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Expression of genes involved in drought stress in two soybean cultivars (*Glycine max*) treated with methyl jasmonate and salicylic acid

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ABSTRACT INFO

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ABSTRACT

Soybean (*Glycine max*) is one of the most important oilseed crops in the world. However, its cultivation is limited in many areas with water shortage, and is affected by drought. This study investigated the expression of genes involved in drought stress in two soybean cultivars, i.e. Williams (tolerant) and L17 (sensitive) after drought stress and treatment with methyl jasmonate (MeJA) and salicylic acid (SA). In addition, the impact of drought and hormone treatments were validated with morpho-physiological evaluation of these two cultivars. Experiment was conducted in a factorial basis with completely randomized design. The results showed that the expression of basic-leucine zipper (*BZIP19),* NAM-ATAF1, 2-CUC2 *(NAC),* dehydration-responsive element-binding *(DREB1),* and vascular plant one zinc finger protein (*VOZ1G) was* higher in the resistant cultivar, i.e. Williams. Gene expression was induced after simultaneous application of SA and MeJA in Williams cultivar. According to the morpho-physiological results, plant height and root length, fresh and dry weight of roots and shoots, nodes and number of lateral roots, number of pods and number of seeds per pod, leaf area, and percentage of relative leaf moisture, number of stem nodes and internode distance, pod weight and harvest index were significantly different between the two cultivars. Increase in the expression of *VOZ* gene, under treatment with SA was more effective on shoot height and nodule formation of Williams than in L17. Results of this investigation should be useful for developing tools for breeding new soybean genotypes with an improved tolerance to drought.

***Key words*: *BZIP*, *DREB*, Methyl jasmonate, Salicylic acid, Soybean, *VOZ*.**

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INTRODUCTION

Soybean is one of the five most important crops worldwide (Kamrava *et al*., 2016). The soybean's global import and export values have outstripped all other major crops, such as maize, wheat, and rice, even without including soybean oil or its other processed forms (Yahoueian *et al.,*2018). Drought stress is one of the main limitations of productivity and crop yield and major environmental stress in the world agriculture. Drought tolerance is a multifaceted and intrinsic trait. Food crop association and candidate gene studies are intensely directed toward selecting favorable alleles for drought resistance (Chakraborty *et al.*, 2023). Water deficiency can negatively affect various aspects of soybean physiological, biochemical, and morphological processes. As a result, it affects soybean development and nitrogen fixation, thereby reducing its growth and yield (Ashraf *et al*., 2011). Meanwhile, climate forecasts are predicted to be extremes by 2050 in Africa, therefore, there is a need to breed high-yielding drought-tolerant genotypes (Wei *et al.,* 2022). Enhancement of drought tolerance is one of the economic strategies to increase the yield and stability of crop production, such as soybean (Nevo *et al*., 2010). Plant regrowth can widely be changed after environmental stress at the molecular level, and the signaling system induces specific genes against the detrimental effects of stresses. Also, some of its products are involved in plant protection and maintaining cell structure (Suprunova *et al*., 2004; Bartels *et al*., 2005).

Hence, plants respond to abiotic stresses through physiological, biochemical, cellular, and molecular processes and adapt to or tolerate environmental conditions (Thomashow, 1999; Bray *et al*., 2000; Shinozaki *et al*., 2003). As soon as intracellular changes occur, different signaling pathways are initiated to convert the physical stress into an appropriate biochemical response, triggering the expression of a set of stress-responsive genes (Xiong and Zhu, 2001; Leonardis *et al*., 2007). The results of these genes protect the cell from stress also regulate the genes involved in stress response signaling (Maruyama *et al*., 2004). These results can be classified into two groups based on their function. The first group includes proteins such as late ginseng proteins (LEAs), blue channel proteins, osmolyte biosynthesis enzymes, chaperones, detoxification enzymes, and membrane lipid-modifying enzymes involved in stress tolerance. The second group includes protein factors involved in regulating gene expression and signaling in response to abiotic stress, such as transcription factors, enzymes involved in phospholipid metabolism, and kinases (Maruyama *et al*., 2004; Yamaguchi and Shinozaki, 2005).

Transcriptomic studies in legumes have been used to reveal responsive genes under drought, heat, or salinity conditions (Yang *et al*., 2021). Transcription factors are trans-acting proteins that amplify or inhibit gene expression by binding to cis-acting located in the promoter region of target genes (Chakraborty *et al*., 2022). Transcription factor genes make up a significant portion of the genomes of all eukaryotes, including organic plants (Riechmann *et al*., 2000).

In plants, a transcription factor can control the expression of many genes by binding specifically to the cis-acting element in the promoter region of target genes (Nakashima and Shinozaki, 2005). The relative expression levels of four basic-leucine zipper (BZIP) transcription factors (JcbZIPs 34, 36, 49 and 50) significantly increased in the drought or salt treatment which was consistent with the expression analysis of RNA sequencing data. Among them, JcbZIP49 and JcbZIP50 were up-regulated in leaves after drought and salt treatments as well as roots after salt treatment (Wang *et al*., 2021). Transcriptome analysis of transgenic rice showed that there were a large number of differentially expressed genes (DEGs) regulated by *Phyllostachys edulis bZIP47* (*PhebZIP47)*, including genes of drought tolerance regulatory pathway and ABA signaling pathway. Meanwhile, a G-box element was significantly enriched in the promoters of the DEGs annotated as 'response to stress', and EMSA experiment suggested that this element could be bound by *PhebZIP47*. In addition, transgenic plants were less sensitive to ABA compared with wild-type plants under exogenous ABA treatment (Lan *et al*., 2023). Digital expression results, in particular, showed that in *Solanum lycopersicum* vascular plant one zinc finger protein *(SlVOZ*s) is not only active during different growth status of tomato but is also involved in abiotic stress response mechanism. Nonetheless, *SlVOZ1* is expressed higher in both developmental stages and under salt stress conditions, confirmed by RT-qPCR (Uluisik *et al*., 2022). The dehydration-responsive element-binding (*DREB*) transcription factor family is found in multiple plant species and has been shown to function in enhancing plant tolerance to various abiotic stresses such as drought stress (Liu *et al*., 1998; Agarwal *et al*., 2006). Many families of these factors are affected by stress, the most important of which being *BZIP* proteins (Uno *et al*., 2000), *DREB* (Sakuma *et al*., 2006), *NAC* (Chen *et al*., 2018), and *VOZ* (Mitsuda *et al*., 2004).

In this study, real-time PCR was used to investigate the expression pattern of *NAC, VOZ*, *DREB*, and *BZIP* genes under drought stress conditions after treatment with Methyl Jasmonate (MeJA) and Salicylic Acid (SA) in two soynean cultivars. Drought tolerance of resistant and sensitive genotypes was then validated with morpho-physiological experiments to associate the gene expression with the level of tolerance.

