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PYRAMIDING GENES FOR RESISTANCE TO MAJOR DISEASES INTO A MARKET-CLASS COMMON BEAN VARIETY IN KENYA

PYRAMIDAGE DE GÈNES DE RÉSISTANCE AUX PRINCIPALES MALADIES DANS UNE VARIÉTÉ COMMERCIALE DE HARICOT COMMUN AU KENYA

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.) production in sub-Saharan Africa (SSA) is persistently threated by three diseases; namely, Anthracnose (*Colletotrichum lindemuthianum*), bean common mosaic and necrosis viruses (BCMV/BCMNV) and common bacterial blight (*Xanthomonas axonopodis* pv. Phaseoli). Under favourable conditions, yield losses of between 50 -100 percent have been reported in susceptible cultivars. The objective of this study was to pyramid resistance genes against major common beans pathogens into a susceptible commercial cultivar. SCAR markers, SAS 13 (anthracnose), SU91 (CBB) and SG-6 (BCMV/BCMNV) were used to track respective resistance genes in the breeding populations and phenotypic screening *via* inoculations to target pathogens was done to confirm resistance among F_2 progenies and pryamids. Segregation ratios in F_2 populations followed expected Mendelian patterns: 3:1 (anthracnose and CBB) and 1:3 (BCMV/BCMNV). Chi-square tests validated these ratios, and disease severity data were subjected to analysis of variance (SAS software). Marker segregation in F_2 populatios for resistance to CBB and anthracnose showed a good fit to the expected ratio for a single dominant gene (3R: 1S); while for BCMV/BCMNV followed that of a reccesive gene (1R:3S). Twelve plants harbouring three-gene combinations were obtained, with nine exhibiting robust resistance upon artificial inoculation to the target pathogens. These results demonstrate successful introgression of anthracnose, BCMV/BCMNV and CBB resistance into a previously susceptible background, confirming the efficacy of marker-assisted selection in breeding common bean varieties with durable resistance.

Key Words: *Colletotrichum lindemuthianum,Phaseolus vulgaris, Xanthomonas axonopodis*

RÉSUMÉ

La production de haricot commun (*Phaseolus vulgaris* L.) en Afrique subsaharienne (ASS) est persistante menacée par trois maladies : l'anthracnose (*Colletotrichum lindemuthianum*), les virus de la mosaïque commune et de la nécrose du haricot (BCMV/BCMNV) et la bactériose commune (*Xanthomonas axonopodis* pv. *phaseoli*). Sous des conditions favorables, des pertes de rendement comprises entre 50 et 100 % ont été signalées chez les cultivars sensibles. L'objectif de cette étude était de pyramider les gènes de résistance contre ces principaux agents pathogènes du haricot commun dans un cultivar commercial sensible. Les marqueurs SCAR SAS 13 (anthracnose), SU91 (bactériose commune) et SG-6 (BCMV/BCMNV) ont été utilisés pour suivre les gènes de résistance respectifs dans les populations de sélection. Un criblage phénotypique par inoculations des pathogènes cibles a été réalisé pour confirmer la résistance parmi les descendances F_2 et les lignées pyramidées. Les ratios de ségrégation dans les populations F_2 ont suivi les modèles mendéliens attendus : 3:1 (anthracnose et bactériose commune) et 1:3 (BCMV/BCMNV). Les tests du Chi-carré ont validé ces ratios, et les données de sévérité de la maladie ont été soumises à une analyse de variance (logiciel SAS). La ségrégation des marqueurs dans les populations F_2 pour la résistance à la bactériose commune et à l'anthracnose a montré un bon ajustement avec le ratio attendu pour un gène dominant unique (3R:1S), tandis que celle pour le BCMV/BCMNV a suivi celle d'un gène récessif (1R:3S). Douze plantes abritant les combinaisons des trois gènes ont été obtenues, dont neuf ont manifesté une résistance robuste lors de l'inoculation artificielle avec les agents pathogènes cibles. Ces résultats démontrent une introgression réussie de la résistance à l'anthracnose, au BCMV/BCMNV et à la bactériose commune dans un fond génétique auparavant sensible, confirmant l'efficacité de la sélection assistée par marqueurs pour la création de variétés de haricot commun dotées d'une résistance durable.

Mots Clés : *Colletotrichum lindemuthianum*, *Phaseolus vulgaris*, *Xanthomonas axonopodis*

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is a major stapple and income earning crop in sub-Saharan Africa (SSA); whose production is purverted by three major diseases; namely, anthracnose (*Colletotrichum lindemuthianum*), bean common mosaic virus (BCMV), bean common mosaic necrosis virus (BCMNV) and common bacterial blight (*Xanthomonas campestris* pv. *phaseoli*) (Mutuku *et al.,* 2018; Ochichi *et al.,* 2018; Mangeni *et al.,* 2019). These diseases appear in complexes of two or more on farmer's fields, causing up to complete crop loss (Rezene *et al.,* 2019; Degu *et al.,* 2020). Control of these diseases is difficult because they are seed transmitted with high survival periods of up to 10 years in seed, crop debris and weeds. Moreover, their control is currently invariably by chemical administration, which is not only costlyy; but also increasingly ineffective on induced resistance (Sofkova *et al.,* 2010). Thus, the most effective and environmentally safe control measure currently advocated is the use of host plant resistance, especially in low input cropping systems (Mekonnen *et al.,* 2017). Presently, there are no suitable varieties with multiple disease resistance (MDR) that are adapted to environments prone to these diseases in the region. Most bean breeding programmes opt for conventional approaches that are not only time and resource-consuming, but also limits the number of genes to be stacked into elite cultivars (Malav *et al.,* 2016). Breeding for one constraint at a time is expensive and time-consuming; thus justifying the need for a multiple constraint breeding (Singh, 1994).

