UNIVERSITY OF NAIROBI

CHARACTERIZATION OF KENYAN COMMON BEAN (*PHASEOLUS VULGARIS* L.) ACCESSIONS FOR RESISTANCE TO COMMON BACTERIAL BLIGHT USING START CODON TARGETED (SCOT) POLYMORPHISM MARKERS

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DECLARATION

I Roselydiah Nasipwondi Makunja declare that this thesis is my original work and has not been presented for award of a degree in any other University.

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DEDICATION

It is my genuine gratefulness and warmest regard that I dedicate this work to the Almighty God, for enabling me to achieve this milestone without whom, I would not have managed. I also dedicate this thesis to my Supervisors, Dr. Evans Nyaboga, Dr. Ezekiel Mecha and Dr. Edward Muge, my father, Mr. Charles Makunja, my mother, Mrs. Doris Mulupi and Mrs. Sandra Kabagenyi, my grandfather Mr. Joseck Mulupi and my grandmothers Mrs. Bilha Mulupi and Mrs. Mactilda Akumu Makunja, my siblings, Fidel Makunja, Aristotle Makunja, Kevin Mulupi and Peris Mulupi

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LIST OF ABREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
APX	Ascorbate Peroxidase
BCMNV	Bean Common Mosaic Necrosis Virus
BCMV	Bean Common Mosaic Virus
CAT	Catalase
CBB	Common Bacterial Blight
CFU	Colony Forming Units
DArT	Diversity Arrays Technology
EDTA	Ethylenediaminetetraacetic Acid
Est. Var	Estimated Variation
GAE	Galic Acid Equivalent
GD	Genetic Diversity
GERRI	Genetic Resources Research Institute
HT	Heterozygozity
I	Shannon Information Index
ISSR	Inter-Simple Sequence Repeat
ISTR	Inverse Sequence-tagged Repeats
MAS	Molecular Marker Assisted Selection
MB	Monomorphic Bands
MCMC	Markov Chain Monte Carlo Methods
MDA	Malondialdehyde
Ne	Effective Number of Alleles

OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PB	Percentage Bands per Loci
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
POD	Peroxidase
POX	Peroxidase gene markers
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
RP	Resolving Power
SCAR	Sequence Characterized Amplification Region
SCoT	Start Codon Targeted
SNP	Single Nucleotide Polymorphism
SRAP	Sequence Related Amplified Polymorphism
SSR	Simple Sequence Repeats
TAB	Total Amplified Band
TBA	Thiobarbitric Acid
TBE	Tris/Borate/EDTA
TCA	Trichloroacetic Acid
TPC	Total Phenolic Content
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

VNTR	Variable Number Tandem Repeat
Xap	*Xanthomonas axonopodis* pv. *Phaseoli*
YPG	Yeast Peptone Glucose
YPGA	Yeast Peptone Glucose Agar
YPG-CC	Yeast Peptone Glucose - Cephalexin and Cycloheximide
µl	Microlitre
µg	Microgram
w/v	Weight Per Volume
×g	Relative Centrifuge Force

ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is one of the most important legume crop used as a source of proteins, vitamins, and other beneficial nutrients among resource-poor populations in Kenya. However, common bean production is limited by several abiotic and biotic constraints. Common bacterial blight (CBB) disease caused by *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) is among the most important biotic constraints affecting both seed quality and yield and has been reported to cause losses of up to 75%. Although the use of resistant varieties is the most effective method of CBB management, limited studies have been carried out to characterize common bean accessions for resistance to CBB using molecular markers. The aim of the present study was to characterize Kenyan common bean accessions using start codon targeted (SCoT) polymorphism markers for resistance to CBB. A total of 30 common bean accessions from farmers' fields in Nyanza and from the Genetic Resources Research Institute (GERRI) were used for molecular characterization. A set of 36 SCoT primers were tested of which 17 primers gave reproducible amplification which was further used for genetic diversity analysis. The 17 SCoT primers generated a total of 224 amplification bands, of which 95% were polymorphic. The mean Shannon information index (I), heterozygosity (HT) and gene diversity (GD) values were 1.28, 0.86 and 0.98, respectively, indicating that the common bean accessions were genetically diverse. Polymorphism information content (PIC) of SCoT markers ranged from 0.601 (SCoT 27) to 0.85 (SCoT 7) with a mean of 0.73 while values of resolving power (RP) ranged from 1.73 (SCoT 24) to 8.13 (SCoT 7) with a mean of 5.09. Such a range suggests that all the primers were informative. Pair-wise genetic similarity among the common bean accessions ranged from 0.26 (between chinchae LRC 08 KSI and GBK 036527, and between Chinchae LRC 08 KSI and GBK 030259) to 0.73 (between GBK 036523 and KAT X69) with a mean of 0.52. Cluster analysis based on the unweighted pair group method with arithmetic mean (UPGMA) and principal component analysis (PCA) showed high genetic variation among the accessions and grouped the common bean accessions into 2 main clusters. Population structure analysis using the Bayesian model-based approach grouped the accessions into 3 subpopulations and showed a high genetic admixture within the subpopulations. Results of the analysis of molecular variance (AMOVA) showed a significant difference across accessions with a high within-population variation of 96%. Based on the clustering in the dendrogram, 20 accessions were selected and screened for CBB resistance in the glasshouse. Out of the 20 common bean accessions screened, 3 were resistant and were randomly distributed in the dendrogram. Three markers including SCoT 7, SCoT 24, 32 and SCoT 36 generated unique bands that were specific to accessions resistant to CBB, these markers can, therefore, be utilized in molecular assisted selection (MAS) for CBB resistance in common beans. Biochemical analysis of 2 resistant and 2 susceptible accessions to CBB, showed significantly higher concentrations of total phenolic compounds (TPCs) and catalase enzyme (CAT) activities in resistant accessions as compared to susceptible ones. The findings of the current study revealed that high genetic variation exists among the Kenya common bean accessions. In addition, CBB-resistant accessions exist within the Kenyan common bean germplasm, which can be exploited for common bean genetic improvement in breeding programs.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the study

Common beans (*Phaseolus vulgaris* L.) play a critical role in food, nutrition, and economic security in Kenya (Katungi *et al*., 2010). The crop is grown by more than 3 million households in Kenya, making it the third staple food after maize and wheat (Katungi *et al*., 2010; Muimui *et al*., 2017). Common beans provide the much needed but affordable alternative source of protein as well as other nutritionally important compounds such as folic acids and carbohydrates. In Kenya, common bean is grown twice a year, due to its short production cycle, mostly inter-cropped with maize, sorghum, coffee, and potatoes, among other crops, thereby providing food while the other crops mature (Jones, 1999; Katungi *et al*., 2010). Common beans also play a significant economic role, providing income to farmers through the sale of surplus produce (Jones, 1999; Katungi *et al*., 2010).

In Kenya, common beans production is predominantly in Eastern, Nyanza, Central, Western and Rift valley regions of the country (Katungi *et al*., 2010), the most popular varieties include; Rosecoco, Red Haricot, Mwitemania and Mwezi Moja (Katungi *et al*., 2010). However, production in these regions is 0.7 t/ha (FAOSTAT, 2018), which is below the potential of 2 t/ha under controlled conditions (Mwang'ombe *et al*., 1994). The poor production is attributed to several biotic and abiotic factors that the crop faces during its production. The chief abiotic factors include poor soil and drought (Kimani *et*

al., 2005b; Lunze *et al*., 2012). On the other hand, biotic factors affecting common bean production in Kenya include; insect pests such as bean fly (*Melanogromyzaphaseoli coq*.), american bollworm (*Heliothis armgera hb*.), spotted borer (*Chilo partellus swinh*.), bean aphid (*Aphis fabae scop*) and bruchid (*Acanthoscelides obtectus Say*). The crop is also affected by various diseases, including; viral diseases namely *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) (Kapil *et al*., 2011; Mutuku *et al*., 2016), fungal diseases such as anthracnose (*Colletotrichum lindemuthianum* (Sacc. and Magn.) (Pastor- Corrales, 2005; Kiryowa *et al*., 2016; Anunda *et al*., 2019) and bacterial diseases like common bacterial blight (CBB) (*Xanthomonas axonopodis* pv. *phaseoli*) (Wortmann *et al*., 1998; Kimani *et al*., 2005b; Belete and Bastas, 2017).

Common bacterial blight (CBB), is caused by a gram-negative bacteria, *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*) and its variant, *Xanthomonas fuscans* subsp. *fuscans* (*Xff*). Common bacterial blight (CBB) is among the most devastating diseases facing global common bean production (Akhavan *et al.*, 2013; Belete and Bastas, 2017). Yield losses of up to 75% have been reported due to CBB disease in Kenya (Makini, 1995). Several methods, such as the use of certified pathogen-free seeds, copper-based bactericides, and crop rotation, have been used in the management of CBB disease (Duncan *et al*., 2011; Ballette and Bastas, 2017). However, these methods are faced with significant challenges that limit their use; for instance, the use of bactericides is not environmentally friendly, and at the same time, costly for the resource-poor farmers (Balete and Bastas, 2017). On the other hand, certified pathogen-free seeds are often expensive, and therefore

inaccessible to resource-poor farmers in the country, placing them at a disadvantage (Yearley, 2012). Therefore, farmers often reuse previously harvested seeds infected with CBB thus, contributing to the progression of the disease (Mkandawire *et al*., 2004; Ballette and Bastas, 2017). Boersma *et al*. (2015) suggested that accessions with durable resistance to CBB offer the most promising long-term and economical means of management of the disease. In Kenya, common bean accessions such as, "Jesca," "MEX 142" and "VCB 81013" have been reported to elicit resistance to CBB disease (Wagara and Kimani, 2007).

Plants respond by activating several defense mechanisms to counter the effect of pathogen-associated stress and eliminate the pathogen. Reactive oxygen species (ROS) act as secondary messengers, thereby signaling the release of plant defense compounds against the pathogen; however, the accumulation of high amounts of ROS may be detrimental to the plant. Lipid peroxidation is an excellent indicator of the extent of cellular damage elicited by a pathogen on the plant and can be accurately estimated by measuring the malondialdehyde (MDA) content in the affected plant (Rosales *et al*., 2012; Awan *et al*., 2018). Disease resistant plants have an excellent mechanism in eliminating the invading pathogen and containing the level of ROS accumulation, thereby inhibiting cellular damage by ROS accumulation. This is achieved through enzymatic mechanisms such as induction of catalase (CAT) and peroxidase (POD) and non-enzymatic mechanisms such as total phenolic compounds (TPCs) which aid in scavenging and elimination of ROS, thereby relieving the plant of the ROS associated stress (Rosales *et al*., 2012).

Genetic diversity is a fundamental requirement for adaptation of species to various environmental changes. Rich genetic diversity among species contributes to a high and wide natural distribution of these species and a robust environmental survival and evolutionary prospects (Liu *et al*., 2015). Traditionally, genetic diversity analysis in plants relied on observation of morpho-agronomic traits such as differences in growth habit, the color of the seeds, yield, among others. However, such methods are not based on gene expression and thus show low or no polymorphism, are affected by environmental changes, and analysis of large samples is nearly impossible (Li *et al*., 2014; Kouam *et al*., 2017). Thus, the information generated is inconclusive, unreliable and, of little use in many genetic diversity studies. These shortcomings can be bridged by the use of molecular markers, which are DNA fragments associated with specific regions within the genome (Idrees *et al*., 2014). These fragments can be used in accurate prediction of the presence of a particular trait within the genome or in highlighting the existing genetic differences within a population. The usefulness of a molecular marker depends on its reproducibility and specificity to the targeted trait (Jiang, 2013).

Molecular markers are broadly categorized into hybridization-, polymerase chain reaction (PCR)- and DNA sequence-based techniques according to the mode of detection (Govindaraj *et al*., 2015). Polymerase chain reaction (PCR)-based techniques are most popular since they are easy to use with a high throughput rate (Varshney *et al*., 2007; Li *et al.,* 2013; Li *et al*., 2014). Despite the benefits of PCR techniques, setbacks such as low consistency and multiplexing output in random amplified polymorphic DNA (RAPD), high cost and multiple steps in simple sequence repeats (SSR) and long and numerous

steps in amplified fragment length polymorphism (AFLP) have limited their use in most diversity studies (Li *et al*., 2001; Li *et al*., 2014). With shortcomings in the use of PCR markers, advances in molecular biology developed newer and superior alternative gene-targeted techniques, for example, start codon targeted (SCoT) polymorphism markers (Kouam *et al*., 2017).

Start codon targeted polymorphism marker is a very sensitive and reproducible single primer technique based on the short conserved region of the ATG transcription start site of a gene in plants (Pakseresht *et al*., 2013; Mahjbi *et al*., 2015). It is linked to functional genes or regions surrounding these genes and their corresponding traits and requires no sequence information (Collard and Mackill, 2007; Pakseresht *et al.*, 2013; Mahjbi *et al*., 2015). Furthermore, SCoT markers have high resolving power (RP), and hence indicate higher polymorphisms in comparison to other common markers such as RAPD. No known study has been undertaken to elucidate the genetic diversity of Kenyan common bean accessions for resistance to CBB using SCoT markers. Therefore, in this study, SCoT markers were used to asses CBB resistance in common bean accessions grown in Kenya.

1.2 Problem statement

Common bean is susceptible to various viral, fungal, and bacterial diseases that affect both the yield and quality of seeds (Boersma *et al*., 2015). In Kenya, CBB is among the major biotic constraints affecting common bean production, resulting to yield losses of up to 75% (Makini, 1995; Belete and Bastas, 2017). The disease is prevalent in all common

bean growing areas in Kenya but more endemic in central, Nyanza and western regions of the country (Muthangya, 1982). Known strategies for management of CBB include the use of pathogen-free seeds, chemical applications and genetic resistance (Akhavan *et al*., 2013; Belete and Bastas, 2017). The use of chemicals is environmentally unfriendly and expensive (Fininsa, 2003). On the other hand, certified disease-free seeds are inaccessible or too expensive for resource-poor farmers in the country; therefore, majority of common bean farmers in Kenya use previously harvested seeds for planting, which may be infected with the CBB disease thereby contributing to the progression of the disease (Okumu *et al*., 2017). Therefore, the use of genetic resistance is the most appropriate, safe and cost-effective method of CBB management. However, there is limited information on the available common bean accessions resistant to CBB. With scarce data, efforts to improve common bean production in the country remain limited and the losses due to CBB increase, leading to a triple effect i.e., food, nutrition and economic insecurity.

Genetic characterization provides the key to unraveling disease-resistant accessions in the population. Genetic characterization of common beans has been widely undertaken by various studies including Singh *et al*. (1991) using allozymes, Kumar *et al.* (2008) using AFLP, Zargar *et al*. (2016) using RAPD and SSR, Velasquez *et al*. (1994) using restriction fragment length polymorphism (RFLP), Svetleva *et al*. (2006) using inter-simple sequence repeat (ISSR), and Gyang *et al*. (2018) using SSR molecular markers. However, these markers are not gene-targeted and unable to highlight existing genetic differences that are linked to gene function.

