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Characterization of Brown Midrib Mutants of Sorghum (*Sorghum bicolor* (L.) Moench)

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ABSTRACT

Twenty brown midrib (*bmr*) mutants of sorghum (*Sorghum bicolor* (L.) Moench) were evaluated for agronomic traits, forage quality traits and the relationships between these traits. Potential fodder quality was assessed by laboratory analysis. Significant differences were observed among the *bmr* mutants (*bmr*1, 3, 6, 7, 8 and two new mutants) for stover yield, plant height, stover nitrogen (N) content, *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME) content and acid detergent lignin (ADL) content. The *bmr* mutants such as IS 23253, IS 21549, IS 23789 and IS 23787 had high stover yield coupled with better forage quality, hence these mutants can be exploited in future *bmr* hybrid breeding programs. All of these had reduced cinnamyl alcohol dehydrogenase (CAD) activity except for the new and uncharacterized *bmr* mutant, IS 23253, whose allelic relationship is yet to be determined.

Keywords: Acid detergent lignin, allelic relationship, biomass, *in vitro* organic matter digestibility, nitrogen content, stover quality, stover yield

Abbreviations: ADL, acid detergent lignin; ***bmr***, brown midrib; **CAD**, cinnamyl alcohol dehydrogenase; **COMT**, caffeic acid *O*-methyl-transferase; **IVOMD**, *in vitro* organic matter digestibility; **ME**, metabolizable energy; **N**, stover nitroge

INTRODUCTION

There has been a great interest in the recent past to develop new renewable sources of energy to partially replace petroleum while mitigating levels of greenhouse gases. Sorghum (*Sorghum bicolor* (L.) Moench) grown for grain, forage, or fuel, has been considered as model biomass feedstock, because it is a diploid C_4 species with heat and drought tolerance. The availability of brown midrib (*bmr*) mutations in sorghum that reduces lignin content and increase forage digestibility in animals also favours this crop as model crop (Cherney *et al.* 1991; Oliver *et al.* 2005; Srinivasa Rao *et al.* 2009, 2010). The value of a crop plant as forage is determined primarily by the degradability of the vegetative tissue and biomass production per time and area unit (Blümmel and Rao 2006). Degradability or digestibility is affected by the property of its cell wall structure as cellulose and hemicellulose in the cell wall provide a major energy source for ruminant animals. Increased forage digestibility is negatively correlated with lignin in many species and is also useful to increase conversion efficiency of biomass into ethanol (Dien *et al.* 2009).

Brown midrib (*bmr*) mutations, both in sorghum and maize, are phenotypically characterized by the presence of brown vascular tissues in the leaf blade and sheath, as well as in the stem. The *bmr* phenotype becomes obvious once plants have reached the four-leaf stage and tends to begin to fade as the plants approach physiological maturity (Porter *et al.* 1978). Although the intensity of the coloration cannot be taken as a measure of reduction in lignin, it is a clear indicator that the *bmr* gene(s) are present. Jung and Fahey (1983) suggested that *bmr* plants have lignin that is less polymerized and contains less phenolic monomers that can affect digestion. Brown midrib silage with or without protein supplements significantly increased milk yield of lactating cows (Frenchik *et al.* 1976; Keith *et al.* 1979; Stallings *et al.* 1982; Cherney *et al.* 1991; Oba and Allen 1999). Similarly, the rate of *in vitro* organic matter digestibility (IVOMD) and rumen bacterium-mediated cell wall degradation of leaf blades from *bmr*-12 sorghum was shown to be significantly higher than those from their respective wild type isolines (Akin *et al.* 1986a, 1986b). Allelism tests on the sorghum *bmr* mutants derived through chemical mutagenesis showed that several of the mutations are allelic, and that the total number of independent *bmr* loci was smaller than the number of mutant lines assembled (Bittinger *et al.* 1981). The effect of the *bmr* mutations on forage quality depends on the genetic background of the line in which the mutation was introduced (Cherney *et al.* 1991; Pedersen *et al*. 2005). Therefore, the effect of each mutation on forage quality and agronomic characteristics needs to be determined. Since the development of diethyl sulphate (DES) induced generation of *bmr* lines (*bmr*1 through *bmr*19) of two (954114 and 954104) grain sorghum lines (Porter *et al.* 1978), additional *bmr* lines from the mutagenized population and a set of spontaneous *bmr* mutants were latter identified (Volger *et al.* 1994; Gupta 1995). Recently, *bmr* 6 plants were shown to have limited cinnamyl alcohol dehydrogenase (CAD) activity, the enzyme that catalyzes the conversion of hydroxycinnamoyl aldehydes (monolignals) to monolignols while *bmr* 12 plants have reduced activity of caffeic acid O-methyl transferase (COMT) that catalyzes the addition of a methyl group to 5-OH-conferyl alcohol in monolignol biosynthetic pathway (Bout and Vermerris 2003; Saballos *et al.* 2008, 2009; Sattler *et al.* 2010).

