

PHYSIOLOGICAL CHARACTERIZATION OF HALOPHYTIC WEEDS GROWING IN SALINE AND NON-SALINE REGIONS OF PAKISTAN

Tamoor-Ul-Hassan and Asghari Bano*

Department of Plant Sciences Quaid-i-Azam University, Islamabad 45320, Pakistan
*Phytohormones lab, Dept of Plant Sciences Quaid-i-Azam University Islamabad Pakistan
Corresponding Author E-mail: banoasghari@gmail.com

ABSTRACT

The difference in salt tolerance mechanism of halophytes with that of glycophytes has already been underlined in the plants of saline land. Present investigation deals with five wild plant species: *Solanum surattense* Burm. f., *Cenchrus ciliaris* L., *Aerva javanica* (Burman f.) A. L. Juss. Ex Schultes, *Peganum harmala* L and *Chrysopogon aucheri* Boiss. collected from Khewra salt range (EC=2.3 dSm⁻¹ and pH: 8.5) to study the physiology of their salt tolerance in comparison with that of plants of same species collected from non-saline soil of Rawalpindi (EC: 0.31 dSm⁻¹; pH: 7.4). Differences exist between plants of saline soil with respect to accumulation of t-zr (trans zeatin riboside), ABA (abscisic acid), SOD (superoxide dismutase), POD (peroxidase), proline, glycine betaine and sugar. The ABA concentration of plants from saline soil was higher in the leaves and roots than that of the plants of non saline area. Proline and glycinebetaine in *Chrysopogon aucheri* (CaKH) from saline soil was significantly higher than other plants of saline soil. All the plants exhibited higher ABA/t-zr ratio as compared to plants of non-saline soil. It is inferred that the ratio of ABA/t-zr and the antioxidant activities of enzymes can serve as index for salt tolerance.

Keywords: Khewra, nutrient contents, phytohormones, transzeatin riboside, weed salt tolerance.

INTRODUCTION

Plant growth and productivity is severely affected by soil salinity (Singh *et al.*, 2011). Over the years comparison of halophytes and glycophytes remains a hot topic in plant physiology. Differential behavior of plants and their adaptations to stress conditions are core part of research studies in biology but researchers are emphasizing on mechanism of salt tolerance in plants Munns and Tester, (2008). Limited data is available on species level about the physiological and biochemical adaptations of plants growing within soil blessed with different type of nutrients.

Salt stress is characterized by ionic, oxidative and osmotic changes in plant structure and for coping these changes, stress tolerant plants are equipped with antioxidants, polyamines, osmolytes and stress responsive proteins Kuznetsov *et al.*, (2006). Numerous studies on salinity and its effect are the keys of our present upgraded knowledge (Jamil *et al.*, (2007) and Duan *et al.*, (2008).

In Pakistan, about 6.3 million hectare of land is being effected by salt, causing loss of about 20 billion Rs per year (Qureshi *et al.*, (2008) and Shahid *et al.*, (2011). Pakistan has vast salt range descending from Kalabagh to Jhelum. Khewra salt range surrounds Khewra salt mine which is the second largest salt mine in the World. Higher concentration of Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻ and HCO₃⁻ was reported in the rhizospheric soil of weeds growing in Khewra salt range (Naz *et al.*, (2009).

Cenchrus ciliaris is perennial herb of Asian and African region. Plant reaches to 50cm in length and spike appeared during flowering season (Clayton *et al.*, 2008). Deep rooting system and higher biomass production (Agrawal (1971) facilitate plant to cope with drought and deserted conditions (Singariya *et al.*, 2012). Additionally, the role of carbon sequestration, Nitrogen cycling and soil binding contributes toward ecosystem stability (Sinha *et al.*, (1996).

Solanum surattense is naturally growing prostrate herb in central Asia and consider as important halophyte of Pakistan (Khan and Qaiser, (2006). The plant bears purplish flower throughout the year. Chemical constituents of *Solanum surattense* are of great medicinal values (Nasir (1985). The weed contains high antioxidant activity, and phenolic contents even when growing in natural unstressed soil (Sridevi *et al.*, (2013). Shahiladevi *et al.*, (2006) also reported the presence of petroleum, benzene in seeds of *Solanum surattense*.

