The effect of salinity stress on Na^+, K^+ concentration, Na^+/K^+ ratio, electrolyte leakage and *HKT* expression profile in roots of *Aeluropus littoralis*

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ABSTRACT: Among abiotic stresses, salinity has been increasing over the time for many reasons like using chemical fertilizers, global warming and rising sea levels. Under salinity stress, the loss of water availability, toxicity of Na^+ and ion imbalance directly reduces carbon fixation and biomass production in plants. K^+ is a major agent that can counteract Na^+ stresses, thus the potential of plants to tolerate salinity is strongly dependent on their potassium nutrition. *HKT*s (High-affinity K^+ Transporters) are a family of transporters that mediate Na^+-specific or Na^+-K^+ transport and play a key role in the regulation of ion homeostasis. In this study, we intended to focus on Electrolyte Leakage, ratio of K^+/Na^+, transcriptomic responses of a subclass two *HKT* in the roots of *Aeluropus littoralis* under salt stress. We investigated a noticeably different expression pattern over studied time points and found a snappy increase of *AlHKT* and rebalance of K^+ concentration. It can be suggested that the early and high response of a Na^+-K^+ coupled transporter acted as a part of *A. littoralis* salt tolerance.

KEYWORDS: *Aeluropus littoralis*, Flame photometry, Membranous HKT, Potassium, Sodium, Real-time PCR

INTRODUCTION

Human population is growing rapidly yearly and it is estimated to reach 9 billion by 2050 (33). The current average increase of agricultural products is 32 million tons (Mt) per year but if we want to meet demand by 2050, the rate of annual increase must be about 44 Mt yearly (33). It means the production should be increased on an unprecedented scale while facing changing climate and dealing with modern nonfood requirements like green fuel.

Abiotic stresses are the most important factors affecting plant growth and their yields (33). Theses stresses are even getting more important because of the climate change and reduction in water quality and availability (33). Among abiotic stresses, Salinity has been increasing over the time for many reasons like using chemical fertilizers, global warming, rising sea levels (16) and lack of drainage system (24). There was only 323 million hectare (MHa) saline land in 1980 but it is estimated to exceed 400 MHa by 2025 (16).

Physiological, biochemical and genetical studies have shown that salinity stress in plants is multifactorial, including osmotic stress (33) and cellular ion toxicity, which inhibits vital enzymes and metabolic processes (19, 26). Photosynthetic processes are among the most sensitive process to salinity; therefore, salinity stress directly reduces carbon fixation and biomass production

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in plants (18). Many policies can be adopted to confront this problem. Improving plant toward salinity tolerance is one of the most reliable methods that has been studying from many aspects. To improve the ability of crops to grow on saline soil, understanding traits and mechanisms that contribute to salt tolerance in wild and tolerant cultivars are necessary (23). However, mechanisms of plant adaptation to abiotic stresses, particularly for drought (33) and salinity are complex but understanding the mechanisms of salt tolerance by studying traits that contribute to tolerance separately and identifying natural occurring variation in varieties or wild relatives are scientifically applied methods to confront the complexity and to improve further salt tolerance in crops (20, 33).

Under salinity stress, the loss of water availability, toxicity of Na^+ and ion imbalance cause growth limitation so plants adopt divert mechanisms to tolerate salinity. It is repeatedly reported that K^+ deficiency and Na^+ toxicity are major restrictions of crop production worldwide (23, 24, 26, 28, 30, 36). K^+ can counteract Na^+ stresses (18) thus the potential of plants to tolerate salinity is strongly dependent on their potassium nutrition (26). K^+ composes about 10% of the plants dry weight and is the most abundant mineral cation in the plants (30). It is involved in many of functions related to enzyme activation, respiration and starch and protein synthesis (6, 30). For instance, about 50mM of K^+ is required for normal starch synthesis and 10-50mM is needed for activation of K^+ dependent enzymes (6). The optimum concentration of K^+ narrows by increasing the amount of Na^+ (26) for many reasons such as similarity of Na^+ and K^+ in their physicochemical structure. This similarity leads to competition of Na^+ and K^+ at transporters or enzymes binding sites that can result in K^+ deficiency and inhibition of biochemical processes that are dependent on K^+ (26). So the capacity of a cell to maintain a high K^+/Na^+ ratio is assumed to be a critical strategy in salt tolerance (26). For instance, it is reported that animal cells maintain the K^+/Na^+ ratio around 20 by regulating the K^+ and Na^+ concentration around 100mM and 5mM respectively (26). In plants, the optimum concentration of K^+ is reported to be about 100-150mM and the minimum value of K^+/Na^+ is about one (26). In contrast, the soil K^+ concentration ranges from 1 to 0.1mM (30, 35) and in some cases, the K^+/Na^+ ratio is less than 0.02 (26). So an efficient and controllable potassium supply system should be available for plants (35).

