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SALT STRESS REDUCES THE PEA GROWTH AND INDUCESTHE EXPRESSION OF SELECTED ANTIOXIDANT GENES

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Pea (*Pisum sativum* L.) is an important edible leguminous seed crop. In the current study, salt stress was induced in Pea and the expression pattern of *Mn-superoxide dismutase*, *Peroxiredoxin*, *Thioredoxin*, and *Alternative oxidase* genes was studied. The research was performed in hydroponic system, with three replicates. Ten days old seedlings of Pea variety "Meteor" were exposed to four different levels of salt stress *i.e.*, 0 mM, 50 mM, 75 mM and 100 mM NaCl. The expression of selected genes was studied by reverse transcriptase polymerase chain reaction (RT-PCR). Results showed that shoot and root length, root and shoot biomass were significantly reduced under salt stress as compared to the control Pea plantlets. Results showed7.77 cm shoot length and 9.89 cm root length under 100 mM NaCl as compared to 22.45 cm shoot length and 24.53 cm root length in control. Fresh shoot biomass of 0.81 g and fresh root biomass of 2.3 g was observed under 100 mM salt stress as compared to 1.5 g fresh shoot biomass and 1.2 g fresh root biomass in control plantlets. *Mn-SOD* gene showed expression in all treatments but high expression was observed under 75 mM NaCl and 100 mM NaCl treatments. *Trxo1* gene showed expression in all treatments but was more pronounced in 75 mM NaCl. The *PrxIIF* gene also showed the expression in all salt treatments and the expression increased with increasing salt stress. *AOX* gene showed high expression in 50 mM and 100 mM NaCl and was less expressed in 75 mM NaCl. As the expression of selected genes was upregulated in Pea leaves under salinity, therefore, the results suggest that these genes are involved in salt stress and these may help to cope salt stress and could be used in developing salt resistant Pea varieties in future.

Keywords: Antioxidant genes, gene expression, hydroponics, NaCl, Pea, *Pisum sativum*, salt stress.

INTRODUCTION

Pea (*Pisum sativum* L.), a diploid plant of family Leguminosae with chromosomes number 2n = 2x= 14, is an essential edible seed crop. Pea seeds contain 23-25% protein, 50% digestible starch, 5% (fiber, soluble sugars, minerals and vitamins), and 10-12% carbohydrates (Bastianelli, *et al.*, 1998). Moreover, Pea is a good source of protein intake for human beings as well as feed for animals all over the world. Pea is also important because it can fix the nitrogen *i.e.*, biological nitrogen fixation, and thus is an important source of nitrogen in both the agricultural and natural ecosystems. Therefore, soil microbial diversity and activities are improved. However, it is not an ideal model for genomic analysis because of the comparatively large and complex genome (Franssen *et al.*, 2011). The genome of Pea crop is ~4.3 Gbp (Humann *et al.*, 2017) and is composed mainly of dispersed large-copy rePeats that apPear to be differentiated into distinct sequence classes.

Pea is growing in 187 countries world-wide with a cultivated area of 11930 thousand hectares and production of 44564thousandtonnes where China is the leading producer with a 11818 thousand tonnes production and with an area of 1477 thousand hectares (FAOSTAT, 2015). It is an important crop of Pakistan and is cultivated over an area of 68614 hectares with a total production of 170970 tonnes (FAOSTAT, 2015).

The Pea plant growth, productivity and yield are affected by different environmental factors that cause severe stress (Yousfi *et al.*, 2007). Salinity is among the abiotic factors that limit drastically the plant productivity (Leonforte *et al.*, 2012). The ions which are responsible for the salinization of soils are: Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^-.

Salinity imposes two kinds of stresses on plants; (1) Osmotic stress; when the salt concentration is high in the soil then the water uptake would become more difficult for plants because it will require energy for uptake. Osmotic stress generally leads to plant water deficit conditions, stomatal closure and

consequently, cessation of carbon assimilation and growth (Munns and Tester, 2008). (2) Ionic stress; ion specific stresses includes the accumulation of higher levels of ions of Na^+ and Cl^- in plant tissues, which ultimately cause ion toxicity. It will also lead to the altered K^+/Na^+ ratio (Munns and Tester, 2008). Sodium and chloride ions are the main ions that cause many physiological disorders in the plants, particularly Cl^-, which is more harmful than Na^+ (Tavakkoli *et al.*, 2010; Tavakkoli,2011).

