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PRE-TREATMENT OF FULVIC ACID PLAYS A STIMULANT ROLE IN PROTECTION OF SOYBEAN (*GLYCINE MAX* L.) LEAVES AGAINST HEAT AND SALT STRESS

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The purpose of this study was to illuminate the effects of fulvic acid in plants' stress signaling pathway. 2.0 mg/l fulvic acid was sprayed on soybean leaves for 3 days at 12 h intervals, followed by treatment of 150 mM NaCl or exposed to heat stress at 35°C for 2 h over 2 days. Pre-treatment with fulvic acid increased the relative water content (RWC), antioxidant enzyme, isoenzyme activities (SOD, APX, GST), as well as alleviated the stress-induced oxidative damage by decreasing the levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). In addition, the application of fulvic acid under salt stress induced *rubisco* expression only at 12 h, while it induced the expression of *cytochrome c oxidase* at 6 h and 12 h. On the other hand, fulvic acid under heat stress induced significant expression of both rubisco and *cytochrome c oxidase* at 6 h and 12 h. However, under high salinity conditions, fulvic acid suppressed the transcript levels of *Hsp70*, while it induced increases in *Hsp70* levels under heat treatment at 6 h. As a result, in this study, fulvic acid played the role of a regulator and stimulant in stress response of soybean leaves.

Keywords: fulvic acid, salt stress, heat stress, antioxidant enzymes, rubisco, cytochrome c oxidase, Hsp70

INTRODUCTION

Fulvic acid is a derivative of humic acid, but it has a smaller molecular size (Grenthe, 1997) and is less stable in soil due to its greater exposure to microbial degradation. It occurs naturally in soil, water and peat like humic acids, and it modifies the soil structure by binding to sand, silt and clay due to its colloidal characteristics (Mayhew, 2005). Due to high ion exchange and hydrolysis capacity of fulvic acid, the resulting excess amounts of amino acids and organic acids increase the soil cation exchange capacity. Fulvic acid decreases soil loss, increases soil fertility and facilitates the transfer of mineral nutrients from the soil to plants. Furthermore, similarly to other organic fertilizers, fulvic acid protects plants against stress conditions by increasing the soil efficiency (Goatley and Schmidt, 1990). The presence of reactive groups in the structure of fulvic acid permits effective chelation of both mineral ions and heavy metals and provides antioxidant properties (Stevenson, 1994).

Fulvic acid can function as a plant hormone (Akıncı and Ongel, 2011). In addition, it decreases plant production under salt stress damage by increasing the buffering properties of the plants against soil acidity and salinity (Gezgin et al., 2012). The application of fulvic acid increases the tolerance of wheat plants under drought stress (Yuling et al., 2000). However, Ni-fulvic acid complexes reduce Ni toxicity (Akıncı and Ongel, 2011) and fulvic acid reduces Pb toxicity (Shahid et al., 2012) in plants. Moreover, Anjum et al. (2011) recently found that fulvic acid induces antioxidant enzyme activity and protects plants against oxidative damage. Similarly, Garcia et al. (2014) showed that humic acid alleviates water stress damage in maize plants.

Although certain physiological effects of fulvic acid have been demonstrated in plant metabolism, little is known regarding the detailed mechanism

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(physiological, biochemical and molecular changes) underlying stress conditions. It is known that salt or heat stress disturbs main metabolism including photosynthesis and respiration in plants. For this reason, behind the physiological parameters, the effects of fulvic acid on antioxidant enzymes, photosynthetic enzyme (rubisco), respiration electron transport system-complex IV (cytochrome c oxidase) and heat shock protein-(Hsp70) were determined. Consequently, the primary subject and aim of the present study was to determine the effects of fulvic acid under stress conditions and illuminate the underlying stress signaling pathway in plants.

MATERIALS AND METHODS

PLANT GROWTH AND EXPERIMENTAL DESIGN

Soybean (*Glycine max* L. Merr.) seeds were obtained from a commercial provider (May, Bursa, TR). The seeds were sterilized in 5% hypochloride solution for 10 min, rinsed three times with distilled sterile water, and then sown in plastic trays (10 cm x 14 cm) filled with soil under dark conditions. After germination, seedlings were taken into a growth chamber at 25°C with 16 h light/8 h photoperiod and light intensity of 500 μmol m^{-2} s^{-1} with Hoagland solution for 3 weeks. The seedlings were divided into 8 groups. In the first group, the soybean leaves were sprayed with 2.0 mg/l fulvic acid (the concentration was determined by pre-experiments) every 12 h for 3 days. The leaves, that were sprayed with fulvic acid or not, were watered with 150 mM NaCl or exposed to heat stress at 35°C for 2 h over 2 days. The other group was exposed to a combination of salt or heat stress treatment. After the stress treatment, the plants were harvested at 0 h, 6 h and 12 h and stored at - 80°C.

RELATIVE WATER CONTENT

The relative water content (RWC) was calculated in accordance with Smart and Bingham (1974). Fresh weights (FW) of seedlings were initially determined. The samples were subsequently oven-dried at 70°C for 72 h and then dry weights (DW) were determined.

LIPID PEROXIDATION

The level of lipid peroxidation in leaf samples was determined in terms of the malondialdehyde (MDA) content according to the method specified by Madhava Rao and Sresty (2000). The MDA content was calculated using the Lambert-Beer law, with extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol MDA per g FW.

PROLINE CONTENT

The proline content of the leaves was determined according to Claussen (2005). For each treatment, 0.5 g leaf sample was ground in a mortar after addition of a small amount of glass powder and 5 mL of a 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was filtered through two layers of glass-fibers. To the filtrate (1 mL), glacial acetic acid and ninhydrin reagent (1 mL each) were added. The closed test tubes containing the reaction mixture were kept in a boiling water bath for 1 h before the reaction was terminated at room temperature (22°C) for 5 min. 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).

HYDROGEN PEROXIDE CONTENT

The H_2O_2 content was determined according to Velikova et al. (2000). Frozen leaf material (0.1 g) was homogenized on ice with 0.1% (w/v) TCA. The homogenate was centrifuged at 15,000 × g for 15 min at 4°C and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the assay mixture was read at 390 nm and the content of H_2O_2 was calculated based on a standard curve of known concentrations of H_2O_2.

ABA LEVEL

The ABA content was determined according to Flores et al. (2011) using UHPLC-MS/MS; (Agilent, 6064), Belgium. Stock solutions of the individual compounds were prepared by exact weighing of the powder and dissolution in methanol (HPLC-grade, Sigma, USA).

