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Morphological, Biochemical, and Gene Expression Responses of Selected Pea (*Pisum sativum* L.) Varieties to Water-deficit Stress

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ABSTRACT

Water deficit is a destructive abiotic stress that limits crops' productivity and survival. In the current study, three pea varieties i.e., Climax, Green Grass, and Meteor were screened under 0% (control), 40%, and 70% water-deficit stress according to field capacity. The effects of stress at the morphological, biochemical, and molecular levels were studied; selected pea varieties showed significant differences in root length, shoot length, leaf area, leaf number, and fresh and dry biomass at 40% and 70% of water deficit. The chlorophyll, carotenoids, lipid peroxidation, and proline content were affected, in all three pea varieties. Overall maximum chlorophyll content was 19.9 mg/g in Climax variety at control while the minimum was 14.3 mg/g in Green Grass variety at 70% water deficit among all three varieties. The lipid peroxidation in all three pea varieties increased at 40% and had a maximum value at 70% water deficit. The maximum proline content of 0.0456 mg/100 g was observed for Meteor variety at 70% water-deficit stress while the minimum was 0.037 mg/100 g for Climax variety at control among all three varieties. Three water-deficit resistance-related genes i.e., *PsDREB2A*, *PsLoxG*, and *PsProC* were expressed differentially in all three pea varieties.

Keywords: abiotic stress, chlorophyll, field capacity, gene expression, pea, proline, water deficit.

INTRODUCTION

Pea (*Pisum sativum* L.) is a well-known leguminous crop, cultivated worldwide including Pakistan in Asia. Pea plant is primarily valued for livestock feed as well as for humans because the seeds of peas are an excellent source of proteins, folic acid, vitamins A, B, C, and K, antioxidants, fiber, starch, and low concentration of oils like phytosterols (Bastianelli et al., 1998; Rungruangmaitree and Jiraungkoorskul, 2017; El-Beltagi et al., 2024a). Pakistan is the seventh largest pea-producing country in the world. About 22.4 thousand hectares are used for pea cultivation with 6.43 tons/ha yield in Pakistan (FAO, 2017).

Due to the sessile nature of plants, pea is extensively subjected to various biotic and abiotic stresses. Water deficit stress affects the germination, survival, chemical composition, and productivity of pea plants (Salter, 1963; Maurer et al., 1968; Stoker, 1973; Pumphrey and Schwanke, 1974; Martin and Tabley, 1981). Water deficit induces physiological and biochemical responses like activation of respiration, stomatal closure, repression of cell growth, and photosynthesis within the plant (Araújo et al., 2015). The plants produce different metabolites to overcome abiotic stresses like water deficit. Moreover, the expression and regulation of various genes involved in water-deficit stress also play a vital role in

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crop protection. In response to water deficit, plants trigger the activation of various protective mechanisms in which the activation of various genes and proteins is inevitable. Several water deficit-induced genes at the transcription level have been recognized in numerous plant species. Multiple transcription factors are involved in the expression of genes for various abiotic factors, among which water-deficit responsible element binding proteins (DREBs) play a crucial part (Nayak et al., 2009). In plants, during water deficiency like various other protective mechanisms proline (amino acid) accumulation is also a well-defined phenomenon. It has been reported that the production and accumulation of proline enhanced during abiotic stresses including water deficit, salinity, elevated temperature, extreme light, and oxidative stress (Meringer et al., 2016). The precursor of amino acid proline is L-glutamic acid in all crop plants. The major enzymes involved in proline synthesis are pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) which is coded by *P5CR* gene. Proline is a useful and reliable parameter for measuring the plant's capability to stress conditions such as water-deficit stress. In addition, it plays a significant role in the osmotic adjustment and stability of different kinds of enzymes and proteins (El-Beltagi et al., 2024b). Lipoxygenases are iron-containing dioxygenases. They are extensively distributed in all plants and animals. PsLoxG gene belongs to the lipoxygenase family LOX and previous researchers have revealed that LOX is basically produced in seeds of plants (Siedow, 1991) and is also frequently produced in germinating seedlings but elevated production of LOX was also observed in plants other parts when subjected to abiotic stresses including water-deficit stress (Forster et al., 1994; Park et al., 1994; Porta et al., 1999). Many other genes are also involved in water deficit stress such as, Flavonoids (PAL1), Superoxide dismutase (SOD), Peroxidase (POX). PAL1 is gene involved in the flavonoids biosynthesis pathway that plays an important role in oxidative stress (Kumar et al., 2018). SOD and POX are involved in the biosynthesis of superoxide dismutase and peroxidase, respectively. Both these act as free radical scavenger and constitute in plant defense system against ROS (Rajput et al., 2021).

The water deficit stress induces morphological and physiological changes in crops and reduces crop yield. The effect of water deficit stress on morphology, physiology, and genes involved in these changes are least studied. The three pea varieties mentioned in current research have not been previously studied for both biochemical and gene expression study at the same time. This is first report as per our knowledge on these three varieties that will be of great importance to the pea growers and researchers. In the current research different levels of water-deficit stress according to the field capacity of soil were applied to three pea varieties i.e., Climax, Green Grass, and Meteor. The response of pea plants to water deficit was studied by considering seed germination, growth parameters, production of the resulting metabolites, and expression of the important genes responsible for producing these metabolites. The current study was designed with the objectives: 1. To evaluate the morphological and biochemical responses of the pea under water deficit stress and 2. To evaluate the expression of selected genes in pea under water deficit stress. These metabolite evaluations are necessary to overcome the detrimental effects of water-deficit stress.

MATERIAL AND METHODS

Soil collection and analysis

The soil was collected from the research area of COMSATS University Islamabad-Abbottabad Campus. The soil parameters like textural class, pH, EC, organic compounds, and moisture content were analyzed. Soil textural was determined by the hydrometer method (Moodie et al., 1959).

The type of soil was determined from a textural triangle (Fernandez-Illescas et al., 2001). Carbon and moisture content were determined by using the ignition method (Wright et al., 2008). Soil pH and electrical conductivity were determined using the pH

meter (model 7110) and the electrical conductivity meter (Combo, H198129), respectively.

