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Physiological and molecular responses of roots differ from those of leaves in spinach plants subjected to short-term drought stress

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ABSTRACT

In this study, physiological responses as well as changes in expressions of specific proteins (dehydrin [DHN] and phospholipase Dα1 [PLDα1]) were determined in leaf and root tissues of *Spinacia oleracea* L.cv. Matador plants under different levels of drought stress. Spinach plants grown in the plant growth chamber were exposed to two levels of drought stress (Moderate Stress [MS]: 50% Field Capacity [FC] and Severe Stress [SS]: 25% FC) and compared with no stress conditions (Control: 100% [FC]) for a period of 10 days. Results revealed that the roots and leaves of spinach plants responded differently to drought stress, probably due to different antioxidant activities and accumulation of specific proteins (DHN and PLDα1). Moderate or severe drought stress did not alter the oxidation parameters in leaves of *S. oleracea* L. cv. Matador plants while significant changes associated with oxidative stress were observed in roots. Dehydrin polypeptides (75 and 50 kDa for leaves and 75 kDa for roots) and PLDα1 polypeptides (22 kDa in leaves; 52 kDa and 28 kDa in roots) have been observed to accumulate following drought exposure. The accumulation of these polypeptides was associated with physiological responses of spinach plants which provide evidence for their contribution to the acclimation process in early drought stress. These data suggest that tissues of spinach plant respond differently against different levels of drought stress and that the response is associated with altered expressions of DHN and PLDα1 polypeptides.

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1. Introduction

Drought stress has become important today due to the increasing global warming as well as declining nature and quantity of water resources around the world. Drought stress is one of the most important environmental factors limiting the cultivation of plant species or varieties. The water supply to the roots is limited or the loss of water through transpiration is very high in drought conditions (Anjum et al., 2011). According to Kirkham (2005), the primary characteristics of plant water relations include relative water contents (RWC), leaf water potential, osmotic potential, pressure potential, and transpiration rate, which are significantly affected by water deficit. Therefore, in drought stress, alterations in metabolic activities (Dinakar et al., 2012; Bravo et al., 2016), reduction in leaf size and stem extension (Farooq et al., 2009) as well as ionic imbalance (Tanveer et al., 2019) may be observed. Responses of plants to drought stress include multiple factors that play a role in the protection of cellular or/and physiological integrity of vegetative tissue and repair of damage caused during drought stress (Oliver et al., 2000; Vicre et al., 2004; Dinakar et al., 2012; Bravo et al., 2016). Plants can adapt to these stress conditions by producing certain metabolites, proteins, enzymes, and by modulating gene expression (Phukan et al., 2014). Acclimation occurs when the plant adjusts itself in physiological, anatomical or morphological manner in order to improve performance or survival in response to stress. These adjustments are usually changeable over a short-term as well as a long-term basis. The complex biochemical mechanisms involved in these processes are diverse in the length of time that plants are exposed to stress. Studies relating to metabolic processes containing the short-term endogenous regulation in plants are important for understanding the long-term structural acclimation to drought stress. Thus, short-term stress responses of plants should be investigated comprehensively, paying special attention to defense systems of cultivated crops. Short-term drought responses include physiological feedback mechanisms for processes and modifications that enable crops to develop in water-limited circumstances and recover quickly after stress termination without suffering yield loss. Therefore, there is a need to generate more information to improve adjustment to drought and maintain the yield of crops with short-term drought studies. At the same time,

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investigating the responses of root and leaf parts during plant exposure to drought stress is important to understand the physiological processes or mechanisms present during drought stress. The first organs to experience drought stress are the roots and they exhibit a higher drop in growth than shoots. Even, it was stated that leaves of different ages under drought stress showed different stomatal conductance and osmotic potential adjustment responses (AbdElgawad et al., 2016). Thus, further studies regarding differences in oxidative stress and antioxidant defenses, in different organs exposed to drought stress and developmental stages are required.

The antioxidative defense system is one of the most important defensive mechanisms at the cellular level of plants against increased levels of Reactive Oxygen Species (ROS) under stress conditions. ROS are produced naturally in plant cells during the metabolic process, but an excessive amount of ROS must be neutralized to prevent damage to cellular organelles and components, such as DNA, proteins, lipids, and carbohydrates (Raja et al., 2017). Minimization of ROS levels in the cell requires redox reactions consisting of different enzyme activities (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, etc.) and non-enzymatic antioxidants (glutathione, α-tocopherol, ascorbate, etc.) (Mittler, 2017). Deleterious ROS formation and antioxidative responses produced at the cellular level during drought stress vary according to plant organs. Results revealed by Signorelli et al. (2013) showed significant changes between plant parts in ROS metabolism, and in comparison with leaves, roots were more susceptible to drought stress. Effective antioxidative responses under these conditions play a major role in stress tolerance of both leaves and roots in many crop species (Al Hassan et al., 2015).

Stress-induced changes are also associated with altered expressions of several genes and their protein products (Allagulova et al., 2003). For example, osmotically active compounds including hydrophilic proteins such as dehydrin (DHN) proteins have an important role in the plant cell responses to various types of stress including dehydration (Kosova et al., 2008; Cansev et al., 2009). Dehydrins are called type II LEA (Late embryogenesis abundant) proteins (Liu et al., 2017) which have a crucial role in maintaining ROS homeostasis against drought, low temperature and salinity stress (Allagulova et al., 2003; Riyazuddin et al., 2022). The accumulation of DHN polypeptides is one of the major components of plant adaptation to these environmental conditions (Yu et al., 2018). Dehydrins can be located in compartments of cells such as the nucleus, mitochondria, chloroplasts, and near the plasma membrane. Although the exact role of dehydrins in the plant is still investigated, studies have shown that such physiological responses as membrane protection, protection from reactive oxygen species, maintenance of photosynthesis rate and prevention of photosynthetic pigments degradation, accumulation of compatible solutes such as proline and soluble sugars, prevention of misfolding of the stress-induced denatured proteins that occur during stress conditions are associated with the presence of dehydrins (Graether and Boddington, 2014; Riyazuddin et al., 2022).

