



Production of Sweet and High Biomass Sorghum Lines with Optimized Cell Wall Components for Increased Biofuel Bioconversion Yield

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJECC/2024/v14i44102

Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/115169>

Original Research Article

Received: 27/01/2024

Accepted: 01/04/2024

Published: 05/04/2024

ABSTRACT

The current research focuses on the estimation of fibre content in sweet and high biomass sorghum lines for Production of sweet and high biomass sorghum lines with optimized cell wall components for increased biofuel bioconversion yield, through partially replicated experimental design for F3 populations based on BLUPs values; this study was carried out for four seasons (from postrainy

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2020 to rainy 2022) to develop F3 populations of sweet and high-biomass *bmr* sorghum lines. The proximate fibre component analysis was done in the matured F3 populations by drying the plant samples and grinding them into a fine powder. Fibre quality components such as cellulose, hemicellulose, acid detergent fibre (ADF), acid detergent lignin (ADL), neutral detergent fibre (NDF), metabolizable energy (ME), nitrogen, in vitro organic matter digestibility (IVOMD), and ash, were assessed using Near-Infrared Reflectance Spectroscopy (NIRS). ICSV18003 had the lowest acid detergent fibre (ADF) percentage at 37.04%, while it also had the highest acid detergent lignin (ADL) percentage at 3.84%. The ash percentage was 3.73% in SSV84 x N609. Moreover, the *bmr* transferred lines of the F3 population exhibited higher levels of cellulose and hemicellulose, while lignin and ash content were decreased. This indicates that the *bmr6* and *bmr12* alleles can be confidently utilized in sorghum breeding for bioenergy production, as they meet the requirements for bioethanol production.

Keywords: F3 populations; *bmr6*; *bmr12*; proximate fibre analysis; *Sorghum bicolor* (L.) Moench.

1. INTRODUCTION

One of the biggest problems facing all nations in the twenty-first century is the world's never-ending need for energy. This circumstance has spurred the search for and implementation of sustainable energy generation options and the growing concern over carbon emissions that cause global warming and the depletion of non-renewable fossil fuel sources [1]. The viability of biofuels as a renewable energy source can depend on several variables, including the feedstock utilized in biofuel production, the economic infrastructure, geographic location and climate. As a result, nations should develop climate-appropriate oil-producing crops and set up incentives that would be simple to implement in their existing infrastructure [2].

Sweet sorghum is an excellent choice for semi-arid and arid marginal regions because it withstands water stress. Its morphophysiological traits confer drought endurance [3] and the C4 photosynthetic system facilitates adequate CO₂ fixation and extraordinary dry matter build-up [4]. Like most other lignocellulosic biomasses, sorghum biomass contains about 20% lignin [5]. High lignin content hinders biofuel synthesis even if it gives the cell wall mechanical strength. Lignin removal is expensive, which challenges manufacturing 2G commercial biofuels. *Bmr6* and *bmr12* play a significant role in the biosynthesis of sorghum lignin and help mutate C-to-T at position 486 relative to the transcription's starting site [5].

The hydrolysis of structural components of biofuel feedstocks is influenced by such as arabinose to xylose ratio, penetration of pretreatment chemicals, crystalline versus amorphous cellulose structure, contents of lignin

and hemicellulose, and their distributions for conversion to fuels [6,7,8]. Significant variations are observed in the chemical composition of soluble and structural components between a non-sweet sorghum hybrid and a sweet sorghum cultivar [9]. The nutritional composition and fraction of cell walls in fodder are directly correlated with the stage of maturity. The progressive maturation increases lignin levels, reducing digestibility, fodder quality, and animal productivity [10]. ADF concentration in forages represents the relative amount of cellulose and lignin in cell walls. The NDF number represents the combined cell wall content, including ADF and hemicellulose components. Lignin adheres to fibre components, such as cellulose and hemicellulose, and is a physical obstacle to microbial enzyme degradation of plant cell walls [11]. Several studies have documented the elevated nutritional content, dry-matter digestibility (DMD), and organic-matter digestibility (OMD) in BMR sorghum [12,13]. Biomass sorghum with mutant genotypes exhibits low lignin - *bmr* (brown midrib) content and sensitive to photoperiod, which differ in terms of cell wall composition, fibre digestibility, followed by quantity and quality compared to conventional materials (Sattler et al. 2010; Cherney et al.1991), are found to be promising for the production of 2G ethanol. Cellulosic ethanol production requires the breakdown of the plant cell wall using chemical and physical methods. Using less resistant materials helps improve the pretreatment process for breaking down the lignocellulosic biomass (Sattler et al. 2016; Oliver et al. 2005). The current research focuses on developing F3 populations through a partially replicated experimental design based on BLUPs values for proximate fibre content analysis of Sorghum [*Sorghum bicolor* (L.) Moench].

