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Evaluation of fitness in F_2 generations of Africa Biofortified Sorghum event 188 and weedy *Sorghum bicolor* ssp. *drummondii*

Titus Magomere [a,b,*], Silas Obukosia [a], Mark Albertsen [c], Florence Wambugu [a], Daniel Kamanga [a], Michael Njuguna [a], Jim Gaffney [c], Zuo-Yu Zhao [c], Ping Che [c], Antony Aseta [a], Esther Kimani [d], Evans Mwasame [d]

[a] *Africa Harvest Biotechnology Foundation International, P.O. Box 642, 00621-Village Market, Nairobi, Kenya*
[b] *Department of Biochemistry & Biotechnology, School of Pure and Applied Sciences, Kenyatta University, P.O. Box 43844, Nairobi, Kenya*
[c] *DuPont Pioneer, 7250 NW 62nd Avenue, Johnston, IA, United States*
[d] *Kenya Agriculture and Livestock Research Organization, P. O Box 57811, 00200 Nairobi, Kenya*

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ABSTRACT

Background: Introgression of transgenes from crops to their wild species may enhance the adaptive advantage and therefore the invasiveness of and weedy forms. The study evaluated the effect of Africa Biofortified Sorghum (ABS) genes from ABS event 188 on the vegetative and reproductive features of the F_2 populations derived from crosses with *Sorghum bicolor* subsp. *drummondii*.
Results: F_1 populations were obtained from reciprocal crosses involving ABS event 188 and its null segregant with inbred weedy parents from *S. bicolor* subsp. *drummondii*. Four F_2 populations and four parental populations were raised in RCBD with 4 replications in a confined field plot for two seasons. Vegetative and reproductive traits were evaluated. The vigour shown in the F_2 populations from the reciprocal crosses involving ABS event 188 and *S. bicolor* subsp. *drummondii* was similar to that in the crosses involving the null segregant and *S. bicolor* subsp. *drummondii*. Differences in vegetative and reproductive parameters were observed between the parental controls and the F_2 populations. Examination of the above and below ground vegetative biomass showed lack of novel weedy related features like rhizomes.
Conclusions: Therefore, release of crops with ABS 188 transgenes into cropping systems is not likely to pose a risk of conferring additional adaptive advantage in the introgressing populations. The interaction of ABS genes in weedy backgrounds will also not have an effect towards enhancing the weedy features in these populations.

1. Introduction

Sorghum was domesticated in Ethiopia and the surrounding countries as early as 4000–3000 BC [1]. Disruptive selection has been important in the evolution of the species resulting in several levels of a particular character being maintained in the population [2,3]. Species belonging in the sorghum genus are described under the family Poaceae, tribe Andropogoneae and subtribe Sorghinae. The genus is separated into five taxonomic subgenera; Eu-Sorghum, Chaetosorghum, Heterosorghum, Para-sorghum and Stiposorghum [4]. Crop sorghums and other important weedy and wild members of the genus belong to the sub genera Eu-Sorghum.

Crop sorghum (*Sorghum bicolor*) is the fifth most important cereal in the world agriculture [2]. Increase in yield (809 kg/ha to 912.9 kg/ha) and production (10 million tons) has been recorded in the last 45 years [5]. Breeding efforts have been focused on reducing several production and utility constraints and improving yield [6]. Currently, genetic transformation techniques are being applied to improve the crop on first generation traits (herbicide and insect resistance) and second generation traits (abiotic, biotic stress tolerance and enhanced nutrient quality) [7,8]. Wild populations in Eu-sorghum include species; *Sorghum propinquum*, *Sorghum halepense* and subspecies Drumondii, Arundinaceum/*verticilliflorum* [9,10].

Transgenic crops growing in traditional agriculture systems may pose dangers to the ecological stability and living organisms inhabiting such ecologies. The engineered genes (transgenes) may be transferred through pollen to wild progenitors, whose hybrid offspring may then be more aggressive and invasive [11,12]. This gene flow may destabilize both the cultivated and wild sorghums

* Corresponding author.
E-mail address: magomeretito@gmail.com (T. Magomere).
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gene pools in secondary and primary centres of origin resulting in reduction of genetic diversity and thus diminishing potential future sources of genes necessary to counter biotic and abiotic stresses.

Uncontrolled movement of crop transgenes into non-target plant populations may have four harm situations and subsequent risk scenarios [13]. These include loss of valuable genetic diversity in crop or compatible species, where there is risk of loss of allelic diversity due to selective sweep and genetic swamping leading to extinction and assimilation. Secondly there is risk of loss of abundance of wild sorghums due to out-breeding depression and pest preference [14,15,16]. Loss in abundance or diversity of valued native flora due to enhanced competition with wild sorghums that have acquired crop transgenes through interspecific hybridization is seen as an important risk [13]. A third risk has been identified as the reduction in abundance or diversity of valued fauna (wildlife or domestic animals) as a result of increased toxicity of the products of the crop transgene within the wild sorghums. The fourth potential harm relates to the significant decrease in yield of crops due to increased reservoirs for crop pests among the wild populations possessing the crop transgene [13,17]. Interactions between the transgene and wild genes in sorghum weeds may increase selective advantage, invasiveness in the wild sorghum populations [13].

The risks associated with transgenic crops have necessitated the development of institutions and regulations to govern their judicious use. These regulations stipulate necessary confines of growing transgenic crops in experimental stations. Confined field trials (CFT) have become popular due to their potential for physical confinement of transgenic crops and associated experimental genetic material [18]. Assessment of gene flow from transgenic crops and the associated effect of the transgenes and other linked genes on the adaptive fitness and changes in fecundity has been noted to be important in risk assessment of genetically modified crops [19]. This is necessary for application for de-regulation of transgenic crops in most bio-safety regulatory institutions.

Studies within Poaceae have shown that F_1 hybrids between round-up-ready corn with teosinte showed significantly higher vegetative vigor than that of teosinte [20]. The F_1 hybrids obtained did not exhibit a direct or negative impact of the transgene on reproductive fitness in the absence of selective pressure from glyphosate [20]. F_1 hybrids between crop sorghum and shattercane exhibited higher vegetative vigor and higher biomass production as compared to the parents [21]. However, the hybrid between shattercane × *S. bicolor* did not show any difference in ecological reproductive fitness with its parents [21].

ABS sorghum (ABS 188) possesses transgenes for high accumulation of beta-carotene (up to 5 μg/g from wild type 0.5 μg/g) in the endosperm and reduction of phytic acid for improved bioavailability of iron and zinc. These traits have not been previously associated with improved vegetative or reproductive success in sorghum. However, the interaction of these alleles in wild backgrounds and possible epistatic effect of the transgenes would act to reduce or enhance the fitness related traits in important sorghum weeds [13].