*HKT*s (High-affinity K^+ Transporters) are a family of transporters that mediate Na^+-specific transport (subclass one) or Na^+-K^+ transport (subclass two) and play a key role in regulation of ion homeostasis and contribute to osmotic adjustment and Na^+ detoxification in plants (20). They have been identified in all studied plants (6). The members of this family were identified in 1994 and were called *HKT* because their mutation in *Xenopus laevis oocytes* had led to defective K^+ uptake (6). According to the studies focused on isolated *HKT*s and their distribution in a variety of crops, it is demonstrated that they are involved in K^+ related salt tolerance responses rather than K^+ nutrition (6). *A. littoralis* is a wild halophyte member of *Poaceae*, native to coastal zones that is salt and drought tolerant (4, 35). Although *A. littoralis* is usually exposed to high saline conditions, it grows normally without any toxic symptoms and can maintain a high K^+/Na^+ ratio under a high salt environment (35).

Retention of a high K^+/Na^+ ratio is defined as a determinative trait in salt tolerance (26). In addition to this, wild relative of crops are important as a naturally selected gene sources. Based on this facts, this study was performed to focus on concentration of Na^+, K^+ and their ratio along with the transcriptomic expression pattern of a *HKT2* of *A. littoralis*'s roots at a 15-days span of salinity stress.

MATERIALS AND METHODS

Plant material

The seeds of *A. littoralis* were purchased from ICRASN (Isfahan Center for Research of Agricultural Science and Natural Resources). The seeds were planted in sand pots in growth chamber (12/12h day/night, temp. 18ºC/22ºC). After 21 days, the seedlings were transferred to continuously aerated hydroponic systems containing Yoshida nutrition medium (10, 15) [pH= 5.5, 16/8h day/night, temp. 25ºC]. The pH was checked and rebalanced every day and the medium was refreshed weekly. The salinity stress started after 21-days establishment period in hydroponic medium. The salinity stress experiment was conducted based on a completely randomized design with 12 sampling concentration/time points and three biological replications. In fact, our study took place in two phases. A pre-experiment was carried out to evaluate some physiological responses of *A. littoralis* to salinity and finding the best concentration/time points for main physiological and transcriptomic studies. For this purpose, we conducted the salinity stress and sampling at 2, 4, 6, 8, 12, 24 and 48 hours of 100, 200, 300, 400, 500 and 600 mM NaCl and

simultaneously with their equivalent controls, all with three replications (7×6×2×3 samples). At the end of pre-experiment, the fresh and dry weights (data not shown), electrolyte leakage (Figure 1), Na^+ (Figure 2) and K^+ concentration (Figure 3) of them was measured. According to these data, the time points with the significant change were chosen for sampling in the main experiment.

Finally, the sampling of roots were done at 6h/100mM, 6h/200mM, 6h/300mM, 48h/300mM, 72h/300mM and 264h/300mM time/concentration points. The sampling of control plants was also done at the same time / concentration points concurrently with treated ones. Collected root tissues were snap-frozen using liquid nitrogen and were stored at -80ºC immediately.

qRT PCR

Real time RT-PCR was performed by isolating RNA from root tissues using Denazist Column RNA Isolation Kit (#S-1020, Iran) followed by DNase digestion (Thermo Scientific DNase I #EN0525, USA). Synthesis of first strand cDNAs was carried out by using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1622). For quantitative real time PCR 5µL of diluted, (1 to16) cDNA were used as templates and the reaction of cDNA synthesis was done using RealQ Plus Master Mix (AMPLIQON #A314402, Denmark) in a Bioer thermo cycler (Applied Bioer, LineGeneK, Hangzhou, China).

