PYRAMIDING BEAN ANTHRACNOSE AND ROOT ROT RESISTANCE GENES INTO SUSCEPTIBLE MARKET CLASS COMMON BEAN VARIETIES USING SCAR MARKERS

By

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DECLARATION

This thesis is entirely my own work and has not been presented for a degree award in any university

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DEDICATION

To my dear late Son Elisha (*Yes, we are fully confident, and we would rather be away from these earthly bodies, for then we will be at home with the Lord. So whether we are here in this body or away from this body, our goal is to please him. 2Cor 5:8-9*)

and

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ABSTRACT

Bean anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. et. Magn) Lams.Scrib., is one of the most widespread and economically important fungal diseases of the common bean. It has a high genetic variability which renders single gene resistance inadequate since it is easily broken down. Root rots on the otherhand are caused by a complex of fungal pathogens of which the *Pythium* spp is the most abundant. This study, therefore, sought to address four objectives namely *a)* Determine the virulence diversity of *Colletotrichum lindemuthianum* in Uganda, *b)* Develop and evaluate advanced common bean lines with multiple resistance genes to anthracnose and *Pythium* root rot using sequence characterized amplified regions (SCAR) markers, *c)* Assess the effectiveness of pyramided genes in conferring broad resistance to bean anthracnose, and *d)* Determine the effect of gene pyramiding of anthracnose and *Pythium* resistance genes on plant agronomic characters.

To determine virulence diversity of *Colletotrichum lindemuthianum* in Uganda, 51 isolates collected from eight purposively selected districts were used to inoculate 12 differential cultivars under controlled conditions. Disease severity scores were used to designate races using the binary nomenclature system ANOVA revealed that variation due to cultivar and isolate effects was highly significant ($P \leq 0.001$). Race designation grouped the 51 isolates into 27 races. The highland districts of Sironko Mbale and Kabarole respectively had the highest race diversity. Races 2047 and 4095 were the most frequentRace 4095 had the broadest spectrum of virulence followed by 2479, 2047 and 2045 respectively. Cultivar G2333 showed the broadest resistance followed by Cornell, TU, and AB136 respectively. Therefore, *C. lindemuthianum* in Uganda was found to be diverse with 27 races samples from eight districts.

A cascading pedigree gene pyramiding scheme was used to pyramid three anthracnose and one *Pythium* resistance genes in four susceptible bean varieties K132, NABE4, NABE13 and NABE14. SCAR markers were used to facilitate the process through marker assisted selection (MAS). Sixty nine (69) $F_{4:6}$ families possessing different number of genes in different combinations were obtained. Out of these, three families inherited all the three anthracnose and one *Pythium* resistance genes. 14 families inherited two anthracnose and one *Pythium* resistance genes. Four *C. lindemuthianum* races namely 2047, 713, 767 and 352 were used to screen for resistance among the pyramided lines. Genotypes possessing all three anthracnose resistance

genes *Co-4*^{2}, *Co-5* and *Co-9* showed high level of resistance towards the four races. Three of four genotypes possessing *Co-4*^{2} and *Co-5* genes were resistant to all four races. Two of four genotypes possessing *Co-5* and *Co-9* were resistant to the four races. Therefore, pyramided lines were successfully developed using SCAR markers and pyramided genes conferred broad resistance to *C. lindemuthianum* although some pyramid groups were more effective than others.

To determine the effectiveness of pyramided genes in conferring broad resistance to bean anthracnose, races 352, 713, 767 and 2047 were used to inoculate 40 F_6 lines using the detached leaf method. Five gene pyramid groups namely *Co-4*^{2}*+Co-5+Co-9*, *Co-4*^{2}*+ Co-5*, *Co-4*^{2}*+Co-9*, *Co-5+Co-9*, Co-*4*^{3}*+Co-9* and four single gene group means were compared using Tukey's test. Results showed that the five pyramid group means were significantly different from each other (*P<0.01*). Of these, *Co-4*^{2}*+Co-5+Co-9* and *Co-4*^{2}*+Co-5* exhibited both a high degree and broad spectrum nature of resistance. The group *Co-4*^{3}*+Co-9* was the least effective. The single-gene groups were significantly different from each other (*P<0.01).* The *Co-4*^{2} and *Co-5* single genes conferred resistance to all the four races. The single gene *Co-4*^{2}was not significantly different from the best pyramid groups *Co-4*^{2}*+Co-5+Co-9* and *Co-4*^{2}*+Co-5* (*P<0.01*) while the *Co-5* was not significantly different from *Co-4*^{2}*+Co-5*, *Co-4*^{2}*+Co-9* and *Co-5+Co-9* gene pyramid groups (*P<0.01*). The study revealed that single genes may be as effective as pyramided genes in conferring broad resistance to anthracnose although pyramided genes may be more effective at suppressing disease symptoms.

To determine effect of pyramided genes on plant agronomic characters correlation and path coefficient analysis were carried out. Results showed that number of pyramided genes was significantly negatively correlated with seed weight per plant (0.17, *P<0.01*) number of pods per plant (-0.24, *P<0.05*), number of seeds per plant (-0.19, *P<0.1*). Path coefficient analysis revealed that only number of seeds per plant and 100-seed weight had significant positive direct effects on seed weight per plant. Number of pyramided genes had a significant negative indirect effect (-0.25) on seed weight per plant via number of seeds per plant. Therefore, a yield penalty may be incurred in gene pyramiding programs.

CHAPTER ONE

Introduction

1.1 Background

Phaseolus vulgaris (L.), the common bean, is a diploid (2n = 22) with a genome size ranging from 450 to 650 mbp/ haploid genome (Bennett and Leitch, 1995; Broughton *et al*., 2003). It was domesticated more than 7,000 years ago in two centers of origin namely Mesoamerica (Mexico and Central America) and the Andean region and was introduced in sub-Saharan Africa several centuries ago by Portuguese traders (CIAT, 2001). 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 (Schwartz and Pastor-Corrales, 1989).

Nutritionists characterize the common bean as a nearly perfect food because of its high protein content and generous amounts of fiber, complex carbohydrates, and other dietary necessities (CIAT, 2001). A single serving of beans provides at least half the US Department of Agriculture's recommended daily allowance of folic acid, one of the vitamins in the B complex that is especially important for pregnant women (CIAT, 2001). It also supplies 25 to 30% of the recommended levels of Iron (Fe) and meets 25% of the daily requirement of Magnesium (Mg) and Copper (Cu) as well as 15% of the Potassium (K) and Zinc (Zn) (CIAT, 2001). While beans are considered a low status food, the "meat of the poor" (Pachico, 1993), they are the most important source of proteins for the Ugandan populations providing up to 45% of the total human dietary requirements (Mauyo *et al*., 2007).

Although beans are largely produced for subsistence, mainly by women farmers, in sub-Saharan Africa (Wortmann *et al.,* 1999), their short maturity period (3 months on average), ease of handling and storability make them a coveted cash crop for small-scale farmers. Thus beans play an essential role in the sustainable livelihoods of smallholder farmers and their families, providing both food security and income. Beans are also highly valued by the poor because all parts of the plant can be consumed; the grain is eaten fresh or dried, the leaves are used as vegetables and the stalk is used to make soda ash (David, 1999).

East Africa has the highest bean production in sub-Saharan Africa at 1,297,000 tons per annum (Wortmann *et al*., 1999), the largest producing countries being Kenya, Uganda, D.R Congo, Burundi, Tanzania, Rwanda and Ethiopia (Pachico, 1993) respectively. In Uganda, the dry bean is the most important legume crop, which occupies an important niche in the Uganda Agricultural sector and farm household economy (Opio *et al*., 2001). The land area planted with beans increased from 495ha in 1991 to 630ha in 1999 (FAO, 1999), however, in the past 10 years, yields of common bean in South West Uganda have declined by about 50% due to declining soil fertility, insect pests and diseases (Spence, 2006), and use of low yielding and disease susceptible varieties. The major bean production constraints in Africa include poor soil fertility with low available nitrogen and phosphorus, aluminum and manganese toxicity, soil moisture deficits pests and diseases (Wortmann and Allen, 1994).

The major fungal diseases of beans in Africa are angular leaf spot, anthracnose, root rots rust, bacterial blight and bean common mosaic (Schwartz and Pastor-Corrales, 1989; (Wortmann and Allen, 1994; Beebe, 2012). Bean Anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. et. Magn) Lams. Scrib., is one of the most important diseases of beans in Uganda (Opio *et al.*, 2001). It is estimated that each 1% increase in anthracnose incidence manifests into seed yield loss of 9kg/ha (Wortmann and Allen, 1994). The disease is spread in nearly all bean growing regions and often causes severe damage, which affects yield, seed quality, and marketability of beans (Bailey *et* al., 1992). Being mainly a seed borne disease (del Rio and Bradley, 2004), bean anthracnose is easily spread in Uganda as farmers depend highly on farm saved seed and exchange of seed is common (Leaky and Simbwa-Bunya, 1972; Opio *et* al., 2001) and as such disease severity on farmer fields is perceived to be high. The disease devastates farmers' fields leading up to 70% marketable yield losses especially when susceptible cultivars such as K132, K20 and Kanyebwa are used (Nkalubo *et al*., 2007). On the other hand, bean root rots are among the most devastating constraints to bean production leading up to 100% yield losses where susceptible varieties are used (CIAT, 2005; Mukankusi, 2007). They are associated with nutrient depleted soils and identified through the scantiness of roots on plants that have died (Spence, 2003). The most important root rots in Uganda are caused by the fungi, *Pythium spp* (Mukalazi, 2004) and *Fusarium spp* (Tusiime, 2003).

Both cultural management and use of fungicides can be used to control the two diseases, however, it is envisaged that the use of resistant varieties and introgression of resistant genes into the popular but susceptible cultivars would be more cost-effective in controlling the diseases among small scale farmers (Nkalubo *et al.*, 2009). If not controlled, bean anthracnose and root rot diseases will gravely undermine the potential of beans as a food security crop, source of income and as a main source of dietary protein and other nutrients for the majority of Ugandans.

1.2 Statement of the problem

Bean anthracnose (*Colletotricum lindemuthianum*) has been reported to possess a high degree of genetic variability in different bean growing regions in the world (Sherman *et al.,* 1999). The highest pathogenic variation of this fungus has been observed in the centers of origin of its host, the Mesoamerican and Andean regions (Pastor-Corrales et al., 1993). Until recent years most sources of resistance to bean anthracnose have sooner or later been overcome by the emergence of new pathotypes. In Uganda, Nkalubo (2006) identified the existence of the delta (23), beta (130) gamma (102) and five other previously undocumented races. Leaky and Simbwa-Bunya (1972) had earlier reported existence of the races α, β, γ, δ, ε and ξ in Uganda. Due to the high degree of pathogen variability and the continual emergence of new races, single gene deployment is not an effective strategy to control bean anthracnose. There are, however, a few available resistant varieties such as G2333, PI207262 and the moderately resistant local variety K131, but these have poor marketability in Uganda, while the available market-class bean cultivars namely K132, NABE 4, NABE 13 and NABE 14 are all susceptible to anthracnose. This has created the need to explore gene pyramiding of multiple anthracnose resistance genes with complementary spectra of resistance as a strategy that may circumvent the problem of *C. lindemuthianum* variability.

Root rots, on the other hand, are among the most devastating constraints to bean production especially in the South Western highland regions of Uganda causing severe damage to the root system of infected plants which may result into 100% yield loss. Root rot caused by *Pythium Spp* is one of the most destructive in East and Central Africa and in Uganda, it is the most predominant in the root rot complex.

Owing to these two diseases some farmer and market-preferred landraces like Kanyebwa, Kahura and Masindi yellow are becoming more difficult to produce and are on the verge of extinction. The co-existence of both anthracnose and root rot diseases on the bean crop in farmers' fields and the ability of these pathogens to overcome incorporated resistances have greatly undermined previous breeding efforts leading to severe yield losses in bean varieties that had been previously released with a single pathogen resistance. This has created the need for pyramiding of anthracnose resistance genes which have complementary spectra as a strategy to circumvent the problem of pathogen variability and simultaneously introgress multiple disease resistance genes within the same variety. The new genotypes created will be resistant to both bean anthracnose and root rot diseases.

Conventional gene pyramiding is extremely time consuming and may fail to detect epistatic genes among segregating progenies. The development of modern plant molecular and quantitative genetics in the last two decades has the potential to revolutionize what has mostly been conventional breeding and has also widened several aspects of the practical application of gene pyramiding. Marker-assisted gene pyramiding is currently the method of choice for inbred line development targeted at improving traits controlled by major genes. Use of molecular markers to track pyramided genes also considerably reduces the breeding period involved in the pyramiding program. Marker-aided selection is, therefore, a very useful approach which helps to pyramid resistance genes for different races or biotypes of a disease or insect pest in order to develop lines with multi-race or multi-biotype resistance. Both conventional and marker assisted selection were used in this study to effectively screen large populations and also increase precision of selecting for materials with multiple resistance genes to bean anthracnose and *Pythium* root rot.

1.3 Justification

Beans are a major food and cash crop for the majority of Ugandan farmers and consumers and it accounts for 7% of national agricultural GDP, ranking fifth behind bananas, cassava, sweet potatoes, and maize (CIAT, 2008). However, beans are notoriously susceptible to diseases

which are responsible for significant yield and economic losses in Africa with an estimated total of 2,288,000 tons lost annually (Wortmann *et al*., 1998).

Bean anthracnose and bean root rots are the two most devastating diseases in Uganda with yield losses of up to 70% (Nkalubo, *et al*., 2007) and 100% (Mukankusi, 2007) respectively. Anthracnose has been reported to have a high genetic variability (Pastor-Corrales *et al*., 1995; Sherman *et al*., 1999), that results in the emergence of new races which easily breakdown single gene resistance. Because of this new sources of resistant germplasm are continually needed (Menezes and Dianese, 1988).

These two pathogens have singly or in combination broken down the resistance in previously released varieties with single gene and single pathogen resistance, thus creating an urgent need for the introgression of multiple disease resistance genes within the same variety such that the new genotypes will have resistance to both bean anthracnose and root rot diseases. There are 10 major genes (*Co-1 – Co-13*) responsible for controlling anthracnose resistance in dry beans (Kelly and Vallejo, 2004) and therefore, there is need to find out which genes are most effective in controlling *C. lindemuthianum* races in Uganda.

Gene pyramiding will make it possible to breed cultivars with broad resistance to different strains of anthracnose. The process of pyramiding genes will be made more efficient through marker assisted selection (MAS) using Sequence Characterized Amplified Region (SCAR) markers. MAS is a proven technology which can provide an effective and efficient breeding tool for detecting, tracking, retaining, combining, and pyramiding disease resistance genes (Miklas and Kelly, 2002). It is, however, proposed as a complementary tool in crop improvement where conventional selection of complex traits has been difficult (Xu, 2002).

Understanding the diversity and distribution of *C. lindemuthianum* races in Uganda is important to bean breeders and pathologists to make better choices on which resistance genes and gene pyramids to deploy in Uganda. Information on genetic variability within segregating populations, effectiveness of pyramided genes in conferring broad resistance and effect of gene pyramiding on plant morpho-agronomic traits is important in guiding breeders in designing

more effective pyramiding strategies. Simultaneous introgression of anthracnose and *Pythium* spp resistance genes is important in generating genotypes that possess multiple disease resistance to the two diseases. This offers bean farmers an opportunity to have varieties that are more productive through resistance to multiple diseases that simultaneously occur on their fields. The new bean genotypes with multiple resistance genes to bean anthracnose and Pythium root rot were developed and are available as germplasm for national and regional evaluation and release and/ or for incorporation into breeding programs of other countries.

1.4 Overall objective

Pyramid multiple anthracnose and *Pythium* root rot resistance genes in susceptible market class genotypes using SCAR markers and assess the effectiveness of pyramided genes in conferring broader resistance to bean anthracnose.

1.5 Specific objectives

1. Determine the virulence diversity of *Colletotrichum lindemuthianum* in Uganda
2. Develop and evaluate advanced common bean lines with multiple resistance genes to anthracnose and *Pythium* root rot through SCAR marker aided gene pyramiding
3. Assess the effectiveness of pyramided genes in conferring broad resistance to bean anthracnose
4. Determine effect of pyramiding anthracnose and *Pythium* root rot resistance genes on plant agronomic characters among different common bean genotypes

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CHAPTER TWO

Literature review

2.1 Introduction

Colletotrichum lindemuthianum (Sacc. & Magnus) Lams. Scrib. is the causal agent of bean anthracnose, a world-wide bean disease which still devastates bean crops in developing countries (Benard-Capelle *et al*., 2006). It is one of the most widespread and economically important fungal diseases of common bean (*Phaseolus vulgaris* L.) with the potential to cause complete yield loss when susceptible genotypes are used and conditions are favorable to the pathogen (Young and Kelly, 1996). In Uganda anthracnose is reported to cause about 70% yield losses to farmers when susceptible cultivars are used (Nkalubo *et al.* 2007).In addition, the incidence of root rots in Uganda is spreading and is increasing as the pressure on the land is increasing. Root rot, caused by the fungi, *Pythium*, *Rhizoctonia* and *Fusarium* species, is such a serious problem that in some seasons it is responsible for entire crop failures (Spence, 2003).

2.2 Pathogen taxonomy and Cytogenetics

The taxonomical classification of *Colletotrichum lindemuthianum* has been a difficult and confused task (Martínez-Pacheco *et al*., 2009). The fungus had been named with different synonyms throughout the years and could hardly be identified through classical taxonomy, because it produces acervuli with or without fruiting body depending on the quality and amount of substrate (Sicard et al., 1997a). The causal agent for bean anthracnose was first designated with several names such as *Gleosporium lindemuthianum* (1878), *Septoria legunimun* (1882), *Septoria legunimun* var *phaseolorum*, *Colletotrichum lindemuthianum* (1894), *C. lagenarium* (1893), *Gleosporium lindemuthianum* (1894) and *Glomerella lindemuthiana* (1993). Recently, the causal agent of the common bean anthracnose disease has been clearly identified as a fungus that presents imperfect and perfect forms called *Colletotrichum lindemuthianum* and *Glomerella cyngulata f.sp phaseoli*, respectively (Martínez-Pacheco *et al*., 2009). Following the proposal by Alexopoulos and Mims (1979), most authors agreed that *C. lindemuthianum* belongs to the Family, Melanconiaceae; Order, Melanconiales; Sub Class, Coelomycetidae; Class, Deuteromycetes; Sub Division, Deuteromycotina; Division Amastigomycota; Kindom Myceteae; Super Kindom, Eucariota.

O'Sullivan et al. (1998) reported that the study of genome structure with molecular cytogenetic analysis, normally conducted during meiosis, was not possible in *C. lindemuthianum* due to absence of the sexual form. Roca et al. (2003) conducted cytogenetic and morphological studies of *C. lindemuthianum*. They revealed evidence of genomic variation and observed sexual reproduction *in vitro*. They observed that nucleus divisions as well as ascospore maturation were asynchronous and also observed chromosomal polymorphism in conidia. O'Sullivan et al. (1998), using Pulse Field Gel Electrophoresis (PFGE), found that total chromosome numbers varied from nine to 12 and suggested that the chromosomes belonged to two distinct classes. The first class had a variable number of small chromosomes (<2.5Mb), some of which were probably below the level of detection by light microscopy. The second class of chromosomes was a set of unresolved chromosomes larger than 7Mb with a variation of four to nine large chromosomes, which could easily be viewed under light microscopy.

2.3 Lifecycle of *Colletotrichum lindemuthianum*

The fungus presents a complex life cycle which has various development phases and two ways to take food. In its imperfect form, *C. lindemuthianum* reproduces asexually with the spores produced inside acervuli and immersed in water soluble pre-formed mucilage (O'Connell *et al*., 1996). The development of fungal spores shows a biphasic behavior which suggests two lifestyles, as a saprophyte and as a biotroph. As a saprophyte, the fungus grows in any carbon source including crystalline cellulose which may be easily converted into fuel molecules by extracellular lytic enzymes. On the other hand, as a biotroph, the fungus has the ability of feeding on nutrients outright off living plants (Martínez-Pacheco *et al*., 2009).

As a saprophyte, the fungal spore germination process begins with the spore adhering to the plant surface under adequate humidity conditions; specifically, correct aqueous content in the spore envelope (mucilage). At this level, the oval spores of the fungus round off due to water absorption and active growth. Later, the germinating tube is formed (germinule phase), and the hyphae elongates to colonize the substrate. The aerial mycelia appear; then the fungal reproductive structures are formed where the spores are stored. Finally, their life cycle is completed and it starts all over again (Martínez-Pacheco *et al*., 2009).

2.4 Disease symptomatology

Symptoms of the attack by *C. lindemuthianum* are commonly known as anthracnose and comprise dark, sunken, lenticular necrotic lesions containing the acervuli of the pathogen. The initial symptoms of anthracnose appear as a dark brown to black lesion along the veins on the underside of the leaves (Buruchara *et al*., 2010). Susceptible genotypes may exhibit symptoms on all aerial parts of the plant (CAB International, 2004). Seed borne infection usually induces dark brown to black eye-shaped lesions longitudinally on the hypocotyls and cotyledons. On the hypocotyls the lesions enlarge and may cause the stem to break. On older stems, lesions are sunken and may reach 5 – 7mm. Earlier signs of leaf infections occur on the petiole and on the lower leaf surface, where small lesions extend along the veins developing a brick red to purple red coloration becoming black. Later similar symptoms appear on the upper leaf surface (CAB International, 2004). The most striking symptoms occur on the pods where pod lesions are typically sunken and are encircled by a slightly raised black ring surrounded by a reddish boarder. Under severe infection, young pods may shrivel and dry prematurely (Buruchara *et al*., 2010). The fungus may also penetrate the seed coat and become firmly established within the seed which, when planted serve as the source of infection in the succeeding crop (Buruchara *et al*., 2010).

2.5 Disease epidemiology

Infected seed supply the primary inoculum for disease development and secondary spread (Tu, 1983). Early seedling infection often leads to high disease severity on the same plant and greater chances for spread and infection of neighboring plants (Zaumeyer and Thomas, 1957). Spread of the disease from a focus to other susceptible plants is influenced by environmental factors responsible for inoculum dispersal, such as rain-splash or wind-driven rain and cultural practices such as intercropping and growing of mixtures (Tu, 1983). The number of foci of the initial inoculum has been shown to be linearly related to disease incidence on leaves but not on pods. *C. lindemuthianum* can survive between seasons on seeds and plant debris (CAB International, 1998). Length of survival is influenced by environment, especially moisture and temperature. The fungus remains viable up to five years in air-dried pods or seeds stored at 4^{o}C. High relative humidity or free water is essential for dissemination and germination of conidia,

infection, incubation and subsequent sporulation (CAB International, 2004). The disease is most severe in moderate temperatures of between 13 - 26^{o}C (Zaumeyer and Thomas, 1957), 17-22^{o}C (Holliday, 1980), 17 – 24^{o}C (Tu and Aylesworth, 1980) and under wet conditions. Good infection is obtained when inoculated plants are incubated at or near 100% relative humidity at 22^{o}C for 5 – 7 days after artificial inoculation (Pastor-Corrales *et al.*, 1985). It thrives in relatively cool and wet regions of the tropics and sub-tropics, and is endemic in southern and central Brazil, the highlands of Peru, Ecuador, Colombia, Costa Rica, Nicaragua, Honduras, Guatemala, Mexico, and Central and East Africa (Pastor-Corrales *et al.*, 1995).

2.6 Diversity of *C. lindemuthianum*

High pathogenic variability in *C. lindemuthianum* has been detected in many areas of Latin America, which is the center of origin of common bean (Pastor-Corrales *et al*., 1995). Recently, three centers of diversity have been reported to have the highest number of *C. lindemuthianum* races. The first is located in Mexico and the southern part of the United States, the second in Columbia, Peru and Brazil, and the third in the Central America countries (Balardin *et* al., 1997; Sicard *et* al., 1997b).

Barrus made the first report of α (alpha) and β (beta) races in 1911, Burkholder described the γ (gamma) race in 1923 while in 1942 Andrus and Wade reported the δ (delta) race; Hubbeling also reported the δ race in 1974; Schnock in 1974 indicated κ (kappa) race presence; and Tu (1992) proposed existence of clearly different physiological races of *C. lindemuthianum*, including α, β, δ, ε, γ, κ and λ (lambda). A standardized binary nomenclature system based on a set of 12 differential cultivars (Table 2.1 below) was identified to characterize virulence (Singh *et al*., 1991). Kelly et al. (1994) reported two races, 7 and 73, in Michigan and North Dakota. The origin of these races was previously unknown and it appears race 73 had existed in Michigan State University bean breeding lines since 1991 but was not detected until 1993. Since race 73 overcomes the *Are* gene and race 7 overcomes the *A* gene, both of which were extensively used in the breeding program, the authors suggested use of gene pyramiding using molecular markers as a disease resistance strategy, since the *A*/*Are* gene combination affords resistance to both races.

Using the binary nomenclature system, 38 races were reported in Mexico (Rodriguez, 1991), Seven races were identified in a group of 10 isolates from Nicaragua (Rava *et al*., 1993), 33 were characterized from a group of 178 isolates from Colombia (Pastor-Corrales *et al*., 1995) and three races were described in the United States (Balardin and Kelly, 1996).

Table 2.1: Binary codes, resistance genes and gene pool of differential cultivars used to characterize *Colletotrichum lindemuthianum*

Differential cultivars	Binary Code	Resistance gene	Gene pool*
Michelite	1	*Co-11*	MA
Michigan Dark Red Kidney	2	*Co-1*	A
Perry Marrow	4	*Co-1*^{3}	A
Cornell 49-242	8	*Co-2*	MA
Widusa	16	*Co-1*^{5}	A
Kaboon	32	*Co-1*^{2}	A
Mexico 222	64	*Co-3*	MA
PI 207262	128	*Co-4*^{3}, *Co-9*	MA
TO	256	*Co-4*	MA
TU	512	*Co-5*	MA
AB 136	1024	*Co-6*	MA
G2333	2048	*Co-4*^{2}, *Co-5*^{2}, *Co-7*	MA

*A = Andean; MA = Meso-American; Source: Awale et al., (2007)

Out of 45 isolates collected from five wild common bean populations located in their South-Andean center of origin, Sicard et al. (1997a) identified 45 polymorphic markers using RAPDS and characterized 15 pathotypes. Sicard et al. (1997b) studied the population subdivision of *C. lindemuthianum* in three regions located in the three centers of diversity of its host, *Phaseolus vulgaris*. They used RAPD markers, restriction endonuclease analysis and virulence on a set of 12 cultivars to assess genetic diversity of *C. lindemuthianum* strains isolated in Mexican, Ecuadorian and Argentinean wild common bean populations. Based on molecular and virulence markers, strains isolated from wild common bean populations were divided into three groups corresponding to host gene pools. Their findings suggested that Andean populations have been

derived from the Mesoamerican center and that different strains adapt on cultivars of the same geographical origin.

Mahuku and Riascos (2004) assessed genetic variability of 200 *Colletotrichum lindemuthianum* isolates collected from the Andean and Mesoamerican bean varieties and regions. They reported high levels of pathotypic (90 pathotypes) and genetic diversity (0.97) among the 200 isolates, revealing that *C. lindemuthianum* is a highly diverse pathogen. Their findings, however, did not reveal clustering of isolates according to common bean gene pools or centers of diversity like the findings of Sicard et al. (1997b) demonstrated. Both studies, however, agree that *C. lindemuthianum* originated and was disseminated from the Mesoamerican region. Mahuku and Riascos (2004) suggested stacking of major resistance genes as the best option for developing cultivars with durable resistance to bean anthracnose.

Padder et al. (2007) analyzed five *C. lindemuthianum* populations for genetic diversity on the basis of allele frequencies of 12 RAPD markers using Nei's genetic diversity formulae. They reported diversity within each population to be high with values ranging from 0.26 – 0.31. A broader array of physiological races has been reported in Europe (Tu, 1992), Latin America (Pastor-Corrales and Tu, 1989; Beebe and Pastor-Corrales, 1991) and Africa (Pastor-Corrales and Tu, 1989).

In Uganda, Leaky and Simba-Bunya (1972) identified races that appeared to conform to the α, β, δ and γ races. Recent work on race identification in Uganda by Nkalubo (2006) confirmed presence of races δ (23), β (130) and γ (102) and reported five new races previously undocumented, 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.

The high genetic variability of *C. lindemuthianum* has, therefore, resulted in continuous breakdown of resistance in commercial cultivars (Menezes and Dianese, 1988; Pastor-Corrales et al., 1993; Kelly et al., 1994), has complicated the use of host resistance genes (Melotto et al.,

2000) and has made it difficult to develop or design effective anthracnose control strategies (Mahuku and Riascos, 2004).

2.7 Disease management and control

Use of clean seed was proposed by Fernandez et al. (1987) as a powerful control measure in areas where strict standards of seed health can be maintained since this reduces the inoculum for secondary spread. Seed treatment with hot water at 50^{o}C for 20 minutes was reported by Hernandez and Mendoza (1987) to be effective in inactivating seed borne *C. lindemuthianum* without affecting seed viability. Burial of plant debris, rogueing of diseased plants, removal of diseased basal leaves at weeding and crop rotation also decrease disease incidence (Truttman and Kayitare, 1991). Use of cultivar mixtures plays an important role in buffering against the disease and stabilizing yields (Truttman et al., 1993). It is commonly practiced in the great lakes region of Central Africa, Malawi, Southern Tanzania and Uganda (CAB International, 1998). Chemical control of anthracnose has also been reported to be effective with satisfactory results reported after use of foliar application of benomyl, carbendazim and difolatan. Other chemicals that have been used against anthracnose include zaneb, captafol and maneb. Chemical control is however, limited by possible development of resistant biotypes, limited effectiveness and high cost of chemicals (Leaky and Simbwa-Bunnya, 1972; Tu and McNaughton, 1980; and Pastor-Coralles and Tu, 1989).

2.7.1 Host-plant resistance as a control method

Host plant resistance is the most effective and appropriate strategy for the control of anthracnose, which has been widely deployed in Europe and Canada (CAB International, 2004). Resistance mechanisms have been identified in the common bean (Pastor-Corrales *et al.*, 1985) and extremely high levels of genetic resistance, controlled by a single or few major genes, have been reported (Peloso *et al*., 1989). However, because of the highly variable nature of the pathogen, which results in the emergence of new races, this resistance is not durable and new sources of resistant germplasm are continually needed (Menezes and Dianese, 1988), and in addition, new cultivars have to be developed continuously (Pastor-Corrales *et al.*, 1985).

Kelly and Vallejo (2004) reported nine major allelic genes, *Co-1* to *Co-10* that condition resistance to anthracnose. Recently, Lacanallo et al. (2010) reported that anthracnose resistance is conditioned primarily by thirteen major independent genes, *Co-1* to *Co-13*. However, of these genes *Co-9/Co-3*^{3} and *Co-7/Co-3* are allelic (Méndez-Vigo et al., 2005; Sousa *et al*., 2009). With the exception of the recessive *co-8* gene, all the others are dominant genes and multiple alleles exist at the *Co-1, Co-3* and *Co-4* and *Co-5* loci. A reverse of dominance at the *Co-1* locus suggests that an order of dominance exists among individual alleles at this locus. The nine resistance genes *Co-2* to *Co-11* are Middle American in origin and *Co-1, Co-12* and *Co-13* are from the Andean gene pool. Eight resistance loci have been mapped to the integrated bean linkage map and *Co-1* resides 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-9/Co-3*^{3} and *Co-10* on B4 but are not linked. The three independent genes in G2333 are allelic to previously mapped genes at the *Co-3, Co-4* and *Co-5* loci. *Co-7* appears to be another allele at *Co-3* locus (Sousa et al., 2009). *Co-4*^{2} is the most broadly resistance allele at the *Co-4* locus (Young et al., 1998); and *Co-5*^{2} is a second allele at *Co-5* locus that has a more restricted resistance pattern than the original *Co-5* allele in TU (Vallejo and Kelly, 2009).Three *Co-* genes map to linkage groups B1, B4 and B11 where clusters with genes for rust resistance are located. In addition there is co-localization with major resistance genes and Quantitative Trait Loci (QTL) that condition partial resistance to anthracnose. Molecular markers linked to the majority of the major *Co*-genes have been reported and these provide an opportunity to enhance disease resistance through marker assisted selection and gene pyramiding.

