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CHARACTERIZATION OF *PSEUDOCERCOSPORA GRISEOLA*, IDENTIFICATION OF LOCAL SOURCES OF RESISTANCE AND EFFECTIVENESS OF GENE PYRAMIDING IN CONTROLLING ANGULAR LEAF SPOT IN COMMON BEAN

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A THESIS SUBMITTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN PLANT BREEDING AND BIOTECHNOLOGY OF MAKERERE UNIVERSITY

MAY 2015

DECLARATION

The work presented in this Thesis has not been submitted to any other institution of learning for any an academic award.

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ACKNOWLEDGEMENTS

I am very grateful to:

The Regional Universities Forum for Capacity Building in Agriculture (RUFORUM), Carnegie Corporation, Borlaug Leadership Enhancement in Agriculture Program (LEAP) and the International Centre for Tropical Agriculture (CIAT) for funding this research.

My doctoral committee including supervisors: Drs. Mildred Ochwo-Ssemakula, Clare Mukankusi Mugisha, Richard Edema, Peter Sseruwagi, Denis Mpairwe and Mentors, Prof. Paul Gepts and Prof. Paul Gibson for the unreserved efforts towards getting this work accomplished.

The Director General, National Agricultural Research Organisation (NARO) and the Director, National Crops Resources Research Institute (NaCRRI) who recommended me for this scholarship. The laboratory staff of CIAT and the National Agricultural Research Laboratories (NARL), Kawanda for the assistance rendered to me during the execution of this research.

My family, particularly my wife Harriet and daughter Gabriella, who endured my absence during the course of carrying out this research. My sincere and heartfelt thanks go to my mother (Maria Nalwoga), brothers [Vincent (RIP), John Bosco, Edward and Joseph] and sisters (Sylvia, Gorreth, Harriet, Margaret and Maurice) for the moral, educational and spiritual support rendered to me in my long education journey ever since our father passed on.

My colleagues especially the pioneer PhD Class of Plant Breeding and Biotechnology at Makerere University, who continuously comforted and supported me especially during the hard moments that came along while conducting this research.

Almighty God, who has given me life despite the many friends that I have lost he has reserved me for a purpose.

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ABREVIATIONS

AFLP	Amplified Fragment Lengths Polymorphism
ALS	Angular Leaf Spot
AUDPC	Area Under Disease Progressive Curve
CBB	Common Bacterial Blight
CBW	Common bean weevil
CIAT	International Centre for Tropical Agricultural
BCMV	Bean Common Mosaic Virus
DNA	Deoxyribonucleic Acid
ERIC	Enterobacterial repetitive inergenic consensus
FAOSTAT	Food and Agriculture Organisation statistical database
FPC	Four parent Cross
GCA	General Combining Ability
GPS	Global Positioning System
ISSR	Inter-Simple Sequence Repeats
KAN	Kanyebwa
LSD	Least significance difference
MAAIF	Ministry of Agriculture Animal Industries and Fisheries
MAS	Marker Assisted Selection
MBW	Mexican bean weevil
MEX54	Mexico 54
MP	Mid Parent
NBP	National Beans Programme
PCR	Polymerase Chain Reaction
RAPDS	Random Amplified DNA Polymorphism
RAMS	Random amplified Microsatellites
RCBD	Randomised complete block design
REP	Repetitive extragenic Palindromic
ReML	Restricted maximum likelihood
SC	Single Cross
SCA	Specific Combining Ability
SED	Standard Error Difference
TC	Triple Cross
UBOS	Uganda Bureau of Statistics
UEPB	Uganda Export Promotions Board
UPGMA	Unweighted Pair Group Method Arithmetic mean

ABSTRACT

Common bean (*Phaseolus vulgaris L.*) is one of the most widely grown legume crops for food and income by small holder farmers in Uganda. Despite its importance, bean production is still constraint by angular leaf spot (ALS), which still cause losses both in quantity and quality of beans. This study was conducted to determine the distribution and variability of *Pseudocercopsora griseola*, identify new sources of resistance and elucidate the effectiveness of pyramided genes in enhancing ALS resistance.

To determine the distribution and variability of *P. griseola*, disease surveys, ALS disease differential bean cultivars and molecular markers were deployed. The results revealed the existence of ALS in all regions and districts surveyed and disease distribution varied depending on region, and altitude. Disease incidence and severity were highest in the north and lowest in south-western Uganda. In terms of districts, the highest disease incidence (68.5%) and severity (score of 7.35 on a 1-9 scale) were recorded in Dokolo while the lowest incidence (29%) and severity (score of 4.07 on a 1-9 scale) were observed in Kisoro. Both disease incidence (60%) and severity (4.95) were higher for bean fields located in low altitude areas than incidence (33.6%) and severity (score of 1.84 on a scale of 1-9) in fields found in high altitude areas. Among the Ugandan *P. griseola* isolates tested for variability, ALS disease differential bean cultivars defined 12 pathotypes and selected molecular markers defined 30 haplotypes which belonged to two major groups: middle-America and Andean group which were highly variable. Pathotypes 61:63 and 17:39 were the most virulent and prevalent respectively, while pathotype 5:55 was specific to high altitude areas. The research findings provided information needed to develop ALS control strategies in Uganda.

On the other hand, eighty Uganda bean landraces were evaluated for ALS resistance using four *P. griseola* pathotypes (1:6, 17:39, 21:39 and 61:63) under screenhouse conditions at Kawanda. The severity of ALS on the bean lines ranged between 4.5 and 40.5 (based on area under disease progressive curve values) being variable depending on the genotype and the pathotype Area under disease progressive curve (AUDPC) values were highest for plants inoculated with pathotype 61:63 compared to the rest of other pathotypes Results also showed that landrace U00297 was the only bean line resistant to all the four pathotypes with a disease severity values ranging between 7.6 and 13.5 on the AUDPC scale. Inheritance of ALS resistance in U00297 and its allelic relationship with other ALS known sources of resistance were also elucidated. Segregation ratios of F_2 populations revealed that U00297

resistance to pathotype 17:39 was conferred by a dominant gene, while digenic epistatic gene interactions were responsible for U00297 resistance to 61:63, 21:39 and 1:6. The allelism test indicated that the dominant gene in U00297 was independent of resistance genes harboured by AND277 and G5686. Hence, U00297 has a potential of being used as an independent source of ALS resistance in breeding programs if its resistance is validated under field conditions.

To study the effectiveness of pyramided genes in enhancing ALS resistance, genes from existing ALS resistance sources (Mexico 54, AND277 and G5686) were pyramided in a cascading scheme. The results showed that single crosses (SC) between resistant parents (R x R) best fitted for 15:1 and 61:3 ratios while the triple cross (TC) best fitted for 249:7 and 247:9 ratios, suggesting that SC segregated for two and three genes while TC segregated for four genes. The combined resistance genes in SC and TC crosses exhibited additive effects within the cross and slightly increased the level of resistance to 61:63 when all the resistance sources were combined. The four parent cross (FPC) exhibited the highest level of resistance to 61:63 compared to the original (Mexico 54, AND277 and G5686) resistance sources. The TC lines with combined resistance were more effective than the individual sources for transferring resistance to susceptible commercial cultivars of major importance in Uganda. Hence, pyramiding was successful in accumulating four ALS resistant genes into the susceptible parents indicating significant progress in breeding for ALS resistance.

PUBLICATIONS FROM THIS STUDY

The work under this Thesis has been published in two peer reviewed Journals and one manuscript accepted as shown below:

1. Ddamulira, G., Mukankusi, C., Ochwo-Ssemakula, M., Edema R., Sseruwagi, P., & Gepts; P (2014). Distribution and Variability of *Pseudocercospora griseola* in Uganda. *Journal of Agricultural Sciences;* 6(6): 1916-9752

2. . Ddamulira, G., Mukankusi, C., Ochwo-Ssemakula, M., Edema R., Sseruwagi P., and Gepts, P .,(2014). Identification of new sources of resistance to angular leaf spot among Uganda common bean landraces. *Canadian Journal of Plant Breeding* 2 (2): 55-65.

3. Ddamulira,G., Mukankusi, C., Ochwo-Ssemakula, M., Edema, R., Sseruwagi P., and Gepts, P (2015). Developing resistance to angular leaf spot through gene pyramiding in common bean *American Journal of experimental Agriculture*; accepted in April 2015

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

1.1.1 Importance and uses of common bean

Common bean (*Phaseolus vulgaris L.)* is the most grown and consumed legume crop globally (Bucheyeki & Mmbaga, 2013). The crop is extensively grown, consumed, and traded in Asia, Africa, Latin America and the Caribbean (Food and Agriculture Organisation [FAO], 2013). Among bean growing regions of the world, Asia ranks first in bean production (10.2 million MT) followed by the Americas (7.1 million MT) while Africa ranks third (4.9 million MT) with most bean growing concentrated in the Eastern and Southern parts of the continent (FAOSTAT, 2013). In these areas, beans provides up to 25% of the total calorie intake (Cortés et al., 2013). In Uganda, Rwanda, Burundi and the Democratic Republic of Congo, the crop is an important source of protein for the poor, supplementing, vitamins and dietary fibre which are important in human nutrition. The leaves are a good source of iron and zinc especially when consumed fresh (Tryphone & Nchimbi, 2010). In addition, the residues from the crop (haulms, stalks and threshed pods) are used as animal feed or fuel for cooking in Africa and Asia (Bruchara, 2006).

In smallholder farming systems, beans amend soil fertility through fixing biological nitrogen. The crop fixes up to 50 Kg N ha^{-1} into the soil thus, enhancing soil nutrient levels (Kabahuma, 2013). Because of the nitrogen fixation nature, common beans are often intercropped with cereals to provide nitrogen in low input farming systems (Legesse et al., 2006). Bean residues are also used as mulch/ or manure in fields to boost soil nutrients and moisture retention, the two practices are suitable for improving smallholder productivity in Uganda.

Economically, beans are important source of income for farmers and traders in Uganda. The crop is mostly grown by women for home consumption but 20% is exported to neighbouring countries (Kilimo Trust, 2012). According to Uganda Export Promotions Board [UEPB], 2010, the crop is ranked fifth behind banana, cassava, indigenous cattle meat and cattle milk in terms of output value (Uganda Bureau of Statistics [UBOS], 2010). Consequently, beans

have the potential to sustain livelihoods of smallholder farmers and also contribute to the national export base (UBOS, 2010).

1.1.2 Production and limitations to productivity of common bean

Annual global bean production was estimated at 22.8 million metric tons (MT), with Myanmar as the leading global producer in 2013 (FAOSTAT, 2013). Africa produced 4.9 million MT of which 75% was from the east African region. Within East Africa, Uganda (419,000 MT) was fourth to Tanzania (967,100 MT) Rwanda (912,900 MT) and Kenya (513,600 MT) in bean production (FAOSTAT, 2013). In the last 15 years, area under bean production in Uganda increased from 669, 000 to 1,100,000 Ha (FAOSTAT, 2013), but production per unit area decreased from 599.4 to 419 kg ha^{-1} during the same period (FAOSTAT, 2013).

On average, common bean yield on smallholder farms has remained below 0.5 t ha^{-1} while potential yield for promising varieties is estimated at 1.5 ha^{-1} (FAOSTAT, 2013). This is due to biotic and abiotic factors that have continued to present major constraints to increased bean production and high yields in Uganda (Beaver & Osomo, 2009). The major abiotic factors include; low soil fertility and acidity (Beebe et al., 2012) arising from soil erosion and degradation (Sanginga & Woolmer, 2009), drought especially in endemic areas of northern Uganda (Yadav et al., 2011). In addition, crop and market related constraints such as limit access to improved seed and lack of market information also continue to constraint bean production in Uganda (Katungi et al., 2010). On the other hand, large bean yield losses are experienced due to biotic factors; pests and diseases. The major bean pests include; bean stem maggot (*Ophiomyia phaseoli*), common bean bruchids (*Acanthoscelides obtectus* and *Zabrotes subfasciatus*), pod borer (*Helicoverpa amigera*), while angular leaf spot (*Pseudocercospora griseola*), anthracnose (*Colletotrichum lindemuthianum*), root rots (*Pythium* spp, *Fusarium solani f. sp. phaseoli*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Macrophomina phaseolina*), common bean mosaic virus and Common Bacterial Blight (CBB): *Xanthomonas phaseoli* , bean common mosaic virus (BCMV)/bean common mosaic necrosis virus (BCMNV) disease are among major diseases of common bean in Uganda (Nkalubo, 2006; Mukankusi et al., 2008).

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1.1.3 Statement of the research problem

Angular leaf spot (ALS) caused by *Pseudocercospora griseola* is considered as one of the most important disease of common bean in Eastern and Southern Africa, the Great lakes region and in Ethiopia (Pastor-Corrales et al., 1998; Strenglein et al., 2003). The disease is favoured by alternating cold and warm temperature; and dry and humid air (Celetti et al., 2006), because such conditions favour disease development stages which include; incubation, extension, foliage defoliation, sporulation and spore dispersal (Monda et al., 2001). *Pseudocercospora griseola* incubation often occurs under a wide range of temperatures (5–33 ^{0}C) (Sindhan and Bose, 1980) but spore germination which follow the incubation period is often delayed at low temperatures (Buruchara, 1983; Bassanezi et al., 1998). Maximum lesion extension is frequently observed at 15 ^{0}C (Verma and Sharma 1984), while sporulation process which depend on temperature and relative humidity, does not occur if relative humidity in air is below 71% (Sindhan & Bose, 1980). The spores that are responsible for pathogen spread once released in the dry atmosphere are readily disseminated by wind (Vieira, 1994),.Under favourable environmental conditions, *P. griseola* causes severe disease symptoms such as leaf premature defoliation, shrivelled pods, shrunken seeds and reduced yield and grain quality (Celetti et al., 2006). In Uganda, ALS is associated with yield losses of up to 50% among released bean varieties (Opio et al., 2001; Namayanja et al., 2006). Elsewhere, yield losses of up to 80% have been reported (Schwartz et al., 1981; Stenglein et al., 2003).

Following the revival of bean research and development activities by the National Beans Programme (NBP) and International Centre for Tropical Agriculture (CIAT) in 1985/1986 after the civil wars from 1979 to 1985, breeding efforts focused mainly on diseases such as ALS, root rots and anthracnose which were the three major diseases infecting the crop in Uganda (Cardon-alvarez and walker, 1956; Opio, 2001; Namayanja et al., 2006; Nkalubo, 2006; Mukankusi et al., 2008). Namayanja et al. (2006) studied the inheritance of ALS in MEX54 and BAT332 using local susceptible checks Kanyebwa, K131 and K132. Like earlier authors (Nietsche et al., 2000; Mahuku et al., 2002a), results suggested that resistance to pathotype 63:39 was conditioned by a single dominant gene in each of the three susceptible backgrounds. The results further revealed that inheritance of ALS was dependent on the genetic background of parents used in the cross. Hence the need to ascertain the mode of inheritance for every new identified sources of resistance before used as parents in breeding programs. Out of this work scar marker OPEO4 was validated and found suitable for use in

Marker-Assisted Selection (MAS) of our local populations. Because of the complex nature of ALS inheritance, this marker has been earmarked for marker assisted selection in Ugandan breeding bean materials.

Despite the contribution from previous research towards developing ALS resistant varieties in Uganda, the breeding process is still constraint by; i) Variability of the casual pathogen (Mahuku et al., 2009), 2) limited information on resistance levels among landraces and failure to exploit already existing ALS resistant sources (Namayanja et al., 2006), 3) Use of home-saved seed and seeds purchased from informal markets by farmers (Mwaniki, 2002) and, iv) poor agronomic practices. Variability of *P. griseola* and limited information on resistance sources limited the process of breeding for ALS resistance while the use of uncertified seed and poor agronomic practices continue to encourage inoculum build up, further rising disease infection levels in bean growing areas. The first two factors limit the process of breeding for ALS resistance and the last two encourage inoculum build up, hence contributing to the existing high occurrence of ALS in bean growing areas. There was therefore, a need for the development of an effective ALS management strategy with a strong component of resistant varieties.

1.2 JUSTIFICATION OF THE STUDY

The process of developing ALS resistant bean varieties requires precise and accurate information on pathogen distribution and variability, resistance levels in locally adapted germplasm and possible existing known sources of resistance. This information forms a basis for developing resistant varieties for major growing areas (Stenglein et al., 2003).

Aggarwal (2004) found that bean line CAL 143 was resistant to ALS in South Africa, Tanzania, and Zambia but not in Uganda. The same author also indicated that the *P. griseola* race which rendered CAL 143 susceptible in Uganda was unique and not found in the three other countries. This suggested the uniqueness of Ugandan *P. griseola* races compared to those in the other three countries. Despite the uniqueness of the Ugandan *P. griseola* its distribution and variability remains unknown, which limit the development of ALS resistant varieties (Mahuku et al., 2002). A better understanding of the variability among populations of *P.griseola* in Uganda was, therefore required in order to facilitate this process.

Previous studies attribute the variability of *P. griseola* to co-evolution with its host which resulted in variation within gene pools (Gepts et al., 1988). Andean bean varieties are the most preferred types in Uganda (Mutetika, 1997) due to their large seed size characteristic and are continually grown with the majority being landraces. It is thus not surprising that bean varieties belonging to Andean gene pool are more susceptible to a wide range of races compared to those from the Mesoamerican gene pool (Sandlin et al., 1999; Araya et al., 2000; Mahuku et al., 2002) and hence continue to escalate the occurrence of ALS. Nevertheless, landraces maintained by farmers have for a long time been known to have useful agronomic traits, indeed most existing resistant sources developed elsewhere, have been derived from landraces (Busogoro et al., 1999). Unlike improved varieties, landraces are endowed with great genetic variability as they have not been subjected to selection over a long period of time making them an important resource of useful traits. For example, G5686, which is a good source of ALS resistance and a member of the ALS differential set, is a landrace that originated from Ecuador (Mahuku et al., 2009). Still, the level of variability of *P. griseola* often compromises the use of ALS resistance derived from landraces (Nietsche et al., 2001) due to continuous emergence of new races, which break down disease resistance (Young et al., 1998). As a result, continuous screening of germplasm is essential to identify new sources of resistance that can regularly be introgressed into commercial varieties (Young & Kelly, 1996). These varieties will counteract the new emerging races and reinforce resistance among existing ALS resistance sources.

Nevertheless, identifying new sources of resistance alone does not offer a lasting solution. A thorough understanding of bean genotype reactions to *P. griseola* races that occur in Uganda is required to facilitate the process of identifying new sources of resistance that are relevant to the Ugandan situation. Unfortunately, such information is not known. In addition, because the mode of inheritance of ALS resistance is dependent on the genetic background used (Namayanja et al., 2006), a clear understanding of resistance inheritance in new sources of resistance is a prerequisite if such sources are to be used as parents in developing resistant lines.

In several studies, bean germplasm was screened for resistance to ALS and resistant lines such as; MEX54, AND277 and G5686 were identified (Mahuku et al., 2003; Aggarwal et al., 2004; Mahuku et al., 2009). Inheritance studies on these resistance lines showed that resistance is controlled by a single dominant gene. MEX 54 carries resistance gene *Phg-2*

which confers resistance to race 63:39 (Namayanja et al., 2006), AND277 carries *Phg-1*, that confers resistance to eight races; 31-17, 31-39, 61-31, 63-19, 63-23, 63-31, 63-35, 61-41 (Caixeta et al., 2005), and G5686 carry gene Phg_{G5686A} that confer resistance to 31-0 races (Mahuku et al., 2009). The knowledge of the exisiting races is hence very important in determining the breeding strategy to be adopted to provide effective and durable reistance. The use of these resistance sources in Uganda is hindered by their low adaptability and undesirable traits. This is because all the three resistance sources were developed from places in the Americas under different environmental conditions with probably different races compared to Uganda. MEX54 and AND277 are medium to small-seeded, an attribute which is disliked by farmers in Uganda and Africa at large (Beebe, et al., 1981).

Preliminary information from surveys of African common bean germplasm suggests that "native" genetic diversity could also provide sources of disease resistance (Kiryowa et al., 2013; Mukalazi, 2004; Mukankusi et al., 2011; Tusiime, 2003) and is consistent with experience in other crops like maize (Burke et al., 2009) and cassava (Abaca et al., 2013), which, like common bean, are not indigenous to Africa. The genes in these resistance sources would hence need to be introgressed into the locally adapted and proffered but susceptible released bean culivars.

On the other hand, the existence of several physiological races of *P. griseola* that evolve from time to time to generate new strains undermines the use of single genes carried by individual resistance sources to effectively control ALS. Pyramiding as a strategy which uses combined gene action is required to offer multiple and durable resistance (Castro et al., 2003). The pyramiding process combines more than two resistance genes in a new cultivar to sustainably enhance its resistance against diseases. But based only on phenotypic evaluation data it is not feasible to track the accumulation of resistance genes in the new line. The use of molecular markers provides a better tool to overcome this problem (Fischer et al., 2004). Therefore marker assisted selection, therefore, becomes useful in combining several genes to accomplish gene pyramiding.

1.3 OBJECTIVES THE STUDY

The aim of this study was to generate information required for development of a management strategy for angular leaf spot of common bean in Uganda. The specific objectives of the study were to:

1. Determine the distribution and variability of *P. griseola* in major bean growing areas of Uganda
2. Identify new sources of ALS resistance among Ugandan bean landraces
3. Determine the effectiveness of pyramided genes in improving levels of resistance to ALS in susceptible bean cultivars.

By understanding the distribution of *P. griseola* it was hoped that steps would be made towards developing breeding materials that responded to the exisiting *P. griseola* races. In addition, studying ALS resistance gene inheritance and pyramiding patterns would be useful in identifying the approporiate selection scheme that would result in improved cultivars with durable resistance to the disease in the right background for easy adoption by bean farmers and consumers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of Common bean

Common bean (*Phaseolus vulgaris L.*) belongs to the genus *Phaseolus*, family *Leguminosae*, sub-family *Papilionoideae* and order *Leguminales*. The crop is widely distributed throughout America, Caribbean, Asia and Africa with over 50 wild growing species. Of the 50 wild species only five are domesticated and include; common bean (*P. vulgaris* L.), runner bean (*P. coccineus L.*), Lima bean (*P. lunatus*), tepary bean (*P. acutifolius A.* Gray) and the year bean (*P. polyanthus* Greenman) (Debouck, 2000). Among the five domesticated species the most adapted and globally cultivated bean type belong to *Phaseolus* spp (Singh, 2001). Most species of *Phaseolus spp* including common bean are diploid with 22 chromosomes (2n=2x=22), though a few cases of aneuploid reduction to 20 chromosomes have been reported in some species (Gepts, 2001).

Common bean bears complete papilionaceous flowers with colours ranging from white to pink and purple and the crop is self-pollinating. The flower structure facilitates self-pollination in that; it carries 10 stamens, with a long ovary, coiled style complimented with a hairy stigma. The stigma is laterally positioned in the inner arc of curved style where it intercepts pollen from its own anthers. Although the crop is less than one percent out-crossing, the crop exhibit considerable variation in growth habit, vegetative growth, flower colour and shape as well as pod and seed colour (Purseglove, 1968).

