Effect of Exogenous Treatment with Nitric Oxide (NO) on Redox Homeostasis in Barley Seedlings (*Hordeum vulgare* L.) Under Copper Stress

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Received: 19 October 2021 / Accepted: 28 December 2021 / Published online: 6 January 2022
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Abstract

The protective mechanism of nitric oxide (NO) in regulating tolerance to Cu-induced toxicity in shoots of barley (*Hordeum vulgare* L.) was studied. The experiment consisted of four treatments based on additions to basal nutrient solutions (BNS): control (CTR), Cu (200 μ M), SNP (500 μ M), and Cu (200 μ M) + SNP (500 μ M) over a period of 10 days. Treatment with Cu significantly reduced seedling growth and photosynthetic efficiency concomitant with an increase in reactive oxygen species contents, lipid peroxidation markers, and antioxidant enzyme activities, indicating that Cu induced oxidative stress. Furthermore, growth inhibition of Cu-treated plants was associated with a reduction in photosynthetic pigments and maximum photosystem II efficiency as well as a strong decrease in levels of glutathione (GSH) and ascorbate (AsA). Addition of a nitric oxide (NO) donor, sodium nitroprusside (SNP), to the growth medium alleviated Cu toxicity by decreasing Cu uptake and enhancing antioxidant capacity, as indicated by increased contents of GSH and AsA. The application of SNP decreased oxidative stress and lipid peroxidation by suppressing lipoxygenase activity and enhancing some antioxidant enzyme activities. The results obtained indicate the potential of exogenously applied SNP in the management of metal toxicity. Hence, NO generating compounds have potential agronomical applications when cultivating in contaminated areas. Our findings indicate that NO can alleviate Cu toxicity by affecting the antioxidant defense system and maintaining the glutathione-ascorbate cycle status, suggesting that SNP treatment protects proteins against oxidation by regulating the cellular redox homeostasis.

Keywords Copper · Oxidative stress · Sodium nitroprusside · Redox status · *Hordeum vulgare* L.

1 Introduction

Globally, heavy metal (HM) contamination is widespread as a result of anthropogenic, technogenic, and geogenic activities (He et al. 2005). Toxic metals such as cadmium (Cd), nickel (Ni), copper (Cu), mercury (Hg), cobalt (Co), and zinc (Zn) are taken up from the soil and water making them bioavailable to plants (Ehlken and Kirchner 2002). Several studies have previously shown that HMs have phytotoxic effects on plant growth and on the underlying physiological,

biochemical, and molecular processes (Gangwar et al. 2011; Wan et al. 2011; Kumar et al. 2012; Chaoui and El Ferjani 2013; Sakouhi et al. 2016; Ben Massoud et al. 2017; Kharbech et al. 2017). Copper, for example, serves as an essential co-factor for several antioxidant metalloenzymes including Cu/Zn superoxide dismutase, peroxidase, catalase, ferroxidases, and cytochrome c oxidases (Connan and Stengel 2011). Furthermore, it is involved in essential biological processes such as photosynthesis, respiration, metabolism, and cell wall lignification (Marschner 1995; Burkhead et al. 2009). Copper exposure results in a range of toxic effects in plants by inducing the accumulation of reactive oxygen species (ROS), which oxidize biomolecules, thereby inhibiting most cellular processes (Karmous et al. 2017; Ben Massoud et al. 2018).

Several strategies have been developed to protect crops against abiotic stresses such as the exogenous application of phytohormones (Ben Massoud et al. 2018), calcium, citrate, or ethylene diaminetetraacetic acid (EDTA) (Sakouhi

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et al. 2016; Ben Massoud et al. 2017; Lwalaba et al. 2017). In addition, nitric oxide (NO), hydrogen sulfide (H₂S), and carbon monoxide (CO) are considered to be gases involved in important signaling processes in plant development and growth in addition to the modulation of defense responses (Kharbech et al. 2020; Li et al. 2012).

Nitric oxide (NO) is one of the smallest endogenous cell-signaling (Laspina et al. 2005; Palavan-Unsal and Arisan. 2009). This hydrophobic bioactive molecule plays a vital role in germination, growth, movement of stomata, and flowering and is involved, as a signaling molecule, in the regulation of responses to environmental stresses (Corpas et al. 2009; Procházková et al. 2013; Kharbech et al. 2017; Rodríguez-Ruiz et al. 2017). NO effects can be studied by treatment with various donors; the most commonly used are sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), S-nitroso N-acetylpenicillamine (SNAP) or diethylamine nonoate (DENO) (Procházková et al. 2013). It is well known that NO can be used to reduce the oxidative stress caused especially by heavy metals (Corpas et al. 2009; Procházková et al. 2013; Kharbech et al. 2017; Rodríguez-Ruiz et al. 2017). In recent years, the importance of signaling molecules in countering abiotic stress by avoiding ROS accumulation and modulating antioxidant activities has been increasingly appreciated. In plants, nitric oxide (NO) is a multifunctional signaling molecule that leads to activation of defensive responses and regulates different metabolic pathways under adverse conditions (Palavan-Unsal and Arisan. 2009; Kharbech et al. 2020). Even though NO is known by its harmful dualistic effects, it has been reported that this gas transmitter, generated at optimum levels, could have either antagonistic or synergistic functions in response to plant stress. In this context, the potential of exogenous application of NO for improving plant tolerance to environmental stress has been extensively reported (Corpas et al. 2009; Demecsová et al. 2019; Kharbech et al. 2020).

