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Introgression of Genes Conferring Resistance Against Angular Leaf Spot (*Pseudosercospora Griseola)* and Anthracnose (*Colletotrichum Lindemuthianum*) in to Common Bean (*Phaseolus Vulgaris L*) *Advanced Line using Marker Assisted Selection*

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Keywords: angular leaf spot, anthracnose, common bean, durable resistance, gene introgression, molecular markers, marker assisted selection.

I. Introduction

Common bean (*Phaseolous vulgaris* L.) is the most important food legume consumed worldwide. It is widely cultivated in tropical and subtropical countries. Its production in sub-Saharan Africa is around 35 million metric tons with 62% being produced in East African countries namely Burundi, DR Congo, Ethiopia, Kenya, Rwanda, Tanzania and Uganda (Broughton *et al*., *2003*).

In Ethiopia, common bean is mainly cultivated in the Eastern, Southern, South-western and Rift valley regions of the country (Habtu *et al*., 1996). Despite its economic significance and wide area of production, current national productivity is 1.5 t/ha which is lower than the potential yield 4.5 t/ha (CSA, 2014). The low national yield could be attributed to various constraints. A recent study revealed that pests and diseases are ranked as the second important production constraints in the Central Rift Valley region, next to drought (Kutangi *et al*., 2010). Moreover, Yesuf (2005) emphasized that diseases are known to be the major factors which threaten the productivity of beans in general and common bean in particular. Anthracnose (*Colletotrichum lindemuthianum*), angular leaf spot (*Phaeoisariopsis griseola*) and common bacterial blight (*Xanthomonas axonopodis pv. phaseoli*) are common diseases of bean in Ethiopia (Habtu *et al*., 1996). It has been confirmed that infection of susceptible cultivars in favorable environmental conditions leading to an epidemic could result in 100% yield loss (Fernandez *et al*., 2000). A study by Tesfaye (1997) showed that yield loss up to 62.8% due to anthracnose (ANT) and angular leaf spot (ALS) is 70% in (*De Jesus Junior et al*., 2001) .The effect of common bacterial blight (CBB) reported to vary

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between 22% and 45% (Wallen and Jackson, 1975; Yoshii 1980).

The current disease prevention and control measures, such as crop rotation, cultivar mixtures and use of fungicides have little impact on the disease (Deeksha *et al*., 2009). Moreover, these prevention and control measures cannot be fully practiced due to land shortage and the high cost of fungicides (Burkett-Cadena *et al*., 2008).

Use of genetic resistance is so far the most effective control measure; and least expensive and easiest for farmers to adopt and use, because resistance would be embedded in genetic makeup of the seed (Mahuku *et al*., 2009). A few resistant genotypes such as AND277, MEX-54 and Vax-6 have been identified for those diseases (Caixeta *et al*., 2005). Genetic studies on these sources revealed that, AND277 is resistant to ALS and ANT (Caixeta *et al*., 2005), Mex- 54 carries gene *Phg-2*, which is responsible for resistance against (ALS) race 63:39 (Namayanja A, *et al*,.2006) and is linked to SCAR marker (Miller T, *et al*, .2018). VAX-6 is similar to the Middle American gene pool and was developed at CIAT, Cali, Colombia &resistant to CBB (*Xanthomonas axonopodis pv. Phaseoli)*(Singh *et al*., 1999). SCAR markers available in screening for CBB are SU91, BC420, SAP 6, BAC 6, R7313 and R4865; SSR markers available are TGA1.1, g796 & SNO-02 screening for ALS and CV542014 is screening for ANT. These sources of resistance can be useful in facilitating the process of transferring ALS, ANT and CBB resistance in to susceptible advanced lines (recurrent parents). The resistant sources are more adapted to conditions in areas where they were developed (Abawi and Pastor-Corrales, 1990). For instance, AND277 and Vax-6 are medium to large-seeded cultivars, their low yields compared to popular landraces and commercial varieties and are not easily accepted by farmers in Africa (Beebe *et al*., 1981; Mukankusi, 2008). All these factors limit the direct release of resistant cultivars from available ALS, ANT and CBB resistance sources in Africa but they are used as sources for resistant gene and during improvement their undesirable character would be reduced by back crossing to recurrent parent and by marker assisted selection. Furthermore, durable resistance within the existing resistance sources is challenged by pathogen variability. Due to *P. griseola, Colletotrichum lindemuthianum* and *Xanthomonas axonopodis pv. Phaseoli* variability, resistance often breaks down as new and more virulent strains of the pathogen evolve and/or the existing strains adapt to the host (Chen *et al*., 1993). Over time resistant cultivars gradually become ineffective. In addition, no single resistant gene is effective against all races of ALS, CBB and ANT; hence protection conferred by a single gene against a hyper variable pathogen is often short lived (Mahuku *et al*., 2002). Considering that there are different P. *griseola, Xanthomonas axonopodis pv. Phaseoli* and *Colletotrichum lindemuthianum* races; the breeding technique such as marker assisted gene pyramiding that can address several constraints or diseases races could probably be the right direction in managing ALS, CBB and ANT in Ethiopia. Pyramiding resistance genes into a single genotype is one of the practical approaches through which durable resistance can be achieved (Castro *et al*., 2003). The work aimed at incorporating resistance genes to angular leaf spot and Anthracnose diseases into one advanced line using molecular marker assisted selection technique.

Lack of improved common bean disease resistant cultivars has been identified as major constrain in production (Pastor-Corrales and Tu, 1989) Common bean bacterial blight, Angular leaf spot and Anthracnose diseases are extremely devastating diseases causing high yield loses in common bean.

Disease management using chemicals is expensive thus; many smallholder farmers cannot afford. Among several control strategies, integration of host plant resistance is the most effective and appropriate method which is affordable to small scale resource poor farmers (Allen *et al*., 1998). Improvement of bean genotypes for single traits is laborious and time consuming, but using genotypes that have resistance to multiple constraints can increase the efficiency of improving bean genotype.

