Research Article
The J. Res. PJTSAU 51(3&4) 42-65, 2023

SCREENING OF SORGHUM (*SORGHUM BICOLOR* L.) GENOTYPES FOR BMR TRAIT BY HISTOCHEMICAL STAINING TECHNIQUE

BRUNO AWIO[1], MALLELA VENKATA NAGESH KUMAR[1], EPHREM HABYARIMANA[2], RAJAN SHARMA[2], ARE ASHOK KUMAR[2] and D. SRINIVASA CHARY[3]

[1]Department of Genetics and Plant Breeding, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad
[2]International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad
[3]Department of Statistics and Mathematics, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad

Date of Receipt: 27-12-2023 Date of Acceptance: 30-12-2023

ABSTRACT

Reddish to pink coloration of brown-midrib (*bmr*) mutant tissues upon histochemical staining with Wiesners' reagent has been associated with accumulation of cinnamaldehydes and acts as an indirect indicator of quality improvement in forage. Cinnamaldehydes are phenolic substrates associated with lignin biosynthesis pathway. This study was planned with an objective of understanding the relationship between Wiesner staining in stem, leaf midrib and forage quality traits. Twenty forage sorghum genotypes of both brown-midrib (*bmr)* and wild-type genotypes were used in the study. The genotypes were planted in a randomized complete block design. Histochemical staining on leaf and stem sections and stem forage quality traits were analyzed at flowering stage. The Wiesners reagent's active ingredient, phloroglucinol (1.0% (w: v)), reacts best in an acidic (10.1 M HCl) environment, and was dissolved in four acid-ethanol (v/v) levels of 35/65, 30/70, 25/75, 20/80. Stained cross-sections images of leaf midrib and stem tissues of the genotypes were captured in flatbed scanner and the staining intensity was scored using traditional histochemical and digitized methods. Forage quality analysis was performed on grounded stem tissue samples using Near Infrared Spectroscopy (NIRS). The analysis of variance revealed significant difference among genotypes for the histochemical staining traits, percent *in vitro* organic matter digestibility (IVOMD), Metabolizable energy (ME mj/kg), and percent neutral detergent fiber (NDF). Orthogonal contrast analysis revealed genotypes carrying *bmr* loci (*bmr6*, *bmr6bmr12* and *bmr12*) had significantly lower ADL (acid detergent lignin) and higher IVOMD than wild-type genotypes. Genotypes BN612, 59353, 59354, N605, N596 N600 had the highest IVOMD and lowest ADL, while wild-type genotypes IS26694, SSV-74, IS11861 and ICSV12006 had the lowest IVOMD and highest ADL. These genotypes had highly red-stained and faintly stained tissues, respectively based on traditional and digitized staining assessment. A highly significant positive correlation between the histochemical staining of leaf midrib and stem tissues was detected suggesting that similar substrates responsible for reaction with phloroglucinol were deposited in both organs. Furthermore, a significantly greater staining in *bmr6>bmr6bmr12>bmr12>Bmr* were detected. These findings shown promise for employing histochemical staining combined with high-through-put phenotyping in selection of *bmr* inbred lines of higher forage quality value. However, further studies are recommended using large number of *bmr* inbred lines to confirm the application of the histochemical staining technique for selection in early and later generation advancement of *bmr* inbred line development.

KEYWORDS: Sorghum, lignin biosynthesis pathway, flavonoid biosynthesis pathway, phloroglucinol, histochemical staining, near infra-red spectroscopy (NIRS), caffeic acid O-methyltransferase, cinnamyl alcohol dehydrogenase

Evolution of lignin in plants was fundamental in the colonization of terrestrial habitat by vascular plants (Rose *et al.,* 2007). Lignin is a product of phenylpropanoid pathway, it is constituted by three monolignol; guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) lignins. The monolignols are polymerized in secondary cell walls of specialized cells/ tissues such as xylem tracheid, vessel elements, and sclereid cell wall, and in spaces in between cellulose, hemicellulose and pectin components (Meents *et al.,* 2018). The dense matrix formed confers mechanical strength to plant cells and whole plant contributing to an erect growth. The inherent hydrophobicity property of lignin (Xin & Herburger, 2021), enables xylem and phloem to translocate water and nutrients to photosynthesizing cells and developing grain. Mutation in the lignin and flavonoid biosynthesis pathways has been associated with reddish brown coloration of the leaf midrib and stem (Bout & Vermerris 2003; Adeyanju et al., 2021). This phenotype called "brown midrib" was isolated in sorghum, maize and pearl millet (Cherney *et al.,* 1988; Sattler *et al.,* 2010). The phenotype appears as early as at three-leaf stage and as late as the sixth leaf stage (Jorgenson, 1931). The central advantage of these *bmr* mutants is their improved biochemical cell wall composition, higher glucose yield (Sattler *et al.,* 2014; Rivera-Burgos *et al.,* 2019) and enhanced fodder digestibility (Oliver *et al.,* 2005). Cattle

Email: awiobruno14@gmail.com

fed with *bmr* fodder show an improved feed intake (Muller *et al.,* 1972), body weight gain (Byers et al., 1964), and milk composition (Byers *et al.,* 1964). The trait also improves process yield of high biomass sorghum for biofuel production (Rivera-Burgos *et al.,* 2019). The first set of 19 *bmr* genotypes in sorghum were isolated and reported by Porter *et al.,* (1978) from mutagenized populations of two genotypes (954114 and 954104). Over the years, a total of 37 *bmr* mutants were isolated from mutagenized populations and naturally occurring spontaneous mutants in existing germplasm materials (Porter *et al.,* 1978; Gupta, 1995; Saballos *et al.,* 2008; Xin *et al.,* 2008; Xin *et al.,* 2009).

The brown midrib mutants have been grouped into eight *bmr* loci (Saballos *et al.,* 2008; Sattler *et al.,* 2014) to foster their utilization in crop breeding programmes. A number of alleles have also been reported within each of these loci (Saballos *et al.,* 2008). *bmr6* and *bmr12* loci and the associated alleles are the most commonly deployed in sorghum improvement. *bmr6,* an orthologous of *bm1* of maize brown midrib has mutations in cinnamyl alcohol dehydrogenase (CAD) gene, that encodes the enzyme involved in the conversion of each monolignol aldehyde to its corresponding alcohol in the last step of monolignol biosynthesis (Saballos *et al.,* 2009), while *bmr12*, an orthologous of *bm3* of maize harbors mutations in caffeic acid O-methyltransferase (COMT) gene that encodes an enzyme involved in the conversion of 5-hydroxy-coniferaldehyde to sinapaldehyde in the synthesis of sinapyl alcohol (Bout & Vermerris, 2003). The single nucleotide mutations in these genes leads to accumulation and incorporation of novel phenolic substrates in the cell wall (Bucholtz *et al.,* 1980; Saballos *et al.,* 2008). Indeed, more coniferaldehyde (Saballos *et al.,* 2008), *p*-coumaric acid and syringyl content (Akin *et al.,* 1986; Fritz *et al.,* 1990), and vanillin and *p*-hydroxybenzoic acid (Akin *et al.,* 1986; Fritz *et al.,* 1990) was reported in cell wall of brown midrib mutants than in corresponding normal genotypes. Ferulic acid has been reported to contribute to bonding between polysaccharide and polysaccharide, and between polysaccharide and lignin (Hatfield *et al.,* 1999). Other lignin components that have been hypothesized to copolymerize with lignin monolignol include hydroxycinnamyl alcohols (Blaschek *et al.,* 2020), benzaldehydes (vanillin), and cinnamyl aldehydes (Kim *et al.,* 2000; Pomar *et al.,* 2002; Blaschek *et al.,* 2020). The mutation of these genes results in changes in chemical composition, which can be detected and quantified using gas chromatography/ Mass spectroscopy (GC/MS) (Ralph and Hatfield 1991; Palmer *et al.,* 2008; Sarath *et al.,* 2006), which are expensive and mostly not accessible by most breeding programmes.

Histochemical staining has been used to monitor changes in cell wall biochemistry, and the characteristics of fundamental tissues, to categorize and evaluate the spatial distribution of cell wall constituents in various plant organs and tissues (Yang *et al.,* 2014; Lux *et al.,* 2005; Sarath; Palmer *et al.,* 2008; de Andrade *et al.,* 2017). Safranin-O.Mäule and Wiesner tests detect regions of plant tissues with low and high aldehyde and syringyl units, respectively (Anderson *et al.,* 2015). In sorghum, Wiesner test is being employed in identification of genotypes with a reduced lignin content. Phloroglucinol, the active ingredient of the reagent, under acidified condition, reacts with the aromatic aldehydes, hydroxyl benzaldehydes and cinnamaldehydes, to form reddish-brown and pink coloration, respectively (Pomar *et al.,* 2002; Blaschek *et al.,* 2020). Cinnamaldehydes was reported to contribute more into the total color production (Pomar *et al.*, 2002). This color formation on staining is independent of the type of lignin monolignol synthesized by a species (Ros *et al.,* 2007). In sorghum, the method has been used to differentiate wild-type allele (*Bmr*) and *bmr2* locus from three other allelic groups (Gorthy *et al*., 2013; Saballos *et al.*, 2008).

Histochemical staining has mostly been qualitatively measured, and subjectively quantified. Akin *et al.,* (1986) using the Wiesner test with visual scale of 1 – 3 to quantify the intensity of staining in epidermal tissue, xylem, parenchyma and sclerenchyma leaf midrib tissues of *bmr12* and the corresponding wild-type genotype. Iji *et al.,* (2022) used a visual scale of 0 – 2 to identify sensitive and less sensitive histochemical staining techniques (Solochrome azurine stain, Walton stain, and the modified Haematoxylin stain) in agar blocks for use in detecting aluminum toxicity in fish. Giaveno and Filho (2000) quantified hematoxylin degree of staining on a 0 (highly tolerant) to 5 (highly sensitive) visual scale to identify aluminum tolerant and sensitive maize genotypes. These staining

quantifications were found to correlate with chemical composition under investigation (Giaveno and Filho. 2000; Choi *et al.,* 2007). However, the lack of appropriate tools to quantify the staining has constrained the progress towards deploying histochemical staining in breeding. Tools such as ImageJ and Adobe Photoshop software have been used to improve accuracy in quantification of the staining (Choi *et al.,* 2007; Blaschek *et al*., 2020). Despite the fact that ImageJ offers a less subjective measurement, the quantification of the area for a specific colour still depends on the thresholds set for image analysis as well as the user's subjective evaluation of the image (Perrier *et al.,* 2017). This introduces random biases that leads to poor differentiation among genotypes (Perrier *et al.,* 2017). Nowadays computer algorithms can be constructed to capture relevant patterns in plant tissues (Da Silva *et al.,* 2015) and application can be built for an automated scanning and quantification of staining. *bmr* and wild-type genotypes leaf midrib and stem tissue staining can vary in the extent and intensity of their staining, and this information could be captured and utilized in breeding. Hence, the present study was conducted to establish the relationship between the staining of leaf midrib and stem tissues with forage quality traits to fast track *bmr* phenotyping in the process of *bmr* cultivar development.