Trxo1 has been reported to play key role in redox regulations (Lázaro *et al.,* 2013). Redox regulation of the enzymatic action is crucial for mitochondrial activity as well as antioxidant capacity by eliminating many free radicals, decrease H_2O_2 content and activating oxidation-inactivated proteins (Handy and Loscalzo, 2012). *Prx* is significant part of plant defense contrary to the oxidative stress as it is capable to detoxify the H_2O_2, alkyl hydroperoxides and per-oxynitrite (Kim *et al.,* 2012). *AOX* is additional target protein of *PsTrxo1*, which has been designated to control decline of the disulfide bonds of this protein accountable for the alternate mitochondrial respiration as well as its capacity (Martı*′ et al.*, 2009). *Trxo1*, *PrxIIF*, *AOX* and *Mn-SOD* activity, as well as particular enzymatic constituents of the ascorbate-glutathione cycle can help to avoid the formation of ROS and oxidative destruction and consequently permit moderate tolerance to salt stress.

The objectives of this research were i). To study the expression of *Mn-superoxide dismutase (Mn-SOD), Thioredoxin (Trxo1), Peroxiredoxin (PrxIIF),* and *Alternative oxidase (AOX)* genes in Pea under salt stress. ii). To compare and find which gene or genes are more important for salt tolerance in Pea. iii). To study the effects of salt stress on phenotypic traits such as root length, shoot length, fresh and dry biomass of Pea.

MATERIALS AND METHODS

This research was performed in the greenhouse and in the Biotechnology Laboratory of COMSATS University Islamabad-Abbottabad Campus, Abbottabad, Pakistan.

***Collection and germination of Seeds*:** Seeds of Pea variety Meteor were obtained from the National Agricultural Research Centre (NARC), Islamabad. For the germination of Pea seeds, they were first soaked overnight in warm water with constant aeration. Pea seeds were germinated by using sandwich method as described by Shahzad *et al.* (2012). After 10 days of incubation when seeds were completely germinated they were placed under the light for one day (Fig. 1A).

***Experimental set up*:** Germinated seedlings were transferred to hydroponic system in greenhouse. Four different NaCl treatments were applied to Pea seedlings; these were50 mM NaCl, 75 mM NaCl, 100 mM NaCl and 0 mM NaCl as control. In hydroponic system, total 12 pots with a capacity of 4 liters volume of each pot, were used. For each treatment, 3 pots were used as replicates *i.e.,* each pot was used as an experimental unit. Four seedlings were placed in each pot which was treated as replication within each experimental unit. Thus a total of 12 pots with 48 seedlings were placed in the green house. An aeration pump was also set for providing oxygen into the pots to avoid eutrophication in the pots.

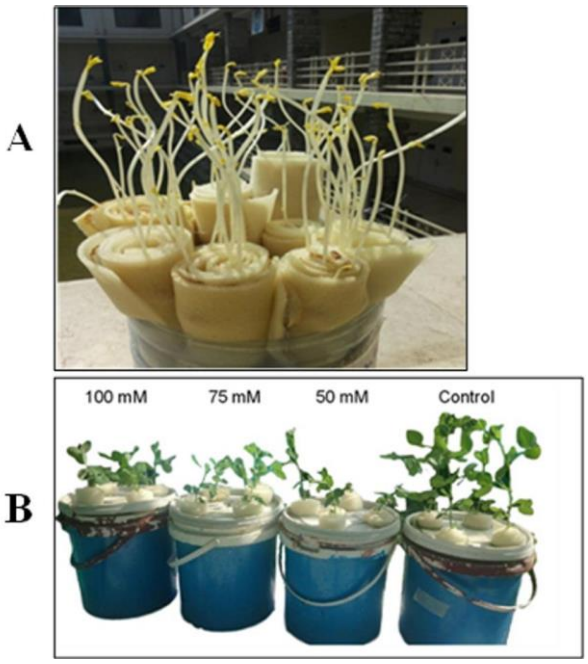

Figure 1. A: Germinated Pea seeds in sandwiches. 10 seeds were placed in each sandwich. B: Pea seedlings after applying the different salt treatments. The seedlings are of 14 days old.

