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Exogenous Nitric Oxide Confers Tolerance to Cr(VI) in Maize (*Zea mays* L.) Seedlings by Modulating Endogenous Oxido-Nitrosative Events

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Abstract

The use of exogenous compounds such as 'gasotransmitter' molecules is a well-established agronomic strategy to improve the crop tolerance to environmental stresses. In this current work, when Cr (200 µM) was combined with the nitric oxide (NO) generator sodium nitroprusside SNP, 500 µM) there was a suppression of metal-induced alterations in embryo growth. Exogenous NO produced by SNP reduced the accumulation of toxic hydrogen peroxide and methylglyoxal and stress-linked proline in Cr-treated seedlings. Chromium increased thiol and S-nitrosothiol levels but this was restored to control levels by SNP, in spite of a competing NO reaction leading to increase of *S*-nitrosoglutathione content. However, added complexity was indicated by addition of arginine analogue N (ω)-nitro-L-arginine methyl ester ($_L$-NAME, 500 µM) in the germinating medium to suppress endogenous NO production. This suppressed endogenous NO production but superoxide dismutase (SOD) was suppressed not enhanced in Cr + $_L$-NAME treatments. In addition, Cr + $_L$-NAME significantly decreased the content of spermidine and spermine in epicotyls as compared to Cr treatment alone. Similarly, exposure to Cr + SNP decreased spermidine and spermine levels in both radicles and epicotyls. This is important as polyamines have been suggested as route for NO production. Thus, our observations suggest that exogenous NO mitigates Cr-induced damage and confers seedling tolerance to Cr through suppression of NADPH oxidase activity and increased GSNO contents. This may act to prevent an excess of methylglyoxal and hydrogen peroxide. However, the reduction in polyamine mediated cellular NO generation could also promote increased viability under Cr stress.

Keywords Chromium · Maize · Nitric oxide · Nitrosative stress · Oxidative stress · Polyamines

Abbreviations

GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione reductase
$_L$-NAME	N_ω-Nitro-L-arginine methyl ester
MG	Methylglyoxal
NO	Nitric oxide
$O_2\cdot^-$	Superoxide radicals
Pas	Polyamines
Pro	Proline
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SNO	S-nitrosothiols
SNP	Sodium nitroprusside
Spd	Spermidine
Spm	Spermine

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Introduction

Environmental contamination with heavy metals can be hazardous to human health by entering the food chain via crop plants. Chromium (Cr) is one of the most toxic metal pollutants in the earth's crust. This transition metal is frequently generated by anthropogenic activities; metallurgy, electroplating, steel, leather and fertilizer production (Tóth et al. 2016). Chromium can adopt several oxidation states,

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but the trivalent Cr(III) and hexavalent Cr(VI) forms are the most dominant and stable in polluted soils. However, Cr(VI) is more toxic than Cr(III) due to its high solubility and mobility through cell membranes (Oliveira 2012; Wakeel et al. 2020).

In plants, excessive accumulation of Cr is harmful and affects germination, growth, development, and metabolism leading to cell death (Wakeel et al. 2020). As a redox active metal, Cr can catalyze the generation of reactive oxygen species (ROS) via Fenton reaction leading to oxidative damage. The reduction of Cr(VI) to Cr(III) is concomitant with the generation of superoxide radicals ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2) (Shahid et al. 2017). To cope with a cascade of negative reactions triggered by metal toxicity, plants have ROS scavenging systems, which include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX) (Shahid et al. 2017). The effects of heavy metal mediated ROS generation may be enhanced by the accumulation of methylglyoxal (MG); a potent cytotoxic compound (Nahar et al. 2016). In addition, high cellular levels of MG affects biological macromolecules and inactivates antioxidant defense systems (Mostofa et al. 2018).

Carbon monoxide (CO), hydrogen sulphide (H_2S) and nitric oxide (NO) are signalling molecules which modulate physiological processes in animals and plants (Hartsfield 2002; Rui 2010; Mukherjee and Corpas 2020). NO is a bioactive endogenous molecule with vital roles in germination, growth and tolerance to biotic and abiotic stresses (Per et al. 2017). NO biosynthesis can be formed via oxidative pathways from L-arginine or polyamines as "reductive pathways" via e.g. nitrate reductase. The oxidative pathway of NO is interconnected with various signalling molecules and metabolites that perform important functions in plant stress responses including polyamines (PAs) and proline (Pro) (Spormann et al. 2020). In plants, the existence of NO synthase (NOS)-like activity is still a controversial issue. PAs and NO share overlapping physiological functions in free radicals and ROS scavenging by activating cellular enzymatic and non-enzymatic systems which confers plant tolerance to metallic stress (Nahar et al. 2016). The PAs are known to act as an agent improving effects of NO (Asghari and Abdollahi 2013). Indeed, an over-accumulation of PAs in response to various environmental constraints can protect cellular structures, and detoxify free radicals, at least in part through the production of NO (Spormann et al. 2020).

The underlying mechanisms by which NO acts in plants to counteract environmental stress are frequently mediated by post-translational modifications (NO-PTMs) of proteins (Begara-Morales et al. 2016). In particular, protein S-nitrosation (also known as S-nitrosylation) is recognized as a key aspect of NO-based signalling. This is based on the reactiveness of NO and protein thiols to generate such S-nitrosothiols (SNO), and S-nitrosoglutathione (GSNO) (Groß et al. 2013; Mukherjee and Corpas 2020). GSNO is formed by the reaction of NO with GSH in the presence of oxygen and can serve as a mobile reserve of bioactive NO in plant cells (Corpas et al. 2019). Another role for GSNO is the S-nitrosylation of cysteine residues to influence a range of protein functions. The degree of protein nitrosation is affected by the cellular level of GSNO which is influenced by *S*-nitrosoglutathione reductase (GSNOR) activity. Nitrosation targets can include antioxidant enzymes such as peroxiredoxin IIE or ROS generating NAD(P)H oxidase and such events can influence cell death mechanisms (Yun et al. 2011).

