

Assessment of antioxidant system and enzyme/nonenzyme regulation related to ascorbate-glutathione cycle in ferulic acid-treated *Triticum aestivum* L. roots under boron toxicity

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Abstract: Ferulic acid (FA; 3-methoxy-4-hydroxycinnamic acid) can eliminate stress-induced damage because of its ability to induce antioxidant activity under stress. The aim of this study was to identify the effects of FA on water status, antioxidant system, and lipid peroxidation in wheat (*Triticum aestivum* L.) roots exposed to boron (B) stress. Plants were grown in hydroponic culture containing the combination or alone form of 25–75 μ M FA and 4–8 mM B. Stress significantly decreased growth (RGR), water content (RWC), proline content (Pro), and osmotic potential (Ψ_{II}). However, FA alleviated the decrease in RGR, RWC, and Pro content. Compared to the control groups, stress decreased the activities of superoxide dismutase (SOD), peroxidase (POX), catalase, and ascorbate peroxidase (APX), but an increase was only observed in glutathione reductase (GR) activity. Hydrogen peroxide (H_2O_2) content accumulated with B stress. Besides, a notable decrease was observed in the scavenging activity of hydroxyl radical (OH^*); thus, wheat roots had high lipid peroxidation (thiobarbituric acid reactive substance content). In response to stress, FA triggered the activities of SOD, POX, and APX. Moreover, when FA was made present in stressed wheat roots, we observed the enhanced activities of dehydroascorbate reductase, and monodehydroascorbate reductase and dehydroascorbate contents which are related to ascorbate-glutathione cycle, so FA could maintain ascorbate (AsA) regeneration. However, when wheat roots were treated with stress, FA did not induce the regeneration of glutathione because of decline in GR activity. Due to successful elimination of H_2O_2 content, the exogenous application of FA alleviated B-induced lipid peroxidation in wheat. Consequently, FA eliminated the damage induced by B stress via the increased POX and the enzymes related to Asada-Halliwell pathway (AsA-GSH cycle) in wheat roots.

Key words: Antioxidant system, ascorbate-glutathione cycle, boron stress, ferulic acid, *Triticum aestivum* L.

1. Introduction

Phenolic compounds, containing ferulic acid (FA; 3-methoxy-4-hydroxycinnamic acid), are widely present in vascular plants especially in cell walls, leaves and seeds (Mathew and Abraham, 2004). FA is synthesized from L-tyrosine or phenylalanine through the shikimate pathway, producing monomeric and polymeric phenols and polyphenols (Lattanzio et al., 2006). FA occurs in varying amounts depending on the plant variety (Hatcher and Kruger, 1997) and has been reported to possess various physiological and biological functions, including antioxidant, hepatoprotective, antiallergic, anticarcinogenic, antiviral, antiinflammatory, antimicrobial, and antithrombotic in animals and plants (Rukkumani et al., 2004). The importance of FA for plants

exposed to the stress conditions is based on its roles as high radical scavengers for free radicals such as superoxide and hydroxyl radical (Acosta-Estrada et al., 2014). Its antioxidant properties come from the numbers and position of the hydroxyl groups in relation to the carboxyl functional group (Amarowicz et al., 2004), metal chelating activity (Balasundram et al., 2006), and induction of proline and soluble sugar (Li et al., 2013).

Boron (B) is an essential micronutrient in plants (Emebiri et al., 2009) and is a metalloids since it shared the properties of both metal and nonmetal (Nable et al., 1997). The amount of B required varies among plant species for optimal growth and development. The primary roles of B in plants are important such as lignification of cell wall, maintenance of plasma membrane function, regulation of

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proton pumps in plasma membrane, nitrogen metabolism, and stimulation of the nucleic acid metabolisms (Reid, 2010). However, an accumulation of B can lead to a toxic level in soils which consequently cause plant disorders and thus affect the quality of crops. The basic reason for B toxicity is to bind to the multiple hydroxyl groups of compounds such as ribose sugar or interference with transcription and translation (Reid, 2010). Excess of B can lead to reactive oxygen species (ROS) generation (Velez-Ramirez et al., 2011) such as hydroxyl radicals (OH^\bullet) and hydrogen peroxide (H_2O_2). Enzymatic/nonenzymatic antioxidants are involved in the defense system against these free radicals (Uluisik et al., 2018). As well as antioxidant activity, phenolic compounds can act as scavengers of free radical and lipid peroxidation inhibitors (Verstraeten et al., 2003).

Phenols and their metabolism against stress help avoid the formation of ROS by limiting the excitation of chlorophyll for the photosynthetic apparatus and greater hydrogen donating and radical stabilization (Michalak, 2006). It is reported that the usage of exogenous substances increases the activity levels of antioxidant in crops (Abu El-Soud et al., 2013). Moreover, Ouyang et al. (2007) reported that salt stress increased the activity of the phenylalanine ammonia lyase (PAL), which plays a role in the biosynthesis of phenolics. However, no reports have focused on the protective roles of FA in plants exposed to B stress. Therefore, our aim was to find out if there is any relationship between exogenous application of FA and stimulation of the antioxidant response under B stress. In this study, wheat (*Triticum aestivum* L.) was chosen as plant material because of its economic importance throughout the world.

2. Materials and methods

2.1. Plant material and experimental design

Wheat seeds (*Triticum aestivum* L. cv. *Dagdas-94*) were obtained from the Bahri Dagdas International Agricultural Research Institute Konya, Turkey. They (*Triticum aestivum* L.) were surface-sterilized and were germinated on the double-layer filter paper wetted with distilled water. The germinated seedlings were hydroponically grown in half strength Hoagland solution under controlled growth conditions for 21 days (d) and the solutions were replaced by fresh half-strength Hoagland solution, twice a week. B and FA were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and for FA and B treatments, they were dissolved in Hoagland solution and were added to the growth medium. In a preliminary experiment for determination of ideal experimental B and FA concentrations, 4, 8, and 10 mM B, and 25, 75 μM and 1 mM FA were applied to wheat plants. However, in 10 mM B and 1 mM FA-treated groups, the plants could not sustain their viability. Therefore, 25

and 75 μM FA were added alone or in combination with B (4 mM and 8 mM). The treatment continued for 7 days and then the plants were harvested.

2.2. Determination of growth, water content, osmotic potential, and proline content

The relative growth rate (RGR) was measured according to Hunt et al. (2002). After harvest on 7 days, six roots were obtained from wheat and their fresh weight (FW) was determined. The roots were floated on deionized water for 6 h and the turgid tissue was blotted dry prior to determining turgid weight (TW). Dry weight (DW) was determined after oven drying at 70 °C. The root relative water content (RWC) was calculated by the following formula (Smart and Bingham, 1974):

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

Roots were extracted by crushing the material with a glass rod. Root osmotic potential (Ψ_{π}) was measured by Vapro Vapor pressure Osmometer 5600. Ψ_{π} was converted to MPa according to Santa-Cruz et al. (2002) by multiplying coefficient of 2.408×10^{-3} .

Proline (Pro) content was determined according to Bates et al. (1973). The roots were homogenized in 3% sulphosalicylic acid and the homogenate was filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the mixture was heated at 100 °C. The mixture was extracted with toluene and the absorbance of fraction with toluene aspired from liquid phase was measured at 520 nm.

2.3. Determination of endogenous ion contents in roots

Finely ground samples (0.5 g) of dried plant material were hydrolyzed with concentrated nitric acid in a microwave reaction system (MARS5; CEM). The contents of B, K^+ , Ca^{2+} , Mg^{2+} , and Fe^{2+} in root extracts were analyzed using Varian Vista-MPX simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES) (Nyomora et al., 1997).

2.4. Determination of ROS accumulation

Hydrogen peroxide (H_2O_2) content was determined according to Liu et al. (2010). The roots were homogenized in cold acetone and centrifuged. The supernatant was mixed with titanium reagent and then ammonium hydroxide was added to precipitate the titanium-peroxide complex. The reaction mixture was centrifuged. The pellet was washed with cold acetone and was dissolved. The absorbance of the solution was measured at 410 nm. H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2 .

