

RESEARCH ARTICLE

Changes in Morpho-physiological Attributes and Gene Expression in Pearl Millet Parental Lines During Seedling Stage Drought Stress

A Govardhani¹, SNCVL Pushpavalli^{1*}, S Vanisri², T Nepolean³ and A Geetha⁴

Abstract

Drought, being a devastating abiotic factor, affects the productivity of many crops. Being a climate-resilient crop, pearl millet's adaptability towards arid regions attracts us to examine morpho-physiological and molecular mechanisms during seedling drought stress. The experimental material consisted of 41 genotypes subjected to drought stress at the seedling stage. Significant differences for morpho-physiological traits such as SL, R/S, RWC, RL (except treatments), and WRC (except-G×T interaction) were observed. Two tolerant and two susceptible genotypes were selected based on RWC under drought conditions. A set of seven genes (ST, NAC, 26S, TD, WD-40, GAUT and ASR) of drought-related pathways were selected and their expression patterns were analyzed in these genotypes. The expression of drought-related genes was in confirmation with the morpho-physiological traits. Our study suggests that drought screening at the early seedling stage for morpho-physiological traits will aid the breeders in the development of drought-tolerant parental lines and hybrids.

Keywords: Relative water content, Root length, Root/shoot ratio, Shoot length, Water retention capacity.

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Received: 23/07/2023 **Revised:** 18/09/2024

Accepted: 20/09/2024

How to cite this article: Govardhani A, SNCVL Pushpavalli, S Vanisri, T Nepolean and A Geetha. (2025). Changes in Morpho-physiological Attributes and Gene Expression in Pearl Millet Parental Lines During Seedling Stage Drought Stress. *Indian J. Plant Genet. Resour.* 38(1), 53-63. DOI: 10.61949/0976-1926.2025.v38i01.06

Introduction

Plants, being sessile, are constantly exposed to different environmental stresses. Among all the abiotic factors, drought is the most important devastating stress that influences plant growth and development. The effect of drought on the plants depends upon the changes in climate conditions and level of water scarcity (Bohnert *et al.*, 1995; Kaya *et al.*, 2006). The impact of drought on crop development depends on the severity and stage of the plant development. Sensitive stages of the plant are more prone to face damage (Bayoumi *et al.*, 2008). Apart from facing the effects of drought, plants also develop numerous acclimatization and adaptive strategies to overcome drought stress, which range from simple morphophysiological traits that serve as markers for stress tolerance to major upheavals in gene expression in which a large number of genes and transcription factors are involved (Bhargava and Sawant, 2013). Transcriptional factors modulate gene expression by binding through cis-regulatory regions in the promoter regions of stress-related genes. Drought-induced gene expression is governed by ABA-dependent and ABA-independent pathways and there is a crosstalk between these pathways in signal transduction by activating a number of TFs and genes (Yoshida *et al.*, 2014; Joshi *et al.*, 2016). The activation of the signal transduction pathways along with biochemical, morphological, or

physiological changes in plants are responsible for tolerance of the genotypes under water stress conditions. So, a better understanding of the interrelation between molecular mechanisms and physiological traits is necessary.

In arid and semi-arid regions of the world mainly Asian and African countries, crop productivity is being limited by water scarcity (Kholová *et al.*, 2010; Yadav *et al.*, 2017). In these regions, climate-resilient crops like pearl millet (*Pennisetum glaucum*) are being cultivated. It is a small-grained panicoid millet with a chromosome complement of $2n = 14$ with genomic size of $\sim 1.7\text{Gb}$. It is a diploid and highly cross-pollinated crop (Varshney *et al.*, 2017; Jaiswal *et al.*, 2018) and the sixth most important crop after rice, wheat, maize, barley and sorghum (Vadez *et al.*, 2012). It plays a vital role in food security in Sub-Saharan countries as it is a nutrient-rich crop (Belton and Taylor, 2004; Varshney *et al.*, 2017; Debieu *et al.*, 2018). Though pearl millet is known for its tolerance to drought, still its sensitive stages of the crop, like the seedling and terminal growth stages, are still being affected by water-deficient conditions (Yadav *et al.*, 2011; Shivhare and Lata, 2017). Therefore, screening for drought tolerance can be done at the sensitive stages of the plants to obtain better-performing parental lines and hybrids. Studies in wheat indicated a higher relationship between the seedling traits and stem-related traits of adult plants (Dodig *et al.*, 2015) and a positive correlation between the seedling root length and grain yield (Abdel-Ghani *et al.*, 2013). Hence, screening of genotypes at the seedling stage by using respective traits was considered to be a cost-effective approach and rapid screening of genotypes can be achieved by the breeders (Badr *et al.*, 2020). Keeping the above in view, the present study was undertaken for assessing the pearl millet genotypes for drought tolerance at the early seedling stage and to analyze the inter-relationship between the physiological traits and molecular mechanisms.

Materials and Methods

The 41 pearl millet genotypes (B and R lines) obtained from the ICRIAT, Hyderabad, were screened for their drought tolerance in 2019-2020 on the basis of morpho-physiological traits.