The rationale behind gene stacking, also known as gene pyramiding, is that combining many resistance genes in the same background would offer broad resistance, and reduce yield losses especially in low input production systems (Kimani and Mwang'ombe, 2007). Gene pyramiding using conventional

approaches may not be effective due to difficulties in selecting genotypes harbouring different resistance genes (Souza *et al.,* 2014), and the need for extensive phenotyping requiring large amounts of time, space and investiment (Kidane, 2021). However, molecular markers accelerate the recovery of the recurrent parent's genome, and enhance efficiency of developing bean cultivars with broad resistance to several pathogens (Souza *et al.,* 2012). The availability of molecular markers linked to anthracnose, BCMV/ BCMNV and CBB resistance provides an opportunity to pyramid these resistance genes into common backgrounds.

The objective of this study was to pyramid resistance genes against anthracnose BCMV/ BCMNV and common bacterial blight into a popular but succeptible common bean cultivar KKCAL 194 using marker-assisted selection.

MATERIALS AND METHODS

Plant materials. Resistance donors were obtained from the International Centre for Tropical Agriculture (CIAT), Uganda and comprised of VAX3 (P1) with SU-91 *QTL* that confers broad resistance against CBB (Singh *et al.* 2001), G2333 (P2) with *Co-4²* gene with resistance against many anthracnose races (Osorno and McClean, 2014) and MCM2001 (P3) with *bc-3* gene that confers resistance to both BCMV and BCMNV strains (Hegey, 2013; Pasev *et al.,* 2013). The recurrent parent was KKCAL 194 (P4), a locally produced commercial variety bred for *Pythium* root rot resistance by the Kenya Agricultural and Livestock Research Organisation, Kakamega (KALRO, 2015).

Study area. The study was carried out at KALRO Kakamega, located at 00°, 17' N latitude and 34°, 17' E longitude with an elevation of 1585 m above sea level (Jaetzold *et al*., 2009). Mean annual rainfall is 1200 mm and average temperatures are 21 to 27°C (KALRO, 2017). The site is a major common bean production area (KALRO, 2017), characterised by high disease pressure, caused by warm and humid conditions that favour development of many common bean pathogens (Kimno *et al.,* 2016).

Generation of breeding populations. Parallel marker assisted backcrossing was used to combine *SU91*, *Co-4²* and *bc-3* resistant genes into a common background (Fig. 1). The recurrent parent (P4) was planted in a screenhouse, alongside donors (P1, P2 and P3) in 3 kg plastic pots filled at ¾ with sterilised soil, two seeds planted in each pot and watered daily. Recipient and donor parents were planted in a staggered manner at four-day intervals.

Six replicate pots were maintained per parental line, to ensure adequate plants for crossing. Artificial cross pollination was performed following the procedures outlined by Bliss (1980).

The minimum population size per generation was derived using Equation 1:

$N = \log_e (1 - P) / \log_e (1- f)$. (Sedcole, 1977).

Where:

N = the minimum number of plants to evaluate;
P = the desired probability (95%); and
f = the event of an individual plant having all desired alleles.

Selection scheme. Marker-assisted selection (MAS) was used to select individuals with desired genes, during the pedigree and fixation steps of the breeding scheme. The SCAR markers used to tag genes of interest are described in Table 1.

DNA extraction and PCR amplicafication. Before crossing, young leaves from two weeks old plants were taken for DNA extraction using procedures of Mahuku (2004). Extracted DNA was quantified on 1% agarose gel using Lamda DNA and stored at 4 °C. The PCR was performed using Taq Ready- To- Go PCR beads containing stabilisers, bovine serum