1.3 Justification of the study

Common bean is the most important legume crop in Kenya because it is a good source of protein and essential minerals, providing quality nourishment to the resource-poor farmers. Per capita consumption of common beans is estimated at 14 kg per year in Kenya, with a high consumption of 66 kg/yr in the western region (Katungi *et al*., 2009). There has been a significant population growth in Kenya, translating to increased demand for food. On the other hand, common bean production in the country has not been keeping up with this trend. The production of the country remains below its maximum potential, while the production area has increased drastically from 689,377 ha in 2010 to 1,171,710 ha in 2016, there is negligible difference in production yield between the same years, (0.57 metric tons/ha in 2010 and 0.72 metric tons in 2016). Consequently, there has been an increase in the country's common bean import to meet the growing demands from 47, 764 in 2013 to 162, 719 metric tons in 2014 while exports have reduced from 7,264 in 2013 to 5,716 metric tons in 2014 despite the high production area in 2014 compared to the previous year (FAOSTAT, 2018). The poor yield of common bean production in Kenya can be attributed to some of the biotic constraints facing the crop, including CBB. Since the use of resistant accession is the only economically feasible solution in managing CBB disease (Duncan *et al*., 2011; Boersma *et al*., 2015), identification of resistant accessions of common beans in Kenya will be useful to smallholder farmers and in breeding programs. A high level of CBB resistance among local common beans will reduce yield losses, minimize dependence to bactericides and accelerate the uptake of an integrated disease management program (Popović *et al*., 2012).

Genetic diversity in common bean accessions is valuable in the conservation and breeding programs. The use of molecular markers in genetic diversity analysis and identifying Kenyan common bean accessions resistant to CBB would help in accelerating the time for the development of resistant accession with other superior agronomic traits. SCoT markers are highly polymorphic and are linked directly to gene function (Rajesh *et al*., 2015) and, therefore, the use of these markers may highlight unique bands that may be directly linked to genes responsible for resistance to CBB in common bean. These markers may be used in breeding programs for fast and efficient genetic improvement of common bean.

The potential roles of plant phenolic compounds and defense enzyme activities in response to pathogen infection have been reported. Total phenolic compounds (TPCs) are toxic towards the invading pathogens and the quantities of TPCs in the plant are directly proportional to the tolerance levels of the plant towards the invading pathogen (Nicholson and Hammerschmidt, 1992). Defense enzymes such as POD and CAT are efficient ROS scavengers, thereby alleviating the damaging effect of ROS on the plant (Bindschedler *et al*., 2006; Shahbazi *et al*., 2010; Mahgoub *et al*., 2015). It is hypothesized that the enzymatic activity responses would be different quantitatively in CBB-susceptible and resistant accessions of common bean, and the activities of these enzymes would be correlated with resistance to CBB. Therefore, an insight into the biochemical responses in common bean and *Xap* interactions would aid in the discrimination of CBB resistant and susceptible accessions.

1.4 Objectives

1.4.1 General objective

To characterize Kenya common bean (*Phaseolus vulgaris* L.) accessions for resistance to common bacterial blight (CBB) using start codon targeted (SCoT) polymorphism markers.

1.4.2 Specific objectives

(i) To determine genetic diversity, variation and population structure of Kenyan common bean accessions using SCoT markers

(ii) To screen Kenyan common bean accessions for resistance to CBB in the glasshouse

(iii) To determine SCoT markers associated with CBB resistance in common bean accessions

1.5 Null hypothesis

(i) There is no genetic diversity and variability in common bean accessions grown in Kenya

(ii) There exist no common bean accessions resistant to CBB in Kenyan accessions.

(iii) There are no SCoT markers associated with CBB resistance in Kenyan common bean accessions

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin, distribution and description of common beans

Common beans are annual plants from the order Fabales, Fabaceae family and genus *Phaseolus* L (OECD, 2016). They consist of nearly 80 cultivated and wild type varieties that originated from Latin America 7,000 - 8,000 years ago. Common bean accessions are divided into two major gene pools, namely; the Mesomerican and Andean gene pools (Asfaw *et al*., 2009; Okii *et al*., 2014). Andean gene pool is the predominant common bean accessions in Kenya. Common beans were established in Africa before the advent of the colonial era. In Eastern Africa, they were first introduced at the coast by the Portuguese traders in the 16^{th} century and later on carried to the interior parts of the countries by Arab slave traders and Swahili merchants (Wortmann *et al*., 2006; Mwaipopo *et al*., 2017). Today, common bean accessions are produced throughout the tropical, sub-tropical and temperate climates/regions of the world. In Kenya, production is mainly by small scale farmers, in Western, Nyanza, Rift Valley, Eastern and Coastal regions of the country (Wortmann *et al*., 1998; Katungi *et al*., 2009).

Common bean is a diploid crop with a $2n=2\times=22$ chromosomal number and bears non-endospermic seeds that differ in both size and color. The cultivated forms of common beans are mainly herbaceous annuals with either indeterminate or determinate growth habits. Species with determinate growth pattern are preferred widely due to their short developmental cycle and their ability to adapt to different environmental conditions.

Common beans have an epigeal germination period of 5 to 7 days (OECD, 2016). On germination, common beans initially have a tap-root system with lateral roots running down to 15 cm below the soil; the roots are later taken over by rhizobium bacteria resulting in an irregular root nodule (Graham and Ranalli, 1997; OECD, 2016).

Flowering in common beans takes 28 days among the non-climbing common bean accessions and 42 days or longer among the climbing accessions. Flowers borne are zygomorphic with bi-petal keel, ten stamens and multi-ovuled ovary that is largely self-pollinating and few but observed instances of cross-pollination. Most flowers produced are shed-off; however, low temperature or water stress leads to the abortion of young fruits and /or developing seeds (OECD, 2016). Common bean also have a largely varied maturation period, which can be as short as 60 to 65 days after planting among the first growing varieties used in areas with short growing cycles or as long as 200 days among the climbing varieties in cool upland areas (Graham and Ranalli, 1997; Katungi *et al*., 2009).

2.2 Economic importance of common beans

Common bean is the most important and multifaceted legume grown and consumed worldwide due to its nutrition and economic value. It contributes about 65% of the total protein consumption and 32% of the total energy. Common beans are one of the principal staple foods in the Eastern and Southern parts of Africa, where they serve as an essential source of dietary protein and calories (Katungi *et al*., 2009). They are grown for their green leaves which, are consumed as vegetables, the immature and dry seeds which are

consumed as canned or boiled, for the immature green pods used as vegetables and for the bean residues used as fodder for animals. The seeds form the most significant economic part of the bean plant, particularly in the developing countries due to their ease of storage and preparation, long storage life, as well as good nutrition properties (Katungi *et al*., 2009).

In Kenya, common bean is the third staple food after maize and wheat, with an annual per capita consumption of 14 kg per person (Katungi *et al*., 2010). Common bean also serves as an affordable protein alternative, rich in essential amino acids such as lysine and Tryptophan (Katungi *et al*., 2010). Moreover, the crop is an excellent supplement to the country's carbohydrate-rich diet. Due to their short production cycle (Kimani *et al.,* 2014), common beans provide alternative food as other crops mature (Wortmann, 1998; Jones, 1999). Besides, the crop also generates foreign exchange to the country through export and income to small scale farmers who sell the crop to urban residents (Katungi *et al*., 2009; Balete and Bastas, 2017).

Common beans offer significant health benefits due to their low cholesterol, triglycerides and fat content. They are also digested slowly and elicit a sustained increase in blood sugar levels. Moreover, common beans are rich in phytochemicals, antioxidants and flavonoids. These factors contribute significantly towards reducing the risk of common diseases such as cancer, diabetes as well as coronary heart disease (Leterme and Munoz, 2002; Katungi *et al*., 2009; Messina, 2014). On the other hand, common bean also

combats constipation, thus preventing risks of colon cancer (Romero-Arenas *et al*., 2013).

Due to their innate ability to fix atmospheric nitrogen, common beans aid in enriching the soil with nitrogen and therefore reducing dependence on the commercial nitrogen fertilizer, which is expensive for the smallholder farmers, who are the major common bean producers in Kenya. Furthermore, common beans also serve as an excellent cover crop, hence preventing soil erosion (Wortmann *et al.*, 1998; Anunda *et al*., 2019).

2.3 Constraints to common bean production in Kenya

Although common beans offer significant economic and nutritional value, they are faced with numerous biotic and abiotic constraints that negatively impact their yield. Abiotic constraints such as extreme drought conditions contribute to 50% of the observed decrease in crop yield in Kenya. Decreased moisture results in poor grain filling and reduced number of seeds per pod, leading to poor seed quality and reduced seed yield (Rodríguez De Luque and Creamer, 2014).

Besides the abiotic constraints, common bean production is also faced with several biotic constraints, particularly insect pests such as aphids; bruchids, pod borers and bean stem maggots that reduce the yield of the crop (Graham and Ranalli, 1997; Wortmann *et al*., 1998). These insect pests also force farmers to sell the crops almost immediately after harvest at low prices resulting in high economic losses. Moreover, common beans are susceptible to various diseases that diminish their yield around the world. Some of the

most common diseases affecting common bean production in Kenya include fungal diseases such as bean root rot and bean stem maggot disease, angular leaf spot (*Phaeoisariopsis griseola* (Sacc.) (Wagara *et al*., 2004; Leitich *et al*., 2016) and anthracnose *(Colletotrichum lindemuthianum*) (Gathuru and Mwangi, 1999), Bacterial diseases such as common bacterial blight (CBB) (Wortmann *et al*., 1998; Kimani *et al*., 2005b; Belete and Bastas, 2017), halo blight (*Pseudomonas syringae* pv. *Phaseolicola*) and bean bacterial wilt (*Curtobacterium flaccumfaciens* pv. f*laccumfaciens*) and viral diseases such as *bean common mosaic virus* (BCMV) and *bean common mosaic necrosis virus* (BCMNV) (Kapil *et al*., 2011; Mutuku *et al*., 2016). Common bacterial blight (CBB) has gained significant attention compared to all the mentioned diseases because it is widespread, seed-borne transmitted, and results to significant yield and economic losses (Wortmann *et al*., 1998; Belete and Bastas, 2007).

2.3.1 Common bacterial blight (CBB)

Common bean bacterial blight is a universal disease that affects the production of common beans globally. The disease is caused by *Xap* from the phylum Proteobacteria, class Gammaproteobacteria, order Xanthomonadeles, family *xanthomonadaceaea*, and genus *Xanthomonas*. It is an aerobic, non-spore forming, gram-negative and rod-shaped bacteria. When grown on glucose-containing media, *Xap* produces yellow, slimy and convex colonies due to the production of xanthan gum on the mucus, and this distinguishes it from its variant, which produces dark brown diffusible colonies on tyrosine containing media (CABI, 2008; Belete and Bastas, 2017).

*Xanthomonas axonopodi*s pv. *phaseoli* alternate between three growth stages, the epiphytic, survival, and pathogenic stages. During the survival stage, the pathogen survives in the seed, organic matter or on volunteer and alternate perennial crops. It remains viable for extended periods (10 - 18 months) in these non-host organs (Akhavan *et al*., 2013). In the pathogenic stage, the bacteria penetrates the tissue and exponential growth is also observed. A single infected crop is enough to cause severe damage to the uninfected crops in the field. On the other hand, the epiphytic stage is a symptomless period characterized by warm environmental conditions, with high humidity and rain, this period is essential for the multiplication and development of substantial populations that would initiate disease during optimum environmental conditions (temperature ranging between 28 °C and 32 °C) (Akhavan *et al*., 2013; Belete and Bastas, 2017).

Common bacterial blight (CBB) contributes to significant losses in all common bean growing regions worldwide. In Kenya, yield losses of between 10 to 75% have been reported (Makini, 1995). The observed losses are associated with the lesions developed on common bean seeds by the *Xap* pathogens that reduce the seeds' quality, thereby negatively affecting their market value and hence significant economic losses to farmers. However, the damage is more pronounced when an infection occurs early in the plant's development stage, since the pathogen leads to reduced plant's translocation capacity and premature defoliation reducing the available photosynthetic area, leading to decreased number and size of common bean seeds and hence poor yield for farmers (Akhavan *et al*., 2013; Belete and Bastas, 2017; CABI, 2018).

2.3.1.1 Symptoms of common bacterial blight

The symptoms due to *Xap* infection in common beans appear on various parts of the plant. They begin as water-soaked spots that later enlarge and merge with neighboring spots and become necrotic. On the leaves, the spots would be surrounded by a yellow zone (Fig. 1A) and severe necrosis on the leaves will lead to defoliation (Akhavan *et al*., 2013). Infected pods will elicit water-soaked lesions with a central yellow or cream-colored bacterial colony; the lesions become sunken and form dark brown blotches (Harveson, 2009) (Fig. 1B). Pod infection leads to poor seed development resulting in seeds that are rotten or shriveled with circular and brownish-red spots, while in severe cases, the pods are shriveled and seedless (Akhavan *et al*., 2013). Symptoms on seeds appear as yellow-brown spots that are randomly distributed around the seed or helium (Fig. 1C); these symptoms are visible, especially on light-colored common bean seeds (Karavina *et al.*, 2011). Severely affected seeds will be shriveled and elicit weak vigor and germination (Belete and Bastas, 2017).

Figure 1: Symptoms caused by CBB on different parts of the bean plant.

(A) Necrotic lesions and yellowing of bean leaf; (B) Water soaked and sunken circular spots on bean pods; (C) Brown spots on bean seeds (on the right). (Source: Akhavan *et al*., 2013)

2.3.1.2 Source of bacterial inoculum, transmission and plant infection

Infected seeds are the primary source of X*ap* inocula, and the pathogen can remain viable for up to 30 years (CABI, 2018). When planted, these infected seeds result in the germination of seedlings with bacterial lesions on the cotyledons, primary leaves, and nodes (Singh and Miklas, 2015). Under optimum conditions, the bacteria will accumulate on the surface of the leaves, and they will be blown to the nearest healthy plants by agents of infection, including splashing and windblown rain as well as overhead irrigation. Insects and human beings are also agents of *Xap* transmission. Long-distance

dissemination of CBB caused by *Xap* pathogen is aided by infected seeds that are carried and established over long distances (Akhavan *et al*., 2013; Belete and Bastas, 2017).

The bacteria infects all the bean plant parts of the susceptible common bean, including; leaves stems as well as seeds (Singh and Miklas, 2015). The infection of common bean leaves occurs through natural openings such as the stomata or the hydathodes. Insects can also create wounds on the plant leaves, thus creating an optimum site for infection. Once inside, the bacteria invade the intracellular spaces multiplying rapidly to produce sufficient populations that would elicit infection symptoms as well as ooze out, causing secondary infection after 10 to 14 days (Karavina *et al*., 2011; Belete and Bastas, 2017; CABI, 2018). However, the pathogen accumulates more in the leaves and is spread faster systemically through vascular tissues in the CBB susceptible accessions than in resistant and partially resistant common bean accessions (Singh and Miklas, 2015). In the stem, the pathogen enters through the stomata of the hypocotyl and epicotyls. It then proceeds to the vascular elements where it builds up and eventually causes wilting of the plant through clogging of the vessel or disintegrating the cell wall. The bacteria in the vascular spaces may enter the developing pod and pass through to the seeds. Once in the seeds, the pathogen remains in the seed coat or cotyledons (Karavina *et al*., 2011; CABI, 2018).

