DISTRIBUTION, SEVERITY AND MOLECULAR VARIATIONS IN ANGULAR LEAF SPOT OF BEANS CAUSED BY *PHAEOISARIOPSIS GRISEOLA* IN SELECTED AREAS OF ZAMBIA

BY

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DECLARATION

I, **Hildah Chimutingiza,** do hereby declare that this dissertation is my own original work and that it has not been previously submitted for any degree or examination at this or any other university.

Signature: ……………………………….. Date: …………………………….

APPROVAL

This dissertation of Hildah Chimutingiza has been approved as partial fulfillment of the requirements for the award of the degree of Master of Science in Plant Pathology by the University of Zambia.

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DEDICATION

To my loving husband Mukunto Mwaba who has been very supportive in my academic work and our three children Ryan Mwaba Mwaba, Twange Jeanette Mwaba and Henry Mwase Mwaba who had to spend less time with me because of my busy school schedule. I also dedicate this work to my mother Jean Chimutingiza and my father Kenneth Chimutingiza who supported me in all my academic endeavours.

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ABSTRACT

Fungal angular leaf spot disease of the common beans is caused by the pathogen *Phaeoisariopsis griseola* (Sacc.) Ferarris synonym, *Pseudocercospora griseola*. The pathogen is estimated to cause about 60-100% yield loss. A survey was carried out to assess the prevalence, severity and incidecnce of angular leaf spot disease and it was established that angular leaf spot was prevalent in all the 41 fields surveyed in the nine districts. The highest percentage severity index was recorded in Mpika (28%) and the lowest was in Kaputa (2.2%). The highest average severity was in Muchinga province. However, ANOVA showed that there was a significant difference in severity among provinces with F=3.36, p=0.045. The incidence was high with eight fields having incidence greater or equal to 80%. There was no significant difference in incidence between provinces with F=2.17, p=0.128. Most of the farmers in the surveyed fields used seed which was recycled and also bought planting seed from the market. Nevertheless, a multiple regression revealed that source of seed and cropping system did not contribute a significant amount of change in disease incidence with $R^2 = 0.076$. The main cropping system which was practiced by the farmers was monocropping where as very few farmers practiced intercropping but there was no significant difference in disease incidence in monocropped and intercropped fields t(39)=1.72, p=0.93.

Symptomatic bean leaf samples were also collected from each field and were cultured on potato dextrose agar, V8- juice agar, and water agar. The resulting condia were identified using published literature. Polymerase chain reaction of the *P. griseola* genomic DNA which was run using selected ISSR markers (UBC 889,888 and 809) revealed high genetic diversity among the 91 isolates. The genetic diversity was assessed using similarity indices which was in the range of 0 – 1, Shannons H =3.16 and the Simpsons 1_D = 0.93 thus showing that indeed there is diversity among the isolates. The concatenated tree dendrogram showed that isolates were clustering according to districts and in some subgroups there was a mixture of isolates from different districts. The results also revealed variation of isolates within the districts as well. This high genetic diversity implies that the pathogen can easily overcome existing resistance in the bean plants thereby leading to higher infections. This calls for regular sceening of existing and new varieites for resistance to angular leaf spot disease. The high genetic variability also means that new pathotypes will arise over time thus creating the need to study the pathogen further using differential cultivars to determine which pathotypes are present in Zambia.

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LIST OF ABREVIATIONS

ALS	Angular leaf spot
ANOVA	Analysis of Variance
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
ISSR	Inter simple sequence repeats
Kb	Kilobase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PIC	Polymorphic information content
PSI	Percentage severity index
UBC	University of British Columbia
UPGMA	Unweighted Pair Group Method with Arithmetic mean

CHAPTER 1

INTRODUCTION

1.1 Importance of common beans

Common bean (*Phaseolus vulgaris L.*) is the most important food legume in the world. It is grown in diverse regions of Latin America, Africa, the Middle East, China, Europe, the United States and Canada (Jones, 1999). Common bean is ranked third after soybeans and groundnuts for oil seed and grain legumes combined. The crop is popular because it is easy to produce and because of its flavor and versatility in its preparation (Myers *et al.,* 2017). The beans are a source of protein for both rural and urban populations and the protein content is variable in landraces and modern varieties. Common bean landraces have been estimated to have a protein content ranging from 16.54% to 25.23% whereas the protein content of modern varieties ranges from 19.70% to 24.30% (Celmeli *et al.,* 2018). The crop is not only considered as a cheap source of protein when compared to animal protein sources, but is also a good source of soluble vitamins and minerals. Inclusion of beans in diets helps to meet the dietary requirements and reduces the risk of diseases (Geil *et al*., 1994). Beans are also a significant source of mineral contents like iron, zinc, copper, phosphorus and aluminum (Hayat *et al.,* 2014).

Most of the bean crop in Zambia is produced in the higher altitude, cooler and high rainfall zones in the Northern, North-Western and Luapula provinces followed by medium rainfall zones of Central and Eastern provinces (ZARI Report, 2002). Beans are mostly grown by small holder farmers in Zambia where the proportion of the small holder farmers was estimated to have remained at 13.1% during 2017 – 2019 growing seasons. The production of beans increased by 12 percent, despite a recorded drop in yield per hectare by 5.4 percent. Common bean production in

Zambia in the 2018 growing season was at 52 thousand metric tonnes (ZASR, 2019) , where as the global bean production was at 30.4 million tonnes.

The area under bean production in Zambia rose from 84, 566 ha in 2018 to 100,279 ha in 2019 while production levels also increased from 52,351 to 85,705 MT. Notwithstanding these increases in both hectarage and production levels, there was a marginal drop in the yield of beans from 0.62 t/ha in 2018 to 0.59 t/ha in 2019 (ZASR, 2019).

The production and yield of beans in Zambia is adversely affected by biotic stress factors like insect pests, viral and fungal diseases and climate. It has been reported that diseases contribute to reduction of beans yield by 25 to 50% in Zambia (ZARI Report, 2002). The predominant diseases in the cooler and wetter regions of Zambia are anthracnose, angular leaf spot, scab, rust and ascochyta blight (ZARI Report, 2002). The management of angular leaf spot and other foliar diseases of beans is hindered by the high cost of chemicals (Ochichi *et al.*, 2018).

1.2 Problem Statement

Angular leaf spot caused by *Phaeoisariopsis griseola L.* Syn. *Pseudocercospora griseola* is a prevalent disease in Zambia and is quiet severe in the northern region of the country. The disease has been estimated to cause substantial yield loss in bean fields. (Launeras *et al*., 2017) affecting all parts of the plants including the leaves, petioles, stems and pods resulting in a variety of symptoms. The causal fungal pathogen is also able to survive on the host debri for eight months and is able to infect new crops. (Altaf *et al*., 2022). Although *Phaeoisariopsis griseola* has been identified as a major pathogen causing significant yield losses in Zambia, its genetic variability is not well understood. It has been reported to have a high genetic variability thus resulting into several races which are able to overcome host resistance. (Pereira *et al*., 2015, Mahuku *et al*., 2002). Furthermore no studies have been carried out in Zambia on the causal pathogen *Phaeoisariopsis griseola*. Therefore this study was undertaken to investigate the prevalence, distribution and genetic variability of *Phaeoisariopsis griseola* in major common bean growing areas of Zambia.

1.3 General Objective

The overall objective of the study was to determine the level of variability of *Phaeoisariopsis griseola* in the major bean-cultivating areas of Zambia and to contribute to possible solutions to the existing disease problem in the area.

1.3.1 Specific objectives

The specific objectives of this research were to determine:

1. The distribution, severity and incidence of angular leaf spot disease in bean-growing parts of Zambia.
2. The molecular variation of *P. griseola* by inter simple sequence repeats markers.

1.4 Research questions

1. What is the distribution, severity and incidence of angular leaf spot disease in the major bean-growing Northern parts of Zambia?
2. What is the level of molecular variation of *P. griseola* as revealed by inter simple sequence repeats markers?

CHAPTER 2

LITERATURE REVIEW

2.1 Production of Beans in Zambia

Common beans are a very important food legume in all parts of Zambia where the crop is harvested as a dry bean and consumed as boiled dry grain, green pods and leaves. Most of the beans are produced in Northern, North-Western and Luapula provinces which fall in the agro-ecological region III of Zambia. Agro-ecological region III of Zambia is ideal for bean production because of the high rainfall of 1000mm per annum that it receives, has cooler weather and high altitude above the sea level which makes it suitable for the crop to mature (Jain, 2007). The other region where bean are grown is region II which encompasses Central and Eastern provinces of Zambia. This region recieves medium rainfall and is a warm Zone. Agro ecological zone II receives approximately about 800 to 1000mm of rainfall, this region also has fertile soils which makes it suitable for agricultural activities. (Jain, 2007)

2.2 Bean Production constraints

Common bean farmers in Zambia face various production constraints including insects and diseases (Hamazakaza *et al.,* 2014). In addition to disease constraints, poor soil fertility, lack of quality certified seed and varying rainfall patterns have also been identified as major production constraining factors (Mwaniki, 2002). Among the diseases that pose a threat to bean production are angular leaf spot disease (ALS), common bacterial blight, Anthracnose, and root rots (Rodríguez *et al.*, 2014). Because of lack of adequate varieties of certified seed, small scale farmers rely on recycling of seed from season to season which tends to carry seed-borne diseases. One such seed-borne disease is Angular leaf spot disease (ALS) which contributes to the reduction in bean production (Mongi *et al.*, 2017). Farmers also do not have knowledge on the management strategies of ALS disease and when they apply fungicides to the crops it doesn’t significantly reduce ALS because the application rates are usually low and late.

2.3 Angular leaf spot disease

2.3.1 Symptoms of Angular leaf spot disease

Angular leaf spot disease is caused by the fungus *Phaeoisariopsis griseola* which was renamed as *Pseudocercospora griseola*. It belongs to the phylum Ascomycota, class Dothideomycetes, order Mycosphaerelles and family Mycosphaerellecea (Braun & Crous, 2006). *Pheaoisariopsis griseola* has been classified as a hemibiotroph and its sexual stage has not been reported. The pathogen survives in overwintering plant debris and soil, which are the primary source of inoculum. The spores infect the leaves which become the pathogen colonization route and on which it produces conidiophores bearing conidia (Swiderska-Burek *et al.,* 2020). In the field the first symptoms of ALS appear during the early stages of plant growth on primary leaves and disease symptoms do not become visible until late flowering or early pod formation (Stenglein *et al.*, 2003). The symptoms of ALS appear as small angular grey lesions on the leaves. The growth of the pathogen is limited by the veins and the lesions appear greyish and contain fruiting body structures**.** They become visible on the lower surface of the leaf (Mckenzie, 2013). The lesions of the disease tend to merge and can cause yellowing of the leaves (Figure 1) and when the disease is severe the leaves fall off (Monda *et al*., 2001). The necrosis and extensive defoliation of leaves prematurely has been observed on plants where no fungicide has been sprayed. Angular leaf spot disease also results in underweight seed of poor quality which are discolored, shriveled and have low market value (Mongi *et al.*, 2017).

Figure 1: **Common bean crop stands in farmers field. A. asymptomatic leaves . B. early infection of anglar leaf spot disease .C. mild infection with fungal angular leaf spot disease.**

2.3.2 Effects of fungal ALS on growth and yield of beans

Fungal angular leaf spot disease causes significant yield loss in beans. Damage to the crop has been reported to be highly variable ranging between 60% and 100%, being more severe in crops where agrochemical treatments have not been applied (Launderas *et al.*, 2017). It has been observed that bean fields which are located at altitudes between 1000 and 1200m above the sea level have a high disease severity and incidence as compared to bean fields which are located in areas with altitudes of over 1500m above the sea level (Ddamulira *et al.*, 2014). In the Democratic Republic of Congo, ALS disease has been reported in farmers' fields with an average severity index of 49.9% (Kijana *et al.*, 2017). In Kenya the disease was identified as one of the most damaging and widely distributed diseases of common bean, causing yield losses that have been estimated to be as high as 80%, and the problem is further compounded by limited information on pathogen distribution and variability thus hindering breeding efforts for angular leaf spot diseases (Leitich *et al.*, 2016). Bean yield is greatly reduced when ALS disease severity is high in a field even in plots which have some level of healthy leaf area. Therefore, it can be said that ALS disease influences the photosynthetic capacity of healthy leaf area (Canteri *et al.*, 2005).

2.4 Diagnostic methods of ALS

2.4.1 Use of differential cultivars

Phaeoisariopsis griseola, the causal pathogen of ALS has been studied using a set of 12 differential cultivars which reveal the virulence and genetic diversity of the pathogen (Nay *et al.*, 2019). The most virulent pathotype based on differential cultivars is pathotype 63-63 which was reported from Kenya (Leitich *et al.*, 2016). This pathotype has also been reported to overcome genetic resistance in all differential cultivars (Silva *et al.*, 2008). However, pathotype 63-63 has also been reported to be highly variable within the race itself (Pereira *et al.*, 2015). A study by Satorato (2002) was carried out to identify *P. griseola* pathotypes using differential cultivars and from the 51 isolates which were tested, seven pathotypes were identified. The most common pathotypes were pathotypes 63-31 and 63-63. Genetic variability of *P. griseola* was also reported by Darmasceno-Silva (2008) who observed 10 pathotypes from the 48 isolates of *P. griseola*. New pathotypes were also observed and were pathotypes 55-15, 63-25 and 63-27.

2.4.2 Use of molecular markers for genetic diversity analysis of *P. griseola*

Different molecular methods have been used to characterize *P. griseola* pathogen and include Random amplified polymorphic DNA (RAPD's), internal transcribed spacer region, actin gene and inter simple sequence repeats (ISSR) markers (Chilagane *et al.,* 2016). Evaluation of the actin gene and the internal transcribed spacer region of *P. griseola* isolates in Tanzania showed that there are 69.7% Andean strains and 30.3% Mesoamerican strains (Chilagane *et al*., 2016). The internal transcribed region was used in a study conducted in Spain to identify three *P. griseola* strains which were identified based on the morphological features. All the three strains were found to be genetically similar (Launderas *et al.*, 2017).

The use of Random amplified polymorphic DNA has shown 99% variability in *P. griseola* genotypes and virulent markers have shown 97% variability (Mahuku *et al.*, 2002). Other molecular markers are also used to study genetic diversity these are inter simple sequence repeat markers (ISSRs). Inter simple sequence repeats (ISSR) involves the detection of polymorphic bands using a single primer. These markers can either be 5´ anchored – consisting of a repeated motif with one or several non motif nucleotides at the 5´end, 3´ anchored – consisting of a repeated motif with one or several non motif nucleotides at the 3´ end or non-anchored – consisting of only a repeated motif (Ng *et al.*, 1994). The 5´and 3´ anchored primers have been reported to be more informative than non-anchored primers because non anchored primers produce inconsistent amplification in every cycle thus making it difficult to reproduce results (Stenglein *et al*., 2006). When carrying out the PCR using ISSR markers, it is important to optimize the annealing temperature because it may vary depending on the base composition of the primer used (Verma *et al.*, 2017). Screening of primers to select working primers has shown that primers which are based on GA, CA and GAA repeats produce good amplification (Kebour *et al.*, 2012). Assessments have been carried out to determine the suitability of ISSR PCR analysis to determine genetic diversity among *P. griseola* isolates. The results show that the *P. griseola* isolates can be discriminated into 27 genotypes. Therefore, ISSR markers can be used to establish the genetic structure of a population of study (Abadio *et al.*, 2012).

2.4.3 Management of angular leaf spot disease

Several methods including the use of resistant bean varieties, crop rotation, intercropping, use of disease free seed and application of fungicides are used for ALS disease management (Pamela *et al.*, 2014). Fungicides have been used in the management of ALS disease to reduce the severity and yield loss (Shiferaw, 2017). Another method that has been used in the control of ALS disease is the supplementation of local bean mixtures with resistant varieties. Results from one study (Pyndji *et al.*, 1992) showed that supplementation of local bean mixtures with 25% of a resistant variety reduced angular leaf spot disease. Bacterial antagonists *Bacillus cereus* and *Pseudomonas putida* have also been assessed to determine their impact on the control of ALS disease of common beans under field conditions (Garcia *et al.*, 2011). These antagonists were effective in reducing the severity of the disease in comparison to the control which was sprayed with water. The bacterial antagonists had the same level of control as the chemical treatment (Garcia *et al.*, 2011).

