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Effectiveness of pyramided genes in conferring resistance to anthracnose disease in common bean populations

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Anthracnose disease (*Colletotrichum lindemuthianum* (Sacc. et. Magn) Lams. Scrib.) is one of the most devastating diseases that constrain common bean production in Uganda. A cascading pedigree pyramiding scheme was used to develop common bean populations to evaluate the effectiveness of pyramided and single resistance genes (*Co-4^2*, *Co-4^3*, *Co-5*, and *Co-9*) on disease development. Detached leaf trifoliates of $F_{4:6}$ genotypes were screened with four *C. lindemuthianum* races (352, 713, 767 and 2047). Disease severity data were subjected to ANOVA. Races, genotypes and Race x Genotype interaction were significant. Genes *Co-4^2* and *Co-5* conferred resistance to the four races and the gene pyramids *Co-4^2+Co-5+Co-9* and *Co-4^2+Co-5 h*ad the lowest severity scores. Gene *Co-4^3* conferred resistance to race 352 and weak resistance to race 713; whereas, gene *Co-9* conferred resistance to race 352. *Co-4^3+Co-9* gene pyramid showed resistance only to race 352. The *Co-4^2* and *Co-5* genes conferred resistance to all the four races 352, 713, 767 and 2047. The single gene *Co-4^2* was not significantly different from the pyramids *Co-4^2+Co-5+Co-9* and *Co-4^2+Co-5* (*P<0.01*). Similarly, the *Co-5* gene was not significantly different from *Co-4^2+Co-5, Co-4^2+Co-9* and *Co-5+Co-9* pyramids. The *Co-9* gene showed antagonism in all pyramids. These results indicate that pyramiding of resistance genes would be effective for disease management in Uganda, but pyramids with *Co-9* gene would be less effective.

Key words: SCAR markers, *Colletotrichum lindemuthianum*, broad-spectrum resistance.

INTRODUCTION

Colletotrichum lindemuthianum (Sacc. et. Magn) Lams. Scrib., the pathogen that causes anthracnose of common beans (*Phaseolus vulgaris* (L.)) has a high degree of pathogenic and genetic variability (Mahuku and Riascos, 2004). New races of the pathogen continually emerge, which has made single resistance gene deployment less

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Table 1. Parents used in the gene pyramiding scheme.

Cultivar	Pedigree	Growth habit	Gene pool	Anthracnose resistance genes	Response to anthracnose
K132[a]	Calima-2 x Argentino 1	B	A	-	Susceptible
NABE 4[a]	Sug 47 x Cal 103	B	A	-	Susceptible
NABE 13[a]	RWR 1946	B	A	-	Susceptible
NABE 14[a]	RWR 2075	B	A	-	Susceptible
G 2333[a, b, c]	Colorado de Teopisca	C	MA	*Co-4*^{2}*, Co-5 & Co-7*	Resistant to most races
PI 207262	Tlalnepantla 64	C	MA	*Co-4*^{3} *& Co-9*	Resistant to some races
K-22 [d]	RWR719	B	MA	-	Susceptible

[a] Officially released varieties in Uganda; [b] Landrace from Mexico; [c] Variety officially released in Rwanda as Umubano and in Uganda as NABE10C [d] Variety officially released in Kenya; A = Andean; MA = Mesoamerica; B = Bush; C = Climbe.

effective as a control strategy. The pathogen is reported to have a reproductive/ telemorphic phase, *Glomerella lindemuthianum* which is partly responsible for its genetic and physiologic variability through genetic recombination (De Silva et al., 2017). This enables it to adapt to new sources of host resistance and easily breakdown host resistance (Melotto et al., 2000). New races continually emerge based on the gene-for-gene hypothesis; this has posed a risk of rendering single resistance gene deployment less effective against the disease. Studies in Uganda have indicated that *C. lindemuthianum* has a high pathogenic variability found to be highest in the Eastern and South Western highland regions (Mwesigwa, 2008; Kiryowa et al., 2016); 24 new physiological races have been reported (Kiryowa et al., 2016).

Resistance to bean anthracnose is conditioned by thirteen (13) major genes *Co-1* to *Co-13* (Lacanallo et al., 2010) with only *co-8* being recessive. Loci *Co-9/Co-3*^{3} and *Co-7/Co-3* are allelic (Méndez-Vigo et al., 2005) and multiple alleles exist at the *Co-1, Co-3, Co-4, Co-5* loci. Genes *Co-1, Co-12* and *Co-13* are Andean in origin while the rest are Mesoamerican (Kelly and Vallejo, 2004). Resistance gene pyramiding, the combination of two or more resistance genes in a cultivar (Ye and Smith, 2008) is recommended as a strategy to increase broad-spectrum resistance against *C. lindemuthianum* pathogen. But the conventional gene pyramiding approach is costly (Joshi and Nayak, 2010) requiring extensive phenotyping with several races of the pathogen over many generations. DNA markers have enormous potential to improve the efficiency and precision of conventional plant breeding through marker-assisted selection (MAS), which is proven to speed up breeding through laboratory based selection of individual plants with the desired trait(s).

Marker assisted selection (MAS) has been extensively used in common bean breeding programs (Miklas et al., 2006) and the availability of molecular markers linked to the major anthracnose (*Co*-) genes provides an opportunity to pyramid multiple disease resistance genes (Kelly and Vallejo, 2004). Kelly et al. (1994) recommended pyramiding resistance genes *Co-1* Andean and *Co-2* Mesoamerican to confer resistance to all known *C. lindemuthianum* races in North America. Young and Kelly (1996) suggested pyramiding major resistance genes *Co-6* and *Co-5* in combination with *Co-1* for durable resistance. Ragagnin et al. (2009) used RAPDs and SCAR markers to pyramid genes *Co-4, Co-6, Co-10* with *Phg-1* angular leaf spot and *Ur-ON* rust resistance genes in a susceptible 'carioca' market class cultivar in Brazil. Genchev et al. (2010) used RAPD and SCAR markers to pyramid *Co-1* and *Co-4* genes to confer effective resistance against *C. lindemuthianum* races in Bulgaria. Ferreira et al. (2012) used SCAR, CAPs and RAPD markers to successfully pyramid *Co-2* and *Co-3/9* genes, along with, *I* and *bc-3* common mosaic virus resistance genes to develop a market class bean genotype. For successful resistance gene pyramiding, there is need to evaluate the different pyramid combinations under a diverse *C. lindemuthianum* population. The purpose of this study therefore is to assess the effectiveness of single and pyramided resistance genes against bean anthracnose disease.