Maize a monocotyledonous plant, is a staple food crop which has a very relevant agronomic and economic importance worldwide (Ranum et al. 2014). In certain areas, maize production could be limited by Cr contamination from source such as industrial production. To help in developing suitable Cr tolerant maize genotypes we aimed to characterize how NO could mitigate the adverse effects of Cr on maize plants. In particular, we here assess the mechanisms through which NO could modulate oxido-nitrosative events with Cr-stress. Based on our observations, we describe and complex interaction between endogenous and exogenous NO, which have effects on (i) the oxidative and nitrosative events, (ii) the suppression of toxic chemicals and (iii) the accumulation of polyamines that act to confer tolerance to Cr.

Material and Methods

Germination Conditions, Treatments and Growth Tests

Maize seeds (*Zea mays* L. cv Agrister), were surface sterilized with 2% (v/v) sodium hypochlorite for 10 min, rinsed and soaked in distilled water at 4 °C for 30 min. Then were germinated in glass petri dishes (23 cm in diameter, 30 seeds/petri dish) at 25 °C in the dark during 9 days between two sheets of filter paper moistened with 30 ml (every 3 days) of the following treatment solutions: (1) H_2O (control), (2) sodium nitroprusside (SNP; NO donor), (3) $Cr(K_2Cr_2O_7)$, (4) Cr + SNP and (5) Cr + N_ω-Nitro-L-arginine methyl ester ($_L$-NAME, an inhibitor of NO generation). The metal salt concentration was selected based on a previous study with *Zea mays* which showed that 200 µM of Cr caused > 50% inhibition embryonic axis growth (Kharbech et al. 2017). In all treatments, the concentration of SNP and $_L$-NAME was 500 µM, which was applied individually or combined with 200 µM of Cr. Harvesting was carried out daily from the third until the ninth day after germination.

The germination stage was selected as it is one of the most vulnerable to heavy metals. At harvest, the germinated seeds were peeled; then the embryonic axis was carefully separated from the cotyledon. This was sequentially washed twice in distilled water for 1 min before drying between two sheets of filter paper. The lengths of the embryonic axes (radicle and epicotyls separately) were measured using a ruler. The samples were weighed and then stored in liquid nitrogen.

Quantification of NO

The NO produced by the cells was estimated in vivo using Griess reagent (Kaur et al. 2015). The latter was composed of 2% (w/v) sulfanilamide and 0.2% (w/v) N-(1-naphthyl) ethylenediamine prepared within 5% (v/v) phosphoric acid solution. NO production from 1 g (fresh weight) of tissue was measured following the methods described by Vitecek et al. (2008). The absorbance of the Griess reagent was read at 540 nm after 15 min of capture of NO carried through the gaseous phase. The NO concentration (as oxidized NO_2^-) was determined from a calibration curve prepared using sodium nitrite as a standard.

Hydrogen Peroxide Determination

H_2O_2 content was evaluated at 390 nm according to the method of Sergiev et al. (1997). 1 g of fresh sample material was homogenized in 10 ml of TCA (0.1%) and then centrifuged at 12,000 × *g* for 15 min at 4 °C. The reaction mixture consists of 1 ml potassium iodide (1 M), 0.5 mL K-phosphate buffer (25 mM, pH 7.0) and the resulting supernatant. Hydrogen peroxide concentrations were calculated using a standard curve.

Protein Extraction, Thiol Levels and Assays of Enzyme Activities

Radicles and epicotyls of maize seedlings were homogenized in liquid nitrogen in Tris–HCl (100 mM, pH 8.0) extraction buffer containing 1 mM EDTA, DTT (5 mM), Triton X-100 (0.02%, v/v) and glycerol (10%, v/v). After centrifugation at 17,000 × *g* for 20 min at 4 °C, the resulting supernatants were recovered and aliquoted for protein, thiol content and enzymatic assays. Protein concentrations were determined using Bradford's (1976) method.

Thiol contents were measured according to the method of Ellman (1959, $\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$). The protein extract was added to DTNB (5–5′-dithiobis acid-2-nitrobenzoic); 4 mg/ml prepared in Tris–HCl (50 mM, pH 8). The reduction of DTNB was detected spectrophotometrically at 412 nm.

The activity of SOD (EC 1.15.1.1.1) in the supernatant was appraised at 490 nm according to the method described by Misra and Fridovich (1972). The reaction mixture contained 1.88 U/mL CAT, sodium carbonate/bicarbonate buffer (62.5 mM, pH 10.4), EDTA (125 µM) and protein extract.

NADPH oxidase activity (EC 1.6.3.1) was determined by monitoring NADPH oxidation, which expressed by the decrease in absorbance at 340 nm (Ishida et al. 1987). The reaction mixture was composed of sodium acetate buffer (100 mM, pH 6.5), $MnCl_2$ (1 mM), acid ρ-coumarate (0.5 mM), NADPH (0.2 mM) and enzyme extract. The activity was calculated using an extinction coefficient of $6.22\ mM^{-1}\ cm^{-1}$.

GSNOR activity (EC 1.2.1.1.1) was determined at 25 °C by following the oxidation of NADH at 340 nm (Barroso et al. 2006). The protein extracts were incubated in Tris–HCl (20 mM, pH 8.0) containing NADH (0.2 mM) and EDTA (0.5 mM). The enzymatic reaction was initiated by adding GSNO (0.4 mM) to the reaction mixture. Enzyme activity was expressed by using $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$.

Determination of S-Nitrosothiol (SNO) Content

Fresh samples were ground in the dark in a phosphate buffer (50 mM, pH 7.2) containing 80 mM S-methyl methanethiosulfonate (MMTS) (Gow et al. 2007) and centrifuged for 20 min at 20,000 × *g* at 4 °C. The supernatants were collected and added to chilled acetone a ratio of 3:1; chilled acetone/extract volume. This was incubated for 60 min at 24 °C and centrifuged again at 20,000 × *g* for 20 min at 4 °C. The supernatant was discarded and the pellets resuspended in the extraction buffer. The SNO content was estimated by the method of Saville (1958). Thus, 300 µL of the protein extract was incubated for 20 min with 300 µL of (3.4%, w/v) sulfanilamide (prepared in 0.4 M HCl) and 250 µL of (0.1%, w/v) N-(1-naphthyl) ethylenediamine, with or without (0.1%, w/v) $HgCl_2$. After incubation for 20 min at room temperature, the absorbance was measured at 540 nm using UV/Visible spectrophotometer, UV-3100PC. The difference in absorbance between samples treated and untreated with $HgCl_2$ was used to estimate the SNO content in the samples.

