SOS1, SERK1 and WEE1, Confer a Defence Response to Salt Stress with Increasing NaCl in *Alfalfa* (*Medicago Sativa* L.) Callus

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Abstract

Alfalfa is an important forage that contributes wildlife habitat and soil conservation worldwide and has a high nutritional feeding quality and N fixation potential. However, *alfalfa* production is seriously reduced by abiotic stress factors. In this study, *SOS1 ,SERK1, WEE1* genes were expressed in *alfalfa* callus cells to control NaCl stress under *in vitro* conditions. The important callus traits in terms of NaCl resistance were found among the genotypes. Higher sugar contents were accumulated in tested genotypes than in control callus when all were exposed to NaCl stress. Based on proline assay, there were inverse relationships between NaCl acclimated-callus and tested genotypes. Na^+ and K^+ showed an increasing trend in response to the increasing concentration of NaCl. The results showed that callus growth increased salt stress related gene expression. *SOS1, SERK1 and WEE1* gene expression levels peaked at 50 mM while highest Na^+ and K^+ and sugar content happened at 110 mM NaCl. As a result of flow cytometry analysis, there were often endopolyploidy in the tested genotypes. Our findings indicated that the *SOS1, SERK1 and WEE1* genes was effective in promoting the alfalfa callus cells in response to salt stress induced by NaCl.

Key Message

SOS1, SERK1 and WEE1 confers a defensive response to salt stress in *alfalfa* callus maintainance. This response confirms with qRT-PCR analysis and cytological analysis.

Introduction

Plant growth and development are greatly affected by biotic and abiotic environmental factors. Salt stress is one of the most common abiotic factors across all plant species and during salt stress it adversely affects agricultural production and yield in many regions of the world (Chinnusamy et al. 2005; Evelin et al. 2019; Erdal et al 2012). Salt stress causes not only productivity loss but also decreases quality of crops. Salinity-induced ionic and osmotic stresses affect photosynthesis, cellular metabolisms, change cytosolic enzyme activity and the rate of carbon assimilation and hence reduce crop productivity (Isayenkov 2012). Plants inhibit the severe stress via different mechanisms in the growth medium. Responses to salt stress differ among crop species throughout their growth season and are primarily observed in the phenological responses in the life cycle of plant growth (Yazıcılar et al. 2021). Furthermore, salt stress involves alterations in different metabolic and physiological processes depending on impact and level of the stress and ultimately restricts plant growth. Level of the salt stress in plants is defined by decreased leaf water content, stomatal dysfunctions, osmotic pressure, and reduced cell division and differentiation. High salinity restricts water uptake by the plants, influences ionic imbalance leading to ionic toxicity and osmotic stress (Rajendran et al. 2009; Roy et al. 2014). Plants withstand salt stress through convenient molecular and cellular responses which attempt to relieve the stress effects. It accumulates compatible solutes such as proline, which decreases the cytoplasmic osmotic potential facilitating water absorption, and scavenges reactive oxygen species molecules (Benavides et al. 2000). Physiological defensive strategies are available to cope with salt stress at developmental stages of the growing period and are therefore thought to play major roles in the plant's

response (Bezirganoglu et al. 2018; Bezirganoglu et al. 2017). All physiological stages of plants such as gaseous exchange, stomata closure, reduced transpiration and distruped electron transport chain owing to osmotic stress were severely affected by salt stress. Under osmotic stress, plants survive the water relations by regulating different active ion compounds including calcium, chloride, proline, sugars, glycine betaine, trehalose and organic acids (Chung et al. 2008; Ruiz Lozane et al. 2012; Auge et al. 2014). Compatible solutes defend the plants from stress damage without any destructive effects on membranes, macromolecules and enzymes even at higher dosages. Proline has been shown to have many roles in environmental stress factors and resistance potential which are the main properties that defend cells by adjusting proteins and organelle membranes (Ashraf and Foolad 2007; Uysal and Bezirganoglu 2017). Proline possess the potential for high salinity injury in salt susceptible plants such as legumes and cereal species. The useful role of proline improving salt resistance in *alfalfa* and *pea* cultivars has been well investigated. Soluble sugars are known as signal molecules, osmotic regulators and contribute to many developmental and metabolic pathways in plants (Yazıcılar et al. 2021). It is also an antioxidant molecule that supports clean plants from injury caused by reactive oxygen species and thus enhances stress resistance in plants (Benavides et al. 2000). Identification of genes encoding a class of transcription factors with maintenance ion homeostasis properties, called salt overly sensitive has been proposed to mediate cellular signaling in improving plant tolerance to salt stress. *SOS1* signal networks cascade to conserve the cells from injury due to excessive ion accumulation and expression of the *SOS1* provides beneficial traits for acclimation to salt stress. The SOS-mediated pathway restricts Na^+ amount in the cytosol in presence of salt stress and adjusts to continue an excess cytosolic K^+/Na^+ ratio, significant for salt resistance (Halfter et al. 2000; Guan et al. 2013; Yue et al.2012). The effects of salt stress on plant development are at least in part mediated by effects on cell cycle regulation and expressions of the cell cycle genes. *WEE1*, belonging to a group of protein kinase participates in the terminal phosphorylation and suppresses cyclin dependent kinase, and plays the main role in regulation of the endoreduplication under stress (De-Schutter et al. 2007). The role of *WEE1* in controlling cell cycle regulation has been shown through a combination of processes including the cell division, expansion, and differentiation (Michael and Newport 1998). *WEE1*, which regulates cell division of plants, can be used strongly to control the DNA replication and DNA damage check-points of the salt stress (Elmaghrabi et al. 2017; Sorrel et al. 2002; Salomonsson et al. 1993). Acclimation is particularly significant in plants subjected to higher concentrations of NaCl during periods of high metabolic or growth activities. Several researchers have obtained somatic embryogenic and organogenic callus by acclimation (Bezirganoglu 2017; Elmaghrabi et al. 2017) Somatic embryogenesis involves various molecular processes including complex signal transduction reprogramming of gene expression patterns for activating or inactivating numerous gene families (Suprasanna and Bapat 2005; Zavattieri et al. 2010). *SERK1* is a beneficial marker with initiation of somatic embryogenesis and stage specific genes required for somatic embryogenesis and whose expression in *Medicago truncatula* was up-regulated in embryogenic callus (Nolan et al. 2003). *Alfalfa* is the main forage crop for dairy producers of many agricultural regions due to its high feeding value. *Alfalfa* is cultivated for its high yield and potential for environment adaptation, wide disease tolerance, and nutritional feeding quality as well as its role on N fixation, wildlife habitat, soil conservation, and bioremediation (El-Ramady et al. 2020; Singer et al 2018). *Alfalfa* is considered

moderately resistant to salt. However, its productivity could be seriously influenced by the salt stress as low as 50 mM NaCl, and also its potential of nitrogen fixation and nodule formation (Bekki et al. 1987; Liu et al. 2011) Therefore, improvement of *alfalfa* cultivars strongly tolerant to salt stress is urgent needed. Although conventional breeding programs have provide remarkable advancement controlling salt stress, most of these programs still have some restrictions, because salt resistance is controlled by multiple genes and involves different physiological and biochemical mechanisms (Flowers 2004). Synergistic expression of salt-related genes mostly adopted as a novel alternative way to defence plants against salt stress. Variation in chromosome count is an significant source of genetic diversity in *alfalfa* and information of the genetic relationship among cultivars of this species could be significant consideration for the diploid lines utilization of this germplasm in new cultivar improvement. *In vitro* culture represents an important strategy in plant science and the major advantage of its as compared with traditional entire-plant cultivation, include overcome seed dormancy, seed viability, independently of external factors (Thorpe 2007). The objective of the current study was to determine the levels of *SERK1, SOS1* and *WEE1* expressions on callus induction and extensions of desirable levels of resistance to salt stress in *alfalfa*. Moreover; sugar, proline and Na^+/K^+ activities were also evaluated in increasing levels of different NaCl in callus cultures.

