

Effect of chromium and nitrogen form on photosynthesis and anti-oxidative system in barley

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Abstract

The effect of nitrogen forms on photosynthesis and anti-oxidative systems of barley plants under chromium stress was studied in a hydroponic experiment. The treatments comprised three chromium concentrations (0, 75, and 100 μM) and three N forms $(\text{NH}_4)_2\text{SO}_4$, urea, and $\text{Ca}(\text{NO}_3)_2$. In comparison with the urea or $(\text{NH}_4)_2\text{SO}_4$ fed plants, the $\text{Ca}(\text{NO}_3)_2$ fed plants had higher net photosynthetic rate, intercellular CO_2 concentration, stomatal conductance, transpiration rate, photosynthetically active radiation utilization efficiency, variable to maximum chlorophyll fluorescence ratio, and the content of chlorophylls and carotenoids. Cr toxicity caused oxidative stress in all plants but the $\text{Ca}(\text{NO}_3)_2$ fed plants had the least oxidative stress. Moreover, the $\text{Ca}(\text{NO}_3)_2$ fed plants had higher activities of anti-oxidative enzymes and content of non-enzymatic antioxidants than the urea or $(\text{NH}_4)_2\text{SO}_4$ fed plants. In addition, the $\text{Ca}(\text{NO}_3)_2$ fed plants had higher N and lower Cr content in all plant tissues than the urea or $(\text{NH}_4)_2\text{SO}_4$ fed plants. The current results indicate that the reasonable choice of N fertilizer is important for barley production on the Cr-contaminated soils.

Additional key words: carotenoids, chlorophylls, *Hordeum vulgare*, oxidative stress, photosynthetic rate, transpiration rate.

Introduction

Chromium is toxic to plants and it may cause a severe damage of many physiological processes (Ali *et al.* 2011c). Disorganization of chloroplast ultrastructure and inhibition of electron transport processes due to Cr toxicity is a possible explanation for Cr-induced decrease in photosynthetic rate (Shanker *et al.* 2005). The overall effect of Cr ions on photosynthesis and energy transfer is also attributed to Cr-induced abnormalities in the chloroplast development, such as a poor lamellar system (Van Assche and Clijsters 1985). In addition, reactive oxygen species (ROS) could be produced through Fenton-type reactions when plants are exposed to Cr stress resulting in severe oxidative damage to plant cells (Panda *et al.* 2003, Ali *et al.* 2011b) reflected by decomposition of proteins, lipids, and nucleic acids (Alia *et al.* 1995, Ali *et al.* 2011a).

There are two main nitrogen forms used by plants in natural conditions: ammonium (NH_4^+) and nitrate (NO_3^-)

ions. In general, most plants use NO_3^- preferentially as N source (Raven *et al.* 1992, Berta *et al.* 2002) whereas plant roots can absorb NH_4^+ when NO_3^- deficiency has occurred in soils. When NH_4^+ is used as the only N source, it may induce toxicity. This phenomenon is widespread but highly variable among plant species (Britto and Kronzucker 2002).

The physiological processes in plants, including photosynthesis, are dramatically affected by N form. Thus, it is possible that uptake and translocation of heavy metals, such as Cr, will be affected by N form in the growth medium. The effect of N fertilizer form on heavy metal toxicity has been investigated and it is reported that the interaction between heavy metals and N forms is dependent on plant species and growth conditions (Schier and McQuattie 1999, Hassan *et al.* 2005). However, the results available up to date in examining the responses of plants to heavy metals and N source are inconsistent.

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Abbreviations: APX - ascorbate peroxidase; ASC - ascorbate; CAT - catalase; c_i - intercellular CO_2 concentration; E - transpiration rate; F_v/F_m - variable to maximum chlorophyll fluorescence ratio (photosynthetic efficiency); GSH - glutathione; GR - glutathione reductase; g_s - stomatal conductance; PAR - photosynthetically active radiation; P_N - net photosynthetic rate; POD - guaiacol peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TCA - trichloroacetic acid.

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For instance, in comparison with NO_3^- , NH_4^+ application enhanced (Schier and McQuattie 1999) or reduced (Fleming 1983) Al tolerance of plants.