Hydroxyl radical (OH^\bullet) scavenging activity was determined according to Chung et al. (1997), with minor changes. The reaction mixture contained 20 mM sodium-phosphate buffer, 10 mM 2-deoxyribose, 10 mM FeSO_4 , 10 mM EDTA, 10 mM H_2O_2 , 0.525 mL H_2O , and 0.075 mL sample. The mixture was incubated at 37 °C. A

mixture of 2.8% (w/v) trichloroacetic acid and 1.0% (w/v) thiobarbituric acid in 50 mM NaOH was added to the test tubes and boiled. After the mixture cooled, absorbance was measured at 520 nm against a blank solution. The OH[•] radical scavenging activity was calculated using the following formula:

$$\text{OH}^{\bullet} \text{ radical scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 was the absorbance of the blank and A_1 the absorbance of the sample.

2.5. Enzyme extraction and determination of isozyme and/or enzyme compositions

For protein and enzyme extractions, 0.5 g of each sample was homogenized in 50 mM Tris-HCl (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol (DTT). For ascorbate peroxidase (APX) activity determination, 5 mM ascorbate (AsA) was added to the homogenization buffer. Samples were centrifuged at $14000 \times g$ for 30 min and the supernatant was used for the determination of protein content and enzyme activities. The total soluble protein content of the enzyme extracts was determined (Bradford, 1976) using bovine serum albumin as a standard. All spectrophotometric analyses were conducted on a Shimadzu spectrophotometer (UV 1800).

Samples containing equal amounts of protein were subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970) with minor modifications. Superoxide dismutase (SOD) activity was detected by photochemical staining using riboflavin and nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). The units of activity for each SOD isozyme were calculated by running a SOD standard from bovine liver (Sigma Chemical Co., St. Louis, MO, USA). The different types of SOD were discriminated by incubating gels with different types of SOD inhibitors before staining: Mn-SOD activity was resistant to both inhibitor treatments and Cu/Zn-SOD activity was sensitive to 2 mM KCN. Cu/Zn-SOD and Fe-SOD activities were inhibited by 3 mM H₂O₂ (Vitória et al., 2001). The total SOD (EC 1.15.1.1) activity assay was based on the method of Beauchamp and Fridovich (1971), which uses spectrophotometric analysis at 560 nm to measure the inhibition of the photochemical reduction of NBT. One unit of specific enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT reduction. Analysis of catalase (CAT) isozymes was completed using the procedure proposed by Woodbury et al. (1971). Total CAT (EC 1.11.1.6) activity was estimated according to the method of Bergmeyer (1970), which measured the initial rate of H₂O₂ disappearance at 240 nm. The decrease in absorption was followed for 3 min, and one unit of CAT was defined as

1 mmol H₂O₂ decomposed min⁻¹ mL⁻¹. Peroxidase (POX) isozymes were detected according to Seevers et al. (1971). Electrophoretic separation of samples was performed on nondenaturing polyacrylamide. Total POX (EC 1.11.1.7) activity was based on the method described by Herzog and Fahimi (1973). The increase in the absorbance at 465 nm was followed for 3 min. One unit of POX activity was defined as mmol H₂O₂ decomposed min⁻¹ mL⁻¹. Electrophoretic ascorbate peroxidase (APX) separation was performed according to Mittler and Zilinskas (1993). Before the samples were loaded, gels were equilibrated with running buffer containing 2 mM AsA for 30 min. Total 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. The concentration of oxidized AsA was calculated by using a 2.8 mM⁻¹ cm⁻¹ extinction coefficient. One unit of APX was defined as 1 mmol AsA oxidized min⁻¹ mL⁻¹. Total GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). Activity was calculated using the extinction coefficient of NADPH (6.2 mM⁻¹ cm⁻¹). One unit of GR was defined as 1 mmol GSSG reduced min⁻¹ mL⁻¹. NADPH oxidase (NOX) isozymes were identified by NBT reduction method as described by Sagi and Fluhr (2001). The samples containing 40 µg protein was loaded per lane. Total NOX (EC 1.6.3.1) activity was measured according to Jiang and Zhang (2002). The assay medium contained 50 mM Tris-HCl buffer, 0.5 mM XTT, 100 mM NADPH, Na₄, and 20 µg of protein sample. After addition of NADPH, XTT reduction was followed at 470 nm. Activity was calculated using the extinction coefficient, 2.16×10^4 M⁻¹ cm⁻¹. One unit of NOX was defined as 1 nmol mL⁻¹ XTT oxidized min⁻¹.

Gels stained for SOD, CAT, NOX, POX, and APX activities were photographed with the Gel Doc XR+ System and then analyzed with Image Lab software v4.0.1 (Bio-Rad, California, USA). Known standard level of enzymes (0.5 unit for SOD, 0.2 unit for POX and CAT) was used.

2.6. Determination of the activity of monodehydroascorbate reductase and dehydroascorbate reductase

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed using the method of Miyake and Asada (1992). The reaction mixture contained 50 mM Hepes-KOH (pH 7.6), 1 mM NADPH, 2.5 mM AsA, 2.5 U AsA oxidase, and enzyme extract. The MDHAR activity was measured by decrease in absorbance as the amount of enzyme that oxidizes 1 mM NADPH per minute at 340 nm. A molar extinction coefficient of 6.2 mM⁻¹ cm⁻¹ was used for the calculation of enzyme activity.

Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was measured according to Dalton et al. (1986). DHAR activity was measured by increase in absorbance at 265 nm due to ascorbate formation. A molar extinction

coefficient of $14.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used for the calculation of enzyme activity.

2.7. Determination of the contents of dehydroascorbate and ascorbate

Total and reduced AsA contents were determined according to the method of Dutilleul et al. (2003) with modifications. Total and reduced AsA contents were assayed spectrophotometrically at 265 nm in 100 mM K-P buffer with 1.0 U of ascorbate oxidase. To calculate AsA, a specific standard curve of AsA was used. The oxidized form of ascorbate (DHA, dehydroascorbate) was measured using the formula: $\text{DHA} = \text{Total AsA} - \text{Reduced AsA}$.

2.8. Determination of the contents of glutathione and oxidized glutathione

Glutathione (GSH) was assayed according to Paradiso et al. (2008), utilizing aliquots of supernatant neutralized with 0.5 M K-P buffer. Based on enzymatic recycling, glutathione is oxidized by DTNB and reduced by NADPH in the presence of GR, and glutathione content is evaluated by the rate of absorption changes at 412 nm. Oxidized glutathione (GSSG) was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used for the quantification.

2.9. Determination of lipid peroxidation levels

The level of lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS) according to Rao and Sresty (2000). TBARS concentration was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The concentration of TBARS was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.10. Statistical analysis

The results were repeated thrice independently. The value was statistically analyzed with SPSS 20.0. Tukey's post hoc was used to compare the treatment groups. Comparisons with $P < 0.05$ were considered significantly different. In all the figures, the error bars represent standard errors of the means.

3. Results

3.1. Growth, water content, osmotic potential, and proline content

As shown in Figure 1a, RGR decreased under stress treatments and the maximum reduction in RGR was at 8 mM B by 49%. However, addition of FA led to the increase in RGR when compared to the stress treatment alone. In comparison to the control group, FA treatment alone caused an enhancement in RGR. Wheat roots had a reduction in RWC under B stress, which represents 6% and 7% decline (Figure 1b). The combination of FA and stress

led to alleviation in RWC. Moreover, an increase in RWC in FA-treated alone groups was observed. Pro content significantly decreased under B stress and reached the minimum levels at 8 mM B (62%) (Figure 1c). Besides the results of FA application alone, a slight increase under FA treatment in wheat roots subjected to B stress was detected in the Pro content compared to the stress treatment alone. The maximum induction of Pro content was at 8B+FA2 (3-fold increase). When wheat was exposed to B stress, Ψ_{Π} decreased to -0.522 and -0.553 MPa from -0.495 MPa at 4 mM and 8 mM B stress, respectively (Figure 1d). However, this reduction by B stress in Ψ_{Π} was triggered by the application of FA. Compared to control groups, Ψ_{Π} showed no major difference between FA treatment alone and control groups in wheat roots.