2.2 Origin, domestication and diversity of common bean

Common bean is believed to have originated from Mexico where it expanded to South America (Bitocchi et al., 2012). It was through domestication that beans reached to other areas such as Peru, Ecaudo and Bolivia which are currently considered as secondary centres of diversity. The crop was introduced into Africa by Portuguese traders in the 16th century and with time it was successfully established in the Great Lakes Region (Trutmann, 1996). The evolutionary history of beans indicate that domestication started in the 7th century, but, even before then two major gene pools of *P.vulgaris* (Mesoamerican and Andean) with partial reproductive isolation existed within distinctive centres of origin (CIAT,1995). It is

anticipated that the domestication process did not interrupt the gene pools because the two gene pools are still evident both in wild and domesticated forms (Angioi et al., 2009).

Still, the genetic diversity in common bean is supported by evidence from several studies based on agronomic traits, morphology and adaptation traits, phaseolin type, isozymes and molecular markers which have indicated the existence of two independent domestication incident (Gepts,1998; Singh, 1994). Based on adaptation Andean type are wild landraces that are kidney shaped whose origin is Andes, while Mesoamerican type are wild landraces and pink seeded cultivars which are rooted from Mexico, central America and Colombia (Gepts, 1986). Morphological studies also recognises the Mesoamerican cultivars as small to medium-seeded (<25 g or 25–40 g/100 seed weight) with S phaseolin type while the Andean are large seeded (>40 g/100 seed weight) (Evans, 1980).

The domesticated/cultivated Mesoamerican and Andean cultivars have been divided into six races: (Durango and Jaliso) (Mesoamerica), Chile, Nueva, Granada and Peru (Andean) (Singh et al., 1991a). However, in Africa nine market class types are grown, the majority of which include; red (large and small) and Calima (Rosecoco or red mottled) types. These beans have high market demand and hence constitute 50% of beans produced on the continent. Others are the navy beans, cream-coloured, brown tan, yellow types, purples, white and black beans (Buruchara, 2006). Similarly, a group of Guatemalan climbing bean accessions which does not group with other members of the Mesoamerican races indicate additional diversity within the group (Beebe et al., 2000).

2.3 Common bean production constraints in Africa

In the last decade area under bean production increased amidst reduction in yield per unit area (FAOSTAT, 2013). This has been attributed to both biotic and a biotic factors (Beaver & Osomo, 2009). Among the abiotic factors low soil fertility resulting from low nitrogen and phosphorus availability, low levels of exchangeable bases and soil acidity (Beebe et al., 2012). Sixty percent of the area under bean cultivation in Africa is deficient in Nitrogen (N) and Phosphorus (P). Ideally, beans are meant to be grown in soils with pH between 5.0 and 6.0 but 23% of beans are grown in soils with pH below 5.0, which compromises yield and nitrogen fixation potential of beans. Similarly, soil physical challenges such as degradation, which is common phenomena in East Africa and Uganda at large, also limit bean yield (Shangiga & Woolmer, 2009). The soil degradation comes with soil erosion and loss of soil

organic matter, which is associated with reduced soil nutrient availability and low water holding capacity leading to decline in soil structure. The low soil fertility related factors mentioned above are commonly observed on most of the farmers' fields in Uganda which possibly affect bean production and productivity.

Drought is another abiotic constraint that affects 60% of bean production in northern Uganda, eastern Kenya and Tanzania where it's endemic. This constraint is likely to continue affecting bean production in Uganda and the neighbouring countries due to change in climatic patterns (Yadav et al., 2011). Climate change is associated with high temperature in lowland areas and low temperatures (below 15^{O}C) in highlands which reduce on pollen fertility and pollination (Porch & Jahn, 2001). In drier parts of Uganda and eastern Africa extreme drought sometimes leads to complete bean crop failure and food insecurity. Similarly, excessive rainfall induced by El Nino phenomena has been held responsible for exacerbating the problem of fungal pathogens, especially soil borne and foliar diseases such as angular leaf spot and anthracnose (Beebe et al., 2011). In addition, excess rainfall and medium to high temperature increase the incidence of angular leaf spot at elevations between 50 and 1400 masl. On the other hand, heat variations due to nocturnal and diurnal temperatures also limit bean production (Yadav et al., 2011). Regions where bean is currently cultivated at the margins of its temperature suffer significant losses due to higher temperatures. Such areas include; lowland Central America and central Brazil, West Africa in general, northern Uganda, and Southern Democratic Republic of the Congo (Beebe et al., 2013).

Insect pests and diseases are also among the biotic stresses which account for reduced yield and market value of beans in Uganda. Major insect pests that attack beans include; bean fly (*Ophiomyia phaseoli, O.spencerella)*, black aphids (*Aphis fabae*), and the common whitefly (*Bemisa tabaci)* which vector the Bean common mosaic virus (BCMV) and golden mosaic causal agent). In addition, insect pests such as flower thrips (*Megalurothrips sjostedti*; Thysanoptera: Thripidae, pod borer (*Maruca testularis*; lepidoptera: Pyralidae) and pod-sucking bugs (*Clavigralla* sp) are very destructive to beans (Byabagambi et al., 1999). Furthermore, storage pests such as bean bruchids are widely distributed in most of bean growing areas. Two species: *Zabrotes subfasciatus* [Mexican bean weevil (MBW)] and *Acanthoscelides obtectus* [Common bean weevil (CBW)] are known. The later is more common in high altitude or cool environments, while former is common in warmer environments. In eastern Africa and Uganda at large CBW is more frequently encountered

(Buruchara et al., 2010). The larvae of both species bore into bean seeds to feed and develop, leaving them perforated with holes. Such seeds lose viability and are unfit for planting or human consumption.

Similarly fungal, bacterial and viral diseases which affect beans also constraint bean production. The major diseases include; angular leaf spot, root rot caused by complex of soil pathogens, particularly Pythium, Fusarium and Rhizoctonia species (Buruchara et al., 1999), anthracnose (*Colletotricum lindemuthiunum*), BCMV, Ascochyta blight (*Ascochyta phaseolorum*) and halo blight (*Psuedomonas syringae pv. phaseoli*). Though some of these diseases such as rot roots are common in highland areas but Ascochyta blight and halo blights are localized in warm-moist and cool-moist environments. But angular leaf spot is widely distributed in bean growing areas of Uganda (Opio et al., 2001), a fact which complicates its management when compared to other diseases.

2.4 Angular leaf spot

2.4.1 Taxonomy, etiology and occurrence

Angular leaf spot (ALS) caused by *Pseudocercospora griseola* (Sacc.) Ferr is considered among the most devastating fungal diseases of common bean in the tropics and subtropics. Its causal pathogen *P.griseola* is an imperfect fungus which belongs to class Dothideomycetes, order capnodiales, family mycosphaerellaceae and genius *Pseudocercospora* (Crous et al., 2006). The disease is more prevalent in tropics due to intermittent cool and warm, wet and dry weather (Celetti et al., 2006), which accelerate disease development (Inglis & Hegedom, 1986). The disease has been reported to cause yield loss of up to 80% (Schwartz et al., 1981), although up to 50% yield loss has been reported in landraces and released varieties (Opio et al., 2001; Namayanja et al., 2006).

2.4.2 Variability of *Pseudocercospora griseola*

Pseudocercospora griseola has a high degree of pathogenic variability (Mahuku et al., 2009). The genetic diversity within its populations has been described using a combination of differential cultivars and molecular markers (Sartorato, 2002; Sebstian et al., 2006). Based on these techniques, two major groups have been identified: Andean and Middle American. This grouping suggests that the evolutionary pattern of the pathogen to be similar to that of its host (Gepts, 1988). The Andean *P. griseola* group is more pathogenic to large seeded bean type while the Middle American group attacks both small and large seeded bean types (Pastor-

Corrales, 1998). In addition, the Middle American group has a much wide virulence range when compared to the Andean group. In Africa, some Andean isolates attack both Andean and Mesoamerican beans and these have been categorised as Afro-Andean (Mahuku et al., 2002) although molecular markers cannot distinguish this group with the Andean type (Wagara et al., 2004).

Although no forms of sexual reproduction has been reported in *P.griseola (*Stenglein et al., 2003*)*, several studies have demonstrated variability among and within populations in different geographical locations. Studies in Argentina identified 13 pathotypes from 45 isolates screened (Sebastian et al., 2006). Similar studies by Busogoro *et al*. (1999); Nietsche et al. (2001) and Mahuku et al. (2002) demonstrated pathogenic variability of 53, 13 and 50 different pathotypes from 54, 30 and 112 isolates obtained from Africa, Brazil and Central America respectively. The high variability exhibited by this pathogen requires that regular monitoring and identification of new sources of resistance be done to sustainably control the disease. For this to be possible a clear understanding of the genetic structure of the pathogen is necessary. Although, the genetic structure of *P. griseola* existing in other countries is known, in Uganda little or no information on the genetic structure is available which limit the development of ALS resistance varieties.

2.4.3 Symptoms and epidemiology of *Pseudocercospora griseola*

Bean plants affected by *P.griseola* are characterised by necrotic angular-like spots on the leaf surface, shrivelling of pods and shrunken of seeds (Schwartz et al., 1982). The epidemic of *P. griseola* is often observed late in the crop cycle (Allen et al., 1998) and entails lesion establishment, extension, foliage defoliation, and sporulation and spore dispersal. Lesion establishment involves germination of *P. griseola spores* on the leaf surface under moist conditions (Monda et al., 2001). Germination occurs under a wide range of temperatures (5–33 ^{0}C), with the optimal range being 18–28 ^{0}C (Sindhan & Bose, 1980). Spore germination is followed by an incubation period, which is reported to be delayed at low temperatures (Buruchara, 1983; Bassanezi et al., 1998), and varies from 12 to 15 days, 9 days, and 10 days at 16 ^{o}C, 24 ^{o}C, and 28 ^{o}C (Cardona-Alvarez and Walker, 1956), respectively. Generally, the incubation period ranges between 10 to 23 days (Correa-Victoria, 1987). Primary inoculum initiates the process whereby healthy sites are infected and latent sites are produced through infection. The latent sites may become infectious after a latency period, which may coincide with the rate of lesion appearance.

Lesion extension is influenced by temperature and host genotype. Verma and Sharma (1984) reported that maximum lesion size is observed at 15 ^{0}C. However, field observations indicate that larger lesions are measured during cooler (18–22 ^{0}C) compared to warmer (28–32 ^{0}C) periods. The largest lesion sizes (about 14 mm^2) and maximal rate of lesion extension (0.34 mm^2 days) are often observed at 24 ^{0}C (Bassanezi et al., 1998). For most isolates, lesion sizes range from 3.8– 20.9 mm^2 and strongly depend on the cultivar (Diaz et al., 1965**;** Correa-Victoria, 1987). A typical isolate produces circular lesions of 10 mm diameter within 6 days after inoculation, with maximum diameters up to 20 mm (Hocking, 1967).

Sporulation of *P. griseola* is also influenced by factors such as temperature, relative humidity and cultivar. The range of temperature where sporulation occurs is very wide (10–30 ^{o}C) (Sindhan & Bose, 1980). The relative humidity of the air is a very strong limiting factor of sporulation, which does not occur below 71% (Sindhan and Bose, 1980). In addition, an early report by Cardona-Alvarez and Walker (1956) indicated that a 24 hr period was required to complete the formation of coremia, and that an additional humid 48 hr period or more was necessary for spore production. Cultivar isolate interactions have been reported to occur, with large effects on sporulation density (5–800 spores mm^2) of lesion area) (Correa-Victoria, 1987).Very little quantitative information is available on spore liberation and dispersal in *P. griseola*. Both rain and wind appear to liberate and disperse spores *of P. griseola*, and wind-blown particles from infested soil, wind-blown spores and rain droplet-borne spores are all effective agents of dissemination according to Cardona-Alvarez and Walker (1956). The pathogen can survive on infected bean residue left on the soil surface, though the pathogen does not survive very long when the infected bean residue is buried and decomposed. The pathogen can also survive the harsh condition by encrusting itself to avoid desiccation and regenerating when conditions become conducive. Similarly, the pathogen can survive between seasons on infested seed, which is one pathway of introduction into the field in the proceeding season.

Figure 1: Symptoms angular leaf spot on different parts of common bean plant; A. on leaves , B. on stems, C.on pod

2.4.4 Factors influencing disease occurrence and severity

Angular leaf spot of common bean occurs in most beans growing areas globally. Occurrence and severity of the disease is governed by a number of physical factors and crop management practices. Some of the physical factors include; temperature, altitude and relative humidity. High ALS severity and occurrence is often experienced under conditions of warm temperatures (24^0C) but it also occurs within a temperature range of 16-28^0C if accompanied by high relative humidity (95-100%) alternating with dry windy conditions (Celleti et al., 2006). But, with temperature conditions below 16 ^{0}C low disease pressure is experienced due to reduced rate of pathogen sporulation which eventually leads to reduced disease development (Inglis and Hegedom, 1988). The occurrence and severity is also known to be influenced by altitude. Mwangombe et al. (2007) reported fewer cases of ALS in high altitude areas in Kenya as compared to mid or low altitude areas. Even then, high altitudes areas experience conditions of high relative humidity which favour pathogen sporulation but with low temperatures disease development is retarded. Such information on the occurrence and severity of ALS is not available in Uganda thus, inhibiting development of disease control strategies.

The type of bean cultivars grown and cropping practices also influence disease occurrence and severity. Bean cultivar preference tends to differ from region to region. For instance in Africa large-seeded beans are more preferred due to size and market demands (David et al., 2000). This brings dominance of certain bean cultivars in certain areas. Over time the races within areas dominated by single cultivar get adapted to the bean cultivar being grown, rendering it susceptible (Sartorato, 2004). It is also known that most resistance sources belong to small- or medium-seeded beans but in Africa and Uganda, large-seeded bean types, which are relatively susceptible to the disease, continue to dominant in most bean growing areas hence escalating disease occurrence.

Since ALS is among fungal diseases that are seed-borne, the type of seed used by farmers also influences its occurrence and severity. Use of un-certified seed contributes to ALS transmission from season to season, especially in areas where exchange of farmer saved seed is a common practice (David et al., 2000). Due to lack of certified seed most smallholder farmers use their own saved seed (Mwang'ombe et al., 2007), which is the surest means of transmitting the disease into the next cropping season. Based on previous research findings it was observed that low disease incidence was recorded in farms which used certified seed

compared to smallholder farms where use of home-saved seed was popular (Wagara et al., 2004). In the same way, poor field sanitary practices such as leaving diseased crop residue or volunteer plants left in the field after harvesting have been linked to increased disease occurrence in subsequent seasons (Celleti et al., 2006). Luckily, ALS occurrence or severity resulting from cropping practices can be controlled if integrated disease management practices are developed, disseminated and adopted by smallholder farmers.

2.4.5 Angular Leaf Spot disease management

Management of ALS is confounded by the variable nature of its causal pathogen, poor field phytosanitary and crop management practices which favour disease prevalence in certain bean growing areas. The pathogen can survive on infested bean residues left in the field, but burying the residue in soil to decompose and rotating crops with non-host crops for at least two years can reduce pathogen survival and disease pressure (Celetti et al., 2006). Nonetheless, use of crop rotation may not be ideal for areas with land shortage. The pathogen can also survive between seasons on infected seed (Wagara et al., 2004), making use of certified seed extremely important in disease management. However in Uganda and Africa at large, use of certified seed is constrained by high seed cost, limited access and availability (Buruchara et al., 2011).

On the other hand, application of effective fungicide during early bloom (10%–30% flowering) when environmental conditions are conducive for disease has been used to control ALS (Celetti, 2006). For this method to be more effective, a second fungicide application is done seven days later at late bloom (50%–70% flowering) if environmental conditions favouring infection and disease development occur between growth stages. Use of fungicide by smallholder farmers to control ALS is still constraint by high cost, environmental and health risks associated with fungicides (Mahuku, 2002). This calls for methods that are affordable to farmers and environmentally friendly. The most effective method to manage ALS is to use genetic resistance. However, attaining genetic resistance has been constrained by *P. griseola* variability (Aggarwal et al., 2004) that causes the developed resistance to break down as new pathotypes that are more virulent than the existing one evolve. Therefore, there is need for continuous identification of new resistance sources to counteract the ever emerging *P. griseola* pathotypes. Alternatively, existing sources of resistance developed elsewhere can be used to enhance resistance in susceptible cultivars that have desired traits.

2.4.6 Tools used to characterise *Pseudocercospora griseola*

Pseudocercospora griseola is a diverse fungus with respect to host range, geographical distribution and location in bean production areas. The methods used to characterise *P. griseola* are dependent on their inherent properties. The methods are broadly divided into phenological and molecular. However due to variable nature of *P. griseola* both are often applied to provide more reliable results. The phenological technique distinguishes isolates based on their reaction with cultivar differentials while the molecular methods are based on their nucleic acid.

2.4.6.1 Bean cultivar disease differentials

Pseudocercospora griseola is variable with large number of pathotypes which differ depending on geographical regions and location. Because of this previously pathologists found it hard to identify *P. griseola* isolates as there was no harmonised method to follow in identification. However, the First International Angular Leaf Spot Workshop held at CIAT in 1995 developed a standardised method for *P. griseola* isolate identification. A set of 12 differential cultivars consisting of six Andean and Mesoamerica were established. Under this method disease evaluation is carried out using a disease assessment scale of 1 to 9 (Schoonhoven & Pastor-Corrales, 1987). On this scale, pathotypes are defined by rating scores of 1-3 as regarded as resistant while rating higher than 3 are considered susceptible. The designation of pathotype is often defined using binary values of the differential genotypes that are compatible with the respective *P. griseola* isolate (Schoonhoven & Pastor-Corrales et al., 1998).The method defines the virulence of pathogenicity level of *P. griseola* but depends on environmental conditions. Therefore when used it is better to be complemented with the molecular method which is not influenced by environmental conditions.

2.4.6.2 Molecular markers

Accurate identification of *P.griseola* pathotypes is among the critical strategies needed to control ALS in common bean. Hence the availability of fast, sensitive and accurate methods for pathogen identification is increasingly becoming necessary to improve disease control decision making. Bean differential cultivars have previously been used to identify ALS isolates but this method depends on environmental conditions for pathogen sporulation. Additionally, this method is often time-consuming, laborious, and requires extensive knowledge of classical taxonomy (Goud & Termorshuizen, 2003). In order to overcome this,

molecular approaches with improved accuracy and reliability have been developed. Molecular methods have been used to identify *P. griseola* isolates and even applied to the study of the genetic variability of pathogen populations. This is because these methods are much faster, more specific, more sensitive, and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise. The advances in molecular methods have indeed provided powerful tools for determining the pathogen variability for instance RAPDS, ISSR and a combination of ISSR-RADPs have all been used to identify and define *P.griseola* pathotypes (Satorato et al., 2004; Strenglein & Ballati, 2006; and Abdio et al., 2012).

2.5 Genetic improvement of common bean

2.5.1 The history and progress of common bean breeding in Uganda

Bean breeding in Uganda started as early as the 1920's, though in the first forty years (1920-1960) no bean variety release was recorded (Mukasa, 1970). In 1960 bean breeding was redirected to address a problem of protein deficiency among infants in banana-based regions of Uganda. This led to release of variety K20 in 1968, which was adopted and grown by farmers in 1970's and presently K20 is still being grown in several parts of Uganda (Kalyebara & Kasozi, 2005). Other varieties such as; K131, K132, MCM2001 and MCM1015 developed from CIAT lines were later released. On the sad note, between 1970 and 1985 no bean variety was released by the breeding program due to civil wars which halted research activities in Uganda (Opio et al., 2001).

Breeding work was resumed in 1986, this time round with the aim of increasing crop productivity through developing acceptable varieties with resistance to pests and diseases (MAAIF, 2003). Under this initiative selections from locally collected cultivars, including introductions from CIAT, Ethiopia, Kenya, Burundi, Democratic Republic of Congo, Malawi, Tanzania, and Rwanda were made (Opio et al., 2001). Through vigorous selection and evaluation 16 bean varieties have been released from 1993 to date (Kiryowa et al., 2015). Fifty percent of bean varieties released are susceptible to ALS (Opio et al., 2001) and those that are tolerant at the time of release, eventually succumb to new pathogen races that evolve over time to break down the resistance.

In the past, breeding for resistance against bean diseases like ALS has been directed towards identifying new sources of resistance or using known sources of resistance from other countries for the purpose of introgressing them into local commercial varieties with desired traits. For instance, Namayanja et al. (2006) studied the inheritance of ALS resistance in Mexico 54 using different genetic background of locally adapted landrace and released bean varieties in Uganda. Presently, CIAT is spearheading work involving using multiple disease resistance approach to manage anthracnose, *Pythium*, BCMV and angular leaf spot (Mukankusi, 2008). In order to consolidate the past achievements in bean improvement, there is need to source for more new resistance sources especially from locally adopted varieties and determine their inheritance pattern. In addition, pyramiding the existing resistant genes into released bean varieties will also provide durable resistance.

2.6 Genetics of angular leaf spot resistance

Developing stable resistance against ALS requires regular monitoring of the causal pathogen, continuous germplasm evaluation to identify new sources of resistance and introgression of resistant genes into adapted cultivars (Iwo, Ittah & Osai, 2012). However, to achieve this, a clear understanding of inheritance of resistance to *P. griseola* is indispensable. This is because it guides the introgression process. Previous research efforts have identified a few sources of resistance but their use in breeding programmes is still low due to limited or no information related to their mode of inheritance. But, for sources where inheritance has been studied, resistance to *P. griseola* has been reported to be controlled by dominant and or recessive genes (Mahuku et al., 2009). Recent studies have demonstrated that resistance in cultivar AND277, G10474 and MAR2 is conditioned by single dominant genes (Mahuku et al., 2002; Mahuku et al., 2009; Nietscle et al., 2000). The dominant resistance genes have been described *Phg-1* and *Phg-2* which are mapped on LGB1 and B8 respectively (Goncalves-vidigal et al., 2011). *Phg-1* is Andean origin identified in the AND277 genotype and *Phg-2* is of Mesoamerican origin identified in genotype Mexico 54. In ouro-negro and G10474 dominant monogenic inheritance for ALS has been described but their relation to *Phg-1* and *Phg-2* is not known (Mahuku et al., 2004). Allelic studies have also revealed three more genes *Phg-3, Phg-4* and *phg-5* with two alleles of each controlling resistance in four bean cultivars. *Phg-3* and *Phg-4* in MAR2 and Cornell 49-242 were identified (Caixeta et al., 2005; Mahuku et al., 2004). In additional major genes, minor genes have also been found to condition resistance of common bean to *P.griseola* (Jara, 2003). Although resistance to ALS is conditioned by major and minor genes, but gene expression is influenced by the

background into which the resistant gene is being studied. This was confirmed by Sartorato et al.(1999) when he used a Mesoamerican cultivar Ruda as susceptible parent and found out that a single dominant gene conferred resistance of Mexico 54 to pathotype 63-19. But, using Mexico 54 as the resistant parent and a snap bean variety as the susceptible parent Mahuku et al. (2002) found that resistance to the same pathotype was conferred by a single recessive gene. Therefore, for new resistance sources it important to test their gene expression in various backgrounds to ascertain their consistency in introgressing their resistance in different genetic background. This provides useful information in deciding the appropriate breeding methods to use when developing resistance against ALS.

