SCREENING FRENCH BEAN GENOTYPES (*Phaseolus vulgaris* L.) USING PHENOTYPIC AND SEQUENCE TAGGED SITE (STS) MOLECULAR MARKER FOR ANGULAR LEAF SPOT (*Pseudocercospora griseola*) RESISTANCE

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A THESIS SUBMITTED TO THE SCHOOL OF AGRICULTURE AND BIOTECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PLANT BREEDING AND BIOTECHNOLOGY OF UNIVERSITY OF ELDORET, KENYA.

2016

DECLARATION

Declaration by the Candidate

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To my family Sharon, Henry and Joy

ABSTRACT

French bean, a sub-group of the common bean (*Phaseolus vulgaris* L.), is grown for its tender green pods and consumed as a green vegetable. It is a major export crop in Kenya but production levels are constrained by both biotic and abiotic stresses. Angular leaf spot (ALS) disease, caused by *Pseudocercospora griseola*, can cause major reductions in yield. Genetic resistance against ALS is a high breeding priority, as effective cultural control is difficult. The aim of this study was to evaluate French bean germplasm for ALS resistance in the field, in controlled conditions with ALS races and using a sequence tagged site (STS) marker. The French bean germplasm panel tested included 26 commercial varieties, three breeding lines and three landraces. The 32 test genotypes were planted in a randomized complete block design with three replicates, in three sites in western Kenya (Eldoret, Kakamega and Kisii) in 2014. Two dry bean varieties—Mexico 54 and GLP X92—were included as resistant and susceptible checks, respectively. Further, the genotypes were screened under greenhouse conditions using 8 races of ALS. The ALS severity on the French bean genotypes was scored based on the CIAT standard scale of 1-9 of disease assessment. Populations derived from a cross between the Mexico 54 (resistant dry bean variety) and Amy (Susceptible French bean variety) were phenotyped and genotyped to study the inheritance of ALS resistance in Mexico 54 and also determine the potential application of STS G796 marker in screening for resistance to ALS. The field results showed significant ($P \leq 0.05$) differences in disease severity between the three sites: the Kakamega site experienced the greatest disease pressure (mean severity of 6.2), followed by the Kisii site (5.7) and the Eldoret site (5.3). Only Mexico 54 out of the 34 entries showed field resistance to ALS. Most exhibited either an intermediate or a susceptible reaction at all three sites. For greenhouse conditions, genotype Manakelly showed resistance to 6 of the 8 races used. Race 63-23 and 63-39 were the most virulent races (6.8) affecting most French bean genotypes. The phenotypic data confirmed that Mexico 54 has a single dominant ALS resistance gene and the co-dominant STS marker was polymorphic between Amy and Mexico 54 and was able to distinguish Mexico 54 from the other 11 ALS differential cultivars and 31 French bean genotypes. The present study has demonstrated that commercial French bean germplasm lacks a source of robust resistance against ALS. However, the exotic variety Mexico 54 could be used to improve ALS susceptible elite varieties. Mexico 54 is an indeterminate dry bean variety, so an intensive backcross program would be needed to restore the necessary pod quality and determinacy. In the meantime, there is a need to identify other sources of resistance, preferably within the French bean genepool, both to increase the diversity of resistance and to simplify the improvement of current elite varieties. The study also showed that STS G796 is a potential molecular marker that can be used for indirect selection during the development of ALS resistant lines using Mexico 54 as one of the progenitors.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABREVIATIONS AND ACRONMYS

ALS	Angular Leafspot
ANOVA	Analysis of Variance
CACO3	Calcium Carbonate
CBB	Common Bacterial Blight
CIAT	International Centre for Tropical Agriculture
DNA	Deoxyribonucleic Acid
FAO	Food and Agricultural Organization of the United Nations
GBK	Gene Bank of Kenya
HCDA	Horticultural Crops Development Authority
KALRO	Kenya Agriculture Livestock Research Organization
MAB	Marker Assisted Backcrossing
MAS	Marker Assisted Selection
MOA	Ministry of Agriculture
MT	Metric Tones
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
SCAR	Sequence Characterized Amplified Region Markers
SSR	Simple Sequence Repeats
STS	Sequence Tagged Site
TE	Tris Ethylene Diamine Tetra Acetic Acid

ACKNOWLEDGEMENT

My special thanks go to my academic supervisors: Dr. Oliver Kiplagat (University of Eldoret), Dr. Esther Arunga (University of Embu), My mentor Prof. Miriam Kinyua , Dr. Billy Makumba and Mr Javan Were for their guidance during the course of the study.

Many thanks go to Ms.Emy Chepkoech and Mr. Ego Amos , for their tireless support and inspiration.

I also extend my gratitude to the institution and staff of CIAT Kawanda –Uganda, Embu University College, KALRO Kakamega and University of Eldoret, Department of Biotechnology for the various contributions and support they made during this study.

I would also immensely thank Kirkhouse trust for funding my studies.

CHAPTER ONE

INTRODUCTION

1.1 Back ground information

French bean also known as snap or green bean comprise a group of common bean (*Phaseolus vulgaris* L.) whose succulent immature pods are consumed as a green vegetable (Myers and Baggett, 1999). The French bean crop in Kenya is mainly grown on small scale at homes, by contracted medium and few large-scale commercialized growers. Kenya, over the years has created a market catchment for its French beans especially in the European Union countries due to high quality beans branded as "Kenya Green Beans" (FAO, 2010). The total World production of French beans is estimated to be at 18 million tones with China, India, Brazil and Spain being the biggest producers and consumers in Asia and Europe respectively. United States of America is also an important producer of French bean in the world while in Africa production of French beans is less than 6 million Tones (FAO, 2010). Higher pod production on French beans is achieved on a cooler weather. Frost, dry winds, long rains and fog periods are harmful to this crop (MOALD, 1995).

French bean production in Kenya like other beans is constrained by diseases and insect pests due to lack of tolerant varieties and stringent export market quality requirements (Monda *et al.,* 2003; KARI, 2005; MOA, 2006). Angular leaf spot (ALS) (*Pseudocercospora griseola (Sacc)*, rust (*Uromyces appendiculatus*), and anthracnose (*Colletotrichum lindemuthianum*) are the economic important and widely distributed diseases of snap beans in East Africa. Angular leaf spot causes severe and premature

defoliation resulting in shriveled pods, shrunken seeds and yield losses of up to 80% (Stenglein *et al*., 2003). Kenya has traditionally followed classical breeding methods in development of improved bean varieties (Kimani and Mwang'ombe, 2007), resulting in long periods of cultivar development. The use of genetic resistance is the most practical and economic way to manage diseases of common bean (Mahuku *et al*., 2002b), and utilization of marker technology presents new opportunities to accelerate cultivar development with more precision and reduce duration to release of improved bean varieties (Miklas *et al*., 2006a). Molecular markers will greatly assist in the transfer and pyramiding of resistance genes to angular leaf spot in French beans.

1.2 Statement of the Problem

French beans are a major export in Kenya to overseas countries. Its production by smallholder farmers in Kenya is unstable compared to that of commercialized large-scale farmers. The trend of instability in production is due to marketing constrains and increased cost of management of pests and diseases e.g. angular leaf spot (CIAT, 2006). Therefore among the biotic constrains, angular leaf spot of beans is a major impediment in French bean production .It is a significant constraint to bean production with annual losses estimated at 374,800 tonnes (Wagara *et al*.,2003). French bean farmers commonly rely on cultural practices such as crop rotation and use of chemical interventions such application of fungicides to limit losses associated with these biotic constrains. The use of chemicals strategies makes the produce less marketable due to the chemical residues (Wasonga *et al.,* 2010). Furthermore, prolonged and uncontrolled use of chemical measures leads to development of chemical resistant organisms including fungal

pathotypes, increased production costs and are unsafe on the environment and human health. There is a high pathogenic variability of the causal agent of ALS (*Pseudocercospora griseola)* in bean growing regions of Kenya. Research has shown that no commercial common bean varieties grown in Kenya are resistant to all ALS pathotypes (Wagara, 2011). Phenotypic markers in dry beans such as purple flower and black seed coat colour is known to be linked XAN 159 and are a source of resistance gene to common bacterial blight (CBB) (Leibenberg *et al*., 2003). There is no known relationship between ALS resistances to growth habit, seed coat colour and stem pigment. However, the only phenotypic traits used to characterize ALS races are host reactions. Currently much focus is on use of polymorphic stable molecular markers such as sequence characterized amplified regions (SCAR) and sequence tagged site (STS) among others. Therefore, there is need to search for angular leaf spot resistance using both phenotypic and molecular markers in French beans genotypes grown in Kenya.

1.3 Justification

French beans is a major income earner in Kenya and accounts for Ksh. 4 billon in the gross domestic product. Angular leaf spot disease affecting beans causes severe and premature defoliation resulting in shriveled pods and subsequently yield reductions. Major reductions in yield is mainly due to decrease of the photosynthetic area though, the pathogen can also reduce the quality of pods by causing lesions (Silva *et al.,* 2008). Losses of 50% to 80% have been reported on bean yields in tropical and subtropical countries (Stenglein *et al*., 2003). The major control method of French bean fungal diseases is scheduled fungicidal sprays. This method although effective is expensive and

leads to destruction of quality of life within the ecosystem. The search for and deployment of resistant cultivars therefore continues to be just as important as its selection for improved pod qualities.

There is high ALS variability in bean growing regions (Wagara *et al.,* 2003). This variability of the pathogen makes it difficult to breed bean genotypes for resistance to only one *P.griseola* pathotype (Monda *et al.,* 2003). At the same time, continued characterization and classification of *P.griseola* physiological races as any other economically important crop disease and the knowledge of its local virulence diversity is an important step in resistance breeding programs. There is therefore need to broaden the base of disease resistance available locally as more complex and virulent races emerge and overcome resistant genes. Different bean genotypes have exhibited varying levels of resistance to angular leaf spot that can be pyramided into appropriate background to provide lasting resistance (Wagara *et al*., 2011). Pyramiding of resistance genes into the one background is therefore, most likely to result in durable resistance to many ALS races (Mahuku *et al*., 2009). The use of host plant resistance is by far the most cost effective and environmentally sustainable method for controlling bean diseases. There are indications that immune reactions against *P. griseola* occurs in some bean varieties whereby varieties show differences in the time when symptoms appear and the extent of disease severity (Wagara, 1996). Molecular markers have been tagged to loci coding for disease resistance in many cultivated crops. Polymerase chain reaction (PCR) based markers such as Random Amplified Polymorphic DNA (RAPDs), Simple sequence Repeats (SSRs), Sequence Characterized Amplified Regions (SCARs) and

Sequence Tagged Sites (STSs) have been utilized in tracking and identifying disease resistance genes (Park *et al* ., 2003, Souza *et al* ., 2011). These PCR based markers have been used as a tool for indirect selection for ALS resistance (*Phg*) genes during the breeding process (Yu *et al.,* 2000). Currently there is scanty documentation on French bean resistance to ALS in Kenya. Therefore, with field screening, use of angular leaf spot races and application of potential molecular markers, sources of ALS resistance can be identified and effectively utilized in future breeding programmes in French beans.

1.4 Broad Objective

To contribute towards management of bean angular leaf spot by identifying new source of resistance in French beans using phenotypic and sequence tagged site G796 molecular marker.

1.5 Specific Objectives

1. To assess the severity of French beans to angular leaf spot under field conditions in western Kenya.
2. To assess the virulence of *P. griseola* races on French beans genotypes under green house conditions
3. To determine the potential application of a Sequence Tagged Site G796 molecular marker to screen for angular leaf spot resistance in French beans.

1.6 Null Hypotheses

1. The French beans genotypes will show significant difference in terms of their severity to angular leaf spot under field conditions in western Kenya.

2. The *Pseudocercospora griseola* races will differ significantly in their virulence on French beans genotypes under green house conditions.
3. The STS marker G796 will be useful in screening French bean lines for angular leaf spot resistance.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin of French beans

French beans also known as green beans, fine beans, string beans, snap beans or squeaky beans are the unripe plant of specific cultivated varieties of the common bean (Myers *et al*., 1999). French bean varieties have been bred especially for the fleshiness, flavor, or sweetness of their pods. Haricots verts, French for "green beans", may refer to a longer, thinner type of green bean than the typical American green bean. French beans evolved in the highlands of Latin America, Mexico, Peru and Colombia from a wild vine over a period of 7000-8000 years. It is estimated that French bean cultivation in Eastern Africa began at the turn of 19th century (Gepts *et al.*, 1986).

2.2 Current French Bean Production

Globally French bean is cultivated on about 28M Ha with a production estimate of 19 million tonnes. Brazil is the leading producer of French beans. Columbia, USA, Canada, Ethiopia, China and Turkey are other major French beans producing countries (FAO, 2010).

