IDENTIFICATION AND VALIDATION OF CODOMINANT MOLECULAR MARKERS FOR SELECTION OF ANTHRACNOSE DISEASE RESISTANCE IN COMMON BEAN

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THIS THESIS IS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE MASTERS OF SCIENCE DEGREE IN CROP SCIENCE OF MAKERERE UNIVERSITY

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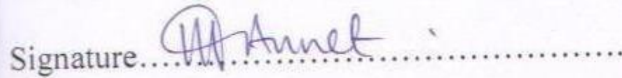
DECLARATION

I, Namusoke Annet, declare that the work contained in this thesis is my original work and it has not been submitted for the award of any degree or its equivalent in this University or in any other institution of higher learning.



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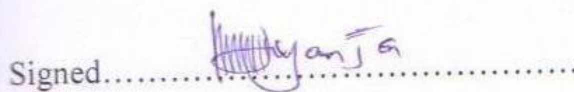

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DEDICATION

To my Dad; Ssetumba Eridard, my husband; Nsereko Innocent and our two sons; Enoch and Elisha

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ABSTRACT

Common bean landrace G2333, of Mesoamerican origin, offers broad resistance to *Colletotrichum lindemuthianum*. The cultivar is pyramided with three genes *Co-4*^{2}, *Co-5 and Co-7.* Of these resistant genes, Co-4^2 has been found to offer the broadest resistance. However, the National legumes breeding program lacks informative and easily assayable markers for Marker assisted selection of *Co-4*^{2} gene. The aim of this study was to identify and validate easily assayable polymerase chain reaction (PCR)-based co-dominant molecular markers for selection of the *Co-4*^{2} gene. The sequence of *CoK-4* gene, earlier on found to be part of the *Co-4*^{2} gene locus was used as a reference and was blasted in the *Phaseolus* gene database to obtain markers close to the gene in a range of + or - 400kbp. Markers were amplified by PCR using primers obtained from database or designed with primer 3 software. Phenotypic segregation of disease resistance in a segregating population was necessary to associate the disease phenotype and the presence of the identified marker alleles. This was done in F_2 populations derived from crosses of G2333 with two susceptible commercial Andean cultivars NABE 13 and NABE 14 with respect to *Colletotrichum lindemuthinum* pathotype, race 7, identified from the study. Results of the polymorphism test indicated that out of 20 identified markers, only two markers, namely BMB 588 and BM 211, behaved co-dominantly. Phenotypic segregation of resistance to susceptibility revealed that duplicate genes controlled disease resistance. Segregation analysis of these two markers tested in the F_2 populations revealed that BM 211 marker did not segregate with disease resistance and could not be used for MAS. However, BMB 588 marker co-segregated with disease resistance with co-dominance of two alleles of 200bp and 400bp. The segregation of BMB 588 marker fitted the expected segregation ratio of 1:2:1 and showed potential for use in marker-assisted selection of the *Co-4*^{2} gene during bean anthracnose resistance breeding.

CHAPTER ONE

INTRODUCTION

1.1. The origin of common bean

Historically dry beans began as one of the weeds in sweet potato and cassava fields in Latin America. After some time, farmers started growing mixtures of bean types as a hedge against drought, disease and pest attacks which later resulted in a wide genetic diversity of beans with a wide variety of sizes, colors, shapes and textures (Purseglove, 1976). The genus *Phaseolus* is now known to comprise over 50 species (Gepts, 2001). Of these, only five of were domesticated and include *Phaseolus acutifolius* (tepary bean), *Phaseolus coccineus* L. (scarlet runner bean), *Phaseolus lunatus* L. (lima bean), *Phaseolus dumosus* Greenman (year-long bean) and *Phaseolus vulgaris* L. (common bean) (Debouck, 2000; Gepts, 2001). Among these species, common bean is the most widely grown, occupying more than 85% of production area sown to all *Phaseolus* species in the world (Singh, 2001).

The crop was domesticated more than 7,000 years ago in Mesoamerica (Mexico and Central America) and the Andean region. It was introduced in sub-Saharan Africa in the 16th century by Portuguese traders (Wortmann *et al.,* 2004). The common bean was introduced to the highlands of Eastern Africa about 400 years ago and the highlands are now a secondary center of genetic diversity (Bellucci *et al.,* 2014).

There are indications of at least two main morphologically distinguishable domestication events for the common bean which in turn gave rise to two main gene pools. These are the large-seeded (> 40 g/100 seeds) beans of Andean origin and small (< 25 g/100 seeds) to medium-sized (25-40 g/100seeds) beans of Middle-American origin (Antonio, 2015). The existence of the two gene pools has been demonstrated by the relationship of seed size (small versus large), the *Dl* genes (*Dl-1* versus *Dl-2*), F_1 hybrid incompatibility, phaseolin seed proteins, allozymes, morphological traits and DNA markers (Singh, 2001; Bellucci *et al.,* 2014). The two gene pools further show landraces of beans that share some agronomic, morphological and adaptive traits but differ from other groups in allelic frequencies of the genes that control differences in those traits. The

Andean and Middle American gene pool are divided into six races where Andean gene pool races include Chile, Nueva Granada and Peru which are all large-seeded constituting of kidney, cranberry and

many snap beans (Phillip *et al*., 2004). Middle American gene pool races include Durango and Jalisco, both medium-seeded and Mesoamerican which are all small-seeded including pinto, pink, black, white, and some snap beans (Singh *et al.*, 2001). Further diversity of beans which was not originally grouped with other races was also found to exist and this composed of Guatemalan climbing beans (Beebe, 2000). For the case of E. Africa, the major commercial seed types that are cultivated and most preferred are red and red mottled and these contribute the highest market share (Namayaja *et al.,* 2001; Katungi *et al.,* 2009; Sibiko *et al.,* 2013). Other important seed types include the navy beans, cream-colored, brown tan, yellow purple, white and black beans.

1.2. Importance of common bean

Common bean is the most important grain legume in the world (Petry *et al.,* 2015). The crop is second only to cowpea in Sub-saharan Africa (Akibode, 2011). Common bean is the most important legume food grown and consumed in Uganda. Over 80% of Ugandan households consume beans every day in both rural and urban areas (Larochelle *et al.,* 2016). Per capita consumption of beans in Uganda is estimated at 9.8kg annually contributing about 12% of total protein and 4% calorie intake consumed per person (Larochelle *et al.,* 2016).

Nutritionists have classified the common bean as a nearly perfect food because the crop has many well-recognized nutritional qualities (Cristiane *et al.,* 2013). The common bean grain has numerous nutritional attributes including high protein content (19-33%), complex carbohydrates (approximately 40%), low fat content, high levels of dietary fibre and a good source of iron, zinc, calcium, thiamine, folic acid and niacin (Jager, 2013). Beans serve as a primary source of dietary protein for low income earners who do not have access to adequate supplies of meat or fish and it is for this reason that they are referred to as a meat for the poor (Akibode, 2011; Romero *et al.,* 2013). In Uganda, beans are estimated to provide about 11.1% and 25.6% of the total calories and proteins respectively per person per day (Larochelle *et al.,* 2016). Beans are therefore vital

for nutritional security and a potential remedy to malnutrition especially for children under five years of age.

Health wise, beans have been found to have nutraceutical properties of decreasing cholesterol and triglycerides which combat and reduce cancers. Beans and other legumes have also been found to be a remedy to cardiovascular and diabetic problems and because of these remedies, beans have been considered as medicinal plants in some pharmaceutical industries (Romero *et al.,* 2013; Zhu *et al.,* 2015).

The common bean is also a crop of high commercial value where millions of small-scale farmers in Africa rely on the production and sale of beans in local markets and urban areas to provide an important source of household income (Sibiko *et al*., 2013). Beans offer good prospects in export markets providing opportunities to earn foreign currency (Mauyo *et al*., 2007). In addition, beans are major contributor to the world trade in pulses and have an important market niche in urban areas. The crop serves as an important source of income for many Ugandan farmers and traders, due to the increasing demand both in the domestic and export markets such as Congo, Sudan and Kenya.

Concerning the farming system, common bean has been described as a very important crop in Sub-Saharan Africa which contributes a great deal in improving and sustaining soil fertility. Common bean has ability as legumes to fix nitrogen in the soil and because of this, the crop is very useful in crop rotations (Stagnari *et al.,* 2017). The crop residues are very important in making compost manure and important poultry and animal feeds.

Beans thus offer a great potential to contribute towards achieving the new sustainable development goals on food security, nutrition, health and poverty eradication. The importance of beans therefore shows that putting efforts to improve the crop yield potential through increased investment in bean research could contribute to significant improvements in the health and well-being of many Ugandans.

1.3. Common bean production

By 2014, annual worldwide production of dry beans was estimated at 23,139,004 tons on almost 19.98 million hectares of land (FAOSTAT, 2014). Continent wise, Asia had a share of bean

production of about 44.6% followed by America with a share of 35%. Africa contributed 17.7% while Europe and Oceania contributed 2.6% and 0.2% respectively (FAOSTAT, 2014). The world leader in production of dry beans is Burma with annual production of 3,800,000 tons followed by India with average annual production of 3,630,000 tons while Brazil took the third position with average annual production of 2,936,444 tons (FAOSTAT, 2014).

According to FAOSTAT (2014) data, about 75% of the total dry bean production in Africa is from E. Africa where Tanzania is ranked highest in Africa and E. Africa with annual production of 1,150,000 tons, followed by Kenya with annual production of 529,265 tons. Uganda takes the third position in E. Africa with annual production of 461,000 tons. Rwanda and Burundi come last in E. Africa with annual production of 438,236 tons and 205944 tons respectively.

Total bean output for Uganda was increasing between 1997 and 2002 from 221000 tons to 535000 tons respectively (FAOSTAT, 2014). This was due to the availability of disease resistant bean varieties that were introduced by NARO during that period (Waluse, 2012). However, there was a great decline in bean production between 2002 to 2011 from 535000 to 4474430 tons (FAOSTAT, 2014) which resulted in a general decline in domestic food supply per capita during the same period. Decline in production was attributed to several biotic and abiotic factors.

The most widely distributed abiotic constraints to common bean production are low soil fertility, particularly low levels of nitrogen, phosphorus and zinc as well as aluminum and manganese toxicity (Singh, 2001). Drought is also among the most important abiotic factors which affect bean production in major bean growing regions of the world. Drought is capable of causing 100% crop failures in some regions when un- tolerant cultivars are planted (Singh, 2001). Biotic constraints to bean production include disease pathogens and insect pests (Buruchara et al., 2010). Breeding programs thus aim at development cultivars with both abiotic and biotic tolerances in order to address the problem of associated yield losses that underrate common bean production (Miklas *etal*.,2006).

1.4. Marker assisted breeding

Marker-assisted selection (MAS) is a method whereby a phenotype is selected based on the genotype of a marker (Collard *et al*., 2005). Selection of lines with target traits is done indirectly

using molecular markers that are closely linked to underlying genes or that have been developed from the actual gene sequences (Xu and Crouch, 2008). This enables the precise identification of a genotype with a specific trait without the confounding effect of the environment thereby increasing heritability (Fregene *et al*., 2006). With MAS, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs of interest (Collard *et al*, 2008).

Marker-assisted breeding greatly increase the efficiency in plant breeding compared to conventional breeding since conventional methods require hundreds of populations which makes them time-consuming and expensive (Collard *et al*., 2005). MAS can also enhance simultaneous introgression of multiple resistance genes into a cultivar which can be impractical with conventional methods which requires screening the lines for many pathogenic races. Application of MAS can avoid the transfer of undesirable or deleterious genes, which increases precision and ultimately reduces the time required to develop a new variety (Ragagnin *et al*., 2009; Costa *et al*., 2010). Markers can also be used to select for traits that are difficult to manage through conventional phenotypic selection because of being difficult to measure, have low penetrance or with complex inheritance. Maintenance of important recessive alleles during backcrossing can also be easily catered for by MAS (Collard and Mackmill., 2008).

However, marker assisted breeding should only be employed using markers that are tightly linked to the genes of interest to avoid loss of the target traits during the selection process. (Yu *et al*., 2000).

Overtime, markers tightly linked to common bean diseases have been reported (Haley etal., 1994, Kelly and Vallejo, 2004, Maricilia, *eta*l., 2000, Sartorato *etal*., 2000). These markers provide an opportunity to enhance disease resistance through marker assisted selection and gene pyramiding. MAS has demonstrated efficiency when breeding for resistance in different regions of the world (Haley etal., 1994, Pastor-corrales, 2003, Blair *etal*., 2006., Miklas *etal*., 2006., Garzon *etal*., 2008, Pastor-Corrales *etal*., 2007, Oliveira etal., 2008). Luseko *etal*., 2013 reported successful incorporation of resistant genes for angular leaf spot and bean common mosaic necrotic virus into adapted market class, farmers and consumers preferred bean genotype using marker assisted selection by the use of MAS in Tanzania. In Kenya, Mwangi, 2014 was able to

use markers to pyramid genes for resistance to angular leaf spot, anthracnose, root rots and bean common mosaic virus into susceptible commercial cultivars. In Uganda, MAS has demonstrated efficiency in pyramiding resistance genes for anthracnose and root rot (Kiryowa e*tal*., 2015). Also resistant pyramided lines for anthracnose, angular leaf spot, root rot and bean common mosaic virus were successfully developed in Uganda using marker assisted selection. (Okii *etal*., 2017). Use of molecular markers linked to resistance genes should therefore be employed as an important alternative technique to ease and speed up the selection of resistant cultivars (Gou-Liang, 2013; Xu and Crouch, 2008).

