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Cold stress in soybean (*Glycine max* L.) roots: exogenous gallic acid promotes water status and increases antioxidant activities

Ceyda Ozfidan-Konakci[1*], Evren Yildiztugay[2], Aysegul Yildiztugay[3] and Mustafa Kucukoduk[3]

1 Department of Molecular Biology and Genetics, Necmettin Erbakan University, 42090, Konya, Turkey
2 Department of Biotechnology, Selcuk University, 42250, Konya, Turkey
3 Department of Biology, Selcuk University, 42250, Konya, Turkey

ABSTRACT: Gallic acid (GLA; 3,4,5- trihydroxybenzoic acid) is a strong antioxidant in plants. In order to clarify the effects of GLA as a pro-oxidant or an antioxidant on cells under stress conditions, soybean (*Glycine max*) was grown under normal conditions or in the presence of cold stress (5 and 10°C) in the absence or presence of gallic acid (GLA; 1 and 2 mM) for 72 h. The soybean roots exposed to stress exhibited a significant decline in growth (RGR), water content (RWC), osmotic potential (Ψ_{II}) and proline content (Pro). However, GLA treatment under stress significantly improved these parameters and alleviated the stress–generated damage. Stress decreased superoxide dismutase (SOD) activity, but GLA effectively mitigated the adverse effects on enzyme activity. After stress treatment, only catalase (CAT) was induced in soybean roots, although it was not sufficient to prevent toxic hydrogen peroxide (H_2O_2) accumulation. Thus, the levels of lipid peroxidation (TBARS content) markedly increased. However, GLA contributed to detoxification of H_2O_2 and lipid peroxidation by enhancing activities of CAT and peroxidase (POX). In addition to these enzymes, SOD activity was able to scavenge superoxide anion radicals, as evidenced by decline in TBARS content. However, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), total ascorbate (tAsA) and glutathione (GSH) showed a decline of content in roots treated with GLA (both concentrations) plus stress. Our results suggest a protective role of GLA, which may strengthen plant tolerance by ensuring efficient water use and enhancing antioxidant systems. In soybean roots, GLA successfully alleviated the toxicity of cold stress by modulating the activities of SOD, CAT and POX rather than enzymes of the ascorbate-glutathione cycle.

Keywords: Antioxidant system, cold stress, gallic acid, *Glycine max*, reactive oxygen species

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INTRODUCTION

Cold is one of the major abiotic stresses limiting the productivity, geographical distribution and yield of many important crops (Zhang *et al.* 2009). Cold stress, defined as temperature in a range low enough to suppress growth without stopping cellular functions, is known to induce several abnormalities at various levels of cell organisation. The primary effect of injury induced by cold stress is impaired reorganisation of the cell membrane (Wu & Zou 2010) and subsequent loss of selective permeability, followed by release of cellular components (Cheng *et al.* 2010). It is generally accepted that environmental stresses like, cold, drought, chilling or heat increase production of reactive oxygen species (ROS) such as superoxide anion radicals, hydrogen peroxide (H_2O_2), singlet oxygen and hydroxyl radicals ($OH^{\bullet}$). To neutralise oxidative stress, plants possess antioxidant

*correspondence: cozfidan@erbakan.edu.tr

enzymes [superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR)] and non-enzymatic antioxidant systems (reduced glutathione, α-tocopherol, ascorbate and phenolics) (Zhang *et al.* 2009).

To eliminate the harmful effects produced by stress conditions and improve crop yields, the application of allelochemicals is promising as an effective agronomic technique (Makoi & Ndakidemi 2012). Among allelochemicals, phenolic compounds can play a role in germination, growth and development of plants at the cellular and molecular levels (Maqbool *et al.* 2013). These compounds are efficient free radical scavengers and inhibitors of lipid peroxidation in the cell membrane (Michalak 2006). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen or decomposing peroxides. This feature has been attributed to the two or more phenolic hydroxyl groups in their chemical structure (Michalak 2006). Tannin, the second most abundant polyphenolic in vascular plant species, can be divided into two major classes, viz., condensed tannins and hydrolysable tannins. Hydrolysable tannins are grouped into gallotannins and ellagitannins, which are composed of gallic acid (GLA, 3, 4, 5-trihydroxybenzoic acid) or hexahydroxydiphenic acid esters, respectively (Kraus *et al.* 2003). The antioxidant ability of GLA provides protection against hydroxyl radical-induced deoxyribose degradation in the Fenton reaction (Kamdem *et al.* 2012).

There are many reports dealing with the protective effects of phenolic compounds in plants exposed to heavy metals, chilling, osmotic stress and salinity (El-Tayeb *et al.* 2006; Li *et al.* 2011b; Ozfidan-Konakci *et al.* 2015). On the other hand, Lara-Nunez *et al.* (2009) reported that some phenolics have a phytotoxic effect on plant physiology. Also, phenolic compounds increase the generation of ROS and cause oxidative stress (Cruz-Ortega *et al.* 2007). Thus, there are a number of sometimes contradictory reports in the literature. At the same time, information is scarce about the protective effects of GLA on the activities of antioxidant enzymes, especially in plants grown under stress conditions. In view of these considerations, the present study was designed to evaluate the effects of GLA on growth, water relations, enzymatic/non-enzymatic antioxidant systems and lipid peroxidation in soybean roots exposed to cold stress.

MATERIALS AND METHODS

Plant materials and experimental design. Seeds of *Glycine max* L. cv. *Mitchell* were obtained from the Bati Akdeniz Agricultural Research Institute, Antalya, Turkey. Seeds were surface-sterilised in 5% sodium hypochlorite for 10 min, rinsed five times with sterile distilled water and then allowed to germinate on double-layer filter paper wetted with distilled water. Germinated soybean seedlings were transferred to half-strength Hoagland solution and grown under controlled conditions (a 16/8 h light/dark regime and a photosynthetic photon flux density of 350 μmol $m^{-2}s^{-1}$). The seedlings were grown in hydroponic culture containing this solution for 21 days, and two concentrations of gallic acid (GLA; 1 and 2 mM) were administered alone or in combination with cold stress (5°C and 10°C). Plants were harvested after 72 h of treatment and the roots stored at −86°C until further analyses.

Determination of growth, water content, osmotic potential and proline content. The relative growth rate (RGR) was computed according to Hunt *et al.* (2002). The root relative water content (RWC) was measured according to Smart & Bingham (1974). The osmotic potential (Ψ_{Π}) of soybean roots was converted from mosmoles kg^{-1} to MPa. Proline accumulation was determined according to Bates *et al.* (1973).

Determination of H_2O_2 accumulation. The method used to determine H_2O_2 content was described in detail in Liu *et al.* (2010).