MATERIALS AND METHODS

Parent materials and Ugandan breeding locations

The cultivars used as parents were obtained from the Legumes Program, National Crops Resources Research Institute (NaCRRI), Namulonge, located 0^0 32'' N of the Equator and 32^0 37'' E, 27Km North of Kampala and elevated at 1,150 meters above sea level. The parents and their traits are presented in Table 1. All crosses and advancement from F1 to F4 generations were conducted under screen house conditions at NaCRRI. Marker-assisted selection (MAS) during gene pyramiding for fixation of alleles and phenotypic screening of advanced lines were conducted at the International Center for Tropical Agriculture (CIAT), based at the National Agricultural Research Laboratories (NARL), Kawanda, located at 0° 24' 38.15" N and 32^o 32' 14.06" E and elevated at 1,147 m above sea level.

Development of populations

A cascading pedigree gene pyramiding scheme (Servin et al.,

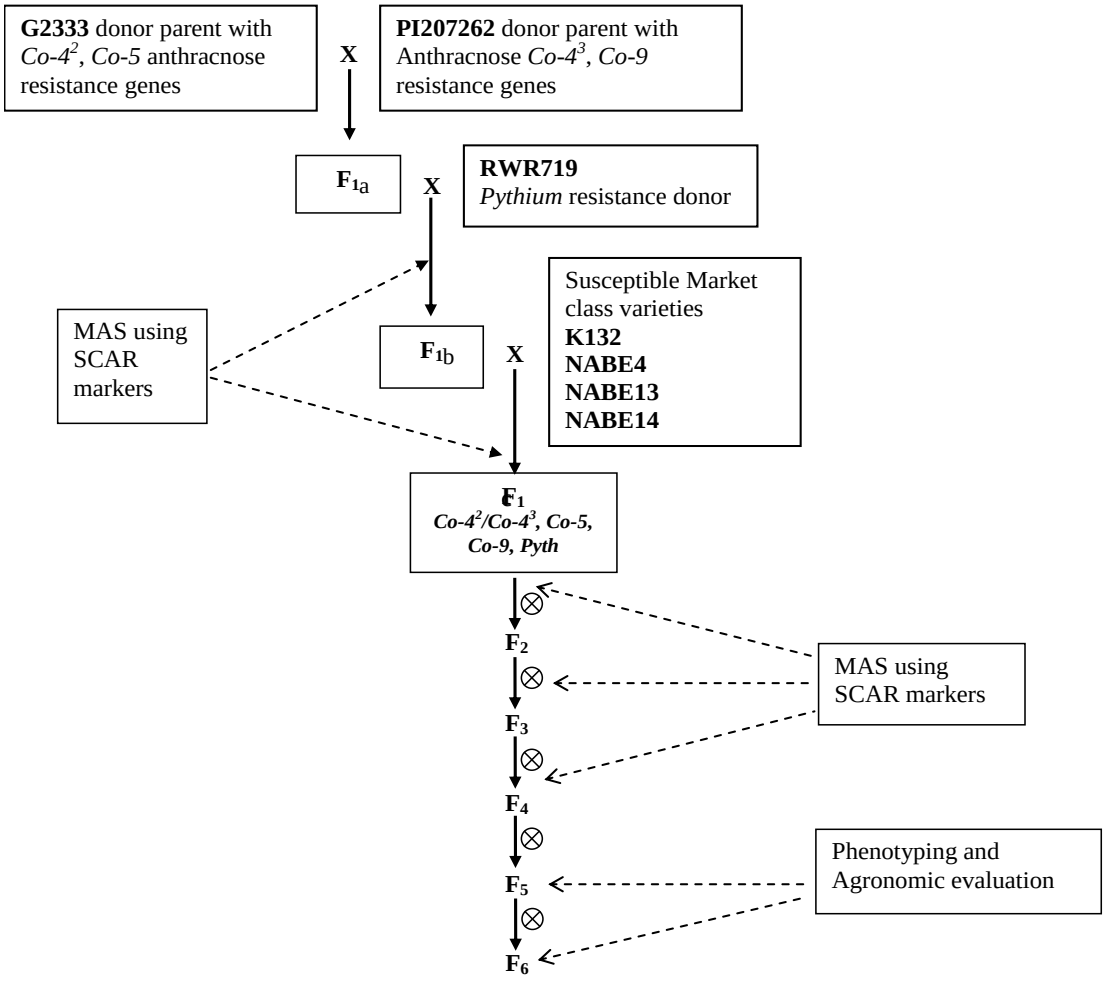

Figure 1. Breeding strategy used to develop common bean populations pyramided with anthracnose resistance genes.

2004) was used to develop populations. In this breeding scheme, only one cross was made at each generation beginning with two founding parents and followed by an intermediate genotype and one founding parent. The root genotype (Servin et al., 2004) combining all the desired resistance genes in heterozygous state was obtained and fixation of these genes was achieved through self-pollination.

Selection scheme

Marker-assisted selection (MAS) was used to select individuals with the desired single and pyramided genes during the pedigree and fixation steps of the breeding scheme.

Pyramiding scheme

The donor parents G2333 and PI207262 for anthracnose resistance were crossed in a screen house to combine the four anthracnose resistance alleles in F1a plants (Figure 1). The F1a plants were crossed with RWR719 to produce F1b plants. The SCAR markers SAS13, SBB14, SAB3 and SB12 run on extracted DNA of 105 F1b plants to identify plants that possessed the target genes. 35 F1b plants showed the fragments associated with resistance. Six of these plants selected and crossed with the susceptible varieties K132, NABE4, NABE13 and NABE14. F1c seeds of the crosses were harvested and planted as individual plants under screen house conditions. Markers SBB14, SAB3, SB12 were run on DNA extracted from 46 F1c plants. Three plants possessing genes in heterozygous state became the root genotype.