Intercropping of common beans with maize has also been evaluated to assess the impact on the severity of the disease under field conditions. However, the reaction of ALS to maize intercropping is not clear-cut but is rather unpredictable and quiet changeable depending on the location and the season. It has been reported that intercropping beans with maize can reduce the disease by 23-33% (Boudreau, 1993). Genetic resistant is the best method for the management of fungal ALS disease because it is cost effective (Wagara *et al*., 2003). However, it is very difficult to breed for ALS resistance because of the extensive virulence of *P. griseola* , new virulent races which keep on appearing and the ability of the pathogen to overcome resistance (Nay *et al.*, 2019). Therefore, screening for disease resistance of beans to angular leaf spot disease calls for understanding of the molecular variability of the causal pathogen *Phaeoisariopsis griseola*.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection and assessment of disease severity

Part of the study was conducted in the major bean-growing areas of Zambia during April/May 2019 where field surveys were conducted in Northern, Luapula and Muchinga Provinces. The mean altitude of the three provinces, based on the GPS altitude readings, were 1162 m for Luapula province, 1469 m for Northern province and 1353 m for Muchinga province. The total number of Districts covered in this study were nine namely; Mbala, Kasama, Mungwi, Mansa, Nchelenge, Kaputa, Mpika and Senga Hill and Luwingu. The bean varieties commonly grown in Northern province are Kabulangeti local, Mandima local, a mixture of common varieties, Kabulangeti improved and Lusaka local. In Muchinga province the varieties grown are similar with preference given to Kabulangeti local, Lusaka local, Mandina local, a mixture of common varieties and Kabulangeti improved (Hamazakaza *et al.*, 2014). The mean size of the small scale farmer fields surveyed ranged from 50m^2 to 2100m^2**.** The three provinces lie in Agro-ecological region III, and receives more than 1000 mm of rainfall per year. The region also has more than 160 effective crop growing days. This region is also suitable for production of millet, sorghum, groundnuts, rice and soybeans.

Forty one fields were selected randomly along motorized roads and were separated by at least a distance of 5km. Fifty plants in each field were examined at one meter interval in an ‘X’ formation and 25 plants were selected in each diagonal for the assessment of incidence and disease severity. Disease severity was assessed by estimating the percentage of the leaf damaged by the disease according to the method of Manandhar *et al.,* 2016 using a scale of 0 – 5, where:

- 0 = No disease,
- 1= 1-10% leaflet area with lesions,
- 2 = 11-25% leaflet area with lesions,
- 3= 25-50% leaflet area with lesions and limited necrosis,
- 4= over 50% or more of the leaflet area with lesions,
- 5 = Extensive necrosis and defoliation.

The bean growth stages targeted were (R6-R8) which are flowering, pod formation and pod filling stages (Manandhar *et al.*, 2016). The severity scores obtained for each field were converted into percentage severity index (PSI) using the formula by Wheeler, (1969);

$$\text{PSI} = \frac{Sum\ of\ numerical\ ratings\ \ x\ 100}{Total\ number\ of\ plants\ x\ maximum\ score\ in\ scale}$$

Disease incidence was calculated as the number of infected plants divided by the total number of plants assessed in the field as a percentage according to the formular of Getachew *et al.*, (2013). The distribution of the disease was determined as the percentage of the number of bean fields in which the disease was present out of the total number of fields surveyed. The latitude, longitude and altitude coordinates for each field were recorded using a Global positioning system hand set. In addition to the disease data, other parameters were recorded for each field such as source of seed used for planting, the type of cropping system practiced, the growth stage of the bean plants as well as the varieties planted. For each field the farmer was asked where the seed for planting was sourced as well as the variety which was planted, the response was recorded on the datasheet. The cropping system practiced and intercops were assessed visually and were also recorded.

Phaeoisariopsis griseola isolates were collected from naturally infected bean leaves at the mid podding stage where the epidemic best manifests itself (Allen *et al.*, 1998). Samples of diseased leaves were collected from different bean varieties to capture a wider representation of pathogen diversity. A minimum of three samples per field were collected and stored in labelled khaki paper envelops indicating the Way point, Latitude, longitude, elevation, variety name, district and province (Figure 2)**.** The collected samples were placed in a cooler box and transported to the Plant Pathology laboratory at Mount Makulu Central Research Station for pathogen isolation and genomic DNA extraction for PCR analysis using primers targeting selected ISSR's.

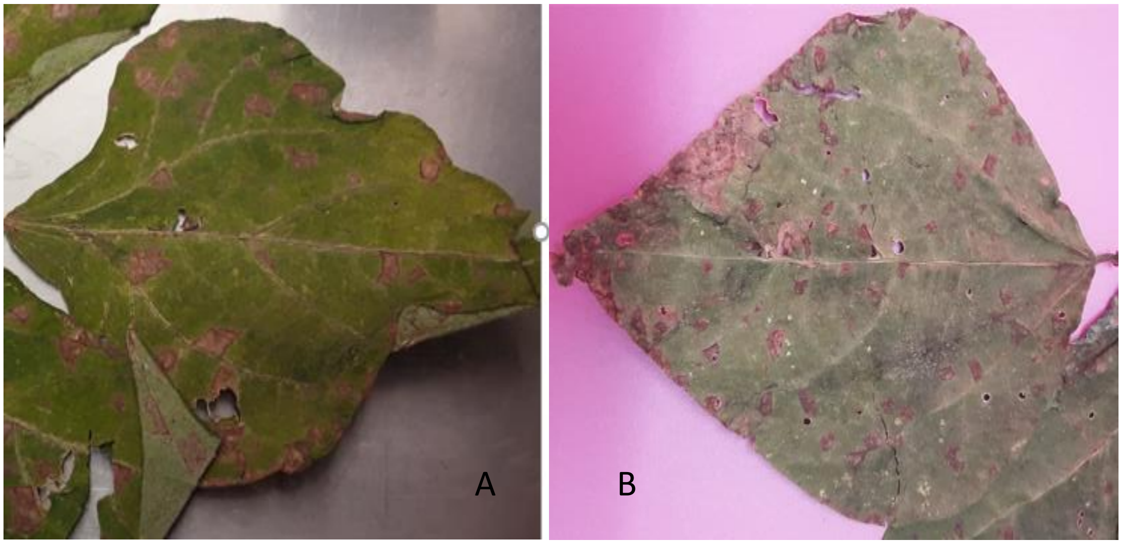

Figure 2: Leaves showing angular leafspot disease symptoms. A. collected from a field in Mbala. B. Collected from a field in Kasama.

3.2 Isolation of *Phaeoisariopsis griseola*

A total of 120 leaf tissue samples were collected from the surveyed 41 fields. However, only 91 out of the 120 isolates were successfully cultured in the laboratory. The effective method which was used to culture the *P. griseola* isolates was streaking on PDA. In the streaking method, a leaf sample showing ALS lesions was placed on a sterilized stage of the stereomicroscope and sporulating synnemata were lifted with the tip of an inoculating needle (Figure 3) and transferred to Petri plates of potato dextrose agar by streaking (Leitich *et al.*, 2016, Aytenfsu *et al.*, 2019).

The spread-plating technique involved picking up spores with a sterile innoculating loop and transferring them on a microscopic slide which had drops of sterile water then the water was transferred on PDA media and a sterile loop was used to spread the water around the petri dish. Less bacterial contamination was observed in the streaking technique as compared to the spreading technique. Inoculated plates were incubated at 24 ^{o}C for 48 hours. After incubation the agar plates were viewed under a stereo microscope to check for sporulation and conidia (Sanglard *et al.*, 2009b). Once the conidia were identified, a sterile blade was used to cut the germinated conidium which were transferred to water agar and V8 juice agar (Castellamos *et al.*, 2016) to obtain monosporic culture for each *Phaeoisariopsis griseola* isolate. Each monosporic isolate obtained from different common bean genotypes was maintained on V8- juice agar at 24^{o}C and water agar (Pastor Corrales *et al.*, 1998).

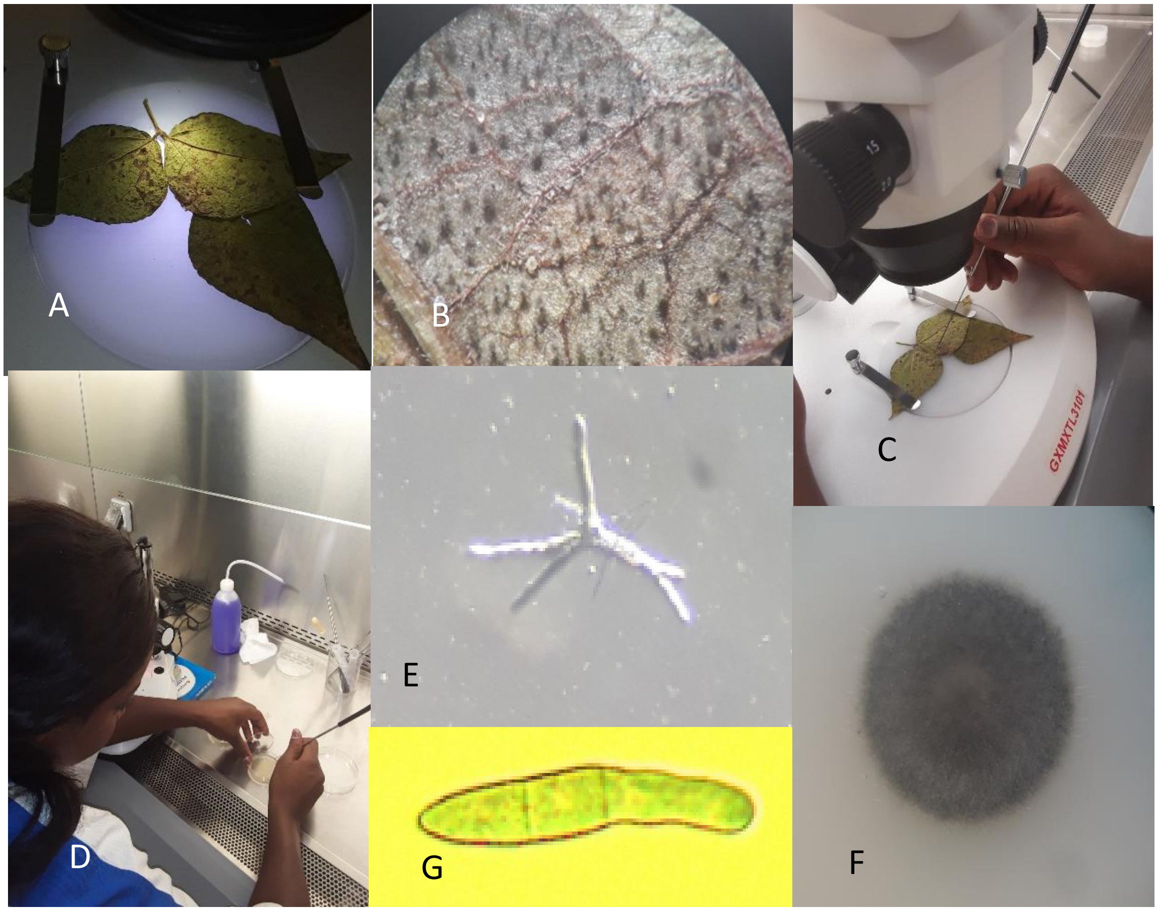

Figure 3: Isolation of *P. griseola* from infected leaves of beans: A. Infected leaf on a stereomicroscope. B. Sporulated synemata on the underside of the leaf. C-D. Isolation of conidia on PDA. E. Germinating conidium. F. Grey *P. griseola* colony and G. Single conidium

3.3 Extraction of genomic DNA from colonies of *Phaeoisariopsis griseola*

Reagents for nucleic acid manipulations including genomic DNA extraction and amplification by the polymerase chain reaction were purchased from Inqaba Biotech Industries LTD, Pretoria, South Africa and HiMedia Laboratories, Mumbai, India. Powder media including potato dextrose agar powder and V8 juice agar were also purchased from HiMedia Laboratoies, India through Kansma Investments LTD, Lusaka, Zambia. Stock solutions and buffers were TES buffer (100Mm Tris at pH 8.0, 10Mm EDTA and 2% SDS), 10% CTAB, 5M ammonium acetate and 5M sodium chloride which were prepared following standard laboratory procedures.

Fungal genomic DNA was extracted following a published procedure (Moller *et al.*, 1992) with minor modifications as follows using one-month old fungal isolates on V8 medium. *Phaeoisariopsis griseola* mycelium (0.3 grams) was cut from V8 media with a sterile scalpel blade and was placed in a mortar to which 500µl of TES buffer and 5µl of proteinase K was added. Mycelium tissue was crushed to a fine slurry with a pestle and each sample slurry was transferred to a labeled 1.5ml centrifuge tube and incubated for one hour at 58^{o}C in an incubator shaker (TS-100 Thermo shaker, Boeco Germany). The salt concentration was adjusted with 5M NaCl followed by 65µl of 10% CTAB and the tubes were incubated for 10 minutes at 65 ^{o}C in a water bath with occasional shaking. After incubation, 700µl of chloroform-isoamyl alcohol (24:1) was added to each tube and mixed gently by inverting. Tubes were placed in a freezer for 30 minutes at 0 ^{o}C followed by centrifugation for 10 minutes at 4 ^{o}C at 14,000 rpm in a benchtop microcentrifuge (MRC Scientific Instruments, England, United Kingdom). The resulting supernatant was transferred to a fresh Eppendorf tube and ammonium acetate was added and the tubes were again placed in the freezer for 30 minutes, afterwhich they were centrifuged at 4 ^{o}C. The supernanant was transferred to a new Eppendorf tube and 510 µl of isopropanol added to each sample. Samples were placed in the freezer for an hour after which they were centrifuged for 5 minutes at 14000 rpm.

The DNA pellets were air dried for one hour and dissolved in 50 µl nuclease free water. The quality of the DNA was checked qualitatively and quantitatively as described below and the samples were stored at -20 ^{o}C.

3.4 Qualitative and quantitative quality analysis of genomic DNA

3.4.1 Quantification of DNA using a Nanodrop

The nucleic acid concentration was measured using a Nanodrop one spectrophotometer (Thermo Fisher Scientific, USA) according to manufacturer's instructions. Briefly pedals of the Nano drop spectrophotometer were cleaned and a blank was loaded using nuclease free water (Desjardins *et al.*, 2010). The pedals were cleaned again and 1µl of DNA was loaded and DNA quantity and quality was determined. The procedure was repeated and the ninety one genomic DNA samples were quantified. The concentration of extracted DNA was in the range of 18 to 389ng/µl. The average absorbance at A260/280 was 1.62 for the 91 samples.

3.4.2 Qualitative analysis using agarose gel electrophoresis

The integrity and quality of the extracted genomic DNA was assessed on 1% agarose gel according to Lee *et al.*, (2012). Briefly, 100 mls of 1X TAE was added to one gram of agarose gel powder in a conical flask and microwaved to melt the agarose after which 5µl of ethidium bromide was added to pre-stain the gel. The gel was poured into a gel-casting tray with a comb used to make loading wells upon solidification of the gel. Samples were electrophoresed at 80 volts for 1 hour and the results were recorded using a BIO RAD gel documentation system (Bio-rad Laboraories Inc, USA), using image lab 4.0 software (Figure 4).

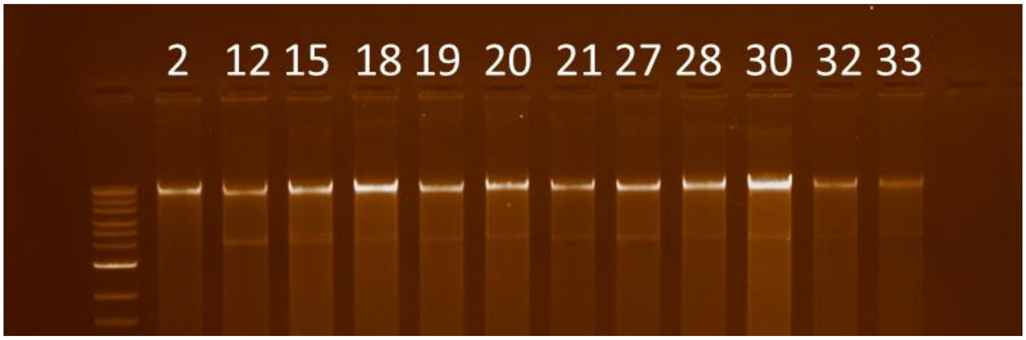

Figure 4: Agarose gel electrophoresis evaluation of genomic DNA quality

3.5 DNA amplification and gel electrophoresis

Eight inter simple sequence repeats (ISSR) markers whose details are given in Table 1 were used to evaluate genetic diversity of *P. griseola* isolates (Abadio *et al.*, 2012).

3.5.1 ISSR primer sequences

A total of 8 inter simple sequence repeats primers (Table 1) developed by The University of British Colombia were purchased from Inqaba Biotec, Hatfield, Pretoria, South Africa. They were preliminarily screened to determine the ones that could be suitable for evaluating the genetic diversity depending on the ones that shows polymorphism.