Since, diseases in common bean are becoming major problems, especially for smallholder farmers. Durable resistant varieties to several disease causing pathogens are becoming a priority for the farmers. Thus, there is a need to produce varieties which are resistance to major pathogens by using marker assisted gene pyramiding technique to speed up the breeding for resistance.

a) Objectives

i. *General objective*

- To develop pyramided lines of common bean with different traits and contribute to the variety development with durable disease resistance.

ii. *Specific objectives*

- To incorporate genes conferring resistance against Anthracnose and Angular leaf spot to selected line with the aid of molecular markers.
- To evaluate the differential reaction of common bean isoline carrying different combinations of angular leaf spot, anthracnose and common bacterial blight resistance genes.

II. Materials and Methods

a) Genotypes used

The genotypes used in this study included one donor genotype (AND277) which contain resistant genes (Phg-1) to angular leaf spot and Anthracnose

(co-1[4]) and one advanced recurrent line KT-ABC 001; breed from Red wolaita *Mex-54 x Red wolaita*Vax-6 which consists of resistant gene (Phg-2) for angular leaf spot common bacterial blight (CBB QTL). It is an elite breeding line developed from RW, MEX & VAX through backcross breeding for the southern region. And which is susceptible to both angular leaf spot (ALS) and anthracnose (ANT) diseases and to which resistance gene is to be incorporated.

b) Marker assisted gene pyramiding and Generation of breeding population

Planting was done in the screen house in pots filled with sterilized forest soil and maintained in the screen house at SARI.

Selected breeding population were developed through hybridization; the crossing procedures used was emasculation and the hook method as described by Bliss (1980) and Buishand (1956) and backcross breeding method was adopted to reduce drag linkage during the recovery of recurrent parent.

The minimum population size to ensure at a predetermined probability of 95% or 99%, that at least one desired genotype is present in the population was derived from the equation; $Nq = \ln(1-q)/\ln(1-p)$ where; N = minimum population size, p = number of individuals of the desirable genotype in a population of size N and q = predetermined probability (Ye and Smith, 2008).

Sequence Characterized Amplified Region (SCAR) & Single sequence repeats (SSR) Molecular Markers were used to check for the presence of the desired character both on the donor and recurrent parents

During crossing, the ALS & ANTH disease resistance genes were incorporated into the advanced line, (KT-ABC001 ×AND277).

The resulting F1 hybrids from the cross were then backcrossed to recurrent & donor parents and BC1F1P1 and BC1F1P2 were obtained, marker assisted selection were applied on resulting back crosses from both parents by using (SAP-6, TGA1.1, CV542014 & g796) molecular markers for the genes resistant for ALS, ANTH & CBB diseases respectively and plants which consists gene of interest were selected and backcrossed to recurrent and donor parents to obtain backcross of parent one and two (BC2F1P1 & BC2F1P2). Finally backcross (BC2F1P1) was allowed for selfing to fix the genes and for evaluating isolines.

c) Markers used for molecular screening

Sequence Characterized Amplified Regions (SCAR) and Simple Sequence Repeat (SSR) markers were used (Table 4.2) to tag anthracnose, common bacterial blight and angular leaf spot resistance genes of interest respectively.

The primers were obtained from the Department of Molecular and Cellular Biology, University of California Davis. A 25/100 bp mixed DNA molecular weight marker (Ladder), specifically designed for determining the size of double strand DNA from 25 to 2,000 base pairs, was used

TGA1.1 and CV542014 markers were used for forward selection to confirm the presence of the desirable genes in which absent in the recurrent parent, SAP6 and g796 markers were used for background selection to confirm the presence of the desirable genes of the recurrent parent.

Table 1: PCR-based markers used in Marker Assisted Selection

No.	Marker	Sequence	Linkage group	Base pair
1	TGA1.1	F 'CAGAGGATGCTTCTCACGGT' R 'AAGCCATGGATCCCATTTG	01	570
2	CV542014	F 'CACTTTCCACTGACGGATT TGAACC' R 'GCACAAGGACAAGTGGTCTGG'	01	450
3	SAP6	F 'GTCACGTCTCCTTAATAGTA' R 'GTCACGTCTCAATAGGCAAA'	10	820
4	g796	F 'GAGAAACTACGGGCTGTTTTACCC' R 'AATTAAAACACCCACCCACTCCAT'	08	230

d) Marker assisted selection (MAS)

i. *DNA extraction, PCR and gel electrophoresis*

a. *DNA extraction*

The recurrent parent (KT-ABC 001), donor parent (AND277), hybrid of parent one and parent two (F1) and backcross to both parents (BC) were planted in pots in the screen house and leaf samples for DNA extraction were collected from 14 days old leaves of seedlings at trifoliate stage.

The DNA was extracted using the Whatman FTA card technology (Whatman FTA Protocol BD08). The leaves were crushed on the FTA plant saver card and the DNA binds to the matrix of the card. The chemical coating on the FTA card can inactivate pathogens, protect the DNA from degradation and allows the cards to be stored at room temperature for extended period of time. To prepare sample for polymerase chain reaction (PCR), a 2 mm disc of the matrix was punched using a Harris Unicore and put in the 0.2 ml PCR tube. Then discs were washed with FTA purification reagent and

Iso-propanol. The washed leaf discs in the PCR tubes were left to dry at room temperature for 5 min. The DNA remains bound to the matrix throughout purification process, thus the matrix provides enough templates for PCR analysis.

b. *DNA amplification*

The PCR reaction was prepared by adding 2 µl of each forward and reverse primers, 21 µl of molecular grade water in the beads (other PCR components), 2 mm disc of DNA (washed FTA card) was added and a total of 25 µl of the reaction mixture were prepared and PCR were run using BIO RAD "MyCycler" thermal cycler machine.

