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Original Scientific Paper

Humic acid protects against oxidative damage induced by cadmium toxicity in wheat (*Triticum aestivum*) roots through water management and the antioxidant defence system

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ABSTRACT: Humic compounds like humic acid (HA) promote ecosystem health by stabilising soil structure and promoting plant development. However, the amount needed is a limiting factor. The use of biostimulants based on HA is an effective way to eliminate oxidative damage caused by heavy metals such as cadmium (Cd) in plants. The aim of this study was to assess the effects of humic acid (HA; 750 and 1500 mg L^{-1}) on growth, the osmotic potential, the antioxidant system, radical content and lipid peroxidation in wheat (*Triticum aestivum*) roots treated alone or in combination with Cd stress (100 and 200 μM). Cadmium-treated wheat roots showed a reduction in growth (RGR) and the osmotic potential (Ψ_Π) and an increase in proline content (Pro). Although 100-μM Cd stress induced the activities of catalase (CAT) and ascorbate peroxidase (APX), hydrogen peroxide (H_2O_2) accumulation in roots exposed to stress was not prevented. The membrane of roots showed stress-dependent lipid peroxidation (TBARS content). Application of HA in combination with stress alleviated RGR and Ψ_Π by promoting water intake. Humic acid reduced levels of H_2O_2 and TBARS through activation of superoxide dismutase (SOD) and CAT. Application of HA under stress also induced enzymes and non-enzymatic substances included in the ascorbate-glutathione cycle such as APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione (GSH), in addition to which it increased GSH/GSSG ratios. These results indicate that HA alleviated the negative effects of Cd-induced oxidative damage in wheat roots through regulation of growth, osmotic adjustment, radical accumulation and the action of antioxidant systems.

Keywords: Ascorbate-glutathione cycle, humic acid, lipid peroxidation, oxidative stress, *Triticum aestivum*

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INTRODUCTION

Excess accumulation of heavy metals in soil has become a critical environmental concern due to their diverse impacts on important metabolic processes. Heavy metals cause various physiological/biochemical alterations and toxicity to basic processes such as uptake and transport of essential elements, photosynthesis and respiration in plants (Adrees *et al.* 2015). As a result of the deleterious effects of heavy metals such as cadmium on energy production and efficiency of other metabolic processes, reactive oxygen species (ROS) are produced in plants. In order to reduce and resist oxidative toxicity of cadmium (Cd), plants have a complex antioxidative defence sys-

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tem that includes both enzymatic [superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR)] and non-enzymatic (ascorbate, glutathione and tocopherol) antioxidants (Gill *et al.* 2015). Superoxide dismutase converts superoxide anion radicals to hydrogen peroxide (H_2O_2), while stress-induced H_2O_2 is eliminated through the activities of CAT, POX, APX and GR.

Besides enhancement of antioxidant activities, use of bio-stimulants is one solution for the elimination of these kinds of oxidative damage. Humic acid (HA), a known bio-stimulant, is the most stable component of soil organic matter (Cozzolino *et al.* 2016). When applied in small amounts, HA promotes plant growth and influences a number of cell processes related to primary (i.e., N assimilation) and secondary (i.e., phenolic production) metabolism (Ramos *et al.* 2015). Studies treating the positive effects of humic acid on plants have generally been focused on growth and indicate that the modes of action of HA can be related to hormone-like activities such as those of auxins, gibberellins and cytokines (Pizzeghello *et al.* 2013). Also, the abundant and diverse functional groups of HA affect solubility and mobility of heavy metals in soil (Ravichandran 2004). Humic acid thereby restricts movement and available concentrations of heavy metals (Ovecka & Takac 2014). Bio-stimulants have also been shown to alleviate other types of stress. For example, Ertani *et al.* (2013) reported that bio-stimulants increased tolerance to salinity stress in maize plants observed through enhancement of Na^+/K^+ and increased synthesis of flavonoids. In plants grown under osmotic stress, changes and disruptions in soil properties, ion exchange capacity, the water retention rate and the photosynthetic apparatus are improved by application of humic compounds (El-Nemr *et al.* 2012). Boehme *et al.* (2005) found that humic substances improve tolerance against diseases and viruses in plants. Conversely, some studies have shown that HA application can have negative effects on plants. For example, Asli & Neumann (2009) reported that HA found in soil water can reduce root hydraulic conductivity and thereby reduce transpiration and growth of maize seedling leaves when applied in high concentrations. The reduction detected in that study may have been due to the uptake of HA by plant roots and accumulation on the surface of the epidermal cell wall. Also, Liu & Cooper (2002) reported that exposure of creeping bentgrass (*Agrostis palustris*) to HA (400 mgL^{-1}) did not aid against salt stress. Overall, the evidence regarding the ability of HA to increase tolerance to salt stress is somewhat contradictory. The effect of HA varies according to the applied concentration, plant species and type of stress conditions. The aims of the present work were to determine: (i) the interaction between growth, the water status and HA treatment; (ii) the effect of HA on photosynthetic parameters; and (iii) the interaction between humic acid application and radical scavenging enzymes in wheat (*Triticum aestivum* L.) roots grown in a Cd-contaminated medium.

MATERIAL AND METHODS

Plant material and experimental design. Seeds of wheat (*Triticum aestivum*) were obtained from the Bahri Dagdas International Agricultural Research Institute, Konya, Turkey. Seeds were surface-sterilised in 5% sodium hypochlorite for 10 min, rinsed five times with sterile distilled water and allowed to germinate on double-layer filter paper wetted with distilled water (50 ml). Germinated wheat seedlings were transferred to half-strength Hoagland solution and grown under controlled conditions (16/8 h light/dark regime at 24°C, 70% relative humidity and 350 μmol m^{-2} s^{-1} photosynthetic photon flux density). The seedlings were grown in hydroponic culture containing this solution for 21 days. Humic acid (HA1 - 750 mg L^{-1}, and HA2 - 1500 mg L^{-1}) was added alone or in combination with cadmium (Cd) (100 μM and 200 μM). Wheat seeds were also grown under normal conditions (irrigated with Hoagland solution) without HA and/or stress, and this group was defined as the control group. Plants were harvested after 7 days of treatment (7d) and the roots stored at −86°C until further analyses. Six plants were used for the control group and for each treatment group. The experiments were repeated three times, making 18 samples in total.