2. MATERIALS AND METHODS

The present research was carried out in four seasons (from post-rainy 2020 to rainy 2022) to develop F3 populations of sweet and high-biomass sorghum lines and one-grain sorghum type; for fibre analysis study, the experiments were conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in Patancheru, Hyderabad, India, at coordinates 17°27'N and 78°28'E.

2.1 Population Development

Parent selection and consecutive cross-breeding for developing F1 hybrids was initiated in the fall of post-rainy 2020. Since sweet and high biomass sorghum lines are photoperiod lines, staggered sowing was followed to get the pollen for on-time crossings for developing hybrids. The reproductive organs (anthers) were removed manually from the recurrent parent plant, which was further pollinated with the pollen collected from *bmr6* and *bmr12* donor parents. The mature F1 seeds were collected from the respective recurrent parent. In the second season (rainy, 2021), few F1 seeds were sown, and the panicles at the base of the shoot were selfed and advanced for F2 populations for *bmr6* and *bmr12* hybrids for observing *bmr* segregations phenotypically. During the third season (post-rainy, 2021), F2 populations were sowed to observe *bmr* and wild type (WT) segregations phenotypically (the segregations for both *bmr6* and *bmr12* hybrids were observed) to confirm the hybridity for F1 through phenotypic approach and advanced for F3 populations. Further, in the fourth season (rainy 2022), the F3 population were sowed for Fiber analysis and advanced for the F4 population.

2.2 Fiber Component Analysis

The F3 populations were sowed for Fiber analysis study by partially replicated experimental design and advanced for the F4 populations. The proximate fibre component analysis was done in the matured F3 population by drying the plant samples and grounding them into a fine powder. Various fibre quality traits, including cellulose, hemicellulose, acid detergent fibre (ADF), lignin (ADL), neutral detergent fibre (NDF), metabolizable energy, nitrogen, in vitro organic matter digestibility (IVOMD), and ash, were assessed using Near-Infrared Reflectance

Spectroscopy (NIRS) following the method adapted from Rivera-Burgos et al. [14]. Near-infrared spectroscopy (NIRS) is now a well-developed and mature technology that is used cost-effectively by the scientific community. NIRS was widely applied as a rapid and non-destructive tool for several products, e.g., meat, fruit, or biomass feedstocks. The samples of the current study were analyzed by the International Livestock Research Institute (ILRI) in their lab at the International Crops Research Institute for the Semi-Arid Tropics, headquarters, India, using FOSS DS 2500 Forage analyzer and WinISI calibration software. The ILRI reported coefficients of determination (R^2) were satisfactory (i.e., 0.83, 0.91, 0.82, 0.91, and 0.9, respectively, for NDF, ADF, ADL, ME, and IVOMD) and more significant than previously reported works [15]. The present investigation was studied using fibre analysis based on the partially replicated BLUP values.

Table 1. List of parents used for developing F3 populations (Source: ICRISAT Germplasm)

S. No.	Parents	<i>bmr</i> /WT	Trait
1	SSV84	WT	SS/HBM
2	ICSV15024	WT	HBM
3	ICSV18003	WT	SS
4	ICSB474	WT	SS/HBM
5	ICSV100324	WT	SS/HBM
6	ICSB38	WT	Grain type
7	N609	<i>bmr</i>	<i>bmr6</i>
8	ICSV101112	<i>bmr</i>	<i>bmr6</i>
9	ICSV101039	<i>bmr</i>	<i>bmr12</i>
10	N600	<i>bmr</i>	<i>bmr12</i>

SS: Sweet Sorghum, HBM: High-biomass sorghum, *bmr* - brown midrib mutant, *Bmr*: wild type (non-*Bmr*)