The PCR conditions were set to correspond to each primer requirements in terms of number of cycles and the temperatures as per Miklas (2009).

c. *Gel electrophoresis and documentation*

A 1.5% agarose was used and gel was prepared by mixing 1.5 gm of agarose with 100ml of 1XTAE buffer and then 10 µl of the PCR products were loaded into the gel for electrophoretic separation of DNA fragments in a solution of 1xTAE buffer with electric potential maintained at 90 V for 70 min run time.

The gel was stained with 0.5 µg/ml ethidium bromide (EtBr) for 30 min then visualized using UV trans-illuminator.

For documentation, digital camera was used to capture the amplified fragments from the gel on the UV trans-illuminator.

e) Inoculum collection, preparation and inoculation

i. *Inoculum collection*

Isolates of ALS, ANT and CBB or the inocula that were used was collected from major common bean growing areas of Ethiopia specifically; Sidama, Wolaita, Gamo gofa and Wolega. Sample collection sheets were used and all important information was recorded. They were collected from naturally infected fields with typical symptoms of the respective disease. The inocula were stored in paper envelopes and then labeled with the description of bean variety and the location where the inoculum was collected. For ALS, the infected part of the leaf indicating the presence of fungal spores were isolated and plated into V8 agar. For inoculum preparation, well established fungal colonies were sub-cultured onto fresh medium. The plates containing isolates of fungal spores were incubated at 24°C for 10 to 15 days to allow for sporulation (Aparício, 1998). Following sufficient sporulation, sterile distilled water containing 0.05% (v/v) Tween 20 was added to the plates and the medium surface scraped smoothly using a sterile syringe needle and the spore suspension was filtered using sterile gauze and the final spore concentration adjusted to 2×10^4 spores/ml using the hemocytometer and made ready for inoculation.

For Anthracnose, Single-spore isolates were placed on fresh Mathur's agar medium in a Petri dish and incubated at 22-24°c for 7 days to allow the fungus enough time to produce conidial spores. For inoculation purposes, conidial spores were scrapped off the growth medium into a small amount of water to make a suspension. Using a hemocytometer the concentration was adjusted to 1.2×10^6 conidia ml^{-1}. 0.1% Tween 20 was added as a surfactant.

For Common Bacterial Blight differential media was prepared following the procedures described by Mortensen (2005). The stored culture of *Xap* was revived by growing it on Yeast Dextrose Carbonate Agar (YDCA) media plates at 28°C for 48 hr. Cell suspensions were made using distilled water and concentration was adjusted to 10^6cfu ml^{-1} using hemocytometer.

ii. *Artificial Inoculation*

The parents (AND277 and KT-ABC001) and progenies (F1, F2, BC1F1P1, BC1F2P1, BC2F1P1, BC2F2P1 and BC1F1P2) in addition Red wolaita (Susceptible check) were planted in five-liter buckets in the screen house and from each 30 seeds were used and evaluated. A randomized complete design (CRD) with three replications was adopted. The technique of artificial inoculation were divided into two set; one set was inoculated with ALS and ANTH isolate using detached leave technique in laboratory (Xu,1986 ; Rezene, Y., *et al* 2018). Starting from 14 days after planting, leaf trifoliate was detached after approximately two thirds of their full development. Each detached trifoliate was inoculated by immersion in the suspension containing *C.lindemuthianum and p.griseala* spores at a concentration of 1.2 x 106 and 2×10^4 ml^{-1} respectively. The inoculated leaf trifoliate were placed inside the Petri dish containing moistened paper towels (cotton) and the petri dish were tightly placed to allow for humidity build-up.

And the other set was inoculated with CBB isolate in green house by wounding the leaves of plants and spraying on the leaves. The isolates were inoculated at a concentration of 2×10^4 conidia ml^{-1}

f) Phenotypic and Genotypic evaluation for inheritance of resistant genes

For segregation study parents (KT-ABC001 and AND277), RW, F1, F2 and backcrosses (F1, F2, BC1F1P1,BC1F2P1, BC2F1P1,BC2F2P1 and BC1F1P2) generations were used and evaluated phenotypically in the screen house for CBB and in laboratory for ALS and ANT in detached leave inoculation. Genotyping for disease resistance was done in the laboratory by extracting DNA of each generation.

For the phenotypic assessment, the data were taken as scores on a 1 to 9 CIAT scale, where by plants with disease score of 1 to 3 are considered to be resistant, 4 to 6; intermediate resistant and 6 to 9; susceptible (CIAT, 1987).

g) Phenotypic and Genotypic evaluation of isolines against ALS, ANT and CBB pathogens

For disease reaction, parents (KT-ABC001 and ND277), 20 isolines (BC2F2) and in addition Red Wolaita as susceptible check were phenotypically evaluated in the screen house for CBB and laboratory for ALS and ANT in detached leaves.

For the phenotypic assessment, the data were taken as scores on a 1 to 9 CIAT scale, where by plants with disease score of 1 to 3 are considered to be resistant, 4 to 6; intermediate resistant and 6 to 9; susceptible (CIAT, 1987).

In genotyping, the evaluation was based on the presence of the gene or absence of the gene as revealed by banding pattern in the gel and this was done by using molecular markers to predict the presence or absence of the gene. A score of 1 is for the presence and a score of 0 is for the absence of the named gene.

Disease symptoms on plants inoculated with ALS and ANTH were evaluated from six to fourteen days after inoculation at three days' interval Disease symptoms on plants inoculated with CBB were evaluated from seven to twenty one days after inoculation at three days interval. A 1–9 scale described by (CIAT, 1987) was used to score disease symptoms.

h) Data collection (scoring) and analysis

Data were collected from the screen house and laboratory as disease score and observation were made to the first appearance of the disease symptoms. Disease scoring was performed starting from the first week at three -day interval for a total of two weeks for ALS and ANTH and three weeks for CBB consecutively.