Table 2.2: Bean anthracnose resistance genes, their sources and linked markers

Gene symbols		Genetic sources	Gene pool	Linked markers	Map location
New	**Original**				
Co-1	*A*	MDRK	Andean	$OF10_{530}$	B1
Co-1^{2}		Kaboon		SE_{ACT}/M_{CCA}	
Co-1^{3}		Perry Marrow			
Co-1^{4}		AND 277			
Co-1^{5}		Widusa		$OA18_{1500}$	

Co-2	*Are*	Cornell 49242	MA	$OQ4_{1440}$, $OH20_{450}$, $B355_{1000}$	B11
Co-3	*Mexique 1*	Mexico 222	MA	NA	B4
Co-3²		Mexico 227	MA		
Co-3³	*Co-9*	BAT 93	MA		
Co-4	*Mexique 2*	TO	MA	SAS13, SH18	B8
Co-4²		SEL 1308	MA	SBB14, OC8	
Co-4³		PI 207262	MA	OY20	
Co-5	*Mexique 3*	TU	MA	$OAB3_{450}$	B7
Co-5²		SEL 1360	MA	SAB3	
Co-6	*Q*	AB 136	MA	$OAH1_{780}$, $OAK20_{890}$	B7
Co-7	*NA*	HI, MSY 7-1, G 2333	MA	NA	NA
co-8	*NA*	AB 136	MA	OPAZ20	NA
Co-9	*NA*	BAT 93	MA	SB12	B4
Co-10	*NA*	Ouro Negro	MA	F10	B4
Co-11	*NA*	Mitchelite	MA	NA	NA
Co-12	*NA*	Jalo Vermelho	Andean	NA	NA
Co-13	*NA*	Jalo Listras Pretas	Andean	OPV20 700	B3

NA = Not Available; MA = Meso American; Source: Kelly (2010)

The 10 *Co*-genes are represented in the anthracnose differential cultivars, but are present as part of a multi-allelic series or in combination with other *Co*-genes, making the characterization of more complex races difficult. Although the *Co*-genes behave as major Mendellian factors, they most likely exist as resistance gene clusters as has been demonstrated on the molecular level at the *Co-2* locus (Kelly and Vallejo, 2004).

The common bean landrace G 2333 carries a three gene pyramid for anthracnose resistance with *Co-4²*, *Co-5* and *Co-7* genes. It is a landrace cultivar called Colorado de Teopisca from Chiapas, Mexico and a result of naturally occurring gene pyramid for anthracnose resistance. The first report on the nature of resistance in G 2333 suggested that two independent dominant genes

were present. A 15:1 (resistant: susceptible) ratio was observed after inoculation of an F2 population of G2333/Pijao with race 521 (Pastor-Coralles *et al*., 1994). Vallejo and Kelly (2009) isolated and characterized the third resistance gene *Co-7* in G2333.

The cultivar PI 207262 (Tlalnepantla 64) is an important source of resistance gene to common bean anthracnose Inheritance studies carried out by Alzate-Marin et al. (2007) using crosses involving this cultivar showed that the two independent genes confer resistance to anthracnose. Allelism tests showed that the genes are located at distinct loci from previously identified resistance genes *Co-1, Co-2, Co-3, Co-5, Co-6* and *Co-10*. They observed no segregation in relation to *Co-4*, *Co-4*^{2}, *Co-9* and to the gene present in cultivar Widusa, indicating that PI 207262 harbors two anthracnose resistance genes, *Co-4* and *Co-9.* The *Co-4* allele of PI 207262 was suggested to be different from *Co-4* and *Co-4*^{2} and they proposed *Co-4*^{3} as the genetic symbol for this resistance allele.

The cultivar AB 136, one of the 12 differentials for anthracnose in common beans has been shown to be resistant to all 25 races of *C. lindemuthianum* identified in Brazil. A single gene designated *Co-6* governs the anthracnose resistance of AB 136 (Alzate-Marin *et al.,* 1997). Vidigal (1994) suggested that a single gene confers anthracnose resistance in AB 136 to pathotypes δ (delta) and κ (kappa). When researchers at CIAT, Cali, Colombia, evaluated a number of common bean lines for resistance to many races of anthracnose, the line AB 136 was found to be one of the most resistant lines (Pastor-Corrales, 1992).

In Uganda, Nkalubo et al. (2009) have shown that one to three major genes were involved in conferring resistance to bean anthracnose. The assessment of the resistance genes carried out by Nkalubo (2006) within the differential cultivars against Ugandan pathotypes has shown that none was pathogenic to differential cultivars AB 136 containing genes *Co-6* and *co-8*; and G2333 containing genes *Co-4*^{2}, *Co-5* and *Co-7*. They therefore, suggested that genes *Co-4* to *Co-8* are responsible for resistance to Ugandan anthracnose pathotypes and further recommended the use of resistant cultivars AB 136 and G2333 in the introgression of anthracnose resistance into susceptible cultivars. The resistance genes in these parents, however, were not dissected to show effect of each individual gene against *C. lindemuthianum*, moreover,

there could be complementary gene action of these genes in combination. There is, therefore, need to carry out molecular dissection of the resistance genes in these parents and determine their individual effect against *C. lindemuthianum*. In the study, cultivars PI 207262 and G2333 were used as the sources of resistance genes. The pyramiding of resistance genes which have complementary spectra of resistance has been suggested as a strategy to circumvent the problem of pathogen variability (Young and Kelly, 1997). There is need, therefore, to investigate gene action of new gene combinations arising from gene pyramiding programs, in order to avoid some gene combinations that may be suppressive.

2.7.2 Inheritance of resistance

The use of genetic resistance to control anthracnose is hindered by existence of various physiological races of the pathogen, which makes it important to keep an up-to-date knowledge to explore the genetic variability in common bean and develop new cultivars (Rava *et al*., 1993). However, to explore the genetic variability in common beans efficiently, there is need to understand how resistance is inherited and the type of gene action possessed by the resistance genes.

There are various sources of resistance to anthracnose as indicated in Table 2 above. The *Are (Co-2)* gene confers resistance to race 69 (epsilon, ε), and has been widely used in common bean breeding programs (Poletine *et al.*, 1999). The dominant resistance gene present in cultivar AB136 was first described by Schwartz et al. (1982) and inheritance studies showed that only the *Q (Co-6)* gene, which was independent of others previously characterized genes, was present in this cultivar (Young and Kelly, 1996; Goncalves-Vidigal *et al*., 1997). Pastor-Coralles *et al*. (1994) reported resistance in cultivar G 2333 to be controlled by two independent dominant genes with equivalent effects. However, Young *et al*. (1998) detected three different dominant resistance genes, *Co-4*^{2}, *Co-5* and *Co-7*. The line G2333 has been reported to be resistant to all Brazillian isolates and all European and North American races (Pastor-Corrales and Tu, 1989; Balardin and Pastor-Corrales, 1990; Balardin et al., 1990; CIAT, 1990).

Poletine et al. (1999) crossed cultivar PI 207262, known to be resistant to race 69, with susceptible cultivars Michelite and Perry Marrow and resistant cultivars Dark red Kidney,

Cornell 49242, AB136 and G2333. Reaction of F1 and F2 populations showed that Dark Red Kidney, Cornell 49242 and AB136 cultivars respectively posses the dominant resistance genes *A (Co-1), Are (Co-2)* and *Q (Co-6)*. Their results indicated segregation of three dominant resistance genes, two of them present in G2333 (*Co-5* and *Co-7*) and another present in PI 207262, which is located in *Co-4* locus. However, they were not able to distinguish between the gene *Co-4*^{2} in G2333 from the gene *Mexique 2* (*Co-4*) in PI 207262, because they are located in the same locus making identification using traditional screening methods impossible. Moreover, Menezes (1985) considered PI 207262 cultivar similar to TO, in relation to the susceptibility to ζ (zeta) race. Based on this affirmation, Gonçalves-Vidigal (1994) also considered PI 207262 to be carrying dominant gene of resistance present in locus *Co-4*.

The allele *Co-4*^{2} in G2333 is already tagged by SCAR markers SAS13 (Young et al., 1998; Kelly et al., 2003), SBB14 and SH18 (Awale and Kelly, 2001; Kelly et al., 2003), while the alleles *Co-4* in TO and *Co-4*^{3} are tagged by the SAS13 marker. In populations where both *Co-4*^{2} and *Co-4*^{3} are segregating, there is need to screen samples using both SBB14 and SAS13 in order to differentiate between the two alleles. There is also need to identify and develop a marker that specifically tags the *Co-4*^{3} allele.

Alzate-Marin *et al*. (1997) studied the inheritance of resistance in differential cultivar AB 136 to races 89, 64 and 73. They made crosses with susceptible cultivars Michelite (89), Mexico 222 (89) and Cornell 49242 (73) and inoculated F1, F2 and backcross-derived plants with the respective race under environmentally controlled greenhouse conditions. Results showed that single dominant genes control resistance to races 89 and 64. For race 73 results suggested that two independent genes may determine resistance in AB 136, one dominant *(Co-6)* and the other recessive *(co-8)*. They therefore concluded that genotypes *Co-6_* or *co-8co-8* would condition resistance whereas susceptibility would be present in genotypes *co-6co-6 Co-8_*. This cultivar, AB 136, should, therefore, be adopted for use in breeding programs aiming at improving resistance to anthracnose.

Goncalves-Vidigal et al. (2007) studied the allelic relationships of anthracnose resistance in the differential cultivar Michelite to races 8 and 64. Crosses were made between Michelite and

Mexico 222 cultivars. The F2 population was inoculated with race 64 and segregation fitted in the ratio 3:1, indicating the presence of a dominant gene conditioning resistance to race 64. Allelism tests carried out with F2 populations derived from crosses between Michelite and AB 136, AND277, BAT93, Cornell 49-242, G2333, Kaboon Mexico 222, Michigan Dark Red Kidney, Ouro Negro, Perry Marrow, PI 207262, To, Tu and Widusa. Results showed that the gene in Michelite is independent from *Co-1, Co-2, Co-3, Co-4, Co-5, Co-6, Co-7, Co-9* and *Co-10* genes. The authors designated the anthracnose resistance gene in Michelite as *Co-11*.

2.8 Bean root rot occurrence, diversity and inheritance of resistance

On the other hand, bean root rots are caused by a complex of fungal pathogens, resident in the soil and includes species of *Pythium*, *Fusarium solani f sp phaseoli*, *Rhizoctonia solani* and *Sclerotium rolfsii* and of these *Pythium* and *Fusarium* species have been reported to be more important, on the basis of spatial distribution, damage and effect on yield, particularly, under high (soil moisture) rainfall and low temperatures, which favor disease development (CIAT, 2003). *Fusarium solani* f.sp. *phaseoli* has been reported to occur in most bean fields throughout the world (Park and Tu, 1994). Tusiime (2003) observed that *Fusarium solani* f sp. *phaseoli* often occurs concurrently with *Pythium* spp. Symptoms associated with the diseases are yellowing and/ or wilting of seedlings occurring two to four weeks after emergence. Surviving plants appear yellow, stunted, and often with little or no pods (CIAT, 2003). *Fusarium solani* f.sp. *phaseoli* kills the tissue around the basal part of the stem and root system, and the characteristic symptom of damage on beans is the reddish discoloration of the tap root and the desiccated lower stem (Erwin *et al*., 1991). *Pythium* root rot was found to be fairly diverse in East Africa with 19 species identified in Rwanda, 16 in Southwestern Uganda and nine in Kenya. Five species were found to be common to all the three countries namely *P. ultimatum, P. salpinophorum, P. nodosum,* and *P. tulorosum* (Buruchara *et al.,* 2005). Resistance to *Pythium* root rot is controlled by a single dominant gene (Otsyula *et al*., 2005) while resistance to *Fusarium* root rot is controlled by many additive genes (Mukankusi, 2007).

2.9 Exploiting plant genetic and genomic resources

There are a large number of collections of Phaseolus germplasm, which include the wild and domesticated genotypes of the Phaseolus species (*P. vulgaris*: common bean; *P. coccineus*: runner bean; *P. dumosus*: year bean; *P. acutifolius*: tepary bean; *P. lunatus*: lima bean) and other wild Phaseolus species. The largest and most diverse in the World Phaseolus collection is at the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia. Other large collections are those of the USDA in Pullman, WA, USA, the Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben, Germany and the Centro Nacional de Recursos Genéticos e Biotecnologia (CENARGEN/ EMBRAPA) in Brasilia. There are also national collections as well as a taxonomic collection focused on wild members of the Phaseolinae subtribe at the National Botanic Garden of Belgium. Together, these collections represent a substantial wealth of genetic diversity that is generally freely available for plant genetic and breeding research (Gepts *et al.*, 2008).

There exists 83,000 ESTs with 11,000 contigs and 9000 singletons (Ramirez et al., 2005; Melotto et al., 2005). Eleven BAC libraries have been reported in the genus Phaseolus (Gepts et al., 2005). Additionally, over 25 linkage genetic maps have been developed for common beans using crosses between domesticated parents belonging to the Andean and Mesoamerican gene pools. To date, there exists several on-line data banks where newly generated information on beans is deposited for exploration and use by researchers and one such data bank is the Legume Information System located at http://www.comparative-legumes.org.

2.10 Resistance gene pyramiding and marker aided selection (MAS)

Gene pyramiding, which aims to assemble multiple desirable genes into a single genotype, is a commonly used method in breeding for self-pollinated crops. Traditionally, the main use of gene pyramiding is to improve an existing elite cultivar through introgression of a few genes of large effects from other sources, since the presence of the target genes has to be monitored by phenotyping, which is only effective for major genes. Depending on the trait and inheritance of targeted genes, gene pyramiding may require much labor, time and material resources. The development of modern plant molecular techniques and quantitative genetics in the last two decades has dramatically widened the applicability of gene pyramiding (Ye and Smith, 2008). It provides enhanced knowledge of the genetics of the breeding traits and of the relative genomic

location of functionally related as well as neutral markers associated with the genes responsible for the traits. It facilitates the identification of genes with large effect of traits which are traditionally regarded as quantitative and not targeted by gene pyramiding program. Marker-based selection reduces/eliminates extensive phenotyping, provides more effective options to control linkage drag, makes the pyramiding of genes with very similar phenotypic effects possible, and reduces the breeding duration. Marker-based gene pyramiding is now the method of choice for inbred line development targeted at improving traits controlled by major genes (Ye and Smith, 2008).

Pyramiding disease resistance genes of Middle American and Andean origin has been used to develop bean germplasm lines with broad and more durable resistance to bean rust (Pastor-Corrales, 2003). Young and Kelly (1996) noted that pyramiding genes for disease resistance requires that virulence patterns of pathogens be monitored and new resistance genes be introgressed into commercial bean cultivars to provide resistance to emerging virulent pathotypes. Nelson (1978) recommended the use of gene pyramiding as a strategy for developing stable resistance and for preventing the outbreak of new pathotypes of a pathogen. It is a practical approach to achieving multiple and durable resistances (Singh *et al*., 2001; Castro *et al*., 2003). Gene pyramiding has been successfully applied in several crop breeding programs, and many varieties and lines possessing multiple attributes have been produced (Huang *et al*., 1997; Wang *et al*., 2001; Samis *et al*., 2002). Roush (2009) found that pyramiding of toxin genes offers what appears to be the most effective way to manage resistance to *Bt* and other insecticidal transgenic toxins. Singh et al. (2001) reported the successful pyramiding of three rice bacterial blight resistance genes, *xa5*, *xa13* and *Xa21*, through Marker Assisted Selection (MAS) in a high-yielding but susceptible rice cultivar, PR106. Their results demonstrated increased and wide-spectrum resistance to the pathogen populations from Punjab and to the Philippine races of *Xoo* in PR106 lines having pyramided genes.

However, traditional breeding procedures are inefficient for pyramiding resistance genes due to the need for multiple inoculations (Michelmore, 1995). Pyramiding resistance genes using molecular markers was proposed as a more efficient selection method for disease-resistant beans (Young and Kelly, 1996; Young and Kelly, 1997). “MAS” permits the indirect selection

of traits in the absence of selection pressure for the trait. For example bean breeders can screen for genes for a disease resistance without running the risk of introducing the disease agent.

MAS has been demonstrated in breeding for resistance to bean common mosaic virus (BCMV) in common bean, where the *I/bc-3* gene combination affords pyramided resistance to all known strains of BCMV (Haley *et al*., 1994). Tagging of genes and QTL in common bean and their application to MAS has been reviewed previously (Kelly *et al*., 2003; Miklas *et al*., 2006). Garzon et al. (2008) investigated the use of MAS in breeding for anthracnose resistance and reported the possibility of exploiting molecular markers that are associated with the *Co-5* and *Co-4*^{2} genes in improving resistance. Pastor-Corrales et al. (2007) used the SW-13 SCAR to confirm the presence of the *I* gene for BCMV resistance in the development of the great northern bean germplasm with multiple disease resistance. Miklas et al. (2006) used MAS to identify plants with the SCAR markers SU-91 and SAP-6 in the development of the common bacterial blight resistant dark red kidney germplasm having the *Co-4*^{2} gene for resistance to anthracnose. Blair et al. (2006) used the SCAR marker SR-2 to confirm the presence of the recessive gene *bgm* for resistance to BGMY in red mottled germplasm after results from screening with the SCAR marker SW-12 suggested that the red mottled germplasm lines did not have a QTL associated with BGYMV resistance. Problems with current molecular markers include the specificity of some markers to only one gene pool (Miklas *et al*., 2006).

However, molecular plant breeding techniques are an additional set of tools available to plant breeders (Broughton *et al*., 2003. The most appropriate combination of conventional and molecular tools will depend upon the specific agricultural problem that needs to be addressed and the available resources (Bernado, 2008).

Since bean anthracnose and root rots occur concurrently in farmers' fields causing serious yield losses, there is need to create new varieties possessing multiple resistances to bean anthracnose and *Pythium* root rot, coupled with farmer preferred agronomic traits in order to effectively control these diseases. This has also created the need to investigate the efficacy of pyramided genes for dual and durable resistance to the two diseases and the need to understand whether stacking of these resistance genes has an effect on other agronomic aspects of the plant. Both

conventional and MAS techniques were used in this study to screen large populations and increase precision of selection for resistant materials to both bean anthracnose and root rot.

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CHAPTER THREE

Virulence diversity of *Colletotrichum lindemuthianum* in Uganda

Abstract

Colletotrichum lindemuthianum is a highly variable pathogen of common beans that easily overcomes resistance in cultivars bred with single-gene resistance. To determine virulence variability of the pathogen in Uganda, samples of common bean tissues with anthracnose symptoms were collected in eight districts of Uganda, namely Kabarole, Sironko, Mbale, Oyam, Lira, Kapchorwa, Maracha and Kisoro. 51 isolates sporulated successfully on Potato dextrose Agar and Mathur's media and were used to inoculate 12 differential cultivars under controlled conditions. Five plants per isolate for each cultivar were inoculated and then evaluated for their reaction using the 1 – 9 severity scale. Data was subjected to analysis of variance and races were classified using the binary nomenclature system proposed by Pastor Corrales (1991). Variation due to cultivar and isolate effects was highly significant ($P \leq 0.001$) for severity. The 51 isolates from eight districts grouped into 27 different races. Sironko district had the highest number of races followed by Mbale and Kabarole. Races 2047 and 4095 were the most frequently found, each with 10 isolates grouped under them. Race 4095 had the broadest spectrum of virulence since it caused a susceptible (S) reaction on all 12 differential cultivars and the susceptible check. This was followed by races 2479, 2047 and 2045 respectively. Two races, 4094 and 2479, caused a susceptible reaction on the differential cultivar G2333, which nevertheless, showed the broadest resistance followed by Cornell, TU, and AB136 respectively. These cultivars are recommended for use in breeding programs aimed at breeding for broad resistance to bean anthracnose in Uganda.

Key words: Bean anthracnose, isolates, races,

3.1 Introduction

Bean anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. and Magn.) Lams.-Scrib, is one of the most important diseases of common beans in Uganda (Opio *et al.*, 2001) devastating farmers' fields causing up to 70% marketable yield losses on susceptible cultivars (Nkalubo *et al*., 2007). The pathogen is mainly seed borne (del Rio and Bradley, 2004) and, therefore, easily spread as farmers highly depend on farm saved seed and exchange of seed is common (Opio *et al*., 2001).

The pathogen has a wide pathogenic variation with various races reported in major bean producing countries such as Mexico and Brazil (Balardin and Kelly, 1998). Mahuku and Riascos (2004) assessed virulence and molecular diversity of 200 *Colletotrichum lindemuthianum* isolates collected from Andean and Mesoamerican bean cultivars and regions. They reported high levels of pathotypic (90 pathotypes) diversity among the 200 isolates. Bigirimana et al. (2000) characterized nine *C. lindemuthianum* races using 12 isolates collected from major bean growing areas in Burundi and12 standard differential cultivars. Races 9, 69, 87, 384, 385, 401, 448, 449 and 485 were identified. Seven of these races were reported for the first time in Burundi.

In Uganda, Leaky and Simbwa-Bunnya (1972) using differential cultivars from Shreiber and Hubberling, identified races 17, 19, 23, 102, 130, and 453 with isolates collected from Central, Western and South Western regions of Uganda. More recently, Nkalubo (2006) reported races 23, 55,102, 130, 227, 375, 511 and 767 from Kabale, Kisoro, Bushenyi and Mpigi, districts with race 767 reported as the most widespread and virulent. Three races of Nkalubo (2006) namely 23, 102 and 130 were similar to those of Leaky and Simbwa-Bunnya (1972). Mwesigwa (2008) reported 21 races (0, 2, 3, 4, 6, 14, 128, 262, 264, 268, 320, 452, 481, 1024, 1536, 1538, 1856, 1857, 1989, 3086 and 4033) out of 47 isolates collected from Kabale, Apac, Mbale, Mpigi and Wakiso districts. None of his races were similar to those of Leaky and Simbwa-Bunnya (1972) and Nkalubo (2006). Two highly virulent races 3086 and 4033 caused symptoms on the highly resistant differential cultivar G2333. Mwesigwa (2008) further reported that nine of the races were virulent to the Mesoamerican cultivars, three races were virulent to the Andean cultivars and seven races were virulent to both groups of cultivars.

There is a wide gap in time from the work of Leakey and Simbwa-Bunnya (1972) to that of Nkalubo (2006) and Mwesigwa (2008) to suspect tremendous change in diversity of the pathogen as a result of increase in bean production, introduction of new varieties from different gene pools and movement of bean seed within and outside the country and region. The differences in physiological races from the earlier studies could be as a result of use of different set of differential cultivars for characterization, sampling differences and emergence or introduction of new races that overcome previously stable resistances among the bean

differential set. The aim of this study was to determine the current virulence diversity of *C. lindemuthianum* in Uganda including bean growing districts that earlier studies had not covered.

3.2 Materials and methods

3.2.1 Sample collection and pathogen isolation

Common bean pods with symptoms of anthracnose disease were collected in from different common bean cultivars from eight districts of Uganda namely Kabarole, Kapchorwa, Kisoro, Lira, Maracha, Mbale, Oyam and Sironko (Table 3.1; Fig. 3.1)). A GPS machine was used to determine altitude and coordinates of fields were samples were picked. Infected tissues from the bean pods were cut into small pieces of up to 5cm long. The tissues were placed into a small beaker and 10ml of Sodium hypochlorite (Jik) bleach was added. After two minutes, the bleach was removed and 10ml of ethanol was added. After two minutes the ethanol was removed and the tissues were rinsed with sterilized water. A pair of forceps was flamed under a sterile laminar flow hood. The tissues were placed on filter paper to remove excess water and dry them. The tissues were placed on PDA media and incubated in darkness at 22 - 24^{o}C. After four days, the different cultures showed growth and were sub-cultured onto modified Mathur's Agar media (500g) 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, to get pure isolates and increase sporulation.

3.2.2 Inoculum preparation

Single-spore isolates were placed on fresh Mathur's agar medium in a Petri dish and incubated at 22-24^{o}C for 7 days to allow the fungus enough time to produce conidial spores. For inoculation purposes, conidial spores were scrapped off the growth medium into a small amount of water to make a suspension. Using a hemocytometer the concentration was adjusted to 1.2 x 10^{6} conidia ml^{-1}. 0.1% Tween 20 was added as a surfactant.

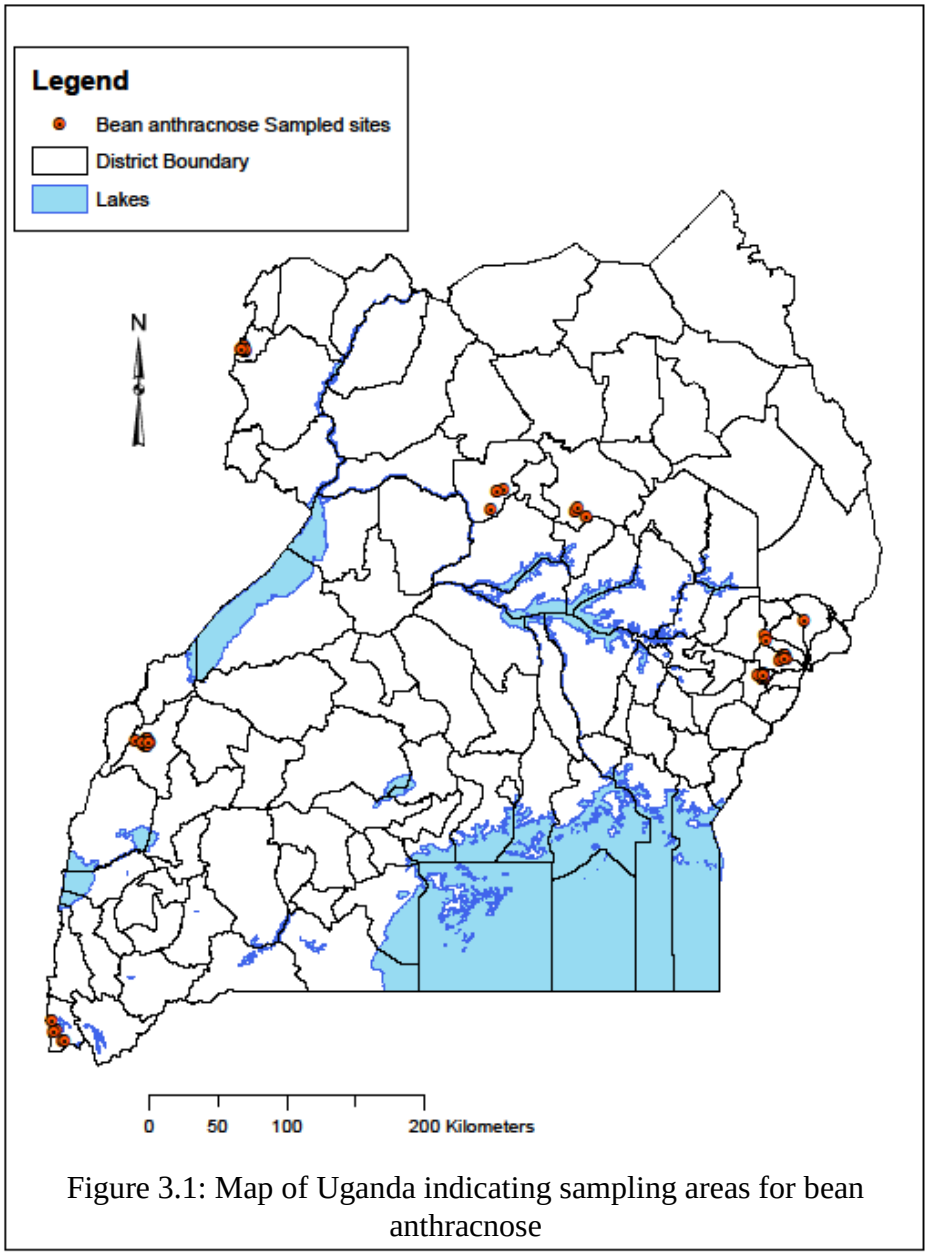

Figure 3.1: Map of Uganda indicating sampling areas for bean anthracnose

3.2.3 Inoculation and disease evaluation

Seed of the 12 differential cultivars were pre-germinated and later soaked for 30 minutes into the inoculum before transplanting into sterilized soil mixed with saw dust, in a controlled screen house at the National Crops Resources research Institute (NaCRRI). Five seeds of each of the 12 differential cultivars were sowed in a tray plus a known susceptible check K132. The screen house conditions were maintained at 95-100% relative humidity and temperature of 22±2^{o}C. Disease severity was scored 10 – 14 days after planting using a 1 – 9 scale (Balardin et al., 1997)

Where; 1 = no symptoms (resistant), 3-4 = very small lesions mostly on primary leaves (resistant) and 5-9 = numerous enlarged lesions or sunken cankers on the lower side of the leaves or hypocotyls (susceptible).

3.2.4 Race characterization and data analysis

To identify races, the binary system was used based on the sum of binary values assigned to each of the 12 differential cultivars proposed by Pastor-Corrales (1991) to characterize anthracnose races. Each differential cultivar had an assigned number 2^n where n corresponds to the place occupied by the cultivar within the differential series. The designation of a race number was obtained by summing the numerical values of all differential cultivars exhibiting susceptible (S) reactions to the isolate used for inoculation. Isolates with similar reactions on the differentials were grouped to form a race. Data was subjected to two-way analysis of variance using GenStat 13th Edition (Payne et al., 2010).

Table 3.1: *C. lindemuthianum* isolates and locations from which infected bean samples were collected in different parts of Uganda.