To confirm the F1 hybrid, these were selfed for obtaining the F2 population, segregations in the 3:1 ratio were observed, and the segregated F2 with *bmr* expressions were advanced for F3 by selfing F2 populations and evaluated by partially replicated design. The partially replicated experimental design [16] was followed for F3 populations, which consist of a total of 96 experimental units, with several treatments of 52, followed by six checks, each check replicating six times and replicated entries with 14 (each entry replicating two times) and un-replicated with 32 entries (replicating only once). There are 4 x 4 blocks, resulting in 6 blocks; each block contains a set of 6 checks. The sub-blocks of 4 x 1

allowed replicated entries to be evenly assigned within the columns [16]. The partially replicated experimental design and F3 populations are mentioned in Fig. 1 below.



Fig. 1. Partially replicated design for F3 populations

Table 2. List of F3 developed lines used for fiber component analysis

S. No	F3 Populations	No of Replications	S. No	F3 Populations	No of Replications
1	ICSB38 × N600	2	27	ICSB474 × ICSV101112	1
2	ICSB38 × N600	2	28	ICSB38 × ICSV101039	1
3	SSV84 × N609	2	29	SSV84 × ICSV101112	1
4	SSV84 × N609	2	30	SSV84 × ICSV101112	1
5	ICSB38 × N600	2	31	ICSV100324 × ICSV101039	1
6	ICSB38 × N600	2	32	SSV84 × N609	1
7	ICSV100324 × ICSV101039	2	33	ICSB474 × ICSV101112	1
8	ICSV18003 × N609	2	34	ICSB474 × N600	1
9	ICSV18003 × N609	2	35	ICSV15024 × N609	1
10	SSV84 × N609	2	36	ICSV15024 × N609	1
11	SSV84 × N609	2	37	ICSB474 × ICSV101039	1
12	SSV84 × ICSV101039	2	38	ICSB474 × ICSV101039	1
13	ICSB38 × ICSV101039	2	39	ICSB474 × ICSV101112	1
14	SSV84 × N609	2	40	ICSB474 × ICSV101039	1
15	ICSB38 × ICSV101039	1	41	ICSB474 × ICSV101112	1
16	ICSV100324 × ICSV101039	1	42	ICSB474 × N600	1
17	SSV84 × ICSV101039	1	43	SSV84 × ICSV101039	1
18	ICSB474 × N609	1	44	SSV84 × ICSV101039	1
19	SSV84 × ICSV101039	1	45	ICSB474 × ICSV101039	1
20	ICSV100324 × ICSV101039	1	46	ICSB474 × ICSV101112	1
21	ICSV15024 × ICSV101112	1	47	Check-1 ICSV18003	6
22	ICSV15024 × ICSV101112	1	48	Check-2 ICSV100324	6
23	ICSB474 × N609	1	49	Check-3 SSV84	6
24	SSV84 × ICSV101039	1	50	Check-4 ICSV15024	6
25	ICSB38 × ICSV101039	1	51	Check -5 ICSV101039	6
26	ICSB474 × ICSV101112	1	52	Check-6 N609 <i>bmr6</i>	6
				Total	96

3. RESULTS PHENOTYPIC DATA

The *bmr6* and *bmr12* hybrid plants were found to have brown midrib colour in the matured plant. However, the brown midrib phenotype was observed only in F2 generations but not in F1, confirming the recessive inheritance of the trait. In the F2 population of *bmr6*, 18.5% of plants were introgressed; in *bmr12*, 23.2% were introgressed. The phenotypic data and the percentage of *bmr* in F2 Populations of *bmr6* and *bmr12* are mentioned in Table 3.

Based on the BLUP values, the study identified that the lowest metabolizable energy (ME) (MJ/kg) was found in ICSV15024 × ICSV101112, followed by ICSB38 × N600 with 5.74 and 5.98, respectively. ICSV18003 had the lowest ADF percentage at 37.04%, while it also had the highest ADL percentage at 3.84%. The ash percentage was 3.73% in SSV84 × N609. Moreover, the *bmr* introgressed lines of the F3 population exhibited higher levels of cellulose and hemicellulose, while lignin and ash content were decreased. Similarly, ash percentage was lowest in SSV84 × N609 at 3.73%, which was comparatively lesser when compared to the respective donor (5.60%) and the recipient (5.33%) parent. Cellulose percentage was lowest in SSV84 × N609 with 32.59 %, and ICSB474 ×