Liquid Chromatography–Electrospray/Mass Spectrometry (LC-ES/MS) Based Measurement of GSNO

For chromatographic measurement of GSNO, the method described by Airaki et al. (2011) was used with slight modifications. Samples (0.3 g) were ground in 1 mL HCl (0.1 M) and the homogenates were centrifuged at 21,000 × *g* for 20 min at 4 °C. The supernatants were collected and passed through 0.45 mm nylon filters, and immediately analyzed. All procedures were performed at 4 °C and protected from light to avoid potential degradation of GSNO.

Samples were assayed by LC-MS using a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific), coupled to an Accela ultra-high performance liquid chromatography (UHPLC) system (Thermo Scientific). Instrument control, data collection, analysis and management were controlled by the Thermo XCalibur 2.0.7 software package. Separation was performed using a Hypersil Gold (1.9 µm, 200 × 2.1 mm) RP-C18 column (Thermo Scientific). GSNO was separated using isocratic conditions using formic Acid (0.1%, v/v) in water for 8 min at 0.4 ml min^{-1}. This was followed by a methanol wash. The effluents from the HPLC were introduced into the mass spectrometer using an electro-spray ionisation (ESI) source. The capillary temperature was 215 °C and the vaporizer temperature was 250 °C. The sheath gas and auxiliary gas settings were 70 psi and 50 psi, respectively. The spray voltage was 4.0 kV. Argon gas (1.5) was in the collision cell. Mass spectrometric parameters were optimized by continuous infusion of 100 ppm of GSNO in 0.1 M HCl. Detection of all the compounds was performed in positive ionization mode. The quantification of the compounds was based on appropriate multiple reaction monitoring (MRM) of ion pairs, using the following transition: GSNO 337.110 > GSNO 232.186.

Targeted Metabolite Measurement by Flow Injection Electrospray High Resolution Mass Spectrometry (FIE-HRMS)

Samples (40 mg) were ground in the presence of 1 mL of the following mixture; chloroform–methanol-H_2O (1: 2.5: 1, v:v:v) at 4 °C. After stirring for 1 h at 4 °C, the homogenates were centrifuged at 5000 × *g* for 5 min at 4 °C. 100 µL of each sample was transferred in mass vials for flow infusion electrospray high-resolution mass spectrometry (FIE-HRMS) fingerprinting analysis. FIE-HRMS were performed using an Exactive HCD mass analyser equipped with an Accela UHPLC system (Thermo Fisher Scientific). Compounds were identified based on ratio mass-to charge (*m/z*); this generated metabolite fingerprints in positive–negative ionization mode, in a single run as described by Kharbech et al. (2020). Electro spray ionization (ESI) source parameters were set according to manufacturer's recommendations. For each ionization mode, mass spectra around the apex of the infusion peak were combined into a single intensity matrix (runs x *m/z*). Metabolomics data analyses used the R-based platform MetaboAnalyst 4.0. Data was normalized based on the percentage total ion count and then log_{10}-transformed from intensity matrix. Each nominal mass spectra (*m/z*) generated matches be a metabolite that was separated from different metabolites by virtue of their molecular mass. KEGG pathway database was used to metabolites annotation; the tolerance on the accurate mass for each mass-ion (*m/z*) was 3 ppm.

Statistical Analysis

The experiments were repeated four times ($n = 4$). The results were analysed by one-way analysis of variance (ANOVA) using Statistica ver. 7 software, with a 5% risk of type I error. Tukey's test was used to assess the degree of significance of the differences between the treatments. Values were given as means ± SE and significant differences were indicated by different letters ($p \leq 0.05$).

Results and Discussion

NO Protects Against Chromium Toxicity in Maize

Maize belongs to the list of plants available for phytoextraction technology, which has high metal accumulating ability (Chiwetalu et al. 2020). However, this ability can be increased if the metal toxic effects are counteracted. The use of exogenous materials such as 'gasotransmitter' molecules is a well-established agronomic strategy to improve the crop tolerance to environmental stresses. Sodium nitroprusside (SNP) is commonly used as an exogenous NO donor to strength crop tolerance against heavy metal (Kaur et al. 2015; Kaya et al. 2019; Kharbech et al. 2020). After 6 days of exposure, the toxic effects of Cr on maize seedlings were indicated by the reduction of radicle and epicotyl elongations reaching 59% and 35% of control respectively (Fig. 1a). After 9 days, Cr effects on cell elongation were more pronounced in radicles (− 70% of control) than epicotyls (− 49% of control). Similarly, radicle fresh weights were severely affected (− 63% of control) after 9 days of Cr treatment (Fig. 1b). These results aligned with those reported in *Zea mays* (Islam et al. 2016).

Under our experimental conditions, when applied alone, SNP (500 µM) did not significantly affect seedling growth (Fig. 1). However, when SNP was combined with Cr, any toxic effects were clearly suppressed (Fig. 1). Focusing on recovery indices, it was clear that, even in Cr-treated seedlings, SNP increased tolerance of radicles from 40 to 74% on day 6 and from 24 to 58% on day 9. In epicotyls, SNP also mitigated growth losses caused by Cr (Fig. 1). These data aligned with reports that NO (often added as SNP) is capable of mitigating the adverse effects of heavy metals stresses, (Per et al. 2017; Singh et al. 2020).

