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Calcium and L-histidine effects on ascorbate-glutathione cycle components under nickel-induced oxidative stress in tomato plants

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Abstract

The effects of $NiSO_4$, calcium, and L-histidine (His) on the components of ascorbate-glutathione cycle, antioxidant enzymes and lipid peroxidation in a tomato cultivar *Early Urbana Y* was investigated. The activities of enzymes including catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), lipoxygenase (LOX), and phenylalanine ammonia lyase (PAL) were measured. In addition, the content of H_2O_2, ascorbate (ASC), dehydroascorbate (DHA), reduced glutathione (GSH), chlorophyll (Chl) *a*+*b*, carotenoids, proteins, malondialdehyde (MDA), membrane aldehydes, and electrolyte leakage (EL) were determined. Results suggest that the excess of Ni increased the content of H_2O_2, MDA, membrane aldehydes and proteins in roots as well as GPX, LOX, APX activities, and EL in leaves, whereas Ca and His ameliorated these effects. Moreover, decreasing leaf GSH and DHA content and GR activity were observed under the Ni stress, but these parameters were raised by Ca plus His treatment. However, no improvement in leaf protein, ASC, root GSH content, and activities of PAL and CAT were observed by using Ca or His under Ni stress.

Additional key words: antioxidant enzymes, ascorbate peroxidase, catalase, electrolyte leakage, glutathione reductase, lipid peroxidation, malondialdehyde, nickel toxicity, superoxide dismutase.

Introduction

Excess of nickel (Ni) cannot produce reactive oxygen species (ROS) directly *via* a Fenton-type reaction. However, Ni can cause oxidative stress as indicated by lipid peroxidation and H_2O_2 generation (Wang *et al.* 2004). Exposure to Ni results in a severe depletion of glutathione (GSH; Madhava Rao and Sresty 2000) which is believed to be a critical step in Ni-induced ROS (Schickler and Caspi 1999). Negative effects of heavy metals (HMs) are often associated with increased production of ROS, such as superoxide anion ($O_2^{\bullet}$), H_2O_2, hydroxyl radical ($HO^{\bullet}$), and singlet oxygen ($^1O_2$) (Gajewska and Sklodowska 2007). ROS can damage cellular structures and macromolecules (Vranova *et al.* 2002).

Under non-stress conditions, ROS are removed by non-enzymatic and enzymatic antioxidants, whereas during a stress, the production of ROS exceeds the capacity of the antioxidative systems (Noctor and Foyer 1998). The non-enzymatic antioxidants include ascorbate (ASC) and GSH, two main constituents of the ASC-GSH cycle which detoxify H_2O_2 in chloroplasts and cytosol (Foyer *et al.* 2001, Sinha and Saxena 2006). Scavenging H_2O_2 by ascorbate peroxidase (APX) is the first step of the ASC-GSH cycle which maintains the ASC pool in its reduced form (Foyer and Halliwell 1976, Asada 1999). Glutathione reductase (GR) is the key enzyme for maintaining the GSH pool (Rennenberg 1982). Catalases also convert H_2O_2 to water and O_2.

Polyunsaturated fatty acids (PUFAs) are considered to be the most susceptible targets to oxidative stress mediated by ROS (Schickler and Caspi 1999). They are converted to oxilipins (Noctor and Foyer 1998) and further to fatty acid hydroperoxides (HPOs) by lipoxygenase (LOX) or by auto-oxidation.

Phenylalanine ammonia lyase (PAL) convert L-phenylalanine to *trans*-cinnamic acid in the phenyl

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Abbreviations: APX - ascorbate peroxidase; ASC - ascorbate; Car - carotenoids; Chl - chlorophyll; CAT - catalase; DHA - dehydroascorbate; EL - electrolyte leakage; GPX - guaiacol peroxidase; GR - glutathione reductase; GSH - reduced glutathione; His - L-histidine; HM - heavy metal; LOX - lipoxygenase; MDA - malondialdehyde; PAL - phenylalanine ammonia lyase; ROS - reactive oxygen species; SOD - superoxide dismutase.
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propanoid pathway also involved in plant defense mechanisms against abiotic stresses including HMs (Chang *et al.* 2009).

