Advances in Bioresearch
Volume 3 [4] December 2012: 53- 61

[ISO 9001: 2008 Certified Organization]
Website: http://www.soeagra.com/abr/abr.htm

ISSN 0976-4585

Original Article

Effects of Salt Stress on Single Strand Preferring Genotype Dependent Nucleases (SSPN) Activity in Wheat Seeds

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ABSTRACT
Crop production has always been affected by soil salinity. Nuclease activity is a major factor that is increased in response to environmental stresses such as salt stress. The changes in the nuclease activities of one salt-tolerant and one salt-sensitive wheat genotypes were studied in response to salt stress.
To evaluate the effect of salt stress on the tolerant and sensitive wheat leaves, the protein and chlorophyll contents as well as the Single Strand Preferring Nunclease (SSPNs) activity were measured in the presence and absence of salt at 2 different pH conditions. Chlorophyll and total protein content of salt tolerant cultivar were significantly increased in response to increased salt concentration from 0 to 120 mM, but were reduced in salt sensitive genotype. SSPNs activity was also increased in sensitive leaves in the presence of salt. Increasing pH from 5.5 to 7.5 increased the SSPNs activity in both genotypes. In tolerant genotype, SSPNs was higher in 120mM only in the presence of Zn^{2+} and Mg^{2+}. Nuclease activity of the leaves was higher in natural pH, whereas, it was decreased in response to lower pH. The decreased SSPN activity in tolerant wheat in the presence of Ca^{2+} could be due to the disappearance of a major 52 kDa protein.
It appears that the Ca^{+2}-dependent endonuclease activity is highly involved in salinity resistance mechanisms. We also conclude that in the presence of Ca ion in soil, tolerant wheat cultivars may be able to better cope with the stressed condition.
Key words: *Salt stress, single strand preferring nuclease, endonuclease, ribonuclease.*

INTRODUCTION

Salinity is a major concern in crop production worldwide [1-3]. A part of detrimental effects of salt stress is related to the activity of nucleases [4]. Induction of plant endonucleases has been observed in response to environmental stresses [5]. Many types of responses to environmental stresses, including salt stress, darkness, phosphate deficiency [6], physical injury [7], water stress [8], chilling stress [9], infectious processes [10] and growth and developmental processes such as cell division are able to cause increased Ribonuclease (RNase) and Nuclease activities [11]. There are several single-strand-preferring nucleases which have not been well-characterized but are known to degrade both RNA and DNA [12]. RNases have been defined as enzymes that are capable of hydrolyzing RNA only, but nucleases are the enzymes capable of hydrolyzing both RNA and DNA [13]. Thus, one enzyme preparation hydrolyzing RNA could be a nuclease, an RNase, or a mixture of both. RNases and nucleases have been reported to be important in plant development because of their ability to modify RNA levels, and thereby influence protein synthesis [14]. There is limited and fragmentary information about the precise number of multiple forms, intracellular location and metabolic function of the enzymes involved in the process of degradation of RNA in plant cells. A number of nucleases have been reported in many different organisms, from microorganisms to mammals [15] and plant tissues but their individual functions *in vivo* are not well defined [11]. They have been classified according to Wilson´s classification [13] as there are four types of enzymes that hydrolyze nucleic acids in plants: RNase I, RNase II, nuclease I and exonuclease I. It has been suggested that nuclease I is involved in several processes, which are strongly regulated during the development and are influenced by many factors such as light, phytohormones, wounding, water stress and infection with some plant pathogens. Nuclease I group is a

heterogeneous group of enzymes with some specificity towards secondary structures of nucleic acids and the ability to cleave different homopolymers [16]. Some of these enzymes are single strand specific (like mung bean nuclease), but there are enzymes with ability to cleave different secondary structures including double-stranded DNA or supercoiled plasmid DNA as well as dsRNA with limited activity [11]. Nuclease induction is strongly associated with different types of plant programmed cell death (PCD) processes [17]. When PCD occurs, activities of nuclease enzymes will increase. This may be associated with DNA fragmentation and nuclear degradation in plants after salt stress [18]. Plant nucleases induced by PCD and biotic or abiotic stresses include two prominent endonucleases, that involves Zn^{2+}-dependent endonuclease [19, 20] and Ca^{2+}-dependent endonuclease [17]. Although the classification and function of various nucleases have been described in detail, but little focus has been made on the role of different types of stresses (including salinity stress) on nuclease activities. In this study, the changes in the nuclease activities of one salt-tolerant and one salt-sensitive wheat genotypes were studied in response to salt stress.

