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Research Article

Unraveling Genotypic Stability of Stem Sweetness in Sweet Sorghum across Growth Stages and Environments

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ABSTRACT

Stem sweetness, that is measured as Brix % is an important trait in sweet sorghum which has direct implication for fodder quality, bioethanol production, and farmer preference. The present study evaluated a diverse panel of 103 sorghum genotypes for stem sweetness in terms of brix% over two consecutive years (2024-2025) at two stages: anthesis and physiological maturity. To address the complexities of environmental variability linear mixed-effects model was used to check the significance of genotype, growth stage, and year along with their interaction effects. The LMM based analysis of variance revealed highly significant effects for genotype, growth stage, and year effect along with their interaction effects. As genotypic effects along with their interactions were significant hence BLUP and WAASB were used to know which genotype is best and more stable. GP-15 and F-902 successfully identified as highly sweet genotypes along with maintaining high brix% regardless of environmental pressure. Furthermore, principal component analysis was exploited that demonstrated a positive correlation of brix% with plant height and internode length, suggesting that sugar storage capacity is linked to vigorous vegetative development. Notably, brix was found to be independent of stem diameter. While checking growth effect brix median progressively increased from ~ 10% to ~ 17% from anthesis to maturity respectively. Further, a potential influence of soil properties on stem Brix expression was also observed. As higher soil nutrients', OM, P, K availability coincides with increase brix (17%) as compared to low soil nutrients where brix remained 15%. Collectively, these results provide a robust, statistically validated framework for the selection of stable, high-sugar sweet sorghum genotypes optimized for high-quality fodder production in variable rainfed agro-ecologies.

Keywords: Sweet sorghum; Stem Brix; Genotype stability; Linear mixed-effects model; BLUP–WAASB analysis; multi-environment evaluation

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INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is a globally important cereal crop cultivated for grain, fodder, and bioenergy purpose (Bakari *et al.*, 2023). It is ranked fourth in the production of major cereals and fifth in acreage after major cereals wheat, rice, maize and barley (Singh and Chauhan, 2017). Regarding agronomic view its commercial cultivars are divided into grain sorghum, forage/fodder sorghum, fiber sorghum, broom sorghum, biomass sorghum and sweet sorghum. Among these, sweet sorghum has gained considerable attention due to its juicy stem rich in sugar contents, often taller, and suitability for fodder production particularly in rainfed and marginal environments where conventional forages often fail to produce dequate biomass (Mokariya and Malam, 2020). Its suitability for fodder enhances palatability, voluntary intake, and digestibility in ruminant animals (Awan *et al.*, 2015; Kumar *et al.*, 2024).

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Stem sweetness, commonly measured as brix percentage/TSS(total soluble solids), is a complex quantitative trait influenced by genetic makeup, growth stage, and environmental conditions (Kawahigashi *et al.*, 2013). Higher stem sugar concentration in sweet sorghum fodder has been positively associated with increased dry matter intake, and high relative feed value better silage quality attributed (Amer *et al.*, 2012; Yucel and Erkan, 2020). Unlike grain sorghum, sweet sorghum retains high biomass, bagass, cane and juice yield, larger leaf area and longer persistent of the green state with stem succulence and juiciness even at later growth stages, making it well-suited for green chop and silage production (Kanbar *et al.*, 2021).

Previously studies were reported concluding that accumulation of sugars in sorghum stem is a highly developmentally regulated process, which not only influenced by growth stage and environmental conditions but also of genotypes (Kanbar *et al.*, 2021; Shukla *et al.*, 2017). Hence genotypes maintaining stable brix expression are therefore particularly valuable for breeding programs which are oriented for sweet fodder of sorghum with higher palatability and digestibility. However, significant genotype × environment (G×E) interactions for brix and related fodder quality traits complicate selection based on single-stage or single-season evaluations (Shiringani *et al.*, 2010).

To resolve these complexities and mitigate the bias introduced by environmental noise, conventional fixed-effects ANOVA was needed to be bypassed by a Linear Mixed-Effects Model (LMM) framework to check the significance of the main effects along with interactions effects. Further, Best Linear Unbiased Prediction (BLUP), were reported to be utilized as it provides more accurate genetic estimates through "shrinkage," which adjusts genotype means based on their reliability across environments (Piepho *et al.*, 2008; Smith *et al.*, 2004). Furthermore, to bridge the gap between high productivity and environmental robustness, use of Weighted Average of Absolute Scores (WAASB) index was reported which was recently developed by Olivoto *et al.*, 2019 with an integrated stability statistic based on AMMI and BLUP models. This statistic is the weighted average of absolute scores from the singular value decomposition (SVD) of the matrix of the best linear unbiased predictions for the genotype × environment interaction effects generated by a linear mixed-effect model. Recently Behera *et al.* (2024) used the WAASB stability index for identifying genotypes with good forage quality in sorghum.

In this context, the present study was undertaken (i) LMM to check significance of main and interactions effects further to quantify the extent of genetic variability for stem Brix content in sorghum using likelihood-based mixed models BLUP was exploited (ii) growth stage, year, and genotype complex interplay on sugar accumulation patterns was also checked and (iii) potential of WAASB-based stability analysis to classify sorghum genotypes based on their sweetness potential and stability was also evaluated . Additionally, Principal Component Analysis (PCA) was utilized to assess the relationships between brix% and other morphological traits. Further it was also checked that either change in soil properties have any impact on brix%. The findings are expected to support the identification of elite and stable sorghum genotypes suitable for high-quality fodder production and bio-industrial utilization under variable rainfed conditions.

