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DEHYDROABIETIC ACID MEDIATED *WRKY71* GENE EXPRESSIONS AND REACTIVE OXYGEN SPECIES REGULATION IN SOYBEAN UNDER SALINITY

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Dehydroabietic acid R=COOH (DHA), a naturally occuring diterpene resin acid, is an activator of systemic acquired resistance (SAR) under biotic stress. However, there had been no report on its functioning under salinity. In the present study, we determined the effects of DHA on salinity and its possible role as a signal transmitter in soybean (*Glycine max* L.) leaves under salinity (200 mM NaCl). Furthermore, physiological parameters, chlorophyll, proline, malondialdehyde (MDA), hydrogen peroxide (H_2O_2) content, superoxide (O_2^-) and hydroxyl radical ($^{\bullet}OH$) scavenge capacity, as well as antioxidant enzymes (SOD, POX, APX and GST) and *GmWRKY-71* gene expressions were investigated in the treated plants at 6 h, 12 h and 24 h. The obtained results showed that pretreatment of DHA caused (1) a reduction in salt-induced damage, (2) improvement in biomass yield, water status, chlorophyll and leaf area, (3) regulation of the proline level and relative electrolyte leakage, (4) increase in reactive oxygen species (ROS) scavenging capacity, (5) induction of SOD and APX enzyme activity at all the investigated periods, while POX only at 6 h, and thus alleviation of the oxidative damage. In addition, the changes in *GmWRKY-71* gene expressions were remarkable in soybean under salinity. To sum up, these results showed that DHA can be used as a ROS inhibitor or a signal molecule in increasing salt tolerance in soybean under salinity.

Keywords: dehydroabietic acid, *GmWRKY-71*, reactive oxygen species, salt stress, soybean

INTRODUCTION

Biological function of terpenoids in plants includes involvement in growth and development, interaction with other organisms, and stress response (Tholl, 2015). Diterpene resin acids are double or triple carboxylic acids of 20 carbons of several skeletal types that exhibit double bond isomers, diastereoisomers, and by way of additional functionalization (Shah, 2016). One of the diterpen family members, dehydroabietic acid R=COOH (DHA), is a naturally occurring resin acid (Ohwada et al., 2003). Firstly, dehydroabietic acid was synthesized by disproportionating pine resin and then reduced to dehydroabietinol (DA) (Gonzales et al., 2010). In recent years, dehydroabietinaline (diterpene) has been found to be an important signaling molecule in plants under biotic stress conditions (Chatuverdi al., 2012). Although certain physiological effects of dehydroabietic acid have been demonstrated in plant metabolism, there is no report on the detailed mechanisms (physiological, biochemical and molecular changes) under stress conditions (e.g., salinity, heat, cold).

It is a known fact that salt stress destroys plants' main metabolism. Salinity causes accumulation of reactive oxygen species (ROS) in plants. ROS are intermediate products of reduction reactions performed by energy or electron transfer to form H_2O in the form of single oxygen (O_2), superoxide molecule (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), perhydroxyl radical. In addition, ROS play critical roles in hormonal signaling, which subsequently plays an important role in plant development, in the change of cell wall polymer structure, in the mechanisms related to environmental perception of the plant, in gene expression, as well as in metabolic and physiological regulation (Swanson

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and Gilroy, 2010). Earlier studies reported that plants maintain ROS balance with enzymatic (SOD, POX, APX, CAT and GST) and non enzymatic (ascorbate, glutathione, dehydroascorbate) antioxidants (Siddiqui et al., 2019).

WRKY transcription factors function as important components in the complex signaling progresses during plant stress responses. Modifications of the expression patterns of *WRKY* genes and/or changes in their activity contribute to the elaboration of various signaling pathways, plant hormones and regulatory networks (Chen et al., 2012). Moreover, a single *WRKY* gene often responds to several abiotic stress factors at the same time and exhibits various regulatory functions during plant stress responses. Furthermore, *WRKY* genes are known to be one of the largest gene families of soybean plants. It has recently been found that 66 of 188 *WRKY* genes are transcriptionally regulated in soybean plants under salt stress. Interestingly, it has been determined that *WRKY-71* gene is inactive in most of these genes in soybean roots under salinity (Yu et al., 2016). However, in the literature, little is known about the association of *WRKY* genes with functional reactive oxygen species antioxidant enzymes, especially in soybean plants under salt stress.

In the light of this information, in this work, we initially investigated the effects of DHA on physiological, biochemical and molecular mechanisms under salinity, and the interaction between DHA and ROS, antioxidant enzymes, *WRKY-71* transcription in soybean plants under salt stress. We noticed that DHA was able to induce *WRKY-71*, antioxidant enzymes to cope with salinity by scavenging ROS.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND PLANT MATERIAL

Soybean (*Glycine max* L. Merr.) SA88 seeds were obtained from a commercial provider (Agrova, Adana, TR). The seeds were sown in plastic trays (10 cm × 14 cm) filled with soil under dark conditions. After germination, seedlings were placed into a growth chamber at 25°C with 16 h/8 h day/night photoperiod and light intensity of 500 μmol m^{-2} s^{-1} with Hoagland solution (1M $Ca(NO_3)_2$, 1M $MgSO_4.7H_2O$, 1M $NH_4H_2PO_4$, 1M H_3BO_3, 0.1M $CuSO_4.5H_2O$, 0.1M $ZnSO_4.7H_2O$, 0.1M MoO_3,1 M KNO_3, 1M $MnSO_4.H_2O$, 1M $FeSO_4.7H_2O$) for 2 weeks. DHA (dehydroabietic acid) was purchased from Sigma Chemical Co. ($C_{20}H_{28}O_2$). In the preliminary experiment, DHA was solved in 99.8% (v/v) 1ml ethanol and (0.206, 0.412, 0.826, 1.65 and 3.3 mM) sprayed on leaves for 3 days at 12 h interval. On the fourth day, the leaves that were exposed to dehydroabietic acid (0.206, 0.412, 0.826, 1.65 and 3.3 mM) and the controls were treated with 200 mM NaCl in Hoagland solution. However, 0.206, 0.412, 0.826, 1.65 mM did not change the MDA levels in them. Therefore, 3.3 mM was the most effective concentration with the lowest MDA level according to the control group in the previous studies at 6 h of stress treatment. Thus, in the main experiment, seedlings were pre-treated with a 3.3 mM concentration of dehydroabietic acid by spraying for 3 days at 12 h intervals. On the fourth day, the leaves that were exposed to dehydroabietic acid (3.3 mM) and the controls were treated with 200 mM NaCl in Hoagland solution. Totally, there were four different experimental groups: controls, DHA, NaCl, DHA + NaCl. After stress treatment, the roots and leaves were harvested at 6 h, 12 h and 24 h and stored at –80°C.

DETERMINATION OF PHYSIOLOGICAL PARAMETERS

Biomass yield. Growth parameters were examined after exposure to salt for 6 h, 12 h and 24 h, then the roots and shoots were harvested. The fresh weight (FW) of the roots and shoots was determined. For dry weight (DW) calculations, the shoots and roots were dried in an oven at 70°C for 48 hours and then weighed (Böhm, 1979).