Calcium (Ca) is a key element in cell wall structure, and by binding to carboxylic groups of polygalacturonic acids (pectins) of the middle lamella, Ca strengthens the cell wall and controls its rigidity (Girija *et al.* 2002). The cell wall is an important chelator of toxic metals, such as Ni, as far as the metal is transported apoplastically in root tissue. Thus, Ca may decrease the uptake, translocation, and accumulation of HMs in plants (Saleh *et al.* 1999, Österås and Greger 2006). Some HMs are supposed to enter into plant cells through cation channels including Ca channels (Wu *et al.* 2009). Moreover, some HMs compete with Ca at sites on intracellular Ca binding protein calmodulin (Girija *et al.* 2002, Min *et al.* 2013). HMs also affect many other cation transporters, such as Zn-regulated transporter (ZRT), Fe-regulated transporter, Zrt/IRT-like protein (ZIP), and cation diffusion facilitator protein (CDF) (Kerkeb and Kramer 2003).

Metal chelators, such as phytosiderophores regulate the metal uptake by plant cells (Curie *et al.* 2001, Shah *et al.* 2001). A crucial component of HMs tolerance is buffering free metal ions in the cytoplasm *via* chelation with high-affinity ligands, and histidine (His) is a strong ligand of Ni (Page and Feller 2005). Therefore, His inhibits toxic effects of Ni on cellular function and structure (Wycisk *et al.* 2004). The positive correlation between a His content and Ni tolerance has been recently confirmed by Wycisk *et al.* (2004).

The aim of this paper was to confirm our hypothesis that Ca and His may have a positive role in controlling and alleviation of Ni-induced oxidative stress by stimulating ASC-GSH cycle components.

Materials and methods

Tomato (*Solanum lycopersicum* Mill. syn. *Lycopersicon esculentum*) cultivar Early Urbana Y widely planted in southeastern Iran was used in this study. Seeds (a gift from the *Falaat Ghaareh Company*, Tehran, Iran) were placed on two sheets of filter paper in Petri dishes moistened with a Hoagland nutrient solution (Hoagland and Arnon 1950). After 7 d, uniform seedlings were selected and transferred into dark polyethylene vessels (two plants per vessel), each supplied with 50 cm^3 of a modified 1/10 Hoagland solution containing 0.5 mM KNO_3, 400 μM $Ca(NO_3)_2$, 10 μM Fe-EDTA, 0.2 mM $MgSO_4$, 0.1 mM KH_2PO_4, 10 μM H_3BO_3, 2 μM $MnCl_2$, 2 μM $ZnSO_4$, 0.1 μM Na_2MoO_4, and 0.2 μM $CuSO_4$, and buffered to pH 5.8 ± 0.1. The growth medium was continuously aerated and nutrient solutions were exchanged once a day. Seedlings were grown for three weeks in a greenhouse with supplementary radiation provided by sodium vapor lamps during the day (a maximum irradiance of 185 μmol m^{-2} s^{-1}), a 16-h photoperiod, day/night temperatures of 25/22 °C, and a relative humidity of 60 %. After this period, the basic nutrient solution was replaced by a 1/10 Hoagland solution containing $NiSO_4$ (0, 150, or 300 μM), L-His (0 and 300 μM), and Ca^{2+} (700 μM) by adding 300 μM $CaCl_2$ to the basic nutrient solution with 400 μM $Ca(NO_3)_2$ (*Merck*, Germany). After 10 d, roots and leaves were washed in deionized water, blotted dry, weighed, frozen in liquid nitrogen and stored at -80°C until analyses.

The content of chlorophyll (Chl) (*a*+*b*) and carotenoids (Car) were measured in an acetone extract from fresh leaves with a spectrophotometer (*Cary 50, Varian,* Australia) at 470, 646.8, and 663.2 nm according to Lichtenthaler (1987). The electrolyte leakage was determined as described by Ben Hamed *et al.* (2007). For the determination of H_2O_2 content, leaf fresh tissue (0.1 g) was extracted with 3 cm^3 of trichloroacetic acid (TCA; 0.1 %, m/v) in an ice bath and centrifuged at 12 000 *g* for 15 min (Velikova *et al.* 2000). The content of H_2O_2 was measured at 390 nm and expressed as μmol g^{-1}(f.m.) using the coefficient of absorbance (ε) of 40 mM^{-1} cm^{-1}. Lipid peroxidation was estimated according to the malon-dialdehyde content measured according to the method of Heath and Packer (1969). Membrane alehydes (other alehydes) were determined according to Meirs *et al.* (1992).