MATERIALS AND METHODS

Experimental Site and Environmental Conditions

The present research was performed at Barani Agricultural Research Station, Fatehjang for consecutively over two cropping seasons that were spanning around 2024 and 2025. The experimental area is characterized as a barani area which permanently reliant on rainfall for the whole crop season. For collecting soil properties related data soil samples were collected from two depths (0–15 cm and 15–30 cm) prior to sowing during both years Soil samples were analyzed for electrical conductivity (EC), pH, organic matter, available phosphorus, and extractable potassium, following standard laboratory procedures (Supplementary Figure S1). To account for inter-annual environmental variation, meteorological data, including minimum and maximum temperature, and total precipitation, were recorded for both years presented in Figure 1.

Plant Materials and Germplasm Diversity

100 sorghum genotypes along with 3 commercially released varieties used as checks were utilized in this study. The germplasm collection included a broad spectrum of genetic resources, comprising local landraces, internationally released varieties, and advanced breeding lines developed for fodder purposes. This diversity was selected to ensure a wide range of variation in stem sweetness (brix %) and morphological architecture, facilitating the identification of stable genotypes for variable environments.

Data Collection

Data collection was carried out at the both growth stages: Anthesis (50% flowering) and physiological maturity. The primary trait, stem sweetness (brix %), was measured using a handheld digital refractometer (0–32% range).

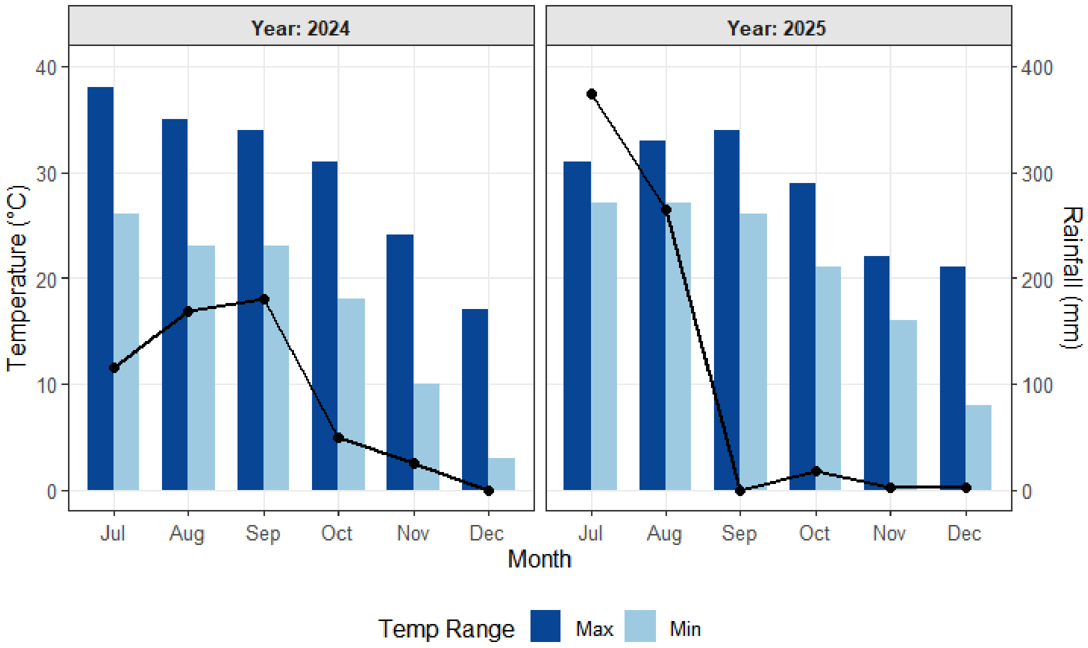

Figure 1. Metrological data of two cropping seasons for monthly rainfall represented as line graph, minimum temperature and maximum temperature represented as clustered columns graph respectively.

Juice was extracted from the middle internode of the main stem from five randomly selected plants per plot. This sampling method ensures a representative measurement of the TSS available in the stalk. To support multivariate analysis, several biomass-related traits were recorded at maturity. Plant height (cm) measured from the soil surface to the tip of the panicle. Stem diameter (mm) measured at the third internode from the base using a digital vernier caliper. Internode length (cm) was measured as the average length of the middle three internodes. Number of green leaves counted at maturity to assess stay-green characteristics.