2.3.1.3 Management of common bacterial blight

Current CBB management options include cultural methods (for example, use of pathogen-free seeds, sanitation, and crop rotation), use of chemicals and biocontrol methods (Balete and Bastas, 2017). However, several challenges face these management

methods. Although the use of pathogen free-seeds is one of the best CBB management option, inaccessibility and high costs associated with these seeds by the smallholder farmers in Kenya limits its wide application. On the other hand, chemicals are quite costly for smallholder farmers and also results in environmental pollution and degradation.

The use of CBB resistant common bean accessions is the most viable and economically feasible option in controlling the disease (Boersma *et al*., 2015). Previous studies have observed CBB resistance in decreasing order from Tepary beans, scarlet runner, and common beans. However, there have been strategies to enhance CBB resistance in common beans by the introduction of resistant genes from other bean species through breeding. To this effect, common beans have been crossed with tepary beans, producing improved breeding lines and accessions such as HR67, VAX 3-6 and HR45 which elicited high levels of CBB resistance (Marquez *et al*., 2007; Boersma *et al*., 2015; Belete and Bastas, 2017). Research on these varieties has been initiated in other countries/regions such as North America and Canada (Boersma *et al*., 2015). However, in Kenya, there exist limited studies on resistance to CBB in locally grown common beans. With the scarcity of this information, efforts to improve common bean production and reduce losses due to CBB in Kenya are limited.

2.4 Molecular markers and their use in crop diversity and genetic improvement of crops

Molecular markers reveal existing polymorphism in nucleotide sequences between different species arising due to mutations on DNA such as insertion, deletions, translocations, substitutions, and errors in replication of tandemly repeated DNA (Collard *et al*., 2005). Available molecular techniques reveal these existing genetic differences, which are then visualized either on a chemical (e.g. Ethidium bromide) stained agarose gel electrophoresis as well as using radioactive or colorimetric probes or automated visualization (Collard *et al*., 2005; Nadeem *et al*., 2018). Molecular markers can be polymorphic, revealing differences between individuals of the same or different species or monomorphic, unable to distinguish between the species. Polymorphic markers can either be co-dominant or dominant based on their ability to distinguish between homozygotes and heterozygotes (Collard *et al*., 2005).

Molecular markers are divided into three groups based on the method of detection. These are; hybridization-, PCR- and DNA sequence-based markers (Govindaraj *et al*., 2015). For the hybridization-based markers such as Restriction Fragment Length Polymorphism (RFLP), polymorphism within the different species is observed in terms of differences in fragment lengths that is due to the molecular changes that occur on the DNA leading to absence or presence of a recognition site (Nadeem *et al*., 2018). However, these markers are laborious and time-consuming and also require a lot of DNA (Garcia *et al*., 2004).

The PCR-based markers such as AFLP, SSRs, and SCoT, amplify particular DNA regions and the amplified products are separated and detected by the use of gel electrophoresis. These techniques obviate the need for hybridization with radio-labeled tags and also save on time (Garcia *et al*., 2004). The latest developments have resulted in third-generation markers like simple nucleotide polymorphisms (SNPs) and diversity array technology (DArT) markers. These are markers with high-throughput performance and automated detection methods. They are based on the determination of the actual sequence of the DNA through sequencing methods such as pyrosequencing and next-generation sequencing and using automated systems to detect existing polymorphisms (Govindaraj *et al*., 2015).

Molecular markers are applied in various studies, for instance in genetic diversity assessment in common beans using various molecular markers such as AFLP (Kumar *et al*., 2008), SSR and RAPD (Zargar *et al*., 2016), RFLP (Velasquez *et al*., 1994) and Inter-Simple Sequence Repeats, (ISSR) (Svetleva *et al*., 2006). Genetic diversity of a particular species gives information on the evolution and the comparative genomics of those species, thus helping in understanding population structure, evolutionary and phylogenetic relationships among and between species. Plant breeders also use molecular markers in the study of heterosis, after making crosses, in order to determine the performance of the progeny and elucidate the best parental crosses that bring forth superior progeny (Pheirim *et al*., 2017)

Molecular marker-assisted selection (MAS) is a superior breeding technique whereby the selection of target breeding lines is carried out based on molecular markers linked to the trait of interest as opposed to the observation of particular traits, as in conventional breeding (Collard and Mackill, 2007). Molecular assisted selection is a time-saving alternative to traditional breeding methods that obviates the need to wait for the phenotypic expression of the desired trait. Moreover, molecular markers are immune to environmental changes and are detected in all stages of plant growth (Collard and Mackill, 2007). Various molecular markers have been used in MAS for the improvement of crops and selection of varieties that are resistant to biotic and abiotic factors. For example, microsatellite and sequence characterized amplified region (SCAR) markers were used in MAS for selection of resistance to cassava mosaic disease in cassava (Carmo *et al*., 2015). In common beans, SCAR markers have been used in MAS for the selection of resistance to BCMV and bean golden mosaic virus diseases (Blair *et al*., 2007). Other markers such as RAPD have also been applied in MAS for improvement of drought resistance in common beans (Schneider *et al*., 1997)

2.4.1 Use of start codon targeted (SCoT) polymorphism marker

Start codon targeted (SCoT) polymorphism marker is a gene-targeted technique based on the nucleotide sequences at the translational start site ATG (Que *et al*., 2014). They are 18-mer primers that differ from each other by at least one nucleotide. The difference occurs mostly at the 3’end, which is believed to control the primer-template specificity; however, the amplification profiles of these primers differ from each other significantly

(Collard and Mackill, 2009). The SCoT marker technique detects the presence of both dominant and co-dominant markers (Nair *et al*., 2016). They are easy to use and less expensive, resulting in wide applications in many laboratories with basic equipment (Zhang *et al*., 2015).

Start codon targeted (SCoT) polymorphism marker was developed to improve and overcome the challenges associated with the use of RAPDs. As a result, they are highly reproducible compared to RAPD markers (Collard and Mackill, 2009). The SCoT markers can also be developed from transcribed regions and therefore, resultant markers may be linked to gene function (Que *et al*., 2014). The amplified products can be converted to a gene-targeted marker system. Therefore, the SCoT marker is an efficient technique in genetic diversity analysis since it is gene-targeted and has low recombination levels compared to ISSR and RAPD. Besides, compared to other markers, SCoT has superior resolving capabilities with a high potential to detect polymorphisms (Zhang *et al*., 2015; Etminan *et al*., 2018).

Initial validation for the use of SCoT marker technique was performed in rice (*Oryza sativa*) (Collard and Mackill, 2009). Following this study, SCoT markers have been applied in various studies, for instance, Al-qurainy *et al*. (2015); Rajesh *et al.* (2015) and Etminan *et al*. (2016) used SCoT markers for genetic diversity analysis in date palms, coconut and wheat respectively. The SCoT makers have also been used in population structure analysis and ancestry determination in ramie (Satya *et al*., 2015) as well as in

polymorphism analysis in sugarcane (Que *et al*., 2014). However, the use of SCoT markers in common bean accessions remains undocumented.

2.5. Plant response to diseases

Infection of plants by invading pathogenic bacteria leads to the induction of the plant defense system due to the recognition of pathogen-associated molecular patterns by the plant's pattern recognition receptors (Silva-Gomes *et al*., 2014). Consequently, a sequence of biochemical events is initiated, leading to the expression of either susceptibility or resistance to disease by the plant (Chatterjee and Ghosh, 2008). The plant-pathogen interaction produces signals which activate defense associated genes, as a result, various plant defense mechanisms are activated, including accumulation of TPCs among other compounds that contribute to plant defense systems (Chatterjee and Ghosh, 2008). Thus based on the response of the plant to the invading pathogen, it can then be categorized as susceptible or resistant. Plant-pathogen interactions lead to a surge in ROS. Although ROS are secondary messengers during pathogen invasion, in high quantities, ROS can be toxic to the plant (Grotto *et al*., 2009). Accumulation of ROS leads to lipid peroxidation which produces MDA as one of its products. Malondialdehyde (MDA) can therefore, be used as a marker for cell membrane damage caused by ROS (Grotto *et al*. 2009). Detoxification of ROS may be achieved enzymatically or through non-enzymatic mechanisms (Sharma *et al*., 2012). Enzymatic detoxification involves enzymes such as CAT and POD, among others.

Peroxidase (POD) is one of the initial enzymes that provide first response and defense against the invading plant pathogens. This is achieved through ROS detoxification and enhancement of the plant's physical defense mechanisms, thereby strengthening its defense ability against the invading pathogen (Siddique *et al*., 2014). On the other hand, CAT is an efficient oxygen-scavenging enzyme; it catalyzes the detoxification of H_2O_2 to water and hydrogen (Sharma *et al*., 2012; Siddique *et al*., 2014).

Phenolic compounds are predominantly involved in plant growth and development, as well as in defense mechanisms. These compounds serve as potent non-enzymatic antioxidants within the cell, they may act nonspecifically against the pathogen through disruption of the structural integrity of the bacterial membrane and specifically by inhibiting the bacterial enzymes involved in electron transport. Phenolic compounds also inhibit lipid peroxidation by stabilizing the membrane, thus limiting the diffusion of free radicals and reduce the peroxidation of membrane lipids (Kulbat, 2016). Also, phenolic compounds form part of the starting molecules required for the synthesis of lignin and suberin, which strengthens plant cell walls, thus preventing further entry of the pathogen and also containing the pathogen within the infected area (Kubalt, 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant materials

Thirty (30) pathogen-free and certified seeds of common bean accessions (Table 1) were used in the current study. Fourteen common bean accessions were obtained from the Genetic Resources Research Institute (GERRI) where they were preserved after collection from farmers' fields in the Nyanza region of Kenya in the year 1998. The other sixteen accessions were collected from farmers' fields in the Nyanza region in the year 2018.

Table 1: Identity and seed characteristics of the common bean accessions characterized using SCoT markers

Sample Id	Accession name	Place of sample collection	Weight in (g)	Color of seed	Seed size
3	KAT X69	Nyanza	0.54	Brown with white specks	Medium
7	GBK 036530	Genebank	0.18	Black	Small
8	GBK 030249	Genebank	0.25	Cream with black specks	Small
9	GBK 036524	Genebank	Not done	Not done	Data not available
19	GBK 036527	Genebank	0.42	Purple	Medium
21	GBK 030178	Genebank	0.29	Brown	Small
22	GBK 030259	Genebank	0.33	Cream with brown specks	Medium
25	GBK 036523	Genebank	0.41	Cream with brown specks	Medium
27	GBK 030171	Genebank	0.30	Brown	Small
28	GBK 0365328	Genebank	0.27	Black	Small
29	GBK 030217	Genebank	Not done	Not done	Data not available
30	GBK 030244	Genebank	0.41	Cream with brown specks	Medium
33	GBK 030227	Genebank	0.43	Brown with cream specks	Medium
35	GBK 030157	Genebank	0.40	Cream with brown specks	Medium
41	Ritinge LRC 20 KSI	Nyanza	0.60	Purple with white specks	Large
45	Osama LRC 23 KSI	Nyanza	Not done	Not done	Data not available
47	Emwamu LRC 22 KSI	Nyanza	0.25	Black	Small
48	Emwetemania LRC 04 KSI	Nyanza	0.24	Cream with brown Specks	Small
49	Girini LRC 05 KSI	Nyanza	0.47	Green	Medium
50	LRC 12 KSI	Nyanza	0.73	purple	Large
51	LRC 11 KSI	Nyanza	0.25	White	Small
53	Chinchae LRC 08 KSI	Nyanza	0.40	Black	Medium
54	Ekoko enyege LRC 15 KSI	Nyanza	0.41	Cream with red specks	Medium
55	Ekoko entabe LRC 24 KSI	Nyanza	0.53	Brown with red specks	Medium
56	Morogi LRC 21 KSI	Nyanza	0.21	Black	Small
58	Manoa LRC 16 KSI	Nyanza	0.81	Black with white specks	Small
60	Royoo LRC 09 KSI	Nyanza	0.20	Brown	Small
61	Makueni 1	Nyanza	0.12	White	Small
63	KAT B1	Nyanza	0.40	Green	Medium
64	GBK 030167	Genebank	0.28	Brown	Small

3.2 Planting of common bean seeds in plastic pots in the glasshouse

Experimental soil was obtained from the forest at the College of Biological and Physical Sciences (CBPS), the University of Nairobi, sterilized by autoclaving and allowed to cool overnight. Then plastic pots, 10 cm in diameter, were obtained. These pots were then clearly labeled using the names of common bean accessions and filled with sterile soil. Seeds of each accession were then established in triplicate in their respective labeled pots, covered with soil and watered. Thereafter, the seeds were watered fortnightly till germination. Three weeks after germination, the leaf samples were taken for DNA extraction.

3.3 Determination of genetic diversity of common bean accessions using SCoT markers

3.3.1 Genomic DNA extraction

Genomic DNA was extracted from young leaves of three-week-old plants using cetyltrimethylammonium bromide (CTAB) protocol, following the Chaudhary *et al*. (2008) method but with minor adjustments made to simplify the protocol and improve on the yield of the DNA. In this modified protocol, the fresh leaf samples were crushed in a CTAB buffer consisting of 1% CTAB powder, 5M NaCl, 1% polyvinylpyrrolidone (PVP), 1M Tris (pH 8.0), 0.5 M Ethylenediaminetetraacetic Acid (EDTA), 10% sodium dodecyl sulfate (SDS). The resulting homogenate was pipetted in a sterile microcentrifuge tube and incubated for 30 min at 65 °C in a water bath (Memmert, Germany). The mixture was then cooled down for 5 min at 25 °C and centrifuged for 10 min at 16060 Relative Centrifuge Force (×g), resulting in a multi-layered solution. The upper supernatant from the heterogeneous mixture was then pipetted into a sterile and labeled micro-centrifuge tube. Then an equal volume of chloroform: Isoamyl alcohol (24:1) was added to the supernatant, gently mixed, and allowed to settle for 5 min at 25

°C before centrifuging for 10 min at 16060 ×g, the resulting supernatant was pipetted into a labeled sterile micro-centrifuge tube. This step was replicated and an equal volume of ice-cold isopropanol was then added to the final supernatant and precipitated overnight at -21 °C. The precipitate was then centrifuged for 10 min at 16060 ×g. The supernatant was pipetted off leaving a clear pellet, 70% ethanol was added to the pellet and centrifuged for 10 min at 160160 ×g, the supernatant was pipetted off and the step repeated. Then the resulting pellet was air drying by inversion of the micro-centrifuge tube on a paper towel for 1 hr at 25 °C. The pellet was re-suspended in 50 µl double-distilled sterile water, followed by RNAse treatment for 1 hr at 37 °C to digest Ribonucleic acid (RNA).