Materials And Methods

Plant material and callus induction

In our study, two cultivars and one ecotype (*Ömer Bey*, *Elçi*, and *Muş)* were used as the material for the response to NaCl acclimation. The mature seeds were sterilized with 22% NaOCl for 5 min, washed several times with sterile distilled water. Leaves were explanted to *in vitro* medium from two weeks old plants onto hormone-free MS medium (Murashige and Skoog 1962). The leaf explants were incubated in total darkness at 25 ± 1°C temperature for one month. After one month, callus formation was assessed and used for NaCl acclimation studies.

Salt stress treatment

The callus were placed onto the co-culture medium containing MS basal medium (pH 5.7) and 0.8 % agar supplemented with 1 mg/L of 2,4-D and 1 mg/L of kinetin without NaCl for 3 months in a growth chamber at 28°C, under a 16/8-h photoperiod condition. Subsequently, the callus were placed to the salt selection medium (MS medium supplemented with 0.02 mg/L of NAA, 0.5 mg/L of BA, 50 mM NaCl), and allowed to develop for 3 months. The callus were sub-cultured for each 3 weeks in the same medium. Salt-resistant callus were transferred to the new selection medium (MS medium supplemented with 1 mg/L of 2,4-D, 1 mg/L of kinetin, 80 mM of NaCl) and allowed to develop for 3 months. After 6 months of salt stress culture on the 80 mM NaCl, healthy green callus were placed to 110 mM NaCl (MS medium supplemented with 1 mg/L of 2,4-D, 1 mg/L of kinetin, 110 mM of NaCl) and allowed to develop for 3 months following the same procedures as described by Elmugrabi et al. (2013), with slight modifications. At each acclimation cycle onto increasing NaCl levels, other embryogenic callus remained continuously

on 50 mM (9 months), 80 mM (6 months), 110 mM NaCl (3 months). On the other hand, control callus were maintained on 0 mM NaCl medium continuously. The control callus were also obtained from leaf explants generated from two-weeks old plantlets in the same way, but without NaCl. Total duration was 12 months.

Cytological analysis

For mitotic chromosome analysis, *Elçi*, *Ömer Bey*, *Muş* root-tip meristems obtained from germinations in Petri dishes were pre-treated with a saturated aqueous solution of α-bromo-naphthalene for 3 h at room temperature. Shortly, mitotic chromosome analysis is prepared Aghayev 1998 method. Zeiss Axiophot microscope and analyzed using a CCD camera (Princeton Instruments, Evry, France) is taken as a single root and 3 preparations are repeated.

Elçi, *Ömer Bey*, *Muş* callus were used in order to examine genome sizes. *Hordeum vulgare* "Cervios" (2 pg/2C) was obtained as a reference standard. All measurements were then analyzed using a Partec CyFlow Space flow cytometer (Munster, Germany) equipped with green laser excitation at 488 nm. The absolute DNA contents of *alfalfa* genotypes were calculated based on the ratios of the G1 peak means of sample and reference standard by using the following formula:

$$\text{Sample 2C DNA content} = \frac{\text{sample G1 peak mean}}{\textit{standard G1 mean}} \text{x2C DNA content (pg)}$$

Nuclear DNA content values were calculated as pg by using formulas. The c-values of the species were compared using t-test.

Proline Estimation

Proline content was detected with the method of Rodriguez and Redman 2005. Briefly, 100 mg of callus tissue was homogenized in 5 mL of 3% aqueous sulfosalicylic acid and centrifuged at 4 °C for 15 min at 4800 rpm. 2 mL of extract was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in test tubes. Samples were kept for 1 h at 100 °C. The reaction was completed in an ice bath. 4 mL of toluene was used for reaction mixture extraction. The absorbance of color reaction product was measured at 520 nm using toluene for a blank. The

proline concentration was measured using a calibration curve.

Soluble Sugar Determination

100 mg callus was homogenized with 5mL 2.5N HCl cold. It was centrifuged at 9000 rpm for 10 minutes. The pellet part was discarded and 2 mL of supernatant was taken and transferred to the glass tube and 2 mL of DNSA (3,5- dinitrosalicylic acid) was added. It was incubated in a 90 ^{0}C water bath for 20 minutes. It was kept in the ice bath until it cools. For each sample, 100 µL per well was added in triplicate to 96 well plate. As a blank, 2 mL of DNSA (3,5- dinitrosalicylic acid) and 2 mL of 2.5N HCl were made in triplicate. Measurements were made at 550 nm at the NanoDrop.

Na^+/K^+ content

10 grams of callus was weighed. It was dried in a 4 ^{0}C oven for 1 night. 10 mL of 15.8M HNO3 was added. It was kept in a sand bath at 240 ^{0}C for 2–3 hours. It was incubated for 15 minutes at room temperature and filtered. 25 mL was completed by adding 15 mL of water. Atomic absorption spectrometry was measured.

RT-qPCR analysis

Total RNA samples were obtained from the callus of control and three genotypes (*Elçi*, *Ömer Bey*, and *Muş*) to determine the levels of gene expression. The total RNA was extracted from the callus using SV Total RNA Isolation System (Promega, Madison, WI, USA). After evaluating the RNA quality, RT-qPCR analysis was performed using 100 ng of total RNA and utilizing *SERK1, SOS1* and *WEE1* primers as described (Spadafora et al. 2011). cDNA was obtained in a 5µ-L volume solution consisting of 1/µL of 0.1mM DTT, 1/µL of 10mM dNTPs, 2/µL of 5U first strand buffer, and 1/µL of reverse transcriptase (5 unit/L). The cycling terms for the RT-qPCR reaction were as follows: 94°C for 5 min, 58°C for 30 s, and 72°C for 50 s, for 30 cycles. The RT-qPCR analysis was carried out using the same primer pairs, as described in the genomic PCR section: *SERK1*-F (GTTGTGGGGGATTTTGGATT) and *SERK1-R* (5 - AGTCGAGCAAGGTCAAAAGC – 3); and *SOS1*-F (5 ATATCCATCTCGCGTTGAGG – 3) and *SOS1*-R (5- CCCTTTGCTCTACCAACCAA-3) and *WEE1*-F (5 TCGATTGAGGAAGGAGATG 3) and *WEE1*-R (5- AATGAATGACCAGGCAGGA-3). Distinctive PCR products were verified using electrophoresis on 1.2 % (w/v) agarose gel with EtBr.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA). The subsequent multiple comparisons were examined based on Duncan's multiple range test. All experiment was repeated three times. All statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS 13). Statistical significance levels were set at $P < 0.05$.

Results

Response of callus on increased levels of NaCl

Highly significant salt-tolerance properties were observed among the genotypes tested in response to increasing NaCl concentrations. Callus developmental stages were almost identical in all genotypes tested at the 50 mM NaCl level compared to the control treatments. The 50 mM NaCl level showed a positive effect, although the developmental processes of acclimizate callus tended to decrease as the NaCl concentration increased. Especially, the growth rate of *Muş* genotype callus significantly decreased under 110 mm NaCl. Although the callus of the genotypes of *Elçi* and *Ömer Bey* showed similar growth rates when exposed to high concentration of NaCl, only NaCl-resistant calluses developed in the *Muş* genotype callus, and have greater densities of necrosis (Figure. 1).