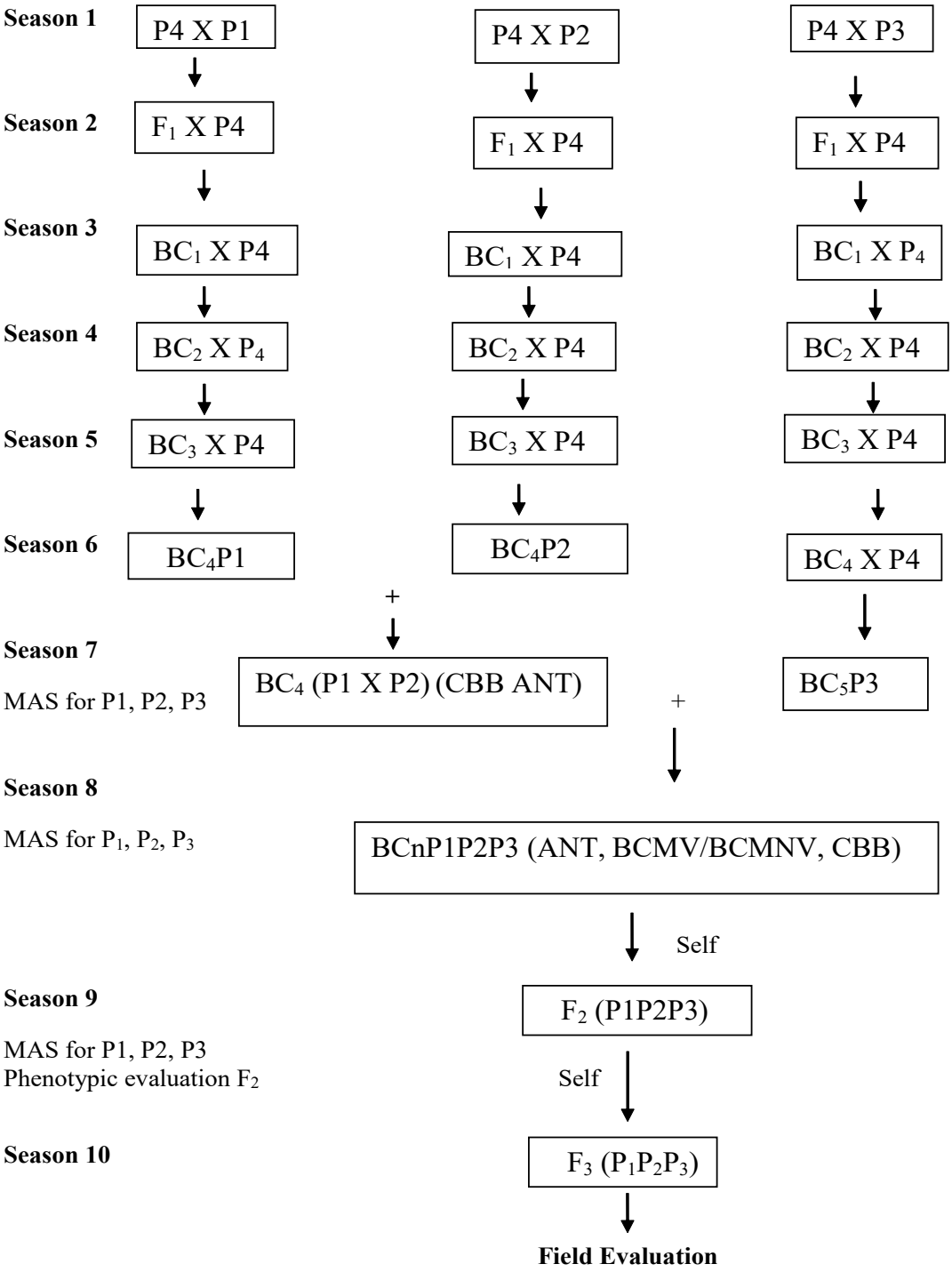

Figure 1. Parallel backcrossing scheme used to pyramid anthracnose, BCMV/BCMNV and CBB resistance into KKCAL 194.

TABLE 1. SCAR Markers used for marker assisted selection in this study

Marker	Locus	Sequence (5' - 3')	Size (bp)	Reference
SU91 (CBB)	SU91	CCACATCGGTTAACATGAGT CCACATCGGTGTCAACGTGA	700*cis*	Singh *et al*. 2001
SAS13 (Anthracnose)	*Co-4*^{2}	CACGGACCGAATAAGCCAC CAACACAC GGACCGAGGAT ACAGTGAAAG	950*cis*	Kelly *et al.* 2003
SG-6 (BCMV/BCMNV)	*bc-3*	GTGCCTAACCGAGTTATCTA GAGTGTGCCTAACCCTCCT	596*cis*	Mukeshimana *et al*. 2005

albumin, 200dNTPs, 2.5 units of pure taq DNA polymerase, 10 mM TrisHCl, 50 mM KCl, 1.5 mM $MgCl_2$ and primers. In twenty-two microliters of molecular biology water, 1µg ml^{-1} of forward and 1µg ml^{-1} of reverse primer were added in each PCR tube containing beads. Then 5 ng µl^{-1} of template DNA was added to make a total reaction volume of 20 µg ml^{-1}. Amplifications were performed in Applied Biosystems 2720 Thermal Cycler with initial DNA denaturation at 94 °C for 40 seconds; followed by denaturation at 94 °C, primer annealing at 58-65 °C (depending on primer) for 40 seconds; polymerisation by DNA polymerase at 72 °C for 120 seconds, of 34 cycles, a final extension at 72 ºC for 5 minutes then held at 4 °C. The PCR products were separated on 1.2% (w/v) agarose gels stained with ethidium bromide (5 µl 100 ml^{-1}) at 100 V in 1×TBE for 90 minutes. The gels were photographed using Benchtop UV transluminator (G110186 model).

Inoculation and phenotypic evaluation. Resistance in 382 $F_{2:3}$ progenies selected from MAS was validated through artificial inoculation, starting with BCMV/BCMNV; followed by CBB under screenhouse conditions. Anthracnose resistance was assessed using a detached leaf assay. Additionally, the twelve plants confirmed to carry all three target resistant genes identified from MAS, were challenged to respective pathogens to confirm pyramided resistance.