3.3.2 DNA quantification and quality assessment

Agarose gel (1%) stained with ethidium bromide was prepared to determine the quality of the DNA. The preparation of this gel proceeded as follows; 1% agarose gel powder 1% (w/v) was weighed and dissolved in 100 ml tris /borate/EDTA (TBE). The mixture was microwaved for 3 min to boil and cooled to approximately 50 to 55 °C at 25 °C. After cooling, 0.6 µg/mL of ethidium bromide stain was added; the mixture was then poured on a casting tray with well-fitted combs to solidify. The solidified gel was submerged in an electrophoresis tank containing 1× TBE buffer and the combs gently removed. Sample DNA was obtained, and from each sample, a volume of 3 µl was obtained and mixed with 2 µl of bromophenol blue dye on a flat sterile surface before pipetting the mixture onto the wells starting with the second well. The lambda DNA ladder (Thermo Scientific) was pipetted into the first well to estimate the DNA size. The gel electrophoresis was allowed to run for 45 min at 70 volts (V). The gel was visualized under anultraviolet (UV) transilluminator and captured using Redmi 4X camera.

Quantification of the DNA of each sample was assayed spectrophotometrically. From each extracted DNA, 5 µl was obtained and diluted to 1000 µl. The absorbance of each sample was taken at 260 and 280 nm. Thereafter, the concentration of each DNA was calculated following a formula described by Sambrook *et al*. (1989) while the A_{260}/A_{280} ratio determined the purity of each DNA sample.

$$\text{DNA concentration} = \frac{OD_{260} \times 50 \text{ (Dilution factor)} \times 50 \text{ µg/ml}}{100}$$

3.3.3 SCoT- PCR amplification

Thirty-six (36) SCoT primers synthesized by MACROGEN (Netherlands) were used. The protocol described by Agarwal *et al*., (2018) was used. The optimum working conditions for each primer were determined (Table 2), the best primers that gave clear and unambiguous amplified bands were selected. The PCR was carried out in a 20 µl reaction volume containing 13 µl master mix 2× PCR AccuPower PCR master mix (Taq buffer and polymerase, dNTPs, and $MgCl_2$) (Bioneer, USA), 0.8 µm of each of the SCoT primers, 2 µl (20 ng/ml) DNA sample and 3 ml sterile distilled water in a 200 µl PCR tubes. The PCR amplification was performed using the Applied Biosystems Veriti 96-well thermal cycler (Singapore). The PCR program was set at an initial denaturation temperature of 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 30 sec, annealing at 46-54 °C (depending on the primer used, Table 2) for 1 min, extention at 72 °C for 1 min, and final extension at 72 °C for 7 min and held at 4 °C till use. Agarose gel (1.5%) stained with 0.5 µg/mL Ethidium bromide in 1× TBE buffer was used to confirm the presence of amplified PCR products via electrophoresis. The electrophoresis was run at 80 V for 2 hrs 30 min. GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) was used

to estimate the band sizes of the amplified PCR products. The resulting agarose gels were visualized and documented by Gel Doc XR system (Bio-rad, Hercules, CA, USA).

Table 2: Descriptions of the 17 SCoT primers used in the study

Marker	Primer sequence	Amplicon size range (bp)	Annealing temperature (°C)	GC content
SCoT 4	CAACAATGGCTACCACCT	300-1000	48	50
SCoT 5	CAACAATGGCTACCACGA	300-1500	46	50
SCoT 7	CAACAATGGCTACCACGG	300-2500	46	55.6
SCoT 11	AAGCAATGGCTACCACCA	350-2500	50	50
SCoT 14	ACGACATGGCGACCACGC	200-1800	54	66.7
SCOT 15	ACGACATGGCGACCGCGA	200-2000	52	66.7
SCoT 16	ACCATGGCTACCACCGAC	200-2000	51	61.1
SCoT 23	CACCATGGCTACCACCAG	350-3000	51	61.1
SCoT 24	CACCATGGCTACCACCAT	400-1500	51	55.6
SCoT 26	ACCATGGCTACCACCGTC	450-2000	51	61.1
SCoT 27	ACCATGGCTACCACCGTG	400-2000	46	61.1
SCoT 29	CCATGGCTACCACCGGCC	250-2000	51	72.2
SCoT 30	CCATGGCTACCACCGGCG	250-1600	54	72.2
SCoT 32	CCATGGCTACCACCGCAC	100-2000	51	66.7
SCoT 34	ACCATGGCTACCACCGCA	400-2000	51	61.1
SCoT 35	CATGGCTACCACCGGCCC	300-2500	53	72.2
SCoT 36	CGCAACAATGGCTACCACC	200-1500	51	55.6

3.3.4 Scoring of amplified DNA bands and statistical data analysis

Only clear and distinct amplified bands on the gel image were scored visually as either present (1) or absent (0), generating a binary matrix data, which was used for subsequent data analysis. The summary of genetic parameters including total amplified bands (TAB), monomorphic bands (MB), percentage bands per loci (PB), RP, heterozygozity (HT), polymorphic information content (PIC), effective number of alleles (Ne), genetic diversity (GD) and shannon information index (I) were computed in PowerMarker V3.25 (Liu *et al*., 2005). Pairwise genetic similarity and dissimilarity between different common bean accessions were determined by calculating Jaccard's dissimilarity coefficient. Unweighted pair group method with arithmetic mean (UPGMA) was used to assess the patterns of diversity among the common bean accessions using DendroUPGMA server (Garcia-Vallvé *et al*., 1999) which was used in calculating matrix distances (Jaccard). A dendrogram was constructed using FigTree software V1.4.2 (Rambaut, 2009). Principal component analysis (PCA) and analysis of molecular variance (AMOVA) for the common bean accessions were assessed using GenAlex V6.5 software (Peakall and Smouse, 2012).

3.3.5 Population structure analysis

The population structure evaluation of the 30 common bean accessions was achieved by STRUCTURE V2.3.4 software (Pritchard *et al*., 2000) using data from 17 SCoT primers. Analyses were performed using admixture model assumptions with correlated alleles; the maximum number of population (K) subgroups were presumed to be between 2 and 10, and were selected after 10 independent runs. The burn-in period for each independent run was 5,000 steps followed by 50,000 Markov Chain Monte Carlo method (MCMC) replicates (Pritchard *et al*.,

2003). Structure harvester (Earl and Vonholdt, 2012) was used to collate the results obtained from STRUCTURE (Evanno *et al*., 2005) and the maximum value of delta K (ΔK) associated with each K value was analyzed to identify the number of clusters that best described the data.

3.4 Screening common bean accessions for common bacterial blight resistance

Based on the dendrogram generated using data obtained from SCoT markers, 20 out of the 30 common bean accessions from each sub-grouping in the dendrogram were selected and screened for CBB resistance in the glasshouse.

3.4.1 Isolation of bacteria from infected leaves of common bean plants

Leaf samples of CBB infected common bean accessions were collected from farmer's fields in Nyanza region and used as a source of inoculum. The *Xap* pathogen was isolated the CBB infected leaf samples as follows; the leaves were sliced into small pieces and transferred to sterile centrifuge tubes. Then 5 ml liquid selective media consisting of; 1% yeast extract, 1% peptone, 1% glucose, 50 mg/l cephalexin and 150 mg/l cycloheximide (pH 7.0) (YPG-CC) was added to the macerated leaf samples. This was then incubated at 28 °C for 48 hrs, thereafter diluted serially to 10^{-3}. An aliquot of 0.1 ml from the bacterial dilution was transferred to 1% yeast extract, 1% peptone, 1% glucose, 1.5% agar (pH 7.0) (YPGA) medium, supplemented with 50 mg/l cephalexin and 150 mg/l cycloheximide, and incubated at 28 °C for 48 hrs. Then a bacterial glycerol stock was prepared and stored at -80 °C till use.

3.4.2 Evaluation of common bean accessions for resistance to CBB

The *Xap* strain in the glycerol stock was revived by taking 10 µl of the bacterial glycerol stock into 15 ml in YPG medium (1% yeast, 1% peptone and 1% glucose), the mixture was incubated overnight at 25 °C. Then the bacteria was then streaked onto the YPGA medium and incubated at 25 °C for 24 hrs. A single colony was then inoculated in a YPG medium and incubated at 28 °C in a shaker at 2,800 rotations per minute (rpm) (Gallenkamp, UK) for three days. The liquid inocula were calibrated at OD 600_{nm} = 0.002, corresponding to 10^6 colony forming units (CFU).

Three-week-old plants of each common bean accession were infected with *Xap* following the procedure by Alladassi *et al*. (2018). The plants were watered 4 hrs before inoculation. The third and fourth fully opened leaves were clipped with a razor blade and dipped in the bacterial solution for 30 sec. The inoculated plants were then covered with a polythene bag for two days. Inoculated plants were observed for disease symptoms, and disease severity was scored between 1 to 5 where: 1 = no symptoms observed; 2 = only leaf spot symptoms visible, i.e., translucent and water-soaked spots; 3 = leaf blight; 10 to 50 % leaf area infected, inoculated trifoliate intact; 4 = severe blight symptoms; more than 50% leaf area infected, inoculated trifoliate intact; and 5 = inoculated trifoliate is shed. In order to determine significant differences between the reactions of the common bean accessions to CBB disease, the disease severity scores of the common bean accessions were subjected to analysis of variance and Duncan Multiple Rank Test (test level 5%) using statistical software for social sciences (SPSS) V21.

3.4.3 Bacteria quantification in leaves of inoculated plants

Each experiment was performed in triplicate, leaves of inoculated and non-inoculated common bean accessions were collected from the greenhouse. Quantification of the bacteria in leaves was performed following the procedure described by Denardin and Agostini. (2013), with minor changes. The leaves were washed under running tap water for 2 min, dipped for 30 sec in 1.5% sodium hypochlorite, rinsed for 1 min in sterile water, dipped for 2 min in 4% H_2O_2, rinsed for 3 min in sterile water, dipped in 70% ethanol for 3 min. The leaf was then washed three times in sterile water for 3 min. Thereafter, each leaf sample was crushed into a fine homogenate in a sterile mortar and pestle. The resultant homogenate was dispensed in well-labeled sterile micro-centrifuge tube and centrifuged at 9500 ×g for 10 min. An aliquot of 80 µl of the supernatant was then spread on the YPGA medium and incubated at 28 °C for 48 hrs. The plates were observed for the growth of *Xap* colonies. Non-inoculated plants and the water used for the last rinse of the leaves were used as controls.

3.4.4 Analysis of biochemical parameters in CBB-resistant and susceptible common bean accessions

Based on the results from the CBB resistance screening experiments, 2 accessions each for resistant and susceptible were selected and used for biochemical analyses.

3.4.4.1 Determination of lipid peroxidation

Lipid peroxidation was estimated from MDAcontent following the Hodges *et al*. (1999) and Chen and Gallie (2006) protocol, based on thiobarbituric acid (TBA). A pod sample weighing

0.4 g was crushed in 4 ml of 0.1% (w/v) trichloroacetic acid (TCA). The suspension was then centrifuged for 5 min at 9500 ×g and 0.5 ml of the supernatant collected. An aliquot of 1 ml 20% (w/v) TCA containing 0.5% (w/v) TBA was added to the supernatant and incubated in a water bath at 95 °C for 30 min before cooling the mixture on ice for 20 min and then spinning for 10 min at 1160 ×g. The absorbance of the supernatant was taken at 600 nm and 532 nm. "Non-specific turbidity was corrected by subtracting the readings obtained at 600 nm from the readings at 532 nm". Malondialdehyde concentration was calculated with its extinction coefficient of 155 mM-1 cm-1 and expressed as nmol malondialdehyde g^{-1} fresh mass using the formula:

Total MDA =Amount of extraction buffer (ml) × Amount of supernatant (ml) ×[Abs 532−Abs 600)/ 155]×1000/ Amount of sample; Where 532 nm =maximum absorbance of the TBA-MDA complex; 600 nm =the correction for non -specific turbidity and 155 $mM^{-1}cm^{-1}$ =specific molar extinction coefficient for MDA.

3.3.4.2 Estimation of total phenolic compounds (TPC)

The concentration of TPC was assayed colorimetrically following the procedure by Bakar *et al*. (2015) based on the reduction of Folin- Ciocalteu. Pod samples, each weighing 0.5g were crushed in 95% ethanol and incubated for 48 hrs at 25 °C. The mixture was then spun for 15 min at 16060 ×g, then 300 µl of the supernatant was taken and mixed with 2250 µl of Folin-ciocalteu and 2250 µl of sodium bicarbonate. The mixture was incubated for 90 min at 25 °C. The absorbance was then read spectrophotometrically at 725 nm and the results recorded. A standard graph using Galic acid was used to estimate the content of phenolic compounds and the results expressed as mg of Galic acid equivalent (GAE) per gram of extracts.

3.4.4.3 Assay of antioxidant enzyme activities

Pod samples obtained from 2 CBB resistant and 2 CBB susceptible common bean accessions were used. The pod samples used, weighed 0.25g; they were then crushed into a fine homogenate in 2 ml of extraction buffer containing 1% w/v PVP, 0.2 mM EDTA and 100 mM potassium phosphate buffer (pH 6.8) on ice. The mixture was then centrifuged for 20 min at 16060 ×g. The supernatants were assayed for catalase and peroxidase enzyme activities.

Peroxidase (POD) activity was determined by adding 50 µl of the homogenized bean pod sample into 2 ml of the reaction mixture containing 25 mM H_2O_2, 25 mM guaiacol and 50 mM sodium acetate buffer (pH 7.0). Peroxidase enzyme activity was determined by recording absorbance readings at 470 nm. The total POD activity was calculated as follows;

> Total peroxidase activity = (Change in absorbance ×Total reaction volume) ÷ (sample volume × Extinction coefficient of the enzyme). The Extinction coefficient of POD is 26.6 $mM^{-1}cm^{-1}$.

The CAT activity in the common bean samples was determined following the Cakmak *et al*. (1993) protocol. To 50 µl of the enzyme extract, 3ml of reaction buffer containing 15 mM H_2O_2 and 50 mM phosphate buffer (pH 7.0) was added. Catalase activity was determined from absorbance readings at 240 nm for 1 minute; the readings decrease with the decay of H_2O_2. The CAT activity was calculated following the formula described by Nakano and Asada, (1981);

> Total catalase activity = (Change in absorbance × Total reaction volume) ÷ (sample volume × Extinction coefficient of the enzyme). The extinction coefficient of CAT is 40 $mM^{-1}cm^{-1}$.

CHAPTER FOUR

4.0 RESULTS

4.1 Molecular characterization of common bean accessions using SCoT primers

4.1.1 Polymorphism and diversity parameters revealed by SCoT markers

A total of 36 SCoT primers were screened for molecular characterization of common bean accessions. Then 17 SCoT primers (Table 2) that produced clear and reproducible bands were selected for the determination of molecular diversity of common bean accessions. A representative amplification profile generated by SCoT 23 and 34 primers for common bean accessions is shown in Fig. 2.

