QTL ANALYSIS FOR RESISTANCE TO ANTHRACNOSE IN BUKOBA X KIJIVU POPULATION OF COMMON BEAN (*Phaseolus vulgaris*)

BY

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DECLARATION

I, **Josephine S.N. Kachapulula**, declare that this dissertation represents my own work and that it has not been previously submitted for a Degree, Diploma or any other qualification at this or any other University.

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APPROVAL

This dissertation by **Josephine S.N. Kachapulula** has been approved as fulfilling the requirements for the award of the degree of **Master of Science in Plant Breeding and Seed Systems - Crops** of the University of Zambia.

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ABSTRACT

The fungus *Colletotrichum lindemuthianum* which causes anthracnose disease in common bean (*Phaseolus vulgaris* L.) has a high genetic variability that requires deployment of loci with resistance to a wide range of *C. lindemuthianum* races. Most of the loci have been sourced from the Middle American genepool which are usually small seeded requiring many generations of back crossing to recover the original big seed size in Andean gene pool varieties which are also the required market classes in Zambia. This requires identification of loci with durable resistance from the Andean gene pool to reduce the time and cost of resistance breeding. Two Andean parents were used in this study Bukoba (ADP 7) and Kijivu (ADP 33). From previous studies by *Sansala et al., (2023*), the Andean variety Bukoba showed resistance to a number of races of *C. lindemuthianum* however the genetic architecture of this resistance was unknown and thus this study. A population of 158 $F_{4:5}$ Recombinant Inbred Lines (RILs) were developed from a cross of two Andean parents Bukoba and Kijivu and evaluated at the University of Zambia, Lusaka, Zambia. The seven races for this study were 19, 38, 51, 167, 263, 1050 and 1105. These were selected based on their prevalence in Zambian bean growing regions and the virulence levels. The population was genotyped using 12000 SNPs out of which 1,838 were polymorphic between the parents and used to build genetic maps. Composite interval mapping was used to identify the quantitative trait loci (QTL). A total of 8 QTLs: ANT01.1, ANT01.2, ANT02.1, ANT04.1, ANT05.1, ANT06.1, ANT10.1 and ANT11.1 with R^2 values ranging from 3.6% for ANT06.1 on Pv06 to 70.1% for ANT01.1 on Pv01 were identified. These were both novel and verified conditioning major and minor resistance to the seven races of *C. lindemuthianum* in this study. ANT01.1 and ANT04.1 were major QTLs while the rest were minor QTLs suggesting the role of both qualitative and quantitative resistance in the mapping population. The major QTLs identified in this study co-localized with previously reported major genes proving their usefulness as target genes for developing durable resistance to anthracnose using gene pyramiding and marker-assisted selection. ANT01.1 co-localised with the *Co-1* locus a major Andean locus that conditioned resistance to the very virulent races 1050 and 1105 in this study while ANT04.1 controlled four moderately virulent races 19, 51, 183 and 263. This locus co-localises with well-known Middle American loci *Co-3, Co-15, Co-16, Co-y* and *Co-z* and the NB-ARC domain cluster that are responsible for resistance to a number of the pathogen's races. Although ANT02.1 was a minor QTL, but due to its co-localisation with the *I*-gene, it should be s considered a candidate for gene pyramiding together with the 2 major QTLs identified as a means of deploying the most durable and cost

effective breeding strategy for controlling *C. lindemuthianum* in Zambia with Bukoba as the source of resistance especially in the yellow bean class. .

DEDICATION

This dissertation is dedicated to my beloved family Dr. Kachapulula, Godfrey, Martha, Paul and Josephine for being super supportive to me in all aspects including this academic achievement.

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CHAPTER ONE

INTRODUCTION

1.1 Common bean production

Produced in the tropical and subtropical regions of the world by smallholder farmers, the common bean constitutes a major staple crop in both developing and developed countries globally making it the most important legume in the human diet (Heuzé et al., 2013). The crop provides proteins, fibre, complex carbohydrates and micro nutrients like iron and zinc for people in the tropics coming second after maize as the major source of calories (OECD iLibrary, 2015). In southern Africa, the common bean is the second most important source of dietary protein and third most important source of calories (Wortmann et al., 1998). Beans are a food source to over one million people in Africa providing an income in excess of US$580 million (SABRN, 2005). The seeds are harvested before physiological maturity as green beans or after physiological maturity as dry beans. Most dry bean varieties are consumed after boiling while dried stems and pods been used as hay for animal feeding Common bean anthracnose caused by the ascomycete hemibiotrophic fungal pathogen *Colletotricum lindemuthianum (C. lindemuthianum)* is a devastating disease of the bean crop in Zambia and many other tropical countries world over where it causes diseases of epidemic proportions. It attacks all aerial parts of the plant causing damage of up to 100% loss if conditions of favourable environment and susceptible host are met (Singh and Schwartz, 2010). The disease is more severe under cool humid conditions.

In Zambia, areas having these cool and humid conditions are most preferable for bean crop production and are also dominated by susceptible unimproved genotypes (Zulu, 2005). Because of the severity and significance of damage caused by this pathogen, development of control and management strategies to reduce crop losses and manage epidemics is warranted. Among the control practices are crop rotation, use of disease free seed, spraying with fungicides and use of resistant varieties. Incorporation of resistance genes into genotypes of important market classes is important to help manage the disease as most farmers of common beans in Zambia are poor and cannot afford to buy fungicides.

The fungus *C. lindemuthianum* has been found to be highly variable in many parts of the world and the deployed resistance genes in the bean crop has been overcome by the pathogen over time limiting the usefulness of deployed resistance genes for long periods of time (Mahuku and

Riascos, 2004). This is because the response of bean plants to the pathogen often exhibits a qualitative mode of inheritance following a gene-gene mechanism of resistance where a particular gene can only protect against specific isolates of the fungus (Ferreira *et.al*., 2013). It is thus important to understand the structure of prevalent *C. lindemuthianum* races to determine the best strategy for deployment of resistance genes. *C. lindemuthianum* has a high degree of pathogen variability (Choudhary *et al.*, 2018) and several races which make breeding difficult due to rapid resistance breakdown or erosion. Currently, over 180 different races of *C. lindemuthianum* have been identified using a set of differential cultivars, a standardized resistance test and a standardized nomenclature system to name the races (Kelly and Vallejo 2004; Ferreira *et al*. 2013; Padder *et al*. 2017).

The resistance of the bean crop to *C. lindemuthianum* is highly race specific (Campa *et al.* 2017). To date, over 20 anthracnose resistance genes, identified by "*Co*" symbol followed by a number, have been described (Ferreira *et al.* 2013, and Castro *et al*., 2017). The resistance genes to anthracnose show complete dominance where the dominant allele conditions the resistance reaction although some dominant epistatic inheritance has been observed in the presence of several resistance genes and a complimentary mode of action between two resistance genes was described by Campa *et al*., (2014). Expression candidate genes associated with resistance loci and early stages of infection (0-72 hours) have been identified (Padder *et al.,* 2016), which has provided important genomic insights into anthracnose resistance at the gene level. Molecular markers linked to resistance genes have also been identified to enable the use of marker-assisted selection (MAS) by breeding programs (Kelly and Bornowski 2018). Also, many resistance genes have been mapped on different chromosomes of the common bean genome, and these mapped genomic regions are often organized in complex clusters comprising many closely linked genes, especially at the sub-telomeric regions of the chromosomes (Ferreira *et al*. 2013; Murube *et al.* 2019). Furthermore, quantitative trait loci (QTL), conferring resistance to different races of *C. lindemuthianum* have also been described (Oblessuc *et al.,* 2014; González *et al.,* 2015; and Choudhary *et al*., 2018; Zuiderveen *et al.,* 2016).

1.2 Justification of the study

Bean anthracnose remains one of the biggest threats to common bean production and productivity in Zambia because all the commercially available varieties are susceptible to the disease. Enhancing the productivity of the bean crop is key to reducing food and nutrition

insecurity in Zambia (Mbewe et al., 1991). *C. lindemuthianum* is a hyper variable pathogen (Mahuku *et al*., 2002) with the capacity to overcome resistance genes (Mc Donald and Linde, 2002). Although there are many different ways of controlling the disease, breeding for resistant varieties is the most economically viable method (Bardas et al., 2007) for resource constrained farmers in Zambia.

Over the years, the small seeded Midle -American bean variety G2333 has been used as the main source of resistance genes (Kelly and Vallejo, 2004). This landrace G2333 has a large number of resistance genes but it being the only source of resistance is a challenge because with time, the pathogen will get to overcome the resistance provided by these genes (McDonald and Linde, 2002). Furthermore, G2333 is small seeded which is a challenge when breeding for large seeded varieties preferable on the Zambian market. This is because it is difficult to recover the original big size of the varieties as multiple generations of back crossing to recover the original seed size are required thus adding onto the costs and time required to generate the new resistant variety. Therefore, novel sources of resistant genes to *C. lindemuthianum* from the Andean gene pool are needed (González et al., 2015) as a cost effective way to maintain the preferred large grain size and offer durable resistance to the crop (Bardas et al., 2007). Finding new resistance sources in Andean beans would provide an additional reservoir of materials within this gene pool, and offer the possibility of pyramiding anthracnose resistance genes from both gene pools, which should result in more durable resistance (Mahuku and Riascos, 2004).

In Sansala *et al.,* (2023) a number of genotypes in the Andean Diversity Panel (ADP) of beans were subjected to a number of races. The East African variety Bukoba (ADP7) was among those that exhibited resistance to a number of *C. lindemuthianum* races. However, the genetic architecture of Bukoba's resistance to *C. lindemuthianum* is still unknown. An understanding of this genetic basis of Bukoba;s resistance could enable its effective use as a source of anthracnose resistance using Marker Assisted Selection (MAS). Therefore, the aim of this study is to identify the genetic regions responsible for the resistance in Bukoba to the selected races of *C. lindemuthianum.*

1.2 Objectives of the study

The overall objective of this study was to determine the genetic architecture of Bukoba's resistance to *C. lindemuthianum.*

1.2.1 Specific objectives are:

1. Evaluate the 158 $F_{4:5}$ recombinant inbred lines derived from across of common bean Andean varieties Kijivu and Bukoba for resistance to the seven races (19, 38, 39, 167, 263 1050 and 1105) of *C. lindemuthianum.*
2. Identify the quantitative trait loci (QTLs) conferring resistance to the selected races of *C. lindemuthianum* in Bukoba variety of common bean.

1.3 Hypothesis

2 There is sufficient genetic variation for resistance in the Recombinant Inbred Line (RIL) population of Bukoba and Kijivu to the seven races (19, 38, 39, 167, 263, 1050 and 1105) of *C. lindemuthianum.*

3 Resistance to anthracnose in the population of RILs derived from Bukoba and Kijivu is controlled mainly by major-effect loci.

CHAPTER TWO

LITERATURE REVIEW

2.1 Phytogeography of the common bean

Common bean (*Phaseolus vulgaris* L) is the most important grain legume used for direct human consumption in many parts of the world and the most accessible source of protein and minerals in many countries (Broughton *et al*. 2003; De Ron *et al.* 2013). It is a member of the legume family (*Leguminosae*) belonging to the tribe *Phaseoli* and sub-family *Papelionoidae*. Beans have two centres of origin that include the Middle American and Andean zones in South America where the crop has been cultivated for centuries (Beebe *et al.*, 2000). The Middle American centre has given rise to the small to medium seeded cultivars, while the large seeded types have risen from the Andean centre of origin. The two gene pools have contrasting agronomics, morphological and biochemical characteristics and are widely distributed world over. In Zambia, the vast majority of farmers cultivate the Andean gene pool varieties because of the preferred large size.

