GENETIC ANALYSIS OF RESISTANCE TO *CERCOSPORA ARACHIDICOLA* HORI IN GROUNDNUT (*ARACHIS HYPOGAEA* L.) USING SIMPLE SEQUENCE REPEAT MARKERS

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF ZAMBIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (PLANT BREEDING AND SEED SYSTEMS)

BY

EVANS TEMBO

UNIVERSITY OF ZAMBIA

LUSAKA

2017

DECLARATION

I hereby declare that this dissertation entitled "Genetic Analysis of Resistance to *Cercospora arachidicola* Hori in Groundnut (*Arachis hypogaea* L.) Using Simple Sequence Repeat Markers" is the result of my own research work. No part of this dissertation has been submitted for a degree or any other qualification. The information derived from literature has been duly acknowledged in the text and explicit references are given.

Signature ……**.....**

Date ……...

APPROVAL

This dissertation of Evans Tembo meets the regulations governing the award of the degree of Master of Science in Plant Breeding and Seed Systems and is approved for its contribution to scientific knowledge and literary.

Examiner's name and signature	Date
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DEDICATION

I hereby dedicate this research work to my mother Muyeko Kondesani Mwale for inculcating the spirit of hardworking in my life.

ABSTRACT

Groundnut (*Arachis hypogaea* L.) is an important global oilseed crop and a major source of protein and vitamins in many rural areas of Africa. In Zambia, the production of groundnut is limited by several factors, among which Early Leaf Spot (ELS) caused by *Cercospora arachidicola* Hori, is a major destructive disease. Development of resistant varieties to ELS remains the most viable disease management strategy. The objectives of this study were to investigate the type of gene action conditioning resistance and to map quantitative trait loci (QTL) associated with resistance to *C. arachidicola* as the first step towards the deployment of marker-assisted breeding for groundnut in Zambia. The laboratory work of the study was conducted at ICRISAT laboratories in Nairobi, Kenya while the field work was conducted at Chitedze Research Station in Malawi. The study was conducted between 2013 and 2014. Parental genotypes (Robut 33-1, susceptible and ICGV-SM 95714, resistant) were screened using 394 Simple Sequence Repeat (SSR) markers. All polymorphic markers (82) were used to screen 113 F_8 recombinant inbred line (RIL) population alongside their parents. Phenotyping of the RIL population was carried out under field conditions supplemented by irrigation and utilizing diseased debris as primary inoculum. The nature of gene action was determined by using a Chi-square test, performed using the area under disease progress curves (AUDPCs) and the result suggested additive gene action. Inclusive composite interval mapping (ICIM) analysis identified two major and one minor QTL associated with resistance to ELS. Two QTLs were mapped on linkage group 2 with phenotypic variation explained (PVE) by the marker values of 37.91% (LOD 15.73) and 7.98% (LOD 3.5) and additive effects of 25.64 and -11.14 respectively. The third QTL was mapped on linkage group 9 with a PVE value of 12.31% (LOD 5.5) and additive effect of 12.92. The two major mapped QTLs were less than 5 cM from the nearest molecular marker. The study thus concluded that the gene action conditioning resistance to ELS was additive and the molecular markers associated with the two QTLs that were identified could possibly be used in marker-assisted breeding for groundnut. There is however, need to validate the detected QTLs in other locations and over seasons.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. L. Tembo, Dr. A. M. Mweetwa and Dr. D. A. Odeny for the support and guidance they provided during the study. I also wish to thank Dr. E. S. Monyo for the mapping population, Dr. S. Njoroge, and Dr. E. Kaimoyo for their valuable advice in conducting the study.

Many thanks also go to the technical staff from ICRISAT laboratories in Kenya and Malawi. These include Mr. V. Njunge, Ms. A. Sairiyoni and Mr. C. Harvey. I also wish acknowledge and greatly appreciate the financial support provided by USAID under the I-FINITE project for me to pursue the program of Master of Science in Plant Breeding and Seed Systems.

Finally, I would like to thank my family and friends for their support and encouragement during my studies.

TABLE OF CONTENTS

LIST OF TABLES

Title **Page**

LIST OF FIGURES

Title **Page**

LIST OF PLATES

Title **Page**

1.0 INTRODUCTION

1.1 Background

Groundnut (*Arachis hypogaea* L.) is a legume which is grown as an annual crop and is one of the most important food and oilseed crops in the tropical and subtropical areas of the world (Asiedu, 2010). Over 100 countries worldwide grow groundnut with developing countries constituting 97% of the global area and 94% of the global production (Ntare *et al*., 2007). The African continent alone accounts for 40% of the global area and 25% of the global production (Nigam *et al.*, 2004). It has an estimated global total production of 38.6 million tons per year over an area of 21.8 million hectares. Zambia produces about 176,000kg per year over an area of 240,000 hectares with 80% being grown in Eastern and Central Provinces of Zambia alone (FAOSTAT, 2015).

Groundnut is an important source of edible oil, dietary protein, carbohydrates and an inexpensive source of vitamins B, E and K. The nutrients found in groundnut supplement diets where maize, rice and cassava are the major energy foods (Monyo *et al*., 2012). The crop is an ideal cash crop that generates income for smallholder farmers in the tropical and subtropical regions. Groundnut haulms are excellent fodder and the cake is used for animal feed while plant roots left behind after harvest add valuable nutrients to the soil (Kedikhar, 2010). The crop has also been utilized as an important source of dyes, plastics and resins (Pandey *et al.*, 2012).

The average yield of groundnut is 500-800 kg/ha and these are considered to be below genetic potential (Ncube *et al.*, 2014). In many producing countries, groundnut production is hampered by both abiotic and biotic stresses. The biotic stresses are mostly attributed to pest and disease infestation during production and some during post-harvest. One of the major constraints in the

high yielding potential of groundnut in Africa has been the prevalence of a fungal disease known as early leaf spot caused by *Cercospora arachidicola* Hori. Early leaf spot disease (ELS) is among disastrous diseases affecting groundnut production and causes yield losses of between 32 to 68% (McDonald *et al*., 1985; Gopal, 2006). The disease also adversely affects pod and haulm yields and their quality. As a consequence of the low yields due to the disease, there is insufficient nutrition to the community and reduced profits to smallholder farmers who do most of the groundnut production in Zambia. Other countries in which early leaf spot has been reported include Niger, Sierra Leone, Nigeria, Democratic Republic of Congo, Malawi, South Africa, India and Pakistan (Sesay, 1992; Tshilenge-Lukanda *et al*., 2012). In Zambia, the disease has been reported in the main groundnut production areas which include eastern, central and southern provinces. In the eastern province, the disease has been estimated to cause up to 60% loss in kernel yield (Chalabesa, 2002).

Besides the yield losses caused by ELS disease, it also has an adverse effect on seed quality and grade characteristics. It deteriorates the quality of plant biomass and thus renders the fodder unsuitable as animal feed. When fungicidal control is not used with susceptible cultivars, yield losses may approach 70% (Nutter and Shokes, 1995). There has been increasing interest in the use of disease resistant genotypes recently. One of the reasons is that most farmers in the developing countries have limited resources and knowledge for safe application of pesticides whereas natural resistance is potentially cheap and efficient. Secondly, pesticides can cause considerable environmental damage. Over 95% of sprayed pesticides affect non-target species, air, water and soil. They are one of the causes of water pollution and contribute to soil

contamination (Miller, 2004). Hence, use of resistant cultivars is considered to be the best means of addressing these issues.

Methods of managing early leaf spot include cultural control, use of fungicides as well as resistant varieties. Development of resistant varieties has been reported to be the long term and economical method of managing the disease (Okello *et al*., 2013). Development of early leaf spot resistant varieties in Zambia would offer the cheapest means of disease control on the part of the farmers as they would not involve any recurring expenditure for example, on fungicides. Resistant varieties are also advantageous in that expression of the trait is not affected by environmental conditions. Farmer preferred varieties of groundnut in Zambia such as MGV4 and MGV5 are susceptible to early leaf spot. These varieties are preferred due to their relatively higher yielding potentials and relatively higher oil content (Ross and de Klerk, 2012).

Conventional breeding for resistance to early leaf spot has been challenging due to low efficiency of selection and long period required for gene introgression. The advent of molecular markers has provided an additional tool to resistance breeding in groundnut. Molecular markers are advantageous over morphological and protein markers because molecular markers are neutral and occur throughout the genome. Additionally, molecular markers are not influenced by the environment, are co-dominant, and are monitored in any tissue and at any stage of the plant and often follow expected Mendelian segregation (Kedikhar, 2010). Marker assisted selection (MAS) can improve the efficiency of selecting for desirable trait, especially in the case of low heritable and recessive traits, where phenotypic selection is difficult, expensive and lack accuracy or precision (Crouch, 2001). Increasingly, breeders have been identifying Quantitative Trait Loci

(QTL) to enhance efficiency and progress in breeding programmes. This approach overcomes some of the common limitations encountered by conventional selection for quantitative traits in many crops (Asins, 2002). For efficient MAS, one requires germplasm with useful traits, suitable mapping populations for the trait of interest, precise screening techniques and efficient marker system, which can detect higher levels of polymorphism. Microsatellites or simple sequence repeat (SSR) markers are considered as potential markers of choice because they are hyper-variable and co-dominant (Gupta and Varshney, 2000). SSR based markers have been found to be quite discriminatory in discerning variations between and among groundnut lines (Oteng-Frimpong *et al.* 2015) and in QTL mapping of leaf spot diseases in groundnut (Kedhikar, 2010).

The current research made use of SSR markers to obtain an understanding of the gene action (i.e. the action and interaction of genes) of early leaf spot resistance in groundnut grown in Africa. A good knowledge on the genetics of resistance will enable groundnut breeders to design an efficient breeding strategy in order to develop early leaf spot resistant groundnut varieties.

1.2 Statement of the Problem

Early leaf spot disease is among the economically important diseases of groundnut in Zambia and causes considerable loss in kernel yield. Gene introgression through interspecific hybridization in groundnut which is a self-pollinated species, has low efficiency, requires a long period and is tedious. There is therefore, a need to understand genetic resistance mechanisms at the molecular level and use specific molecular techniques to hasten and enhance disease resistance breeding programs for the crop.

1.3 Study Justification

The justification of the study was that an understating of the gene action and mapping of quantitative trait loci linked to resistance to early leaf spot will contribute to knowledge that will be useful to plant breeders in breeding for resistance to early leaf spot in groundnut.

1.4 Study Objectives

1.4.1 General Objective

The main objective of the investigation was to determine gene action for early leaf spot resistance in groundnut.

1.4.2 Specific Objectives

The specific objectives were:

1. To investigate the type of gene action conditioning resistance to *Cercospora arachidicola*
2. To map quantitative trait loci linked to *Cercospora arachidicola* resistance

1.5 Hypothesis tested in the study

This study was carried out on the hypothesis that resistance to early leaf spot, as in many other disease resistance traits in plants, is controlled by polygenes. Polygenes are responsible for quantitative traits and if it is established that ELS is controlled by polygenes, it would then be possible to conduct mapping of quantitative trait loci linked to early leaf spot resistance.