2.7 Gene-pyramiding to improve disease resistance

Presently, gene pyramiding assisted with molecular marker technology has been integrated into existing plant breeding programmes to allow researchers to access, transfer and combine genes with a precision which was not previously possible. Although use of resistant varieties is seen as the most economical and practical control strategy for most fungal pathogens, but its effectiveness in offering durable resistance is complicated by the high pathogenic variability of *P. griseola* (Jerônimo et al., 2011). In such situations, gene pyramiding may be used to achieve durable and broad spectrum resistance (Kelly et al., 2003). This is because gene pyramiding has the capacity to combine several genes into a single cultivar. Quite often most virulent pathogens can overcome single gene resistance but pyramiding delays resistance break down or reduces the evolution of resistance against *P. griseola* (Ferre & Van Rie, 2002). Based on previous pyramiding work done in other crops, there is no doubt that combined resistance can fail to offer durable resistance compared to single gene resistance (Bates et al., 2005). Because of this comparative advantage over other methods in the recent past gene pyramiding has been a target for most crop improvement programs including beans. It has been successfully applied to combine multiple genes not only for one disease but to more than two (Ragagnin et al., 2005). For instance, bean improvement programs in the past have developed bean lines with resistant genes for angular leaf spot (*Phg-1*), anthracnose (Co-o' and Co-4) and rust (Ur-ON) through a process assisted by molecular markers (Ragagnin et al., 2005). In the same way for cases where a disease caused by multiple races, durable resistance has been achieved through gene-pyramiding approach. Zheng et al. (2006) successfully pyramided three genes Rsv1, Rsv3, and Rsv4 for soya bean mosaic virus with the aid of microsatellite markers. Pyramiding of resistance genes has been also effectively used as a promising strategy to create more durable resistance to major strains of barley

yellow mosaic virus which is a major threat to winter cultivation in Europe (Werner et al., 2005). Based on previous results arising from pyramiding, there is hope that the same method may be used to manage ALS with numerous races which cannot be controlled by single gene approach.

2.8 Tools used to study inheritance and pyramiding

Pseudocercospora griseola is a diverse fungus with respect to host range, geographical distribution and location in bean production areas. The methods used to characterise *P. griseola* are dependent on their inherent properties. The methods are broadly divided into phenological and molecular. However due to variable nature of *P. griseola* both are often applied to provide more reliable results. The phenological technique distinguishes isolates based on their reaction with cultivar differentials while the molecular methods are based on their nucleic acid.

2.8.1 Diallel mating design

The diallel cross refers to a set of all possible mating between several genotypes (Hayman, 1954). The advantage of this cross is that it can be used in both self and cross-pollinating plant species, clones, homozygous and inbred and non-inbred parents (Griffing, 1956, Gardener and Eberhart, 1966). The diallel analysis helps to obtain genetic information on inheritance of traits that may require improvement because it is capable of predicting the performance of trait in subsequent generations through assessing the potential of different crosses in F_1 and F_2, (Dabholkar, 1992). It is due to this predictive ability that Christie and Shattuck. (1992) established that diallel analysis is important in progeny testing that it can generate information which other analytical methods cannot deliver and hence often used by breeders to aid selection. But despite its usefulness, Sokol and Baker (1977) suggested that genetic interpretation of data from diallel experiments is valid only if the following assumptions about the parental material are true: diploid segregation, homozygous parents, gene frequencies are equal to one-half at all segregating loci; genes are independently distributed between parents, and no non-allelic interaction.

Diallel analysis methods with modifications have been described by Jinks and Hayman (1953), Griffing (1956b), Gilbert (1958), and Gardner and Eberhart (1966). But each method has its own assumptions which may limit its use or interpretations of its results. Hence if these assumptions are not adhered to there is likelihood of misinterpretation of results (Baker,

1978). Nonetheless, these methods are of great importance to breeders and are extensively used to test genetic attributes in plant populations and complement the interpretation of results (Hayman, 1954). So far diallel analysis has been effectively used to determine the combining ability and heritability of many crop traits.

Griffing's analysis determines the combining ability of genotypes and provides reliable information on general combining ability (GCA) and specific combining ability (SCA) of parents (Nienhus & singh, 1986). Combining abilities assist breeders to determine appropriate breeding and selection strategy to use while developing better cultivars (Zhang et al., 2001). This is possible because GCA and SCA can identify parents and crosses that are responsible for certain type of gene action observed in subsequent generations (Dabholkar, 1992). General combining ability literally refers to mean performance of a line in all its crosses and it is expressed as a deviation from the mean of all crosses (Falconer and Mackay, 1996). It is the average value of all F_1s having this line as one parent, the value being expressed as a deviation from the overall mean of crosses. Any particular cross has an expected value which is the sum of the general combining abilities of its two parental varieties. However, the cross may deviate from this value to a greater or lesser extent. This deviation is called the SCA of the two varieties in combination (Falconer & Mackay, 1996). Differences in GCA have been attributed to additive, additive x additive and higher order interactions of additive genetic effects in the base population, while differences in SCA have been attributed to non-additive genetic variance (Baker, 1978).

2.8.2 Marker-assisted gene pyramiding

The success of gene pyramiding is heavily aided by molecular markers because to achieve durable resistance, it's desirable for breeders to combine as many genes as possible within the same genotype. However, based on the conventional phenotypic evaluation it is hardly feasible to track the accumulation of resistance genes in the intended genetic background. But molecular markers have been used efficiently by breeders to overcome this problem (Fischer et al., 2004). In line with this research efforts have been geared towards identifying markers that are linked to ALS resistant genes. Through this effort a number of molecular markers linked to resistant genes have been identified. For instance Maria et al. (2011) showed that marker TGA1.1 segregated with gene *Phg-1* found in bean accession AND 277. Further still, other authors like Ferreira et al. (2000) and Namayanja et al. 2006 reported that amplification with primer OPE04 generated a 500-bp fragment distinguished the resistant

from the susceptible bulk populations, in crosses of Ruda x MAR 2 and Kanyebwa x Mexico 54 respectively. In the same way, Mahuku et al. (2002) reported other AFLP markers linked to recessive gene in Mexico 54.

On the other hand, three microsatellite markers, Pv-ag004, Pv-at007 and Pv-ctt001 have been reported to segregate in coupling phase with the resistance genes in G5686 (Mahuku et al., 2009). Microsatellites Pv-ag004 and Pv-ctt001, located on opposite ends of linkage group B04 segregate with resistance genes Phg_{G5686A}, Phg_{G5686B} at 0.0 and 17.1 cM, respectively, while marker Pv-at007, localized on linkage group B09 segregate with resistance gene Phg_{G5686C} at 12.1 cM. These genes are polymorphic in both Andean and Mesoamerica background which makes them useful in pyramiding (Mahuku et al., 2009). The identification of these genes gives a lay way for breeders to stack ALS resistant genes into the same background and their detection through marker assisted selection.

2.9 Literature review summary

Previous research indicates how variable *P. griseoala* is in different geographical areas, locations and seasons. This calls for proper and regular characterisation of pathogen in order to develop appropriate disease monitoring and control strategies for specific bean production areas. The study will determine the distribution and variability of *P. griseola* in Uganda as well as identifying new sources of resistance among adapted landraces.

Most breeders have suggested that for any source of resistance to be effective, there is need to understand its resistance inheritance pattern before resistance genes are incorporated into other susceptible cultivars. The diallel method used in the study is one of the suggested mating designs used to facilitate the understanding of disease resistance inheritance pattern. Reviewed literature indicates that durable resistance against variable pathogens have been developed in other crops. In such crops single gene resistance has been observed to offer less effective durable resistance against highly variable fungal diseases compared to when multiple genes are deployed. The study targeted the use of multiple genes concentrated in one genotype to offer more reliable durable resistance against ALS.

CHAPTER THREE
VARIABILITY AND DISTRIBUTION OF *PSEUDOCERCOSPORA GRISEOLA* IN UGANDA

3.1 INTRODUCTION

Angular leaf spot caused by *Pseudocercospora griseola Sacc.* (Crous and U. Braun) is one of the most destructive diseases of common bean (*Phaseolus vulgaris*) in tropical and subtropical regions (Oblessuc et al., 2012). The disease is also a major constraint to bean production in the Great Lakes Region (Kenya, Uganda, Tanzania and Rwanda) where bean production is popular (Wortmann et al., 1998). The wide disease spread within this region is attributed to a number of biotic and abiotic factors. Some of the biotic factors include; use of susceptible cultivars and poor field sanitary practices (Wagara et al., 2003). On the other hand, abiotic factors, such as intermittent dry-wet and warm-cool weather which characterise weather patterns in most bean growing areas in Uganda, accelerate the pathogen sporulation process (Correa-Victoria et al., 1989). The dry-wet conditions coupled with poor disease management practices encourage *P. griseola* proliferation leading to heavy infection (Celleti, 2006).

Control of ALS is complicated by pathogenic variation of the casual fungus. Based on morphological and molecular markers; two gene pools (groups) related to common bean origin have been defined namely; Andean, and the Middle American. The Andean isolates are pathogenic to large seeded, while the Middle Americans are pathogenic to both small and large seeded beans (Pastor-Corrales et al., 1998). Nonetheless *P. griseola* pathotype structure might also be associated with the compatibility of cultivated bean genotypes. Whereas most large seeded commercial varieties in Uganda are susceptible to ALS (Namayanja et al., 2006), cultivars such as AND277, G5686 and Mexico 54 have been identified as valuable sources of resistance (Aggarwal et al., 2004; Nietsche et al., 2001; Pastor-Corrales et al., 1998). A number of studies have demonstrated that the level of variability among and within populations of *P. griseola* is considerably high, even though the sexual form of the fungus has not been found (Leibenberg & Pretorius, 1997). Previous studies demonstrated variation in pathogenicity of fungal isolates such, as 53, 13 and 50 different pathotypes described among 54, 30 and 112 isolates that where obtained from Africa, Brazil and Central America, respectively (Busogoro et al., 1999; Mahuku et al., 2004a; Nietsche et al., 2001). Though ALS has been identified in Uganda, the pathotype structure of the fungus remains unknown

and probably as a consequence of this there is hardly any commercial cultivar either tolerant or resistance which has been developed. In fact this is evidenced by 50% yield loss due to ALS reported among commercial varieties in Uganda (Opio et al., 2001).

In countries such as Brazil and Argentina where ALS also occurs, yield loss due to ALS has been minimised through practising crop rotation, using certified seed and fungicide (Sartorato, 2002; Stenglein et al., 2003). Currently, such control measures are not viable in Uganda due to limited land holdings per farmer hence low investment in bean production as an enterprise, poor access to and high cost of quality seed and fungicides (Nkonya et al., 2001). In such a situation, breeding for resistance is seen as the most practical and economic approach to manage ALS under farmers' conditions. However, the process of designing an effective ALS breeding program requires precise and accurate knowledge on population dynamics and spatial and temporal distribution of the pathogen (*P. griseola*) (Stenglein et al., 2003). Past studies have indicated that many races of *P. griseola* occur and vary in time and space; a bean cultivar which is resistant in one location, season or year may be susceptible in another (Aggarwal et al., 2004). There is limited information on pathogen variability and distribution in Uganda (Mahuku et al., 2004) hindering breeding for ALS resistance. Therefore, a study to understand the pathogen variability and distribution of *P. griseola* as a first step in designing strategic breeding interventions for resistance against ALS in Uganda (Sartorato, 2004) is required.

Several techniques have been used to study the variability and distribution of fungi; some of them include; disease surveys, differential cultivars and molecular techniques (Sartorato, 2004; Sebastian & Balatti, 2006). Disease surveys allow pathogen distribution to be determined in terms of incidence, severity, spatial and temporal spread. Such surveys have been used in Kenya to determine ALS incidence and severity (Mwang'ombe et al., 2007), and generate information needed to design integrated disease management strategies (Wagara et al., 2003). On the other hand, *P. griseola* virulence is assessed based on reaction of isolates on a standard differential set of 12 common bean cultivars established during the International angular leafspot workshop hosted by International Centre for Tropical Agriculture (CIAT) in 1995. In this technique, a binary system based on the position of each cultivar within the series is used to define the virulence level of isolates under study (Pastor-Corrales et al., 1998). Use of differential cultivars generates a true picture of virulence structure and reveals the pathogen properties related to host selection effect on the pathogen

population (Sebastian and Balatti, 2006). But the major limitation for this method is its heavy dependence on environmental conditions (Kolmer et al., 1995).

However, challenges in using differential technique has been overcome, through the use of molecular techniques as alternative options for detecting variability in pathogen populations. Available genetic markers such as random amplified microsatellites (RAMS), repetitive sequences such as enterobacterial repetitive intergenic consensus sequence (ERIC) and repetitive extragenic palindromic (REP) which have all indicated how *P. griseola* is extremely diverse (Sebastian & Balatti, 2006). Such makers have been reported to be reliable, reproducible when used to assess fungus strain diversity especially when combined with computer-assisted data analysis (Louws et al., 1999). Depending on the reliability, markers have been previously used to determine genetic variation of *P. griseola* and divided it into two Andean and middle American groups (Pastor-Corrales et al., 1998). The existence of two groups suggest that developing durable resistance requires incorporation of resistance genes from Andean into a mesoamerican cultivar, a process best complemented by a clear understanding of how variable *P.griseola* is. Hence, this study aimed at assessing the distribution and variation of *P. griseola* isolates from Uganda using both the differential cultivar and molecular techniques in order to facilitate breeding for ALS resistance.

3.2 MATERIALS AND METHODS

3.2.1 Assessing the incidence and severity of angular leaf spot in major bean producing areas of Uganda

A disease scouting survey was conducted in ten districts of Uganda including; Mbale, Sironko, Lira, Dokolo, Apac, Bukomansibi, Lwengo, Rakai, Kabale and Kisoro. The districts were selected based on their bean production intensity and location in terms of altitude. Dokolo, Lira and Apac districts represented low altitude areas (1000-1200 metres above sea level m.a.s.l), Bukomansibi, Lwengo, Rakai mid altitude (1200-1500 m.a.s.l) and Mbale, Sironko, Kabale, Kisoro high altitude (>1500 m.a.s.l). Observations were made during August 2011 for Eastern and Northern and December 2011 for Central and South Western districts respectively. The period the surveys were conducted concided with the second rain season that were characterised by alternating wet and warm temperatures with high relative humidity conducive for ALS development. The period also represented the mid-podding stage for beans when ALS epidemics best manifests itself (Allen et al., 1998).

During the survey, random sampling of ALS infected plants was done in 20 randomly selected fields that were 5 km apart per district. Within the fields, disease incidence and severity were assessed by taking a transact walk across the field, three sites which were 10 m apart along the transcant were earmarked and 20 plants observed for presence of ALS symptoms.

Disease incidence was expressed as the percentage of infected plants over the 20 plants picked. Disease severity evaluation was conducted using a 1-9 visual scale (van Schoonhoven & Pastor-Corrales, 1987) as follows: 1, no visible symptoms (0% lesions on the leaf area); 3, lesions on 5-10% of the leaf area of the plants; 5, lesion sporulation on 20% of the leaf area; 7, lesions and sporulation, associated with chlorosis and necrosis on 60% of the leaf area; and 9, lesions frequently associated with early loss of leaves and plant death, on 90% of leaf area. Severity was estimated as percent leaf area diseased per plant for five plants per field rather than the individual plant score.

For each sampled site a Global Positioning System (GPS) reading was taken to ascertain its location in terms of latitude, longitude and elevation. In addition, two diseased leaf samples at each site were placed between two papers in a counter book, labelled and taken to the laboratory for pathogen isolation.

Table 1. Origin and pathotype nomenclature of *Pseudocercospora griseola* isolates used in this study

Isolate code	Bean cultivar	District	Pathotype
MB001	Kanyebwa	Mbale	21:38
MB014	Wayirima	Mbale	17:23
MB015	Kaki	Mbale	5:55
MB017B	Kanyebwa	Mbale	15:39
MB020	Kamwanyi	Mbale	17:23
MB025	Kamwanyi	Mbale	17.23
MB023	Kanyebwa	Mbale	17:23
MB026	Kanyebwa	Mbale	17:23
MB024	Kamwanyi	Sironko	17:23
D0041	Mudugavu	Apac	41:6
D0047	Mudugavu	Apac	15:39
D0051	Mudugavu	Apac	15:39
D0053	Mudugavu	Apac	15:39
D0056	Mudugavu	Apac	15:39
D0058	Mudagavu	Dokolo	17:23
L0064	Mudugavu	Lira	17:23
L0065	Mudugavu	Lira	17:23
L0068	Mudugavu	Lira	17:23

L0069	Owakwak	Lira	5:6
L0070	Mudugavu	Lira	17:23
L0066	Mudugavu	Lira	15:39
L0072	Mudugavu	Lira	15:39
L0073	Mudugavu	Lira	5:30
MA081D	Nambale	Masaka	17:39
RA06/2	Kanyebwa	Rakai	17:39
RW032C	White beans	Lwengo	5:30
KA036	Kakira	Kabale	13:13
KA033	Kakira	Kabale	13:55
KA041C	Kakira	Kabale	17:39
KA034C	Bishara	Kabale	5:31
KA044	Bishara	Kabale	13:55
KA045	Bishara	Kabale	13:13
KA047	Biashara	Kabala	5:31
KA0 48C	Bishara	Kabale	5:55
KA060	Bishara	Kabale	61:63
KA084	Muzahura	Kabale	21:38
KIS039B	Muzahura	Kisoro	15:39
KIS039D	Sugar	Kisoro	21:38
KIS061A	White beans	Kisoro	21:38
KIS062	Sugar	Kisoro	5:30
KIS048	Sugar	Kisoro	5:30
KIS064B	Sugar	Kisoro	17:39
KIS066	Sugar	Kisoro	41.6
KKIS075	Sugar	Kisoro	5:6
KIS074	Sugar	Kisoro	17:39

3.2.2 Fungal isolation and inoculum preparation

Forty five single spore *P. griseola* isolates obtained from ten bean growing districts of Uganda were isolated from the diseased bean leaf samples collected during the observatory survey (Table 1). Two additional characterised isolates (Middle American and Andean) obtained from CIAT at Kawanda were also included to elucidate the relationship between isolates belonging to the middle American and those from Andean among the Ugandan isolates. Isolation, monosporic culture production and inoculum preparation were done according to Pastor-Corrales et al. (1998). Spore concentration in the inoculum was estimated using a haemocytometer and adjusted to a final concentration of 2×10^4 conidia ml^{-1} using sterile distilled water.

3.2.3 Inoculation and pathotype determination

In order to determine, the pathotype of each isolate a set of 12 differential cultivars consisting of six Andean (Don Timoteo, G 11796, Bolon Bayo, Montcalm, Amendoin, G 5686) and six Mesoamerica (Pan 72, G 2858, Flor de Mayo, Mexico 54, BAT 332, Cornell 49–242) were

used. Five seeds of each differential cultivar were planted in 5-litre buckets containing black soil, manure and sand in a ratio of 3:1:1. The experiment was laid out in a randomised complete block design (RCBD) with three replications. The 21-days old bean plants were spray-inoculated with inoculum and kept in a humid chamber at 22-28 °C and 95% relative humidity. Four days after inoculation, plants were transferred into the screen house and watered regularly.

Disease severity on the inoculated plants was evaluated using 1-9 visual score scale (Schoonhoven and Pastor-Corrales, 1987) for 21 days at an interval of three days. Pathotypes were defined by rating scores of 1-3 to be incompatible or resistant, while ratings >3 were compatible or susceptible. Pathotype designation was executed by adding binary values of the differential genotypes that were compatible with the respective *P. griseola* isolates. For instance, for pathotype 15:39 (virulence phenotype abcdef-ghijkl) in Table 5, the first value was obtained by adding the binary values of the susceptible Andean differential genotype abcdef (1+2+4+8 =15) and the second value was obtained by adding the binary values of the susceptible Mesoamerica genotypes ghijkl (1+2+4+32 = 39) in table 5 (Pastor- Corrales et al., 1998). For consistent results, the experiment was repeated twice.

3.2.4 Molecular characterisation of *Pseudocercospora griseola*

Apart from using ALS differential bean cultivars, genetic variability of *P. griseola* was assessed using amplified fragment length polymorphism involving use of PCR as part of the molecular tools. The molecular tools comprised of random amplified microsatellites (RAMS), enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) elements also known as palindromic units and the BOX element (George et al., 1997). The consensus DNA sequences in the conserved inverted repeats of RAMS, REP, BOX and ERIC type elements have previously been used to design specific oligonucleotide primers that can be used to probe fungal genomes and other organisms (Bruijn, 1992).

3.2.5 DNA Extraction and Primer Analysis

The mycelium used in DNA extraction was generated on V8 agar growth media by cutting three disks of 1.5 mm diameter on the edges of actively growing fungi and inoculated on growth media contained in Erlenmeyer flasks (250ml). The cultures were placed on a rotary shaker (120 rpm) at room temperature for 14 days. Mycelium was harvested through filtration

with a cheese-cloth, washed in sterile deionised water, freeze-dried and stored at -24ºC. DNA was extracted according to Mahuku (2004b) and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). The DNA was adjusted to a standard concentration of 10 ng/µl before using it in the PCR reaction. To analyse the primers, four random amplified microsatellites and five conserved sequences (Table 2) were used to amplify the fungal DNA extracted. The PCR reactions were carried out in 20µl volumes containing 1× DNA polymerase buffer (100mM Tris-HCl, 400 mM KCl, 15 mM $MgCl_2$, pH 9.0), 3mM $MgCl_2$, 0.4mM dNTPs, 1µM of each primer, 0.3U Taq DNA polymerase (Bioneer Inc. Korea) and 50ng of genomic DNA. A water control (DNA replaced with sterile water) was included with each set of 10 isolates. In additional DNA from isolate RU7 with a known PCR reaction was also included as a positive PCR control. DNA amplification was performed in a Mycycler thermal cycler (Bioneer Inc, Korea) under a program of one cycle at 94 ºC for 5 min, followed by 35 cycles at 94 ºC for 20s, 50 ºC for 40 s and 65 ºC for 8 min and a final 16 min extension at 65 ºC (Table 2). The DNA amplicons were electrophoresed in 1.5% agarose gel for 1h at 90V in 1XTris–borate–EDTA buffer (89mM Tris base, 89mM boric acid–borate and 2mM EDTA pH 8.0) and later stained for 20 min in 0.5 µg/ml ethidium bromide. Gel images were captured using the GeneSnap gel documentation system (SynGene, Frederick MD).

Table 2. Sequences and annealing temperatures of RAMS and conserved primers used to amplify *Pseudocercospora griseola* DNA

Primers	Sequence (5′ to 3′)	Annealing temperature (0°C)	Total number bands	Reference
RAMS 4	AGGGTGTGTGTGTG	40	23	Hantula et al., 1996
RAMS 2	TGCCGAGCTG	40	11	“
RAMS 5	GGGTAACGCC	40	11	“
RAMS 6	GTGATCGCAG	40	25	“
BOX A1R	CTACGGCAAGGCGACGCTGACG	50	20	Versalovic et al.,1991
ERIC 1R	ATGTAAGCTCCTGGGGAT	50	22	Coenye et al., 2002
ERIC 2	AAGTAAGTGACTGGGGGTGAGC	50	22	“
REP1R	IIIICGICGICATCIGGC	40	14	Seurink et al., 2003
REP 2	ICGICTTATCIGGCCTAC	40	14	“

3.3 Statistical analysis for phenotypic data

For the phenotypic data a homogeity test was performed according to Cochran’s test to assess the difference in disease scores between the experimental repeats. The test revealed no

significance (P > 0.05) differences in disease scores for the two repeats and the data was pooled. In addition, Kurtosis-Skew tests were conducted on disease incidence and severity data and found to be significantly different from the normal. To improve on the non-normality arising from real observed data points the data was transformed using arcsine transformation of arcsine percentage (Gomez and Gomez, 1984) and analysed using in Genstat edition 14 (Payne et al., 2011). Incidence and severity means were separated using Fisher's protected Least Significant Difference (LSD) test at P < 0.05.