Due to the absence of *A. littoralis HKT's* gene sequence in databases, primers for the amplification of membranous *AlHKT* were designed based on the membranous *HKT*2s (subfamily 2) of *Poaceae*, specially CDs of *Zea maize* and *Setaria italica* using Bioedit® v7.0.9 to align sequences and Primer Premier© v5 to design primers. Finally, the following primers were used for amplification of membranous *AlHKT* (product size 369bp):

AlHKT	5' GAAACCGAGCAACCCTGAC 3'
	5' AATCCTAAGTATCTAACGCTC 3'

In order to normalize the qPCR data, primers of *βActin* (product size 113bp) and *elongation factor* (product size 90bp) genes were designed in the following sequences based on the above mentioned method and examined.

βActin	5' TTGCTGGCCGAGACCTTAC 3'
	5' GGCGAGCTTTTCCTTGATG 3'
elfactor	5' ACCTTCTCTGAATACCCTCCTCTG 3'
	5' CTTCTCCACACTCTTGATGACTCC 3'

The PCR condition for *AlHKT* was 94ºC/10sec, 63ºC/20sec and 72ºC/20sec and for *βActin* it was 94ºC/10sec, 55ºC/20sec and 72ºC/20sec. The calculation of relative gene expression was done based on methods that explain expression ratio equal to $2^{-\Delta\Delta Ct}$ (31).

Sodium and Potassium Concentration

The measurement of Na^+, K^+ concentration was performed using flame photometry (#pfp7, Jenway. UK). Because tissue mass of some samples were very low, the preparation of the samples was done using 15mg of root tissues according to a wet ashing method (11). In this method, instead of direct heat of oven, heat and concentrated nitric acid are used to degrade organic material of tissues.

Statistical analysis

As mentioned above, our experiment was performed based on a completely randomized design with three biological replications in the samplings. To increase the reliability of gene expression analysis, real time PCR experiments were done with two identical technical replications. The statistically analyzes of the data were done using Tukey's range test ($\alpha = 0.05$; MINITAB v16.).

RESULTS AND DISCUSSION

The PCR production results of all primers were coincided with bioinformatically predicted lengths. After examination of housekeeping genes, *βActin* selected as the reference gene for normalizing the qPCR data because it showed less influence under salinity stress. The expression pattern of *AlHKT* showed that it differs noticeably over concentration/time points; in fact its expression reduced to ⅓ at the initiation of stress ($6h^{**}$ – Figure 4) but increased snappily after 54h (Figure 4). The highest expression ratio of *AlHKT* was observed at 6h/200mM NaCl equal to 54h from initiation of stress (Figure 4). The highest expression at this time point was more than eleven (11.57) times higher than controls but the expression was reduced and remained equal to untreated samples after that ($102h^{**}$ - Figure 4). The report of Horie et al. (19) on rice also showed similar result that low-K^+ concentrations (less than 3 mM) induced the expression of all *OsHKT* genes in roots, but mRNA accumulation was inhibited by the presence of 30 mM Na^+. This drop in gene expression can be attributed

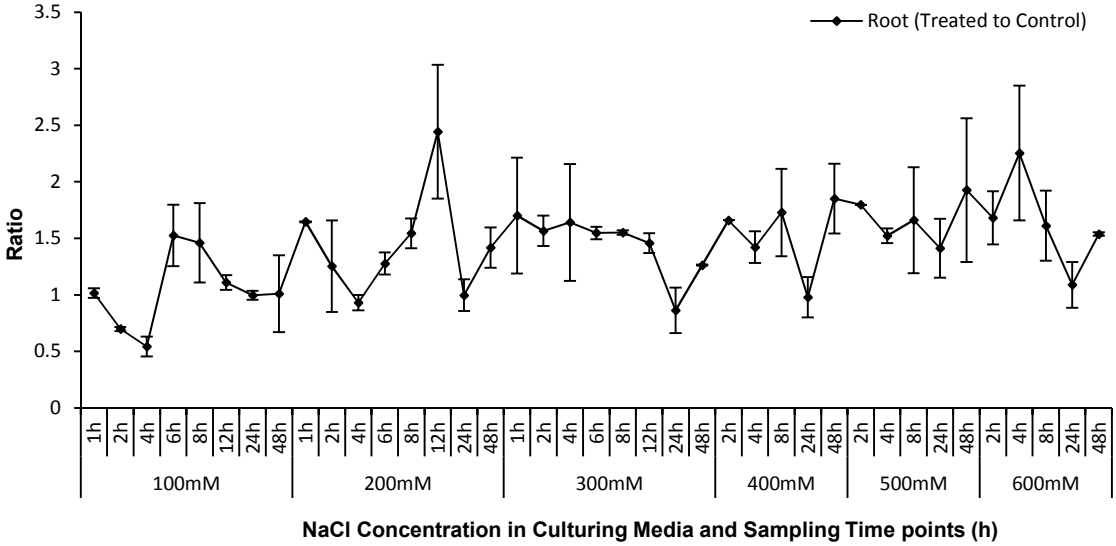

Figure 1. Treated to Control Electrolyte leakage ratio in roots of *Aeluropus littoralis* under salinity stress of pre-test.