The release of genetically modified crops will expose transgenes to wild biotypes commonly found in East Africa. *S. bicolor* ssp. *drummondii and S. bicolor* ssp. *verticilliflorum* are important weeds in cropping systems in East Africa [22]. These weeds have similar agronomic requirements as the crop thus they are found to grow in sympatry in the tropics. This situation increases the risk of introgressing crop transgenes in the weeds which may confer adaptive advantage to the weeds making it hard to control them in agricultural field [15]. Thus regulation of the use of transgenic varieties needs to be informed by the nature of specific transgenes, information of hybridization and introgression of crop genes in weeds, relative fitness of the crop/weed hybrids and possible gene flow and introgression mitigation strategies.

The goal of the study was to evaluate the effect of ABS genes from ABS event 188 (*S. bicolor*) on the vegetative and reproductive features of the F_2 populations derived from crosses with wild sorghums.

2. Materials and methods

2.1. Genetic materials used in the experiment

2.1.1. ABS sorghum event 188

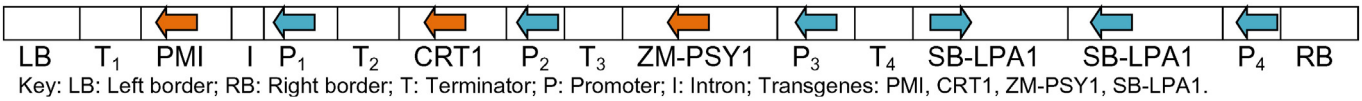
ABS sorghum (ABS 188) possessing transgenes for high accumulation of beta-carotene in the endosperm [23] and reduction of phytic acid [24] for improved bioavailability of iron and zinc were obtained from DuPont/Pioneer. The transgene construct (Fig. 1) was introduced into inbred lines of TX430 through *Agrobacterium tumefaciens* mediated transformation of immature sorghum embryos [25]. The transformed calli was selected on medium containing mannose (a hexose sugar). Calli containing the phospho-mannose isomarese (PMI) enzyme from *E. coli* grew on the selection medium due to the conversion of mannose-6-phosphate to fructose-6-phosphate. Without this enzyme the non-transformed calli experiences a block in glucolysis due to accumulation of mannose-6-phosphate that inhibits the enzyme phosphoglucose isomerase. The accumulation of M-6-P also depletes the cell inorganic phosphate required for ATP synthesis and inhibits plant cell growth in the untransformed calli.

Regenerated plantlets possessing the ABS transgenes were transplanted into pots and acclimatized to obtain T_0 seed. The T_1 and T_2 populations were raised and the null segregants isolated from the T_2 generation using QPCR, southern blot and western blot assays. The T_2 seeds and their null segregants were shipped to Kenya for the hybridization experiments after the necessary regulatory documents were processed. Two sorghum accessions were used as control populations in hybridization and evaluation of F_2 population for the effect of ABS alleles in wild and weedy sorghums. The two accessions included; Non transgenic *S. bicolor* ssp. *bicolor* (TX430) and Null segregates from transgenic ABS *S. bicolor* ssp. *bicolor*. Both were similar to the ABS event 188 but lacked the ABS alleles.

2.2. Selection of weedy parents

Wild sorghum accessions were selected from the materials obtained from the germplasm banks. The selected materials demonstrated wide genetic and morphological variation from the crop. These material were well adapted to both greenhouse and field conditions in Kenya. The materials were potentially inter-fertile with crop sorghum. The selected material were known locally sympatric species and had synchrony or closest synchrony in dates to first bloom. The diploid wild sorghum used as female parents for the study included the following;

- PI 330271 — *Sorghum bicolor* ssp. *drummondii* — IS 11988 — Ethiopia
- PI 532565 — *Sorghum bicolor* ssp. *verticilliflorum* — TCD 050 — Chad
- PI 153867 — *Sorghum bicolor* ssp. *verticilliflorum* — MN 1273 — Kenya



Key: LB: Left border; RB: Right border; T: Terminator; P: Promoter; I: Intron; Transgenes: PMI, CRT1, ZM-PSY1, SB-LPA1.

Fig. 1. ABS 188 event Ti plasmid insert showing the selectable marker and transgenes on the β-carotene pathway and phytate repression.

These wild sorghums have significant agricultural importance; they show potential of being weeds in agricultural fields. The wild populations possess important weedy structures like tillering and high fecundity. The accessions have average height for ease of manoeuvrability in the greenhouse and confined field trials (CFT) structures.

2.3. Inbreeding of S. bicolor, S. bicolor ssp. drummondii and S. bicolor ssp. verticilliflorum for 5 generations

Fourty seeds from each of the four species (*S. bicolor* ssp. bicolor, *S. bicolor* ssp. *drummondii*, *S. bicolor* ssp. *verticilliflorum* — PI 532565 and *S. bicolor* ssp. *verticilliflorum* — PI 153867) obtained from USDA were germinated and raised in a greenhouse at the College of Agriculture and Veterinary Sciences (CAVS) (-1° 14′ 59.72″, +36° 44′ 30.79″) of the University of Nairobi from January 2010 to December 2012. Planting was done in the greenhouse in pots for 5 generations (S_1–S_5). Seedlings were raised in sterile soil mixture in the greenhouse where the temperatures were maintained at 28/22°C day/night. A weekly fertilization regime of 300 mg/L Nitrogen, 250 mg/L phosphorus and 220 mg/L potassium was applied. Insect pests e.g. cutworms, spider mites and stem borers were controlled using pesticides. On the onset of bolting panicles of all plants were bagged and allowed to self pollinate and set seed. S_3 homozygous populations were used for hybridization while S_5 populations were used for comparison with the F_2 population from wild sorghums and ABS event 188.

2.4. Evaluation of homozygosity in the inbred populations

Codominant loci were used to provide a rapid method for identifying homozygous materials in the weedy S_3 populations that were used for hybridization. A protocol for extraction of DNA from the four accessions was optimized. Genomic DNA was extracted from 0.3 g of young leaves ground in liquid nitrogen using a CTAB extraction procedure modified from Doyle and Doyle, [26]. Cell lysis was done using 500 μL of buffer at 65°C (100 mM Tris pH 8.0; 1.4 M NaCl; 20 mM EDTA pH 8.0; 2% CTAB) for 30 min. Extraction was accomplished by 200 μL of chloroform/isoamyl alcohol (24:1), centrifuged at 6000 rpm for 15 min at 20°C. The supernatant was precipitated using isopropanol (1/10 vol at -20°C). The pellet was washed with 300 μL of 70% ethanol, dried and eluted in TE buffer. Finally, 3 μL of the extract was loaded on 2% agarose gels and run for 1 h at 100 V.

2.5. PCR of selected microsatellite loci

Primers for the study were selected based on the position of loci on the *S. bicolor* ssp. *bicolor BTX623* chromosomes, number of repeats, type of repeat, size of the amplified product, number of possible alleles among and within species, presence or absence, melting temperature and their codominance condition. The twenty identified primer pair sequences were sent to invitrogen for synthesis. The primer pairs obtained were resuspended in nuclease free water to make a stock solution of 100 μM and stored at -20°C in functional aliquots until PCR. Annealing temperatures differed due to the inherent difference among the melting temperature of different sets of primers. Annealing temperatures for the primer pairs ranged 1–2°C below each of their melting temperatures. The PCR denaturation and elongation temperatures were maintained at 94 and 72°C respectively and all programmes were set for 35 cycles. An initial denaturation step of 94°C for 3 min and a final elongation cycle of 72°C for 10 min were added. The results were scored on the absence or presence of alleles. Polymorphisms were also scored on the differential size of the bands amplified per reaction.