Determination of lipid peroxidation. Lipid peroxidation in soybean roots was determined as the content of thiobarbituric acid- reactive substances (TBARS) according to the method described by Rao & Sresty (2000).

Protein content and enzyme extraction. For protein and enzyme analysis, 0.5 g of soybean roots was homogenised in 50 mM Tris-HCl containing ethylenediaminetetraacetic acid (EDTA), Triton X-100, phenylmethylsulphonyl fluoride and dithiothreitol (DTT). Total protein content was determined according to Bradford (1976).

Determination of antioxidant enzyme and isozyme composition. Samples were subjected to polyacrylamide gel electrophoresis (PAGE) as described by Beauchamp & Fridovich (1971). The total SOD (EC 1.15.1.1) activity was analysed according to Beauchamp & Fridovich (1971). Total CAT (EC 1.11.1.6) activity was determined by the method of Bergmeyer (1970). Isozymes of POX (EC 1.11.1.7) and total POX activity were determined according to Seevers *et al.* (1971) and Herzog & Fahimi (1973), respectively. Electrophoretic APX separation was analysed according to Mittler & Zilinskas (1993). Total activities of APX (EC 1.11.1.11) and GR (EC 1.6.4.2) were ascertained according to Nakano & Asada (1981) and Foyer & Halliwell (1976), respectively.

Gels stained for the activities of SOD, POX and APX were monitored with the Gel Doc XR+ System and measured with Image Lab software, ver. 4.0.1 (Bio-Rad, California, USA). Known standard concentrations of

enzymes (0.5 units of SOD and 0.2 units of POX) were used. The unit of isozyme activity for each group was established by comparison with the standard. Average values (shown with the same symbol) were not significant at $p > 0.05$ using Tukey's post hoc test.

Determination of the activities of monodehydroascorbate reductase and dehydroascorbate reductase. The activities of monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) were determined as described in detail by Miyake & Asada (1992) and Dalton *et al.* (1986), respectively.

Determination of the content of dehydroascorbate and ascorbate. The concentrations of total and reduced ascorbate (AsA) were assayed according to the method of Dutilleul *et al.* (2003). The oxidised form of ascorbate (DHA, dehydroascorbate) was analysed using the formula DHA content = total AsA – reduced AsA in soybean roots.

Determination of the content of glutathione and oxidised glutathione. Glutathione (GSH) content was determined according to Paradiso *et al.* (2008). Oxidised glutathione (GSSG) content in soybean roots was measured after removal of GSH by 2-vinylpyridine.

Statistical analysis. The experiments were repeated thrice. All data obtained were subjected to one-way analysis of variance (ANOVA). Statistical analysis of the data was performed using SPSS 20.0. The treatment groups were compared using Tukey's post hoc test. The results are presented as the mean with error bars indicating the standard error of the mean.

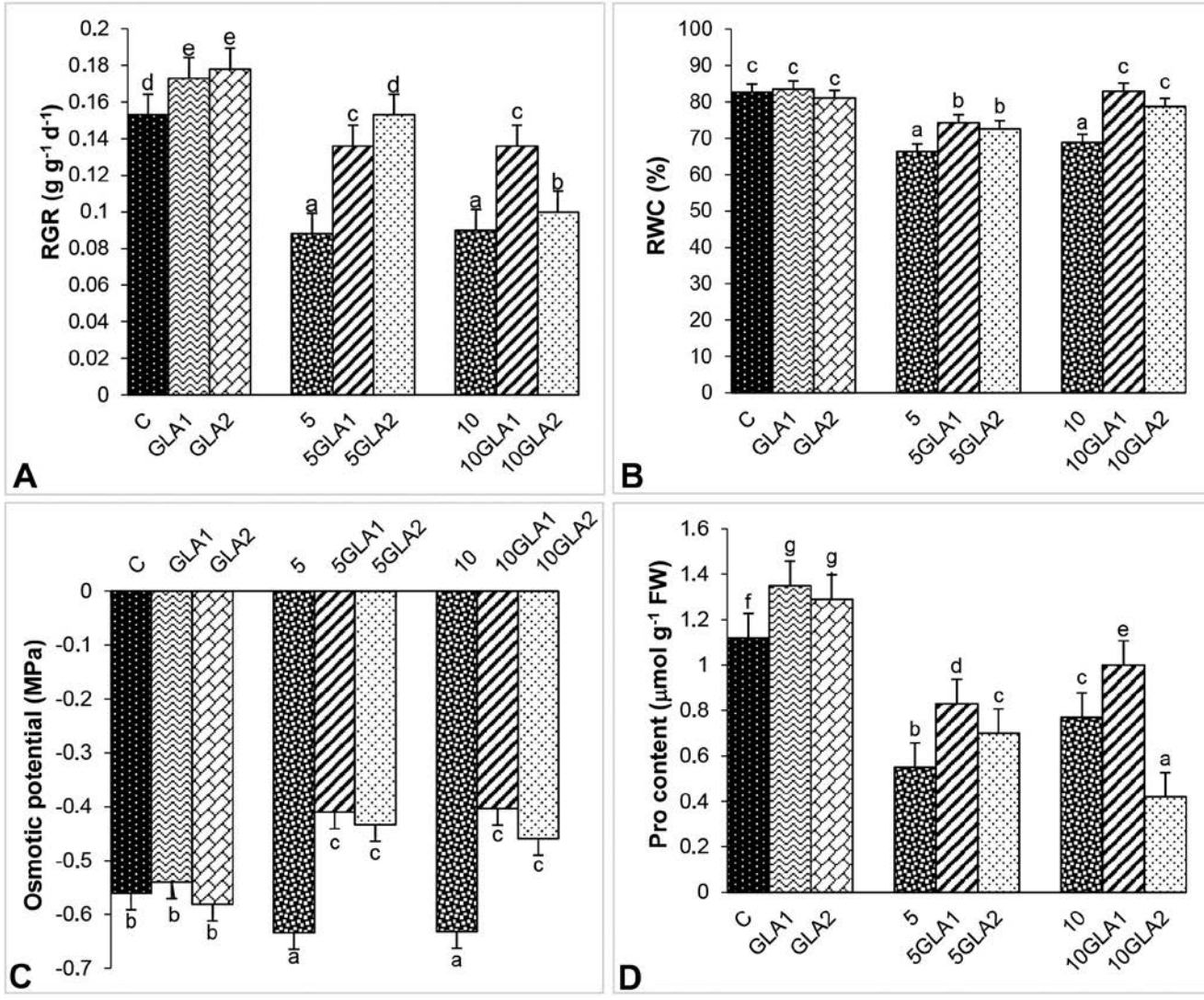

Fig. 1. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on growth (RGR) (**A**), water content (RWC) (**B**), osmotic potential (Ψ_{Π}) (**C**) and proline content (Pro) (**D**) in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h. Columns with different letters are significantly different ($P < 0.05$). The columns represent the mean ± SE ($n = 6$).

