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Research Article/Araştırma Makalesi

Biochemical and molecular tolerance of *Carpobrotus acinaciformis* L. halophyte plants exposed to high level of NaCl stress

NaCl stresine maruz bırakılan Carpobrotus acinaciformis L. halofit bitkisinin biyokimyasal ve moleküler tepkileri

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ABSTRACT

Carpobrotus acinaciformis L. plant is a kind of halophyte that is able to survive in high salt conditions. It is important to determine its physiological, biochemical and molecular limit of NaCl stress if one aims to use it for phytoremediation purpose. In this study, the alkaline protocol of the modified plant comet assay were used for rapid detection of DNA damage in C. acinaciformis L. plants exposed to a series of NaCl stress concentrations (0-, 50-, 100-, 200-, 300-, 400 and 500 mmol L^{-1}) in hydroponic conditions for 2 weeks. DNA damage was measured as the values of percentage of DNA in tails and tail length. The halophyte C. acinaciformis L. did not show any dose response up to 400 mmol L^{-1} NaCl level in terms of DNA damages. DNA integrity measured via comet assay showed that DNA preserved its original shape up to 400 mmol L^{-1} NaCl level. However, the very high concentrations of NaCl (400 and 500 mmol L^{-1}) caused DNA damages. When physiological and biochemical parameters such as proline, chlorophyll a and b, peroxidase (POX), catalase (CAT), H_2O_2, malondialdehyde (MDA) contents were examined, oxidant molecules such as H_2O_2 (0.912-3.72 µmol g^{-1} Fwt) and MDA (7.1-34 nmol g^{-1} Fwt) gradually increased along with the increase of NaCl concentrations, $p<0.05$. On the other hand, antioxidant enzyme POX and an osmolyte molecule proline slightly increased up to 400 mmol L^{-1} NaCl level then slightly decreased after that. Similar issues were obtained from that of protease enzyme which indicates the power of protein hydrolysis in which a slight decrease (182-95 Unit mg^{-1} protein) over a dose of NaCl was evident. Chlorophyll contents and CAT activity were not affected upon increase of NaCl concentrations. This study showed that the halophyte C. acinaciformis L. can be easily used to remove salt up to 400 mmol L^{-1} NaCl concentrations from a saline-affected soil. Measuring DNA damage is concluded as a very useful parameter to find out what level of NaCl could be tolerated if a halophyte plant is aimed to remediate the saline soils.

Key Words: Halophyte, NaCl stress, Comet assay, SCGE, DNA damage

ÖZ

Carpobrotus acinaciformis L. bitkisi yüksek tuz koşullarında yaşayabilen bir çeşit halofit bitkidir. Bu bitki fitoremediasyon çalışmaları için kullanılmak üzere planlandığında, bu bitkinin tuz stresine karşı fizyolojik, biyokimyasal ve moleküler sınırlarını belirlemek önem arz etmektedir. *C. acinaciformis* L. bitkisinde DNA hasar seviyesini belirlemek için hidroponik koşullarda 2 hafta süre ile tuz stresine (0-, 50-, 100-, 200-, 300-, 400 and 500 mmol L^{-1}) maruz bırakılan bitkilerde modifiye edilmiş alkali bitki comet assay metodu kullanılmıştır. DNA hasarı kuyruk uzunluğu ve kuyrukta DNA yüzdesi olarak ölçülmüştür. Halofit *C. acinaciformis* L 400 mmol L^{-1} NaCl seviyesine kadar DNA hasarı ile ilgili olarak doz tepkisi göstermemiştir. Comet assay ile ölçülen yönteme göre halofit bitkilerin DNA bütünlüğünü 400 mmol L^{-1} NaCl seviyesine kadar