Determination of growth rate, osmotic potential and proline content. Root fresh weights (FW) were obtained. After the samples were dried, their dry weights (DW) were recorded. Reduction in growth (RGR) was calculated according to the following formula published by Hunt *et al.* (2002):

$$RGR = [\ln (DW2) - \ln (DW1)] / (t2 - t1),$$

where DW1 = dry weight (g) at t1; DW2 = dry weight (g) at t2; t1 = initial harvest; and t2 = final harvest.

Roots were homogenised with a glass rod. After centrifugation (12000 × g) for 10 min, the extract was directly used for Ψ_Π determination. Root osmolarity (c) was measured with a Vapro 5600 vapour pressure osmometer and converted from mosmoles kg^{-1} to MPa using the formula: Ψ_Π (MPa) = −c (mosmoles kg^{-1}) × 2.58 × 10^{-3}, according to the Van't Hoff equation.

Proline (Pro) content was measured according to Bates *et al.* (1973). The roots were homogenised in 3% sulphosalicylic acid and the homogenate was filtered through filter paper. After addition of acidic ninhydrin and glacial acetic acid, the mixture was heated at 100°C. The mixture was then extracted with toluene. Absorbance of the toluene fraction was measured at 520 nm on a Shimadzu spectrophotometer (UV 1800), the instrument used for all spectrophotometric analyses in this experiment.

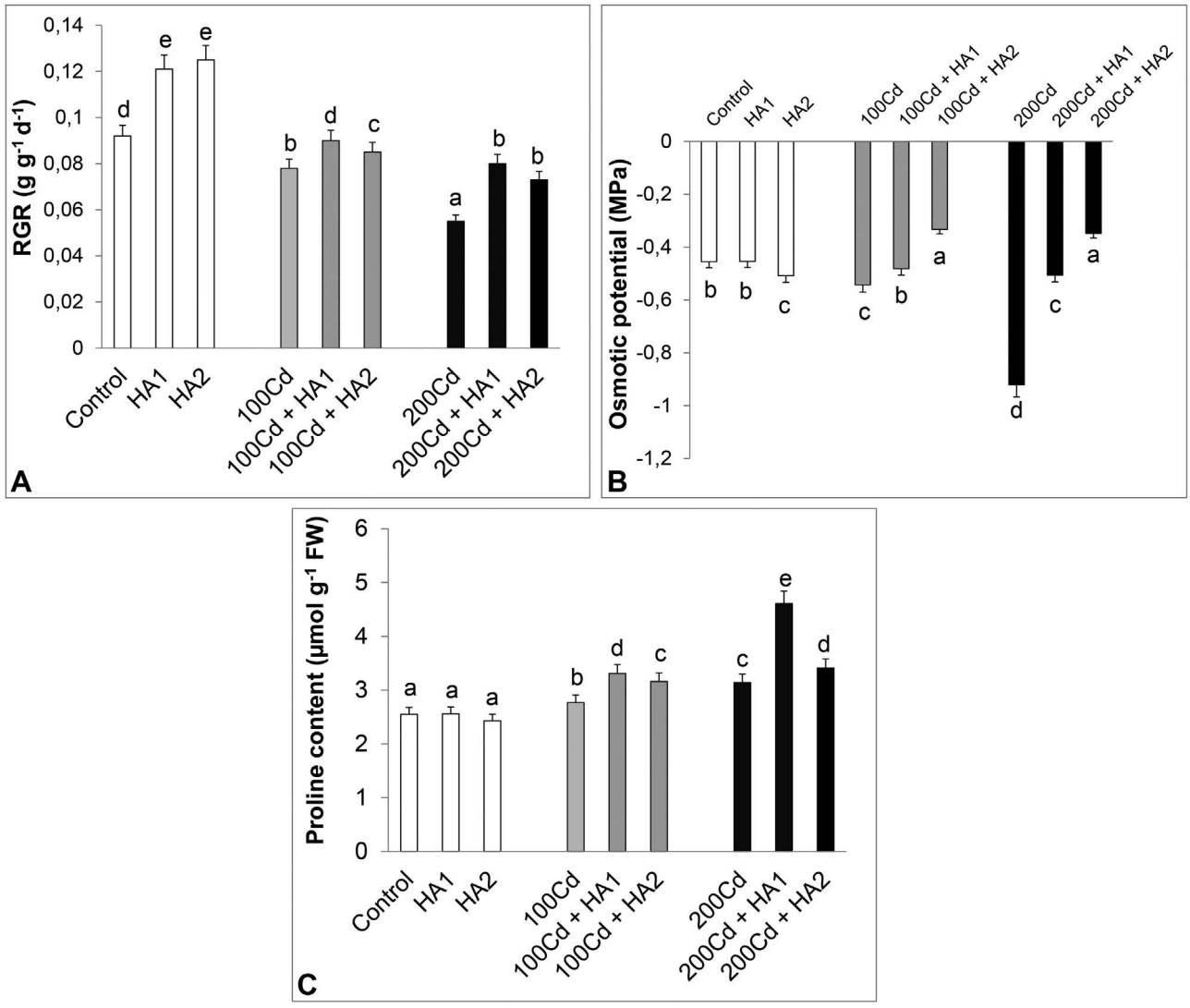

Fig. 1. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on the relative growth rate (RGR, **A**), osmotic potential (Ψ_π, **B**) and proline content (Pro, **C**) in wheat roots exposed to 100 µM Cd (100Cd) and 200 µM Cd (200Cd) for 7 days.

Determination of ROS accumulation. Determination of H_2O_2 content was performed according to Liu *et al.* (2010). Roots were homogenised in cold acetone and centrifuged. The supernatant was mixed with titanium reagent, after which ammonium hydroxide was added to precipitate the titanium-peroxide complex. The reaction mixture was centrifuged. The pellet was washed with cold acetone and dissolved. Absorbance of the solution was measured at 410 nm. Concentrations of H_2O_2 were calculated using a standard curve prepared with known concentrations of H_2O_2.

