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Responses of *Spinacia oleracea* L. cv. Matador Plants to Various Abiotic Stresses Such as Cadmium Metal Toxicity, Drought and Salinity[A]

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Abstract: *Spinacia oleracea* L. cv. Matador plants produced in many regions are exposed to many abiotic stresses from drought to metal toxicity. In this study, the effects of drought (control: 100% field capacity (FC), D1: 50% FC and D2: no-watering), salinity (100 and 200 mM NaCl) and cadmium (Cd; 100 and 200 µM $CdCl_2$) metal toxicity, which are among the most common abiotic stress factors, on spinach plants were determined at the cellular level. There was not determined any alterations along 10 days' drought, salinity and Cd stress in dry and fresh weights of spinach plants grown in plant growth chamber, in which there is a 16-hour photoperiod under a light intensity of 1200 lux at 24°C/20°C (day/night). However, all treatments caused oxidative stress. Cd treatments were more structurally damaging than drought and salinity treatments. In drought and salinity treatments, chlorophyll content and dry weight did not change despite the increased superoxide dismutase (SOD) and catalase (CAT) activities. The highest values in SOD activity were obtained at D2-drought treatment and 200 mM salinity treatment. SOD activity determined in Cd treatments was also increased compared to control, but this increase was lower than the other treatments. Thus, it can be considered that CAT enzyme is primarily scavenger of reactive oxygen species (ROS) in spinach plants under Cd stress. As a results, spinach plants had an ability to cope with this stresses. The different responses of spinach seedlings to various stress factors provide

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for estimate of the plant's powerful physiological mechanism. In the continuation of this study, it is recommended to conduct molecular studies and to investigate of the cellular responses to long-term stress on *Spinacia oleracea* L. cv. Matador plants, which we found to be tolerant to short-term stresses.

Keywords: Abiotic stress, antioxidative defense system, cadmium, drought, salinity, *Spinacia oleracea* L. cv. Matador.

Tuzluluk, Kuraklık, Kadmiyum Metal Toksitesi gibi Çeşitli Abiyotik Streslere Matador Çeşidi *Spinacia oleracea* L. nın Verdiği Yanıtlar

Öz: Birçok bölgede üretilen Matador çeşidi *Spinacia oleracea* L. bitkisi, kuraklıktan metal toksisitesine kadar birçok abiyotik strese maruz kalmaktadır. Bu çalışmada Matador çeşidi ıspanak bitkilerinde en yaygın abiyotik stres faktörlerinden kuraklık (kontrol: %100 tarla kapasitesi, D1: %50 tarla kapasitesi ve D2: tam kuraklık koşulları), tuzluluk (100 ve 200 mM NaCl) ve kadmiyum (Cd; 100 ve 200 µM $CdCl_2$) metal toksisitesinin hücresel düzeyde etkileri belirlenmiştir. Elde edilen sonuçlarda 10 günlük kuraklık, tuzluluk ve Cd stresi boyunca 24°C/20°C'de (gündüz/gece) 1200 lüks ışık yoğunluğu altında 16 saatlik fotoperiyodun bulunduğu bitki büyüme odasında yetiştirilen Matador çeşidi ıspanak bitkilerinin kuru ve yaş ağırlıklarında herhangi bir değişiklik tespit edilmemiştir. Bununla birlikte, tüm stres uygulamaları oksidatif strese neden olmuştur. Cd uygulamalarının, ıspanak bitkilerinde kuraklık ve tuzluluk uygulamalarına göre yapısal olarak daha zarar verici olduğu tespit edilmiştir. Kuraklık ve tuzluluk uygulamalarında, klorofil içeriği ve kuru ağırlık, artan süperoksit dismütaz (SOD) ve katalaz (CAT) aktivitesine rağmen değişmemiştir. SOD aktivitesinde en yüksek değerler, D2-kuraklık uygulamasında ve 200 mM konsantrasyonunda tuzluluk uygulamasında elde edilmiştir. Cd uygulamalarında belirlenen SOD aktivitesi de kontrole göre artmıştır ancak bu artış, diğer uygulamalardan daha düşüktür. Bu nedenle, Matador çeşidi ıspanak bitkilerinde Cd stresi altında CAT enziminin esas olarak reaktif oksijen türleri (ROS) nin temizleyicisi olduğu düşünülebilir. Sonuç olarak, Matador çeşidi ıspanak bitkilerinin bu streslerle başa çıkma yeteneği vardır. Çeşitli stres faktörlerine ıspanak fidelerinin verdiği farklı yanıtlar, bitkinin güçlü fizyolojik mekanizmasının tahminini sağlar. Bu çalışmanın devamında, kısa süreli streslere toleranslı olduğunu tespit ettiğimiz Matador çeşidi *Spinacia oleracea* L. üzerinde moleküler çalışmaların yapılması ve uzun vadeli strese hücresel tepkilerin araştırılması önerilmektedir.