Chrysopogon aucheri is widely distributed in Asia and Africa. It is perennial herb (30-60cm in height) blossoms during June and July (Ahmed *et al.*, 2000). *Chrysopogon aucheri* leaves contained higher Na⁺ and Ca⁺ contents. Agriculturally important root colonizing halophytic bacteria has also been isolated from *Chrysopogon aucheri* growing under salt stress of Khewra salt range (Bano *et al.*, (2009).

Erva javanica is perennial herb commonly found in sandy soils. Plant height varies (0.4-1.8 m) and often blossomed with white flowers between January to

October. The plant is important medicinally and gargle is used as tooth paste (Samejo et al., (2011) and Charboneau et al., (2013).

Pegnum harmala is perennial herb known for its medicinal values and often used for its diuretic, analgesic, disinfectant, antihelmintic and anti-inflammatory activities (Tarhrouch et al., (1998), Shahverdi et al., (2005) and Monsef et al., (2004). Different studies reveal that *Solanum surattense*, *Pegnum harmala*, *Erva javanica* and *Cenchrus ciliaris* have remarkable antifungal potential against certain fungal pathogens (Sheeba and Palanivel, (2013) and Srinivas and Reddy, (2012). Additionally, these weeds have also potential to inhibit some bacterial strains (Minan (2010) and Singariya et al., (2012).

Present study deals with the investigation of physiological and biochemical parameters of five salt tolerant plants from saline soil of Khewra salt range and compared with the same species growing in the non-saline area of plain land (Rawalpindi). Our focus was to investigate the phytohormonal modulation of weeds with changing nutrients availability in different types of soils.

MATERIALS AND METHODS

Collection of soil and plants samples: During the present study five weed species were collected at their vegetative stage along with their rhizospheric soil from 0-15cm depth in three different locations of Khewra salt range (Kh) [pH: 8.5; EC: 2.3 dSm⁻¹; 32°56'00" N; 73°44'00" E] from an altitude of 300-395 m.a.s.l with minimum human interference and district Rawalpindi (RWP) (pH 7.6; EC 0.31 dSm⁻¹; 37°35' N, 73°23' E) during July 2011. Leaves and roots were stored at -70°C till further analysis. The examined weed species were *Aerva javanica* (EjKH for KH; EjRWP for RWP), *Cenchrus ciliaris* (CcKH for KH; CcRWP for RWP), *Peganum harmala* (PhKH for KH; PhRWP for RWP), *Chrysopogan aucheri* (CaKH for KH; CaRWP for RWP) and *Solanum surattense* (SsKH for KH; Ss for RWP).

Physiological and Biochemical analysis of plants

Relative water contents of leaves: Relative water contents of leaves were determined following the method given by (Gupta (1996). Relative water content was calculated by applying the formula of (Weatherly (1950).

Leaf protein contents: Protein content of leaves was determined following the method of (Lowry et al., (1951) using BSA as standard. Fresh leaves 0.1 g were ground in phosphate buffer (pH 7.5), centrifuged for 10 min at 3000 rpm. The supernatant (0.1 ml) was taken in test tube and final volume (1 ml) was made by adding distill water. Sample was treated with reagents (folin phenol) and absorbance of each sample was recorded at 650 nm after

30 min incubation.

Sugar estimation: Sugar estimation of fresh leaves was done following method of (Dubo et al., (1956). Homogenate (0.5 g plant tissue + 10 ml distill water) was centrifuged at 3000 rpm for 5 min. Supernatant (0.1 ml) was treated with 5 ml concentrated sulphuric acid. After incubation (4 hrs) absorbance was measured at 420 nm.

Chlorophyll content of leaves: Chlorophyll content of leaves was determined by the method of (Arnon (1949). Homogenate of plant tissue and dist. Water (1 ml) was mixed with 4 ml of 80% (w/v) acetone and kept in dark. After centrifugation (2000 rpm for 5 min), absorbance of supernatant was read at 645 nm (chlorophyll a) and at 663nm (chlorophyll b). Total chlorophyll was calculated by formula:

$$\text{Total chlorophyll (mg/l)} = (20.2 \times A_{645}) + (8.02 \times B_{663})$$

Osmotic potential: Osmotic potential of the cell sap was measured from leaves of plants with a freezing point osmometer (Roebing Messtechnik. Berlin, Germany) following (Capell and Doerffling 1993), and using the formula of (Bigot and Boucaud, (1996):

$$\text{Osmotic potential (MPa)} = \text{Osmolarity (milliosmol)} \times 0.831 \times 10^{-5} \times T (^{\circ}\text{K})$$

Electrolyte leakage: Electrolyte leakage from leaves was measured following the method of (Lutts et al., (1995).