ICSV101039 were 32.6% compared to other F3 populations and respective parents. Further, the least NDF digestibility was found in ICSB474 × ICSV101112 with 60.66%, and the highest was seen in SSV84 × ICSV101039 with 82.66% compared to its respective recipient, donor and wild type plants, IVOMD% was least in ICSV15024 × N609 with 36% and highest in ICSV15024 × ICSV101112 with 64%. In addition, the lowest hemicellulose percentage was observed in ICSB474 × N600 at 19.29% and the highest in ICSV15024 × N609 at 29.51%. Details of the individual BLUP's (Best Linear Unbiased Predictions) mean values are mentioned in Table 4.

4. DISCUSSION

In this study, the lignin concentration was found to be the least in *bmr6* lines of the F3 population (ICSV18003 × N609, SSV84 × N609), which was in agreement with the studies done by other researchers [17], Ceballos et al. 2009). Our study outcomes are based on earlier research findings, which can be attributed to the combined influence of the sweet stem and *BMR* traits. Research by Oliveira et al. [18] and Espinal [19] indicates that genotypes with both the sweet stem trait and the *bmr* mutation (SS/HBM genotypes) synergistically work to decrease lignin content in the stover.

Table 3. Phenotypic data for F2 populations

<i>bmr6</i>							
S. No.	Cross Combinations	Stage	<i>Bmr</i>	<i>bmr</i> plants	WT Plants	Total Plants	% of <i>bmr</i>
1	SSV84 × N609	F2	<i>bmr6</i>	7	15	22	31.8
2	SSV84 × ICSV101112	F2	<i>bmr6</i>	3	19	22	13.6
3	ICSV18003 × N609	F2	<i>bmr6</i>	3	19	22	13.6
4	ICSV15024 × N609	F2	<i>bmr6</i>	3	18	21	14.2
5	ICSV15024 × ICSV101112	F2	<i>bmr6</i>	4	17	21	19
6	ICSB474 × N609	F2	<i>bmr6</i>	3	19	22	13.6
7	ICSB474 × ICSV101112	F2	<i>bmr6</i>	5	16	21	23.8
Total				28	123	151	18.5
<i>bmr12</i>							
1	SSV84 × ICSV101039	F2	<i>bmr12</i>	6	15	21	28.6
2	ICSB474 × ICSV101039	F2	<i>bmr12</i>	5	16	21	23.8
3	ICSB474 × N600	F2	<i>bmr12</i>	3	17	20	15
4	ICSV100324 × ICSV101039	F2	<i>bmr12</i>	5	16	21	23.8
5	ICSB38 × N600	F2	<i>bmr12</i>	5	16	21	23.8
6	ICSB38 × ICSV101039	F2	<i>bmr12</i>	5	16	21	23.8
Total				29	96	125	23.2

Table 4. Partially replicated experimental design (BLUP's Values)

