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Bhanu Kumar Tiwari
Department of Agricultural Biotechnology and Molecular Biology, (Dr. RPCAU) Pusa, Samastipur, Bihar, India

Ahmed Aquib
Department of Agricultural Biotechnology and Molecular Biology, (Dr. RPCAU) Pusa, Samastipur, Bihar, India

Rahul Anand
Department of Agricultural Biotechnology and Molecular Biology, (Dr. RPCAU) Pusa, Samastipur, Bihar, India

Corresponding Author:
Bhanu Kumar Tiwari
Department of Agricultural Biotechnology and Molecular Biology, (Dr. RPCAU) Pusa, Samastipur, Bihar, India

Analysis of physiological traits and expression of NHX and SOS3 genes in bread wheat (*Triticum aestivum* L.) under salinity stress

Bhanu Kumar Tiwari, Ahmed Aquib and Rahul Anand

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Abstract
A hydroponic experiment was conducted in a phytotron to study the effect of salinity on physiological traits and qRT_PCR analysis for gene expression was also done in bread wheat. Wheat genotype HD-2687, was grown in hydroponic under different salt concentrations of 100 mM NaCl, 200 mM NaCl & 250 mM NaCl along with control treatment. Various parameters were recorded during the experiment, viz., chlorophyll content, carotenoid content, relative water content, root area and root diameter. Based on the experiment, it can be concluded that salinity stress negatively impacted various physiological traits such as relative water content, chlorophyll and carotenoid contents and root traits in bread wheat. The decline in various traits was exacerbated as the stress level was increased. Expression of genes involved in sodium ion homeostasis (NHX, SOS3) were up-regulated in root tissues of wheat subjected to salinity stress.

Keywords: Salinity, bread wheat, SOS3, NHX, HD-2687

Introduction
Wheat (*Triticum aestivum* L.) is the soul of the food crop of India. It pertains to the family of Gramineae. It is the second major food crop after the rice, cultivated mainly in the state of Uttar Pradesh, Madhya Pradesh, Punjab, Haryana, Bihar, Gujarat and Maharashtra. Among them, Punjab ranked first in terms of productivity while Uttar Pradesh ranked first in the area and total production. Wheat requires a well-pulverized soil with a proper balance of moisture and air for growing. Several biotic and abiotic stresses result in the depletion of food production in today's world. Salt stress severely affects the growth and yield of the crop. Soil salinity is created due to the accumulation of excess minerals such as Na^+ and Cl^- ions in the soil, and is one of the serious limiting factor for crop growth and production. According to FAO, around 6% of the world's land is affected by salinity. It is estimated that about 20% of the irrigated land in the present world is affected by the salinity that is exclusively classified as arid and desert land comprising 25% of the total land of our planet (Yeo 1999). Crops are categorized as halophytes (tolerate high salinity) and glycophyte (tolerate low concentration of salt).

Effect of Salinity
Salinity affects the crop in two ways (Munns 2005) [1], Osmotic or water deficit effect of salinity, reduce the water potential of soil and disturb the capacity of roots to extract water [2]. Effect of salinity due to increase in ions, in which the concentration of the salt increases within the plant and produces a disturbance in ion homeostasis & leads to toxicity. Salt stress is ultimately an osmotic effect, which affects germination, photosynthetic pigmentation, photosynthesis, water relation, nutrient imbalance, and yield.
The sensitivity of the wheat crop to salinity due to the inability for maintenance of Na^+ and Cl^- ions out of the transpiration stream (Munns *et al*. 2002) [8]. The excess salt enters the plant in the transpiration stream, does injury to cells in the transpiring leaves and may cause a further reduction in growth. Na^+ interferes with K^+ ion uptake & disrupts stomatal regulation that causes water loss while Cl^- disturbs the chlorophyll production cause chlorate toxicity. Salinity affects the vital process of germination in wheat, alters the imbibition of water by the seed due to the lower osmotic potential of germinating media (Khan, 2000) [5] generate toxicity which changes the activities of enzymes of nucleic acid metabolism, alters protein metabolism (Dantas *et al*. 2007), disturbs hormonal balance (Khan & Rizu; 1994) and reduces the utilization of seed reserves (Othman *et al*. 2008).

