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Research article

Pyramiding aphid resistance genes into the elite cowpea variety, Zaayura, using marker-assisted backcrossing

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ABSTRACT

The cowpea aphid (*Aphis cracivora*) is a cosmopolitan insect pest that causes economic damage on cowpea. Although the pest persists at all the growth stages of the crop, in West Africa, aphids are the only major insect pests that farmers regularly control at the vegetative stage. Thus, deploying aphid-resistant crop varieties can reduce farmers' expenditure on insecticide. The availability of different biotypes of the pest and reports of resistance breakdown necessitates pyramiding of sources of aphid resistance to develop a more robust genotype for durable resistance. Two aphid-resistance genes, sourced from SARC-1-57-2 and IT97K-556-6, were introgressed through gene pyramiding technique into a farmers' preferred cowpea variety, Zaayura, using marker-assisted backcrossing. A simple sequence repeat (SSR) marker, CP 171F/172R, and an allele-specific single nucleotide polymorphism (SNP) marker, 1_0912, were used for foreground selection of the SARC-1-57-2 and IT97K-556-6 aphid resistance genes, respectively. A stepwise backcross approach was used to introgress the major aphid resistance QTL (*QAc-vu7.1)* from IT97K-556-6 into Zaayura using the marker 1_0912 coupled with intermittent screening under artificial aphid infestation. After the fourth backcross generation, three heterozygous BC_4F_1 of Zaayura/TT97K-556-6 were intercrossed to Zaayura Pali to develop intercross F_1 (ICF_1). Three true ICF_1 hybrids allowed to self to produce ICF_2. Five (5) out of 48 ICF_2 plants which were genotyped with the two foreground markers had the two aphid resistance genes fixed in the double homozygous dominant state. For background selection, out of 192 allele-specific markers screened, only 47 polymorphic markers were identified and used for the background analysis of the pyramided lines. The recurrent parent genome recovery ranged from 72 to 93.8 %. ICF_2_Zaa/556/SARC-P6 had the highest recurrent parent genome and the least heterozygosity among the five improved lines. The five pyramided lines showed superior resistance under artificial aphid infestation as compared to the two donor parents with damage scores ranging from 2.0 to 2.3. On the field, however, there were no significant differences between the pyramided lines and their recurrent parent for all the agronomic traits measured except for grain yield. The pyramided lines do not only stand the chance of being released as new varieties but are also valuable genetic resources for other breeding programs that seek to improve cowpea for aphid resistance.

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1. Introduction

Cowpea [*Vigna unguiculate* (L.) Walp.] is a diploid (2n = 2x = 22) legume with a genome size of 620 megabases (Mb) [1]. Although the crop is believed to have an African origin, it is cultivated in Africa and other parts of the world including South America, Asia, and the United States of America (USA) [2]. The crop is normally cultivated for its fresh and dry grains, fresh leaves and fodder. The cowpea grain contains protein that serves as a cheap source of dietary protein in most diets in West and Central Africa [3,4].

Despite the high quantity of cowpea grains produced annually in sub-Saharan Africa, the yields on smallholder farmers' fields are comparatively low (less than 600 kg/ha) relative to the yield potential of the crop (more than 2000 kg/ha) [2]. The yields on farmers' fields are, thus, about 30–40 % less of the potential. This disparity in yield can be attributed to several abiotic and biotic factors affecting the profitable production of cowpea. Some of the important abiotic factors that impede cowpea production include heat, drought and low soil fertility stress. Biotic factors such as disease pathogens, parasitic weeds (*Striga gesnerioides* and *Alectra vogelii*), nematodes and insect pests cause significant damages to cowpea-production in sub-Saharan Africa and other cowpea producing regions across the globe [5].

Among all the insect pests bedeviling cowpea production, the cowpea aphid (*Aphis craccivora* Koch), is considered the most important [6,7]. It is a sap-sucking pest that is found in all cowpea-growing regions around the world causing substantial yield losses at the seedling or podding stage of the plants growth [7–11]. Estimated yield losses in cowpea up to 35 % and 40 % due to aphids have been reported in Africa and Asia, respectively [9]. Damage caused by aphids exacerbates with dry spells following seedling emergence.

In attempts to control cowpea aphids, farmers usually employ cultural, biological and chemical methods on affected cowpea fields [6,12]. Biological control of the aphids using natural enemies has been found to be ineffective primarily due to the rapid multiplication of the pest in the presence of its natural enemies [6]. Although the use of pesticides is very common and quick, the continuous use of pesticides has led to resurgence of pests that are resistant to pesticides, non-target destruction of beneficial insects and human health complications [6,10]. The emergence of aphids with resistance to some pesticides has resulted in the need for multiple combinations and rotations of different pesticides to effectively control cowpea aphid. The development of host - plant resistant cultivars through breeding has been suggested as the most sustainable strategy that guarantees an effective, environmentally friendly, and a relatively cheap method of controlling cowpea aphids [5,13].

Aphid resistant cowpea genotypes have been reported by several authors. For instance, Tvu 2897 and TVNu 1158 were found to be resistant to cowpea aphids [6]. The basis for resistance in these lines was antibiosis [6]. Studies by some authors have also revealed that, resistance to cowpea aphids is controlled by single dominant genes [6,14,15]. This assertion thus, suggests that breeding for aphid resistance will be easier since resistant individuals can be selected from a segregating population [14,15]. Nevertheless, the major challenges to successful breeding of cowpea cultivars that are resistant to aphids is the emergence of possible biotypes and resistance breakdown. In an aphid screening study, it was reported that the aphid biotypes in Ghana were more aggressive than those in Nigeria when an aphid resistant cowpea line from Nigeria (IT99k-499-35) was found to be susceptible to aphids in Ghana [7].

To develop cowpea cultivars with durable resistance to aphids, it is imperative to pyramid multiple resistance genes that are non-allelic into a single cultivar. The cowpea breeding line SARC 1-57-2 was found to have stable resistance to aphids in all the cowpea growing regions in Ghana [16]. The resistance in this genotype is conditioned by a single dominant gene [14]. Similarly, the aphid resistant line IT97K-556-6 [10,17] was also found to be resistant to aphids in Ghana. In an allelism study (unpublished data), it was found that the resistance in SARC 1-57-2 and IT97K-556-6 was controlled by two dominant genes (one from each parent) that segregated independently. Gene-associated markers also showed that the aphid resistance in SARC 1-57-2 was mapped to linkage group (LG) 10 [14] while the aphid resistance QTLs in IT97K-556-6 were mapped to LG 1 (chromosome 5) and LG 7 (Chromosome 2) [10]. Thus, resistance genes in these two lines are non-allelic and segregate independently.

Modern breeding efforts thrive on a combination of molecular and classical methods. Molecular breeding techniques, such as marker assisted selection (MAS), enable precision breeding targeting specific genes of interest. The two resistance sources can therefore be harnessed, using available molecular resources, to develop a robust and durable aphid-resistant cowpea genotype through gene pyramiding technique.