to this assumption that the studied *HKT* genes do not carry regulation elements related to rapid and continuous expression. It can be assumed that any mechanisms other than continues *HKT* expression helped them to overcome salinity stress. In our study, it was assumed that because Yoshida medium's K^+ content (2 mM) is lower than other common hydroponic mediums like Hoagland (4-5 mM), so this high accumulation of *HKT* seems to be necessary to obviate K^+ deficiency.

It is thought that Na^+ and K^+ homeostasis are crucial for salt-tolerance in plants (19; 8). Most plant species are sensitive to high concentrations of sodium (Na^+), which produce combined Na^+ toxicity and osmotic stress (18). It is also successively reported that K^+ is the most efficient monovalent cation in both biophysical and biochemical processes (6). Thus, plants designed and gathered efficient acquisition, redistribution and homeostasis systems for Na^+ sequestration (5, 8, 14) or increasing cytoplasmic potassium (K^+) levels relative to Na^+ that would lead to increased salt tolerance in plants (18). Up to now, plant membranous potassium transporters are categorized into three families (14) but according to the reports of Horie *et al* (2009), the major salt-tolerance trait in monocot crops is based on some of the *HKT*-mediated mechanisms.

In line with the changes of *HKT* expression, we also investigated the ratio of sodium and potassium concentration in samples. The Na^+ concentration in treated samples increased constantly from 10 to 35 mg g^{-1}DW over the stress span (Figure 5). However, it seemed that the concentration of Na^+ had been constantly maintained about 5-10mg g^{-1}DW in untreated samples. In contrast, as it is illustrated in figure 6, the concentration of the K^+ in root tissue of control samples increased and maintained about 45mg g^{-1}DW, however it decreased gradually after 48 hours from initiation of stress that could be because of the increasing of tissue mass (in vegetative growth). It is also reported that Aeluropus root biomass increases under salt stress (4). The concentration of potassium ion in treated samples decreased in contrast to control samples. This decrease in concentration has continued until 54 hours since initiation of salinity stress and then increased and continued in a fluctuation like control samples but in a statistically non-significant lower level. It seems that the reduction of accessible K^+ or lower expression of HKT (Figure 1) led to the early reduction of K^+ in treated samples; as mentioned, the expression of membranous HKT increased dramatically (about 11.5 times) in 54 hours after the initiation of stress (= 6h*/200mM, Figure 5).

The increase of HKT proteins as a result of the increases in transcriptomic level may lead to accumulation of K^+ in roots after 72 hours in stressed samples (= 24h/300mM). The deferment between the transcriptomic increase of HKT and accumulation of K^+ could be attributed to the time for translation and activation of HKT proteins.

The concentration of Na^+ maintained about 5-10mg g^{-1}DW in control samples but increased significantly in stressed samples and led to the decrease of K^+/Na^+ ratio in treated samples (Figure 7).