However, the benefits of using MAS need to be critically compared to those achieved or expected from any existing conventional breeding methods. Although conventional breeding methods have several limitations, they have been used by breeders to successfully develop good varieties. Application of molecular marker tools should therefore not replace conventional breeding methods completely. The two approaches should instead complement one another so as to achieve the benefits of both tools in crop breeding programs (Muhamba *etal*., 2013).

1.5. Problem statement

Bean anthracnose is one of the most destructive diseases to bean production especially in cool and wet production areas of tropical and temperate regions. Use of resistant cultivars is the most effective, economic and practical way to manage the disease. However, the conventional methods of selection for resistance to diseases based on phenotypes are limited by the lengthy screening procedures, reliance on favourable environmental factors and being unreliable for some traits. .

Molecular markers (Marker-assisted selection: MAS) linked to resistance genes ease and speed up screening for resistance. However, there are no molecular markers that fulfill all requirements needed by researchers. For example Markers that are appropriate for MAS in one breeding program may not be suitable for another breeding program because of problems such as lack of polymorphism, robustness and reproducibility. Several molecular markers identified to date are insufficient to monitor all the useful resistance genes in *P. Vulgaris* and their use requires validation outside the mapping population.

Markers such as SH18, SAS13 and SSB14 have been developed for selection of the *Co-4*^{2}, the broadest gene for resistance to *C. lindemuthianum* in *P.Vulgaris* (Kelly and Vallejo, 2004). However SH18 and SAS13 are dominant markers and cannot discriminate between heterozygous and homozygous plant lines. SBB14 has been reported as codominant marker but it is hardly reproducible and it is not robust for polymerase chain reactions. There was, therefore, a need to identify new and more informative co-segregating polymorphic marker(s) that can be used in marker-assisted breeding for anthracnose resistance. This study therefore targeted the identification and validation of polymorphic InDel, SSR and STS markers for potential use in selection of anthracnose disease resistant lines.

1.6. Justification of the study

The control measures of bean anthracnose include regular spraying with fungicides, planting of disease free seed, removal of plant debris, crop rotation and breeding for resistance (Mohammed, 2013). Fungicide use is expensive, not user-friendly to farmers and leads to environmental damage. Other control measures are not easily adoptable to farmers thus making them inappropriate for management of anthracnose. Use of resistant cultivars is apparently the most appropriate strategy to enhance the crop's productivity through controlling pests and diseases (Oliviera *et al.,* 2008).

The development of anthracnose resistant bean varieties is highly challenged due to high pathogenic variability coupled with occurrence of newly evolving virulent pathotypes which overcome the resistance in the released cultivars (Mahuku *et al.*, 2002). Breeders should therefore continuously breed for new varieties to avoid decline in bean production. However, the phenotypic conventional methods of selection for resistance to diseases are slow, due to reliance on environmental factors. In addition, these phenotypic methods of selection are not feasible for introgression of some traits. This delays bean breeders in development and dissemination of the anthracnose resistant and adapted cultivars to bean growers. Therefore, tools usable by breeders for speeding up the breeding work should be employed.

Use of molecular markers shorten the time needed for variety development thus enhancing their availability to farmers in a shortest time possible. Use of molecular markers is therefore a technology worthy of incorporation in breeding programs for better gains.

1.7. General objective

To contribute to the development of anthracnose resistant bean varieties through identification and validation of molecular markers that can be used to select for anthracnose resistance genes.

1.7.1. Specific objectives

1. To identify polymorphic codominant markers from *Phaseolus* gene database that can be used for anthracnose resistance selection.
2. To test the co-segregation of anthracnose disease resistance and the polymorphic marker(s) and determine their utility in selecting anthracnose resistant lines.

1.8. Hypotheses

i. There are polymorphic co-dominant markers identifiable from Phaseolus genes database.

ii. The co-dominant markers co-segregate with anthracnose disease resistance in common bean.

CHAPTER TWO

LITERATURE REVIEW

2.1. Importance of anthracnose disease to bean production

Anthracnose is a serious seed-borne fungal disease of common bean. The disease causes losses of up to 100% particularly on susceptible varieties under favorable conditions and especially when infected seed is used (Mohammed, 2013). For the case of Uganda, bean anthracnose has been reported to cause up to 70% yield loss (Nkalubo *et al*., 2007).

The spread and increased effect of anthracnose disease in Uganda has been enhanced by the fact that anthracnose is a seed borne disease and yet there is no emphasis of use of certified seeds. For most farmers, the seed is retained from previous season, purchased from local produce markets and sharing of seed with fellow farmers (David, 2004; Mastenbroek and Ntare, 2016). Diseased seed serves an important role in the long distance distribution of the anthracnose pathogen (Mohammed and Somsiri, 2005). In Uganda, the disease is particularly important in the high-altitude areas including Kabale, Kapchorwa, Mbale, Busenyi and Kisoro districts that are characterized by cool temperatures and high relative humidity yet these are the major bean growing regions in the country. Of recent, bean anthracnose disease has also been seen to exist in farmers' fields in low altitude areas and this might be due to adaptation of the disease-causing pathogen.

Bean anthracnose disease reduces bean production due to poor seed germination, poor seedling vigor, and low yields. There are also marketing losses which are attributed to seed spots and blemishes which lower their quality rating and salability (Kelly and Vallejo, 2004). Because of its importance, bean anthracnose is a major subject of research by several national bean programs in Africa with the objective of developing technologies for combating the disease.

2.2. Classification of the causative agent of bean anthracnose

Colletotrichum lindemuthianum (Sacc. and Magn, Scrib) is known to be the causal agent of anthracnose disease of legumes including common beans (*Phaseolus vulgaris* L.), climbing bean (*Phaseolus coccineus*), lima bean (*Phaseolus lunatus*), tepary bean (*Phaseolus acutifolius*), cowpea (*Vigna unguiculata*) and broad bean (*Vicia faba*) (Mohammed, 2013). However, the

major host of *C. lindemuthianum* is common bean whereas the rest of the bean types are slightly susceptible (Mohammed, 2013).

Anthracnose disease causing agent presented a lot of difficulty in taxonomical grouping and naming to several scientists since it can form acervuli with or without fruiting bodies depending on the quality and amount of substrate onto which it grows (Mohammed, 2013). After several taxonomical names were given to anthracnose disease causing agent by several scientists, it was found out that the fungus could exist in both imperfect and perfect forms which were confirmed as *Colletotrichum lindemuthianum* and *Glomerella cingulata* f.sp *phaseoli* respectively (Martínez-Pacheco *et al*., 2009). The pathogen's perfect stage*, G. cingulata* reproduces sexually and has perithecia containing asci and ascopores and it is very rarely observed in nature (Camargo *et al.,* 2007). The pathogen commonly exists in nature in imperfect stage, *C. lindemuthianum*. In the imperfect stage, the reproduction of the pathogen is asexual and the spores are produced inside acervuli and immerse in water-formed mucilage (Mohammed, 2013). *Colletotrichum lindemuthianum* forms dark brown oval shaped conidia on growth medium (Mwesigwa, 2008) and on the host, it forms spots which turn brown and black specks. Each of the black specks contains a mass of pinkish spores (Mohammed, 2013). *C. lindemuthianum* differs from *G. cingulata* by its slow growth and a dark pigmentation on culture media and is characterized by hyaline to grey hyphae which become dark with very compact aerial mycelium on maturity (Mota *et al.,* 2016).

Taxonomically, the pathogen belongs to the genus *Colletotrichum*, Family, Melanconiaceae; Order, Melanconiales; Sub Class, Coelomycetidae; Class, Deuteromycetes; Sub Division, Deuteromycotina; Division Amastigomycota; Kingdom Myceteae; Super Kingdom, Eucariota(Mohammed, 2013). Characteristics of *C. lindemuthianum* on growth media and its conidial appearance should clearly be identified to avoid confusion of the pathogen with other fungi that show similar growth patterns during its isolation and inoculum preparation.

2.3. Symptoms and epidemiology of anthracnose disease

Anthracnose is a seed borne disease and the causative pathogen is primarily spread by infected seed. The disease can move into new fields with infected seed which leads to the introduction of new races of the pathogen into different geographic regions (Tesfaye, 2003). The infested seeds

give rise to diseased seedlings that act as a source of inoculum of the anthracnose fungal spores that are spread to adjacent plants by splashing rain, wind, humans or contaminated machinery and equipment (Markell *et al.,* 2012).

Disease development is favored by relatively cool and humid conditions. Conidia production which later leads to plant infection is always favored by temperatures between 17 to 24°C and relative humidity greater than 92% (Silver *et al.,* 2013; Padder *et al.,* 2016). Frequent rainfall is the most critical environmental factor needed for the development and spread of anthracnose. Free moisture on the plant surface is needed for spores (conidia) to infect the plant and cause a lesion and splashing is needed for spore dispersal (Rio and Bradley, 2002). High temperatures above 30°C temporarily will stop fungal growth. However, the high prevailing temperatures may not limit the infection and disease spread provided sufficient precipitations occur during this period. Under favorable environmental conditions, the time from infection to production of new spores is 5 to 7 days (Markell *et al.,* 2012) and during this period, pathogen infects all aerial parts like leaves, stems and pods (Mohammed, 2013).

Severe symptoms develop on the underside of the leaf and appear as dark brown-black slender lesions that follow the leaf veins (Buruchara *et al*., 2010). Older lesions may appear sunken and can occur on both sides of the leaves. On petioles and branches, lesions are brown-black and are commonly slender to oval-shaped. As the disease progresses, discoloration appears on the upper leaf surface and heavy infection of the leaves often results in early defoliation. On stems, an early sign of infection is dark brown eye-spots that develop longitudinally. On young seedlings, bean anthracnose infection may cause the stem to rot and die prematurely (Buruchara *et al*., 2010).

The most striking symptoms develop on the pods. When the pathogen infects the pods, the symptoms begin with rusty-brown spots that later will appear as circular and sunken cankers about 1/8 inch in diameter, although the size of lesions can vary significantly (Figure.1). The margins of the cankers are slightly raised, well-defined black ring often surrounded by a thin red-brown halo. The interior of the lesion will appear light to dark brown (Mohammed, 2013). When lesions are wet for a prolonged period, white to salmon-colored spore masses may appear as ooze in the center. When spore masses dry, they may appear as gray to black grains of sand. When

pod infection is visible, the seeds likely are infected (Markell *et al*., 2012). When seeds are infected in early development, they may be shriveled, discolored, and have dark brown to black blemishes and sunken lesions. When it is moist, white fungal growth may be visible on infected seeds. However, seeds infected late in development, may not express any visible symptoms of the disease.

Seedlings grown from anthracnose-infected seeds often have dark brown to black sunken lesions on the cotyledons and stems. Severely infected cotyledons die prematurely. Growth of the plants is retarded and diseased areas may girdle resulting in the death of the plant. Spores produced on cotyledon and stem lesions may also spread to the leaves (Kelly and Vallejo, 2004).

After harvesting, infected and overwintered crop residues can act as a source of field infection in the following seasons. The *C. lindemuthianum* pathogen is able to overwinter in infested crop residues for up to two years and can also survive in infected seed for up to five years. Infections resulting from infected seed may be apparent at emergence, while infection occurring from residue may not appear until favorable conditions like free moisture and cool to moderate temperatures are available (Rio and Bradly, 2002).

Figure 1. Anthracnose symptoms on leaves and pods of susceptible market class bean cultivar, NABE 14.

2.4. Pathogen diversity and race designation

Colletotrichum. lindemuthianum pathogen has many races that vary from country, region, location and variety (Mohammed, 2013) and because of this, new races are continuously reported. The continuous development of new races is due to sexual reproduction, mutations and other chromosomal variations that occur in the pathogen (Ishikawa *et al.,* 2012). Different pathotypes differ in pathogenicity.

Barrus (1911) gave the first report of *C. lindemuthianum* diversity and he and reported the existence of α (alpha) and β (beta) races. Later another report of existence of the δ (delta) race was given (Hubbeling, 1957). Tu (1992) reported the existence of different physiological races of *C. lindemuthianum* including α, β, δ, ε, γ, κ and λ. Previously, many bean cultivars were used as differentials for *C. lindemuthianum* race designation (Vallejo and Kelly, 2004). More confusion came in when many different systems of race identification were used. Because of this confusion, some anthracnose races were known by different names depending on the differential cultivars used and the system of nomenclature employed (Vallejo and Kelly, 2004). However, it was noted that some races remain un- characterized since they cannot fit into either of these groupings (Ombiri *et al*., 2002).

Scientists later came up with a system of *C. lindemuthianum* race designation by the use of a set of twelve international common bean differentials and binary coding system to ease information and germplasm exchange (Kelly and Vallejo, 2004). With the utilization of the twelve differential cultivars coupled with binary system for race designation, it is possible to characterize all to the existing races by designating them Arabic numerals. This method of *C. lindemuthianum* race identification that was agreed upon by the scientists is called the binary system.