To the best of our knowledge, it is the first study on interaction of N fertilizer form and Cr toxicity on any

Materials and methods

Plants and treatments: Seeds of a barley (*Hordeum vulgare* L.) genotype Hua 30 were surface sterilized in 3 % (v/v) H_2O_2 for 30 min, rinsed with distilled water, submerged in deionized water in the dark overnight, and germinated in sterilized moist quartz sand in a controlled chamber at a 16-h photoperiod, irradiance of $225 \pm 25 \mu\text{mol m}^{-2} \text{s}^{-1}$, light/dark temperatures of 22/18 °C, and 85 % relative humidity. Uniform seedlings (14-d-old, second leaf stage) were transplanted to pots, which were covered with a polystyrol-plate with seven evenly spaced holes (in each hole two seedlings) and placed in a greenhouse. The composition of the basic nutrient solution was [mg dm^{-3}]: NaH_2PO_4 37.24, K_2SO_4 127.96, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 308.17, CaCl_2 177.58, Fe citrate 6.8, $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$ 0.9, $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.11, $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ 0.04, H_3BO_3 2.9, and H_2MoO_4 0.01. After 18 d, $\text{K}_2\text{Cr}_2\text{O}_7$ was added into the solution to obtain three Cr concentrations: 0 (control), 75, and 150 μM , and N as $(\text{NH}_4)_2\text{SO}_4$, urea, or $\text{Ca}(\text{NO}_3)_2$ was added at the 1.6 mM N concentration. Calcium was correspondingly reduced in the culture solution where $\text{Ca}(\text{NO}_3)_2$ was used as N fertilizer, thus keeping Ca concentration the same for all N treatments. The pH of the culture solution in each pot was adjusted to 5.7 every other day with 1 M HCl or NaOH as required. The solution was continuously aerated with an air pump and renewed every 4 d. There were 6 replications for each treatment and the plants were sampled for all measurements at the day 30 after treatments.

Photosynthetic pigments and gas exchange: Content of chlorophyll (Chl) *a*, Chl *b*, and carotenoids (Car) were determined spectrophotometrically (type 721, Yonghen Co., Shanghai, China) in 85 % (v/v) acetone extract of the topmost fully expanded leaves at three wavelengths of 452.5, 644, and 663 nm.

Gas exchange parameters were measured by a LICOR-6400 portable photosynthesis system (LICOR, Lincoln, NE, USA). Photosynthetic rate (P_N), intercellular CO_2 concentration (c_i), stomatal conductance (g_s), and transpiration rate (E) were measured on the topmost fully expanded leaf after 2 h of acclimation in a growth cabinet under irradiance of $1\,200 \mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity of 60 %, and CO_2 concentration of $500 \mu\text{mol mol}^{-1}$. Six leaves were measured for each replication.

Antioxidant enzymes and oxidation stress: The second fully expanded leaves (0.5 g) were homogenized in 0.05 M phosphate buffer (pH 7.8) by grinding with a mortar and pestle in liquid nitrogen. The homogenate was

filtered through four layers of muslin cloth and centrifuged at 12 000 g and 4 °C for 15 min. The supernatants were stored at 4 °C and used for measurements of various antioxidant enzyme activities.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by the nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich 1971). Guaiacol peroxidase (POD, EC 1.11.1.7) activity was measured according to the method of Putter (1974) with some modifications. The reaction mixture (3 cm^3) consisted of 0.1 cm^3 of enzyme extract, 0.1 cm^3 of guaiacol (1.5 %, v/v), 0.1 cm^3 of 300 mM H_2O_2 , and 2.7 cm^3 of 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0). Increase in the absorbance due to oxidation of guaiacol was measured spectrophotometrically at 470 nm ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi (1984). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to the method of Nakano and Asada (1981). Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to Garcia-Limonos *et al.* (2002).

To determine non-enzymatic antioxidants (reduced glutathione and ascorbate), the leaf samples (0.4 g) were ground with a mortar and pestle in 4 cm^3 of 5 % (m/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 10 000 g and 4 °C for 15 min. The supernatants were used for the assays of reduced glutathione (GSH) content according to the method of Ellman (1959) and ascorbate (ASC) content according to Law *et al.* (1983).

The lipid peroxidation was expressed as MDA content determined according to Wu *et al.* (2003). H_2O_2 content was determined colorimetrically as described by Jana and Choudhuri (1981). O_2^- was measured as described by Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of O_2^- .