In Ghana, farmers prefer to cultivate high yielding cowpea varieties with large seed size, cream or white seed coat color, black or brown eye color and easy to cook seed characteristics preferred by consumers. Cowpea with large seeds cost more than those with small seeds [18]. Zaayura is a popularly cultivated cowpea variety because of its long pods, heavy pod load and large seed size that translate into high yields. Although it has previously been improved with aphid resistance from SARC 1-57-2 [14] and released as Zaayura Pali (improved Zaayura), single gene resistance may easily be overcome; therefore, an additional aphid resistance gene from IT97K-556-6 will make resistance of Zaayura to aphids more robust and durable.

This study aimed at pyramiding cowpea aphid resistance genes from two different sources into a farmer-preferred cowpea variety, Zaayura, using marker-assisted backcrossing.

2. Materials and methods

2.1. Experimental site and plating material

The research was conducted at Savannah Agricultural Research Institute of the Council for Scientific and Industrial Research (CSIR-SARI), Ghana. The screenhouse and field experiments were carried out at the Manga Research Station of the CSIR-SARI. The molecular aspect of the research was conducted at the biotechnology lab at the main station of CSIR-SARI at Nyankpala. The pyramided lines

were developed between January 2018 to December 2020. The aphid screening and field evaluation of the pyramided lines were carried out in the rainy season of 2021. The Manga Station lies between latitude 11.01° North and longitude 0.26° West. The area is within the Sudan-Savannah zone and is characterized by a unimodal rainfall pattern lasting 4–5 months, from June to October, and a dry period lasting 7–8 months, with an annual mean rainfall of 800–1000 mm (Manga weather station data). The soil in the area is well-drained sandy soil. The characteristics and description of the parental lines used in this study are presented in Table 1.

2.2. Pyramiding aphid resistance into Zaayura

A stepwise backcross approach was used to pyramid the IT97K-556-6 and SARC-1-57-2 genes into Zaayura. In the stepwise approach, Zaayura served as the recurrent parent while IT97K-556-6 served as the initial donor parent for four backcrosses after which the gene positive BC_4F_1 was intercrossed with Zaayura Pali which carries the SARC 1-57-2 aphid resistance gene (Fig. 1).

2.3. Hybridization procedure and population development

All crosses were done in the screenhouse at the CSIR-SARI Manga station. Crossing blocks were established by planting seeds of the parents in pots filled with soil. The dimensions of the pots were 35 × 30 × 20 cm for height x top diameter x base diameter respectively. Two seeds of the same genotype were planted in each pot. Plants were watered and weeds controlled by hand pulling from the pots. Seedlings were tagged and given unique plant identification numbers. Leaves were sampled from each plant for DNA extraction and genotyping. In the case of backcrossing, selected plants based on molecular markers were used for the crosses. Plants that were not selected were discarded from the crossing block to avoid overcrowding.

At flowering, the resistant genotypes were used as the pollen donors and crossed to the recurrent parent. Hybridization was usually carried out early in the morning from 6 to 8 a.m. when most of the flowers were open. Occasionally, some crosses were carried out during late afternoon from 5 to 6 p.m. For the early morning crosses, mature buds on the female plant that were to open the next day were the recipient and the opened flowers on the male parent were picked for the pollen source. Emasculation was carried out using a blade to cut open the flower buds just at the tip and carefully removing the stamens, leaving the pistil. The careful removal was to avoid self-pollination. The opened flower of the male plant was picked and cut transversely. The upper part containing the pollen in the cup was retained and the lower part discarded. The cup with the pollen was used to cover the exposed pistil of the female bud. The covering was done such that the pollen had contact with the stigma of the pistil. The cross-pollinated buds were tagged and labeled to differentiate them from the selfed pods. For the late afternoon pollination, the open flowers were collected in the morning into well labeled petri dishes and stored in a refrigerator (4 °C) until when needed in the late afternoon. At the late afternoon, the stored flowers were used to pollinate the recipients. The blade used in the crosses was cleaned with rubbing alcohol (70 % v/v) when moving from one plant to the other.

2.4. Marker - assisted backcrossing

The marker, 1_0912, which is a flanking marker of the major aphid resistance QTL (*QAc-vu7.1*) in IT97K-556-6 [10], was used to confirm the hybridity of the F_1s and also for foreground selection at each backcross stage. The marker is located at 21.7 cM while the QTL is at 22 cM on chromosome 2 of the cowpea genome [10]. Despite the closeness of the foreground marker 1_0912 to the aphid resistance QTL, it is not a functional marker; thus, intermittent phenotyping of the BC progenies under artificial aphid infestation was used to confirm the presence of the aphid resistant gene. Through phenotyping, it is impossible to differentiate between aphid resistance from IT97K-556-6 and SARC 1-57-2. Using Zaayura as the recurrent parent in the backcrossing allowed for the phenotypic confirmation of the aphid resistance genes from IT97K-556-6.

After four backcrosses, the BC_4F_1 from Zaayura/IT97K-556-6 was intercrossed to Zaayura Pali to generate the intercross F_1 (ICF_1). After the intercross, the SSR marker CP 171F/172R [14] was used to select the aphid resistance genes from SARC 1-57-2. Double heterozygous individuals were selected based on the foreground markers and their resemblance to Zaayura. The selected ICF_1 plants were advanced to ICF_2. The ICF_2s were planted and genotyped with the foreground markers, CP 171F/172R and 1_0912, to select double homozygous individuals. After selection of the double homozygous individuals, recurrent – parent genome recovery analysis was carried out using foreground markers (Fig. 1).

2.5. DNA extraction, PCR and electrophoresis

Total genomic DNA was extracted using the CTAB protocol by Doyle and Doyle, [19]. A 2 % agarose gel (2 g agarose, 100 ml of ×1

Table 1
Parental lines, their pedigree and their aphid resistance status.

Genotype	Parentage/pedigree	Genotype type	Source of seed
SARC 1-57-2	Apagbaala/UCR 01-11-52.	Breeding line in CSIR-SARI. Resistant to aphids [7,14,16]	CSIR-SARI, Ghana
IT97K-556-6	A breeding line from IITA, Ibadan, Nigeria.	Resistant to aphids [10,17]	IITA, Nigeria
Zaayura	Marfo-Tuya/UCR 01-15-127-2	Aphid susceptible released in 2008 [16]	CSIR-SARI, Ghana
Zaayura Pali	Zaayura/SARC 1-57-2	Aphid resistant released in Ghana in 2016 [14]	CSIR-SARI, Ghana