Experimental layout

After soil analysis, the analyzed soil was transferred to twenty-seven different pots. Each pot of size 10x10 inches was filled with 2 kg of soil containing three-fourth part of soil and one-fourth of sand. The current research was conducted in the research field of COMSATS University Islamabad-Abbottabad Campus in 2022. The experiment was performed in the controlled conditons in green house in pots. Temperaure was kept as 25+2°C. Humidity was also properly controlled.

Seed collection and germination

Seeds of three pea cultivars i.e., Meteor, Green Grass, and Climax were collected from NARC (National Agriculture Research Centre, Islamabad) Pakistan. These seeds were surface sterilized with 70% ethanol and sown in 27 pots with 5 seeds in each pot. Three replicates of each variety (Climax, Green Grass, and Meteor) were sown in three pots. After nine to ten days seeds germinated and were left growing until they reached a growth stage for water-deficit stress (21 days).

Application of water-deficit treatments

Water-deficit treatments (40% water-deficit, 70% water-deficit, and control) were applied when seedlings reached 6-7 cm in height and had 2-3 leaf pairs on a shoot on 21 days of germination. The cultivar pots were divided into three groups and then irrigated according to different field capacities. The pots were set up as a completely randomized design.

Phenotypic traits analysis

The leaves were collected in the sixth week after water-deficit treatment and stored at -80°C until further analysis. Phenotypic data of all plants of three varieties (Climax, Green Grass, and Meteor) were recorded. Shoot and root lengths of pea varieties were measured using a transparent scale and mean shoot length and root length were calculated. The plants were uprooted carefully and washed with tap water, and root length was measured using a transparent ruler. The leaves were counted visually to find the number of leaves per plant. The leaf area (cm^2) was calculated by multiplying length and width. Each plant was weighed on a digital balance for fresh biomass, then dried in an oven at 60°C for three days and reweighed for dry biomass. The over view of the phenotypic presentation of the experiment is shown in Figure 1.

Figure 1. Overview of phenotypic presentation of the experiment;
seeds of pea (**A**), pea seedlings at 21 days (**B**), and root and shoot of pea varieties (**C**)

Biochemical analysis

Analysis of proline and chlorophyll content

Leaf samples were ground in sulphosalicylic acid (3%) and centrifuged at 3000 rpm for 10 min, then 2 ml of supernatant was collected. The glacial acetic acid, orthophosphoric acid (6M), and ninhydrin reagent (2 ml each) were added to the supernatant. The mixture was placed in the water bath at 100°C for an hour, given the ice shock for a few minutes, and left at room temperature. Optical density was measured at 520 nm and pure proline was used as standard. The proline content was estimated by comparing obtained values with the standard curve (Bates et al., 1973).

For chlorophyll, leaf samples were homogenized in mortar with 80% acetone. The extract was centrifuged at 5000 g for 5 min.

The absorbance of supernatants was recorded at wavelengths of 663, 645, and 470 nm spectrophotometrically (Mapada UV 1200), Shanghai Mapada Instruments Co., Ltd (Lichtenthaler, 1987). The quantification of chlorophyll a, chlorophyll b, and carotenoids was done using the formulae given below:

$$Chlorophyll\ a = 12.7(A663) - 2.69(A645)$$

$$Chlorophyll\ b = 22.9(A645) - 4.68(A663)$$

$$Total\ carotenoids = \frac{1000A470 - 1.82Ca - 85.02Cb}{198}$$

where C_a is chlorophyll a while C_b is chlorophyll b.

Analysis of lipid peroxidation

Leaves were ground using pre-chilled 1 ml of 5% trichloroacetic acid (TCA) to make a homogenized mixture. This homogenate was centrifuged for 10 min at 10000 g, and 1 ml of thiobarbituric acid solution was added to the collected supernatant. The mixture was heated at 95℃ for 30 min, then transferred to ice for 1min, and centrifuged for 10 min at 10000 g. The quantification was done by spectrophotometer, readings were taken at the absorbance of 450, 532 and 600 nm wavelength (Venkatachalam et al., 2017). Malondialdehyde content was determined using the formula given below:

$$MDA = \frac{[6.45 \times (A532 - A600)] - [(0.56 \times A450)]}{W} \times VT$$

where V_T = total volume taken as 0.001L and W = 0.1 g.

Molecular Analysis

RNA extraction and cDNA Synthesis

RNA was extracted by the CTAB method (Irfan et al., 2013). RNA was analyzed using 1% agarose gel electrophoresis. The gel was prepared by dissolving 0.3 g of agarose powder into 1 X TBE buffer (30 ml). 2-3 µl ethidium bromide (EtBr) was used to visualize the bands under UV light in a trans-illuminator. RNA was quantified at λ = 260 nm using a spectrophotometer (Mapada UV 1200), Shanghai Mapada Instruments Co., Ltd. Distilled water was used as a blank. The ratio of 260/280 was used to determine the purity of RNA using the following formula:

$$RNA\ conc.\left(\frac{ng}{\mu l}\right) = OD260 \times DF \times 40$$

where, $DF = \frac{total\ volume}{volume\ of\ RNA}$

cDNA was synthesised from RNA using the Enzynomics cDNA synthesis kit (TOPsccript^{TM}). cDNA was synthesized as 1000 ng/µl from the total RNA. RNA extract was mixed with autoclaved/sterilized water and incubated at 70℃ for 5 min. Oligo dT primers were added and the mixture was placed on the ice. The master mix used for all the reactions was made once, the amount of master mix used for each sample was 5.5 µl and the final volume was made as 20 µl. After adding the master mix to each sample, it was homogenized and incubated twice for 1 hour at 50℃ temperature and 5 min at 95℃. Then cDNA was stored at -80℃ for later use.

Designing primers and PCR analysis

Specific primers were designed for three genes i.e., pyrroline 5 carboxylase synthetase *(ProC,* accession number X62842.1), lipoxygenase (*LoxG,* accession number X76124.1), and dehydration-responsive element binding proteins *(*DREB2A, accession number HM229349.1), by using primer3 plus online available software. The *actin* gene was used as a reference gene. Primer sequences are given in Table 1. PCR was performed with cDNA as a template to check the gene expression. The total reaction volume for PCR was 10 µl containing cDNA (1 µl), each primer (0.8 µl), 6X Master mix having Taq DNA polymerase, $MgCl_2$, reaction buffer (2 µl), and ddH_2O (5.4 µl). The PCR product was run on 1% agarose gel prepared in TAE/TBE buffer. Samples were run for 45 min at 100 volts and the band size and intensity were compared with 1 kb ladder.