We hypothesized that SNP application could be acting to amplify an innate NO response to heavy metal in order to make it more effective. To confirm this, NO production was measured in epicotyls and radicles following various treatments using a Griess reagent-based assay (Fig. 2). In the control treatment, the slight NO production elicited over the 9-day measuring period noted is likely to be a sampling artefact. Addition of Cr increased the production

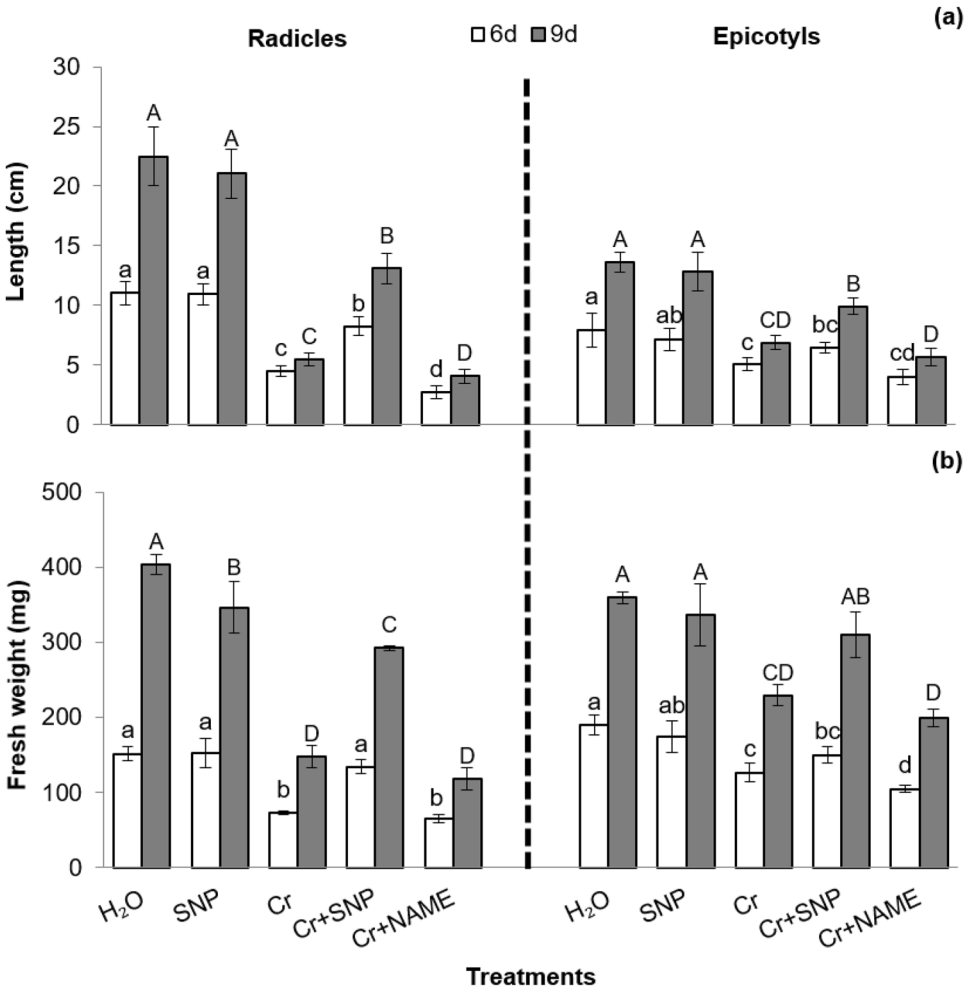

Fig. 1 Length (**a**) and fresh weight (**b**) of radicles and epicotyls of 6 and 9-day-old maize seedlings grown in the presence of H_2O (control), 500 µM SNP and 200 µM Cr(VI) individually or in combination with 500 µM SNP or $_L$-NAME. Values are given as the means ± SE of six replicates ($n = 6$). Bars followed by different letters indicate significant differences ($p \leq 0.05$). Lowercase and uppercase letters were used for 6 and 9-day-old seedlings respectively

of NO which was further increased with SNP and even more with SNP + Cr. These results could be linked with the effectiveness of exogenous NO application in improving growth processes and NO levels.

To indicate the effects of endogenous NO production on any anti-Cr action, the experiments were repeated in the presence of an inhibitor of NO biosynthesis: $_L$-NAME which acts by inhibiting nitric oxide synthase (NOS) in animal systems (Astier et al. 2018). The addition of $_L$-NAME effectively abolished the increase in NO previously seen with Cr alone (Figs. 2 and 3). Similarly, NO produced with Pb (Kaur et al. 2015) and Cr (Singh et al. 2020) stresses could confer tolerance but was quashed after $_L$-NAME addition (Phillips et al. 2018). In plants, NOS-like activity (if not NOS) has been widely involved in generating endogenous NO but $_L$-NAME could also be affecting several possible NO-generating pathways (Geng et al. 2019; Singh et al. 2020). This was not assessed in our study but the $_L$-NAME treatments clearly show a role for NO in Cr tolerance.

NO Affects Oxido-Nitrosative Metabolism in Responses to Chromium

Cr is one of the highly ecotoxic borderline metals. Cr(VI) stimulates ROS generation (Oliveira, 2012) and this was supported by this present study. Thus, Cr has considerably increased H_2O_2 levels by 268% and 237% respectively in both radicles and epicotyls (Fig. 4a). However, exogenously applied, SNP significantly attenuated H_2O_2 levels in both organs (Fig. 4a). Indeed, SNP/Cr co-administration could considerably alleviate Cr accumulation in maize radicles (about 30%; Kharbech et al. 2017). Equally, the ROS-inducing effect of Cr (VI) was clearly apparent when $_L$-NAME was combined with Cr (Fig. 4a). These data are fully in agreement with earlier studies with NO scavengers to reverse the protective effects of NO with resulting effect on both H_2O_2 generation and metal accumulation (Per et al. 2017; Kováčik et al. 2019).