MATERIALS AND METHODS

Plant material and tissue sampling

Seeds of drought-tolerant (Williams) and drought-sensitive (Line L17) were prepared by the Research Institute of Environmental Sciences. Seeds were sowed in plastic pods in the greenhouse at 25 °C and 16/8 h daylight condition. Two levels of normal and drought treatment were applied. Drought stress treatment was applied with 50% of crop capacity. Drought stress started at six leaf stage and lasted for 7 days. Then hormonal treatment of SA (1.5 mM) and MeJA (10 µM) were performed based on the literature review (Menkens *et al*., 1995; Verma *et al*., 2016). After 12 h, young/mature leaves collected from different parts of the plant were used to study the expression patterns of *VOZ, DREB*, *NAC*, and *BZIP* genes. Leaves from control (without drought stress and hormonal treatment) and drought-treated sampled were immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. The relative water content of leaves was measured by harvesting leaf samples in each replication according to Ritchie *et al.* (1990) report.

After drought stress and plant maturity, plants were removed from the pot, and roots were washed with water. Then, each plant aerial organs and roots were separated to measure their morphological parameters. Plant height and root length, wet and dry weights of shoot and root organs, internodes, leaf area, number of pods, seeds per pod, nodule, and lateral roots, and relative water content of leaves were measured in five replicates. RWC was calculated using this formula:

$$RWC=FW-DW/\ TW-DW\times100\% \quad (1)$$

Where FW, DW, and TW were the fresh weight, dry weight, and turgid weight of the leaflet, respectively.

RNA extraction and cDNA synthesis

Total RNA was extracted from 300 mg of frozen sample ground in liquid nitrogen using the CTAB method according to Gasic *et al*. (2004) (Figure 1). RNA samples were quantified with 1% agarose gel and a ScanDrop 100 spectrophotometer (Analytik Jena, Germany). Two µg of RNA was treated with 1 µL of DNase I and 1 µL of DNase buffer 10× to remove any possible DNA contamination according to the manufacturer's instruction (Fermentas Co.). cDNA library was made using 1.5 µg of DNase-treated samples, 1 µL of Oligo(dT) primer, 1 µL of 50 µM dNTP, 5 µL of 5x M-MLV buffer, 1 µL of RNasin Inhibitor, and 1 µL of M-MLV in a total volume of 12 µL according to the manufacturer's instruction (Yekta Tajhiz Co.). cDNAs were stored at –20 °C until further analysis.

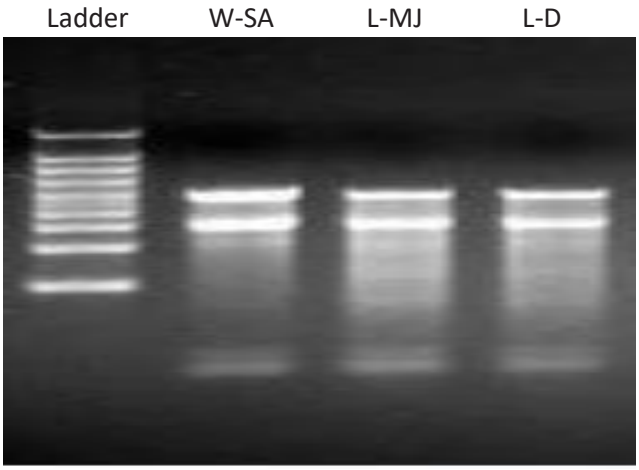

Figure 1. Total RNA extraction from leaves of soybean by Gasic *et al.* (2004) method. (W: Williams, SA: Salicylic acid, L: L17, D: Drought, MJ: Methyl Jasmonate).

Validation of the gene expression by quantitative Reverse-Transcription PCR (qRT-PCR)

qRT-PCR was used to evaluate *BZIP*, *VOZ*, *DREB*, and *NAC* expression levels under different treatments using specific primers. For the target genes, the specific primers were selected from the related publications in peer-review journals, and the selected primers were double checked in phytozome and NCBI (Uno *et al*., 2000; Mitsuda *et al*., 2004; Sakuma *et al*., 2006; Chen *et al*., 2018). in order to ensure they span the exon-intron junction, and to prevent the amplification of genomic DNA (Table 1).

After extracting RNA and confirming its quality, cDNA was synthesized according to the protocol. Negative control was used to confirm the absence of contamination and confirm the synthesis of cDNA in PCR reaction. The specificity of each primer pair was checked de novo (based on the gene sequence and blast to the genome). In addition, single product amplification was checked with qRT-PCR using melting curve analysis for each gene. Efficiency of selected primers was checked using the method proposed by Pfaffl (2001).

qPCR reaction contained 5 µL SYBR Green, 2 µL cDNA (diluted 20 times from the original cDNA), and

Table 1. Sequence of Oligonucleotides and their features. The Annealing temperature around 60 was taken into consideration when selecting the primers.

Primers	Annealing temperature (°C)	Product size (bp)	Sequences (5'-3')	GC (%)
F- BZIP19	57.89	109	GGAAACTGCCAACCTGAAATG	47.62
R- BZIP19	57.97	109	ATCCGTCTTGAGATGCAGATG	47.62
F- VOZ1G	56.82	150	AACTTGTATGGAGGGCACATAA	40.91
R- VOZ1G	56.5	150	ATAGAAGTGGCCTTGTAACGAA	40.91
F- DREB1	60.13	367	CGATGAAACCTTACCGTGGAA	47.62
R- DREB1	56.85	367	AAGTCGGGCTTGAGATTGAG	50
F- NAC8	59.86	396	TGCAATTTCCCCAACACCAAC	47.62
R- NAC8	61.06	396	CTGATTTCCCAACCCAACACGTA	47.83
F- ACTIN	61.78	126	AGCCACACTGTCCCTATCTA	41.67
R- ACTIN	59.9	126	GCTGAGGTGGTGAAGGAATAA	68.42

0.2 µM of each specific primer for *BZIP*, *VOZ*, *DREB*, and *NAC*, in a total volume of 10 µL. *Actin* gene was used as the internal control for the expression normalization. qRT-PCR was performed as following: 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The CT for each sample/treatment was recorded and ratio between treatment/control were calculated based on the reference gene (Table 1) Fold changes in expression were calculated by the $2^{-\Delta\Delta Ct}$ method using *actin* as the reference gene according to the method proposed by Pfaffl (2001).

Validation of effect of exogenous application of hormones on two cultivars in drought condition

To validate the impact of two hormones on the drought tolerance, an experiment was carried out with William and L17. Plants were treated with, 1.5 mM of SA and 10 µM MeJA, respectively while control plants were sprayed with water. The hormonal concentration was chosen based on the literature review of similar experiments. Similar experiment was carried out in drought stress condition. Plants characteristics including plant height, root length, nodules, number of pods, lateral branches, stem nodes, shoots dry weight, shoots wet weight, roots wet weight, roots dry weight, pods weight, leaf area, percentage of relative leaf moisture, and harvest index were evaluated. All data were measured in three technical repeats, and data were analyzed with Minitab ver. 16. Mean comparison was analyzed with Tukey's test at 5% and presented as bar charts when a significant difference was observed.

RESULTS

RNA extraction

RNA was extracted from leaf tissue of Williams and L17 genotypes. After concentrating RNA samples with the Nano Drop device, sample quality was examined with 1% agarose gel. The extraction yield was ranged between 10-20 µg /300 mg of leaf samples. The 260/280 and 260/230 ration were between 1.8-2.1.