The incidence map was developed using survey data points that were geo-referenced with a GPS and incidence means generated from the analysis. Correlation between incidence and severity means was done according to Payne et al. (2011). Data points were transformed into a point map using Ilwis 3.2 software (Toxopeus, 1997) and the map exported and visualised in Arc View® GIS3.2 software (Rockware Inc). Virulence and molecular data analysis was done separately. Isolate virulence was determined by considering each differential as a marker and information on virulence phenotype generated by considering incompatible interaction (rating ≤ 3) as absence of a virulence(-) and compatible interactions (rating > 3) as presence (+).

3.3.1 Statistical analysis of molecular data

However, for molecular data, markers were scored as either (1) for the presence of a band or (0) absence of the band. Only strong and reproducible bands were scored and weak ones discarded. Genetic similarities between all the pairs of isolates were computed using; S= Jaccard coefficient (Sneath and Sokal, 1973) in DARwin5. Dissimilarities were computed as genetic distance = 1-S and based on this data a dendogram was constructed by un-weighted pair group method with arithmetic mean (UPGMA) hierarchical clustering using MEGA 5.0. The gene diversity across all loci was estimated (Nei, 1987).

3.4 RESULTS

3.4.1 Incidence of angular leaf spot in the major bean growing areas of Uganda

Generally there was significant ($P < 0.05$) variation of ALS incidence across regions. The northern region with the highest incidence, ranged between 55.5 and 69.5 while south western which had the least incidence was within a range of 18.5-30.5. In the same way ALS varied significantly ($P < 0.05$) across districts. High disease incidence were recorded in Dokolo (68.5%), Lira (65.5 %) and Apac (57%) districts, while Kisoro registered the least

disease incidence (29%) (Fig.2). Angular leaf spot disease incidence ranged between 36.5-49.5% in the central districts of Bukomansibi, Lwengo and Rakai, but disease incidence above 49.5 % was observed in Mbale and Sironko districts (Fig. 2).

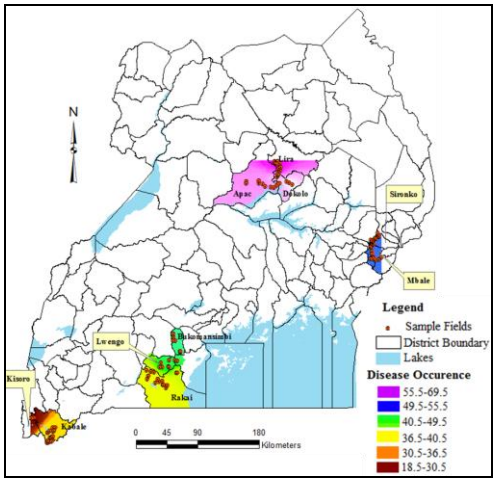

Figure 2. Incidence of *Pseudocercospora griseola* in ten common bean growing districts of Uganda in 2011

3.4.2 Severity of angular leaf spot in major bean growing areas of Uganda

Angular leaf spot severity varied significantly (*P<0.05*) among the surveyed districts. Severity was highest in Dokolo averaging 7.4 on a 1-9 scale and lowest in Kisoro districts averaging a score of 4 on a 1-9 scale (Table 3). The high ALS severity in Dokolo was observed to be accompanied with heavy leaf defoliation before reaching physiological maturity. Lira and Apac equally had high disease severity, followed by Sironko and Mbale (Table 3). In terms of regions south west had significantly ($P < 0.05$) low disease severity compared to northern and eastern.

Table 3. Severity of *Pseudocercospora griseola* in four regions and ten common bean growing districts of Uganda in 2011

Regions	Districts	Severity (1-9 scale)
Eastern	Mbale	6.44
Eastern	Sironko	6.37
Northern	Dokolo	7.35
Northern	Apac	6.92
Northern	Lira	6.78
Central	Bukomansibi	5.34
Central	Rakai	5.38
Central	Lwengo	5.54
South west	Kabale	5.03
South west	Kisoro	4.07
Mean		6.67
LSD (P=0.05)		1.23

Angular leaf spot incidence and severity varied significantly ($P < 0.05$) across altitudes (Table 4). Low altitude (1000-1200 m) areas had the highest disease incidence and severity followed by mid altitude (1200-1400 m) areas. High altitude (above 1500 m) areas exhibited the least disease incidence and severity.

Table 4. Incidence and severity of angular leaf spot at three different altitudes in Uganda in 2011

Altitude M (a.s.l)	Incidence (%)	Severity (1-9 scale)
1000-1200	60.0^{c}	4.95^{c}
1200-1400	45.4^{b}	3.53^{b}
>1500	33.6^{a}	1.84^{a}
Mean	46.3	3.44
$LSD_{(0.05)}$	2.4	1.07

Figures followed by the same letter indicate lack of significance while those with different letter indicate significant difference.

3.4.3 Angular leaf spot disease management practices

The study also considered crop stand (single and mixed varieties) as one of the crop management practices that are known to contribute to disease spread. Crop stand significantly ($P < 0.05$) influenced the disease incidence and severity across the regions (Table 5). Both disease incidence and severity were highest in fields surveyed in north and lowest in fields found in south west. In the north the high disease incidence and severity were mostly observed in fields planted with single varieties compared to those planted with mixed varieties

Table 5. The incidence and severity of angular leaf spot under different crop management in four bean growing regions of Uganda

Region	Single stand		Mixed stand	
	Incidence (%)	**Severity (1-9 scale)**	Incidence (%)	**Severity (1-9 scale)**
South west	27.5	4.03	27.5	3.82
Central	43.5	6.12	42.7	5.53
Eastern	52.5	6.08	51.3	6.02
Northern	62.5	6.86	50.2	5.49
Mean	45.7	5.77	37.7	5.21
LSD 0.05	11.8	0.52	10.1	1.25

3.4.4 Race characterisation of *P. griseola* based on pathogenicity levels on angular leaf spot differential set

Pseudocercospora griseola pathotypes are often defined based on pathogenicity reaction to a set of 12 bean differential cultivars or molecular markers (Mahuku et al., 2009). In this study, the reaction of 45 *P. griseola* isolates on differential cultivars revealed the existence of pathogenic variability in this fungus. In total 12 pathotypes were defined out of the 45 isolates studied (Table 6). Most isolates where pathogenic to both Andean and Mesoamerica differentials with only two isolates that were more pathogenic to Andean than Mesoamerica differentials (Table 6). Based on isolates' pathogenic reactions on the differentials, 43 isolates were classified as Middle America and two as Andean groups respectively. Pathotype 61:63 was compatible with eleven differentials tested, while 17:23 and 21:39 were the most prevalent in the ten districts which were surveyed. It was worth noting that the most prevalent pathotypes (17:23 and 21:39) identified in this study had not been previously reported in Uganda, and pathotype 5:55 was observed to occur only in high altitude districts (Table 1).

Table 6. Response of a set of bean differential cultivars to inoculation with *Pseudocercospora griseola* isolates collected from Uganda.

Andean group[a]						Mesoamerican group[b]						Pathotype[c]	Number of isolates
a	b	c	d	e	f	g	h	I	j	k	l		
1	2	4	8	16	32	1	2	4	8	16	32		
+	-	+	-	+	-	+	+	+	-	-	+	21:39	8
+	-	-	-	+	-	+	+	+	-	-	+	17:39	5
+	-	-	+	-	+	-	+	+	-	-	-	41:6	2
+	-	+	-	-	-	+	+	+	+	+	-	5:31	2
+	-	+	-	+	-	+	+	+	-	-	+	21:38	4
+	-	+	-	-	-	+	+	+	-	+	+	5:55	2
+	-	+	-	-	-	+	-	+	+	-	-	13:13	2
+	-	+	-	-	-	-	+	+	+	+	-	5:30	4
+	-	+	-	-	-	-	+	+	-	-	-	5:6	2
+	-	-	-	+	-	+	+	+	-	+	-	17:23	11
+	-	+	+	-	-	+	+	+	-	+	+	13:55	2
+	-	+	+	+	+	+	+	+	+	+	+	61:63	1

[ab]Andean groups included cultivars: (a) Don Timoteo; (b) G 11796; (c) Bloom Bayo; (d) Montcalm; (e) Amendoin; (f) G 5686. Middle American group included cultivars: (g) Pan 72; (h) G 2858; (i) Flor de Mayo; (j) Mexico 54; (k) BAT 332; (l) Cornell 49–242. [c]Pathotype designation is based on the sum (binary values) of bean cultivars with 10 scale value. (+), Compatible reaction; (-), Incompatible reaction. All pathogenicity tests included three replicates per isolate.

3.4.5 Molecular characterisation of *P. griseola*

The pathogenic variability of *P. griseola* was analysed using nine primers (Table 2). The isolates were grouped into two main clusters (Fig. 3) with an average dissimilarity of 0.98. The Middle America and Andean groups constituted 25 and 20 isolates with one control for each group respectively. The two main groups were clustered into sub-groups; the Andean group constituted two sub-groups with average dissimilarity of 50%, while the Middle American was divided into three sub-groups with 98% dissimilarity (Fig. 3). Nonetheless, grouping of isolates was not based on their places of origin. The combination of markers used in the study identified 30 halophytes. Analysis of molecular variance (AMOVA) indicated that most of the variation resulted from genetic differences within Middle American and Andean group (68.4%, $P<0.05$) rather than among main groups (31.6%). Analysis of genetic diversity indicated that the pathogen was highly variable as supported by the high polymorphic DNA bands shown in Figure 4. The genetic diversity of the entire population was 90.1% indicating the most isolates that constituted the population were different. The

genetic diversity index of the Middle American and Andean isolates were 0.450 (SD 0.098) and 0.443 (SD 0.061), respectively.

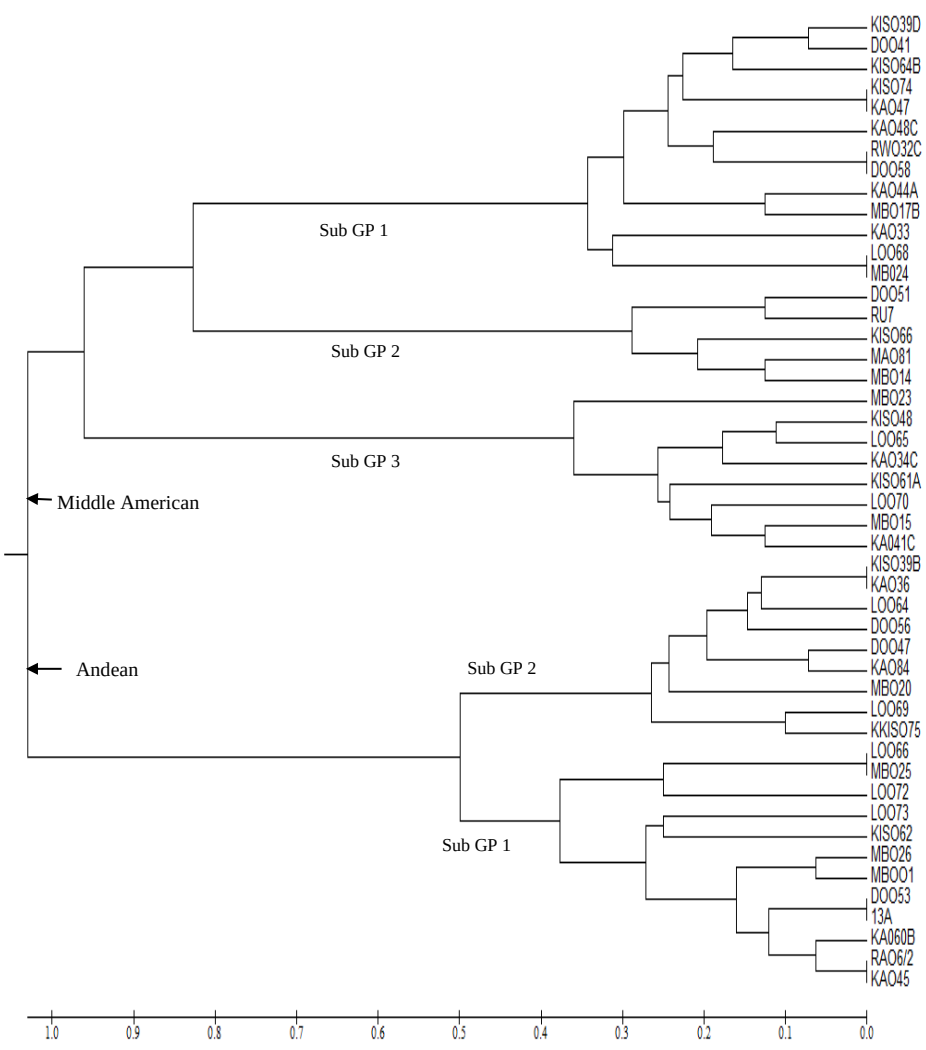

Figure 3. Dendogram generated based on RAMS and REP bands from 45 *P. griseola* isolates from Uganda and control isolates (RU7 and 13A) from CIAT, a representative group 1 (Middle American) and representative group 2 (Andean). Sub GP1, 2, 3=Subgroup 1, 2, 3 for Middle American isolates, Sub GP1, 2=Sub group 1, 2 of Andean isolate

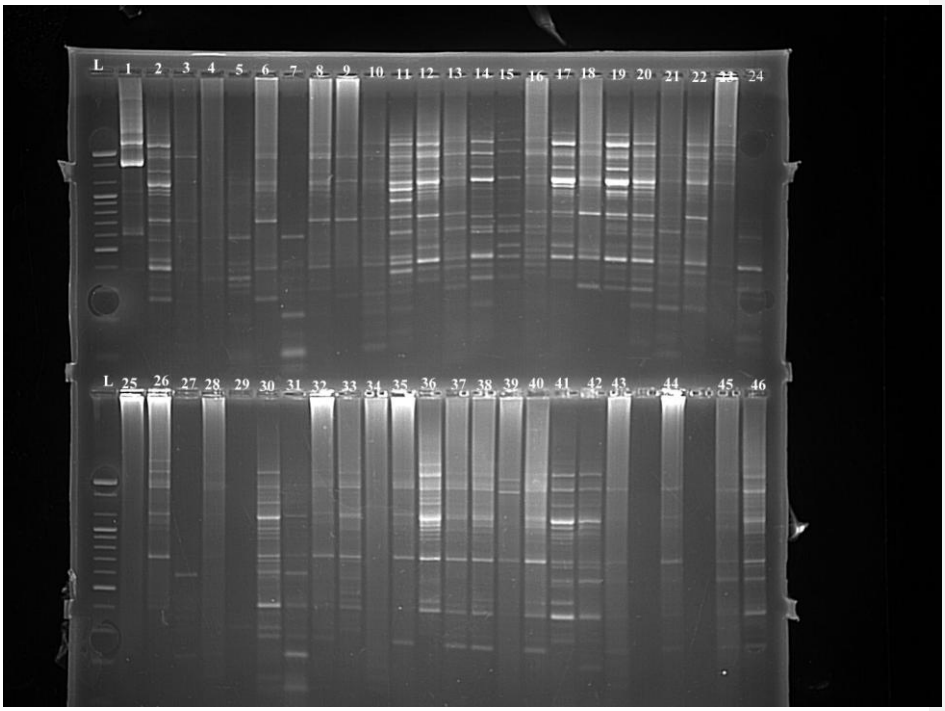

Figure 4. Amplification pattern of 47 *P. griseola* isolates by repetitive extragenic palindromic (REP) and Random amplified micro-satellites (RAMS). Numbers on top represent the different isolates and the first lanes are DNA ladders, top bands (1-24) and bottom bands (25-46) represents amplication of isolate DNA by REP and RAMS, respectively

3.5 DISCUSSION

Generally, the results confirmed the presence of ALS (*P. griseola*) in Uganda with varying pathotypes defined by phenotypic and molecular markers. The disease was found to occur at varying levels in all regions, districts and at different altitude sampled under the study. This indicated that bean growing areas in Uganda have conditions that favour ALS development.

Most often ALS incidence and severity are influenced by environmental factors and cropping practices. In the current study, environmental factors such as temperature and relative humidity possibly explained the findings. The low ALS incidence and severity in Kisoro may be attributed to cold temperatures. Among the districts surveyed, Kisoro is the coldest with temperatures as low as 10 °C at night (Mugisha, 2008). Temperatures below 16 °C, which is

the threshold for *P. griseola* infection and sporulation, are known to retard ALS development (Inglis & Hagedom, 1986). Similarly, high altitude (>1500 m) areas have high relative humidity above the threshold (70%) for ALS development, but they are characterised by cold temperatures, which are also known to delay disease development (Inglis & Hagedom, 1986). This was clearly exhibited in the study by the low ALS incidence and severity recorded in Kabale, Kisoro and for other high altitude areas such as Mbale and Sironko, which are characterised by cold conditions. On the other hand, the slightly high disease incidence and severity observed in high altitude areas of Mbale and Sironko when compared to other areas with similar altitude indicated that cold temperatures might not be the only factor contributing to the low disease incidence and severity in all high altitude areas. Other factors such as bean cultivar type grown may also influence disease incidence. For instance it was observed that most fields sampled in Mbale and Sironko had Kanyebwa a popular landrace in Uganda, which is known to be highly susceptible to ALS (Namayanja *et al.*, 2006). This probably explained the high disease incidence and severity observed in Sironko and Mbale compared to other high altitude areas studied.

Angular leaf spot development is stimulated under temperature conditions of 18-22°C and relative humidity of 70-100 % (Stenglein et al., 2003). Such conditions are experienced in low altitude bean growing areas in Uganda (Mugisha, 2008). Similarly, disease samples were collected during the bean growing period when wet- and dry weather conditions are experienced in low altitude areas. Since these environmental conditions are known to favour ALS development (Correa-Victoria et al., 1989), they possibly explained the high disease incidence and severity observed in low altitude areas.

On the other hand, the high ALS incidence and severity observed in Dokolo, Lira and Apac districts was partly attributed to bean cultivar preference. In these districts farmers prefer small seeded beans (Nkonya, 2001). This is because small sized beans are comparable to pigeon pea a common delicacy in the region. Cultivar preference was evidenced in the findings by the majority of samples collected from the three districts which were small seeded cultivars. The using similar bean cultivar over time possibly led to pathogen adaptation to the commonly used small seeded cultivars and hence increased disease incidence and severity. Pathogen adaptation to popular bean cultivars has been previously reported by Sartorato (2004) in Brazil where *P. griseola* adaption to carioca bean type, increased cases of ALS occurrence in the region

Most fungal pathogens that affect common beans are highly variable (Mahuku et al., 2009) and this study also confirmed the variability in *P. griseola.* Based on pathogenicity reaction of *P. griseola* on standard bean differential cultivars, the pathogen was divided into Middle American and Andean, corresponding to two gene pools of common bean. These results were consistent with Pedro et al. (2006) who reported that the causal organism for ALS underwent a micro evolution with its host, leading to two distinct groups: the middle-America and Andean. This was also evidenced in the study when the reactions of isolates to differentials of known Andean and Middle American origin were compared. Andean isolates were mostly virulent to large seeded (Andean) bean types while the middle-American isolates were more virulent to small seeded (Mesoamerican) bean types, based on this we confirmed that the standard differential cultivars categorised *P. griseola* isolates from Uganda into two major groups.

Among the two groups defined by differentials, most isolates belonged to Middle America group, yet in Uganda beans of Andean origin are the predominantly grown bean type. This indicated that some of the isolates that were recovered from Andean genotypes might not have been necessarily of the Andean type. This was possible because Middle America isolates are also known to attack bean genotypes from Andean gene pool (Araya et al., 2004). In addition previous studies by Guzman et al. (1995) in Malawi indicated the possibility of Middle America isolates occurring on Andean host genotype, implying that in Uganda the existing *P. griseola* race may not be dependent on the bean type predominantly grown.

The study further identified pathotype 61:63 among Ugandan isolates, which was compatible with and overcome the resistance genes differential cultivars. Similar pathotypes have been reported in Brazil and Argentina (Sartarota, 2002; Sebstian et al., 2006) and their presence in Uganda and other countries implies that new sources of resistance need to be identified regularly to mitigate resistance break down. On the other hand, pathotypes that overcome resistance in differential cultivars are potential candidates for use in screening germplasm against ALS by breeding programs.

The common occurrence of pathotypes 17:23 and 21:39 indicated an overlap of pathotypes across bean production areas. Such pathotype overlaps could possibly be as a result of seed exchange, which is a common practice among small holder farmers in Uganda (David et al., 2000). Due to the high cost of certified seed, farmers source seed from fellow farmers or

informal markets whose supplies come from different parts of the country. Such a seed system enhances transmission of different *P. griseola* races across bean production areas.

The occurrence of pathotype 5:55 in highland areas only could be attributed to complex interactions between the environment and crop management practices rather than virulence. Crop management practices such as continued use of one cultivar overtime lead to adaptation of pathotypes to particular bean types (Sartorato, 2004). In Uganda, farmers in highland areas prefer climbing beans to bush types due to their high yields and low disease pressure associated with the cool environment (MAAIF, 2003). The continued use of climbing bean cultivars in highlands may be responsible for presence of pathotype 5:55 only in highlands which over time could have got adapted to climbing beans dominantly grown in highlands. However, this requires further validation studies. It was also worth noting that the frequently occurring pathotypes 17:23 and 21:39 were reported for the first time in Uganda. This could be attributed to the nature of studies done on ALS previously which were continental/regional based without comprehensive/substantial coverage of bean producing areas in Uganda. For instance, among the seven and sixteen Ugandan isolates studied by Aggarwal et al. (2004) and Mahuku et al. (2002) in a regional study in eastern and southern Africa none of the isolates were in the same pathogenic class like 17:23, 21:39 identified in this study. In this study we studied 45 isolates from ten bean production districts and molecular analysis identified 30 haplophytes. This means that with more extensive isolate collection covering the whole country more pathogen variability could be revealed.