The agronomic characterization besides forage quality assessment of the *bmr* mutants will help to breed productive cultivars with high forage quality, however such studies were not reported yet in the literature. Hence, the objective of this study was to provide an agronomic and fodder quality assessment of the different *bmr* mutants.

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Table 1 List of brown midrib mutants/sources used in the study.

Germplasm accession/ Genetic stock	Brown midrib gene	Reference
IS 21887	*bmr* 1	Porter *et al.* 1978
IS 21888	*bmr* 3	Porter *et al.* 1978
IS 21889	*bmr* 6	Porter *et al.* 1978
IS 21890	*bmr* 7	Porter *et al.* 1978
IS 21891	*bmr* 8	Porter *et al.* 1978
IS 40602	*bmr* 12	Porter *et al.* 1978
IS 23253[a]	NA	ICRISAT Gene bank
IS 11861[a]	NA	ICRISAT Gene bank
IS 21549	*bmr* 6	Gupta 1995
IS 23765	*bmr* 6	Gupta 1995
IS 23787	*bmr* 6	Gupta 1995
IS 23789	*bmr* 6	Gupta 1995
N 592	Rox Orange *bmr* 6	Pederson *et al.* 2006
N 593	Rox Orange *bmr* 12	Pederson *et al.* 2006
N 594	Kansas Collier *bmr*-6	Pederson *et al.* 2006
N 595	Kansas Collier *bmr* 12	Pederson *et al.* 2006
N 596	Early Hegari *bmr* 6	Pederson *et al.* 2006
N 597	Early Hegari *bmr* 12	Pederson *et al.* 2006
N 598	Atlas *bmr* 6	Pederson *et al.* 2006
N 599	Atlas *bmr* 12	Pederson *et al.* 2008
RSSV 9	SS variety	MPKV
ICSV 93046	SS variety	ICRISAT

[a]Germplasm accession in ICRISAT gene bank, not yet reported; NA: not available; MPKV: Mahatma Phule Krishi Vidyapeeth

MATERIALS AND METHODS

Materials and experimental site

A total of twenty different *bmr* mutant sources, namely *bmr*1, 3, 6, 7, 8 and 12 developed at Purdue University, USA; two new *bmr* sources (IS 11861 and IS 23253) collected from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) genebank; four natural *bmr* mutants reported in Malawi (Gupta 1995); eight *bmr* 6 or *bmr* 12 introgressed lines developed at USDA (Pederson *et al.* 2006) along with two white midrib sweet sorghum varieties as controls *viz*, RSSV 9 and ICSV 93046 (**Table 1**) were evaluated during rainy and post rainy seasons of 2010 in a randomized complete block design (RCBD) with two replications at ICRISAT, Patancheru (latitude: 17° 27' N; longitude: 78° 28' E). Each cultivar was planted in 2 rows of 4 m length in 6 m^2 plots with a spacing of 75 cm between the rows and 15 cm within the row. Fertilizer dosage of 80 Kg ha^{-1} nitrogen and 40 Kg ha^{-1} P_2O_5 was applied with 50% of N as basal and the balance on 35 days after emergence as side-dressing. Hand weeding was done twice followed by hoeing and inter-cultivation.

