Genome Wide Marker Trait Association Study, Molecular Characterization and Pathogenic Variability Among *Pseudocercospora griseola* (Sacc.) Crous & Braun, Isolates the Causal Agent of Angular Leaf Spot Disease of Common Bean in Ethiopia

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A Dissertation

Submitted in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy (PhD) in Biology (Applied Genetics) to the Department of Cellular Microbial and Molecular Biology Graduate School,

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DECLARATION

I declare that the thesis hereby submitted by me for the degree of Doctor of Philosophy (PhD) in biology (Applied Genetics) to the School of Graduate Studies of Addis Ababa University is my own independent work and has not previously been submitted by me or anybody else at another university. The materials obtained from other sources have been duly acknowledged in the thesis.

PhD Candidate ______________________

Yayis Rezene

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LIST OF PUBLICATIONS

This dissertation is based on the work contained in the following research papers which were submitted for publications and currently under review,

I. Simple and Rapid Detached Leaf Technique for Screening Common Beans (*Phaseolus Vulgarise* L.) *In vitro* Against Angular Leaf Spot (*Pseudocercospora griseola*) Disease

Yayis Rezene[1,3], Kassahun Tesfaye[2,3], Clare Mukankusi & Paul Gepts[4]

II. Pathotype Characterization and Virulence Diversity of *Pseudocercospora griseola* the Causal Agent of Angular Leaf Spot Collected from Major Common Bean (*Phaseolus vulgaries* L.) Growing Areas of Ethiopia

Yayis Rezene[1,3], Kassahun Tesfaye[2,3], Clare Mukankisu & Paul Gepts[4]

III. Genetic Diversity and Population Structure Among *Pseudocercospora griseola* the Causal Agent for Angular Leaf Spot Pathogen of Common Beans (*Phaseolus vulgaries* L.) Collected from Ethiopia as Revealed by REP-PCR Genomic Fingerprinting

Yayis Rezene[1,3], Kessahun Tesfaye[2,3], Allan Male[4], Mukankusi Clare[4], and Paul Gepts[5]

IV. Genome-Wide Marker Trait Association Study of Angular Leaf (*Pseudocercospora griseola*) Resistance in Common Bean (*Phaseolus Vulgarise* L.)

Yayis Rezene[1], Kessahun Tesfaye[2], Karen Cichy[3], Mukankusi Clare[4], and Paul Gepts[5]

V. Marker Assisted Pyramiding Resistance Genes Against Angular Leaf Spot (*Pseudocercospora griseola*) and Common Bacterial Blight (*Xanthomonas campestris pv phaseoli X. campestris pv. phaseoli var. fuscans*) in to" REDWOLAITA" Popular Common Bean cultivar

Yayis Rezene[1], Kessahun Tesfaye[2], Bodo Raatz[3], Mukankusi Clare[4], and Paul Gepts[5]

DISSERTATION OUTLINE

Chapter 1 is the general introduction referring the importance of common bean production and its major role in the lives of small-scale farmer as nutritional, food security and country's economy whereas, *Pseudocercospora griseola* the most economically important disease of this important crop, includes the general and specific objectives of this study.

Chapter 2 is literature review part where many scientific reports of related work were reviewed to support the study.

Chapter 3 is the research report on the pathotype characterization and virulence diversity of *Pseudocercospora griseola* the causal agent of angular leaf spot from major common bean (*Phaseolus vulgaries* L.) growing in various localities of Ethiopia. Hence, this chapter determines the pathotype and its diversity and its distribution across bean growing areas Ethiopia.

Chapter 4 is the about the genetic diversity and population structure among *Pseudocercospora griseola* the causal agent for angular leaf spot pathogen of common beans (*Phaseolus vulgaries* L.) collected from Ethiopia as revealed by REP-PCR Genomic fingerprinting. Here Rep-PCR finger printing were used to determine the genomic diversity and estimate the population structure of *P. griseola* collected from diverse common bean areas of Ethiopia.

Chapter 5 is about the genome wide marker trait association study of angular leaf spot resistance in common bean (*Phaseolus vulgarise* L.). Common bean genotypes including 288 genotypes were phenotyped under the naturally endemic ALS pathogen. After filtering 3,332SNPs genotypic data were used for this specific study. This chapter reports a total of 18 significant marker-trait associations, distributed on chromosomes Pv04 and Pv08. The locus on chromosome Pv04 was the most saturated locus with 11 SNP markers followed by chromosome Pv08 with 7 significant SNPs markers.

Chapter 6 is a marker assisted pyramiding resistance genes against angular leaf spot (*P. griseola*) and common bacterial blight (*Xanthomonas campestris* pv *phaseoli X. campestris pv. phaseoli var. fuscans*) in to ''REDWOLAITA'' common bean cultivar. Monogenic Near Isogenic Lines (MNILs) with R genes linked to SAP6, g796 & SU91 molecular markers $MNIL^{SAP6}$, $MNIL^{SU91}$ & $MNIL^{g796}$, and polygene PNILs with different gene combination includes polygene $PNILs^{SAP6/g796}$, $PNILs^{SU91/g796}$, $PNILs^{SAP6/SU}$, $PNILs^{SAP6/g796/SAP6}$, were developed with 97% genome recovery from the RW genetic background.

Chapter 7 covers summary and conclusions of the results of the study and its implication for future resistance Breeding.

ACRONYMS AND ABBREVIATIONS

ABC	= African Bean Consortium
AFLP	= Amplified Fragment Length Polymorphism
ALS	= Angular Leaf Spot
ANT	= Anthracnose
AMOVA	=Analysis of molecular variance
ANOVA	= Analysis of variance
BC	= Backcross
CBB	= Common Bacterial Blight
CIAT	= International centre for Tropical Agriculture
CMLM	= Compressed Mixed Linear Model
CV	= Coefficient of variance
CSA	= Central Statistics Agency
GAPIT	= Genome Association and Prediction Tool
GWAS	=Genome Wide Association Study
ERIC	= Enterobacterial repetitive intergenic census
FAO	= Food and Agriculture Organization
MABC	= Marker Assisted Backcrossing
MAGP	= Marker Assisted Gene Pyramiding
MSE	= Mean square error
MNILs	=Monogenic Near Isogenic Lines
PNILs	=Polygenic Near Isogenic Lines
PCR	= Polymerase chain reaction
RAMS	= Randomly Amplified
REPs	= Repetitive Extragenic Palindromes
RQTL	= Resistance Quantitative Trait locus
SMV	= soybean Mosaic Virus
SNP	= Single Nucleotide polymorphism
QRL	= Quantitative Resistance Locus
LD	= Linkage Disqulibriem

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Genome Wide Marker Trait Association Study, Molecular Characterization and Pathogenic Variability Among *Pseudocercospora griseola* (Sacc.) Crous & Braun, Isolates the Causal Agent of Angular Leaf Spot Disease of common Bean n Ethiopia

Abstract

By

Yayis Rezene (Addis Ababa University, 2018)

Common bean (*Phaseolus vulgarise* L.) is the most important pulse crop grown for its nutritional, food security and for its economic value across all the regional states of Ethiopia. *Pseudocercospora griseola* the causal agent for the angular leaf spot of the common bean is one of the major disease affecting the productivity as much as by 50-80%. The development and use of resistance cultivars are the most effective, economic and environmentally sound strategy of disease control. Knowledge of pathotype variation and its population structure among the isolates of *P. griseola* is important to any common bean improvement program to guide the deployment of resistance genes. However, there is no information regarding *P. griseola* pathotype variability of the Ethiopian *P. griseola* isolates, which puts the common bean improvement program under challenging situation to develop resistance varieties with wider adaptation. Therefore, this study was aimed at (i) to determine the pathotype and virulence variability and its geographic distribution (ii) to determine the genetic variability and population structure of *P. griseola* with rep-PCR genomic finger printing (iii) studying genome wide marker trait association and determine genomic loci significantly associated with the angular leaf spot resistance (iv) to develop multiple resistance common bean cultivar resistance to angular leaf spot and common bacterial disease through Marker-assisted gene pyramiding breeding techniques. To achieve these objectives both *P. griseola* pathotype and molecular characterization were conducted. The study included a single spore isolates of *P. griseola* of 39 and 78 for pathotypes determination and rep-PCR genomic finger printing study respectively. The single spores were obtained from the infected leaves with lesions of the angular leaf spot of the common bean collected from the diverse common bean growing areas of Ethiopia. From the study a total of twenty-one pathotypes of *P. griseola* (63:63, 63:59, 63:23, 61:51, 56:36, 55:39, 49:7, 48:60, 42:59, 41:10, 34:53, 23:61, 19:33, 17:45, 8:18, 8:0, 4:16, 1:24, 1:10, 16:18. and 4:37) were determined among 39 isolates using the reaction with sets of 12 common bean differentials set. The result revealed the existence of high and diverse pathogenic variability of *P. griseola* in common bean growing areas of Ethiopia. Among the identified pathotypes 63:59 and 19:33 were found to be the most frequently appeared. The study also identified the most pathogenic pathotype (63:63) which was compatible and overcomes the resistance genes in all differential bean genotypes. The molecular characterization the gDNA was extracted from each of the 79 monosporic isolates of *P. griseola* and the diversity were studied with sets of rep-PCR families (BOX-, ERIC- & REP-PCR), genomic finger printing patterns. Thus, rep-PCR fingerprinting grouped the 79 single spore isolates into 25 distinct clusters. These

clusters showed the existence of high genetic differentiation within and among the isolates of *P. griseola* collected from the diverse common bean growing regions of Ethiopia. The genetic structure of *P. griseola* population from Ethiopia showed no evidence of geographic differentiation. Genome wide marker trait associations was conducted with a set of 288 diverse common bean genotypes collected all over the world using 3,332 SNP markers. The genome wide association analysis was performed with the most powerful compressed mixed linear model (CMLM) including Kinship matrix genome association and prediction integrated tool (GAPIT) to dissect the complex architecture of quantitatively inherited common bean angular leaf spot disease resistance and to provide effective molecular marker that could be used in the common bean improvement program. This study determined a total of 18 significant marker-trait associations, which were distributed on chromosomes Pv04 and Pv08. The locus on chromosome Pv04 was the most saturated locus with 11 significantly associated SNP markers, followed by chromosome Pv08 with 7 significant SNPs. This genomic region was previously reported to have B4 R gene cluster that encode Nucleotide Binding Site Leucine Rich Repeats (NBS-LRR) proteins. This large family is encoded by hundreds of genes and were known to respond pathogen attach in plants. Hence, this genomic region will be validated and used for the future marker assisted breeding program. Marker-assisted pyramiding multiple resistance genes in to the back ground of popular 'REDWOLAITA'(RW) cultivar was performed using Marker Assisted Parallel Back Crossing (MAPBC) breeding schemas. Three molecular markers namely g796, SU91 and SAP6 linked to *Phg-2* R gene and 2 R QTLs were used to track independently from multiple donor parents with known sources of resistance. The donor parents include VAX 6 for two independent RQTLs linked to SAP6 and SU91 genetic markers and Mex 54 for *Phg-2* R gene and linked to g796 genetic marker till BC4 generation. After BC4 progenies that had known markers of SU91 were inter-crossed with progenies with g796 and progenies that combines both SU91 and g976 were then selected for further crosses to combine with SAP6 markers so that this made possibility of selecting progenies with all desirable traits having all combined R genes and major QTLs. Monogenic Near Isogenic Lines (MNILs) with R genes tagged by the SAP6, g796, and SU91 molecular markers and polygenic PNILs with different gene combination includes MNIL^{SAP6}, MNIL^{SU91} & MNIL^{g796}, polygenetic PNILs $^{SAP6/g796}$, PNILs $^{SU91/g796}$, PNILs $^{SAP6/SU}$, PNILs $^{SAP6/g796/SAP6}$, with more than 97% genome recovered from the RW genetic background were developed. The developed lines showed high level of disease resistance under the screening conditions and with comparable 100 seed weight(gm) and seed colour. Hence, developed MNILs and PNILs will be used as a potential source of resistance and parental lines for the future gene pyramiding activities. Pyramided common bean lines with different gene combination should be multiplied and tested under multiple environment before varietal release and wider production.

Key words: *Pseudocercospora griseola,* Pathotype variability, rep-PCR fingerprinting, marker trait association, Marker-assisted gene pyramiding, common bean

CHAPTER ONE

GENERAL INTRODUCTION

1.2. Background and Justifications

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume for human consumption in East, Central and Southern Africa. It occupies more than 4 million hectares of land with annual production of 4.3 tons in Africa providing food for more than 100 million people (Wortmann *et al.,* 1998; FAOSAT, 2013). Ethiopia is the fourth largest common bean producer in Eastern Africa, next to Tanzania, Kenya and Uganda (FAOSAT, 2013). Among the most important legumes grown in Ethiopia, common bean ranks second, next to faba bean, both in area coverage and production (CSA, 2015). The major production areas are Oromia with average hectarage of (152,151.74 ha), Southern Nations, Nationalities, and Peoples' (SNNPR) (96,200.12 ha), and Amhara (65,918 ha) (CSA, 2015). It is the second most important source of dietary protein after animal proteins and the third most important source of calories for lower income African households after cassava and maize (Broughton *et al*., 2003). Although common beans are playing an important role in stabilizing the farming system, livelihoods of many people, and the national economy at large, the productivity of the crop in Ethiopia is very low. Under optimal management conditions, common bean production can reach to 2.5 to 3.0 ton per hectare in Ethiopia (CSA, 2015). However, common bean production under farmers' conditions is estimated between the ranges of 0.6 to 0.7 ton per hectare. This is mainly attributed to a number of abiotic and biotic stresses. The situations become even worse considering with effects of climate change, such as increasing temperatures and changes in precipitation in some parts of Ethiopia. Low soil fertility (mainly nitrogen and phosphorus), drought, low soil pH conditions (especially in western and south-western parts of the country) are the major abiotic

stresses in Ethiopia (Wortmann *et al*., 1998; Rubyogo, 2011). Among the biotic stresses, diseases and insect pests play a significant role in bean production system of the country. The most important and widely distributed common bean fungal and bacterial diseases include rust (*Uromyces appendiculatus*), angular leaf spot (*Pseudocercospora griseola*, previously *Phaeoisariopsis griseola*), anthracnose (*Colletotrichum lindemuthianum*), common bacterial blight (*Xanthomonas campestris* pv *phaseoli*) and halo blight (*Pseudomonas syringae* pv *phaseolicola*) (Fininsa, 1996; Yesuf and Sangchote, 2005; Tadesse *et al.,* 2006; Tefera 2006).

1.3. Statement of the problem

Among the biotic stress angular leaf spot (ALS) caused by the imperfect fungus *Pseudocercospora griseola* is one of the most damaging and widely distributed diseases of common bean, causing yield losses as high as 80% (Liebenberg and Pretorius, 1997; Lemessa *et al*., 2011). Breeding for resistance is the most practical and economic approach to manage ALS under farmers' condition in Africa. However, the process of designing an effective ALS breeding program requires precise and accurate knowledge on population dynamics and spatial and temporal distribution of *P. griseola* (Stenglein *et al*., 2003). Most of the previous studies have indicated that many races of *P. griseola* occur and vary in time and space. A common bean cultivar, which is resistant in one location, season or year, may be susceptible in another location, season and year (Aggarwal *et al.*, 2004; Ddamulira *et al*.,2014). This could be explained by seasonal variation coupled with pathogen race differences and resistance breakage. In the Ethiopian bean improvement program, there is still limited information of pathogen distribution, variability, virulence and source of resistances, and that hinder breeding for ALS resistance. A study to understand the pathogen distribution, virulence and

variability of *P. griseola* as the first step in designing strategic breeding interventions to develop resistance common bean varieties against ALS in Ethiopia is highly required.

The QTL approach which is specific to the genetic population is not suitable to identify the tremendous phenotypic variation within the scope of the whole genome (Wang, 2014). The genome wide association study(GWAS) has emerged as a powerful approach for simultaneously screening genetic variation underlying complex phenotype. GWAS was applied for the first time in 2005 to a human disease (Edwards *et al*., 2005). Subsequently, a series of research results on GWAS have been published (Hindorff *et al*., 2009) however, GWAS applied to dissection of complex traits is just only beginning because of the lack of effective genotyping techniques and the limited resource for developing high density haplotype maps. For both QTL approaches and GWAS, genetic transformation is greatly required to identify the candidate genes. In plants association mapping, where unlike conventional QTL mapping, populations of un-structurally related individuals are employed, it is important to consider population structure and kinship among individuals, because false associations may be detected due to the confounding effects of population admixture (Oraguzie *et al*.,2007). By exploiting broader genetic diversity, GWAS offers several advantages over linkage mapping, such as mapping resolution, allele number, time saved in establishing marker-trait associations, and application in breeding programs.

Therefore, this study was executed to establish the pathogen distribution across different agro-ecological zones, characterize the virulence and molecular diversity of existing races and identify potential novel sources of resistance to the *P. griseola* population in Ethiopia. The research was carried out at the Southern Agricultural Research Institute (SARI), Hawassa, Ethiopia, and was part of the African Bean Consortium (ABC) project, which was aimed at the identification of the extent of

pathogenic and genetic variability and geographical distribution in Ethiopia and identification of new sources of bean disease resistance and pyramiding genes conferring resistance against angular leaf spot and common bacterial blight resistance in to the popular common bean cultivar, The results will contribute to an increase of the production of this important crop under farmer condition in Ethiopia and will have a significant benefit and implication for regional and national common bean improvement programs and the ABC region.

1.4. Objectives

1.4.1. General Objective:

The main goal of this research was to determine pathotype and genetic diversity of *Pseudocercospora griseola* isolates obtained from Ethiopia and study the genome wide and marker trait associations, identification of the genomic region linked to angular leaf spot disease resistance trait and to pyramid angular leaf spot and common bacterial blight resistance genes in to the background of 'REDWOLAITA' cultivar.

1.4.2. Specific objectives:

This research was designed with the following specific objectives

- To determine the pathogen diversity and virulence variability in *P. griseola*
- To reveal the geographic distribution and the population structure of *P. griseola*;
- To study marker trait association and determine loci significantly associated with the angular leaf spot resistance in common bean (*Phaseolus vulgaries* L.) genotypes
- To develop durable resistance common bean cultivar resistance to angular leaf spot and common bacterial disease through Marker assisted pyramiding breeding techniques

CHAPTER TWO

REVIEW OF LITERATURE

2.1. Taxonomy of common bean

Common bean (*Phaseolus vulgaris L.*) belongs to the genus *Phaseolus*, family *Leguminosae*, sub-family *Papilionoideae* and order *Leguminales*. The crop is widely distributed throughout America, Caribbean, Asia and Africa with over 50 wild growing species. Of the 50-wild species only five are domesticated and include; common bean (*P. vulgaris* L.), runner bean (*P. coccineous L.*), Lima bean (*P. lunatus*), Tepary bean (*P. acutifolius A.* Gray) and the year bean (*P. polyanthus* Greenman) (Debouck, 2000; Gepts and Debouck.2001). Among the five-domesticated species the most adapted and globally cultivated bean type belong to *Phaseolus spp* (Singh, 2001; Gepts and Debouck, 2001). Most of the *Phaseolus spp* including common bean are diploid with 22 chromosomes (2n=2x=22), though a few cases of aneuploid reduction to 20 chromosomes have been reported in some species (Gepts, 2001). Botanically, the common bean varies in growth habit that ranges from determinant upright bush (Type 1), indeterminant upright bush (Type II), indeterminate prostrate non-climbing or semi climbing (Type III) and indeterminate strong climbers (Type IV) (Sing, 1982). The leaves are trifoliate, developing from terminal buds but the first two true leaves are unifoliate (Bailey, 1969). Common bean bears complete papilionaceous flowers with colors ranging from white to pink and purple and the crop is self-pollinating. The flower structure facilitates self-pollination in that; it carries 10 stamens, with a long ovary, coiled style complimented with a hairy stigma. The stigma is laterally positioned in the inner arc of curved style where it intercepts pollen from its own anthers. Although the crop is less than one percent out-crossing, the crop exhibit considerable variation in

growth habit, vegetative growth, flower color and shape as well as pod and seed color (Purseglove, 1968).

2.2. Origin, Domestication Genetic Resources of common bean

Common bean is believed to have originated from Mexico where it expanded to South America (Bellucci et al., 2014; Gepts and Debouck.2001). It was through domestication that beans reached to other areas such as Peru, Ecuador and Bolivia which are currently considered as secondary centers of diversity (Gepts and Debouck.2001). The crop was introduced into Africa by Portuguese traders in the 16th century and with time it was successfully established in the Great Lakes Region (Trutmann, 1996). The evolutionary history of beans indicate that domestication started in the 7th century, but, even before then two major gene pools of *P. vulgaris* (Mesoamerican and Andean) with partial reproductive isolation existed within distinctive centers of origin (CIAT,1995).

2.3. Common bean: cultivations and major disease in Ethiopia

Common bean (*Phaseolus vulgaris* L.) is one of easily available source of dietary protein and income-generating crop for the majority of the rural poor farmers (Broughton *et al*., 2003). Its production is agronomically diverse, being grown in many crop associations. Common beans are adapted to various climatic and agronomic conditions and exhibit considerable variation in growth habit. The wide geographic range, cropping practices, growers' preferences and sources of seeds are responsible for the wide range of bean types in the country (Asrat *et al*., 2013). In Ethiopia, common bean is grown primarily by small-scale farmers who have limited resources and usually produce the crop under adverse conditions such as low agricultural input use, marginal lands, without intensive cultural practices and intercropping with competitive crops like maize and sorghum (Asrat *et al*., 2013). Even though common beans are playing an important role in the farming system, livelihood of many people, and the national

economy at large, the productivity of the crop in Ethiopia remains very low (CSA, 2015). Under optimal management conditions, common bean production can reach to 2.5 to 3.0 ton per hectare in Ethiopia (CSA, 2015).

Various production constraints contribute to the low yield of common beans and include diseases (Habtu *et al*., 1996; Belete and Bastas, 2017); insects (Ferede and Tsedeke, 1986) drought (Asrat, 2014) and low soil fertility (Kidane, 1987). Diseases are known to be the major factors, which directly or indirectly affect the production of this crop in Ethiopia (Habtu *et al*., 1996; Belete and Bastas, 2017). The major diseases that are threatening common bean production in Ethiopia are angular leaf spot (*Pseudocercospora griseola*), anthracnose (*Colletotrichum lindemuthianum*), common bacterial blight (*Xanthomonas* sp.) (Belete and Bastas, 2017), rust (*Uromysis appendiculatus*), and ascochyta blight (*Ascochyta phaseolorum*). Among others, angular leaf spot, common bacterial blight, anthracnose and rust are more important and widely distributed while the remaining are also important but much more restricted in their distribution. Except bean rust, all the diseases mentioned are known to be seed borne in various degree of transmission (Habtu *et al*., 1996; Belete and Bastas, 2017).

2.4. Effect of ALS on yield and yield components of common beans

Angular leaf spot of common bean, which is caused by the imperfect fungus *Pseudocercospora griseola*, is one of the most damaging and widely distributed diseases of common bean, causing yield losses as high as 80% (Wortmann *et al.*, 1998; Liebenberg and Pretorius, 1997). In Africa, particularly in Ethiopia, Uganda, Tanzania, Kenya, Malawi and the Great Lakes Region, where beans constitute the most important source of dietary protein, ALS is a significant constraint to bean production with annual yield losses estimated at 374,800 tones (Wortmann *et al.*, 1998). Apart from Africa, it is generally of major importance in tropical and subtropical areas, causing yield losses

of up to 80 % (Saettler 1991; Liebenberg & Pretorius, 1997). The disease mainly affects pods and foliage, and is particularly destructive in warm, humid areas (Saettler, 1991). Pod symptoms consist of circular to elliptical red-brown lesions, while leaf lesions start as small, brown or grey spots that become angular and necrotic, being confined by leaf veins. Leaf spots eventually coalesce, causing premature defoliation (Correa-Victoria *et al.,* 1989, Saettler 1991). Furthermore, the disease also affects the quality and marketability of seed across bean-producing areas of the world and contributes to income reduction among common bean producing farmers (Pastor-Corrales *et al.* 1998).

2. 5. Biology of *Pseudocercospora griseola*

Studies indicate *P. griseola* can survive winters in infested bean residue left on the soil surface; however, the pathogen does not survive very long when infested bean debris is buried in soil and decomposes. The pathogen can also survive between seasons on infested seed, which is one pathway of introduction into fields (Saettler and Corea, 1988). Spores from lesions on stems, leaves and pods, as well as on crop debris, only develop after continuous high humidity or wet conditions for 24-48 hours. Spores produced on infested debris or seed are rain-splashed and/or wind-blown onto healthy tissue after planting. When spores land on susceptible bean tissue, they germinate and infect through stomata. Disease develops rapidly during periods of warm temperatures (24^{0}C) but can occur over a range of moderate to warm temperatures (16^{0}C-28^{0}C) when accompanied by wet weather or high humidity alternating with dry, windy conditions. ALS tends to develop and spread quickly during late summer on late seeded snap bean crops when day temperatures are warm and night temperatures become cool resulting in dew formation on plants. However, significant disease development and yield loss

can also occur on earlier seeded crops if moderate temperatures coincide with prolonged periods of wet weather (Sindhan and Bose, 1979).

2.6. Pathogenic variability of *Pseudocercospora griseola*

Previous studies have revealed high levels of pathotypic variation in *P. griseola* (Guzman *et al*., 1995; Pastor-Corrales *et al*., 1998;Wagara *et al*., 2003; Ddamulira *et al*, 2014; Chilagane *et al*., 2016). For example, Marin-Villegas (1959) identified 13 pathotypes among 33 isolates from Colombia, while with Brazilian isolates; Paula & Pastor-Corrales (1996) identified 21 pathotypes among 27 isolates. A high occurrence of pathogenic variability has also been reported in the neighboring Kenyan and Ugandan isolates of *P. griseola* (Wagara, 1996; Wagara *et al*., 2003; Ddamulira *et al*., 2014). Despite the high pathotype diversity, all *P. griseola* pathotypes have frequently been divided into Andean and Mesoamerican groups that correspond to the two common-bean gene pools (Guzmán *et al.*, 1995; Boshoff *et al*., 1996; Pastor-Corrales *et al*., 1998; Ddamulira *et al.*, 2014). The Andean group consists of *P. griseola* isolates recovered from large-seeded common-bean genotypes of Andean origin that infect Andean genotypes only; the Mesoamerican group contains isolates that are more virulent on Mesoamerican bean genotypes (Guzmán *et al.* 1995; CIAT, 1995; Gepts, 1988; Pastor-Corrales *et al*., 1998).

2.7. Characterization of *Pseudocercospora griseola*

Several techniques have been used to study the distribution and variability of *P. griseola* fungi; some of them include; disease surveys, morphological and biochemical, differential cultivars and molecular techniques (Sartorato, 2004; Sebastian & Balatti, 2006; Mahuku *et al*., 2009: Ddilumera *et al*.,204; Luseko *et al.,*2017). Disease surveys allow pathogen distribution to be determined in terms of incidence, severity, spatial and temporal distribution.

2.7.1. Morphological Markers

Traditional identification and characterization of *fungal isolate* has relied primarily on differences in morphological features such as colony color, size and shape of conidia and appressoria, optimal temperature for growth, growth rate, presence or absence of setae, and existence of the *Glomerella* teleomorph (Sutton, 1992). Due to environmental influences on the stability of morphological traits and the existence of intermediate forms, these criteria are not always adequate for reliable differentiation among fungal species. The overlap of morphological characters and phenotypes among species makes classification difficult.

2.7.2. Biochemical Marker - Allozymes (Isozyme)

Isozymes analysis has been used for over 60 years for various research purposes in biology, viz. to delineate phylogenetic relationships, to estimate genetic variability and taxonomy, to study population genetics and developmental biology, to characterization in plant genetic resources management and plant breeding (Bretting & Widrlechner 1995, Staub & Serquen 1996). Isozymes were defined as structurally different molecular forms of an enzyme with, qualitatively, the same catalytic function. Isozymes originate through amino acid alterations, which cause changes in net charge, or the spatial structure (conformation) of the enzyme molecules and also, therefore, their electrophoretic mobility. After specific staining the isozyme profile of individual samples can be observed (Soltis & Soltis 1989). Allozymes are allelic variants of enzymes encoded by structural genes. Enzymes are proteins consisting of amino acids, some of which are electrically charged. As a result, enzymes have a net electric charge, depending on the stretch of amino acids comprising the protein. When a mutation in the DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change.

Because of changes in electric charge and conformation can affect the migration rate of proteins in an electric field, allelic variation can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt (e.g. nitro-blue tetrazolium). The strength of allozymes is simplicity. Because allozyme analysis does not require DNA extraction or the availability of sequence information, primers or probes, they are quick and easy to use. Some species, however, can require considerable optimization of techniques for certain enzymes. Simple analytical procedures, allow some allozymes to be applied at relatively low costs, depending on the enzyme staining reagents used. Isoenzyme markers are the oldest among the molecular markers. Isozymes markers have been successfully used in several crop improvement programmes (Baes & Custsem 1993). The main weakness of allozymes is their relatively low abundance and low level of polymorphism. Moreover, proteins with identical electrophoretic mobility (co-migration) may not be homologous for distantly related germplasm. In addition, their selective neutrality may be in question (Krieger & Ross 2002).

2.7.2. *P. griseola* Virulence and Differential Lines in Common bean

P. griseola virulence is assessed based on reaction of isolates on a standard differential set of 12 common bean cultivars established during the International Angular Leaf Spot Workshop hosted by International Centre for Tropical Agriculture (CIAT) in 1995. In this technique, a binary system based on the position of each cultivar within the series is used to define the virulence level of isolates under study (Pastor-Corrales *et al.,* 1998). Use of differential cultivars generates a true picture of virulence structure and reveals the pathogen properties related to host selection effect on the pathogen population (Sebastian and Balatti, 2006). But the major limitation for this method is its heavy dependence on environmental conditions (Kolmer *et al*., 1995). However,

challenges in using the differential technique have been overcome through the use of molecular techniques as alternative options for detecting variability in pathogen populations.

2.7.3. Molecular Markers

A molecular marker is a DNA sequences that is readily detected and whose inheritance can be easily be monitored. The uses of molecular markers are based on the naturally occurring DNA polymorphism, which forms basis for designing strategies to exploit for applied purposes. A marker must to be polymorphic i.e. it must exist in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosomes with the normal gene by a marker it also carries. The first such DNA markers to be utilized were fragments produced by restriction digestion the restriction fragment length polymorphism (RFLP) based genes marker. Consequently, several markers system has been developed. Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labelled probe, which is a DNA fragment of known origin or sequence. PCR based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermos table DNA polymerase enzyme.

Molecular markers are necessary in pathogen identification, especially to studies genetic diversity and gene mapping. The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) or microsatellites, single nucleotide polymorphisms (SNPs), and more recently

next-generation DNA sequencing (Staub *et al*., 1996; Gupta & Varshney, 2000; Xu and Crouch, 2008). Genetic markers such as random amplified microsatellite (RAMS), repetitive sequences such as enterobacterial repetitive intergenic consensus sequence (ERIC) and repetitive extragenic palindromic (REP) have all been used to describe the diversity of *P. griseola* (Sebastian & Balatti, 2006). Such markers are reliable and reproducible when used to assess fungus strain diversity, especially when combined with computer-assisted data analysis (Louws *et al.,* 1999). Depending on their reliability, markers have been previously used to determine genetic variation of *P. griseola* and divided it into two Andean and Middle American groups following the two major gene pool sources (Guzmán *et al*., 1995; Pastor-Corrales *et al*., 1998). The major limitations of some of these methods are low reproducibility of RAPD, high cost of AFLP, and the need to know the flanking sequences to develop species specific primers for SSR polymorphism.