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

Enzyme extraction and determination of isozyme and/or enzyme compositions. For protein and enzyme extractions, 0.5 g of each sample was homogenised in 50 mM Tris-HCl (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, 1 mM phenylmethylsulphonyl fluoride and 2 mM dithiothreitol (DTT). For determination of APX activity, 5 mM ascorbate (AsA) was added to the homogenisation buffer. Samples were centrifuged at 14000 × g for 30 min and the supernatant was used for determination of protein

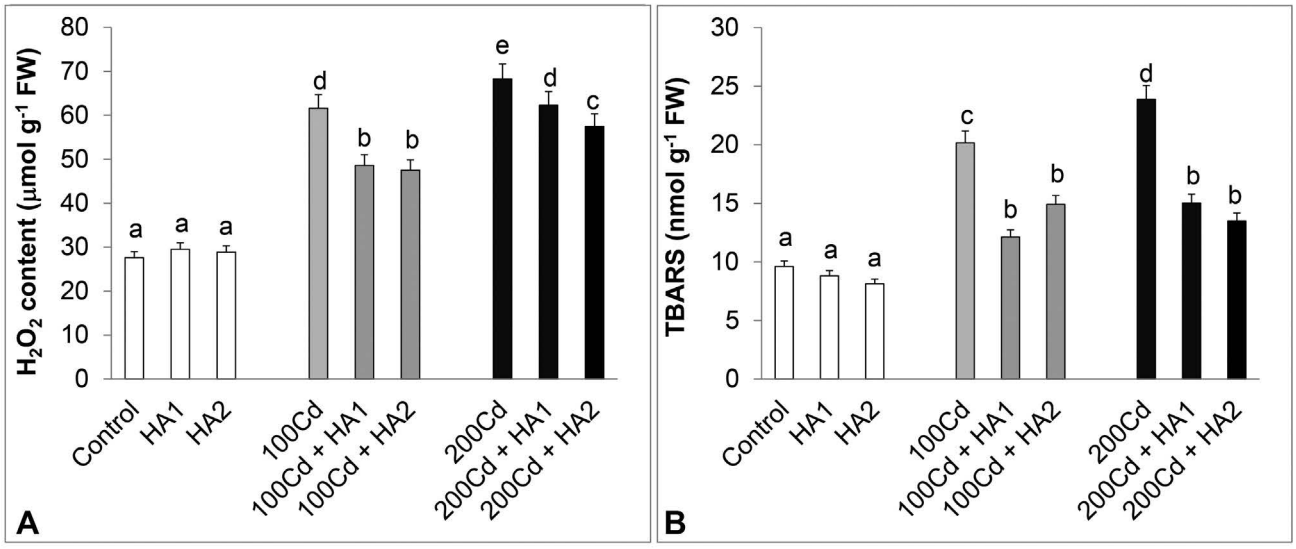

Fig. 2. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on hydrogen peroxide (H_2O_2, **A**) and lipid peroxidation (TBARS, **B**) in wheat roots exposed to 100 μM Cd (100Cd) and 200 μM Cd (200Cd) for 7 days.

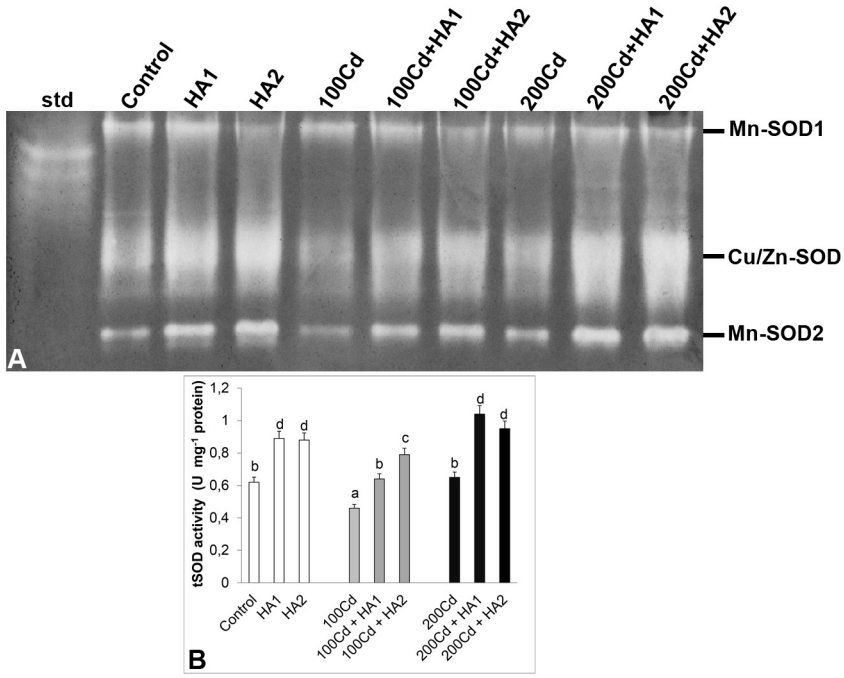

Fig. 3. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on relative band intensity of different types of superoxide dismutase isoenzymes (SOD, **A**) and total SOD activity (**B**) in wheat roots exposed to 100 μM Cd (100Cd) and 200 μM Cd (200Cd) for 7 days.

content and enzyme activities. The total soluble protein content of enzyme extracts was determined (Bradford 1976) using bovine serum albumin as a standard.

Samples containing equal amounts of protein (35 μg) were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970) with minor modifications. Activity of SOD was detected by photochemical staining using riboflavin and NBT (Beauchamp & 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, while Cu/Zn-SOD activity was sensitive to 2 mM KCN. The activities of Cu/Zn-SOD and Fe-SOD were inhibited by 3 mM H_2O_2 (Vitória *et al.* 2001). The total SOD (EC 1.15.1.1)

activity assay was based on the method of Beauchamp & Fridovich (1971), which uses spectrophotometric analysis at 560 nm to measure inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). One unit of specific enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT reduction.