Table 1. Primers of genes PsProC, PsDREB2A, PsLoxG, and PsActin used in the current research work

Gene name	Accession Number	Forward primer	Reverse primer	Expected band size (bp)
PsProC	X62842	5' GGCTGATGGAGGAGTAGCAG 3'	5' GCAGCAACAACAGCATTCAT 3'	219
PsDREB2A	HM229349	5' ACAGAGGACTTGGGGGAAAT 3'	5' GTTAAGTCGCGCAGAAGGAC 3'	15
PsLoxG	X76124	5' CCTAAACAAGGGCCACAAG 3'	5' CTGATCAGCTGGAGGGAG 3'	192
PsActin	X90378	5’ CCACTTCTGCAGAGCGAGAA 3'	5' CGGAGATTCCATGCCGATCA 3'	215

Statistical Analysis

The experiment was carried out in a completely randomized design (CRD) with three biological replicates, each with three explants (n=3). The collected data were subjected to a one-way analysis of variance (ANOVA) using the IBM-SPSS software (version 26), with treatments and varieties as the main factors. The treatment means were compared using the least significant difference (LSD) test at $p<0.05$. Correlation coefficients, heatmap and principal component analysis (PCA) were performed with MetaboAnalyst software V. 5.0.

RESULTS AND DISCUSSION

Soil Analysis

The results of physio-chemical analysis of soil are summarized in Table 2. By using the International Soil Science Society (ISSS) classification system, the soil textural class was determined as loamy sand having more sand as compared to other particles (Table 2). The pH, EC_e, carbon content and field capacity are also given in Table 2.

Table 2. Characteristics of soil used in the experiment

Parameter	Value (Unit)
Soil textural class	Loamy sand (78% sand, 10% clay, 12% silt)
Soil pH	7.9
Electrical conductivity (µS)	275
Carbon content (%)	2.5
Field capacity (%)	37

Phenotypic Traits Analysis

Shoot and root length

The length of the longest root was measured and the mean was calculated. The shoot and root length of the three pea varieties i.e., Climax, Green Grass, and Meteor was decreased for 70% and 40% water-deficit stress. The maximum (14.7±1.7 cm) and the minimum shoot length (11±0.9 cm) were in Climax in control and Meteor in 70% water deficit, respectively (Figure 2A). The maximum root length (3.9±0.3 cm) was observed for Climax in control while the average minimum root length (2.3±0.5 cm) was observed for variety Meteor in 70% water deficit (Figure 2B).

Leaf number and size

The maximum 12 leaves per plant were in the variety Climax while the minimum 10 leaves per plant were in Green Grass (Figure 2C). Among the three varieties of pea plant the maximum leaf area was attained by variety Climax i.e., 2.6±0.36 cm^2 while the minimum leaf area was attained by variety Green Grass which was 1.8±0.28 cm^2. In all the three varieties of pea i.e., Climax, Green Grass and Meteor the maximum average leave size was for Climax i.e., 2.6±0.36 cm^2 while the average minimum leaf size of Climax was 1.9±0.11 cm^2 (Figure 2D).

Fresh and dry biomass

Variety Meteor and Green Grass both showed average maximum biomass of 1.33±0.01 g at control while this decreased to average minimum biomass of 1.03±0.13 g at 70% water-deficit stress. Variety Climax had the maximum average fresh biomass of 1.27±0.02 g in control, and an average minimum fresh biomass of 1.05±0.03 g at

70% of the water-deficit stress level (Figure 2E). The average maximum dry biomass of the Climax was found to be 0.13±0.01 g while the average minimum dry biomass for it was found to be 0.11±0.03 g in the variety Meteor. The maximum dry biomass was observed in the variety Green Grass while the minimum dry biomass was attained by the variety Meteor (Figure 2F).