The binary system designates a race after summing up all binary values for cultivars which show susceptible reactions to a given race. A binary number is equal to 2^n, where n is the place of the cultivar within the differential series order (Table 1). For example, the position of Perry marrow is 2 and that of Widusa is 4. The sum of all binary numbers of cultivars with susceptible reactions will equal to 2n + 2(n+1) +2(n+2) +……. 2(n+11); where n=0 gives a specific race number or name. This method is currently used by all other researchers over the world in

identifying races of *C. lindemuthianum* (Kelly and Vallejo, 2004). The standard system of race designation is very useful in giving similar grouping of the pathogen across different regions of the world. This is important to breeders when exchanging germplasm and information.

Since then, researchers have continuously reported new pathogenic variations and new races of the pathogen in Europe, United States, Latin-America, Asia and Africa. In East Africa, twelve isolates were collected from different regions of Burundi and these were characterized into nine races and of these, two had already been reported there while seven were reported for the first time in the country (Bigirimana *et al*., 2000). Musyimi (2014) collected thirty one *C. lindemuthianum* strains from western rift valley, central, eastern and coastal regions of Kenya. These isolates were characterized into twelve pathogenic races and of these, seven (1, 2, 17, 23, 38, 55 and 485) had been previously identified while five (65, 73, 81, 87 and 89) were new. She reported races 65 and 73 to be most frequent in Kenya. Of recent, Kazimot (2016) collected thirty two isolates from five districts of Tanzania and these were characterized into five races. Amongst the isolates she collected, she identified race 161 which had not been reported by earlier studies in Tanzania or elsewhere in the world.

In Uganda, Leaky and Simbwa-Bunya (1972) reported the existence of *C. lindemuthianum* races that conformed to earlier classification into α, β, δ and γ races. Nkalubo (2006) confirmed presence of races δ (23), β (130) and γ (102) that earlier had been reported by Leaky and Simbwa-Bunya (1972). In addition, Nkalubo (2006) also reported five new races that had not been previously documented which he attributed to introduction into the country of already infected seed and/or due to pathogenic variability of *C. lindemuthianum*. He reported race 767 to be the most widespread and virulent of all the races. Mwesigwa (2008) collected forty seven *C. lindemuthianum* isolates from five districts of Uganda which he characterized into twenty one races and of these, none of them were similar to earlier studies of Leaky and Simbwa-Bunya (1972) and Nkalubo (2006). Kiryowa (2015) collected fifty one pathogen isolates from eight districts of Uganda and when these were inoculated onto the standard differentials, he characterized them into twenty seven races. Only three races in his study were similar to races 0 and 128 characterized by Mwesigwa (2008) and race 767 reported by Nkalubo (2006). Kiryowa (2015) also reported the emergence of new virulent races 2479 and 4095 that overcame the

resistance in cultivar G2333. These highly virulent pathotypes that have been reported to overcome resistance in cultivar G2333 in Uganda have not yet been reported elsewhere in the world (Mwesigwa, 2008; Kiryowa, 2015).

The tremendous variability of the pathogen has contributed to economic importance of anthracnose in many countries and has resulted in continuous breakdown of resistance in commercial cultivars. In addition, this great genetic diversity of the pathogen has complicated the use of host resistance genes (Melotto *et al*., 2000) and has made it difficult to implement effective anthracnose control strategies (Mahuku and Riascos, 2004). The great variability of *C. lindemuthianum* pathogen thus calls for continuous monitoring of new pathogen races and identification of new sources of resistance for the continuous anthracnose resistance breeding.

Table 1. Description of the 12 standard differentials for *Colletotrichum lindemuthianum*

Differential cultivar	Known resistance genes	Place of cultivar in the series	Gene pool	Binary number	Growth habit
Mitchelite	Co-11	0	MA	1	II
MDRK	Co-1	1	A	2	I
Perry Marrow	Co-13	2	A	4	II
Cornel 49242	Co-2	3	MA	8	II
Widusa	Co -15 , Co3^{3}	4	A	16	I
Kaboon	Co-12	5	A	32	II
Mexico 222	Co-3	6	MA	64	I
PI 207262	Co -4^{3},Co-3^{3}	7	MA	128	III
TO	Co-4	8	MA	256	I
TU	Co-5	9	MA	512	III
AB 136	Co-6, Co-8	10	MA	1024	IV
G 2333	Co-4^{2}, Co-5, Co-5^{2}, Co-7	11	MA	2048	IV

N.B Growth habit: I: Determinate; II: Indeterminate bush; III: Indeterminate bush with weak main stem and prostrate branches; IV**:** Indeterminate climbing habit. MA: Middle American

gene pool; A: Andean gene pool of *Phaseolus Vulgaris*. The binary number is equivalent to $2^{\mathbf{n}}$, where n is the place of the cultivar within the differential cultivars' series (0-11). The sum of cultivars with susceptible reaction will give the binary number of a specific race. For example if the pathogen is virulent to cultivars Perry marrow and Widusa, then its race will be 20. That is, Perry marrow ($2^n = 2^2=4$) and Widusa ($2^n =2^4=16$). The race of the pathotype =4+16=20.

2.5. Bean anthracnose disease management

Bean anthracnose management calls for a combination of strategies since there is no single effective method. The following are some of management strategies of bean anthracnose disease.

2.5.1. Soil solarization

Soil solarization is a practice used to manage weeds, nematodes, fungal diseases and insect pests in soil or soil surface. *Colletotrichum lindemuthianum* spores that disperse onto the soil surface and bean debris from the previous seasons can be managed by this method. The previous bean field is covered with transparent plastic paper which allows sunlight to pass through and heat up the surface to temperatures that are lethal to the *C.lindemuthianum* spores. Soil solarization has been identified as an environmentally sound, pesticide free, low cost method of controlling a wide variety of soil-borne fungal plant pathogens, parasitic nematodes and weeds (Mohammed *et al.,* 2013). Soil solarization can be one of the useful methods to control this fungus in farmers' fields.

2.5.2. Crop rotation

Anthracnose has been shown to overwinter on crop residues in fields where beans are continuously cropped since previous seasons' inoculum can initiate epidemics of anthracnose (Mohammed, 2013). The pathogen spores can survive on infested residue for up to two years, although persistence of the pathogen may be longer in dry environments. Rotation with non-host crop species may reduce the development of bean anthracnose mainly due to the reduction of initial infection that arises from the initial inoculum source (Rio and Bradley, 2002). Additionally, dry beans should not be planted into fields adjacent to a field infected in the previous year since the inoculum can easily be spread between adjacent fields with the help of wind. Rotation of beans with other crops for at least three years is an affordable control which reduces the pathogen inoculum in farmer's fields.

2.5.3. Limiting field to field spread

Limiting field to field spread of the disease is very important in the disease control. Disease spread from one field to another can be reduced by removal of bean debris from the garden after harvest since most of the disease-causing agents can survive in plant debris. Tillage of infested residues will reduce the risk of disease transmission to dry beans planted in adjacent fields the subsequent year. With fewer residues remaining on the soil surface, less disease transmission occurs to the new crop and fewer residues are available for wind-borne dispersal to adjacent fields (Rio and Bradly, 2002). If infection is found in a field, spores may attach to equipment as a sticky ooze and be spread throughout the farm. Power-washing equipment to remove debris likely will remove most of the inoculum but if anthracnose is suspected in a field cultivation, spraying and harvesting of the infested field should be done last (Rio and Bradly, 2002). Limiting field to field spread is a practice that bean farmers can easily adapt. However, it requires sensitization from agriculturalists.

2.5.4. Fungicide use

Fungicides may be useful for reducing anthracnose. Seed treatment with fungicides like Mancozeb at a rate of 3g/kg seed can control the pathogen in infected seeds while foliar fungicides like Carbendazim at a rate of 0.5kg/hectare can control the pathogen on the shoots (Mohammed *et al.,* 2013). However, appropriately timed foliar fungicide applications do not confer complete control of anthracnose but they reduce seed discoloration to between 75 and 25% of the level found in the non-treated checks and increase yields by approximately 10 to 50%. Also the fungicide seed treatments are not a sure deal that eliminates seed to seedling transmission but reduce the initial severity. This can delay the onset of an epidemic that results from infected seed but they cannot be relied upon for preventing disease transmission (Markell *et al*., 2012). In addition, chemical control is characterized by several problems including environmental degradation, possible human poisoning and development of resistant biotypes (Holger *et al*., 2008; Padder *et al.,* 2010). Careful consideration should therefore be taken before one selects a given fungicide to use and should have considerable knowledge about its effectiveness and when to use it.

2.5.5. Use of disease free seed

Use of certified disease-free seed of known high quality is critical strategy for management of anthracnose (Rio and Bradley, 2002.) Even if anthracnose is not observed, planting bin-run seed is still risky. If anthracnose is introduced into a field at low levels when the weather is unfavorable for disease development, the disease may go undetected. In such cases, the occurrence of anthracnose may be limited to a few isolated spots in the field and disease severity may be low. However, even low levels of anthracnose result in infected seed. If the seed is planted, disease levels almost certainly will be higher than the subsequent years. Farmers should therefore not use the harvested seed for the next season planting (Rio and Bradley, 2002). Infected seed can be symptomless since they are not always discolored. The use of disease free seeds has been one of the most effective management strategies of seed borne diseases in the developed world. If the use of disease free seed is emphasized in Africa, it will reduce bean production losses due to seed borne diseases like anthracnose.

2.5.6. Use of resistant bean genotypes

Resistant varieties can be a solution for problems related to fungicide use and lack of certified seed especially for small holder farmers who are unable to continue buying chemicals for disease control (Oliviera *et al*., 2008; Grady *et al.,* 2016). The use of desired resistant cultivars is the best way of reducing the yield losses associated with anthracnose disease (Nkalubo *et al*., 2007). It is considered that the use of resistant cultivars is an efficient, safe and inexpensive and sustainable technique which can easily be accessed by bean farmers (Freira *et al*., 2000). However, different races of the pathogen exist and can change over time since the pathogen has a wide genetic diversity. Plants resistant to one race may be susceptible to another and yet it is still a challenge to produce cultivars that are resistant to multiple races (Rio and Bradley, 2002). In addition, there is always the possibility of breakdown of resistance in anthracnose resistant cultivars due to the adaptation of the pathogen to the host resistance (Chengdao and Fernando, 2016). This creates continuous need for new sources of resistant and continuous breeding for new resistant cultivars.

Pyramiding genetically diverse resistance genes using MAS and deploying different gene combinations in different regions has been proposed as the most practical and realistic approach to provide efficient long-term control of bean anthracnose (Goncalves-Vidigal *et al.,* 2011).

However, this should be accompanied by regular evaluation of cultivars when selecting for resistance in a particular region where they are to be grown to determine their tolerance to the locally prevalent races. Also seed companies that supply certified seeds should regularly consult for the most up to date information when considering resistance as a management tool.

2.6. Sources of resistance and resistance genes for anthracnose bean disease

Lacanallo *et al.* (2010) reported the existence of thirteen genes that condition anthracnose resistance which were designated as *Co-1* to *Co-13*. Freira *et al.* (2013) reported over 20 anthracnose resistance loci including *Co-1* to *Co-7*, *Co-8*, *and Co-9* to *Co-14* and *Co-u* to *Co-z*. These genes conferred resistance to specific races in common bean. It was found that some genes exist as allele forms. For example *Co-3* and *Co-9* reported by Lacanallo *et al.* (2010) were found to be alleles of each other and *Co-7* gene has been reported to be an allelic form of *Co-3* (Costa *et al*., 2017). All anthracnose resistance genes described, except for *Co-8*, exhibit complete dominance. Most of the *Co* genes were mapped onto the integrated bean linkage map. *Co-1* was found on linkage group B1, *Co-2* on B11, *Co-3* on B4, *Co-4* on B8, *Co-6* on B7, *Co-13* on B3. *Co-3* or *Co-9* and *Co-10* are located on linkage group B4 (Table.2). The *Co* genes that control resistance to bean anthracnose disease are all represented by the 12 standard differential cultivars (Table.1). Most of *C. lindemuthianum* differentials have been used as sources of resistance to anthracnose in breeding programs (Kelly and Vallejo, 2004; Kiryowa, 2015; Kazimot, 2016). In Andean races, anthracnose resistance loci from Mesoamerican beans are very important since the Andean and Mesoamerican races of *C. lindemuthianum* have evolved separately. In the same way Andean resistance loci are very important in Mesoamerican beans (Goncalves-Vidigal *et al*., 2011).

Though several resistance sources to bean anthracnose have been suggested, the available resistance is not effective against all known races from the same or different regions (Mahuku *et al*., 2002). For example, some of the well-known resistance genes that are effective in Europe (*Co-2*, *Co-3*, *Co-4*, and *Co-5*) may not be effective in Colombia, Brazil, Costa Rica, and Mexico and viseversa (Mahuku *et al*., 2002).

