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Impact of Salt Stress on Biochemical and Morphological Traits as well as Expression of *GR*, *GST* and *MapK3* Genes in Three Pea (*Pisum sativum* L.) Varieties Under Hydroponic System

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ABSTRACT

Salt stress is a global issue for agricultural crops including pea. In this research, the effects of three different levels of salt stress (NaCl) i.e., 50 mM, 75 mM and 100 mM were applied to these varieties of pea namely Climax, Green grass and Meteor in hydroponic system. Morphological and biochemical traits were investigated. The expression of important salt responsive i.e., *GR*, *GST*, *MapK3* genes was also studied. Green grass depicted maximum reduction in plant height (9.03 ± 0.55 cm) while, Climax showed more plant height (13.67 ± 0.58 cm) on the 100 mM salt stress while root length (5.40 ± 0.26 cm) was minimum in Green grass variety and maximum (7.37 ± 0.12) in Climax in response to 100 mM NaCl. The interecation response to plant height and root length was statistically non significant. Variations in total phenolics, flavonoids and proline contents were statistically significant for the ineterction effects. Total phenolics content was the highest in Green grass variety (1.5 ± 0.07 mg GA/100g FW) while minimum (1.05 ± 0.08) in variety Climax at 100 mM treatment, while elevated levels of total flavonoids and proline contents were found in Climax variety i.e., 0.20 ± 0.004 and 0.037 ± 0.001 respectively) at 100 mM. Proline content (0.021 ± 0.0004) in low amount was found in Climax at 0 mM NaCl. The lowest total flavonoids content was found in Green grass at 50 mM. The expression level of *GR*, *GST*, *MapK3* genes increased significantly under higher salt stress (100 mM) compared to control. *MapK3* gene showed a higher expression in all the treatments as compared to the other two genes. The salt stress-responsive *GR*, *GST*, *MapK3* genes also showed an upregulation with increasing salt stress that probably help pea plant to survive under such unfavourable environmental conditions by producning the underlying metabolites.

Keywords: flavonoids, genes, gene expression, pea, phenolics, proline, salt stress.

INTRODUCTION

Pea (*Pisum sativum* L.) is an edible winter crop and is used as a biochemical and genetics model for plant studies (Bräutigam et al., 2008). The pea was the second most cultivated crop in 2017 with an estimated annual production of 33290.6 thousand tonnes on an area of 4241 thousand hectares globally, while in Pakistan the production was 151.7 thousand tonnes with an area of 23.8 thousand hectares (FAOSTAT, 2019). Pea seeds are a major source of different types of nutrients i.e., carbohydrates, fibers and proteins (10-50, 15-32, 6-13%, respectively), along with different minerals, and vitamins (A, B and C), therefore, pea is a major component of human diet and animal

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feed (Arnoldi et al., 2015). Secondary metabolites such as isoflavonoids having anti-cancer and health-promoting role accumulate in legume plants (Dixon et al., 2003). Pea is a significant legume crop in the Pakistan as it plays an important role in the economy with an enormous commercial demand because of its nutritional value and pleasant taste (Zia-Ul-Haq et al., 2013).

Salinity, amongst other stresses, is one of the main abiotic stress that decreases plant growth, yield, and quality across the world (Qin et al., 2010). About 20% of the total cultivated land and 33% of the irrigated land is affected from salinity in the world (Rui and Ricardo, 2017). There is 10% increase in saline area annually due to low precipitation, high surface evaporation, weathering of innate rocks, irrigation with salty water, and poor cultural practices. It is predicted that by 2050, 50% arable land will become saline (Ashraf, 2009; Jamil et al., 2011) and an estimated loss of around 12 billion US$ has already been reported in agricultural production from those lands, which are affected by salinity (Flowers et al., 2010).

In Pakistan, salinity affects around 25% of all irrigated land, out of which about 7% (1.5 million ha) is highly saline and causing a crop loss of approximately 15-55 billion rupees (Corbishley and Pearce, 2007). Pea plant is more salt-sensitive as compare to soybean and faba beans (Nenova, 2008). It has been reported that 100 mM of salt stress (NaCl) decreases pea crop production by 50% (Subbarao and Johansen, 1999), and as the amount of salt stress rises, the crop production decreases (Najafi et al., 2007). Furthermore, 80 mM NaCl stress causes deficiency of K^+ and accumulation of Na^+ in the leaves of pea plant (Pandolfi et al., 2012).

Phenolics have shown an affinity towards scavenging free radicals such as reactive oxygen and nitrogen species and protect and strengthen plants against different pathogens (Dai and Mumper, 2010; Hussain et al., 2013). Similarly, flavonoids being signal molecule, UV filters, and freezing tolerator, provide protection against different biotic and abiotic stresses including drought and salinity (Scandalios, 2004; Farooq et al., 2021). On the other hand, proline accumulation in plants is a common adaptive response in several abiotic stresses that helps to maintain different metabolites, redox balance and to control the expression of various genes hence acts as a signaling and regulatory molecule (Szabados and Savoure, 2010).

Different genes such as *Glutathione reductase (GR), Glutathione transferases*, and *MAP kinases* in prokaryotes and eukaryotes, not only control the responses against salinity stress but also provide tolerance (Zhang et al., 2013). Multiple forms of *GR* are found in plants, i.e., eight different forms of GR in pea (Edwards et al., 1990) and two forms in wheat (Dalal and Khanna-Chopra, 2001). *GR* isoforms are localized in chloroplasts, cytosol, and mitochondria (Ashraf, 2009), and are stimulated by diverse environmental signals and perform various functions under various environmental stresses (Rao and Reddy, 2008). For example, GR participates in the maintenance of a negative E_{GSH} (i.e., high GSH/GSSG) to support basic processes in the cytosol, mitochondria, and in chloroplast stroma during light. Also, it is essential for the proper function of the glutathione/ascorbate cycle (Foyer and Noctor, 2011; Gill et al., 2013) and provides GSH that is needed for MG detoxification and for deglutathionylation of proteins by glutaredoxins.