2.6. Confined field trial (CFT) for the hybridization experiment

To ensure confinement of seed, pollen and other possible underground propagation features a screenhouse with a 15 cm concrete floor was constructed within the National Biosafety Authority approved CFT facility at KARLO Kiboko (2°13′29.1″S 37°43′13.5″E). The experiments were undertaken from 24th April 2012 to 21st of September 2012. Pots were placed within the CFT and irrigated with drip pipes.

The screenhouse ensured confinement all the seed from crosses and the parental populations, barred birds from entering and leaving and kept roots and rhizomes away from the ground soil. In addition the structure ensured low human traffic in and out of the screenhouse, contained all vegetative material that were destroyed and burnt to ash after the experiment and improved control of soil and panicle insects.

2.7. Hybridization among ABS event 188, S. bicolor ssp. drummondii and S. bicolor ssp. verticilliflorum

Inbred seed that had been evaluated for homozygosity at polymorphic loci was harvested and prepared for planting. Three seeds were planted in 45 cm pots with Kiboko sandy-loam soils and individually labeled. The soil in the pots and the surrounding areas in and out of the screenhouse were drenched to control cut worms and other soil insects. The weedy species; *S. bicolor* ssp. *drummondii* and *S. bicolor* ssp. *verticilliflorum* which flower earlier than the ABS event and the nulls were staggered to allow uniform flowering for pollination. Flowering tillers were also used in increasing the pollination period. In the hybridization experiment both ABS event 188 and the ABS nulls were utilized to generate F_1s. The ABS genetic materials were used both as pollen donors and female parents. Giving rise to eight F_1 populations (Table 1), that were assayed for successful hybridization and advance to F_2 for the fitness assay.

Both hand emasculation and plastic bag emasculation methods were important in obtaining hybrid seed. The ready panicles were trimmed and 15 to 30 florets were emasculated. Ready stigmas were exposed to pollen from male parents twice to ensure pollination and bagged till seed set. The seed was harvested and stored till the next planting.

2.8. Evaluation of F_1 crosses from ABS event 188, S. bicolor ssp. drummondii and S. bicolor ssp. verticilliflorum

Seeds obtained from crosses between sorghum and weed species and their reciprocals (Table 2) were planted in pots at the Kiboko CFT site from 2nd of October 2012 to Jan 2013. The first evaluation involved establishing successful crossing among ABS genetic materials and wild sorghums. A PMI test was applied for crosses involving ABS event 188 while visual examination of the vegetative biomass for hybrid vigor was utilized for crosses involving ABS nulls (controls) where the PMI gene was absent. This evaluation was important where ABS event 188 was the female parent and tracking with the PMI kit would yield positives with or without hybridization. The second evaluation was done on possible variation in germination of seeds

Table 1
Crosses made between the crop and wild sorghums.

No.	Cross
1	*Sorghum bicolor* ssp. *drummondii* × Tx430 (ABS 188)
2	Tx430 (ABS 188)× *Sorghum bicolor* ssp. *drummondii*
3	*Sorghum bicolor* ssp. *Drummondii* × Tx430 (Null ABS 188)
4	TX430 (Null ABS 188) × *Sorghum bicolor* ssp. *drummondii*
5	*Sorghum bicolor* ssp. *verticilliflorum* × Tx430 (ABS 188)
6	Tx430 (ABS 188) × *Sorghum bicolor* ssp. *verticilliflorum*
7	*Sorghum bicolor* ssp. *verticilliflorum* × Tx430 (Null ABS 188)
8	TX430 (Null ABS 188) × *Sorghum bicolor* ssp. *verticilliflorum*

NB: *Sorghum bicolor* ssp. *verticilliflorum* population 2 was used.

Table 2
F_1 and F_2 populations, parents and control accessions evaluated.

1	TX430	7	ABS 188 × *Sorghum bicolor* ssp. *drummondii*
2	ABS 188 Null	8	*Sorghum bicolor* ssp. *verticilliflorum* × ABS 188
3	ABS 188	9	*Sorghum bicolor* ssp. *drummondii* × ABS 188 Null
4	*Sorghum bicolor* ssp. *verticilliflorum*	10	*Sorghum bicolor* ssp. *verticilliflorum* × ABS 188 Null
5	*Sorghum bicolor* ssp. *drummondii*	11	ABS 188 Null × *Sorghum bicolor* ssp. *drummondii*
6	ABS 188 × *Sorghum bicolor* ssp. *verticilliflorum*	12	ABS 188 Null × *Sorghum bicolor* ssp. *verticilliflorum*

NB: *Sorghum bicolor* ssp. *verticilliflorum* population 2 was used.

from crosses and reciprocal crosses involving ABS genetic material and weedy accessions. The parent genotypes and seeds obtained from hand crosses were raised on germination racks for 14 d. The germination racks were raised 1 cm from the cement floor and top covered with a clear polyethylene sheet to maintain relatively high soil temperatures and moisture to break any inherent dormancy. Germination data was taken and the seedlings were transplanted into 15 cm wide pots with sandy loam soil.

Evaluation for presence of ABS transgenes in the F_1s using PMI lateral flow dip sticks (Agrastrip® PMI Strip Test — Seedchek from Romer Labs) was done on leaves discs retrieved from 30 d old seedlings within the CFT. The F_1s obtained from crosses having ABS 188 as one of the parents were labeled and the young leaves were identified. Eppendorf tubes 1.5 mL were used to collect leaf discs from the sampled leaves. A wooden rod (5 cm long) was utilized to grind the leaf discs in 500 μL 1X PBST buffer (8.0 g sodium chloride, 1.15 g sodium phosphate, 0.2 g potassium phosphate, 0.2 g potassium chloride 0.5 mL Tween-20 in 1000 mL, pH 7.4). Once the buffer was green, a PMI dip stick was inserted into the Eppendorf tube and incubated in room temperature for 10 min before taking data. Non-transgenic material (TX430) and the null segregates from the T_2 population were used as the negative controls while the ABS event 188 was the positive control.

2.9. Evaluation of F_2 Africa Biofortified Sorghum Hybrid with wild sorghums for changes in fitness related traits

Test genotypes and controls (Table 2) were raised in the NBA approved CFT, both in the screenhouse (with concrete slab) and field (unenclosed in the CFT). The field experiments were carried out in RCBD with 4 replications for two seasons at KALRO Kiboko from 20th February 2013 to 18th January 2014 to evaluate the vegetative parameters. A CRD design was adopted for the screenhouse experiments where reproductive traits were evaluated for two seasons. The F_2 populations were evaluated for any obvious differences in weediness or invasiveness between the populations with the transgenes and those without.

