DEVELOPMENT OF MULTIPLE DISEASE RESISTANT COMMON BEAN (*Phaseolus vulgaris* L.) CULTIVARS USING MARKER ASSISTED SELECTION

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EXTENDED ABSTRACT

The work was undertaken to develop lines with multiple disease resistance incorporating ALS, BCMNV and CBB and characterization of resistance to ALS from bean landraces to improve yield of common bean in Tanzania. Single, double and three-way crosses were made between the susceptible recurrent parent, Kablanketi and resistant parents (Mexico 54, Vax 4 and MCM 5001). The F_2 were screened for the presence of resistance markers through marker assisted selection using SNO2, SAP6 and ROC11 SCAR markers. Thirty five F_2 lines had a combination of genes for ALS, CBB and BCMV while 11 had a combination of two genes for resistance which forms a good source for folia disease resistance in breeding programmes. Those lines making 120 plants were validated for the resistance by phenotyping with three pathogens (ALS, CBB and BCMV) in the screen house and 31 plants were found resistant to the three pathogens (*P. griseola, Xap* and *BCMV*). The performance of genotypes varied significantly for the disease reactions and yields. Yield varied from 484 to 3305 $kgha^{-1}$ with a mean of 2042 $kgha^{-1}$. The genotypes with combined resistance and high yields are recommended further for evaluation for release as a variety. Results also demonstrated that the heritability for disease in this study was moderate to higher indicating that the selection can be performed in early generation for some of the pathogens like ALS and BCMV traits. The study confirmed further that resistance of *P. griseola* is due to monogenic dominant gene for ALS and single recessive gene for BCMV. The CBB resistance was found to be conditioned by one major gene which had effects of partial resistance. The inheritance of ALS resistance in Beti-10, Nanka, Nanavala and Nkanamna landraces indicated that one single dominant gene controls the resistance. The heritability for ALS in the landraces was high indicating the additive genetic effect for that trait. SSR marker Pv-ag004 was found to be linked to resistance in Beti-10. This landrace can be used in pyramiding the ALS resistant genes in

the farmers preferred varieties. However, detailed investigation is needed for the mechanisms of resistance of the four landraces.

DECLARATION

I, GEORGE MUHAMBA TRYPHONE, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work and that it has neither been submitted nor concurrently submitted for a degree award in any other institution.

10.06.2014

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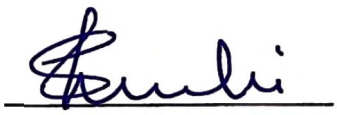
Date

The above declaration is confirmed



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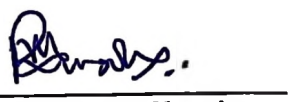

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DEDICATION

To Almighty God, My Mother, Clara Kaliganya Tryphone and beloved son Humphrey

Mwemezi George

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LIST OF ABBREVIATIONS AND SYMBOLS

AFLP	Amplified Fragment Length Polymorphism
ALS	Angular Leaf Spot
CBB	Common Bacterial Blight
BCMV	Bean Common Mosaic Virus
BCMNV	Bean Common Mosaic Necrosis Virus
CIAT	Centro Internacional de Agricultura Tropical
DNA	Deoxy-ribo Nucleic Acid
DAI	Days After Inoculation
DAP	Days After Planting
ER	Extreme Resistance
GLR	Great Lake Region
HR	Hypersensitive Reaction
MAS	Marker Assisted Selection
PCR	Polymerase Chain Reaction
SCAR	Sequence Characterized Amplified Regions
SSR	Simple Sequence Repeat
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SUA	Sokoine University of Agriculture
YDCA	Yeast Destrose Carbonated Agar

CHAPTER ONE

1.0 General Introduction

1.1 Economic Importance of Common Bean

Common bean (*Phaseolus vulgaris* L.) is an important grain legume for human consumption (Wortmann *et al.*, 1998). It is a staple food for more than 100 million people in Africa, with per capita consumption of 60 kg/person/year in the Great Lakes Regions (GLR) (CTA, 2010). The crop represents one of the principal crops in East Africa in terms of total area planted and number of farmers involved in production (Kelly, 2004). It is an important source of dietary protein and starch in Africa and a primary staple in parts of the GLR. It is estimated that over 75% of rural households in Tanzania depend on it for daily dietary requirements (CIAT, 2008). Bean production also provides farm households with both income and food for nutrition (Wortmann *et al.*, 1998). Bean is an income earner crop where the dry seeds and fresh pods attract a higher market price (Broughton *et al.*, 2003). Production of common beans in Tanzania is higher than any other pulses estimated at 300 000 tonnes annually, representing 82% of the total pulse production (NBS, 2006). It complements cereals and other carbohydrate rich foods by providing near perfect nutrition to people of all ages. Common bean has the nutritional benefits such as high source of proteins and high mineral contents especially Fe and Zn which combat high prevalence related micronutrient deficiencies (Tryphone and Nchimbi-Msolla, 2010). Consuming beans also have medicinal benefits that contribute to treating human aliments like cancer, diabetes, and heart diseases (Singh, 2000; Hangen and Bennink, 2003). Common beans have an important market niche in urban areas and fetches good price and generate income to farmers (Hillocks *et al.*, 2006; CIAT, 2008). Beans are also highly valued by the poor because all parts of the plants can be consumed i.e. the grain is eaten fresh or dried, the leaves are plucked for vegetables and the stalk is used to make soda ash and animal feeds

(Wortmann *et al.*, 1999; David *et al.*, 2000). Dry beans are raised mostly by women for subsistence and the market on more than 3.5 million hectares, accounting for a quarter of global output (CIAT, 2001). The production of beans is largely subsistence, concentrated in densely populated eastern Africa and the GLR and highlands of southern Africa (Beebe *et al.*, 2000). Approximately 40% of total bean production in sub-Saharan Africa is marketed at an average annual value of USD 452 million (Wortmann *et al.*, 1999; Hillocks *et al.*, 2006). Tanzania ranks 6th among top 10 bean producers worldwide and is the first larger producer in Africa with 850,000 MT produced per year which is equivalent to a commercial value of US$ 246 583 000 (FAO, 2010).

1.2 Constraints to Common Bean Production

Despite the importance of common bean in Tanzania and other developing countries, its production mostly relies on local cultivars (Gepts and Debouck, 1999; Miklas *et al.*, 2006; Chataika *et al.*, 2011). The local cultivars, however, are commonly known to produce low yields as they are highly constrained by several biotic and abiotic factors, including diseases, insect pests, poor seed quality, drought, low soil fertility and poor crop management (Wortmann *et al.*, 1998; Mkandawire *et al.*, 2004; Hillocks *et al.*, 2006; Mwang'ombe *et al.*, 2007). The major diseases affecting bean production in Tanzania include bean common mosaic necrosis, common bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli*), halo bacterial blight (*Pseudomonas syringae* pv. *phaseolicola*), angular leaf spot (*Psedocercospora griseola*), anthracnose (*Colletotricum lindemuthianum*) and rust (*Uromyces phaseoli*) (Hillocks *et al.*, 2006). On sandy soils the root-knot nematodes (*Meloidogyne incognita* and *M. javanica*) are the main problems (Ijani *et al.*, 2000).

Yield losses caused by bean diseases are very significant and devastating in the bean industry (Kelly and Miklas, 1998; Ferreira *et al.*, 2000; Coyne *et al.*, 2003; Hillock *et al.*, 2006). The economic losses caused by diseases result from reduction of seed quality and yield (Yu *et al.*, 2000). The diseases which are considered to be of significant economic importance are angular leaf spot (ALS). common bacterial blight (CBB), Bean Common Mosaic Virus (BCMV) and Bean Common Mosaic Necrosis Virus (BCMNV) diseases occurring across low, mid and high altitude bean production zones (Wortmann *et al.*, 1998). Since most local landraces and improved cultivars grown in Tanzania are susceptible to these diseases, there is a need, therefore, to incorporate resistance against diseases in adapted cultivars.

Currently, none of the commercial bean genotypes has multiple resistances to common bean diseases. Using classical breeding, significant strides have been made in crop improvement through phenotypic selections for agronomically important traits. Considerable difficulties however, are often encountered during this process, due to genotype-environment interactions (Tar'an *et al.*, 2002). With the exception of BCMV and BCMNV diseases, resistance to ALS and CBB is complex as they are quantitatively inherited making it difficult to achieve rapid progress through classical breeding (Kelly and Miklas, 1998). In addition, breeding is complicated by the pathogens variability and different genes conditioning resistances (Kelly and Miklas, 1998; Wortmann *et al.*, 1998). The identification of plants carrying two or more resistance alleles of different genes using standard inoculation test is impractical because several races would be needed to screen for specific alleles (Yu *et al.*, 2004). Thus classical breeding is limited by the length of screening procedures and reliance on the environmental factors. Hence, deployment of the molecular markers linked to resistance genes would be an alternative, more reliable screening procedure.

The molecular markers linked to genes and or quantitative trait loci (QTL) conditioning resistance are available and have increased the efficiency of breeding for diseases using marker assisted selection (MAS). These include 23 random amplified polymorphic DNA (RAPD) and five sequence characterized amplified region (SCAR) markers linked to 15 different resistance genes in addition to QTL conditioning resistance to seven major pathogens of common bean (Kelly and Miklas, 1998; Miklas *et al.*, 2006). The use of DNA molecular markers will improve understanding of the genetic factors conditioning these traits and is expected to assist in the selection of superior genotypes (Tar'an *et al.*, 2002; Bezawada *et al.*, 2003). From these studies, marker assisted selection can be used to simultaneously screen for resistance to these three diseases (ALS, CBB, and BCMNV) without affecting the growth of the plants. Selection for genetic markers linked to resistance genes and QTL can accelerate development of multiple resistant varieties and increase efficacy (Ferreira *et al.*, 2000; Babu *et al.*, 2004; Semagn *et al.*, 2006). Thus, the use of disease resistant cultivars in combination with appropriate cultural practices is essential for the management of bean diseases (Singh *et al.*, 2001; Ferreira *et al.*, 2004; Gomez, 2004).

1.3 Breeding for Disease Resistance

The low bean yields in developing countries among others are due to lack of effective diseases management practices including lack of disease resistant cultivars and when such cultivars are available, they are not integrated in the disease management packages. The development of cultivars with improved resistance to biotic and abiotic stresses has long been a primary goal for many bean breeding programs (Miklas *et al.*, 2006). It is considered that the use of resistant cultivars is an efficient, safe and inexpensive technique accessible for bean growers (Ferreira *et al.*, 2000; Yu *et al.*, 2000). In fact, this strategy is the most effective and sustainable method for controlling bean diseases (Oliveira *et al.*,

2008). Resistant varieties therefore provide distinct channels for achieving productivity increase through productivity maintenance, where benefits are not derived from the avoidance of yield losses associated with disease pressure and the yield gains the resistant varieties can give under disease pressure (Mooney, 2007). The use of resistant varieties leads to a reduction in both production costs especially pesticide cost and lower the quantity of pesticides or their residues released into the environment (Ferreira *et al.*, 2000). Thus, varieties with improved disease resistance can reduce reliance on pesticides in high input systems, avert the risk of yield loss from diseases in low- and high-input systems, and enable more stable bean production across diverse and adverse environments and poor soil conditions (Mooney, 2007). However, the development of resistant variety is an everlasting obstacle for breeders as most pathogen exhibits a great variability for pathogenicity which mostly overcomes the resistance in the released cultivars. Breeders are thus continuously forced to look for new sources of resistances. The screening procedures to ascertain resistance is another setback as pathogenicity tests need to be reliable by exhibiting comparable and reproducible results (Kelly and Miklas, 1998). The other constraint is whatever resistances detected with those tests should be efficient in controlling the target diseases in the field. Finally, methods usable by breeders for speeding up the breeding work should be developed. Genomics of *P. vulgaris* appear to be promising in discovering and tagging novel alleles (Collard *et al.*, 2005; O'Boyle et *al.*, 2007). If closely linked to resistant genes, molecular markers such as Sequence Characterized Amplified Region (SCAR), Simple Sequence Repeats (SSR), Amplified fragment length polymorphism (AFLP) can enhance the efficiency of breeding programs especially in the so-called marker assisted selection (MAS) and can be used in initial and intermediate stages of the breeding process. The target traits can be achieved indirectly using molecular markers closely linked to underlying genes or that have been developed from the actual gene sequences (Xu and Crouch, 2008). MAS can be used to

simultaneously screen for resistance to without affecting the growth of the plants. Selection for genetic markers linked with resistance genes and QTL can accelerate development of multiple resistant varieties and increase efficacy (Babu *et al.*, 2004; Semagn *et al.*, 2006). The uses of MAS enable the introgression of resistance genes into a cultivar, decreases population size and ultimately reduce the time required to develop a new variety.

1.4 Molecular Markers

Genetic markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as signs or flags and they are used as chromosome landmarks to facilitate the introgression of chromosome regions with genes associated with economically important traits. However, such markers themselves do not affect the phenotype of that trait of interest because they are located only near or are linked to genes controlling the target traits (Kelly and Miklas, 1998). Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as either hybridization-based or polymerase chain reaction (PCR)-based markers (Miklas, 2005). DNA markers are useful particularly if they can reveal difference between individuals of the same species or different species.

There are three types of genetic markers: morphological (or classical or visible) markers, which themselves are phenotypic traits or characters (morphological markers), biochemical markers which include allelic variants of enzymes called isozymes and DNA (or molecular) markers, which reveal sites of variation in DNA (Collard *et al.*, 2005). Morphological markers are characterized by phenotypic characters such as flower colour, seed shape, growth habits or pigmentation. Isozyme markers are differences in enzymes that are detected by electrophoresis and specific staining. However, those markers are

influenced by environmental factors and/or developmental stages of the plants (Collard *et al.*, 2005).

Properties desirable for ideal DNA markers in MAS as suggested by Collard and Mackill (2008) and Xu and Crouch (2008) are: highly polymorphic nature, codominant inheritance (determination of homozygous and heterozygous states of diploid organisms), quality and quantity of DNA required, frequent occurrence in genome (reliability), selective neutral behaviour (the DNA sequences of any organism are neutral to environmental conditions or management practices), easy access (availability), easy and fast assay, high reproducibility and easy exchange of data between laboratories. However, it is not easy to find a molecular marker which meets all these criteria. Depending on the type of study undertaken, a marker system can be identified that would fulfil at least a few of these criteria.

1.5 Marker Assisted Selection (MAS)

By using DNA markers to assist in plant breeding, efficiency and precision could be greatly increased. Use of markers in plant breeding is called marker-assisted selection (MAS) and is a complement of the discipline of molecular breeding (Collard and Mackill, 2008). Marker assisted selection is the novel approach in which individuals for intercrossing are selected using selection index based on phenotypic data controlled by few or several genes (Quantitative linked traits or QTL). The gain from selection using such index is expected to be higher than phenotypic selection used in conventional recurrent methods (Semagn *et al.*, 2006). Significant progress has been made through phenotypic selections for agronomic traits. However, difficulties are often encountered due to the genotype x environment interactions (Tar'an *et al.*, 2002). For example, significant progress has been achieved in selecting BCMV and BCMNV resistant lines (Kelly and Miklas, 1998). Bean angular leaf spot and CBB diseases resistances are complex traits.

These traits are typically controlled by multiple genetic loci (Quantitative Trait Linked Loci or QTLs) and display a strong interaction with the environment. However, molecular markers linked to major genes and QTLs for ALS, CBB and BCMN diseases are available and have increased the efficiency of breeding for diseases using MAS (Kelly and Miklas, 1998). The use of DNA molecular markers will improve understanding of the genetic factors conditioning these traits and is expected to assist in the selection of superior genotypes (Bezawada *et al.*, 2003)

Molecular marker assisted selection is an approach designed to avert problems related to conventional plant selection and phenotying by changing the selection criteria, selecting phenotypes through the selection of genes that control the traits of interest. This is because molecular markers are clearly not influenced by environment and are detectable at all stages of plant growth. With the availability of an array of molecular markers and genetic maps, marker assisted selection has become possible for traits governed by single gene or QTLs. Marker assisted selection is a good approach for bean breeders who also work to improve bean disease resistance. For MAS to be highly successful, a high correlation and/or tight linkage must exist between the genes for resistance to diseases and molecular markers, and the markers must be stable, reproducible and easy to assay (Yu *et al.*, 2004).

Molecular marker assisted selection can provide an effective and efficient breeding tool for detecting, tracking, retaining, combining and pyramiding disease resistance genes (O'Boyle *et al.*, 2007). DNA based markers can be effectively utilized for the following basic purposes (i) tracing favourable alleles (dominant or recessive) across generations and (ii) identifying the most suitable individual (s) among the segregating progeny, based on allelic composition across a part or the entire genome (Babu *et al.*, 2004; Xu and Crouch, 2008).

1.6 Use of Marker Assisted Selection in Plant Breeding

Justifications for the development and use of MAS in plant breeding fall into four broad areas that are relevant to almost all target crops (Ribaut and Hoisington, 1998; Xu *et al.*, 2005): (i) traits that are difficult to manage through conventional phenotypic selection because they are expensive or time-consuming to measure, or have low penetrance or complex inheritance; (ii) traits whose selection depends on specific environments or developmental stages that influence the expression of the target phenotypes; (iii) maintenance of recessive alleles during backcrossing or for speeding up backcross breeding in general; and (iv) pyramiding multiple monogenic traits (such as insect pest and disease resistances or quality traits) or several QTL for a single target trait with complex inheritance (such as drought tolerance or other adaptive traits). Introgression and pyramiding of multiple genes affecting the same trait is a great challenge to breeding programs. The target cropping environments of many breeding programs require a combination of diverse biotic stress resistances, agronomic and quality trait profiles, plus abiotic stress tolerances to improve performance, yield stability, and farmers' acceptability. The greatest impact from MAS will only be realized when breeding systems are adapted to make best use of large-scale genotyping for both multiple target traits and the genetic background. The greatest benefits from this type of integrated molecular breeding approach will be to achieve the same breeding progress in a much shorter time than through conventional breeding, and from pyramiding combinations of genes that could not be readily combined through other means (Collard *et al.*, 2005; Xu and Crouch, 2008).