The molecular data was scored in lab from the gel. A score of 1 was for the presence of marker and a score of 0 was for the absence as revealed by the banding pattern in the agarose gel.

The disease score data were subjected to the SAS for phenotypic data analysis and the *chi*-square test was used to test the phenotypic segregation of the populations from the crosses between recurrent and donor parents.

Molecular data were also subjected to AMOVA for molecular data and ANOVA for initial and final disease severity were done using GenAlex (6.51) and SAS (9.2) Software respectively. Means among each score were separated using least significant difference (LSD) at 5% probability level.

Molecular data were also subjected to the *chi*-square test to assess the gene segregation pattern of the populations derived from the two crosses using GenAlex (6.51). A correlation study was done to compare the molecular marker data with the phenotypic expression of the diseases. The heritability was also studied from the variances from the distribution of the score data for the diseases.

III. Result and Discussion

a) Marker assisted gene pyramiding and Generation of breeding population

The highly resistant parent AND277 (Co-1^4&Phg-1) was crossed with KT-ABC001 line (with known locus *Phg-2* and *CBB QTL*) under screen house conditions and 50 seeds of F1 plants were obtained. Before crossing with the recurrent parent, the molecular markers SAP-6, g796, TGA1.1 and CV542014 were run on extracted DNA of 30 F1 plants of the cross [(KT-ABC001 x AND277]. DNA of 20 F1 plants positively amplified all the four desired angular leaf spot anthracnose and Common bacterial blight resistance genes (*Phg-1&Phg-2,Co-*$1^4$and CBB QTL) respectively. Those 20 F1 plants were selected and crossed with the recurrent parent to raise BC1. 45 seeds of the back cross [KT-ABC001 x F1] were harvested and 30 seeds replanted for next crossing. Molecular markers SAP-6, g796, TGA1.1 and CV542014 were run on DNA extracted from 30 plants BC1F1. Five plants possessing all anthracnose, angular leaf spot and common bacterial blight resistance genes in heterozygous state were obtained and those five plants were selfed to advance and fix the genes and 42 seeds of BC2F2 harvested. From total harvest; 20 seeds (isolines) were planted for genotypic and phenotypic evaluation against ALS, ANT and CBB pathogens. Three plants possessing all the four desired genes, five plants possessing three desired genes, four plants possessing two desired genes and six plants possessing single desired gene and two plants possessing none of desired genes were identified by combined evaluation of phenotypic and genotypic techniques.

Selfing continued up to only F2 generation due shortage of time to complete thesis and it would be continued by mega project to ensure that the genes were fully fixed in homozygous state.

b) Phenotypic and molecular marker evaluation of Angular leaf spot resistant gene inheritance

The disease symptom development started during the first week after inoculation. The symptoms were observed to be skewed on different ends among the parents, while in AND277 the score were in the resistant side (1 to 3) and in recurrent parent (KT-ABC001) line and Red wolaita, on the susceptible side (4 to 9). In the F1 populations, the score was also in the resistant side with few susceptible plants as it was expected. The F2 populations, the scores were distributed along the scale but it skewed to the left side showing more of the resistant plants than the susceptible ones. In backcrosses, the distribution was almost normal in showing equal distribution of plants among the susceptible and resistant ones and in BC1F1P1. The frequency of phenotypic classes in the disease score for resistance and susceptibility reaction

to the pathogen in F1, F2 and backcross populations obtained from the cross between KT-ABC001 with AND277 and the segregation classes as per molecular marker g796 for *Phg-2* and CV542014 for Phg-1 gene for ALS resistance are shown in Table 3. Segregation ratios were 3:1 (resistant: susceptible) for the F2 populations in both the phenotypic evaluation and genotypic evaluation, 1:1 for the back crosses and 1:0 for donor parent. These results showed that the observed ratios and the calculated ratios are not significantly different at the 0.05 level of significance; where $P < 0.896$ for F2 and $P < 0.655$ for the BC2F2 in phenotypic screening and $P < 0.776$ for BC2F2 in molecular marker screening. These ratios and the results are in agreement with the previous work on ALS using Mexico -54 as the donor parent that suggested that the gene for ALS follows the single gene inheritance pattern (Namayanja et al., 2006; Chataika et al., 2010; Sartorato et al., 2000).

The study by Tryphone et al. (2012) working on ALS using Mexico 54 as resistant parent, confirmed the single gene inheritance. However, other studies showed that the resistance to different *P. griseola* pathotypes may be controlled by one, two or even three dominant or recessive genes (Carvalho et al., 1998). More recently, studies have also demonstrated that the resistance of the cultivars AND 277, Cornell 49242, MAR 1, G 10474 and MAR 2 to some pathotypes were each conditioned by a single dominant gene (Nietsche et al., 2000; Ferreira et al., 2000; Mahuku et al., 2002).

Table 2: Frequencies of phenotypic and genotypic/marker classes for ALS resistance

Parent/Cross/Marker	Generation	Number of plant		Expected ratio	x^2c	x^2t at df(1)=3.841
		Resistance	susceptible			
KT-ABC	P1	7	23			
AND277	P2	24	6			
P1 x P2	F1	17	13	1:1		
F1 x F1	F2	21	9	3:1	0.18	3.841
P1 x F1	BC1F1	14	16	1:1		
P2 x F1	BC1F1	27	3	1:0	1.16	
P1 x BC1F1	BC1F2	17	13	1:1		
P1 x BC1F2	BC2F1	18	12	1:1		
P1 x BC2F1	BC2F2	16	14	1:1	0.03	
g796(Phg-2)	BC2F2	12	8	1:1	0.45	
CV542014(Phg-1)	BC2F2	10	10	1:1	0.05	