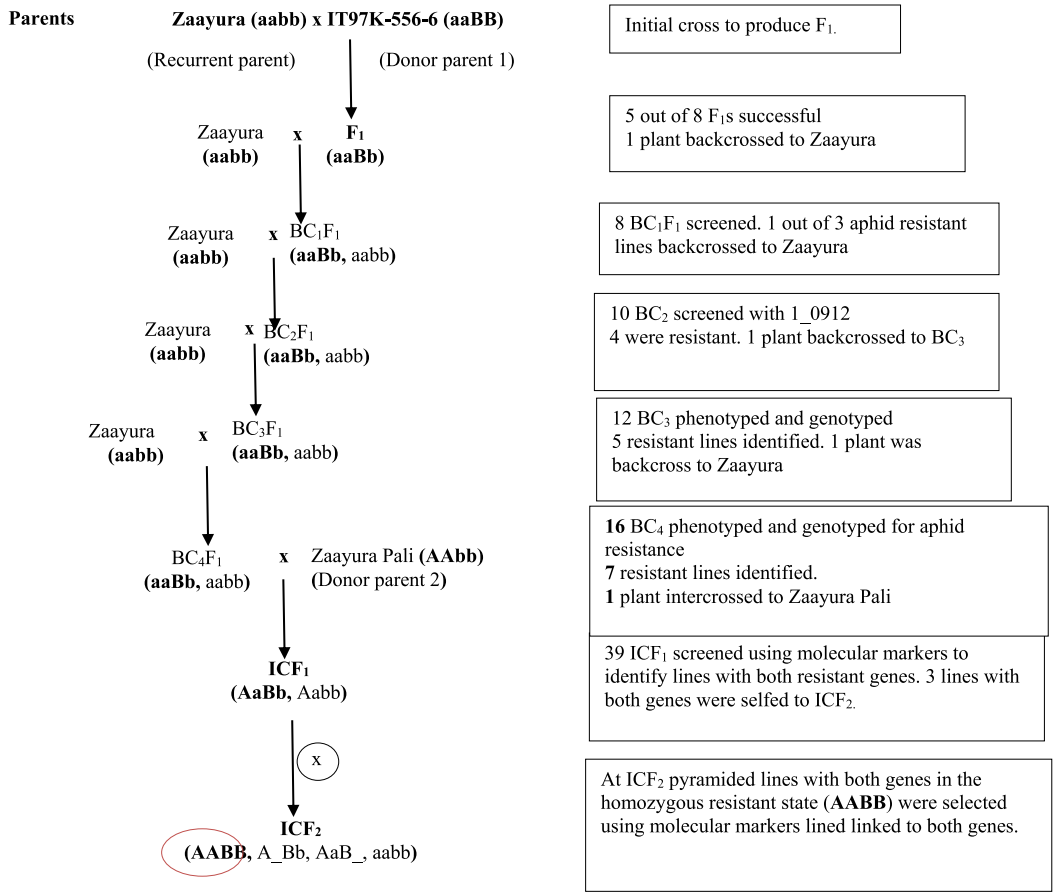

Fig. 1. Stepwise backcrossing scheme. The "**A**" and "**B**" represent the resistant alleles from SARC 1-57-2 and IT97K-556-6 respectively. The "**a**" and "**b**" are susceptible/recessive alleles form the donor parents. The possible genotypes at each stage are in the brackets. The selected genotype at each stage is in Bold font. The final genotype of interest is the "**AABB**" the has been circled in red.

TAE buffer and 5 μl of ethidium bromide) was used in checking the quality of DNA obtained. Polymerase chain reaction (PCR) of the extracted DNAs was carried out using the following conditions: denaturation at 94 °C for 30 s, annealing at X °C (depending on primer, Supplementary Table 1) for 30 s and extension at 72 °C for 30 s for 35 cycles in a reaction volume of 10 μl (3 μl of ddH_2O, 1 μl of DNA, 1 μl of primer and 5 μl of premix). The premix used was the illustra™ puReTaq Ready-To-Go PCR Beads.

The PCR products were resolved on a 6 % horizontal polyacrylamide gel at a voltage of 120 V, running time of 3 h. The gel was stained with ethidium bromide for 30 min. The gels were visualized and photographed under a UV *trans*-illuminator.

2.6. Marker-assisted background analysis of improved lines

The parental genotypes were genotyped with molecular markers that span through the entire cowpea genome to identify polymorphic markers among the cowpea genotypes. A total of 192 allele-specific primers were screened. The allele-specific primers were developed from SNP markers that flank QTL regions in cowpea. The names of the markers, their sequence, their synthesis report, and references are presented in Supplementary Table 1. The markers that were polymorphic between the donor parents and the recurrent parent were used for the background analysis. These polymorphic markers were used to genotype the pyramided (double homozygous) lines and their parents. After the PCR, the amplicons were resolved on a 6 % horizontal polyacrylamide gel (hPAGE) at a voltage of 120 V and a running time of 2 h. The gels were stained with ethidium bromide and visualized under a UV *trans*-illuminator. The gel images of the pyramided lines and their parents were scored for the background analysis. Homozygous bands were scored as 'A' or 'B' when the allele was like the recurrent parent or donor parent, respectively, and heterozygous bands were scored 'H'.

2.7. Screening pyramided lines under artificial aphid infestation

The pyramided lines, Zaayura, SARC 1-57-2, IT97K-556-6 and Apagbaala were evaluated using the seedling-stage aphid-screening method [16]. IT97K-556-6 and SARC 1-57-2 served as resistant checks while Zaayura (recurrent parent) and Apagbaala served as susceptible checks. The evaluation was carried out in an insect-proof screen house. Five seeds of each genotype were planted in plastic

pots filled with loamy soil. After emergence, 5 days after planting, the seedlings were thinned to 4 uniform plants per pot. Each pot with the 4 plants served as a replicate for the genotype. The pots were arranged in a completely randomized design with ten replications for each genotype. Seven (7) days after planting, the seedlings were infested with 5 (fourth instar) aphids collected from aphid cultures in the Insectary. Infestation was carried out using a soft camel-hair brush. The seedlings were monitored after 24 h, the seedlings with less than 5 aphids were re-infested to make up for the deficit. The plants were watered regularly by pouring the water at the base of the plants to avoid washing off the aphids. The aphid fed and multiplied on the seedlings. Three weeks after the infestation, when the susceptible checks were severely damaged by the aphids, seedlings were scored for aphid damage and plant vigour after which insecticides were sprayed to get rid of the aphids. Aphid damage was rated on a 5-point scale where 1 = seedling with no aphid (control pots) and no aphid damage, 2 = seedling with aphids and slight symptoms of damage (slight yellowing of lower leaves without capping), 3 = seedling showing symptoms of aphids' damage (yellowing of lower leaves and slight capping), 4 = seedling with weak stem and leaves with symptoms of aphid damage (severe capping of leaves, stunted plants, yellowing of all leaves) and 5 = dead seedling due to aphids' damage.

2.8. Evaluation of pyramided lines for agronomic traits

The pyramided lines, their recurrent parent (Zaayura), and two aphid resistance donor parents, IT97k-556-6 and SARC 1-57-2 were evaluated at the research field of the CSIR-SARI Manga Research Station. The experiment was carried out during the rainy season (July to October) of 2022. The experiment was laid out in a randomized complete block design with three replications. Seeds of each genotype were planted on a 4-row plot of 4 m in length. Three seeds were planted per hill at a spacing of 20 cm and 60 cm for intra and inter row respectively. The space between plots was 1 m. Two weeks after planting, the seedlings were thinned to two per hill. Weed control was done manually using hoes. Insect pests such as thrips, aphids, bean pod borer and pod-sucking bugs were controlled by spraying a synthetic pyrethroid, lambda – cyhalothrin (Lambda Super ®), at a rate of 500 ml/ha. Data collected include plant height and canopy spread at flowering, number of pods per plant and number of pods per peduncle prior to harvesting. These data were collected as an average of 5 randomly tagged plants. Post-harvest data collected included pod length, seeds per pod, hundred-seed weight and grain yield.