Experimental Design

The seeds were surface sterilized and sown in plastic glasses filled with autoclaved red soil and allowed to germinate until the third leaf emergence. The experiment was carried out in two treatments, i.e., control and water-stressed (drought) conditions and three replications. Water was withheld for the plants grown under drought conditions (at the 11th DOS) while control was watered daily till the drought symptoms (wilting and slight yellowing of leaves) were noticed (15 DOS). The plants were removed for recording physiological traits data and total RNA isolation for gene expression studies.

Physiological Traits

Root length (RL) and shoot length (SL) data were noted in centimeters. The root/shoot length (R/S) ratio was obtained by dividing root length by shoot length. Relative water content (RWC) of seedlings subjected to drought and control was measured as per the procedure of Barrs and Weatherley (1962). The RWC of the samples was calculated as per the following formula:

$$\text{RWC} = (\text{Fresh weight} - \text{Dry weight}) / (\text{Turgid weight} - \text{Dry weight}) \times 100$$

The chlorophyll stability index (CSI) was estimated using the method of Koleyoreas (1958) in both control and drought samples continuously for 3 days after withholding water by SPAD 502 chlorophyll meter.

$$\text{CSI} = (\text{Total chlorophyll content of stressed plant}) / (\text{Total chlorophyll content of plant}) \times 100$$

Water retention capacity (WRC) was calculated as per the procedure of Sangakkara *et al.* (1996) in control and drought samples by the formula

$$\text{WRC} = \text{Turgid weight} / \text{Dry weight}.$$

Statistical Analysis

Two factorial completely randomized designs were performed to find out the significant differences among the genotypes, treatments, genotypes treatments interaction of the traits using Infostat software. Correlation analysis was performed using Pearson's correlation method to find out the strength of the relationship among all the traits (SPSS_v20 software).

Gene Expression Studies

The total RNA was isolated using a NucleoSpin RNA Plant kit (Macherey-Nagel). cDNA synthesis was carried out using a cDNA synthesis Kit (Genetix, USA). The transcriptional factors and genes that cover ABA-dependent and ABA-independent MAPK pathways sequences were downloaded from the pearl millet drought transcriptome database (PMDTDb) developed by Jaiswal *et al.*, 2018 and primers were designed using Primer 3 software (Table 1). β -actin served as internal control. The PCR products were detected by running on a 3% agarose gel. Gene expression was calculated by capturing the fluorescence intensity of agarose gel by using Image Lab Software. The relative gene expression (fold change) is calculated as a ratio of the mean value of normalized gene expression under drought to that of control.

Result and Discussion

To develop superior hybrids with drought tolerance, it is necessary to screen the parental lines and utilize them in

Table 1: Details of genes and primers analyzed by semi-quantitative RT-PCR

Gene	Primer (5'-3')	Annealing temperature (°C)
Serine-threonine kinase receptor-associated protein (ST)	FP: GGATGTGAGAAGCTGGAAAAA RP: ACCGTGATGTCCTTTGTTAC	52
Stress-induced transcription factor nac1 (NAC)	FP: AAGAAAAGGGAAGGAGAGG RP: AGGGGTCGAACTTGTAGAG	53
26s proteasome regulatory particle triple-a atpase subunit4 (26S)	FP: AGATTGAGATTCCACTACCC RP: AGCTCCCTGACGATACAC	54
Tonoplastdicarboxylate transporter-like (TD)	FP: GGGAGTCTAATTGTGCTATG RP: GGATAGGATGTCAGTCAGG	53
Wd-40 repeat family expressed (WD-40)	FP: GTTCCAGAGAGCAAGAGA RP: GTTGGTGATGGAGTAGTTG	50
Galacturonosyltransferase (GAUT)	FP: GCCTGTAGAGAAGAGATGGA RP: ATGAGAAGGCGGAATGTAG	52
Abscisic Stress-Ripening Protein 2-like (ASR)	FP: AGAAGAAGCAGGACCACAA RP: AAACACACACATGACACACC	50
β -actin	FP: GTTCGTGACATCAAGGAGAA RP: ACCATCAGGCAATTCTGATG	51

breeding programs. Differential responses of genotypes to drought at the seedling stage could be utilized and exploited by the plant breeders to identify drought-tolerant genotypes before performing comprehensive field studies. Drought screening at the seedling stage is not only a cost-effective and time-saving approach (Badr *et al.*, 2020) but has also shown a positive relationship with the stem-related traits and grain yield in wheat and maize (Abdel-Ghani *et al.*, 2013; Dodig *et al.*, 2015). On the basis of these studies, we have carried out morpho-physiological screening of pearl millet parental lines for seedling drought tolerance, followed by gene expression studies.

Genotypic Variation for Morpho-physiological traits

Physiological traits such as RL, SL, R/S, RWC, WRC and CSI were investigated in 41 genotypes after subjecting to early seedling stage drought stress. The effect of drought stress on treatments, genotypes, and their interactions was highly significant for SL, R/S ratio and RWC ($p < 0.001$) (Table 2). Our results are in accordance with the reports in wheat wherein PEG-induced drought showed significant differences with respect to genotypes, treatments, and genotype \times treatment interaction (Belay *et al.*, 2021). Significant differences were not observed for RL with treatments and genotype \times treatment interactions for WRC.