MATERIALS AND METHODS

Seeds of wheat genotypes were obtained from the Agricultural Biotechnology Research Institute of Iran (ABRII). Two genotypes were used; Atila (a salt sensitive genotype) and Karchia (a salt tolerant genotype). Surface–sterilized seeds were germinated on moist filter papers under dark condition at 25^{o}C. After 3 days, healthy seedlings were grown hydroponically in washed sand and Hogland solution. Sodium chloride solution (120 mM) was used to create salt stress treatment. Control treatment did not contain any salt. To apply salt stress, regenerated seedlings were transferred to media containing 120 mM NaCl starting one week after germination and continued for 4 weeks. All experiments were carried out with three replicates. Leaf tissue was collected from each genotype, fixed in liquid nitrogen and kept in minus 80º C until used.

Protein extraction

About 1 g of the frozen leaf tissues were ground in liquid nitrogen using mortar and pestle to make a fine powder. The powder was transferred into 15 ml screw-capped Falcon tubes containing 4 ml of extraction buffer (0.5 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM N-ethylmalemide) [12]. Total protein content was measured using the standard Bradford assay [21].

Chlorophyll content measurement

Chlorophyll was measured according to the standard method of Arnon [22]. Absorbance was measured at Wavelengths of 645 and 663 nm and chlorophyll content was calculated as:

$Ct\ (mg/mL) = (20/2 \times A_{645}) + (8/20 \times A_{663})$

Spectrophotometric DNase activity assay

The assay was carried out in 1.5 ml tubes, as described by Blank and McKeon [12] with some modifications. Briefly, 300 µl reaction containing 30 µl of 500 mM citrate buffer (pH 5.5 and 7.5), 30 µl of one of the divalent cations Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+} or EDTA, 30 µl of 1% (w/v) single stranded (ss) calf thymus DNA, 30 µl of 1 % (w/v) BSA and 20 µl of protein extract was used for each assay. Control reaction was stopped at 0 min and the other reaction was stopped after 20 min incubation at 37 °C by adding 1 ml of 3.4 % (v/v) perchloric acid and placing the tubes on ice. The tubes were centrifuged at 20000 x *g*. Then, 1 mL of the supernatant was transferred into a quartz cell and absorbance was measured at 260 nm. Means of measurements of 3 replicates for the nuclease activity and protein content were presented. Each unit of enzyme activity was calculated as: ΔA_{260}/mg of protein/20 minutes [12]. All data were analyzed using SPSS computer program version 10.

Native-PAGE assay

Nuclease gel activity assays were performed using standard Native PAGE protocol as described by Blank and McKeon [12] with some modifications. Native-PAGE was performed using a 12.5% (w/v) resolving gel that contained 100 µg mL^{-1} BSA. To identify Single Strand Preferring Nunclease (SSPNs) activity, gels contained 100 µg mL^{-1} calf thymus DNA. The DNA was made single-stranded by boiling for 10 min and cooling immediately on ice. Seventy-five µg of total protein extracts were loaded on the stacking gel consisting 4.5%. Gels were run at 120 V for 2 h at 25 °C. After electrophoresis, nucleases were renatured by incubating gels in renaturation buffer [0.5 M citrate buffer (pH 7.5), 1% Triton X-100] at 37 °C with gentle shaking for 1 h. Gels were rinsed twice with

0.5 M citrate buffer (pH 5.5 or 7.5) and incubated in developing buffer [0.5 M citrate buffer (pH 7.5), 20 mM NaCl] complemented with 1 mM of one of the Ca^{2+}, Mg^{2+}, Zn^{2+} or Cu^{2+} at 37 °C, overnight. Then gels were stained for 1 h in a solution of 2 % (w/v) acid orcein and destained in destaining solution containing 10 % (v/v) acetic acid and 50 % (v/v) methanol.

Plasmid extraction

Bacterial growth and multiplication were carried out in the LB medium containing the ampicillin antibiotic at 37 °C with 110 rpm shaking. Plasmid DNA was extracted by alkaline lysis method with SDS (Mini preparation) [23].