Kenya has been exporting vegetables to the European markets since twentieth century. Kenya's original success in exporting vegetables, especially French beans is based on its climatic and geographic competitive advantage (HCDA, 2008). The French beans production was estimated at 120,000 MT and accounted for KSh. 4 billion of Kenya's earnings from vegetable exports in the year 2012. A kilogram of French beans cost an

estimated Sh320 ($4) in the US market, depending on the quality of the beans (HCDA, 2010).

In Kenya the major French bean production areas are Central (16,526 mt), Rift valley (4,419 mt), Eastern (3,396 mt), Western (980 mt) and Coast (320 mt) (HCDA 2009; 2010). The fresh market class varieties include Amy, Pekera, Teresa, Paulista, Rexas, Samantha and Cupvert. The Processing varieties are mainly Julia, Vernandon and Sasa. However, processing of the beans, including canning and freezing, is steadily increasing (HCDA, 2008).

The French bean production in Kenya is by smallholder farmers estimated at 50,000 growers. These are households with less than 2 acres of land. The average profit being US$750 per year (HCDA, 2010). Domestic fresh vegetables supply is irregular and faces many imports. There is minimal local consumption of French beans in Kenya due to lack of promotion of green bean utilization and lack of ready demand in the local markets. The export market requires freshly, straight, long, rounded in cross-section beans (Ndegwa *et al*., 2009).

2.3 Agronomy and Climatic Requirements

The optimum temperature range for growing French beans is 20-25°C, but has been grown in temperatures ranging between 14-32°C. Extreme temperatures result in poor flower development and poor pod set. The crop can be grown between 1,000-2,100 m.a.s.l. However, French beans mature faster in warmer areas. Cultivation is possible in areas with well-distributed medium to high annual rainfall (900-1200 mm p.a.). Irrigation through furrow or overhead irrigation is very essential during off-season. Up to 50 mm of

water per week is required. French beans grows best on well drained, silty loam to heavy clay soils rich in organic matter contents and a slightly acidic to slightly alkaline reaction (pH 6.5-7.5) but can tolerate pH 4.5 to 5.5 (HCDA 2005). Seed rate is partly determined by variety. The beans rows should be on the contours or at least parallel to the slope to reduce soil erosion. (www.nafis.go.ke June 2014).

2.4 Constrains in French production

French bean production in Kenya is constrained by several biotic and abiotic factors. The most important of which are fungal, bacterial and viral diseases, insect pest, decreasing soil fertility, periodic water stress and lack of market (Wasonga *et al*., 2010). Bean angular leafspot caused *by Pseudocercospora griseola (Sacc) ferraris*, bean rust caused by *Uromyces appendiculatus* (Pers.pers.) Unger, common bacterial blight caused by *Xanthomonas campestris.*pv.phaseoloi (Erw.smith), Dowson and anthracnose caused by *Colletotricum lindemuthianum* (sacc. and Magn.) are the major diseases in tropical Africa including Kenya (Mwang'ombe *et al.,* 2007). Of these major diseases, ALS is the among the most important constraint to common bean production in Africa (Pastor-corrales *et al.,*1998) with an estimated annual loss at 374,800 Tonnes (Wortmann *et al.,* 1998).

2.5 Angular Leaf Spot

2.5.1 Taxonomy and epidemiology of angular leaf spot

Pseudocercospora griseola, the causative pathogen of ALS is an imperfect fungus belonging to family *Stibaceae,* class *Hyphomycete* and order *Moniliales*. *Pseudocercospora griseola* produces synnemata, which are 20-40 μm wide, and consist of joined long conidiophores. The conidium formed at the tips of the conidiophores are

smooth, obclavate, 2-6 septate, and pale olive to olivaceous brown, measuring 30 to 70 µm in length, 5 to 8 µm wide, and thinning to 1.5 to 2.0 µm at the base. Different variations in length and width of the synnemata are evident among isolates (Liebenberg and Pretorius, 1997).

Plate 1: Angular leaf spot lesions on the underside of snap bean leaf and spores protruding from leaf spot lesions (Author, 2015)

2.5.2 **Host range and Occurrence**

The main host of *P. griseola* is the *Phaseolus* species including common bean, scarlet, runner bean, snap beans as well as wild species of *Phaseolus* species (Leibenberg, 1997). Infected seeds, plant debris, volunteer plants and offseason crops have been documented as important reservoir sources of *P. griseola* inoculum (Stenglein *et al.*, 2003). Even in the absence of the living host, the *P. griseola* pathogen has the potential to survive for up to 19 months on host plant debris (Liebenberg and Pretorius, 1997). Upon infection and destruction of plant tissues the pathogen forms stroma that causes it to survive in dormant condition until favorable conditions prevails (Monda *et al*., 2001). Infected seed, water and air currently play an important role in the spread and dispersion of *P.griseola* spores (Liebenberg and Pretorius, 1997).

2.5.3 Symptoms

Angular shaped spots on the leaves characterize the diseased plants. Initially the lesions are tannish gray and later turn dark brown or black. As they increase in size, several may coalesce and large proportions of the leaf area become chlorotic (Aggarwal *et al*., 2004). In the periods of high humidity, the undersurface of the leaves may have a black felt like appearance due to formation of spores by the pathogen and premature defoliation of the plant occurs. Diseased pods show circular shaped spots with reddish brown centers, severely diseased plants have distinctively reduced vigor and poor yield (Stengelin *et al.*, 2003).

2.5.4 Environmental factors

2.5.4.1 Temperature

There is general consensus on the temperature requirements of ALS pathogen worldwide, with very little variation even in isolates under controlled environment (Sartorato, 1988). Optimum temperature for spore germination lies between 18 and 24°C. Infection has been reported to decrease above and below 24 °C, ceasing above 36 °C and below 5 °C. Chlorosis and defoliation develops most rapidly at 24 °C, and at a slower rate at 20, 28 and 16°C. Retardation of chlorosis and defoliation occurs at 16 °C. Under field conditions, the rate of disease development is maximum at temperature ranges of 20-25 °C, on a relative humidity above 82 % (Stenglein *et al.*, 2003). Delay in disease development occurs in cool temperatures of 16 °C and below (Inglis & Hagedorn, 1986).

2.5.4.2 Humidity

Adequate moisture is a critical requirement for the successful infection of the host and sporulation of the pathogen. For successful ALS infection, initial period of exposure to high humidity (optimum 95-100 %) is a prerequisite. A minimum period of three hours in a dew chamber is reported to be sufficient for infection to take place; the severity of infection can increase in 24 hours to few days under field conditions. An epidemic development is favoured by alternate periods of high and low relative humidity while sporulation of the fungus ceases at RH below 49 % (Sartorato, 1988).

2.5.4.3 Light

The effect of light has been reported not have adverse effects on *Pseudocercospora griseola* conidium germination and sporulation . However, Santos Filho *et al*. (1976a) found germination to be slightly better in light, although this factor did not have an effect on pathogenicity. Different light has almost no effect on incubation period and defoliation Cardona-Alvarez (1956). However, necrosis was more severe on plants exposed to medium shading, and initial symptoms are more pronounced on uncovered than covered plants (Liebenberg *et al*., 2003). Exposure to different day lengths also has a minimal effect on incubation period. The highest disease severity is obtained on a 12-hour photoperiod and the lowest on photoperiods of 16 and of 4 hours (Cardona-Alvarez, 1956). Sporulation in culture has been reported to be better under 12 hour's light or continuous darkness than under continuous light (Santos Filho *et al*., 1976a). Cultures are generally been grown in the dark (Schwartz et al. 1982; Buruchara 1983).

2.5.5 Angular leaf spot control

2.5.5.1 Cultural control

Several cultural control practices such as crop rotation, planting pathogen free seed in well drained have been employed in control of ALS (Stenglein *et al.*, 2003). The use of seed mixtures and multi lines has been proposed as a control strategy of ALS and emergence of new races (Wagara, 2005). Effective weed control and wide spacing is documented to discourage formation of dense canopy, which creates a humid microclimate that is conducive for foliar diseases of bean including ALS (Mcmillian *et al*, 2003). Crop rotation of every two years or more between bean crops is widely recommended especially in areas where the sexual stage is known to occur but for its effectiveness it must be complimented with good sanitation practices (Allen *et al*., 1996). Cultural practices have minimal effect on ALS disease severity and together with other control measures play a big role towards reducing severity of ALS in beans (Wagara *et al.*, 2005).

2.5.5.2 Chemical control

The chemical fungicides benzimidazole, thiabendozole, strobilurin and benomyl have been shown to provide control of ALS in beans (Ploper *et al.*, 2003). Although there are no significant differences among the timing and frequency of fungicide application, an earlier application of an efficacious fungicide effectively reduces disease severity and incidence more than a later application. Fungicide evaluation experiments showed that are most efficient in inhibiting mycelial growth and decreasing colony dry weight of ALS pathogen (Sartorato *et al*., 2009). There is no apparent advantage of two applications of a fungicide compared to one under the moderate level of disease infection. However,

with higher disease levels, two applications may be required for effective disease management (Jesus Junior *et al*., 1999). The fungus can be eradicated from seed by chemical treatment of benomyl at 6g/Kg seed and a captan-Zineb combination at 3.7g/Kg seed (Correa Victoria .,1985). The development of a disease predictive model for ALS may help growers decide on the proper timing and frequency of fungicide application. The disadvantage of fungicide application include expense, technical knowhow in application and chemical residues in green beans (Liebenberg *et al*., 2003, Wagara, 2005)

2.5.5.3 Biological control

Synthetic fungicides have harmful effects to ecosystem, they are best recommended for use as part of integrated approach or when other safer methods of control have been found inadequate (Islam *et al*., 2004). Use of biological chemicals and fungicides is therefore, preferred as a cheaper and environmentally friendly alternative. Several reports have documented the use crude extract *in vitro* to confirm their fungicidal activity against different plant pathogenic fungi (Singh and Prithiviraj, 2002). Fungicidal and inhibitory effects on *Alternaria Spp* and *Fusarium Spp* have been reported on extracts from garlic bulbs, while bacterial strains of genera *Bacillus* and *Erwinia* have also been documented to antagonize the growth of the fungi rusts (Gowdu and Balasubramanian, 1998). Mizubuti *et al*., (1995) found *Bacillus subtilis* were most effective in inhibiting urediniospores germination and reducing uredinia formation of bean rust. The efficacy of leaf extracts from *Azadirachta indica*, *cannabis sativa* and *Aegle marmelos* in controlling leaf spot in faba beans caused by *Alternaria alternate* was determined in field and laboratory experiments in Bihar India 2001 (Singh and Prithiviraj 2002). *Allium*

sativum and *Warburgia ugandensis* crude extacts have been reported in Kenya to have bio-fungicide of activity against pathotypes of ALS (Karwitha M. *et al*., 2009).

2.5.8 Pathogenic variability of *Pseudocercospora griseola*

Pathogenic variation in *P. griseola* dates back to early 1950‘s when Brock exhibited indications of pathogenic differences between 13 Australian isolates. Marin Villegas in (1959) characterized 13 pathotypes in Colombia. These early reports however, have been found inconclusive, as Brock did not use single spore isolates while the purity of differential cultivars used by Marin Villegas is disputed (Wortmann *et al.*, 1998). In early 1980’s, Buruchara while working using six differential cultivars found variation among 21 isolates in Colombia and grouped them into seven races (Mahuku *et al.,* 2002). High pathogenic variability in *P. griseola* have been continuously reported in other parts of the world. In Latin America and African countries, 333 pathotypes were obtained from 433 isolates of *P. griseola* (Pastor-Corrales *et al.* 1998). Fifty races of *P. griseola* were reported from 112 isolates collected from Central America (Mahuku *et al.,* 2002). Pyndji (1992) documented 21 isolates from the Great Lakes region using 11 differential cultivars. In Kenya, existence of wide pathogenic diversity in P. *griseola* has also been reported, 19 isolates of *P. griseola* from different areas in Kenya were grouped into 14 races using 11 differential cultivars (Monda, 1995; Wagara, 1996). Wagara (1996) managed to group 15 races from 18 isolates collected from 15 districts in Kenya. The first report of a systematic collection and race typing of *P. griseola* isolates in Kenya using the 12 international bean differentials identified 44 races from 100 isolates obtained in 5 districts (Wagara *et al*., 2005). Fifty seven isolate collected in bean growing regions

of Kenya in 2013 yielded 23 different physiological races of *Pseudocercospora griseola* based on their virulence reactions on the 12 bean differentials (Mwangi, 2014). In the past, numerous methodologies were used to determine *P. griseola* physiological specialization (Correa-Victoria, 1987). Four cultivars (Brown Beauty, Stringless Black, Valentine and Red Mexican) were reportedly used by Brock while Alvarez-Ayala and Schwartz in 1979 used five cultivars that included, Caraota 260, Alabama 1, Red Kidney, ICA-Duva and Cauca 27 (Mahuku *et al.,* 2002). Buruchara (1983) utilized eight cultivars, three of which had previously been used by Alvarez-Ayala and Schwartz (1979) i.e. Alabama 1, Caraota 260, and ICA-Duva, which together with G01805-1P-1C, G02575-1OP-2C and G02858 were selected as the most suitable differential cultivars. Correa-Victoria (1987) used 21 differential cultivars, which included four used by Buruchara (1983), and two used by Alvarez-Ayala and Schwartz (1979). Eight of these cultivars namely Montcalm, Seafarer, BAT 332, Pompadour Checa, G05686, Cornell 49-242, A 339 and BAT 1647, were selected because of their ability to differentiate between the isolates used (Liebenberg and Pretorius, 1997). Pyndji (1992) used these eight cultivars, plus G02858, Caraota 260 and A 285, and reported Montcalm, A 285, A 339, Caraota 260 and BAT 1647 to be the best indicators of the occurrence of new pathotypes in a given area.

