Genetic architecture for drought tolerance in pearl millet (*Pennisetum glaucum* (L.) Br.) germplasms

by

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Abstract

Drought and heat stress are major challenges to crop productivity, especially in semi-arid regions with limited water and erratic rainfall. Pearl millet, the sixth most widely cultivated cereal for food, forage, and feed, has emerged as a promising climate-resilient crop to address these challenges. However, its potential remains underexplored due to limited breeding efforts. This thesis focuses on identifying drought-tolerant pearl millet germplasms and uncovering genomic regions associated with drought resilience to support the development of improved forage hybrids, particularly for drought-prone areas like western Kansas. Two experiments were conducted to achieve these objectives. The first experiment evaluated 188 diverse pearl millet accessions under irrigated and rainfed conditions at Kansas State University's Agricultural Research Center in Hays in summer 2023 and 2024. Agronomic traits such as days to 50% flowering, chlorophyll index, plant height, number of productive tillers, staygreen, and biomass were measured. Accessions were grouped into early, medium, and late maturity classes based on flowering time. Principal component analysis using genotyping-by-sequencing single nucleotide polymorphism (SNPs) data revealed broad genetic diversity within maturity groups. Drought-tolerant accessions were identified using multiple selection indices including Rank Summation Index, BLUP-based GGE biplot, MTSI, MGIDI, and FAI-BLUP. Nine stable and widely adapted drought-tolerant accessions were selected, with chlorophyll content, staygreen, and biomass proving to be reliable drought resilience indicators due to their moderate to high heritability. The second experiment involved genome-wide association studies (GWAS) on 187 accessions genotyped using GBS, yielding 35,071 high-quality SNP markers. Phenotypic data on ten traits and calculated stress tolerance indices were used to identify quantitative trait nucleotides (QTNs) linked to drought tolerance. Sixty-two QTNs were mapped across all seven chromosomes using

four GWAS models (MLMM, FarmCPU, Blink, and 3vRMLM). These QTNs co-localized with 77 candidate genes, including PMF1G04719, PMF2G07960, and PMF1G07862, many of which are involved in drought response mechanisms, such as reactive oxygen species (ROS) scavenging, abscisic acid pathway and so on. After validation, together, these findings will serve as valuable genetic and genomic resources for improving pearl millet breeding programs and to advance climate resilience in dryland agriculture.

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Chapter 1 - Literature Review

1.1 Drought and Agriculture

In the twenty-first century, there are several concerns about agriculture and food security, including urbanization, population increase, water shortages, rising food prices, and global warming. It is expected that by 2030, climate change will cause 132 million people to fall into extreme poverty in developing countries, with an additional 72 million people undernourished by 2050 (Sedithippa Janarthanan, 2024). To address these issues and end poverty and hunger, researchers must investigate alternate food sources at every step of the process—from production to processing to consumption. Drought is one of the major concerns to global agricultural production and food security. Drought and water scarcity are the main obstacles to agricultural productivity in many developing countries; also, also cause yield loss in many areas in developed countries. In 2012, $30 billion was lost in agriculture due to drought that spread from the US Corn Belt to the West Coast (Rippey et al., 2015). Wheat (*Triticum aestivum* L.) faces the highest drought vulnerability, particularly in the US and Canada, with an >80% yield loss risk under exceptional droughts; maize (*Zea mays* L.) is most at risk in India, rice (*Oryza sativa* L.) in Vietnam and Thailand, and soybeans (*Glycine max* L.) in the USA, Russia, and India (Leng and Hall, 2019). A modeling study compiling irrigation data at the county level for four crops (soybean, maize, wheat, and grain sorghum (*Sorghum bicolor* L. Moench)) in Kansas, US predicted a yield decrease of 2.2 to 12.4% for maize, soybean, and grain sorghum induced by drought at the state level (Zhang and Lin, 2016).

Several studies have proposed farmers to switch from rainfed to irrigated crops or increase or intensify irrigation as a way to cope with climate change (Suttles et al., 2024). For

climate-impacted food-producing zones, irrigation is essential because it provides a consistent water supply and reduces heat stress (Zaveri and B. Lobell, 2019). In the US, irrigation reduced the crop yield variations from year to year by roughly 41% (Kukal and Irmak, 2020). Expanding irrigation, however, puts more strain on the world's freshwater supplies, frequently resulting in their unsustainable use (Dalin et al., 2017). Groundwater storage worldwide has decreased in all significant irrigation regions within the last 20 years (Gleick, 2000). According to a report, the quantity of groundwater extracted in the US for irrigation increased by 16% between 2010 and 2015 (Dieter et al., 2018). Arkansas, California, Idaho, and Nebraska were the four states that contributed most to this rise. These states already used a significant amount of groundwater for irrigation, further increasing the use by 26% to 59% (Dieter et al., 2018). The Southern Ogallala aquifer in the Great Plains has been slowly declining over the last two years, making it one of the aquifers that has been emptying the fastest over the last 20 years (Yamazaki and Pierce, 2024). This depletion resulted in the loss of the aquifer's most agriculturally productive areas extending from Texas north through Kansas (Smidt et al., 2016). Climate change will exacerbate this problem significantly. Between 2010 and 2060, it has been predicted that Kansas's groundwater storage might drop by 25% as a result of climate change-induced precipitation decreases and rising groundwater withdrawals (Steward et al., 2013). In response to this depletion, Kansas State has recently enacted two laws to impose stricter limitations on groundwater usage for agricultural purposes (Condos, 2023). The Kansas Water Plan of 2022 strongly emphasized locally driven approaches to water management, suggesting increased crop water use efficiency, better irrigation efficiency, and the use of alternative crops to alleviate water scarcity (Kansas State Water Office, 2022).

In this regard, millets are considered as the most suitable crops for sustaining agriculture and food security on low-fertility marginal lands (Habiyaremye et al., 2017). Due to their drought tolerance, pearl millet [*Pennisetum glaucum* (L) Br.] and sorghum are considered to be an ecologically preferred crop for semi-arid regions, which may account for their recent popularity over maize and other important crops (Dube et al., 2018). To further emphasize the significance of millets, the UN declared 2023 to be the International Year of Millet (Harish et al., 2024). Furthermore, to acknowledge the noteworthiness of millets, the Ministry of Agriculture and Farmers Welfare India has designated major millets (sorghum and pearl millet), two pseudo-millets (buckwheat (*Fagopyrum esculentum*) and amaranth (*Amaranthus hypochondriacus*)), and minor millets (including finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), kodo millet (*Paspalum scrobiculatum*), barnyard millet (*Echinochloa colona*), little millet (*Panicum sumatrense*), and brown top millet (*Brachiaria ramosa* (Syn. *Urochloa ramosa*))) as "Nutri-cereals" for production, trade and consumption (Harish et al., 2024). According to the Statistical Database of the Food and Agriculture Organization of the United Nations (FAO), global millet production in 2018 amounted to around 31 million metric tons. Africa and Asia contributed 51% and 46% of the total output, respectively, while the Americas accounted for only 1% of global millet production (Crookston et al., 2020).

1.2 Pearl Millet - A Climate Resilient Crop

Pearl millet [Pennisetum glaucum (L.) Br., Syn. Cenchrus americanus (L.) Morrone] is a diploid (2n =14), C4, warm season, cross-pollinated crop. It has a short lifecycle and is protogynous. Having originated in Western Africa some 4000 years ago, it differentiated into two races- the globosum race that moved to the western side and the typhoides race that reached

Eastern Africa and spread to India and southern Africa 2000-3000 years ago. The genus Pennisetum is the largest genera belonging to the section: Panicillaria; subtribe: Panicinae; tribe: Paniceae; subfamily: Panicoideae of the family Poaceae (Watson and Dallwitz, 1992; S.K Pattanashetti et al., 2016). The Pennisetum genus includes over 80-140 species (Brunken, 1977, Clayton and Renvoize, 1986; S.K Pattanashetti et al., 2016) which differ in their duration (annual or perennial), reproduction (sexual or asexual/ apomictic), somatic chromosome number (2n=10-78), basic chromosome number (x=5,7,8 or 9) (Jauhar, 1981a, Jauhar, 1981b; S.K Pattanashetti et al., 2016) chromosome size, genome size and ploidy levels (diploid to octaploid) (Martel et al., 1997; S.K Pattanashetti et al., 2016). Pearl millet biology includes all growth and developmental features from germination to seed formation, including three well-defined growth phases i.e. vegetative, reproductive, and grain filling. The vegetative phase (0-21) includes- the emergence stage (2-3 days after emergence (DAE)), three-leaf stage (3-7 DAE), five-leaf stage (7-14 DAE), and panicle initiation stage (14-21 DAE). Reproductive/ Panicle development phase (21-42) includes- flag leaf stage (21-28 DAE), boot stage (28-35 DAE), and half bloom stage (35-42 DAE). Grain filling phase (42-77 DAE) includes- milk stage (42-49 DAE), dough stage (49-56 DAE), and black layer formation or physiological maturity (56-63 DAE) (http://www.aicpmip.res.in/pmbiology.pdf).

1.3 Production and Potential Uses

Globally, pearl millet is considered the sixth most important cereal crop, accounting for more than half of the worldwide millet production (Serba et al., 2017). Majority of the crop is grown in Africa (~18 million ha) and Asia (> 10 million ha) (Pattanashetti et al., 2016), especially India leading in area (6.93 million ha) and production (8.61 million tons) (Ramalingam et al., 2024). However, it is increasingly being acknowledged in the semi-arid

region of the Southern Great Plains, USA, as a promising forage crop that can be incorporated into animal feed cropping systems alongside grain sorghum and winter wheat by substituting summer fallow (Bhattarai et al., 2019; Crookston et al., 2020). It is considered a high-quality forage crop in the USA and Australia and is being experimented as a new forage crop in South America and Korea. There is a new interest in the USA in growing pearl millet as a grain crop because of its drought tolerance and high-quality grain (Khairwel et al. 2007). According to reports, gluten-free dietary preferences as well as the demand for millet flour by some African and Asian immigrant communities has further boosted the market for pearl millet grain in the United States.

Pearl millet, a climate-resilient and protein-rich cereal crop has proven its potential as an alternative crop for semi-arid regions yielding approximately 80% of sorghum yields (Christensen et al., 1984). It is known to provide high-yielding, high-quality forage for summer grazing (Burton and Forston, 1966). Research in North Dakota highlights pearl millet as a high-yielding, palatable forage with exceptional crude protein content. It is ideal for hay, silage, or pasture, curing more slowly than foxtail millet but similarly to sudangrass (*Sorghum sudanense*), and benefits from using a crimper. Unlike sudangrass and sorghum, it poses no risk of prussic acid poisoning and performs well on sandy soils, excelling on fertile, moist soils (Sedivec et al., 1991). A field study found that irrigating pearl millet with 75% of its water requirement preserved forage yield and quality while improving water-use efficiency under higher water stress (Sasani et al., 2004). An in-vitro study on digestibility found that pearl millet cultivars exhibited digestibility values similar to maize, but greater than sorghum (Ejeta et al., 1987). Pearl millet has the potential to feed the world's expanding population, which is predicted to

reach 9.1 billion people by 2050, as cereal production has to increase from 2.1 to 3 billion tons (Alexandratos, 2009).

Pearl millet has high photosynthetic efficiency and dry-matter production capacity (Satyavathi et al., 2021). The WUE of forage pearl millet (280 kg dry matter ha-1 mm-1) is better than forage sorghum (310 kg dry matter ha-1 mm-1) (Chapman and Carter, 1976). Dry-matter digestibility of forage pearl millet (64-69%) is lower than corn (73%) but similar to forage sorghum (63%). Pearl millet produces an excellent leaf-to-stem ratio, resulting in around 3.5-6.3% lignin compared to 4.9% in corn and 8.3% in sorghum (Hassanat, 2007). Production of pearl millet silage (31t/ha) is higher than corn (27/ha) and sorghum(19/ha) (Kichel et al., 1999). It is being cultivated as a forage crop for livestock grazing, hay production, silage, and green fodder (Harinarayana et al., 2005). Pearl millet is nutritionally rich in proteins, vitamins, minerals, antioxidants, dietary fibers, crude fibers, fats, and essential micronutrients (Satyavathi et al., 2021). It is easily digestible, gluten-free (Satyavathi et al., 2021), and is used in many foods and beverages like couscous, flatbreads, doughs, porridges, nonalcoholic beverages, and beer. The pearl millet flour can be used as a substitute for wheat flour in whole-grain breads, crackers, pretzels, and dry and creamed cereals (Dahlberg et al., 2003). Being suitable for monogastric animals, it is used as the main component in poultry feed and cattle feed (Serba et al., 2017).

Pearl millet is adaptable for double cropping and fall grazing at any stage and can also fit well as a cover crop in water-limited environments. Due to its high water use efficiency (WUE), pearl millet can produce sufficient aboveground biomass to minimize weeds, improve soil health, and provide forage/silage resources to increase the profitability of the dryland cropping and livestock system (Bhattarai et al., 2019). Pearl millet can be one of the potential alternative

climate-resilient crops needed to fill the production gaps in the semi-arid harsh drought and other varying stress environments at these critical stages. However, the importance and value of the underutilized pearl millet crop is in an unexplored situation due to limited crop breeding efforts for the forage cultivar development besides a lack of marketing infrastructure and awareness among growers (Serba et al 2017).

1.4 Genetic Diversity and Germplasm Utilization in Pearl Millet

The genetic diversity study of *Pennisetum* species identified three distinct gene pools: primary, secondary, and tertiary (Serba et al., 2017). The primary gene pool included all forms of cultivated, weedy, and wild diploids (2n=2x=14); the secondary poll consisted solely of tetraploid *P. purpureum* (Shum) (2n=4x=28); and the tertiary pool included distantly related *Pennisetum* species of various ploidy levels (Harlan and de Wet, 1971; Hanna & Dujardin, 1986). There are 1283 active collections of pearl millet accessions maintained at GRIN (Germplasm Resource Information Network) (Reddy et al., 2024). By the year 2007, ICRISAT had successfully collected more than 20,800 cultivated pearl millet accessions and 750 wild relatives through 76 missions across 28 different countries (Serba et al., 2017). A total of 56,580 accessions (including possible duplicates) of pearl millet in 70 genebanks of 46 countries across the world are available. Landraces represent the largest part of pearl millet germplasm, followed by breeding/research material and wild relatives. The Indian national collection includes 7,059 accessions at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. Global collections managed by ICRISAT comprise of 22,888 pearl millet accessions from 51 countries. However, only a very small fraction of these accessions has been utilized so far. (Yadav et al., 2017). The reproductive characteristics of pearl millet make it the most versatile and most exciting of the major cereals for genetic and plant breeding research (Singh et al.,

2023). These include the production of 1000-3000 seeds per panicle, the ability to exploit protogyny to cross-pollinate the crop without emasculation, the ability to exploit both population and pure-line plant breeding techniques, and the existence of significant heterosis and multiple cytoplasmic-genetic male-sterility systems (Bidinger et al., 2004).

1.5 Drought-Responsive Traits and Pearl Millet Adaptability

Pre-breeding is a crucial step in crop breeding to introduce novel features and genetic diversity into elite germplasm. Despite substantial advancements in overall production and productivity, primarily driven by a strong emphasis on yield and its components, biotic and abiotic stresses remain major challenges, leading to considerable yield losses. Even though pearl millet is well suited to drought, salinity, high temperatures, and unfavorable soil conditions, abiotic factors have a detrimental effect on its growth and potential output (Shivhare & Lata, 2017). Duration, intensity, and timing of the drought stress determine its effect on the crop (Prasad et al., 2008). Many studies have been conducted to understand how pearl millet responds to and adapts to water scarcity at different phases of its developmental stages. For instance, water stress during the germination or seedling emergence stage causes seedling death, resulting in poor crop establishment (Farooq et al., 2009; Lata et al., 2015). Significant moisture stress during the seedling stage is the primary cause of pearl millet's poor production in arid and semi-arid regions (Soman et al., 1987). Studies related to water stress have been performed on pearl millet during its three primary stages: vegetative, panicle growth, and grain-filling (Shivhare et al., 2020), revealing resistance mechanisms such as drought avoidance, tolerance, escape, and recovery. Studies revealed that leaf rolling, reduced canopy leaf area, and decreased transpiration rate as adaptive traits of pearl millet under water stress (Srivastava et al., 2022). In pearl millet, the response to short-term drought involves precise regulation of osmotic adjustment, stomatal

conductance, and reactive oxygen species (ROS) scavenging, along with ABA and ethylene signaling pathways. Equally crucial are long-term adaptive strategies, including flexibility in tillering, root growth, leaf modifications, and flowering time, which help mitigate severe water stress and partially compensate for yield losses through asynchronous tiller production (Shrestha et al., 2023). Drought stress at the vegetative phase shows little to almost no decline in crop growth and yield of pearl millet because of its asynchronous tillering and rapid growth rate, which allow it to recover quickly (Bidinger et al., 1987). The stress results in delayed flowering time of the main shoot. Pearl millet has the ability to delay flowering until unfavorable conditions have improved (Henson & Mahalakshmi, 1985). However, post-flowering or terminal drought stress has the most negative impact on pearl millet grain and stover yield as well as yield stability (Bidinger & Hash, 2004; Mahalakshmi et al., 1987).

The global pearl millet germplasm contains a vast amount of genetic variation for constitutive and drought-responsive characteristics. Grain productivity under water stress is determined by early flowering (Bidinger et al., 1987). Thus, earliness is an important drought escape feature of pearl millet and is a crucial component of genotype-plus-genotype-by-environment interaction. (Vadez et al., 2012). A huge amount of genetic variation in earliness is available in the germplasm (Yadav et al., 2017). Murty et al., (1967) evaluated 1,532 accessions and documented variation in flowering time, ranging from 52 to 77 days in Indian collections and 53 to 85 days in exotic collections. Pearl millet produces primary tillers, which are followed by secondary ones. In the event of a mid-season drought, pearl millet can compensate for the possible failure of the main and primary tillers due to its tillering and developmental plasticity (Vadez et al., 2012). The drought-tolerant genotypes change their crop phenology by developing late-season tillers that flower and produce grains after the dry spell or when stress is relieved

(Craufurd & Bidinger, 1988). Developmental plasticity is connected with growth plasticity. Pearl millet is mainly grown for fodder, silage, and as a dual purpose for both grain and stover (Harinarayana et al., 2005). It is a promising fodder crop due to its nutritional composition, great tillering, and leafiness, along with high digestibility, crude protein, and biomass production (Sedivec et al. 1991). Various fodder-related traits are divided into two main groups based on the methods of observation and their relationship: morphologically observed biomass-related traits (such as the number of tillers per plant, plant height, number of leaves, stem girth, leaf length and breadth, number of internodes, total leaf and stem weight), and quality-related characteristics (like crude protein content, in vitro digestibility, neutral detergent fiber content, acid detergent fiber content, lignin content, and cellulose and hemicellulose content) as determined by biochemical analysis (Daduwal et al., 2024). Pearl millet can store up to 70% of its biomass in tillers (Azam-Ali et al., 1984). NBPGR and ICRISAT evaluations of different accessions from multiple nations showed significant variation in several fodder components, including plant height (49–443.3 cm), number of tillers plant-1 (1–9.3), stem thickness (6–31.2 mm), number of leaves (4.3–37), leaf length (19.3–130 cm), and leaf width (1.1–8.6 cm) (Harinarayana et al., 2005). Whether cultivated for grain and stover (dual- purpose) or solely for forage, integrating the stay-green trait significantly enhances fodder quality (Akplo et al., 2023). Furthermore, studies have reported that traits such as number of tillers per plant, chlorophyll index, grain yield per plant, 1000 grain weight, and harvest index showed high heritability and genetic advance under drought stress, thus could be utilized for direct selection of genotypes as well as for improvement of drought tolerance (Singh et al., 2014). Similar findings were also reported by Gupta et al. (2005) for 1000 grain weight, grain yield, and flag leaf area; El-Kareem

and El-Saidy (2011) for 1000 grain weight, HI, and grain yield per plant; and Mondal and Kour (2004) for flag leaf area, number of tillers per plant, and grain yield.

1.6 Multi-trait Based Evaluation Methods

The varying responses of landraces and elite genetic materials in drought and non-drought conditions highlight the need to combine the drought tolerance of landraces with the high yield potential of elite varieties (Yadav & Rai, 2011). Selection of specific landraces, elite cultivars, and superior germplasm for hybrid production is possible through the characterization of their phenotypic traits (such as days to flowering, tillering, plant height, etc.), drought adaptation, and yield potential (Patil et al., 2020). However, the selection of superior genotypes is hindered by factors such as genotype (G), environment (E), management (M), and more importantly the genotype-plus-genotype-by-environment interaction (GGE) (Quintero et al., 2018). Multi-environmental trials (MET) are important for examining the GEI and selecting superior and stable genotypes based on multiple trait evaluation (Abebe et al., 2024). Multi-trait-based stability evaluation methods including best linear unbiased prediction (BLUP)-based GGE biplot, multi-trait mean performance and stability index (MTSI), multi-trait genotype-ideotype distance index (MGIDI), and multi-trait index based on factor analysis and genotype-ideotype distance (FAI-BLUP) have been proposed for selection of stable genotypes across different environments (Pour-Aboughadareh et al., 2022; Olivoto et al., 2019; Olivoto & Nardino, 2021; Rocha et al., 2018).

GGE biplots are used to visualize the relationships between G, E, and G×E (Patel et al., 2023). The GGE biplot's distinctive feature is that it allows one to determine which genotype has the most potential in a given environment or subgroup based on the plots (Farshadfar et al., 2012). MTSI uses the mean performance and stability of the selected for multiple trait

evaluation, addressing the limitations in univariate analysis (Authrapun et al., 2021). The accession with the lower MTSI exhibits high mean performance and stability across all variables analyzed (Olivoto et al., 2019). Zuffo et al., (2020) reported the use of MTSI for selecting drought and salinity-tolerant genotypes in soybean. Likewise, Sellami et al., (2021) used MTSI for selecting suitable lentil genotypes under rainfed conditions. MGIDI is a multivariate selection indicator that incorporates different characteristic information into a single value and ranks genotypes based on their distance from an ideal genotype, addressing multicollinearity issues (Olivoto & Nardino (2021). MGIDI has been instrumental in crop improvement and breeding, contributing to advancements in salinity and waterlogging tolerance, stability analysis, drought adaptation, agronomic and tuber quality, nutritional enrichment, productivity, adaptability, essential amino acid enhancement in grain, yield increase, early maturity, and stress resistance (Debnath et al., 2024). FAI-BLUP combines factor analysis and genotype-ideotype design by using a multi-trait approach free from multicollinearity and has been successfully used for multi-trait selection of genotypes for several purposes (Silva et al., 2018).