3.2. Endogenous ion contents in roots

The plants treated with stress had higher B content than the control groups (Figure 2a). B content of FA-treated plants in response to stress decreased; the decrease was more severe at FA2 plus B stress. After exposure to FA alone, endogenous B content was similar to those of the control groups. On the other hand, the contents of K^+ (Figure 2b), Ca^{2+} (Figure 2c), Mg^{2+} (Figure 2d), and Fe^{2+} (Figure 2e) decreased or were similar to those of the control groups under B stress. Addition of FA to B-treated plants significantly increased these ion contents (except for 4B+FA1 in Fe^{2+} content) when compared with the stress treatment alone.

3.3. ROS accumulation

The scavenging activity of hydroxyl radical (OH^{\bullet}) was declined with 4 mM and 8 mM B (Figure 3a). FA in combination with B stress displayed an enhancement in this activity. On the other hand, the scavenging activity of this radical was similar to the those of control groups under FA alone. The level of H_2O_2 content in FA-treated plants under control conditions did not show significant change (Figure 3b). However, B toxicity caused an increase in H_2O_2 content and it reached the maximum levels (1.7-fold increment) at 8 mM B. On the other hand, H_2O_2 content was markedly lowered in wheat exposed to FA plus stress than stress treatment alone. The presence of 4 mM and 8 mM B caused a prominent increase in TBARS content (1.1- and 1.7-fold, respectively) in roots compared to the nonstress conditions (Figure 3c). After FA application to stress-treated wheat, TBARS content decreased during experimental period. Besides, the impact of added FA alone to roots was not significant in TBARS content.

3.4. The activation of enzymatic and nonenzymatic anti-oxidant molecules

As it is evident from Figure 4a, three SOD isozymes were defined with gel assays and were determined as one Mn-SOD and two Fe-SOD1-2. Compared with control groups,

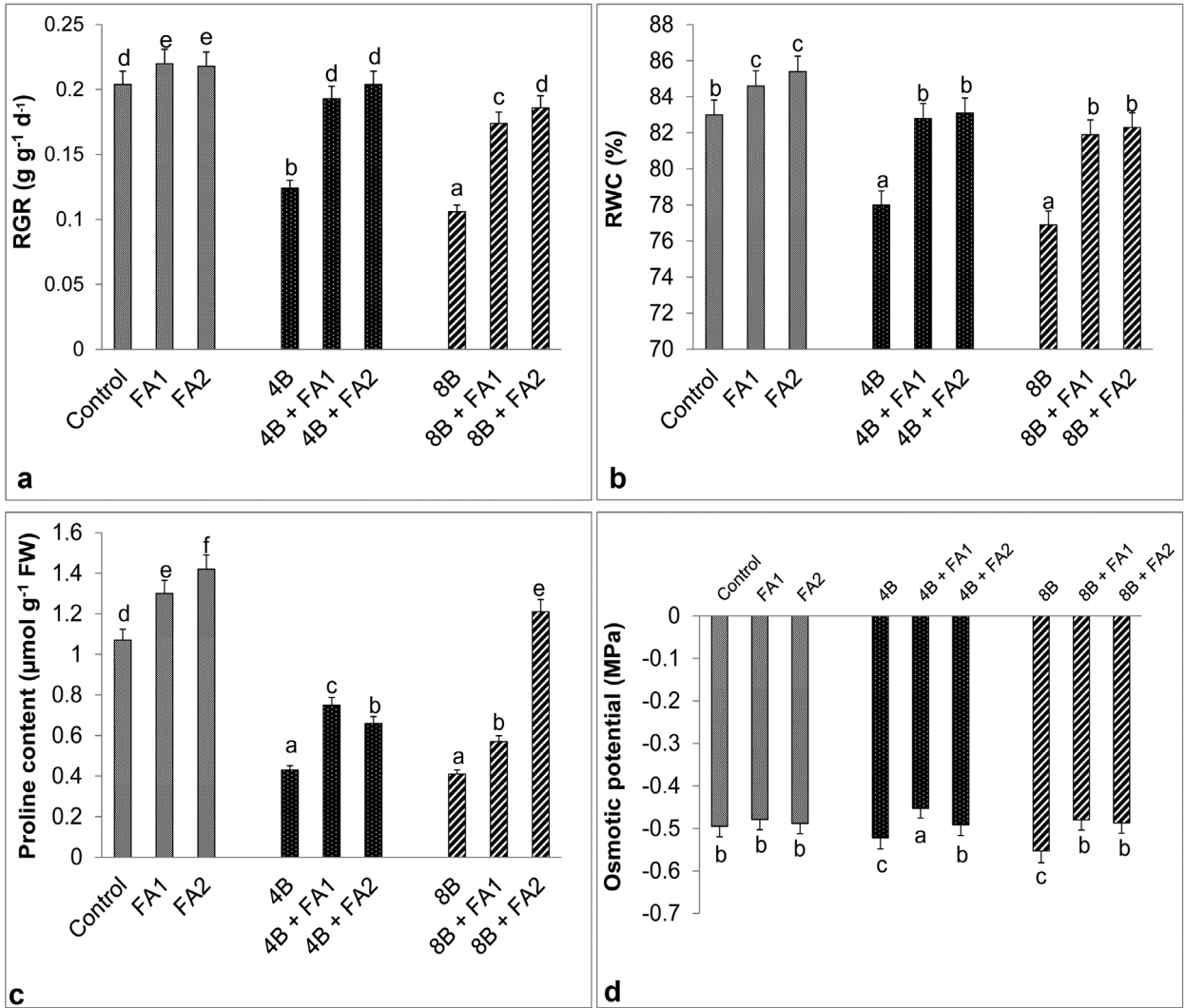


Figure 1. Effects of exogenous 25 (FA1) and 75 μM (FA2) FA treatment on growth (RGR, **a**), relative water content (RWC, **b**), proline content (Pro, **c**), and osmotic potential (Ψ_{π} , **d**) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate \pm SE and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

a reduction in total SOD activity observed under B stress (Figure 4b) depending on the intensities of Mn-SOD and Fe-SOD1. On the other hand, this decline caused by stress in SOD activity was prevented by FA treatment in comparison to the stress treatment alone. The maximum rate of this increase in SOD was by 2.7-fold at 8 mM B plus 75 μM FA (Figure 4b) with regard to the all intensities of SOD isozymes (Figure 4a). In the present study, only one CAT isozyme was identified (Figure 5a). Total CAT activity decreased under B stress (Figure 5b) which was paralleled to the intensity of CAT isoform (Figure 5a). While any change in CAT activity was observed under FA-alone treatment, the wheat roots with FA plus B stress exhibited a decrease in total CAT activity. Gel analysis revealed

that three POX isozymes (POX1-2-3) were observed in treatment groups (Figure 6a). B-treated plants had a reduction in total POX activity (Figure 6b). When stress and FA were applied together, FA prevented the decline in POX in terms of especially POX3 isoform (Figure 6a). As shown in Figure 7a, the isoforms of APX1 and APX2 were detected in the all treatment groups. B stress decreased total APX activity (Figure 7b). Moreover, both FA alone treatment and FA plus stress application showed a similar pattern which increased APX activity as suggested by the isozymes of APX1-2 (Figure 7a). Besides, wheat with B stress had an increase in GR activity (Figure 7c). Except for 4 μM B plus 25 mM FA, the exogenously applied FA with stress decreased total GR activity as compared to B