korunduğu gözlenmiştir. Fakat, çok daha yüksek NaCl konsantrasyonları (400 ve 500 mmol L^{-1}) DNA hasarına yol açmıştır. Prolin, klorofil *a* ve *b*, peroksidaz (POX), katalaz (CAT), H_2O_2, malondialdehid (MDA) içerikleri gibi fizyolojik ve biyokimyasal parametreler incelendiğinde, oksidant moleküllerden H_2O_2 (0.912-3.72 µmol g^{-1} taze ağırlık) and MDA (7.1-34 nmol g^{-1} taze ağırlık) artan tuz konsantrasyonu ile paralel olarak sıralı artış göstermiştir, $p<0.05$. Diğer yandan, antioksidant enzimlerden POX ve bir osmolit olan prolin 400 mmol L^{-1} NaCl' e kadar hafifçe artış göstermiş daha sonra tekrar düşmüştür. Benzer durumlar protein hidrolizini belirlemede kullanılan proteaz enzim (182-95 Unit mg^{-1} protein) seviyesinde de görülmüş, artan NaCl dozuna bağlı olarak enzim miktarı kademeli olarak azalmıştır. Klorofil miktarı ve CAT enzim seviyesi NaCl konsantrasyon artışına bağlı olarak değişim göstermemiştir. Bu çalışma, *C. acinaciformis* L. bitkisinin tuzdan etkilenmiş topraklarda 400 mmol L^{-1} NaCl' e kadar olan tuz konsantrasyonunu uzaklaştırmada rahatlıkla kullanılabileceğini ortaya koymuştur. DNA hasarını ölçmek, tuzlu alanları ıslah etmede kullanılacak halofit bitkinin hangi seviyede NaCl stresine dayanabileceğini belirlemede çok kullanışlı bir parametre olarak kaydedilmiştir.

Anahtar Kelimeler: NaCl stres, Comet assay, SCGE, DNA hasarı

Introduction

Salinity is a major factor limiting crop production in the world. Salinity has an effect on almost every aspect of the physiology and biochemistry of plants and significantly reduces yield (Pirasteh-Anosheh et al., 2016; Suo et al., 2017). Salinity induces ion imbalance, osmotic stress, oxidative damage, and other subsequent disturbances (Pirasteh-Anosheh et al., 2016). Plants under stressful conditions produce reactive oxygen species (ROS), however, they are able protect their cells and subcellular systems against the effects of (ROS) by enzymes such as superoxide dismutase, catalase, peroxidase, glutathione reductase, polyphenol oxidase and non-enzymatic antioxidants such as ascorbate and glutathione (Agarwal and Pandey, 2004). ROS types such as superoxide (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), and singlet oxygen (O^-) have capacity to cause oxidative damage in proteins, DNA and lipids (Sharma et al., 2012; Tripathy and Oelmüller, 2012). Malondialdehyde (MDA) content, a product of lipid peroxidation, has been considered as an indicator of oxidative damage. Similarly, H_2O_2 has been regarded as a good marker for oxidative stress. These molecules could decrease the membrane stability and reduce the metabolic functions of cells (Miller et. al., 2010). By using above markers, we could easily differentiate the salt-tolerant and salt-sensitive plants species. Recently, DNA damage studies via the use of comet assay have enabled the plant scientists more straight forward approach. Conditions of plants under stress could be evaluated via the measurement of DNA integrity. The comet assay (single-cell gel electrophoresis) is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. It determines single and double-strand breaks in DNA.

Halophytes are salt-resistant or salt-tolerant plants and they have remarkable ability to complete their life cycle in saline conditions (Flowers and Colmer, 2008; Grigore et al., 2014). Therefore, halophytes have been explored for saline agriculture in many studies (Flowers and Colmer, 2015; Karakas et al., 2017). However, little information is available on well-defined molecular defense mechanisms against salt stress (Anjum et al., 2012; Joshi et al., 2015). Determination of defense or tolerance mechanisms of halophytes in a molecular level would enable us to decide whether the selected halophyte is suitable that have potential remediation capacity in terms of salinity and heavy metal toxicity.

Carpobrotus acinaciformis L. is a fast growing, succulent perennial plant belonging to the family of Azioaceae. It is commonly inhabited in the Mediterranean, all of which share a similar climate; it was oriented from South Africa. It is resistant to harsh conditions such as salt and drought (Suehs et al., 2003; Traveset et. al., 2008). Its growth is rapid and the coloniziation capacity is high in the ground (D'Antonio, 1993; Price and Marshall 1999).

In this study, the responses of *C. acinaciformis* L. to NaCl stress (0-, 50-, 100-, 200-, 300-, 400 and 500 mmol L^{-1}) were tested to characterize its salt tolerance capacity for the possible phytoremediation approach.

Material and Methods

The halophyte plants (*C. acinaciformis* L.) were collected at the clonal growth stage from the gardens of Harran University, Turkey. The plants were cut at equal length and were exposed to series of NaCl stress concentrations (0-, 50-, 100-, 200-, 300-, 400 and 500 mmol L^{-1}) in hydroponic conditions for 2 weeks. Control plants were treated with dH_2O only.