Nitrogen and chromium content: Twenty barley plants in each treatment were harvested and carefully washed with tap water, distilled water, and deionized water 3 times, respectively, and then immersed into 20 mM EDTA- Na_2 for 3 h to remove any bound metals from plant surface. Plant samples were divided into roots, stems, and leaves, dried at 80 °C in an oven for 72 h, and then ground into powder. One gram of each sample was dry-ashed in a Muffle furnace, digested with HNO_3 - HClO_4 (2:1, v/v). The content of Cr in leaves, stems, and roots was determined by inductively coupled plasma atomic absorption spectroscopy (AA6300, Shimadzu, Tokyo, Japan). For total nitrogen, 0.5 g sample was

digested in 8 cm³ of H₂SO₄ and N quantified according to the micro-Kjeldahl method using *Kjeflex K-306* (BUCHI, Switzerland).

Statistical analysis: All values reported in this study are

Results

There were significant differences in P_N, c_i, g_s, E, photosynthetically active radiation utilization efficiency (PAR-UE), and variable to maximum Chl fluorescence ratio (F_v/F_m) among three N forms under no Cr addition; the Ca(NO₃)₂ treated plants had the highest values (Fig. 1). Cr addition significantly reduced P_N, c_i, g_s, E, PAR-UE, and F_v/F_m in all the plants, with more reduction at higher Cr concentration than at lower one, and the

means of at least three replicates. A two-way analysis of variance (ANOVA) was performed by using a statistical package *SPSS v. 16.0* (SPSS, Chicago, IL, USA). Duncan's multiple range test was done to determine the significant difference between means.

reduction was also dependent on N forms used for plant fertilization. Ca(NO₃)₂ treated plants were less affected and had significantly higher values of all measured parameters than (NH₄)₂SO₄ fed plants at both Cr concentrations (Fig. 1).

There were significant differences in Chl *a*, Chl *b*, total Chl, and Car content among the three N forms, with Ca(NO₃)₂ fed plants and (NH₄)₂SO₄ fed plants having the