Total chlorophyll content. The chlorophyll content of leaves was measured in accordance with the method specified by Lichtenthaler and Wellburn (1983). The pigments of 0.1 g fresh leaves were extracted in 80% (v/v) acetone. The absorbance of chlorophyll content was measured at 645 and 663 nm using 10S UV VIS spectrophotometry.

The equations used for the calculation are presented below:

Chlorophyll a (μg/ml) = 12.25 A663 – 2.798 A645
Chlorophyll b (μg/ml) = 21.5 A645 – 5.1 A663
Total Chlorophyll (μg/ml) = chlorophyll a + chlorophyll b

Leaf area. The leaf area was measured on the harvest day using a CID Bio-Science CI-201 portable laser leaf area meter. The data were collected

from triplet leaves of the plant. The calculations were made by the program contained in the device and the leaf area was calculated in cm^2.

Relative water content. The relative water content (RWC) was calculated in accordance with Smart and Bingham (1974). The leaves were floated on deionized water for 5 h in low irradiance; then the turgid tissue was quickly blotted to remove excess water, and the turgid weights (TW) were determined. DW was determined after seedlings had been dried in an oven at 70°C for 72 h, the time point at which a constant weight was reached. Relative water content was calculated using the folowing formula:

$$\text{RWC (\%)} = \text{FW} - \text{DW}/\ \text{TW} - \text{DW} \times 100$$

Relative electrolyte leakage. Leaf tissue was vibrated for 30 min in deionized water, followed by measurement of conductivity of bathing medium (C_1). The samples were boiled for 15 min and again the conductivity (C_2) was measured (Singh et al., 2008). The percentage of relative electrolyte leakage (REL) was determined using the following formula:

$$REL = (\frac{C1}{C2}\%)$$

DETERMINATION OF BIOCHEMICAL PARAMETERS

Malondialdehyde content. The level of lipid peroxidation in leaf samples was determined in terms of the malondialdehyde (MDA) content according to the method specified by Rao and Sresty (2000). The MDA content, an end product of lipid peroxidation, was determined using the thiobarbituric acid reaction. The MDA concentration was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. An extinction coefficient of 155 mM^{-1} cm^{-1} was used to determine the MDA concentration.

Hydrogen peroxide content. The H_2O_2 content was determined according to Velikova et al. (2000). Fresh leaves (0.1 g) were homogenized in 5 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 15 minutes. The supernatant (0.5 ml) was then mixed with 0.5 ml of buffer (10 mM potassium phosphate, pH 7) and 1 ml of 1M KI. The absorbance reading was taken at 390 nm.

Proline content. The proline content of the leaves was determined according to Claussen (2005). The absorbance of the reaction mixture was determined at 546 nm. The proline concentration was determined from a standard curve and calculated on fresh weight basis (μg proline g^{-1} FW).

Hydroxyl radical scavenging activity. The ability of fusiformis extracts to scavenge the hydroxyl radical generated by Fenton reaction was measured according to the modified method given by Kim et al. (1997), where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of resveratrol sample. The scavenging activity on hydroxyl radicals:

$$[(\text{A0} - \text{A1})/\text{A0} \times 100]$$

Superoxide radical scavenging activity. The scavenging ability of superoxide anion radical was evaluated by the method of negative staining technique (De Rosa et al., 1979). The absorbance of the reaction mixture was measured at 560 nm and the inhibition rate was calculated by measuring the amount of formazan that was reduced from NBT by superoxide.

Determination of antioxidant enzyme activity. All operations were performed at 4°C. For protein and enzyme extractions, 0.5 g of fresh leaf samples were homogenized in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.8). The samples were centrifuged at 14,000 × g for 40 min, and the supernatants were used for determination of the protein content and enzyme activities. The total soluble protein contents of the enzyme extracts were determined according to Bradford (1976) using bovine serum albumin as a standard. Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed based on its ability to inhibit the photochemical reduction of nitrotetrazolium blue chloride (NBT) at 560 nm (Beauchamp and Fridovich, 1973). Peroxidase (POX; EC 1.11.1.7) activity was determined according to the method of Herzog and Fahimi (1973). The ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). Glutathione-*s*-transferase (GST; EC 2.5.1.18) activity was determined according to Habig et al. (1974) by following the increase in absorbance at 340 nm due to the formation of the 1-chloro-2,4-dinitrobenzene (CDNB) conjugate using reduced glutathione (GSH) as the substrate.

Equal amounts of protein were subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE), as described by Laemmli (1970), excluding the omission of sodium dodecyl sulfate. For the separation of SOD isoenzymes, 4.5% stacking and 12.5% separating gels under constant current (60 mA) at 4°C were used. Electrophoretic POX separation was done according to Seevers et al. (1971). The separation of APX isoenzymes was performed by non-denaturing PAGE at 4°C with 4% stacking and 12.5% separating gels under a constant current (30 mA) and supported by 10% glycerol with a carrier buffer containing 2 mM ascorbate (Navari-Izzo et al., 1998). Equal amounts of protein were run in 10% (w/v) native PAGE stained for GST activity using the method of Ricci et al. (1984).

DETERMINATION OF *GMWRKY-71* GENE EXPRESSION, RNA ISOLATION, CDNA SYNTHESIS, AND REAL-TIME RT-PCR ASSAY

RNA extraction was performed using Tripure reagent (Roche) according to the manufacturer's instructions. The integrity of total RNA was checked spectrophotometrically using a NanoDrop Spectrophotometer ND-2000 (Labtech International), followed by gel electrophoresis. cDNA synthesis was performed from 4 μg total RNA using a Transcriptor 1st strand cDNA synthesis kit (Roche) according to the manufacturer's instructions and cDNAs of independent biological replicates (n = 3) from same treatments were pooled into single samples. Subsequently, transcript levels were analyzed in a LightCycler 480II real-time PCR cycler (Roche) using a Fast Start Essential DNA Probes Master kit (Roche) according to the manufacturer's instructions. Reaction conditions were 95°C for 600 s, followed by 45 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 1 s. Relative quantification of gene expression and statistical analysis of all qRT-PCR data (pairwise fixed reallocation randomization test) were performed using the REST software according to Pfaffl et al. (2002). GmWRKY71-specific products were obtained using the following primers: forward primer, CATCCAATGAAGCTGAAGCA and reverse primer, ACACGCTTTTGGCTGCTTAT (Table 3). Primer design was made using Acs number XM003547534.3, Acs number NM001251745.1, NCBI and ensemble gene banks.

STATISTICAL ANALYSIS

The experiment was conducted in a completely randomized design and the measurements were performed with 6 replicates (n = 6). Statistical variance analysis of the data was performed using ANOVA and the differences among treatments were compared using Tukey's post-hoc analysis with the least significant differences at the 5% level. In all the figures, the spread of the values is shown as error bars representing standard errors of the means.