A sub-set of six *bmr* germplasm lines (*bmr*1, 3, 6, 7, 8 and 12) available with ICRISAT genebank (No. 1 to 6 in **Table 1**) were also used in order to study the allelic relationship among these *bmr* mutants, and evaluated in a replicated RCBD trial to compare their forage quality (as explained in following paragraph) with that of a standard grain sorghum B-line, BTx623. For allelism testing, the six *bmr* lines were crossed in all possible combinations (excluding reciprocals) using hand emasculation during rainy seasons of 2008 and 2009. The F_1 seeds were sown during late post-rainy seasons of 2008 and 2009. Few typical F_1 hybrids were selfed during late post-rainy season of 2008 to study the inheritance of *bmr* loci. The F_2 populations were sown during late post-rainy season of 2009. Presence or absence of brown coloration of midrib, both for F_1 and F_2 plants, was used for scoring.

Measurements and biochemical analysis

The days to 50% flowering (DF) was recorded at 50% anthesis; plant height (PHT) was measured to the top of the mature panicle before harvest in each plot. Ten mature plants were randomly selected from the centre four rows of each plot, and the panicles were cut for estimations of grain yield. Stover quality analyses were conducted on samples harvested from each plot. All samples were analyzed by near infrared spectroscopy (NIRS; FOSS Forage Analyzer 5000 with software package Win SI) calibrated for this experiment against conventional chemical and *in vitro* analyses. Stover nitrogen (N) was determined by Auto Analyzer and acid detergent lignin (ADL) content was analyzed according to Goering and Van Soest (1970). The IVOMD and metabolizable energy (ME) contents were determined and calculated according to Menke and Steingass (1988) as modified by Blümmel and Ørskov (1993).

Statistical analysis

General linear model (GLM) was used for analysis of variance and to calculate significant differences among improved varieties (SAS computer program 1988). GraphPad Prism (1994) software was used for simple linear regression analysis between traits. To confirm the inheritance, chi-square test was used to test the data for goodness of fit.

RESULTS AND DISCUSSION

Allelic relationship and inheritance

The F_1 plants from crosses of *bmr*1 with *bmr*3, 8 and *bmr*3 with *bmr*8 were uniformly possessing brown midrib. The results were as per previously established allelic groups (Bittinger *et al.* 1981; Saballos *et al.* 2008). The F_1 crosses involving *bmr*6 with *bmr*1, 3, 8 were with normal midrib indicating non-complementary interaction. Further, the F_1 plants from cross *bmr*6 and *bmr*12 were all white in colour, and also the phenotype of *bmr* 6 is taller than originally reported (Porter *et al.* 1978). As *bmr* 6 and *bmr*12 were already established as two distinct loci (Saballos *et al.* 2008), it can be suspected that seed stock of *bmr*6 accession available at ICRISAT genebank is not true *bmr*6. Similar inconsistencies were observed for germplasm line with *bmr*7. All the crosses of *bmr* 7 with *bmr*1, 3, 8 were uniformly brown, while crosses of *bmr*7 with *bmr* 6, 12 were with normal midrib. To confirm discrepancies in allelic relationship of *bmr*6 and *bmr*7, we selfed typical F_1 plants derived from crossing of *bmr*6 with *bmr*1, 8, 12; and *bmr*7 cross with *bmr*3. All the crosses produced individuals in expected ratio (nine wild type: 7 *bmr*) (Gupta 1995; Saballos *et al.* 2008), except for cross *bmr*6 × *bmr*12 (data not shown). All the F_2 plants were scored from this cross were with brown midrib. Similarly, *bmr*7 cross with *bmr*3 produced a ratio of 9 wild type: 7 *bmr* for two complementary loci; further confirming the *bmr*7 source used in the study as not true-to-type.