The genes *Glutathione transferases*/ *Glutathione S-transferases* (*GSTs)* play an important role in the detoxification of herbicide, hydrogen peroxide, hormone homeostasis, metabolism of tyrosine, apoptosis regulation, and responses to biotic and abiotic stresses (Dixon et al., 2010). The *Mitogen Activated Protein Kinases* (*MAPKs)* belong to a large serine threonine genes kinases family and cascade involves in transducing extracellular signals to the nucleus for an appropriate cellular adjustment (Sinha et al., 2011). They contribute in various signaling pathways induced by biotic and abiotic stresses including; low temperature, drought, salt and pathogens (Ding et al., 2009).

It is important to study the effects of salinity on morphological, biochemcail and

molecular parameters of pea. Expression of *GR*, *GST* and *mutagen-activated kinase 3 (MapK3)* genes, determination of phenolics and flavonoids under salt stress in pea have not been investigated yet to our knowledge that need to be studied.

MATERIAL AND METHODS

Seeds collection, germination and hydroponic culture

Seeds of three pea varieties i.e., Climax, Green grass and Meteor were purchased from the certified Kisan Agro Seed and Fertilizer shop, Mansehra, Pakistan. Seeds were soaked for 12 hrs in 0.5 mM $CaSO_4$ at room temperature before germination. Sandwich method described by Shahzad et al. (2012) was used for germination. Briefly, soaked seeds were placed on the foam with wet tissue paper on it at an equal distance and foam was rolled. The sandwiches were placed in the bottles half-filled with 0.5 mM $CaSO_4$ solution and incubated at 28°C for approximately 5 days. After five days, seedlings were placed in sunlight for one day before shifting to the hydroponic system. Hydroponic setup comprised of 36 pots (4 litres capacity of each pot) for three varieties of pea plants, five seedlings were placed in each pot and each pot was connected to an aeration pump through a pipe. The micro and macronutrients were supplied to plants, on the first day one-fourth of full concentration while, second day, the concentration was increased to one-half and left the media for one day. On the fourth day media from each pot was replaced with fresh media with full concentration of growth nutrients and plants were left for two days without changing any media. After each week media were replaced with fresh one with full nutrients composition. This process was repeated until the plants were grown enough and ready for salt stress application after 20 days. The nutrients composition of hydroplonic system are given in Table 1.

Table 1. Details of nutrients in hydroponics system

Nutrient	Substance	Molecular weight (g)
Phosphorous (P)	KH_2PO_4	136.09
Potassium (K)	K_2SO_4	174.27
Chlorine (Cl)	KCl	74.5
Calcium (Ca)	$Ca(NO_3)_2$	236.15
Magnesium (Mg)	$MgSO_4$	246.48
Sodium (Na)	NaCl	58.4
Iron (Fe)	Fe-EDTA	367.05
Boron (B)	H_3PO_3	61.83
Manganese (Mn)	$MnSO_4$	169
Zinc (Zn)	$ZnSO_4$	287.54
Copper (Cu)	$CuSO_4.5H_2O$	249.68
Molybdenum (Mo)	$(NH_4)_2Mo_7O_{24}$	1235.86

Preparation and application of salt treatments

Stock solution of NaCl (5M) was prepared and used to prepare three diluted concentrations i.e., 50 mM, 75 mM, and 100 mM. These concentrations of salt solution were applied as stress treatments to plants in triplicate and applied in increments in the same way as for nutrients. Plants were left for approximately one week after the application of full salt treatments. A control (without NaCl) treatment was also there for comprasion purpose.

Morphological traits data collection

The plant height, root/shoot length were measured by using a scale and total leaf area of each variety (Climax, Meteor, and Green grass) was calculated by using imageJ software after one week of full salt stress treatment.

To measure moisture content and dry biomass, fresh plants of each variety were taken and weighed and fresh plants were wrapped into the labeled paper bag and placed into the incubator at 70°C for 3 days till constant mass was obtained. Samples were completely dried and weighed by a previously reported method of Guo et al. (2012). The percentage decrease in weight was shown as percentage moisture content and was calculated by using the formula given below:

$$\text{MC (\%)} = \frac{W_1 - W_2}{W_1} \text{X } 100$$

where, MC is (% of moisture content), W_1 (fresh sample weight), W_2 (dried sample weight). Likewise, percentage of dry content was estimated using formula:

$$\%\text{Dry content (DC)} = 100 - \text{MC}$$

Molecular analysis

RNA extraction

After one week of stress treatment, the leaves of each plant were pricked by using a sterilized blade and placed in the labeled zipper bags on ice to avoid enzymatic degradation before storing at -80°C. Leaves from each treatment were taken and were ground to fine powder in liquid nitrogen using autoclaved pestle and mortar. Then ground samples were put into the labeled 1.5 ml eppendorf tubes and stored at -80°C. Total RNA extraction from leaves was done by using CTAB protocol as reported by Irfan et al. (2013). Briefly, 200 mg of plant material was taken into the 1.5 ml eppendorf tube and 600 µl of 10% preheated CTAB buffer was added and incubated at 65°C for 15 min. The mixture was centrifuged at 12000 rpm for 15 min at 4°C and supernatant was collected in the new eppendorf tube and 600 µl of chilled absolute chloroform: Isoamylalcohol (24:1) was added to it. This mixture was recentrifuged at the same conditions as mentioned above, supernatant was collected and one-fourth of 10 M lithium chloride was added to supernatant and kept at -20°C for overnight. On the next day, the sample was again centrifuged at 6500 rpm at 4°C for 15 min, supernatant was discarded and 500 µl of 70% ethanol was added to wash the pellet and centrifuged at 6500 rpm for 5 min. Then ethanol was evaporated, and the pellet was air dried by placing eppendorf tubes on tissue paper. Pellet was resuspended into 40 µl of DEPC water.