1.7 Genomic Approaches for Pearl Millet Improvement

Over the last ten years, the use of omics/genomic techniques to improve pearl millet's resistance to drought has become more popular. The molecular genetic traits of pearl millet make it suitable for the application of molecular techniques of crop improvement. These include its diploid nature and large chromosomes, its moderate haploid DNA content and relatively low recombination rates (resulting in a short genetic map), its high degree of polymorphism at both phenotypic and molecular levels, plus the ease with which both self- and cross-pollinated progenies can be generated (Bidinger et al., 2004). Sequencing-based methods allow the identification of novel variations for a large number of genes through genotype-phenotype

associations (D'Agostino et al., 2017). The 1.76 Gb genome of pearl millet has been sequenced with 38,579 genes, 88,256 simple sequence repeats (SSRs), and 4,50,000 single nucleotide polymorphisms (SNPs) and those markers offers a valuable source for creating precise genetic maps. (Varshney et al., 2017). The publicly accessible draft genome sequence offers a genomic resource for enhancing the plant's resistance to abiotic stressors to further advance pearl millet research efforts Different genetic and genomic tools such as molecular markers, genetic maps, genome-wide association studies (GWAS), quantitative trait loci (QTL) mapping, genomic selection, and genome editing have been developed, aiding in the genetic improvement of pearl millet against biotic and abiotic stresses, particularly drought.

Genotype by sequencing is a reduced representation high-throughput sequencing method that simultaneously detects and genotypes SNPs in multiplexed samples, each of which has a distinct nucleotide barcode (Baird et al., 2008). GBS depends on using restriction enzymes for cutting genomic DNA, followed by high-depth sequencing of the flanking regions. Several studies have been reported utilizing GBS for GWAS and other association studies (Babu et al., 2020; Pan et al., 2017). Significant genotype-phenotype associations can be detected through GWAS, a statistical-genetic approach. GWAS explores the genetic basis of natural phenotypic variation in complex traits by utilizing diverse genetic panels (Aswath et al., 2023). The core principle of association mapping depends on linkage disequilibrium (LD), which is a non-random association between genes, markers, or QTLs (Alvarez et al., 2014). Utilizing historical recombination events, built up over thousands of years for trait associations, enables higher resolution in GWAS as compared to the traditional QTL mapping strategy that uses RIL or DH mapping populations relying on crossover events over a few generations (Soto-Cerda et al., 2022).

1.8 Association Mapping for Drought Tolerance in Pearl Millet

Leveraging genomic tools has enabled the identification of SNP markers, which has been pivotal in detecting QTL related to drought tolerance in diverse pearl millet accessions (Sehgal et al., 2012). Association mapping through its potential to identify promising QTL and detect causal polymorphisms at the gene level, has emerged as a powerful strategy for marker-trait associations (Palaisa et al., 2003). Studies have been reported employing GWAS to identify QTL associated with agronomic and nutritional traits in pearl millet. For instance, Saidou et al. (2009) studied the flowering time and morphological variations in 90 pearl millet inbred lines and identified significant associations between the *PHYC* gene and flowering time, spike length, and stem diameter in the inbred line panel. Kambara et al (2024) conducted association mapping using GWAS on 107 pearl millet parental lines and identified genomic regions associated with culm height and pigmentation of the shoot basal part. The region associated with pigmentation was found to be a homolog of the Phenylalanine ammonia-lyase 2 (PAL2) gene. Likewise, Pujar et al (2020) employed GWAS on a diversity pearl millet panel and identified four SNPs co-segregated for Fe and Zn across chromosomes Pgl04, Pgl05, and Pgl07 and eight SNPs encoded promising genes such as ‘late embryogenesis abundant protein’, ‘Myb domain’, ‘pentatricopeptide repeat’, and ‘iron ion binding’.

However, few studies have focused on employing GWAS for drought tolerance in pearl millet. Debieu et al. (2018) utilized GWAS for drought tolerance in 188 pearl millet inbred lines and reported QTL related to biomass and staygreen in early drought stress conditions. Genes involved in the sirohaem and wax biosynthesis pathways were found to be co-located with the identified QTL. Thus, GWAS offers a more effective strategy for dissecting complex traits, overcoming several constraints associated with conventional linkage mapping. Several studies on

pearl millet utilizing this platform have yielded significant findings, reinforcing the value of this technology.

Chapter 2 - Characterization of genetic diversity and selection of drought tolerant and potential pearl millet (*Pennisetum glaucum* (L.) Br.) germplasms for forage breeding

2.1 Introduction

A high crop yield variability due to inevitable environmental changes is becoming a common challenge for the long-term sustainability of global food and feed production. Increased environmental variability affects grain yield, quantity and quality of biomass and forages for grazing, and profitability of farm operations. The unpredictable and variable rainfall and precipitation and abiotic stresses like drought and high temperature (heat) are increasing the rate of conversion of irrigated crop acres to dryland, reducing the crop yields and lowering the land values (Suttles et al., 2024). The variation in rainfall distribution during the growing season in different areas around the globe impacts forage production (Giridhar et al., 2015). In the United States (US), irrigation has reduced crop yield variability by 41% (Kukal et al., 2020). Expanding irrigation, however, puts more strain on the world's freshwater supplies, frequently resulting in their unsustainable use (Dalin et al., 2017). The Ogallala aquifer in the Central Great Plains (CGP) has been declining rapidly, making it one of the aquifers that has been emptying the fastest over the last 20 years (Famiglietti and Ferguson (2021)). The Kansas Water Plan strongly emphasizes locally driven approaches to water management, suggesting focused efforts to increase crop water use efficiency (WUE) and the use of alternative crops to alleviate water scarcity (Kansas Water Plan, 2022).

Pre-breeding plays a crucial role in improving yield, and stress tolerance by assembling key traits or trait combinations, in new germplasms providing essential genetic diversity

(Sukumaran et al., 2022; Prasanth et al., 2021). These challenges to meet the global demand will be dependent on the continuous research efforts on screening germplasms to identify potential accessions for developing stress tolerant and high yielding hybrids involving both classical and molecular pre-breeding approaches. The pre-breeding multi-environment evaluation strategies for a stable yield with minimum drought impact will also be dependent on the available genetic resources and the subsequent selection for adaptation to challenging environmental stresses.

Existing forage crops in the US, like alfalfa (*Medicago sativa* L.) is known for its nutritional value and palatability but lacks to thrive in poorly drained soils and acidic conditions (Undersander et al., 2021). Clovers (*Trifolium spp.* L.) are good to improve soil fertility, but can have a high bloat potential in grazing animals if not managed carefully (Forage Facts). Corn silage (*Zea mays* L.) is also used as high-energy forage for livestock (Karnatam et al., 2023), however it requires more water and proper moisture content for optimal fermentation (Muck et al., 2010). These challenges associated with the forage crops need to be addressed. Alternative forage crops like sorghum (*Sorghum bicolor* L. Moench) and pearl millet (*Pennisetum glaucum* [L.] Br.) are being investigated by researchers and farmers for their drought tolerance and high silage yields requiring less water than corn (Sattler et al., 2010). Substituting fallow with forages in the dryland wheat (*Triticum aestivum* L.) farming system reduced the risk of wheat crop failure by enhancing water-use efficiency (Holman et al., 2018; Nielsen et al., 2005; Peterson et al., 1996; Carr et al., 2021). Pearl millet, one of the alternative climate resilient crops, can be adapted as dryland grain, forage, and cover crop, suitable feed for poultry and livestock particularly for monogastric animals and humans (Davis et al., 2003). Having evolved in Africa's drought-prone arid and semi-arid areas, pearl millet outperforms other annual grass crops under non-irrigated and low rainfall conditions (Hassan et al., 2014), maintaining high yields with 381

to 584millimeters of annual rainfall (Lee et al., 2009). Hassanat (2007) reported that under dryland conditions, pearl millet produced 6322 and 97511 kg ha^{-1} of forage dry matter harvested at the heading stage with 203 and 279millimeters of rainfall respectively.

In the 1990s, TifLeaf and Ghai forage cultivars of pearl millet were in cultivation for grazing, hay, cover crops, and forage purposes with 0.50 M ha^{-1} (Andrews et al., 1992) and a slight increase of 0.61 M ha^{-1} (Myers et al., 2002) acreage in 2002 in the southeastern USA. Pearl millet has been gaining importance again since 2019 as a promising forage crop substituting summer fallow in grain sorghum-winter wheat cropping systems in the southeastern USA (Baumhardt et al., 2006; Bhattarai et al., 2019). Unlike sorghum, pearl millet genetically possesses several unrealized forage attributes: short growing season with more tillers and high photosynthetic efficiency, thinner stems, and more feed value due to low lignin content and high palatability (Nagaraja et al., 2024). Above all, the absence of prussic acid (Sedivec et al., 1991) makes pearl millet safe for summer grazing at any crop stage with high-quality forage (Burton et al., 1966). The highest water-use efficiency (WUE) of forage pearl millet (6.13 Mg ha^{-1} mm^{-1}) was reported by Crookston (2020) in tilled soil than in no-till and makes pearl millet a suitable alternative for drought-sensitive forage/feed crops. Sasani (2004) reported that irrigating pearl millet with 75% of its water requirement does not change its forage yield and quality while its WUE keeps increasing with higher water stress. However, the dry matter digestibility of forage pearl millet (64.0-69.0%) is slightly lower than forage corn (73.0%) but similar to forage sorghum (63.0%) (Hassanat et al., 2007). Also, pearl millet forage yields (4.5 to 6.5 t ha^{-1}) are lower than the 8 to 16 t ha^{-1} reported for forage sorghum in Western Kansas (Holman et al., 2022).

Pearl millet is a cross-pollinated species, possessing a wide range of genetic variations, and the available diversified germplasm resources are not fully exploited for improving the agronomic traits, stress tolerance, and for high productivity in diverse agro-climatic conditions (Passot et al., 2016; Shivhare et al., 2017; Kanfany et al., 2020). Globally, the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Research and Development Institute, and Global Genebank Information System (GRIN) have more than 66,000 diversified accessions that represent more than 140 pearl millet species. Unfortunately, limited research has been done for the development of genetic and genomic resources for forage traits in pearl millet (Daduwal et al., 2024). Yadav and Bidinger (2008) reported that improved pearl millet varieties demonstrate high biomass production and superior forage quality compared to other cereal crops, making it a valuable alternative under water-limited conditions. Sustained breeding efforts are needed for increased forage yield potential with enhanced drought tolerance in pearl millet hybrid development, particularly for tropical and sub-tropical regions where water resource is limited.

Rank summation index (RSI) described by Mulamba and Mock (1978) is an ideal method to identify high-yielding and drought-tolerant germplasms, as it enables efficient multi-trait selection by ranking and summing germplasms across key traits. However, selecting superior genotypes for forage yield might be challenging since it is affected by multiple factors such as genotype (G), environment (E), and management (M) practices. This is further complicated by the genotype-by-environment interaction (GEI), thus slowing the genetic progress in breeding programs[38]. As a result, it is essential to understand how environmental variations influence genotypic traits and their adaptability in a specific stress condition. Different stability models such as ANOVA-based (Yates and Cochran, 1938; Shukla, 1972), regression-based (Eberhart

and Russel, 1966) methods were used earlier, and additive main effects and multiplicative interaction (AMMI) and genotype plus genotype-by-environment interaction (GGE) biplot (Yan et al., 2007) were developed to apprehend the effects of the GEI for trait-based stability assessment. In recent times, multi-environment trial (MET) analysis has been developed, increasing the use of linear mixed-effect models such as best linear unbiased prediction (BLUP) to analyze the MET data (van Eeuwijk et al., 2016). Multi-trait-based stability evaluation methods including multi-trait mean performance and stability index (MTSI), multi-trait genotype-ideotype distance index (MGIDI), and multi-trait index based on factor analysis and genotype-ideotype distance (FAI-BLUP) index are the robust statistical tools for effective selection of stable accessions across different environmental conditions (Rocha et al., 2018; Olivoto et al., 2019; Olivoto and Nardino, 2021). However, few studies addressing the selection of stable pearl millet cultivars using multi-trait-based stability evaluation methods have been reported (Naveen et al., 2024; Khandelwal et al., 2024; Ramalingam et al., 2024) and not for traits for forage improvement. The current research grounded in this context, and proposes a framework by using RSI, GGE biplot, MTSI, MGIDI, and FAI-BLUP indices for the selection of drought-tolerant and stable pearl millet germplasms that would be the basis for the parental lines and heterotic forage hybrid development with improved yield potential and drought tolerance across diverse environmental conditions.

We hypothesized that the selected germplasm collection for different maturity groups has accessions with different drought stress tolerance under field conditions. The objectives of the study were to: (a) select different maturity groups from the diversified germplasm accessions and assess their genetic distribution (b) quantify the impact of drought stress on agronomic traits in different germplasms and (c) exploit genetic variability and identify promising drought-tolerant

accessions to be used as potential parental lines for forage hybrids development with improved yield potential.

2.2 Materials and methods

2.2.1 Planting materials

Out of 309 pearl millet germplasms received from International Crop Research in Semi-Arid Tropics (ICRISAT), Niger, West Africa (Kanfany et al., 2020), 188 photo-insensitive accessions from 24 countries were used in this study (Supplementary Table 2.1). These accessions are being maintained through repeated selfed generations at the Kansas State University Agriculture Research Center, Hays (ARCH), Kansas, USA.

2.2.2 Field experiments

The accessions were evaluated under irrigated (control) and rainfed (stress) conditions at ARCH, KS (latitude 38.9798 N, longitude 99.3268 W; soil type: Harney silt loam), in the summer 2023 and 2024. Two-row plots with a length of 3 meters and a 1.5 meter alley were arranged in an augmented block design across the four environments. The irrigated field received water at three critical growth stages: vegetative, flowering, and grain filling, while no irrigation was provided to the rainfed field experiments throughout the cropping period. Sprinkler was used for the irrigation Weather parameters like temperature and precipitation were recorded using the Kansas Mesonet (https://mesonet.k-state.edu/weather/historical/). Teros 12 soil moisture sensor was used to record the hourly soil moisture content in summer 2024 irrigated and rainfed field experiments.

2.2.3 Data collection and statistical analysis

Three plants were randomly selected from each plot for data collection. The agronomic traits such as days to 50% flowering (DF), chlorophyll index (CL), plant height (PH), staygreen

(SG), number of productive tillers (PT), and biomass (BM) were measured at different developmental stages of the plants. The days to 50% flowering were recorded as the days from planting until 50% of the accessions had female flowers. Chlorophyll meter SPAD-502 Plus was used to measure the chlorophyll index 10 days after the anthesis. The average of five measurements taken from different areas of the flag leaf was calculated and recorded as a single reading per plant. Plant height expressed in centimeters was measured from the soil surface to the tip of the main stem. Plot-based visual scoring of the stay green rating was done at or shortly after physiological maturity. A scale of 1 to 10 was used to assign scores based on the percentage of green leaves (Xu et al., 2000). A rating of 1 indicated 10% green leaves, 5 indicated 50% green leaves and 10 indicated 100% green leaves. The number of productive tillers was counted per plant and the biomass was recorded by weighing the harvested above-ground plant material in grams (g) per plant.

2.2.4 Analysis of variance

A combined analysis of variance using a general linear model in R was performed on the measured traits (Ramalingam et al., 2024). The genotype, replication, treatment (irrigated and rainfed), and environment (year) were considered sources of variation. Least significant differences (LSDs) were used to compare the mean of each treatment across germplasms and traits under study.

2.2.5 Percent reduction and rank summation index

All 188 accessions were classified into three maturity groups based on the average data of days to 50% flowering from irrigated field experiments in summer 2023 and 2024 as: early maturing (55 to 65 days), medium maturing (66 to 75 days), and late maturing (>75 days).

Equation (1) was used to calculate percent reduction for each trait using the average values of the three plants per plot.

$$Percent\ reduction = \frac{IR-RF}{IR} \times 100 \qquad (1)$$

Where, *IR* and *RF* indicate the average value of the trait under irrigated and rainfed treatments, respectively. Based on the percent reduction, a multi-criteria selection procedure known as rank summation index (RSI) was employed to identify the highest-ranked germplasms by simultaneously evaluating all five traits (CL, PH, SG, PT, and BM) together (Ramalingam et al., 2024). Germplasms with the least percent reduction were ranked first while those with the highest were ranked last. The thirty highest-ranked accessions were selected within each maturity group and advanced for further analysis. The corrplot package in R was used to perform the correlation (Wei et al., 2017) using the percent reduction values for the traits under study for 2023 and 2024.

2.2.6 Genetic structure

We used single nucleotide polymorphism (SNP) markers developed through genotype by sequencing (GBS) method to verify the genetic variation in the selected germplasms in this present investigation. Fresh leaf tissues (50mg) were collected from 188 pearl millet germplasms at the 5-leaf stage and genotyped at the University of Minnesota Genomic Center (UMGC). The deoxyribonucleic acid (DNA) was isolated from the samples and genotyped using the GBS sequencing technique at UGMC. DNA samples were digested using the restriction enzyme *Pst*l (CTGCAG) and barcode adapters were ligated to individual samples. The sequence data was trimmed using the GBStrim.pl software and the quality control of reads was verified before and after trimming using FASTQC (Andrews 2010). Burrows wheeler alignment (BWA) (Li and Durbin, 2009) was used to align reads to reference genome Tift 23D2BI-P1-P5 (Salson et al.,

2023), and SNP calling was done using the Freebayes. SNP filtering was done using Tassel v.5 (Bradbury et al., 2007), and SNPs with missing rate > 20%, minor allele frequency <0.05, and heterozygosity >0.1 were removed (Ramalingam et al., 2023). The retrieved SNPs were first imputed using Beagle (Browning et al., 2018) and later pruned using Plink (Purcell et al., 2007), yielding 13576 high-quality SNPs. Principle component analysis (PCA) was performed where two PCA axes were built with the 188 pearl millet accessions and the selected germplasms from each maturity group were projected on these axes using the ggplot2 package in R.

2.2.7 Multi-traits-based stability analysis

Four different multi-trait-based models - BLUP-based GGE biplot, MTSI, MGIDI, and FAI-BLUP were used to identify the reliable and consistent stable accessions across diverse environments by considering all the measured traits together. The gge () function from the metan package in R was used to perform the GGE biplot analysis using the BLUP values (Olivoto et al., 2019). For other three models (MTSI, MGIDI, and FAI-BLUP) the metan package in R was used to calculate the different indices using different functions (Olivoto and Lucio, 2020).

2.2.7.a BLUP-based GGE biplot

Best linear unbiased predictions (BLUP) were predicted by considering year and treatment as fixed effects while the genotype and replication nested within the year and treatment as random effects. Equation (2) was used to calculate the linear mixed-effects model using the lme4 package in R (Bates et al., 2003)

$$Y_{ijklm} = \mu + Year_i + Treatment_j + G_k + R_{l(ij)} + \in_{ijklm} \quad (2)$$

where Y_{ijklm} is the observed value of the trait, μ is the overall mean, $Year_i$ and $Treatment_j$ are fixed effects for the ith year and jth treatment, respectively, G_k is the random effect of the

kth genotype, and $R_{l(ij)}$ is the random effect of the lth replication nested within the ith year and jth treatment combination. The residual error is represented by $\mathcal{E}_{ijklm}$.

2.2.7.b Multi-trait mean performance and stability index (MTSI)

Equation (3) was used to estimate the multi-trait mean performance and stability index.

$$\mathrm{MTSI}_i = \sqrt{\left(\sum_{j=1}^{f}(\mathrm{F}_{ij} - \mathrm{F}_j)^2\right)} \qquad (3)$$

Where, $MTSI_i$ is the multi-trait stability index of the ith genotype, F_{ij} is the jth score of the ith genotype, and F_j is the jth score of the ideotype. The MTSI was calculated using the *mtsi ()* function from the metan package (Olivoto and Lucio, 2019).

2.2.7.c Multi-trait genotype-ideotype distance index (MGIDI)

The MGIDI was determined as described by Olivoto and Nardino (2021) in four steps: (a) rescaling the traits (b) using factor analysis (c) planning ideotype and (d) computing the MGIDI index. Equation (4) was used to calculate MGIDI.

$$MGIDI_i = \sqrt{\left(\sum_{j=1}^{f}(Y_{ij} - Y_j)^2\right)} \qquad (4)$$

Where, $MGIDI_i$ is the multi-trait genotype-ideotype for the ith genotype, Y_{ij} is the score of the ith genotype in the jth factor *(i = 1, 2... g; j=1, 2… f; g* and *f* being number of genotypes and factors respectively), and Y_j is the jth score of ideotype. The "*gamem*" and "*mgidi*" functions in the metan package were used to calculate the MGIDI index (Olivoto and Nardino, 2020).

2.2.7.d Multi-trait index based on factor analysis and genotype-ideotype distance (FAI-BLUP)

After defining the ideotype, the distance of each genotype from the ideotype was determined and transformed into a spatial probability, which in turn allowed the genotype ranking. Equation (5) was used to estimate the FAI-BLUP index.

$$P_{ij} = \left(\frac{1/d_{ij}}{\sum_{i=1;j=1}^{i=n;j=m} \left(1/d_{ij}\right)} \right) \qquad (5)$$

where P_{ij} is the probability that the ith ($i=1,2,\ldots,n$) genotype is the same as the jth ($j=1,2,\ldots,m$) genotype; d_{ij} is the genotype-ideotype distance from the ith genotype to the jth ideotype, based on the normalized Euclidean distance mean (Rocha et al., 2018). The FAI-BLUP index was estimated using the fai_blup () function in the metan package (Olivoto and Lucio, 2020).

Venn diagram was created using the venn_plot () function using the metan package in R (Olivoto and Lucio, 2020).

2.2.8 Selection differential, heritability, and selection gain

Equation (6) was used to calculate the selection differential (ΔS) that was computed using the percentage of population mean (Falconer, 1981).

$$\Delta S\% = \frac{(X_s - X_o)}{X_o} \times 100 \qquad (6)$$

Equation (7) was used to calculate the broad sense heritability (h^2) based on mean performance.

$$h^2 = \left(\frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_i^2}{e} + \frac{\sigma_e^2}{eb}} \right) \qquad (7)$$

Where, σ^2_g, σ^2_i, and σ^2_e are variances related to genotypes; genotype-environment interaction, and error respectively; e and b refer to the number of environments and blocks per environment, respectively.

Equation (8) was used to compute genetic gain (ΔG) percentage under selection.

$$\Delta G\% = \frac{(X_s - X_o)}{X_o} \times h \times 100 \qquad (8)$$

Where, X_o is the mean for WAASBY index of the original population; X_s is the mean for WAASBY index of selected genotypes; h is heritability.