Anahtar Kelimeler: Abiyotik stres, antioksidatif savunma sistemi, kadmiyum, kuraklık, Matador çeşidi *Spinacia oleracea* L., tuzluluk.

Introduction

Spinach (*Spinacia oleracea* L.) is a one-year culture plant and is cultivated with seeds (Bender Özenç and Şenlikoğlu, 2017). According to FAO (2018), spinach cultivation in the world is 27.9 million tons. China is the first in this production; Turkey is in the 4th place with approximately 225,000 tons of production. Spinach production is carried out in all regions of Turkey except the Eastern Black Sea Region (Sertkaya, 2015). It is exposed to many stress factor like that drought, salinity, heavy metal stress due to it is grown almost everywhere. Extreme temperatures, water scarcity (drought), and ion toxicity (salinity, heavy metals) are the major causes which adversely affect the many plant growth and productivity in worldwide (Mahajan and Tuteja, 2005; Tuteja, 2007; Khan and Singh, 2008).

Drought is the biggest factor among these stress factors with its 26% rate (Farooq et al., 2009; Bayram Erdoğan, 2018). It affects all plant development stages from germination and vegetative growth to reproductive (Hossain et al., 2012; Ahmad et al., 2018). Many growth variables and functions at these stages are affected by stress caused by water deficiency (Nezhadahmadi et al., 2013). Like most vegetables, water deficit for also spinach is a significant limitation (Xu and Leskovar, 2015). There was reported that it a sensitive species to water stress (Yurtyeri et al., 2014). Increasing drought levels due to global warming on earth causes an increase in salinity of agricultural land. Therefore, salinity is other major environmental stress factor that have serious negative effects on plant growth and development (Bartels and Sunkar, 2005; Liang et al., 2018). Salinity not only severely restrict agricultural production in hot-arid and semi-arid regions (Belmecheri-Cherifi et al., 2019), but is also big problem for greenhouses (Du et al., 2015). Both drought and salinity cause stress by disrupting the ionic and osmotic balance (Mahajan and Tuteja, 2005). These conditions reduce leaf cell expansion and stomatal conductance. While reduction in the intake of CO_2 and increased level of oxygen primarily results in decrease of the photosynthesis rate, it causes production of reactive oxygen species (ROS) via electron transport chain (ETS) which operates in chloroplasts (Taiz and Zeiger, 2002; Anjum et al., 2011; Osakabe et al., 2014; Ahmad et al., 2018).