Table 1: Description of the ISSR primers, sequences, melting temperature and sequence length

PRIMERS	**SEQUENCE (5'→3')***	**Melting Temperature**	**Length of Sequence**
UBC 809	AGAGAGAGAGAGAGAGG	47.05	17
UBC 836	AGAGAGAGAGAGAGAGYA	45.77/48.04	18
UBC 842	GAGAGAGAGAGAGAGAYG	48.04/50.32	18
UBC 880	GGAGAGGAGAGGAGA	44.67	15
UBC 888	BDBCACACACACACACA	42.23/49.46	17
UBC 889	DBDACACACACACACCA	42.23/49.46	17
UBC 890	VHVGTGTGTGTGTGTGT	42.23/49.46	17
UBC 891	HVHTGTGTGTGTGTGTG	42.23/49.46	17

*****Y= (C, T), B= (C, G, T), D= (A, G, T), H= (A, C, T), V= (A, C, G)

Polymerase chain reaction was performed in 20 µl reaction volumes containing 50 ng total DNA polymerase, 1 µl dNTPs, 0.1 µl *Taq* DNA polymerase, 1 µl primers and 2 µl 10X buffer and 1 µl of $MgCl_2$ (Yugander *et al.*, 2015).

The PCR conditions for each primer varied depending on the optimized annealing temperature. The annealing temperatures tested on the eight primers was in the range of 45 °C to 52 °C. Polymerase chain reaction was performed using a thermorcycler with the first cycle at 94 °C for 5 minutes followed by 35 cycles at 1 minute, temperature range of 45 – 52 °C for 1 minute, 72°C for 2 minutes and a final extention of 7 minutes at 72 °C.

The PCR products were analysed by gel electrophoresis on a 2.5% agarose gel in 0.5XTAE buffer under constant voltage of 60V/cm. The fragments were visualized by ethidium bromide staining

(5µl). The DNA ladders that were used as molecular size markers were 1kb and 50bp, the products were visualized in a BIO RAD gel documentation system (Bio-rad Laboratories Inc, USA).

3.5.2 Data Analysis

3.5.2.1 Summaery statistics, regression and T-test

The maps for disease incidence and severity were generated using Diva GIS 7.5.0 to show the distribution of the fields for the selected variable classes. Charts for incidence and severity, seed source and cropping system were created using IBM SPSS statistics 22. The t- test to assses if there was a significant difference in incidence in monocropped and intercropped fields was carried out using Datatab (https://datatab.net). Multiple regression analysis was carried out using Genstat 19th Edition, to accertain to what extent cropping system and source of seed contribute to incidence of ALS. An ANOVA was also carried out using Genstat 19th Edition to determine if there was a significant difference in disease severity and incidence among provinces.

3.5.2.2 Molecular analysis

The gel images which were obtained using the three primers 889, 809 and 888 were printed and the copies were used to score for presence (1) or absence (0) of reproducible bands using a binary method. The amplified ISSR bands for the three primers ranged from 300 bp to 1900 bp, as estimated using 1kb ad 50kb molecular size markers. The Jaccards similarity index, Shannons –H and Simpsons 1-D were determined using Past 4.03 software. To estimate the genetic relationship among the isolates, dendrograms were constructed from the binary data using the unweighted pair-group method using arithmetic average (UPGMA). The genetic similarity between isolates was constructed using the Jaccards similarity coefficient with a cophen correlation of 0.9438. The polymorphic information content was computed using the formular in Microsoft Excel®:

PIC $= 2 * f * (1 - f)$ where f = frequency.

The binary data for all the three markers was combined to generate a concatenated phylogenetic tree using Past 4.03 software. The phylogenetic tree was constructed using the Jaccards similarity coefficient and genetic diversity among isolates was determined with the help of the Simpson 1-D and Shannnons H diversity indices.

CHAPTER 4

RESULTS

4.1 Angular leaf spot disease incidence and severity

4.1.1 Angular leaf spot severity

The angular leaf spot percentage severity index (PSI) was assessed for the samples from 41 fields with the highest PSI being (40.8%) and the lowest (0.4%). Mpika had the highest mean PSI of 28% followed by Kasama which has a PSI of 23.9%. The lowest PSI was 2.2% which was recorded in Kaputa (Table 2). Two districts surveyed only had one field each recording a PSI of 22% in Chiengi and 7.2% in Mwense. Out of the 41 fields surveyed, 9 had a severity greater than 25%, whereas the remaining 32 fields had a severity less than 25% (Figure 5)**.**

Table 2: Angular leaf spot (*Phaeoisariopsis griseola*) severity in the nine districts

DISTRICT	MEAN (PSI)
Kasama	23.9
Mpika	28
Luwingu	17.7
Mbala	8.5
Mansa	17.9
Senga Hill	12.1
Kaputa	2.2

The median was considered for this data set because it represents the central value of the observations better. The median of severity for the three provinces were Muchinga (1.84), Luapula (1.51) and Northern (1.25). All the median values for the three provinces were below 2 on a scale of 0 – 5 (Table 3).

Table 3: Average severity of angular leaf spot (*Phaeoisariopsis griseola*) per province

PROVINCE	MEDIAN SEVERITY	AVERAGE SEVERITY
Muchinga	1.84	1.67
Luapula	1.51	1.53
Northern	1.25	1.3

An analysis of variance carried out, resulted in the tabulated F-ratio of 3.36 and an F- critical of 3.25. The average LSD for the dataset was 0.35 and the means were separated as shown (Table 4).

Table 4: Significance testing for disease severity among the three provinces

MEAN PAIR	DIFFERENCE	CONCLUSSION
1.67 – 1.53	0.14	Not significant
1.67 – 1.3	0.37	Significant
1.53 – 1.3	0.23	Not significant

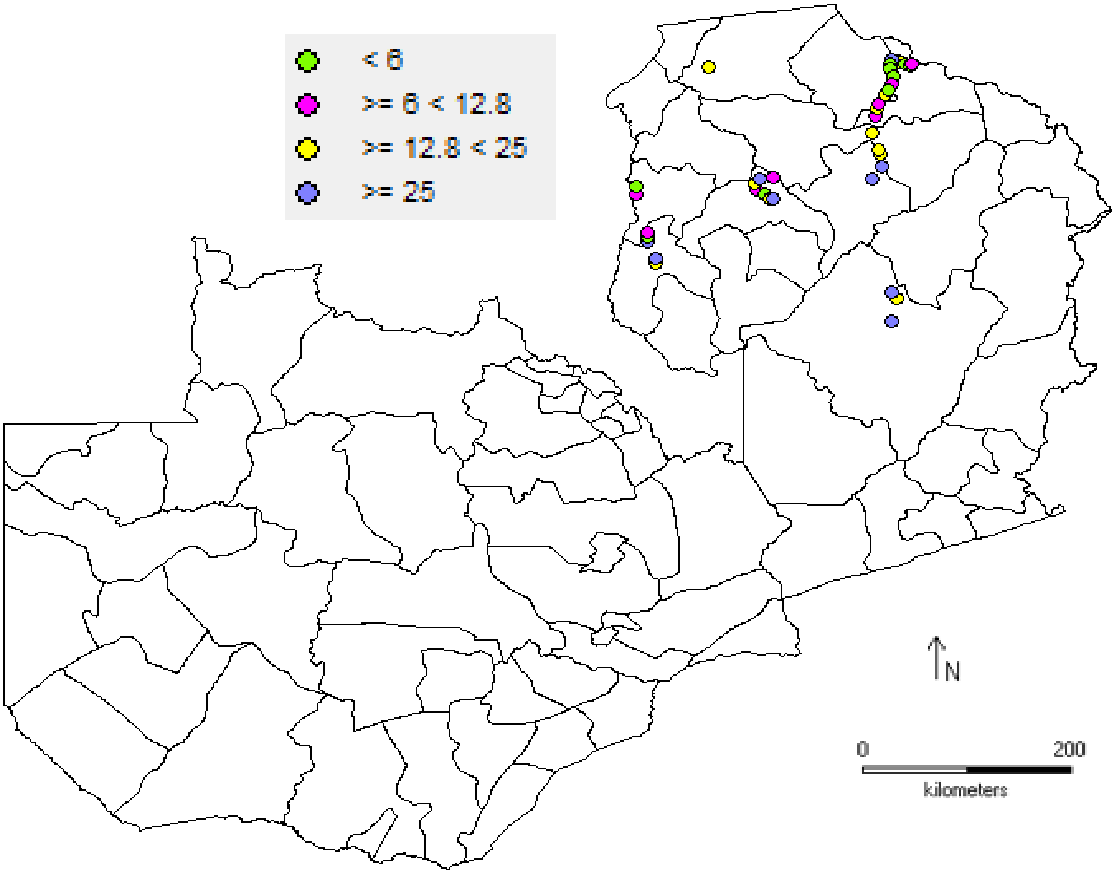

Figure 5: Angular leaf spot disease severity of *Phaeoisariopsis griseola* observed in April 2019 in Northern, Luapula and Muchinga provinces of Zambia.

4.1.2 Angular leaf spot disease incidence

The range of angular leaf spot disease incidence in the nine districts surveyed was 2 – 100% . Generally, ALS disease incidence in the surveyed fields was high with 19 fields having incidence greater than 58% whereas 22 fields had incidence less than 58% (Figure 6). The highest mean incidence was from Mpika district (81.3%) followed by Kasama district (71.6%) and the lowest incidence was from Kaputa (15%). Chiengi and Mwense which had one surveyed field each recorded mean disease incidence of 70% and 30% respectively (Table 5)

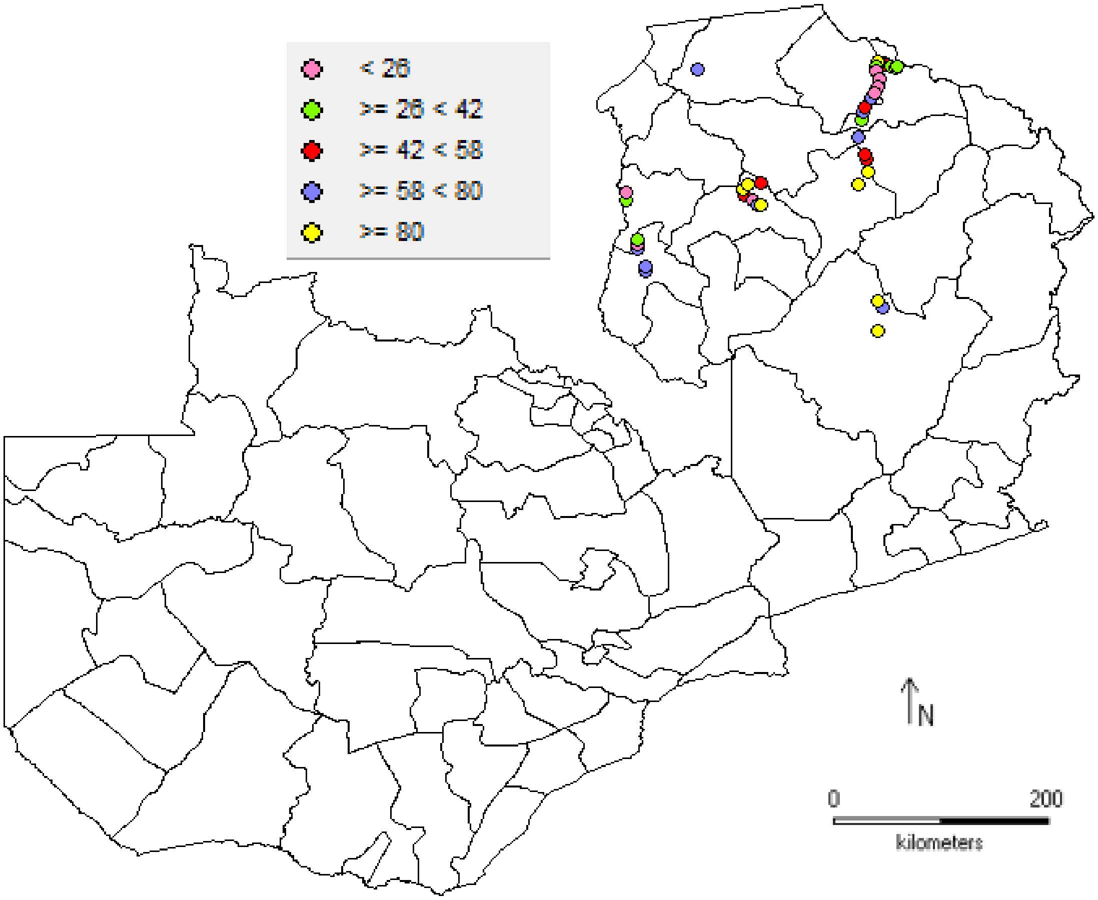

Figure 6: Angular leaf spot (*Phaeoisariopsis griseola*) disease incidence observed in Northern, Luapula and Muchinga provinces.

Table 5: Angular leaf spot (*Phaeoisariopsis griseola*) incidence in the nine districts.

DISTRICT	MEAN
Kasama	71.6
Mpika	81.3
Luwingu	60.5
Mbala	31
Mansa	53
Senga Hill	47.3
Kaputa	15

The median for disease incidence in the three provinces was as follows Muchinga (86%), Luapula (59%) and Northern (47%) (Table 6). The results show that the median ALS incidence was high in muchinga province (86%). However, it was lower in Luapula (59%) and Northern province (47%). When the average incidence was considered for each province, Luapula province had a higher incidence of 85.5% as compared to Muchinga and Northern province with disease incidence of 81.3% and 65.5%.

Table 6: Average incidence of angular leaf spot (*Phaeoisariopsis griseola*) per province.

PROVINCE	MEDIAN INCIDENCE	AVERAGE INCIDENCE
Muchinga	86%	81.33%
Luapula	59%	52.25%
Northern	47%	46.2%

Analysis of variance resulted in the F- ratio of 2.17 and the F- critical of 3.24. The LSD for the data set was 38.47 and the means were separated as shown (Table 7).

Table 7: Significance testing for disease incidence among the three provinces.

MEAN PAIR	DIFFERENCE	CONCLUSSION
81.33– 52.25	29.08	Not significant
81.33 – 46.2	35.13	Not Significant
52.25 – 46.2	6.05	Not significant

4.1.3 Angular leaf spot disease incidence and severity at different variable classes

Disease incidence and severity was compared to assess how many fields were in the different variable class combinations (Figure 7). There were seven fields in the severity range 0-10% and incidence range 0-20% whereas nine fields were in the incidence range of 21-40% and severity range of 0-10%. The highest number of fields (n=11) were in the severity range of 11-20% and incidence range of 41 to 60%. Two fields fell in the incidence range of 61-80% and severity range of 11-20%. There were only six fields which were in the highest severity and incidence classes of 31-40% and 81-100%.

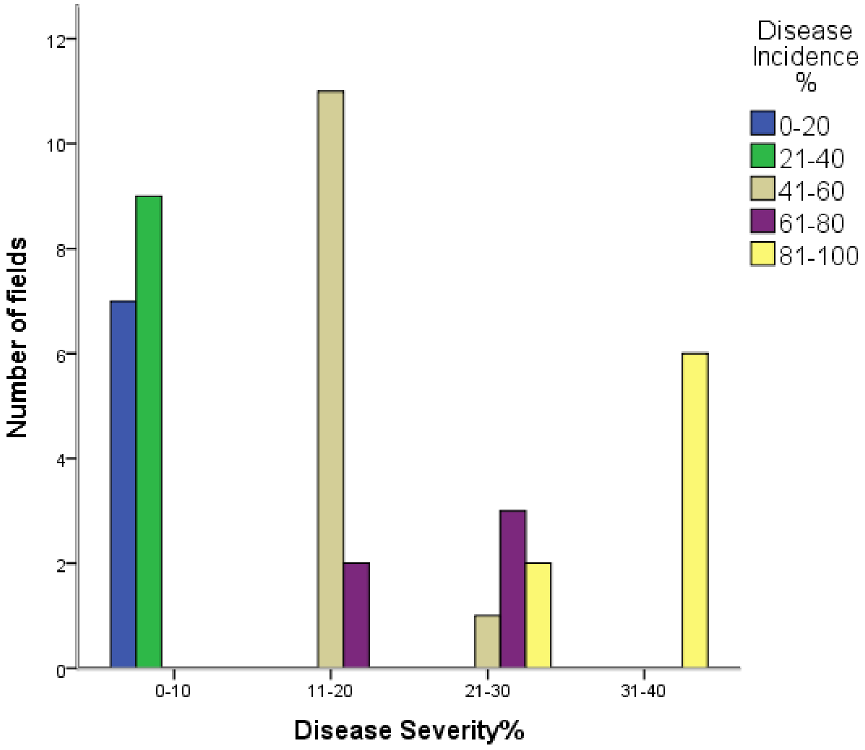

Figure 7: Incidence and severity of angular leaf spot disease at different variable classes

4.1.4 Angular leaf spot incidence vs source of seed

The results of the study show that the seeds for planting used by the farmers were sourced either from the local market, recycled from the previous season or bought from seed companies and some householders did not know where the seed was sourced. In the disease incidence range of 0-20%, 21-40% and 60-80% the highest number of fields were those where farmers used recycled seed for planting followed by seeds bought from the local local market. However for the disease incidence range of 41-60% the highest number of fields were those where farmers planted seed bought from the local market, followed by recycled seed and only 2 farmers planted seeds bought from seed companies. In the highest disease incidence range of 81 – 100%, there was one field where disease incidence was high despite the farmer planting seed which was bought from a seed company (Figure 8).