Previous data have shown that NO-mediates up-regulation of antioxidant enzymes to detoxify H_2O_2 and $O_2{\cdot}^-$ which

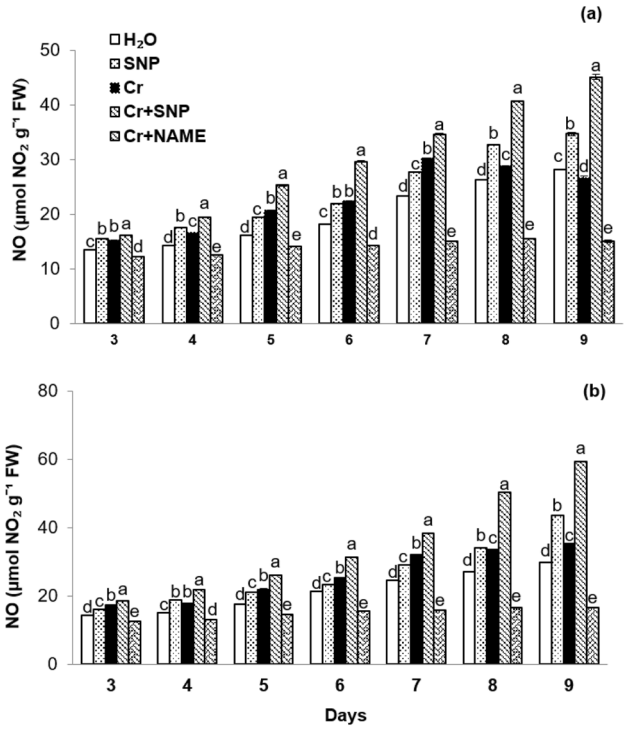

Fig. 2 Nitric oxide content in the radicles (**a**) and epicotyls (**b**) of maize seedlings treated with H_2O (control), SNP (500 µM) and Cr(VI) (200 µM) individually or in combination with SNP or $_L$-NAME (500 µM). Values were determined daily from the 3rd to the 9th day. Values are given as the means ± SE of four replicates ($n = 4$). Bars followed by different letters indicate significant differences ($p \leq 0.05$)

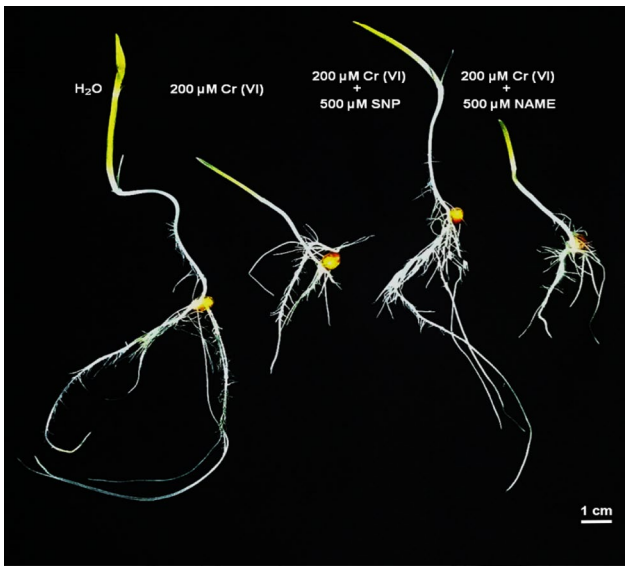

Fig. 3 The impact on phenotype in radicles and epicotyls of 9-day-old maize seedlings germinated and grown in the presence of H_2O (control), and 200 µM Cr(VI) individually or in combination with 500 µM SNP or $_L$-NAME

are generated following Cr treatments (Kharbech et al. 2017; 2020). To explore how far SNP could be acting to quench ROS through increased antioxidant enzyme activities and/or suppressing their generation, SOD and NADPH oxidase activities were assessed (Fig. 4). Under Cr stress, SOD activity was increased by 64% and 26% in radicles and epicotyls, respectively and greatly augmented in both organs with SNP (e.g. 2.5 fold increase in radicles compared to Cr-treated only) (Fig. 4b).

Endogenous metal accumulation may affect antioxidant defenses. In this context, Kharbech et al. (2017) reported that Cr accumulation in maize seedling radicles elevated catalase, glycolate oxidase, and peroxidase activities. Given, $O_2{\cdot}^-$ is recognised as a precursor for H_2O_2 which can be formed by SOD so that the up-regulation of SOD (Fig. 4b) may contribute to Cr-increased H_2O_2 levels (Fig. 4a). Several previous investigations were demonstrated that NO supply might trigger the endogenous NO synthesis and quench ROS by stimulating the antioxidant enzymes system especially under stress conditions (Kaur et al. 2015; Nahar et al. 2016; Kaya et al. 2019). However, this could reflect an effect of endogenous NO production since Cr + $_L$-NAME treatment reduced SOD activities more than 35% compared to Cr in both radicles and epicotyls. Indeed inhibition of antioxidant enzymes, including SOD, is a widely accepted consequence of $_L$-NAME treatment (Phillips et al. 2018; Singh et al. 2020), which suggest that NO is essential for strengthening antioxidant defence systems. These changes need not associated to direct inhibitory effects of $_L$-NAME but can seem likely to be a consequence of a shift in endogenous NO levels. NO can directly or indirectly react with pro- and antioxidants eventually by regulating the expression of pro- and anti- oxidant enzyme activities (Groß et al., 2013).