Statistical Analysis

Due to the environmental heterogeneity and the nature of the augmented block design, a linear mixed-effects model (LMM) was implemented using the lme4 and metan packages in R (Olivoto *et al.*, 2019). In the present model to check the significance of the genotype, stage and year they were set as fixed effect while blocks were considered as random. As fixed effects in the model represents the factors whose levels are of primary interest while random effect represents that source of variation which is not of interest but are included to check for experimental variability. The model partitioned the total phenotypic variance into its constituent components that are genotype, growth stage, year, and their subsequent interactions (genotype × stage, genotype × year, and genotype × stage × year) (Smith *et al.*, 2004). The modal equation is presented below.

$$Y_{ijkl}=\mu+G_i+S_j+Y_k+(GS)_{ij}+(GY)_{ik}+(SY)_{jk}+(GSY)_{ijk}+B_l(Y_k)+\varepsilon_{ijkl}$$

Here in the model equation Y_{ijkl} represent brix value, μ is overall mean, G_i is the effect of the i^{th} genotype, S_j effect of the j^{th} stage, Y_k is the effect of k^{th} year, GS_{ij} is the genotype × stage interaction, GY_{ik} is the genotype × year interaction+ SY_{jk} stage × year interaction, GSY_{ijk} is the interaction of genotype × stage × year, $B_l(Y_k)$ is the effect of block with in each year, ε_{ijkl} is residual error.

To validate the necessity of the mixed model, a Likelihood Ratio Test (LRT) was performed. The BLUP approach was used to predict the genetic merit of each genotype using adjusted means which were accompanied with 95% confidence interval. BLUPs are superior to arithmetic means in multi-environment trials as they "shrink" estimates toward the mean, effectively reducing the impact of environmental noise and providing more reliable adjusted means for genotype ranking (Piepho *et al.*, 2008). Further, to ensure the validity of the statistical inferences, the residuals of the mixed models were subjected to rigorous diagnostic checks: Normality was assessed via Normal Q-Q plots to ensure the residuals followed a Gaussian distribution. Homogeneity of variance was evaluated using Residuals vs. Fitted values and Scale-Location plots to confirm that error variance was constant across different levels of the predictor variables. Independence was confirmed via Residuals vs. Factor-levels plots to ensure no systematic bias was introduced by specific genotype or blocks. Model diagnostics assess the adequacy of the linear mixed-effects model for stem Brix content were presented in (Supplementary Figure S2).

Genotypic stability for stem Brix across years and stages was quantified using the WAASB index. The WAASB is calculated based on the SVD of the BLUP-based interaction matrix. A lower WAASB score indicates higher stability (minimal interaction with the environment), while a higher score suggests environment-specific sensitivity. Genotypes were plotted on a 4-quadrant biplot according to (Olivoto *et al.*, 2019). To explore the relationships between sweetness and morphological development, Principal Component Analysis (PCA) was performed on the adjusted genotype means. (Yan and Tinker, 2006).

RESULTS

LMM ANOVA showed significant variations for all the main effects genotype, stage, and year along with their interaction's effects i.e., genotype: stage, genotype: year, stage: year, genotype: stage: Year as P value for all these is < 0.001 (Table 1). Hence, stem Brix percentage is a complex trait governed by strong genetic factors, environmental fluctuations, and developmental timing.

Table 1. Analysis of variance for stem brix content in sorghum genotypes evaluated across environments.

Effect	Sum Sq	DF	F value	P value
Genotype	2596.2	102	19.74	2.2×10^{-16} ***
Stage	4184.2	1	3244.60	2.0×10^{-16} ***
Year	280.7	1	217.64	2.1×10^{-16} ***
Genotype: Stage	1294.8	102	9.84	1.24×10^{-14} ***
Genotype: Year	1299.3	102	9.88	1.16×10^{-14} ***
Stage: Year	19.4	1	15.08	0.000315 ***
Genotype: Stage: Year	625.6	102	4.80	1.35×10^{-8} ***

In LRT the genotype main effect was highly significant ($P = 9.24 \times 10^{-7}$), confirming substantial genetic diversity among the evaluated sweet sorghum genotypes for stem brix% (Table 2). More strikingly, the G×E interaction term was extremely significant ($P = 2.04 \times 10^{-15}$), indicating that genotype performance for Brix is strongly modulated by environmental conditions across the tested years (and potentially locations).

Table 2. LRT for genotype main effect and genotype × environment (G×E) interaction on stem Brix content in sweet sorghum genotypes evaluated across environments.

Model	P-value for Brix
Genotype	9.24×10^{-7}
G×E interaction	2.04×10^{-15}

Using BLUP adjusted mean Brix values of maturity averaged over two years genotypes performance regarding Brix % were classified into low, medium, and high sweetness groups using quantile-based grouping. The low-sweetness group represented as beige color showed a broad distribution ranged from 5 to 15% Brix on the graph, with a peak around 11–13%, showing a greater variability but with poor sugar accumulation ability. The 2nd group identified as medium-sweetness group showed a relatively narrow and concentrated distribution centered between 15 and 17% Brix. In contrast, the 3rd group high-sweetness group showed a clearcut right-shifted distribution that spans from 17 to 22% Brix, with its peak density around 18–20%. The graphical distribution of genotypes across sweetness categories is shown in (Figure 2).

The top-ranking genotypes using BLUP based adjusted means were presented in Table 3. Pak-47 with brix (18.0%) was the highest followed by GP-15, SGD-011-2, and Sweet Sorghum (17.0%), while PARC-2009, Asil, F-902, Kohe Suleman, PVK-801, and FJSS-03 ranged from 16.25% to 16.75% Brix. Complete list of BLUP-adjusted means for all 103 genotypes across 2024 and 2025 presented in (Supplementary Table S2). WAASB model was used to evaluate sorghum genotypes mean brix and their brix stability across different environments. Quadrant based WAASB plot was presented in Figure 3 that differentiated stable and high brix genotypes. WAASB score/ stability score was presented on x-axis and mean brix on y-axis. All the four quadrants were represented with different colors of the genotypes i.e. orange, green, red and blue present in each quadrant.