Barley (*Hordeum vulgare* L.) is one of the four most important cereals in the world, after wheat, maize, and rice. It is one of the most versatile cereals and known to be well adapted to various global climates. In addition, it has the ability to cope with diverse stress conditions. For example, it is one of the most salt-tolerant cereal species (Munns et al. 1995). However, several studies have shown that HMs in the growth medium affect the growth and metabolism of barley seedlings (Tamás et al. 2008; Lwalaba et al. 2017, 2019). Here, we investigate at the biochemical level how the exogenous application of NO ameliorates the detrimental effect of Cu on barley seedling development, cell viability, and redox state. The results show the capacity of this gaseous signaling molecule to reverse the negative effects of Cu on barley seedling growth by maintaining redox homeostasis and increasing AsA and GSH contents, thereby alleviating

damage to the photosynthetic apparatus and decreasing cell death.

2 Materials and Methods

2.1 Plant Material and Hydroponic Culture

Seeds of barley (*Hordeum vulgare* L.) were surface sterilized for 10 min with 2% sodium hypochlorite (v/v) before thorough rinsing with distilled water. For germination, seeds were placed onto two sheets of filter paper moistened with distilled water. The growth room was maintained with a photoperiod of 14-h light/10-h dark and a temperature of 20 °C (throughout both the light and dark periods). Plants were grown at PFD (photon flux density) of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Four days after germination, equally sized seedlings were transplanted into 1-L plastic containers for hydroponic culture, which were filled with basal nutrient solutions (BNS) composed of (NH₄)₂SO₄ 48.2 mg l⁻¹, MgSO₄·7H₂O 154.8 mg l⁻¹, K₂SO₄ 15.9 mg l⁻¹, KNO₃ 18.5 mg l⁻¹, KH₂PO₄ 24.8 mg l⁻¹, Ca(NO₃)₂·4H₂O 86.17 mg l⁻¹, FeC₆H₆O₇ 335.03 mg l⁻¹, MnCl₂·4H₂O 0.9 mg l⁻¹, ZnSO₄·7H₂O 0.11 mg l⁻¹, CuSO₄·5H₂O 0.04 mg l⁻¹, H₃BO₃ 2.9 mg l⁻¹, and H₂MoO₄ 0.01 mg l⁻¹. The growth solution was renewed daily, with the pH being corrected to 5.8, using HCl or NaOH as required. The experiment was laid out with four independent biological replicates for each treatment.

2.2 Treatments and Sampling

The experiment consisted of four treatments based on additions to BNS: control (CTR), Cu (200 μM), SNP (500 μM), and Cu (200 μM) + SNP (500 μM). Treatments were initiated with seedlings from the two-leaf stage, with Cu and SNP being administered as solutions of CuCl₂ and Na₂(Fe(CN)₅NO)·0.2H₂O in distilled water, respectively. Initial experiments showed 200 μM Cu to be the level at which seedling growth decreased to approximately half that of the control value (Fig. 1a, b). This concentration was therefore chosen as the basis for the subsequent experiments. Plants were harvested after 10 days of treatment, separated into roots and shoots, and weighed. Samples were stored at -80 °C for biochemical analyses.

2.3 Copper Concentration

Cu concentrations were determined using an atomic absorption spectrophotometer (Perkin Elmer, Waltham MA, USA) after digesting the oven-dried seedlings with 10 ml per 0.1 g dry tissue of an acid mixture of HNO₃:HClO₄ (4:1 v/v). Sigma Diagnostic Standards (Copper Atomic Absorption Solutions, Sigma-Aldrich) were used for calibration after

Fig. 1 Seedling leaf length (cm) and seedling fresh weight (mg/plant) of shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either different CuCl₂ concentrations (100, 150, 200, and 300 μM) (a, b) or 200 μM CuCl₂ with different SNP (sodium nitro-

prusside) concentrations (400, 500, and 600 μM) (c, d). Mean values $\pm$ standard error (SE ($n=5$)) followed by a common letter are not different at the $\alpha 0.05$ level of significance, following analysis of variance (ANOVA) and multiple pairwise comparison by Tukey's honestly significant difference test

dilution to the appropriate ranges with 0.1 N HNO₃ (diluted from 65% HNO₃, Sigma-Aldrich).

2.4 Estimation of Cell Viability

Cell viability was determined according to the method of Romero-Puertas et al. (2004) by spectrophotometric determination of Evans Blue uptake. After infiltration with an aqueous solution of 0.25% (w/v) Evans Blue, the roots and shoots were boiled in 80% (v/v) ethanol for 5 min and then oven-dried. After drying, they were incubated in 50% (v/v) methanol with 1% (w/v) sodium dodecyl sulfate (SDS) at 60 °C for 30 min to extract the dye bound to dead cells and to quantify the dye by measuring the absorbance at 600 nm.

2.5 Protein Extraction and Enzyme Activity Assays

Fresh seedling tissue was homogenized in 50 mM potassium phosphate buffer (pH 7.0), supplemented with 1 mM

ethylenediaminetetraacetic acid (EDTA). After centrifugation of the homogenate at 10,000 $\times g$ for 15 min at 4 °C, the resulting supernatant was used as the soluble protein fraction for assays of enzyme activities.