Some differential cultivars have demonstrated superior potential sources of resistance to a broad range of *C. lindemuthianum* pathotypes. For example cultivar G2333 has the broadest known

resistance against *C. lindemuthianum* race. Mahuku *et al*., (2002) showed that only the G 2333 line was resistant to 380 isolates of *C. lindemuthianum*. For the case of Uganda, differential cultivars AB 136 and G 2333 were resistant to all eight races of *C. lindemuthianum* pathogen and it was suggested that these cultivars could be utilized in the introgression of resistance genes into susceptible local cultivars (Nkalubo, 2006). Resistance in G 2333 was reported to be controlled by three different dominant resistance genes, *Co-4*^{2}, *Co-5* and *Co-7* (Kelly and Vallejjo, 2004). The *Co-4*^{2} is the broadest resistance allele at the *Co-4* locus (Silverio *et al.,* 2002). It is believed that the natural pyramiding of these 3 genes is responsible for giving it a broad resistance to most of the *C. lindemuthianum* pathotypes.

Table 2. Different *Co-* genes, their genetic sources and the markers linked to the genes.

Co-gene	Genetic source	Gene pool	Linkage group	Marker allele linked to the gene
Co-1	Michigan red kidney	Andean	B1	$OF10_{530}$
$Co\text{-}1^2$	Kaboon	Andean	B1	SE_{ACT}/MOCA
$Co\text{-}1^3$	Perry-marrow	Andean	B1	
$Co\text{-}1^4$	AND277	Andean	B1	
$Co\text{-}1^5$	Widusa	Andean	B1	$OA18_{1500}$
Co-2	Cornell 49242	Meso-American	B11	$OQ4_{1440}$,$OH20_{450}$,$B355_{1000}$
Co-3	Mexico 222	Meso-American	B4	
$Co\text{-}3^2$	Mexico 227	Meso-American	B4	
$Co\text{-}3^3$	BAT 93	Meso-American	B4	
Co-4	To	Meso-American	B8	Co8,Y20
$Co\text{-}4^2$	SEL1308, G2333	Meso-American	B8	SBB14, SAS13, SH18
$Co\text{-}4^3$	PI207262	Meso-American	B8	OY20
Co-5	Tu	Meso-America	B7	OAB_{3450}
$Co\text{-}5^2$	SEL 1360	Meso-American	B7	SAB3
Co-6	AB136	Meso-American	B7	OAHI780, $OAK20_{890}$
Co-7	G2333, MSY7-1	Meso-American	B4	None

Co-8	AB136	Meso-American	None	OPZ20
Co-9	BAT 93	Meso-American	B4	SB12
Co-10	Ouro-Negro	Meso-American	B4	F10
Co-11	Michelite	Meso-American	None	None
Co-12	Jalo Vemelho	Andean	None	
Co-13	Jalo Listras	Andean	B3	$OPV20_{700}$

2.7. Molecular markers

Molecular markers reveal sites of variation in DNA thus making them enormously useful in plant and animal breeding. They arise from different classes of mutations such as substitution mutations, rearrangements or errors in replication of tandem repeated DNA (Collard *et al*., 2005). Molecular markers are selectively neutral because they are usually located in non-coding regions of DNA (Collard *et al*., 2005). Unlike morphological and biochemical markers, molecular markers are practically unlimited in number and are not affected by environmental factors and/or the developmental stage of an organism (Muhamba etal.,2013).

Molecular markers should not be considered as normal genes, as they usually do not have any biological effect and instead can be thought of as constant landmarks in the genome. They are identifiable DNA sequences found at specific locations of the genome. Molecular markers are transmitted by the standard laws of inheritance from one generation to the next (Collard *et al*., 2005). Polymorphism is one of the most significant developments in the field of molecular genetics. Molecular markers are useful only if they are polymorphic in nature with an ability to discriminate between genotypes (Collard and Mackmill, 2008). Polymorphic markers can be dominant or co-dominant depending on whether a marker can discriminate between homozygotes and heterozygotes. Co-dominant markers show different band sizes depending on the various alleles that exist whereas dominant markers are either present or absent (Piepho and Koch, 2000)

Existence of various types of molecular markers and differences in their principles, methodologies and applications require careful consideration in choosing one or more of such markers. A single molecular marker cannot fulfill all requirements needed by researchers

(Semagn *et al*., 2006). One can choose among the variety of molecular techniques, each of which combines at least some desirable properties depending on the nature of the study. Common molecular marker types applied by breeders include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSRs), sequence characterized regions (SCARs), sequence tag sites (STSs), cleaved amplified polymorphic sequences (CAPS), microsatellites or simple sequence repeats (SSRs), expressed sequence tags (ESTs), single nucleotide polymorphisms (SNPs), InDels and other maker types (Muhammad and Muhammad, 2014).

2.7.1. Amplified fragment length polymorphism (ALFP).

This technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity. The fingerprints are produced without any prior knowledge of sequence using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. AFLP technique is reliable since stringent reaction conditions are used for primer annealing. This technique thus shows a combination of RFLP and PCR techniques and is extremely useful in detection of polymorphism between closely related genotypes. For example Vipin *eta*l., (2008), applied an AFLP technique to assess the genetic diversity among 44 common bean accessions which conclusively gave a good insight of the genetic diversity available in Indian bean accessions. AFLP markers are useful in assessing diversity of common bean and incase the marker is linked to a trait of importance then it can be useful for MAS in selecting or screening genotypes for that particular trait (Sustar *et al*., 2006). However, AFLP markers' use is too difficult and troublesome and need to be converted to other types of markers like SCAR or STS which is also a complicated process to achieve.

2.7.2. Simple Sequence Repeats (SSRs) and inter sequence simple sequence repeats (ISSR)

Simple sequence repeats or microsatellites are stretches of DNA sequence consisting of short tandem repeats of nucleotides (Collard *et al*., 2005). Simple sequence repeats are widely distributed throughout genomes and have been found in all prokaryotic and eukaryotic genomes (Viera *et al*., 2016).

ISSR involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly inter simple sequence repeats of different sizes (Ng *et al.,* 2015). The microsatellite repeats used as primers for ISSRs can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The primers used can be either unanchored or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Kalita *et al.,* 2007).

ISSRs exhibit the specificity of microsatellite markers but need no sequence information for primer synthesis enjoying the advantage of random markers (Joshi *et al*., 2000). SSR markers usually show high polymorphism (Anne, 2006) although the level of polymorphism has been shown to vary with the detection method used. SSR markers are advantageous to use for MAS because they are highly reliable (i.e. reproducible), co-dominant in inheritance, relatively simple, cheap to use and generally highly polymorphic. This type of markers requires very small quantities of DNA for assaying (Collard *et al*., 2005) and is amenable to automated allele detection by capillary electrophoresis. In addition, some SSR markers are genic and play functional roles in gene expression and regulation and they are easy to analyze at a moderate cost (Lu *et al.,* 2014).

For example, (Manasa ,2013) used 11SSRs and 3 ISSR markers in genotypic evaluation of French bean germplasm and this type of markers played a big role in anthracnose resistance selection. SSR markers play a big role in gene discovery for important genes that can be used for anthracnose breeding. Neeraj *etal*. (2018) used SSR markers to locate a quantitative trait loci for anthracnose breeding in North-western Himalaya. Oliviera *etal*., (2008) applied microsatellite/SSR markers that successfully enhanced simultaneous transfer of resistance genes for rust, anthracnose and angular leaf spot into Brazilian commercial cultivars.

However, use of SSR markers is complicated due to the fact that in most cases they require polyacrylamide gel electrophoresis and generally give information only about a single locus per assay, although multiplexing of several markers is possible. These problems have been overcome

in many cases by selecting SSR markers that have large enough size differences for detection in agarose gels, as well as multiplexing several markers in a single reaction (Elfath *et al*., 2000).

2.7.3. Sequence characterized regions

Sequence characterized regions (SCARS) are DNA fragments amplified by PCR using specific 15-30 bp primers designed from nucleotide sequences established from cloned RAPD fragments linked to a trait of interest. By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs. Obtaining a co-dominant marker may be an additional advantage of converting RAPDs into SCARs although SCARs may exhibit dominance when one or both primers partially overlap the site of sequence variation (Segman *et al.,* 2006).

The main advantage of SCARs is that they require low quantities of template DNA for PCR, have high reproducibility since they are locus-specific making them quick and easy to use. Because of these merits, SCARS have been applied in gene mapping studies and marker assisted selection. In Bulgaria, SCAR markers were applied in marker assisted breeding program to achieve a complex resistance against ten *C. lindemuthianum* races (Genchev *etal*;2010). Gilmar *etal*. (2012) pyramided anthracnose and angular leaf spot resistance genes in Carioca common bean cultivar from which he reported that selection assisted by SCAR molecular markers was an important tool in common breeding for resistance to *C.lindemuthianum* and *P.griseola*. In Uganda, Kiryowa.(2015) introgressed at least two anthracnose and one *Pythium* root rot resistance genes into susceptible market class common bean varieties using SCAR marker assisted selection. He reported that the tightly linked SCAR markers greatly enhanced the efficiency of marker based gene pyramiding. However, he expressed challenges from the use of SCAR markers in his study since they provided limited information at the loci they tag because they are bi-allelic dominant in nature. This implied that it was only possible to tell whether a given allele was present or not at a given locus but could not distinguish between the heterozygous from the homozygous genotypes in a segregating population. He suggested the use of codominant markers since they allow the distinction of homozygous and heterozygous genotypes through gel electrophoresis. In addition, SCARs are disadvantageous in a way that there is a need for sequence data to design the Primers for polymerase chain reaction use which can limit the use of SCARs in MAS (Collard *et al.,* 2005).

2.7.4. Sequence tag sites a and expressed sequence tags

Sequence tag site (STS) is a short, unique sequence whose exact sequence is found nowhere else in the genome. Two or more clones containing the same STS must overlap and the overlap must include STS. Any clone that can be sequenced may be used as STS provided it contains a unique sequence. In plants, STS is characterized by a pair of PCR primers that are designed by sequencing either an RFLP probe representing a mapped low copy number sequence (Muhammad and Muhammad, 2014) or AFLP fragments.

Expressed sequence tags or ESTs are short and single pass end sequences (either 5′ or 3′ end) of the cDNA clones which represents the coding region of the genome. EST markers are obtained by sequencing a few hundred nucleotides from either the 5' or 3' end to create 5' expressed sequence tags (5' ESTs) and 3' ESTs respectively (Piyarat *et al.,* 2016). A 5' EST is obtained from the portion of a transcript (exons) that usually codes for a protein. These regions tend to be conserved across species and do not change much within a gene family. The 3' ESTs are likely to fall within non-coding (introns) or untranslated regions (UTRs) and therefore tend to exhibit less cross-species conservation than do coding sequences. ESTs also allow a computational approach to the development of SSR and SNP markers (Choa *et al.,* 2000; Eujayl *et al*., 2002; Piyarat *et al*., 2016) for which previous development strategies have been expensive. Often EST-based RFLP markers allow comparative mapping across different species because sequence conservation is high in the coding regions. Hence, marker development and map based cloning in one species will profit directly from data which is available in any other species. Pattern-finding programs can be employed to identify SSRs in ESTs. ESTs (EST-RFLP) have been extensively used for the construction of high-density genetic linkage maps (Durand *et al*., 2010). STS markers are co-dominant, highly reproducible, suitable for high through put and automation and technically simple for use (Reamon-Buttner and Jung, 2000). For example**,** Lorenna *etal*. (2015) characterized the genetic reistance in Andean common bean cultivar corithiano to *C. lindemuthianum* races 8,65,89 nd 2047 using STS marker g2685.This STS marker enabled the successful characterization and mapping of anthracnose resistance in the common bean landrace. Souza et al. (2014). Identified highly linked sequence tagged site marker, g12333250, located at a distance of 1.2cM from the *Co-5* gene. This marker was reported to have a potential to increase

the efficiency of selection for the *Co-5 t*hat conditions resistance to anthracnose. Thus STS and EST markers are some of markers of choice that can be applied in breeding.

2.7.5. Single nucleotide polymorphism (SNP)

Most SNP markers are just a single base change in a DNA sequence with alternative of two possible nucleotides at a given position (Collard *et al*., 2005). SNPS are the most abundant source of genetic polymorphism and distributed throughout the genome in various species including plants and animals (Maughan *et al.,* 2010; Mohd and Mohammad, 2013). Single nucleotide polymorphisms are evolutionarily stable with low mutation rates (Burgner *et al*., 2003) which makes SNPs excellent markers for studying complex genetic traits and as a tool for understanding genome evolution (Chagne *et al.,* 2008). In plants, the ability to screen large numbers of samples for a range of SNP variants enables the prediction of resistance or susceptibility to a wide range of diseases (Gowda *et al.,* 2015; Osorio-Guarin *et al.,* 2016). However, the SNP marker technique requires the availability of large amounts of biological sequence data in public databases and the advent of next-generation sequencing which raises the cost of the technology (Zhou *et al.,* 2015).