We calculated selection differential, heritability, and selection gain from only three stability models (MTSI, MGIDI and FAI-BLUP).

2.3 Results

2.3.1 Weather parameters of the experimental site

The average temperature recorded during the cropping season of 2023 and 2024 were 22.0 and 22.9°C, respectively. The amount of rainfall received in 2023 and 2024 was 254.5 and 271.5 mm respectively. The rainfall distribution was accompanied with long dry spells Also, the rainfall received during the critical flowering period in August was the highest in 2024 (106.9 mm) when compared to 2023 (95.3 mm) (Figure 2.1a). Irrigation was provided during the critical growing stages (after planting, flowering, and grain filling stage) in the irrigated (control) field experiments in both years showing a significant difference in the moisture content between the treatments (irrigated and rainfed). The moisture sensor used in summer 2024 recorded a cumulative moisture content of 1.28 cubic meters of water per cubic meter of soil ($m^3m^{-3)}$ in the irrigated as compared to the rainfed condition ($0.694m^3m^{-3}$) from June to October. (Figure 2.1b).

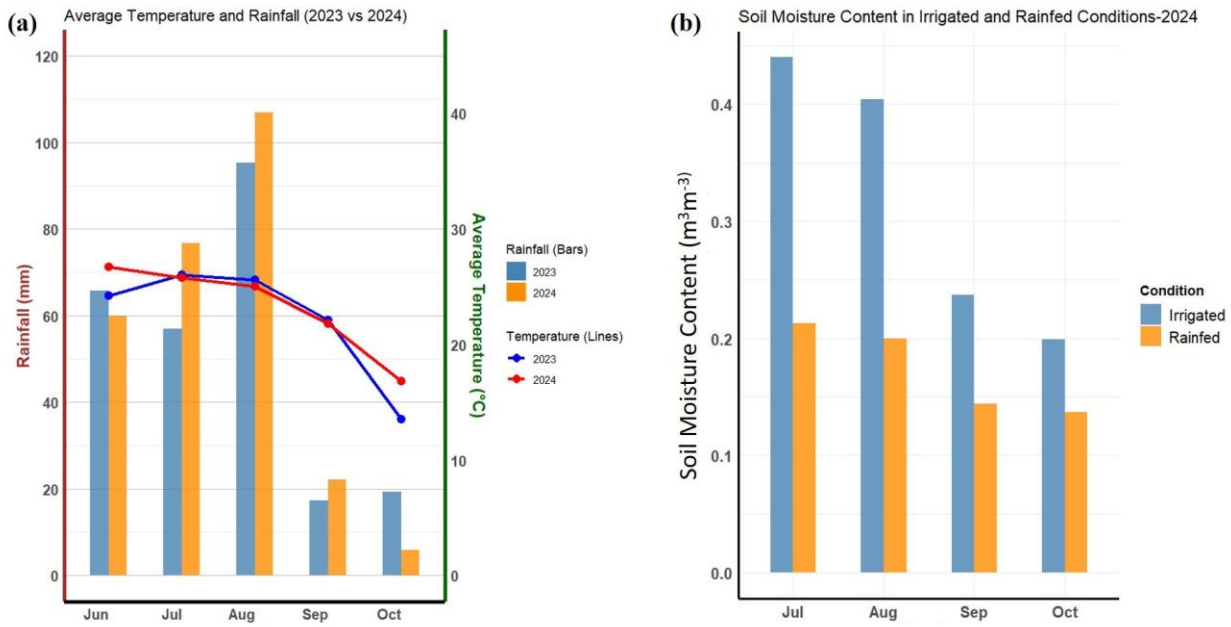

Figure 2.1. Weather parameters. (a) Rainfall and average temperature variation during the cropping period of summer 2023 and 2024 and (b) soil moisture content recorded in summer 2024 at Agricultural Research Center, Hays, Kansas.

2.3.2 Variation and mean performance

A combined analysis of variance revealed a significant difference between treatments (irrigated and rainfed), years, among genotypes, and GEI on all drought-related traits under study (Table 2.1). The LSD test between treatments of each year indicated that traits like DF, PH, PT, and SG showed significant differences in both years. However, no significant difference in CL and BM was observed under the rainfed treatment for both years. A significant effect of treatment was observed on the performance of the germplasms. The treatment was the main source of phenotypic variation, calculated using the mean square values for all traits except SG (97.2% for DF, 91.1% for CL, 85.9% for PH, 75.9% for PT, and 97% for BM) (Figure 2.2). For SG, the environmental (year) contribution to phenotypic variance was the highest followed by genotype, explaining 34.8 and 20.1%, respectively. The mean performance of the germplasms under irrigated and rainfed conditions in both years across different maturity groups is given in Table 2.2. It was lower in rainfed conditions across the years within each maturity group for all traits except DF. Flowering was delayed for early and medium maturity groups in drought stress conditions across each year. However, there was no difference in the average DF mean performance for the late maturing germplasms under control and drought stress conditions.

Table 2.1 Combined analysis of variance (ANOVA) for drought-related traits of pearl millet germplasms under irrigated and rainfed conditions.

Source	DF	CL (SPAD)	PH (cm)	PT	SG	BM (g)
Replication (R)	32563	24	1373*	27.5*	26.9*	37250*
Genotypes (G)	512*	538*	6546*	7.8*	8.4*	8881*
Treatment (T)	34412*	126957*	2165196*	2746.1*	4.7*	1804556*
Environment (E)	0*	11324*	340811*	850.3*	14.5*	31808*
G × T	125*	218*	2568*	4.4*	4.7*	5254*
G × E	227*	269*	2665*	4.6*	5.9*	6479*
E × T	469*	10274*	235844*	601.6*	528*	62360*
G × T × E	119*	130*	2269*	3.8*	3.5*	3953*

Environment (E) - Summer 2023 and 2024; Treatment (T)- irrigated (control) and rainfed (stress) conditions (*$p \leq 0.001$). DF, days to 50% flowering; CL, chlorophyll content; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass.

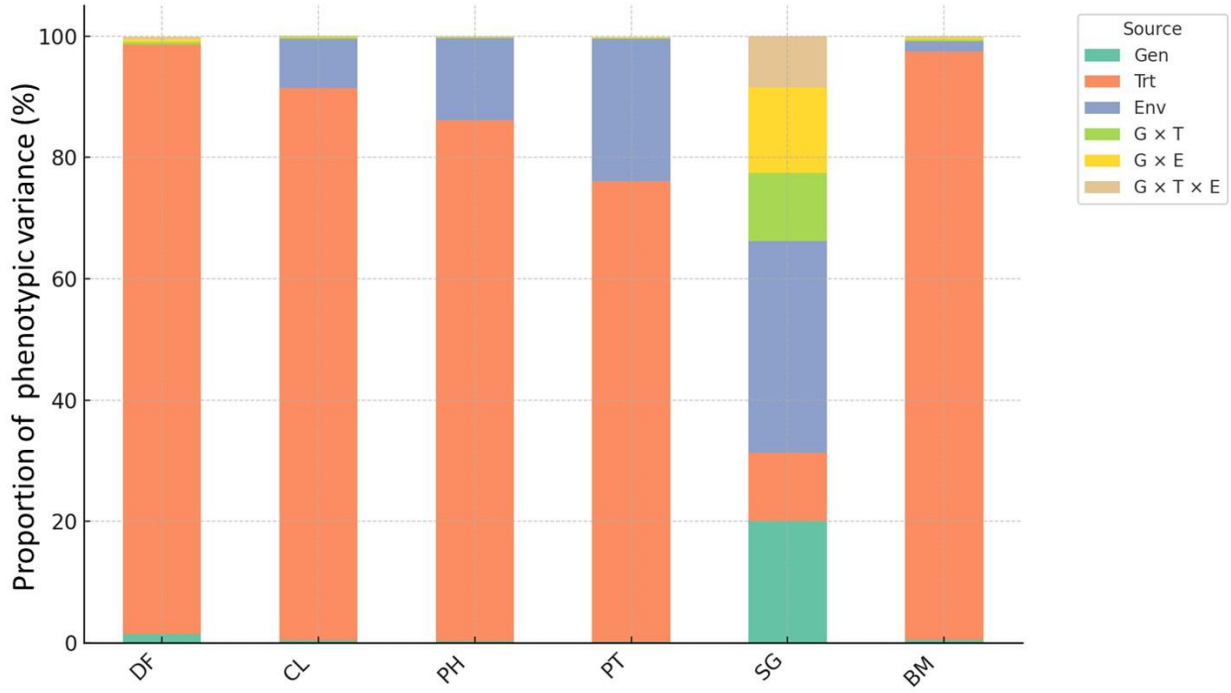

Figure 2.2 Proportion of phenotypic variance contributed by given sources of variation across 188 pearl millet germplasms under irrigated (control) and rainfed treatments for summer 2023 and 2024.

DF, days to 50% flowering; CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass.

Table 2.2. Mean performance of pearl millet germplasms within three different maturity groups.

Maturity group	Treatment	Year	DF	CL	PH	PT	SG	BM
		2023	63.24±0.42	47.08 ±1.24	181.49 ± 5.84	3.89± 0.3	3.39 ± 0.18	154.9±12
	irrigated	2024	61.1±0.52	51.06±1.06	213.91±5.21	5.2±0.22	3.44±0.27	136.1±6.94
Early	(control)	Mean	62.17±0.35	49.07±0.84	197.7±4.29	4.55±0.2	3.42±0.16	145.51±6.95
(41 accessions)		2023	74.39±1.3	35.32±1.54	140.43±4.81	2.02±0.19	2.61±0.21	80.76±8.2
	Rainfed	2024	69.9±0.86	35.78±1.41	145.87±3.86	1.98±0.14	1.39±0.10	76.73±4.87
		Mean	72.15±0.81	35.55±1.04	143.15±3.08	2.00±0.12	2±0.14	78.74±4.75
		Overall mean	67.16±0.59	42.31±0.85	170.42±3.39	3.27±0.15	2.71±0.12	112.13±4.94
		2023	73.02±0.74	40.36±1.07	182.93±4.01	2.94±0.17	4±0.15	157.01±8.19
	irrigated	2024	67.84±0.59	48.407±0.8	226.38±3.87	4.993±0.142	4.165±0.172	157.45±5.98
Medium	(control)	Mean	70.43±0.51	44.38±0.73	204.66±3.21	3.97±0.13	4.08±0.11	157.23±5.06
(91 accessions)		2023	82.45±0.95	28.14±1.07	136.66±3.74	1.49±0.07	2.88±0.14	85.8±4.22
	Rainfed	2024	75.86±0.73	28.3±1.07	139.24±2.47	1.76±0.1	1.77±0.98	84.11±3.47
		Mean	79.15±0.64	28.22±0.75	137.95±2.24	1.62±0.06	2.32±0.09	84.96±2.72
		Overall mean	74.79±0.47	36.30±0.67	171.3±2.62	2.79±0.1	3.20±0.09	121.09±3.44
		2023	89.21±1.02	35.44±0.86	161.49±4.57	1.63±0.15	4.63±0.23	185.1±14.1
	irrigated	2024	72.04±0.86	45.3±1.15	211.48±5.71	4.51±0.23	4.13±0.26	149.68±8
Late	(control)	Mean	80.63±1.05	40.37±0.86	186.49±4.34	3.07±0.19	4.38±0.18	167.41±8.25
(56 accessions)		2023	88.89±0.9	25.5±1.28	124.31±4.22	1.2±0.07	3.11±0.23	92.34±5.26
	Rainfed	2024	80.45±1.05	25.62±1.29	130.01±3.44	1.48±0.11	2.02±0.15	87.95±4.58
		Mean	84.67±0.8	25.56±0.91	127.16±2.72	1.34±0.07	2.56±0.15	90.14±3.48
		Overall mean	82.65±0.67	32.97±0.8	156.83±3.24	2.20±0.12	3.47±0.13	128.78±5.16

DF, days to 50% flowering; CL, chlorophyll content; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass.

2.3.3 Inter-relationship analysis

An inter-relationship analysis was performed among the drought-related traits using the percent reduction for 2023 and 2024. All traits showed significant positive correlations between the years (2023 and 2024): CL (r=0.35), SG (r=0.17), and BM (r=0.17). CL showed significant positive correlations with PH in both 2023 (r=0.19) and 2024 (r=0.25), and with PT (r=0.32) only in 2024. PH and PT showed a positive correlation across the years (r=0.25 in 2023 and r=0.15 in 2024) (Figure 2.3).

2.3.4 Rank summation index

The RSI was calculated separately for different maturity groups using the percent reduction values by considering all the evaluated traits for both 2023 and 2024 together (Figure 2.4). Based on the RSI results, 90 germplasms were selected (30 from each maturity group) (Supplementary Table A.1) for stability analysis.

2.3.5 Genetic structure

PCA of the 188 germplasms (showed a cumulative variance of 16.82% using the first two PCs (9.07 and 7.75%). The selected 90 germplasms in three maturity groups each with 30 accessions in this PCA were widely distributed, indicating distinct genetic differences of the selected accessions between and within each maturity group (Figure 2.5).

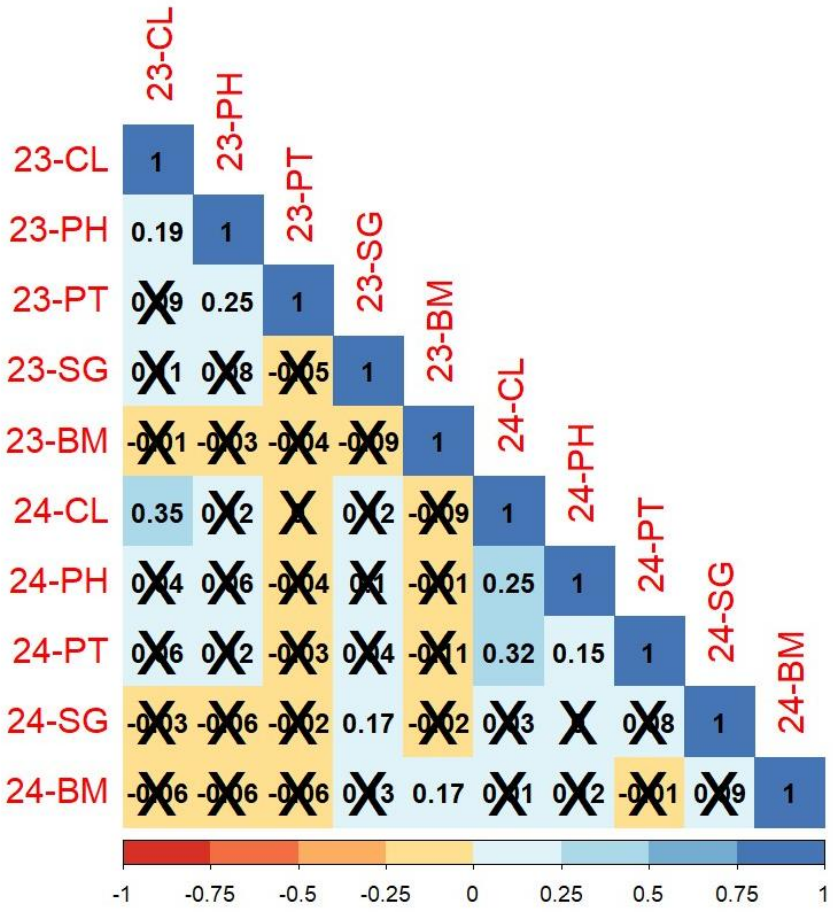

Figure 2.3. Interrelation among drought-related traits using percent reduction for summer 2023 and 2024. X indicates non-significant correlations. CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass. Other values are significant at $p < 0.01$

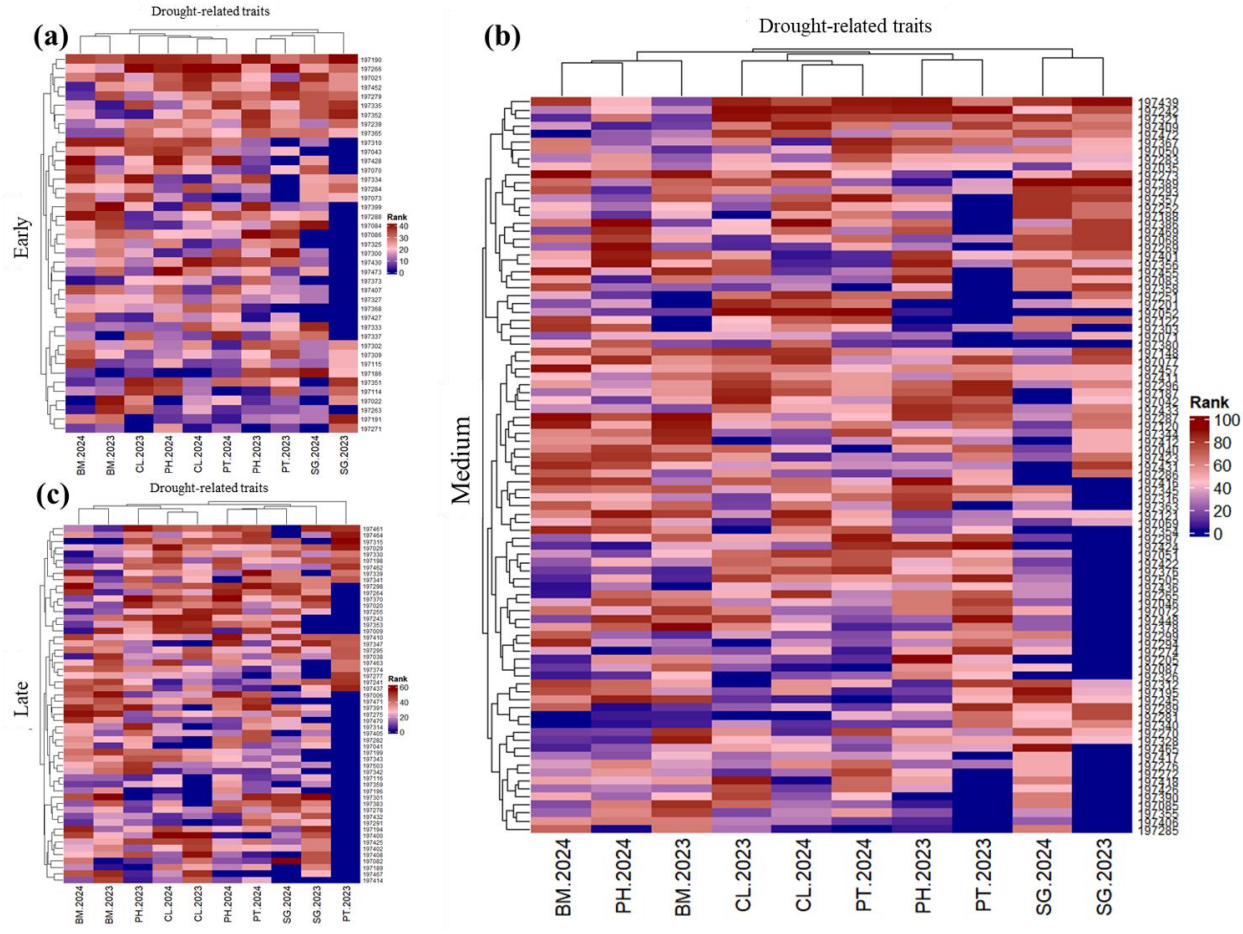

Figure 2.4. Heatmap of rank summation index based on the percent reduction of 2023 and 2024 for (a) early (b) medium, and (c) late maturing pearl millet germplasms for all the traits together. Blue cells: germplasms with a lower percent reduction, thus being drought-tolerant; Red cells: indicate germplasms with a higher percent reduction, thus being drought-susceptible. CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass.

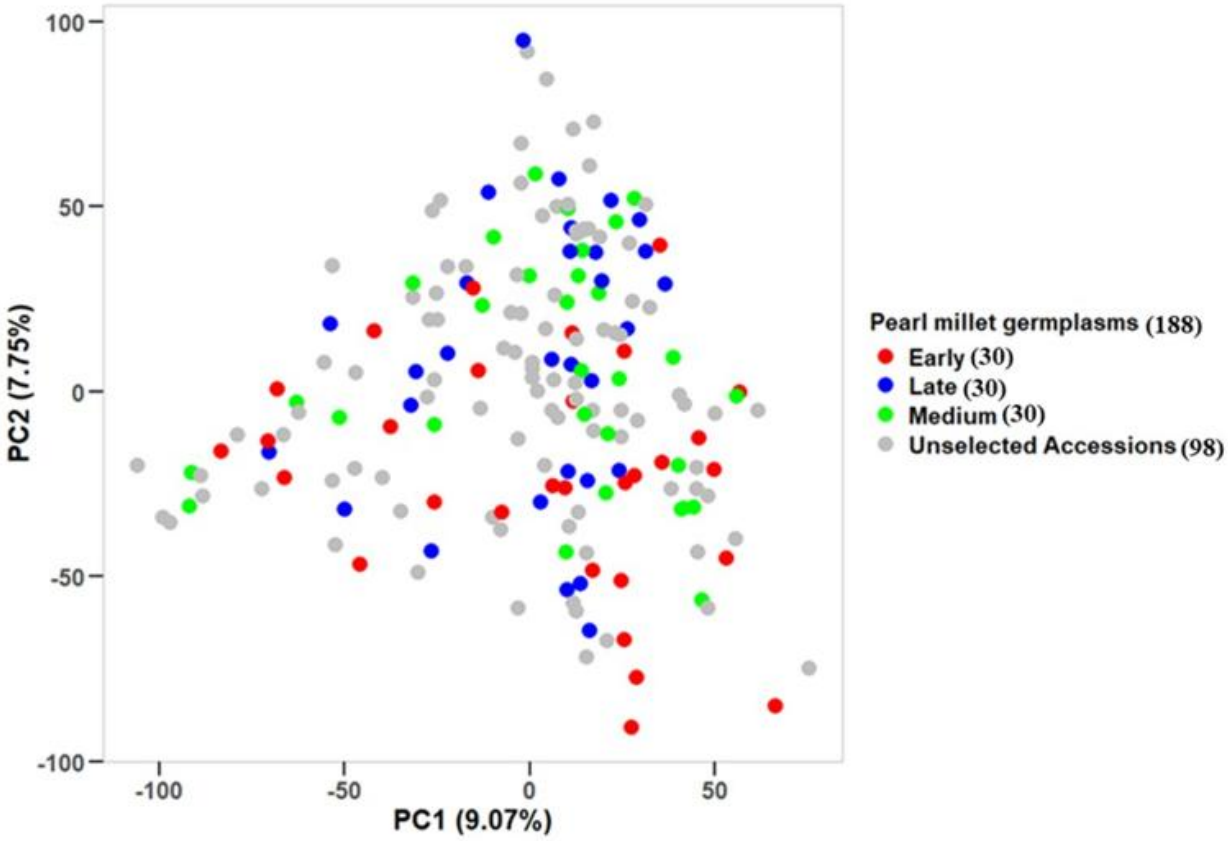

Figure 2.5. Principal component analysis (PCA) of three maturity groups of pearl millet germplasms each with 30 diversified accessions.