The genetic diversity of *P. griseola* fungus was studied based on molecular markers. At DNA level high diversity was exhibited, with markers defining 30 haplotypes compared to virulence, which defined 12 pathotype. This was expected because molecular markers are unrelated with pathotype diversity (Sebastian et al., 2006) suggesting that isolates of the same pathotypes might not necessarily be closely related based on DNA analysis. These results are consistent with findings by Sicard et al. (1993) and Leibenberg and Pretorius (1997) who provided evidence that there is high haplotypic diversity in *P. griseola*. The high genetic differentiation among Andean and Middle America groups revealed a strong influence of host specialisation on the population structure of *P. griseola*. It also confirmed that the sub-groups of this pathogen are highly variable and structured along host gene pools (Pastor-Corrales *et al.,* 1998). Genetic differentiation within each group was high (68.4%), indicating that sufficient genetic diversity is being maintained in the fungal population. But the sources of

genetic diversity observed among isolates were uncertain for a fungus like *P. griseola* with no sexual cycle reported (Leibenberg & Pretorius, 1997). However, factors such as mutations, migration and parasexuality (Anderson & Kohn, 1995; Brown & Wolfe, 1990; McDonald & McDermott, 1993; Zeigler et al., 1995) can interact to create or maintain high levels of genetic diversity as observed in *P. griseola*. Zeigler et al. (1995) showed evidence that high levels of haplotypic diversity can be maintained in asexually reproducing fungi through parasexual reproduction. Chromosomal diversions, deletions and loss of chromosome segement (Kristler & Miao, 1992), and the presence of transposons (Kempken & Kuck, 1998) all have the capacity to increase the diversity in fungi and contribute to high haplotypic diversity (Leibenberg & Pretorius, 1997). Seed transmission of *P. griseola* has been adequately documented (Sartorato, 2000), and introductions of new haplotypes through contaminated seed cannot be ruled out as a source of high diversity (Leibenberg & Pretorius, 1997). Since this study was unable to identify the actual cause of the high differentiation, we therefore suggest that further research is undertaken to validate whether the observed population differentiation resulted from the factors mentioned above.

The molecular results further revealed that isolate grouping was not based on places of origin. In most fungal pathogens, presence of genetically differentiated populations within the same location is as a result of geographical isolation or ecological niche preference (Klaassen et al., 2012). However, in this study *P. griseola* grouping based on place of origin was not observed probably because races from two gene pools can occupy the same ecological niche (Araya et al., 2004). Similar findings have been reported by several authors, such as Sebastian et al. (2006) who reported that different pathotypes coexisted in the production and/or geographical areas in Argentina. In additional, findings by Sartorato (2000) indicated isolates collected from the same location showed difference in their grouping pattern. This implies that different strategies that are not location specific should be employed when managing ALS in Uganda.

Several studies have reported the high genetic variability of *P. griseola* defined by different markers. For instance, studies by Sartorato (2004) estimated high genetic variability of 96 *P.griseola* using RAPD markers though the grouping was not based on geographical origin. Other studies on estimation of genetic variability have been conducted by Sebastian and Balatti (2006) in Argentina; out of 45 *P. griseola* isolates a combination of ISSR and RAPD defined 18 halophytes. Similarly, in Brazil Abdio et al. (2012) defined 27 halopytes out of the

27 isolates using ISSR markers, in our study a combination of RAMS and REP has revealed 30 halophytes indicating high variability among *P. griseola* isolates existing in Uganda.

3.6 CONCLUSION

The study confirmed the occurrence of angular leaf spot in Uganda and associated the disease with *P. griseola* as the casual agent. The disease is distributed in most bean growing regions, districts and at different altitudes. The variation in *P. griseola* was revealed by the existence of 12 pathotypes and 30 halophytes, which were defined by standard bean differential cultivars and molecular makers. Molecular markers detected more variation than differentials cultivars. The variation exhibited by this pathogen indicates that different sources of resistance will be required to manage ALS in Uganda among which will include broad host resistance. The findings of the current study do not only provide fundamental information on the distribution of *P.griseola* in Uganda, but also provide a basis for breeding strategies aimed at developing disease resistance against angular leaf spot.

CHAPTER FOUR
IDENTIFICATION OF NEW SOURCES OF RESISTANCE TO ANGULAR LEAF SPOT AMONG UGANDAN COMMON BEAN LANDRACES

4.1 INTRODUCTION

Common bean (*Phaseolus vulgaris L*.) is widely grown and consumed in Latin America, Asia and Africa. The crop is a good source of dietary protein consumed wholly without processing compared to other staple crops such as maize, rice and cassava (Buruchara et al., 2006). It is also rich in dietary fibre, minerals and vitamins (Gepts et al., 2008). Bean production is greatly affected by a number of diseases that occur in bean growing areas. Angular leaf spot (ALS), caused by a fungus *Pseudocercospora griseola* (Sacc.) is one of the most destructive diseases of common bean. It is ranked second among biotic and abiotic factors that constrain bean production in Africa (Aggarwal et al., 2003). In the Great Lakes Region, where beans are a major source of protein and calories for several communities, annual bean production is estimated at 3.67 million MT (FAOSTAT, 2013). Of the total metric tonnes of beans produced 374,800 MT are lost due to ALS (Wagara et al., 2003). Leaf and stem infections by *P. griseola* result in premature defoliation, shrivelled pods and shrunken seeds, thus reducing the yield potential of beans (Strenglein et al., 2003). In Uganda, yield losses of up to 50% have been reported among commercial varieties (Opio et al., 2001). Furthermore, late infection on pods and seeds also cause scars that reduce on seed quality and market value (Mahuku et al., 2003).

Presently, in smallholder farming systems, ALS is managed through cultural practices such as crop rotation and cultivar mixtures. However, these have limited potential in managing the disease, because land scarcity cannot allow crop rotation to be practiced (Stenglein et al., 2003). Moreover, effective methods of ALS control such as use of fungicide are far beyond the means of low resource endowed farmers. This is because of the high cost and long term consequences fungicides pose to human health and the environment (Mahuku, 2002).

The use of genetic resistance is the most appropriate, safe and cost-effective way to control ALS among smallholder farmers (Wagara et al., 2003). A number of exotic sources of ALS resistance do exist and have been utilised in breeding programs targeting ALS and they include among others Mexico 54, MAR1, MAR2, AND277, G5686, G10909 and G10474 (Mahuku et al., 2003; Caixeta et al., 2005). But their use is limited by; low adaptability and

undesirable traits. They are adapted to only environments in which they originated or were developed; this limits their use in other environments where they are not acclimatised to (Holbrook et al., 2000). Besides, majority of resistance sources are small-seeded with a climbing growth pattern; such attributes are not readily accepted by farmers in Uganda, and Africa at large (Beebe et al., 1981). But landraces maintained by farmers have for a long time been known to have useful agronomic traits. Indeed most existing resistant sources developed elsewhere, have been derived from landraces (Busogoro et al., 1999). For instance G5686, which is a good source of ALS resistance and a member of the ALS differential set, is a landrace that originated from Ecuador (Mahuku et al., 2009).

Though resistance may exist in some landraces, the high degree of genetic variability of *P. griseola* often compromises the use of ALS resistance derived from landraces (Nietsche et al., 2001). This is due to continuous emergency of new races, which break down disease resistance (Young et al., 1998). Hence, the need for continuous screening of germplasm to identify new sources of resistance that can regularly be introgressed into commercial cultivars (Young & Kelly, 1996). This will counteract the new emerging races and reinforce resistance in existing ALS resistance sources.

Nonetheless, identifying new sources of resistance alone cannot guarantee full protection of beans against ALS since resistance often breaks down (McDermott, 1993). Moreover, *P. griseola* is a highly variable pathogen with no known single resistance gene that is effective against all races. One way of developing stable resistance against such a variable pathogen, is by pyramiding several identified resistant genes into a single genotype with desirable traits. But pyramiding depends heavily on information related to inheritance and allelic relationship between resistance sources (Namayanja et al., 2006). Therefore, the study aimed at identifying new sources of resistance to ALS among common bean landraces. In addition, the study determined the mode of inheritance in the identified resistance sources, and the allelic relationship between the new and existing sources of resistance.

4.2 MATERIALS AND METHODS

Common bean germplasm used in the study was obtained from Uganda National Bean Programme (UNBP), Namulonge, and the International Centre for Tropical Agriculture (CIAT), Kawanda, in Uganda. A total of 80 bean lines (74 landraces, two checks and four commercial varieties) constituted the germplasm which was evaluated for *P. griseola*

resistance under screenhouse conditions. The landraces that were used in the study were collected from major bean growing areas in Uganda (Okii et al., 2014). The resistance and susceptible checks used included: BAT332 which is a Mesoamerican domesticated line, small-seeded and routinely used as one of the differentials for ALS (Mahuku, 2002). It is also resistant to race 61:41 (Nietsche et al., 2000) and to most Andean and Middle American *P. griseola* races (Buruchara and Buah, 1999). 'Kanyebwa', a popular landrace in Uganda, which is susceptible to ALS (Namayanja et al., 2006) was included. Commercial varieties included: K131, K132, NABE4 and NABE13, which are commonly grown in Uganda. Furthermore, four bean lines: U00297, Mexico 54, AND277 and G5686 were also used for the inheritance and allelic tests. U00297 is a small- sized (25g/100 seeds), cream-seeded landrace with determinate growth habit. K131 (MCM5001) and K132 (CAL96) are CIAT-bred lines belonging to the Mesoamerican and Andean gene pools, respectively. G5686, AND277 and Mexico 54 are resistant to races 31:0, 63:31, 63:39 of *P. griseola* with one to three genes that condition resistance and the genes are inherited in a dominant manner (Caixeta et al., 2005; Carvalho et al., 1998; Mahuku et al., 2009).

4.2.1 Screening for *Pseudocercospora griseola* resistance

4.2.1.1 Fungal isolates

Four *P. griseola* pathotypes (1:6, 21:39, 17:39 and 61:63) sourced from CIAT were used in the screening of bean lines for resistance. The pathotypes were previously characterised using a set of 12 ALS International bean differential cultivars (CIAT, 1995; Ddamulira et al., 2014). The first three isolates were Middle American type while 61:63 was Andean. These isolates also varied in terms of virulence with 1:6 and 61:63 being the least and most virulent pathotypes respectively. On the other hand, 61:63 and 17:39 are some of most virulent and prevalent *P. griseola* pathotypes in major bean growing areas in Uganda, respectively (Ddamulira et al., 2014).

4.2.1.2 Inoculum preparation

The inoculum used was extracted from monosporic cultures of four pathotypes grown on V8 agar media as described by Pastor-Corrales et al. (1998). Pure isolates were sub-cultured onto fresh media by adding 100µl of sterile water onto each plate. The spore suspension was spread onto the fresh media containing V8 agar media and incubated for 14 days at 24^0C to allow more sporulation. Plates on which isolates were grown were flooded with 100µl of sterile distilled water, and the surface scraped with a glass rod to release the conidia. The

dislodged spores in suspension were filtered through a sterile cheese cloth and conidial concentration in the suspension adjusted to 2×10^4 conidia ml^{-1} in the final inoculum suspension.

4.2.1.3 Trial set up

To identify possible sources of resistance, 80 bean lines were evaluated for ALS resistance under screenhouse conditions. Five seeds of each bean line were sown in 5-litre plastic buckets containing forest soil, lake sand, and animal manure in a ratio of 3:1:1 and replicated three times. After attaining three trifoliate leaves, plants were inoculated with spore suspension (2×10^4 spores/ml). The suspension was applied on the lower and upper surface of a leaf using a hand sprayer. The inoculated plants were placed in a humid chamber at approximately 22-28 ^{0}C with relative humidity of 95% for 4 days to allow infection to take place. The plants were then transferred into the screenhouse and watered one to two times daily depending on the sunshine intensity. The plants were evaluated for ALS resistance according to Schoonhoven and Pastor-Corrales (1987). The area under disease progress curve (AUDPC) was calculated to determine their reaction type.

4.2.1.4 Confirmation of resistance

The best way to confirm resistance to fungal pathogens is through repeated screening of materials that prove to be resistant and moderately resistant in the preliminary screening stages. Hence, out of 74 landraces, 34 bean lines which were primarily identified to be resistant and moderately resistant to the four pathotypes including, four commercial varieties and two checks (BAT332 and Kanyebwa) where re-screened twice in replicated trials. Similar isolate preparation, inoculation, plant management procedures and disease assessment used during preliminary evaluation where adhered to in confirmatory evaluation. For statistical data analysis, the area under the diseases progress curve (AUDPC) was calculated for each accession using the midpoint rule method (Campell and Madden, 1990) as shown in Equation .1 below.

$$\text{AUDPC} = \sum_{i=1}^{n=1} [(y_i + y_{i-1})/2](t_{i+1} - t_i) \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (1)$$

where “t” is time in days of each evaluation, “y’ is the disease percentage representing the infected foliage at each evaluation, and ‘n’ is the number of evaluations. Means were

generated in Genstat (Payne et al., 2011) and separated by least significant difference (LSD) at P = 0.05.

4.2.2 Inheritance of ALS resistance in landrace U00297

Based on the results from the screening, a study was designed to elucidate the resistance inheritance mechanisms in U00297. A 4 × 4 partial diallel mating design involving four parents (U00297, K132, K131, and Kanyebwa) was executed according to the Griffings (1956) method I, model I, where the parents were crossed in all possible combinations with reciprocals and ignoring selfs to generate 12 families. U00297 is resistant and the other lines are susceptible to ALS. Different susceptible parents were used to ascertain the nature of resistance genes contained in U00297 under different genetic backgrounds. Part of the generated F_1 seed was used to plant in a backcrossing program to U00297 (BC_RF_1), and to K132, K131 and Kanyebwa (BC_SF_1). Another part of the seed was selfed to generate F_2 seeds. Thereafter, seeds from the parents, F_1, F_2 and backcross populations were planted for evaluation under screenhouse conditions. The seeds were sown in 5-litre plastic pots containing forest soil, lake sand, and animal manure in a ratio of 3:1:1. The experiment was replicated three times and watered to provide moisture. Between 14-30 seeds of each parent and F_1 individuals were evaluated depending on seed availability. The number of F_2 individuals evaluated also ranged from 98 to 166 for each cross and 16 to 97 for each backcross population depending on seed availability. To determine the inheritance pattern, a Chi squared goodness-of-fit test was performed on data from crosses between U00297, K131, K132, and Kanyebwa to verify if observed segregation ratios of resistant and susceptible plants fitted the expected Mendelian 3:1, or epistatic 9:7 and 15:1 phenotypic ratios, respectively. Further still, combining ability effects and variance were also calculated according to Griffin's (1956) method 1 model 1. Parents and crosses were considered fixed effects, while replications were considered as random factors. The following model was used:

$$Y_{ij} = \mu + g_i + g_j + s_{ij} + r_{ij} + e_{ijk} \quad \cdots\cdots\cdots\cdots (2)$$

where y_{ij} is the mean phenotypic value; µ is general mean; g_i and g_j are the GCA effects of the i^{th} and j^{th} parents respectively; s^{ij} is SCA effects of the ij^{th} cross; r_{ij} is the reciprocal effect associated with the ij^{th} cross and e_{ijk} is the residual effect.

4.2.3 Allelism of resistance in landrace U0029 to existing resistances

U00297 was crossed with G5686, AND277, BAT332 and MEX54 to generate F_1 and F_2 populations. These four bean genotypes possess resistant and complementary genes, which are responsible for their resistance action. The resistance is controlled by one, two or three dominant genes (*Phg*$_{G5686A}$, *Phg*$_{G5686B}$, *Phg*$_{5686C}$, *Phg-1*, and *Phg-2*) depending on the genotype (Caixeta et al., 2005; Carvalho et al., 1998; Mahuku et al., 2009; Namayanja et al., 2006). Most of these genes are inherited in monogenic and dominant manner (Caixeta et al., 2003; Namayanja et al., 2006). In the course of crossing, the crosses involving Mexico 54 and BAT332 failed and only F_1s of U00297 × G5686 and U00297×AND277 were obtained. It is probable that Mexico 54 and BAT 332 were not compatible with U00297. Seeds from parents, F_1, F_2 and backcross populations were planted for evaluation under screenhouse conditions. The seeds were sown in 5-litre plastic pots containing forest soil, lake sand, and animal manure in a ratio of 3:1:1. The experiment was replicated and watered regularly to provide the required moisture for proper growth. Thirty to forty plants of each parent, BC_1F_1 and F_1 individuals were evaluated. The number of F_2 individual plants ranged from 50-157 depending on seed availability.

To test for allelic relationship between resistance sources, segregation ratios for each R × R progeny were computed. Genetic hypotheses were tested for significance for each population using the chi- squared goodness-of-fit test to determine the deviation of observed frequencies from the hypothesized ratios.

4.2.4 Data collection and analysis

Disease severity data was collected on the disease progress development for 21 days at an interval of three days, using the CIAT 1–9 visual scale (Schoonhoven and Pastor-Corrales 1987), described as follows: 1, plants with no visible disease symptoms; 3, presence of a few small non- sporulating lesions that cover approximately 2% of the leaf surface; 5, plants with several small lesions with limited sporulation and covering approximately 5% of leaf surface; 7, plants with abundant and generally large sporulating lesions covering approximately 10% of leaf surface and associated with chlorosis and necrosis; 9, 25% or more of leaf surface with large sporulating and often coalescing lesions, frequently associated with chlorosis resulting in severe and premature defoliation. The data was evaluated using the method of Redman et al., 1969 where the disease foliage of several plants were converted to single values. The area under disease progressive curves (AUDPC) was calculated from the single

values. Individual plants for each bean line were considered resistant (R) for AUDPC value symptom scores ≤ 13.5, intermediate resistant (IR) for AUDPC 13.5-27 and susceptible (S) for AUDPC > 27. The data obtained was subjected to analysis of variance using Genstat compute package (Payne et al., 2011) mean values were separated using the least significance difference at the 5% level of probability and the two mean values were declared significantly different when the difference between them was greater than the LSD. Segregation data of F_2 population were subjected to qualitative genetic analysis using a chi-square test (x^2). The observed phenotypic segregations were compared to the expected Mendelian segregation ratios.

4.3 RESULTS

4.3.1 Screening for *Pseudocercospora griseola* resistance

In a first screening, AUDPC ranged from 30.2 - 40.5 among 74 landraces. Analysis of variance for AUDPC among these landraces indicated that there were significant AUDPC differences ($P < 0.05$) among these landraces for each of the four *P. griseola* pathotypes (1:6, 17:39, 21:39 and 61:63) (Table 6). Out of 74 landraces screened,14% were rated as resistant (< 13.5) with no symptoms observed on the leaves, 22% were moderately resistant (13.5-27.0) having small lesions on leaves with limited sporulation, while 54% were considered to be susceptible (> 27.0) to *P. griseola*. On the other hand, significant ($P < 0.05$) differences for AUDPC among commercial varieties were observed only for two pathotypes, 1:6 and 17:39 (Table 7).

Table 7. Analysis of angular leaf spot severity on 74 landraces and 4 commercial bean varieties under screenhouse conditions at Kawanda based on four *Pseudocercospora griseola* isolates in 2011.

Pathotype	Landraces					Commercial varieties				
	DF	MS Severity	AUDPC	SED =0.05	CV %	DF	MS Severity	AUDPC	SED =0.05	CV %
1:6	73	688.8**	30.2	3.39	13.8	3	689.1**	28.4	2.90	12.5
17:39	73	381.6**	32.7	8.29	31.1	3	111.1**	42.2	2.34	20.1
21:39	73	334.1**	34.5	5.53	19.6	3	NS	29.3	9.70	40.5
61:63	73	169.6**	40.5	6.45	19.5	3	NS	28.1	6.20	25.0

** P<0.01, NS- not significant P >0.05, CV-coefficient of variation, MS- Mean square of ALS severity, DF-Degrees of freedom, SED-standard error of difference,AUDPC-area under disease progressive curve

Reaction of 34 landraces (which were resistant or moderately resistant to four pathotypes in the first screening trial) to inoculation of individual pathotypes was significantly ($P < 0.05$) different (Table 8). The AUDPC values for pathotypes 1:6, 21:39, 17:39 and 61:63 ranged from 4.5-40.5, 9- 32.8, 5.8-36.9 and 12.9-35.2, respectively. Most landraces (62.5%) were resistant (<13.5) to pathotype 1:6; but majority were susceptible (70%) to pathotype 61:63, which is among the most virulent pathotype in Uganda. Forty-seven percent of the screened bean lines were moderately resistant (rating 13.5 - 27.0) to pathotype 21:39, while 17.5% of screened bean lines were moderately resistant to 1:

Table 8. Reaction of 40 common bean lines to inoculation with four *Pseudocercospora griseola* pathotypes under screenhouse conditions at Kawanda in 2010

Bean lines	**ALS REACTION**							
	1:6		**21:39**		**17:39**		**61:63**	
	AUDPC	**RC**	**AUDPC**	**RC**	**AUDPC**	**RC**	**AUDPC**	**RC**
Landraces								
U0041	9.0	R	22.5	I	14.0	I	27.6	S
U0074	4.5	R	9.0	R	9.0	R	34.2	S
U351	9.0	R	13.5	R	13.5	R	28.3	S
U0066	9.0	R	13.5	R	31.5	S	28.7	S
U1-9	7.2	R	13.5	R	10.8	R	33.1	S
U0077	9.0	R	18.0	I	18.9	I	33.2	S
U614	13.5	R	14.8	I	21.6	I	31.6	S
U620	9.0	R	25.2	I	14.4	I	29.2	S
U0082	14.8	I	13.5	R	9.0	R	22.3	I
U204	19.3	I	25.2	I	31.5	S	32.3	S
U00335	9.0	R	13.5	R	15.5	I	28.4	S
U0043	11.7	R	19.5	I	8.55	R	18.9	I
U284	5.8	R	20.7	I	8.55	R	17.4	I
U608	10.4	R	20.7	I	30.1	S	30.8	S
Masindi yellow	7.2	R	32.8	S	14.4	R	23.1	I
U650	38.7	S	16.2	I	36.9	S	32.4	S
U342	13.5	R	23.8	I	27.5	S	24.4	I
U00297	9.0	R	13.5	R	7.6	R	12.9	R
U00101	5.9	R	31.5	S	19.8	I	32.4	S
U274	14.8	I	23.8	I	24.7	I	28.9	S
U0049	14.4	I	14.9	I	12.2	R	14.7	I
U0068	36	S	30.1	S	20.3	I	32.2	S
U0070	9.0	R	37.3	S	18.0	I	35.2	S
U0080	11.7	R	27.0	I	24.3	I	33.2	S
U0083	13.5	R	28.3	S	29.7	S	32.7	S
U0085	16.2	I	30.1	S	5.82	R	20.8	I
U00212	9.0	R	16.2	R	16.2	R	15.7	I
U609	13.5	R	22.5	I	27.0	I	27.8	S

U653	19.4	I	34.2	S	24.7	I	29.1	S
U659	40.5	S	31.5	S	27.5	S	33.3	S
U0010	40.5	S	14.8	I	29.3	S	34.8	S
U635	11.7	R	19.3	I	17.6	I	29.1	S
U0053	4.5	R	18.0	I	17.1	I	24.4	I
U1-5	25.2	I	23.8	I	36.4	S	32.4	S
Checks								
BAT332	4.5	R	4.5	R	5.5	R	4.0	R
Kanyebwa	27.4	S	30.2	S	37.8	S	32.2	S
Commercial varieties								
K131	37.3	S	27.4	S	28.5	S	32.7	S
K132	28.4	S	29.7	S	28.8	S	33.2	S
NABE13	10.8	R	16.7	I	12.6	R	18.4	I
NABE4	28	S	31.2	S	32.9	S	33.7	S
Mean	15.7		21.8		20.5		27.5	
$LSD_{(0.05)}$	6.3		12.1		15.3		16.0	
CV%	26.4		37.6		34.8		46.2	

AUDPC=Area under disease progress curve, RC=Resistance conditions, R=Resistant, S=Susceptible, I=intermediate resistance

Apart from the resistant check (BAT332), only landrace U00297 was resistant to all the four pathotypes. Three landraces (U0074, U351 and U1- 9) were resistant to three pathotypes, but susceptible to the most virulent pathotype 61:63. It was also observed that most commercial varieties were susceptible to the four pathotypes, except one recently released commercial variety NABE13, which was resistant to 1:6 and 17:39, moderately resistant to 21:39, but susceptible to 61:63.