2.9. Data analysis

The gel scoring data of the pyramided lines and their parents were subjected to analysis using the genetic software Graphical Genotype (GGT 2.0) software [20]. The output of the analysis was percentages for marker homozygous for recipient parent (%A), the parent donor allele (%B and %C) and heterozygous plant (%D).

All phenotypic data were analyzed using the GenStat statistical program (12th edition). Aphid damage scores were square-root transformed to ensure homogeneity of their variances before subjecting them to analysis of variance (ANOVA). The agronomic data were also subjected to analysis of variance (ANOVA) and where there were significant differences, means were separated using Tukey Honestly significant difference (HSD) test at 5 % probability level.

3. Results

3.1. Introgression of Qac-vu7.1 aphid resistance QTL into Zaayura

The hybridity of the F_1s were confirmed using the SNP marker 1_0912 linked to the aphid resistance in IT97K-556-6 and is polymorphic between the parental pairs. The bands of interest were the ~150bp and ~165 bp which were amplified in Zaayura and IT97K-556-6 respectively. Five (5) out of the 7 F_1s were heterozygous whilst 2 had bands just like Zaayura (Fig. 2).

The true hybrids were backcrossed to Zaayura to develop BC_1F_1. The gel image of the backcross progenies genotyped with the SNP 1_0912 marker is presented in Fig. 3. Three out of the eight progenies were heterozygous for the foreground marker used. Out of these, one plant that showed the highest resistance level to aphid phenotypically was used for the next backcross. The cycle of selection and backcrossing was continued to BC_4F_1.

The BC_4F_1 progenies that SNP 1_0912 identified as resistant were intercrossed with Zaayura Pali to develop the ICF_1. The gel pictures of the ICF_1s genotyped with CP 171F/172R and SNP 1_0912 are presented in Figs. 4 and 5. Because Zaayura Pali is

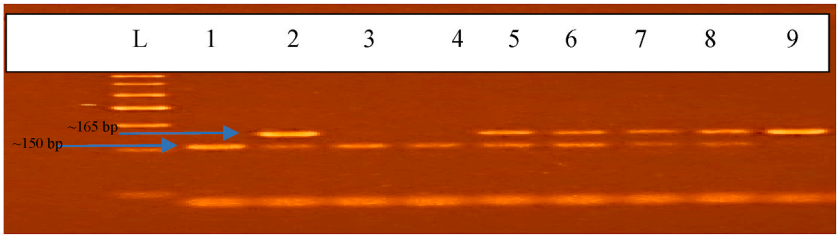

Fig. 2. Confirming success of hybridization using SNP 1_0912. L = 50 bp ladder; 1 = Zaayura; 2-8 = F_1[Zaayura/IT97K-556-6] 9 = IT97K-556-6.

homozygous dominant for the CP 171F/172R marker, almost all the ICF_1 progenies were heterozygous for the marker (Fig. 4). A few individuals had bands like SARC-1-57-2. In Fig. 5, the individuals segregated into heterozygous and recessive band patterns. Out of the 39 plants, 20 were heterozygous, while 12 were recessive. The SNP 1_0912 marker did not amplify in seven plants. The segregation of the progenies fit a 1:1 heterozygous: recessive ratio with a chi-square of 2.0 ($p = 0.157$), which is expected when a heterozygote is crossed to a homozygous recessive plant. Considering both markers, 14 out of the 39 plants combined both genes in the heterozygous state. These plants were samples 2, 3, 9, 10, 12, 13, 14, 15, 19, 20,28, 29,31 and 32. Out of these plants, 5, 10 and 29 were selected to produce the ICF_2 population based on their close resemblance to Zaayura.

3.2. Fixation of aphid resistance genes

The gel pictures of the 48 ICF_2 genotyped with CP171/172 and SNP1_0912 are presented in Figs. 6 and 7. In Fig. 6, the CP 171F/172R differentiated the plants into homozygous dominants, heterozygotes and homozygous recessives. A total of 14 were dominant, 25 were heterozygous, and 9 were recessive. This observation fit a 1:2:1 segregation ratio with a chi-square value of 1.125 ($p = 00.569$). Plants ICF_2_Zaa/556/SARC-P6, -P8, P10, P11, P17, P18, P24, P30, P31, P33, P34, P38, P44, P46, P47 and P48 were homozygous dominant for the CP 171F/172R marker.

In Fig. 7, the SNP 1_0912 amplified 11 dominants, 12 heterozygous and 14 recessives. Twelve samples were not amplified by the marker. The homozygous plants dominant for the SNP 1_0912 were ICF_2_Zaa/556/SARC-P6, -P7, P26, P27, P35, P36, P38, P39, P46, P47 and P48. The double dominant homozygous plants were five, namely ICF_2_Zaa/556/SARC-P6, P38, P46, P47 and P48.

3.3. Marker-assisted background selection

Out of the 192 allele-specific markers (converted SNPs) screened (Supplementary Table 1), 47 were polymorphic between the recurrent and donor parents. The distribution of the markers on the chromosomes are presented in Table 2. The number of polymorphic markers per chromosome ranged from 3 on chromosomes 1, 7 and 8, to 7 on chromosome 3. The number of polymorphic markers represents 24.5 % of the total allele-specific markers screened. None of the markers on chromosome 6 that were screened showed polymorphism among the parents. The polymorphic markers were used for the background analysis of the double homozygous ICF_2 plants identified based on the foreground markers and phenotypic resistance to the recurrent parent.

The recurrent parent genome (RPG) recovery ranged from 72 % (in ICF_2_Zaa/556/SARC-P47) to 93.8 % in (ICF_2_Zaa/556/SARC-P6). The average RPG recovery was 87 %. The summary of RPG recovery and homozygous segments of the double homozygous aphid resistant lines identified are presented in Table 3.

The chromosome-wise recurrent parent genome recovery of the selected ICF_2 lines is presented in Fig. 8. The background analysis observed that chromosomes 1 and 8 were fully recovered in all selected double homozygous resistant individuals. Segments of the donor parent genome were distributed on the other chromosomes. The percentage of the segments of the donor parent, IT97K-556-6, chromosome substituted in the selected lines ranged from 1.1 % to 4.8 %. The segments from SARC 1-57-2 substituted in the selected ICF_2 lines ranged from 0 to 8.9 %. The segments of IT97K-556-6 were retained on chromosomes 5 and 7, while aphid resistant segments from SARC-1-57-2 were retained on chromosome 10, which carry the aphid resistant genes in the respective donors (Fig. 9).

The best individual with the highest RPG recovered and the lowest heterozygous and donor parent segments substitution was ICF_2_Zaa/556/SARC-P6. The chromosome-wise RPG recovered for the best individual is presented in Fig. 10. All the chromosomes of ICF_2_Zaa/556/SARC-P6 were fully recovered except for chromosomes 5, 7 and 10, which retained segments of the donor parents (Fig. 10). No heterozygous segments were retained in any of the chromosomes of ICF_2_Zaa/556/SARC-P6.