Like that, heavy metal stress also causes ionic stress in plants. Especially Cd is become one of the major constraints for food safety and soil quality via liquid and soil manure (or their derivate, compost, or sludge) or fertilizers derived from phosphate rocks mostly used in agriculture (Atafar et al., 2010; Roberts, 2014; Younis et al., 2016). Vegetables like spinach are grown in pre-urban soils interacted with raw city effluents containing Cd (Khan et al., 2015; Liu et al., 2015). Spinach is a leafy vegetable comprising broad with green leaves with large surface area, relatively higher growth rate, and metal accumulation ability (Bagheri et al., 2013; Alia et al., 2015). Recently, *S. oleracea* L. and its varieties have been researched in a number of scientific studies to observe their growth and toxicity responses to Cd contaminations (Dhongade and Nandkar, 2011; Alia et al., 2015; Pinto et al., 2017). It was indicated in some researches that Cd accumulation is in spinach and translocate to its up ground parts (Verma et al., 2007; Rezakhani et al., 2013). However, there is still a lack of information about physiological responses of *Spinacia oleracea* L. cv. Matador such as biomass, yield, chlorophyll content, stomatal size and transpiration rate, the water and nutrient uptake and ROS production, against not only Cd, but also other ionic and organic stress. Therefore, it is necessary to reveal each of the responses to these stress factors in *Spinacia oleracea* L. cv. Matador plants. The responses of spinach to these stress conditions will create important data to be used in the cultivation of these plants, which are frequently used by people and grown widely in the world and in our country. In this study, we aimed to determine the responses of spinach at the cellular level under various abiotic stress conditions such as drought, salinity and Cd concentrations. For this purpose, drought (control: 100% field capacity (FC), D1: 50% FC and D2: no-watering), salinity (100 and 200 mM NaCl) and cadmium (100 and 200 µM $CdCl_2$) were applied to spinach seedlings grown under the same conditions.

Material and Method

Plant material and growth conditions

In this research, *Spinacia oleracea* L. cv. Matador, widely grown in Turkey as plant material, was used. Seeds was planted to 72-cell viols containing peat (Klasmann Rec119 Potgrond H) as one seed in each eye. After 4 weeks, seedlings were transferred to 14 x 12 cm pots containing a mixture of peat / perlite (1:1). Seedlings were grown in plant growth chamber, in which there was a 16-hour photoperiod under a light intensity of 1200 lux at 24°C/20°C (day/night). Growing seedlings were irrigated with Actagro (7-7-7) Nutrient Solution.

Abiotic stress treatments

The 8 weeks-old seedlings were divided into four basic groups to investigate responses to salt, drought and Cd stresses. All plants were fertilized in Actagro (7-7-7) Nutrient Solution. The first group: Control group; the second group: The experimental groups exposed to two different Cd concentration (100 and 200 µM, supplied as $CdCl_2$), the third group: The experimental group created according to gravimetric method (Khorasaninejad et al., 2011), so; control: 100% field capacity (FC), D1: 50% FC and D2: no-watering. And last group: The experimental groups exposed to two different NaCl (100 and 200 mM) concentration. The all treatments were maintained for 10 days.

Growth measurement

The measurements after the plants are harvested, was stated as fresh weight (FW, g). The dry weight (DW, g) was measured after oven-drying of the samples at 80ºC until the weight was constant.

Leaf relative water content (RWC, %) and turgor loss (%)

The analyses related to water status of the leaves were measured according to the method described by Arefian and Shafaroudi (2015). Approximately 1 cm leaf discs were weighed (Fresh Weight, FW) and so put in a petri dish containing deionised water for 4 h. Then blotted and weighed (Turgid Weight, TW). For the dry weight (DW), leaf discs were oven-dried (48 h, 70°C). Leaf RWC and turgor loss were estimated as follows: $RWC= [(FW-DW)/ (TW-DW)] \times 100$, $Turgor\ loss= (TW-FW)/TW x100$.

Chlorophyll assay

The leaf chlorophyll values in the spinach plants was determined by a portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Japan) and given as SPAD value. SPAD measurements were made on the fully expanded leaves, then averaged.

Ion leakage

Percent of ion leakage for spinach leaf tissue was measured by using the procedure of Arora et al. (1998), with some modifications of Gulen and Eris (2003). Leaf discs were cut (2cm) and lightly rinsed in distilled water, gently blotted with paper, and one disc per tube containing water (20mL) was placed. Samples were then vacuum infiltrated to allow uniform diffusion of electrolytes and incubated for 4 h at room temperature. After incubation, electrical conductivity of each solution (EC1) was measured using a conductivity meter (WTW Cond 315i, Weilheim, Germany). Leaf discs were then autoclaving in the same solution, and total conductivity was measured at room temperature (EC2). Percentage of leakage each treatment was calculated using the equation: %Ion leakage=(EC_1/EC_2) x 100.