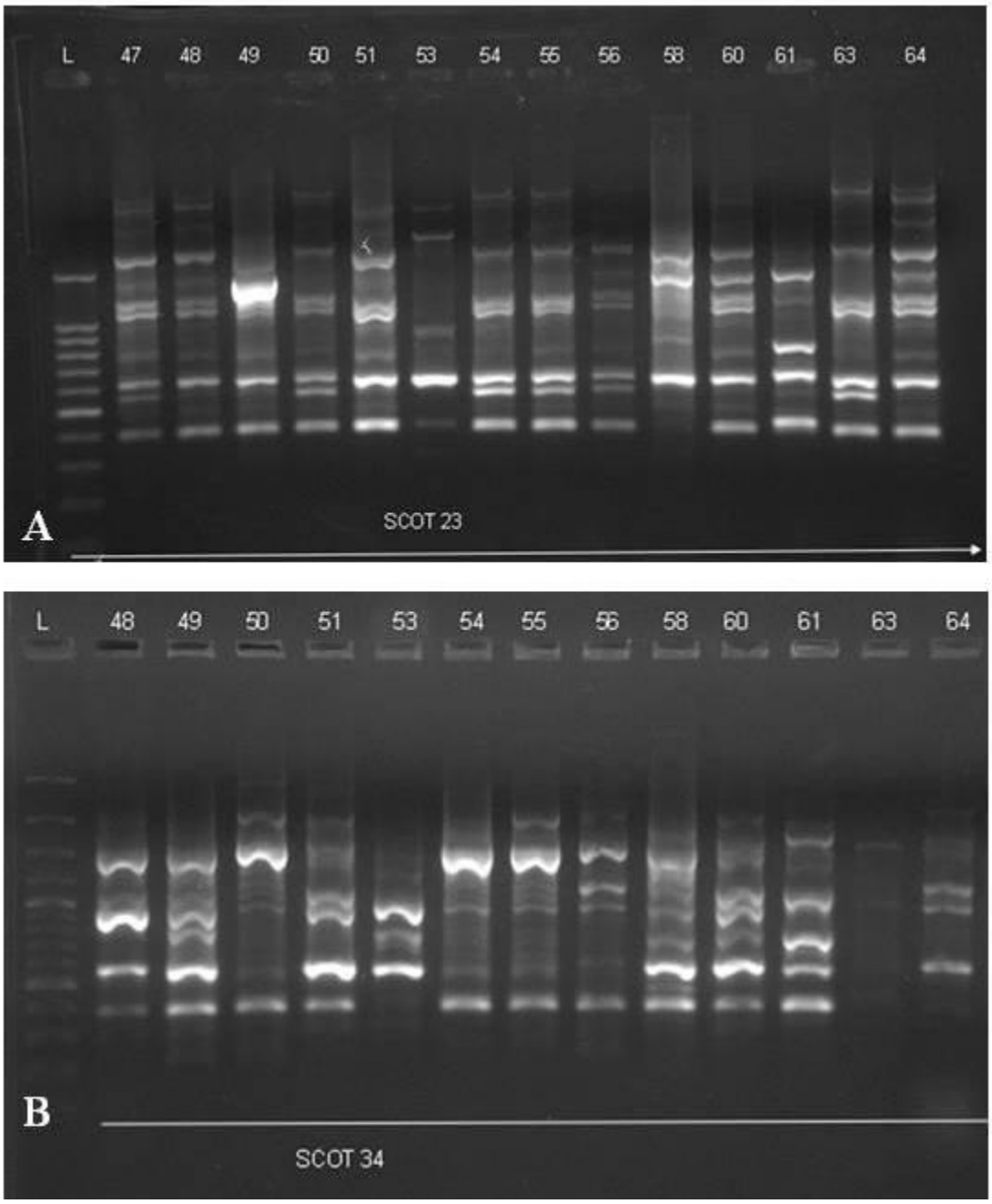

Figure 2: Electrophoresis gel image of PCR amplicons using SCoT 23 and SCoT 34 for common bean accessions.

A and B represent PCR amplification profile of SCoT 23 and SCoT 34 respectively. Lanes 47 – 64 and 48 - 64 in A and B, respectively, represent amplified DNA samples of common bean accessions. L is GeneRuler 1 kb Plus DNA Ladder (Fisher ThermoScientific).

The 17 SCoT markers amplified a total of 224 reliable and unambiguous bands ranging from 100 – 3000 bp, the average bands per primer was 13, ranging from 6 (SCoT 24) to 18 (SCoT 7) (Table 3), 95% of the amplified bands were polymorphic.

The GD of the assessed common bean accessions was between 0.93 (SCoT 24) and 0.99 (SCoT 4,7,32 and 35) with a mean of 0.98 (Table 3). On the other hand, I ranged from 0.90 (SCoT 4) to 1.54 (SCoT 27) with a mean of 1.24. Minimum (0.74) and maximum (0.91) HT values were recorded by SCoT 24 and SCoT 7, respectively, with a mean of 0.86. The minimum (3.77) and maximum (11.03) Ne was observed for markers SCoT 24 and SCoT 7, respectively, with a mean of 7.68. The average PIC for the 17 SCoT markers was 0.73, the lowest PIC value (0.60) was observed for marker SCoT 27 while the highest (0.85) was observed for marker SCoT 7. The mean RP was 5.09, the RP values recorded for the 17 SCoT markers ranged from 1.73 (SCoT 24) to 8.13 (SCoT 7) (Table 3).

Table 3: Amplification and polymorphism parameters of common bean accessions revealed by SCoT markers

No	Primer	TAB	MB	PB Per loci	Gene diversity	I	HT	Ne	PIC	RP
1	SCoT 4	13	1	0.92	**0.99**	**0.90**	0.81	5.35	0.81	2.93
2	SCoT 5	9	1	0.89	0.97	1.21	0.84	6.09	0.72	4.47
3	SCoT 7	**18**	0	1.00	**0.99**	1.13	**0.91**	**11.03**	**0.85**	**8.13**
4	SCoT 11	12	2	0.83	0.98	1.11	0.85	6.46	0.76	4.13
5	SCoT 14	13	0	1.00	0.98	1.35	0.85	6.63	0.76	7.00
6	SCoT 15	11	0	1.00	0.98	1.36	0.88	8.40	0.74	6.00
7	SCoT 16	14	1	0.93	0.98	1.35	0.84	6.09	0.74	6.07
8	SCoT 23	17	2	0.88	0.98	1.35	0.90	9.56	0.68	5.40
9	SCoT 24	**6**	1	0.83	**0.93**	1.07	**0.74**	**3.77**	0.60	**1.73**
10	SCoT 26	9	0	1.00	0.96	1.31	0.84	6.09	0.63	3.00
11	SCoT 27	14	1	0.93	0.97	**1.54**	0.89	9.28	**0.60**	4.40
12	SCoT 29	16	0	1.00	0.98	1.24	0.88	8.54	0.74	4.93
13	SCoT 30	16	0	1.00	0.98	1.32	0.89	8.94	0.71	4.47
14	SCoT 32	17	1	0.94	**0.99**	1.29	**0.91**	10.63	0.77	7.73
15	SCoT 34	14	1	0.93	0.98	1.12	0.86	7.14	0.76	4.13
16	SCoT 35	13	0	1.00	**0.99**	1.13	0.88	8.16	0.82	5.73
17	SCoT 36	12	0	1.00	0.98	1.26	0.88	8.41	0.78	6.20
Mean	-	13	4	0.95	0.98	1.24	0.86	7.68	0.73	5.09

Total amplified band (TAB) Monomorphic bands (MB) percentage bands per loci (PB) Shannon information index (I) Heterozygozity (HT) Effective alleles(Ne) Polymorphic information content (PIC) Resolving power (RP)

4.1.2 Similarity coefficient among the 30 common bean accessions

Estimation of Jaccards' coefficient of genetic similarity among the assessed common bean accessions revealed an average similarity index of 0.52. The least similarity was between Chinchae LRC 08 KSI (53) and GBK 036527(19) and between Chinchae LRC 08 KSI (53) and GBK 030259 (22) with a similarity index of 0.26 while the highest similarity was between GBK 036523 (25) and KAT X69 (3) with a similarity index of 0.73 (Table 4).

Table 4: Pairwise genetic similarity among 30 common bean accessions as revealed by 17 SCoT primers

	3	7	8	9	19	21	22	25	27	28	29	30	33	35	41	45	47	48	49	50	51	53	54	55	56	58	60	61	63	64
3	1.00	0.53	0.40	0.65	0.63	0.60	0.57	0.73	0.58	0.55	0.58	0.68	0.55	0.70	0.60	0.57	0.57	0.57	0.47	0.60	0.50	0.31	0.61	0.69	0.59	0.33	0.53	0.44	0.50	0.56
7		1.00	0.51	0.54	0.48	0.61	0.47	0.51	0.62	0.45	0.54	0.45	0.43	0.47	0.53	0.63	0.57	0.60	0.54	0.46	0.53	0.27	0.48	0.50	0.47	0.32	0.59	0.42	0.43	0.51
8			1.00	0.45	0.37	0.46	0.44	0.37	0.48	0.36	0.43	0.37	0.34	0.41	0.42	0.49	0.41	0.41	0.46	0.35	0.44	0.33	0.41	0.38	0.39	0.29	0.45	0.32	0.40	0.42
9				1.00	0.60	0.56	0.54	0.61	0.58	0.55	0.60	0.62	0.49	0.64	0.58	0.54	0.56	0.52	0.50	0.60	0.49	0.29	0.56	0.66	0.51	0.32	0.50	0.38	0.50	0.50
19					1.00	0.52	0.44	0.57	0.52	0.51	0.61	0.58	0.52	0.58	0.55	0.49	0.50	0.49	0.44	0.48	0.47	0.26	0.55	0.56	0.44	0.32	0.51	0.38	0.44	0.46
21						1.00	0.48	0.53	0.65	0.47	0.57	0.53	0.41	0.57	0.50	0.53	0.62	0.56	0.54	0.54	0.53	0.33	0.52	0.56	0.50	0.33	0.54	0.41	0.52	0.60
22							1.00	0.57	0.52	0.51	0.50	0.50	0.54	0.58	0.51	0.51	0.51	0.47	0.41	0.47	0.48	0.26	0.56	0.55	0.49	0.32	0.48	0.35	0.51	0.47
25								1.00	0.56	0.47	0.58	0.64	0.48	0.65	0.56	0.54	0.55	0.52	0.50	0.59	0.47	0.29	0.61	0.62	0.52	0.33	0.51	0.39	0.47	0.49
27									1.00	0.50	0.55	0.56	0.46	0.58	0.46	0.54	0.55	0.54	0.56	0.51	0.56	0.28	0.51	0.55	0.50	0.33	0.59	0.36	0.44	0.58
28										1.00	0.53	0.54	0.51	0.56	0.51	0.49	0.48	0.42	0.39	0.44	0.43	0.27	0.55	0.48	0.45	0.29	0.45	0.33	0.47	0.41
29											1.00	0.57	0.45	0.60	0.55	0.50	0.53	0.49	0.46	0.45	0.46	0.28	0.53	0.51	0.50	0.34	0.44	0.35	0.44	0.49
30												1.00	0.54	0.64	0.56	0.47	0.48	0.47	0.45	0.56	0.44	0.29	0.54	0.58	0.44	0.36	0.51	0.37	0.51	0.46
33													1.00	0.52	0.49	0.47	0.44	0.44	0.38	0.48	0.43	0.29	0.44	0.46	0.41	0.32	0.46	0.34	0.43	0.39
35														1.00	0.63	0.58	0.55	0.54	0.46	0.59	0.45	0.33	0.60	0.58	0.57	0.36	0.51	0.38	0.56	0.52
41															1.00	0.59	0.54	0.54	0.40	0.53	0.46	0.31	0.54	0.55	0.48	0.34	0.46	0.34	0.55	0.51
45																1.00	0.57	0.55	0.49	0.49	0.51	0.29	0.48	0.53	0.52	0.32	0.54	0.38	0.46	0.51
47																	1.00	0.67	0.54	0.57	0.52	0.33	0.51	0.57	0.58	0.32	0.49	0.37	0.46	0.58
48																		1.00	0.55	0.58	0.54	0.33	0.50	0.55	0.55	0.39	0.53	0.43	0.49	0.57
49																			1.00	0.49	0.55	0.30	0.44	0.50	0.46	0.30	0.54	0.36	0.43	0.50
50																				1.00	0.52	0.39	0.57	0.69	0.54	0.33	0.52	0.40	0.53	0.55
51																					1.00	0.31	0.47	0.55	0.57	0.30	0.60	0.38	0.46	0.54
53																						1.00	0.31	0.32	0.31	0.33	0.28	0.36	0.35	0.30
54																							1.00	0.65	0.50	0.31	0.48	0.38	0.53	0.47
55																								1.00	0.56	0.35	0.55	0.47	0.53	0.57
56																									1.00	0.34	0.45	0.41	0.53	0.56
58																										1.00	0.33	0.35	0.32	0.31
60																											1.00	0.44	0.41	0.55
61																												1.00	0.41	0.40
63																													1.00	0.51
64																														1.00

3= KAT X69, 7= GBK 036530, 8= GBK 030249, 9= GBK 036524, 19= GBK 036527, 21= GBK 030178, 22= GBK 030259, 25= GBK 036523, 27= GBK 030171, 28= GBK 0365328, 29= GBK 030217, 30= GBK 030244, 33=GBK 030227, 35=GBK 030157, 41= LRC20, 45= LRC 23, 47= LRC22, 48=LRC04, 49=LRC05, 50=LRC11, 51=LRC08, 53=LRC15, 55=LRC24, 56=LRC21, 58=LRC16, 60= LRC09, 61= Makueni 1, 63=KATB1, GBK030167

4.1.3 Cluster analysis

Based on the UPGMA clustering algorithm from the 17 SCoT markers, the 30 common bean accessions were categorized into 2 clusters, A and B (Fig. 3) at 44% with an index length of 0.3-0.7. The first and second clusters were composed of 24 and 2 common bean accessions, respectively. The common bean accessions from the two different collection sites were randomly clustered in the dendrogram with no specificity to the site of seed collection (Table 5). Four outgroups consisting of accessions from the Genebank and Nyanza were also observed in the dendrogram (Fig. 3; Table 5).

Table 5: Description of the distribution of 30 common bean accessions in the dendrogram and their respective sites of collection.