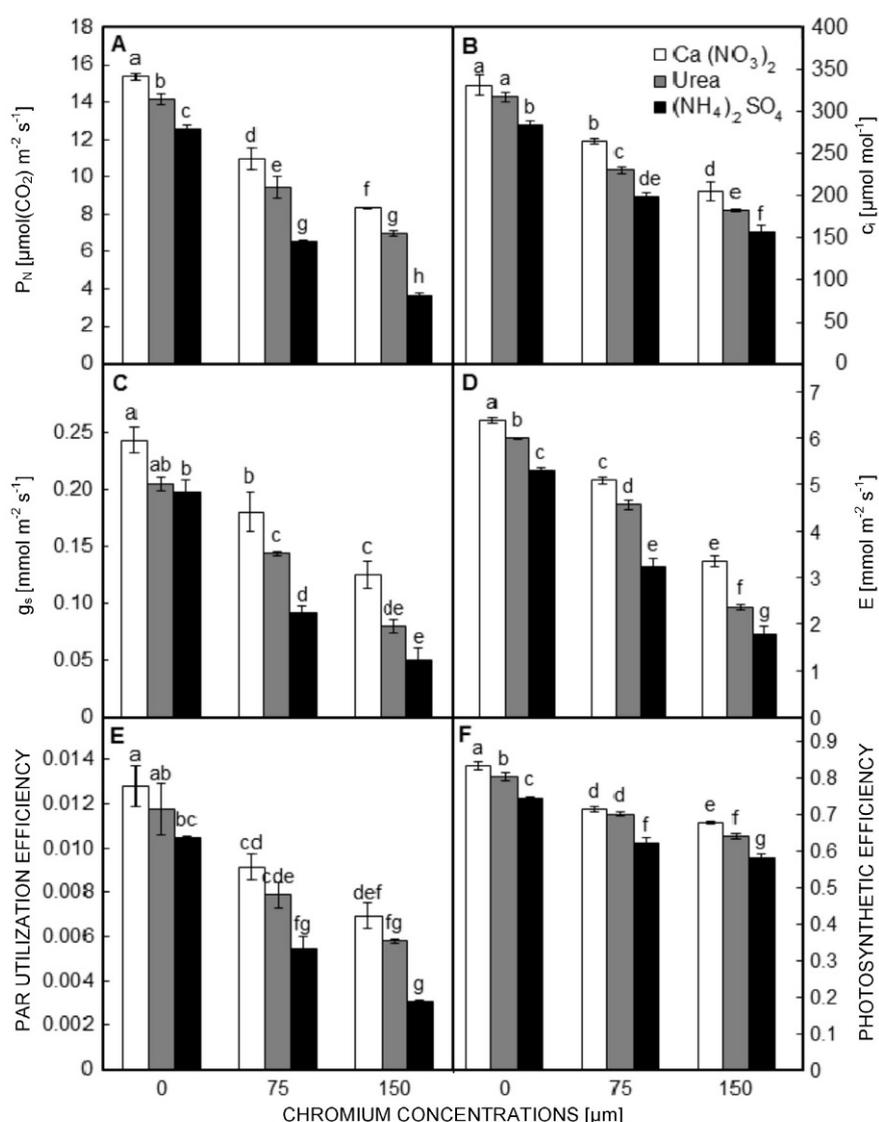


Fig. 1. The effect of different N forms and Cr concentrations on net photosynthetic rate (A), intercellular CO₂ concentration (B), stomatal conductance (C), transpiration rate (D), PAR utilization efficiency (E), photosynthetic efficiency measured as F_v/F_m (F) of barley leaves. Bars represent SE (n = 3). The same letters means no significant difference at 95 % probability level.

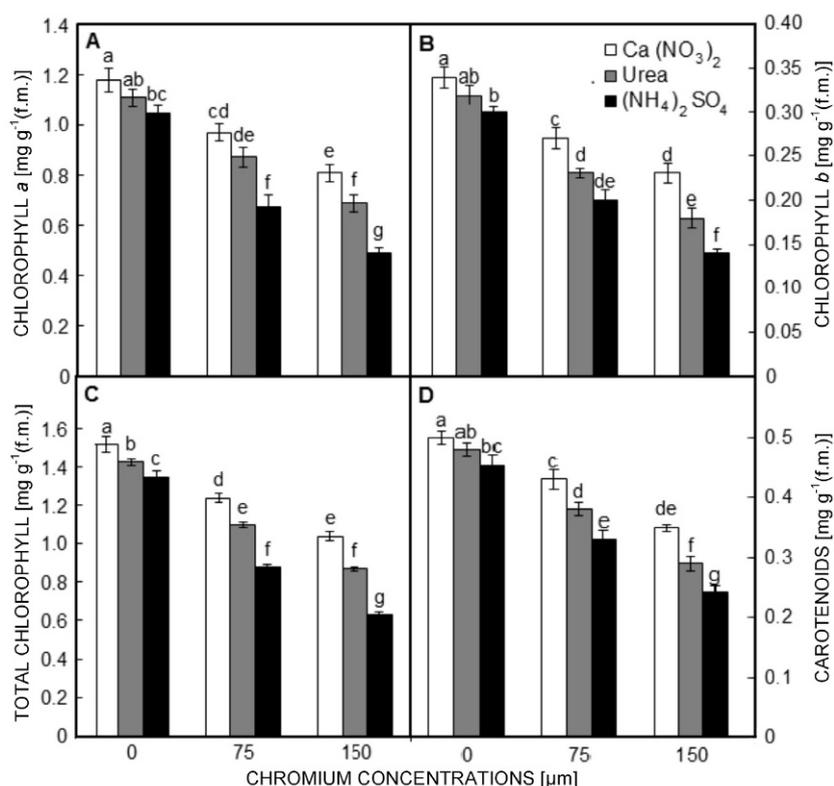


Fig. 2. The effect of different N form and Cr concentrations on Chl *a* (A), Chl *b* (B), total Chl (C), and Car (D) content of barley leaves. Bars represent SE ($n = 3$). The same letters means no significant difference at 95 % probability level.

Table 1. The effect of different N forms and Cr concentrations on content of malondialdehyde (MDA), H_2O_2 , O_2^- , reduced glutathione (GSH), and ascorbate (ASC) in barley plants. The same letters after the data within a column means no significant difference at 95 % probability level.

Cr [μM]	N Form	MDA [$nmol g^{-1}(f.m.)$]	H_2O_2 [$nmol g^{-1}(f.m.)$]	O_2^- [$nmol g^{-1}(f.m.) min^{-1}$]	GSH [$mg g^{-1}(f.m.)$]	ASC [$mg g^{-1}(f.m.)$]
0	Ca(NO ₃) ₂	13.17 h	9.78 f	4.96 g	6.68 ef	2.07 d
	urea	15.85 g	10.09 f	5.78 g	5.93 fg	1.72 e
	(NH ₄) ₂ SO ₄	17.32 f	12.79 f	6.70 g	5.44 g	1.61 e
75	Ca(NO ₃) ₂	23.44 e	17.35 e	14.44 f	12.71 a	4.94 a
	urea	28.99 d	22.28 d	17.99 e	10.59 b	3.85 b
	(NH ₄) ₂ SO ₄	33.88 c	27.75 c	21.08 d	8.54 d	2.94 c
150	Ca(NO ₃) ₂	34.12 c	29.86 c	23.96 c	9.59 c	4.14 b
	urea	38.37 b	34.18 b	28.57 b	7.17 e	3.02 c
	(NH ₄) ₂ SO ₄	45.38 a	43.15 a	33.62 a	6.38 efg	2.33 d