Proline and content Glycine betaine (GB) of leaves: Proline was measured by the method of (Bates et al. (1973). For proline estimation 0.5 g of plant material was homogenized in 10 ml of 3% aqueous sulphosalicylic acid. 2 ml of filtrate was treated with 2 ml acid ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 hr at 100°C. The reaction mixture was extracted with 4ml toluene and stirred for 15-20 sec. The absorbance read at 520 nm against toluene as blank.

Glycine betaine contents were estimated according to the method of (Grieve and Grattan, (1983). Filtrate of plant extract in water (24 hrs old) was diluted to 1:1 with H₂SO₄ (0.25ml each). 0.5 ml of the mixture was incubated in ice water for 1 h and treated with cold KI-I₂ reagent (0.2 ml). The tubes were stored at 4 °C for 16 h and centrifuged (10,000 rpm for 15min at 0 °C). The per iodide crystals were dissolved in 9 ml of 1, 2-dichloroethane and absorbance was measured at 365 nm after 2 h incubation.

Extraction for Peroxidase: POD activity was measured by method of (Vetter et al, 1958). Fresh leaves (5g) were homogenized with 15ml of 0.05M phosphate buffer (pH 7.0) containing 10% polyvinyl polypyrrolidone and 0.1 M Ethylene diamine tetra acetate (EDTA).

Assay for Superoxide Dismutase Activity (SOD): SOD activity was determined by measuring inhibition of

photochemical reduction of nitrobluetetrazolium (NBT) using method of Beauchamp and Fridovich, (1971).

Determination of ABA: The extraction and purification for ABA was made following the method of Kettner and Doerhung, (1995). Plant leaves (1g) were grounded in 80% methanol at 4°C with an antioxidant, Butylated hydroxyl Toluene (BHT). The leaves and roots were extracted at 4°C in dark for 72 hrs with subsequent change of solvent. The extracted samples were centrifuged and the supernatant was reduced to aqueous phase using rotary thin film evaporator (RFE). The pH of aqueous phase was adjusted to 2.5-3.0 and partitioned four times with ½ volume of ethyl acetate. The ethyl acetate was dried down completely using rotary thin film evaporator. The dried samples were re-dissolved in 1 ml of methanol 100% and analyzed on HPLC (Shimadzu, C-R4A Chromatopac; SCL-6B system controller) using UV detector and C-18 column (39x300mm) for identification of hormones. 100 µl samples filtered through 0.45 millipore filter were injected in column. Pure ABA (sigma, USA) was used as standard and ABA was identified on basis of retention time and peak area. Methanol, acetic acid and water (29: 1: 70) were used in and flow rate 0.5 ml/min was adjusted for an average run of 15 min/sample. The wavelength used for detecting ABA was adjusted at 280nm.

Determination of Trans-ZeatinRiboside (t-zr): The trans-zeatinriboside (t-zr) was extracted and analyzed following the method of Tien *et al.*, (1979). Centrifuged (8,000 rpm for 20 min at 4°C) leaves and root extract was reduced using rotary thin film evaporator (RFE). Aqueous phase (pH 2.5) was partitioned four times with the same volume of acetic acid and ethyl acetate (1% v/v). The acidic ethyl acetate was evaporated by RFE. The residues were dissolved in 1000µl methanol/water (30:70). The samples were analyzed on HPLC (Agilent 1100) using UV detector adjusted to 254nm for t-zr determination and C₁₈ column (39 x 300mm).

Statistical analysis: Statistical analysis was done by analysis of variance (ANOVA) using Statistix program, version 8.1. Two Factorial design was followed and four replicates were taken for each plant species. Mean values were separated according to LSD test P=0.05, DF = 21 with ±SE (as shown over error bars).

RESULTS

The osmotic potential of leaves (Table 1) of plants from saline soil was in general 2-fold less negative than that of collected from non-saline soil. The relative water contents and electrolyte leakage (Figure 1) of plants from saline soil were 13-33% lower and 14-20% higher respectively than that of plants from same species of non-saline soil. Among the plants of saline soil

electrolyte leakage was very high in CaKH and relative water content was lowest in EjKH.