2.7.3.1. Rep-PCR fingerprinting

The rep-PCR technique was chosen because this technique is simple, can differentiate between closely related strains of bacteria and shows good reproducibility. Rep-PCR has been applied successfully in the classification and differentiation of strains of many Gram-positive and -negative bacteria. Different molecular techniques (e.g. RAPD, ISSR, RFLP, SSR and rep-PCR) have frequently been used for genotyping of fungal species to determine the variability at intraspecific level, but some authors applied them to assessment variability at interspecific level for detection and differentiation of fungal species (Mohali *et al*., 2007). Among these, rep-PCR is a well-known molecular method based on PCR amplification of regions between short interspersed repetitive elements that are dispersed throughout the genome of prokaryotes and eukaryotes (Versalovic *et al*., 1991; Olive & Bean, 1999). A large number of repetitive DNA

sequences are found in multiple sites in the genomes of numerous bacteria, archaea and fungi. While the functions of many of these repetitive sequence elements are unknown, they have proven to be useful as the basis of several powerful tools for use in molecular diagnostics, medical microbiology, epidemiological analyses and environmental microbiology. The repetitive sequence-based PCR or rep-PCR DNA fingerprint technique uses primers targeting several of these repetitive elements and PCR to generate unique DNA profiles or 'fingerprints' of individual microbial strains. Although this technique has been extensively used to examine diversity among variety of prokaryotic microorganisms, rep-PCR DNA fingerprinting can also be applied to microbial ecology and microbial evolution studies since it has the power to distinguish microbes at the strain or isolate level. Recent advancement in rep-PCR methodology has resulted in increased accuracy, reproducibility and throughput. There are three main sets of repetitive DNA elements used for typing purposes. The repetitive extragenic palindromic (REP) elements are palindromic units, which contain a variable loop in the proposed stem-loop structure (Stern *et al*., 1984). ERIC sequences are characterized by central, conserved palindromic structures (Hulton *et al.,* 1991). BOX elements consist of differentially conserved subunits, namely boxA, boxB, and boxC (Martin *et al*., 1992). Only the boxA-like subunit sequences appear highly conserved among diverse bacteria (Versalovic *et al.,* 1994). BOX elements were the first repetitive sequences identified in a Gram-positive organism (Streptococcus pneumoniae) (Martin *et al.,* 1992). REP- and ERIC-sequences were originally identified in Gram-negative bacteria and then found to be conserved in all related Gram-negative enteric bacteria and in many diverse, unrelated bacteria from multiple phyla (Versalovic *et al*., 1994; Olive and Bean, 1999).

REP-PCR, the (18-mer) primers REP 1R-I (59-IIIICGICGICATCIGGC-39) and REP 2-I (59-ICGICTTATCIGGCCTAC-39) and for ERIC-PCR, the (22-mer) primers ERIC 1R (59-ATGTA AGCTCCTGGGGATTCAC-39) and ERIC 2 (59-AAGTAAGTGACTGGGGTGAGCG-39) (Versalovic *et al*. 1991) were used. The REP 1R-I and REP 2-I primers contain the nucleotide inosine (I) at ambiguous positions in the REP consensus (Versalovic *et al*. 1991). Inosine can form Watson-Crick base pairs with A, T, G, or C. PCRs were carried out as described by Versalovic *et al*. (1991). Rep-PCR fingerprinting is a highly reproducible and simple method to distinguish closely related microbial strains, deduce phylogenic relationships, and study their diversity in different ecosystems (Ishii and Sadowsky, 2009). Currently, rep-PCR has been proved as a useful molecular method to identify and study the genetic variability in the fungal species (González-Mejía *et al*., 2005).

2.8. Host pathogen interaction analysis

The interaction between a plant and a pathogen activates a wide variety defense response. The recent development of microarray-based expression profiling methods, together with the availability of genomic and expressed sequence tags sequence data for some plants species allowed significant progress in the characterization of plant pathogenesis related response (Wan *et al.,* 2002). Studies on the variability of *P. griseola* isolates revealed the existence of two major groups of the pathogen, Andean and Mesoamerican, which correspond to and have co-evolved with the Andean and Mesoamerican host gene pools of common bean (Guzman *et al.,* 1995; Pastor-Corrales *et al*., 1998; Crous *et al*., 2006). Mesoamerican strains of this pathogen are considered more virulent as compared to Andean strains and they tend to affect both Mesoamerican and Andean beans while Andean strains are less virulent, affecting mostly Andean host genotypes (Pastor Corrales *et al*., 1998). Apart from these two distinct sets of host and

pathogen based interaction on geographical origin, another group of pathogen was found peculiar to Africa designated as Afro–Andean. This group of pathogen has characteristics typical of the isolates of Andean origin but it was found to be pathogenic on Mesoamerican common bean host which is unusual (Mahuku *et al*., 2002; CIAT, 1997). Different methods have been applied in attempting to host pathogen analysis and characterize the ALS pathogen including virulence testing, where isolates are classified according to the reaction they cause to a set of differential cultivars (Luseko *et al*.,2016).

2. 9. Genome wide and Marker Trait Association studies

Recent development of high density markers such as SNPs enables identification of trait-marker association through association mapping, and GWAS for many important agronomic traits have been reported. With the decreased genotyping cost and improved statistical methods, genome-wide association study (GWAS) and genomic selection (GS) present promising prospects for genetic improvement of complex traits in crop species. GWAS with a population of unrelated lines and high density single nucleotide polymorphism (SNP) markers is capable of identifying causal genes for a broad range of complex traits in different crops (Huang *et al*., 2010; Li *et al*., 2013; Morris *et al*., 2013). Since the advent of high-throughput genotyping methods, genome-wide association (GWAS) is the emerging tool for studying the genetics underling natural phenotyping variation. GWAS have been applied across a wide range of species where it enabled fine-scale genetic mapping (Welter *et al*. 2014). Genome-wide association studies (GWAS) or association mapping (AM) and linkage disequilibrium (LD) offer high resolution through historical recombination accumulated in natural populations and collections of landraces, breeding materials, and varieties (Rossi *et al.,* 2009). By exploiting broader genetic diversity, GWAS offers several advantages over linkage

mapping, such as mapping resolution, allele number, time saved in establishing marker-trait associations, and application in breeding programs (Yu *et al.,* 2006). The strength of the correlation between two markers is a function of the distance between them: the closer two markers are, the stronger the LD. The resolution with which a QRL can be mapped is a function of how quickly LD decays over distance. Selfing reduces opportunities for recombination; thus, in self-pollinating species such as rice (Oryza Sativa), LD may extend to 100 Kb or more (Flint-Garcia *et al*.,2007). In general, high LD is expected between tightly linked loci, since the mutation should have eliminated LD between loci that are not in close proximity to one another (Breseghello and Sorrells, 2006). In common bean, little information is available on the extent of LD (Kwak and Gepts, 2009; Rossi *et al.,* 2009; Blair *et al.,* 2010). Some groups have performed AM studies for common bean, but few of the studies have focused on CBB, ANT and ALS resistance (Shi *et al*., 2011; Cichy *et al*., 2015; Kamfwa *et al*.,2015; Perseguini *et al*.,2016; Kamfwa *et al.,* 2017).

2.10. Marker Assisted Backcrossing(MAB) for Disease Resistance

Backcrossing is a plant breeding method most commonly used to incorporate one or few genes into an adapted or elite variety. In most cases, the parent used for backcrossing has a large number of desirable attributes but is deficient in only a few characteristics (Allard, 1999). It has been a widely used technique in plant breeding for a century. The use of DNA markers in backcrossing greatly increase the efficiency of selection. The main purpose of MABC is to transfer the desired character/or targeted gene along with recovering the recurrent parent characters or genes. MABC is now playing an important role for the development of disease resistance cultivars (Sundaram *et al.,* 2009) and it is superior to conventional backcrossing in precision and efficiency and time saving. Molecular markers which are tightly linked with important traits are

used in MABC. Therefore, molecular markers are the tools that can be used to detect the presence of desired character in backcrossing and greatly increases the efficiency of selection (Ashkani *et al*., 2015). There are three general levels of marker assisted backcrossing(MAB) that have been described (Holland 2004). In the first level, markers can be used in combination with or to replace screening to target genes or QTL. This is referred as 'foreground selection' (Hospital & Charcosset, 1977). This may be particularly useful for traits that have laborious or time consuming phenotypic screening procedures. It can also be used to select for reproductive stage traits in the seedling stage, allowing the best plants to be identified for backcrossing (Hospital & Charcosset, 1977). Furthermore, recessive alleles can be selected, which is difficult to do using conventional methods. The second level involves selecting BC progeny with the target gene and recombination events between the target locus and linked flanking markers & referred 'recombinant selection'. The purpose of recombinant selection is to reduce the size of the donor segment containing the target locus (i.e. size of the introgression). This is important because the rate of decrease of this donor fragment is slower than for unlinked region and many undesirable genes that negatively affects crop performance may be linked to the 'linkage drag' (Hospital 2005). Using conventional breeding methods, the donor segment can remain very large even with many BC generations (Salina *et al*.,2003). By using markers that flank a target gene (e.g. less that 5cM on either side), linkage drag can be minimized. Since double recombination events occurring on both sides of target locus are extremely rare, recombinant selection is usually performed using at least two BC generations (Frisch *et al*.,1999). The third level of MAB involves selecting BC progeny with the greatest proportion of recurrent parent(RP) genome, using markers that are unlinked to the target locus-referred 'background selection. In the literature, background selection refers to the use of tightly

linked flanking markers to select for the RP (Frisch *et al.,*1999). Background markers are markers that are unlinked to the target gene/QTL on all other chromosomes, in other words, markers that can be used to select against the donor genome (Hasan *et al.,* 2015). This is extremely useful because the RP recovery can be greatly accelerated. With conventional backcrossing, it takes a minimum of six BC generations to recover the RP and there may still be several donor chromosome fragments unlinked to the target gene. Using markers, it can be achieved by BC_4, BC_3 even BC_2 (Visscher *et al*. 1996; Hospital & Charcosset 1997; Frisch *et al*. 1999). Thus, saving two to four BC generations. The use of background selection during MAB to accelerate the development of RP with an additional or few genes has been referred to as 'complete line conversion' (Ribaut *et al*.,2002).

2.11. Marker-assisted Selection and Gene pyramiding

Gene pyramiding is defined as a method aimed at assembling multiple desirable genes from multiple parents into a single genotype. Conventional gene pyramiding is extremely time consuming and may fail to detect epistatic genes among segregation progenies. The development of modern plant molecular and quantitative genetics in the last two decades has potential to revolutionize what has mostly been conventional breeding and has widened several aspects of practical applications of gene pyramiding. Marker Assisted Gene Pyramiding is currently the method of choice for inbred line development targeted at improving traits controlling by major genes (Joshi *et al*.,2010). Use of molecular markers to track pyramided genes also considerably reduces the breeding period involved in pyramiding program (Joshi *et al*.,2010). Markers, assisted backcrossing has been successfully applied in gene pyramiding programs for targeted transferring and pyramiding resistance loci to create more durable and broad specific resistance in different crops (Joshi and Nayak 2010). In the wheat cultivar "Yang", Liu

et al. (2000) successfully combined three powdery mildew resistance gene combinations *pm2 + pm4a, pm2+pm211*, and *pm4a+pm21* using restriction fragment length polymorphism (RFLP) markers. In soybean for mosaic virus disease resistance (SMV), researchers successfully pyramided three genes *Rsv1*, *Rsv3* and *Rsv4* with the aid of microsatellite markers in order to develop new soybean lines containing multiple resistance genes for soybean mosaic virus (SMV) resistance. Marker-assisted selection (MAS) and gene pyramiding have been reported in common bean research (Kelly *et al.*, 2003, Miklas *et al.,* 2006; Ragagnin et al. 2009); Ferreira et al 2012; Kumar et al. 2017). Recently, Ddamulira *et al,* (2015) reported the efficiency and effectiveness of gene pyramiding in improving angular leaf spot resistance in susceptible common bean cultivar.

CHAPTER THREE

Pathotypes Characterization and Virulence Diversity of *Pseudocercospora griseola* the Causal Agent of Angular Leaf Spot Disease Collected from Major Common Bean (*Phaseolus vulgaries* L.) Growing Areas of Ethiopia

Abstract

Angular leaf spot caused by the fungus *Pseudocercospora griseola* is one of the most economically important disease affecting common bean production in Ethiopia. Until now no information has been generated regarding the pathotype and pathogenic variability and its distribution in Ethiopia. Hence, the aim of this study was to characterize and determine the diversity among 39 isolates of *P. griseola* collected from diverse common bean growing areas of Ethiopia. A total of 21 pathotypes of *P. griseola* (63:63, 63:59, 63:23, 61:51, 56:36, 55:39, 49:7, 48:60, 42:59, 41:10, 34:53, 23:61, 19:33, 17:45, 8:18, 8:0, 4:16, 1:24, 1:10, 16:18. and 4:37) were determined among 39 isolates using 12 sets of Angular leaf spot common bean differentials and the result revealed the presence of high and diverse pathogenic variability of this economically important fungus in Ethiopia. Among the determined *P. griseola* pathotypes 63:59 and 19:33 were the most frequently appeared. The occurrence of pathotype 63:63 the broadest virulence spectrum was also determined in the study this pathotype was the most virulent that caused leaf spot on all 12 sets of common bean differential genotypes. Among the determined *P. griseola* pathotypes the occurrence of 63:63, 63:59 and 63:23 pathotypes in central America and Argentina were also reported. This will be the first comprehensive report of *P. griseola* pathotypes existing in the common bean growing areas of Ethiopia. Except for the pathotype that were compatible with Andean groups (8:0), most of the isolates were pathogenic to both Andean and Mesoamerican gene pools and based on that pathogens were grouped and classified as Mesoamerican origin pathotypes. This specific study provided major information about the pathogenic diversity and pathotype of *P. griseola* the causal agent for the angular leaf spot disease of common bean endemic to Ethiopia. Hence, the information obtained will also help to breeders working in common bean improvement program to develop cultivars with enhanced tolerance and/or resistance through gene deployment and marker aided pyramiding technique.

Keywords: *Pseudocercosporea griseola,* Pathotype variability, Pathogenic variability

3.1. Introduction

Common bean (*Phaseolus vulgarise* L.) is the most important grain legume next to Faba bean and which is produced all of the regions of Ethiopia with different intensity for its nutritional and economic values (CSA, 2016). Among several factors affecting common bean yield the incidence of disease is one of the principal factor affecting the common bean production. Angular leaf spot (ALS) caused by the fungus *P. griseola* (Miklas *et al*., 2006), which is caused by the fungus *Pseudocercospora griseola* (*Sacc*.) Crous & Braun (*sin. Phaeoisariopsis griseola (Sacc*.) Ferraris) is one of the most important disease affecting the common bean production. This specific disease can cause necrotic lesions on the aerial parts of the plant, reducing the productivity and quality of common bean seed (Crous *et a*l., 2006) by hindering common bean production in eastern and central Africa (Crous *et al*., 2006; Singh and Schwartz, 2010). Yield loss of more than 374,800 tonnes annually have been reported by Wortmann *et al*., (1998). The pathogen is found in nature in the form of mycelia or conidia on living tissues of the host plant (susceptible on and off-season crop, volunteer plants), undecomposed infected bean residues and infected soils.

The pathogen is a seed born in most cases external contamination may occur on seed during harvesting and the pathogen has been associated with the hilum area of the seed coat (Correa *et al.,* 1989). This specific fungus is highly variable and has the ability to infect both Andean and Mesoamerican gene pools. Previous studies have revealed high levels of pathotypic variation in *P. griseola* (Guzman et al., 1995; Pastor-Corrales *et al.,* 1998; Wagara *et al*., 2003; Ddamulira *et al*, 2014; Chilagane *et al*., 2016; Luseko *et al.,* 2017). In addition of that many of the scientists working on this pathogen reported as the presence of high pathotype diversity hence, Marin-Villegas (1959) reported from Colombian isolates and he identified 13 pathotypes among 33 isolates, while from the

Brazilian isolates; Paula & Pastor-Corrales (1996) identified 21 pathotypes among 27 isolates. In recent studies high occurrence of pathogenic variability has also been reported in the neighboring Kenyan and Ugandan isolates of *P. griseola* (Wagara, 1996; Wagara *et al*., 2003; Ddamulira *et al*., 2014). In spite of the high pathotype diversity, all *P. griseola* pathotypes have been divided into Andean and Mesoamerican groups that correspond to the two common-bean gene pools (Guzmán *et al.*, 1995; Boshoff *et al.,* 1996; Pastor-Corrales et al., 1998; Ddamulira *et al.*, 2014 Chilagane *et al.,* 2016). The Andean group consists of *P. griseola* isolates recovered from large-seeded common bean genotypes of Andean origin that infect Andean genotypes only; the Mesoamerican group contains isolates that are more virulent on Mesoamerican bean and Andean genotypes (Guzmán *et al.*, 1995; CIAT, 1995; Gepts, 1988; Pastor-Corrales *et al.,* 1998).

To assess the degree of diversity of *P. griseola*, a set of 12 differential cultivars carrying different resistance genes to ALS diseases were proposed in the first ALS workshop held at CIAT in 1993 to standardize the methodology for *P. griseola* pathotype identification (Pastor-Corrales and Jara, 1995) which was two sets Andean and Mesoamerican with six common bean genotypes included in each set. The common bean differential genotypes and their binary numbers includes, Don Timoteo (1), G1179 (2), Bolon Bayo (4), Montcalm (8), Amendoin (16), G5686 (32), PAN 72 (1), G2858 (2) Flor de Mayo (4), Mexico 54 (8), BAT 332 (16) and Cornell 49242 (32). The number or race designation given to an isolate is determined by the cultivars of the differential set that are infected by that isolate. For example; if an isolate infects Andean common bean genotype Amendoin and Montcalm (binary value, 16 and 8 respectively) and the Mesoamerican variety BAT332 (binary value 16) and Cornell49242 (32) the race would be designated by adding the values (16 + 8): (16+32) which is 24:42.

Knowledge of pathotype variation among the isolates of *P. griseola* is important to the common bean improvement program to guide the deployment of resistance genes to angular leaf spot disease. However, there is no information regarding *P. griseola* pathotype variability from the Ethiopian *P. griseola* isolates, which puts the common bean improvement program under challenging situation to develop durable disease (ALS) resistance varieties with wider adaptation. Therefore, this specific study used the international sets of ALS differential common bean cultivars to determine and characterize the *P. griseola* pathotypes occurring in bean growing areas of Ethiopia. The objectives of the study were to determine the pathotype variability and the virulence pattern of *P. griseola* in the bean growing agro-ecologies of Ethiopia and to identify predominant pathotypes that exist in the major common bean growing areas of Ethiopia

3.2. Materials and Methods

3.2.1. Sampling Strategy and collection

Samples of infected leaves with symptoms of ALS obtained were collected from the field survey during 2015 and 2016 from diverse agro-ecological zones in six diverse areas of Ethiopia (Areka, Dolla, Chano, Omo, Wondo and Goffa) these locations are known for its major common bean production and for its high common bean disease severity especially the angular leaf spot. The sampling locations were selected based purposively on the intensity of bean production, especially ecological locations representing diverse conditions under which common beans are produced. Random sampling technique were adopted in which infected leaves with lesion symptoms usually appear as a brown spot with a tan or colliery centre that were initially confined to tissue between major veins, that gives angular appearance were collected from 10

random farmers field from each location. Then collected leaf samples were kept in a paper bag and transported to the lab

3.2.2. *P griseola* Isolation and Inoculum preparation

Selected fresh diseased common bean leaf samples from the collections were directly used and lesions were thereafter examined under a dissecting microscope (Motic BA210) to view the synemata and assess the quality of the sporulation. Conidia of individual lesions were picked from the clean synemata by gently brushing the tips with a small piece of agar at the tip of an inoculating needle and transferred to a drop of sterile water placed on water agar as described by Mahuku *et al*., (2002). The inoculated petri dishes were allowed to grow the pathogen evenly. The Petri dishes were incubated at 24^0C for 24 hr and then the germinated spores were picked and immediately transferred to V8 media. This specific media was prepared using 200ml V8 juce with 3g $CaCO_3$, 20g of Bacto Agar in about 800ml dd H_2O to make 1000ml and autoclaved at 121°C for 15 mins. Each Petri dishes of V8 was inoculated with a single conidium avoiding mixtures during race identification and determination. Each colony that grew from a single conidium was treated as an isolate and a single spore isolates are considered pure during race determination. For the colonies to develop and multiply the Petri dishes were allowed for about 14 days. Then each colony was treated as monosporic isolate. Based on that single spores from sampled locations includes Areka (12), Dolla (2), Chano (10), Omo(1), Wondo (7) and Goffa(7) were considered in the study.

3.2.3. Inoculation and pathotype determination

A total of 39 single spore spores were isolated from infected and diseased leaves collected from the diverse common bean growing regions using methods developed by

CIAT (Schoonhoven *et al*.,1987). Isolation, monosporic culture and inoculation were done according to the method developed by Pastor Coralles *et al*. (1998). Before the inoculation the spore concentration was adjusted in distil water at 10 x 10^4 per ml using haemocytometer. After monosporic culture of the *P. griseola* the fungus was grown in petri dishes containing V8 medium (which was prepared using 200ml V8 juce with 3g CaCO3, 20g of Bacto Agar in 800ml dd H2O autoclave at 121oC for 15 mins). The resulting spores and mycelia were scrapped smoothly with a spatula and filtered through gauze and the spore concentration was adjusted to 2.0 X $l0^{-4}$ conidia/ml. Detached leaves 18-days old from the 12-common bean differentials (Don Timoteo, G 11796, Bolon Bayo, Montcalm, Amendoin, G 5686, Pan 72, G 2858, Flore de mayo, Mexico 54, BAT 332, Cornell 49-242) grown in the screening house were used for pathotype determination. For the detached leaf method, which was based on developed method (Yayis *et al.,* 2108 unpublished) and Tu (1986) with minor modifications, 18 days after germination the middle follicle of the first trifoliate leaves of each common bean differential plants were removed or detached when they had reached approximately two-thirds of their full development. The detached leaves were inoculated by immersion into a spore suspension and placed in petri dishes (90 x 15 mm) on a cotton moistened with 3.0 mL of tap water. The experiment was replicated three times for consistent result. The Petri dishes were watered regularly to maintain about 95% relative humidity to allow the growth of the pathogen. Disease severity on the inoculated plants was evaluated using 1-9 visual score scale (Schoonhoven*et al*., 1987) for 21 days at an interval of three days. Pathotypes were defined by rating scores of 1-3 to be incompatible (-) or resistant, while ratings >3 were compatible (+) or susceptible. Pathotype designation was executed by adding binary values of the

differential genotypes that were compatible with the respective *P. griseola* isolates (Table 3.1& Fig 3.1).

Table 3.1 Common bean ALS differential cultivar and binary system for pathotype determination study

CODE	CULTIVAR ID	SEED SIZE	BEAN RACE	BINARY VALUE	R Gene present
A	Don Timoteo	Medium	Chile	1	1 dominant
B	G 11796	Large	Peru	2	
C	Bolon Bayo	Large	Peru	4	
D	Montcalm	Large	Nueva Granda	8	2 recessives
E	Amendoim	Large	Nueva Granda	16	2 recessives
F	G 5686	Large	Nueva Granda	32	1 dominant
G	Pan 72	Small	Mesoamerica	1	1 dominant
H	G 2858	Medium	Durango	2	1 dominant
I	Flore De Mayo	Small	Jalisco	4	2 duplicates
J	Mexico 54	Medium	Jalisco	8	*Ph-2, Ph-5, ph-6,*
K	BAT332	Small	Mesoamerica	16	*ph-6*^{2}
L	Cornell 49242	Small	Mesoamerica	32	*Ph-3*

Source: Caixeta et al., 2002; Mahuku et al., 2004; Sartorato et al., 2002

Figure 3.1. Different disease scoring of leaves inoculated with *Pseudocercospora griseola*, the causal agents of angular leaf spot disease, using the proposed detached-leaf inoculation method, where 1 = no visible symptoms; 3 10 to 20% of plants infected and/or about 5% of the total plant area affected by the pathogen; 5 = 40 to 50% of plants infected and/or about 20% of plant area affected; 7 = 60 to 70% of plants infected and/or about 40% of plant area affected; and 9 = >80%.

3.3. Results

3.3.1. Pathotype diversity and distribution of *P. griseola* isolates from Ethiopia

Pathotypes of the *P. griseola* were identified based on pathogenicity on a series of 12 common bean differentials (Pastor-Coralles *et al.,* 1998). The result revealed the existence of 21 *P. griseola* pathotypes among the 39 isolates occurring in Ethiopia the result determined highly pathogenic isolates on both Andean and Middle American common bean differentials because of this most of the isolates from the diverse bean growing regions were considered as the Middle American group of fungus. The result is in in agreement with common bean cultivars predominantly grown in Ethiopia which is from the Mesoamerican genepool. The remaining few number of isolates were highly pathogenic only on the Andean differentials and these isolates infecting only the Andean differentials were considered members of the Andean groups. Among the 39 isolates twenty-one pathotypes were identified. Two pathotypes which includes 63:59 and 19:33 occurred most frequently than others (Figure 3.2). Different pathotypes co-exist in certain bean production areas of Ethiopia. Isolates from similar common bean areas or geographic location varied in their pathogenicity when they were inoculated on the 12 sets of common bean differentials. From the result, two isolates coded with *Pg01* & *Pg02* from Areka district induced similar pathogenicity on some of the differentials but not on all the differential genotypes. Whereas, there were cases like isolates coded *Pg20* and *Pg21* obtained from similar common bean growing areas induced similar pathogenicity (Table 3.2). In most cases *P. griseola* isolates obtained from the same geographic locations varied in their pathogens confirming presence of pathotype variability in Ethiopian isolates. The existence of higher pathogenic variation in *P. griseola* populations collected from diverse common bean growing areas of Ethiopia supports earlier findings elsewhere on the existence of *P. griseola* variability

by AlvarezAyala & Schwartz (1979), Buruchara (1983), CorreaVictoria (1987), and Marín-Villegas (1959); Ddulimra *et al.,*2014; Luseko et al., 2016). The source of such high variability is not clear, because *P. griseola* has no known sexual cycle (Liebenberg and Pretorius 1997). The mechanisms that generate variation in pathogen populations include mutation, recombination and migration were also reported (Leung *et al.,* 1993; Wagara *et al*.,2005; Ddamulira *et al*.,2014).

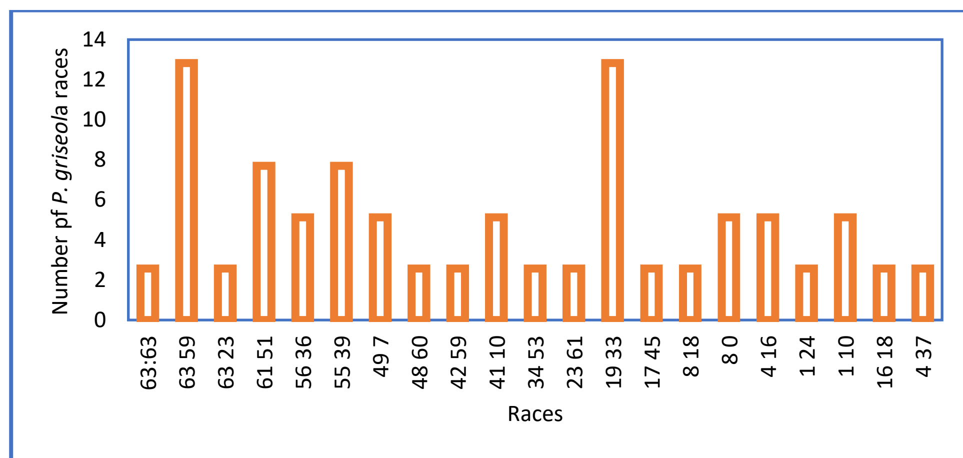

Figure 3.2 Frequency of Pseudocercospora griseola in Ethiopia

3.3.2. Reaction of common bean differentials to *P. griseola* pathogen

All the international differential sets of common bean genotypes reacted differently for the monosporic *P. griseola* isolates obtained from the diverse common bean growing regions of Ethiopia. The 39 monosporic pure isolates *P. griseola* obtained from the infected leaves collected from the diverse common bean growing regions of Ethiopia caused lesions of angular leaf spot symptoms in some or all of the host differential host common bean genotypes. Symptoms in the leaves were brown spots that appeared on the primary detached leaves as angular brown spot limited by veins were observed. The fungal growth on the underside of the spots were observed as clusters of synemata

which bared pores or conidia. Under the electron microscope, conidian were observed as obclavate cylindrical with two to four septate (Figure 3.3)

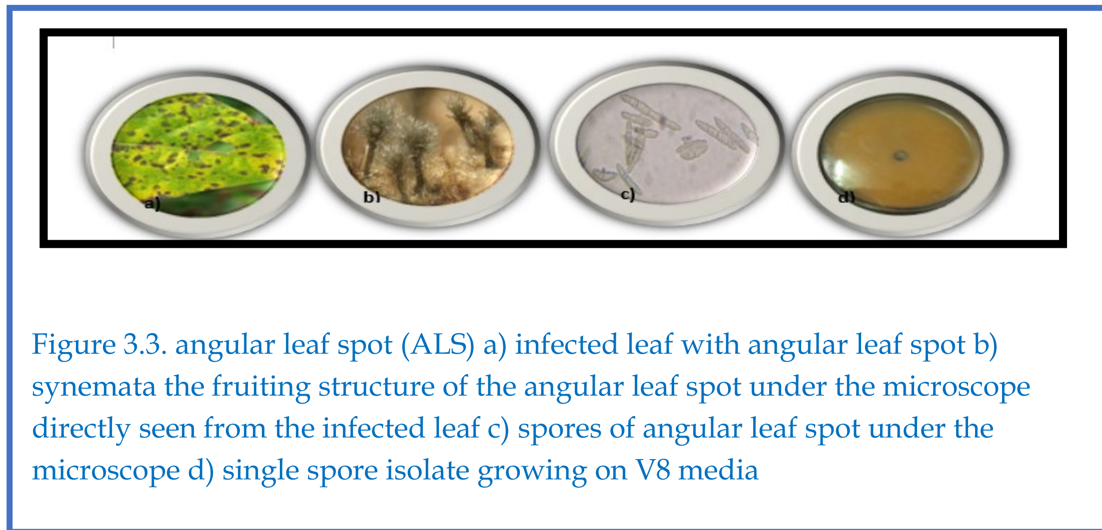

Figure 3.3. angular leaf spot (ALS) a) infected leaf with angular leaf spot b) synemata the fruiting structure of the angular leaf spot under the microscope directly seen from the infected leaf c) spores of angular leaf spot under the microscope d) single spore isolate growing on V8 media

3.3.3. Virulence analysis and pathotype identification

During sample collection angular leaf spot disease was observed in most of the common bean fields with regardless of the common bean cultivars grown and in all of the twelve sets of differentials inoculated with the isolates collected from the diverse common bean areas of Ethiopia. Variations in response of the angular leaf spot differentials cultivars were observed irrespective of the genepool in which they were originated. *P. griseola* pathotypes are defined based on the pathogenicity reaction to a set of 12 common bean differential genotypes. In this study the reaction of 39 single spore isolates on a set of differential common bean genotypes revealed the existence of pathotype variability in Ethiopian *P. griseola* isolates (Table 3.2). Based on the virulence reaction, twenty-one pathotypes were characterized and except pathotype 8:0 all of the pathotypes were virulence to both the Andean and Mesoamerican common bean sets of differentials. The result also confirmed the presence of both Andean and Mesoamerican origin pathotypes in Ethiopia (Table 3.2).

Table 3.2. Pathotype determination *Pseudocercospora griseola* from diverse common bean growing areas of Ethiopia

Isolate Code	Location	Isolate	Pathotype	Isolate Code	Location	Isolate	Pathotype
Pg01	Areka	PG 37A	49:07	*Pg21*	Areka	R4P2B	8:0
Pg02	Areka	R7P8	63:59	*Pg22*	Areka	R1P8C	4:16
Pg03	Areka	R6P14B	61:51	*Pg23*	Areka	R6P11	4:16
Pg04	Gofa	PG44A	63:59	*Pg24*	Wondo	B6P57	56:36
Pg05	Gofa	PG32C	34:53	*Pg25*	Wondo	B3P46	41:10
Pg06	Gofa	CD45A	49:07	*Pg26*	Wondo	ADP-0095	61:51
Pg07	Gofa	CD50B	55:39	*Pg27*	Wondo	B6P57	56:36
Pg08	Gofa	CDPG33C	55:39	*Pg28*	Wondo	ADP-0675	41:10
Pg09	Omo/Jinka	om418	63:23	*Pg29*	Dolla	D 13	42:59
Pg10	Chano	pg384	16:18	*Pg30*	Dolla	B6P14	61:51
Pg11	Wondo	p11	23:61	*Pg31*	Chano	C D PG32	19:33
Pg12	Wondo	p67	63:63	*Pg32*	Chano	C D PG38	19:33
Pg13	Gofa	PG112	63:59	*Pg33*	Chano	C D PG38	19:33
Pg14	Areka	R7P8	48:60	*Pg34*	Chano	C DPG 41	1:10
Pg15	Areka	R1P35	63:59	*Pg35*	Chano	C D PG 44	1:24
Pg16	Areka	R1P5C	63:59	*Pg36*	Chano	C DPG 45	19:33
Pg17	Areka	R1P8B	08:18	*Pg37*	Chano	C D PG46	41:10
Pg18	Areka	R1PBA	17:45	*Pg38*	Chano	C D PG 50	55:39
Pg19	Gofa	PG 110	4:37	*Pg39*	Chano	C D PG50	19:33
Pg20	Areka	R3P3A	8:0				

The study identified pathotype 63:63 among the Ethiopian isolates which is compatible to all of the 12 sets of differential common bean genotypes (Table 3.3). Pathotype 63:63 overcomes resistance genes in 12 known sources of resistance that constitute differential sets of sets. Similar pathotypes were reported in Brazil, Argentina and central America (Sartarota 2002; Sabstian et al.,2006; Mahuku *et al.,* 2004).