RESULTS

EFFECTS OF DEHYDROABIETIC ACID ON PHYSIOLOGICAL PARAMETERS

In the present study, salt treatment did not change the fresh and dry weight (FW and DW) of shoots in soybean plants at 6 h, compared to the control group, while significiant decreases in FW and DW were recorded after 12 h and 24 h (Table 1). On the other hand, DHA pretreatment alone did not cause any change in these values. However, DHA application increased the fresh weight of shoots under salinity by 28.3% at 12 h and 34.16% at 24 h. Also, the dry weight of shoots was elevated by salt stress plus DHA treatment by 21.42% at 12 h and 18.8% at 24 h. Table 1 shows that the root fresh and dry weights (FW and DW) of soybean were reduced by salinity at all periods. However, there was an increase caused by DHA treatment alone in the roots. As in the case of shoots, there was a notable increase in the fresh weight resulting from DHA treatment under salinity but it was the highest at 24 h (FW- 55.5%), (DW – 71.4%) (Table 1).

Our results showed that after salt treatment leaf area levels were reduced at all periods but the highest reduction was 62.82% at 24 h (Table 2). On the other hand, DHA pretreatment alone did not change leaf area significiantly in soybean but combined effects of DHA and salinity increased this value by 16.95%, 47.80% and 65.95% at 6 h, 12 h and 24 h, respectively. Relative water content is an important indicator of the plant water status under stress to establish the adequate amount of water needed for metabolism. In the present study, salt stress inhibited relative water content in soybean leaves at all periods but DHA alone did not change it significiantly (Table 1). Nevertheless, DHA pretreatment alleviated reduction in RWC

Table 1. Time course effects of dehydroabietic acid pretreatment on fresh and dry weight of roots and shoots of soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

		DRY WEIGHT (g)			FRESH WEIGHT (g)		
		6h	**12h**	**24h**	**6h**	**12h**	**24h**
ROOT	**C**	0.004±0.001^a	0.008±0.004^a	0.004±0.01^a	0.15±0.004^a	0.24±0.06^a	0.12±0.01^a
	DHA	0.009±0.002^b	0.012±0.004^b	0.008±0.02^b	0.23±0.005^b	0.28±0.04^b	0.15±0.03^b
	NaCl	0.002±0.001^c	0.006±0.001^c	0.003±0.001^c	0.12±0.01^c	0.16±0.01^c	0.09±0.02^c
	DHA + NaCl	0.004±0.001^a	0.008±0.001^a	0.005±0.001^a	0.14±0.003^a	0.24±0.03^a	0.14±0.01^b
SHOOT	**C**	0.101±0.006^a	0.11±0.006^a	0.096±0.007^a	1.07±0.01^a	1.09±0.1^a	1.03±0.03^a
	DHA	0.11±0.02^a	0.12±0.01^a	0.098±0.01^a	1.28±0.2^a	1.16±0.1^a	1.02±0.02^a
	NaCl	0.11±0.006^a	0.09±0.01^b	0.085±0.01^b	1.10±0.1^a	0.78±0.2^b	0.80±0.01^b
	DHA + NaCl	0.101±0.01^a	0.119±0.3^a	0.101±0.03^a	1.08±0.1^a	1.01±0.2^a	1.08±0.02^a

Table 2. Time course effects of dehydroabietic acid pretreatment on leaf relative water content (RWC), chlorophyll (CHL), relative electrolyte leakage (REL) and leaf area (LA) of soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

	CHL (mg)			LA (cm^2)			RWC (%)			REL (%)		
Periods / Groups	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**	**6h**	**12h**	**24h**
C	24.19 ±1.7^a	20.31 ±1.0^a	16.37 ±1.2^a	7.71 ±0.53^a	6.74 ±0.74^a	8.77 ±0.51^a	47.22 ±3.78^a	56.36 ±3.53^a	54.04 ±10.94^a	15.24± 3.3^a	18.77 ±1.6^a	16.70 ±0.4^a
DHA	23.56 ±2.4^a	19.56 ±5.5^a	15.78 ±1.1^a	8.16 ±1.22^a	7.83 ±0.66^a	8.06 ±0.83^a	46.39 ±3.65^a	55.73 ±4.14^a	53.84 ±4.03^a	14.68 ±0.8^a	16.42 ±3.0^a	16.34 ±1.9^a
NaCl	19.55 ±1.3^b	15.51 ±7.2^b	12.63 ±1.0^b	5.13 ±0.88^b	4.10 ±0.54^b	3.26 ±0.20^b	36.56 ±2.18^b	22.99 ±10.46^b	26.51 ±7.49^b	59.17 ±3.1^b	70.11 ±7.7^b	76.73 ±3.5^b
DHA + NaCl	24.53 ±1.1^a	19.33 ±2.1 ^{a}	17.45 ±1.2^a	6.00 ±1.02^c	6.06 ±0.59^c	5.41 ±0.23^c	40.44 ±5.18^c	38.46 ±6.34^c	31.51 ±5.71^c	48.43 ±5.3^c	64.58 ±0.3^a	67.02 ±13.3^c

Table 3. Primers used in the study. Gene identification number (gene ID number), forward (OR), reverse and primer sequences, probe number and expected amplicon length (Bp).

	Gene ID No	Forward Primer	Reverse Primer	Probe Number	Bp
WRKY 71	XM_003518461.3	CATCCAATGAAGCTGAAGCA	ACACGCTTTTGGCTGCTTAT	#UPL143	74
GmAct	XM_003547534.3	GAGCTATGAATTGCCTGATGG	CGTTTCATGAATTCCAGTAGC	#UPL 61	118

values caused by salinity at all periods, while the highest increase was at 12 h (67.29%) (Table 2).

Proline is an important amino acid in plants needed to reduce harmful effects of oxidative stress. In our results, the proline level was decreased by DHA plus salt treatment by 18.80% and 24.45% at 12 h and 24 h. On the contrary, DHA treatment alone did not affect proline levels at any periods. In our research, ~ 5 fold increase, in comparison to the control group, was observed in salt treated leaves at 12 h and 24 h (Fig. 1). The increase in electrolyte leakage through cell membranes is commonly considered an indicator of membrane damage or deterioration. According to our results, plants treated with salinity, increased their relative electrolyte leakage, compared to the control groups, at all pariods (Table 2). However, DHA alone did not reduce REL values, compared to the control groups. On the other hand, plants treated with DHA under salinity were able to maintain 22.17%, 7.88% and 12.6% at 6 h, 12 h and 24 h of their REL values. As shown in Table 2, in soybean leaves, chlorophyll content was reduced under salt stress by 19.18%, 23.63% and 22.84%. However, DHA pretreatment under stress increased this value at all periods (max at 12 h), in comparison to salt treatment alone, while DHA treatment alone did not change this level at any periods.