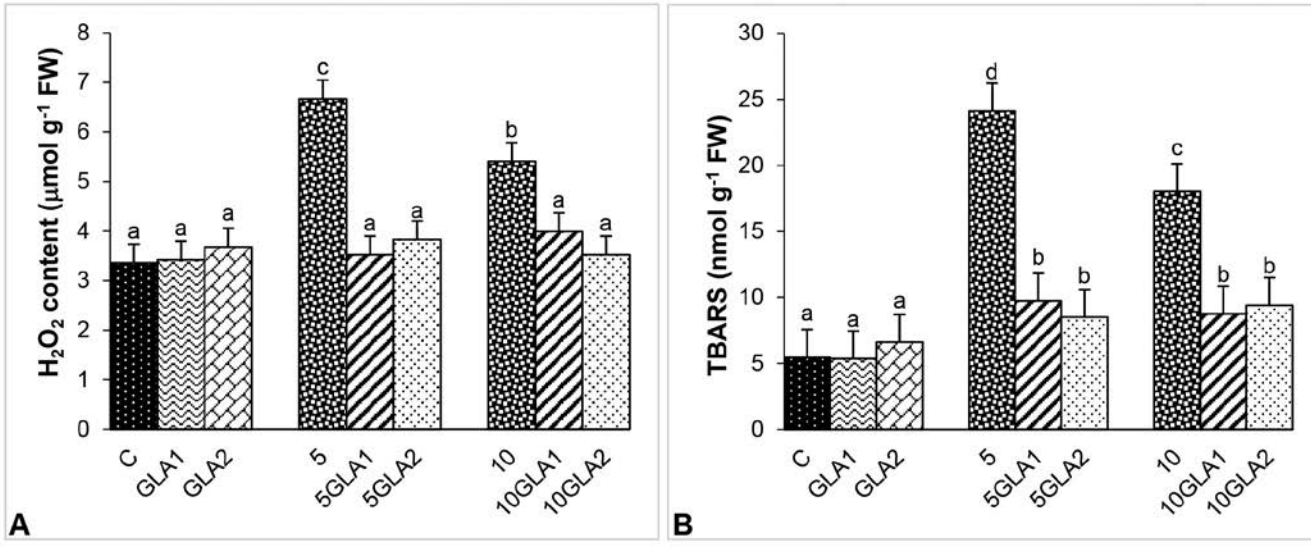

Fig. 2. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on H_2O_2 content (μmol g^{-1} of FW) (**A**) and content of thiobarbituric acid-reactive substances (TBARS; nmol g^{-1} of FW) (**B**) in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h. Columns with different letters are significantly different ($P < 0.05$). The columns represent the mean ± SE (n = 6).

RESULTS

Growth, water content, osmotic potential and proline content. Exposure of soybean roots to 5 and 10°C resulted in decrease of RGR, by 42 and 41%, respectively, as compared to the control group (Fig. 1A). Addition of GLA under conditions of cold stress significantly prevented the stress-induced decrease of RGR recorded in roots subjected to stress treatment alone. Maximum induction of RGR was observed in soybean treated with GLA2 plus 5°C. In soybean roots, GLA alone significantly increased RGR during the period of experimentation.

As shown in Fig. 1B, RWC of soybean roots was slightly reduced by cold stress and its levels in plants with the lowest temperature treatment were reduced by 20%. In the roots of soybean supplemented with GLA under conditions of stress treatment, the increase in RWC was slight when compared to the stress treatment alone. The best improvement of RWC was at GLA1+10°C (an increase of 20%). After 72 h of GLA treatment alone, RWC was not significantly affected compared to the control.

As indicated Fig. 1C, cold stress significantly lowered the osmotic potential (Ψ_{Π}) as compared to the control groups. For example, Ψ_{Π} dropped from -0.560 MPa to -0.634 MPa in 5°C-treated plants. Both GLA concentrations led to increase in Ψ_{Π} of plants exposed to stress treatments. The value of Ψ_{Π} was not significantly influenced by GLA application under control conditions.

When cold stress was applied to soybean, a decrease in proline content (Pro) was observed in comparison with the control group (Fig. 1D). Except in the case of the 10°C+GLA2 group, the addition of GLA to the stress-treated plants increased Pro content. The highest induction of Pro as compared to the stress treatment alone (51%) was in the 5°C+GLA1 variant. Also, a significant increase of Pro was observed in roots exposed to GLA applications alone (GLA1 and GLA2), by 20.5 and 15.1%, respectively.

H_2O_2 content. As shown in Fig. 2A, 5 and 10°C stress significantly enhanced H_2O_2 content (6.67 and 5.41 μmol g^{-1} of FW, respectively) as compared to the control (3.36 μmol g^{-1} of FW). The stress-induced enhancement of H_2O_2 content decreased significantly with the application of GLA and reached the control levels. It was also observed that H_2O_2 content in roots treated with GLA alone was similar to that in the control groups.

Lipid peroxidation. The content of TBARS is shown in Fig. 2B. Cold stress increased lipid peroxidation in soybean roots. The highest increase (4.4-fold) in TBARS content was observed in 5°C-treated plants. In contrast, TBARS content sharply decreased with GLA treatments under stress conditions. By itself, GLA had no significant effect on TBARS content.

Antioxidant enzyme and isozyme composition. In the conducted native-page analysis, seven SOD isoenzyme bands, including three Mn-SODs and four Cu/Zn-SODs, were detected in roots (Fig. 3A). Total SOD activity decreased under cold treatments (Fig. 3B). Furthermore, GLA also distinctly enhanced SOD activity under stress as compared to stress treatment alone, and the highest activity increase (39%) occurred in the variant with 5°C+GLA2, as indicated by the intensities of Mn-SOD2-3 and Cu/Zn-SOD1-4 activities (Table 1). It turned out that GLA treatment alone resulted in 1.23-

Table 1. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on relative contribution of SOD isozymes in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h (nd: not defined). Columns with different letters are significantly different ($P < 0.05$). Columns represent the mean ± SE ($n = 6$).

