

Production of sweet and high biomass sorghum lines with optimized cell wall components for increased biofuel bioconversion yield

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 F_3 populations based on BLUP values; this study was carried out for four seasons (from post-rainy 2020 to rainy 2022) to develop F_3 populations of sweet and high-biomass *bmr* sorghum lines. The proximate fibre component analysis was done in the matured F_3 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 × N609. Moreover, the *bmr* transferred lines of the F_3 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: F_3 populations, *bmr6*, *bmr12*, Proximate fibre analysis, *Sorghum bicolor* (L.) Moench

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 (Sentanuhady *et al.* 2021). 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 (Khan *et al.* 2021).

Sweet sorghum is an excellent choice for semi-arid and arid marginal regions because it withstands water stress. Its morphophysiological traits confer drought endurance (Zegada-Lizarazu & Monti, 2013), and the C₄ photosynthetic system facilitates adequate CO₂ fixation and extraordinary dry matter build-up (Mastrorilli *et al.*, 1999). Like most other lignocellulosic biomasses, sorghum biomass contains about 20% lignin (Bout & Vermerris, 2003). 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 (Bout & Vermerris, 2003).

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 (Kurakake *et al.*, 2001; Park *et al.*, 2010; Vandenbrink *et al.*, 2010). Significant variations are observed in the chemical composition of soluble and structural components between a non-sweet sorghum hybrid and a sweet sorghum cultivar (Dolciotti *et al.*, 1998). 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 (Beck *et al.*, 2013). 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 (Salama & Nawar, 2016). Several studies have documented the elevated nutritional content, dry-matter digestibility (DMD), and organic-

matter digestibility (OMD) in BMR sorghum (Li *et al.*, 2015; Wahyono *et al.*, 2019). 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 material 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].

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.

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 was sowed for fiber analysis and advanced for the F₄ population.

Table1-Listofparents usedfordevelopingF₃populations (Source: ICRISATGermplasm)

S.No.	Parent	<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

UNDER PEER REVIEW

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*)

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 grinding 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.*, (2019). 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 (e.g., Otero *et al.*, 2023). The present investigation was studied using fibre analysis based on the partially replicated BLUP values.

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

S. No	F ₃ Populations	No of Replications	S. No	F ₃ 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-3SSV84	6
24	SSV84 ×ICSV101039	1	50	Check-4 ICSV15024	6
25	ICSB38×ICSV101039	1	51	Check-5ICSV101039 <i>bmr</i> 12	6
26	ICSB474×ICSV101112	1	52	Check-6N609 <i>bmr</i> 6	6
				Total	96

To confirm the F₁ hybrid, these were selfed for obtaining the F₂ population, segregation in the 3:1 ratio were observed, and these segregated F₂ with *bm* expressions were

advanced for F₃ by selfing F₂ populations and evaluated by partially replicated design. The partially replicated experimental design (Cullis *et al.*, 2006) 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 (Gilmour *et al.*, 1997). The partially replicated experimental design and F₃ populations are mentioned in Figure 1 below.

Fig.1-Partially replicated design for F₃ populations

Partially Replicated Experimental Design.																							
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
35	52	23	49	3	1	52	51	11	43	4	51	6	13	50	14	12	48	29	47	2	30	5	48
50	17	47	40	6	39	7	10	28	47	5	27	52	46	9	15	8	22	52	31	12	51	50	33
26	48	37	2	42	48	44	20	48	1	34	13	24	49	21	47	45	14	38	11	52	36	32	49
7	4	8	51	50	18	49	47	41	52	49	50	51	16	48	25	50	51	10	49	19	9	47	3

Legend

- Checks replicated 6 times
- Unreplicated test entries
- Test entries replicated twice

Results Phenoty

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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.