Chromosome number and ploidy level

Out of the 3 genotypes tested, *Muş* genotype was found to be diploid with basic chromosome number 2n = 2x = 16. Although *Ömer Bey* cultivar was observed to be tetraploid, basic chromosome numbers were detected as *2n = 4x = 30*. Only *Elçi* was found to be entirely tetraploid with basic chromosome number *2n = 3x = 32* (Figure. 2).

The genome sizes content of the tested genotypes ranged from 5.08 pg in *Elçi* to 6.50 pg in *Muş*, which is a polyploid. Genome size was 6.50 pg for *Muş*, 6.02 pg for *Ömer Bey*, and 5.08 pg for *Elçi*, respectively. There was no intraspecific diversity in nuclear DNA content among tested. Overall, tested varieties demonstrated a small different value of nuclear DNA content (Figure. 2).

Proline Assay

Proline level was significantly different in callus of all the *alfalfa* genotypes in *in vitro conditions* after acclimation. Proline values varied slightly between genotypes and 50, 80, 110 mM concentrations in vitro. It is ranged from 0.107 to 0.377 nmol g^{-1} FW. Proline values were found from 0.250 to 0.377 nmol g^{-1} FW in 3 months and it were found from 0,147 to 0,241 FW in the 6. months. It was found from 0.107 to 0.140 nmol g^{-1} FW in the last 9th month.

There was no significant difference between NaCl concentrations and months. The highest proline value was 0.337 nmol g^{-1} FW at the 50 mm NaCl level in 3 months. On the other hand, the lowest proline level was found at 80 mm NaCl level 0.107 nmol g^{-1} FW at 9th month (Figure. 3).

Sugar Content

Sugar content varied widely between genotypes and NaCl concentrations of 50, 80, 110 under in vitro conditions, ranging from 1,272 to 2,708 nmol g^{-1} FW. Sugar content at 3. months was found from 0,146 to 1,807 nmol g^{-1} FW and at 6. months was found from 1,272 to 2,308 FW.

It was found from 2,161 to 2,178 FW nmol g^{-1} in the last 9th month. The sugar content was significantly higher compared to other treatments at 110 mm concentration at 3 months. Although there was no significant difference in control (1,272 nmol g^{-1} FW) treatment with 50 mm NaCl (1,285 nmol g^{-1} FW) concentrations at 6 months. A significant difference was found between 80 mM NaCl (1,852 nmol g^{-1} FW) and 110 (2,308 nmol g^{-1} FW) mM. Sugar content 80 mM (2,718 nmol g^{-1} FW) and 110 (2,708 nmol g^{-1} FW) significant changes were found compared to control (2,302 nmol g^{-1} FW) and 50 mM (2,161 nmol g^{-1} FW) at 9 months. The highest sugar content was found at 80 mm NaCl level (2,718 nmol g^{-1}) in the 9th month. while, the lowest sugar content was found at 50 mm NaCl level (0,146 nmol g^{-1} FW) in the 3rd month (Figure .4).

Na^+/K^+ Content

Significant differences (0,233 with 3,518 nmol g^{-1} FW) were found between genotypes (*Elçi, Muş* and *Ömer Bey*) and NaCl (50, 80, 110 mM) in Na^+ and K^+ ratio in acclimizated callus. Significant difference between control treatments (0,233 nmol g^{-1} FW), in NaCl concentrations 50 mM NaCl (1,170 nmol g^{-1} FW), 80 mM NaCl (2,095 nmol g^{-1} FW) and 110 mM NaCl (3,201 nmol g^{-1} FW) in the *Elçi* genotype. Although there was no significant difference between 80 mM (1,390 nmol g^{-1} FW) and 50 mM (1,390 nmol g^{-1} FW) in the *Muş* genotype, a significant difference was found between control (0,3 nmol g^{-1} FW) and 110 mM (2,060 nmol g^{-1} FW). There are significant differences control (1,695 nmol g^{-1} FW) and 50 mM (1,340 nmol g^{-1} FW), 80 mM NaCl (2,325 nmol g^{-1} FW) and 110 mM NaCl (3,518 nmol g^{-1} FW) in NaCl concentrations in the *Ömer Bey* genotype. The most abundant Na^+ and K^+ accumulation was observed at 110 mm NaCl level in the *Ömer Bey* genotype. On the other hand, the lowest Na^+ and K^+ accumulation was observed in the control callus of the *Elçi* genotype. Na^+ and K^+ ratio increased with increasing NaCl concentration in acclimated callus (Figure. 5).

Expression of salt stress related-genes (SERK1, SOS1 and WEE1)

SOS1 gene expression level was a significant difference (from 0,346 to 10,71) between genotypes (*Elçi, Muş* and *Ömer Bey*) and NaCl (50, 80,110 mM) in the acclimated callus (Fig. 6). There was significant difference was found at 50 mm concentration *Elçi* genotype in *SOS1* gene expression. Similar results was observed for *Ömer Bey* genotype. Although there is no significant different between higher concentrations and control. There was significant differences between 50 mM NaCl concentrations and control in *Muş* genotype. The most abundant *SOS1* expression level was found in the *Muş* genotype at control (10,71) treatment, while the lowest expression level was found in the 80 mM treatment (0,346) of the same genotype (Figure. 6). *SERK1* gene expression level had a significant difference (from 2,92 to 10,173) between genotypes (*Elçi, Muş* and *Ömer Bey*) and NaCl (50, 80, 110 mM) in the acclimated callus. Although there was no significant difference between 80 mM NaCl and 110 mM NaCl. There was significant difference between control (4,58) and 50 mM NaCl (3,140) in *Elçi* genotype. There was significant different between 50 mM NaCl, 80 mM NaCl, 110 mM NaCl in *Ömer Bey* genotype compared to control. Although there was no significant difference between 80 mM NaCl and 110 mM NaCl. There was significant different between control and 50 mM NaCl in *Muş* genotype. The most abundant *SERK1* expression level was found in the *Muş* genotype at 50 mM NaCl (10,73) concentration, while the lowest expression level was found in the control treatment (2,92) of *Ömer Bey* genotype (Figure. 7). *WEE1* gene expression level showed a significant difference between genotypes (*Elçi, Muş* and *Ömer Bey*) and NaCl (50, 80, 110 mM) concentrations in the acclimated callus. Although there was no significant difference between control and NaCl treatments in *Elçi* genotype. There was a significant difference between control and 50 mM NaCl treatments in *Ömer Bey* and *Muş* genotype. The most abundant *WEE1* expression level was found in the *Ömer Bey* genotype at 50 mM NaCl (9,166) concentration, while the lowest expression level was found in the control treatment (1,461) of the same genotype (Fig. 8).

Discussion

Under experimental analysis in our study, assessment of stress tolerance in the presence of NaCl was based on the determination of soluble carbohydrates, free proline and Na^+/K^+ as well as *SOS1, SERK1* and *WEE1* expression. The results indicated that the soluble sugar accumulation rate of *alfalfa* callus was highly increased by 80–110 mM NaCl with the treatment of long term period. Soluble sugar accumulation also slightly increased with the 50 mM NaCl with prolonged exposure time, which was in agreement with the change in the cell growth rate when compare to control callus (Fig. 4). In our cases, the results verified that the accumulation of soluble sugars is dependent on increasing salt levels and this was confirmed by the statistical analysis. Moreover, the period of treatment had great influence on callus stressed at 80 mM, which accumulated more sugars than all other treatments. The function of soluble sugars in *in vitro* acclimization is explosive, and even their accumulation can be harmful in the lower salt levels such as 50 mM (Fig. 4). The results of Arefian et al. (2014) are evident that a considerable collection of sugar weakens the osmotic potential of cells and decreases injury of turgidity in resistant genotypes of chickpea. Tested genotypes higher concentrations NaCl treated *alfalfa* callus indicated a remarkably increased of sugar contents. It is proved that higher concentrations NaCl induced the sugar metabolism; thus eleminating NaCl severity.