Effect of Drought Stress on Morpho-physiological Traits

Under water-deficient conditions, roots are the first to respond by increasing their length (Kano-Nataka *et al.*, 2011). In normal conditions, root length (RL) varied from 17.22 to 6.41 cm, whereas under drought conditions, it ranged from 16.35 to 6.91 cm. Notably, the pearl millet B line ICMX 1410698-SB-11-1-1-2 exhibited the highest increase in root length (83.7%) under seedling drought conditions. Seedlings

or adult plants with longer roots are better at sensing and responding to water stress compared to those with shallower root systems, thus indicating greater tolerance (Asch *et al.*, 2005; Khodarahmpour, 2011). Root length increases have been utilized to screen drought-tolerant lines in crops like chickpeas (Serraj *et al.*, 2004) and rice (Madabula *et al.*, 2016). However, while many genotypes showed increased root length in response to drought, some also experienced a reduction compared to control conditions. For instance, a 39.8% decrease in root length was observed in the TT-1 pearl millet genotype under PEG-induced drought stress (Shivhare and Lata, 2019), and similar reductions have been reported in maize (Avramova *et al.*, 2016) and citrus (Zaher-Ara *et al.*, 2016). Increased root growth often reflects a genotype's ability to withstand water stress. Under stress, plants activate various metabolic pathways to survive, with rapid root growth being a key adaptive response in more tolerant genotypes, while sensitive ones may not exhibit this trait.

Shoot length is a crucial trait affected by water-deficient conditions and is an important criterion for selecting drought-tolerant genotypes (Schubert *et al.*, 1995; Ahmed *et al.*, 2019). In our study, shoot length (SL) ranged from 19.35 to 5.91 cm under control conditions and from 17.35 to 6.96 cm under drought stress. While some genotypes, such as ICMB 100173 and ICMX 1410509-SB-7-1-1-1, exhibited a decrease in SL, others, like ICMX 1410506-SB-1-4-1-B and ICMX 1410698-SB-11-1-1-2, showed an increase. Notably, the genotype ICMP 100230 displayed the most significant relative decrease in SL, indicating potential drought tolerance. Previous research has also observed reduced shoot length under drought stress in crops such as rice, pea, and wheat (Junfeng *et al.*, 2004; Asch *et al.*, 2005; Okçu *et al.*, 2005; Almaghrabi, 2012). This reduction in shoot length is often due to decreased cell

Table 2: Analysis of variance of morpho-physiological traits

Source of variation	Df	Mean sum of square				
		RL	SL	R/S	RWC	WRC
Genotypes	40	27.61**	22.65**	0.30**	418.12**	20.58**
Treatments	1	6.60	35.90**	0.05**	3957.63**	319.77**
Genotypes x Treatments	40	7.31**	7.24**	0.06**	78.11**	3.68
CD		1.07	1.05	0.05	4.48	1.85
CV (%)		7.89	7.57	4.78	5.54	12.52

Note: * significant at 5% ** significant at 1%

division and cell enlargement, as drought stress negatively impacts growth by limiting cell division and elongation (Kramer, 1983; Shao *et al.*, 2008; Ahmed *et al.*, 2019).

In response to drought, an increase in the root-to-shoot (R/S) ratio has been observed in crops such as mungbean and rice, and this ratio is used as a criterion for drought screening (Wade *et al.*, 2000; Haider *et al.*, 2012; Aslam *et al.*, 2013). Under control conditions, the R/S values ranged from 1.55 to 0.51. When exposed to drought, these values shifted to a range of 1.69 to 0.50. Genotypes exhibited varied responses regarding the R/S ratio; some showed no significant change, while others experienced a decrease (e.g., ICMX 1410506-SB-1-4-1-B, ICMR 100221). Conversely, genotypes such as ICMB 1502 and ICMB 100173, which had higher R/S ratios, indicated better drought tolerance. An increase in the R/S ratio under drought conditions has also been reported in other crops like wheat (Liu *et al.*, 2004) and rice (Cui *et al.*, 2008). This increase is attributed to the translocation of carbohydrates from shoot tissues to the roots, promoting root growth and enhancing drought resilience.

Water retention capacity (WRC) has been a key parameter for assessing drought-tolerant genotypes in wheat (Sandhu and Laude, 1958; Salim *et al.*, 1969; Kirkham *et al.*, 1980) and is thus used in this study to identify drought-tolerant genotypes in pearl millet. Under control conditions, WRC ranged from 22.38 to 10.59, whereas under drought conditions, it ranged from 16.16 to 7.55. The decrease in water retention capacity compared to control conditions indicates damage to the cell structure. The genotype ICMX 1410848-B-9-2-2 exhibited the greatest relative decrease in WRC (41.4%), suggesting substantial cell damage and greater drought susceptibility. These findings align with previous reports in soybeans, where cultivars showed significant reductions in relative water content (RWC), exudation rate, and WRC during drought (Chowdhury *et al.*, 2017).