Measurement of physiological and biochemical parameters

A chlorophyll determination was performed according to the method of Arnon (1949). For the analysis, a 0.5 g leaf sample was homogenized in 5 mL acetone:water (80:20% v/v) mixture. The homogenate was prepared via filtering the samples through Whatman No.1 filter paper and centrifuged at 10000 g for 5 min to minimize the residual pellet. The supernant was then read against the blank containing 80% acetone control alone. The measurement was made at 663 nm for chl a and 645 nm for chl b in a UV spectrophotometer (UV–1700, Shimadzu).

The proline measurement was conducted according to the method of Bates et al. (1973). Acid-ninhydrin was employed as a reagent, which was obtained by dissolving (warming and agitating) 1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid. A quantity of 0.5 g of leaf material was homogenized in 10 mL of 3% w/v sulfosalicylic acid, then the homogenate was filtered through Whatman No. 2 filter paper. A 2 mL of filtrate was mixed in a test tube with 2 mL of acid ninhydrin reagent and boiled at 100°C for one hour. The reaction was terminated in an ice bath. The reaction mixture was extracted with 5 mL of toluene. Tubes were thoroughly shaken for 15 - 20 seconds and left for further 20 min in order to achieve separation of the two layers. The chromophore containing toluene was removed and allowed to warm to room temperature. Absorbance of the solution was measured at 515 nm using a toluene blank as a reference in a spectrophotometry (UV–1700, Shimadzu). The results were expressed as µmol g^{-1} fresh tissue.

The malondialdehyde (MDA) content was determined according to the method of (Sairam and Saxena, 2000). A 0.5 g leaf tissue sample was homogenized using 10 mL of a 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000 *g* for five minutes. Four mL of 20% v/v TCA containing 0.5% v/v thiobarbituric acid (TBA) was added to 1 mL of the supernatant. The solution was heated at 95^{o}C for 30 min and then quickly cooled on ice. The mixture was centrifuged once again at 10,000 *g* for 5 min and the absorbance of the clean supernatant was determined at 532 and 600 nm. The MDA content of leaves is expressed as nmol g^{-1} fresh tissue.

Catalase enzyme activity (CAT, E,C. 1.11.1.6) was determined by monitoring the decomposition of H_2O_2 according to the method of Milosevic and Slusarenko (1996). For the analysis, 0.5 g of plant material was homogenized in 10 mL of a 50 mM Na-phosphate buffer solution, then 50 µL of plant extract was added to a 2.95 mL (10 mM H_2O_2, 50 mM Na-phosphate buffer and 4 mM Na_2EDTA) reaction mixture and measured for 30 seconds at 240 nm with a UV spectrometer (UV-1700, Shimadzu). One CAT activity unit (U) is defined as a change of 0.1 absorbance unit per minute. Activity is expressed as enzyme units per gram fresh weight.

Peroxidase enzyme activity (POX, E.C.1.11.1.7) was determined by monitoring the increase in absorbance due to the tetraguaiocol formation at 470 nm according to the method of Cvikrova et al. (1994). For the analysis, 100 µL of extract (obtained as above) was added to 3 mL of the reaction mixture (13 mM guaiacol, 5 mM H_2O_2, and 50 mM Na-phosphate, pH 6.5). The reaction was initiated with a H_2O_2 addition and was measured at 470 nm, using a UV spectrophotometer (UV-1700, Shimadzu) at one-minute interval until 3rd minute. One unit of POX activity is defined as a change of 0.1 absorbance unit per minute at 470 nm. Activity is expressed as enzyme units per gram of fresh weight.

Protease activity was determined according to the method of Girard and Michaud (2002). One g plant was placed into 2 mL Eppendorf tube and then 500 µL of 1% (w/v) azocasein in 50 mmol L^{-1} Tris-HCl, pH 8.8. Azocasein hydrolysis was initiated by incubating the tubes 2 h at room temperature. Proteolysis was stopped by adding 300 µl of 10% (w/v) cold trichloroacetic acid (TCA). After centrifugation for 10 min at 15,000 g, 350 µl of the supernatant was collected and mixed with 300 µl of 1 N NaOH. Protease activity was then determined by reading the optical density of the resulting solution at 440 nm using a UV spectrophotometer. A change of 0.01 units per minute in absorbance was considered to be equal to one unit protease activity, which was expressed as U (unit) mg^{-1} protein.