Although these *bmr* loci were not in similar genetic background, the replicated RCBD trial of these germplasm lines revealed significant higher IVMOD values in *bmr* lines than check grain sorghum entry (data not shown). The germplasm lines described as *bmr* 6 and *bmr* 12, had non-significant differences in IVMOD values.

ANOVA for agronomic and biochemical traits

The combined ANOVA (**Table 2**) reveals highly significant variability for all the traits among the *bmr* sources, and their interaction with season were also highly significant except for nitrogen content and neutral detergent fibre. The genotype × season interaction is highly significant because of photoperiod sensitivity as indicated by the significant differences in flowering behavior during rainy and post rainy season. Most of the lines took 2 to 21 days more to reach 50% flowering stage, particularly so in the four Malawian spontaneous *bmr* mutants where the difference is more than two weeks (data not shown).

The means performance of *bmr* mutants for DF, PHT, N, ME, IVOMD and ADL are presented in **Table 3**. The range among the *bmr* mutants for DF is 53 to 88 days; fresh fodder yield is 7.21 to 31.6 t ha^{-1}; stover yield is 2.48 to 11.61 t ha^{-1} indicating huge genetic variability that can be directly exploited to derive productive cultivars. The *bmr* 6 and 12 introgressed Early Hegari lines are earliest to flower at 53-55 days while the new *bmr* source IS 11861 reaches DF by 88 days. The background effects of same mutant *bmr* 6 are

Table 2 Combined ANOVA table for brown midrib source varieties for fresh fodder yield (t ha^{-1}), stover yield (t ha^{-1}), nitrogen content (N), acid detergent lignin (ADL), metabolizable energy (ME) MJ kg^{-1}, *in vitro* organic matter organic matter digestibility (IVOMD) and agronomic traits.

Source of variation	df	Plant height (m)	Days to 50% flowering	Fresh fodder yield (t ha^{-1})	Stover yield (t ha^{-1})	N (%)	ADL (%)	ME (MJ kg^{-1})	IVOMD (%)
Replication	1	0.12	5.50	0.06	0.30	0.03	0.2	0.2	1.2
Season	1	2.63	0.00	1074.1	10.50	0.15	1.8	11.2	31.6
Genotype	21	1.21 **	273.70 **	218.97 **	22.79 **	0.15 **	2.1**	1.0 **	32.5 **
Genotype x season	21	0.21 **	166.04 **	122.17 **	10.25 **	0.06	0.5 **	0.3 **	9.2 **

[a] df = degrees of freedom; * Significant at P≤0.05; ** Significant at P≤0.01

Table 3 Mean performance of brown midrib sources for fresh fodder yield (t ha^{-1}), stover yield (t ha^{-1}), nitrogen content (N), acid detergent lignin (ADL), metabolizable energy (ME) MJ kg^{-1}, *in vitro* organic matter organic matter digestibility (IVOMD) and agronomic traits.