The ability of plants to endure drought also depends on chlorophyll availability, which is crucial for dry matter production and increasing photosynthetic rates. The chlorophyll content is described by the chlorophyll stress index (CSI), which is a screening method used to identify tolerant genotypes (Aparna *et al.*, 2017). Previous studies

have utilized CSI for drought screening in chickpeas and wheat (Gupta *et al.*, 2000; Ulemale *et al.*, 2013). In this investigation, no significant differences were observed among genotypes, treatments, or genotype × treatment interactions.

RWC is widely recognized as one of the most straightforward and crucial parameters for screening drought tolerance in agriculture. It assesses tissue sensitivity and dehydration tolerance of cells. Research has shown that drought-resistant genotypes retain more water in their leaves compared to sensitive ones, as observed in wheat (Rampino *et al.*, 2006; Ahmed *et al.*, 2019) and sugarcane (Silva *et al.*, 2007). Our study found that drought stress significantly reduced RWC; in control samples, RWC ranged from 88.3 to 49.1%, while in drought conditions, it ranged from 82.0 to 47.5%. RWC is a critical criterion for selecting genotypes for gene expression studies. Previous research has also highlighted RWC as a key selection criterion for drought-tolerant genotypes in wheat (Schonfeld *et al.*, 1988), snap bean (Omae *et al.*, 2005), and soybean (Chowdhury *et al.*, 2017). Among the various parameters studied, RWC is considered one of the best criteria for selecting drought-tolerant genotypes in crop plants (Rad *et al.*, 2013; Maheswari *et al.*, 2016).

For gene expression studies, we selected two of the best-performing genotypes (ICMX 1410852-B-23-2-2 and ICMP 100230) and two poor-performing genotypes (ICMX 1410848-B-9-2-2 and ICMX 1410506-SB-1-4-1-B) based on their RWC. Gene expression was analyzed using semi-quantitative RT-PCR with the following markers: ST (Hu *et al.*, 2011), NAC (McGrann, 2015), 26S (Kurepa *et al.*, 2009), TD (Yıldırım *et al.*, 2018), WD-40 (Maksup *et al.*, 2014), GULT (Cheng *et al.*, 2018), and ASR (Feng *et al.*, 2016). The physiological response of genotypes (B and R lines) to control and drought stress is presented in Table 3 and Figure 1.

Correlation Among Different Physiological Traits

To identify best performing genotypes under drought stress correlation studies have been carried out. In the present investigation, RL exhibited a significant positive correlation with R/S ratio (control [0.40**] & drought [0.47**]) and

Table 3: Morpho-physiological changes of pearl millet genotypes during seedling drought stress

Genotypes	RL (cm)		SL (cm)		R/S		RWC (%)		WRC		CSI		
	C	D	C	D	C	D	C	D	C	D	1 st day	2 nd day	3 rd Day
ICMB 100173 (B)	9.62	10.91	14.72	12.78	0.82	0.92	69.7	64.95	15.85	15.42	94.78	96.87	128.56
ICMX 1410719-SB-1- 2-1-2(B)	8.12	8.75	12.82	12.12	0.65	0.66	65.48	60.65	22.38	14.22	99.80	106.85	96.08
ICMX 1410509-SB-7- 1-1-1(B)	9.64	10.82	19.35	14.64	0.79	0.79	71.29	68.36	14.86	12.25	76.44	83.13	116.62
ICMX 1410722-SB-5- 7-2-3(B)	10.14	8.24	10.76	12.13	0.74	0.95	70.86	68.29	18.14	16.16	124.64	168.41	148.23
ICMX 1410506-SB-1- 4-1-B(B)	6.41	6.91	11.83	13.71	0.55	0.50	49.07	47.45	15.48	12.24	45.73	108.71	99.59
ICMX 1410488- SB-2-1-5-1 (B)	14.33	13.61	11.34	12.39	1.15	1.12	69.60	57.87	12.36	10.79	100.59	97.94	98.48
ICMX 1410495-SB-8- 2-1-B(B)	9.44	8.22	16.54	15.06	0.54	0.56	66.08	65.00	14.31	13.57	101.63	109.94	125.25
ICMX 1410843- B-8-1-2 (B)	8.70	9.28	15.23	13.41	0.51	0.71	78.64	76.13	15.67	13.21	64.05	86.17	130.61
843B(B)	9.92	11.74	10.95	12.26	1.09	0.99	84.03	74.53	13.42	11.5	104.88	109.74	100.56
ICMX 1410723-SB-3- 3-1-B(B)	10.41	12.50	11.26	11.92	0.99	1.18	71.99	57.03	12.50	10.01	85.67	107.35	114.02
ICMX 1410698-SB-11- 1-1-2(B)	7.35	13.50	10.12	12.00	0.65	1.09	69.63	54.61	14.85	14.11	104.58	97.09	92.60
ICMB 101724(B)	10.32	13.11	14.12	12.41	1.17	1.17	66.48	56.48	11.29	10.85	104.60	84.99	102.71
ICMX 1410852-B-1- 5-3(B)	13.31	13.76	14.35	10.33	1.01	1.29	80.19	78.71	14.12	13.06	85.31	82.81	95.81
ICMX 1410848-B-9- 2-2(B)	15.83	14.08	13.20	11.05	1.25	1.35	59.72	49.41	12.85	7.55	94.10	97.44	105.21
ICMX 1410849-B-11- 1-1(B)	12.24	11.91	13.16	11.33	1.12	0.99	66.28	53.02	14.69	11.11	96.72	90.67	100.97
ICMB 02333(B)	9.46	12.60	13.13	11.82	1.25	1.22	73.37	60.62	15.98	12.61	92.79	97.11	100.13
ICMB 04222(B)	15.61	16.35	18.42	17.35	1.00	0.94	73.61	52.25	12.83	10.33	90.41	88.18	93.10
ICMB 1502(B)	7.03	10.93	12.45	10.60	0.91	1.22	73.64	66.23	11.68	9.00	103.13	82.60	93.59
ICMB 101572(B)	11.93	9.32	11.12	12.50	1.16	1.08	61.83	61.10	13.56	12.98	109.90	104.41	99.13
ICMX 1410852-B-23- 1-1(B)	11.2	12.28	14.20	12.31	0.76	0.89	82.96	67.06	13.83	11.56	99.67	97.53	97.75
ICMX 1410852-B-23- 2-2(B)	15.73	15.06	14.14	13.09	1.11	1.07	85.47	81.95	11.50	9.25	93.31	85.05	81.74
ICMB 04999 (R)	12.60	11.40	10.02	11.04	1.39	1.21	79.63	61.67	15.93	12.88	81.69	84.83	89.08
ICMR 100221(R)	15.76	12.00	13.52	14.50	1.05	0.67	61.47	60.59	13.76	11.75	106.86	97.57	97.09
ICMR 100218(R)	13.63	11.85	11.85	12.73	1.14	0.83	72.93	65.97	15.29	13.31	113.71	116.07	104.62
ICMX 1510531-SB-7- 1-4(R)	10.75	13.85	14.64	12.23	1.07	1.13	85.29	63.21	13.50	10.03	97.46	90.87	93.43