Table 2 shows that among 30 samples from individual F1, F2, BC1F1P1, BC1F2P1, BC2F1P1, BC2F2P1 and BC1F1P2 plants for phenotypic data, the ratio of 17:13, 21:9, 14:16, 17:13, 18:12, 16:14 and 27:3 for resistant (R) and susceptible (S) and among 20 DNA sample from individual BC2F2 for genotypic data, the ratio of 12:8 and 10:10 was observed for g796 and CV542014 markers respectively. The Chi square value from these findings, $X^2 = 0.18$ for F2 and 0.03 for BC2F2 individuals for phenotypic data, shows that there is no significant difference between the observed and expected ratios at probability of 0.776 or $X^2t=3.841$ (that is, $P < 0.776$) as shown in Table 3. This analysis also revealed a segregation ratio of 1:1 for CV541420 as a Co-dominant marker associated to Phg-*1* gene fit to the expected ratios and g796 co-dominant marker associated to gene linked to ALS resistance, was fitted expected ratios at $X^2c=0.05$ and $X^2c=0.45$ at $X^2t=3.841$ CV541420 and g796 markers respectively .

Other previous studies using molecular markers reported similar observations that resistance to specific isolates of *P. griseola* is simply inherited (Mahuku *et al*., 2004; Ferreira *et al*., 2000; Carvahlo *et al*., 1998; Larsen et al., 2005). It was also found in the genotype AND 277 that its resistance to ALS is being conferred by single dominant gene *phg-1* (Ragagnin, 2005). When the genes are simply inherited then one can easily pyramid genes for resistance to ALS, thus overcoming the issue of high degree of pathogenic variability as reported by Sartorato (2002).

i. *Heritability of Angular leaf spot*

The estimated narrow sense heritability using the F2 and backcross generations for the cross KT-ABC001 × AND277 was found to be 0.975; this indicates that almost 95.7 % of the trait from the parents has been transferred to the offspring and the contribution of genes is higher than the environmental effects. These results agree with what is reported by Amaro et al. (2007) that the resistance to angular leaf

spot normally has a relatively high heritability values and plants can be phenotypically selected for recombination in the early generation.

Some other studies revealed that the heritability for reaction to angular leaf spot was varied from 44.44 to 58.86% and it was suggested that in the case of low heritability, marker assisted selection is more advantageous (Melo *et al*., 2002 cited by Teixeira *et al.,* 2005). It has also been reported that there is a complex interaction between genotype and environment which also gives the basis in deciding on the adoption of indirect selection based on molecular markers (Teixeira *et al*., 2005).

ii. *Correlation between molecular and phenotypic screening to Angular leaf spot resistance*

The data from phenotyping was compared with the data from the molecular marker to assess the reliability of MAS as a tool in breeding for disease resistance. The correlation coefficient between phenotypic and molecular screening for ALS was found to be -0.658 for CV542014 marker. This high and negative correlation coefficient indicates that one has high chance of success in using molecular markers for screening for disease resistance. The probability occurring by chance is $P < 0.001$, which indicates high correlation thus in early generations when number of segregating plants are high; one can rely on molecular marker data to equally determine the phenotype.

This correlation is also supported by the chi square test that both phenotypic and genotypic data presented single gene inheritance. This has also been observed in previous studies using marker assisted selection where both ratios from the molecular marker analysis and that from the field screening were not significantly different from those of Mendelian inheritance (Mukeshimana *et al*., 2005; Namayanja et al., 2006). It has been reported that the recovery of superior individuals based solely on phenotyping is insufficient because some traits are of low heritability or it is difficult to create favorable conditions for selection as the case for disease resistance. In contrast to this, the molecular markers are highly heritable and unaffected by the environment.

This indicates, there is a need to incorporate the molecular marker techniques in selection process in breeding programs in order to hasten the breeding work especially when the trait that is to be selected is of low heritability or its conditions for selection can hardly be set as the case for disease resistance. Apart from all that, there is a need to prove that there is high correlation between the molecular marker and the trait to be selected; then, one can be confident and sure of using MAS in the following generations of selection. It has also been cautioned that one should never assume that MAS is necessarily superior to phenotypic selection which for some trait can be as effective and efficient as the use of molecular markers (Blair et al., 2007). Apart from these correlations being useful in selection, also since there is no one to one correlation, then, the possibilities that there exist some variability among the isolates used that reacts differently to the genotype irrespective of the presence of the genes for resistance to the disease in question.

This necessitates the study of the pathogen variability in order to come up with an integrated management that will consider pathogen variability as part of the breeding strategy for disease resistance.

c) *Phenotypic and molecular marker evaluation of Anthracnose resistant gene inheritance*

The disease symptom development started during the fourth day after inoculation. The symptoms were observed to be skewed on different ends among the parents and progenies while in AND277 the score were in the resistant side (1 to 3) and in recurrent parent (KT-ABC001) line and Red wolaita (susceptible check) on the susceptible side (4 to 9). In the F1 populations, the score was also in the resistant side with few susceptible plants as it was expected and in the F2 populations the scores were distributed along the scale but it skewed to the left side showing more of the resistant plants than the susceptible ones. In the backcrosses, the distribution was almost normal in BC1F1P1 showing equal distribution of plants among the susceptible and resistant ones and in BC1F1P1, the scores concentrated to the resistant sides. The frequency of phenotypic classes in the disease score for resistance and susceptibility reaction to the pathogen in F1, F2 and backcrosses population obtained from the cross between KT-ABC and AND277 and the segregation classes as per molecular marker TGA1.1 gene for ANT resistance was shown in Table 3. Segregation ratios were 3:1 and 1:1 (resistant: susceptible) for the F2, BC2F2 populations in phenotypic evaluation respectively. Genotypic evaluation, 1:1 ratio for the BC2F2 (resistant to susceptible). These results show that the observed ratios and the calculated ratios are not significantly different at the 0.05 level of significance; where $X^2=0.71$for F2 and $X^2=0.03$ for BC2F2 in phenotypic screening.