The seeds were first germinated on racks in the screenhouse to break dormancy and ensure uniformity. Fifty plants were transplanted per plot in each of the twelve plots representing the twelve species (treatments) per block. Planting was done at a spacing of 0.5 m × 0.5 m in 5 m × 2 m plots. A border row was maintained with two rows of *S. bicolor* (TX430). Diammonium phosphate; DAP (18% N, 46% P_2O_5, 0% K_2O) was applied on the rows (120 kg/ha) during transplanting and Calcium ammonium nitrate; CAN ($5Ca(NO_3)_2 \times NH_4NO_3 \times 10H_2O$ — 8% calcium and 21–27% nitrogen) was applied (135 kg/ha) when plants attained knee height.

Insect pests, such as cutworms, spider mites, shoot flies and stem borers were controlled using miticide Ortus®, SC 5% (fenpyroximate), pesticides Dursban® 50W (chlorpyrifos), Polytrin® P440 EC. Harvested seed was dressed with Thiram ®50WP. The plants were drip irrigated twice a week to ensure uniform soil moisture in all plots throughout the growing season. All panicles in the field experiment were destroyed at booting stage, while plants in the screenhouse were allowed to flower and set seed while bagged. Vegetative materials and extra seeds were destroyed within the CFT after harvesting.

2.10. Data collection and analysis

Data were collected on a fortnight basis throughout the growth phase on all vegetative traits from each individual plant in the plots. Reproductive data were collected at physiological maturity from each individual plant in plots in the screen-house. Plants from the outermost row of each of the plots were not used in the analysis to reduce variability due to low competition (more space) on the edges. A linear model was applied for the analysis of variance considering the treatment effect, block effect, treatment x block effect and a random element of variation. Analysis of variance (ANOVA), analysis of differences among means, discriminant analysis and Mahalanobis analysis of D^2 intergroup distances was done in GENSTAT 15.

3. Results and discussion

3.1. Homozygosity in inbred populations of wild sorghums

Selfed parental genotypes from *S. bicolor* ssp. *verticilliflorum* and *S. bicolor* ssp. *drummondii* exhibited similar banding profiles on agarose gels within the species, they were also morphologically similar. Plants from selfed populations gave a monomorphic banding profile on loci that show polymorphisms in unselfed populations.

Fragments of 300 bp and 260 bp were amplified from *S. bicolor* ssp. *drummondii* plants from inbred populations on loci SB1764 and SB5293 respectively, but did not amplify alleles from loci SB960 and SB297 (Fig. 2a, b). *S. bicolor* ssp. *verticilliflorum* (population 1) samples from inbred populations had the 200 bp allele, 840 bp allele, 280 bp allele, and 270 bp from SB960, SB1764, SB5293 and SB297 (Fig. 2a, b). *S. bicolor* ssp. *verticilliflorum* (population 2) samples from inbred populations had the 200 bp allele, 840 bp allele and 300 bp allele from SB960, SB1764, SB5293, but did not amplify alleles from loci SB297 (Fig. 2a, b). Specific SSR analysis has shown utility in several genomic studies in sorghums [27,28] due to the inherent polymorphism from disruptive selection [2,3] and presence or sequence data in gene banks.

3.2. Hybridization among ABS event 188, S. bicolor ssp. drummondii and S. bicolor ssp. verticilliflorum

The F_1 plants where ABS188 was the female parent and crosses that involved the Null segregant where the PMI gene was absent had enhanced phenotypic characteristics due to heterosis (data not presented). These included wider leaves, plants showing increase in height as compared with the crop, plants with increased tillers, F_1 plants with crop like (greenish) midrib and crop like wider culms. The F_1 plants also set flowers earlier (Fig. 3).

F_1 plants from crosses involving ABS event 188 were positive for the PMI gene (Fig. 4). However, varying degrees of hybridization were observed in the populations. ABS188 which was used as the positive control had two bands on the PMI strip while the null segregant had

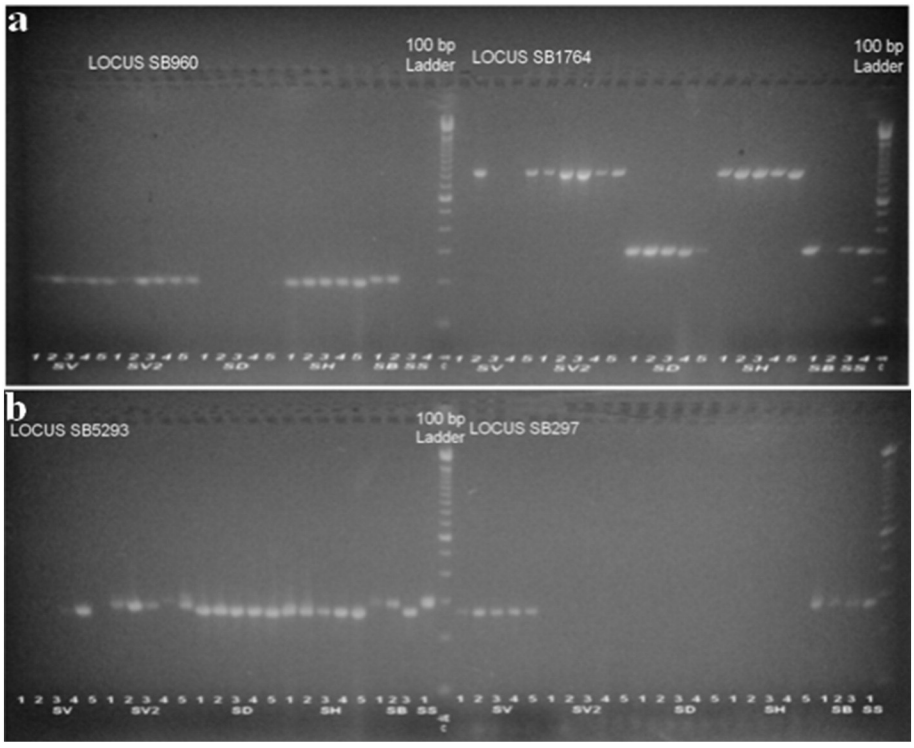


Fig. 2. 4% agarose gel electrophoresis showing (a) alleles and null alleles in wild sorghum populations on loci SB960 and SB1764, (b) homozygosity in wild sorghum populations on loci SB5293 and SB297. SV — *S. bicolor* ssp. verticilliflorum, SD — *S. bicolor* ssp. drummondii, SH — *S. halepense*, SB — *S. bicolor*, SS — *S. sudanense*.

Fig. 3. Phenotypic characteristics of the F_1s showing differences between the hybrids on the left and the non hybrids on the right.

one band as a negative control. Hybrids between crop and wild sorghums were recovered as shown in Table 3. Non-hybrids were all uprooted and stored in biohazard bags awaiting disposal by burning.