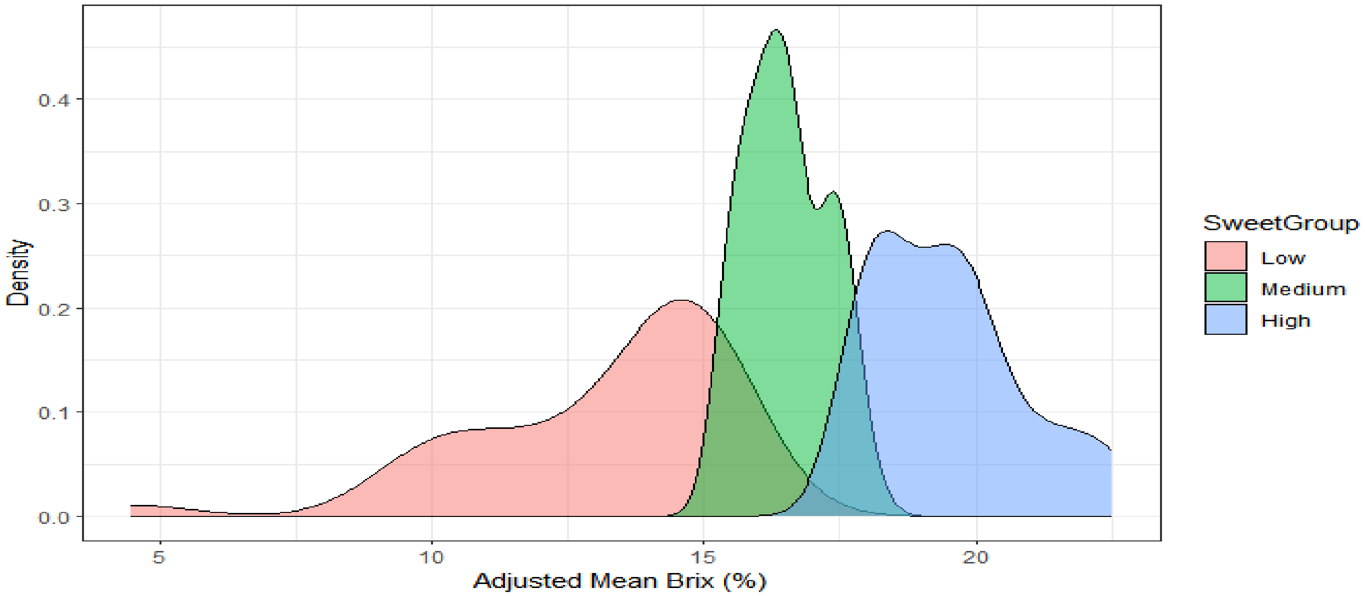

Figure 2. Density distribution curve of stem Brix % with respect to grouping of genotypes into low, medium and high sweetness group. X-axis shows adjusted mean brix% and Y-axis represents density distribution of sorghum genotypes Based on the BLUPs at maturity, genotypes were ranked to identify the top genotypes regarding Brix%.

Table 3. Top 10 Ranked Genotypes based on Adjusted mean Brix using BLUP

Rank	Genotype	Adjusted Mean Brix (%)	95% Confidence Interval
1	Pak-47	18.00	16.76 – 19.24
2	GP-15	17.00	15.76 – 18.24
3	SGD-011-2	17.00	15.76 – 18.24
4	Sweet Sorghum	17.00	15.76 – 18.24
5	PARC-2009	16.75	15.51 – 17.99
6	Asil	16.50	15.26 – 17.74
7	F-902	16.50	15.26 – 17.74
8	Kohe Suleman	16.50	15.26 – 17.74
9	PVK-801	16.25	15.01 – 17.49
10	FJSS-03	16.25	15.01 – 17.49

The quadrant I was the most desirable as it contained high mean and high stability genotypes regarding brix. GP-15 and F-902 which consistently maintained high brix levels exceeding the 13.5 average with high stability, were present in this quadrant. The II quadrant where genotypes represented with green color features genotypes like Pak-47, SGD-011-2 and Asli that also had high brix but their stability was low as WAASB score high for this quadrant, suggesting they were sweet, but performance would be fluctuate with environments. The bottom quadrants represent genotypes with below-average brix levels, with the red highlighted genotypes into III quadrant showed high stability with below average brix (e.g., IS-10281C) and the blue highlighted genotypes in to IV quadrant contained the least ideal genotypes, such as IS-12550 C, which had low-brix value and also unstable. Full WAASB scores and rankings for every genotype in the study was presented in Supplementary Table S3.

The PCA biplot employed to check interrelationship among the studied morphological traits along with differentiation among sorghum genotypes regarding these traits. The principal component 1 (PC1) captured 35.4% of total variation and found a strong positive association among brix, plant height, and internode length. PC2 captured 19.8% of the total variation and further this source of variation was mainly driven by stem diameter which was farthest from rest of the traits making a right angle to little obtuse angle. On the biplot genotypes projected farthest along the brix vector e.g., those near or beyond the tip of the brix arrow, such as 102, 75, 78 are likely stable high-sweetness performers. The PCA biplot illustrating trait relationships and genotype distribution is presented in Figure 4. Further contribution of all PCs showed in Supplementary Figure S3.