Table 3.3. Pathotype identification and reaction of differential common bean genotypes to the 39 isolates of *P. griseola* collected from diverse common bean growing areas of Ethiopia

Andean						Mesoamerican						Pathotype	No of Isolates
a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*		
1	2	4	8	16	32	1	2	4	8	16	32		
+	+	+	+	+	+	+	+	+	+	+	+	63:63	1
+	+	+	+	+	+	+	+	-	+	+	+	63:59	5
+	+	+	+	+	+	+	+	+	-	+	-	63:23	1
+	-	+	+	+	+	+	+	-	-	-	+	61:51	3
-	-	-	+	+	+	-	-	+	-	-	+	56:36	2
+	+	+	-	+	+	+	-	+	-	-	+	55:39	3
+	-	-	-	+	+	+	+	+	-	-	+	49:7	2
-	-	-	-	+	+	-	-	+	+	+	+	48:60	1
-	+	-	+	-	+	+	+	-	+	+	+	42:59	1
+	-	-	+	-	+	-	+	-	+	-	-	41:10	2
-	+	-	-	-	+	+	-	+	-	+	+	34:53	1
+	+	+	-	+	-	+	-	+	+	+	+	23:61	1
+	+	-	-	+	-	+	-	+	-	-	+	19:33	5
+	-	-	-	+	-	+	-	+	+	-	+	17:45	1
-	-	-	+	-	-	-	+	-	-	+	-	8:18	1
-	-	-	+	-	-	-	-	-	-	-	-	8:0	2
-	-	+	-	-	-	-	-	-	-	+	-	4:16	2
+	-	-	-	-	-	-	-	-	+	+	-	1:24	1
+	-	-	-	-	-	-	+	-	+	-	-	1:10	2
-	-	-	-	+	-	+	-	+	-	-	-	16:18	1
-	-	+	-	-	-	+	-	+	-	-	+	4:37	1
													39

a= Don Timoteo, b= G 11796, c= Bolon Bayo, d=Mont calm, e=Amendoin, f=G 5686, g=Pan 76, h= G2858, i=Flore de Mayo, j=Mex 54, k=BAT 332, l= Corell49-242

3.3.4. Race distribution and frequency of occurrence

The result of race and frequency distribution of *P. griseola* collected from the diverse common bean growing areas were analysed as percentages from the total number of isolates were presented in Figure 3.4. A wide frequency variation revealed in which differential MEX-54 had the lowest frequency with 38.47% while cultivar Don Timoteo and Amendoin with 69.23% and 66.67% respectively scored the highest.

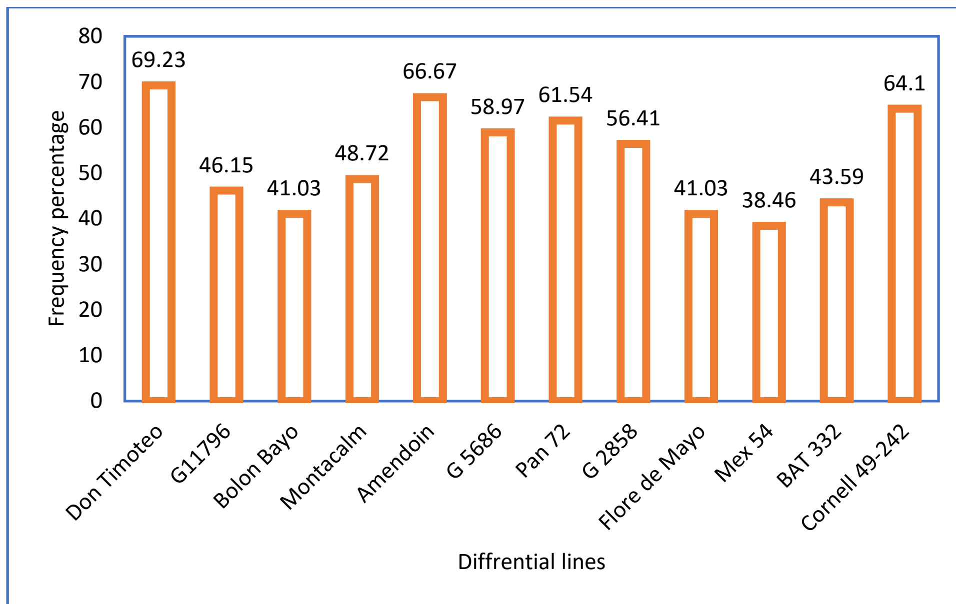

Figure 3.4. Frequency of compatible reaction between sets of differential common bean genotypes and evaluated isolates

3.4. **Discussion**

This study revealed that the presence of both Andean and Mesoamerican pathotypes in Ethiopia where the Mesoamerican pathotypes predominant the Andean and this result supports that Mesoamerican common bean genotypes were dominantly cultivated in Ethiopia (Asrat *et al.,* 2009). Although, previously no sexual reproduction was confirmed (Ddamulira *et al*., 2014), the *P. griseola* isolates from the diverse common bean growing regions showed high variation in virulence pattern. The virulence variation was high in such way that isolates collected from the same location showed differences in their virulence patterns. Several different pathotypes were found from the same farm, which is not surprising as many of the previous report, because this specific pathogen has high race variability. These results are in agreement with the findings of Pastor-Corrales *et al.* (1998), Busogoro *et al*. (1999) and Wagara *et al.* (2005). This result was also supported by many authors including from countries such as Mexico, Brazil, Kenya, Uganda, Tanzania (Busogoro *et al.,* 1999; Sartorato, 2002; Mahuku *et al.,* 2009; Wagara *et al.,* 2005; Ddamulira *et al.,*1914; Castellamos *et*

al.,2016). The high difference in virulence patterns observed during the study indicates that the majority of the resistance genes in the host differentials common bean cultivars were effective against most of the *P. griseola* isolates from Ethiopia. This might suggest *P. griseola* isolates from Ethiopia probably have isolates with different virulence genes which might not matched with the resistance genes in the host differentials common bean cultivars. This is new report of virulence pattern of *P. griseola* isolates from the common bean growing areas of Ethiopia that includes twenty-one (63:63, 63:59, 63:23, 61:51, 56:36, 55:39, 49:7, 48:60, 42:59, 41:10, 34:53, 23:61, 19:33, 17:45, 8:18, 8:0, 4:16, 1:24, 1:10, 16:18. and 4:37) among which, pathotypes 63:59 and 19:33 were the most frequently observed new pathotypes of *P. griseola* reported for the first time in Ethiopia. The majority of common bean cultivars grown in diverse common bean growing areas of Ethiopia have shown severe infections of *P. griseola*, confirming the existence of new pathotype of *P. griseola.* However, the sources of new race are currently not known and it might probably be farmers practice of growing diverse common bean cultivars in adjacent field and the informal seed exchange which is the common practice of seed system dominantly practice by farming community. These may lead in the introduction of new pathotypes which leads to the pathotype variability. In addition of that and as many of the previous reports the pathogen might undergone par asexual that facilitates exchange of genetic material within and between isolates. It might be also because of chromosomal inversion, deletion and presence of transposons because all are reported to have capability to increase the variability in *P. griseola* (Kristler and Miao, 1992; Kempken and Kuck, 1998). Mex-54 with low frequency percentage of pathogen infection could be used as parental lines with potential source of resistance gene in the common bean breeding program. The number of isolates obtained from the Middle American groups were more than obtained from the Andean

group hence this was in lines with the dominantly common bean production in Ethiopia is from the Middle America group and since the *P. griseola* were co-evolved with common bean gene pool.

3.5. Conclusion

This specific study revealed the existence of *P. griseola* pathogen in diverse common bean growing areas of Ethiopia. The *P. griseola* isolates existing in the diverse common bean growing areas of Ethiopia were most predominantly from the Middle American gene pool that affects mostly the Middle American gene pool common beans and Andean gene pool common bean. This is expected and in line with the dominantly grown common bean type which is the middle American gene pool have been known to be grown in Ethiopia. From the study it was determined that the existence of large pathogenic and pathotype variability *P. griseola* and these pathotypes were distributed across diverse regions of Ethiopia. *P. griseola* isolates which were obtained from the same geographic locations showed different pathogenicity due to the district differences in response common bean differential genotypes. The observed variation among the *P. griseola* isolates was probably due to the complex gene constitution of the differential common bean genotypes used in the study. Hence, this would be the first report and the result from this study confirmed the presence of high pathotype and pathogenic variability of *P. griseola* across major common bean growing regions of Ethiopia. The information generated from this study has a significant implication for the bean improvement program, because the *P. griseola* isolates existing in Ethiopia are with wider virulence spectrum. Hence these pathogens must be taken into consideration when developing and deploying bean cultivars with resistance to ALS disease. Hence the common bean breeding program could plan at developing bean cultivars with non-pathotype specific or non-race specific resistance.

CHAPTER FOUR

Rep-PCR Genomic Fingerprinting Revealed Genetic Diversity and Population Structure Among Ethiopian Isolates of *Pseudocercospora griseola* the Causal Agent for Angular Leaf Spot Disease of the Common Bean (*Phaseolus vulgaris* L.)

Abstract

Common bean (*Phaseolus vulgarise* L.) is the most widely grown pulse crop in Ethiopia. Angular leaf spot (ALS; *Pseudocercospora griseola*) is one of the most devastating diseases affecting common bean production in most parts of Ethiopia. Thus, use of common bean varieties with durable resistance is the most effective and economical control measure against ALS. Knowledge about the genetic variability and the population structure of the pathogen populations is important for a successful common bean improvement program that aims to develop varieties with stable resistance. The specific objective of this study was to determine the genomic diversity existing among and between *P. griseola* isolates obtained from the diverse common-bean growing regions of Ethiopia. The repetitive extragenic palindromic elements-polymerase chain reaction protocol was used to fingerprinting DNA sequence diversity. Thus, 78 single-spore isolates of the *P. griseola* pathogen, collected and isolated from diverse common bean growing regions of Ethiopia were analyzed. Molecular Analysis of Variance (AMOVA) and cluster analysis revealed the existence of high genetic diversity within the isolates of *P. griseola* and among the populations. ERIC PCR produced 17 different patterns of clusters, and REP-PCR 11 different patterns of clusters and BOX PCR produced five different patterns. Some isolates that shared the same BOX patterns could be distinguished by the ERIC and REP finger printing patterns. The ERIC-, BOX- and REP-PCR fingerprinting patterns discriminated 25 different patterns among the 79 monosporic isolates of *P. griseola* at cut-off 77% genetic similarity matrix were produced. The discriminated clusters revealed the existence of high genetic diversity within and among the isolates of *P. griseola* collected from the diverse common bean growing regions of Ethiopia. The lack of isolation by distance indicates the *P. griseola* fungi have efficient dispersal at the common bean growing areas of the region. Due to the informal common bean seed exchange practices coupled with spore dispersion and for the long-distance gene flow with human and non-human interference. This study is the first report using rep-PCR genomic finger printing on genomic variation and population structure of *P. griseola* isolates from diverse common bean growing regions of Ethiopian. The results revealed that *P. griseola* in Ethiopia demonstrates with high level of genomic diversity. The common bean improvement programs in Ethiopia should also give priorities for gene deployment and marker aided gene pyramiding techniques in developing broad and multiple disease resistance common bean varieties.

Keyword: Rep-PCR, Repetitive elements, Palindromic unit, *P. griseola*, Genetic diversity

4.1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the most cultivated pulse crop worldwide. In Ethiopia it is an important food for direct human consumption and export. Several biotic and abiotic stress are limiting the productivity of common bean of which Angular leaf spot (ALS) caused by *Pseudocercospora griseola* is the most devastating disease its yield reduction is estimated to reach 80% (Schwartz *et al*., 1981). The use of resistance common bean varieties with durable resistance is the most effective and economical control measure of ALS. However, getting common bean varieties with durable resistance is not easy. Knowledge about the genetic variability of the pathogen populations is important for a successful common bean improvement program that aims to develop disease resistant varieties (McDonald and Linde, 2002).

Genetic structure is defined as the amount and distribution of genetic variation within and among populations (Schmid *et al.,*2006). Thus, knowledge of genetic structure gives insight into the evolutionary processes that shaped a population in the past. It is useful to differentiate between the two types of genetic diversity that contribute to genetic structure: gene diversity and genotype diversity, Gene diversity increases as the number of alleles increases and the relative frequencies of those alleles become more equal. Genotype diversity refers to the number and frequencies of multi-locus genotypes, or genetically distinct individuals, in a population. Genotype diversity is an important concept for plant pathogens that have a significant component of asexual reproduction in their life history (McDonald and Linde, 2002)

The genomes of microbes contain a variety of repetitive DNA sequences, accounting for up to 5% of the genome (Ussery *et al.,* 2004). Many of these repetitive DNA elements are of unknown function and have been localized to both intergenic and extragenic regions of the microbial genome. The Palindromic Units (PU) or Repetitive

Extragenic Palindromes (REP) constitute the characterized family of bacterial repetitive sequences. PUs are present in about 500–1000 copies in the chromosome of *Escherichia coli* and of *Salmonella typhimurium*. PU sequences consist of a 35–40bp inverted repeat and are found in clusters. A second family of repetitive elements, called IRU (Intergenic Repeat Units) or ERIC (Enterobacterial Repetitive Intergenic Consensus), has been described (Versalovic *et al.*, 1991). IRU are 124–127 bp long in which successive copies (up to six) are arranged in alternate orientation (Gilson *et al.,*1984; Martin *et al.,* 1992). Both PU and IRU families are similarly located in non-coding, probably transcribed, regions of the chromosome.

Repetitive Element Polymorphism PCR (rep-PCR) fingerprinting has become a frequent method to discriminate bacteria species analyzing the distribution of repetitive DNA sequences in prokaryotic genomes (Versalovic *et al.,* 1991). Rep-PCR is based on the observation that outwardly facing oligonucleotide primers, complementary to interspersed repeated sequences, enable the amplification of differently sized DNA fragments, consisting of sequences lying between these elements (Versalovic *et al.,* 1994). Multiple amplicons of different sizes can be resolved by electrophoresis, establishing DNA-fingerprint-specific patterns for bacterial strains (Rademaker & De Bruijn 1997). Several of these interspersed repetitive elements are conserved in diverse genera of bacteria and, therefore, enable single primer sets to be used for DNA fingerprinting in many different microorganisms (Versalovic *et al.*, 1994; Rademaker & De Bruijn 1997).

Although rep-PCR primers were developed for repetitive elements in prokaryotic genomes, these primers have been applied with success in the fingerprinting of eukaryotic genomes as well (van Belkum *et al.,* 1998). Thus, rep-PCR primers have been used to characterize the variability of several fungal genera

(McDonald *et al.,* 2000; Mehta *et al.,* 2002; de Arruda *et al.,* 2003; Abdollahzadeh and Zolfaghari, 2014 & Ddamulira *et al.,* 2014). Endogenous repetitive DNA elements have been identified in fungi and used to generate genomic fingerprints (van Belkum *et al.,* 1998). For example, repetitive sequences like microsatellites were shown to be useful targets for DNA-based typing because of their length variation and widespread occurrence (Taylor *et al*., 1999).

Rep-PCR fingerprinting is a highly reproducible and simple method to distinguish closely related microbial strains, deduce phylogenic relationships, and study their diversity in different ecosystems (Ishii and Sadowsky, 2009). However, few studies have been performed regarding the applicability of rep-PCR to the discrimination of fungal species. Currently, rep-PCR has been proved as a useful molecular method to identify and study the genetic variability in the fungal species. Thus, the objective of the study was to study the genetic diversity and the population structure of *P. griseola* isolates obtained from the collected infected leaves of common bean from the various areas of Ethiopia using rep-PCR molecular finger printing methods.

4.2. Materials and Methods

4.2.1. Sample collection and isolation of *P. griseola*

The experiment was conducted in the Molecular Biotech Lab at the Southern Agricultural Research Institute (SARI), Hawassa, Ethiopia. Leaves with lesions of ALS were sampled and collected from fields of common bean during the field survey in 2016 and 2017 in diverse agroecological zones of Ethiopia that are known major common bean production areas (Fig 4.1). A total of 78 pure and single spores were isolated from infected and diseased leaves collected from the various common-bean growing regions using methods developed by CIAT (Table 4.1). Moreover, one additional isolate already characterised isolate from an Andean gene pools that was obtained from CIAT-Uganda was included in the study to differentiate the Ethiopian isolates into Middle American and Andean groups (Ddamulira *et al*., 2014). Isolation and monosporic

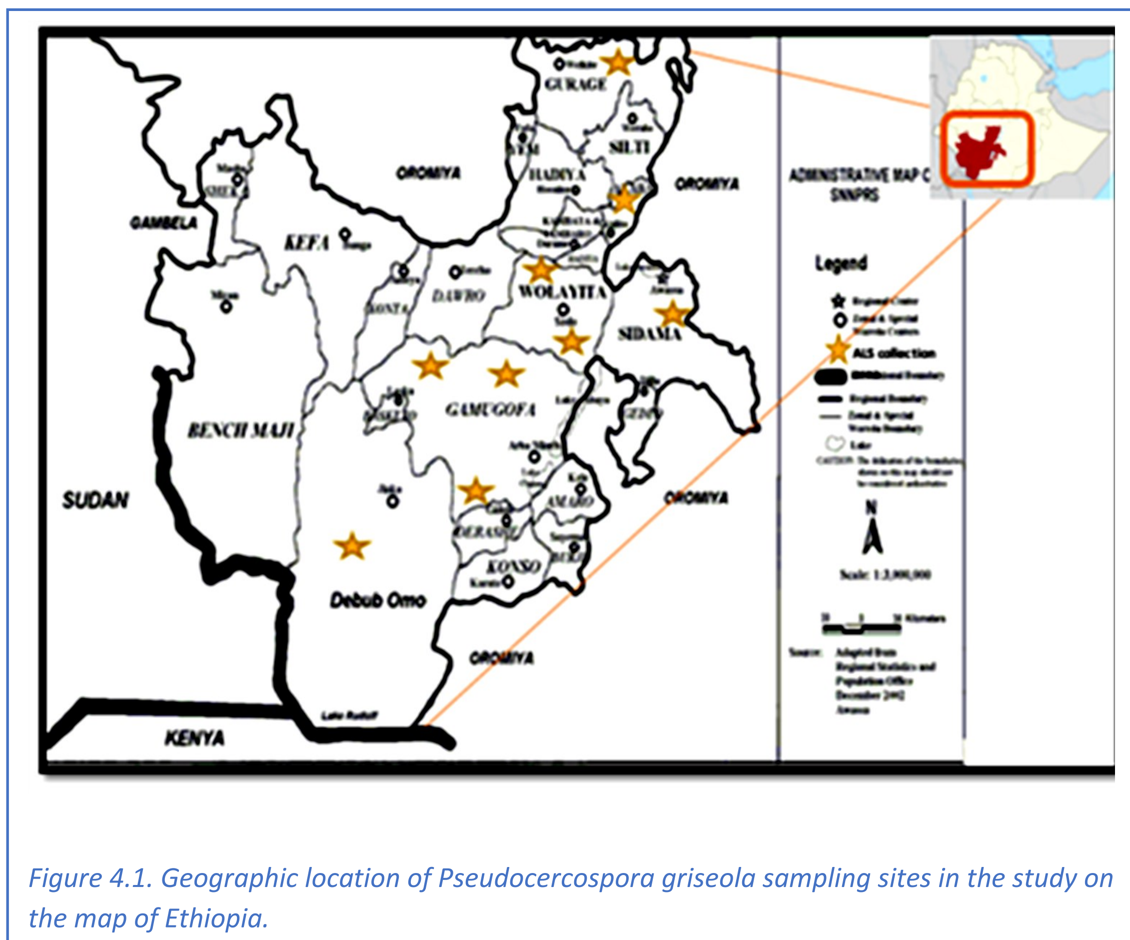

Figure 4.1. Geographic location of Pseudocercospora griseola sampling sites in the study on the map of Ethiopia.

culture were done according to the method developed by Pastor- Corrales *et al.* (1998). Accordingly, freshly infected leaves of common bean were used and single spore were transferred from fungal structures formed on lesions to culture media, using a sterilized fine needle under a dissecting microscope (Motic compound microscope). Monosporic cultures of *P. griseola* were grown on V8 culture media in 12h dark and light incubator for 20 days at 25ºC until genomic DNA extraction.

Table 4.1 Single spore isolates of *P. griseola* from the diverse bean growing areas of Ethiopia

Isolates CODE	Location	Altitude	Host	Genepool	Year of Collection
pget001	WONDO	1742masl	PIC6	A	2017
pget002	WONDO	1742masl	ADP-0100	A	2017
pget003	GOFA	1400masl	SMALL RED	M	2017
pget004	WONDO	1742masl	ADP-0095	A	2017
pget005	WONDO	1742masl	ADP-0468	A	2017
pget006	GOFA	1400masl	SMALL RED	M	2017
pget007	HALABA	1872masl	TATU	A	2017
pget008	GOFA	1400masl	HDUME	M	2017
pget009	WONDO	1742masl	ADP-0668	A	2017
pget010	WONDO	1742masl	ADP-0518	A	2017
pget011	WONDO	1742masl	ADP-0037	A	2017
pget012	WONDO	1742masl	ADP-0037	A	2017
pget013	WONDO	1742masl	ADP-0675	A	2017
pget014	WONDO	1742masl	ADP-0675	A	2017
pget015	WONDO	1742masl	ADP-0675	A	2017
pget016	WONDO	1742masl	ADP-0675	A	2017
pget017	DOLLA	1865masl	REDWOLAITA	M	2017
pget018	DOLLA	1865masl	REDWOLAITA	M	2017
pget019	DOLLA	1865masl	REDWOLAITA	M	2017
pget020	DOLLA	1865masl	REDWOLAITA	M	2017
pget021	DOLLA	1865masl	REDWOLAITA	M	2017
pget022	DOLLA	1865masl	REDWOLAITA	M	2017
pget023	DOLLA	1865masl	REDWOLAITA	M	2017
pget024	DOLLA	1865masl	REDWOLAITA	M	2017
pget025	DOLLA	1865masl	REDWOLAITA	M	2017
pget026	DOLLA	1865masl	REDWOLAITA	M	2017
pget027	DOLLA	1865masl	RED WOLAITA	M	2017
pget028	DOLLA	1865masl	RED WOLAITA	M	2017
pget029	DOLLA	1219masl	NASIER	M	2017
pget030	CHANO DORGA	1219masl	NASIER	M	2017

Isolates CODE	Location	Altitude	Host	Genepool	Year of Collection
pget031	SOUTH OMO	1363masl	RED WOLAITA	M	2017
pget032	SOUTH OMO	1363masl	RED WOLAITA	M	2017
pget033	SOUTH OMO	1363masl	H DUME	M	2017
pget034	SOUTH OMO	1363masl	H DUME	M	2017
pget035	SOUTH OMO	1363masl	H DUME	M	2017
pget036	SOUTH OMO	1363masl	H DUME	M	2017
pget037	SOUTH OMO	1363masl	H DUME	M	2017
pget038	CHANO MILE	1219masl	NASIER	M	2017
pget039	BAKO GAZAR	1363masl	SMALL RED	M	2017
pget040	BAKO GAZAR	1363masl	SMALL RED	M	2017
pget041	SOUTH OMO	1363masl	HDUME	M	2017
pget042	AREKA	1802masl	ADP	A	2017
pget043	AREKA	1802masl	ADP	A	2017
pget044	CHANO DORGA	1219masl	NASIER	M	2017
pget045	CHANO DORGA	1219masl	NASIER	M	2017
pget046	CHANO DORGA	1219masl	NASIER	M	2017
pget047	CHANO DORGA	1219masl	NASIER	M	2017
pget048	CHANO DORGA	1219masl	NASIER	M	2017
pget049	CHANO DORGA	1219masl	NASIER	M	2017
pget050	CHANO DORGA	1219masl	NASIER	M	2017
pget051	CHANO DORGA	1219masl	NASIER	M	2017
pget052	CHANO DORGA	1219masl	NASIER	M	2017
pget053	CHANO DORGA	1219masl	NASIER	M	2017
pget054	CHANO DORGA	1219masl	NASIER	M	2017
pget055	CHANO DORGA	1219masl	NASIER	M	2017
pget056	CHANO DORGA	1219masl	NASIER	M	2017
pget057	CHANO DORGA	1219masl	NASIER	M	2017
pget058	CHANO DORGA	1219masl	NASIER	M	2017
pget059	CHANO MILE	1212masl	NASIER	M	2017
pget060	CHANO MILE	1219masl	NASIER	M	2017
pget061	CHANO MILE	1219masl	NASIER	M	2017
pget062	CHANO MILE	1219masl	NASIER	M	2017
pget063	CHANO MILE	1219masl	NASIER	M	2017
pget064	CHANO MILE	1219masl	NASIER	M	2017
pget065	CHANO MILE	1219masl	NASIER	M	2017
pget066	CHANO MILE	1212masl	NASIER	M	2017
pget067	CHANO MILE	1212masl	NASIER	M	2017
pget068	CHANO MILE	1212masl	NASIER	M	2017
pget069	CHANO MILE	1212masl	NASIER	M	2017
pget070	HALABA	1872masl	HDUME	M	2016
pget071	GURAGE	1604masl	NASIER	M	2016
pget072	GURAGE	1700masl	NASIER	M	2016
pget073	GURAGE	1772masl	NASIER	M	2016

Isolates CODE	Location	Altitude	Host	Genepool	Year of Collection
pget074	AREKA	1884masl	REDWOLAITA	M	2016
pget075	GURAGE	1742masl	NASIER	M	2016
pget076	KAO60 /CIAT			A	2016
pget077	240				2016
pget078	220				2016
pget079	224				2016

M=Middle American gene pool, A=Andean gene pool, m.a.s.l. =meter above sea level

4.2.2. Genomic deoxyribonucleic acid (DNA) extraction

Genomic DNA was extracted using a protocol described by Mahuku (2004) with minor modification. The harvested fresh fungal mycelium was transferred to sterilized 1.7 ml tube containing 500 µl of TES extraction buffer (0.2 m Tris–HCl pH 8.0; 10 mm EDTA, pH 8.0, 0.5 m NaCl, 1% SDS); sterilized sand was added and grinded using mortar and pistil. The samples were mixed using vortex for 30s and then incubated in the water bath at 65 ºC for 30 minutes before it was centrifuged for 15 minutes at 20,800g. The supernatant was transferred to a new tube and an equal volume of ice cold isopropanol was added. Tubes were then incubated at -20ºC for 1 hour, followed by centrifugation for 10 minutes at 20,800g to pellet the DNA. The supernatant was eliminated and the DNA pellet was washed with 800 µl of cold 70% ethanol; the tubes were then turned upside down on clear sterile paper towel for 45 minutes to air dry. Then the dried DNA pellet was diluted with 1 x TE buffer and ready for the PCR.

4.2.3. Rep-PCR fingerprinting

In rep-PCR, fingerprinting three families of repetitive sequences were used (Table 4.2). They included 1) the repetitive extragenic palindromic (REP) sequence REP1R-1/REP2-1 (18 nucleotides in length), as described by Versalovic *et al* (1991); 2) the enterobacterial repetitive intergenic consensus (ERIC) in which two oligonucleotide primer pairs used for PCR amplification ERIC1R/ERIC2 (22 nucleotides in length) and 3) BOX elements (22 nucleotides in length) (Lupski *et al.,* 1992). Optimal PCR conditions for each of the primer sets used were as described by Versalovic *et al.* (1994) with minor modification of the annealing temperature. The reproducibility of rep-PCR was tested by amplifying DNA two times from ten randomly chosen strains. The PCR amplifications were performed with a thermal cycler 2710 using PCR premix (GEillustra),(https://us.bioneer.com/products/accupower/accupower-premix-overview.aspx).

The PCR products were electrophoresed in a 1.5% agarose gel for 2 h at a constant voltage of 90 V in 1×TAE buffer (40 mMTris–Acetate, 1 mM EDTA, pH 8.0) at 4 °C. Gels were stained in ethidium bromide (0.5 µg/ml) and the rep-PCR profiles and fingerprinting patterns were visualized under UV light, and the image was captured using a Canon digital camera mounted on the visualization hood.

Table 4.2 Molecular markers used to amplify PCR fingerprinting products of *Pseudocercospora griseola*

Genetic markers	Sequences 5' to 3'	T_a ^{0}C	GC %	Number of nucleotides	References
REP 1R	IIIICGICGICATCIGGC	49	52.9	18	Seurink et al.2003
REP 2	IIICGNCGNCATCNGGC	58	52.9	17	Seurink et al., 2003
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	58	50	22	Coenye et al., 2002
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	42	54.5	22	Coenye et al., 2003
BOX A1R	CTACGGCAAGGCGACGCTGACG	50	68.2	22	Versalovic et al 1991

I = Inosine

4.2.4. Data analysis and interpretation

4.2.4.1 Analysis of genetic similarity and dissimilarity

The results of PCR fingerprinting with ERIC, BOX, and REP primers were collected into matrices with scored presence (1) or absence (0), of banding pattern in each PCR analysis lane. In each case, a simple matrix was obtained by comparing pairs of isolates of *P. griseola* using a simple matching coefficient (SM). A dendrogram was constructed with all parameters together after cluster analysis with the Dice similarity matrix, the Jaccard dissimilarity matrix and the Euclidean distance (Rencher, 1995). As suggested by the Kosman diversity and distance measures (Kosman and Leonard 2007) for populations with an asexual and mixed mode of reproduction were considered in this specific study to measure the genetic diversity with populations and distance between populations. The Kosman distance and diversity measures for populations were calculated using different measures of dissimilarity between individuals (Jaccard, and Dice coefficients of dissimilarity). Similarity among the profiles was calculated using the Dice similarity matrix. The clustering was based on an average linkage or unweighted pair group method with arithmetic averages (UPGMA).

4.2.4.2. Analysis of molecular variance (AMOVA) and genetic diversity

An analysis of molecular variance (AMOVA) was performed using GenAlEx6.1 (Peakall and Smouse, 2006) to assess genotypic variations across all the populations studied (Tables 4.3 & 4.4). The analysis included partitioning of total genetic variation into within-groups and among groups variance components, hence, it provided a measure of intergroup genetic distance as proportion of the total variation residing among populations. The significance of analysis was tested using 999 random permutations.

4.5. Results

4.5.1. Analysis of molecular markers

4.5.1.1. Rep-PCR amplification in Pseudocercospora griseola

Rep-PCR analysis using primer sets REP, ERIC and BOX of highly conserved repetitive sequences resulted in differential banding patterns among and within *P. griseola* populations collected from the diverse common bean growing regions of Ethiopia. In Rep-PCR, three families of repetitive sequences were used, including the repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive extragenic palindromic sequence, enterobacterial repetitive intergenic consensus (ERIC), and BOX elements (Lupski *et al.,*1992).

Amplification of genomic DNA from the *P. griseola* isolates collected from the diverse common bean growing regions of Ethiopia with rep-PCR resulted in complex fingerprint patterns (Figs 4.2, 4.3 & 4.4). Rep-PCR fingerprint patterns for isolates of *P. griseola* were examined. The size of amplification products ranged from 100bp to 1500bp. Analysis of the ERIC PCR fingerprinting patterns by unweighted pair group method with arithmetic mean (UPGMA) using Dice similarity coefficient resulted 17 distinct groups among the *79 P. griseola* at 77% similarity cut of level (Fig 4.2). While BOX and REP PCR fingerprinting pattern discriminated 5 and 11 distinct groups among the 79 *P. griseola* at cut-off 60 and 66% similarities level respectively (Figs 4.3 & 4.4). Hence, ERIC-PCR was the most informative to differentiate isolates of *P. griseola* collected from the diverse common bean growing regions of Ethiopia. The dendrogram obtained from the cluster analysis using combined ERIC-, REP- and BOX-PCR genomic fingerprints revealed the overall grouping of the *P. griseola* isolates collected from the diverse areas of Ethiopia. Thus, combined REP-

PCR fingerprinting discriminated 26 distinct groups among the 79 isolates of *P. griseola* at a cut off 77% similarity molecular level (Figure 4.5).