Germplasm accession/ Line	Plant height (m)	Days to 50% flowering	Fresh fodder yield (t ha^{-1})	Stover yield (t ha^{-1})	N (%)	ADL (%)	ME (MJ kg^{-1})	IVOMD (%)
IS 21887	0.9	70	7.39	2.67	1.26	4.25	7.89	52.58
IS 21888	0.9	69	8.04	2.48	1.66	6.09	6.75	47.10
IS 21889	1.3	77	12.45	4.65	1.13	3.89	7.71	52.55
IS 21890	1.6	70	7.70	3.53	0.86	3.39	8.24	55.40
IS 21891	1.0	69	7.21	2.98	1.11	4.40	7.97	53.72
IS 40602	1.3	82	12.59	4.54	1.48	4.75	7.48	51.37
IS 23253	2.3	71	22.57	5.56	0.99	3.39	8.61	56.02
IS 11861	2.9	88	28.44	11.61	0.91	5.01	7.73	50.93
IS 21549	2.0	78	23.69	9.81	1.03	4.16	8.29	54.59
IS 23765	1.7	81	15.37	6.12	0.99	4.08	7.93	55.10
IS 23787	2.5	70	31.60	9.11	1.06	4.19	8.04	54.21
IS 23789	2.5	71	22.20	5.21	0.87	3.02	8.98	58.18
N 592	2.1	64	22.73	7.54	1.01	4.27	8.19	54.48
N 593	2.2	68	10.20	4.00	0.94	3.54	7.75	51.00
N 594	1.8	70	14.68	4.80	1.04	4.04	7.96	53.78
N595	1.9	68	10.67	3.65	0.98	3.78	8.42	54.21
N596	1.3	53	7.89	3.83	1.17	4.40	7.78	51.25
N597	1.5	55	17.57	3.58	1.11	5.83	6.91	46.45
N 598	2.2	68	18.24	5.50	1.05	4.72	7.70	51.06
N599	1.8	66	15.52	5.72	1.37	4.02	8.17	53.72
RSSV 9	2.3	66	26.64	7.60	1.14	4.62	7.46	50.71
ICSV 93046	2.3	82	13.16	5.32	1.03	5.35	7.21	48.44
Mean	1.8	71	16.21	5.45	1.10	4.31	7.88	52.63
Minimum	0.9	53	7.21	2.48	0.86	3.02	6.75	46.45
Maximum	2.9	88	31.60	11.61	1.66	6.09	8.98	58.18
LSD (p<0.005)	0.237	3.523	3.222	1.917	0.398	0.875	0.481	2.022
CV %	6.3	2.5	10.1	17.8	18.2	10.3	3	2.022

LSD: Least significant difference; CV: Coefficient of variation

evident even for DF as it ranged from 53 days in Early Hegari (N596) to 70 days in Kansas Collier (N594), indicating the earlier reports of Cox and Cherney (2001) and Oliver *et al.* (2005). Similarly, background effects were noticed in *bmr* 12 introgressed lines also.

The range for many of the forage quality traits among the studied *bmr* mutants is high, i.e. 0.86-1.66% for N; 3.02-6.09% ADL and 46.45-58.18% for IVOMD (**Table 3**). Among the white midrib controls, ICSV 93046 recorded 5.35% ADL and 48.44% IVOMD while RSSV 9 had 4.62 ADL and 51.71% IVOMD. The fresh fodder yield is significantly higher in IS 23787 (*bmr*6) at 31.6 t ha^{-1} while new *bmr* mutant collected from the ICRISAT gene bank IS 11861 with 28.44 t ha^{-1} is at par with the best white midrib control RSSV 9 (26.64 t ha^{-1}). The stover yield is highest in IS 11861 (11.61 t ha^{-1}) followed by IS 21549 (9.81 t ha^{-1}) and IS 23787 (9.11 t ha^{-1}) compared to the white midrib control, RSSV9 (7.6 t ha^{-1}). For IVOMD, the *bmr* mutant lines IS 21890 (*bmr*7), IS 21891 (*bmr*8), IS 23253 (*bmr* gene not known), IS 21549 (*bmr*6), IS 23765 (*bmr*6), IS 23787 (*bmr*6), IS 23789 (*bmr*6), N 592 (Rox Orange *bmr*6), N 594 (Kansas Collier *bmr*6), N595 (Kansas Collier *bmr*12) and N599 (Atlas *bmr*12) had a range of 53.72% to 58.12% and are superior to the white midrib control RSSV 9 (50.71%). The *bmr* mutant lines, IS 23789 (*bmr*6), IS 23765 (*bmr*6); IS 23253 (new *bmr* source) and IS 21890 (*bmr*7) had recorded over 55% IVOMD while IS 21888 (*bmr*3) and N597 (Early Hegari with *bmr*12) had lowest IVOMD of 47.1 and 46.45%, respectively. These two *bmr* lines had highest ADL of about 6%, similar to the observations of earlier reports (Cherney *et al.* 1991; Oliver *et al.* 2005; Pedersen *et al.* 2006). Most of the mutants with low IVOMD have recorded over 4% ADL content. The four Malawian *bmr* mutants have recorded ADL between 3.02 and 4.19% and the IVOMD varied between 54.21 to 58.18%. Hence these mutants are of particular importance for the breeders owing to their better forage quality coupled with high stover yield of over 6 t ha^{-1}.