2.3.6 Multi-traits-based stability analysis

2.3.6.a BLUP-based GGE biplot

The BLUP-based GGE biplot selected stable and high-ranking germplasms from different maturity groups for traits CL, PH, PT, SG, and BM based on their close relatedness to the ideal germplasm (Figure 2.6). The first two PCs accounted for 63.46% of the total variation for the early, 64.08% for medium, and 58.19% for the late maturity group. For the early maturing, germplasms E12, E11, E3, E5, E26, and E2 were positioned closer to the center of the concentric circle, indicating greater stability (Figure 2.6a). Similarly, germplasms M29, M1, M21, M19, M5, and M28 in the medium maturity group (Figure 2.6b), and L4, L19, L1, L13, L5, L16, and L2 in the late maturity group (Figure 2.6c) were closer to the center, reflecting their stability.

2.3.6.b Multi-trait mean performance and stability index (MTSI)

MTSI index ranks the germplasms by employing information from the drought-related traits CL, PH, PT, SG, and BM in and across the environments. Utilizing a selection intensity of 20%, six superior germplasms with high mean performance and stability were selected among the early, medium, and late maturity groups (Figure 2.7). Germplasms E11, E5, E8, E12, E22, and E10 were selected among the early maturing, indicating a low MTSI index and high stability (Figure 2.7a). Similarly, M19, M5, M1, M28, M15, and M29 in the medium (Figure 2.7b), and L17, L16, L5, L19, L12, and L2 in the late maturity group were selected as superior germplasms (Figure 2.7c).

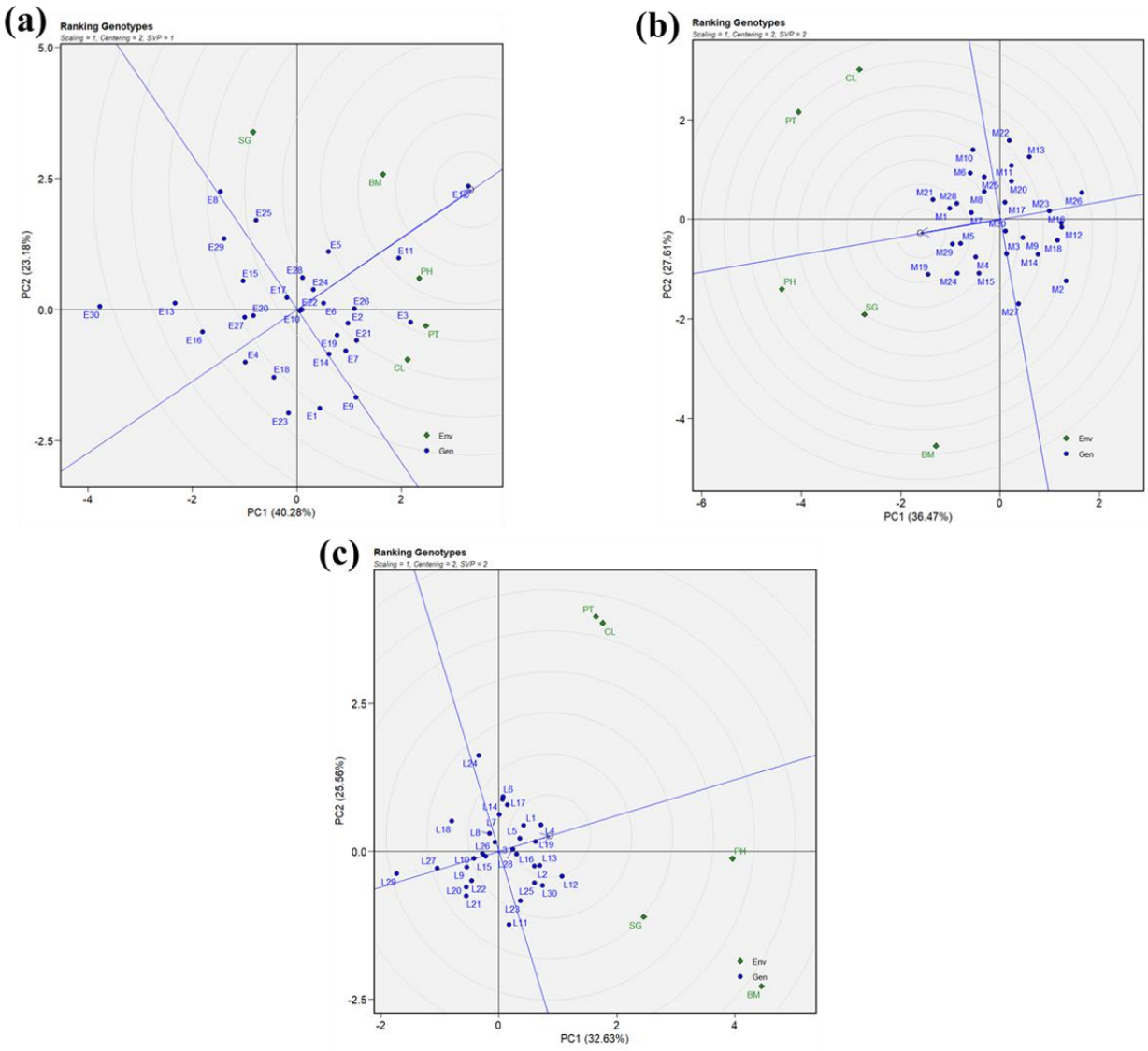

Figure 2.6. Genotype plus genotype-by-environment biplot graph showing the ranking of the tested germplasms for (a) early (b) medium and, (c) late maturity groups.

CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass.

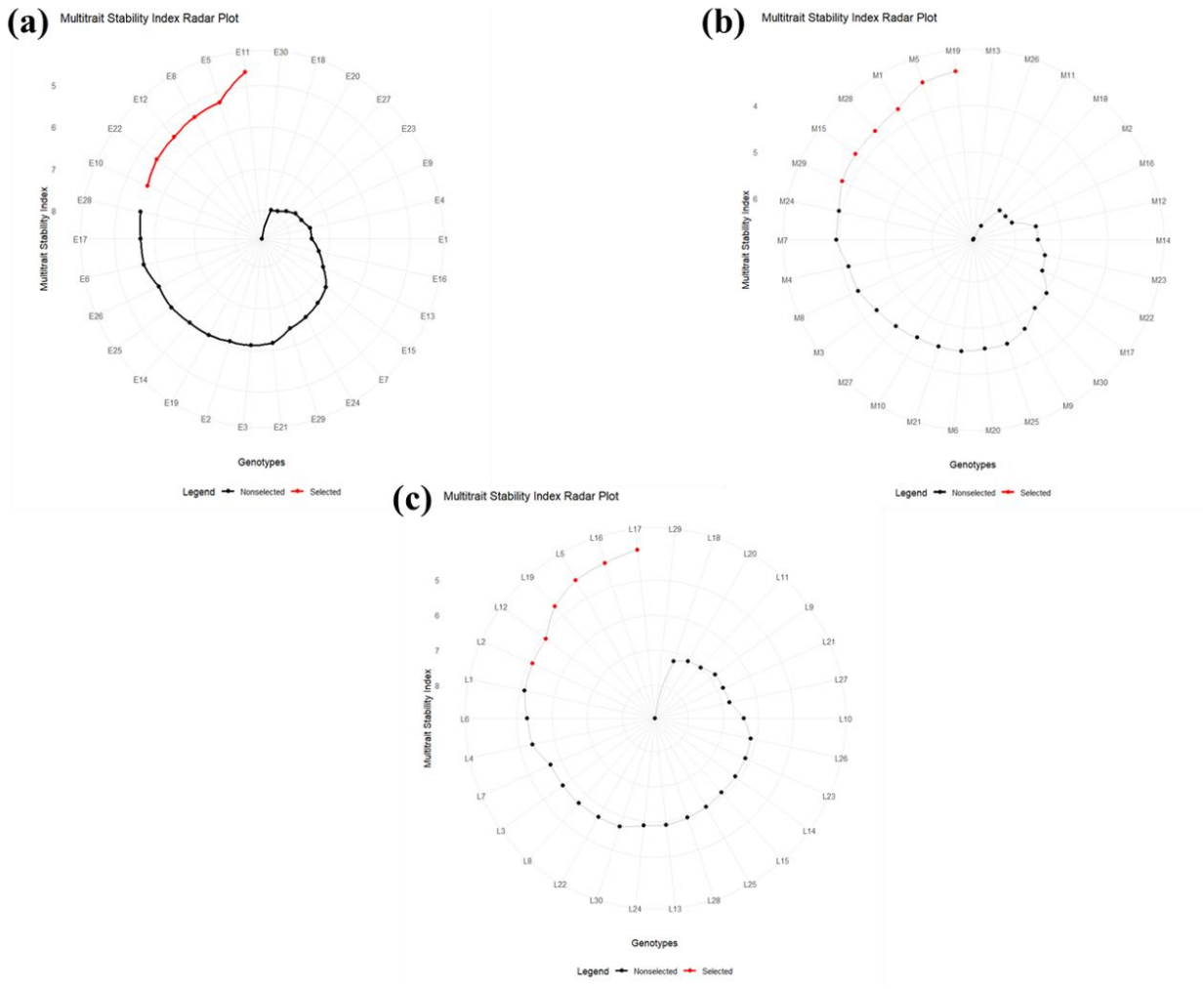

Figure 2.7. Multi-trait stability index (MTSI) for (a) early (b) medium and (c) late maturity groups.

Selected accessions are highlighted in red.

2.3.6.c Multi-trait genotype-ideotype distance index (MGIDI)

Utilizing a selection intensity of 20%, six germplasms were selected by the MGIDI index within each maturity group. The selected germplasms exhibited a low MGIDI index, indicating more closeness to the ideal ideotype. For the early maturing, germplasms E12, E11, E5, E25, E3, and E28 were selected based on their low MGIDI scores (Figure 2.8a). Germplasms M19, M21, M29, M24, M1, and M5 were selected from the medium maturing (Figure 2.8b). For late maturing, L12, L4, L5, L17, L19, and L1 were selected as the ideal germplasms (Figure 2.8c).

2.3.6.d Multi-trait index based on factor analysis and genotype-ideotype distance (FAI-BLUP)

Based on a selection intensity of 20%, FAI-BLUP selected six superior germplasms within each maturity group with a low FAI-BLUP score. For early maturing, E12, E11, E5, E3, 26, and E28 were selected as ideal germplasms (Figure 2.9a). Germplasms M19, M21, M29, M1, M5, and M28 were ideal for medium maturing (Figure 2.9b). For late maturing, L12, L5, L4, L17, L16, and L19 were selected as the ideal germplasms (Figure 2.9c).

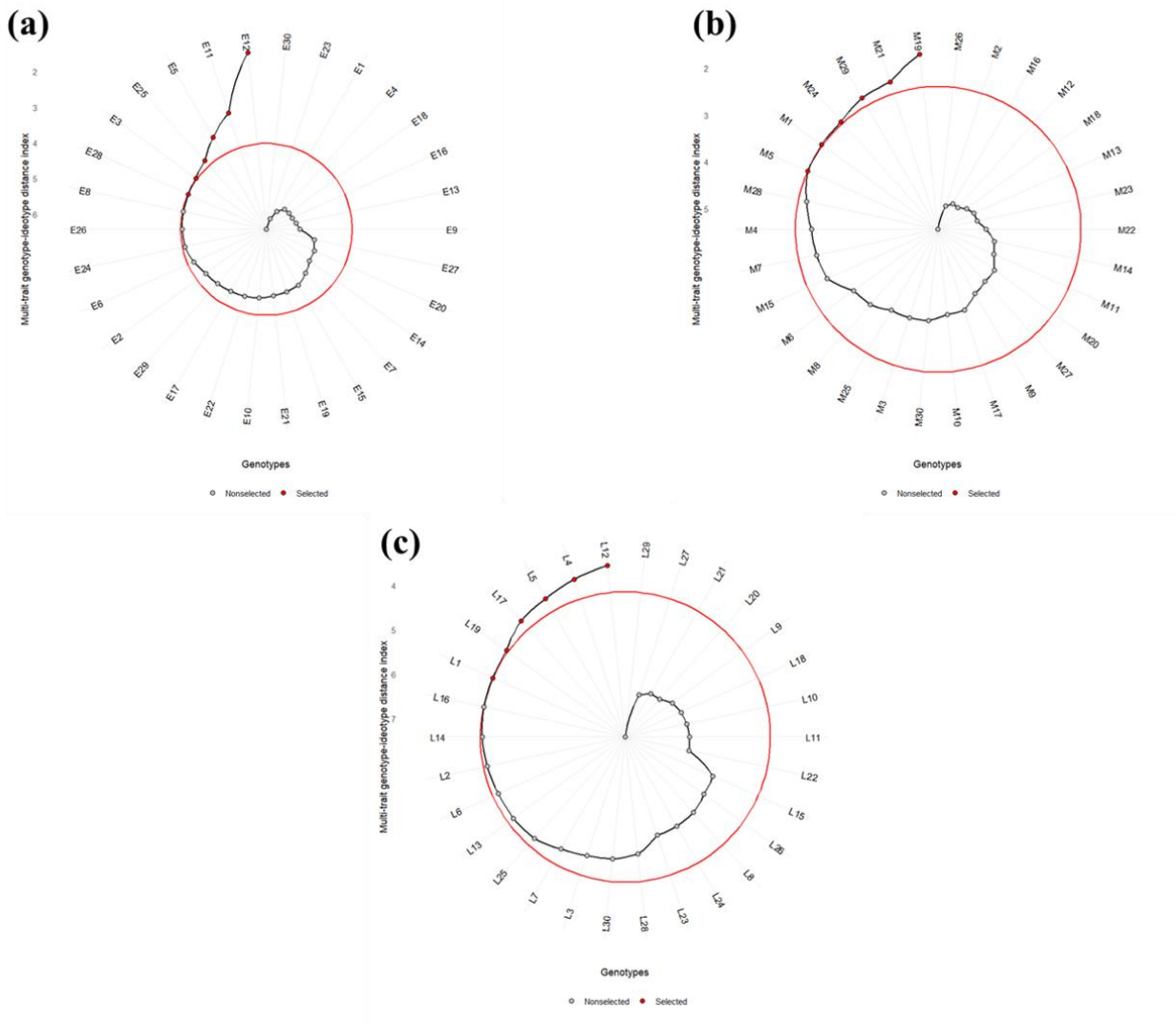

Figure 2.8. Multi-trait genotype-ideotype distance index (MGIDI) for (a) early (b) medium and (c) late flowering. Selected accessions are highlighted in red.

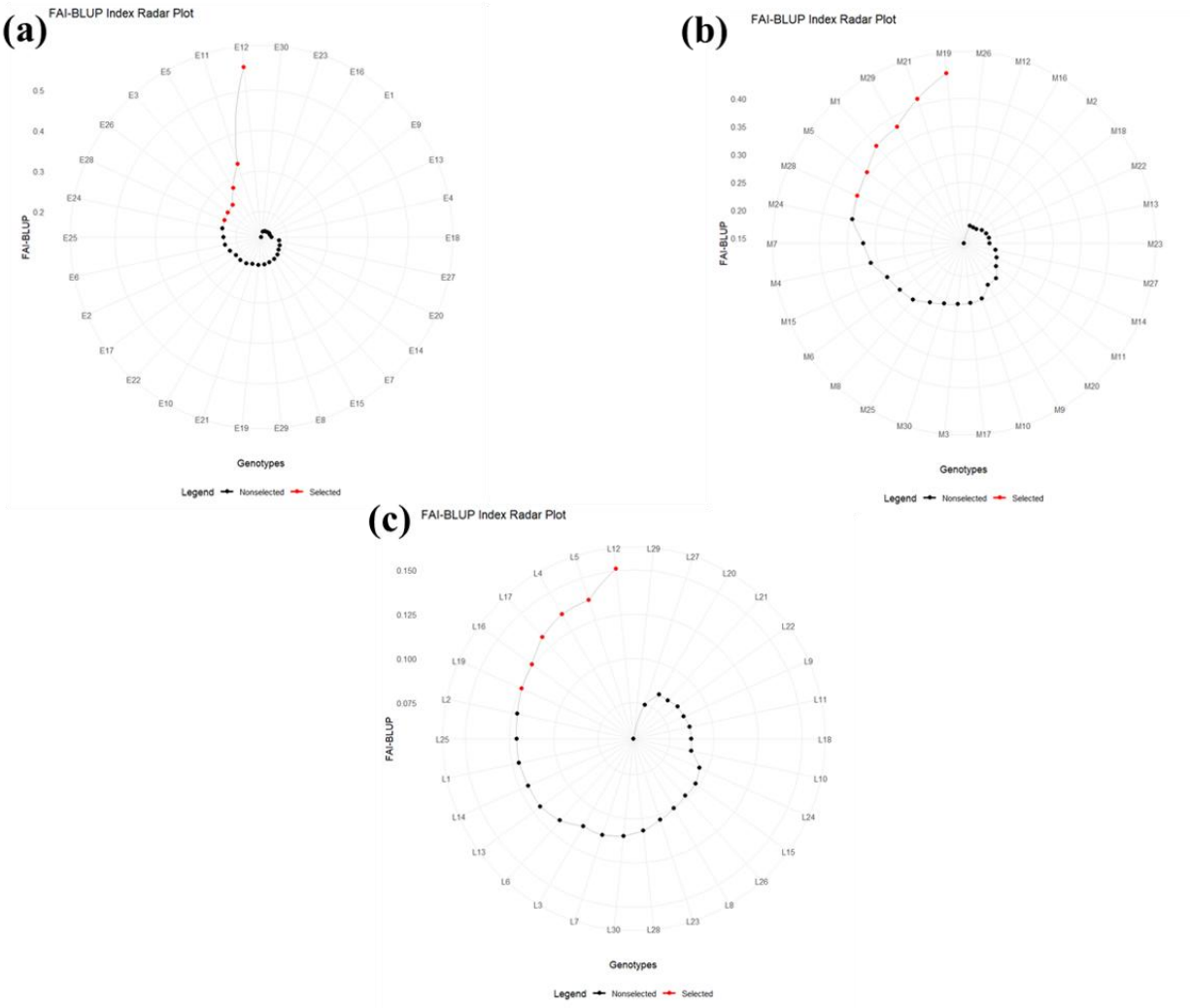

Figure 2.9. Multi-trait index based on factor analysis and genotype-ideotype distance (FAI-BLUP) for (a) early (b) medium and (c) late flowering. Selected accessions are highlighted in red.

2.3.7 Selection differential, heritability, and selection gain

The mean values of selection differential percentage (SD%), heritability (h^2), and selection gain percentage (SG%) were calculated using the estimates obtained from the three stability indices (MTSI, MGIDI, and FAI-BLUP). The mean SD% for CL was low in early (2.71%), and high (11.24%) in late maturing germplasms, while a moderate mean h^2 (0.53) was observed in medium maturing germplasms. PH and BM exhibited higher mean SD% and h^2 in medium-maturing compared to early and late maturing germplasms. For PT, mean SD% ranged from 1.4% in late maturing to 11.1% in medium maturing germplasms, with low to moderate h^2 across each maturity group. For SG, higher mean SD% values were observed across all maturity groups with 15.22% for early, 14.5% for medium, and 24.23% for late maturing. However, h^2 for SG was moderate in early and medium but high in late maturing germplasms. A positive mean SG% percentage was recorded for all the traits studied across each maturity group (Table 2.3).

Table 2.3. Selection differential, heritability and selection gains of the drought-related traits for the germplasms within different maturity groups.

Trait	Maturity group	Index	Xo	Xs	SD%	H^2	SG%
Chlorophyll content	Early	MTSI	42.60	43.60	2.49	0.41	1.02
		MGIDI	42.60	43.10	1.26	0.28	0.36
		FAI-BLUP	42.60	44.40	4.39	0.28	1.25
		Average	**42.60**	**43.70**	**2.71**	**0.33**	**0.88**
	Medium	MTSI	37.90	39.90	5.42	0.65	3.55
		MGIDI	37.90	40.30	6.28	0.48	2.98
		FAI-BLUP	37.90	41.30	9.05	0.48	4.30
		Average	**37.90**	**40.50**	**5.19**	**0.53**	**3.61**
	Late	MTSI	34.80	39.70	14.10	0.53	7.44
		MGIDI	34.80	37.90	8.82	0.28	2.48
		FAI-BLUP	34.80	38.60	10.80	0.28	3.05
		Average	**34.80**	**38.73**	**11.24**	**0.36**	**4.32**
Plant height	Early	MTSI	172.00	177.00	3.21	0.49	1.57
		MGIDI	172.00	183.00	6.74	0.34	2.26
		FAI-BLUP	172.00	181.00	5.31	0.34	1.78
		Average	**172.00**	**180.33**	**5.90**	**0.39**	**1.87**
	Medium	MTSI	174.00	203.00	16.70	0.64	10.60
		MGIDI	174.00	193.00	10.80	0.40	4.32
		FAI-BLUP	174.00	192.00	10.40	0.40	4.16
		Average	**174.00**	**196.00**	**12.63**	**0.48**	**6.36**
	Late	MTSI	153.00	162.00	6.11	0.72	4.40
		MGIDI	153.00	163.00	6.18	0.46	2.82
		FAI-BLUP	153.00	162.00	5.52	0.46	2.52
		Average	**153.00**	**162.33**	**5.94**	**0.55**	**3.25**
Number of productive tillers	Early	MTSI	3.40	3.68	8.16	0.32	2.63
		MGIDI	3.40	3.75	10.30	0.23	2.38
		FAI-BLUP	3.40	3.74	10.00	0.23	2.32
		Average	**3.40**	**3.72**	**9.49**	**0.26**	**2.44**
	Medium	MTSI	2.86	3.21	12.10	0.42	5.08
		MGIDI	2.86	3.17	10.70	0.20	2.16
		FAI-BLUP	2.86	3.16	10.50	0.20	2.11
		Average	**2.86**	**3.18**	**11.10**	**0.27**	**3.12**
	Late	MTSI	2.16	2.08	-3.35	0.64	-2.15
		MGIDI	2.16	2.34	8.49	0.33	2.82
		FAI-BLUP	2.16	2.14	-0.94	0.33	-0.31
		Average	**2.16**	**2.19**	**1.40**	**0.44**	**0.12**
Staygreen	Early	MTSI	2.69	3.26	21.10	0.51	10.70
		MGIDI	2.69	3.17	17.50	0.49	8.58

		FAI-BLUP	2.69	2.88	7.07	0.49	3.46
Trait	**Maturity group**	**Index**	**Xo**	**Xs**	**SD%**	**H^2**	**SG%**
		Average	**2.69**	**3.10**	**15.22**	**0.50**	**7.58**
	Medium	MTSI	3.26	3.79	16.20	0.44	7.04
		MGIDI	3.26	3.69	13.20	0.40	5.22
		FAI-BLUP	3.26	3.72	14.10	0.40	5.59
		Average	**3.26**	**3.73**	**14,5**	**0.41**	**5.95**
	Late	MTSI	3.28	4.25	29.40	0.75	22.10
		MGIDI	3.28	3.94	20.00	0.68	13.60
		FAI-BLUP	3.28	4.05	23.30	0.68	15.90
		Average	**3.28**	**4.08**	**24.23**	**0.71**	**17.20**
Biomass	Early	MTSI	115.00	156.00	36.00	0.52	18.80
		MGIDI	115.00	142.00	23.50	0.48	11.30
		FAI-BLUP	115.00	150.00	30.50	0.48	14.60
		Average	**115.00**	**149.33**	**3.00**	**0.49**	**14.90**
	Medium	MTSI	117.00	136.00	16.60	0.76	12.60
		MGIDI	117.00	141.00	20.90	0.68	14.10
		FAI-BLUP	117.00	131.00	12.40	0.68	8.43
		Average	**117.00**	**136.00**	**16.63**	**0.71**	**11.71**
	Late	MTSI	120.00	138.00	14.50	0.15	2.12
		MGIDI	120.00	130.00	8.37	0.32	2.65
		FAI-BLUP	120.00	131.00	8.46	0.32	2.68
		Average	**120.00**	**133.00**	**1.44**	**0.26**	**2.48**

Xo, mean for WAASBY index of the original population; Xs, mean for WAASBY index of selected genotypes; H^2, heritability; SD%, selection differential percentage; SG%, selection gain percentage.