	Control	GLA1	GLA2	5	5GLA1	5GLA2	10	10GLA1	10GLA2
Mn-SOD1	0.086±0.004 ^{d}	0.082±0.001 ^{d}	0.021±0.008 ^{a}	0.075±0.004 ^{c}	0.048±0.004 ^{b}	0.056±0.001 ^{b}	0.073±0.007 ^{c}	0.057±0.004 ^{b}	0.019±0.007 ^{a}
Mn-SOD2	0.152±0.005 ^{b}	0.149±0.005 ^{b}	0.169±0.007 ^{c}	0.112±0.005 ^{a}	0.123±0.001 ^{a}	0.165±0.007 ^{c}	0.137±0.007 ^{b}	0.161±0.005 ^{c}	0.149±0.006 ^{b}
Mn-SOD3	0.312±0.004 ^{b}	0.433±0.009 ^{c}	0.435±0.004 ^{c}	0.298±0.007 ^{a}	0.458±0.001 ^{c}	0.45±0.007 ^{c}	0.292±0.006 ^{a}	0.434±0.005 ^{c}	0.404±0.006 ^{c}
Cu/Zn-SOD1	0.047±0.005 ^{b}	0.055±0.008 ^{c}	0.033±0.004 ^{a}	0.037±0.007 ^{a}	0.044±0.008 ^{b}	0.051±0.006 ^{c}	0.035±0.005 ^{a}	0.05±0.001 ^{c}	0.03±0.003 ^{a}
Cu/Zn-SOD2	0.006±0.001 ^{a}	0.017±0.007 ^{d}	0.026±0.002 ^{e}	0.011±0.008 ^{b}	0.013±0.007 ^{c}	0.013±0.003 ^{c}	0.012±0.002 ^{b}	0.018±0.007 ^{d}	0.012±0.007 ^{b}
Cu/Zn-SOD3	0.012±0.008 ^{a}	0.028±0.002 ^{b}	0.038±0.008 ^{c}	0.016±0.002 ^{a}	0.031±0.008 ^{c}	0.024±0.002 ^{b}	0.013±0.001 ^{a}	0.026±0.009 ^{b}	0.025±0.001 ^{b}
Cu/Zn-SOD4	nd	0.009±0.002 ^{c}	0.014±0.006 ^{d}	0.005±0.002 ^{a}	0.009±0.002 ^{c}	0.007±0.002 ^{b}	nd	nd	0.01±0.004 ^{c}

Table 2. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on relative contribution of POX isozymes in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h. Columns with different letters are significantly different ($P < 0.05$). Columns represent the mean ± SE ($n = 6$).

	Control	GLA1	GLA2	5	5GLA1	5GLA2	10	10GLA1	10GLA2
POX1	0.023±0.002 ^{c}	0.026±0.005 ^{c}	0.069±0.001 ^{e}	0.032±0.004 ^{d}	0.023±0.004 ^{c}	0.022±0.004 ^{c}	0.016±0.002 ^{b}	0.005±0.002 ^{a}	0.008±0.007 ^{a}
POX2	0.033±0.005 ^{b}	0.027±0.006 ^{a}	0.073±0.001 ^{d}	0.037±0.001 ^{b}	0.033±0.002 ^{b}	0.036±0.003 ^{b}	0.034±0.001 ^{b}	0.032±0.005 ^{b}	0.046±0.004 ^{c}
POX3	0.007±0.007 ^{b}	0.006±0.006 ^{a}	0.014±0.008 ^{c}	0.007±0.001 ^{b}	0.012±0.005 ^{c}	0.013±0.001 ^{c}	0.008±0.001 ^{b}	0.005±0.008 ^{a}	0.012±0.004 ^{c}
POX4	0.05±0.003 ^{a}	0.047±0.001 ^{a}	0.053±0.006 ^{b}	0.049±0.005 ^{a}	0.06±0.004 ^{c}	0.066±0.001 ^{c}	0.058±0.004 ^{b}	0.047±0.007 ^{a}	0.054±0.001 ^{b}
POX5	0.002±0.004 ^{a}	0.003±0.007 ^{a}	0.002±0.006 ^{a}	0.005±0.009 ^{c}	0.002±0.008 ^{a}	0.002±0.006 ^{a}	0.004±0.007 ^{c}	0.002±0.007 ^{a}	0.003±0.003 ^{a}
POX6	0.177±0.004 ^{e}	0.123±0.002 ^{b}	0.134±0.005 ^{c}	0.18±0.004 ^{e}	0.158±0.003 ^{d}	0.135±0.003 ^{c}	0.106±0.007 ^{a}	0.12±0.001 ^{b}	0.123±0.008 ^{b}
POX7	0.027±0.003 ^{d}	0.014±0.003 ^{a}	0.02±0.001 ^{c}	0.024±0.007 ^{c}	0.027±0.004 ^{d}	0.016±0.001 ^{b}	0.016±0.003 ^{b}	0.013±0.002 ^{a}	0.013±0.007 ^{a}
POX8	0.018±0.009 ^{c}	0.013±0.004 ^{b}	0.015±0.009 ^{b}	0.015±0.002 ^{b}	0.02±0.003 ^{c}	0.011±0.002 ^{a}	0.016±0.004 ^{b}	0.015±0.006 ^{b}	0.014±0.008 ^{b}
POX9	0.023±0.004 ^{a}	0.033±0.003 ^{d}	0.025±0.007 ^{b}	0.021±0.001 ^{a}	0.031±0.008 ^{d}	0.032±0.008 ^{d}	0.025±0.008 ^{b}	0.028±0.007 ^{c}	0.025±0.008 ^{b}
POX10	0.008±0.001 ^{a}	0.023±0.001 ^{c}	0.023±0.007 ^{c}	0.01±0.006 ^{a}	0.022±0.004 ^{c}	0.022±0.002 ^{c}	0.009±0.007 ^{a}	0.015±0.009 ^{b}	0.015±0.002 ^{b}

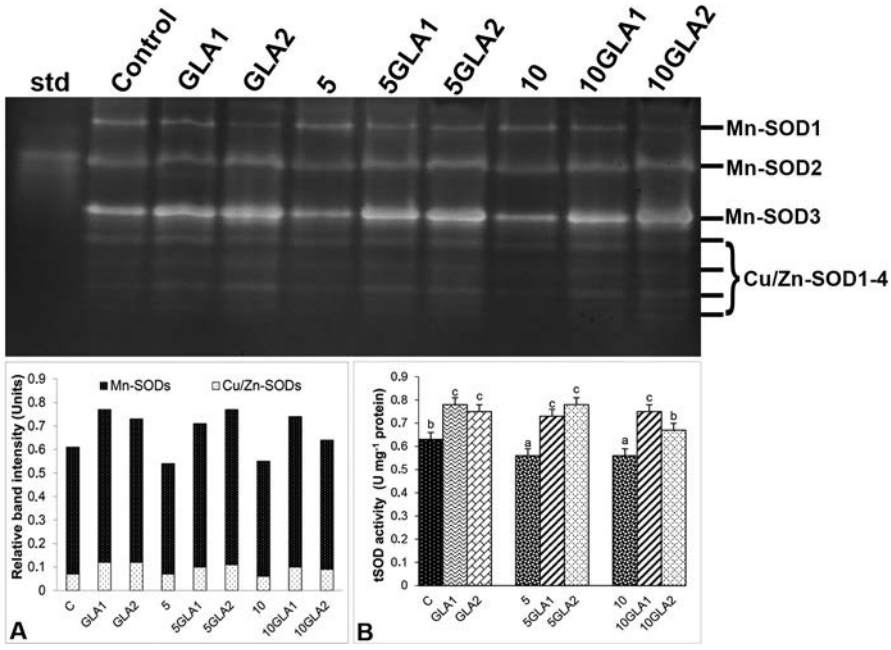

Fig. 3. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on activity staining of SOD isozymes (**A**) and total SOD activity (U, mg^{-1} of protein) (**B**) in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h. Columns with different letters are significantly different ($P < 0.05$). The columns represent the mean ± SE ($n = 6$).