The ASC and dehydroascorbate (DHA) content were measured as described by De Pinto *et al.* (1999). Briefly, total ASC was determined after reduction of DHA to ASC with dithiotreitol (DTT), and the content of DHA was estimated by the difference between the total ASC pool (ASC plus DHA) and ASC. The GSH content was determined by the spectrophotometric method of Ellman (1959) where GSH was oxidized in 2.6 cm^3 of a sodium phosphate buffer (pH 7.0) containing 0.2 cm^3 of a sample extract and 0.2 cm^3 of 6 mM 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). The absorbance was monitored at 412 nm. The GSH content was calculated from a standard curve constructed using GSH over the range 0 - 100 μM.

For protein extraction and analysis, the extracts of frozen samples prepared in a 50 mM potassium phosphate buffer (pH 7) containing 1mM phenyl-methane sulfonyl fluoride (PMSF), 1 mM sodium ethylene diaminetetraacetic acid (Na_2EDTA), and 1 % (m/v) polyvinylpyrrolidone (PVP) were centrifuged at 15 000 *g* and 4 °C for 15 min and supernatants were used for the estimation of protein content and enzyme activities. The total protein content was measured according to the method of Bradford (1976) using bovine serum albumin as standard.

All spectrophotometric analyses were conducted in a final volume of 3 cm^3 by using a *Cary 50* UV/visible spectrophotometer. The catalase (CAT; E.C 1.11.1.6) activity was measured according to the modified

method of Dhindsa *et al.* (1981). The reaction mixture consisted of a 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 0.1 cm^3 of the enzyme extract. The decomposition of H_2O_2 was followed by measuring the decrease in absorbance at 240 nm. The activity was calculated using $\varepsilon = 40\ mM^{-1}cm^{-1}$. One unit (U) of CAT activity was defined as the amount of enzyme that decomposed 1 mmol of H_2O_2 per minute.

The quaiacol peroxidase (GPX; EC 1.11.1.7) activity was determined in a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.3 % (v/v) H_2O_2, 1 % (v/v) guaiacol and 0.02 cm^3 of the enzyme extract by a method derived from Plewa *et al.* (1991). The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm and the enzyme activity was determined using $\varepsilon = 25.5\ mM^{-1}cm^{-1}$. One U of GPX activity was defined as the amount of enzyme that produced 1 mmol of tetraguaiacol per minute.

APX (EC 1.11.1.11) was assayed by monitoring the decrease in absorbance at 290 nm due to ASC oxidation (Nakano and Asada 1987). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 0.15 mM H_2O_2, 0.5 mM ASC, and 0.15 cm^3 of the enzyme extract. The activity of APX was calculated using $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One U of APX activity was defined as the amount of enzyme that decomposed 1 mmol of ascorbate per minute.

The superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to the inhibition of reduction of nitroblue tetrazolium (NBT) to furmazone at pH 7.0 (Giannopolitis and Ries 1977). The reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM Na_2EDTA, 75 μM riboflavin, 13 mM methionine, and 0.05 cm^3 of the enzyme extract. One unit of SOD activity was defined as the amount of the enzyme that inhibited the NBT photoreduction by 50 %.

For the measurement of the LOX (EC 1.13.11.12) activity, we used the Minguez-Mosquera *et al.* (1993) method. To 2.92 cm^3 of a phosphate buffer (200 mM, pH 6.5), 0.06 cm^3 of the crude extract and 0.02 cm^3 of 100 mM linoleic acid containing 0.1 % (v/v) *Tween-20* were added. The increase in absorbance at 234 nm between 10 and 40 s of the reaction was used and the enzyme unit was defined as 1 μmol of the product formed per min.

The glutathione reductase (GR; EC1.6.4.2) activity was determined following the decrease in absorbance at 340 nm associated with the oxidation of NADPH (Foyer and Halliwell 1976). The assay contained 50 mM Tris-HCl (pH 7.8), 150 μM NADPH, 500 μM oxidised glutathione (GSSG) and 0.05 cm^3 of the enzyme extract. One U of GR was defined as the amount of enzyme that oxidized 1 μmol of NADPH per minute.

The phenylalanine ammonia lyase (PAL; EC 4.3.1.24) activity was measured by a modified method of Tanaka *et al.* (1974). The reaction mixture was 0.4 cm^3 of a 50 mM Tris-HCl buffer (pH 8.8), 0.24 cm^3 of 99 % (v/v) mercaptoethanol, 0.2 cm^3 of 10 mM phenyl-alanine and 1 cm^3 of the enzyme extract. The reaction mixture was incubated at 37 °C for 60 min, and the reaction was terminated by adding 0.2 cm^3 of 10 % (m/v) TCA. In the control, the same amount of phenylalanine was added after termination. To remove precipitated proteins, the assay mixture was centrifuged at 10 000 *g* and 4 °C for 15 min and the absorbance of the supernatant was measured at 290 nm relative to the control. One U of PAL activity was defined as the amount of enzyme that increased the absorbance by 0.01 per min under the assay conditions.