Nutrients required for the growth of Pea were applied in specific concentrations as in natural environment. The details of nutrients along with their concentrations are given in Supplementary Table 1.

Supplementary Table 1. Details of nutrients applied to Pea seedlings in hydroponics system

Nutrients	Substance	Molecular weight (g)
Phosphorous (P)	KH_2PO_4	136.09
Potassium (K)	K_2SO_4	174.27
Chlorine (Cl)	KCl	74.50
Calcium (Ca)	$Ca(NO_3)_2$	236.15
Magnesium (Mg)	$MgSO_4$	246.48
Sodium (Na)	NaCl	58.40
Iron (Fe)	Fe-EDTA	367.05
Boron (B)	H_3PO_3	61.83
Manganese (Mn)	$MnSO_4$	169.00
Zinc (Zn)	$ZnSO_4$	287.54
Copper (Cu)	$CuSO_4.5H_2O$	249.68
Molybdenum Mo)	$(NH_4)_2Mo_7O_{24}$	1235.86

These nutrients were given indifferent doses, after seedlings were transferred to hydroponics culture, the nutrients were given in 1/4thconcentration on first day, on second day, the concentration of nutrients was increased to ½ of the total concentration. The hydroponics medium was left for one day as such and on the fourth day; nutrients with full concentration were applied, and kept for one day.

NaCl of three different concentrations *i.e.,* 50 mM NaCl, 75 mM NaCl and 100 mM NaCl solutions were prepared. These different concentrations were then applied as treatments to the seedlings in triplicate. The control (without NaCl) was also run in triplicate. As like nutrients, salt treatments were also given in doses of 1/4, (for one day) and 1/2 (for one day) and then full concentrations of salt were applied. After the application of full concentrations of treatments, the plants were kept in this medium for one week.

***Phenotypic traits study*:** The seedlings of Pea variety 'Meteor' were harvested after one week of salt treatment and the data for shoot length, root length, root and shoot fresh biomass, and root and shoot dry biomass were recorded.

For dry biomass the plant of each sample (treatment) was placed separately in envelops. These samples were then put in a microwave oven at 60°C for 48 hours., dry weight of all samples was recorded by using weighing balance.

***Plant leaves harvesting and total RNA extraction*:** After one week of salt treatments, Pea plant leaves were harvested in liquid nitrogen. Total ribonucleic acid (RNA) was extracted from the Pea leaves by using CTAB method (Jordon-Thaden *et al.,* 2015). Briefly, leaves were ground in liquid nitrogen and 1000 µl CTAB buffer containing 1% β-mercaptoethanol was added. This solution was heated in a water bath at 65 °C for 30 minutes and then 600 µl of CTAB extraction buffer and 600 µl chloroform-isoamyl alcohol in 24:1 was added. Samples were centrifuged at 12000 rcffor 15 minutes at 4 °C, supernatant was taken and 600 µl chloroform-isoamyl alcohol was again added to it. These were again centrifuged at 1200 rcf for 15 minutes at 4°C, supernatant was taken and 1/4 10M $LiCl_2$ was added. Samples were then stored at -20°C for overnight and next day samples were centrifuged at 12000 rcf for 15 minutes at 4 °C. Supernatant was discarded and 500 µl of ethanol (70%) was added and again centrifuged at 6000 rpm for 5 minutes at 4 °C. Finally, supernatant was discarded and pellet was allowed to air dry for 30 minutes and re-suspended in 80 µl of DEPC-treated water and stored at -80 °C.

***Determination of RNA quality and quantity*:** The extracted RNA was analyzed on 1.5% agarose gel by running 10 µl of RNA sample including 2 µl of loading dye. Further, RNA for each sample was quantified on Nano drop (Titertek Berthold Colibri spectrometer) apparatus.

***Complementary DNA (cDNA) synthesis*:** cDNA synthesis was carried out through TOPscript™ cDNA synthesis kit (Enzynomics). For this purpose, 2 µg of the RNA sample was taken and mixed with1µl of Oligo dt_{18} primer. The solution was incubated at 70 °C for 5 minutes and samples were chilled on ice and 2 µl 10x TOPscript™ RT buffer, 1µl TOPscript™ reverse transcriptase (200 units/µl), 2 µl dNTPs, 0.5 µl RNase inhibitor and 11.5 µl sterile water (to make final volume up to 20 µl) was added to the mixture. Reaction mixture was then incubated at 50 ˚C for 60 minutes and then at 95˚C for 5 minutes to stop the reaction. Samples were stored at -20 ˚C until further process.