Salinity also leads to ion toxicity in wheat and other crops. Increased salt uptake induces specific io; toxicities that decrease the uptake of essential nutrients like *N,P, K* and Ca (Zhu 2004) [14]. The high concentration of Na^+ competes with uptake of K^+ and Ca^{2+} and decrease rate of photosynthesis by reducing stomatal conductance and high Cl^- reduced photosynthesis due to chlorophyll degradation (Tavakkoli *et al*. 2001). Salinity cause production and accumulation of ROS. It is one of the main causes for the decrease in crop productivity (Haliwell & Gutteridge 1989, Asada 1994). It causes peroxidation of lipids, oxidation of proteins, inactivation of enzymes and DNA damage.

Response to Salinity

Response to salinity by crops occurs correspondingly as stress in two phases. Shoot ion-independent response occurs first within minutes or days after stress associated with the Na^+ sensing and signalling (Gilroy *et al*. 2014; Roy *et al*. 2014) and the second phase of the response occurs after a longer period of stress involve a build-up of ions in the shoot to toxic concentration causing premature senescence of leaves or even plant death.

Mechanism of Salinity Tolerance

Crops exhibit tolerance to salinity in three ways (Munns and Tester 2008) [9], (1) Ion exclusion mechanism- in which toxic ions are excluded from the shoot (2) Tissue tolerance: ions are compartmentalized into specific tissues, cells, and subcellular organelles (3) Shoot ion- independent tolerance: which allows the growth and water uptake, independent of extent of Na^+ accumulation in root. Sodium ions in saline soils are toxic to plants by negatively affecting K^+ nutrition, photosynthesis, and enzyme activities.

Accumulation of Na^+ in the cytoplasm can be curtailed by reduced Na^+ influx, increased Na^+ efflux, and vacuolar sequestration of Na^+ ions. At the plasma membrane, a family of P-type H^+-ATPases serves as the primary pump that generates a proton motive force driving the active transport of other solutes, including K^+ and Na^+. In wheat roots, high rates of Na^+ efflux were inferred because net uptake was very low relative to unidirectional influx. The Na^+/H^+ antiporter, salt overly sensitive SOS1 is the only Na^+ efflux protein at the plasma membrane of plants characterized so far. Mutants of Arabidopsis thaliana lacking SOS1 are extremely salt sensitive and have combined defects in Na^+ extrusion and in the long distance transport of this ion from root to shoot. SOS3 is a myristoylated Ca^{2+} sensor belonging to calcineurin B-like (CBL) protein family. Mutant plants deficient in SOS3 share the salt-sensitive phenotype of sos1 plants (Zhu, 2001). Vacuolar sequestration of Na^+ is an important and cost-effective strategy for osmotic adjustment that also reduces the Na^+ concentration in the cytosol. In the ion exclusion mechanism of salt tolerance, tonoplast (NHX1 $Na^+(K^+)/H^+$ exchanger is important for vacuolar Na^+ sequestration and salinity tolerance (Munns and Testerl 2008) [9]. Two loci Nax1 (on chromosome 2A) and nax2 (on chromosome 5A) were discovered in durum wheat (James *et al*., 2006) [7], these loci with transporters HKT1; 4 and HKT1; 5 (candidate genes) respectively retrieves Na^+ from xylem. Nax loci confer two highly complementary mechanisms, both of which contribute towards reducing Na^+ content from xylem, in which Nax1 gene confers a reduced rate of transport of Na^+ from root to shoot and retention of Na^+ in the leaf sheath, thus giving a higher sheath to blade Na^+ concentration ratio. The Nax2 confer a lower rate of transport of Na^+ from root to shoot and has a higher rate of K^+ transport, resulting in enhanced K^+ versus Na^+ discrimination in leaf (James *et al*. 2006) [7].

Material and Methods

Plant material and salinity treatment protocol

Seeds of bread wheat genotype HD-2687, surface sterilized with 0.1% mercuric chloride ($HgCl_2$) were germinated in petri-dishes lined with moist filter paper 20-22 °C in a BOD (Biological Oxygen Demand) incubator. Four days after germination, uniform-sized seedlings were transplanted in the germination sheet as cigar rolls (Fig. 1) in hydroponics system supplemented with half-strength Hoagland solution with salinity treatment of 100mM NaCl, 200mM NaCl & 250mM NaCl at optimum temperature regime 22/18 °C (day/night) with relative humidity 60%, photo-period 16/8 hr and light intensity 350 mol $m^{-2}s^{-1}$. Salt stress treatment was given for two weeks, seedling not exposed to salinity treatments were used as control plants and were grown normally in Hoagland solution. Three treatments and control, all were grown in the replication of three. Sampling for physiological observations were done after two weeks of treatment, whereas for gene expression studies, leaf and root tissues from control and salinity treatments were collected after two days of treatment.