In *alfalfa* callus, proline content was irregularly detected under salt stress acclimization and the effects of acclimization on proline accumulation are negatively correlated to the salt-tolerance ability. Although the proline content of tested callus subjected to 50 mM showed statistically significant increase in the short term (50 mM) salt treated callus compared with controls, there was a declining trend between the groups in all treatments (Fig. 3). These results are in agreement with the result obtained in two *tobacco* cultivars under salt stress (Wang et al. 2013). Previous studies indicated that a positive relationship between proline rates and callus cultures by salt stress is present in many plant species. Patnaik et al. (1997) used palmarosa callus cells produced from cultured nodal explants as their experimental resources and different dosage of NaCl to select quickly growing callus; NaCl-resistant callus cells indicated adaptive mechanisms including increased proline content. The results are in disagreement with other reports that suggested the increase in proline content under increasing salinity in callus cultures. However, our results are very far from expected situation due to the degrees of proline decreased with increasing NaCl concentration and exposure time. It is possible that 50 mM NaCl induced the proline activity, thereby affecting acclimization capacity, callus are properly growing in lower NaCl concentration *in vitro* conditions.

The degree of salt resistance appeared to be directly correlated to the *SOS1* expression levels of the genotypes, as determined by RT-PCR analysis, suggesting that the resistance is manifested by a $Na^+/K+$ content resistance mechanism (Fig. 6). Expression of *SOS1* was induced by NaCl in callus, where high levels of *SOS1* expression were detected in callus cells of 50 and 110 mM treatments (Fig. 5). This explains that increased *SOS1* expression supported a mechanism that led to withstand stress severity in the presence of NaCl. In terms of the result of the *SOS1* gene expression, there was considerable variation between the three genotypes because of the effects of the physiological alterations in conditions of exogenous NaCl. For example, *Elçi* and *Ömer Bey* genotypes exhibited similar values of *SOS1* gene

expression, but Na^+/K^+ content did not show similar values in the lower NaCl. In contrast, the *Muş* ecotype did not show the expected expression rates in the callus after treatment with salt stress. Interestingly, *Muş* genotype exhibited the highest expression level of *SOS1* in 0 mM NaCl and a lowest expression level at 80 mM NaCl. The reduction in *SOS1* gene expression level in the *Muş* genotypes ranged from 10,71 to 6,34 in the 50 mM NaCl treatment (Fig. 6). Similarly, the proline level detected in the same genotype showed significantly reduced from that detected in control callus. This suggests that the significant reduction in callus of *alfalfa* genotype is not solely a Na^+/K^+ effect of exogenous NaCl, but that several regulation factors exist in the callus cells (Luo et al 2020). It is conceivable that 50 mM NaCl promoted physiological activity; thereby adjusting Na^+/K^+ exclusion capacity, *SOS*-mediated pathway restricts Na^+ amount by epidermal cells in the cytosol. This result was also detected and resolved to maintain optimal cytosolic Na^+/K^+ homeostasis in *barley* (Shi et al. 2002) and *arabidopsis* (Chen et al.2008) subject to salt stress. Darko et al. (2015) showed that in wheat/barley addition lines, the treatments of salt could induce various genes related to Na^+ uptake and transport was not linked with the salt resistance of the genotypes.

In Arabidopsis, *SERK1, SERK3* and *SERK4* participated in stress resistance and are activated by brassinosteroid (Albrecht et al. 2008). The rice *SERK1* have been reported to be stimulated in ABA signaling which promotes oxidative stress (Hu et al. 2005). Li et al. (2017) reported that response to the salt stress stimulates the expressions of *SERK1* and *SERK3* in barley. Our results displayed that *SERK1* exhibits high levels of expression at 50 mM of the salt stress, similarly to what had already been reported by Pérez-Núñez et al. (2009). *SERK1* is a gene that has been well-known and used as a marker gene for somatic embryogenesis formation (Mahdavi-Darvari et al. 2015; Montalvo et al. 2020). Moreover, the observations obtained at 50 mM of the salt stress are in agreement with the induction of embryogenic calluses (Fig. 1). The same basal response content was detected for *SERK1* in *Elçi* and *Ömer Bey* genotypes under normal and NaCl conditions. *Muş* displayed a strong expression of *SERK1* in the acclimated state with respect to non-acclimated state. It is likely that the degree of *SERK1* expression is associated with the degree of embryogenic callus activation which is linked to the optimizations by 2,4-D, a known somatic embryogenesis inducer. It is clearly detected from *Elçi* and *Ömer Bey* genotypes that *SERK1* induced undifferentiated cells and strong stability. This results displayed that embryogenic induction capacity is a genotypic trait and suggested the presence of gene or supression of genes participated in embryogenic callus initiation (Gandonou et al. 2005). Our results in *alfalfa* propose that the somatic cell induction of callus is accompanied by 50 mM NaCl, which could be caused by the synergistic functions of the endogenous auxins and of the 2,4-D present in the medium and could support the embryogenic callus initiation. The remarkable rise in the expression of *SERK1* at 50 mM and the decrease in its expression under 80 and 110 mM conditions seem to confirm this hypothesis (Fig. 7). This could be attributed to fact that NaCl at lower dosage acted like an auxin and promoted the regular growth of callus on media, which in turn improved the overall embryogenic capacity as detected in *rice SERK1* (Hu et al. 2005). This gene is inducible by exogenous treatment including abscisic acid, salicylic acid and jasmonic acid which is *SERK*1 in two rice cultivars to lead an enhancement in host resistance against blast fungus (Hu et al. 2005). Somatic cells induce callus differentiation rapidly and then lose

mitotic and morphological changes in *alfalfa* callus (Fig. 1). The adverse impact of long-term treatments with a high dosage of NaCl has been reported and observations are also consistent with the results obtained in callus of *triticale* by Yazıcılar et al. (2021). The increase in the dosage of NaCl in the present study is an indicative of a decreased *SERK1* gene expression response stress severity that resulted in reduction cell viability. These results indicate that the NaCl on controlling cell differentiation could be a necessary step to precisely modulate the activity of somatic embryogenesis-related genes in *alfalfa*, which is in agreement with the earlier findings required for somatic embryogenesis (Nolan et al. 2003; Elmaghrabi et al. 2013; Luo et al 2020).