2.2 Importance of the Common bean

Common bean (*Phaseolus vulgaris*) is widely cultivated food legume (Choudhary et al., 2018) and a cheaper source of proteins and nutrients (Beebe et al., 2013). In Southern Africa where Zambia is located, common bean is the second most important source of dietary protein and third most important source of calories (Wortmann et al., 1998). It is also an important source of dietary fibre and other micro nutrients like iron, zinc and complex carbohydrates (Chirwa, 2014; Kamfwa, Cichy and Kelly, 2015). The grain is cooked and eaten as dry beans with maize which is the main staple or as an immature green pod or fresh seeds fried in cooking oil while leaves are consumed as a vegetable leaves. (Broughton et al., 2003; Padder et al., 2017). The haulms are used as forage (Purseglove, 1988). Soups that are canned and frozen for export are another product. This crop is used by subsistence farmers to alleviate soil fertility through nitrogen fixation (SABRN, 2005). In 2018, global exports of common bean were about 3.9 million tonnes (Index Box, 2020). Additionally, beans is a major source of revenue and livelihood for many small scale farmers in Zambia and those in the value chain (Sichilima et al., 2016). As of 2020, an annual income of $30.1 billion dollars has been reported from global trade in the common bean IndexBox, 2020).

2.3 Bean production constraints in Zambia

The production and productivity of common bean in Zambia is hampered by both biotic and abiotic constraints leading to mean yields of 0.5t/ha which are comparatively lower than the mean yields from North America of 1.8t/ha (Hillocks *et al.,* 2006)). The low productivity is further still in contrast with the yields realized at research stations ranging from 2-3t/ha (Mbewe *et al.,* 1991). In Zambia, resource-constrained small scale farmers are usually the major producers of common beans (Beebe *et al*., 2014).

Among the abiotic factors affecting bean productivity are heat, low soil fertility, aluminium toxicity, acidic soils and unpredictable rainfall. Drought is another major contributing factor to low bean yields globally with over 60% of bean production being on lands that experience drought stress frequently (Beebe *et al.,* 2011).

The biotic constraints include poor genotypes which even though adapted to the Zambian local environments, have low productivity as they are susceptible to diseases (Costa *et al.,* 2017). Other biotic factors are pests like bean stem maggots *Oophiomyia phaseoli*) and the common bean weevil (*Acanthoscelides obtectus).* An estimated reduction in productivity of 25-50% is attributed to viral, bacterial and fungal diseases (Greenberg *et al.,* 1986) which include anthracnose, angular leaf spot, common bean mosaic virus and common bean blight.

Seed recycling is also a major problem. The low income farmers cannot afford to buy new clean seed thus further perpetuating seed-borne diseases (Choudhary *et al.,* 2018)). Additionally, common bean farmers in Zambia have relatively small pieces of land on which they grow all their food annually which makes crop rotation difficult. Late planting by farmers is also another constraint to productivity. Because of the many pests and diseases that the bean crop encounters if planted in the rain season, the resource-constrained farmers resort to late planting to avoid these challenges.

2.4 Bean anthracnose epidemiology and symptoms

The bean anthracnose disease is one of the well-studied diseases of common bean with well documented literature.

2.4.1 Epidemiology

Anthracnose caused by the ascomycete fungus *Colletotricum lindemuthianum* is the most devastating disease of common bean attacking all above-ground parts of the plant and severely

affects pods (Agrios, 2005). This disease has the ability to cause 100% loss if the conditions of a susceptible host, virulent race and favourable environment are present (Singh and Schwartz, 2010) and the infection occurs during the phase of active growth cycle in a susceptible host (Mahuku and Riascos, 2004, Fernandez *et al*., 2000). Cool temperature of 16-22^{0}C and high relative humidity RH > 90% favour infection and disease development (Mahuku *et al.,* 2002, Pastor-Corrales, 2005 and Bardas *et al*., 2009). Bean anthracnose is the most important disease in the wet and cool areas of Northern, North-western and Luapula provinces of Zambia where most of the beans are produced (Mbewe *et al.,* 1991).

Anthracnose is seed-borne (Zuiderveen *et al.,* 2016) and long distance spread of new races of the pathogen to different geographical regions is primarily through infected seed. As long as the infected seed remains viable, the pathogen can survive as dormant mycelium within the seed coat and cotyledon cells or as spores between cotyledons or elsewhere in the seed. When seedlings emerge from planting infected seed, they become a source of inoculum of the pathogen. Once lesions begin forming on the seedlings, the spores can then be spread by rain splash and wind drift from diseased to neighbouring health plants. This rain splash also moves the disease further up the plant and onto pods (Melotto *et al*., 2000). The pathogen can also survive on infected old bean debris under field conditions for more than two years (Sikora *et al*., 2004) through formation of sclerotia (Melotto *et al*., 2000).

2.4.2 Bean anthracnose disease pyramid

The bean anthracnose disease develops due to a combination of susceptible host, a virulent pathogen and favourable environmental conditions over a relatively long period of time (Agrios, 2005). Through growing the crop in cool and wet weather, use of infected seed, recycling of infected seed and monoculture, humans have played a great role in perpetuating this epidemic (Pastor-Corrales et al., 2009; Sikora *et al*., 2004). If appropriate control measures are adopted, humans then would play a very important role in stopping the epidemic through their interventions.

2.4.3 Disease development in the bean crop

Anthracnose affects all the above ground parts of a plant with pods particularly being the most severely affected, Figure 1A

A B

Figure 2.1. Bean pods affected by anthracnose disease, A and disease symptoms on leaves, B

Leaf symptoms appear initially on the lower surface as brick-red to purplish-red discolorations along the veins. With time, this discoloration develops on the upper leaf surface as well and simultaneously brown lesions of different sizes bearing brown, black or even purplish margins develop around small veins.

On larger leaf veins, lesions expand into sunken cankers within which acervuli bearing conidia are produced and subsequently leaves become dark-brittle, rugged and reduced in size (Allen, 1991). Lesions also develop on the cotyledon petioles, branches, stems and pods. Pod lesions are typically sunken and contain masses of salmon-pink conidia (Melotto *et al*., 2000 and Allen, 1991). On seed, symptoms include appearance of rusty brown spots with small brown specks on pods. Seeds acquired from seriously infected pods generally reveal brown to light chocolate spots on the seed coat. If these lesions extend into the cotyledon, serious blemishes that make the seed unfit for utilization occur (Tu, 1988).

2.4.4. Management of Bean anthracnose

In the process of preventing losses caused by bean anthracnose, use of disease-free seed is a critical component (Corner *et al*., 2001). The clean seed should preferably be produced under arid conditions as seed produced under wet humid conditions is most susceptible to infection.

Seed treatment with fungicides has also been used to reduce transmission of anthracnose to seedlings. However, it should be noted that fungicides do not completely eliminate the disease from heavily infected seed and therefore, foliar application of fungicides and quarantine have also been used (Corner *et al*., 2004). Fungicides such as Chlorothalonil,,

ethylenebisdithiocarbamate, Farmaneb, Zineb, and Benomyl, have been recommended at first sight of the disease and then reapplied weekly during the season (Agrios, 2005).

Proper field cultural practices are also important in anthracnose disease management. Effective crop rotation of 3year crop mixtures and management of crop debris by deep ploughing have been shown to be effective in managing the disease (Ntahimpera *et al*., 1997 and Sikora *et al.*, 2004). Also, fields should not be worked when plants are wet as fungal spores easily spread from diseased to healthy plants.

Although the above methods seem to be effective, in the case of endemic pathogens cultivation of resistant cultivars is the safest, cost-effective and ecological friendly way of handling anthracnose disease (Bardas *et al*., 2007).

2.5 *Colletotricum lindemuthianum* biology, host range and resistance breeding

2.5.1 Biology, Diversity and Host range

Colletotricum lindemuthianum belongs to the genus Colletotricum classified under Deuteromycetes, order Melanconiales, family Melanconiaceae and section Hyalospae. The genus is characterised by production of acervuli which are disc shaped waxy sub-epidermal structures with dark setae at edges or among conidiophores (Bailey, 1997). It differs from other species in the genus by its growth characteristic and production of a dark pigmentation on cultures (Tesfaye, 2003).

The pathogen has a sequential biotrophic and necrotrophic –infection process to invade and colonize the plant host that involves the transition from asymptomatic biotrophic phase characterised by intracellular thick primary hyphae to a destructive necrotrophic phase characterised by thin filamentous secondary hyphae known as biotrophynecrotrophy switch which is essential for disease development (Bhadauria *et al*., 2011). *C. lindemuthianum* causes a hypersensitive response in bean resistant plants. This manifests in groups of red-brownish wounds of different sizes that are produced by the plant to limit the spread of the fungus (Martinez-pacheco *et al*., 2009.

The bean anthracnose fungus is highly variable (Mahuku et al., 2002; Mahuku and Riascoa 2004). To date, over 180 different races of *C. lindemuthianum* have been identified using a set of differential cultivars, a standardized resistance test and a standardized nomenclature system to name the races (Kelly and Vallejo 2004; Ferreira *et al*. 2013; Padder *et al.* 2017). This high

rate of pathogen variability may be attributed to chromosomal losses and duplications as well as gene exchange with similar pathogen species hosted by different crops (Bailey, 1997). The extensive virulence diversity of *C. lindemuthianum* is the most important reason for the absence of durable resistance to anthracnose in common bean. Sexual and asexual recombination mechanisms have been reported for this pathogen (Souza *et al.,* 2010, Ishikawa *et al*., 2012, Barcelos *et al.,* 2014 and Martins *et al*., 2019). In Zambia, thirteen races designated 255, 342, 407, 510, 382, 247, 485, 65, 207, 53, 39, 84 and 73 have been identified so far (Zulu, 2005).

C. lindemuthianum is mostly found on dry and snap beans (*P. vulgaris)* but has also been isolated from lima beans (*P. lunatus L.*), scarlet bean (*P. muliflorus* Wild.), mung bean (*P. aureus* Roxb.), cowpea (*Vigna sinensis Savi*.) and broad bean (*Vicia faba L.*) (Mahuku *et al.,* 2002)

2.5.2 Pathogen race identification

Identification of *C. lindemuthianum* races has been internationally standardized based on the disease reaction of the 12 differential common bean cultivars (table 1) and a binary nomenclature system (Pastor-Corrales 1991).

Table 2.1: Characteristics of bean differential cultivars used in assessing races of C. lindemuthianum in the current study.

Differential Cultivar	Host genes	Gene Pool	Position of Cultivar	Binary[3] Number	Growth Habit
Mitchelite	…	M[1]	0	1	II
MDRK	*Co* - 1	A[2]	1	2	I
Perry Marrow	Co -13	A	2	4	II
Cornell 49242	*Co* - 2	M	3	8	II
Widusa	*Co* -9	A	4	16	I
Kaboon	*Co* - 12	A	5	32	II
Mexico 222	*Co* - 3	M	6	64	II
PI 207262	*Co* - 43, *Co* - 9	M	7	128	III
TO	*Co* - 4	M	8	256	I
TU	*Co* - 5	M	9	512	III
AB 136	*Co* - 6, *Co* - 8	M	10	1024	IV
G2333	*Co* - 42, *Co* - 5, *Co* - 7	M	11	2048	IV

Source: Kelly and Vallejo (2004).