The activity of NADPH oxidase was boosted by Cr more than 2.7 and fourfold in both radicles and epicotyls, respectively (Fig. 4c). Chromium elicited NADPH oxidase activity was significantly suppressed following SNP treatment (Fig. 4b). Unlike with SOD, suppression of endogenous generation of NO using $_L$-NAME had no significant effect on this activity. This agreed with our recent finding (Kharbech et al. 2020) that NADPH oxidase activity, as opposed to the induction of antioxidant enzymes, are a source of exogenously applied SNP –mediated Cr tolerance (Figs. 1 and 3). Arasimowicz-Jelonek et al. (2012) observed a similar response of NADPH oxidase in relation in lupine roots treated with 2–4-Carboxyphenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) under Cd conditions. In addition, Ding et al. (2009) found that pretreatment with $_L$-NAME and DMTU (dimethylthiourea, a suppressor of H_2O_2 generation) abolished signalling transduction of mitogen-associated protein kinases triggered by NO and ROS which to affect Cr (VI) toxicity in maize roots. In plant immunity; S-nitrosylation of NADPH oxidase has

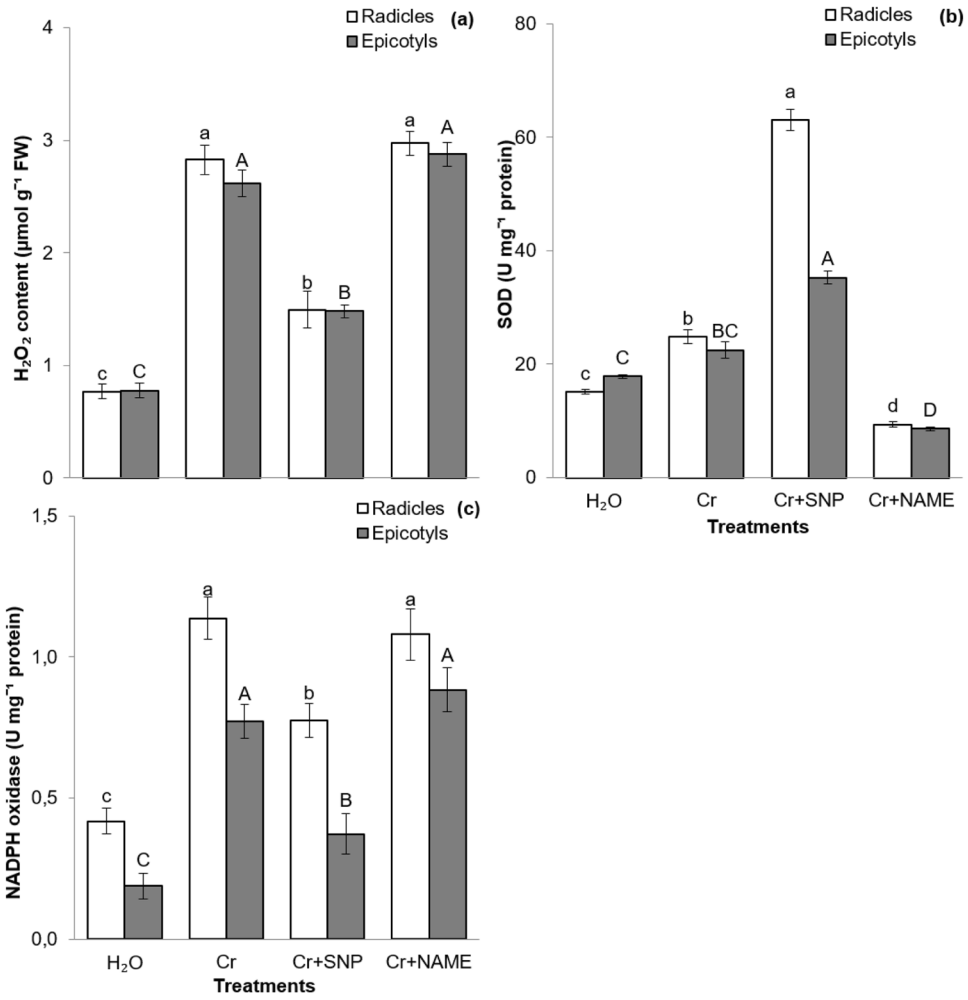

Fig. 4 Hydrogen peroxide content (**a**) and activities of superoxide dismutase (**b**) and NADPH oxidase (**C**) in radicles and epicotyls of 9-day-old maize seedlings treated with H_2O (control) and Cr(VI) (200 µM) individually or in combination with SNP or $_L$-NAME (500 µM). Values are given as the means ± SE of four replicates ($n = 4$). Bars followed by different letters indicate significant differences ($p \leq 0.05$). Lowercase and uppercase letters were used for radicles and epicotyls respectively

been shown to limit its activity (Yun et al. 2011) and a similar mechanism could be acting with Cr/SNP treatment.

SNP Influences GSNO in Response to Chromium

Total protein -SH is often used as a marker of protein oxidation state under metallic stress which reflects the chemical affinity between thiols and metal ions (Woolhouse, 1983). Quantitative changes in protein -SH were observed in bean, chickpea and pea treated with Cd or Cu (Sakouhi et al. 2016; Ben Massoud et al. 2018). Such data exhibit a major capacity of plants to protect the redox status of protein thiols in response to heavy metal. Chromium was found to induce a significant increase of -SH levels in epicotyls which was reduced by 46% with SNP, but not with $_L$-NAME (Fig. 5a). However, the lower levels of –SH seen with SNP treatment could reflect the outcome of S-nitrosylation.

Protein S-nitrosation consists of the covalent addition of a NO group to the -SH leading to SNO generation and consequently might modify the function of a broad spectrum of proteins (Barroso et al. 2006). S-nitrosation is reversible and selective NO-PTMs that might also highly interfere with cell signalling mechanisms (Corpas et al. 2019). Endogenous SNO reduction is substantially influenced by varying processes including transnitrosylation and denitrosylation. Glutathione and thioredoxin (Trx), and their associated redox systems are particularly involved in cellular redox homeostasis and signalling (Benhar, 2015). Thioredoxin/thioredoxin reductase systems is increasingly recognized as a 'denitrosylases' as well as GSNOR activity; thus influencing the cellular balance between nitrosylation and denitrosylation (Begara-Morales et al. 2016). Cr greatly stimulates S-nitrosylation, which is lowered by combination with SNP (Fig. 5b). In contrast, under stress, $_L$-NAME did not significantly affect SNO levels compared to Cr and Cr + SNP treatments. Thus, $_L$-NAME treatment reduced NO levels (Fig. 2) and was linked to low levels (equivalent to controls) of SNO under Cr toxicity (Fig. 5b). Thus, Cr-increased SNO reflect the effects of endogenous NO production. This clearly does not equate to a mitigatory mechanism (Figs. 1 and 3) and so that the effects of SNP cannot be clearly linked to S-nitrosylation events despite a high availability of free NO (Fig. 2).