After electrophoresis of samples containing 35 μg protein, CAT isozymes were detected according to Woodbury *et al.* (1971). Total CAT (EC 1.11.1.6) activity was estimated according to the method of Bergmeyer (1970), which measures the initial rate of H_2O_2 disappearance at 240 nm. The decrease in absorption was followed for 3 min. One unit of CAT was defined as 1 mmol of H_2O_2 decomposed min^{-1} mL^{-1}.

Isozymes of POX were detected according to Seevers *et al.* (1971). Electrophoretic separation of samples containing 35 μg protein was performed on non-denaturing polyacrylamide. Determination of total POX (EC 1.11.1.7) activity was based on the method described by Herzog & Fahimi (1973). The increase in absorbance at 465 nm was followed for 3 min. One unit of POX activity was defined as 1 mmol of H_2O_2 decomposed min^{-1} mL^{-1}.

Electrophoretic APX separation was performed according to Mittler & Zilinskas (1993). Before the samples (35 μg protein) were loaded, gels were equilibrated with a running buffer containing 2 mM AsA for 30 min. Total APX (EC 1.11.1.11) activity was measured according to Nakano & Asada (1981). The assay depends on the decrease in absorbance at 290 nm. The concentration of oxidised AsA was calculated by using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX was defined as 1 mmol of AsA oxidised min^{-1} mL^{-1}.

Total GR (EC 1.6.4.2) activity was measured according to Foyer & 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 of GSSG reduced min^{-1} mL^{-1}.

Gels stained for SOD, CAT, POX and APX activities were photographed with the Gel Doc XR System and analysed with Image Lab software v4.0.1 (Bio-Rad, California, USA). Known standard amounts of enzymes (0.5 units of SOD and CAT, and 0.2 units of POX) were loaded onto gels. The units of isozyme activity for each group were calculated by comparison with the standard and are given in graphic form below each gel photo.

Determination of the activity of monodehydroascorbate reductase and dehydroascorbate reductase. Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed by the method of Miyake & 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 activity of MDHAR was measured from decrease in absorbance as the amount of enzyme that oxidises 1 mmole of NADPH per minute at 340 nm. A molar extinction coefficient of 6.2 mM^{-1} cm^{-1} was used for calculation of enzyme activity.

Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was measured according to Dalton *et al.* (1986). Activity of DHAR was measured from increase in absorbance at 265 nm due to ascorbate formation. A molar extinction coefficient of 14.6 mM^{-1} cm^{-1} was used for calculation of enzyme activity.

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

Statistical analysis. The experiments were repeated three times independently and each data point was the mean of six replicates. All data obtained were subjected to a one-way analysis of variance (ANOVA). Statistical analysis of the values was performed using SPSS 20.0. Tukey's post-test was used to compare the treatment groups. Comparisons with $p < 0.05$ were considered significantly different. Average values marked with the same letter were not significantly different at $p > 0.05$ using Tukey's post-test. In all figures, error bars represent standard errors of mean values.

RESULTS

A significant reduction of RGR in wheat roots with Cd stress was observed when compared to non-stress conditions (Fig. 1A). The maximum reduction of 40.2% was detected at 200 μM Cd. This reduction was alleviated by exogenous HA application. Both HA concentrations caused increases in RGR. Cadmium stress resulted in a notable decrease of Ψ_Π (Fig. 1B). On the other hand, Ψ_Π was higher in Cd plus HA treatments than in Cd-treated plants alone. The value of Ψ_Π under 750 mg L^{-1} HA alone was similar to that of the control group. The Pro content of Cd-treated plants increased by 8.6 and 23.1% (Fig. 1C) as compared to the control group. After HA treatment with Cd application, an increase of Pro content was observed compared with Cd alone. The maximum inducible response was detected in Cd stress plus low concentration HA treatment. Furthermore, the application of HA alone did not cause any change in Pro content of roots compared with non-stress conditions.

Cadmium stress (100 and 200 μM) in the growth medium caused dramatic increases of H_2O_2 content in roots compared with non-stress conditions, by 123.1 and 147.3%, respectively (Fig. 2A). However, Cd+HA signifi-

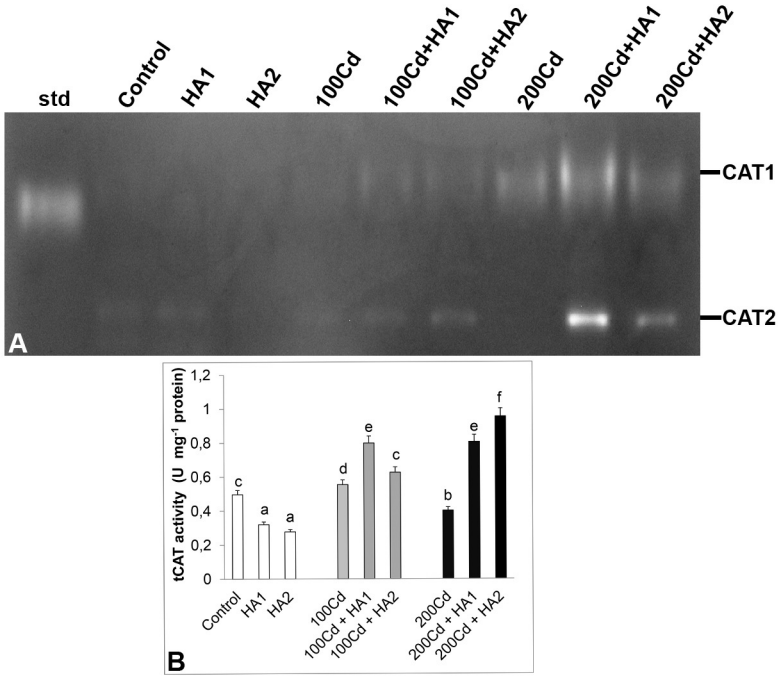

Fig. 4. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on relative band intensity of different types of catalase isoenzymes (CAT, **A**) and total CAT activity (**B**) in wheat roots exposed to 100 μM Cd (100Cd) and 200 μM Cd (200Cd) for 7 days.