Isolate I.D	District	Sub-county	Village	Longitude	Latitude	Altitude
025A	Kabarole	Mugusu	Kibede	00°36.152N	030°12.976E	1596
028A	Kabarole	Mugusu	Kirugu	00°37.461N	030°13.135E	1605
34A	Kabarole	Mugusu	Kisaru	00°37.506N	030°12.528E	1562
36A	Kabarole	Karambi	Karambi	00°38.107N	030°13.785E	1563
37A	Kabarole	Karambi	Karambi	00°38.107N	030°13.785E	1563
38A	Kabarole	Karambi	Karambi	00°38.107N	030°13.785E	1563
40A	Kabarole	Karambi	Karambi	00°38.107N	030°13.785E	1563
41A	Kabarole	Karambi	Karambi	00°38.100N	030°14.842E	1553
44A	Kabarole	Karambi	Karambi	00°38.100N	030°14.842E	1553
46A	Kabarole	Karambi	Karambi	00°37.923N	030°14.643E	1553
52A	Kabarole	Karambi	Karambi	00°37.923N	030°14.643E	1555
55A	Kabarole	Karambi	Karambi	00°37.923N	030°14.643E	1558
77A	Kapchorwa	Binyinyi	-	01°25.042N	034°31.741E	1929
001A	Kisoro	Busanza	Nyakababi	01°10.066S	029°35.152E	1689
007A	Kisoro	Nyarubuye	Mwalo	01°15.127S	029°38.902E	1790
008A	Kisoro	Nyarubuye	Mwalo	01°15.127S	029°38.902E	1790
012A	Kisoro	Nyarusiza	Nyamushungwa	01°18.691S	029°40.856E	1985
016A	Kisoro	Nyarusiza	Buhangura	01°19.457S	029°40.987E	2045
56A	Lira	Amac	Acuma-roma	02°09.939N	033°01.885E	1071

57A	Lira	Amac	Adyeri	02°05.683N	033°02.392E	1060
59A	Lira	Amac	Corner Ariti	02°06.751N	032°59.934E	1060
64A	Maracha-Terego	Oluvu	Anderu	03°12.316N	030°51.911E	1221
65A	Maracha-Terego	Oluvu	Anderu	03°13.747N	030°50.747E	1238
66A	Maracha-Terego	Oluvu	Anderu	03°13.628N	030°51.030E	1254
67A	Maracha-Terego	Oluvu	Anderu	03°12.427N	030°52.328E	1203
69A	Maracha-Terego	Oluvu	Asuru	03°12.529N	030°51.891E	1212
71A	Maracha-Terego	Oluvu	Asuru	03°12.041N	030°50.730E	1240
72A	Maracha-Terego	Oluvu	Asuru	03°12.305N	030°51.912E	1228
73A	Maracha-Terego	Oluvu	Asuru	03°12.111N	030°52.182E	1218
91A	Mbale	Budwale	Bukumi	01°03.581N	034°15.572E	1862
92A	Mbale	Wanare	Bugeheme	01°03.158N	034°14.834E	1891
94A	Mbale	Wanare	Bugeheme	01°03.204N	034°14.618E	1871
95A	Mbale	Wanare	Bugeheme	01°03.451N	034°14.960E	1842
96A	Mbale	Budwale	Bukumi	01°03.520N	034°15.375E	1518
97A	Mbale	Wanare	Bugeheme	01°03.266N	034°14.982E	1854
98A	Mbale	Bungokho	Namashere	01°03.383N	034°13.907E	1187
99A	Mbale	Wanare	Bugeheme	01°03.197N	034°14.626E	1887
100A	Mbale	Budwale	Bukumi	01°03.621N	034°15.413E	1837
61A	Oyam	Loro	Alut-kot	02°13.793N	032°28.989E	1058
62A	Oyam	Loro	Adyeda	02°14.953N	032°33.087E	1051
63A	Oyam	Loro	Awe-ikwo	02°13.822N	032°29.041E	1061
75A	Sironko	Bukhalu	Bwerucra	01°17.537N	034°17.481E	1089
76A	Sironko	Bukhalu	Buyaga	01°16.478N	034°16.453E	1132
81A	Sironko	Masifwa	Jewa	01°10.236N	034°21.437E	1292
82A	Sironko	Masifwa	Lubenya	01°11.207N	034°22.697E	1549
83A	Sironko	Masifwa	Bugibulungi	01°09.068N	034°22.517E	1690
84A	Sironko	Masifwa	Bugimwera	01°11.472N	034°22.926E	1685
85A	Sironko	Masifwa	Gimbubuni	01°10.643N	034°22.584E	1485
86A	Sironko	Masifwa	Bunagani	01°10.529N	034°22.109E	1314
88A	Sironko	Masifwa	Lubenya	01°11.329N	034°22.810E	1690
90A	Sironko	Masifwa	Bunamono	01°09.173N	034°22.675E	1364

*Presented above are the 51 *C. lindemuthianum* isolates that cultured and sporulated successfully. The rest were eliminated due to their poor sporulation

3.3 Results and discussion

Analysis of variance (Table 3.2) revealed highly significant differences ($P \leq 0.001$) among the cultivars and isolates. Variation due to the interaction between cultivars and isolates was also highly significant ($P \leq 0.001$). Isolate contributed most (49.8%) to the total variation followed by cultivars (46.2%).

Table 3.2: Analysis of variance for bean anthracnose severity on the 12 differential cultivars

		Severity	
Source of variation	**d.f**	**Mean square**	**% Contribution[a]**
Rep	4	1.10	1.51
Cultivar	12	33.78*	46.20
Isolate	50	36.41*	49.79
Cultivar x Isolate	600	1.33*	1.82
Residual	2647	0.50	0.69

[a] Percentage contribution of each source of variation to the total variation
* Significant at P≤0.001

The 51 isolates were screened for virulence using 12 standard differentials (see their reaction in appendix i) and were grouped into 27 races (Table 3.4). Races 2047 and 4095 were the most abundant. Race 4095 had the broadest virulence spectrum causing a susceptible (S) reaction on all of the 12 differential cultivars and the susceptible check. This was followed by races 2047, 2045 2039 and 2023. Race 2047 was from 10 isolates collected from five districts of Kabarole, Kisoro, Maracha, Mbale and Sironko.

Table 3.3: Race designation, isolate groupings and susceptible and resistant reactions of common bean differentials to 51 *C. lindemuthianum* isolates collected from Eastern, Northern and South Western Uganda

	Differential cultivars and their respective resistance genes [a]												Isolates
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
Race*	Co-11	Co-1	Co-1^3	Co-2	Co-1^5	Co-1^2	Co-3	Co-4^3, Co-9	Co-4	Co-5	Co-6, co-8	Co-4^2, Co-5^2, Co-7	
0	R	R	R	R	R	R	R	R	R	R	R	R	38A, 59A, 57A, 25A
1	S	R	R	R	R	R	R	R	R	R	R	R	82A
42	R	S	R	S	R	S	R	R	R	R	R	R	85A
81	S	R	R	R	S	R	S	R	R	R	R	R	92A
128	R	R	R	R	R	R	R	S	R	R	R	R	36A
352	R	R	R	R	R	S	S	R	S	R	R	R	84A
386	R	S	R	R	R	R	R	S	S	R	R	R	61A
503	S	S	S	R	S	S	S	S	S	R	R	R	88A
704	R	R	R	R	R	R	S	S	R	S	R	R	34A
713	S	R	R	S	R	R	S	S	R	S	R	R	86A
767	S	S	S	S	S	S	S	S	R	S	R	R	69A
784	R	R	R	R	S	R	R	R	S	S	R	R	56A
1023	S	S	S	S	S	S	S	S	S	S	R	R	62A
1094	R	S	S	R	R	R	S	R	R	R	S	R	12A
1169	S	R	R	R	S	R	R	S	R	R	S	R	72A
1175	S	S	S	R	S	R	R	S	R	R	S	R	91A
1334	R	S	S	R	S	S	R	R	S	R	S	R	94A
1471	S	S	S	S	S	S	R	S	S	R	S	R	90A, 100A
1527	S	S	S	R	S	S	S	S	S	R	S	R	16A, 99A
1791	S	S	S	S	S	S	S	S	R	S	S	R	41A
1834	R	S	R	S	R	S	R	R	S	S	S	R	81A
2023	S	S	S	R	R	S	S	S	S	S	S	R	007A
2039	S	S	S	R	S	S	S	S	S	S	S	R	97A
2045	S	R	S	S	S	S	S	S	S	S	S	R	95A, 40A
2047	S	S	S	S	S	S	S	S	S	S	S	R	65A, 08A, 64A, 75A, 98A, 52A, 55A, 37A, 46A,71A
2479	S	S	S	S	R	S	R	S	S	R	R	S	83A
4095	S	S	S	S	S	S	S	S	S	S	S	S	66A, 63A, 44A, 67A, 76A, 28A, 001A, 77A, 73A, 96A

*Races characterized using the binary system (Pastor Coralles, 1991); Resistant reaction (R); Susceptible reaction (S)

[a] Differential cultivars: 1 = Michelite; 2 = MDRK; 3 = Perry Marrow; 4 = Cornell 49-242; 5 = Widusa; 6 = Kaboon; 7 = Mexico 222; 8 = PI 207262; 9 = TO; 10 = TU; 11 = AB136; and 12 = G 2333

Race 2479 was from from one isolate collected from Sironko district, while race 4095 was made of 10 isolates collected from seven districts of Kabarole, Kapchorwa, Kisoro, Oyam, Mbale, Maracha and Sironko. This makes race 4095 the most widely distributed followed by race 4027. (Figure 3.2).

The Eastern region of Uganda (Sironko and Mbale) had the highest number of races including the most virulent ones followed by the Western (Kabarole district) and South western (Kisoro district) regions. These are all high altitude bean growing regions ranging from 1429 – 1860m above sea level (Table: 3.1) and therefore offer favorable conditions for the development and spread of bean anthracnose.

Only three races in this study were similar to races reported in earlier studies in Uganda namely race 0 and 128 (Mwesigwa, 2008) and race 767 (Nkalubo 2006). This lack of consistency may be attributed todifferences in areas sampled, phenotying techniques and to some extent mutation of the pathogen to produce diverse pathotypes. However, employing molecular analysis techniques such as Eric-Rep-Box PCR could further uncover interesting diversity patterns of *C. lindemuthianum* in Uganda. This technique was used by Bardas et al. (2009) to characterize molecular diversity of *C. lindemuthianum* isolates collected in Greece.

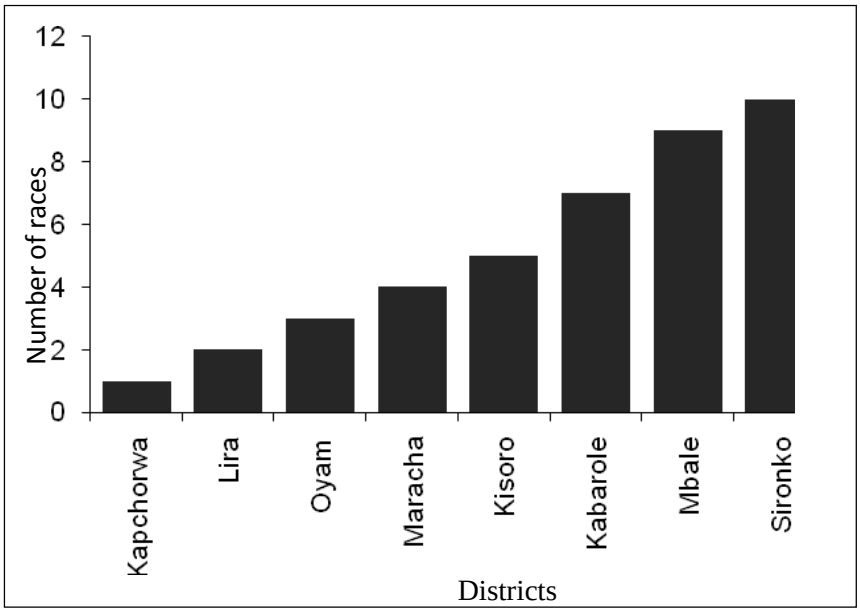

Figure 3.2: Number of *C. lindemuthianum* races by district

The differential cultivar G2333 showed the highest number of resistant (R) reactions followed by cultivars Cornell 49-242, TU and AB 136 (Figure 3.3 above). This implies that the cultivars in their respective order confer broad resistance to the anthracnose races in Uganda. The cultivars TU, AB136 and G2333 were also reported to be resistant against 12 isolates collected in major bean-growing areas in Burundi (Bigirimana *et al*., 2000). The similarity in trend with Ugandan isolates indicates that the four cultivars G2333, Cornell 49-242, TU and AB 136 are respectively the best parents to use in a hybridization program aiming at breeding for broad resistance against bean anthracnose. The highly effective resistance in cultivar G2333 is mostly attributed to its naturally occurring resistance gene pyramid comprising of *Co-4*^{2}, Co-5 and Co-7 genes. The allele *Co-4*^{2} is recognized as being among the most effective resistance genes described in common beans (Balardin and Kelly, 1998; Silverio *et al*., 2002). Mwesigwa (2008), on the other hand, reported that the differential cultivar Widusa did not succumb to any isolate in his collection from which two races, 3086 and 4033, overcame the resistance in G2333. The cultivars PI 207262, MDRK and Michelite, respectively had the lowest number of Resistant (R) reactions, implying that their resistance is more specific in nature since they showed exceptionally effective resistance to certain isolates. They could still be useful in breeding programs targeting resistance against certain races or pyramiding

genes to obtain broader resistance against a range of races. The cultivar Michelite, contrary to reports in the literature, was not the most susceptible and seemed to confer resistance in a more effective manner than the popularly known resistant cultivar PI207262. Mwesigwa (2008) observed the same about Michelite, which is usually reported as a universal susceptible differential cultivar. The resistance in this cultivar can be attributed to the *Co-11* gene reported by Gonçalves-Vidigal et al. (2007).

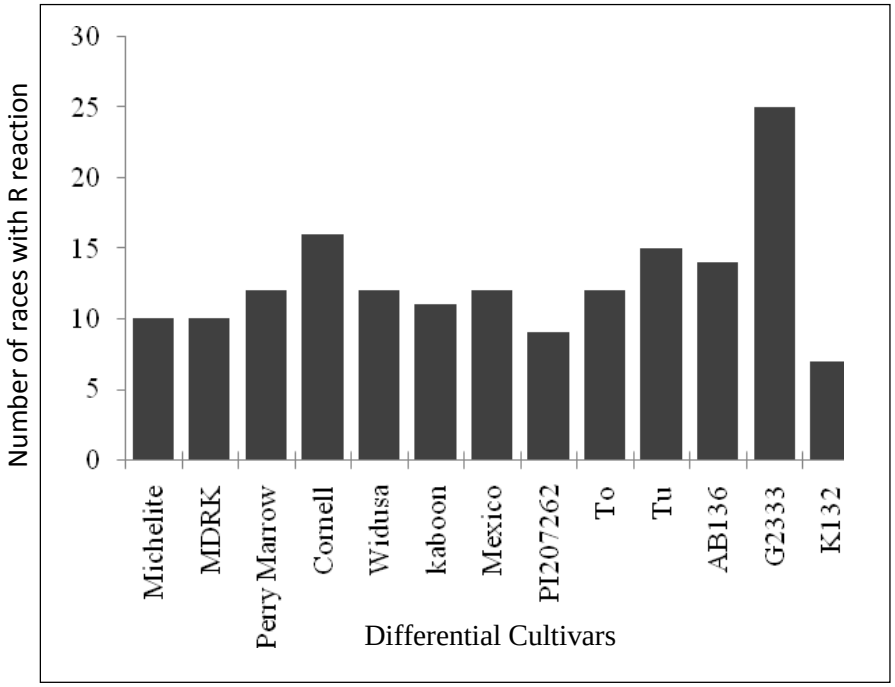

Figure 3.3: Pathogenicity of 27 races on 12 differential common bean cultivars and a susceptible check, K132 (CAL96).

Leaky and Simbwa-Bunnya (1972) observed that the immune nature of resistance in the cultivar Cornell 49-242, conferred by a single dominant gene *Co-2* (Mastenbroek, 1960), reduced the disease to a status of no importance in Holland but when tested in Uganda it showed clear anthracnose symptoms with five Ugandan isolates. In our study the cultivar Cornell 49-242, which ranked second after G2333, showed clear anthracnose symptoms with 11 races.

The cultivar PI207262 (Tlalnepantla 64) ranked the lowest of all the 12 differential cultivars (Fig 3.3). Bigirimana et al. (2000) ranked it second lowest after Michelite out of all the 12 differential cultivars. PI207262 is reported to possess a very specific breeding value against Andean races of *C. lindemuthianum* and is recommended for use only to diversify the resistance in gene pyramids because of its independence and potential value in controlling highly virulent Andean races (Kelly and Vallejo, 2004).

3.4 Conclusion

The results of this study conducted under controlled conditions indicate that*Colletotrichum lindemuthianum* in Uganda is diverse. 51 isolates were grouped into 27 diverse physiological races .

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CHAPTER FOUR

Marker-aided pyramiding of multiple resistance genes conferring resistance to anthracnose and *Pythium* root rot into popular susceptible common bean cultivars

Abstract

Bean anthracnose and root rots are two of the most devastating diseases that constrain common bean production especially in the highland areas of Uganda. Sequence characterized amplified regions (SCAR) markers were used to facilitate the process of pyramiding three anthracnose and one *Pythium* root rot resistance genes in to four popular but susceptible market-class bean varieties K132, NABE4, NABE13 and NABE14. A cascading pedigree gene pyramiding scheme was used to develop a root genotype which was then selfed for six generations to fix the target genes. Advanced lines were screened with four races of *Colletotrichum lindmeuthianum* and tested for yield performance. Sixty nine $F_{4:6}$ families possessing different number of genes in different combinations were obtained. The pyramids *Co-4*^{2}*+Co*-5+*Co-9* and *Co-4*^{2}*+Co-5* conferred resistance to all the four races 2047, 713, 767 and 352 while lines that inherited none of the resistance genes were susceptible to all the races. Three pyramided lines mamely 16.3.3.11.160.5.6 with three genes; 44.1.4.5.142.4 and 16.1.3.8.136.2 with two genes were among the best ten based on seed weight per plant. Marker-aided gene pyramiding of anthracnose and *Pythium* resistance genes was successfully achieved for the first time in Uganda.

Key words: Gene pyramiding, Marker Assisted Selection (MAS), SCAR markers, *Colletotrichum lindmeuthianum, Pythium spp.*

4.1 Introduction

The common bean *Phaseolus vulgaris* (L.), is an important crop grown and consumed throughout Uganda and a major source of income for the rural smallholder farmers. The crop is the most important source of protein for the Ugandan population providing up to 45% of the total daily human dietary protein intake (Mauyo *et al*., 2007). Beans provide a cheap source of protein to most vulnerable groups such as children below five years, pregnant mothers and AIDS patients. Common bean is also reported to contain a protein that inhibits the HIV-1 reverse transcriptase (Wang et al., 2006), in addition to being a

rich source of zinc and iron, two of the most important nutrients in delaying progression of AIDS (Savarino *et al.*, 1999; ADA, 2004). It is, therefore, important that production and productivity of the common bean in Uganda and the region is increased.

One of the factors that accounts for the low yields in common bean is the occurrence of several diseases that affect the common bean crop posing a challenge for plant breeders who must develop cultivars having multiple disease resistance (Beaver and Osorno, 2009). It is estimated that 10% yield loss in common bean is caused by bean pathogens (Schwartz *et al*., 2005).

Among the diseases, bean anthracnose disease caused by *Colletotrichum lindemuthianum* (Sacc. et. Magn) Lams. Scrib, is the second most important disease after root rots in the highland cool areas of Uganda (Opio *et al*., 2001). It is estimated that every one percent increase in disease incidence manifests into seed yield loss of 9kg/ha (Wortmann and Allen, 1994). The pathogen *C. lindmeuthianum* is known to be highly variable both pathogenically and genetically (Mahuku and Riascos, 2004; Kelly and Vallejo, 2004), which consequently makes single gene resistance short lived and highly vulnerable to dynamic and diverse plant pathogen populations.

Bean root rots, on the other hand, are reported to be among the most devastating constraints to bean production especially in South Western highland regions of Uganda (CIAT, 2005). They damage the root system of infected plants and are usually associated with nutrient depleted soils (Spence, 2003). They can cause yield losses of up to 100% on susceptible varieties (Tusiime, 2003: Mukankusi, 2010). Root rots are caused by a complex of soil-borne pathogens including *Fusarium solani*, *Pythium spp, Rhizoctonia solani* and *Macrophomina phaseoli* (Rusuku et al., 1997). Root rot caused by *Pythium Spp* is one of the most destructive in East and Central Africa (Wortman et al., 1998) and in Uganda, *Pythium spp* was found to be the most predominant pathogen in the root rot complex (Mukalazi, 2004).

Several methods are recommended in the control of these two diseases including crop rotation, elimination of contaminated debris, enhancing soil fertility, use of chemical fungicides and use of resistant varieties (Pastor-Corrales *et al*., 1994). Chemical control of the diseases is not widely adopted by farmers because it raises production costs and is not eco-friendly. The use of resistant cultivars offers farmers an efficient, safe and inexpensive alternative for managing the diseases and boosting their production.

In most cases, however, bean cultivars will be resistant to some races but not to others (CIAT, 1997) leading to easy breakdown in its resistance. Through gene pyramiding, a method aimed at assembling multiple desirable genes from multiple parents into a single genotype (Ye and Smith, 2008), it is possible to combine several anthracnose and *Pythium* resistance genes into a common background of susceptible bean varieties. This would offer a broad and multiple-resistance to the two pathogens and their races. Gene pyramiding, however, is a long and costly affair (Joshi and Nayak, 2010) requiring extensive disease screening with several races of the pathogen for many generations (Ye and Smith, 2008). In addition, different loci may be involved in the genetic control of a resistant phenotype, and the expression of specific genes can be masked due to epistatic interactions (Ferreira et al., 2012).

Nevertheless, the development of modern plant molecular techniques and quantitative genetics in the last two decades has dramatically widened the applicability of gene pyramiding because marker based selection reduces extensive phenotyping thus reducing the breeding duration (Ye and Smith, 2008).

Marker assisted selection (MAS) has a long history in common bean research that is second to no other plant species (Kelly et al. 2003, Miklas et al. 2006). Molecular markers linked to the majority of major anthracnose (*Co*-) genes have been widely reported and these provide the opportunity to enhance disease resistance through MAS (Kelly et al., 2003; Kelly & Vallejo, 2004). MAS has been used successfully to breed for enhanced resistance to anthracnose in the cultivar Perola in Brazil (Ragagnin et al., 2009). Miklas et al. (2003) used sequence characterized amplified regions (SCAR)

marker SAS13 to develop Pinto bean germplasm having the *Co-4*^{2} gene for anthracnose resistance. Additionally, SAS13 SCAR marker, tightly linked with the *Co-4*^{2} gene was used as a starting point for cloning gene sequences associated with the *Co-4* locus that conditions resistance to anthracnose (Melotto & Kelly, 2001). The SCAR marker, PYAA 19_{800} was characterized as being associated with *Pythium* root rot resistance gene in RWR 719 and AND 1062 (Mahuku *et al*., 2007). The marker was successfully used by Nzungize et al. (2011) to introgress *Pythium* root rot resistance into Rwandan susceptible bean cultivars. Kelly et al. (2003) and Miklas et al. (2006) provide further numerous examples of the effectiveness of molecular markers in the selection of bean lines with enhanced resistance to disease and pests and greater tolerance to abiotic stresses.

MAS has been successfully applied in gene pyramiding programs to create more durable and broad specific resistance in other crops. For instance, Liu et al. (2000) successfully integrated three powdery mildew resistance gene combinations *Pm2+Pm4a*, *Pm2+Pm211* and *Pm4a+Pm21* into the wheat cultivar Yang 158 using Restriction Fragment Length Polymorphism (RFLP) markers. In soybean, Shi et al. (2008) successfully pyramided three genes *RsV1*, *Rsv3* and *Rsv4* for soybean mosaic virus (SMV) resistance with the aid of microsatellite markers in order to develop new soybean lines containing multiple resistance genes. In cotton, second generation pyramided dual *Bt* gene cottons Bollgard^{R} II (*Cry1Ac+Cry2Ab*) and WideStrike^{TM} (*Cry1Ac+Cry1F*), which express two *Bt* endotoxins were successfully developed by Monsanto to raise the level of control of *Helicoverpa Zea*, which was not satisfactorily controlled by *Cry1Ac* toxin alone (Ferry *et al*., 2004; Bates *et al*., 2005; Gahan *et al*., 2005).

In common beans, Ragagnin et al. (2009) used random amplified and polymorphic DNA (RAPD) and SCAR markers to pyramid *Co-4, Co-6, Co-10* anthracnose, *Phg-1* angular leaf spot and *Ur-ON* rust resistance genes in to a susceptible 'carioca' market class cultivar called Ruda. Ferreira et al. (2012) used SCAR, CAPs and RAPD markers to successfully pyramid *Co-2, Co-3/9* anthracnose, *I* and *bc-3* common mosaic virus resistance genes into a market class fabada A25 bean genotype. There is need, therefore,

to pyramid anthracnose and root rot resistance genes into susceptible but adapted market class bean varieties that are popular among farmers in Uganda.

The objective of this study was to introgress at least two anthracnose and one *Pythium* root rot resistance genes into susceptible market class common bean varieties using SCAR marker assisted selection. The efficiency of SCAR marker selection system, reaction of pyramided lines to different *C. lindmeuthianum* races and field agronomic performance of advanced lines are discussed.

4.2 Materials and methods

4.2.1 Plant materials and study sites

Parental plant materials used are indicated in Table 4.1. All plant materials were obtained from the Legumes Program, National Crop Resources Research Institute (NaCRRI) located 00 32" N of the Equator and 320 37" E, 27Km North of Kampala and elevated at 1150 meters above sea level (asl). All crosses and F_1 - F_4 progeny testing were conducted under screen house conditions at NaCRRI. Fixation of alleles up to F_6, genotyping and phenotyping of lines was conducted at the International Center for Tropical Agriculture (CIAT) research facilities at the National Agricultural Research Laboratories (NARL), Kawanda, Uganda, located 0º 24' 38.15" N and 32º 32' 14.06" E and elevated at 1147 meters above sea level.

Table 4.1: Parental materials used in making crosses

Cultivar	Pedigree	Growth habit	Gene pool	Response to anthracnose and root rots
K132	Calima-2 x Argentino 1	Bush	Andean	Susceptible to anthracnose and root rots
NABE 4	Sug 47 x Cal 103	Bush	Andean	Susceptible to anthracnose and root rots
NABE 13	RWR 1946	Bush	Andean	Susceptible to anthracnose and tolerant to root rots
NABE 14	RWR 2075	Bush	Andean	Susceptible to anthracnose and tolerant to root rots
G 2333*	Colorado de Teopisca	Climber	Mesoamerican	Resistant to anthracnose (*Co-4*^{2}, *Co-5*, *Co-7*)
PI 207262	Tlalnepantla 64	Climber	Mesoamerican	Resistant to some races of anthracnose (*Co-4*^{3}, *Co-9*)
RWR719	Omo-95	Bush	Mesoamerican	Resistant to *Pythium* root rot

* Landrace called Colorado de Teopisca from Mexico, released in the great lakes region as Umubano in Rwanda and NABE10C in Uganda. The allele *Co-4*^{2} in this cultivar is allelic with the allele *Co-4*^{3} in cultivar PI207262

4.2.2 Pyramiding resistance genes and line development

A cascading pedigree gene pyramiding scheme was used, where by only one cross is made at each generation involving an intermediate genotype and one founding parent (Servin, et al., 2004). The minimum population size to ensure at a predetermined probability of 95% or 99%, that at least one desired genotype is present in the population was derived from the equation; N_q = In(1-*q*)/ In(1-*p*) where; *N* = minimum population size, *p* = number of individuals of the desirable genotype in a population of size *N* and *q* = predetermined probability (Ye and Smith, 2008). After obtaining the root genotype, which possesses all the target genes in heterozygous state, the fixation of these genes was conducted through selfing. Pedigree breeding method was used to advance selected individuals that possessed desired number and combination of genes. Marker Assisted selection (MAS) was used to select for individuals with the desired pyramided genes both during the pedigree/crossing scheme and fixation scheme.

4.2.3 Molecular markers

Sequence Characterized Amplified Regions (SCAR) markers were used (Table 4.2) to tag anthracnose and root rot resistance genes of interest. The primers were obtained from the Department of Molecular and Cellular Biology, University of Cape Town, Randebosch, South Africa. Later batches were obtained from Bioneer Corporation, Munpyeong-dong, Daejeon, South Korea. A 25/100 bp mixed DNA molecular weight marker (Ladder), specifically designed for determining the size of double strand DNA from 25 to 2,000 base pairs, was used.

Table 4.2: PCR-based markers used in Marker Assisted Selection

Marker/ locus tagged	Original marker*	Linkage group	Size (bp)/ orientation	Primer Sequence	Reference
SBB14/ *Co-4*^{2}	BB14	B8	1150/1050, codominant	*Forward:* GTG GGA CCT GTT CAA TAA TAC *Reverse:* GTG GGA CCT GGG TAG TGT AGA AAT	Awale and Kelly, 2001
SAS13/ *Co-4*	AS13	B8	950, *Cis*	*Forward:* CAC GGA CCG AAT AAG CCA CCAACA *Reverse:* CAC GGA CCG AGG ATA CAG TGA AAG	Young et al., 1998

SAB3/ *Co-5*	AB-3	B7	400, *Cis*	*Forward:* TGG CGC ACA CAT AAG TTC TCA CGG *Reverse:* TGG CGC ACA CCA TCA AAA AAG GTT	Vallejo and Kelly, 2001
SB12/ *Co-9*	B-12	B4	350, *Cis*	*Forward:* CCT TGA CGC ACC TCC ATG *Reverse:* TTG ACG ATGGG TTG GCC	Mendez de Vigo et al., 2002
$PYAA19_{800}$/ *Pythium*	AA19		800, *Cis*	Forward: TTA GGC ATG TTA ATT CAC GTT GG Reverse: TGA GGC GTG TAA GGT CAG AG	Muhuku, 2007

*Original markers were Random Amplified Polymorphic DNA (RAPDs) markers converted to SCARs

4.2.4 Marker Assisted Selection

4.2.4.1 DNA extraction and amplification

Leaf samples were picked from 14-day old plants in the screen house. Genomic DNA was extracted using the Cetytrimethylammoniumbromide (CTAB) method according to Rogers and Bendich (1988). Genomic DNA was stored in 50µl of 0.1 X TE (10mM Tris-HCl, PH8; 1mM EDTA) buffer at – 20ºC. To carry out the amplification, a 20µl volume PCR premix (Bioner Inc,Korea) containing 2mM $MgCl_2$,0.2mM dNTPS ,0.2U Top DNA polymerase and tracking dye was used onto which was added 40ng of DNA, 0.5µM of both forward and reverse primer and 17µl of double distilled water. When the Premix was not used, PCR reagents were used as follows; 50mM $MgCl_2$ (1.2ul/20ul), 10mM dNTPs (0.8/20ul), 10X Buffer (2.0/20ul), 10mM forward primer (1.0//20ul), 10mM reverse primer (1.0/20ul), and 5U/ul *Taq* polymerase (0.4ul/20ul).

DNA amplification, was carried out in a Bioneer Mycycler Thermal cycler (Bioneer Inc,Korea) with an initial denaturation step at 95ºC for 5 minutes and 35 cycles each of a denaturation step at 94ºC for 20 seconds, an annealing step at 64ºC (SBB14), 68ºC (SAS13), 65ºC (SAB3), 65ºC (SB12), 64ºC (SH18) and 63ºC ($PYAA19_{800}$) for 40 seconds; an extension step at 72ºC for 1 minute followed by a final extension for 10 minutes at 72ºC. The amplicons were resolved on 1.5% agarose gels in 1X TBE (0.045 M Tris–borate and 1 mM EDTA, pH 8.2) at 100V for 90 minutes and stained with 0.5µg/ml ethidium bromide for 10 minutes. The image was captured using the SynGene G: BOX gel documentation system (Syngene, Frederick, MD,USA).