MATERIAL AND METHODS

Experimental Material

Twenty (20) sorghum genotypes were used in the study; the genotypes included 6 introgression *bmr* lines carrying *bmr6* and 3 introgression lines carrying *bmr12*, 3 double mutant (*bmr6bmr12*) lines, and 8 white colored midrib (*Bmr*) wild-type genotypes (Table 1). The genotypes were selected from the breeding material at Sorghum Breeding Programme, ICRISAT, Patencheru, India. The wild-type genotypes included four improved sorghum genotypes and four landraces. The genotypes (*bmr* and wild-type) were chosen to evaluate the relationships between histochemical staining and fodder quality variables. The *bmr* genotypes used in the study were developed at USDA-ARS. Details for the development of these *bmr* introgression lines have been described in the works of Pedersen *et al.,* (2006a); Pedersen *et al.,* (2006b); Pedersen *et al.,* (2006c) and Pedersen *et al.,* (2008). These introgression lines were developed through four cycles of backcross and selfing. The *bmr6* and *bmr12* alleles were later pyramided through four cycles of backcross and selfing to generate four double *bmr* mutant lines under the genetic backgrounds of Atlas, Wheatland, and RT x 430 (Pedersen *et al.,* 2008). The current study made use of ten *bmr* near isogenic lines developed from the USDA-ARS and two new genetic backgrounds developed at ICRISAT (Table 1).

Experimental design

The genotypes were planted in post-rainy (*Rabi*) season 2021 in randomized complete block design of two replications. The experiment was conducted at ICRISAT, Patancheru, Telangana, India, in sand-clay soil. Genotype ICSV100305 did not germinate in both replication leaving a total of nineteen genotypes evaluated in the study. All the recommended crop management practices were followed to maintain good crop stand. At 50% flowering stage, six random plants per plot were cut from the base of the plants. The second leaf from the top of these plants were clipped off along with the last internode from the top-most part of the stem for use in the study. The leaves and the stems in uppermost part of the plants were chosen as suggested in earlier studies that reported the region to have high amounts of phenolic compounds in *bmr* genotypes (Palmer *et al.,* 2008; Dowd & Sattler, 2015; Dowd *et al.,* 2016). In laboratory, leaf midrib from three plants were carefully excised using a two-edged razor blade. Five-cm tissue sections at the base of the node were also obtained and were enclosed in plastic bags. These leaf midrib and stem samples were stored in refrigerator at -20^0 C until time for grinding and forage quality analysis. However, only the stem samples were later analyzed for forage quality.

Histochemical staining

Leaf midrib and stem tissues from the remaining three plants were used in the histochemical staining study. The sorghum midrib samples were dissected longitudinally to expose the inner tissues. Twenty-one (21) sections of leaf midrib tissues from three plants measuring ~ 2.5 mm in length were sectioned using two-edged razor blade. The histochemical staining protocol was adopted from the works of Saballos *et al.,* (2008). Here, stem and leaf

midrib tissues were soaked in deionized water twice for thirty minutes to remove water-soluble compounds. The samples were then immersed in acetone twice for one hour each to remove unbound phenolics compounds. These pieces were later soaked in 1.0% (w:v) phloroglucinol in 10.1 M hydrochloric acid-ethanol level (25/75, v/v) for 5 minutes as described by Saballos *et al.* (2008). Eight different proportions of acid-ethanol levels; 35/65, 30/70, 25/75, 20/80, 15/85, 10/90, 5/95 and 0:100 were prepared for the validation study. A set of three midrib pieces for each of the genotypes were submerged in the prepared phloroglucinol solution for 5 minutes. In the acidified solution, phloroglucinol reacts with aldehyde end-groups (Clifford, 1974), forming a stable red-brown pigments (Pomar *et al*., 2002). The three pieces of midrib tissues from the same leaf per genotype obtained for the seven proportions of acid-ethanol were loaded on microscopic glass slides (Fig.1). The same methodology was repeated in the stem sections staining. They were first sliced into ~1 mm thickness from the base of the node of the internode to generate 21 slices.

The stained tissues on the glass slides were scanned on graphic arts model flatbed scanner manufactured by Epson to generate 24-bit colored (std) images at a resolution of 200 (dpi) and scaling of 13% (Figure 1). The scanned images were imported in Adobe Photoshop (EPSON expression Version 1640XL), and trimmed at boundary of the plant tissues so that only the regions of leaf midrib and stem tissues were analyzed in python. The trimmed plant tissues were imported and analyzed in python using in-house script which has been provided in Appendix. A total of 1396 images for leaf tissues and 1256 images for stem tissues were obtained from the twenty genotypes.

Image analysis

The stained images of stem and leaf midrib of the nineteen genotypes were separately analyzed in Python (Spyder) version 3.9 (Van Rossum & Drake, 2009). Python packages such Panda (McKinney, 2010), Numpy (Harris et al., 2020) and cv2 (Bradski., 2000) were used in executing the analysis. Total number of pixels in the images, and the number of pixels in the highly stained regions were generated using cv2 library. The boundaries for the RED, GREEN, BLUE (RGB) colors were captured at 70, 50, 50 for the lower boundary, and 255, 255, 255 in the upper boundary for highly red-stained regions. The regions for medium and lowly red-stained regions were as well captured. These were optimized from online color picker (https://www.kapwing.com) and color codes chart (https://www.rapidtables.com/web/color/RGB_Color.html). The number of pixels in the highly red-stained portion per sample (Figure 1) were converted into percent number of pixels, by dividing the respective number of pixels by total number of pixels multiplied by 100. Number of pixels in the medium and lowly red-stained regions were as well recorded. However, there were mostly zero pixels in the medium red-stained regions, while, number of pixels in the low red-stained regions where as expected highly negatively correlated with number of pixels in the highly red-stained regions. Furthermore, the images were scored using a traditional scoring scale. A scale of 1-3 was used, where 1, implied no staining, 2 faint red staining and 3 highly red-stained (Akin *et al.,* 1986).

Forage quality analysis

The leaf midrib and stem tissues stored in the refrigerator were dried in a lyophilizer. The dried samples were grounded to a fine powder in a mix grinder and sieved to obtain ~ 0.75 mm particle size. However, only the stem samples were analyzed for forage quality. Samples of two genotypes (ICSV100453 and ICSV100309) were not analyzed due to the small quantity of biomass samples obtained. The forage quality variables were analyzed using NIRS at the ILRI laboratory, based at the ICRISAT campus, Patancheru, India. The spectral data of the dried, grounded (0.75 mm) samples for forage quality were obtained using the FOSS Forage Analyzer 5000 monochromator spectrophotometer, equipped with a rotation module that performs reflectance measurements in the spectral region between 400 and 12500 nm, at 2 nm intervals. The spectral data and the chemometric analysis were carried out using the WinISI II v program 1.5. “Outliers” samples were determined based on the Global Mahalanobis distance (GH) and NH values. Samples that had GH values greater than 3 and NH values greater than 1 had spectral information outside the calibration model range, and these were re-analyzed by wet chemistry method. The following variables were measured by the NIRS: percent dry matter (%dm), percent nitrogen (nit%dm), percent neutral detergent fiber (NDF%dm), percent acid detergent fiber

(ADF%dm), percent acid detergent lignin (ADL%dm), metabolizable energy (ME) (mjkg) and percent *in vitro* organic matter digestibility (invomd%). Estimates of percent hemicellulose and percent cellulose content were calculated by subtracting ADF from NDF, and ADL from ADF, respectively. Percent crude protein was obtained by multiplying percent nitrogen content by 6.25.

Data analysis

The raw datasets for the staining quantification for the individual genotypes were visualized in scatter plot in excel. The average percent number of pixel in highly red-stained portions of plant tissues per plot at each acid-ethanol level were obtained in excel. The data for percent number of pixel in the highly red-stained areas was square root transformed because the percent data were within the range 0 – 30% (Gomez & Gomez, 1984). Similarly, average scores of staining per plot were also obtained from the traditional method in excel. However, data from acid-ethanol levels of 35/65, 30/70, 25/75 and 20/80 where considered for presentation in this article. The staining at 0:100, 5/95, 10/90, 15/85 was only conducted in *bmr* genotypes, and the response of the genotypes were similar as in four acid-ethanol levels.

Forage quality variables were examined in excel, and three plot values were excluded from the analysis because of an unexpected forage variable values. These genotypes had forage quality estimates above the expected performance for wild-type genotypes. Analysis of variance for the histochemical staining quantification, traditional staining visual scores and forage quality traits were conducted following Randomized Complete Block Design model, considering genotypes as a fixed factor according to the model $Y_{ij} = ì + á_{i.} + â_{.j} + õ_{ij}$, where Y_{ij} is the performance of genotype in i^{th} replication and j^{th} genotype, ì is the mean performance of genotype across replication, $á_{i.}$ is the effects of i^{th} replication across genotypes, $â_{.j}$ is the effects of j^{th} genotype across replications, and $õ_{ij}$ is the measurement error associated with the i^{th} replication and j^{th} genotype. Orthogonal contrast analysis was performed to determine *bmr* (*bmr6*, *bmr12*, *bmr6bmr12*, and *Bmr*) effects on histochemical staining using digitized staining quantification, traditional visual stain scores and forage quality traits to fast track *bmr* phenotyping. Furthermore, correlation and partial regression analysis were performed using the genotype mean values to determine the relationships among genotypes, histochemical staining quantification and forage quality variables. The analysis of variance and correlation analysis were conducted in R software version 4.2.1 (R Core Team 2016), while the partial regression analysis was conducted in excel function.