M^1 = Meso American genepool; A^2 = Andean genepool. [3] (Binary numbers): 2^n, where n is equivalent to the place of the cultivar within the series (0-11). The sum of the cultivars with susceptible reaction will give the binary number of specific race. For example, race 6 is virulent on MDRK ($2^n = 2^1$=2) and Perry Marrow ($2^n = 2^2 = 4$). Growth habit: I = Determinate, II = Indeterminate bush, III = Indeterminate bush with weak main stem and prostrate branches; IV = Indeterminate climbing habit.

2.5.3 Pathogen Evolution, Co-evolution and Resistance Breeding

C. lindemuthianum has co-evolved with the Andean and Middle-American gene pools respectively. As a result, some races are more virulent on Andean beans but less virulent on Middle-American beans while other races are more virulent on Middle American but less virulent on Andean beans. However, some studies have reported a richer diversity in the virulence of Middle American races as they have been reported to be virulent on both Andean and Middle American geneotypes. The high variability of *C. lindemuthianum* and its co-evolution with the genepools of beans present unique challenges in the deployment of resistance and durability of the deployed resistance due to continuous resistance breakdown Melotto *et al*., (2000).

The process of co-evolution between the fungus and bean resistant plants has led *C. lindemuthianum* to produce new pathogenic variants which can be detected on the basis of the phenotypic response to anthracnose infection shown by different varieties of common bean (Melotto *et al*., 2000; Rodriguez-Guerra *et al.*, 2003). New pathotypes emerge with the introduction of new varieties of crops thus accurate and rapid detection of new races help in formulating strategies for deploying resistant cultivars in particular regions and countries. Due to the high and rapid variability in the pathogen, pyramiding of resistance genes in the host is the best option for developing cultivars with durable anthracnose resistance (Mahuku and Riascos, 2004).

2.6 Bean host Evolution and Variability and Resistance to disease

2.6.1 Host Evolution and Variability

Beans are leguminous plants of the genus phaseolus, family legumonosae, subfamily papilionoidae, tribe phaseoleae and subtribe phaseolinae. They belong to a genus with more than fifty wild-growing species (Gepts, 2001. Common beans are believed to have been

domesticated in two centres of origin, one in Meso-America and the other in Central America (Duran *et al*., 2005). These two centres of origin have resulted in two separate gene pools, the Middle-American and the Andean that are distinguished at the morphological, physiological and molecular levels (Duran *et al*., 2005).

The Andean genotypes are large seeded and consist of kidney, cranberry and many snap beans (Phillip *et al.,* 2004). They also have large leaves with ovate or lanceolate central leaflets and lanceolate or triangular bracteoles, longer internodes in the main stem, a stripe less standard on white flowers and a central pod beak position (Duran *et al.,* 2005). The Middle-American genotypes are medium and small-seeded consisting of pinto, pink, black, white and some snap beans (Phillip *et al*., 2004). They also have striped standards and coloured flowers, a placental pod beak position and cordate or ovate bracteoles (Duran *et al*., 2005).

The high genetic variability existing in beans could be an indicator of variability resident at disease resistance loci and thus useful in disease resistance breeding efforts as evidenced by Kelly and Vallejo, (2004).

2.6.2 Bean Host resistance to anthracnose

Resistance of the common bean to anthracnose follows a qualitative mode of inheritance with very specific gene interactions so that one resistance gene protects against specific isolates of the fungus (Ferreira *et al*., 2013). Different anthracnose resistance loci conferring resistance to specific races (Co- followed by a number or letter) have been described in common bean. Generally, anthracnose resistance shows complete dominance where the dominant allele conditions the resistance reaction. However, in the presence of several resistance genes, dominant epistatic inheritance has been observed and a complimentary mode of action between two resistance genes was described by Campa *et al.,* (2011 and 2014). Most of these anthracnose resistance loci have been mapped and the markers tagging these genes have been identified.

2.6.3 Genes for Anthracnose Resistance and their Location in the genome.

In order to reduce the effect of *C. lindemuthianum* in common bean, incorporation of genetic resistance is the research area that is most promising. Currently, over 100 pathogenic races have been globally reported using the 12 differential bean cultivars and the binary system for race identification (Ferreira *et al*., 2013). Resistance to anthracnose is dominant at all loci

except the *Co*-locus. However, multiple alleles have been identified at the *Co-1*, (Rodriguez-Suarez *et al*., 2007), *Co-3 (*Rodriguez-Suarez et al., 2007, 2008; Sousa *et al*., 2013), *Co-4* (Melotto *et al*., 2004); and *Co-5* loci (Campa *et al*., 2009). Novel resistance genes have been identified in two different Brazilian landraces with gene *Co-I5* found in Carinthiano and *Co-16* found in Crioulo 159. Both genes have been mapped to Pv04. *The Co-13* and *Co-17* genes were recently mapped to Pv03 (Trabanco *et al*., 2015). Also the co-localization of the major *Co-1* gene (*Co*-1^4) with the angular leaf spot gene phg-1 was confirmed on Pv01 (Meziadi et al., 2016). Furthermore, the co-segreagation of the C0-10 gene with the Phg-3 for angular leaf spot in Ouro Negro was confirmed on Pv04 (Gonçalves *et al*., 2013). Also the Co-3 locus, a major resistance cluster with five alleles is also confirmed on Pv04. The alleles are *Co-*3^3*, Co-*3^4*, Co-*3^5*, Co-15*, *Co-16* along with *Ur-5*, *Ur-14*, and *Phg-3* genes for rust and angular leaf spot (Valentini *et al.,* 2015). *Co-13* has been mapped to Pv03 and *Co-u* gene is mapped to Pv02 (Campa *et al*., 2014).

Allelic variants of *Co*-1, *Co*-3 and *Co*-4 have been proposed from the differences observed in the resistance spectrum between different bean genotypes. From the genetic mapping of genes conferring resistance to specific races of *C. lindemuthianum*, Ferreira *et al*., (2013) indicated that these *Co*-genes are not distributed randomly in the genome but are organized in clusters of race-specific resistance genes. Quantitative resistance loci (QRL) have also been confirmed with the major gene Co-u on Pv02 and *Co-5* on *Pv07* (Oblessuc *et al*., 2014). Other confirmed QRL are on *Pv01*, *Pv03*, *Pv04*, *Pv05*, *Pv07*, *Pv08* and Pv09 (González *et al*., 2015). The *Co-x* gene has been fine mapped to *Pv01*, independent of the *Co*-1 locus while fine mapping of the Co-4 (COK-4) locus on Pv08 revealed 18 copies of the COK-4 gene (Oblessuc *et al.,* 2015). Therefore, with all the information on resistance sources in the common bean that have been tagged with molecular markers, breeders are able to pyramid these genes in order to control the rapid evolution of new races of the *C. lindemuthianum* pathogen. Majority of anthracnose resistance genes in Andean beans reside on Pv01 and Pv04, making the identification of additional resistance sources on other chromosomes critical for future gene pyramiding (González *et al.,* 2015).

Genes *Co*-1, *Co*-x and *Co*-w were mapped on Pv01(Rodriguez-Suarez *et al*., 2007; Geffroy *et al*., 2008); *Co*-u on Pv02 (Geffroy *et al*., 2008); *Co*-13 on Pv03 (Concalves-Vidigal *et al*.,2009); *Co*-3, *Co*-9, *Co*-y, *Co*-z, *Co*-10 and *Co*-15 on Pv04 (Geffroy *et al*., 1999; Alzante-Marin et al., 2003; Mendez-Vigo *et al*., 2005; Rodriguez-Suarez et al., 2007, 2008; Sousa *et*

al., 2013). *Co*-5, *Co*-6 and *Co*-v on Pv07 (Geffroy, 1997 and Camps *et al.*, 2009); *Co*-4 on Pv08 (Melotto *et al.*,2004); *CoPv09* on Pv09 (Campa *et al*., 2014) and *Co*-2 on Pv11 (Adam-Blondon *et al*., 1994).

2.6.4.1 Chromosome Pv01

This chromosome is known to condition broad resistance and specific resistance to *C. lindemuthianum* races 65 and 73, 3481 and 3993 (Zuiderveen *et al*., 2016). The Co-1 gene originally known as the A gene was the first disease resistance gene described in common bean and the first genotype by pathogen race interaction was demonstrated with this particular gene and isolates of anthracnose (Barrus, 1915). The *Co-1* locus is present in the majority of Andean beans surveyed, with allelic differences existing between cultivars. This locus, however, is very valuable in the breeding of beans of MA origin, particularly in those countries where MA races of the anthracnose fungus predominate (Balardin and Kelly, 1998). The *Co-1* gene is the known source of resistance in Michigan Dark Red Kidney (MDRK) and A193 widely used in Mexican breeding programs (Mendoza *et al.*, 2001). Alleles of the *Co-1* present in the Andean gene pool are: $Co\text{-}1^2$ in Kaboon, $Co\text{-}1^3$ in Perry marrow, $Co\text{-}1^4$ in AND277 and $Co\text{-}1^5$ in Widusa (Melotto and Kelly, 2000). Among the four confirmed alleles described at the *Co-1* locus, the $Co\text{-}1^2$ allele offers the broadest-based resistance (Mendoza *et al*., 2001). Genetic studies carried out by Vallejo *et al*., (2003) confirmed that *Co-x* and *Co-1* are the same gene. This *Co-1* locus is unique in importance to bean breeders developing gene pyramids with complementary genes from both Andean and Mesoamerican gene pools.

2.6.4.2 Chromosome Pv02.

The *Co-2* gene was originally known as the Are gene and was first described by Mastenbroek (1960). It is known to condition resistance to four races of *C. lindemuthianum* race 17 (alpha), race 130 (beta), race 102 (gamma) and race 23(delta). The gene is considered to have no alleles although a study by Muhalet *et al*., (1981) indicates a high probability of another tightly linked resistance gene as the F_2 in the study did not segregate according to the expected 3:1 ratio but rather a 15: 1 ratio expected of two resistance genes. This gene was a widely deployed resistance gene in breeding of both dry and snap beans (Tu, 1992) and was initially considered to possess a horizontal type of resistance because it provides protection against many races of the pathogen (Tu, 1992). However, with the rapid emergence of new virulent races, race specificity of the *Co-2* was detected by Hubbeling (1977). Also, due to its extensive

deployment, the *Co-2* gene later failed to provide satisfactory resistance in breeding programmes world over. (Kelly *et al.*, 1994, Balardin *et al*., 1997, Hubbeling, 1976, Leakey and Simbwa-Bunnya, 1972.

Currently, the Pv02 chromosome is known to host the Quantitative Resistance Locus (QRL) ANT ANT02.1^{UC} (Oblessuc *et al.*, 2015) or the *Co-u* gene (Geffroy *et al.*, 2008) which major gene confers resistance to bean anthracnose races 38, 39 and 55 where the latter two are known to be very virulent races to the Andean gene pool. The locus responsible for this resistance is the same locus as the *Co-u* gene. Other races whose resistance is conferred on the Pv02 chromosome are 3, 19 and 449 but the resistance is conferred on another locus away from the *Co-u* gene (Geffroy *et al.*, 2008)

2.6.4.3 Chromosome Pv04.

This chromosome hosts the *Co-3* genes which was originally called Mexique 1 in the genoype Mexico 222 (Bannerot, 1965). Two alleles have been speculated at this locus (Kelly and Vallejo, unpublished data). The breeding value of this locus is very specific to given locations as it affords resistance to highly virulent Mesoamerican races 3481, 3977 and 3993 in Costa Rica (Kelly and Vallejo, 2003). In other areas, the gene has registered failure to protect the host against those same particular races. Therefore, where markers are available, this gene should only be included in a breeding programme as part of a pyramid.