Na and K measurement

Organic matter of leaves and roots was destroyed by combustion at a temperature of 70°C for 48 hr in the presence of air. The residue ash was then dissolved in a dilute acid (6M HCl) to bring the mineral elements into the solution. The solution was filtered with Whatman 42 filter paper. Potassium and Na contents of the extract were subsequently analyzed with a flame photometer (Model 410, Corning, England). The Na+ and K+ contents were measured in triplicate using flame photometer after extraction with 500 mM HNO3 [24].

Statistical Analysis

Results are presented as means ± SEM. Statistical analysis used Instat software to do ANOVA followed by a Student Newman-Keuls post hoc test. Significance differences are based on $P < 0.05$.

RESULTS

Total chlorophyll content

In the sensitive genotype, chlorophyll content (a, b and total) decreased from 0 to 120 mM NaCl concentrations. However, this decrease was not significant in the case of chlorophyll a and b contents. In the tolerant genotype, chlorophyll content (a, b and total) was significantly increased in 120 mM NaCl compared to 0 mM NaCl concentration at 5% confidence level (Fig. 1).

Total protein content

Figure 2 shows protein content of fresh leaves extracted from sensitive and tolerant wheat genotypes under the control and salt stressed conditions. In the sensitive genotype protein content was decreased in response to salt stress. However, in the tolerant genotype, the protein content showed an increase from 0 to 120 mM NaCl concentration (Fig. 2). Changes in protein content between 0 and 120 mM NaCl concentrations and between two genotypes were significant at 5% confidence level.

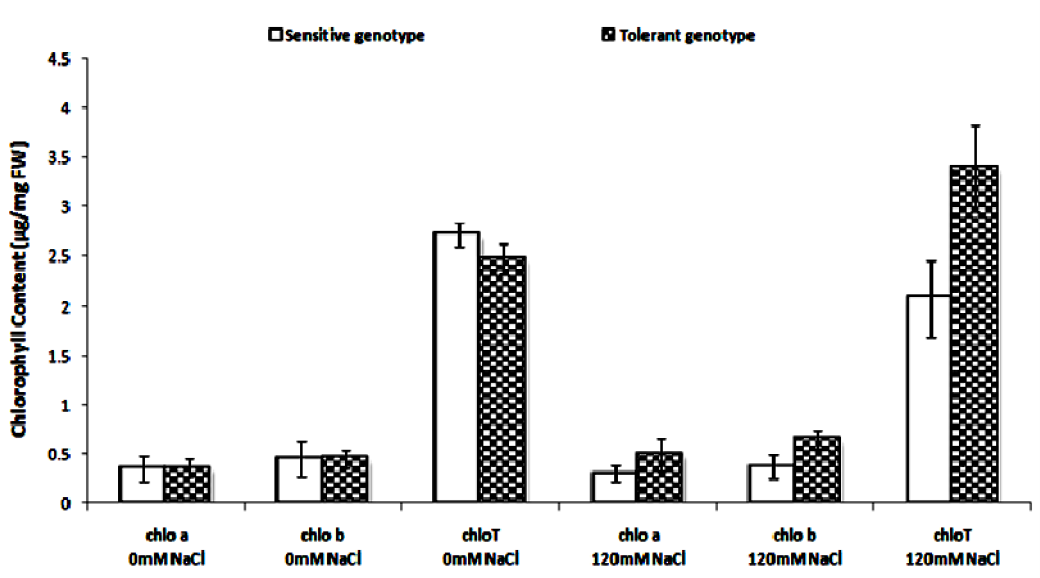

Figure 1. Chlorophyll content of salt sensitive and salt tolerant wheat genotypes in response to 0 and 120 mM NaCl. While chlorophyll content is significantly increased in tolerant genotype under salt condition, it is reduced in the salt sensitive genotype under the same condition.