ICMX 1411014-B-7- 3-1(R)	13.10	12.89	10.25	8.98	1.33	1.57	70.44	66.8	11.53	8.01	102.60	123.30	108.38
ICMX 1510541-SB-3- 4-2(R)	11.43	13.62	10.80	12.20	1.13	1.17	84.58	76.83	12.75	12.49	99.55	117.35	104.64
ICMX 1510531-SB-7- 1-2(R)	13.82	11.52	12.72	6.96	0.92	0.83	77.07	73.03	13.42	12.61	104.55	107.96	91.13
ICMX 1510541-SB-3- 4-5(R)	14.05	14.93	13.7	13.49	1.04	1.06	85.34	80.73	17.59	11.83	90.88	91.20	91.67
ICMR 100029(R)	13.60	13.73	12.55	9.74	1.08	1.27	76.90	74.13	15.20	11.16	115.91	98.64	91.91
ICMR 100591(R)	11.30	12.99	9.40	11.65	1.14	1.25	88.30	79.32	13.32	10.97	108.33	122.03	112.75
ICMP 100230(R)	8.73	9.11	5.91	9.84	1.01	1.39	81.34	80.84	12.57	11.54	102.46	111.07	98.45
ICMX 1410857-B-17- 3-1-2(R)	11.75	10.76	15.51	9.90	0.96	1.06	81.56	78.25	11.15	10.91	133.85	102.15	101.48
ICMX 1411007-B-16- 2-3(R)	13.66	15.52	13.76	12.62	1.31	1.15	86.85	63.97	13.46	11.76	109.26	102.05	91.45
ICMX 1411016-B-1- 2-2(R)	9.82	14.07	9.95	12.13	1.03	1.00	69.55	68.3	14.46	11.33	83.68	107.81	118.39
ICMX 1411004-B-37- 2-1(R)	12.51	14.10	10.45	8.16	1.29	1.69	77.20	75.63	13.05	9.12	98.47	91.26	100.53
ICMX 1510532-SB-2- 7-7(R)	15.72	12.62	11.92	14.1	1.55	1.08	84.43	67.29	10.59	9.93	100.92	94.88	89.16
ICMX 1510552-SB-9- 6-2(R)	14.10	11.73	12.82	10.64	1.13	1.16	75.38	74.58	11.16	8.81	81.99	101.86	96.12
ICMX 1410826-B-1- 3-2(R)	17.22	13.73	12.84	11.45	1.33	0.98	83.72	80.65	14.00	12.59	98.08	104.32	95.44
ICMR 100068(R)	13.06	11.32	9.94	8.63	1.32	1.22	75.73	72.36	11.74	10.98	105.10	108.64	109.25
ICMX 1410827-B-1- 3-3(R)	10.95	12.12	11.48	9.73	0.97	1.24	84.57	57.43	14.64	12.30	159.75	123.59	118.76
MEAN	11.81	12.14	12.62	11.85	1.03	1.06	74.93	66.91	13.93	11.61	99.22	101.96	103.04
RANGE	10.81	9.44	13.44	10.39	1.04	1.19	39.23	34.5	11.79	8.61	114.02	85.81	66.49