4.3.2 Inheritance of resistance to *Pseudocercospora griseola*

U00297 was resistant (AUDPC < 13.5) to pathotypes 17:39, 21:39 and 61:63, while parents K131, K132 and Kanyebwa were all susceptible (AUDPC >13.5) to the same pathotypes (Table 8). Pathotype 1:6 was excluded from those used for inheritance study due to loss of viability that led to no observable disease symptoms appearing on plants inoculated with it. Most F_1 plants grew healthy with no visible disease symptoms', suggesting that ALS resistance is inherited in a dominant manner. Nonetheless, the F_1 cross U00297×K131 was susceptible to 61:63 and U00297×K131 and U00297 x Kanyebwa were susceptible to 21:39 (Table 9).

Table 9. Reaction of parents and F_1 progenies to inoculation of pathotypes 61:63, 17:39 and 21:39 under screenhouse conditions at Kawanda in 2012.

Parents	Pathotype	Resistant	Susceptible	Total
U00297	61:63	30	0	30
K131	61:63	0	29	29
K132	61:63	0	30	30
Kanyebwa (KB)	61:63	0	31	31
U00297	17:39	30	0	30
K131	17:39	0	29	29
Kanyebwa (KB)	17:39	0	29	29
U00297	21:39	30	0	30
K131	21:39	0	29	29
Kanyebwa (KB)	21:39	0	30	30
F_1(K131 x U00297)	61:63	0	26	26
F_1(K132 x U00297)	61:63	23	0	23
F_1(KB x U00297)	61:63	19	0	19
F_1(K131 x U00297)	17:39	17	0	17
F_1(K132 x U00297)	17:39	24	0	24
F_1(KB x U00297)	17:39	26	0	26
F_1 (K131 x U00297)	21:39	0	14	14
F_1 (KB x U00297)	21:39	0	17	17

The chi-square test indicated that segregation of ALS resistance in F_2 population of crosses KB × U00297 and K131 × U00297 when inoculated with 61:63 and 17:39 fitted the tested ratio 9:7, respectively (Table 10). The best fit to 9:7 in these crosses suggests that they segregated for at least two genes. In contrast crosses K132 × U00297 and K131 × U00297 when inoculated with 61:63 and 21:39, respectively, exhibited segregation ratio of 7:9, suggesting the presence of complementary epistatic gene interactions (Table 9). F_2 populations (K132 × U00297 and KB × U00297) fitted the test ratio of 3:1 when inoculated with 17:39. But cross KB × U00297 failed to fit the same test ratio when it was inoculated with 21:39 (Table 10). This possibly suggests test ratios differ depending on the pathotype being used. The segregation ratios in the backcross populations fitted the expected segregation ratios 1:1 and 1:0 respectively, except for the back cross with resistant parent (U00297) when it was inoculated with 17:39 (Table 10).

Table 10. Reaction of F_2, back cross progenies to inoculation of three *P. griseola* pathotypes under screenhouse conditions at Kawanda

Populations	Pathotypes	Total no. of plants	Observed plants		Expected ratio		
			R	S	R:S	x^2	P-value
F_2 (K131 x U00297)	61:63	157	72	85	7:9	0.2839	0.5940
F_2 (K132 x U00297)	61:63	166	74	92	7:9	0.0462	0.8296
F_2 (KB x U00297)	61:63	77	47	30	9:7	0.7176	0.3969
BC_{K132}	61:63	58	27	31	1:1	0.2759	0.5994
BC_{K131}	61:63	53	25	28	1:1	0.1698	0.6803
BC_{KB}	61:63	61	32	29	1:1	0.8251	0.3637
BC_{U00297}	61:63	47	45	2	1:0	0.0957	0.1915
F_2 (K131 x U00297)	17:39	98	62	36	9:7	1.9598	0.1615
F_2 (K132 x U00297)	17:39	98	70	28	3:1	0.6663	0.4142
F_2 (KB x U00297)	17:39	157	123	34	3:1	0.9363	0.3332
BC_{K132}	17:39	59	28	31	1:1	0.0763	0.1525
BC_{K131}	17:39	67	35	32	1:1	0.1343	0.7140
BC_{KB}	17:39	54	29	25	1:1	0.2963	0.5862
BC_{U00297}	17:39	97	95	2	1:0	0.0000	0.0412
F_2 (K131 x U00297)	21:39	102	41	61	7:9	0.5234	0.4693
F_2 (KB x U00297)	21:39	111	72	39	3:1	6.0810	0.0136
BC_{K132}	21:39	47	25	22	1:1	0.0000	0.0851
BC_{K131}	21:39	64	30	34	1:1	0.2500	0.6171
BC_{KB}	21:39	81	42	39	1:1	0.1111	0.7389
BC_{U00297}	21: 39	16	16	0	1:0	0.0000	1.0000

R: resistant, S: susceptible, Chi-square P- values greater than 0.05 indicate that the observed values were not significantly different from the expected value

The results indicated both general combining ability (GCA) and specific combining ability (SCA) were highly significant ($P < 0.001$). The GCA effects associated with resistant parent revealed that the effect of U00297 was negative and highly significant for resistance to both 61:63 and 17:39 (Table 11). U00297 exhibited good combining ability for resistance to both pathotypes. Crosses K131 × U00297 and K132 × U00297 had significant negative SCA effects for resistance to 61:63 and 17:39 pathotype (Table 11), which confirmed their tendency to resist the two pathotypes. On the other hand, cross Kanyebwa × U00297 was a specific cross for resistance to 61:63 and 17:39 as evident from its significant and positive SCA effects (Table 11).

Table 11. GCA and SCA effects of parental lines of crosses with their reciprocal values for resistance to angular leaf spot

	Pathotype 61:63				Pathotype 17:39			
Male	**U00297**	**K131**	**K132**	**Kanyebwa**	**U00297**	**K131**	**K132**	**Kanyebwa**
Female								
Parent means	**2.00**	**6.50**	**6.17**	**6.67**	**2.00**	**5.92**	**5.42**	**6.33**
U00297	-0.58	-0.65*	-0.04**	0.03**	-0.92	-0.88*	-0.33*	0.24**
K131	*0.04*	-0.58	-0.65	0.03	*-0.13*	-0.92	-0.88	0.24
K132	*-0.04*	*0.04*	-0.51	0.04	*0.34*	*-0.13*	-0.03	-0.33
Kanyebwa	*-0.08*	*0.13*	*0.04*	-0.51	*-0.13*	*0.13*	*-0.34*	-0.03
Parental GCA's	**-1.68****	**0.23**	**0.07**	**0.25**	**-1.92****	**1.36****	**0.07**	**4.40**

SCA and reciprocal values appear in the upper and lower triangles in italics respectively. *, ** Significance of the effect from zero at 0.05 and 0.01 levels of probability

4.3.3 Testing allelic relationship between resistance genotypes

The allelic relationship between angular leaf spot resistance gene in landrace U00297 and other resistance genes previously characterised in cultivars G5686, AND277 and Mexico 54 are presented in Table 12. The segregation of ALS resistance in the allelism test fitted 15 resistant: 1 susceptible and 63 resistant: 1 susceptible ratio, which exhibited the action of dominant genes conferring resistance to 17:39, 21:39 and 61:63. The chi-square x^2 values showed a good fit for a segregation ratio of 15 resistant to 1 susceptible in three F_2 populations from crosses U00297×G5686, U00297×AND 277 and G5686×AND277 (Table 12), which demonstrates the presence of two dominant genes that confer resistance to pathotypes 17:39 and 21:39 of *P. griseola*. These results support the hypothesis that the gene conferring resistance to pathotypes 17:39 and 21:39 of this fungal pathogen, present in U00297, is independent from other genes (*Phg-1, Phg_{G5686A}*), harboured in AND277 and G5686, respectively.

In addition, the allelism test applied to the cross AND277 × G5686 had a segregation ratio of 63R:1S, which exhibited the action of three dominant genes that confer resistance to pathotype 61:39. This also indicated independence of AND277 genes from *Phg_{G5686A}*, *Phg_{G5686B}* and *Phg_{G5686C}* genes. No susceptible plants were observed in the population from the cross Mexico 54×AND277, which indicated that the resistance gene in the two cultivars co-segregate and are either in same locus or are closely linked genes. On the other hand, all the G5686×Mexico 54 crossed flowers aborted probably due to incompatibility as described by Gepts and Bliss (1985).

Table 12. Reaction of F_2 progenies derived from resistant parents to inoculation of 61:63, 17:39 and 21:39 *Pseudocercospora griseola* pathotypes under screenhouse conditions at Kawanda

F_2 populations	Pathotypes	Total No. plants	Observed plants		Expected ratio		
			R	S	R:S	x^2	P-value
G5686 x U00297	61:63	104	102	2	63:1	0.1049	0.7460
AND 277 x U00297	61:63	111	105	6	15:1	0.0567	0.8119
AND 277 x Mexico 54	61:63	85	85	0	15:1	5.6667	0.0173
AND 277 x G5686	61:63	103	97	6	15: 1	0.0026	0.9791
G5686 x U00297	17:39	100	92	8	15:1	0.9131	0.3393
AND 277 x U00297	17:39	94	88	6	15:1	0.0456	0.8308
AND 277 x Mexico 54	17:39	97	97	0	15:1	6.4667	0.0110
AND 277 x G5686	17:39	98	92	6	15:1	0.0110	0.9163
G5686 x U00297	21:39	56	51	5	15:1	1.0735	0.3002
AND 277 x U00297	21:39	108	101	7	15:1	0.0735	0.7835
AND 277 x Mexico 54	21:39	96	96	0	15:1	6.4000	0.0114
AND 277 x G5686	21:39	105	98	7	15:1	0.1269	0.7216

R: resistant, S: susceptible, chi-square P values greater than 0.05 indicate that the observed values were not significantly different from the expected value

4.4 DISCUSSION

Developing resistant bean cultivars partly depends on nature of genotypes as well as variability expressed by the disease-causing pathogen. Since most fungal diseases are spread by highly variable pathogens, it is important to continuously diversify sources of resistance as a strategy to control these diseases and rationalise the breeding process; angular leaf spot is one of such diseases. In this study, landrace U00297 was identified to be resistant to four *P. griseola* pathotypes 1:6, 17:39, 21:39 and 61:63 under screenhouse conditions. In some genetic backgrounds, resistance in U00297 is conferred by a single dominant gene, which is independent of resistance genes found in cultivars AND277 and G5686, while in others, resistance was due to epistatic gene interaction involving two or three genes. Resistance in U00297 was successfully transmitted into certain F_2 progenies. This is evident in the study by F_2 plants which were resistant to *P. griseola* pathotype 17:39.

The screening process revealed variation in reaction of bean lines to Ugandan *P. griseola* pathotypes. Only U00297 was resistant to four pathotypes, indicating low levels of resistance in other bean lines evaluated. Nonetheless, U00297 can be a good source of resistance, which can supplement other existing resistance sources in developing durable ALS resistance. Given the fact that U00297 is resistant to pathotypes 17:39 and 61:63, which are the most prevalent and virulent pathotypes in Uganda, it constitutes a resistant source that can provide the desired resistance to commercial bean varieties in Uganda, which are known to be susceptible to ALS (Opio et al., 2001). These findings were in line with earlier studies by Mahuku et al. (2002), which also identified four bean accessions in a core bean collection that were resistant to pathotype 63:63 (one of the most known virulent pathotypes that overcomes resistance in all 12 differential bean cultivars) under screenhouse conditions. In the same way, Wagara et al. (2003) identified 13 bean genotypes that were resistant to at least 40 *P. griseola* pathotypes in Kenya. Therefore, U00297 has a potential of being used to improve resistance against ALS among susceptible commercial bean varieties in Uganda.

Though commercial varieties are routinely screened for ALS resistance during variety development, findings in this study revealed that most of them were susceptible to *P. griseola*. This was in support of earlier work by Opio et al. (2001), which indicated that 50% of commercial varieties in Uganda were susceptible to ALS. Susceptibility among commercial bean varieties was possibly attributed to breakdown of host resistance by the pathogen as commercial varieties become increasingly used by farmers (McDermott 1993). Because of the inherent evolutionary variability of *P. griseola*, over time new strains develop that overcome the resistance in commercial varieties (Pedro et al., 2006). This was reflected in this study when newly released variety NABE13 was resistant to two pathotypes, while popular varieties, such K132, K131 and NABE 4, which were released much earlier and are commonly used by farmers (Kalyebara et al., 2005), were all susceptible to the four pathotypes. This implied that even with newly released varieties, resistance breakdown is likely to be experienced over time. Hence, the need for regular monitoring of disease resistance in released varieties. This could facilitate the process of genetic improvement of newly released bean varieties for resistance against ALS.

One approach that ensures continued improvement of ALS resistance in bean varieties is through understanding the inheritance and segregation pattern in new sources of resistance. This is pertinent in breeding because it offers breeders an opportunity to design strategies that

maximise efficiency in developing improved resistant cultivars. This study showed that F_1 plants were resistant to most pathotypes, suggesting that resistance in U00297 is inherited as a dominant trait. The monogenic inheritance of resistance indicates that pedigree or backcross breeding would be adequate to transfer resistance to susceptible lines. Similarly, segregation for resistance in F_2 K132 × U00297 and KB × U00297 populations were consistent with a ratio 3:1 as resistant: susceptible, which further confirmed that U00297 resistance to pathotype 17:39 was due to a single dominant gene. The dominant nature of resistance in U00297 cultivar revealed that resistance transfer into KB and K132 is possible through conventional breeding provided that both alleles to be transferred are dominant alleles. Muthoni et al. (2011) and Caixeta et al. (2003) reported similar resistance inheritance pattern in other ALS resistant sources. Similarly, inheritance to *P. griseola* in Mexico 54 and BAT332 is also reported to be monogenic with a single dominant gene effect. Mahuku et al. (2009) also reported that ALS resistance in bean cultivar G5686 to pathotypes 31-0 was conditioned by a single or three dominant genes. However, previous inheritance studies have revealed that resistance to *P. griseola* is conditioned by few genes that can either be recessive or dominant depending on the cultivar used as a susceptible parent (Carvalho et al., 1998). In this study it was observed that segregation ratios in F_2 population deviated from the expected ratios indicating that resistance of U00297 to pathotypes 21:39 and 61:39 involved digenic epistatic gene interactions. The GCA and SCA were significant determinants of resistance for some parents. This indicated the critical role both additive and dominance or epistatic components play in the inheritance of ALS resistance. GCA was more pronounced than SCA for resistance, thus procedures that emphasise use of additive effects for the incorporation of resistance should enhance genetic gain from selection during bean improvement. But it should be noted that both additive and dominance appear to be effective in transmitting genes conditioning ALS resistance. Analysis of GCA for parents provides breeders with useful information on the average performance of a line in hybrid combinations (Ana and Staub, 2002). Such analyses are important because they provide an indication of genetic difference that exist among lines being evaluated and the importance of genes with largely additive effects. Earlier studies have shown the influence of additive and dominance effects on ALS resistance expression in bean cultivars BAT322 and KBT (Fivawo et al., 2013). The same authors reported the predominance of additive variance over dominance variance in ALS resistance expression, which concurs with this study findings.

Furthermore, parents with higher GCA estimates for other traits such as yield are used for the constitution of new populations, aiming at attaining high genetic progress in breeding programs. However, for the case of disease resistance evaluation, the interest concentrates on genotypes with lower severity of the disease, or either, genotypes that contribute to diminishing character expression through showing negative estimates of GCA (Cruz & Regazzi, 2001). In this study, the negative GCA values indicated the contribution towards *P. griseola* resistance in common bean, as observed in resistant parent U00297. In contrast, positive estimates were observed on susceptible (K132, K131 and Kanyebwa) parents. Kanyebwa presented the most unfavourable general combining abilities estimate. It is therefore, one of the parents with the lowest capacity to contribute to resistance alleles to the genetic pool under study. Specific combining ability is the deviation from the performance predicted on the basis of general combining ability. The SCA effects are an important criterion for the evaluation of crosses that will eventually be used to develop hybrids. Two crosses had high negative and significant SCA effects: K131 × U00297 and K132 × U00297, which indicated the presence of non–additive gene effects for resistance to pathotype 61:63 and 17:39, respectively. It is probable that either one of the parents in theses crosses possesses some dominant resistance genes or that epistasis among disease resistance loci was involved. U00297 possesses a dominant gene for resistance in these crosses as exhibited earlier by the segregation ratios observed in F_2 plants. The SCA effects for Kanyebwa × U00297 were positive and significant, indicating non-additive, epistatic gene action governing susceptibility to 61:63 and 17:39 resistance. In our study, G5686 × U00297 (R × R) yielded a ratio of 63R:1S in the F_2 generation, when inoculated with pathotypes 61:63, suggested a segregation of three unlinked resistance genes. Because Mahuku et al. (2009) posited the existence of three resistance genes in G5686 and one of them being shared between G5686 and U00297.

4.5 CONCLUSION

The study identified the landrace U00297 as a potential source of resistance to two *P. griseola* pathotypes, including the two most virulent and prevalent pathotypes found on common bean in Uganda. Resistance to pathotype 17:39 in landrace U00297 is inherited in a dominant manner. It is possible to adequately transfer this resistance into genotypes K132 and Kanyebwa using pedigree breeding. Based on GCA results, U00297 is a good combiner and an effective source of resistance to pathotype 17:39 and 61:63, while SCA values for

U00297 crosses with genotypes K131 and K132 indicated presence of non-additive gene effects for resistance to pathotypes 61:63 and 17:39. The resistant gene which confers resistance in U00297 is independent of resistance genes harboured by genotypes AND277 and G5686. This information will aid breeding programs targeting improving resistance to ALS using U00297 as the parent.

CHAPTER FIVE
DEVELOPING RESISTANCE TO ANGULAR LEAF SPOT THROUGH GENE PYRAMIDING IN COMMON BEAN

5.1 INTRODUCTION

Angular leaf spot (ALS) caused by *Pseudocercospora griseola* is a major fungal disease limiting common bean (*Phaseolus vulgaris L.*) production (Jarvie, 2002). The disease often occurs under mild temperature (16-28^{0}C) and high relative humidity (75-100%). Such conditions, coupled with the use of susceptible cultivars, predispose beans to ALS attack, leading to yield losses of up to 50% among released varieties and popular landraces in Uganda and elsewhere (Opio et al., 2001; Sanglard et al., 2013). Despite the high prevalence of ALS, the current disease control measures, such as crop rotation, cultivar mixtures and use of fungicides (Deeksha et al., 2009) have little or no impact on the disease. Moreover, these 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 is already embedded in the seed that farmers plant (Mahuku et al., 2009).

A few ALS resistant sources such as genotypes AND277, Mexico 54, and G5686 have been identified (Caixeta et al., 2005) and utilised in improving resistance globally. Genetic studies on these sources revealed that genotype AND277 is resistant to eight races of *P. griseola*, that is; 31:17, 31:39, 61:31, 63:19, 63:23, 63:31, 63:35, and 61:41 (Caixeta et al., 2005), while Mexico 54, BAT332, and G5686 are resistant to pathotypes 63:39 and 31:0 (Caixeta et al., 2003; Namayanja et al., 2006; Mahuku et al., 2009). These sources of resistance can be useful in facilitating the process of transferring ALS resistance into susceptible farmer preferred Ugandan bean cultivars. But despite the existence of such sources of ALS resistance, their use is limited because some of them like Mexico 54 and BAT332 are of Mesoamerican gene pool with low adaptability and undesirable traits. The sources of resistance are more adapted to conditions in areas where they were developed (Abawi & Pastor Corrales, 1990). For instance, Mexico 54 is a climber and BAT332 is small-seeded; such traits are not accepted by farmers in Africa (Beebe et al, 1981; Mukankusi, 2008) and not easily camouflaged in breeding populations. Although genotypes AND277 and G5686 belong to the Andean gene pool, which are medium to large-seeded cultivars, their use is constrained by the low yields they produce, compared to popular landraces and commercial varieties (Anonymous, 2009).

All these factors consequently limit the development and release of resistant cultivars from available ALS resistance sources in Uganda.

Furthermore, durable resistance within the existing resistance sources is challenged by pathogen variability. Due to *P. griseola* 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 pathotypes of ALS; hence protection conferred by a single gene against a hypervariable pathogen is often short lived (Mahuku et al., 2002). Considering that Uganda has several pathotypes (Ddamulira et al., 2014), a breeding technique such as gene pyramiding would be appropriate in addressing pathogen variability as well as other constraints such as pest resistance.

Pyramiding resistance genes into a single genotype is one of the practical approaches through which durable resistance can be achieved (Castro et al., 2003). Gene pyramiding has been applied successfully in concentrating multiple genes into single cultivars to control diseases such as bacterial blight (Huang et al., 2004) and blast (Hittalmani et al., 2000) in rice;and soybean mosaic virus in soybean (Shi et al., 2008). The technique has, however, not been explored in developing durable resistance against ALS in common bean. In other crops, through gene pyramiding, synergistic interactions between genes may occur such that resistance gene combinations are higher than the sum of resistance conditioned by individual genes (Obala et al., 2012, Shi et al., 2008, Huang et al., 2004). Presently, it is not known whether resistance genes from the different ALS resistance sources, once pyramided into a single bean cultivar, would increase the level of resistance in susceptible cultivars or would have negative effects on other parameters of the crop. The aim of the study was therefore, to determine the effectiveness of pyramided resistance genes in improving levels of ALS resistance in susceptible common bean cultivars and how they interact with each other.

5.2 MATERIALS AND METHODS

The study was conducted at CIAT, Kawanda, in Uganda from 2010-2014. Three bean genotypes (Mexico 54, AND277, and G5686) that were previously characterised for ALS resistance and two susceptible parents (K132 and Kanyebwa) were used in this study. Genotype Mexico 54 carries gene *Phg-2*, which is responsible for resistance against race 63:39 (Gonçalves-Vidigal et al., 2011) and is linked to SCAR marker OPE04 (Sartorato et al.,

1999). AND227carries gene *Phg-1*, which is responsible for resistance against eight *P. griseola* races; 31:17, 31:39, 61:31, 63:19, 63:23, 63:31, 63:35, and 61:41 (Caixeta et al., 2005) and is linked to SSR marker TGA1.1 on chromosome Pv01 (Gonçalves-Vidigal et al., 2011). G5686 is an Andean large-seeded landrace whose origin is Ecuador (Mahuku et al., 2009). The resistance genes (Phg_{G5686A}, Phg_{G5686B} and Phg_{G5686C}) found in G5686, confer resistance to race 31:0; these genes are linked to SSR markers Pv-ag004 and Pv-cct001 on chromosome Pv04 (opposite ends) and Pv-at007 on chromosome Pv09 (Mahuku *et al.*, 2009). The two genes *Phg-2* and Phg_{G5686A} associated with resistance in AND277 and G5686, respectively, have been mapped on chromosomes Pv01 and Pv04 by markers TGA1.1 at 1.3 cM and Pv-ag004 at 0.0 cM (Gonçalves-Vidigal et al.,2011, Blair et al., 2012), respectively. The resistance in AND277 and Mexico 54 (Maria et al., 2010; Namayanja et al., 2006) is dominant while for G5686 resistance is conditioned by dominant or recessive with complementary or epistatic effects that act alone or in combination genes (Mahuku et al., 2003). One of the resistant parent used; Mexico 54 belongs to Mesoamerica gene pool while the two (AND277, and G5686) belong to the Andean. The three parents used in pyramiding are also resistant to Uganda *P. griseola* isolates 61:63 and 17:39 (Ddamulira et al., 2014). K132 commonly known and databased as CAL96 is a large-seeded red mottled variety developed by CIAT which belongs to Andean gene pool while Kanyebwa is a popular medium seeded sugar bean landrace in Uganda. Both K132 and Kanyebwa are susceptible to ALS (Namayanja et al., 2006).