The new set of standardized 12 differential cultivars namely Don Timoteo, G11796, Boloom Bayo, Montcalm, Amendoin, G05686, Pan 72, G02858, Flor de Mayo, Mexico 54, BAT 332 and Cornell 49-242, in use (Liebenberg and Pretorius, 1997). This has enabled meaningful comparison of results over time and from different parts of the world.

Two *P. griseola* pathogenic groups (Mesoamerican and Andean) appear to have co-evolved with the Andean and Mesoamerican bean gene pools, respectively (Chacon *et al* 2005). The diversity has led to the characterization of two major groups of *P. griseola* that are defined as 'Andean' (*Pseudocercospora griseola P.griseola)* and 'Mesoamerican' (*Pseudocercospora griseola f. Mesoamericana*) (Crous *et al*., 2006). Pastor-Corrales and Jara (1995) suggested that the strategy for developing new angular leaf spot resistant bean genotypes requires understanding of the genetic variation of the pathogen with incorporation of resistant genes from a given gene pool into cultivars of the other gene pool.

Studies have shown that several independent genes, which possess one or more alleles resistant to several races of the pathogen control resistance to angular leaf spot (Carvalho *et al.,* 1998; Mahuku *et al.,* 2003; Caixeta *et al*., 2005). Resistance of cultivars AND 277, Cornell 49-242, G10474 and MAR 2 to pathotypes 63-23, 31-17, 63-63 and 63-39 are conditioned by a single dominant gene (Nietzsche *et al*., 2000). Cultivars with only one resistance gene or allele can control the disease for only a few years, until the appearance of new races of the fungus (Pastor-Corrales *et al.,* 1998; Stenglein *et al.,* 2003).

2.5.9 Durable resistance

Developing resistant genotypes is the best way for managing ALS. Taking into account that the ALS pathogen is highly variable, the use of resistance that is conditioned by few major genes may not be effective for a long time (Mahuku *et al.,* 2002). It is important to utilize new strategies of breeding bean varieties to ensure durability of resistance. One such strategy is to use sources with resistance to a wide range of *P. griseola* races

(Wagara *et al*., 2004). The most effective durable resistance would be achieved with minor genes. Gamete selection is one of the methods of breeding that could be used to combine the minor genes and favorable alleles contributing to resistance in a single genotype. Gamete selection ‘‘selection of self-fertilized lines’’ was proposed by Singh (1994) as a method to simultaneously improving multiple traits in common bean, by crossing multiple parents followed by early generation testing and selection. Gamete selection was used to improve resistance to white mould disease in common beans (Teran and Singh, 2009) and to improve resistance to common and halo bacterial blights in common bean (Asensio *et al*., 2006). Gamete selection has also been used to combine resistance to different bacterial, fungal and viral diseases into one cultivar (Teran *et al.,* 2013).

2.6 Marker Assisted Selection.

Molecular (DNA) markers used in plant breeding greatly assist in increasing efficiency and precision. Use of these molecular markers in plant breeding is marker-assisted selection (MAS). It is a complement of the new discipline of molecular breeding (Collards *et al*., 2008). Marker assisted selection is the novel approach in which individuals for intercrossing are selected using selection index based on genotypic data controlled by few or several genes (Quantitative linked traits). The gain from selection using such index is higher than phenotypic selection used in conventional recurrent methods (Queller *et al.,* 1993). There has been significant progress made through phenotypic selections for agronomic traits (Taran *et al*., 2002). Additionally, some of the traits are controlled by multiple genetic loci (QTL) and displays a strong interaction with the environment. Molecular markers linked to such traits are available and have increased

the efficiency of breeding for diseases in MAS programmes (Kelly *et al.,* 1998, Collards *et al*., 2005). The use of DNA molecular markers improves the understanding of the genetic factors conditioning the traits and assists in the selection of superior genotypes (Biswas *et al.,* 2003). The use of disease resistant cultivars in combination with appropriate cultural practices is essential for the management of bean diseases (Singh *et al.,* 2000).

2.6.1 Morphological Markers.

Phenotypic identification of plants is a powerful tool used in the classification of plant genotypes. Morphological characterization is used for various purposes including; studies of genetic variations patterns, identification of duplicates and correlation with characteristics of agronomic importance (CIAT, 1984). Morphological markers are visible characters, such as flower color, pigmentation, and seed shape and growth habits. Morphological markers have been utilized in the study of genetic diversity in beans, inheritance studies of traits and for gene mapping (Koinange *et al.,* 1996). These markers have been used to map the XAN 159 source of common bacterial blight (CBB) *Xanthomonas campestris* resistance that is known to be linked to purple flower colour and black seed coat (Jung *et al.,* 1997). Morphological markers are limited in number and are influenced by environmental factors and developmental stage of the plant (Winter and Kahl, 1995).

2.8.2 Molecular Markers

Molecular markers are distinguished into two types; hybridization based that rely on hybridization between probe and homologus DNA segments within the genome (RFLP),

and secondly semi arbitrary Polymerase Chain Reaction (PCR) based molecular markers such as amplified fragment length polymorphism and arbitrary polymerase chain reaction based molecular markers such as simple sequence repeats (Semagn *et al.,* 2006). Molecular markers are stable, genetically informative and useful for genotype discrimination. DNA markers are selectively neutral because they are located in the non-coding regions of DNA (Collard *et al.,* 2005)

2.5.3 Simple Sequence Repeats (SSRs).

Simple sequence repeats or microsatellites are arbitrary polymerase chain reaction molecular markers. They are tandem repeats of short (2-5) sequences (Queller *et al.,* 1993). Agarose gels or polyacrylamides are used to detect such differences, where repeat lengths migrate distances according to sizes (Robinson and Harris, 1999). Simple sequence repeats are now the markers of choice in most areas of molecular genetics as they are highly polymorphic even in closely related lines (Gupta *et al.,* 1999). Simple sequence repeat markers have been developed from known sequence libraries (Blair *et al.,* 2003). Diversity surveys of beans conducted have determined race structures in beans .SSR markers are used to construct PCR based genetic maps, to evaluate intra-specific diversity within genus and to fingerprint genetic diversity in bean varieties in the world (Yu *et al.,* 2000; Blair *et al.,* 2003).

2.5.4 Sequence Characterized Amplified Region markers (SCARs).

In SCAR markers, the RAPD marker termini are sequenced and longer primers are designed (22–24 nucleotide bases long) for specific amplification of a particular locus.

The presence or absence of the band indicates variation in sequence. These are better reproducible than RAPDs (Yu *et al.,* 2000).

Usually, SCARs are dominant markers, however, some of them can be converted into co-dominant markers by digesting them with tetra cutting restriction enzymes and polymorphism can be deduced by using simple non denaturing gels to detect whether the products has different restriction sites for the different alleles. The SCARs exhibit several advantages in mapping studies, map-based cloning as they can be used to screen pooled genomic libraries by PCR, physical mapping, locus specificity (Yu *et al.*,2000). It also enables comparative mapping or homology studies among related species, thus making it an extremely adaptable tool in the near future (Yu *et al.,* 2000; Collard *et al.,* 2005). These markers have been widely used in breeding for disease resistance especially to diseases controlled by dominant genes since these markers are dominant in nature (Milkas *et al.*, 2009).

2.5.5 Sequence Tagged Sites (STS) markers

Sequence tagged sites (STS) molecular analysis is comprised of PCR amplification of a genomic region by using a primer set (18-22 bp), which directs the amplification of a sequence for a specific locus (Olson *et al.*, 1989). The STS primers are designed based on cloning and sequencing mapped RFLP (Brown 2006), AFLP (Brown 2006; Shan, Blake & Talbert 1999) and RAPD products (Brown 2006; Olson *et al.*, 1989). STS polymorphisms can be read directly from an agarose gel (Tragoonrung *et al*., 1992). The STS is easy to detect with moderate-resolution analytical techniques and the sequence-tagged-sites provide co-dominant markers. Using this technique, tedious hybridization procedures involved in RFLP analysis can be overcome (Olson *et al*., 1989). This

approach is extremely useful for studying the relationship between various species. These markers have been used in particular for disease resistance against viral and fungal pathogens and also for insect and nematode pests and have the potential of easily being integrated into MAS of the trait of interest and of pyramiding of resistance genes for effective breeding programs (Joshi B. K. *et al* .,2009) .

2.5.6 Molecular marker assisted selection for ALS in beans

Resistance genes against *Pseudocercospora griseola* the causal agent of angular leaf spot is controlled by major genes that are either dominant or recessive. The genes can be acting individually or in duplication and may interact in an additive manner with or without epistasis (Mahuku *et al.,* 2003). Inheritance of ALS resistance is complex, involving both dominant and recessive genes that are either dominant or recessive with complementary or epistatic effects and acting alone or in combination (Mahuku *et al*., 2004). Major and minor genes mediate ALS resistance in beans and a number of sources for these resistance genes have been identified (Mahuku *et al.,* 2003.) Examples of resistant cultivars include A 75, A 140, A 152, A 175, A 229, BAT 76, BAT 431, BAT 1432, BAT 1458 and G5686, MAR 1, MAR 2 (Ferreira *et al*., 2009). Cornell 49-242 has *Pgh-2* that confers resistance to *P. griseola* pathotype 31:17 (Nietsche *et al.,* 2009). Resistance to ALS in Mexico 54 is due to a single dominant gene that confers resistance and G 06727 has resistance to *P. griseola* pathotype 63:59 (Mahuku *et al.*, 2004). G5686 and Mexico 54 display fairly good levels of resistance to nearly all races so far characterized (Ferreira *et al*., 2000). These cultivars are good sources of resistance to *P.griseola*. Mexico 54 has been found to be resistant to some *P. griseola* isolates so far characterized in Africa (Mahuku *et al*., 2002).

Sequence characterized amplified region markers used for selecting genes for resistance to ALS include SH13 for *phg-1* gene in linkage group *6* and SNO2 for *Phg-2* gene in linkage group 8 (Miklas *et al.,* 2002, Nietsche *et al.,* 2000). Others include SAA19, SBA16, and SMO2, which is ouro negro dominant gene (Queiroz *et al.,* 2004). STS G796 in linkage group 8 and has a 24 base pair insertion in the allele of the ALS resistant parent Mexico 54. This marker was developed following screening of 16 breeding parents for angular leaf spot resistance using next generation sequencing. The screening identified polymorphic, co-dominnant single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs), with an average number occurring across the 11 bean chromosomes of 2566 and 431000 respectively. This use of whole genome sequences identified G796 STS polymorphic marker closely linked to SNO2 whereas none of the susceptible parents has the same sequence (Tamara *et al.,* 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Field Evaluation

3.1.1 Plant Germplasm

The germplasm panel comprised a set of 26 commercial genotypes, 3 advanced University of Eldoret (U.O.E) French bean breeding lines and 3 landraces provided by the National Gene Bank of Kenya. Two genotypes Mexico 54 and Mwitemania (GLPX-92) were included in the experiment as resistant and susceptible checks respectively.

3.1.2 Study sites

The 34 genotypes were planted under natural infection conditions at the University of Eldoret (0^{o}34'N,35^{o}18'E), Kisii (0^{o}48'S,34^{o}58'E) and Kakamega (0^{o}16'S34^{o}45^{o}E) in 2014. The Eldoret site has an elevation of 2153 m above sea level (m.a.s.l) predominantly a LH3 zone and experiences one rainfall season between march and September. This site is a minor been growing region although it borders major bean growing county of Kakamega. The annual average rainfalls is 1085 mm and mean temperatures range between 11-24 ^{o}C .The soils in Eldoret are *rhodic ferralsols* (Jaetzold *et al.,* 2006).