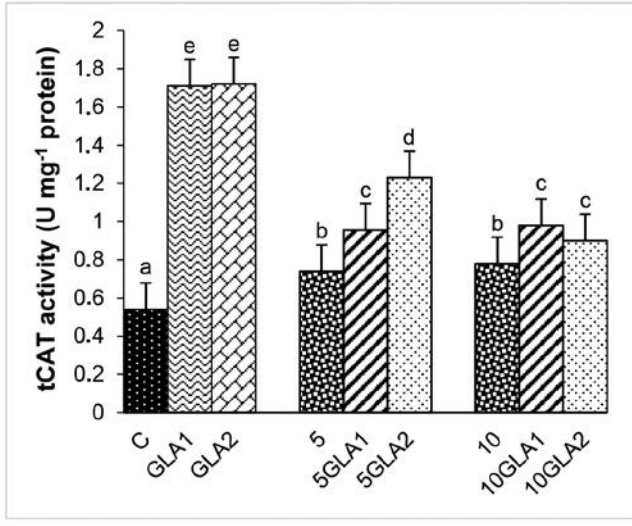

Fig. 4. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on total CAT activity (U mg^{-1} of protein) in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h. Columns with different letters are significantly different ($P < 0.05$). The columns represent the mean ± SE ($n = 6$).

and 1.19-fold increases in total SOD activity as compared to the control. This change was dependent on the intensities of Mn-SOD3 and Cu/Zn-SOD activities.

Increases of about 37 and 44% in total CAT activity were detected in roots of the variants with 5 and 10°C, respectively (Fig. 4). Application of GLA increased CAT activity in plants exposed to stress treatments, which exhibited a higher value (a 2.49-fold increase in the variant with 5°C+GLA2) than that of the plants grown under stress conditions alone. Similarly, after GLA1 and GLA2 treatments under non-stress conditions, the plants had higher activity of this enzyme (3.18 and 3.19 times higher, respectively) than in the control group.

The zymogram of POX showed 10 distinct bands (POX1-10) in soybean roots (Fig. 5A). The roots of soybean exhibited a marked decrease of POX activity, down to 18% at the lowest temperature (Fig. 5B). The addition of GLA to stress-treated plants significantly improved POX activity, with the highest induction (by 26%) in the 5°C+GLA2 group. Analysis of total POX revealed that GLA1 application alone did not change the isozyme pattern, but both total POX activity and activities of POX isozymes ncreased in GLA2-exposed roots, this effect being more pronounced in the intensities of POX1, 2, 3, 4, 9 and 10 (Table 2).

Four activity bands of APX isozymes (APX1-4) were detected in enzyme preparations from soybean roots (Fig. 6A). Cold stress lowered total APX activity (Fig. 6B). Also, GLA applied to the soybean roots grown under stress caused a decline in the activation of APX, consistent with decreased intensities of APX2, 3 and 4. Application of GLA alone decreased total APX activity as compared with the control, the most notable activity being recorded in the APX2-3 isoforms.

Roots treated with stress had a lower GR activity than in the control plants (Fig. 6C). However, GLA application mitigated the inhibitory effect of cold stress on GR activity. The treatments with GLA application alone showed no significant promotional effect on total GR activity compared with that of the control.

When compared to the control, a slight decrease of DHAR activity was observed in response to stress (Fig.

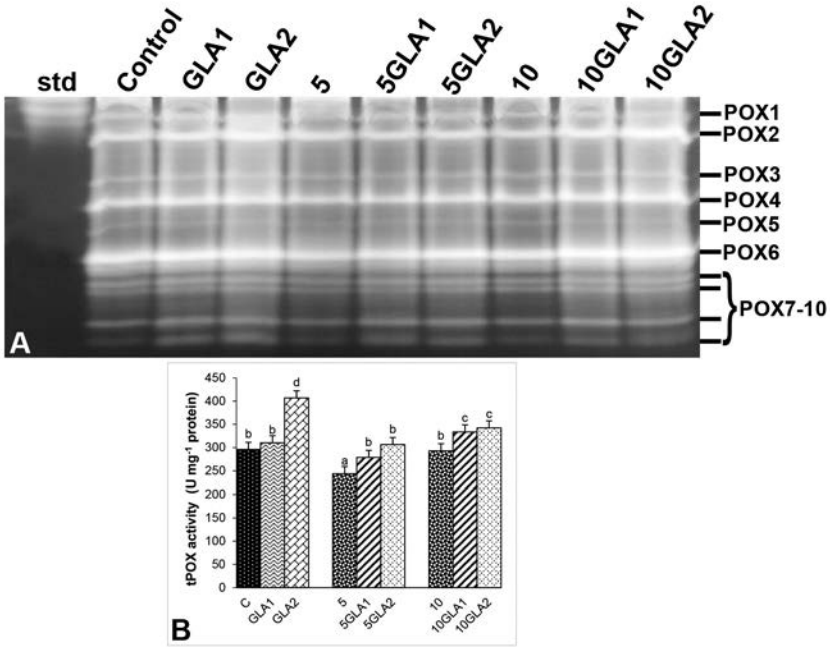

Fig. 5. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on activity staining of POX isozymes (**A**) and total POX activity (U mg^{-1} of protein) (**B**) in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 hours (h). Columns with different letters are significantly different ($P < 0.05$). The columns represent mean ± SE (n = 6).