NA^+, CL^-, AND CA^{2+} CONTENT

The ion content was determined by flame photometry according to Mathis (1956). The Cl^- concentration was obtained by wet oxidation of dried leaf tissue with nitric and perchloric acids in accordance with the method adapted by Johnson and Ulrich (1959). The digest was diluted in 0.1 N perchloric acid, and Cl concentrations were determined by atomic absorption spectrophotometry.

ANTIOXIDANT ENZYMES AND ISOENZYMES

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). Ascorbate peroxidase (APX; EC 1.11.1.11)

activity was measured according to Nakano and Asada (1981). The assay depends on the decrease in absorbance at 290 nm as ascorbate is oxidized. Glutathione *S*-transferase (GST; EC 2.5.1.18) activity was determined by the method of 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. The total soluble protein contents of the enzyme extracts were determined according to Bradford (1976) using bovine serum albumin as a standard.

SOD ISOENZYME ACTIVITY

Equal amounts of protein were subjected to non-denaturing 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. SOD activity was detected by photochemical staining with riboflavin and NBT, as described by Beauchamp and Fridovich (1971).

APX ISOENZYME ACTIVITY

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). The APX isoenzymes were detected in the gels as reported by Mittler and Zilinkas (1993).

GST ISOENZYME ACTIVITY

Equal amounts of protein were separated by 10% (w/v) native PAGE according to the method described by Laemmli (1970) and stained for GST activity according to Ricci et al. (1984). Briefly, after electrophoresis, the gel was equilibrated in 0.1 M potassium phosphate buffer (pH 6.5) for 10 min and transferred to a reaction mixture containing 4.5 mM GSH, 1 mM CDNB and 1 mM nitroblue tetrazolium in 0.1 M potassium phosphate buffer (pH 6.5) at 37°C for 10 min. Next, the gel was incubated at room temperature in 0.1 M Tris/HCl (pH 9.6) containing 3 mM phenazine methosulfate.

The gels that were stained for enzyme activities were photographed using Image Acquisition and Analysis Software (4.6.00.0; UVP BioImaging systems, UK). For the densitometric analyses of SOD, APX and GST activity, the activities of control plants were considered as 100%, and the percentages of the control values for each treatment are shown in Figs. 3, 4 and 5.

GENE EXPRESSION ANALYSIS
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 quality and integrity of total RNA was checked spectrophotometrically using a Nano Drop Spectrophotometer ND-1000 (Thermo Scientific), followed by gel electrophoresis. Prior to cDNA synthesis, total RNA samples were pretreated with a RNase-free DNase I to eliminate any contaminating genomic DNA. cDNA synthesis was performed from 4 μg total RNA using a Transcriptor High Fidelity cDNA synthesis kit (Roche) according to the manufacturer's instructions with the oligo (dT) primer and cDNAs of independent biological replicates (n = 3) from the same treatments were pooled into single samples. Subsequently, the Cq was automatically determined for each reaction by the Light Cycler Nano real-time PCR cycler system (Roche) using a FastStart Essential DNA Probes Master kit (Roche) according to the manufacturer's instructions. Primer sequences and probe numbers used in the reaction are given in the following Tab. 3, designed with Roche Universal Probe Library Assay Design Center. Reaction conditions were 95°C for 10 m, followed by 45 cycles of 95°C for 10 s, 59°C for 30 s, and 72°C for 10 s. The comparative ΔΔCt method (Livak and Schmittgen, 2001) was used to evaluate the relative quantification of gene expression in the samples. Gene expression levels were normalized with the expression levels of a housekeeping gene (Cons4, ATP binding cassette transporter: ABC transporter) and also with the unstressed control sample. Statistical analyses of all qRT-PCR data were performed using the SPSS 16 software. The results were subjected to One Way ANOVA (Analysis Of Variance) Dunnet T3 and Post-Hoc test (Dunnet, 1955; Roscoe, 1975). Primer sequences designed for gene reasons and probe numbers are shown in Tab. 3.

STATISTICAL ANALYSIS

All analyses were conducted in accordance with a completely randomized design. The data were subjected to a non-parametric Kruskal Wallis test. Each data point is the mean of six replicates (n=6), and $p < 0.05$ was considered statistically significant.

RESULTS

ION CONTENT

In the present study, pre-treatment with fulvic acid followed by salt stress results in a decrease in Na^+, Cl^- ions and induces Ca^{2+} ions significantly

after 6 h and 12 h compared to salt stress alone (Tab. 1). Nevertheless, pre-treatment with fulvic acid under heat stress did not change Na^+ and Cl^- but decreased Ca^{2+} at 6 h in comparison to heat stress alone. In addition, pre-treatment with fulvic acid under salt and heat stresses increased the levels of Na^+ at 6 h and 12 h compared to control leaves, while the levels of Ca^{2+} increased and Cl^- decreased at 6 h (Tab. 2).

RELATIVE WATER CONTENT (RWC) AND PROLINE CONTENT

According to our data, pre-treatment with fulvic acid increased the RWC content of soybean leaves compared to salt or heat treatment alone (Tab. 1). In addition, pre-treatment with fulvic acid under salt and heat stresses increased RWC at 6 h; however, no change was detected at 12 h. Pre-treatment with fulvic acid under heat stress decreased the proline content compared to heat treatment alone, while it was increased in comparison to salt treatment alone. However, pre-treatment with fulvic acid under salt and heat stresses reduced the proline content by 23% at 6 h and by 42.5% at 12 h compared to heat and salt treatment alone (Fig. 1).

MALONDIALDEHYDE AND HYDROGEN PEROXIDE CONTENT, ABA LEVEL

The data presented clearly show that pre-treatment with fulvic acid under all the three stresses (salt, heat or combination) decreased the MDA and H_2O_2 content at all of the assessed time points (Fig. 1). Our results also showed that pre-treatment with fulvic acid under salt stress increased ABA levels at 6 h but decreased these levels significantly at 12 h in comparison to stress treatment alone. Pre-treatment with fulvic acid under heat stress increased ABA levels by 53.5% at 6 h and 28.5% at 12 h compared to heat treatment alone. Pre-treatment with fulvic acid increased ABA levels under salt and heat stresses in comparison to salt and heat treatment alone (Fig. 2).