Fixation steps of the pyramiding scheme

F2 seed from the root genotype was planted and DNA extracted from 69 plants. MAS was conducted to identify plants that inherited 0, 1, 2, 3 target resistance genes. Selfing continued up to F6 generation to ensure that the genes were fully fixed in homozygous state, but MAS was conducted up to F4 generation of the fixation scheme. The F6 advanced lines were classified into 10 groups according to the alleles inherited as indicated in Table 4.

Molecular markers used in MAS

The target genes, linked DNA markers used to tag the genes

Table 2. PCR-based markers used in marker assisted selection.

Marker	Locus/ gene tagged	Original marker*	Linkage group	Size (bp)/ orientation	Primer sequence	References
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$_{800}$	*Prr*	AA19	-	800, *Cis*	Forward: TTA GGC ATG TTA ATT CAC GTT GG Reverse: TGA GGC GTG TAA GGT CAG AG	Mahuku et al. (2007)

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

Table 3. Ingredients of a PCR master mix using PCR reagents and a Bioneer PCR premix.

PCR reagents	Reaction volume (µl)	Stock concentration	Final concentration
ddH_2O	10.6		
dNTPs	0.8	10 mM	0.1 mM
Primer 1	1.0	10 mM	0.1 µM
Primer 2	1.0	10 mM	0.1 µM
$MgCl_2$	1.2	50 mM	1.5 mM
PCR Buffer	2.0	10X	1X
Taq polymerase enzyme	0.4	5U/µl	0.2U
	PCR premix		
ddH_2O	17		
Primer 1	1.0	10mM	0.1 µM
Primer 2	1.0	10 mM	0.1 µM

amplified fragment sizes, orientation and primer sequences are presented in Table 2. Sequence characterized amplified regions (SCAR) markers were used to tag the resistance genes. The primers were obtained from the Department of Molecular and Cellular Biology, University of Cape Town, Randebosch, South Africa. Later batches in premix form were obtained from Bioneer Corporation, Munpyeong-dong, Daejeon, South Korea. A 25/100 base pairs (bps) mixed DNA molecular weight marker, specifically designed for determining the size of double strand DNA from 25 to 2,000 bps was used to estimate fragment sizes.

DNA extraction

Leaf samples were collected from 14-day old plants raised in a screen house. Genomic DNA was extracted using the Cetyltrimethylammoniumbromide (CTAB) method adapted from Doyle and Doyle (1987).

DNA amplification, gel electrophoresis and imaging

To prepare a polymerase chain reaction (PCR) master mix using PCR reagents, double distilled water (ddH2O), deoxy nucleoside triphosphates (dNTPs including dATP, dGTP, dCTP and dTTP), forward and reverse primers, MgCl2, PCR buffer and Taq polymerase enzyme were mixed based on the concentrations in Table 3. Nineteen microliters (19 µl) of the master mix were pipetted into individual PCR tubes and 1.0 µl of 40 ng plant DNA was added to each tube to make a total PCR reaction volume of 20 µl. When the PCR Bioneer Premix (Bioneer Inc, Korea) was used instead of the PCR reagents, the PCR master mix was prepared by

Figure 2. Bean leaf trifoliate placed on paper towel inside a transparent plastic container

adding ddH_2O to a 1.5ml Eppendorf tube, followed by the forward and reverse primers according to the working concentrations in Table 3. Amplification of DNA was carried out in a Bioneer Thermal cycler (Bioneer Inc, Korea) with an initial denaturation step at 95°C for 5 min and 35 cycles each of a denaturation step at 94°C for 20 s, an annealing step at 64°C (SBB14), 68°C (SAS13), 65^{o}C (SAB3), 65^{o}C (SB12), 64^{o}C (SH18) and 63°C (PYAA19800) for 40 s; an extension step at 72^{o}C for one (1) min followed by a final extension for 10 min at 72^{o}C. 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 min and stained with 0.5 µg/ml ethidium bromide for 10 min. Gel images were captured using the SynGene G: BOX gel documentation system (Syngene, Frederick, MD, USA).

Phenotypic screening

Seeds of F6 lines were sown in five liter plastic pots filled with top soil mixed with sand and sterilized manure in the ratio of 5:3:2 respectively. Diammonium Phosphate (DAP) fertilizer was applied prior to sowing and watering was done daily. Four *C. lindemuthianum* races 352, 713,767 and 2047 were cultured on Potato Dextrose Agar (PDA) media and incubated in darkness at 22 - 24°C for four days before sub-culturing onto modified Mathur's Agar media (500 g) (Champion et al., 1973). Inoculum was prepared by scrapping germinated conidia off the growth media into a jar with small amounts of distilled water to form a suspension. A hemocytometer was used to adjust the concentration to 1.2 x 10^6 conidia ml^{-1} (Inglis et al., 1988) and 0.1% Tween 20 was added as a surfactant. The detached leaf technique (Tu, 1986) was used to differentiate the bean families. Leaf trifoliates were detached 14 days after planting and immersed in the suspension containing *C. lindemuthianum* spores. The inoculated leaf trifoliates were placed in transparent plastic containers with moistened paper towels and covered with transparent covers to maintain humid conditions (Figure 2). The containers were placed on wooden shelves fitted with PhillipsR TLT 18-20W/75RS Fluorescents tubes that supplied approximately 50 µmoles m-2s-1 of light to enable prolonged physiological processes of the detached leaves up to 28 days. A 12 h day light and 12 h night regime, and room temperatures were maintained between 22 and 25°C, which is recommended for successful infection of *C. lindemuthianum* (Awale et al., 2007)

Disease symptoms were scored after a seven day incubation period using a modified 1 – 9 scale (Balardin et al., 1997) where; 1 = no symptoms (resistant), 2 – 3 = very small lesions mostly on primary leaves (resistant), 4 – 9 = numerous enlarged lesions or sunken cankers on the lower side of the leaves (susceptible). This experiment was designed in a Randomized Complete Block Design (RCBD) with three replicates.