The excessive increase in levels of ROS formed during drought stress is a leading cause of cell membrane damage. Phospholipase D (PLD) is a second messenger that has a regulatory role in stress conditions (Meijer and Munnik, 2003). PLD mediates membrane rearrangement and re-modeling events in order to prevent changes in membrane fluidity and osmotic balance caused by stress (Bargmann and Munnik, 2006). PLDs in plants are subdivided into six classes; the α- and δ- classes of PLDs are the most abundant ones which are linked to dehydration, freezing, wounding and salt stresses (Bargmann and Munnik, 2006; Hong et al., 2016). PLDα1 alters the ratios of membrane lipids in adaptation to the stress condition (Wang, 2005). At the same time, PLDα1 mediates the abscisic acid (ABA) signal transduction for maintaining hydration status in leaves (Zhang et al., 2004).

In this study, we aimed to determine the physiological responses and expressions of specific proteins at different levels of drought stress in spinach (*Spinacea oleracea* L. cv. Matador) plants. For this purpose, spinach seedlings grown under the controlled conditions were exposed to two levels of drought stress including moderate stress (50% Field Capacity [FC]) and severe stress (25% FC) conditions during the short-term period (10 days) and data were compared with no stress conditions (Control; 100% FC). Total soluble protein (TSP) contents as well as expressions of dehydrins (DHN) and phospholipase Dα1 (PLDα1), the stress-associated polypeptides, were examined. In addition, the extent of oxidative stress (hydrogen peroxide [H_2O_2] and malondialdehyde [MDA] contents) and the antioxidative defense system (superoxide dismutase [SOD] and catalase [CAT]), chlorophyll and relative water contents as well as turgor loss was determined. To the best of our knowledge, besides physiological findings, our study provides the first data with regard to involvement of DHN and PLDα1 proteins in spinach plants in response to different drought conditions. In addition, a number of physiological responses produced by leaf and root tissues in spinach plants exposed to moderate (MS) or severe (SS) drought stress have been presented. Studying the behavior of spinach plants in response to drought will provide us with valuable information for future physiological studies changes such as protein and/or enzyme profiles to understand and explain the mechanisms of drought tolerance.

2. Material and methods

2.1. Plant material and experimental design

Seeds of *Spinacia oleracea* L. cv. Matador were planted in 72-cell plastic trays containing seedling medium (Klasmann Rec119 Potgrond H) and grown for 4 weeks. Seedlings were then transferred to pots (14 × 12 cm) containing a mixture of peat/perlite (1:1) and grown in plant growth chambers (16 h photoperiod, 1200 lux at 24 °C/20 °C [day/night]) for an additional 4 weeks. Actagro (7-7-7) Nutrient Solution was used to irrigate growing seedlings. Drought stress was induced in 8 week-old plants (10–14 true leaves stage) by the gravimetric method (Samarah et al., 2009) by exposing the plants to moderate stress (MS: 50% FC) or severe stress (SS: 25% FC) for 10 days (Kovar and Olsovska, 2020) while control plants received no stress (100% FC). Pots were irrigated at 2-day intervals. On completion of experiments, leaves of plants in each pot were scored for rolling followed by analyses of chlorophyll content, relative water content and turgor loss. Plants were then harvested, their leaves and roots were frozen in liquid nitrogen and kept at −80 °C until further analysis.

2.2. Leaf rolling score

The scoring of leaf rolling was done through visualization using the scale of 1–3 according to Gana (2011). Score 1 indicates the leaf is not rolled up (control plants), score 2 means rolled shaped like a reversed V letter, and score 3 indicates rolled shaped like leaves margins touching.

2.3. Chlorophyll assay

Leaf chlorophyll contents of spinach plants were determined by using a portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Japan) and chlorophyll values were given as SPAD value. Measurements were performed on fully expanded leaves and reported as the average of 10 measurements from each plant.

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

Relative water contents of leaves were measured according to the method described by Arefian and Shafaroudi (2015). Leaf discs with 1 cm diameter were weighed initially (fresh weight, FW) and then placed in a petri dish containing deionized water for 4 h and weighed once more (turgid weight, TW). To examine the dry weight (DW), leaf discs were weighed after oven drying for 48 h at 70 °C. Leaf RWC and turgor loss were estimated as follows:

$$\mathrm{RWC} = \left[\frac{\mathrm{FW}-\mathrm{DW}}{\mathrm{TW}-\mathrm{DW}}\right]100,\ \text{Turgor loss} = \left[\frac{\mathrm{TW}-\mathrm{FW}}{\mathrm{TW}}\right]100$$

2.5. Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide was measured spectrophotometrically according to Alexieva et al. (2001). The reaction mixture consisted of 0.1% trichloroacetic acid (TCA, 0.5 mL), plant samples, 100 mM K-phosphate buffer (0.5 mL) and KI reagent (1 M KI w/v in fresh dd-water H_2O, 2 mL). The blank consisted of 0.1% TCA in the absence of samples. The mixtures were incubated for 1 h in dark and absorbance of samples was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve with H_2O_2 concentrations.