SOD (EC 1.15.1.1) activity was assayed according to the method of Misra and Fridovich (1972), with minor modifications. The inhibition of epinephrine auto-oxidation was followed as an increase in absorbance at 480 nm. CAT (EC 1.11.1.6) activity was measured according to the method of Aebi (1984). CAT activity was estimated by monitoring the decrease in the absorbance of H₂O₂ reduction at 240 nm, using an extinction coefficient of $36 \times 10^{-6} \text{ M}^{-1} \text{ cm}^{-1}$. APX (EC 1.11.1.11) activity was measured as described by Nakano and Asada (1981). APX activity was determined by following the decrease in ascorbate absorbance at 290 nm. The extinction coefficient of $2.8 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ was used. GPX (EC 1.11.1.9) activity was measured according to the method described by Nagalakshmi and Prasad (2001). The decrease of absorbance indicating the oxidation of NADPH

was followed at 340 nm ($\epsilon = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). GPOX (EC 1.11.1.7) activity was measured as described by Fielding and Hall (1978). GPOX activity was estimated by measuring the increase in absorbance of tetraguaiacol at 470 nm, using an extinction coefficient of $26.6 \text{ M}^{-1} \text{ cm}^{-1}$. GR (EC 1.6.4.2) activity was determined according to the method described by Foyer and Halliwell (1976), by measuring the rate of NADPH oxidation/decrease by monitoring absorbance at 340 nm.

LOX (EC 1.13.11.12) activity was extracted in 50 mM sodium phosphate buffer (pH 7.0) with 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2% (w/v) polyvinylpyrrolidone (PVP), 1% glycerol, and 0.1% Tween-20. The extract was centrifuged for 20 min at $15,000 \times g$ and 4°C . 2.9 mL of the assay solution (1 mM linoleic acid in 0.1 M sodium acetate buffer) was then added to 0.1 mL of the supernatant. Absorbance at a wavelength of 240 nm was measured, and LOX activity was calculated using the extinction coefficient ($\epsilon = 25 \text{ mM}^{-1} \text{ cm}^{-1}$) of conjugated dienes (Ramakrishna and Rao 2012).

DHAR (EC 2.5.1.18) activity was determined by monitoring the reduction of dehydroascorbate (DHA) into reduced ascorbate (AsA) at a wavelength of 265 nm, using an extinction coefficient of $7.0 \text{ mM}^{-1} \text{ cm}^{-1}$ (Nakano and Asada 1981).

MDAR (EC 1.6.5.4) was assayed by measuring NADH oxidation as a decrease in absorbance at 340 nm, generating monodehydroascorbate by the ascorbate/ascorbate oxidase system (Arrigoni et al. 1981). The rate of monodehydroascorbate-independent NADH oxidation in the absence of ascorbate and ascorbate oxidase was subtracted from the monodehydroascorbate-dependent NADH oxidation rate in the presence of ascorbate and ascorbate oxidase to determine the MDAR activity.

2.6 Stress Biomarker Concentrations (H_2O_2 , MDA, $\text{O}_2^{\bullet-}$, and $\text{OH}^\bullet$)

Hydrogen peroxide (H_2O_2) was determined according to the method of Sergiev et al. (1997). The H_2O_2 concentration was estimated based on the absorbance of the supernatant at 390 nm. Malondialdehyde (MDA) concentration was determined according to the method described by Tong et al. (2014). Hydroxyl radical ($\text{OH}^\bullet$) contents were quantified using hydroxyl radical-induced oxidative degradation of deoxyribose and the resultant malondialdehyde (MDA) production by fluorometric quantitation of the 2-thiobarbituric acid (TBA) adducts (Dunaevsky and Belozersky, 1993). Levels of superoxide anion ($\text{O}_2^{\bullet-}$) were determined based on the reduction of the tetrazolium compound 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) following to Dunaevsky and Belozersky (1993) method.

2.7 Photosynthetic Pigment Concentrations

Chlorophyll was extracted from leaf tissue by incubation in 80% (v/v) acetone. Absorbance was measured at 750, 663, 645, and 453 nm to determine the concentrations of chlorophyll a, chlorophyll b, and total carotenoids, respectively, using the equations of Arnon (1949).

2.8 SPAD Measurement of In Situ Leaf Chlorophyll Concentration

A SPAD-502 Plus chlorophyll meter (Konica Minolta, Inc.) was used to determine the leaf chlorophyll concentration in situ by measuring the leaf absorbance in the red (650 nm) and near-infrared (940 nm) regions. The resulting numerical SPAD value gives the relative concentration of chlorophyll on a leaf area basis (Konica Minolta Optics 2012).

2.9 Maximum Photosystem II Efficiency

Maximum photosystem II efficiency (F_v/F_m) was determined by measuring chlorophyll fluorescence with a FluorPen (Photon Systems Instruments) after dark adaptation of leaves for at least 20 min.

2.10 Estimation of Glutathione, Ascorbate and Proline Concentrations

Reduced (AsA) and oxidized ascorbate (DHA) were measured using the method of Ghorbani et al. (2018), based on the formation of a red chelate between Fe^{2+} and 2,2'-bipyridyl after reduction of Fe^{3+} into Fe^{2+} with ascorbic acid in acid solution.

Oxidized glutathione (GSSG) and reduced glutathione (GSH) concentrations were determined according to the methods described by McGill and Jaeschke (2015).

The free proline concentration in shoots was determined by the method described by Bates et al. (1973). The fresh tissue sample was homogenized with sulfosalicylic acid (3% [w/v]) and centrifuged at $12,000 \times g$ for 10 min. Glacial acetic acid and acid ninhydrin solutions were added to the supernatant. The reaction mixture was heated in a water bath at 100°C for 1 h and then cooled in an ice bath. Toluene was added to this mixture and then the absorbance at 520 nm was determined.

2.11 Statistical Analysis

All experiments were performed with five biological replicates and at least in three technical replicates for each sample. Values are presented as mean $\pm$ standard error (SE), of four biological replicates. Significance of differences was tested at $p < 0.05$, using analysis of variance (ANOVA) test

followed by multiple comparisons by Tukey's honestly significant difference (HSD) test. One-way ANOVA followed by Tukey's post hoc multiple comparison tests was performed using the software package Statistica 8.0.