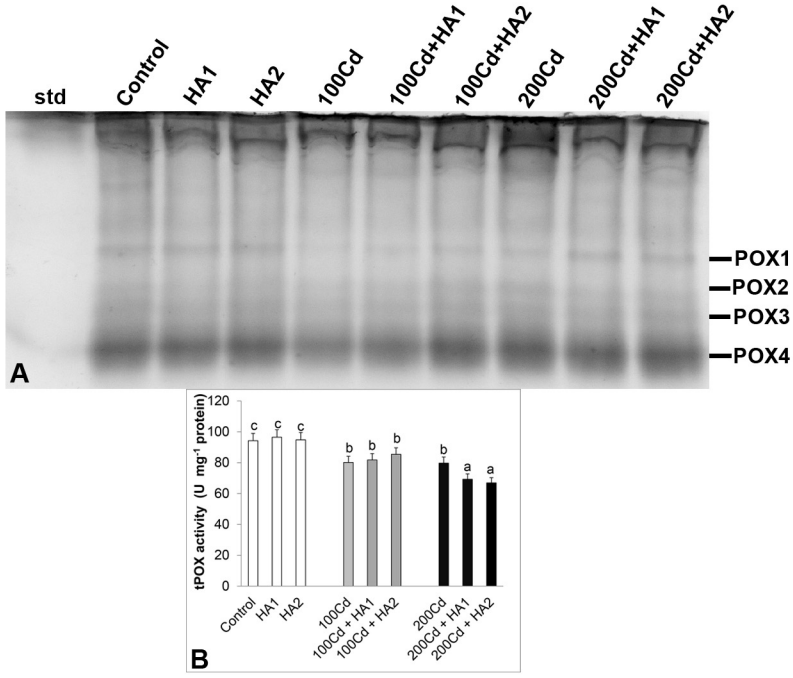

Fig. 5. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on relative band intensity of different types of peroxidase isoenzymes (POX, **A**) and total POX activity (**B**) in wheat roots exposed to 100 μM Cd (100Cd) and 200 μM Cd (200Cd) for 7 days.

cantly decreased H_2O_2 content compared with Cd treatment. The impact of HA-only treatment was not significant in regard to H_2O_2 content. Cadmium led to a dose-dependent increase of TBARS content, indicating lipid peroxidation (Fig. 2B). This increase of TBARS content was as great as 2.48-fold under 200 μM Cd treatment. Addition of HA together with Cd significantly alleviated this increase of TBARS content. Application of HA alone showed no significant change in TBARS compared with the control group.

Gel assays for detecting SOD activity revealed that the three isozymes of SOD were two Mn-SODs and one Cu/Zn-SOD (Fig. 3A). It turned out that Fe-SOD did not appear in any treatment group. During the experimental period, Cd stress did not lead to any increase in total root SOD activity (Fig. 3B). The isoforms Cu/Zn-SOD and Mn-SOD2 were similar or decreased under Cd exposure. These Cd-induced changes were alleviated by HA application. Also, the total SOD activity in wheat treated with HA alone was higher than that of the control group.

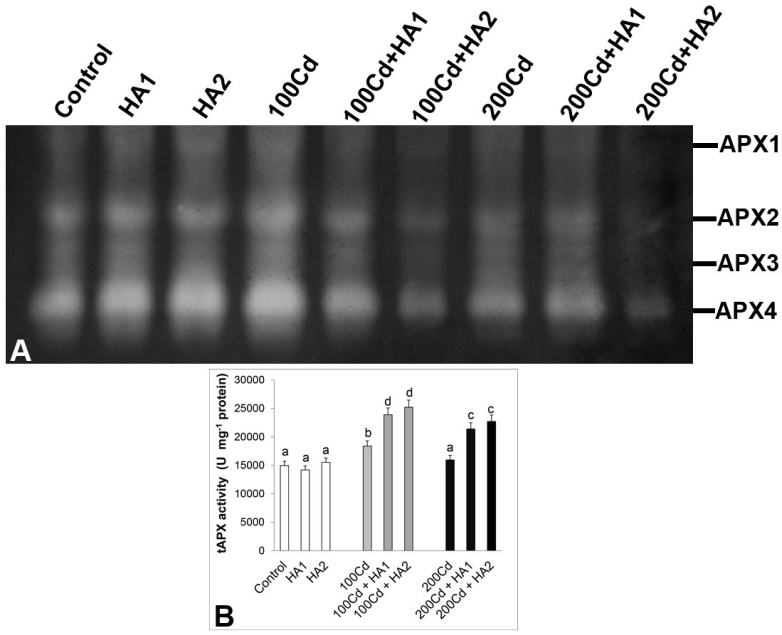

Fig. 6. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on relative band intensity of different types of ascorbate peroxidase isoenzymes (APX, **A**) and total APX activity (**B**) in wheat roots exposed to 100 µM Cd (100Cd) and 200 µM Cd (200Cd) for 7 days.

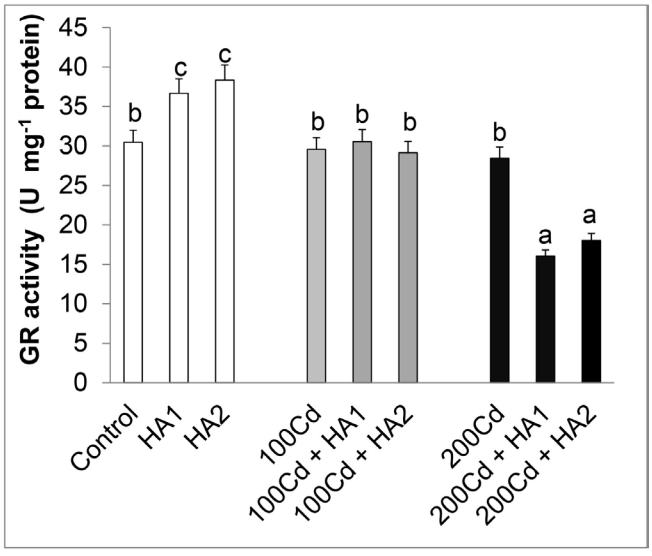

Fig. 7. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on total glutathione reductase activity (GR) in wheat roots exposed to 100 µM Cd (100Cd) and 200 µM Cd (200Cd) for 7 days.