2.0 LITERATURE REVIEW

2.1 Introduction

Groundnut (*Arachis hypogaea* L.) is a legume which is cultivated in the tropical and subtropical areas and the warmer areas of temperate regions of the world (Asiedu, 2010). The cultivated tetraploid groundnut is a member of the genus *Arachis* and belongs to the family *Leguminosae*, subfamily *Fabaceae*, tribe *Aeschynomeneae*, subtribe *Stylosanthenae* (Krapovickas and Gregory, 1994). It has a chromosome length ranging from 1.4 to 3.9 µm with a chromosome number 2n=4x=40 and a genome size of 2,891 Mbp (Aquaah, 2007; Holbrook *et al.*, 2003). The domesticated groundnut is an amphidiploid or allotetraploid, with two sets of chromosomes from two different species. The wild ancestors have been confirmed as *A. duranensis* and *A. ipaensis* (Seijo *et al.*, 2007).

The cultivated peanut is divided into two subspecies, one with two and the other with four botanical varieties. In the subspecies *hypogaea var. hypogaea* (Virginia and Runner market types) and *var. hirsuta*, the varieties have long duration cycle and seeds undergo dormancy. The subspecies *fastigiata var. fastigiata* (Valencia market class) and *var. vulgaris* (Spanish market class), the varieties are early maturing with generally no fresh seed dormancy (Krapovickas and Gregory, 1994).

Groundnut will grow in almost any type of soil except heavy clay soils which are low in organic matter. The crop grows well in warm climates with temperatures of 24-30^{o}C with moderate rainfall (500 - 1,250mm) or overhead irrigation (Anochili, 1984). Sandy loams well supplied

with calcium and a moderate amount of organic matter are the best soils. Production of groundnut is limited to areas between 40^{o}N and 40^{o}S (Mothilal, 2012).

Groundnut is currently grown on over 22.2 million hectares worldwide with a total production of over 35 million tonnes. India and China are the world's largest producers of groundnut accounting for over 41% and over 18% of world production respectively (Integrated Breeding Platform, 2013). In Zambia, groundnut is the second most common field crop grown by smallholder farmers. The crop is produced by nearly half of the estimated 1.4 million rural smallholder households, making it the second largest after maize, in terms of production volume and hectares cultivated. Approximately 8.8% of the total land cultivated in Zambia is planted to groundnut (Mukuka and Shipekesa, 2013).

The varieties grown in Zambia are mainly used for confectionery and oil extraction purposes. These varieties are adapted to different agro-ecological conditions and have varying characteristics which include high yielding, disease resistance, early/late maturity, drought tolerance, high/low oil content availability and peanut butter making. The varieties grown include Chishango, Katete, MGV 5, MGV 4, Luena, Chipego, Champion, Chalimbana, Natal Common, Makulu Red and Comet (Ross and de Klerk, 2012).

The main constraints hampering higher yields of groundnut in Zambia and Africa at large include intermittent drought due to erratic rainfall patterns and terminal drought during maturation. It is also affected by heavy weed pressure and calcium deficiency with the latter causing unfilled shells known as pops (Integrated Breeding Platform, 2013). The crop is also

affected by several diseases such as leaf spots, collar rot, rust, bud and stem necrosis, rosette virus and others. Other production constraints include shelling which is labour intensive, lack of pesticides, insect pests, lack of certified seed, foraging pigs and aflatoxins (Mukuka and Shipekesa, 2013).

2.2 Early Leaf Spot disease

Early Leaf Spot (ELS) disease is caused by *Cercospora arachidicola* Hori a soil borne intracellular fungal pathogen that is dispersed directly from conidia that emerge from mycelia in conditions of high relative humidity and warm temperatures ranging from 25–30°C (McDonald *et al.,* 1985). The pathogen survives intercrop periods in crop residue. As soon as the crop emerges from the soil, spores disseminated by wind and rain splash are deposited on groundnut leaf surfaces. When the weather is favourable for infection, spores will germinate and penetrate host tissue primarily through stomata. Lesions and halos can be seen in 6-8 days. Epidemics are favoured by temperatures of 16 to 25 ^{0}C and long periods of high relative humidity over several days (Nutter and Shokes, 1995). The pathogen can reproduce and spread disease very rapidly. This causes severe epidemics which occur quickly and cause significant defoliation and yield loss. Early leaf spot is probably most destructive when it occurs on the leaves but all of the above ground plant parts are subject to infection. Lesions appear on petiole, stems, pegs, central stems and lateral branches.

During the initial stages of development, lesions appear as tiny, pinpoint, yellowish specks. The specks develop into irregularly shaped to circular spots whose colour varies from dark brown to almost black on the upper surfaces of leaflets and is generally tan to reddish tan on the lower

surfaces. The brown lesion colour is most distinct on the lower leaf surface. The fungus produces tufts of silvery, hair-like spores on lesions during humid weather (Shew, 2012). A yellow halo often surrounds the dark spot and is more pronounced on the upper surface. The yellowing is caused by a toxin produced by the fungus. The toxin kills cells in advance of the fungal growth, resulting in the yellow halo. Spores (conidia) are produced on stalks (conidiophores) mainly on the upper surfaces of the lesions but few may be found on the lower surfaces of older spots.

The pathogen causes plants to defoliate, starting with the lower leaves and progressing to the upper canopy. In severe epidemics, plants are completely defoliated and the bare stems may also have lesions. The defoliation that occurs reduces the total Leaf Area Index (LAI) and Leaf Weight Ratio (LWR), subsequently reducing yield (McDonald *et al.*, 1985). The pathogenicity of *Cercospora arachidicola* is linked to the production of photo activated cercosporin, a perylenequinone, which leads to the production of highly toxic reactive oxygen species (Herrero *et al.*, 2007). The photo-oxidation therefore, results in damage to nuclear membranes and cell organelles of the host plant enhancing symptoms and disease severity (Tsanko *et al*., 2006; Daub *et al*., 2005). It should be noted that the disease severity effect on host plants will vary depending on type and components of host plant resistance.

2.3 Components of resistance

Identification of genotypes with desirable level of disease resistance and knowledge of components of resistance are prerequisite for an effective implementation of a resistance breeding program. Cultivars exhibiting disease resistance have been found to have a number of components that constitute the resistance. These components have been identified as longer

latent periods, reduced sporulation, and less defoliation when compared to the susceptible ones (Neville, 1982). Other components reported are maximum percentage of lesions that sporulate, longer incubation period, smaller lesion diameter and lower infection frequencies (Waliyar *et al*., 1994).

2.4 Control measures

To minimize losses due to the disease, several methods of disease control have been developed and these include host plant resistance, cultural control, biological control, use of plant products and chemical control (Ghewande *et al*., 1993; Subrahmanyam *et al*., 1997; Pande *et al*., 2001). Since the leaf spot pathogens survive mainly in crop debris, cultural practices such as crop rotation, burying crop debris during land preparation and early sowing have been reported to reduce the incidence of the disease (Nyambok, 2011). In some countries such as India, the secondary spread of the disease in the field is controlled by spraying with a fungicide (Vashishta and Sinha, 2007). However, these control measures are considered to be unsustainable, uneconomical or short term. Development of leaf spot resistant varieties has been indicated to be the long term and economical method of managing the disease (Okello *et al.*, 2013). In Zambia, the methods of managing the disease has been through the use of host plant resistance and to a less extent, the use of fungicides (Sandhu *et. al*., 1985; Syamasonta, 1992). Screening for leaf spot resistance in Zambia begun in 1981 but up to now, the commonly cultivated and preferred groundnut varieties such as MGV4 and MGV5 are still susceptible to the disease (Sandhu *et. al*., 1985; Kannaiyan and Haciwa, 1990; Ross and de Klerk, 2012).

2.5 Resistance breeding efforts to *Cercospora arachidicola*

The variation in ploidy levels of groundnut varieties complicates the introgression of resistance traits by conventional breeding from wild diploid species to tetraploid cultivars by any method other than interspecific hybridisation (Pandey *et al.,* 2012). This limits the diversity of resistance traits in cultivated groundnut. In addition, groundnut being a self-pollinated species requires additional time, labour and skill in emasculation and crossing (Singh and Oswalt, 1992). Natural polyploidisation and self-pollination have resulted in a narrow genetic base of tetraploid varieties (Pandey *et al.,* 2012).

Inspite of these limitations, varieties that have resistance to foliar diseases in groundnuts have been identified, and though with difficulty, have been successfully utilized for resistant gene/trait introgression by conventional breeding methods (Garcia *et al.,* 2006). Additionally, conventional breeding methods have posed challenges such as the long time required for gene introgression through hybridization (Varshney *et al*., 2010). Given the predominantly inbreeding nature of groundnut, the most commonly used breeding methods are pedigree selection, bulk-pedigree selection, and single seed descent. Backcross breeding has not been extensively utilized because most of the economically important traits such as disease resistance and yield components in the crop have complex inheritance pattern. Mutation breeding, using both physical and chemical mutagens has also been extensively used in groundnut breeding to induce variability (Wynne and Gregory, 1981; Knauft and Wynne, 1995; Pasupuleti *et al*., 2013).

Some varieties with resistance to diseases have been developed using conventional breeding such as resistance to late leaf spot and rust (Gowda *et al.,* 2002) and peanut bud necrosis (Ghewande

et al., 2002). Due to the afore mentioned difficulties in conventional breeding, newly emerging biotechnological tools like marker-assisted selection can contribute to the success of disease resistance breeding in groundnut. Currently in groundnut, a molecular breeding approach known as Marker-Assisted Back-Crossing (MABC) is used for improvement of resistance to foliar fungal diseases such as late leaf spot and rust while Marker-Assisted Recurrent Selection (MARS) has been suggested to improve complex traits such as nutrient content and yield components (Pasupuleti *et al*., 2013). In Zambia, breeding for resistance to *Cercospora arachidicola* has mainly followed conventional methods. Since the commonly cultivated groundnut varieties are still susceptible to the disease (Ross and de Klerk, 2012), there is need to use marker assisted selection as an additional tool in breeding for resistance to early leaf spot.