Physiological parameters

Physiological parameters such as chlorophyll-content, relative water content (RWC), carotenoid content, root diameter, surface area, and volume were recorded.

Relative Water Content

RWC was determined by the method as described in Weatherly, 1950. For the estimation, topmost leaf sample was collected from each treatment and control. The fresh weight was taken by weighing an equal number of leaves from each sample immediately after collection. The leaves were incubated in distilled water for 4h to get the turgid weight. The leaf samples were then dried in an oven to measure the dry weight of the sample.

Estimation of chlorophyll and carotenoid content (mg g^{-1} DW).

Chlorophyll content was determined according to Hiscox and Israelstam (1979). Freshly sampled, uppermost fully expanded leaves were put in test tubes containing dimethyl sulphoxide (DMSO). The test tubes were then kept in dark at room temperature for overnight to facilitate the extraction of chlorophyll into the solution. The absorbance was measured at 470, 645 and 663 nm using UV-visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). Chlorophyll 'a', chlorophyll 'b', total chlorophyll and total carotenoids were calculated according to Arnon (1949) and expressed as mg g^{-1} DW. Ratio of Chlorophyll a/b and total chlorophyll/carotenoids was also calculated.

Measurement of root surface area (cm^2) and other root parameters

Fresh roots were used for root scanning by Win-RHIZO, Regent Instruments Inc. Two representative plants were taken for each replication and scanning was done in triplicates for each treatment. Root scanning data were retrieved to calculate average diameter, volume of main root (diameter >0.5mm), volume of lateral roots (diameter ≤0.5mm), surface area of main root (diameter >0.5mm) and surface area of lateral roots (diameter ≤0.5mm), total root volume, total root surface area,

Isolation of total RNA
Total RNA isolation of freshly harvested leaf and root sample material from control and stress treated plants (250mM NaCl) was done using the TRIzol method (Invitrogen, Life Technologies, USA).

Agarose gel electrophoresis
Agarose gel was prepared by melting 0.8-1g agarose (Pronodisa) in 100 ml 1X TAE. To check the presence of RNA, 4 µl of the extracted sample was loaded with 2 µl of 6x DNA loading dye (Fermentas) along with 1.5 µl of 100bp plus DNA ladder (Fermentas, Gene ruler) as a marker and electrophoresed for ½ hour at 120 V in 0.5x TAE buffer. Presence of RNA was confirmed by two specific bands.

Quantification of RNA
RNA was quantified using thermo nanodrop 2000c spectrophotometer and purity was also confirmed by checking the ratio of A260/A280 and A260/230.

cDNA preparation
RNA was reverse transcribed using Takara, Bio prime script 1st strand cDNA synthesis kit. Required amount of RNA was taken in PCR tubes, to it oligo primer and dNTP was added with DNase/RNase free water. RNase inhibitor and reverse transcriptase enzyme were then added to all PCR tubes. After preparation, tubes were taken out and stored in freezer until further use.

Real Time qRT-PCR
To know the expression level of above mentioned genes qRT-PCR was carried using Power SYBR®. Green Master Mix (Applied Biosystems, USA) on real time PCR detection system (Applied Biosystems). The qRT-PCR was performed using cDNA as template. Normalization of the data for each transcript was carried out using wheat TaActin as an internal control and level of expression were analyzed using 2-DDCt method (Livak and Schmittgemittgen 2001). Thermal cycling consisted of initial denaturation at 95 °C for 10 min followed by 40 cycles of each PCR step: 95 °C for 15 secs and 60 °C for 1 min.

Results and Discussions
Effect of salinity (NaCl) stress on physiological parameters of wheat seed (var HD 2687).
Salt stress (250mM NaCl) led to decrease in RWC by 16.5%. It led to decrease in Chlorophyll/Carotenoid ratio by 12.47% and increase in Chlorophyll A/ Chlorophyll B content by 7.5%. Total Surface Area was also decreased by 37% and Average Diameter was increased by 46%. in stress treated plants (Fig. 3).
The results obtained in the present study conducted with breadwheat genotype revealed stress induced variation in various physiological parameters. The most important process that is affected in plants growing under saline conditions is photosynthesis. Reduced photosynthesis under salinity is not only attributed to stomata closure leading to a reduction of intercellular CO_2 concentration, but also to non stomatal factors. There is strong evidence that salt stress affects photosynthetic enzymes, chlorophylls and carotenoids (Stepien *et al.*, 2006). Our study also showed that salinity treatment significantly affected the total chlorophyll and total carotenoid content and root traits in bread wheat.