EFFECTS OF DEHYDROABIETIC ACID ON BIOCHEMICAL PARAMETERS

In soybean leaves, 200 mM NaCl application increased MDA value by 76.46% at 6 h and ~ 2 fold at 12 h and 24 h, compared to the control groups, although DHA application alone did not affect it at all periods (Fig. 2). In the present study, DHA pretreatment under salt stress led to a 41.2%, 33% and 31.5% decrease in MDA values at all

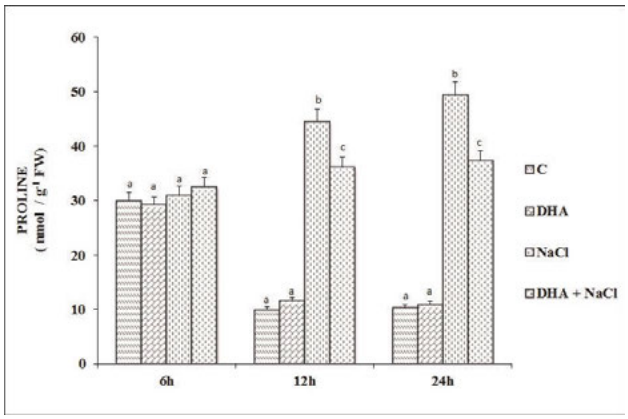

Fig. 1. Time course effects of dehydroabietic acid pretreatment on proline content of soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

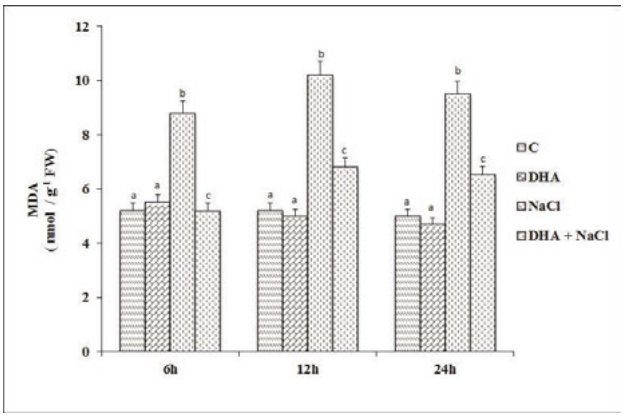

Fig. 2. Time course effects of dehydroabietic acid pretreatment on leaf malondialdehyde (MDA) content of soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

periods respectively, as compared to the salt treatment alone (Fig. 2). Figure 3 demonstrates that hydrogen peroxide level was increased (~ 2 fold) significiantly under salt treatments at all periods. When DHA was applied to salt treated plants, DHA prevented this increase at all periods, respectively, while there was a noticeable decrease at 24 h (by 34.76%).

Figure 4 illustrates that plants treated with salt showed low superoxide radical scavenger capacity, in comparison to the control groups, at all periods (8.29%, 11.38%, 24.11%). Moreover, this capacity increased significiantly (36.1%, 16.76% and 17.4%) under DHA plus salt treatment, as compared to the salt treatment alone. Salt stress alone caused a decrease in hydroxyl radical scavenger activity in soybean leaves (12.6%, 15.56%, 16.82%) at 6 h, 12 h and 24 h. Moreover, DHA pre-application under salt stress increased hydroxyl anion radical scavenging activity (22.6%, 22.4%, 53.89%) at all periods (Fig. 5).

In the present study, a significant increase (by 15.62% at 6 h) in SOD enzyme activity under salinity was detected, whereas this level was decreased at 12 h (20.67%) and was not changed at 24 h (Fig. 6). Otherwise, DHA pretreatment alone did not influence the SOD enzyme activity, compared to the control groups. Moreover, combined effects of salt and DHA application increased this activity by 28.45%, 19.67% and 24.92%, as compared to the salt groups (Fig. 6). Six isoenzymes were observed in the evaluation of SOD isoenzyme

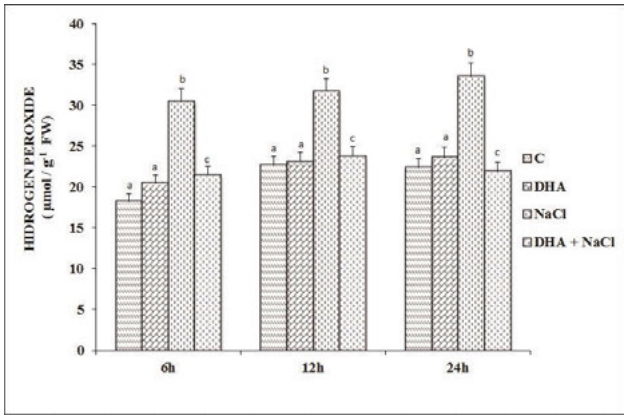

Fig. 3. Time course effects of dehydroabietic acid pretreatment on leaf hydrogen peroxide (H_2O_2) content of soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

profiles (Fig. 6). SOD 1, 5, 6 were remarkly upregulated with by salt application at 6 h; SOD 1, 2, 5, 6 were reduced at 12 h. Furthermore, SOD 2, 3, 4 isoenzymes were induced at 12 h and 24 h, while SOD 1 and SOD 6 were induced at 6 h under salt and DHA application, as determined by the measurement of total SOD activity. According to our results, POX enzyme activity was increased by salinity by 41.34%, 23.25%, 41.98% and these

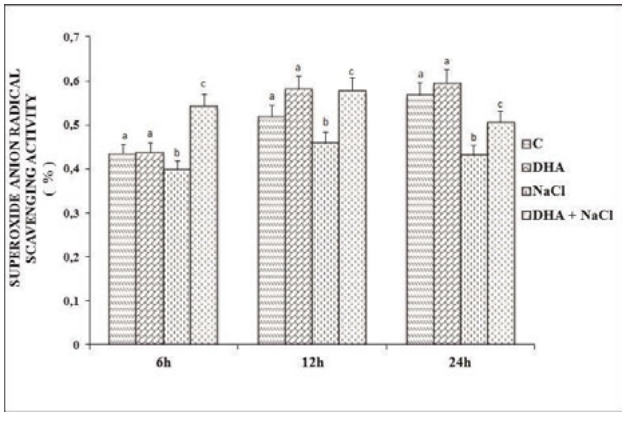

Fig. 4. Time course effects of dehydroabietic acid pretreatment on superoxide anion radical ($O_2^{\bullet-}$) scavenging activity of soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