Table 3-Phenotypic data for F₂ populations

<i>bmr6</i>							
S. No.	Cross Combination	Stage	<i>Bmr</i>	<i>bmr</i> plants	WT Plants	Total Plants	% of <i>bmr</i>
1	SSV84×N609	F ₂	<i>bmr6</i>	7	15	22	31.8
2	SSV84×ICSV101112	F ₂	<i>bmr6</i>	3	19	22	13.6
3	ICSV18003×N609	F ₂	<i>bmr6</i>	3	19	22	13.6
4	ICSV15024×N609	F ₂	<i>bmr6</i>	3	18	21	14.2

5	ICSV15024× ICSV101112	F ₂	<i>bmr6</i>	4	17	21	19
6	ICSB474 ×N609	F ₂	<i>bmr6</i>	3	19	22	13.6
7	ICSB474×ICSV101112	F ₂	<i>bmr6</i>	5	16	21	23.8
Total				28	123	151	18.5
<i>bmr12</i>							
1	SSV84×ICSV101039	F ₂	<i>bmr12</i>	6	15	21	28.6
2	ICSB474×ICSV101039	F ₂	<i>bmr12</i>	5	16	21	23.8
3	ICSB474 ×N600	F ₂	<i>bmr12</i>	3	17	20	15
4	ICSV100324× ICSV101039	F ₂	<i>bmr12</i>	5	16	21	23.8
5	ICSB38 ×N600	F ₂	<i>bmr12</i>	5	16	21	23.8
6	ICSB38×ICSV101039	F ₂	<i>bmr12</i>	5	16	21	23.8
Total				29	96	125	23.2

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.

Table4-Partiallyreplicatedexperimentaldesign(BLUP'sValues)

S.No	Category F ₃ , (RP & DPParents)	bmr/WT	Adf %	ME (MJ/kg)	adl%	Ash %	Ndf %	ivomd %	Cellulose(%)	HemiCellulose(%)
1	ICSB38×N600	F _{3bmr12}	49.89	5.98	5.10	8.07	77.89	44	43.53	28.86
2	ICSB38 ×N600.	F _{3bmr12}	47.15	6.92	5.60	10.86	69.73	43	40.61	23.11
3	SSV84 ×N609	F _{3bmr6}	45.39	6.83	4.99	9.38	70.06	47	39.84	25.08
4	SSV84 ×N609	F _{3bmr6}	46.07	6.95	4.87	10.17	70.81	47	40.27	24.99
5	ICSB38 XN600	F _{3bmr12}	45.56	6.72	4.18	9.77	71.10	47	40.70	27.19
6	ICSB38 XN600	F _{3bmr12}	44.85	7.19	4.91	9.94	69.56	43	39.84	25.18
7	ICSV100324× ICSV101039	F _{3bmr12}	45.40	7.30	5.04	8.36	69.58	45	39.73	25.26
8	ICSV18003×N609	F _{3bmr6}	40.84	7.77	3.91	5.99	66.49	53	37.57	26.02
9	ICSV18003×N609	F _{3bmr6}	45.26	7.54	4.85	6.61	71.05	45	40.51	26.12
10	SSV84 ×N609	F _{3bmr6}	47.55	6.77	4.55	9.90	73.38	48	43.87	24.77
11	SSV84 ×N609	F _{3bmr6}	45.19	6.88	4.71	10.80	70.99	42	41.81	23.46
12	SSV84 ×ICSV101039	F _{3bmr12}	46.82	6.63	5.10	9.24	75.52	49	41.64	27.69
13	SSV84 ×ICSV101039	F _{3bmr12}	49.51	7.06	5.02	10.77	73.09	43	44.14	22.95
14	SSV84 ×N609	F _{3bmr6}	38.07	8.19	3.91	8.87	62.68	40	34.24	23.20
15	ICSB38 ×ICSV101039	F _{3bmr12}	48.61	6.51	5.57	9.26	76.70	57	43.15	28.22
16	ICSV100324× ICSV101039	F _{3bmr12}	47.32	6.61	4.48	10.33	71.94	49	42.16	25.22
17	SSV84 ×ICSV101039	F _{3bmr12}	49.98	6.16	7.18	7.35	78.73	47	42.91	28.87
18	ICSB474×N609	F _{3bmr6}	51.62	6.30	4.85	13.11	74.26	44	46.93	22.27
19	SSV84 ×ICSV101039	F _{3bmr6}	53.73	6.59	5.54	8.44	82.56	61	48.27	29.34
20	ICSV100324× ICSV101039	F _{3bmr12}	49.38	6.43	5.00	13.75	69.41	48	42.22	22.09
21	ICSV15024× ICSV101112	F _{3bmr6}	48.83	6.97	5.48	10.66	74.58	38	42.79	25.38
22	ICSV15024 × ICSV101112	F _{3bmr6}	48.94	5.74	5.97	6.26	77.17	64	43.74	29.23
23	ICSB474×N609	F _{3bmr6}	43.28	8.01	4.04	9.11	63.71	46	35.42	23.18
24	SSV84 ×ICSV101039	F _{3bmr12}	59.69	6.69	4.08	11.75	82.66	44	51.52	23.11
25	ICSB38 ×ICSV101039	F _{3bmr12}	45.23	7.21	4.89	8.39	70.82	49	40.26	26.39