For BC2F2 in molecular marker (TGA1.1) screening, $X^2=0.05$. These ratios and the results are in agreement with the previous work on ANT using AND277 as the donor parent that suggested that the gene for ANT follows the single gene inheritance pattern, Alzate-Marin et al. (2003)

This result is also consistent with those of Alzate-Marin et al. (2003) and Carvalho et al. (1998) showing that resistance to the ANT and ALS pathogens in AND 277 is monogenic and dominant.

Pyramiding ANT and ALS resistance genes are the main focus of many bean breeding programs

throughout the world (Ragagnin et al. 2003; Miklas and Singh 2007). Co-1^4 is one of the main genes used in ANT resistance breeding programs due to its ample resistance spectrum. Similarly, Phg-1 is used for resistance to ALS. Because of their physical linkage and—cis configuration, Co-1^4 and Phg-1 tend to be inherited together yet can be indirectly monitored with the CV542014 and TGA1.1 markers. The results were obtained using marker TGA1.1 for the genefor Co-1^4 ANT resistance.

Table 3: Frequencies of phenotypic and genotypic/marker classes for ANT resistance

Parent/Cross/Marker	Generation	Number of plant		Expected ratio	X^2c	X^2t at df=1
		Resistance	Susceptible			
KT-ABC	P1	0	30			
AND277	P2	30	0			
P1 x P2	F1	17	13	1:1		
F1 X F1	F2	20	10	3:1	0.71	3.841
P1 x F1	BC1F1P1	15	15	1:1		
P2 XF1	BC1F1P2	28	2	1:0	1.19	3.841
P1 x BC1F1	BC1F2P1	16	14	1:1		
P1 x BC1F2	BC2F1P1	17	13	1:1		
P1 x BC2F1	BC2F2P1	16	14	1:1	0.03	3.841
TGA1.1(Co-1^4)	BC2F2P1	10	10	1:1	0.05	3.841

Table 3 shows that among 30 samples from individual F2, BC2F2P1 and BC1F1P2 plants for phenotypic data, the ratio of 20:10, 16:14 and 28:2 for resistant (R) and susceptible (S) respectively. And among 20 DNA sample from individual BC2F2P1 for genotypic data, the ratio of 10:10 was observed. The Chi square value from these findings, X^2c = 0.71 for F2 and 0.03 for BC2F2 individuals shows that there is no significant difference between the observed and expected ratios at X^2=0.05 where X^2t at df =1 is 3.841 as shown in Table 3. This analysis also revealed a segregation ratio of 1:1 for TGA1.1 as a Co-dominant marker associated to Co-1^4 gene fit to the expected ratios linked to ANT resistance, at X^2c=0.05.

It was also found in the genotype AND 277 that its resistance to ANT is being conferred by single dominant gene Co-1^4(Ragagnin, 2005).

i. *Heritability of Anthracnose*

The estimated narrow sense heritability using the F2 and backcross generations for the cross KT-ABC 001× AND277 was found to be 0.95 this indicates that almost 95% of the trait from the parents has been transferred to the offspring and the contribution of genes is higher than the environmental effects. This result is in agreement with the findings of Ragagnin *(2005*). The resistance to anthracnose disease normally has a relatively high heritability values and plants can be phenotypically selected for recombination in the F2 generation.

Various studies revealed the heritability for reaction to anthracnose was varied from 55.8 to 84.5% and it was suggested that in the case of low heritability, marker assisted selection is more advantageous *(*Alzate-Marin *et al.,* 1997*).* It has also been reported that there is a complex interaction between genotype and environment which also gives the basis in deciding on the adoption of indirect selection based on molecular markers (Teixeira *et al*., 2005).

ii. *Correlation between molecular and phenotypic screening to Anthracnose*

The result from phenotypic screening was compared with the result from the molecular marker screening to assess the reliability of MAS as a tool in breeding for disease resistance. The correlation coefficient between phenotypic and molecular screening for ANT was found to be, -0.739.This high and negative correlation coefficient indicates that one has high chance of success in using molecular markers for screening for disease resistance. The probability of this occurring by chance is P < 0.001 which indicates when number of segregating plants is high; one can rely on molecular marker data to equally determine the phenotype. This correlation is also supported by the chi square test that both phenotypic and genotypic data presented single gene inheritance. This has also been observed in previous studies using marker assisted selection where both ratios from the molecular marker analysis and that from the field screening were not significantly different from those of Mendelian inheritance (Mukeshimana *et al*., 2005; Namayanja *et al*., 2006).It has been reported that the recovery of superior individuals based solely on phenotyping is insufficient because some traits are of low heritability or it is difficult to create favorable conditions for selection as the case for disease resistance. In contrast to this, the molecular

markers are highly heritable and unaffected by the environment.

This implies, there is a need to incorporate the molecular marker techniques in the selection process of breeding programs. Molecular markers can hasten the breeding work especially when the trait to be selected is of low heritability or its conditions for selection can hardly be set as the case for disease resistance. The presence of high correlation between the molecular marker and the trait to be selected; indicates assurance of using MAS in selection process of the following generations. It has also been cautioned that one should never assume that MAS is necessarily superior to phenotypic selection which for some trait can be as effective and efficient as the use of molecular markers (Blair et al., 2007). Apart from these correlations being useful in selection, also since there is no one to one correlation, then, the possibilities that there exist some variability among the isolates used that reacts differently to the genotype irrespective of the presence of the genes for resistance to the disease in question.

This necessitates the study of the pathogen variability in order to come up with an integrated management that will consider pathogen variability as part of the breeding strategy for disease resistance.

d) Selection of plants with combined resistance to ALS, ANTH and CBB.