Leaves of the plants collected from saline soil contained significantly lower (P=0.05) protein (Figure 2) ranging from 34-93% and chlorophyll content 32-48% respectively but the sugar content was higher than that of plants collected from non-saline soil. The sugar contents of EjKH, CcKH, PhKH, CaKH and SsKH were 39%, 28%, 43%, 30% and 30% more than EjRWP, CcRWP, PhRWP, CaRWP and SsRWP respectively. Among plants from saline soil, maximum sugar content was found in PhKH while SsKH contained least sugar content. EjRWP has highest protein and chlorophyll contents.

The proline and glycine betaine contents of leaves (Figure 3) of plants from saline soil were 25-55% and 17-57% higher respectively than that of plants from non-saline soil. The concentration of proline and glycinebetaine in CaKH was significantly higher than other plants of saline soil.

The superoxide dismutase (SOD) and peroxidase (POD) activities of leaves (Figure 4) of plants from saline soil was significantly (25-59%) higher. The SsRWP had lowest SOD and POD activity while PhKH contained highest SOD activity. Similarly, EjKH has maximum POD activity.

Table. 1. Osmotic potential (Mpa) and chlorophyll contents of plants leaves collected from Khewra salt range and Rawalpind.

Treatments	Osmotic potential	Chlorophyll (mg/g)
EjKH	-3.95A	4.28E
EjRwp	-7.35C	8.13B
CcKH	-4.16A	6.67C
CcRWP	-8.59D	8.48B
PhKH	-3.24A	5.16D
PhRWP	-5.95B	8.51B
CaKH	-4.04A	8.17B
CaRWP	-8.35D	13.54A
SsKH	-4.23A	4.3E
SsRWP	-8.31D	6.34C
MS	-5.11	3.13
LSD 0.5%	-3.89	1.62

Values followed by different letters in a column are significantly different (P = 0.05). Results are means of four replicates.

The t-zr concentration was higher in roots and ABA concentration was higher in leaves of all plants from saline soil (Figure 5). The ABA concentration of plants from saline soil varied from 11-98% in leaves whereas the roots (Figure 6) had 16-47% higher ABA than the plants of non- saline soil. Among the plants from saline soil SsKH has significantly lower ABA contents in leaves of salt tolerant plants. Greater concentration (2 fold) of trans-zeatinriboside (t-zr) was observed in roots

(Figure 6) and 2-5 times in leaves (Figure 6) of plants of non- saline soil as compared to the plants from saline soil. The ABA/t-zr ratio (results not shown) was higher in

leaves than in the roots of all plants of salt range, maximum being in CcKH while the ABA/t-zr ratio was lower in roots of halophytes.

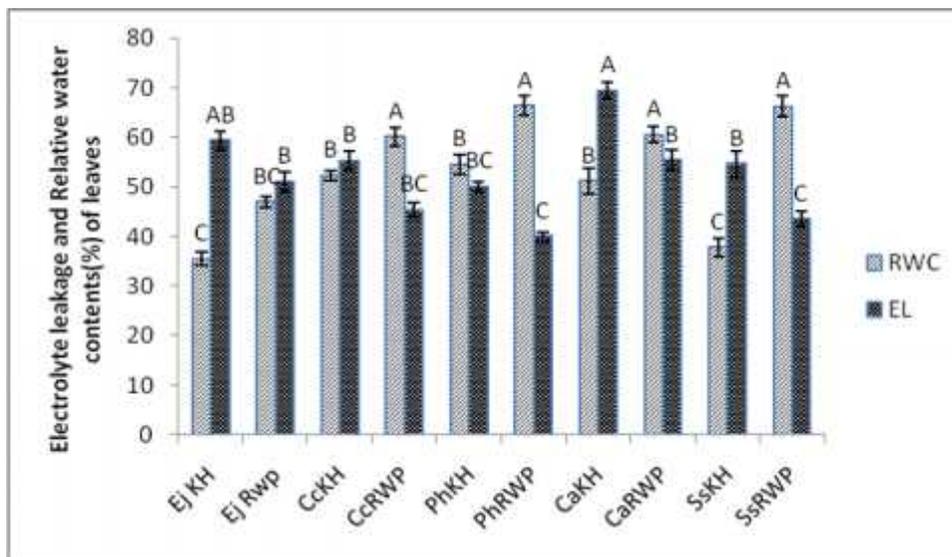


Figure 1. Relative water contents and electrolyte leakage (%) of leaves of plant species (as on Y-axis) collected from Khewra salt range and non-saline soil of district Rawalpindi. *Aerva javanica* (EjKh for Kh; EjRWP for RWP), *Cenchrusciliaris* (CcKh for Kh; CcRWP for RWP), *Peganum harmala* (PhKh for Kh; PhRWP for RWP), *Chrysopogan aucheri* (CaKh for Kh; CaRWP for RWP) and *Solanum surattense* (SsKh for Kh; Ss for RWP).