Hydrogen peroxide (H_2O_2) content was determined according to the method of Velikova et al. (2000). A tissue sample of (0.1 g) was homogenized in an ice bath with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min and 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI were added. The absorbance was read at 390 nm and the H_2O_2 level was calculated as µmol g^{-1} FW.

Assessment of DNA damage caused by NaCl was made via the comet assay method for plants (Gichner et al. 2008; Kassaye et al., 2013). Nuclei were isolated from leaves of *C. acinaciformis* L. cuttings which were exposed to NaCl in hydroponic conditions. A 50-100 mg fresh weight of leaf material was mixed with 200 µl cold phosphate buffered saline (PBS) containing 50 mM Na_2EDTA on a 60-mm Petri dish placed on ice. Leaf tissues were quickly chopped rather than slicing with a sharp scalpel under dim light in a dark room to collect nuclei (Pourrut et al., 2015). DNA damage was examined after the assay protocol in both control and exposed groups. Ethyl methanesulfonate (EMS) was used as a positive control.

The assay was briefly outlined as following:; Cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis performed at high pH (pH ≥13) results in structures resembling comets when observed under fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks (Collins, 2004).

Result and Discussion

Assessment of Physiological parameters

The fresh weight (FW) and dry weight (DW) of the halophyte *C. acinaciformis* increased at 50 and 100 mmol L^{-1} NaCl, then returned to the control values at 200 and 300 mmol L^{-1} NaCl, and then decreased at about 400 and 500 mmol L^{-1} NaCl. The growth of *C. acinaciformis* was promoted at low levels of NaCl. However, these levels have been regarded as toxic and inhibit the growth of many salt-tolerant glycophytes and many salt-tolerant crop plants (Lei et al., 2018). The halophyte *C. acinaciformis* was able to grow in the presence of 500 mmol L^{-1} NaCl and remained alive at 500 mmol L^{-1} NaCl which is a higher dose than salt concentration of sea water. Low levels of NaCl improved the growth and development of *C. acinaciformis* (Figure 1).

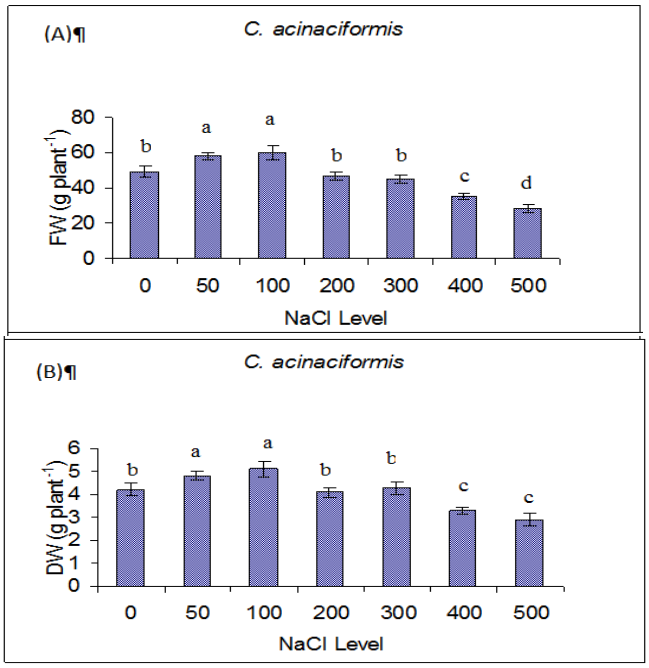

Figure 1. (A) FW and (B) DW of *C. acinaciformis* under NaCl stress. Different letters indicate significant differences (P<0.05).

Şekil 1. Tuz stresi altında C. acinaciformis bitkisinin (A) Taze ağırlık, TA ve (B) Kuru ağırlık, KA değerleri. Farklı harfler istatistik olarak önemli farklılıkları ifade etmektedir (P<0.05).

Assessment of biochemical parameters

Chlorophyll *a* and chlorophyll *b* contents were not affected under NaCl stress at all NaCl concentrations, Figure 2. In some studies, photosynthesis was shown not to be affected by salinity and even stimulated at low salt concentrations (Kurban et al., 1999, Parida et al., 2004). However, high salinity tolerance, in general, do not affect the chlorophyll synthesis in halophytes even at high concentrations (Flowers and Colmer, 2008).