Note: (B) – B line, (R)- R line, C-Control, D-Drought, RL- Root length, SL- Shoot length, R/S- Root/Shoot ratio, RWC- Relative water content, WRC- Water retention capacity, CSI-Chlorophyll stability index

RWC (0.29** control conditions) but a significant negative correlation with WRC (control [-0.29**] & drought [-0.33**]). No significant association was observed between root length (RL) and shoot length (SL). A similar lack of association was reported in wheat (Ahmed *et al.*, 2019). SL was negatively correlated with R/S ratio and RWC under both control and drought stress. A similar result of a negative correlation between the RL and R/S was observed in rice (Haider *et al.*, 2012). A significant positive correlation was observed between the R/S ratio and RWC but a negative correlation with WRC under both control and drought stress. There was

no significant association between the RL and SL in both control and drought stress (Table 4).

Expression of Drought Responsive Genes

To elucidate molecular changes in response to drought stress, gene expression studies were carried out in selected (two susceptible and two tolerant) pearl millet parental lines at the early seedling stage by semi-quantitative PCR analysis. Seven drought-responsive genes were shortlisted for the expression studies such that they include MAPK pathway, ABA-dependent, and independent pathways, and also those involved in secondary metabolism, programmed cell

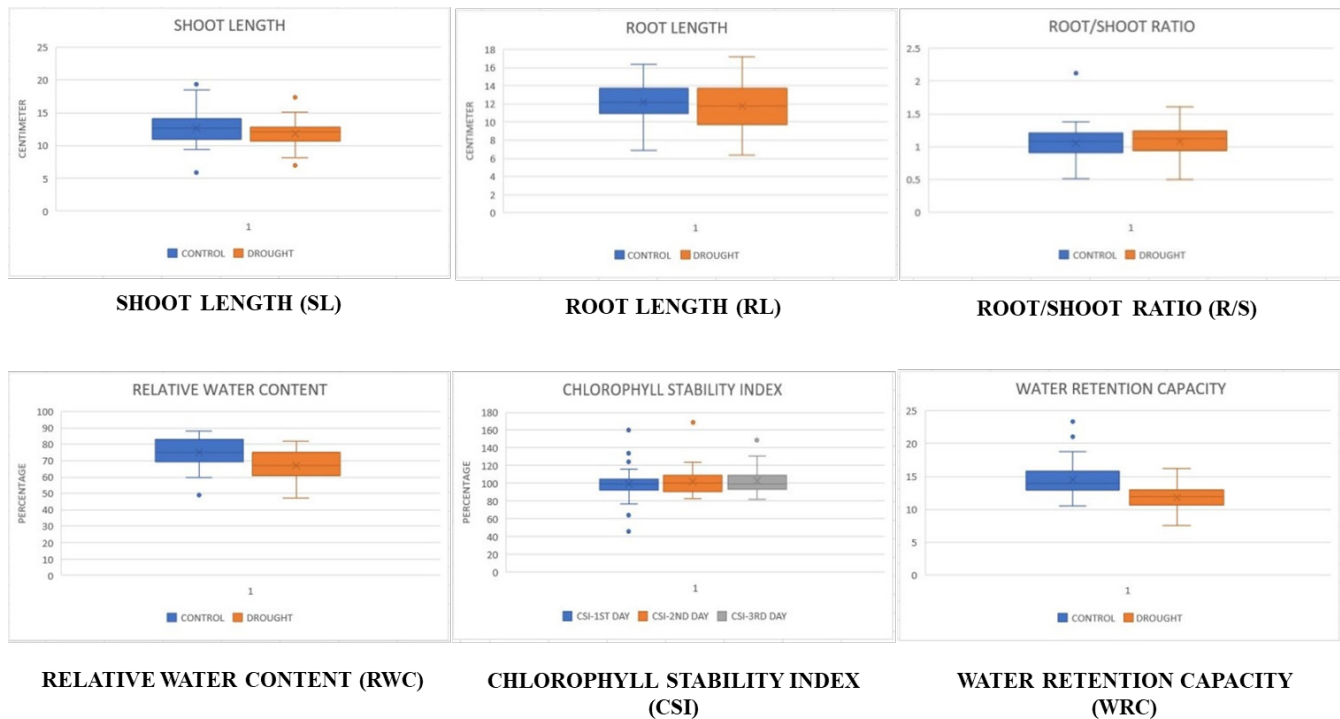


Figure 1: Box and whisker charts showing mean values of RL, SL, R/S, RWC, CSI and WRC of pearl millet genotypes under drought stress

Table 4: Pearson's correlation coefficients matrix of pearl millet genotypes under drought stress

		Root length	Shoot length	R/S	RWC	WRC
Shoot length	C	0.134				
	D	0.037				
R/S	C	0.40**	-0.414**			
	D	0.479**	-0.581**			
RWC	C	0.299**	-0.164	0.401**		
	D	0.103	-0.254**	0.303**		
WRC	C	-0.293**	0.075	-0.295**	-0.186*	
	D	-0.33**	0.16	-0.408**	-0.017	