2.4 Discussion

This study evaluated the drought tolerance of 188 pearl millet germplasms for forage yield potential. There was no considerable difference in the average temperatures recorded but with notable variations in the amount and distribution of rainfall received during the cropping period in 2023 and 2024, resulting in significant variations in the performance of the germplasms across the years. The increase in precipitation in summer 2024 as compared to 2023 enhanced the soil moisture levels, which is crucial for facilitating essential physiological processes like photosynthesis and transpiration during the crop's growth cycle (Blum, 2010). Also, the lower CL, PH, and PT in 2023 compared to 2024 can be attributed to the relatively low rainfall during the critical flowering period and overall cropping season in 2023. Drought stress, especially during the flowering stage reduces pollen viability and seed production, resulting in lowered yields and performance (Bidinger et al., 1987). The prevalence of water stress in both years impacted the agronomic traits, as reflected by the ANOVA results and the overall mean performance of the germplasms across each year. Drought stress in the rainfed condition showed significant differences as it contributed maximum phenotypic variations for all the traits except stay green in both years (Figure 2.2) which is mainly due to the irrigation provided during the critical crop growth periods in the controlled field experiments. The resulting water-limited rainfed stress condition and environmental interactions caused significant variations and delayed DF and lowered CL, PH, PT, and BM in both years. These findings emphasize the need to understand rainfall variability and its influence on pearl millet performance. Breeding efforts aimed at improving drought tolerance and adaptability to varying rainfall distribution could strengthen the crop's resilience to environmental fluctuations (Li et al., 2021; Serraj et al., 2004; Hash et al., 2000).

A significant positive correlation within chlorophyll index, staygreen, and biomass was observed over the years. Ali (2023) reported similar results with a significant positive relationship between chlorophyll and staygreen in wheat. These three traits exhibited moderate to high heritability across each maturity group, indicating a high genetic influence on these traits showing adaptable performance across years, highlighting the importance of chlorophyll index, staygreen, and biomass as reliable indicators for drought resilience (Ochieng, 2022; Xu et al., 2000; Kumar et al., 2022; Ramkumar et al., 2019). PH and PT also showed a positive association with each other across the years. Interestingly, the positive correlation of CL with PH and PT, indicates that chlorophyll retention might contribute to improved plant height and panicle traits under stress conditions (Wasaya et al., 2021; Singh et al., 2016). Fodder quality and biomass are influenced by various factors (Minson, 1990), with most of them showing a positive correlation with each other (Daduwal et al., 2024). Saygidar (2024) reported a positive correlation between biomass yield, plant height, number of tillers per plant, dry matter digestibility rate, relative feed value, and crude protein in pearl millet. The use of percent reduction values for correlation analysis provides a reliable depiction of the relative impact of drought on the studied traits, as it accounts for the degree of stress-induced variability. It provides valuable insights into the interrelationships among the component traits, which can aid in selecting drought-tolerant germplasms. Xu et al. (2023) reported the use of drought tolerance index (DI) for analyzing the correlation of eight yield-related traits in spring wheat and observed a positive relationship between DIs of chlorophyll index and PH.

For selecting drought-tolerant germplasm, we used RSI based on the percent reduction (Jumrani and Bhatia, 2019) by considering all the studied traits (CL, PH, PT, SG, and BM) for both 2023 and 2024 together. We narrowed down the preliminary selection by using RSI from

188 to 90 germplasms and classified into three maturity groups (early, medium, and late), each with the top thirty ranked germplasms, exhibiting low percent reduction and high drought tolerance. The genetic structure analysis also confirmed wide variation among the selected accessions within each maturity group. This classification is based on flowering time, a crucial trait for adaptation to climatic variability as it ensures the crop's development coincides with the rainy period (Diack et al., 2020). Ramalingam et al. (2024) used RSI using percent reduction to select drought-tolerant seed and pollinator parental lines in pearl millet. Similarly, Okoli (2021) reported the selection of green maize hybrids based on RSI.

The significance of GEI effect for the traits under study revealed they are controlled by polygenes and is challenging to make an effective selection of stable germplasms across diverse environments. It is clearly demonstrated through the difference in the mean performance of the studied germplasms with a significant genotype, treatment, and year interaction for all the traits under study. RSI was used to identify drought tolerant accessions considering the difference in control and stress environments performance. However, identifying stable accessions adaptable to multi-environments by tackling the limitations of GEI necessitates robust methods to address these challenges (Cabello et al., 2013). Lee (2023) highlighted the use of multi-environmental trials and stability analysis for evaluating yield-related traits across different maturity groups for the selection of stable genotypes in rice (*Oryza sativa* L.). Mohammadi (2016) investigated the effectiveness of different yield-based drought-tolerant indices in durum wheat and reported that the stress susceptible index (SSI) (Fischer and Maurer, 1978), tolerance index (TOI) (Rosielle and Hamblin, 1981), and yield stability index (YSI) (Bouslama and Schapaugh, 1984) models were inaccurate in identifying drought-tolerant stable genotypes particularly in dryland areas. Stress susceptibility index (SSI) used by Yadav and Bidinger (2008) in pearl millet had a

limitation as it considered change in genotype performance from control to stress treatment. For drought screening, Yadav (2010) suggested not to use the drought response index (DRI) to identify drought-tolerant pearl millet population due to many limitations.

By considering these limitations reported in the earlier studies, we used four advanced stability models: BLUP-based-GGE biplot (Sood et al., 2020), MTSI (Olivoto et al., 2019), MGIDI (Olivoto and Nardino, 2021), and FAI-BLUP (Rocha et al., 2018) in this investigation which have been used in recent studies to justify the significance and reliability of the selection of stable genotypes. BLUP-based-GGE biplot has the greatest relative ability to distinguish among accessions selected for consistent performance across diverse environments (Sood et al., 2020). Zuffo (2020) recommended MTSI as this model is more efficient for the selection of stable genotypes across adverse drought and saline stress environments. A simulation study by Olivota (2022) clearly indicated that MGIDI is a multi-trait-based framework highlighting the uniqueness and robustness of the model to analyze multivariate data across diverse environments. Progeny selection using FAI-BLUP index was effective in soybean (*Glycine max* L.) for stability performance as this model was balanced with desirable genetic gains for all traits simultaneously (Volpato et al., 2020).

The results from these four robust models facilitated more efficient and reliable selection of accessions from each maturity group with stable performance across four environments. These four advanced stability models clearly justified the efficiency of each model to delineate the G×E interactions and is clearly revealed in the selection of most of the stable genotypes identified by each model were different except few in all three maturity groups (Figure's 2.6, 2.7, 2.8 and 2.9 and Table 2.4). Several studies in other cereals have reported the use of combination of these indices for consistently selecting stable genotypes in sorghum (Behera et al., 2024), maize

(Singamseti et al., 2023; Subramani et al., 2024), wheat (Mohammadi, 2016), and barley (*Hordeum vulgare* L.) (Ghavidel et al., 2024; Ghazvini et al., 2024)

Table 2.4. List of selected drought tolerant and stable germplasms within each maturity group by different analyses.

Maturity groups	BLUP-based GGE biplot	MTSI	MGIDI	FAI-BLUP
Early	PI197373 (E2), PI197302 (E26), PI197427 (E3), PI197115 (E11), PI197368 (E5), PI197327 (E12)	PI197186 (E8), PI197309 (E22), PI197073 (E10), PI197115 (E11), PI197368 (E5), PI197327 (E12)	PI197399 (E25), PI197086 (E28), PI197427 (E3), PI197115 (E11), PI197368 (E5), PI197327 (E12)	PI197086 (E28), PI197302 (E26), PI197427 (E3), PI197115 (E11), PI197368 (E5), PI197327 (E12)
Medium	PI197299 (M21), PI197276 (M28), PI197465 (M19), PI197436 (M5), PI197326 (M1), PI197069 (M29)	PI197390 (M15), PI197276 (M28), PI197465 (M19), PI197436 (M5), PI197326 (M1), PI197069 (M29)	PI197424 (M24), PI197299 (M21), PI197465 (M19), PI197436 (M5), PI197326 (M1), PI197069 (M29)	PI197299 (M21), PI197276 (M28), PI197465 (M19), PI197436 (M5), PI197326 (M1), PI197069 (M29)
Late	PI197196 (L1), PI197414 (L4), PI197405 (L13), PI197359 (L2), PI197291 (L5), PI197278 (L19)	PI197503 (L16), PI0197359 (L2), PI197282 (L17), PI197291 (L5), PI197278 (L19), PI197277 (L12)	PI197196 (L1), PI197414 (L4), PI197282 (L17), PI197291 (L5), PI197278 (L19), PI197277 (L12)	PI197414 (L4), PI197503 (L16), PI197282 (L17), PI197291 (L5), PI197278 (L19), PI197277 (L12)

BLUP-based GGE biplot - Best linear unbiased predictions based genotype plus genotype-by-environment interaction (GGE) biplot; MTSI - Multi-trait mean performance and stability; MGIDI - Multi-trait genotype-ideotype distance index; FAI-BLUP - Multi-trait index based on factor analysis and genotype-ideotype distance

Venn diagrams, as a visualization tool was used to bring all the selected accessions together by different stability indices and identified the commonly shared accessions by all four models for each maturity group. Three accessions (E11 - PI197115, E5 - PI197368 and E12 - PI197327) from early, four (M19 - PI197465, M5 - PI197436, M1 - PI197326, and M29 - PI197069) from medium and two (L5 - PI197291 and L19 - PI197278) from late maturity exhibited wide adaptation, demonstrating stable performance across four environments as these selected drought tolerant accessions were commonly identified in all four stability models (Figure 2.10). Naveen (2024) conducted similar study by using four different stability models, assessed the selection efficiency and identified ideal and stable genotypes from a diverse global set of 248 pearl millet genotypes evaluated in diverse environments. The selected nine drought tolerant pearl millet germplasms from this study will be further used for quality traits' analyses for the development of hybrids with improved forage yield potential and quality traits.

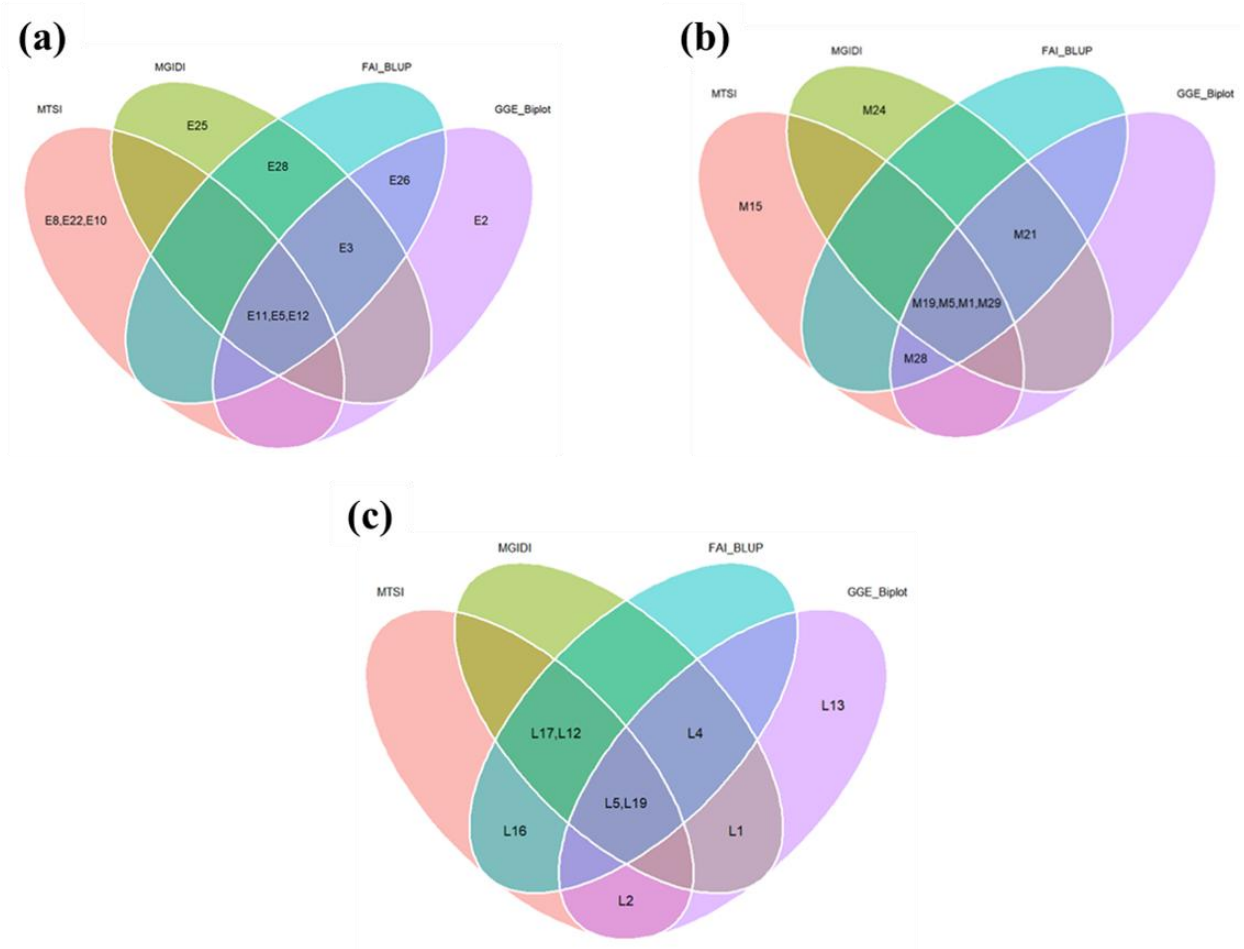

Figure 2.10. Venn diagram with the accessions selected by MTSI, MGIDI, FAI-BLUP indices, and GGE-Biplot for (a) early (b) medium, and (c) late maturing germplasms.

MTSI: Multi-trait stability index; MGIDI: Multi-trait genotype-ideotype distance index; FAI_BLUP: factor analysis genotype-ideotype distance- best linear unbiased predictions; GGE_Biplot: genotype plus genotype-by-environment biplot. Common accessions selected by different indices: Early maturity: PI197115 (E11), PI197368 (E5), PI197327 (E12) Medium maturity: PI197465 (M19), PI197436 (M5), PI197326 (M1), PI197069 (M29) Late maturity PI197291 (L5), PI197278 (L19).

2.5 Conclusions and future research

This study primarily focused on traits such as chlorophyll index, stay-green, plant height, productive tillers, and biomass which are related to drought tolerance. The stability performance cross-validated through four stability models (BLUP-based GGE biplot, MTSI, MGIDI and FAI-BLUP) selected nine drought-tolerant germplasms (Early: PI197115, PI197368, PI197327; Medium: PI197465, PI197436, PI197326, PI197069; Late: PI197291, PI197278) from different maturity groups. These selected nine drought tolerant accessions recorded higher mean performance for chlorophyll index, productive tillers and biomass across 188 germplasms indicating as potential source for parental lines and hybrid development with increased forage yield potential under drought stress environments. The findings of this study also suggest that traits such as chlorophyll index and staygreen could serve as reliable indicators of drought tolerance in pearl millet for improved forage yield potential. Additionally, biomass-related traits like plant height and productive tillers, despite exhibiting variability, hold the potential for enhancing biomass production per unit area. These insights contribute to forage breeding strategies aimed at improving drought resilience and biomass yield in pearl millet under water-limited conditions. The decision to limit the scope to these traits was due to the extensive screening of 188 germplasms, making the inclusion of forage-quality traits in these large collections practically cumbersome and challenging. Future research is required to address this gap by characterizing the selected nine germplasms from all three maturity groups in this study for key forage quality traits, including wet biomass at different stages followed by quality traits like crude protein content, in vitro digestibility, neutral detergent fiber content, acid detergent fiber content, lignin content, and cellulose and hemicellulose content. These evaluations will enhance the understanding of forage potential and nutritional value, contributing to the

development of high yielding pearl millet parental lines and forage hybrids with improved yield potential.

Chapter 3 - Unravelling Drought Tolerance in Pearl Millet Germplasms using Genome-Wide Association Studies

3.1 Introduction

Pearl millet [*Pennisetum glaucum* (L.) Br.] is the sixth most important cereal crop in the world, followed by rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), and sorghum (*Sorghum bicolor* L. Moench)). Globally, pearl millet is cultivated on > 30 million hectares across 30 countries, primarily in Africa and Asia (Yadav and Rai (2013)), with India leading in cultivation (6.93 million ha) and production (8.61 million tonnes) (Directorate of Millet Development, 2020). It is the most economical energy source among food crops including cereals and pulses, providing 361 kcal per 100g. It is a valuable source of minerals such as phosphorus (296–360 mg/100g), iron (8–11 mg/100g), zinc (3.1–6.6 mg/100g), calcium (40–42 mg/100 g), magnesium (97–137 mg/100g), and vitamins such as vitamin A, E, riboflavin, thiamine, vitamin K and niacin, etc. (Nibhoria et al., 2024). Pearl millet is grown for various purposes as grain, forage, and fodder in India (Yadav et al., 2013), as a fodder crop in Africa (Boote et al., 2022), and as a forage crop in the USA (Myers et al., 2002; Bhattarai et al., 2019). There is a new interest in the USA in growing pearl millet as a grain crop because of its drought tolerance and high-quality gluten-free grain (Andrews et al. 1993). According to reports, gluten-free dietary preferences and the demand for millet flour by some African and Asian immigrant communities have further boosted the market for pearl millet grain in the United States.

Drought is one of the major abiotic stresses reducing pearl millet production. Studies of the effects of drought on pearl millet include genetic analyses of variation in flowering time (Yadav et al., 2004), tillering (Mahalakshmi et al., 1987), grain yield (Bidinger et al.,

1987); Yadav et al., 2002; Yadav et al., 2004), biochemical (Choudhary et al., 2021), osmolyte (Kusaka et al., 2005), proteomic (Ghatak et al., 2016) and gene expression (Choudhary and Padaria, 2015; Dudhate et al., 2018) analyses. However, in terms of genetic improvement pearl millet remains behind, when compared to other cereals, resulting in its low average yields (Debieu et al., 2018). However, there is considerable scope to enhance its resilience against environmental stresses (Srivastava et al., 2022). The availability of whole genome sequence in pearl millet (Varshney et al., 2017), makes it possible to utilize its wide genetic diversity to develop cultivars and hybrids tolerant to environmental challenges (Debieu et al., 2017).

Advances in genetic and genomic technologies including marker-assisted breeding, development of genetic linkage maps, association mapping like genome-wide association studies (GWAS), quantitative trait loci (QTL) mapping, genomic selection, and genome editing have been employed to facilitate the genetic enhancement of pearl millet, particularly for improving its tolerance to biotic and abiotic (drought) stresses. The emergence of next-generation sequencing (NGS) technologies: genotyping-by-sequencing (GBS), skim sequencing, exome-capture etc. have revolutionized pearl millet research by enabling rapid genome decoding and providing a robust repository of genome-wide single nucleotide polymorphism (SNP) markers to enhance trait mapping, selection, and genetic gains (Srivastava et al., 2022). These markers serve as essential genomic tools in genetic studies aimed for pearl millet crop improvement through marker assisted breeding (Srivastava et al., 2020).

Compared to linkage mapping, association mapping offers higher resolution, utilizing historical genetic variations and recombinations facilitating the identification of markers closer to key genes (Liu et al., 2016). GWAS uses the principle of linkage disequilibrium (LD) to pinpoint association between DNA marker and target trait (Gupta et al., 20). However, due to the

considerable heterogeneity and heterozygosity in pearl millet germplasm accessions, only a limited number of association mapping strategies have been successfully developed (Kannan et al., 2014). In pearl millet, several studies have been conducted to dissect genomic regions affecting agronomic and grain nutritional traits through GWAS (Saidou et al., 2009; Kambara et al., 2024; Pujar et al., 2020). Limited research has been done to unravel the QTNs associated with abiotic stress tolerance. With this as background, the current research utilizes GWAS to detect the SNP markers and candidate genes associated with drought tolerance in pearl millet germplasm collection. The identified candidate genes will be used for trait introgression to improve future pearl millet breeding program.

We hypothesized that understanding the genetic structure and nucleotide diversity in pearl millet germplasms under drought stress allows the identification of QTNs associated with drought tolerance. The aim of the study was (a) to assess the genetic structure and diversity of the pearl millet germplasms (b) to characterize the impact of drought and assess the genetic variability of agronomic traits under field conditions, and (c) to identify QTNs and candidate genes associated with drought tolerance for all the traits under study.

3.2 Materials and Methods

3.2.1 Plant materials and field experiments

The materials and field experiments are described in chapter 2. However, for the GWAS analysis presented in this chapter, only 187 pearl millet accessions were used, as one genotype was excluded due to poor-quality genotypic data.