The experiment in Kisii was set up in a small holder farm . The site had an elevation of 1936 m.a.s.l at LH3-UM2 and experiences a bimodal rainfall with long rains occurring between March and May and short rains occurring in September to November. It is therefore a major common bean growing region .The annual average rainfall is 2130 mm, mean temperature ranges between 16^{o}C and 26^{o}C (Jaetzold *et al.,* 2006). The predominant soil types in this study site is *luvic phaoezem*. Kenya Agricultural Livestock

Research Organization (KALRO) Kakamega experimental site is major bean growing and breeding site, located at UM2 with an elevation of 1585 m.a.s.l. The predominant soil type in this study site is *ferralo orthic acrisols*. This experimental site experiences a bimodal rainfall with long rains in April to June, Short rains in August to October and annual rainfall average of 1250mm per year and temperature ranges of 12^{o}C to 24^{o}C (Jaetzold *et al.,* 2006).

3.1.3 Experimental Procedures

At the three sites, the thirty four germplasm were planted in a Randomized Complete Block Design with three replications. The experimental plots were 3 m long single rows of 30 cm x 15 cm. At planting, fertilizer (di-ammonium phosphate, 80 Kg Ha^{-1}) was applied. In addition, weeding was done in all sites thrice: two weeks after seedling emergence, before flowering and after podding and pesticide confidor was applied to control insect pests in the sites.

3.1.4 Disease assessment

The data for angular leaf spot disease severity on the French beans was taken at trifoliate, flowering, pod formation and the scores averaged. The 1-9 CIAT scale of disease assessment was used in data collection with scores of ≤ 3 considered as resistant, 4 to 6, intermediate and 7 to 9 as susceptible (Vanschoonhoven and Pastor Corrales,1987) as represented in Table 1.

Table 1: CIAT scale (1-9) for angular leaf spot disease severity

Reaction Rating	Category	Description
1	Resistant	No visible symptom
2	Resistant	Lesions on up to 3% of leaf area
3	Resistant	Lesions on up to5% of leaf area
4	Intermediate	Lesions and sporulation on up to 10% of leaf area
5	Intermediate	Lesions and sporulations with 2–3 mm in diameter on 11–15% of leaf area
6	Intermediate	Lesions and sporulations >3 mm in diameter on 16–20% of leaf area
7	Susceptible	Lesions and sporulations >3 mm in diameter on 21–25% of leaf area
8	Susceptible	Lesions and sporulations >3 mm in diameter on 26–30% of leaf area
9	Susceptible	Lesions, frequently associated with early loss of leaves and plant

Source: Van Schoonhoven and Pastor-Corrales (1987)

3.1.5 Data analysis

Data for disease assessment scores from the experimental sites were subjected to analysis of variance (ANOVA) using SAS software and effects declared significant at 5% level of significance. Tukey's test was used to separate the means.

3.1.6 Statistical Equation

$X_{ijklm} = \mu + B_i + G_j + E_k + EG_{jk} + £_{ijkl}$

Where: μ=Mean;

B_i = Effect due to the i^{th} Block;

G_j=Effect due to the j^{th} genotype;

E_k = Effect due to the k^{th} environment

GE_{jk} =Interaction between the j^{th} genotype and k^{th} environment;

$£_{ijkl}$ =Residual effect

3.2 Characterization of *Pseudocercospora griseola* isolates

A total of 36 angular leaf spot infected leaves were collected as isolates from farmers' fields in Uasin gishu (2153 m.a.s.l, 0°34'N, 35°18'E), Kakamega (1585 m.a.s.l, 0°16'S, 34°45'E) and Kisii (1936 m.a.s.l, 0°48'S,34°58'E) counties to be used for screening. The isolates were collected randomly from farms near experimental sites by walking along a diagonal line from one side of the farm to the other. All isolates were collected from susceptible bean genotypes, wrapped in a paper towel, placed in an envelope and the corresponding label firmly attached to a paper bag and taken to the University of Eldoret, Biotechnology laboratory for fungal isolation.

3.2.1 Isolation and purification of isolates.

Pseudocercospora griseola was isolated from the infected bean leaves showing characteristic ALS symptoms. Infected leaves were incubated in a sterile moist chamber to induce sporulation (Appendix 3). Using a dissecting microscope, well sporulating lesions were selected and conidia picked using a tiny piece of agar placed at the tip of a sterile mounted needle (Wagara *et al.,* 2011). The conidia were transferred to a drop of sterile distilled water on a microscope slide and the suspension was stirred and streaked onto water agar plates using a sterile wire loop. The plates were incubated at 24°C in a non-illuminated incubator and conidia germination was monitored using a dissecting microscope. An agar block with a single germinating conidium was removed and transferred onto V-8 medium, (200ml V8 juice, 3g $CaCO_3$, 18g Bacto agar and 800ml ddH_2O) to obtain monosporic cultures for each isolate. The individual germinated conidium was then transplanted onto V8 medium to obtain monosporic cultures for each

P. griseola isolate. Isolates were maintained on V8 juice agar and kept in a dark incubator at 21 °C for up to 21 days to promote sporulation. To prepare the inoculum, sterile water was poured onto the growing colonies, they were gently scraped and the suspension filtered through a double muslin cloth to remove the mycelia mass ((Wagara *et al.,* 2011,Pastor -Corrales *et al*., 1987).

3.2.3 Characterization of *P. griseola* isolates

Characterization of *P. griseola* isolates were performed using a set of 12 differential bean cultivars (Wagara *et al.,* 2005). Six of the differentials were large-seeded varieties of Andean origin and the other six were small- or medium-seeded Middle American genotypes all sourced from CIAT –Uganda (Table 2). Seeds were surface-sterilized in 2.5% sodium hypochlorite for five minutes and rinsed in three changes of sterile distilled water. The seeds were planted in sterile soil mixture composed of soil, sand and manure in the ratio of 2:1:1. (Wagara *et al.,* 2011). Three seeds of each cultivar were sown per polythene pot. After 14 days, concentrated inoculum was prepared by adding 1000 µl of sterile distilled water to each plate and scraping the surface of culture. Three-week-old seedlings were inoculated with the pathogen until runoff using a hand sprayer. Inoculated plants were incubated on the greenhouse bench and maintained in high humidity through frequent mist sprays to facilitate disease development .The experiment was replicated thrice using three pots of plants. Disease reactions were scored 21 days after inoculation for compatible reaction (+) or incompatible reaction (-) (CIAT 2005, Pastor-Corrales 2005,Wagara *et al.,* 2011). Isolates that induced similar disease reactions were grouped together and comprised a pathotype. To denominate a pathotype, two numbers separated by a dash were used. The first number was obtained by adding the binary values of the

susceptible Andean differential cultivars, each of which was given a letter a, b, c, d, e or f corresponding to 1, 2, 4, 8, 16 and 32, respectively. The second number was obtained by adding the binary values of the susceptible Middle American differential cultivars, each of which was given a letter g, h, i, j, k or l corresponding into 1, 2, 4, 8, 16 and 32, respectively as indicated in Table 2. For example, for race 30-11 (virulence phenotype a b c d e f g h j), the first value (30) was obtained by summing the binary values of the susceptible Andean differential cultivars Don Timoteo, G11796, BolonBayo, Montcalm, Amendoin and G 5686 (1+2+4+8+16+32=30). The second value (11) was obtained by summing the binary values of the susceptible Middle American varieties PAN72, G2858, Flor de Mayo and Mexico 54 (1 + 2 + 8 =11). (CIAT 2005, Pastor-Corrales 2005, Wagara *et al.*, 2011).

Table 2: Characteristics of differential genotypes used to identify races of *P. griseola* isolates

Differential cultivar	Notation[a]	Seed size[b]	Gene pool	Binary values	Resistance genes
Don Timoteo	A	L	Andean	1	
G 11796	B	L	Andean	2	
Bloom Bayo	C	L	Andean	4	
Montcalm	D	L	Andean	8	
Amendoin	E	L	Andean	16	
G 5686	F	L	Andean	32	
PAN 72	G	S	Middle American	1	
G 2858	H	M	Middle American	2	
Flor de Mayo	I	S	Middle American	4	
Mexico 54	J	M	Middle American	8	*Phg-2, Phg-5, Phg-6*
BAT 332	K	S	Middle American	16	
Cornell 49-242	L	S	Middle American	32	*Phg-3*

Source: Pastor-Corrales and Jara (1995), Nietsche *et al*. (2001), Mahuku *et al*. (2002b), Miklas *et al*. (2006a). [b](L) Large ;(M) Medium ;(S) Small

3.2.4 Screening French bean germplasm for resistance to *P.griseola* in the greenhouse.

Reactions of the thirty two French bean germplasm to races of *P. griseola* were assessed under greenhouse conditions at University of Eldoret. The Seeds were surface-sterilized in 2.5% sodium hypochlorite for five minutes and rinsed in three changes of sterile distilled water. Seeds were sown in plastic trays with sterile soil mixture composed of forest soil, sand and fine manure in the ratio of 2:1:1. Six seeds per germplasm were sown in polythene pot and thinned to three plants two weeks after sowing. Three pots were used for each genotype and arranged in a completely randomized design at the greenhouse. After 14 days, concentrated inoculum of the successfully isolated races was prepared by adding 1000µl of sterile distilled water to the petri dish the surface of culture scrapped using glass rod to dislodge the fungal pathogen. Three-week-old seedlings were inoculated with the inoculum at a concentration of $2x10^4$ conidia ml^{-1} with each specific race until runoff using a hand sprayer. Inoculated plants were then transferred to the greenhouse bench and maintained in high humidity by frequently mist spraying to facilitate disease development. The plants were watered twice daily depending on the intensity of the sun. Disease development on inoculated plants was monitored daily until at 21 days post inoculation. Data for disease reactions were collected according to procedures earlier described by Van Schoonhoven and Pastor-Corrales, (1987). Reaction type categories were determined from the mean of disease scores for each race-host genotype combination. Plants showing disease scores of ≤ 3 were resistant, 4 to 6 as intermediate resistant and host responses of 7 to 9 as susceptible. The experiment was repeated once to validate the findings. Data on disease severity of

each isolate host genotype interaction were analysed by the analysis of Variance (ANOVA) considering completely randomized design using SAS Version 9.1. Separation of means was done using least significant difference (LSD) test at $P < 0.05$.

3.3 Screening using STS G796 molecular marker

3.3.1 Population development

The experimental plant material consisted crosses of Amy a commercially preferred genotype with white seeds and forms round, straight, and smooth pods but susceptible to ALS with Mexico 54 (medium size seeds of pink colour) which is an exotic variety resistant to ALS. A total of 144 F2 progeny were derived from crosses involving Amy and Mexico 54 as the susceptible and resistant parents, respectively. The emasculation and pollination technique was carried by hand as described by Viera (1967). The method consisted mechanical emasculation of the female parent, by removing the flower buds with tweezers one day before flowering. Emasculation was followed by cross pollination, for which ripe pollen grains from open flowers of male parent were used to perform crosses. A total of 50 crosses were made for each hybrid combination to enhance a sufficient quantity of seeds to form F1 populations. The F1 seeds, obtained through crossing, were multiplied under green house conditions to obtain F2, that were subsequently used in the experiment. 140 F_2 plants were inoculated with ALS following the method described in 3.2.4 above. The phenotypic data for ALS infection were taken as scores on a 0 to 9 CIAT scale, whereby 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).

3.3.2 Extraction of Genomic DNA

Plants used for genotyping were non-inoculated with fully expanded trifoliate leaves. The leaves were collected from 100 F_2 plants alongside their parents, French bean genotypes and ALS differentials cultivars 28 days after planting. The harvested leaves were crushed using sterilized mortar and pestle to form a paste and placed on a marked position of whatman FTA® plant card. The cards were air dried at room temperature (20-25°C) for one and a half hours. The cards were washed with FTA purification reagent as per manufacturer's procedure. Ten 2 mm FTA® discs were obtained using a Harris ® core punch and placed in 1.5 ml micro –centrifuge tubes. Five hundred microlitres of FTA® purification reagent were then added to the discs, and incubated at room temperature for five minutes with moderate mixing. The procedure repeated twice. The discs were washed twice with 500 µl TE following the same procedure before drying them for one and a half hour at room temperature. However, DNA quantification was not done for FTA discs because they are estimated to contain between 5–50 ng/µl DNA and can be used direct for amplification. The discs were then stored at 4°C ready for polymerase chain reaction.