Comparison of normal and drought conditions on gene expression in two soybean cultivars

Comparing the expression of *BZIP19*, *DREB1*, *NAC8*, and *VOZ1G* genes under drought treatment, *DREB1* gene showed a greater increase in expression. The highest expression in *DREB1* gene under drought stress was 8.2 fold increase in Williams, while the lowest expression in *NAC8* gene was observed in cultivar L17 compared to the control. Results showed that *BZIP19* was upregulated under drought stress. The expression of the *BZIP* gene increased compared to normal conditions. In this experiment, the highest expression of this gene was observed in Williams cultivar. The expression of *BZIP* gene in Williams, under drought stress was 2.6 times higher than the normal condition. In L17 the expression of *BZIP* was lower than Williams (Figure 2).

The expression of the *VOZ1G* gene increased compared to normal conditions. In this experiment, the highest expression of this gene was observed in Williams cultivar. The expression of *VOZ1G* gene in Williams, was 6.8 times more in drought stress than the normal condition, while it was 3.4 times more in drought condition compared to the normal condition (Figure 2). Similar results were obtained for *NAC8* gene in Williams, in which a 2 fold increase in gene expression was observed under drought stress relative to normal condition (Figure 2).

DREB1 was also significantly induced by drought stress in Williams cultivar. The expression of this gene in Williams was 8.1 times upregulated after drought stress than in the normal condition. The expression was also induced 1.1 times more in L17 cultivar than

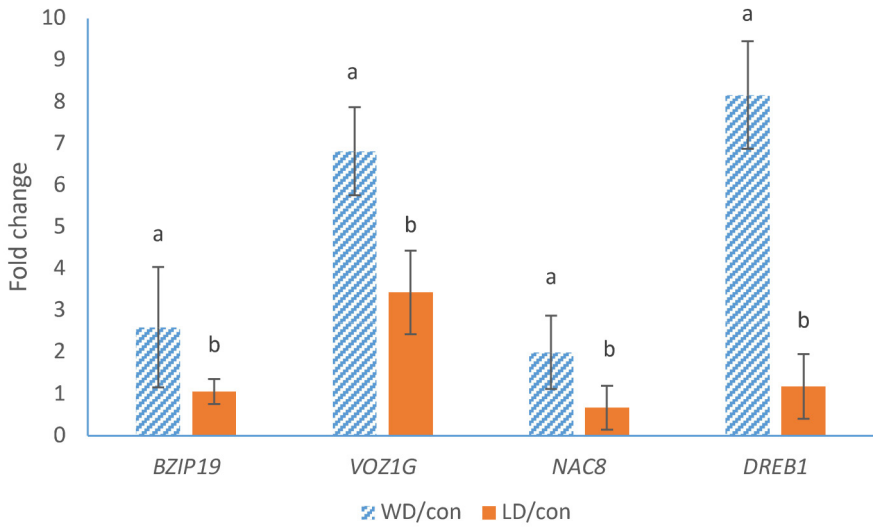

Figure 2. Comparison diagram of expression of *BZIP19, VOZ1G, NAC8* and *DREB1*, genes under drought stress. (D: Drought, con: Control (no- drought), W: Williams, L: L17).

in drought stress (Figure 2).

Impact of hormones on gene expression in two soybean cultivars in normal and drought conditions

The expression of *BZIP19* gene was significantly induced by hormone treatment. Simultaneous treatment of soybean using SA and MeJA produced the highest expression in two cultivars (Figures 3A and 3B). In drought-stress condition, the expression of *BZIP19* gene was 5.3 and 4.4 times higher after simultaneous treatment of two hormones, for William and L17, respectively. (Figure 3A).

Similar results were obtained in normal condition, in which the simultaneous application of two hormones caused 4.3 and 3.6 times higher expression than the control (no hormone), in Williams and L17, respectively (Figure 3B). *VOZ1G* was significantly induced by hormone treatment. Simultaneous treatment of SA and MeJA produced the highest expression in two cultivars (Figures 3C and 3D). In drought stress condition, *VOZ1G* was highly induced after hormone treatments, especially in Williams cultivar, in which the expression was 19.4 times higher in SA-MJ treatment than in the no-hormone condition. However, a similar induction was also observed for L17, but a lower expression, in SA/MJ treatment was observed than the no-hormone treatment (Figure 3C). In normal condition, the expression of *VOZ1G* gene was 3.4 and 2 times higher after simultaneous treatment of two hormones, for William and L17, respectively (Figure 3D).

The expression of *DREB1* was induced by hormone treatment. Simultaneous treatment using SA and MeJA produced the highest expression in two cultivars (Figures 4E and 4F). In drought stress condition, *DREB1* expression was highly induced after drought stress compared to the control, where a 16.9 and 11.2 times higher expression was observed, for Williams and L17, respectively (Figure 4E). Similar results were obtained in no drought-stress condition, thus the expression of *DREB1* gene was 12.7 and 9.1 times higher after simultaneous treatment of two hormones, for William and L17, respectively (Figure 4F).

The expression level of *NAC8* was upregulated following the hormone treatment with MeJA and SA. The expression of *NAC8* was induced after hormone treatment in drought-stress condition. However, the difference between SA, MJ and SA-MJ treatments was less noticeable for both cultivars (Figure 4G). However, the resistant cultivar showed more level of upregulation for this gene than the susceptible cultivars. Similarly, in no drought-stress condition, the expression of *NAC8* was 4.2 and 2 times higher after simultaneous treatment of two hormones, for William and L17, respectively (Figure 4H).

Comparison of *BZIP19*, *VOZ1G*, *NAC8* and *DREB1* genes in two cultivars after treatment with hormones and drought

Comparing the graphs showed that the two hormones SA and MeJA induced the expression of these genes in the plants under drought stress, which is more noticeable in the Williams cultivar. The highest expression in *VOZ1G* gene was observed under drought stress and two hormone treatment compared to the control condition, i.e. 19.4 and 6.1, higher in hormone treated than control for Williams and L17