Data analysis

Disease severity data were 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 that all gene group means are equal; Tukey Test statistic; HSD = q√MSE/nc. Where; q = value from studentized range table, MSE = Mean Square for Error from ANOVA table, nc = number of replicates per treatment. Standard error of pyramid group means (SEM) was computed using the formula; SEM = s/√n, where; s = sample standard deviation and n = sample size. Sample standard deviation (s) was computed using the formula; s = √1/N-1 ∑Ni=1(xi – x)2, where; x1 …., xN = the sample data set, x = mean value of the data set, N = size of sample data set.

Table 4. Grouping of the F_6 advanced lines based on number and type of genes inherited.

Group	Genes inherited	Number of genes inherited	Number of lines
Group 1	*Co-4²+Co-5+Co-9*	3	9
Group 2	*Co-4²+Co-5*	2	8
Group 3	*Co-4²+Co-9*	2	1
Group 4	*Co-4³+Co-9*	2	1
Group 5	*Co-5+Co9*	2	7
Group 6	*Co-5*	1	9
Group 7	*Co-4²*	1	11
Group 8	*Co-4³*	1	5
Group 9	*Co-9*	1	3
Group 10	No gene inherited	0	15
Total			**69**

RESULTS

F4:6 populations with pyramided and single resistance genes

Sixty nine F4:6 lines were obtained after the fixation steps (Table 5). Nine lines inherited three anthracnose resistance genes, 17 inherited two alleles, 28 inherited a single allele; while 12 did not inherit any of the target alleles. Twenty seven possessed *Co-4²* allele, eight possessed *Co-4³* allele, 33 possessed *Co-5* allele, while 21 possessed *Co-9* allele. Images of the amplified DNA fragments for the four dominant SCAR markers SAS13, SAB3, SB12 and one codominant marker SBB14 are presented on Figure 3.

Evaluation of families for resistance to anthracnose

Five lines that possessed alleles *Co-4²*, *Co-5* and *Co-9* were resistant to the four races. Three of the four lines possessing *Co-4²* and *Co-5* alleles were also resistant to the four races. Four of the five lines possessing allele *Co-4²* were resistant to the four races. Two families that possessed allele *Co-9* and the family that possessed *Co-4³+Co-9* pyramid showed resistance to only race 352. Six families that did not inherit any resistance alleles were susceptible to the four races. Three of four families possessing *Co-5* allele were resistant to the four races. Presence of the *Co-9* gene was always associated with symptoms except in the three-gene pyramid.

Effectiveness of single and pyramided genes in conferring resistance to diverse *C. lindemuthianum* races

Data from the analysis of variance for anthracnose disease severity are presented in Table 6. Gene-groups, races and interaction between gene-groups and races were all highly significant ($P < 0.01$) with gene-groups contributing highest total variation followed by races.

Severity scores of gene groups inoculated with diverse *C. lindemuthianum* races are presented in Table 7. Race 2047 had the highest overall mean score followed by races 767, 713 and 352. The mean scores for pyramid and single-gene groups respectively were not significantly different from each other (Lsd 0.05). Among the two-gene pyramid groups, the *Co-4³+Co-9* group had the highest overall mean severity score across the four races, followed by *Co-5+Co-9* and *Co-4²+Co-9*, both of which succumbed to race 2047. The pyramid group *Co-4²+Co-5* had the lowest severity score and did not succumb to any race.

The mean severity score for the three-gene pyramid *Co-4²+ Co-5+Co-9* was significantly lower than the two-gene pyramid groups. The mean score for *Co-4²+Co-5* pyramid group was significantly lower than scores for the *Co-5+Co-9* pyramid group, but was not significantly different from the *Co-4²+Co-9* pyramid group. The three-gene pyramid *Co-4³+Co-5+Co-9* was not developed for comparison. Overall severity scores of single-gene groups were significantly different from each other and from the no-gene group. The *Co-4²* gene group had the lowest severity score followed by *Co-5*, *Co-4³* and *Co-9* gene groups. The mean severity score of *Co-9* gene-group was significantly higher than mean scores of all the single-gene groups and was the only group with severity score falling in the susceptible range.

The mean score of the *Co-4²* single-gene group was not significantly different from the mean scores of the *Co-4²+Co-5+Co-9* and *Co-4²+Co-5* pyramid groups, but was significantly lower than the mean scores of the *Co-4²+Co-9*, *Co-4³+Co-5* and *Co-5+Co-9* pyramid groups. The mean score of the *Co-5* single-gene group was not significantly different from mean scores of the *Co-4²+Co-5*, *Co-4²+Co-9* and *Co-5+Co-9* pyramid groups, but was significantly higher than the score for the three-gene pyramid *Co-4²+Co-5+Co-9*. The mean score of the *Co- 4³* single-gene group was significantly less than the Co-