3 Results

3.1 Choice of Copper and Nitric Oxide (SNP) Concentrations

Copper concentrations of 100 μM , 150 μM , 200 μM , and 300 μM reduced the length of barley shoots by around 18%, 23%, 51%, and 80% (Fig. 1a) and the fresh weight by 11%, 28%, 63%, and 81%, respectively, compared with the control (Fig. 1b). According to these results, we chose a concentration of 200 μM CuCl_2 for subsequent experiments. After identifying the metal concentration to be used for the rest of the testing, we identified the most appropriate concentration of the exogenous effector (SNP) to use in combination with Cu (200 μM). SNP concentrations (400, 500, and 600 μM)

resulted in recoveries of Cu-induced growth inhibition of approximately 62%, 91%, and 93%, respectively, in terms of the length of barley shoots (Fig. 1c) and by approximately 49%, 75%, and 77% in recovery of barley fresh weight, respectively, in Cu + SNP treatment compared with Cu treatment (Fig. 1d).

3.2 Effect of NO on Seedling Growth and Cu Accumulation

After 10 days of exposure, Cu (200 μM) significantly reduced barley seedling growth by approximately half in length, fresh weight, and dry weight compared to the control (Table 1). Under our experimental conditions, SNP (500 μM), when applied alone, did not significantly affect seedling growth (Table 1). However, the addition of SNP to Cu hydroponic medium significantly improved seedling growth compared to Cu treatment (Fig. 2), with SNP restoring most of the growth inhibition exhibited in seedlings in Cu treatment, to 91% (length), 75% (fresh weight), and 81% (dry weight) compared to the control (Table 1).

Table 1 Length, fresh weight, dry weight, and copper concentration in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 μM CuCl_2 , 500 μM SNP (sodium nitroprusside), or 200 μM CuCl_2 and 500 μM SNP (SNP + Cu). Mean values $\pm$ standard

Treatment	Length (cm)	Fresh weight (mg plant ⁻¹)	Dry weight (mg plant ⁻¹)	Cu concentration ($\mu\text{g g}^{-1}$ DW)
CTR	18.37 $\pm$ 1.06 ab	210.37 $\pm$ 8.29 a	20.49 $\pm$ 1.49 a	18.22 $\pm$ 3.14 c
CuCl_2	09.02 $\pm$ 1.51 c	78.38 $\pm$ 3.81 c	11.65 $\pm$ 1.65 c	62.54 $\pm$ 7.58 a
SNP	20.25 $\pm$ 1.28 a	227.06 $\pm$ 6.18 a	21.53 $\pm$ 1.83 a	16.32 $\pm$ 5.15 c
SNP + Cu	16.62 $\pm$ 1.07 b	157.51 $\pm$ 6.52 b	16.61 $\pm$ 1.39 b	41.61 $\pm$ 6.44 b

error (SE ($n=5$)) followed by a common superscript letter in a column are not different at the $\alpha 0.05$ level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test

Fig. 2 Morphology of barley seedlings grown hydroponically for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 μM CuCl_2 , 500 μM SNP (sodium nitroprusside), or both 200 μM CuCl_2 and 500 μM SNP (SNP + Cu)

The Cu concentration in barley seedlings increased significantly when the seedlings were exposed to Cu treatment (Table 1). Shoots of barley seedlings exposed to 200 μM CuCl_2 accumulated more than 3.5 times the level of Cu accumulated by the control. On the other hand, following addition of SNP to Cu treatment (i.e., Cu + SNP treatment), there was a 33% decrease in Cu accumulation, compared with the seedlings in Cu treatment, an effect associated with the reduction in Cu-induced growth damage in seedlings exposed to Cu + SNP treatment (Table 1). These data indicate that NO has beneficial effects on Cu-induced stress.

3.3 Effect of NO on Cell Viability and Lipid Peroxidation Marker

The increase in Cu accumulation in Cu-stressed barley seedlings (Table 1) was associated with a greater capacity (a twofold increase) to absorb the dye in comparison with the control, indicating that the number of dead cells increased in the Cu-stressed shoots (Fig. 3a), whereas NO application significantly alleviated the damaging effect of Cu on cell viability (Fig. 3a).

The ameliorative action of SNP on Cu-induced cell death could be, at least in part, a direct consequence of a lowering of LOX activity. In the present investigation, exposure to Cu resulted in a strong stimulation of LOX activity by up to 68%, compared with the minus-Cu control, whereas the simultaneous application of SNP and Cu reduced this increase to 46% (Fig. 3b). LOX activity in barley seedlings was positively related with the levels of MDA (stimulated by more than threefold compared to control level, whereas SNP lowered this Cu-induced lipid damage) (Fig. 3c).

3.4 Effect of NO on Antioxidant Defenses and Oxidative Damage

The activities of the enzymatic antioxidants tested in shoots of barley increased considerably under Cu stress showing an increase of 54% in SOD, 64% in CAT, 51% in GPOX, and 65% in GPX, as compared with the control activities (Fig. 4). However, when SNP was combined with Cu, the activities of SOD, CAT, GPOX, and GPX decreased significantly by 40%, 48%, 34%, and 43%, respectively, as compared with Cu treatment (Fig. 4). In the current study, stress marker concentrations also showed significant increases, with concentrations of H_2O_2 , $\text{O}_2^{\cdot-}$, and $\cdot\text{OH}$ in Cu-stressed barley seedlings increasing by around the half, compared with the control

Fig. 3 Cell death estimation (a), lipoxygenase (LOX) activity (b), and malondialdehyde (MDA) concentration (c) in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with 200 μM CuCl_2 , 500 μM SNP (sodium nitroprusside), or both 200 μM CuCl_2 and 500 μM SNP (SNP+Cu). Mean values $\pm$ standard error (SE ($n=5$)) followed by a common letter are not different at the $\alpha 0.05$ level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test

(Fig. 5), whereas the exogenous application of SNP in the hydroponic environment reduced significantly this increase in the stress markers studied (Fig. 5).