The aim of evaluation was to find plants with combined markers or multiple genes for resistance of three diseases, ALS, ANT and CBB. Selection was done among the segregating BC2F2 population by using phenotypic data and using molecular markers. Having found that the genes for ALS, ANT and CBB are dominant and have simple inheritance pattern. This is an indication that there is a high probability of selecting plants with; all the four desirable genes, three of the genes, two of genes and single gene for each disease, and none of the genes (Table:4). During selection process, the g796 and CV542014 marker were used to screen for *Phg-2 and Phg-1* genes respectively that confers resistance to ALS. TGA1.1 marker was used to screen co-1^4 gene, that confer resistance ANT and SAP-6 marker was used to screen CBB QTL that confer resistance to CBB.

Table 4: Gene pyramid groups and their severity mean with three pathogens

Pyramid groups	Mean severity of isolates			
	ALS	ANT	CBB	Grand mean
Phg-1+phg-2+co-1^4+cbb qtl	1±0.00	1±0.00	1±0.00	1±0.00
Phg-1+phg-2+co-1^4	1.66±0.57	1.66±0.57	7.33±0.57	3.55±0.57
Phg-1+ co-1^4 + cbb qtl	1±0.000	1.33±0.57	1±0.00	1.11±0.19
Phg-1+ co-1^4	1.33±0.57	2.33±0.57	7.33±0.57	3.66±0.57
phg-2+ cbb qtl	4.33±0.57	6±0.00	2±0.00	4.11±0.19
phg-2	8.33±0.57	7.66±0.57	7.66±0.57	7.89±0.57
cbb qtl	6.33±0.57	7±0.00	1.33±0.57	4.89±0.38
No-gene	8±0.00	8±0.00	8±0.00	8±0.00
HSD (0.05)	0.52	0.68	0.78	0.66
S.E.D	0.33	0.31	0.34	
CV	11.94	17.21	17.76	

There were five gene pyramid groups with two to four pyramided resistance genes namely, Phg-1+phg-2+co-1^4+cbb qtl, Phg-1+phg-2+co-1^4, Phg-1+ co-1^4 + cbb qtl, Phg-1+ co-1^4 and phg-2 +cbb qtl (Table 4 & Figure 1). The Phg-1+phg-2+co-1^4+cbb qtl and the Phg-1+ co-1^4 + cbb qtl groups conferred resistance to all the four isolates (ALS, ANT & CBB). The Phg-1+phg-2+co-1^4 and Phg-1+ co-1^4pyramid groups conferred resistance to ALS and ANT but were overcome by CBB while the phg-2 +cbb qtl pyramid group was overcome by ALS and ANT but conferred resistance to only CBB.

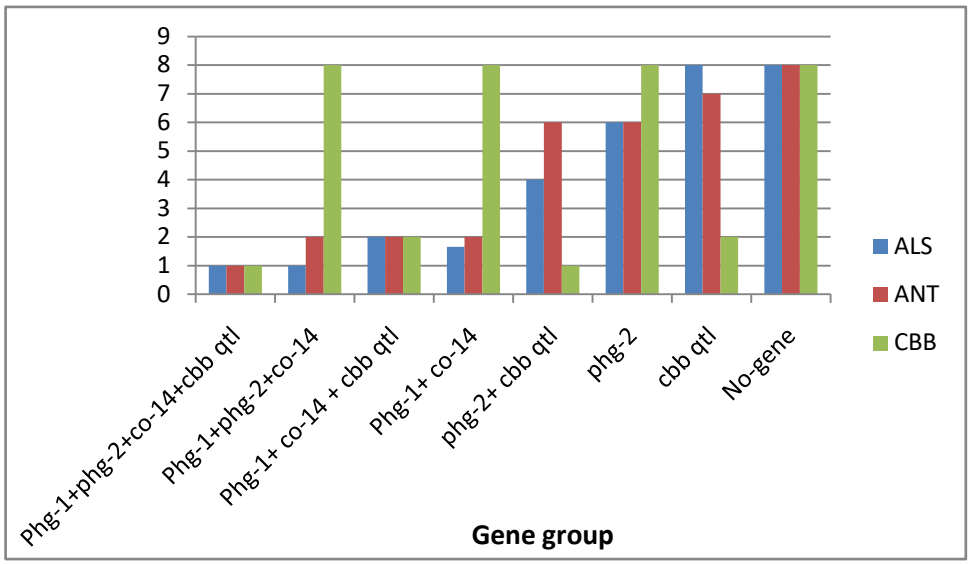

Figure 1: Severity levels of the different pyramid-gene groups screened with four isolates of ALS, ANT and CBB

The pyramid group "Phg1+phg2+co1^4+cbb qtl" had the least mean severity 1±0.00 followed by group "Phg-1+co-1^4+cbb qtl" with a score of 1.11±0.0 while group "No-gene" had the highest mean severity of 8±0.00

In general, gene pyramid "Phg1+phg2+co1^4 +cbb qtl" recorded the lowest mean disease severity compared to the triple, double and single gene combinations implying that pyramiding was a beneficial strategy in reducing leaf severity across the three pathogens. This is evident in the amount of leaf symptoms expressed on leaves from plants with "Phg-1+co-1^4+cbb qtl" combination compared with those expressed on leaves with "Phg1+phg2+co1^4+cbb qtl" combination. Symptoms of ALS, ANT & CBB were not significantly different in the former than in the later, though both were deemed highly resistant reactions. Among the two gene pyramids Phg1+phg2+co1^4+cbb qtl and Phg-1+co-1^4+cbb qtl, had similar mean effect although phg-2+ cbb qtl was overcome by the ALS. The "phg-2+ cbb qtl" combination was not effective for ALS and ANT case since it only afforded resistance to the CBB, Rather it is the most effective to combine phg-1 with others because it is linked with Co-1^4 and is broad resistant for most of African races. Kelly and Vallejo (2004) reported that the *Co-1^4*gene possesses a high breeding value against Andean races of *C. lindemuthianum*, but is susceptible to weaker Mesoamerican races and the linked phg-1 possess resistance to most of Andean *p.greaseola*. This could explain the good resistance associated with those genes both singly and in combination with other genes.