3.2.2 Phenotyping

Measurement of most traits are described in chapter 2. Additionally, Seed weight was recorded as a single plant yield, and the number of seeds were counted. Thousand seed weight

was calculated as (seed weight/seed count)*1000. Harvest index was calculated as the ratio of seed weight and biomass.

3.2.3 Phenotyping data analysis

Best linear unbiased predictions (BLUP) were predicted by considering the year as a fixed effect while genotype and replication (nested within the year) were random effects. The following linear mixed model was fitted using the lme4 package in R,

$$Y_{ijk} = \mu + Y_i + G_j + (YR)_{ik} + \in_{ijk}$$

where Y_{ijk} is the observed value of the trait for the *jth* genotype in the *ith* year and *kth* replication, μ is the overall mean, Y_i is the fixed effect of the ith year and G_j is the random effect of *jth* genotype and YR_{ik} is the random effect of the kth replication nested within the year. The residual error is represented by $\mathcal{E}_{ijk}$ (Henderson et al., 1953).

BLUPs were calculated separately for the irrigated and rainfed conditions, considering both year data together. The BLUPs were then used as observed (phenotypic values) in subsequent analyses (Wondifraw et al., 2024). Descriptive statistics (minimum, maximum, and average values) for the BLUP values were calculated (Table 1). Pearson correlation among irrigated and rainfed BLUPs (Xue et al., 2013) for ten traits was done and visualized using the corrplot package in R (Wei et al., 2017). To estimate the variance components and calculate heritability, genotypes and environment were assigned as a random effect and analyzed using the lme4 package in R. The following linear mixed model was used:

$$Y_{ijk} = \mu + G_i + E_j + \in_{ijk}$$

where Y_{ijk} is phenotypic observation of the i_{th} genotype in the j_{th} environment (replicate k); μ is overall mean; G_i is genotypic effect, treated as a random effect; E_j is environmental

effect, treated as a random effect; and ε_{ijk} is residual error (Piepho et al., 2008). Broad-sense heritability was then calculated as:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$$

where σ_g^2 is genotypic variance and σ_e^2 refers to environmental variance.

The drought stress tolerance index STI (Li et al., 2018) was calculated using the BLUPs for all the traits under study as follows,

$$STI_i = \frac{(Y_{ir})(Y_{rf})}{Y^2{}_{m,ir}}$$

Where STI_i is the stress tolerance index of a genotype for trait i, and Y_{ir} and Y_{rf} are the BLUP values for the trait under irrigated and rainfed conditions, respectively. For STI, the higher the value, the more the drought tolerance of that genotype.

3.2.4 Genotyping and quality control

In this study we used single nucleotide polymorphism (SNP) markers developed through genotype by sequencing (GBS) method to assess the genetic variation in the studied germplasms. Fresh leaf tissues (50mg) were collected from 188 pearl millet germplasms at the 5-leaf stage and genotyped at the University of Minnesota Genomic Center (UMGC). The deoxyribonucleic acid (DNA) was isolated from the samples and genotyped using the GBS sequencing technique at UGMC. DNA samples were digested using the restriction enzyme *Pst*l (CTGCAG) and barcode adapters were ligated to individual samples.

The sequence data was trimmed using the GBStrim.pl software and the quality control of reads was verified before and after trimming using FASTQC (Andrews 2010). Burrows wheeler alignment (BWA) (Li and Durbin, 2009) was used to align reads to reference genome Cenchrus. americanus Tifleaf3 cultivar, GCA_020739585.1 (Yan et al., 2023), and SNP calling was done

using the Freebayes, producing a total of 654001 SNPs. SNP filtering was done using *Tassel 5.2.95* (Bradbury et al., 2007), and SNPs with missing rate > 20%, minor allele frequency <0.05, and heterozygosity >0.1 were removed (Ramalingam et al., 2023). The retrieved SNPs were first imputed using Beagle (Browning et al., 2018), yielding a total of 35071 filtered SNPs and later pruned using Plink (Purcell et al., 2007), yielding 11981 high-quality SNPs.

3.2.5 Population structure and linkage disequilibrium estimation

The pruned SNPs were used for the genetic structure analysis. The population structure was estimated through principal component analysis (PCA) and a model-based maximum likelihood method. PCA was performed where two PCA axes were built with the 187 pearl millet accessions, and the origin data for each accession was projected on these axes using the ggplot2 package in R. Model-based maximum likelihood approach implemented by ADMIXTURE v1.23 were performed for inferring population structure of the panel using ~12k pruned SNPs. Pairwise LD values (r^2) were calculated and plotted against the physical distance (bp) in R, using the ~35k SNPs.

3.2.6 Genome-wide association study

STI of all traits was integrated with the genotyping data of 35071 SNPs to perform genome-wide association studies on ten drought-related traits viz. DF, CL, PH, PT, SG, BM, SW, SC, TSW, and HI. GWAS was conducted using four models - multiple loci mixed model (MLMM) (Segura et al., 2012), fixed and random model circulating probability unification (FarmCPU) (Liu et al., 2016), bayesian information and linkage-disequilibrium iteratively nested keyway (Blink) (Huang et al., 2019), by genome association and prediction integrated tool (GAPIT, v.3) and 3 variance-component multi-locus random-SNP-effect mixed linear model (Li et al., 2022) using IIVmrMLM package in R. Bonferroni correction was calculated to adjust the

significance thresholds for multiple testing. Significant QTNs from all traits under study, exceeding the threshold (5.8), were compared against the known QTLs for the related traits, and with the LD decay values of $r^2 = 0.1$ as guideline, candidate genes near significant QTNs or within a proximity of 20kb distance were searched using milletdb. (http://milletdb.novogene.com).

3.3 Results

3.3.1 Phenotypic evaluations and correlations

A violin plot represents the distribution of the phenotypic value (BLUPs) for key traits under different moisture conditions (Figure 3.1). Each violin plot combines a box plot (representing the median) and a density plot (representing the distribution), showing the spread of the BLUP values. The violin plot also shows a clear difference between irrigated and rainfed conditions, meaning water stress play a role in trait expression. The descriptive statistics calculated for the studied traits are presented in Table 3.1.

Pearson's correlation coefficients ranged from r = -0.66 to r = 0.91 (Figure 3.2). Interestingly intra-trait correlations were observed between irrigated and rainfed conditions. Also, CL showed positive correlations with the grain traits including PT, SW, SC, TSW, and HI under both rainfed condition as well as between irrigated and rainfed condition.

All traits showed low to moderate heritability, with the lowest recorded for BM (h^2 = 0.13) and highest recorded for HI (h^2 = 0.7) (Table 3.1). Low heritability was observed for BM (0.13), SW (0.2), SC (0.21), and PT (0.22); moderate heritability was observed for CL (0.24) and PH (0.24); and high heritability was observed for TSW (0.56), DF (0.54), and HI (0.7).

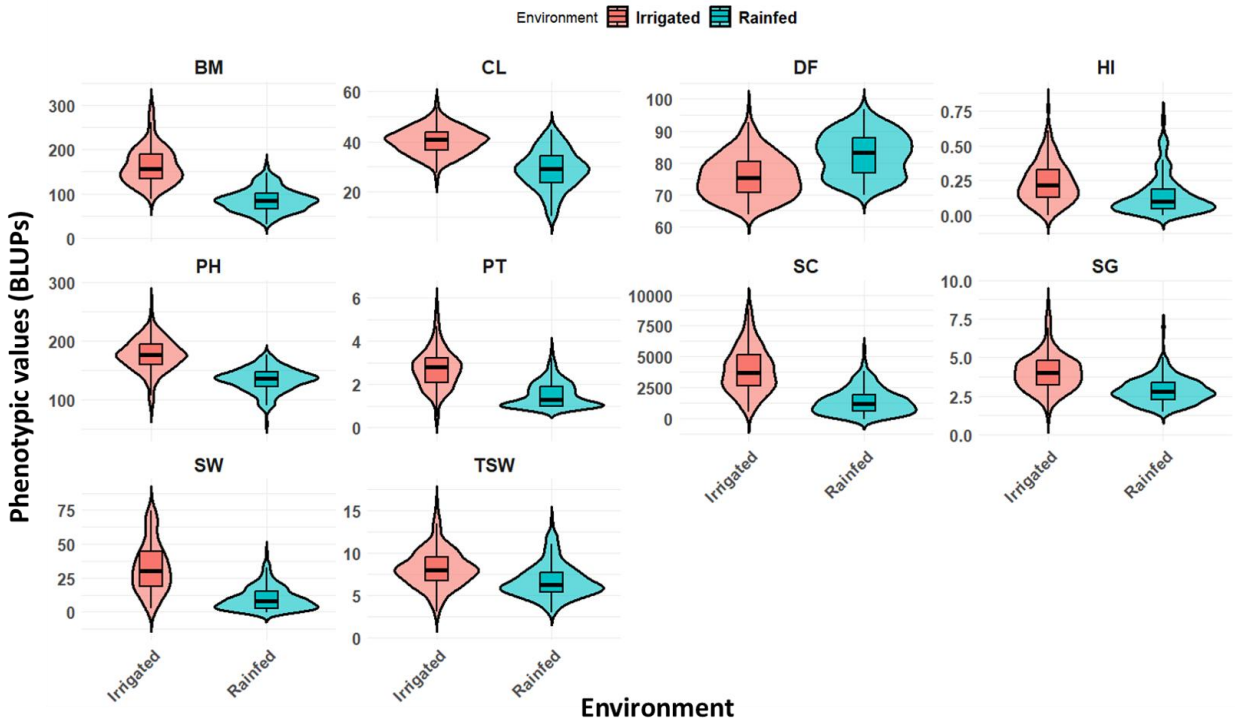

Figure 3.1. Violin plots showing the distribution of phenotypic values (BLUPs) for key traits under irrigated and rainfed conditions.

BM, biomass; CL, chlorophyll index; DF, days to 50% flowering; HI, harvest index; PH, plant height; PT, number of productive tillers; SC, seed count; SG, staygreen; SW, seed weight; TSW, thousand seed weight.

Table 3.1. Summary of phenotypic variation and heritability of drought-related traits in pearl millet germplasms.

Trait	Irrigated		Rainfed		Heritability (h^2)
	range	mean	range	mean	
DF	63.7-96.4	75.78±0.49	70-96.9	82.57±0.49	0.54
CL	24.3-56.1	40.34±0.39	10.3-44.6	28.85±0.57	0.24
PH	85-266.2	176.32±2.05	58.7-176.1	133.97±1.48	0.24
PT	0.5-5.7	2.73±0.07	1-3.6	1.53±0.04	0.22
SG	1.2-8.4	4.04±0.09	1.5-7	2.87±0.06	0.39
BM	88.5-298.5	163.99±3.05	31.5-164.7	86.32±1.9	0.13
SW	2.4-74.8	32.92±1.35	(-)0.2-43.9	10±0.62	0.2
SC	538-8843.4	3961.71±134.71	(-)56.6-5630.9	1427.49±76.73	0.21
TSW	2.6-15.9	8.18±0.16	3-13.9	6.78±0.15	0.56
HI	0-0.77	0.24±0.01	0-0.71	0.15±0.01	0.7

DF, days to 50% flowering; CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass; SW, seed weight; SC, seed count; TSW, thousand seed weight; HI, harvest index; IR, irrigated; RF, rainfed; h2, broad sense heritability.

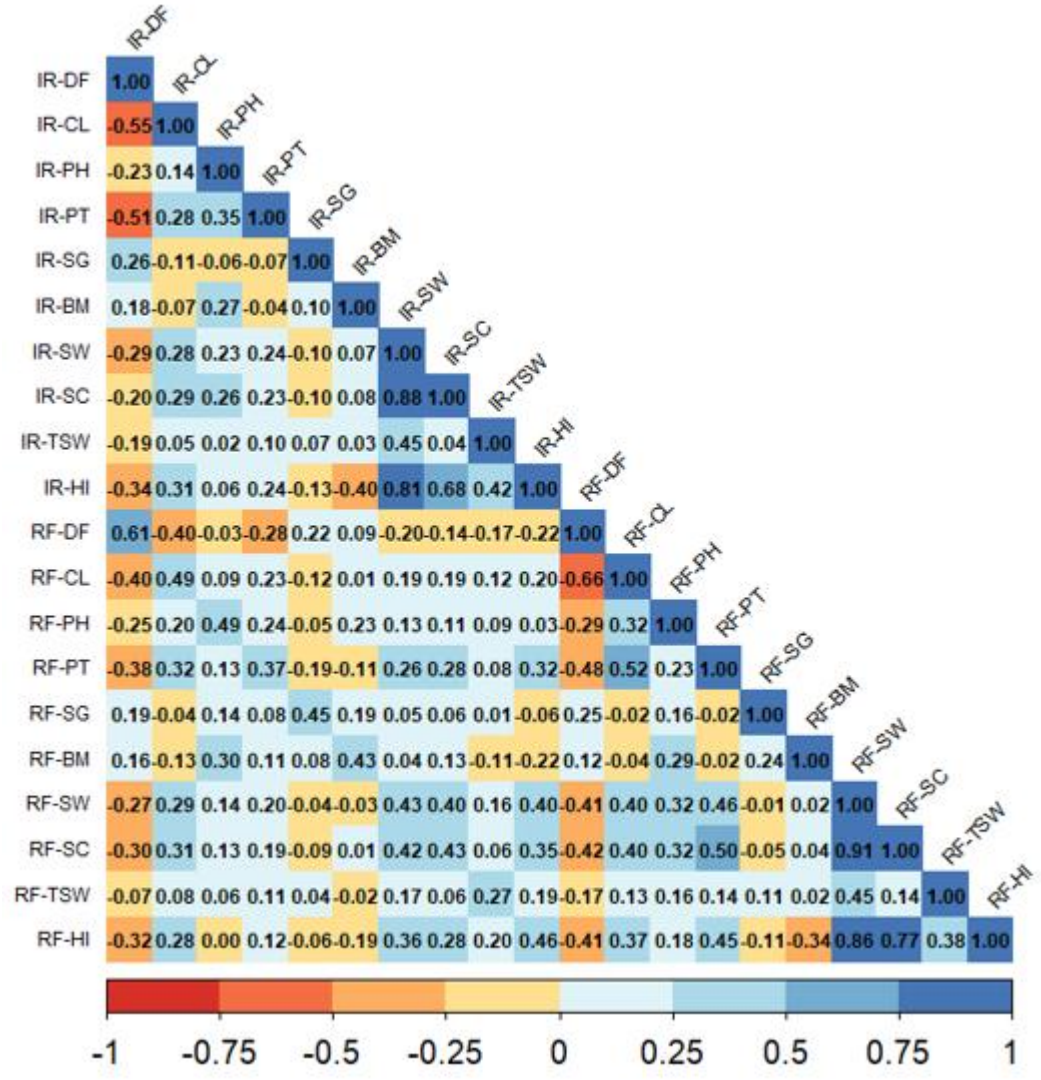

Figure 3.2. Pearson correlation among drought-related traits using BLUPs under irrigated (IR) and rainfed (RF) conditions.

DF, days to 50% flowering; CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass; SW, seed weight; SC, seed count; TSW, thousand seed weight; HI, harvest index. Values are significant at $p < 0.01$

3.3.2 Population structure and linkage disequilibrium

PCA was performed to identify and adjust for population stratification. The first two PCs contributed a cumulative genetic variance of 17.21% (PC1=9.47%; PC2=7.74%), indicating a wide genetic diversity among the pearl millet germplasms (Figure 3.3). The scattered distribution of the germplasms suggests that no strong population structure exists among the different origins. However, higher PC values might reveal a hidden population structure. Utilizing the tenfold cross-validation error (CV), ADMIXTURE analysis was performed with values of K ranging from 1 to 10. The population structure analysis revealed that the optimal number of subpopulations (K) was three (showing minimum CV error value) (Figure 3.4). At K=3, we grouped all the accessions into three subpopulations, however, this grouping was not based on the geographical origin of the germplasms (Figure 3.5). The initial average genome-wide LD (r^2) value in the population was reduced to 0.1 at ~18kb (Figure 3.6).

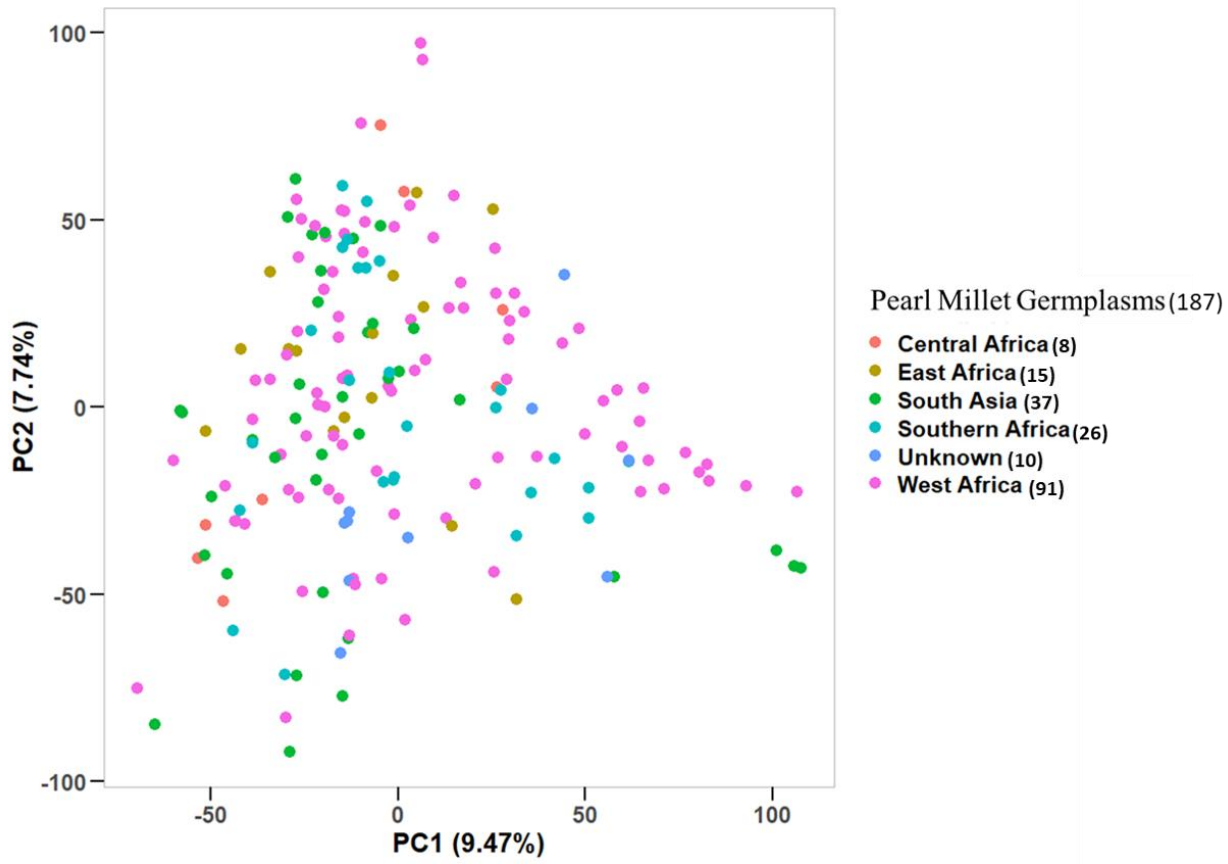

Figure 3.3. Principal component analysis (PCA) of pearl millet germplasms each with diversified origin.

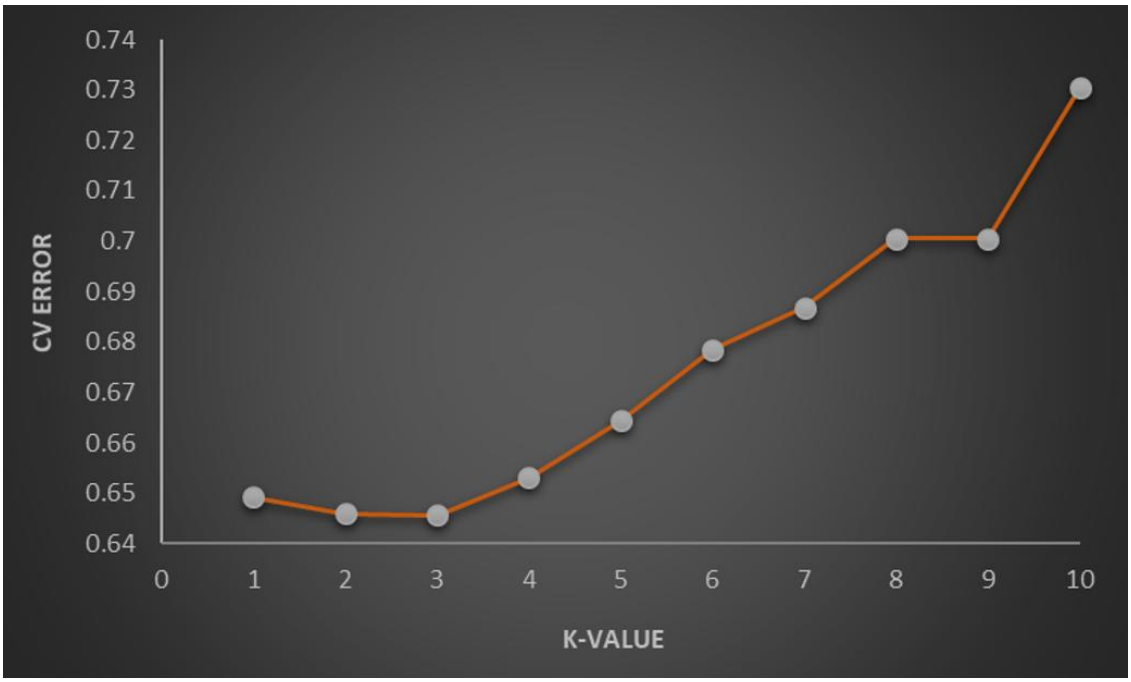

Figure 3.4. Cross validation (CV) error plot with values of K ranging from 1 to 10. Minimum CV observed at K = 3 (0.64572).