4.3.1 Marker assisted gene pyramiding

The gene pyramiding and marker assisted selection strategy used is illustrated in Figure 4.1. The highly resistant parent G2333 (*Co-4*^{2}, *Co-5* and *Co-7*) was crossed with PI207262 (*Co-4*^{3} and *Co-9*) under screen house conditions. The F_1 plants were crossed with the line RWR719 (donor for *Pythium* root rot resistance) to develop the genotype [(G2333 x PI207262) x RWR719]. The F_1 seeds of the cross [(G2333 x PI207262) x RWR719] were planted together with four released but susceptible market class varieties namely K132, NABE4, NABE13 and NABE14. Before crossing with the susceptible varieties, the SCAR markers SAS13, SBB14, SAB3 and SB12 were run on extracted DNA of 105 F_1 plants of the cross [(G2333 x PI207262) x RWR719]. DNA of 35 F_1 plants positively amplified all the three desired anthracnose resistance genes (*Co-4*^{2}*/Co-4*^{3}, *Co-5* and *Co-9*). Six of the 35 F_1 plants were selected and crossed with the four susceptible market class varieties. F_1 seeds of the crosses [((G2333 x PI207262) x RWR719) x K132, NABE4, NABE13, NABE14] was harvested and replanted. Markers SBB14, SAB3, SB12 and PYAA19$_{800}$ were run on DNA extracted from 46 F_1 plants. Two plants possessing all three anthracnose and one *Pythium* resistance markers and one plant possessing all three anthracnose resistance markers but lacking the *Pythium* resistance marker were identified. The two plants possessing all the four desired genes in heterozygous state became the root genotype (Servin et al., 2004). F_2 seeds from the root genotype was harvested and replanted. DNA extracted from 69 F_2 plants was amplified using SBB14, SAS13, SAB3, SB12 and PYAA19$_{800}$ markers. Molecular data revealed an array of genotypes among the F_2 segregating populations ranging from genotypes with all four desired genes to those that did not inherit any of the desired genes.

The target of the study was to develop individuals with at least two anthracnose and one *Pythium* resistance genes in homozygous state. Nine plants possessing all the four desired genes were identified and advanced to F_3 generation along with several other plants with desirable gene combinations.

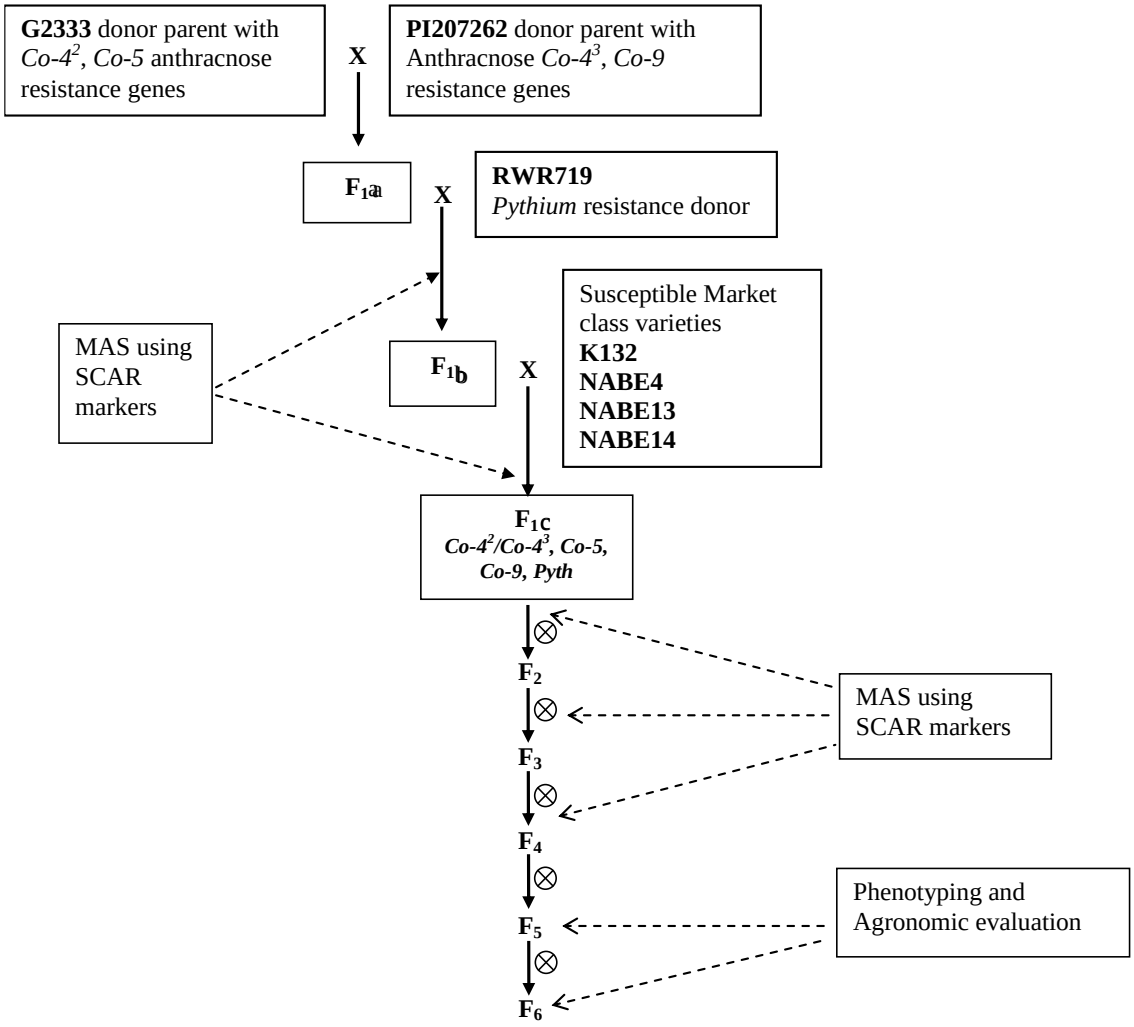

Figure 4.1: Breeding strategy used to develop advanced common bean lines pyramided with anthracnose and *Pythium* resistance genes

DNA samples of 156 F_3 plants were amplified using SBB14, SAS13, SAB3, SB12 and $PYAA19_{800}$ SCAR markers and out of these six plants inherited all the four target genes. Selfing continued up to F_6 generation to ensure that the genes were fully fixed in homozygous state, however, due to limited resources, MAS was conducted up to F_4 generation of the fixation scheme.

4.2.6 Phenotypic screening

Four races of *C. lindemuthianum* namely 352, 713, 767 and 2047 were used to inoculate detached leaves (Tu, 1986) of the pyramided lines in order to screen for their resistance spectrum. Starting from 14 days after planting, leaf trifoliates were detached after approximately two thirds of their full development. Each detached trifoliate was inoculated by immersion in the suspension containing *C. lindemuthianum* spores at a concentration of 1.2×10^6 ml^{-1}. The inoculated leaf trifoliates were placed in transparent plastic containers containing moistened paper towels and transparent covers were tightly placed to allow for humidity build-up. The containers were placed on wooden shelves fitted with Phillips^R TLT 18-20W/75RS Fluorescents tubes that supplied approximately 50µmoles $m^{-2}s^{-1}$ of light. A light timer was connected to enable 12 hour daily light regime. Room temperatures were maintained between 22^{o}C and 25^{o}C with the help of an air conditioner. Disease symptoms were scored after a seven day incubation period using the 1 – 9 scale by Balardin et al. (1997) where; 1-2 = no symptoms (resistant), 3-4 = very small lesions mostly on primary leaves (resistant), 5– 9 = numerous enlarged lesions or sunken cankers on the lower side of the leaves or hypocotyls (susceptible).

4.3 Results and discussion

4.3.1 Pyramided lines development and yield evaluation

The pedigree steps lasted for 12 months while the fixation steps lasted for 20 months making a total of 32 months of implementing the gene pyramiding scheme. 53 $F_{4:5}$ and 69 $F_{4:6}$ familes were obtained. However, a total of 101 families with varying gene combinations were not advanced beyond F_4 generation due to costs involved in handling many families. Out of 69 $F_{4:6}$ families, three families inherited all the four target genes, 18 families inherited three genes, 21 families inherited two genes, 15 families inherited one gene and 12 famileies inherited none of the target genes. Thirty families possessed both anthracnose and *Pythium* resistance genes. Of these 14 families inherited two anthracnose and one *Pythium* resistance genes and two families inherited three anthracnose and one *Pythium* resistance genes.

Plate 4.1 shows seeds of parents used in the pyramiding scheme and some of the bulked F_6 lines The overall best five genotypes/ families in terms of seed weight per plant were line 16.1.2.3.7.1 (45.8gms), anthracnose resistance donor parent G2333 (37.1gm), susceptible parent K132 (36.1gm), *Pythium* root rot resistance donor parent RWR719 (34.3gm), line 44.7.8.2.91.11 (31.6gm). Three of these were parents while the other two were advanced lines each with a single gene inherited. There were three lines with a four-gene pyramid namely 44.7.9.8.102.1 (8.4gm), 44.7.2.2.76.4 (7.9gm) and 44.7.2.2.76.1 (1.8gm) with a yield range of 1.8gm – 8.4gm per plant. There were fourteen genotypes with a three-gene pyramid with a yield range of 11.0gm and 28.4gm. Eighteen genotypes had two-gene pyramids with a yield range of 1.8gm and 27.8. Nine genotypes inherited only a single gene and had a yield range of 4.3gm and 45.8gm.

Among pyramided lines, the line 16.3.3.11.160.5.6 with a three-gene pyramid performed best in terms of yield per plant (28.6gm) followed by 44.1.4.5.142.4 (27.8gm) and 16.1.3.8.136.2 (26.8gms) both with a two-gene pyramid. These three lines were among the overall best ten genotypes in terms of seed weight per plant. Other gene pyramiding studies have reported successful gene pyramiding of resistance genes without significant yield penalties. For instance, Ragagnin et al. (2009) successfully used molecular markers to multiple resistance genes for bean rust, anthracnose and angular leaf spot into carioca market-type bean cultivar 'Ruda' and yield tests indicated that pyramided lines were as productive as the best carioca-type cultivars. Similarly, Suh et al. (2013), used marker assisted backcross gene pyramiding with forward and background selection to successfully pyramided three resistance bacterial blight resistance genes *Xa4, Xa5* and *Xa21* into a popular but susceptible elite rice cultivar 'Mangeumbeyo'. Agronomic evaluation did not reveal any yield penalty among the pyramided lines. Results from this study did not indicate otherwise.

However, the efficiency of a gene pyramiding program can be improved through strategies that enable recovery of the ideotype in the shortest duration and in the cheapest way. Servin et al. (2004) recommended crossing the root genotype with a blank parent to obtain a genotype carrying all favorable alleles in coupling phase then selfing the F_1 to

produce the ideotype with fixed target genes in one generation. They further stated that selfing the root genotype and the following offspring would be counter productive and span too many generations, as observed during this study.

Plate 4.1: Plates *a* and *b* are donor parents for *Co-4*^{2}, *Co-5* and *Co-4*^{3}, *Co-9* anthracnose resistance genes respectively; Plate 'd' is a donor parent for *Pythium* root rot resistance. Plates *c* and *e* are market-class released varieties susceptible to anthracnose and *Pythium* root rot while Plates *f* and *g* are market-class varieties susceptible to anthracnose but resistant to root rot. Plates h to p are F7 bulked families with pyramided genes for anthracnose and *Pythium* resistance as follows; Plate i = family 44.7.8.3.92.3.5 with Co-42 gene; Plate j = family 16.1.2.3.7.1.1 possessing *Pyth* gene; Plate k = family 44.5.2.3.28.4.5 possessing *Co-4*^{2}*+Co-5+Co-9+Pyth* pyramid; Plate l = family 44.1.4.5.145.4.6 possessing *Co-5+Pyth* pyramid; Plate m = family 16.1.3.3.136.1.4 possessing *Co-4*^{3}*+Pyth* pyramid; Plate n = family16.3.3.9.158.1.3 possessing *Co-4*^{2}*+Co-5+Pyth* pyramid; Plate o = family 16.3.3.11.160.2.2 possessing *Co-4*^{2}*+Co-5+Pyth* pyramid; and Plate p = family 42.12.4.4.161.1 possessing *Co-5+Co-9+Pyth* pyramid

4.3.2 Specific molecular marker analysis and polymorphism

Table 4.3 below summarizes frequencies of the different allele combinations over four MAS cycles.

Table 4.3: Allele combination frequencies over four MAS cycles

#	Allele combinations*	Allele frequencies				Total
		MAS 1	MAS 2	MAS 3	MAS 4	
1	*Co-4*+Co-5+Co-9+Py*	0	2	9	6	17
2	*Co-4+Co-5+Py*	0	2	2	12	16
3	*Co-4+Co-9+Py*	0	3	12	2	17
4	*Co-5+Co-9+Py*	0	3	5	4	12
5	*Co-4+Py*	0	1	2	16	19
6	*Co-5+Py*	0	1	1	12	14
7	*Co-9+Py*	0	7	4	0	11
8	*Co-4+Co-5+Co-9*	35	1	7	18	61
9	*Co-4+Co-5*	5	1	4	15	25
10	*Co-4+Co-9*	41	5	6	11	63
11	*Co-5+Co-9*	1	2	3	12	18
12	*Co-4*	7	4	1	9	21
13	*Co-5*	0	1	1	1	3
14	*Co-9*	4	7	2	6	19
15	*Py*	0	3	1	13	17
16	*No gene inherited*	8	3	9	19	39
Total individuals		101	46	69	156	372

MAS = Marker assisted selection; * *Co-4* locus with either *Co-4*^{2} or *Co-4*^{3} tagged by SAS13 & SBB14 markers; *Co-5* tagged by SAB3 marker; *Co-9* tagged by SB12 marker; and *Py* tagged by PYAA19$_{800}$ marker.

The four dominant markers SAS13, SAB3, SB12, PYAA19$_{800}$ and one codominant marker SBB14 were polymorphic between the resistant and susceptible parents and were successfully used to distinguish genotypes where target gene was present from those where the gene was absent (Plate 4.2). A total of 1,704 PCR reactions were performed with the five markers in four MAS cycles out of which 893 were positive reactions. SAS13 was the most frequent marker with 64.2% of 372 reactions followed by SB12 (58.6% of 372 reactions), SBB14 (47.0% of 317 reactions), PYAA19$_{800}$ (45.4% of 271 reactions) and SAB3 (44.1% of 372 reactions).

It was observed that frequency of appearance of a marker in a population increased as the linkage distance of the marker from the target gene decreased. Among the four markers, SAB3 is reported by Vallejo and Kelly (2001) and Campa et al. (2005) to be the most distant marker from the gene of interest at 12.98cM and 14.4cM respectively. This is

followed by SBB14 linked to the *Co-4*^{2} allele at 5.89cM (Awale and Kelly, 2001). SB12 is reported to be linked to the *Co-9* gene at a distance of 2.9cM (Mendez de Vigo *et al*., 2002) while SAS13 is reported to be the most tightly linked to the *Co-4* locus (Young *et al*., 1998). As a general rule, 1cM of genetic distance is approximately equal to 1% recombination and hence, as the distance between the marker and the gene of interest increases, there is a greater chance of recombination between gene and marker (Byrne and Richardson, 2005). This implies that the chances of recombination leading to false positives were highest with SAB3 and least with SAS13.

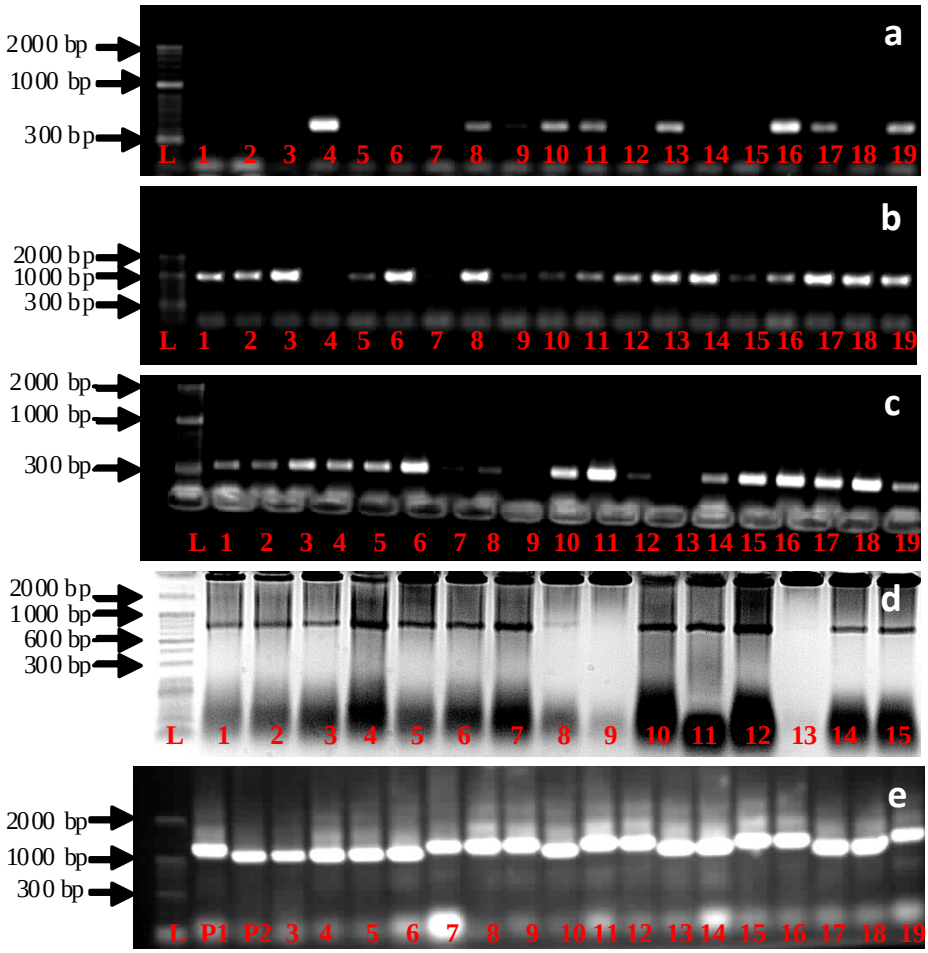

Plate 4.2: Plate a; DNA fragment at 400bps amplified with SAB3 primer linked to *Co-5* gene. Plate b; DNA fragment at 950bps amplified with SAS13 primer linked to *Co-4* locus. Plate c; DNA fragment at 350bps amplified with SB12 primer linked to *Co-9* gene. Plate d; DNA fragment at 800bps amplified with KL1KR1 primer linked to *Py* gene. Plate e; Two DNA fragments at 1150bps for upper fragment and 1050bps for lower fragment amplified with SBB14 codominant primer linked to allele *Co-4*^{2}. Lanes: *L* 2000bps molecular weight marker ladder; *1 – 19* segregating lines; *P1* Donor parent; *P2*

The breeder's capacity to identify the desired genotype is one of the factors that determine the success of gene pyramiding. The use of tightly linked markers has greatly enhanced this capacity. The efficiency of marker based gene pyramiding will increase substantially if the markers are tightly linked with the target gene. In the absence of tightly linked markers the use of a pair of markers flanking the target gene can be effective (Ye and Smith, 2008). A new highly linked sequence tagged site (STS) marker, $g1233_{3250}$, located at a distance of 1.2cM from the *Co-5* gene, has been mapped and reported by Souza et al. (2014). This marker will increase the efficiency of selection for the *Co-5* gene in MAS programs.

The two donor parents G2333 and PI207262 have different alleles at the *Co-4* locus namely $Co\text{-}4^2$ and $Co\text{-}4^3$ respectively. There was a challenge using a single marker to detect which of the two alleles was present in a sample. This is because the SAS13 marker is reported to amplify consensus sequences common to all alleles at the *Co-4* locus. However, we found that the codominant marker SBB14 specifically amplified the $Co\text{-}4^2$ allele which agrees with the findings of Awale and Kelly (2001). Therefore, by running both SAS13 and SBB14 markers on the same samples it was possible to differentiate between genotypes possessing $Co\text{-}4^2$ allele from those possessing $Co\text{-}4^3$ allele. This implies that the cost of MAS involving the two alleles would be higher since two markers have to be run on samples to differentiate between the two alleles. All breeding programs are operated within the limits of available operating capital and therefore reducing the overall cost is always an important consideration when choosing a strategy (Ye and smith, 2008).

Another major challenge encountered was that the SCAR markers SAS13, SAB3, SB12, $PYAA19_{800}$ provided limited information at the loci they tag because they are bi-allelic dominant in nature. This implies that it is only possible to tell whether a given allele is present or not at a given locus but cannot distinguish between the heterozygous from the homozygous genotypes in a segregating population. Codominant markers are preferred to dominant markers due to the larger information content since they allow the distinction of homozygous and heterozygous genotypes through gel electrophoresis (Piepho and Koch,

2000). The optimal strategy of gene pyramiding is one involving a minimum number of generations required to fix all the loci for the desirable alleles with the shortest duration and within a fixed expected investment (Ye and Smith, 2008). Codominant markers will be useful to achieve such a strategy since they enable early generation identification and selection of homozygous genotypes at a given locus and therefore eliminate the necessity of further genotyping for the fixed allele which allows the breeder to subsequently focus on fewer segregating alleles in subsequent generations.

4.3.3 Resistance evaluation for anthracnose

Thirty six genotypes possessing varying number of genes in different combinations, based on molecular marker data, were evaluated for resistance using four races of *C. lindemuthianum* (2047, 713, 767 and 352) under controlled conditions. All the five genotypes that possessed all the three anthracnose pyramided genes *Co-4*^{2}, *Co-5* and *Co-9* were resistant to all the four races. Three of four genotypes possessing *Co-4*^{2} and *Co-5* genes were also resistant to all the four races. Two genotypes possessing *Co-5*+*Co-9* pyramid were resistant to all races while two genotypes with the same pyramid succumbed to the aggressive races 2047 and 767. Four of five genotypes possessing single gene *Co-4*^{2} were resistant to all the four races, while genotypes with only *Co-4*^{3} allele succumbed to aggressive races 2047 and 767. The two genotypes that possessed single gene *Co-9* and the genotype that possessed *Co-4*^{3}*+Co-9* pyramid succumbed to three aggressive races 2047, 713 and 767 and showed resistance to only race 352. The donor parents PI207262 possessing the *Co-4*^{3}*+Co-9* pyramid was only resistant to race 352.

All the six genotypes that did not inherit any anthracnose resistance gene were susceptible to all the four races. Three of four genotypes possessing *Co-5* single gene were resistant to all the four races. It is probable that the one genotype of the four which was susceptible to all the four races was a case of recombination of the SAB3 marker. It is worth noting that genotypes with single genes *Co-4*^{2} and *Co-5* were resistant to all the four races just like genotypes with the *Co-4*^{2}*+Co-5+Co-9* and *Co-4*^{2}*+Co-5* pyramids. This observation is opposed to findings of studies done on bacterial blight in rice where

four resistance genes showed a broadspectrum and higher resistance than the lines with a single resistance gene (Gu *et al*. 2005; Jeung *et al*. 2006; Kim *et al*. 2009; Singh *et al*. 2001; Suh *et al*. 2009; Suh *et al*. 2013).

4.4 Conclusion

The study demonstrated that SCAR markers can be used to successfully pyramid multiple anthracnose and Pythium resistance genes in susceptible varieties. Pyramided genes effectively confered resistance to the four races of *C. lindemuthianum*, however, single genes *Co-4*^{2} and *Co-5* demonstrated effective resistance against the races as well.

Table 4.4: Reaction of pyramided lines and parents to four races of *C. lindmeuthianum* and four SCAR markers

Genotype	[a]Pedigree	Phenotypic data				Genotypic data			
		Races				Markers			
		2047	713	767	352	SAS13	SAB3	SB12	$PYAA19_{800}$
44.5.7.1.1.1.1	12x8xRWR719xK132	S	S	S	R	0	0	1	0
16.1.2.1.6.3.3	12x8xRWR719xK132	S	S	S	S	0	0	0	0
16.1.2.1.6.3.5	12x8xRWR719xK132	S	S	S	S	0	0	0	0
16.1.2.3.7.1.4	12x8xRWR719xK132	S	S	S	S	0	0	0	1
16.6.1.6.25.1.2	12x8xRWR719xK132	S	R	R	R	1	0	0	0
44.5.2.1.26.1.4	12x8xRWR719xK132	S	S	S	S	0	0	0	0
44.5.2.3.28.1.1	12x8xRWR719xK132	S	R	S	R	0	1	1	0
44.5.2.3.28.1.2	12x8xRWR719xK132	S	R	S	R	0	1	1	0
44.5.2.3.28.4.5	12x8xRWR719xK132	R	R	R	R	1	1	1	0
44.5.2.3.28.4.8	12x8xRWR719xK132	R	R	R	R	1	1	1	0
44.7.2.2.76.1.1	12x8xRWR719xNabe 4	R	R	R	R	1	1	1	1
44.7.2.2.76.4.1	12x8xRWR719xNabe 4	R	R	R	R	1	1	1	1
89.5.1.1.81.1.1	12x8xRWR719xNabe 4	R	R	R	R	1	0	0	1
44.7.8.2.91.10.3	12x8xRWR719xNabe 4	R	R	R	R	1	0	0	0
44.7.8.2.91.10.4	12x8xRWR719xNabe 4	R	R	R	R	1	0	0	0
44.7.9.5.92.2.3.4.6	12x8xRWR719xNabe 4	R	R	R	R	1	0	0	0
44.7.9.8.102.1.1	12x8xRWR719xNabe 4	R	R	R	R	1	1	1	0
44.7.5.8.112.1.3	12x8xRWR719xNabe 4	S	S	S	S	0	0	0	0
89.5.2.7.117.1.1	12x8xRWR719xNabe 4	S	R	R	R	1	0	1	0
89.5.2.10.118.15.3	12x8xRWR719xNabe 4	S	S	S	R	1*	0	1	0
44.1.6.7.130.1.1	12x8xRWR719xNabe 13	S	S	S	S	0	0	0	0
16.1.3.8.136.1.4	12x8xRWR719xNabe 13	R	R	S	R	1*	0	0	1
16.1.3.8.136.2.7	12x8xRWR719xNabe 13	S	R	S	R	1*	0	0	1
16.1.3.8.136.2.8	12x8xRWR719xNabe 13	S	R	S	R	1*	0	0	1
44.1.4.3.141.1.2	12x8xRWR719xNabe 13	R	R	R	R	0	1	1	1
44.1.4.3.141.1.4	12x8xRWR719xNabe 13	R	R	R	R	0	1	1	1
44.1.4.5.142.4.2	12x8xRWR719xNabe 13	R	R	R	R	0	1	0	1
44.1.4.5.143.1.4	12x8xRWR719xNabe 13	R	R	R	R	0	1	0	0
16.3.3.1.151.1.1	12x8xRWR719xNabe 14	S	S	S	S	0	1	0	1
16.3.3.1.151.1.2	12x8xRWR719xNabe 14	R	R	R	R	0	1	0	1
16.3.3.2.152.1.2.2	12x8xRWR719xNabe 14	S	S	S	S	0	0	0	0
16.3.3.9.158.1.2	12x8xRWR719xNabe 14	R	R	R	R	1	1	0	1
16.3.3.9.158.1.3	12x8xRWR719xNabe 14	R	R	R	R	1	1	0	1
16.3.3.11.160.2.2	12x8xRWR719xNabe 14	S	R	R	R	1	1	0	1
16.3.3.11.160.2.3	12x8xRWR719xNabe 14	R	R	R	R	1	1	0	1
42.12.4.1.161.4	12x8xRWR719xNabe 14	S	S	S	R	0	0	1	1

Parents									
PI207262	Tlalnepantla 64	S	S	S	R	1*	0	1	0
Nabe 4	Sug 47 x Cal 103	S	S	S	S	0	0	0	0
RWR719	Omo-95	S	S	S	S	0	0	0	1
G2333	Colorado de Teopisca	R	R	R	R	1	1	0	0
K132	Calima-2 x Argentino	S	S	S	S	0	0	0	0
Nabe 13	RWR 1946	S	S	S	S	0	0	0	1
Nabe 14	RWR 2075	S	S	S	S	0	0	0	1

* *Co-4*^{3} allele was identified in segregating populations by simultaneously running SBB14 and SAS13 markers on the samples

[a] 12 is the position number of cultivar G2333 while 8 is position number of cultivar PI207262 in the anthracnose differential series

R = resistant reaction, S = Susceptible reaction; 1 = marker present, 0 = marker absent

SAS13 marker tags *Co-4* locus (both *Co-4*^{2} & *Co-4*^{3} alleles), SAB 3 marker tags *Co-5* gene, SB12 marker tags *Co-9* gene and PYAA19$_{800}$ marker tags *Pythium* resistance gene

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CHAPTER FIVE

Effectiveness of pyramided genes in conferring broad resistance to bean anthracnose disease

Abstract

Single gene deployment against *Colletotrichum lindemuthianum* has been rendered ineffective due to continual emergence of new races. Gene pyramiding has been proposed as a strategy to increase spectrum and durability of resistance to the diverse pathotypes. Using Sequence characterized amplified regions (SCAR) markers, three anthracnose resistance genes; *Co-4^2*, *Co-5* from cultivar G2333 and *Co-4^3*, *Co-9* from cultivar PI207262 were pyramided in market class susceptible bean varieties K132, Nabe 4, Nabe 13 and Nabe 14. The genes *Co-4^2* and *Co-4^3* are allelic. A cascading pyramiding scheme was used and crossing was conducted as follows; (((G2333 x PI207262) x RWR719) x Susceptible). Thereafter selfing was done for five generations up to F6 to fix the genes. Segregation produced different pyramids with different number and combination of genes. Using the detached leaf method, 40 F6 advanced genotypes were screened with four races of *C. lindemuthianum* namely 352, 713, 767 and 2047. Data was subjected to ANOVA and means were subjected to Tukey's Honest Significant Test (HSD) to test the hypothesis that all gene pyramid groups react equally to *C. lindemuthianum* races. Results showed that there were significant differences (P<0.001) among races and among genotypes and their interaction. Five gene pyramid groups were developed and evaluated namely *Co-4^2+Co-5+Co-9*, *Co-4^2+ Co-5*, *Co-4^2+Co-9*, *Co-5+Co-9*, and Co-*4^3+Co-9*. In addition, four single-genes groups; *Co-4^2*, *Co-4^3*, *Co-5*, *Co-9* and a "*no-gene*" group were developed. The five gene pyramid group means were significantly different from each other (*P<0.01*) of which Of these, *Co-4^2+Co-5+Co-9* and *Co-4^2+Co-5* exhibited the lowest mean disease score to all the four races indicating both a high degree and a broad spectrum of resistance. The group *Co-4^3+Co-9* had the highest mean disease severity. The single-gene groups were significantly different from each other (*P<0.01).* The *Co-4^2* and *Co-5* gene groups both conferred resistance to all the four races 352, 713, 767 and 2047, while the *No-gene* (5.9±0.21) group was overcome by all the four races. *Co-4^2* group had the least mean severity across races followed by *Co-5*, Co-*4^3*, *Co-9* and *No-gene*. The single gene *Co-4^2*was not significantly different from the best pyramid groups *Co-4^2+Co-5+Co-9* and *Co-4^2+Co-5* (*P<0.01*) and was better than the other three pyramid groups. Similarly the *Co-5* single gene was not significantly different from *Co-4^2+Co-5*, *Co-4^2+Co-9* and *Co-5+Co-9* gene pyramid groups (*P<0.01*). The study suggests that the two single genes *Co-4^2* and *Co-5* have factors that promote broad-spectrum and an increased degree of resistance to *C. lindemuthianum* and are therefore recommended for combining with other single genes to develop highly effective gene pyramids.