3.3.3 Genotypic screening using STS G796 marker

The F_2 lines, parents (Amy and Mexico 54), 31 French bean cultivars and other 11 ALS differential genotypes were screened at University of Eldoret biotechnology laboratory using STS G796 marker (forward primer GAGAAACTACGGGCTGTTTTACCC and reverse primer AATTAAAACACCCACCCACTCCAT) to confirm the presence of the *Phg-2* gene for *P. griseola* resistance (Olson *et al*., 1989 ,Tamara *et al*., 2015) .The amplification of the STS marker was done using puReTaq ready to go PCR beads®. The PCR reaction

was prepared by adding 1 µl of each forward and reverse primers to lyophilized PCR beads 1 U of Taq polymerase, 250 µM of dNTPs, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM MgCl2 and stabilizer and tracking dye. 23 µl of sterile PCR molecular water was added into the PCR beads® PCR tubes to a total volume of 25µl. The lyophilized white pellet was then dissolved by vortexing, and briefly spinned down. Finally the template DNA (2mm FTA disc) was added , and PCR amplification of the samples performed in eppendorf Thermal Cycler® with initial DNA denaturation at 94°C for 30s; followed by 35 cycles primer annealing at 65°C for 45s; polymerization by DNA polymerase at 72°C for 30s, and a final extension at 72ºC for 5 minutes and a final rest at 4°C. After 35 cycles, the amplification products were separated by gel electrophoresis (2325 Galileo Unit, Galileo Bioscience) on a 1.2% (w/v) agarose gel, stained with 5µl/100ml ethidium bromide at 100V in 1× TBE buffer for one hour (Blair *et al* .,2005). The DNA bands were visualized under ultraviolet light transilluminator and photographed using a mounted digital camera. Selection of the presumed resistant line was done by observing amplification of the molecular marker, exhibited by the presence of a band with the right size (Tamara *et al*., 2015)

3.3.4 Statistical analysis

The *chi*-squared test was used to test goodness-of-fit to a 3:1 ratio in the F_2 generation in order to confirm the hypothesis of a single dominant gene conferring resistance. The following formula was used:

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Where O = observed value and E = expected value.

The co-segregation analysis between the marker and the resistance gene was analyzed using a analysis of variance in STATGRAPHICS programme. The genetic marker data was assigned as the independent variable and ALS disease rating as the dependent variable. An association between an STS marker and the trait was considered significant where the probability was ≤ 0.05. Segregation of the STS marker in the F_2 population was tested for its goodness-of-fit to 1:2:1 using the chi-squared test.

CHAPTER FOUR

RESULTS

4.1 Reaction of French beans to ALS in experimental sites.

The results of angular leaf spot severity in French beans in Western Kenya are represented in Table 3. Significant differences ($P \leq 0.05$) were revealed between entries with respect to disease severity, and also between sites. The 34 French bean genotypes were all susceptible to ALS with a mean severity of 5.8. The mean disease severity in Kakamega, Kisii and Eldoret was 6.2, 5.7 and 5.3, respectively. Genotypes: Monel, GBK 032952 and GLP X92 (susceptible check) were the most susceptible genotypes across all sites with means of 7.0, 7.3 and 7.3 respectively .. The French bean entries all exhibited an intermediate to susceptible reaction ranging from 4.0 to 7.5. Genotypes: andate, Serengeti and Samantha with average severity mean of (5.0-5.2) exhibited an overall significant difference among the commercial genotypes screened in this experiment. Of the 34 entries, only Mexico 54 was resistant to ALS in the experimental sites with average score of 3.2. On genotype by environment (GXE) there was minimal variation on genotypes reaction to ALS except in Kakamegawherecommercialgenotypes:tana2,star2053,venda,Julia,monel,T19,Paulista,Morlane,

Organdia, Superviolet, Castore, Teresa, Amy and bravo and all three U.O.E French bean lines and landrace GBK032921 and 032952 that exhibited significant difference in susceptibility all the genotypes recorded mean intermediate resistance. Commercial genotypes andate ,Serengeti and samantha were significantly different to other genotypes screen in all the three sites having a moderate resistance score of 5.0 .Landrace

GBK032952 was the most significantly susceptible in genotype by environment interactions while Mexico 54 with a mean of 3.2 was significantly resistant in the all the three sites.

Table 3: Mean severity of angular leaf spot on French bean genotypes in the study sites in Western Kenya.

Genotype	Eldoret	Kakamega	Kisii	GXE
Manakelly	5.5bdac	5.7ba	5.3ba	5.5bdc
Tana2	5.5bdac	6.1a	5.5ba	5.7bdac
Star2053	5.2bdac	6.3a	6.5ba	6.0bdac
Venda	4.5bdac	6.7a	6.6ba	5.9bdac
Julia	4.0dc	6.3a	6.1ba	5.5bdc
Monel	7.3ba	7.3a	6.5ba	7.0bac
Morgan	4.8bdac	5.3ba	5.5ba	5.3dc
Andate	4.3bdc	5.5ba	5.3ba	5.0d
Venadon	4.7bdac	5.6ba	6.1ba	5.5bdc
T19	4.6bdac	6.5a	6.1ba	5.7bdac
Paulista	4.6bdac	6.5a	6.1ba	5.7bdac
Konza	5.3bdac	5.8ba	5.6ba	5.6bdac
Strada	4.8bdac	5.8ba	5.6ba	5.4bdc
Kendo	5.8bdac	5.6ba	5.5ba	5.6bdac
Serengeti	5.2bdac	6.0ba	4.5ba	5.2d
Morlane	6.3bac	7.1a	6.3ba	6.6bdac
Organdia	7.0ba	6.3a	4.5ba	5.9bdac
Superviolet	5.8bdac	6.5a	5.6ba	6.0bdac
Morelli	5.6bdac	6.5ba	5.5ba	5.9bdac
Castore	5.0bdac	6.8a	6.0ba	5.9bdac
Samantha	5.0bdac	5.6ba	4.5ba	5.0d
Teresa	6.0bdac	7.1a	6.5ba	6.5bdac
Ducato	5.0bdac	6.0ba	5.5ba	5.5bdc
Amy	5.3bdac	6.5a	5.5ba	5.8bdac
Tana	6.0bdac	6.0ba	6.5ba	6.2bdac
Bravo	6.0bac	6.3a	5.1ba	5.8bdac
French bean lines				
U.O.E#2	4.8bdac	6.1a	5.1ba	5.3dc
U.O.E#10	5.0bdac	6.5a	4.5ba	5.3dc
U.O.E#13	6.0bac	6.0a	6.0ba	6.0bdac
Landraces				
GBK032805	4.8bdac	5.8ba	5.6ba	5.4bdc
GBK032921	4.2dc	7.2a	6.1ba	5.8bdac
GBK032952	7.5a	7.5a	7.0a	7.3a
Checks				
GLPX- 92	7.3ba	7.1a	7.5a	7.3ba
Mexico54	3.0d	3.3b	3.3b	3.2e
Mean	5.3c	6.2a	5.7b	5.8
CV%	18.0	13.8	18.5	17.2

Means sharing the same letter are not different at $P \leq 0.05$ according to Tukey's test

4.1 Race typing of angular leaf spot isolates based on the reaction of 12 differential cultivars

The reaction of differential cultivars to isolates angular leaf spot collected from the three regions of Western Kenya revealed existence of pathogenic variability of the ALS fungus (Table 4). Eight races were identified out of a total of 36 isolates. In Eldoret races 31-23, 63-39, 63-23 and 24-48 were isolated, races 63-39,30-55,36-39,and 63-3 were race typed in Kakamega while in Kisii races 30-23, 63.39 and 63-3 were race typed. Most of the races were isolated in one study site apart from races 63-39 which was isolated in all the study sites and 63-3 that was isolated in Kakamega and Kisii . The race distribution per experimental sites ranged between 25% and 33% as represented in fig1.

Table 4: Race typing of angular leaf spot isolates based on response to differential bean cultivars

	DIFFERENTIAL CULTIVAR													
Location	**Andean Group[a]**						**Mesoamerican Group[b]**						**Race**	**No of Isolates**
	a	b	c	d	E	f	g	h	i	j	k	l		
Eldoret	+	+	+	+	+	-	+	+	+	-	+	-	**31-23**	3
Eldoret	+	+	+	+	+	+	+	+	+	-	-	+	**63-39**	2
Eldoret	+	+	+	+	+	+	+	+	+	-	+	-	**63-23**	2
Eldoret	-	-	-	+	+	-	-	-	-	-	+	+	**24-48**	4
Kakamega	+	+	+	+	+	+	+	+	+	-	-	+	**63-39**	5
Kakamega	-	+	+	+	+	-	+	+	+	-	+	+	**30-55**	1
Kakamega	-	-	+	-	-	+	+	+	+	-	-	+	**36-39**	3
Kakamega	+	+	+	+	+	+	+	+	-	-	-	-	**63-3**	4
Kisii	-	+	+	+	+	-	+	+	+	-	+	-	**30-23**	5
Kisii	+	+	+	+	+	+	+	+	+	-	-	+	**63-39**	3
Kisii	+	+	+	+	+	+	+	+	-	-	-	-	**63-3**	4

[a]Andeangroups:(a)DonTimoteo;(b)G11796;(c)BloomBayo;(d)Montcalm;(e)Amendoin;(f) G5686.[b]Middle American groups: (g)Pan72;(h)G2858; (i) Flor de Mayo;(j)Mexico 54;(k) BAT332;(l)Cornell49-242.(+) Compatible reaction; (-) Incompatible reaction.

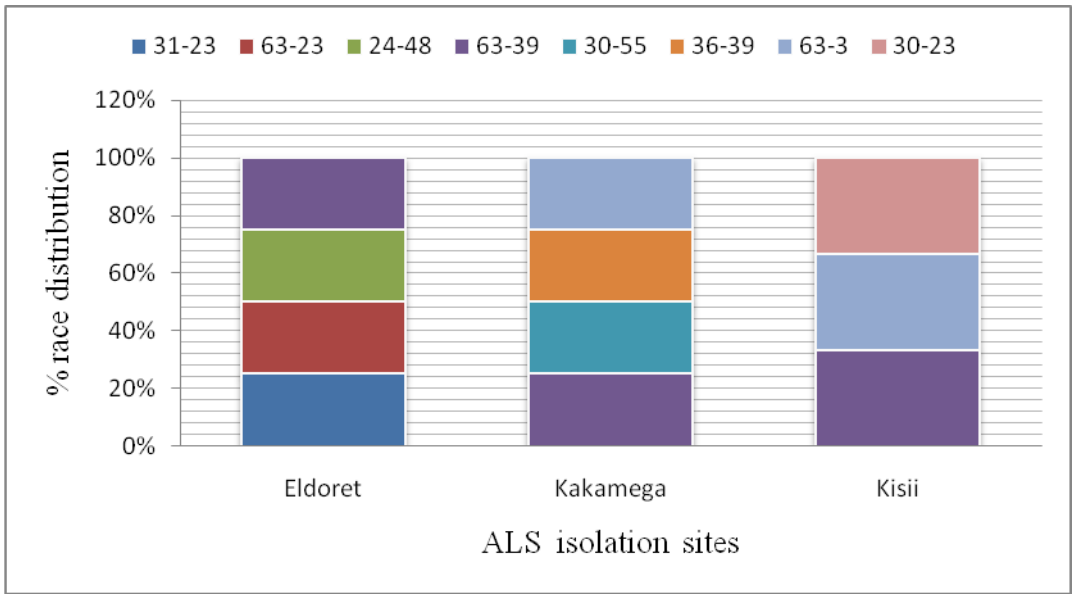

Figure 1: Percent distribution of eight *P. griseola* races in experimental sites in western Kenya

4.2 Virulence of *Pseudocercospora griseola* Races on French beans.

The virulence of *P.griseola* races on the French bean genotypes are presented in Table 5 and in Fig 2, 3 and 4. The *P.griseola* races showed significant differences in their ability to infect the French bean genotypes ($P< 0.05$). Races 63-39 and 63-23 were the most virulent with scores of 6.8 while 63-3 was the least virulent with average severity 4.5. The genotypes however, were either resistant (scores 1 to 3) or moderately resistant (scores 4 to 6) to most of the races and only susceptible (scores 7 to 9) to a few races. Out of the 34 bean genotypes inoculated with *P. griseola* races, Manakelly was resistant to 6 races : 31-23, 36-39, 24-48, 30-55, 30-14 and 63-3, Venda (24-48, 30-55) with scores of :3.3,3.8, 3.7,3.8,3.2,3.2 respectively , while were resistance to 2 races were recorded in Paulista 24-48 and 63-3 with scores of 3.8 and 3.5, Kendo with scores of 3.3 on 24-48 and 30-14 , Morelli recorded resistance in 31-23 and 30-14 with score of 3.8 and 3.3

respectively , resistance to 30-14 and 63-3 were exhibited in Bravo with scores of 3.8 and 3.7 respectively, UoE#2 recorded resistance in 31-23 and 24-48 with scores of 3.3 and 3.8 and UoE# recorded resistance in races 24-48 and 63-3 with scores of 3.8 . In addition the following genotypes exhibited resistance to one race each : Tana 2, 24 -48 (3.2) , Julia 63-3 (3.2), Morgan 63-3 (3.5), Venadon 63-3 (3.8), Strada 63-3 (3.0) Castore 31-23 (3.8), Ducato 63-3 (3.8), Amy 24-48 (3.0), UoE #13 63-3 (3.3), GBK032805 63-3 (3.5), and GBK032921 31-23 (3.3). 12 commercial genotypes: Star2053, monel, andate, T19, konza, Serengeti, Morlane organdia, supervolet, Samantha, teresa and tana (fig 2) and landrace GBK 032952 (fig 4) did not record any resistance to any of the eight races. Resistance to race 63-23 and 63-39 were only recorded by Mexico 54 (3.0) the resistant check which also recorded resistance in all the other six races whereas Mwitemania (GLPX-92) was susceptible to all the characterized races .