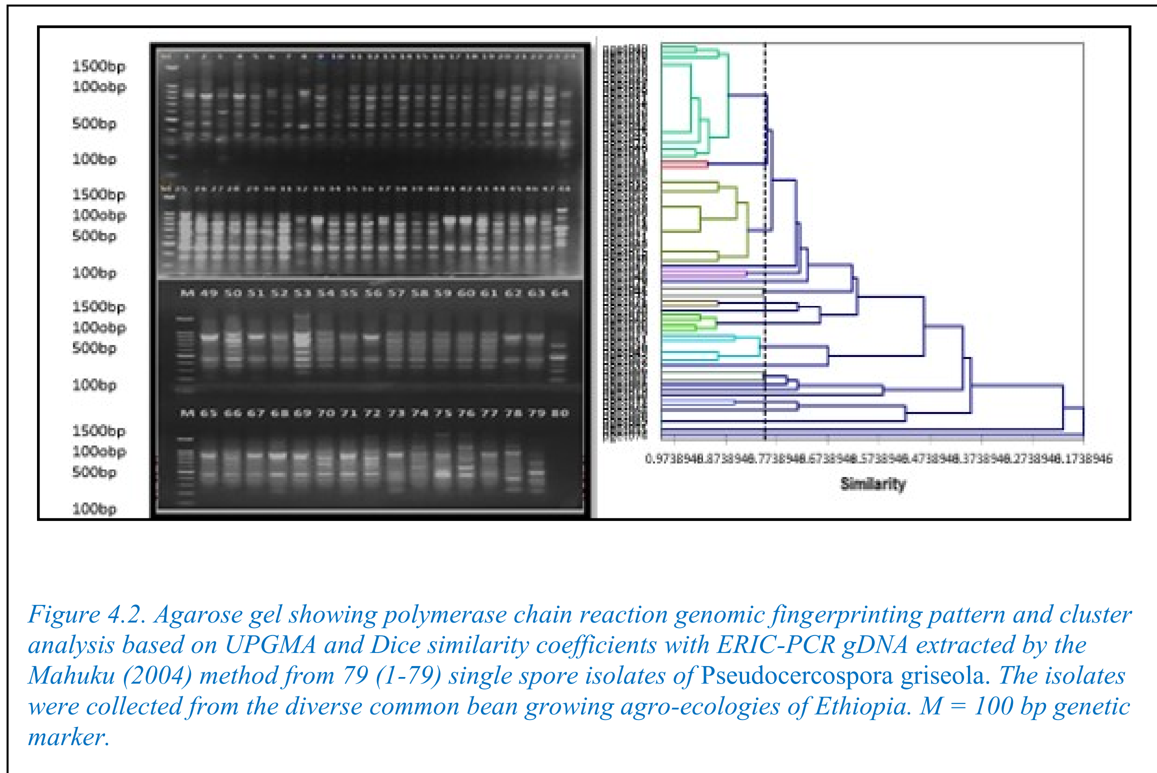

Figure 4.2. Agarose gel showing polymerase chain reaction genomic fingerprinting pattern and cluster analysis based on UPGMA and Dice similarity coefficients with ERIC-PCR gDNA extracted by the Mahuku (2004) method from 79 (1-79) single spore isolates of Pseudocercospora griseola. *The isolates were collected from the diverse common bean growing agro-ecologies of Ethiopia. M = 100 bp genetic marker.*

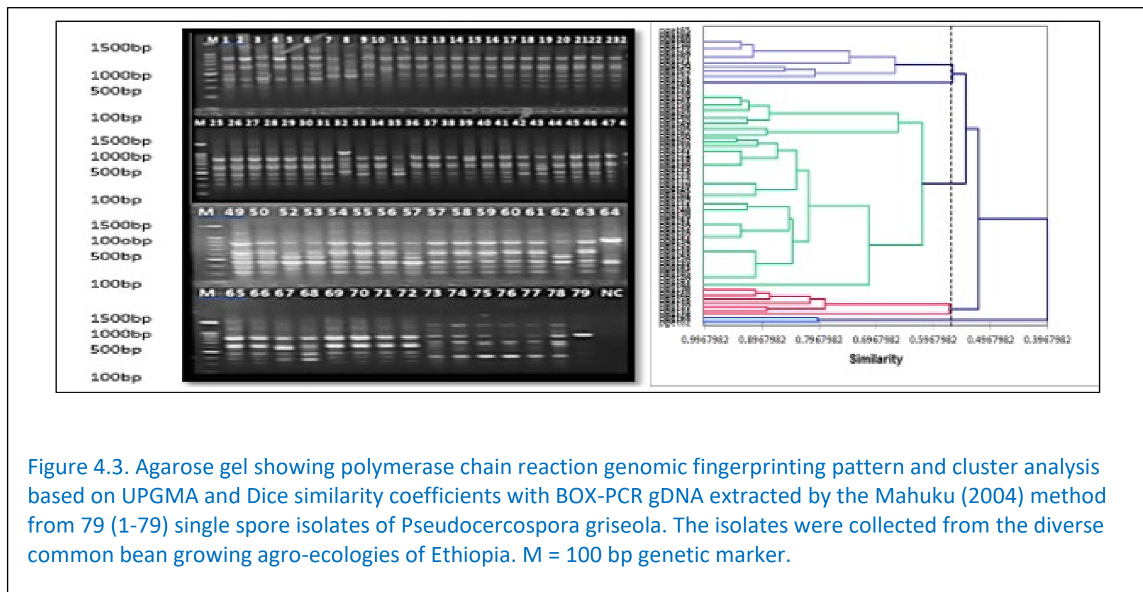

Figure 4.3. Agarose gel showing polymerase chain reaction genomic fingerprinting pattern and cluster analysis based on UPGMA and Dice similarity coefficients with BOX-PCR gDNA extracted by the Mahuku (2004) method from 79 (1-79) single spore isolates of Pseudocercospora griseola. The isolates were collected from the diverse common bean growing agro-ecologies of Ethiopia. M = 100 bp genetic marker.

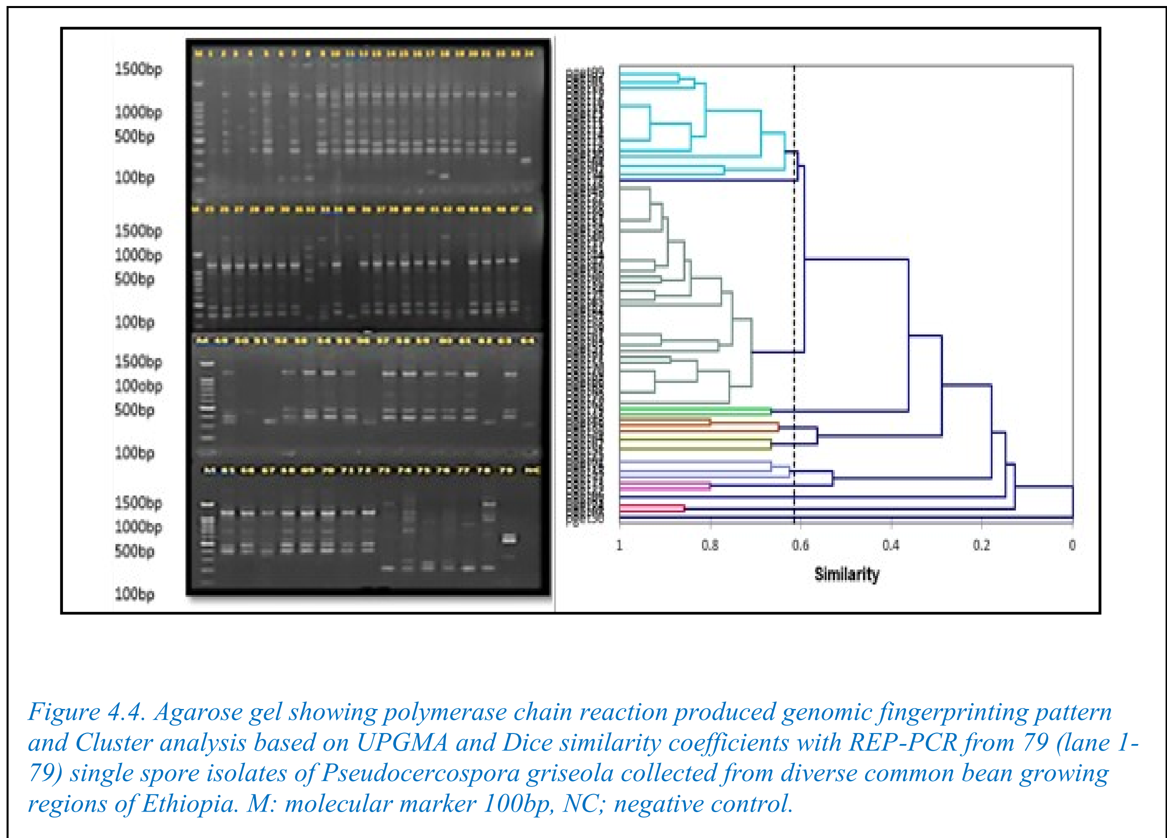

Figure 4.4. Agarose gel showing polymerase chain reaction produced genomic fingerprinting pattern and Cluster analysis based on UPGMA and Dice similarity coefficients with REP-PCR from 79 (lane 1-79) single spore isolates of Pseudocercospora griseola collected from diverse common bean growing regions of Ethiopia. M: molecular marker 100bp, NC; negative control.

4.5.1.2. Analysis of molecular variance (AMOVA)

The analysis of molecular variance (AMOVA), which revealed 83% and 17% genetic variations ($P<0.05$) within and among the monosporic isolates of *P. griseola* obtained from the collections of the diverse common bean growing areas of Ethiopia (Fig 4.5).

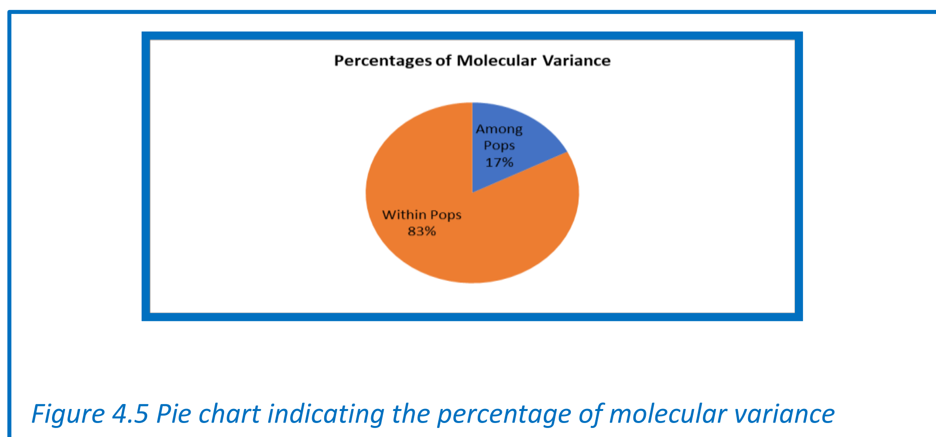

Figure 4.5 Pie chart indicating the percentage of molecular variance

4.5.3. Cluster analysis of BOX, REP and ERIC-PCR fingerprinting pattern

Cluster analysis was performed on the combined DNA fingerprints produced from BOX, REP and ERIC PCR products (Figure 4.5). The dendrogram obtained from the cluster analysis of combined (REP/BOX/ERIC) Rep-PCR fingerprinting patterns discriminated the entire monosporic *P. griseola* isolates, that were collected from various common bean regions of Ethiopia into 25 distinct types among the 79 *P. griseola* isolates. The results of the present study determined primarily the usefulness of Rep-PCR genomic fingerprinting as complimentary or as an alternative strategy to other methods in studying the genomic diversity of *P. griseola*. The genetic structure of *P. griseola* revealed no geographical differentiation (Fig 4.6)

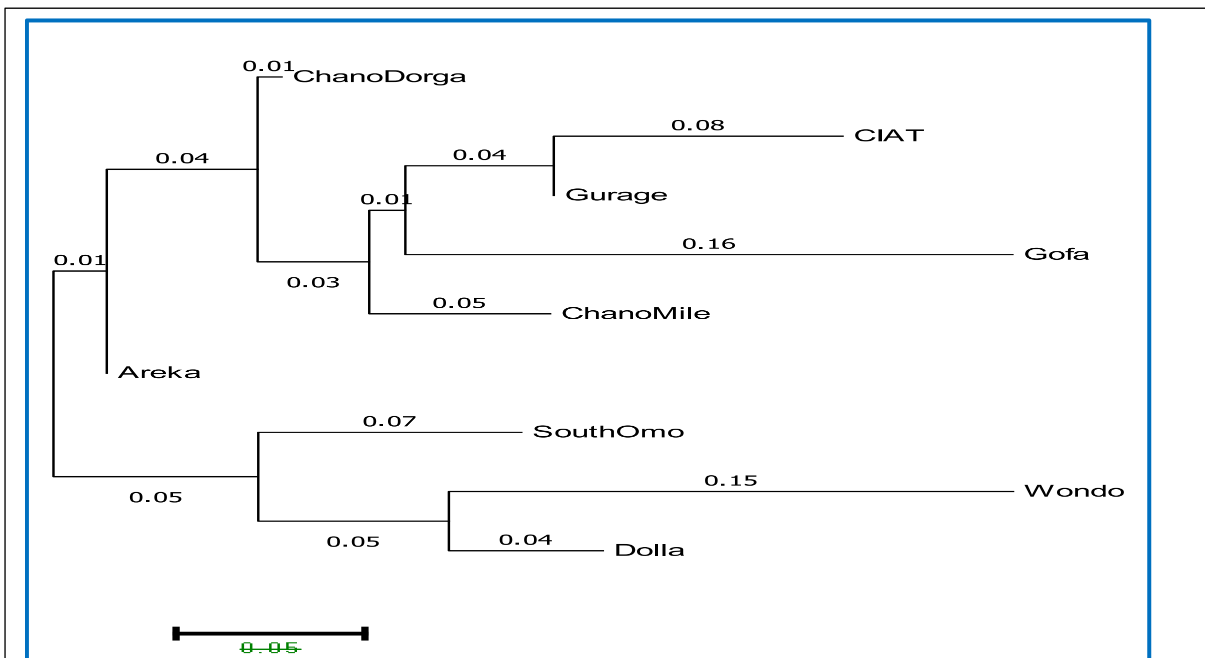

Figure 4.6. The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.75085937 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ME tree was searched using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1. The Neighbour-joining algorithm was used to generate the initial tree. Evolutionary analyses were conducted in MEGA6.

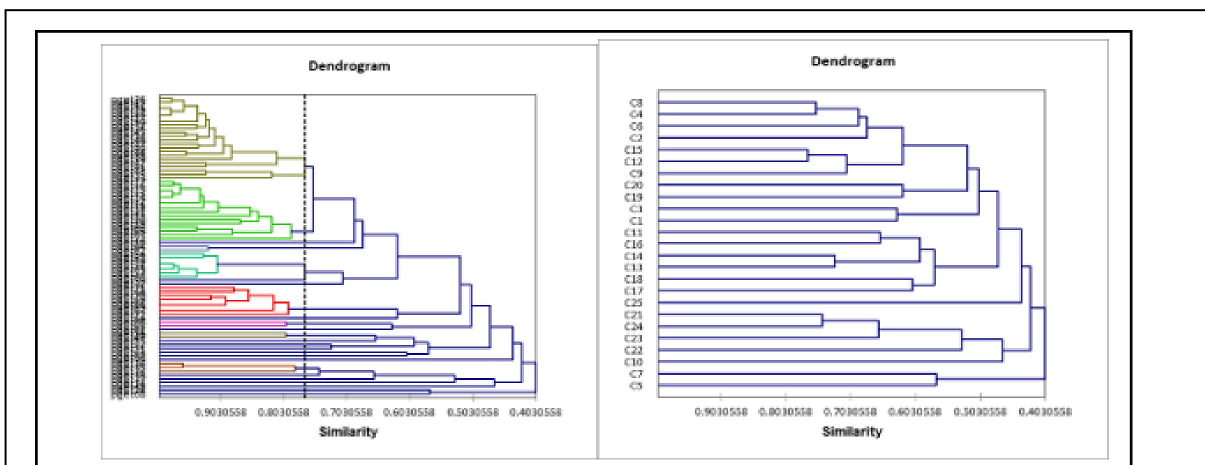

Figure 4.7. Cluster analysis based on UPGMA and Dice similarity coefficients obtained from the combined REP, BOX and ERIC genomic fingerprinting patterns of 79 single-spore isolates of Pseudocercospora griseola collected from diverse common bean growing regions of Ethiopia. Same Colors within the cluster indicates genetic similarity of P. griseola isolates and the dendrogram in the right side indicates the genetically discriminated 25 clusters among the 79 monosporic isolates

4.5. Discussion

The genomic DNA fingerprinting patterns found among the *P. griseola* isolates obtained from infected common bean leaves collected from various common bean growing areas of Ethiopia were found to be varied in size and number depending on each *P. griseola* isolates indicating the existence of diverse genetic variability within each isolate. However, some of the isolates showed similar DNA fingerprinting patterns with only minor differences; hence, these isolates with similar genomic DNA fingerprinting pattern were clustered in the same group. The Rep-PCR primers set families of ERIC, REP and BOX generated multiple distinct DNA genomic fingerprints ranging from 100bp to 1500pb (Figs 4.1, 4.2, & 4.3). The results of genomic DNA fingerprint profiles obtained from monosporic isolates complement with the many of the previous reports and can be reproducible from one experiment to another (McDonald *et al*., 2000; Mehta *et al*., 2002; de Aruba *et al.*, 2003; Abdollahzadeh and Zolfaghari 2014 & Ddamulira *et al.,* 2014). Previously the distribution of ERIC, REP and BOX elements has been examined and reported in diverse prokaryotic genomes (Versalovic *et al.,* 1991). The observed significant variation within the monosporic isolates of *P. griseola*, among the isolates of the same geographic locations were due to the co-existence of diverse host genotypes and based on many of the reports the pathogen might undergone par asexual that facilitates exchange of genetic material within and between isolates. It might also be because of chromosomal inversion, deletion and presence of transposons since all are reported to have capability to increase the variability in *P. griseola* (Kristler and Miao, 1992; Kempken and Kuck, 1998). The genetic structure of *P. griseola* revealed no geographical differentiation. The small reds & white coloured beans from the Mesoamerican gene pool have been predominantly cultivated in Ethiopia with the exception of a few areas known for the cultivation large

and speckled red beans from the Andean gene pool (Asrat *et al.,* 2009). Therefore, geographical specialization was not evident. This has important implications for the deployment of angular leaf spot resistance genes and the development of common bean cultivars for the ALS disease resistance. High genetic variability of *P. griseola* was observed in areas typically cultivating Mesoamerican common bean. Since Mesoamerican common beans are predominantly cultivated in Ethiopia, the greatest challenge to manage angular leaf spot of the common bean is in areas that are known for the cultivation of beans from the Mesoamerican genepools.

The lack of isolation by distance among the isolates of *P. griseola* from the diverse common bean growing areas of Ethiopia indicates the *P. griseola* fungi have efficient dispersal at the common bean growing areas of the region. From our study we confirmed that the genetic divergence between the populations was very low which was 13% whereas, 87% of the molecular variance was attributed to the variation within populations. This results were indicated with sharing of rep-PCR genomic finger printing pattern between geographic populations from distinct locations of common bean growing areas of Ethiopia, (for example genomic finger printing patterns between Dolla and south Omo the two locations that are far away for about 450 km from each other). The observed gene flow and sharing of same genomic fingerprints between isolates of the two distinct locations could be due to different possibilities; one of the possibilities for the long-distance gene flow might be due to the long-distance gene flow nature of the pathogen and second due to spore dispersal without human interference because of the wind and other natural influence. Moreover, the long distance gene flow over hundreds to thousands of kilometers has been reported in many of fungi (James *et al.,* 1999; Kuyper, 2003; Edman and Gustafson, 2003) or the other possibility for the long-distance gene flow could be also be due to the human

involvement and the seed born nature of *P. griseola.* The informal seed system, which is common practice and is associated with movement of infected planting materials between different locations or common bean growing areas, including wind dispersal could be the main causes for the observed genomic fingerprinting pattern between distinct locations. This was explained with the presence of *P. griseola* isolates from different geographical regions in the same branch of the dendrogram. Human activities were reported and found to be responsible for the long-distance dispersal of may fungi and pathogens (Fry *et al.,* 1992; Roche *et al.,* 1995; Milgroom *et al.,* 1996; Zeng and Luo, 2006). This study is the first report using rep-PCR genomic finger printing on genomic variation and population structure of *P. griseola* isolates that were collected from diverse common bean growing regions of Ethiopian. The results revealed that *P. griseola* in Ethiopia demonstrates high level of genomic diversity. As previously reported, Rep-PCR fingerprinting was a highly reproducible and a simple method to distinguish closely related fungal isolates. To infer the phylogenic relationships and to study their diversity in different ecosystems (de Bruijn *et al.,* 1992; Ishii and Sadowsky, 2009). The majority of our *P. griseola* samples were from the southern parts Ethiopia which is known for its wider and potential common bean production areas of Ethiopia. The area is known for its hotspot area for the angular leaf spot and majority of the isolates of *P. griseola* from this area were confirmed to be genetically very diverse and this area might not represent other parts of Ethiopia. The analysis of additional samples from other areas as well as more genes might allow to define the population structure of *P. griseola* existing in Ethiopia. The result from this study represents an important step towards understanding the presence of high genetic diversity within the *P. griseola* existing in common bean production areas of Ethiopia and hence the common bean breeding program aiming to develop durable resistance varieties should consider this

information during the deployment of resistance genes to develop resistance common bean varieties.

4.6. Conclusion

This study was the first report on the genomic variation and population structure of *P. griseola* that were collected from the diverse common bean growing regions and the result revealed that *P. griseola* in Ethiopia displays with high level of genomic diversity. The genetic structure of *P. griseola* reveals no geographic differentiation. Moreover, the result from this specific study compliments with many of the reports that confirms the sources of genomic variability existed within and among the monosporic isolates of *P. griseola* obtained from the diverse common bean growing areas of Ethiopia might be the informal seed system that was dominantly practiced with common bean seed system within the small-scale farming community. In addition of that the movement of infected planting materials between different location in the common bean growing areas including wind dispersal of spores also the main contributors to the presence of *P. griseola* isolates from the different geographic regions in the same group and for the absence of geographic differentiation between common bean growing locations. Rep-PCR fingerprinting was a highly reproducible and a simple method to distinguish closely related fungal isolates. The regional and national common bean improvement programs in Ethiopia should also give priorities for gene deployment and marker aided gene pyramiding techniques in developing broad and multiple disease resistance common bean varieties along with identification of new sources of resistance common bean cultivar.

CHAPTER FIVE

Genome-Wide Marker Trait Association Study of Angular Leaf Spot (*Pseudocercospora griseola)* Resistance in Common Bean (*Phaseolus Vulgaris L.*)

Abstract

Angular leaf spot (ALS), caused by *Pseudocercospora griseola*, is one of the major diseases that affect common bean production all of the world. The fungus is highly diverse and variable and has the ability to infect bean varieties from both Mesoamerican and Andean gene pools. Identification of specific resistance loci and alleles variants contributing to improve resistance may be useful to breeders by allowing selection of resistance alleles with favourable agronomic characteristics. Genome wide association studies (GWAS) have been widely used in genetic dissection of complex traits, especially with the development of advanced genomic sequence technologies. Association analysis was performed using the most powerful compressed mixed linear model (CMLM) including kinship matrix using the GAPIT R computer program. The chromosomal location of ALS resistance genes was established using a genome-wide association study based on genotyping 3,335 SNPs variants across 288 diverse common bean genotypes. We observed a total of 18 significant marker-trait associations, which were distributed on chromosomes Pv04 and Pv08. The locus on chromosome Pv04 was the most saturated locus with 11 significantly associated SNP markers, followed by chromosome Pv08 with 7 significant SNPs. Hence, two candidate genes were detected for further verification. The result also indicates a quantitative and complex inheritance pattern for resistance to ALS disease in common bean, confirming earlier results. Our results demonstrate the great potential of genome-wide association studies to identify quantitative resistance loci for angular leaf spot of the common bean.

Keywords*: Pseudocercospora griseola*, resistance quantitative locus, genomic region, GWAS, significant markers

5.1 . Introduction

Common bean (*Phaseolus vulgarise L.*) is widely grown for food and income by small-scale farmers in Ethiopia. It is frequently affected by the angular leaf spot (ALS) caused by *Pseudocercospora griseola*, which is one of the major diseases affecting common bean production in Ethiopia and other tropical areas of the world. The disease can lead to 80% yield loss. The use of resistance is the main means for disease control. However, the pathogen is highly diverse, which makes genetic control quite challenging (Lemessa *et al*., 2011; Ddamulira *et al*., 2014).

The QTL approach based on bi-parental populations provides results specific to each population. Linkage mapping based on a segregating population from a cross between two parents with different phenotypes is well known approaches to locate quantitative trait loci(QTL). They are statistically inferred, generally vis linear regression and maximum likelihood estimate methods (Zeng, 1994), and based on a genetic linkage map (lander and Bostein, 1989). Only a few QTLs are generally detected vis linkage mapping in each experiment. Further fine mapping of QTL to more narrowly precise genetic position and cloning of the underlying gene, as large secondary population are required to achieve sufficient map resolution (Dinka *et al.,* 2007), these procedures are resource and time-consuming process. It is therefore, not suitable to survey genotypic variation across the entire genome in a gene pool. In contrast, genome wide association studies (GWAS) have emerged as a powerful approach for screening genetic variation underlying complex phenotypes. GWAS using a diverse population provides another strategy to effectively fine map QTL due to a large number of historical recombination event that lead to rapid decay of linkage disequilibrium(LD) (Flint-Garcia *et al.,* 2003). This strategy of GWAS was applied for the first time in 2005 to a human disease (Edwards *et al.,* 2005). Subsequently, an increasing series of research results on GWAS

have been published (Hindorff *et al.,* 2009; Korte and Farlow 2013; Huang and Han 2014) and stimulated by the increasing availability of high-throughput marker systems. For both QTL and GWAS approaches, further research is required to validate the results and eventually identify the candidate genes. Association mapping, unlike conventional QTL mapping, does not rely on populations of known pedigree; therefore, it is important to consider population structure and kinship among individuals, because they may lead to the confounding effects (Oraguzie *et al.,* 2007)

Genome-wide association studies (GWAS) or association mapping (AM) offer higher resolution because historical recombination accumulated in natural populations and collections of landraces, breeding materials, and varieties, has reduced linkage disequilibrium (LD) (Rossi *et al.,* 2009). LD analysis, which effectively incorporates the effects of many past generations of recombination, has often been instrumental in the final phases of gene localization (Feder *et al.,* 1996). By exploiting broader genetic diversity, GWAS offers several advantages over bi-parental mapping, such as mapping resolution, allele number, time saved in establishing marker-trait associations, and application in breeding programs (Yu *et al.,* 2006). The strength of the correlation between two markers is a function of the distance between them: the closer two markers are, the stronger the LD. The resolution with which a QRL can be mapped is a function of how quickly LD decays over distance. Selfing reduces opportunities for recombination; thus, in self-pollinating species such as rice (*Oryza sativa*), LD may extend to 100 Kb or more (Flint-Garcia *et al.,* 2007). In common bean, information is available on the extent of LD, especially since the development of whole-genome DNA reference sequences (Kwak and Gepts, 2009; Rossi *et al.,* 2009; Blair *et al.,* 2010; Schmutz *et al.,* 2014).

Genome-wide association studies have proven to be one of the most efficient methods relative to time, cost, and precision for identifying candidate genes that control agriculturally important traits. However, besides insufficient computing speed, low statistical power and false positives are also factors that influence GWAS performance and reliability (Atwell *et al.,* 2010; Yang *et al.,* 2014). A typical GWAS can have an inflated false positive rate if the statistical model used includes only a tested genetic marker, such as a single-nucleotide polymorphism (SNP), as an explanatory variable. Indeed, associations between a genetic marker and a phenotype occur for many reasons, in addition to the genetic linkage between the tested genetic markers and functional causal polymorphisms. For example, population structure and relatedness among individuals are two common sources of false positives (Falush *et al.,* 2007). Consequently, population structure and individuals' total genetic effects are often fitted as covariates in a mixed linear model (MLM) to reduce the false discovery rate (FDR) (Yu *et al.,* 2006). Unfortunately, this reduction of false positives can also increase false negatives through confounding phenotypes with population structure and individuals' total genetic effects (Atwell *et al.,* 2010). Therefore, new analysis methods with greater statistical power are critical for resolving these confounding issues and improving interpretive reliability (Yang *et al.,* 2014). Several methods have been developed to improve the computing speed of Mixed linear models(MLMs) including efficient mixed-model association (EMMA) (Kang *et al.,* 2014), EMMA accelerated and population parameter previously determined (Kang *et al.,* 2010; Zhang *et al.,* 2010), genome wide EMMA (Zhou and Stephens, 2012) FaST-LMM (Lippert *et al*., 2011), and Gen ABEL (Svishcheva *et al*., 2012). The compressed mixed linear model (CMLM) replaces the individual's genetic effects with those the of the group to which each individual belongs (Zhang *et al.,* 2010). That is, individuals are clustered into

groups on the basis of their relationships derived from all available genetic markers. Simulation demonstrate that CMLM improves statistical power by 5 to 15 % compared to regular MLM (Zhang *et al.,* 2010) additionally CMLM includes a dramatic reduction in computing time. The CMLM approach was implemented in the first release of GAPIT (Lipka *et al.,* 2012).

In this specific study, the genomic region of ALS resistance was examined by a genome-wide association study (GWAS) based on genotyping of 3,335 SNP variants across 288 diverse common bean genotypes. This study was designed to identify any loci related to ALS resistance that could be important for common bean production and improvement.

5.2. Materials and Methods

5.2.1. Plant materials

A very diverse common bean germplasm sample was established by Cichy *et al.* (2015), which included a collection of 288 landraces collections and breeding lines, collectively referred to as the Andean Diversity Panel (ADP) (Appendix Table 2). The ADP includes entries from the United States National Plant Germplasm Collection, East Africa, U.S., Canadian, and Ecuadoran bean breeding programs, African breeding programs collected by CIAT (Cali, Colombia), the CIAT Germplasm Collection, Angolan and Caribbean *Phaseolus* collections, landrace cultivars used as checks or parents in African breeding programs, and lines from a set of ALS resistance differentials. The large majority of these entries originated from the Andean domestication center of common bean (Cichy *et al.,* 2015).

5.2.2. Field experimental design and phenotyping and statistical analysis

The 288 entries were planted in an augmented randomized block design in which each block contains an incomplete set of entries and including 12 international sets of ALS differential common bean genotypes which were replicated in each block as standard checks within individual plots consisting of two rows of 3m long and planted during the main seasons of 2016 and 2017. The materials were evaluated under natural disease epidemics in two different environments, at the Wondogenet and Areka research stations located in southern Ethiopia, respectively at N07"03.968' E037"41.124' and 1803 m.a.s.l and 7°4′N 37°42′E and 1774 m.a.s.l with an average precipitation 1290mm. These two locations are known for its high infestation of both angular leaf spot and common bacterial blight. Disease severity was recorded at 55 and 66 days after planting using the 1-9 CIAT scale. The statistical analysis for the field phenotypic data was conducted using the Augmented SAS macro in SAS 9.4 (SAS Institute, 2011; Scott and Milliken, 1993). Each single environment was separately analysed for the angular leaf spot resistance/ response using a mixed model with genotypes as fixed and replications and incomplete blocks.

5.2.3. Genotyping of common bean germplasm and association mapping

We have used the SNP marker data from the ADP previously genotyped by Cichy et al. (2015) with the Illumina (Illumina Inc., San Diego, CA) BARCBean6K_3 SNP array with 5398 SNPs (Song *et al*., 2015). After filtering for low quality, monomorphic SNPs and for minor allele frequency (MAF>0.05), a total of 3,335 SNPs were retained for PCA, kinship matrix calculation, and genome wide association analysis. The kinship matrix developed using identity-by-descent method was included in the association analysis to correct for ambiguous relatedness. In addition, we applied a GWAS approach using GAPIT (Tang *et al.,* 2016) including a compressed mixed linear model

(CMLM) to calculate genomic best linear unbiased prediction (gBLUP) (Zhang *et al.,* 2010). The CMLM equation used in the analysis was as follows:

$Y = \mu + X\alpha + P\beta + Zu + \varepsilon$

where Y is N x 1 vector of BLUPs of genetic effect (N is the number of line), μ is the overall mean, **X** is the incidence matrix relating the individuals to the fixed marker effect **α**, ***P*** is the incidence matrix relating the individuals to the fixed principal component (**PC**) effects ***β***, and **Z** is the incidence matrix relating the individual to the random group effect **u** obtained from the compression algorithm. The random group effect u follows a multivariate normal distribution with mean 0 and variance-covariance matrix **2KVg**, where K is the kinship matrix, and Vg is the genetic variance component. The random error term ε follows a multivariate normal distribution with mean 0 and variance and co-variance matrix $\mathbf{IV_e}$, where I is the identity matrix and $\mathbf{V_e}$ the error variance component. Compressed Mixed Model (CMLM) and regular MLM were applied according to GAPIT (You Tang *et al.*, 2016). The first four PCs were involved in models as covariate variables according to the Bayesian Information Criterion (BIC) test of the model fitness. The significance threshold for SNP-trait associations was determined by false discovery rate (q value) < 0.05 or $P < 7.9 \times 10^{-5}$. The conservative Bonferroni corrected $p = 1.0 \times 10^{-5}$ (for $\alpha = 0.05$ and 3335 SNPs) was used to determine the significance threshold for SNPs.