Key words: *Colletotrichum lindemuthianum*, Gene pyramiding, durable resistance, Molecular markers

5.1 Introduction

Bean anthracnose caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magn.) Bri. & Cav. (Martínez-Pacheco *et al*., 2009) is regarded as the most severe disease of common bean worldwide causing up to 44% total yield loss and 72% marketable yield loss on farmers' fields (Nkalubo *et al*., 2007). *Colletotrichum lindemuthianum* has a high degree of pathogenic and genetic variability (Melloto et al., 2000; Mahuku and Riascos, 2004; Kelly and Vallejo, 2004). New races that break down resistance in commercial cultivars continually emerge (Kelly *et al*., 1994). This has made single gene deployment as a strategy to control the disease, ineffective and complicated. Preliminary findings suggest that 27 races exist in Uganda. However, genetic resistance still remains the most cost-effective means to control the disease on a large scale for resource-poor farmers, given that agrochemical use increases production costs and has severe long term environmental impacts.

Through gene pyramiding, it is possible to breed for resistance to a broad range of bean anthracnose races and reduce the problem of pathogen variability (Young and Kelly, 1997). Gene pyramiding is based on the probabilities hypothesis which states that "cultivars possessing multiple race-specific resistance genes, owe their broad-spectrum/ durable resistance to a low probability of the pathogen independently mutating to virulence at multiple avirulent loci corresponding to the host resistance genes" (Mundt, 1990). Wheeler and Diachun (1983), in support of the hypothesis postulated that a variety with four to five resistance genes might provide stable resistance for centuries. According to Mundt (1990, 1991), however, this hypothesis does not sufficiently explain why and how pyramids work because it lacks supportive empirical data, assumes that mutations to virulence at different loci are independent and also assumes that mutation is the only source of genetic variation for plant pathogens. The presence of certain single genes such as the *Sr6* resistance genes for wheat stem rust was found to be more closely associated with broad-spectrum/ durable resistance than number of genes possessed by a cultivar, implying that the choice of genes to be deployed may be as important as the number of genes to be pyramided (Mundt 1990).

Conventional gene pyramiding requires extensive phenotyping with several races of the pathogen at every crossing generation, due to the race specificity of many of these resistance genes (Ye and Smith, 2008). Pyramiding genetically diverse resistance genes using MAS and deploying different gene combinations in different geographic regions is probably the most practical approach incontrolling the diverse *C. lindemuthianum* pathogen (Balardin and Kelly, 1998). DNA markers greatly facilitate selection of plants with desired traits as they are non-destructive allowing selection for multiple specific genes from one DNA sample without phenotyping (Collard and Mackill, 2008). Molecular markers have extensively been used in the selection and characterization of single anthracnose resistance genes found in naturally occurring pyramids such as in cultivar G2333 (Young et al., 1998). Vallejo and Kelly (2009) used a RAPD marker $AB3_{450}$ to dissect the *Co-5, Co-7* gene pyramid in line SEL 111. Miklas et al. (2003) used the SCAR marker SAS13 (Young *et al*., 1998) to develop Pinto bean germplasm having the *Co-4*^{2} anthracnose resistance gene.

Different authors studying bean anthracnose have suggested the use of gene pyramiding as a strategy to increase broad resistance to bean anthracnose. Kelly et al. (1994), suggested that pyramiding *Co-1* (*A*) and *Co-2* (*Are*) genes would afford protection against all known *C. lindemuthianum* races in North America. The *A* gene is of Andean origin while *Are* gene is of Meso-American origin. Young and Kelly (1996) suggested that deployment of major genes such as *Co-6* and *Co-5* (*Mexique 3*) in different combinations with *Co-1* (*A*) gene should contribute to more durable anthracnose resistance in common beans. Mahuku and Riascos (2004), after observing a higher pathotype diversity of *C. lindemuthianum* in regions cultivating Mesoamerican cultivars than in regions cultivating Andean cultivars, suggested pyramiding resistance genes which have a greater effect against Mesoamerican races in these regions. Kelly and Vallejo (2004) further suggested that due to the limited use of *Co-5* gene offers breeders a valuable resource for use in in pyramiding programs with other independent resistance genes.

However, much as the use of gene pyramiding for broad-spectrum resistance to bean anthracnose has been suggested and recommended, efforts by breeders to undertake gene pyramiding are still limited and in addition there is need to understand the nature of action of single resistance genes and their combined effect against an array of *C. lindemuthianum* races in Uganda in order have a

successful gene pyramiding program. The aim of this study, therefore, was to assess the effectiveness of resistance conferred by different gene pyramids involving *Co-4*^{2}, *Co-4*^{3}, *Co-5* and *Co-9* genes against different *C. lindmuthianum* races.

5.2 Materials and methods

5.2.1 Pyramided line development

5.2.1.1 Cascading Pedigree scheme

The gene pyramiding scheme illustrated in Figure 4.1 was used. All parental materials used were obtained from the Bean breeding program at the National Crops Resources Research Institute (NaCRRI), located 00 32" N of the Equator and 320 37" E, 27Km North of Kampala and elevated at 1150 meters above sea level (asl). The parent G2333 bearing three resistance genes; *Co-4*^{2}, *Co-5* and *Co-7* was crossed with PI207262 (*Co-4*^{3} and *Co-9*) under screen house conditions and F_{1a} seeds harvested. Harvested F_{1a} plants were crossed with the line RWR719 a donor parent for *Pythium* root rot resistance to develop the genotype [(G2333 x PI207262) x RWR719] and the F_{1b} seed harvested. The F_{1b} seed was planted together with four released but susceptible varieties namely K132, NABE 4, NABE 13 and NABE 14. The SCAR markers SAS13, SBB14, SAB3, SH18 and SB12 markers were run on 105 F_{1b} plants of the cross [(G2333 x PI207262) x RWR719] and plants positive for the five desired anthracnose and *Pythium* resistance genes (*Co-4*^{2}*/Co-4*^{3}, *Co-5*, *Co-9 and Py*) were crossed with each of the four susceptible parents K132, Nabe 4, Nabe 13 and Nabe 14 to develop the F_{1c} root genotype.

5.2.1.2 Fixation scheme

The F_{1c} root genotype (Servin *et al*., 2004) seed was advanced to F_2 and MAS conducted to select for anthracnose resistance and *Pythium* resistance genes. Plants positive for the markers of interest were advanced up to F_6 with MAS being done at each generation upto F_4 to identify genotypes with desired gene combinations. At each generation selected individual plants were allowed to self in order to fix the genes of interest. All crosses and F_1 - F_4 progeny advancement and testing were conducted under screen house conditions at NaCRRI. Fixation of alleles up to F_6, genotyping and phenotyping of lines was conducted at the International Center for Tropical Agriculture (CIAT) research facilities at the National Agricultural Research Laboratories (NARL), Kawanda, Uganda.

5.2.2 Genotyping using SCAR Marker Assisted Selection system

Leaf samples were picked from 14-day old plants in the screen house. Genomic DNA was extracted using the Cetytrimethylammoniumbromide (CTAB) (Rogers and Bendich, 1988). Genomic DNA was stored in 50µl of 0.1 X TE (10mM Tris-HCl, PH8; 1mM EDTA) buffer at –20^{o}C. Sequence Characterized Amplified Region (SCAR) markers were obtained from the Department of Molecular and Cellular Biology, University of Cape Town, Randebosch, South Africa (see Table 4.2 for primer details). DNA amplification, gel electrophoresis and gel viewing were done as described in section 4.2.4.1.

5.2.3 Phenotyping anthracnose resistance levels in different pyramid groups

Four of the 27 *C. lindemuthianum* races namely 352, 713, 767, 2047 characterized as shown in Table 3.3 were used for screening bean lines with different gene combinations.

5.2.3.1 Inoculum preparation

Single spore isolates of each race were placed on fresh Mathur's agar medium in separate Petri dishes and incubated in total darkness at a controlled temperature between 22-25^{o} C for 4-7 days to allow the fungus enough time to produce conidial spores. For inoculation purposes, 10mls of distilled water were poured 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 adjust conidial concentration to 1.2 x 10^{6} conidia ml^{-1} and 0.1% Tween 20 was added to the suspension as a surfactant.

5.2.3.2 Raising bean lines to be screened

Seed of test lines/ cultivars was sown in five liter plastic pots filled with top soil mixed with sand and sterilized manure in the ratio of 5:3:2 respectively under greenhouse conditions. Diammonium Phosphate (DAP) fertilizer was applied prior to sowing and watering was done daily.

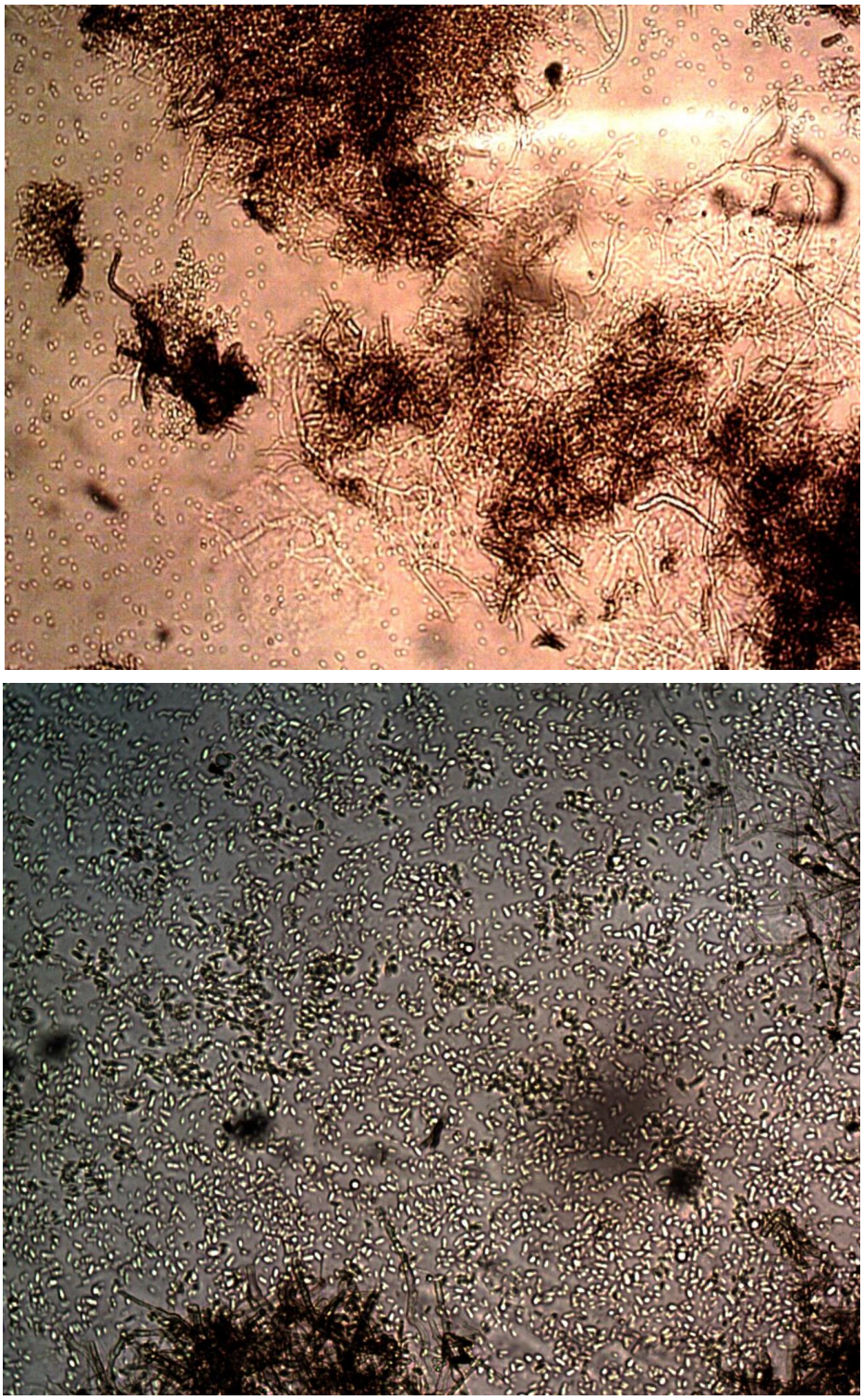

Plate 5.1: Photos of *Colletotrichum lindemuthianum* in sporulation taken using Optikam Pro 3 microscope digital camera; *above)* Race 713; *below)* Race 2047

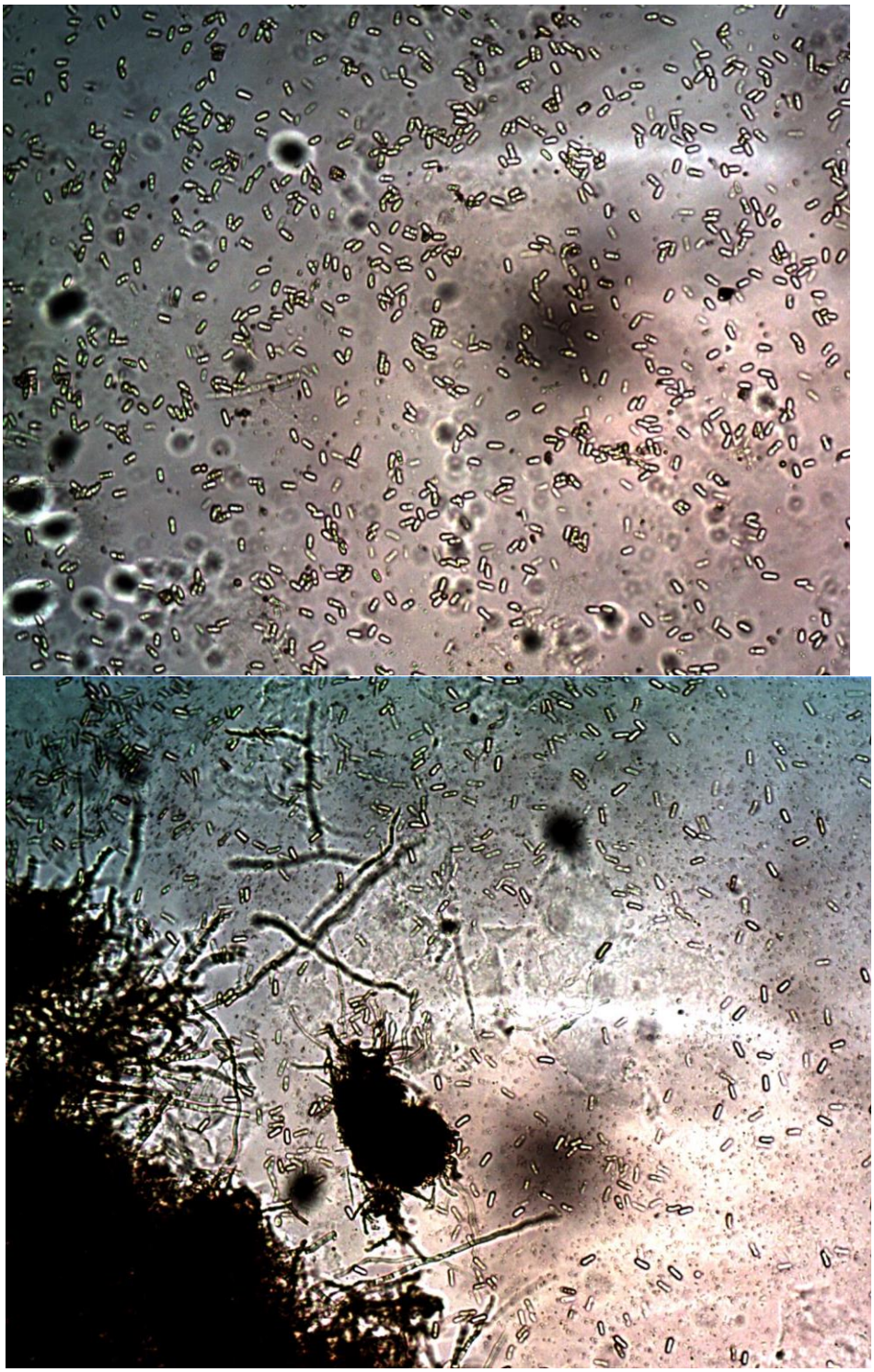

Plate 5.2: Photos of *Colletotrichum lindemuthianum* in sporulation taken using Optikam Pro 3 microscope digital camera; *above)* Race 767; and *below)* Race 352

5.2.3.3 Inoculation and screening using detached leaf method

Starting from 14 days after planting, leaf trifoliates were detached after approximately two thirds of their full development. Each detached trifoliate was inoculated by immersion in the suspension containing *C. lindemuthianum* spores. The inoculated leaf trifoliate was placed in a transparent plastic container, of 1 - 3 liters capacity containing moistened paper towels and then tightly covered to allow for humidity build-up. The containers were placed on wooden shelves fitted with Phillips^{R} TLT 18-20W/75RS Fluorescents tubes that supplied approximately 50µmoles $m^{-2}s^{-1}$ of light. A light timer was connected to enable 12 hour daily light regime. Room temperatures were maintained between 22^{o}C and 25^{o}C with the help of an air conditioner. Anthracnose disease symptoms were scored after a seven day incubation period using a modified 1 – 9 scale based on Balardin et al. (1997) where; 1 = no symptoms (resistant), 3-4 = very small lesions mostly on primary leaves (resistant), 5 – 9 = numerous enlarged lesions or sunken cankers on the lower side of the leaves or hypocotyls (susceptible).

Incubation process using the detached leaf method of screening for anthracnose resistance. Above: Sealed transparent container with detached leaf trifoliate showing humidity build up. Below) improvised screening chamber fixed with Flourescent tubes and a light timer

5.2.4 Data analysis

Disease severity data was subjected to Analysis of Variance (ANOVA) using GenStat Discovery, 12th Edition (Anonymous, 2009). To determine whether pyramid group means were significantly different with respect to anthracnose resistance levels, a Tukey's Honest Significant Difference (HSD) test was carried out to test the null hypothesis H_o; all gene pyramid group means are equal; $H_o : \mu_i = \mu_j$, $H_a : \mu_i \neq \mu_j$. Tukey Test statistic; $HSD = q\sqrt{MSE/n_c}$. Where; q = value from studentized range table, MSE = Mean Square for Error from ANOVA table, n_c = number of replicates per treatment. Standard error of pyramid group means (SEM) was computed using the formula; $SEM = s/\sqrt{n}$, where; s = sample standard deviation and n = sample size. Sample standard deviation (s) was computed using the formula; $s = \sqrt{1/N-1} \sum^{N}_{i=1}(x_i - x)^2$, where; x_1 …., x_N = the sample data set, x = mean value of the data set, N = size of sample data set.

5.3 Results and discussion

5.3.1 Effectiveness of gene pyramid groups in reducing anthracnose disease levels

Genotypes, races and genotype by race interactions were found to be significantly different at $p<0.001$ (Table 5.1) indicating their relative importance in development and progress of disease symptoms on the host plant.

Table 5.1: Analysis of variance for severity of four races on different Gene-groups

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	0.175	0.087	0.75	
Gene-group	9	276.121	30.680	264.14	<.001
Race	3	76.547	25.516	219.68	<.001
Gene-group x Race	27	53.581	1.985	17.09	<.001
Residual	78	9.060	0.116		

There were five gene pyramid groups with two to three pyramided resistance genes namely, *Co-4²+Co-5+Co-9*, *Co-4²+ Co-5*, *Co-4²+Co-9*, *Co-5+Co-9*, and Co-*4³+Co-9* (Table 5.2 and Figure 5.1). The *Co-4²+Co-5+Co-9* and the *Co-4²+Co-5* groups conferred resistance to all the four races 352, 713, 767 and 2047. The *Co-4²+Co-9* and *Co-5+Co-9* pyramid groups conferred resistance to races 352, 713 and 767 but were overcome by race 2047 while the *Co-4³+Co-9* pyramid group was overcome by races 713, 767 and 2047 but conferred resistance to only race 352.

Table 5.2: Gene pyramid groups and their mean severities with four races of *C. lindemuthianum*

	Mean severity of races				
Pyramid group	**352**	**713**	**767**	**2047**	**Group mean**
Co-4^2+Co-5+Co-9	1.0±0.00	1.1±0.07	1.1±0.07	1.1±0.07	1.1±0.03
Co-4^2+Co-5	1.1±1.1	2.2±0.12	1.9±0.18	3.1±0.24	2.1±0.41
Co-4^2+Co-9	1.0±0.08	2.0±0.08	2.0±0.29	4.3±0.29	2.3±0.70
Co-4^3+Co-9	1.5±0.29	5.8±0.17	6.3±0.44	7.0±0.29	5.2±1.24
Co-5+Co9	1.4±0.00	2.3±0.00	3.3±0.00	4.3±0.33	2.8±0.70
Co-5	2.0±0.00	2.5±0.25	2.8±0.08	3.0±0.25	2.6±0.23
Co-4^2	1.0±0.00	1.4±0.12	1.2±0.12	2.4±0.12	1.5±0.31
Co-4^3	1.9±0.11	3.3±0.51	4.3±0.38	4.2±0.11	3.4±0.56
Co-9	2.3±0.17	5.2±0.17	5.5±0.00	4.8±0.17	4.5±0.73
No-gene	5.4±0.08	5.9±0.07	6.3±0.13	6.3±0.10	5.9±0.21
Lsd (0.05) = 0.55, S.e.d = 0.26, C.V = 7.8%					

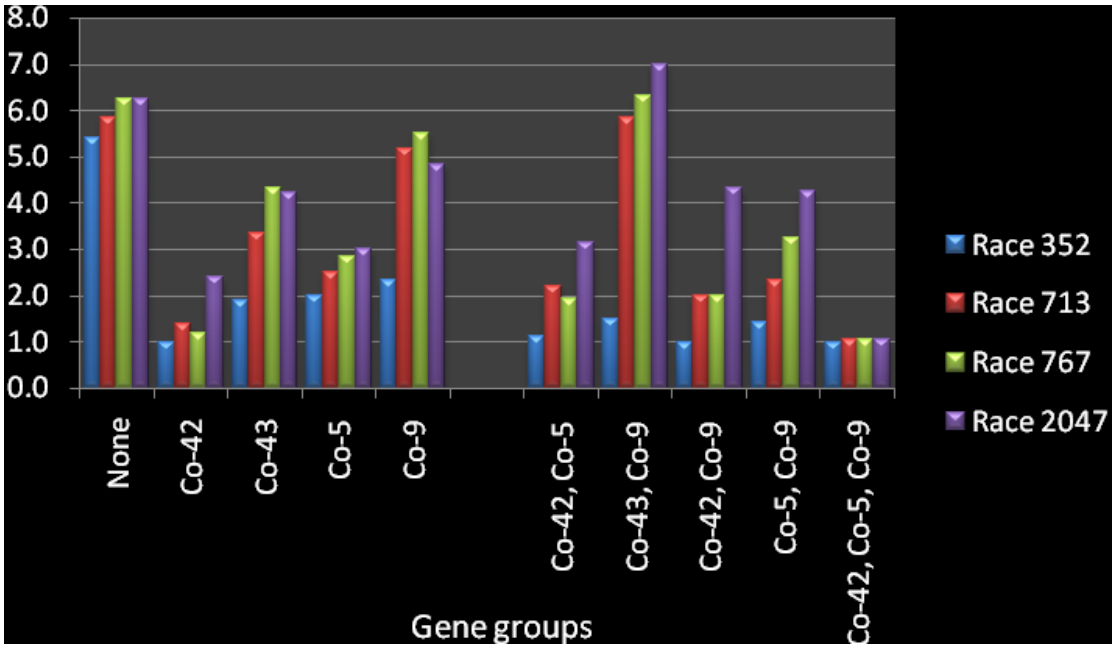

Figure 5.1: Severity levels of the different single-gene and pyramid-gene groups screened with four races of *C. lindemuthianum*

Pair-wise comparisons of gene group means on resistance to anthracnose are presented in Table 5.3. Disease severity means across the four races were significantly different from each other (*Lsd*

= 0.05, P<0.01, $HSD_{0.05}$ = 0.63, $HSD_{0.01}$ = 0.78) for all the pyramid groups, with the exception of groups *Co-4^2+Co-5* (2.1±0.41) and *Co-4^2+Co-9* (2.3±0.70).

Table 5.3: Pair-wise comparison of group means using Tukey's HSD test

		Co-4^2+Co-5+Co-9	*Co-4^2+Co-5*	*Co-4^2+Co-9*	*Co-4^3+Co-9*	*Co-5+Co-9*	*Co-5*	*Co-4^2*	*Co-4^3*	*Co-9*	*None-gene*
	Means	**1.1**	**2.1**	**2.3**	**5.2**	**2.8**	**2.6**	**1.5**	**3.4**	**4.5**	**5.9**
Co-4^2+Co-5+Co-9	**1.1**										
Co-4^2+Co-5	**2.1**	P<0.01									
Co-4^2+Co-9	**2.3**	P<0.01	n/s								
Co-4^3+Co-9	**5.2**	P<0.01	P<0.01	P<0.01							
Co-5+Co-9	**2.8**	P<0.01	P<0.05	n/s	P<0.01						
Co-5	**2.6**	P<0.01	n/s	n/s	P<0.01	n/s					
Co-4^2	**1.5**	n/s	n/s	P<0.01	P<0.01	P<0.01	P<0.01				
Co-4^3	**3.4**	P<0.01	P<0.01	P<0.01	P<0.01	n/s	P<0.01	P<0.01			
Co-9	**4.5**	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01		
None-gene	**5.9**	P<0.01	P<0.01	P<0.01	P<0.05	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01	

Critical values $HSD_{0.05}$ = 0.63, $HSD_{0.01}$ = 0.78, n/s = not significant

The pyramid group "*Co-4^2+Co-5+Co-9*" had the least mean severity 1.1±0.03 followed by group "*Co-4^2+Co-5*" with a score of 2.1±0.41 while group "*Co-4^3+Co-9*" had the highest mean severity of 5.2±1.24.

In general, the triple-gene pyramid "*Co-4^2+Co-5+Co-9*" recorded the lowest mean disease severity compared to the double and single gene combinations implying that pyramiding was a beneficial strategy in reducing leaf severity across the four pathogen races. This is evident in the amount of leaf symptoms expressed on leaves from plants with "*Co-4^2+Co-5*" combination compared with those expressed on leaves with "*Co-4^2+Co-5+Co-9*" combination. Symptoms were more pronounced in the later than in the former, though both were deemed highly resistant reactions. Among the two gene pyramids, *Co-4^2+Co-9* and *Co-4^2+Co-5* had similar mean effect although *Co-4^2+Co-9* was overcome by the aggressive race 2047. The "*Co-4^3+Co-9*" combination was not effective since it only afforded resistance to the least aggressive race, 352, of the four races. Kelly and Vallejo (2004) reported that the *Co-9* gene possesses a very specific breeding value against Andean races of *C. lindemuthianum*, but is susceptible to weaker Mesoamerican races. This could explain the poor resistance associated with this gene both singly and in combination with other genes especially *Co-4^3*.

5.3.2 Effectiveness of single-gene groups in reducing anthracnose disease levels

There were four groups with single genes *Co-4*^{2}, *Co-4*^{3}, *Co-5*, *Co-9* one group with no resistance genes "*no gene*" group. Both the *Co-4*^{2} and *Co-5* genes conferred resistance to all the four races 352, 713, 767 and 2047. The *Co-4*^{3} group conferred resistance to races 352 and 713 but was overcome by races 767 and 2047. The *Co-9* group conferred resistance to only race 352 while the *No-gene* group was overcome by all the four races. Mean severity means across the four races were significantly different (*Lsd=0.05, P<0.01, HSD*$_{0.05}$ *= 0.63, HSD*$_{0.01}$ *= 0.*78) among the four single gene-groups and the *No- gene* group (Table 3). *Co-4*^{2} group had the least mean severity (1.5±0.31) across races followed by *Co-5* (2.6±0.23), *Co-4*^{3} (3.4±0.56) and *Co-9* (4.5±0.73) groups respectively while the *No-gene* group had the highest mean severity (5.9±0.21). The mean severity, 4.5±0.73, of the *Co-9* single gene was significantly different from all the single genes and pyramids and was the only single gene with a mean severity falling in the susceptible range

5.3.3 Comparison between single-gene, two-gene and three-gene pyramid groups

The mean severity of all the pyramid groups was 2.7±0.70 while the mean severity of all the single gene groups was 3.0±0.63. These two means were not significantly different from each other although both were significantly different from the 5.9±0.21 mean score of the *No-gene* group (*P*<0.01, *HSD*$_{0.05}$ = 0.58, *HSD*$_{0.01}$ = 0.72). The mean score, 1.5±0.31, of *Co-4*^{2} single gene group was not significantly different from the mean scores, 1.1±0.03 and 2.1±0.41 of *Co-4*^{2}*+Co-5+Co-9* and *Co-4*^{2}*+Co-5* pyramid groups respectively, but was significantly different from the mean scores of other pyramid groups (Table 5.3). The mean score, 2.6±0.23, of the *Co-5* single gene was not significantly different from scores 2.1±0.41, 2.3±0.70 and 2.8±0 of the *Co-4*^{2}*+Co-5*, *Co-4*^{2}*+Co-9* and *Co-5+Co-9* pyramid groups respectively (*P<0.01,* HSD$_{0.05}$ *= 0.63, HSD*$_{0.01}$ *= 0.78*). The mean score 3.4±0.56 of the *Co-4*^{3} single gene was not significantly different from 2.8±0.70 recorded for "*Co-5+Co-9*" gene pyramid but was significantly different from 5.2±1.24 recorded for the "*Co-4*^{3}*+Co-9*" gene pyramid.

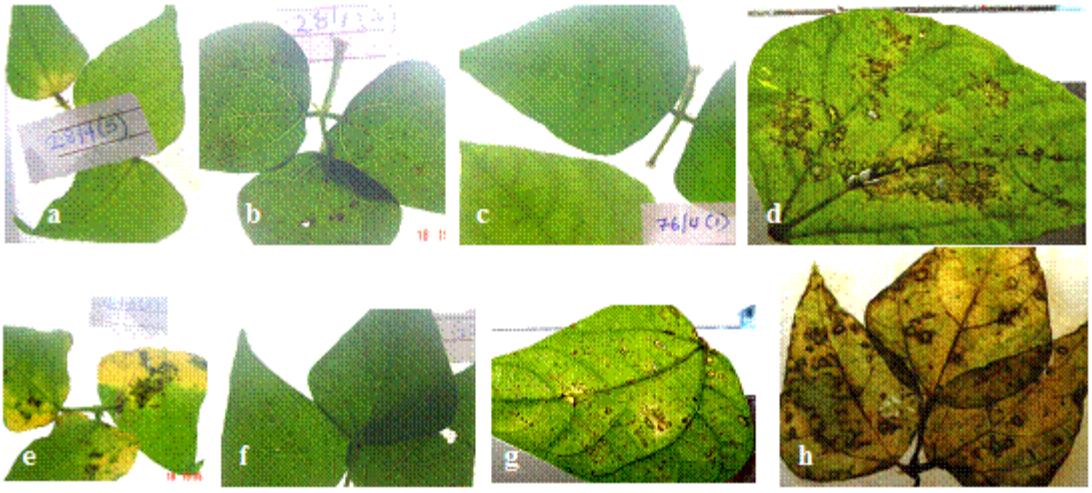

Plate 5.2: Anthracnose symptoms on detached leaves at seven days post inoculation (dpi) with race 767. Genotypes *a)* 28/4(5) with *Co-4*^{2}, *Co-5* & *Co-9* genes; *b)* 28/1(2) with *Co-5* & *Co-9* genes; *c)* 76/4(1) with *Co-4*^{2}, *Co-5* & *Co-9* genes; *d)* Parent PI207262 with *Co-4*^{3} & *Co-9* genes; *e)* 118/15(3) with *Co-4*^{3} & *Co-9* genes; *f)* parent G2333 with *Co-4*^{2} & *Co-5* genes; *g)* 136/2(7) with *Co-4*^{3} allele; and *h)* Susceptible parent K132 with no resistance gene.