Pair-wise comparisons of gene group means on resistance to ALS, ANT & CBB are presented in Table Disease severity means across the four isolates were significantly different from each other (*HSD0.05* =0.52, 0.68 and 0.78) for ALS,ANT and CBB respectively for all the pyramid groups, with the exception of groups Phg1+phg2+co1^4+cbb qtl (1±0.00) and Phg-1+co-1^4+cbb qtl (1.1±0.19), Phg-1+ co-1^4(3.66±0.57) and Phg1+ph2+co-1^4(3.55±0.570.00).

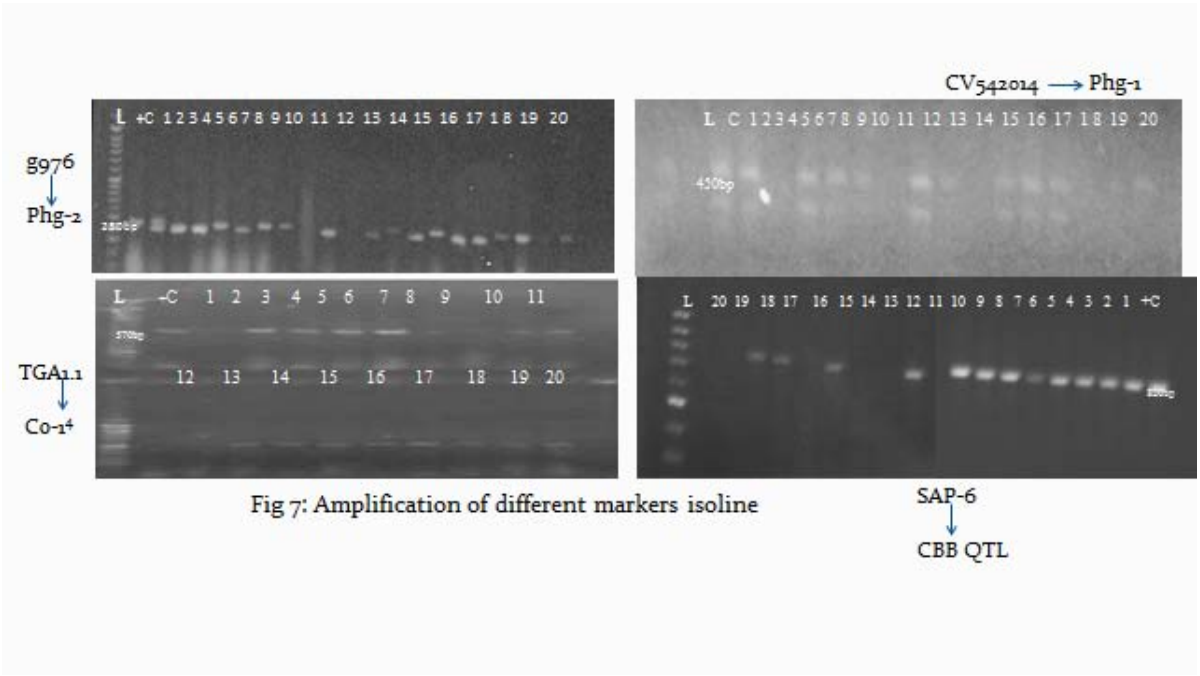

Fig 7: Amplification of different markers isoline

Figure 2: Sample gel for multiple gene selection ALS (*Phg-2&Phg-1 gene*) , ANT (*Co-1⁴gene*) and CBB QTL.*of paretents and BC2F2genereation*

IV. Conclusions and Recommendations

This study clearly demonstrated that, gene pyramids increase degree and spectrum of resistance against the three diverse pathogen; however, not all pyramids were effective against the pathogens. Some double genes as well demonstrated a broad spectrum nature of resistance against three pathogens and in some cases conferred a more effective resistance than some four or three gene pyramids. Therefore, before embarking on a long and costly gene pyramiding program caution should be taken in choosing genes that will result in effective gene pyramids offering increased level and spectrum of resistance against different pathogen in a given target location. In some cases the mere deployment of a single broad-spectrum gene such as Co-1⁴&Phg-1may suffice, but added benefits may be realized when it is in combination with other complementary genes whose modes of single and combined action are well understood.

This work has also substantiated the use of MAS in crop improvement in Ethiopia in order to increase the pace of breeding. From the studies, it was found that, inheritance for pathogens is simple and stable utilizing single dominant gene for ALS and ANT. These results provides strong basis for their use in improvement of common bean in Ethiopia. The correlation between the phenotypic and genotypic data showed strong correlation which makes the use of molecular marker more valid. Apart from that, it was found that there was no one to one relationship (that is, r < 1) suggesting that MAS should not be used alone rather being integrated with phenotypic screening in the field/strong classical breeding at some points to validate the continuing molecular marker use, this will aim at breeding for more adapted common bean lines.

Generally from 20 isolines evaluated with the combination of phenotypic and genotypic techniques, three plants were found with four desired genes and those plants shall be advanced and incorporated to breeding program in order to release variety with resistant to ALS,ANT and CBB pathogens.

The following recommendations were made based on the current results:-

- More studies should be done to check with the pathogen variability and validate the stability of these markers with time as the pathogen as well as the genotypes changes; this will promote improvement of the existing tools to suit the growing conditions, present genotypes and possibly new pathogen races.
- Pathogen characterization variability should be done for common bean Anthracnose in Ethiopian case.
- Donor parent (AND277) should be maintained to use in common bean improvement program as source of resistance genes for Angular leaf spot and Anthracnose diseases.

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