Table 3: Response of French beans to *P.griseola* races

Genotypes	31-23	36-39	24-48	63-23	30-55	63-39	30-14	63-3
Manakely	3.3	3.8	3.7	4.3	3.8	4.7	3.2	3.2
Tana2	5.0	6.5	3.2	5.0	5.5	6.5	5.8	5.8
Star2053	5.2	4.0	5.2	6.0	5.7	6.7	5.0	3.8
Venda	6.2	4.3	3.5	7.7	3.8	5.3	6.8	5.3
Julia	5.8	6.5	5.0	7.0	5.3	5.5	5.8	3.2
Monel	6.7	7.2	5.7	8.0	6.0	6.2	6.8	4.3
Morgan	5.3	5.8	5.3	8.0	6.5	7.7	4.8	3.5
Andate	4.3	5.7	5.5	7.7	6.8	8.0	4.5	5.3
Venadon	5.3	4.3	5.5	7.7	6.7	7.8	5.8	3.8
T19	5.7	5.7	4.7	7.7	6.3	7.5	5.0	5.5
Paulista	4.5	6.5	3.8	7.0	6.0	5.7	4.8	3.5
Konza	4.5	4.3	4.5	7.0	6.0	5.8	5.2	4.3
Strada	5.3	6.2	5.3	6.7	5.8	6.5	5.2	3.0
Kendo	4.2	4.2	3.3	6.3	5.7	7.2	3.3	4.2
Serengeti	6.5	5.3	6.3	6.3	6.7	7.5	6.8	6.3
Morlane	6.2	7.7	6.2	7.7	5.5	7.0	7.2	6.5
Organdia	6.3	6.3	5.5	7.7	5.8	7.2	6.0	5.8
Supervolet	7.5	7.0	6.8	7.0	6.5	7.0	6.8	5.0
Morelli	3.8	6.3	5.8	7.3	5.3	7.5	3.3	6.2
Castore	3.8	4.2	4.8	7.3	4.5	7.0	5.7	4.0
Samantha	4.5	6.2	5.5	6.7	5.5	7.0	5.5	4.8
Teresa	6.5	4.5	6.7	7.3	7.5	7.2	6.8	4.8
Ducato	5.3	4.5	4.8	6.7	5.8	7.2	5.2	3.8
Amy	4.3	6.5	3.0	5.3	5.5	7.8	4.8	5.0
Tana	5.3	5.7	5.7	5.8	5.3	7.7	4.2	4.5
Bravo	6.2	6.3	4.5	6.2	6.2	7.8	3.8	3.7
French bean lines								
U.O.E#2	3.3	5.3	3.8	7.0	5.8	5.7	4.8	5.2
U.O.E#10	5.8	4.5	3.8	7.3	5.0	7.0	4.5	3.8
U.O.E #13	5.8	4.3	5.3	6.3	6.7	7.3	5.3	3.3
Landraces								
GBK032805	5.0	5.3	5.0	6.7	5.5	7.8	4.5	3.5
GBK032921	3.3	4.3	6.0	7.7	6.8	6.2	4.8	4.5
GBK032952	7.5	6.3	6.5	7.7	7.2	8.0	6.8	4.8
Checks								
GLPX- 92	7.3	6.7	5.5	7.3	6.7	7.5	6.8	5.5
Mexico 54	3.0	3.0	2.3	3.0	2.7	3.0	2.3	2.3
Mean	5.3dc	5.5c	4.9d	6.8a	5.8b	6.8a	5.2dc	4.5e
S.E	1.8	1.1	1.5	1.4	1.2	2.5	2.0	1.5
CV%	20.4	12.2	18.8	13.0	13.1	22.8	23.7	21.1

Means with the same letter indicate lack of significance

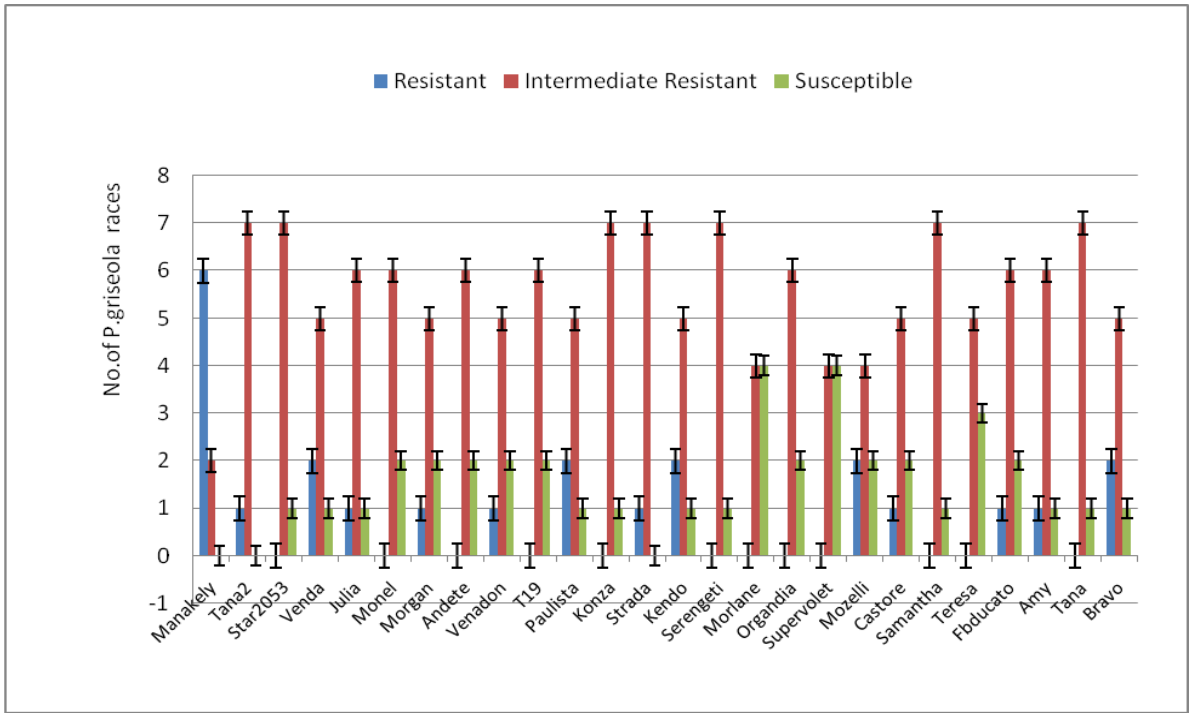

Figure 1: Reaction of twenty six French bean genotypes to eight races of *Pseudocercospora griseola*

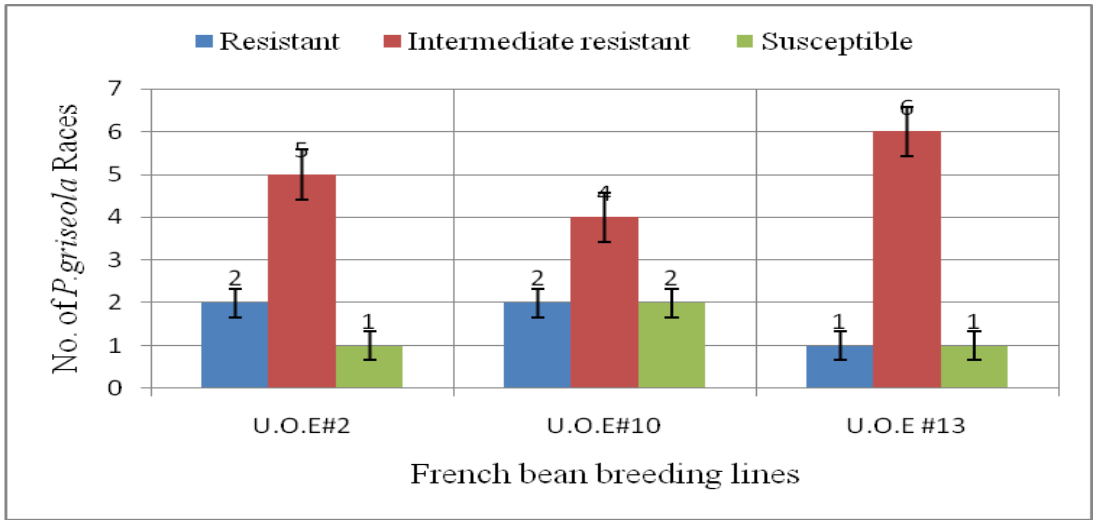

Figure 2 :Reaction of breeding lines to eight races of *Pseudocercospora griseola*.

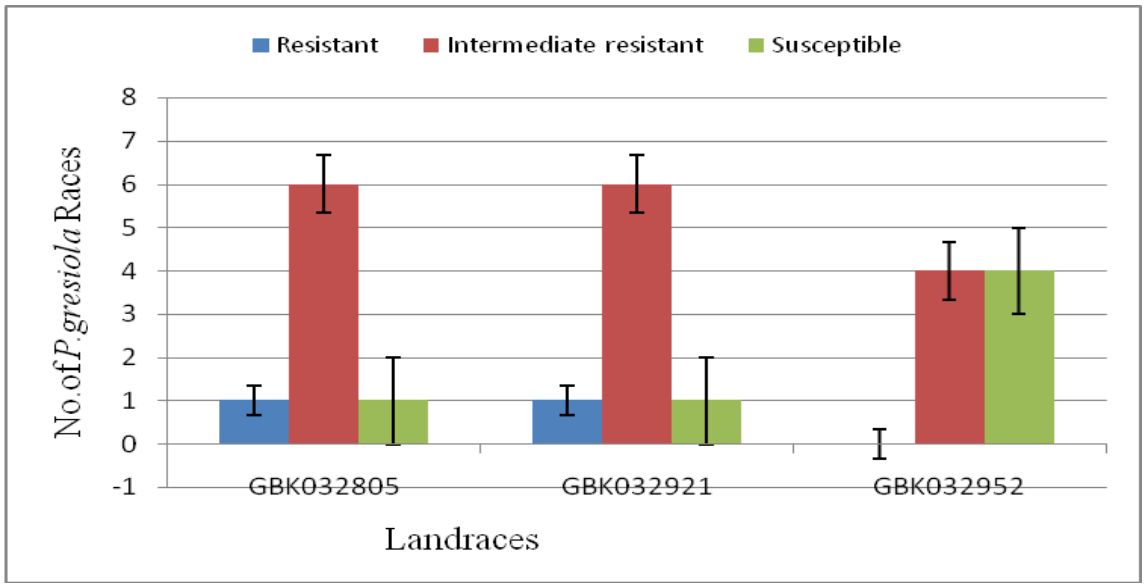

Figure 3: Reaction of landraces to eight races of *Pseudocercospora griseola*.

4.4 Marker Assisted Selection using STS G796

The frequency of phenotypic classes in the disease score for resistance and susceptibility reaction to the pathogen in F_1 and F_2 populations obtained from the cross between Amy with Mexico 54 and the segregation classes as per molecular marker STS G796 for *Phg-2* gene for ALS resistance are shown in Table 10 and plate 1. Segregation ratios were 3:1 (resistant: susceptible) for the F_2 populations in phenotypic evaluation and 1:2:1 in genotypic evaluation. In the F_1 populations, the scores were in the resistant class 20:0.

The F_2 populations, scores were distributed with 106 of plants being resistant and 38 being susceptible. The molecular analysis of the F_2 population showed that STS marker G796 is a co-dominant and is polymorphic between genotypes Mexico 54 and Amy as well as the differential cultivars and the French bean varieties evaluated (plate 2 , 3, and 4). The marker was able to distinguish 24 susceptible, 33 resistant and 43 heterozygotes in 100 F_2 plants although 3 plants that were considered susceptible presented two for

heterozygotes .The association between the marker and disease resistant scores showed that the STS was significantly (P = 0.00) associated with the disease score (R^2 = 0.75).