Recycled seed accounted for the highest number of fields infected with anguar leaf spot disease in each disease incidence range except in the 41-60% incidence range where seed sourced from the local market accounted for higher number of fields infected (Figure 8). It was noted that most of the seed planted by farmers was recycled seed and seed from the local market. Only three farmers planted seed from seed companies but these farms were also infected with angular leaf spot disease.

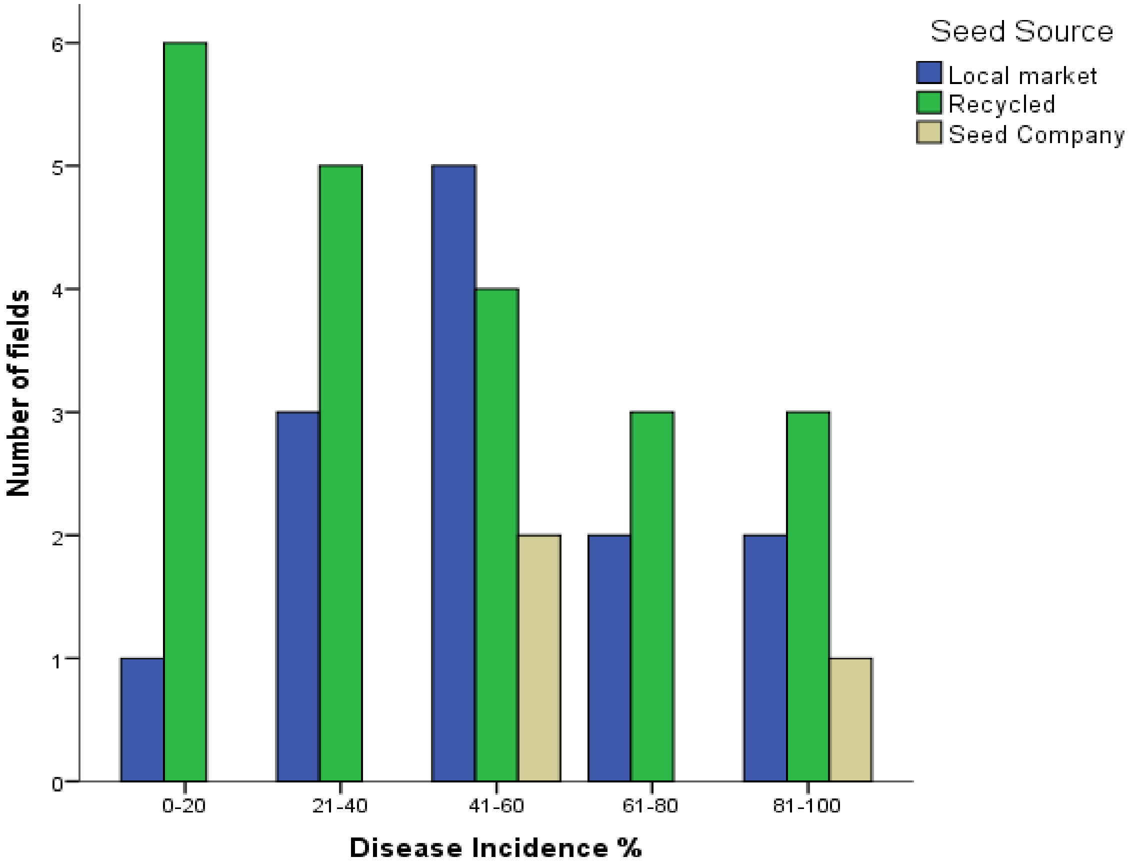

Figure 8: **Bar chart showing source of the common bean seed planted by farmers and disease incidence.**

4.1.5 Angular leaf spot disease incidence and cropping system

The survey showed that most of the farmers in the assessed fields practiced monocropping, where the common bean was the sole crop in the field. Only five farmers practiced intercropping where as thirty-six farmers practiced monocropping (Figure 9). The monocropped fields were present in all the disease incidence variable classes whereas the intercropped fields were only present in three disease incidence variable classes (0-20%), (21-40%) and (41-60%).

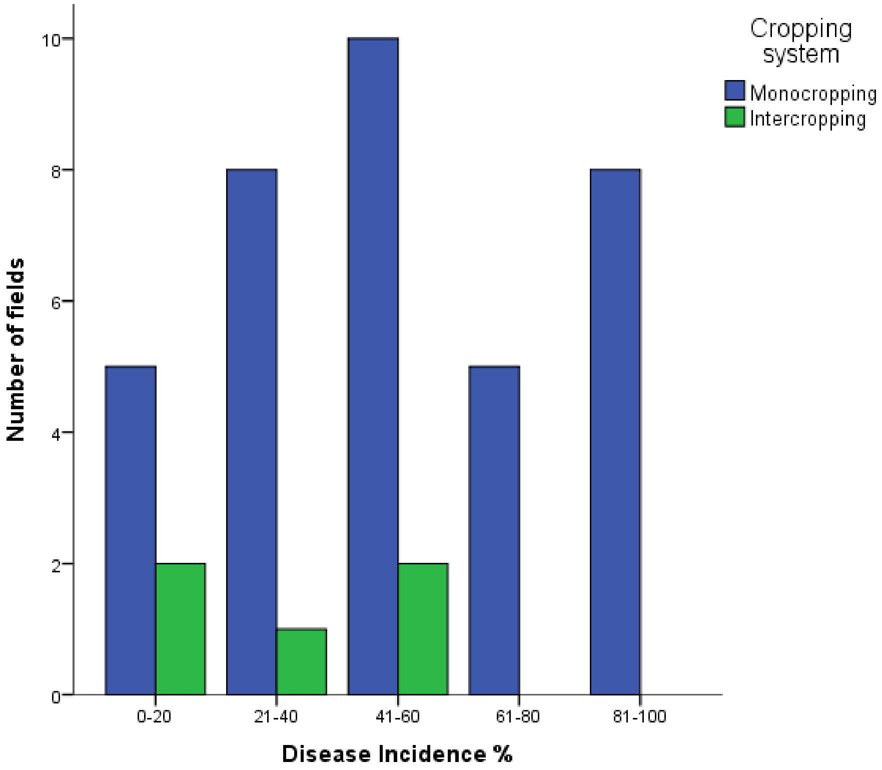

Figure 9: Incidence of angular leaf spot disease against cropping system practiced by the farmers.

4.1.6 Statistical analysis

4.1.6.1 Regression analysis between ALS disease incidence, cropping system and seed source

A multiple regression analysis carried out resulted in a *p*-value of 0.222 (Table 8) which is not significant when compared 0.05 significance level. R^2 value was 0.076 (Table 10), whereas the t-probability value for cropping system was 0.093 and 0.639 for source of seed (Table 9).

Table 8: Regression analysis results on disease incidence, cropping system and seed source.

Response variate: DISEASE_INCIDENCE_%

Fitted terms: Constant, cropping system codes and seed source codes

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	2	2540.	1270.2	1.57	0.222
Residual	38	30816.	810.9		
Total	40	33356.	833.9		

Table 9: Estimates of parameters for the regression analysis

Parameter	estimate	s.e.	t(38)	t pr.
Constant	72.5	17.5	4.15	<.001
CROPPING_SYSTEM_CODES	-23.5	13.6	-1.72	0.093
SOURCE_OF_SEED_CODE	1.55	3.27	0.47	0.639

Table 10: Multiple regression model summary for disease incidence, source of seed and cropping system.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.276[a]	.076	.028	28.4769	.076	1.566	2	38	.222

a. Predictors: (Constant), SOURCE OF SEED CODE, CROPPING SYSTEM CODES

b. Dependent Variable: DISEASE INCIDENCE (%)

4.1.6.2 T- test for disease incidence in intercropped fields vs monocropped fields

The monocropped group had higher values for the depended variable disease incidence with M=52.78, SD=29.39 than the intercropped group where M=29.6, SD= 13.8 (Table 11). A two tailed test for independent samples resulted in t- value of 1.72 at 39 degrees of freedom at 95% confidence interval with p=0.93 (Table 12).

Table 11: Summary statistics for disease incidence in intercropped vs monocropped fields

		N	Mean	Std. Deviation	Std. Error Mean
DISEASE INCIDENCE (%)	Monocrop	36	52.78	29.39	4.9
	Intercrop	5	29.6	13.81	6.18

Table 12: Test for equality of sample variances under the null hypothesis of equal and unequal variances.

		t	df	p (2-tailed)
DISEASE INCIDENCE (%)	Equal variances	1.72	39	.093
	Unequal variances	2.94	10.15	.015

4.1.7 Isolation of Phaeoisariopsis griseola

The isolates were cultured using steak and spread plating techniques with the streaking technique proving to be more suitable for pathogen isolation. Monosporic isolates were also obtained (Figure 10). The isolated conidium had more than one septa and were either straight or slightly curved (figure 11).

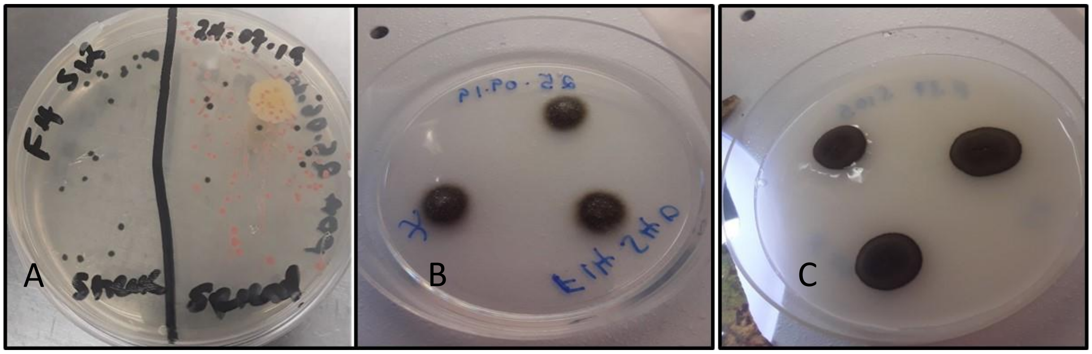

Figure 10: ***Phaeoisariopsis griseola*** **isolates: (A) Cultured using streak method on the left and spread-plating method on the right; (B) monosporic isolates on water agar and (C) monosporic isolates on V8 agar.**

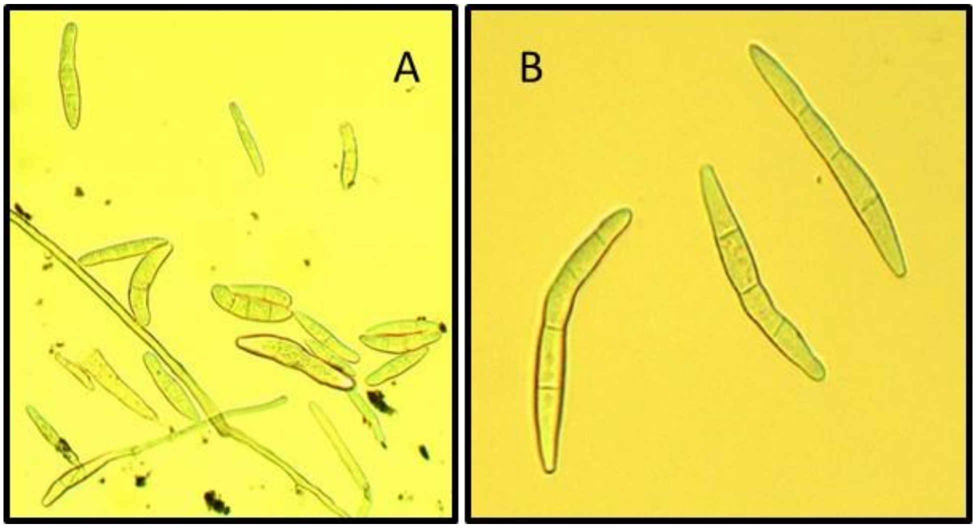

Figure 11: Conidia of *P. griseola* on water agar medium (400X magnification). Conidia was identified using a Motic BA310 digital microscope (Motic, Kowloon, Hong Kong).

4.1.8 Genetic diversity of *P. griseola* using ISSR markers

4.1.8.1 Primer ubc 889

The similarity indices computed for *Phaeoisariopsis griseola* for primer UBC 889 was in the range of 0.11 to 1.0. The Shannons H divesity index was 3.33, Simpsons 1-D was 0.933 and the polymorphic information content (PIC) was 0.35 (Table 8). At the similarity coefficient of 0.11 the isolates grouped into two major clusters, one cluster with one isolate and the second cluster with 82 isolates which further branched into two subgroups (Figure 13). The other eight isolates outside the two clusters were not amplified by the primer hence the grouping together, these are isolates 3, 4, 49, 63, 77, 98, 101 and 106.

Ten major clades were identified from the dendrogram with four isolates (8, 15, 27 and 109) individually standing out. The second clade following isolate 109, had four *P. griseola* isolates which were closely related to each other and these were isolates 5, 11,7 and 2. Within the same clade there were isolates (7 and 19) which were only related to each other with a genetic similarity coefficient 0.77, one was from Mwense and another from Mansa. The fourth clade showed that 7 isolates were related to each other with a genetic similarity coefficient of 1. However there were also isolates which had a similarity coefficient of 0.71 such as isolates 68, 69 and 0.85 for isolates 71, 93.

In the fifth clade there were 8 isolates which had high genetic similarity of 1. The genetic similarity in this group ranged from 0.833 to 1. In clade six, 24 isolates had a similarity coefficient of 1, the range of similarity for isolates in this group was from 0.55-1 . This group had isolates which are genetically similar originating from Mwense, Luwingu, Kasama, Mbala and Mpika.

In clade seven isolates which were genetically similar were isolates 28, 42, 66, 107, 112, 75, 81, 84, 86, 88 and 96. The genetic similarity range for this group was 0.42 to 1. Clade eight had 5 isolates which were genetically similar with a coefficient of similarity of 1. Isolate 55 has a genetic similarity coefficient of 0.33 to isolates 62 and 74 which shows that it's the most distant to these isolates. The genetic similarity was even lower, 0.25 when isolate 55 was compared to isolate 83. The genetic similarity range for this group was 0.25 - 1. Overall UBC primer 889 has shown that there is genetic diversity among the 91 *P. griseola* samples and this can be seen in the gel image showing the banding patterns for representative samples (Figure 12).

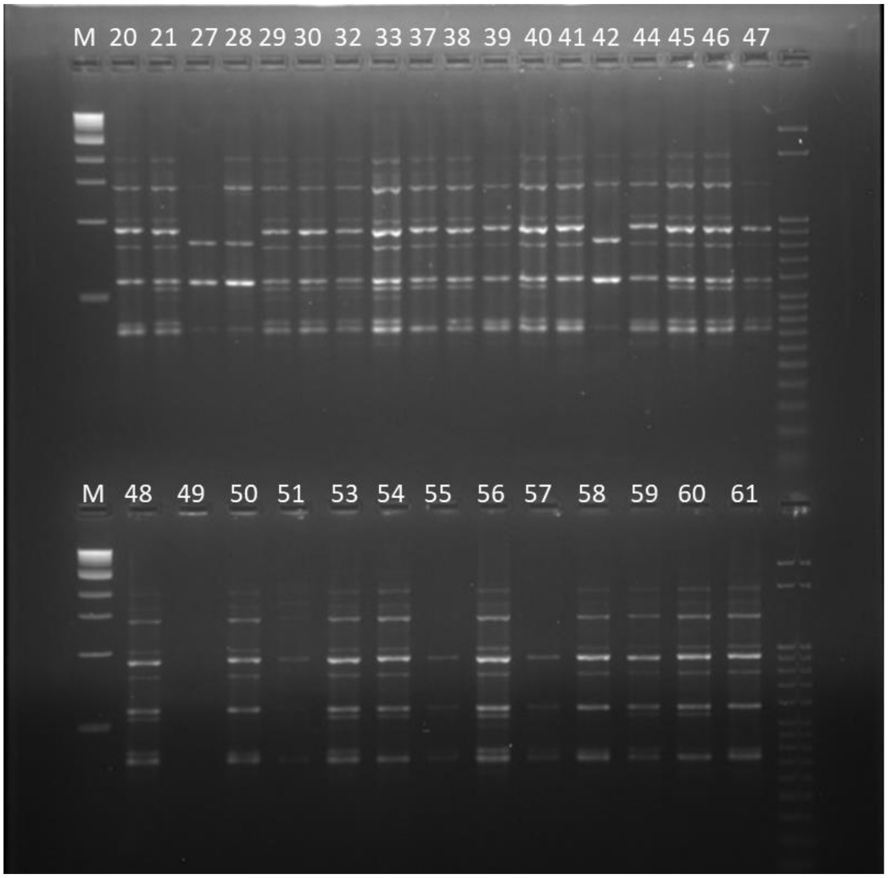

Figure 12: Polymorphic banding patterns for 24 representative *P. griseola* isolates using UBC 889. M is a 1kb extended molecular marker.