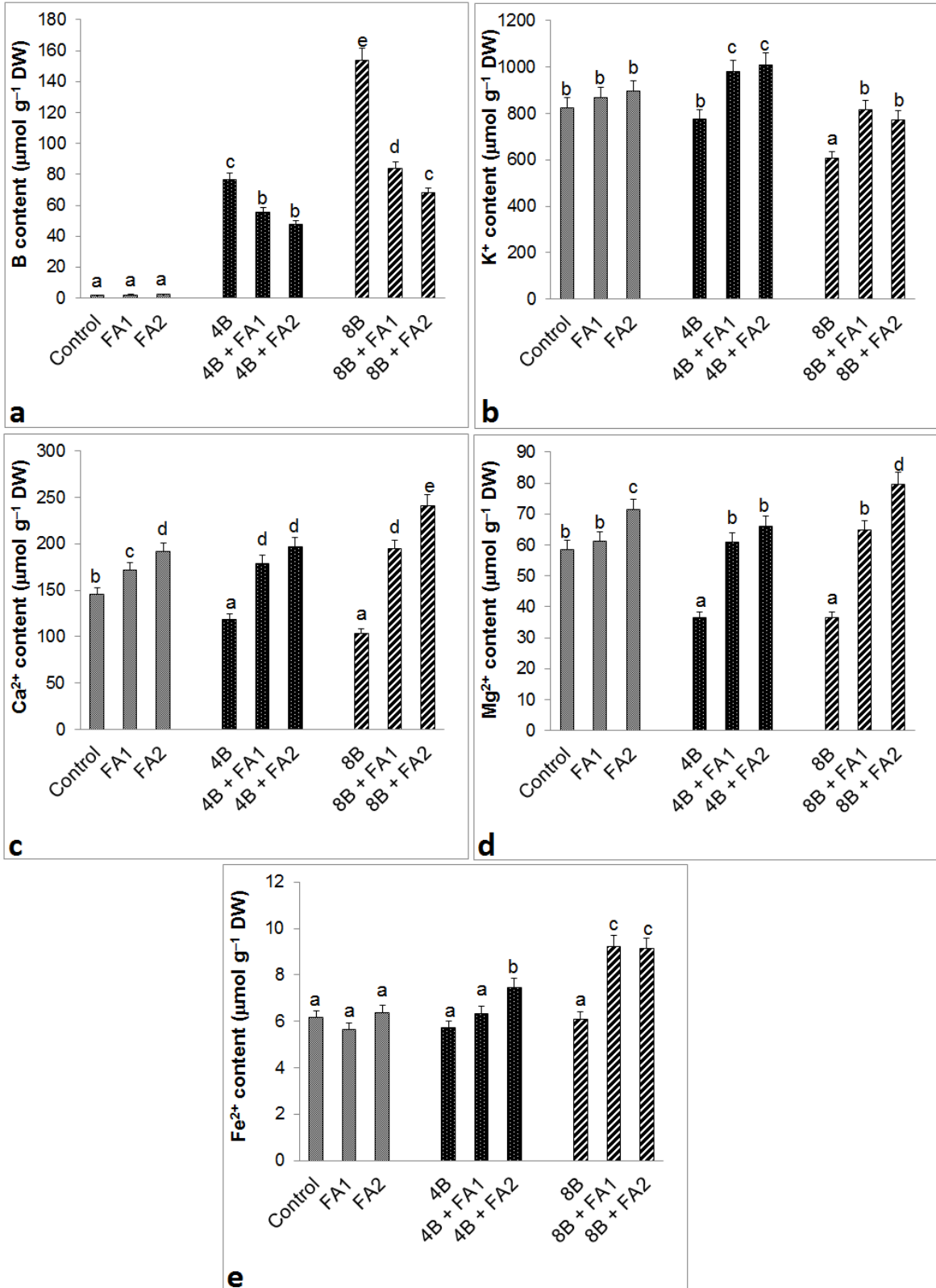


Figure 2. Effects of exogenous 25 (FA1) and 75 μM (FA2) FA treatment on the contents of B (a), K⁺ (b), Ca²⁺ (c), Mg²⁺ (d), and Fe²⁺ (e) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate \pm SE and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

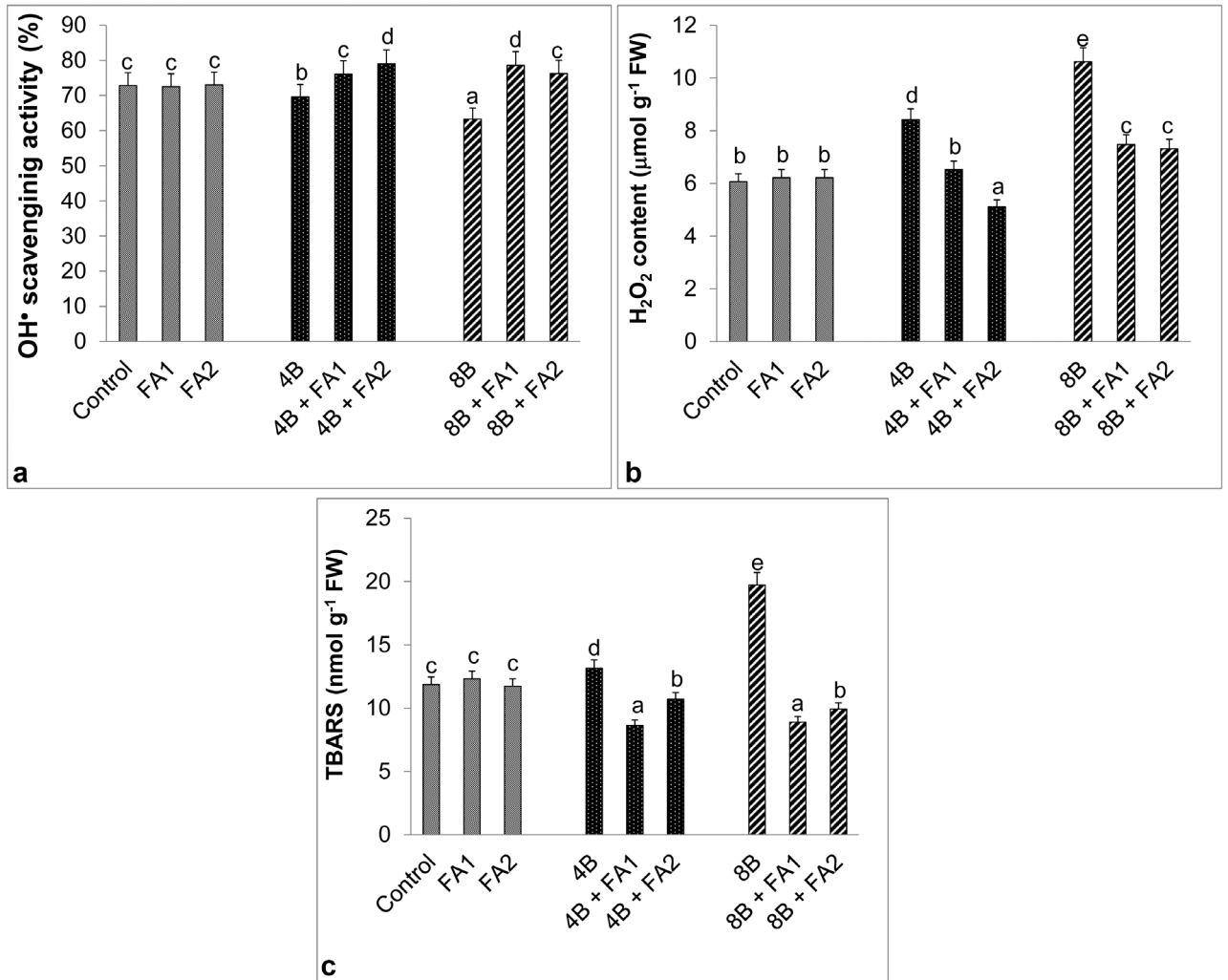


Figure 3. Effects of exogenous 25 (FA1) and 75 μM (FA2) FA treatment on the scavenging activity of hydroxyl radical (OH•, a) hydrogen peroxide (H₂O₂, b), and lipid peroxidation (TBARS, c) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate ±SE and the different lowercase letters are significantly different (P < 0.05) values according to the Tukey test. The error bars represent standard errors of the means.