ANTIOXIDANT ENZYME AND IZOENZYME ACTIVITES

Pre-treatment with fulvic acid under salt treatment increased and decreased SOD enzyme activity at 6 h and 12 h, respectively, compared to salt treatment alone. This treatment also induced SOD2 isoenzyme activity at 6 h compared to salt treatment alone. Pre-treatment with fulvic acid under heat stress decreased SOD enzyme activity at 6 h and increased at 12 h. In agreement with the total activity, pre-treat-

TABLE 1. Time course effects of fulvic acid pretreatment on leaf Na^+, Cl^- and Ca^{2+} content of soybean (*Glycine max* L.) seedlings under salt and heat stress. Control (C), Fulvic acid (FA), Salt stress (S), Heat stress (H), Salt + Heat stress (S+H), Fulvic acid + Salt stress (FA+S), Fulvic acid + Heat Stress (FA+H) Fulvic acid + Salt + Heat stress (FA+S+H). Columns with different letters represent significantly different ($P < 0.05$) values.

	0 h			6 h			12 h		
	Na^+ %	Cl^- %	Ca^+ %	Na^+ %	Cl^- %	Ca^+ %	Na^+ %	Cl^- %	Ca^+ %
GROUPS									
C	0.019± 0.004^a	0.175± 0.001^b	0.638± 0.021^a	0.010± 0.004^a	0.173± 0.001^b	0.553± 0.024^a	0.015± 0.001^a	0.235± 0.002^b	0.722± 0.019^b
FA	0.022± 0.005^b	0.098± 0.002^a	0.743± 0.023^b	0.025± 0.008^b	0.172± 0.001^b	0.600± 0.037^b	0.021± 0.001^b	0.211± 0.002^b	0.718± 0.013^b
S				0.015± 0.006^b	0.442± 0.002^d	0.640± 0.026^b	0.020± 0.001^b	0.302± 0.002^c	0.645± 0.017^a
H				0.008± 0.004^a	0.174± 0.001^b	0.726± 0.014^c	0.013± 0.001^a	0.233± 0.003^b	0.628± 0.016^a
FA+S				0.012± 0.001^a	0.173± 0.001^b	0.777± 0.029^c	0.013± 0.001^a	0.096± 0.001^a	0.960± 0.011^c
FA+H				0.016± 0.002^a	0.172± 0.000^b	0.661± 0.010^b	0.015± 0.001^a	0.094± 0.002^a	0.721± 0.013^b
S+H				0.021± 0.001^b	0.272± 0.001^c	0.646± 0.009^b	0.025± 0.001^b	0.321± 0.002^b	0.798± 0.016^c
FA+S+H				0.027± 0.002^c	0.131± 0.001^a	0.735± 0.016^c	0.023± 0.002^b	0.102± 0.001^a	0.656± 0.029^a

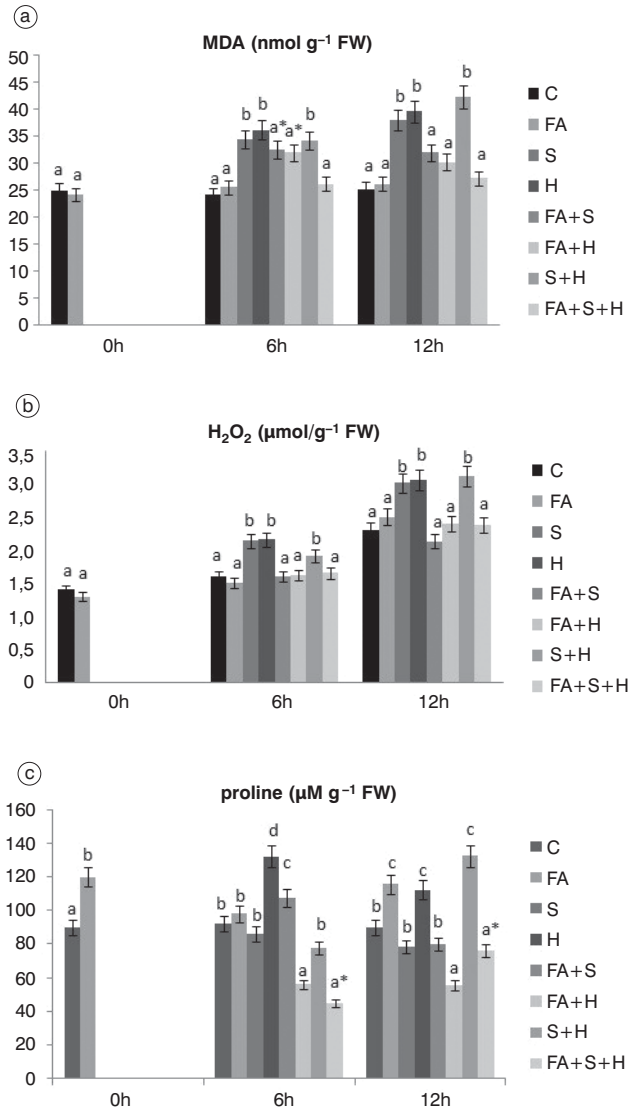

Fig. 1. Time course effects of fulvic acid pretreatment on leaf. (**a**) malondialdehyde (MDA), (**b**) hydrogen peroxide (H_2O_2), (**c**) proline content of soybean (*Glycine max* L.) seedlings under salt and heat stresses. Control (C), Fulvic acid (FA), Salt stress (S), Heat stress (H), Salt + Heat stress (S+H), Fulvic acid + salt stress (FA+S), Fulvic acid + heat stress (FA+H) Fulvic acid + Salt + Heat stress (FA+S+H). Columns with different letters represent significantly different ($P < 0.05$) values.

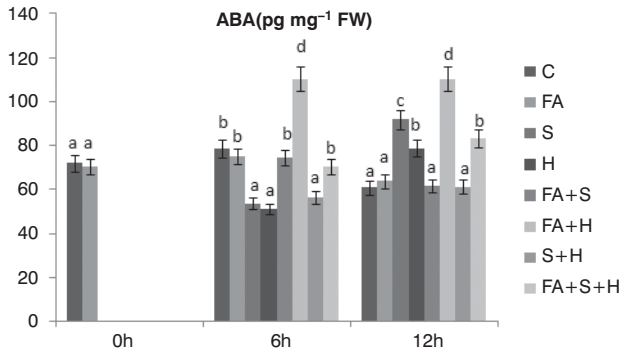

Fig. 2. Time course effects of fulvic acid pretreatment on leaf abscisic acid (ABA) level of soybean (*Glycine max* L.) seedlings under salt and heat stresses. Control (C), Fulvic acid (FA), Salt stress (S), Heat stress (H), Salt + Heat stress (S+H), Fulvic acid + Salt stress (FA+S), Fulvic acid + Heat stress (FA+H), Fulvic acid + Salt + Heat stress (FA+S+H). Columns with different letters represent significantly different ($P < 0.05$) values.

ment with fulvic acid under heat stress decreased SOD5, 6 and 7 isoenzyme activities but did not change the activity of the other enzymes at 6 h, while all of the isoenzymes were up-regulated except SOD1 at 12 h in comparison to heat treatment alone. Combined (salt and heat) treatment decreased and increased SOD enzyme activity at 6 h and 12 h, respectively. Pre-treatment with fulvic acid under salt and heat stresses increased SOD enzyme activity at 6 h and decreased at 12 h compared to salt and heat stresses alone. Pre-treatment with fulvic acid under salt and heat stresses increased the levels of all of the isoenzymes except SOD1 and SOD2, while SOD1, 6 and 7 decreased at 12 h (Fig. 3).