Antioxidative Enzymatic Assay

Plant extraction were performed according to the method by Ardıç et al. (2009). One g fresh leaf samples were homogenized with buffer solution [50 mM Na-phosphate buffer (pH 7.8), 2% (w/v) polyvinylpolypyrrolidone (PVP), 1 mM EDTA] in an ice bath. Then they were centrifuged at 14.000 g (40 min, 4°C). The supernatants were used in SOD and CAT activity analyses. The SOD activity (EC 1.15.1.1) was determined according to the method of Beuchamp and Fridovich (1971). This method is based on the inhibition of the nitroblue-tetrazolium at 560 nm (Novaspec II LKB Biochrom). SOD assay kit (SOD S7446, Sigma-Aldrich, USA) was used in the preparation of standards. The activity of enzyme was determined according to the linear equation which was obtained from the curve after the calculation of % inhibition, it was expressed as units per mg protein (U/mg protein).

The CAT activity (EC 1.11.1.6) was assayed with some modification (Lester et al., 2004) and the activity of both enzyme was also expressed as units per mg protein (U/mg protein). For CAT activity, 0.1 mL supernatant was added to 20 mM sodium phosphate buffer (pH 6.8) and 15 mM H_2O_2. The change in absorbance was measured at 240 nm for 3 minute (Shimadzu UV-2100).

Statistical analysis

The experiment was arranged in a randomized block design with three replications and for 4 plants in each repeat. ANOVA were tested by SPSS 13.0 for Windows program.

Results

In the present, different concentrations of cadmium (Cd) (100 and 200 µM), drought (D1: 50% FC and D2: no-watering) and salinity (100 and 200 mM) treatments were separately applied to spinach plants grown under the same controlled conditions. Figure 1 shows the alterations occurred in dry and fresh weights of *Spinacia oleracea* L. cv. Matador plants. According to this, there was no negative effect on dry and fresh weight in both two different Cd treatments. As shown in Figure 1, whereas salinity treatments did not cause any decrease in the dry weight of spinach plants, fresh weight decreased statistically in only 200 mM NaCl treatment ($p<0.05$). In the drought conditions, both treatments caused a similar decrease in fresh weight (Fig. 1), and it was statistically significant ($p<0.05$) findings. Reduced in growth under drought are caused by altered water relations. Because, in dry weight of spinach plants, statistically a significant decrease was not determined in any treatments (Fig. 1).