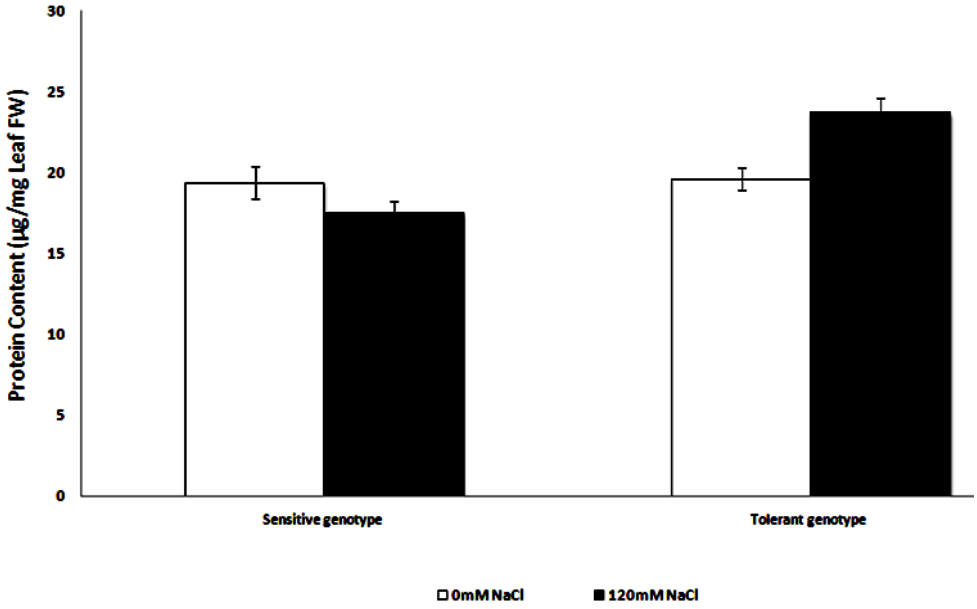

Figure 2. Comparison of total protein content of a salt sensitive and a salt tolerant wheat genotypes in response to different concentrations of NaCl.

SSPN activity

Effects of salt stress, divalent cations and two pHs were investigated on SSPN activity. The effect of pH x salt stress x cations on the enzyme activity of the sensitive and tolerant genotypes are shown in Fig. 3. SSPN activity was higher in presence of cations Ca^{2+}, Mg^{2+} and Zn^{2+} under different pH and NaCl concentrations. In sensitive genotype, SSPN activity was not significantly different between 0 and 120 mM NaCl in presence to Mg^{2+} at pH 5.5, but at pH 7.5 SSPN activity was significantly higher at 120 mM NaCl, in comparison to 0 mM NaCl. The same trend was observed in the presence of Ca^{2+} and Zn^{2+}. Cu^{2+} showed an inhibitory/decreasing effect on SSPN activity at pH 7.5 (Fig. 3A).

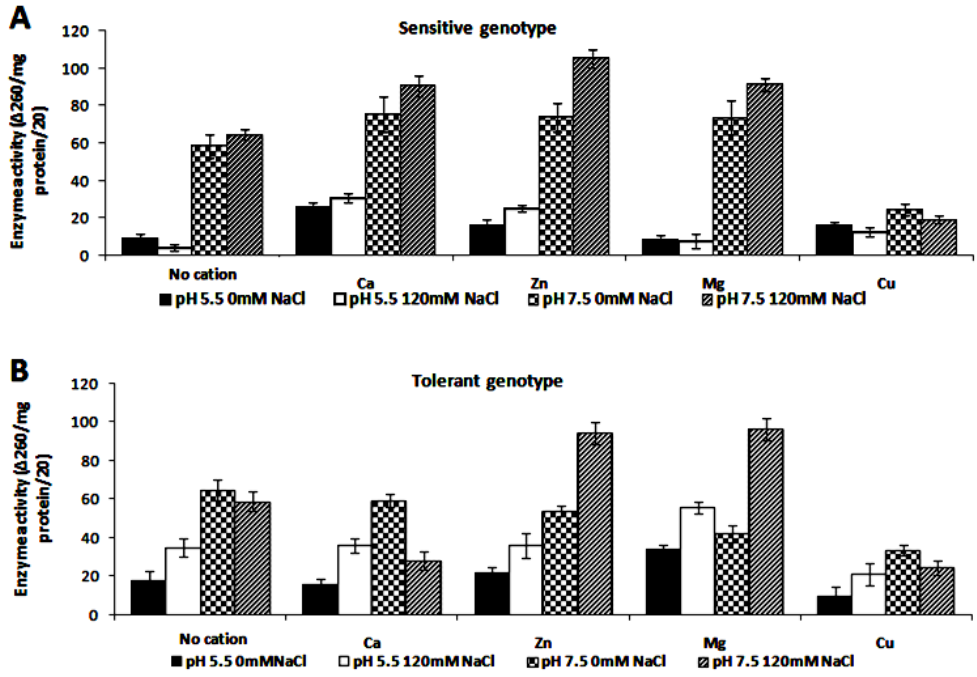

Figure 3. Effect of salt stress, divalent cations and two pHs on SSPN activity in a salt sensitive (A) and a salt tolerant (B) wheat genotypes. SSPN activity was measured spectrophotometrically from crude proteins extracted from leaves of wheat genotypes under 0 and 120 mM NaCl.