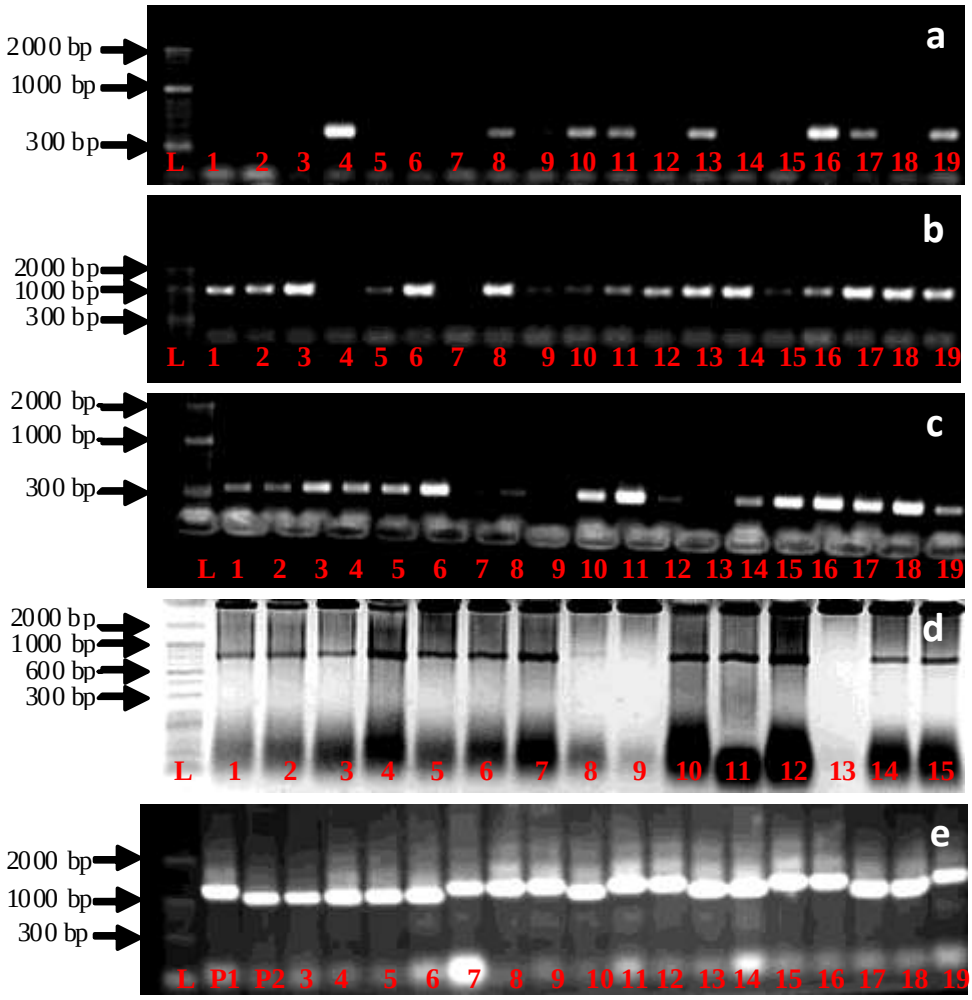

Figure 3. Gel photos showing banding patterns of DNA fragments amplified with the different SCAR markers. a = DNA fragment at 400bps amplified with *SAB3* primer linked to *Co-5* gene; b = DNA fragment at 950bps amplified with *SAS13* primer linked to *Co-4* locus; c = DNA fragment at 350bps amplified with *SB12* primer linked to *Co-9* gene; d = DNA fragment at 800 bp amplified with *KL1KR1* primer of the $PYAA19_{800}$ marker, e = Two DNA fragments at 1150bps for upper fragment and 1050 bps for lower fragment amplified with *SBB14* codominant primer linked to allele $Co\text{-}4^2$. Lanes: *L* 2000 bps molecular weight marker ladder; *1 – 19* segregating lines; *P1* Donor parent; *P2* Susceptible parent.

43+Co-9 pyramid group. The mean score of the Co-9 gene group was significantly higher than scores for all the pyramid gene groups.

DISCUSSION

Marker analysis and polymorphism during gene pyramiding

SAB3 was reported by Campa et al. (2005) to be the most distant marker from the gene of interest at 14.4cM, followed by SBB14 linked to the $Co\text{-}4^2$ allele at 5.89cM (Awale and Kelly, 2001). SB12 was reported to be linked to the *Co-9* gene at a distance of 2.9cM (Mendez de Vigo et al., 2002) while SAS13 was reported to be the most tightly linked to the *Co-4* locus at 0.01cM (Young et al., 1998). Chances of recombination leading to false positives were highest with SAB3 and least with SAS13.

Effectiveness of single and pyramided genes in conferring broad resistance to bean anthracnose

The significance of 'Gene-group x Race' interaction indicates that the effect of *C. lindemuthianum* races on disease severity highly depended on number and combination of resistance genes in the bean cultivars, which is in agreement with Davide and Souza (2009).

Table 5. Sixty nine $F_{4:6}$ families with their profile of inherited resistance genes.

Pedigree[a]	Family codes	Markers and Genes screened				
		SAS13	***SBB14***	***SAB3***	***SB12***	***PYAA19$_{800}$***
		Co-4^2/ Co-4^3	***Co-4^2***	***Co-5***	***Co-9***	***Prr***
12x8xRWR719xK132	44.5.7.1.1.1.1	-	-	-	+	-
12x8xRWR719xK132	44.5.7.1.1.1.4	-	-	-	+	-
12x8xRWR719xK132	44.5.7.1.1.1.7	-	-	-	+	-
12x8xRWR719xK132	16.1.2.1.6.3.1	-	-	-	-	-
12x8xRWR719xK132	16.1.2.1.6.3.2	-	-	-	-	-
12x8xRWR719xK132	16.1.2.1.6.3.3	-	-	-	-	-
12x8xRWR719xK132	16.1.2.1.6.3.5	-	-	-	-	-
12x8xRWR719xK132	16.1.2.3.7.1.1	-	-	-	-	+
12x8xRWR719xK132	16.1.2.3.7.1.3	-	-	-	-	+
12x8xRWR719xK132	16.1.2.3.7.1.4	-	-	-	-	+
12x8xRWR719xK132	16.6.1.6.25.1.2	+	+	-	-	-
12x8xRWR719xK132	44.5.2.1.26.1.4	+	+	+	-	-
12x8xRWR719xK132	44.5.2.3.28.1.1	+	+	+	+	-
12x8xRWR719xK132	44.5.2.3.28.1.2	+	+	+	+	-
12x8xRWR719xK132	44.5.2.3.28.4.3	+	+	+	+	-
12x8xRWR719xK132	44.5.2.3.28.4.5	+	+	+	+	-
12x8xRWR719xK132	44.5.2.3.28.4.6	+	+	+	+	-
12x8xRWR719xK132	44.5.2.3.28.4.8	+	+	+	+	-
12x8xRWR719xNABE4	44.7.9.8.102.1.1	+	+	+	+	-
12x8xRWR719xNABE4	44.7.5.8.112.1.2	-	-	-	-	-
12x8xRWR719xNABE4	44.7.5.8.112.1.3	-	-	-	-	-
12x8xRWR719xNABE4	44.7.5.8.112.1.4	-	-	-	-	-
12x8xRWR719xNABE4	44.7.5.8.112.2.1	-	-	-	-	-
12x8xRWR719xNABE4	89.5.2.7.117.1.1	+	+	-	+	-
12x8xRWR719xNABE4	89.5.2.10.118.15.2	+	-	-	+	-
12x8xRWR719xNABE4	44.7.2.2.76.1.1	+	+	+	+	+
12x8xRWR719xNABE4	44.7.2.2.76.4.1	+	+	+	+	+
12x8xRWR719xNABE4	89.5.1.1.81.1.1	+	+	-	-	+
12x8xRWR719xNABE4	89.5.1.1.81.2.1	+	+	-	-	+
12x8xRWR719xNABE4	44.7.8.2.91.10.1	+	+	-	-	-
12x8xRWR719xNABE4	44.7.8.2.91.10.3	+	+	-	-	-
12x8xRWR719xNABE4	44.7.8.2.91.10.4	+	+	-	-	-
12x8xRWR719xNABE4	44.7.8.2.91.11.2	+	+	-	-	-
12x8xRWR719xNABE4	44.7.8.2.91.11.3	+	+	-	-	-
12x8xRWR719xNABE4	44.7.9.5.92.2.3.4.6	+	+	-	-	-
12x8xRWR719xNABE4	44.7.8.3.92.3.2	+	+	-	-	-
12x8xRWR719xNABE4	44.7.8.3.92.3.5	+	+	-	-	-
12x8xRWR719xNABE13	44.1.6.7.130.1.1	-	-	-	-	-
12x8xRWR719xNABE13	44.1.6.7.130.3.2	-	-	-	-	-
12x8xRWR719xNABE13	16.1.3.8.136.1.4	+	-	-	-	+
12x8xRWR719xNABE13	16.1.3.8.136.2.5	+	-	-	-	+
12x8xRWR719xNABE13	16.1.3.8.136.2.7	+	-	-	-	+
12x8xRWR719xNABE13	16.1.3.8.136.2.8	+	-	-	-	+
12x8xRWR719xNABE13	16.1.3.8.136.2.9	+	-	-	-	+
12x8xRWR719xNABE13	44.1.4.3.141.1.2	-	-	+	+	+
12x8xRWR719xNABE13	44.1.4.3.141.1.3	-	-	+	+	+
12x8xRWR719xNABE13	44.1.4.3.141.1.4	-	-	+	+	+
12x8xRWR719xNABE13	44.1.4.3.141.2.1	-	-	+	+	+
12x8xRWR719xNABE13	44.1.4.5.142.3.6	-	-	+	-	+