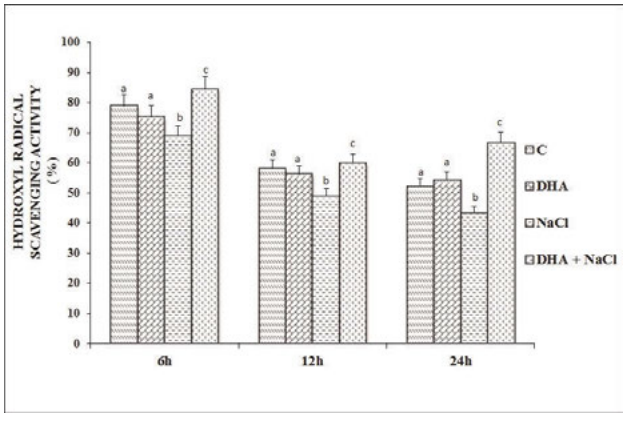

Fig. 5. Time course effects of dehydroabietic acid pretreatment on hydroxyl radical (OH^-) scavenging activity of soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

results are in agreement with the native PAGE analysis. In gel analyses, 5 POX isoenzymes were detected in all groups. Salt stress increased POX 1, 2, 3 isoenzyme pattern at 6 h and 24 h, while it was POX 1, 3 at 12 h. However, no changes were observed with DHA pretreatment alone at 12 h and 24 h, while POX activity it was increased by 33.56% only at 6 h. Otherwise, DHA and salt application did not change this level, compared to salt stress alone at 12 h and 24 h but this was raised by 18.18% at 6 h (Fig. 7). APX enzyme activity was not changed by salinity in soybean leaves at any periods. Also, DHA pretreatment alone did not affect the APX enzyme activity at any periods. However, salt and DHA pretreatment increased this activity by 6.68%, 24.42% and 16.9% at all periods (6, 12, 24, respectively). Similarly to total activity of APX, 7 isoenzyme patterns were determined in the treated plants (Fig. 8). APX 1, 3, 4 isoenzymes were induced at all periods under DHA and salinity, compared to salt treatment alone. On the basis of our results, Figure 9 shows that total GST enzyme activity was induced by salt treatment at all periods. This result is in agreement with the one observed in the isoenzyme gels, which showed increased values. In native PAGE, 9 GST isoenzymes were obtained at all periods (Fig. 9). GST 2, 3, 5, 6, 7 were increased by salinity at 12 h, while GST 1, 2, 5, 7, 8 and 9 were raised at 24 h. However, all GST isoenzymes, except GST 4, were induced at 6 h.

EFFECTS OF DEHYDROABIETIC ACID ON *WRKY-71* GENE EXPRESSION

Surprisingly, in the present study, *WRKY-71* gene expression was upregulated by DHA pretreatment under NaCl stress at 12 h and 24 h hours, while it was down regulated by both NaCl treatment alone and DHA treatment alone at all periods (Fig. 10).

DISCUSSION

According to the results, salt stress reduced root FW and DW by confirming the inhibition of plant growth. This phenomenon clearly indicated that salt stress affected water status in soybean. Other-

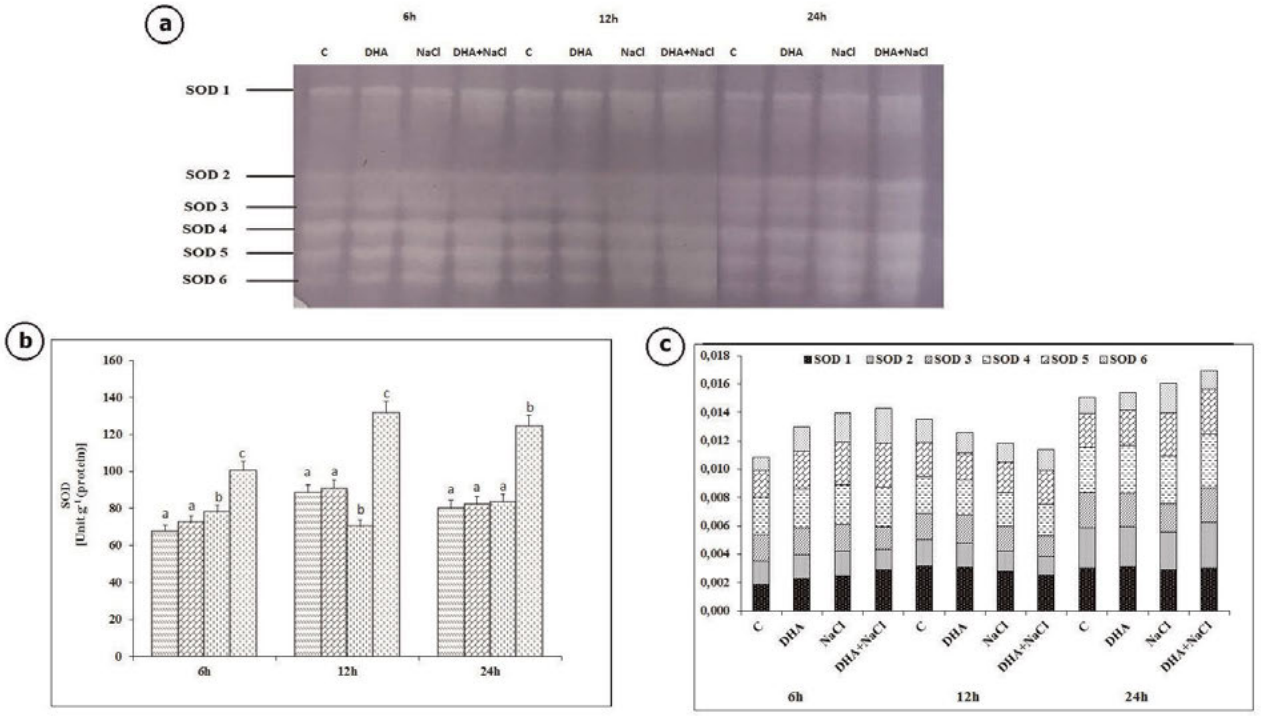

Fig. 6. Time course effects of dehydroabietic acid pretreatment on leaf activity staining and % induction of SOD (**a-b-c**), isoenzymes and total activity in soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

wise, DHA plus salt treatment caused an increase of these values in both roots and shoots (Table 1). Parallel to our results, giberellins and brassinostereoids, which also orginate from terpenes, prompt plant growth under stress conditions (Vince and Zoltan, 2011). It can be suggested that DHA induced water uptake and minerals from soil and increased the weight of roots. Similarly, Wang et al. (2019) reported that exogenous giberellic acid (GA_3), which is a diterpenoid compound, increased FW and DW of *Abelmoscus esculentus* seedling under salt stress. Furthermore, DHA application alone increased FW and DW in roots at all periods but, suprisingly, not in shoots as stated above (Table 1). Based on this result, it could be claimed that under non stress conditions, DHA can behave only as a diterpene and may be able to increase water or minerals (e.g K^+, Ca^{+2}) movement from soil to roots.

In the present study, leaf area levels were reduced at all periods (Table 2). This was one of the deleterious effects of salinity in plants. In agreement with these results, Kao et al. (2006) determined that different NaCl concentrations decreased leaf area in soybean plants. In the present study, combined effects of DHA and salinity increased leaf area level. So, it could be suggested that DHA was able to prompt soybean growth under salinity. However, it was interesting that DHA pretreatment alone did not affect leaf area. This finding supports our results of the fresh and dry weights of shoots. This result showed that when stress is perceived by soybean roots, DHA may play a role in signal transmission and affect the root metabolism firstly. As it is shown in Table 2, salt stress inhibited RWC content in soybean leaves at all periods but DHA alone did not change it significiantly. Similiar results were reported by Liu et al. (2017) who demonstrated that salinity reduced RWC values in soybean plants. However, DHA application under salinity alleviated RWC value at all periods. These results were also in accordance with the results obtained for FW and leaf area.