In the tolerant genotype, SSPN activity was not significantly different at different NaCl concentrations in presence of Ca^{2+} at pH 5.5. Ca^{2+} did not have any significant effect in this case. However, at pH 7.5 and 120 mM NaCl concentration SSPN activity was significantly reduced by Ca^{2+}. Zn^{2+} had an increasing effect on SSPN activity at pH 5.5 and 7.5 at 120 mM NaCl. In presence of Zn^{2+} increasing pH from 5.5 to 7.5 also significantly increased the SSPN activity both at 0 and 120 mM NaCl concentrations. The most prominent effect of Zn^{2+} was observed at pH 7.5 and 120mM NaCl concentration that significantly increased the SSPN activity compared to the absence of this cation. Mg^{2+} had an increasing effect on the SSPN activity at pH 5.5 at both NaCl concentrations, and a similar effect to that of Zn^{2+} at pH 7.5 and 120 mM NaCl concentration. Presence of Mg^{2+} significantly reduced the SSPN activity at pH 7.5 under 0mM salt concentration. Cu^{2+} had an inhibitory effect on SSPN activity (Fig. 3B) at both pHs and salt concentrations in tolerant genotype and at pH 7.5 in sensitive genotype. Reduction in SSPN activity was significant between two genotypes in presence of Cu^{2+} at 1% level.

Cation-dependent SSPN activity

***Ca^{2+}-dependent SSPN activity*:** Two Ca^{2+} dependent SSPNs of 52 and 28 KD were revealed on native PAGE in both genotypes at 0 and 120 mM NaCl, under pH 5.5 (data not shown) and pH 7.5 (Fig. 4A). However, the 52-KD enzyme was not observed in the tolerant genotype at pH 7.5 and 120 mM NaCl (Fig. 4A).