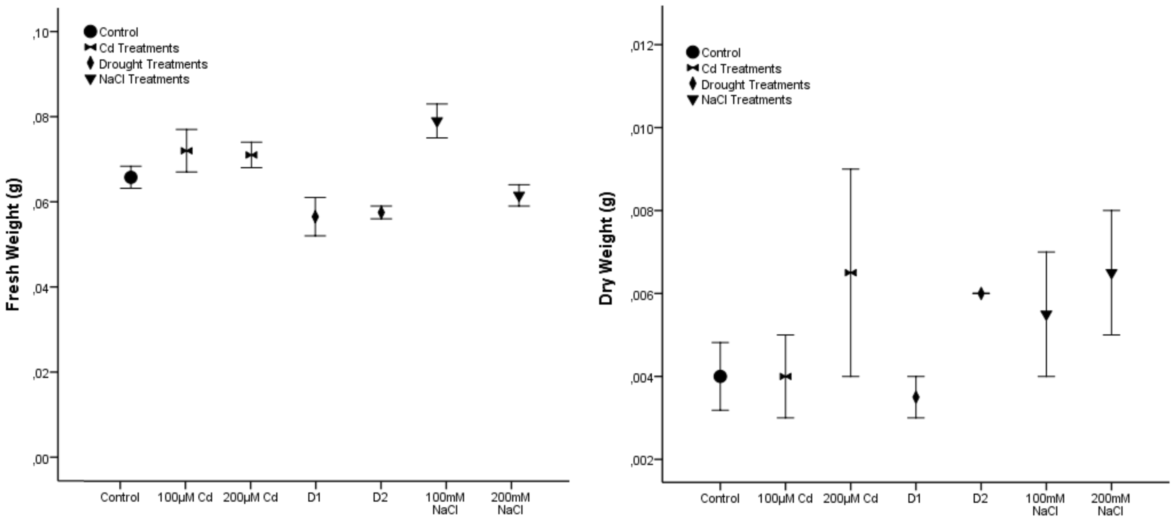

Figure 1. *Fresh Weight (FW) and Dry Weight (DW) in Spinacia oleracea L. cv. Matador plants exposed to Cd, drought and salinity treatments. Data points represent means and standard errors (n=4).*

The changes in leaf relative water content (RWC, %) and turgor loss (%) of spinach plants was shown in Figure 2. No change in leaf RWC was observed in Cd treatments. These results are compatible with leaf dry and fresh weight values in this study. When the average leaf turgor loss values were evaluated, it was not also determined any change in Cd treatments (Fig. 2). In both drought and salinity treatments, while the leaf relative water content (%) in spinach plants decreased, turgor loss (%) increased similarly together with increasing in concentration ($p<0.05$).

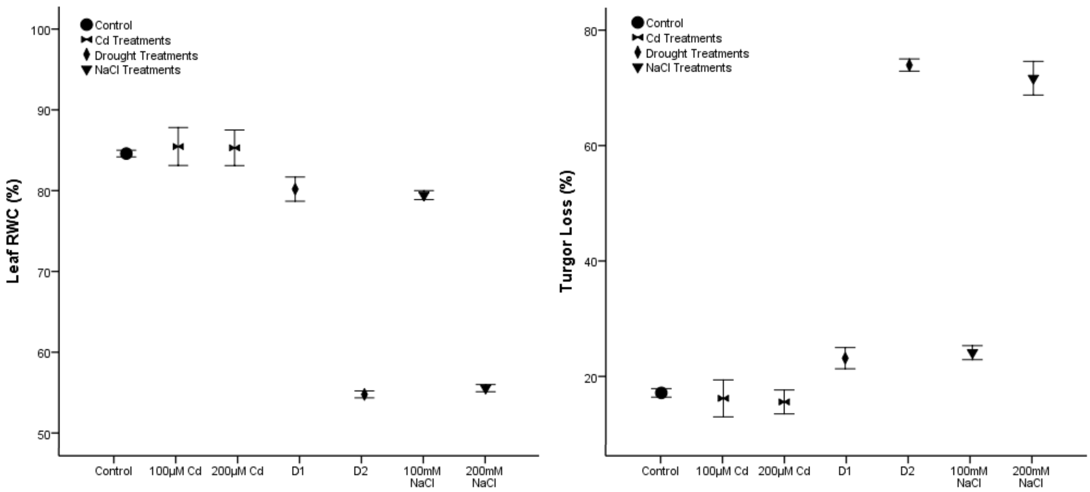

Figure 2. *Leaf relative water content (RWC, %) and turgor loss (%) in Spinacia oleracea L. cv. Matador plants exposed to Cd, drought and salinity treatments. Data points represent means and standard errors (n=4).*

Chlorophyll content in spinach plants exposed to these abiotic stresses was shown in Table 1. In Cd treatments, chlorophyll content of spinach plants decreased with increasing Cd concentration ($p<0.05$). The results obtained in salinity and drought stress were similar each other ($p<0.05$, Table 1). Both types of stress were associated with dehydration stress and a clear correlation was not found between chlorophyll content and drought or salinity treatments in spinach plants.

Table 1. Chlorophyll content (SPAD value) in *Spinacia oleracea* L. cv. Matador plants exposed to Cd, drought and salinity treatments. Data points represent means and standard errors (n=4).