1.7 Objectives

1.7.1 Overall objective

The overall objective of this study was to develop bean lines with multiple disease resistance by incorporating ALS, BCMNV and CBB resistance genes in local bean

landraces and characterization of resistance to ALS in local bean landraces or varieties with a view to improving yield of common bean in Tanzania.

1.7.2 Specific objectives

(i) To develop bean genotypes resistant to ALS, CBB and BCMN disease pathogens

(ii) To validate by phenotyping the resistant bean genotypes for ALS, CBB and BCMN diseases that combined with desired agronomic traits

(iii) To characterize the genetic resistance of ALS in selected landraces in Tanzania

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CHAPTER TWO

Introgression of ALS, CBB and BCMNV Resistance using Advanced Lines with Single Resistance and Incorporation of Resistance in Farmers Preferred Market Class Bean Varieties of Tanzania

2.1 Abstract

The low bean yields in developing countries as well as others are due to lack of effective disease management practices including the lack of disease resistant cultivars. When such cultivars are available, they are not integrated in the disease management packages. The development of cultivars with improved resistance to biotic and abiotic stresses has long been a primary goal for many bean breeding programs. It is considered that the use of resistant cultivars is an efficient, safe and inexpensive technique accessible for bean growers. This study was undertaken to combine the resistance genes for ALS, CBB and BCMNV into one background market class variety and selection of resistant lines using molecular markers. The crosses were made using Mexico 54, Vax 4 and MCM 5001 as sources of resistance for respectively ALS, CBB and BCMV. Marker assisted selection was used to identify plants with resistance genes from the introgressed lines involving Kablanketi or advanced bean lines. To transfer resistances into recurrent parent (*Kablanketi*) single cross, double cross and three way crosses were employed. SCAR markers SNO2, ROC11, SW13 and SAP6 were used to select the F_2 that possessed markers for the resistance to the target diseases. A total of 38 breeding lines were identified with the combined introgression of resistance to diseases on target. Seeds were harvested and subsequently tested against ALS, BCMNV and CBB pathogens in the screen house to validate resistance.

2.2 Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important food staples produced in the East African region. They hold the key to improving incomes of EA farmers comprising around 6 million small holder households (Kilimotrust, 2012). They are produced as stable food by about 87% and consumed by over 41% of the population of East Africa providing the most affordable source of protein, fibre, minerals and calories (Mahuku *et al.*, 2009). Bean is the environmentally friendly crop as it replaces nitrogen in the soil which it extracts providing up to 50% of its own nitrogen requirements as compared to other crops (Kilimotrust, 2012). Thus, beans play an essential role in the sustainable livelihoods of smallholder farmers and their families, providing both food security and income.

In many areas there are several bean growing seasons per year, thus crops are grown with minimal rotation and limited fallow period. This has led to an increase in insect pest and disease pressure (Buruchara *et al.*, 2013; Tryphone *et al.*, 2013). Pan Africa Bean Research Alliance (PABRA) and International Centre for Tropical Agriculture (CIAT) estimate that insect pest and disease problems are the second biggest constraints to bean production after low soil fertility (Hillocks *et al.*, 2006). Annual losses vary from 20 to 100% in both bean yields and incomes of bean growers (Buruchara *et al.*, 2013). This reduction in yield has been attributed to the effect of insect pests and diseases, most specifically ALS, CBB, BCMNV and BCMNV which can lead to significant yield losses (Hall, 2000; Ferreira *et al.*, 2000; Coyne *et al.*, 2003; Hillocks *et al.*, 2006). These factors are accelerated by availability of seeds the local cultivars available to farmers which are of poor quality and perform poorly due to their susceptibility to the factors mentioned. A reduction in these bean diseases will contribute to increased yield, providing increased stability in food

markets and a reduction in food shortage, benefitting both farmer and the landless poor in both rural and urban locations.

However, losses from diseases may also be reduced by cultural, chemical and genetic measures (Mahuku *et al.*, 2009). Cultural measures reduce the losses to some extent but are not absolute control measures, while the use of pesticides to control the diseases has several disadvantages, particularly the cost of pesticides and their residual effects (Mooney, 2007). Furthermore, conducive environments to many diseases pose a constant threat to bean production (Wortmann *et al.*, 1998). The use of host resistance to address the risks posed by those diseases is the most effective and practical strategy, especially for smallholder, resource limited farmers (Singh, 2001). The advantage of host plant resistance is that once the variety has been developed, the seed is easy to disseminate and deploy, and does not require any additional or specialized handling on the part of the farmers, other than what they normally do to grow their crops. Therefore, major emphasis may be given to the development of disease resistant variety. A variety having resistance against two or more diseases is known as a multiple disease resistant variety. The development of multiple disease resistant varieties so far has not been given due attention in this country. Most of common bean varieties are resistant to only one or two diseases and are attacked by more other diseases. Hence, development of multiple disease resistant varieties is the best solution to control or avoid spray of pesticides and makes it difficult for a pathogen to evolve that would overcome all the resistance genes simultaneously. Thus consequently breeding cultivar with multiple disease resistance would help sustain bean productivity.

To achieve the high level of cultivar resistance with multiple disease resistance, CIAT developed bean genotypes which are resistant to the pathogens that cause ALS, CBB and BCMV and BCMNV diseases (Singh and Munoz, 1999). Those genotypes include cultivar

Mexico 54 which is an indeterminate strong climber resistant to most African isolates of ALS disease (Mahuku *et al.*, 2002). Other resistant genotypes are VAX 3 and VAX 4 lines, which have high level of resistance to CBB (Singh *et al.*, 2001) and MCM 5001 a line with the *bc-3* gene which confers resistance to BCMNV (Singh and Munoz, 1999). Therefore, those genotypes have been reported to deliver good level of resistance to common bean diseases in Africa. SCAR markers have been developed which are linked to genes/QTLs within the resistant genotypes. The molecular markers linked to the genes include SNO2 for *Phg-2* gene for ALS, ROC11 for *bc-3* for BCMNV, SW13 for *I* gene for BCMV and SU91and SAP6 for QTLs for CBB. With the help of molecular markers, pyramided lines are obtained with resistance alleles to several pathogens, by means of marker assisted selection (Ragagnin *et al.* 2009, Costa *et al.* 2010). The selected lines are extremely useful in crossing programs to incorporate alleles with resistance to pathogens in elite lines and commercial cultivars. Selection assisted by molecular markers for resistance to pathogens can also help to identify plants with desirable characters and prevent less promising plants from being submitted to later stages of selection. Therefore, the objective of this study was to combine the resistance genes for ALS, CBB and BCMNV into one background farmers preferred market class variety and selection of resistant lines using molecular markers.

2.3 Materials and Methods

2.3.1 Bean germplasm

Bean lines resistant to ALS, BCMNV and CBB disease were obtained from Sokoine University of Agriculture (SUA), Department of Crop Science and Production and are as shown in Table 2.1. Other resistant parents were imported from the Kawanda National Agricultural Research Institute, Uganda which included Mexico 54 for angular leaf spot (ALS) and MCM 5001 for bean common mosaic necrosis virus (BCMNV). The resistant parents to common bacterial blight (CBB) used was VAX 4 were obtained from the

University of California, Davis – USA. The susceptible adapted bean parent, *Kablanketi* was collected from farmers' fields in Mbeya Region.

2.3.2 Description of parental genotypes used

The bean lines at SUA in the Department of Crop Science and Production germplasm were developed under the CRSP/Bean project at SUA. Those lines were developed by crossing different local cultivars Kablanketi, Canadian Wonder, Masai Red and Njano with resistant parents to ALS, BCMNV and CBB. The resistant parents included MAR-1, MAR-2, Mexico 54, G5686, Vax 3, Vax 4, MCM5001 and UBR (92) 25. The developed lines were phenotypically screened and advanced at different stages. The selected resistant lines were either of F_5 or F_7. The lines with the score of ≤ 3 based on CIAT 1-9 rating scale (van Schoonhoven and Pastor-Corrales, 1987) were selected and used in this study.

The cultivar Mexico 54 is an indeterminate strong climber resistant to most African isolates of ALS disease (Mahuku *et al.*, 2002). This cultivar is, therefore, a potential source of resistance in breeding work. Kablanketi is the landrace which has a good market class and displays other good culinary traits but is universally susceptible to various diseases of common bean. The seeds are medium sized which are gray in colour with the type II growth habit and gives low yields. It fetches high price because of its colour, short cooking time and good palatability (Wortmann *et al.*, 1998; Hillocks *et al.*, 2006). Genotype VAX 4 is redish medium sized seed with the type 1 growth habit and was used due to its high level of resistance to CBB (Singh *et al.*, 2001) while MCM 5001 is the small cream/brown stripped with resistance to BCMNV. The genes present in the resistant parents are as follows, Mexico 54 has *phg-2*; VAX 4 has *QTL* linked to
group 10 and MCM 5001 has *bc-3* gene.

Table 2.1: Selected sources of resistance from SUA germplasm list

S/No.	BEAN GENOTYPES/LINES		
	BCMNV (*bc-3* gene)	ALS (*phg-2*)	CBB (QTL)
1	PS04-220A**	PS04-336AB-7**	PS04-585A-21**
2	PS04-245B**	PS04-139BA-1**	PS04-585A-11**
3	PS04-245A **	PS04-139BB-1**	PS04-148A-15**
4	PS04-244A	PS04-139BB-5**	PS04-148A-11**
5	PS04-263A	PS04-207Ab-6	PS04-90A-5**
6	PS04-265A	PS04-335B-12	PS04-120A-10**
7	PS04-263B	PS04-333Aa-18	PS04-122D-15
8	PS04-265B	PS04-126Aa-8	PS04-120A-10
9	PS04-220B	PS04-139Ab-10	PS04-557C-15
10	PS04-221A**	PS04-139Aa-10	PS04-585A-20
11	PS04-26A	PS04-106Ba-2	PS04-90C-16
12	PS04-221B	PSO4-139Bb-4	PS04-92D-14

** Genotypes demonstrating presence of alleles for resistance to the target disease

2.3.3 Validation of sources of resistant parents

Existing lines in the bean germplasm collection at SUA possess resistance to only one of the diseases (ALS, BCMNV or CBB). Before making crosses, each resistant parent was screened to confirm the presence of marker alleles associated with resistance genes (Table 2.1). Also, resistant parents Mexico 54, Vax 4 and MCM 5001 were screened for validation of the presence of resistant genes based on the marker for each gene.

2.3.4 Planting conditions

Planting was done in pots previously filled with sterilized loam soil where one seed was planted at the centre of each pot. Re-planting was done one week after germination for some of the seeds which failed to germinate. Irrigation was done daily to maintain the required moisture. Urea (20kg Nhectare^{-1} (i.e. 0.04g Npot^{-1}) was applied at flowering to improve plant vigour. For the crossing block establishment, the plants (recipient and donor parents) were staggered to ensure that there are constant flowers for both parents. Control of insect pests (Spider mites and white flies) was done by spraying Thionex 35 EC (40mls/20litres of water).

2.3.5 Hybridization

2.3.5.1 Using elite germplasm at SUA

A three way cross was conducted in the screen house at Sokoine University of Agriculture (SUA) to incorporate resistance genes into bean genotypes. The crossing procedure involved emasculation of female flowers and transfer of pollen from just opened flowers to the stigma of emasculated plants. The crossing was done in the morning and evening when the temperatures were optimum between 18 °C and 27°C. This is because at higher temperatures, the pollen and stigma desiccate (Bliss, 1980). The mean monthly Temperature were 25 °C and 28 °C with daily mean minimum of 26 °C and mean maximum of 30 °C. Actual mean monthly Temperatures when conducting experiment range between 25°C and 30°C with daily mean minimum 25°C and mean maximum 34 °C. The initial step involved crossing bean lines (Table 2.1) having ALS resistance with those possessing resistance to BCMNV to form F_1s. The resultant F_1 plants to the ALS x BCMNV crosses were selfed to obtain F_2 which were assayed for the presence of resistance markers linked to ALS and BCMNV resistance genes. Selected F_2 lines possessing the resistance markers for ALS and BCMNV were then crossed to lines

possessing CBB resistance to generate three-way F_1 plants (Fig. 2.1). These F_1 plants were advanced to F_2 population. The resultant F_2 population progenies were further screened against the presence of ALS, BCMNV and CBB markers linked to resistance genes. Phenotyping for disease resistance was carried out using F_3 populations derived from selected F_2 plants in the screen house based on marker information.

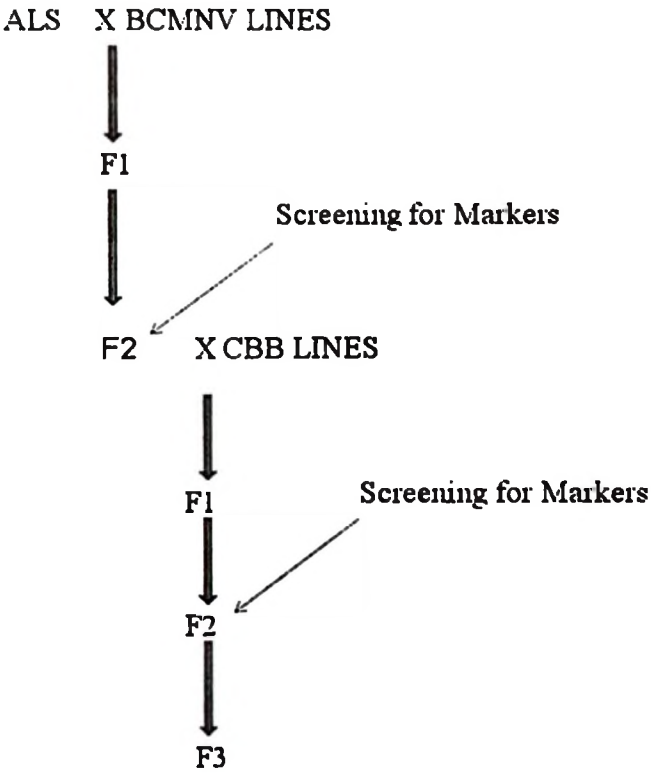

Figure 2. 1: Crossing scheme to introgress the resistance to diseases

2.3.5.2 Introgression of ALS, BCMNV and CBB into farmers' preferred bean variety, Kablanketi

The crosses were made using Mexico 54, Vax 4, and MCM 5001 as sources of resistance. Marker assisted selection was used to transfer the resistance traits into recurrent parents (*Kablanketi*) where single cross, double cross and three way crosses were employed in the process to incorporate the resistant genes (Fig. 2.2). The F_2 population for incorporation of

each disease (A and B) were assayed for presence of molecular markers. The identified F_2 plants from a single cross of ALS (*Kablanketi* x Mexico 54) were crossed with the one from a single cross of BCMNV (*Kablanketi* x MCM 5001) to get F_1. The F_1 populations were self pollinated to get F_2. The F_2 lines were screened for presence of molecular marker alleles and plants with markers for ALS and BCMNV were crossed to resistant parents of CBB (Vax 4) to produce F_1 of the three-way cross. The F_1 of this cross were self pollinated to get F_2 and the F_2 that possessed the resistance linked markers were harvested and subsequently tested against ALS, BCMNV and CBB pathogens in the screen house.

2.3.6 Extraction of DNA

Total genomic DNA was extracted from young trifoliolate leaves collected from F_2 plants in the screen house using the Whatman FTA card technology/protocol (Appendix 2.1). The leaves were crushed on the FTA Plant saver card and the DNA binds to the matrix of the card. The chemical coating on the FTA card inactivates pathogens, protects the DNA from degradation and allows the cards to be stored at room temperature for extended period of time. To prepare sample for PCR, a 2 mm disc of the matrix was punched using a Harris Unicore, put in the 0.2 ml PCR tube. Then disc was washed with FTA purification reagent (Whatman® FTA® card technology) and Iso-propanol. The washed leaf disc in the PCR tube was left to dry at room temperature for five minutes. The DNA remains bound to the matrix throughout purification process, thus the matrix provides enough templates for PCR analysis.