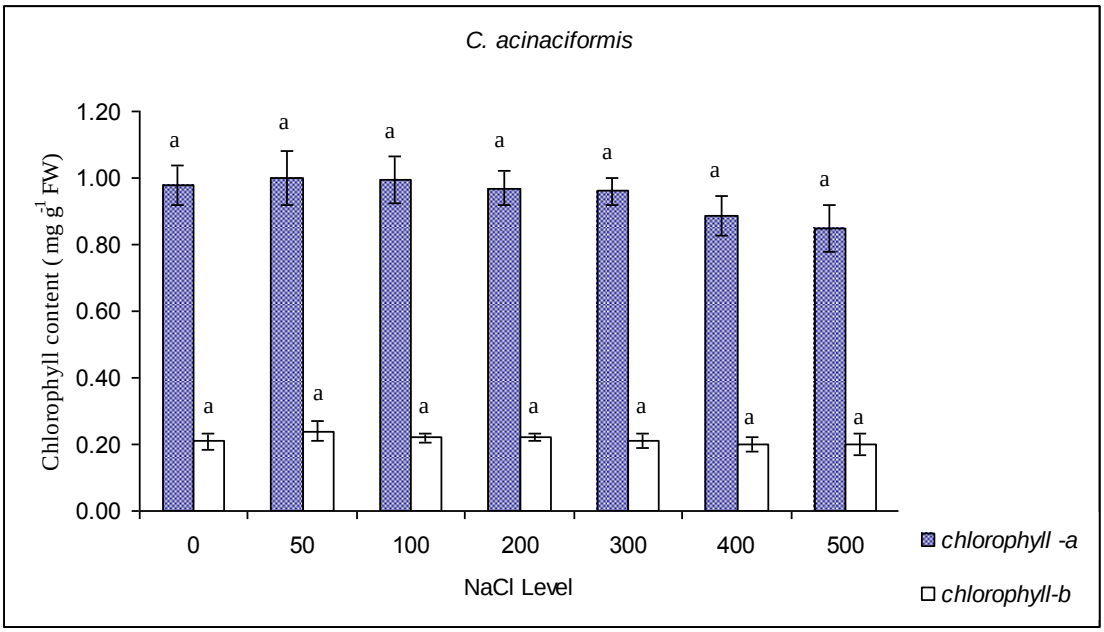

Figure 2. Content of chlorophyll *a* and *b*.
Şekil 2. Klorofil a ve b içeriği.

An osmolyte molecule proline slightly increased up to 300 mmol L^{-1} NaCl level, then slightly decreased. Plants sensitivity and leaf proline concentration showed that high proline contents were related to their reactivity to salt (Chérifi et al., 2016). Several plants accumulate higher level of proline in contrast to other amino acids when exposed to high salt content in the soil (Heidari et al., 2011). Proline acts as a mediator of osmotic adjustment stabilizing the effect of salt accumulation in the vacuole (Heidari et al., 2011) to protect cell membranes, several different enzymes and metabolic machinery (Zadehbagheri et al., 2014).

Oxidant molecules such as H_2O_2 (0.91-3.72 µmol g^{-1} FW) and MDA (7.1-34 nmol g^{-1} FW) gradually increased with dose of NaCl, $P<0.05$, Figure 3. Oxidant molecules did not show any significant differences up to 50 mmol L^{-1} NaCl concentration for the accumulation of stress metabolites. The remarkable accumulation of stress metabolites was evident at 400 and 500 mmol L^{-1} NaCl conditions. On the other hand, proline, an anti-stress metabolite, was accumulated right after 50 mmol L^{-1} NaCl level indicating an adaptive characteristic of the halophyte. Similarly, Rubio et al. (2009) has observed greater oxidative damage in *Lotus japonicus* exposed to a high saline concentration, despite the maintenance of antioxidant levels.

Some authors consider that H_2O_2 accumulation under high saline concentrations may be a signal for an adaptative response to stress (Foyer et al., 1997). It has been demonstrated that H_2O_2 accumulation was involved in stomata closure induced by abscisic acid (ABA) signalling (Zhang et al., 2001).