RESULTS AND DISCUSSION

Evaluation for forage quality of the genotypes

There was no significant difference among the genotypes for most of the forage quality traits (percent DM, percent ASH, percent Nit., percent NDF, percent ADF, percent hemicellulose, percent cellulose and percent CP) (Table 2). Significant difference among the genotypes were detected for percent ADL (p=0.008), percent IVOMD (p=0.04), and percent ME (mj/kg) (p=0.03) (Table 2). Orthogonal contrast analysis revealed a significantly higher percent Ash, percent CP, percent IVOMD and ME (mj/kg) in *bmr* loci groups (*bmr6, bmr12, bmr6bmr12* and *Bmr*) than in the wild-type genotypes (Table 3). On the other hand, genotypes carrying *bmr* loci (*bmr6, bmr12* and *bmr6bmr12*) had a significantly lower percent ADL and percent ADF than in the wild-type genotypes (Table 3). However, no significant difference between genotypes carrying *bmr* loci (*bmr6, bmr12* and *bmr6bmr12*) and wild-type genotypes for percent NDF, percent hemicellulose and percent cellulose were detected (Table 3).

The genotypes were compared for percent ADL, percent IVOMD and ME that were found to have significant genotypic effects. *bmr* genotypes BN612 (mean IVOMD 66.7%, mean ME 9.81), 59353 (mean IVOMD 65.76%, mean ME 9.63), 59354 (mean IVOMD 65.13%, mean ME 9.82), N605 (mean IVOMD 64.38, mean ME 9.56), N608 (mean IVOMD 63.19, mean ME 9.08) and N600 (mean IVOMD 62.87, mean ME 9.44) had the highest percent digestibility and metabolizable energy (Table 4). On the other hand, wild-type genotypes IS26694 (mean IVOMD 58.89, mean ME 8.68), IS93025 (mean IVOMD 58.57, mean ME 8.77), IS11861 (mean IVOMD 57.15, mean ME 8.42) and ICSV12006 (mean IVOMD 56.63, mean ME 8.35), and a *bmr* genotype N610 (mean IVOMD 56.63, mean ME 8.27), N606 (mean IVOMD 59.25, mean ME 8.94), N597 (mean IVOMD 59.29, mean ME 8.51) had the least percent digestibility and

metabolizable energy (Table 4). Wild-type genotypes IS93025 (mean ADL 3.31), IS11861 (mean ADL 2.96), SSV-74 (mean ADL 2.53) and ICSV12006 (mean ADL 2.53), and *bmr* genotypes N606 (mean ADL 2.97) and RN613 (mean ADL 2.57) had the highest percent ADL while *bmr* genotypes N596 (mean ADL 1.77), BN612 (mean ADL 1.36), 59353 (mean ADL 1.08), BN611 (mean ADL 0.96), N597 (mean ADL 0.79) and N608 (mean ADL 0.60) had the least percent ADL (Table 4).

Evaluation for histochemical staining of the genotypes

Digitized image staining quantification and traditional scoring of degree of staining of the images were employed in both the leaf midrib and stem tissue histochemical staining assessment (Table 5). A highly significant genotypic difference for histochemical staining were detected at each of the four acid-ethanol levels (20:80, 25:75, 30:70 and 35:65) using both methods (Table 5). Mean performance of the genotypes were compared for histological staining. A general increase in the intensity of red staining in most of the genotypes with increases in acid levels under both the traditional and digitization method were observed (Table 6 and Table 7). Leaf midrib and stem tissues for genotypes N600 and 59353 carrying *bmr12*, 59354, N596 and N605 carrying *bmr6*, and RN613 and BN612 carrying *bmr6bmr12* were highly red-stained, while wild-type genotypes ICSV100453, IS11861, IS26694, ICSV12006 and SSV74 had no or were faintly red-stained (Table 6). Un expectedly, *bmr* genotypes N610 and N606 carrying *bmr12* were faintly red-stained as the wild-type genotypes across the four acid-ethanol levels (Table 6). Similar results were also found using the digitized method (Table 7).

Relationships between leaf midrib and stem tissues histochemical staining quantification

There was highly significant positive correlation for histochemical staining between stem and leaf midrib tissues at the four acid-ethanol levels assessed using both traditional and digitized method (Table 8). Similarly, a highly significant difference was detected for histochemical staining among the acid-ethanol levels within leaf midrib and stem tissues (Table 8).

Application of histochemical staining in breeding

The *bmr* loci effects were analyzed to demonstrate the usefulness of the histochemical staining in breeding. The comparison among *bmr* loci groups were made using both traditional and digitized methods (Table 9 and Table 10). In the traditional method, leaf midrib tissues of genotypes carrying *bmr6* had a significantly higher intensity of red-stains than leaf midrib tissues of genotypes carrying *bmr6bmr12*, followed by genotypes carrying *bmr12* and wild-type genotypes at acid-ethanol level of 20:80 (Table 9). Similar trend was detected at acid-ethanol levels of 25:75, 30:70 and 35:65 (Table 9). A similar trend was detected in the stem tissues of the genotypes (Table 9). However, there was no significant difference in the intensity of staining between stem tissues of genotypes carrying *bmr12* and wild-type genotypes at all the acid-ethanol levels, except at acid level of 20:80 (Table 9).

On the other hand, the digitized method, revealed leaf tissues for genotypes carrying *bmr6* had a significantly higher intensity of staining than genotypes carrying the other loci (*bmr12, bmr6bmr12* and *Bmr*) at acid-ethanol levels of 20:80, 25:75 and 30:70 (Table 10). However, there were no significant difference in the intensity of staining among genotypes carrying *bmr* loci (*bmr12, bmr6bmr12* and *Bmr)* (Table 10). Similar trend where observed in the staining of stem tissues (Table 10). The difference in the intensity of histological staining was in the order of *bmr12>bmr6bmr12>bmr12>Bmr* in the stem tissues (Table 10).

There are several histochemical staining techniques that have been developed for use in quick diagnosis and preliminary analysis of samples before applying more involving and expensive techniques (Pradhan Mitra & Loqué, 2014). Priority in choosing a staining method is based on sensitivity of the staining technique to detect the slightest quantity of the substrate responsible in the reaction to lead in staining of the tissues (Iji *et al.,* 2022). False negative detection may arise from using a lower concentration of the active ingredient involved in the staining or following an inappropriate tissue preparation procedure before staining (Lux *et al.,* 2005). Mäule and Wiesner tests are the major staining methods being employed in *bmr* screening, and these techniques identify regions of low and high aldehyde and syringyl unit contents, respectively (Anderson *et al.,* 2015). It has been empirically determined that phloroglucinol, the active

ingredient in the Wiesner' reagent reacts with coniferaldehyde and related aldehyde residues to form colored pigmentation (Clifford, 1974; Davidson et al., 1995; Pomar *et al*., 2002). As a result, it was recommended that this method can be used for the detection of the hydroxycinnamyl aldehyde end units contained in lignins (Davidson *et al.,* 1995; Pomar *et al*., 2002). This method has therefore been employed in differentiating *bmr* from wild-type genotypes (Saballos *et al.*, 2008; Gorthy *et al*., 2013). The *bmr* genotypes stain reddish-brown in Wiesner' solution when submerged in the solution for five minutes (Saballos *et al.*, 2008; Gorthy *et al*., 2013). However, the staining has been subjectively assessed limiting their usage in breeding. Computer algorithms capable of capturing staining intensities can be constructed to quantify the staining intensities. The current study uses Wiesner test to determine the relationship between Wiesner staining in stem, leaf midrib and forage quality variables with a goal of contributing to improve the method to fast-track the phenotyping of *bmr* trait in early and later generations in sorghum breeding pipelines.

The genotypes used in the study had statistically similar performance for forage quality traits; percent DM, percent ASH, percent NDF, percent ADF, percent hemicellulose, percent cellulose and percent CP (Table 2). These were associated with the less diverse materials evaluated in the study. Similar findings have been reported by de Aguilar *et al.,* (2014) who also found no significant genetotypic difference for NDF and ADF between near isogenic *bmr6* and wild-type genotypes under two genetic backgrounds. Another study, found statistically similar performance for percent CP content in *bmr* hybrids and wild-type hybrids from sorghum x sudan-grass hybrids (Sattler *et al.,* 2010). However, significant genotypic difference where detected for percent ADL, percent IVOMD, and ME (mj/kg) (Table 2). These traits are the most important in forage quality improvement. As a result, the genetic materials are ideal for histochemical staining studies. As expected, orthogonal contrast analysis revealed that there was a significantly higher percent IVOMD and ME (mj/kg) in genotypes carrying *bmr* mutants (*bmr6, bmr12,* and *bmr6bmr12*) than in wild-type genotypes (*Bmr*) (Table 3) which was reported in other studies (Oliver *et al.,* 2005; Dien *et al.,* 2009; Sattler *et al.,* 2010). The increased percent IVOMD and ME has been associated to the reduction in lignin content (Oliver *et al.,* 2005; Dien *et al.,* 2009; Sattler *et al.,* 2010). Indeed, genotypes carrying *bmr* mutants (*bmr6, bmr12*, and *bmr6bmr12*) were found to have a significantly lower percent ADL and percent ADF than in wild-type genotypes (*Bmr*) (Table 3). *bmr6* and *bmr12* mutants carry mutations in the CAD and COMT genes. The *Bmr6* and *Bmr12* loci, encodes cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT) enzymes, respectively. CAD catalyses the conversion of monolignol aldehyde (*p*-coumaraldehyde and coniferaldehyde) to its corresponding alcohol (Saballos *et al.,* 2009), while a COMT gene converts 5-hydroxy-coniferaldehyde to sinapaldehyde in the synthesis of sinapyl alcohol (Bout & Vermerris, 2003). These enzymes catalyses the last step of monolignol biosynthesis. As a result, the mutations of these genes results in a reduced lignin content.