2.7.6. InDel (Insertion and deletion) markers

InDels are either an insertion or deletion of bases in the genome of a given species. InDels can be used as genetic markers in natural populations especially in phylogenetic studies. It has been shown that genomic regions with multiple InDels can also be used for species-identification procedures. Low levels of polymorphism limit the application of marker-assisted selection in plant breeding programs. It has been reported that insertion and deletion (InDels) markers are more polymorphic than SSRs in some crops (Liu *et al*., 2015). InDel markers are the most frequently used in marker-assisted selection and genetic diversity studies (Hayashi and Yoshida, 2006). InDel markers remain useful even in a population with low genetic diversity and can be utilized in genetic mapping and diagnostics (Vali *et al.,* 2008).

InDel markers are highly abundant and distributed throughout the genome in various species of plants. For instance in comparing sequences from Japonica and Indica rice cultivars, there was at least one InDel in every 540 bp in the genome of rice (Yu *et al*., 2005). The abundance of polymorphisms of InDels in plant genomes makes them an attractive tool for marker assisted

selection and map based cloning (Zhou *et al.,* 2015). In addition, InDel markers can be amplified using regular PCR instruments and genotyped on agarose gel electrophoresis system of HRM (high resolution technology) (Zhou *et al.,* 2015). Samira etal. (2014) developed market class specific InDel markers from next generation sequence data in *Phaseolus vulgaris* selected from both Andean and Middle American gene pools .These markers were reported to be user friendly and can have wide applications for MAS and genomic studies in common bean breeding.

The advantages and disadvantages of the different marker types thus suggest that there is no DNA marker technology which meets all the required conditions. Pros and cons must be considered when planning to use any given marker. A marker technology to be used in a given study should thus be selected depending on the requirement and the major objective of the study (Yang *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Areas where the study was done

Diseased samples that were used in this study were collected from three districts; Bushenyi, Kabale and Kisoro. *Colletotrichum lindemuthianum* isolation was done at Center for International Agriculture (CIAT) at Kawanda-Uganda while disease screening and genotypic characterisation was conducted in the screen house and biotechnology laboratory at National Crops Resources Research Institute (NaCCRI). NaCRRI is located at Namulonge village, 27km North East of Kampala along Kampala-Zirobwe road. NaCRRI lies along 0.5288^{o}N and 32.6123^{o}E at an elevation of 1,160M above sea level while CIAT lies at 024'25N and 32 32'7''E north of Kampala at an elevation of 1,300m above sea level and 13Km along Kampala Gulu road.

3.2. Identification of polymorphic codominant markers from the Phaseolus gene database for anthracnose resistance.

3.2.1. Marker search from the *Phaseolus* gene database

The *CoK-4* sequence earlier on confirmed to be part of the *Co-4 gene* locus conditioning resistance to *C. lindemuthianum* in bean (Melotto *et al.,* 2000) was used as a reference sequence for marker search. The sequence of the *CoK-4* gene was obtained from the national center for biotechnology information (NCBI) (https://www.ncbi.nlm.nih.gov/nuccore/979647) and was the reference sequence which was blasted in the *Phaseolus* gene database (phaseolusgenes.bioinformatics.ucdavis.edu/).

The best hint which had an e' value of less than '0' indicated a similar region with linkage on chromosome 8 where the *Co-4*^{2} gene locus that conditions resistance to *C. lindemuthianum* is found. A total of 20 co-dominant markers including SSR, STS and InDeL were selected from the *Phaseolus* gene database. Of these, 18 markers were selected from the region that ranged between 2367884 and 2370892bp on the database. The other two markers were outside this range.

3.2.2. Primer designing

The SSR and STS markers which were selected from the *Phaseolus* gene database were provided together with primer sequences (Table 3). However, for the InDel markers, primer sequence pairs were not provided from the *Phaseolus* gene database and these were designed using Primer3 software (http://www.simgene.com/Primer3) (Table 3).

3.2.3. Polymorphism test

Genomic DNA was extracted from five parental genotypes NABE 13, NABE 14, G 2333, NABE 12C and K 132 using a modified CTAB protocol (Afanador and Haley,1993). Leaf samples were collected from young leaf lobes weighing about 0.2 to 0.3g and ground in liquid nitrogen. The ground powder was transferred into 2ml sterile eppendorf tubes and 800µl of CTAB extraction buffer at 65°C was added (2% CTAB, 100mM Tris-HCl, 20mM EDTA at pH 8.0, 1.4mM NaCl and 1% freshly prepared β-mercaptoethanol). The mixture was shaken vigorously for a minute to disperse the tissue in the buffer and then incubated at 65°C for 30 minutes.

Proteins and polysaccharides were then precipitated by the addition of 800µl of chloroform: Iso-amyl alcohol (24:1) and mixed by inversion for 15 minutes. The mixture was then centrifuged at 13,000 revolutions per minute (rpm) for 10 minutes to allow separation of the supernatant with DNA from the leaf particles. The upper aqueous phase was removed and transferred to a new sterile 1.5ml eppendorf tube. Isopropanol at 0°C was added to the supernatant and mixed gently by inversion to precipitate genomic DNA and the tubes were incubated on ice for 15 minutes to allow precipitation of DNA. This was followed by centrifugation at 13,000 rpm for 10 minutes. The Isopropanol was then poured off to obtain the DNA pellet on the bottom of the eppendorf tube. The DNA pellet was washed using 500µl of 70% ethanol and then centrifuged for 5 minutes. The ethanol was poured off and the tubes were inverted to allow the DNA pellets to dry completely for an hour.

The pellets were then dissolved in 100µl of sterile distilled water and 4µl of 10mg/µl RNase A was added in each tube and the tubes were incubated in a water bath at 37°C for 15 minutes. Ice cold 100% ethanol was then added in each tube to precipitate out DNA. The tubes were then centrifuged at 13000 rpm for 10 minutes to recover the DNA pellet. The ethanol was then poured off leaving behind the DNA pellets. The tubes were then inverted to allow the pellets to dry

completely. The pellets were then dissolved in 1X TE buffer and later the quality of DNA was tested by electrophoresis of 10µl of each sample on 1% Agarose gel in 1x TAE buffer (40mM Tris-HCl and 1mM EDTA at pH 8.3 adjusted with hydrochloric acid). The gel was stained with 1 µg/ml Ethidium bromide. The concentration of each sample was measured by the use of a Nanodrop 2000 spectrophotometer, thermo scientific model and the A260/A280 ratio was used to provide an additional estimate of DNA purity. The DNA concentration was standardized to a uniform working concentration of 200ng/µl by addition of the appropriate amount of sterile water.

The PCR amplification conditions were optimized for all the marker primers. For each 10 PCR reaction volume, the components included: solid premix [2.5U of Taq DNA polymerase, 200µM dNTPs, 10 mM Tris HCl, 50mM KCl, 1.5mM $MgCl_2$], 0.5 µ1forward primer, 0.5µl reverse primer, each with a final concentration of 0.5µM, 1µl of DNA with the final concentration of 20ng/µl and 8µl of sterile double distilled water. The PCR profile that was used included: An initial denaturation at 95°C for 5 minutes, denaturation at 94°C for 20 seconds, extension at 72°C for 2 minutes and elongation at 72°C for 10minutes. The annealing temperatures differed between the different primers (Table 3). The PCR reactions were kept at 4°C and later were loaded onto 6% HPAGE gel.

Table 3. A list of markers selected from the *Phaseolus* gene database

Marker	Position in database (bp)	Primer sequences (5' to 3')	Annealing temperature
CP 110$_{STS}$	Chr08 2400144-2402503	F-GTTCGCGGTTACCCTTTCAGTCT R-TTGCTGGCCTTGCTGTGATAGATA	58°C
CP 112$_{STS}$	Chr08:2400227-2402142	F-CGTCCGTGGATACCCTTTCAGTC R-CAACCAGCCCACATATCATCGTA	
BMC 294 $_{SSR}$	Chr08:242072-2462134	F-GGTCGTGATGTCTCCATTT R-TACCTCCCATCATGCACTTAC	
CP 109 $_{STS}$	Chr08:2401565-2401737	F- CTGCCAAAAGAAGCTACCACTGT R- CCATCCGATCTCTAACCTTCAAAC	
CP 108 $_{STS}$	Chr08:2401565-2401737	F- CACGAGCAAACAAACGCACAC R- ATGGGGTGGTGGCGACAAA	

G 1879 $_{STS}$		F- TGAATCTGATCCGTGCAAAGAGTA R- GAGAATGGAACATCAAGCAACTCA	52ºC
NDSU_IND_8_2.7546 $_{Indel}$	Chr08:2754585-2754612	F- ATTGTAAGCGCCTACGAACCTA R- AGGTCGAGCGAAAAGAAAAGTT	52ºC
INDSU_IND_8_2.83623 $_{Indel}$	Chr08:2836336-2836351	F- TTTCCCTTCCATTTTAACATCTG R- TTTCCCTTCCATTTTAACATCTG	
IAC 79 $_{SSR}$	Chr08:2318067-2318197	F- TGTTGCCTATTGCTTCCTAA R- CCTCCAACCGGTGTAACTT	
BM 98 $_{SSR}$	Chr08:2321326-2321471	F- GCATCACAAAGGACTGAGAGC R- CCCAAGCAAAGAGTCGATTT	
BMD 48 $_{SSR}$	Chr08:2745334-2758168	F- CCCCACCAACTCTTTCTTCC R- CAGAATTGACTTGGCGAGAA	
BMB 588 $_{SSR}$	Chr08:2654219-2696201	F- ATTATCCAAACATGATCCGT R- TCATACTGAAGTCATGCCAA	
INDSU_IND_8_2.8360 $_{Indel}$	Chr08:2836021-2836036	F- CCCACATCAGAAACGAGCTT R- GATGCCAAATCAACAAACCC	56ºC
BM 211 $_{SSR}$	Chr08:10710392-10710868	F- ATACCCACATGCACAAGTTTGG R- CCACCATGTGCTCATGAAGAT	
Bng 71 $_{STS}$	Chr08:2,316,796-2,317,585	F- AGCAAGTGAGGTCCACCAAG R- CAGTGGCCATGGTATCAGAA	
BM 189 $_{SSR}$	Chr08:47,874,118-47,874,148	F- CTCCCACTCACCCTCACT R- GCGCCAAGTGAAACTAAGTAGA	
BM 25 $_{SSR}$	Chr08:6,590,061-6,590,139	F- GCAGATCGCCTACTCACAAA R- CGTTGACGAGAAGCATCAAG	54ºC
BMD 201 $_{SSR}$	Chr08:2462056-2462140	F- TGCAAAGAATCACCTACACA R- CACAAACGATCAACTTGCTA	
BM 151 $_{SSR}$	Chr08:10385859-10386068	F- CACAACAAGAAAGACCTCCT R- TTATGTATTAGACCACATTACTTCC	48ºC
BMD 422 $_{SSR}$	Chr08:2857300-2857395	F-ATTCGAATTTGGATCTGATT R- ACTTCACAAACGCTGTCTCT	

F: Forward primer, R: Reverse primer, Chro8: Chromosome 8, bp: Base pairs, STS: sequence tagged site marker, SSR: Simple sequence repeat marker, Indel: Insertion or deletion marker

3.3. Testing the co-segregation of the anthracnose disease resistance and co-dominant marker(s) and determine their utility in selecting anthracnose resistant lines.

3.3.1. Identification of a pathogenic *C.lindemuthianum* pathotype for the study.

This part of the study required identification of a pathogenic *C. lindemuthianum* isolate for inoculating the F_2 segregating population. An association between the phenotypic reaction of the F_2 progenies and marker alleles for the marker(s) under test would give a basis for testing the usability of the marker(s) in selection for anthracnose resistance.

3.3.1.1. Collection of diseased materials

Anthracnose diseased materials were collected from three districts: Bushenyi, Kabale and Kisoro (Figure 2) where the environmental conditions greatly favor the growth of the pathogen. Samples were collected from three sub-counties of each of the three districts. From Bushenyi district, Rushinya, Kabaare and Nyabubare sub-counties were sampled. From Kabaale district, Nyukyonga, Kamuganguzi and Kitumba sub-counties were sampled. From Kisoro district, Nshora, Gahembe and Muramba sub-counties were sampled. In each sub county, at least six farmers' fields were considered and ten diseased pods were picked randomly per farmer's field. The infected bean pods were placed in labeled brown paper bags and were transported to NaCRRI where they were allowed to dry for two days and later transported to CIAT- Kawanda were isolation was done. The three sub-counties per district and the six farmers' fields per sub-county were selected basing on the presence of NABE14 or NABE13 infected plants. At least six farmers' fields in each of the three sub-counties had planted NABE14 or NABE13 varieties that season. Diseased pods were only picked from either of the two varieties to ensure that an isolate to which these two varieties could succumb to was obtained. NABE14 and NABE13's production has declined greatly in South west regions of Uganda where it was originally grown on a large scale. This has been due to susceptibility of the two varieties to anthracnose disease despite of their high market in south west Uganda. The National legumes program is currently carrying out introgression of anthracnose resistance genes in these two released varieties and this gave a basis for their selection for this study.