Cluster	Sub-cluster	No. of accessions	Accession ID	Site of accession collection
A	1	14	3, 25, 35, 30, 9, 50, 55, 54, 41, 19, 29, 28, 22, 33	Genebank and Nyanza
	2	10	7, 45, 47, 48, 21, 27, 64, 49, 51, 60	Nyanza and Genebank
B		2	56, 63	Nyanza
Outgroups		4	8, 61, 58, 53	Nyanza and Genebank

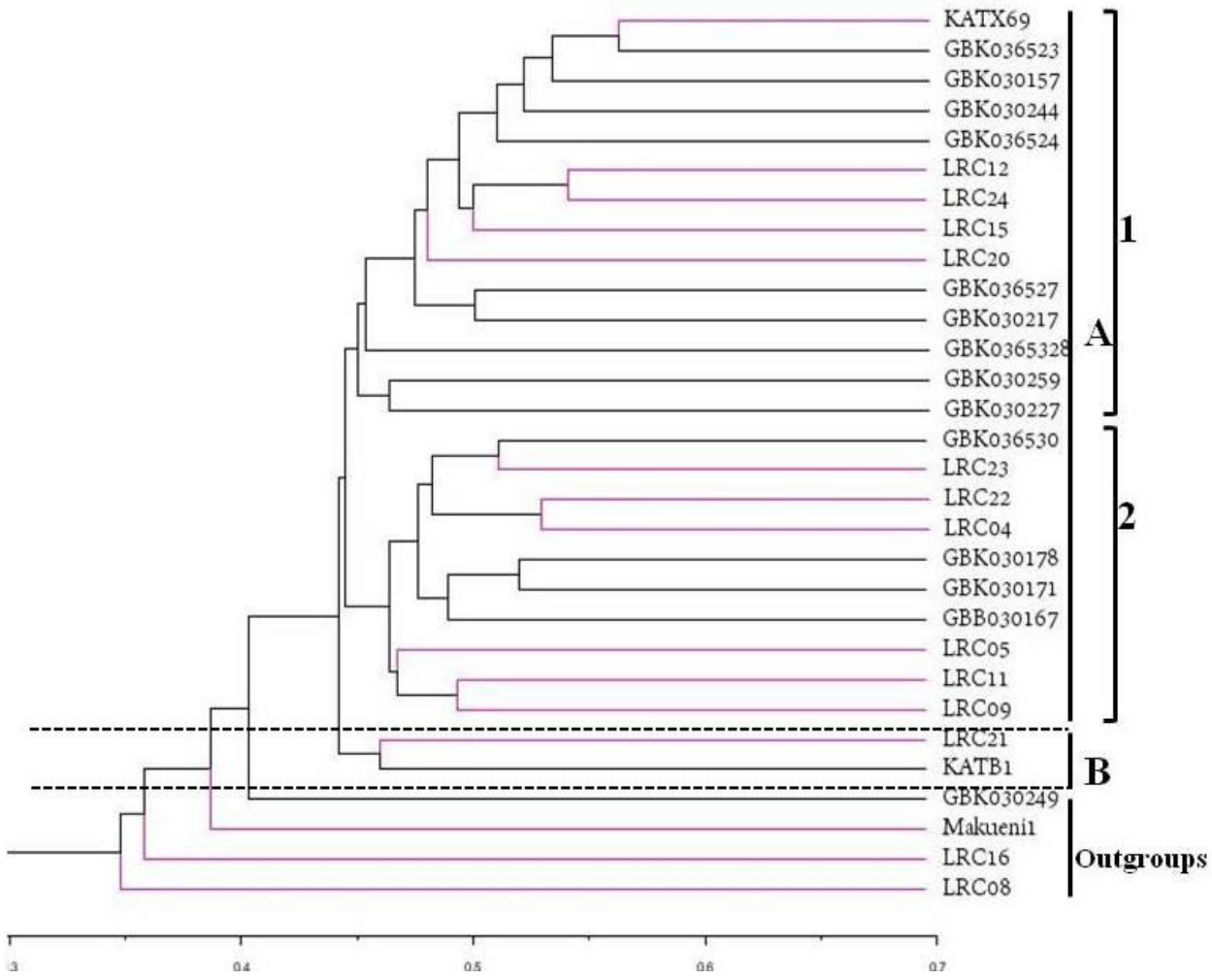

Figure 3: Dendrogram of 30 Kenyan common bean accessions obtained from 17 SCoT markers polymorphisms using UPGMA.

Black and Purple colors indicate common bean accessions collected from Genebank and Nyanza, respectively.

4.1.4 Principal component analysis (PCA)

The PCA using the 17 SCoT markers clustered the 30 common beans into two clusters and four single outgroups (Fig. 4). The common bean accessions were randomly distributed across the groups irrespective of place of collection. The generated scatter plot highlighted a total variation of 45.92% with the first and second components composed of 33.54% and 12.38% of the total variations, respectively.

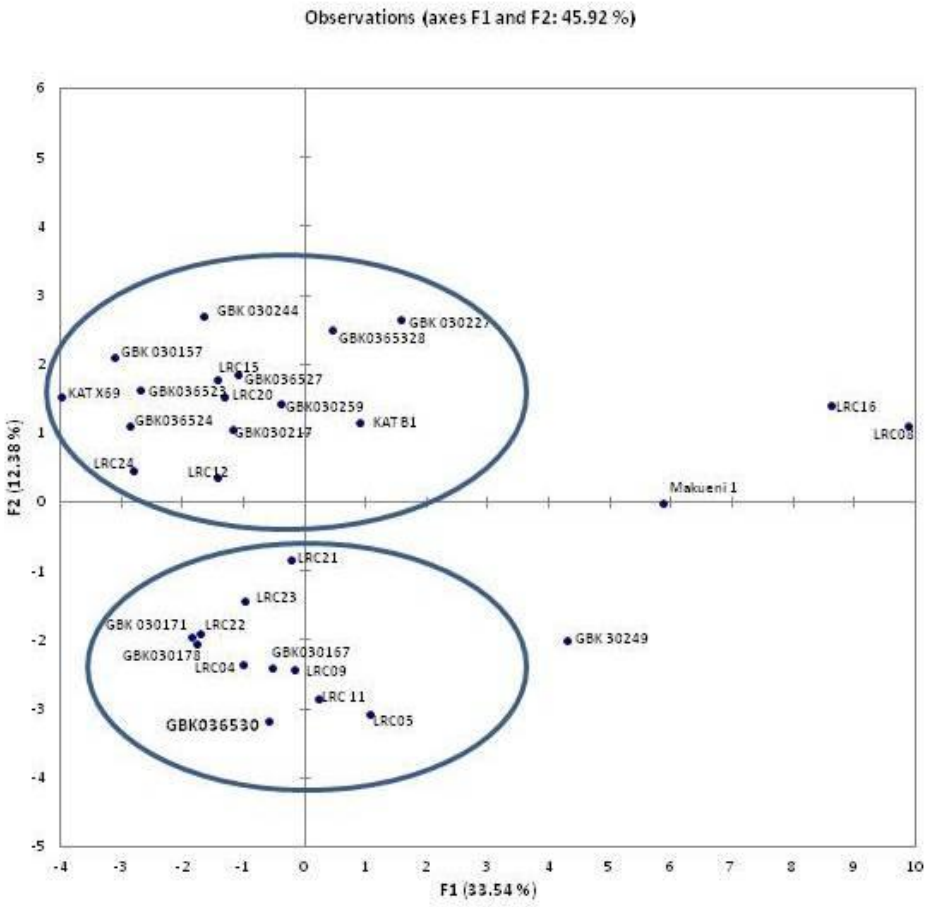

Figure 4: Principal component analysis (PCA) of 30 common bean accessions as revealed by 17 SCoT markers.

FI = first principal component and F2 = Second principal component

4.1.5 Population structure

The maximum ΔK value (136.374) was highest at K=3, indicating that the optimal number of subpopulations between the common bean accessions was 3 (Figure 5). The separation of the subpopulations at K = 3 in structure is presented in Figure 5. Sub-population 1 (red), 2 (green) and 3 (blue) consisted of 3, 12 and 15 common bean accessions, respectively. However, 22 common bean accessions comprised of admixtures of the three main subpopulations (Fig. 6).

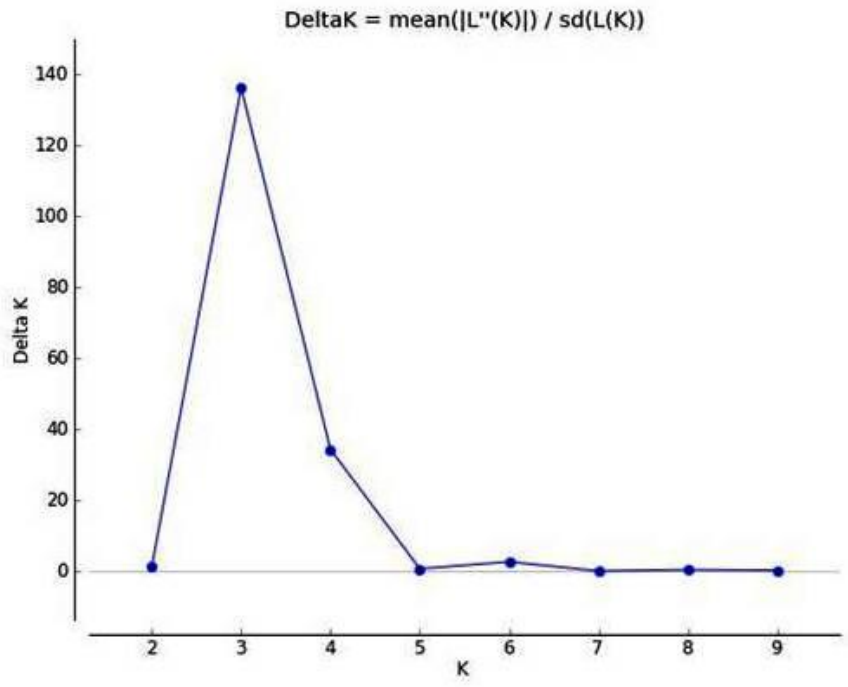

Figure 5: Plot of delta K (ΔK) values obtained from the Structure analyses of 30 common bean accessions, obtained using Structure harvester.

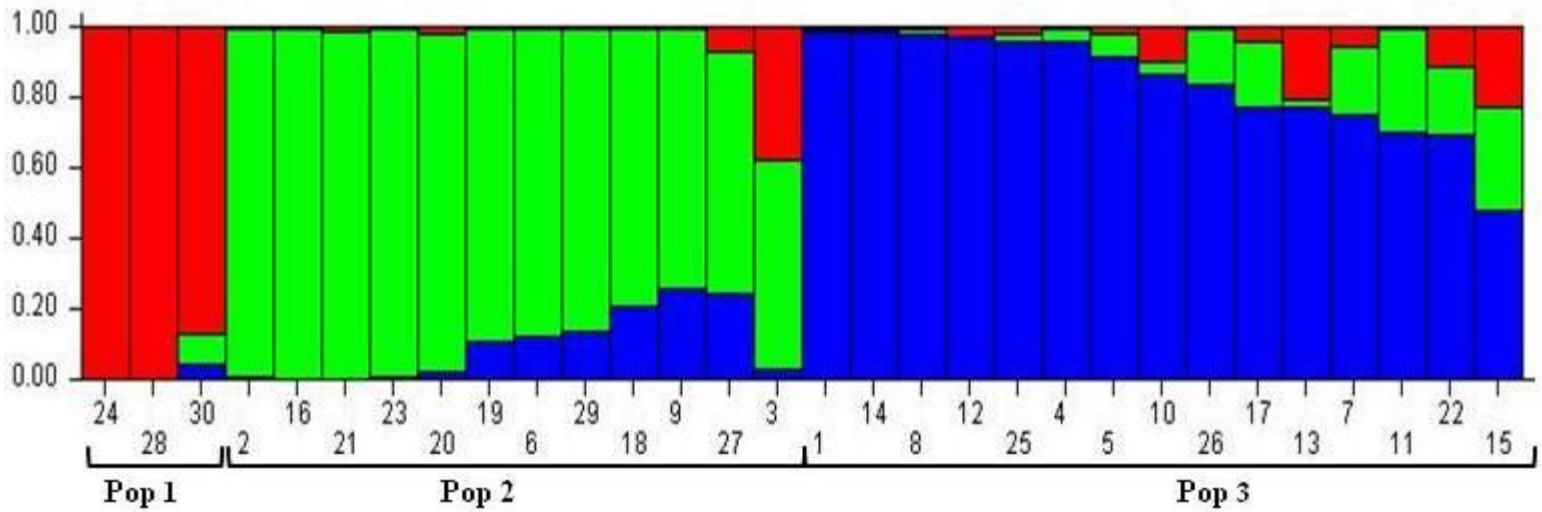

Figure 6: Population structure analysis of 30 common bean accessions for K=3 groups obtained by structure software V2.3.4.

Each solid bar represents single accession; the proportions of the color bars represent the admixtures in the accessions. The coordinate axis shows the estimated ancestry of each accession from a particular subpopulation while the horizontal axis shows every common bean accession on its individual bar.

1= KAT X69, 2= GBK 036530, 3= GBK 030249, 4= GBK 036524, 5= GBK 036527, 6= GBK 030178, 7= GBK 030259, 8= GBK 036523, 9= GBK 030171, 10= GBK 0365328, 11= GBK 030217, 12= GBK 030244, 13= GBK 030227, 14= GBK 030157, 15= KAT B1, 16= GBB 030167, 17= LRC 20, 18= LRC 23, 19=LRC 22, 20= LRC 04, 21= LRC 05, 22= LRC 12, 23= LRC 11, 24= LRC 08, 25= LRC 15, 26= LRC 24, 27= LRC 21, 28= LRC 16, 29= LRC 9 and 30= Makueni 1

4.1.7 Analysis of Molecular Variance (AMOVA)

AMOVA was used to assess the existing differences within and among the common bean accessions under study (Table 6). The results revealed a significantly high genetic variation within the population (96%) compared to the 4% among the two populations ($p<0.001$).

Table 6: Analysis of molecular variance (AMOVA) based on SCoT markers for 30 common bean accessions

Source	Degrees of freedom	Sum of squares	Mean of squares	Estimated variation	Percent variation	P. value
Among population	1	50.358	50.358	1.332	4%	<0.001
Within population	28	852.875	30.460	30.460	96%	<0.001
Total	29	903.233		31.792	100%	

4.2 Screening of 20 selected common bean accessions for resistance to CBB

The plants of susceptible common bean accessions exhibited CBB symptoms 14 days post-inoculation. Initially, the CBB symptoms were observed as water-filled spots on leaves, which enlarged to irregular brown necrotic lesions at 28 days post-inoculation. The symptoms on the leaves are shown in Fig. 7. Similar symptoms were also observed on the pods of CBB susceptible common bean accessions (Fig. 8).

Figure 7: Symptoms of CBB on leaves of susceptible common bean plant at 21 days following *Xap* inoculation.

A and B represents non-inoculated control and inoculated plants, respectively, of common bean accession 28 (GBK 0365328); C and D represents non-inoculated control and inoculated plants, respectively, of common bean accession 48 (Emwetemania LRC 04 KSI). Necrotic tip with a narrow yellow zone in Fig. B and D represent the CBB symptoms

Figure 8: Symptoms of CBB on pods of susceptible common bean plant at 21 days following *Xap* inoculation.

A and B represent non-inoculated control and inoculated common bean pods, respectively, of common bean accession 28 (GBK 0365328); C and D represents non-inoculated control and inoculated common bean pods, respectively, of common bean accession 48 (Emwetemania LRC 04 KSI). Sunken and dark reddish-brown blotches in Fig. B and D indicate the CBB symptoms.