Expression of genes regulating salinity tolerance in leaf and root tissues
Salinity stress upregulated the gene expression by 1.64 and 14.5 times with respect to control treatment. Salinity stressed root tissues showed comparatively higher expression of vacuolar sodium proton antiporter gene, NHX. In case of Salt Overly Sensitive 3(*SOS3),* salinity stress up regulated the gene expression by 1.5 times with respect to control treatment in roots.
The salt tolerance gene *SOS3* is predicted to encode a calcium binding protein with an N-myristoylation signature sequence. Gene product of *SOS3* is required for *SOS2* and *SOS3*. Present study revealed an increase in mRNA expression of *SOS3* gene with increase in salinity treatment in wheat (Fig. 4). The *SOS3* gene encodes an EF-hand type calcium-binding protein with similarities to animal neuronal calcium sensors and the yeast calcineurin B subunit. Mutations in calcineurin B lead to increased sensitivity of yeast cells to growth inhibition by Na^+ and Li^+ stresses.
The AtNHX1 gene encodes a tonoplast Na^+/H^+ antiporter and functions in compartmentalizing Na^+ into the vacuole. Over-expression of AtNHX1 enhances the salt tolerance of Arabidopsis plants. Our study showed that salt treatment sharply increased gene expression of NHX1 in wheat (Fig. 4). Apse *et al.* (1999) suggested that under high soil salinity condition plants tend to compartmentalise toxic Na^+ into vacuole, which is one of its defence response against salt stress.

Fig 1: Transplantation of plants in Cigar rolls

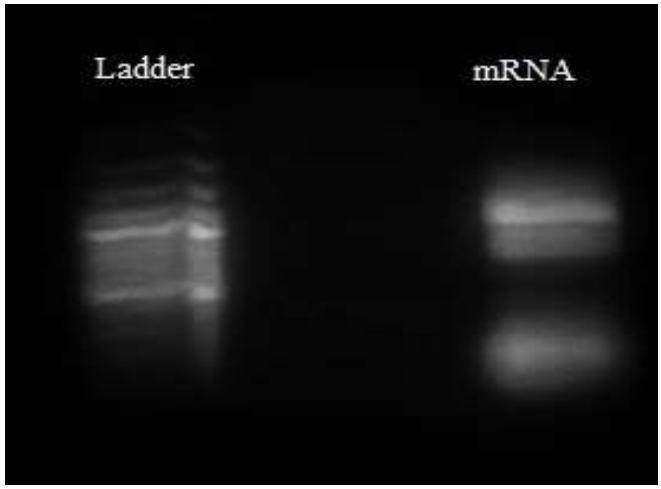

Fig 2: Presence of RNA after isolation was confirmed by gel electrophoresis.