2.5.1 Marker assisted selection

Marker assisted selection involves using the presence/absence of a marker as a substitute for or to assist in phenotypic selection, in a way which may make the phenotyping process more efficient, effective, reliable and cost-effective thereby supplementing conventional plant breeding (Collard *et al*., 2005). Marker assisted selection as an additional tool of breeding, has proven to expand useful genetic diversity for crop improvement, increase action of favourable genes and increase the efficiency of selection (Moose and Mumm, 2008). Molecular markers are useful in disease resistance breeding as they can supplement phenotypic screening in a breeding program. The molecular markers can also be used to identify resistant lines during early breeding stages which can save time and cost of screening (Park *et al.,* 2009; Kumar *et al.,* 2009). This helps in easy phenotype identification, monitoring of gene introgression and aids in eliminating undesirable traits in a much shorter time frame than that expected through conventional breeding

programs. Molecular markers are advantageous for traits where conventional phenotypic selection is difficult, expensive, or lacks accuracy. These traits include resistance to pests and diseases as well as tolerance to abiotic stresses, quality parameters and complex agronomic traits with low heritabilities (Crouch, 2001). Some of the traits that justify application of MAS in groundnuts are resistance to *Cercospora arachidicola*, rust, nematode resistance, leaf minor and *Spodoptera* (Khedikar, 2010). Examples of specific traits that have been associated with molecular markers in groundnut include rust resistance, late leaf spot resistance and aflatoxin contamination (Khedikar 2010; Sujay *et al*., 2012; Lei *et al*., 2006).

2.5.1.1 Molecular marker techniques

Molecular markers are specific regions on chromosomes that serve as reference points to the location of other genes of interest when a genetic map is constructed (Acquaah, 2007). The application of molecular markers has allowed the genetics of quantitative resistance to be determined and quantitative trait loci involved in resistance to be identified. Molecular markers have also contributed to improvement of breeding strategies for monogenic resistance genes which involves combining them through gene pyramiding in order to come up with a more durable resistance (Slusarenko *et al.,* 2000). Markers that have been used in groundnut studies are Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) in linkage mapping, Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeats (SSR) in detection of polymorphisms i.e. differences between genotypes. Other markers developed and used in groundnut studies include Diversity Arrays Technology (DArT) and Single Nucleotide Polymorphism (SNP) which are among the most recent of the genotyping based markers (Jiang, 2012). However, SSR markers are frequently

used for genetic and breeding studies in groundnut because they are multi-allelic, co-dominant and easy to use (Hong, 2008; Pandey *et al*., 2012). The process of detecting polymorphism involves the identification of DNA markers that reveal differences between parents. It is critical that sufficient polymorphism for a trait of interest exists between parents in order to construct a linkage map (He *et al.*, 2003).

Simple Sequence Repeats (SSRs) or Microsatellites is an extensively used marker system and detects highest polymorphism in groundnut (Jiang, 2012). SSR markers are often chosen as the preferred markers for a variety of applications in breeding because of their multi-allelic nature, co-dominant inheritance, relative abundance and extensive genome coverage (Gupta and Varshney, 2000). The SSR marker system includes DNA polymorphism using specific primers designed from the sequence data of a specific locus. Primers complementary to the flanking regions of the simple sequence repeat loci yield highly polymorphic amplification products (Weber *et al.*, 1989). Polymorphisms appear because of variation in the number of tandem repeats in a given repeat motif. This method is technically simple, robust, reliable and transferable between populations. Higher levels of DNA polymorphism by SSR markers have been reported in cultivated groundnut as compared to other DNA markers (He *et al.*, 2003; Mace *et al.*, 2006). Upadhyaya *et al*., (2007), studied genetic diversity in composite collection containing 916 accessions with 21 SSR markers and revealed considerable variation among the accessions (0.819 PIC value; 490 alleles) Nimmakayala *et al.*, (2007) used 96 SSR primers to screen 30 species representing A, B and D genomes of Arachis with various ploidy levels (18 diploid, 9 tetraploid and one aneuploid) along with two cultivated groundnut varieties. Of these, 50 (52.08 %) were found to be polymorphic. Gimenes *et al*., (2007) isolated thirteen

microsatellite loci and characterized 16 accessions of *A. hypogaea*. The level of variation found in *A. hypogaea* using microsatellites was higher than with other markers.

2.5.1.2 Linkage and QTL mapping efforts in groundnut

Using molecular markers to genotype materials and thereafter, selecting by associating to Quantitative Trait Loci (QTL) maps, has the potential to hasten the intensity of selection for early leaf spot resistance. This therefore, advances the populations within a reduced timeframe (Varshney *et al*., 2010). Construction of a linkage or genetic map is an essential step for breeders in order to use molecular breeding strategies for improving biotic and abiotic stress resistance (Azhaguvel *et al.,* 2006). This facilitates identification of potential genomic regions and transfer into important varieties. Simple Sequence Repeat marker-based genetic linkage maps have been developed in diploid wild species (Moretzsohn *et al*., 2005; Gobbi *et al*., 2006) and in the cultivated tetraploid (AABB) (Khedikar *et al.,* 2010; Ravi *et al*., 2011; Sarvamangala *et al*., 2011; Sujay *et al*., 2012). A reference consensus genetic map for the cultivated groundnut was developed by Gautami *et al.,* in 2012. This map is comprised of 897 marker loci including 895 SSR markers and 2 cleaved amplified polymorphic sequence (CAPS) loci distributed on 20 linkage groups spanning a map distance of 3, 863.6 cM with an average map density of 4.4 cM. Quantitative trait loci identified for some economically important disease traits in groundnut include resistance to late leaf spot and rust (Sujay *et al*., 2011; Khedikar *et al.,* 2010) and aphid vector of rosette disease (Herselman *et al.,* 2004). Pandey *et al*., (2012) reported that mapping populations segregating for early leaf spot have been developed at some universities and research stations in the U.S.A and Malawi. However, there is need to develop specific populations for

Zambia so as to ensure that the populations have resistance or are segregating for the prevailing pathotypes of the disease.

2.5.1.3 Quantitative Trait Loci mapping

A quantitative trait locus (QTL) is a region within a genome that contains genes associated with a particular quantitative trait (Collard *et al.,* 2005). QTL mapping is based on the basic principle that if there is linkage disequilibrium between the causal factor and a marker locus, mean values of the trait under study will differ among genotype groups with different genotypes at the marker locus (Zou and Zeng, 2008). In other words, QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers (Collard *et al*., 2005). The key requirements for mapping QTLs are trait phenotype, polymorphic markers and genetic structure of populations (Acquaah, 2007). According to Semagn *et al.,* (2010) and Acquaah (2007) QTL mapping involves the following summarized steps:

(i) Constructing a mapping population from two parents;

(ii) Identifying candidate markers and screening them for polymorphism;

(iii) Constructing a linkage map;

(iv) Analyzing for QTL-trait association using QTL detection methods.

(i) Mapping population

The choice of the mapping population is critical in QTL mapping. The breeder generates a segregating population by crossing lines with extreme phenotypic performance for the

quantitative trait of interest (Semagn *et al.*, 2010). The most frequently used populations are derived from crossing two inbred lines that are assumed to be homozygous with different alleles at both QTLs and genetic markers. These materials include F_2, backcrosses, recombinant inbred lines, and doubled haploids (Acquaah, 2007). The mapping population is genotyped for segregating markers targeted to specific chromosome regions and/or markers evenly distributed over a genome-wide genetic map. The segregating genotypes are also characterized phenotypically for quantitative and/or qualitative traits (Alonso-Blanco *et al.,* 2005). Such populations have pronounced strong linkage disequilibrium between loci, allowing the detection of linkage between markers and the trait of interest.

F_2 populations, derived from F_1 and backcross populations (derived from F_1 or F_2 plants crossed to one or both parents) are the simplest types of mapping populations developed for self-pollinating species such as groundnut (Collard *et al.*, 2005). Their main advantages are that they are easy to construct and require only a short time to produce. Inbreeding from individual F_2 plants forms recombinant inbred lines, which consist of a series of nearly homozygous lines each containing a unique combination of chromosomal segments from the original parents (Butruille *et al.*, 1999). Evaluation of highly homozygous families, such as recombinant inbred lines, offers several advantages when compared to the evaluation of F_2 plants or F_3 families. The advantages include: (1) Very limited heterozygosity, which in turn allows for the more effective use of dominant markers; (2) Greater genetic variability among families due to stronger expression of additive effects; (3) Higher mapping resolution due to the higher number of crossover events and (4) the opportunity to more consistently reproduce the phenotypic evaluations across space and time (Tuberosa *et al.,* 2003). The length of time needed for producing recombinant inbred populations is the major disadvantage, because usually six to eight generations are required

(Collard, 2005). Recombinant inbred lines have been used in groundnut to map QTL's associated with resistance to diseases (Khedikar *et al.*, 2010) and agronomic traits (Varshney *et al*., 2009; Ravi *et al*., 2011).

(ii) Identification of polymorphism

Polymorphism refers to the differences between parents. An important step in QTL mapping is to identify DNA markers that are polymorphic (Singh, 2009). It is critical that sufficient polymorphism exist between parents in order to construct a linkage map (Young, 1994). In most cases parents that provide adequate polymorphism are selected on the basis of their level of genetic diversity. Once polymorphic markers have been identified, they must be screened across the entire mapping population, including the parents (Collard *et al*., 2005).

(iii) Linkage analysis

A linkage map indicates the position and relative genetic distances between markers along chromosomes, which is analogous to signs or landmarks along a highway (Paterson, 1996). After identifying polymorphic markers, a linkage map is constructed by recording genotype data for each DNA marker on each individual of a mapping population and then using computer programmes to analyze for linkage between markers and phenotypic traits. The likelihood that particular markers are linked is usually expressed using the odds ratio, i.e., the ratio of the probability of linkage versus the probability of no linkage expressed as the logarithm of the ratio called a Logarithm of Odds (LOD) (Collard *et al*., 2005). Linked markers are grouped together into linkage groups which represent chromosomal segments or entire chromosomes. In a study to

map QTLs for resistance to Late Leaf Spot, Varshney *et al*., (2010) screened 1,089 SSR markers and developed a partial groundnut linkage map with 56 SSR loci from which 11 QTLs for the disease were identified. A reference consensus linkage map for the cultivated groundnut was developed by Gautami *et al*., (2012).

(iv) Methods of detecting Quantitative Trait Loci

There are three widely used methods for detecting QTLs namely single marker analysis, simple interval mapping and composite interval mapping (Tanksley, 1993; Acquaah, 2007; Wang *et al*., 2012).

Single marker analysis is used for detecting QTLs associated with single markers and does not require a linkage map. It is based on the principle of detecting an association between phenotypic expression and the genotype of the DNA markers. DNA markers are used to partition the mapping population into different genotypic groups in order to determine whether significant differences exist between groups with respect to the trait being measured (Collard, *et al.*, 2005). A significant difference between phenotypic means of the groups indicates that the marker locus is linked to a QTL controlling the trait. Three essentially equivalent statistical methods are used for single marker-analysis: T-test, analysis of variance and linear regression (Collard, *et al.*, 2005). The contribution to the total phenotypic variance of the genetic effect attributed to a single locus (indicated as R^2) is estimated through standard regression approach (Tuberosa *et al.*, 2003). The limitation of single marker analysis is that it produces ambiguities regarding both location of QTLs and the estimates of their effects (Acquaah, 2007).