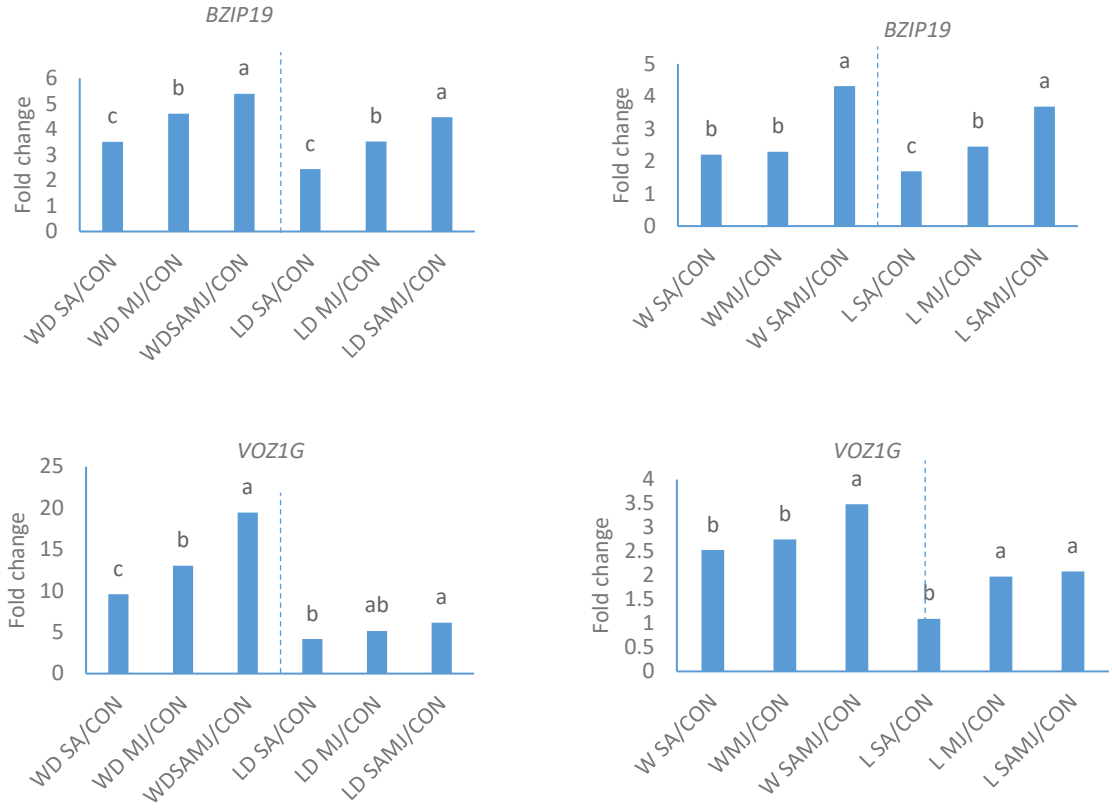

Figure 3. Comparison charts of the *BZIP19* and *VOZ1G,* genes expressions (D: Drought treatment, SA/CON: Gene expression ratio in SA treatment to hormone-free treatment, MJ/CON: Gene expression ratio in MeJA treatment to hormone-free treatment, SAMJ/CON: Gene expression ratio in simultaneous treatment by SA and MeJA to hormone-free treatment, W: Williams, L: L17).

cultivars, respectively (Figure 5). Considering that the increase in the expression of these genes in Williams cultivar was higher than that observed in L17 cultivar, Williams cultivar was more tolerant to drought stress.

DREB1 was the second most induced gene after hormone and drought -stress treatment, where the gene expression was 16.9 and 7.2 fold higher, for Williams and L17 cultivars, respectively. *NAC8* and *BZIP19* were also induced by drought and hormone treatment, in which the expression was less induced in compared to the *VOZ1G* and *DREB1*. Interestingly, all four genes showed higher expression patterns in Williams than L17, after drought and hormone treatment (Figure 5).

Validation of drought and hormone impact on Williams and L17 traits

To validate the impact of drought and hormones on two cultivars, we performed an experiment in greenhouse with Williams and L17 after exposure to drought and treating with SA and MeJA. After watering was ceased for 7 d, plants showed obvious drought stress symptoms like leaf rolling. Such a difference was not observed when plants were grown under normal conditions.

Analyses of morphological traits of two cultivars in normal condition

The results showed a statistically significant difference between the two cultivars for plant height, number of root nodes, number of pods, lateral roots, shoots wet weight, shoots dry weight, pod weight, leaf area, relative leaf moisture content, and harvest index (p-value<0.001), (Tables 2 and 3).

For these traits, the differences between the number of stem nodes, root wet weight, and root dry weight were significant at 1% level for these two cultivars. Also, the cultivars showed a significant difference in root length trait at 5% level. However, there was no statistically significant difference between cultivars in terms of seed in the pod, lateral branch, and internode distance (Figure 6).

Comparison of Williams and L17 in normal and drought condition

According to the analysis of variance (ANOVA), drought can significantly affect all traits (Table 2).

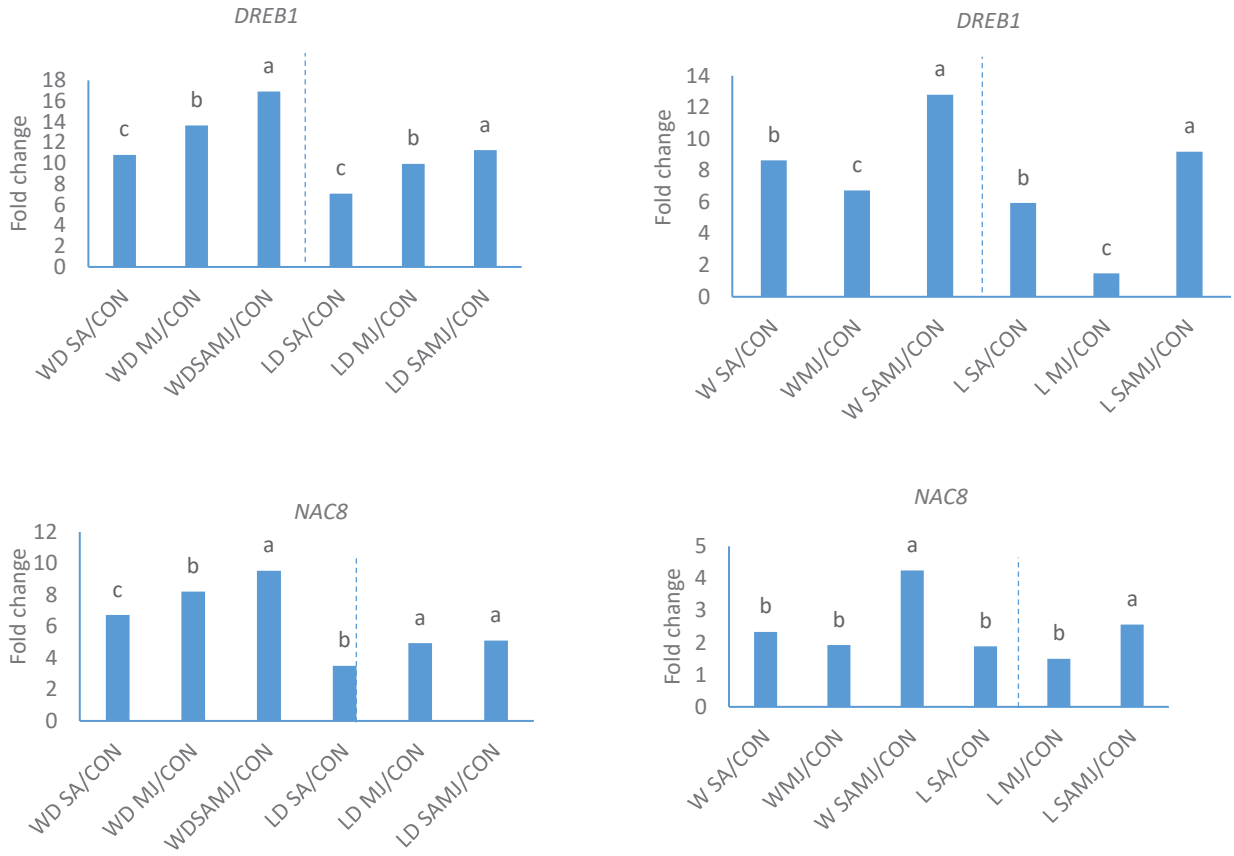

Figure 4. Comparison charts of the, *DREB1* and *NAC8,* genes expressions (D: Drought treatment, SA/CON: Gene expression ratio in SA treatment to hormone-free treatment, MJ/CON: Gene expression ratio in MeJA treatment to hormone-free treatment, SAMJ/CON: Gene expression ratio in simultaneous treatment by SA and MeJA to hormone-free treatment, W: Williams, L: L17).