Two CAT isozymes (CAT1-2) were defined by densiometric analysis in native PAGE (Fig. 4A). No bands appeared in plants treated with 1500 mg L^{-1} HA alone. Compared with the control group, an increase in total CAT activity (Fig. 4B) was only observed at low Cd concentration, as a result of increased concentration of the CAT2 isozyme (Fig. 4A). Addition of HA to Cd-exposed plants led to increased expression of both CAT isozymes. Interestingly, HA treatment alone led to a decline of total CAT activity.

Four POX isozyme bands (POX1-2-3-4) were detected (Fig. 5A). Total POX activity decreased with Cd treatment (Fig. 5B). This decline in activity reached a maximum level of 15.4% with 200 µM Cd stress. Roots of plants exposed to Cd+HA showed lower or unchanged POX activity. No significant differences were observed in the isozymes POX1, POX3 and POX4 in HA-treated plants without Cd.

Gel analysis revealed two APX isozymes (APX1-2) in all treatment groups (Fig. 6A). While 100 µM Cd stress caused an increase of total APX activity (Fig. 6B) as reflected by enhancement of APX1-2 (Fig. 6A), no changes in APX isoforms or total intensity were observed under 200 µM Cd stress. Addition of HA together with Cd triggered an increase of APX activity. Treatment with HA alone produced a similar increase in APX activity.

Neither Cd stress alone nor stress plus HA led to any significant increase of total GR activity (Fig. 7). However, the roots of wheat plants treated with HA alone showed higher GR activity.

There was a decline of MDHAR activity in Cd-exposed plants (Fig. 8A). However, when Cd and HA were applied together, an increase in this activity was observed. As in the Cd group, growth with HA alone (750 and 1500 mg L^{-1}) led to notable decreases of MDHAR activity, by 26.6 and 43.2% as compared to Cd stress alone, respectively. The changes in MDHAR and DHAR were similar across all the treatment groups (Fig. 8A-8B). Both Cd-only and HA-only treatments resulted in decreased DHAR activity (Fig. 8B). However, this reduction in DHAR activity was prevented in plants exposed to HA plus Cd. Both Cd concentrations resulted in decline of GSH content, greatest at 100 µM Cd (32.7%; Fig. 8C). Application of HA under Cd or non-Cd conditions

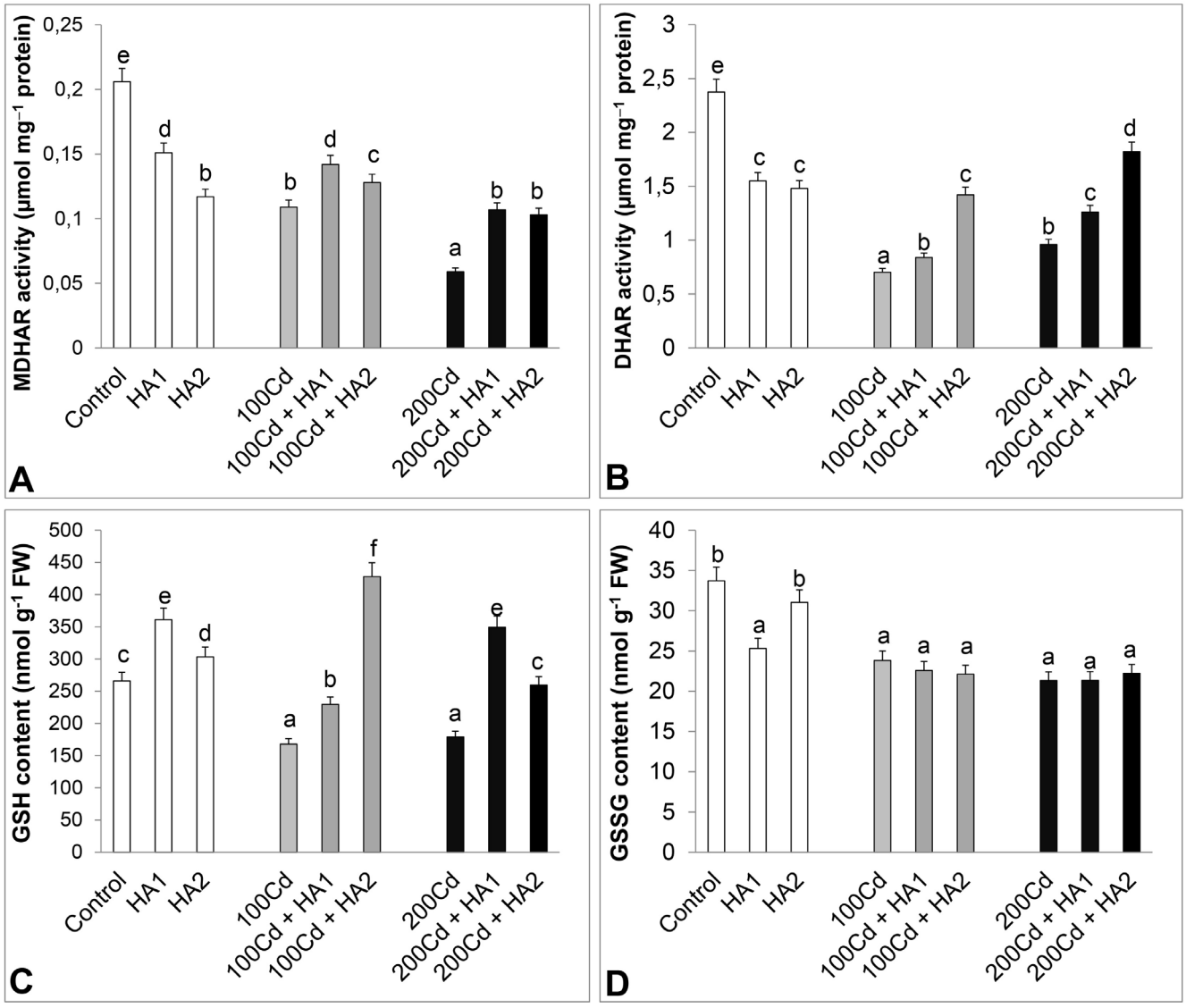

Fig. 8. Effects of exogenous 750 mg L^{-1} humic acid treatment (HA1) and 1500 mg L^{-1} humic acid treatment (HA2) on monodehydroascorbate reductase activity (MDHAR, **A**), dehydroascorbate reductase activity (DHAR, **B**), reduced glutathione content (GSH, **C**) and oxidised glutathione content (GSSG, **D**) in wheat roots exposed to 100 µM Cd (100Cd) and 200 µM Cd (200Cd) for 7 days.

led to increases in GSH content. Cadmium exposure led to a decrease of GSSG content (Fig. 8D). However, when Cd and HA were applied together, no change of GSSG content was observed. A decrease of GSSG content was observed in roots subjected to HA alone.