The experimental design was completely randomized with 12 treatments, one cultivar and four replications per treatment. Samples were collected from two plants per culture vessel. Data were analyzed by using the analysis of variance (*ANOVA*) followed by the least significance difference (LSD) and Duncan tests at a 0.05 probability level. All statistical analyses were done using the software *SPSS* package, *v. 18.0 for Windows*.

Results

The activity of leaf GR and the content of leaf and root GSH decreased under the Ni application compared to the control (Fig. 1*A*), but they were less decreased after the addition of Ca, His, or Ca + His. The effect of His alone was mostly lower than that of Ca + His. The combination of 300 μM Ni + Ca + His increased the leaf GSH content compared to the control and at 300 μM Ni alone, but at 150 μM Ni, the effect of Ca + His was similar to effects of Ca or His individually. However, the root GSH content increased under 150 μM Ni + Ca compared to only 150 μM Ni. However, under 300 μM Ni + Ca or His, the root GSH content was lower than that under the control conditions (Fig. 1*B,C*).

Generally, the LOX activity increased under the Ni stress but less under the 300 μM Ni + Ca + His treatment (Table 2). Similar results were found under 150 μM Ni. However, the highest LOX activity was observed under 300 μM Ni + 700 μM Ca (Table 2).

In the presence of Ni (150 and 300 μM), a higher accumulation of total proteins was observed in roots and leaves than under the control conditions. The addition of Ca + His reduced the proteins content in both roots and leaves compared to Ni alone (Table 1). A very low protein content in roots was observed at 150 μM Ni + Ca and 0 or 150 μM Ni + His (Table 1).

Our results show that the Ni treatments decreased the leaf ASC content significantly compared to the control. However, under 150 μM Ni + Ca and/or His, the ASC

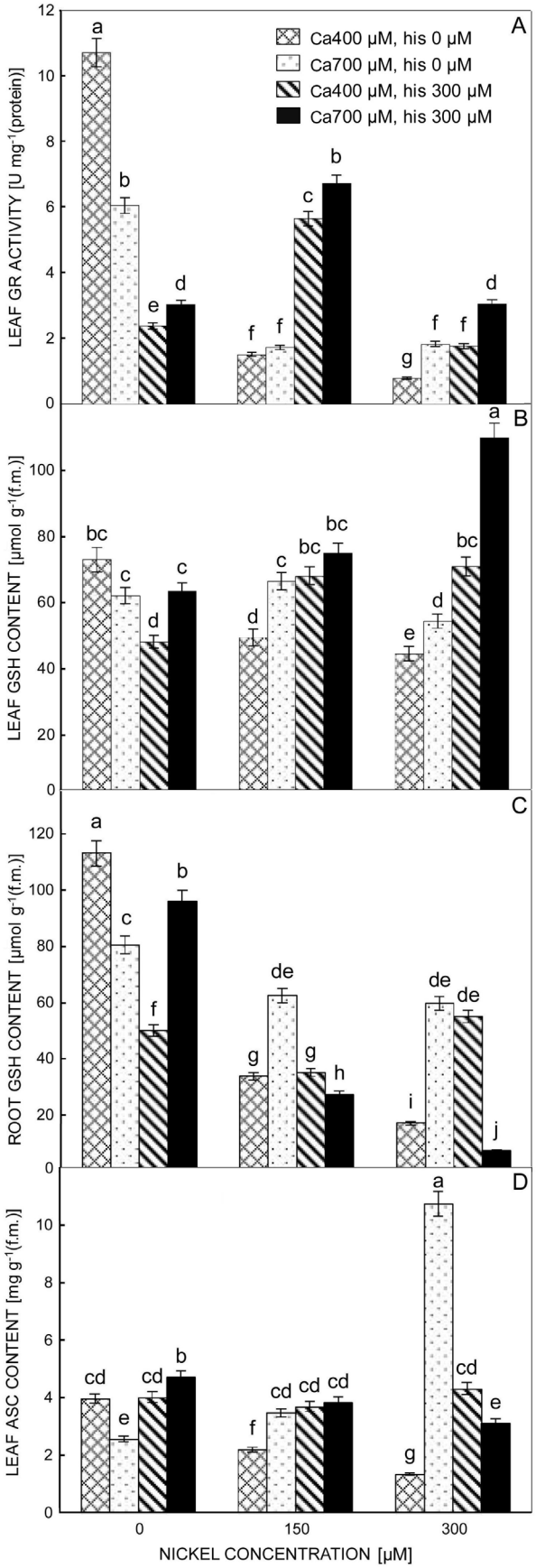

Fig. 1. The leaf GR activity (*A*), leaf GSH content (*B*), root GSH content (*C*), and leaf ASC content (*D*) in tomato plants treated with 0, 150, or 300 µM Ni in combination with 400 (the concentration in nutrient solution) or 700 µM Ca and/or 300 µM His. Means ± SE, n = 4. Different letters indicate significantly different means between the treatments ($P \leq 0.05$).