Note: * significant at 5% ** significant at 1%

death, ion transport and cell growth. ST showed increased expression in all genotypes under drought stress. ST and NAC genes displayed 3.5 and 5.9 fold increases under drought stress in tolerant genotype ICMP 100230, while it was only 2.03 and 3.62 fold in ICMP 1410852-B-23-2-2. The physiologically susceptible genotypes, ICMP 1410848-B-9-2-2 ICMP 1410506-SB-1-4-B have shown expression levels of 1.41-fold for ST, 2.28-fold for NAC and 1.04-fold for ST, 1.02 fold for NAC gene. ST is a positive regulator of plant response to drought and is involved in abiotic stress signal transduction in plants (Hrabak *et al.*, 2003). The expression

of ST was higher in the tolerant genotypes ICMP 100230 attributing to a low relative decrease in RWC and higher RL. Tolerant genotypes showed higher expression compared to the susceptible ones, representing activation of SNF1-related protein kinase 2 pathways (ABA-dependent pathway) in response to drought. Our observations were in accordance with previous reports on maize and soybean (Hu *et al.*, 2011; Sun *et al.*, 2013). NAC transcriptional factors, which are involved in the ABA-independent pathway, are involved in the regulation of many developmental processes, including secondary cell wall biosynthesis, senescence, and biotic and abiotic stress tolerance (Puranik *et al.*, 2012). NAC expression ranged from 3.6 to 5.9 folds in the tolerant genotypes, representing activation of ABA independent pathway and drought stress-responsive genes. Earlier studies also indicated similar results in rice and wheat (Hu *et al.*, 2006; Saad *et al.*, 2013).

Galacturonosyltransferase is a component of the cell wall and is involved in pectin synthesis, flower and fruit pigmentation, hormone homeostasis, and defense responses (Atmodjo *et al.*, 2011; Fangel *et al.*, 2011). Genotype ICMP 1410852-B-23-2-2 expressed a higher fold change of 2.68, followed by ICMP 100230, expressing fold changes of 2.32. Higher expression in tolerant genotypes may be attributed to negligible changes in structure and cell wall composition. This is supported by Crombie *et al.* (2003), and Parre and Geitmann (2005) and our observations are

consistence with those reported in rice, eucalyptus, and tea (Liu *et al.*, 2016; Zheng *et al.*, 2016; Cheng *et al.*, 2018).

ASR is crucial for regulating plant responses to ABA and various stresses, including osmotic, salinity, and drought (Çakir *et al.*, 2003; Hu *et al.*, 2013). Genotype ICMX 1410852-B-23-2-2 exhibited a fold change of 2.93 for ASR, which was higher than ICMP 100230, which showed a fold change of 2.04 for ASR. More than 2 fold increase was noted in the tolerant genotypes, attributing to higher RWC and increased expression of antioxidants and enzymes such as superoxide dismutase, catalase, and peroxidize. The reports in rice further supported this (Philippe *et al.*, 2010) and tomato (Maskin *et al.*, 2001).

The expression of 26S proteasome was higher in susceptible ones ICMX 1410848-B-9-2-2 (2.46), ICMX 1410506-SB-1-4-1-B (2.75) compared to the tolerant genotypes ICMP 100230 (1.64), ICMX 1410852-B-23-2-2 (1.80). Lower expression of 26S indicates a relative increase of 20S proteasome, which imparts oxidative stress tolerance under drought conditions and plays an important role in ubiquitin-dependent proteolysis as in the case of upland and lowland rice cultivars subjected to water stress (Wang *et al.*,

et al., 2007). The TD gene expression was comparatively higher in tolerant genotypes ICMP 100230 and ICMX 1410852-B-23-2-2 with a fold change of 6.06 and 5.84, respectively. In response to drought, several signal transduction pathways operate, including vacuolar transport proteins such as tonoplast dicarboxylate transporter (TD), leading to the effective accumulation of solutes in vacuoles, resulting in the maintenance of cell turgor. Higher expression of TD in the tolerance genotype, ICMP 100230 ICMX 1410852-B-23-2-2 can be attributed to a minimum relative decrease in the RWC values.

Cell death is one of the commonly adapted defensive mechanisms by plants under abiotic stress. WD-40 repeat proteins are usually related to E3 ubiquitin ligase enzymes, which are involved in cell cycle control and apoptosis (Yee and Goring, 2009). The sensitive genotype ICMX 1410506-SB-1-4-1-B, ICMX 1410848-B-9-2-2 expressed a higher fold change of 5.29 and 3.79, representing high-level oxidative damage of cells, leading to necrosis compared to tolerant genotypes ICMP 100230 (1.69), ICMX 1410852-B-23-2-2 (1.49). Drought-sensitive rice variety IR20 expressed increased WD-40 repeat protein by 2.5 times under drought