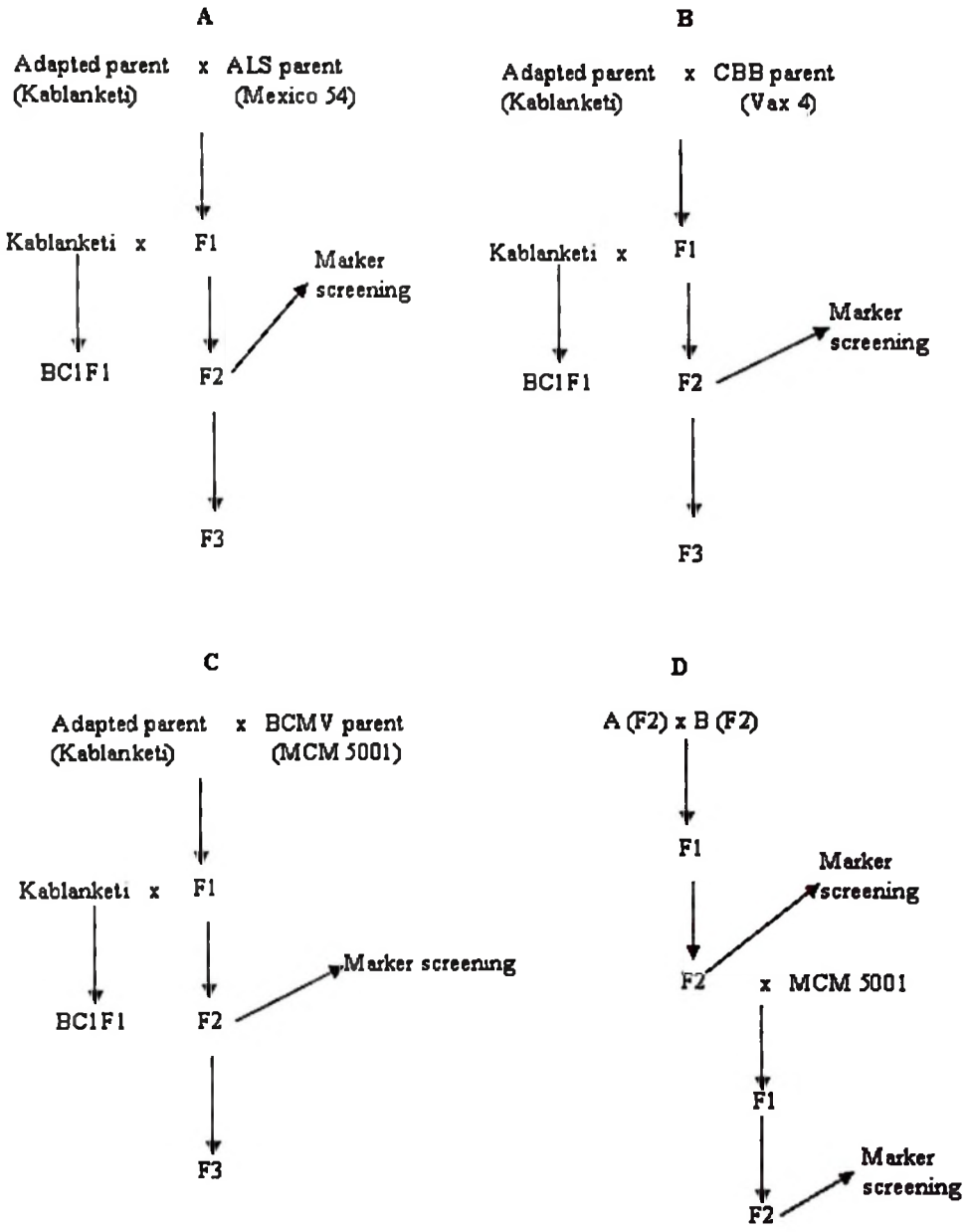

Figure 2. 2: Crossing scheme for transfer of ALS, BCMNV and CBB disease resistance genes and screening for multiple disease resistance

2.3.7 Amplification of DNA

The PCR reaction was prepared by adding 1µl of each forward and reverse primers, 18 µl of PCR water in the PCR premix and 20µl of this reaction mixture being mixed with the washed FTA card ready for PCR. The PCR conditions were set to correspond to each

primers requirement in terms of number of cycles and the temperatures as per Miklas, (2009). Samples for ALS were amplified using the primer SNO2 and for BCMNV; ROC11 and for BCMV; SW13. The PCR conditions for the primer are as shown in Appendix 2.2.

2.3.8 Electrophoresis and gel documentation

Amplification products were separated through electrophoresis in a 1.5% agarose gel with 6.0 µL DNA ladder in 0.5X TBE (Tris-Borate EDTA) buffer under a voltage of 100 V for 80 min. The gel was stained in ethidium bromide (EtBr) with concentration of 0.5µg/ml for 30 minutes, de-stained for 30 minutes by using distilled water. The stained gel were lighted with ultraviolet light, the bands present on the gel were observed and the mounted digital camera was used to capture the amplified fragments for documentation and scoring according to specific base pair of SAP6 – 820 bp, SNO2-890bp, SW13-690bp and ROC11-460bp by comparing with a reference molecular weights of the 100bp DNA ladder.

2.4 Results and Discussion

2.4.1 Production of breeding lines

The crosses were made and the plants screened for the presence of linked markers to the genes. The success and efficiency of developing breeding lines (crosses) were determined by a number of factors including weather/environmental conditions, sunlight, temperature and humidity. More success was observed when the seeds of the same size were used in hybridization process than when small seeded were crossed with large seeded types. This is attributed to the difference in the background of the seeds used and compatibility effect since the small seeded are of Mesoamerican origin while large seeded is of Andean type. Also, the success of pollination depends on the skills of the pollinator to avoid injuring stigmas and styles of the emasculated flowers and thus to avoid their desiccation after

pollination. Bliss (1980) pointed out that a combination of factors can lead to efficiency and success in the hybridization process.

2.4.2 Incorporation of resistance using SUA germplasm

Results of screened F_2 using molecular markers for the three diseases are presented in Table 2.2a and Plate 2.1. The lines PSO4-220A, PSO4-245B, PSO4-120A-10, PSO4-585A-21, PSO4-148A-21, PSO4-336AB-7 and PSO4-139BB-1. These are the lines that had single resistance and when combined produced lines with multiple disease resistance. The bean lines with combination of genes for disease resistance are presented in Table 2.2b and the seed types of some of the harvested F_2 are shown in Plate 2.2. Results show that there was success in developing the breeding lines with resistance genes to angular leaf spot, bean common mosaic virus/ bean common mosaic necrosis virus and common bacterial blight diseases.

Table 2.2a: Common bean lines screened for different SCAR markers for resistance to ALS, CBB, BCMV and BCMNV

CROSSES	RESISTANT MARKER PRESENT				
	SAP6-QTL	ROC11-bc-3	SNO2-phg-2	SW13 - *I* gene	% gene present
(245B x 585A-21) x 139AB-7	+	–	+	–	50
(245B x5 85A-21) x 139AB-7	+	–	+	–	50
(148A-15 x 220A) x 139BB-5	+	–	+	–	50
(148A-15 x 220A) x 139BB-5	–	–	+	–	25
(244A x 148A-11) x 139BA-1	–	–	+	+	50
(585A-11 x 254A) x 139BB-5	–	–	+	–	25
(585A-11 x 254A) x 139BB-5	–	–	+	+	50
(245A x 585A-11) x 139BA-1	–	+	+	–	50
(245A x 585A-21) x 336Ab-7	+	–	+	–	50
(245A x 585A-21) x 336Ab-7	+	+	+	+	100
(245A x 585A-21)x 336Ab-7	+	+	–	–	50
(148A-21 x 220A) x 139Bb-1	+	+	+	+	100
(245A x 585A-11) x 139BA-1	–	+	–	–	25
(244A x 336Ab-7) x 585A-11	–	+	–	+	50
(336AB-7 x 220A) x 148A-15	–	+	+	–	50
(336AB-7 x 220A) x 148A-15	–	+	–	+	50
(244A x 585A-21) x 139BB-1	–	+	+	–	50
(244A x 585A-21) x 139BB-1	–	–	+	–	25
(245B x 336Ab-7) x 120A-10	–	+	–	+	25
(245B x 336Ab-7) x 120A-10	+	+	+	+	100

Key: + refers to presence of resistance marker; and – refers to absence of resistance marker with respect to disease in question

Table 2.2b: Breeding lines with different combination of genes for four diseases

CROSS	REISTANT MARKER PRESENT			
	ROC11 – *bc-3*	SNO2-*Phg-2*	SAP6 - *QTL*	SW13 - *I gene*
(245AX585A-21)X 336Ab-7	+	+	+	+
(148A-21 X 220A)X 139Bb-1	+	+	+	+
(245B X 336Ab-7) X 120A-10	+	+	+	+

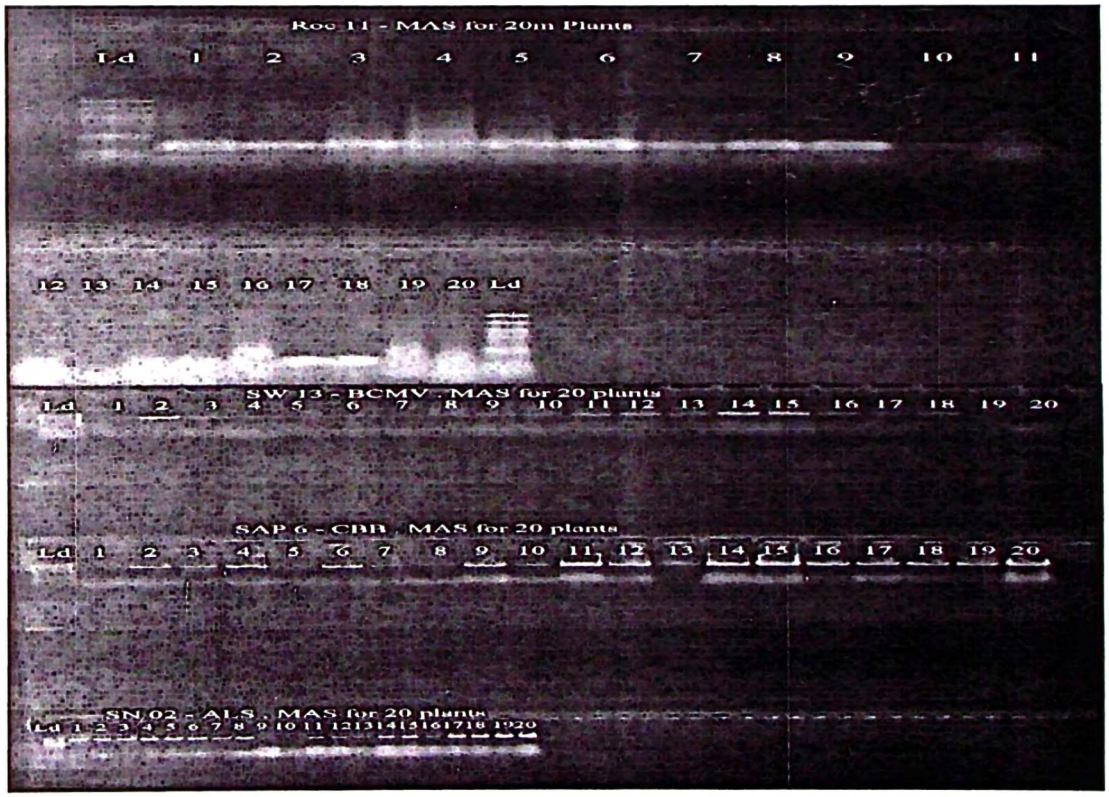

Plate 2. 1: Amplified products for 20 F2 common bean lines with SCAR marker ROC11, SW13, SAP6 and SNO2 SCAR as observed on 1.5% agarose gels.

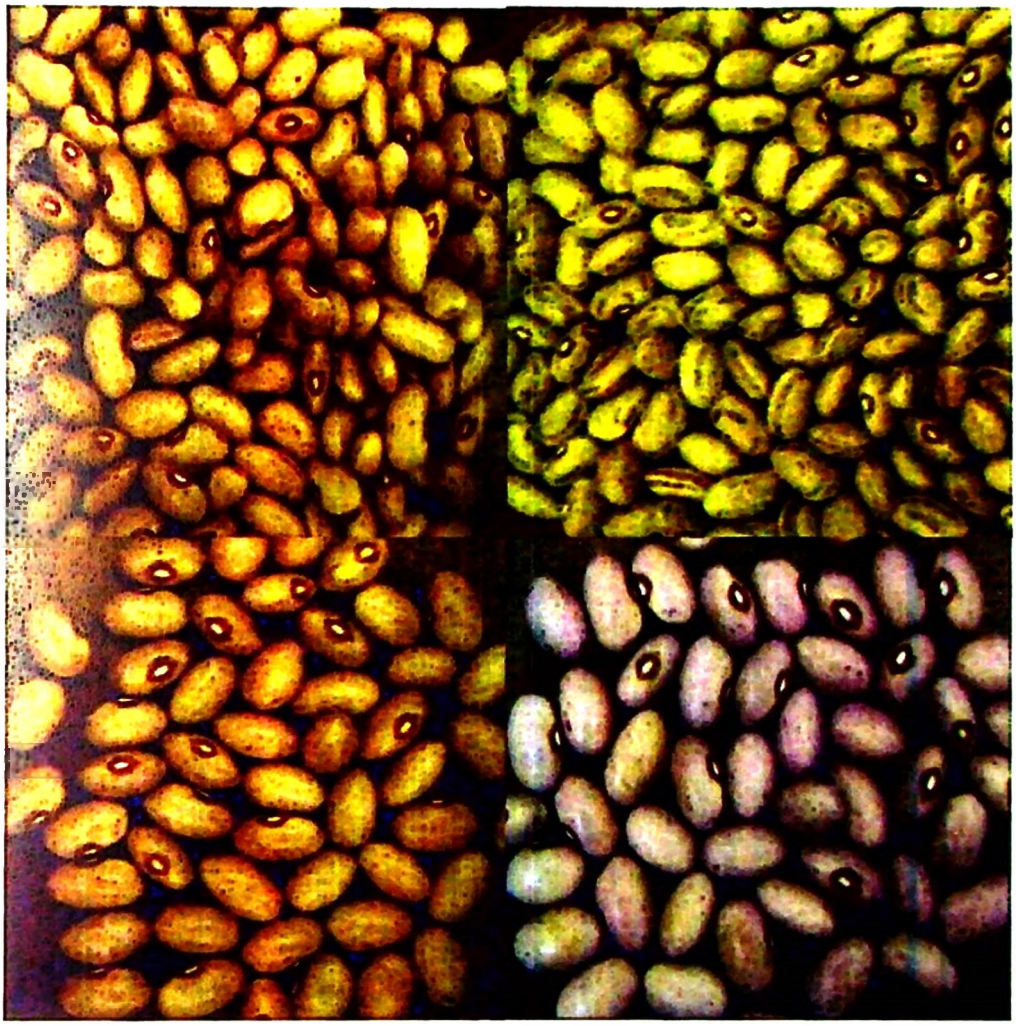

Plate 2. 2: Different seed types from harvested F2 bean lines containing multiple resistance generated from cross (245A x 585A-21) x 336Ab-7)

2.4.3 Transfer of resistance into adapted cultivar Kablanketi

Results of the crosses made using Mexico 54, Vax 4 and MCM 5001 as sources of resistance to transfer into Kablanketi are presented in Table 2.3a and Plate 2.3. Twelve F_2 lines had a combination of four genes, 23 had three genes, 11 had two genes and eight had only one resistant gene. Table 2.3b shows the breeding lines with combination of resistance to BCMNV, ALS and CBB summarized from Plate 2.3. There were some lines with the combination of two resistance genes of ALS and CBB, ALS and BCMNV and CBB and BCMNV. These lines could also be useful in the breeding programme. Seed

types of some of those lines are shown in Plate 2.4 for F_2 and Plate 2.4 for F_4 of the resistant lines.

Many plants genotyped (Table 2.3a, b) showed to have resistance to ALS, CBB and BCMV and less to BCMNV diseases. These results indicate that the markers for ALS (SN02), CBB (SAP6) and BCMV (SW13) are reproducible and easy to work with as they are dominant SCAR markers. The marker for BCMNV (ROC11) is a complex marker and it detects the recessive gene (*bc-3*). The presence of the band showed that there is a dominant gene and absence of a band from the gel meant that there is a recessive gene ***bc-3*** (Plate 2.3). However, this marker was associated with poor reproducibility and difficult to optimize in some case. Therefore, there is a need for alternative and more informative markers which can work better like SNP or SSR markers.

Twelve F_2 plants identified with multiple resistance genes were selected and harvested, then advanced to F_4 and screened for the presence of molecular markers associated with the resistance gene (Plate 2.5). The harvested lines were further advanced and verified for consistence for the presence of the resistance genes. This indicates that the plants/lines with DNA markers associated with resistance gene can be selected in early generation and still maintain its resistance in later generations. At the same time it is important to eliminate plants which do not have resistance genes in early generation. This saves time, lower cost of rescreening, labour and space competition.