Table 13: Simpsons, Shannons diversity indices and PIC values for UBC 889.

Simpson_1-D	0.932884615384615
Shannon_H	3.33323076923077
PIC value	0.35

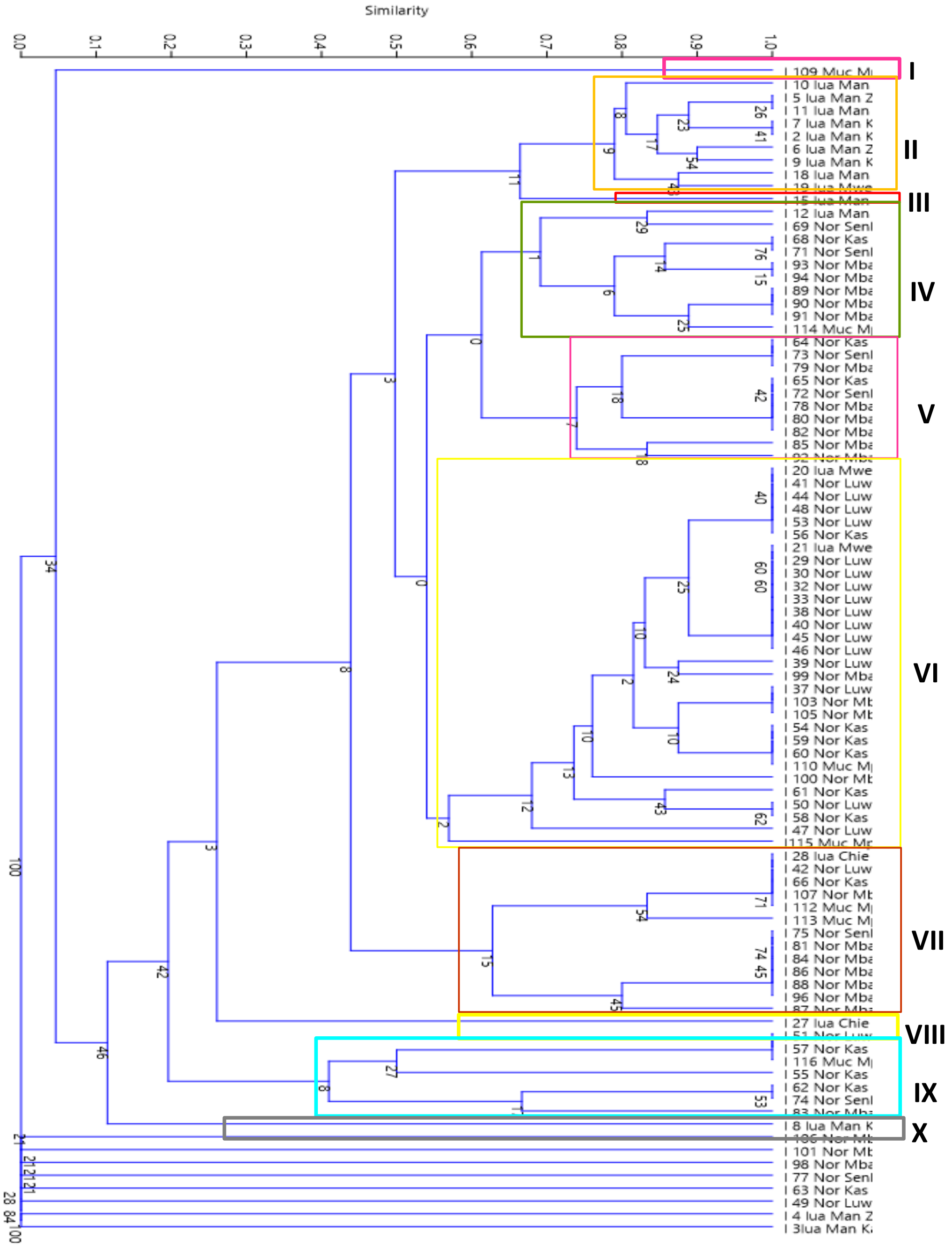

Figure 13: Dendrogram for UBC 889 showing the genetic relationship among the 91 *Phaeoisariopsis griseola* isolates analysed using UPGMA. (Isolate number, Province and District are included)

4.1.8.2 Primer ubc 809

When primer UBC 809 was analysed, it was noted that there were four outlier samples which were not amplified and these were isolates 3 from Mansa, 29 and 48 from Luwingu and 114 from Mpika. The other 87 isolates were grouped into two major clusters, and the first cluster had one isolate (116) from Mpika and the other 86 isolates were in the second major cluster (figure 14). The second major cluster branched into further sub clusters and this cluster showed great genetic variabiity for *P. griseola* of common beans in Zambia. The Shannons diversity index was found to be 2.96, Simpsons 1-D was 0.923 and PIC value was 0.30 hence supporting the high diversity of the pathogen (Table 9). The dendrogram showed that there were 16 groups of isolates which had a close relationship with a similarity index of 1. However it was noted that diversity varied as well between these groups,for example isolate 5 and 30 had a similarity coefficient of 0.71, isolate 5 and 37 were not closely related to each other and had a similarity coefficeint of 0.57. Isolate 105 from Mbala and isolate 28 from Chiengi did not share any genetic similarity because they had a similarity coefficient of 0. However there were isolates which were also related between these groups such isolates 37, 39 and isolates 69, 77 (figure 15). Other isolates which were not genetically similar included isolates 115,10 and 98,79 with a coefficient of similarity ranging from 0 to 0.375. It was observed that there were also isolates which were classified as similar but are from different districts such as isolates 75 from Senga Hill and isolate 66 from Kasama. Isolates 112 from Mpika and isolate 96 from Mbala were also very closely related with a jaccards coefficient similarity of 1 which is 100% (Figure 15).

Sample 116 which appeared to belong to a separate branch alone showing similarity to only 10 other isolates, but the coefficient of similarty was very low in the range of 0.11 to 0.33, this just shows that sample 116 was different from the other samples.

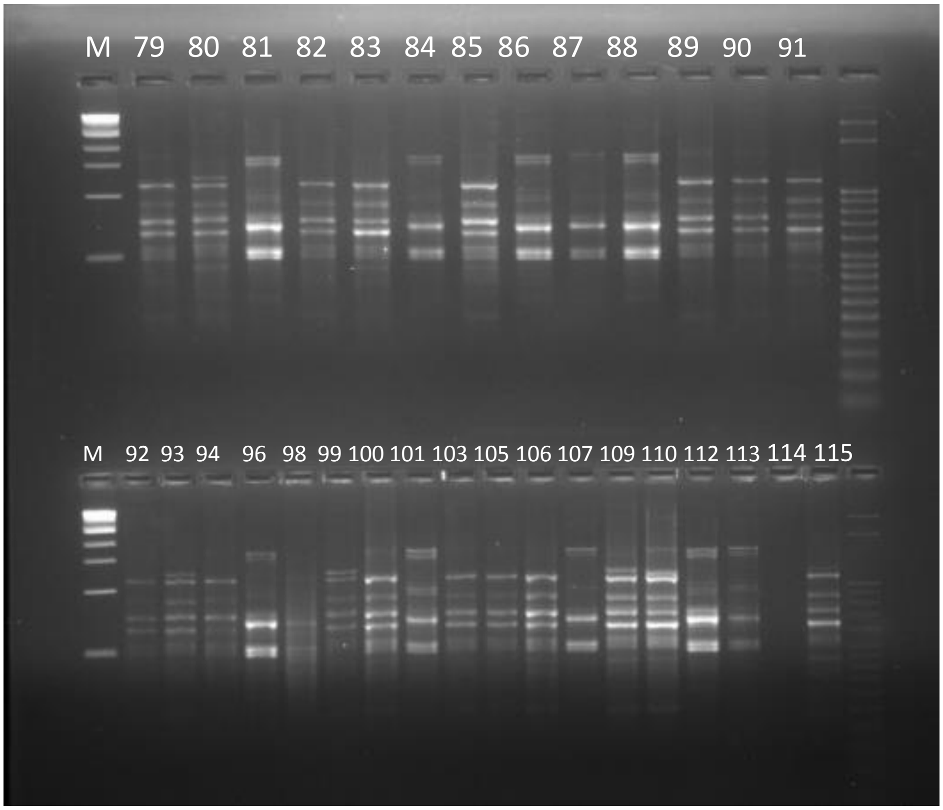

Figure 14: Polymorphic banding patterns for 31 representative *P. griseola* isolates using Primer UBC 709. M is a 1kb extended molecular marker.

Table 14: Simpsons, Shannons diversity indices and PIC value for UBC 809 tabulated using past 4.03 software

Simpson_1-D	0.923171
Shannon_H	2.957529
PIC value	0.30

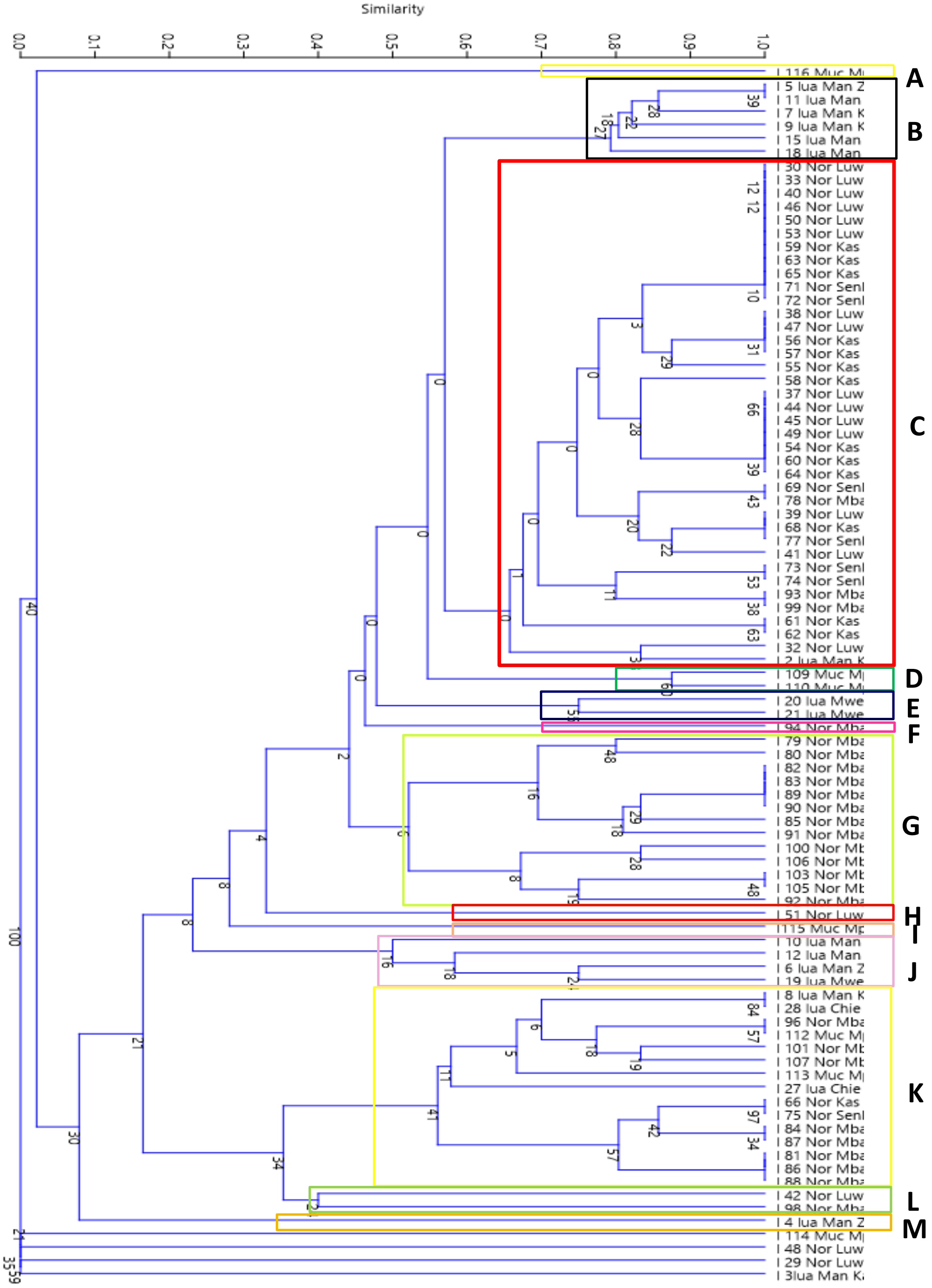

Figure 15: Dendrogram (UPGMA) for UBC 809 representing the genetic relationship among the 91 *Phaeoisariopsis griseola* isolates using the jaccards similarity coefficients.

4.1.8.3 Primer ubc 888

The samples segregated into two major clusters at 0.2 similarity. One cluster had two clades (E and F) with sixteen *P.griesola* isolates and the second cluster had seventy isolates which were distributed into four clades A, B, C and D (Figure 17). Clade B had two groups of isolates which had a similarity coefficient of 1; however, the isolates between these groups had a similarity coefficient of 0.8 such as isolates 82 and 94. Cluster C had eighteen isolates which had a similarity index of 1, within this group isolates 39, 2 had similarity index of 0.71, and isolates 18, and 39 which had a similarity index of 0.63. Clade D had three groups of isolates which had a similarity index of 1.

Cluster 2 separated into two clades and these were G and H. Clade G had one isolate from Mbala and clade H had 15 isolates, among the 15 isolates, 13 isolates had a similarity coefficient of 1. Isolates 27,28,75,86 were closely related as well as isolates 86,88, and 98,101,107,113. The isolates which segregated into this cluster originated from Chiengi, Senga Hill, Mbala,Kasama and Mpika. Within this cluster there were isolates which were very distant from each other genetically such as isolate 87 and 27 with the similarity coefficient of 0.286. Isolate 87 and 8 were also not closely related genetically with a similarity coefficient of 0.125. Isolates 65 and 66 with a genetic coefficient of 0.091 were not genetically similar as well as isolates 65 and 88 which had a genetic similarity of 0.1 (Figure 17). The samples which were not amplified using this primer include samples 4, 10, 89, 90 and 112, sample 4 was also not amplified by primer UBC 889. The Shannons diversity index (H) for UBC 888 was 3.2, the Simpsons 1-D was 0.925 and the PIC value was 0.31 (Table 10).

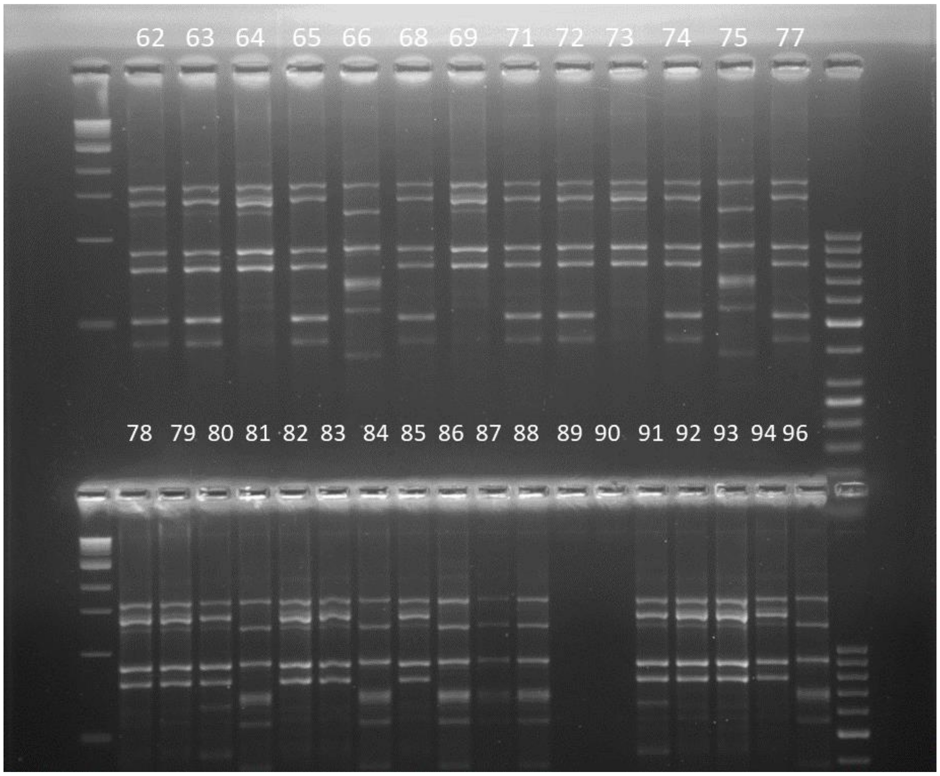

Figure 16: Polymorphic banding patterns for 31 representative *P. griseola* isolates using Primer UBC 888. M is a 1kb extended molecular marker.