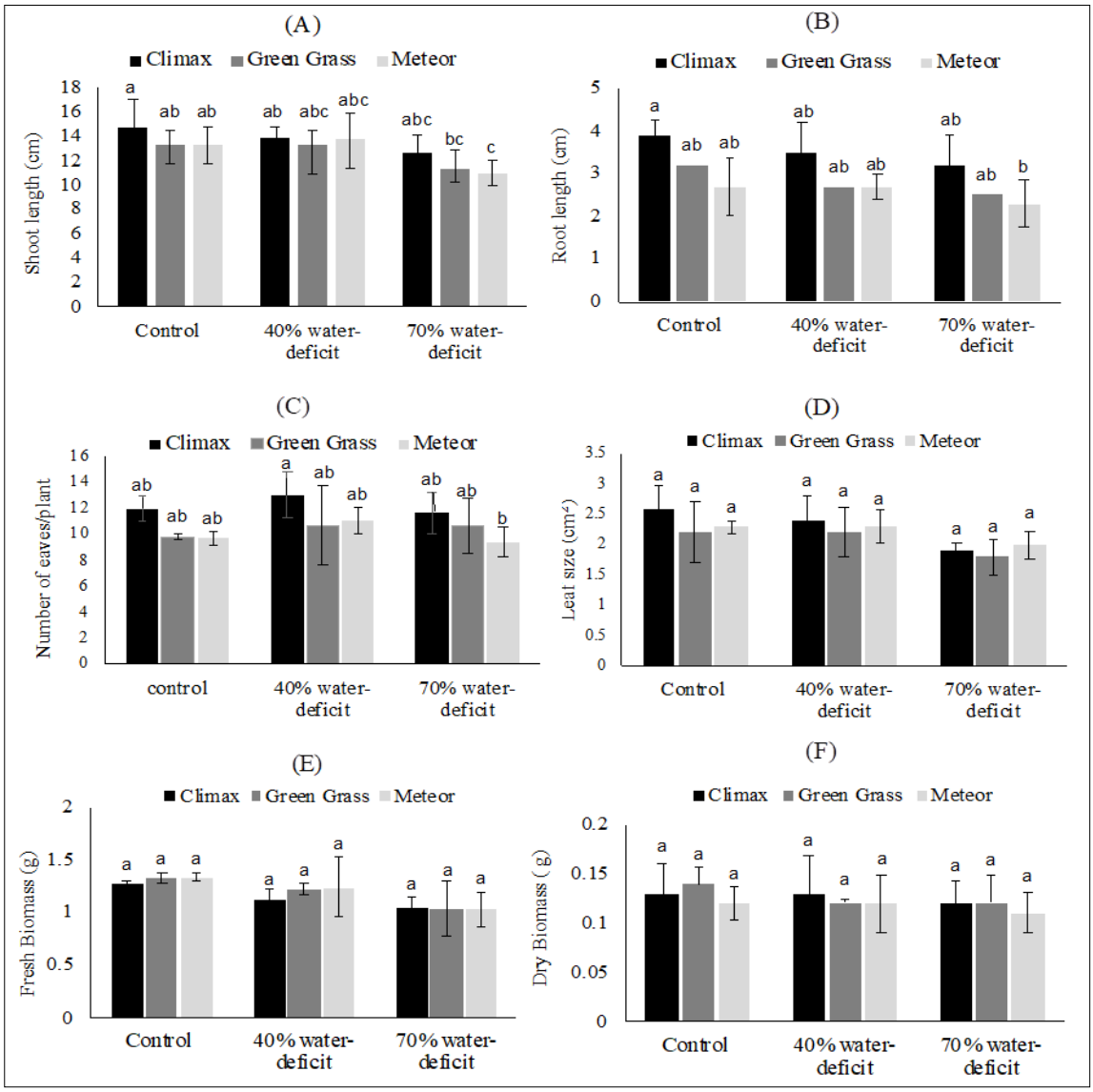

Figure 2. (**A**) shoot length; (**B**) root length; (**C**) number of leaves; (**D**) leaf size; (**E**) fresh weight; (**F**) dry biomass of pea (*Pisum sativum* L.) seedlings in response to water-deficit stress levels of 70% and 40%. Values within columns represent the mean of three replicates and means were separated using the least significant difference (LSD) test at $p<0.05$. The error bars are standard deviations of the mean among three replicates. Bars marked by different letters differ significantly ($p<0.05$) as revealed by the LSD test.

Biochemical Analysis

The average chlorophyll content was decreased slightly with 40% and 70% stress levels in all three pea varieties. Maximum chlorophyll a was 19.9±0.03 mg/g in Climax while the minimum 14.3±0.12 mg/g was in the variety Green Grass (Figure 3A). The chlorophyll b showed variations in all three levels of water deficit stress. The average maximum chlorophyll b 17.98±0.06 mg/g was in the variety Green Grass at the stress level of 70% and the average minimum chlorophyll b 9.37±0.00 mg/g was in the variety Climax (Figure 3B). The maximum average of carotenoids in the variety Green Grass was 4.6+0.08 mg/g in control while minimum average carotenoids were 1.98±0.08 mg/g in the variety Meteor (Figure 3C).

Analysis of proline and lipid peroxidation

The average maximum proline content in the variety Climax at 70% of water-deficit stress level was 0.044 mg/g (FW) and the average minimum proline content in the Green Grass at the control level was 0.037±0.002 mg/g (FW) (Figure 3A). With the increase in water-deficit stress the lipid peroxidation level increases as well. Hence the least value of MDA is shown by all the three varieties of pea used for experimentation at control. Climax possessed MDA concentrations of 0.0021 µM, 0.0023 µM and 0.0024 µM at control, 40% water-deficit and 70% water-deficit treatment, respectively. Green Grass contains MDA content of the concentration of 0.0025 µM at control while this amount fairly increased to 0.0032 µM at 70% water-deficit stress. Similarly, the lipid peroxidation content in Meteor was found to be 0.0028 µM at the control and this concentration fairly increased to the amount of 0.0032 µM at 40% water-deficit stress and to the amount of 0.0035 µM at 70% water-deficit level (Figure 3B).