3.3. Germination of hybrids among ABS event 188, S. bicolor ssp. drummondii and S. bicolor ssp. verticilliflorum

The planted seed had variations in their germinations. The crop seeds including *S. bicolor* Tx430 (ABS 188) and *S. bicolor* Tx430 (Null ABS 188) had higher germinations than the weedy species (12–15%) and the F_1s where the weedy sorghums were the female parents (0–1.5%), (Table 4). This could be attributed to seed dormancy. The dormancy in the F_1s reduces viability of the seed which rots due to exposure of the endosperm (not protected by glumes). F_1s where the crop sorghums were the female parents had higher germination (Table 4).

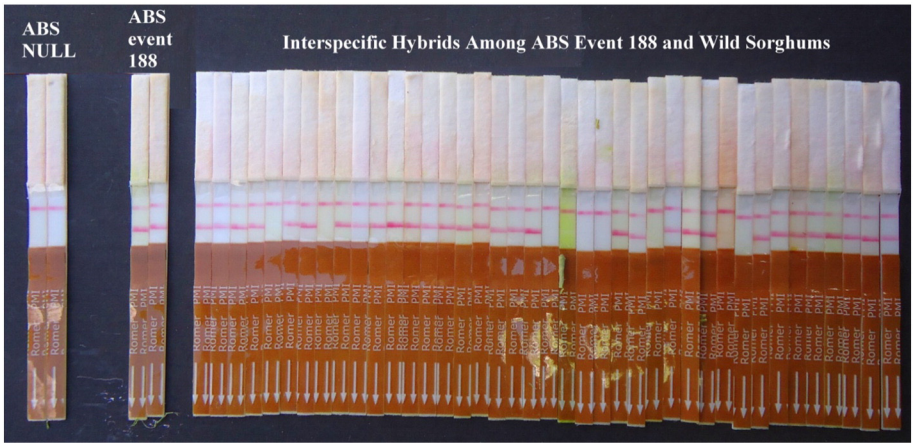


Fig. 4. PMI lateral flow strip test on parental and F_1 populations of *S. bicolor* ABS genetic material and *S. bicolor* ssp. *drummondii*.

Table 3
Evaluation of F_1s with the PMI kit.

Population	No. evaluated	No. With PMI
ABS 188 × *Sorghum bicolor* ssp. *drummondii*	24[a]	20
ABS 188 × *Sorghum bicolor* ssp. *verticilliflorum*	14[a]	2
Sorghum bicolor ssp. *verticilliflorum* × ABS 188	63	6
ABS 188	1	1
ABS 188 Null	2	0

[a] ABS 188 was used as the female parent thus both PMI and phenotypic (visual) characterization was applied to maintain the F_1s.

Table 4
Germination percentages of the F_1s among ABS event 188, *Sorghum bicolor* ssp. *drummondii* and *Sorghum bicolor ssp. verticilliflorum* (mean of 164 seeds per population).

	Population	% Germination
1	ABS 188 × *Sorghum bicolor* ssp. *drummondii*	100
2	ABS 188 × *Sorghum bicolor* ssp. *verticilliflorum*	100
3	ABS 188 null × *Sorghum bicolor* ssp. *drummondii*	100
4	ABS 188 null × *Sorghum bicolor* ssp. *verticilliflorum*	100
5	*Sorghum bicolor* ssp. *drummondii* × ABS 188	0
6	*Sorghum bicolor* ssp. *verticilliflorum* × ABS 188	1.4
7	*Sorghum bicolor* ssp. *drummondii* × ABS 188 Null	1.2
8	*Sorghum bicolor* ssp. *verticilliflorum* × ABS 188 Null	0
9	ABS 188 — *Sorghum bicolor* (Tx430)	77.4
10	ABS 188 null — *Sorghum bicolor* (Tx430)	73.8
11	*Sorghum bicolor* ssp. *drummondii*	12.4
12	*Sorghum bicolor* ssp. *verticilliflorum*	14.8

3.4. Vegetative features of the F_2 progenies from crosses between S. bicolor — ABS event 188/ABS null and S. bicolor ssp. drummondii

Evaluation of the vegetative features in the F_2 populations was based on plant height, total number of leaves, culm width and number of tillers at 47 d from germination. From the analysis the ABS event 188 population had a mean height of 96 cm (Fig. 5), which was not significantly different from the ABS Null (99.1 cm). The *S. bicolor* (TX430) population had a mean height of 109.4 cm, while *S. bicolor* ssp. *drummondii* attain a height of 180.3 cm. The F_2 populations involving crosses from *S. bicolor* ssp. *drummondii* and ABS Null were similar to those involving ABS event 188 and *S. bicolor* ssp. *drummondii*. This shows that in competitive situation the F_2s with ABS alleles may not out shade the populations without ABS alleles.

The *S. bicolor* parental populations had more leaves than those of the *S. bicolor* ssp. *drummondii* with ABS event 188, ABS Null and TX 430 showing means of 10, 11 and 11 leaves respectively at 47 d from germination (Fig. 5). The *S. bicolor* ssp. *drummondii* population had a mean of 7.35 leaves. The F_2 populations were not significantly different on the number of leaves. Crosses involving ABS event 188 had means of 9.2 leaves while those involving the ABS Nulls had means 8.8 leaves. Therefore implying lack of significant differences among the F_2 populations.

The culm width in the parental *S. bicolor* population were similar with ABS event 188, ABS Null and *S. bicolor* (TX430) having means of 1.8, 1.96 and 2.1 cm respectively (Fig. 5). The *S. bicolor* ssp. *drummondii* culms were thin with width means of 0.8 cm. However, the F_2 populations from crosses involving the ABS event 188 and the ABS Null were not significantly different. The increase in mean width due to ABS event 188 genes and non ABS genes in the ABS Null was similar.

Tiller expression was low in the *S. bicolor* populations, where ABS event 188, ABS Null and *S. bicolor* (TX430) had means of 2.8, 2.4 and 3.5 tillers respectively (Fig. 5). The *S. bicolor* ssp. *drummondii* population had high means of up to 12.5 tillers. The mean number of tillers did not differ significantly among the F_2 derived from crosses involving ABS event 188 or ABS Null parents with *S. bicolor* ssp. *drummondii*. Therefore the ABS event 188 alleles did not have a conditioning effect on the vegetative biomass in F_2 population.

Multivariate discriminant analysis using plant height, number of leaves, culm width and number of tillers, showed three clusters involving *S. bicolor* subsp. Drummondii, *S. bicolor* and the F_2 population (Fig. 6). Mahalanobis analysis of D^2 intergroup distances showed similarity on vegetative parameters among ABS event 188 vs ABS Null (0.726) ABS event 188 vs *S. bicolor* (TX430) (0.633) and ABS Null vs *S. bicolor* (TX430) (0.283) (Table 5). Comparison between *S. bicolor* ssp. *drummondii* vs ABS event 188 (28), *S. bicolor* ssp. *drummondii* vs ABS Null (33.8) or *S. bicolor* ssp. *drummondii* vs *S. bicolor* (TX430) (29.9) gave high D^2 distances on the vegetative parameters. Comparison of the F_2 population with parental populations also had high D^2 values ranging from 15 to 17.88. However, comparison of the F_2 population involving ABS Null or ABS event 188 parents with *S. bicolor* ssp. *drummondii* had very low D^2 values, suggesting that the population were similar. In the D^2 analysis ABS Null × *S. bicolor* ssp. *drummondii* vs ABS event 188 × *S. bicolor* ssp. *drummondii* had 0.446, *S. bicolor* ssp. *drummondii* × ABS Null vs ABS event 188 × *S. bicolor* ssp. *drummondii* had 0.063 while *S. bicolor* ssp.