Novel phenolic substrates such as coniferaldehyde (Saballos *et al.,* 2008), *p*-coumaric acid and syringyl content (Akin *et al.,* 1986; Fritz *et al.,* 1990), vanillin and *p*-hydroxybenzoic acid (Akin *et al.,* 1986; Fritz *et al.,* 1990) are deposited in cell wall of the *bmr* mutants. Phloroglucinol, the active ingredient in the Wieners' test reacts with these components of the plant cell wall (Pomar *et al.,* 2002; Blaschek *et al.,* 2020). Our study detected an increase in intensity of the staining on stem and leaf midrib tissues with the increase in the proportion of HCl in acid-ethanol solution using both the traditional and digitized method (Table 6 & 7). The active ingredient shows a favorable reaction under a more acidic condition (Clifford, 1974; Pomar *et al*., 2002). There was no major change in the ranking of genotype performance for histochemical staining at the four levels detected (Table 8). Earlier studies detected strong reddish-brown coloration of tissues for reaction with phloroglucinol at 20:80 HCl: Ethanol (Clifford, 1974) and 30:70 HCl: Ethanol levels (Saballos *et al.*, 2008; Gorthy *et al*., 2013). Therefore, a favorable result on staining of plant tissues with acidified phloroglucinol can be attained at levels of HCl: Ethanol in the range 20:80 to 35:65. The staining method shows promise for deployment in selection as noted from the significant genotypic differences at each of the four acid-ethanol levels (Table 5). One obvious utility of this staining that has been demonstrated is the generally greater intensity of the staining detected on *bmr* genotypes

than wild-type genotypes. Historically, these method has been qualitatively employed to confirm *bmr* status (Gorthy *et al*., 2013) and has been used in differentiating *bmr2* loci from *bmr6*, *bmr12* and *bmr19* allelic groups (Saballos *et al.*, 2008). *bmr* genotypes used in the study carry the *bmr6* and *bmr12* alleles. *bmr6* alleles are dysfunctional for the cinnamyl alcohol dehydrogenase (CAD) enzyme and are unable to convert monolignol aldehyde to its corresponding alcohol in the last step of monolignol biosynthesis (Saballos *et al.,* 2009), while *bmr12* alleles are dysfunctional in caffeic acid O-methyltransferase (COMT) enzyme hence the enzyme fails to catalyze conversion of 5-hydroxy-coniferaldehyde to sinapaldehyde in the synthesis of sinapyl alcohol (Bout & Vermerris, 2003). The defect in these enzymes leads to a buildup of hydroxycinnamyl aldehyde, which get integrated in plant cell wall (Bucholtz *et al.,* 1980; Saballos et al., 2008). However, these substrates (hydroxycinnamyl aldehyde) are converted into lignin monolignol in the presence of the fully functional enzymes. This explains why stem and leaf midrib tissues from the wild-type genotypes showed no stain or just faint red staining at the four levels of HCl: Ethanol (Table 6 & 7).

The degree of reddish-brown coloration of stem and leaf midrib in *bmr* genotypes depend on the growth stage and environment condition. An intense reddish-brown coloration is normally detected on the midrib of younger leaves and along the stem in some genotypes. Leaf and stem tissues from these regions of *bmr* genotypes have been reported to confer an antibiosis resistance to insect pest (Dowd & Sattler, 2015; Dowd *et al.,* 2016) and are toxic to disease pathogens (Palmer *et al.,* 2008; Funnell-Harris *et al.,* 2017). The current study found strong positive, but significant correlation between the staining of the leaf midrib and stem tissues using both traditional and digitized methods (Table 8), suggesting that the substrates needed for the phloroglucinol reaction accumulated similarly in the stem and leaf midrib tissues. The intensity of red stain was more pronounced in the stem than in the leaf midrib tissues. The chemical composition of upper internodes has been reported as complex, having array of soluble phenolic and associated aromatic chemicals compared to the bottom stem internodes (Sarath *et al.,* 2007; Palmer *et al.,* 2008). Our results suggest that the chemical composition of stem tissues could also be more complex than that of leaf midrib tissues. This result is in accordance with previous studies in sorghum. In sorghum, Akin and colleagues (1989) reported stem to have more specialized cells that can be lignified than in leaf tissues. In their study, xylem, sclerenchyma, epidermis, and parenchyma tissues in stem were extensively and intensely stained using Wiener's test, but only xylem and sclerenchyma tissues were stained in leave tissues. In another study, Pillonel *et al.,* (1991) observed that the shoot tissues of 20-day-old *bmr6* mutant had more intense pink staining in comparison to the shoot tissues of wild-type sorghum genotypes. Their analysis found guaiacyl aldehyde and syringyl aldehyde were present in high concentrations in *bmr6* mutant than in corresponding wild-type. As a result, either stem or leaf midrib tissues can provide a reliable means for histochemical investigation of *bmr* expression in sorghum.

This is the first attempt in the quantification of Wiesner's staining, and the results has demonstrated the possibility that the staining method can be improved and deployed in breeding. The intensity of the staining *bmr* loci was found greater in the order *bmr6*> *bmr6bmr12*> *bmr12*> *Bmr* using both approaches (Table 9 & 10). Mutations in *bmr6* mutants affects two stages in the lignin biosynthesis pathway; the conversion of monolignol aldehyde (*p*-coumaraldehyde and coniferaldehyde) to its corresponding alcohol (Saballos *et al.,* 2009; Palmer *et al.,* 2008), as compared to *bmr12* mutants that affects the conversion of 5-hydroxy-coniferaldehyde to sinapaldehyde in the synthesis of sinapyl alcohol (Bout & Vermerris, 2003; Palmer *et al.,* 2008). It is therefore expected that *bmr6* would have a greater effect on lignin reduction and alteration in cell wall chemistry than in *bmr12* mutants. Earlier studies such as the works of Palmer *et al.,* (2008) and Dowd and Sattler (2015) found higher soluble phenolic substrates and antibiosis resistance associated with mutants of *bmr6* over *bmr12*. These findings show that histochemical staining could provide an alternative approach in the preliminary selection of brown midrib in sorghum. Digitization of the staining quantification would encourage its deployment in breeding programs.

In summary, the genetic materials of our study provided a good assessment for deployment of

histochemical staining in breeding. *bmr* genotypes had low lignin content over the wild-type genotypes. Both approaches, traditional and digitized histochemical quantification had corroborated with variation in percent lignin content and percent IVOMD. The staining for leaf midrib and stem tissues were found high in *bmr6* mutants, followed by genotypes carrying double mutant (*bmr6bmr12*), then *bmr12* and *Bmr* (wild-type genotype) using both traditional and digitized method. These findings shown promise for employing histochemical staining combined with high-through-put phenotyping in breeding programs. However, further studies are recommended using a large number of *bmr* inbred lines to determine the relationships between histochemical staining quantification for forage quality assessment. The python algorithms for determining the estimates of staining intensity used in the study once improved can be incorporated in a user friendly device and deployed in large scale phenotyping of the *bmr* trait in breeding programs.

Table 1. List of sorghum genotypes used in the study

S. No.	Genotype	*Bmr* locus	Pedigree	References
1	N597	*bmr12*	Early Hegari-Sart x F220	Pedersen *et al.* (2006)
2	59353	*bmr12*	((IS 21893 x N 595) x IS 21893) x IS 21893	ICRISAT, Sorghum Program
3	N600	*bmr12*	Atlas(NSL 3986) x F220	Pedersen *et al.* (2006)
4	N606	*bmr12*	BTx630 x F220	Pedersen *et al.* (2006)
5	N608	*bmr12*	BTx630 x F220	Pedersen *et al.* (2006)
6	N 610	*bmr12*	RTx430 x F220	Pedersen *et al.* (2008)
7	BN 612	*bmr6bmr12*	N 599 x N 600 (Wheatland bg)	Pedersen *et al.* (2008)
8	BN 611	*bmr6bmr12*	N 598 x "Atlas *bmr12*"	Pedersen *et al.* (2008)
9	RN 613	*bmr6bmr12*	N609 x N610 (RT x 430 bg)	Pedersen *et al.* (2008)
10	59354	*bmr6*	((IS 23789 x IS 8813) x IS 23789) x IS 23789	ICRISAT, Sorghum Program
11	N596	*bmr6*	Early Hagari-sart x N 121	Pedersen *et al.* (2006)
12	N605	*bmr6*	BTx630 x N 121	Pedersen *et al.* (2006)
13	ICSV100305	*Bmr*	(IS 903 x SP 4511-2) -1-1-3-2-1-2	ICRISAT, Sorghum Program
14	ICSV 100453	*Bmr*	(IS 903 x SP 4511-2) -1-1-6-2-1-2	ICRISAT, Sorghum Program
15	ICSV100309	*Bmr*	(IS 903 x SP 4511-2) -1-1-6-5-1-1	ICRISAT, Sorghum Program
16	ICSV12006	*Bmr*	(Ch-1 x (DSV 4 x IS 23568) -1-2-1-1)-13-1-1-4	ICRISAT, Sorghum Program
17	IS 93025	*Bmr*	IS 93025	ICRISAT, Sorghum Program
18	IS 26694	*Bmr*	IS 26694	ICRISAT, Sorghum Program
19	IS 11861	*Bmr*	IS 11861	ICRISAT, Sorghum Program
20	SSV-74	*Bmr*	IS 23558-2-1	ICRISAT, Sorghum Program

Where bg = background

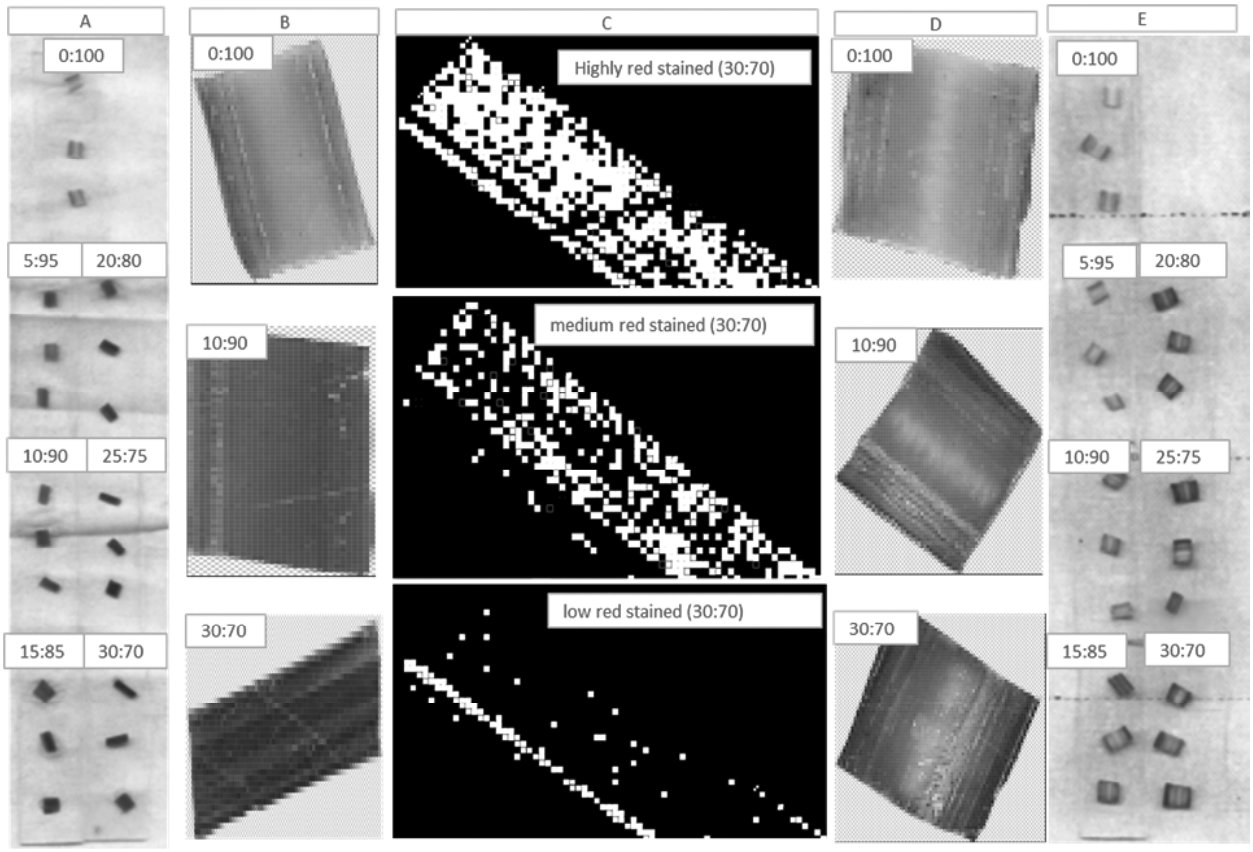

Fig. 1: leaf midrib pieces scanned by flatbed scanner (A & E), trimmed and magnified in Adobe photoshop (B & D) and image analysis in pthyon for highly, medium and low red stained areas (C). A, B & C represents bmr genotype, BN 612 and D & E represent non-bmr genotype, ICSV100305. Where 0:100, 5:95, 10:90, 15:85, 20:80, 25:75, 30:70 are HCl : Ethanol levels.