cDNA synthesis and designing of primers

cDNA synthesis was done from RNA by using the Enzynomics cDNA synthesis kit (*TOPsccript*™). cDNA was synthesized as 1000 ng/µl from the total RNA. RNA extract was mixed with autoclaved/sterilized water and incubated at 70°C for 5 min. Oligo dT primers were added and mixture was placed on the ice. The amount of master mix for each sample was 5.5 µl and the final volume was made as 20 µl. After adding the master mix into each sample, it was first homogenized and incubated twice for 1 hr at 50°C temperature and 5 min at 95°C. Then cDNA was stored at -80°C for later use. Specific primers of three genes i.e., *Glutathione reductase (GR), Glutathione S-transferase (GST)* and *Mitogen activated protein kinase 3 (MapK3)* were designed with the help of primer3 plus online software. These primers sequences are given in Table 2.

Table 2. Primers used for expression of *GR*, *GST* and *MapK3* genes

Gene name	Accession number	Forward primer	Reverse primer
Glutathione Reductase	X98274.1	5' ATATGGGCTGTGGGTGATGT 3'	5' CTGCTCTACTGCCTGCTCCT 3'
Glutathione S-transferase	AB087837.1	5' CTTGGAGAATGCCCTTGGTA 3'	5' CTTGTTCAGCTCCTCGATCC 3'
Mitogen-activated Kinase 3	AF153061.1	5' TGGCCGGAGTTAATCAAAAC 3'	5' CGTTTGTTCGCTCTTGAACA 3'

The expected PCR product size for *GR* (X98274.1) gene was 192 bp, for *GST* (AB087837.1) gene was 181 bp and for *MapK3* (AF153061.1) gene was 174 bp. *Actin* gene was used as a control. To check the gene expression, semi-quantitative PCR

was performed with cDNA as template. 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 double distilled water 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 was compared with 1 kb ladder.

Determination of phenolics, flavonoids and proline contents

Extract Preparation

To prepare methanolic extracts, 1 g of each fresh sample was ground in liquid nitrogen to make a fine powder and transfered to a falcon tube (15 ml) containing 10 ml of methanol and kept in a shaker overnight. Falcon tubes were vortexed for 5 min and centrifuged for 5 min at 9000 rpm. The extract was collected in a labeled falcon tube and the whole process was repeated three to four times as described by Vaghasiya et al. (2011).

Phenolics content

Total phenolic content was determined by FolinCiocalteu method as reported by Lin and Tang (2007). Briefly, 5 ml of FolinCiocalteu reagent was added to 1 ml of plant extract followed by 2 ml of 6% Na_2CO_3 solution and incubated for 90 min in dark. The total phenols were determined at 760 nm wave length, change in color showed the presence of phenolics (Mc Donald et al., 2001). Three replicates of each sample were taken and measured levels of total phenolic content (TPC) was expressed as mg Gallic acid equivalents in 100 g of the fresh sample (mg GAE/100 g, FW). Different concentrations of Gallic acid (100 ppm in methanol) i.e., 5, 10, 20, 40, 60, 80 and 100 ug/ml were used to make a standard curve.

Total Flavonoids content

For the flavonoids determination, colorimetric method was used as explained before (Lin and Tang, 2007). Briefly, 1 ml of plant extract was mixed with 0.5 ml sodium nitrate and 0.5 ml of aluminum chloride and mixture was incubated at room temperature for 5 min followed by 2 ml of NaOH solution. UV-visible spectrophotometer was used to record absorbance at 510 nm. The total flavonoid content (TFC) in triplicates was recorded as milligram quercetin equivalent per 100 g (mg QE/100 g) of the sample. Different concentrations of quercetin solution (100 ppm in methanol) i.e., 5, 10, 20, 40, 60, 80 and 100 ug/ml were used to make a standard curve.

Proline content

Proline content in three varieties of pea plant was determined by using spectrophotometric analysis as reported by Bates et al. (1973). Different proline concentrations i.e., 10, 20, 40, 60, 80, 100 ppm were made in 3% sulphosalicylic acid. To 1 ml of each standard solution taken into the new 15 ml falcon tube, 2 ml of each acetic acid, ninhydrin reagent and orthophosphoric acid were added. These falcon tubes were placed into the water bath at 100℃ for 45 minutes and then left on ice for 30 minutes. Plant sample (250 mg) ground in liquid nitrogen was homogenized with 10 ml of 3% sulphosalicylic acid, centrifuged at 3000 rpm for 10 mins, and 1 ml of supernatant was collected in a new falcon tube. The mixture containing 1 ml supernatant, 2 ml of acetic acid, 2 ml of orthophosphoric acid and 2 ml of ninhydrin reagent was kept in a water bath at 100℃ for 1 hr and cooled on ice for 5 minutes. Then 4 ml of toluene were added to each falcon tube and shake properly, two clear layers were formed, the upper layer was decanted, and the optical density of each sample was measured at 520 nm wavelength. After this proline content was estimated by comparing obtained values with the standard curve.