This chromosome is responsible for the resistance against anthracnose races 7 and 109 in the Andean genepool. One or both sources of resistance could be associated with the major Co-3 locus on Pv04 whose alleles are *Co-3* from Mexico 222, *Co-3*^{2} from Mexico 227, *Co-3*^{3} from PI 207262 and BAT93, *Co-3*^{4} from Ouro Negro and *Co-3*^{5} from MSU-7 (Sousa *et al.*, 2014). Additionally, the Andean *Co-z* resistance gene has been identified to co-localize with the *Co-3*^{4} (Geffroy *et al.*, 2000). Most of the genes and alleles located on this chromosome have been previously reported from the Mesoamerican germplasm thus having Andean sources of the same gene are crucial in the bean breeding programmes that are focused on the Andean type (Sousa *et al*., 2014).

2.6.4.4 Chromosome Pv08

The chromosome Pv08 hosts the Co-4 locus originally known in Mexique 2 and was first described in the genotype TO. Another locus Co-4^{2} from G_{2333} has been described to offer the

broadest based resistance genes in common bean against anthracnose (Barladin and Kelly, 1998). Additional alleles at the *Co-4* locus identified in the anthracnose differential cultivar PI 20726 are independent of the *Co-1* and *Co-2* loci.

2.6.4.5 Chromosome Pv10.

This chromosome is also considered to have minor resistance to race 7 and 385 (Lopez *et al*., 2003). This region appears to be distinct from the *Co-2* gene cluster that is a common source of resistance to race 7 in Mesoamerican beans (Ferreira *et al*., 2013). Previous studies indicate that this gene co-localizes with the Phvul.011G021500 gene which encodes for Phospholipid scramblase enzyme that results in outward translocation of phosphatidylserine from the cell, a major signal for macrophages to eliminate apoptotic cells that is known to interact with cytochrome C (Collazo *et al*., 2006). Therefore, there is a high probability that this gene plays a complimentary role in resistance to race 7 as the candidate on Pv04 which encodes for cytochrome P450 as both genes are involved in programmed cell death to prevent further spread of the disease as witnessed in the studies on the common bean cultivars Xana and Cornell 49242 on Pv04 and Pv11 (Campa *et al*., 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study locations

The study was carried out at the University of Zambia, school of Agricultural Sciences in Lusaka, Zambia. The isolates in the study were collected from seven districts: Mbala, Kasama, Luwingu, Mporokoso, Lusaka, Mufulira and Mansa. A field survey was carried out in these major bean-growing districts of Zambia located within two agro-ecological zones (II and III). With the exception of Lusaka district, all the other districts lie within Agro-ecological zone III characterised by high annual rainfall in excess of 1000mm, low temperatures during bean growing season averaging 16ºC and a long growing season of 120-150 days. These conditions are conducive for bean anthracnose disease development and spread. Lusaka district lies in Agro-ecological zone II, which is characterised by medium annual rainfall of about 800-1000mm, medium temperatures averaging 23-25ºC and a growing season of 100-140 days (Bunyoro et al., 1995).

Preliminary studies for *C. lindemuthianum* anthracnose race identification in Zambia were done by Sansala *et al.,* (2023) from which seven races were picked based on their virulence and prevalence levels in Zambia as well as their discriminate behaviour on the parents. The selected seven races for this study were 19, 38, 51, 167, 263, 1050 and 1105 which had already been named according to the standardized system of virulence on the 12 differential bean cultivars were selected based on their *C. lindemuthianum* discriminating scores on the parents (Bukoba and Kijivu) as well as their frequency and distribution over the production areas.

3.2 Plant Materials

In this study, a population of 158 $F_{4:5}$ Recombinant Inbred Lines (RILs) developed from a cross between two Andean parents Bukoba (ADP 7) and Kijivu (ADP 33) were used. Both Bukoba and Kijivu are Andean, and were developed in Tanzania. Both Bukoba and Kijivu have type 1 growth habit (bush type). Bukoba has a yellow coloured seed coat while Kijivu's seed coat is purple speckled. The RILs were developed using Single Seed Descent method.

3.3 Disease Sample Collection

A hierarchical strategy was used to collect bean anthracnose diseased leaf samples from the seven districts within two agro-ecological zones. The sampling structure consisted of three hierarchical levels of district, farming blocks within district and farms within farming block.
In the 2017-2018 farming season, bean anthracnose samples were collected from farmer's fields growing both local and improved varieties of common bean in March, 2018. In each district three farming blocks were chosen from which three farms were sampled at a distance of 5-15 km. At each farm, three to four diseased pods or leaves displaying characteristic anthracnose symptoms were picked along a transect of 20 metres interval. The samples were put in paper envelopes, labelled and taken to the University of Zambia Biotechnology laboratory where they were stored at room temperature before isolation of the pathogen.

3.4 Disease Sample Isolation

Following identification of the races of the specific isolates from the study of Sansala *et al.,* (2023), the plant tissue samples were retrieved in the laboratory. The interface of the healthy and diseased parts of the pods/leaves/stems where cut into small pieces and surface sterilized using 70% ethanol, followed by 1% sodium hypochlorite solution for 2 minutes and then rinsed in sterilized distilled water followed by drying on filer paper for 10 minutes.
The diseased tissue pieces were then incubated in Petri dishes with either Potato Dextrose Agar (PDA) 39 g/l or Modified Mathur's agar culture medium made with dextrose (8 g/l), $MgSO4 \cdot 7H2O$ (2.5 g/l), KH2PO4 (2.7 g/l), neopeptone (2.4 g/l), yeast extract (2.0 g/1), and agar (16 g/1). The reason for using either PDA or Mathur's agar was because some isolates sporulated better on PDA while others on Mathur's agar.
The margin of the growing fungus from the tissue on the Petri dish was excised using a cork borer and transferred to a new plate and incubated in the dark at room temperature until sporulation. Single spore/monosporic cultures were made on PDA and Modified Mathur's agar and incubated in the dark for 10-15 days at room temperature.

3.5 Disease Inoculum preparation

When the seven isolates had sporulated, cultures containing the typical morphological characteristics of *C. lindemuthianum* (brownish-white, uniform, circular, fluffy growth in centre with concentric rings and appressed growth at periphery on potato dextrose agar) were used to create a dilute spore suspension for seedling inoculation.

The spore suspension was prepared by flooding plates with distilled water and left to stand for about 30 minutes to allow the culture to soften. The culture was then scraped with a sterile glass rod to dislodge spores into spore suspension. The spore suspension on the plate was then filtered through a double-layered cheesecloth and the concentration adjusted to 1.2×10^6 spores per ml using a hemocytometer. Tween 20 was then added to the inoculum to a final concentration of ~ 0.01% (1 drop of tween 20 from a Pasteur pipet for every 100 ml of inoculum).

3.6 Inoculation and Scoring of RILs

Styrofoam trays containing 60 wells were used. Each well was 5 cm wide, 5 cm long and 5 cm deep. These wells were filled with non-sterile soil in a greenhouse at the University of Zambia in Lusaka. Two seeds of the 158 RILs, parents and checks were planted in each well in a completely randomized design (CRD) and each genotype was replicated thrice (a total of six seeds per genotype per disease race). The checks were G_{2333} (resistant) and Kabulangeti (susceptible). These were watered daily for germination and growth until the primary leaf stage (7-10 day old seedlings) when the 158 RILs, parents and checks were inoculated individually with a standardized spore suspension of a particular race of *C. lindemuthianum* by wetting both surfaces (upper and lower) of the unifoliate leaves and stems until run off using a hand sprayer. After inoculating the plants with a selected pathogenic race among the seven 19, 38, 39,167, 263, 1050 and 1105 in this study, the plants were air dried for a few hours and then transferred to a humidity chamber to meet the conditions of high humidity (>90%) with 12hr light and 12 hr darkness at room temperature (25°C) for 72 hours (3days) for disease infection on susceptible plants.

The plants were then removed from the humidity chamber and allowed to air dry prior to being transferred to the benches in the green house for five days for further disease development.

Disease assessment was done 8-10 days from inoculation on a scale of 1-9 (Barladin *et al.*, 1990) where 1- Plant with no visible symptoms, 2- Plant with few isolated small lesions on mid-veins in the lower leaf surface, 3-Plant with a higher frequency of small lesions on mid-veins in the lower leaf surface, 4- Plant with lesions in the mid-vein and occasionally in secondary leaf veins, 5- Plant with many small lesions scattered on mid- and secondary veins, 6- Plant with many small lesions as described in grade 5 in the lower and upper leaf surface and stem, 7- Plant with many large lesions scattered over the leaf blade and stem, 8- Plant with many large, coalesced lesions accompanied by tissue breakdown and chlorotic or abscised leaf

and stem, 9- Severely diseased or dead plant. In summary, plants that scored 1-3 were considered resistant, those scoring 4-6 were considered moderately resistat while those scoring 7-9 were considered susceptible. For the success of this genetic study, specific phenotypic data of the recombinant inbred lines (RILs) that is directly related to a particular race was used. Such data would not be acquired in nature where a mixture of *C.lindemuthianum* races would be in existence hence the need for a controlled environment.

3.7 Statistical Analysis

Statistical analyses for phenotypic data were conducted in JMP 9.0.0 (SAS Institute, 2010). An independent T-test was conducted on the parents to determine variation in their score responses to the different races of *C lindemuthianum*. Analysis of variance (ANOVA) was done on the RILs to determine if there were significant variation in the score response of the RILs to the different races of *C. lindemuthianum*. The statistical model used was $Yik=\mu+\alpha i+\beta k+\varepsilon ik$, where: *Yik* was anthracnose severity score for the i^{th} genotype and k^{th} replication, αi was the fixed variable effect of the genotype (RIL), βk was the random variable effect of the k^{th} replication and εik was the residual error associated with the k^{th} replication of the i^{th} genotype. Yik was a random variable, which was assumed to be normally distributed with mean equal to 0 (zero).

3.8 DNA Extraction and Genotyping

DNA was extracted from newly emerged trifoliate leaves of each of the 158 $F_{4:5}$ RILs and from 3 plants of each parent and checks which were raised in the greenhouse at Washington State University, Prosser, USA. Using a modified CTAB (Hexadecyl Trimethyl Ammonium Bromide) protocol described by Doyle and Doyle (1987). The CTABextraction buffer was made of 2% CTAB (hexadecytTrimethylammoniumbromide), 100mM TrisHCl (at pH 8), 20Mm EDTA, 1.4M NaCl.

An estimate of 200mg of tender bean leaf tissue samples were individually collected and cryogenically ground into a fine powder with a pestle in a mortar and individually transferred to a properly marked polypropylene tube. 1000 µl of CTAB Buffer were added, mixed properly and immersed in a water bath at 60°C for 30 minutes.