Table 15: Simpsons, Shannons diversity indices and PIC values for UBC 888.

Simpson_1-D	0.924654
Shannon_H	3.247854
PIC value	0.31

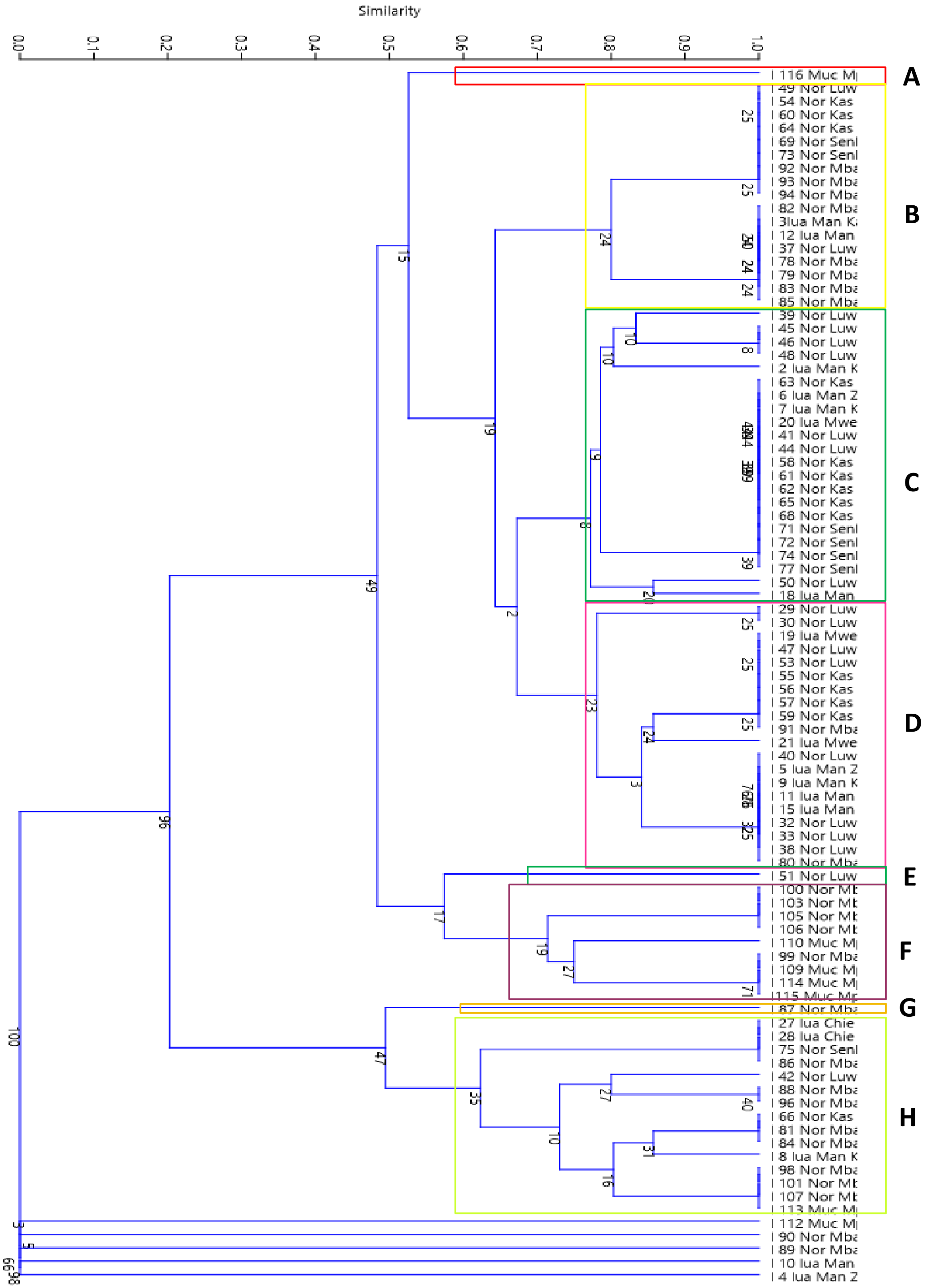

Figure 17: Dendrogram for UBC 888 generated using UPGMA showing the relationship among the 91 *Phaeoisariopsis griseola* isolates using Jaccards similarity coefficients.

4.1.8.4 Concatenation of primers ubc 889, ubc 888 and ubc 809

The coefficients of similarity for the concatenated primers was in the range of 0 - 1. The simpson 1- D was high with a value of 0.93, the simpsons (H) was 3.16 and the polymorphic information content was 0.32 (Table 11). Ultimately the isolates were separated into two groups at 0.4 coefficient of similarity with isolate 4 from Mansa standing out alone and the rest of the 90 isolates were further divided into subgroups. Isolates were clustering according to the place of origin in some cases such as isolates 5,11,9,15,9,6,18,7 and 2 (Figure 18). Isolate 5 and 11 from Mansa has a similarity coefficient of 1 which means that they are closely related. Isolates from Kasama and Luwingu were also grouped together in a clade. Isolate 65 from Kasama and 72 from Sengahill also had a similarity coefficient of 1, these were from different districts but were similar. Isolate 116 from Mpika and isolate 3 from Mansa had a lower coefficient of similarity of 0.33 which shows that these isolates are not closely related genetically. Isolate 30 and 40 from Luwingu were closely related with a coefficient of similarity of 0.95, this was also the same for isolate 40 and 32which had a similar coefficient of similarity. Generally some isolates from different districts were clustering together and some from within the districts were also clustering together.

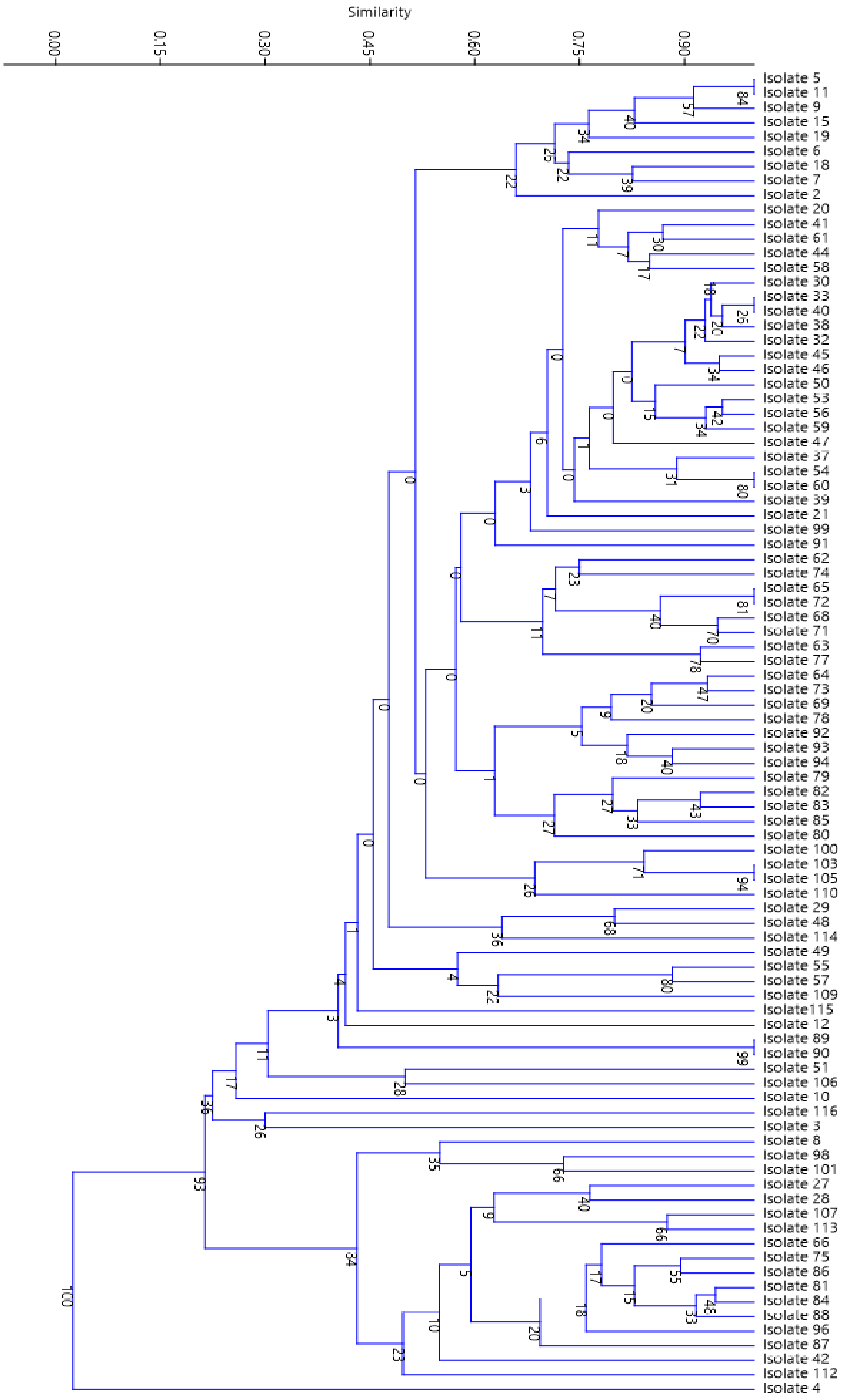

Figure 18: Tree dendrogram based on concatenation of ISSR markers, the binary data was aligned using classical clustering

Table 16: Shannons 1-D, Shannons_H and PIC values for concatenated ISSR markers

Simpson_1-D	0.926556
Shannon_H	3.158886
PIC value	0.32

CHAPTER 5

DISCUSSION

5.1 Prevalence, severity and incidence of angular leaf spot disease

The survey conducted in the Northern part of Zambia showed that fungal angular leaf spot disease was present in all the districts and was recorded in 100% of the farms which were assessed. Such high prevalence of angular leaf spot has also been reported in Kenya where angular leaf spot was present in 89% of the fields visited (Mwang'ombe *et al.*, 2007). The highest PSI was recorded in Mpika (28%) and the lowest was 2.2% in Kaputa. There were only nine fields which had a percentage severity index greater than 25% whereas 32 fields had a PSI less than 25%. The province with the highest average severity of 1.67 was Muchinga province. An analysis of variance showed that there was a significant difference of severity between provinces with F=3.36 and p=0.045. The most significant difference in the means was between Muchinga and Northern provinces, with Muchinga having a higher mean severity as compared to Northern province. The low disease severity observed in the study can be attributed to climatic conditions which could have had a hindrance on disease progression (Degu *et al.*, 2020). Disease incidence on the other hand was very high with eight fields having disease incidence greater than or equal to 80%. Some studies have reported higher disease incidence ranges of 77-100% and 82-100% (Canpolat *et al.*, 2021). An analysis of variance carried out of disease incidence between provinces revealed that there was no significant difference in disease incidence between provinces with F=2.17 and p=0.128. The bean varieties grown in the fields where high disease incidence was recorded included white beans, Lusaka beans, Kabulangeti and a combination of white,yellow and white,red beans. These are varieties which have been classified to be resistant to angular leaf spot disease by bean breeders, but the results of the survey shows that the resistant has either been broken or the seeds have been over recycled hence leading to higher disease incidence (Muimui *et al.*, 2021). Furthermore the causal pathogen of angular leaf spot *Phaeoisariopsis griseola* is highly variable therefore durable resistance is very difficult to achieve in resistant varieties (Pastor – Corrales *et al.*, 1998).

The study highlighted that farmers obtained their seed from the local market, some used recycled seed and others bought planting seed from seed companies. A multiple regression carried out to establish to what extent the source of seed and cropping system contributed to disease incidence showed that cropping system and seed source did not contribute to a significant amount of variance in disease incidence. The R^2 was equal to 0.076 which is 7.6%, this means that source of seed and cropping system only brought about 7.6% change in disease incidence which is not quie a significant change. The P-value for cropping system amd seed source were 0.639 and 0.093 respectively which is not significant when compared to 0.05 confidence level. However, a study conducted by Ochichi (2018) on quality of bean seeds revealed that farmers saved seed is usually infected with bacterial and fungal pathogens, therefore recycling seed from season to season could make it the primary source of inoculum for disease. This is centrally to the results of Icishahayo (2014) whos finding showed the absence of the fungal pathogen *Phaeoisariopsis griseola* in home saved seed. This low infection in the planted seed was attributed to low disease pressure in the field during the growing season.

The main cropping system which was practiced in these common bean-growing areas was monocropping (87.8%) whereas 12.2% of the farmers practiced intercropping. A t- test which was carried out to determine if there was a significant difference in disease incidence in monocropped fields as compared to intercropped fields was not significant $t(39) = 1.72$, $p = 0.93$. This implies that there was no significant difference in disease incidence in monocropped fields and in intercropped fields. This can be attributed to the seed which is used by the farmers, it could already be infected with the pathogen hence disease would develop irrespective of the cropping sytem being practiced. Another reason could be that the three provinces lie in the same ecological region hence there would be not much of a difference in the infection rate of the pathogen. Futhermore the causal pathogen of ALS is able to survive in the host debri for 8 months so there is re-infection in the next cropping season which falls within the 8 months period (Altaf *et al*., 2022) . These results are contrary to the results which were reported by Kijana (2017) who found that disease incidence was more severe in farms where intercropping was practiced as compared to farms where there was monocropping; therefore, it was established that the crops which were intercropped with the beans in these areas provided a conducieve environment for the growth of the pathogen.

5.2 Molecular diversity of *Phaeoisariopsis griseola*

Three primers UBC 889, 888 and 809 (Abadio *et al*., 2012) were used to assess genetic diversity of *Phaeoisariopsis griseola* isolates from the study area. Primer UBC 889 showed high genetic diversity because the similarity indices ranged from 0.11-1.0. The Shannons diversity index was also the highest with a value of 3.33 as compared to UBC 888 which had a diversity index of 3.24 and UBC 809 which had a diversity index of 2.96. Primer UBC 889 also gave the highest polymorphic information content among the isolates with a PIC value of 0.35. This is closer to 0.5 which has been demonstrated to be the highest for dominant markers (De Riek *et al.*, 2001). Selected individual isolates were compared to determine their genetic relatedness. Results indicated that one isolate (55) from Kasama was quiet distant from either isolate 62 also from Kasama and 74 from Senga Hill with a coefficient of similarity of 0.33. When isolate 55 was also compared with isolate 83 from Mbala an even lower similarity coefficient of 0.25 was obtained.

These differences could be due to independent mutations or recombination events that occurred in nature in each of the respective isolates (De Almeida *et al*, 2021).

Another primer UBC 888 identified 75 isolates which had a similarity coefficient of 1. However this primer also detected isolates which were not genetically similar such as isolates 87, 27 which had a similarity coefficient of 0.125. Isolates 65,66 were from the same district but were not genetically similar with a similarity coefficient of 0.091 and isolates 65,88 had a genetic similarity of 0.1 which means that they are not genetically related to each other. When isolates 87, 27 were compared using primer UBC 889 there was also a low relationship between the two samples because the diversity index was 0.166. However, with primer UBC 889 the isolate 65,66 and 65,88 were said to be 50% related to each other because the similarity coefficient was 0.5.

Primer UBC 809 had the lowest shannons diversity index of 2.96 among the three primers. The isolates which had a similarity index of 1 were 52; genetic diversity also varied within the groups. This high similarity index observed in the study shows that that the *P. griseola* isolates are closely related whereas the low similarity index shows that the isolates are not closely related (Jundi *et al*., 2020) There were isolates which were not genetically similar such as isolate 115,10, and 98,79 which had a coefficient of similariy ranging from 0 - 0.375. However, some *P. griseola* isolates failed to cluster according to the region of origin such as isolates 87 and 86 from Mbala and isolates 47 and 50 from Luwingu. The results from the current study are comparable to those reported by Busogoro *et al.* (1999) who showed that *P. griseola* isolates were not able to cluster according to the region of origin even though their results demonstrated that there was high similarity among isolates collected from within the same site. However, it was observed in this study that there were isolates which were genetically similar but were from different districts such as isolate 75 from Senga Hill and Isolate 66 from Kasama. Isolate 112 from Mpika and isolate 96 from Mbala were also closely related and had a jaccards similarity coefficient of 1. This is not surprising because farmers use recycled seed and seed bought from the local market which could have been sourced from another district becase of trade. Isolate 116 which was clustered alone using this primer was related to only 10 *P. griseola* isolates but the coefficient of similarity was very low in the range of 0.11 – 0.33 which shows that it was indeed different from other isolates. The implication is that this could be a new race which has resulted in order to break the resistance of the plants in this particular area. Therefore it imperative to use such an isolate in pathotype studies to establish its virulence.