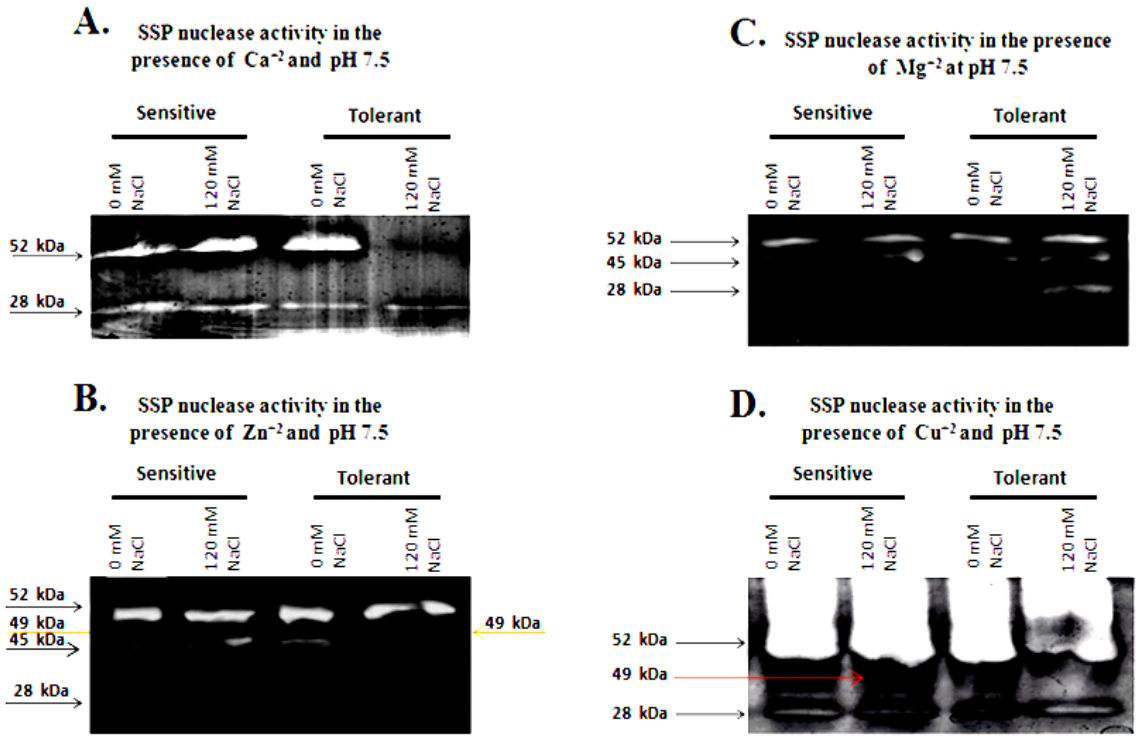

Figure 4. Characterization of SSPN activity on NaDodSO4 activity gels. Leaf extracts were heated in buffer containing 2% NaDodSO4 and were loaded on native polyacrylamide gels containing denatured DNA in the matrix and were incubated in 0.5 M-Citrate buffer (A-D). Ca^{2+} dependent SSPNs of 52 and 28 KD are shown. The 52-KD enzyme was not observed in the tolerant genotype at pH 7.5 and 120 mM NaCl (A). Zn^{+2} dependent SSPNs of 52, 45 and 28-KD are found in both genotypes, at 0 and 120 mM NaCl and 49 KD observed in the tolerant genotype at pH 7.5 and 120 mM NaCl (B). Mg^{+2} dependent SSPN activity in both salt sensitive and salt tolerant genotypes, at 0 and 120 mM NaCl conditions at pH 7.5 is shown. The three observed bands are similar to those Zn^{+2} dependents SSPNs (C). Cu^{+2} dependent activities in salt sensitive and salt tolerant genotypes, at 0 and 120 mM NaCl conditions at pH 7.5 were different. The two minor bands observed in salt sensitive genotype under 120 mM NaCl are absent in the salt tolerant genotype under the same condition (D). Gels were loaded with 75 µg of crude protein extracts.

Zn²⁺ dependent SSPN activity: In both genotypes, three Zn^{2+} dependent SSPNs with the molecular weights of 52, 45 and 28-KDa were observed at 0 and120 mM NaCl at pH 7.5 (Fig. 4B) in presence of 10 mM Zn^{2+}. Additionally, a 49 KDa SSPN activity was observed in presence of 120 mM NaCl in tolerant genotype only.

Mg²⁺ dependent SSPN activity**:** In both genotypes, at both 0 and 120 mM NaCl concentrations at pH 7.5, three SSPNs of 52, 45 and 28-KDa were observed in presence of 10mM **Mg^{2+}** similar to those observed for Zn^{2+} dependent SSPNs (Fig. 4C). SSPN activity was more prominent at 120 mM NaCl in tolerant genotype compared to that in the sensitive genotype.

Cu²⁺ dependent SSPN activity**: Two** Cu^{2+} dependent SSPN activities at the molecular weights of 52, and 28 KDa were detected under different treatments for the two salt tolerant and sensitive genotypes at both 0 and 120 mM NaCl concentrations at pH 7.5 (Fig. 4D). However, an additional SSPN activity at 49-KDa was observed in salt sensitive genotype at 120 mM NaCl only.