The effect of the *bmr* mutations on forage quality varies depending on the genetic background of the line in which the mutation is introduced (Cherney *et al.* 1991; Pedersen *et al.* 2006; Palmer *et al*. 2008). This suggests the need to either identify a suitable genetic background that allows for optimal impact of the mutation.

Correlation among agronomic and forage quality traits

The correlation coefficients of agronomic characters with candidate forage digestibility traits in *bmr* mutants is shown in **Table 4**. Plant height (m) has significant negative correlation with N (-0.62) (**Fig. 1A**), while it has positive correlation with fresh fodder yield (0.75) and stover yield (0.79) as expected. The DF has positive correlation with stover yield, i.e. late maturing lines yields more stover and it had no correlation with IVOMD (**Fig. 1B**). There is no significant correlation between stover yield neither with IVOMD (**Fig. 1C**) nor N (**Fig. 1D**). This aspect needs to be exploited to improve stover yield without compromising on stover N. High stover N is positively correlated with protein content. The IVOMD has significant negative correlation with ADL (-0.88) while it has recorded significant positive

Table 4 Correlation of agronomic traits (days to 50% flowering, plant height, fresh fodder yield and stover yield) with candidate forage digestibility traits [nitrogen content (N), acid detergent lignin (ADL), metabolizable energy (ME) MJ kg^{-1}, *in vitro* organic matter organic matter digestibility (IVOMD)] in source lines.

Trait	ADL (%)	Days to 50% flowering	Stover yield (t ha^{-1})	Fresh fodder (t ha^{-1})	IVOMD (%)	ME (MJ kg^{-1})	N (%)	Plant height (m)
ADL (%)	-							
Days to 50 % Flowering	0.01	-						
Stover yield (t ha^{-1})	0.00	0.44*	-					
Fresh fodder (t ha^{-1})	-0.04	0.21	0.87**	-				
IVOMD (%)	-0.88**	0.14	0.21	0.23	-			
ME (MJ kg^{-1})	-0.89**	0.05	0.22	0.26	0.95**	-		
N (%)	0.57*	-0.10	-0.34	-0.33	-0.48*	-0.56**	-	
Plant height (m)	-0.23	0.29	0.75**	0.79**	0.26	0.37	-0.62**	-