Fig. 4 Activities of antioxidant enzymes: superoxide dismutase (SOD) (a), catalase (CAT) (b), guaiacol peroxidase (GPOX) (c), and glutathione peroxidase GPX (d) in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 μM CuCl_2 , 500 μM SNP (sodium nitroprusside), or both 200 μM CuCl_2 and 500 μM SNP (SNP + Cu). Mean values $\pm$ standard error (SE ($n=5$)) followed by a common letter are not different at the $\alpha 0.05$ level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test

3.5 Effect of NO on Photosynthetic Pigment Concentrations, Chlorophyll Content and Photosystem II Efficiency Under Copper Stress

The effects on photosynthetic pigment concentrations in Cu-treated barley seedlings are presented in Table 2, showing a similar response to that of growth parameters in plants treated with copper. The Cu-treated seedlings contained less chlorophyll a, chlorophyll b, and total carotenoids than did the control seedlings. In the current study, we noted a significant decline in the chlorophyll a/b ratio under Cu stress, meaning that chlorophyll a was more affected than was chlorophyll b.

Photosystem II efficiency is considered to be an important indicator of abiotic stress, and our findings in Table 2 showed a decrease both the maximum quantum yield of PSII (F_v/F_m ratio) and in chlorophyll concentration of the plant leaves, measured using a SPAD meter, indicating photo-oxidative damage in line with the ROS accumulation measured here (Fig. 5). On the other hand, SNP addition to the hydroponic culture medium partly alleviated the negative effects of Cu treatment on chlorophyll concentration and F_v/F_m .

3.6 Effect of NO on GSH-AsA Cycle Enzymes and Non-enzymatic Antioxidants Under Copper Stress

In order to investigate the ameliorative effects of exogenous NO application on Cu toxicity, we measured the antioxidant activities of GSH-AsA cycle enzymes in barley seedling shoots. In Cu-treated seedlings APX, MDAR, DHAR, and GR activities increased significantly

compared with control seedlings (Fig. 6). However, exogenously applied SNP significantly corrected this increase (Fig. 6). The concentrations of several non-enzymatic antioxidants were also determined in shoots of barley seedlings exposed to Cu and/or SNP. Copper was found to induce a significant decrease in AsA and GSH concentrations, which were reduced by around 49% and 39%, respectively, compared with control seedlings, with the inclusion of SNP allowing Cu-treated seedlings to partly overcome this decrease, with a recovery rate of 44% in AsA and 23% in GSH (Table 3). Our results also showed a significant increase in AsA/DHA and GSH/GSSG ratios under Cu stress. However, a significant re-balancing of the AsA/DHA and GSH/GSSG ratios was also observed in seedlings treated with both Cu and SNP, compared with control seedlings (Table 3). The other enzymes of the GSH-AsA cycle (MDHAR, DHAR, and GR) also play a crucial role in the elimination of ROS and the minimization of oxidative stress. The activities of these enzymes increased significantly in response to Cu treatment, which indicates that the barley seedlings were stressed (Fig. 6). However, the application of SNP significantly reduced the activities of these enzymes, which explains the homeostatic re-balance at the level of the GSH-AsA cycle (Fig. 6).

In our study, GSH content was reduced in seedlings exposed to Cu. Table 3 shows the concentrations of GSH and GSSG, as well as the GSH/GSSG ratio, in the shoots of barley seedlings subjected to different treatments. The concentrations of both forms of glutathione (reduced [GSH] and oxidized [GSSG]) decreased significantly, by approximately 39% and 51%, respectively, relative to the control in seedlings exposed to Cu treatment. However, application

Fig. 5 Concentrations of hydrogen peroxide (H_2O_2) (a), superoxide anion ($\text{O}_2^{\bullet-}$) (b), and hydroxyl radical ($\text{OH}^{\bullet}$) (c) in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 μM CuCl_2 , 500 μM SNP (sodium nitroprusside), or 200 μM CuCl_2 and 500 μM SNP (SNP+Cu). Mean values $\pm$ standard error (SE) ($n=5$) followed by a common letter are not different at the $\alpha 0.05$ level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test

of SNP induced restoration of levels of both forms of glutathione (Table 3).

Figure 7 shows a significant 42% increase in proline concentration following exposure to Cu compared with the control. Treatment with SNP decreased this increase in barley shoots by 15%, compared with Cu treatment alone (Fig. 7).

4 Discussion

The present study revealed that, at the early stages of the barley life cycle, Cu treatment resulted a concentration-dependent reduction in seedlings' biomass. According to these results, a concentration of 200 μM CuCl_2 was chosen for subsequent experiments. This corresponds to the concentration of metal salts used for various species such as Cr for *Zea mays* (Kharbech et al. 2017); Cd for *Cicer arietinum* (Sakouhi et al. 2016), *Phaseolus vulgaris* (Karmous et al. 2017), and *Pisum sativum* (Jaouani et al. 2018); and Cu for *Pisum sativum* (Ben Massoud et al. 2018). On the other hand, the choice of metal concentrations varies according to species tolerance, as for example for Cd (100 μM), *Medicago truncatula* (Rahoui et al. 2015); Al (50 μM), *Anabaena* PCC 7120 (Tiwari et al. 2019); and As (50 μM), *Pisum sativum* (Rodríguez-Ruiz et al. 2019). Similar results were reported in previous studies showing the effect of HMs on various plant species, such as Cu in *Pisum sativum* (Ben Massoud et al. 2018) and *Lens culinaris* Medik (Chaoui and El Ferjani 2013), for Cr in *Zea mays* (Kharbech et al. 2017), and for Cd in *Cicer arietinum* (Sakouhi et al. 2016), *Phaseolus vulgaris* (Karmous et al. 2017), and *Pisum sativum* (Jaouani et al. 2018).