Table 7: Common bacterial blight disease severity and rating of the 20 common bean accessions

Accession ID	Name of the accession	Disease severity	Disease rating	*Xap* Present/Absent
41	Ritinge LRC 20 KSI	4.67±0.333 a	S	+
28	GBK 0365328	4.33±0.667 ab	S	+
19	GBK 036527	4±0 bc	S	+
56	Morogi LRC 21 KSI	3.67±0.333cd	S	+
3	KAT X69	3±0e	S	+
7	GBK 036530	3±0de	S	+
8	GBK 030249	3±0de	S	+
25	GBK 036523	3±0de	S	+
27	GBK 030171	3±0de	S	+
30	GBK 030244	3±0de	S	+
47	Emwamu LRC 22 KSI	3±0de	S	+
48	Emwetemania LRC 04 KSI	3±0de	S	+
49	Girini LRC 05 KSI	3±0de	S	+
50	LRC 12 KSI	3±0de	S	+
54	Ekoko enyege LRC 15 KSI	3±0de	S	+
55	Ekoko entabe LRC 24 KSI	3±0de	S	+
33	GBK 030227	2.33±0.333e	S	+
58	Manoa LRC 16 KSI	1±0f	R	–
61	Makueni 1	1±0f	R	–
64	GBK 030167	1±0f	R	–

Mean disease severity values followed by the same letter (s) are not significantly different at $P \leq 0.005$, using Duncan multiple rank test.

Disease severity score was used to rate the accessions as resistant (R) or susceptible (S), where; R= ≤ 1.5 and S= ≥ 1.5. + and – represent the presence and absence of *Xap* , respectively

According to the disease severity scale used, 15% and 85% common bean accessions were classified as resistant and susceptible, respectively (Table 7). The disease severity was significantly different among the common bean accessions ($F= 4.69$, $P>0.05$). The most susceptible accessions to CBB were GBK 036527, GBK 0365328 and Ritinge LRC 20 KSI (19,

28, and 41) while CBB resistant accessions were Manoa LRC 16 KSI, Makueni 1 and GBK 030167 (58, 61 and 64) (Fig. 8, Table 7).

The resistance or susceptibility to CBB was confirmed by re-isolation of *Xap* bacteria from inoculated plants on YPGA. *Xap* colonies were obtained in all plants of susceptible common bean accessions (Fig. 9 A). However, the *Xap* colonies were absent among the resistant common bean accessions (Fig. 9 B).

Figure 9: Yeast glucose peptone agar (YPGA) plates for common bean plant leaf extracts infected with *Xanthomonas axonopodis pv. phaseoli.*

A and B represent plates of leaf extracts from plants of non-inoculated and inoculated common bean accession susceptible to CBB, respectively.

4.3 Biochemical analyses in resistant and susceptible common bean accessions

4.3.1 Estimation of malondialdehyde (MDA) content

The MDA content was significantly different between susceptible and resistant common bean accessions. The MDA content was significantly higher ($P \leq 0.05$) in inoculated common bean accessions of susceptible plants as compared with the non-inoculated plants. However, resistant

common bean accessions did not show any significant difference between the inoculated and non-inoculated plants (Fig. 10).

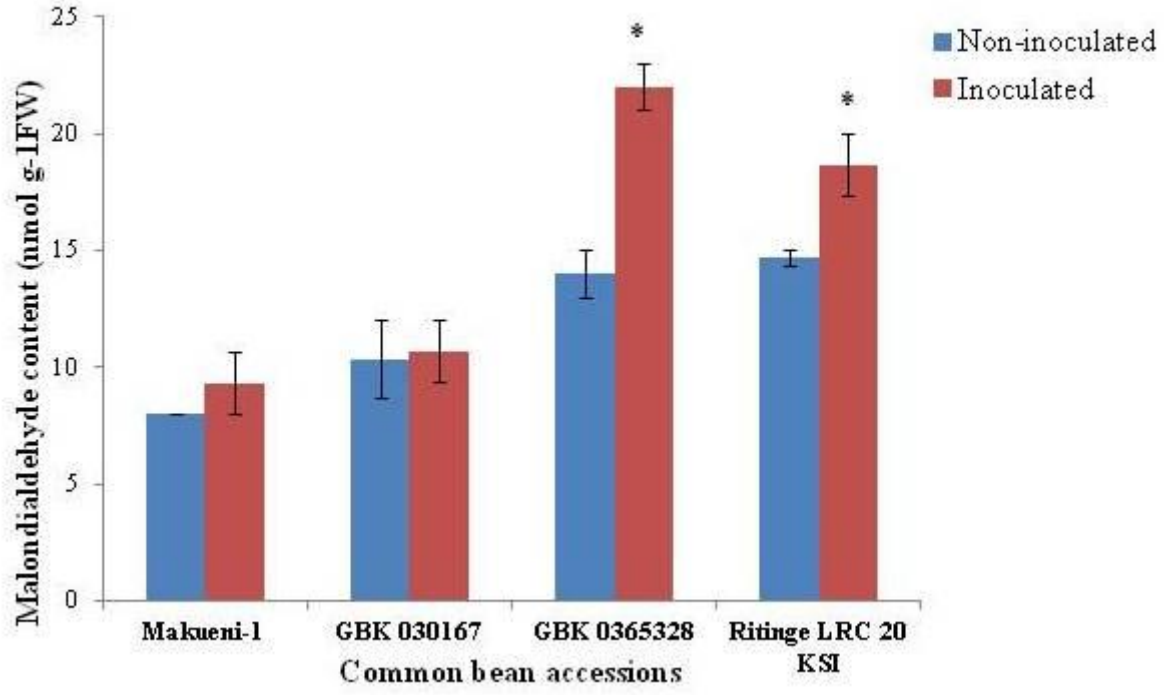

Figure 10: The content of malondialdehyde (MDA) in pods of susceptible and resistant plants of common bean accessions at 30 days post-inoculation with *Xanthomonas axonopodis* pv. *phaseoli*.

Makueni 1 and GBK 030167 represent CBB resistant accession; GBK 0365328 and Ritinge LRC 20 KSI represent CBB susceptible common bean accessions. * Indicate significant difference between non-inoculated and inoculated plants of the same accessions ($P \leq 0.05$). Bars represent standard error of means.

4.3.2 Determination of total phenolic content

The TPCs were significantly high ($P \leq 0.05$) among the CBB-resistant common bean accessions as compared to the susceptible ones. Among the CBB resistant common bean accessions, there was a significant ($P \leq 0.05$) difference between the inoculated and non-inoculated CBB

accessions. In the susceptible common bean accessions, there was a significant difference between the inoculated and non-inoculated GBK 0365328, while the inoculated and non-inoculated Ritinge LRC 20 KSI did not record any difference (Fig. 11).

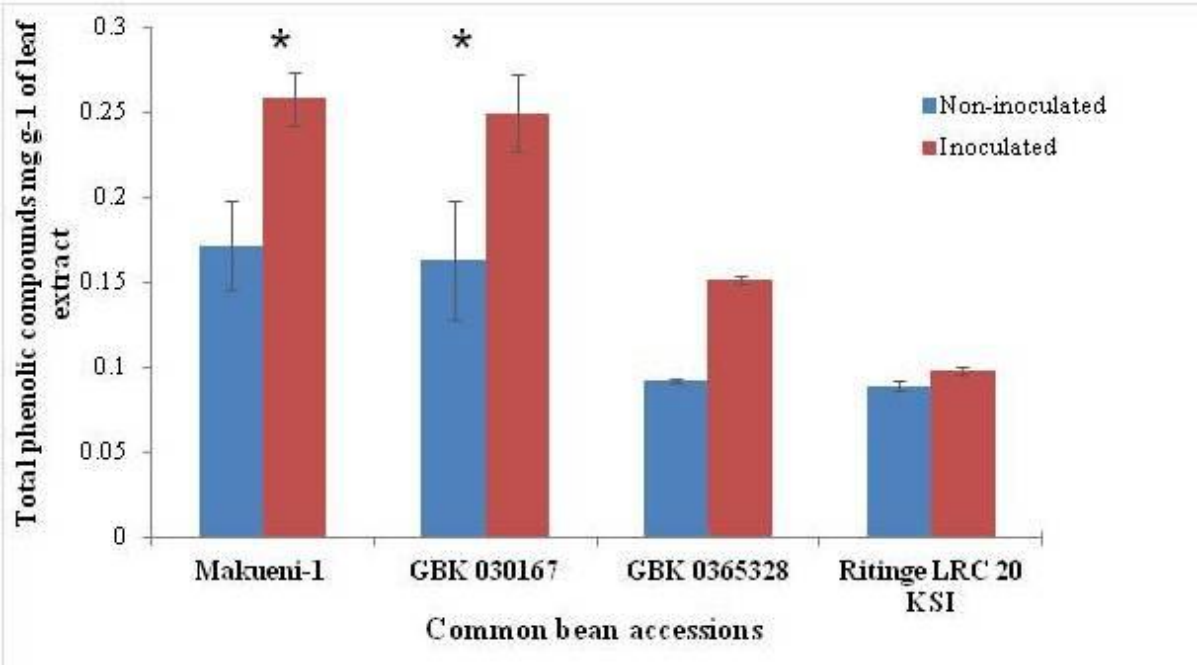

Figure 11: The content of phenolic compounds in pods of susceptible and resistant plants of common bean accessions at 30 days post-inoculation with *Xanthomonas axonopodis* pv. *phaseoli*.

Makueni 1 and GBK 030167 represent CBB resistant accession; GBK 0365328 and Ritinge LRC 20 KSI represent CBB susceptible common bean accessions. *Indicate significant difference between non-inoculated and inoculated plants of the same accessions ($P \leq 0.05$). Error bars represent standard error of means.

4.3.4 Antioxidant enzyme activities

Significant changes in the antioxidant enzyme activities were observed in CBB-resistant and susceptible common bean accessions following inoculation with *Xap* pathogen.

4.3.4.1 Peroxidase activity

There was no significant difference in POD activity between the inoculated and non-inoculated plants of both resistant and susceptible common bean accessions (Fig. 12). However, POD levels were notably high in GBK 030167, compared to the other accessions, while Makueni 1, a resistant common bean accession, recorded the least POD enzyme activity.

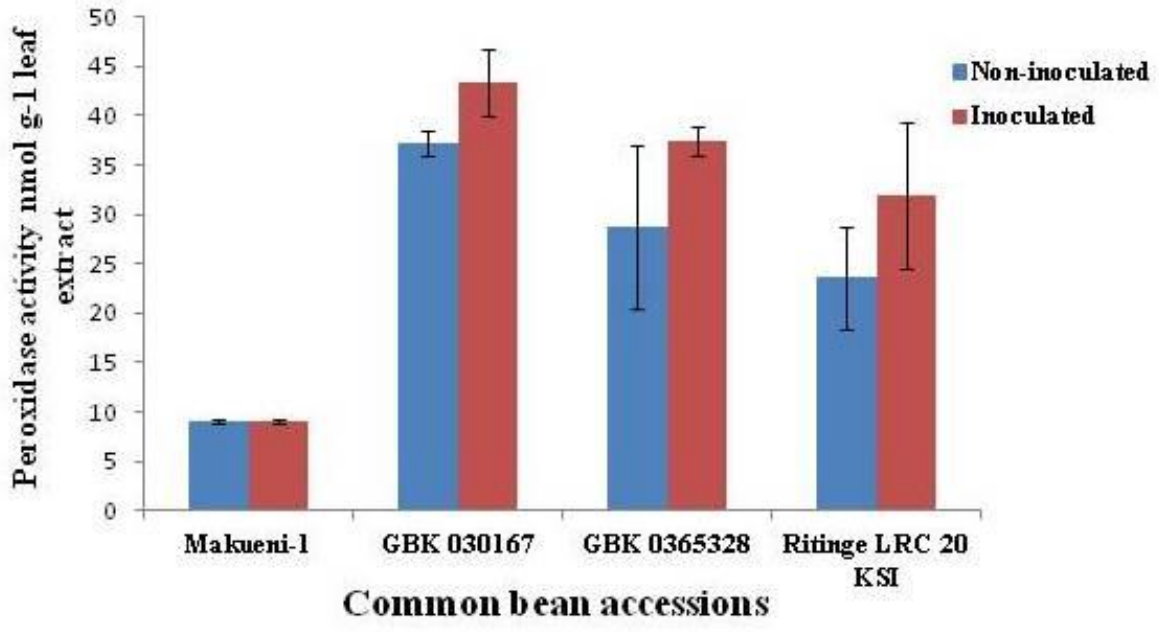

Figure 12: Peroxidase (POD) enzyme activity in pods of susceptible and resistant plants of common bean accessions at 30 days post-inoculation with *Xanthomonas axonopodis* pv. *phaseoli*.

Makueni 1 and GBK 030167 represent CBB resistant accession, GBK 0365328, and Ritinge LRC 20 KSI represent CBB susceptible common bean accessions. Error bars represent standard error of means.

4.3.4.2 Catalase activity

Catalase activity was notably higher among the inoculated plants compared to the non-inoculated plants of both resistant and susceptible common bean accessions. There was no significant

difference in CAT activity between the susceptible and resistant common bean accessions under study (Fig.13).

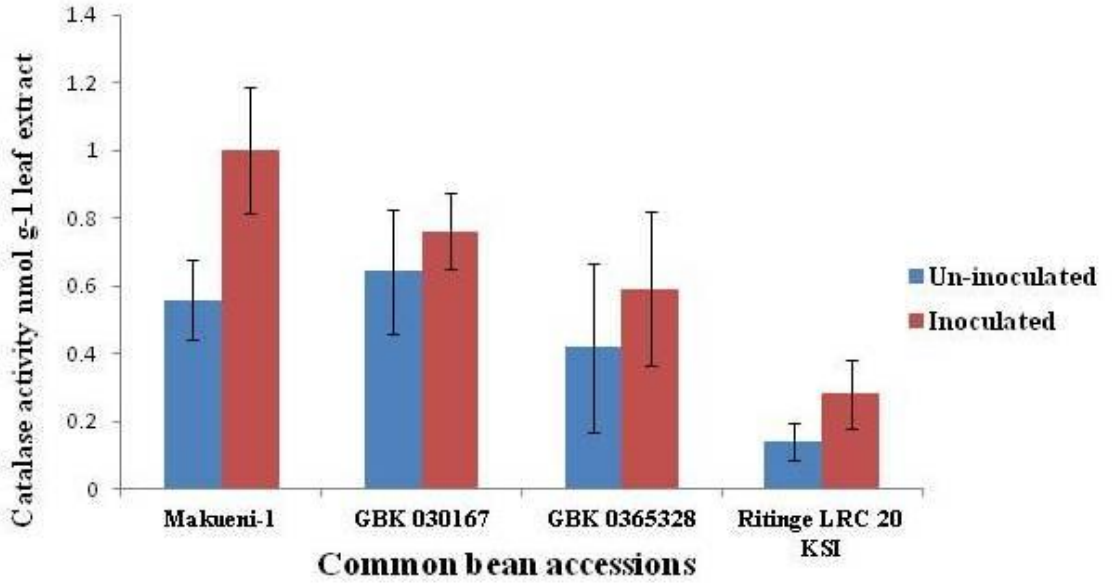

Figure 13: Catalase (CAT) enzyme activity in pods of susceptible and resistant plants of common bean accessions at 30 days post-inoculation with *Xanthomonas axonopodis* pv. *phaseoli*.