2.6. Lipid peroxidation

In determination of lipid peroxidation, malondialdehyde (MDA) content was defined by thiobarbituric acid (TBA) reaction according to Heath and Packer (1968) with some modifications. Leaf and root samples were homogenized in (0.5 mL) 0.1% (w/v) trichloroacetic acid (TCA) and then was centrifuged (for 10 min at 15.000 g). The reaction mixture (0.5 mL from the supernatant, 1.5 mL from the mixture of 20% TCA and 0.5% TBA) was added and the samples were centrifuged (15 000 g, 4 °C, 5 min) following incubation at 95 °C for 30 min. The absorbance was measured at 532 and 600 nm. Extinction coefficient (ε) used in calculation was 155 mM^{-1} cm^{-1} (Kwon et al., 1965).

2.7. Superoxide dismutase (SOD) and catalase (CAT) activities

In antioxidative enzymatic activity assay for determining SOD and CAT activities; plant extraction was performed according to the method by Ardıç et al. (2009). Samples were homogenized with buffer solution (50 mM Na-phosphate buffer [pH 7.8], 2% polyvinylpolypyrrolidone [PVP; w/v], 1 mM EDTA) in an ice bath. Then they were centrifuged at 14.000 g for 40 min at 4 °C. The supernatants were used in SOD activity analyses.

The SOD activity was determined according to the method of Beauchamp and Fridovich (1971) using SOD standard from bovine erythrocytes (SOD S7446, Sigma-Aldrich, USA). This method is based on the inhibition of the nitroblue-tetrazolium at 560 nm. SOD activity was defined according to the linear equation which was obtained from the curve after the calculation of% inhibition, and was expressed as units per mg protein (U/mg protein).

CAT activity was assayed according to the method of Lester et al. (2004) with minor modifications. 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 min spectrophotometrically. CAT activity was expressed as units per mg protein (U/mg protein).

2.8. Total soluble protein (TSP) content

TSP extraction was made using the procedure determined by Arora et al. (1992) with modifications according to Eris et al. (2007). Samples were ground in a mortar with liquid nitrogen. Then, 1 g leaf sample were homogenized in borate buffer (pH 9.0, 50 mM ascorbic acid, 50 mM sodium tetraborate, 1 mM phenylmethylsulphonylfluoride [PMSF],%1 β-mercaptoethanol) and insoluble PVPP paste made with this buffer using a 1:2:5 (tissue: PVPP paste:buffer) extraction ratio at 4 °C. Then samples were shaken on a gyratory shaker (15 min at 4 °C) followed by centrifugation for 1.5 h at 26 000 g. The supernatant was filtered with 0.4 and 0.2 μm filters (Millex; Millipore Co., Bedford, MA, USA). Protein content was measured according to the Bradford assay method (Arora and Wisniewski, 1994).

2.9. Western blot analysis

Proteins were precipitated according to the method reported by Lim et al. (1999), by adding TCA (100 ml/ml) to the supernatant. Samples were incubated for 30 min at 4 °C, then centrifuged (16.000 g for 30 min at 4 °C). Firstly, pellets were washed (3 times with cold acetone) and re-centrifuged. After the first acetone wash, they were broken physically using a sealed pipette tip. Dried pellets were resuspended in the sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) sample buffer containing 65 mM Tris-HCl, 20 mg/ml (w/v) SDS, 100 ml/ml (v/v) glycerol, pH 6.8 and 50 ml/ml b-mercaptoethanol with Bromophenol Blue. Discontinuous SDS-PAGE was made using 0.04 stacking gel and 0.125 separating gel with a PROTEAN III vertical electrophoresis unit (Bio-Rad, Hercules, CA, USA). Approximately 30 mg total protein for each sample was loaded, then gels were stained with Coomassie Brilliant Blue G-250. For immunoblotting, proteins from unstained gels were separated and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, USA). The remaining binding sites were blocked with 0.03 gelatin solved in Tris-buffered saline Tween-20 (TBST) for 30 min. After rinsing with TBST buffer, membranes were immersed in TBST solution containing a 1:1000 dilution of a polyclonal dehydrin antibody (AGRISERA, AS07 206). This antibody is directed to the synthetic peptide of the 19 amino acid (TGEKKGIMDKIKEKLPGQH, KLH-conjugated peptide sequence TGEKKGIMDKIKEKLPGQH of K-segment) consensus region of dehydrin family of proteins. For Phospholipase Dα1, TBST solution containing a 1:1000 dilution of a PLDα1 antibody (ACRIS, AP21377BT-N) were used. The membranes were incubated overnight and rinsed five times in TBST buffer, then blots were incubated for 1 h with the appropriate peroxidase-linked secondary antibody. After rinsing in TBST buffer with 10 mg/ml gelatin for five times, protein antibody complexes were defined and then visualized using the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ, USA) and Kodak X-AR film. Films were digitized with a Supervista S-12 scanner (UMAX Technologies, Freemont, CA, USA). Immunoreactive bands were compared densitometrically with the Public Domain NIH Image program (http://rsb. info.NIH.gov/nih-image/ (verified 28 July 2008)), and were expressed as arbitrary units.

2.10. Statistical analysis

The experiment was arranged in a randomized plot design with 3 replications consisting of four plants per replicate. Data were expressed as mean ± standard deviation of means. Statistical analyses were performed using SPSS 24.0 for Windows program. Comparisons between groups were made by ANOVA followed by post-hoc Tukey test. Significance level was set at $p < 0.05$.

3. Results and discussion

Plants tend to develop various adaptive mechanisms depending on the level of drought stress via complex morphological, physiological, biochemical and molecular changes (Farooq et al., 2009; Pandey and Shukla, 2015). Leaf morphological parameters such as leaf rolling have been reported as criteria reflecting acclimation responses to

Table 1
Leaf rolling score, RWC (%), turgor loss (%) and chlorophyll content of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. Data points represent means and standard deviations ($n = 4$). Means within a column followed by the different letters indicate significant differences at $p < 0.05$ related to drought level using one-way ANOVA followed by post hoc Tukey test.