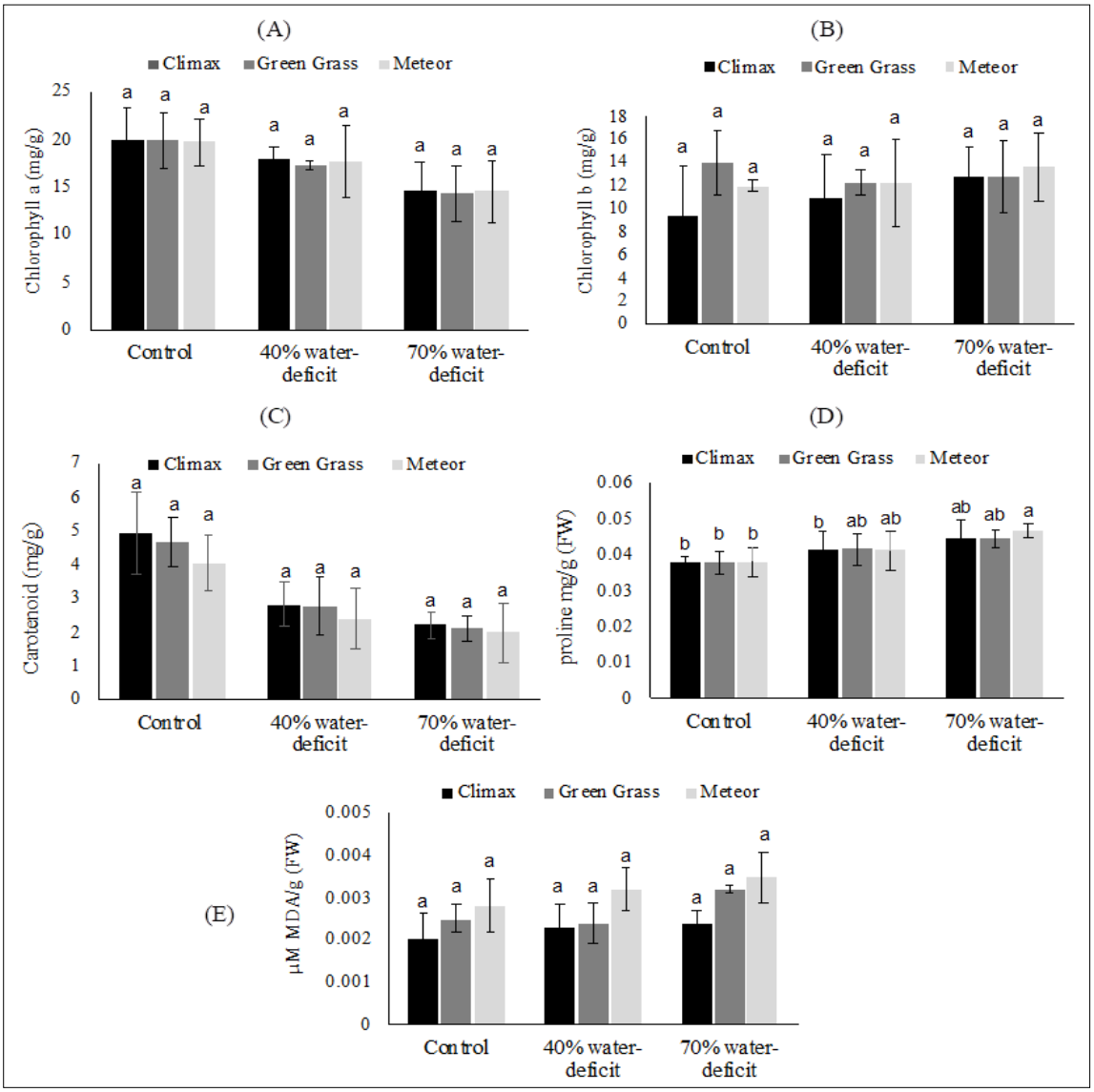

Figure 3. (**A**) chlorophyll a; (**B**) chlorophyll b; (**C**) carotenoids; (**D**) proline; (**E**) malondialdehyde (MDA) levels in pea (*Pisum sativum* L.) seedlings in response to water-deficit stress levels of 70% and 40%. Values within columns represent the mean of three replicates and means were separated using the least significant difference (LSD) test at $p<0.05$. The error bars are standard deviations of the mean among three replicates. Bars marked by different letters differ significantly ($p<0.05$) as revealed by the LSD test.

Gene Expression Analysis

Total RNA was extracted from pea leaves (Figure 4A). The purity of RNA was evaluated by reading taken from a spectrophotometer at 260 nm and 280 nm wavelength. The expression of three water-deficit stress-related genes i.e., *PsLoxG*, *PsDREB2A*, and *PsProC* is shown in Figure 4. The expression of gene *PsLoxG* is shown in Figure 4B, which showed that in variety Meteor its expression is increased in high water-deficit stress compared to control. Green grass shows not much change in expression of this gene while Climax showed increased expression at 70% water-deficit conditions. The expression of gene *PsDREB2A* was not prominent in Meteor in all treatments (Figure 4C) while Green grass showed increased expression at 40% and 70% water-deficit stress. The variety Climax also showed stable expression of gene *PsDREB2A* (Figure 4C). PsProc gene was also not differentially expressed in varieties Meteor and Green grass at various water deficit stresses, however in Climax there was increased expression at the highest level of water deficit stress i.e., 70%. Figure 3D indicates the amplified product of gene *PsDREB2A* in all three varieties of pea at control 40% and 70% of stress levels. Expression of the *PsProC* gene was analyzed in the pea leaves of all three varieties at control as well as 40 and 70% of water-deficit stress levels (Figure 4). The housekeeping gene *PsActin* was used as a reference gene (Figure 4E).