Interval mapping is the method of using flanking markers and is based on the hypothesis that a QTL lies between linked marker loci (Acquaah, 2007). It uses an estimated genetic map as the framework to discover the location of the QTL (Collard, *et al.,* 2005). The intervals, defined by ordered pairs of markers, are searched in increments and statistical methods are used to test whether a QTL is likely to be present within that interval.

Composite interval mapping (CIM) combines interval mapping with linear regression by evaluating a statistical model that includes both the adjacent pair of linked markers being evaluated for interval mapping and one or more additional genetic markers at other chromosomal positions (Jansen and Stam, 1994). The main advantage of composite interval mapping is that it is more precise and effective at mapping QTL than is single-marker analysis or interval mapping, especially when linked QTL are involved (Collard *et al.,* 2005). More recently, the use of Inclusive Composite Interval Mapping (ICIM) was proposed and has been found to have increased detection power, reduced false detection rate and less biased estimates of QTL effects compared to CIM in additive and dominance mapping (Wang *et al*., 2012). A list of the major QTLs detected in groundnut using SSR markers for several traits such as drought resistance, rust resistance, late leaf spot resistance, bacterial wilt resistance etc., has been reported by Janila *et al*. (2016).

2.5.2 Nature of gene action

Identification of resistance sources, knowledge of components, mechanism of resistance and the number of loci contributing to resistance are the prerequisites for the success of a disease resistance breeding program (Dwivedi *et al.,* 2002). Studies have shown that there are two different mechanisms for disease resistance: monogenic and polygenic or quantitative resistance.

Monogenic resistance is based on single genes whereas quantitative resistance depends on two or more genes. Monogenic resistance is relatively easy to study because there are a few numbers of genes involved and each gene effect is large. On the other hand, quantitative resistance is controlled by many genes and most of them with a small effect and are significantly influenced by the environment (Mackay *et al*., 2009). The genetic characterization of disease resistance in plants has been essential for the understanding of plant-pathogen interactions (Slusarenko *et al.,* 2000). Gene action of a trait refers to the action and interaction of genes in expressing a trait. There are four types of gene actions: additive, dominance, epistatic and overdominance (Singh, 2009). Additive gene action is the sum effects of each allele. Dominance gene action describes the relationship of alleles at the same locus. Dominance effects are deviations from additivity that make the heterozygote resemble one parent more than the other. Overdominance exists when each allele at a locus produces a separate effect on the phenotype and their combined effect exceeds the independent effect of the alleles. Epistatic effects are non-allelic gene interactions (Acquaah, 2007).

The gene actions are also broadly classified as additive and non-additive gene actions (Conner and Hartl, 2004). The additive gene action is the most important for sexually reproducing species, because only the additive effects of genes are passed on directly from parents to offspring. The dominance and epistatic effects are not passed on (with the exception of tight linkage), because only one allele at each locus is transferred from each parent to create new dominance relationships in the offspring, and similarly independent assortment of alleles at different loci creates new epistatic effects (Conner and Hartl, 2004). In order to develop a variety, it is important to understand the gene action of the trait under consideration.

A study conducted on genetic analysis for resistance to *Cercosporidium personatum* a pathogen which causes late leaf spot in interspecific groundnuts suggested that resistance to late leaf spot is controlled by a combination of both nuclear and maternal gene effects. Among nuclear gene effects, additive effect controlled majority of the variation (Pasupuleti *et al*., 2013). Earlier studies (Anderson, 1991) indicated that additive gene action was significant for both early and late leaf spot disease resistance. Green and Wynne, (1986) suggested that non-additive gene effects are also important for early leaf spot disease resistance.

3.0 MATERIALS AND METHODS

The experimental material consisted of a mapping population obtained from ICRISAT- Malawi and consisted of 113 Recombinant Inbred Lines (RILs) segregating for early leaf spot (ELS) resistance. This population was derived from a biparental cross between Robut 33-1 × ICGV 95714. Filial generation one (F1) plants were selfed to produce F2s seed and advanced through single plant selection until F8s generation. This was done between 2005 and 2014 at Chitedze Research Station in Malawi. The research station is located on longitude 13^0 85' S and latitude 33^0 38' E and lies at an altitude of 1146m above sea level. Chitedze has a mean annual temperature of 20^0C and receives a mean annual rainfall of 892mm with 85% falling between November and March.

3.1 Salient features of parental material

Robut 33-1 is an early maturing Virginia type from India (Table 1). It is susceptible to ELS disease and was used as the female line in the cross. ICGV-SM 95714 is an early maturing Valencia type which is resistant to ELS disease and was used as the male line in the cross.

3.2 Phenotyping of population for resistance to Early Leaf Spot

Phenotyping of RILs was carried out at Chitedze Research Station in Lilongwe, Malawi. This is a known hotspot for groundnut and other crop foliar diseases. The study was conducted during the 2013/14 rain season with supplementary irrigation.

3.2.1 Production of Early Leaf Spot inoculum

The inoculum was produced in a groundnut nursery using a highly susceptible groundnut variety, JL 24 in the 2012/13 cropping season. The inoculum consisted of infected plant debris. The infected plant materials were harvested at 4 months after planting and kept in a screen house with

controlled environment set at high humidity and temperature to maintain viability of the spores. Based on a visual disease scoring scale of 1-9 with 1 being the score for no symptom and 9 being the score for very severe disease infestation (Chiteka *et al*., 1988), the plants had an average ELS disease score of 8 by the time of harvest.

Table 1: Summary of traits of the parents used to create the mapping population

	Trait/Aspect	**Robut 33-1**	**ICGV-SM 95714**
1	Origin	India	Breeding line developed in Malawi
2	Type	Virginia	Valencia
3	Morphological description	Profuse branching, alternate flowering pattern, medium sized pods	3 seeded
4	Yield	1200-1500 kg/ha	1700-2000kg/ha
5	Seed colour	Tan	Tan
6	Maturity	Early (115 days)	Early (90-100 days)
7	Disease resistance	Bud necrosis	Early leaf spot
8	Disease susceptibility	Early leaf spot	-
9	Oil content	Not done	Not done
10	Seed dormancy	Long	No seed dormancy
11	Sex in Cross	Female	Male
12	Countries in which released as a variety	India as Kadiri 3.	Not yet released but used as an ELS resistant source

3.2.2 Management of the experimental plot

The 113 RILs were sown randomly in the experimental plot. Ten seeds of each RIL were planted in 1 m rows with 75 cm and 10 cm inter and intra-row spacing, respectively. The two parents were also sown as controls. All the necessary agronomic practices were followed to raise a healthy crop. Inoculation was done uniformly 25 days after planting. Inoculation involved

spreading the infected debris in the rows. A humid environment as required by the pathogen for disease infection was provided by irrigating the field using sprinklers.

3.2.3 Data collection

The phenotyping of the population was carried out at Chitedze Research Station in Lilongwe, Malawi and commenced 60 days after planting. It was conducted by visual disease scoring using a 1-9 scale (Chiteka *et al*., 1988) where 1 means highly resistant and 9 means highly susceptible (Table 2).

Table 2: Early Leaf Spot description and scoring scale (Chiteka *et al*.,1988)

Leaf spot Score[1]	Description	Disease severity (%)
1	No disease	0
2	Lesions largely on lower leaves; no defoliation	1-5
3	Lesions largely on lower leaves; very few lesions on middle leaves; defoliation of some leaflets evident on lower leaves	6-10
4	Lesions on lower and middle leaves, but severe on lower leaves; defoliation of some leaflets evident on lower leaves	11-20
5	Lesions on all lower and middle leaves; over 50% defoliation of lower leaves	21-30
6	Lesions severe on lower and middle leaves; lesions on top leaves but less severe; extensive defoliation of lower leaves; defoliation of some leaflets evident on middle leaves	31-40
7	Lesions on all leaves but less severe on top leaves; defoliation of all lower and some middle leaves	41-60
8	Defoliation of all lower and middle leaves; lesions severe on top leaves and some defoliation of top leaves evident	61-80
9	Defoliation of almost all leaves leaving bare stems; some leaflets may be present, but with severe leaf spots.	81-100

[1] ≤3 = resistance, 3 - 4 = tolerance, 5 - 9 = susceptible

A rating of three or lower was regarded as an indication of resistance, a rating of between three and four as an indication of tolerance and a rating of five to nine as susceptible (Pretorius, 2006).

Scoring was done at 60, 75, 90 and 100 days after planting. For each RIL, initial plant stand was noted and four plants were selected at random and tagged from which data was collected during the four scoring intervals.

3.3 DNA extraction and genotyping of RIL population

DNA extraction and genotyping of RIL population was carried out at ICRISAT laboratories in Nairobi, Kenya. The seeds of the RIL population were planted in a screen house and leaf samples for DNA extraction were collected at 14 days after planting.

3.3.1 DNA extraction

For the Simple Sequence Repeat (SSR) marker screening, DNA was extracted using the Zymo DNA extraction kit (www.zymoresearch.com) while for genotyping the 113 RILs, DNA was extracted using Isolate II Plant DNA extraction kit (www.bioline.com). The principle in the extraction process involves cell lysis in a buffer, removal of other cell constituents followed by DNA precipitation and washing and finally DNA elution. The DNA quality was checked using 0.8% (w/v) agarose gel under Ultra Violet (UV) light. The principle of the process uses the flourescence of a chemical (gel red) when subjected to UV light. The DNA quantification was done using an apparatus called Nanodrop which quantifies DNA using spectophotometry. Due to the large set of SSR markers and RILs, a number of equipment available at ICRISAT – Nairobi laboratories were made use of in order to hasten the extraction of DNA. The equipment included agitators (for cell lysis), centrifuge machines (for DNA precipitation) and multichannel electronic pipettes. The agitators and centrifuge machines had capacities of handling up to 96 test tubes during one operation. The multichannel pipettes had capacities of transferring liquids from ordinary test tubes and eppendorf tubes which were as many as 16 during one operation.

3.3.2 Screening developed SSR markers for polymorphism

Three hundred and ninety four (394) SSR markers available at the ICRISAT-Nairobi laboratories were used for initial detection of polymorphism between the two parents (Robut 33-1 and ICGV-SM 95714). The overall methodology for screening for polymorphism was adapted from procedures developed by the ICRISAT – Nairobi laboratories (De Villiers, 2015, Ncube *et al.*, 2014). A universal fluorescent labeling strategy was used to label the 394 SSR markers as described by Schuelke (2000). PCR reactions were performed in 10µl volumes containing 1x PCR buffer, 2mM $MgCl_2$, 0.16mM dNTPs, 0.16pM of Fluorescent label (Vic, Fam, Pet and Ned), 0.04pM of the forward primer (with the M13 sequence extension), 0.2pM of the reverse primer, 0.2U of Taq polymerase and 15ng of DNA. The thermocycler was programmed as follows: initial denaturation at 94^0C for 5 minutes, 30 cycles of denaturation at 94^0C, annealing at 59^0C and extension at 72^0C for 30 seconds, 1 minute and 2 minutes respectively. The final extension was set at 72^0C for 12 minutes after which the machine would hold infinitely at 15^0C. In order to hasten the amplifications, the PCR reactions were performed on thermocyclers that were handling up to 4 plates of 384 well PCR plates.