treatments alone. Similar to this result, 25 μM FA alone treatment decreased total GR activity. The only one NOX band was identified in wheat roots (Figure 8a). Similar to the total NOX activity (Figure 8b), under B stress, the intensity of NOX isoform was weaker than those of the control groups (Figure 8a). However, the wheat roots possessed the highest total and isozyme activity of NOX after the combination treatment of FA and stress, as well as in FA-treated plants without stress. Compared with control group, B stress caused a reduction in total ascorbate (tAsA) content (Figure 9a). However, this change reduced by B stress in tAsA was prevented by exogenous FA application. There is no difference between 25 μM FA and control group in tAsA content, but the high FA concentration decreased this content. As illustrated in Figure 8b, similar

to the results of tAsA content, there was a decline in DHA content under B stress. On the other hand, stress plus FA led to a significant induction in DHA content as compared to B stress treatment alone. This change was also observed at 25 μM FA alone treatment. Besides, effect in DHAR activity was not observed under B stress and FA-alone treatment (Figure 9c). The enhancement in DHAR activity was triggered in wheat roots with FA plus stress and it was more at B+FA2 stress. While MDHAR activity declined under B stress compared to the control groups, after FA application under stress it was higher than stress treatment alone (Figure 9d). The results of MDHAR activity were similar between FA alone and FA+stress groups. As given in Figure 9e, B stress had an induction in GSH content. However, except for 4B+FA1, when stress and FA were

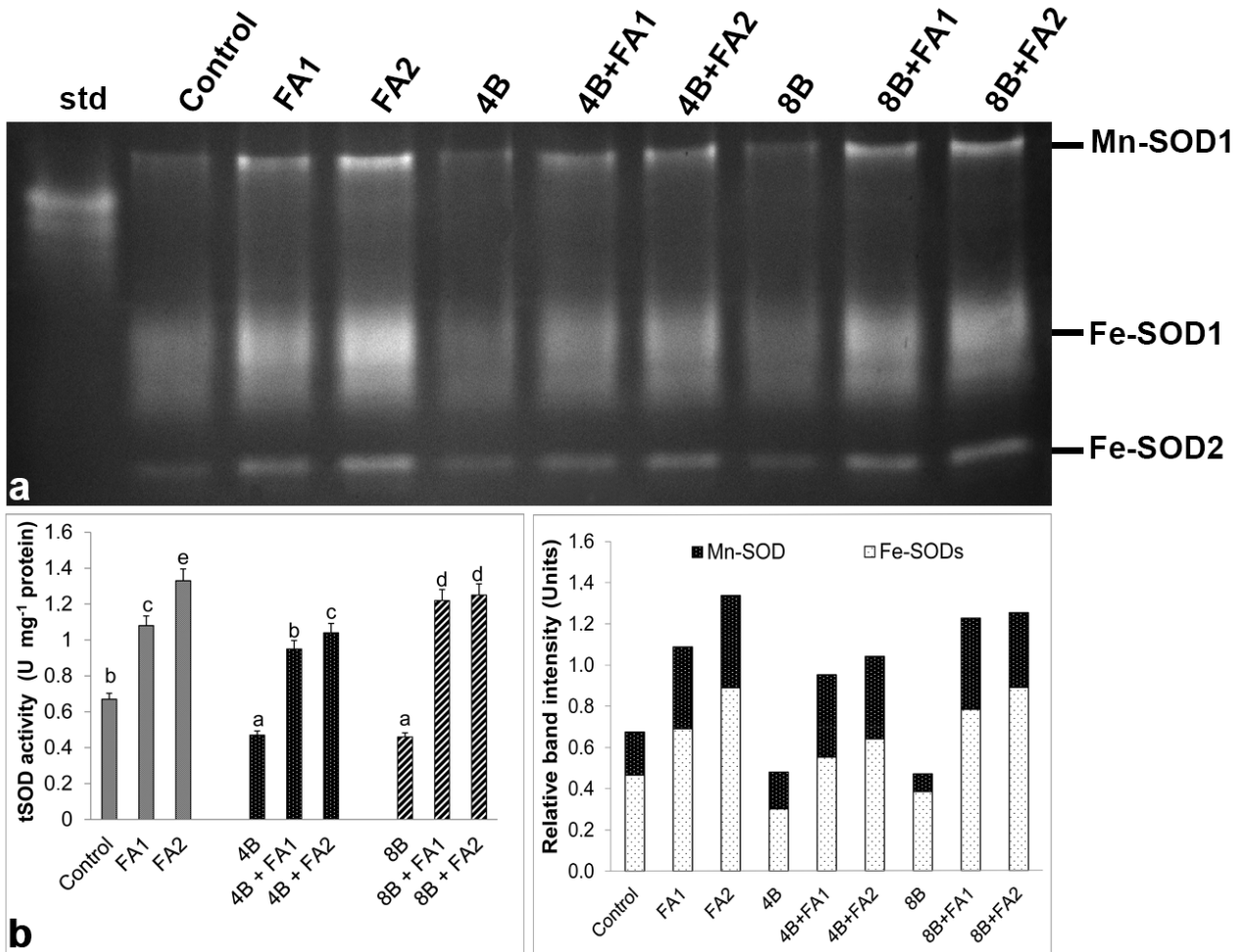


Figure 4. Effects of exogenous 25 (FA1) and 75 μ M (FA2) FA treatment on relative band intensity of different types of superoxide dismutase isoenzymes (SOD, **a**) and total SOD activity (**b**) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate \pm SE and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

applied together, a large decrease in GSH content was detected in wheat roots. Unlike stress plus FA, FA-alone treatment resulted in an augmentation in GSH content. As shown in Figure 9f, no change in GSSG content was observed in roots with B toxicity. However, an increase in GSSG content was observed at FA+stress. Besides, FA alone did not modify GSSG content.

4. Discussion

Phenolic compound is considered a great factor for plant growth especially under stress conditions. For example, Abu El-Soud et al. (2013) reported that the foliar treatments of FA to *Cicer arietinum* exposed to osmotic stress caused a significant increase of all growth (RGR). This result is in

agreement with our findings that in B-treated wheat roots, FA caused an induction in RGR as well as in FA-treated alone. On the other hand, there was a decrease in RGR at B stress. The inhibition of RGR induced by B stress might be associated with damaged photosynthesis system in wheat; therefore, a reduction in translocated nutrients from leaf to root might be considered. One of the plant nutrients, K^+ , can play role in growth (Sarafı et al., 2018). In the current study, there was a correlation between RGR and the endogenous content of K^+ under B toxicity. The decline in the K^+ content of the roots under B toxicity could be attributed to membrane damage, which results in K^+ -leakage into the apoplast, as shown in Sarafı et al. (2018). Similarly, B-treated plants also showed reductions in RWC;