Antioxidant enzyme POX slightly increased up to 300 mmol L^{-1} NaCl level, then slightly decreased (Figure 4A). Protease activity, on the other hand, gradually decreased upon increase of NaCl concentrations (Figure 4B) indicating depletion of proteins under stress conditions. Decrease in protein concentrations has been

regarded as a defense response (Gupta and Huang, 2014). Since antioxidant or phenolic enzymes require proteins to synthesize enzymes and biochemical compounds etc. therefore, a gradual decrease in protease activity was evident. Similar reports were also made by Simova-Stoilova et al. (2006) who showed a gradual decrease of protease in wheat plants under drought stress conditions.

enzyme expressions were not observed after 2-week incubation in all NaCl conditions. This also indicated that the halophyte species showed similar response in all NaCl concentrations. Also, proteins have been reported to be separated into subunits, amino acids, under stress conditions (He, 2005). This might have also reduced the concentrations of proteins. Similar reports were also made by Shetti and Kaliwall (2017).

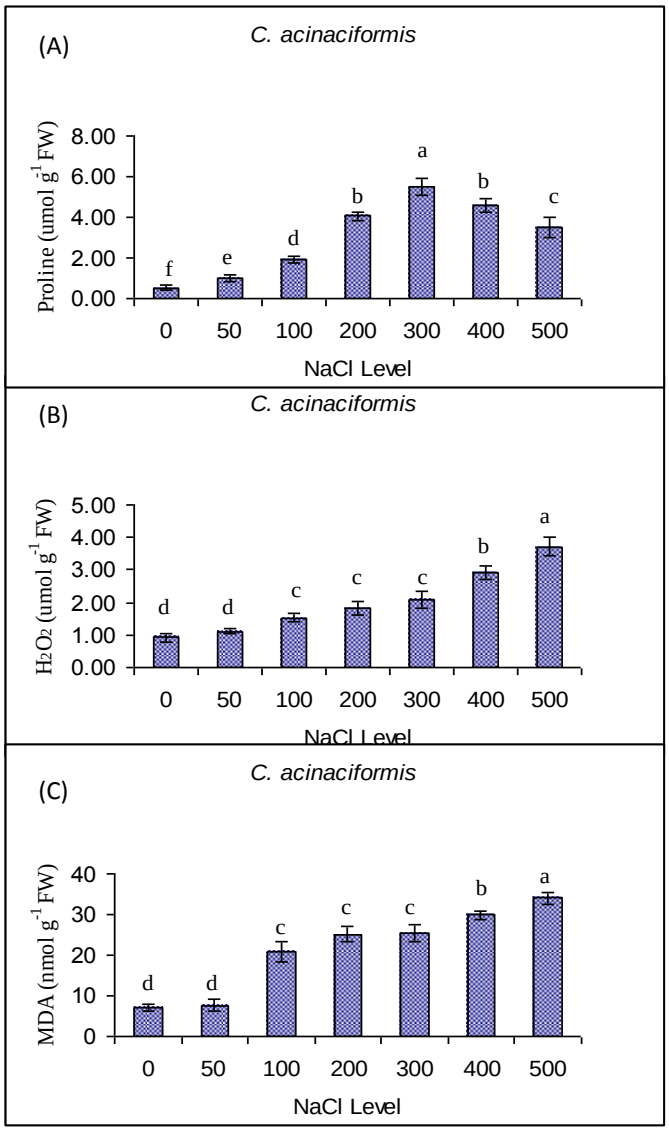

Figure 3. (A) Proline, (B) H_2O_2 and (C) MDA contents of *C. acinaciformis* under NaCl stress. Mean ± SE of three replication. Different letters indicate significant differences (P<0.05).

Şekil 3. NaCl stresi altında C. acinaciformis bitkisinin (A) Prolin, (B) H_2O_2 ve (C) MDA içerikleri. Sonuçlar Ortalama ± SH olarak ifade edilmiştir. Farklı harfler istatistik olarak önemli farklılıkları ifade etmektedir (P<0.05).