The tubes were then placed in a centrifuge for 5 minutes at 13,600rpm and 500 µl of the supernatant were transferred to a properly marked new tube. Then, 0.8mL of chloroform/isoamyl alcohol (24:1) were added in a fume hood and mixed gently on a rotary

mixer for 15 minutes followed by centrifuging for 5 minute at 13,600rpm to separate the phases. The aqueous upper clear phase was then transferred to a new tube and 50 µl of 3M sodium acetate were added to purify the DNA followed by addition of an equal amount of isopropanol and incubated at -20°C overnight to precipitate the DNA. The samples were then centrifuged at 13,600rpm for 5minutes followed by decanting of the supernatant without disturbing the pellet.

Subsequently, the pellet was dissolved in TE buffer pH of 8and 6 µl of RNaseA were added followed by incubation for 30 minutes to clean out the RNA. The pellet was then washed with 500µl of ice-cold 100% ethanol and incubated at room temperature for 1 hour then centrifuged for 5 minutes to get the pellet free of RNA. The remnant alcohol was further removed by air drying the pellet without drying the DNA and the alcohol-free DNA pellet was then dissolved in 20µl TE buffer (10 mM Tris, pH 8, 1 mM EDTA) and temporarily kept at 4°C for full dissolution of the DNA followed by storage at -20°C. DNA concentrations were measured using a Nano-drop spectrophotometer and DNA quality checked on 1.5%agarose gel.

The DNA samples were then genotyped at Beltsville Agricultural Research Centre, Maryland, USA in the Soybean Genomics and Improvement USDA Laboratory using the Illumina BARCBean6k_3 BeadChip with 12,000 Single Nucleotides Polymorphism (SNP) (Song et al. 2015). SNP genotyping was conducted on the Illumina platform following the Infinium HD Assay Ultra Protocol (Illumina Inc.). An Illumina BeadArray Reader was used to measure the intensity of the fluorescence signal at every marker locus and automatically, SNP alleles were called using GenomeStudio Software from Illumina, Inc. the allele calling data was visually inspected for each locus and the SNPs which were polymorphic between parents were used to construct the linkage map.

3.9 Genetic Map Construction

Out of the 12000 SNPs genotyped, 1,838 were polymorphic between the parents Bukoba and Kijivu and were used to build 11 linkage groups (Pv01-Pv11) for the RILs using the software JoinMap version 4.1 (Van Ooijen, 2011). Linkage maps were drawn using the software MapChart (Voorrips, 2002). The total genetic distance of the 11 linkage groups was 888.2 cM. The average genetic distance per linkage group was 80.7cM. The smallest linkage group was Pv11 with 40.1 cM. and the largest was Pv01 with 106.9 cM. The average number of markers per linkage group was 167. The average genetic distance between markers was 0.5 cM. The number of markers per linkage group ranged from 53 on Pv06 to 262 on Pv10.

3.10 QTL Analysis

For this study, composite interval mapping (CIM) was conducted to map the QTL for resistance to the seven races of *C. lindemuthianum* using the software Windows QTL Cartographer version 2.5-011 (Wang et al., 2012). CIM was conducted using the following parameters: Settings: (i) model 6 (Standard model), (ii) 5 control/background markers, (iii) 10 cM window size, (iv) forward and backward multiple regression model, and (V) 1 cM walk speed (genome scan interval).

A permutation test (Doerge and Churchill, 1996) for races was conducted in QTL Cartographer (1000 permutations) to determine a genome-wide LOD threshold of 3 at $p = 0.05$ was used for declaring a QTL significant. The QTL were named based on guidelines provided by the Genetics Committee of Bean Improvement Cooperative (Miklas and Porch, 2010). The superscript BK in the QTL names represent the mapping population developed from the cross between Bukoba and Kijivu.

CHAPTER FOUR

RESULTS

4.1 Anthracnose Evaluation

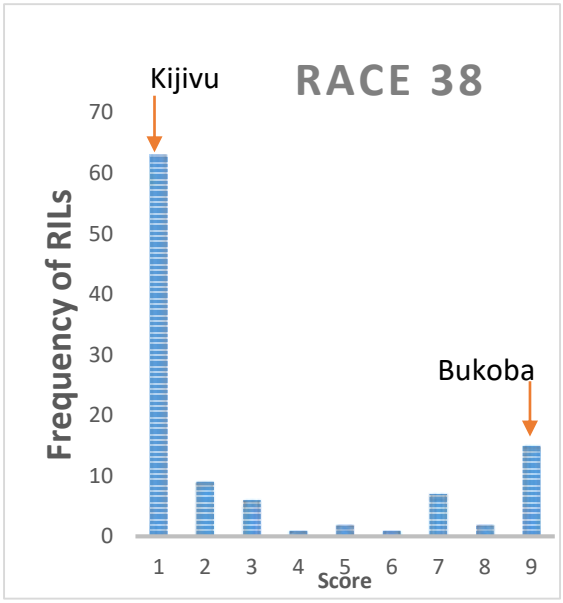
The races showed varying degrees of virulence on parents, checks and the RILs. The isolates were identified as *C. lindemuthianum* based on their typical characteristic of brick red lesions on the plant tissue. Genotypes that scored 1-3 were considered resistant, that which scored 4-6 were considered moderately resistant and those with scores of 7-9 were considered susceptible. The reaction of parents (Bukoba and Kijivu), checks and RILs are shown in table 4.1. Both parents showed varying resistance levels to race 19. Bukoba was distinctively highly resistant (score 1-3) to races 38, 51, 167 and 263 while Kijivu was highly resistant (score 1-3) to races 1050 and 1105 (table 4.1). The t-test results revealed highly significant differences between parents in their reaction to the seven races used in the current study (table 4.1). The checks, resistant (G2333) and susceptible (Kabulangeti) performed as expected except for the races 263 where Kabulangeti was resistant with a score of 1.0. Significant differences ($p<0.001$) in disease severity scores were observed between RILs for all seven races used in the current study (Table 4.1) where transgressive segregation was observed, with several RILs being more resistant (races 167 and 1050) or susceptible (races 19, 1050 and 1105) than either parent (Figure 4.1).

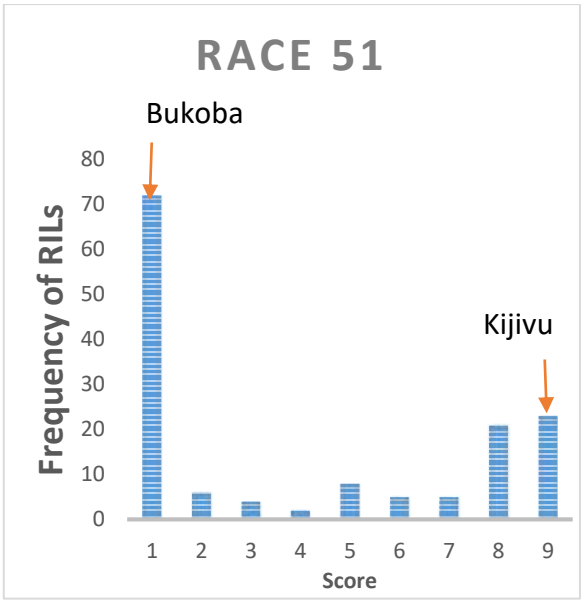
Table 4.1: Means and ranges for anthracnose severity measured on parents (Bukoba and Kijivu), checks (G2333 and Kabulangeti) and 158 recombinant inbred lines inoculated with seven strains of Colletotricum lindemuthianum

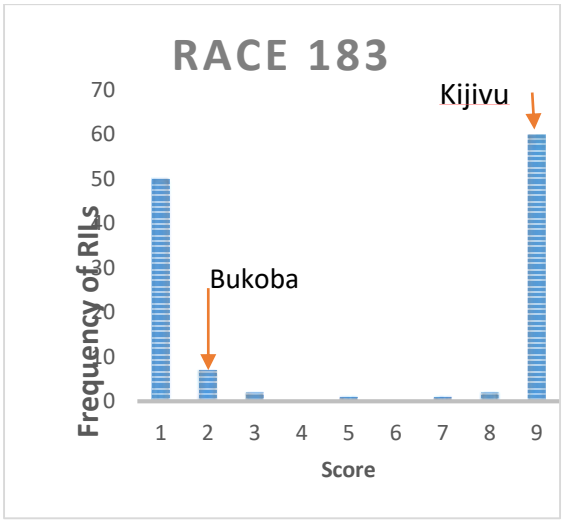
	PARENTS			CHECKS		RILs (158)		
RACE	Kijivu	Bukoba	t-test	G2333	Kabulangeti	Mean	Range	Anova
19	2.2±0.2	1.2±0.3	**	1.0	9.0	3.1±0.1	1.0-9.0	***
38	1.3±0.3	8.75±0.3	***	1.0	9.0	3.2±0.2	1.0-9.0	***
51	8.8±0.2	1.4±0.3	***	1.0	9.0	4.0±0.1	1.0-9.0	***
183	8.75±0.3	1.5±0.3	***	1.0	9.0	5.9±0.2	1.0-9.0	***
263	5.7±0.2	1.2±0.2	***	1.0	9.0	3.2±0.2	1.0-9.0	***
1050	7.5±0.2	1.75±0.3	**	1.0	9.0	3.6±0.2	1.0-9.0	***
1105_	5.0±0.3	1.3±0.2	***	1.0	9.0	2.0±0.1	1.0-9.0	***

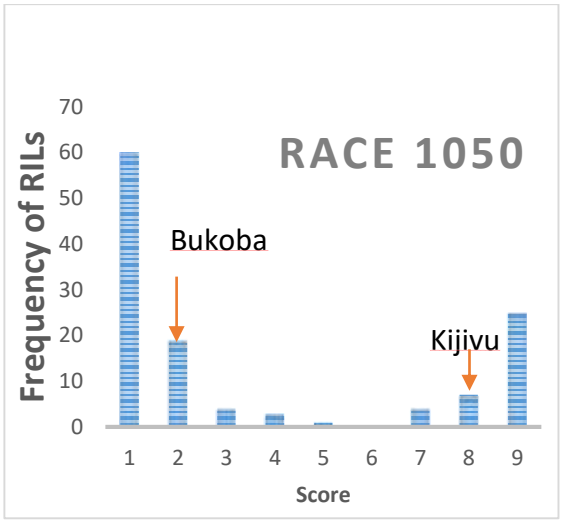
RILs =recombinant inbred lines; G2333 = resistant check; Kabulangeti = susceptible check; ± S.E the Mean; t-test represent the level of significance for the p-value of a t-test between parental means; ANOVA = Analysis of Variance; * = 0.05 (level of significance); ** = 0.01 (level of significance) and *** = 0.001 (level of significance). G2333 = resistant check; Kabulangeti = susceptible check; ± S.E the Mean; t-test represent the level of significance for

the p-value of a t-test between parental means; ANOVA = Analysis of Variance; * = 0.05 (level of significance); ** = 0.01 (level of significance); *** = 0.001 (level of significance).