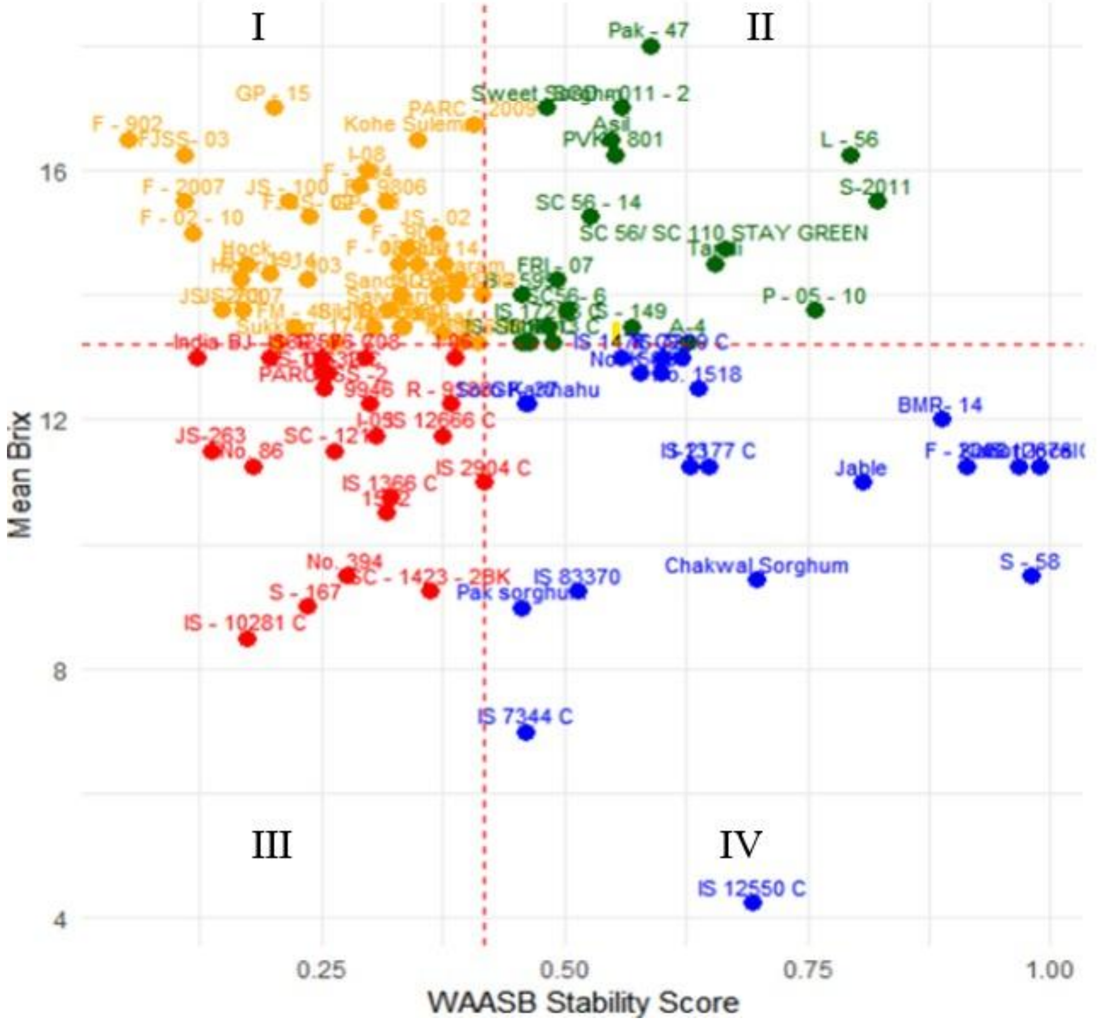

Figure 3. WAASB based quadrant plot for checking the mean and stability of sorghum genotypes regarding Brix %.

As the stage effect was significant $P < 0.001$ in the linear mixed model effect hence year was shuffle in brix across both growth stages was checked to further explore how much actual change occurred. The boxplot was used to illustrate the change in stem Brix (%) from anthesis to maturity across the two growing seasons. Stem Bix increased markedly from anthesis to maturity across both crop seasons. In 2024, Brix values at anthesis ranged approximately from 2-16%, with a median around 10-11%, whereas at maturity they increased substantially, ranging from about 8-24% with a median near 17%. A similar trend was observed in 2025 crop season, where anthesis values ranged from approximately 1-18% (median ≈10%), while maturity values ranged from 5-24% (median ≈16%) (Figure 5).

The genotypic response of stem Brix (%) across both developmental stages (anthesis to maturity) over both years was shown in Figure 6. In this Figure, each of the lines represented an individual genotype and the direction of line showed brix content changed across both stages. In both years, most of the genotypes exhibited an increase in brix from anthesis to maturity, while few show decrease while constant was identified only in few genotypes.