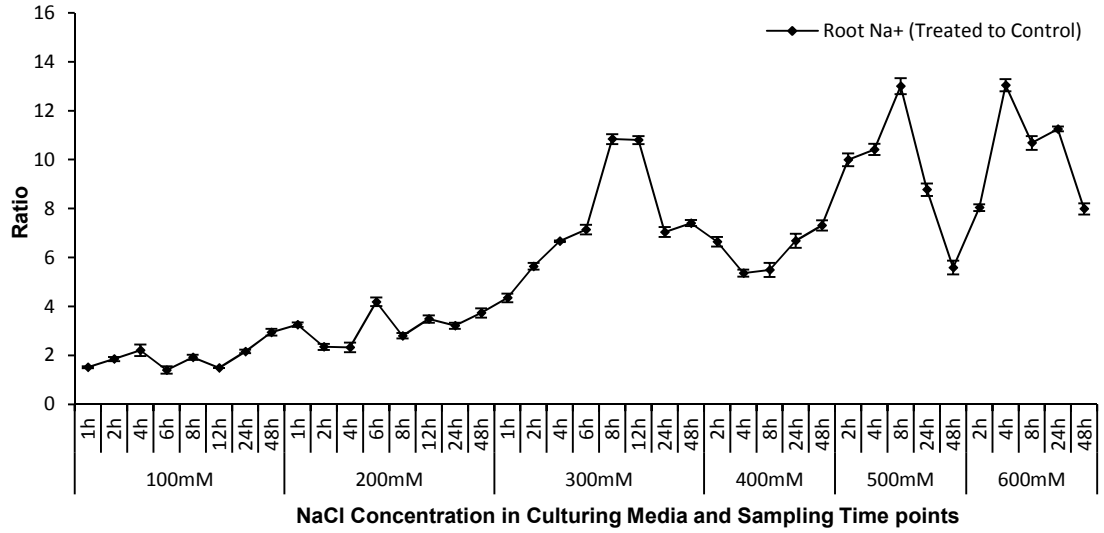

Figure 2. Treated to Control Na^+ Concentration ratio in roots of *Aeluropus littoralis* under salinity stress of pre-test.

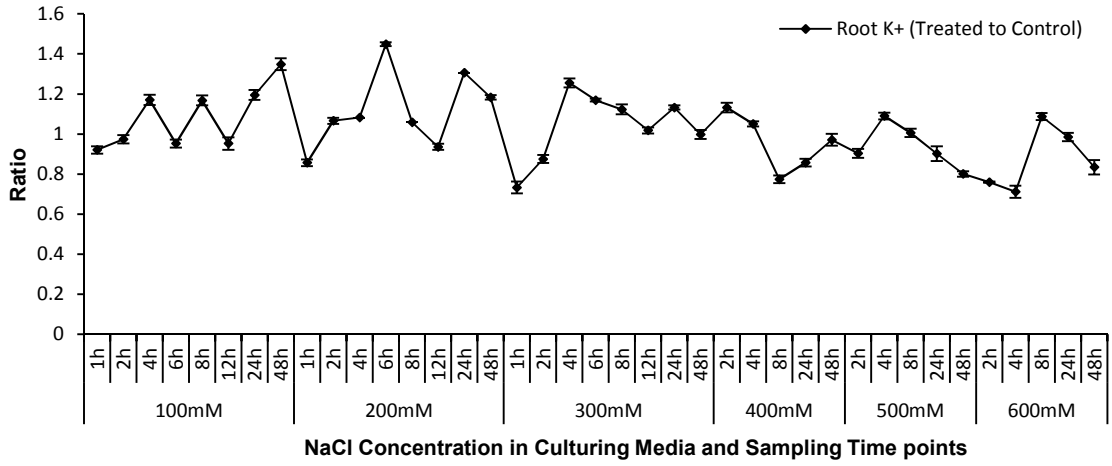

Figure 3. Treated to Control K^+ Concentration ratio in roots of *Aeluropus littoralis* under salinity stress of pre-test.

This increase of Na^+ can be attributed to multiple reasons. The first reason can be the competition of Na^+ and K^+ to influx into the root cells; The electrophysiological similarity of Na^+ and K^+ (6), in addition to the higher accessibility of Na^+, may lead to its higher seepage into root cells. It is also reported that in the absence of potassium, certain potassium channels can conduct sodium (25). Secondly, it could be assumed that Na^+/ K^+ simporters have been activated to increase the concentration of K^+ in response to the decline of K^+. This may have happened along with compartmentization of Na^+ in organelles like vacuoles (however it needs further studies to be approved). *A. littoralis* is a member of the four genera (*Spartina, Aeluropus, Distichlis* and *Chloris*) of Poacea family that are able to excrete salts and *A. littoralis* do it with a high selectivity in favor of sodium opposing K^+ and Ca^+ (4). So the Na+ may be transported to shoot tissues to be excreted via specific glands. Third, because of electrophysiological similarity of sodium and potassium (6), in potassium deficiency, it can be replaced with Na^+ for some biological processes with lower specificity to potassium (12). Cramer et al. (9) and Mäser et al. (28) also reported that K^+ counteracts Na^+ stress, while Na^+, in turn, can to a certain degree, alleviate K^+ deficiency. So, the gradual increase in Na^+ concentration and HKT expression observed in our study could be assumed as a strategy of *A. littoralis* using Na^+ to maintain the biological process (33). Fourth, as it is reported, high-affinity potassium transporters (HKTs) are a large super family of transporters in plants, bacteria, and fungi (2, 14, 28, 32) It has been suggested that these transporters play crucial roles in salinity tolerant via removal of Na^+ from