26	ICSB474×ICSV101112	F _{3bmr6}	42.18	7.86	4.54	5.68	70.43	44	38.13	25.98
27	ICSB474×ICSV101112	F _{3bmr6}	42.18	6.93	4.47	13.09	60.66	52	37.07	21.57
28	ICSB38×ICSV101039	F _{3bmr12}	50.08	6.44	4.23	11.12	72.90	58	44.16	24.28
29	SSV84×ICSV101112	F _{3bmr6}	51.23	6.30	5.51	9.11	76.69	41	44.70	25.34
30	SSV84×ICSV101112	F _{3bmr6}	49.96	6.17	4.79	10.71	76.07	52	45.58	25.33
31	ICSV100324× ICSV101039	F _{3bmr12}	42.85	6.84	4.07	8.00	66.44	52	37.10	25.52
32	SSV84 ×N609	F _{3bmr6}	34.59	8.15	4.84	3.73	64.63	46	32.59	27.29
33	ICSB474×ICSV101112	F _{3bmr6}	45.03	6.80	4.53	7.97	69.01	45	40.01	25.12
34	ICSB474×N600	F _{3bmr12}	45.54	7.06	4.94	5.15	73.10	43	39.53	26.31
35	ICSV15024×N609	F _{3bmr6}	50.47	6.37	4.66	8.43	74.67	44	44.08	25.77
36	ICSV15024×N609	F _{3bmr6}	44.27	7.01	4.39	7.89	76.75	36	41.60	29.51
37	ICSB474×ICSV101039	F _{3bmr12}	43.05	7.58	4.61	8.84	68.30	43	39.46	23.72
38	ICSB474×ICSV101039	F _{3bmr12}	41.86	7.58	5.03	4.56	62.67	41	32.83	24.46
39	ICSB474×ICSV101112	F _{3bmr6}	54.32	6.30	4.22	7.85	79.35	48	48.14	24.61
40	ICSB474×ICSV101039	F _{3bmr12}	46.46	6.76	4.27	11.85	68.84	50	41.05	22.71
41	ICSB474×ICSV101112	F _{3bmr6}	43.21	6.88	4.26	10.84	67.53	45	39.50	24.71
42	ICSB474×N600	F _{3bmr12}	53.68	6.18	4.98	11.39	75.37	43	47.92	19.29
43	SSV84×ICSV101039	F _{3bmr12}	45.07	6.77	5.41	9.39	71.40	51	39.13	26.30
44	SSV84×ICSV101039	F _{3bmr12}	51.11	6.34	5.49	10.12	79.06	37	48.16	24.48
45	ICSB474×ICSV101039	F _{3bmr12}	39.36	8.18	4.02	6.51	63.29	42	32.60	25.76
46	ICSB474×ICSV101112	F _{3bmr6}	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-RecurrentParent,WT-WildType,DP-DonorParent,BLUPs-BestLinearUnbiasedPredictions.