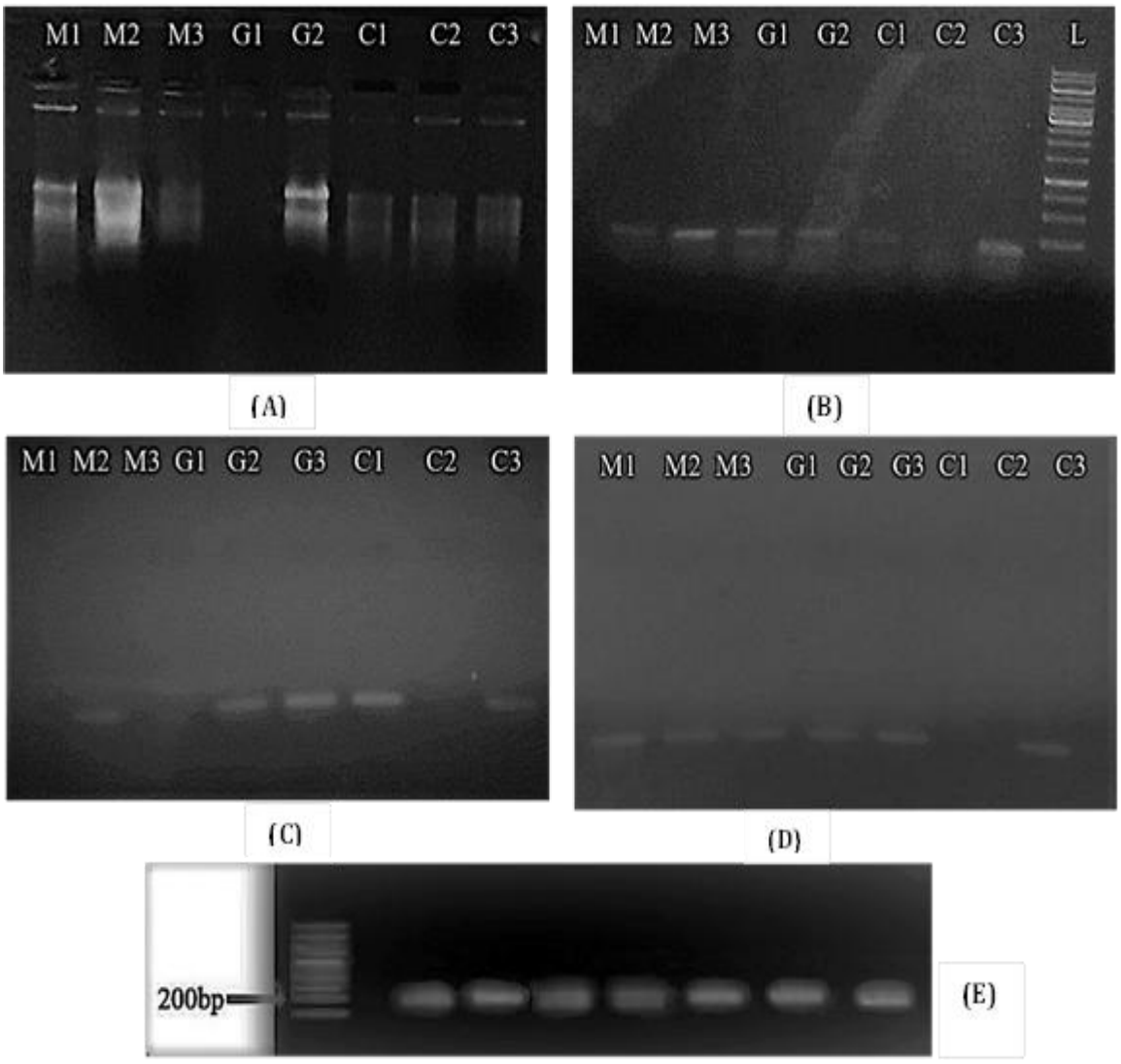

Figure 4. Gene expression analysis of water-deficit responsive genes in pea varieties Climax, Green Grass, and Meteor: (**A**) Total RNA extracted from pea leaves; (**B**) PCR product of PsLoxG gene at 40% and70% water-deficit stress level; (**C**) PCR product of gene PsDREB2A in pea at different water-deficit levels; (**D**) PCR product of gene PsProC in three varieties of pea under water-deficit stress; (**E**) PCR product of the housekeeping gene PsActin as reference gene.

Correlation among related traits

The relationship between various traits related to pea varieties is measured by correlation coefficient analysis as illustrated in Table 3. In the present study, correlation analysis indicated a significant positive association between different agronomic and biochemical traits. There was a strong positive correlation between MDA and Ch.b (r=0.94**) while a medium positive relationship exists between; CAR with Ch.b (r=0.71**); CAR with MDA (r=0.74**); Ch.a (r=0.57**) and FW with Ch.a (r=0.52**) as well as LS with SL (r=0.63**). The traits MDA showed moderate positive correlation with PR (r=0.40**), FW with PR (r=0.47**) Ch.a with Ch.b (r=0.38**) and with MDA (r=0.44**) and FW (r=0.52**). The RL showed a moderate positive correlation with PR (r=0.38**) and SL (r=0.43**) as well as DW with No.L (r=0.52**). While a lower to moderately significant association was observed in PR with Ch.b (r=0.32**); MDA (r=0.40**); and RL (r=0.38**); Ch.a with Ch.b (r=0.39**); FW with CAR (r=0.39**).

Table 3. Correlation analysis among the investigated agronomical and biochemical traits of three pea varieties under 40 and 70% of water-deficit

	PR	Ch.b	MDA	FW	Ch.a	CAR	SL	LS	RL	No.L	DW
PR	1.00										
Ch.b	0.32*	1.00									
MDA	0.40**	0.94**	1.00								
FW	-0.47**	-0.14	-0.21	1.00							
Ch.a	-0.30*	-0.38**	-0.44**	0.52**	1.00						
CAR	-0.43**	-0.71**	-0.74**	0.40**	0.57**	1.00					
SL	-0.22	0.16	0.12	0.31*	-0.13	-0.06	1.00				
LS	0.04	0.04	0.08	0.24	0.22	0.05	0.63**	1.00			
RL	-0.38**	-0.02	-0.06	0.29*	0.26	0.10	0.43**	0.31*	1.00		
No.L	-0.24	-0.30*	-0.21	0.09	-0.09	0.04	0.11	0.14	0.38**	1.00	
DW	-0.45**	-0.12	-0.18	0.45**	0.03	0.24	0.24	0.12	0.29	0.52**	1.00

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).
SL, shoot length; Ch.a, chlorophyll a; Ch.b, chlorophyll b; PR, proline; CAR, carotenoids; MDA, lipid peroxidation level; DW, dry weight; FW, fresh weight; RL, root length; No.L, number of leaves and LS, leaves size.

Heatmap and hierarchical clustering

Heatmap hierarchical clustering was carried out to identify the pea varieties-water-deficit stress association (Figure 5). Pea varieties under water-deficit stress were placed on the Y-axis, whereas all investigated traits were placed on X-axis (Figure 5). All pea varieties were grouped into two major clusters (A and B), and the A cluster was subdivided into two sub-clusters labelled 1 and 2 (Figure 5). The varieties Green Grass (GG), Climax (Cl) and Meteor (M) under 40% of water-deficit stress were clustered together. Whereas, GG control and M control were clustered together, and CI control clustered alone in a single cluster. The investigated traits were grouped into two major clusters and these clusters were sub-grouped into four sub-clusters labeled 1, 2, 3 and 4 (Figure 5). The results of clustering indicated a distinct separation of pea varieties under water-deficit stress in comparison to the control.