Pre-treatment with fulvic acid under salt stress, compared to salt treatment alone, increased APX enzyme activity at 6 h and decreased at 12 h (Fig. 4). In parallel with total activity, pre-treatment with fulvic acid under salt stress, compared to salt treatment alone, increased APX1, 3 and 4 isoenzyme activity at 6 h and decreased APX1, 2, 3 and 4. In addition, pre-treatment with fulvic acid under heat stress increased the total APX enzyme activity at 6 h but decreased this activity at 12 h, compared to heat treatment alone. Pre-treatment with fulvic acid under salt and heat stresses, compared to salt and heat treatment alone, did not change APX activity at 6 h but decreased it by 10% at 12 h.

Pre-treatment with fulvic acid under salt and heat stresses increased GST activity at 6 h and 12 h (Fig. 5). On the other hand, pre-treatment with fulvic acid under combined stress decreased only GST2 isoenzyme activity at 6 h. Only GST1, 2 and 3 decreased at 12 h, while the other enzymes increased. Pre-treatment with fulvic acid under salt stress did not modify GST enzyme activity at 6 h and decreased it at 12h. Pre-treatment with fulvic acid under salt stress, compared to salt treatment alone, induced GST1 and 2. Pre-treatment with fulvic acid under heat stress, compared to heat stress alone, increased GST enzyme activity at 6 h, but no change was observed at 12 h. Similarly to the total

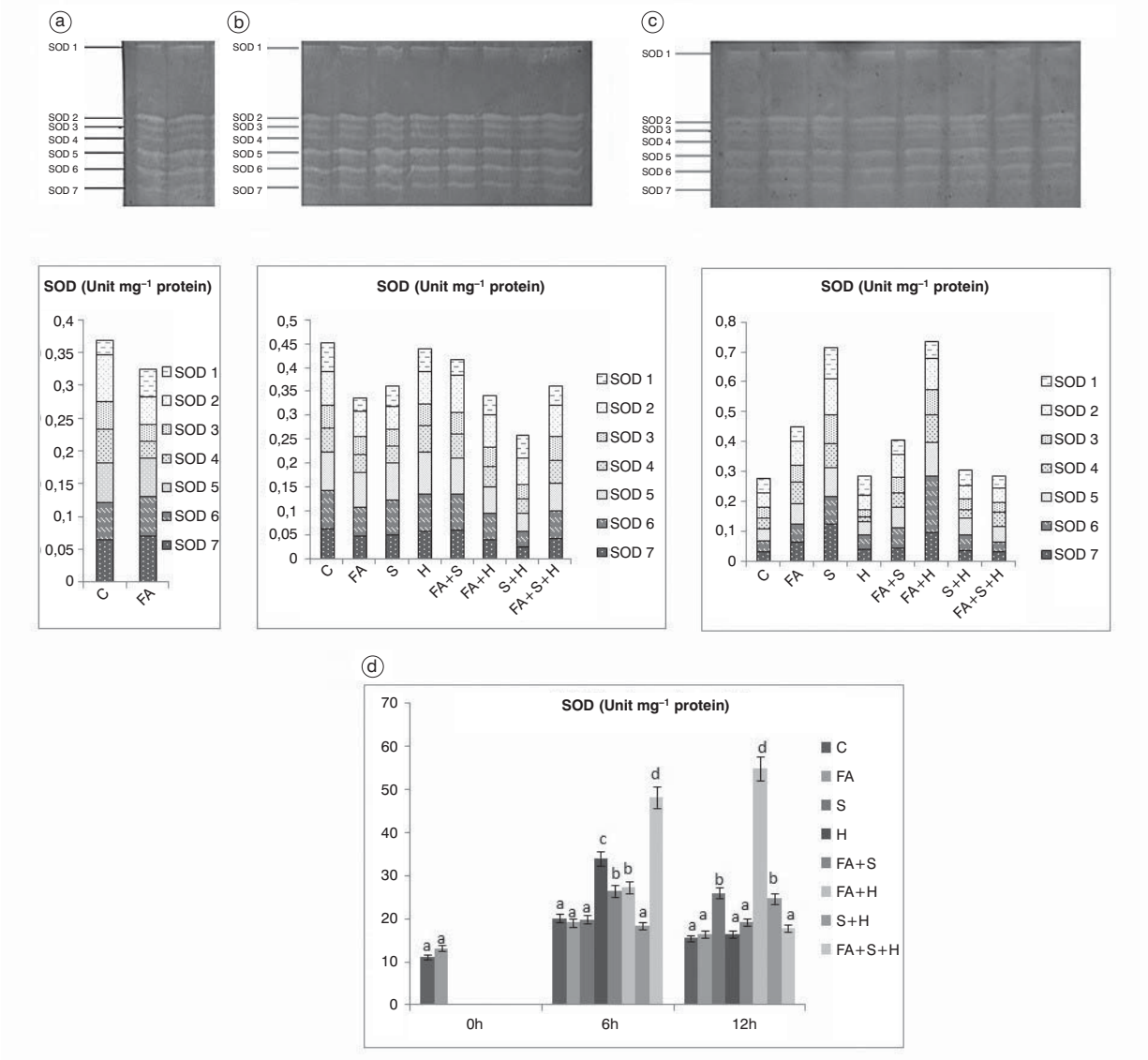

Fig. 3. Time course effects of fulvic acid pretreatment on leaf activity staining, % induction of SOD isoenzymes (**a-b-c**) and total superoxide dismutase (SOD) activity (**d**) in soybean (*Glycine max* L.) seedlings under salt and heat stresses. Control (C), Fulvic acid (FA), Salt stress (S), Heat stress (H), Salt + Heat stress (S+H), Fulvic acid + Salt stress (FA+S), Fulvic acid + Heat stress (FA+H), Fulvic acid + Salt + Heat stress (FA+S+H). Columns with different letters represent significantly different ($P < 0.05$) values.