Table 2. Analysis of variance for 11 forage quality analysis conducted on stem tissues

	Df	DMC	Ash.	Nit	C P	NDF	ADF	ADL	IVOMD	ME	Hemi-cellu-lose	Cellu lose
Rep	1	0.14	0.33	0.09	3.42	2.56	0.37	0.01	0.04	0.000	4.89	0.28
Gen	16	0.549	1.686	0.119	4.658	10.548	9.736	1.138 * *	14.086 *	0.323 *	2.236	6.503
Resi-duals	8	0.211	1.016	0.059	2.313	7.528	7.478	0.191	4.15	0.083	0.931	6.405

DMC = percent dry matter content, Ash. = percent ash content, Nit = percent nitrogen content, CP = percent crude protein, NDF = percent neutral detergent fibre, ADF = percent acid detergent fibre, ADL = percent acid detergent lignin, IVOMD = percent *in vitro* organic matter digestibility, ME = metabolizable energy (mj/kg), Hemicellulose = percent hemicellulose, Cellulose = percent

Table 3. Orthogonal contrast analysis for bmr loci effects on 11 forage quality traits

	DMC					Ash.				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	90.006	0.1983	453.933	<2e-16	***	6.494	0.3561	18.237	3.58E-15	***
bmr6 vs *bmr12*	0.5115	0.3709	1.379	0.1812		0.0835	0.6662	0.125	0.9013	
bmr6bmr12 vs *bmr12*	0.274	0.3434	0.798	0.4331		-0.766	0.6168	-1.242	0.2268	
Bmr vs *bmr12*	0.7715	0.2974	2.594	0.0162	*	-1.4752	0.5342	-2.762	0.0111	*
	CP					**NDF**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	6.675	0.7334	9.101	4.38E-09	***	57.358	1.146	50.037	2.00E-16	***
bmr6 vs *bmr12*	-1.7125	1.3721	-1.248	0.2246		-0.578	2.144	-0.27	0.7899	
bmr6bmr12 vs *bmr12*	-2.717	1.2704	-2.139	0.0433	*	2.28	1.986	1.148	0.2626	
Bmr vs *bmr12*	-2.6825	1.1002	-2.438	0.0229	*	3.142	1.72	1.827	0.0807 .	
	ADF					**ADL**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	27.584	1.091	25.289	2.00E-16	***	1.249	0.2253	5.544	1.22E-05	***
bmr6 vs bmr12	0.481	2.041	0.236	0.8157		0.711	0.4214	1.687	0.105111	
bmr6bmr12 vs bmr12	2.36	1.889	1.249	0.2242		0.431	0.3902	1.105	0.28075	
Bmr vs bmr12	3.793	1.636	2.319	0.0297	*	1.481	0.3379	4.383	0.000217	***
	IVOMD					**ME (mj/kg)**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	62.473	0.9657	64.69 2	.00E-16	***	9.109	0.1459	62.452	2e-16	***
bmr6 vs bmr12	0.807	1.8067	0.447	0.65929		0.2535	0.2729	0.929	0.3625	
bmr6bmr12 vs bmr12	-0.639	1.6727	-0.382	0.70595		0.027	0.2526	0.107	0.9158	
Bmr vs bmr12	-4.4242	1.4486	-3.054	0.00563	**	-0.5152	0.2188	-2.355	0.0274	*
	Hemicellulose					**Cellulose**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	29.773	0.4228	70.414	2.00E-16	***	26.333	0.9815	26.83	2.00E-16	***
bmr6 vs *bmr12*	-1.0555	0.791	-1.334	0.195		-0.2305	1.8362	-0.126	0.901	
bmr6bmr12 vs *bmr12*	-0.077	0.7323	-0.105	0.917		1.931	1.7	1.136	0.268	
Bmr vs *bmr12*	-0.6518	0.6342	-1.028	0.315		2.317	1.4722	1.574	0.129	

DMC = percent dry matter content, Ash. = percent ash content, Nit = percent nitrogen content, CP = percent crude protein, NDF = percent neutral detergent fibre, ADF = percent acid detergent fibre, ADL = percent acid detergent lignin, IVOMD = percent *in vitro* organic matter digestibility, ME = metabolizable energy (mj/kg), Hemicellulose = percent hemicellulose, Cellulose = percent cellulose content

Table 4. Mean performance of 11 forage quality traits for *bmr* and wild-type genotypes obtained from top most stem internode sections

Genotype	*DMC	ASH	NIT	NDF	ADF	ADL	Hemicellulose	Cellulose	CP	ME	IVOMD
BN612	90.67	7.07	0.77	55.79	27.41	1.36	28.38	26.05	4.83	9.81	66.66
59353	90.01	7.46	0.98	54.49	25.75	1.08	28.74	24.67	6.12	9.63	65.76
59354	90.97	5.72	0.27	56.45	28.42	2.11	28.04	26.30	1.72	9.82	65.13
N605	91.22	5.73	0.46	58.08	30.19	2.20	27.89	27.99	2.86	9.56	64.38
N608	89.90	6.11	1.02	57.08	25.54	0.60	31.55	24.94	6.40	9.08	63.19
N600	89.00	5.09	0.75	56.66	28.34	1.93	28.31	26.41	4.72	9.44	62.87
N596	89.94	7.43	1.22	56.30	26.83	1.77	29.47	25.06	7.64	9.04	61.81
BN611	89.60	6.05	0.62	59.38	28.86	0.96	30.53	27.90	3.87	8.99	61.41
RN613	90.77	4.74	0.58	61.82	32.30	2.57	29.53	29.74	3.61	8.95	59.85
N597	90.19	7.19	0.98	62.03	31.82	0.79	30.21	31.03	6.09	8.51	59.29
N606	90.75	4.15	0.56	59.77	31.84	2.97	27.93	28.87	3.49	8.94	59.25
SSV-74	90.42	4.91	0.66	57.56	28.90	2.53	28.65	26.38	4.15	8.75	59.09
IS26694	91.01	5.36	0.73	61.09	30.63	2.34	30.46	28.30	4.56	8.68	58.89
IS93025	91.23	4.73	0.52	59.92	31.79	3.31	28.13	28.48	3.23	8.77	58.57
IS11861	90.86	5.43	1.00	61.85	33.05	2.96	28.80	30.10	6.24	8.42	57.15
N610	90.89	6.13	1.26	62.78	31.37	2.15	31.40	29.22	7.90	8.27	56.80
ICSV12006	90.23	4.82	0.48	61.29	32.12	2.53	29.18	29.59	3.00	8.35	56.63
Mean	90.16	6.33	0.76	56.78	27.67	1.50	29.11	26.17	4.77	9.42	63.90
LSD	N/A	N/A	N/A	N/A	N/A	1.03	N/A	N/A	N/A	0.28	1.96
CV%	0.51	17.25	31.61	4.65	9.22	22.68	3.20	3.35	3.28	9.13	31.66

*** DMC = percent dry matter content, Ash. = percent ash content, Nit = percent nitrogen content, CP = percent crude protein, NDF = percent neutral detergent fibre, ADF = percent acid detergent fibre, ADL = percent acid detergent lignin, IVOMD = percent *in vitro* organic matter digestibility, ME = metabolizable energy (mj/kg), Hemicellulose = percent hemicellulose, Cellulose = percent cellulose content**

Table 5. Analysis of variance for Wiesner test staining conducted on leaf midrib and stem tissues assessed using traditional method at each of the four acid-ethanol levels (20/80, 25/75, 30/70 and 35/65)

Visual scoring of the histochemical staining									
		Leaf midrib tissue				Stem tissue			
	Df	LF_20:80	LF_25:75	LF_30:70	LF_35:65	ST_20:80	ST_25:75	ST_30:70	ST_35:65
Replications	1	0.54 **	0.21	0.09	0.17	0.04	0.00	0.03	0.02
Genotypes	18	0.86 ***	1.08 ***	1.12 ***	1.13 ***	0.97 ***	0.86 ***	1.00 ***	0.90 ***
Residual error	13	0.06	0.05	0.07	0.14	0.05	0.06	0.03	0.05
Histochemical staining quantification using python									
		Leaf midrib tissue				Stem tissue			
	Df	LF_20:80	LF_25:75	LF_30:70	LF_35:65	ST_20:80	ST_25:75	ST_30:70	ST_35:65
Replications	1	0.43	0.38	1.47	1.89	0.28	0.28	0.02	0.23
Genotypes	18	2.51 ***	3.71 ***	4.02 ***	5.09 ***	0.52 ***	0.68 ***	0.75 ***	0.86 ***
Residual error	15	0.29	0.21	0.78	1.01	0.05	0.02	0.06	0.03

Table 6. Mean histochemical staining on leaf midrib and stem tissues of 19 genotypes assessed using traditional method (score scale of 1 – 3) at acid-ethanol levels of 20:80, 25:75, 30:70 and 35:65