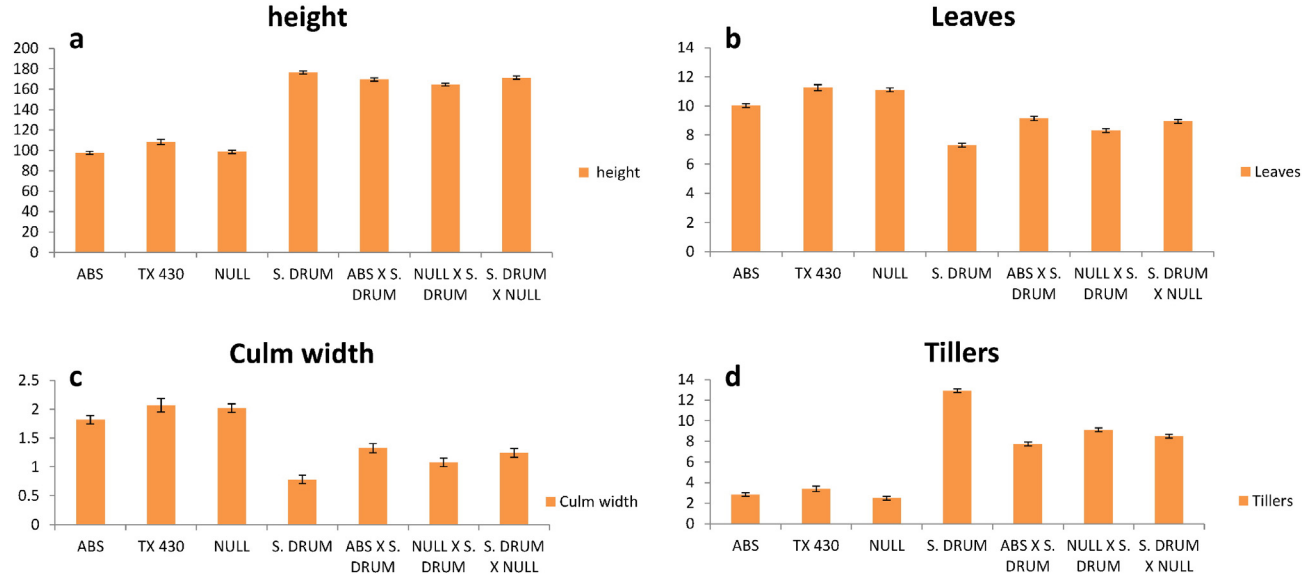


Fig. 5. Vegetative features of F_2 populations from crosses involving ABS event 188, *Sorghum bicolor* ssp. *drummondii*.

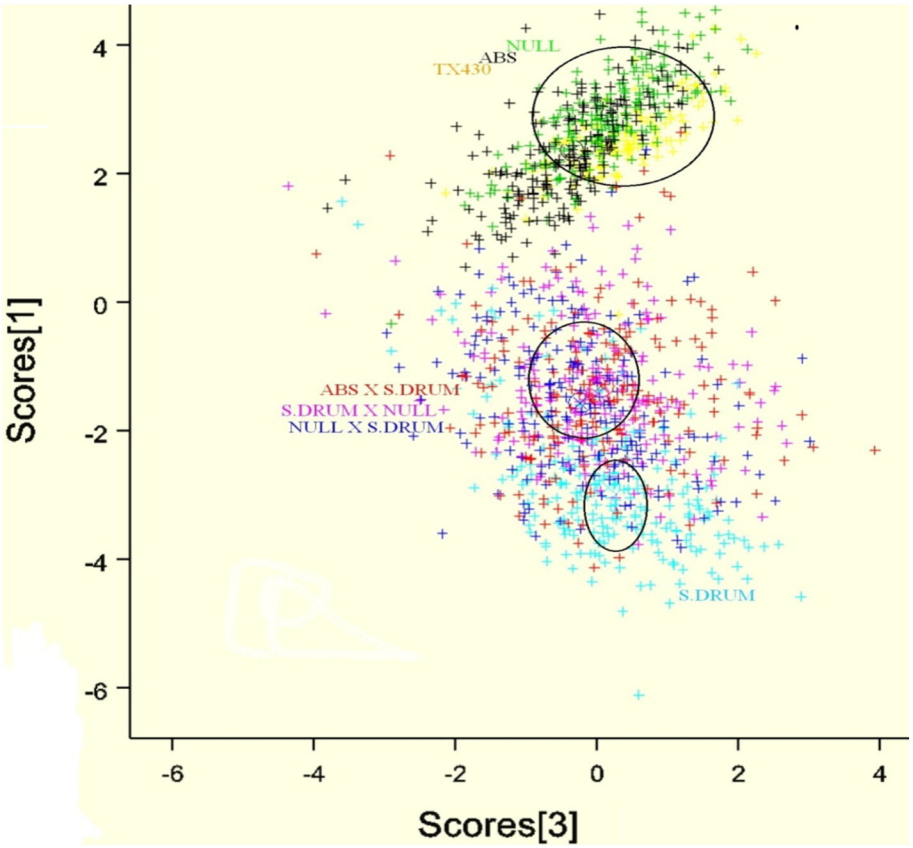


Fig. 6. Multivariate discriminant analysis using plant height, number of leaves, culm width and number of tillers.

drummondii × ABS Null vs ABS Null × *S. bicolor* ssp. *drummondii* had 0.1910 in the vegetative trait. This invalidates the possible effect of differential transgressive segregation in the F_2 populations derived since populations with and those without the ABS transgenes show lack of significant differences on the vegetative trait. This is vital while considering that the vigor observed at the F_1 diminishes in subsequent generations as its contribution to general vegetative diminishes [29,30,31].

3.5. Reproductive features of the F_2 progenies from crosses between *S. bicolor* — *ABS event 188/ABS null and S. bicolor ssp. drummondii*

Reproductive fitness of the F_2 and parental populations was evaluated on mean 100 seed weight, mean total seed number and mean total seed weight. From the analysis of 100 seed weight, the ABS event 188 population had 3.7 g. This was similar to the ABS Null 3.7 g, but significantly different from the *S. bicolor* (TX430) control population where 100 seed weight was observed to be 4.6 g. The *S. bicolor* ssp. *drummondii* parental population had only 1.4 g due to the seed possessing small endosperms. The reciprocal crosses involving ABS event 188 or *S. bicolor* ssp. *drummondii* as parental populations did not show significant differences (1.7 g) (Fig. 7).