GST enzyme activity, pre-treatment with fulvic acid under heat stress increased the expression of all of the isoenzymes, compared to heat treatment alone; however, no changes were observed at 12 h.

RUBISCO, CYTOCHROME C OXIDASE, HSP70 GENE EXPRESSION

In addition, pre-treatment with fulvic acid under salt stress suppressed *rubisco* expression at 6 h but induced it significantly at 12 h, compared to salt stress alone (Fig. 6). Furthermore, *cytochrome c oxidase* expression was induced by fulvic acid treatment under salt stress (maximum at 6 h), while salt stress increased and decreased *Hsp70* transcript levels at 6 h and 12 h, respectively. Moreover, pre-treatment with fulvic acid under heat stress induced *rubisco* and *cytochrome c oxidase* enzyme expression (maximum at 12 h), in comparison to heat treatment alone. However, *Hsp70* was significantly up-regulated in plants that were pre-treated with fulvic acid and heat stress at 6 h, while it was

TABLE 2. Time course effects of fulvic acid pretreatment on leaf RWC content of soybean (*Glycine max* L.) seedlings under salt and heat stress. Control (C), Fulvic acid (FA), Salt stress (S), Heat stress (H), Salt + Heat stress (S+H), Fulvic acid + Salt stress (FA+S), Fulvic acid + Heat Stress (FA+H), Fulvic acid + Salt + Heat stress (FA+S+H). Columns with different letters represent significantly different ($P < 0.05$) values.

		0 h	6 h	12 h
	GROUPS			
RWC (%)	C	73.510± 11.725^{a}	77.721± 2.329^{b}	75.704± 4.040^{b}
	FA	74.688± 2.826^{a}	75.465± 29.364^{b}	72.502± 6.996^{b}
	S		66.790± 29.679^{a}	61.460± 13.695^{a}
	H		64.426± 8.509^{a}	61.767± 12.954^{a}
	FA+S		72.477± 6.585^{c}	67.437± 9.638^{c}
	FA+H		72.084± 10.983^{c}	70.952± 8.763^{c}
	S+H		69.841± 7.564^{a}	71.117± 4.828^{a}
	FA+S+H		75.889± 5.838^{b}	73.622± 13.985^{b}

TABLE 3. Genes used in this study, The National Center for Biotechnology Information (NCBI) gene identification number (gene ID), forward (F) and reverse primer sequences, probe numbers and expected amplicon length

Gene	Gene ID Number	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Probe Number (Roche Cat. No)	Amplicon length (bp)
Rubisco	3989271	CGAGTAACTCCTCAACCAGGA	AGTAGAAGATTCGGCGGCTA	#124 (04693582001)	69
Cytochrome c oxidase	100782702	CCCTACAATAGCCCCTTGTG	TGCTGCATCTTGAAACCCTA	#84 (04689089001)	61
Hsp70	100816111	TTCCCAGAAACACAACCATTC	GGTTGTCGGAATAGGTCGAA	#68 (04688678001)	63
Cons 4 (Libault et al., 2008)	BU578186	GATCAGCAATTATGCACAACG	CCGCCACCATTCAGATTATGT	#74 (04688970001)	106

suppressed at 12 h. Fulvic acid application induced *cytochrome c oxidase* at 6 h but suppressed it at 12 h. Salt and heat treatment increased the levels of *Hsp70* transcript at 6 h and suppressed them at 12 h, as compared to control groups. However, fulvic acid treatment induced this transcription both at 6 h and 12 h, compared to the combined group.

DISCUSSION

In the present study, pre-treatment with fulvic acid followed by salt stress results in a decrease in Na^{+}, Cl^{-} ions and induces Ca^{2+} ions significantly after 6 h and 12 h (Tab. 1). In agreement with these findings, Fernandes et al. (2009) reported that leonardite, which is a derivative of humic acid, induced Ca^{2+} ions significantly in olive trees. However, pretreatment with fulvic acid under heat stress did not change Na^{+} and Cl^{-} but decreased Ca^{2+} at 6 h. This comparative result shows that fulvic acid induces a different signaling network in response to different stress conditions (salt or heat) that may depend on the requirements of the plants under stress conditions. Fulvic acid may be capable of maintaining the ion balance, in particular Na^{+} and Cl^{-} ions, under salt compared to heat stress. These results clearly showed that fulvic acid might provide protection against the adverse effects of stress treatment by maintaining Ca^{2+} levels.

According to our data, fulvic acid application alleviated the salt or heat stress alone and combined stress induced damage (reduced RWC) in the leaves of soybean (Tab. 2). This alleviation may be related to an increase in water uptake and mineral ions by roots from the soil and the chelation of toxic ions due to the antioxidant properties of fulvic acid. Overall, it can be suggested that fulvic acid increased the tolerance of soybean leaves to stress in this experiment. In agreement with this result, it has been reported that a fertilizer containing K^{+} increases the water content of leaves under drought