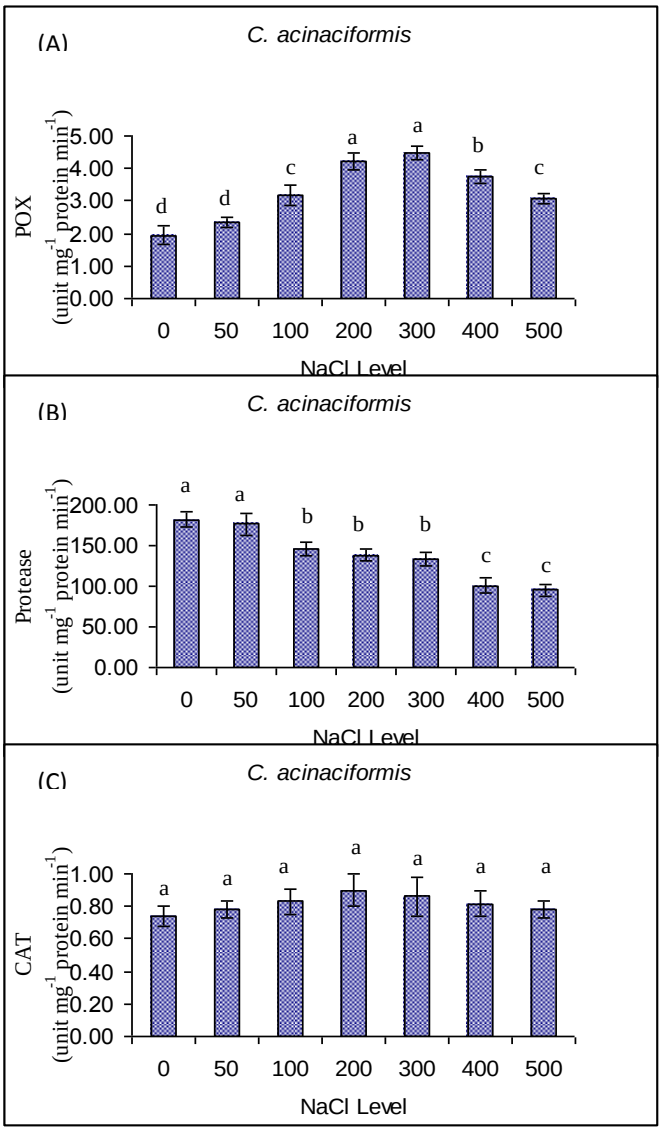

Figure 4. A) Peroxidase (POX), B) proteaz and C) catalase (CAT) of *C. acinaciformis* under NaCl stress. Mean ± SE of three replication. Different letters indicate significant differences (P<0.05).

Şekil 4. NaCl stresi altında C. acinaciformis bitkisinin (A) Peroksidaz (POX), (B) proteaz ve (C) katalaz (CAT) değerleri. Sonuçlar Ortalama ± SH olarak ifade edilmiştir. Farklı harfler istatistik olarak önemli farklılıkları ifade etmektedir (p<0.05).

CAT activity was not affected upon increase of NaCl concentrations (Figure 4C). Since CAT enzyme has a very short half-life, differences in

Assessment of DNA damage caused by NaCl

C. acinaciformis did not show any dose response to NaCl stress up to 400 mmol L^{-1} NaCl

level with respect to DNA damages. DNA integrity measurement showed that DNA of the halophyte preserved its uniform shape and was not affected by the toxicity of NaCl as the other components of cell material (Figure 5). It is important to note that DNA of any organism is quite protected and surrounded by many layers. Therefore, it could be expressed that the only 400 mmol L^{-1} and above NaCl concentrations were able to cause DNA damages. It was observed that at high NaCl level, the halophyte was able to grow and produce FW and DW. Similarly, Karakas et al. (2017) tested four levels of saline soils which were cultivated with the halophyte species *S. soda* L. and *P. oleracea* L. in pots. The FW and DW of the halophytes were significantly greater in the moderately saline and highly saline soil types than in slightly saline and non-saline soil types. *S. soda* produced 43 g DW per pot while *P. oleracea* produced 40 g DW per pot in the highly saline soil type after 100 days of cultivation. The halophytes produced almost twice as much DW compared to non-saline soil treatment.

It was reported that DNA damages of potato plants were evident at 200 mmol L^{-1} concentrations. However, defense enzymes and metabolites responded at much lower concentrations indicating that DNA was the most resistant part of the cell components (Dikilitas et al., 2015). Therefore, DNA damages occurring under *abiotic stress (*such as salinity, drought, extreme temperatures) biotic stress (such as bacteria, viruses, fungi, parasites, weeds and insects) at their combination show the level of stress threshold that cause breaks in DNA. Stress that causes breaks in DNA structure is able to cause biochemical and physiological changes. A stress level that does not cause any breaks in DNA structure could be tolerated and biochemical or physiological changes could be normalized upon removal of stress factor. In this study, measurement of DNA damages via comet assay enabled us to determine what level of NaCl could be tolerated by *C. acinaciformis* halophyte species that is planned to use for phytoremediation purposes (Figure 6.).