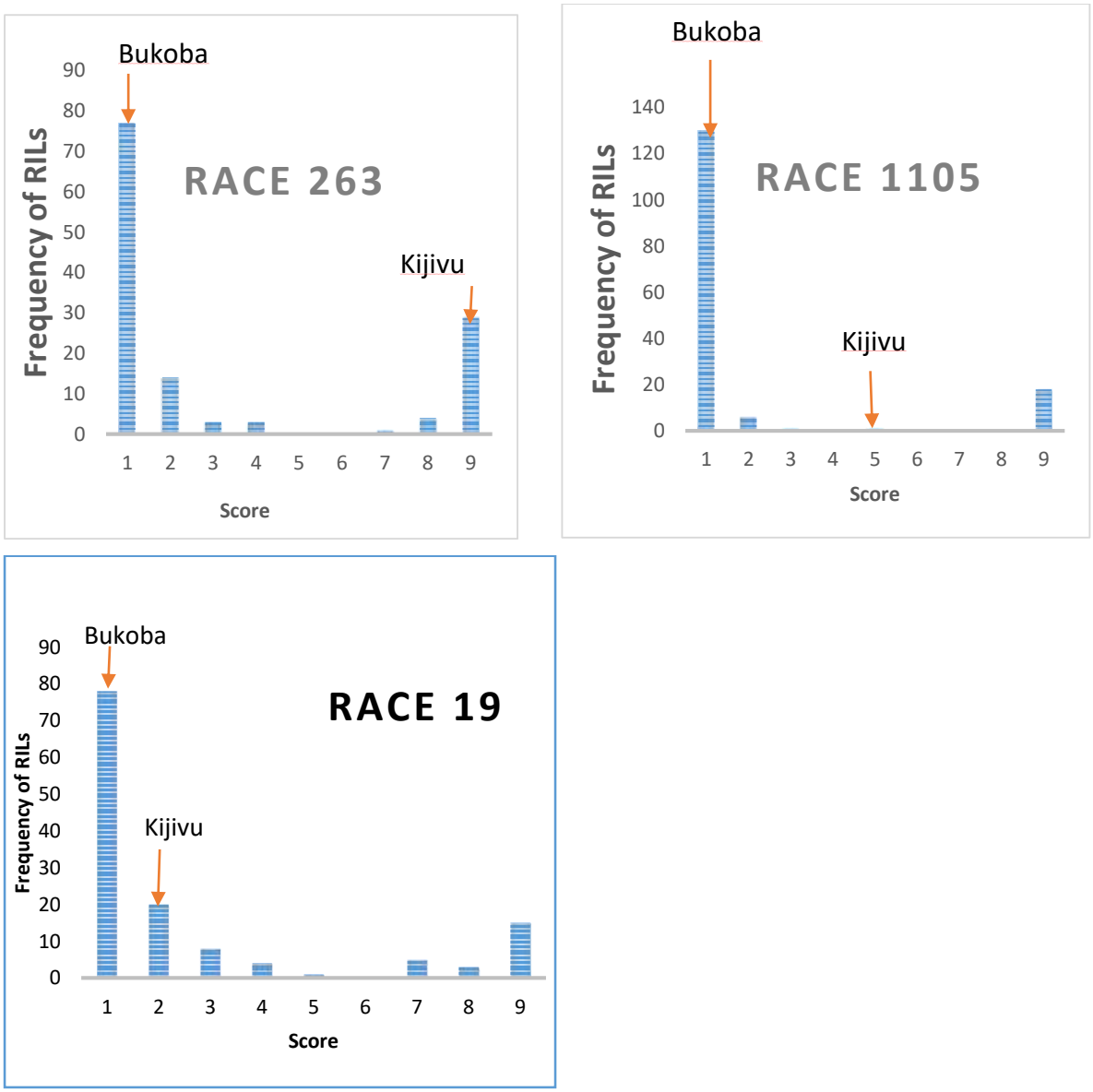


Figure 4.1: Frequency distributions of recombinant inbred lines for severity scores for races 19, 38, 51, 167, 263, 1050 and 1105. The scores for parents Bukoba and Kijivu are also indicated on the graphs.

4.2 QTL Analysis

A total of 12 QTLs for resistance to seven (7) races of *C. lindemuthianum* were identified on seven chromosomes including Pv01, Pv02, Pv 04, Pv05, Pv 06, Pv10, Pv11 (Table 4.2).

Chromosome PV01

A proportion of 1.9%, 3.1% and 0.8% of the variation of the RILs to races 1105, 1050 and 263 respectively was explained by QTL ANT01.1 located on chromosome PV01 and mapped to 2.9 to 50.5 Mbp. The respective R^2 values of the above races were 34.2%, 70.1% and 5.6% with LOD scores ranging from 4.3 to 33.9 depending on the race. This was a major resistance

QTL for both races 1105 and 1050. The source of resistance alleles for races 1105 and 1050 was Bukoba while Kijivu was responsible for the resistance alleles controlling the resistance in race 263.

Chromosome PV02

An estimate of 0.9% of the variation of the RILs to race 38 was explained by QTL ANT02.1 located on chromosome PV02 and mapped to 47.7 to 48.3Mbp with an R^2 value of 7%. The resistant alleles at this locus were contributed by Kijivu.

Chromosome PV04

QTL ANT04.1 was identified on chromosome PV04 and mapped to 0 - 48.3Mbp. This QTL was responsible for resistance to four races 19, 51, 183 and 263 with their respective R^2 values of 14.7%, 27.5%, 10.8 and 25.4% making it a major resistance QTL for all the four races. The LOD score for this QTL ranged from 4.6to 16.2 depending on the race. Bukoba was the parent responsible for all the resistance witnessed at this locus for all the races.

Chromosome PV05

QTL ANT05.1 was identified on chromosome PV05 and mapped to 2.1-2.2Mbp. This QTL was responsible for resistance to race 19 with an R^2 value of 5.2% and a LOD score of 3.1 with Kijivu as the resistant parent contributing the resistance alleles at this locus.

Chromosome PV06

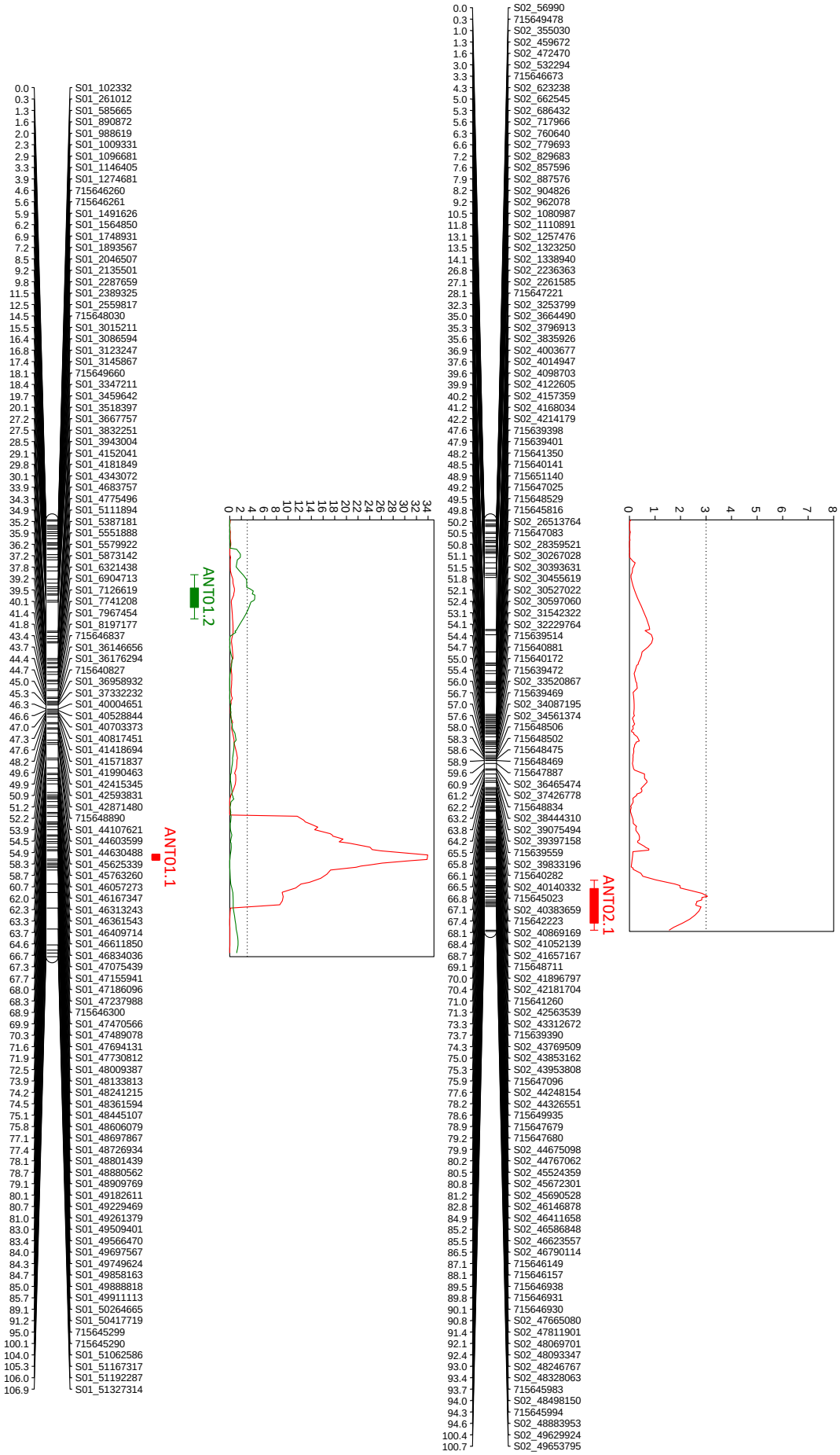
QTL ANT06.1 was identified on chromosome PV06 and mapped to 23.8-24.1Mbp. This QTL was responsible for resistance to isolate Mpika1 with an R^2 value of 3.6% and a respective LOD score of 3.1. Kijivu was the parent contributing the resistant alleles at this locus.

Chromosome PV10

QTL ANT10.1 was identified on chromosome PV08 and mapped to 42.2-42.7Mbp. This QTL was responsible for resistance to 38 with an R^2 values of 7.3 and LOD score of 3.5. Kijivu was the parent contributing the resistant alleles at this minor resistance locus for race 38.