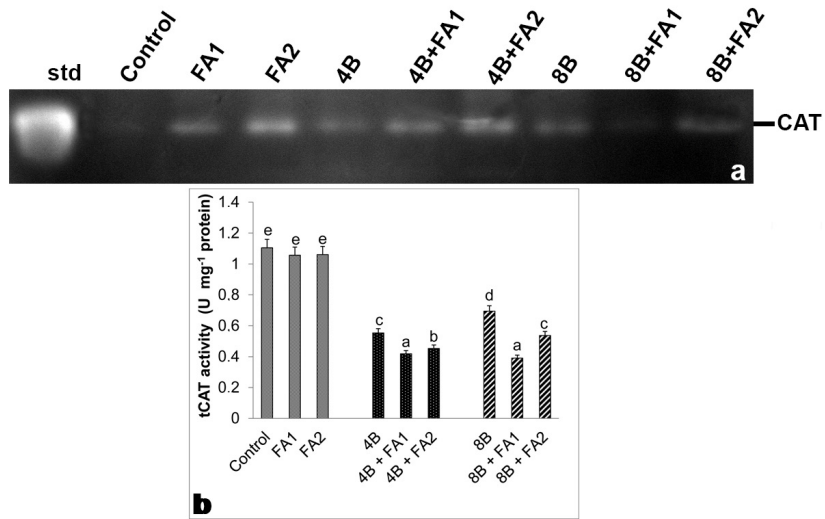


Figure 5. Effects of exogenous 25 and 75 mM FA treatment on relative band intensity of different types of catalase isoenzymes (CAT, a) and total CAT activity (b) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate \pm SE and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

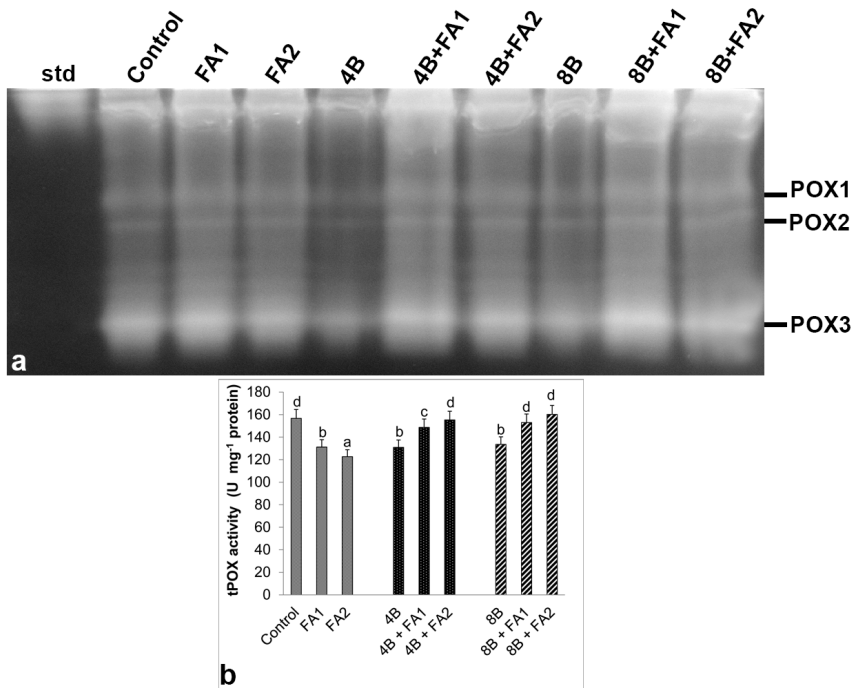


Figure 6. Effects of exogenous 25 (FA1) and 75 μ M (FA2) FA treatment on relative band intensity of different types of peroxidase isoenzymes (POX, a) and total POX activity (b) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate \pm SE and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

a maximum decline was observed at 8 mM B as shown by Wang et al. (2016). However, FA application improved the water uptake as suggested by the results of RWC levels. In this study, the increased RWC under FA plus stress might be related to RGR. On the other hand, Abu El-Soud et al.

(2013) detected that the chickpea seedlings exposed to ellagic acid application showed the enhanced growth due to the high accumulation osmolytes. Similar to this result, in our study, FA application induced Pro content in wheat roots treated with stress, which B stress caused a reduction

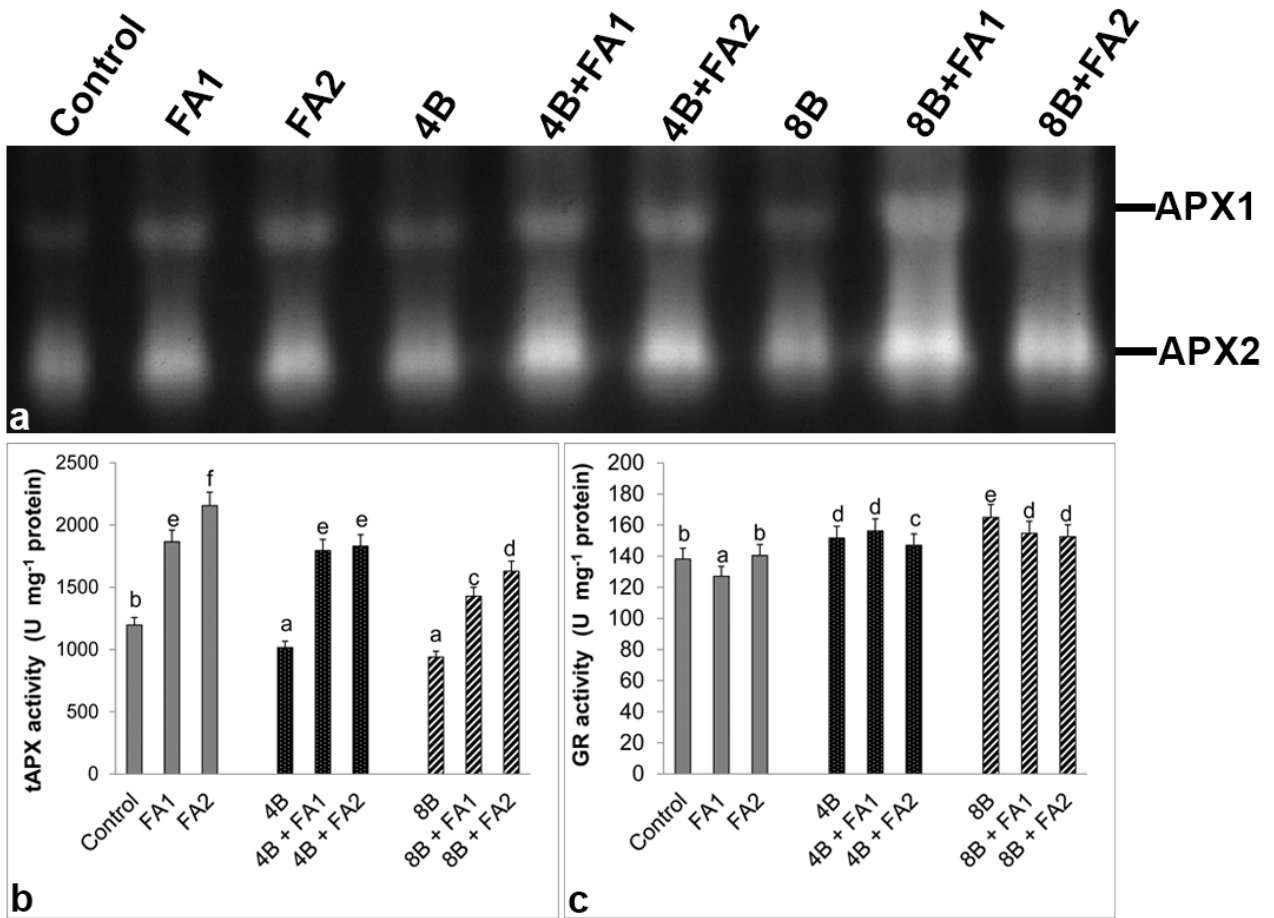


Figure 7. Effects of exogenous 25 (FA1) and 75 μM (FA2) FA treatment on relative band intensity of different types of ascorbate peroxidase isoenzymes (APX, **a**), total APX activity (**b**), and total GR activity (**c**) in wheat roots exposed to 4 and 8 mM B stress for 7 days (**d**). For each group, vertical bars indicate $\pm\text{SE}$ and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

in Pro content. This situation might be connected to FA treatment resulting in lower water potential in wheat; therefore, it supplied more water uptake into the roots as shown by Li et al. (2013). Moreover, FA-induced Pro accumulation might stimulate the activity of antioxidant enzymes in wheat roots as suggesting by Abu El-Soud et al. (2013). In our study, under B stress, Y_p induced in wheat roots treated with FA which is related with improved RGR and RWC. This pattern of Pro content had contributed in the osmotic potential adjustment. Thus, the results of the increased RGR and RWC, Ψ_{II} and Pro content were compatible with each other. Sun et al. (2002) revealed that the cucumber seedlings with FA had protected themselves from PEG-induced dehydration by increasing their RWC and Pro content. The reason behind the improvement in FA-induced RGR, RWC, and Pro might be the decline of B uptake (as observed in decreased endogenous B content), improvement for K^+ concentration (osmoregulation) and regulation of aquaporin in plasma membrane. FA might

be responsible for reduced B accumulation through its carriers in the membrane.