Inoculation of BCMV / BCMNV. The $F_{2:3}$ progenies were inoculated with BCMV and BCMNV inoculum prepared using the modified protocol of (Chilagane *et al.,* 2013). The inoculum was derived from symptomatic leaf tissue of systemically infected plants (Chilagane *et al.,* 2013). Young leaves (1 g) exhibiting BCMV/BCMNV symptoms, were homogenised in 5 ml of ice-cold 0.01 M phosphate buffer (pH 7.0), containing 0.1% (v/v) Tween 20. The homogenate was filtered through sterile cheesecloth, and the resulting supernatant was used as the inoculum.

At ten days after germination, the primary leaves were dusted with carborundum powder by gentle rubbing the inoculum onto the entire leaf surface. Inoculated plants were maintained under controlled conditions, and symptom development was monitored daily. Disease assessment commenced seven days post-inoculation.

Inoculation of CBB. Bacterial strains used for inoculation were collected from plants exhibiting typical CBB symptoms and cultured on semi-selective medium, following the protocol of Sheppard *et al.* (2007). The inoculum was suspended in distilled water and adjusted to a density of 1.7 ×10^8 CFU ml^{-1}

(Duncan *et al.,* 2012). At trifoliate stage, the plants were inoculated using a hand spray procedure (Viteri *et al.* 2014). Inoculated plants were maintained in the screen house at 25 ^{O}C. Common bacterial blight severity was assessed at 7, 14 and 21 days post inoculation, using the scale described by Lema *et al.* (2014).

Anthracnose inoculation process. Pods exhibiting anthracnose symptoms, were collected from infected plants for pathogen isolation. Isolates were cultured on potato dextrose agar (PDA), following the methodology described by Vallejo and Kelly (2009). Race identification was performed using a twelve cultivar differential set described by Van Schoonhoven and Pastor-Corrales (1987). Four isolates (38, 65, 73, and 485) were obtained, with isolate 485 selected for inoculation due to its high prevalence and virulence (Padder *et al.,* 2017). The selected isolate was grown on PDA and incubated at 22 °C in darkness to promote sporulation. Spores were harvested by scraping the culture into an aqueous solution containing 0.01% Tween 20. A spore suspension of 1.2 x 10v spores ml^{-1} was prepared for inoculation using the detached leaf method described by Rezene *et al.* (2019). Disease symptoms were assessed at 7, 10, and 14 days post-inoculation and scored according to the 1-9 CIAT scale (Pastor-Carlos *et al.,* 1998).

Data analysis. Disease severity data from the phenotypic evaluation, were analysed using analysis of variance (ANOVA) in SAS (Version 9.3; PROC GLM). For anthracnose and CBB, the progenies were classified as resistant (scores 1-3) or susceptible (scores 4-9); while scores of 0 (resistant) and 1 (susceptible) were adopted for BCMV/BCMNV.

For MAS, amplified fragments were resolved on agarose gels and compared against a 1000-bp DNA ladder. Chi-square (χ^2) tests were conducted to assess deviations of phenotypic and genotypic segregation ratios from expected Mendelian ratios in backcross and F_2 populations.

RESULTS

Introgression of CBB resistance. Multiple disease resistance genes to *bc-3* (BCMV/ BCMNV), (*Co-4^2*) anthracnose and *SU-91* (CBB) were successfully pyramided into the commercial common bean cultivar KKCAL 194. The observed segregation ratios for CBB resistance in the developed populations, did not deviate significantly ($P < 0.05$) from the expected Mendelian ratios (Table 2). The F_2 progenies exhibited a 3R:1S (resistant:susceptible) ratio; while backcross populations showed a 1R:1S ratio.

Introgression of anthracnose resistance. Segregation analysis revealed a 3:1 resistant-to-susceptible (R:S) ratio in F_2 populations; and a 1:1 ratio in backcross progenies exhibited a dominat inheritance pattern. Molecular marker analysis further supported this genetic model, with the SAS13 marker displaying a segregation pattern of 287 resistant (+) to 95 susceptible (-) plants in the F_2 population, fitting the expected 3:1 ratio ($\chi^2 = 0.34$, $P > 0.05$) (Table 3). Marker segregation and phenotypic screening of F_2 populations when inoculated with race 485 of *C. lindemuthianum* adhered to the predicted Mendelian ratios. Furthermore, genotyping with the SAS13 marker in the F_2 populations yielded a 287 resistant and 95 succeptible plants, which was not significantly different from the expected 3:1 ($\chi^2 = 0.34$, $P > 0.05$).

Introgression of bc-3 gene linked to BCMV/ BCMNV resistance. Introgression of *bc-3* gene that confers resistance to both BCMV and BCMNV virus strains into a previously susceptible genetic background, was successful using the SG-6 marker (Table 4). Segregation analysis confirmed that the phenotypic ratios in backcross and F_2 populations, as well as the genotypic ratio for SG-6 marker, fit the expected Mendelian ratios for a recessive allele. This was evidenced by a 1R:3S ratio observed in F_2 progenies and its corresponding marker data, that aligned

TABLE 2. Evaluation of parents, backcross and F_2 progenies for SU91Marker

Parent/cross/ marker	Generation	Observed plants		Expected ratio	Goodness of fit	
		R	S	R:S	X^2	P
VAX 3 (P_1)	P_1	20	0			
CAL 194 (P_4)	P_4	20	0			
$P_4 \times P_1$	F_1	11	9			
$F_1 \times P_4$	BC_1F_1	13	8	1:1	1.190	0.275
$BC_1P_1 \times P_4$	$BC2F_1$	14	12	1:1	0.150	0.695
$BC_2P_1 \times P_4$	BC_3F_1	18	11	1:1	1.690	0.194
MSU 91	F_2	276	106	3:1	1.540	0.215