In the process of pyramiding resistance from different sources, Mexico 54 was crossed with AND277 and the F_1 plants were crossed with G5686 to generate triple cross (TC) populations (Figure 5). The F_1 plants from the TC were grown in screenhouse, harvested and the seed bulked. Two sets of one hundred fifty F_2 seeds each were planted in the screenhouse and inoculated with two *P. griseola* isolates (17:39 and 61:63) independently. These two isolates used because they are the most prevalent and virulent isolates in Uganda, respectively (Ddamulira et al., 2014a). The inoculums was applied at a concentration of 2×10^4 conidia ml^{-1} at leaf stage V3 (first trifoliate leaf open and the second trifoliate leaf appears) as described by Mahuku et al. (2004). Disease symptoms on the inoculated plants were evaluated from 6 to 21 days after inoculation at three days interval. The disease response was assessed based on 1–9 rating scale, where 1 is immune and 9 is highly susceptible. Ratings of 1-3 are considered resistant, 3-6 intermediate and 6 as susceptible (Schoonhoven and Pastor-Corrales, 1987). Fifty plants that were resistant to both 17:39 and 61:63 were further screened

with molecular markers (OPEO4, TGA1.1, Pv-ctt001, Pv-ag004 and Pv-at007) to confirm the presence of five genes.

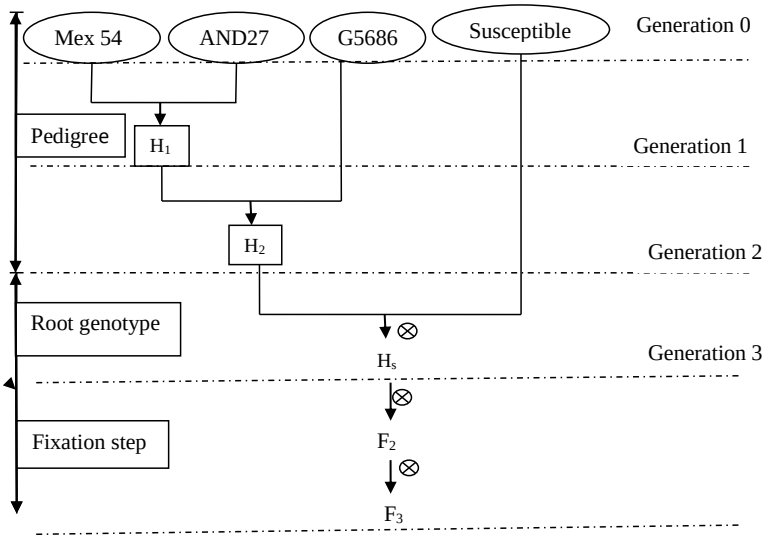

Figure 5. Cascading pedigree gene pyramiding scheme used in developing pyramided lines of common bean. H refers to hybrid created by the cross, Mex 54=Mexico 54, AND27=AND277

5.2.1 Molecular Analysis

In order to confirm the presence of five genes (*Phg-2, Phg-1* Phg_{G5686B}, Phg_{G5686A}, and Phg_{G5686C}) molecular markers SCAR OPE04 (for *Phg-2*), SSR-TGA1.1 (for *Phg-1*), SSR - Pv-ctt001 (for Phg_{G5686B}), pv-ag004 (for Phg_{G5686A}) and Pv-at007 (for Phg_{G5686C}) were used to tag the pyramided genes in the F_2 progenies. DNA was extracted from young leaves of 50 TC plants following procedures described by Mahuku et al. (2004). The extracted DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, USA). DNA concentration was adjusted to a standard concentration of 10 ng/ µl before used in the PCR reaction. PCR reactions were carried out in 20µl volumes containing 1 × DNA polymerase buffer (100 mMTris-HCl, 400 mMKCl, 15 mM $MgCl_2$, pH 9.0), 3mM $MgCl_2$, 0.4mM dNTPs, 1µM of each primer, 0.3U Taq DNA polymerase (Bioneer Inc. Korea) and 50ng of genomic DNA. DNA amplification was performed in a My cycler thermal cycler (BioneerInc, Korea) under a program of one cycle at 94 ^{0}C for 5 min, followed by 35 cycles at 94 ^{0}C for 20s, 50 ^{0}C for 40 s and 65^{0}C for 8 min, and a final 16 min extension at 65 ^{0}C.

The DNA amplicons were electrophoresed in 1.5% agarose gel for 1 h at 90V in 1XTris–borate–EDTA buffer (89mM Tris base, 89mM boric acid–borate and 2mM EDTA pH 8.0) and later stained for 20 min in 0.5µg/ml ethidium bromide. Gel images were captured using the GeneSnap gel documentation system (SynGene, Frederick, MD, USA).

Table 13. Parents and F_2 population with and without angular leaf spot resistant genes

No. entries	Pedigree	Molecular makers				
		OPE04	***TGA1.1***	***Pv-ctt001***	***Pv-ag004***	***Pv-at007***
3	F_2TC	+	+	+	+	-
37	F_2TC	+	-	+	-	-
10	F_2TC	-	+	+	+	-
15	Mexico 54	+	-	-	-	-
15	G5686	-	-	+	+	+
15	AND277	-	+	-	-	-
15	K132	-	-	-	-	+
15	Kanyebwa	-	-	-	-	-

(+) presence, (-) absence of the gene, TC triple cross

Through combined phenotypic and molecular screening, three plants were found to have four genes (*Phg-2, Phg-1*, $Phg_{G5686B,}$ Phg_{G5686A}), all detected by races 17:39 and 61:63 and genes linked with markers OPEO4, TGA 1:1, Pv-ctt001 and Pv-ag004. Nonetheless gene Phg_{G5686C} was not detected because marker Pv-at007 linked to it was polymorphic when evaluated on G5686 and K132 but when it was used to amplify DNA from F_2 individuals of TC the results were not conclusive. The three selected plants with four genes were advanced to F_3 by single seed descend and then crossed with susceptible parents; K132 and KAN to form four-parent cross (FPC) populations: KAN x [(Mexico 54 x AND277 x G5686)] and K132 x [(Mexico 54 x AND277 x G5686)]. In all crosses, the susceptible cultivars were used as female parents. Part of FPC F_1 seeds were retained for planting F_1controls and the other portion was advanced to the F_2 through selfing.

Besides, in generating single crosses (SC), each resistant parent; AND277, G5686, Mexico 54 used in gene pyramiding were crossed between themselves (AND277 x Mexico 54, AND277 x G5686 and Mexico 54 x G5686) to generate R x R crosses, while resistant and susceptible parents were also crossed to generate S x R crosses: (K132 x AND277, K132 x

Mexico 54, K132 x G5686, Kanyebwa x AND277, Kanyebwa x Mexico 54 and Kanyebwa x G5686. In all crosses involving resistant and susceptible parents, K132 and Kanyebwa were used as female parents. Part of the F_1 from SC was retained and the rest was advanced to F_2 generation for phenotypic evaluation.

5.2.2 Phenotypic screening for angular leaf spot resistance

The parents (Kanyebwa, K132, AND277, G5686 and Mexico 54) involved in all the populations, SC, TC, FPC, F_1 and F_2 progeny seeds were planted in 5-litre bucket in the screenhouse. A randomised complete block design with three replications was adopted for parents and SC F_1. The plants were divided into two equal sets; one set was inoculated with isolate 17:39 and the other with 61:63. The isolates were inoculated at a concentration of 2 x 10^4 conidia ml^{-1} as described by Mahuku et al. (2004). Disease symptoms on plants inoculated with 61:63 were evaluated from 6-21 days after inoculation at 3 day interval. A 1–9 scale described by Schoonhoven and Pastor-Corrales, 1987 was used to score disease symptoms. However, disease symptoms score data on plants inoculated with 17:39 was not reported because no symptoms were observed on plants inoculated with this race. Even the susceptible parents (Kanyebwa and K132) inoculated with this isolate did not express disease symptoms an indication that over time 17:39 isolate could have lost its viability.

5.2.3 Data analysis

To estimate the number of pyramided genes among the progeny lines a Mendelian analysis of segregating populations of plants was carried out. The F_1 and F_2 progenies in R x R crosses were categorized into resistant (score of 1-3) and susceptible (score of 4-9). Two, three and four-gene models were developed by taking into consideration the differences in the segregation patterns of the SC F_1 and F_2, as well as TC F_1 and F_2 generations.

Before conducting the χ^2 goodness-of-fit tests, homogeneity of ratios test was performed to assess the difference in segregation between the three replications. The χ^2 test of homogeneity was based on the Mather (1957) model. Where the homogeneity of ratios test indicated no difference in the segregation pattern of a cross between the two replications, data from the replications were pooled prior to χ^2 goodness of-fit test. The χ^2 value for goodness-of-fit test was calculated using the model of Mather (1957). Means of parents and progenies were compared to provide insight on the types of gene action conditioning ALS resistance in both the R x R and S x R populations. Comparisons between means of FPC and SC (S x R)

populations were used to determine the effect of pyramided resistance genes. Means were computed using the restricted (residual) maximum likelihood (ReML) analysis in GenStat (Payne et al., 2011). Where the mean squares from ReML analysis indicated significant genotype effects, means were compared using a Student t-test for each pairwise comparison of interest, based on the standard error of the difference (SED) for that specific pair of entries. The Student t-test was used due to unequal number of individuals among genotypes tested (Gomez & Gomez, 1984).

5.2 RESULTS

5.2.1 Estimate of resistance genes in R x R crosses

In single (SC) and triple (TC) crosses the distribution was normal, though skewed towards resistance (Fig. 6). In all the crosses the distribution of plant resistance against isolate 61:63 grouped the tested plants in two distinct phenotypic classes.

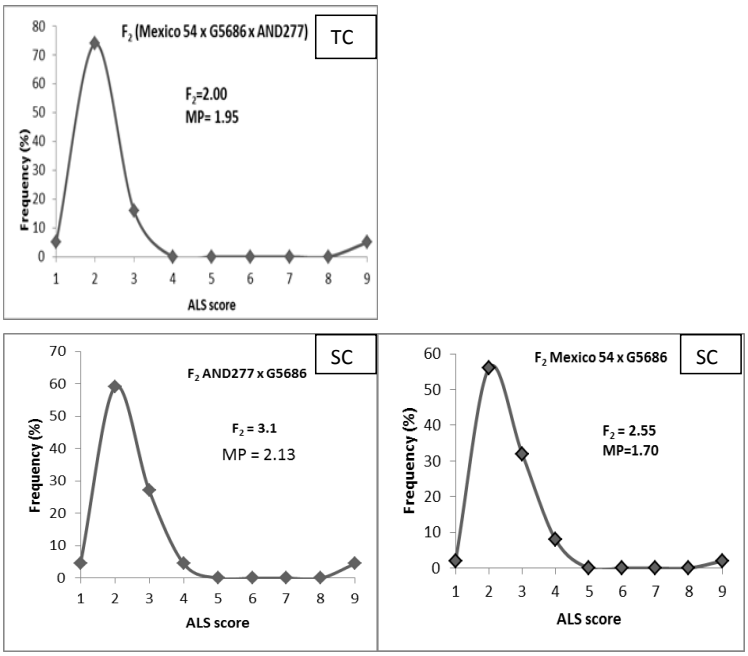

Figure 6. Frequency distribution of angular leaf spot scores in populations of TC (triple cross) and SC (single cross) cross mating of common bean genotypes resistant to angular leaf spot.

Bean plants were categorized into resistant (R) and susceptible (S) classes (Table 13). The F_2 populations of AND277 x G5686 and Mexico 54 x G5686 single crosses fitted 15:1 and 61:3

segregation ratios; accept Mexico 54 x AND277 population which did not fit either ratios (Table 14). Similarly F_2 population of [(Mexico 54 x AND277) x G5686)] did not fit 15:1 and 63:1 ratios but fitted 247:9 and 249:7 segregation ratios (Table 14).

Table 14. Observed vs hypothesized phenotypic class frequencies for resistant and susceptible reaction to *Pseudocercospora griseola* in F_1 and F_2 single and triple R x R cross populations

Cross	Number of plants			Observed ratio R:S	Expected ratio R:S	Number of R genes	Goodness of fit	
	Total	R	S				X^2	P-value
F_2SC_1	220	190	15	13.6:1	15:1	2 genes with duplicate dominant epistasis	1.40	0.24
					61:3	2 dominant genes and 1 recessive gene	4.00	0.05
F_2SC_2	240	220	10	23.0:1	15:1	2 genes with duplicate dominant epistasis	1.80	0.18
					61:3	2 dominant and one recessive gene	0.50	0.49
F_1TC	150	140	10	14.0:1	15:1	2 genes with duplicate dominant epistasis	0.00	0.83
					61:3	2 dominant and one recessive gene	1.30	0.25
					247:9	2 dominant and 2 recessive genes	4.4	0.04
					249:7	2 dominant and 2 complementary genes	8.7	0.03
F_2TC	485	460	25	18.4:1	15:1	2 genes with duplicate dominant epistasis	9.9	0.02
					61:3	2 dominant and one recessive gene	4.2	0.04
					247:9	2 dominant and 2 recessive genes	1.1	0.29
					249:7	2 dominant and 2 complementary genes	0.3	0.59

SC_1= (AND277 x G5686), SC_2= (Mexico 54 x G5686), TC = [(Mexico 54 x AND 277) x G5686)]; R= resistance and S = Susceptible. Chi-square P-values greater than 0.05 indicate that the observed values were not significantly different from the expected.

5.2.2 Interaction among pyramided resistance genes in single crosses

It was observed that all the R x R crosses showed non-significant deviations (P > 0.05) of the F_1 mean from MP, the F_2 mean from MP, and the F_2 mean from the average of MP and F_1 (Table 15).

Table 15. Angular leaf spot symptom severity means scores of parental, F_1 and F_2 genotypes and their comparisons in R x R crosses

Crosses	P_1	P_2	P_3	MP	F_1	F_2	F_1-MP	F_2-MP	F_2-(MP +F_1)/2)
SC_1	1.60	1.79	-	1.70	2.20	2.55	-0.50^{ns}	0.85^{ns}	0.60^{ns}
SC_2	-	1.79	2.46	2.13	2.40	3.10	0.27^{ns}	0.97^{ns}	0.83^{ns}
SC_3	1.60	-	2.46	2.03	1.99	2.12	-0.04^{ns}	0.09^{ns}	0.11^{ns}
TC_1	1.60	1.79	2.46	1.95	1.79	2.00	-0.16^{ns}	0.05^{ns}	0.13^{ns}

P1 = Mexico 54, P2 =G5686, P3 = AND277, SC1 = G5686 x Mexico 54, SC2 = (AND 277 x G5686), SC3=Mexico 54 x AND277, TC1 = [(Mexico 54 x AND 277) x G5686]

But S x R crosses exhibited both insignificant ($P > 0.05$) and significant ($P < 0.05$) deviations from the means (Table 16). The F_2 populations of KAN x Mexico 54, KAN x AND277 and KAN x G5686 exhibited non significant deviation ($P > 0.05$) of F_1 mean from MP and F_2 mean from the average of MP and F_1 while F_2 populations of K132 x Mexico 54, K132 x AND277 and K132 x G5686 exhibited a significant ($P < 0.05$) deviation of the F_1 mean from MP and a significant F_2 mean from the average of MP and F_1 (Table 14). In the same way, the four-parent crosses- FPC exhibited a significant negative deviation ($P < 0.001$) of F_1 mean from MP and F_2 mean from the averages of MP and F_1 (Table 15). The parental genotypes of the R x R and S x R crosses were significantly different ($P < 0.05$) in both F_1 and F_2 generations (Table 16). Hence, it was necessary to understand the types of gene action that controlled ALS resistance in the studied crosses.

Table 16. Angular leaf spot symptom severity mean scores of parental and four-parent cross F_1 and F_2 and their comparisons

Crosses	P_S	P_R	MP	F_1	F_2	F_1-MP	F_2-(MP +F1)/2	FPC_{F1}-SC_{F1}	FPC_{F2}-SC_{F2}
Mexico 54 x KAN	7.97	2.38	5.18	5.62	4.76	-0.45^{ns}	-0.64^{ns}	-2.29^{**}	-2.04^{*}
AND277x KAN	7.97	2.46	5.18	6.12	6.10	-0.91^{ns}	0.45^{ns}	-1.03^{**}	-3.38^{*}
G5686 x KAN	7.97	2.66	5.78	4.98	4.49	-0.34^{ns}	-0.89^{ns}	-2.38^{**}	-1.77^{*}
FPC_{KAN}	7.97	1.41	4.37	2.61	2.72	-1.76^{**}	-0.77^{**}		
Mexico 54 x K132	7.33	2.38	6.05	3.52	4.81	-2.53^{**}	0.03^{*}	-0.06^{ns}	-2.15^{*}
AND277 x K132	7.33	2.46	4.89	3.53	5.00	-1.36^{**}	0.79^{*}	-3.51^{ns}	-2.34^{*}
G5686 x K132	7.33	2.66	4.98	3.55	4.93	-1.43^{**}	-0.67^{*}	0.04^{ns}	-2.27^{**}
FPC_{K132}	7.33	1.41	4.69	2.49	2.66	-2.20^{**}	-0.93^{**}		

FPC = Four -parent cross; FPC_{Kan} = Kan x [(AND 277 x G5686) x Mexico 54]; FPC_{K132}= K132 x [(AND 277 x G5686) x Mexico 54)]; Kan = Kanyebwa; P_R and P_S = means of resistant and susceptible parents, respectively; P_S for the FPC was the mean for the triple-cross F_1; F_1 and F_2 = means of F_1 and F_2 generations, respectively; MP = mid-parent value; F_1-MP = F_1 deviation from MP; F_2-(MP+F_1)/2 = mean deviation of F_2 from the average of MP, ns = not significant at P = 0.05; * and ** = significant at P = 0.05 and P = 0.01, respectively

5.2.3 Effect of pyramided resistance genes in S x R crosses *P. griseola*

In both the Kanyebwa and K132 populations the F_2 mean of both FPC had significant negative deviation from the SC means, indicating lower ALS symptom severity in the FPC than in the SC (Table 16). The F_2 frequency distributions also showed that the FPC in both Kanyebwa and K132 populations had higher proportions of resistant plants than any of the single crosses in the respective populations (Figs. 7 and 8).

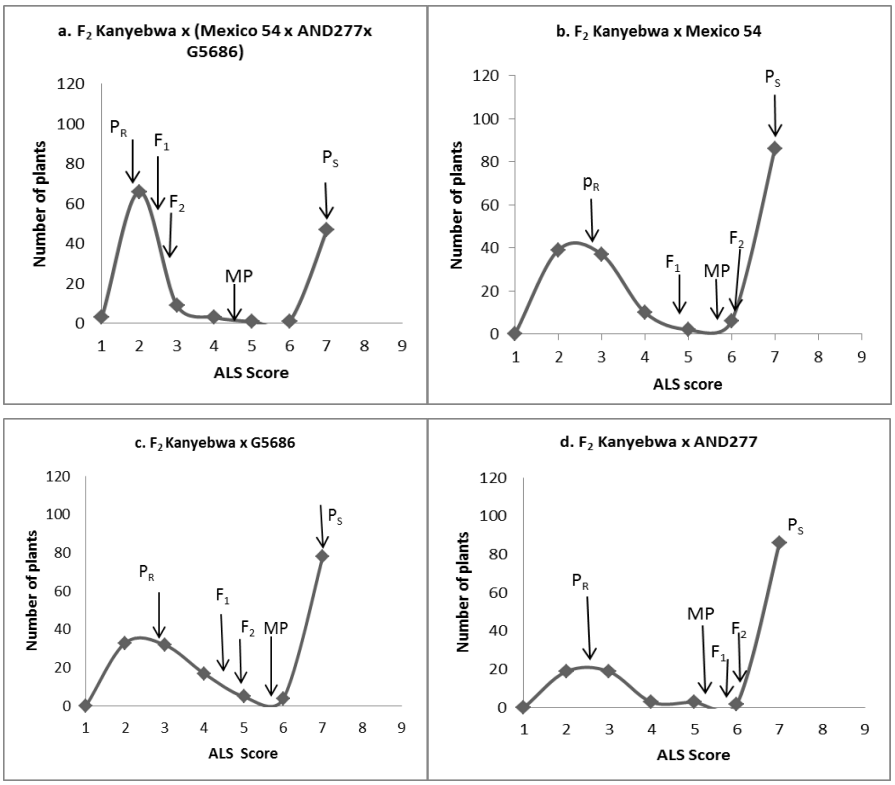

Figure 7. Frequency distribution of F_2 population in KAN population

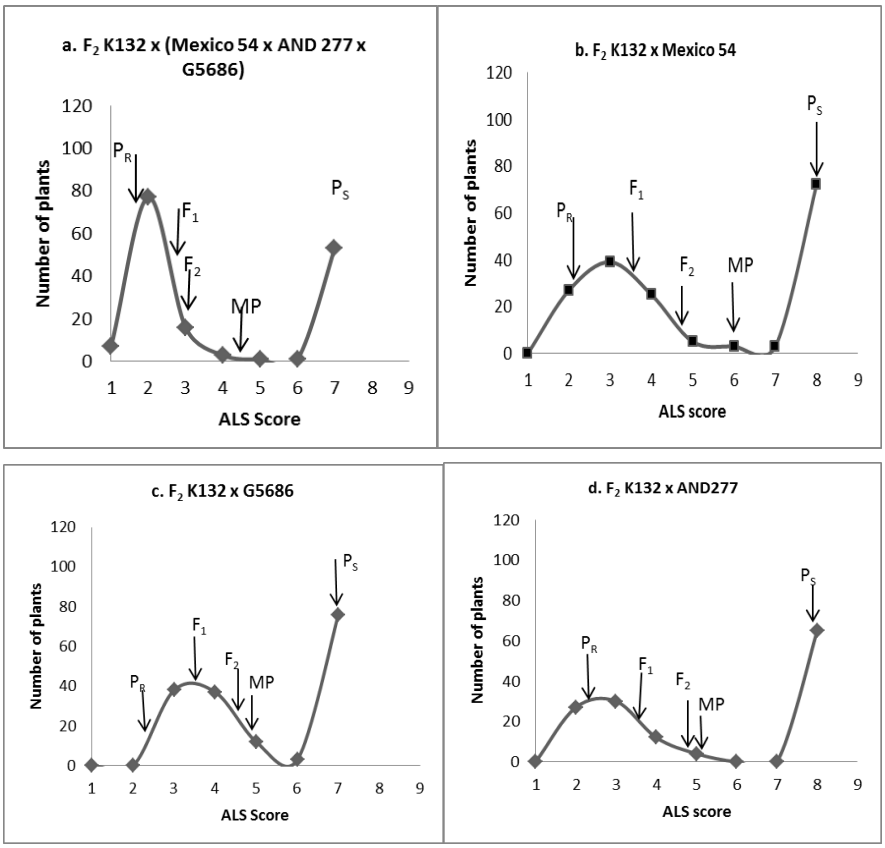

Figure 8: Frequency distribution of F_2 population in K132 population

5.3 DISCUSSION

In this study, three different ALS-resistant bean genotypes were used to improve the level of resistance against pathotype 61:63 in two common bean cultivars; K132 and Kanyebwa through gene pyramiding. The study revealed that combining resistance genes from different sources increased the level of resistance against pathotype 61:63. This supports earlier observation made by Morales and Singh, (1993) and Obala et al. (2012) that combining resistant gene from different sources enhances resistance against common bean diseases. Thus, pyramiding resistance through hybridisation of different ALS resistance sources possibly is one of the strategies that can enhance resistance against ALS and also increase the genetic base of ALS resistance in common bean.