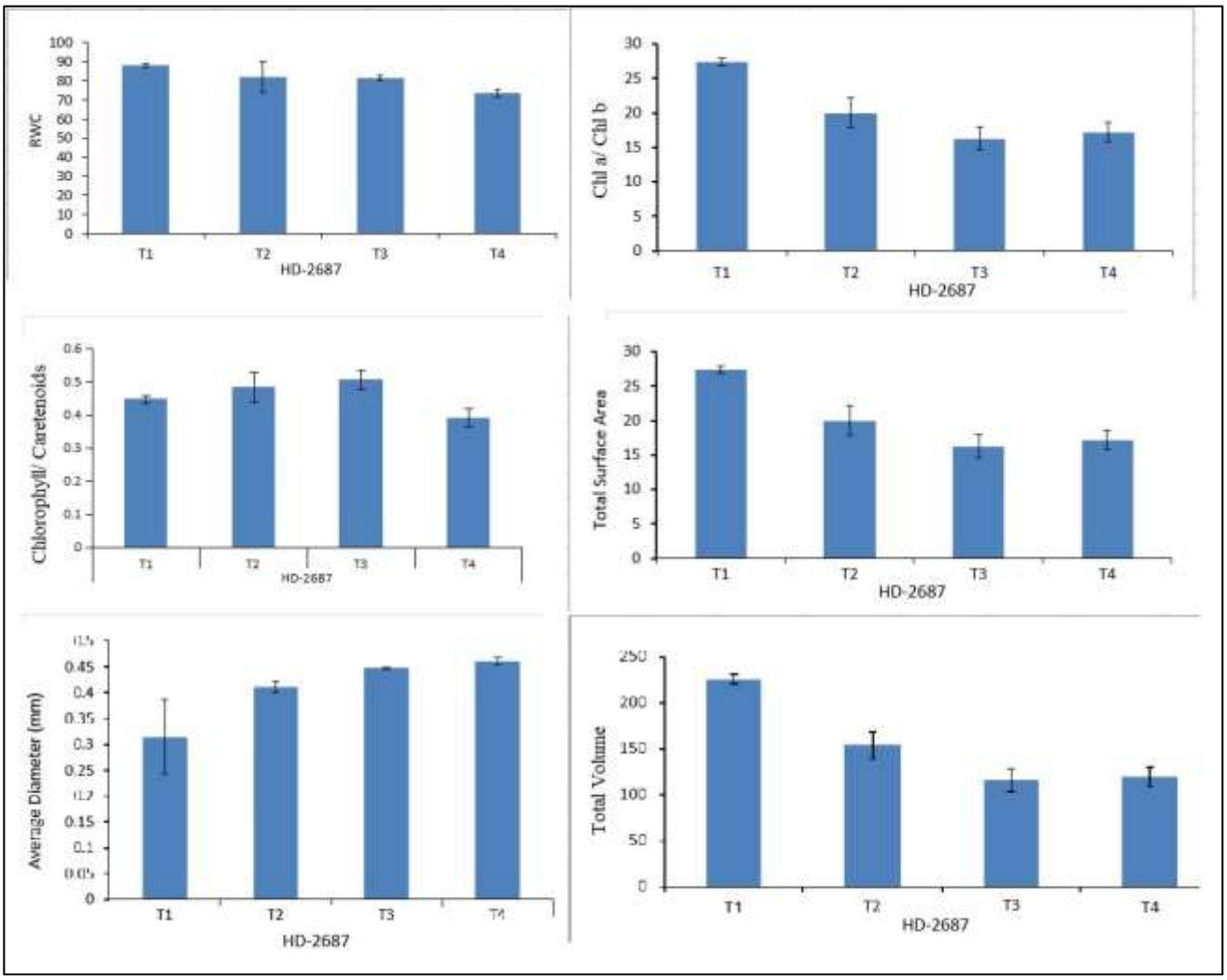

Fig 3: Effect of salinity stress on Relative water content, Chlorophyll a/b, cholorophyll/caretonoid, surface area, average diameter and total volume

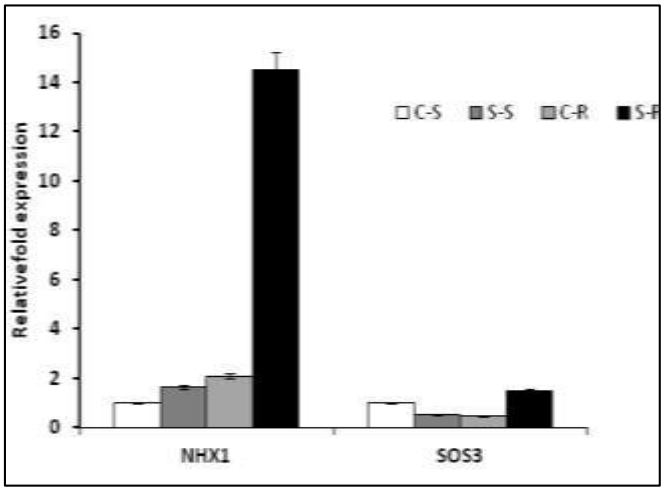

Fig 4: Effect of control (C) and salinity stress (S, 200mM NaCl) treatments on expression of vacuolar sodium antiporter (NHX1) and salt overly sensitive 3 (SOS3) gene in shoot (S) and root (R) tissues of bread wheat analysed by quantitative real-time PCR

Conclusion

Based on the experiment it can be concluded that salinity stress negatively impacted various physiological traits such as relative water content, chlorophyll and carotenoid contents, and root traits in bread wheat. The decline in various traits was exacerbated as the stress level was increased. Expression of genes involved in sodium ion homeostasis (NHX, SOS3) were up-regulated in root tissues of wheat subjected to salinity stress.

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