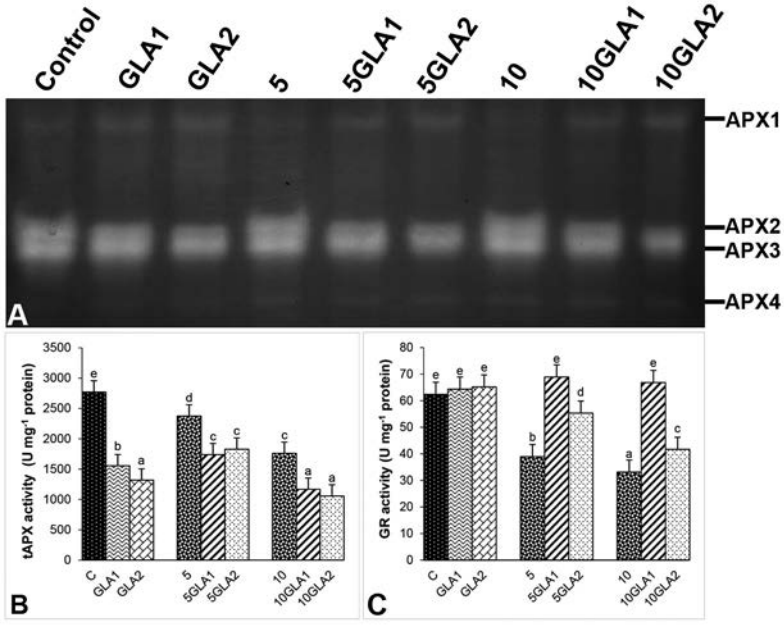

Fig. 6. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on activity staining and relative contribution of APX isozymes (**A**), total APX activity (U mg^{-1} of protein) (**B**) and total GR activity (U mg^{-1} of protein) (**C**) in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h. Means followed by different letters are significantly different ($P < 0.05$). Columns with different letters are significantly different ($P < 0.05$). The columns represent the mean ± SE (n = 6).

7A). Also, DHAR activity in roots subjected to stress was notably reduced by GLA treatments. For example, in the case of this activity, the maximum reduction (down to 27%) was in 10°C+GLA1-treated plants. On the other hand, a significant improvement of DHAR activity was observed in soybean roots with GLA treatment alone. A significant reduction of MDHAR activity was detected in soybean plants subjected to stress treatment with/without GLA application (Fig. 7B). There was no enhancement of MDHAR activity following GLA treatment alone.

The content of DHA and that of AsA were not stimulated by either of the stresses alone or by stress with GLA application (Fig. 7C and D). Their content remained at the control level or decreased. However, GLA exposure increased the levels of DHA and AsA when compared with control group. For example, DHA and AsA concentrations reached the maximum levels (6 and 13%, respectively) in roots with GLA1.

The content of GSH and GSSG decreased or did not change under both stress conditions or conditions of treatment with GLA alone (Fig. 7E and F). Similarly, the

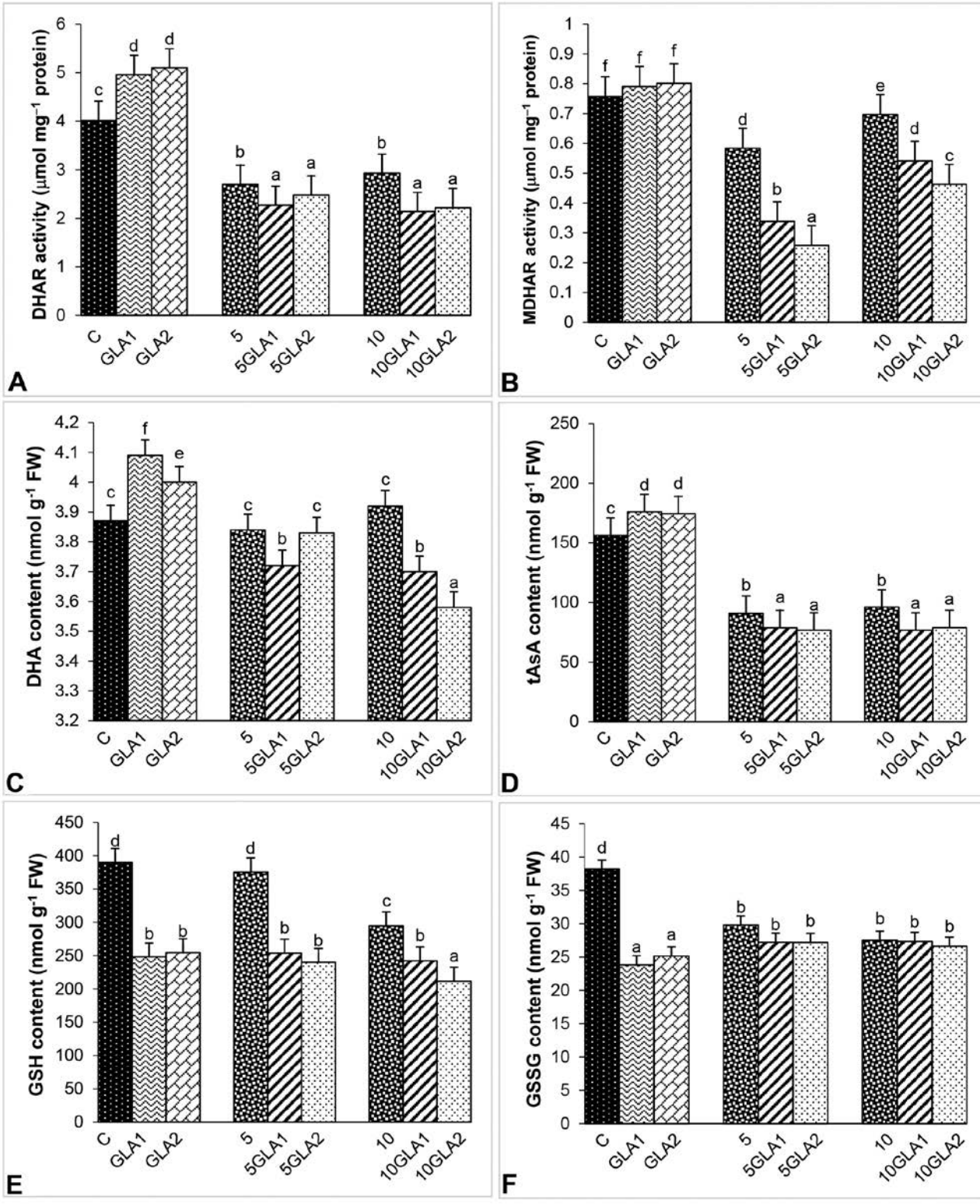

Fig. 7. Effects of gallic acid (GLA1 and GLA2, 1 and 2 mM) on DHAR activity (U mg^{-1} of protein) (**A**), MDHAR activity (U mg^{-1} of protein) (**B**), DHA content (**C**), AsA content (**D**), glutathione content (GSH, **E**) and GSSG content (**F**) in roots of *Glycine max* exposed to cold stress (5 and 10°C) for 72 h. Columns with different letters are significantly different ($P < 0.05$). The columns represent the mean ± SE (n = 6).

combination of GLA treatment with stress caused no increase iof GSH and GSSG content.