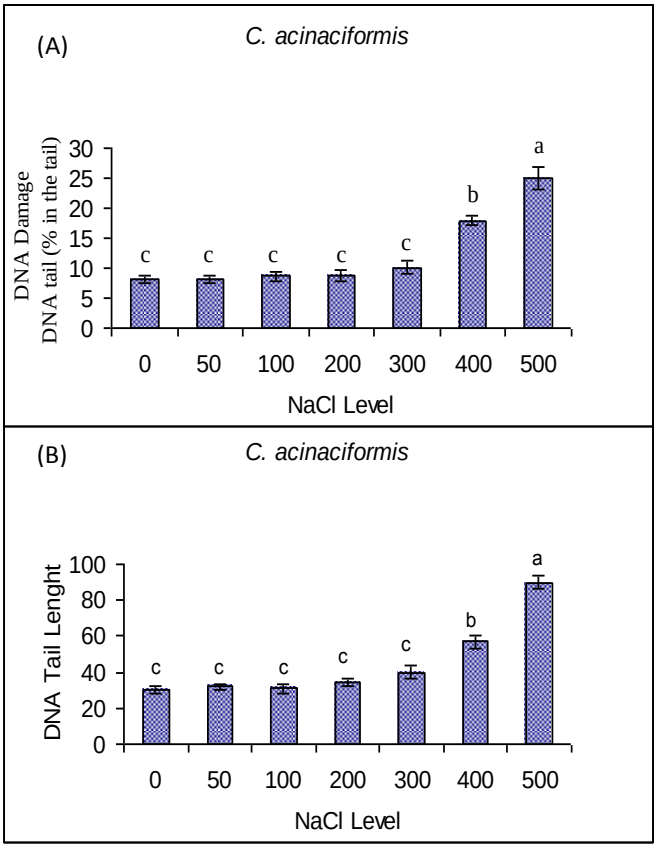

Figure 5. A) Percentage DNA damage and B) DNA tail length of *C. acinaciformis* under NaCl stress. Mean ± SE of three replication. Different letters indicate significant differences (P<0.05).

Şekil 5. NaCl stresi altında C. acinaciformis bitkisinin (A) üzde DNA hasar ve (B) DNA kuyruk uzunluğu. Sonuçlar Ortalama ± SH olarak ifade edilmiştir. Farklı harfler istatistik olarak önemli farklılıkları ifade etmektedir (P<0.05).

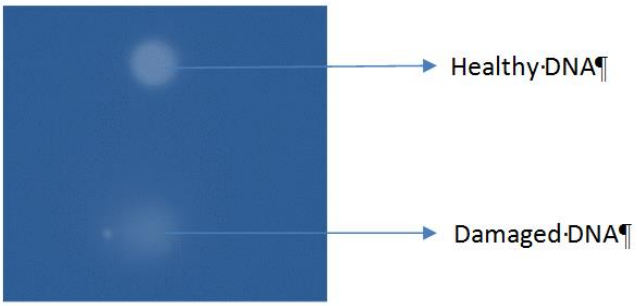

Figure 6. DNA damages of *C. acinaciformis* halophyte under NaCl stress.

Şekil 6. NaCl stresi altında C. acinaciformis bitkisinin DNA hasarları.

Conclusions

Halophyte plant *C. acinaciformis* grew rapidly at moderate salt concentrations and was able to survive at extreme saline conditions almost close to seawater salt concentrations. The present study showed that salinity triggered some solutes (proline, MDA and antioxidant enzymes). The

plant could be easily used to remove salt up to 400 mmol L^{-1} NaCl concentrations from the saline soil. Measuring DNA damage could be very useful to find out up to what level of NaCl could be tolerated if the halophyte is aimed to remediate the saline soils. This study is the first of its kind to measure the response of a halophyte in a molecular level measuring its DNA uniformly through assessing DNA damage or breaks. Our next study is underway which aims to find out the correlation among stress parameters and DNA health. We plan to determine what sorts of or what levels of stress metabolites would be able to affect DNA health and integrity.

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