Results indicate that the *Co-4*^{2}and *Co-5* single genes, which effectively conferred resistance to all the four races, have factors that promote broad-spectrum resistance and can, therefore, be deployed individually and still afford effective control in multi-race environments. The *Co-4*^{2} gene has been reported in literature to exhibit the most broad-based resistance against *C. lindemuthianum* in common beans (Young and Kelly, 1996; Balardin and Kelly, 1998; Awale and Kelly, 2001). It is located at the *Co-4* locus which has been reported to control up to 97% of all currently identified races of *C. lindemuthianum* (Melloto *et al*., 2000). Young et al. (1998) reported that the *Co-4* locus is a complex gene family and presumably the site of a multi-allelic series. Three anthracnose resistance alleles are reported to reside at this locus and include *Co-4*^{2} in cultivars G2333 and SEL 1308, *Co-4* allele in cultivar TO (Young et al., 1998) and *Co-4*^{3} in cultivar PI207262 (Alzarte-Marin *et al*., 2007). Although the *Co-4*^{3} gene group showed a susceptible reaction to races 767 and 2047 with an average mean score of 4.3 and 4.2 respectively, overall mean severity across the four races still fell under the resistant range implying that it could still be beneficial to specific races and further add value in combination with other single genes. The *Co-4*^{3} gene was reported to possess a narrower anthracnose resistance spectrum than *Co-4* in cultivar TO and *Co-4*^{2} in cultivar G2333 (Balardin et al., 1999;

Shama et al., 1999; and Alzarte-Marin et al., 2007), which is consistent with findings of this study. Melloto and Kelly (2001) carried out genetic characterization of the *Co-4* locus and reported it a complex locus. They further revealed presence of retro-elements clustered with putative resistance and defense related genes in the region of this locus and according to Ronald (1998), these may be involved in the appearance of new resistance specificities, which may partly explain its broad-based resistance.

The *Co-5* gene is also reported to be among the most effective genes in Central America and Mexico, however, its use in breeding has been limited thus making it even more valuable to breeders (Kelly and Vallejo, 2004). Several other single genes with a broad-based resistance spectrum to *C. lindemuthianum* have been reported. For example, the multiple allelic Andean resistance gene *Co-1* has four alleles (*Co-1*^{1}, *Co-1*^{2}, *Co-1*^{3} and *Co-1*^{4}) of which the *Co-1*^{2} in cultivar Kaboon has the most broad-based resistance spectrum. Other studies have also reported single genes with broad resistance. The *Rpg1* gene was reported by Steffenson (1992) to possess durable resistance to many pathotypes of *Puccinia graminis* f.sp *tritici* that attack barley.

The results further suggest that pyramided genes may not necessarily and obviously confer better resistance against a broad array of *C. lindemuthianum* races as compared to single genes. It appears that the single resistance genes *Co-4*^{2}, *Co-4*^{3} and *Co-5* conferred similar levels or better resistance levels than some pyramids. This implies, therefore, that indiscriminate combination of genes in gene pyramiding programs may be ineffective against some pathogen populations. Similar observations have been made by different authors. Kousik and Ritchie (1999) found that pyramiding *Bs2* and *Bs3* resistance genes in the isoline ECW-23R did not consistently provide resistance against race 5 of *Xanthomonas campestris* pv. *Vesicatoria* that causes bacterial spot disease in Bell pepper, as compared with the same genes deployed individually. Kloppers and Pretorius (1997) observed that the degree of increase in resistance conferred by lines combining three wheat leaf rust resistance genes *Lr13, Lr34* and *Lr37* compared with that shown by lines with single genes, depended greatly on the genes involved and the pathogen isolate, a trend that the results of the current study are consistent with.

Therefore, the combination of major genes if properly deployed after studying the population structure of the pathogen and its resistance spectrum should be of immense importance (Hittalmani et al., 2000). Balardin and Kelly (1998) proposed pyramiding genetically diverse resistance genes and deploying different gene combinations in different regions as the most practical and realistic approach to provide effective long term anthracnose resistance. Kelly (2004) proposed combination of *Co-4*^{2}, *Co-5* and *Co-6* genes in North America and *Co-1*^{2} and *Co-4*^{2} gene pair for Central America.

5.4 Conclusion

This study clearly demonstrated that, gene pyramids may increase degree and spectrum of resistance against the four diverse races, however, not all pyramids were effective against the races. Some single genes as well demonstrated a broad spectrum nature of resistance against the four races and in some cases conferred a more effective resistance than some gene pyramids. Therefore, before embarking on a long and costly gene pyramiding program caution should be taken in choosing genes that will result in effective gene pyramids offering increased level and spectrum of resistance against *C. lindemuthianum* races in a given target location. In some cases the mere deployment of a single broad-spectrum gene such as *Co-4*^{2} may suffice, but added benefits may be realized when it is in combination with other complementary genes whose modes of single and combined action are well understood.

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CHAPTER SIX

Effect of marker aided pyramiding of anthracnose and *Pythium* root rot resistance genes on plant agronomic characters among advanced common bean genotypes

Abstract

One of the factors that accounts for the low yields in common bean is the simultaneous occurrence of diseases on the common bean crop. Bean root rots and anthracnose are the most important common bean diseases that simultaneously occur on the bean crop in Uganda. Moreover, *Colletotrichum lindemuthianum*, the pathogen that causes bean anthracnose, possesses a high genetic variability which makes it easily break down single gene resistance. Pyramiding resistance genes for both diseases in commercial varieties would ensure reduction of yield losses resulting from the two diseases. However, the effect of marker assisted gene pyramiding on plant agronomic characters is not well understood. Three-way crosses were made to pyramid three anthracnose and one *Pythium* root rot resistance genes in four susceptible market class varieties. Sequence characterized amplified regions (SCAR) markers were used to facilitate the pyramiding scheme. Correlation analysis and Path coefficient analysis were used to assess the association between number of pyramided genes and different plant agronomic characters. Number of pyramided genes was negatively correlated with number of pods per plant (-0.32), number of seeds per plant (-0.25), number of seeds per pod (-0.18), pod length (-0.17), days to 50% flowering (-0.09) and 100-seed weight (-0.02). Path coefficient analysis showed that number of pyramided genes, plant height, days to 50% flowering, number of seeds per pod and number of pods per plant had negative direct effects on seed weight per plant. Number of seeds per plant had the highest positive direct effects (0.98) followed by 100-seed weight (0.28) while days to maturity had the least positive direct effect (0.03) on seed weight per plant. Only number of seeds per plant had its correlation coefficient (0.94) almost equal to the direct path coefficient (0.97). Number of pyramided genes had significant (P<0.05) negative indirect effect on seed weight per plant only through number of seeds per plant (-0.25). Therefore, pyramiding higher numbers of resistance genes may cause a grain yield reduction via number of seeds per plant. Therefore, it is important for breeders to simultaneously select for number of pyramided genes with number of seeds per plant and other highly associated traits.

Key words: *Phaseolus vulgaris*, correlation analysis, path analysis, pyramided genes, grain yield

6.1 Introduction

The common bean, *Phaseolus vulgaris* (L.) is the most important legume crop in Uganda occupying an important niche in the Agricultural sector and farm household economy (Opio *et*

al., 2001). Common beans provide up to 45% of the total human dietary requirements of proteins (Mauyo *et al*., 2007) for resource poor farmers and in addition provide appreciable levels of B vitamins, iron and Magnesium among other micro-nutrients. Common bean production in Uganda is, however, greatly constrained by the simultaneous occurrence of bean anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. et. Magn) Lams. Scrib., and bean root rots. *Colletotrichum lindmeuthianum* is a highly variable pathogen both pathogenically and genetically (Mahuku and Riascos, 2004; Kelly and Vallejo, 2004) consequently making single gene resistance short lived and highly vulnerable to dynamic and diverse plant pathogen populations. Bean anthracnose can cause yield losses of upto 44% when susceptible varieties are used (Nkalubo et al., 2007). Root rots on the other hand usually occur as a complex of many pathogens, with *Pythium spp* being the predominant pathogen in the complex (Mukalazi, 2004), Root rots are capable of causing yield losses of up to 100% on susceptible varieties (Tusiime, 2003: Mukankusi *et al*., 2010).

Chemical control of the two diseases is not widely adopted by farmers in Uganda because it raises production costs and is not eco-friendly. Therefore, the use of resistant cultivars offers farmers an efficient, safe and inexpensive alternative for managing the two diseases. With the help of molecular markers, it is possible to combine both bean anthracnose and *Pythium spp* resistance genes in a common susceptible background. The use of molecular markers to select desirable plants eliminates extensive phenotyping thus reducing the breeding duration (Ye and Smith, 2008). Marker assisted gene pyramiding has been successfully used to pyramid resistance genes to create broad and durable resistance in different crops (Ferreira *et al*., 2012; Ragagnin *et al*., 2009; Shi *et al*., 2008; Bates *et al*., 2005; Liu *et al*., 2000). However, the effect of gene pyramiding on plant agronomic characters is not well elucidated.

Generally, direct selection for yield may not be effective as it is a complex trait and depends upon the component traits. Therefore, understanding the association of characters is crucial in developing an efficient breeding program that aims at developing high yielding and stable varieties. More than one trait should be considered in a common bean breeding program using pure lines. In addition to grain yield, other traits such as 100-seed weight, pods per plant, disease resistance, seeds per pod, pod length, yield per plant should be observed and measured to complement the objectives of the program (Gonzalves *et al*., 2003). Correlation coefficients are

used to show relationships among independent characteristics and describe relationships in a simple manner (Yucel et al., 2006). An analysis of correlation between yield and yield components is essential in determining selection criteria. The estimated correlation measured from the association among the character enables the breeder to understand the changes that occur in a determined trait in function of the selection practiced on another correlated trait (Gonzalves *et al*., 2003). However, the quantification and interpretation of the magnitude of a correlation can result in errors when indirect selection strategy is used based on the effect of a third trait or of a group of other traits on them (Gonzalves *et al.,* 2003). Path coefficient analysis permits the partitioning of the correlation coefficients into direct and indirect effects of a set of independent variables on the dependent variable (Guler *et al*., 2001) and, therefore, plays an important role in determining the degree of relationship between yield and its components (Ciftci et al., 2004). There is need, therefore, to determine relationship between number of pyramided genes inherited and grain yield, and its component characters. The purpose of this study was to determine degree of association between number of pyramided genes and yield characters.

6.2 Materials and methods

A cascading pedigree gene pyramiding scheme was used to cross three donor parents namely G2333 (anthracnose resistance genes *Co-4*^{2}, *Co-5*), PI207262 (anthracnose resistance genes *Co-4*^{3} *& Co-9*) and RWR719 (*Pythium* resistance gene) with four susceptible commercial varieties K132, NABE 4, NABE 13 and NABE 14 (Refer to Figure 4.1). SCAR markers were used in marker assisted selection with *SAS13* tagging *Co-4*^{2} & *Co-4*^{3}alleles, SBB14 tagging *Co-4*^{2} allele, SAB3 tagging *Co-5* allele, SB12 tagging *Co-9* allele and *PYAA19*$_{800}$ tagging the *Pythium* resistance allele. Plants with different numbers of genes ranging from 0 to 4 were selected using markers and the genes fixed through selfing individual plants up to F6 generation. This experiment was carried out for two seasons using F5 and F6 seeds.. The experiment was conducted during the second season of 2012 with 45 families and the first season of 2013 with 67 families at the National Agricultural Research Laboratories (NARL), Kawanda, located 0^o 24’ 38.15” N and 32^o 32’ 14.06” E and elevated at 1147 meters above sea level. A nested randomized complete block design (RCBD) with three replicates was used. Single row plots of 1.5m length were used with a 50cm between row and 15cm within row spacing.

Data was collected on plant vigor at two weeks after planting (1 – 5 where 1 = poor vigor and 5 = high vigor), number of pods per plant, number of seeds per pod, pod length (cm), number of seeds per plant, seed weight per plant (gm), 100-seed weight (gm), plants at harvest per family, days to 50% flowering, days to maturity and number of pyramided genes.

The degree of association of different characters was done through correlation analysis with correlation coefficients calculated using the formula by Weher and Moorthy (1952);

$r_{xy} = \text{Cov}XY/\sqrt{\sigma_p^2 x.\sigma_g^2 y}$

Where; r_{xy} = Correlation between characters *x* and *y*; Cov *XY* = Covariance between characters *x* and *y* $\sigma_p^2 x.\sigma_g^2 y$ = Phenotypic variance of characters *x* and *y* respectively.

Path analysis was done according to the method described by Akintunde (2012). Path coefficients were obtained by solving the simultaneous equations which express the basic relationships between correlations and path coefficients as follows;

$$r_{x1.y} = P_{x1.y} + r_{x1.x2}P_{x2.y} + r_{x1.x3}P_{x3.y} + r_{x1.x4}P_{x4.y} + r_{x1.x5}P_{x5.y} + r_{x1.x6}P_{x6.y} + r_{x1.x7}P_{x7.y} + r_{x1.x8}P_{x8.y} + r_{x1.x9}P_{x9.y} + r_{x1.x10}P_{x10.y}$$

$$r_{x2.y} = r_{x2.x1}P_{x1.y} + P_{x2.y} + r_{x2.x3}P_{x3.y} + r_{x2.x4}P_{x4.y} + r_{x2.x5}P_{x5.y} + r_{x2.x6}P_{x6.y} + r_{x2.x7}P_{x7.y} + r_{x2.x8}P_{x8.y} + r_{x2.x9}P_{x9.y} + r_{x2.x10}P_{x10.y}$$

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$$r_{x10.y} = r_{x10.x1}P_{x1.y} + r_{x10.x2}P_{x2.y} + r_{x10.x3}P_{x3.y} + r_{x10.x4}P_{x4.y} + r_{x10.x5}P_{x5.y} + r_{x10.x6}P_{x6.y} + r_{x10.x7}P_{x7.y} + r_{x10.x8}P_{x8.y} + r_{x10.x9}P_{x9.y} + P_{x10.y}$$

Where; $r_{x1.y}$ to $r_{x10.y}$ denotes the correlation coefficients between independent characters *x1* to *x10* and the dependent character *y*; $r_{x1.x2}$ to $r_{x10.x9}$ denotes the correlation coefficients between all possible combinations of independent characters; $P_{x1.y}$ to $P_{x10.y}$ denotes the direct effects of characters *x1* to *x10.*

6.3 Results and discussion

6.3.1 Correlation analysis

Table 1 shows the correlation matrix of 11 plant growth and yield characters of beans. Number of seeds per plant (0.92), number of pods per plant (0.82), pod length (0.56), plant vigor (0.56) and

100-seed weight (0.42) respectively exhibited the highest positive correlation with seed weight per plant implying the dependence of plant yield on these characters. Number of seeds per pod (0.46), plant height (0.45) and days to maturity (0.17) were also positively correlated with seed weight per plant but with lower correlation coefficients. These results are consistent with the findings of Cokkizgin et al. (2001), who found seed yield to be significantly correlated with all yield traits except plant height and 100-seed weight. Number of pyramided genes exhibited negative correlation (-0.17) with seed weight per plant suggesting a slight yield penalty for pyramiding.

Table 6.1: Correlation matrix for seed weight per plant and different plant growth and yield characters

Variables	*SWP*	*NPG*	*PV*	*PH*	*DF*	*DM*	*NSP*	*PL*	*NPP*	*NSPT*	*100SW*
SWP	1.00										
NPG	-0.17***	1.00									
PV	0.56***	-0.02ns	1.00								
PH	0.45***	0.00	0.33***	1.00							
DF	-0.03ns	-0.09ns	-0.24**	0.30**	1.00						
DM	0.17ns	0.08ns	-0.03ns	0.39***	0.63***	1.00					
NSP	0.46***	-0.18ns	0.25**	0.56***	0.25**	0.18ns	1.00				
PL	0.56***	-0.17ns	0.36***	0.50***	0.13ns	0.11ns	0.86***	1.00			
NPP	0.82***	-0.24**	0.39***	0.40***	-0.05ns	0.15ns	0.31**	0.45	1.00		
NSPT	0.92***	-0.19*	0.39***	0.49***	0.07ns	0.25**	0.52***	0.56***	0.85***	1.00	
100SW	0.42***	-0.02ns	0.42***	0.12ns	-0.19*	-0.12ns	0.01ns	0.23*	0.03ns	-0.09ns	1.00

*** Highly significant (P<0.01), ** Significant (P<0.05), * Significant (P<0.1). ns = not significant. *SWP*= seed weight per plant, *NPG* = number of pyramided genes, *PV*= plant vigor, *PH*= plant height, *DF* = days to 50% flowering, *DM* = days to maturity, *NSP* = number of seeds per pod, *PL* = pod length, *NPP* = number of pods per plant, *NSPT* = number of seeds per plant and *100SW* = 100-seed weight.

Additionally, Number of pyramided genes significantly negatively correlated with number of pods per plant (-0.24) and number of seeds per plant (-0.19).This implies that gene pyramiding may affect plant yield through its negative effect on number of pods per plant and number of seeds per plant.

Yield determining characters were highly positively correlated with each other. For instance, number of seeds per pod was highly correlated with pod length (0.86). Number of pods per plant

was highly correlated with number of seeds per plant (0.85) and number of seeds per plant (0.52). Plant vigor was highly correlated with 100-seed weight (0.42).

The results are consistent with findings of Verma et al. (2004), Dursun (2007) and Cokkizgin et al., (2013) who reported that pod length showed positive and significant correlation with number of seeds per pod, number of pods per plant and number of seeds per plant. They further revealed that number of seeds per pod had a positive and significant relationship with number of seeds per plant and seed yield per plant and that number of pods per plant had a strong positive relationship with number of seeds per plant.

6.3.2 Path coefficient analysis

Path coefficient analysis provides an effective means of separating direct and indirect cause of association and permits critical examination of the specific forces acting to produce a given correlation and measures the relative importance of each causal factor (Singh et al., 2013). Table 2 gives estimates of direct and indirect path coefficients using seed weight per plant as the response variable. Number of seeds per plant (0.98), pod length (0.06), days to maturity (0.03), 100-seed weight (0.28) and plant vigor (0.08) had positive direct effects on seed weight per plant, but only effects of number of seeds per plant and 100-seed weight were significant ($P<0.01$; $P<0.5$ respectively). Only number of seeds per plant had its correlation coefficient (0.94) almost equal to the direct path coefficient (0.98) implying that number of seeds per plant mostly correlated directly with seed weight per plant.

All characters except days to 50% flowering had significant ($P<0.01$, $P<0.05$) positive indirect effects on seed weight per plant only through number of seeds per plant, with number of pods per plant having the most significant ($P<0.01$) effect (0.88), followed by pod length (0.55), number of seeds per pod (0.51), plant height (0.48), plant vigor (0.37), 100-seed weight (0.27) and days to maturity (0.24). Therefore, grain yield can best be improved through direct selection for number of seeds per plant and by indirectly selecting for number of pods per plant, pod length, number of seeds per pod, plant height and plant vigor respectively. Results are consistent with findings of Tunturk and Ciftici et al. (2004), Peksen and Gulumser (2005), and Ahmadzadeh et al. (2012).

Of all characters considered in the study, only number of pyramided genes had a significant (P<0.05) negative indirect effect on seed weight per plant through number of seeds per plant (-0.25). This implies that gene pyramiding for higher numbers of resistance genes could indirectly affect plant grain yield by causing a reduction in number of seeds per plant.

Table 6.2: Direct and indirect path coefficients for seed weight per plant

Variables	*NPG*	*PV*	*PH*	*DF*	*DM*	*NSP*	*PL*	*NPP*	*NSPLT*	*100SW*	***SWP (r)***
NPG	**-0.012**	0.013	-0.000	0.004	0.002	0.012	-0.011	0.050	-0.246**	-0.006	**-0.194***
PV	-0.002	**0.082**	-0.006	0.010	-0.001	-0.017	0.022	-0.052	0.371***	0.143	**0.549*****
PH	-0.000	0.027	**-0.018**	-0.012	0.010	-0.038	0.031	-0.063	0.478***	0.033	**0.447*****
DF	0.001	-0.020	-0.005	**-0.040**	0.016	-0.017	0.008	0.008	0.070	-0.054	**-0.034**
DM	-0.001	-0.002	-0.007	-0.025	**0.025**	-0.012	0.007	-0.024	0.240**	-0.034	**0.165**
NSP	0.002	0.021	-0.010	-0.010	0.005	**-0.067**	0.053	-0.048	0.511***	0.002	**0.456*****
PL	0.002	0.030	-0.009	-0.005	0.003	-0.058	**0.061**	-0.070	0.548***	0.063	**0.565*****
NPP	0.002	0.026	-0.007	0.002	0.004	-0.021	0.027	**-0.157**	0.882***	0.105	**0.867*****
NSPLT	0.003	0.031	-0.009	-0.003	0.006	-0.035	0.035	-0.142	**0.975*****	0.076	**0.937*****
100SW	0.000	0.042	-0.002	0.008	-0.003	-0.000	0.014	-0.060	0.268**	**0.276****	**0.544*****

*** Highly significant (P<0.01), ** Significant (P<0.05), * Significant (P<0.1). *SWP (r)* = seed weight per plant, *NPG* = number of pyramided genes, *PV* = plant vigor, *PH* = plant height, *DF* = days to 50% flowering, *DM* = days to maturity, *NSP* = number of seeds per pod, *PL* = pod length, *NPP* = number of pods per plant, *NSPLT* = number of seeds per plant and *100SW* = 100-seed weight. Residual = 1 - $\sqrt{R^2}$ = 0.0519. The sum of direct and indirect path coefficients appears in bold in the last column of the table to the right. Direct path coefficients appear diagonally in bold and underlined. The rest of the coefficients are indirect coefficients.

6.4 Conclusion

The study demonstrated that selection for higher numbers of pyramided genes may indirectly cause a yield reduction via number of seeds per plant. Therefore, breeders undertaking gene pyramiding should weigh the benefits against the potential cost of yield foregone. Additionally a lower number of genes pyramided may incur a lower yield loss. This, therefore, would require a careful selection of fewer but effective and complementary genes to pyramid.

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CHAPTER SEVEN

Overview, implications and discussion

7.1 Introduction

The common bean is the most important legume crop in Uganda occupying an important niche in the Uganda Agricultural sector and farm household economy. Its production is however severely constrained by diseases key among which are the bean anthracnose disease caused by *Colletotrichum lindemuthianum* and root rot caused by *Pythium spp*. *Colletotrichum lindemuthianum* is a highly diverse pathogen making single gene resistance of low effectiveness towards controlling the diseases due to easy breakdown. These two diseases simultaneously exist on the bean crop in farmers' fields causing severe yields losses. This study therefore, purposed to a) determine the virulence diversity of *Colletotrichum lindemuthianum* in Uganda; b) develop through gene pyramiding and evaluate bean lines with multiple resistance genes to anthracnose and *Pythium* root rot with the aid of marker assisted selection; c) assess the effectiveness of pyramided genes in conferring broad resistance to bean anthracnose; and d) determine effect of gene pyramiding on plant agronomic characters. The study made some key findings as follows;

7.2 Summary of findings

a) Pathogenicity tests on twelve differentials cultivars revealed that *Colletotrichum lindemuthianum* diversity was high and 27 races were characterized from 51 isolates. Races 2047 and 4095 were the most widely distributed in the sampled regions, Races 4095, 2479 and 2047 respectively were the most virulent with race 4095 causing a susceptible reaction on all the 12 differentials.

b) Pathogenicity testing further revealed that the cultivars G2333, Cornell, TU, and AB136 respectively showed the broadest resistance to all the 27 races.

c) Gene pyramiding for broad resistance against *C. lindemuthianum* with at least two anthracnose resistance genes and one *Pythium spp* root rot resistance gene was successfully achieved using SCAR markers. Disease resistance screening of advanced

pyramided lines using four races showed that pyramided genes effectively conferred resistance to multiple *C. lindemuthianum* races.

d) Out of the five pyramids created only the *Co-4^2+Co-5+Co-9* and *Co-4^2+Co-5* conferred resistance to all the four *C. lindemuthianum* races 352, 713, 767 and 2047. It was, however, observed that the single genes *Co-4^2* and *Co-5* as well conferred resistance to all the four races 352, 713, 767 and 2047

e) Correlation analysis revealed that number of genes pyramided had a significant negative correlation with seed weight per plant, number of pods per plant and number of seeds per plant, while path analysis revealed that number of genes pyramided had a significant negative indirect effect (-0.25) on seed weight per plant via number of seeds per plant.

7.3 Implications and discussion

The Central and Eastern Africa great lakes region is a secondary center of diversity for common bean. Bean production and per capita consumption is reported to be highest in this region with the Andean bean varieties being the commonest in Uganda. Therefore, due to a wide host diversity, the pathogen *Colletotrichum lindemuthianum*, through co-evolution with its host has also become diverse. Additionally, given the seed-borne nature of bean anthracnose disease, it is highly probable that new races from other areas within the region have been introduced to Uganda through the unregulated cross boarder seed trade. This, therefore, necessitates a change in strategy by bean breeders to be able to breed for broad and durable resistance to the pathogen. Durable resistance based on major genes has not always been effective when resistance genes are deployed one at a time. Therefore, combining complementary resistance genes through marker assisted gene pyramiding is a strategy that would confer a more long-term resistance. However, the choice of genes to pyramid should be informed by prior investigations in the mode of action of individual genes in a host background and action while in combination with other resistance genes.

The longevity of resistance is to a large extent dependent on the variation displayed by the pathogen. Therefore, with a highly variable pathogen like *C. lindemuthianum*, it is necessary to

diversify the sources of resistance to effectively manage the disease. In this study, the cultivars G2333, Cornell 49-242, TU and AB136 respectively conferred the broadest resistance against the 27 races. This implies that the resistance genes *Co-4*^{2}, *Co-5*^{2} from cultivar G2333, *Co-2* from cultivar Cornell 49-242, *Co-5* from cultivar TU and *Co-6, co-8* from cultivar AB136 are the best sources of resistance to bean anthracnose disease in Uganda. However, all six genes are from the Mesoamerican gene pool and to develop effective gene pyramids, it is recommended to combine resistance genes from both the Mesoamerican and Andean gene pools. The Andean gene *Co-1* is multiple allelic having four different alleles namely *Co-1, Co-1*^{2}, *Co-1*^{3} & *Co-1*^{5} from cultivars Michigan Dark Red Kidney, Kaboon, Perry marrow and Widusa respectively. Based on results of this study the alleles *Co-1*^{3} & *Co-1*^{5} were more effective and therefore, any of them may be combined with the above Andean genes for a more durable resistance against *C. lindemuthianum* in Uganda.

The SCAR markers used were dominant in nature except SBB14 tagging *Co-4*^{2} and did not provide information enabling identification of heterozygotes. The marker SB12 tagging *Co-5* gene is 14.4cM away from target gene and as such had the least frequency due to its high recombination rate. This implies that for a successful and fast gene pyramiding program in Uganda there is need to use or develop more efficient, informative and cost-friendly molecular markers tagging the key genes of interest. SNP based markers are most ideal because they are more stable, abundant and have a high precision.

Obtaining the root genotype and the ideotype with all the target genes fixed was a lengthy ordeal during the gene pyramiding due to selfing the root genotype directly to fix target genes. This implies that new approaches of obtaining the ideotype faster need to be incorporated into future gene pyramiding schemes. Two are proposed in literature namely a) generating a population of doubled haploids from the root genotype and selecting the ideotype; b) crossing the root genotype with a blank parent containing none of the target alleles to obtain offspring carrying all alleles in coupling phase, then selfing the offspring to give the ideotype in one generation.

Comparison of effectiveness of pyramided genes with single genes against four races revealed that the single genes *Co-4*^{2} and *Co-5* were as effective as the best two pyramids *Co-4*^{2}*+Co-5+Co-*

9 and *Co-4*^{2}*+Co-5*. This implies that single resistance genes may be adequate for a long period of time but only in environments less conducive to the pathogen in which the disease occurs at moderate or low severity. This therefore saves on time and resources and still yields same results as would be expected with pyramided genes. However, in more dynamic environments where pathogen diversity is high, with high disease pressure and conditions are highly favorable for the pathogen to flourish, gene pyramiding is inevitable since there is a high probability that a new race will soon overcome the single gene resistance in such environments. Gene pyramiding has been successful in the control of wheat stem rust for over 50 years but the same pyramids are threatened by the new stem rust race Ug99, implying that gene pyramids are not necessarily permanent (Mundt, 2014) but at least confer resistance for a longer time than single genes. Therefore, both single and pyramided genes still have their place of importance when it comes to disease control, although the choice of which strategy to use heavily relies on knowledge of pathogen diversity and the interaction of different races with different resistance genes.

Correlation and path analysis revealed that number of genes pyramided had a negative effect on final plant yield. This implies that targeting pyramids with higher numbers of genes may drastically impact on plant yield. Moreover, according to Mundt (2014), there is no strong evidence for gene number *per se* as the dominant mechanism for the durability of pyramids. This was observed in this study since some single genes performed as effectively as the best pyramids. Therefore, pyramids with fewer but highly effective and complementary resistance genes should be targeted by breeders to minimize on the potential yield penalty and save on resources involved in pyramiding many genes. Breeders should therefore judge and weigh the potential benefits of gene pyramiding against the potential yield penalty in the different circumstances in which the pathogen/ disease may present itself and the resources available.