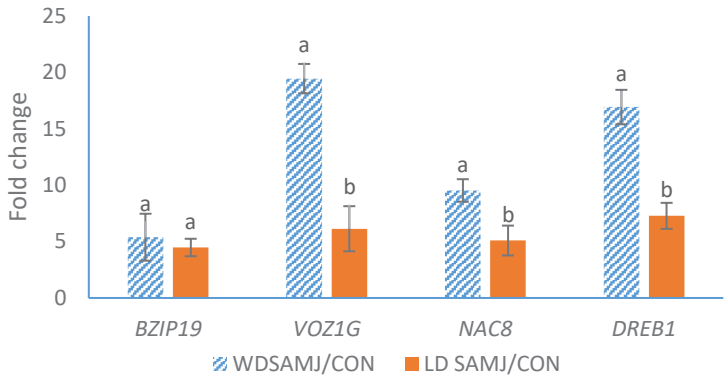

Figure 5. Comparison diagram of expression of *BZIP19, VOZ1G, NAC8*, and *DREB1* genes under drought stress and hormone treatment (D: Drought, W: Williams, L: L17, SAMJ/con: Gene expression ratio in combined treatment of SA and MeJA to hormone-free treatment).

Plant height, root length, nodules, number of pods, lateral branches, stem nodes, shoots wet weight, shoots dry weight, roots wet weight, roots dry weight, pods weight, leaf area, percentage of relative leaf moisture, and harvest index showed a very significant difference between the drought and sensitive cultivars (p-value<0.001). Also, seed in the pod, internode, and number of lateral roots showed a significant difference between cultivars at 5% level (Figure 7).

Table 2. ANOVA results for morphological and physiological traits.

Sources of variation	df	Mean of square								
		Plant height	Root length	Nodule	Pod	Seed in pod	Lateral branch	Inter node	Node	Lateral root
Cultivar	1	282.949***	27^{ns}	243***	40.333***	0.020^{ns}	0.333^{ns}	1.140^{ns}	9.187**	46.020***
Drought	1	328.339***	336.021***	252.083***	234.083***	1.687*	4.083***	2*	31.687***	9.187*
Hormone	3	98.611***	163.361***	644.389***	34.361***	2.409***	0.972**	3.477***	18.409***	19.298***
Cultivar×drought	1	22.059*	1.021^{ns}	102.083***	0.333^{ns}	0.020^{ns}	0.120^{ns}	0.460^{ns}	3.520^{ns}	0.520^{ns}
Cultivar×hormone	3	3.719^{ns}	9.833^{ns}	23.833***	20.389***	0.131^{ns}	0.222^{ns}	0.732^{ns}	0.520^{ns}	1.576^{ns}
Drought×hormone	3	3.860^{ns}	13.410^{ns}	8.250^{ns}	4.694*	0.020^{ns}	0.305^{ns}	0.620^{ns}	0.687^{ns}	0.409^{ns}
Cultivar× drought×hormone	3	2.240^{ns}	1.521^{ns}	15.250**	27.389***	0.020^{ns}	0.111^{ns}	0.507^{ns}	0.409^{ns}	0.076^{ns}
Error	32	3.792	6.219	3.167	1.523	0.229	0.187	0.436	1.95	1.46
Coefficient of variation (%)		21.45	23.26	15.55	22.91	20.30	24.64	17.87	20	14.78

ns: Not significant, *, ** and ***: Significant at 0.05, 0.01 and 0.001.

Table 3. ANOVA results for morphological and physiological traits.

Sources of variation	df	Mean of square							
		Pod weight	Leaf area	Shoot wet weight	Shoot dry weight	Root wet weight	Root dry weight	RWC	Harvest I
Cultivar	1	138.041***	196.263***	162.068***	20.527***	25.259**	1.360**	95.87***	78.306***
Drought	1	37.206***	138.856***	140.699***	7.704***	59.674***	6.571***	256.55***	439.967***
Hormone	3	71.340***	710.357***	54.425***	2.414**	69.663***	1.753***	1209.94***	196.470***
Cultivar×drought	1	0.743^{ns}	0.546^{ns}	27.241***	1.119^{ns}	0.554^{ns}	0.035^{ns}	1.35^{ns}	15.497***
Cultivar×hormone	3	9.296***	20.300^{ns}	4.108^{ns}	0.594^{ns}	4.322^{ns}	0.193^{ns}	14.13*	0.591^{ns}
Drought×hormone	3	1.189^{ns}	13.999*	2.502^{ns}	0.675^{ns}	0.310^{ns}	0.910***	87.77***	4.719*
Cultivar× drought×hormone	3	0.427^{ns}	1.597^{ns}	10.211**	0.423^{ns}	3.170^{ns}	0.305^{ns}	2.80^{ns}	15.765***
Error	32	0.732	5.127	1.967	0.418	2.126	0.136	4.01	1.097
Coefficient of variation (%)		16.85	13.32	12.49	21.13	19.56	22.67	11.31	13.76

ns: Not significant, *, ** and ***: Significant at 0.05, 0.01 and 0.001.