***Designing of primers and PCR analysis*:** Primers were designed from the previous study of Martı´ *et al.* (2009) and the forward and reverse sequence of each gene primer used in the PCR are given in Table 1. Polymerase chain reaction was carried out on cDNA with a total reaction volume of 20 µl to check the expression of different genes. The total number of PCR cycles was set as 30 with a touchdown (T.D) of 65 to 55ºC. The detail of the PCR program for all the primers is given in Supplementary Table 2.

***Phylogenetic analysis of the proteins of selected genes*:** The protein sequences for the four selected genes from other leguminous plants were retrieved from the NCBI database by using the online available BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The proteins sequences of each gene were then aligned and phylogenetic tree was constructed using the online bioinformatics tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical Analysis: The experiment was performed

Table 1. Details of genes and primers used in this study

Gene Name	Primer Sequence	Melting temperature (Tm)
Peroxiredoxin (*PrxIIF*)*;* accession Number: AJ717306	**F:**5'- ACCGACTTCGTTCTTCCTCA- 3'	**F (Tm):** 55.8 ºC
	R:5'- GAAACTTGGATGCAGCAGA- 3'	**R (Tm):** 53.1ºC
Manganese superoxide dismutase (*Mn-SOD*)*;* accession number: X60170	**F:** 5' - AAAACCCTATCCTCCGTGCT-3'	**F (Tm):** 56.1ºC
	R: 5'-ATGATTTCGCCGCTAATGAC-3'	**R (Tm):** 53.3 ºC
Alternative oxidase (*AOX*)*;* accession number: Q07185.1	**F:**5'-GAGCG **(**AT**)**ATGCACCTAATGAC-3'	**F (Tm):**53.6 ˚C
	R:5'-TGCATTGAAGAAAACTCCCGTGTAC-3'	**R (Tm):**57.6 ˚C
Thioredoxin (*Trxo1*); accession number: AM235208	**F:** 5'- CCTTGCAGGTTCATTTCTCC-3'	**F (Tm):**53.8 ºC
	R: 5'-CTGCAACCTGCTCAATGTGT-3'	**R: (Tm):**56.0 ºC
Actin (X90378)	**F:**5'-TGGTGTTAGTCACACGGTTC-3'	**F (Tm):**54.8 ˚C
	R: 5'-CTGCAGAAGTGGTGAAAGTG-3'	**R (Tm):**53.9 ˚C

Supplementary Table 2. Primers used along with their PCR program

Primers used	Denaturation (Tm/second)	Annealing (Tm/second)	Elongation (Tm/second)
Peroxiredoxin (PrxII)	94°C for 5 min and then 95°C/ 30	54.45 °C/ 30	72 °C/ 30
Manganese superoxide dismutase (Mn-SOD)		54.70 °C/ 30	
Alternative oxidase (AOX)		55.60 °C/ 30	
Thioredoxin (Trx)		54.90 °C/ 30	
Actin		54.35 °C/ 30	

according to completely randomized design with three replications. The data were analyzed by general linear model and Tukey test ($p \leq 0.05$) using the SPSS software (Version 11, SPSS Inc., Chicago, IL). The significant differences were indicated with the help of small and capital alphabets at top of each bar. Error bars in the figures indicate the standard error of mean (SE).

RESULTS

Seed germination: Seeds of Pea variety "Meteor" were germinated through a sandwich method. The seeds started to germinate after three days in the incubator at 28 °C and were transferred to hydroponic system after 10 days.

Phenotypic variability due to salt stress: The plantlets in the hydroponic system were exposed to the salt stress of 50mM, 75mM, and 100mM along with the control. Four plantlets per pot were used as replicates for each of the four treatments. After one week of stress exposure the plantlets showed considerable phenotypic variations as shown in Figure1B and Figure 2.

Figure 2. Phenotypic variability due to salt stress. The differences in shoot length and root length due to salt stress are clearly shown.