content was similar as in the control. The maximum ASC content was observed under 300 µM Ni + Ca (Fig. 1*D*). The DHA content decreased under 300 µM Ni but less under 300 µM Ni + Ca + His (Fig. 2*A*). The results indicate that the application of Ca and/or His improved the ASC pool especially under 300 µM Ni.

The content of Chl and Car were significantly decreased under the Ni stress. The application of Ca increased the Chl content not only under the Ni stress but also under the control conditions. The application of His or Ca + His increased the Chl content under 150 or 300 µM Ni compared to the Ni stress alone. Under both Ni treatments, the application Ca + His also elevated the Car content (Table 2).

When the plants were exposed to 300 µM Ni, the leaf CAT activity was 102.3 % higher than that in the control (Table 2). The activity of this enzyme decreased compared to the control in the Ni + Ca + His treated plants (Table 2). The CAT activity under 150 µM Ni was lower compared to the control, whereas the control + His showed the highest value.

The GPX activity slightly increased due to the Ni stress but it considerably increased by the Ca or His applications. However, the combination Ca + His decreased the GPX activity (Fig. 2*B*).

An increase in the APX activity was observed almost in all treatments compared to the control. The APX activities under 300 µM Ni + His and 150µM Ni + His were higher than in the other treatments. On the other hand, the combination of Ca + His decreased the APX activity (Table 2).

Relative reductions in the SOD activities were 63.5 and 66.8 %, respectively, under 150 and 300 µM Ni compared to the control plants. Ca or His increased the SOD activity compared to that at the respective Ni concentrations. The independent effects of Ca or His on elevating the SOD activity was more pronounced in comparison to the combination of both (Fig. 2*C*).

Similarly to SOD, independent effects of Ca and His on increasing the PAL activity was higher compared to the effect of combined Ca + His. The application of 300 µM Ni + Ca + His even decreased the PAL activity by 19.6 % more than 300 µM Ni alone. On the other hand, the PAL activity at 150 µM Ni + Ca + His was by 93 % higher compared to 150 µM alone. However, the maximum PAL activity was in 0 µM Ni + Ca (Table 2).

Our data show that the content of MDA and membrane aldehydes increased significantly under 150 and 300 µM Ni compared to the control, however, less when Ca or His independently or in combination were added (Table 1).

There was observed a significant increase in the H_2O_2 content under 150 and 300 μM Ni compared to the control. The highest H_2O_2 content was after 700 μM Ca addition at all Ni concentrations. However, His and Ca + His decreased the H_2O_2 content under Ni toxicity (Fig. 2*D*).

EL of leaf increased by 10.2-fold and 13.6-fold under Ni concentrations 150 and 300 μM, respectively, in comparison to the control. This negative effect of Ni was partially ameliorated by the Ca, His, or Ca + His applications (Table 1).

Table 1. The content of leaf and root total proteins [mg g^{-1}(f.m.)], of leaf MDA and membrane aldehydes [nmol g^{-1}(f.m.)], and of leaf EL [%] in tomato plants treated with 0, 150, or 300 μM Ni alone or in combination with 400 (the concentration in nutrient solution) or 700 μM Ca and/or 300 μM His. Means ± SE, $n = 4$. Different letters indicate significantly different means between the treatments. According to *ANOVA*: * - the effect of the total treatments on the parameter change is significant at $P < 0.05$, ** - the interactive effect of three factors (Ca, His, and Ni) on the parameter change is significant.