5.3. Results

5.3.1. Phenotypic data analysis and the response of common bean to the *P. griseola*

The analysis of variance (ANOVA) for the genotypes tested for the angular leaf spot indicated that there was a highly significant (P< 0.001) genotype difference for the angular leaf spot under the field conditions. Hence all of the tested common bean

genotypes showed different reactions for the ALS disease. Severity of ALS for the combined sample ranged from resistance score (1.9) scale to susceptible scores (7.9). Distribution of phenotypes ranged from susceptible to resistance across the tested 288-common bean germplasm. Analysis of variance revealed the variance components for the genotype were highly significant (P< 0.001) differences between the common bean genotypes among the common bean and sets of differential and within the ALS differentials for the natural angular leaf spot infestation suggesting the presence of genotypic diversity for the angular leaf spot resistance among the 288 common beans germplasm (Table 5.1and Fig 5.1).

Table 5.1. Mean squares of analysis of variance for the 288 common bean genotypes evaluated under naturally endemic angular leaf spot disease during 2016 and 2017 across two locations Wondogenet and Areka, Ethiopia (ALSW16, ALSW17ALSA17 and ALSCOM) which are known for the disease pressure

		Mean square			
Source	**DF**	**ALS W16**	**ALSW17**	**ALSA17**	**ALSCOMB**
Block	5	0.222222	0.088889	2.280556	0.134333
Treatment	299	3.648644**	2.10195**	1.774703**	1.167315**
Tests	287	2.217615**	1.718745**	1.491113**	0.869834**
Controls	11	32.76767**	11.40767**	9.316919**	6.245606**
Tests vs Controls	1	94.04444**	9.71867**	0.200694**	30.683361**
Error	55	0.210101	0.088889	0.880556	0.089121**
* - Significant at 5% (level of significance opted by user), NS - Non Significant					
p-Value < 0.05 - Significant at 5% (*), p-Value < 0.01 - Significant at 1% (**)					
R-square		0.99	0.99	0.92	0.99
CV		8.47	5.99	19.33	5.92
ALS Mean		5.41	4.97	4.85	5.04

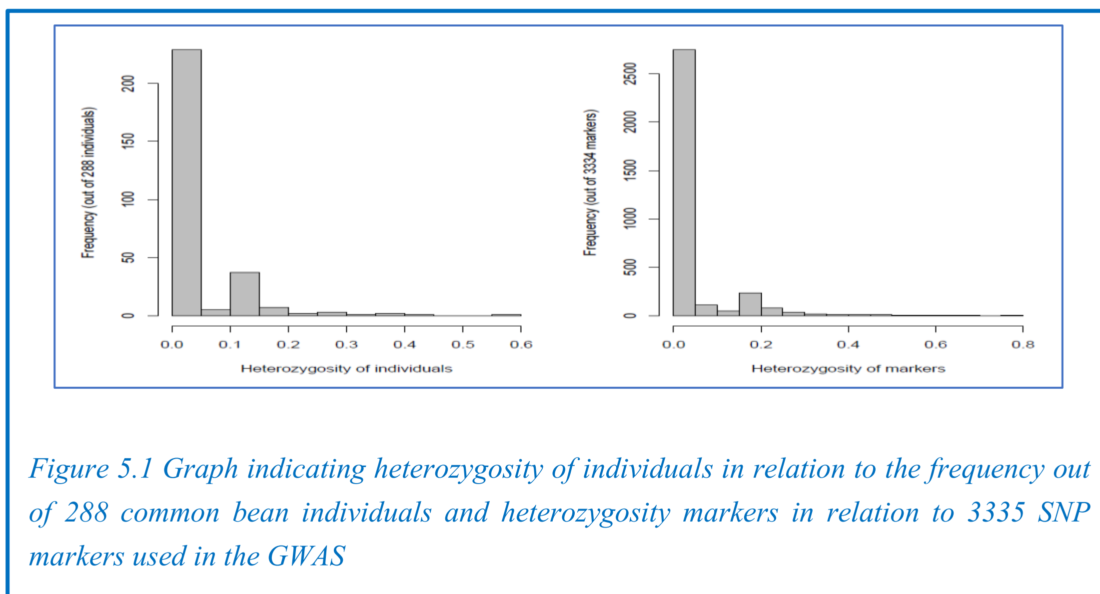

Figure 5.1 Graph indicating heterozygosity of individuals in relation to the frequency out of 288 common bean individuals and heterozygosity markers in relation to 3335 SNP markers used in the GWAS

5. 3.2. Principal component(PCs) and kinship analysis

Principal component analysis conducted using the GAPIT program indicated the first tow PCs explained the largest portions of the genetic variance, as shown in Figure 5.2.

The Misxed Model Association (EMMA) algorithm was used to establish a kinship matrix, a heat map of values in kinship matrix is show on in Figure 5.3(a). The kinship matrix is displayed as heatmap where red indicate the highest correlation between individuals and yellow indicate the lowest correlation hence the heat map showed low level of relatedness among the common bean genotypes. The displayed hierarchy tree was based on their kinship relation (Fig 5.3a). A heat map of the kinship matrix with genetic relatedness among the 288 common bean genotypes was included in GWAS. Since the observed and expected *P*-values differed substantially only for a few SNPs, the quantile-quantile (QQ) plot supported the appropriateness of the GWAS model (Table 5.2)

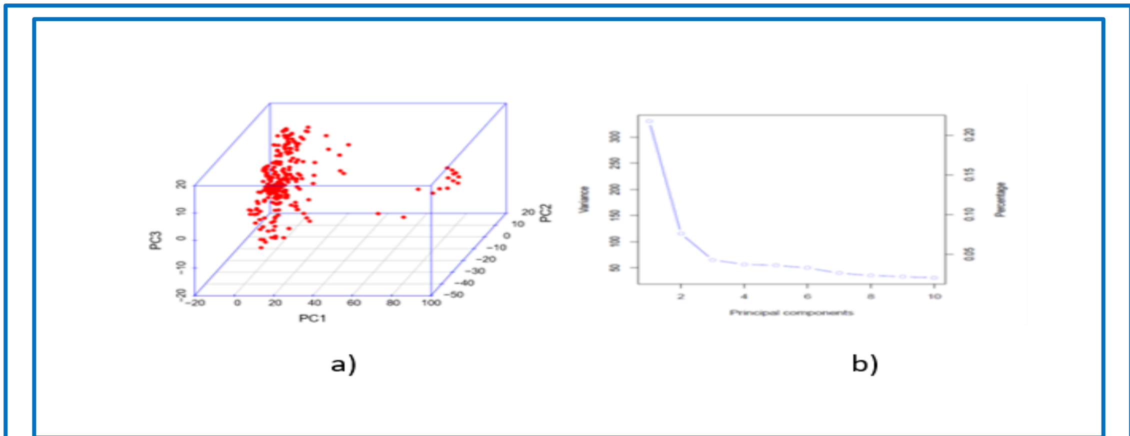

Figure 5.2. Principal Component analysis derived from genetic marker which revealed population structure a) the first three principal components of the 3335SNPs used in the GWAS indicates indicate little population structure among the 288 common bean genotypes. B) the genetic variance explained by each principal component, up to 10

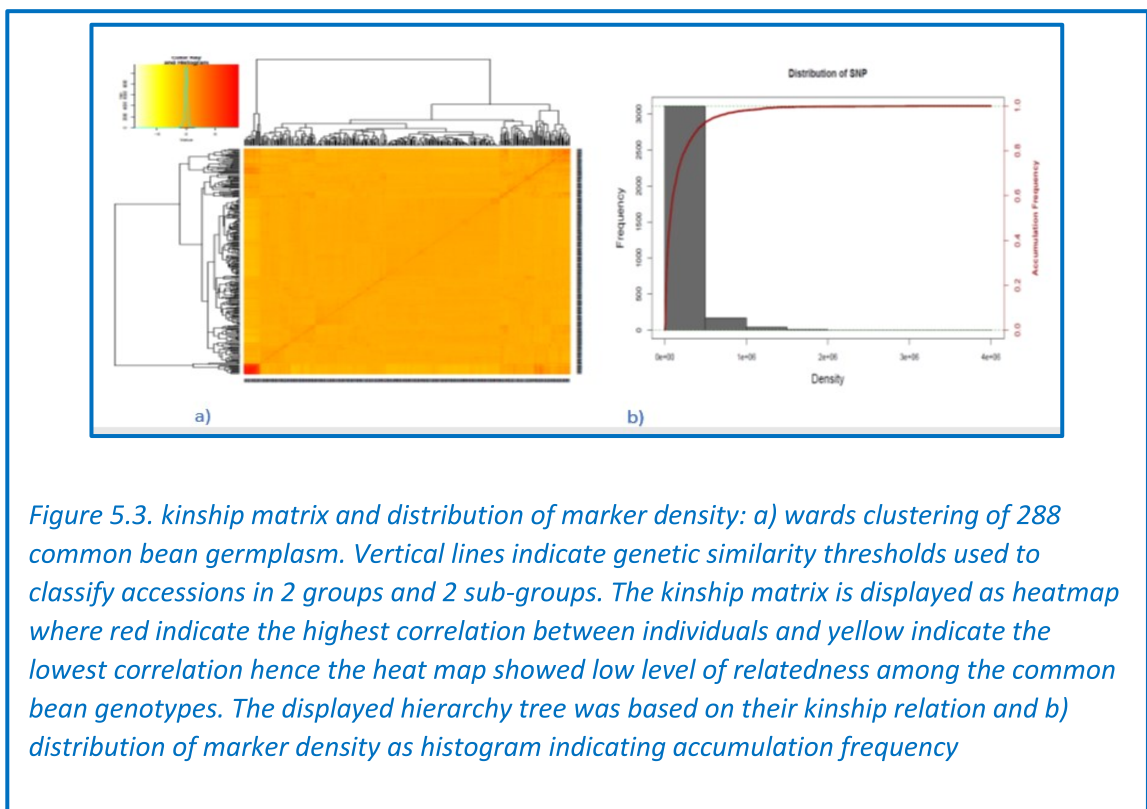

Figure 5.3. kinship matrix and distribution of marker density: a) wards clustering of 288 common bean germplasm. Vertical lines indicate genetic similarity thresholds used to classify accessions in 2 groups and 2 sub-groups. The kinship matrix is displayed as heatmap where red indicate the highest correlation between individuals and yellow indicate the lowest correlation hence the heat map showed low level of relatedness among the common bean genotypes. The displayed hierarchy tree was based on their kinship relation and b) distribution of marker density as histogram indicating accumulation frequency

5.3.3. Marker trait association for angular leaf spot resistance

The association analysis in this study were compared in terms of quantile-quantile(QQ) plot of *P* values and optimum compression. In the QQ plot, the observed $-\log_{10}(P)$ values were plotted against the expected $-\log_{10}(P)$ values. Thus, the SNP markers under the null hypotheses of no association between the SNP markers and the trait would have laid diagonal line in the QQ-plot. Based on the result majority of the SNP markers that were not associated with the trait of interest fit the null hypothesis. The SNP marker deviated from the diagonal line indicated potential association, truly associated with the angular leaf spot disease resistance trait (Fig 5.4). Hence from this study a total of 17 significant marker-trait associations were detected using the CMLM method. The markers were located on chromosomes Pv04 and Pv08 (Table 5.2 and Fig. 5.4) representing 17 potential QTLs of which two highly significant marker associations one QTL were detected on Pv04 and the other one was detected on Pv08 with the k method. The statistical power as the preparation for the genetic marker with additional genetic variance was indicated (Fig 5.5)

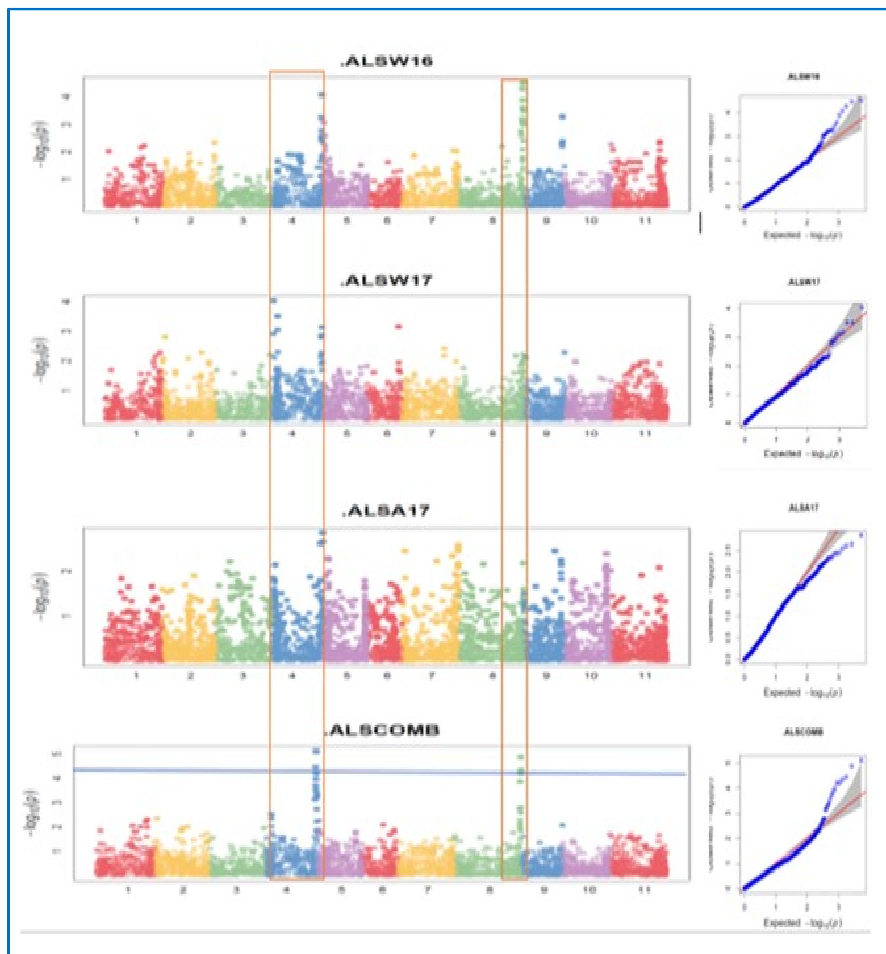

Figure 5.4. Manhattan plots with their corresponding Q-Q plots indicating significant SNPs and their P-values from GWAS using CMLM for Angular leaf spot resistance QTL on chromosome Pv.04 and Pv.08 for the data from ALSW16, ALSW17, ALSA17 and ALSCOMB. Horizontal line after Bonferroni correction of α =0.05 the area indicated in vertical lines indicates the most saturated RQTL

Table 5.2 Association mapping result for the angular leaf spot resistance using Compressed Mixed Linear Model (CMLM)

SNPs	Chro No_	Position	Allele	*P value*	MAF	nobs	R^2	FDR_ Adjusted	Allele effect
ss715645805	4	44739881	G/T	7.81E-06	0.088542	288	0.320094	0.0362583	0.539778
ss715645811	4	44332730	G/A	3.62E-05	0.064236	288	0.312017	0.056201	0.541993
ss715649811	4	44892790	G/A	6.22E-05	0.078125	288	0.309205	0.056201	0.519045
ss715645801	4	44563487	C/T	0.000107	0.071181	288	0.306403	0.081361	0.460855
ss715645803	4	44509705	G/T	0.0002	0.076389	288	0.303203	0.1181196	0.437985
ss715645813	4	44687942	G/A	0.000307	0.067708	288	0.301004	0.1637622	0.459743
ss715642594	4	45414195	G/A	0.000456	0.138889	288	0.299008	0.2208665	0.39644
ss715642595	4	45426135	C/T	0.000504	0.140625	288	0.298502	0.2238621	0.390988
ss715645802	4	44553360	C/T	0.000688	0.076389	288	0.296939	0.2751911	0.36853
ss715645798	4	43773443	A/G	0.000723	0.277778	288	0.296692	0.2751911	0.239911
ss715647356	4	45349784	T/C	0.001867	0.192708	288	0.291985	0.5850104	-0.30541
ss715647399	8	59248128	G/A	1.36E-05	0.109375	288	0.317151	0.0362583	0.49317
ss715647403	8	59451465	T/C	4.61E-05	0.151042	288	0.310762	0.056201	-0.37298
ss715647397	8	59278982	T/C	6.33E-05	0.126736	288	0.309111	0.056201	0.404474
ss715646761	8	58772554	T/C	0.00014	0.185764	288	0.305022	0.0931348	-0.33163
ss715647406	8	59421333	T/G	0.001629	0.184028	288	0.292657	0.5783935	0.250532
ss715646120	8	58463221	G/A	0.001741	0.135417	288	0.29233	0.5795186	-0.29562

5.3.4. Comparison of significant QTLs with previously reported genes

Our result provides an overview of the relationships between the loci identified in this GWAS and the previously identified and reported genes and QTLs for the angular leaf spot resistance. Until now, different authors reported ALS resistance QTL and these QTLs have been found in 10 common bean chromosomes except chromosome Pv11 (Pv01, Pv02, Pv03, Pv04, Pv05, Pv06, Pv07, Pv08, Pv09 and Pv10) and by (Carvalho *et al*.,1998; Mahuku *et al.,*1998; Sartorato *et al.,* 2000; Mahuku., 2009; Mahuku *et al*., 2011; Keller *et al.,* 2015) In the Mesoamerican common bean gene pool, the ALS resistance gene *Phg-2* was identified in cultivar Mexico-54 on chromosome Pv08

(Sartorato *et al.,* 2000; Mahuku *et al.*, 2011). The gene *Phg-1*of the Andean cultivar AND277 (Carvalho *et al.,*1998) was mapped on chromosome Pv01. From an Andean gene pool accession, G5686, Mahuku *et al*. (1998) identified a major resistance locus on Pv04, which was confirmed later by Oblessuc *et al*. (2012) and named ALS4.1GS^{UC}. Keller et al. (2015) also reported a major QRL on Pv04. In addition, two genetic markers were co-localized near the QRL ALS3.1^{uc}. In addition, there are also reports of QTLs controlling resistance to ALS. Five QTLS were mapped and reported on linkage group Pv04, one on Pv08, and one Pv09. Three QTLs were mapped on linkage group Pv10 (Lopez *et al.*, 2003; Mahuku *et al.*, 2009 and Mahuku *et al.*, 2011).

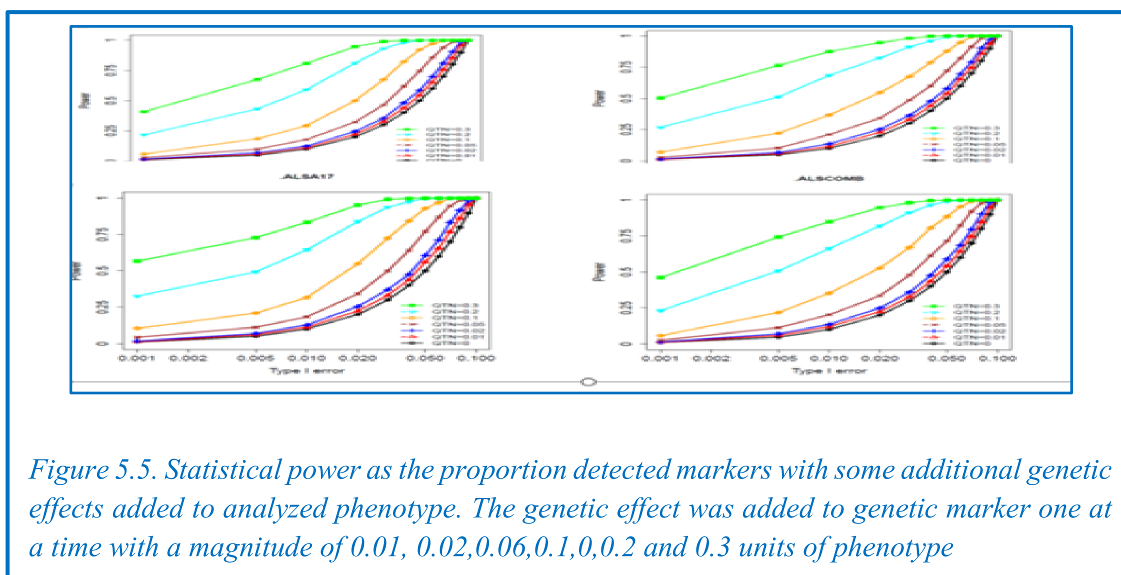

Figure 5.5. Statistical power as the proportion detected markers with some additional genetic effects added to analyzed phenotype. The genetic effect was added to genetic marker one at a time with a magnitude of 0.01, 0.02,0.06,0.1,0,0.2 and 0.3 units of phenotype

Table 5.3. The most resistance common bean genotypes for the endemic strains under Ethiopia

Taxa	ALS (1-9)	ID	Genotype		SeedColour	Growth Habit	Source
ADP0740	1.9	Malawi-CIAT	CIM-RM05-ALS-82	RD MOTT	MED	Vine	E Africa
ADP0540	2.1	CL-17	3449-3454	AFR 708	red mottled	Bush	CIAT AF
ADP0735	2.1	Malawi-CIAT	KG 27-13	RD MOTT	LG	Vine	E Africa
ADP0732	2.6	Malawi-CIAT	GCI-CAL-270-AR-3	RD MOTT	MED	Bush	E Africa
ADP0166	2.7	AF-30		NABE 4	red mottled	Bush	Africa
ADP0551	2.7	CL-28	3533-3538	AFR 612	d. red mottled	Bush	CIAT AF
ADP0576	2.9	CL-53	3719-3724	SAB618	Red mottled	Bush	CIAT AF
ADP0125	3.0	AFC-6		CHEUPE	white	Ag. Climber	Africa
ADP0554	3.0	CL-31	3551-3556	AND279	red mottled	Bush	CIAT AF
ADP0716	3.0	Malawi-CIAT	SUGAR 131	CRAN	LG	Vine	E Africa
ADP0107	3.1	AF-12	OT1127-43	Mshindi	pur mot	Bush	Africa
ADP0561	3.1	CL-38	3599-3604	DAB246	red mottled	Bush	CIAT AF
ADP0731	3.1	Malawi-CIAT	BILFA UYOLE	RD MOTT	MED	Bush	E Africa
ADP0556	3.2	CL-33	3563-3568	BRB194	d. red	Bush	CIAT AF
ADP0120	3.4	AFV-25	OT1127-29	Tygerberg	cran	Vine	Africa

5.4. Discussion

Angular leaf spot caused by the fungus *P. griseola* is one of the major and economically significant disease of the common bean production in Latin America and eastern African countries where common bean is largely produced for nutritional supplementary and as food security crop. This specific disease is also a major problem devastating in the common bean production of Ethiopia. The disease can be managed with different strategies including use of fungicide treatment, crop rotation, use of clean planting materials and adoption of use of resistance common bean varieties. Currently in most of the common bean breeding programs the use of resistance varieties is one of the priorities of controlling strategies of angular leaf spot disease. ALS resistance is now considered an important trait for new variety development. Genetic diversity is an important to all crop improvement programs and efforts to find new sources of ALS resistance and develop molecular tools to support conventional breeding is currently a priority for the common bean breeding in SARI, Ethiopia. Previously the genetic basis of ALS resistance in common bean were investigated and QTL explaining resistance identified in bi-parental mapping populations have been reported (Mutlu *et al*.,2006; Gonçalves-Vidigal *et al.,* 2011; Oblessuc *et al.,* 2012; Gonçalves-Vidigal *et al*.,2013; Keller *et al*.,2015; Perseguini *et al*., 2016) however; the large size of QTL regions identified has limited their application in marker assisted selection due to disassociation, and linkage drag which can cause unexpected genetic back ground (Collard *et al*., 2005). In this study the result provides an overview of the relationships between the loci identified in this GWAS. Hence from this study a total of 17 significant marker-trait associations were detected using the CMLM method. The markers were located on chromosomes Pv04 and Pv08 (Table 5.2, Fig. 5.4) representing 17 potential

QTLs of which two highly significant marker associations one QTL were detected on Pv04 and the other one was detected on Pv08 with K method. The previously identified and reported genes and QTLs for the angular leaf spot resistance were using the bi parental populations. Many authors reported ALS resistance QTL and these QTLs have been found in 10 common bean chromosomes except chromosome Pv11 (Pv01, Pv02, Pv03, Pv04, Pv05, Pv06, Pv07, Pv08, Pv09 and Pv10) and by (Carvalho *et al*.,1998; Mahuku *et al.,*1998; Sartorato *et al.,* 2000; Mahuku., 2009; Mahuku *et al.,* 2011; Keller *et al.,* 2015: from the Mesoamerican gene pool, the resistance gene *Phg-2* was identified in cultivar Mex54 on chromosome Pv08 (Sartorato *et al.,* 2000; Mahuku *et al.,* 2011). The resistance gene *Phg-1* was identified from the Andean cultivar AND277 (Carvalho *et al*.,1998) was mapped on chromosome Pv01. From an Andean gene pool accession, G5686, Mahuku *et al*. (1998) identified a major resistance locus on Pv04, which was confirmed later by Oblessuc *et al*. (2012) and named ALS4.1GS^{UC}. Keller *et al*. (2015) also reported a major QRL on Pv04. In addition, two genetic markers were co-localized near the QRL ALS3.1^{uc}. In addition, there are also reports of QTLs controlling resistance to ALS. Five QTLS were mapped and reported on linkage group Pv04, one on Pv08, and one Pv09. Three QTLs were mapped on linkage group Pv10 (Lopez *et al*., 2003; Mahuku *et al*., 2009 and Mahuku *et al*., 2011). This specific genome wide association and marker trait study identified two genomic regions highly associated with the angular leaf spot resistance (Pv04 and Pv08) using 3335 SNPs and 288 common bean genotype's. This genomic region was previously reported to have B4 R gene cluster that encode Nucleotide Binding Site Leucine Rich Repeats (NBS-LRR) proteins. This large family is encoded by hundreds of genes and were known to respond pathogen attach in plants. Hence, this genomic region will be validated and used for the future marker assisted breeding program. The result from this study showed that marker

trait association analysis can be a powerful tool for assessing genetic potential of the common bean germplasm collection, identifying the potential loci responsible to the disease resistance identification of potential and significant molecular markers to the angular leaf spot disease resistance to be used in future marker-assisted breeding program.

5.5. Conclusion

The study was successful in identifying significant QRL and SNPs associated and responsible for the angular leaf spot resistance, which is increasing in importance worldwide and constitutes a challenge due to the high variability of the pathogen on chromosome 4 and chromosome 8. The use of GWAS resulted in the identification of Quantitative Resistance Locus (QRL) with higher statistical significance. From the result the primary advantage of GWAS is the high-resolution power compared to the linkage mapping. This feature enabled GWAS to further narrow down the chromosomal regions of candidate QTLs and predict casual genes. Among the significant SNPs at both Pv04 and Pv08 chromosomes two of the SNPs (ss715645805 and ss715647399) one on each of the chromosome were above the threshold level (Table 5.2, Fig 5.3 & Fig 5.8). This study not only discovered two RQTLs and significant SNPs associated with the ALS disease resistance but also demonstrated the potential of GWAS and marker trait association for genetic analysis.

CHAPTER SIX

Marker-assisted Pyramiding Resistance Genes Against Angular Leaf Spot and Common Bacterial Blight Disease Into Preferred Common Bean Cultivar ''REDWOLAITA''

Abstract

Angular leaf spot (ALS) caused by *Pseudocercospora griseola* and common bacterial blight (CBB) caused by *Xanthomonas campestris* pv *phaseoli X. campestris pv. phaseoli var. fuscans* are the most economically important diseases of common bean production in Ethiopia. This research aims at pyramiding the *Phg-2* R gene for angular leaf spot resistance and two CBB major resistance quantitative trait loci (RQTLs) into the background of the most popular and susceptible common bean cultivar "REDWOLAITA" (RW) with the aid of marker-assisted breeding method. Marker-assisted Parallel Back Crossing (MAPBC) breeding scheme with three separate parallel backcrossing streams were adopted for tracking three independent resistance loci linked to g796 (*Phg-2* for ALS resistance) and, SU91 and SAP6 genetic markers from two different donor parents to the REDWOLAITA recurrent parent. The two donor parental lines VAX6 (with known RQTLs tagged by the SAP6 and SU91 genetic markers on linkage groups 10 and 8, respectively) and MEX54 (with the *Phg-2* R gene tagged by the g796 genetic marker at the linkage group 8 were used in the gene pyramiding program. After the BC4 generation, progenies that combined SAP6 and g796 genetic markers were created and selected from the BC4 inter-crossing of progenies. Then, further inter-crossing was made between selected progenies that combined the SAP6 and g796 genetic markers with selected progenies with the SU91 genetic marker. Finally, from this study we developed Monogenic Near Isogenic Lines (MNILs) with R genes tagged by the SAP6, g796, and SU91 molecular markers and polygenic PNILs with different gene combination includes $\text{MNIL}^{\text{SAP6}}$, $\text{MNIL}^{\text{SU91}}$ & $\text{MNIL}^{\text{g796}}$, polygenetic PNILs $^{\text{SAP6/g796}}$, PNILs $^{\text{SU91/g796}}$, PNILs $^{\text{SAP6/SU}}$, PNILs $^{\text{SAP6/g796/SAP6}}$, with more than 97% genome recovered from the RW genetic background. Marker-assisted backcrossing facilitated selection of progenies that combined good agronomic traits with resistance loci were constructed from the RW common bean cultivar genetic background and tested under the screening house condition. The developed lines showed high level of disease resistance to the strains of CBB and ALS present under the screening conditions. They were selected to be multiplied and tested under multiple environment, before varietal release and wider production. Developed MNILs with good agronomic background will also be used as alternative donor parent for the future gene pyramiding program.

Keywords: Gene pyramiding, parallel backcrossing, RQTLs, inter-crossing, Isogenic lines

6.1.Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume for direct human consumption and used as main food and /or food component in Latin America and eastern and southern Africa. Common bean is seed-propagated and a diploid (2n = 2x = 22) with a relatively small genome (650 Mb) (Broughton *et al.,* 2003), originated in the Neotropics, with at least two major centers of domestication in Mesoamerica and the Andes (Gepts, 1988).

Common bean is believed to have been introduced together with maize via the east coast of Africa by Portuguese and Spanish traders in the sixteenth and seventeenth century (Greenway 1945; Gentry 1969). In Ethiopia, common bean is the principal food and nutrition security legume crop providing dietary protein and a source of cash income for resource-poor farmers. Among a number of factors that could attribute to their low yield, diseases especially angular leaf spot (ALS) caused by *Pseudocercospora griseola* and common bacterial blight (CBB) caused by *Xanthomonas campestris* pv *phaseoli* cause the most significant harvest losses in common bean in farmer's field (Belete and Bastas, 2017). The impact of disease on crop production in Ethiopia and beyond may be worsening with the current and predicted climate change scenarios of rising temperatures and variability and changes in precipitation. These has been observed in disease incidence and severity of common beans in Ethiopia (Belete and Bastas, 2017).

Using host resistance has been proven to be the most effective and economical method to control disease in common bean and other crops. Therefore, to obtain a durable and broad-spectrum resistance variety, pyramiding multiple R genes/RQTLs into a recurrent common bean cultivar is an important and practicable breeding strategy to control angular leaf spot and common bacterial blight (de Mendonça *et al.,* 2003).

The backcrossing approach to deploy one or more genes into an elite line was proposed by Harlan and Pope (1992). Since then, backcrossing has become a widely used plant breeding approach in diverse crop species (Hasan *et al.,*2015; Hansan *et al* 2016). This method is most commonly used to incorporate one or a few highly heritable traits into an adapted or elite variety. In most cases, the elite variety used for backcrossing has a large number of desirable attributes but is deficient in only a few characteristics. The other parent, called the 'donor parent', possesses one or more genes controlling an important trait, which is lacking in the elite variety (Hansan *et al* 2016). Traditional backcrossing programs are designed on the assumption that the proportion of the recurrent parent genome is recovered at a rate of $1 - (1/2)^{t+1}$ for each of t generations of backcrossing. Thus, after four backcrosses, we expect to recover $1 - (1/2)^5 = 96.9\%$ of the recurrent parent genome (Babu *et al.,*2004). However, any BC progeny individual will deviate from this expectation due to chance and to linkage between the gene from the donor parent being selected for and nearby genes (Muhammad *et al.,* 2014).