3.4. Screening for aphid resistance

Apagbaala was the most susceptible genotype to the cowpea aphids while the pyramided lines ICF_4_Zaa/556/SARC-P6 and -P38 showed the highest resistance to aphid damage. The recurrent parent, Zaayura, was also susceptible to aphids with a mean damage

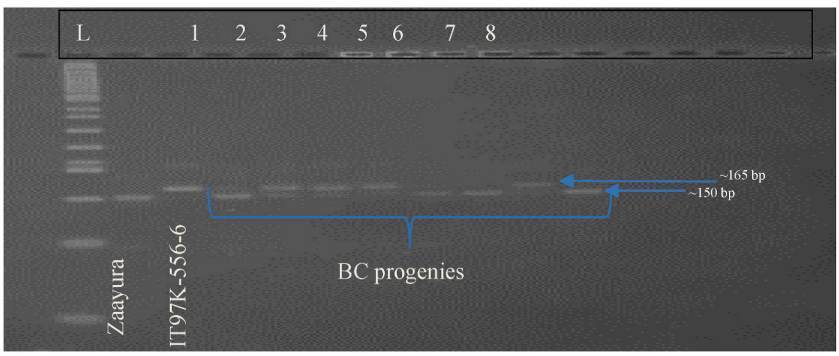

Fig. 3. Foreground selection of resistant BC progenies using SNP 1_0912. L = 50 bp ladder; 1-8 = BC_1F_1[Zaayura/IT97K-556-6].

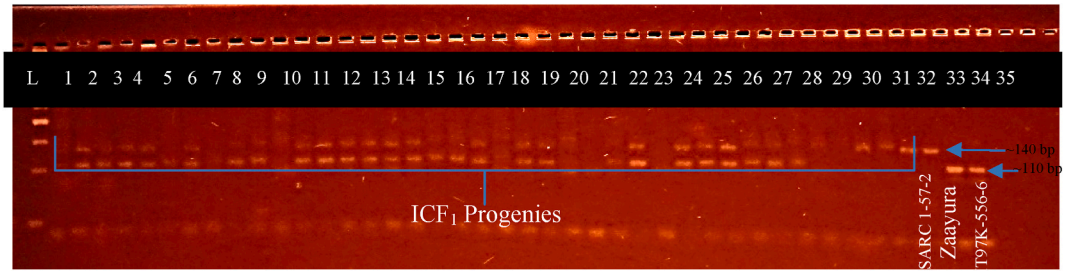

Fig. 4. hPAGE picture of ICF1 [Zaayura x 556/SARC] and parents genotyped with CP171/172. L = 50 bp ladder.

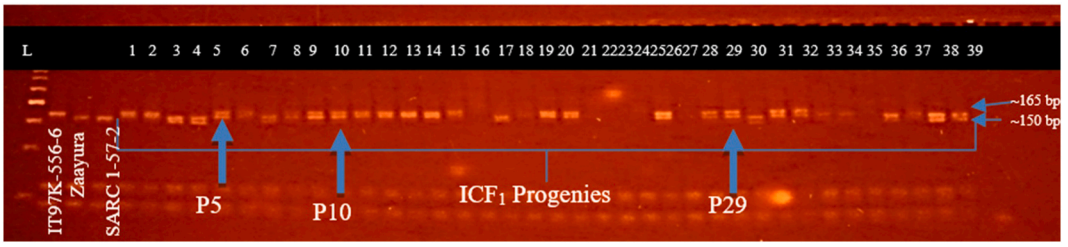

Fig. 5. hPAGE picture of ICF1 [Zaayura x 556/SARC] and parents genotyped with SNP 1_0912. L = 50 bp ladder.

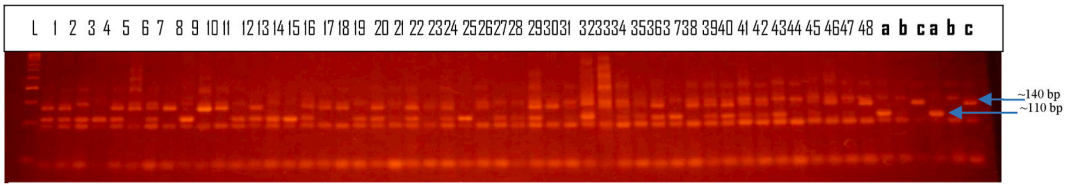

Fig. 6. hPAGE picture of ICF2 [Zaayura x 556/SARC] and parents genotyped with CP171/172. L = 50bps ladder; 1 – 48 = ICF_2_Zaa/556/SARC progenies; a = Zaayura; b = IT97K-556-6; c = SARC-1-57-2.

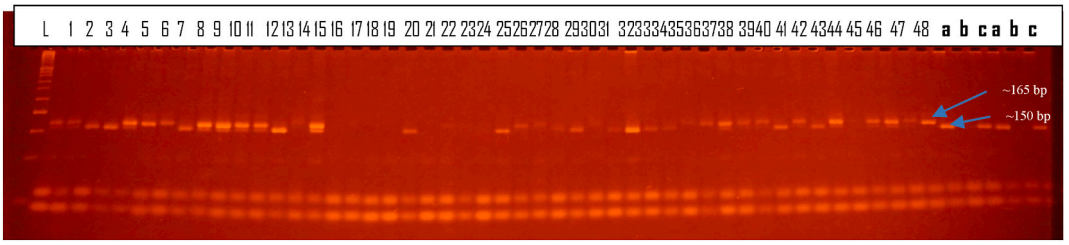

Fig. 7. hPAGE picture of ICF2 [Zaayura x 556/SARC] and parents genotyped with SNP1_0912. L = 50bps ladder; 1 – 48 = ICF2_Zaa/556/SARC progenies; a = Zaayura; b = IT97K-556-6; c = SARC-1-57-2.

score of 3.76 (Table 4). There were significant differences between the Pyramided lines and the parental lines. The resistant checks, IT97K-566-6 and SARC 1-57-2 had aphid damage scores of 3 and 2.69 respectively which were significantly different from the susceptible checks.

3.5. Agronomic performance of pyramided lines

There were significant differences ($P < 0.05$) among the genotypes for all the traits measured with the exception of plant height and canopy spread (Table 5). The plant height recorded ranged from 32.40 cm to 42.87 cm and averaged at 39.25 cm. On the average, canopy spread was 58.72 cm and ranged from 56.73 cm to 61.60 cm for the genotypes. The highest number of pods per plant was produced by ICF_4_Zaa/556/SARC-P38 (30 pods) which was significantly higher than the lowest number of pods (12) produced by IT97K-556-6. Unlike the number of pods per plant, IT97K-556-6 had the longest pods (18.66 cm) which was significantly different

Table 2
Description of polymorphic markers used for the background selection.