Statistical Analysis

The collected data were subjected to analysis of variance (ANOVA) to observe the main effects of varieties, treatments and their interaction. Furthermore, to differentiate among the significance of means, Duncan

multiple range (DMR) test was applied at p value≤0.05.

RESULTS AND DISCUSSION

Morphological traits evaluation under salt stress

Seeds of pea varieties Climax, Green grass and Meteor were germinated fully in 5 days and germination rate was about 40-60%. Different phenotypic symptoms were observed in these varieties of pea in response to salt stress. The high concentration of salt stress (75 mM and 100 mM) induced leaf wilting in all the three varieties (Figure 1), and reduced the plant height, root/shoot length, and number of leaves per plant. The higher NaCl concentrations (75 mM and 100 mM) showed severe effects on the morphological parameters in all the three varieties (Figure 2). Variety Climax showed an average of 20 leaves per plant as compared to Meteor 18 and Green grass 14 leaves per plant.

The reduction in root length was observed in selected pea varieties under all NaCl concentrations. There was statistically significant (p≤0.05) variations among the varieties and also among the treatments. However, the interaction effects was statistically non significant for root length for the difreent varieties under various salt stress. The maximum root length of 14.27 ± 0.95 cm was found for variety Climax in the control while minimum root length of 5.40 ± 0.26 cm was that for variety Green grass at 100 mM NaCl as compare to control (Figure 3A). A significant effect of salt stress was observed in all the three treatments 50, 75 and 100 mM NaCl as compare to control, plants with 100 mM treatment showed the lowest root length 6.30 ± 0.20 cm followed by 75 mM and 50 mM NaCl in the variety Meteor, respectively (Figure 3A).

As for roots, there was statistically significant (p≤0.05) variations among the varieties and also among the treatments for shoot length. However, the interaction effects was statistically non significant for shoot length for the difreent varieties under various salt stress. The lowest shoot length was observed in treatment 100 mM followed by 75 mM and 50 mM NaCl in selected pea varieties. Variety Green grass showed more reduction in shoot length followed by Meteor and Climax. 100 mM NaCl showed the lowest shoot length of 6.90 ± 0.56 cm for variety Green grass while maximum shoot length 14.47 ± 0.15 cm was observed for variety Climax in control (Figure 3B).

For plant height, there was statistically significant (p≤0.05) variations among the varieties and also among the treatments. However, the interaction effects was statistically non significant for plant height for the difreent varieties under various salt stress. When concentration of NaCl was increased, the plant height was reduced in all three pea varieties as shown in Figure 3C. The maximum plant height of 24.33 ± 0.87 cm was observed for variety Climax in the control while minimum plant height of 9.03 ± 0.55 cm was that for variety Green grass at 100 mM NaCl. The surface area of leaves was reduced with the increasing NaCl concentration in all three varieties as shown in Figure 3D.

There was statistically significant (p≤0.05) variations among the varieties, treatments and also the interaction effects for leaf surface area for the difreent varieties under various salt stress. The maximum leaf surface was observed for variety Meteor as 4.36 ± 0.20 cm^2 in control while the minimum leaf surface area 1.20 + 0.07 cm^2 was for variety Climax at 100 mM NaCl.