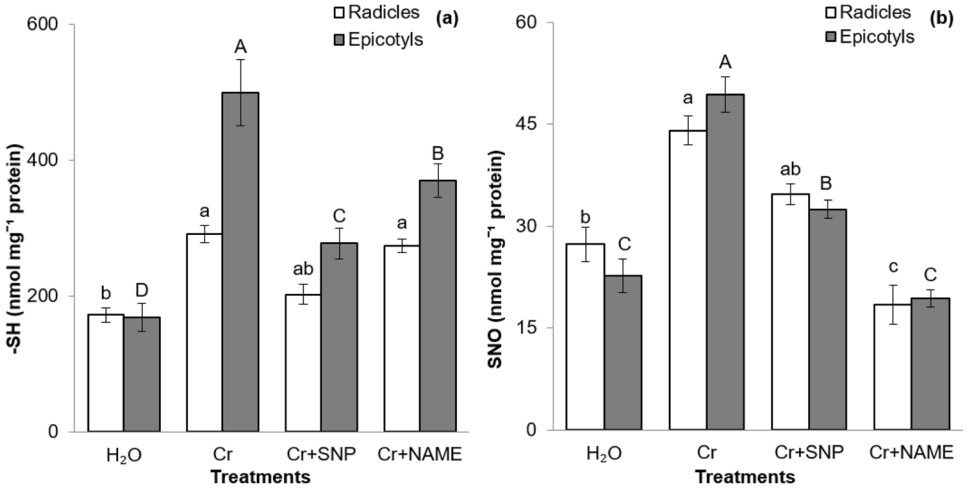

Fig. 5 Thiol (**a**) and *S*-nitrosothiol (**b**) in radicles and epicotyls of 9-day-old maize seedlings germinated in the presence of H_2O (control) and Cr (VI) (200 µM) individually or in combination with SNP or $_L$-NAME (500 µM). Values are given as the means ± SE of four replicates ($n = 4$). Bars followed by different letters indicate significant differences ($p \leq 0.05$). Lowercase and uppercase letters were used for radicles and epicotyls respectively

In maize seedlings treated with Cr + SNP, the increase of NO contents (Fig. 2) induced a significant nitrosylation of GSH leading to an almost two fold increase in GSNO levels in the epicotyls; although this occurred to a lesser extent in radicles (Fig. 6a). Besides acting as a mobile reservoir of bioactive NO, GSNO also exists in a balance with S-nitrosylated proteins through a trans-nitrosylation process (Corpas et al. 2019). In Cr-treated seedlings, cellular levels of NO were near to the control (H_2O) and even less in radicles at the 9th day (Fig. 2). After Cr application, a large amount of NO can interact with -SH to generate SNO (Fig. 5b). With Cr treatment, low levels of GSNO were detected and correlated with increasing activity of GSNOR (Fig. 6). GSNOR activity appeared to be elevated with Cr treatment (Fig. 6b) which correlated with the reduction in GSNO (Fig. 6a) and increase in SNO content (Fig. 5b). However, with co-treatment Cr + SNP, maize seedling maintained their high concentration of GSNO (Fig. 6a), with little appreciable change in GSNOR activity as compared to treatment with Cr alone (Fig. 6b). The importance of GSNO suggests the question of how this acts to confer SNP-induced tolerance to Cr. Given the reduction in ROS and RNS levels may reflect the result of NADPH oxidase activity reducing, GSNO increase could be acting as a "sponge" to soak up excess NO to prevent wide-ranging impacts on protein function via S-nitrosylation. The increase in GSNOR activity, concomitantly with reduction in GSNO cell reserve, was related to the abnormal decrease in NO content caused by the inhibitory effect of $_L$-NAME with Cr (Fig. 2). He et al. (2018) have revealed the same behaviour in plants under various stress conditions.

The GSNOR activity protects against nitrosative stress by affecting the degree of nitrosylation on both GSH and other substrates. The presence of -SH in GSH makes this molecule a redox switch of proteins facilitating their regulation through processes such as S-glutathionylation and S-nitrosylation (Groß et al. 2013). Thus, current data suggests that maize seedlings exposition to Cr can enhance the catabolism reaction of GSNO possible to: (i) to compensate for a NO

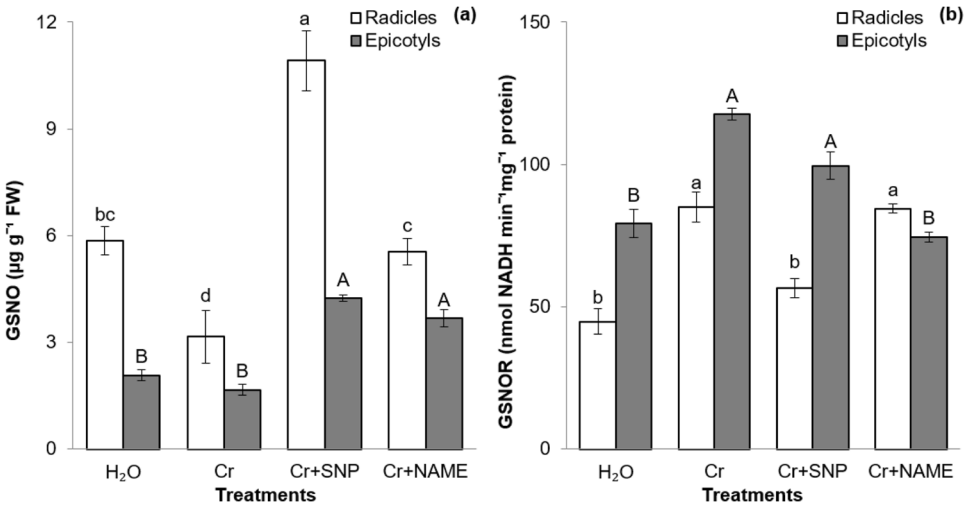

Fig. 6 *S*-nitrosoglutathion (**a**) contents and *S*-nitrosoglutathion reductase activity (**b**) in radicles and epicotyls of 9-day-old maize seedlings germinated in the presence of H_2O (control) and Cr(VI) (200 µM) individually or in combination with SNP or $_L$-NAME (500 µM). Values are given as the means ± SE of four replicates ($n = 4$). Bars followed by different letters indicate significant differences ($p \leq 0.05$). Lowercase and uppercase letters were used for radicles and epicotyls respectively