Values given are mean of four replicates ± SE (represented with error bars). Values followed by different letters heading the bars represents significant differences (P<0.05) LSD.electrolyte leakage =1.46, LSD Relative water content = 7.03

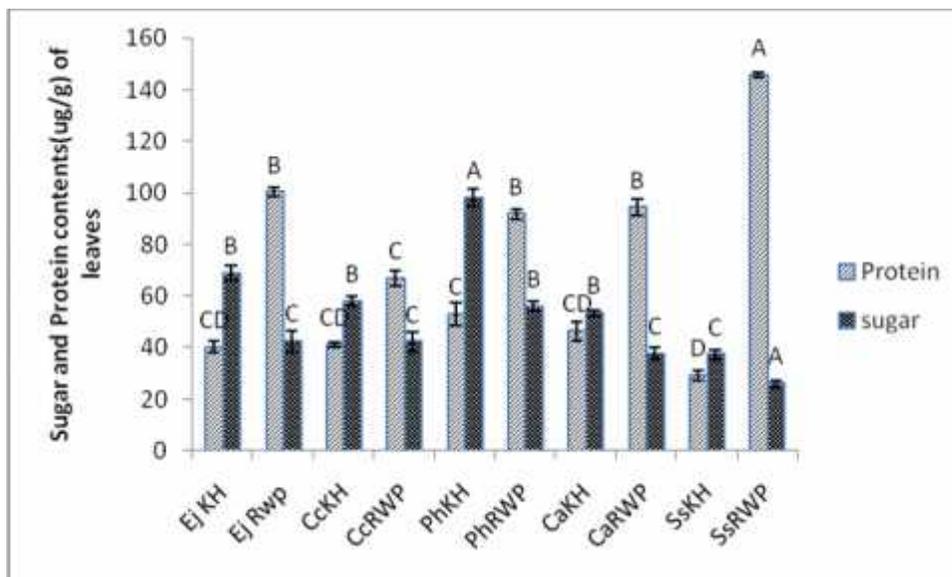


Figure 2. Protein (ug/g) and sugar (ug/g) of leaves (as in Y-axis) of plant species collected from Khewra salt range and non-saline soil of district Rawalpindi. *Aerva javanica* (EjKh for Kh; EjRWP for RWP), *Cenchrusciliaris* (CcKh for Kh; CcRWP for RWP), *Peganum harmala* (PhKh for Kh; PhRWP for RWP), *Chrysopogan aucheri* (CaKh for Kh; CaRWP for RWP) and *Solanum surattense* (SsKh for Kh; Ss for RWP). Values followed by different letters heading the bars represents significant differences (P<0.05), LSD protein =2.63, LSD sugar = 3.48.