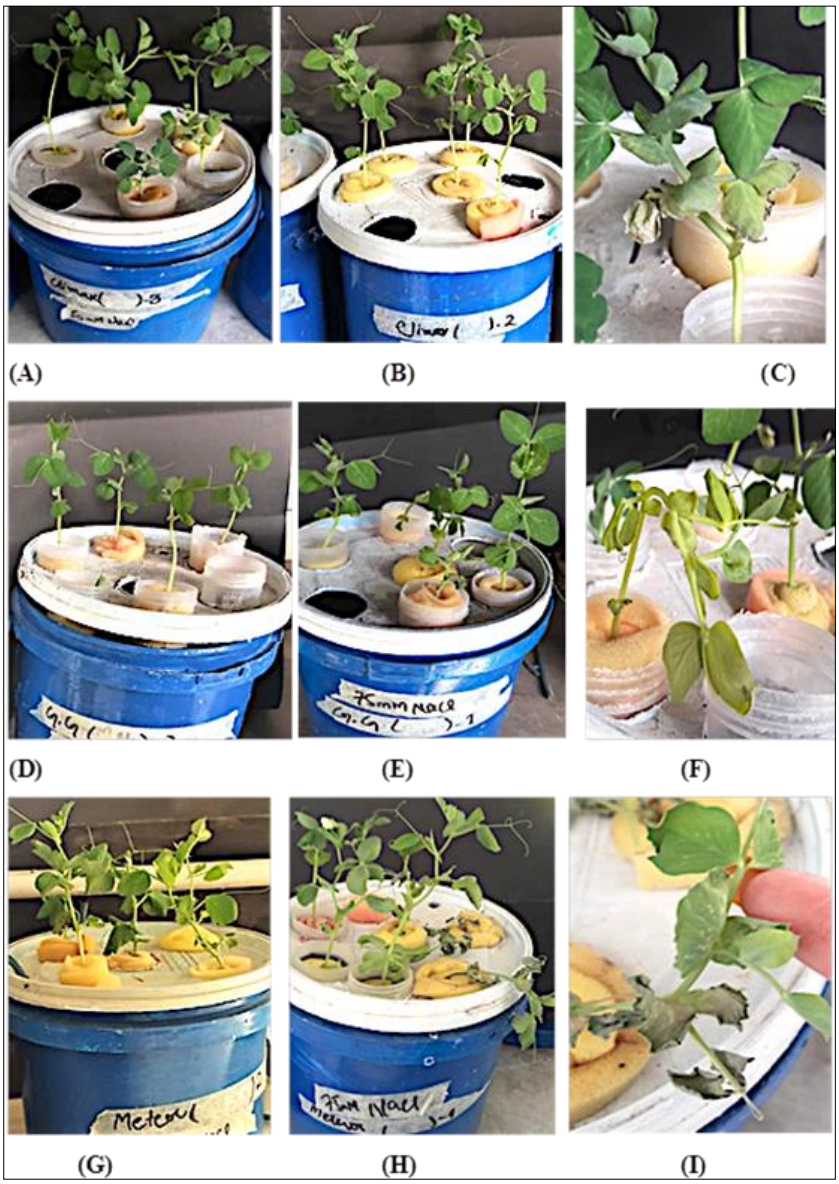


Figure 1. Different symptoms after the salt stress application in three pea varieties Climax (**A**=50 mM, **B**=75 mM, **C**=100 mM), Green grass (**D**=50 mM, **E**=75 mM, **F**=100 mM) and Meteor (**G**=50 mM, **H**=75 mM, **I**=100 mM)

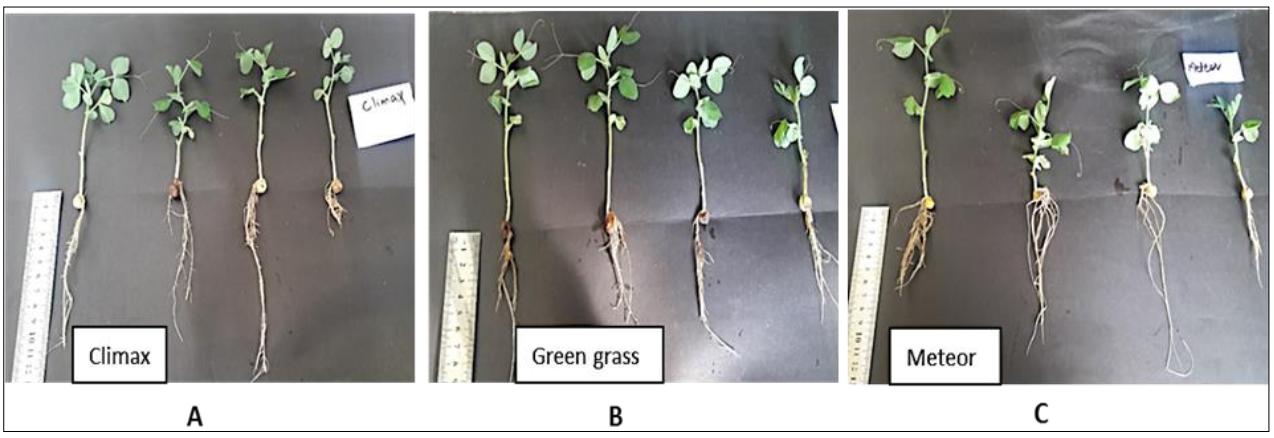


Figure 2. Phenotypic effects of salt stress on the roots, shoots and leaves of (**A**) Climax, (**B**) Green grass, and (**C**) Meteor. In each figure from left to right: 0 mM, 50 mM, 75 mM and 100 mM NaCl.

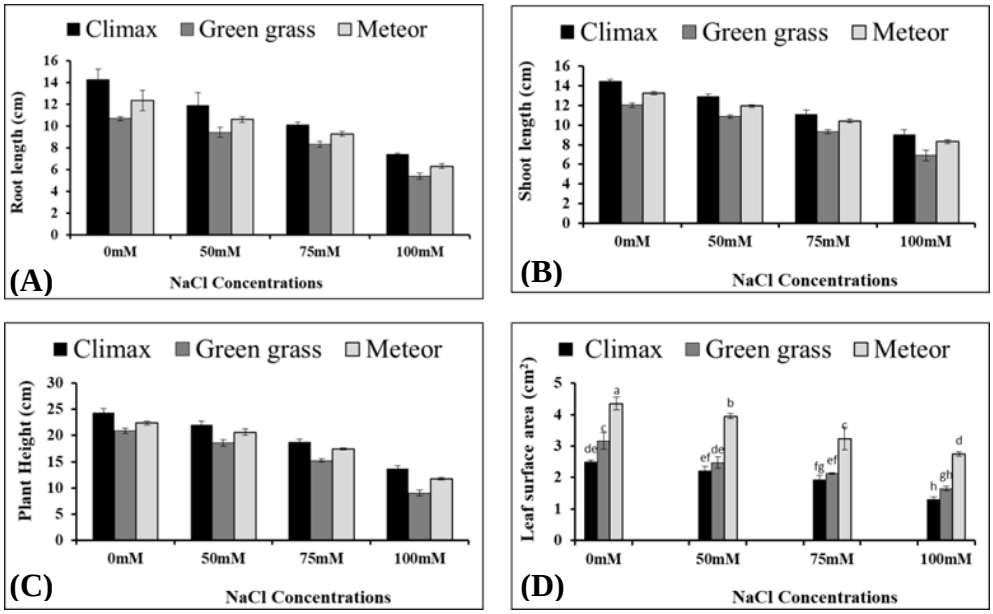


Figure 3. Effects of salt stress on (**A**) Root length, (**B**) Shoot length, (**C**) Plant height and (**D**) Leaf surface area of Climax, Green grass, and Meteor varieties