Amplification products were separated on 2.0% (w/v) agarose gel alongside a 100bp ladder and visualized under UV light. All SSR products showing distinct single bands per sample were submitted for capillary electrophoresis (ABI 3500 Genetic Analyzer) by co-loading 1.5 – 3.5µl of each sample multiplexed for 4 SSR markers (each having a different fluorescent label) together with the internal size standard, GeneScan™ –500 LIZ® (Applied Biosystems). Allele scoring was done using Gene Mapper Software (Version 4.0, Applied Biosystems) and all polymorphic markers further selected for genotyping the RIL population.

3.3.3 Genotyping of mapping population

All the 113 RILs were genotyped alongside the parental lines using all polymorphic markers identified from the marker screening of parental lines (Section 3.3.1). The appendix lists all the polymorphic markers used in the study. The result of the DNA analysis was scored for each RIL family and this constituted the genotypic data. Amplification of DNA used the master mix and thermocycler programmes as outlined in Section 3.3.2 with few adjustments. The adjustments were for the thermocycler whose timings for extension and final extension was set for 1 minute and 7 minutes respectively.

3.3.4 Data analysis

Analysis of phenotypic data involved the calculation of the area under disease progress curves (AUDPCs) and the generation of the distribution curve. The AUDPC was calculated using the trapezoidal method below:

$$AUDPC = \sum_{i=1}^{n-1}\left(\frac{X_i + X_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where X_i is the disease incidence, n is the number of evaluations and $(t_{i+1} - t_i)$ is the time interval between two consecutive evaluations (Campbell and Madden, 1990). To investigate the type of gene action, a Chi-square test was performed using the AUDPCs by determining whether the phenotypic data conforms to the expected ratios. In order to generate a distribution curve, the AUDPCs were put into categories. Since the RIL population was developed from two parents contrasting for resistance to the disease, the two extreme ends of the distribution were categorized as resistant and susceptible while the categories in-between were either medium susceptible or medium resistant.

The linkage map was constructed using JoinMap version 4 software (Stam, 1993) and made use of the genotypic data. For QTL analysis, QTL IciMapping version 3.2 software (Wang *et al.,* 2012) was used and this made use of phenotypic as well as genotypic data. QTL analysis was performed using Inclusive Composite Interval Mapping (ICIM) and was set at the likelihood of odds (LOD) score threshold of 2.5.

4.0 RESULTS

4.1 Phenotyping

Average Early Leaf Spot disease scores of the RILs ranged from 2 to 8 suggesting the presence of both susceptible and resistant phenotypes. Other diseases observed in the experimental plot were leaf blotch and late leaf spot. These other diseases were not scored but care was taken so as not to affect the scoring for ELS. Plate 1 shows the symptoms of Early Leaf Spot on the susceptible RILs at 60 days after planting. The AUDPCs which were calculated from mean scores ranged from 72.5 to 225 score-days with a mean of 147.75 and a population standard deviation of 36.69. Based on the AUDPCs, the RIL families were categorized as resistant or susceptible to facilitate performance of the Chi-square Test (Table 3). The results showed that the segregation of the F_{8S} RILs to ELS followed the Mendelian ratio of 1:1. Frequency distribution of the AUDPCs indicated a near-normal distribution pattern (Figure 2).

Plate 1: Symptoms of Early Leaf Spot disease on susceptible lines (60 days after planting)

Plate 2: Susceptible line (top) and resistant line (bottom)

Table 3: Observed categories of areas under Disease Progress Curves (AUDPCs)

AUDPC Range	Frequency	Phenotype Category	Chi-square Category
70_90	10	Resistant	57 (Resistant)
91_110	12		
111_130	12	Medium Resistant	
131_150	23		
151_170	24	Medium Susceptible	53 (Susceptible)
171_190	17		
191_210	6	Susceptible	
211_230	6		

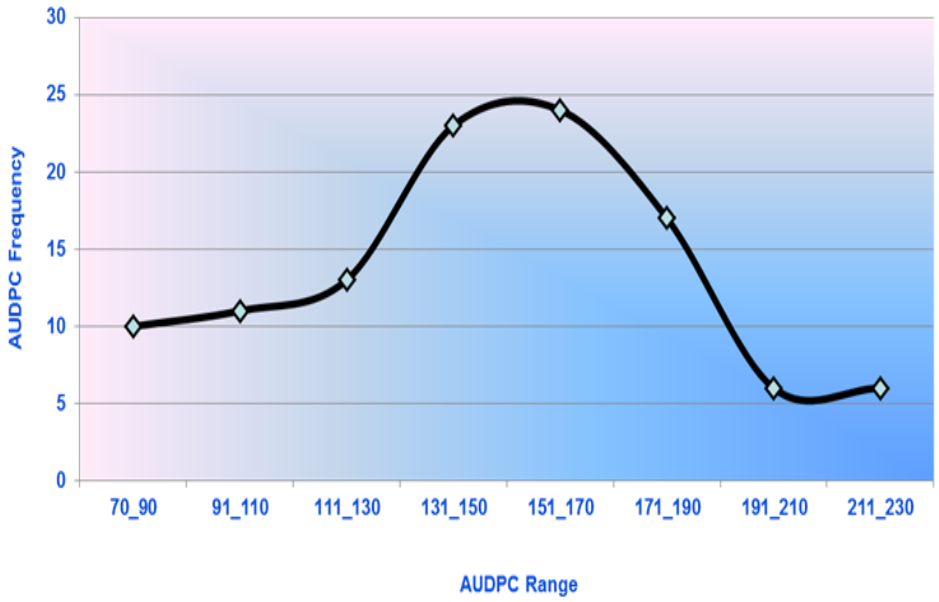

Figure 1: Frequency distribution of areas under disease progress curves (AUDPCs)

4.2 Genetic linkage map construction

Eighty two (82) SSR markers, which were polymorphic between the parents (Robut 33-1 and ICGV 95714) were used to generate a linkage map. Plate 3 shows a sample of the results of the screening of SSR markers for polymorphism between the two parents. The plate shows an agarose gel partitioned into three sections on which bands of PCR products of the two parents were visualized. The agarose gel was portioned in order to make maximum use of the material because of the many gels which were done. The PCR products were loaded as pairs per marker on the gel in order to facilitate observation of any possible polymorphism. A 100bp ladder is shown on the sides to give an indication of the size of the PCR products.

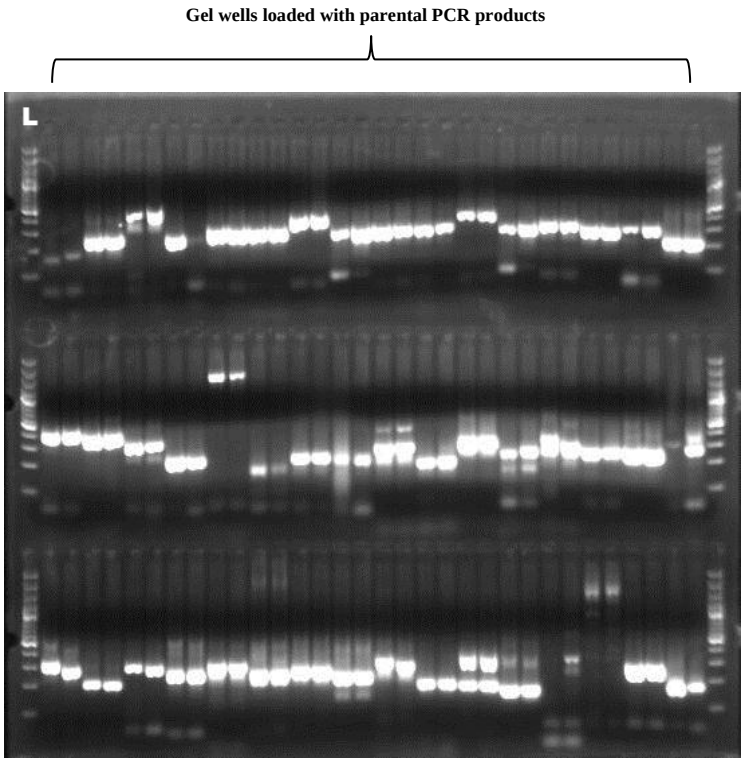

Plate 3: DNA profiles of two parental accessions (Robut 33-1 and ICGV 95714) with SSR markers. L: 100bp ladder

The linkage map generated had thirteen (13) linkage groups or chromosomes as shown in Figures 2a and 2b. The figures show the genetic linkage map of the cultivated groundnut using SSR markers. The SSR marker names are indicated together with their relative position on each chromosome with the distance measured in cM. The linkage groups spanned a total distance of 1,034.58cM with an average distance of 12.62cM between two markers. The biggest linkage group was 198.17cM with 15 markers located on chromosome 3 and the smallest was 15.6cM with 3 markers and located on chromosome 8 on the groundnut consensus map.