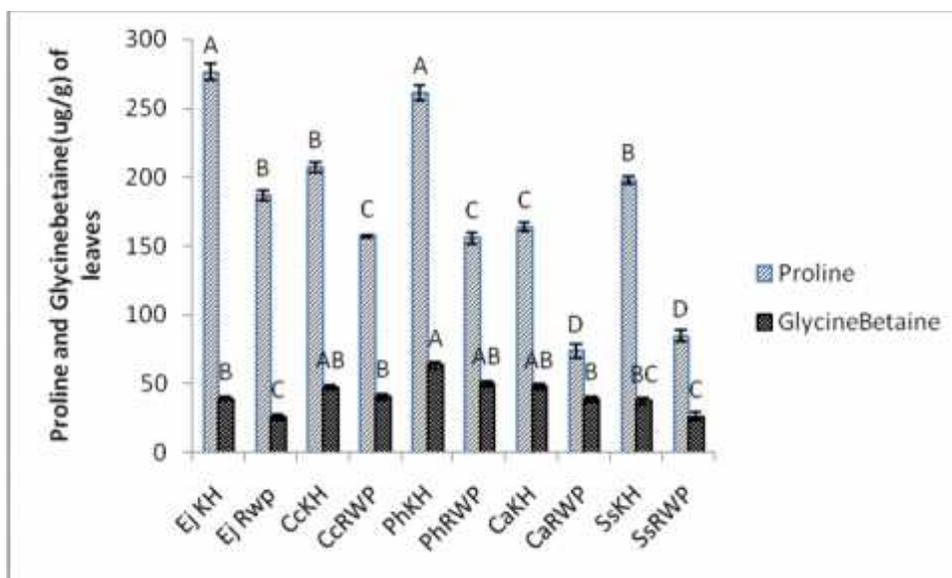


Figure 3. Proline and Glycine betaine (ug/g) contents (as in Y-axis) of leaves of plant species collected from Khewra salt range and non-saline soil of district Rawalpindi. *Aerva javanica* (EjKh for Kh; EjRWP for RWP), *Cenchrusciliaris* (CcKh for Kh; CcRWP for RWP), *Peganum harmala* (PhKh for Kh; PhRWP for RWP), *Chrysopogan aucheri* (CaKh for Kh; CaRWP for RWP) and *Solanum surattense* (SsKh for Kh; Ss for RWP). Values followed by different letters heading the bars represents significant differences ($P < 0.05$) LSD proline = 4.73, LSD glycine betaine = 5.56

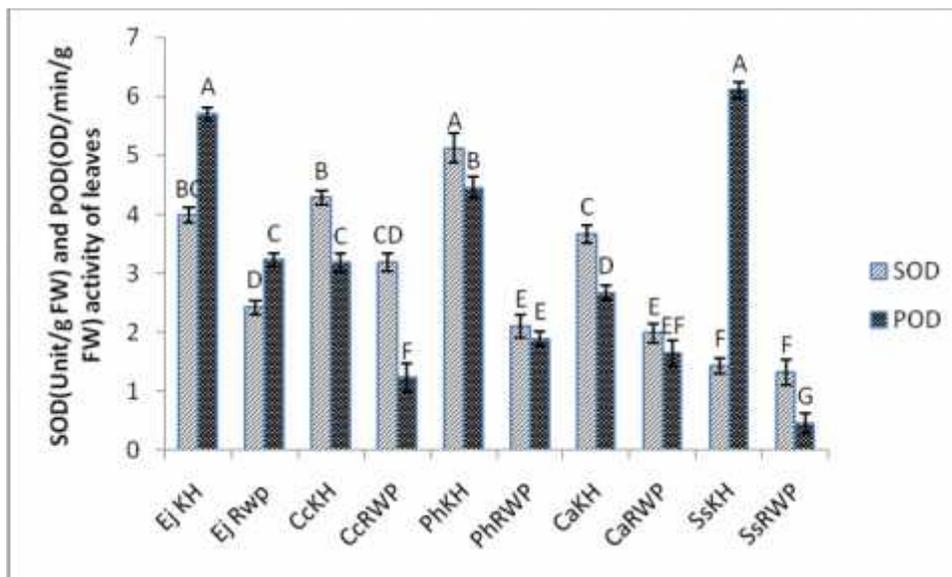


Figure 4. Superoxide dismutase (Unit/g FW) and Peroxidase (OD/min/g FW) activity of leaves of plant species collected from Khewra salt range and non-saline soil of district Rawalpindi. *Aerva javanica* (EjKh for Kh; EjRWP for RWP), *Cenchrusciliaris* (CcKh for Kh; CcRWP for RWP), *Peganum harmala* (PhKh for Kh; PhRWP for RWP), *Chrysopogan aucheri* (CaKh for Kh; CaRWP for RWP) and *Solanum surattense* (SsKh for Kh; Ss for RWP). Values followed by different letters heading the bars represents significant differences ($P < 0.05$). LSD SOD = 3.35, LSD POD = 4.25.