Plate 2: Interaction of ALS pathogen isolate with french bean genotypes showing susceptible and resistant

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

Parent/ Marker	Generation	No. of plants	Expected ratio [a]	Observed ratio	X^2	P(%)
Amy	Parent (s)	15	0(R):1(S)	0(R):15(S)	-	-
Mexico 54	Parent	15	1(R):0(S)	15(R):0(S)	-	-
Phg-2 gene	F1	20	1(R):0(S)	20(R):(S)	-	-
Phg-2 gene	F2	144	3(R):1(S)	106(R):38(S)	0.14	0.70
G796	F2	100	1(R):2(S+R):1(R)	33(R):43(S+R):24(S)	3.58	1.66

[a] Resistant plants (R),Susceptible plants (s) , resistant allele (R) and susceptible allele(S).

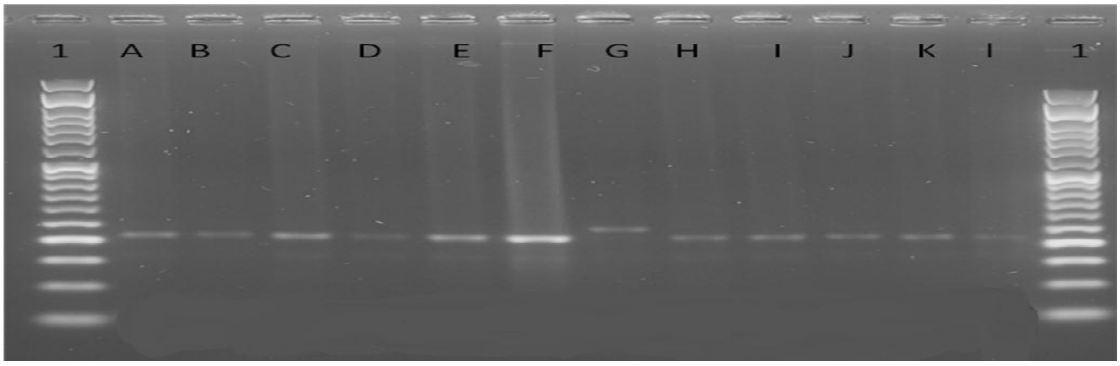

Plate 3: PCR amplification products of DNA between the two parents and differentials amplified with G796 STS primer showing polymorphism.

Lane1:100bpLadder,A:pan72,B:Dontimoteo,C:Flordemayo,D:Amedoin,E:BAT332,F:Amy,G:Mexico 54,H:Montcalm,I:Balon bayo,J:G11796,K:G5686,L:G2858

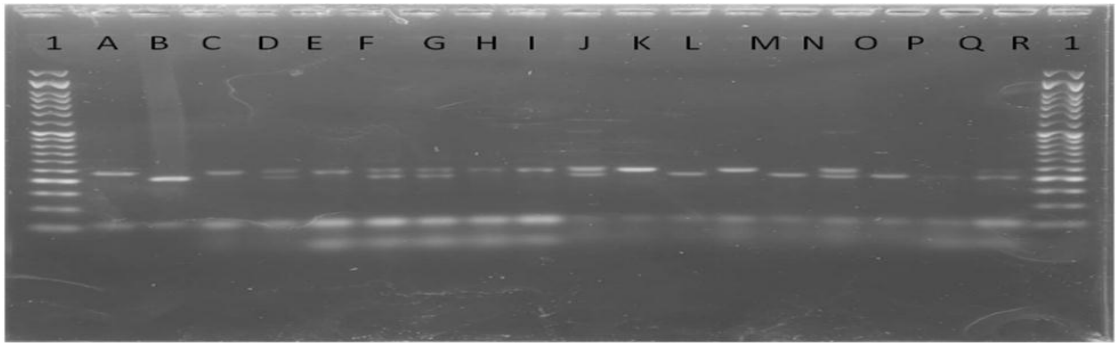

Plate 4: PCR amplification products of genomic DNA from Resistant(R), Heterozygotes (H) and susceptible(S) F2S French bean lines with their respective parents following amplification with STS G796 primer.

Lane**1**:100bpladder,A:Mexico54(**R**),B:Amy(**S**),C:A188(**R**),D:A186(**H**),E:A181(**R**),F:A172(**H**),G:A166(**H**),H:A160(**R**),I:A157(**R**),J:A155(**H**),K:A152(**R**),L:A147(**S**),M:A144(**R**),N:A143(**S**),O:A141(**H**),P:A139(**S**),Q:A125(**S**),R:A1219 (**H**).

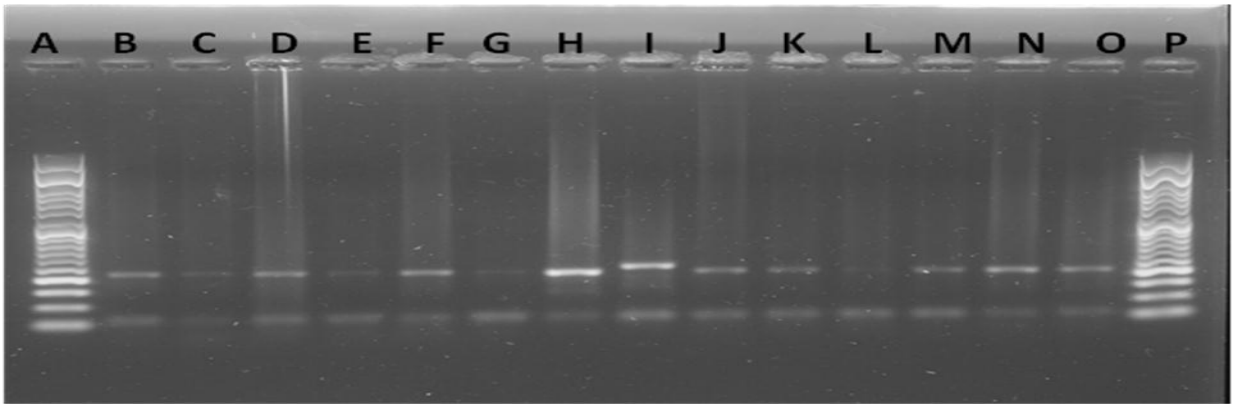

Plate 5: PCR amplification products of French bean germplasm and Mexico 54 genomic DNA amplified with STS G796 primer showing polymorphism.

A:100bpLadder,**B**:Ducato,**C**:GBK032805**,D**:Paulista,**E**:UOE#7,**F**:Konza,**G**:Organdia, **H**:Amy,**I**:Mexico 54, **J**:T19,**K**:Samantha, **L:**Mara, **M**:Bravo,**N** :Morelli **O:** Teresa **P:** 100bpLadder

CHAPTER FIVE

DISCUSSION

5.1 Reaction of French beans to ALS in Western Kenya

The study has described the severity of ALS in Kenyan-grown French beans. The lowest disease scores were recorded in Eldoret, which reflects the lower temperatures resulting from its high altitude, an environmental factor that is unfavourable for disease development. Previous studies have suggested that the ALS pathogen requires a relative humidity of 70-100% and a temperature range 18-22ºC to successfully infect and sporulate and temperatures below 16ºC have been documented to retard fungal development (Stenglein *et al*., 2003). At both Kakamega and Kisii, where the disease pressure was higher, some of the entries experienced premature leaf drop contributes to significant yield loss due to poor podding, lesions on pods and eventual shrivelling of the pods development this has greatly reduces marketability of these fresh produce both in the local markets and overseas. These two sites are among the most important bean growing regions in Kenya, since their bimodal rainfall and moderate temperatures allow for continuous cropping. As a result, the inoculum load is high and the disease is endemic. Genotype by environment effect indicated minimal variable rate of ALS infection on the genotypes except for Kakamega site which almost all genotypes experienced significant susceptibility.This highlights Kakamega as an ALS hotspot. Disease hotspot sites are useful for screening for field resistance to disease therefore any new breeding for ALS lines could be therefore tested in this sites. On this basis both Kakamega and Kisii, appear to be favourable sites for ALS screening. A similar recommendation applies to bean rust and similar studies on ALS (Arunga *et al.,* 2007,

Mwang'ombe *et al.*, 2007). The three commercial genotypes andate, Serengeti and Samantha that were moderately susceptible could be promoted for planting in the three sites.The fungal population is not significantly restricted by chemical intervention, because smallholder farmers tend to use little fungicide during common bean production. None of the French bean accessions tested exhibited full resistance, but some showed a moderate level of resistance against ALS; the moderate resistance to susceptibility presumably reflects a degree of selection pressure imposed by breeders for pod quality not focused to this biotic constraint.

5.2 Race typing of ALS Races in Western Kenya

Most fungal pathogens have been documented to affect beans have wide variability (Mahuku *et al*., 2009). This study also agrees that there is a high virulence variability of ALS causing pathogen *P. gresiola* in Uasin-gishu, Kakamega and Kisii. Based on the pathogenicity reaction of *P.gresiola* on 12 standard differentials, 8 races were identified from 36 isolates obtained from major bean growing regions of western Kenya. The 12 differential cultivars namely Don Timoteo, G11796, Boloom Bayo, Montcalm, Amendoin, G05686, Pan 72, G02858, Flor de Mayo, Mexico 54, BAT 332 and Cornell 49-242, used in the study enabled meaningful comparison of results of ALS isolates from the three experimental sites. This variation of races in western Kenya supports earlier findings by various authors (Pastor-Corrales *et al*., 1998; Mahuku *et al*., 2002a; Stenglein *et al*., 2003; Wagara *et al*., 2004; Silva *et al*., 2008) who indicated that a wide pathogenic variability of ALS exists whether isolates are collected from different geographical areas or from a given locality. Race 63-39 has been isolated in Machakos Kenya (Wagara *et al*., 2011) it has also been identified in Uganda, Rwanda, Malawi and Brazil

(Ddamulira *et al.*,2014) while race 30-55 has been identified in Embu, Kenya (Ngayu-wanjau.,2013). The common occurrence of the races at the locations of study exhibits an overlap of pathotypes. This findings are consistent with findings by David *et al*., 2000 who reported fungual pathogens are passed across localities of bean growing regions during seed exchange among the farmers or by suppliers from different parts of the country. In the current, study different races were characterized per experimental site . Similar observations on race variation have been reported even on single leaf samples (Mahuku *et al*., 2002).

5.3 Reaction of French beans genotypes to *P.griseola* races.

Based on data from the pathogenicity tests on French beans it was observed that genotypes exhibited resistance, intermediate resistance and susceptibility to races of *P. griseola* . This finding concurs with previous studies that there are landraces and other Phaseolus genotypes e.g. *P. coccineus* with independent genes or more alleles resistant to some races of angular leaf spot (Carvalho *et al.,* 1998; Mahuku *et al.,* 2003; Caixeta *et al*., 2005). Cultivars e.g. AND 277, Cornell 49-242, G10474 and MAR 2 are documented to have resistance conditioned by a single dominant gene to races: 63-23, 31-17, 63-63 and 63-39 (Nietzsche *et al*., 2000). Susceptibility in some of the thirty-two genotypes in this experiment to some races could be attributed to lack of resistant genes and alleles that can control the disease or subjection to new races of the eight races of the fungus that caused breakdown of their resistance. These results are consistent with those of Wagara *et al*., (2005) who reported that none of the fifty common bean genotypes she evaluated were resistant to all forty four ALS races. Appearance of new races of the fungus have been found to breakdown resistance in some bean cultivars while cultivars with only one

resistance gene or allele are found to control the disease for only a limited period of time (Pastor-Corrales *et al.,* 1998; Stenglein *et al.,* 2003). Ngayu-wanjau (2013) reported that incorporation of angular leafspot resistance has not been a major breeding goal in common beans and this could be same case with French bean genotypes. Resistance in Mexico 54 during this study concurred with previous studies conducted by CIAT that found Mexico 54 to be most resistant cultivar to ALS races in Africa (Mahuku *et al.,* 2003).

5.4 Phenotypic and molecular marker evaluation of ALS resistance

The disease symptoms were observed to be skewed on different ends among the parents while in Mexico 54, the score were in the resistant side and in Amy, on the susceptible side. The segregation results in F_2, supports 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. Chataika *et al*., (2010) and Tryphone *et al*., (2012) confirmed that resistant to ALS in Mexico 54 is conditioned by a single gene. The data obtained from the present study showed that the use of STS G796 is suitable for monitoring resistance of ALS since it can indicate differences in allele sizes and differentiate heterozygotes from homozygotes. This results indicates that the gene *Phg-2* for ALS resistance transferable and supports previous studies of ALS resistance using Mexico 54 as a donor parent. Other previous studies using SNO2 SCAR molecular markers reported the same observations in Mexico 54 (Mahuku *et a*l., 2004; Ferreira *et al*., 2000; Carvahlo *et al*., 1998; Larsen *et a*l., 2005). The STS G796 was developed from RFLP specifically in linkage group 8 and has a 24 base pair insertion in the allele of the ALS resistant parent Mexico 54 whereas none of the susceptible parents have the same sequence (Tamara *et*

al., 2015). This marker was reproducible and easy to score on agarose gel similar to the report of other STS (Olson *et al*., 1989). This type of resistance in plant is easily transferable by conventional plant breeding methods with marker-assisted breeding.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1. Conclusions

The study determined that all French bean genotypes screened for ALS resistance were susceptible to angular leaf pot under field conditions. Although promising intermediate genotypes across the test sites, i.e. Andate, Serengeti and Samantha, and outstanding resistant genotypes under controlled conditions e.g. manakelly ,venda, Morelli were identified as resistant to some ALS races.