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Appendix i: Reactions of 51 *Colletotrichum lindemuthianum* isolates on the 12 bean differential cultivars

		Differential cultivars*[1] and resistance genes possessed											
		1[b]	2[a]	3[a]	4[b]	5[a]	6[a]	7[b]	8[b]	9[b]	10[b]	11[b]	12[b]
Isolate No.	Isolate ID	Co-11	Co-1	Co-1[3]	Co-2	Co-1[5]	Co-1[2]	Co-3	Co-4[3], Co-9	Co-4	Co-5	Co-6, co-8	Co-4[2], Co-5[2], Co-7
1	12A	R	S	S	R	R	R	S	R	R	R	S	R
2	34A	R	R	R	R	R	R	S	S	R	S	R	R
3	91A	S	S	S	R	S	R	R	S	R	R	S	R
4	92A	S	R	R	R	S	R	S	R	R	R	R	R
5	88A	S	S	S	R	S	S	S	S	S	R	R	R
6	65A	S	S	S	S	S	S	S	S	S	S	S	R
7	94A	R	S	S	R	S	S	R	R	S	R	S	R
8	56A	R	R	R	R	S	R	R	R	S	S	R	R
9	38A	R	R	R	R	R	R	R	R	R	R	R	R
10	08A	S	S	S	S	S	S	S	S	S	S	S	R
11	66A	S	S	S	S	S	S	S	S	S	S	S	S
12	90A	S	S	S	S	S	S	R	S	S	R	S	R
13	72A	S	R	R	R	S	R	R	S	R	R	S	R
14	64A	S	S	S	S	S	S	S	S	S	S	S	R
15	81A	R	S	R	S	R	S	R	R	S	S	S	R
16	100A	S	S	S	S	S	S	R	S	S	R	S	R
17	82A	S	R	R	R	R	R	R	R	R	R	R	R
18	59A	R	R	R	R	R	R	R	R	R	R	R	R
19	57A	R	R	R	R	R	R	R	R	R	R	R	R
20	63A	S	S	S	S	S	S	S	S	S	S	S	S
21	25A	R	R	R	R	R	R	R	R	R	R	R	R
22	44A	S	S	S	S	S	S	S	S	S	S	S	S
23	75A	S	S	S	S	S	S	S	S	S	S	S	R
24	84A	R	R	R	R	R	S	S	R	S	R	R	R
25	69A	S	S	S	S	S	S	S	S	R	S	R	R
26	41A	S	S	S	S	S	S	S	S	R	S	S	R
27	98A	S	S	S	S	S	S	S	S	S	S	S	R
28	52A	S	S	S	S	S	S	S	S	S	S	S	R
29	86A	S	R	R	S	R	R	S	S	R	S	R	R
30	67A	S	S	S	S	S	S	S	S	S	S	S	S
31	55A	S	S	S	S	S	S	S	S	S	S	S	R
32	95A	S	R	S	S	S	S	S	S	S	S	S	R
33	76A	S	S	S	S	S	S	S	S	S	S	S	S
34	28A	S	S	S	S	S	S	S	S	S	S	S	S
35	62A	S	S	S	S	S	S	S	S	S	S	R	R
36	37A	S	S	S	S	S	S	S	S	S	S	S	R

37	16A	S	S	S	R	S	S	S	S	S	R	S	R
38	83A	S	S	S	S	R	S	R	S	S	R	R	S
39	97A	S	S	S	R	S	S	S	S	S	S	S	R
40	99A	S	S	S	R	S	S	S	S	S	R	S	R
41	001A	S	S	S	S	S	S	S	S	S	S	S	S
42	77A	S	S	S	S	S	S	S	S	S	S	S	S
43	73A	S	S	S	S	S	S	S	S	S	S	S	S
44	46A	S	S	S	S	S	S	S	S	S	S	S	R
45	61A	R	S	R	R	R	R	R	S	S	R	R	R
46	40A	S	R	S	S	S	S	S	S	S	S	S	R
47	71A	S	S	S	S	S	S	S	S	S	S	S	R
48	007A	S	S	S	R	R	S	S	S	S	S	S	R
49	85A	R	S	R	S	R	S	R	R	R	R	R	R
50	36A	R	R	R	R	R	R	R	S	R	R	R	R
51	96A	S	S	S	S	S	S	S	S	S	S	S	S

*****Differential cultivars; 1 = Michelite; 2 = MDRK; 3 = Perry Marrow; 4 = Cornell 49-242; 5 = Widusa; 6 = Kaboon; 7 = Mexico 222; 8 = PI 207262; 9 = TO; 10 = TU; 11 = AB136; and 12 = G 2333

[a] Andean gene pool; [b] Mesoamerican gene pool

[1] Position numbers and Binary codes: Michelite (0)1; MDRK (1)2; Perry Marrow (2)4; Cornell 49-242 (3)8; Widusa (4)16; Kaboon (5)32; Mexico 222 (6)64; PI 207262 (7)128; TO (8)256; TU (9)512; AB136 (10)1024; and G 2333 (11)2048

Appendix ii: Season one standardized data for correlation analysis

y	*x1*	*x2*	*x3*	*x4*	*x5*	*x6*	*x7*	*x8*	*x9*	*x10*
1.26696	-1.60908	0.40679	-0.11280	-1.12318	-0.21160	-1.07384	0.19996	1.63025	0.20838	2.33833
3.53006	-0.74765	1.03474	0.04571	-0.65322	-0.45364	0.82620	0.94829	3.47493	3.79739	0.47706
1.04694	-0.74765	-0.84912	0.26625	-0.18326	0.03044	-0.77949	-0.10276	1.69869	1.24512	1.06697
-1.49402	0.97521	-2.10502	-1.84267	0.28670	-0.21160	-1.84522	-2.13705	-1.87590	-1.77085	0.91129
-0.31084	-0.74765	-0.43048	-0.51024	-1.43648	-1.58317	0.18473	-0.19963	-0.11756	-0.10937	-0.88713
-0.34893	0.11378	-0.22116	-1.42226	-1.12318	-1.90590	-0.09946	-0.59922	-0.18073	-0.41636	-1.03377
-0.63458	0.11378	-0.43048	1.68139	-0.18326	0.51453	0.33651	-0.19378	-0.43019	-0.18311	-1.32777
-0.49624	0.97521	-0.43048	1.17139	-0.02660	0.35317	-0.33270	-0.69597	-0.38078	-0.24940	-1.24831
-1.26876	-0.74765	0.40679	-1.62213	-2.06310	-1.90590	-1.60163	-1.43473	-0.83353	-1.25652	-0.79299
-1.28547	0.11378	-0.84912	-1.53943	-2.06310	-1.90590	-1.08737	-1.28943	-1.13887	-1.31845	-1.12848
-0.19928	0.97521	-1.26775	0.62463	0.28670	-0.29228	-0.07916	-0.35705	0.14567	0.28109	-0.96957
-0.75833	0.11378	-2.10502	-0.06456	0.44336	0.51453	0.23981	0.03907	0.45803	0.17068	-2.27290
-0.93937	-1.60908	-0.84912	-1.31888	-1.12318	-0.45364	-1.84522	0.02438	0.04038	-0.88491	0.39240
-0.18617	0.97521	-0.84912	-0.02321	-2.06310	-0.93773	-0.28216	-0.22990	-0.25443	-0.47021	0.89375
-0.79320	1.83664	-0.22116	-0.07834	0.52168	1.48270	-0.12265	0.21722	-0.29129	-0.93203	0.09869
0.07586	-1.60908	1.03474	-1.03632	-0.18326	-1.17977	0.64147	-0.12395	0.44574	0.15183	0.88314
-1.26854	0.11378	-1.47707	-1.26375	0.75666	0.51453	-0.89791	-1.26117	-1.21257	-1.16137	-1.09247
-0.56094	0.11378	0.82542	0.00895	0.44336	0.51453	0.96119	0.65318	-0.56153	-0.01782	-1.51137
-0.88777	0.11378	0.40679	-0.24375	0.75666	0.27249	0.68382	0.57534	-1.08154	-0.82584	-0.53935
0.03815	-0.74765	0.40679	0.77625	-0.02660	0.51453	-0.39299	-0.31919	0.60543	0.01569	0.68500
0.78260	-0.74765	0.82542	1.23112	-2.06310	-0.29228	0.80725	0.55118	0.56858	0.87441	0.08192
0.20457	-0.74765	1.03474	-0.06456	-1.12318	-0.29228	-0.36770	-0.11954	0.39415	0.06889	0.51526
-0.97045	-1.60908	-0.22116	-0.91226	0.75666	-1.42181	-0.91144	-0.91405	-1.17046	-1.00878	-0.80537
-0.56594	0.11378	-0.84912	-0.64348	0.75666	0.51453	-1.27683	-1.28943	0.26149	-0.31941	-0.84323
-1.60715	0.11378	-2.10502	-1.69794	1.22662	0.51453	-2.41361	-3.32372	-2.09701	-1.86510	-0.12937
-0.84933	1.83664	-0.22116	-1.45328	-0.18326	0.51453	-0.42425	-0.56895	-1.21257	-0.45136	-1.32801
-0.41415	0.11378	-0.22116	1.03815	1.22662	0.75657	0.42833	-0.14514	-0.18073	-0.25658	-0.28818
-1.55289	1.83664	0.40679	-1.01564	-0.18326	1.48270	-1.27683	-1.37419	-1.94960	-1.63890	-1.91164
-0.85683	0.11378	-0.84912	0.51436	1.22662	0.03044	0.71252	0.32105	-0.62295	-0.65871	-1.07616
1.88555	-0.74765	1.66269	0.79004	-0.18326	0.51453	1.31643	1.46534	0.22463	1.30167	0.67360
-0.43320	-0.74765	0.40679	-0.18861	0.28670	-0.93773	0.00204	0.23629	-0.51239	-0.83778	1.14443
0.87408	-1.60908	-0.84912	1.11625	1.53993	0.83725	0.99671	0.49057	1.66184	1.64097	-0.54120
0.39926	-1.60908	-0.84912	1.45855	1.85324	0.67589	1.32827	0.73073	0.16322	0.69848	-0.30547
1.06627	-1.60908	-0.43048	0.27544	1.69658	2.45087	0.69476	0.60712	1.40388	1.58442	-0.31993
0.23025	0.11378	0.40679	0.02504	-0.18326	-0.69568	0.30202	0.44348	0.22873	-0.05970	0.84766
1.33585	0.11378	1.66269	0.79233	0.91332	2.28951	0.47205	0.66010	0.37488	0.66948	1.28874

0.76895	0.97521	1.24406	1.02896	-0.33991	1.32134	-0.44455	-0.71305	0.95640	0.70745	0.66030
-0.36163	0.97521	1.03474	-0.21618	-1.12318	-0.45364	-1.27683	-1.35300	-0.53082	-0.63515	1.01336
0.99482	0.11378	0.40679	0.40409	1.69658	2.12815	0.22939	0.11762	1.04274	1.15841	0.17334
-0.14358	0.11378	0.40679	-0.41375	0.60001	-0.13092	-0.80791	-0.67914	-0.38710	-0.38350	0.49391
1.44518	0.11378	0.40679	-0.33334	-1.12318	0.27249	0.00204	-0.13455	1.66184	1.56557	0.32058
0.62049	0.11378	0.40679	1.47234	0.52168	0.51453	-0.01088	-0.20293	0.42229	0.60594	0.18629
0.00141	0.11378	-0.22116	-1.02391	-0.18326	-0.29228	0.71252	1.44415	0.33519	-0.06494	0.37655
0.23182	0.97521	0.82542	-0.92834	-0.80987	-1.09909	0.27044	0.33989	-0.39365	-0.25239	1.54630
0.11597	0.97521	0.40679	-0.81807	-0.80987	-1.42181	0.28623	0.66010	0.00353	-0.17333	0.86956
0.54644	0.97521	0.40679	0.39031	-0.18326	0.03044	0.71252	1.39470	0.06495	0.49741	0.30074
0.93957	0.97521	1.24406	1.64923	-0.18326	-0.45364	1.28091	1.26839	-0.04200	0.51885	1.34856
-0.11287	0.97521	1.66269	1.46544	0.28670	-0.93773	-0.89791	-0.47006	0.21235	-0.30056	0.68899
-0.72224	-1.60908	-2.10502	-0.50909	0.75666	-0.21160	0.65568	1.05000	-0.74087	-0.40235	-1.43239
1.53608	0.97521	1.66269	0.43166	-0.18326	-0.45364	1.33775	1.35515	0.55630	1.12071	1.32524
0.86426	0.97521	0.40679	-0.06456	0.75666	-0.45364	-0.14006	1.16867	0.26149	0.62308	0.81574
-0.29352	-1.60908	-0.84912	0.55571	0.75666	0.51453	2.98607	1.76200	-0.77035	-0.20631	-0.24449
-0.48514	0.97521	0.40679	2.14774	0.75666	0.51453	1.28091	1.50772	-0.32814	-0.39481	-0.38816
0.0[a]	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
1.0[b]	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**

[a] Mean, [b] Standard Deviation

Appendix iii: Season two standardized data for correlation analysis

y	*x1*	*x2*	*x3*	*x4*	*x5*	*x6*
.397432	0.480622	-0.6222	0.261856	0.902343	1.155284	-0.68125
1.066157	-0.01031	0.729671	-0.29087	1.008167	1.198478	-0.68125
-0.17044	-0.01031	0.729671	0.017379	0.367502	-0.30611	-0.68125
1.463182	1.807458	0.690296	0.54885	1.282738	1.061697	-1.55174
1.093974	1.369602	-0.18251	1.112209	0.57915	0.464183	-1.55174
0.967532	4.023273	-0.18251	1.832351	1.131152	0.075439	-1.55174
-0.00606	0.042767	-0.83876	0.732207	-0.40473	-0.39249	-1.55174
1.240645	0.440817	-3.18156	-0.0278	2.832919	2.29992	-0.68125
0.476941	0.918478	0.473735	-0.43437	1.271297	0.737744	-0.68125
0.56545	0.918478	0.690296	-0.55395	1.125431	0.867325	-0.68125
0.044512	-0.40836	0.257173	-0.05703	0.167294	0.039444	-0.68125
-1.95073	-0.84621	1.346544	-0.93661	-1.55449	-1.79629	0.189237
-1.47278	-1.28407	-0.18251	-1.97298	-1.15694	-0.87483	0.189237
-0.25642	-0.84621	-0.83876	-1.83745	0.041449	0.730545	0.189237
-0.88609	-0.84621	-0.39907	-1.90389	-0.54487	-0.06854	1.059726
-1.31347	-0.84621	0.913421	-1.79228	-0.99963	-0.70925	1.059726
-1.28565	-0.84621	0.690296	-1.61158	-1.00821	-0.73804	1.059726
-0.14009	-0.40836	0.034049	-0.89675	0.101511	0.291408	1.059726
-1.23507	-0.84621	-0.6222	0.070526	1.139732	-1.29237	-1.55174
-0.78494	-1.14475	-2.15126	-0.00919	-0.38185	-0.79564	-1.55174
0.28475	0.038786	0.257173	0.264513	0.370362	0.097036	0.189237
-0.66609	0.480622	0.257173	0.362835	-1.05683	-0.75964	0.189237
-0.05158	1.365621	1.346544	0.883676	-0.83661	-0.44289	0.189237
0.474412	1.365621	0.257173	-0.11283	0.370362	0.48578	0.189237
-0.09457	0.480622	-0.18251	-0.19521	-0.46479	-0.02535	0.189237
-0.66103	-0.40438	0.690296	-0.6443	1.045348	-0.48608	1.059726
0.95236	0.038786	-1.27845	-0.71605	1.122571	1.32086	1.059726
-0.40056	0.038786	0.690296	0.054582	0.433284	-0.48608	1.059726
-0.25389	0.038786	0.473735	-0.91269	1.031048	0.169025	1.059726
-0.28423	0.480622	0.257173	0.421297	-0.06152	-0.50768	0.189237
0.365673	0.922458	-0.39907	0.530248	0.736456	0.032245	0.189237
-0.33987	0.038786	0.034049	-0.33871	0.101511	-0.24851	0.189237
-0.03641	0.922458	0.690296	-0.68682	0.441864	0.284209	0.189237
-1.26036	0.480622	0.913421	-1.08277	-0.64784	-0.89642	0.189237
0.378317	0.922458	0.473735	-0.35997	0.164434	0.536173	0.189237
1.442951	0.038786	0.473735	-0.25101	1.811858	1.587222	0.189237
-1.41715	-1.28805	0.034049	0.663116	-1.81762	-1.63072	0.189237
-0.55988	-0.40438	0.473735	0.788012	-1.1312	-0.88202	0.189237
0.653958	0.038786	0.034049	-0.73731	0.287418	1.198478	-1.55174

-1.13645	-0.84621	-0.39907	-0.86486	-1.18268	-0.85323	-1.55174
-0.00101	0.480622	0.034049	1.779204	-0.55917	-0.72365	1.059726
-0.13503	-0.84621	-0.83876	-1.04025	-0.07582	0.320204	1.059726
1.721121	1.807458	0.690296	1.319482	1.411443	0.874524	1.059726
-0.27918	1.807458	1.346544	0.867732	-1.1884	-0.63726	1.059726
0.684304	0.922458	0.473735	1.114866	-0.45621	0.169025	1.059726
0.570507	-0.40438	-3.02407	0.623256	-0.56203	0.269811	1.059726
0.289808	0.038786	-1.49501	1.016544	-0.87951	0.62256	1.059726
-0.06676	0.038786	0.257173	-0.25899	0.624912	-0.00375	1.059726
0.798101	-0.40438	-1.27845	-0.30682	1.065369	0.888922	1.059726
1.040868	-0.40438	0.257173	0.891648	0.490486	0.600963	1.059726
1.447647	-0.84621	-0.18251	0.093043	0.440639	1.341429	1.930215
-1.22432	-0.84621	0.473735	0.017036	-1.3929	-1.27077	-1.55174
-1.36376	-0.84621	1.129982	0.70209	-1.54782	-1.58272	-1.55174
-0.11164	-0.84621	0.473735	0.452632	-0.57062	-0.3439	-1.55174
3.020936	0.480622	-0.83876	-0.7066	2.551674	3.636528	-1.55174
-1.91785	-0.84621	1.78623	-0.31973	-1.32855	-1.88988	0.189237
-0.96702	-0.84621	-1.49501	-1.44573	-0.38471	-0.42129	0.189237
-0.76808	-2.17305	-2.8075	3.758047	-0.40378	-1.70271	1.930215
-0.92543	-0.84621	1.129982	0.618803	-1.54782	-1.15878	1.930215
-1.46351	-0.84621	-0.83876	-0.90146	-1.64316	-1.23477	0.189237
-0.70627	-0.84621	1.129982	-0.69471	-0.88046	-0.47088	0.189237
1.396168	0.480622	0.473735	-0.12195	0.501927	1.428845	-0.68125
0.316903	-0.84621	-0.18251	-0.79402	-0.04967	0.71409	-0.68125
-0.44987	-0.84621	0.473735	0.22036	-0.89238	-0.57787	-0.68125
1.916684	1.807458	-0.83876	1.768326	0.501927	0.852927	-0.68125
0.530046	-0.84621	0.473735	0.672818	0.251667	0.160027	-0.68125
0.173201	0.480622	1.129982	0.016634	0.168247	0.121032	-0.68125
-0.31892	-0.84621	-0.18251	-0.11369	-0.4174	-0.31433	-0.68125
0.587848	0.480622	-0.18251	1.455007	0.154627	-0.10865	-0.68125
0.0[a]	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
1.0[b]	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**

[a] Mean, [b] Standard Deviation

Appendix iv: First cycle of marker assisted selection among F1 progenies of the cross (((G2333 X PI207262) x RWR719)

Sample No.	Pedigree	Genes screened for & markers used			
		SBB14	SAS13	SAB3	SB12
		Co-4[2]	Co-4[2]	Co-5	Co-9
1	RWR719 x (8x12)	–	+	–	+
2	RWR719 x (8x12)	–	+	+	+
3	RWR719 x (8x12)	–	+	–	+
4	RWR719 x (8x12)	–	+	–	+
5	RWR719 x (8x12)	+	+	–	+
6	RWR719 x (8x12)	+	+	–	+
7	RWR719 x (8x12)	–	+	–	–
8	(8x12) x RWR719	–	+	+	–
9	(8x12) x RWR719	–	–	–	–
10	(8x12) x RWR719	–	+	–	+
11	(8x12) x RWR719	–	+	+	+
12	(8x12) x RWR719	+	+	–	–
13	RWR719 x (12x8)	–	–	–	–
14	RWR719 x (12x8)	–	+	–	+
15	RWR719 x (12x8)	–	+	–	+
16	(12x8) x RWR719	+	+	+	+
17	(12x8) x RWR719	–	+	–	+
18	RWR719 x (8x12)	–	+	–	+
19	RWR719 x (8x12)	+	+	–	+
20	RWR719 x (8x12)	–	–	–	–
21	RWR719 x (8x12)	–	+	+	+
22	RWR719 x (8x12)	–	–	–	–
23	RWR719 x (8x12)	–	–	–	–
24	RWR719 x (8x12)	–	+	–	–
25	(8x12) x RWR719	+	+	+	+
26	(8x12) x RWR719	–	+	+	–
27	(8x12) x RWR719	–	+	+	+
28	(8x12) x RWR719	–	+	+	–
29	(8x12) x RWR719	–	–	–	–
30	(8x12) x RWR719	–	+	+	+
31	RWR719 x (12x8)	+	+	–	+
32	RWR719 x (12x8)	+	–	–	+
33	RWR719 x (12x8)	–	+	+	+
34	RWR719 x (12x8)	–	+	+	+

35	RWR719 x (12x8)	–	–	–	–
36	RWR719 x (12x8)	–	+	+	+
37	RWR719 x (12x8)	+	+	–	–
38	RWR719 x (12x8)	–	+	+	–
39	(12x8) x RWR719	–	+	–	+
40	(12x8) x RWR719	–	+	–	+
41	(12x8) x RWR719	+	+	+	+
42	(12x8) x RWR719	–	+	+	+
43	(12x8) x RWR719	+	+	+	+
44	(12x8) x RWR719	+	+	+	+
45	(12x8) x RWR719	+	+	–	+
46	RWR719 x (8x12)	+	+	–	+
47	RWR719 x (8x12)		+	–	–
48	RWR719 x (8x12)		–	–	+
49	RWR719 x (8x12)		+	+	+
50	RWR719 x (8x12)		+	–	–
51	RWR719 x (8x12)		+	+	+
52	RWR719 x (8x12)		+	–	+
53	RWR719 x (8x12)		–	–	+
54	(8x12) x RWR719		+	–	–
55	(8x12) x RWR719		+	–	+
56	(8x12) x RWR719		–	–	+
57	(8x12) x RWR719		+	+	+
58	(8x12) x RWR719		+	+	+
59	(8x12) x RWR719		+	+	+
60	(8x12) x RWR719		+	+	+
61	(8x12) x RWR719		+	–	+
62	(8x12) x RWR719		+	–	+
63	(8x12) x RWR719		+	–	+
64	RWR719 x (12x8)		+	–	+
65	RWR719 x (12x8)		*	*	*
66	RWR719 x (12x8)		*	*	*
67	RWR719 x (12x8)		+	+	+
68	RWR719 x (12x8)		+	+	+
69	RWR719 x (12x8)		*	*	*
70	RWR719 x (12x8)		+	–	+
71	RWR719 x (12x8)		+	–	+
72	RWR719 x (12x8)		+	+	+
73	RWR719 x (12x8)		+	–	+
74	RWR719 x (12x8)		+	+	+
75	RWR719 x (12x8)		+	+	+

76	RWR719 x (12x8)	+	–	+
77	(12x8) x RWR719	+	–	+
78	(12x8) x RWR719	+	–	+
79	(12x8) x RWR719	+	–	+
80	(12x8) x RWR719	+	–	+
81	(12x8) x RWR719	+	–	+
82	(12x8) x RWR719	+	–	+
83	(12x8) x RWR719	+	+	+
84	(12x8) x RWR719	–	–	–
85	RWR719 x (8x12)	+	–	+
86	RWR719 x (8x12)	+	–	+
87	RWR719 x (8x12)	+	+	+
88	RWR719 x (8x12)	–	–	+
89	RWR719 x (8x12)		+	+
90	RWR719 x (8x12)		–	+
91	RWR719 x (8x12)		–	+
92	RWR719 x (8x12)		–	+
93	(8x12) x RWR719		+	+
94	(8x12) x RWR719		+	+
95	(8x12) x RWR719		*	*
96	(8x12) x RWR719		+	–
97	(8x12) x RWR719			+
98	(8x12) x RWR719			+
99	(8x12) x RWR719			+
100	(8x12) x RWR719			+
101	(8x12) x RWR719			+
102	(8x12) x RWR719			+
103	RWR719 x (12x8)			+
104	RWR719 x (12x8)			+
105	RWR719 x (12x8)			+
106	PI207262 (8)	+		+
107	RWR719	–	–	–
108	G2333 (12)	+	+	–

Highlighted individuals were selected for advancement

Appendix v: Second cycle of marker assisted selection among F1 progenies of the cross (((G2333 X PI207262) x RWR719) x Susceptible)

Sample No.	Pedigree	Genes screened for & Primers used			
		SBB14	SAB3	SB12	$PYAA19_{800}$
		Co-4[2]	Co-5	Co-9	*Py (Pythium)*
1	NABE14	-	-	-	+
2	G2333 (12)	+	+	-	-
3	PI207262 (8)	-	-	+	-
4	NABE 13	-	-	-	+
5	RWR719	-	-	+	+
6	RWR 719X8X12XK132 (87.5)	+	-	+	+
7	12X8XRWR 719XNABE4 (44.1)	-	-	+	-
8	12X8XRWR 719XK132 (44.10)	-	-	+	-
9	12X8XRWR 719XNABE4 (44.7)	+	+	+	+
10	12X8XRWR 719XNABE4 (44.10)	-	-	+	+
11	12X8XRWR 719XNABE4 (44.2)	-	-	-	+
12	RWR 719X8X12XNABE4 (89.4)	+	-	-	+
13	12X8XRWR 719XNABE14 (42.11)	-	-	+	-
14	12X8XRWR 719XNABE14 (42.9)	-	+	+	+
15	12X8XRWR 719XNABE14 (43.2)	+	+	-	+
16	NABE4	-	-	-	-
17	K132	-	-	-	-
18	12X8XRWR 719XNABE14 (16.1)	-	-	+	+
19	12X8XRWR 719XNABE14 (42.10)	-	-	+	+
20	12X8XRWR 719XNABE14 (42.12)	-	+	+	+
21	RWR 719X8X12XNABE4 (89.5)	+	-	+	+
22	12X8XRWR 719XNABE4 (44.12)	+	-	+	-
23	12X8XRWR 719XNABE4 (44.13)	+	+	-	-
24	12X8XRWR 719XNABE4 (44.8)	-	+	-	+
25	12X8XRWR 719XNABE4 (16.4)	+	-	-	-
26	12X8XRWR 719XK132 (16.6)	+	-	-	-
27	12X8XRWR 719XK132 (16.7)	-	-	-	+
28	12X8XRWR 719XK132 (16.12)	-	-	+	+
29	12X8XRWR 719XK132 (44.3)	+	-	+	-
30	12X8XRWR 719XK132 (44.5)	+	+	+	-
31	12X8XRWR 719XK132 (44.11)	-	-	+	+
32	12X8XRWR 719XK132 (16.3)	-	-	-	-
33	12X8XRWR 719XK132 (16.2)	+	-	+	+
34	12X8XRWR 719XK132 (16.1)	-	+	+	+

35	12X8XRWR 719XK132 (16.8)	-	-	+	+
36	12X8XRWR 719XK132 (16.9)	+	-	+	-
37	12X8XRWR 719XK132 (44.1)	-	-	-	-
38	12X8XRWR 719XK132 (44.2)	-	+	+	-
39	12X8XRWR 719XK132 (44.7)	+	-	-	-
40	12X8XRWR 719XK132 (44.9)	-	-	-	+
41	RWR 719X8X12XK132 (87.4)	-	-	+	-
42	12X8XRWR 719XNABE4 (44.6)	-	-	+	-
43	12X8XRWR 719XNABE4 (44.4)	-	-	+	+
44	12X8XRWR 719XNABE14 (16.3)	+	+	+	+
45	12X8XRWR 719XNABE14 (16.2)	+	-	+	-
46	12X8XRWR 719XNABE4 (44.15)	+	-	-	-
47	12X8XRWR 719XNABE13 (44.1)	+	+	-	+
48	12X8XRWR 719XNABE13 (44.2)	-	+	+	-
49	RWR 719X8X12XNABE14 (43.3)	+	-	+	-
50	8X12XRWR 719XNABE13 (59.2)	-	-	+	-
51	8X12XRWR 719XNABE13 (59.1)	-	-	+	-
52	12X8XRWR 719XNABE13 (16.2)	-	-	-	-
53	12X8XRWR 719XNABE13 (16.1)	-	+	-	-

Highlighted individuals were selected for advancement

Appendix vi: Cycle three of marker assisted selection among F2 progenies of the cross (((G2333 X PI207262) x RWR719) x Susceptible)

Sample No	Genotype/Pedigree	Markers and Genes screened					
		SH18	SAS13	SBB14	SAB3	SB12	$PYAA19_{800}$
		Co-4[2]	Co-4[2]	Co-4[2]	Co-5	Co-9	Pyth
1	K132	-	-	-	-	-	-
2	NABE 4	-	-	-	-	-	-
3	PI207262	-	+	-	-	+	+
4	NABE 14	-	-	-	-	-	+
5	NABE 13	-	-	-	-	-	+
6	RWR 719	-	-	-	-	+	+
7	RWR 719	-	-	-	-	+	+
8	RWR 719	-	-	-	-	+	+
9	RWR 719	-	-	-	-	+	+
10	RWR 719	-	-	-	-	-	+
11	G2333	+	+	+	+	-	-
12	12X8XRWR719XNABE4 (44.13.2)	-	-	-	-	-	-
13	RWR719X8X12XNABE4 (89.5.1)	+	+	+	-	-	+
14	RWR719X8X12XNABE14 (42.12.3)	-	-	-	+	+	+
15	12X8XRWR719XNABE14 (42.12.4)	-	-	-	+	+	+
16	RWR719X8X12XK132 (87.5.2)	-	-	-	-	-	-
17	12X8XRWR719XNABE13 (16.1.5)	-	-	-	-	-	-
18	12X8XRWR719XNABE4 (44.15.3)	+	+	+	-	+	+
19	12X8XRWR719XK132 (16.12.5)	-	+	-	+	-	-
20	12X8XRWR719XNABE13 (16.1.4)	-	+	-	-	+	+
21	12X8XRWR719XNABE4 (44.15.4)	+	+	+	-	+	+
22	12X8XRWR719XNABE13 (16.1.3)	-	+	-	-	+	+
23	12X8XRWR719XNABE4 (44.15.5)	+	+	-	-	+	-
24	12X8XRWR719XK132 (16.12.2)	-	-	-	-	-	-
25	12X8XRWR719XK132 (16.12.1)	-	-	-	-	+	+
26	12X8XRWR719XNABE4 (44.1.2)	-	-	-	-	-	+
27	12X8XRWR719XNABE4 (44.1.3)	-	-	-	-	+	+
28	12X8XRWR719XK132 (16.6.3)	+	+	+	-	+	+
29	12X8XRWR719XK132 (16.6.1)	+	+	-	-	-	+
30	12X8XRWR719XNABE14 (42.11.2)	-	+	-	-	+	+
31	12X8XRWR719XNABE14 (42.11.1)	-	+	-	-	+	+
32	RWR719X8X12XNABE4 (89.5.4)	+	+	+	-	+	+
33	12X8XRWR719XK132 (44.3.4)	+	+	+	-	+	-
34	12X8XRWR719XK132 (44.3.2)	-	-	-	-	+	-
35	12X8XRWR719XK132 (44.3.1)	-	-	-	-	+	-
36	12X8XRWR719XK132 (16.1.3)	-	-	-	+	+	-
37	12X8XRWR719XNABE13 (44.1.4)	-	-	-	+	+	+
38	12X8XRWR719XK132 (16.1.2)	-	-	-	-	+	+
39	12X8XRWR719XK132 (16.1.1)	-	-	-	+	+	+