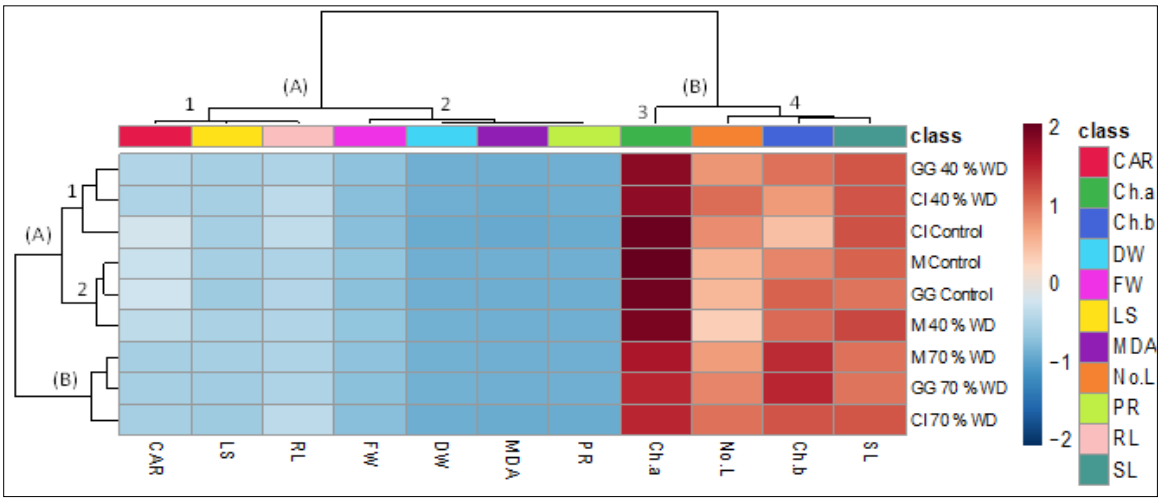

Figure 5. Heatmap hierarchical cluster analysis based on eight phenotypic and biochemical traits of three pea varieties under 40 and 70% of water-deficit. Pea varieties under water-deficit stress were placed on the Y-axis, whereas all nine traits were placed on X-axis. Colors indicate high (red) and low (blue) associations between pea varieties and investigated traits. GG, Green Grass; Cl, Climax; M, Meteor; WD, water-deficit; SL, shoot length; Ch.a, chlorophyll a; Ch.b, chlorophyll b; PR, proline; MDA, lipid peroxidation level; CAR, carotenoids; DW, dry weight; FW, fresh weight; RL, root length; No.L, number of leaves and LS, leaves size.

Multivariate analysis of the phenotypic and biochemical parameters

To better understand the correlation between different water-deficit stress treatments and measured traits, an integrative analysis was carried out by applying a Principal Component Analysis (PCA) based on all the parameters measured as shown in Figure 6, which is consistent with the results obtained in the heatmap and hierarchical clustering illustrated in Figure 5. Two PCA clusters were generated relative to the data regarding parameters (Figure 6A) and reaction of pea varieties to water-deficit stress (Figure 6B). The two principal components (PC) contributed to 96.3% (in which 92.8% belongs to PC1 and 2.5% belongs to PC2) of the observed variability for measured parameters and 77.5% (in which 52.8% belongs to PC1 and 24.7% belongs to PC2) of the reaction of pea varieties to water-deficit level.

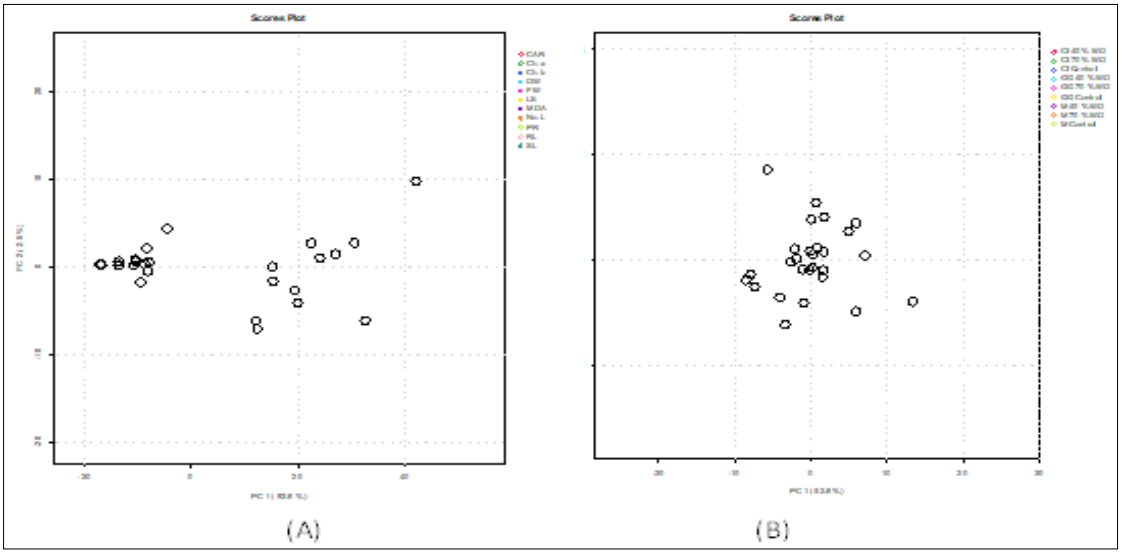

Figure 6. PCA clustering generated using all measured parameters collected from the three pea varieties in response to water-deficit stress. (**A**) PCA plot for the measured parameters; (**B**) PCA plot for pea varieties-water-deficit stress. GG, Green Grass; Cl, Climax; M, Meteor; WD, water-deficit; SL, shoot length; Ch.a, chlorophyll a; Ch.b, chlorophyll b; PR, proline; MDA, lipid peroxidation level; CAR, carotenoids; DW, dry weight; FW, fresh weight and RL, root length; No.L, number of leaves and LS, leaves size.

Crop yield is affected by various biotic and abiotic factors, there is a need to select the varieties of crops with higher yield under these stresses to meet the increased demand for food with an increasing human population (Ximénez-Embún et al., 2016). The low annual precipitation is a major abiotic stress that affects the growth and productivity of plants due to low soil moisture contents. The reactive oxygen species (ROS) cause oxidative damage in many plants and impairment of many metabolic functions of plants like protein and carbohydrate metabolism. Water-deficit stress like all abiotic stresses stimulates the overproduction of ROS (Gill and Tuteja, 2010).

Water-deficit stress causes stomatal closure to reduce extensive water loss, this decreases photosynthesis rate which affects many physiological functions of plants. Water-deficit is the main abiotic factor and reduces crop yield in many parts of the world (Boyer, 1982). It has been observed that abiotic stresses cause more than 50% reduction in crop yield (Vinocur and Altman, 2005), and about 45% of total world agricultural land is affected adversely by water stress (Ashraf and Foolad, 2007). Many plants produce self-protective mechanisms to cope with environmental stresses such as the production of many osmolytes like glycine betaine, proline, polyols, and phytohormones like abscisic acid and jasmonic acid (Sharma et al., 2012).

Pakistan is the seventh largest pea-growing country, pea is cultivated in an area of about 22.4 thousand hectares with an average yield of about 6.43 tons/ hectare (FAOSTAT, 2017). Pea plant when exposed to drought i.e. the leaf water potential of approximately -1.3 MPa indicates about a 78% decrease in photosynthesis and approximately 83% decline in transpiration and an 18% reduction in the carotenoids, chlorophyll a, and soluble proteins concentration (Moran et al., 1994). It has been observed that proper watering of pea plants enhances 26% of their yield (Podsiadlo et al., 1999).