DISCUSSION

It has been reported that Cd-induced oxidative stress decreases the growth of plants by limiting biochemical activities and by altering cell structure (Kandeler *et al.* 2000). In our study, roots of wheat plants grown with Cd alone showed significant decreases in RGR. However, addition of HA alleviated these decreases. This is in accordance with the results of Elmongy *et al.* (2018), who reported that HA-induced root enhancement might be associated with increased rooting percentage, root number and root length in azalea plants (*Rhododendron* subgenus *Tsutusi*, cultivar Zihudie). Humic acid enhances plant growth due to the content of functional groups such as phenol and carboxylic acid groups and polar molecular bio-fragments which preserve hydrophobic aggregates (Tahir *et al.* 2011; Canellas *et al.* 2012). The improvement of root length observed with HA application might also be due to cellular elongation caused by activation of plasma membrane H^+-ATPase, which causes the acidification of apoplasts by protons, stimulating an increase in extension of the cell wall (Elmongy *et al.* 2018). This improvement could be due to lowered Cd bioavailability, increased auxin content and decline

of stomatal conductance via chelation and modification of soils (Yu *et al.* 2017). Previous studies have demonstrated that accumulation of Pro under Cd exposure enhances tolerance by regulating cell Ψ_Π and protecting plasma membrane stability (Xu *et al.* 2010). In the present study, Cd stress caused an increase of Pro content. Besides its impact on osmotic adjustment processes, Pro can play a role in protection of enzymes and cellular structures against heavy metal damage thanks to formation of Cd–proline complexes (Sharma & Dietz 2006). In wheat roots, despite its positive effects, Pro did not eliminate the toxic accumulation of radicals produced by Cd stress. On the other hand, similar to the inducing effect of HA on RGR, HA also contributes to regulation of N metabolism and N-containing compounds like Pro (Craigie 2011). In the present study, Pro accumulation was induced in roots of wheat plants grown under HA plus Cd, most likely as a mechanism of osmotic regulation. It has been proposed that under stress conditions, HA serves to modulate osmotic adjustment (Zhang *et al.* 2013). Proline indirectly acts as an antioxidant due to maintenance of the glutathione redox state (Siripornadulsil *et al.* 2002). In parallel with this, under the combined treatment of HA and Cd there was a correlation between increased GSH content and Pro in wheat roots. However, it was previously reported that D-pyrroline-5-carboxlate synthase (P5CS1), which is involved in Pro biosynthesis, did not change expression after treatment with PEG and HA (Garcia *et al.* 2016). There is a negative effect of Cd stress on the osmotic potential in plants (Lefevre *et al.* 2009). In line with our results, the same researcher reported that the presence of Cd reduced leaf Ψ_Π values. That study reported a positive correlation between accumulation of Pro and reduction of Ψ_Π in response to Cd stress in *Atriplex halimus*. In the present study, HA application prevented the Ψ_Π decrease caused by Cd. In addition to having antioxidative properties, HA modifies expression of tonoplast intrinsic proteins (TIPs) responsible for the flow of water between cytoplasm and vacuole (Kaldenhoff & Fischer 2006). These proteins regulate turgor pressure and the cell osmotic balance (Hohmann *et al.* 2000).

Cadmium stress leads to ROS accumulation and induces oxidative stress (Zhang *et al.* 2009). To protect against the harmful effects of Cd-induced radicals on organic molecules, plants have evolved antioxidant systems (Saidi *et al.* 2013). The first step in the system of protection against oxidative damage is activation of the enzyme SOD, which converts superoxide anion radicals to H_2O_2 and oxygen (Zhang *et al.* 2009). Wheat roots grown under Cd stress did not show an increase in SOD activity, consistent with a report published by Ulusu *et al.* (2017) indicating the same thing in parsley (*Petroselinum crispum*) plants treated with 300 µM Cd. Although SOD activity did not increase under conditions of Cd exposure, H_2O_2 content did increase. Other possible sources of increased H_2O_2 content are oxidase enzymes such as glycolate, glucose, amino-acid and sulphite oxidases (Asada 1999). Our results are in accordance with those of Zouari *et al.* (2016), who reported induction of H_2O_2 production reflecting oxidative stress in olive (*Olea europaea*) tissues under conditions of Cd exposure. On the other hand, the presence of phenols, carboxylic acids and quinones in the structure of HA impart antioxidant activity (Siddiqui *et al.* 2009). Exogenously applied HA significantly increased SOD activity, as reported by Zhang *et al.* (2013) and Elmongy *et al.* (2018). Catalase and POX are key enzymes responsible for the decomposition of H_2O_2 produced by SOD. In the present study, Cd-induced H_2O_2 was scavenged by the activation of CAT and APX (only at 100 µM Cd stress). Similarly, it has been reported that CAT and APX play a crucial role in decreasing Cd-induced radical accumulation in barley seedlings (Hegedus *et al.* 2001). In the Asada-Halliwell cycle, ascorbate and glutathione are also used as cofactors in reactions catalysed by peroxidases (APX and GPX) to reduce H_2O_2 to water. This cycle includes non-enzymatic antioxidants (AsA and GSH) and antioxidant enzymes (APX, GR, DHAR and MDHAR) (Zhang *et al.* 2013). The regeneration of ascorbate from monodehydroascorbate and dehydroascorbate is catalyzed by monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (de Tullio *et al.* 1998). Asadi karam *et al.* (2017) and Arora *et al.* (2008) reported a decrease in the activities of APX, MDHAR and DHAR in As-treated plants, similar to the results of our study. Also, lack of change in GR activity altered the decrease in GSH content under Cd stress in this study, parallel to the results of Saeid *et al.* (2014). GSH can directly reduce H_2O_2, resulting in glutathione disulfide (GSSG), which can be regenerated by glutathione reductase (GR) using NADPH as an electron donor (Jozefczak *et al.* 2014). In the present study, declining contents of GSH and GSSG were observed in Cd-treated plants, an indication of oxidative stress. Similarly, a recent report by Hasanuzzaman *et al.* (2013) showed a decrease in GSH and the GSH/GSSG ratio in As-treated wheat. In the present study, there was positive correlation among induced H_2O_2 content, TBARS content (as a marker in lipid peroxidation) and reduced ROS scavenging system. These results are compatible with the findings of Rui *et al.* (2016), who observed that Cd stress stimulated lipid peroxidation levels in *Vicia sativa* roots. On the other hand, an induction in CAT activity under HA plus Cd was related to the reduction of H_2O_2 content in wheat. This is in accordance with a previous study where HA increased CAT activity in rice (García *et al.* 2012). Combination of HA and Cd stress enhanced APX, MDHAR, DHAR, GSH content and GSH/GSSG ratio in wheat roots exposed to Cd stress. Interestingly, after HA application under Cd stress in wheat leaves,