The choice of the exogenous effector concentration (SNP) was based on selecting the lowest dose of SNP which achieved the greatest recovery of barley growth, compared with the control and Cu treatments. The two concentrations, 500 and 600 μM SNP, achieved recovery levels, compared with the control, which were not significantly different from one another. Based on this experiment, 500 μM SNP was chosen for the remainder of the study as the lowest dose of SNP that supported the highest recovery compared to control and treatment with Cu alone. Interestingly, the same SNP concentration was used by Kharbech et al. (2017) in order to alleviate the toxicity of Cr in *Zea mays*. Several other studies have reported that NO is able to mitigate the harmful effects of different stresses (Corpas et al. 2013; Kharbech et al. 2017). Our results are in agreement with those findings previously reported in pea (Corpas et al. 2008), maize (Kharbech et al. 2017), and barley (Demecsová et al. 2019; He et al. 2019). In agreement with our findings, it has also been reported that the exogenous application of NO could reduce the accumulation of heavy metals in the different tissues of plants of different species exposed to metallic stress, such as Cr/maize (Kharbech et al. 2017), Cd/barley (Demecsová et al. 2019), Ni/rice (Rizwan et al. 2018), and Cu/ rice (Mostofa et al. 2014).

The viability of barley seedling cells in response to the various treatments was evaluated by the Evans Blue technique. Two mechanisms of cell death in plants have been

Table 2 Chlorophyll and carotenoid concentrations (mg/g FW), maximal photochemical efficiency of PSII (F_v/F_m), and chlorophyll concentration of plant leaves (Chl.P.L) in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 μ M CuCl_2 , 500 μ M SNP (sodium nitroprusside), or 200 μ M CuCl_2 and 500 μ M

SNP (SNP+Cu). Mean values $\pm$ standard error (SE ($n=5$)) in a row followed by a common superscript letter are not different at the $\alpha 0.05$ level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test. *Chl a*, chlorophyll a; *Chl b*, chlorophyll b; *Chl (a+b)*, total chlorophyll; *Car*, carotenoids; *Chl.P.L*, chlorophyll concentration of plant leaves

Variables	CTR	CuCl_2	SNP	SNP+Cu
Chl a	$1.15 \pm 0.06\text{b}$	$0.66 \pm 0.09\text{c}$	$1.42 \pm 0.08\text{a}$	$1.12 \pm 0.09\text{b}$
Chl b	$0.6 \pm 0.06\text{b}$	$0.39 \pm 0.03\text{c}$	$0.77 \pm 0.05\text{a}$	$0.61 \pm 0.05\text{b}$
Chl (a+b)	$1.75 \pm 0.12\text{b}$	$1.05 \pm 0.12\text{c}$	$2.19 \pm 0.13\text{a}$	$1.73 \pm 0.14\text{b}$
Chl (a/b)	$1.92 \pm 0.16\text{a}$	$1.69 \pm 0.2\text{c}$	$1.84 \pm 0.13\text{b}$	$1.84 \pm 0.24\text{b}$
Car	$0.37 \pm 0.04\text{b}$	$0.22 \pm 0.03\text{c}$	$0.49 \pm 0.02\text{a}$	$0.34 \pm 0.03\text{bd}$
F_v/F_m	$0.82 \pm 0.01\text{a}$	$0.75 \pm 0.02\text{b}$	$0.82 \pm 0.01\text{a}$	$0.83 \pm 0.01\text{a}$
Chl.P.L	$36.14 \pm 1.65\text{b}$	$22.94 \pm 1.96\text{c}$	$35.91 \pm 1.51\text{b}$	$40.71 \pm 2.42\text{a}$

Fig. 6 Activities of antioxidant enzymes: ascorbate peroxidase (APX) (a), monodehydroascorbate reductase (MDHAR) (b), dehydroascorbate reductase (DHAR) (c), and glutathione reductase (GR) (d) in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 μ M CuCl_2 , 500 μ M SNP (sodium nitroprusside), or 200 μ M CuCl_2 and 500 μ M SNP (SNP+Cu). Mean values $\pm$ standard error (SE ($n=5$)) followed by a common letter are not different at the $\alpha 0.05$ level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test

Table 3 Concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), reduced ascorbate (AsA), and dehydroascorbate (DHA) in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 μ M CuCl_2 , 500 μ M SNP (sodium

nitroprusside), or 200 μ M CuCl_2 and 500 μ M SNP (SNP+Cu). Mean values $\pm$ standard error (SE ($n=5$)) in a row followed by a common superscript letter are not different at the $\alpha 0.05$ level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test