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 (Barrière *et al.*, 2007 and 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.* (2020) and Espinal (2009) 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.

The highest and lowest ash percentages were observed in *bmr* traits ICSB474 × 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 (Carrillo *et al.*, 2014). 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. (Carrillo *et al.*, 2014). 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 (Habyarimana *et al.*, 2016; Tse *et al.*, 2021).

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.

References

1. Barrière, Y., Riboulet, C., Méchin, V., Maltese, S., Pichon, M., Cardinal, A., & Martinant. (2007). Genetics and genomics of lignification in grass cell walls based on maize as model species. *Genes Genomes Genomics*, 1, 133–156.
2. Bout, S., & Vermerris, W. (2003). A candidate-gene approach to clone the sorghum Brown midrib gene encoding caffeic acid O-methyltransferase. *Molecular Genetics and Genomics: MGG*, 269(2), 205–214. <https://doi.org/10.1007/s00438-003-0824-4>
3. Beck, P., K., Poe, B., Stewart, P., Capps, & H. Gray. 2013. Effect of brown midrib gene and maturity at harvest on forage yield and nutritive quality of Sudan grass. Japanese Society of Grassland Science, *Grassland Science*. 59:52-58. <https://doi.org/10.1111/grs.12007>
4. Carrillo, M.A., Staggenborg, S.A., & Pineda, J.A. (2014). Washing sorghum biomass with water to improve its quality for combustion. *Fuel*, 116, 427-431.
5. Cullis, B.R., Smith, A.B. & Coombes, N.E. On the design of early generation variety trials with correlated data. *Journal of Agricultural, Biological, and Environmental Statistics* 11, 381–393 (2006). <https://doi.org/10.1198/108571106X154443>.
6. Dolciotti, I., Mambelli, S., Grandi, S., & Venturi, G. (1998). Comparison of two Sorghum genotypes for sugar and fibre production. *Industrial Crops and Products*, 7(2–3), 265–272. [https://doi.org/10.1016/s0926-6690\(97\)00057-5](https://doi.org/10.1016/s0926-6690(97)00057-5).
7. Espinal, A. I. M. S. (2009). *Genetic dissection of sorghum traits important for biofuel production* (Doctoral dissertation, Purdue University).
8. Gilmour, Arthur R., Brian R. Cullis, and Arunas P. Verbyla. Accounting for Natural and Extraneous Variation in the Analysis of Field Experiments. *Journal of Agricultural, Biological, and Environmental Statistics* 2, no. 3 (1997): 269–93.
9. Habyarimana, E., Lorenzoni, C., Marudelli, M., Redaelli, R., & Amaducci, S. (2016). A meta-analysis of bioenergy conversion relevant traits in sorghum landraces, lines and hybrids in the Mediterranean region. *Industrial Crops and Products*, 81, 100–109. <https://doi.org/10.1016/j.indcrop.2015.11.051>
10. Kurakake, M., Kisaka, W., Ouchi, K., & Komaki, T. (2001). Pretreatment with ammonia water for enzymatic hydrolysis of corn husk, bagasse, and switchgrass. *Applied Biochemistry and Biotechnology*, 90(3), 251–

259.<https://doi.org/10.1385/abab:90:3:251>.