Though both the F_1 and F_2 populations had higher 100 seed weight, the vigor in the control crosses from the ABS Null parents did not differ from the ABS event 188 suggesting that the ABS transgenes did not confer better adaptive traits in the progeny. The endosperms in the F_2 populations were similar and thus the ABS transgenes could not improve germination or enhance vigor (Fig. 7).

Total seed number from the ABS event 188 parents (372) significantly differed from that of the ABS Null (778) and *S. bicolor* (TX430) parents (872) but was similar to the *S. bicolor* ssp. *drummondii* parental population (444). However, the F_2 populations derived from the reciprocal crosses were all significantly similar (Fig. 7) suggesting that the presence or absence of the ABS event 188 transgenes did not have any effect on the total seed number in the F_2 populations. This implies lack of enhanced fitness in the introgression progenies from the crosses involving the ABS transgenes.

Further to this, the low seed number seen in the ABS event 188 parent is compensated in the crosses with *S. bicolor* ssp. *drummondii*, showing that backcrosses of the ABS transgenes in local material will

Table 5
Mahalanobis analysis of D^2 intergroup distances on vegetative parameters. Showing; Africa Biofortified Sorghum; ABS — ABS event 188, ABS null — null segregate, Wild Sorghum; S. Drum — *S. bicolor* ssp. *drummondii*, and control cultivar TX430 of *S. bicolor*.

ABS	0						
ABS × S. DRUM	14.547	0					
NULL	0.726	17.883	0				
NULL × S. DRUM	15.685	0.446	19.833	0			
S. DRUM	28.017	4.492	33.841	2.735	0		
S. DRUM × NULL	15.04	0.063	18.598	0.191	3.7	0	
TX 430	0.633	15.208	0.283	17.201	29.994	15.948	0
	ABS	ABS × S. DRUM	NULL	NULL × S. DRUM	S. DRUM	S. DRUM × NULL	TX 430

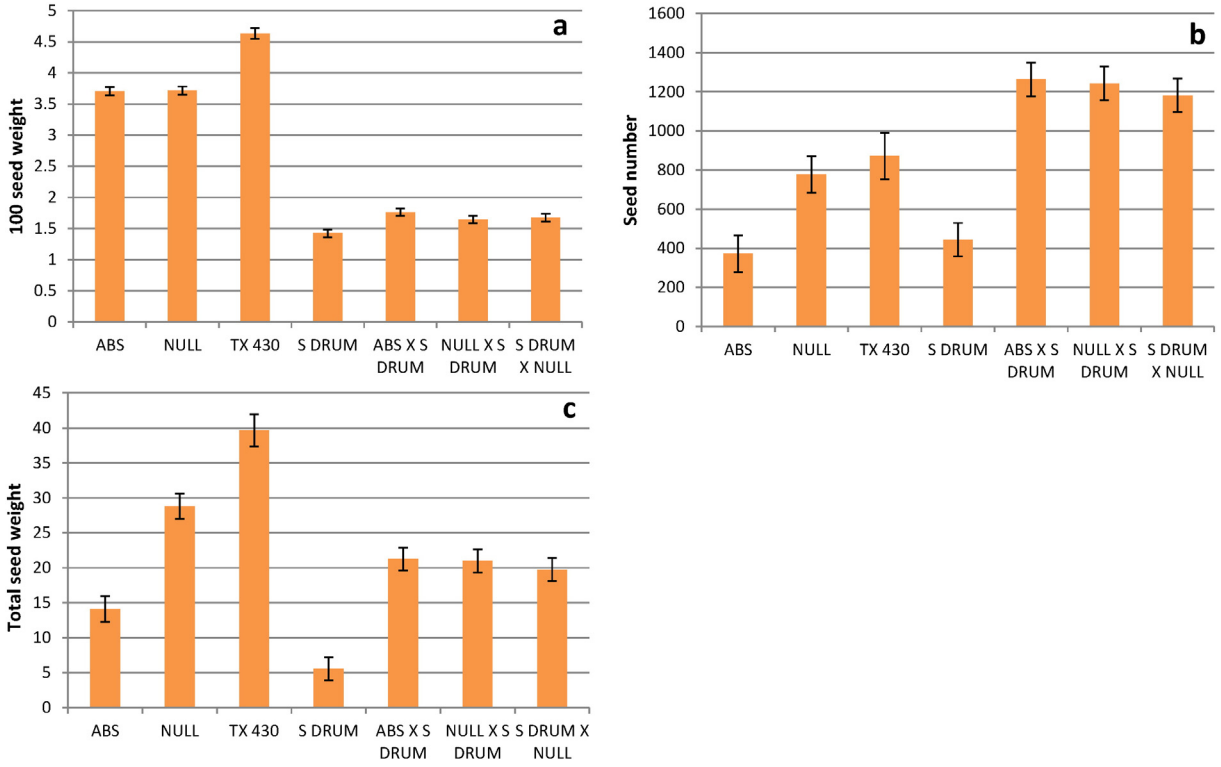


Fig. 7. Reproductive features of F_2 populations from crosses involving ABS event 188, *Sorghum bicolor* ssp. *drummondii*.

probably maintain high seed yield and the ABS traits. The elevated seed number in the F_2 could be attributed to transgressive segregation obtained from wide crossing.

Total seed weight per plant was high in the *S. bicolor* (TX430) (39.6 g) and the ABS Null (28.8 g) parents. The ABS event 188 parents had a mean of 14.1 g while the *S. bicolor* ssp. *drummondii* parent had a mean of 5.5 g. The F_2 population with or without the ABS transgene had significantly similar total seed weight. In addition they had maintained smaller endosperms from the *S. bicolor* ssp. *drummondii* parent thus low seed weight (Fig. 7). The differences in seed weight between the ABS event 188 and the ABS Null controls had diminished in the F_2 population, suggesting that, the differential expression of the ABS alleles on total seed weight was dependant on the genetic background.

Multivariate discriminant analysis using the mean 100 seed weight, mean total seed weight and mean total seed number, showed significant differences among the parental ABS event 188, *S. bicolor* ssp. *drummondii*, ABS Null and *S. bicolor* (TX430) populations (Fig. 8). Low Mahalanobis D^2 intergroup distances were observed between ABS event 188 vs ABS Null (2.1), ABS Null vs *S. bicolor* (TX430) (3.2) and *S. bicolor* (TX430) vs ABS event 188 (7.4) (Table 6). Conversely, high values were observed between ABS event 188 vs *S. bicolor* ssp. *drummondii* (15.6), ABS Null vs *S. bicolor* ssp. *drummondii* (17.1) and *S. bicolor* (TX430) vs *S. bicolor* ssp. *drummondii* (35.1). Significant differences were also observed between ABS event 188 vs ABS Null (2.1) ABS Null vs the F_2 from the reciprocal crosses with a D^2 value ranging from 12.5 to 13.652. These differences were also observed between ABS Null and the reciprocal crosses with D^2 values ranging between 12.7 and 13.5 (Table 6).