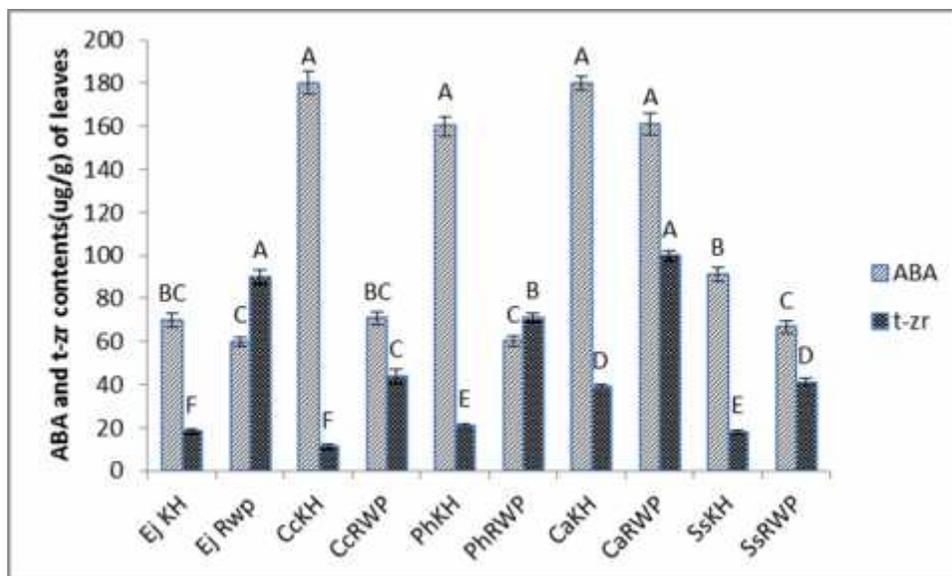


Figure 5. ABA and t-zr (µg/g) contents (as in Y-axis) of leaves of plant species collected from Khewra salt range and non-saline soil of district Rawalpindi. *Aerva javanica* (EjKh for Kh; EjRWP for RWP), *Cenchrusciliaris* (CcKh for Kh; CcRWP for RWP), *Peganum harmala* (PhKh for Kh; PhRWP for RWP), *Chrysopogon acheri* (CaKh for Kh; CaRWP for RWP) and *Solanum surattense* (SsKh for Kh; Ss for RWP). Values followed by different letters heading the bars represents significant differences (P<0.05), LSD ABA = 3.39, LSD t-zr = 2.33.

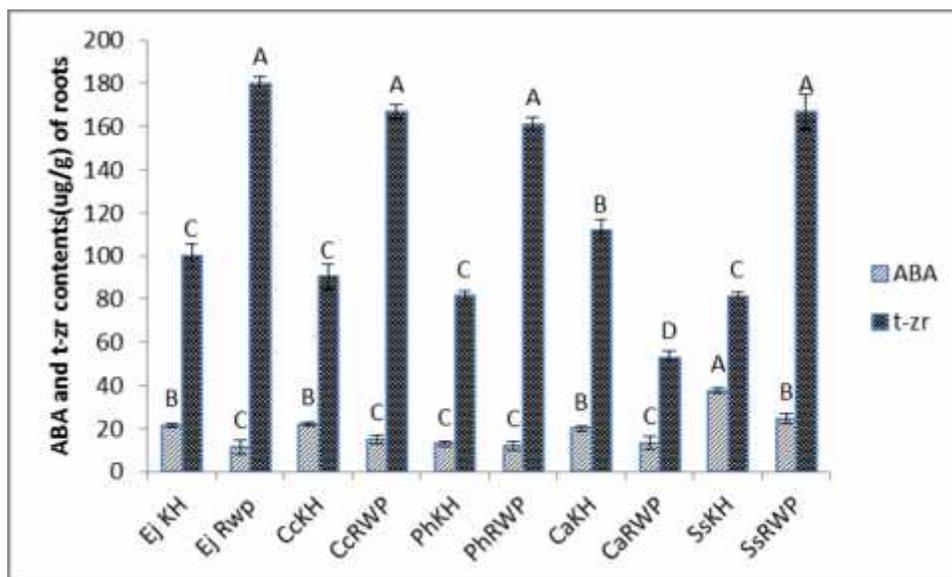


Fig 6. ABA and t-zr (µg/g) contents of roots (as in Y-axis) of plant species collected from Khewra salt range and non-saline soil of district Rawalpindi. *Aerva javanica* (EjKh for Kh; EjRWP for RWP), *Cenchrusciliaris* (CcKh for Kh; CcRWP for RWP), *Peganum harmala* (PhKh for Kh; PhRWP for RWP), *Chrysopogon acheri* (CaKh for Kh; CaRWP for RWP) and *Solanum surattense* (SsKh for Kh; Ss for RWP). Values followed by different letters heading the bars represents significant differences (P<0.05) LSD ABA=7.39, LSD t-zr = 1.44.