WEE1 was expressed in the *alfalfa* callus tissue, suggesting that it plays a role in cell cycle besides its involvement in DNA replication. Similarly, After applying the salt stress, 50 mM NaCl of long-term salt stress displayed a clear increase of *WEE1* expression. It's expression in the presence of NaCl was remarkable at 50 mM and 110 mM rather than 80 mM concentration (Fig. 8). Elmaghrabi et al. (2017) detected high expression of *WEE1* (*Medicago truncatula* L.) in the PEG treatments, thus verifying the regulation of the cell cycle, which is also confirmed as necessary to defend the cells from DNA damage induced by the PEG-related osmotic stress treatment. Our results do not support the claim that there has been reversible DNA injury due to the higher dosage NaCl exposed on callus. It is likely that the level of salt stress tolerance is independent with the level of *WEE1* expression, which is correlated to the regulation in plant cell cycle progression. Previous researchers showed that *WEE1* had been characterized as a role in plant genome endoreduplication, considering its expression in endoreduplication tomato and maize cells (Salomonsson et al. 1993; Sunarpi et al. 2005). These results are in agreement with our findings, which demonstrated that there are some specific correlations between *WEE1* and on ploidy identification of *alfalfa*. The amount of nuclear DNA was the highest in *Muş* (6.50 pg^{-1} C) and had almost similar values with *Ömer bey* (6.02 pg^{-1} C), whereas *Elçi* (5.08 pg^{-1} C) genotype had low nuclear DNA amounts (Fig. 2). Barow and Meister (2003) revealed that an inversely correlation exists between the endoreduplication and genome size and, in most conditions, the species with a small genome display higher levels of endoreduplication. Our findings partially confirm this phenomenon; *Muş* ecotype displayed the highest peak value in endoreduplication followed by *Elçi* and *Ömer Bey* which is also confirmed by the presence of diploid genome with basic chromosome number 2n = 2x = 16. *Muş* (6.50 pg^{-1} C) callus somaclones indicated higher genome sizes *Muş* (6.50 pg^{-1} C) than that of the leaf (3.80 pg^{-1} C) somaclones. This can be explained that a longer period of *in vitro* cultivation seemed to increase somaclonal variations, which involves the interaction of auxin, can act directly to induce polyploidy events. These findings are consistent with those published by De Schutter et al. (2007) in the study on ploidy distribution profile of *Arabidopsis thaliana* in various tissues on cell cycle regulation of *WEE1*. Their results demonstrated that *WEE1* expression levels could induce responses to types of stress stimuli and inhibit plant growth by arresting dividing cells in the G2 phase of the cell cycle after DNA stress. This can be explained as the *WEE1*, which involves in the regulation of programme of endoreduplication, can act as a main regulator of the mitosis to endocycle transition.

Conclusions

One recent study used cytological, physiological, analysis to show that the expression of salt stress genes strongly correlates with higher NaCl treatments (Elmaghrabi et al. 2013). This report is one of the recent works involving the cytological, physiological, and salt stress-related gene expression of the *alfalfa* genotypes. Our results showed that callus growth was declined due to the high NaCl concentrations resulted from the the promoted ion imbalance of callus tissues. The activities of cytological analysis changed to regulate the cell cycle induced by NaCl treatments. Increased the accumulation of osmoprotectants after higher NaCl treatments. Level of expression *SERK1, SOS1* and *WEE1* obtained in this study seem to be beneficial in the salt stress studies of *alfalfa*.

Declarations

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

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Figures

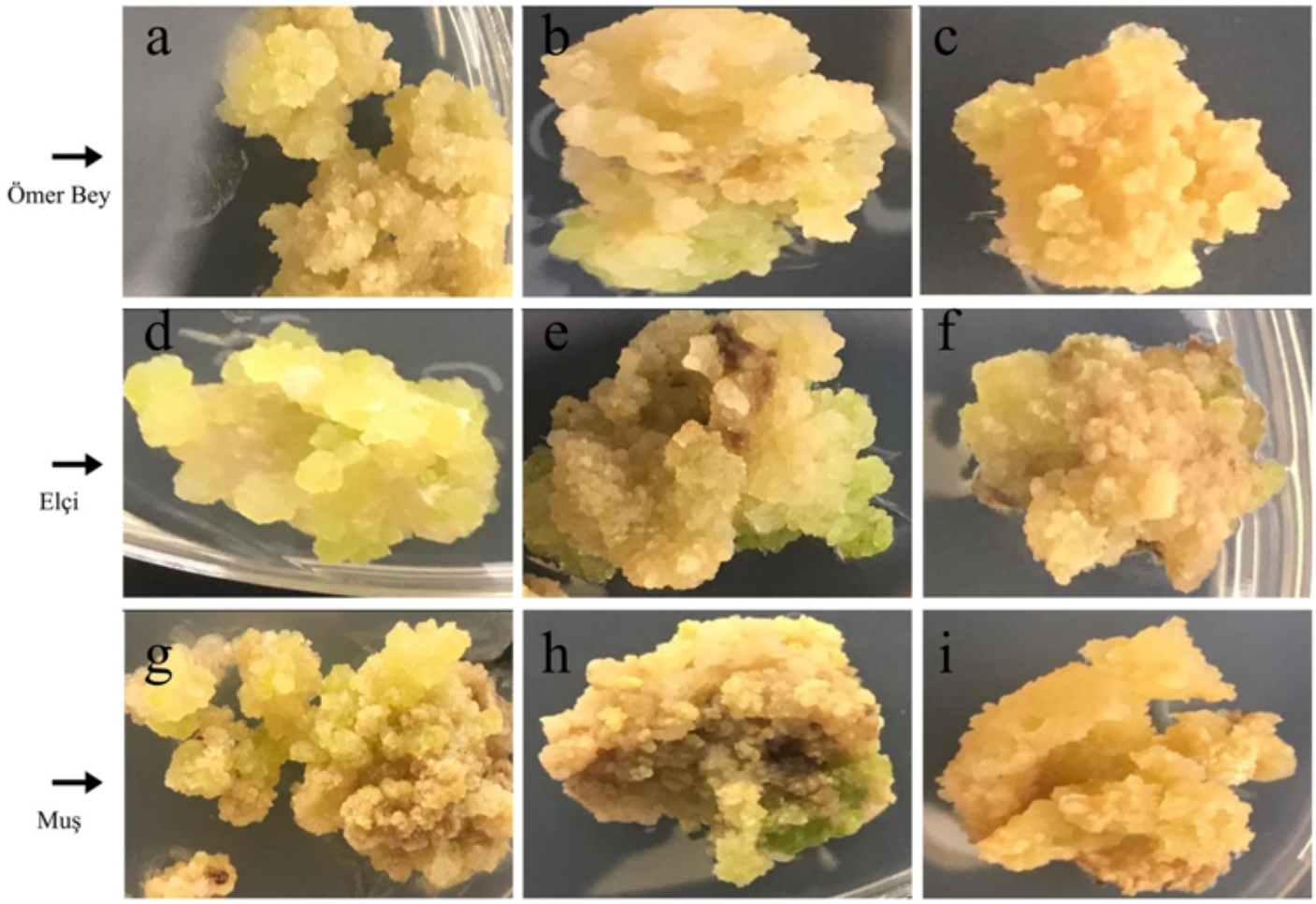

Figure 1

Response to salt treatments in three alfalfa cultivars callus a: 50 mM NaCl of Ömer Bey, b: 80 mM NaCl of Ömer Bey c: 110 mM NaCl of Ömer Bey d: 50 mM NaCl of Elçi e: 80 mM NaCl of Elçi f: 110 mM NaCl of Elçi g: 50 mM NaCl of Muş h: 80 mM NaCl of Muş i: 110 mM NaCl of Muş

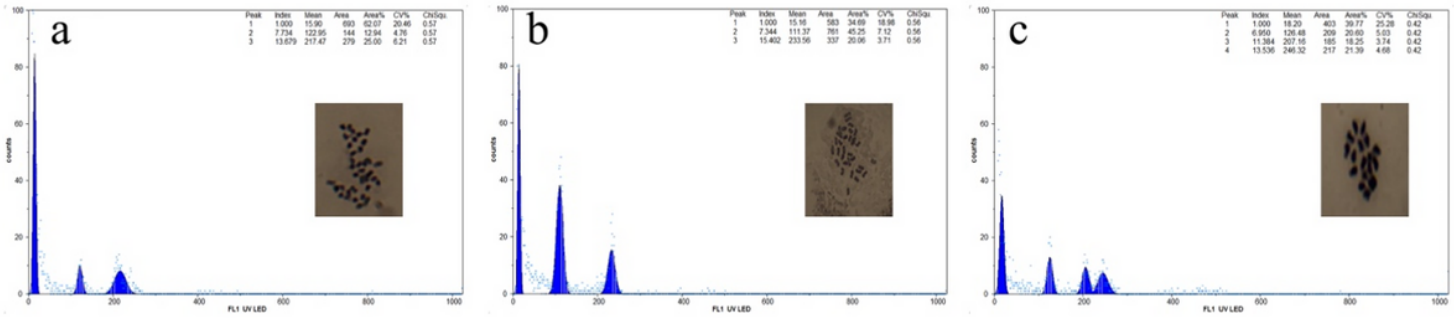

Figure 2

Flow cytometric analysis of DAPI stained nuclei Alfalfa using Barley as internal standart and chromosome numbers analysis of alfalfa genotypes using microscopy. Representative of the ploidy