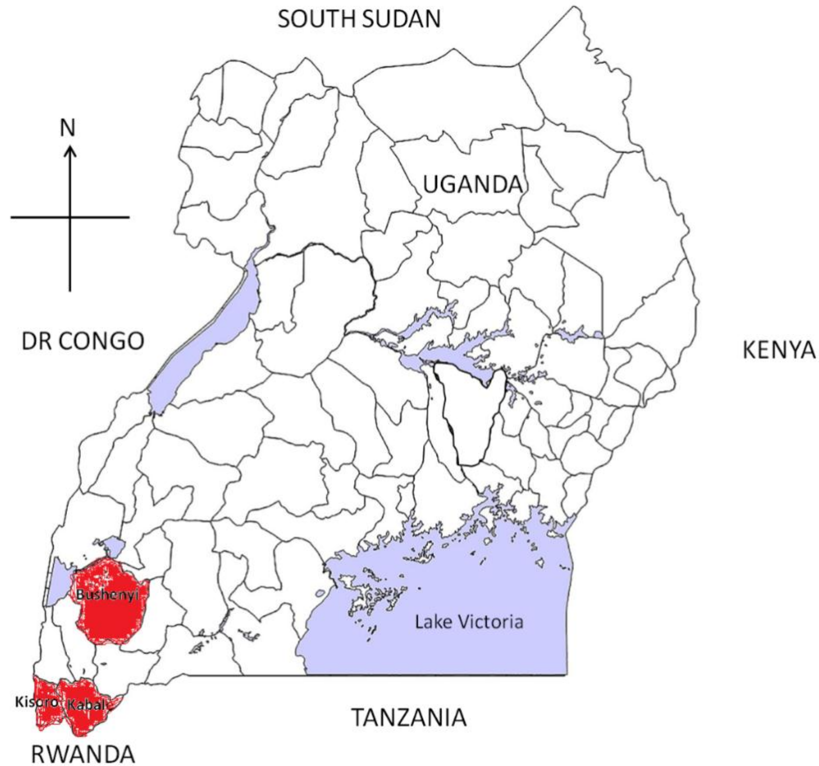

Figure.2. Location of the three districts where the *C. lindemuthianum* isolates were collected in Uganda

3.3.1.2. Pathogen isolation

Colletotrichum. lindemuthianum was isolated on potato dextrose agar (PDA). Standard PDA medium was prepared by mixing 39g of PDA in one liter of distilled water. The media was autoclaved at 121°C for 15 minutes. Chloromphenical (CAM) and Ampicillin (AMP) antibiotics at concentrations of 40µl/ml and 13µl/ml respectively were added to the cooled medium to prevent bacterial contamination. The medium together with antibiotics were mixed thoroughly and there after poured into 9cm petri-dishes and allowed to set overnight. Well-developed pod lesions were carefully cut to a size of 1cm long and surface sterilized with 1% sodium hypochlorite for 2 minutes, then surface sterilized with 70% ethanol for 2 minutes. The diseased tissues were then rinsed with sterilized distilled water and were dried using sterilized filter papers before plating on PDA medium. The symptomatic tissues were placed on medium in sterile petri-dishes under laminar flow hood. The plates were then placed upside down in an incubator at 22-

23ºC. The fungus was sub-cultured from the original plates after five days by cutting small pieces of agar with the fungus and placing them onto new plates containing PDA media. Mono-conidial isolates were obtained by dissolving little mycelia in sterile distilled water; 1ml of the pathogen suspension was plated on tap water agar media and incubated at 22ºC for 1 day. The single spores were then picked by a sterile pin-point needle under a dissecting microscope and then sub-cultured on fresh PDA medium.

3.3.1.3. Pathogen preservation

Pieces of filter paper were cut, wrapped in aluminium foil and then steam sterilized in an autoclave. The sterilized paper pieces were aseptically laid onto PDA plates. Pieces of the pathogen mycelia were cut and sub cultured onto the PDA plates with sterile filter paper pieces. The plates were incubated at 23ºC for two weeks to allow the pathogen to grow and cover up the paper pieces. The filter paper discs with mycelia were picked off the plates aseptically using sterile forceps and were wrapped into sterilized aluminium foil and desiccated in silica gel and were stored at -20°C for future use.

3.3.1.4. Inoculum preparation

Single spores of each isolate were placed on fresh Mathur's agar medium in separate petri-dishes and incubated in total darkness at a controlled temperature between 20-22ºC for 21 days to allow the fungus enough time to produce conidial spores. The modified Mathur's agar medium (500ml) was made up of 4g of dextrose, 1.25g of magnesium sulfate, 1.35g of potassium phosphate, 1.2g of Neopeptone, 1g of yeast extract and 8g of agar (Tu,1985).

Inoculum suspension was done by adding 5mls of distilled water onto the plate with sporulating culture. Conidial spores were scrapped off the growth medium using a spatula into a small amount of water to make a suspension. A haemocytometer was used to count the spores and the spore concentration was adjusted to 1.2 x 10^6 conidia ml/L and 0.1% Tween 20 was added to the suspension as a surfactant.

3.3.1.5. Pathogenicity tests

Seven bean genotypes were used to include; G 2333, NABE 13, NABE 14, K 132, NABE 12C, Mexico 54, and RWR 719. G 2333 is a small red seeded climbing bean genotype, highly

resistant to anthracnose. NABE 13 (large red seeded), NABE 14 (large red seeded) and K 132 (large red mottled) are bush bean genotypes commercially grown in Uganda but highly susceptible to anthracnose. The National legumes program is currently introgressing anthracnose resistance in NABE14 and NABE13 which partly gave a basis for the selection of these two varieties for this study. K 132 also known as CAL 96 is the susceptible check for most of the dry bean diseases. NABE 12C, a climbing bean with a cranberry (sugar) seed type is also commercially grown in Uganda and is currently being improved for multiple disease resistances by the National Legumes Program. RWR 719 (small red) and Mexico 54 (medium pink) are bush bean genotypes used as sources of root rot and angular leaf spot resistance genes respectively in the breeding program. The pathogenic test was useful for identifying a pathogenic isolate that could show clear phenotypic differences between; G2333 (resistant cultivar) and the susceptible cultivars, NABE14, NABE13 and K132 (susceptible control). NABE 12C is being introgressed for multiple bean diseases by the National legumes program and there was an interest to observe the NABE12C cultivar's response to anthracnose in a controlled environment. RWR719 and Mexico 54 resistant sources were included in the experiment to see how they also respond to anthracnose disease.

Twenty one day-old potted seedlings of the 7 bean genotypes above were inoculated with each of the successfully obtained *C. lindemuthianum* isolates using a hand sprayer. The inoculum suspension containing *C. lindemuthianum* spores was sprayed on both abaxial and adaxial surfaces of leaves until runoff. The inoculated plants were tied with transparent polythene bags to obtain the required humidity for infection (Mwesigwa, 2008). The experiment was then maintained in the growth chamber at room temperatures maintained between 20°C and 22°C with the help of an air conditioner and light supplied by Phillips TLT 18-20W/75RS fluorescent tubes. A light timer was connected to enable 12 hour daily light regime (Kiryowa, 2015). Anthracnose disease symptoms were scored after 6 days of incubation period using a modified 1 - 9 scale (Balardin *et al.,* (1997). The experiment was repeated in a succession of 1 week. Analysis of variance was done to determine an isolate with the highest of severity levels per genotype. The isolate that gave the highest severity level was selected for the study.

Amongst the 6 isolates of *C. lindemuthianum* that were successfully obtained together with 2 additional uncharacterized isolates from CIAT-Kawanda, isolate K B2 was selected for the

study. KB2 pathotype was a collection from Kabaale district and it infected three out of the four susceptible bean varieties used in this study. In addition, this isolate could clearly differentiate between the resistant parent (G 2333) and the susceptible parents (NABE 13 and NABE 14) by not causing symptoms on G 2333 while showing severe symptoms on NABE 13 and NABE 14. Testing an association between the marker(s) and disease resistance required a segregating population from parental genotypes that have highly contrasting phenotypes. From the Pathogenicity test, G2333 could serve as a donor parent while NABE14 and NABE13 were selected to be female parents for the next objective of the study.

3.3.1.6. Race determination of the most virulent pathotype (KB2) with the anthracnose standard differentials.

Twenty one day old potted seedlings of the 12 standard anthracnose differentials namely; Michelite, Michigan red kidney, Perry marrow, Cornell 49242, Widusa, Kaboon, Mexico 222, PI 207262, To, Tu, AB 136 and G 2333 inoculated and maintained in a growth chamber as in section **3.3.1.5** above.

The standard system of pathogen characterization that uses the 12 bean cultivars was employed in characterization of K B2 pathotype (Kelly and Vallejo, 2004). This system designates a race after summing up all binary numbers for cultivars which show susceptible reactions to that given race. A binary number is equal to 2^n, where n is the place of the cultivar within the differential series of 0-11 (Table. 1).

3.3.2. Generation of the study population

Parental genotypes NABE 13, NABE 14 and G 2333 were grown in pots containing soil each containing 1g of NPK fertilizer. The genotype G 2333 (resistant to anthracnose) was crossed with each of the two susceptible cultivars NABE 13 and NABE 14 to generate F_1 hybrid seeds i.e. F_1 (NABE 13 X G 2333) and F_1 (NABE 14 X G 2333). One hundred seeds of each F_1 cross were grown in pots containing soil with NPK fertilizer and were allowed to self-fertilize to obtain the segregating F_2 study population.

3.3.3. Phenotypic characterization of the F_2 population

200 seeds of F_2 progenies and 15 F_1 seeds of each cross (NABE 13 X G 2333 and NABE 14 X G 2333) were grown in completely randomized design in the screen house in 10cm diameter

plastic containers with sterilized soil containing NPK fertilizer. At 21 days of growth, the seedlings were transferred to the inoculation chamber and were inoculated with $1.2X10^6$ spores/ml of K B2 isolate spores by spraying. Leaves were sprayed on both the abaxial and adaxial surfaces until runoff. The inoculated plants were tied with transparent polythene bags to obtain the required humidity for infection (Mwesigwa, 2008). The plants were then maintained in the inoculation chamber with conditions as above (section 3.3.1.5). Disease severity was scored 7 days after inoculation using the 1-9 as above (section 3.3.1.5).

3.3.4. Genotypic characterization of the F_2 segregating population with BMB 588 and BM 211 markers

BMB 588 and BM 211 markers were selected for this study because they showed codominance with two bands. Genomic DNA was extracted from all the successfully germinated F_2 progenies of the crosses NABE 14 X G 2333 and NABE 13 X G 2333 using modified CTAB protocol (Section 3.2.3.) (Afanador and Haley, 1993). The concentration of each sample was measured using a Nano-drop 2000 spectrophotometer, thermo scientific model and the A260/A280 ratio was used to provide an additional estimate of DNA purity. The DNA concentration was standardized to a uniform working concentration of 200ng/µl by addition of the appropriate amount of sterile water.

The PCR reaction mixture and PCR thermo cycles were as above (Section 3.2.3). Marker BMB 588 primer pair (F-5′ATTATCCAAACATGATCCGT′, R5′TCATACTGAAGTCATGCCAA3′) and BM211 marker primer (F-5′ATACCCACATGCACAAGTTTGG3′, R-5′CCACCATGTG CTCATGAAGAT 5′) were used in the PCR reaction. The two markers had annealing temperatures of 52°c and 56°c respectively. The PCR reactions were kept at 4°C and later the PCR products resulting from BM 211 marker amplication were electrophoresed on 6% HPAGE gel while that of BMB 588 marker was electrophoresed on 1.5% agarose gel. The alleles of BM588 marker had a large separation between them and could clearly be separated on an agarose gel. On the other hand, marker BM 211 alleles were very close to each other and needed to be separated on an acyrlamide gel since acyrlamide has a higher resolution than agarose. The gel products were viewed with a gel Genius-syngene documentation system and the banding patterns of individual F_2 individual plants were coded.

3.3.5. Data analysis.

The co-segregation of the markers and anthracnose disease severity was subjected to chi-square test using the 14th edition of Genstat software at a 5% significant level.

CHAPTER FOUR

RESULTS

4.1. **Identification of codominant markers for anthracnose resistance selection**

Out of the 20 markers (11 SSR, 6 STS and 3 InDels; Table 3) that were selected from the *Phaseolus* gene database, three SSR markers (1AC 79, BM 211 and BMB 588) showed polymorphism in respect to genotype G 2333, NABE 13, NABE 14, NABE 12C and K 132 (Figure 4). Marker IAC 79 showed dominance while markers BM 211 and BMB 588 showed co-dominance.