DISCUSSION

One part of the damage induced by cold stress in soybean roots was growth reduction, as evidenced by decreased values of RGR. Similarly, Lin & Saltveit (2005) reported growth reduction in mung bean plants under low-temperature stress. As noted by Silva *et al.* (2011), root growth is much reduced under cold stress because of the prolonged cycle of cell division. The reduced growth in plants exposed to stress is also due to the greater amount of energy required for osmoregulation and leaf senescence and subsequent abscission (Gheyi *et al.* 2005). However, GLA application can reverse the negative effects of cold stress on RGR in soybean roots. Singh *et al.* (2017) demonstrated that an increment in growth was observed in rutin- and GLA-treated rice as compared to the control groups. Improvement of the RGR of soybean roots might be connected with higher photosynthetic rates due to greater amounts of photosynthetic pigments, as observed by Yildiztugay *et al.* (2017) and Singh *et al.* (2017) in GLA-treated soybean and rice seedlings, respectively. Similar to the values of RGR, RWC of soybean roots decreased significantly after cold stress, confirming the results obtained by Tang *et al.* (2014). GLA prevented worsening of the water status in root cells when soybean plants were exposed to stress. It is quite likely that the stability of the RWC values after GLA application to stress-treated soybean roots (except in the case of for 10°C+GLA2) reflected induction of Pro accumulation, which allowed osmoregulation and the maintenance of hydration (Willadino & Camara 2004). This result is consistent with findings in cucumber (Li *et al.* 2013) subjected to ferulic acid plus PEG-induced osmotic stress. In the present study, the enhancement in Pro accumulation following GLA treatment under cold stress increased $\Psi_{\Pi,}$ enabling the soybean roots to realise higher water uptake, which resulted in a positive influence. Application of GLA to cold-stressed plants prevented the reduction in Ψ_{Π} caused by stress. On the other hand, cold stress decreased Ψ_{Π} of plants in the study of Xiong *et al.* (2002). The lowering of Ψ_{Π} in soybean roots under stress might be a result of osmoregulation in spite of the decline in values of root RWC. It has been reported that cold stress causes an increase in Pro content (Szabados & Savoure 2010). Conversely, in the present study, stress resulted in a decrease of Pro content in soybean roots, as was previously observed by Lv *et al.* (2011). On the other hand, GLA application induced accumulation of Pro in wheat roots, except in the case of 10°C+GLA2. Its accumulation might have lowered the potential ability of roots cells to absorb more water and improve the RGR under stress conditions. Also, this result might indicate that other compatible osmolytes (including soluble sugars) could play a role in osmotic regulation, thus contributing to stress tolerance (Willadino *et al.* 2011). The high Pro content might be related to decrease of lipid peroxidation, as indicated by lower TBARS content. Dar *et al.* (2016) asserted that Pro plays roles in protection of membrane integrity and stabilisation of organic molecules.

The metabolism of ROS is controlled by various antioxidant enzymes such as SOD, CAT, POX, APX and GR. One of the first steps in scavenging of ROS is conversion of superoxide anion radicals to H_2O_2, which is catalysed by SOD activity (Keunen *et al.* 2013). Under chilling stress, SOD activity increased in chickpea seedlings (Genisel *et al.* 2013). To the contrary, in the present study cold stress caused decline of total SOD activity in soybean roots, which appeared to be due to preferential reduction in activity of the isozymes Mn-SOD1,2 and 3 and Cu/Zn-SOD1 in spite of increased activation of Cu-Zn-SOD2 and 3 and a newly identified isoform (Cu/Zn-SOD4). Although SOD activity was not stimulated under stress treatments, H_2O_2 accumulation was observed. This is supported by Li *et al.* (2014), who reported that cold stress significantly increased H_2O_2 concentration in *Torreya grandis* leaves. This result could be linked with a repressed capacity of scavenging systems for H_2O_2 under stress or another source of H_2O_2 such as glycolate oxidases, glucose oxidases, sulphite oxidases and plasma membrane NADPH oxidases (NOX) (Slesak *et al.* 2007; Keunen *et al.* 2013). Interestingly, in a study on soybean leaves (Yildiztugay *et al.* 2017) , the same result was observed, namely that there was no increase of SOD activity in stress-treated soybean leaves, although an increase of H_2O_2 content was induced. As detected by Yildiztugay *et al.* (2017), enhancement of H_2O_2 content in stress-treated roots of soybean plants was related to increased NOX activity in their leaves. On the other hand, GLA application prevented the decline of SOD activity induced by stress. Our observations are in agreement with earlier reports that SOD activity was enhanced by addition of GLA to NaCl-treated rice roots (Ozfidan-Konakci *et al.* 2015).

The toxic levels of H_2O_2 produced after stress treatment are able to inactivate enzymes by oxidising their thiol groups, thereby causing stress (Gill & Tuteja 2010). Hydrogen peroxide is decomposed to water and oxygen by CAT and POX, together with APX and GR, which play important roles in scavenging of ROS under stress conditions (Keunen *et al.* 2013). However, in the present study only one antioxidant enzyme activity, that of CAT, was induced by cold stress in soybean roots. The activation of only one antioxidant enzyme was insufficient for scavenging of H_2O_2 content and thus H_2O_2 markedly increased in soybean roots under stress. On the other hand, GLA treatment in cold-stressed soybean roots significantly induced CAT and POX activity and raised levels of GR as compared to cold stress alone, as

reported by Yildiztugay *et al.* (2017). This enhancement of their activities under conditions of GLA plus stress was consistent with the decreased H_2O_2 content. Also, GLA alone increased the activities of SOD, CAT, POX and GR. RiceEvans *et al.* (1997) maintained that the antioxidant activity of GLA might be connected with the number and position of hydroxyl groups in relation to the carboxyl functional group. Gallic acid has three –OH groups and one –COOH (carboxylic acid) group, and its hydroxyl groups are able to bind iron and copper in particular (Kumaran & Karunakaran 2007). They may inactivate iron ions by chelating them and additionally by suppressing the superoxide-driven Fenton reaction, which is a source of ROS (RiceEvans *et al.* 1997).

