-established in the literature as a powerful tool used in the evaluation of genetic diversity and relationships among germplasm collections (Lokko et al., 2005).

Herein, the use of SCoT markers provided deeper insights into the genetic makeup of the cassava varieties, revealing patterns of relatedness and population structure. These genetic markers, in combination with phenotypic data, are valuable for effective germplasm conservation and guiding breeding strategies for desired traits (Bryne et al., 2018). Interestingly, in terms of their reaction to Cassava bacterial blight (CBB) infection, the varieties Migyera, Fumbachai, NASE 14, Ebwanatereka 2, and MM97/0293 showed tolerance to CBB in both field experiments and the greenhouse experiment. It is noteworthy that these tolerant varieties shared several common morphological traits, such as the colour of apical leaves, leaf lobe number, prominence of foliar scars, colour of end branches of adult plants, length of stipules, and stipule margin.

However, it is important to consider that these traits were also present in some susceptible varieties, suggesting that they cannot serve as reliable distinguishing factors for CBB resistance. These findings emphasize the complex nature of host resistance to CBB in cassava plants and highlight the need for further investigation into the underlying genetic mechanism of resistance. The fact that tolerant varieties shared morphological traits with susceptible ones suggests that other genetic factors, such as specific genes or gene combinations, may play a significant role in conferring CBB resistance. Thus, there is need for future research to focus on exploring the underlying genetic mechanisms and identifying reliable molecular markers linked to CBB-resistance. The lack of clear phenotypic distinctions between resistant and susceptible varieties underscores the importance of utilizing genotypic characterization methods, such as marker-based analysis, to gain deeper insights into the genetic variation and relatedness within available cassava germplasm.

Previous studies have supported the notion that multiple genetic factors contribute to a plant's resistance to diseases like CBB (Bart et al., 2012), as it is well-known that the traits responsible for CBB resistance are complex and influenced by various genetic loci and environmental factors. Therefore, solely relying on morphological traits to predict CBB resistance may not be adequate or accurate.

Notably, the genotypes Fumbachai, Nase14, Migyera, and Ebwanatereka 2 clustered together in Subcluster A of cluster I, indicating a potential genetic similarity among these varieties. This suggests that they may share common genetic markers associated with tolerance to CBB. However, the clustering of MM97/0293 with MM96/2480, Karembo, Tajirika, MM96/9308, and KME 1 in subcluster B of cluster I, despite the varying disease response patterns observed, raises an interesting point regarding their genetic similarity and the potential factors influencing disease response.

It is worth noting that MM97/0293 exhibited tolerance to CBB in the greenhouse experiment, whereas the rest of the genotypes in the subcluster were susceptible. MM97/0293 might possess specific genetic factors or gene combinations that contribute to its CBB tolerance. These genetic factors may not be present or may differ in the other varieties, leading to their susceptibility.

In addition, it is possible that MM97/0293 escaped infection in the greenhouse experiment, leading to its observed tolerance to Cassava bacterial blight (CBB). Despite its clustering with other susceptible genotypes in sub cluster B of cluster I, the greenhouse environment may have provided conditions that limited or prevented CBB infection in MM97/0293 specifically. Therefore, the clustering analysis based on SCoT markers and the observed differences in disease response among the cassava genotypes in sub cluster B highlight the need for further studies to validate and understand these findings. The distinct clustering of genotypes in clusters II, III, IV, and V (Kisimbani, Ex-ndoro and Mariakani, KCA 2, and KME 2, respectively) further highlights the genetic diversity among the cassava varieties assessed under this study. These distinct clusters suggest variations in their genetic makeup and potentially different mechanisms of disease response.

The clustering results based on the SCoT markers were consistent with the responses of different varieties to CBB that were observed in both the greenhouse and field experiments. This indicates that the SCoT markers were effective in capturing the genetic variations associated with CBB resistance in cassava. However, the susceptibility patterns from the greenhouse versus the field experiments revealed some discrepancies, suggesting the influence of environmental conditions on disease expression.

The findings obtained from this study demonstrate the potential of molecular markers, such as SCoT markers, in supporting the clustering of cassava genotypes and identifying potential genetic markers associated with CBB resistance. However, it is crucial to consider that the SCoT markers may not capture the full complexity of CBB resistance, as other genetic factors and environmental influences can also contribute to the observed variations in disease response.

5.2 Conclusions

This study serves as a valuable resource by providing baseline data for breeders working towards developing cassava varieties that have enhanced resistance to CBB. The clustering analysis utilizing morphological traits and SCoT markers provided insight into the genetic variation and relatedness among the cassava varieties studied. This diversity provides breeders with a foundation to strategically select parents that maximize genetic variability, ultimately improving the potential for developing CBB-resistant varieties.

Furthermore, the findings of this study offer insights into potential genetic similarities among cassava varieties exhibiting different disease response patterns. The identification of distinct clusters and sub-clusters suggests the presence of genetic markers associated with CBB tolerance. These markers can serve as valuable tools for breeders, aiding in the selection and breeding of cassava varieties with improved resistance to CBB.

Additionally, this study underscores the importance of taking into account the influence of environmental conditions on disease expression. The discrepancy in disease response within specific sub-clusters, such as the contrasting tolerance of MM97/0293 in the greenhouse compared to other genotypes, highlights the need for breeders to account for the environmental context in which these genotypes will be grown. By considering both genetic markers and environmental conditions, breeders can design more targeted and effective breeding strategies to develop CBB-resistant cassava varieties.

5.3 Recommendations

This study recommends the following:

i. Further research should be conducted to validate and expand upon the findings of this study. This includes conducting additional studies with larger sample sizes to confirm the genetic clustering patterns and identify more precise genetic markers associated with Cassava bacterial blight (CBB) resistance. The validation of such markers enables access of breeders to more reliable tools for selecting and breeding CBB-resistant cassava varieties.

ii. There is need for conducting a comprehensive assessment of the biochemical traits contributing to Cassava bacterial blight (CBB) resistance. As a result, valuable insights can be gained into the underlying mechanisms controlling CBB resistance. A particular focus should be placed on developing crosses between CBB-resistant and susceptible materials to establish the nature and mode of CBB inheritance. Through this approach, an in-depth understanding of the underlying mechanism of biochemical resistance can be achieved, enabling breeders to design more precise and effective breeding strategies for developing CBB-resistant cassava varieties. Such assessments will not only enhance the knowledge of CBB resistance mechanisms but also contribute to the development of more resilient and sustainable cassava crops.

iii. There is need for identifying and molecular profiling genes associated with cassava resistance to CBB. Future research should focus on employing advanced molecular tools, including next-generation sequencing and genome-wide association studies (GWAS), to identify and characterize the genes linked to CBB resistance. These techniques can provide comprehensive insights into the genetic variations and specific markers associated with resistance.

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