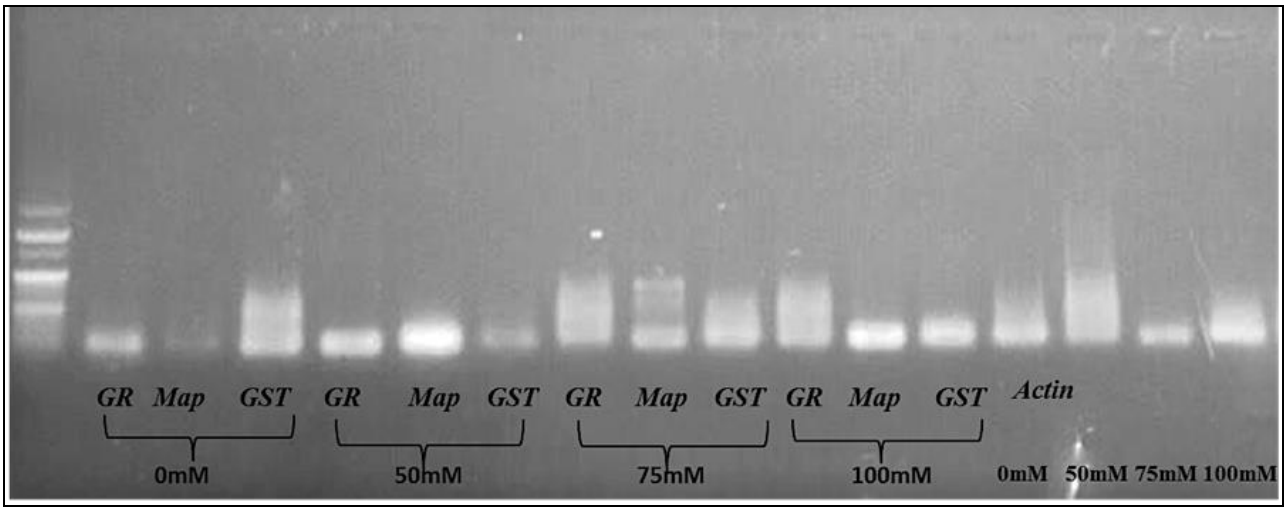
Among three varieties and four treatments i.e., 0 mM, 50 mM, 75 mM, 100 mM, highest moisture contents 93.3% and 91.0% were shown by variety Climax in 50 mM and 100 mM NaCl as compared to control (Table 1). Variety Meteor in 100 mM and 50 mM NaCl, showed 90% and 77.7% while lowest value i.e., 40.0 % was observed for variety Green grass in 75 mM NaCl plants. Similarly, in variety Green grass 70.5% moisture content was measured in 100 mM (Table 1). The highest dry content in variety Climax was observed in control as 20% and lowest value of dry content was 9% in 100 mM NaCl treated plants, while in variety Green grass dry content was similar in control and 50 mM NaCl treatments i.e., 60%. 100 mM NaCl showed the lowest of all which was 29.5%. 68.8% was the highest dry contents showed in variety Climax in 75 mM NaCl treated plants followed by the 100 mM NaCl with lowest value of 10% (Table 3).

Table 3. Moisture content (%) and dry content (%) of three different varieties of pea plant after salt stress treatments

Varieties	0 mM NaCl	50 mM NaCl	75 mM NaCl	100 mM NaCl
Moisture content (%)				
Climax	80.0	93.3	87.3	91.0
Green grass	55.2	62.5	40.0	70.5
Meteor	68.8	77.7	50.3	90.0
Dry content (%)				
Climax	20.0	6.7	12.7	9.0
Green grass	60.0	37.5	60.0	29.5
Meteor	31.2	22.3	68.5	10.0

Response of *GR*, *GST* and *MapK3* genes to salt stress

The expression of genes *Glutathione reductase (GR), Glutathione s-transferase (GST)* and *Mitogen-activated kinase3 (Mapk3)* in three varieties of pea are shown in the Figure 4. It was found that the expression was increased with the increase in the concentration of salt stress. Further, there was a differential expression pattern among the different genes for the given different levels of salt stress. The expression of *Mapk3* and *GR* genes was more at 50 mM and 100 mM salt stress. *GST* gene was highly expressed at 100 mM salt stress conditions as compare to the other levels of stress. *Mapk3* gene showed a significant expression than *GST* and *GR* genes.



L = Ladder. *GR*: *Glutathione reductase*, *Map: Mitogen activated kinase3*, *GST*: *Glutathione s-transferase*

Figure 4. Expression of selected genes under different salt stress treated pea plants in three varieties i.e., Climax, Green grass and Meteor.

Metabolic analysis under salt stress

The total flavonoid content (TFCs) in all the varieties were increased with increases in the salt concentration as shown in Figure 5A. There was statistically significant ($p \leq 0.05$) variations among the varieties, treatments and also the interaction effects for TFCs for the difreent varieties under various salt stress. Among which Climax variety showed the highest total flavonoid content 0.20 ± 0.004 mg/100 gFW at 100 mM NaCl followed by varieties Meteor and Green grass. The lowest TFC 0.21 ± 0.001 mg/100 g FW was found in variety Green grass at 50 mM salt stress. It has been found that upon several NaCl concentrations the Climax variety showed a significant response to all the four levels of stress treatments and plants releases a high amount of TFCs when compared with the TFCs production in Meteor and Green grass (Figure 5A).