Then, since the advent of molecular genetic markers, assisted backcrossing has been successfully applied in gene pyramiding programs for targeted transferring and pyramiding resistance loci to create more durable and broad specific resistance in different crops (Joshi and Nayak 2010). In the wheat cultivar "Yang", Liu *et al*. (2000) successfully combined three powdery mildew resistance gene combinations *pm2 + pm4a, pm2+pm211*, and *pm4a+pm21* using restriction fragment length polymorphism (RFLP) markers. In soybean for mosaic virus disease resistance (SMV), researchers successfully pyramided three genes *Rsv1*, *Rsv3* and *Rsv4* with the aid of microsatellite markers in order to develop new soybean lines containing multiple resistance genes for soybean mosaic virus (SMV) resistance. Marker-assisted selection (MAS) and gene

pyramiding have been reported in common bean research (Kelly *et al.,* 2003, Miklas *et al.*, 2006; Ragagnin *et al.,* 2009); Ferreira *et al.,* 2012; Kumar et al. 2017). Recently, Ddamulira *et al*, (2015) reported the efficiency and effectiveness of gene pyramiding in improving angular leaf spot resistance in susceptible common bean cultivar.

This specific research aims at cumulating the *phg-2* R gene for ALS and two major RQTL's for CBB resistance into the background of popular common bean cultivar 'REDWOLAITA' through the aid of molecular and conventional breeding techniques. The breeding strategy consisted of marker-assisted backcrossing (MABC) in transferring disease resistance in the adapted common bean cultivar 'REDWOLAITA', which belongs to the Mesoamerican gene pool.

6.2. Materials and Methods

6.2.1. Experimental location

The study was conducted in the Molecular Biotech Lab and screen house at the Southern Agricultural Research Institute (SARI), located 7° 4' N latitude and 38° 31' E longitude and an altitude of 1700 m.a.s.l in Hawassa, Ethiopia from 2015 to 2017. After the backcross pyramided progenies were generated, they were genotyped to identify those progenies with the required molecular markers; the progenies were also phenotyped for their performance and disease reaction.

6.2.2. Plant materials

The materials under study included three parents: 'REDWOLAITA' (RW) as the recurrent parent and VAX-6 and MEX 54 as sources of disease resistance (Table 6.1). REDWOLAITA (RW) common bean cultivar from the Mesoamerican gene pool was the most popular and widely grown for its colour and cooking quality and high preference by most of farmers was selected as a recurrent parent. This common bean

cultivar, although, it was the most preferred by famers, for its good quality but the cultivar was the most susceptible to common bacterial blight caused by *Xanthomonas campestris* pv *phaseoli X. campestris pv. phaseoli var. fuscans* and angular leaf spot caused by *Pseudocercospora griseola* endemic to Ethiopia. Therefore, this common bean cultivar was selected as recurrent parent to be improved through marker-assisted gene pyramiding program. The other two parents including VAX-6 and MEX-54 with known sources of major sources of resistance QTL and *Phg-2* R gene for bacterial blight pathogen caused by *Xanthomonas campestris pv. phaseoli* and *X. campestris pv. phaseoli* var. *fuscans* and the angular leaf spot caused by *Pseudocercospora griseola* respectively for these disease endemics and widely distributed to common bean growing areas of Ethiopia were selected to be used as a donor parent in this specific study (Caixeta *et al.,* 2005).

Table 6.1. Characteristics of Common Bean Parental Lines Which Were Used in Marker Assisted Gene Pyramiding Program(MABCP)

Parents used in MABCP	Gene pool	Seed size & colour	Growth Habit/type	Disease Reaction	
				ALS	CBB
REDWOLAITA	Mesoamerican	Small red	II	+	+
VAX-6	Mesoamerican	Small pale red	I	+	-
MEX-54	Mesoamerican	Small pink	IV	-	+

CBB= common bacterial blight, ALS= angular leaf spot, += compatible(susceptible) disease reaction, - = incompatible (resistance) disease reaction

For this study the best resistance donors and most reliable and polymorphic markers were used to deploy resistance into the background donor. The parents were tested for marker polymorphism and usefulness for MAS (Table 6.3). Crosses were made and advanced through the application of molecular markers. The resistance gene transfer was confirmed with aid molecular marker linked to the R gene /RQTL and through reliable screening techniques in screening house.

6.2.3. Molecular Markers

Sequence Characterized Amplified Regions (SCAR) markers were used (Table 6.2) to tag angular leaf spot and common bacterial resistance genes of interest. The original oligonucleotide markers were obtained from Eurofins Genomics. A 50 / 100 bp mixed DNA molecular weight marker (Ladder) specifically designed for determining the size of double strand DNA from 25 to 300bp was used. The presence of $SU91_{700}$ (Pedraza *et al.,* 1997) linked a resistance QTL located on B8, $SAP6_{820}$ (Miklas *et al*., 2000) linked to a resistance QTL on whereas the *Phg-2* resistance locus were B10, $g796_{220}$ (Miller *et al*.,2018) were determined using genetic markers.

Table 6.2. Polymorphic molecular markers used in gene pyramiding and selection

Gene /Locus	Linked Molecular markers	linkage group	Primer sequences	Expected band Size/orientation	Reference
QTL	**SAP6**	**10**	**F GTCACGTCTCCTTAATAGTA** R GTCACGTCTCAATAGGCAAA	**806/cis**	Milklas *et al.*, 2000
QTL	**SU91**	**8**	**F CCACATCGGTTAACATGAGT** R CCACATCGGTGTCAACGTGA	**669/cis**	Pedraza *et al.*, 1997
Phg2	**g796**	**8**	**F GAGAAACTACGGGCTGTTTTACCC** **R AATTAAAACACCCACCCACTCCAT**	**220**	Miller *et al.*, 2018
Phg2	**SN02**	**8**	**F ACCAGGGGCATTATGAACAG** **R ACCAGGGGCAACATACTATG**	**890/cis**	Nietsche *et al.* 2000

F=Forward, R= Reverse

6.3. Marker-assisted Selection

6.3.1. DNA Extraction and Amplifications

Genomic deoxyribonucleic acid (gDNA) was isolated using FTA card matrix technology following the manufacturer's procedure with minor modification from fresh leaves of 12-day-young plantlets. Common bean progenies were sampled from each succeeding generation, i.e., BC1F1, BC2F1, BC3F1, and BC4F1, and including the

progenies created through inter-crossings of BC4s and BC4F2. FTA is a paper-based technology, which was designed for the collection of nucleic acids, either in their purified form or within pressed samples of fresh tissue. Proprietary chemicals impregnated into the paper act to lyse cellular material and fix and preserve DNA within the fibre matrix. As described in the manufacturer's protocol with minor modification (www.gelifesciences.com/whatman) in which captured nucleic acids were ready for purification when taken with a punch from the FTA card, purification reagents were added, and the paper was rinsed with TE-1 (10mM Tris-HCl, 0.1mM EDTA, pH8) buffer. The DNA markers SAP6 (829bp), SU91 (700bp) and g796 (233bp) were used to select plants with linked resistance loci, which were then backcrossed to the recurrent parent. After washing the punched discs, the DNA was eluted and tested for its quality using agarose gel (0.98%) for use in PCR.

6.3.2. Polymerase chain reaction (PCR)

Sequence characterized amplified region (SCAR) markers used in selection for CBB resistance were dominant and were scored as the presence or absence of a single band on an agarose gel. The INDEL marker used in ALS resistance selection was co-dominant. DNA amplification was performed under ABI 2720 Thermal cycler under the program for SU91, SAP6 and g796 were 34 cycles of 10s at 94 °C, 40s at 58 °C (for SAB6), 40s at 60^0C for SU91 &30s at 44^0C for g796, 2 min at 72 °C, and 5 min at 72 °C for the final extension (Table 6.3). PCR results were analyzed using a 1.4% agarose gel stained with ethidium bromide (0.02 $\mu g \cdot mL^{-1}$). Bands present on the gel were compared by size to a 100-bp molecular marker.

Table 6.3. DNA Marker Validation for Selecting Polymorphic Markers to be Used in the Marker Assisted Gene Pyramiding

DNA markers	Annealing T^0C	RW	VAX6	MEX54	Description of Marker
SAP6	58	-	+	-	[1]SCAR, Linked to CBB Resistance QTL
SU91	60	-	+	-	[2]SCAR, Linked to CBB Resistance QTL
g796	44	-	-	+	[3]STS, linked to *phg-2* ALS R gene
SN02		-	-	-	[4]STS, linked to *phg-2* ALS R gene
OPE4		-	-	+	[5]STS, linked to *phg-2* ALS R gene

R= Resistance, SCAR=sequence characterized amplified region, STS= sequence tagged site

1 Miklas *et al.,* 2000; 2 Pedraza *et al.,*2000; 3 Miller *et al*.,2018;

4 Nietzsche *et al.,*2000

6.3.3. Marker-Assisted Back Crossing and Gene Pyramiding

The gene pyramiding and marker-assisted backcrossing breeding selection strategy used is illustrated in Figure 6,1. The marker-assisted backcrossing program was planned in such a way that three independent crossing streams were undertaken to track R/RQTLs loci tagged by SAB6, SU91 and g796 molecular markers on the linkage groups 10 and 08, respectively (Table 6.2). The resistance donor parents MEX54 with R gene (*Phg-2*) for ALS and VAX6 (with two RQTLs loci for CBB) were independently crossed with the recurrent parent 'REDWOLAITA' common bean cultivar under screening house conditions (Table 6.3).

The F_1 were crossed with the RP to produce the first backcross generation (BC_1F_1 or just BC_1). Markers closely linked to the resistance loci were then used to check targeted genes from each crossing streams of BC1F1 populations. Based on the plan, molecular markers, which included SAB6 in crossing stream one, g796 in crossing stream two, and SU91 in crossing stream three were used. Then, the succeeding

backcross generations were made by crossing selected BC_1 F1plants (that had been screened for the targeted resistance trait (*Phg*-2 and CBB RQTL loci) from each crossing streams with the RP to produce the BC_2F1 populations. Subsequent backcross populations were made by repeatedly crossing the selected backcross (BC) plants with the RP. That backcross progeny with the target trait were selected based on phenotype during each round of backcrossing. To further identify targeted homozygous plants at the backcross four (BC4) from each backcross streams selected plants were selfed to get targeted homozygous plants. Then, plants with homozygous genotypes for the targeted R genes were selected randomly from each segregating population. BC4F3 seeds were then harvested individually from each selected BC4F2 [RW/RW/VAX(+SAP6)], [RW/RW/MEX(+g796)] and [RW/RW/VAX(+SU91)] these created lines were monogenic near isogenic lines, constituting $MNIL^{SAP6}$, $MNIL^{SU91}$ and $MNIL^{g796}$ respectively.

Then intercrossing was made to further combine the resistance genes into a single background. The pyramid lines with different gene combinations were created combining SAP6 with g796 genes $PNILs^{SAP6/g796,}$ $PNILs^{SAP6/SU91}$ combining genes SAP6 with SU91, $PNILs^{SAP6/g796}$ combining genes SAP6 with g796, and further crossing was made to create polygenic line with good agronomic background combining all of the R genes $PNILs^{SAP6/SU91/g796}$ polygenic lines that combined three R loci linked to SAP6, g796 & SU91 were developed with help of Marker-assisted Selection (MAS).

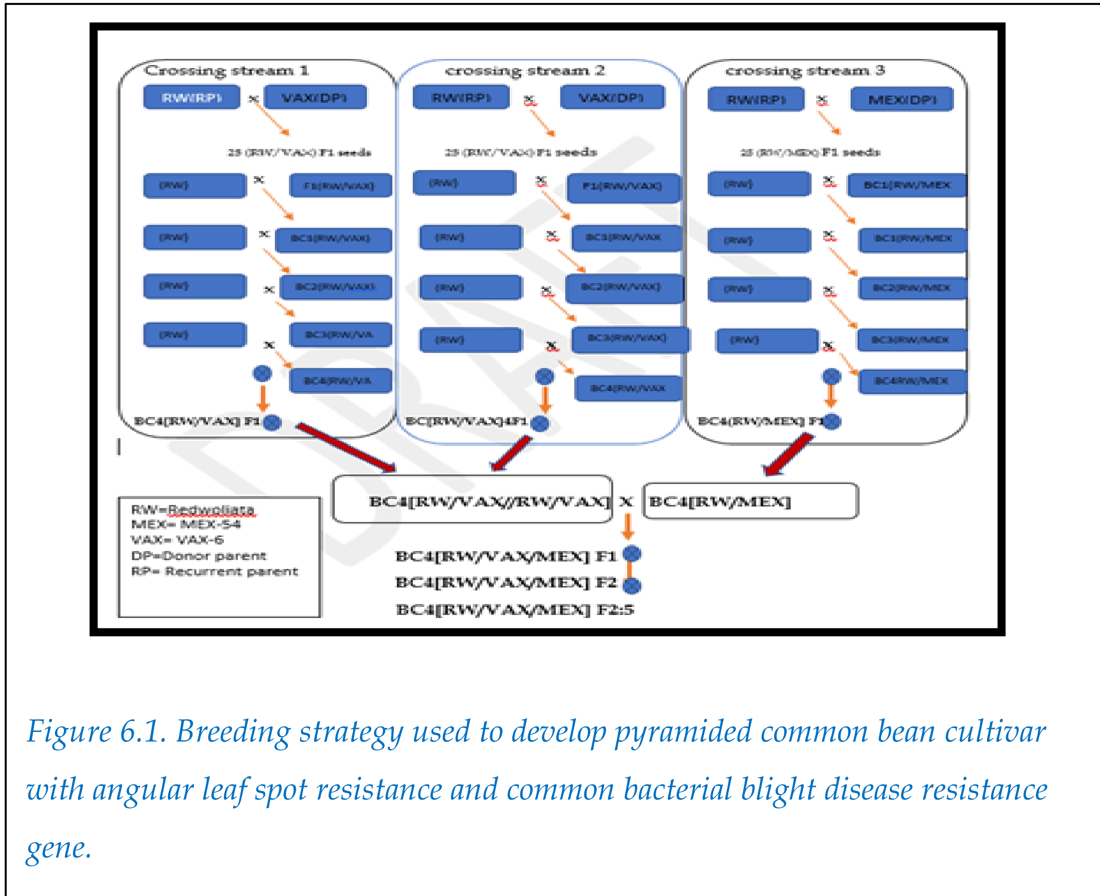

Figure 6.1. Breeding strategy used to develop pyramided common bean cultivar with angular leaf spot resistance and common bacterial blight disease resistance gene.

6.4. Selection of Near Isogenic and Polygenic Pyramided Resistance Lines

The isogenic and polygenic near isogenic pyramided lines were evaluated for their reaction to both common bean angular leaf spot and bacterial blight under the screening house using the most virulent pathogens collected from Ethiopia. Fourteen days old plants were inoculated with angular leaf spot suspension 10 x10^6 spore concentration and CBB which were virulent to common bean growing areas of Ethiopia. in this experiment evaluation for R genes and appropriate traits were based on the reaction of selected lines with virulent pathogen and morphological characteristics like the selection was based on the growth habit and the seed colour of the plant.

6.5. Results

6.5.1. Marker-assisted Backcross Breeding

Three independent and separate parallel back crossing schemes were adopted to track resistance loci from two donor parents. In our back-crossing program, three polymorphic DNA-based molecular markers were used during marker-assisted parallel backcrossing (MABC) breeding program to deploy CBB and ALS resistance gene/QTLs into the farmer-preferred cultivar but susceptible bean variety REDWOLAITA. The molecular markers (SU 91, SAP6 and g796) allowed us to conduct early selection of bean lines with resistance to the fungal and bacterial pathogens (Fig 6.2, 6.3 and 6.4).

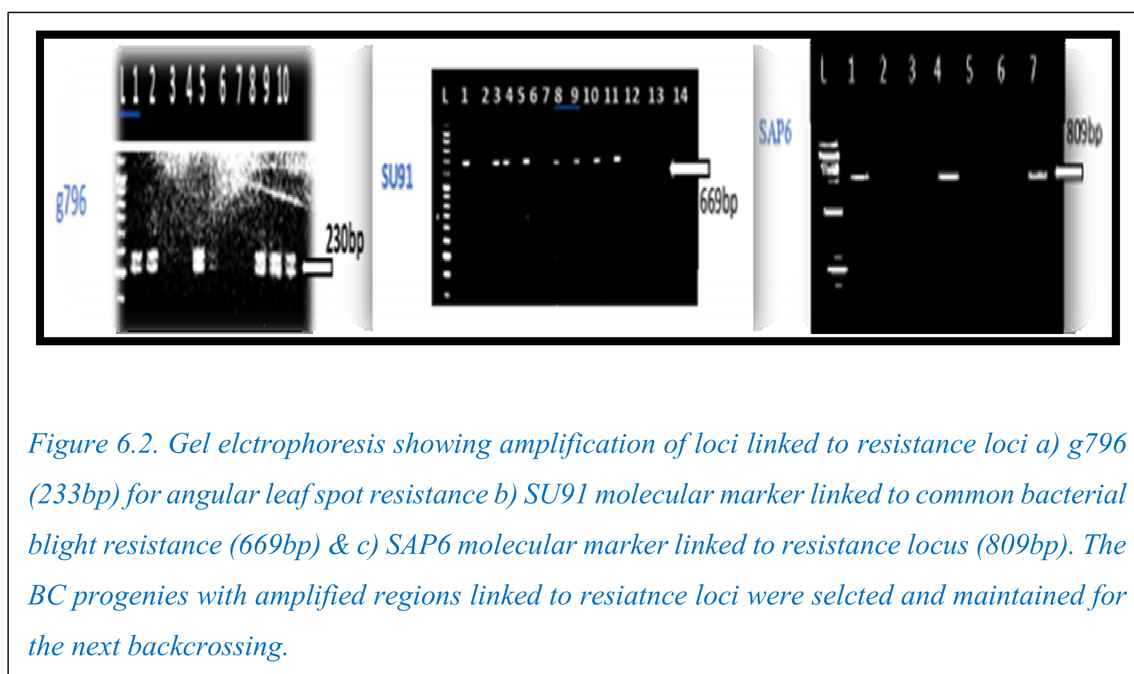

Figure 6.2. Gel elctrophoresis showing amplification of loci linked to resistance loci a) g796 (233bp) for angular leaf spot resistance b) SU91 molecular marker linked to common bacterial blight resistance (669bp) & c) SAP6 molecular marker linked to resistance locus (809bp). The BC progenies with amplified regions linked to resiatnce loci were selcted and maintained for the next backcrossing.

Table 6.4. Selected Progenies from the Successive Parallel Backcrossing Program in Each Generation Based on the Target Locus Linked to Molecular Marker

Generation	Progenies from Stream one MABC	Target (+SAP6)	Progenies from Stream two MABC	Target (+g796)	Progenies from Stream three MABC	Target +(SU91)
1	F1 [RW/VAX]		F1 [RW/MEX]		F1 [RW/VAX]	
2	BC1[RW/VAX] F1	3:24	BC1[RW/MEX] F1	7:24	BC1[RW/MEX] F1	2:24
3	BC2[RW/VAX] F1	5:19	BC2[RW/MEX] F1	7:20	BC2[RW/MEX] F1	11:15
4	BC3[RW/VAX] F1	5:12	BC3[RW/MEX] F1	3:7	BC3[RW/MEX] F1	5:12
5	BC4[RW/VAX] F1	5:12	BC4[RW/MEX] F1	8:17	BC4[RW/VAX] F1	5:10
	Inter-crossing isogenic lines			**Target**		
6	BC4[RW/VAX]/BC4[RW/MEX]			(+SAP6/+g796) 5/14 (2homoz, 3 heteroz)		
7	BC4[RW/VAX/MEX] F1 / BC4[RW/VAX] F1			(+SAP6/+g796/+SU91) 6:10 (g/SU/SAB) / 10:10 (g796+SAP)		
8	BC4[RW/VAX/MEX/VAX] F1			(+SAP6/+g796/+SU91)		
9	BC4[RW/VAX/MEX/VAX] F2:4			(+SAP6/+g796/+SU91)		

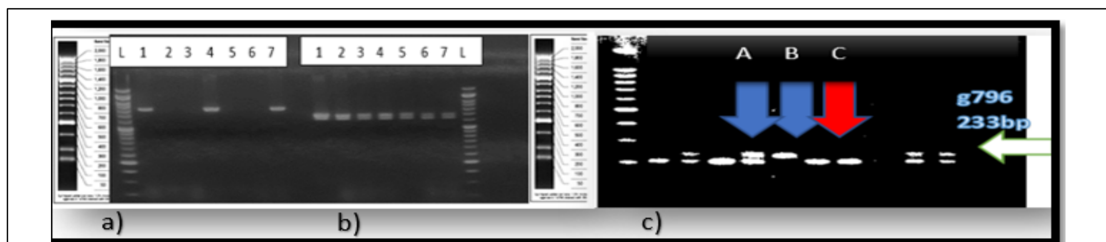

Figure 6.3. Polymerase chain reaction amplification (PCR) obtained using markers a) $SAP6_{809}$ & b) $SU91_{669}$ and identification and selection of BC progenies with loci linked to SAP6 and SU91 for major RQTL CBB resistance. Plants 1 ,4 & 7 have both $SAP6_{809}$ & $SU91_{669}$ genetic marker for common bacterial blight, whereas BC progenies 1, 2, 3, 5 and 6 have only SU91 major RQTL marker c) Gel amplification obtained by g796 molecular marker. BC bean progenies A: B: C with different banding patterns, at 200bp & ~233bp: progeny A with co-dominant banding pattern indicating heterozygous resistance, progeny B with homozygous resistance, and progeny C with homozygous recessive susceptibility for g796 R locus

There result revealed that the successful gene pyramiding of three R genes (*Phg-1* R gene for the angular leaf spot & two major RQTLs for CBB resistance) through DNA-based marker-assisted gene pyramiding into the popular and farmer-preferred but susceptible common bean cultivar "REDWOLAITA" (Table 6.4; Fig 6.5). Disease resistance screening for the advanced lines with strains of ALS and CBB showed that

single gene and poly gene pyramided lines with R genes showed effectively conferred resistance to both ALS and CBB strains (Table 6.5).

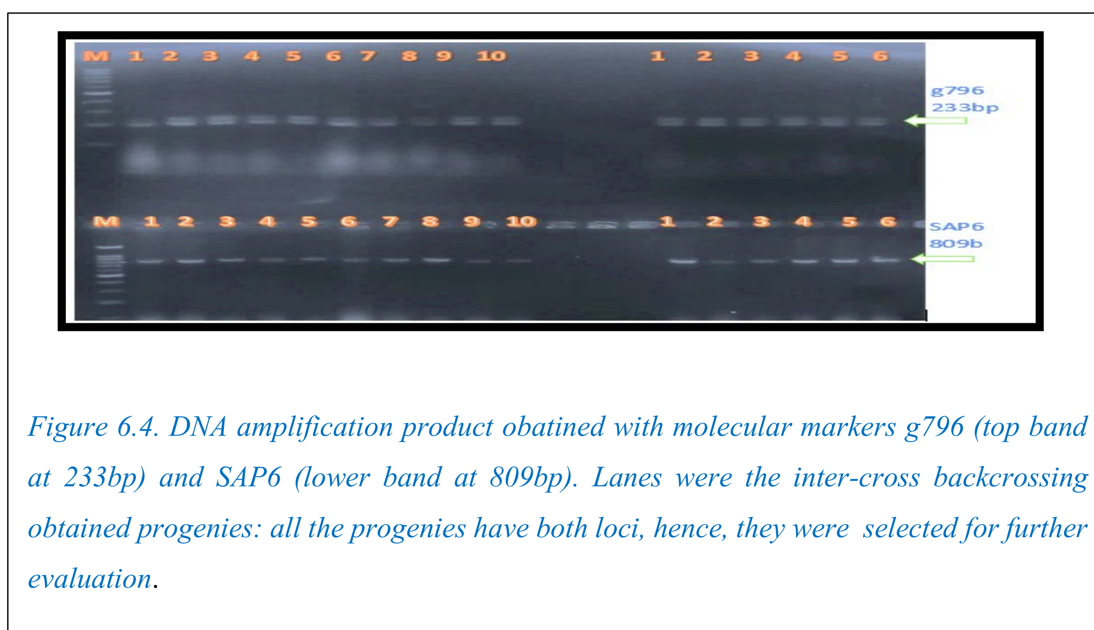

Figure 6.4. DNA amplification product obatined with molecular markers g796 (top band at 233bp) and SAP6 (lower band at 809bp). Lanes were the inter-cross backcrossing obtained progenies: all the progenies have both loci, hence, they were selected for further evaluation.

Table 6.5. Agronomic performance of pyramided MNILs, PNILs and parental lies under screen house

Progenies	Pedigree	**ALS (1-9)**	**CBB (1-9)**	Seed HSW(gm)	Seed colour
ETKT01	BC4 [RW/VAX(+SAP6)]	S	MR	25.7	SR
ETKT02	BC4 [RW/VAX (+SU91)]	S	MR	23.3	SR
ETKT03	BC4 [RW/MEX54(+g796)]	R	S	16.8	SR
ETKT04	BC4 [RW/VAX(+SAP6/+SU91)]	S	R	17.4	SR
ETKT05	BC4 [RW/VAX/MEX(+SAP6/+g796)]	R	MR	18.4	SR
ETKT06	BC4 [RW/VAX/MEX(+SU91/+g796)]	R	MR	11.4	SR
ETKT07	BC4[RW/VAX/MEX/VAX(+SAP6/+g796/+SU91)]	R	R	21.3	SR
P1	RW (RP)	S	S	18.8	SR
P2	MEX54 (DP)	R	S	28.2	SPP
P3	VAX6 (DP)	S	R	17.2	SR
	CV			3.3	
	LSD			1.12	

MNILs=monogenic near isogenic lines, PNILs= polygenic near isogenic lines, R=Resistance, MR=Moderately resistance, SR=small red, SPR =small pal red, SP=small pink, HSW=hundred seed weight (gm), ALS= angular leaf spot, CBB=common bacterial blight, HSW=hundred seed weignt (gm), S= susceptible.

6.6. Discussion

6.6.1. Gene Pyramiding and Cultivar Development

Among the pyramided polygenic NILS /pyramided lines, the lines that combined three R loci PNILs$^{SAP6/g796/SU91}$ performed best followed by pyramided lines PNILs$^{SAP6/g796}$, PNILs$^{SU91/g796}$, both with two disease resistance lines in terms of disease reaction, hundred seed weight (HSW) (gm) and seed colour. Among the developed near-isogenic pyramided polygenic line, the PNILs$^{SAP6/SU91}$ line with two R loci on chromosomes Pv08 and Pv10 for common bacterial blight, performed best under the CBB disease but showed susceptible reaction to angular leaf spot. Hence, this specific line with more than 76% with RW genetic background recovery and with good agronomic trait will be used for a future gene pyramiding program, which includes monogenic NILs, including NILs^{SAP6}, NILs^{SU91} & NILs^{g796}.

Angular leaf spot caused by *Pseudocercospora griseola* and common bacterial blight caused by *Xanthomonas campestris* pv *phaseoli* and *X. campestris pv. phaseoli var. fuscans* are the major destructive diseases of common bean (*Phaseolus vulgarise* L.) in Ethiopia. Pyramiding resistance genes/QTLs has been becoming an effective strategy to develop new variety with long-lasting and wide spectrum resistance. Marker-assisted selection (MAS) and gene pyramiding has been reported before in common bean research (Kelly *et al.,* 2003, Miklas *et al.,* 2006). Molecular markers linked to major angular leaf spot resistance loci (e.g., *Phg-2*) genes and common bacterial blight resistance QTLs have been widely reported (Namayanja et al.,2006; Miklas *et al.,* 2006; Miller *et al*.,2018). The gene pyramiding approaches of this study complements that of Ragagnin *et al.* (2009) who succeeded using random amplified and polymorphic DNA (RAPD) and sequence-characterized amplified regions (SCAR)

markers to pyramid resistance genes *Co-4, Co-6,* and *Co-10* against anthracnose, *Phg-*1 against angular leaf spot, and *Ur-ON* for rust into the susceptible 'carioca' market class cultivar Rudá. Ferreira et al. (2012) used SCAR, CAPS, and RAPD markers to successfully pyramid *Co-2, Co-3/9* anthracnose and *I* and *bc-3* common mosaic virus resistance genes into the 'fabada' market class A25 genotype. Ddamulira *et al*. (2015) reported the effectiveness of gene pyramiding in improving angular leaf spot resistance in susceptible common bean cultivar. Recently, Kumar *et al.* (2017) reported marker-assisted pyramiding of bacterial blight and gall midge resistance genes (*Gm4, Gm8*, *and Xa21*) into 'RPHR-1005' the restorer line of the popular rice hybrid 'DRRH-3' and the variety developed with cumulating thee genes were better yield and increased disease resistance trait.

This study also demonstrated that molecular markers can be used to successfully pyramid angular leaf spot and common bacterial blight resistance genes/QTLs into susceptible common bean varieties. This study introduced *Phg-2* and 2 RQTLs into REDWOLAITA resulting in monogenic and polygenic near isogenic lines (MNILs & PNILs) with different gene combinations for the resistance to CBB and ALS. The lines under screen house study showed significantly enhanced levels of resistance. Further inter-crossing and gene pyramiding was conducted in order to combine resistances. Pyramided NILs with R genes/RQTLs linked to SAP6, g796 & SU91 molecular markers including $\text{MNIL}^{\text{SAP6}}$, $\text{MNIL}^{\text{SU91}}$ and $\text{MNIL}^{\text{g796}}$ for the CBB and ALS disease resistance with good agronomic trait were constructed from the RW common bean cultivar genetic background and tested under screening house conditions. The resulted polygene-pyramided isogenic lines ($\text{PNILs}^{\text{SAP6/SU91/g796}}$) effectively conferred resistance to most frequently appeared pathotypes (63:59 and 19:33) of angular leaf spot and common bacterial blight pathogens that are endemic to Ethiopia. The

developed pyramided lines with different gene combinations showed increased level of disease resistance compared to the parental lines. Those lines will be used for the future gene Pyramiding program

Monogenic Near Isogenic Lines (MNILs) with R genes linked to SAP6, g796 & SU91 molecular markers were developed. These include $MNIL^{SAP6}$, $MNIL^{SU91}$ & $MNIL^{g796}$ and polygenic $PNILs^{SAP6/g796}$, $PNILs^{SU91/g796}$, $PNILs^{SAP6/SU91}$, $PNILs^{SAP6/g796}$ and $PNILs^{SAP6/g796/SAP6}$, with more than 97% RW genetic background were created. The lines will be multiplied and tested under multiple environment and will be tested as a candidate variety for official varietal release.

In this particular study, we have developed seven resistance lines from the 'REDWOLAITA' common bean cultivar to both common bacterial blight and angular leaf spot diseases through marker-assisted gene pyramiding techniques (Table 6.5). Phenotypic background selection implemented during marker-assisted gene pyramiding accompanying molecular forward selection could be a reliable improvement strategy in the marker-assisted back cross breeding. Therefore, it could be suitable for less well-equipped breeding laboratories, as marker-mediated background selection which is costly strategy. As genetic resistance is an effective strategy for the famers to grow and reduce yield loss due to these economically important diseases and stabilize common bean production.

6.7. Conclusion and Implication for the Common Bean Improvement Program

Common bean (*Phaseolus vulgaris* L.) production in Ethiopia is becoming the most and predominantly cultivated pulse crop. Although, it is traditional food and nutritional security crop, it is important as source of foreign currency and cash as income for smallholder farmers is increasing whereas, productivity under farmer's field declining

due to the frequent occurrence of the major bacterial and fungal disease. *P. griseola* with high pathogenic diversity and its seed born nature, it would be very important to change the common bean improvement strategy in Ethiopia to be able to breed for broad and durable resistance to the pathogen. Durable resistance based on the major genes has not been effective when resistance genes deployed one at a time. Therefore, cumulating complimentary resistance genes through marker-assisted gene pyramiding is a strategy that would confer a long-term resistance. In problems with multiple disease infection with different pathogens on common bean affecting its productivity and cause complete crop loss in susceptible varieties. PCR based molecular markers will be the key to success of MAS and gene pyramiding in common bean improvement. Therefore, gene pyramiding using marker-assisted breeding stagey and back crossing will provide a cost effective controlling measures to bean diseases. The developed lines with the R genes could be evaluated under multi-location in the future to release best performing lines for the famers. The lines with good genetic background consisting R genes also will be used as parental lines in the future breeding program.