the xylem during salinity stress (21, 22). Based on the amino acid sequences, *HKTs* are categorized into two subfamilies called 1 and 2 (32). The members of two subfamilies are different in their cation (Na^+ or K^+) selection (6). Members of subfamily 1 are all Na^+ specific transporters (6, 20) but the other subfamily members are Na^+/K^+ uniporters (20). It is demonstrated that they are more permeable to K^+ compared with the subfamily 1of *HKTs* and the transcript level of them has increased in K^+ starvation stress in wheat (*Triticum aestivum*), rice (*O. sativa*) and barley (*Hurdeum vulgare*) (19). However, according to the reports of Bañuelos et al. (3), the uniport and symport activities of HKTs are also dependent on their protein density in the membrane. For instance, high level expression of barley's HKT1 (*H. vulgaris*) in yeast led to K^+ or Na^+ uniport activity, but when protein expression decreased significantly, the transporter acted in the K^+/Na^+ symport mode (6). So, the other reason for Na^+ accumulation in *A. littoralis* during salinity stress in this study on can be attributed to K^+ or Na^+ uniport activity of HKTs. However, physiological data indicate that plants have a higher capacity for K^+ uptake and distribution than for Na^+ (1, 37). The fifth reason that may explain the accumulation of sodium ion in our study is that photosynthesis is vulnerable to salinity (18) so to protect this vital process via maintaining high K^+/Na^+ ratio in photosynthetically active tissues. *A. littoralis,*

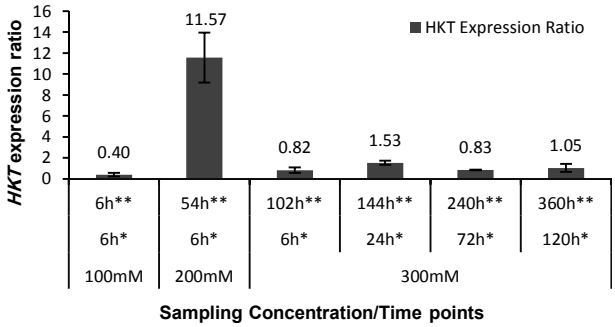

Figure 4. The treated to control expression pattern of HKT in *Aeluropus littoralis* root under salinity stress.

* Shows hours from the initiation of mentioned concentration.

** Shows hours from the initiation of stress.

alike many other species (7, 17) adopted a strategy to transport Na^+ via the phloem from shoot tissues to roots. As it is approved, Na^+/K^+ homeostasis is a key parameter in plant salinity tolerance (16, 36, 37) and the HKT family members are the most important transporters related to this balance (29) but little is known about their behavior in wild plants (2). In conclusion, our study has provided an expression analysis of *AlHKT* transporter for six concentration/time points of *A. littoralis* under salt stress by real-time PCR (Figure 4). Because it showed the highest level of expression at early point, and the increase of K^+ concentration occurred after that, we predict that K^+ inwardly flux in this species is one of the main salinity