Races 63-39, 63-3 were the most prevalent races observed among the ALS isolates western Kenya. Race 63-39 showing that it is the most predominant race in western Kenya was isolated in all the 3 sites while 63-3 was isolated in Kisii and Kakamega. Kakamega and Eldoret recorded the highest number of isolates. One new race, 31-23 was reported for the first time in Kenya. These findings provide new and important information on diversity and virulence of ALS on French beans in Kenya.

In this study STS G796 marker was found to be potentially valuable for use in marker assisted breeding and tracking the success of introgression of ALS resistance genes in French bean lines developed from crosses with Mexico 54. Though the selected molecular Marker played a role in resistance breeding, the role of suitable virulent pathotypes should not be overlooked.

6.2 Recommendations

1. Promising French bean genotypes e.g. andate, Serengeti and Samantha under field condition and Manakely in controlled condition identified in this study to possess varying levels of resistance should be promoted for inclusion in farmers' varietal mixtures to boost management of ALS.
2. Introgression of resistance genes in French beans genotypes should be broadened to include landraces which are genetically diverse*;* the diversity can be exploited positively to identify potential sources of resistance to angular leaf spot and incorporated in breeding of French beans.
3. The Kenyan population of *P.griseola* be regularly monitored for emergence of new pathotypes.
4. Linkage analysis of the STS G796 molecular marker and *Phg-2* gene for ALS resistance in the developed F_2 lines should be carried out.

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APPENDICES

Appendix I. ANOVA tables for Host Genotype Interactions

Source	df	ssq	Meansq	f value	pr>f
Model	35	91.52451	2.614986	2.86	<.0001
Error	66	60.29902	0.913622		
Corrected Total	101	151.8235			

r-ssq	cv	rootmse	Incident mean
0.602835	17.95492	0.955836	5.323529

Source	Df	Anovass	Meansq	Fvalue	Pr>F
Env	33	90.15686	6172.631	2.99	<.0001
Rep	2	1.367365	0.683824	0.75	<.4771

ANOVA table of ALS disease severity data from KARLO Kakamega, Kakamega County

Source	df	ssq	Meansq	f value	pr>f
Model	35	57.27206	1.636345	2.2	<.0029
Error	66	49.06372	0.74339		
Corrected total	101	106.3358			

	r-ssq	cv	rootmse	mean
	0.538596	13.83862	0.862201	6.230392

source	Df	anovass	meansq	fvalue	pr>f
VAR	33	53.50245	1.621864	2.18	0.0036
BLK	2	3.769608	1.884804	2.54	0.0869

ANOVA table of ALS disease severity data from Kisii County

Source	Df	Ssq	Meansq	F Value	Pr>F
Model	35	82.42892	2.355112	2.11	<.0045
Error	66	73.70098	101166815		
Corrected total	101	156.1299			

	r-ssq	cv	rootmse	mean
	0.527951	18.53596	1.056732	5.70098

Source	Df	Anovass	Meansq	Fvalue	Pr>F
env	33	62.6299	1.8978758	1.7	0.0339
BLK	2	19.79902	9.8995098	8.87	0.0004

ANOVA of ALS disease severity data from all experimental Sites

Source	df	ssq	Meansq	f value	pr>f
Model	35	191.4592	5.470261	5.57	<.0001
Error	270	265.1603	0.982093		
Correctedtotal	305	456.6242			

	r-ssq	cv	rootmse	mean
	0.419293	17.22999	0.991006	5.751634

Source	df	anovass	meansq	fvalue	pr>f
Env	33	149.1242	4.518915	4.6	<.0001
BLK	2	42.33497	21.16748	21.55	<.0001

ANOVA table of ALS disease severity data of race 31-23

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	147.8652	4.480764	3.88	<.0001
Error	68	78.5	1.154412		
Corrected Total	101	226.3652			

R-Square	Coeff Var	Rootmse	Mean1
0.653215	20.42729	1.074436	5.259804

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	147.8652	4.480764	3.88	<.0001
Race	0	0	0		

ANOVA table of ALS disease severity data of race 36-39 on French beans.

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	130.754902	3.9622698	8.98	<.0001
Error	68	30	0.4411765		
Corrected Total	101	160.754902			

R-Square	Coeff Var	Rootmse	Mean
0.653215	12.18517	0.664211	5.45098

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	130.754902	3.9622698	8.98	<.0001
Race	0	0	0		

ANOVA table of ALS disease severity data of race 24-48 on French bean

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	124.9534	3.786468	4.4	<.0001
Error	68	58.5	0.860294		
Correctedtotal	101	183.4534			

R-Square	Coeff Var	Rootmse	Mean
0.681118	18.75264	0.92752	4.946078

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	124.9534	3.786468	4.4	<.0001
Race	0	0	0		

ANOVA table of ALS disease severity data of race 36-39 on French bean

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	97.57843	2.956922	3.66	<.0001
Error	68	55	0.808824		
Corrected Total	101	152.5784			

R-Square	Coeff Var	Rootmse	Mean
0.63953	13.21806	0.899346	6.803922

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	97.57843	2.956922	3.66	<.0001
Race	0	0	0		

ANOVA table of ALS disease severity data of race 30-55 on French bean

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	93.71814	2.839944	4.83	<.0001
Error	68	40	0.588235		
CorrectedTotal	101	133.7181			

R-Square	Coeff Var	Rootmse	Mean
0.63953	13.24817	0.766965	5.789216

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	93.71814	2.839944	4.83	<.0001
Race	0	0	0		

ANOVA table of ALS disease severity data of race 63-23 on French bean

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	63.12745	1.912953	0.71	0.8634
Error	68	184.3333	2.710784		

Corrected Total	101	247.4608	

R-Square	Coeff Var	Rootmse	Mean
0.255101	23.45496	1.646446	7.01608

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	63.12745	1.912953	0.71	0.8634
Race	0	0	0		

ANOVA table of ALS disease severity data of race 30-23 on French bean

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	114.4804	3.4691028	2.19	0.0033
Error	68	107.8333	1.5857843		
CorrectedTotal	101	222.3137			

R-Square	Coeff Var	Rootmse	Mean
0.51495	24.09878	1.259279	5.22549

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	114.4804	3.4691028	2.19	0.0033
Race	0	0	0		

ANOVA table of ALS disease severity data of race 63-3 on French bean

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	33	99.02206	3.000668	3.34	<.0001
Error	68	61.16667	0.89951		
Corrected Total	101	160.1887			

R-Square	Coeff Var	Root mse	Mean
0.618159	20.96194	0.948425	4.52451

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	99.02206	3.000668	3.34	<.0001
Race	0	0	0		

ANOVA of ALS disease severity interaction on French bean

Source	Df	Ss	Meansq	Fvalue	Pr>F
Model	40	964.4902	24.11226	17.48	<.0001
Error	775	1068.755	1.379039		
Corrected Total	815	2033.245			

R-Square	CoeffVar	Rootmse	Mean
0.47436	20.86779	1.174325	5.627451

Source	Df	Anovass	Meansquare	Fvalue	Pr>F
Genotyp	33	418.0784	12.66903	9.19	<.0001
Race	7	546.4118	78.05882	56.6	<.0001

ANOVA table of genotypic data of STS G796 marker

Source	**df**	**Sum of Squares**	**Mean Square**	**F**	**Sig.**
Genotype	1	16.25	16.25	288.77	0.00
Error	98	5.52	0.56		
Total	99	21.76			

Appendix 2 : DNA Electrophoresis Buffers

EDTA 0.5M pH 8 at 25°C (1L)

- EDTA 186g
- NaOH 20g

Add distilled water, dissolve, adjust pH and adjust final volume to 2L

TBE 10X (2L) (Tris Boric acid EDTA)

- Tris 216g
- Boric acid 110g
- EDTA 0.5M pH 8 80ml
- Distilled water top to 2L
- TE (Tris –EDTA buffer)
- 1ml of Tris HCl 1M pH=8
- 200µl of EDTA 0.5 M pH=8, volume make to 100ml

dNTPs 5mM

- dATP 100mM 50µl
- dGTP 100mM 50µl
- dTTP 100mM 50µl
- dCTP 100mM 50µl

Added double distilled water to make 1000µl

Appendix 3

Isolation and culture of P.gresiola from infected an bean leaf.

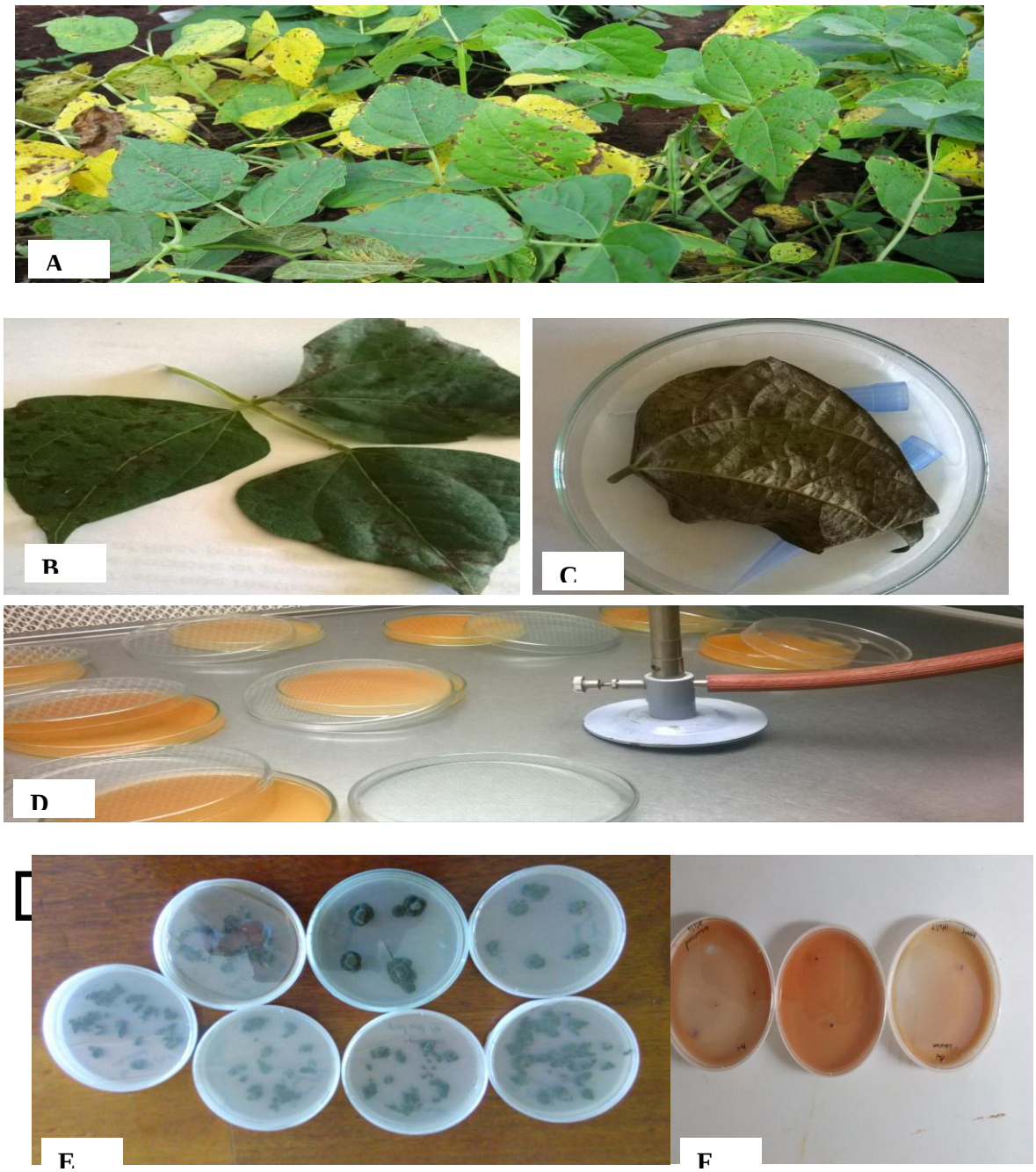

Plate 6: An ALS infected french bean ,B: isolated ALS infected leaves , C:Isolation of ALS from infected leaf,D;V8 media, E:ALS in PDA media,F:ALS in V8 media.Source : (Author, 2015)