In developing disease resistance, it is important to ascertain the number of genes responsible for resistance for a particularly kind of disease you are dealing with. In this study, segregation for ALS resistance in single crosses (SC) showed that the F_2 population of the AND277 x G5686 cross best fitted a 15:1 ratio. This indicated that the SC segregated for two genes with duplicate dominant epitasis gene action and one dominant gene present in each parent (Estakhr and Assad, 2002). On the other hand, the F_2 population of the Mexico 54 x G5686 cross best fitted the ratio 63:1 suggesting that this F_2 population most likely segregated for at least two dominant genes and one recessive gene for resistance. However, the F_2 population of Mexico 54 x AND277 cross did not fit for both segregation ratios, indicating that either genes in genotypes AND277 and Mexico 54 are possibly found on the same locus or closely linked to each other. This concurred with earlier studies by Caixeta et al. (2005) which indicated that genes in Mexico 54 and AND277 co-segregate upon inoculation with *P. griseola* pathotypes 63:23 and 63:19. On the other hand, F_2 population of three-way cross [(Mexico 54 x AND 277) x G5686)] best fitted the ratio 249:7, suggesting that the population segregated for four genes, two dominant and two complementary genes.

In all R x R crosses, results indicated insignificant deviations of F_2 means from the mid-parent means and F_1 suggested that ALS resistance in such crosses was primarily additive in nature. These results support earlier findings by Borel et al. (2011), who showed that genetic control of angular leaf spot reaction in common bean leaves and pods of cross Carioca MG x ESAL 686, was dominated by additive gene effects. In addition, populations of KAN x Mexico 54, KAN x AND277 and KAN x G5686 also exhibited additive gene action. Similar results were reported from Tanzania in crosses between resistant genotypes (Mexico 54 and G5686) and susceptible local bean genotypes Kablanketi and Spenjeli (Fivawo et al., 2013).

In several previous studies it has been reported that alleles that interact at a single locus in an additive manner are responsible for resistance in most of the ALS resistance sources (Mendonca et al., 2003). However, in this study significant deviation of the F_1 means from MP, and F_2 means from the average of MP and F_1 for K132 x Mexico 54, K132 x AND277 and K132 x G5686 and FPC populations, reflected that epistatic interaction was responsible for resistance in single crosses involving K132 as a susceptible parent and in the complex crosses. Furthermore, the inheritance for the three sources was different in the Kanyebwa and K132 parents which indicated that inheritance was sensitive to genetic background. This concurred with earlier work by Namayanja et al., 2006 which showed that inheritance of ALS

resistance depend on the genetic background of the parents used in a cross. This phenomena is further explained by Sartorato et al., 1999 who reported that, resistance to pathotype 63-19 was due to a dominant allele at a single locus when Mexico 54 was crossed with the Rudá cultivar (Mesoamerican), but when the same parent was crossed with a snap bean cultivar, Mahuku et al. (2000) observed that resistance was due to a recessive allele at a different single locus.

In terms of effectiveness of pyramided genes, FPC plant population had low ALS symptom severity compared to SC populations. The low disease severity in FPC was attributed to epistatic interaction because in Kanyebwa population, FPC was the only cross with significant ($P < 0.05$) negative F_2 deviation from the average of MP and F_1. Similarly though all crosses in K132 population had significant ($P < 0.05$) F_2 deviations, it was only FPC that showed a significant negative deviation ($P < 0.01$) of the F_2 mean from the average of the MP and F_1. Most times the effectiveness of epistatic interactions depends on whether the F_2 deviation from the average of MP and F_1 is positive or negative. The negative deviation of F_2 from the average of MP and F_1, in FPC suggested that epistatic interaction had beneficial effects, which contributed to effective resistance in FPC crosses while the positive deviations of F_2 mean from the average of MP and F_1 observed for most of the SC suggested that epistatic interaction had detrimental effect to ALS resistance through favouring susceptibility (Fenster and Galloway, 2000). From these observations it's seen that epistatic effects contributed to better resistance of FPC population against isolate 61:63 than SC. This was likely to be due to more beneficially interacting loci in FPC than SC. The result also further confirms, and is consistent with, the additive nature of resistance indicated in the R x R crosses. The better performance of the FPC over the SC demonstrates that combining resistance genes from different ALS resistance sources can provide a better source of resistance than using single sources of resistance.

5.4 CONCLUSION

Resistance present in the three sources, Mexico 54, AND277 and G5686 to ALS pathotype 61:63 is complex. The sources of resistance did not exhibit dominant inheritance against this pathotype but rather epistatic inheritance. The inheritance or resistance for the three sources was different in the Kanyebwa and K132 parents, which indicated that inheritance was sensitive to genetic background. Susceptible F_2 individual plants observed from the F_2

crosses between resistance sources confirmed independence of the resistance genes from among the different sources. The combined resistance genes in SC and TC crosses exhibited additive effects within the cross and slightly increased level of resistance to an individual ALS race when all three sources were combined. Markers and resistance to pathotype 61:63 was used to identify F_2 plants with all four putative genes (*Phg-2*, *Phg-1*, Phg_{G5686B}, Phg_{G5686A}). The F_3 lines from these F_2 plants exhibited the highest level of resistance to an individual race compared to the original resistance sources. The F_3 lines with combined resistance were more effective than the individual sources for transferring resistance to susceptible commercial cultivars of major importance in Uganda. Future studies need to be conducted to determine how broad and effective combined resistances in these newly developed lines have against the variability of the ALS pathogen sampled in Uganda.

CHAPTER SIX
GENERAL DISCUSSIONS, CONCLUSIONS, PRACTICAL APPLICATION OF RESULTS AND FUTURE RESEARCH NEEDS

6.1 DISSCUSSION AND CONCLUSIONS

The study was conducted with the aim of generating additional information important for developing a management strategy for angular leaf spot of common bean in Uganda. To achieve this, three specific objectives were set out; i) to determine the distribution and variability of *P. griseola* in major bean growing areas of Uganda, ii) to dentify new sources of ALS resistance among Ugandan bean landraces, and iii) to determine the effectiveness gene pyramiding as a strategy for improving levels of resistance to ALS in susceptible commercial bean varieties.

Findings from the study, showed that *P. griseola* is present throughout the major bean growing areas in Uganda and is highly variable with 12 pathotypes and 30 halophytes as defined by bean differentials and molecular markers respectively. The observed disease distribution suggests an efficient spread pattern and appears to support previous work on factors that may influence ALS incidence and severity (Mwangombe et al. 2007). Some of these factors are environmental in nature (temperature and relative humidity) and others are practices such as cultivar preference (Wagara et al., 2003). Low disease incidence and severity was observed in Kisoro, Kabale and high altitude areas where low temperatures (16 ^{0}C) are experienced (Mugisha, 2008). In contrast, relatively high disease severity occurred in Lira and Dokolo where temperatures are moderately high (Mugisha, 2008). Temperature may therefore play a critical role in ALS distribution in bean growing areas of Uganda. This finding is consistent with previous reports by several authors. For instance, Inglis and Hagedom 1986 reported that low temperatures below the threshold (16 ^{0}C) affect *P. griseola* infection and sporulation process, hence retarding disease development. On the other hand, Stenglein et al., 2003 reported that ALS development is stimulated by temperature within a range of (18-22^{0}C).

In addition, to the moderately high temperatures in Dokolo and lira, cultivar preference possibly also contributed to the high disease incidence and severity observed in the two districts. The dominant cultivation of only small-seeded beans is due to the fact that they are bean cultivars most preferred by farmers in Lira and Dokolo (Nkonyi, 2001). This might have

led the pathogen to get adapted to its host. Increased ALS incidence arising from pathogen adaptation due to over use of popular bean cultivars (cultivar preference) has been earlier reported by Sartorato (2004) in Goias state in Brazil.

Besides, *P. griseola* being broadly distributed in Uganda, it was also observed to be highly variable. By using molecular markers and ALS bean differentials, it was possible to confirm that 30 halophytes and 12 *P. griseola* pathotypes exist in Uganda. Based on markers; RAMS 2,4,5,6, ERIC 1,2, REP 1,2 and BOX A1R, two major groups (Andean and Middle American) which were structured along gene pools were defined. This indicated that *P. griseola* evolved with its host leading to formation of two distinct groups (Pedro et al., 2006). More variability was observed within Andean and Middle America groups, but genetic variability within groups expressed by molecular markers was much higher due to the fact that makers are unrelated with pathogen diversity (Sebastian et al., 2006). The results clearly indicated that genetic variability was still maintained in *P. griseola* but the source of variation was uncertain for a fungus like *P. griseola* with no sexual cycle. Nonetheless, processes such as mutations, migrations and parasexual can also interact to create or maintain genetic variability. Zeigler et al. (1995) showed that high levels of haplotypic diversity can be maintained in asexually reproducing fungi through parasexual reproduction. Further investigation into the cause of variability in *P. griseola* with no sexual cycle is required. It was also worth noting that among the 12 pathotypes confirmed by bean differentials, 17:39 and 21:39 were the most wide spread pathotypes, found in all bean regions where the pathogen were collected. This indicated an overlap of the two pathotypes across bean production areas. Pathotype overlap was possibly as a result of seed exchange which is a common practice among small holder farmers in Uganda (David et al., 2000). Because of the high cost of certified seed, farmers source seed from fellow farmers or informal markets whose supplies come from different parts of the country. Wagara et al. 2003 reported that seed exchange by farmers encourages transmission of *P. griseola* races across bean production regions

Based on the variability expressed by *P. griseola*, its management requires diverse and new sources of resistance. Through artificial inoculation of potential landraces with the most dorminant and virulent pathotypes in Uganda (Ddamulira et al., 2014) it was possible to confirm ALS resistance in landrace U00297 that was collected from Masaka district in Central Uganda. U00297 was found resistant to four *P. griseola* pathotypes and the resistance

could partially be attributed to its small seed size/genepool. Previous studies indicate that most resistance to fungal diseases (fusarium root rot, angular leaf spot and pythium root rot) is associated with small size and Mesoamerican genepool (Beebe, 1981; Caixetal et al., 2005). But also a few large-seeded resistant genotypes such as CAL143, MEX 54 and G5686 do exist (Aggarwal et al., 2004; Mahuku et al., 2009). The landrace (U00297) as a new source resistance to ALS could probably replace the exotic lines that are currently being used. The use of U00297 as a sorces of resistance in breeding for resistance to ALS is more likely to result in easily adaptable germplasm bearing traits preferred by farmers for easy adoption as it being a landrace it is belived to to be adapted to the growing conditions in Uganda and possibly Masaka in particular. Angular leaf spot resistance in U00297 was established to be conferred by three genes but one of the genes was found to be independent of the established resistance genes found in existing resistant sources; AND277 (*Phg-1*) and G5686 (*Phg-G5686*). The study also revealed that pyramiding ALS resistance genes obtained from Mexico 54 (*Phg-2*), AND277 (*Phg-1*) and G5686 (*Phg-G5686*) into popular susceptible K132 and Kanyebwa enhanced their resistance against ALS as evidenced by high proportions of resistant plants in FPC than in SC population.

The inheritance of resistance in U00297 varied among the three *P. griseola* pathotypes; 61:63, 17:39 and 17:39. This was evidenced by the difference in modes of inheritance expressed when different pathotypes where inoculated on the same crosses. For instance a similar reaction of F_1 plants and resistant parents to pathotype 17:39 was observed suggesting that dominance mode of inheritance was involved. This was further confirmed by F_2 chi-square values which were significantly consistence with the expected 3:1 phenotypic ratio. A similar mode of inheritance was reported by Caixetal et al. (2005) in Cornell 49-242 genotype.

In contrast, when pathotypes 21:39 and 61:63 were inoculated on similar crosses, deviations from the expected ratios were observed indicating that more than one gene (epistatic gene interactions) was responsible for resistance to 21:39 and 61:63. This therefore demonstrated that the type of pathotype used influenced the mode of resistance inheritance in U00297. This finding was consistent with earlier findings by Namayanja et al. (2006) which specified that inheritance of ALS resistance differs depending on the pathotypes/ races and the genetic background used.

For the of number of genes responsible for resistance in U00297, the allelic studies demonstrated a good fit for segregation ratio of 15:1 suggesting that resistance in U0029 was conferred by three genes. But one of the genes that confer resistance to pathotypes 17:39 and 21:39 is independent of genes harbored in AND277 and G5686. However, the lack of segregation in F_1 and F_2 progenies of AND277 x Mexico 54 cross indicated a common locus conditioning ALS resistance in both parents. Caxieta et al. (2002) and Maria et al. (2011) showed that cultivar Mexico 54 had three dominant genes and AND277 had one dominant gene respectively. One of the genes out of the three genes in Mexico 54 is probably the same as the one in AND277.

Nonetheless the resistant genes can be put to better use when they are combined into one susceptible cultivar. The concept of combining resistance genes from three sources provided better resistance to ALS than individual source could offer. Improved ALS resistance was attributed to increased number of genes that accumulated during the pyramiding process. Single crosses like AND277 x G5686 and Mexico 54 x G5686 segregated for two and three genes with average disease scores of 5.25 and 4.37 on a 1-9 scale respectively. AND277 x G5686 cross segregated with duplicate dominant epistasis and one dominant gene present in each parent (SEAKR and Assad, 2002) whereas Mexico 54 x G5686 cross segregated for three genes; two dominant genes with one recessive gene. On the other hand, pyramided cross [(Mexico 54 x AND 277) x G5686)] segregated for four resistance genes with an average disease score of 1.97 on a 1-9 scale. The apparent segregation of more genes exhibited by pyramided cross compared to single crosses provided evidence that accumulation of resistance genes in the same genetic background may improve resistance against ALS.

In terms of gene action, additive gene effect was dominant in most single crosses except crosses involving K132 as a susceptible parent. This finding concurred with what earlier authors had reported. Boral et al. (2011), showed that genetic control of angular leaf spot reaction in common bean leaves and pods of cross Carioca MG x ESAL 686, was dominated by additive gene effects. Similar results were also reported from Tanzania in crosses between resistant genotypes (Mexico 54, BAT 332, Agendum and G5686) and susceptible local bean genotypes (Kablanketi and Spenjeli) (Fivawo et al., 2013).

On the other hand, epistatic gene action was majorly responsible for resistance in single crosses (SC) involving K132 as a susceptible parent and the four parent crosses (FPC). Although resistance in both crosses were due to epistatic gene action but epistatic contribution to resistance differed depending on their mean deviation of F_2 from the mid-parent mean. The four parent cross negative deviation of F_2 from the mid-parent mean had beneficial effects which favored ALS resistance, while the positive deviation of F_2 from the mid-parent exhibited by single crosses involving K132 as susceptible parent had detrimental effect to ALS resistance through favoring susceptibility (Fenster & Galloway, 2000). Similar results on the effect of negative and positive deviations of F_2 from the mid-parent mean to resistance contribution in fungal diseases such as fusarium root rot have been reported by Obala et al. (2012). The effectiveness of the pyramided genes or low ALS symptom severity in FPC compared to SC was due to epistatic gene action. This was depicted by the highly negative deviation which had a beneficial effect that favored resistance. Hence, epistatic effects seemed to have made more contributions than additive effects exhibited in SC to improve the performance of FPC relative to the SC as indicated by the predominance of beneficial epistatic effects in the FPC than in SC.

6.2 Recommendations

i. The distribution and variability information generated should be used in designing ALS disease management strategies in Uganda
ii. ALS resistance identified in U00297 needs further validation under natural infection in the field
iii. Future studies to determine how effective the lines developed from four parent crosses are against the variable Ugandan *P. griseola* pathotypes.

6.3 Practical applications of research results and future research needs

The information generated on the distribution and variability of *P. griseola* from the study can be used by breeders as a guide in designing strategic breeding interventions that can deployed to manage angular leaf spot the disease. The findings indicated that the most virulent pathoypes were obtained from south west (Kabale) and the least virulent from the North (Dokolo). Based on this information, the National Bean Breeding Program can develop varieties that are resistant to the most virulent Ugandan *P. griseola* pathotypes for Kabale and varieties with less resistance to ALS can be developed for Dokolo. Nonetheless, developing resistant varieties alone is not enough to contain ALS in Uganda. The resistant bean varieties

developed for specific geographical areas need to be complemented with integrated disease management options. The information generated from the study can be applied by pathologists to develop integrated diseases management options that can effectively control ALS and improve bean production in Uganda.

The most virulent pathotype 61:63 indentified in the study is a potential candidate pathotype that can be used in screening germplasm during ALS resistant variety development. Unlike other sources of ALS resistance, U00297 which is adapted to Ugandan conditions and has been proved to be resistant to four *P. griseola* races with dominant resistance inheritance pattern. It can be used to accumulate ALS resistant genes into Ugandan commercial varieties that are susceptible to ALS, and progenies selected using simple pedigree breeding methods such as backcrossing. Due to these attributes, the newly identified sources of ALS resistance U00297 have a potential of being used by breeding programs to improve bean germplasm against ALS. However, the potential use of U00297 will require further validation under natural infection in the field.

The enhanced resistance through gene pyramiding has opened a way for bean breeders to utilise resistance sources developed from other regions under different climatic conditions to improve susceptible commercial bean varieties in Uganda. Even though resistance in the pyramided populations is anticipated to last longer before its matching pathogen that occur in Uganda appear, studies can be done to determine the effectiveness of pyramided lines against variable Ugandan *P. griseola* pathotypes.

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6.5 APPENDICES

Appendix 1: Landraces and commercial common bean varieties used in the screen for resistance against angular leaf spot

No.	Entry	Origin	Seed size	Seed Colour	Growth habit
1.	U0041	Sironko	large	Red	Bush
2.	U0074	Kapchorwa	Large	Red mottled	Bush
3.	U351	Mukono	Large	Red mottled	bush
4.	U0066	Kapchorwa	Large	Red mottled	Climber
5.	U1-9	Bushenyi	Large	Purple	Bush
6.	U0077	Kamuli	Large	Red mottled	Bush
7.	U614	Iganga	Large	Red	Bush
8.	U620	Mbarara	Large	Cream	Bush
9.	U0082	Kamuli	Large	cream	Climber
10.	U204	Mubende	Large	Black	Bush
11.	U00335	Masaka	Medium	Yellow	Bush
12.	U0043	Sironko	Medium	Yellow	Bush
13.	U284	Mbale	Medium	Brown	Bush
14.	U608	Mbale	Medium	Yellow	Climber
15.	Masindi yellow	Masindi	Medium	Yellow	Bush
16.	U650	Mebende	Medium	Cream	Bush
17.	U342	Iganga	Medium	Cream	Bush
18.	U00297	Masaka	Small	cream	Bush
19.	U00101	Masaka	Medium	Yellow	Bush
20.	U274	Kapchorwa	Medium	Purple	Bush
21.	U0049	Sironko	Small	Cream	Bush
22.	U0068	Kapchorwa	Small	Cream	Bush
23.	U0070	Kapchorwa	Small	Cream	Climber
24.	U0080	Kamuli	Small	Brown	Bush
25.	U0083	Kamuli	Small	White	Bush
26.	U0085	Kamuli	Small	Yellow	Bush
27.	U00212	Sironko	Small	Red	Bush
28.	U609	Masindi	Small	White	Bush
29.	U653	Kisoro	Small	black	Climber
30.	U659	Iganga	Small	Red mottled	Climber
31.	U0010	Masaka	Small	White	Climber
32.	U635	West Nile	Small	White	Climber
33.	U0053	Sironko	Small	Red	Climber
34.	U1-5	Nebbi	Small	Brown	Bush

35.	BAT332	CIAT	Small	Brown	Bush
36.	Kanyebwa	CIAT	Medium	Red mottled	Bush
37.	K131	CIAT	Small	Red mottled	Bush
38.	K132	CIAT	Large	Red mottled	Bush
39.	NABE13	Namulonge	Large	Red-white Mottled	Bush
40.	NABE4	Namulonge	Large	Red mottled	Bush
41.	U8-4	Nebbi	Large	Brown	Bush
42.	U234	Iganga	Medium	Brown	Bush
43.	U241	Iganga	Small	White	Bush
44.	U352	Kabale	Large	Cream mottled	Climber
45.	U366	Kabale	Large	red mottled	Climber
46.	U335	Kabale	Large	Purple	Climber
47.	U369	Kabale	Small	Cream	Bush
48.	U392	Kabale	Small	Purple	Bush
49.	U369	Kabale	Small	White	Bush
50.	U612	Kabale	Large	White	Climber
51.	U610	Kabale	Small	Cream	Climber
52.	U630	Mbarara	Large	Red	Bush
53.	U322	Mbarara	Large	Yellow	bush
54.	U88	Mbarara	Large	Purple	Bush
55.	U76	Mbarara	Medium	Cream	Bush
56.	U66	Northen Uganda	Small	White	Bush
57.	U94-1	Northen Uganda	Large	Red mottled	bush
58.	U10-3	Northen Uganda	Large	Yellow	Bush
59.	U9-1	Northen Uganda	Medium	Red mottled	Bush
60.	U313	Northen Uganda	Small	Brown	Bush
61.	U76	Northen Uganda	Small	Black	bush
62.	U4-1	Northen Uganda	Medium	Cream	Bush
63.	U64-4	Northen Uganda	Big	Yellow	Bush
64.	U125	Northen Uganda	Small	Cream	Bush
65.	U260	Northen Uganda	Medium	Grey	bush
66.	U5-1	Northen Uganda	Medium	Cream	Bush
67.	U34	Arua	Medium	Cream	Bush
68.	U8-5	Arua	Medium	Yellow	bush
69.	U10-7	Arua	Large	Brown	Bush
70.	U340	Arua	Medium	White	Bush
71.	U3-5	Arua	Small	Yellow	Bush
72.	U337	Arua	Medium	Cream	bush
73	U13-1	Arua	medium	Brown	Bush
74.	U338	Arua	Large	Yellow	Bush
75	U4-9	Arua	Small	Cream	bush
76.	U75	Kamuli	Small	Brown	Bush
77.	U82	Masaka	Large	Brown	Bush
79.	U323	Mpigi	Large	Brown	Bush
80	U323	Mukono	Large	Yellow	bush