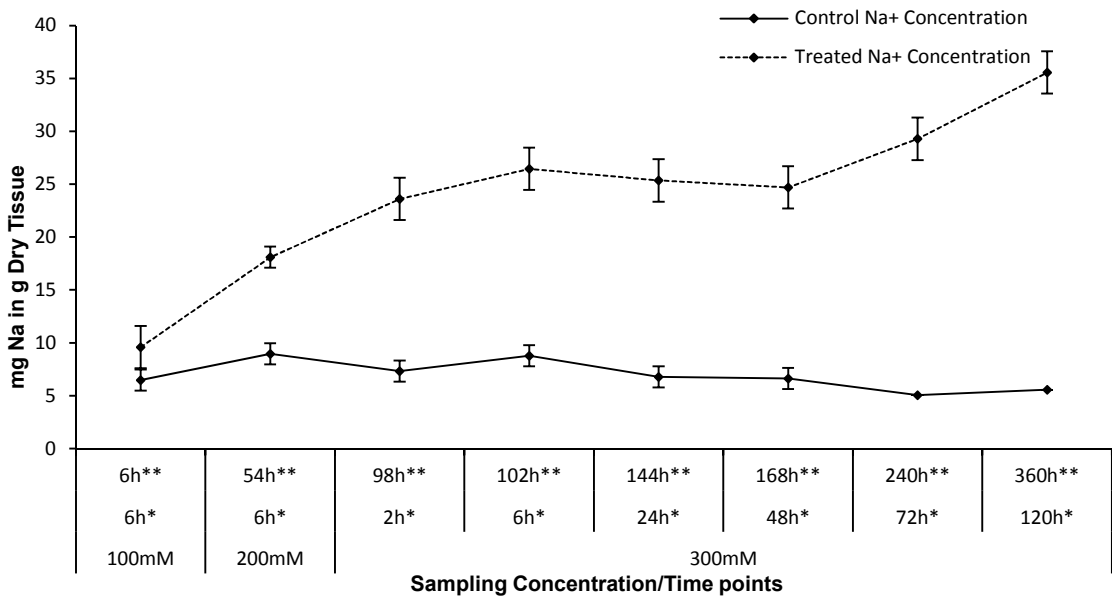

Figure 5. The fluctuation pattern of Na^+ concentration in *Aeluropus littoralis* root of control and under salinity stress samples.

* Shows hours from the initiation of mentioned concentration.

** Shows hours from the initiation of stress.

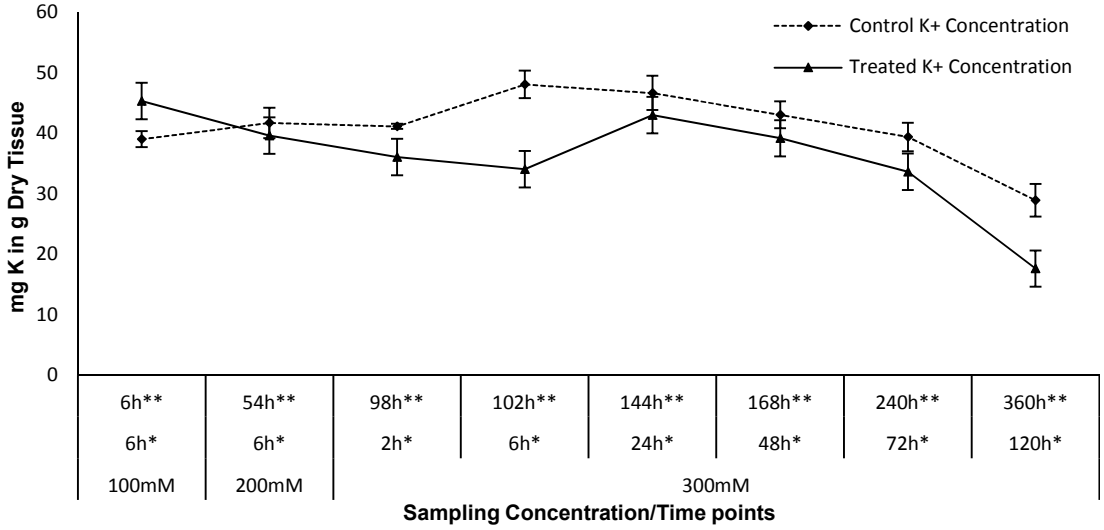

Figure 6. The fluctuation pattern of K^+ concentration in *Aeluropus littoralis* roots of control and under salinity stress samples.

* Shows hours from the initiation of mentioned concentration.