highest and lowest values, respectively (Fig. 2). Cr addition significantly reduced all photosynthetic pigments and the (NH₄)₂SO₄ fed plants showed the greatest reduction relative to the control at both Cr levels, whereas the Ca(NO₃)₂ fed plants had the smallest reduction.

The Ca(NO₃)₂ fed plants had a significantly lower MDA content than the plants fed by other two N forms. However, in case of H_2O_2 and O_2^- content, there was no significant differences among the plants treated with the three N forms. Cr addition to the culture solution markedly increased MDA, H_2O_2 , and O_2^- content. When

the plants were subjected to Cr stress, the effect was dependent on N forms. The Ca(NO₃)₂ fed plants had the lowest MDA, H_2O_2 , and O_2^- content, followed by the urea-fed plants (Table 1).

There were significant differences in APX activity and ASC content between Ca(NO₃)₂ and (NH₄)₂SO₄ fed plants growing in the solution without Cr whereas for other enzymes and antioxidants, no significant differences were found among the plants fed with various N forms. When plants were exposed to Cr stress, a dramatic increase in activities of SOD, POD, APX, CAT, and GR, and in content of GSH and ASC were observed and the

Table 2. The effect of different N forms and Cr concentrations on superoxide dismutase (SOD) [$\text{U g}^{-1}(\text{f.m.})$], peroxidase (POD) [$\text{A}_{470} \text{g}^{-1}(\text{f.m.}) \text{min}^{-1}$], ascorbate peroxidase (APX) [$\text{mmol g}^{-1}(\text{f.m.}) \text{min}^{-1}$], catalase (CAT) [$\text{mmol g}^{-1}(\text{f.m.}) \text{min}^{-1}$], and glutathione reductase (GR) [$\text{mmol g}^{-1}(\text{f.m.}) \text{min}^{-1}$] activities in barley leaves. The same letters within a column means no significant difference at 95 % probability level.

Cr [μM]	N Form	SOD	POD	APX	CAT	GR
0	$\text{Ca}(\text{NO}_3)_2$	175.0 \pm SE de	107.2 g	40.17 g	0.75 ef	0.53 de
	urea	166.6 de	111.5 fg	46.03 fg	0.69 ef	0.44 e
	$(\text{NH}_4)_2\text{SO}_4$	155.3 e	125.7 efg	51.37 ef	0.63 f	0.38 e
75	$\text{Ca}(\text{NO}_3)_2$	271.1 a	200.8 a	84.40 a	1.71 a	1.23 a
	urea	226.2 b	163.2 bc	73.54 b	1.50 b	0.96 b
	$(\text{NH}_4)_2\text{SO}_4$	207.8 bc	149.3 cd	62.09 cd	1.21 cd	0.63 cd
150	$\text{Ca}(\text{NO}_3)_2$	254.6 a	169.6 b	69.57 bc	1.28 c	1.06 b
	urea	199.5 c	141.8 de	59.86 d	1.09 d	0.75 c
	$(\text{NH}_4)_2\text{SO}_4$	176.9 d	129.0 ef	55.66 de	0.87 e	0.51 de

Table 3. The effect of different N forms and Cr concentration on Cr and N content in barley plants. The same letters within a column means no significant difference at 95 % probability level.

Cr [μM]	N form	Cr content [$\mu\text{g g}^{-1}(\text{d.m.})$]			N content [$\mu\text{g g}^{-1}(\text{d.m.})$]		
		roots	stem	leaves	roots	stem	leaves
0	$\text{Ca}(\text{NO}_3)_2$	2.91 \pm SE g	1.25 f	0.85 g	19.8 a	21.8 a	31.3 a
	urea	2.84 g	1.21 f	1.11 g	18.3 b	20.5 b	30.2 a
	$(\text{NH}_4)_2\text{SO}_4$	4.78 g	1.73 f	1.67 g	17.1 b	18.0 c	28.0 b
75	$\text{Ca}(\text{NO}_3)_2$	791.87 f	54.91 e	25.24 f	14.5 c	17.7 c	27.2 b
	urea	1084.56 e	67.84 d	32.66 e	12.8 d	15.1 d	24.2 c
	$(\text{NH}_4)_2\text{SO}_4$	1330.93 d	94.73 c	42.22 d	11.7 d	13.3 e	21.1 d
150	$\text{Ca}(\text{NO}_3)_2$	1445.59 c	100.19 c	46.38 c	9.5 e	12.7 e	20.2 d
	urea	1655.23 b	143.73 b	56.78 b	8.5 e	10.5 f	16.2 e
	$(\text{NH}_4)_2\text{SO}_4$	1846.54 a	186.30 a	76.8 a	6.8 f	8.6 g	13.0 f