Cluster analysis resulting from the three primers showed that the *P.griseola* isolates were clustering according to province in some subgroups where as in other subgroups the isolates were mixed from different provinces in terms of clustering. Similar results were also observed where subgroups were formed by isolates from the same local origin and where some subgroups had a mixture of isolates from other origins (Sartorato, 2004). The reason why isolates could be mixed from different districts and provinces is because of interchange of bean seeds among farmers. The pathogen is also seed born so isolates from one district can be introduced into another district due to the informal seed system which is used by the farmers.

The coefficients of similarity for the concatenated primers was in the range of 0 - 1. Which shows that the combination of the three primers shows that there is high genetic diversity among the *P.griseola* isolates. Isolate 4 from Mansa was standing out where as the rest of the 90 isolates were in another cluster which formed subgroups. There were isolates which were closely related to each other with a similarity coefficient of 1. Examples of these were isolates 5 and 11 from Mansa, 33 and 40 from Luwingu, 54 and 60 from Kasama, 65 from Kasama and 72 from Sengahill, 103 and 105 from Mbala and 89 and 90 from Mbala. It was observed that some sub-clades had isolates which were clastering together according to district such as isolates from Mansa, Mbala and Luwingu whereas in some subgroups the isolaes were mixed from different districts such as isolates 20, and 41, 61, and 44 and 58 which are from Mwense , Luwingu and Kasama respectively. Within the districts there were also isolates which were not closely related genetically and had a low coefficient of similarity such as isolate 87 and 98 from Mbala. This shows that there is also variation of *P. griseola* within the districts. This high genetic variability within *P.griseola* shows that the majority of the isolates present in the Northern part of Zambia are unique (Mahuku *et al.*,2002).

Genetic variability has also been studied in other species using ISSR markers such as *Lycium* species which indeed confirms the capability of ISSR markers to establish genetic variability (Liu *et al* 2020). This high genetic variability was observed in this study by the concatenated primers which resulted in a Shannons diversity index of 3.2 which is closer to 3.3 which was obtained in another study of *Phaeoisariopsis griseola* (Abadio *et al*., 2012). However a study of ALS pathogens genetic diversity using SSR markers demonstrated moderate diversity among the analysed *P. griseola* isolates (De Almeida *et al*, 2021).

CHAPTER 6

6.1 Summary

6.1.1 Distribution, incidence and severity of angular leaf spot disease

The study showed that angular leaf spot disease caused by *Phaeoisariopsis griseola* was present in all the nine districts surveyed in the northern part of Zambia that is Mbala, Kasama, Mansa, Mwense, Chiengi, Kaputa, Mpika, Senga Hill and Luwingu; the disease was recorded in 100% of the farms surveyed. The highest PSI was recorded in Mpika (28%) and the lowest in Kaputa (2.2%). Muchinga province had the highest average severity of 1.67 and analysis of variance showed that there was a significant difference of severity among the provinces, the most significant being Muchinga and Northern. Disease incidence was high with eight fields having incidence greater than 80%. The ANOVA carried out showed that there was no significant difference in disease incidence between provinces. Most of the farmers used recycled seed for planting nevertheless, a multiple regression carried out showed that the cropping system and source of seed did not contribute to variance in disease incidence. The main cropping system practiced by the farmers was monocropping, However a t-test carried out revealed that there was no significant difference in disease incidence in monocropped fields and in intercropped fields.

6.1.2 Molecular variations of *Phaeoisariopsis griseola* using inter simple sequence repeats markers

The molecular diversity evaluated using three inter simple sequence repeats primers showed that UBC 889 exhibited the highest diversity index with the value of 3.33, followed by UBC 888 with a diversity index of 3.24 and UBC 809 which had a diversity index of 2.96. Primer 889 divided the 91 isolates into 10 clades with four isolates standing out that is *P. griseola* isolates 8, 15, 27 and 109. The genetic similarity range for these isolates was 0.25 – 1. There were also isolates which were identified to be genetically similar but were from different districts such as Mwense, Luwingu, Kasama, Mbala and Mpika. The primer UBC 809 identified some isolates which were similar but were from different districts such as *P. griseola* isolates 75 from Senga Hill and isolate 66 from Kasama. Isolate 112 from Mpika was also related to isolate 96 from Mbala. The *P. griseola* isolate 116 which was clustered alone was only related to 10 other isolates with a very low coefficient of similarity ranging from 0.1- 0.33. This just shows that this isolate was different from the others. Primer UBC 888 identified some *P. griseola* isolates which were not related to each other such as isolates 87,8 and 65,88. There were also isolates which were identified as being related to each other such as isolates 27, 28, 75, 86 and isolates 94,101,107,113. These isolates were originating from Chiengi, Senga Hill, Mbala, Kasama and Mpika. Thr concatenated primers showed that *P.griseola* isolates were clustering according to districts in some subgroups where as in some subgroups. The isolates were mixed from different districts this could be due to interchange of bean seeds among farmers. The genetic diversity was also very high among isolates because the coefficients of similarity ranged from 0 – 1. Some isolates from some districts were genetically similar such as isolates 5, 11 from Mansa and isolates 33, 40 from Mansa as well. Within the districts there were isolates which were not genetically similar such as isolates 87 and 98 from Mbala. The variation within the *P. griseola* shows that the majority of the isolates present in the Northern part of Zambia are unique.

6.2 Conclussion

Fungal angular leaf spot was present in 100% of the fields surveyed. Severity was genrally low with the highest PSI being 28%. However there was a significant difference in severity among the provinces. The incidence was high with eight fields having incidence greater than 80%, however there was no significant difference in incidence among the provinces. The cropping system which was mostly practiced was monocropping whereas intercropping was practiced by very few farmers. This did not have an effect on the disease incidence observed. The source of seed did not significantly contribute to the variance in disease incidence. The individual primers and concatenated primers showed that there was genetic diversity in *P. griseola* isolate. The isolates were clustering according to districts in some subgroups there was a mixture of isolates from different districts. The Shannons 1-D index (0.926) was high this implies that there was a high level of genetic diversity of *P. griseola* isolates in the Northern part of Zambia.

6.3 Recommendations

The following recomendaions have been made based on the findings of the study:

- There is a need to use the genetically different *Pheaoisariopsis griseola* isolates which have been identified by the primers to study their virulence on the common bean varieties used by farmers in the major bean growing areas.
- There is also a need for the use of race differential cultivars in future studies to further develop a better understanding on the different races of *P. griseola* that exists in Zambia.
- Further surveys can be carried out in other parts of Zambia where beans is grown such as Eastern province to also assess the prevalence of ALS disease
- Collection and screening of farmer saved seed for presemce of *Phaeoisariopsis griseola*.

REFERENCES

Abadio, A.K.R., Lima, S.S., Santana, Mateus., Salomão, T.M.F., Sartorato, A., Mizubuti, Eduardo., Araújo, E.F., and Queiroz, Marisa. (2012). Genetic diversity analysis of isolates of the fungal bean pathogen *Pseudocercospora griseola* from central and southern Brazil. *Genetics and molecular research*: GMR. 11. 1272-9. 10.4238/2012.May.14.1.

Akçali Giachino, R.R. (2020). Investigation of the genetic variation of anise (*Pimpinella anisum L.*) using RAPD and ISSR markers. *Genet Resour Crop* Evol 67, 763–780. https://doi.org/10.1007/s10722-019-00861-y

Allen, D. J., Buruchara, R. A., and Smithson, J. B. (1998). Diseases of common bean. In D. J. Allen, & J. M. Lenne´ (Eds.), *the Pathology of Food and Pasture Legumes* (pp. 179-265). Walligford, CABI, UK.

Altaf, A.W., Farooq, A. B., Raheeba ,T. N., Tanveer, A. W., Waseem, R., Shaheen K. Jan., Tariq, R. R., Nahida, A., Mohammad, A. W., and Peerzada, M. M. (2022). Bean angular leaf spot (*Phaeoisariopsis griseola*): Epidemiology and yield loss assessment. *The Pharma Innovation Journal*; 11(2S): 291-297.

Aytenfsu, M., Terefe, H., and Ayana, G. (2019). Aggressiveness of *Phaeoisariopsis griseola* isolates and reaction of common bean varieties to the isolates under greenhouseS conditions. *African Journal of Agricultural Research*, 14(32), 1553-1567.

Boudreau, M., A (1993). Effect of intercropping beans with maize on the severity of angular leaf spot of beans in Kenya. *Plant pathology* 42(1):16-25. DOI: 10.1111/j.1365-3059.1993.tb01467.x

Buruchar, R. A., (1983). Determination of pathogenic variation in *Isariopsis griseola* Sacc. and *Pseudomonas syringae pv. phaseolicola* (Burk., 1926) Young, Dye & Wilkie 1978. Ph.D. thesis, University of Nairobi (1983)

Busogoro, J., Jijakli, M., and Lepoivre, P. (1999). Virulence Variation and RAPD Polymorphism in African Isolates of *Phaeoisariospis griseola* (Sacc.) Ferr., the Causal Agent of Angular Leaf Spot of Common Bean. *European Journal of Plant Pathology* 105, 559–569. https://doi.org/10.1023/A:1008707101645

Canpolat, S., and Maden, S. (2021). Characterization of Turkish isolates of *Pseudocercospora griseola* the causal agent of angular leaf spot of common beans. *Journal of Plant Protection Research*, 61(1), 95-102. https://doi.org/10.24425/jppr.2021.136273

Canteri M., G and Godoy C., V. (2005). Photosynthetic efficiency of healthy leaf Area influenced by angular leaf spot severity in bean field. Ciêncius Agrárias, Londrina, v.26, n.2, p. 179-186, abr./jun.2005.

Castellamos, Guillermo; Jara, Carlos; Mosquera, Gloria (Eds.). 2016. Bean pathogens: practical guide for Lab and greenhouse work. 2nd ed. Centro Internacional de Agricultura Tropical (CIAT), Cali, CO. 257 p. (Publicación CIAT No. 420)

Celmeli, T., Sari, H., Canci, H., Sari, D., Adak, A., Eker, T., and Toker, C. The Nutritional Content of Common Bean (Phaseolus vulgaris L.) Landraces in Comparison to Modern Varieties. *Agronomy* 2018, 8, 166.

Chikoti P.C and Muimui K.K. Occurrence and distribution of Angular leaf spot of common bean in Zambia. Zambia Agricultural Research Institute, Mount Makulu, (Unpublished data).

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

Crous, P. W., Liebenberg, M. M., Braun, U., and Groenewald, J. Z., (2006). Re-evaluating the taxonomic status of *Phaeoisariopsis griseola*, the causal agent of angular leaf spot of bean. *Studies in Mycology*, Volume 55, Pages 163-173, ISSN 0166-0616, https://doi.org/10.3114/sim.55.1.163.

Dagnew, K., Haileselassie, T., and Feyissa, T. (2014). Genetic diversity study of common bean (*Phaseolus vulgaris L.*) germplasm from Ethiopia using inter simple sequence repeat (ISSR) markers. *African Journal of Biotechnology*, 13(36), 3638-3649.

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

Ddamulira, G., Mukankusi, C., Ochwo-Ssemakula, M., Edema, R., Sseruwagi, P., and Gepts , P. (2014). Distribution and variability of *Pseudocercospora griseola* in Uganda. *Journal of Agricultural Science* 6(6): 16-29.

De Almeida, C. P., Arruda, N., de Carvalho Paulino, J.F. et al (2021). Genetic diversity of *Pseudocercospora griseola* resistance loci in common beans. *Trop. plant pathol*. 46, 129–138 (2021). https://doi.org/10.1007/s40858-020-00395-0

De Riek, J., Calsyn, E., Everaert, I.G., Van Bockstaele, E., and de Loose, M. (2001). AFLP based alternatives for the assessment of Distinctness, Uniformity and Stability of sugar beet varieties. *Theoretical and Applied Genetics, 103*, 1254-1265.

Degu, T., Yaregal, W., and Gudisa, T. (2020). Status of common bean (*Phaseolus Vulgaris L.*) Diseases in Metekel zone, North West Ethiopia. *J Plant Pathol microbial* 11:494.doi:10352481/2157-7471.20.11.494.

Deighton, F. C., (1990). Observations on *Phaeoisariopsis. Mycological Research*, 94, pp. 1096-1102

Desjardins, P., and Conklin, D. (2010). Nanodrop microvolume quantitation of nucleic acids. *Journal of visualized experiments:* JoVE, (45), 2565. https://doi.org/10.3791/2565

Garcia, F. A. O and Romeiro, R. S. (2011). Biocontrole da mancha-angular do feijoeiro por antagonistas bacterianos. *Pesquisa Agropecuária Brasileira*, v. 46, n. 12, p. 1603-1608.

Geil P. B., and J W Anderson J. W. (1994) Nutrition and health implications of dry beans: a review., *Journal of the American College of Nutrition*, 13:6, 549-558, DOI: 10.1080/07315724.1994.10718446

Getachew, G., Tesfaye, A., and Kassahun, T. (2013). Evaluation of disease incidence and severity and yield loss of finger millet variety and mycelial growth inhibition of *Pyricularia grisea* isolates using biological antagonists and fungicide in vitro condition. *J .Appl. Biosci.,* 73: 5883-5901.

Hammer, Ø., Harper, D. A. T., and Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica,* 4, 9 p.

Hamzakaza, P., Katungi, E., Reyes, B., Maredia,M., Muimui, K., and Ojara, M (2014). Assessing access and adoption of common bean improved varieties in Zambia. Research Technical Report. Zambia Agriculture Research Institute, International Center for Tropical Agriculture (CIAT) and Michigan state University. Lusaka, Zambia.

Hayat, I., Amad .A., Masud, T., Ahmed, A., and Bashir, S (2014) Nutritional and Health Perspectives of Beans (*Phaseolus vulgaris L*.): An Overview, Critical Reviews in Food Science and Nutrition, 54:5, 580-592, DOI: 10.1080/10408398.2011.596639

Icishahayo, David (2014) Management of Common Bean (*Phaseolus vulgaris*) Angular Leaf Spot (*Pseudocercospora griseola*) Using Cultural Practices and Development of Disease-Weather Models for Prediction of the Disease and Host Characteristics. PhD thesis, University of Zimbabwe.

Jain, S. 2006. 'An empirical economic assessment of impacts of climate change on Agriculture in Zambia'. *CEEPA Discussion paper number .27,* Centre for Environmental Economics and Policy in Africa- CEEPA University of Pretoria.

Jain, S.(2007) An Empirical Economic Assessment of climate Change on Agriculture in Zambia. Policy Research Working Paper 4291. World Bank.

Jones A.L, and Mejia, D. (1999). PHASEOLUS BEANS: Post Harvest Operations, Centro Internacional de Agricultura Tropical (CIAT) www.cgiar.org/ciat.

Jundi, A., Dyah, S., Daryono, B. and Purnomo. (2020). Genetic diversity of Johar (Senna siamea (Lam.) H.S. Irwin & Barneby) in five Islands in Indonesia based on ISSR molecular characters. *AIP Conference Proceedings*. 2260. 020012. 10.1063/5.0016281.

Kebour, D., Boutekrabt, A., Melfti, M., and Abdull Husaain, A.S. (2012).Use of the ISSR markers for the study of genetic polymorphism of the pistachio fruit *pistachio vera* L. in Algeria. UPB Sci. Bull. Ser. B Chem. Mater. Sci. 74, 191-200.

Kijana, R., Abang, M., Edema, R., Mukankushi, C., and Buruchara, R. (2017). Prevalence of angular leaf spot disease and source of resistance in common bean in Eastern Democratic Republic of Congo. *African Crop Science Journal*, 25(1): 109-122.

Landeras, E., Trapiello, E., Braña, M., and González, A. J. (2017) "Short communication: Occurrence of angular leaf spot caused by *Pseudocercospora griseola* in Phaseolus vulgaris in Asturias, Spain". *Spanish Journal of Agricultural Research*, 15(3), p. e10SC03. doi: 10.5424/sjar/2017153-10798.