** Shows hours from the initiation of stress.

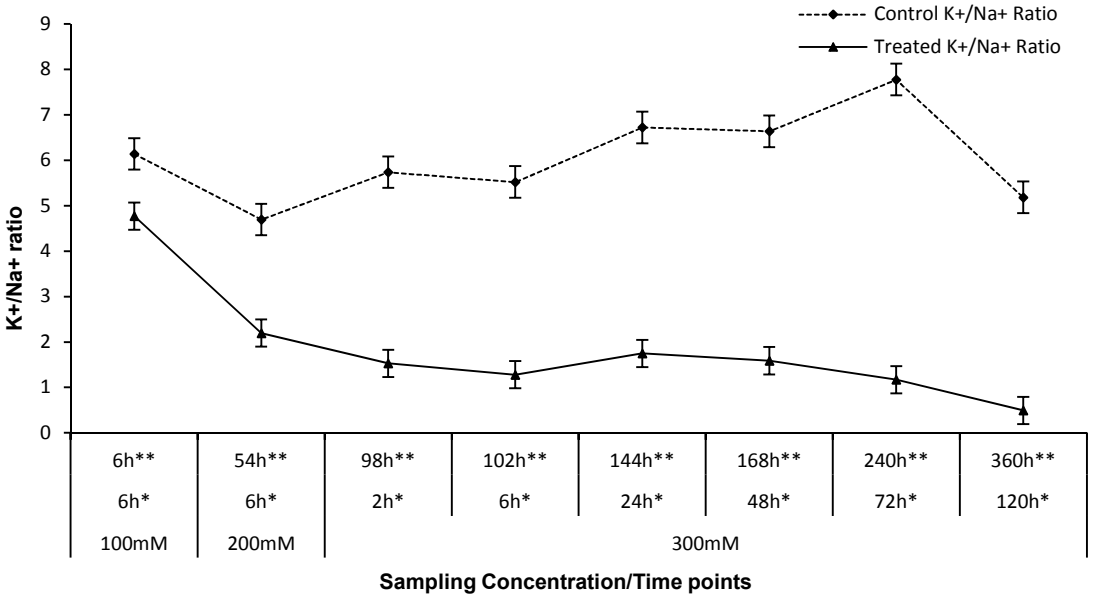

Figure 7. The fluctuation pattern of K^+/Na^+ ratio in *Aeluropus littoralis* root under salinity stress.

* Shows hours from the initiation of mentioned concentration.

** Shows hours from the initiation of stress.

tolerance mechanism too and its HKT proteins were able to uptake K^+ efficiently in the presence of high concentration of Na^+ (Figure 6). However, the relationship between the primary structure of this transporter and Na^+ insensitivity needs to be further investigated but different Na^+ sensitivity of high-affinity K^+ uptake transporters among species is approved and attributed to their amino acid sequence and structure (35). For instance, when the *Capsicum annum* HAK1 was functionally characterized in yeast, it showed sensitivity to millimolar concentrations of Na^+ (27). HAK of *Cymodocea nodosa*, (*CnHAK1*) was insensitive to Na^+ (13) and the expression of HAKs from *Mesembryanthemum crystallinum* (*McHAK1* and *McHAK4*) led to growth of yeast in 150 mM NaCl (34). The early and highly expression of HKT gene studied in our experiment and its permanency and lasting activity in high concentration of Na^+ make its sequence and

regulatory elements as a prospective candidate for future empirical studies as well as transforming it and improving salinity tolerance in many Poaceae crops.

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اثر تنش شوری بر غلظت سدیم و پتاسیم، نسبت K^+/Na^+، نشت الکترولیت و الگوی بیان ژن *HKT* در ریشه آلوروپوس لیتورالیس (*Aeluropus littoralis*)

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چکیده

از میان تنش‌های غیر زیستی، تنش شوری به دلایلی همچون افزایش استفاده از کودهای شیمیایی، گرم شدن زمین و بالا آمدن سطح آب دریاها، رو به افزایش است. تحت شرایط تنش شوری کاهش آب در دسترس، سمیت یون سدیم و عدم تعادل یونی مستقیماً باعث کاهش تثبیت کربن و تولید زیست توده در گیاهان می‌شود. یون پتاسیم یکی از اصلی‌ترین عواملی است که می‌تواند تنش حاصل از سمیت سدیم را خنثی کند به همین دلیل توانایی تحمل تنش شوری در گیاهان به نسبت بالایی به جذب پتاسیم آنها بستگی دارد. HKTها خانواده‌ای از ناقلین پروتئینی هستند که ناقلین اختصاصی Na^+ یا Na^+ و K^+ هستند و نقش کلیدی در تنظیم تعادل یونی سلول بازی می‌کنند. در پژوهش حاضر نشت الکترولیت، روند تغییر شاخص K^+/Na^+ و الگوی بیان ژنی از زیر خانواده *HKT2* در ریشه گیاه *Aeluropus littoralis* تحت تنش شوری مورد ارزیابی قرار گرفت. نتایج تفاوت معناداری را در الگوی بیان ژن در طی زمان و غلظت‌های مورد مطالعه و افزایش شدید *AlHKT* و بازیابی تعادل دوباره K^+ را نشان داد. بطوری که می‌توان پیشنهاد داد که پاسخ سریع و افزایشی بیان هم ناقلین Na^+ و K^+ در تحمل به تنش شوری آلوروپوس نقش آفرینی می‌نماید.

کلمات کلیدی: آلوروپوس، بیان ژن، پتاسیم، سدیم، ناقل غشایی، هالوفیت