B may affect the absorption and accumulation of some plant nutrients such as Ca^{2+} , K^+ , and Fe^{2+} . While Ca^{2+} has the roles in cell wall metabolism and transport process (Tariq and Mott, 2007), Mg^{2+} is related to alterations in the mesophyll cells and the differences in chlorophyll concentration (Papadakis et al., 2004). In the present study, the data about the contents of Ca^{2+} , K^+ , and Fe^{2+} under B stress are in line with that of Sarafi et al. (2018). The reason behind the decline in RGR, RWC, and Ψ_{II} was the decreased contents of these ions. However, the accumulation of these ions was improved by FA application after stress treatment. These data indicate that FA might be linked to an induction of chlorophyll concentration (the increased Fe^{2+} content, as shown in our study) and regulation of transport in membrane, which was of significance for growth (RGR). Furthermore, the ameliorative effect of FA might be related with repairing

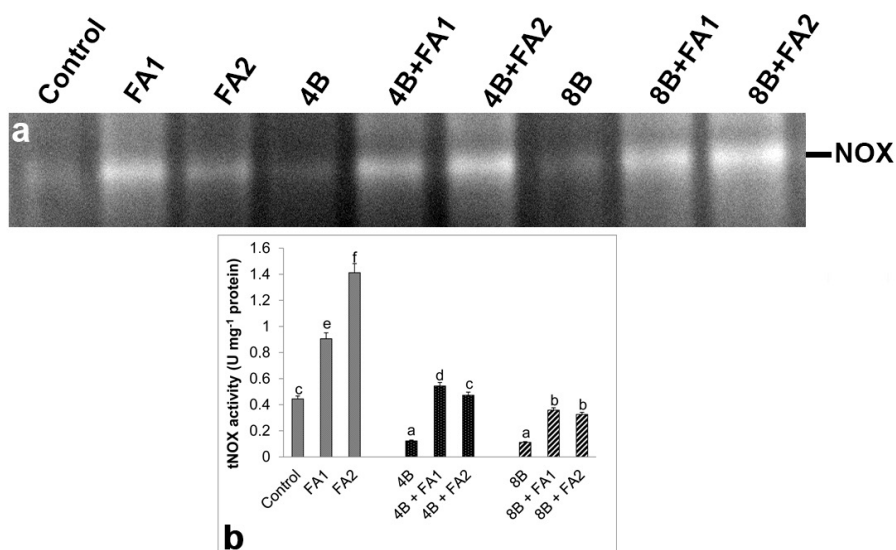


Figure 8. Effects of exogenous 25 (FA1) and 75 μM (FA2) FA treatment on relative band intensity of different types of NADPH oxidase isoenzymes (NOX, **a**) and total NOX activity (**b**) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate $\pm\text{SE}$ and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

effect on photosynthetic apparatus or preventing effect on photo-inhibition in leaves (Yildiztugay et al., 2019).

The toxicity of B stress leads to an increase in oxidative damage on DNA and membrane functions, or the inhibition of protein process, protein structure and activities (Ulusik et al., 2018). Several ways are in place to eliminate free radicals and avoid oxidative stress, including increase of the activity of ROS scavenging enzymes (Rezaee et al., 2013). As a major scavenger, SOD breaks superoxide anions to H_2O_2 and oxygen. In the present study, the total SOD activity decreased under B stress, depending on the low intensities of Mn-SOD and Fe-SOD1. It might be due to the disturbance of SOD enzyme subunits or binding/absence of cofactors to SOD such as Fe^{2+} and Mn^{2+} by B treatments. In contrast to this, Kayihan et al. (2016) reported that *Arabidopsis thaliana* subjected to B toxicity had an enhancement in SOD activity. On the other hand, the production of ROS is triggered extracellularly through membrane NADPH-dependent oxidase (NOX), peroxidases present in cell wall or amine, diamine and polyamine oxidase (Bolwell et al., 2002). However, in the current study no relationship was observed with ROS accumulation and NOX activity under stress because both total and isozyme activity of NOX did not trigger with B stress. However, after FA application under B stress, the reduced SOD activity improved, providing all intensities of SOD isozymes. In this study, the increased SOD activity contributed to eliminate radical-induced damage in wheat roots. Supportively, it has been shown that SOD enzyme could be activated by FA application in cucumber (Li et

al., 2013). Moreover, there was a relationship between inhibition of the lipid peroxidation and the increased SOD activity. Although NOX activity increased in roots with FA plus stress, it did not cause any damage to the roots because of the process of radical scavenging was done effectively.

B stress-induced H_2O_2 content was scavenged by the activities of CAT, POX, APX, and GR (Kaya and Ashraf, 2015). While, in the current study, no increase in the total/ isozyme activities of CAT, POX, and APX was observed under B toxicity, only GR activity showed an increase during the experimental period. In accordance with these data, B excess caused a reduction in CAT, POX, and APX (Shah et al., 2017), and an induction in GR activity (Karabal et al., 2003). In the current study, despite increased GR activity, toxic level of H_2O_2 under B treatments could not be scavenged. The wheat roots treated with B stress showed an induction in H_2O_2 content and a decline in OH^\bullet scavenging activity. This result is compatible with the study conducted by that Cervilla et al. (2007) who showed that an enhancement in H_2O_2 content in tomato is reported under high B concentration. The toxic levels of ROS are linked with lipid peroxidation (Singh et al., 2017b); in our study there was a similarity between TBARS content and H_2O_2 content in stress-treated wheat. This result is in keeping with the data of Pizzeghello et al. (2013), who detected that B stress stimulated TBARS content in the maize leaves. On the other hand, FA treatment in combination with B stress induced the activities of POX and APX. Similarly, Bhardwaj et al. (2017) revealed that gallic acid-treated wheat cultivars had upregulation of POX and APX