Table 2.3a: Summary of crosses (F_2) with combinations of different genes

Cross	# of plant with 4 genes	# of plant with 3 genes	# of plant with 2 genes	# of plant with *1* genes
(Kab x Vax 4)(Kab x MCM 5001) x Mexico 54	3	8	3	3
(Kab x Mexico 54)(Kab x MCM 5001) x Vax 4	1	9	4	2
(Kab x Vax 4)(Kab x Mexico 54) x MCM 5001	8	6	4	4
Total	12	23	11	8

Table 2.3b: Breeding lines (F2) with different combination of linked markers for the ALS, CBB, BCMV and BCMNV diseases

CROSSES	MARKER-GENE PRESENT			
	ROC11-*bc-3*	SNO2-*phg-2*	SAP 6-*QTL*	SW 13- I gene
(Kab x Vax 4) (Kab x MCM 5001) x Mex 54	-	+	+	+
(Kab x Vax 4) (Kab x MCM 5001) x Mex 54	-	+	+	+
(Kab x Vax 4) (Kab x MCM 5001) x Mex 54	-	+	+	+
(Kab x Mex 54) (Kab x MCM 5001) x Vax 4	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+
(Kab x Vax 4) (Kab x Mex 54) x MCM 5001	-	+	+	+

Key: + refers to presence of resistance marker; and – refers to absence of resistance marker with respect to disease in question

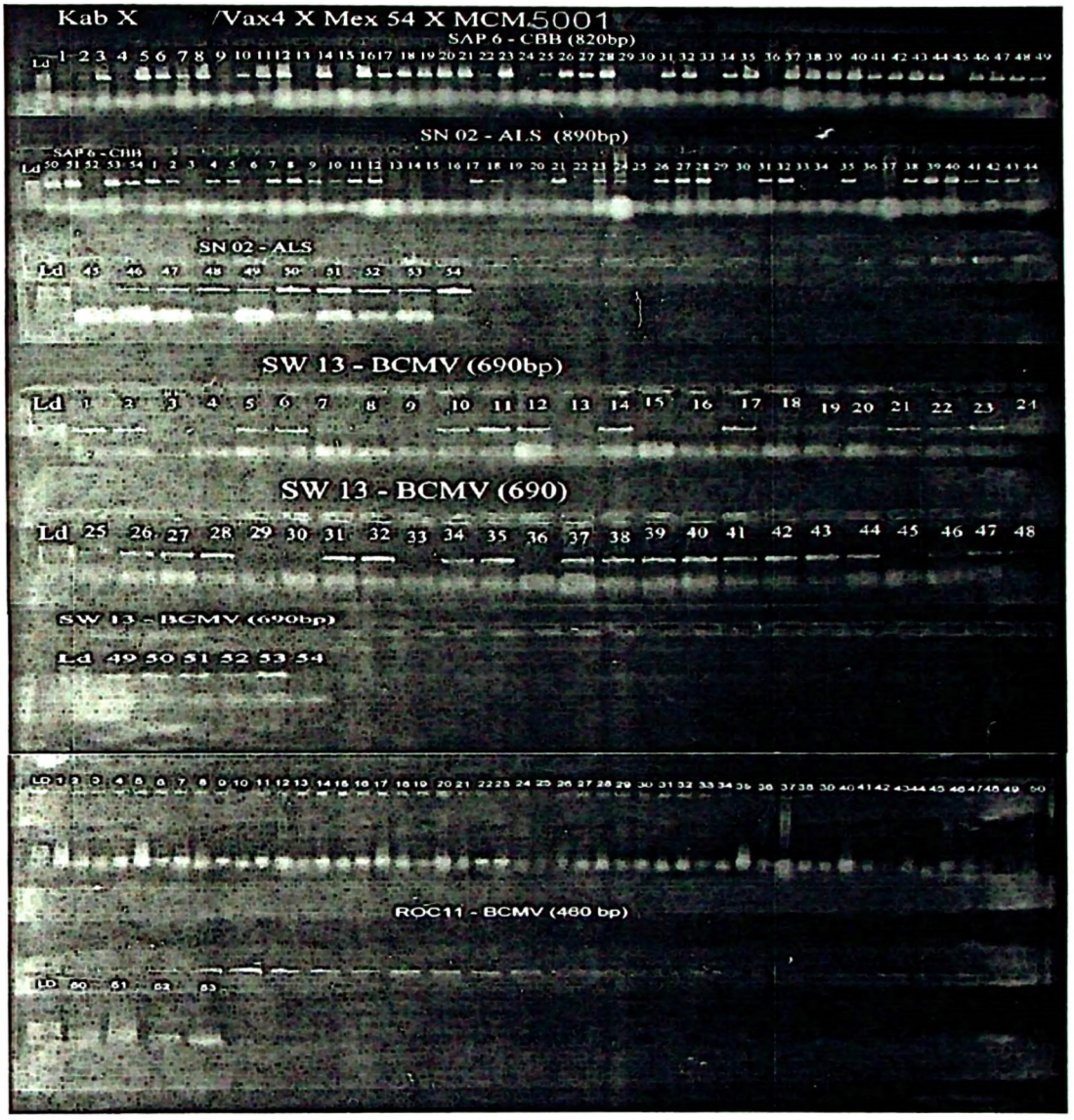

Plate 2. 3: Amplified products for F2 common bean lines with SCAR markers SAP6, SNO2, SW13 and ROC11 as observed on 1.5% Agarose gels

Key: absence of band refers to presence of intended gene; presence of band refers to absence of gene for SCAR marker ROC11

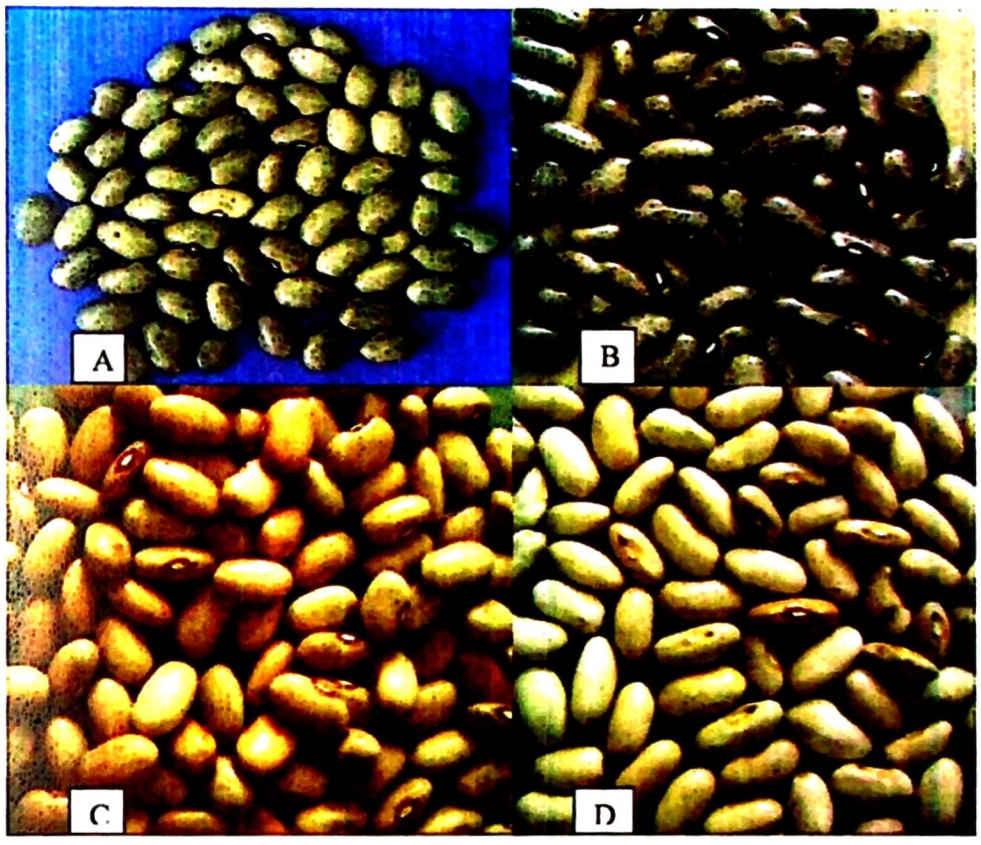

Plate 2. 4: Different seed types of F2 bean lines harvested with resistance for ALS, CBB, BCMV and BCMNV ((Kab x Vax 4)(Kab x Mexico 54) x MCM 5001 A-B; (Kab x Vax 4)(Kab x MCM 5001) x Mexico 54 C-D)

Plate 2. 5: Different seed types for F4 bean lines harvested with combined resistance for diseases (Kab x Vax 4)(Kab x Mexico 54) x MCM 5001 (A-C), (Kab x Vax 4)(Kab x MCM 5001) x Mexico 54 (D-F)

The resistant genes were successfully transferred into the crosses made. However, the success depends on optimization of the environment and time of performing the cross. The molecular markers are a useful tool in identifying and selecting the resistant plants (Miklas

et al., 2006). Molecular marker assisted selection offers a simpler and more efficient and accurate way to breed improved cultivars. It is especially helpful for breeding disease resistance compared with selection based on phenotypic screening. Marker assisted pyramiding of major genes has helped in the past to introduce genes for major diseases of common beans. Such diseases included pythium root rot (Nzungize *et al.*, 2011) and CBB (Miklas *et al.*, 2007; Mutlu *et al.* 2008). The technique used for field screening and multiple needle inoculations in the screenhouse, combined with MAS for the SCAR markers SAP6 and SU91, to develop CBB resistant. These include dark red kidney, white kidney, cranberry and pinto bean germplasm lines resistant to CBB. Also, Provvidenti (2001) developed lines resistant to BCMV. There are no reports of the transfer of genes conferring resistance to multiple diseases in the common bean in Tanzania despite the advances made in common bean genomics and breeding that combine resistance to multiple diseases.

The application of markers to introgress multiple resistance genes is a possible contributor to achieving a higher level of resistance in the advanced lines. However, there are risks being accompanied by the possibility of bringing undesirable traits into the advanced breeding lines due to linkage drag (Gepts *et al.*, 2005). MAS in early selection has been advocated for the quick recovery of recurrent parent genome by use of flanking markers linked to the resistant genes to identify successful superior breeding lines (Amaro *et al.*, 2007). However, the use of MAS will not completely eliminate the need for direct phenotypic selection but can be used to reduce the number of lines that require direct screening.

Molecular marker assisted selection is useful in rapid reconstitution of recovery parent genome, it can be very demanding in terms of application in breeding programme due to

several constraints, including identifying useful polymorphisms, its cost and timely execution (Singh *et al.*, 2001). Therefore, a more practical approach involving MAS for trait (s) of interest, in combination with phenotypic selection for specific characters unique to the recurrent parent, would help not only in transferring the traits of interest into recurrent parent but also in developing superior lines with additional desirable traits from the donor parent. MAS is the novel approach in which individuals for intercrossing are selected using selection index based on genotypic data controlled by few or several genes (Costa *et al.*, 2010). The gain from selection using such index is expected to be higher than phenotypic selection used in conventional recurrent methods as it was possible to select more lines with resistance at early generation. MAS offers significant advantages in cases where phenotypic screening is expensive, difficult or impossible or traits are of low heritability.

This is the first report of successful marker assisted transfer of genes conferring resistance to different diseases in common bean in Tanzania. These diseases were angular leaf spot, common bacterial blight and bean common mosaic virus. The genes incorporated and verified in farmers preferred variety (Kablanketi) are *phg-2* for ALS, major/resistant QTL for CBB, *bc-3* for BCMNV, and *I* gene for BCMV. These resistance genes have been combined through MAS to lead to development of lines that are resistant to both of the diseases. Based on phenotypic evaluation data, it has always been hard to track the accumulation of resistance genes in new breeding lines, hence the use of molecular marker provides a new tool for breeders and may help to overcome this problem. The improved lines have desirable market class and in tandem with inbuilt resistance to diseases and possibly to yields. The plants which are resistant to the three/four diseases were identified for further work which is validation of the resistance by phenotyping them in screen house and in the field by challenging them with pathogens.

2.5 Conclusion

It was possible to transfer resistance genes to the adapted cultivar Kablanketi and do selection using molecular markers. Twelve bean lines with multiple genes for disease resistance to angular leaf spot, common bacterial blight and bean common mosaic virus were developed. This indicates there are greater chances of selecting resistant individuals using molecular markers at early generation stage (F_2). Thirty eight breeding lines were identified with resistance to ALS, CBB and BCMV by molecular markers that were further evaluated with artificial inoculation and/or with field screening results to be presented in another paper. However, the breeding strategies that combine markers and phenotypic selection are the most effective in developing lines with improved resistance to diseases. Therefore, in case of many segregating plants, use of markers can be appropriate to select individuals with target allele. Furthermore, phenotypic data alone is not sufficient for some traits especially the ones with low heritability. Breeding for multiple disease resistance requires markers that have high level of correlation and/or linkage existing between the resistance and molecular markers. This kind of marker however, must be stable, reproducible and easy to assay. Molecular marker assisted selection is used to reduce the number of lines that require phenotypic screening. This work has proven that use of molecular marker can assist in doing selection to various traits including disease resistance genes. This will give significant contribution to improving the selection and save resources which could have been used in advancing the generation to gain gene fixation.

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CHAPTER THREE

Inheritance and Validation of Multiple Disease Resistant Bean Genotypes for ALS, BCMNV and CBB Combined with Desired Agronomic Traits

3.1 Abstract

The study was carried out to validate the resistance of the newly developed genotypes by MAS by determining the reaction of bean genotypes to ALS, CBB and BCMV and identifying genotypes with high levels of resistance to these diseases both in screen house and field conditions as described in Chapter 2 of this thesis. This work validates co-segregation of markers to multiple disease resistance among genotypes by artificial inoculation and/or with field screening. Results also demonstrated that the heritability for disease in this study is moderate to higher indicating that the trait was transferred to the offspring and selection could be performed in early generation for some of the pathogens like ALS and BCMV traits. The study revealed further that resistance of *P. griseola* is due to monogenic dominant gene for ALS and single recessive gene for BCMV. The CBB resistance was found to be conditioned by one major gene with partial resistance. The genotypes identified as resistant by inoculation in the screen house were also found to be resistant in the field and gave high yield when compared to disease susceptible control. This is the first report in Tanzania to confirm this kind of multiple disease resistance in *P. vulgaris*. The genotypes with resistance to all diseases are recommended for evaluation for release as varieties with multiple disease resistance.

3.2 Introduction

Common bean diseases are devastating the bean industry worldwide where common beans are cultivated. In Tanzania the diseases are endemic in all common bean producing ecologies (Wortmann *et al.*, 1998; Hillocks *et al.*, 2006). However, the most prominent

diseases include angular leaf spot (ALS) caused by *Pseudocercospora griseola*, common bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv *phaseoli (Xap)* and Bean common mosaic necrosis disease caused by *Bean common mosaic necrosis virus* (BCMNV). These diseases can simultaneously reduce yield and quality within and across different producing regions and yield losses can range from 10 - 100% depending on the diseases involved, time of infection and susceptibility of the cultivar used (Hall, 2000).

Angular leaf spot disease development occurs over a wide range of temperature with the optimum development at 24 °C (Bassanezi *et al.*, 1998). High relative humidity (90 - 100%) favours the growth of the fungi. Infection may not occur if the humidity is below 85% (Wortimann *et al.*, 1998). The disease affects all aerial plant parts especially leaves, pods, seeds, leaf petioles and lower surface of the leaflets. This leads to premature leaf drop, foliar and stem necrosis which often result to poorly filled seeds and reduce seed quality (Liebenberg and Pretorius, 1997). The lesions on leaves can cause severe defoliation and decreased leaf area index (de Jesus *et al.*, 2001) resulting to yield losses of up to 80% under severe infection (Hall, 2000; Namayanja *et al.*, 2006).

Common bacterial blight disease causes severe damage under warm temperatures, high rainfall and high humidity with maximum disease development at around 28°C (Wortmann *et al.*, 1998). Many infected seeds fail to germinate, abort or shrivel and get discoloured as they mature (Allen and Lenne, 1998). It has been estimated that each 1% increase in blight severity causes yield loss of about 10.5 - 78 kg ha^{-1}, depending on the season and crop growth stage (Allen and Lenne, 1998). The losses due to CBB disease are estimated to be 20 - 75% (Opio *et al.*, 1996; Mahuku *et al.*, 2003; Lema-Marquez *et al.*, 2007; Mutlu *et al.*, 2008). However, the extent of yield and quality losses is determined by weather conditions,

susceptibility of the cultivars in use and disease pressure (Allen and Lenne, 1998; Lema-Marquez *et al.*, 2007).

Bean Common Mosaic Virus (BCMV) and *Bean Common Mosaic Necrosis Virus* (BCMNV) are the most damaging viruses of bean that are naturally transmitted by aphids in a non persistent manner and through seed and pollen. It belongs to the genus Potyvirus in the family Potyviridae (Abtahi *et al.*, 2009). Infected bean plants and seeds are the main sources of primary inoculum for infection of the bean crop (Naderpour *et al.*, 2010). The disease causes a significant reduction in the absolute growth rate, which is attributed to decrease in plant height, number of branches per plant and number of leaves per plant (Provvidenti, 2001). The relative growth rate is also reduced and this is associated with a reduction in the net assimilation rate (Sengooba, 1994; Provvidenti, 2001; Mavrič and Šuštar-vozlič, 2004) leading to low yields in bean.

Control strategies for these diseases include chemical applications, cultural practices, and genetic resistance. Use of pathogen free seed is the most feasible cultural control method (Hampton, 1983), but such seed is not available to small-scale farmers in Tanzania (David *et al.*, 2006). However, among the existing strategies, genetic resistance is considered to be the most appropriate, environmentally safe and cost effective for small scale bean growers (Liu *et al.*, 2005). Selection of bean genotypes with resistance against ALS, CBB, BCMV and BCMNV for improving common bean production in terms of quality and quantity as well as prevention of post infection is paramount (Wortmann *et al.*, 1998; Broughton *et al.*, 2003). Therefore, the objective of this work was to determine the reaction of bean genotypes to ALS, CBB, BCMV and BCMNV and identify genotypes with high levels of resistance to these diseases both in screen house and field.

3.3 Materials and Methods

3.3.1 Location

The study was conducted at Sokoine University of Agriculture (SUA). The University is located at latitude 6°5′ South and Longitude 37°39′ East and 549 meters above sea level on the foot of Uluguru mountains.

3.3.2 Experimental plant material

The experimental plant material consisted of seeds of locally adapted bean cultivar Kablanketi, which is susceptible to CBB, ALS, and BCMNV. The CBB, ALS and BCMNV resistant parents i.e. Vax 4 *(QTL)*, Mexico 54 *(Phg-2)* and MCM 5001 (*bc-3*) respectively, their progenies F_1, F_2, and backcrosses to both parents (Fig. 2, Chapter 3). The progeny derived from backcrossing the F_1 to the female parent was designated BC_1F_1-P_1 while those resulting from the backcrossing to the male parent was referred to as BC_1 F_1-P_2. Kablanketi was used as a female parent. This cultivar has medium sized seeds, grayish in colour and it is a semi climber. It fetches high prices in the local markets because of its desirable seed coat colour, short cooking time and good palatability (Wortmann *et al.*, 1998; Hillocks *et al.*, 2006). Vax 4, Mexico 54 and MCM 5001 obtained from CIAT, Uganda were used as male parents due to their high levels of resistance to CBB, ALS and BCMNV respectively (Singh *et al.*, 2001).

One bean seed of each parent and their respective generations were planted per pot in screen house and the germinating seedlings were inoculated with the respective pathogens when they were 17 days old for ALS and CBB but at 10 DAP for BCMNV pathogens.