Ni [μM]	His [μM]	Ca [μM]	Leaf protein	Root protein	MDA	Aldehydes	EL
0	0	400	3.82±0.84 c	3.97±0.12 cd	0.20±0.01 d	1.72±0.11 e	4.03±0.24 g
150	0	400	9.18±0.44 a	5.81±0.25 a	0.64±0.012 b	3.74±0.15 b	40.96±0.18 b
300	0	400	10.47±0.52 a	5.56±0.24 a	0.79±0.011 a	4.15±0.21 a	54.92±0.23 a
0	0	700	1.65±0.06 e	3.64±0.13 cd	0.26±0.02 d	1.55±0.05 e	4.75±0.61 f
150	0	700	6.00±0.29 b	0.79±0.02 e	0.43±0.02 c	2.74±0.12 cd	16.22±0.84 c
300	0	700	5.93±0.22 b	5.14±0.18 a	0.39±0.01 c	1.48±0.16 e	31.11±1.41 c
0	300	400	2.61±0.12 d	0.51±0.01 g	0.22±0.013 d	1.52±0.13 e	5.68±0.88 f
150	300	400	4.32±0.19 c	0.60±0.02 f	0.34±0.01 c	3.20±0.12 b	32.83±1.58 c
300	300	400	3.32±0.16 c	3.06±0.16 cd	0.20±0.01 d	1.62±0.05 e	18.23±0.89 d
0	300	700	5.37±0.26 b	4.01±0.24 b	0.23±0.010 d	2.21±0.11 cd	5.75±0.77 f
150	300	700	4.34±0.21 c	4.01±0.19 b	0.37±0.03 c	1.20±0.28 f	5.67±1.18 f
300	300	700	5.77±0.24 b	2.98±0.10 d	0.19±0.01 e	1.24±0.24 f	10.34±0.76 e
Ni or Ca or HIS			0.02*	0.03*	0.02*	0.03*	0.001*
Ni + Ca + HIS			0.053	0.004**	0.049**	0.018**	0.046**

Table 2. The leaf content of Chl and Car [mg g^{-1}(f.m.)], and the activities of CAT, APX, LOX, and PAL [U mg^{-1}(protein)] in tomato plants treated with 0, 150, or 300 μM Ni alone or in combination with 400 (the concentration in nutrient solution) or 700 μM Ca and/or 300 μM His. Means ± SE, $n = 4$. Different letters indicate significantly different means between the treatments. According to *ANOVA*: * - the effect of the total treatments on the parameter change is significant at $P < 0.05$, ** - the interactive effect of three factors (Ca, His, and Ni) on the parameter change is significant.

Ni [μM]	His [μM]	Ca [μM]	Chl content	Car content	CAT	APX	LOX	PAL
0	0	400	24.02±1.21 c	3.32±0.05 b	13.35±0.54 c	8.33±0.34 f	0.51±0.02 g	19.70±0.88 e
150	0	400	5.98±1.05 g	1.55±0.04 g	4.90±0.24 f	14.08±0.67 d	1.63±0.01 f	10.42±0.51 g
300	0	400	4.58±1.24 g	1.77±0.06 f	27.01±1.59 b	13.73±0.61 d	4.66±0.18 c	8.66±0.39 h
0	0	700	35.61±1.03 a	2.67±0.06 c	10.20±0.51 d	35.15±1.16 b	4.12±0.19 c	45.80±2.20 a
150	0	700	13.12±1.05 f	1.30±0.05 h	0.23±0.01 g	22.88±1.26 cd	1.31±0.01 f	19.87±0.98 e
300	0	700	15.46±1.28ef	1.59±0.08 f	8.87±0.35 e	28.99±1.50 c	12.86±0.58 a	31.34±1.24 bc
0	300	400	20.06±1.68 d	3.31±0.05 b	71.42±2.84 a	12.29±0.55 e	10.44±0.47 b	30.44±1.27 bc
150	300	400	17.79±1.64 e	2.00±0.04 e	6.96±0.26 ef	34.33±1.24 b	2.22±0.11 e	28.67±1.11 d
300	300	400	18.64±1.54 e	2.10±0.09 d	8.54±0.42 e	45.32±2.14 a	5.14±0.26 c	32.37±1.42 bc
0	300	700	24.48±1.68 c	2.49±0.07 c	11.02±0.41 d	7.75±0.32 f	3.86±0.14 d	13.25±0.66 f
150	300	700	28.42±0.87 b	3.93±0.08 a	5.12±0.19 f	9.51±0.45 f	3.39±0.14 d	20.11±1.05 e
300	300	700	22.86±1.11 c	3.37±0.09 b	9.66±0.42 e	12.94±0.59 e	0.53±0.02 g	6.96±0.32 i
Ni or Ca or HIS			0.015*	0.049*	0.015*	0.049*	0.003*	0.030*
Ni + Ca + HIS			0.010**	0.030**	0.061	0.022**	0.017**	0.022**