CHAPTER SEVEN

Conclusion and Implication for Resistance Breeding

7.1. Introduction

Common bean (*Phaseolus vulgarise* L.) is an annual legume crop with relatively small genome of 473Mb. It is the most important grain legume for direct human consumption as a major sources of quality protein, which is high in lysine and therefore complements most cereals. In addition, common beans are high in carbohydrates, fibre, and minerals (calcium, potassium, phosphors, iron, and magnesium). Angular leaf spot (ALS) caused by *Pseudocercospora griseola* and common bacterial blight which is caused by *Xanthomonas campestris pv. phaseoli* and *X. campestris pv. phaseoli* var. *fuscans* are the two economical important diseases that have the greatest impact on crop yield reduction. Yield loss on common bean production under farmer's field due to the disease caused by angular leaf spot (*P. griseola*) pathogen are extremely high reaching up to 100%. *P. griseola* has been reported with a high degree of variability (Mkandawire *et al*., 2004; Mahuku *et al*., 2009). Use of resistance genetic materials has been the best controlling strategy for the disease management. However, until now no information on the pathotype, genetic and its distribution throughout the bean growing areas of Ethiopia. This studies therefore, was proposed to a) Determine the pathotype and pathogenic variability b) to study the genetic diversity population structure of *P. griseola* the causal agent of angular leaf spot of common bean in Ethiopia c) to study the Genome wide and marker trait association for angular leaf spot resistance d) develop multiple resistance common bean variety through Marker-assisted gene pyramiding molecular breeding technique.

7.2. Major finding

7.2.1. Pathotype and pathogenic variability of *P. griseola* in Ethiopia

The knowledge of pathotype and pathogenic variability of *P. griseola* for the common bean improvement program is vital and as far as pathotype and pathogenic variability in Ethiopia considered no report have been except few, hence this specific study confirmed the presence of high pathotype and pathogenic variability of *P. griseola*. Hence, the pathotype and pathogenic variability was characterized using 39 *P. griseola* isolates obtained from the diverse common bean growing agroecologies of Ethiopia. Pathogenicity test on the set of 12 common bean differential revealed that *P. griseola* revealed the existence of high pathotype variability hence, 21(63:63, 63:59, 63:23, 61:51, 56:36, 55:39, 49:7, 48:60, 42:59, 41:10, 34:53, 23:61, 19:33, 17:45, 8:18, 8:0, 4:16, 1:24, 1:10, 16:18. and 4:37) different physiological pathotypes were characterized among 39 *P. griseola* isolates. The result revealed the presence of high pathogenic variability of this economically important pathogenic fungus in Ethiopia. All of the pathotypes were not reported in Ethiopia before and therefore, this will be the first report of *P. griseola* pathotypes existing in common bean growing areas of Ethiopia. Except one pathotype that was compatible with Andean groups (8:0), most of the isolates were pathogenic to both Andean and Mesoamerican gene pools and based on that they were grouped and classified as Mesoamerican origin pathotypes. Among the 21 pathotypes among them two of the pathotypes (63:59 and 19:13) found most frequently than the others. The study also identified pathotype 63:63 among Ethiopian isolates which is pathogenic and compatible to the 12 sets of differential common bean genotypes. This pathotype overcomes the resistance genes constituting all of the differential sets of common bean genotypes. This pathotype were also reported in Argentina, Brazil, central America and eastern African countries including Kenya,

Uganda and Tanzania. Pathogenicity test revealed among the 12 sets of the international differential common bean genotypes Don Timo and Amendoin were widely infected by 69.23 and 66.6 % respectively and whereas Mex-54 with low percentage of infestation rates to the *P. griseola* isolates existing in Ethiopia.

7.2.2. Genetic variability and population structure of *P. griseola*

Molecular markers like Rep-PCR (Repetitive Extragenic Palindromic) to determine the genetic variability revealed the presence of high genetic variability of *P. griseola* at molecular level and hence DNA based genetic markers and the rep-PCR finger printing is the choices of genetic markers to study the genetic variability of pathogens. Based on the molecular study the isolates were grouped based on two groups the Mesoamerican and Andean origins hence these results were in lines with the earlier reports confirming the *P. griseola* which were identified in Ethiopia were co-evolved with Mesoamerican and Andean gene pools. Genetic diversity at the DNA level among isolates of *P. griseola* based on rep-PCR molecular markers defined 25 haplotypes while virulence defined only 21 different pathotypes indicating that the rep-PCR are neutral markers unrelated with pathotype diversity. The result obtained are consistence with other finding findings suggesting that isolates of the same pathotype are not necessarily related based on DNA analysis. Rep-PCR DNA marker showed higher levels of diversity than pathogenicity.

The study confirmed the existence of large pathogen variability among the isolates of *P. griseola*. The existence of high variability among the isolates indicates, P. *griseola* fungus has great potential to generate variability. The information obtained from this specific study has significant implications for resistance breeding and resistance gene deployment through marker-based gene pyramiding techniques that

facilitate cumulating of resistance genes/ QTLs in to the back ground of common bean cultivar generating durable resistance.

7.2.3. Genome wide marker trait association study for the Angular leaf spot (*P. griseola)* disease resistance

The result examined angular leaf spot resistance in genome wide association study based on genotyping 3,335 SNPs variants across 288 diverse common bean genotypes. The study confirmed a total of 18 marker trait significant associations that were distributed across chromosome Pv04 and Pv08 in which chromosome Pv04 with the most saturated genomic region with 11 significant associated SNP markers followed by chromosome Pv08 with 7 significant SNPs. Hence, chromosome Pv04 and Pv08 were found with the greater number of significant loci associated with angular leaf spot disease resistance and these both of which two marker traits association were above the threshold level hence, two genomic regions were detected for further verification. Our results demonstrate the great potential of genome wide association study to identify quantitative resistance loci (QRL) related to the angular leaf spot of the common bean.

7.2.4. Marker-assisted pyramiding genes conferring resistance to angular leaf spot and common bacterial blight in to popular common bean cultivar

Developing varieties within creased level of disease resistance is the main and most important goal for the common bean breeding program in Ethiopia. Marker assisted gene pyramiding has been successfully applied in gene pyramiding programs for targeted transferring and pyramiding resistance loci and to create more durable and broad specific resistance in different crops. This research targeted at pyramiding *Phg-2* R gene for angular leaf spot resistance and CBB major RQTLs in the background of the most popular common bean cultivar ''REDWOLAITA'' but with susceptible trait

to the bacterial and fungal pathogen with the help of molecular marker assisted breeding techniques.

From this study we developed Monogenic Near Isogenic Lines (MNILs) with R genes linked to SAP6, g796 & SU91 molecular markers and polygene Near Isogenic Lines (PNILs) with different gene combination includes $MNIL^{SAP6}$, $MNIL^{SU91}$ & $MNIL^{g796}$, polygene $PNILs^{SAP6/g796}$, $PNILs^{SU91/g796}$, $PNILs^{SAP6/SU}$, $PNILs^{SAP6/g796/SAP6}$, with more than 97% genome recovery from the RW genetic background. Marker assisted backcrossing were facilitated to select progenies that combined good agronomic trait with resistance loci were constructed from the RW common bean cultivar genetic background and tested under the screening house condition. The developed lines showed high level of disease resistance for the CBB and ALS strains under the screening conditions and selected lines to be multiplied and tested under multiple environment before varietal release and wider production. Developed MNILs with good agronomic background will be also used as an alternative donor parent for the future gene pyramiding program.

2.2.5 Implication for future common bean resistance bean breeding

The common bean breeding program aiming to develop resistance varieties should consider the identified *P. griseola* pathotypes to have good result for future breeding and wider production. The result regarding the genetic diversity within each group of the *P. griseola* fungus in Ethiopia was considerable. Genetic diversity at both pathotype and molecular level among isolates of *P. griseola* based on rep-PCR fingerprinting pattern defined 25 haplotypes while virulence defined only 21 among 39 single spore isolates which is not surprising considering that rep-PCR are neutral markers unrelated with pathotype diversity. The result obtained were consistence with other findings and recent reports in different areas suggesting that isolates of the same pathotype are not

necessarily closely related based on the DNA analysis. It is interesting to mention that rep-PCR DNA fingerprinting pattern showed higher level of diversity than pathogenicity. This result revealed the presence of high level of pathotype and genetic diversity in the *P. griseola* isolates collected from the diverse common bean growing regions. The high virulence variability and wider distribution of pathogen races in the *P. griseola* population in Ethiopia has an implication in ALS disease management practices. Hence, use of single source of resistance would not be sufficient management practice to control the challenges of this important economic disease. It also has an implication on using single location to test common bean genotypes is not sufficient because different pathotypes of *P. griseola* exists at different common bean growing locations. Among the sets of differential common bean cultivars Mex54 showing with low level of infection and high level of resistance to most of the isolates would therefore, be a good source of resistance to ALS in common bean breeding program for gene deployment. Marker assisted pyramiding through marker assisted back crossing provides an opportunity to create host plant with multiple resistance genes to angular leaf spot and common bacterial blight. This technique also accelerates breeding of cultivars with multiple resistance to several economically important pathogens. The created Pyramided monogenic and polygene isogenic lines from this specific study should be advanced to develop and release common bean varieties with multiple resistance for resource poor farmers in high disease spot areas in Ethiopia.

References

Abate, T. (1991). The bean fly, *Ophiomyia phaseoli* (Tryon) (Dipt., Agromyzidae), and its parasitoids in Ethiopia. *Journal of Applied Entomology*, *111*(1-5), 278-285.

Abdollahzadeh, J., & Zolfaghari, S. (2014). Efficiency of rep-PCR fingerprinting as a useful technique for molecular typing of plant pathogenic fungal species: *Botryosphaeriaceae* species as a case study. *FEMS microbiology letters*, *361*(2), 144-157.

Aggarwal, V. D., Pastor-Corrales, M. A., Chirwa, R. M., & Buruchara, R. A. (2004). Andean beans (*Phaseolus vulgaris* L.) with resistance to the angular leaf spot pathogen (*Phaeoisariopsis griseola*) in southern and eastern Africa. *Euphytica*, *136*(2), 201-210.

Allen, D. J., Dessert, M., Trutmann, P., & Voss, J. (1989). Common beans in Africa and their constraints. *Bean production problems in the tropics. CIAT, Cali, Colombia*, 9-31.

Ariani, A., y Teran, J. C. B. M., & Gepts, P. (2016). Genome-wide identification of SNPs and copy number variation in common bean (Phaseolus vulgaris L.) using genotyping-by-sequencing (GBS). *Molecular breeding*, *36*(7), 87.

Asfaw, A., & Blair, M. W. (2014). Quantification of drought tolerance in Ethiopian common bean varieties. *Agricultural Sciences*, *5*(02), 124.

Asfaw, A., Almekinders, C. J., Struik, P. C., & Blair, M. W. (2013). Farmers' common bean variety and seed management in the face of drought and climate instability in southern Ethiopia. *Scientific research and essays*, *8*(22), 1022-1037.

Asfaw, A., Blair, M. W., & Almekinders, C. (2009). Genetic diversity and population structure of common bean (Phaseolus vulgaris L.) landraces from the East African highlands. *Theoretical and Applied Genetics*, *120*(1), 1-12.

Babu, R., Nair, S. K., Prasanna, B. M., & Gupta, H. S. (2004). Integrating marker-assisted selection in crop breeding–prospects and challenges. *Current Science*, 607-619.

Baes, P. G., & Van Cutsem, P. J. (1993). Isozyme polymorphism in three gene pools of cultivated chicory (Cichorium intybus L.). *Euphytica*, *71*(1-2), 143-150.

Belete T, and Bastas K.K. (2017) Common Bacterial Blight (*Xanthomonas axonopodis* pv. phaseoli) of Beans with Special Focus on Ethiopian Condition. *Journal of Plant Patholog yandMicrobiol* 8:403. doi: 10.4172/2157-7471.1000403

Bellucci, E., Bitocchi, E., Rau, D., Rodriguez, M., Biagetti, E., Giardini, A., ... & Papa, R. (2014). Genomics of origin, domestication and evolution of Phaseolus vulgaris. In *Genomics of plant genetic resources* (pp. 483-507). *Springer*, Dordrecht.

Blair, M. W., Prieto, S., Díaz, L. M., Buendía, H. F., & Cardona, C. (2010). Linkage disequilibrium at the APA insecticidal seed protein locus of common bean (*Phaseolus vulgaris* L.). BMC *plant biology*, 10(1), 79.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics*, 23(19), 2633-2635.

Breseghello, F., & Sorrells, M. E. (2006). Association analysis as a strategy for improvement of quantitative traits in plants. *Crop Science*, *46*(3), 1323-1330.

Bretting, P. K., & Widrlechner, M. P. (1995). Genetic markers and plant genetic resource management.

Broughton, W. J., Hernandez, G., Blair, M., Beebe, S., Gepts, P., & Vanderleyden, J. (2003). Beans (Phaseolus spp.)–model food legumes. *Plant and soil*, *252*(1), 55-128.

Caixeta, E. T., Borém, A., Alzate-Marin, A. L., de Azevedo Fagundes, S., e Silva, M. G. D. M., de Barros, E. G., & Moreira, M. A. (2005). Allelic relationships for genes that confer resistance to angular leaf spot in common bean. *Euphytica*, *145*(3), 237-245.

Castellamos, G., Jara, C., & Mosquera, G. (2016). Bean pathogens: practical guide for Lab and greenhouse work.

Chilagane, L. A., Nchimbi-Msolla, S., Kusolwa, P. M., Porch, T. G., Diaz, L. M. S., & Tryphone, G. M. (2016). Characterization of the common bean host and Pseudocercospora griseola, the causative agent of angular leaf spot disease in Tanzania. *African Journal of Plant Science*, *10*(11), 238-245.

Cichy, K. A., Porch, T. G., Beaver, J. S., Cregan, P., Fourie, D., Glahn, R. P., ... & Mndolwa, E. (2015). A *Phaseolus vulgaris* diversity panel for Andean bean improvement. *Crop Science*, *55*(5), 2149-2160.

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B., & Pang, E. C. K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. *Euphytica*, *142*(1-2), 169-196

Correa-Victoria, F.J., Pastor-Corrales, M.A. and Saettler, A.W., (1989). Angular leaf spot. *Bean production problems in the tropics*, *59*, p.75.

Crous PW, Liebenberg MM, Braun U and Groenewald JZ (2006). Re-evaluating the taxonomic status of *Phaeoisariopsis griseola*, the causal agent of angular leaf spot of bean. *Stud. Mycol.* 55: 163-173.

CSA (Central Statistics Agency of Ethiopia). (2016). Report on area and crop production of major crops for 2016 *Meher* season, volume 1; 125 pp. Addis Ababa, Ethiopia.

Dalirsefat, S. B., da Silva Meyer, A., & Mirhoseini, S. Z. (2009). Comparison of similarity coefficients used for cluster analysis with amplified fragment length polymorphism markers in the silkworm, Bombyx mori. *Journal of insect science*, *9*(1).

Ddamulira, G., Mukankusi, C., Ochwo-Ssemakula, M., Edema, R., Sseruwagi, P., & Gepts, P. (2015). Gene pyramiding improved resistance to angular leaf spot in common bean. *American Journal of Experimental Agriculture*, *9*(2), 1-12.

Ddamulira, G., Mukankusi, C., Ochwo-Ssemakula, M., Edema, R., Sseruwagi, P., & Gepts, P. (2014). Distribution and Variability of Pseudocercospora griseola in Uganda. *Journal of Agricultural Science*, *6*(6), 16.

Debouck, D. G. (2000). Biodiversity, ecology and genetic resources of Phaseolus beans-seven answered and unanswered questions. In *The Seventh Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan, International Workshop on Genetic Resources, Ibaraki, Japan, 13-15 October, 1999: Part 1, wild legumes.* (pp. 95-123). National Institute of Agrobiological Resources (NIAR).

de Mendonça, H. A., dos Santos, J. B., & Ramalho, M. A. P. (2003). Genetic control of

common bean reaction to angular leaf spot. *Crop Breeding and Applied Biotechnology*, *3*(3).

De Bruijn, F. J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of Rhizobium melilot isolates and other soil bacteria. *Applied and environmental microbiology*, *58*(7), 2180-2187.

Deshmukh, U. C., Verma, R. K., Saxena, R. R., Mohan, P., & Verulkar, S. B. (2017). Marker assisted selection for bacterial leaf blight resistance in segregating populations of Karma Mahsuri. *Vegetos 30: 1. doi: 10.4172/2229*, *4473*.

Dice, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology*, *26*(3), 297-302.

Edwards, A. O., Ritter, R., Abel, K. J., Manning, A., Panhuysen, C., & Farrer, L. A. (2005). Complement factor H polymorphism and age-related macular degeneration. *Science*, *308*(5720), 421-424.

Ferreira, J. J., Campa, A., Pérez-Vega, E., Rodríguez-Suárez, C., & Giraldez, R. (2012). Introgression and pyramiding into common bean market class fabada of genes conferring resistance to anthracnose and potyvirus. *Theoretical and Applied Genetics*, *124*(4), 777-788.

Fininsa, C. (1996). Effect of intercropping bean with maize on bean common bacterial blight and rust diseases. *International Journal of Pest Management*, *42*(1), 51-54.

Flint-Garcia, S. A., Thornsberry, J. M., & Buckler IV, E. S. (2003). Structure of linkage disequilibrium in plants. *Annual review of plant biology*, 54(1), 357-374.

Frisch, M., Bohn, M., & Melchinger, A. E. (1999). Comparison of selection strategies for marker-assisted backcrossing of a gene. *Crop Science*, *39*(5), 1295-1301.

Garris, A. J., Tai, T. H., Coburn, J., Kresovich, S., & McCouch, S. (2005). Genetic structure and diversity in Oryza sativa L. *Genetics,* 169(3), 1631-1638.

Geil, P. B., & Anderson, J. W. (1994). Nutrition and health implications of dry beans: a review. *Journal of the American College of Nutrition*, *13*(6), 549-558.

Gentry, H. S. (1969). Origin of the common bean, *Phaseolus vulgaris*. *Economic*

Botany, *23*(1), 55-69.

Gepts, P. and Debouck, D. (1991). Origin, domestication, and evolution of the common bean (Phaseolus vulgaris L.). *Common beans: research for crop improvement*, *7*, p.53.

Gepts, P. (1988). A Middle American and an Andean common bean gene pool. In *Genetic resources of Phaseolus beans* (pp. 375-390). *Springer*, Dordrecht.

Gepts, P. (2004). Crop domestication as a long-term selection experiment. *Plant breeding reviews*, *24*(2), 1-44.

Gevers, D., Huys, G., & Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. *FEMS Microbiology Letters*, *205*(1), 31-36.

Gilson, E., Clément, J. M., Brutlag, D., & Hofnung, M. (1984). A family of dispersed repetitive extragenic palindromic DNA sequences in E. coli. *The EMBO journal*, *3*(6), 1417-1421.

Glaubitz JC, Casstevens TM, Lu F, Harriman J, Elshire RJ, Sun Q, Buckler ES. (2014) TASSEL-GBS: A High Capacity Genotyping by Sequencing Analysis Pipeline. PLoS ONE 9(2): e90346

Godwin ID, Aitken EAB, Smith LW (1997). Application of inter-simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis* 18:1524-1528.

Gonçalves-Vidigal, M. C., Cruz, A. S., Lacanallo, G. F., Vidigal Filho, P. S., Sousa, L. L., Pacheco, C. M. N. A., ... & Pastor-Corrales, M. A. (2013). Co-segregation analysis and mapping of the anthracnose Co-10 and angular leaf spot Phg-ON disease-resistance genes in the common bean cultivar Ouro Negro. *Theoretical and applied genetics*, *126*(9), 2245-2255.

Gonçalves-Vidigal, M. C., Cruz, A. S., Garcia, A., Kami, J., Vidigal Filho, P. S., Sousa, L. L., & Pastor-Corrales, M. A. (2011). Linkage mapping of the *Phg-1* and Co-1 4 genes for resistance to angular leaf spot and anthracnose in the common bean cultivar AND 277. *Theoretical and applied genetics*, *122*(5), 893-903.

Greenway, P. J. (1945). Origins of Some East African Food Plants: Part III. *The East African Agricultural Journal*, *10*(3), 177-180.

Gupta, P. K., & Varshney, R. K. (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica*, *113*(3), 163-185.

Guzmán, P., Gilbertson, R. L., Nodari, R., Johnson, W. C., Temple, S. R., Mandala, D., ... & Gepts, P. (1995). Characterization of variability in the fungus Phaeoisariopsis griseola suggests coevolution with the common bean (*Phaseolus vulgaris*). *Phytopathology*, *85*(5), 600-607.

Hasan, M. M., Rafii, M. Y., Ismail, M. R., Mahmood, M., Rahim, H. A., Alam, M. A., ... & Latif, M. A. (2015). Marker-assisted backcrossing: a useful method for rice improvement. *Biotechnology & Biotechnological Equipment*, *29*(2), 237-254.

Habtu, A., Zadoks, I. S. J., & Thiverval-Grignon, F. (1996). A survey of cropping practices and foliar diseases of common beans in Ethiopia. *Epidemiology of bean rust in Ethiopia*, 23.

Hanson, P., Lu, S. F., Wang, J. F., Chen, W., Kenyon, L., Tan, C. W., ... & Ledesma, D. (2016). Conventional and molecular marker-assisted selection and pyramiding of genes for multiple disease resistance in tomato. *Scientia horticulturae*, *201*, 346-354.

Hasan, M. M., Rafii, M. Y., Ismail, M. R., Mahmood, M., Rahim, H. A., Alam, M. A., ... & Latif, M. A. (2015). Marker-assisted backcrossing: a useful method for rice improvement. *Biotechnology & Biotechnological Equipment*, *29*(2), 237-254.

Harlan, H. V., & Pope, M. N. (1922). The use and value of back-crosses in small-grain breeding. *Journal of Heredity*, *13*(7), 319-322.

Hindorff, L. A., Sethupathy, P., Junkins, H. A., Ramos, E. M., Mehta, J. P., Collins, F. S., & Manolio, T. A. (2009). Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proceedings of the National Academy of Sciences,* 106(23), 9362-9367.

Huang X, Han B (2014) Natural variations and genome-wide association studies in crop plants. *Annual Review of Plant Biology* 65:531-551

Hulton, C. S. J., Higgins, C. F., & Sharp, P. M. (1991). ERIC sequences: a novel family of repetitive elements in the genomes of Escherichia coli, Salmonella typhimurium

and other enterobacteria. *Molecular microbiology*, *5*(4), 825-834.

Ishii, S., & Sadowsky, M. J. (2009). Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. *Environmental microbiology*, *11*(4), 733-740.

Joshi, R. K., & Nayak, S. (2010). Gene pyramiding-A broad spectrum technique for developing durable stress resistance in crops. *Biotechnology and Molecular Biology Reviews*, *5*(3), 51-60.

Kamfwa, K., Cichy, K. A., & Kelly, J. D. (2015). Genome-wide association analysis of symbiotic nitrogen fixation in common bean. *Theoretical and applied genetics*, *128*(10), 1999-2017.

Kamfwa, K., Cichy, K. A., & Kelly, J. D. (2015). Genome-wide association study of agronomic traits in common bean. *The Plant Genome*, *8*(2).

Keller, B., Manzanares, C., Jara, C., Lobaton, J. D., Studer, B., & Raatz, B. (2015). Fine-mapping of a major QTL controlling angular leaf spot resistance in common bean (Phaseolus vulgaris L.). *Theoretical and Applied Genetics*, *128*(5), 813-826.

Kempken, F., & Kück, U. (1998). Transposons in filamentous fungi facts and perspectives. *BioEssays*, *20*(8), 652-659.

Kistler, H. C., & Miao, V. P. (1992). New modes of genetic change in filamentous fungi. *Annual review of phytopathology*, *30*(1), 131-153.

Kolmer, J. A., Liu, J. Q., & Sies, M. (1995). Virulence and molecular polymorphism in Puccinia recondita f. sp. tritici in Canada. *Phytopathology*, *85*(3), 276-285.

Korte A, Farlow A (2013) The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods* 9:29

Kosman, E., & Leonard, K. J. (2005). Similarity coefficients for molecular markers in studies of genetic relationships between individuals for haploid, diploid, and polyploid species. *Molecular ecology*, *14*(2), 415-424.

Krieger, M. J., & Ross, K. G. (2002). Identification of a major gene regulating complex social behavior. *Science*, *295*(5553), 328-332.

Kumar, V.A., Balachiranjeevi, C.H., Naik, S.B., Rekha, G., Rambabu, R., Harika, G.,

Pranathi, K., Hajira, S.K., Anila, M., Kousik, M. and Kale, R., (2017). Marker-assisted pyramiding of bacterial blight and gall midge resistance genes into RPHR-1005, the restorer line of the popular rice hybrid DRRH-3. *Molecular Breeding*, *37*(7), p.86.

Kumar, G. A., Hanchinal, R. R., Desai, S., & Biradar, S. (2017). Marker Assisted Gene Pyramiding of Leaf Rust Resistance Genes Lr24 and Lr28 in the Background of Wheat Variety DWR 162 (Triticum aestivum L.). *Int. J. Curr. Microbiol. App. Sci*, *6*(9), 1883-1893.

Kwak, M., & Gepts, P. (2009). Structure of genetic diversity in the two major gene pools of common bean (*Phaseolus vulgaris* L., Fabaceae). *Theoretical and Applied Genetics*, 118(5), 979-992

Lemessa, F., Sori, W., & Wakjira, M. (2011). Association between angular leaf spot (Phaeoisariopsis griseola (Sacc.) Ferraris) and common bean (Phaseolus vulgaris L.) yield loss at Jimma, Southwestern Ethiopia. *Plan. Pathol. J*, *110*(2), 57-65.

Leung, H., Nelson, R. J., & Leach, J. E. (1993). Population structure of plant pathogenic fungi and bacteria. *Advances in plant pathology*

Liebenberg, M. M., & Pretorius, Z. A. (1997). A review of angular leaf spot of common bean (Phaseolus vulgaris L.). *African Plant Protection*, *3*(2), 81-106.

Lipka, A. E., Tian, F., Wang, Q., Peiffer, J., Li, M., Bradbury, P. J., ... & Zhang, Z. (2012). GAPIT: genome association and prediction integrated tool. *Bioinformatics*, 28(18), 2397-2399.

Liu, J., Liu, D., Tao, W., Li, W., Wang, S., Chen, P., ... & Gao, D. (2000). Molecular marker-facilitated pyramiding of different genes for powdery mildew resistance in wheat. *Plant Breeding*, *119*(1), 21-24.

Louws, F. J., Rademaker, J. L. W., & De Bruijn, F. J. (1999). The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Annual review of phytopathology*, *37*(1), 81-125.

Mahuku, G. S., Henríquez, M. A., Montoya, C., Jara, C., Teran, H., & Beebe, S. (2011). Inheritance and development of molecular markers linked to angular leaf spot resistance genes in the common bean accession G10909. *Molecular*

Breeding, 28(1), 57-71.

Mahuku, G. S., Iglesias, Á. M., & Jara, C. (2009). Genetics of angular leaf spot resistance in the Andean common bean accession G5686 and identification of markers linked to the resistance genes. *Euphytica*, *167*(3), 381-396.

Mahuku, G. S. (2004). A simple extraction method suitable for PCR-based analysis of plant, fungal, and bacterial DNA. *Plant Molecular Biology Reporter*, *22*(1), 71-81.

Mahuku, G. S., Jara, C., Cajiao, C., & Beebe, S. (2003). Sources of resistance to angular leaf spot (Phaeoisariopsis griseola) in common bean core collection, wild Phaseolus vulgaris and secondary gene pool. *Euphytica*, 130(3), 303-313.

Mahuku, G. S., Jara, C., Cuasquer, J. B., & Castellanos, G. (2002). Genetic variability within Phaeoisariopsis griseola from Central America and its implications for resistance breeding of common bean. *Plant pathology*, *51*(5), 594-604.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., & Morrison, D. A. (1992). A highly conserved repeated DNA element located in the chromosome of Streptococcus pneumoniae. *Nucleic acids research*, *20*(13), 3479-3483

McDonald, B. A., & Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annual review of phytopathology*, *40*(1), 349-379.

McDonald, J. G., Wong, E., & White, G. P. (2000). Differentiation of *Tilletia* species by rep-PCR genomic fingerprinting. *Plant Disease*, *84*(10), 1121-1125.

Mehta, A., Mehta, Y. R., & Rosato, Y. B. Homology and Sequencing of Fragments Amplified by Eric And Rep-PCR In *Stemphylium solani* Of Cotton.

Mehta, Y. R., Mehta, A., & Rosato, Y. B. (2002). ERIC and REP-PCR banding patterns and sequence analysis of the internal transcribed spacer of rDNA of *Stemphylium solani* isolates from cotton. *Current Microbiology*, *44*(5), 323-328.

Miller, T., Gepts, P., Kimmo, S., Arunga, E., Chilagane, L. A., Nchimbi-Msolla, S., ... & Tedla, Y. R. (2018). Alternative markers linked to the Phg-2 angular leaf spot resistance locus in common bean using the Phaseolus genes marker

database. *African Journal of Biotechnology*, *17*(26), 818-828.

Miklas, P. N., Smith, J. R., Riley, R., Grafton, K. F., Singh, S. P., Jung, G., & Coyne, D. P. (2000). Marker-assisted breeding for pyramided resistance to common bacterial blight in common bean. *ANNUAL REPORT-BEAN IMPROVEMENT COOPERATIVE*, *43*, 39-40.

Mkandawire, A. B., Mabagala, R. B., Guzmán, P., Gepts, P., & Gilbertson, R. L. (2004). Genetic diversity and pathogenic variation of common blight bacteria (*Xanthomonas campestris pv. phaseoli* and *X. campestris pv. phaseoli var. fuscans*) suggests pathogen coevolution with the common bean. *Phytopathology*, *94*(6), 593-603.

Mohali, S., Slippers, B., & Wingfield, M. J. (2007). Identification of *Botryosphaeriaceae* from Eucalyptus, Acacia and Pinus in Venezuela. *Fungal Diversity*, *25*(25), 103-125.

Mutlu, N., Miklas, P. N., & Coyne, D. P. (2006). Resistance gene analog polymorphism (RGAP) markers co-localize with disease resistance genes and QTL in common bean. *Molecular breeding*, *17*(2), 127-135.

Namayanja, A., Buruchara, R., Mahuku, G., Rubaihayo, P., Kimani, P., Mayanja, S., & Eyedu, H. (2006). Inheritance of resistance to angular leaf spot in common bean and validation of the utility of resistance linked markers for marker assisted selection outside the mapping population. *Euphytica*, *151*(3), 361-369.

Nordborg, M., & Tavaré, S. (2002). Linkage disequilibrium: what history has to tell us. *TRENDS in Genetics*, 18(2), 83-90.

Oblessuc, P. R., Baroni, R. M., Garcia, A. A. F., Chioratto, A. F., Carbonell, S. A. M., Camargo, L. E. A., & Benchimol, L. L. (2012). Mapping of angular leaf spot resistance QTL in common bean (Phaseolus vulgaris L.) under different environments. *BMC genetics*, 13(1), 50.

Olive, D. M., & Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of clinical microbiology*, *37*(6), 1661-1669.

Oraguzie, N.C., Gardiner, S.E., Rikkerink, E.H. and Silva, H.N. eds.

(2007.) Association mapping in plants (p. 277). Berlin, Germany: *Springer*.

Pastor-Corrales, M.A., (2004). Review of coevolution studies between pathogens and their common bean hosts: implication for the development of disease-resistant beans. *Annual Report-Bean Improvement Cooperative*, *47*, pp.67-68.

Pastor-Corrales, M.A., Jara, C. and Singh, S.P., (1998). Pathogenic variation in, sources of, and breeding for resistance to *Phaeoisariopsis griseola* causing angular leaf spot in common bean. *Euphytica*, *103*(2), pp.161-171.

Perseguini, J. M. K. C., Oblessuc, P. R., Rosa, J. R. B. F., Gomes, K. A., Chiorato, A. F., Carbonell, S. A. M., ... & Benchimol-Reis, L. L. (2016). Genome-wide association studies of anthracnose and angular leaf spot resistance in common bean (*Phaseolus vulgaris* L.). *PLoS One*, *11*(3), e0150506.

Singh, S.P. and Schwartz, H.F., (2010). Breeding common bean for resistance to diseases: a review. *Crop Science*, *50*(6), pp.2199-2223.

Stern, M. J., Ames, G. F. L., Smith, N. H., Robinson, E. C., & Higgins, C. F. (1984). Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell*, *37*(3), 1015-1026.

Peakall, R. O. D., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Resources*, *6*(1), 288-295.

Perseguini, J. M. K. C., Oblessuc, P. R., Rosa, J. R. B. F., Gomes, K. A., Chiorato, A. F., Carbonell, S. A. M., ... & Benchimol-Reis, L. L. (2016). Genome-wide association studies of anthracnose and angular leaf spot resistance in common bean (Phaseolus vulgaris L.). *PLoS One*, *11*(3), e0150506.

Pinkal, A., Korobeinikova, M., Kotsyubinskaya, O., Marakayeva, T., Kazydub, N. and Kuzmina, S., (2017). Chemical composition of seeds and green beans of common bean varieties, breeded in Omsk *State Agrarian* University under conditions of southern forest-steppe zone of Western Siberia.

Purseglove, J.W., (1968). Tropical crops. Dicotyledons 1 and 2. *Tropical crops.*

Dicotyledons 1 and 2.