Shoot and root length: Shoot and root length showed a clear difference under salt stress. As compared to the control (22.45 cm) the shoot length was significantly less under 50 mM =17.14 cm, 75 mM=11.49 cm, and 100 mM=7.77 cm. The root length was also significantly affected by the salt stress. Root length in control was 24.53 cm and in 50 mM = 19.27 cm, 75 mM= 14.37 cm, and in 100 mM=9.89 cm. The 50 mM treatment was less severe as compared to the higher salt treatments *i.e.,* 100 mM NaCl. The salt treated plants showed increased root length as compared to shoot length (Fig. 2, 3).

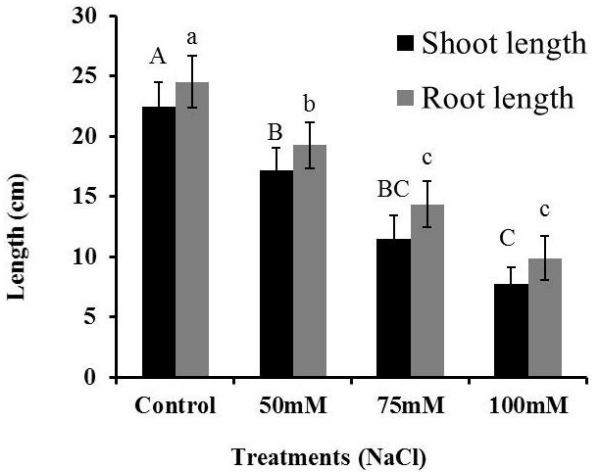

Figure 3. Effects of salt stress on shoot and root length of Pea. The significant differences are indicated with the help of small alphabets at top of each bar. Error bars in the figures indicate the mean standard error (S.E.).

Fresh and dry biomass: Results showed significant differences in fresh and dry biomass of the root and shoots for different NaCl treatments (Fig. 4). The 100 mM salt treatment showed the least fresh shoot biomass of 0.81 g and dry shoot biomass of 0.56 g. The maximum 1.3 g for fresh and 1.1 g for dried shoot biomass was observed under 50 mM NaCl. Control showed 1.5 g for fresh shoot biomass and 1.2 g for dried shoot biomass.

The lowest value of 1.6 g for 50 mM and highest value of 2.3 g for 100 mM NaCl in case of fresh root biomass was observed. For, dried root biomass maximum weight of 1.97 g was recorded for 100 mM NaCl and the lowest value of 1.33 g was observed for 50 mM NaCl (Fig. 4). Control showed 1.2 g for fresh root biomass and 0.97 g for dried root biomass.

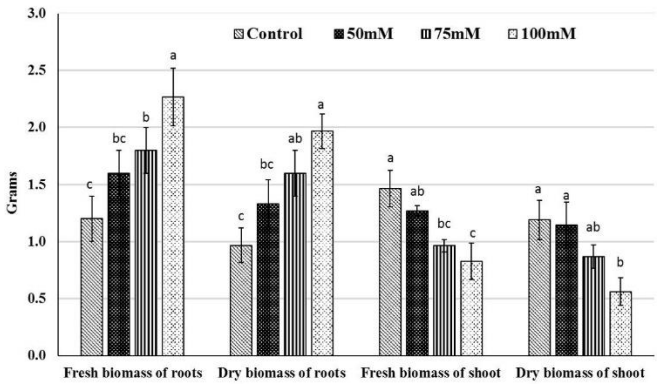

Figure 4. Effects of salt stress on fresh and dry biomass of Pea. The significant differences are indicated with the help of small alphabets at top of each bar. Error bars in the figures indicate the mean standard error (S.E.).

Expression pattern of different genes under salt stress conditions: The expression pattern of various genes involved in salt stress was studied by RT-PCR. The expression pattern of *Mn-SOD* (accession number: X60170), *Trxo1* (accession number: AM235208), *PrxIIF* (accession Number: AJ717306), *AOX* (accession number: Q07185.1) and *Actin* (accession Number: X90378) genes was analyzed as shown in Figure 5. *Mn-SOD* gene showed expression in all treatments but more expression in 75 mM and 100mM as compared to control. *Trxo1* gene showed expression in all treatments but more expression in 75mM was observed. *PrxIIF* gene was also expressed in all the treatments but pronounced more in 100mM salt treatment. However, *AOX* gene showed more expression in 50mM and 100mM treatments and was less expressed in 75mMtreatment and control