	Visual scoring of the histochemical staining							
	Leaf midrib tissue				Stem tissue			
Genotype	**20:80**	**25:75**	**30:70**	**35:65**	**20:80**	**25:75**	**30:70**	**35:65**
59353	2.7	2.8	2.9	3.0	2.7	2.8	3.0	3.0
59354	3.0	3.0	3.0	3.0	2.7	2.8	2.9	3.0
BN611	1.6	2.0	2.4	3.0	2.1	2.4	2.6	3.0
BN612	2.0	2.5	2.8	2.8	2.9	2.9	3.0	2.9
ICSV100309	1.5	1.6	1.8	2.0	1.5	2.0	1.9	2.0
ICSV100453	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0
ICSV12006	1.0	1.0	1.0	1.0	1.0	1.9	2.0	1.9
IS11861	1.0	1.3	1.2	1.5	1.0	1.0	1.2	1.5
IS26694	1.0	1.0	1.0	1.0	1.1	1.7	2.0	2.0
IS93025	1.7	2.0	2.0	2.3	1.7	2.0	2.0	2.2
N596	2.5	2.9	3.0	3.0	2.6	3.0	3.0	3.0
N597	1.2	1.3	1.6	1.8	1.0	1.0	1.0	1.0
N600	2.3	2.7	2.7	2.8	2.7	2.6	2.9	3.0
N605	2.7	2.9	3.0	3.0	1.8	2.0	3.0	3.0
N606	1.0	1.0	1.2	1.3	1.7	1.9	1.9	2.0
N608	1.6	1.7	1.8	1.8	1.3	1.8	1.7	2.0
N610	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
RN613	2.5	2.8	2.8	2.8	2.8	3.0	3.0	3.0
SSV74	1.0	1.0	1.0	1.0	1.0	1.9	2.0	1.9
Mean	1.7	1.9	2.0	2.0	1.8	2.0	2.2	2.3
LSD	0.4	0.4	0.4	0.6	0.3	0.4	0.3	0.3
CV%	13.9	11.9	13.4	17.8	12.1	11.8	7.8	9.4

Table 7. Mean histochemical staining on leaf midrib and stem tissues of 19 genotypes assessed using digitized method (square root transformed percent number of pixels) at acid-ethanol levels of 20:80, 25:75, 30:70 and 35:65

	Histochemical staining qualification							
	Leaf midrib tissue				Stem tissue			
Genotype	**20:80**	**25:75**	**30:70**	**35:65**	**20:80**	**25:75**	**30:70**	**35:65**
59353	2.81	3.69	4.44	4.84	3.6	5.4	6.4	8.1
59354	3.47	3.74	3.44	1.91	5.7	5.7	6.3	7.6
BN611	0.74	1.06	1.05	0.71	1.2	1.7	3.4	4.2
BN612	1.88	2.21	3.18	2.17	6.4	7.8	8.2	8.3
CSV100309	0.81	1.00	0.87	0.71	1.8	2.5	2.4	2.5
ICSV100453	0.71	0.71	0.71	0.71	1.0	1.0	1.0	1.0
ICSV12006	0.71	0.71	0.71	0.71	1.1	2.6	2.0	1.9
IS11861	0.71	0.71	0.71	0.71	1.0	1.0	1.0	1.0
IS26694	0.71	0.71	0.71	0.71	1.2	1.7	1.7	2.3
IS93025	1.90	2.46	2.63	2.96	2.2	3.1	3.0	2.7
N596	2.26	3.89	3.85	4.17	5.4	8.2	8.1	8.1
N597	0.71	0.91	0.99	0.91	1.0	1.0	1.0	1.0
N600	3.67	3.90	2.92	4.59	6.5	5.7	6.8	7.2
N605	3.39	4.06	4.43	4.94	4.4	6.5	7.9	8.0
N606	0.71	0.71	0.78	0.78	2.0	2.4	2.5	2.1
N608	0.85	0.92	0.94	1.10	1.4	1.7	2.7	3.7
N610	0.71	0.71	0.71	0.71	1.0	1.0	1.0	1.0
RN613	2.97	3.17	3.28	2.98	6.8	7.4	7.2	7.0
SSV74	0.71	0.71	0.71	0.71	1.0	1.4	1.6	1.9
Mean	1.60	1.89	1.95	1.95	2.9	3.5	3.9	4.2
LSD	0.51	0.44	0.85	0.96	1.3	0.83	1.38	0.95
% CV	32.42	23.11	43.68	49.70	30.1	15.3	23.4	14.9

Table 8. Correlation analysis for histochemical staining among four acid-ethanol levels within leaf midrib and stem tissues using both traditional and digitized method

	*LT_20.80	LT_25.75	LT_30.70	LT_35.65	*ST_20.80	ST_25.75	ST_30.70	ST_35.65
LT_20.80	1	0.97***	0.91***	0.87***	0.88***	0.83***	0.85***	0.86***
LT_25.75	0.98***	1	0.96***	0.93***	0.87***	0.87***	0.89***	0.89***
LT_30.70	0.96***	0.99***	1	0.92***	0.84***	0.89***	0.91***	0.91***
LT_35.65	0.91***	0.96***	0.98***	1	0.74***	0.79***	0.82***	0.81***
ST_20.80	0.86***	0.89***	0.90***	0.88***	1	0.95***	0.93***	0.90***
ST_25.75	0.79***	0.81***	0.82***	0.78***	0.92***	1	0.98***	0.94***
ST_30.70	0.84***	0.86***	0.86***	0.82***	0.88***	0.95***	1	0.98***
ST_35.65	0.83***	0.85***	0.85***	0.83***	0.87***	0.89***	0.94***	1

The upper diagonal is the leaf midrib and stem digitized histochemical staining quantification and lower diagonal is the leaf midrib and stem traditional histochemical staining scores. *LT _ leaf midrib tissues, *ST_ upper internode stem tissues

Table 9. Orthogonal contrast analysis for leaf midrib and stem histochemical staining assessed using traditional method at acid-ethanol levels of 20:80, 25:75, 30:70 and 35:65

	Leaf histochemical staining									
	Conc 20:80					**Conc 25:75**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	1.62	0.16	9.94	0.00	***	1.74	0.17	10.01	0.00	***
bmr6 vs *bmr12*	1.10	0.29	3.78	0.00	***	1.20	0.31	3.88	0.00	***
bmr6bmr12 vs *bmr12*	0.40	0.27	1.45	0.16		0.70	0.29	2.39	0.02	*
Bmr vs *bmr12*	-0.41	0.23	-1.78	0.09	.	-0.41	0.25	-1.67	0.11	
	Conc 30:70					**Conc 35:65**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	1.86	0.17	10.90	0.00	***	1.94	0.19	10.29	0.00	***
bmr6 vs *bmr12*	1.14	0.31	3.72	0.00	***	1.06	0.34	3.16	0.00	**
bmr6bmr12 vs *bmr12*	0.79	0.29	2.73	0.01	*	0.9	0.32	2.83	0.01	**
Bmr vs *bmr12*	-0.50	0.24	-2.07	0.05	*	-0.44	0.27	-1.64	0.11	
	Stem histochemical staining									
	Conc 20:80					**Conc 25:75**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	1.75	0.17	10.60	0.00	***	1.85	0.17	10.71	0.00	***
bmr6 vs *bmr12*	0.69	0.30	2.32	0.03	*	0.83	0.31	2.71	0.01	*
bmr6bmr12 vs *bmr12*	0.83	0.28	2.98	0.01	**	0.89	0.29	3.06	0.00	**
Bmr vs *bmr12*	-0.55	0.23	-2.33	0.03	*	-0.17	0.24	-0.71	0.48	
	Conc 30:70					**Conc 35:65**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	1.92	0.18	10.88	0.00	***	1.99	0.17	11.73	0.00	***
bmr6 vs *bmr12*	1.02	0.32	3.24	0.00	**	0.99	0.30	3.26	0.00	**
bmr6bmr12 vs *bmr12*	0.93	0.30	3.14	0.00	**	0.98	0.29	3.42	0.00	**
Bmr vs *bmr12*	-0.18	0.25	-0.73	0.47		-0.07	0.24	-0.30	0.76	

Conc_ = concentration

Table 10. Orthogonal contrast analysis for leaf midrib and stem histochemical staining assessed using digitized method at acid-ethanol levels of 20:80, 25:75, 30:70 and 35:65

	Leaf histochemical staining									
	Conc 20:80					**Conc 25:75**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	1.58	0.29	5.51	0.00	***	1.81	0.31	5.83	0.00	***
bmr6 vs *bmr12*	1.47	0.50	2.95	0.01	**	2.09	0.54	3.89	0.00	***
bmr6bmr12 vs *bmr12*	0.29	0.50	0.58	0.57		0.34	0.54	2.64	0.53	
Bmr vs *bmr12*	-0.65	0.41	-1.61	0.12	.	-0.76	0.44	-1.73	0.09	
	Conc 30:70					**Conc 35:65**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	1.80	0.36	4.93	0.00	***	2.16	0.46	4.69	0.00	***
bmr6 vs *bmr12*	2.11	0.63	3.35	0.00	**	1.52	0.80	1.91	0.07	
bmr6bmr12 vs *bmr12*	0.71	0.63	1.12	0.27		-0.20	0.80	-0.26	0.80	
Bmr vs *bmr12*	-0.74	0.51	-1.43	0.16		-0.07	0.65	-1.65	0.11	
	Stem histochemical staining									
	Conc 20:80					**Conc 25:75**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	2.56	0.52	4.94	0.00	***	2.83	0.55	5.20	0.00	***
bmr6 vs *bmr12*	2.74	0.96	2.87	0.01	**	4.01	0.01	3.99	0.00	***
bmr6bmr12 vs *bmr12*	2.23	0.90	2.48	0.02	*	2.77	0.94	2.93	0.01	**
Bmr vs *bmr12*	-1.20	0.73	-1.64	0.11		-0.84	0.77	-1.09	0.28	
	Conc 30:70					**Conc 35:65**				
	Estimate	**Std.**	**Error**	**t_value**	**Pr(>|t|)**	**Estimate**	**Std.**	**Error**	**t_value**	**Pr(>|t|)**
(Intercept)	3.38	0.55	6.14	0.00	***	3.84	0.58	6.59	0.00	***
bmr6 vs *bmr12*	3.95	0.01	3.89	0.00	***	4.02	1.07	3.74	0.00	***
bmr6bmr12 vs *bmr12*	2.88	0.95	3.02	0.01	**	2.63	1.01	2.60	0.01	*
Bmr vs *bmr12*	-1.51	0.78	-1.94	0.06		-1.88	0.82	-2.27	0.03	*

Conc_ = concentration

REFERENCES

Adeyanju, A. O., Sattler, S. E., Rich, P. J., Rivera-Burgos, L. A., Xu, X and Ejeta, G. 2021. *Sorghum brown* midrib19 (*Bmr19*) gene links lignin biosynthesis to folate metabolism. Genes. 12(660): 1-18 https://doi.org/10.3390/genes12050660