3.3.3 Inocula collection

To obtain inoculum for each pathogen, diseased leaves were collected from different bean planting areas viz. crop museum, SUA; Mgeta, Morogoro; Lushoto, Tanga; and Uyole,-Mbeya. Leaf and pod specimens were collected from naturally infected fields with typical disease symptoms. The diseased leaves and pods were detached from bean plants and preserved as follows: For ALS and CBB specimens, the collected leaf samples were placed between two layers of absorbent brown paper and then put in envelopes labelled with the name of the bean variety, date and location from where the sample was collected. For the BCMNV specimens, the fresh samples were placed on ice in plastic bags ready for inoculums preparation. The samples were then brought to the plant pathology laboratory in the Department of Crop Science and Production at SUA for isolation and characterization of the *Xap and P. griseola* pathogens.

3.3.4 Pathogen isolation, preparations of inoculum and inoculation

3.3.4.1 Angular leaf spot

Isolation of *P. griseola*

Tissue segments with ALS lesions were picked by sterile forceps and placed in petri dishes lined with three moistened filter papers. The plates were incubated in darkness at room temperature for 48h in order to induce sporulation. Plates were examined for sporulation and conidia from individual lesions were picked from clean synnemata, by gently brushing the synnemata with a small piece of agar at the tip of slightly bent inoculating needle. The conidia were transferred to two drops of sterile distilled water placed onto Potato Dextrose Agar (PDA). Drops of sterile distilled water were immediately spread over the surface of agar media using a sterile L-shaped glass rod. Thereafter the agar plates were incubated at 22°C for 24h after which the individual germinating conidia were transferred to fresh agar media. The plates were stored in the refrigerator until when required for inoculum

preparation. In order to obtain a pure culture of the pathogen, the fungi were cultured on V8 media without an antibiotic and kept in the inoculation room (24°C) for 14 days. Thereafter, the conidia were removed by adding 10 mL of sterile distilled water to each petri dish, followed by scraping the surface with a sterile glass rod. Then the suspension was filtered through a double layer of sterile gauze. The suspension was adjusted to 10^4spores ml^{-1} using haemocytometer and 0.1% Tween 20 was added to enable spores stick on the leaf surface.

Inoculation

Plants were inoculated at 17 days after planting (DAP) when they had 2-3 sets of fully expanded trifoliolate leaves with isolate from SUA-Crop Museum. The inoculated plants were covered with plastic sheets for 72h to create humidity. After 72h, the plastic sheets were removed and the plants were put on screen-house benches made of meshed steel, one metre high. Plants were observed daily for disease development.

Disease resistance rating

The disease severity was assessed on all leaves weekly from seven days after inoculation for a maximum of four weeks. The diseased leaf area was estimated in percentage according to CIAT 1-9 scale (van Schoonhoven and Pastor-Corrales, 1987) (Table 3.1), where 1 represents no visible symptoms and 9 simply means severe symptoms and disease expression. Plants that had a score of 1 - 3 were considered resistant, 4 – 6 intermediate and scores greater than 6 were considered susceptible.

Table 3. 1: General scale used to evaluate the reaction of bean germplasm to fungal and bacterial blight

Rating	Category	Description	Comments
1-3	Resistant	No visible symptom to light symptoms	Germplasm useful as a parent or commercial variety
4-6	Intermediate	Visible and conspicuous symptoms resulting only in limited economic damage	Germplasm can be used as commercial variety or source of resistance to disease
7-9	Susceptible	Severe to very severe symptoms causing useful yield losses or plant death	Germplasm in most cases not useful as parent or commercial variety

Source: van Schoonhoven and Pastor-Corrales (1987)

3.3.4.2 Common bacterial blight

Isolation of *Xap* the causal agent of common bacterial blight

Bacterial blight differential media was prepared following the procedures as described by Mortensen (2005). A section from the advancing margin of healthy and diseased leaf tissue was macerated in sterile distilled water, and the macerate was diluted to 10-fold dilution with sterile distilled water and streaked onto plates of Milk Tween (MT) agar medium selective for *X. axonopodis* pv *phaseoli.* Cell suspensions were made using sterile distilled water and its concentration was adjusted to 10^6cfu/ml using haemocytometer. Plates were incubated in an oven at 28°C for 4 to 5 days. After 5 days yellow mucoid colonies surrounded by zones of starch hydrolysis were sub-cultured onto yeast dextrose carbonate agar (YDCA) media. The recovered colonies of *Xap* were preserved in 40% glycerol and stored at -20°C for further characterization and identification. The storage culture of *Xap* was revived by growing it on Yeast Dextrose Carbonate Agar (YDCA) media at 28°C for

48h. Cell suspensions were made using distilled water and its concentration was adjusted to 10^6cfu ml^{-1} using haemocytometer.

Inoculation

Plants were inoculated at 17 DAS when they had 2 to 3 sets of fully expanded trifoliolate leaves by spraying the inoculums on the leaves using a miniature hand pump sprayer. Thereafter, the inoculated plants were covered with plastic sheets for 72h to increase relative humidity. After 72h, the plastic sheets were removed and the test plants placed in screen-house benches made of meshed steel, one metre high for symptoms development.

Disease rating

The disease severity was assessed on all leaves weekly from seven days after inoculation for a maximum of four weeks. The disease severity rating was estimated according to CIAT 1-9 scale (van Schoonhoven and Pastor-Corrales, 1987) (Table 3.1).

3.3.4.3 Common mosaic necrosis virus

Inoculum preparation and inoculation for BCMNV

Four seeds of each genotype were sown in sterile soil in 3 litre plastic pots placed on concrete benches in screen house and BCMNV inoculum was prepared as follows. One gram of infected leaf was ground using a mortar and pestle in 5 ml Potassium phosphate buffer containing 0.1% Tween 20. The resulting sap was filtered through a double layer of sterilized cheese cloth to remove large plant debris. A 10 g of carborundum powder (300 mesh) and sterile PBS was put into the clarified sap and stirred before inoculation. The final dilution was 1:10 (w/v).

Inoculation

Mechanical inoculation was performed using a cotton swab by lightly rubbing the young leaves of each test bean plant on both surfaces of leaves. Inoculation was done on the same day by dipping the index finger into the inoculum and then rubbing the sap on the primary leaves of 10 days old plants. Control seedlings were inoculated with distilled water only.

Disease severity rating

Disease assessment was done at 15 days after inoculation (DAI) using the disease severity evaluation scale of van Schoonhoven and Pastor-Corrales (1987) as described below in Table 3.2.

Table 3. 2: General evaluation scale for viral diseases

Rating scale	Symptoms	Incidence (%)	Yield
1	Absent	0	Excellent
2	Doubtful	1-10	
3	Weak	11-25	Good
4	Moderate	26-40	
5	Intermediate	41-60	Intermediate
6	General	61-75	
7	Intense	76-90	Poor
8	Severe	91-99	
9	Dead	100	Very poor

Source: van Schoonhoven and Pastor-Corrales (1987)

3.3.5 Multiple inoculation experiment

3.3.5.1 Planting and inoculation

The experiment was composed of 120 plants of common bean genotypes inoculated with *P. griseola*, *Xap* and *BCMV*. The plants were inoculated with the three pathogens in sequential way, starting with BCMV at 10 days after planting (DAP). At 16 days after inoculation (DAI), the susceptible plants were removed while the resistant plants were inoculated with both *P. griseola* and *Xap* pathogens as described above in section 3.3.4.1 and 3.3.4.2 for *P. griseola* and *Xap*, respectively.

3.3.5.2 Disease resistance rating

Diseases were assessed at 7, 14, 21 and 28 days after inoculation on trifoliolate leaves. The disease rating was done based on assessment of the severity of disease and general appearance of the diseased plant for both ALS and CBB using CIAT 1-9 rating scale (van Schoonhoven and Pastor-Corrales, 1987) (Table 3.1). Genotypes that had a score of 1 - 3 were considered resistant, 4 – 6 intermediate and scores greater than 6 were considered susceptible. For BCMV, disease assessment was done at 15 DAI using the modified evaluation scale of van Schoonhoven and Pastor-Corrales (1987) as shown Table 3.2.

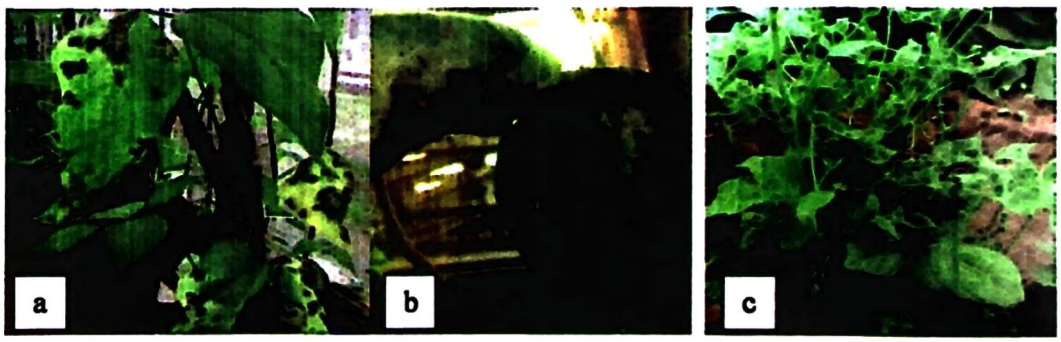

Plate 3.1: Symptoms of bean on the bean leaves inoculated with (a) ALS (*P. griseola*), (b) CBB (*Xap*) and (c) BCMV

3.3.6 Field experiment

The study was conducted at Crop Museum, Sokoine University of Agriculture (SUA), located at latitude of 6°45'S, longitude of 37°40'E and an altitude of 547m above sea level (masl), Morogoro, Tanzania. Site clearing was done manually following by harrowing. P was applied to the soil before sowing in the form of TSP at a rate of 25 kg P ha. Nitrogen was applied in form of urea at a rate of 40 kg/ha two weeks after emergence. A Randomized Complete Block Design (RCBD) was used with 35 genotypes replicated three times. Sowing was done at a spacing of 50 cm x 20 cm between and within rows, respectively. A plot had one row per treatment and 20 plants per row which gave a plot size of 0.5 x 8.4 m = 4.2 m^2. Each treatment made a single row plot, so there were 35 plots. Individual treatment was randomly assigned to plots.

After emergence, irrigation was carried out regularly to maintain the moisture content required by the crop at or above field capacity. Three weeding were performed throughout the study and diseases and insect pests were not controlled to suppress the treatment for better judgment

3.3.7 Data collection and analysis

Data were collected on the reaction (severity) of bean lines for ALS, CBB, and BCMNV diseases respectively and analyzed using the 14th Edition GENSTAT statistical package to generate means, variance, standard deviation, standard error and coefficient of variation. The variances of parents, F_1, F_2 and backcrosses were generated and used to estimate the heritability in narrow sense based on scaling test as described by Falcon and Mackay (1996). Narrow sense heritability (h^2) was determined from the variances of the parents, F_1's, F_2's and the backcross generations where $h^2 = 1/2D/VF_2$. V_{F2} is the total variance of F_2 and 1/2D is the additive genetic component of variance of F_2 calculated from the

variances of the backcrosses and the total variance of F_2 and $1/2D = 2(V_{F2}) - (V_{B1} + V_{B2})$. Inheritance was calculated based on the crosses generated. MS Excel 2007 was used to generate the frequency graphs and estimate Chi-square (χ^2). The chi-square values were computed on numbers of resistant and susceptible plants in each generation to determine whether the observed ratios deviated from the expected Mendelian ratios for segregation expectations according to gene action type and number of genes controlling resistance to the diseases.

For field experiment, data collected include days to 50% flowering (DF), days to maturity (DPM), plant height (PHT), pods/plant (PP), seeds per pod (SDPD), ALS, CBB BCMV severity, 100 seed weight, yield per plant (YDPL) and yield per ha (YDHA). These data were subjected to analysis of variance using GenStat statistical package 14th Edition. Means were separated by LSD at $P \leq 0.05$

3.4 Results and Discussion

3.4.1 Heritability for disease resistance

3.4.1.1 Angular leaf spot resistance

The narrow sense heritability estimates for diseases are present on Table 3.3. The heritability (h^2) estimated for progenies from the cross Kablanketi x Mexico 54 for angular leaf spot was 72% implying that additive effect for the genes controlling disease resistance exists in F_2 population. However, other authors have reported the heritability for this trait (angular leaf spot resistance) to range from medium to high. Amaro *et al.* (2007) reported the heritability for the reaction of common bean to angular leaf spot ranging from 44 to 59%. Results obtained in this study show that the heritability for ALS is high which implies a higher contribution of the genes to this trait (additive gene effects) than the environmental effects.

Table 3. 3: Heritability estimates in narrow sense for the reaction to ALS, CBB, and BCMV in common bean leaves

Crosses	Organ assessed	Estimated heritability (h^2)
Kablanketi x Mexico 54 (ALS)	Leaves	0.719
Kablanketi x Vax 4 (CBB)	Leaves	0.320
Kablanketi x MCM5001(BCMV)	Leaves	0.453

High h^2 usually reflects good general combining ability suggesting that resistance could be improved by selection. Estimate of heritability from segregating population is useful in understanding the genetic consequences of hybridization and inbreeding. They can help the breeder in selecting and utilizing the superior individuals from a population. Heritability is the most important parameter in the prediction of selection response since the effectiveness of selection depends on the additive portion of genetic variance in relation to total variance (Falconer and Mackay, 1996). Traits with relatively high heritability have been reported to respond highly to selections for effectiveness in crop improvement at early generation (Ali and Wynne, 1994; Falconer and Mackay, 1996). It is suggested that phenotypic selection of the promising plants in large F_2 population followed by progeny testing would increase productivity. However, low heritability can be obtained depending on the susceptible parents used. In such situations the application of molecular marker linked to the genes to be transferred to the susceptible background is appropriate (Amaro *et al.*, 2007).

3.4.1.2 Common bacterial blight resistance

The estimated heritability in narrow sense for common bacterial blight resistance trait was 32%. This heritability is classified as moderate according to Falconer and Mackay (1996). The low to moderate heritability has been reported for leaf reaction to *Xap* in dry bean by other authors (Arnaud-Santana *et al.*, 1994; Ariyarathne *et al.* 1999). Breeding for

resistance using quantitative genes involves shifting the population mean towards resistance (Bonos, 2006). Breeding programs relying on additive genetic variation for successful population improvement towards more resistant phenotypes could be advantageous. The moderate heritability implies that resistance is conditioned by few major genes with mean effects of partial dominance (Singh, 1991; Falconer and Mackay 1996; Fourie *et al.*, 2011). It was found that additive gene action was significant for leaf reaction with heritability in narrow sense ranging from 18 to 87% (Silva *et al.*, 1989), 30 - 60% (Ariyarathne *et al.*, 1999), 0.52-0.60 (Arnaud-Santana *et al.*, 1994) and from 9 to 93% (Singh, 1991). Low heritability to CBB in leaf and pod reactions means the gene is inherited quantitatively (Arnaud-Santana *et al.*, 1994). Usually heritability values depend on different aspects such as the population in consideration, environmental conditions and experimental design, precision of data collection and genetic complexity of the trait under study. Therefore, differences in heritability results for the same trait are quite common (Jung *et al.*, 1996). Selection efficiency for the resistance to *Xap* may be increased using molecular markers such as SCAR markers in early generations (Ariyarathne *et al.*, 1999). However, Ferreira *et al.* (2004) reported high heritability of 80% for F_6 and 88.3% for F_7. This demonstrates that evaluations being carried out with advanced materials contributed to its increase, enabling a more accurate selection of superior genotypes. Results have been reported to vary with environmental conditions, study populations as well as the inoculation methods, in addition to pathogen complexity. This calls for concerted efforts to explore more on the behaviour of the pathogen. It also, demonstrates potential of existence and possibility to discover promising genotypes within the advanced populations based on their genetic variability. Singh and Munoz (1999) reported that low to moderate heritability was accompanied by the complex nature of resistance and the environmental effect on symptom development which makes screening for CBB resistance difficult.

3.4.1.3 Bean common mosaic necrosis virus resistance

Heritability in narrow sense for this disease as estimated was 45.3% indicating that additive components play a significant role for this trait. Since the estimated heritability is moderately high, it is associated with additive gene action. Similar results have been observed by other researchers who worked with this disease. For example, Mike, (2008) reported the heritability in narrow sense of 64% while Peyambari *et al.* (2006) reported the heritability in narrow sense ranging from 62 - 64%. All those results indicate the importance of additive components for this trait and the need to focus on high resistance during selection.