* Significant at P≤0.05; ** Significant at P≤0.01

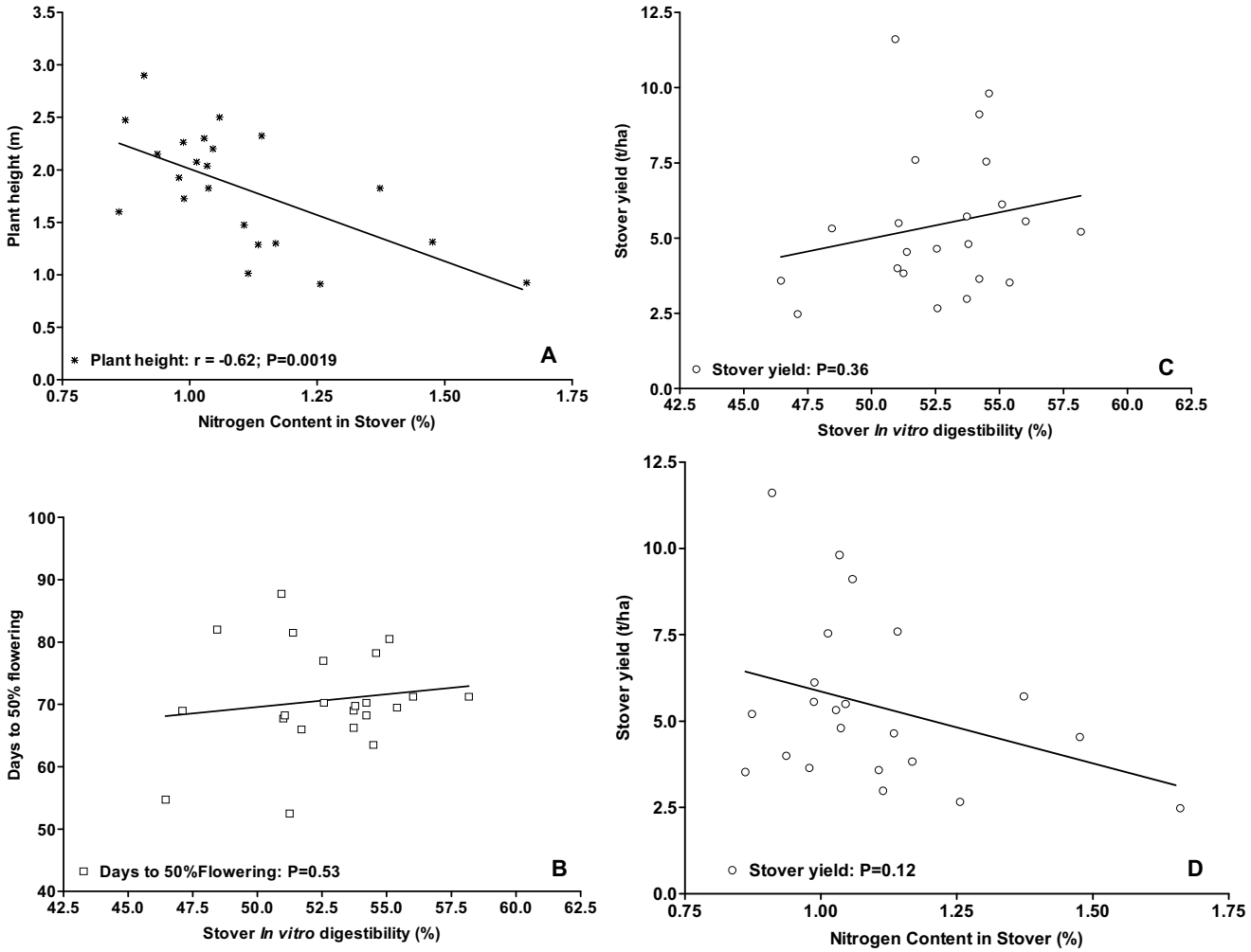

Fig. 1 Relationships between agronomic and forage quality traits. (**A**) Relationships between plant height and nitrogen content of stover in *bmr* mutants. (**B**) Relationships between days to 50% flowering and stover *in vitro* digestibility in *bmr* mutants. (**C**) Relationships between stover yield and stover *in vitro* digestibility in *bmr* mutants. (**D**) Relationships between stover yield and nitrogen content in *bmr* mutants.

correlation with ME (0.95) as reported in the number of previous studies (Rook *et al.* 1977; Cherney *et al.* 1991; Oliver *et al.* 2005).

CONCLUSION

There is enormous variability for agronomic traits (DF, PHT and stover yield) and also for forage quality traits (ADL, ME and IVOMD) among the 20 *bmr* mutants. The high biomass yielding *bmr* mutants with better forage quality, such as IS 23253, IS 21549, IS 23789 and IS 23787, can be exploited by converting them to male sterile by introgressing *ms3* genes for developing high biomass yielding hybrids without compromising the forage quality involving locally adapted cultivars.

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