	Leaf Rolling Score	RWC(%)	Turgor Loss(%)	Chlorophyll content (SPAD Value)
Control	1	82.70 ± 1.27^{a}	16.30 ± 1.20^{c}	55.13 ± 2.28^{b}
MS	2	73.09 ± 5.44^{b}	34.17 ± 9.03^{b}	58.86 ± 1.81^{ab}
SS	3	58.91 ± 3.09^{c}	37.80 ± 3.65^{a}	62.22 ± 1.61^{a}

avoid drought stress (Saruhan et al., 2012; Pandey and Shukla, 2015). In the present study, drastic changes like wilting were not observed among the application groups exposed to drought stress. However, increasing degrees of leaf rolling were detected in response to MS and SS (Table 1). Leaf rolling is a water-saving regulatory mechanism (Sirault et al., 2015) which leads to reduced transpiration, leaf dehydration and light interception under drought stress. Moreover, it is accepted as a stress avoidance mechanism for plants (Kadioglu et al., 2012; Pandey and Shukla, 2015). Therefore, our data indicated that spinach plants employed a regulatory mechanism to avoid stress by leaf orientation changes within short term depending on the severity of drought stress. Table 1 shows the alterations that occurred in chlorophyll content of *S. oleracea* L. cv. Matador plants subjected to different levels of drought stress. Our results revealed that leaf chlorophyll content increased significantly ($p < 0.05$) by SS, but not by MS, application compared with control group. The chlorophyll content is negatively affected by various stress factors (Guo et al., 2016), but these alterations vary according to the plant species and the severity and duration of the stress (Demirevska et al., 2009). For example, short-term drought stress in lettuce caused increased chlorophyll content on day 4, while decreasing on day 8 (Shin et al., 2021). This case might be caused by a state in which an organism's response to an abiotic stressor varies with its level of exposure. Thus, stress levels may either elicit a stimulatory/beneficial response or could cause inhibition/toxicity (Calabrese and Baldwin, 2003; Costantini et al., 2010). Therefore, the increased chlorophyll content observed in our study might have occurred as a tolerance mechanism against short-term drought stress.

Average leaf relative water contents (RWC) of spinach plants in control group (82.70%) were reduced significantly by exposure to MS (73.09%; $p < 0.05$) or SS (58.91%; $p < 0.05$) (Table 1). Our findings demonstrate that RWC is maintained in spinach plant leaves under short-term, severe drought stress at a minimum of about 60%. The RWC is a reliable indicator reflecting drought response (Khanna et al., 2014). The capacity of a plant to maintain RWC during drought stress is an important strategy for acquiring resistance to drought (Selote and Khanna-Chopra, 2006). Drought stress is also reflected by loss of turgor in leaves of plants due to water loss from cells. Consistently, we found in this study, increased turgor loss (%) in leaves of spinach plants from 16.30% to 37.80% following SS ($p < 0.05$, Table 1). Tolerance of plants to water deficiency is related with the maintenance of RWC ability which varies within plant species (Bravo et al., 2016). While drought damage occurs by only 10% water loss in some plants, others can survive at extreme desiccation levels up to 90% (Gechev et al., 2012).

Drought is considered to be caused primarily by osmotic stress, resulting in the disruption of homeostasis balance and ion distribution in the cell (Zhu, 2001; Ors and Suarez, 2017). This can cause oxidative stress in plant cells and, if it increases further, cellular damage. Thus, the amount of hydrogen peroxide (H_2O_2), one of the common ROS, was also analyzed in our study (Fig. 1B). While MS or SS caused significant increases in H_2O_2 amounts (5.87 ± 0.45 and 6.50 ± 0.24 μmol/g fresh weight, respectively) compared to Control group (4.55 ± 0.43 μmol/g fresh weight) in the roots, H_2O_2 amounts in leaves of spinach plants exposed to two drought stress levels were increased only by SS (15.25 ± 2.33 μmol/g fresh weight; $p < 0.05$) application. Two-way ANOVA revealed a significant effect on drought stress levels, plant organs but not the interaction of drought stress levels, plant organs on H_2O_2 content (Table 2). The ROS formed during stress conditions may either play a signaling role or become cytotoxic (Huang et al., 2019). MDA is the end product of lipid peroxidation and is one of the indicator molecules of oxidative stress that occurs in case the ROS becomes cytotoxic (Morales and Munné-Bosch, 2019). Our study provides evidence for cell membrane damage demonstrated by analyses of MDA content in roots of spinach plants under short-term drought stress. Changes in MDA content of spinach plants under varying drought stress levels were given in Fig. 1A. According to our results, MDA contents in the roots were increased by MS (0.92 ± 0.13 μM/g fresh weight; $p < 0.05$) or SS (1.33 ± 0.14 μM/g fresh weight; $p < 0.05$) compared to control group (0.57 ± 0.08 μM/g fresh weight), although no significant differences were found for MDA levels among the experimental groups in the