Lee, P. Y., Costumbrado, J., Hsu, C. Y., and Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. Journal of visualized experiments: *JoVE*, (62), 3923. https://doi.org/10.3791/3923

Leitich, R., Omayio, D. O., Mukoye, B., Mangeni, B. C., Wosula, D. W., Arinaitwe, W., Otsyula, R. M., Were, H. K., and Abang, M. M. (2016). Pathogenic Variability of Angular Leaf Spot Disease of Common Bean in Western Kenya. *International Journal of Applied Agricultural Sciences*. 2. 92-98. 10.11648/j.ijaas.20160206.13.

Liu, X., Du, J., Khan, M., Cheng, J., Wei, C., Mei, Z., and Fu, J. (2020). Analysis of genetic diversity and similarities between different *Lycium* varieties based on ISSR analysis and RAMP-PCR markers. *World Academy of Sciences Journal*, 2, 83-90. https://doi.org/10.3892/wasj.2020.39

Liu, X., Du, J., Khan, M., Cheng, J., Wei, C., Mei, Z., Chen, H., He, T., and Fu, J (2020)."Analysis of genetic diversity and similarities between different *Lycium* varieties based on ISSR analysis and RAMP-PCR markers". *World Academy of Sciences Journal* 2.2: 83-90. https://doi.org/10.3892/wasj.2020.39

Luque, J., and Creamer, B. (2014). Major constraints and trends for common bean production and commercialization; establishing priorities for future research. *Agronomía Colombiana*, 32 (3):423. http://dx.doi.org/10.15446/agron.colomb.v32n3.46052

Mahuku, G. S., Henríquez, M. A., Munõz, J., Buruchara, R. A. (2002). Molecular Markers Dispute the Existence of the Afro-Andean Group of the Bean Angular Leaf Spot Pathogen, *Phaeoisariopsis griseola*. *Phytopathology*. 92(6):580-9. doi: 10.1094/PHYTO.2002.92.6.580. PMID: 18944253.

Mahuku, G. S., Jara, C., Cuasquer, J. B., and Castellanos, G. (2002). Genetic variability within *Phaeoisariopsis griseola* from Central America and its implication for resistance breeding of common bean. *Plant Pathology*. 51. 594 - 604. 10.1046/j.1365-3059.2002.00742. x.

Manandhar, H. K., Timila, R. D., Sharma, S., Joshi, S., Manandhar, S., Gurung, S. B., Sthapit, S., Palikhey, E., Pandey, A., Joshi, B. K., Manandhar, G., Gauchan, D., Jarvis, D. I., and Sthapit, B. R. (2016). A field guide for identification and scoring methods of diseases in the mountain crops of Nepal. Pokhara, Nepal, India: *Bioversiy International*, p. 20.

McKenzie, E. (2013) *Phaeoisariopsis griseola (Phaeoisariopsis griseola)* Updated on 4/16/2014 11:16:01 PM Available online: PaDIL - http://www.padil.gov.au.

Melzer, M. S and Boland G. J. (2001). First report of angular leaf spot caused by *Phaeoisariopsis griseola* on bean in Ontario, Canada. *Plant Dis*. 85:919. doi:10.1094/PDIS.2001.85.8.919D - DOI - PubMed

Moller E. M., Bahnweg, G., Sandermann, H., and Geiger, H. H. (1992). A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected tissues. *Nucleic Acids Research* 20, 6115-6.

Monda, E. O., Sanders, F. E., and Hicks, A. (2001). Infection and colonization of leaf by *Phaeoisariopsis griseola*. *Plant Pathol*. 50:103-110. https://doi.org/10.1046/j.1365-3059.2001.00537.x Crossref, ISI, Google Scholar

Mongi, R., Tongoona, P., Shimelis, H., and Sibiya, J. (2017). Agronomic Performance and Economics of Yield Loss Associated with Angular Leaf Spot Disease of Common Bean in the Southern Highlands of Tanzania. *Plant Disease*. 102. 10.1094/PDIS-04-17-0547-RE.

Muimui, K., Mulube, M., Mukuma, C., Lungu, R., and Chikuma, P. (2021). Bean variety descriptor 3rd edition. Zambia Agriculture Research Institute.

Mwangombe, A.W., Wagara I.N, Kimenju J.W, and Buruchara, R. A. (2007). Occurrence and severity of angular leaf spot of common bean in Kenya as influenced by geographical location, altitude and agroecological zones. *Plant Pathol. J*., 6: 235-241

Mwaniki A.W. (2002). Assessment of bean production constraints and seed quality and health of improved common bean seed. M.Sc Thesis, University of Nairobi Kenya. Abstract.

Myers J. R., and Kmiecik, K. (2017) Common Bean: Economic Importance and Relevance to Biological Science Research. In: Pérez de la Vega M., Santalla M., Marsolais F. (eds) The Common Bean Genome. Compendium of Plant Genomes. Springer, Cham. https://doi.org/10.1007/978-3-319-63526-2_1

Nay, M. M., Souza, T. L., Raatz, B., Mukankusi, C. M., Goncalves- Vidigal, M.C., Abreu, A. F., and Pastor-Corrales, M. A. (2019). A review of Angular leaf spot resistance in common bean. *Crop science,* 59, 1376 -1391.

Ng, W. L., and Tan, S. G. (1994). Inter- simple sequence repeat (ISSR) makers. *ASM Science Journal* 9:30-31

Ochichi, P.B., Muthomi, J., and Wagacha, J. (2018). Contribution of Seed Quality to the Occurrence of Fungal and Bacterial Diseases of Farmer Produced Common Bean in Varied Agro-Climatic Zones of Western Kenya. *American Journal of Science.* Vol.5, NO:2, 2018, pp.19-27.

Pamela, P., Mawejje, D., and Ugen, M. (2014). Severity of angular leaf spot and rust diseases on common beans in Central Uganda. *Uganda Journal of Agricultural Sciences*, *15*, 63-72.

Pastor-Corrales, M.A., Jara, C., and Singh, S.P. Pathogenic variation in, sources of, and breeding for resistance to *Phaeoisariopsis griseola* causing angular leaf spot in common bean. *Euphytica* 103, 161–171 (1998). https://doi.org/10.1023/A:1018350826591

Pereira, R., Souza, E. A., Barcelos, Q. L., Abreu, A. F. B., and Librelon, S. S. (2015). Aggressiveness of Pseudocercospora griseola strains in common bean genotypes and implications for genetic improvement. *Genet. Mol. Res.* 14, 5044–5053. doi: 10.4238/2015.May.12.7

Pyndji, M., and Trutmann, P. (1992). Managing Angular Leaf Spot on Common Bean in Africa by Supplementing Farmer Mixtures with Resistant Varieties. *Plant Disease*. 76. 1144-1147. 10.1094/PD-76-1144.

Rodríguez De Luque, J., and Bernardo,C. (2014). Major constraints and trends for common bean production and commercialization; establishing priorities for future research. *Agronomía Colombiana*. 32. 423-431. 10.15446/agron.colomb.v32n3.46052.

Salim, R., Aly, S., Abosereh, N.A., Hathout, A.S., and Sabry, B.A. (2019). Molecular Identification and Inter-Simple Sequence Repeat (ISSR) Differentiation of Toxigenic Aspergillus Strains.

Sanlard, D. A., Balbi, B. P., Barros, E. G., and Moreira, M. A. (2009b). An efficient protocol for isolation, sporulation and maintenance of *Pseudocercospora griseola. Annual Report of the Bean Improvement Cooperaive,52, 62-63.*

Sartorato, A. (2002) Identification of *Phaeoisariopsis griseola* pathotypes from five states in Brazil. *Fitopatol Bras* 27: 78– 81.

Sartorato, A. (2004). Pathogenic variability and genetic diversity of *Phaeoisariopsis griseola* isolates from two counties in the State of Goias, Brazil. *Phytopathologische Zeitschrift*, 152, 385-390. doi: 10.1111/j.1439-0434.2004.00858.x

Shiferaw Abebe (2017). Evaluation of Fungicides and Fungicidal Spray Schedule for the Management of Angular Leaf Spot of Common Bean. *Canadian Journal of Agriculture and Crops*. 2. 74-83. 10.20448/803.2.2.74.83.

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

Stenglein, S. A., and Balatti, P. A. (2006). Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed pathogenic and molecular markers. *Physiol. Mol. Plant Pathology*. 68:158-167. doi: 10.1016/j.pmpp.2006.10.001

Stenglein, S., Ploper, L., Vizgarra, O., and Balatti, P. (2003). Angular Leaf Spot: A Disease Caused by the Fungus *Phaeoisariopsis griseola* (Sacc.) Ferraris on Phaseolus vulgaris L.*Advances in applied microbiology*. 52. 209-43. 10.1016/S0065-2164(03)01009-8.

Swiderska-Burek, U., Daub, M. E., Thomas, E., Jaszek, M., Pawlik, A., and Janusz, G. (2020). Review of Phytopathogenic Cercosporoid Fungi-From Taxonomy to Modern Biochemistry and Molecular Biology. *International Journal of Molecular Sciences*, 21(22). https://doi.org/10.3390/ijms21228555

Verma, K. S., Ul Haq, S., Kachhwaha, S., and Kothari, S. L. (2017). RAPD and ISSR marker assessment of genetic diversity in *Citrullus colocynthis* (L) Schrad: a unique source of germplasm highly adapted to drought and high- temperature stress. 3 *Biotech*, 7:288.doi: 10.1007/s13205-017-0918-z.

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

Wheeler, B.E.J. (1969). An Introduction to Plant Diseases. *Wiley*, London: 347p.

Yugander, A., Kiran, S., Surekha, M., Babu, P., Laha, G. S., Madhav, M. S., and Reddy, S. (2015). Determination of genetic variation by using ISSR markers in toxigenic strains of Aspergillus in paddy from Telangana State, India. *International Journal of Agricultural Science and Research, 5*, 193-202.

Zambia Agriculture Research Institute report (2002). BEANS Production Guide, Soils and Crops Research Branch, Ministry of Agriculture and Cooperatives. (ZARI, 2002).

Zambia Agriculture Status Report (2019). Indaba Agricultural Policy Research Institute (IAPRI), Lusaka, Zambia

APPENDICES

Appendix 1: DNA concentration for the 91 *Phaeoisariopsis griseola* isolates

Sample Number	**Concentration ng/µl**	**Sample number**	**Concentration ng/µl**	**Sample number**	**Concentration ng/µl**
2	236.3	47	241.5	83	140.9
3	234.7	48	256.7	84	81
4	77	49	230.4	85	121.8
5	154.2	50	191.1	86	74.5
6	166.4	51	17.7	87	51.7
7	216.5	53	161.5	88	178.2
8	274	54	130.9	89	134
9	310.5	55	198.4	90	132.4
10	389.5	56	72.3	91	78.9
11	220.1	57	65.6	92	150.2
12	325.7	58	343.6	93	94.8
15	307.9	59	224.3	94	72.9
18	181.8	60	168.4	96	103.5
19	147.5	61	131.7	98	88.9
20	149.5	62	222.6	99	111.5
21	233.7	63	110.8	100	121.099
27	284.2	64	107	101	116.262
28	324.1	65	187.3	103	84.23

29	155.1	66	90.6	105	99.915
32	217.2	68	215.5	106	68.185
39	216.5	69	296.6	107	225.53
42	191.4	71	78.1	109	96.463
45	155.4	72	64.6	110	112.064
30	249.5	73	55.2	112	81.262
32	220.9	74	78	113	87.083
33	127.4	75	72.5	114	89.364
37	212.9	77	303.9	115	100.604
38	113.5	78	167.5	116	63.313
40	157.9	79	90.7	117	93.576
41	296.9	80	80.1	118	85.543
44	254.4	81	121.9	119	94.417
46	252.9	82	103.3	120	78.975

Appendix 2: Regression analysis for disease incidence, seed source and cropping system

```
REGRESSION ANALYSIS

Response variate:       DISEASE_INCIDENCE_%

Fitted terms: Constant, CROPPING_SYSTEM_CODES AND SOURCE_OF_SEED_CODE

Summary of analysis


Source              d.f.  s.s.  m.s.    v.r.     F pr.

Regression            2           2540.          1270.2          1.57    0.222

Residual             38          30816.           810.9

Total                40          33356.           833.9


Percentage variance accounted for 2.8

Standard error of observations is estimated to be 28.5.

 Estimates of parameters

 Parameterestimate           s.e.            t(38)        t pr.

Constant                              72.5               17.5          4.15    <.001

CROPPING_SYSTEM_CODES        -23.5               13.6         -1.72    0.093

SOURCE_OF_SEED_CODE           1.55               3.27          0.47    0.639
```

```
 CORRELATIONS BETWEEN PARAMETER ESTIMATES

Parameter          ref correlations

Constant             1 1.000

CROPPING_SYSTEM_CODES
                       2   -0.853    1.000

SOURCE_OF_SEED_CODE
                       3   -0.417   -0.044    1.000

                             1           2           3
```

Multiple regression model summary generated using IBM spss

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.276[a]	.076	.028	28.4769	.076	1.566	2	38	.222

a. Predictors: (Constant), SOURCE OF SEED CODE, CROPPING SYSTEM CODES

b. Dependent Variable: DISEASE INCIDENCE (%)

Appendix 3: T-test for disease incidence and cropping system

TWO-SAMPLE T-TEST

```
 Variate: DISEASE_INCIDENCE_%

 Group factor: CROPPING_SYSTEM

 Test for equality of sample variances

 Test statistic F = 4.53 on 35 and 4 d.f.

 Probability (under null hypothesis of equal variances) = 0.15
 Summary

                                               Standard    Standard error

Sample        Size      Mean     Variance     deviation           of mean

Intercrop        5     29.60        190.8         13.81             6.177

Monocrop        36     52.78        863.8         29.39             4.899

Difference of means:            -23.18

Standard error of difference:    13.46

95% confidence interval for difference in means: (-50.39, 4.038)

Test of null hypothesis that mean of DISEASE_INCIDENCE_% with CROPPING_SYSTEM =
Intercrop is equal to mean with CROPPING_SYSTEM = Monocrop

Test statistic t = -1.72 on 39 d.f.

Probability = 0.093
```

Appendix 4: Origin of the *P. griseola* isolates used in the study

SAMPLE NUMBER	FIELD NUMBER	DISTRICT	LATITUDE	LONGITUDE	ALTITUDE	FIELD SIZE m^2
2,3	1	Mansa	-15.54758	28.24913	1155	100
4,5,6	2	Mansa	-11.1844	28.88683	1219	150
7,8,9	3	Mansa	-11.11591	28.88321	1258	1500
10, 11,12	4	Mansa	-10.93684	28.80187	1268	300
15	5	Mansa	-10.8805	28.7956	1253	100
18	6	Mansa	-10.82001	28.79422	1257	200
19,20,21	7	Mwense	-10.38553	28.67556	938	150
27,28	10	Chienge	-8.94241	29.43398	947	200
29,30	11	Luwingu	-10.28329	29.93786	1308	150
32,33	12	Luwingu	-10.34409	29.95441	1332	1200
37	13	Luwingu	-10.38971	30.03228	1314	600
38,39,40,41	14	Luwingu	-10.43645	30.08748	1276	1200
42,44	15	Luwingu	-10.45074	30.12969	1296	2000
45,46,47	16	Luwingu	-10.26057	29.9248	1385	375
48,49,50	17	Luwingu	-10.21795	29.98575	1469	600
51,53	18	Luwingu	-10.19288	30.12444	1365	800

54,55,56	19	Kasama	-10.21093	31.1654	1349	600
57,58,59	20	Kasama	-10.07781	31.27212	1412	1000
60,61,62	21	Kasama	-9.93974	31.2495	1389	450
63,64,65	22	Kasama	-9.88954	31.24608	1407	100
66, 68	23	Kasama	-9.68984	31.17286	1462	450
69,71	24	Senga Hill	-9.50731	31.21194	1551	400
72,73,74	25	Senga Hill	-9.41787	31.2295	1607	300
75, 77	26	Senga Hill	-9.36868	31.2391	1586	450
78,79,80	27	Mbala	-9.25561	31.30237	1556	400
81,82,83	28	Mbala	-8.87084	31.45214	1696	300
84,85,86	29	Mbala	-8.89893	31.46643	1690	200
87,88,89	30	Mbala	-8.90229	31.51257	1654	600
90,91,92	31	Mbala	-8.92935	31.56155	1593	900
93,94	32	Mbala	-8.90354	31.58902	1657	1600
96,98	33	Mbala	-8.85246	31.38464	1672	600
99,100,101	34	Mbala	-8.90713	31.35521	1676	600
103	36	Mbala	-9.0568	31.38732	1684	2100
105	37	Mbala	-9.13199	31.37963	1576	300
107	38	Mbala	-9.20499	31.3499	1510	800

109,110	39	Mpika	-11.83266	31.38002	1299	100
112,113	40	Mpika	-11.5688	31.43384	1401	2000
114,115,116	41	Mpika	-11.50596	31.37251	1360	200