Akin, D. E., Hanna, W. W ., Snook, M. E., Himmelsbach, D. S., Barton, F. E and Windham, W. R. 1986. Normal 12 and *Brown Midrib 12 Sorghum.* II. Chemical Variations and Digestibility 1. Agronomy Journal. 78(5): 832-837.https://doi.org/10.2134/agronj1986.00021962007800050018x

Akin, D. E. 1989. Histological and Physical Factors Affecting Digestibility of Forages. Agronomy Journal. 81(1): 17–25. https://doi.org/10.2134/agronj1989.00021962008100010004x

Anderson, N. A., Tobimatsu, Y., Ciesielski, P. N., Ximenes, E., Ralph, J., Donohoe, B. S., Ladisch, M and Chapplea, C. 2015. Manipulation of guaiacyl and syringyl monomer biosynthesis in an arabidopsis cinnamyl alcohol dehydrogenase mutant results in atypical lignin biosynthesis and modified cell wall structure. Plant Cell. 27(8): 2195–2209. https://doi.org/10.1105/tpc.15.00373

Blaschek, L., Champagne, A., Dimotakis, C., Nuoendagula, C., Decou, R., Hishiyama, S., Kratzer, S., Kajita, S and Pesquet, E. 2020. Cellular and Genetic Regulation of Coniferaldehyde Incorporation in Lignin of Herbaceous and Woody Plants by Quantitative Wiesner Staining. Frontiers in Plant Science. 11 March. 1–17. https://doi.org/10.3389/fpls.2020.00109

Bout, S and Vermerris, W. 2003. A candidate-gene approach to clone the sorghum Brown midrib gene encoding caffeic acid O-methyltransferase. Molecular Genetics and Genomics. 269(2): 205-214. https://doi.org/10.1007/s00438-003-0824-4

Bucholtz, D. L., Cantrell, R. P., Axtell, J. D and Lechtenberg, V. L. 1980. Lignin Biochemistry of Normal and Brown Midrib Mutant Sorghum. Journal of Agricultural and Food Chemistry. 28(6): 1239–1241. https://doi.org/10.1021/jf60232a045

Byers, J. H., Davis, C. L and Baylor, C. E. 1964. Feeding Value of Limestone-Treated Corn Silage for Lactating Dairy Cows. Journal of Dairy Science. 1(10): 1062–1064. https://doi.org/10.3168/jds.S0022-0302(64)88846-9

Cherney, J. H., Axtell, J. D., Hassen, M. M and Anliker, K. S. 1988. Forage Quality Characterization of a Chemically Induced Brown Midrib Mutant in Pearl Millet. Crop Science. 28(5): 783–787. https://doi.org/10.2135/cropsci1988. 0011 183 x0028 00050012x

Choi, E. Y., Graham, R and Stangoulis, J. 2007. Semi-quantitative analysis for selecting Fe- and Zn-dense genotypes of staple food crops. Journal of Food Composition and Analysis. 20(6): 496-505. https://doi.org/10.1016/j.jfca.2007.01.004

Clifford, M. N. 1974. Specificity of acidic phloroglucinol reagents. Journal of Chromotograph. 94: 321-324.

Davidson, R. S., Choudhury, H., Origgi, S., Castellan, A., Trichet, V and Capretti, G. 1995. The reaction of phloroglucinol in the presence of acid with lignin-containing materials. Journal of Photo chemistry and Photobiology A: Chemistry. 91: 87–93.

Da Silva, N. R., Florindo, J. B., Gómez, M. C., Rossatto, D. R., Kolb, R. M and Bruno, O. M. 2015. Plant identification based on leaf midrib cross-section images using fractal descriptors. PLoS ONE.10(6):https://doi.org/10.1371/journal.pone.0130014

De Andrade, E. A., Folquitto, D. G., Luz, L. E. C., Paludo, K. S., Farago, P. V and Budel, J. M. 2017. Anatomy and histochemistry of leaves and stems of Sapium glandulosum. Revista Brasileira de Farmacognosia. 27(3): 282–289. https://doi.org/10.1016/j.bjp.2017.01.001

Dien, B. S., Sarath, G., Pedersen, J. F., Sattler, S. E., Chen, H., Funnell-Harris, D. L., Nichols, N. N and Cotta, M. A. 2009. Improved sugar conversion and ethanol yield for forage sorghum (sorghum bicolor L. Moench) lines with reduced lignin contents. Bioenergy Research. 2(3), 153–164. https://doi.org/10.1007/s12155-009-9041-2

Dowd, P. F., Funnell-Harris, D. L and Sattler, S. E. 2016. Field damage of sorghum (*Sorghum bicolor)* with reduced lignin levels by naturally occurring insect pests and pathogens. Journal of Pest Science. 89(4): 885–895. https://doi.org/10.1007/s10340-015-0728-1

Dowd, P. F and Sattler, S. E. 2015. Helicoverpa zea (Lepidoptera: Noctuidae) and Spodoptera frugiperda (Lepidoptera: Noctuidae) Responses to Sorghum bicolor (Poales: Poaceae) Tissues from Lowered Lignin Lines. Journal of Insect Science. 15(1): https://doi.org/10.1093/jisesa/ieu162

Fritz, J. O., Moore, K. J and Jaster, E. H. 1990. Digestion Kinetics and Cell Wall Composition of Brown Midrib Sorghum x Sudangrass Morphological Components. Crop Science. 30(1): 213–219. https://doi.org/10.2135/cropsci1990.00111 83x003000010046x

Funnell-Harris, D. L., O'Neill, P. M., Sattler, S. E., Gries, T., Berhow, M. A and Pedersen, J. F. 2017. Response of sorghum stalk pathogens to brown midrib plants and soluble phenolic extracts from near isogenic lines. European Journal of Plant Pathology. 148(4): 941–953. https://doi.org/10.1007/s10658-017-1148-2

Gabriel, K. R., 1971. The biplot graphic display of matrices with application to principal component analysis. Biometrika. 58(3): 453 – 467. https://doi.org/10.1093/biomet/58.3.453

Giaveno, C. D and Filho, J. B. M. 2000. Rapid screening for aluminum tolerance in maize. Genetics and Molecular Biology, 23(4), 847–850.

Gomez, K. A and Gomez, A. A. 1984. Statistical Procedures for Agricultural Research (Second Edition).

Gorthy, S., Mayandi, K., Faldu, D and Dalal, M. 2013. Molecular characterization of allelic variation in spontaneous brown midrib mutants of sorghum (*Sorghum bicolor* (L.) *Moench*). *Molecular Breeding*, *31*(4), 795–803. https://doi.org/10.1007/s11032-012-9834-4

Gupta, S. C. 1995. Allelic Relationships and Inheritance of Brown Midrib Trait in Sorghum. Journal of Heredity. 86(1): 72–74. https://doi.org/10.1093/oxfordjournals.jhered.a111533

Harris, C. R., Millman, K. J., van der Walt, S. J., Gommers, R., Virtanen, P., Cournapeau, D., Wieser, E., Taylor, J., Berg, S., Smith, N. J., Kern, R., Picus, M., Hoyer, S., van Kerkwijk, M. H., Brett, M., Haldane, A., del Río, J. F., Wiebe, M., Peterson, P and Oliphant, T. E. 2020. Array programming with Num Py. Nature. 585(7825): 357–362. https://doi.org/10.1038/s41586-020-2649-2

Hatfield, R. D., Ralph, J and Grabber, J. H. 1999. Cell Wall Cross-Linking in Grasses by Ferulates and Diferulates. ACS Symposium Series. 79: 403–407. https://doi.org/10.1021/bk-1998-0697.ch016

Iji, O. T., Myburgh, J., Mokonoto, P. M and McGaw, L. J. 2022. Evaluating Three Histochemical Stains (Solochrome Azurine Stain (Asa), Walton Stain, and Modified Hematoxylin) used in Tissue Aluminium Detection. Nigerian Veterinary Journal. 42(3):189–196. https://doi.org/10.4314/nvj.v42i3.2

Jorgenson, L. R. 1931. Brown Midrib in Maize and its Linkage Relations1. Agronomy Journal. 23(7): 549.https://doi.org/10.2134/agronj1931.00021962002300070005x

Jung, H. G and Deetz, D. A. 1993. Cell wall lignification and degradability. Forage Cell Wall Structure and Digestibility. 315–346. https://doi.org/10.2134/1993.foragecellwall.c13

Kim, H., Ralph, J., Yahiaoui, N., Pean, M and Boudet, A. M. 2000. Cross-coupling of hydroxycinnamyl aldehydes into lignins. Organic Letters. 2(15): 2197–2200. https://doi.org/10.1021/ol005906o

Lux, A., Morita, S., Abe, J and Ito, K. 2005. An improved method for clearing and staining free-hand sections and whole-mount samples. Annals of Botany. 96(6): 989–996. https://doi.org/10.1093/aob/mci266

McKinney, W. 2010. Data Structures for Statistical Computing in Python. Proceedings of the 9th Python in Science Conference. 56–61. https://doi.org/10.25080/majora-92bf1922-00a

Meents, M. J., Watanabe, Y and Samuels, A. L. 2018. The cell biology of secondary cell wall biosynthesis. In Annals of Botany. 121(6): pp. 1107–1125. Oxford University Press. https://doi.org/10.1093/aob/mcy005

Mori, B and Bellani, L. M. 1996. Differential Staining for Cellulosic and Modified Plant Cell Walls.

Muller, L. D., Lechtenberg, V. L., Bauman, L. F., Barnes, R. F and Rhykerd, C. L. 1972. in Vivo Evaluation of brown midrib mutant of Zea mays L. Journal of Animal Science. 35(4): 883–889.

O'brien, T. P., Feder, N and Mccully, M. E. 1964. Polyehromatic Staining of Plant Cell Walls by Toluidine Blue O.