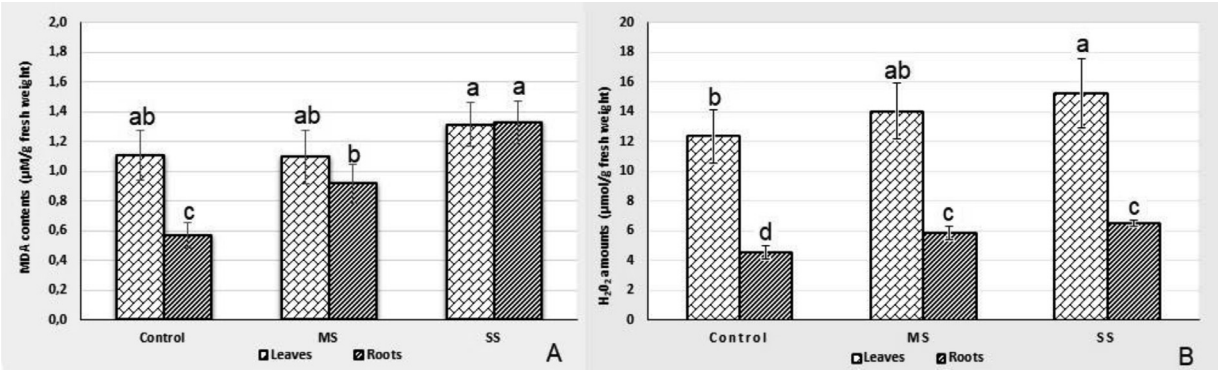

Fig. 1. Malondialdehyde (MDA) contents (μM/g fresh weight) (A) and H_2O_2 amounts (μmol/g fresh weight) (B) in the leaves and roots of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. Data points represent means and standard deviations ($n = 4$). Different capital letters (for leaves) and lower case letters (for roots) indicate significant differences at $p < 0.05$ related to drought level by post hoc Tukey test.

Table 2
Results of analyses of variance (two-way ANOVA) of drought stress levels (DSL), plant organs (PO), and their interactions (DSL x PO) for malondialdehyde (MDA) content, hidrogen peroxide (H_2O_2) content, superoxide dismutase (SOD) activity, catalase (CAT) activity and total soluble protein (TSP) content. Numbers represent F values at 0.05 level.

Dependent variable	Independent variable		
	DSL	PO	DSL x PO
MDA content	21.431 ($p < 0.01$)	29.603 ($p < 0.01$)	27.946 ($p < 0.01$)
H_2O_2 content	32.986 ($p < 0.01$)	1172.301 ($p < 0.01$)	0.917 ($p = 0.418$)
SOD activity	35.575 ($p < 0.01$)	92.107 ($p < 0.01$)	31.016 ($p < 0.01$)
CAT activity	3.061 ($p = 0.072$)	75.398 ($p < 0.01$)	12.156 ($p < 0.01$)
TSP content	150.709 ($p < 0.01$)	4726.931 ($p < 0.01$)	159.485 ($p < 0.01$)

leaves. Two-way ANOVA revealed a significant effect on drought stress levels, plant organs, and the interaction of drought stress levels, plant organs on MDA content (Table 2).

Our data suggest that oxidative damage occurred in roots of spinach plants exposed to short-term moderate or severe drought. It is clear that water deficiency in the rhizosphere of plants leads to enhanced ROS generation in the roots of spinach. Under stress conditions, peroxidation of the cell membrane caused by high ROS levels leads to MDA production, which is followed by impaired stability and permeability of the cell membrane (Sairam et al., 2001; Sharma et al., 2012). On the other hand, SS drought stress increased H_2O_2 content in leaves but it did not result in an increase in MDA. As a result, during short-term drought stress, oxidative stress was observed while oxidative damage did not occur in the leaves under SS.

Tissue damage involving enhanced ROS contents may be mitigated by activation of members of free radical scavenging system such as the antioxidant enzymes SOD and CAT, which play a protective role in removing ROS and maintaining redox homeostasis in order to provide drought tolerance (Hameed et al., 2013; Fan et al., 2014). The SOD enzyme takes part in the first step of the enzymatic defense system that can be activated in the plant in case of oxidative stress (Alscher et al., 2002). In our study, we found that SOD activity in the roots of *S. oleracea* L. cv. Matador plants was increased significantly by MS (1.11 ± 0.14 U/mg protein; $p < 0.05$) or SS (2.00 ± 0.35 U/mg protein; $p < 0.05$) compared to control group (0.68 ± 0.04 U/mg protein), although no significant difference was found for SOD activity among the experimental groups in the leaves (Fig. 2A). Two-way ANOVA revealed a significant effect on drought stress levels, plant organs, and the interaction of drought stress levels, plant organs on SOD activity (Table 2). On the other hand, SS ($p < 0.05$) but not MS significantly enhanced CAT activity in the roots of spinach plants compared to control group (Fig. 2B). CAT activity is essential for the removal of H_2O_2 produced in the peroxisomes and cytosol (Noctor et al., 2000). Our data suggest that activities of SOD and CAT enzymes in roots of spinach plants are enhanced in response to increased ROS content due to drought stress for providing a protective role in ROS scavenging. However, CAT activity in leaves was surprisingly reduced in MS and SS applications compared to the control group ($p < 0.05$). Two-way ANOVA revealed a significant effect on drought stress levels, plant organs, and the interaction of drought stress levels, plant organs on CAT activity (Table 2). Although many abiotic stresses like drought stress can increase the photorespiratory production of H_2O_2 and emerge to CAT activity inhibition, hence decreasing its activity (Voss et al., 2013), CAT inhibition and the physiological role of that alteration remain unclear (Luna et al., 2005; Sousa et al., 2015). Our results show no oxidative damage in leaves via other many parameters such as SOD, MDA, and chlorophyll contents. Although H_2O_2 content in leaves of spinach was enhanced under SS application, but not by MS application probably because other peroxisomal peroxidases that are capable of overlapping with CAT activity under such stressful conditions may have been effective in the removal of H_2O_2. To date, there is no consensus on whether different proteins of the peroxisomal redox network can compensate for each other or not (Narendra et al., 2006; Sousa et al., 2015, 2019). H_2O_2 content in leaves may have been involved in signaling functions because CAT serves to limit excessive H_2O_2 accumulation (Schroeder et al., 2001; Kohler et al., 2003).