levels of genotypes A. Ömer Bey 2n=2x=30 B. Elçi 2n=2x=32 C. Muş 2n=2x=16

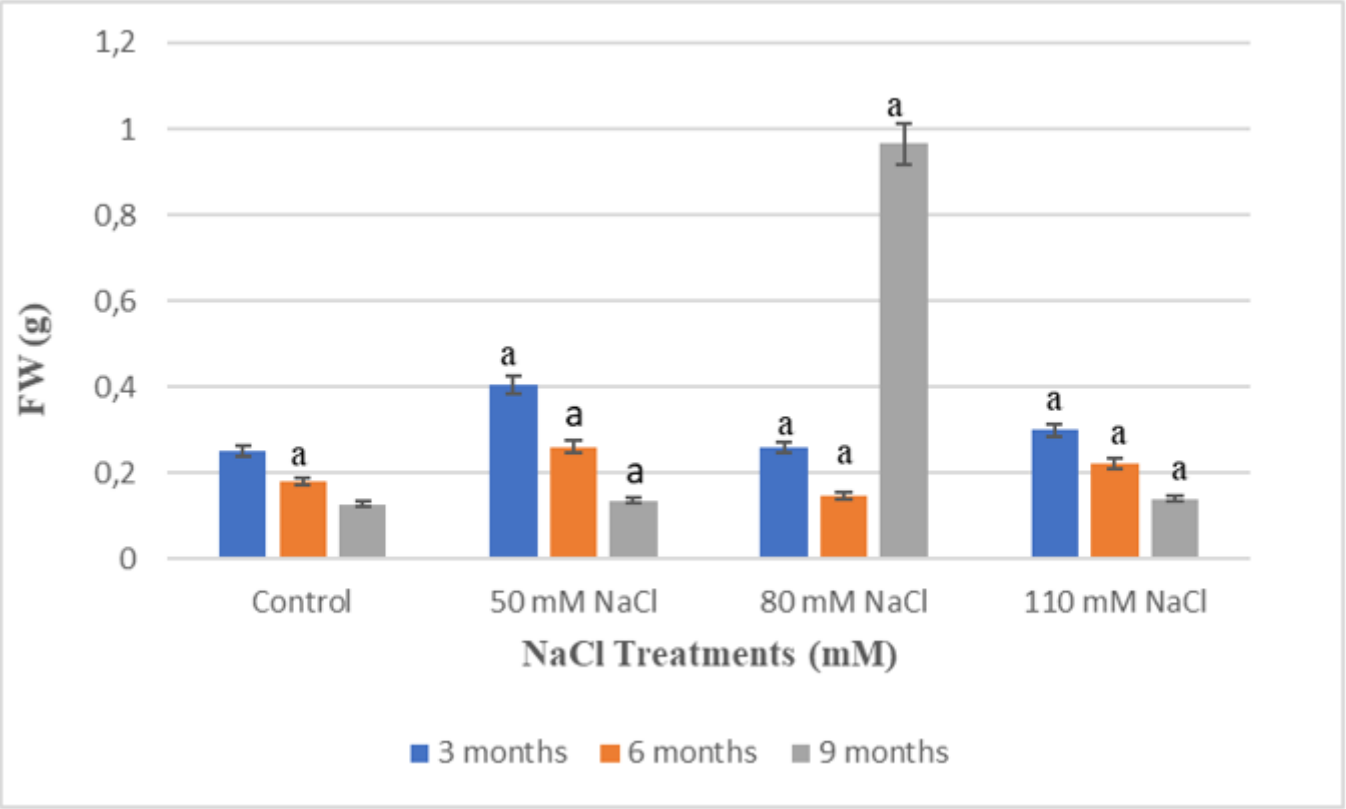

Figure 3

Changes in proline activity in alfalfa genotypes treated NaCl stress.

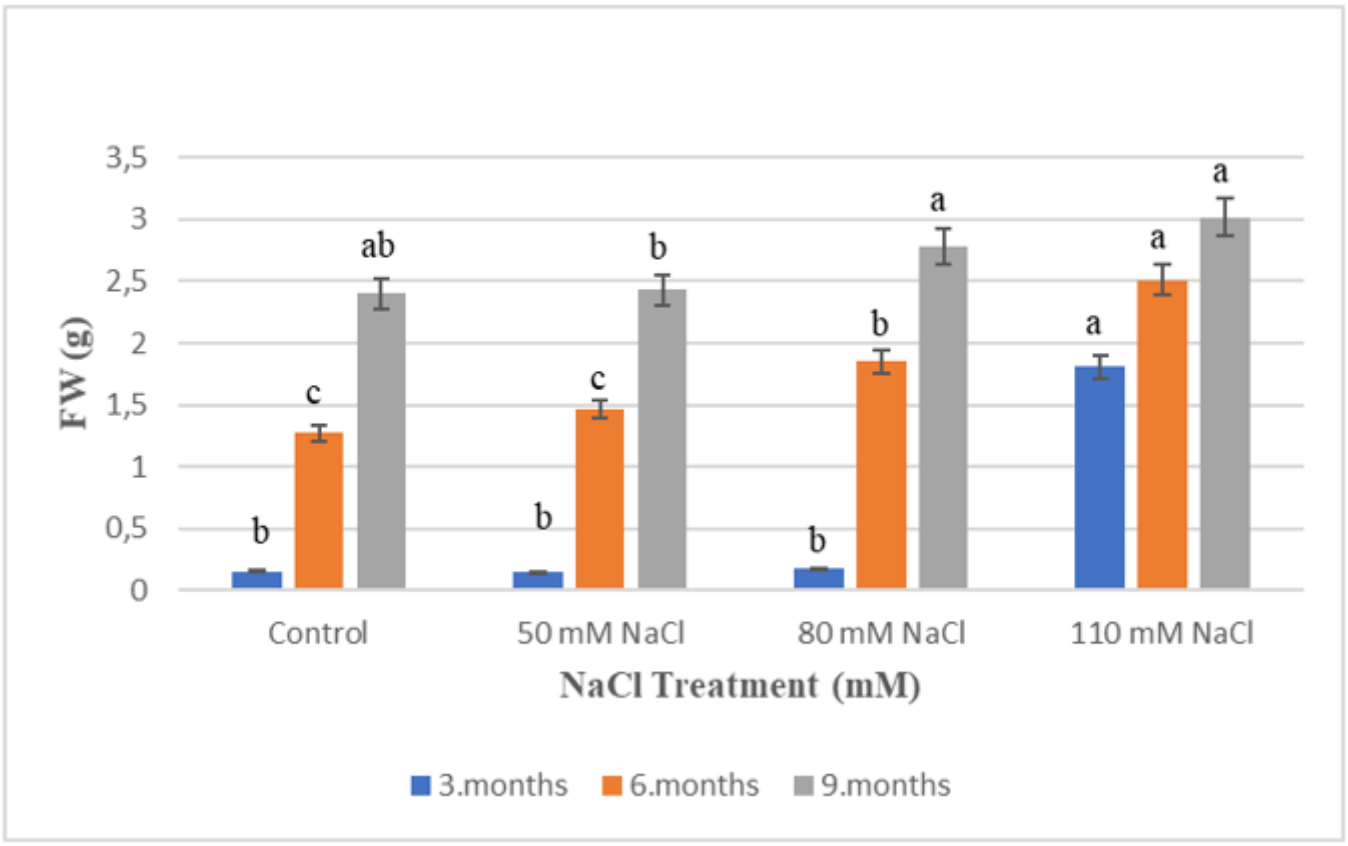

Figure 4

Changes in sugar contents in alfalfa genotypes treated NaCl stress.