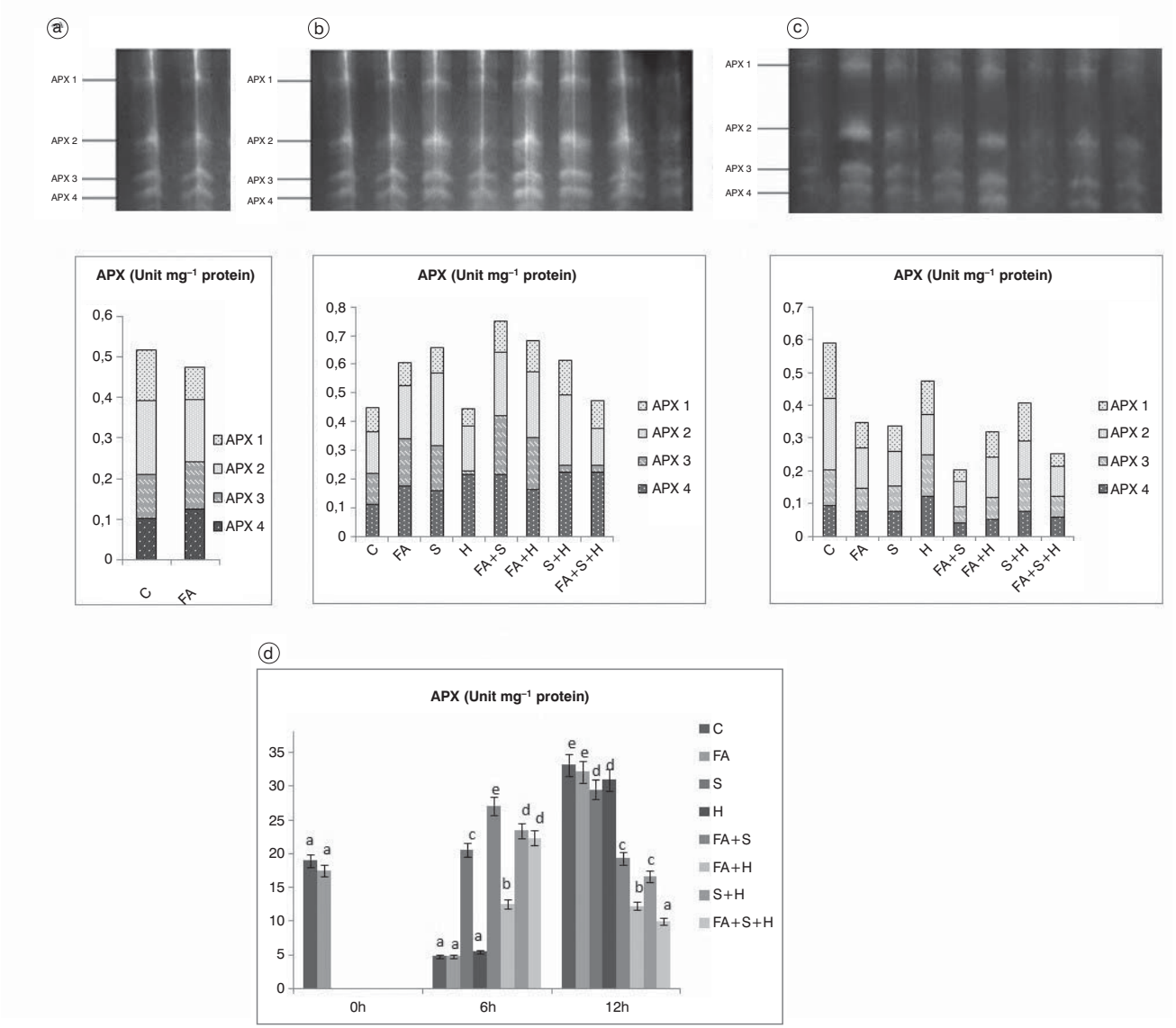

Fig. 4. Time course effects of fulvic acid pretreatment on leaf activity staining, % induction of APX isoenzymes (**a-b-c**) and total ascorbate peroxidase (APX) activity (**d**) in soybean (*Glycine max* L.) seedlings under salt and heat stresses. Control (C), Fulvic acid (FA), Salt stress (S), Heat stress (H), Salt + Heat stress (S+H), Fulvic acid + Salt stress (FA+S), Fulvic acid + Heat stress (FA+H), Fulvic acid + Salt + Heat stress (FA+S+H). Columns with different letters represent significantly different ($P < 0.05$) values.

stress in beans (Nandwal et al., 1998). Moreover, these results are consistent with those of Anjum et al. (2011), who also reported that fulvic acid treatment increased RWC in wheat plants.

Many studies have shown that proline, which is one of the biocompatible accumulating solutes, shows differences in a time frame of hours in plants (Balibrea et al., 1997). In the present study, pretreatment with fulvic acid increased proline content of soybean leaves, compared to salt treatment alone, but pre-treatment with fulvic acid under heat stress resulted in a different response, compared to salt treatment. This finding also showed that fulvic acid may play an important role in the increase in proline content but also functions with proline to maintain the water balance. Yet, heat and salt treatment did not affect the proline content at 6 h but increased it at 12 h, compared to the control group (Fig. 1). Kuznetsov et al. (1997) reported that heat and salt stress increased the proline content of