R = resistant, S = susceptible, Chi-square P- values greater than 0.05 indicate observed values are not significantly different from the expected values

TABLE 3. Response of parents, backcross and F_2 progenies for SAS13 marker

Parent/cross/ marker	Generation	Observed plants		Expected ratio	Goodness of fit	
		R	S	R:S	X^2	P
G 2333 (P_2)	P_2	15	0			
CAL 194 (P_4)	P_4	0	15			
$P_4 \times P_2$	F_1	14	8		0.370	0.542
$P_1F_1 \times P_4$	BC_1F_1	13	10	1:1	0.390	0.532
$BC_1P_2 \times P_4$	BC_2F_1	14	9	1:1	1.090	0.297
$BC_2P_2 \times P_4$	BC_3F_1	12	8	1:1	0.800	0.371
SAS13	F_2	287	95	3:1	0.274	0.953

R = resistant, S = susceptible, Chi-square P- values greater than 0.05 indicate that the observed values are not significantly different from the expected value

perfectly with the expected Mendelian ratios for a single recessive allele (*bc-3*). Specifically, the F_2 populations exhibited a 1 resistant (R):3 susceptible (S) segregation ratio ($\chi^2 = 0.42$, P = 0.52); while backcross generations followed the predicted 1R:1S ratio ($\chi^2 = 0.18$, P = 0.67). In the subsequent F2 generation (n = 382), both phenotypic screening and marker analysis followed the recessive inheritance pattern, with observed ratios of 1R:3S (94 resistant:288 susceptible; $\chi^2 = 0.28$, P = 0.68) that matched the expectated Mendelian ratios (Table 4).

Marker assisted backcrossing for development of breeding populations. The SCAR markers SAS13 (anthracnose), SU-91 (CBB) and SG-6 (BCMV/BCMNV) allowed the selection and tracking of bean plants with respective alleles. Genotypic screening of F_2 progenies confirmed the successful pyramiding

TABLE 4. Marker of parents, backcross and F_2 progenies for BCMV and BCMNV resistance

Parent/cross/ marker	Generation	Observed plants		Expected ratio	Goodness of fit	
		R	S	R:S	X^2	P
MCM 2001 (P_3)	P_3	20	0			
CAL 194 (P_4)	P_4	0	20			
P_4 x P_3	F_1	3	16			
P_3F_1 x P_4	BC_1F_1	5	18	0:1	3.5902	0.0581
BC_1P_3 x P_4	BC_2F_1	4	9	0:1	2.6591	0.0103
BC_2P_3 x P_4	BC_3F_1		5	18	0:1	3.5902
0.0581						
SG-6	F_2	94	288	1:3	4.280	0.0390

R = resistant, S = susceptible, Chi-square P- values greater than 0.05 indicate that the observed values are not significantly different from the expected values

TABLE 5. Phenotypic and genotypic response of three gene pyramids to BCMV/BCMNV, CBB and anthracnose

S/N	Phenotypic reaction			Presence or absence of markers		
	CBB	ANT	BCMV/BCMNV	SU91	SAS13	SG-6
1	R	R	R	+	+	+
2	S	R	R	+	+	+
3	R	R	R	+	+	+
4	R	R	R	+	+	+
5	R	R	R	+	+	+
6	R	R	R	+	+	+
7	R	R	R	+	+	+
8	R	R	R	+	+	+
9	R	R	S	+	+	+
10	R	R	R	+	+	+
11	R	S	S	+	+	+
12	S	R	S	+	+	+
Donors						
VAX3	R	S	R	+	-	+
G2333	S	R	S	-	+	-
MCM2001	R	S	R	+	-	+
KKCAL194	S	S	S	-	-	-

R = resistant, S = susceptible, + = marker present, - = marker absent

of three target resistance genes (*bc-3, Co-4*^{2}, and *SU-91*) with twelve individuals for all three loci. Ten plants inherited CBB resistance QTL (SU-91); 11 plants inherited anthracnose resistance gene (*Co-4*^{2}) and 9 plants inherited the *bc-3* allele for BCMV/BCMNV resistance (Table 5). Images of the amplified DNA fragments for the three dominant markers, markers are presented in Figure 2. Phenotypic evaluation, following artificial pathogen inoculation, confirmed that nine of these twelve plants with pyramided genes, exhibited resistant reactions to all three diseases. Phenotypic disease evaluations conducted on $F_{2:3}$ progenies, revealed that most plants exhibited resistance to anthracnose, followed by CBB resistance; while BCMV/BCMNV resistance was displayed in least number of plants (Fig. 3).