		Chlorophyll Content (SPAD Value)
	Control	39.91 ± 1.76
Cd Treatments	100 µM Cd	43.58 ± 3.96
	200 µM Cd	37.17 ± 2.95
Drought Treatments	D1	35.20 ± 2.80
	D2	40.27 ± 3.22
Salinity Treatments	100 mM NaCl	40.80 ± 2.46
	200 mM NaCl	48.03 ± 4.63

The changes in ion leakage (%) in spinach plants exposed to Cd, drought and NaCl treatments were given in Figure 3. Accordingly, all treatments increased ion leakage values in spinach plant ($p<0.05$). The highest membrane damage in spinach plants was achieved in D2-drought treatment. As the drought and Cd concentrations, the value of ion leakage also increased, but there was no difference between salinity treatments. Salinity concentrations used in the present study caused a 4-fold increase in ion leakage of spinach plants.

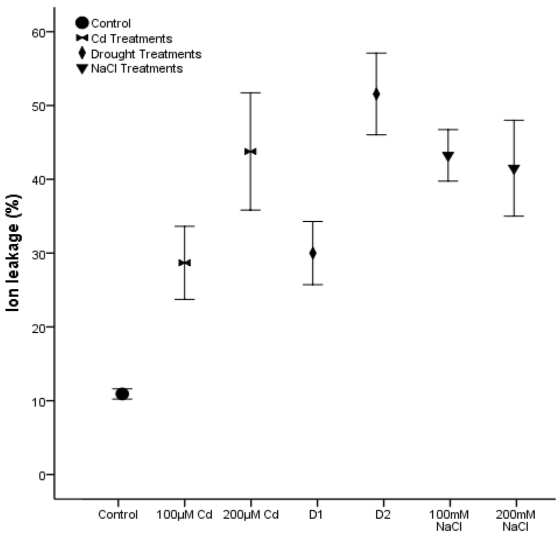

Figure 3. *Ion leakage (%) in Spinacia oleracea L. cv. Matador plants exposed to Cd, drought and salinity treatments. Data points represent means and standard errors (n=4).*

SOD and CAT activity values occurring in spinach plants exposed to Cd, drought and salinity stress were given in Figure 4. Both antioxidative enzyme activity increased significantly in all treatments of drought, salinity and Cd stresses. Although the highest value in SOD activity was obtained at D2-drought treatment and 200 mM concentration salinity treatment ($p<0.05$). The lowest values belonged to Cd treatments. In the Cd treatments, there were evoked a remarkable increase in CAT activity. This increase in CAT activity was 2-fold higher than in control ($p<0.05$).

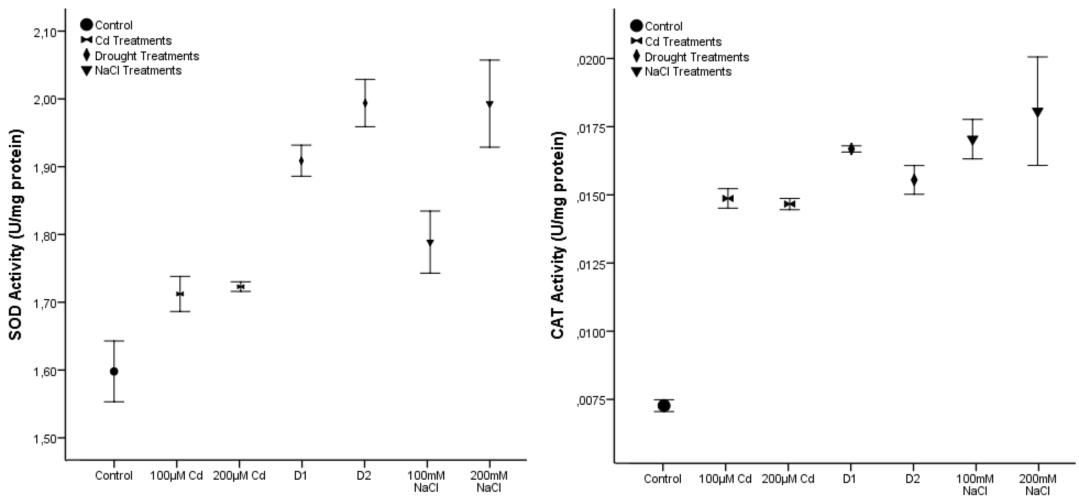

Figure 4. *Superoxide dismutase (SOD) and catalase (CAT) activity (U/mg protein) in Spinacia oleracea L. cv. Matador plants exposed to Cd, drought and salinity treatments. Data points represent means and standard errors (n=4).*