Treatments	Glutathione			Ascorbate		
	GSH	GSSG	GSH/GSSG	AsA	DHA	AsA/DHA
	Concentration (nmol g ⁻¹ FW)			Concentration (nmol g ⁻¹ FW)		
CTR	$727.5 \pm 26.3 \text{ a}$	$45.13 \pm 5.23 \text{ a}$	$16.12 \pm 5.03 \text{ b}$	$102.25 \pm 9.03 \text{ b}$	$21.12 \pm 1.36 \text{ b}$	$4.84 \pm 0.08 \text{ b}$
CuCl_2	$445 \pm 33.17 \text{ c}$	$22.12 \pm 4.18 \text{ c}$	$20.11 \pm 7.93 \text{ a}$	$52.13 \pm 8.28 \text{ d}$	$9.78 \pm 2.13 \text{ c}$	$5.33 \pm 0.13 \text{ a}$
SNP	$772.5 \pm 42.72 \text{ a}$	$48.21 \pm 6.12 \text{ a}$	$16.02 \pm 6.98 \text{ b}$	$140.52 \pm 9.24 \text{ a}$	$32.22 \pm 3.45 \text{ a}$	$4.36 \pm 0.09 \text{ c}$
SNP+Cu	$580 \pm 33.66 \text{ b}$	$36.12 \pm 7.23 \text{ b}$	$16.06 \pm 4.65 \text{ b}$	$93.51 \pm 7.41 \text{ c}$	$19.12 \pm 2.98 \text{ b}$	$4.89 \pm 0.15 \text{ b}$

Fig. 7 Proline concentration in shoots of barley seedlings grown in hydroponic culture for 10 days in the presence of nutrient solution (CTR) alone or in combination with either 200 µM CuCl₂, 500 µM SNP (sodium nitroprusside), or 200 µM CuCl₂ and 500 µM SNP (SNP+Cu). Mean values ± standard error (SE (*n*=5)) followed by a common letter are not different at the α0.05 level of significance, using analysis of variance (ANOVA) followed by Tukey's honestly significant difference test

reported. The first mechanism of cell death is a passive process, known as necrosis (Van Breusegem and Dat 2006), whereas the second is an active genetically controlled process, called apoptosis or programmed cell death, which involves shrinkage of the cell, condensation of the nucleus, and chromatin fragmentation (Lam 2004). In apoptosis, the cells are selectively eliminated by a process involving specific nucleases and proteases (Petrov et al. 2015).

LOX activity is considered to be a reflection of lipid peroxidation, as a result of the oxygenation of polyunsaturated fatty acids into lipid hydroperoxides during responses to stress (Li et al. 2012; Ramakrishna and Rao 2012). MDA production is considered to be evidence of oxidative stress (Karmous et al. 2017; Ben Massoud et al. 2018). A previous study had reported that NO could protect maize against lipid peroxidation under Cr stress (Kharbech et al. 2017).

One of the main strategies of plant metal tolerance is elimination of the elevated levels of ROS by antioxidant enzymes (Apel and Hirt. 2004; Karmous et al. 2017). Indeed, previous studies had reported that a strong stimulation of the activities of antioxidant enzymes was observed following exposure to biotic or abiotic stress (Chaoui and El Ferjani 2013; Ben Massoud et al. 2018). In this context, researchers have focused on finding treatments to combat this stress, including the exogenous application of phytohormones (Ben Massoud et al. 2018); ionic competitors, such as calcium (Ben Massoud et al. 2017; Lwalaba et al. 2019); chelators, such as ethyleneglycoltetraacetic acid (EGTA; Sakouhi et al. 2016); and citrate (Ben Massoud et al. 2017),

with the application of NO being effective at balancing oxidative status (Corpas et al. 2009; Kharbech et al. 2017).

As predicted, the application of NO abolished, completely, the Cu-imposed rise in H₂O₂, O₂^{•−}, and •OH concentrations, indicating the recovery of the cellular redox state. This protective role should be assigned, at least in part, to the restriction of the accumulation of Cu ions in cell tissues as judged by previous investigation (Mostofa et al. 2014).

The results suggest Cu-induced damage of the photosynthetic apparatus. Similar responses had been reported in bean plants subjected to high Mn concentrations, showing severe chlorophyll losses, which might be due to photosynthetic disruption and/or nutrient imbalance (Mahjoubi et al. 2020). Ashraf and Mehmood (1990) reported a decrease in the chlorophyll a/b ratio exhibited by three out of four *Brassica* species (Ashraf and Harris 2013). However, the decrease in the maximum quantum yield of PSII (*F_v/F_m* ratio) and in chlorophyll concentration of the plant leaves, indicating photo-oxidative damage in line with the ROS accumulation measured here (Fig. 5), which can be explained by the affected functionality of both PSII and PSI, particularly PSII (Ashraf and Harris 2013). The effect of SNP may be due to its effects as a signaling molecule in reversing stress damage. It has been shown that SNP moderates ROS effects under stress conditions, protecting chlorophyll pigments and ensuring normal photosynthesis (Ahmad et al. 2018). Hence, this molecule protects the quantum yield of photosystem II, which can explain the ameliorative effect of SNP on chlorophyll fluorescence (Hasanuzzaman et al. 2018).

The plant antioxidant machinery includes many non-enzymatic antioxidants (GSH, AsA, phenolic compounds, flavonoids, carotenoids, alkaloids, proline, non-protein amino acids, and α-tocopherols) and several antioxidant enzymes (APX, MDHAR, DHAR, GR, GPX, CAT, GPOX, and GST) which work in a coordinated way to eliminate ROS and minimize oxidative stress (Apel and Hirt 2004; Hasanuzzaman et al. 2012).