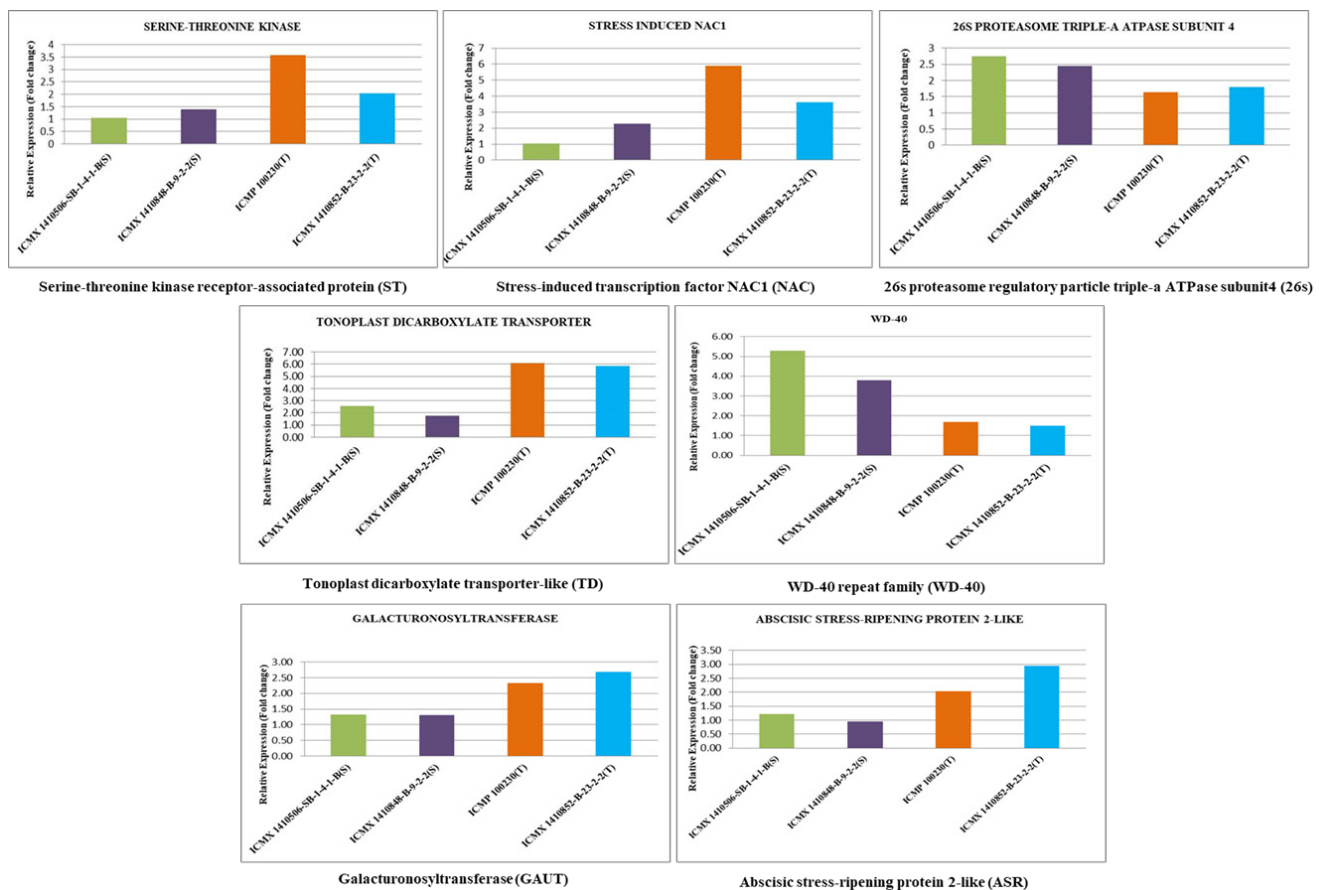


Figure 2: Relative expression (Fold change) of selected genes (ST, NAC, 26S, TD, WD-40, GAUT, ASR) in four pearl millet [tolerant (T) and susceptible (S)] genotypes under control and drought stress conditions

stress (Maksup *et al.*, 2014) and in maize (Zheng *et al.*, 2004). The bar diagrams depicting the relative expressions in four pearl millet genotypes are presented in Figure 2.

Conclusion

Considering our results, we concluded that pearl millet genotypes (B and R lines) respond differentially in response to drought by bringing changes in their morpho-physiological traits and operating different stress-responsive pathways. There exists a correlation between the morphological and molecular behavior of plants in response to seedling drought stress. Physiological traits like RL, SL, R/S and RWC can be considered for the screening of drought-tolerant lines. Based on these traits, five drought-tolerant genotypes were identified (ICMX 1510541-SB-3-4-5, ICMX 1410852-B-1-5-3, ICMX 1410843-B-8-1-2, ICMX 1411004-B-37-2-1 and ICMR 100029). The information generated is useful for breeders in the development and improvement of parental lines and hybrids.

Acknowledgment

We would like to express our sincere gratitude to the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) for generously providing the seed material essential for this research. We also extend our heartfelt thanks to Professor Jayashankar Telangana State Agricultural University (PJTSAU) for offering the necessary infrastructure and facilities that greatly facilitated our research activities. Their contribution played a crucial role in the execution and progress of this work.

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