3.4.2 Inheritance patterns for disease resistance

3.4.2.1 Angular leaf spot

Average disease severity data for angular leaf spot of all generations from the parents and three different crosses (F_1, F_2, and backcrosses) were recorded (Fig. 3.1). The frequency distributions of observed leaf lesion reactions of the Mexico 54, Kablanketi, F_1, F_2, Kablanketi x F_1 and Mexico 54 x F_1 populations illustrated several aspects of the genetic system conditioning ALS resistance in Mexico 54. Majority of the plants in the Mexico 54 population exhibited a resistant reaction (leaf lesion score $\leq$3). All plants in the Kablanketi population exhibited a susceptible reaction (leaf lesion score $\geq$4). Majority of plants in the F_1 population exhibited a resistant reaction with the scores of 1-3, suggesting that the ALS resistance is inherited as a dominant trait. The distribution frequencies in the F_2 population and Kablanketi x F_1 backcross population indicated that ALS resistance is mostly conditioned by qualitative genetic system (Fig.3.1). The monogenic inheritance of resistance indicates that pedigree breeding would be adequate for transferring the resistance to the susceptible genotypes. The disease grade distributions (Fig.3.1) clearly indicated a monogenic type of inheritance of resistance to *P. griseola.*

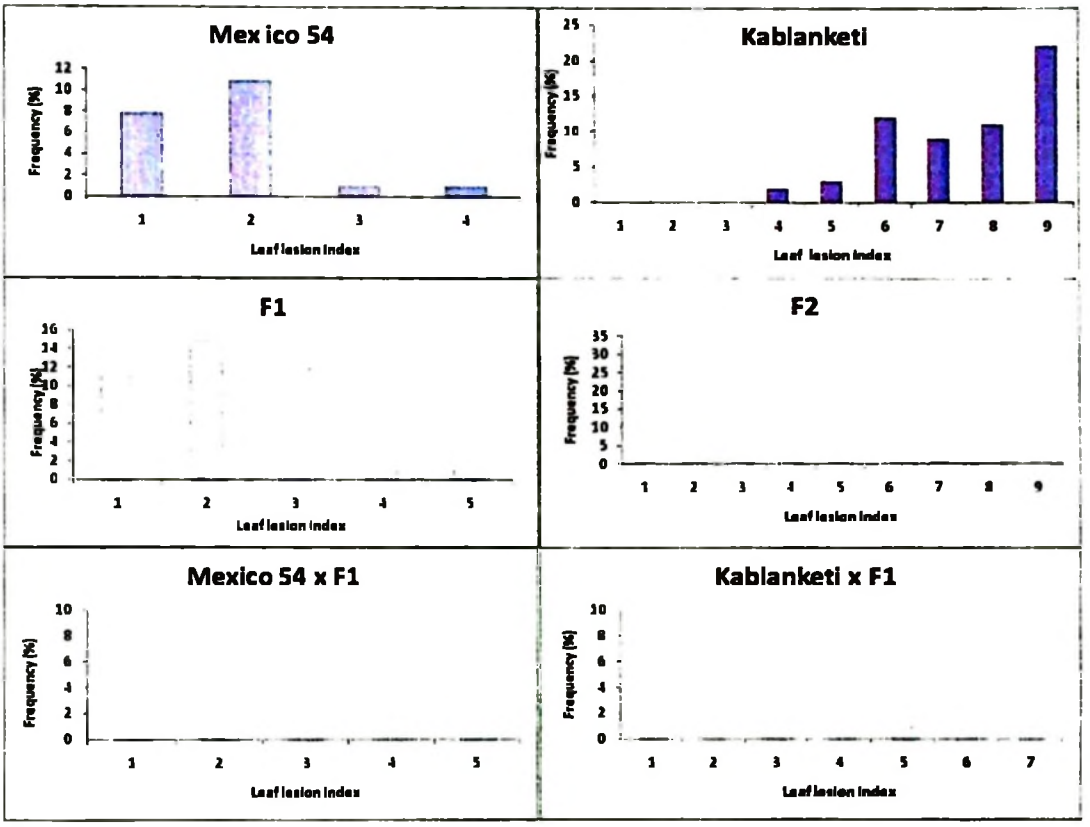

Figure 3. 1: Comparative frequency distributions of reaction to *P. griseola* for parental, F_1, F_2 and backcross populations of Kablanketi (♀) x Mexico 54 (♂) grown in screenhouse (1= no visible symptoms/lesions (resistant), 9= chlorotic leaf tisue susceptible)

The segregation data indicated that ALS resistance in Mexico 54 is conditioned by a major gene (Table 3.4). Segregation for resistance in the F_2 population was consistent with a ratio of 3:1 as resistant: susceptible plants in a population (χ^2 = 0.015; p>0.05) and that the backcross to susceptible parent (Kablanketi) fits a 1:1 ratio (χ^2 = 0.177; p>0.05) (Table 3.4). These results support the hypothesis that a single dominant gene in Mexico 54 controls resistance to *P. griseola* as reported by Muthomi *et al.* (2011). Similar results were reported using the same source of resistance on modes of inheritance to *P. griseola* in Mexico 54 indicating a single dominant gene effect that controls resistance (Namayanja *et al.*, 2006). Caixeta *et al.* (2003) identified monogenic inheritance using BAT 332 as a source of resistance to *P. griseola.*

However, several studies in common bean cultivars have reported that *P. griseola* resistance may be controlled by one, two or three dominant or recessive genes (Carvalho *et al.*, 1998; Caixeta *et al.*, 2003; Mahuku *et al.*, 2003;). It has been established however, that genetic resistance to *P. griseola* in different cultivars as source of resistance is monogenic and dominant (Muthomi *et al.*, 2011). This implies that it is possible to transfer this trait to elite cultivars by backcrossing. The cultivars that are resistant sources to *P. griseola* include AND 277 (Carvalho *et al.*, 1998), MAR 2 (Ferreira *et al.*, 2000), Cornell 49-242 (Nietsche *et al.*, 2000), Mexico 54 (Sartorato *et al.*, 2000; Mahuku *et al.*, 2004), BAT 332 (Caixeta *et al.*, 2003; Mahuku *et al.*, 2004) and CAL 143 (Chataika *et al.*, 2010). A total of nine genes have been identified in some of the resistant cultivars after allelism tests. These genes include *Phg-1*^{a}, *Phg-2*^{2}, *Phg-3*^{2}, and *Phg-4*^{2} for cultivar AND 227, *Phg-2*, *Phg-5* and *Phg-6* for Mexico 54 and MAR 2 has *Phg-4* and *Phg-5* resistance genes (Caixeta *et al.*, 2002).

Table 3.4: Segregation for resistance to angular leaf spot (*P. griseola*) in parental, F_1, F_2, and backcross populations of the cross Kablanketi x Mexico 54

Parent/Cross	Generation	Number of plants		Expected	χ^2
		R	S	Ratio	
Kablanketi	P1	0	59		
Mexico 54	P2	61	2		
Kablanketi x Mexico 54	F_1	65	6		
Kablanketi x Mexico 54	F_2	67	23	3:1	0.015
Kablanketi x F1	BC_1F_1-P_1	27	24	1:1	0.177
Mexico 54 x F1	BC_1F_1-P_2	34	6	1:0	

Resistant (R): number of resistant plants (leaf lesion index ≤3); Susceptible (S): number of susceptible plants (leaf lesion index ≥4); on CIAT scale of 1-9 (1987)

Selection for traits having high heritability would be very effective as there would be a close correspondence between genotype and phenotype. This work has demonstrated that the heritability for ALS disease is higher indicating that the trait was transferred to the offspring and selection can be performed in early generation. High heritability coupled with high expected genetic advance is considered to be more useful in predicting the outcome of selection. The study also revealed that resistance of *P. griseola* is due to monogenic dominant gene using Mexico 54 as source of resistance. The screening technique coupled with the simple inheritance of angular leaf spot resistance would lead to easy selection of breeding lines when the source of resistance is independent of inocula density. The breeding lines identified to have ALS resistance with scores of 1-3 and those lines can be selected as breeding lines for multiplexing other resistance genes into Kablanketi.

3.4.2.2 Common bacterial blight resistance

Results obtained with F_1 show that 54 plants were resistant while six of them were susceptible suggesting that resistance is rather dominant. The six susceptible plants within the F_1s were treated as false cross because no segregation is expected at F_1. The hypothesis that only one dominant gene is segregating was confirmed by the F_2 and backcross populations show the single dominant gene inheritance with F_2 segregation of 3:1 and the backcross segregation of 1:1 (Fig. 3.2 and Table 3.5). The plants of Kablanketi were susceptible and all the plants of Vax 4 were resistant as expected. The backcrosses to susceptible parent, showed the segregation of 1:1 and F_2 segregation of 3:1 resistant to susceptible. However, the backcross to Vax 4 had few susceptible individuals. The F_2 progenies showed segregation patterns ranging from complete resistance to susceptibility (Table 3.5). The phenotypic segregation of F_2 progenies for the reaction to *Xap* largely segregated in the ratio of 3:1 ($\chi^2 = 0.47$; $P>0.05$) suggesting the presence of dominant

genes controlling resistance *Xap* in Vax 4. This result is in conformity with the results of other authors. For example, Muimui *et al.* (2011), indicated that resistance to *Xap* is controlled by dominant genes in Vax 4. The Vax 4 has been reported to have good level of resistance to common bacterial blight (Singh and Mufioz, 1999). Results by Miklas *et al.* (2006) and Chataika *et al.* (2011) showed that the resistance to CBB is quantitatively inherited with major gene effect. Resistance to CBB, quantitative patterns of inheritance, differential leaf and pod reaction has been reported (Jung *et al.*, 1996). The complex inheritance to *Xap* makes the transfer of quantitatively inherited disease resistance genes into elite cultivars difficult (Jung *et al.*, 1996). The nature of inheritance greatly depends on the genotype used as the susceptible parent among other factors (Pastor–Corrales *et al.*, 1998). For example, it has been established that inheritance and gene action to *Xap* is influenced by plant architecture which includes growth habit influencing disease severity (Beebe and Pastor-Corrales, 1991). In addition, Silva *et al.* (1989) reported that inheritance of resistance to common bacterial blight in trifoliate leaf and plant canopy was controlled by a single major gene. Therefore, resistance to common bacterial blight is different depending on the source of resistance and may be determined by both major and minor genes (Singh, 1991). Since Kablanketi is semi determine it could have effect on the inheritance and gene action of *Xap*.

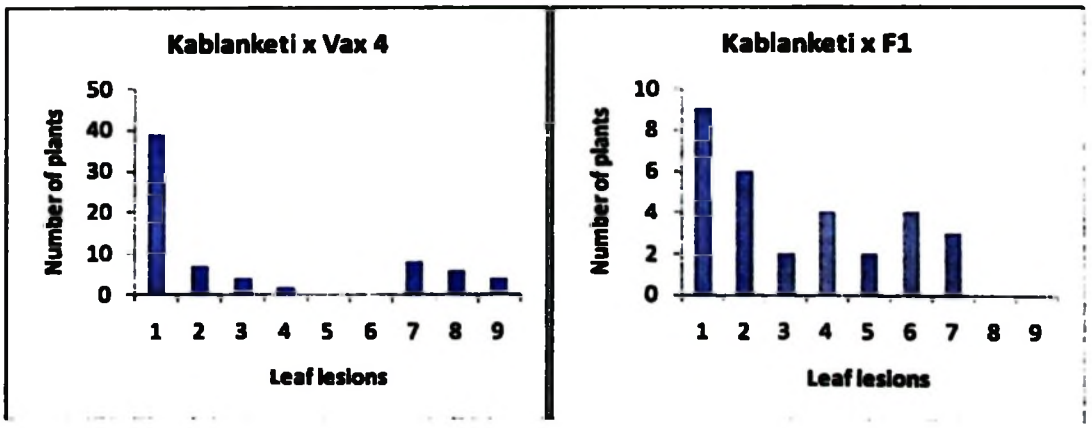

Figure 3. 2: Distributions of F_2 (Kablanketi x Vax 4) and backcross to Kablanketi (Kablanketi x F_1) plants for the reaction to *Xap*

Table 3. 5: Analysis of segregation ratios for resistance to susceptible in parental genotypes *Kablanketi*, Vax 4 and their crosses inoculated with *Xap*

Parent/Cross	Generation	Number of plants		Expected	χ^2
		R	S	Ratio	
Kablanketi	P1	0	70		
Vax 4	P2	80	0		
Kablanketi x Vax 4	F_1	54	6		
Kablanketi x Vax 4	F_2	50	20	3:1	0.47
Kablanketi x F1	BC_1F_1-P_1	17	13	1:1	0.53
Vax 4 x F1	BC_1F_1-P_2	44	9	1:0	

3.4.2.3 Bean common mosaic necrosis virus

Results for segregation of the F_2 and backcross generations are presented in Table 4.6. The susceptible plants responded with green mottle, downwards leaf rolling, reduction of leaf size and partial stunting. The F_1 plants of cross between Kablanketi x MCM 5001 reacted, some of them with local infection but all the plants were systematically resistant. The F_2 population segregated with recessive resistance factor near the ratio of 1 resistant: 3 susceptible. The progenies of the backcrosses to resistant parent MCM 5001 segregated in a ratio of 1 resistant: 1 susceptible. The analysis conforms to their goodness of fit with 1:3 and 1:1 resistant to susceptible segregation ratios using the χ^2-test. Results are not significantly different from the expected ratios. For F_2·s and backcross segregation data were not significantly different ($\chi^2 = 0.003$, $p = 0.957$) and (0.15, $p = 0.701$), respectively. This means that the gene responsible for the resistance is recessive and its inheritance is determined by single recessive gene which is *bc-3* gene for MCM 5001. A similar observation has been reported by Mukeshimana *et al.* (2005) working with F_2 population. The authors confirmed the segregation ratio of 1:3 by phenotypic screen of the F_2 which supports the presence of a single recessive gene conferring resistance to the disease. Similarly, Provvidenti (2001) found that the resistance to BCMNV is controlled by single

recessive gene with the segregation ratios of 1:3 and 1:1 for F_2 and Backcross generations respectively. The recessive bc-3 gene and dominant *I* gene have distinctly different mechanisms of resistance and their combination through MAS offers durable resistance. The *bc-3* gene confers resistance to all known strains of BCMNV and BCMV in the presence of the dominant *I* gene (Kelly *et al.*, 1999; Mukeshimana *et al.*, 2005). This gene is constitutively expressed and act by restricting virus replication or movement within the host plant (Kelly *et al.*, 2003). There are also other loci which confer resistance in common bean for BCMNV and they include *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2} and bc-u in presence of dominant *I* gene.

Table 3. 6: Segregation analysis for BCMNV resistance in common bean genotypes

Parent/Cross	Generation	Number of plants		Expected ratio	χ^2
		R	S		
Kablanketi	P1	0	80		
MCM 5001	P2	75	0		
Kablanketi x MCM 5001	F_1	0	60		
Kablanketi x Vax 4	F_2	28	85	1:3	0.003
MCM 5001 x F1	BC_1F_1-P_1	32	29	1:1	0.150
Kablanketi x F1	BC_1F_1-P_2	5	35	0:1	

Resistant: rr = (*bc-3, bc-3*); Susceptible: R- (*Bc-3, bc-3*), RR (*Bc-3, Bc-3*).

3.4.4 Evaluation of 120 genotypes for BCMNV, ALS and CBB diseases

Simultaneous evaluation was made with *Pg*, *Xap* and *BCMNV* to all parents. Kablanketi showed to be susceptible to three pathogens, Mexico 54 was susceptible to *Xap*, *BCMNV* but resistant to *P. griseola*, Vax 4 was susceptible to *P. griseola*, *BCMNV* but resistant to *Xap*, MCM 5001 were susceptible to *Xap*, *P. griseola*, but resistant to *BCMNV*. All the parental genotypes exhibited reaction similar to when they were inoculated with only one pathogen. This implies that no cross protection was observed among the resistant parents.

Out of 120 screened plants, 36 plants were dead due to necrosis (BCMNV) and 84 surviving plants were later screened for ALS and CBB in combination. Out of 84 plants, 31 (36%) plants were resistant to the three diseases and the remaining 53 plants were resistant to either ALS, CBB, BCMNV or to two of the diseases. In this study, it was observed that there was no incompatibility interaction of common bean genotypes inoculated simultaneously with the three pathogens. During evaluation for diseases one needs to be very careful because symptoms of two diseases like ALS and CBB may be found interacting on the same leaflet. These results allow deducing that in common bean breeding programmes aiming at ALS, CBB and BCMNV resistance, simultaneous inoculation of distinct pathogens may be accomplished, reducing time and cost in identifying resistant lines/cultivars. The 31 plants identified to have three resistance genes were harvested and their seeds planted later tested in natural infection in the field.

3.4.5 Preliminary field evaluation of identified genotypes resistant to ALS, BCMNVand CBB

The performance of genotypes of F_4 generation varied significantly for the disease reactions and yield as indicated in Table 3.7. All genotypes tested showed to be resistant to angular leaf spot except the susceptible parent (Kablanketi). For common bacterial blight, the genotypes varied from resistant to moderate resistant for genotype entries 2, 17, and 31 (susceptible parent). The reaction of genotypes for BCMV varied widely from resistant to susceptible. Mexico 54 and Vax 4 showed to be a good source for both ALS and CBB respectively. The evaluation of genotypes was favoured with environmental conditions and the presence of pathogens allowed the fast and homogeneous development of the diseases in the trial under evaluation.