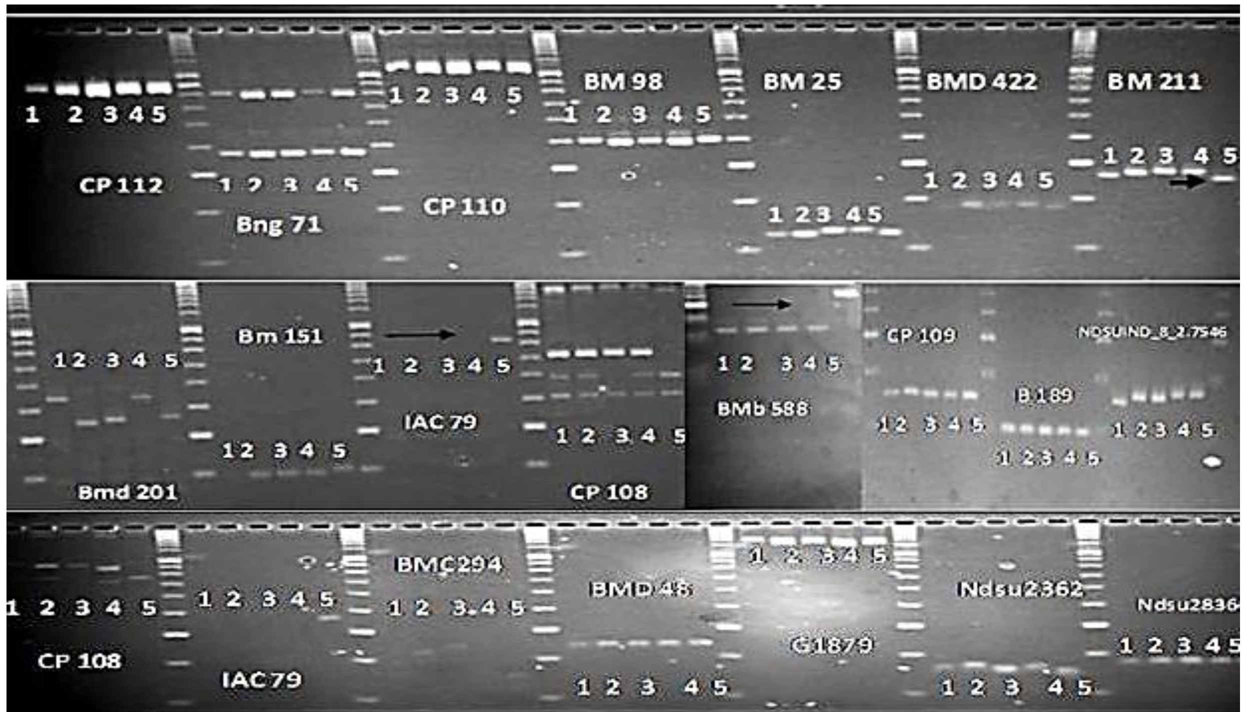

Figure. 3. Banding pattern of the 20 markers on 6% HPAGE (Polyacrylamide gel). 1 = K 132, 2 = NABE 12C, 3 = NABE 14, 4 = NABE 13, 5 = G 2333. The markers that showed polymorphism IAC 79, BM 211 and BMB 588 are shown by arrows**.** DNA ladder of 50bp was used between the markers.

4.2. Co-segregation test of the co-dominant markers and disease resistance

4.2.1. Identification of a pathogenic *C.lindemuthianum* isolate for the study

Six monosporic cultures were successfully obtained: two from Kabaale district (K B1, K B2), two from Bushenyi district (B U1, B U2) and two isolates from Kisoro district (KI-1 and KI-2). Two additional uncharacterized isolates (KB 33B, KIS 18) from CIAT-Kawanda were also included in the study. From the pathogenicity of each isolate on the seven bean breeding genotypes; G 2333, NABE 13, NABE 14, NABE 12C, K 132, Mexico 54 and RWR 719, isolate K B2 showed broadest virulence. K B2 differentiated between resistant parent (G 2333) and susceptible parents (NABE 14, NABE 13 and K 132) by not showing any visible symptoms on the resistant genotype while causing severe symptoms on susceptible genotypes. (Table. 4).

Table 4. Anova for *C. lindemuthianum* isolates' severity levels on seven bean genotypes

	Genotypes						
Isolates	G2333	K132	MEXICO54	NABE12C	NABE13	NABE14	RWR 719
BU1	2.000	4.500	2.000	2.000	4.500	2.500	2.000
BU2	2.000	4.500	3.000	2.500	4.000	2.500	2.000
K11	1.000	1.500	2.000	2.000	1.500	1.500	2.000
K12	1.500	3.000	1.000	1.500	3.500	1.500	1.000
KB1	1.000	2.000	4.000	2.000	2.000	4.500	2.000
KB2	1.000	7.500	3.000	3.000	7.500	8.000	2.500
KB33B	1.000	2.000	1.000	2.000	2.000	2.000	1.000
KIS18	1.500	2.000	5.500	4.500	4.000	2.500	2.000
Average	1.375	3.375	2.688	2.438	3.625	.3.125	1.812
Lsd 0.43							
CV 21.8							

From ANOVA table above, isolate KB2 showed the highest severity level at a significance level of 0.5 (Lsd=0.43,Cv=218). Isolate KB2 was selected for use in this study.

4.2.2. Race determination for *C. lindemuthianum* (KB2) isolate selected for the study

The first three differentials in the differential series; Mitchelite, Michigan red kidney and Perrymarrow with binary numbers 1, 2 and 4 respectively succumbed to the disease, giving severity levels of 4 and above. Using the binary system of classification, these numbers summed up to 7. Isolate KB2 was therefore designated race 7 (Table 5).

Table. 5. Reaction of the standard differentials to the selected isolate (KB2)

Differential genotype	Binary number	Severity level
Mitchelite	1	4
Michigan red kidney	2	8
Perrymarrow	4	8
Cornell 49-242	8	1
Widusa	16	2
Kaboon	32	1
Mexico 222	64	1
PI 207262	128	1
To	256	1
Tu	512	2
AB136	1024	1
G2333	2048	1

1; no symptoms (resistant), 2-3; very small lesions mostly on primary leaves (resistant), 4-9; numerous enlarged lesions or sunken cankers on the

4.2.3. Phenotyping of the F_2 segregating population

After 21 days of inoculation, all plants of the parental genotype G 2333 showed resistance. All plants of the parental genotypes, NABE 13 and NABE 14 showed susceptibility. All the F_1 NABE 13 X G 2333 and F_1 NABE 14 X G 2333 plants showed resistance. On the other hand, out of the 174 F_2 NABE 13X G 2333 plants, only 12 individuals succumbed to the disease and out of

188 F_2 NABE 14 X G 2333 plants, only 12 individual plants showed susceptibility (Table 6). The observed disease resistance segregation in the F_2 population fitted the 15:1 segregation ratio for two independently assorted genes (χ^2=0.124, P=0.72).

Table 6. χ^2 test for the segregation of F_2 generations to race 7 of *C. lindemuthianum*

Pedigree	Total number	Number of resistant plants	Number of susceptible plants	Expected ratio	χ^2 Value	Probability Value
NABE 13	7	0	7			
NABE 14	7	0	7			
G 2333	6	6	0			
F_1(NABE 13 X G 2333)	35	35	0			
F_1(NABE 14 X G 2333)	31	31	0			
F_2(NABE 13 X G 2333)	174	162	12	15:1	0.124	0.72

4.2.4. Genotyping of F_2 segregating population

4.2.4.1. Testing F_2 NABE 13 X G 2333 and F_2 NABE 14 X G 2333 with BMB 588 marker

DNA amplification products obtained with BMB 588 marker showed that, out of 174 F_2 NABE 13 X G 2333, 48 individual plants had a single band of about 400bp which was similar to the band present in G 2333 (resistant parent); 84 individuals had two bands of 400bp and 200bp similar to G 2333 and NABE 13 respectively; and 42 individuals had a single band of 200bp similar to one present in NABE 13 (susceptible parent). On the other hand, out of 188 F_2 NABE 14 X G 2333, 49 individuals showed a single band of about 400bp which was similar to the band present in G 2333 (resistant parent); 94 individuals showed two bands of 400bp and 200bp similar to G 2333 and NABE 14 respectively; and 45 individuals showed a single band of 200bp similar to one present in NABE 14 (Table 7, Figures 4a and 4b). The segregation of marker BMB 588 fitted the expected segregation ratio of 1:2:1 of a codominant marker in both segregating populations of F_2 NABE 13 X G 2333 and F_2 NABE 14 X G 2333 (χ^2=0.58, P= 0.75 and χ^2=0.17, P=0.92) respectively (Table 7).

Table 7. χ^2 test for the segregation of BMB 588 marker in the F_2 progenies of NABE 13 X G 2333 and NABE 14 X G 2333.

Pedigree	Total number of plants	RR	Rr	rr	Expected ratio	χ^2 value	Probability value
G2333	5	5	0	0			
NABE13	5	0	0	5			
NABE14	5	0	0	5			
F1(NABE13xG2333)	5	0	5	0			
F1(NABE14xG2333)	5	0	5	0			
F2(NABE13xG2333)	174	48	84	42	1:2:1	0.58	0.75
F2(NABE14xG2333)	188	49	94	45	1:2:1	0.17	0.92

RR: Plants with only one band of 400bp, Rr: Plants with both 400bp and 200bp bands, rr: Plants with only one band of 200bp

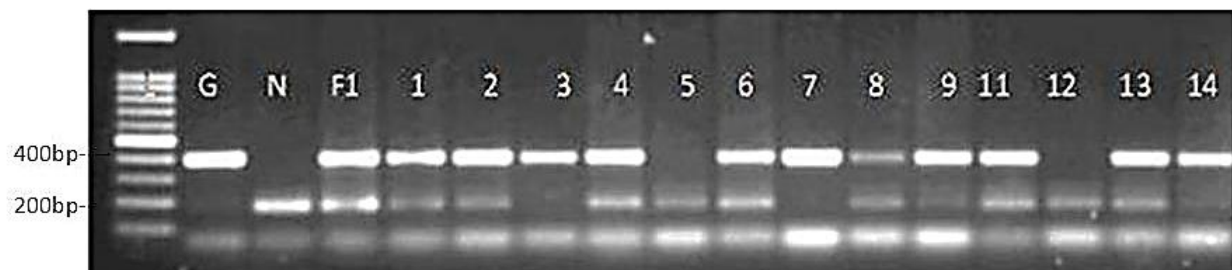

Figure 4a. Banding pattern of marker BMB 588 in the resistant parent G (G 2333), susceptible parent, N (NABE 13), F_1 progeny and the first 14 F_2 (NABE 13 X G2333) progenies on 1.3% Agarose gel. A 100bp ladder was used.

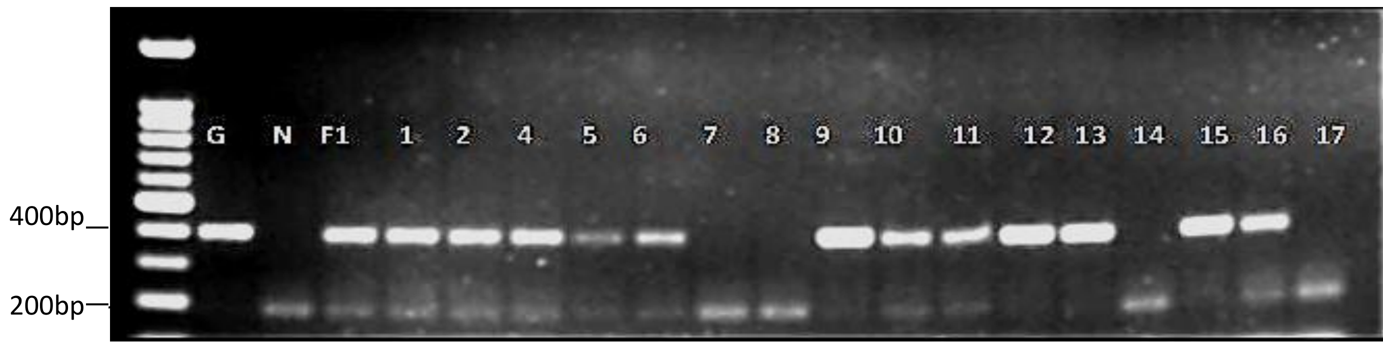

Figure 4b. Banding pattern of marker BMB 588 in the resistant parent G (G2333), susceptible parent N (NABE 14), F_1 progeny and 17 F_2 (NABE 14X G2333) progenies on 1.3% Agarose gel**.** A 100bp ladder was used.

4.2.4.2. Testing F_2 NABE 13 X G2333 and F_2 NABE 14 X G 2333 with BM 211 marker.

DNA amplification products obtained with BM211 marker, revealed that out 174 F_2 NABE 13 X G 2333, 35 individuals had a single band of about 110bp which was similar to one which was present in NABE13 (susceptible parent); 102 individual plants had two bands of 110bp and 100bp similar to NABE13 and G2333 respectively; and 37 plants had a single band of 100bp similar to one present in G2333 (resistant parent). Out of 188 F_2 NABE 14 X G 2333, 37 individuals showed a single band of about 110bp which was similar to one which was present NABE14 (susceptible parent); 121 individuals showed two bands of 110bp and 100bp similar to NABE14 and G2333 respectively; and 30 individuals showed a single band of 100bp similar to one present in G2333 (Table 8, Figures 5a and 5b).

The segregation of marker BM 211 segregation in F_2 (NABE 13 X G 2333) was not significantly different from the expected ratio (χ^2=5.22, p=0.0735) while it did not fit the expected 1:2:1 ratio for a codominant marker in F_2 (NABE 14 X G 2333), χ^2 =16.03, p=0.0003) at a significance level of 5%. (Table 8).

Table 8: χ^2 square test for the segregation of BM 211 marker in the F_2 progenies of NABE 13 X G 2333 and NABE 14 X G2333.