Chromosome PV11

QTL ANT11.1 was identified on chromosome PV11 and mapped to 50.4-51.7. This QTL was responsible for resistance to race 19 with an R^2 value of 7.3 and a LOD score of 3.9 with Bukoba as the parent contributing the resistance alleles at this locus.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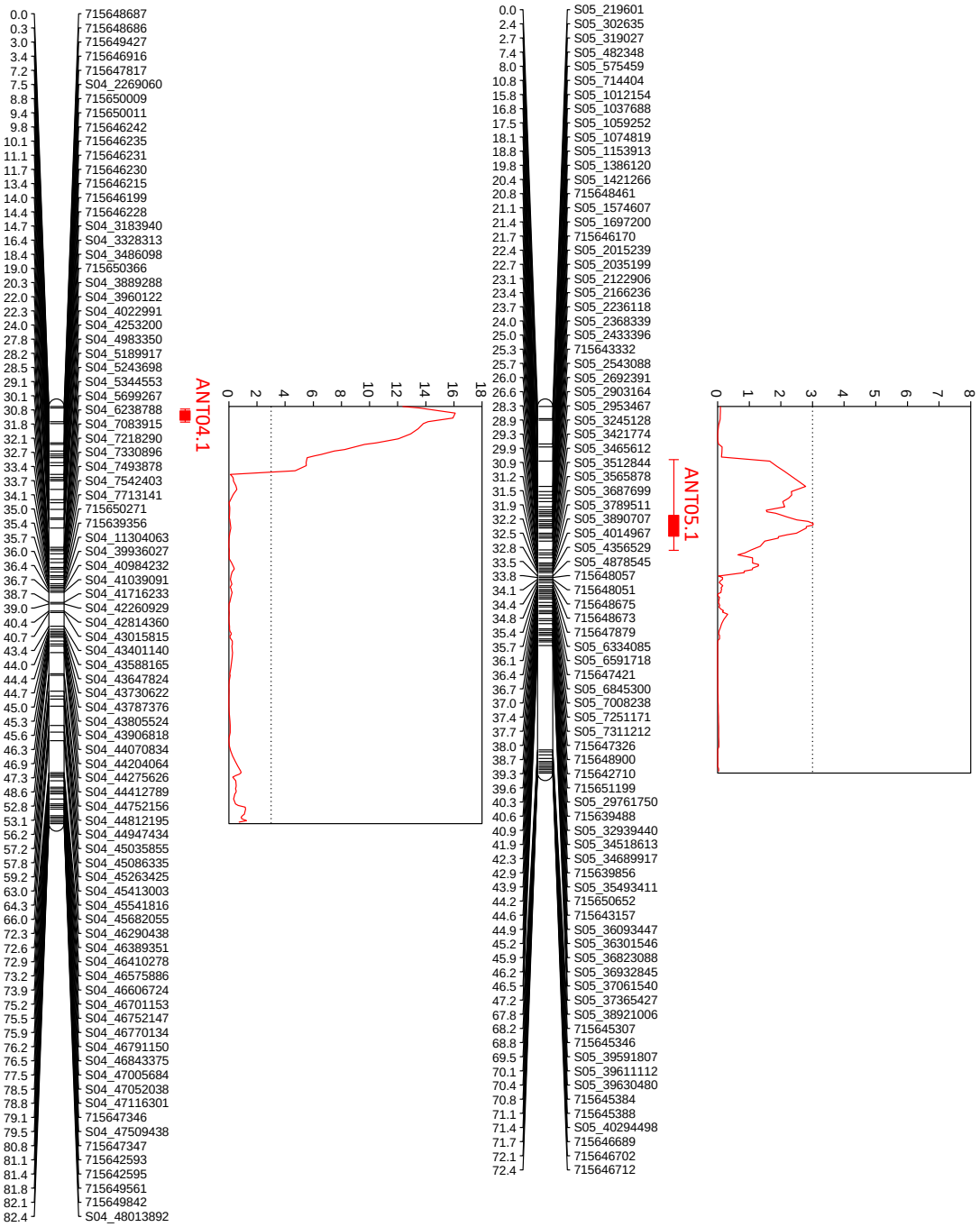
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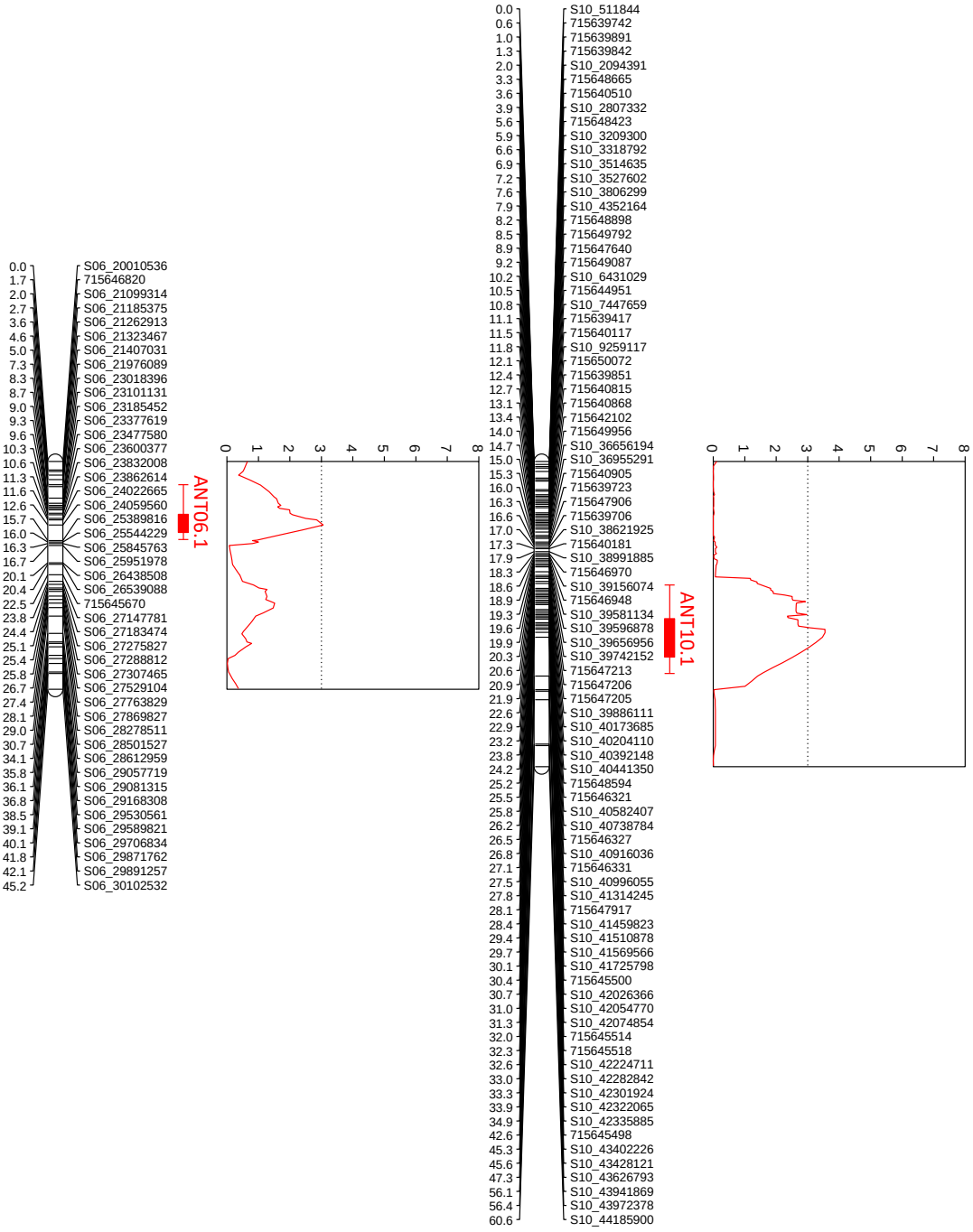
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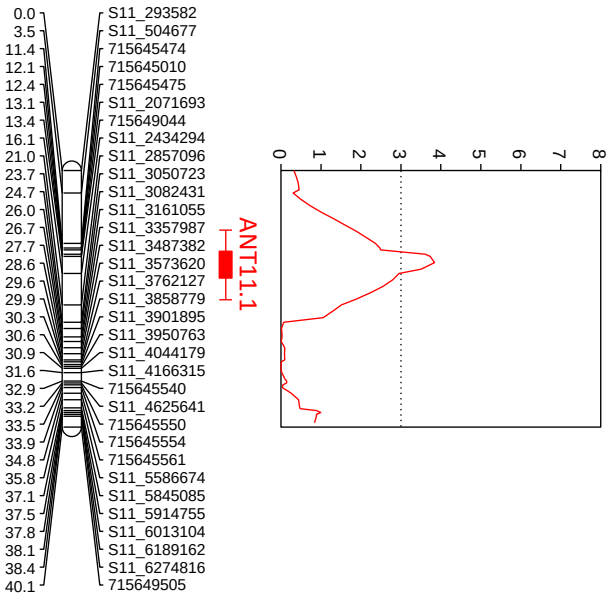

Figure 4.2: Linkage maps on which quantitative trait loci were identified for resistance to C. lindemuthianum in a Bukoba x Kijivu mapping population.

Table 4.2: QTL positions for resistance to Colletotricum lindemuthianum races (19, 38, 51, 183, 263, 1050 and 1105) identified using 155 RILs derived from Bukoba and Kijivu.

QTL Name	CHR	Race/Isolate	QTL Peak (Mbp)	QTL Interval (Mbp)	LOD	R2 (%)	Add	Source
ANT01.1	Pv01	1105	49.7	48.2-50.2	23.1	34.2	1.9	BK
	Pv01	1050	49.5	48.1-50.5	33.9	70.1	3.1	BK
ANT01.2	Pv01	263	3.4	2.9 - 3.5	4.3	5.6	0.8	KJ
ANT02.1	Pv02	38	48.1	47.7-48.3	3.1	7	0.9	KJ
ANT04.1	Pv04	19	0.5	0-2.6	7.8	14.7	1.1	BK
	Pv04	51	1.2	0-2.6	14.6	27.5	1.9	BK
	Pv04	183	0.3	0-2.6	4.6	10.8	0.9	BK
	Pv04	263	0.3	0-2.8	16.2	25.4	1.7	BK
ANT05.1	PV05	19	2.1	2.1-2.2	3.1	5.2	0.6	KJ
ANT06.1	Pv06	263	24.1	23.8-24.1	3.1	3.6	0.7	KJ
ANT10.1	Pv10	38	42.3	42.2-42.7	3.5	7.3	0.8	KJ
ANT11.1	Pv11	19	51.1	50.4-51.7	3.9	7.3	0.8	BK

CHAPTER FIVE

DISCUSSION

The parents Bukoba and Kijivu, check and RILs showed variable resistance to the seven races of *C. lindemuthianum* suggesting that both parents have resistance and susceptible alleles at the anthracnose resistance loci. Transgressive segregation was shown for all races thus proving presence of both susceptible and resistance alleles for both parents. A bi-modal distribution was observed for the races 19, 38, 39, 167, 263 1050 and 1105 (Fig 4.1) confirming a qualitative inheritance to these races within the mapping population.

Although both parents Kijivu and Bukoba were resistant Race 19 with mean scores of 1 and 2 respectively, this race was chosen because it is the most widely spread race found in all bean growing areas in Zambia. Also, the difference in the scores of both parents although resistant, indicates inherent differences in the alleles responsible for resistance in both parents (Figure 4.1). The RILs were transgressive segregants to anthracnose resistance The results in table 4.2 further confirms this by having QTL ANT04.1 and ANT11.1 being responsible for the resistance in Bukoba with additive values of 1.1 and 0.8 while their respective R2 values were 14.7% and 7.3%. This indicates that ANT04.1 is a major QTL thus Bukoba's resistance to race 19 is controlled by both a major and minor QTL. QTL ANT05.1 is responsible for Kijivu's resistance to race 19 with an additive value of 0.6 and R2 value of 5.2 indicating that this particular resistance to race 19 is controlled by a minor QTL only. The resistance for race 38 was from the susceptible parent Kijivu as indicated in both tables 4.1 and 4.2. this means although Bukoba was generally had more resistance QTLs to C.lindemuthianum races, it was devoid of the QTLs ANT02.1 and ANT10.1 which were responsible for the resistance to race 38 that is witnessed in Kijivu.

Protection of crops against pathogens with high virulence diversity like *C. lindemuthianum* needs a broad genetic base thus, the utilization of Andean QTLs identified on PV01 and Pv04 which are major resistance QTLs protecting the bean crop against six races (19,51, 183,263, 10050 and 1105) of C. lindemuthianum out of the seven in this study is especially important given that previous emphasis has been primarily on the use of Meso-American common bean germplasm as sources of resistance.