Marker name	chromosome	location	Marker name	chromosome	location
2_32753	1	37.2	2_23058	7	10.8
2_04147	1	40.3	2_28580	7	21.1
2_04219	1	46.9	2_24046	7	56.9
2_02471	2	34.4	2_10843	8	61.7
2_13136	2	35.9	2_02661	8	41.6
2_21023	2	3.7	2_09959	8	38.0
2_32890	2	14.7	2_05151	9	12.8
2_14148	3	15.4	2_24923	9	6.4
2_15464	3	2.6	2_17305	9	86.6
2_51968	3	78.9	2_48326	9	41.3
2_10882	3	62.7	2_16425	10	51.9
2_10954	3	54.3	2_40097	10	46.8
2_17476	3	50.8	2_54013	10	54.2
2_20995	3	25.5	2_05766	10	21.6
2_02870	4	30.6	2_23117	10	15.1
2_08233	4	35.2	2_44318	10	58.5
2_07872	4	24.8	2_22867	11	6.0
2_27951	4	20.1	2_23307	11	20.9
2_32586	4	63.8	2_26050	11	15.6
2_11663	5	1.7	2_24219	11	58.8
2_13411	5	20.6	2_41050	11	58.2
2_21226	5	5.3	2_44580	11	25.6
2_05752	5	63.8			
2_08249	5	82.2			
2_16911	5	72.3			

Table 3
Analysis of introgressed segments and background of selected ICF_2 lines and their parents.

Alias	NA (%)	A (%)	B (%)	C (%)	D (%)	Total (cM)
Zaayura (A)	0	100	0	0	0	488.7
IT97K-556-6 (B)	0	0	100	0	0	488.7
SARC 1-57-2 (C)	0	0	0	100	0	488.7
ICF_2_Zaa/556/SARC-P6	0	93.8	2.9	3.2	0	488.7
ICF_2_Zaa/556/SARC-P38	0	91	1.1	0	7.9	488.7
ICF_2_Zaa/556/SARC-P46	0	92.5	2.1	0	5.4	488.7
ICF_2_Zaa/556/SARC-P47	10.6	72	4.8	8.9	3.6	488.7
ICF_2_Zaa/556/SARC-P48	4.7	85.7	2.5	4.8	2.3	488.7

NA = no amplification; A = zaayura genotype; B = IT97K-556-6 genotype; C = SARC-1-57-2 genotype and D = heterozygous genotype.

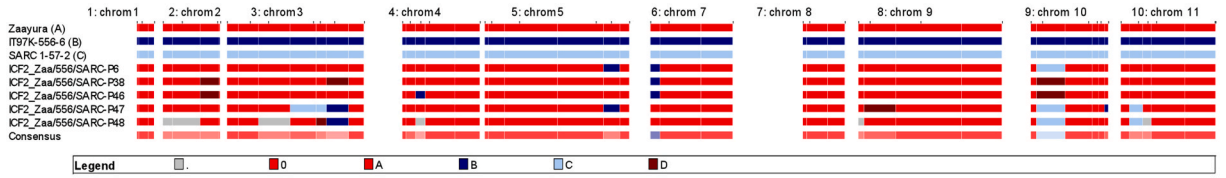

Fig. 8. Chromosome-wise analysis of recurrent parent genome recovery of pyramided ICF_2 plants and their parents across 10 cowpea chromosomes. . = no amplification; A = zaayura genotype; B = IT97K-556-6 genotype; C = SARC-1-57-2 genotype and D = heterozygous genotype.

from the pod length of Zaayura (14.56 cm) which were the shortest. The average length of pods in this study was 16.23 cm. A similar trend was observed for seeds per pod. The longer pods of IT97K-556-6 had the highest number of seeds (13 seeds) and the Zaayura produced the lowest number of seeds per pod (9 seeds). There were no significant differences between the Zaayura and SARC 1-57-2 in terms of number of seeds per pod (Table 5).

Significant variation ($p < 0.001$) was observed between the genotypes with respect to hundred seed weight. SARC 1-57-2 had the lowest hundred seed weight of 19.20 g whilst ICF_Zaa/556/SARC-P46 had the highest hundred seed weight of 23.21 g. Grain yield ranged from 1701 kg/ha to 4565 kg/ha and averaged at 2605 kg/ha. The highest yielding genotype, ICF_Zaa/556/SARC-P38, was significantly different from all the other genotypes tested. Generally, there were no significant differences among the pyramided lines and Zaayura for all the traits measured except for grain yield. Likewise, there were no significant difference among the donor parents and Zaayura for all the traits except pod length and seeds per pod. (Table 5).

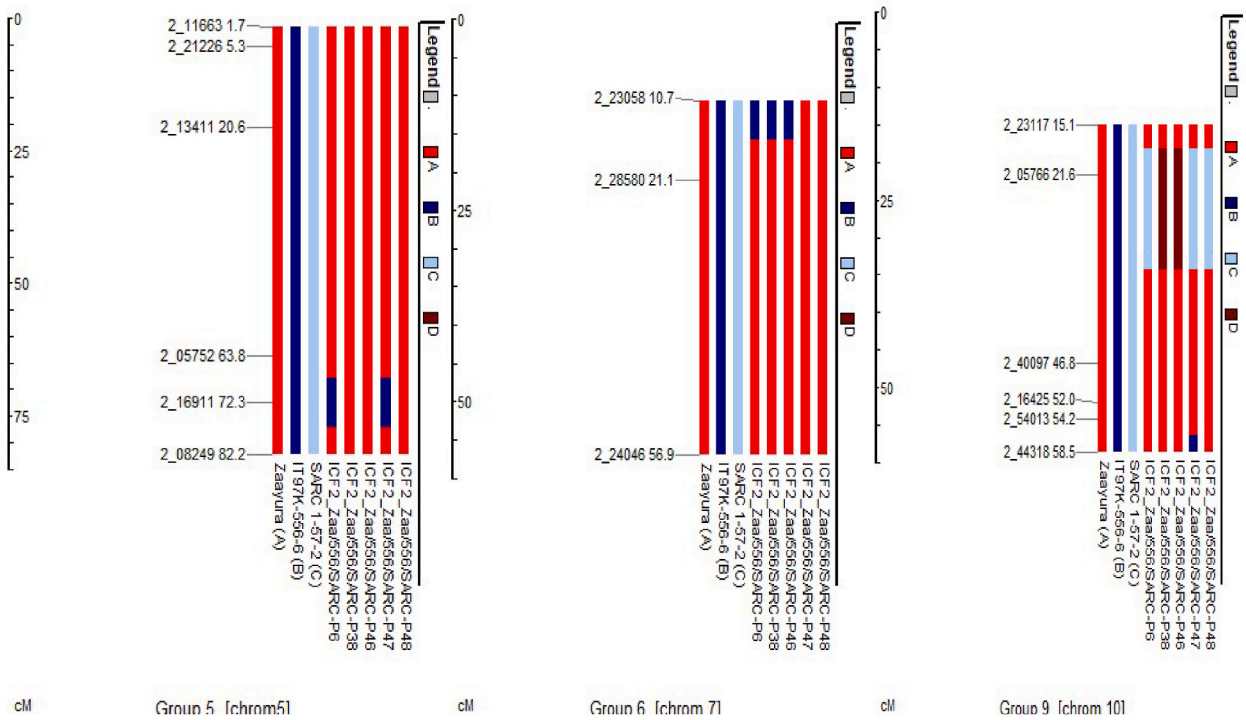

Fig. 9. Analysis of introgression segments associated with aphid resistance in chromosomes 5, 7 and 10. . = no amplification; A = zaayura genotype; B = IT97K-556-6 genotype; C = SARC-1-57-2 genotype and D = heterozygous genotype; Group 5 [chrom5] = chromosome 5; Group 6 [chrom 7] = chromosome 7; Group 9 [chrom 10] = chromosome 10.