Discussion

Abiotic stresses have also crucial effect in spinach production, like in other agricultural products. These stresses are often drought, temperature extremes (heat, cold chilling/ frost), radiation (UV, ionizing radiation) and edaphic factors which include chemical (nutrient deficiencies, excess of soluble salts, salinity, alkalinity, heavy metal contaminants) in today (Minhas et al., 2017). Therefore, we observed effects of drought, salinity and Cd metal toxicity in *Spinacia oleracea* L. cv. Matador and determined it's responses to these stresses. It was demonstrated that although all treatments cause oxidative stress (Fig. 3), any decreases were not found in growth of *Spinacia oleracea* L. cv. Matador (Fig. 1). Hina et al. (2019) expressed that the 100 µM Cd concentration did not cause any changes in the dry or fresh weight of the spinach plant. However, it was shown that 200 µM Cd also gave similar results in the current study (Fig.1). It is understood that the *Spinacia oleracea* L. cv. Matador plants are resistant to short-term treatments of high Cd concentrations. Similarly, it was found in the study conducted by Bagheri et al. (2017) that the Cd treatment (40 µg $CdCl_2$/g soil) during 2 and 4 days did not cause any change in dry weight of spinach.

In salinity treatments in *Spinach oleracea* L cv. Matador, Seven and Sağlam (2020) was found a significant reduction in fresh weight of plants treated with 150 mM NaCl. However, a high reduction in weight (60%) of fresh weight of spinach plants was noted, as the study was conducted in a hydroponic environment and the plants were kept in maximum contact with the stressor in hydroponic conditions. In our study, an 8% reduction was obtained in the 200 mM salinity treatment. This decrease in 200 mM salinity treatment is similar to the results we obtained in both drought treatments. Reduced in growth under drought stress are caused by altered water relations. Because, in dry weight of spinach plants, statistically a significant decrease was not determined in any treatments (Fig. 1). Reduction in fresh weight of plants with green leaves due to drought stress were showed up after the 5th day of stress, as seen in some studies (Petropoulos et al., 2008; Bandurska and Jozwiak, 2010; Rahdari et al., 2012). As a matter of fact, water loss of spinach plants in the highest drought and salinity treatments was quite remarkable (Fig. 2). Decrease in the leaf RWC (%) of *Spinach oleracea* L. cv. Matador plants after salinity treatments was also recorded by Kaya et al. (2001) even in 60 mM NaCl treatment to *Spinach oleracea* L. cv. Matador. The basic physiology of salinity stress overlaps with drought stress (Mahajan and Tuteja, 2005). Decrease in leaf RWC is the most visible effect of drought (Jaleel et al., 2009; Farooq et al., 2009). Because they have been lead to cellular dehydration by causing osmotic stress. As a result, removal of water from the cytoplasm into the extracellular space have been resulting in turgor loss.

Measurements of ion leakage in leaf tissues are also contribute to revealing the damage caused by these stress factors. In our study, both salinity concentrations caused a 4-fold increase in ion leakage of spinach plants; when in drought and Cd treatments, ion leakage raised parallel to the increase in applied concentration (Fig. 3). This is an indicator of oxidative stress and the formation of large amounts of ROS (Mishra et al., 2006). It is known that increased ROS in cells induces membrane damage due to the removal of hydrogen from unsaturated fatty acids and impairs membrane stability (Blokhina et al., 2003). A similar increase for *Spinacia oleracea* L. cv. Matador plants was indicated by Kaya et. al. (2001) in 60 mM NaCl concentration.