S. No	Category F ₃ , (RP & DP Parents)	bmr/WT	Adf %	ME (MJ/ kg)	Adl %	Ash %	Ndf %	Ivomd %	Cellulose (%)	Hemi Cellulose (%)
1	ICSB38 × N600	F ₃ <i>bmr12</i>	49.89	5.98	5.10	8.07	77.89	44	43.53	28.86
2	ICSB38 × N600.	F ₃ <i>bmr12</i>	47.15	6.92	5.60	10.86	69.73	43	40.61	23.11
3	SSV84 × N609	F ₃ <i>bmr6</i>	45.39	6.83	4.99	9.38	70.06	47	39.84	25.08
4	SSV84 × N609	F ₃ <i>bmr6</i>	46.07	6.95	4.87	10.17	70.81	47	40.27	24.99
5	ICSB38 X N600	F ₃ <i>bmr12</i>	45.56	6.72	4.18	9.77	71.10	47	40.70	27.19
6	ICSB38 X N600	F ₃ <i>bmr12</i>	44.85	7.19	4.91	9.94	69.56	43	39.84	25.18
7	ICSV100324 × ICSV101039	F ₃ <i>bmr12</i>	45.40	7.30	5.04	8.36	69.58	45	39.73	25.26
8	ICSV18003 × N609	F ₃ <i>bmr6</i>	40.84	7.77	3.91	5.99	66.49	53	37.57	26.02
9	ICSV18003 × N609	F ₃ <i>bmr6</i>	45.26	7.54	4.85	6.61	71.05	45	40.51	26.12
10	SSV84 × N609	F ₃ <i>bmr6</i>	47.55	6.77	4.55	9.90	73.38	48	43.87	24.77
11	SSV84 × N609	F ₃ <i>bmr6</i>	45.19	6.88	4.71	10.80	70.99	42	41.81	23.46
12	SSV84 × ICSV101039	F ₃ <i>bmr12</i>	46.82	6.63	5.10	9.24	75.52	49	41.64	27.69
13	SSV84 × ICSV101039	F ₃ <i>bmr12</i>	49.51	7.06	5.02	10.77	73.09	43	44.14	22.95
14	SSV84 × N609	F ₃ <i>bmr6</i>	38.07	8.19	3.91	8.87	62.68	40	34.24	23.20
15	ICSB38 × ICSV101039	F ₃ <i>bmr12</i>	48.61	6.51	5.57	9.26	76.70	57	43.15	28.22
16	ICSV100324 × ICSV101039	F ₃ <i>bmr12</i>	47.32	6.61	4.48	10.33	71.94	49	42.16	25.22
17	SSV84 × ICSV101039	F ₃ <i>bmr12</i>	49.98	6.16	7.18	7.35	78.73	47	42.91	28.87
18	ICSB474 × N609	F ₃ <i>bmr6</i>	51.62	6.30	4.85	13.11	74.26	44	46.93	22.27
19	SSV84 × ICSV101039	F ₃ <i>bmr6</i>	53.73	6.59	5.54	8.44	82.56	61	48.27	29.34
20	ICSV100324 × ICSV101039	F ₃ <i>bmr12</i>	49.38	6.43	5.00	13.75	69.41	48	42.22	22.09
21	ICSV15024 × ICSV101112	F ₃ <i>bmr6</i>	48.83	6.97	5.48	10.66	74.58	38	42.79	25.38
22	ICSV15024 × ICSV101112	F ₃ <i>bmr6</i>	48.94	5.74	5.97	6.26	77.17	64	43.74	29.23
23	ICSB474 × N609	F ₃ <i>bmr6</i>	43.28	8.01	4.04	9.11	63.71	46	35.42	23.18
24	SSV84 × ICSV101039	F ₃ <i>bmr12</i>	59.69	6.69	4.08	11.75	82.66	44	51.52	23.11
25	ICSB38 × ICSV101039	F ₃ <i>bmr12</i>	45.23	7.21	4.89	8.39	70.82	49	40.26	26.39
26	ICSB474 × ICSV101112	F ₃ <i>bmr6</i>	42.18	7.86	4.54	5.68	70.43	44	38.13	25.98
27	ICSB474 × ICSV101112	F ₃ <i>bmr6</i>	42.18	6.93	4.47	13.09	60.66	52	37.07	21.57
28	ICSB38 × ICSV101039	F ₃ <i>bmr12</i>	50.08	6.44	4.23	11.12	72.90	58	44.16	24.28
29	SSV84 × ICSV101112	F ₃ <i>bmr6</i>	51.23	6.30	5.51	9.11	76.69	41	44.70	25.34
30	SSV84 × ICSV101112	F ₃ <i>bmr6</i>	49.96	6.17	4.79	10.71	76.07	52	45.58	25.33
31	ICSV100324 × ICSV101039	F ₃ <i>bmr12</i>	42.85	6.84	4.07	8.00	66.44	52	37.10	25.52
32	SSV84 × N609	F ₃ <i>bmr6</i>	34.59	8.15	4.84	3.73	64.63	46	32.59	27.29
33	ICSB474 × ICSV101112	F ₃ <i>bmr6</i>	45.03	6.80	4.53	7.97	69.01	45	40.01	25.12
34	ICSB474 × N600	F ₃ <i>bmr12</i>	45.54	7.06	4.94	5.15	73.10	43	39.53	26.31
35	ICSV15024 × N609	F ₃ <i>bmr6</i>	50.47	6.37	4.66	8.43	74.67	44	44.08	25.77
36	ICSV15024 × N609	F ₃ <i>bmr6</i>	44.27	7.01	4.39	7.89	76.75	36	41.60	29.51