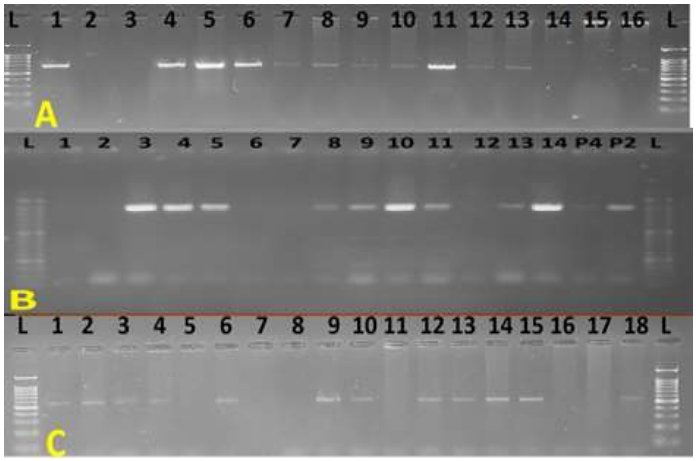

Figure 2. Amplification of markers in breeding populations: Plate A: $SU91_{700bp}$ marker tags SU91; L=ladder and lanes 1-16 are F_2 plants. Plate B. $SAS13_{950bp}$ marker tagging *Co-4*^{2} L=ladder; lanes 1 -14 are F_{2s}; P_4=KKCAL 194 and P_2=G2333. Plate C. $SG\text{-}6_{595bp}$ marker tags *bc-3* gene L =ladder; lanes 1 -18 are F_2 plants.

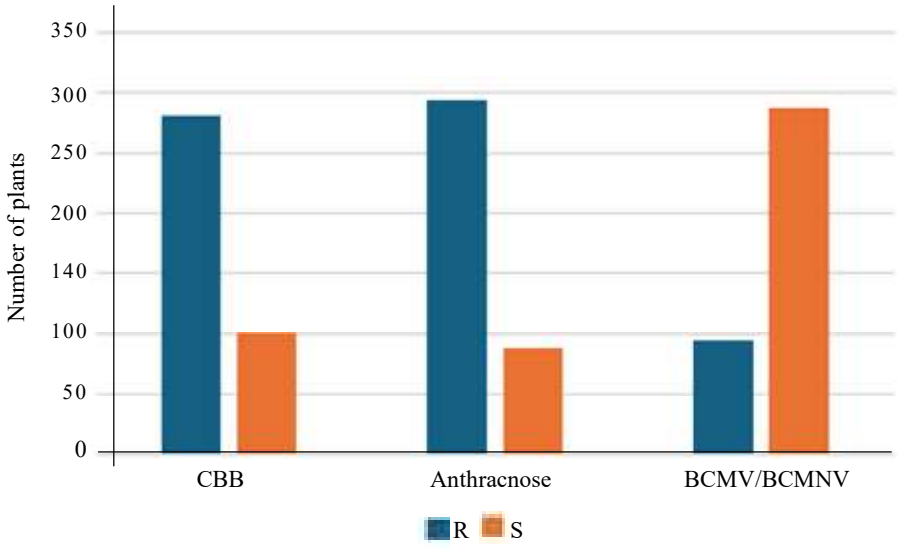

Figure 3. Reaction of F2:3 progenies to pathogens upon artificial inoculation.

DISCUSSION

Marker assisted introgression of anthracnose, BCMV/BCMNV and CBB resistance. The success in pyramiding the multiple disease resistance genes against anthracnose, BCMV/BCMNV and CBB into the commercial common bean cultivar KKCAL 194 is demonstrated by data in Table 5. This is potentially a turning point in the efficacious control of these burdensome diseases of common bean, often difficult to control singly (Rezene *et al.,* 2019). Resistance in bean cultivars to two or more diseases reportedly renders better protection than single disease resistance, since the occurrence of more than one pathogen on a single crop is a well-known phenomenon in the tropics (Fininsa and Tefera, 2006). When breeding for disease-resistant cultivars, the technique of gene pyramiding (i.e., introducing many disease-resistance genes into one genotype) can be used to lengthen the lifespan of new cultivars (Dormatey *et al*., 2020).

The lack of significant deviation of the observed segregation ratios for CBB resistance in the developed populations, from the expected Mendelian ratios signifies that SU91 is a dominant QTL (Table 2). On the other hand, the exhibition of F_2 progenies of a 3R:1S (resistant:susceptible) ratio; while backcross populations showed a 1R:1S ratio, confirms the dominant inheritance of the SU91 QTL derived from the donor parent VAX3 (Table 2).

Our findings demonstrate that the SU91 QTL, derived from VAX3, exhibits a simple inheritance pattern, facilitates its straightforward introgression and selection, making it ideal for marker-assisted breeding. Our findings are consistent with the work of O'Boyle and Kelly (2007), who demonstrated that the simply inherited nature of the *SU91* QTL from VAX3, facilitates its efficient deployment in breeding for CBB resistance. As noted by Ambachew *et al.* (2021), CBB resistance in common bean is conditioned by SU91; a major QTL that is quantitatively inherited. Phenotypic screening validated SU91 as a valuable source of resistance, with the majority of pyramided lines displaying enhanced tolerance.

Although two pyramided lines succumbed to *Xap* inoculation, this is consistent with previous reports that the expression of SU91 can be modulated by the genetic background or environment (Singh and Miklas, 2015). Two lines with pyramided genes succumbed to *Xap* inoculation. However, phenotypic screening confirmed successful deployment of SU91 QTL as a good source of resistance for combating CBB.