In the present study, we focused on the effect of Cu on the GSH-AsA cycle (Table 3) and the key enzymes of the cycle (APX, MDHAR, DHAR, and GR) as well as the role of NO in maintaining cellular homeostatic balance (Fig. 6). APX is the key enzyme in the GSH-AsA cycle and plays an important role in plant defense by catalyzing the conversion of H₂O₂ to water at the expense of AsA (Bashri and Prasad 2016). APX activity increased in response to Cu treatment (Fig. 6), a phenomenon which has been reported in previous studies (Ben Massoud et al. 2017; Karmous et al. 2017), while several other studies have confirmed similar results with other metals, such as Al (Sharma and Dubey 2007) and Cd (Sakouhi et al. 2016). This finding corroborates previous studies which showed a role of NO in achieving cellular balance by reducing antioxidant activities (Corpas et al. 2007; Li et al. 2013; Kharbech et al. 2017). Several studies have

shown that the application of exogenous effectors, such as phytohormones (Ben Massoud et al. 2018), EGTA (Sakouhi et al. 2016), Ca and citrate (Ben Massoud et al. 2017), SNP (Kharbech et al. 2017), and H₂S (Li et al. 2013) could reduce the accumulation of ROS and hence minimize oxidative stress.

Given the importance of the GSH–AsA cycle in achieving redox cell homeostasis, we studied the effects of Cu on the concentrations of GSH and AsA. Ascorbate is one of the most effective substrates for scavenging H₂O₂. AsA reduces the state of metal ions in prosthetic groups and maintains the activity of antioxidant enzymes (Hasanuzzaman et al. 2019). In plant tissues, AsA, which serves as an electron donor in numerous reactions, is one of the most abundant water-soluble antioxidants (Foyer and Noctor 2011). After oxidation of AsA to monodehydroascorbate and then to DHA, it can be reduced back to AsA by the AsA–GSH cycle (i.e., the Foyer–Halliwell–Asada pathway; Foyer and Halliwell 1976; Asada, 1999). Previous studies have shown that the AsA concentration in plants decreases under stress conditions; for example at the whole leaf level, drought stress has been shown to cause a general reduction in AsA concentration (Munné-Bosch and Alegre 2003; Seminario et al. 2017). The results obtained in the current study show a significant decrease in the concentration of major non-enzymatic antioxidants in response to Cu stress, which could be corrected by the addition of SNP (Table 3). Taken together, these results suggested that the functionality of the AsA–GSH cycle could be limited, which could explain the increase in H₂O₂ concentration observed in pea shoots exposed to Cu (Ben Massoud et al. 2017).

Glutathione is a very important antioxidant for various physiological processes, especially during abiotic stress. It is oxidized to GSSG in close coordination with AsA turnover (Hasanuzzaman et al. 2018). Similar results have also been observed in different plant species under Cd stress (Romero-Puertas et al. 2007). In the present study, NO might have played a significant role in maintaining higher GSH concentration through efficient recycling. The increased concentration of the GSH pool is considered to protect against oxidative stress (May and Leaver 1993), so that the increased GSH content in response to SNP application in the present study protects the seedlings against Cu stress.

The production of high concentrations of proline in plants is a typical non-enzymatic response to several biotic and abiotic stresses (Szabados and Savaure 2010; Shahzad et al. 2018). On the other hand, the addition of this amino acid can mitigate the harmful effects of HMs in plants (Hayat et al. 2013; Shahid et al. 2014). Several roles have been attributed to proline in terms of ameliorating the phytotoxicity of HMs (Aslam et al. 2017), in particular the elimination of ROS, the achievement of redox homeostasis (Mourato et al. 2012), and the chelation of metal ions, and protein stabilization (Mishra and Dubey 2006).

Fig. 8 Schematic model showing the involvement of NO (nitric oxide) in the mitigation of Cu toxicity by enhancing enzymatic and non-enzymatic antioxidants, which might reduce photo-oxidative stress. APX, ascorbate peroxidase; Pro, proline; SOD, superoxide dismutase; CAT, catalase; GPOX, guaiacol peroxidase; MDA, malondialdehyde; LOX, lipoxygenase activity; MDAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; H₂O₂, hydrogen peroxide; OH•, hydroxyl radical; O₂^{•-}, superoxide anion; AsA, reduced ascorbate; DHA, oxidized ascorbate; GSSG, oxidized glutathione; GSH, reduced glutathione

5 Conclusion

The present investigation was performed in order to (1) better understand the NO-induced modulation of Cu toxicity, with special focus on the involvement of the AsA–GSH cycle, and to (2) test the role of nitric oxide (NO) in plant–heavy metal interactions. The addition of copper (CuCl₂) to the growth medium significantly reduced the growth, carotenoids, chlorophyll, and maximum photosystem II efficiency of plants. There was a strong increase in the contents of reactive oxygen species (ROS), and antioxidant enzyme activities associated with a decrease in levels of the non-enzymatic antioxidants glutathione (GSH) and ascorbate (AsA) as a result of CuCl₂ treatment. However, exogenous application of the NO donor of sodium nitroprusside (SNP) considerably counteracted the inhibitory effects of CuCl₂. On the one hand, NO reduced lipid peroxidation when applied with CuCl₂ or alone, and on the other hand, it decreased the activities of antioxidant enzymes while increasing the content of ascorbate and glutathione compared to CuCl₂ treatment in the absence of SNP (Fig. 8). In summary, we show that NO restores the cellular redox homeostasis, photosynthesis, and

antioxidant defense systems by reducing Cu-induced toxicity in the shoots of barley seedlings.

Acknowledgements The authors wish to thank Ms. Eileen Daly (University College Cork, Ireland), for her technical support and assistance.

Author Contribution MBM carried out the experiments and data analysis and wrote the manuscript. OK and YM helped in designing the experiment. AC and AW reviewed, improved the final version, and supervised the project. All authors approved the final manuscript.

Funding Open Access funding provided by the IReL Consortium. This work was supported by the Tunisian Ministry of Higher Education and Scientific Research (LR18ES38).

Availability of Data and Material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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