Soil properties were further examined to check either they impacted the brix%. In the 2024 soil was enriched with soil parameters while the next year low soil nutrients were recorded. Regression analysis was performed to check the cause-and-effect relationship and a positive association between soil nutrient status (K, OM, and P) and mean stem Brix (%) was recorded.

Higher nutrient availability of K =70 mg kg^{-1}, OM = 0.90%, P =15.7 mg kg^{-1} corresponded with elevated brix% at maturity (~17%), compared to 2025 where reduced nutrient levels i.e., K = 65 mg kg^{-1}) ^{1}, OM = 0.78%, P = 12.0 mg kg^{-1}were low and associated with lower maturity Brix (~15%). The positive regression slopes across all parameters indicate that soil fertility plays a critical role in enhancing stem sugar accumulation (Figure 7).

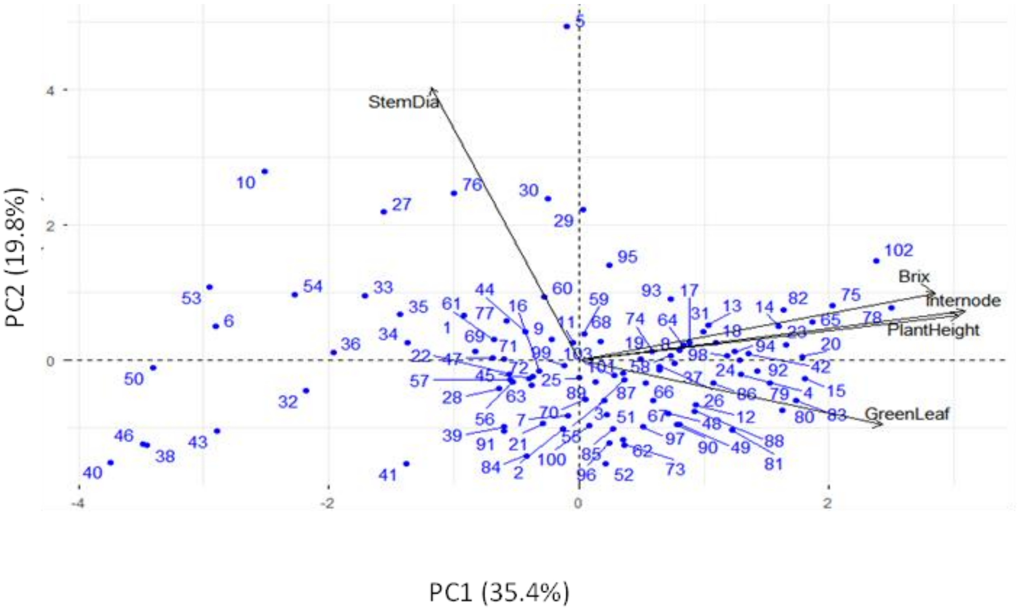

Figure 4. PCA Biplot showing relationship of Brix% with other morphological traits. PC1= principal component 1 and PC2= principal component 1. Blue dots represented the genotypes used in the study. Black arrows originating from origin represent the studied traits. Angle between these arrows represent the relationship i.e., acute angle < 90˚= positive correlation, right angle 90˚= independent, and obtuse angle > 90˚= negative correlation while 180˚= strong negative correlation.

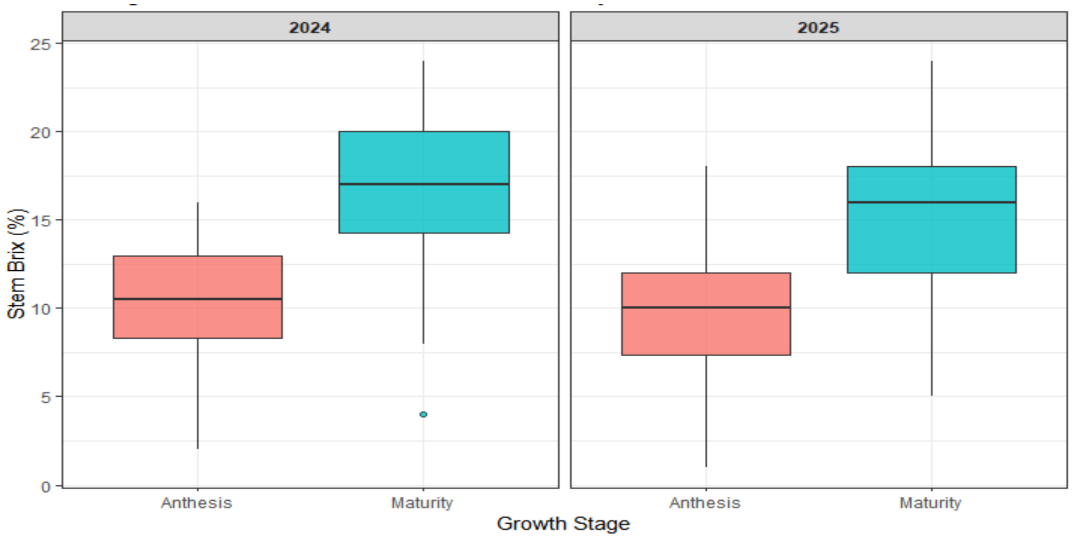

Figure 5. Box plots representing shift in brix% from anthesis to maturity across both crop seasons.