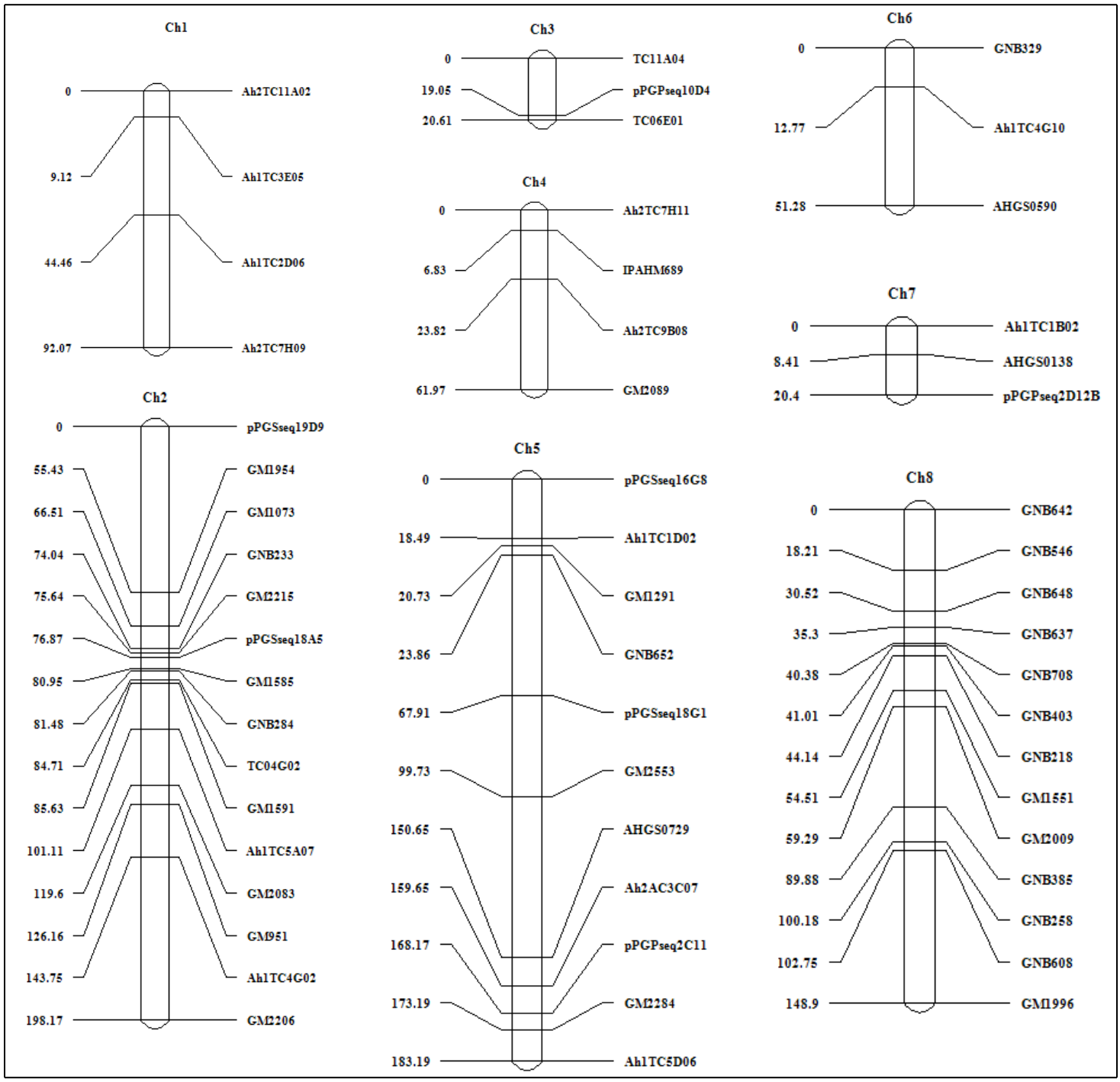

Figure 2a: Genetic linkage map of cultivated groundnut (Linkage groups 1 to 8)[1]

[1]Left side of chromosome (Ch) – Distance in cM
Right side of chromosome (Ch) – Marker name

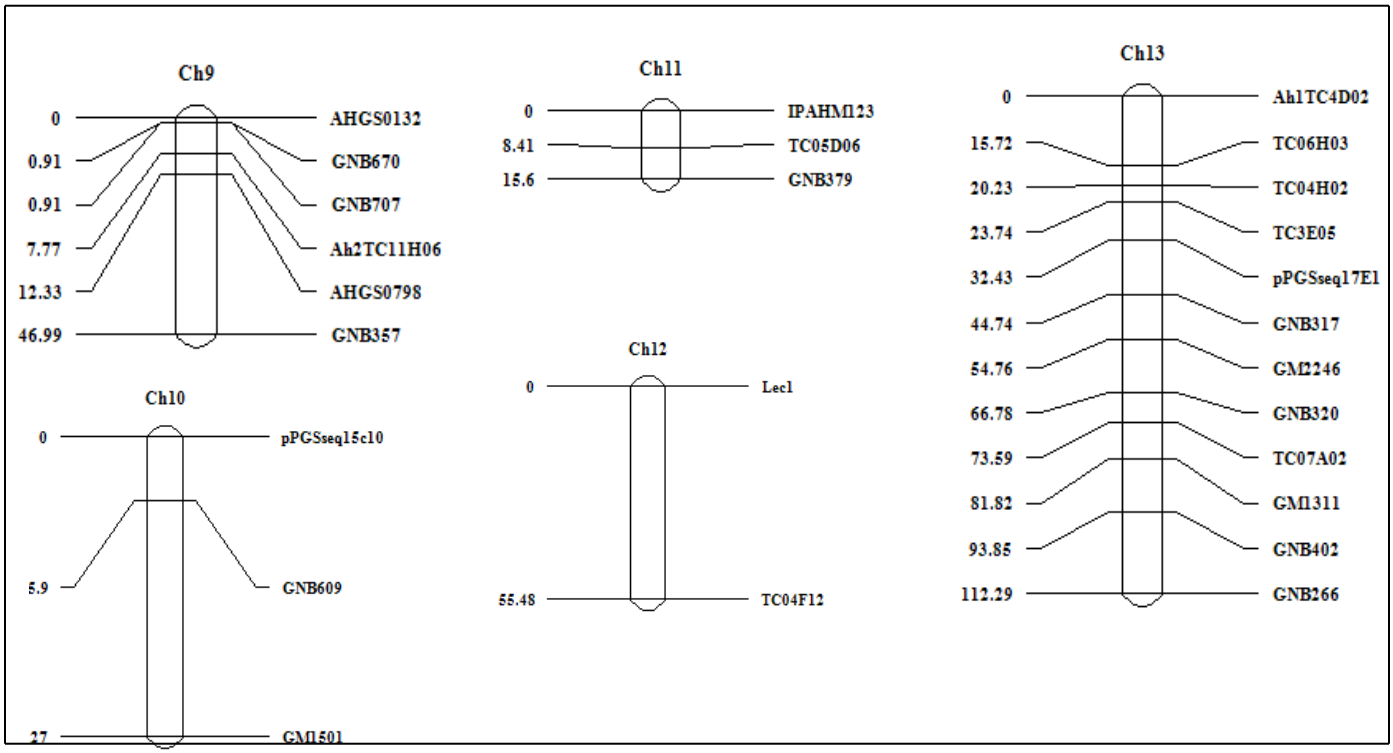

Figure 2b: Genetic linkage map of cultivated groundnut (Linkage groups 9 to 13)[1]

[1]Left side of chromosome (Ch) – Distance in cM
Right side of chromosome (Ch) – Marker name

4.3 Quantitative Trait Loci analysis

QTL detection using Inclusive Composite Interval Mapping revealed three QTLs located on two linkage groups. The first QTL was mapped on linkage group 2 with phenotypic variation explained (PVE) by the marker of 37.91% and a likelihood of odds (LOD) score of 15.73. The other QTL on the linkage group is a minor QTL with a PVE value of 7.98% and LOD score of 3.5. The two QTLs had additive effects of 25.64 and -11.14 respectively. The third QTL was mapped on linkage group 9 with a PVE value of 12.31%, LOD score of 5.5 and additive effect of 12.92. The first QTL was flanked by two markers namely GM1585 to the left and GNB284 to the right. The second QTL was flanked by markers GM951and Ah1TC4G02 whilst the third had AHGS0798 and GNB357. The linkage groups with detected QTLs are shown in Figures 3a and

3b while the summarized information on QTL analysis is presented in Table 4**.** Figures 3a and 3b shows linkage groups 2 and 3 respectively with the detected QTLs. On the sides of the linkage groups are the LOD scores which indicate the positions of the QTLs and their flanking markers. Table 4 provides summarized details on the detected QTLs which include the category (whether major or minor), the position on the linkage group on which the QTL is located, the flanking markers, LOD score, PVE and additive effect. A QTL is considered to be major if it has a PVE of more than 10% (Collard *et al*., 2005).

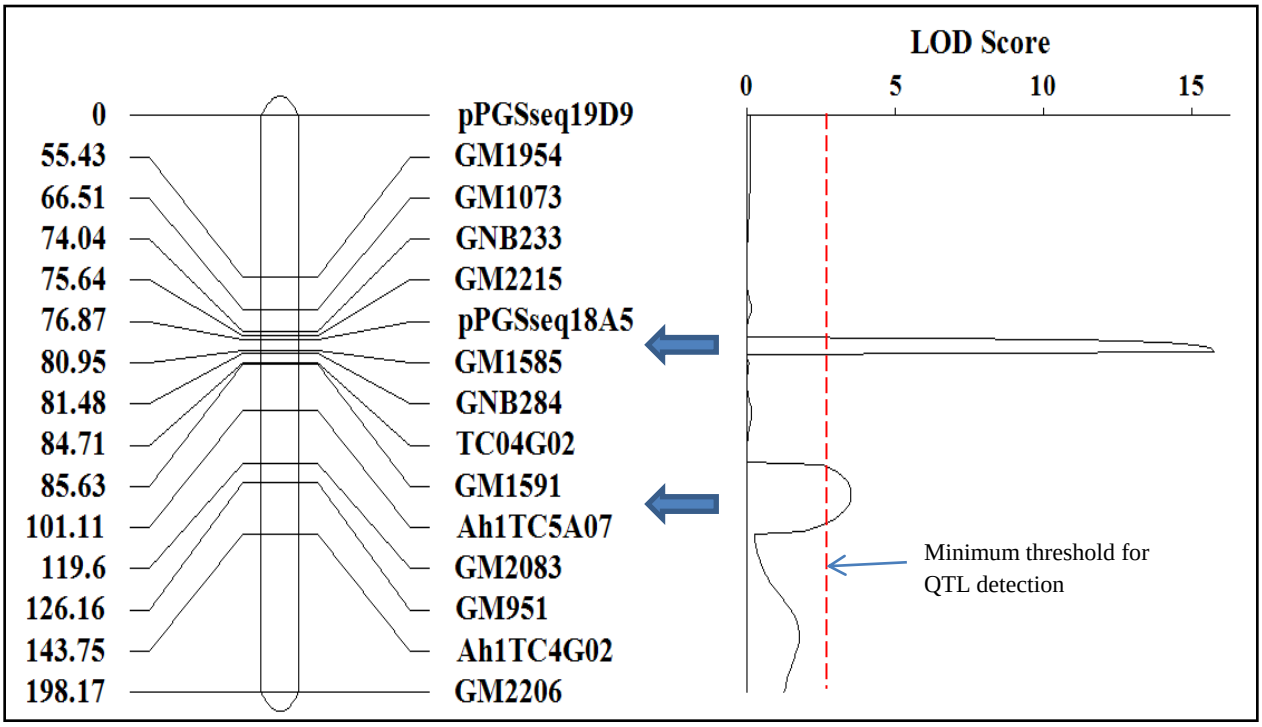

Figure 3a: Linkage group 2 with detected Quantitative Trait Loci (Indicated by arrows)[1,2]

[1]Left side of chromosome – Distance in cM
Right side of chromosome – Marker name

[2]Graph to the right side of chromosome – LOD score showing the positions of the detected QTL