Two QTLs ANT01.1 and ANT 01.2 were identified on either distal ends of PV01. ANT01.1 conditioned major resistance to races 1105 and 1050 was mapped to 48.1 - 50.5 Mb. This region has been previously reported by Young and Kelly (1997a), Geffroy et al. (2008), Vallejo and

Kelly (2008), Campa et al., (2009) and Ferreira et al., (2013) and based on its relative position, this QTL has been prescribed to contain the anthracnose resistance locus *Co-1*. ANT01.2 provided minor resistance to race 263 was mapped to 2.9 - 3.5 Mb corresponding to the *Co-x*. same region (Vallejo et al., 1992). Geffroy et al., (2008) identified race specific resistance genes in this region with *Co-w* conditioning resistance to isolates 40, 82, 100, and 3616 of *C. lindemuthianum* and *Co-x* conditioning resistance against strain E25 while Pastor-Corrales et al. (1994) identified that the Andean *Co-x* resistance gene conferred resistance to strain 100 which was virulent to G 2333. This *Co-1* locus was first described as the A factor (Burkholder 1918) and (McRostie 1919) and was assumed to be the same gene present the differential cultivar MDRK and *Co-12* from Kaboon (Melotto et al 2000). Within this same region, Campa et al., (2011) identified three race-specific dominant genes for races 31, 81 and 1545 further suggesting that this *Co-1* gene is actually a cluster of closely linked race-specific resistance genes. The relative position of the QTL in this study identifies, Bukoba variety as a valuable source of race-specific resistance to very virulent races 1105 and 1050 of *C. lindemuthianum* making it a potentially important in breeding of common bean genotypes of Andean origin which are of specific market interest in Africa.

The QTL ANT02.1, which provided resistance to races 38 was mapped to 47.7 - 48.3Mbp. This QTL overlapped with previously reported QTL for resistance to anthracnose including QTL ANT02.1$^{UC, SA}$ (Mungalu *et.al*, 2020) which was mapped to 48.0 - 49.0Mbp thus further validating the previously identified QTLs at this locus by Oblessuc et al., 2014 (QTL ANT02.1UC), Geffroy *et al.*, 2008 (*Co-u*). Following a GWAS study, 2 significant SNPs co-localising with ANT02.1 were identified (Zuiderveen et al., 2016) to be overlapping with the *I* gene for resistance to Bean Common Mosaic Virus (Bello et al., 2014; Ferreira et al., 2017). These SNPs were ss715642306 (48 617342 bp) and ss715642306 (48 606517 bp) for resistance to races 39 and 55 respectively. Mungalu *et.al.* (2020) reported this locus to be responsible to five races of *C. lindemuthianum (*38, 39, 45, 55 and 566) indicating a possibility of a cluster of race-specific resistance genes at this locus. The R^2value at ANT02.1 for race 38 was 7% indicating that this was a minor QTL. This contrasts with results by Mungalu *et.al*., (2020) who reported this QTL located at 48.0-48.4 Mbp as a major resistance locus for the same race 38 with an R^2 value of 79%. The difference in these R2 values could be attributed to the difference in the parents and thus the difference in allelic differences of the contributing parents Kijivu in this study verses AO in Mungalu et.al., (2020). Because the resistance locus in this study was mapped near the *I* gene which is known to control resistance of viral diseases to 10 related

potyviruses, 1 comovirus and the bean common mosaic virus (Vallejo et al., 2006), there is a possibility of clustering or co-localization of disease resistance genes on the maps (Marczewski et al. 2006). Meyers et al. (2003) and McHale et al. (2006) both noted that a cluster of resistance genes in plants encode NBS-LRR proteins sequences that are critical in pest and disease recognition. From this study, Geffroy et al., (2007) and Mungalu et al., (2020), it can be assumed that this locus on PV02 is a cluster of race-specific genes due to the so many strains controlled by the same locus and thus a candidate for gene pyramiding in order to build durable resistance against *C. lindemuthianum.*

A major QTL ANT04.1 conditioning resistance to four races 19, 51, 183 and 263 was identified at the distal end of chromosome PV04 and mapped to 0 - 2.6 Mb. This region co-localises with Mungalu et al., (2020) who identified a locus at 1.0 - 2.1Mb which controlled resistance to six races: 19, 49, 55, 530, 566 and 1331 making it plausible for this locus to be a cluster of race-specific resistance genes. Geffroy et al., (2007) further confirmed the complexity of this gene cluster in a study where the same locus conditioned resistance to seven randomly chosen strains from various geographical regions world-over in which some strains showed to have resistance near the *Co-9* Meso-American gene and the *Co-y* gene identified in Geffroy et al., (1999). Molecular studies confirmed this locus as a composition of tightly linked specific resistance genes (Ferreir Cana et al., 2003 and 2005). Two loci located at 0.45 and 0.53Mb responsible for resistance to races 7 and 109 respectively of *C. lindemuthianum* and indicated a possibility of either any or both resistance to be associated with the *C0-3* locus on PV04 (Zuiderveen et al., (2016). The alleles at the *CO-3* locus and their respective members of the differential series have been identified as: Mexico 222 (*Co-3*), Mexico 227 now extant (*Co-3*^{2}), PI 207262 (*Co-3*^{3}), BAT93 (*Co-3*^{3}), Ouro Negro (*Co-3*^{4}) and MSU-7 (*Co-3*^{5} (Meziadi et al., 2016). Studies have mapped the *Co-3*^{4} allele near the genomic marker g2303 located at 3.36 Mb on the physical map thus making this locus to be slightly away from the region identified in this study (Gonçalves-Vidigal et al., 2012). Also, Sousa et al., (2015) identified an Andean-gene *Co-15* on PV04 to be tightly linked to the genomic marker g2685 located at 9.08 Mb which is still very far away from the identified locus in this study.

A minor resistance QTL 05.1 for resistance to race 19 was identified on the distal end of PV05 and mapped to 2.1 -2.2 Mbp. This is a chromosome that has been under studied for resistance factors (Garzon and Blair, 2014).

A minor resistance QTL ANT06.1 for resistance to race 263 was identified on PV06 and mapped to 23.8–24.1Mbp. This is a novel QTL that has not been identified before.

A minor QTL ANT10.1 was identified on Pv10 for resistance to race 38. The QTL was located between 42.2- 42.7Mb closer to the minor QTL on this chromosome reported by Mungalu et. al., (2020) for resistance to race 1331of *C. lindemuthianum* suggesting that this may actually be a cluster of loci conditioning minor race-specific resistance to this pathogen. Currently, no major anthracnose resistance QTL has been reported on Pv10 although a number of minor effect QTLs have been reported (Zuiderveen et al. 2016), (López et al. 2003) and (Wu et al. 2017).

A minor resistance QTL11.01 for resistance to race 19 was identified on PV11 and mapped to 50.4-51.7Mbp. Lopez et al., (2003) earlier supported the existence of a QTL on Pv11 for resistance to race 385 (Cl43) in the Andean accession G19833. However, the locus identified in this study is separate from the *Co-2* gene and that identified by Zuiderveen et al., (2016). The *Co-2* gene, originally known as the *Are* gene, was first described as a single dominant gene conferring resistance against strains alpha, beta, gamma, and delta (Mastenbroek 1960). This cluster of linked resistance genes is linked to the SCAreoli and SQ4 markers (Awale et al. 2008).

The QTL ANT01.1 a major Andean locus was responsible for controlling the most virulent races 1050 and 1105 because the resistance source parent Bukoba is from the Andean genepool. QTL04.1 was responsible for major resistance to four mixed races 19, 51, 263 and 183 this is a major Middle American locus. The resistance from Bukoba on QTLs ANT01.1 and ANT04.1 was sufficient to have significant control of all races except race 38 whose resistance is conditioned by loci found in the susceptible parent Kijivu.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATION

From this study, a total of 12 QTLs for resistance to seven races of *C. lindemuthianum* were identified with some being novel while others overlapped with previously reported QTLs. QTLs ANT01.1 and ANT04.1 were major effect QTLs providing resistance to six out of the seven races under study while the rest were minor effect QTLs. This further confirms the importance of qualitative and quantitative resistance in this BK mapping population for resistance to *C. lindemuthianum.*

Although ANT02.1 was a minor resistance locus, it overlaps with the I gene and has also been reported to condition major resistance to other races of *C. lindemuthianum* not in this study meaning that incorporation of this locus during breeding does not only control anthracnose but also major viral diseases like the common bean mosaic virus.

It can also be deduced that anthracnose resistance genes are organized in groups or gene clusters and are located in specific regions of the genome with each gene conferring resistance to one specific race. The organisation of these closely linked genes into clusters give evidence for recombination between resistance genes that has happened over time.

6.1 Recommendation

In Zambia, the loci ANT01.1, ANT02.1 and ANT04.1 appear ideal for Marker assisted selection as they host major-resistance genes that would be strong targets for gene pyramiding in order to develop Andean common bean varieties with broader resistance to *C. lindemuthianum.* However, further research is needed to validate these identified QTLs and markers directly linked to them need to be developed in order to ease selection. From this study, I recommend that Bukoba can be used as a source of re of resistance for anthracnose especially in the yellow bean class.

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APPENDICES

Appendix 1: Analysis of variance for severity of *C. lindemuthianum* for race 1050 in the study.

Source of variation	d.f	Sum of squares	Mean squares	Fratio	Prob > F
RIL	122	4388.62	35.97	200.25	<0.0001*
Error	257	46.17	0.18		
C.Total	379	4434.79			

Appendix 2: Analysis of variance for severity of *C. lindemuthianum* for race 38 in the study.

Source of variation	d.f	Sum of squares	Mean squares	Fratio	Prob > F
RIL	105	2216.75	21.11	23.32	<0.0001*
Error	111	72.5	0.65		
C.Total	216	2289.25			

Appendix 3: Analysis of variance for severity of *C. lindemuthianum* for race 167 in the study.

Source of variation	d.f	Sum of squares	Mean squares	Fratio	Prob > F
RIL	122	4880.95	40.01	973.9	<0.0001*
Error	213	8.75	0.04		
C.Total	335	4889.7			

Appendix 4: Analysis of variance for severity of *C. lindemuthianum* for race 19 in the study.

Source of variation	d.f	Sum of squares	Mean squares	Fratio	Prob > F
RIL	145	4508.2	31.09	21.96	<0.0001*
Error	260	368.25	1.42		
C.Total	405	4876.45			

Appendix 5: Analysis of variance for severity of *C. lindemuthianum* for race 51 in the study.

Source of variation	d.f	Sum of squares	Mean squares	Fratio	Prob > F
RIL	147	10565.99	71.88	1026.14	<0.0001*
Error	715	50.08	0.07		
C.Total	862	10616.07			

Appendix 6: Analysis of variance for severity of *C. lindemuthianum* for race 263 in the study.

Source of variation	d.f	Sum of squares	Mean squares	Fratio	Prob > F
RIL	130	5238.79	40.3	33.03	<0.0001*
Error	350	427	1.22		
C.Total	480	5665.79			

Appendix 7: Analysis of variance for severity of *C. lindemuthianum* for race 1105 in the study.

Source of variation	d.f	Sum of squares	Mean squares	Fratio	Prob >F
RIL	155	6092.87	39.31	190.13	<0.0001*
Error	761	157.33	0.21		
C.Total	916	6250.2			