Table 3. 7: Preliminary field evaluation of developed F_4 common bean lines in 2012 season

Entry	DF*	DPM*	PHT*	PP*	SDPD*	ALS*	CBB*	BCMV*	100WT*	YDPL*	YDHA*
1	30	70	79 9	24.73	3.33	1.0	3.00	4.00	30.93	21 51	3267
2	32	64	103.3	14.20	3.00	1 7	4 00	6.33	35.17	14.71	1843
3	34	73	74.3	20.20	3.33	1.0	2 67	4 33	23 63	11.81	2482
4	38	60	56 5	14.67	3 33	1 0	3 00	7 00	29.13	11 59	917
5	32	70	111 4	23 33	4.33	1 0	2.67	4.33	31 30	25.11	2500
6	33	60	84 7	19 00	3 33	1 0	2 67	4 33	25 73	14 74	1727
7	32	67	92 0	19 73	4 00	1.0	3.33	4.67	33.93	24.69	2365
8	33	66	80 7	23 93	4.67	1 0	3 33	4.67	28 83	17.75	3205
9	33	63	73 3	22 13	5 00	1 0	2 67	4 33	28 00	25 60	2607
10	32	61	87 1	21 67	2.67	1.0	1.67	4 67	24 20	9 63	1276
11	31	60	84 7	19 07	3 33	1 0	2 33	3 00	34 73	16 12	2210
12	33	60	81 6	22 93	4 33	1.0	3.00	4.00	27 30	23.96	3072
13	34	68	74 6	13.40	3 00	1.0	3.00	5.67	34 27	10.40	1375
14	32	70	98 8	25 47	4 33	1.0	3.00	4.00	27.70	23.46	3521
15	35	63	96 1	23 07	3.67	1.0	2.67	4.33	25.20	19.35	2883
16	32	66	80 1	23.53	3.33	1 7	3 00	6.67	31.37	18.20	800
17	31	59	67 1	21.07	3 00	1 0	4.00	6 33	30.90	15.59	1814
18	32	63	78 3	16 53	2.67	1 0	2.67	6.33	25.43	9.97	484
19	31	66	88 7	24.93	3.33	1.0	3.33	4.67	31 10	20.19	2306
20	31	61	93 3	19 07	4.00	1 7	3.00	4 33	29.50	17.55	1605
21	35	61	69 9	22 53	3 00	1 0	3 00	6 00	30.87	18.26	1747
22	33	62	81 6	20.60	4.00	1 0	2 67	4 33	28.00	20.77	2672
23	31	58	83 2	16 47	4 00	1 0	2 00	3.00	27 53	17 99	2324
24	31	65	92 5	15 00	2 33	1 0	3 00	7 33	38 53	10 94	1109
25	31	68	88 7	19 80	3 00	1 0	2.67	4 33	37.40	19.07	1756
26	32	60	87 3	17 33	2.67	1 0	3.33	4.67	37 80	14.16	2291
27	32	58	86 0	16 60	2.67	1 0	2.67	4.33	33.77	17.17	2529
Mexico 54	36	77	115.3	22 93	3 00	1.0	2 33	4.00	32.47	16.37	1198
MCM 5001	35	74	76 6	20.20	6.00	1 0	3.00	2.67	19.63	22.79	3305
Vax 4	38	71	64 1	23.20	4.33	1 3	2.33	4 33	23.70	19.93	1738
Kablanketi	32	67	87 7	13.13	3.00	7.0	6.33	8.00	39.33	13.46	676
32	32	67	104.7	19.33	2.33	1.0	3.33	4.67	34.17	15.67	2046
33	32	61	69.5	20.67	4.00	1.0	2.67	4.33	31.07	17.10	2260
34	32	73	83.1	22.13	3.33	1.0	3.33	4.67	19.17	14.23	1401
35	34	77	86.7	29.07	3.00	1 0	3 00	4.00	23.03	16.65	2162
Mean	32.8	65.4	84.7	20.33	3.51	1.3	2.99	4.82	29.85	17.33	2042.0
SE±	2.2	4.5	11.9	3.54	0.51	0.3	0.55	0.88	3.52	3.46	595.8
$LSD_{(0.05)}$	4.4	9.1	23.8	7.06	1.01	0.6	1.10	1.76	7.02	6.90	1188.9
CV (%)	8.3	8.5	17.2	21.30	17.70	28.8	22.60	22.50	14.40	24.50	35.7

SE± refers to standard error; $LSD_{(0.05)}$ refers to least significant difference; and CV (%) refers to coefficient of variation

* Refers to Days to 50% flowering (DF), days to maturity (DPM), plant height (PHT), pods/plant (PP), seeds per pod (SDPD), ALS, CBB BCMV severity, 100 seed weight, yield per plant (YDPL) and yield per ha (YDHA)

Performance of the genotypes were significantly different and varied from 484 to 3305 kg/ha with a means of 2042 kg/ha (Table 3.7). The genotype entry 14 and 29 were however outstanding with mean of 3521 and 3305 kg/ha respectively. However, more than half of the entry performed below the mean which is 2042 kg/ha. The large variations among the genotypes were observed with entry 14 yielding the highest and entry 18 yielding the least. Other genotypes clustered around the mean across genotypes (Table 3.7).

The poor performance for the majority of the entries was associated with presence of diseases which included ALS, BCMNV, CBB and rust. Plants infected with fungi, bacteria and virus usually exhibit a reduced photosynthetic rate and stunted growth to some genotypes. Occurrence of the two or more diseases/pathogens on the same host at the same time is frequent especially in tropical crops (de Jesus, 2005). The effect of pathogen on yield reduction could be explained in part by decrease in stomatal conductance and net photosynthetic rate of diseased bean leaves (de Jesus, 2001). However, disease control does not increase yield but rather reduces crop losses and sustain the performance of the crop potential. The simultaneous occurrence of diseases can lead to combined effects on crop yield and on the population dynamics of the pathogens. Generally the interactions of the pathogens complicate the control of the diseases and the portioning of the causes of yield loss. The interactions may alter the occurrence and speed of epidemics and can have significant implications to assess the crop losses, to diagnose the causes of losses, to select appropriate management strategies and forecast, model and simulate epidemics (Pastor-Corrales *et al.*, 1998; de Jesus, 2001). The genotypes with resistance to all diseases may be strongly recommended for evaluation for release as varieties with multiple disease resistances. Several backcrosses must be considered as well since the seed quality differs widely from genotype to genotype.

3.5 Conclusion

The objective of this work was to validate the resistance by determining the reaction of bean genotypes selected using MAS for resistance to ALS, CBB, BCMV and BCMNV and eventually identify genotypes with high levels of resistance to these diseases both in screen house and field conditions. The genotypes were developed by marker assisted selection. Results demonstrated that the heritability for diseases that is ALS, CBB and BCMV in this study is moderate to high indicating that the trait was transferred to the offspring and selection can be performed in early generation for some of the pathogens like ALS and BCMV traits. Selection for traits having high heritability would be very effective as there would be a close correspondence between genotype and phenotype. The study revealed further that resistance of *P. griseola* is due to monogenic dominant gene for ALS and single recessive gene for BCMV. The CBB resistance was found to be conditioned by one major gene which has effects of partial resistance.

The genotypes identified as resistant by inoculation in the screen house were also found to be resistant in the field when compared with resistant and susceptible control. The genotypes with resistance to all diseases are recommended for evaluation for release as varieties with multiple disease resistance. Since this was one season evaluation of these genotypes, it is suggested that those genotypes be tested and evaluated for more seasons with additional replications and locations to further verify the results obtained. This is because they displayed appreciable yields under the presence of the diseases. Several backcrosses must be considered since the seed quality differs widely from genotype to genotype for variety release. These genotypes could significantly contribute to the improvement of multiple disease breeding programmes in common bean with a view of improving food and nutrition security as well as income generation to small holder farmers.

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CHAPTER FOUR

Genetic Characterization of ALS Resistance in Selected Common Bean Landraces from Tanzania

4.1 Abstract

Angular leaf spot disease caused by the fungus *Pseudocercospora griseola* is one of the most important bean diseases in Tanzania. The landraces Beti-10, Nanka, Nanavala and Nkanamna were identified as resistance sources for ALS but the nature and inheritance in those potential sources have not been elucidated. This information is crucial for breeding programmes, hence the need for characterization towards the identification of mechanisms of resistance to ALS. The objective was to study the inheritance of ALS in four landraces and identification of genetic resistance using SSR markers. The crosses were made between resistant landraces and susceptible Kablanketi. The parents, F_1, F_2 and backcrosses derived plants were inoculated with *P. griseola* in the screenhouse. The results indicate that one single dominant gene control resistance in the four landraces. The DNA from landraces was screened with 30 SSRs markers for polymorphism and linked resistance. The SSR marker Pv-ag004 was found to be polymorphic for Beti-10 and Kablanketi. The resistance were validated by checking the F_2 population of the cross between Kablanketi x Beti-10. Therefore, since marker Pv-ag004 was linked to ALS resistance and was found to amplify in Beti-10, the Beti-10 landrace might be a good source for ALS resistance. However, there has been no detailed study with more markers on those landraces, there are possibilities of identifying new markers linked to ALS resistance and limited SSR markers could be fine tuned to explore the possibilities of mapping genes associated with resistance to ALS. Also, the allelism test is needed to confirm the resistance between the landraces whether they are similar or different.

4.2 Introduction

Common bean (*Phaseolus vulgaris* L.) is an important grain legume for direct human consumption (Wortmann *et al.*, 1998). Common bean production also provides farm households with both a source of income and food for nutrition (Tryphone and Nchimbi-Msolla, 2010). Despite the importance of the common bean crop, pest and disease outbreak are among the main problem reducing bean yield world wide that limit the genetic potential of the crop (de Jesus *et al.*, 2001). In Africa pest and disease problem is the second biggest constraints to bean productivity with an estimated annual yield loss of 228,800 t/ha of which 348,000 t/ha (17%) is due to angular leaf spot (ALS) disease caused by the fungus *Pseudocercospora griseola* (Sacc.) Ferr. pathogen (Mahuku *et al.*, 2003). Therefore, ALS is currently the most economically important disease that affects common bean production (de Jesus *et al.*, 2001; Correa *et al.*, 1994) and widely distributed disease (Steiglein *et al.*, 2003). In Tanzania the disease is endemic in low to high altitude bean producing zones (Tryphone *et al.*, 2012).

Angular leaf spot affects all above ground parts of the bean plant but most notable symptoms are on leaves, where the pathogen induces premature leaf defoliation that results in shriveled seeds of reduced size and quality (Saettler, 1991). At the same time the lesions on leaves causes severe defoliation and decreased leaf area index which has implication on crop performance. The disease can reduce yield as much as 50-80% when susceptible cultivar is grown (de Jesus *et al.*, 2001; Correa *et al.*, 1994). It has been estimated that ALS disease cause yield loss of 389 900 t/year in sub-Saharan Africa, 263 600 t/year in East Africa, Tanzania inclusive and 125 300 t/year in Southern Africa (Wortmann *et al.*, 1998). At the same time, the durable resistance to ALS is hardly achievable due to variability of the pathogen then the need to pyramid more than one gene from diverse background to overcome the variability of the pathogen (Mahuku *et al.*, 2003). Albeit, the trait is typically

vertical as well as horizontally controlled and display a strong interaction with the environment (Kelly and Miklas, 1998; Wortmann *et al.*, 1998). Selection and identification of resistant cultivar as well as the mechanism of resistance in resistant cultivars is paramount to deal with such a disease.

The large variability of the pathogen necessitates the constant identification and characterization of resistance genes and the subsequent development of resistant cultivars so as to minimize the risk associated with epidemics. The high pathogenic variability of *Pseudocercospora griseola*, the causal agent of ALS the search and characterization of new sources of resistance is indispensable as well as understanding of the genetics of the reaction to the pathogen (Borel *et al.*, 2011). Information about genetic control is very useful, because it helps breeders to choose the most efficient strategies for development of resistant and productive cultivars. Through bean breeding programme at Sokoine University of Agriculture, local cultivars resistant to ALS pathogen were identified under phenotypic screening in the field. The resistant cultivars include Nkanamna, Beti 10, Nanavala and Nanka. These cultivars are well adapted to Tanzanian bean growing ecologies and succumb less infection due to ALS (Fivawo and Msolla, 2011). Although resistance sources for ALS resistance have been identified, the nature and inheritance in those potential sources have not been elucidated although this information is crucial for breeding programmes, hence the need for characterization towards the identification of mechanisms of resistance to ALS. Furthermore, the use of such resistant cultivars requires information on nature of resistance genes in breeding to transfer resistance and gene pyramiding and the QTL/ genes conditioning their resistance. This information is therefore currently not well understood thus the need for working on it. Therefore, molecular markers were used to elucidate the ALS resistance among the local resistant cultivars.

Molecular markers linked to genes that control resistance to ALS disease are available (Nietsche *et al.*, 2000; Queiroz *et al.*, 2004; Miklas, 2005). Among the useful molecular markers are simple sequence repeats (SSR) to explore the potential of SSR in detecting linkage between a marker and disease resistance. However, simple sequence repeat markers have been extensively used to identify angular leaf spot disease resistance genes in common bean (Blair *et al.*, 2000). They provide several advantages over other two markers when applied in plant breeding programmes. Markedly, they are based on the polymerase chain reaction (PCR) technique; they are codominant, represent single loci and can detect high level of polymorphism and reproducibility once obtained with a large and specific primer. The SSR are also closer to the resistance alleles of some angular leaf spot disease (Collard and Mackill, 2008). The relatively low recombination frequency between the SSR and the locus can be characterized as useful marker for indirect selection. For example, the expected frequency of susceptible plants selected as resistant in F_2 population is 4.5 % (Silva *et al.*, 2003). Therefore, parents and the F_2 populations were screened to ascertain the resistance and identification of promising markers for resistance. The objective of this work was to study the inheritance of ALS in four landraces and identification of genetic factors for resistance using DNA molecular markers.

4.3 Materials and Methods

4.3.1 Plant materials

The susceptible adapted bean parent, Kablanketi was collected from farmers' fields in Mbeya, Region. Landraces Nanka, Nanavala, Beti 10 and Mkanamna obtained from SUA collection and those cultivars that have been identified to be resistant to ALS both in screen house and in the field (Fivawo and Msolla, 2011).

4.3.2 Population development

Kablanketi was crossed with resistant parents; Nanka, Beti 10, Nkanamna and Nanavala to generate F_1 populations. The F_1 seeds were backcrossed to susceptible and to resistant (resistant backcross). The remaining F_1 plants were self pollinated to produce the F_2 population.

4.3.3 Plant evaluation

The seeds from the parents, F_1, F_2, and backcross populations were planted for evaluation of ALS resistance. *Pseudocercospora griseola* isolation collected from SUA-Museum, inoculum preparation and inoculation procedures were adopted as described by Tryphone *et al.* (2012).

4.3.4 Heritability and inheritance patterns

The F_2 and backcross population were used to study the heritability of resistance genes as well as the inheritance patterns for ALS in the landraces (Nanka, Beti 10, Nkanamna and Nanavala).

4.3.5 Extraction and amplification of DNA

Total genomic DNA extraction was done as described by Delapotar *et al.* (2003) with some modifications. As, sodium acetate was used instead of potassium acetate, incubation stage was done at 60^0C for 30 min. instead of 65^0C for 45 min., centrifuging was done at 16 000 g for 10 min instead of 20 min. at 3 000g. For DNA amplification, PCR was set as per specification primers Tm. and resulting fragments of each primer were detected using agarose gel electrophoresis.

4.3.6 Gel electrophoresis and documentation

Amplification products were separated through electrophoresis migration in a 3% agarose gel, 1X TBE (Tris-Borate EDTA) buffer. The gel was stained in EtBr at 0.5µg/ml for 30 minutes, de-stained for 30 minutes by using distilled water. The bands present on the gel were observed and the mounted digital camera was used to capture the amplified fragments for documentation and scoring

4.3.7 ALS characterization

Screening for the markers closely linked to the ALS resistance in common bean landraces (Beti-10, Nanka, Nanavala and Nkanamna) by genotyping the parents using 30 SSR markers.

The F_2 populations from each cross of the susceptible x resistant parents were planted in the screen house for evaluation to the ALS reaction. The F_2 population was inoculated with *P. griseola* suspensions. The plants were evaluated for the reaction to ALS in the screen house. The CIAT evaluation scale of 1-9 was applied (Van Schoonhoven and Pastor-Corrales, 1987).

Deoxyribonucleic acid (DNA) for each individual plant was extracted and amplified as in section 5.3.5 in this thesis. Two DNA pools of ALS resistant and ALS susceptible individuals [resistant (landrace cultivar) x Kablanketi (susceptible)] F_2 population were constituted using 10 plants of each pool to make two samples. Then, the individual DNA samples were made for both susceptible and resistant plants. Those DNA samples were used to detect the linked markers to the ALS resistance. DNA from each bulk was used to confirm the polymorphism for potential markers. Thirty microsatellites markers were screened in for polymorphism among parents and the F_2 populations.

4.3.8 Data collection and analysis

Data obtained on disease score were processed and analysed by the 14th Edition GenStat statistical package. The chi-square test was used to test the phenotypic segregation of the populations from the crosses between Kablanketi and the respective parents for the inheritance study. The heritability calculated from the variances from the distribution of the score data for ALS disease.

4.4 Results and Discussion

4.4.1 Inheritance of ALS resistance

Segregation analysis of the F_2 generation for the crosses between Kablanketi and Beti 10, Nanka, Nkanamna and Nanavala landraces, showed a ratio of three resistant plants to one susceptible (3:1). For all cases, the Chi-square test was not significant which indicated the occurrence of monogenic inheritance where the dominant allele is responsible for the resistance (Table 4.1).

Improvement of ALS resistance have shown that in F_2 and backcross generations to Beti 10, Nanka, Nkanamna and Nanavala have managed to introgress ALS resistance in the adapted genotype in single crosses as a well as in backcrosses. Each of them demonstrated its specific and effective way of introgression of ALS resistance genes to the adapted bean genotype (Kablanketi). They, however, differed in the number of plants produced and to both resistant and susceptible ones. The success in deploying ALS resistance is likely due to the number of genes controlling the disease.

Table 4. 1: Segregation for ALS resistance of F_2 populations derived from Kablanketi x resistant parents

Crosses	No. of plants assessed	Expected ratio	Observed ratio	χ^2	*P*
Kablanketi x Nanka	135	3:1	98:37	0.417	0.518
Kablanketi x Beti 10	120	3:1	87:33	0.400	0.527
Kablanketi x Nanavala	140	3:1	102:38	0.343	0.558
Kablanketi x Nkanamna	138	3:1	105:33	0.087	0.768

These parents used are Mesoamerican type of common bean. The F_2 populations segregated in 3R: IS ratio showing monogenic inheritance, with resistance being due to one dominant allele. Similar results have been observed from other Mesoamerican resistance sources like Mexico 54 (Sartorato *et al.*, 1999; Mahuku *et al.*, 2004; Tryphone *et al.*, 2012; cultivars AND 277 (Carvalho *et al.*,1998) and that resistance of MAR 2 to ALS was due to one dominant gene (Ferreira, 1998). The identification of these genes is extremely important to bean breeding programmes aiming at developing cultivar resistant to this pathogen.