UNDER PEER REVIEW

11. Khan, M. A. H., Bonifacio, S., Clowes, J., Foulds, A., Holland, R., Matthews, J. C., ...& Shallcross, D. E. (2021). Investigation of biofuel as a potential renewable energysource. *Atmosphere*, 12(10), 1289
12. Li, Y., P. Mao, W. Zhang, X. Wang, Y. You, H. Zhao, L. Zhai, & G. Liu. 2015. Dynamic expression of the nutritive values in forage sorghum populations associated with white, green and brown midrib genotypes. *Field Crops Res.* 184:112-122. <https://doi.org/10.1016/j.fcr.2015.09.008>
13. Mastrorilli, M., Katerji, N., & Rana, G. (1999). The soil water deficit at different vegetative growth stages affects sweet sorghum's productivity and water use efficiency. *European Journal of Agronomy: The Journal of the European Society for Agronomy*, 11(3-4), 207-215. [https://doi.org/10.1016/s1161-0301\(99\)00032-5](https://doi.org/10.1016/s1161-0301(99)00032-5).
14. Oliveira, D.M., Mota, T.R., Grandis, A., de Moraes, G.R., de Lucas, R.C., Polizeli, M.L.T.M., Marchiosi, R., Buckeridge, M.S., Ferrarese-Filho, O., & dos Santos, W.D. (2020). Lignin plays a key role in determining biomass recalcitrance in forage grasses. *Renewable Energy*, 147, 2206-2217. <https://doi.org/10.1016/j.renene.2019.10.020>.
15. A. Otero, C. Segura, L. Tey, G. Masferrer, J. Comaposada, B. Fernández, (2023). Near-infrared spectroscopy for fast characterization of animal by-product feedstocks for biogas
16. Park, S., Baker, J.O., Himmel, M.E., Parilla, P.A., & Johnson, D.K. (2010). Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for Biofuels*, 3(1), 10. <https://doi.org/10.1186/1754-6834-3-10>.
17. Rivera-Burgos, L. A., Volenec, J. J., & Ejeta, G. (2019). Biomass and bioenergy potential of brown midrib sweet sorghum germplasm. *Frontiers in Plant Science*, 10, 1142. <https://doi.org/10.3389/fpls.2019.01142>
18. Saballos, A., Ejeta, G., Sanchez, E., Kang, C., & Vermerris, W. (2009). A genome-wide analysis of the cinnamyl alcohol dehydrogenase family in sorghum [*Sorghum bicolor* (L.) Moench] identifies *SbCAD2* as the brown midrib6 gene. *Genetics*, 181(2), 783-795.

19. Sentanuhady, J., Atmaja, G.P.S.G., & Muflikhun, M.A. (2021). Challenges of Biofuel Applications in Industrial and Automotive: A Review. *Journal of Engineering Science & Technology Review*, 14(4).
20. Salama, H.S.A. & A.I. Nawar. 2016. Variations of the cell wall components of multi-cut forage legumes, grasses and leucaena-grass binary mixtures grown in Egypt. *Asian J. Crop Sci.* 3:96-102. <https://doi.org/10.3923/ajcs.2016.96.102>
21. Tse, T. J., Wiens, D. J., & Reaney, M. J. (2021). *Production of bioethanol—A review of factors affecting ethanol yield*. *Fermentation*, 7(4), 268.
22. Vandenbrink, J.P., Delgado, M.P., Frederick, J.R., & Feltus, F.A. (2010). A sorghum diversity panel biofuel feedstock screen for genotypes with high hydrolysis yield potential. *Industrial Crops and Products*, 31(3), 444–448. <https://doi.org/10.1016/j.indcrop.2010.01.001>.
23. Wahyono, T., I. Sugoro, A. Jayanegara, K. G. Wiryawan, & D. A. Astuti. 2019. Nutrient profile and in vitro degradability of new promising mutant lines sorghum as forage in Indonesia. *Adv. Anim. Vet. Sci.* 9:810-818. <https://doi.org/10.17582/journal.aavs/2019/7.9.810.818>
24. Zegada-Lizarazu, W., & Monti, A. (2013). Photosynthetic response of sweet sorghum to drought and re-watering at different growth stages. *Physiological Plantarum*, 149(1), 56–66. <https://doi.org/10.1111/ppl.12016>