S. No	Category F3 , (RP & DP Parents)	bmr/WT	Adf %	ME (MJ/ kg)	Adl %	Ash %	Ndf %	Ivomd %	Cellulose (%)	Hemi Cellulose (%)
37	ICSB474 × ICSV101039	F3 <i>bmr12</i>	43.05	7.58	4.61	8.84	68.30	43	39.46	23.72
38	ICSB474 × ICSV101039	F3 <i>bmr12</i>	41.86	7.58	5.03	4.56	62.67	41	32.83	24.46
39	ICSB474 × ICSV101112	F3 <i>bmr6</i>	54.32	6.30	4.22	7.85	79.35	48	48.14	24.61
40	ICSB474 × ICSV101039	F3 <i>bmr12</i>	46.46	6.76	4.27	11.85	68.84	50	41.05	22.71
41	ICSB474 × ICSV101112	F3 <i>bmr6</i>	43.21	6.88	4.26	10.84	67.53	45	39.50	24.71
42	ICSB474 × N600	F3 <i>bmr12</i>	53.68	6.18	4.98	11.39	75.37	43	47.92	19.29
43	SSV84 × ICSV101039	F3 <i>bmr12</i>	45.07	6.77	5.41	9.39	71.40	51	39.13	26.30
44	SSV84 × ICSV101039	F3 <i>bmr12</i>	51.11	6.34	5.49	10.12	79.06	37	48.16	24.48
45	ICSB474 × ICSV101039	F3 <i>bmr12</i>	39.36	8.18	4.02	6.51	63.29	42	32.60	25.76
46	ICSB474 × ICSV101112	F3 <i>bmr6</i>	47.35	6.78	5.01	9.17	67.29	46	41.07	22.02
47	ICSV18003 (RP)	WT	37.04	8.56	5.84	5.11	61.68	45	33.95	24.08
48	ICSV100324 (RP)	WT	42.24	7.68	5.08	7.19	66.90	45	37.28	24.03
49	SSV84 (RP)	WT	45.68	6.79	5.33	8.30	72.39	48	40.60	26.49
50	ICSV15024 (RP)	WT	40.95	7.54	5.92	4.08	67.21	50	35.92	26.01
51	ICSV101039 (DP)	<i>bmr12</i>	46.31	6.70	5.64	10.74	70.41	48	41.57	24.47
52	N609 (DP)	<i>bmr6</i>	42.86	7.18	5.60	11.83	66.50	48	37.82	23

ADF-Acid Detergent Fibre, ADL- Acid Detergent Lignin, NDF-Neutral Detergent Fibre, IVOMD-In-Vitro Organic Matter Digestibility, ME- Metabolizable Energy, DM-Dry Matter, RP-Recurrent Parent, WT-Wild Type, DP-Donor Parent, BLUPs-Best Linear Unbiased Predictions

The highest and lowest ash percentages were observed in *bmr* traits IC5B474 × N609 and SSV84 × N609, respectively, compared to WT populations. The natural alkalinity of biomass ash reduces the fusion point of ashes, resulting in fouling, slagging, and compromised performance of combustion systems [20]. On the other hand, alkaline ash containing phosphorus, potassium, calcium, boron, and other mineral elements *bmr6* and *bmr12* sorghum ash by-products can be used to grow plants healthily. In the current work, however, the ash percentage was at most 13.75%.

The *bmr* lines meet the bioethanol production requirement of having an ash content of 10% or less. Exceeding this limit, leading to higher ash concentrations, which is undesirable as it could hinder the fermentation process and cause the tool crust during distillation process [20]. Therefore, it can be inferred that the *bmr6* and *bmr12* mutations can safely be deployed in sorghum breeding for the bioenergy production. Interestingly, an improved cellulose content relative to WT genotypes was observed in *bmr12*, which can be considered in sorghum breeding to boost the 2G (Second Generation) bioethanol bio-conversion [21,22,23,24]

5. CONCLUSION

The *bmr* traits differed in the proximate fibre component analysis compared to WT lines. Considering the reduced lignin and ash percentage in *bmr* lines meeting the bioethanol production requirement, the *bmr6* and *bmr12* genes can safely be deployed in sorghum breeding for bioenergy production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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