Results of the present study demonstrate that anthracnose resistance allele *Co-4²,* introgressed from G2333, is monogenic with dominance inheritance pattern. Segregation analysis in F2 and backcross populations inoculated with race 485 fits the expected pattern for a single dominant gene, confirming the dominant nature of Co-4^2 (Table 3). The genetic simplicity of this trait facilitates its direct introgression into elite genetic backgrounds like the (KKCAL 194) to enhance anthracnose resistance. The dominat nature of the *Co-4²* allele enabled efficient selection through phenotypic screening and MAS accelerating the development of resistant lines. This finding is consistent with previous studies, indicating that anthracnose resistance in common bean is often monogenic and dominant (Vieira *et al*., 2018) and that it can be readily introgressed during breeding, which simplifies the selection process in segregating populations (Souza *et al*., 2015).

However, a major challenge in breeding for anthracnose resistance is the frequent breakdown of resistance due to the emergence of new pathogenic races as well as diversity within existing races. In this context, the identification of durable, broad-spectrum resistance genes is paramount. However, the *Co-4²* allele is recognised as one of the most effective allele providing, broad-spectrum resistance against diverse *Collectotrichum*

lindemuthianum populations; hence its widespread deployment in most breeding programmes (Kiryowa *et al*., 2021). The *Co-4²* is one of the most effective allele against a wide variety of virulent races, including race 485 (Padder *et al.,* 2017). The cultivar G2333 has a three gene pyramid (*Co-4²*, *Co-5*, and *Co-7*) that offer resistance to most anthracnose races (Vallejo and Kelly, 2009). This translated to most of the lines developed being resistant to anthracnose, hence illustrating the value of G2333 as a donor for anthracnose resistance.

In common bean, the recessive *bc-3* allele, provides broad-spectrum and durable resistance against BCMV/BCMNV. Due to its effectiveness, bc-3 has become an essential component of gene pyramiding strategies and has been successfully introgressed into numerous dry and snap bean cultivars, to offer stable resistance thereby reducing yield losses (Munubi *et al*., 2026). The inheritance nature of *bc-3* is monogenic and recessive (Ruhimbana and Mutlu, 2019), a pattern that conforms to results of our study that fit expected genetic ratios for phenotypic and marker segregation data in backcross and F_2 populations (Table 4). This recessive nature presents a practical breeding challenge; such that the heterozygous plants harbor the allele but exhibit a susceptible phenotype (Mukeshimana *et al*., 2005).

Being a recessive gene**,** individual plants must have two copies of homozygous recessive, to express the resistant phenotype while a plant with only one copy (heterozygous) is susceptible (Tang and Feng, 2022). To overcome this, the breeder must self pollinate individuals segregating for the recessive allele before conducting MAS. This process is time consuming (adds at least one exra generation) and labourious.

Validation of SCAR markers for selection of resistant progenies. The successful introgression of *bc-3*, *Co-4²* and *SU91* into KKCAL 194, demonstrates the potential of MAS in developing resilient bean cultivars to multiple pathogens. The SCAR markers (SAS 13 (*Co-4²*), SG-6 (*bc-3*) and SU-91 (*SU - 91*)) were polymorphic between resistant and succeptible progenies (Fig. 2). These markers were effectively used to aid the pyramiding process and the development of progenies with anthracnose, BCMV/BCMNV and CBB resistance. Moreover, the tracking and pyramiding of genes using the aforementioned molecular markers, have been reported in many previous studies (Miklas *et al*., 2006; Arunga and Odikara, 2020).

Through marker-assisted selection, twelve lines were developed by pyramiding resistance genes for anthracnose, BCMV/BCMNV and CBB, into a susceptible genetic background with market-class qualities (KKCAL 194). Nine of these twelve lines exhibited a resistant reaction to all three target diseases when challenged to target pathogens (Table 5). These successful introgression of the multiple resistance genes, enhances the genetic resilience of the new lines, which is expected to contribute to stable yield performance in disease-prone environments such as western Kenya. The developed lines represent a significant step toward creating a durable resistant cultivar with desirable market qualities.

Our results conform to previous studies that demonstrated the efficacy of MAS in pyramiding resistance genes to enhance disease resilience in common beans (Assefa *et al*., 2019; Rezene *et al*., 2019; Lema *et al*., 2021). The gene pyramiding approaches of this study complements that of Ragagnin *et al.* (2009) using SCAR and RAPD markers to pyramid resistance genes against anthracnose, angular leaf spot and rust into the susceptible 'carioca' market class cultivar Ruda. In another related study, Ferreira *et al*. (2012) used SCAR, CAPS and RAPD markers to successfully pyramid anthracnose (*Co-2*, *Co-3/9*) and common mosaic virus (*I* and *bc-3*) resistance genes into the 'fabada' market class A25 genotype. Recently, Munubi *et al.* (2026) reported successful use MAS to pyramid resistance genes against anthracnose (*Co-4²*),

ALS (*Phg-2*), BCMV and BCMNV (*I* and *bc-3*) into advanced French bean breeding lines in Kenya.

Successful gene pyramiding of multiple genes has also been reported in many other crop plants such as wheat, barley and soybean (Li *et al*., 2010). It can be concluded with doubt that marker-assisted gene pyramiding is a rapid, competent, cost-effective and simple strategy useful in plant breeding to pyramid genes of interest to develop different stress tolerances in crops (Kumar *et al.,* 2019; Angeles-Shim *et al*., 2020).