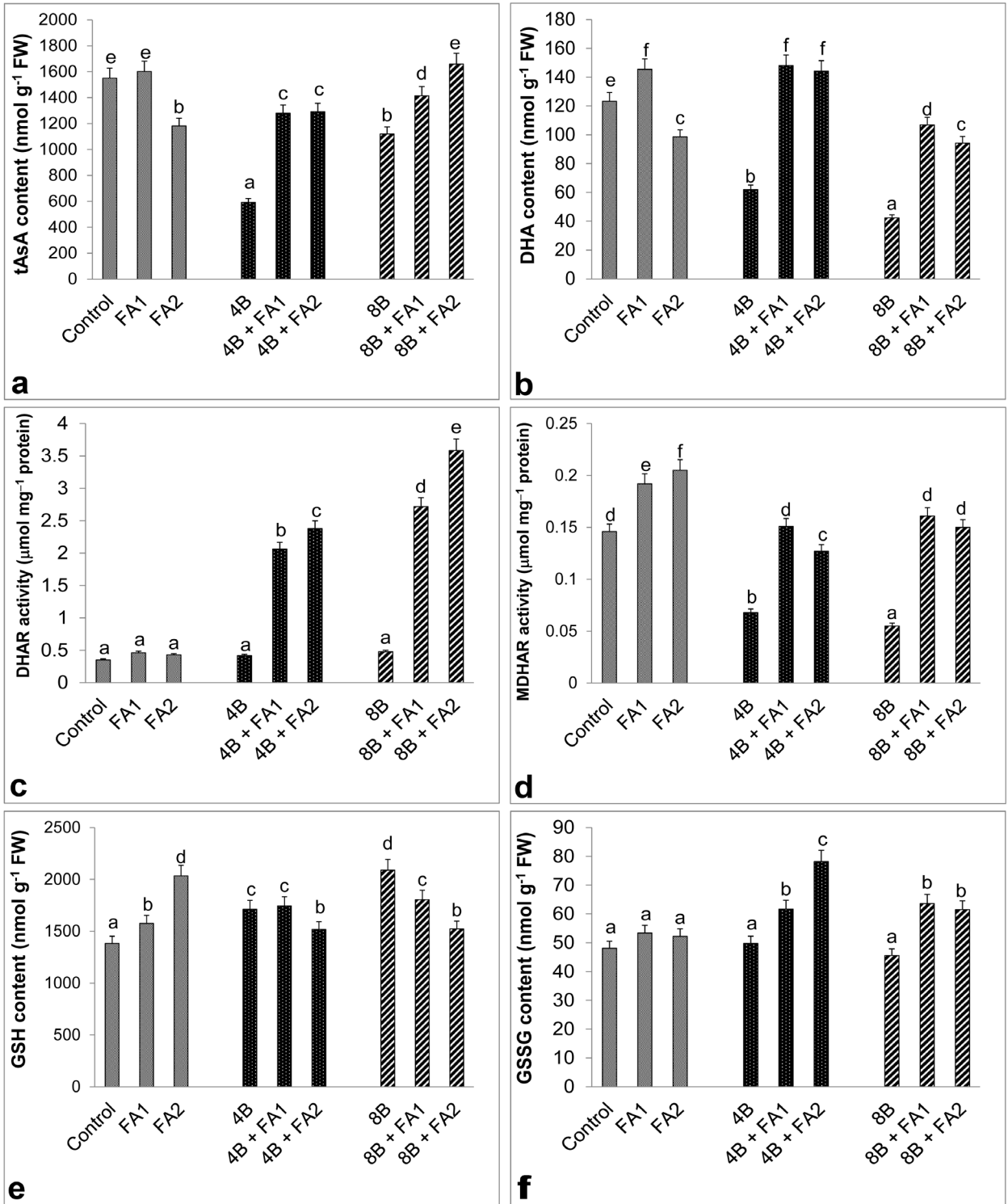


Figure 9. Effects of exogenous 25 (FA1) and 75 μM (FA2) FA treatment on total ascorbate content (tAsA, **a**), dehydroascorbate content (DHA, **b**), dehydroascorbate reductase activity (DHAR, **c**) monodehydroascorbate reductase activity (MDHAR, **d**), reduced glutathione content (GSH, **e**), and oxidized glutathione content (GSSG, **f**) in wheat roots exposed to 4 and 8 mM B stress for 7 days (d). For each group, vertical bars indicate ±SE and the different lowercase letters are significantly different ($P < 0.05$) values according to the Tukey test. The error bars represent standard errors of the means.

under drought stress. Besides, when evaluated according to Yildiztugay et al. (2019), there was a different response mechanism of exogenous FA applied to the roots and leaves of wheat. In other words, the wheat roots exposed to FA plus B stress had a lower CAT activity than stress treatment alone, but the increased activities of CAT and POX were detected by FA application under 4 mM B in wheat leaves.

APX, GR, AsA, and GSH play an important role in Asada-Halliwell pathway (AsA-GSH cycle) in plants (Cervilla et al., 2007). The activities of MDHAR, DHAR, and GR stimulate the regeneration of AsA, which is a significant step in antioxidant response for tolerance against stress conditions (Noctor et al., 2012). In the present study, B stress decreased tAsA content, a finding also reported by Wang et al. (2011). Besides, DHA content, which converts to AsA by DHAR activity, decreased in roots treated with B stress (Figure 8b). Under stress, DHAR activity was similar to the control groups. Thus, the decline in tAsA content was compatible with decreasing in MDHAR activity (Figure 8d), DHA content, and unchanged DHAR activity. When we interpret all these results, we can say that B stress disrupted the regeneration of AsA in wheat roots. As well as AsA content, GSH is a functional metabolite in this pathway and preserved thiols of protein from oxidation under stress (Noctor et al., 2012). In the present study, because of nonchanging DHAR activity, the conversion of GSH to the oxidized form of GSH (GSSG) was not maintained under B stress. Thus, in response to B toxicity, GSH content was increased and GSSG content was similar to the control groups in wheat roots. Our result is in accordance with the findings obtained by Ayvaz et al. (2016), who confirmed the increase in GSH content at the high B concentration. On the other hand, FA application to stress-treated wheat roots triggered the regeneration of AsA content. In wheat roots, FA-induced AsA accumulation was related to enhance in the activities of MDHAR and DHAR and DHA content. Besides, FA-induced APX could easily get the substrate AsA. Moreover, Wan et al. (2014) reported that there is a positive correlation among elevated contents of AsA, enhanced activities of MDHAR and DHAR in cucumber seedlings treated with caffeic acid. Furthermore, the turnover of GSH to GSSG was generated via the increased DHAR activity in wheat with the combine treatments of FA and B, as compared to B treatment alone. However, FA could not provide the conversion of GSSG to GSH content because of decline in GR activity (except for 4B+FA1), which catalyzes the conversion of GSSG to GSH, and GSH/GSSG decreased. Therefore, FA could not recycle GSH when FA-treated wheat roots were exposed to B excess, but caused AsA regeneration. On the other hand, in

wheat leaves, 25 μ M FA in response to B stress maintained the regeneration of both AsA and GSH (Yildiztugay et al., 2019). Because of the activated antioxidant enzymes/nonenzymes, FA-treated wheat roots exposed to stress had low H_2O_2 content and rising scavenging activity of OH^\cdot . These results are consistent with the data obtained by Abu El-Soud et al. (2013) who indicated that the application of ellagic acid to chickpea plants mitigated effects of stress by reducing ROS content. Moreover, some of the recent studies showed that the application of phenolic acids such as gallic and cinnamic acid alleviated the oxidative damage induced by stress via lessening H_2O_2 and TBARS contents and increasing antioxidant capacity (Ozfidan-Konakci et al., 2015; Singh et al., 2017a).

In conclusion, B stress inhibited RGR, RWC, Ψ_{IT} , and Pro content. However, FA improved growth, water status, and Pro content of wheat roots subjected to the stress conditions. B stress decreased the activities of SOD, CAT, POX, and APX, but only GR activity increased after B stress treatment to wheat roots. As connecting with these changes, the toxic ROS levels (increased H_2O_2 content and decreased scavenging activity of OH^\cdot) were affected by B stress in roots. Therefore, B stress resulted in lipid peroxidation (increased TBARS content). On the other hand, the combination of FA and stress increased the activities of SOD, POX, and APX during experimental period. Thus, FA could achieve the low H_2O_2 content in wheat. Besides, FA triggered the activities of APX, MDHAR, and DHAR, the accumulation of DHA content, so FA could maintain the AsA regeneration. However, when wheat roots treated with stress, FA did not maintain the turnover of GSH (to GSH from GSSG) because of decline in GR activity (except for 4B+FA1). On the other hand, due to successful eliminating of H_2O_2 content, B-induced lipid peroxidation (TBARS content) was alleviated through the exogenous application of FA in wheat. Consequently, exogenous of FA together with stress significantly ameliorated B-induced toxic effects in wheat roots, which coincided with the decreased levels of H_2O_2 and TBARS, through the increased POX activity and the enhanced enzyme activity and AsA regeneration related to AsA-GSH cycle as well as improved water management and osmotic regulation.

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