Ozfidan-konakci *et al.* (2018) reported that only 750 mg L^{-1} HA increased the activities of MDHAR and DHAR but, in roots the both HA applications did. This result is consistent by Tartoura *et al.* (2014) who reported that organic compost application increased the activities of APX, MDHAR and DHAR in *Solanum lycopersicum* subjected to salt stress. A high level of GSH/GSSG protects GSH function in the AsA-GSH cycle. As indicated in our results, elevated GSH content and GSH/GSSG ratio were positively correlated with the capacity of plants to resist Cd-induced oxidative damages (Semane *et al.* 2007) through regulating GSH biosynthesis or by regenerating the reactive cysteine residue of GSH (Espinosa-Diez *et al.* 2015). Therefore, HA supplementation promoted enzyme and non-enzyme activity and hence it successfully alleviated stress-induced H_2O_2 and TBARS content. These results are compatible with studies by García *et al.* (2014) and (Lotfi *et al.* 2015) which exhibited lower H_2O_2 content and TBARS in HA-treated plants grown under stress.

CONCLUSION

Cd stress disrupted growth, turgor and osmotic balance and lipid structure of membrane in wheat roots as indicated by RGR, Ψ_Π, Pro accumulation and TBARS content. Only 100 μM Cd stress triggered the activation of CAT and APX enzymes, although these enzymes did not prevent the increase of H_2O_2 content. On the other hand, addition of HA to stress-treated plants led to increased activities of SOD, CAT, APX, MDHAR, DHAR and GSH content and GSH/GSSG ratio. The activation of these protective molecules by exogenous HA was correlated with decreases of H_2O_2 and TBARS contents. As well as antioxidant activities, there was a positive interaction between HA application and RGR, Ψ_Π and Pro content. HA could eliminate the oxidative damage induced by Cd stress through the enzymatic/non-enzymatic antioxidants related to ascorbate-glutathione cycle.

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Botanica SERBICA

REZIME

Huminska kiselina štiti od oksidativnog oštećenja indukovanog toksičnošću kadmijuma u korenu pšenice (Triticum aestivum) kroz upravljanje vodama i antioksidativni sistem odbrane

Evren Yildiztugay, Ceyda Ozfidan-Konakci, Fevzi Elbasan, Aysegul Yildiztugay i Mustafa Kucukoduk

Huminski sastojci, kao što je huminska kiselina (HA) podstiču zdravlje ekosistema stabilizacijom strukture tla i podsticanjem razvoja biljke. Međutim, korišćena količina predstavlja limitirajući factor. Upotreba biostimulanasa baziranih na HA je efikasan način da se eliminiše oksidativno oštećenje nastalo kao posledica teških metala u biljakama, kao što je Cd. CIlj ovog rada je da se ispita efekat huminske kiseline (HA; 750 and 1500 mg L^{-1}) na rast, osmotski potencijal, antioksidativni sistem, sadržaj radikala i lipidnu peroksidaciju u korenu pšenice (*Triticum aestivum*) tretiranih samih ili u kombinaciji sa Cd stresom (100 and 200 μM). Korenovi tretirani kadmijumom pokazuju redukciju rasta (RGR) i osmotskog potencijala (Ψ_Π) i indukciju sadržaja prolina (Pro). Iako je 100 μM Cd stres indukovao aktivnosti katalaze (CAT) i askorbat peroksidaze (APX), u korenu izloženom stresu akumulacija vodonik peroksida (H_2O_2) nije sprečena. Membrana korena pokazuje stres-zavisnu lipidnu peroksidaciju (sadržaj TBARS). Primena HA u kombinaciji sa stresom ublažava RGR i Ψ_Π podsticanjem usvajanja vode. Huminska kiselina smanjuje nivoe H_2O_2 i TBARS kroz aktivaciju superoksid dismutase (SOD) i CAT. Primena HA pri stresu, takođe, indukuje enzimske i neenzimske supstance uključene u ciklusaskorbat-glutationa, kao što su APX, monodehidroaskorbat reduktaza (MDHAR), dehidroaskorbat reduktaza (DHAR) i glutation (GSH) pored povećanja odnosa GSH/GSSG. Ovi rezultati pokazuju da u korenu pšenice HA ublažava negativne efekte oksidativnog oštećenja izazvanog Cd, regulacijom rasta, osmotskim podešavanjem, akumulacijom radikala i dejstvom antioksidativnih sistema.

Ključne reči: Ciklus askorbat-glutationa, huminska kiselina, lipidna peroksidacija, oksidativni stres, *Triticum aestivum*