In the present study, ~ 5 fold increase was observed in proline content in salt treated leaves, compared to the control group, at 12 h and 24 h

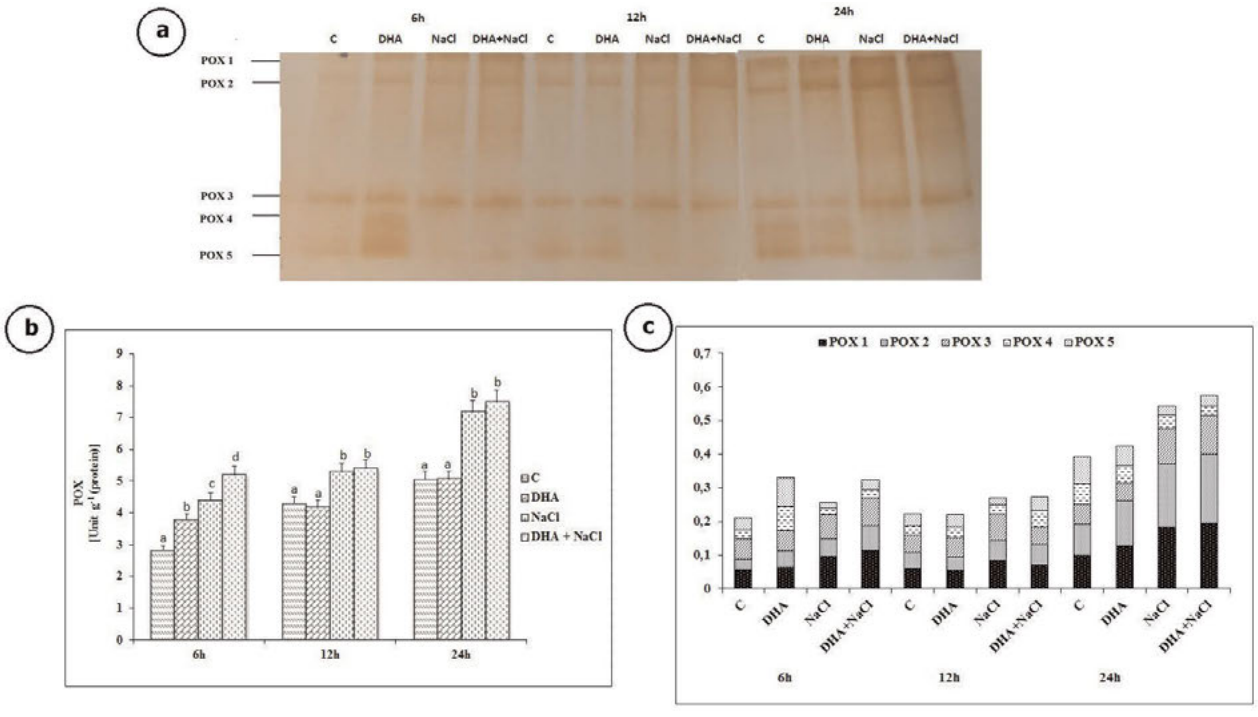

Fig. 7. Time course effects of dehydroabietic acid pretreatment on leaf activity staining and % induction of POX (**a-b-c**) isoenzymes and total activity in soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

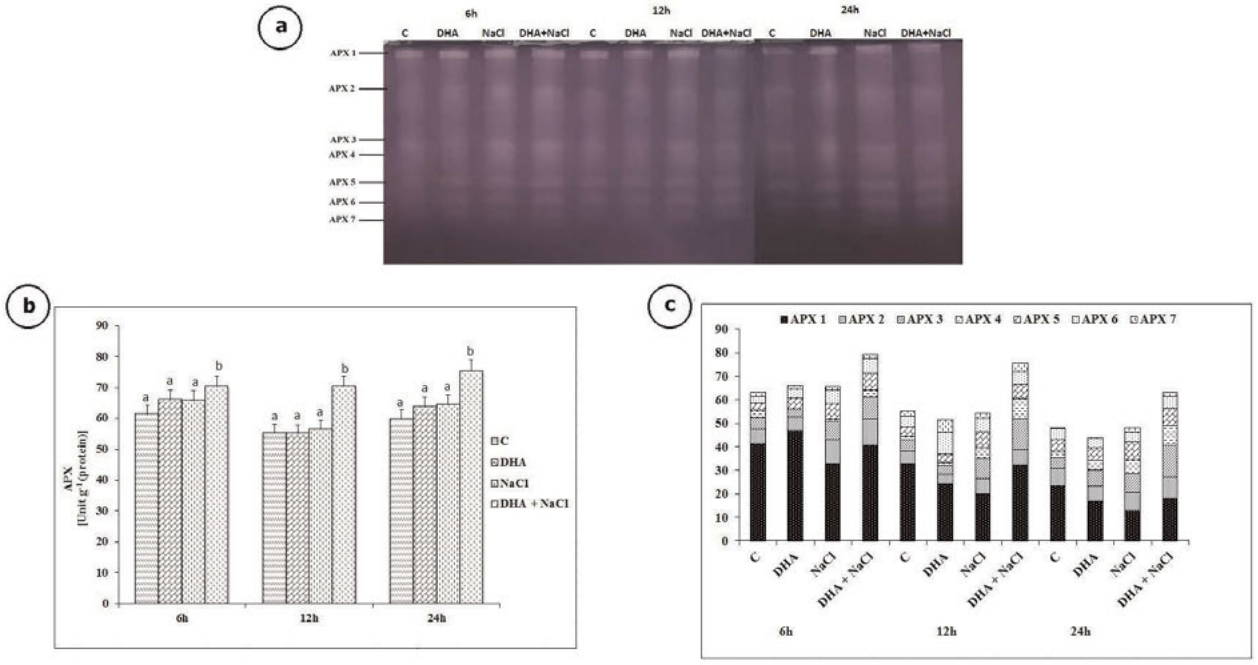

Fig. 8. Time course effects of dehydroabietic acid pretreatment on leaf activity staining and % induction of APX (**a-b-c**) isoenzymes and total activity in soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