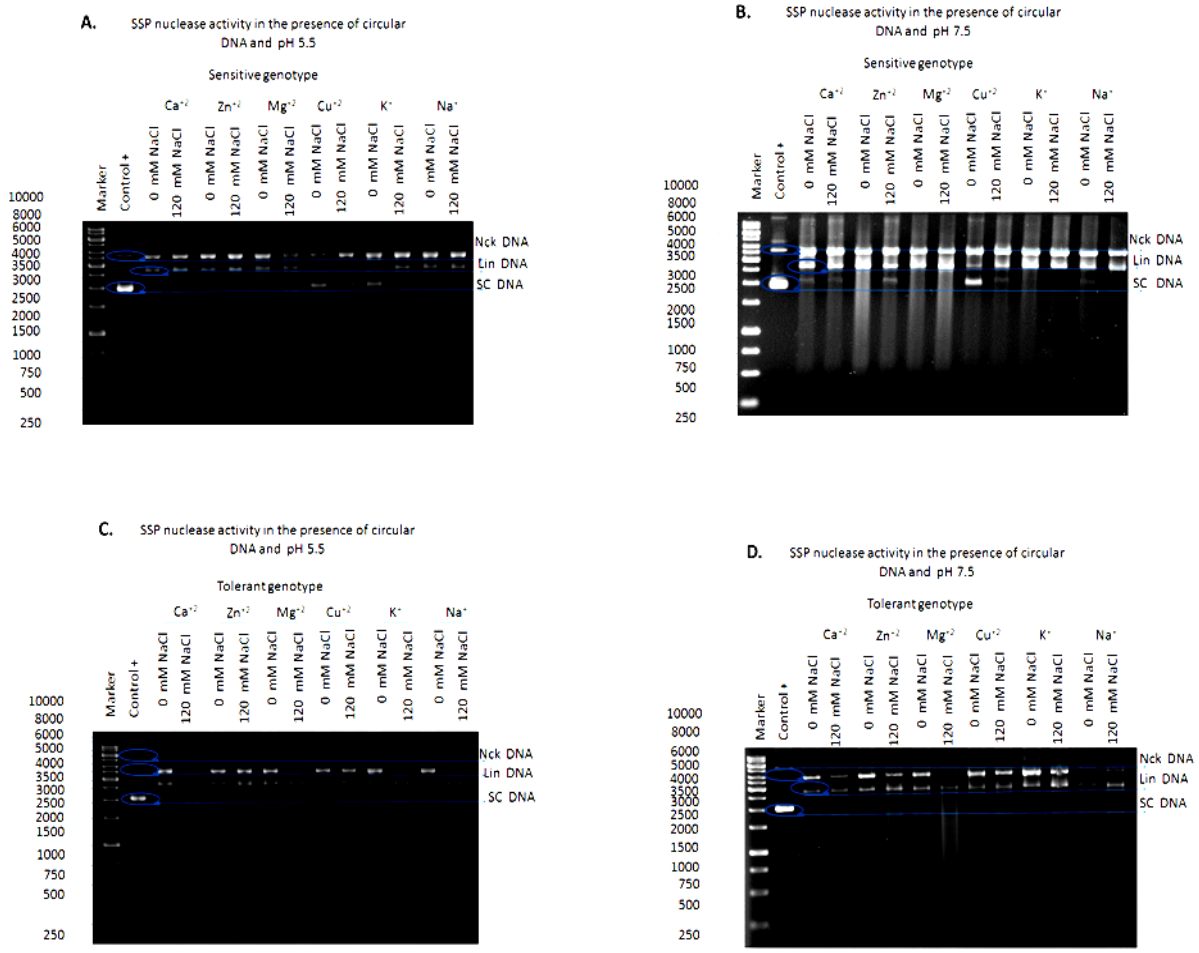

Figure 5. Ethidium bromide staining of an agarose gel after electrophoresis of pUC19 DNA and its hydrolyzed products after incubation with SSPN crude protein extracted from leaves of 45 days old sensitive wheat seedlings at pH 5.5 (A) and 7.5 (B) and tolerant what seedlings at pH 5.5 (C) and 7.5 (D). Circular DNA was used as substrate and the reaction time was 20 min

DNase activity on plasmid DNA

The plasmid DNA (pUC19) was treated with protein extract of both genotypes at both pH 5.5 and 7.5 and in the presence of Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, K^{+} and, Na^{+}.

Figure 5 (A and B) shows the ethidium bromide stained agarose gels containing the intact circular pUC19 plasmid (lane 2), and the same plasmid treated with different protein preparations. Three bands were observed as expected for supercoiled, linearized and nicked plasmids. This is a characteristic feature of circular plasmids. If the pUC19 plasmid is digested at one site, one band of

2.7 kb is expected to appear on the gel. Therefore, the presence of a 2.7 kb band was interpreted as a result of DNase activity.

At pH 5.5, 0 and 120 mM NaCl and in the presence of Ca^{2+}, Zn^{2+}, Mg^{2+}, K^{+} and, Na^{+}, incubated with protein extracted from the salt sensitive genotype, DNase activity was observed as judged by linearized plasmid (2.7 kb band) (Fig.5.A). The linearization was more apparent in the presence of Ca^{2+}. Very little or no DNAse activity was observed in presence of Cu^{2+} (Fig 5.A.lanes, lanes 9-10). Presence of a background smear may suggest that some more DNase activity may have occurred at more than one site on the plasmid DNA randomly.

At pH 7.5, the same phenomenon was observed. At this pH, the DNAse activity was observed even at the presence of Cu^{2+} (Fig.5.B).

The linearization of the circular plasmid substrate by protein extracted from the salt tolerant wheat genotype was also observed in the presence of all cations, at 0 and 120 mM NaCl and at pH 5.5 and 7.5 (Fig 5.C and D respectively).