DISCUSSION

The high electrolyte leakage accompanied by low relative water contents of leaves particularly in plants of saline

soil represents active replacement of electrolytes and maintenance of high turgidity by lowering the relative water contents, their adaptability to saline condition as described by Maaouia-Houimli *et al.*, (2010).

Rajasekaran *et al.*, (2001) demonstrated that a sharp decline in osmotic potential compared with the total water potential led to turgor maintenance in plants under progressive or prolonged NaCl stress. The higher decrease in osmotic potential and increase in electrolyte leakage in salt tolerant plants might be compensated by the accumulation of compatible solutes proline and glycine betaine as observed during the present investigation and are in accordance with the findings of (Hasegawa *et al.*, (2000). Under osmotic stress the proline appears to play more active role of osmoprotectant than glycine betaine as demonstrated previously by Hayat *et al.*, (2012).

The osmotic potential in leaves of plants growing under saline condition appears to be correlated with ABA concentration. Verslues and Zhu, (2005) also reported that an increased level of ABA under salinity and water stress balances the osmotic potential. The sugar content ($\mu\text{g/g}$) of plants from salt range was greater than plants from non-saline area. Salt stress increases carbohydrates such as sugars (glucose, fructose, sucrose, fructans) and starch in plants (Parida *et al.*, (2002). The low chlorophyll and protein contents (soluble) of plants from salt range than that of plants from non-saline area is in line with previous findings (Doganlar *et al.*, (2010).

Proline and glycine betaine help in the replacement of electrolytes, regulation of water uptake and in the stability of membranes. Proline plays a major role in providing protection to macromolecules as well as act as an energy and nitrogen source during salt stress (Nazarbeygi *et al.*, (2011). Seraj and Sinclair, (2002) also reported a positive correlation between leaf osmotic potential, glycine betaine and proline.

The higher SOD and POD activities in the plants from salt range suggest that plants exhibit operation of better scavenging mechanism for ROS. Plants protect cell and sub cellular systems from the cytotoxic effects of reactive oxygen species by induction of SOD, POD and catalase activities for scavenging reactive oxygen species (Misra *et al.*, (2006).

Plants adaptive responses may be modulated by phytohormones (Kuiper *et al.*, 1990). Plant stress hormone abscisic acid play major role in plant responses (Zhang *et al.*, (2006). ABA concentration was higher in leaves than that of roots which reveals that leaves are the major site for ABA biosynthesis. It appears that plants living in saline environment synthesize ABA at higher concentration in their leaves as observed in tolerant plants of salt range. The ABA/t-zr ratio was higher in halophytes as compared to those of non-saline plants. Noteworthy, the cytokinin (CK) content measured as t-zr decreases in the leaves of salt tolerant plants under saline condition but not in roots which indicate the putative mechanism of CK under similar condition.

The presence of higher t-zr contents in the root of the plants of saline land is noteworthy. Root is the first

organ exposed to salt stress. Presence of higher t-zr concentration in root tissue possibly is an adaptive mechanism to maintain supply of growth promoting hormone t-zr, to delay senescence and prevent dormancy induction in roots caused by high ABA. The maintenance of higher CK content as hormone, preventing senescence or retaining chlorophyll, and protein content may be beneficial as an adaptive mechanism for the plants to continue growth under salt stress as high t-zr/ABA ratios.

During salt stress, reduction of cytokinin supply from the root alters gene expression in the shoot, thereby elicits appropriate responses to ameliorate the effects of stress (Hare *et al.* (2004). Roots of plants from salt range have higher concentration of t-zr as compared to plants from non-saline area. It appears that halophytes have higher t-zr ratio in roots to ensure the supply of CK as required. (Polanska *et al.*, (2007) evaluated that t-zr counteract many processes induced by ABA.

It is inferred from the result that greater ratio of t-zr/ABA can be regarded as one of the selection criterion for tolerance of plants exposed to salt stress. Noteworthy, ABA, proline contents, and SOD activity define the underlying tolerance mechanism of plants from saline soil. The role of ABA and t-zr cannot be overlooked as the maintenance of high t-zr in roots is another adaptive mechanism that prevails in halophytes.

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