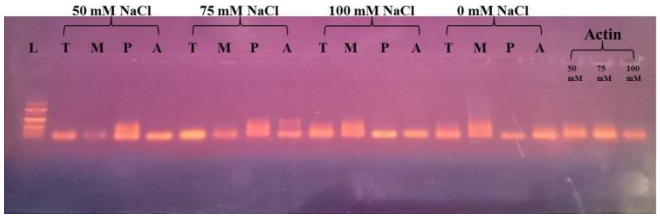

Figure 5. Expression of antioxidant genes in 50 mM, 75 mM, 100 mM and control (0 mM) NaCl treatments. T: *Trxo1* (accession number: AM235208), M: *Mn-SOD* (accession number: X60170), P: *PrxIIF* (accession number: AJ717306), A: *AOX* (accession number: Q07185.1) and *Actin* (accession number: X90378).

Phylogenetic relationship of the antioxidant genes studied in this study: The phylogenetic study of *PrxIIF* gene revealed that it is more closely related to *PrxIIF* gene of *Medicago truncatula* followed by *Cicer arietinum PrxIIF* gene. It is at more distance from *Vigna angularis* and *Vignar adiata PrxIIF* gene (Fig. 6).The phylogenetic study for *Mn-SOD* gene revealed that it is more related to *ViciasativaMn-SOD* gene and much different from *Phaseolus vulgaris* and *Lotus japonicus Mn-SOD* gene (Fig. 6).The *AOX* gene revealed that it is much relevant to *Cicerarietinum AOX* gene followed by *Caja nuscajan AOX* gene while it is much different from *Vigna radiate* and *Vigna angularis AOX* gene. For *Trxo1* gene, the phylogenetic study revealed that it is more related to *Medicago truncatulaTrxo1* as compared to all the other relatives of Pea crop. The *Trifolium subterraneum* and *LotusjaponicusTrxo1* gene is far apart from the Pea crop (Fig. 6).

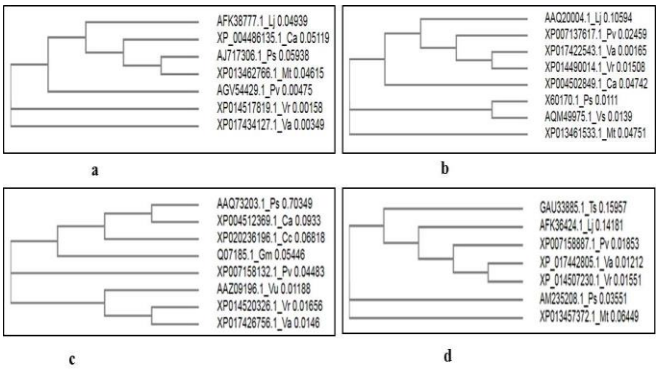

Figure 6. Phylogenetic tree of the four important genes involved in salt stress. a. peroxiredoxin (*PrxIIF*), b. manganese superoxide dismutase (*Mn-SOD*), c. *alternativeoxidase* (*AOX*), andd. *thioredoxin* (*Trxo1*). *Ah: Arachis hypogaea, Ca: Cicer arietinum, Cc: Cajanuscajan, Gm: Glycine max, Lj: Lotus japonicas,Mt: Medicago truncatula, Ps*: *Pisumsativum, Pv: Phaseolus vulgaris, Ts: Trifoliumsubterraneum,Va: Vigna angularis, Vr: Vigna radiata, Vs: Vicia sativa*, and *Vu: Vigna unguiculata*.

DISCUSSION

Salinity stress is the main cause of reduction in yield of major crops and more than 50% yield reduction is reported every year worldwide (Mahajan and Tuteja, 2005; Satir andBerberoglu,2016).Currently, around 77 million hectares of cultivated land is salt affected throughout the world which is approximately 40% of the total cultivated land (Jadhav *et al.*, 2010; Evelin *et al.*, 2009).