Makueni 1 and GBK 030167 represent CBB resistant accession, GBK 0365328, and Ritinge LRC 20 KSI represent CBB susceptible common bean accessions. Error bars represent standard error of means.

4.4 Unique bands revealed by SCoT markers among the assessed 30 common bean accessions

Twelve out of the seventeen SCoT markers generated eight unique bands that were present in susceptible and resistant common bean accessions. SCoT 7, 24, 32, and 36 revealed 6 unique bands only present in CBB resistant plants, while SCoT 5 and 26 amplified two unique bands present in CBB susceptible common bean accessions. However, SCoT 11, 16, 23, 29, 30 and 34 amplified eighteen unique bands present in both CBB-resistant and susceptible accessions (Table 8).

Table 8: Unique bands amplified using SCoT primers and the ID of the associated common bean accessions

Marker name	No. of unique amplified bands	Accession name
SCoT 5*	1	Girini LRC 05 KSI
SCoT 7**	3	GBK 030167
SCoT 11	2	LRC 12 KSI and Manoa LRC 16 KSI
SCoT 16	2	Ritinge LRC 20 KSI and Manoa LRC 16 KSI
SCoT 23	3	Manoa LRC 16 KSI, Chinchae LRC 8 KSI and GBK 036527
SCoT 24**	1	Manoa LRC 16 KSI
SCoT 26*	1	GBK 0365328
SCoT 29	2	LRC 11 KSI and GBK 030167
SCoT 30	5	Ekoko Enyege LRC 15 LSI, Manoa LRC 16 KSI and Makueni 1
SCoT 32**	1	Manoa LRC 16 KSI
SCoT 34	4	Morogi LRC 21 KSI, Chinchae LRC 8 KSI, Girini LRC 05 KSI, GBK 030167
SCoT 36**	1	Makueni 1

** represent SCoT markers that amplified unique bands in only CBB resistant common bean accessions, * represent SCoT markers that amplified unique bands in only CBB susceptible common bean accessions. Without * represent markers that amplified unique bands in both CBB susceptible and resistant common bean accessions.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Genetic diversity of crop plants is a pre-requisite step in plant breeding (development of superior accessions), utilization as well as conservation of superior cultivars (Xanthopoulou *et al*., 2015; Chai *et al*., 2017). Molecular markers provide a simple and effective strategy for studying the genetic diversity of common bean cultivars based on DNA polymorphisms (Gyang *et al*., 2017). Genetic diversity in common bean has been previously analyzed by molecular markers such as RAPD, ISSR, AFLP, and SSR (Dagnew *et al*., 2014; Nyakio *et al*., 2014; Bukhari *et al.*, 2015; Gyang *et al*., 2017). However, all these markers are not linked to gene function. Gene targeted markers are superior for diversity analysis since they measure genetic diversity from the gene regions and directly reveal functional diversity present in the studied species; thus, they are suitable for marker-assisted breeding (Collard and Mackill, 2009; Paliwal *et al.*, 2013). Studies using gene-targeted markers such as SCoT for genetic diversity analysis in Kenyan common bean accessions were reported for the first time in the current study.

In the current study, the 17 SCoT makers amplified 224 bands, ranging between 100 and 3000 bp, with an average of 13 amplicons per marker. The amplified bands were significantly high as compared to 65 bands amplified by RAPD (Razvi *et al*., 2013). This difference can be attributed to the high GC-content of the SCoT markers used, which plays a significant role in the stability of GC-complementation with respect to A-T pairing and hence, a higher number of bands (Bukhari *et al.*, 2015). In this study, 95% of the amplified SCoT loci were polymorphic, which was high compared to the polymorphism rates detected by SCoT analysis in mango (76.19%), peanut (36.76%) and ramie (87.5%) (Luo *et al*., 2010; Xiong *et al*., 2011; Satya *et al*., 2015).

Similarly, mean values of other diversity indicator parameters such as; gene diversity (0.98), I (1.24), HT (0.86) and Ne (7.68) were also high compared to mean values of 0.33, 0.19 and 0.47of I, HT and Ne respectively obtained by Gyang *et al*., (2018). The high polymorphism rates observed in this study indicates a rich genetic diversity among the assayed common bean accessions.

The mean PIC value of 0.73, which ranged from 0.60 to 0.85, was obtained in this study; these values are in agreement with findings by (Agarwal *et al*., 2018), who recorded an average PIC of 0.78 in 29 Rose (*Rosa gallica*) accessions using 32 SCoT markers. The PIC value indicates the usefulness of a marker for linkage analysis as well as highlighting its resolvability. The value is directly proportional to the marker's informativeness and is a fundamental factor in identifying genotypic variation (Bukhari *et al*., 2015). According to Botstein *et al*. (1980), a PIC value of less than 0.25 reflects low polymorphism, whereas between 0.25 and 0.50 indicates average polymorphism, and a value of more than 0.5 indicates high polymorphism. In the current study, the high PIC value obtained indicates the efficiency of SCoT markers in genetic diversity analysis. Previous studies in Kenya used SSR (Gyang *et al*., 2017) and POX markers (Anunda *et al*., 2019) and recorded a mean PIC value of 0.60 and 0.28, respectively, in their analysis of genetic diversity in common bean accessions. This suggests a rich genetic diversity among the common bean accessions grown in Kenya. The resolving power (RP), which indicates the discriminatory power of a marker, was found to be 5.09. The SCoT markers used in the current study recorded high RP values in comparison to the RP values reported by Zargar and Sharma (2016) for RAPDs (RP of 3.86) and SSRs (RP of 4.96). This is because SCoT markers are highly polymorphic and gene-targeted; hence they might highlight more variations than non-targeted markers such as RAPD, and SSR as reported by Etminan *et al*. (2016) in their study on the genetic diversity analysis in durum wheat genotypes using different molecular markers.

Analysis of genetic similarity of the Kenyan common bean accessions using similarity coefficient and UPGMA revealed two main clusters among the 30 common bean accessions at a genetic similarity of 44%. The common bean accessions from the two regions of collection were randomly distributed in the dendrogram. Similarly, in the PCA plot, the 30 common bean accessions formed two clusters and exhibited random distribution of common bean accessions collected from the two different sites. These findings illustrated the existence of a genetic relationship between the accessions collected from farmer's fields and those obtained from the Genebank. The maximum genetic similarity was between GBK 036523 and KAT X69. Since KAT X69 is a breeders' line, it is possible then that GBK 036523 was used as a parent accession in the breeding program. On the other hand, the least genetic similarity was observed between Chinchae LRC 8 KSI and GBK 036527, this is expected since the two accessions are from two different collection regions, thereby implying high genetic diversity between the two accessions.

Analysis of molecular variance (AMOVA) showed that 96% of the variation was apportioned within the populations, and 4% of the differences were apportioned among the populations. The within-population genetic variation obtained in the current study was higher than that reported by Gyang *et al*. (2017), who showed 87% genetic variation within common bean accessions using SSR markers. The low significant ($P < 0.001$) genetic differentiation between populations, as demonstrated by AMOVA in the current study, might be due to low selection pressure among farmers over a long period (Kimani *et al*., 2001). Clustering among the 30 common bean accessions was also established by the Bayesian clustering algorithm using STRUCTURE software. Bayesian clustering approach aids in the determination of the existing population structure and allocation of individual or portion of genetic information to several clusters based on multilocus accessions (Chen *et al*., 2007). The delta K method was found to be best at $K = 3$, which explained the types of diversification in the clustering. The population structure divided

the accessions from the two collection sites into three subpopulations. There was a high genetic admixture, observed among the common bean accessions, illustrating the existence of considerable genetic variability in the common beans under study. Within the three subpopulations identified in the structure analysis, more accessions from Nyanza were present in the admixture as compared to the ones from the Genebank, indicating a rich genetic diversity in the common bean accessions from the farmer's field compared to the accessions from the Genebank.

The degree of genetic relationship and differentiation provide information about the different genetic background of common bean accessions. Therefore, the selection of genetically distant accessions for hybridization in common bean breeding programs will potentially lead to elite varieties with broadened genetic bases. These results indicated the enormous potential of accelerating the genetic improvement in the future common bean-breeding programs by MAS. The broader range of genetic diversity among the accessions may indicate the potential gene pool for future common bean breeding programs. It will not only lead to the genetic improvement of common bean accessions but will also be used to explore the new alleles for valuable agronomic traits.

Common bacterial blight disease is prevalent in all bean growing regions in Kenya. Reports indicate that the disease contributes to significant economic and yield losses among common bean producing farmers in Kenya. With the high losses associated with CBB coupled with existing management options that are difficult to integrate with subsistence production systems among resource-poor farmers, the losses due to CBB will continue to affect common bean growing farmers. Studies report that the use of resistant varieties is the only economical and efficient mechanism in the management of CBB (Osdaghi *et al.*, 2009). Identification of CBB

resistant common bean accessions, therefore, will have a considerable positive effect in minimizing CBB associated yield and economic losses experienced by farmers in Kenya.

In the current study, three out of twenty accessions screened in the glasshouse did not elicit CBB associated symptoms. On the other hand, the remaining seventeen common bean accessions elicited CBB symptoms, characterized by a narrow lemon-yellow halo surrounding necrotic lesion on the leaves and a dark-brown sunken lesion on pods. Further analysis by re-isolation of the *Xap* pathogen from each inoculated common bean sample confirmed that the three common bean accessions were resistant to CBB while the other seventeen accessions were susceptible. The different responses of the common bean accessions to *Xap* inoculation exhibited during this study demonstrates the varying levels of resistance to CBB among the common bean accessions grown in Kenya. This may be associated with the existing genetic diversity among the common beans under study. Interesting to note, Manoa LRC 16 KSI (58) reported to be resistant to *Pythium* root rot disease (Anunda *et al*., 2019), was also found to be resistant to CBB in this study. Therefore, one common bean accession may be resistance to multiple diseases.

Malondialdehyde (MDA) content, a product of lipid peroxidation is a prominent indicator of membrane damage in plants exposed to biotic stresses (Ibrahim and Jaafar, 2012). Malondialdehyde is produced upon the breakdown of membrane lipids and is an indicator of cellular damage caused by pathogen infection (Siddique *et al*., 2014). In the present study, the levels of MDA increased significantly in inoculated plants of susceptible common bean accessions than in the resistant accessions. This is in agreement with the findings by Islam *et al.* (2017) who observed elevated levels of MDA in *Brassica napus* (Rapeseed) plants, indicating oxidative stress in response to *Xanthomonas campestris* pv. *campestris* infection. The susceptible common bean

accessions challenged with *Xap* in this study recorded the highest levels of MDA compared to the resistant accessions thus explaining the severity in cellular damage in the susceptible accessions than in the resistant ones. It may be possible that the low lipid peroxidation in the resistant accessions is an indication that the plant defense system is activated to suppress ROS production.

Phenolic compounds are vital functions in the protection of plants against oxidative damage. The concentrations of phenolic compounds, in the present study, increased significantly in *Xap*-inoculated plants. However, there was a significantly high amount of phenolic compounds in both inoculated and non-inoculated plants of CBB resistant common bean accessions as compared to the susceptible accessions. It is possible that the phenolic compounds in the common bean accessions resistant to CBB might be involved in inducing resistance. Production of phenolic compounds has been shown to induce the release of host-pathogenesis related protein that may aid in relieving disease-associated stress (Awan *et al*., 2018).

Antioxidant enzymes such as CAT and POD play an essential role in protecting plants from oxidative damage (Mittler *et al.* 2004; Gill and Tuteja, 2010). Catalases and peroxidases scavenge H_2O_2. Results from this study indicated that infection by *Xap* led to the substantial changes in the antioxidant status of plants of common bean accessions. Catalase enzyme plays a vital role in H_2O_2 homeostasis and ROS signaling in response to plant pathogen infection (Magbanua *et al*., 2007). The results of CAT enzyme in non-inoculated plants of resistant and susceptible common bean accessions were lower compared with inoculated plants, indicating a possible role of this enzyme during *Xap* infection and common bean CBB resistance. There was high catalase enzyme activity among the plants of common bean accessions resistant to CBB compared to the plants of susceptible accessions. Similar findings were reported by Kumar *et al*. (2011), who

showed an increase in CAT activity in sweet orange leaves following infection with *Xanthomonas axopodonis* subsp *citri.*

Peroxidase enzyme plays two roles during infection, i.e., scavenging high levels of H_2O_2 and enhancing instant defense response against pathogen infection (Sulman *et al.,* 2001). Peroxidases are a member of a large multigenic family that are involved in a vast range of physiological processes throughout the life cycle of a plant (Almagro *et al.,* 2009). This participation in the physiological processes is thought to be so, due to the wide range of enzymatic isoforms in PODs and also because of the versatility of their enzyme-catalyzed reactions. Peroxidases are involved in lignin formation in plants, cross-linking of cell wall components and ROS metabolism. The cross-linking creates a physical barrier to inhibit pathogen colonization (Almagro *et al.,* 2009). In this study, plants of both resistant and susceptible common bean accessions recorded high POD activities following *Xap* inoculation. However, Makueni 1 recorded the least POD enzyme activities compared to the other common bean accessions. These differences could be attributed to the genetic differences between Makueni 1 and the other resistant accession (GBK 03067), indicating genotypic differences in POD enzyme activity induction following *Xap* inoculation. Therefore, in this study, it is difficult to explain exactly the role of POD enzyme following *Xap* infection.

A total of eight useful and informative bands were amplified by SCoT markers, which were linked to common bean accessions resistant to CBB. Since SCoT markers are gene-targeted, it may be possible, therefore, that the unique bands are linked directly to functional genes involved in conferring resistance to CBB in common beans. Consequently, the unique bands and CBB resistant common beans can be incorporated in breeding programs for the improvement of

susceptible but farmer preferred common bean accessions. These unique bands may also be used in MAS for fast and efficient identification of CBB resistant common bean accessions.

5.2 Conclusions

The results of the current study showed that there exists rich genetic diversity among common bean accessions in Nyanza region and the Genebank of Kenya. Start codon targeted (SCoT) polymorphism markers recorded high PIC and RP values; therefore they are efficient markers for genetic diversity analysis of common beans.

Three out of twenty common bean accessions were resistant to CBB. Inoculation of common bean accessions with *Xap* changed the oxidative metabolism (production of phenolics and antioxidant enzymes) differently in plants of susceptible and resistant common bean accessions.

Unique SCoT bands were identified in both CBB resistant and susceptible common bean accessions.

5.3 Recommendations

The following are the recommendations:

(i) There is a need to determine the genetic diversity of common bean accessions from other regions of Kenya using SCoT polymorphism markers.

(ii) There is a need to screen all common bean accessions grown in Kenya for CBB resistance to identify more CBB resistant accessions for use in future breeding programs.

The identified three common bean accessions can be used to initiate breeding programs for genetic improvement of susceptible but farmer-preferred accessions.

(iii) There is a need to use the identified unique bands in CBB resistant accessions to develop SCAR primer pairs that could be used to rapidly, effectively and reliably identify and differentiate CBB resistant common bean accessions in breeding programs.

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