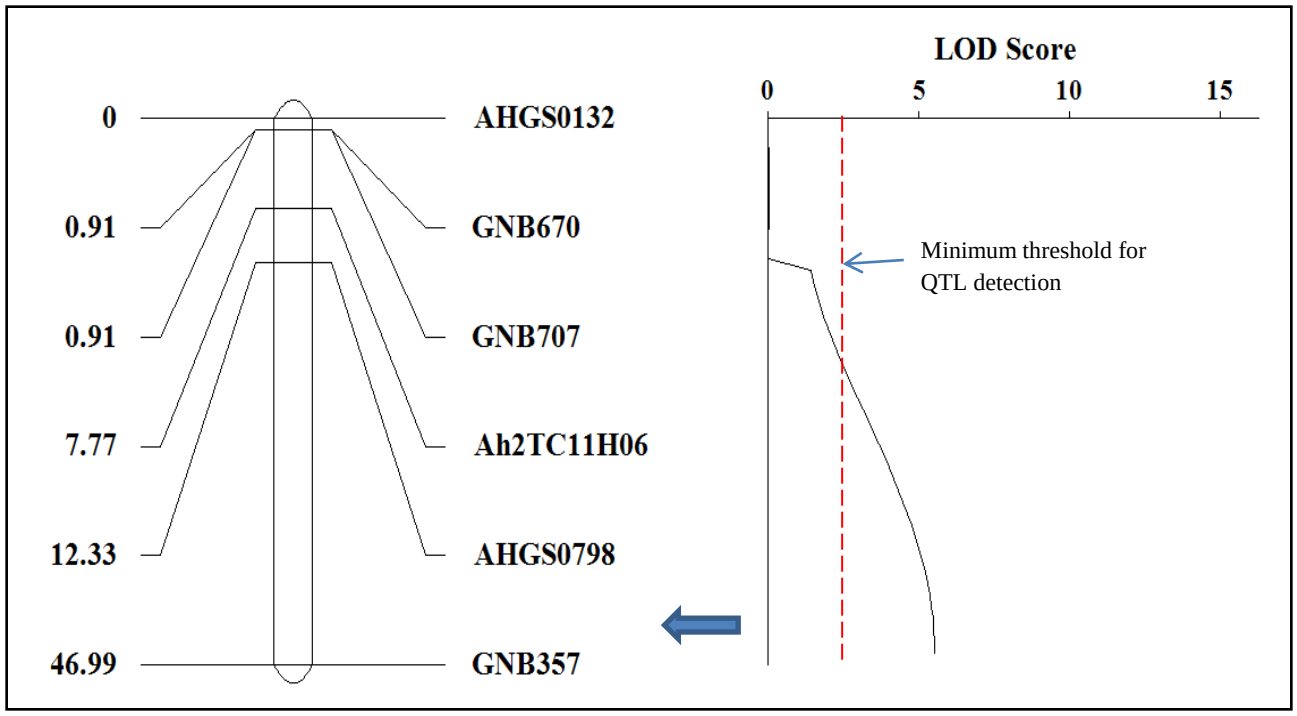

Figure 3b: Linkage group 9 with detected Quantitative Trait Loci (Indicated by arrow) [1,2]

[1]Left side of chromosome – Distance in cM
Right side of chromosome – Marker name

[2]Graph to the right side of chromosome – LOD score showing the positions of the detected QTL

Table 4: Summary on Quantitative Trait Loci Analysis

QTL	Category	Linkage group	Position (cM) [1]	Left Marker	Right Marker	LOD score[2]	PVE[3] (%)	Additive Effect
1	Major	2	81	GM1585	GNB284	15.74	37.91	25.65
2	Minor	2	130	GM951	Ah1TC4G02	3.51	7.98	-11.15
3	Major	9	45	AHGS0798	GNB357	5.52	12.31	12.93

[1]cM – Distance in centi Morgans on the chromosome
[2]LOD score – Logarithm of odds score
[3]PVE – Phenotypic variance explained by the marker in %

5.0 DISCUSSION

5.1 Phenotyping

In this study, the gene action of resistance to *Cercospora arachidicola*, the pathogen that causes Early Leaf Spot (ELS) disease in groundnut was investigated and QTLs for resistance to the pathogen were successfully mapped. Although ELS occurs naturally in the field, it does not spread uniformly. Therefore, artificial inoculation which was done 25 days after planting was aimed at ensuring that there is uniform spreading of the disease so as to produce reliable phenotypic data. Average disease scores of the RILs ranged from 2 to 8 on a scale of 1 to 9 with 1 being highly resistant and 9 being highly susceptible (Chiteka *et al*., 1988). The presence of average scores that are close to the two extremes (resistant and susceptible) suggests the presence of both susceptible and resistant phenotypes in the population. This is expected as the population is from a biparental cross of parents contrasting in disease resistance. Furthermore, with the hypothesis that resistance to the disease is quantitative, the phenotypic data was expected to be a continuous variation and not discrete classes of resistant and susceptible lines (Conner and Hartl, 2004). In this study, the phenotypic data was put into classes purely for the purpose of conducting the Chisquare test.

The phenotypic data obtained as AUDPCs, had a range of 72.5 to 225 score-days with a mean of 147.75. The data showed that the RILs were distributed continuously over the range of the AUDPC values in an approximately normal shape suggesting a quantitative nature of resistance in the (Robut 33-1 × ICGV-SM 95714) population. This suggests that the trait may be controlled by polygenes. This result is in line with earlier studies that indicated that additive gene action was significant for both early and late leaf spot disease resistance (Anderson, 1991). The discovery of additive gene action or inheritance by polygenes justifies the need to conduct QTL

analysis. The phenotypic results also showed that the segregation of the F_8 RILs to the disease followed the Mendelian ratio of 1:1. This is the expected ratio in RILs at such a generation because segregation would have stabilized (Collard *et al.,* 2005). The fact that additive gene action was found to determine the nature of gene action, implies that this trait can be transferred from parent to offspring. Breeding schemes such as pedigree and single seed decent can be used (Singh, 2009; Acquaah, 2007) in breeding for resistance to *Cercospora arachidicola*.

Two other diseases were observed in the experimental plot and these were leaf blotch and late leaf spot. Leaf blotch was observed early and was present during the initial scoring (60 days) but the symptoms of the disease were very different from ELS and therefore, did not affect the scoring of ELS. Late leaf spot infestation was not severe and care was taken not to affect ELS scoring by distinguishing the disease symptoms.

5.2 Genetic linkage map construction and Quantitative Trait Loci Analysis

Three hundred and ninety four (394) SSR markers were used for initial detection of polymorphism between the two parents (Robut 33-1 and ICGV-SM 95714) out of which eighty two (82) representing 20.81% were polymorphic. This is a relatively high proportion of polymorphic markers for cultivated tetraploid peanut genomes considering that scarce genetic variability at the DNA marker level has been repeatedly reported within *A. hypogaea* species in the past (Subramanian *et al.,* 2000; Gimenes *et al.,* 2007; Milla *et al.,* 2005). A possible explanation for this relatively high rate of polymorphism is that the markers used were selected from a larger set which already had been found to be polymorphic between at least two cultivated peanut genomes in a previous study (Ncube *et al.,*2014).

Two methods of DNA extraction were employed in the study and these made use of Zymo DNA extraction kit and Isolate II Plant DNA extraction kit. This was due to the large number of DNA

extractions that were performed that exhausted the materials for the initial method and the same materials could not be procured in time. Therefore, a method that was similar was identified and employed.

A total of thirteen linkage groups were mapped using the genotypic data at a Logarithm of Odds (LOD) value of 2.5. The biggest linkage group was 198.17cM with 15 markers located on chromosome 3 and the smallest was 15.6cM with 3 markers and located on chromosome 8 on the groundnut consensus map. In each linkage group, the number of loci ranged from two to fifteen loci. Three QTLs were detected on two linkage groups (2 and 9) out of which two QTLs were considered to be major QTLs due to their high PVE (phenotypic variation explained by the marker) or R^2 of 12.31 and 37.91%. A QTL is considered to be major if it has a PVE of more than 10% (Collard *et al*., 2005). The QTLs detected had high additive effects of 12.93 and 25.65 respectively. The high additive effects indicate the reliability of the QTLs as contrasted to QTLs with lower additive effects. The minor QTL had a PVE of 7.98% and additive effect of -11.15. The finding on negative additive effect implies that the substitution effect of a non-favorable allele (susceptible) with a favorable allele (resistant) reduced severity at that locus (Tembo *et al*., 2014). The markers associated with the first major QTL were GM1585 and GNB284 while for the second major QTL the markers were AHGS0798 and GNB357. Marker-assisted selection works best when mapped QTLs are tightly linked to the markers and the tighter the linkage, the higher the probability for a marker to be inherited together with the detected QTL. For the marker to be efficient, a distance of less than 5 cM between the marker and the QTL is recommended (Collard *et al*., 2005; Bertrand *et al*., 2008).

In this study, the closest marker to a mapped QTL was less than 5cM. In the first major QTL, there is a possibility of using flanking markers since the two markers on each side of the QTL are less than 5cM away from the QTL. Using a pair of flanking markers can greatly improve reliability compared to the use of a single marker (Bernardo, 2010). The reason for the increased reliability is that there is a much lower chance of recombination between two markers and QTL compared to the chance of recombination between a single marker and QTL. Therefore, since the markers are tightly linked to the detected QTLs and the QTLs are major, the markers can be considered for use in marker-assisted selection.

6.0 CONCLUSION

It is concluded that this study has provided information on the nature of early leaf spot disease in groundnut by suggesting that resistance to the pathogen that causes the disease is determined by additive gene action. This is due to the near normal distribution of the AUDPC of the biparental population used in the study. Breeding schemes such as pedigree and single seed decent can be used in breeding for resistance to ELS. Three QTLs were detected and mapped on two linkage groups with two of them being major QTLs. With the detection of QTLs, markers that are linked to resistance to ELS have been identified. The identified markers can be used in marker assisted selection in breeding for resistance to ELS. This would hasten groundnut breeding programmes and provide the farmer with ideal genotypes for cultivation within a reduced time frame.

7.0 RECOMMENDATIONS

The study suggested that additive gene action determines the nature of gene action of resistance to ELS indicating that breeding schemes such as pedigree and single seed decent can be employed. However, whether to apply early or late generation selection will be determined by the narrow sense heritability estimate value, which was not determined in this study. Therefore a further study should be done to determine the heritability estimates.

The phenotypic assessment of the trait was based on one evaluation and this may not establish the stability of the QTL. There is therefore, need to ascertain the stability by evaluating the mapping population over seasons and locations. There is also need to validate the putative QTLs in other populations before consideration in marker assisted breeding. Additionally, use of a controlled environment such as a screen house and quantified inoculum (cultured inoculum) can validate the data from phenotyping by reducing environmental error. In this experiment, inoculum was not quantified because it was in the form of infected debris were the quantification related to the amount of debris and not necessarily the amount of spores.