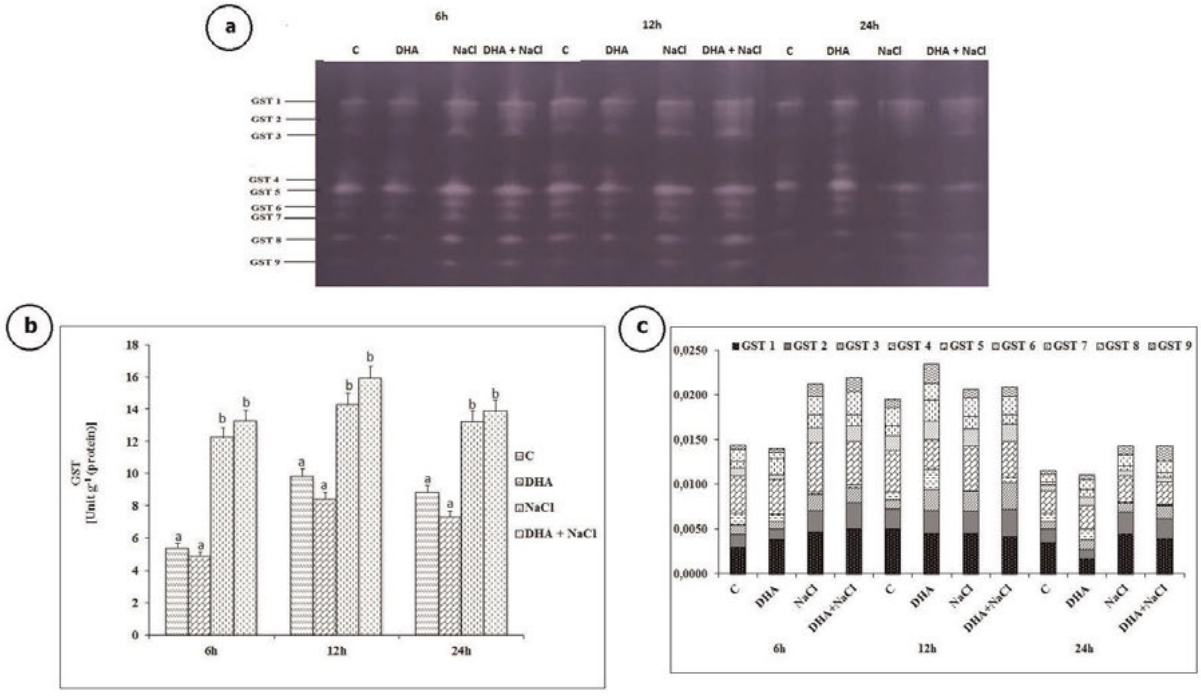

Fig. 9. Time course effects of dehydroabietic acid pretreatment on leaf activity staining and % induction of GST (**a-b-c**) isoenzymes and total activity in soybean (*Glycine max* L.) seedlings under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

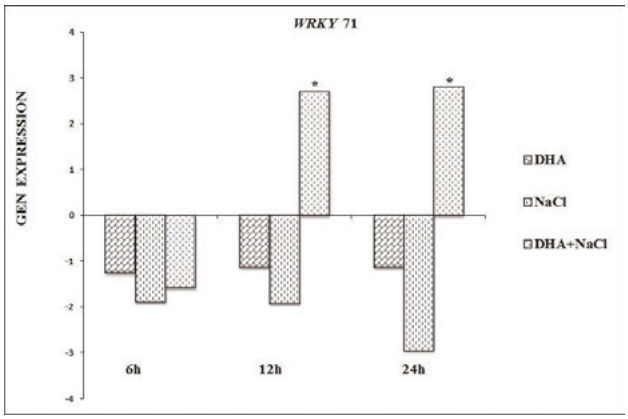

Fig. 10. Time course effects of dehydroabietic acid pretreatment on relative (*WRKY-71*) gene expression determined by qRT-PCR in leaves of soybean (*Glycine max* L.) under salt stress. Control (C), Dehydroabietic acid (DHA), Salt stress (NaCl), Dehydroabietic acid + Salt stress (DHA + NaCl). Columns with different letters represent significantly different ($P < 0.05$) values.

(Fig. 1). Also previous studies reported that salt treatment increased proline in soybean leaves (Sarisoy et al., 2018). Nevertheless, this level was decreased with DHA plus salt treatment at 12 h and 24 h. Otherwise, DHA treatment alone did not affect proline levels at any all periods. These findings showed that soybean leaves have their own capacity to increase proline level with their defence system to salt stress. It can be argued that DHA preapplication might maintain the proline level as a stress inhibitor and DHA can induce stress-related amino acids or other compounds and phytohormones under stress conditions. However, there was a reduction in proline levels caused by DHA application under stress. Based on this result, it could be suggested that these compounds were probably phytohormones and they need to be investigated in future.

In our study, salt stress increased relative electrolyte leakage compared to the control groups at all periods (Table 2). In accord with our results, *OsMYB6*-overexpressing transgenic rice plants had low REL content which increases tolerance to salt and drought stress (Tang et al., 2019). Nevertheless, DHA alone did not reduce REL values, compared to the control groups. On the other hand, plants treated with DHA under salinity reduced their REL values at all periods. In agreement with this result, 24-epibrassinolide (a terpene) application reduced REL in rice under chilling stress (Clouse and Sasse, 1998). In the present study, the DHA pretreatment may have protected the membrane structure against oxidative damage by decreasing lipid peroxidation and increasing antioxidant enzyme activities in leaf membranes (Figs. 6, 8).

In our research, salinity decreased chlorophyll content at all periods. This reduction could be explained by degradation of the existing chlorophyll molecules. Nevertheless, DHA pretreatment under stress increased this value at all periods (max at 12 h), compared to salt treatment alone, although DHA treatment alone did not change this level at any periods (Table 2). Our results are in agreement with the findings of Tounekti et al. (2011) who reported that phenolic diterpene-kinetin improves salt tolerance by increasing pigment content in *Salvia officinalis*. DHA might reduce the activity of chlorophyllase which is responsible for the chlorophyll degradation which was determined the effects of brassinosteroids (terpene) in *Brassica juncea* (Wani et al., 2019). Otherwise, this finding was also in accord with the results of REL, which were increased by salinity in soybean leaves.

Malondialdehyde (MDA) is a product of peroxidation of unsaturated fatty acids in phospholipids and it is responsible for cell membrane damage. It is clear that in this study NaCl caused oxidative damage and increased lipid peroxidation (Fig. 2). In parallel to our findings, many reports have proven that NaCl stress leads to increase in MDA level in soybean (Lu et al., 2016). In the present study, DHA pretreatment under salt stress reduced the MDA values at all periods, as compared to the salt treatment alone (Fig. 2). This result showed that DHA alleviated salt induced damage as an activator in the treated leaves. In the past years, it was reported that diterpenes protect biological membranes from oxidative damage and inhibit superoxide radical production in isolated chloroplasts and microsomes (Haraguchi, 1998). Similarly, carnosic acid, which is one of the diterpenes, plays a role in oxidative damage prevention in rosemary plants under drought stress (Munne-Bosch et al., 2001). Consequently, DHA, a diterpene, functions as a potential antioxidant to scavenge ROS and reduces lipid peroxidation levels under stress conditions in soybean. This results is also in agreement with the findings for REL (Table 2).