This research was conducted to investigate the effects of water deficit on the plant morphology, stress-related metabolites, and expression of the associated genes on the three pea varieties i.e., Meteor, Green Grass and Climax. Enhanced water-deficit stress causes a decrease in stomatal conductance due to the secretion of ABA and reduces the size of all vegetative parts of a plant like the leaf area. in the small leaf area results in stunted plant growth due to reduced photosynthesis. The three pea varieties showed a decrease in the root length, shoot length, and leaf area with increased stress level for field capacity i.e., 40% and 70% of water-deficit stress.

The fresh and dry mass of all three pea varieties Climax, Green Grass, and Meteor decreased considerably with an increase of 40% and 70% in water-deficit stress compared to the control. During applied stress, stomata close to keep an internal osmotic environment. This slows down photosynthesis and other metabolic processes and decreases plant biomass. Water-deficit stress lowers the most important metabolic and physiological functioning of all plants including peas and the fresh and dry biomass are reduced (Gill and Singh, 1985; Ravi et al., 2011).

There was an increase in proline in all three pea varieties with the increasing water-deficit stress. Researchers have pointed out that proline metabolism affects the signaling process by affecting reactive oxygen species production in the mitochondria through the electron transport chain (Xinwen et al., 2013). The average maximum proline content was observed in the pea variety Meteor at 70% water-deficit stress and the average minimum proline content was observed in Climax and Green Grass at control level. Current research work confirms the results of many previous researchers that proline content shows elevation in its quantity at stress conditions (Kiyosue et al., 1996; Ahmad et al., 2017).

The chlorophyll content was observed under water-deficit stress in three pea varieties i.e., Climax, Green Grass, and Meteor at control as 40% and 70% of water-deficit stress and it was investigated that chlorophyll “a” content

also showed a decrease in its amount with an increase in stress level i.e., in all the three varieties of pea. The maximum chlorophyll concentration was 19.9 mg/g at control for Climax and Green Grass while the minimum chlorophyll "a" content was 14.3 mg/g for Green Grass at 70% water-deficit stress. However, chlorophyll "b" indicates variation in its results that is maximum chlorophyll content was observed in the variety Green Grass at 70% of water-deficit stress, and the minimum chlorophyll "b" was found in variety Climax at the control level. The maximum carotenoid content was calculated to be 4.64 mg/g at control while its minimum concentration was found to be 1.98 mg/g at 70% water-deficit stress. It was investigated that water-deficit stress causes a decline in the chlorophyll content and causes stomatal closure to prevent extensive water loss, which consequently reduces the gaseous exchange thus by reducing chlorophyll content and lower stomatal conductance the functioning of the whole plant more proficiently.

Lipid peroxidation was also analyzed in all three pea varieties at control, 40%, and 70% of water-deficit stress and a considerable increase in MDA contents in all three varieties of peas i.e. Meteor, Green Grass, and Climax at 40% and 70% of water-deficit stress as compared to control. In the current research work the effects of water-deficit stress were investigated the maximum MDA contents were observed in variety Meteor which is about 0.0035 at 70% of water-deficit stress other two varieties also have increased production at 40% and 70% of stress level compared to control. The increase in MDA levels during stress as well. Thus the current research work confirms the previous findings of many researchers that MDA content shows an increase during water-deficit stress (Nikolaeva et al., 2010; El-Beltagi et al., 2022a,b).

Expression analysis of three water-deficit related genes i.e., *PsDREB2A, PsLoxG,* and *PsProC* in all the three pea varieties i.e., Climax, Green Grass, and Meteor was analyzed at control and under 40% and 70% of water-deficit stress according to field capacity. The gene *PsDREB2A* belongs to the dehydration-responsive element binding proteins DREBs which was studied as it has a vital role in signaling during many abiotic stresses (Nayak et al., 2009). The DRE dehydration-responsive element was recognized as *a cis-acting* promoter binding element which is involved in the regulation of many genes in response to abiotic stresses including water-deficit stress (Sakuma et al., 2006). In the current research work the expression of this gene *PsDREB2A* was studied under water-deficit stress of different treatments this gene is expressed equally in the variety Climax, Green Grass, and pea variety Meteor, in Meteor at 40% of water-deficit stress no clear result was obtained but PsDREB2A showed expression in all the three pea varieties. Thus, *PsDREB2A* is strongly induced during water shortages in pea leaves. The gene *PsLoxG* was also analyzed in all three pea varieties at control 40% and 70% of stress levels. *PsLoxG* belongs to the lipoxygenase family LOX and previous research has revealed that LOX are produced in seeds of plants (Siedow, 1991) and also frequently produces in germinating seedlings but elevated production of LOX was also observed in other parts of the plant when subjected to wounds or abiotic stresses including water-deficit stress (Forster et al., 1994; Park et al., 1994; Porta et al., 1999). In the current research work LOX gene was analyzed in three pea varieties i.e., Climax, Green Grass, and pea variety Meteor the water-deficit stress at the control level as well as at 40% and 70% of water-deficit stress level and *PsLoxG* was expressed in the leaves of all the three pea varieties high level of expression was shown by Meteor at 40% of water-deficit stress, while the expression level was less in variety Green Grass at control level while at 40% of stress, Green Grass also exhibit more expression. The gene *ProC* responsible for proline content and was given the name *PsProC* was also investigated in all three pea varieties named Climax, Green Grass, and Meteor at control level as well as 40% and 70% of water-deficit stress concerning field capacity. The *ProC* gene is responsible for P5CR pyrroline-5-carboxylase reductase which is responsible for proline content. It was revealed that this amino acid

accumulation occurs in water deficit or salt enabling plants to withstand harsh environmental conditions. It has osmo regulatory functions and was observed that expression of this gene increased to many folds in salt and water-deficit stress conditions (Williamson and Slocum, 1992).

CONCLUSIONS

It is concluded that water affects pea crops at morphological, biochemical, and molecular levels. The three varieties of peas i.e., Climax, Green Grass, and Meteor used for this research showed a decrease in phenotypic traits, especially at 70% stress. A decrease in chlorophyll content while an increase in proline content was observed under the high level of water deficit. In molecular studies, the three genes showed differential expressions in all three varieties.

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