Phenotypic evaluation of progenies with pyramided genes. The lines developed through the introgression of resistant genes using SCAR markers were further validated for resistance based on artificial inoculation. Phenotypic evaluation of the twelve lines with pyramided genes, revealed that nine of them displayed resistant reaction to pathogens upon inoculation (Table 5). The SCAR markers used in MAS demonstrated 75% selection accuracy, correctly predicting the resistant phenotypes in progenies (Fig. 2). These results imply the possible reliance on genotypic data to predict the phenotype. This conforms to findings by Misango *et al*. (2023), who reported that one has high chance of success when using molecular markers for screening for disease resistance; thus, one can rely on molecular marker data to equally determine the phenotype. Our findings confirm the utility of these markers in marker assisted breeding for multiple disease resistance. This inference is supported by findings of Chukwu *et al.* (2020) who reported that if 75% or more of the genotypes that performed well phenotypically show the preferred marker allele, the marker is useful in marker-assisted breeding.

The presence of markers in the progenies, indicates that the transfer of resistance from the donors into the recurrent parent was achieved (Fig. 2); signifying that the gene pyramiding process was successful. Phenotypic and molecular results suggest that both genotypic and phenotypic selection methods complement one another, reinforcing the importance of integrating both approaches in breeding programmes. The incorperation of artificial inoculation besides MAS further streangthened the selection process.

However, some markers were found in succpetible progenies, where 9 out of 12 progenies were correctly predicted upon artificial inoculation. The observed discrepancy between genotypic data and disease phenotype, can be attributed to the fact that resistance alleles may not have been fixed during the breeding process, due to recombination events between the marker and the actual gene during meiosis such that the marker and the gene will be separated (Jiang, 2013); resulting in genotypes that are false positives depending on association between target gene and the marker (Liu *et al.,* 2005).

The distance between markers and gene (s) of interest were 0.5 cM (SU 91), 0.1 cM (SAS 13) and 3.7 cM (SG-6), implying that there is a high chance that some markers had been lost in the progenies due to recombination. As the distance between maker and gene of interest increases, there is a greater chance of recombination (Ashkani *et al.,* 2013), such that the genotype carrying the marker (s) could be a false positive (Jiang, 2013). Recombination frequencies may be higher due to different genotype segregation patterns and events in the development of backcross populations (Kumari *et al*., 2023). Similar reactions of succeptible reactions to pathogens despite marker expression have been reported (Kidane, 2021).

The expression of resistance genes can be influenced by genetic background and the environment, signifying some epistatic interaction between the QTLs (Kachulu *et al*., 2011); this is the case of SU91 QTL that tags CBB resistance. The marker will always be present, but the resistant phenotype may not be expressed under certain conditions. To

overcome this, it is desirable to use a tightly linked marker which reliably predicts the phenotype (Gazal *et al.,* 2016).

In addition to MAS, the presence of target genes must further be confirmed by phenotyping with artificial inoculation (Kumar *et al.,* 2019). The availability of phenotypic succeptible progenies having markers provides evidence for the need to combine phenotypic besides marker assisted selection when selecting for disease resistance and that, both phenotypic and genotypic data should be used in selecting resistant genotypes. Nonetheless, the fact that phenotypic resistance was observed among the populations confirmed the utility of molecular markers in predicting phenotypes.

It is worth nothing that, most resistant progenies among F2 plants dispalyed anthracnose resistance (Fig. 5). This situation may be explained by presence of 'other genes' inherited besides the *Co-4*^{2} that was traced by the SA13 marker. The G2333 cultivar has a three gene pyramid; *Co-4*^{2}*, Co-5*^{2} and *Co-7* (Souza *et al.,* 2015, Vieira *et al.,* 2018). The present study focused on *Co-4*^{2} because it confers broad resistance to most anthracnose strains, although *Co-5*^{2}*,* and *Co-7* genes could also have been inherited. Additionally, the SAS13 marker that tags the *Co-4*^{2} locus is tightly linked at 0.01 cM; hence, minimal chances of recombination (Young *et al.,* 1998). This close linkage between the *Co-4*^{2} gene and the SAS13 marker, ensured effective selection. There could also be a possibility of dominat alleles masking the recessive alleles during the gene pyramiding making it impossible to select for the recessive allele based on the phenotype alone hence, the need for use of marker assisted selection to efficiently combine such genes in a pyramid (Singh *et al*., 2013). Thus, it is not surprising that plants presumed to have inherited all the pyramided resistant genes (3 out of 12) succumbed to BCMV/BCMNV viral strains upon artificial inoculation.

CONCLUSION

This study has demonstrated the potential of marker-assisted backcrossing to pyramid multiple disease resistance genes into a preferred Kenyan common bean variety. It represents a significant breakthrough in the developement of robust dry bean cultivars resistant to multiple pathogens affecting its productivity and cause complete crop loss in susceptible varieties. The integration of conventional breeding with molecular tools accelerated the breeding process leading to faster genetic gains.

Future work should focus on advancing these breeding populations through further selection, multi-location field trials in disease hotspots to confirm resistance stability and agronomic performance. Yield penalties associated with gene pyramiding reported in literature should be evaluated on the developed lines before the release of a superior, resilient bean variety to the farming community.

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