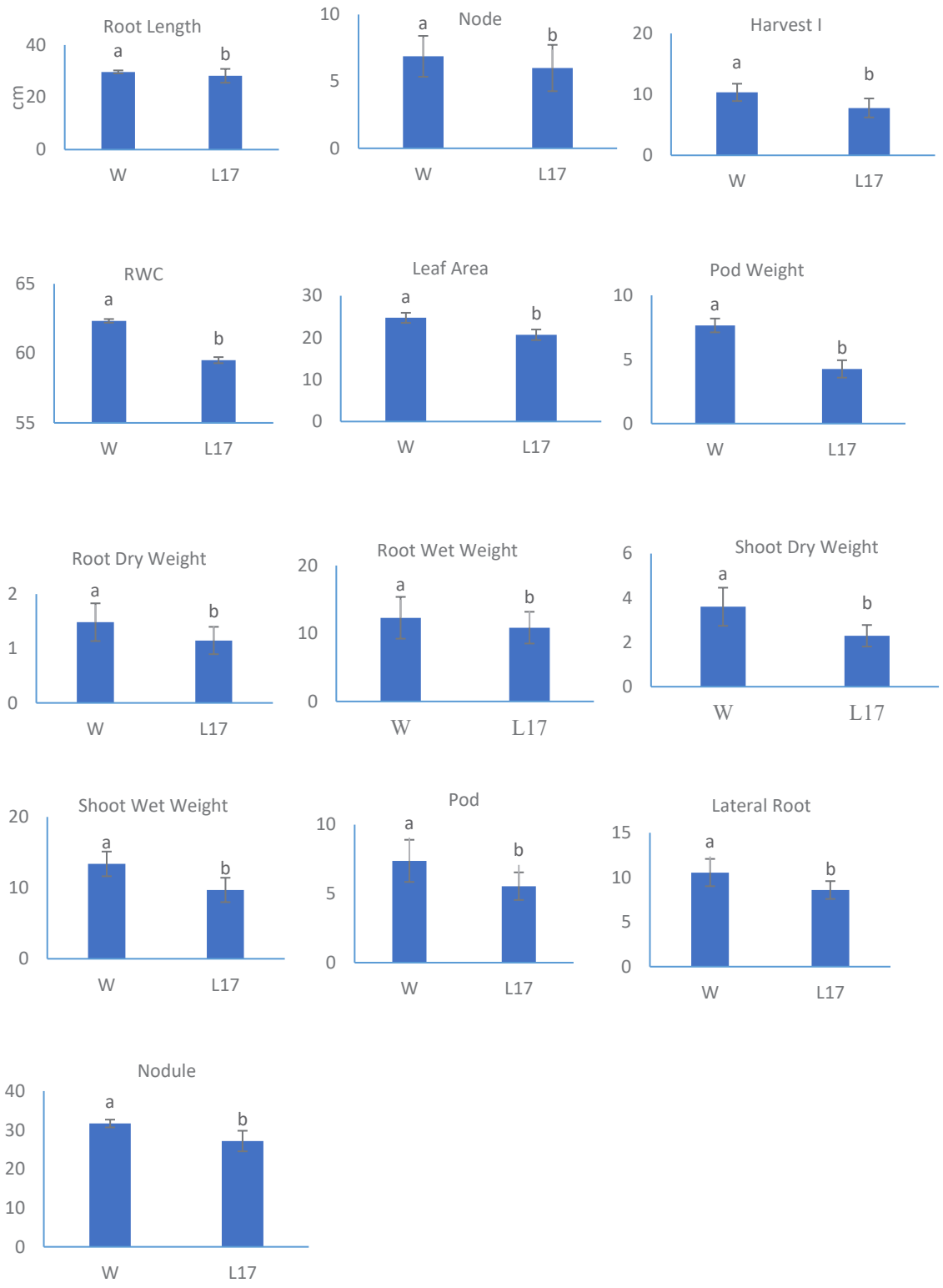

Figure 6. Analyses of different parameters in Williams (tolerant) and L17 (sensitive) in normal condition using ANOVA and Tukey test. The letters at the top of the graphs indicate a significant difference between the treatments (W: Williams, L17: L17).

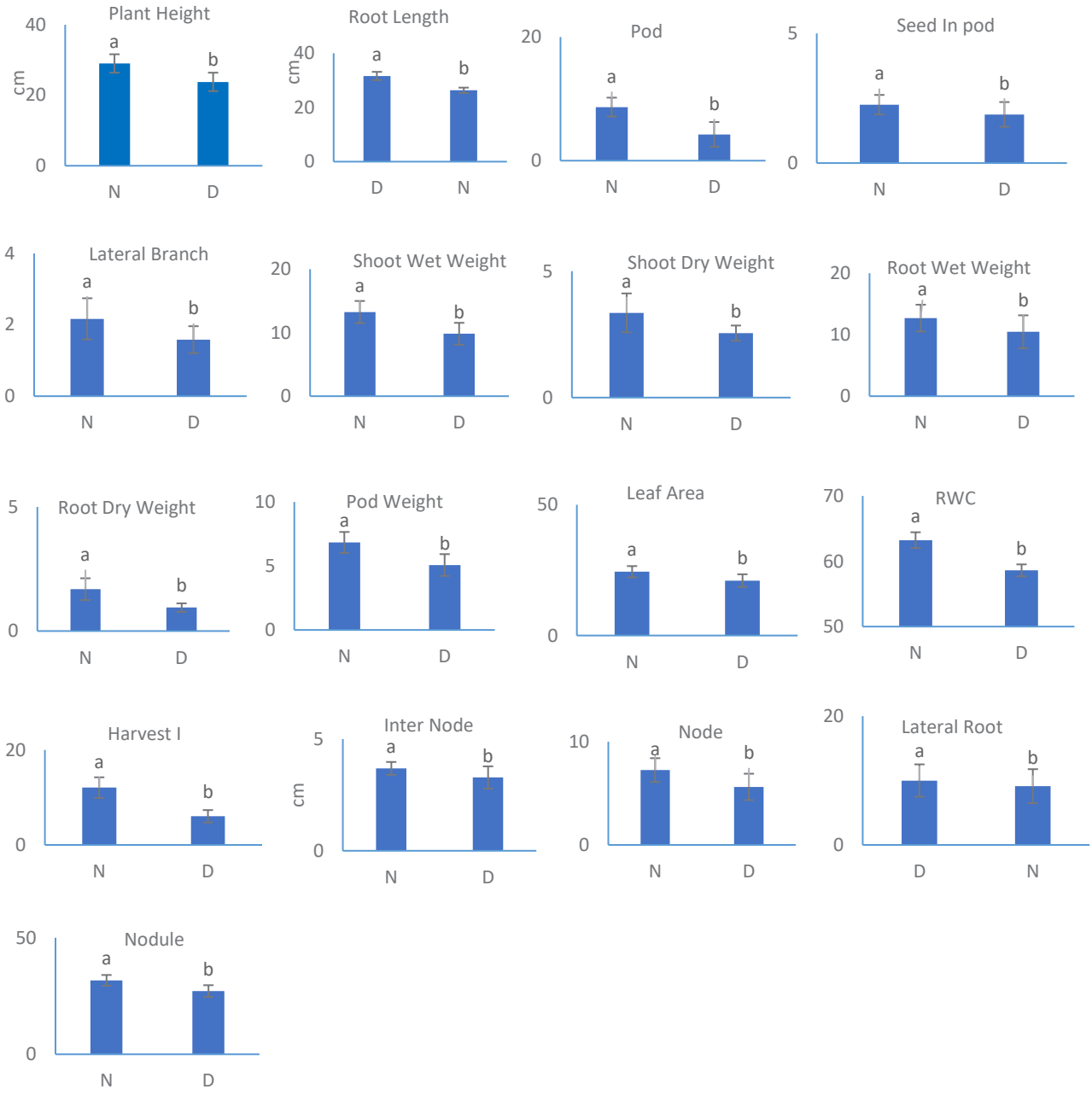

Figure 7. Comparison of morphological parameters in Williams (tolerant) and L17 (sensitive) in normal and drought condition using ANOVA and Tukey test. The letters at the top of the graphs indicate a significant difference between the treatments (D: Dry treatment, N: Control treatment).

Analyses of morphological traits of two cultivars after hormonal treatment

The results of treatment with two hormones of the tolerant and sensitive cultivars showed a significant difference between the two cultivars regarding different morphological characteristics. Plant height, number of root nodes, number of pods, lateral roots, shoots wet weight, shoots dry weight, pod weight, leaf area, relative leaf moisture content, and harvest index (p-value<0.01) was significantly different in drought and normal condition (Figure 8). The cultivar interaction on hormone was significant for nodule number, pod number, and pod weight (p-value<0.001). In addition, the relative moisture content of leaves was significantly (p-value<0.001) different between control and hormone treated samples (Figure 9).