However, there were no differences among the F_2 populations obtained from reciprocal crosses between ABS event 188 or ABS Null and *S. bicolor* ssp. *drummondii*. They had low D^2 values of between 0.02 and 0.06. Based on the seed characteristics of the F_2 populations derived from crosses between ABS event 188, ABS Null and the wild *S. bicolor* ssp. *drummondii* (Fig. 9), it can be deduced that the ABS transgenes do not confer enhanced fitness. Thus, the use of such genes in conventional cropping systems may not pose a significant danger or risk of enhancing weediness of local wild species.

3.6. Testing for novel morphological features on F_2 populations derived from *S. bicolor* — ABS event 188/ABS null and *S. bicolor* ssp. drummondii

The F_2 plants from crosses involving ABS event 188 and *S. bicolor* ssp. *drummondii* did not demonstrate existence of special features found in uncultivated member of the Sorghum genus. Enhanced tillering was demonstrated in F_2 populations from both ABS event 188 and the null thus could not be attributed to the ABS genes (Fig. 10). However, vegetative propagation structures like rhizomes or their vestigial forms were not demonstrated in the ABS event 188 and

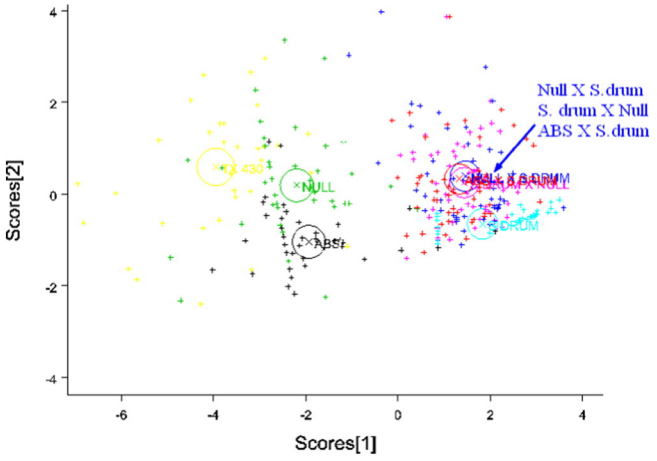


Fig. 8. Multivariate discriminant analysis using the mean 100 seed weight, mean total seed weight and mean total seed number.

Table 6
Mahalanobis analysis of D^2 intergroup distances on vegetative parameters. Showing; Africa Biofortified Sorghum; ABS — ABS event 188, ABS Null — Null segregate, Wild Sorghum; S. Drum — *S. bicolor* ssp. *drummondii*, and control cultivar TX430 of *S. bicolor*.

ABS	0						
ABS × S DRUM	12.528	0					
NULL	2.126	12.728	0				
NULL × S DRUM	13.682	0.058	13.517	0			
S DRUM	15.62	2.121	17.109	1.8	0		
S DRUM × NULL	13.187	0.055	13.359	0.02	1.547	0	
TX 430	7.378	28.265	3.229	29.434	35.052	29.328	0
	ABS	ABS × S DRUM	NULL	NULL × S DRUM	S DRUM	S DRUM × NULL	TX 430

Null crosses with *S. bicolor* ssp. *drummondii*. This analysis is vital based on the origin of *S. halepense*, which is the most important weed in the genus. This species has been shown to be a rhizomatous progenitor of *S. bicolor* and *S. propinquum* after disomic or segmental polyploidization [2,32].

Absence of fitness enhancing features was demonstrated when non-transgenic crop sorghum BTX623 was crossed with wild sorghums belonging to *S. sudanense* and *S. halepense* and the F_1s grown in competitive mixtures [29]. Sahoo et al. [21], also did not obtain rhizomes or other vegetative structures that could enhance fitness or fecundity in F_1 progenies derived from shattercane and crop sorghums.

4. Conclusions

Transgenic crops have the potential of introgressing with their wild progenitors culminating in harm to the ecology and traditional cropping systems through enhancing the fitness and invasiveness of the weedy materials. The composition of the transgene construct and trait of focus in a given transgenic event determines the impact of the harm. Introgression of ABS transgenes into wild sorghums did not confer significant changes on either the vegetative or reproductive parameters of the F_2 generation. Heterosis was demonstrated in the F_1 generation and it diminished in both ABS crosses and those without the ABS transgenes in the F_2 generation. This implies lack of differential epistasis or gene interaction that could be attributed to the presence of the ABS transgenes. This confirms that nutrition related traits engineered in the ABS sorghums could be integrated into cropping systems with little or no harm due to low invasiveness of its wild progenitors.

Conflict of interest statement

The authors declare no conflicts of interest.

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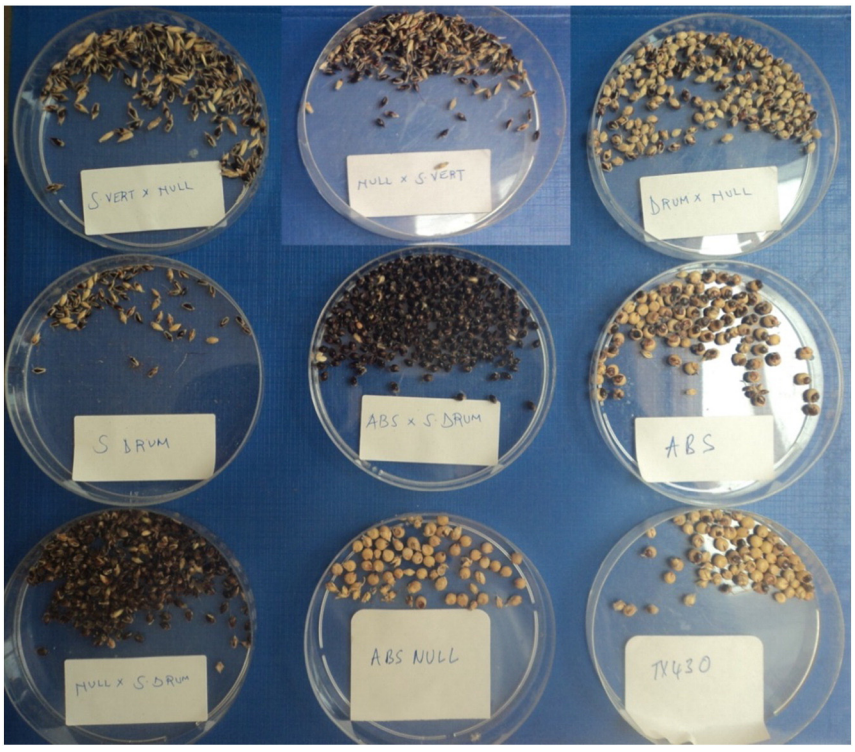

Fig. 9. Seed characteristics of parental and F_1 populations of *S. bicolor and S. bicolor* ssp. *drummondii*. Showing; Africa Biofortified Sorghum; ABS — ABS event 188, ABS null — null segregate, Wild Sorghum; S. Drum — *S. bicolor* ssp. *drummondii*, and control cultivar TX430 of *S. bicolor*.

Fig. 10. Root and vegetative biomass characteristics of F_2 populations of *S. bicolor* (ABS 188) *and S. bicolor* ssp. *drummondii*.

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