Discussion

It has been demonstrated that HMs, such as Ni, induces oxidative stress in plant tissues, and lipid peroxidation has been used as indicator of oxidative stress when plants are subjected to Ni (Boominathan and Doran 2002). Our

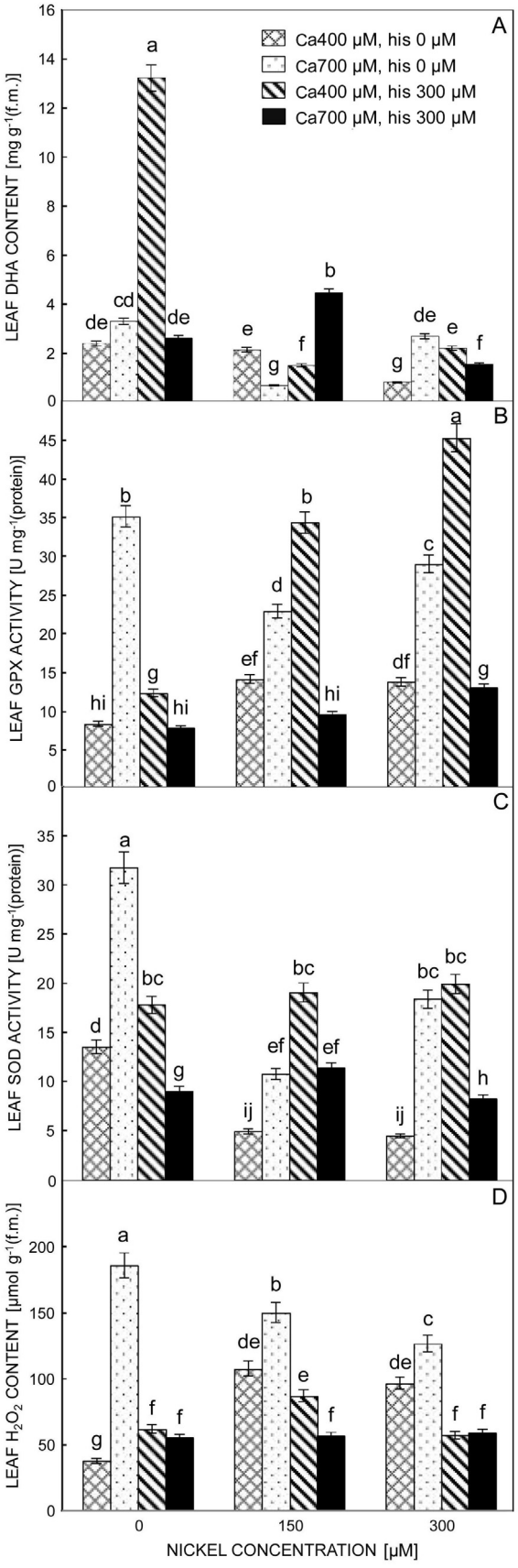

Fig. 2. The leaf DHA content (*A*), the GPX (*B*), and SOD (*C*) activities and the leaf H_2O_2 content (*D*) in tomato plants treated with 0, 150, or 300 µM Ni alone or in combination with 400 (the concentration in nutrient solution) or 700 µM Ca and/or 300 µM His. Means ± SE, n = 4. Different letters indicate significantly different means between the treatments ($P \leq 0.05$).

data are in agreement with this idea because we found a significant increase in the H_2O_2 content and leaf lipid peroxidation marker MDA under 150 or 300 µM Ni (Table 1 and Fig. 2*D*). Therefore, it was the possibility of oxidative damage of photosynthetic pigments. The decrease in the Chl and Car content in the tomato leaves as result of Ni toxicity found in our experiments (Table 2) and also in *Miscanthus* species (Hsu *et al.* 1992) could be related to the Ni-induced oxidative damages.

Relatively higher activities of ROS-scavenging enzymes have been reported in tolerant plants suggesting that the antioxidant system plays an important role in plant tolerance against HMs-induced oxidative stress. The activities of APX and LOX (Table 2) from the tomato plants increased with the excess of Ni compared to the control. The APX activity further increased by the Ca or His application which might be important for H_2O_2 scavenging. Higher activities of antioxidant enzymes under the stress conditions were found also in other plants (Mittova *et al.* 2002). Also the overexpression of cytosolic APX in tobacco chloroplasts enhances tolerance to HMs stress (Badawi *et al.* 2004). LOX-derived HPOs could be converted into more stable compounds like jasmonic acid (JA) which participates in defense reactions and tolerance to different stresses, such as Ni (Bueno *et al.* 2001), and it is a mediator in antioxidant enzymes induction. Clarke *et al.* (2002) reported that the treatment of *Phaseolus vulgaris* plants with 2.5 nM JA greatly increases GR and peroxidase activities but only slightly CAT and SOD activities.