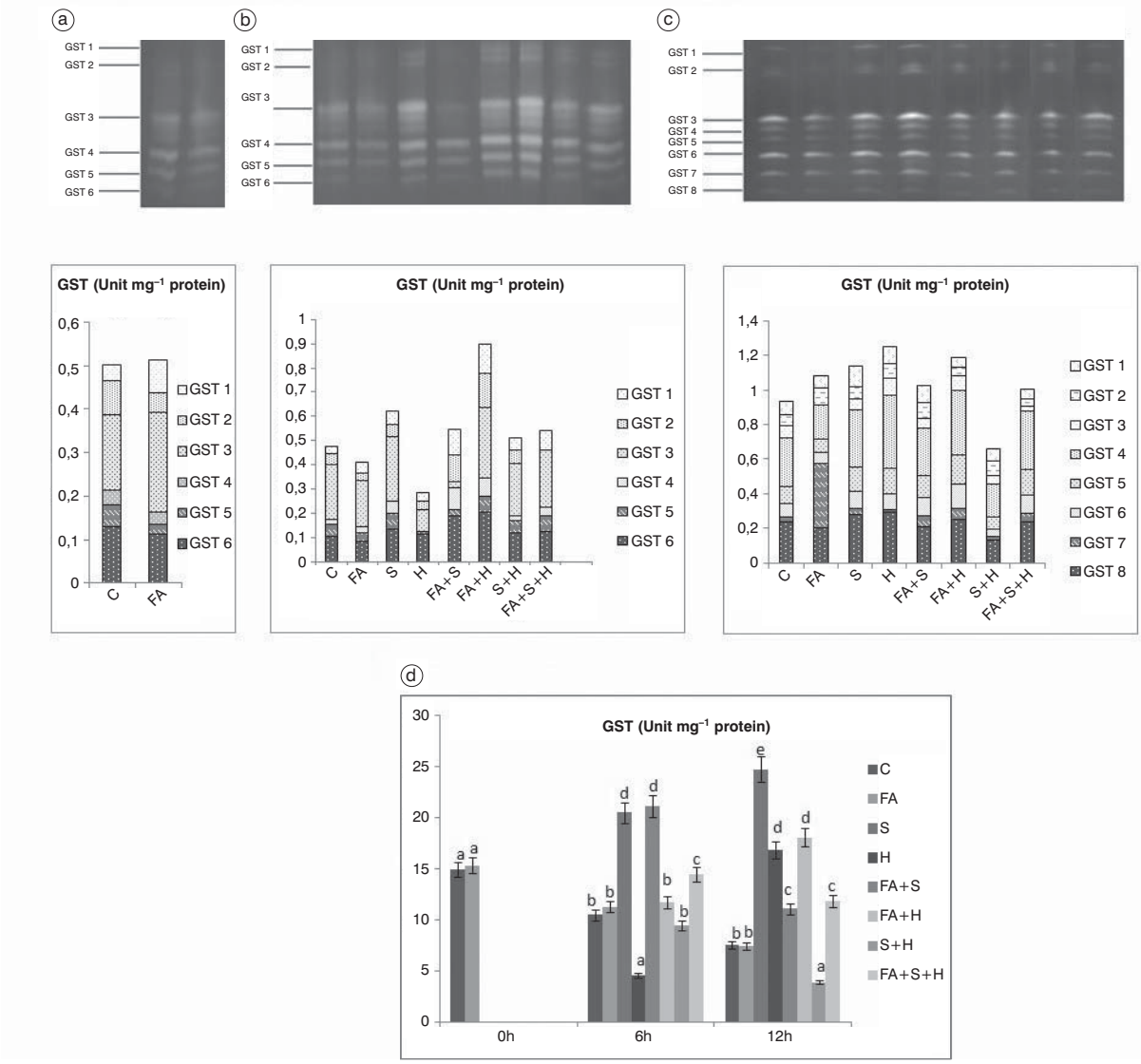

Fig. 5. Time course effects of fulvic acid pretreatment on leaf activity staining, % induction of % GST isoenzymes (**a-b-c**) and total glutatione S-transferase (GST) activity (**d**) in soybean (*Glycine max* L.) seedlings under salt and heat stresses. Control (C), Fulvic acid (FA), Salt stress (S), Heat stress (H), Salt + Heat stress (S+H), Fulvic acid + Salt stress (FA+S), Fulvic acid + Heat stress (FA+H), Fulvic acid + Salt + Heat stress (FA+S+H). Columns with different letters represent significantly different ($P < 0.05$) values.

tobacco plants. Pre-treatment with fulvic acid under salt and heat stresses reduced the proline content at 6 h and 12 h.

Membrane lipid peroxidation in soybean leaves was determined based on the content of malondialdehyde (MDA). As it is shown in Fig. 1, pre-treatment with fulvic acid under stress decreased the MDA and H_2O_2 content at all of the assessed time points. In agreement with this result, the application of humic acid has also been shown to decrease the MDA content in rice plants under water stress (Garcia et al., 2014) and in grapes in response to biotic stress (Kesba and El-Baltagi, 2012).

Initially, exogenous fulvic acid application was reported to maintain the water balance by causing the closure of the stomata in wheat plants (Xudan, 1986). This closure could be achieved by ABA, which is known as a signal hormone for plants (Zhang et al., 2006). Nevertheless, our results showed that pre-treatment with fulvic acid under salt stress increased ABA levels at 6 h but decreased these levels significantly at 12 h, despite

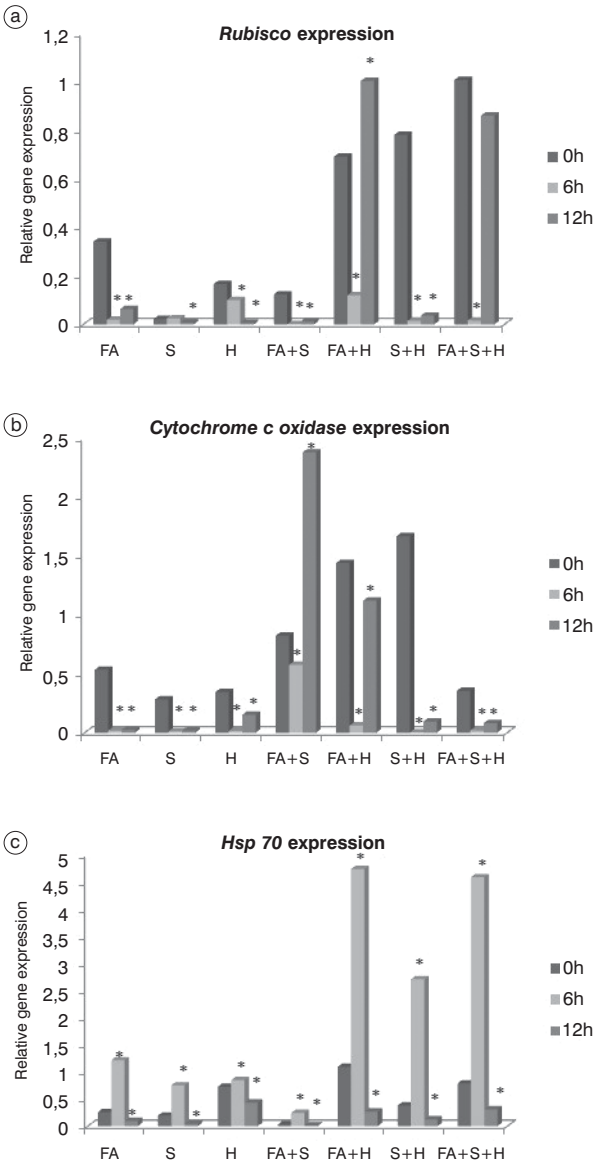

Fig. 6. Time course effects of fulvic acid pretreatment on relative (**a**) *rubisco*, (**b**) *cytochrome c oxidase* and (**c**) *Hsp 70* gene expression determined by qRT-PCR in leaves of (*Glycine max* L.) under salt and heat stresses. Data are means ± SE of three replications. Asterisks denote statistically different samples at $P < 0.05$ according to pairwise fixed reallocation randomization test.

providing protective effects against oxidative damage (reduced MDA content) at 6 h and 12 h (Fig. 2). Based on this finding, it can be suggested that fulvic acid may have changed the ABA levels and stomatal conductance in soybean leaves at an earlier stress time point (6 h) but maintained the ion content and water balance independently of the ABA content and stomatal closure at later stress time points (12 h). Otherwise, pre-treatment with fulvic acid increased ABA levels under both heat and combination (salt and heat) stresses. This comparative result shows that fulvic acid induces a different signaling network in response to different stress conditions that may depend on the requirements of the plants under stress conditions. Fulvic acid may be capable of maintaining the ion balance, in particular Na^+ and Cl^- ions, under salt compared to heat stress. Consistent with this result, Morard et al. (2010) showed that humic acid, which has similar properties to ABA, decreased transpiration in roots. It can be suggested that fulvic acid plays a role similar to ABA hormone in soybean leaves.