DISCUSSION

Increase in Brix from anthesis (~10%) to physiological maturity (~18%) is a primary finding of this study, supported by a highly significant growth stage effect (F = 3244.60). This increase from anthesis to physiological maturity where median values nearly doubled confirms that the post-anthesis period is the primary window for non-structural carbohydrate sequestration. During anthesis, the plant's physiological priority is the maintenance of reproductive organs and the initiation of grain filling. However, as the plant reaches physiological maturity, the stem acts as a primary sink for surplus photosynthates that are not utilized by the developing panicle. This "sink-source" relationship is a hallmark of sweet sorghum, where the stems continue to accumulate sucrose even as the grain matures. Our results align with (Shukla *et al.*, 2017) who also observed increase in sugar concentration in sorghum as the plants move to maturity after anthesis. The highly significant effect of genotype (F=19.74), alongside the distinct right-skewed distribution observed at maturity, suggests that sugar accumulation follows a genetically programmed threshold.

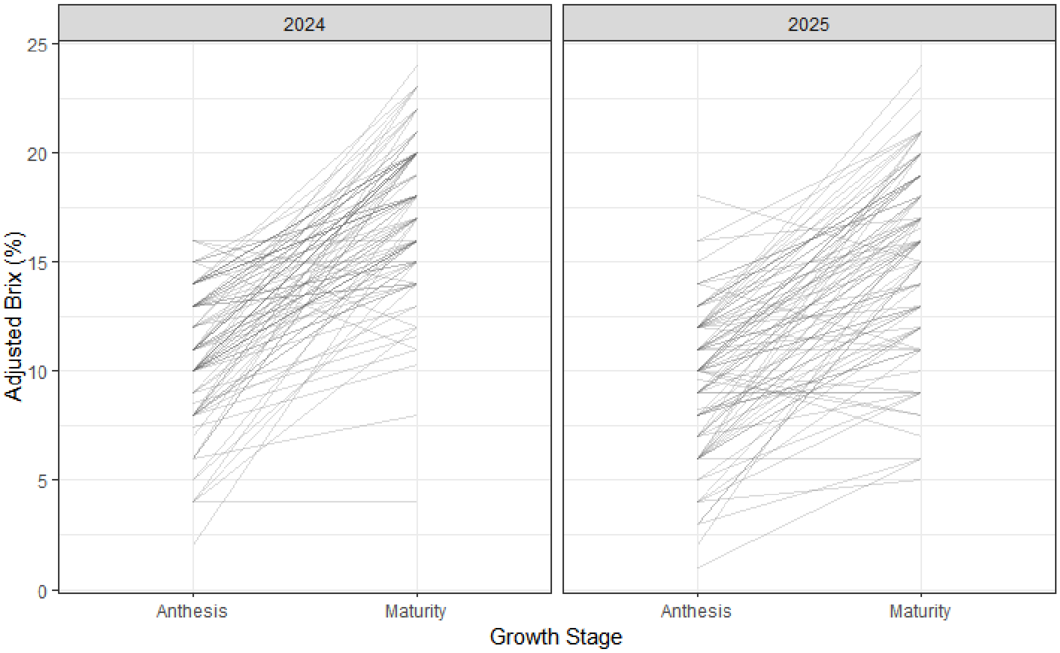

Figure 6. Genotypic performance regarding Brix accumulation at anthesis and maturity in both crop season 2024 and 2025. Lines represent the shift in performance of genotypes from anthesis and maturity.

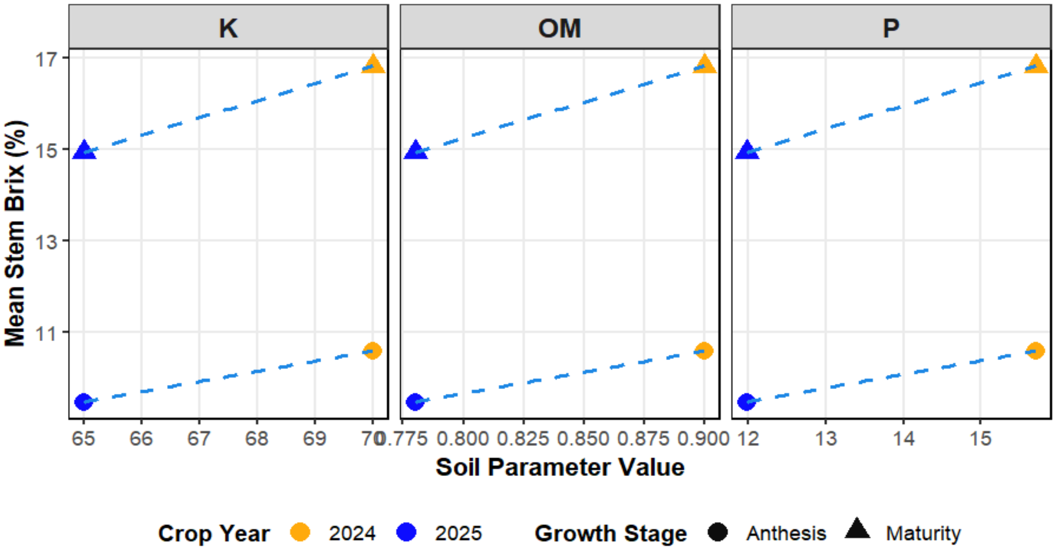

Figure 7. Relationship between selected soil fertility parameters x-axis labels to: K (mg kg^{-1})), OM (%), and P (mg kg^{-1}).and mean stem Brix (%) on y-axis.