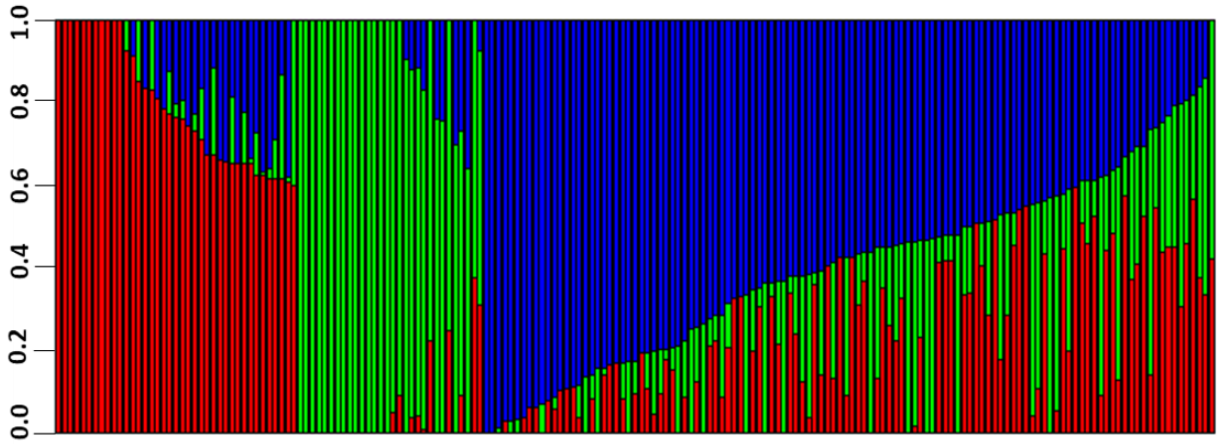

Figure 3.5. Population stratification using model based maximum likelihood approach at K = 3, shows separation of the pearl millet germplasms into four subpopulations.

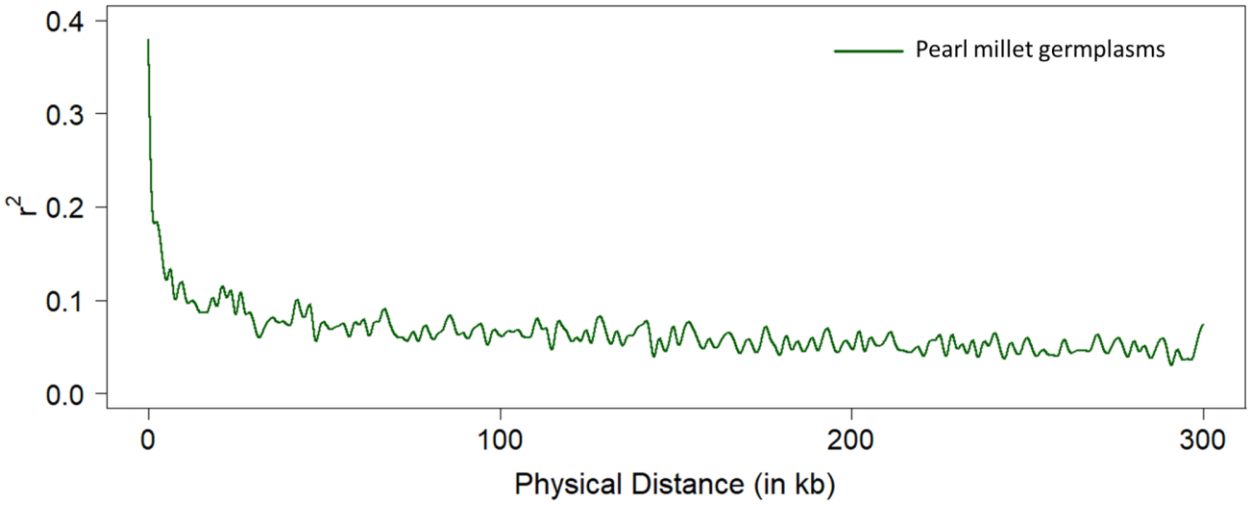

Figure 3.6. Linkage disequilibrium (LD) decay indicating decay to the background level (r^2 < 0.1) within ~18kb in the pearl millet germplasms.

3.3.3 Genome-wide association studies

Considering the ten drought-related traits and the stringent Bonferroni-adjusted significance threshold of 5.8 [$-\log_{10}(p)$], a total of 62 QTNs (within 20kb distance) were identified across 7 chromosomes using four GWAS models. Some QTNs were identified by multiple models and associated with more than one trait, while others were specific to a single model and a trait.

QTNs associated with traits like CL, PT, SG, SW, and HI were identified by more than one model (Figure 3.7), while those associated with DF, PH, BM, SC, and TSW were identified by the 3vmrMLM model only (Figure 3.8). From the selected subset of 62 drought-related QTNs, 3 were associated with DF, 12 with CL, 10 with PH, 4 with PT, 5 with SG, 3 with BM, 11 with SW, 9 with SC, 5 with TSW, and 6 QTNs were associated with HI. Several QTNs were associated with more than one trait. For instance, QTN SCM036296.1_219279663 was shared by DF and CL; SCM036295.1_66576426 by CL, SW, and HI; SCM036291.1_305871832 by SW and HI; SCM036293.1_892599 by SW and SC; SCM036295.1_62627244 by SC and HI (Table 3.2).

To confirm the significance of the newly identified QTNs, annotation was performed using milletdb database. A total of 77 candidate genes were searched colocalized with the identified 62 QTNs. From the set, 37 QTNs were found inside the candidate genes, the remaining were detected in the up and downstream of the candidate genes. For instance, for CL, QTNs such as SCM036291.1_209078226 and SCM036295.1_82539289 were found inside PMF1G04719 encoding aldo-keto reductase family 4 and PMF5G03975 encoding G-type lectin S-receptor-like serine/threonine-protein kinase genes, respectively; for PH, QTNs SCM036292.1_62937009 and SCM036292.1_240495105 were found inside PMF2G01922 encoding cyclic nucleotide-gated

ion channel 1 and PMF2G07960 encoding Rubisco large subunit-binding protein subunit alpha genes, respectively. Likewise, SG QTNs SCM036292.1_1143479 and SCM036293.1_95645749 were present inside PMF2G00146 and PMF3G03185 genes, encoding serine carboxypeptidase 3 and glycerophosphodiester phosphodiesterase GDPD1, respectively. The summary of the QTNs colocalized with drought-related candidate genes can be found in Table 3.3.

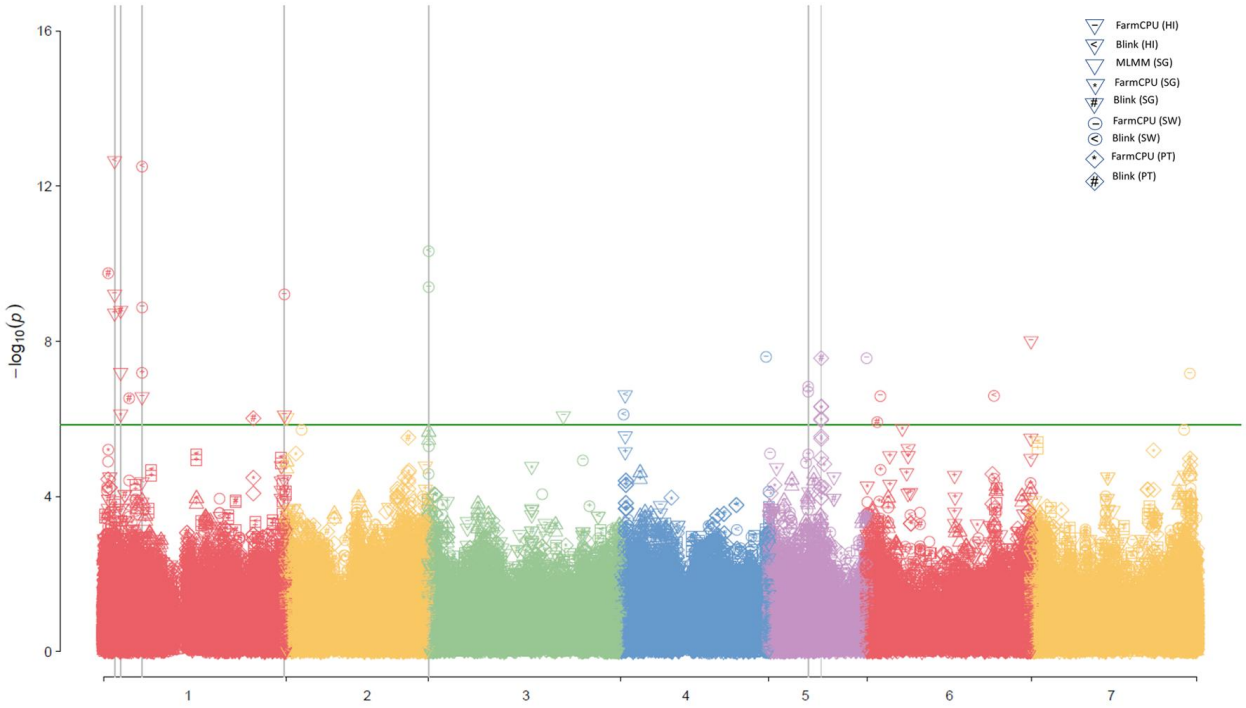

Figure 3.7. Multiple traits/methods symphysis Manhattan plot showing QTNs identified by MLMM, FarmCPU, and Blink models, associated with key traits. The solid line indicates common significant markers detected by more than two methods or traits.

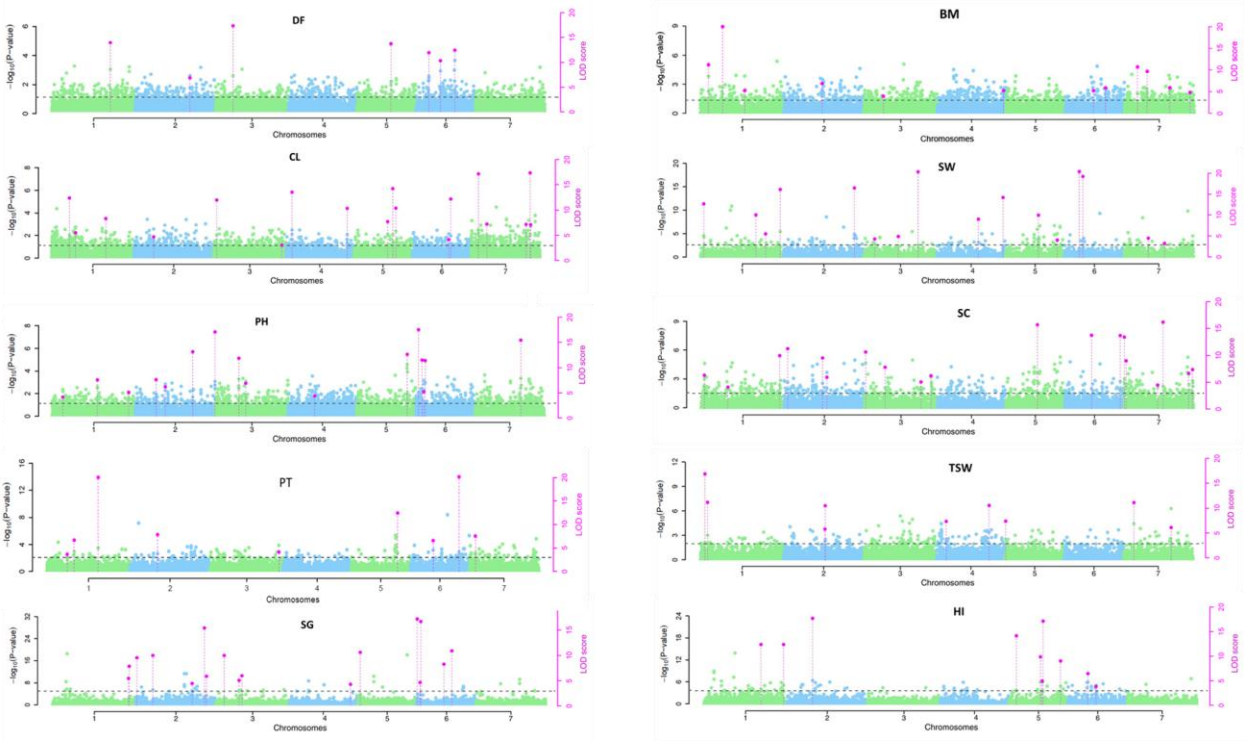

Figure 3.8. Manhattan plot displaying QTNs identified by 3VmrMLM model associated with the key traits.

DF, days to 50% flowering; CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass; SW, seed weight; SC, seed count; TSW, thousand seed weight; HI, harvest index.

Table 3.2. Significant marker-trait associations identified from genome-wide association studies for key traits in pearl millet germplasms using stress tolerance index.

QTN	Trait	Chrom	Model	Position	[-log(*p*)]
SCM036291.1_28440583	SG	1	1,2,3	28440583	8.8
SCM036292.1_1143479	SG	2	2,4	1143479	10
SCM036293.1_95645749	SG	3	4	95645749	6.21
SCM036295.1_1749587	SG	5	4	1749587	11.11
SCM036296.1_2485912	SG	6	4	2485912	17.58
SCM036291.1_305871832	SW,HI	1	2,4	305871832	15.86, 12.86
SCM036293.1_892599	SW,SC	3	2,3,4	892599	10.32,10.97
SCM036294.1_246146619	SW	4	2	246146619	7.6
SCM036295.1_66576426	SW,HI,CL	5	2,3,4	66576426	6.83, 17.9, 8.58
SCM036295.1_165561232	SW	5	2	165561232	7.56
SCM036292.1_228693239	SW	2	4	228693239	16.26
SCM036293.1_273059611	SW	3	4	273059611	22.26
SCM036294.1_132841060	SW	4	4	132841060	8.97
SCM036294.1_245164402	SW	4	4	245164402	14.01
SCM036295.1_65477411	SW	5	4	65477411	9.93
SCM036296.1_215662441	SW	6	4	215662441	6.59
SCM036296.1_278151464	HI	6	2	278151464	8.01
SCM036292.1_97954025	HI	2	4	97954025	18.47
SCM036296.1_46577044	HI	6	4	46577044	6.68
SCM036295.1_62627244	HI,SC	5	4	62627244	10.25, 16.21
SCM036293.1_60213101	DF	3	4	60213101	18.26
SCM036296.1_19351831	DF	6	4	19351831	11.87
SCM036296.1_219279663	DF,CL	6	4	219279663	12.38, 13.9
SCM036291.1_36577535	CL	1	4	36577535	14.14
SCM036291.1_209078226	CL	1	4	209078226	9.28
SCM036293.1_3177677	CL	3	4	3177677	13.64
SCM036294.1_11236950	CL	4	4	11236950	15.5
SCM036294.1_219804481	CL	4	4	219804481	11.7
SCM036295.1_82539289	CL	5	4	82539289	11.73
SCM036297.1_10753100	CL	7	4	10753100	19.8
SCM036297.1_32274307	CL	7	4	32274307	8.03
SCM036297.1_261260305	CL	7	4	261260305	20.54
SCM036297.1_262052975	CL	7	4	262052975	7.87
SCM036291.1_172351176	PH	1	4	172351176	8.41
SCM036292.1_62937009	PH	2	4	62937009	8.47

QTN	Trait	Chrom	Model	Position	[-log(*p*)]
SCM036292.1_122757008	PH	2	4	122757008	6.81
SCM036292.1_199003788	PH	2	4	199003788	14.92
SCM036292.1_240495105	PH	2	4	240495105	19.51
SCM036293.1_84543563	PH	3	4	84543563	13.46
SCM036293.1_114713950	PH	3	4	114713950	7.64
SCM036296.1_6403278	PH	6	4	6403278	13.01
SCM036296.1_13188834	PH	6	4	13188834	12.9
SCM036297.1_208051192	PH	7	4	208051192	17.61
SCM036291.1_10851054	BM	1	4	10851054	11.2
SCM036291.1_47338733	BM	1	4	47338733	20.56
SCM036297.1_66359887	BM	7	4	66359887	9.66
SCM036292.1_150093930	SC	2	4	150093930	9.79
SCM036293.1_68054255	SC	3	4	68054255	8.01
SCM036296.1_275930590	SC	6	4	275930590	14.12
SCM036297.1_205146	SC	7	4	205146	13.82
SCM036297.1_2353845	SC	7	4	2353845	9.25
SCM036297.1_138042108	SC	7	4	138042108	16.71
SCM036297.1_269911430	SC	7	4	269911430	6.84
SCM036291.1_6282493	TSW	1	4	6282493	15.64
SCM036292.1_157801935	TSW	2	4	157801935	9.9
SCM036294.1_23465293	TSW	4	4	23465293	7.03
SCM036295.1_392165	TSW	5	4	392165	7.1
SCM036297.1_16038638	TSW	7	4	16038638	10.44
SCM036295.1_93783576	PT	5	4	93783576	17.34
SCM036296.1_256574902	PT	6	4	256574902	9.94
SCM036297.1_24127033	PT	7	4	24127033	13.37
SCM036295.1_88647891	PT	5	2	88647891	6

QTN, quantitative trait nucleotide; Chrom, chromosome; DF, days to 50% flowering; CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass; SW, seed weight; SC, seed count; TSW, thousand seed weight; HI, harvest index.

Table 3.3. Summary of colocalized quantitative trait nucleotides (QTNs) identified in this study and annotated candidate genes.

Gene ID	QTNs	Chrom	Trait	Distance (kbp)	Position (QTN)	Description
PMF1G01057	SCM036291.1_36577535	1	CL	7.53	-	1-aminocyclopropane-1-carboxylate oxidase homolog 1
PMF1G04719	SCM036291.1_209078226	1	CL	0	intergenic	Aldo-keto reductase family 4 member C9
PMF1G03773	SCM036291.1_172351176	1	PH	0.13	-	Mevalonate kinase
PMF1G00790	SCM036291.1_28440583	1	SG	0.25	+	Putative disease resistance protein At4g19050
PMF1G00412	SCM036291.1_10851054	1	BM	0	intergenic	Laccase-19
PMF1G01367	SCM036291.1_47338733	1	BM	0	intergenic	Zinc finger CCCH domain-containing protein 43
PMF1G07862	SCM036291.1_305871832	1	SW, HI	0.22	-	Actin-7
PMF1G07861	SCM036291.1_305871832	1	SW; HI	1.24	+	Pentatricopeptide repeat-containing protein At4g15720
PMF1G00264	SCM036291.1_6282493	1	TSW	0	intergenic	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha
PMF2G01922	SCM036292.1_62937009	2	PH	0	intergenic	Cyclic nucleotide-gated ion channel 1
PMF2G03073	SCM036292.1_122757008	2	PH	11.06	-	Xyloglucan endotransglucosylase/hydrolase protein 2
PMF2G05725	SCM036292.1_199003788	2	PH	14.1	+	GDSL esterase/lipase At5g55050
PMF2G07960	SCM036292.1_240495105	2	PH	0	intergenic	RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment)
PMF2G00146	SCM036292.1_1143479	2	SG	0	intergenic	Serine carboxypeptidase 3

PMF2G07113	SCM036292.1_228693239	2	SW	0.77	-	Probable LRR receptor-like serine/threonine-protein kinase At2g16250
PMF2G03860	SCM036292.1_150093930	2	SC	0	intergenic	Starch synthase 1, chloroplastic/amyloplastic
PMF2G04108	SCM036292.1_157801935	2	TSW	0	intergenic	Early nodulin-93
PMF2G02560	SCM036292.1_97954025	2	HI	0	intergenic	hypothetical protein OsI_32741 [Oryza sativa Indica Group]
PMF3G02263	SCM036293.1_60213101	3	DF	6.18	-	Probable WRKY transcription factor 2
PMF3G00306	SCM036293.1_3177677	3	CL	0	intergenic	Protein IQ-DOMAIN 32
PMF3G00305	SCM036293.1_3177677	3	CL	1.32	+	Zinc finger CCCH domain-containing protein 30
PMF3G02919	SCM036293.1_84543563	3	PH	0.89	-	Aluminum-activated malate transporter 1
PMF3G03614	SCM036293.1_114713950	3	PH	0	intergenic	uncharacterized protein LOC101765475 [Setaria italica]
PMF3G03615	SCM036293.1_114713950	3	PH	1.05	-	uncharacterized protein LOC101765084 [Setaria italica]
PMF3G03613	SCM036293.1_114713950	3	PH	6.21	+	Protein EMBRYO DEFECTIVE 1674
PMF3G03185	SCM036293.1_95645749	3	SG	0	intergenic	Glycerophosphodiester phosphodiesterase GDPD1, chloroplastic
PMF3G00102	SCM036293.1_892599	3	SW, SC	0	intergenic	Phosphatidylinositol N-acetylglucosaminyltransferase subunit P
PMF3G06509	SCM036293.1_273059611	3	SW	2.16	+	LOB domain-containing protein 12
PMF3G02522	SCM036293.1_68054255	3	SC	0	intergenic	hypothetical protein SORBIDRAFT_06g018430 [Sorghum bicolor]
PMF4G00348	SCM036294.1_11236950	4	CL	0	intergenic	Disease resistance protein RPS2

PMF4G05989	SCM036294.1_219804481	4	CL	14.14	-	Salt stress-induced protein
PMF4G06577	SCM036294.1_246146619	4	SW	0	intergenic	5'-3' exoribonuclease 3
PMF4G04522	SCM036294.1_132841060	4	SW	0	intergenic	Cytochrome b561 and DOMON domain-containing protein At3g25290
PMF4G06545	SCM036294.1_245164402	4	SW	0	intergenic	Putative pentatricopeptide repeat-containing protein At4g17915
PMF4G00927	SCM036294.1_23465293	4	TSW	0	intergenic	Cytochrome P450 714C3
PMF5G03358	SCM036295.1_66576426	5	CL, SW, HI	1.2	+	Calmodulin-interacting protein 111
PMF5G03975	SCM036295.1_82539289	5	CL	0	intergenic	G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130
PMF5G04288	SCM036295.1_93783576	5	PT	2.88	-	Protein STRUBBELIG-RECEPTOR FAMILY 8
PMF5G04289	SCM036295.1_93783576	5	PT	9.72	-	Nudix hydrolase 2
PMF5G04177	SCM036295.1_88647891	5	PT	0	intergenic intergenic	4-hydroxy-tetrahydrodipicolinate synthase, chloroplastic
PMF5G00179	SCM036295.1_1749587	5	SG	6.39	+	MADS-box transcription factor 50
PMF5G00180	SCM036295.1_1749587	5	SG	4.74	-	Probable receptor-like protein kinase At5g24010
PMF5G05899	SCM036295.1_165561232	5	SW	0	intergenic	uncharacterized protein LOC101757072 [Setaria italica]
PMF5G05898	SCM036295.1_165561232	5	SW	1.35	+	BTB/POZ domain-containing protein FBL11
PMF5G03310	SCM036295.1_65477411	5	SW	0.9	+	Plastidal glycolate/glycerate translocator 1, chloroplastic
PMF5G03311	SCM036295.1_65477411	5	SW	0.78	-	Choline-phosphate cytidylyltransferase 2