There was statistically significant ($p \leq 0.05$) variations among the varieties, treatments and also the interaction effects for total phenolic content for the difreent varieties under various salt stress. Green grass variety showed the high total phenolic contents (1.97 ± 0.080 mg/100 g FW) followed by Meteor 1.79 ± 0.060 mg/100 g FW) (Figure 5B). There was statistically significant (p value$\leq$0.05) variations for total phenolic contents under different salt treatments. TPCs in variety Green grass was found to be increased with the increase in stress level (Figure 5B). In the 100 mM salinity stress treatment lowest value of TPCs (0.91 ± 0.05 mg/ 100 g FW) was estimated in Green grass as compared to control and other treatments.

The Proline concentration was found to increase with increasing levels of salt stress in all the three varieties. There was statistically significant ($p \leq 0.05$) variations among the varieties, treatments and also the interaction effects for proline content for the difreent varieties under various salt stress. The comparison of proline is given in three varieties (Figure 5C), among which variety Climax exhibited the high accumulation of proline (0.037 ± 0.001 mg/ 100 g FW) at 100 mM NaCl followed by Green grass (0.024 ± 0.001 mg/ 100 g FW) and Meteor (0.025 ± 0.004 mg/ 100 g FW). The interaction effects of various salt treatments and varieties were statistically significant (p value<0.05). The lowest proline was found to be accumulated in the control plants, which means that the plant showed a significant response to salt stress as the proline accumulation raised with increase in the NaCl.

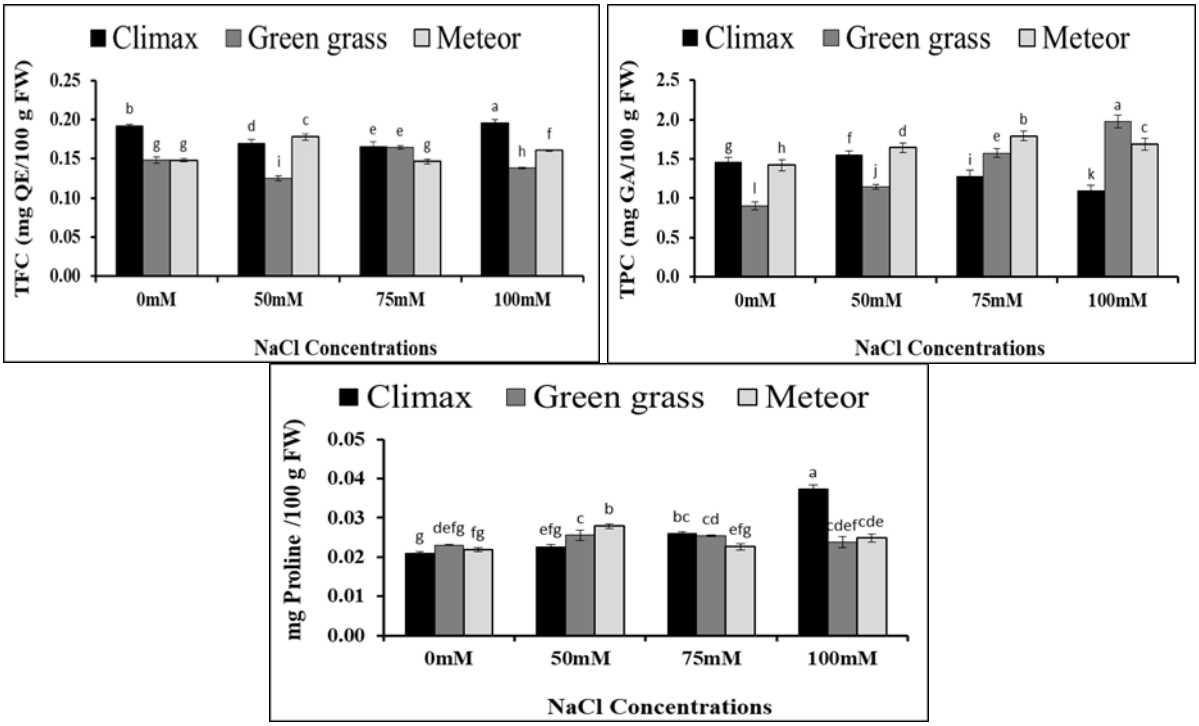


Figure 5. (**A**) TFCs, (**B**) TPCs and (**C**) Proline content in Climax, Green grass and Meteor pea varieties treated with four different salt stress treatments

To produce more food to feed the ever-growing population, the production of crops with better quality should be increased. However, agriculture faces the challenge of increased production because crops growth is limited by many biotic and abiotic stresses (Ximénez-Embún et al., 2016). Salinity is one of the abiotic factors that reduces the crop production and causing nutritional problem in rural parts of the world (Machado and Serralheiro, 2017). About 50% of arable land around the globe will be salinity affected by 2050 (Bartels and Sunkar, 2005). It has also been estimated that worldwide, 33% of the irrigated agricultural and about 20% of the cultivated land is under salinity stress (Shrivastava and Kumar, 2015). It is reported that the global demand for food would be expected to increase almost by 70% in 2050 as the current population will increase by 30% (Davies and Bowman, 2014). For this reason, the current agriculture production needs to be expanded despite the limited water and land resources (Davies and Bowman, 2014; Ahmad et al., 2017). In Pakistan, salinity problem is growing due to long-term mismanagement of irrigation system and human-induced soil erosion. About 25% of all irrigated land in Pakistan is affected by salinity, causes the loss of approximately 15-55 billion Pakistani rupees (Corbishley and Pearce, 2007).