Plants have the antioxidative defense system for cope with this damage (Lombardi and Sebastiani, 2005; Boojar and Goodarzi, 2007). Superoxide dismutase (SOD), which takes place in the first step in the antioxidant defense system, neutralizes free radicals by converting them into hydrogen peroxide and oxygen molecules. CAT enzyme is involved in the removal of H_2O_2 formed by SOD by converting it into water and oxygen (Upadhyay and Panda, 2009). Increased SOD activity shown in Figure 4 give important knowledge about *Spinach oleracea* L. cv. Matador's enzymatic antioxidative defense system. The SOD activity obtained in drought and salinity treatments is higher than Cd treatments in spinach plants (Fig. 4). This situation may be attributed that H_2O_2 production at high Cd concentrations originated from a number of non-enzymatic and enzymatic processes in different cellular compartments (Dixit et al., 2001; Schützendübel et al., 2002). As a matter of fact, the increase in CAT activity of *Spinach oleracea* L. cv. Matador under Cd stress also indicate it (Fig. 4). In this case, it can be considered that CAT is primarily scavenger of excess H_2O_2 in *Spinach oleracea* L. cv. Matador under Cd stress. Chloroplastic and mitochondrial electron transport chains are main ways in ROS production. Free radical generation is increase via these ways in drought and salinity stress conditions (Noctor et al., 2014). However, it was seen in this study that the formation of free radicals in spinach plants under Cd stress come from different sources. At the same time, Wang et al. (2010) stated that the defense mechanisms of the plant are effective in SOD / CAT cycle in short-term stress. As much as that we have observed this situation in drought and salinity treatments to the spinach plants, but did not see in the Cd treatments (Fig. 4).

It is seen from Table 1 that salinity and drought treatments do not cause structural damage in chloroplasts. It was also reported by Zaeifizade and Goliov (2009) that the amount of chlorophyll content and superoxide dismutase (SOD) in drought tolerant cultivars increases during drought stress. For this reason, the *Spinacia oleracea* L. cv. Matador plants can be tolerant to these short-term drought stresses. Cd treatments cause a decrease in the chlorophyll content of spinach plants (Table 1). Since Cd is a toxic metal (Karapınar and Kılıçel, 2020), it affected negatively the chlorophyll content in high concentrations. Cd is also known to reduce chlorophyll content in different plant species (Mishra et al., 2006; Mohan and Hosetti, 2006; Arslan et al., 2014). Pinto et al. (2017) also stated that Cd causes a decrease in the chlorophyll content of spinach plants. However, their Cd treatment was in hydroponic water culture and more low concentration than our study.

Conclusion

In this study, spinach (*Spinacia oleracea* L., cv. Matador) was exposed to drought, salinity and Cd stresses at increasing concentrations under same conditions. According to our results, plant dry weight was not changed statistically in applied stresses. In fresh weight, decreases were observed related to the change in water content. All treatments in this study, caused oxidative stress in the spinach plants. However, it was shown that the *Spinacia oleracea* L., cv. Matador plants can cope with this stress. As a matter of fact, we have seen in our results that chlorophyll content and dry weight did not change at severe drought and salinity concentrations despite the increased SOD and CAT activity. Thus, the *Spinacia oleracea* L. cv. Matador plants can be tolerant to these short-term drought stresses. It is important to know the physiological responses of spinach plants, which is widely consumed by humans, to stress conditions, which are today's basic problems such as drought, salinity and heavy metal pollution. The applied stress conditions are the main problems experienced today and are expected to increase even more in the coming years. The different responses of spinach seedlings to various stress factors provide an estimate of the plant's powerful physiological mechanism. Therefore, current study provides important information to the literature in order to enlighten a current issue. It is recommended to conduct molecular studies and to investigate of the cellular responses to long-term stress on *Spinacia oleracea* L. cv. Matador plants, which we see to be tolerant to short-term stresses.

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