The effect of drought on hormones on root dry weight and relative moisture content of leaves indicated a significant difference (p-value<0.001). Also, its effect on leaf area, number of pods, and harvest index

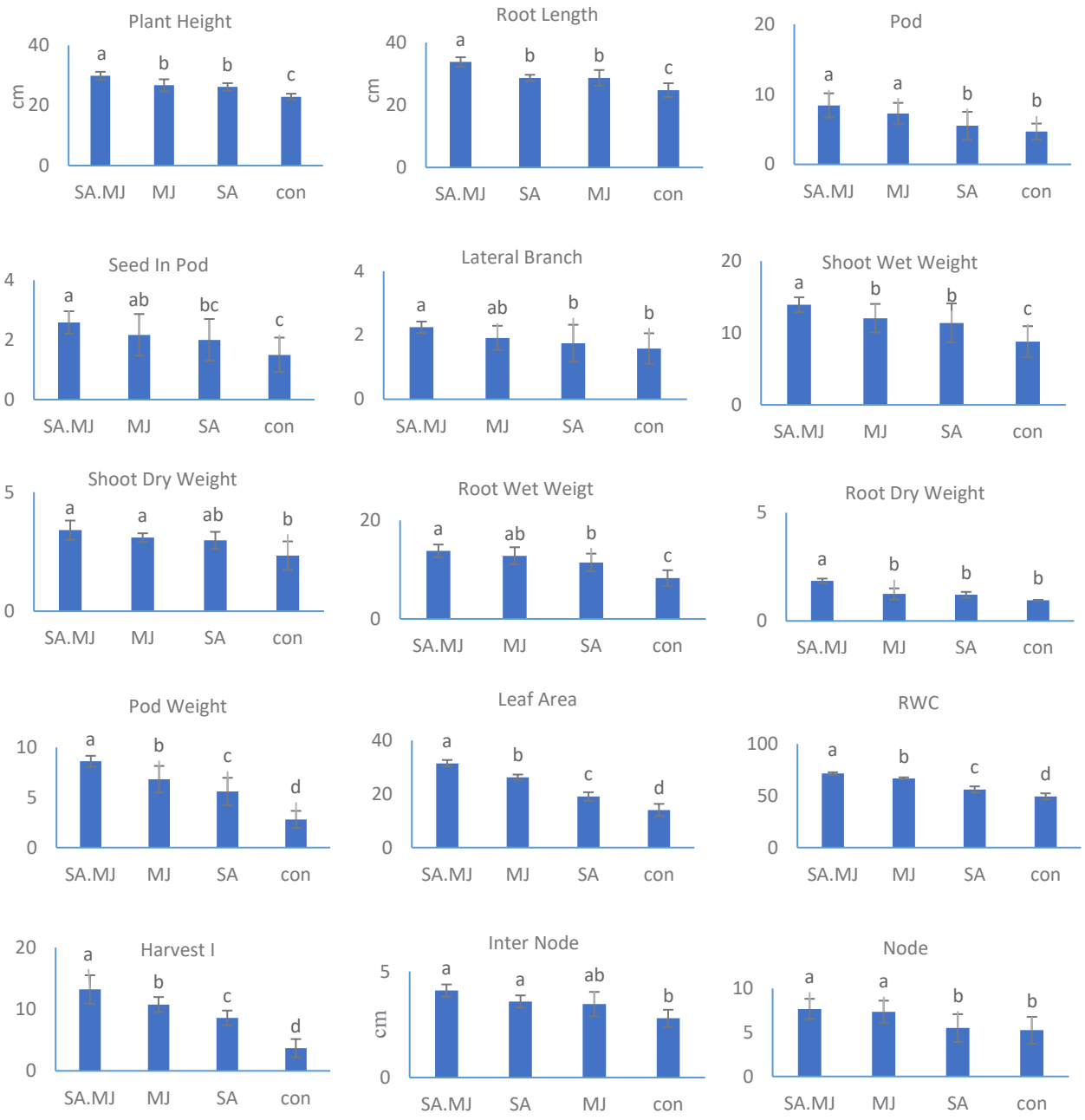

Figure 8. Analyses of different parameters in Williams (tolerant) and L17 (sensitive) after hormonal treatment using ANOVA and Tukey test. The letters at the top of the graphs indicate a significant difference between the treatments (SA: Salicylic acid, MJ: Methyl jasmonate, con: Control treatment).

traits were significant at 5% level. Meanwhile, the interactions for plant height, root length, seed per pod, lateral branch, internode, stem node, lateral root, shoot dry weight, shoot wet weight, root wet weight, and pod weight were not significant (Table 2).

Interactions between cultivar and drought and hormone were significant between pod number and harvest index (p-value<0.001), and the number of nodules and shoot wet weight traits were significant (p-value<0.01). However, these interactions were not significant between plant height, root length, seed per pod, lateral branch, internodes, stem node, lateral root, dry weight of shoot, root weight, weight pod, leaf area, and relative moisture content.

DISCUSSION

Legumes can stabilize nitrogen through the nitrogen fixation process using bacteria resulted in nodule