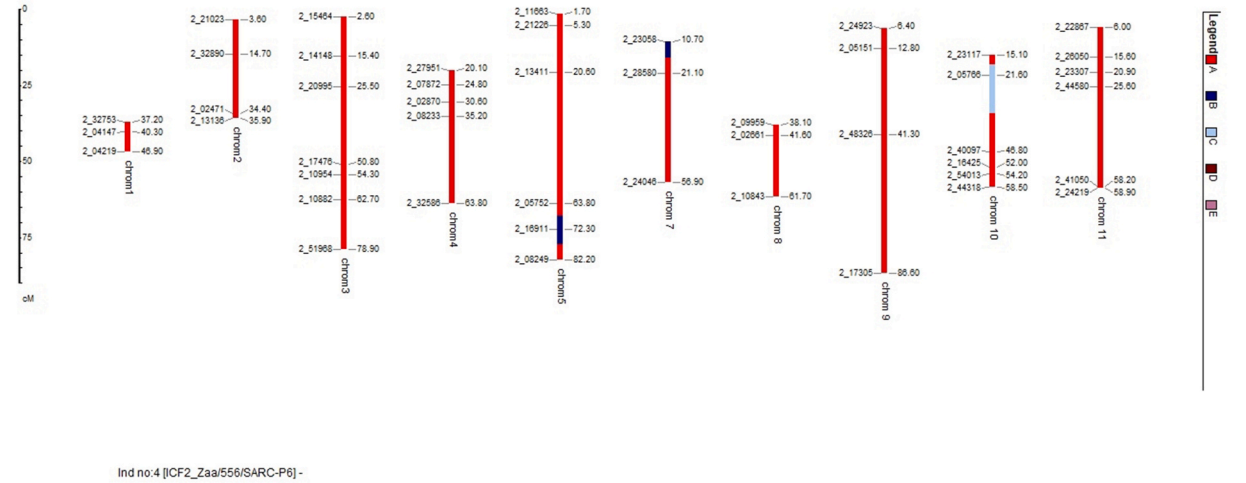

Fig. 10. Graphical genotype view of the best pyramided plant ICF2_Zaa/556/SARC-P6. . = no amplification; A = zaayura genotype; B = IT97K-556-6 genotype; C = SARC-1-57-2 genotype and D = heterozygous genotype.

4. Discussion

In a marker-assisted backcross breeding program, polymorphic molecular markers between the parental pairs are critical for success. Markers are utilized for both foreground and background selections to achieve remarkable success within a short period, contrary to conventional backcrossing that does not rely on markers. After intercrossing the BC_4F_1 of Zaayura/556 and Zaayura Pali, it was impossible to phenotypically identify the individual which combined both resistance genes under artificial aphid infestation. However, the use of CP 171F/172R and SNP 1_0912 linked to the aphid resistance in SARC-1-57-2 and IT97K-556-6, respectively,

Table 4
Reaction of pyramided lines and checks under aphid infestation.

Genotype	Aphid damage score	Aphid reaction
Apagbaala	4.7 ± 0.2	Susceptible
Zaayura	3.8 ± 0.1	Susceptible
IT97K-556-6	3.0 ± 0.2	Resistant
SARC 1-57-2	2.7 ± 0.1	Resistant
ICF_4_ ZAA/556/SARC-P6	2.0 ± 0.1	Very resistant
ICF_4_ZAA/556/SARC-P38	2.0 ± 0.2	Very resistant
ICF_4_ZAA/556/SARC-P46	2.3 ± 0.2	Very resistant
ICF_4_ZAA/556/SARC-P47	2.2 ± 0.1	Very resistant
ICF_4_ZAA/556/SARC-P48	2.1 ± 0.2	Very resistant
Mean	2.7	
CV (%)	18.8	
HSD (5 %)	0.6	

Aphid damage score means ± standard error of means; CV = coefficient of variation; HSD = Tukey honest significant difference.

Table 5
Agronomic performance of pyramided lines and parents.

Genotype	Canopy (cm)	Plant height (cm)	pod length (cm)	Pods/plant	Seeds/pod	HSW (g)	Grain yield (kg/ha)
Zaayura	57.5	39.1	14.6	17.3	8.9	22.9	2154
SARC 1-57-2	57.7	32.4	16.1	15.5	11.1	19.2	2017
IT97K-556-6	61.3	40.1	18.7	12.5	13.4	19.3	1701
ICF4_ZAA/556/SARC-P6	61.6	42.9	15.5	20.8	10.6	24.1	2134
ICF4_ZAA/556/SARC-P48	60.4	41.6	16.2	27.5	11.5	25.0	2738
ICF4_ZAA/556/SARC-P47	56.8	39.4	16.6	21.9	11.0	22.7	2595
ICF4_ZAA/556/SARC-P46	56.7	36.6	16.9	23.2	11.5	26.9	2937
ICF4_ ZAA/556/SARC-P38	57.7	42	15.5	30.3	10.8	25.6	4565
Mean	58.7	39.3	16.3	21.1	11.1	23.2	2605
CV (%)	6.4	12.2	5.8	28.6	9.5	7.1	11.9
HSD (5 %)	10.7	13.8	2.7	17.4	3.0	4.7	890.8

HSW = hundred seed weight; CV = coefficient of variation; HSD = Tukey honest significant difference.

allowed individuals with both resistance genes to be selected. The heterozygous individuals with close phenotypic resemblance to Zaayura were allowed to self to ICF_2. Among the ICF_2s, only five plants, namely ICF_2_Zaa/556/SARC-P6, -P38, -P46, -P47 and -P48, were homozygous resistant for both genes.

The breeders' aim in a backcross breeding program is to select progenies with the trait of interest without compromising on the desired traits of the recurrent parent. Background selection using molecular markers ensures the recovery of the recurrent parent genome within the shortest possible time [21]. Molecular markers can be used to select against linkage drag, thus reducing the number of backcrossing by 10-fold [22]. As a general practice, the more the markers used in marker-assisted selection, the more efficient the selection [23,24]. Although background selection is a necessary procedure in marker-assisted backcross breeding, its application is hampered by the low detection of polymorphic markers, cost and execution time [25,26].

The number of polymorphic markers used for the background selection in this study, 47, which represents 24.5 %, was relatively low. These markers, however, could satisfactorily reveal the recurrent parent genome recovery in the ICF_2 double homozygous lines. None of the markers on chromosome 6 that were screened was polymorphic and thus omitted from the background analysis. The low rate of polymorphic markers among a crop species or a collection indicates its narrow genetic base among the lines. According to Sundaram et al. [27], an adequate recurrent genome was recovered when a limited number of polymorphic markers were used for background selection in a fourth backcross generation in addition to the target trait in rice. Similarly, Linh et al. [28], and Arunakumari et al. [29], detected a low rate of polymorphic markers of 18.7 % and 12.6 %, respectively for their background selections.