Ragagnin, V. A., De Souza, T. L. P. O., Sanglard, D. A., Arruda, K. M. A., Costa, M. R., Alzate-Marin, A. L., ... & De Barros, E. G. (2009). Development and agronomic performance of common bean lines simultaneously resistant to anthracnose, angular leaf spot and rust. *Plant Breeding*, *128*(2), 156-163.

Rademaker, J. L., & de Bruijn, F. J. (1997). Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis. *DNA markers: protocols, applications and overviews*, *1*, 151-171.

Rencher, A. C. (1995). Multivariate analysis of variance. *Methods of Multivariate Analysis, Second Edition*, 156-247.

Rossi, M., Bitocchi, E., Bellucci, E., Nanni, L., Rau, D., Attene, G., & Papa, R. (2009). Linkage disequilibrium and population structure in wild and domesticated populations of Phaseolus vulgaris L. *Evolutionary Applications*, 2(4), 504-522.

Rubyogo, J. C., Gebeyehu, S., Tumsa, K., Negash, K., Habte, E., Katungi, E., ... & Wozemba, D. (2011, November). Increased bean productivity through increased access to improved seeds and use of improved bean management techniques in Ethiopia. In *conference "Increasing Agricultural Productivity and Enhancing Food Security in Africa* (pp. 1-3).

Saettler, A.W., (1991). Angular leaf spot. *Compendium of bean diseases*, *15*, p.16.

Saettler, A. W., & Corea, F. J. (1988). Transmission of *Phaeoisariopsis griseola* by bean seed. *Journal of Seed Technology*, *12*(2), 133-142.

Sartorato, A., & Alzate-Marin, A. L. (2004). Analysis of the pathogenic variability of Phaeoisariopsis griseola in Brazil. *ANNUAL REPORT-BEAN IMPROVEMENT COOPERATIVE*, *47*, 235-236.

Schmutz, J., McClean, P. E., Mamidi, S., Wu, G. A., Cannon, S. B., Grimwood, J., ... & Torres-Torres, M. (2014). A reference genome for common bean and genome-wide analysis of dual domestications. *Nature genetics*, *46*(7), 707.

Schoonhoven, A. V., & Pastor-Corrales, M. A. (1987). Standard system for the evaluation of bean germplasm. *CIAT, Cali, Colombia*, *56*.

Schwartz, H. F., Correa, V., Pineda, D., Mercedes Otoya, M., & Katherman, M. J. (1981). Dry bean yield losses caused by Ascochyta, angular, and white leaf spots in Colombia. *Plant Disease*.

Scott, R. A., & Milliken, G. A. (1993). A SAS program for analyzing augmented randomized complete-block designs. *Crop Science*, *33*(4), 865-867.

Shi, C., Navabi, A., & Yu, K. (2011). Association mapping of common bacterial blight resistance QTL in Ontario bean breeding populations. *BMC Plant Biology*, *11*(1), 52.

Silva, E.K.J.D., Damasceno, K.J., De Souza, E.A. and Sartorato, A., (2008). Pathogenic variability of isolates of *Pseudocercospora griseola*, the cause of common bean angular leaf spot, and its implications for resistance breeding. *Journal of Phytopathology*, *156*(10), pp.602-606.

Sindhan, G. S., & Bose, S. K. (1979). Perpetuation of *Phaeoisariopsis griseola* causing angular leaf spot of French beans. *Indian Phytopathology*, *32*(2), 252-254.

Singh, S. P. (2001). Broadening the genetic base of common bean cultivars. *Crop Science*, *41*(6), 1659-1675.

Singh, S. P., & Munoz, C. G. (1999). Resistance to common bacterial blight amongPhaseolus species and common bean improvement. *Crop Science*, *39*(1), 80-89.

Staub, J. E., Serquen, F. C., & Gupta, M. (1996). Genetic markers, map construction, and their application in plant breeding. *HortScience*, *31*(5), 729-741.

Stenglein, S. A., & Balatti, P. A. (2006). Genetic diversity of Phaeoisariopsis griseola in Argentina as revealed by pathogenic and molecular markers. *Physiological and Molecular Plant Pathology*, *68*(4-6), 158-167.

Stenglein, S. A., & Balatti, P. A. (2006). Genetic diversity of Phaeoisariopsis griseola in Argentina as revealed by pathogenic and molecular markers. *Physiological and Molecular Plant Pathology*, *68*(4-6), 158-167.

Stenglein, S., Ploper, L. D., Vizgarra, O., & Balatti, P. (2003). Angular leaf spot: a disease caused by the fungus Phaeoisariopsis griseola (Sacc.) Ferraris on Phaseolus vulgaris L. *Advances in applied microbiology*, *52*, 209-243.

Tadesse, N., Ahmed, S., Gorfu, D., Beshir, T., Fininsa, C., Abraham, A., ... & Meles, K. (2006). Review of research on diseases food legumes. *Increasing Crop Production through Improved Plant Protection–Volume I*, 85.

Tefera, T. (2006). Effect of common bacterial blight severity on common bean yield. *Tropical science*, *46*(1), 41-44.

Miller, T., Gepts, P., Kimmo, S., Arunga, E., Chilagane, L. A., Nchimbi-Msolla, S., ... & Tedla, Y. R. (2018). Alternative markers linked to the *Phg-2* angular leaf spot resistance locus in common bean using the Phaseolus genes marker database. *African Journal of Biotechnology*, *17*(26), 818-828.

Tang, Y., Liu, X., Wang, J., Li, M., Wang, Q., Tian, F., ... & Buckler, E. S. (2016). GAPIT Version 2: an enhanced integrated tool for genomic association and prediction. *The plant genome*, 9(2).

Trutmann, P., Voss, J., & Fairhead, J. (1996). Local knowledge and farmer perceptions of bean diseases in the Central African Highlands. *Agriculture and Human Values*, *13*(4), 64-70.

Tsumura, Y., Yoshimura, K., Tomaru, N., & Ohba, K. (1995). Molecular phytogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. *Theoretical and applied genetics*, *91*(8), 1222-1236.

Turner, S. D. (2014). qqman: An R package for visualizing GWAS results using QQ and manhattan plots. BioRxiv, 005165.

Ussery, D. W., Binnewies, T. T., Gouveia-Oliveira, R., Jarmer, H., & Hallin, P. F. (2004). Genome update: DNA repeats in bacterial genomes. *Microbiology*, *150*(11), 3519-3521.

Versalovic, J., Schneider, M., De Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in molecular and cellular biology*, *5*(1), 25-40.

Versalovic, J., Koeuth, T., & Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic acids research*, *19*(24), 6823-6831.

Van Belkum, A., Scherer, S., Van Alphen, L., & Verbrugh, H. (1998). Short-sequence

DNA repeats in prokaryotic genomes. *Microbiology and Molecular Biology Reviews*, *62*(2), 275-293.

Villordo-Pineda, E., González-Chavira, M. M., Giraldo-Carbajo, P., Acosta-Gallegos, J. A., & Caballero-Pérez, J. (2015). Identification of novel drought-tolerant-associated SNPs in common bean (*Phaseolus vulgaris*). *Frontiers in plant science*, *6*, 546.

Wagara, N., Mwang'ombe, A. W., Kimenju, J. W., Buruchara, R. A., & Kimani, P. M. (2003). Pathogenic variability in *Phaeoisariopsis griseola* and response of bean germplasm to different races of the pathogen. *African Crop Science Journal Society*, *6*, 352-357.

Wagara, I. N., Mwang'ombe, A. W., Kimenju, J. W., & Buruchara, R. A. (2005). Virulence, variability and physiological races of the angular leaf spot pathogen Phaeoisariopsis griseola in Kenya. *African Plant Protection*, *11*(1), 23-31.

Wortmann, C.S., (1998). *Atlas of common bean (Phaseolus vulgaris L.) production in Africa* (No. 297). CIAT.

Wan, J., Dunning, M. F., & Bent, A. F. (2002). Probing plant-pathogen interactions and downstream defense signaling using DNA microarrays. *Functional & integrative genomics*, *2*(6), 259-273.

Wang, C., Yang, Y., Yuan, X., Xu, Q., Feng, Y., Yu, H., & Wang, Y. (2014). Genome-wide association study of blast resistance in indica rice. *BMC plant biology*, *14*(1), 311.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.* 18:6531-6535.

Wortmann, C. S. (1998). *Atlas of common bean (Phaseolus vulgaris L.) production in Africa* (No. 297). CIAT.

Xu, Y., & Crouch, J. H. (2008). Marker-assisted selection in plant breeding: from publications to practice. *Crop science*, *48*(2), 391-407.

Ye C, Yu Z, Kong F, Wu S, Wang B (2005). R-ISSR as a new tool for genomic fingerprinting, mapping and gene tagging. *Plant Mol. Biol. Rep*. 23:167-177.

Yesuf, M., & Sangchote, S. (2005). Occurrence and distribution of major seedborne fungi associated with Phaseolus bean seeds in Ethiopia. *Kasetsart Journal*, 216.

Yu, J., Pressoir, G., Briggs, W. H., Bi, I. V., Yamasaki, M., Doebley, J. F., ... & Kresovich, S. (2006). A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nature genetics*, 38(2), 203-208.

Taylor, J. W., Geiser, D. M., Burt, A., & Koufopanou, V. (1999). The evolutionary biology and population genetics underlying fungal strain typing. *Clinical Microbiology Reviews*, *12*(1), 126-146.

Zhang Z, Ersoz E, Lai C-Q, Todhunter RJ, Tiwari HK, Gore MA, Bradbury PJ, Yu J, Arnett DK, Ordovas JM, Buckler ES. (2010) Mixed linear model approach adapted for genome-wide association studies. *Nature Genetics* 42:355-360

Wang, C., Yang, Y., Yuan, X., Xu, Q., Feng, Y., Yu, H., & Wang, Y. (2014). Genome-wide association study of blast resistance in Indica rice. *BMC plant biology*, 14(1), 311.

Appendix I

Appendix-Table 1 Angular leaf spot isolates collected from diverse bean growing regions of Ethiopia introduced from CIAT Uganda

CODE	Location	Altitude	Host	Genepool	Origin	Year of Collection
pget001	WONDO	1742masl	PIC6	A	Ethiopia	2017
pget002	WONDO	1742masl	ADP-0100	A	Ethiopia	2017
pget003	GOFA	1400masl	SMALL RED	M	Ethiopia	2017
pget004	WONDO	1742masl	ADP-0095	A	Ethiopia	2017
pget005	WONDO	1742masl	ADP-0468	A	Ethiopia	2017
pget006	GOFA	1400masl	SMALL RED	M	Ethiopia	2017
pget007	HALABA	1872masl	TATU	A	Ethiopia	2017
pget008	GOFA	1400masl	HDUME	M	Ethiopia	2017
pget009	WONDO	1742masl	ADP-0668	A	Ethiopia	2017
pget010	WONDO	1742masl	ADP-0518	A	Ethiopia	2017
pget011	WONDO	1742masl	ADP-0037	A	Ethiopia	2017
pget012	WONDO	1742masl	ADP-0037	A	Ethiopia	2017
pget013	WONDO	1742masl	ADP-0675	A	Ethiopia	2017
pget014	WONDO	1742masl	ADP-0675	A	Ethiopia	2017
pget015	WONDO	1742masl	ADP-0675	A	Ethiopia	2017
pget016	WONDO	1742masl	ADP-0675	A	Ethiopia	2017
pget017	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget018	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget019	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget020	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget021	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget022	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget023	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget024	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget025	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget026	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget027	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget028	DOLLA	1865masl	REDWOLAITA	M	Ethiopia	2017
pget029	DOLLA	1219masl	NASIER	M	Ethiopia	2017
pget030	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget031	SOUTH OMO	1363masl	REDWOLAITA	M	Ethiopia	2017
pget032	SOUTH OMO	1363masl	REDWOLAITA	M	Ethiopia	2017
pget033	SOUTH OMO	1363masl	H DUME	M	Ethiopia	2017

CODE	Location	Altitude	Host	Genepool	Origin	Year of Collection
pget034	SOUTH OMO	1363masl	H DUME	M	Ethiopia	2017
pget035	SOUTH OMO	1363masl	H DUME	M	Ethiopia	2017
pget036	SOUTH OMO	1363masl	H DUME	M	Ethiopia	2017
pget037	SOUTH OMO	1363masl	H DUME	M	Ethiopia	2017
pget038	CHANO MILE	1219masl	NASIER	M	Ethiopia	2017
pget039	BAKO GAZAR	1363masl	SMALL RED	M	Ethiopia	2017
pget040	BAKO GAZAR	1363masl	SMALL RED	M	Ethiopia	2017
pget041	SOUTH OMO	1363masl	HDUME	M	Ethiopia	2017
pget042	AREKA	1802masl	ADP	A	Ethiopia	2017
pget043	AREKA	1802masl	ADP	A	Ethiopia	2017
pget044	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget045	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget046	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget047	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget048	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget049	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget050	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget051	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget052	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget053	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget054	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget055	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget056	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget057	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget058	CHANO DORGA	1219masl	NASIER	M	Ethiopia	2017
pget059	CHANO MILE	1212masl	NASIER	M	Ethiopia	2017
pget060	CHANO MILE	1219masl	NASIER	M	Ethiopia	2017
pget061	CHANO MILE	1219masl	NASIER	M	Ethiopia	2017
pget062	CHANO MILE	1219masl	NASIER	M	Ethiopia	2017
pget063	CHANO MILE	1219masl	NASIER	M	Ethiopia	2017
pget064	CHANO MILE	1219masl	NASIER	M	Ethiopia	2017
pget065	CHANO MILE	1219masl	NASIER	M	Ethiopia	2017
pget066	CHANO MILE	1212masl	NASIER	M	Ethiopia	2017
pget067	CHANO MILE	1212masl	NASIER	M	Ethiopia	2017
pget068	CHANO MILE	1212masl	NASIER	M	Ethiopia	2017
pget069	CHANO MILE	1212masl	NASIER	M	Ethiopia	2017
pget070	HALABA	1872masl	HDUME	M	Ethiopia	2016
pget071	GURAGE	1604masl	NASIER	M	Ethiopia	2016

CODE	Location	Altitude	Host	Genepool	Origin	Year of Collection
pget072	GURAGE		NASIER	M	Ethiopia	2016
pget073	GURAGE	1772masl	NASIER	M	Ethiopia	2016
pget074	AREKA	1884masl	REDWOLAITA	M	Ethiopia	2016
pget075	GURAGE	1742masl	NASIER	M	UGANDA	2016
pget076	KAO60 /CIAT			A	UGANDA	2016
pget077	240					2016
pget078	220					2016
pget079						

SR=small red, RW=REDWOLAITA MA=Mesoamerican

Appendix-Table 2 Diverse Common bean genotypes included in the genome wide study

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0006	W6 16465	red	Bush	Africa
ADP-0008	Nyayo	red mottled	Bush	Africa
ADP-0010	CANADA	red	Bush	Africa
ADP-0011	KIBOROLONI	red	Bush	Africa
ADP-0012	W6 16489	red	Bush	Africa
ADP-0013	KIBUMBULA	red	Bush	Africa
ADP-0014	KIANGWE	yellow	Bush	Africa
ADP-0015	W6 16495	red	Bush	Africa
ADP-0016	GOLOLI	red	Bush	Africa
ADP-0018	SODAN	red	Bush	Africa
ADP-0019	KASUKANYWELE	zebra	Bush	Africa
ADP-0021	MBULAMTWE	yellow	Bush	Africa
ADP-0022	KISAPURI	red	Bush	Africa
ADP-0023	MSHORONYLONI	red	Bush	Africa
ADP-0028	Sisi	yellow	Bush	Africa
ADP-0029	RH No. 2	red	Bush	Africa
ADP-0030	RH No. 6	blk	Bush	Africa
ADP-0032	RH No. 21	brown	Bush	Africa
ADP-0033	KIJIVU	purple spec	Bush	Africa
ADP-0034	KIJIVU	purple spec	Bush	Africa
ADP-0035	Kokola	red mottled	Bush	Africa
ADP-0036	Lyamungu 85	red mottled	Bush	Africa
ADP-0037	W6 16488	brn	Bush	Africa
ADP-0038	Moono	red	Bush	Africa
ADP-0040	KATWELA	red	Climber	Africa
ADP-0041	MRONDO	red	Vine	Africa

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0042	MKOKOLA	red	Vine	Africa
ADP-0043	BWANA SHAMBA	red	Vine	Africa
ADP-0044	KIJIVU	purple spec	Vine	Africa
ADP-0047	MSOLINI	brn	Vine	Africa
ADP-0048	W6 16534	red	Climber	Africa
ADP-0049	W6 16546	red	Vine	Africa
ADP-0052	RH No. 9	purple spec	Climber	Africa
ADP-0053	MAHARAGE MAKUBWA	red	Climber	Africa
ADP-0054	W6 16447	cran	Vine	Africa
ADP-0055	KABUKU	red spec	Vine	Africa
ADP-0056	SOYA	purple spec	Vine	Africa
ADP-0057	KIJIVU	red	Vine	Africa
ADP-0060	CANADA	red	Vine	Africa
ADP-0061	Maulasi	brn cran	Bush	Africa
ADP-0062	MAULASI	red spec	Vine	Africa
ADP-0063	Soya	purple. Spec	Climber	Africa
ADP-0064	W6 16500	yellow	Vine	Africa
ADP-0065	W6 16501	red	Vine	Africa
ADP-0066	NJANO	yellow	Vine	Africa
ADP-0067	NJANO	yellow	Climber	Africa
ADP-0069	SOYA	purple spec	Vine	Africa
ADP-0070	Msafiri	red	Vine	Africa
ADP-0071	NJANO-DOLEA	gray	Vine	Africa
ADP-0072	MASUSU	brn	Vine	Africa
ADP-0074	KABLANKETI	purple spec	Vine	Africa
ADP-0075	MABUKU	brn	Vine	Africa
ADP-0076	KABLANKETI	purple spec	Vine	Africa
ADP-0077	NAMWANGA	purple spec	Climber	Africa
ADP-0081	KABLANKETI	purple spec	Vine	Africa
ADP-0082	KABLANKETI	purple spec	Climber	Africa
ADP-0083	W6 16547	purple spec	Climber	Africa
ADP-0084	KABLANKETI NDEFU	drk purple spec	Climber	Africa
ADP-0085	KABLANKETI	purple spec	Climber	Africa
ADP-0086	NYAMHONGA MWEKUNDU	purple spec	Climber	Africa
ADP-0088	KABLANKETI	purple spec	Vine	Africa
ADP-0092	MORO	yellow	Vine	Africa
ADP-0093	MORO	yellow	Vine	Africa
ADP-0094	LUSHALA	yellow	Vine	Africa
ADP-0095	CANADA	zebra	Vine	Africa
ADP-0096	Rojo	red	Bush	Africa
ADP-0097	Bilfa 4	brn	Bush	Africa
ADP-0098	Selian 97	red	Bush	Africa
ADP-0100	EG 21	pur mot	Bush	Africa

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0101	Witrood	cran	Bush	Africa
ADP-0103	Pesa	red	Bush	Africa
ADP-0105	Sewani 97	red	Bush	Africa
ADP-0106	Zawadi	pur mot	Bush	Africa
ADP-0107	Mshindi	pur mot	Bush	Africa
ADP-0108	Njano	yellow	Vine	Africa
ADP-0109	Kablanketi	pur mot	Vine	Africa
ADP-0111	Uyole 98	yellow	Vine	Africa
ADP-0113	OPS-RS4	cran	Vine	Africa
ADP-0114	OPS-RS1	cran	Vine	Africa
ADP-0117	A483	pur mot	Vine	Africa
ADP-0118	Werna	cran	Vine	Africa
ADP-0119	A193	spec red	Vine	Africa
ADP-0120	Tygerberg	cran	Vine	Africa
ADP-0121	Kranskop HR-1	cran	Vine	Africa
ADP-0122	Kranskop	cran	Vine	Africa
ADP-0125	CHEUPE	white	Climber	Africa
ADP-0127	SELIAN 06	pink	Climber	Africa
ADP-0166	NABE 4	red mottled	Bush	Africa
ADP-0180	G 433	cran	Bush	CIAT core
ADP-0186	G 1368	red	Vine	CIAT core
ADP-0188	G 1375	yellow	Vine	CIAT core
ADP-0199	G 3452	pink	Bush	CIAT core
ADP-0204	G 4474	off-white	Vine	CIAT core
ADP-0206	G 4499	white	Vine	CIAT core
ADP-0207	G 4564	j. cattle	Bush	CIAT core
ADP-0208	G 4644	red mottled	Bush	CIAT core
ADP-0212	G 4970	tan	Bush	CIAT core
ADP-0213	G 5034	grey	Vine	CIAT core
ADP-0214	G 5087	black	Vine	CIAT core
ADP-0224	G 6239	j. cattle; tan on white	Vine	CIAT core
ADP-0247	G 9975	cran	Climber	CIAT core
ADP-0271	G 13167	white	Bush	CIAT core
ADP-0272	G 13336	purple cran	Climber	CIAT core
ADP-0303	G 17913	cream	Bush	CIAT core
ADP-0324	G 20729	d. red	Vine	CIAT core
ADP-0345	G 22147	red	Bush	CIAT core
ADP-0346	G 22246	red mottled	Vine	CIAT core
ADP-0354	G 22502	purple spec	Vine	CIAT core
ADP-0368	G 23093	white pinkish	Vine	CIAT core
ADP-0379	PI 203934	cream	Bush	US core
ADP-0390	PI 307808	red	Bush	US core
ADP-0391	PI 308894	red kidney	Bush	US core

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0392	PI 309701	cran	Vine	US core
ADP-0395	PI 310511	red mottled	Bush	US core
ADP-0417	PI 451906	red kidney	Bush	US core
ADP-0427	Badillo	red kidney	Same as ADP-626	Caribbean
ADP-0430	PR1013-3	pink mottled	Bush	Caribbean
ADP-0433	PR9745-232	red mottled	Bush	Caribbean
ADP-0434	PR0737-1	red mottled	Vine	Caribbean
ADP-0438	46-1	red mottled	Vine	Caribbean
ADP-0439	754-3	red mottled	Vine	Caribbean
ADP-0441	91-1	yellow	Vine	Caribbean
ADP-0442	Larga Comercial	red mottled	Vine	Caribbean
ADP-0443	Vazon 7	red mottled	Vine	Caribbean
ADP-0445	Chijar	red mottled	Vine	Caribbean
ADP-0446	Raz 25	red mottled	Vine	Caribbean
ADP-0449	INIAP 420	yellow	Bush	Ecuador
ADP-0450	INIAP 422	white	Bush	Ecuador
ADP-0452	INIAP 425	white	Bush	Ecuador
ADP-0453	INIAP 428		Vine	Ecuador
ADP-0454	INIAP 429	red mottled	Climber	Ecuador
ADP-0455	INIAP 430	red mottled	Bush	Ecuador
ADP-0456	INIAP 480		Vine	Ecuador
ADP-0457	INIAP 481	red mottled	Climber	Ecuador
ADP-0458	INIAP 483	red mottled	Bush	Ecuador
ADP-0466	PI449430	Purplele speckled	Bush	East Africa
ADP-0467	PI209808	Purplele speckled	Vine	East Africa
ADP-0468	PI527538	yellow	Bush	East Africa
ADP-0470	PI527508	Cranberry	Bush	East Africa
ADP-0471	PI527537-C	yellow/ brown	Vine	East Africa
ADP-0473	PI527537-F	gray	Vine	East Africa
ADP-0474	PI527519	purplele mottled	Vine	East Africa
ADP-0479	PI527530	yellow/ brown	Vine	East Africa
ADP-0480	PI209804	Purplele mottled	Bush	East Africa
ADP-0481	PI449428	red/white mottled	Bush	East Africa
ADP-0482	PI209802	Purplele mottled	Bush	East Africa
ADP-0510	Ohlio de perdiz	Jacobs Cattle	Vine	Angola
ADP-0512	Ervilha	manteca	Vine	Angola
ADP-0515	Katarina, Kibala	Cranberry	Bush	Angola
ADP-0516	Mantega, Kibala	manteca	Vine	Angola
ADP-0517	Carioca, Kibala	carioca	Vine	Angola
ADP-0518	Mantega blanca, Kibala	manteca	Bush	Angola
ADP-0519	Katarina, Cela	Cranberry	Bush	Angola
ADP-0520	Chumbo, Cela	green	Vine	Angola
ADP-0521	Cebo, Cela	manteca	Bush	Angola

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0523	Canario, Cela	yellow	Vine	Angola
ADP-0524	KAT B1	green yellow	Bush	CIAT Africa
ADP-0529	LYAMUNGO 90	d red mottled	Bush	CIAT Africa
ADP-0530	SELIAN 94	pink mottled	Vine	CIAT Africa
ADP-0532	A 197	light tan	Bush	CIAT Africa
ADP-0534	G 22501	lt tan grey	Bush	CIAT Africa
ADP-0538	RWR 221	LRK	Bush	CIAT Africa
ADP-0540	AFR 708	red mottled	Bush	CIAT Africa
ADP-0543	G 16157	LRK	Bush	CIAT Africa
ADP-0544	PVA 773	red mottled	Bush	CIAT Africa
ADP-0546	RED CANADIAN WONDER	dark red-pur	Vine	CIAT Africa
ADP-0549	RWR 10	red	Vine	CIAT Africa
ADP-0550	RANJONOMBY		Bush	CIAT Africa
ADP-0551	AFR 612	d. red mottled	Bush	CIAT Africa
ADP-0553	AND277	red mottled	Bush	CIAT Africa
ADP-0554	AND279	red mottled	Bush	CIAT Africa
ADP-0555	BRB191	red mottled	Bush	CIAT Africa
ADP-0556	BRB194	d. red	Bush	CIAT Africa
ADP-0558	DAB 528	red	Bush	CIAT Africa
ADP-0560	DAB230	red mottled	Bush	CIAT Africa
ADP-0561	DAB246	red mottled	Bush	CIAT Africa
ADP-0564	G 5164	pink mottled	Vine	CIAT Africa
ADP-0567	G 4523	d. red mottled	Bush	CIAT Africa
ADP-0569	MDRK	dark red	Bush	CIAT Africa
ADP-0571	NUA45	d red mottled	Bush	CIAT Africa
ADP-0572	NUA56	d red mottled	Vine	CIAT Africa
ADP-0576	SAB618	Red mottled	Bush	CIAT Africa
ADP-0581	SAB629	CRAN	Bush	CIAT Africa
ADP-0584	SAB659	red mottled	Bush	CIAT Africa
ADP-0586	SAB691	CRAN	Bush	CIAT Africa
ADP-0588	SAP 1	d. red mottled	Bush	CIAT Africa
ADP-0592	AND 1005	red mottled	Vine	CIAT Africa
ADP-0595	G13094	yellow	Bush	CIAT Africa
ADP-0597	G23829	black	Vine	CIAT Africa
ADP-0598	Charlevoix	DRK	Bush	N American
ADP-0602	Sacramento	LRK	Bush	N American
ADP-0603	Wallace 773-V98	LRK	Bush	N American
ADP-0605	1132-V96	LRK	Bush	N American
ADP-0607	NY 105	LRK	Bush	N American
ADP-0608	UI-51	CRAN	Bush	N American
ADP-0613	02-385-14	LRK	Bush	N American
ADP-0615	Litekid	LRK	Bush	N American
ADP-0617	Red Rider	cran	Bush	N American

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0618	AC Elk	LRK	Bush	N American
ADP-0619	UCD 0906	Jacobs Cattle	Bush	N American
ADP-0620	UCD0405	red spec	Bush	N American
ADP-0621	Jalo EEP558	Yellow	Vine	N American
ADP-0622	UCD 0701	Jacobs cattle	Bush	N American
ADP-0623	Drake	DRK	Bush	N American
ADP-0624	Dolly	CRAN	Bush	N American
ADP-0625	Micran	CRAN	Bush	N American
ADP-0628	H9659-27-7	LRK	Vine	N American
ADP-0629	H9659-27-10	LRK	Vine	N American
ADP-0635	OAC Redstar	DRK	Bush	N American
ADP-0636	Montcalm	DRK	Bush	N American
ADP-0637	Isabella	LRK	Bush	N American
ADP-0638	Red Hawk	DRK	Bush	N American
ADP-0639	Chinook 2000	LRK	Bush	N American
ADP-0641	Capri	CRAN	Bush	N American
ADP-0642	Taylor Hort	CRAN	Bush	N American
ADP-0644	Fox Fire	LRK	Bush	N American
ADP-0645	Lassen	WK	Bush	N American
ADP-0648	Red Kloud	LRK	Bush	N American
ADP-0650	K-42	LRK	Bush	N American
ADP-0651	K-59	LRK	Bush	N American
ADP-0653	USDK-CBB-15	DRK	Bush	N American
ADP-0654	USDK-4	DRK	Bush	N American
ADP-0655	Fiero	DRK	Bush	N American
ADP-0656	Royal Red	DRK	Bush	N American
ADP-0657	Kardinal	LRK	bush	N American
ADP-0659	USLK-1	LRK	Bush	N American
ADP-0660	Krimson	CRAN	Bush	N American
ADP-0663	USCR-CBB-20	CRAN	Bush	N American
ADP-0664	Silver Cloud	WK	Bush	N American
ADP-0665	USWK-CBB-17	WK	Bush	N American
ADP-0666	USWK-6	WK	Bush	N American
ADP-0667	VA-19	LRK	Bush	N American
ADP-0670	AC Calmont	DRK	Bush	N American
ADP-0671	AC Elk	LRK	Bush	N American
ADP-0672	CDRK	DRK	Bush	N American
ADP-0674	UCD 0704	WK	Bush	N American
ADP-0675	UCD 0801	CRAN	Bush	N American
ADP-0676	CELRK	DRK	Bush	N American
ADP-0679	Red Rover	DRK	Bush	N American
ADP-0680	Clouseau	LRK	Bush	N American
ADP-0682	UI-686	CRAN	Vine	N American

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0683	IJR	pink mottle	Vine	N American
ADP-0716	LG	CRAN	Vine	E Africa
ADP-0717	LG	CRAN	Vine	E Africa
ADP-0718	LG	RD MOTT	Bush	E Africa
ADP-0719	MED	PUR MOTT	Vine	E Africa
ADP-0720	SM	CRAN	Vine	E Africa
ADP-0721	MED	DRK	Vine	E Africa
ADP-0722	SM	CRAN	Vine	E Africa
ADP-0724	MED	DRK	Vine	E Africa
ADP-0725	MED	CRAN	Bush	E Africa
ADP-0727	LG	TAN	Bush	E Africa
ADP-0728	MED	LRK	Vine	E Africa
ADP-0729	LG	STRIP PUR	Vine	E Africa
ADP-0730	SM	PUR MOTT	Vine	E Africa
ADP-0731	MED	RD MOTT	Bush	E Africa
ADP-0732	MED	RD MOTT	Bush	E Africa
ADP-0733	SM	RD MOTT	Bush	E Africa
ADP-0734	MED	RD MOTT	Bush	E Africa
ADP-0735	LG	RD MOTT	Vine	E Africa
ADP-0737	MED	RD MOTT	Vine	E Africa
ADP-0739	LG	TAN/PUR	Bush	E Africa
ADP-0740	MED	RD MOTT	Vine	E Africa
ADP-0741	brown	Brown	Bush	E Africa
ADP-0742	pur red	Pure red	Vine	E Africa
ADP-0743	d. red	Dark Red	Vine	E Africa
ADP-0744	pink mottled	Pink Mottled	Vine	E Africa
ADP-0745	dark pur	Dark Pure	Vine	E Africa
ADP-0746	dark pur	Dark Pure	Vine	E Africa
ADP-0750	dark red	Drak red	Bush	E Africa
ADP-0751	red mottled	red mottled	Bush	E Africa
ADP-0753	black and white	Black & white	Bush	E Africa
ADP-0754	red mottled	red mottled		E Africa
ADP-0760	Wanja	Brown	10	E Africa
ADP-0769	ACUG12-C1	CRAN	10	N America
ADP-0770	ACUG12-C2	CRAN	10	N America
ADP-0771	ACUG13-C1	CRAN	10	N America
ADP-0774	ACUG13-L2	red kidney	10	N America
ADP-0775	HR202-4973	CRAN	10	N America
ADP-0776	Dynasty	Dark Red Kidney	10	N America
ADP-0777	AC-Darkid	Dark Red Kidney	10	N America
ADP-0778	Yeti	White Kidney	10	N America
ADP-0779	CDC-Sol	Yellow	10	N America
ADP-0781	L11YL012	Yellow	10	N America
ADP-0784	PS11-006C-8-B	CRAN	10	N America

ADP ID	Genotype	seed colour	Growth Habit	Country
ADP-0785	PS11-006C-1-B	CRAN	10	N America
ADP-0789	PR0313-3	Pink stri		
ADP-0791	PR1146-123	medium		