The response of plants to high salinity stress is very complex and involves numerous modifications in the physiology, morphology and metabolism (Munns and Tester, 2008). Prior studies have revealed that NaCl causes oxidative stress with reduction in the growth parameters such as fresh and dry biomass, shoot and root length, decrease in the relative water

content and the K^+ concentrations, and decrease in photosynthetic pigments such as chlorophyll content (Ahmad and Jhon, 2005; Kapoor and Pande, 2015). NaCl stress has been reported to significantly reduce the growth and development of Pea crop (Jamil *et al.*, 2016). Also in different maize cultivars it has been shown that salt stress reduces the plant vegetative growth by upto 40-60% (Pitann *et al.*, 2009). In the present study, expression pattern of four important genes *i.e., peroxiredoxin* (*PrxIIF*), *manganese superoxide dismutase* (*Mn-SOD*), *alternative oxidase* (*AOX*), and *thioredoxin* (*Trxo1*) was evaluated. These four genes are mitochondrion based. Mitochondria play an important role in the reactive oxygen species signal transduction during salt stress. *Trxo1* is involved in disulphide/dithiol interchange and thus have an important role in redox regulation in salt stress. *Mn-SOD* controls the $O_2^{\cdot -}$ content in the organelle, and the complete ascorbate-glutatione pathway that is an important pathway in redox regulation (Jiménez *et al.*, 1997). *Mn-SOD* gene allows mitochondrion organelle to regulate the internal H_2O_2 concentration, including that produced in other cell compartments. Moreover, a thioredoxin/peroxiredoxin (Trx/Prx) system which includes an NADPH-dependent thioredoxin reductase and glutaredoxin has been described in plant mitochondria (Finkemeier *et al.*, 2005). AOX is suggested to prevent over-reduction of the ubiquinone pool, thus avoiding the formation of reactive oxygen species (Maxwell *et al.*, 1999). *AOX* has been shown to be regulated by the mitochondrial *PsTrxo1* (Martí *et al.*, 2009), and a strong interaction occurs between *PsTrxo1* and *PsPrxIIF*. It is evident from the above discussion that all these four selected genes have diverse functions under salt stress as they are involved in various important metabolic pathways yet these are correlated strongly with one another in the whole process of ROS generation and subsequent rescue of plants from the adverse effects of the ROS. In view of the above, the hypothesis of current research was that whether these genesare involved in the salt stressor not in Pakistani local Pea variety 'Meteor'. The results of current study revealed that salt stress has severe effects on plant phenotype and the associated phenotypic parameters such as shoot and root length, and biomass (Fig. 3-4).

However, the selected genes expression was also upregulated with an increasing salt stress (Fig. 5). Under salt stress the plant produce the reactive oxygen species that reduce the growth of plants by reducing the photosynthesis and nutrient uptake. However, plant antioxidant genes and proteins detoxify these reactive oxygen species. Among the different antioxidant genes, *PrxIIF*, *Mn-SOD*, *AOX*, and*Trxo1* are the most important in protecting the cells against the deleterious effects of reactive oxygen species (ROS). *PrxIIF* proteins detoxify H_2O_2, alkyl hydroperoxides and peroxinitrite and protect plants from different abiotic stresses (Dietz *et al.*, 2006). Similarly *Mn-SOD, AOX,* and*Trxo1* genes and proteins increase the salt stress tolerance in plants by overexpressing themselves (Tanveer and Shabala, 2018). This research suggests that although increased salt stress reduces the phenotypic performance of the Pea crop but at the genetic level the expression of these genes increased. This up-regulation of above mentioned genes could indicate that they may have protected the plants but there is possibility of limited translation of these genes into protein and thus could not fully rescue the plant from this stress condition. Another possible reason for decrease in growth of Pea plants is that more ROS may be produced as compared to antioxidant genes products. In order to know the evolutionary relationship of these genes/proteins involved in salt stress, phylogenetic analysis was performed (Fig. 6). The phylogenetic analysis revealed that these four different genes have strong evolutionary relationship with species of Leguminosae family plants. It further shows that the role of these antioxidant genes is very diverse in salt stress and salt stress phenomenon is very complex and involved several different biochemical pathways. Therefore, the biochemical and translational study of these genes should be studied in future to understand their full role in salt stress.

***Conclusion*:** Salt stress affects negatively the morphological traits of Pea plants particularly shoot length, root length and total biomass. Root and shoot lengths decreased with increasing the level of salts stresses. The four selected genes were found up-regulated with increasing salt stress that shows that these genes are involved in salt stress. In future, these genes should be studied at protein level to further confirm their role in salt stress. This is the first step towards finding the involvement of these four genes in Pakistani Pea varieties. In future, more work is needed to evaluate the role of these important genes in the screening of other Pea varieties for salt stress.

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