In addition to CAT and POX, the AsA-GSH cycle--including APX, DHAR, MDHAR and GR--is part of the first line of defense against the harmful effects of H_2O_2 (Keunen *et al.* 2013). As shown in *Jatropha curcas* by Pedranzani *et al.* (2015), APX activity in the current study was rapidly lost under cold stress, which might be due to the low concentration of AsA. It is known that MDHAR and GR are responsible for AsA regeneration and for the reduction of GSSG to GSH using NADPH, respectively. The main function of MDHAR is to limit the amount of MDHA radicals undergoing non-enzymatic disproportionation for DHA generation. Moreover, DHAR also utilises GSH to reduce DHA and regenerate AsA (Cai *et al.* 2011). It is reported in the literature that increased activities of MDHAR, DHAR, APX and GR contribute to chilling tolerance in plants (Pastori *et al.* 2000; Hasanuzzaman *et al.* 2009; Cai *et al.* 2011; Wu *et al.* 2015). However, in our study, both cold stress treatments alone and GLA plus stress lowered the activities of MDHAR, DHAR and GR in soybean roots. This might explain, at least partially, why the content of tAsA and that of DHA were not enhanced by cold stress or GLA with stress. Similarly, Abu El-Soud *et al.* (2013) observed that MDHAR and DHAR levels were not different in ellagic acid-treated and untreated seedlings. Also, Yen *et al.* (2002) reported that the scavenging effect of GLA toward H_2O_2 was greater than that of AsA. In addition to AsA, GSH has an important function in maintaining the cellular redox status and plays a protective role based on induction of the plant´s antioxidant capacity and signaling (Cai *et al.* 2011). Oxidised glutathione (GSSG) is reconverted to GSH by GR activity (Keunen *et al.* 2013). In the present study, although GR activity increased in GLA-treated soybean roots exposed to stress, an increase of GSH content was not observed. Also, GLA application did not cause any increase of the GSH/GSSG ratio in soybean roots subjected to cold stress. As indicated by Semane *et al.* (2007), an elevated GSH/GSSG ratio is positively correlated with the ability of plants to protect themselves against stress-induced damage. However, in the present study, GLA did not contribute to elimination of the damage caused by cold stress through alteration of the reduced/oxidised GSH ratio under stress conditions. On the other hand, GLA application in soybean leaves treated with stress (5°C) activated the ascorbate-glutathione cycle (Yildiztugay *et al.* 2017). These results suggest no apparent role for the ascorbate-glutathione cycle in damage alleviation by GLA in soybean roots under cold stress. Moreover, defensive reactions in response to GLA under stress conditions vary according to the plant part (root or leaf).

Finally, TBARS is a product of lipid peroxidation caused by ROS, and TBARS accumulation indicates an increase of membrane injury under stress (Hsu & Kao 2007). In the present study, TBARS accumulation was induced by both temperature treatments and was at its highest levels at the lower temperature. This result is comparable with results obtained in a previous study by Li *et al.* (2011a). However, lower TBARS content in GLA-treated soybean roots under stress could be due to induced activity of antioxidant enzymes. Gallic acid was also able to lower the toxic H_2O_2 levels associated with stress thanks to its antioxidant activity. Similarly, Fabiani & Morozzi (2010) concluded that phenolics with two or more phenolic hydroxyl groups have antioxidant activity and can play a role in DNA protection in olive oil. Also, Kumaran & Karunakaran (2007) showed that the protective role of GLA might be attributable to its hydroxyl radical-scavenging effects. The higher antioxidant activity induced by GLA under stress might be mainly due to its $OH^{\bullet}$-scavenging activity. The $OH^{\bullet}$-quenching ability of the tested phenolic compounds seems to be directly correlated with the number of hydroxyl groups.

CONCLUSION

These results suggest that cold stress caused negative effects on soybean roots, as evidenced by decreased RGR, RWC and Pro content. The soybean roots attempted to withstand the toxic ROS accumulation induced by stress through the action of only one antioxidant enzyme, CAT. However, the increased activity of CAT did not ensure sufficient protection by scavenging of H_2O_2, and thus the levels of lipid peroxidation increased markedly. On the other hand, exogenous GLA treatment under cold stress was found to be capable of eliminating H_2O_2 and lipid peroxidation by increasing activities of SOD, CAT and POX, as well as by enhancing RGR, RWC and Ψ_{Π}. Although no increase of MDHAR, DHAR, tAsA and GSH was observed under conditions of GLA treatment plus stress, SOD, CAT and POX were able to compensate, as evidenced by decrease of TBARS content. There is no apparent role for the ascorbate-glutathione cycle in GLA alleviation of damage in soybean roots under cold stress. Future molecular analyses will aim at elucidating the precise molecular link between GLA and expression of the genes controlling antioxidant enzymes.

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REZIME

Hladni stres u korenu soje (*Glycine max* L.): Egzogena galna kiselina pospešuje vodni režim i povećava antioksidativne aktivnosti

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

Galna kiselina (GLA; 3,4,5- trihidrobenzoična kiselina) je jak antioksidans kod biljaka. Kako bi razjasnili ulogu GLA kao prooksidansa i oksidansa na ćelije u uslovima stresa, soja (*Glycine max*) je gajena u uslovima hladnog stresa (5 i 10°C) i/ili galne kiseline (GLA; 1 i 2 mM) tokom 72 h. Korenovi soje izloženi stresu pokazali su značajno smanjenje rasta (RGR), sadržaja vode (RWC), osmotskog potencijala ($Y_{\dot{O}}$) i sadržaja prolina (Pro). Međutim, tretman GLA u uslovima stresa je značajno popravio ove parametre i ublažio štetu nanetu stresom. Stres je smanjio aktivnost superoksid dismutaze (SOD), ali GLA je efektivno ublažila negativne efekte na aktivnost enzima. Nakon tretmana stresom, u korenovima soje samo je podstaknuta katalaza (CAT), iako to nije bilo dovoljno kako bi se sprečila akumulacija vodonik peroksida (H_2O_2). Na taj način se nivo lipidne peroksidacije (TBARS content) značajno povećao. Međutim, GLA doprinosi detoksifikaciji H_2O_2 i lipidne peroksidacije podsticanjem aktivnosti katalaze i peroksidaze (POX). U prilog ovim enzimima, aktivnost superoksid dismutase je mogla da izbriše superoksidni anjonski radikal, o čemu svedoči pad sadržaja TBARS. Međutim, monodehidroaskorbat reduktaza (MDHAR), dehidroaskorbat reduktaza (DHAR), ukupni askorbat (tAsA) i glutation (GSH) pokazuju smanjenje sadržaja u korenovima trentiranim sa GLA (obe koncentracije) plus stress. Naši rezultati ukazuju na zaštitnu ulogu GLA što može povećati toleranciju biljaka omogućavanjem efikasnog korišćenja vode i podsticanjem antiokvidativnih sistema. Stoga, u korenovima soje, GLA uspešno ublažava toksičnost hladnog stresa moduliranjem aktivnosti SOD, CAT i POKS, više nego enzima askorbat-glutation ciklusa.

Ključne reči: antioksidativni sistem, hladni stres, galna kiselina, *Glycine max*, reaktivne vrste kiseonika