In our study, increases in total soluble protein (TSP) contents of leaves as well as roots were detected in SS application ($p<0.05$; Fig. 3). Two-way ANOVA revealed a significant effect on drought stress levels, plant organs, and the interaction of drought stress levels, plant organs on TSP content (Table 2). Several stress-induced proteins have been identified in plant species and accumulate in response to heat, cold, waterlogging, drought and salt stress (Ashraf and Harris, 2004). For this reason, an increase in the content of TSP is thought to be associated with the synthesis of certain stress proteins. Ashraf and Harris (2004) suggested that certain proteins could be considered as stress-tolerance indicators depending on the nature of the plant species or cultivar. Thus, dehydrin (DHN) proteins in spinach were also investigated in this study (Figs. 4 and 5). Dehydrin proteins are stress-inducible proteins as well as they are constitutively present with a broad distribution in various tissues of plants (Kosova

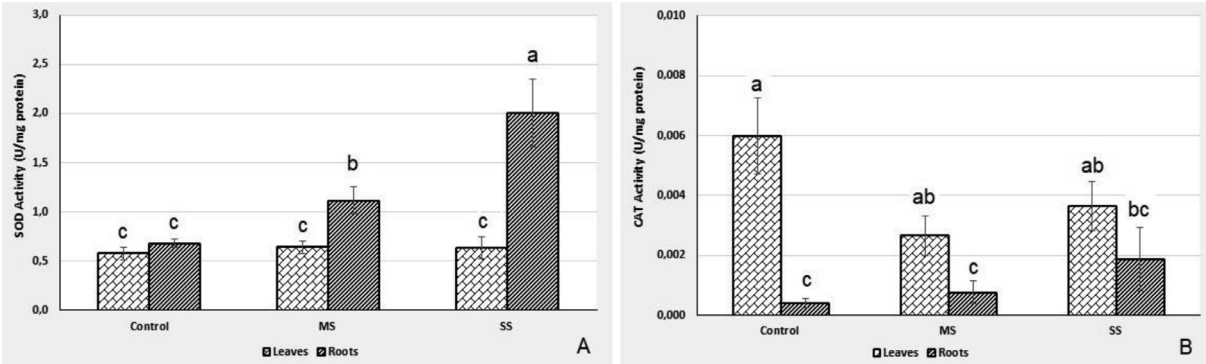

Fig. 2. (**A**) Superoxide dismutase (SOD) (U/mg protein) activity and (**B**) Catalase (CAT) activity (U/mg protein) in the leaves and roots of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. Data points represent means and standard deviations ($n = 4$). Different capital letters (for leaves) and lower case letters (for roots) indicate significant differences at $p < 0.05$ related to drought level by post hoc Tukey test.

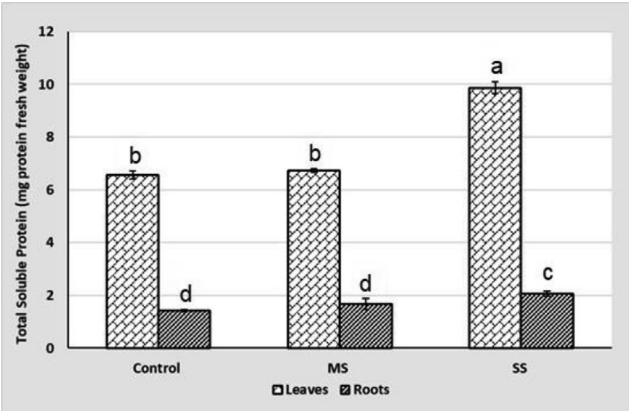

Fig. 3. Total Soluble Protein (TSP) (mg protein fresh weight) contents in the leaves and roots of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. Data points represent means and standard deviations (n = 4). Different capital letters (for leaves) and lower case letters (for roots) indicate significant differences at $p < 0.05$ related to drought level by post hoc Tukey test.

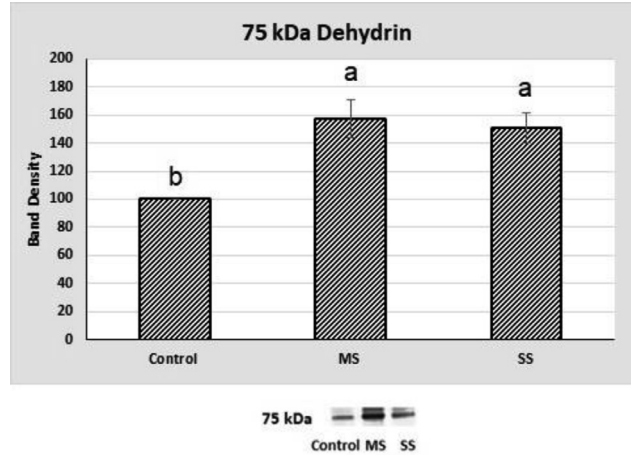

Fig. 5. Levels of dehydrin (DHN) proteins in the roots of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. Dehydrin 75 kDa were assayed by Western blot using total soluble protein homogenates of roots. Areas were expressed as arbitrary units and normalized as percentages of those generated in the same blot using samples from 100% FC of control plants. Data points represent means and standard deviations (n = 4). Different letters indicate significant differences at $p < 0.05$ related to drought level by post hoc Tukey test.