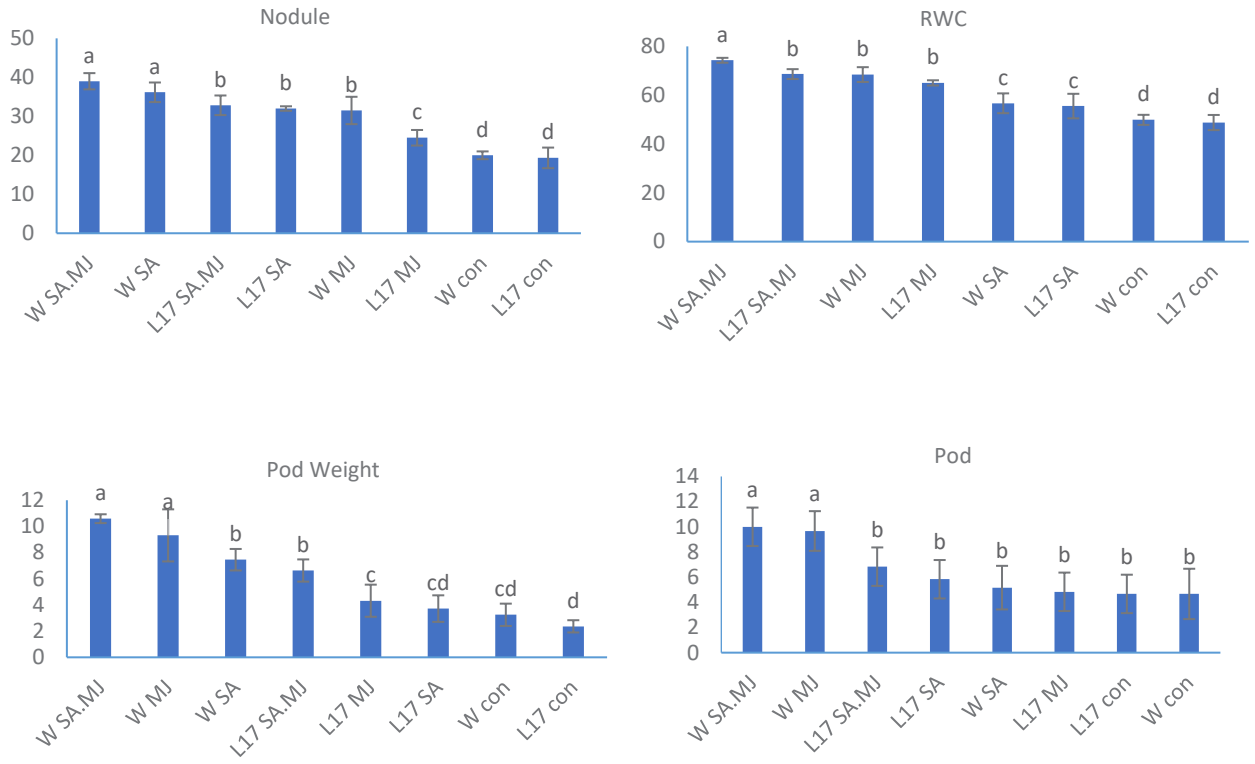

Figure 9. Comparing the parameters including nodule, RWC, pod weight, and the number of the pod in terms of the interaction of cultivar on hormone with using ANOVA and Tukey test. The letters at the top of the graphs indicate a significant difference between the treatments (W: Williams, L17: L17, SA: Salicylic acid, MJ: Methyl jasmonate).

production in the root. The intercropping-based legumes enhance the chemotaxis and the behavior of beneficial root-associated bacteria in the rhizosphere (Chamkhi *et al*., 2022). Although nitrogen is the most abundant element (~79%) in the atmosphere, most plants are unable to directly utilize atmospheric nitrogen gas (Yang *et al*., 2022). Therefore, application of different hormones may help the legumes not only to better fix the nitrogen, but also to enhance the resistance/tolerance to stresses. Plants are subjected to various stresses, such as salt, drought and pathogens but drought stress has received increased attention as it inhibits plant growth and development (Zhu, 2016). This phenomenon affects agricultural production. Hence, achieving soybean varieties with increased drought tolerance was a major goal of soybean breeding programs (Kumudini *et al*., 2001). Drought stress provokes plants to change their growth pattern and biochemical contents to overcome adverse situations (Fatema *et al*., 2023). Drought tolerance is a complex trait governed by multiple genes and is highly affected by environmental factors (Chitkara *et al*., 2022). In order to investigate the genetic mechanism of drought tolerance, we studied the level of expression of four genes after drought stress and challenged with SA, JA and simultaneous application of two hormones.

This research focuses on drought stress, hormones effect on the expression of genes involved in drought stress, and the drought stress effect on physiological traits and function of soybean cultivars. The results showed the application of two hormones (i.e., JA and SA) caused an increase in the expression of *VOZ1G* gene in Williams cultivar. Also, application of SA and MeJA caused a significant difference in some of the morphological traits. These morphological characteristics did not change significantly in the control plants, which did not receive any hormones. Increase in the expression of *VOZ1G* gene, under treatment with salicylic acid was more effective on shoot height and nodule formation of the Williams than the L17. A recent study has shown that *VOZ* proteins are relocated to the nucleus and rapidly degraded via the ubiquitin/26S proteasome system under certain stress conditions (Koguchi *et al*., 2017; Selote *et al*., 2018). In another study, several stress-related cis-acting elements were identified in the promoter region of *VOZ* genes. Based on the obtained results, these cis-elements are involved in responding to drought, salinity, low temperature, and pathogen attack. These observations suggest the

diverse roles of soybean *VOZ* transcription factors in plant responses to abiotic and biotic stresses. *VOZ1/2* also participates in regulating MeJA- and SA-mediated defense responses to pathogens in Arabidopsis (Nakai *et al*., 2013).

The results showed that overexpressing *DREB1* gene in soybean enhanced tolerance to drought. Application of two hormones increased the expression of *DREB1* gene under drought stress. The results showed the application of two hormones can increase the expression of *DREB1* gene in the resistant (Williams) cultivar more than the susceptible cultivar (L17). Increase in the expression of *DREB1* gene, under treatment with SA and JA was more effective on pod weight and harvest index of Williams cultivar than L17 cultivar. Recent studies have shown that *DREB* act as a transcription factor that interacts with the DRE/CRT (C-repeat) cis-acting element in the promoter region of stress-inducible genes. This interaction is involved in the expression of many stress-inducible genes in plants and can increase plants tolerance to drought, low temperature, high salt, and heat (Cai, *et al*., 2018). In another study, two genes DREB3a and DREB3b were reported as the key components involved in salt stress tolerance in soybean. Hou *et al.* (2022) reported 103 DREB gene in this family. These findings may indicate the importance of this family in stress tolerance of soybean.

In general, salicylic acid was more effective on shoot height and nodule formation of Williams than L17. In addition, using MeJA had a more significant effect on pod weight and leaf area than SA. Simultaneous use of both hormones had the most significant effect on important morpho-physiological traits, i.e., dry and wet weight of roots and shoots, and harvest index of the soybean cultivars. In regards to the gene expression results, the *BZIP* transcription factors were reported to associate with plant responses to hormones such as ethylene, abscisic acid (ABA), (SA), and (MeJA), which play important roles in plant resistance (Menkens *et al*., 1995). Similar results were observed in rice (*Oryza sativa* L.) which the drought-sensitive genotype raised with primed seeds with 100 µM of MeJA retained its morphology better than the tolerant genotype under drought condition (Sasi *et al*., 2021). Previous study showed that the expression of 15 members of the soybean *BZIP* family could be induced by salt and drought stresses. These results indicate that *BZIP* genes in soybeans play an important role in plant resistance to abiotic stresses (Wang *et al*., 2015).

GmNAC8 acts as a positive regulator of drought tolerance in soybean and inferred that GmNAC8 probably functions by interacting with another positive regulatory protein, GmDi19-3 (Yang *et al.,* 2020). The role of *NAC* gene in the regulation of drought stress response in soybean has also been studied by Hussain *et al*. (2017). Our study verified the response of the *NAC8* gene by analyzing the expression pattern of *NAC8* in leaf tissues under drought stress at the initiation of the reproductive growth stage in soybean. It was also observed that expression of the *NAC8* gene increased significantly in tissues and leaves by prolonging the duration of drought stress. Based on the results, it might be hypothesized that *NAC8* might respond to drought stress by regulating phytohormone signaling pathways. The role of the *NAC8* gene in increasing drought tolerance in Arabidopsis was investigated. The results revealed that transgenic plants have an increase in *NAC8* expression, they also showed an increase in drought tolerance compared to non-transgenic plants. The roles of the phytohormones SA, ABA, MJ and ethylene in the adaptation of plants to various abiotic stresses and pathogenic infection have been well characterized (Le Hir *et al*., 2003; Verma *et al*., 2016). Moreover, in a recent study, Wang *et al*. (2022) proposed the Triticale NAC transcription factor (*TwNAC01*) as a novel candidate transcription factor gene that can improve plant stress tolerance by increasing root length, regulating the water content of plant leaves by reducing MDA and H_2O_2 content, and adjusting respiration rate Wang *et al*. (2022).

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Conflicts of interest

Authors declare that they have no conflict of interest.

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