Table 5. Contd.

12x8xRWR719xNABE13	44.1.4.5.142.4.2	-	-	+	-	+
12x8xRWR719xNABE13	44.1.4.5.142.4.4	-	-	+	-	+
12x8xRWR719xNABE13	44.1.4.5.142.4.6	-	-	+	-	+
12x8xRWR719xNABE13	44.1.4.5.142.4.7	-	-	+	-	+
12x8xRWR719xNABE13	44.1.4.7.143.1.4	-	-	+	-	-
12x8xRWR719xNABE13	44.1.4.7.143.1.8	-	-	+	-	-
12x8xRWR719xNABE14	16.3.3.1.151.1.1	-	-	+	-	+
12x8xRWR719xNABE14	16.3.3.1.151.1.2	-	-	+	-	+
12x8xRWR719xNABE14	16.3.3.2.152.1.2.1	-	-	-	-	-
12x8xRWR719xNABE14	16.3.3.2.152.1.2.2	-	-	-	-	-
12x8xRWR719xNABE14	16.3.3.8.157.3.3	+	+	+	-	+
12x8xRWR719xNABE14	16.3.3.9.158.1.2	+	-	+	-	+
12x8xRWR719xNABE14	16.3.3.9.158.1.3	+	-	+	-	+
12x8xRWR719xNABE14	16.3.3.10.159.6.2	+	+	+	-	+
12x8xRWR719xNABE14	16.3.3.11.160.2.1	+	+	+	-	+
12x8xRWR719xNABE14	16.3.3.11.160.2.2	+	+	+	-	+
12x8xRWR719xNABE14	16.3.3.11.160.2.3	+	+	+	-	+
12x8xRWR719xNABE14	16.3.3.11.161.1	-	-	+	+	+
12x8xRWR719xNABE14	16.3.3.11.161.3	-	-	+	+	+
12x8xRWR719xNABE14	16.3.3.11.161.4	-	-	+	+	+

[a] 12 = Donor parent G2333; 8 = Donor parent PI 207262; + = presence of band on gel; - = absence of band on gel.

Table 6. Analysis of variance for severity of four races on different Gene-groups.

Source of variation	d.f.	S.S	M.S	%Contribution	F pr.
Rep	2	0.175	0.087	0.15	
Gene-groups	9	276.121	30.680*	52.55	<.001
Race	3	76.547	25.516*	43.70	<.001
Gene-group x Race	27	53.581	1.985*	3.40	<.001
Residual	78	9.060	0.116	0.20	

Races 2047 and 767 were the most aggressive and resulted in the highest disease scores on cultivars possessing the *Co-4*^{3}*+Co-9* and moderate susceptibility on those possessing *Co-4*^{2}*+Co-9* and *Co-5+Co-9* pyramids and *Co-4*^{3} and *Co-9* single genes. This implies that these gene pyramids and single genes do not effectively confer resistance to the two races. Darben et al. (2017) reported race 2047 to be one of the most aggressive *C. lindemuthianum* races in Brazil that could overcome anthracnose resistance conferred by seven resistance genes *Co-1*, *Co-2*, *Co-3*, *Co-4*, *Co-5*, *Co-6* and *Co-1*^{1}; and five alleles namely *Co-1*^{2}, *Co-1*^{3}, *Co-1*^{5}, *Co-3*^{3} and *Co-4*^{3}. Nkalubo (2006) found race 767 to be the most widespread out of eight identified studies in Uganda

Race 713 caused moderate symptoms on cultivars possessing *Co-4*^{3}*+Co-9* pyramid and Co-9 single gene implying that the pyramid combination and the single gene *Co-9* were not effective. Race 352 did not cause symptoms on any cultivar apart from cultivars with no resistance gene suggesting that all the single genes and the different pyramid combinations were effective against it (Figure 4).