Oliver, A. L., Pedersen, J. F., Grant, R. J and Klopfenstein, T. J. 2005. Comparative Effects of the Sorghum *bmr -6* and *bmr-12* Genes: 1. Forage Sorghum Yield and Quality. Crop Sc. 45: 2234–2239. https://doi.org/10.2135/cropsci2004.0644

Perrier, L., Rouan, L., Jaffuel, S., Clément-Vidal, A., Roques, S., Soutiras, A., Baptiste, C., Bastianelli, D., Fabre, D., Dubois, C., Pot, D and Luquet, D. 2017. Plasticity of sorghum stem biomass accumulation in response to water deficit: A multiscale analysis from internode tissue to plant level. Frontiers in Plant Science. 8. https://doi.org/10.3389/fpls.2017.01516

Palmer, N. A., Sattler, S. E., Saathoff, A. J., Funnell, D., Pedersen, J. F and Sarath, G. 2008. Genetic background impacts soluble and cell wall-bound aromatics in brown midrib mutants of sorghum. Planta. 229(1): 115–127. https://doi.org/10.1007/s00425-008-0814-1

Pedersen, J. F., Funnell, D. L., Toy, J. J., Oliver, A. L., and Grant, R. J. 2006a. Registration of Seven Forage Sorghum Genetic Stocks Near Isogenic for the Brown Midrib Genes *bmr6* and *bmr12*. Crop Science. 46(1): 490–491. https://doi.org/10.2135/cropsci2005.06-0095

Pedersen, J. F., Funnell, D. L., Toy, J. J., Oliver, A. L and Grant, R. J. 2006b. Registration of Twelve Grain Sorghum Genetic Stocks Near isogenic for the Brown Midrib Genes *bmr6* and *bmr12*. Crop Science. 46(1): 491–492. https://doi.org/10.2135/cropsci2005.07-0183

Pedersen, J. F., Funnell, D. L., Toy, J. J., Oliver, A. L and Grant, R. J. 2006c. Registration of 'Atlas *bmr12*' Forage Sorghum. Crop Science. 46(1): 478. https://doi.org/10.2135/cropsci2005.06-0094

Pedersen, J. F., Toy, J. J., Funnell, D. L., Sattler, S. E., Oliver, A. L and Grant, R. A. 2008. Registration of BN611, AN612, BN612 and RN613 Sorghum Genetic Stocks with Stacked *bmr6* and *bmr12* Genes. Journal of Plant Registrations. 2(3): 258–262. https://doi.org/10.3198/jpr2008. 01.00 65crs

Pillonel, C., Mulder, M. M., Boon, J. J., Forster, B and Binder, A. 1991. Involvement of cinnamyl-alcohol dehydrogenase in the control of lignin formation in *Sorghum bicolor* L. *Moench.* In Planta. 185: 538-544

Pomar, F., Merino, F and Barceló, A. R. 2002. O-4-linked coniferyl and sinapyl aldehydes in lignifying cell walls are the main targets of the Wiesner (phloroglucinol-HCl) reaction. Protoplasma. 220(2): 17–28. https://doi.org/10.1007/s00709-002-0030-y

Porter, K. S., Axtell, J. D., Lechtenberg, V. L and Colenbrander, V. F. 1978. Phenotype, Fiber Composition, and in vitro Dry Matter Disappearance of Chemically Induced Brown Midrib (*bmr*) Mutants of Sorghum1. Crop Science.18(2): 205. https://doi.org/10.2135/cropsci1978.0011183x 001800020002x

Pradhan Mitra, P and Loqué, D. 2014. Histochemical staining of Arabidopsis thaliana secondary cell wall elements. Journal of Visualized Experiments. 87: 1-11. https://doi.org/10.3791/51381

Ralph, J and Hatfield, R. D. 1991. Pyrolysis-Gc-Ms Characterization of Forage Materials. Journal of Agricultural and Food Chemistry. 39(8): 1426–1437. https://doi.org/10.1021/jf00008a014

Rivera-Burgos, L. A., Volenec, J. J and Ejeta, G. 2019. Biomass and Bioenergy Potential of Brown Midrib Sweet Sorghum Germplasm. Frontiers in Plant Science. 10: 1-12. https://doi.org/10.3389/fpls.2019.01142

Ros, L. V. G., Gabaldón, C., Pomar, F., Merino, F., Pedreño, M. A and Barceló, A. R. 2007. Structural motifs of syringyl peroxidases predate not only the gymnosperm-angiosperm divergence but also the radiation of tracheophytes. New Phytologist. 173(1): 63–78. https://doi.org/10.1111/j.1469-8137.2006.01898.x

R Core Team 2016 R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Saballos, A., Ejeta, G., Sanchez, E., Kang, C and Vermerris, W. 2009. A genomewide 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. https://doi.org/10.1534/genetics.108.098996

Saballos, A., Vermerris, W., Rivera, L and Ejeta, G. 2008. Allelic Association, Chemical Characterization and Saccharification Properties of brown midrib Mutants of Sorghum (*Sorghum bicolor* (L.) Moench). BioEnergy Research. 1(4): 193–204. https://doi.org/10.1007/s12155-008-9025-7

Sarath, G., Baird, L. M., Vogel, K. P and Mitchell, R. B. 2007. Internode structure and cell wall composition in maturing tillers of switchgrass (*Panicum virgatum*. L). Bioresource Technology. 98(16): 2985–2992. https://doi.org/10.1016/j.biortech.2006.10.020

Sattler, S. E., Saballos, A., Xin, Z., Funnell-Harris, D. L., Vermerris, W and Pedersen, J. F. 2014. Characterization of novel sorghum brown midrib mutants from an EMS-mutagenized population. G3: Genes, Genomes, Genetics. 4(11): 2115-2124. https://doi.org/10.1534/g3.114.014001

Sattler, S. E., Funnell-Harris, D. L and Pedersen, J. F. 2010. Brown midrib mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues. Plant Science. 178(3): 229–238. https://doi.org/10.1016/j.plantsci.2010.01.001

Tetreault, H. M., Gries, T., Liu, S., Toy, J., Xin, Z., Vermerris, W., Ralph, J., Funnell-Harris, D. L and Sattler, S. E. 2021. The Sorghum (*Sorghum bicolor*) Brown Midrib 30 Gene Encodes a Chalcone Isomerase Required for Cell Wall Lignification. Frontiers in Plant Science. 12: 1-18. https://doi.org/10.3389/fpls.2021.732307

Xin, A and Herburger, K. 2021. Mini Review: Transport of Hydrophobic Polymers Into the Plant Apoplast. In Frontiers in Plant Science. 11: 1-8 Frontiers Media S.A. https://doi.org/10. 3389/fpls.2020.590990

Xin, Z., Li Wang, M., Barkley, N. A., Burow, G., Franks, C., Pederson, G and Burke, J. 2008. Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. BMC Plant Biology. 8(103): 1-14. https://doi.org/10.1186/1471-2229-8-103

Xin, Z., Wang, M. L., Burow, G., & Burke, J. 2009. An induced sorghum mutant population suitable for bioenergy research. Bioenergy Research. 2(2): 10-16. https://doi.org/10.1007/s12155-008-9029-3

Yang, C., Zhang, X., Li, J., Bao, M., Ni, D and Seago, J. L. 2014. Anatomy and histochemistry of roots and shoots in wild rice (Zizania latifolia Griseb.). Journal of Botany. 2014. 1-9. https://doi.org/10.1155/2014/181727

Appendix

The in-house script used

```
# -*- coding: utf-8 -*-
“””
Spyder Editor
This is a temporary script file.
“””
# -*- coding: utf-8 -*-
“””
Bruno’s Research
@author: xenificity
“””
print(“Red staining is divided into three levels\n\n”,”Please wait!\n\n\n”)
#Importing libraries
import cv2
import numpy as np
import pandas as pd
import os
#directory=os.chdir(“C:/Users/Bruno/Desktop/Leaf - Shared”)
directory=os.chdir(“C:/Users/Bruno/Desktop/Leaf - Shared/leaf - plot 7”)
list_dir=os.listdir(directory)
def identify_stain():
   result = pd.DataFrame(columns=[‘Image’,’Total_pixels’,’High_red’,’medium_red’,’low_red’,’perc_high_red’,’perc_med_red’,’perc_low_red’])
   for i in list_dir:
   #importing image -representing your original image
      image = cv2.imread(str(i))
      #cv2.imshow(“image”,image)
       #cv2.waitKey(0)
       #cv2.destroyAllWindows()
       #Converting image RGB into HSV -Gives more insights from your original image
       img_hsv = cv2.cvtColor(image,cv2.COLOR_BGR2HSV)
      #cv2.imshow(“image”,img_hsv)
       #cv2.waitKey(0)
      #cv2.destroyAllWindows()
     def red_intensity_high():
         lower_bound = np.array([151,87, 95])
         upper_bound = np.array([166,94, 108])
         msk = cv2.inRange(img_hsv, lower_bound, upper_bound)
         #cv2.imshow(“image”,msk)
          #cv2.waitKey(0)
          #cv2.destroyAllWindows()
          height, width = msk.shape[:2]
          num_pixels = height * width
         count_white = cv2.countNonZero(msk)
         percent_high_red_pixels = (count_white/ num_pixels) * 100
         percent_high_red_pixels = round(percent_high_red_pixels,2)
         high_red_pixels=count_white
          return num_pixels,high_red_pixels,percent_high_red_pixels
     def red_intensity_medium():
         lower_bound = np.array([102,51, 0])
         upper_bound = np.array([255,229, 204])
         msk = cv2.inRange(img_hsv, lower_bound, upper_bound)
         #cv2.imshow(“image”,msk)
          #cv2.waitKey(0)
          #cv2.destroyAllWindows()
```

```
        height, width = msk.shape[:2]

        num_pixels = height * width

       count_white = cv2.countNonZero(msk)

      percent_medium_red_pixels = (count_white/
num_pixels) * 100

       percent_medium_red_pixels =
round(percent_medium_red_pixels,2)

       medium_red_pixels = count_white

     return
medium_red_pixels,percent_medium_red_pixels

   def red_intensity_low():

       lower_bound = np.array([255,51, 51])

       upper_bound = np.array([255,204, 204])

       msk = cv2.inRange(img_hsv, lower_bound,
upper_bound)

       #cv2.imshow("image",msk)

        #cv2.waitKey(0)

       #cv2.destroyAllWindows()

        height, width = msk.shape[:2]

        num_pixels = height * width

       count_white = cv2.countNonZero(msk)

       percent_low_red_pixels = (count_white/
num_pixels) * 100

       percent_low_red_pixels =
round(percent_low_red_pixels,2)

       low_red_pixels=count_white

       return
low_red_pixels,percent_low_red_pixels

   a,b,c=red_intensity_high()

   d,e=red_intensity_medium()

   f,g=red_intensity_low()

     data =
[[str(i),int(a),int(b),int(c),int(d),int(e),int(f),int(g)]]

     data_new =
pd.DataFrame(data,columns=['Image','Total_pixels','
High_red','m

edium_red','low_red','perc_high_red','perc_med_red','perc

_low_red']

   result = pd.concat([result,data_new])

   print(result)

    #print(xyz)

   #print("Results are:
High",result_1,"Medium",result_2,"low",result_3)

  return result

   #result.to_csv('C:/Users/Bruno/Desktop/Leaf -
Shared/results.csv')

output=identify_stain()

output.to_csv(".....")
```