SOD (EC 1.15.1.1) is a major scavenger of superoxide ($O_2^{-\cdot}$) and plays a role in the formation of H_2O_2 and O_2 (Meloni et al., 2003). Pre-treatment with fulvic acid under salt treatment or combination (salt and heat) increased enzyme activity at 6 h, while it increased under heat stress at 12 h (Fig. 3). Consistent with this result, fulvic acid increased SOD enzyme activity in wheat under drought stress (Yuling et al., 2000). Similarly, Sun et al. (2004) showed that SOD enzyme activity increased in response to humic acid application in pepper plants.

Hydrogen peroxide is scavenged by ascorbate peroxidase (APX, EC 1.11.1.11) through the use of ascorbate as an electron donor (Asada, 1992). Pre-treatment with fulvic acid under salt or heat stress, compared to salt treatment alone, increased APX enzyme activity at 6 h and decreased at 12 h, while it was not efficient under the combination (Fig. 4). Consistent with these results, Kesba and Beltagi (2012) showed that humic acid induced APX enzyme activity in grape plants under biotic stress. GSTs (EC 2.5.1.18) catalyze the nucleophilic attack of the thiol group of the tripeptide glutathione (GSH) by various electrophilic molecules (Sheehan et al., 2001). Pre-treatment with fulvic acid under salt stress did not modify GST enzyme activity at 6 h and decreased at 12h. Pre-treatment with fulvic acid under heat stress, compared to heat stress alone, increased GST enzyme activity at 6 h, but no change was observed at 12 h. Pre-treatment with fulvic acid under salt and heat stresses increased GST activity at 6 h and 12 h. Because there are no reports regarding the effects of fulvic acid on GST enzyme activity, the present study is the first to demonstrate that fulvic acid has an inductive role in GST enzyme activity (Fig. 5).

Rubisco 1,5 bisphosphate carboxylase/oxygenase (RuBP, EC.4.1.1.39) plays a key role in the reduction of photosynthetic CO_2. Pre-treatment with fulvic acid under salt or heat stress induced *rubisco*

expression significantly at 12 h, while this expression was induced under combination at 6 h and 12 h. This result clearly suggested that fulvic acid induces photosynthesis mechanism under stress conditions. Consistent with this result, 200 mM NaCl and heat stress has been shown to reduce *rubisco* enzyme activity in rice plants (Sivakumar et al., 2000) and in cotton plants (Crafts-Brandner et al., 2000).

Cytochrome c oxidase enzyme (EC. 1.9.3.1), which is localized in the electron transport system of mitochondria, produces two water molecules via the transfer of electrons to O_2. Deficiency or lack of this enzyme is lethal for plants because mitochondria, but not chloroplasts, also produce reactive oxygen species (Dahan et al., 2014). Furthermore, *cytochrome c oxidase* expression was induced by fulvic acid treatment under salt stress (maximum at 6 h), while it was maximum at 12 h under heat stress. Similarly to this result, drought and heat stress induced photosynthesis and cytochrome respiration pathway in drought tolerant *Capsicum annuum* L. plants (Hu et al., 2010). This result demonstrated that fulvic acid promoted the conversion of oxygen to water in soybean leaves under stress conditions. This finding could also be related to the reduced MDA content and alleviated oxidative damage provided by fulvic acid. To date, no studies have investigated this phenomenon. Fulvic acid application induced *cytochrome c oxidase* at 6 h but suppressed it at 12 h under combination. Based on this observation, it can be suggested that the application of fulvic acid under salt and heat stresses does not affect respiration at 12 h. Regarding the results obtained for MDA and *cytochrome c oxidase* at 12 h, fulvic acid had a more positive effect at 6 h, compared to 12 h.

Hsp70 has essential functions in preventing aggregation and in assisting with the refolding of non-native proteins under both normal and stress conditions (Hartl, 1996). It is known that over-expression of *Hsp70* genes correlates positively with the acquisition of thermotolerance and results in an enhanced tolerance to salt, water and high temperature in plants (Wang et al., 2004). Under high salinity conditions, fulvic acid treatment significantly suppressed *Hsp70* transcript levels at 6 h and 12 h. Heat stress increased and decreased *Hsp70* transcript levels at 6 h and 12 h, respectively. In agreement with this result, it has also been reported that in transgenic *T. harzianum* the expression of the *Hsp70* gene increased in response to heat tolerance (Montero-Barrientos et al., 2010). However, in the present study, *Hsp70* was significantly up-regulated in plants that were pre-treated with fulvic acid and heat stress at 6 h, while it was suppressed at 12 h. As mentioned previously, fulvic acid protected soybean leaves against heat stress in a manner that differed from its function in response to high salinity. Salt and heat treatment increased the levels of *Hsp70* transcript at 6 h and suppressed them at 12 h. However, fulvic acid treatment induced this transcription both at 6 h and 12 h. These findings demonstrated that fulvic acid protected soybean leaves by maintaining the water and ion balance independently of *Hsp70* signaling under high salinity based on the results for MDA.

CONCLUSIONS

In the past, fulvic acid was known only as a "fertilizer" which protects plants by increasing the soil efficiency and fertility, having hydrolysis capacity, chelating of mineral ions and buffering properties. Nowadays, it begins to attract attention in plant stress response by only inducing some antioxidant enzyme but there is no detailed study in literature. The present results indicated that the foliar application of fulvic acid protected soybean plants against stress-induced damage not only by maintaining the water balance, proline content and ABA levels but also by reducing toxic ion contents (Na^+, Cl^-, Ca^{2+}) and inducing antioxidant enzyme activities and some important gene expression in soybean leaves. Given the observed antioxidant enzyme responses, the results of this study clearly showed that fulvic acid induced antioxidant enzyme during decreased or unchanged stress conditions. However, while no changes were detected, the soybean leaves had already induced these enzyme levels. In addition, fulvic acid application under salt stress induced *rubisco* expression only at 12 h, while under heat stress, fulvic acid significantly induced both *rubisco* and *cytochrome c oxidase* expression at 6 h and 12 h. On the other hand, the application of fulvic acid under high salinity conditions suppressed the *Hsp70* transcript levels; however, it induced the levels of these transcripts under heat stress at 6 h. In conclusion, "fulvic acid" application protected soybean leaves during stress conditions via stimulant and regulatory mechanisms.

AUTHORS' CONTRIBUTIONS

The following declarations about authors' contributions to the research have been made: supervision of the research design and manuscript preparation: BSD, physiological and biochemical analysis: BSD, molecular analysis: EGG and TT. The authors declare that they have no conflicts of interest.

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