In the current study, the effects of salt stress on the morphology of three different varieties of pea., Climax, Green grass, and Meteor were determined as well as the expression of important genes involved in the salinity stress tolerance was performed to evaluate which gene or genes show stress tolerance, and check which vital metabolite or metabolites expressed when plant is exposed to different levels of salt stress. It has been reported that salinity stress widely affects the plant yield and productivity, reduction in the water potential and causes reduced plant growth.

The morphological data were collected from each plant which showed a significant reduction in the plant height, root/shoot length and leaf surface area in those plants which were treated with 75 mM and 75 mM NaCl treatments. It was found in the current study that the plant height of variety Green grass shown to be more affected compare to the other two varieties and the significant effects of salinity were at the 75 mM and 100 mM NaCl treatments. Under severe salinity stress plants root/shoot length was also found

to be decreased as compared to control plants and most prominent reduction was found in variety green grass and Meteor at the 75 mM and 100 mM treatments, which means that variety Climax showed least effects on root/shoot length under same stress conditions. Among morphological parameters, the leaf surface area is a major parameter and considered to be important because reduced leaf surface area and welting of leaves are major symptoms plants show under abiotic stresses specifically in drought and salinity. All varieties showed a reduced leaf surface area at the treatment of 75 mM and 100 mM NaCl as compared to control and other treatments.

To find out the salt tolerance in pea at genetic level towards the different concentrations of salt, the expression level of genes was studied. For this, total RNA from all treatments of selected three pea varieties was extracted, and cDNA was synthesized which acts as a template for the primers to evaluate the expression of related genes. *Glutathione reductase (GR), Glutathione s-transferase (GST)* and *Mitogen-activated kinase3 (MapK3)* were amplified and their level of expression was studied in the NaCl treated pea plants. As *GR* is the constituent of AsA-GSH cycle, it plays an important role in detoxification of reactive oxygen species (ROS), regeneration of GSH and tolerance to abiotic stresses in plants (Hasanuzzaman et al., 2012). Increased activity of GR gives tolerance in stress conditions and it can manipulate the redox state of vital components of the electron transport chain. Under salinity stress conditions, *GR* provides tolerance through GSH recycling and regulation of GSH/GSSG ratio in the plant cell (Pang and Wang, 2010) and *GSTs* detoxify herbicide and H_2O_2. It also has functions like hormone homeostasis, metabolism of tyrosine, apoptosis regulation, and in plant responses to biotic and abiotic stresses (Dixon et al., 2010). *MAPKs* contribute to various signaling pathways induced by abiotic and biotic stresses including; low temperature, drought, salt and pathogens (Ding et al., 2009). *GR, GST* and *MapK3* showed significant expression in all the treatments (0, 50, 75 and 100 mM NaCl). The level of expression of all the genes increases with the increase in the level of salt concentration i.e., at 75 and 100 mM NaCl, the expression of *GR, GST* and *MapK3* was significantly increased when compared with control and 50 mM NaCl.

Besides the role of these genes in the defense mechanism of plant against salt stress, some metabolites such as Phenolics, Flavonoids, and Proline help the plant to withstand the abiotic stresses and help in the scavenging of several harmful reactive oxygen species produced when plant encounters salt stress and hence prevents the plant from oxidative damage. Phenolics, produced in plants provides defense against various environmental stresses, they are chelators of metals and show an affinity for scavenging free radicals (Dai et al., 2010). They also protect and strengthen the plants against different pathogens, while polyphenolics have scavenging activity as they suppress the reactive oxygen species (ROS) (Hussain et al., 2013). Total phenolic contents (TPCs) in variety Climax exhibited highest values at control lowest values were recorded in variety Green grass at 100 mM NaCl. In plants, flavonoids also provide protection against different biotic and abiotic stresses including drought and salinity and act as a signal molecule, UV filters, phytoallexins, and detoxifying agents (Scandalios, 2004). Among three varieties Climax, Green grass and Meteor the highest total flavonoid (TFCs) content was reported in variety Climax at the 100 mM NaCl treatment compared to other treatments, and lowest TFCs were estimated in variety Climax at the 0 mM NaCl treatment. It has been concluded in our previous study that when the concentration of NaCl is increases, the plant accumulates more TFC_S which helps the plants to survive under severe abiotic stress i.e., NaCl (Farooq et al., 2021). In literature, it is reported that of proline shows complex effects on plant development, and on the tolerance to stress conditions. Plants under salinity stress (EC of 16-20 dS/m)

show a higher accumulation of proline compare to control or less than EC of 16 dS/m stress (Muchate et al., 2016). Under stressful conditions, proline is rapidly degraded which generates the energy as it releases ATP which is used for the recovery and repairment of damages caused by stress (Chakraborty et al., 2016). Under oxidative stress, proline production increases in maize (Molazem and Bashırzadeh, 2015), and in the pea leaves there was a proline up-regulation under salinity stress (50 mM) (Jamil et al., 2016; Manzoor et al., 2020). In current study, the proline content was also determined in which the variety Climax at 100 mM and Green grass at 100 mM and 75 mM NaCl treatments showed the highest proline while variety Meteor accumulated comparatively low proline content.

CONCLUSIONS

The current research concluded that salt stress affects the growth and yield of pea plants as the level of induced salinity stress increases. Plants under a high concentration of NaCl i.e., 75 mM and 100 mM showed more damages as compared to control and 50 mM NaCl. Moreover, it has been concluded that the *GR*, *GST* and *MapK3* genes that are related to salt stress tolerance play a crucial role in helping the plant to withstand under the high concentration of toxic ions. Plant metabolites phenolics also increased with increasing salt stress, and so as flavonoids, and proline showed increase under salinity stress and prevents the plants from severe damages.

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