Pedigree	Total number of plants	RR	Rr	Rr	Expected ratio	χ^2	Probability value
G2333	5	5	0	0			
NABE13	5	0	0	5			
NABE14	5	0	0	5			
F_1 (NABE 13 X G 2333)	5	0	5	0			
F_1 (NABE 14 X G 2333)	5	0	5	0			
F_2 (NABE 13 X G 2333)	174	35	102	37	1:2:1	5.22	0.0735
F_2 (NABE 14 X G 2333)	188	37	121	30	1:2:1	16.03	0.0003

RR: Plants with only one band of 110bp, Rr: Plants with both 110bp and 100bp bands, rr: Plants with only one band of 100bp

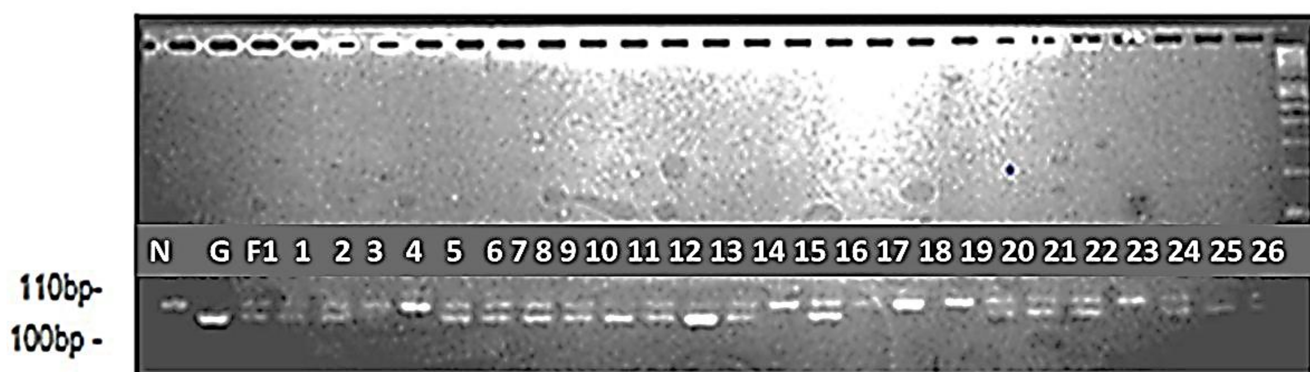

Figure 5a. Banding pattern of marker BM 211 in the susceptible parent N (NABE13), Resistant parent G(G2333), F_1 progeny and the first 26 F_2 (NABE 13 X G2333) progenies on 6% polyacrylamide gel. A 50bp ladder was used.

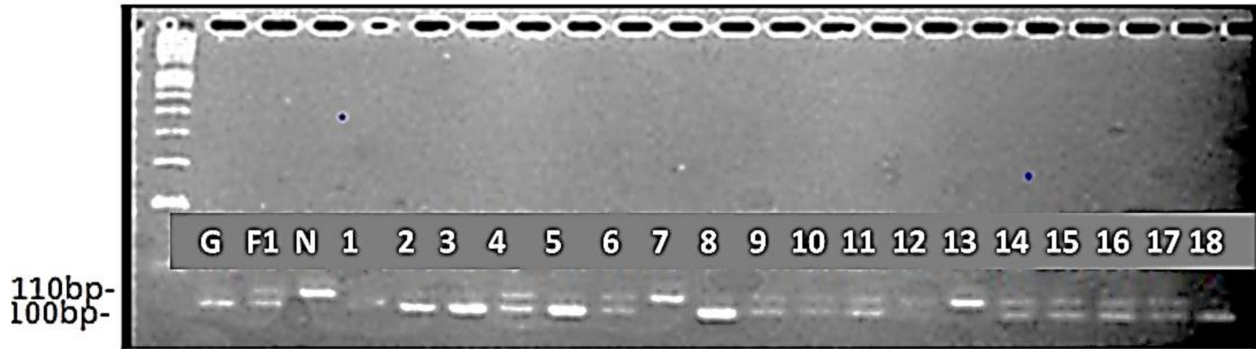

Figure 5b. Banding pattern of marker BM 211 in the susceptible parent N (NABE 14), F_1 progeny, resistant parent G (G2333), and the first 18 F_2 (NABE 14X G2333) progenies on 6% polyacrylamide gel. A 50bp ladder was used.

CHAPTER FIVE

DISCUSSION

Use of the molecular markers linked to resistance genes (marker assisted selection) is a reliable screening procedure to increase the efficiency of breeding for disease resistance. In order for MAS to be successful, there should be a tight linkage between the disease resistance gene, disease phenotype and the molecular marker used.

This study therefore aimed at the identification of a PCR-based co-dominant marker for *Co-4*^{2} anthracnose resistance gene from the alternative markers provided by the *Phaseolus* gene database. In order to achieve this, an association between the genotypes and the phenotypes of the segregating populations was determined.

In the course of searching for a pathogenic *C. lindemuthianum* race which would give the disease phenotype, a new pathotype of race 7 that severely infected susceptible cultivars was revealed. This pathotype had not been reported in earlier diversity studies in Uganda (Ssimbwa Bunya1972, Nkalubo 2006, Mwesigwa, 2008 and Kiryowa, 2015). Elsewhere, this race has been reported in the USA, Arigentina and Mexico (Balardin *et al*., 1997), Brazil (Silver *et al*., 2007), Europe (Ansari *et al*., 2004) and South Africa, (Muth and Lienberg, 2009). The pathotype of race 7 was reported as one of the most virulent pathogenic races of *C. lindemuthianum* (Ansari *et al.,* 2004; Vidigal *et al*., 2006). The failure of earlier studies to identify race 7 in Uganda may suggest that it is a new introduction. There is constant starter of seeds to the country through informal and formal routes which might have led to the introduction of new *C. lindemuthianum* races since this fungus is seed-borne (DIMAT, 2012). Freira *eta*l., (2003) also observed that new races of the pathogen with different genetic and virulence factors are constantly introduced to new places. Since the pathogen co-evolves with its host, this leads to great variability of the pathogen. The existence of race 7 might have not been reported by earlier studies because of failure of earlier scientists to sample this race from the fields where diseased samples were collected. The contrasting results also explain that the pathogen might have evolved over time through mutations and recombination resulting from sexual reproduction. Souza *et al.* 2010 found *C. lindemuthianum* to be highly evolving due to many mutants that had

been formed. The finding of the severe race 7 and the diversity of the pathogen calls for the need for extensive collection and diversity analysis for *C.lindemuthianum* in the country.

The severity of race 7 pathotype of *C. lindemuthianum* on segregating populations that fitted a 15:1 (resistant: susceptible) explains an epistasis gene interaction referred to as a duplicate gene action where two genes control the same phenotype (Miko, 2008). This implies that resistance from resistant parent G 2333 into susceptible parents with respect to race 7 is controlled by two dominant, independently assorted genes. This agrees with results from earlier studies (Pastor-Corrales *et al.,* 1994; Poletine *et al*., 2000; Alzate-Marin *et al.,* 2001; Castro *et al.,* 2017). However, studies by Young and Kelly, (1996) and Kelly and Vallejo, (2004) reported resistance to bean anthracnose in G 2333 to be controlled by three dominant genes (*Co-4*^{2}, *Co-5* and *Co-7*). The difference in reports of either two or three genes in cultivar G2333 might be due to differences in genotypes and pathotypes used. Parental plants used in one study might have different genes which might be resistant for one pathogenic race yet succumb to another (Pastor-Corrales *et al.,* 1994). For this study, G 2333 was used as a donor parent because it is known to be highly resistant to most *C. lindemuthianum* pathotypes in Uganda (Nkalubo, 2006). In addition, cultivar G 2333 is recommended in the introgression of anthracnose resistance into susceptible cultivars since it shows highly negative general combining ability which qualifies it as a suitable parent for easily transferring its genes into its progenies (Nkalubo *et al* ., 2009).

From the BM 211marker banding pattern ratios of RR/35: Rr/102: rr/37 and RR/37: Rr/121: rr/30 F_2 (NABE 13 X G 2333) and F_2 (NABE 14 X G 2333) respectively, all progenies that showed a 100bp allele were expected to be resistant to race 7 of *C. lindemuthianum*. These results were as expected since all the F_2s that had a 100bp band similar to one present in resistant parent G 2333, (RR and Rr) were phenotypically resistant.

However, a total of 37 F_2 (NABE 13 X G 2333) and 30 F_2 (NABE 14 X G 2333) showed a single band of 110bp allele which was similar to the band observed in the susceptible parents NABE 13 and NABE 14 (Fig 5a and 5b). This would mean that all these progenies that carried a 110bp band of this marker were susceptible to bean anthracnose yet only 12 individuals of each population were phenotypically susceptible. In addition, segregation of BM 211 marker did not fit the expected ratio of a codominant marker (Table 6 and 8). This marker showed segregation

distortions where a marker tends not to segregate in Mendelian fashion which may render it useless for MAS (Jinjin *etal*., 2011).

From the BMB 588 marker banding pattern ratios of RR/48: Rr/84: rr/42 and RR/49: Rr/94: rr/45 F_2 (NABE 13 X G 2333) and F_2 (NABE 14 X G 2333) respectively, all progenies that showed a 400bp allele were expected to be resistant to race 7 of *C. lindemuthianum*. These results were as expected since all the F_2s that had a 400bp band similar to one present in resistant parent G2333 (RR and Rr) were phenotypically resistant.

However, a total of 42 F_2 (NABE 13 X G 2333) and 45 F_2 (NABE 14 X G2333) showed a single band of 200 bp allele which was similar to the band observed in the susceptible parents NABE 13 and NABE 14 (Fig 4a and 4b). This would mean that all these progenies that carried a 200 bp band of this marker would be susceptible to bean anthracnose yet only 12 individuals of each population were phenotypically susceptible (Table 6 and 7). These unexpected phenotypic results are explained by the fact that G2333 which was the source of resistance carries more than one gene for anthracnose resistance presenting a possibility of gene interaction in its F_2 progenies (Pastor-Corrales *et al.,* 1994, Young and Kelly, 1996, Kelly and Vallejo, 2004).

Therefore, because of the possible epistasic gene interaction, the other 30 F_2 (NABE 13X G 2333) and 33 F_2 (NABE 14 X G 2333) that were expected to be susceptible did not succumb to the disease. On the other hand, genotypic results were as expected; marker BMB 588 segregated co- dominantly with a ratio of 1RR:2Rr:1rr and gave a good fit to expected χ^2 value for a co-dominant marker in both F_2 segregating populations. These results have been supported by work done by Geneticists. For example Miko, 2008 had similar observations for colored Kernel production in wheat. He explained that epistatic interactions may be limited at phenotypic level while the genes involved in a specific epistatic interaction may still show independent assortment at a genotypic level. The fact that marker BMB 588 was selected from chromosome 8 where the *Co-4*^{2} gene is located and it had the ability to be passed on to F_1 and segregate in F_2 progenies as expected, is shows that this marker co- segregated with the *Co-4*^{2} gene. However, correlating the phenotype and the genotype resulting from marker alleles is a prerequisite to estimate how much the marker is linked to the gene of interest. This gives a prediction of whether the marker will or not lost be lost in subsequent generations during selection. This Estimation requires

across of parental genotypes without phenotypic anomalies caused by gene interactions. Other similar studies have overcome these anomalies by replacing cultivar G 2333 with cultivars with one gene of resistance that originated from crosses of G 2333 and other susceptible cultivars (Awale and Kelly, 2001; Sousa *et al* ., 2014).

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1. CONCLUSION

This study gives the first report of the existence of a virulent *C. lindemuthianum* pathotype of race 7 in Uganda. Earlier diversity and characterization studies of *C. lindemuthianum* did not show the existence of this pathotype in the country. This has demonstrated the need for routine monitoring of emerging pathotypes and a wider characterisation of the pathogen.

Genotypic screening using molecular markers requires markers that are tightly linked with the gene and trait of interest by showing ability to co-segregate with the trait and reliability to predict the phenotype. The segregation of marker BM 211 and disease resistance did not fit the expected ratios and the marker did not seem appropriate for anthracnose disease selection. However, marker BMB 588 could clearly discriminate between homozygotes and heterozygotes with two bands of 200bp and 400bp which could separate on a normal agarose gel. In addition, this marker showed co-segregation with disease resistance by fitting the χ^2 expected ratio of 1:2:1. Marker BM 588 gave promising results as a marker tool that can rapidly be used for selection and transfer of the *Co-4*^{2} gene into other susceptible cultivars in marker assisted breeding programs.

6.2. RECOMMENDATIONS

In order to make the best use of findings of this study, the following are recommended;

1. The finding of the new severe race 7 of *C. lindemuthianum* in Uganda with such a limited collection calls for routine monitoring of emerging *C.lindemuthianum* and carrying out a wider characterization of the pathogen.

2. The linkage analysis of marker BMB 588 with disease resistance needs to be determined further to ensure its ability to select the gene in subsequent generations without being lost during genetic recombination. This could not be done in the segregating population which was used in this study because the donor parent, G 2333 had several resistance genes.

3. Marker BMB 588 should be validated in other bean cultivars outside this study scope. Marker presence should be tested on a range of cultivars and other important genotypes.

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