PMF5G03205	SCM036295.1_62627244	5	SC, HI	8.38	-	NAC domain-containing protein 45
PMF5G00052	SCM036295.1_392165	5	TSW	3.39	-	Homeobox-leucine zipper protein HOX10
PMF6G00941	SCM036296.1_19351831	6	DF	0	intergenic	hypothetical protein SORBIDRAFT_01g012540 [Sorghum bicolor]
PMF6G00942	SCM036296.1_19351831	6	DF	6.49	-	Gamma-glutamyltranspeptidase 3
PMF6G05087	SCM036296.1_219279663	6	DF, CL	0	intergenic	uncharacterized protein LOC101757322 [Setaria italica]
PMF6G05086	SCM036296.1_219279663	6	DF, CL	2.1	+	Putative pentatricopeptide repeat-containing protein At5g40405
PMF6G00438	SCM036296.1_6403278	6	PH	2.7	-	NADP-dependent malic enzyme, chloroplastic
PMF6G00731	SCM036296.1_13188834	6	PH	0	intergenic	Protein BREAST CANCER SUSCEPTIBILITY 2 homolog A
PMF6G00730	SCM036296.1_13188834	6	PH	13.73	+	Dehydration-responsive element-binding protein 2A
PMF6G06151	SCM036296.1_256574902	6	PT	12.03	+	LOB domain-containing protein 15
PMF6G00219	SCM036296.1_2485912	6	SG	2.91	+	Basic leucine zipper 2
PMF6G00220	SCM036296.1_2485912	6	SG	1.74	-	Ubiquitin carboxyl-terminal hydrolase 18
PMF6G04983	SCM036296.1_215662441	6	SW	0	intergenic	RNA polymerase II transcriptional coactivator KELP
PMF6G07007	SCM036296.1_275930590	6	SC	0	intergenic	Protein YLS7
PMF6G07145	SCM036296.1_278151464	6	HI	0	intergenic	uncharacterized protein LOC101757203 isoform X1 [Setaria italica]

PMF6G01769	SCM036296.1_46577044	6	HI	0	intergenic	Probable splicing factor 3A subunit 1
PMF7G00663	SCM036297.1_10753100	7	CL	0	intergenic	Os03g0105300 [Oryza sativa Japonica Group]
PMF7G01569	SCM036297.1_32274307	7	CL	0	intergenic	Protein TRANSPARENT TESTA 12
PMF7G06861	SCM036297.1_261260305	7	CL	0	intergenic	Disease resistance protein RGA2
PMF7G06889	SCM036297.1_262052975	7	CL	0	intergenic	fiber protein Fb2 [Zea mays]
PMF7G06890	SCM036297.1_262052975	7	CL	1.79	-	Probable receptor-like protein kinase At5g39030
PMF7G05494	SCM036297.1_208051192	7	PH	3.71	-	Subtilisin-like protease SBT3.3
PMF7G01297	SCM036297.1_24127033	7	PT	0	intergenic	na
PMF7G01296	SCM036297.1_24127033	7	PT	2.85	+	Polygalacturonase
PMF7G02669	SCM036297.1_66359887	7	BM	1.86	-	Ankyrin repeat domain-containing protein 2
PMF7G00005	SCM036297.1_205146	7	SC	9.42	-	na
PMF7G00006	SCM036297.1_205146	7	SC	10.38	-	Zinc finger CCCH domain-containing protein 53
PMF7G00203	SCM036297.1_2353845	7	SC	9.46	+	Sodium/hydrogen exchanger 2
PMF7G04423	SCM036297.1_138042108	7	SC	0	intergenic	receptorWall-associated kinase 5
PMF7G07135	SCM036297.1_269911430	7	SC	0	intergenic	Ferredoxin--NADP reductase, embryo isozyme, chloroplastic
PMF7G00948	SCM036297.1_16038638	7	TSW	0.49	-	Eukaryotic peptide chain release factor subunit 1-3

Chrom, chromosome; DF, days to 50% flowering; CL, chlorophyll index; PH, plant height; PT, number of productive tillers; SG, staygreen; BM, biomass; SW, seed weight; SC, seed count; TSW, thousand seed weight; HI, harvest index; +, upstream; -, downstream.

3.4 Discussion

The present study attempted to utilize 187 pearl millet germplasm sources and performed GWAS utilizing four models viz. multiple loci mixed model (MLMM) (Segura et al., 2012), fixed and random model circulating probability unification (FarmCPU) (Liu et al., 2016), Bayesian information and linkage-disequilibrium iteratively nested keyway (Blink) (Huang et al., 2019), and 3 variance-component multi-locus random-SNP-effect mixed linear model (3VmrMLM) (Li et al., 2022) to explore the QTNs related with drought tolerance for ten drought-related traits viz. days to flowering (DF), chlorophyll index (CL), plant height (PH), number of productive tillers (PT), staygreen (SG), biomass (BM), seed weight (SW), seed count (SC), thousand seed weight (TSW), and harvest index (HI). The water stress in the rainfed conditions resulted in delayed flowering and lowered mean performance of the pearl millet accessions when compared to control as seen from the significant mean differences (Blum et al., 2010). The positive intra-trait correlation of the studied traits between irrigated and rainfed treatments indicates selection based on one condition could predict performance under another. Also, the high heritability of DF, PH, CL, SG, HI, and TSW, shows a strong genetic control, and offers indirect selection for biomass and yield traits (Xu et al., 2023; Singh et al., 2014; Wasaya et al., 2021). Thus, could serve as reliable traits for developing stable germplasms across diverse environments.

Genetic diversity forms the foundation for crop improvement in plant breeding. Without enough genetic variation, achieving significant improvements in crop productivity becomes challenging. Utilizing SNP markers to study germplasms provides deeper insights into genomic diversity and population structure (Serba et al., 2019). The population structure analysis at K=3 clustered the pearl millet accessions into three subgroups, however this clustering was not based

on geographical origin. Serba et al. (2019) used higher values of K, and found the clustering was based on the geographical origin of the accessions studied. We conducted PCA to assess diversity within and among subgroups, and observed that the accessions were widely scattered and did not follow any clustering or subgrouping with PC1 and PC2. The non-random association of the alleles at different loci in the genome (linkage disequilibrium), is a key to understand the historical recombination events, and can aid in mapping genes associated with complex quantitative traits. The LD decay to 0.1 at ~18kb observed for the pearl millet germplasms indicates a rapid gene flow. The weak population structure and rapid LD decay in the pearl millet accessions make it a good population for genetic dissection of complex quantitative traits using GWAS. Our results were supported by previous studies to understand the population structure and genetic diversity of global pearl millet accessions (Hu et al., 2015; Serba et al., 2019; Budak et al., 2003; Mariac et al., 2006).

MLMM, FarmCPU, and Blink are multiple loci models and considered to have high statistical power compared to the other GWAS models available. Within multiple loci model category, FarmCPU is superior to MLMM (Liu et al., 2016) and BLINK is superior to FarmCPU (Huang et al., 2019). MLMM use forward stepwise regression to identify pseudo QTNs as covariates and employs backward stepwise regression to estimate marker effects (Wang et al., 2022). FarmCPU has high computational power and is better at maintaining balance between false positives and false negatives. Blink uses linkage disequilibrium information, enhancing the detection of QTNs while reducing confounding effects. Recently, 3VmrMLM, a more efficient MLM was developed, integrating three variance components for improved computational efficiency. This model is useful for identification of main-effect QTNs and QTN-by-environment and QTN-by-QTN interactions (QEIs and QQIs) for complex and multi-omics traits. It provides

high accuracy and low false positive rate even in small mapping population (Li et al., 2022). Several GWAS studies have employed using one or a combination of MLMM, FarmCPU, Blink and 3VmrMLM (Xiong et al., 2023; Singh et al., 2023; Singh et al., 2023; Paire et al., 2024; Yang et al., 2024) to dissect QTNs and candidate genes associated with agronomic, grain quality traits and stress tolerance in different crops.

Considering the advantages as reported in above studies, we utilized MLMM, FarmCPU, Blink, and 3VmrMLM models for conducting GWAS. More specifically, a GWAS was completed using 35071 SNPs on ten drought-related traits. Finally, 62 QTNs and 72 genes were identified (Table 3) distributed across the seven chromosomes of pearl millet genome. On chromosome 1, nine co-located QTNs were detected for CL, PH, SG, BM, SW, HI, and TSW. Chromosome 2 also harbored nine co-located QTNs for PH, SG, SW, SC, TSW, and HI. Likewise, eleven co-located QTNs were detected on chromosome 3 for DF, CL, PH, SG, SW, and SC; six on chromosome 4 for CL, SW, and TSW; thirteen on chromosome 5 for CL, SW, HI, PT, SG, SC, and TSW; fourteen on chromosome 6 for DF, CL, PH, PT, SG, SW, SC, and HI; and fifteen on chromosome 7 for CL, PH, PT, BM, SC and TSW. The identified QTNs colocalized with 77 potential candidate genes. The majority of these candidate genes were involved in regulating response to drought stress such as aldo-keto reductases involved in scavenging cytotoxic aldehydes and enhancing reactive oxygen species (ROS) clearance pathways; serine carboxypeptidase 3 involved in abscisic acid pathway and so on. These candidate genes were also reported by earlier studies (Saleem et al., 2021; Sehgal et al., 2012). In general, the location of QTNs from the current study with annotated genes and previously reported QTNs and the homologues could be the confirmation of the reliability of the trait associations in this study.

3.5 Conclusions and future research

This study identified 62 QTNs associated with drought tolerance in pearl millet for ten key agronomic and physiological traits, including days to 50% flowering, chlorophyll index, plant height, number of productive tillers, staygreen, biomass, seed weight, seed count, thousand seed weight, and harvest index. These associations were detected using four GWAS models: MLMM, FarmCPU, BLINK, and 3VmrMLM, enhancing confidence and confirmation of the results. The co-localization of several QTNs with known or predicted genes highlights key genomic regions potentially involved in drought adaptation. These loci offer valuable insights into the genetic basis of drought tolerance and represent promising targets for integration into genomic selection pipelines. Their relevance to stress-responsive traits makes them a useful resource for breeding programs focused on enhancing resilience and yield stability in water-limited environments.

To advance the practical application of these findings, further validation of the identified QTNs and candidate genes is essential. This includes GO/KEGG pathway enrichment to uncover associated biological processes, validation through expression analysis like RNA-seq; functional validation such as overexpression or gene knockout. Integration of multi-omics approaches: such as transcriptomics, proteomics, and metabolomics, can offer a holistic understanding of gene function and regulatory networks. Ultimately, validated QTNs could be incorporated into marker-assisted selection (MAS) to enable trait introgression and accelerate the development of drought-tolerant pearl millet cultivars tailored for arid and semi-arid environments.

Chapter 1- References

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Supplementary Table for Chapter 2

Appendix A Table Error! No text of specified style in document..**1.** Pedigree details of 188 pearl millet germplasms.

X Selected 30 pearl millet accessions for detailed analyses from each maturity group.

Pedigree	Type	Origin	Days to Flowering	Rank summation Index	Selected Germplasms for analyses	Code for the selected germplasms
			Early Maturing (41 accessions)			
PI-197271	Landrace	India	62	99	✓	E1
PI-197373	Landrace	ICRISAT-Mali	62	117	✓	E2
PI-197427	Landrace	Togo	65	121	✓	E3
PI-197263	Landrace	Senegal	61	134	✓	E4
PI-197368	Landrace	Zimbabwe	63	146	✓	E5
PI-197022	Landrace	ICRISAT-Mali	62	149	✓	E6
PI-197191	Improved	India	60	156	✓	E7
PI-197186	Improved	India	63	157	✓	E8
PI-197325	Landrace	ICRISAT-Mali	55	157	✓	E9
PI-197073	Improved	Togo	65	162	✓	E10
PI-197115	Improved Cultivar	Zimbabwe	63	162	✓	E11
PI-197327	Landrace	ICRISAT-Mali	61	164	✓	E12
PI-197337	Landrace	ICRISAT-Mali	62	169	✓	E13
PI-197300	Landrace	Niger	64	179	✓	E14
PI-197333	Landrace	ICRISAT-Mali	62	179	✓	E15
PI-197114	Improved Cultivar	Zimbabwe	64	189	✓	E16
PI-197473	Breeding Material	Unknown	64	191	✓	E17
PI-197284	Landrace	Uganda	62	193	✓	E18
PI-197070	Improved	Togo	59	194	✓	E19
PI-197084	Improved	Togo	55	195	✓	E20
PI-197407	Landrace	Niger	63	195	✓	E21
PI-197309	Landrace	Malawi	64	197	✓	E22
PI-197043	Improved	Niger	61	200	✓	E23
PI-197428	Landrace	India	62	209	✓	E24
PI-197399	Breeding Line	Nigeria	59	211	✓	E25
PI-197302	Breeding Line	Niger	63	216	✓	E26
PI-197351	Landrace	Togo	65	221	✓	E27
PI-197086	Improved	Togo	64	225	✓	E28
PI-197288	Landrace	Niger	63	225	✓	E29
PI-197334	Breeding Line	ICRISAT-Mali	65	226	✓	E30
PI-197365	Breeding Line	Nigeria	61	230	×	–

PI-197239	Landrace	Niger	62	232	×	–
PI-197430	Landrace	Zimbabwe	63	233	×	–
PI-197310	Landrace	Malawi	64	235	×	–
PI-197352	Improved Cultivar	Ghana	64	235	×	–
PI-197335	Landrace	ICRISAT-Mali	63	243	×	–
PI-197279	Landrace	India	62	261	×	–
PI-197452	Landrace	Namibia	63	266	×	–
PI-197021	Landrace	ICRISAT-Mali	59	280	×	–
PI-197266	Landrace	India	63	321	×	–
PI-197190	Unknown	India	64	353	×	–
			Medium Maturing (91 accessions)			
PI-197326	Landrace	ICRISAT	66	212	✓	M1
PI-197285	Landrace	Nigeria	75	236	✓	M2
PI-197380	Landrace	Burkina Faso	72	239	✓	M3
PI-197340	Landrace	ICRISAT	70	258	✓	M4
PI-197436	Landrace	Togo	66	265	✓	M5
PI-197281	Breeding Line	Senegal	69	273	✓	M6
PI-197417	Landrace	Zimbabwe	66	273	✓	M7
PI-197406	Breeding Line	Mali	71	287	✓	M8
PI-197071	Improved	Togo	70	296	✓	M9
PI-197087	Improved	Togo	69	299	✓	M10
PI-197205	Landrace	Cameroon	70	315	✓	M11
PI-197355	Breeding Line	Ghana	75	316	✓	M12
PI-197274	Landrace	India	72	317	✓	M13
PI-197426	Improved Cultivar	Tanzania	69	321	✓	M14
PI-197390	Landrace	Sudan	68	326	✓	M15
PI-197272	Landrace	India	75	332	✓	M16
PI-197294	Landrace	Nigeria	70	333	✓	M17
PI-197052	Landrace	Mali	69	341	✓	M18
PI-197465	Improved Cultivar	ICRISAT	69	346	✓	M19
PI-197085	Improved	Togo	68	351	✓	M20
PI-197299	Breeding Line	Central African Republic	67	351	✓	M21
PI-197358	Landrace	Nigeria	68	355	✓	M22
PI-197201	Unknown	Niger	71	360	✓	M23
PI-197424	Landrace	Burkina Faso	74	373	✓	M24
PI-197286	Landrace	Nigeria	68	379	✓	M25
PI-197188	Unknown	India	74	380	✓	M26
PI-197376	Landrace	Sudan	74	382	✓	M27
PI-197276	Landrace	India	69	388	✓	M28
PI-197069	Improved	Togo	66	390	✓	M29
PI-197269	Landrace	India	75	394	✓	M30

PI-197412	Landrace	Tanzania	75	400	×	–
PI-197083	Improved	Togo	73	403	×	–
PI-197303	Landrace	Niger	66	405	×	–
PI-197251	Landrace	Niger	74	408	×	–
PI-197035	Landrace	Mauritania	72	414	×	–
PI-197289	Landrace	Niger	71	414	×	–
PI-197328	Landrace	ICRISAT	66	416	×	–
PI-197354	Landrace	Ghana	69	418	×	–
PI-197363	Landrace	Mali	73	419	×	–
PI-197422	Landrace	India	72	419	×	–
PI-197418	Landrace	Cameroon	70	420	×	–
PI-197245	Landrace	Niger	74	424	×	–
PI-197378	Breeding Line	Kenya	68	427	×	–
PI-197448	Landrace	Namibia	74	438	×	–
PI-197505	Breeding Material	Unknown	67	439	×	–
PI-197283	Landrace	Uganda	67	441	×	–
PI-197316	Landrace	Kenya	68	443	×	–
PI-197046	Landrace	Niger	70	448	×	–
PI-197122	Improved Cultivar	Zimbabwe	71	450	×	–
PI-197195	Improved	Unknown	67	452	×	–
PI-197050	Landrace	Mali	69	456	×	–
PI-197252	Landrace	Niger	67	462	×	–
PI-197270	Landrace	India	75	462	×	–
PI-197265	Improved Cultivar	Nigeria	72	465	×	–
PI-197051	Landrace	Mali	66	467	×	–
PI-197042	Landrace	Niger	71	472	×	–
PI-197431	Landrace	Zimbabwe	70	474	×	–
PI-197297	Breeding Line	Senegal	66	475	×	–
PI-197072	Improved	Togo	66	479	×	–
PI-197344	Landrace	Botswana	69	486	×	–
PI-197472	Breeding Material	Unknown	75	490	×	–
PI-197423	Breeding Line	Burkina Faso	66	498	×	–
PI-197469	Breeding Material	Unknown	72	498	×	–
PI-197367	Landrace	Zimbabwe	75	500	×	–
PI-197356	Landrace	Ghana	74	501	×	–
PI-197040	Landrace	Mauritania	74	504	×	–
PI-197433	Landrace	Zimbabwe	71	505	×	–
PI-197312	Landrace	Malawi	70	507	×	–
PI-197409	Landrace	Nigeria	67	507	×	–
PI-197293	Breeding Line	Nigeria	67	508	×	–

PI-197416	Landrace	Zimbabwe	66	512	×	–
PI-197401	Landrace	Nigeria	66	519	×	–
PI-197411	Improved Cultivar	Sudan	75	519	×	–
PI-197068	Improved	Togo	74	526	×	–
PI-197121	Improved Cultivar	Zimbabwe	73	529	×	–
PI-197357	Landrace	Nigeria	70	531	×	–
PI-197386	Improved Cultivar	Burkina Faso	67	541	×	–
PI-197455	Landrace	Niger	75	541	×	–
PI-197187	Improved	India	70	544	×	–
PI-197389	Landrace	United Kingdom	74	552	×	–
PI-197120	Improved Cultivar	Zimbabwe	74	560	×	–
PI-197457	Landrace	Niger	73	568	×	–
PI-197345	Landrace	Botswana	75	573	×	–
PI-197077	Improved	Togo	74	586	×	–
PI-197273	Landrace	India	72	588	×	–
PI-197296	Landrace	Senegal	69	591	×	–
PI-197321	Landrace	India	68	621	×	–
PI-197287	Landrace	Niger	71	634	×	–
PI-197148	Improved Cultivar	Zimbabwe	73	655	×	–
PI-197242	Landrace	Cameroon	73	666	×	–
PI-197439	Landrace	Pakistan	66	719	×	–
			Late Maturing (56 accessions)			
PI-197196	Improved	Niger	81	127	✓	L1
PI-197359	Landrace	Nigeria	84	132	✓	L2
PI-197116	Improved Cultivar	Zimbabwe	89	133	✓	L3
PI-197414	Breeding Line	Burkina Faso	77	161	✓	L4
PI-197291	Landrace	Niger	91	166	✓	L5
PI-197041	Landrace	Niger	81	170	✓	L6
PI-197342	Landrace	Nigeria	78	176	✓	L7
PI-197189	Improved	India	86	182	✓	L8
PI-197432	Landrace	Zimbabwe	82	188	✓	L9
PI-197470	Breeding Material	Unknown	76	191	✓	L10
PI-197467	Landrace	Niger	78	205	✓	L11
PI-197277	Landrace	India	83	208	✓	L12
PI-197405	Improved Cultivar	Malawi	80	213	✓	L13
PI-197082	Improved	Togo	81	223	✓	L14
PI-197314	Landrace	Kenya	82	224	✓	L15

PI-197503	Breeding Material	Unknown	80	232	✓	L16
PI-197282	Landrace	Uganda	78	237	✓	L17
PI-197408	Landrace	Niger	78	243	✓	L18
PI-197278	Landrace	India	78	247	✓	L19
PI-197343	Landrace	Botswana	83	260	✓	L20
PI-197255	Landrace	Niger	83	262	✓	L21
PI-197199	Unknown	Niger	78	264	✓	L22
PI-197402	Landrace	South Africa	88	269	✓	L23
PI-197437	Landrace	Togo	78	271	✓	L24
PI-197009	Landrace	Cameroon	82	272	✓	L25
PI-197347	Landrace	Togo	83	272	✓	L26
PI-197383	Landrace	Burkina Faso	77	272	✓	L27
PI-197374	Improved Cultivar	Mali	79	274	✓	L28
PI-197400	Landrace	Nigeria	77	275	✓	L29
PI-197330	Landrace	Sudan	77	278	✓	L30
PI-197463	Breeding Line	ICRISAT	84	278	×	–
PI-197471	Breeding Material	Unknown	82	279	×	–
PI-197006	Landrace	Cameroon	77	287	×	–
PI-197341	Landrace	ICRISAT	80	288	×	–
PI-197038	Landrace	Mauritania	76	290	×	–
PI-197020	Landrace	Cameroon	77	293	×	–
PI-197241	Landrace	Niger	80	294	×	–
PI-197464	Breeding Line	ICRISAT	79	295	×	–
PI-197275	Landrace	India	82	297	×	–
PI-197301	Landrace	Niger	84	297	×	–
PI-197353	Landrace	Ghana	88	297	×	–
PI-197339	Breeding Line	ICRISAT	82	298	×	–
PI-197462	Breeding Line	ICRISAT	79	301	×	–
PI-197194	Improved	Unknown	78	302	×	–
PI-197243	Landrace	Niger	77	302	×	–
PI-197198	Improved	Niger	77	308	×	–
PI-197264	Landrace	India	78	308	×	–
PI-197425	Landrace	Tanzania	87	311	×	–
PI-197391	Landrace	South Africa	80	321	×	–
PI-197315	Landrace	Kenya	76	330	×	–
PI-197295	Breeding Line	Senegal	79	331	×	–
PI-197029	Landrace	Mali	79	358	×	–
PI-197370	Landrace	Senegal	76	358	×	–
PI-197461	Landrace	Niger	77	363	×	–
PI-197410	Landrace	Sudan	77	365	×	–
PI-197298	Landrace	Central African Republic	88	392	×	–