et al., 2008). Dehydrins are accumulated in response to stress caused by cellular dehydration in conditions such as drought, low temperature and salinity (Nylander et al., 2001; Allagulova et al., 2003; Yu et al., 2018). The accumulation of dehydrin proteins has been reported to increase the tolerance of the plant to drought stress (Pour-Benab et al., 2019). Dehydrin proteins occur in various polypeptide sequence and thus, it is important to determine which DHN polypeptides are accumulated under cellular dehydration conditions for developing new strategies in drought management. In the present study, expressions of 75 kDa and 50 kDa DHN polypeptides were significantly ($p < 0.05$) increased in leaves (Fig. 4) and expression of 75 kDa DHN polypeptides was significantly ($p < 0.05$) increased in roots (Fig. 5) of spinach plants exposed to short-term MS or SS. When the 75 kDa and 50 kDa DHN polypeptides in leaves of spinach were plotted against turgor loss, there was a linear and positive correlation in MS and SS applications (r^2: 0.99 and r^2: 0.96, respectively). In addition, there was a negative correlation compared to the plant relative water content (RLWC) (r^2: 0.86 and r^2:0.72, respectively). The presence of the DHN polypeptides in root tissues promotes the maintenance of water and molecule transport to the leaves via the vascular system for acclimation to drought by providing water influx into parenchymal cells of the root meristem (Vaseva et al., 2012). In present study, short-term drought exposure did not cause oxidative damage in the leaves since MDA contents of leaves were not changed significantly in MS and SS applications. Hence, our data support the association of these polypeptides with drought acclimation of spinach plants. Especially, the expression of 75 and 50 kDa dehydrin polypeptides for leaves might be considered to contribute the protection of membrane lipids against drought stress in spinach. DHN polypeptides may be observed in different molecular weights depending on the plant species as well as tissues and cell organelles as shown in a previous study by Malik et al. (2017) who stated that DHN polypeptides with various molecular masses are overexpressed during certain environmental stress conditions. The roots may be expected to respond differently to drought stress than leaves since drought exposure initially affects the roots. In the present study, we observed that MDA and H_2O_2 contents were increased in the roots subjected to short-term MS and SS while these increases did not affect the plants' survival. This observation suggests that the 75 kDa DHN polypeptides

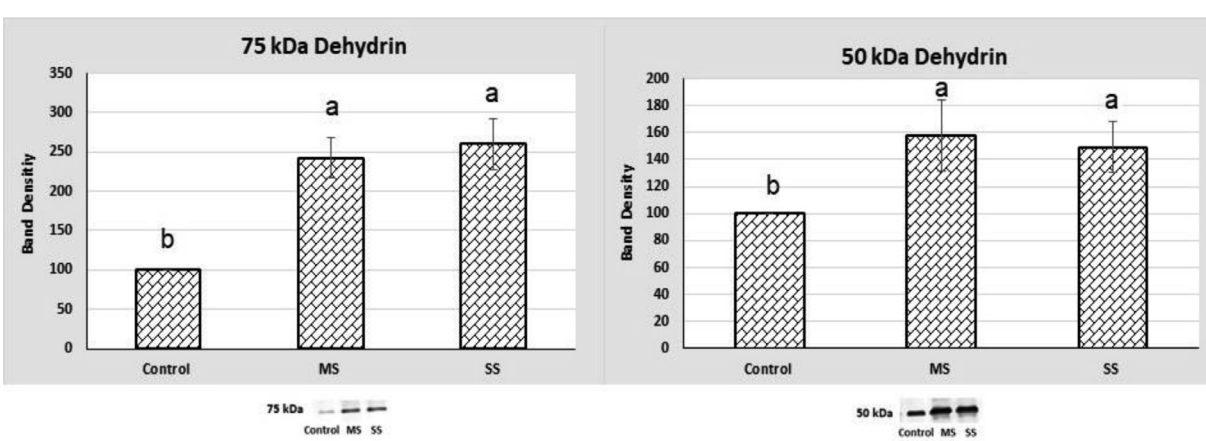

Fig. 4. Levels of dehydrin (DHN) proteins in the leaves of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. Dehydrin 75 kDa (A), 50 kDa (B) were assayed by Western blot using total soluble protein homogenates of leaves. Areas were expressed as arbitrary units and normalized as percentages of those generated in the same blot using samples from 100% FC of control plants. Data points represent means and standard deviations (n = 4). Different letters indicate significant differences at $p < 0.05$ related to drought level by post hoc Tukey test.

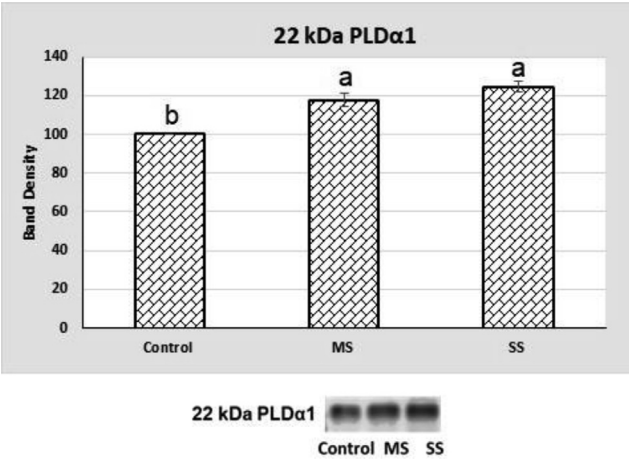

Fig. 6. Levels of phospholipaseDα1 (PLDα1) polypeptides in the leaves of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. 22 kDa PLDα1were assayed by Western blot using total soluble protein homogenates of leaves. Areas were expressed as arbitrary units and normalized as percentages of those generated in the same blot using samples from 100% FC of control plants. Data points represent means and standard deviations (n = 4). Different letters indicate significant differences at $p < 0.05$ related to drought level by post hoc Tukey test.

determined in the roots of spinach might have a protective role that limits the extent and severity of drought damage by stabilizing membranes and other drought sensitive systems.