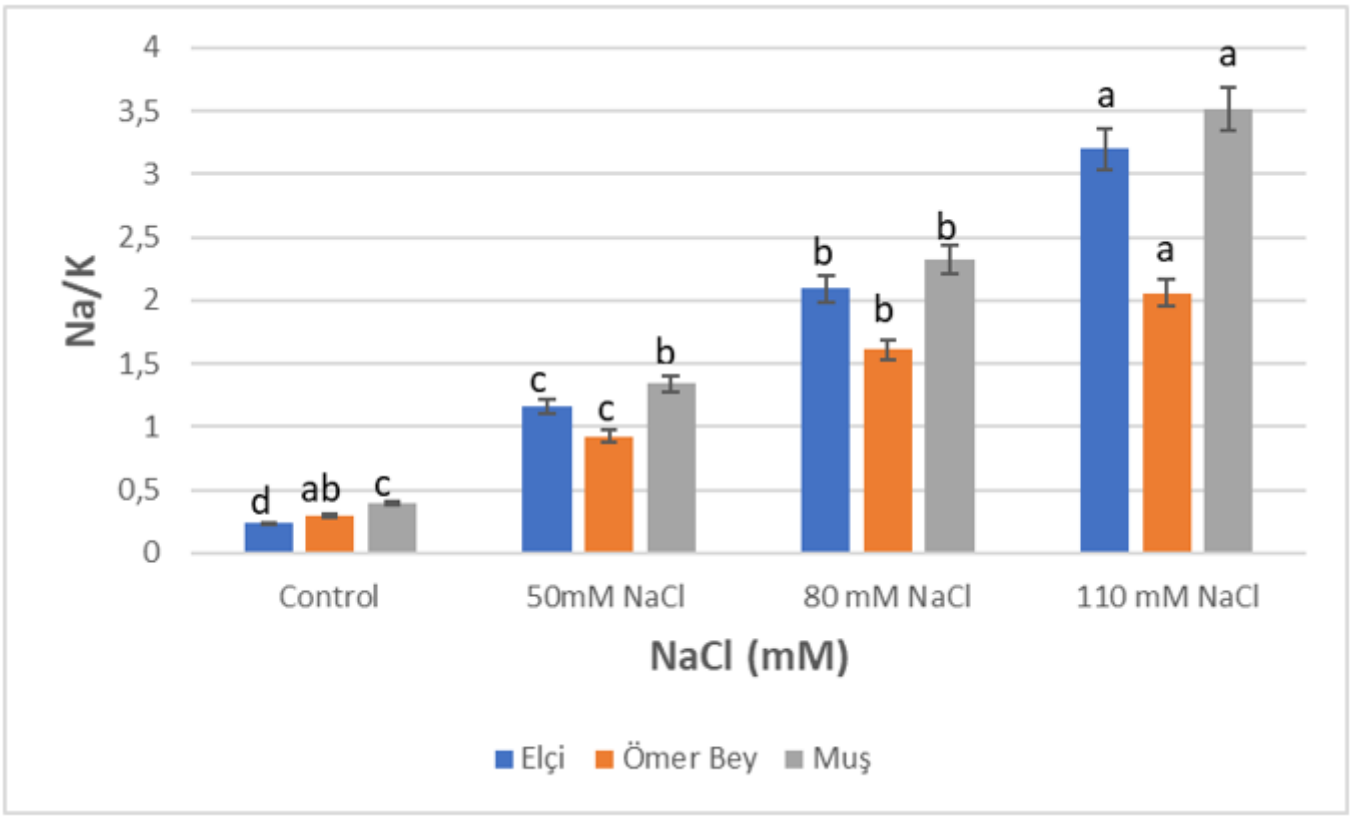

Figure 5

Changes in Na+/K+ in alfalfa genotypes treated NaCl stress.

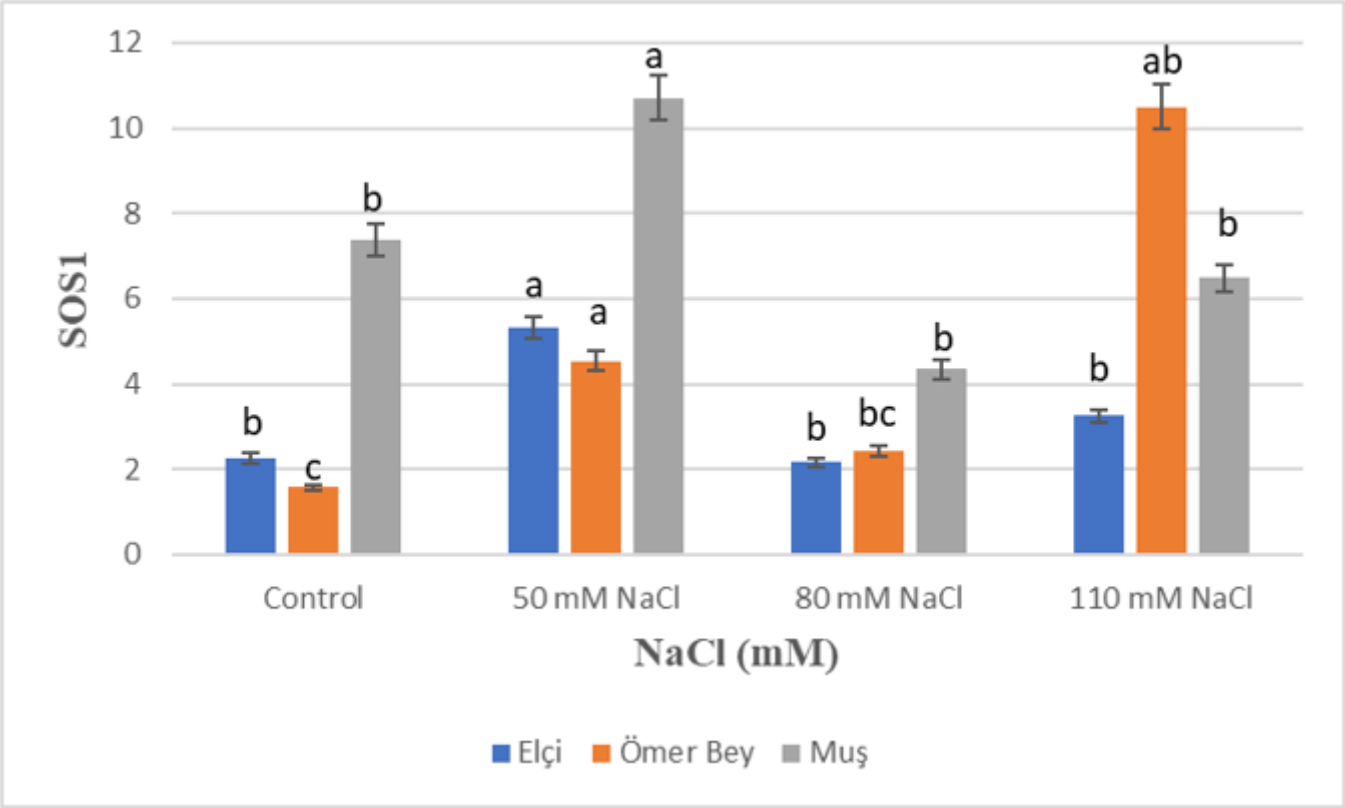

Figure 6

Levels of SOS1 gene expression in alfalfa genotypes treated NaCl stress.