However, inheritance of the common bean resistance to angular leaf spot has shown to be complex in some situations. The genetic control of reaction in some lines was observed to vary according to the susceptible parent used. A single gene with dominant allele was observed for the resistance to pathotype 63-19 when the line Mexico 54 was crossed with the Ruda cultivar (Mesoamerican) (Sartorato *et al.*, 1999). Otherwise, Mahuku *et al.* (2002) described the resistance of line Mexico 54 to pathotype 31-55 as a single gene with the resistance due the recessive allele, when crossed with a snap bean cultivar. Another fact that stands out is the continuous response to phenotypic recurrent selection for resistance to the angular leaf spot (Amaro *et al.*, 2007). In the genetic control of resistance to the

angular leaf spot minor genes could be involved that were environmentally influenced, in addition to major genes or modifier genes. Therefore, both parents involved in this study were of Mesoamerican gene pools which provided high compatibility effect and thus the results obtained.

4.4.2 Heritability studies for ALS resistance

Heritability in narrow sense was estimated for the crosses and the results were as shown in Table 4.2. The heritability in narrow sense ranged from 0.46 for a cross between Kablanketi x Nkanamna to 0.73 for Kablanketi x Nanka. Estimating narrow sense heritability among crosses made with Kablanketi and Beti 10, Nanka, Nkanamna and Nanavala cutivars are moderate to high with regard to ALS resistance implying that these donor parents are suitable parents to be used for improvement of ALS resistance breeding programmes compared to the other parents. In a study conducted by Borel *et al.* (2011) heritability in narrow sense estimates ranged from 0.19 to 0.68 in common bean. The heritability is not immutable, it depends on the population and environmental conditions in which individuals were grown (Bernardo, 2002). The degree to which the variability of a quantitative character may be transmitted to the progeny is referred to as heritability. Heritability is among the most important genetic parameters in plant breeding (Allard, 1960). Fehr, (1980) categorized heritability estimates as low or weak (0-0.2), moderate or medium (0.21-0.39) and high or strong (0.4-1.0). However, low heritability estimates suggest that selection in early generation would not be effective since no improvement would result for the trait, this could be caused by environmental effects (Amaro *et al.*, 2006).

Table 4. 2: Heritability in narrow sense estimated for the crosses between Kablanketi x 4 landraces

Population	Organ Assessed	Disease score (Range)	Estimated Narrow sense Heritability (h^2)
Kablanketi x Nanka	Leaves	1-4	0.73
Kablanketi x Beti 10	Leaves	1-8	0.68
Kablanketi x Nanavala	Leaves	1-6	0.53
Kablanketi x Nkanamuna	Leaves	1-8	0.46

High heritability estimates indicate that the additive gene effects play an important role for that trait. It implies that this trait or character was not largely influenced by environment. Traits with relatively high heritability or additive gene variance have been reported to respond highly to selection and cross breeding (Falconer and Mackay, 1996).

The potential of a cross in common bean can be predicted on the basis of the performance of parents or that of the progeny of early generation (Tryphone *et al.*, 2012). Thus, estimating heritability is important because it enables the breeder to base selection on the phenotypic performance for improving a particular trait.

4.4.3 Angular leaf spot resistance and molecular markers

Among the four resistant landraces viz. Nanka, Beti-10, Nanavala and Nkanamna, Beti-10 was selected for molecular screening for ALS resistance. The choice for Beti-10 was based on the compatibility and production of crosses compared to other landraces.

Out of 30 SSR primer pairs, one SSR was polymorphic in Beti-10 and Kablanketi (Plate 5.1). Co-segregation analysis of the polymorphic marker and disease reaction in the F_2 population derived from Kablanketi x Beti-10 confirmed that SSR marker was associated

with resistance to ALS in landrace Beti-10 and F2 individuals. There has a consistence of phenotypic evaluation and marker based on Pv-ag004.

Simple sequence repeat (SSR) marker (Pv-ag004) segregate with resistant gene in common bean. It is polymorphic in parents with 270bp for resistant parent and 240bp for susceptible parent (Plate 4.1). The SSR Pv-ag004 segregates with resistant individuals. From previous studies by Mahuku *et al.* (2009), the microsatellite marker Pv-ag004 segregated with resistant gene Phs_{G5686A}, linked with ALS resistance on linkage group B04 of the consensus molecular linkage map of common bean (Mahuku *et al.*, 2009). This marker is co-dominant and polymorphic to both resistant and susceptible parent. Pv-ag004 marker amplified and it happened to be polymorphic between Beti-10 and Kablanketi, this can be explained that the Beti-10 carry the gene Phg_{G5686} which is resistance to ALS and the cultivar Beti-10 identified in this study carrying this gene for resistant. Therefore, Beti-10 is identified as the potential donor of resistance to ALS and this is explained by 68% heritability (Table 4.2).

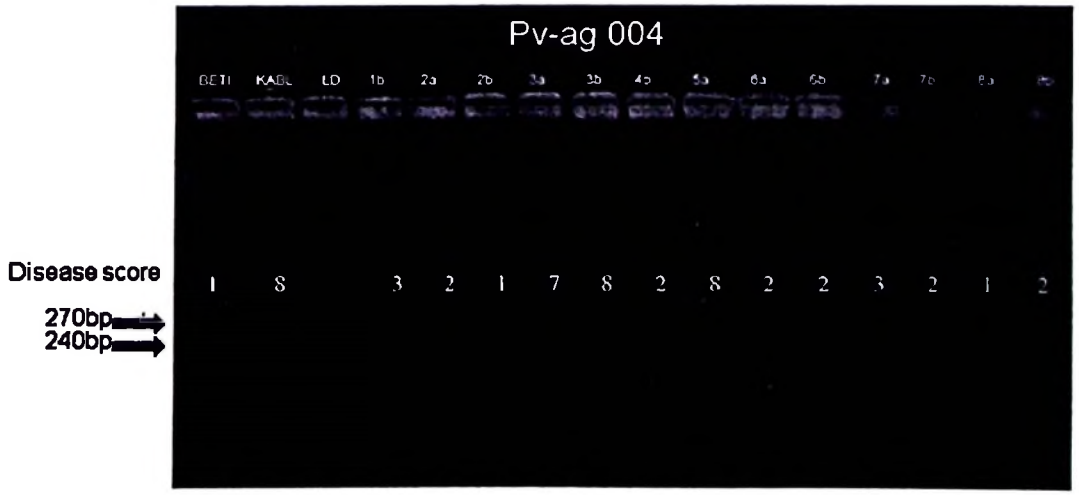

Plate 4. 1: Amplified products for two parents and F_2 individuals with Pv-ag004 SSR marker. Lane 1, resistant parent (Beti-10), Lane 2, susceptible parent (Kablanketi) Lane 100bp ladder and Lane 4-16 are the segregating F_2 individuals

However, it is not apparent whether these genes are the same or different from those presents in Mexico 54 or MAR 2. It has been observed that Beti-10 was resistant to many isolates of *P. griseola* that were to cause disease on other genotypes (Fivawo and Msolla, 2011), signifying that the resistance genes in the two cultivars are distinct. Nevertheless, mapping of marker linked to the resistant parent will provide a better understanding of the relationship between of ALS resistance from these sources. This information will allow breeder to make an informed selection of resistant parent to use in their programmes and to avoid over deployment of a single locus from different sources for resistance genes. Also, to avoid the loss of these genes during selection, it is essential to identify molecular markers tightly linked to the genes of interest that will permit the identification and detection of these genes and permit their use in marker assisted selection (Kelly, 1995). In absence of allelism tests, locating the markers on the linkage map would help to determine gene independence and their relationship to genes from other sources of resistance (Miklas *et al.*, 2006). Such information will help breeder to make informed selection of resistance genes to use in their breeding programmes.

Integrating molecular markers in plant breeding has the potential to increase the efficiency of crop breeding above that reached by classical breeding methods alone. The efficiency of MAS is a function of the distance between the gene of interest and the markers (Mahuku *et al.*, 2009). For a marker to be useful in molecular marker assisted breeding, it must be located within 5 cM of the resistance gene but ideally, a marker that is <1 cM is the most useful (Kelly and Miklas, 1999). The SSR Pv-ag004 linked to PhgG5686 has the greatest potential for molecular marker assisted breeding as it is mapped 0.0cM from the resistance gene (Mahuku *et al.*, 2009).

4.5 Conclusion

The inheritance of angular leaf spot disease resistance in common bean landraces, Beti-10, Nanka, Nanavala and Nkanamna were established. The results indicate that one single dominant gene controls the resistance in the studied landraces. Also, the heritability for the angular leaf spot trait in the landraces was high indicating the additive genetic effect for that trait. SSR marker Pv-ag004 was found to be linked to resistance in Beti-10. This landrace can be used as resistant parent in pyramiding the ALS resistant genes in the farmers preferred varieties. This calls for detailed analysis of the mapping of all the identified resistant parents for resistance with many and robust markers. Furthermore, this may come-up with more new markers because few molecular markers have been developed for a few ALS resistance genes. At the same time, to come up with the clear picture of the genes present, whether they are similar or distinct to already existing ones, allelism test is important for resistant parents. Also, there should be a way of comparing the identified landrace resistant parents with Mexico 54 because it is known to be resistant to all African isolates of ALS.

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CHAPTER FIVE

General Conclusion and Recommendations

5.1 General Conclusion

This investigation attempted to provide information on the integration of molecular markers in developing new varieties, overlaps of the molecular as well as phenotypic selection, inheritance of the pathogen as well verifying the molecular marker results from laboratory to the field. Therefore, the study has been able to incorporate multiple resistances into farmers preferred variety. Phenotyping has resulted in selecting 31 bean lines resistant to diseases. Furthermore, the characterization of landraces for angular leaf spot disease in Tanzania and their usefulness as sources of resistance. . Inheritance of donor parents has confirmed and established for local cultivars (Nanavala, Beti-10, Nkamumna and Nanka). The Pv-ag004 SSR marker was identified to be linked to resistance in local cultivar Beti-10. Hence, Beti-10 was identified as potential source of resistance source to ALS. The molecular markers were found to be useful in developing resistant genotypes and selecting the resistant genotypes in early generation. This calls for the use of molecular markers to speed up the breeding processes especially MAS to bred for resistance and improve yields of common bean in Tanzania. Results for markers were reflected in the field performance of the selected genotypes. Furthermore, the inheritance for the studied pathogens was documented across the work.

5.2 Recommendations

(i) It is recommended that for the purpose of variety development, classical breeding coupled with molecular marker screening should be considered. This will serve time and money that would be spent in the series of

phenotypic screening both in the screen house and in the field for each pathogen.

(ii) Application of molecular markers for MAS should be validated for intended population and the selected markers should be as close as possible for the gene of interest.

(iii) The genotypes with resistance to all diseases are recommended for evaluation in more seasons and locations for release as varieties with multiple disease resistances.

(iv) The identified genotypes displayed high yields in the presence of the diseases with different seed quality, several backcrosses must be considered for some of the genotypes to attain appropriate/preferred seed type.

(v) It is recommended that more studies be conducted to establish the mechanisms of resistance in Tanzanian landraces by screening more populations with more markers for ALS.

(vi) There is a need to sequence the allele identified in Beti-10 linked to PV-ag004

(vii) Establish the long term application of resistant genotypes and durability in reducing yield losses.

PUBLISHED PAPERS (SUMMARY)

Paper 1: Inheritance of angular leaf spot (Phaeoisariopsis griseola (Sacc.) Ferr) resistance in common bean (Phaseolus vulgaris L.) population. Paper published in Journal of Agricultural Science and Technology A 2(7): 856 – 862

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Abstract

The genetic resistance to angular leaf spot (ALS) caused by *Phaeoisariopsis griseola* in the common bean cultivar Mexico 54 was investigated on disease reactions in parental, F_1, F_2 and backcross generations derived from crosses between a resistant cultivar Mexico 54 and a susceptible cultivar Kablanketi under screen house conditions. The heritability (h^2) estimate was as high as 0.719 indicating a successful transfer of ALS resistance among progenies and thus selection can be performed in early generation. High heritability coupled with high expected genetic advance of 39.5% is considered to be more useful in predicting the outcome of selecting the best individuals. Chi-square values were computed to determine whether the observed ratios for disease reactions deviated from expected Mendelian ratios for a single, dominant gene controlling resistance to angular leaf spot in common bean. Based on the resistance of the F_2, and the backcross generation to the

resistant parent, a 3 resistant: 1 susceptible segregation ratio in the F_2 and a 1 resistant: 1 susceptible segregation ratio in the backcross generation to the susceptible parent was obtained implying that resistance to the isolate of *Phaeoisariopsis griseola* is governed by a single, dominant gene.

Key words: *Phaeoisariopsis griseola*, heritability, genetic advance, inheritance, common bean.

Paper 2: Introgression of common bacterial blight (Xanthomonas axonopodis pv .phaseoli) resistance to common bean (Phaseolus vulgaris L.) adapted toTanzania facilitated by marker assisted selection. Paper published in International Journal of Agricultural Science 2(10): 285 - 290.

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Abstract

Common bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv *phaseoli (Xap)* is an important disease of common bean in Tanzania causing severe damage. This study was carried out to introgress resistance to CBB to the adapted common bean in Tanzania with the facilitation of molecular markers along with determining the inheritance and heritability of the disease. Crosses were made between the adapted parent Kablanketi and the resistant parent Vax 4 and their F_1, F_2 and the backcrosses to both parents generated. The phenotypic evaluation was carried out after inoculation with *Xap* and the molecular marker was applied on the F_2 generations using the SCAR marker SAP 6 linked to a QTL for CBB resistance. The result shows no significant deviation from the expected 3:1 (χ^2 = 0.47; P>0.05) in the F_2 population and 1:1 for the backcross to the susceptible parent. These results that resistance in Vax 4 to *Xap* is conditioned by the presence of dominant genes. The moderate heritability of 0.32 was estimated implying that resistance is

conditioned by one major gene which has effects of partial resistance. There were significant correlation between the phenotypic reaction and molecular marker screening (resistant QTL) ($r = 0.502$: $p<0.05$). This indicates there are greater chances of selecting resistant individuals using molecular markers which also exhibited resistance under field conditions.

Key words: Common bacterial blight, marker assisted selection, *Xanthomonas axonopodis* pv *phaseoli*, Tanzania.

Paper 3: Marker Assisted Selection for Common Bean Diseases Improvements in Tanzania: Prospects and Future Needs. Chapter five published in a Book: Plant Breeding From Laboratory to Fields (Edited by Sven Bode Andersen), Intech Publisher, Croatia. pp. 121 – 147. (http://dx.doi.org/10.5772/52823)

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Summary

In Tanzania yield losses of common bean are due to a great number of diseases affecting the crop. Angular leaf spot, common bacterial blight, bean common mosaic virus/bean common mosaic necrosis virus, root rot as well as anthracnose are diseases which are prominent in Tanzania occurring across all bean production ecologies. They can cause yield loss up to 100% depending on the environment and the cultivars used. The low bean yields among others are due to a lack of effective diseases management practices including the lack of disease resistant cultivars and when such cultivars are available, they are not integrated in the disease management packages. The development of cultivars with improved resistance to biotic and abiotic stresses has long been a primary goal for many bean breeding programs. It is considered that the use of resistant cultivars is an efficient, safe and inexpensive technique accessible by resource poor farmers. There is thus a need to breed for high resistance levels and one option is the introgression of multiple resistance genes in adapted cultivars grown locally or into one line. The application of DNA molecular markers assist in plant breeding, efficiency and precision for variety

development could be greatly increased. The use of molecular markers in plant breeding is called marker-assisted selection (MAS) and is a complement of the new discipline of molecular breeding. MAS is the novel approach in which individuals for intercrossing are selected using selection index based on genotypic data controlled by few or several genes. The gain from selection using such index is expected to be higher than phenotypic selection used in conventional recurrent methods. MAS offer significant advantages in cases where phenotypic screening is expensive, difficult or impossible or traits are of low heritability. This chapter focuses on the importance of MAS and how it can be integrated into breeding programs for enhancing selection efficiency in developing disease resistant bean varieties in Tanzania with emphasis of molecular markers available for such bean diseases; what has been done by use of MAS and their implication in plant breeding.

APPENDICES

Appendix 2. 1: Leaf sample preparation and deoxyribonucleic acid (DNA) extraction using FTA saver card protocol

i) A 2 mm leaf disc was excised using a Harris unicore borer;

ii) The disc was washed two times using FTA purification reagent (100 µl);

iii) The material was incubated for 3 min at room temperature;

iv) The disc was also washed again two times by using isopropanol (100 µl);

v) The material was incubated for 3 min at room temperature;

vi) The disc was dried in PCR tube for 5 min.;

vii) The DNA remains bound to the matrix throughout purification process, thus the matrix provides enough templates for PCR analysis thus sample discs were thus ready for addition of PCR master mix.

Appendix 2. 2: PCR conditions for the primers used

Primer	Conditions
ROC11	34 cycles of 10s at 94°C, 40s at 58°C and 120s at 72°C; followed by one cycle of 10 min at 72°C
SW13	34 cycles of 10s at 94°C, 40s at 67°C and 120s at 72°C; followed by one cycle of 5min at 72°C
SNO2	30 cycles of 30s at 94°C, 60s at 65°C and 90s at 72°C
SAP6	34 cycles of 10s at 94°C, 40s at 55°C and 120s at 72°C; followed by one cycle of 5min at 72°C

Source: Miklas (2009)

Appendix 4. 1: Mean severity of the angular leaf spot in five common bean cultivars used as parents

s/no.	Parents (Landraces)	Scores (CIAT scale 1-9)
1	Nanka	1
2	Beti-10	1
3	Nanavala	1
4	Nkanamna	2
5	Kablanketi	8

Appendix 4. 2: Amplified products for five parents of common bean landraces with some SSR markers showing monomorphic as observed on 3% Agarose gels