The Co-9 gene was the least effective against the pathogen and was also associated with increased severity when combined with other genes implying it was antagonistic when combined with other genes and therefore should be avoided in gene pyramiding programs. Its presence in the three gene pyramid *Co-4*^{2}*+Co-5+Co-9*, however, was not antagonistic probably because of the combined effectiveness of the *Co-4*^{2} and *Co-5* genes. Kelly and Vallejo (2004) reported the *Co-9* gene to possess a very specific breeding value against Andean races of *C. lindemuthianum*. Alzate-Marin et al. (2003) reported the *Co-9* gene to be susceptible to even the weak Mesoamerican races 65 and 69. The cultivar PI207262 possessing the *Co-9* gene was reported to be overcome by many anthracnose races (Kelly, 2004)

Table 7. Mean severity scores of cultivars in the different gene groups inoculated with diverse *C. lindemuthianum* races.

Group	Pyramid groups	Races				Overall group mean
		352	713	767	2047	
1	*Co-4²+Co-5+Co-9*	1.0±0.00	1.1±0.07	1.1±0.07	1.1±0.07	**1.1±0.03**
2	*Co-4²+Co-5*	1.1±0.13	2.2±0.12	1.9±0.18	3.1±0.24	**2.1±0.41**
3	*Co-4²+Co-9*	1.0±0.00	2.0±0.00	2.0±0.00	4.3±0.33	**2.3±0.69**
4	*Co-4³+Co-9*	1.5±0.29	5.8±0.17	6.3±0.44	7.0±0.29	**5.2±1.24**
5	*Co-5+Co-9*	1.4±0.07	2.3±0.07	3.3±0.29	4.3±0.29	**2.8±0.63**
Mean		**1.2±0.10**	**2.7±0.81**	**2.9±0.92**	**4.00±0.96**	**2.7±0.68**
	Singe-gene groups					
6	*Co-5*	2.0±0.00	2.5±0.23	2.8±0.07	3.0±0.27	**2.6±0.22**
7	*Co-4²*	1.0±0.00	1.4±0.12	1.2±0.12	2.4±0.12	**1.5±0.31**
8	*Co-4³*	1.9±0.10	3.3±0.49	4.3±0.38	4.2±0.11	**3.4±0.56**
9	*Co-9*	2.3±0.17	5.2±0.17	5.5±0.00	4.8±0.17	**4.5±0.73**
Mean		**1.8±0.28**	**3.1±0.80**	**3.5±0.93**	**3.6±0.55**	**3.0±0.63**
10	**No-gene group**	5.4±0.07	5.9±0.07	6.3±0.12	6.3±0.09	**5.9±0.21**
Overall mean for races		**1.9±0.42**	**3.2±0.57**	**3.5±0.64**	**4.1±0.56**	**3.1±0.50**

Lsd (0.05) = 0.55, S.e.d = 0.26, C.V = 7.8%.

implying the ineffectiveness of the *Co-9* gene as a resistance source. This explains the poor resistance spectrum observed with the *Co-9* gene in this study. Kelly and Vallejo (2004) recommended its use only to diversify resistance in gene pyramids because of its independence and potential value in controlling Andean races.

In this study, the single gene *Co-4²* was found to be as effective as the best pyramid combinations *Co-4²+Co-5* and *Co-4²+Co-5+Co-9* and the single gene *Co-5* was as effective as pyramid combinations *Co-4²+Co-5* and *Co-4²+Co-9*. This implies that the two single genes possess factors that promote broad-spectrum resistance and that these factors are higher in *Co-4²* gene. These two genes would confer effective and broad-spectrum resistance in single deployment against a diverse *C. lindemuthianum* population. The *Co-5* gene was reported to be among the most effective genes in Central America and Mexico but with limited use by bean breeders (Kelly and Vallejo, 2004). It was reported to possess a wide resistance spectrum conferring resistance to 31 races (Balardin et al., 1997). The *Co-4²* gene was reported to exhibit the most broad-spectrum resistance against *C. lindemuthianum* in common beans (Young and Kelly, 1996; Balardin and Kelly, 1998; Awale and Kelly, 2001) and controls of up to 97% of all currently identified races of *C. lindemuthianum* (Melloto et al., 2000). Young et al. (1998) reported the *Co-4* locus to be a complex gene family with three anthracnose resistance alleles residing at the locus namely *Co-4²* in cultivars G2333 and SEL 1308, *Co-4* in cultivar TO (Young et al., 1998) and *Co-4³* in cultivar PI207262 (Alzarte-Marin et al., 2007). Melotto and Kelly (2001) fine mapped the complex *Co-4* locus to reveal an open reading frame designated as *COK-4* that encodes for protein kinase and was strongly associated with anthracnose resistance. The predicted amino-acid sequence of *COK-4* had a high degree of similarity with expressed sequences generated by resistance genes in other crops such as Pto in *Lycopersicon pimpinellifolium* and *L. esculentum*; and Xa21 in O. Sativa. Further genetic analysis by Melotto and Kelly (2001) revealed that other genes may be tightly clustered with *Co-4²*, although segregation data indicated a single gene. These tightly clustered genes could be responsible for the broad spectrum resistance associated with the *Co-4* locus.

In this study, the *Co-4³* allele showed a mildly susceptible reaction to races 767 and 2047, however, it is still a highly beneficial allele to specific races and has the potential to add value if combined with other compatible single genes in pyramid. The *Co-4³* allele was, however, reported to possess a narrower anthracnose resistance spectrum than the *Co-4* allele in cultivar TO and the Co-42 allele in cultivar G2333 (Alzate-Marin et al., 2007).