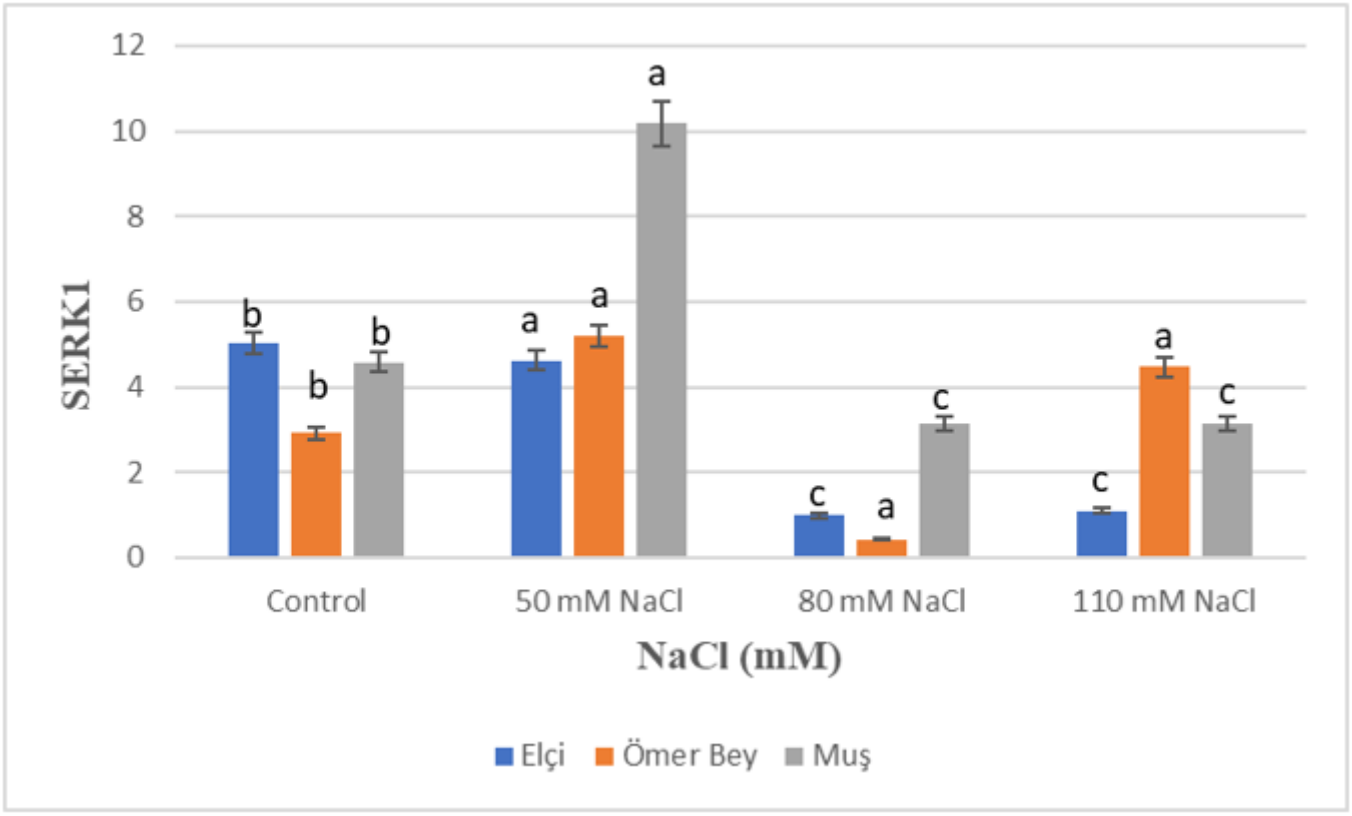

Figure 7

Levels of SERK1 gene expression in alfalfa genotypes treated NaCl stress.

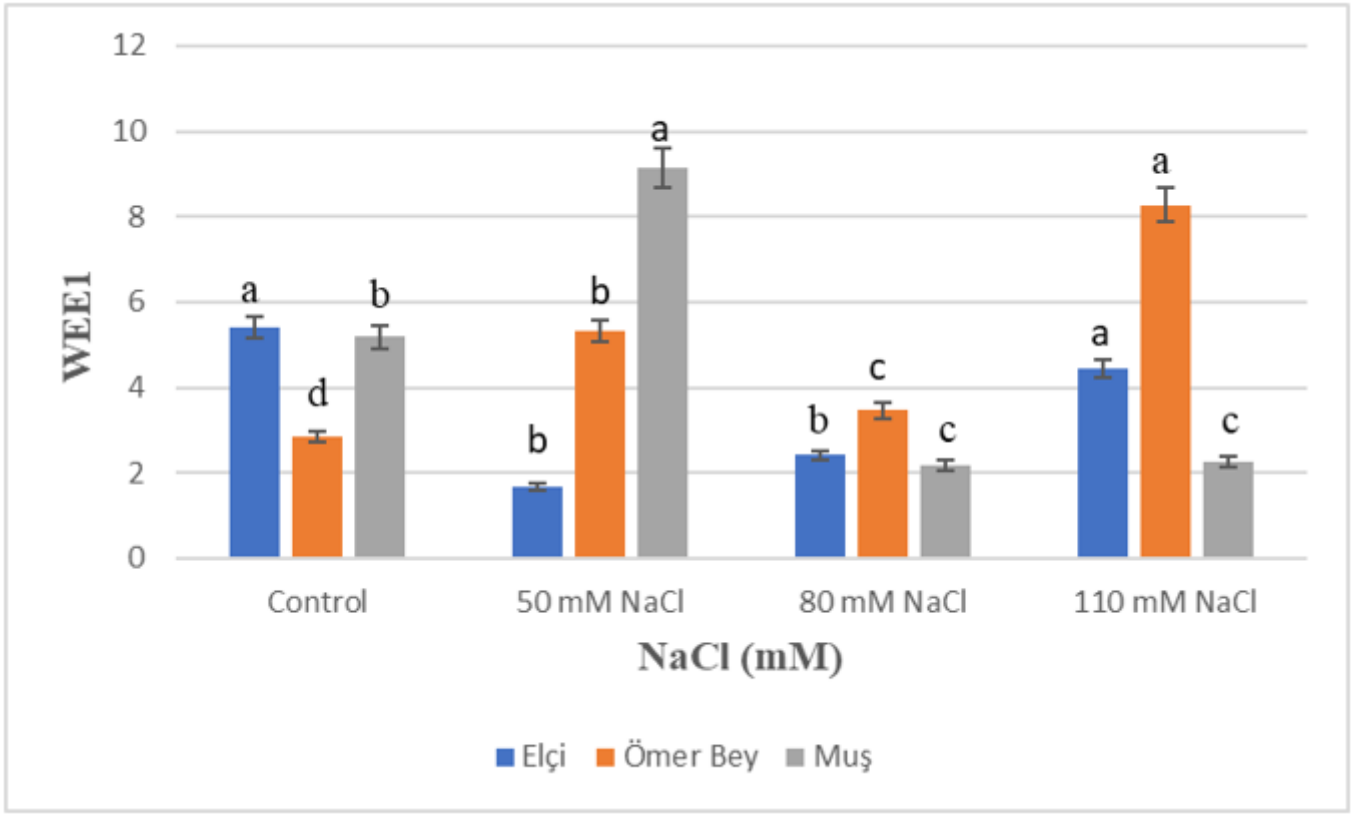

Figure 8

Levels of WEE1 gene expression in alfalfa genotypes treated NaCl stress.