This skewness towards higher Brix values in elite genotypes reflects a specialized capacity for stem sink loading that distinguishes sweet types from common forage sorghum, a phenomenon noted by (Kanbar *et al.*, 2021) regarding the differential sugar accumulation patterns in sweet vs. grain sorghum types .The grouping of genotypes into high, medium, and low sweetness categories demonstrates that while all genotypes follow a similar developmental trajectory, the capacity of the stem sink is genetically determined.

The presence of a significant genotype × year × stage interaction ($P = 1.35 \times 10^{-8}$) highlights the challenges faced by breeders in rainfed environments. The high expressivity of brix in 2024 likely resulted from more favorable post-anthesis solar radiation or temperature regimes along with higher soil fertility, which enhanced the source capacity. (Sher *et al.*, 2016) similarly reported that environmental factors, particularly moisture availability and temperature significantly alter the brix-to-biomass ratio in sorghum. This phenomenon necessitates the use of BLUPs, which "shrink" the mean performance of genotypes towards the population mean in unstable environments, thereby preventing the selection of genotypes that only performed well due to localized environmental advantages (Piepho *et al.*, 2008)

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A central finding of this study was the identification of stable, high-sugar genotypes using the WAASB 4-quadrant biplot. Because genotypes are frequently chosen in traditional breeding based only on mean performance, "opportunistic" cultivars that fail under pressure may be promoted. According to our analysis, GP-15 and F-902 have a strong genetic buffering mechanism because they are in the "I quadrant" (high mean, high stability).

The principal component analysis provided critical insights into the ideotype of a high-sugar sorghum plant. The strong positive correlation between Brix, plant height, and internode length suggests that larger vegetative structures provide a larger "storage tank" for soluble carbohydrates. This is consistent with the findings of (Murray *et al.*, 2009), who while mapping QTLs for brix and plant height found positive relationship among both traits. However, some recent studies found negative relation among plant height and brix% (Disasa *et al.*, 2017; Shukla *et al.*, 2017). However, the most significant result is the independence of brix from stem diameter. In many cereal crops, there is a fear that increasing sugar content (which fills the stem with juice) might lead to thinner, weaker stalks that are prone to lodging. The independent relationship between stem diameter and brix% was also reported by (Disasa *et al.*, 2017) while evaluation sorghum varieties for brix and sugar content. Our PCA shows that it is entirely possible to select for high-sugar genotypes that also maintain a thick, robust stem diameter. This allows for the development of high-sugar ideotypes that retain structural integrity and lodging resistance, a concern previously addressed by (Kawahigashi *et al.*, 2013).

Moreover, the transition from anthesis to maturity serves as a guide for harvest timing. While maximum biomass is often the goal, our data shows that harvesting too early (at anthesis) results in a significant sugar loss up to 8% in median brix. The outcomes are in line with earlier research by Oyier *et al.* (2017). According to Kanbar *et al.* (2021), there was a gradual rise in brix content in the sweet program from anthesis to physiological maturity. Delaying harvest until the early dough stage or physiological maturity is crucial for farmers looking to increase milk yield or weight gain in order to collect the stem's maximal energy density. Beyond genetic and phenological factors, variations in soil nutrient status can account for a portion of the significant year influence on Brix (F = 217.64), with a higher brix percentage in the year with high soil fertility. This study shows that sorghum stem sweetness is an environmentally sensitive but heritable characteristic. We have advanced from straightforward yield-based selection to a more complex stability performance framework by combining mixed models with the WAASB stability index. The discovery of stable, high-brix genotypes GP-15 and F-902 offers a fundamental genetic resource for improving sorghum in rainfed areas. To support marker-assisted selection MAS, future studies should concentrate on the molecular markers linked to these stable "orange quadrant" genotypes. It would also be advantageous to investigate the connection between stem Brix and stay-green characteristics, since genotypes that retain green leaves until maturity are probably going to have a longer duration of sugar loading.

CONCLUSION

The current study showed significant genetic variation in sorghum stem sweetness and emphasized the significant impact of development stage and year on Brix expression. In sweet sorghum breeding, significant genotype × stage × year interactions highlight the importance of multi-environment and multi-stage testing. A number of genotypes showed high and consistent stem Brix values, which made them attractive options for improvement initiatives focused on bioenergy and fodder. Future sorghum breeding initiatives can reliably employ the use of mixed linear models, adjusted means, and classification techniques for the accurate identification of superior genotypes. Future multi-year studies with greater soil variability are advised to validate this apparent association, given the observed increase in maturity-stage Brix from ~15% in 2025 to ~17% in 2024 along with higher soil nutrient levels.

AUTHOR CONTRIBUTIONS

Saba Aleem: Writing - original draft, data analysis & formal analysis. Ayesha Malik: data collection, Muhammad Zeeshan: review & editing. Ghulam Ahmad & Suleman Raza: Resources (provided the germplasm). Muhammad Saqib: Validation, Visualization. Muhammad Imran Khan and Waheed Arshad: Conceptualization, Methodology, Supervision, Resources, Validation.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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