DISCUSSION

Salinity is one of the major limitations on crop productivity and quality in the world. The negative effects of salinity include: increased nuclease activity, reduction in growth rate and biomass, shorter stature, smaller leaves, osmotic effects, nutritional deficiency and mineral disorders [25].

A number of nucleases accumulate in plants exposed to saline stress. When tolerant and sensitive seedlings are sown under saline conditions both show an increase in Zn^{2+}-dependent endonuclease and Mg^{2+}-dependent endonuclease activity [26]. In this research work, we obtained very similar results to their findings. In some situations, the increase in divalent cations-dependent endonuclease are related to *de novo* synthesis [27], whereas in others this increase is either partially dependent upon *de novo* synthesis [28] or independent of it. However, the mechanism by which salinity affects this process is not known. Gomes-Filho and Sodek [8] showed that by the developmental progress of salt tolerant wheat seedlings, decreased Ca^{2+}-dependent endonuclease activity was stimulated by salt-stress. This was further confirmed in our research where Ca2+ reduced significantly the SSPN activity of salt tolerant wheat genotype at 120 mM NaCl.

Chlorophyll content and total protein content of salt tolerant cultivar were significantly increased in response to increased salt concentration from 0 to 120 mM, but were reduced in salt sensitive genotype. This indicates better adoptability of the tolerant cultivar to salt stress and its continued photosynthesis under the stressed condition.

In general 120 mM salt increased SSPN activity in sensitive genotype. Our data are similar to those reported previously [29]. In tolerant genotype, SSPN was higher in 120mM only in the presence of Zn^{2+} and Mg^{2+}.

SSPN activity was significantly increased in response to increasing pH from 5.5 to 7.5 in both genotypes. This increased activity was more pronounced in the case of sensitive genotype. The only exceptions were 1) the interaction between pH and Ca, where the SSPN activity was decreased at pH7.5 compared to that at pH 5.5 in presence of Ca cation and 2) the presence of Cu that decreased the SSPN activity in response to increased salt at both pH 5.5 in salt tolerant genotype and pH 7.5 in both genotypes. The decreased SSPN activity at pH7.5 and 120 mM NaCl in presence of Ca may be correlated with the absence of the 52 KDa protein in the native PAGE assay (Fig 4A). Presence of an extra 49 KDa SSPN activity in presence of Zn2+ at 120 mM NaCl and pH7.5 (Fig 4B) could justify the pronounced SSPN activity observed spectrophotometrically (Fig 3). Overall, there was a perfect matching in the results obtained for SSPN activity using two different approaches ie, the Native PAGE assay and spectrophotometric measurement of the DNAse activity.

In general, nuclease activity of the leaves was higher in natural pH. It was decreased in response to lower pH. SSPN activity was still higher in salt stressed condition. In the tolerant genotype Ca^{+2}-dependent endonuclease activity was significantly lower in response to salt stress at higher pH compared to that observed in the salt sensitive genotype. It has been reported that at high concentrations, sodium can displace membrane-associated Ca^{2+} [30] and cause Ca^{2+} deficiency. This effect may be reduced when the Ca^{2+} concentration in the external solution is higher. There was a complete agreement between the spectrophotometric DNase activity assay and the native-PAGE assay in our study of the effect of Ca^{2+} on SSPN activity. The decreased SSPN activity in tolerant

wheat in presence of Ca^{2+} (Fig. 3B) may be attributed to the disappearance of the major 52 kDa protein with SSPN activity (Fig. 4A). Whether the absence of this protein is the result or the cause of salt tolerance requires further studies.

NaCl-salinity reduced the Ca^{+2}-dependent endonuclease activity in tolerant genotype, but increased its activity in sensitive genotype.

Nucleases extracted from both genotypes under the control and stressed conditions, showed two bands on SDS-PAGE with a molecular mass of (52 and 28 kDa) but in tolerant genotype under salinity condition in the presence of CaCl2, the 52 kDa band was obviuosly blocked (Fig. 4A). It appears that the Ca^{+2}-dependent endonuclease activity is highly involved in salinity resistance mechanisms. We also conclude that in presence of Ca ion in the soil, tolerant wheat cultivars may be able to better cope with the stressed condition. However, this needs to be verified in field trials.

ACKNOWLEDGEMENT

The authors report no conflicts of interest in this work.

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[Received 25.90.12; Accepted: 3.12.12]