The expression levels of phospholipase Dα1 (PLDα1) polypeptides in the *Spinacia oleracea* L. cv. Matador plants were given in Figs. 6 and 7. Phospholipase D(PLD) is a major family of phospholipase in plants and hydrolyses membrane phospholipids to phosphatidic acid (PA). The levels of PA in plant cells change according to various abiotic stress factors such as salinity, drought, wounding, cold, freezing, and pathogen attack and provide regulation by interacting specifically with their protein targets. Therefore, changes in PLD activity have been implicated in signaling and/or catabolic functions (Hong et al., 2008). ROS are continuously produced in cellular metabolism and plant cells have a scavenging system to protect from ROS toxicity. Although high ROS production on cellular components have harmful effects (Møller et al., 2007), low concentration of ROS in cells is firmly established to have a signaling function (Foyer and Noctor, 2009; Choudhury et al., 2017; Mittler, 2017). Within PLD isoforms, especially PLDα1 was shown to have a signaling function by promoting ROS production in response to drought stress (Guo et al., 2012) leading to decreased transpirational water loss by stoma closure (Zhang et al., 2009). In our study, it was observed that PLDα1 expression increased in spinach leaves, while MDA levels did not change statistically. This suggests that PLDα1 has a signaling function for spinach leaves during MS and SS. We found dramatic increases in levels of 22 kDa PLDα1 polypeptide ($p < 0.05$, Fig. 6) in leaves of spinach plant under drought stress which were correlated with increased turgor loss (r^2:0.96) and decreased RWC (r^2:0.72). It was stated by Hong et al. (2008) that PLDα1 expression increased in short-term drought stress and participate to maintain RWC via induction of stomatal closure in leaves of tobacco. Hence the increases in 22 kDa PLDα1 polypeptide can be considered to be associated with dehydration in spinach leaves and may contribute to the unchanged oxidation parameters, as well. 28 kDa PLDα1 polypeptide was significantly enhanced following both MS and SS ($p < 0.05$, Fig. 7B) in the roots while 52 kDa PLDα1 polypeptide only enhanced following MS ($p < 0.05$, Fig. 7A) which might be associated with increased catabolism of this isoform by the enhancement in the severity of drought stress deserving a detailed investigation.

4. Conclusion

We conclude that roots and leaves of spinach plants respond differently to drought stress, probably due to differences in antioxidant activities and accumulation of certain polypeptides which might be associated with stress acclimation. This study provided novel information with regard to the spinach plant's tolerance to drought stress and the physiological roles of dehydrin (DHN) and PLDα1 in relation to stress damage across different stages of drought stress. Varying soil desiccation in the *S. oleracea* L. cv. Matador plants did not cause structural changes like the chlorophyll or protein content, and oxidative damage. Furthermore, we observed that DHN and PLDα1 proteins are effective in the adaptation of the *S. oleracea* L. cv. Matador plants to different levels of water scarcity in short term. Hence, the roles of plant cellular and molecular components with regard to drought tolerance deserve further investigation. Future physiological studies investigating photosynthetic performance, accumulation of compatible osmolytes, alterations in plant growth regulators and inorganic ions would provide significant information with regard to mechanisms involved in response to drought stress in plants.

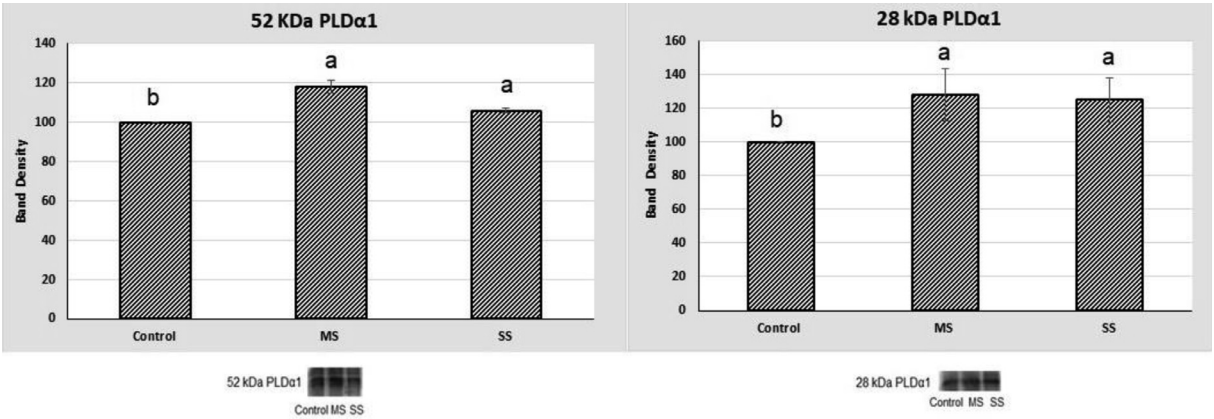

Fig. 7. Levels of phospholipaseDα1 (PLDα1) polypeptides in the roots of *Spinacia oleracea* L. cv. Matador plants under two drought stress levels [Moderate Stress (MS): 50% FC and Severe Stress (SS): 25% FC] and non-stress [Control: 100% Field Capacity (FC)]. 52 kDa (A) and 28 kDa (B) PLDα1 were assayed by Western blot using total soluble protein homogenates of roots. Areas were expressed as arbitrary units and normalized as percentages of those generated in the same blot using samples from 100% FC of control plants. Data points represent means and standard deviations (n = 4). Different letters indicate significant differences at $p < 0.05$ related to drought level by post hoc Tukey test.

Declaration of Competing Interest

No conflicts declared.

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