Some researchers found a correlation between an increase in GPX activity and Ni tolerance (Schickler and Caspi 1999). In the tomato leaves, the GPX activity was higher under 150 and 300 µM Ni than in the controls. Also, exogenous Ca or His increased the GPX activity (Fig. 2*B*).

Tolerance of some plants to HMs is associated with increases in both APX and GR activities (Madhava Rao and Sresty 2000) but we observed a decrease in the GR activity under the Ni stress (Fig. 1*A*) which was alleviated by the Ca, His, or especially Ca + His applications. On the other hand, an increase in GR activity and a decline in protein thiols has also been shown by Maheshwari and Dubey (2009) in rice seedlings under Ni stress.

The accumulation of GSH and ASC decreased under 150 and 300 µM Ni in comparison with the control, and the addition of Ca and/or His influenced differently the GSH content in leaves and roots. A suitable GSH and ASC content could be important in protection against the Ni-induced oxidative stress, and the Ca + His application increased the GSH content in leaves under 150 and especially 300 µM Ni considerably (Fig. 1*B*).

Our findings show that the CAT activity increased under 300 μM Ni compared to the control, whereas it decreased in the presence of 300 μM Ni + Ca + His in comparison to the Ni stress conditions (Table 2). Similar results have been reported in barley leaves and roots (Kumar *et al.* 2007). A decrease in the SOD activity under the Ni stress and a significant increase in the SOD activity due to the Ca or His applications in comparison with the Ni stress alone (Fig. 2*C*) are important for scavenging superoxide radical to H_2O_2 (Alscher *et al.* 2002). Recent studies have demonstrated that overexpression of mitochondrial Mn-SOD in transgenic *Arabidopsis thaliana* (Wang *et al.* 2004), and chloroplastic Cu/Zn-SOD in transgenic *Nicotiana tabacum* (Badawi *et al.* 2004) can enhance tolerance to stresses. Similar results have been found in pigeon pea seedlings (Madhava Rao and Sresty 2000). However, Gajewska and Sklodowska (2007, 2008) showed an inhibition of SOD activity under Ni exposure in barley and pea plants similarly as we found it in the tomato leaves. Although SOD is not the only H_2O_2-producing enzyme in plant tissues, the balance between the SOD activity and activities of the H_2O_2-scavenging enzymes is crucial (Badawi *et al.* 2004). SOD/(CAT+APX+GPX) activities ratio in the tomato plants increased with the Ni stress (the data not shown) leading to an increased H_2O_2 content under the Ni stress. The SOD/(CAT+APX+GPX) activities ratio increased with the application of Ca + His in the tomato plants exposed to the Ni stress.

It was shown that PAL is generally stimulated in plant tissues exposed to HMs stresses (Kovacik *et al.* 2009). In contrast, the PAL activity in the tomato leaves decreased under the Ni stress and this decrease was ameliorated by the Ca or His application but mostly not by Ca + His.

Ca can change MDA content and stimulate activities of GPX, SOD, and CAT which could be the reasons for the improvement of abiotic stress tolerance (Waraich *et al.* 2012). Ca activates ATPase in the plasma membrane which pumps back the nutrients lost during cell membrane damage. Calmodulin (CaM), a ubiquitous Ca-binding protein, can activate CAT and so down-regulate H_2O_2 accumulation. Ca-dependent protein kinases (CDPKs) are triggered by biotic and abiotic stresses. CDPK2 signaling can trigger enhancing the content of JA and ethylene but not salicylic acid (Quan *et al.* 2008).

In conclusion, the rather high tolerance of the tomato cultivar Early Urbana Y to the Ni-induced oxidative stress seems to be dependent upon the efficiency of the antioxidant system which maintained the redox homeostasis and integrity of cellular components. Thus, the antioxidant enzymes GR, SOD, GPX, and APX played major roles in the Ni stressed tomato plants. On the whole, our findings support the hypothesis that the higher efficiency of the antioxidants after the application of Ca and/or His could be responsible for increasing tomato tolerance to the Ni stress. These data also suggest that the ratio of the SOD/H_2O_2-scavenging enzyme activities might be a useful marker for HMs tolerance in tomato. Finally, the ASC-GSH cycle enzymes under the Ni stress and Ca and/or His applications may have a significant role in tomato HMs tolerance.

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