increase varied with Cr concentration and N forms. At 75 μM Cr, the $\text{Ca}(\text{NO}_3)_2$ fed plants had significantly higher activities of all studied enzymes and content of GSH and ASC than the urea or $(\text{NH}_4)_2\text{SO}_4$ fed plants. However, at 150 μM Cr, the decrease in enzyme activities and also in content of GSH and ASC was observed in all three N forms used to fertilize the plants relative to those at 75 μM Cr, except SOD activity in the $\text{Ca}(\text{NO}_3)_2$ fed plants. Moreover, at both Cr concentrations, the $\text{Ca}(\text{NO}_3)_2$ fed plants had higher enzyme activities and content of GSH and ASC than the urea and $(\text{NH}_4)_2\text{SO}_4$ fed plants (Tables 1 and 2). Cr addition significantly increased Cr content in all plant parts irrespective of N

forms used for fertilization, and roots had the highest Cr content followed by a stem (Table 3). The Cr content was higher in the $(\text{NH}_4)_2\text{SO}_4$ fed plants than in the urea fed plants and the $\text{Ca}(\text{NO}_3)_2$ fed plants had the lowest values.

The N content in all plant parts was dependent on both Cr concentration and N form (Table 3). Under no Cr, the $\text{Ca}(\text{NO}_3)_2$ fed plants had higher N content than the $(\text{NH}_4)_2\text{SO}_4$ fed plants. Under Cr stress, there was a marked reduction in N content of all plant parts. The effect was more severe at 150 μM of Cr than at 75 μM . The $\text{Ca}(\text{NO}_3)_2$ fed plants were least affected among the three N form treatments.

Discussion

The interaction of N forms and heavy metal stress, including Cd, Al, and Zn, on plant growth has been investigated (Hassan *et al.* 2005, Zhao *et al.* 2009, Monsanto *et al.* 2010). It was reported that the interaction between heavy metals and N forms was highly specific to plant species, pH, and heavy metal. Monsanto *et al.* (2010) found that NO_3^- fed *Noccaea caerulescens* had greater biomass than NH_4^+ fed plants under Al stress, but Zhao *et al.* (2009) and Hassan *et al.* (2005) obtained the

opposite results using rice under Cd stress. It may be concluded that interaction between heavy metals and N forms varies with plant species.

Addition of Cr into the nutrient solution induced oxidative stress, resulting in increased content of H_2O_2 and O_2^- . Moreover, malondialdehyde (MDA) content was also increased which is a product of membrane lipid peroxidation commonly considered as an indicator of oxidative stress under Cr toxicity (Malmir 2011).

Similarly, Cr-induced oxidative stress was also found in sunflower (Gallego *et al.* 2002) and mustard (Pandey *et al.* 2005). In this study, we found that barley plants changed the activities of SOD, POD, CAT, APX, and GR, and the content of GSH and ASC in response to Cr stress. The current results showed that N forms had a great influence on oxidative stress caused by Cr toxicity. Hence, the Ca(NO₃)₂ fed plants had less oxidative stress than the plants fed with other two N forms reflected by the higher activities of the antioxidative enzymes and the higher content of the non-enzymatic antioxidants in these plants.

In addition, there were significant differences among

the three N treatments in Cr and N amounts of the plants subjected to Cr stress, with the Ca(NO₃)₂ fed plants having the highest N and the lowest Cr content in all plant tissues indicating the possibility of reducing Cr accumulation in plants.

In conclusion, plant growth, photosynthesis, oxidative stress, and Cr and N uptake in the barley plants exposed to Cr stress were dependent on N form. The Ca(NO₃)₂ fed plants had less oxidative damage and growth reduction under Cr stress than the urea and ammonium fed plants indicating that the suitable use of N fertilizer form is important for alleviating Cr stress in Cr contaminated soils.

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