Hydrogen peroxide level was increased by salinity at all periods (Fig. 3). This result is also parallel to the reports of Klein et al. (2015). DHA pretreatment alone did not lead to any change at this level but DHA prevented this increase under salinity at all periods. In accord with our results,

Khan et al. (2010) reported that GA_3, which is known as a diterpene, decreased H_2O_2 content in *Linum usitatissimum* L. plants under salt stress. To the best of our knowledge, there is no report about the effects of DHA on hydrogen peroxide level in plants, although it is known only as a mobile signal molecule in systemic acquired resistance under biotic stress (Chatuverdi et al., 2012). This interesting finding firstly showed us that DHA could motivate antioxidant enzyme activities to increase salt tolerance in soybean.

Both superoxide radical scavenger and hydroxyl radical scavenger capacity were reduced by salt stress in soybean leaves. Nonetheless, DHA application increased them at all periods (Figs. 4,5). These results are also consistent with the decrease of MDA, H_2O_2 level under DHA and salt treatment (Figs. 2,3). However, DHA pretreatment alone did not affect this level at any periods. In literature, terpenoids have been shown to possess antioxidative properties in different situations (Teissedre and Waterhouse, 2000), particularly against lipid peroxidation as a result of their high lipophilicity, while it is not clear in plants. As a result, in this study, DHA might behave as a scavenger because of its high lipophilicity capacity.

Noctor et al. (1998) reported that superoxide radical can be chemically reduced or dismutated to H_2O_2, a reaction that is accelerated by superoxide dismutases (SODs). In our research, Figure 6 shows that SOD enzyme activity was increased at 6 h but decreased at 12 h, while it was not affected at 24 h. Although SOD enzyme was not increased at 12 h and 24 h, there was a remarkable increase in hydrogen peroxide content at all periods. This could be explained by other sources of hydrogen peroxide such as NADPH oxidases. Similar results were reported by (Ji et al., 2016) who demonstrated that salinity increased SOD enzyme activity in soybean leaves. Moreover, combined effects of salt and DHA application increased this activity at all periods. These results are also compatible with the radical scavenger activities of this molecule in soybean leaves (Figs. 4, 5). Otherwise, decreased hydrogen peroxide and MDA levels in DHA plus salt treated groups were observed while they were higher under salinity, as it is discussed below (Figs. 2, 3). Besides the induction of SOD activity, this might be related to increase in APX activity to scavenge H_2O_2 by DHA effect in soybean leaves (Fig. 8). Consequently, DHA can improve this enzyme activity to scavenge superoxide radical which was produced by salt stress.

POX can scavenge hydrogen peroxide in plants cell. In the present study, POX enzyme activity was increased by salinity. The findings obtained by Ji et al. (2016), who established that POX enzyme activity was increased by stress treatment in soybean plants, are consistent with our results. Only at 6 h, there was an increase in POX enyzme activity by DHA treatment alone. Similarly, DHA plus salt stress induced this enzyme activity at 6 h (Fig.7). This could be explained by the role of POX enzyme in cell wall lignificiation but not scavenging of hydrogen peroxide. It could be suggested that DHA might help lignin biosynthesis by inducing POX activity in soybean leaves at the early stage of stress (6 h).

H_2O_2 is detoxified into water and oxygen by ascorbate peroxidase (APX) through a cycle, called the ascorbate-glutathione cycle (Asada, 1999). In the present study, salt treatment did not cause any change in APX enzyme activity at any periods. In contrast with this result, Cicek and Cakırlar (2008) reported that APX enzyme activity was induced in soybean cultivar (SA88) under salt stress but within several days. This difference might be related to the term of stress application, comparing with this study. However, salt and DHA pretreatment induced this activity, while DHA pretreatment alone did not affect the APX enzyme activity at any periods. This result might be related to the decrease in reactive oxygen species level and MDA content under DHA plus salinity. Consequently, it could be claimed that DHA could induce two important enzyme activities such as SOD and APX in soybean leaves under stress conditions.

Glutathione-*s*-transferase (GST; EC 2.5.1.18) plays a role in oxidative stress tolerance in plants. According to our results, salt stress induced total GST enzyme activity at all periods (Fig. 9). It is in agreement with what was observed in isoenzymes gels, which showed increased values. In parallel to our results, Dinler et al. (2014) found that NaCl treatment induced GST enzyme activities in soybean plants. In the present study, although there was a slight increase in GST enzyme activity under salinity, there was an increase in MDA and ROS content. This could be explained by the unchanged SOD and APX activity caused by salinity. Otherwise, there was no change in the leaves pretreated with DHA alone and under salinity. These findings suggested that DHA was not effective on GST enzyme to protect soybean leaves from salt stress within the investigated periods.

The *WRKY* gene family has been suggested to play important roles in the regulation of transcriptional reprogramming associated with plant stress responses (Bakshi and Oelmüller, 2014). In literature, there are many reports with regard to *GmWRKYs* in plants under stress conditions. For example, it was reported that the *WRKY27* gene was induced in 200 mM NaCl-stressed soybean seedlings (Wang et al., 2015). In our research, *WRKY-71* gene expression was upregulated under NaCl stress at 12 h and 24 h, although it was down regulated by both NaCl treatment alone and DHA treatment alone at all periods (Fig. 10). These results showed that DHA might induce salt stress signaling pathway in soybean by changing *WRKY-71* gene expressions.

On the other hand, it was established that *WRKY* gene family also plays a role in ROS signalingin plants. The key proponents of the ROS signaling cascade are ascorbate peroxidases (APX), NADPH oxidases, and Zn fingerproteins. The TF *TaWRKY10* of wheat, when over-expressed in transgenic tobacco, decreased the accumulation of MDA and lowered the levels of superoxide radical and hydrogen peroxide formation on exposure to salinity and drought stresses (Banerjee and Roychoudhur, 2015). It has been also reported that transgenic tobacco plants, *GhWRKY39,* have capacity to increase in ROS related enzymes (SOD, POX, CAT) under stress (Chu et al., 2015). However, in the present study, there was an increase in *WRKY-71* gene expression, although antioxidant enzymes (POX, GST) were unchanged by DHA application under stress. It could be argued that DHA might induce hormones (salicylic acid, abscisic acid, nitric oxide) as a signal transmitter to increase *WRKY-71* expressions and lead to increase in the activities of two important enzymes such as SOD and APX.

CONCLUSIONS

Salinity is an important stress factor for plant growth. Based on this study, it can be suggested that DHA treatment can be used as a plant activator, an antioxidant and signal molecule to protect plants from salt stress and stress-induced damage, such as decreased chlorophyll, leaf area, fresh and dry weight, distorted water status, membrane injury, changed proline level, oxidative damage and possibly yield loss. The results firstly showed that DHA was also actually effective on increasing salt tolerance in soybean leaves by changing antioxidant enzymes activities and *WRKY71* gene expressions, especially when this gene was not upregulated under salinity. More research and detailed analysis are needed to illuminate the of this interesting molecule, especially its communication with phytohormones.

AUTHORS' CONTRIBUTIONS

ET and BSD carried out experiment, performed analysis. BSD wrote the manuscript. The authors declare no competing interests.

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