Despite the low number of markers used for the background selection, the high recovery rate could also be due to the four generations of backcrossing used in the study. According to Sundaram et al. [27], a combination of four backcross generations and limited markers adequately yielded the high recovery of recurrent parent genome while introgressing bacterial blight resistance in the Samba Mahsuri rice variety. Ideally, background selection should be carried out at each backcross generation to ensure effective and rapid recovery of the recurrent parent genome [24,25]. However, background selection was only carried out after the improved lines were identified at the intercross F_2 stage due to scarce resources. In the absence of background selection at the backcross stages, a rigorous phenotypic selection based on qualitative traits of Zaayura was carried out after the foreground selection.

Theoretically, at BC_4, the least recurrent parent genome (RPG) expected to be recovered is 96.8 %. However, when near-isogenic lines (NILs) are intercrossed, the expected least RPG recovery at ICF_2 would be less than the RPG of the parental NILs. The least expected RPG of the ICF_2 is given by the formula $\boldsymbol{A} = \left[\frac{(\boldsymbol{P1}+\boldsymbol{P2})}{2}\right] \times \left[\mathbf{1} - \left(\frac{\mathbf{1}}{\mathbf{2}}\right)^{\boldsymbol{n}}\right]$, where $\boldsymbol{A}$ is the expected RPG, *P1* and *P2* are the RPG of the

intercross parents NILs, and ***n*** is the intercross generation. The $\left(\frac{1}{2}\right)^{n}$ component of the formula is the proportion of heterozygosity at the ***n***th generation of the intercross. Thus, for both intercross parents having RPG of 96.8 % (BC_4), the least expected RPG of the ICF_2 is 72.6 %. The number of recurrent parent genome (RPG) recovered in this study ranged from 72 to 93.8 % among the improved ICF_2 (double dominant homozygous) lines. Similarly, Arunakumari et al. [30], intercrossed lines with RPG of 90 % and 92 % and reported a recovery ranging from 82 % to 92 % among the pyramided ICF_2 rice lines. Apart from the ICF_2-16-59, which recovered 92 % of the RPG, all the pyramided lines identified had RPG less than the parental NILs [30].

The great extent of recurrent parent genome recovery over the expected 72.6 % at ICF_2 in this study could be attributed to the stringent phenotypic selection for qualitative traits following the foreground selection for the aphid resistance trait at each backcross stage. A modification to marker-assisted selection was suggested by Singh et al. [31], in which a rigorous phenotypic selection for desirable traits of the recurrent parent follows foreground selection of the target trait. This approach of marker-assisted selection, combined with phenotypic selection, was adopted in this study, and thus yielded the high recurrent parent genome recovery. This approach is economical and practical where resources are limited [26].

During the aphid resistance screening, the seedlings of the pyramided lines grew vigorously despite the presence of aphid colonies on them. These lines and the resistant checks sustained minimal aphid damage. According to Kusi et al. [16], seedlings of resistant genotypes maintained green leaves, grew vigorously, and had higher survival despite being infested with aphids. On the other hand, susceptibility was marked by stunted growth, capping of upper leaves and the yellowing of lower leaves due to aphid feeding. The susceptible checks sustained severe aphid damage including weak stem and even the death of some of the seedlings. In the present study the pyramided lines showed stronger resistance to aphid than the two donor parents (resistant checks). The significant difference in aphid damage score between the pyramided lines and the resistant checks may be due to the selection of transgressive segregants during the backcross stage. These pyramided lines do not only show higher resistance than the donor parents but are a promise of durable resistance in the case of resistance breakdown.

The evaluation of the agronomic performance of the pyramided lines was to elucidate their agronomic similarities with their recurrent parent. The success s of a backcross breeding program is when the trait of interest is introgressed without compromising the desired traits of the recurrent parent [21]. From the field evaluation, there were no significant differences between the pyramided lines and their recurrent parent, Zaayura, for all the traits except grain yield. This indicates that there were no tradeoffs or penalties for those traits as far as pyramiding the aphid resistant genes were concerned. Similar observations were made by Huynh et al. [32], when the aphid resistant cowpea had qualities like its recurrent parent.

Interestingly, the grain yield of ICF_Zaa/556/SARC-P38 was significantly higher than that of Zaayura. The high number of pods per plant translated into the yield that was observed for the line. This pyramided line is thus a transgressive segregant since it out-yielded its' parents. The no significant differences between the recurrent and the donor parents for all the traits measured is an indication of how similar the agronomic performance of the parents are. The results from this study demonstrate that in a backcross program, the level of progress can the improved and linkage drags reduced when both donor and recurrent parents are elite lines and do not differ much in terms of traits of interest.

5. Limitations and future direction

The molecular markers used for the foreground selection are not functional markers (they are not located within the gene) and that necessitated the intermittent aphid resistance screening under artificial infestation to confirm the aphid resistance status of the progenies. In this study, no polymorphic markers were identified on chromosome 6 for the background selection which is an indication of how conserved the regions on that chromosome are and how related the lines are. The exploitation of more molecular markers may lead to identification of more polymorphic markers for the background selection. Finally, the generalization for aphid resistance made in this study is with respect to seedling or vegetative stage resistance of the cowpea plant and did not consider the aphid resistance at the reproductive stage. This is because aphids are the main pest at the vegetative stage of the crop that need controlling. At the reproductive stage many insect pests attack the crop and any attempt to control one of them will automatically control aphids.

6. Conclusion

In this study, Zaayura was improved with two aphid resistance genes using CP 171F/172R and SNP 1_0912 as the foreground markers through markers–assisted backcrossing. These foreground markers allowed for the identification of five pyramided lines (double homozygous) among the ICF_2 plants. The extent of recurrent parent genome recovery among the five pyramided lines ranged from 72 to 93.8 %. Although the number of polymorphic markers for the background analysis was low, a high rate of recovery was achieved because of the high number of backcrossing and rigorous phenotypic selection at each backcross stage. Evaluation of the lines showed that the pyramided lines exhibited strong resistance to aphids relative to the donor parents. However, there were no significant differences between the pyramided lines and the recurrent parent for all the agronomic traits measured except yield. The pyramided lines developed in this study may be released as new varieties or serve as a valuable genetic resource for other breeding programs that seek to improve cowpea for aphid resistance.

Data availability

Data are available upon request by writing to the corresponding author.

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CRediT authorship contribution statement

Patrick Attamah: Writing – original draft, Methodology, Investigation, Formal analysis. **Francis Kusi:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Alexander Wireko Kena:** Supervision, Software, Resources, Data curation. **Frederick J. Awuku:** Investigation, Formal analysis, Data curation. **Salim Lamini:** Methodology, Investigation, Formal analysis, Data curation. **Gloria Mensah:** Writing – review & editing, Methodology, Investigation, Data curation. **Mukhtaru Zackaria:** Investigation, Formal analysis, Data curation. **Emmanuel Yaw Owusu:** Writing – review & editing, Methodology, Investigation, Data curation. **Richard Akromah:** Supervision, Software, Resources, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Francis Kusi reports financial support and equipment, drugs, or supplies were provided by Kirkhouse Trust. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31976.

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