40	12X8XRWR719XNABE13 (44.1.6)	+	+	+	+	+	+
41	12X8XRWR719XK132 (44.5.1)	-	-	-	+	+	-
42	12X8XRWR719XNABE14 (16.3.1)	+	+	+	+	+	+
43	12X8XRWR719XK132 (44.5.2)	+	+	+	+	+	+
44	12X8XRWR719XNABE14 (16.3.3)	+	+	-	+	-	-
45	12X8XRWR719XK132 (44.5.3)	+	+	+	+	+	-
46	12X8XRWR719XK132 (44.5.4)	+	+	+	+	+	-
47	12X8XRWR719XK132 (44.5.5)	+	+	+	+	+	-
48	12X8XRWR719XK132 (44.5.6)	+	+	+	+	-	-
49	12X8XRWR719XK132 (44.5.7)	-	-	-	-	-	-
50	12X8XRWR719XK132 (44.5.8)	+	+	+	+	+	-
51	12X8XRWR719XK132 (44.5.9)	+	+	+	+	+	-
52	12X8XRWR719XK132 (44.5.10)	+	+	+	+	-	-
53	12X8XRWR719XNABE4 (44.7.11)	+	-	-	+	-	-
54	12X8XRWR719XNABE4 (44.7.1)	+	+	+	+	+	+
55	12X8XRWR719XNABE4 (44.7.2)	+	+	+	+	+	+
56	12X8XRWR719XNABE4 (44.7.9)	+	+	+	+	+	+
57	12X8XRWR719XNABE4 (44.7.3)	+	+	+	-	+	-
58	12X8XRWR719XNABE4 (44.7.8)	+	+	+	-	+	+
59	12X8XRWR719XNABE4 (44.7.4)	-	-	-	-	-	-
60	12X8XRWR719XNABE4 (44.7.5)	+	+	+	+	-	+
61	12X8XRWR719XNABE4 (44.7.6)	-	-	-	-	-	-
62	12X8XRWR719XNABE4 (44.7.7)	+	+	+	+	+	+
63	12X8XRWR719XNABE4 (44.13.5)	+	+	+	+	+	-
64	12X8XRWR719XNABE4 (44.13.4)	+	+	+	-	+	-
65	12X8XRWR719XK132 (44.2.3)	-	-	-	-	-	-
66	12X8XRWR719XK132 (44.2.4)	-	-	-	+	+	-
67	12X8XRWR719XNABE13 (44.2.1)	+	+	+	+	+	+
68	12X8XRWR719XK132 (44.2.2)	-	-	-	-	-	-
69	12X8XRWR719XNABE13 (44.1.2)	+	+	+	+	-	+
70	12X8XRWR719XNABE4 (44.8.2)	-	-	-	+	+	+
71	12X8XRWR719XNABE13 (44.1.3)	+	+	+	+	+	+
72	12X8XRWR719XNABE4 (44.13.1)	+	+	+	+	+	-
73	12X8XRWR719XK132 (44.3.5)	+	+	+	-	+	-
74	RWR719X8X12XNABE4 (89.5.2)	+	+	+	-	+	-
75	12X8XRWR719XNABE14 (42.12.1)	-	-	-	-	+	+
76	RWR719X8X12XNABE14 (42.12.2)	-	-	-	+	-	+
77	RWR719X8X12XK132 (87.5.3)	+	+	+	-	+	+
78	RWR719X8X12XK132 (87.5.5)	+	+	+	-	+	+
79	RWR719X8X12XNABE4 (89.4.2)	+	+	+	-	-	-
80	12X8XRWR719XNABE4 (44.6.3)	+	+	+	-	+	+

Appendix vii: Cycle four of marker assisted selection among F3 progenies of the cross (((G2333 X PI207262) x RWR719) x Susceptible)

Sample No.	Pedigree	Genotype	SAS13 Co-4	SBB14 Co-4^2	SAB3 Co-5	SB12 Co-9	PYAA 19$_{800}$ *Py*
1	12X8XRWR719XK132 (44.5.7.1)	None	-	-	-	+	-
2	12X8XRWR719XK132 (44.5.7.3)	None	-	-	-	+	-
3	12X8XRWR719XK132 (44.5.7.4)	None	-	-	-	+	-
4	12X8XRWR719XK132 (44.5.7.5)	None	-	-	-	+	-
5	12X8XRWR719XK132 (44.5.7.6)	None	-	-	-	+	-
6	12X8XRWR719XK132 (16.1.2.1)	Co9/Py	-	-	-	-	-
7	12X8XRWR719XK132 (16.1.2.3)	Co9/Py	-	-	-	-	+
8	12X8XRWR719XK132 (16.1.2.5)	Co9/Py	-	-	-	-	+
9	12X8XRWR719XK132 (16.1.2.6)	Co9/Py	-	-	-	-	+
10	12X8XRWR719XK132 (16.1.2.7)	Co9/Py	-	-	-	-	+
11	12X8XRWR719XK132 (16.1.2.8)	Co9/Py	-	-	-	-	+
12	12X8XRWR719XK132 (16.1.2.9)	Co9/Py	-	-	-	-	+
13	12X8XRWR719XK132 (16.1.2.10)	Co9/Py	-	-	-	-	+
14	12X8XRWR719XK132 (44.5.9.1)	Co4^2/5/9	-	+	+	+	-
15	12X8XRWR719XK132 (44.5.9.2)	Co4^2/5/9	+	+	+	+	-
16	12X8XRWR719XK132 (44.5.9.3)	Co4^2/5/9	-	-	-	-	-
17	12X8XRWR719XK132 (44.5.9.4)	Co4^2/5/9	+	+	+	+	-
18	12X8XRWR719XK132 (44.5.9.5)	Co4^2/5/9	+	+	+	+	-
19	12X8XRWR719XK132 (44.5.9.7)	Co4^2/5/9	-	+	+	+	-
20	12X8XRWR719XK132 (44.5.9.8)	Co4^2/5/9	-	+	+	+	-
21	12X8XRWR719XK132 (16.6.1.1)	Co4^2/Py	-	-	-	-	-
22	12X8XRWR719XK132 (16.6.1.2)	Co4^2/Py	+	+	-	-	+
23	12X8XRWR719XK132 (16.6.1.3)	Co4^2/Py	+	+	-	-	+
24	12X8XRWR719XK132 (16.6.1.5)	Co4^2/Py	+	+	-	-	+
25	12X8XRWR719XK132 (16.6.1.6)	Co4^2/Py	+	+	-	-	-
26	12X8XRWR719XK132 (44.5.2.1)	Co4^2/5/9/Py	+	+	+	-	-
27	12X8XRWR719XK132 (44.5.2.2)	Co4^2/5/9/Py	+	+	+	-	-
28	12X8XRWR719XK132 (44.5.2.3)	Co4^2/5/9/Py	+	+	+	+	-
29	12X8XRWR719XK132 (44.5.2.4)	Co4^2/5/9/Py	+	+	+	+	-
30	12X8XRWR719XK132 (44.5.2.5)	Co4^2/5/9/Py	+	+	+	+	-
31	12X8XRWR719XK132 (44.5.2.7)	Co4^2/5/9/Py	+	+	+	-	-
32	12X8XRWR719XK132 (44.5.2.9)	Co4^2/5/9/Py	+	+	+	+	-
33	12X8XRWR719XK132 (44.5.2.10)	Co4^2/5/9/Py	+	+	+	+	-
34	12X8XRWR719XK132 (16.1.2.3)	Co9/Py	-	-	-	-	+
35	12X8XRWR719XK132 (16.1.2.4)	Co9/Py	-	-	-	-	+
36	12X8XRWR719XK132 (16.1.2.5)	Co9/Py	-	-	-	-	-
37	12X8XRWR719XK132 (16.1.2.6)	Co9/Py	-	-	-	-	+
38	12X8XRWR719XK132 (44.5.1.1)	Co5/9	-	-	+	+	-

39	12X8XRWR719XK132 (44.5.1.2)	Co5/9	-	-	-	-	-
40	12X8XRWR719XK132 (44.5.1.3)	Co5/9	-	-	+	+	-
41	12X8XRWR719XK132 (44.5.1.5)	Co5/9	-	-	+	+	-
42	12X8XRWR719XK132 (44.5.1.7)	Co5/9	-	-	+	+	-
43	12X8XRWR719XK132 (44.5.1.8)	Co5/9	-	-	-	+	-
44	12X8XRWR719XK132 (44.5.1.9)	Co5/9	-	-	+	+	-
45	12X8XRWR719XK132 (44.5.1.10)	Co5/9	-	-	+	+	-
46	12X8XRWR719XK132 (44.5.1.11)	Co5/9	-	-	+	+	-
47	12X8XRWR719XK132 (44.5.1.12)	Co5/9	-	-	+	+	-
48	12X8XRWR719XK132 (44.5.1.13)	Co5/9	-	-	+	+	-
49	12X8XRWR719XK132 (44.5.1.15)	Co5/9	-	-	+	+	-
50	12X8XRWR719XK132 (44.5.1.16)	Co5/9	-	-	+	+	-
51	12X8XRWR719XK132 (44.3.1.1)	Co9	-	-	-	-	-
52	12X8XRWR719XK132 (44.5.6.1)	$Co4^2/5$	+	+	+	-	-
53	12X8XRWR719XK132 (44.5.6.2)	$Co4^2/5$	+	+	+	-	-
54	12X8XRWR719XK132 (44.5.6.3)	$Co4^2/5$	+	+	+	-	-
55	12X8XRWR719XK132 (44.5.6.4)	$Co4^2/5$	+	-	+	-	-
56	12X8XRWR719XK132 (44.5.6.5)	$Co4^2/5$	+	+	+	-	-
57	12X8XRWR719XK132 (44.5.6.7)	$Co4^2/5$	+	+	+	-	-
58	12X8XRWR719XK132 (44.5.6.9)	$Co4^2/5$	+	+	+	-	-
59	12X8XRWR719XK132 (44.5.6.11)	$Co4^2/5$	+	+	+	-	-
60	12X8XRWR719XK132 (44.5.6.12)	$Co4^2/5$	+	+	+	-	-
61	12X8XRWR719XK132 (44.5.6.13)	$Co4^2/5$	+	+	+	-	-
62	12X8XRWR719XK132 (44.5.6.14)	$Co4^2/5$	+	-	+	-	-
63	12X8XRWR719XK132 (44.5.4.3)	$Co4^2/5/9$	+	+	+	+	-
64	12X8XRWR719XK132 (44.5.4.4)	$Co4^2/5/9$	+	+	+	+	-
65	12X8XRWR719XK132 (44.5.4.5)	$Co4^2/5/9$	+	+	-	-	-
66	12X8XRWR719XK132 (44.5.4.6)	$Co4^2/5/9$	+	+	-	-	-
67	12X8XRWR719XK132 (44.5.4.7)	$Co4^2/5/9$	+	+	+	+	-
68	12X8XRWR719XK132 (44.5.4.11)	$Co4^2/5/9$	+	+	-	+	-
69	12X8XRWR719XK132 (44.5.4.12)	$Co4^2/5/9$	+	+	+	+	-
70	12X8XRWR719XK132 (44.5.4.13)	$Co4^2/5/9$	+	+	+	+	-
71	12X8XRWR719XK132 (44.5.4.14)	$Co4^2/5/9$	+	+	+	-	-
72	12X8XRWR719XK132 (44.5.4.15)	$Co4^2/5/9$	+	+	-	+	-
73	12X8XRWR719XK132 (44.5.4.16)	$Co4^2/5/9$	+	+	-	+	-
74	12X8XRWR719XK132 (44.5.4.17)	$Co4^2/5/9$	+	+	+	+	-
75	12X8XRWR719XNABE4 (44.7.2.1)	$Co4^2/5/9/Py$	-	-	+	+	+
76	12X8XRWR719XNABE4 (44.7.2.2)	$Co4^2/5/9/Py$	+	+	+	+	+
77	12X8XRWR719XNABE4 (44.7.2.3)	Co42/5/9/Py	-	-	-	-	-
81	12X8XRWR719XNABE4 (89.5.1.1)	$Co4^2/Py$	+	+	-	-	+
82	12X8XRWR719XNABE4 (89.5.1.3)	$Co4^2/Py$	-	-	-	-	-
83	12X8XRWR719XNABE4 (89.5.1.4)	$Co4^2/Py$	-	+	-	-	+
84	12X8XRWR719XNABE4 (89.5.1.5)	$Co4^2/Py$	+	+	-	-	+

85	12X8XRWR719XNABE4 (89.5.1.6)	Co4²/Py	+	+	-	-	+
86	12X8XRWR719XNABE4 (89.5.1.7)	Co4²/Py	-	+	-	-	+
87	12X8XRWR719XNABE4 (89.5.1.9)	Co4²/Py	+	+	-	-	-
88	12X8XRWR719XNABE4 (89.5.1.10)	Co4²/Py	+	+	-	-	+
89	12X8XRWR719XNABE4 (89.5.1.11)	Co4²/Py	+	+	-	-	+
90	12X8XRWR719XNABE4 (44.7.8.1)	Co4²/9/Py	-	-	-	-	-
91	12X8XRWR719XNABE4 (44.7.8.2)	Co4²/9/Py	+	+	-	-	-
92	12X8XRWR719XNABE4 (44.7.8.3)	Co4²/9/Py	+	+	-	-	-
93	12X8XRWR719XNABE4 (44.7.8.5)	Co4²/9/Py	+	+	-	-	+
94	12X8XRWR719XNABE4 (44.7.8.6)	Co4²/9/Py	-	-	-	-	-
95	12X8XRWR719XNABE4 (44.7.8.7)	Co4²/9/Py	+	+	+	+	+
96	12X8XRWR719XNABE4 (44.7.9.1)	Co4²/5/9/Py	-	-	+	+	+
98	12X8XRWR719XNABE4 (44.7.9.4)	Co42/5/9/Py	-	-	-	-	-
99	12X8XRWR719XNABE4 (44.7.9.5)	Co4²/5/9/Py	-	-	+	+	-
101	12X8XRWR719XNABE4 (44.7.9.7)	Co4²/5/9/Py	+	+	+	+	+
102	12X8XRWR719XNABE4 (44.7.9.8)	Co4²/5/9/Py	+	+	+	+	-
103	12X8XRWR719XNABE4 (44.7.9.9)	Co4²/5/9/Py	+	+	-	+	-
104	12X8XRWR719XNABE4 (44.7.9.10)	Co4²/5/9/Py	+	+	-	+	+
105	12X8XRWR719XNABE4 (44.7.9.11)	Co4²/5/9/Py	+	+	+	+	+
106	12X8XRWR719XNABE4 (44.7.9.12)	Co4²/5/9/Py	+	+	+	+	+
107	12X8XRWR719XNABE4 (44.7.5.1)	Co4²/5/Py	+	-	+	+	+
108	12X8XRWR719XNABE4 (44.7.5.3)	Co4²/5/Py	+	+	-	-	+
109	12X8XRWR719XNABE4 (44.7.5.4)	Co4²/5/Py	+	+	-	-	+
110	12X8XRWR719XNABE4 (44.7.5.5)	Co4²/5/Py	+	+	-	-	-
111	12X8XRWR719XNABE4 (44.7.5.7)	Co4²/5/Py	-	-	-	-	-
112	12X8XRWR719XNABE4 (44.7.5.8)	Co4²/5/Py	-	-	-	-	-
113	12X8XRWR719XNABE4 (89.5.2.1)	Co4²/9	+	+	-	+	-
114	12X8XRWR719XNABE4 (89.5.2.2)	Co4²/9	+	+	-	-	-
115	12X8XRWR719XNABE4 (89.5.2.3)	Co4²/9	-	-	-	-	-
116	12X8XRWR719XNABE4 (89.5.2.6)	Co4²/9	-	-	-	-	-
116	12X8XRWR719XNABE4 (89.5.2.7)	Co4²/9	+	+	-	+	-
117	12X8XRWR719XNABE4 (89.5.2.9)	Co4²/9	+	+	-	+	-
118	12X8XRWR719XNABE4 (89.5.2.10)	Co4²/9	+	-	-	+	-
119	12X8XRWR719XNABE4 (89.5.2.11)	Co4²/9	+	+	-	+	-
120	12X8XRWR719XNABE4 (89.5.2.12)	Co4²/9	+	+	-	+	-
121	12X8XRWR719XNABE4 (89.5.2.13)	Co4²/9	+	+	-	+	-
122	12X8XRWR719XNABE4 (89.5.2.14)	Co4²/9	-	-	-	-	-
123	12X8XRWR719XNABE4 (89.5.2.15)	Co4²/9	+	+	-	-	-
124	12X8XRWR719XNABE13 (44.1.6.1)	Co4²/5/9/Py	+	+	+	-	+
125	12X8XRWR719XNABE13 (44.1.6.2)	Co42/5/9/Py	+	+	+	-	+
126	12X8XRWR719XNABE13 (44.1.6.3)	Co4²/5/9/Py	+	+	+	-	+
127	12X8XRWR719XNABE13 (44.1.6.4)	Co4²/5/9/Py	+	+	+	-	+
128	12X8XRWR719XNABE13 (44.1.6.5)	Co4²/5/9/Py	-	-	+	-	+

129	12X8XRWR719XNABE13 (44.1.6.6)	Co4^{2}/5/9/Py	-	-	+	-	+
130	12X8XRWR719XNABE13 (44.1.6.7)	Co4^{2}/5/9/Py	-	-	-	-	-
131	12X8XRWR719XNABE13 (16.1.3.1)	Co4^{3}/9/py	+	-	-	+	+
132	12X8XRWR719XNABE13 (16.1.3.3)	Co4^{3}/9/py	-	-	-	-	+
133	12X8XRWR719XNABE13 (16.1.3.5)	Co4^{3}/9/py	-	-	-	-	+
134	12X8XRWR719XNABE13 (16.1.3.6)	Co4^{3}/9/py	+	-	-	-	+
135	12X8XRWR719XNABE13 (16.1.3.7)	Co4^{3}/9/py	+	-	-	-	+
136	12X8XRWR719XNABE13 (16.1.3.8)	Co4^{3}/9/py	+	-	-	-	+
137	12X8XRWR719XNABE13 (16.1.3.9)	Co4^{3}/9/py	-	-	-	-	-
138	12X8XRWR719XNABE13 (16.1.3.10)	Co4^{3}/9/py	-	-	-	-	+
139	12X8XRWR719XNABE13 (44.1.4.1)	Co5/9/Py	-	-	+	-	+
140	12X8XRWR719XNABE13 (44.1.4.2)	Co5/9/Py	-	-	+	-	+
141	12X8XRWR719XNABE13 (44.1.4.3)	Co5/9/Py	-	-	+	+	+
142	12X8XRWR719XNABE13 (44.1.4.5)	Co5/9/Py	-	-	+	-	+
143	12X8XRWR719XNABE13 (44.1.4.7)	Co5/9/Py	-	-	+	-	-
144	12X8XRWR719XNABE13 (44.1.4.9)	Co5/9/Py	-	-	+	-	+
145	12X8XRWR719XNABE13 (44.1.4.10)	Co5/9/Py	-	-	+	-	+
146	12X8XRWR719XNABE13 (44.1.4.11)	Co5/9/Py	-	-	+	-	+
148	12X8XRWR719XNABE13 (44.1.4.13)	Co5/9/Py	-	-	+	-	+
149	12X8XRWR719XNABE13 (44.1.4.14)	Co5/9/Py	-	-	+	-	+
150	12X8XRWR719XNABE13 (44.1.4.15)	Co5/9/Py	-	-	+	-	+
151	12X8XRWR719XNABE14 (16.3.3.1)	Co4^{2}/5	-	-	+	-	+
152	12X8XRWR719XNABE14 (16.3.3.2)	Co4^{2}/5	-	-	-	-	-
153	12X8XRWR719XNABE14 (16.3.3.3)	Co4^{2}/5	+	+	+	-	+
154	12X8XRWR719XNABE14 (16.3.3.5)	Co4^{2}/5	+	+	+	-	+
155	12X8XRWR719XNABE14 (16.3.3.6)	Co4^{2}/5	+	+	+	-	+
156	12X8XRWR719XNABE14 (16.3.3.7)	Co4^{2}/5	+	+	+	-	+
157	12X8XRWR719XNABE14 (16.3.3.8)	Co4^{2}/5	+	+	+	-	+
158	12X8XRWR719XNABE14 (16.3.3.9)	Co4^{2}/5	+	-	+	-	+
159	12X8XRWR719XNABE14 (16.3.3.10)	Co4^{2}/5	+	+	+	-	+
160	12X8XRWR719XNABE14 (16.3.3.11)	Co4^{2}/5	+	+	+	-	+
161	12X8XRWR719XNABE14 (42.12.4.1)	Co5/9/Py	-	-	+	+	+

Appendix viii: Cycle five of marker assisted selection among F4 progenies of the cross (((G2333 X PI207262) x RWR719) x Susceptible)

FTA card No	Sample No.	FTA Code	SAS13	SAB3	SB12	$PYAA19_{800}$
1	1	01/1	-	-	+	-
1	2	06/1	-	-	-	-
1	3	06/2	-	-	-	-
1	4	06/3	-	-	-	-
1	5	07/1	-	-	-	-
1	6	07/2	-	-	-	-
1	7	07/3	-	-	-	+
1	8	07/4	-	-	-	+
1	9	07/5	-	-	-	+
1	10	07/6	-	-	-	+
1	11	07/7	-	-	-	-
1	12	07/8	-	-	-	-
1	13	07/9	-	-	-	+
1	14	08/1	-	-	-	+
1	15	08/2	-	-	-	-
1	16	08/3	-	-	-	+
2	17	08/4	-	-	-	+
2	18	08/5	-	-	-	+
2	19	09/1	-	-	-	-
2	20	09/2	-	-	-	-
2	21	09/3	-	-	-	-
2	22	09/4	-	-	-	-
2	23	09/5	-	-	-	+
2	24	09/6	-	-	-	-
2	25	09/7	-	-	-	+
2	26	09/8	-	-	-	+
2	27	10/1	-	-	-	-
2	28	10/2	-	-	-	+
2	29	10/3	-	-	-	-
2	30	11/1	-	-	-	-
2	31	11/2	-	-	-	+
2	32	12/1	-	-	-	-
3	33	12/2	-	-	-	+
3	34	14/1	+	+	+	-
3	35	14/2	+	+	+	-
3	36	14/3		+	+	-
3	37	20/1	+	+	+	-
3	38	21/1	+	-	-	-
3	39	21/2	+	-	-	-
3	40	21/3		-	-	-
3	41	21/4	+	-	-	-
3	42	25/1		-	-	-
3	43	25/2		-	-	-
3	44	26/1	+	-	-	-
3	45	26/2	+	+	-	-
3	46	26/3		+	-	-

3	47	26/4	+	+	-	-
3	48	27/1	+	+	-	-
4	49	27/2	+	+	-	-
4	50	27/3	+	+	-	-
4	51	27/4	+	+	-	-
4	52	28/1	-	+	+	-
4	53	28/2	+	+	+	-
4	54	28/3	+	+	+	-
4	55	28/4	+	+	+	-
4	56	30/1	+	+	+	-
4	57	30/2	+	+	+	-
4	58	30/3	+	+	+	-
4	59	31/1	+	+	+	-
4	60	32/1	-	-	-	+
4	61	32/2	-	-	-	+
4	62	32/3	+	-	-	+
4	63	33/1	-	+	+	-
4	64	33/2	+	+	-	-
5	65	34/1	-	-	-	+
5	66	34/2	-	-	-	+
5	67	34/3	-	-	-	-
5	68	34/4	-	-	-	-
5	69	34/5	-	-	-	+
5	70	35/1	-	-	-	-
5	71	35/2	-	-	-	-
5	72	35/3	-	-	-	-
5	73	35/4	-	-	-	+
5	74	35/5	-	-	-	+
5	75	36/1	-	-	-	+
5	76	36/2	-	-	-	+
5	77	36/3	-	-	-	+
5	78	36/4	-	-	-	+
5	79	36/5	-	-	-	+
5	80	36/6	-	-	-	+
6	81	36/7	-	-	-	+
6	82	36/8	-	-	-	+
6	83	38/1	-	+	+	-
6	84	38/2	-	+	+	-
6	85	38/3	-	+	+	-
6	86	39/1	-	+	+	-
6	87	39/2	-	+	-	-
6	88	39/3	-	+	+	-
6	89	40/2	-	+	-	-
6	90	40/1	-	-	+	-
6	91	40/3	-	-	-	-
6	92	40/4	-	-	+	-
6	93	40/5	-	-	+	-
6	94	41/1	-	-	+	-
6	95	41/2	-	-	+	-
6	96	41/3	-	-	+	-

7	97	41/3	-	-	+	-
7	98	41/4	-	+	+	-
7	99	41/5	-	+	+	-
7	100	41/6	-	-	+	-
7	101	41/7	-	-	+	-
7	102	41/8	-	-	+	-
7	103	44/1	-	-	+	-
7	104	44/2	-	+	+	-
7	105	44/3	-	+	+	-
7	106	44/4	-	-	+	-
7	107	45/1	-	+	+	-
7	108	45/2	-	-	+	-
7	109	46/1	-	+	+	-
7	110	46/2	-	+	+	-
7	111	46/3	-	+	+	-
7	112	46/4	-	+	+	-
8	113	46/5	-	+	+	-
8	114	46/6	-	+	+	-
8	115	47/1	-	+	-	-
8	116	47/2	-	+	-	-
8	117	47/3	-	+	-	-
8	118	47/4	-	+	-	-
8	119	48/1	-	+	+	-
8	120	48/2	-	+	+	-
8	121	49/1	-	+	+	-
8	122	49/2	-	-	+	-
8	123	49/3	-	+	+	-
8	124	49/4	-	+	+	-
8	125	49/5	-	+	+	-
8	126	49/6	-	+	+	-
8	127	49/7	-	+	+	-
8	128	49/8	-	+	+	-
9	129	49/9	-	+	+	-
9	130	50/1	-	+	+	-
9	131	50/2	-	+	+	-
9	132	50/3	-	+	+	-
9	133	50/4	-	+	+	-
9	134	50/5	-	+	+	-
9	135	53/1	+	-	-	-
9	136	53/2	+	-	-	-
9	137	53/3	+	+	-	-
9	138	53/4	+	+	-	-
9	139	55/1	+	+	-	-
9	140	55/2	+	+	-	-
9	141	58/1	-	+	-	-
9	142	58/2	+	+	-	-
9	143	61/1	+	+	-	-
9	144	64/1	+	+	+	-
10	145	64/2	+	+	+	-
10	146	64/3	+	-	+	-

10	147	64/4	+	+	+	-
10	148	64/5	+	+	+	-
10	149	64/6	+	-	+	-
10	150	65/1	+	-	-	-
10	151	65/2	+	-	-	-
10	152	66/1	-	-	-	-
10	153	66/2	+	-	-	-
10	154	66/3	+	-	-	-
10	155	69/1	+	-	+	-
10	156	69/2	-	+	+	-
10	157	69/3	+	-	+	-
10	158	69/4	-	+	+	-
10	159	70/1	-	+	+	-
10	160	70/2	-	+	+	-
11	161	70/3	+	-	+	-
11	162	70/4	-	+	+	-
11	163	70/5	-	+	+	-
11	164	71/1	-	+	-	-
11	165	71/2	+	+	-	-
11	166	71/3	+	+	-	-
11	167	71/4	+	+	-	-
11	168	71/5	-	+	-	-
11	169	75/1	-	+	+	+
11	170	75/2	-	+	+	-
11	171	76/1	-	+	+	+
11	172	76/2	-	+	-	+
11	173	76/3	+	+	+	+
11	174	76/4	-	+	-	+
11	175	76/5	-	+	-	+
11	176	77/1	-	-	-	-
12	177	81/1	Repeat	-	-	+
12	178	81/2	Repeat	-	-	+
12	179	81/3	Repeat	-	-	+
12	180	83/1	Repeat	-	-	-
12	181	84/1	Repeat	-	-	+
12	182	86/1	Repeat	-	-	+
12	183	86/2	Repeat	-	-	+
12	184	86/5	Repeat	-	-	+
12	185	86/4	Repeat	-	-	+
12	186	86/3	Repeat	-	-	+
12	187	86/6	Repeat	-	-	+
12	188	87/1	Repeat	-	-	-
12	189	88/1	Repeat	-	-	+
12	190	88/2	Repeat	-	-	+
12	191	88/3	Repeat	-	-	+
12	192	88/4	Repeat	-	-	+
13	193	89/1	Repeat	-	-	
13	194	89/2	Repeat	-	-	
13	195	90/1	Repeat	-	-	
13	196	90/2	Repeat	-	-	

13	197	91/1	-	-	-
13	198	91/2	-	-	-
13	199	91/3	+	-	-
13	200	91/4	+	-	-
13	201	91/5	+	-	-
13	202	91/6	+	-	-
13	203	91/7	+	-	-
13	204	91/8	-	-	-
13	205	91/9	-	-	-
13	206	91/10	+	-	-
13	207	91/11	+	-	-
13	208	92/1	+	-	-
14	209	92/3	+	-	-
14	210	96/1	+	-	+
14	211	98/1	-	-	+
14	212	99/1	-	-	-
14	213	99/2	-	-	+
14	214	99/3	-	-	+
14	215	99/4	-	-	+
14	216	102/1	+	-	+
14	217	103/1	+	-	+
14	218	104/1	+	-	+
14	219	104/2	-	-	-
14	220	104/3	+	-	+
14	221	104/4	+	-	+
14	222	104/5	+	-	+
14	223	104/6	+	-	+
14	224	108/1	+	-	-
14	225	108/2	+	-	-
14	226	109/1	+	-	-
15	227	110/1	+	-	-
15	228	110/2	+	-	-
15	229	110/3	+	-	-
15	230	110/4	+	-	-
15	231	112/1	-	-	-
15	232	112/2	-	-	-
15	233	112/3	-	-	-
15	234	115/1	-	-	-
15	235	115/2	-	-	-
15	236	115/3	-	-	-
15	237	117/1	+	-	-
15	238	117/2	+	-	-
15	239	118/1	+	-	+
15	240	118/2	+	-	+
15	241	118/3	+	-	+
15	242	118/4	+	-	
16	243	118/5	+	-	+
16	244	118/6	+	-	+
16	245	118/7	+	-	
16	246	118/8	+	-	+

16	247	118/9	+	-	+
16	248	118/10	+	-	+
16	249	118/11	+	-	+
16	250	118/12	+	-	+
16	251	118/13	+	-	+
16	252	118/14	+	-	+
16	253	118/15	+	-	+
16	254	119/1	+	-	+
16	255	119/2	+	-	+
16	256	120/1	+	-	+
16	257	120/2	+	-	+
16	258	120/3	+	-	+
17	259	120/4	+	-	+
17	260	121/1		-	+
17	261	121/2	+	-	+
17	262	121/3	+	-	-
17	263	121/4	+	-	-
17	264	123/1	+	-	-
17	265	123/2	+	-	-
17	266	123/3	+	-	-
17	267	123/4	-	-	-
17	268	130/1	-	-	-
17	269	130/2	-	-	-
17	270	130/3	-	-	-
17	271	132/1	-	-	-
17	272	132/2	-	-	-
18	273	132/3	-	-	-
18	274	132/4	-	-	-
18	275	132/6	-	-	-
18	276	132/5	-	-	-
18	277	132/7	-	-	-
18	278	133/1	-	-	-
18	279	133/2	-	-	-
18	280	133/3	-	-	-
18	281	133/4	-	-	-
18	282	133/5	-	-	-
18	283	133/6	-	-	-
18	284	133/7	-	-	-
18	285	133/8	-	-	-
18	286	133/9	-	-	-
18	287	134/1	+	-	-
18	288	134/2	-	-	-
19	289	135/1	-	-	-
19	290	135/2	-	-	-
19	291	136/1	+	-	-
19	292	136/2	+	-	-
19	293	136/3	-	-	-
19	294	141/1	-	+	-
19	295	141/2	-	+	+
19	296	142/1	-	+	-

19	297	142/2	-	+	-
19	298	142/3	-	+	-
19	299	142/4	-	+	-
19	300	142/5	-	+	-
19	301	143/1	-	+	-
19	302	154/1	+	+	-
19	303	156/1	+	-	-
19	304	156/2	+	-	-
20	305	157/1	+	-	-
20	306	157/2	+	-	-
20	307	157/3	+	+	-
20	308	158/1	+	+	-
20	309	158/2	+	+	-
20	310	159/1	+	+	-
20	311	159/2	+	+	-
20	312	159/4	-	+	-
20	313	160/1	-	-	-
20	314	160/2	+	+	-
20	315	160/3	+	-	-
20	316	160/4	+	-	-
20	317	160/5	+	-	-
20	318	160/6	+	-	-
20	319	161/1	-	-	+
20	320	Unknown	-	-	