The *Co-4²+Co-5+Co-9* and *Co-4²+Co-5* pyramid had the lowest severity scores and did not succumb to any of the races implying that these pyramids are highly effective in conferring broad-spectrum resistance to *C. lindemuthianum* in Uganda. Kelly (2004) proposed combination of *Co-4²*, *Co-5* and *Co-6* genes in North America and *Co-1²* and *Co-4²* gene pair for Central America. The *Co-1²* gene confers resistance against Andean races while *Co-4²* confers resistance against Mesoamerican races. Resistance gene pyramids whichincorporate at least two unique modes of action are reported to delay the evolution of virulent pathotypes (Roush, 1998). Results further revealed that gene pyramids, depending on number and the specific single

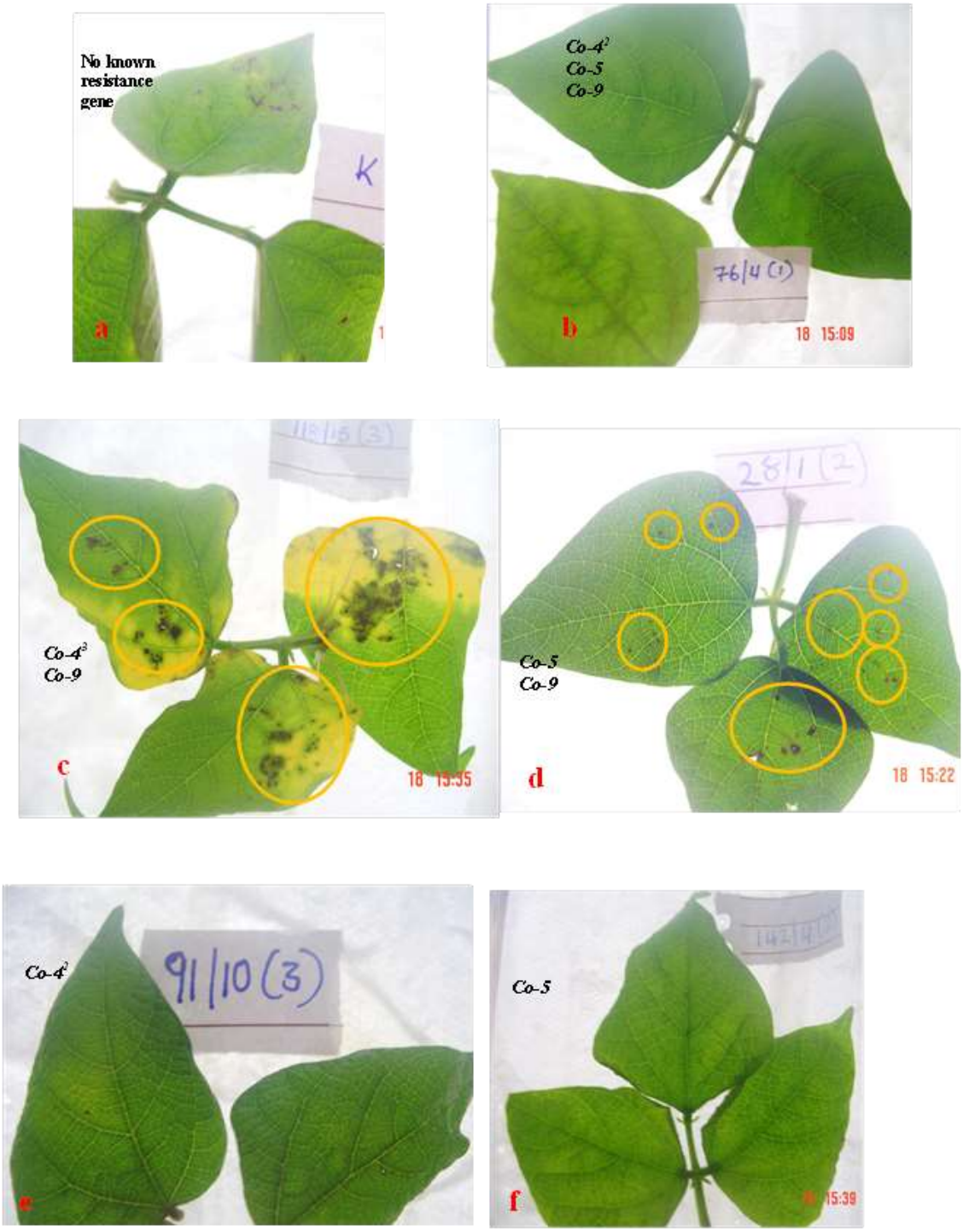

Figure 4. Symptom expression on Leaf trifoliates of; **a** = susceptible cultivar K132 with moderate symptoms; **b** = cultivar with the three-gene pyramid *Co-4²+Co-5+Co-9* with no symptoms; **c** = cultivar with the two-gene pyramid *Co-4³+Co-9* with severe symptoms; **d** = cultivar with the two-gene pyramid *Co-5+Co-9* showing mild symptoms; **e** = cultivar with single gene *C-o4²* with no symptoms; and **f** = cultivar with single gene *Co-5* with no symptoms

genes combined were not consistent in conferring broad-spectrum resistance. It was observed that a higher number of genes in a pyramid significantly reduced the severity of symptoms on the host implying that gene pyramiding has the potential of increasing the potency of resistance. According to Wheeler and Diachun (1983);

Schafer and Roelfs (1985); and Kolmer et al. (1991), pyramids with higher resistance gene numbers are most likely to remain effective over long periods of time before breaking down. Their argument is based on the probability hypothesis (Mundt, 1991) which states that "cultivars possessing multiple race-specific resistance genes (pyramided genes) owe their durable resistance to a low probability of the pathogen independently mutating to virulence at multiple avirulent loci corresponding to the host resistance genes". According to this hypothesis the pyramids *Co-4*^{2}*+Co-5+Co-9* and *Co-4*^{2}*+Co-5*, which were the most effective in conferring resistance, are likely to remain effective over a longer period of time. However, the mechanisms by which gene pyramids increase durability is still unknown and there is no strong evidence for gene number as the dominant mechanism for the durability of pyramids (Mundt, 1991).

Conclusions

Resistance gene pyramiding was effective in conferring broad-spectrum resistance in some cases and was ineffective in other cases. Effectiveness of pyramided resistance genes heavily depended on the fitness of individual resistance genes combined. It is therefore crucial that plant breeders identify and use favorable resistance gene combinations as opposed to mere accumulation of resistance genes in a cultivar in gene pyramiding programs. The gene combinations Co-4^2+Co-5+Co-9 and Co-4^2+Co-5 and the single genes Co-4^2 and Co-5 were the most effective resistance options against *C. lindemuthianum* and are recommended for control of the bean anthracnose disease in Uganda through development of resistant varieties. The races 2047 and 767 are recommended for use in screening for resistance to bean anthracnose disease. Further investigations would be beneficial in identifying new effective gene combinations for against *C. lindemuthianum* based on other available resistance genes.

CONFLICT OF INTERESTS

There are no competing interests.

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