8.0 REFERENCES

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Appendix: List of Polymorphic Markers Used in the Study

Marker	Forward Primer (5'-3')	Reverse Primer (5'-3')
GNB233	GACCCATTAACCAGCAAGGA	TGCCATTTTCCCAGTCCTAC
Ah2TC11A02	CACGACGTTGTAAAACGACAATCGGAATGGCAAGAGACA	AGAGCAAAGGGCGAATCTATG
GNB608	CGAGCTGGCTCAAATAATAAGAA	AAGTTCAGCTCATTAACTCGTAAAC
GNB637	AAACTTCGCAGGCTGTGACT	GCATTCACAGACGATGGCTA
GNB652	CAAAGTCGCACAAAGTGGAA	AACTCCGCAGGCTGTGACTA
GNB266	AGATGTAAACAACTGATGCCCA	TTCATGAGGTGGTCAAACGA
GNB284	AATGCGTCATTTAGGCAAGG	GCTCTGCATGGTAGGGTGTT
Ah1TC4D02	CACGACGTTGTAAAACGACAAGTTGTTCCCGTTGCACTC	AAAACACCATAAGGTGAATCAAA
GNB707	CGTTTCTGTTGAAGTCCTTGC	CACCACTCATATTGGTGGGAA
GNB546	GCAAAGATTGGATCAAGGGA	ATAATAGTGGGCCACGGACA
GM1073	CACGACGTTGTAAAACGACTCCCATACTACCCCTTAGCTTTT	GAAAACAACCAAACCGAAGTT
GNB642	ACCACTGAGCTATGACGGCT	CACCCATATAGCCGAATCCA
GNB609	GCGTCTTAAAGTTTCTGCGG	CTAACTCAGCCTGCAAAGCC
AHGS0138	CACGACGTTGTAAAACGACCATATTGACGGTGATGGCAG	CCGACCCTAATCCTAATACAACA
GNB258	TTGAAATGGTTAAGTTTAAATGCC	CGCACTCAAGTCAAGCACAT
GM2083	CACGACGTTGTAAAACGACTCACCGATCATCATCATCAAA	ATTGGGGTTGTTTCCATTCTC
Ah1TC5D06	CACGACGTTGTAAAACGACGAAATTTTAGTTTTCAGCACAGCA	TTTTCCCCTCTTAAATTTTCTCG
GM2246	CACGACGTTGTAAAACGACGCAATTTATGTGCACCCTTTT	CGCTTGACACCAATGAAGTCT
GM1501	CACGACGTTGTAAAACGACTCTGCAGTGTGTGTGTGATGA	TAAGAACCAAAATTGCGACCA
GNB329	CCCTTTTTCGCTTTCTTCCT	GTTCTCGTTTGTGCCCTCTC
pPGSseq19D9	CACGACGTTGTAAAACGACTGTTGCCCACTGTTCTAATCA	TCAAATGGCATAGTCTCCCC
GNB670	ATTTCTGGCTAGTCCGCTCA	TGGAATTGATTGCTAATTGCC
GM1551	CACGACGTTGTAAAACGACTCACTGTCTCGGTGTTTTCCT	TCAGCCTTCTCTCACCGTAAA
GM1311	CACGACGTTGTAAAACGACCACATGATGATGGATCTTTGTTC	AACCATGACACCAGCTACACC
pPGSseq18G1	CACGACGTTGTAAAACGACAATAGGTTGTGAAGCACGCA	TTCGGTGGTACTTTTAAGGCA
AHGS0132	CACGACGTTGTAAAACGACCAAATGTACCTTCGGCGATT	TTACGAACACCCCCTTTCTG
pPGPseq2C11	CACGACGTTGTAAAACGACTGACCTCAATTTTGGGGAAG	GCCACTATTCATCGCGGTA
GNB317	GAAAAGCTTGCAAAATCGAGA	TCCTTCCATGTTGGTGAATG
pPGSseq16G8	CACGACGTTGTAAAACGACCTCAAAAAGCGCTTAGCCAC	CTGCCTACTGCCTACTGCCT
GNB708	GCAATGCTTTTACCACACCA	CCTCAACACCTCTTCCCAAA
GM2284	CACGACGTTGTAAAACGACACACCCCAAATAGCTTCGTCT	TCCACAACACCAACCTTCTTC

Marker	Forward Primer (5'-3')	Reverse Primer (5'-3')
GNB648	AGGGGGAGAGGAATAAACGA	CCGGGGATTGATTGTGATTA
GM2206	CACGACGTTGTAAAACGACTTCCTTCTCCAAAGTCCAAGC	GGAGGAGGGATGTAAGTACGG
TC04H02	CACGACGTTGTAAAACGACACCGCAAACTCATCCATCTC	GATAGCGTCAGAGGCAGAGG
Ah2TC9B08	CACGACGTTGTAAAACGACGGTTGGGTTGAGAACAAGG	ACCCTCACCACTAACTCCATTA
pPGSseq18A5	CACGACGTTGTAAAACGACTGATTCGATTTACTCATGCACA	GAGGATTCTTGAGCCTCGAC
TC05D06	CACGACGTTGTAAAACGACGAAATTTTAGTTTTCAGCACAGCA	TTTTCCCCTCTTAAATTTTCTCG
GM2009	CACGACGTTGTAAAACGACCAAACGCATACACCCCATAAC	TTTGGTTCTCGTTTGTGTTTT
AHGS0590	CACGACGTTGTAAAACGACAAGCCCTTCTCCCTCACTTC	ATAGTGGACCTCAACCACGG
Ah1TC4G10	CACGACGTTGTAAAACGACTTCGGTCATGTTTGTCCAGA	CTCGAGTGCTCACCCTTCAT
GNB402	CGCTTCGCTTTTCGTAATTC	AACACCCCGTTACCCTAAGC
GM2215	CACGACGTTGTAAAACGACGAAATCGGAGTCGGAGAGGT	TCCCCTTCTTTCTTCGTTCTT
GNB218	GCCATATTTCTGTCAAATCAAAA	TACCATCTGGTTTACCCCCA
GNB403	TAATGCTGGATCAGTGGTGC	CCCTAAATCCTAAACCAACGAA
Ah1TC4G02	CACGACGTTGTAAAACGACGATCCAACTGTGAATTGGGC	CACACCAGCAACAAGGAATC
pPGPseq2D12B	CACGACGTTGTAAAACGACAAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA
Ah1TC1D02	CACGACGTTGTAAAACGACGATCCAAAATCTCGCCTTGA	GCTGCTCTGCACAACAAGAA
GM1996	CACGACGTTGTAAAACGACCATCCCATCATTTTCCCTCTT	TACAGTGAAGGTGGGATCCTG
GM2089	CACGACGTTGTAAAACGACATCGCGCAGTTAAAGAAGTGA	ATCTGAGTTCCGAGCAGTTCA
IPAHM689	CACGACGTTGTAAAACGACGATGACAATAGCGACGAGCA	GTAAGCCTGCAGCAACAACA
Ah2AC3C07	CACGACGTTGTAAAACGACGGGGGTTTAGGAGCAAGATTT	CAAGGTGAGAACAAAGGCAAAG
GM951	CACGACGTTGTAAAACGACCCACCACCACCATTCAATAAC	TTGCAGACATGTGTGGAGAAC
TC06H03	CACGACGTTGTAAAACGACTCACAATCAGAGCTCCAACAA	CAGGTTCACCAGGAACGAGT
TC07A02	CACGACGTTGTAAAACGACCGAAAACGACACTATGAAACTGC	CCTTGGCTTACACGACTTCCT
AHGS0729	CACGACGTTGTAAAACGACTGGTTGTTCTAACCCTTCGG	TCACTATCCCATCCCTGCTC
GM1954	CACGACGTTGTAAAACGACGAGGAGTGTGAGGTTCTGACG	TGGTTCATTGCATTTGCATAC
GM1591	CACGACGTTGTAAAACGACTCATCACATTTGATTGCTTGTG	TGCCTTAATAAGCTGGCCTTT
GM1585	CACGACGTTGTAAAACGACAGAAAGGGCATGATGAAACTG	TAACCGCCGCTAAATCAAAAT
Ah1TC2D06	CACGACGTTGTAAAACGACAGGGGGAGTCAAAGGAAAGA	TCACGATCCCTTCTCCTTCA
GNB379	CCGTGGTATGATCGTTCCTT	GCGTGGGGTGTTCTTTTCT
AHGS0798	CACGACGTTGTAAAACGACCGTAGTTGGTGGTAGCCGAT	GAACCGTTAACCCTCTTCCC
TC04F12	CACGACGTTGTAAAACGACGATCTTTCCGCCATTTTCTC	GGTGAATGACAGATGCTCCA

Marker	Forward Primer (5'-3')	Reverse Primer (5'-3')
Ah1TC3E05	CACGACGTTGTAAAACGACCACCACTTGAGTTGGTGAGG	CTTCTTCTTCTCCCGCAATG
GNB385	TCGTTGCTATTGTTGCTAGGG	TGGTCATCTTCTCCACCCTC
IPAHM123	CACGACGTTGTAAAACGACCGGAGACAGAACACAAACCA	TACCCTGAGCCTCTCTCTCG
GNB357	AGGTTTGCTTTGGGATGATG	CCGATAAAACCAGGCAAGAA
TC3E05	CACGACGTTGTAAAACGACCACCACTTGAGTTGGTGAGG	CTTCTTCTTCTCCCGCAATG
GM2553	CACGACGTTGTAAAACGACTCCCTTCAGTTCCGTTGAATA	GCCCCTTCCTCTTTTGTTATG
TC06E01	CACGACGTTGTAAAACGACCTCCCTCGCTTCCTCTTTCT	ACGCATTAACCACACACCAA
TC04G02	CACGACGTTGTAAAACGACGATCCAACTGTGAATTGGGC	CACACCAGCAACAAGGAATC
Ah2TC7H11	CACGACGTTGTAAAACGACAGGTTGGAACTATGGCTGATTG	CCAGTTTAGCATGTGTGGTTCA
TC11A04	CACGACGTTGTAAAACGACACTCTGCATGGATGGCTACAG	CATGTTCGGTTTCAAGTCTCAA
Ah2TC11H06	CACGACGTTGTAAAACGACCCATGTGAGGTATCAGTAAAGAAAGG	CCACCAACAACATTGGATGAAT
Lec1	CACGACGTTGTAAAACGACCAAGCATCAACAACAACGA	GTCCGACCACATACAAGAGTT
Ah2TC7H09	CACGACGTTGTAAAACGACAACTTTATGCCAGTCCCCTCTT	GGATGATGACAAGGGTGATTTC
Ah1TC5A07	CACGACGTTGTAAAACGACGTTTGGTTCTCCCTCCTCCT	AGCCTCTTCATTCCCCTCAT
GM1291	CACGACGTTGTAAAACGACTCCTTGCTATTTTCCCCAAAT	GACGACCCACTTCCTTACAGA
Ah1TC1B02	CACGACGTTGTAAAACGACAACATGCATGCAAATGGAAA	GCCAAAGTCACTTGTTTGCTT
pPGSseq15C10	CACGACGTTGTAAAACGACATTCCCATGTCGTCAAGACC	GCGACGGTATTGGCTTTTAG