deficiency resulting from the ROS and/or –SH interaction, (ii) to boost antioxidants enzymes such as SOD and (iii) to generate endogenous GSH (Corpas et al. 2019). Additionally, GSNOR plays a key role in preventing or reversing nitrosylation of varying thiols of proteins, thus indirectly controlling SNO levels. It is also plausible that there is crosstalk between NO and thiol, which involve GSH/GSNOR and Trx/TrxR systems. If this were the case, this is interaction should be investigated in the future. The downregulation of GSNOR activity by SNP could indicate that the denitrosylation processes of SNO was mediated more by the Trx/TrxR than GSH/GSNOR.

SNP-Derived NO Influences Cr-Elicited Stress Metabolites

Beyond effects on oxido-nitrosative stress, we also considered additional mechanisms through which SNP acts to confer Cr tolerance. In one, the possibly of SNP derived NO affected MG accumulation which is formed in plants as one result of metal toxicity. In maize seedlings, Cr induced MG accumulation in both radicles and epicotyls (Figs. 7a and b). In radicles, this could be minimized by 23% with SNP but achieved controls levels with $_{L}$-NAME (Fig. 7a). This could suggest that the MG increase was dependent on NADPH oxidase, ROS and endogenous NO production which could not countered by exogenous SNP effects. The over-accumulation of MG can cause the deterioration of different physiological and metabolic processes (Mostofa et al. 2018). Previous studies have established that NO is a major actor in MG detoxification in heavy metal contaminated plants, but either directly by improving the glyoxalase system (Nahar et al. 2016; Kharbech et al. 2020) or indirectly by quenching the ROS production by activating the antioxidant defence systems as we also noted in our investigation (see SOD activity, Fig. 4b).

In both radicles and epicotyls of maize, increased levels of Pro were registered in response to Cr (Figs. 7c and d). Co-addition of SNP with Cr significantly reduced Pro accumulation relatively with NO contents (more than 50% with SNP compared to Cr alone). These results suggest that SNP, i.e. high amounts of NO, but not endogenous levels affected Cr-induced accumulation of Pro in epicotyls. A minor effect could be occurring in radicles and indicated by $_{L}$-NAME (Fig. 7c and d). Proline accumulation patterns could reflect wider effects of Cr on amino acid/ amine metabolism. The production of high amounts of Pro in plants is a typical non-enzymatic response to several biotic and abiotic constraints (Shahzad et al. 2018). Proline has been linked to several roles against metal toxicity including ROS removal, redox homeostasis, metal ion chelation and protein stabilization (Aslam et al. 2017). Kováčik et al. (2019) reported that NO intensifies accumulation of Pro. Such wider impacts have been noted in NO effects on proline and polyamines (PAs) metabolism (e.g. Wang et al. 2020).

In plants, the most common free PAs are Spd and Spm as well as putrescine (Asghari and Abdollahi, 2013; Spormann et al. 2020). Chromium was found to increase the accumulation of PAs in both radicles (2 and 5 times, compared to control, for Spd and Spm, respectively) (Fig. 8a

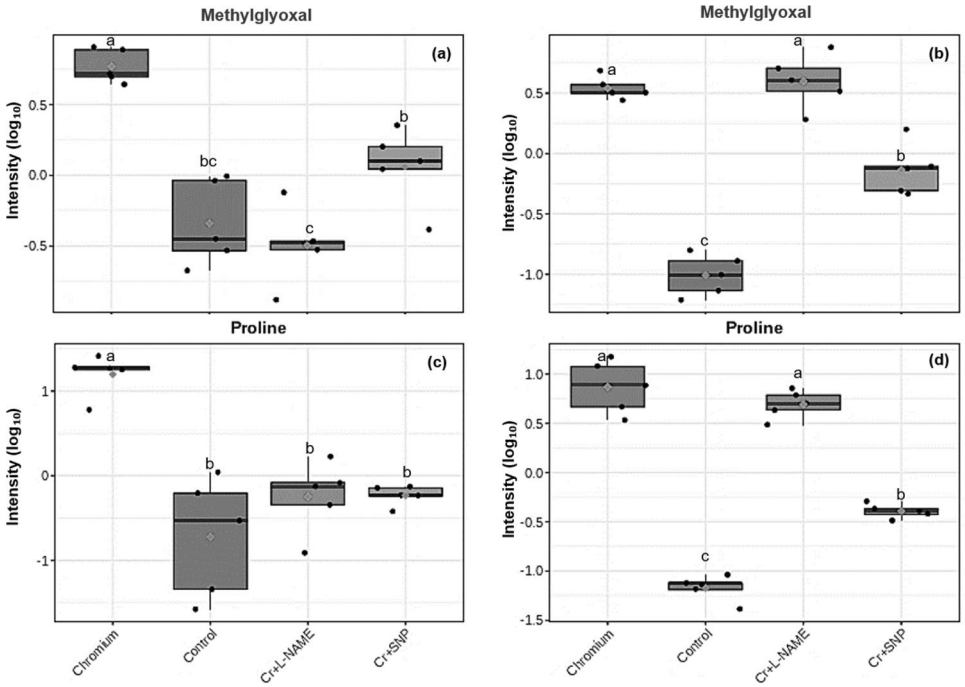

Fig. 7 Accumulation of methylglyoxal (**a**, **b**) and proline (**c**, **d**) in radicles (**a**, **c**) and epicotyls (**b**, **d**) of 9-day-old maize seedlings treated by H_2O (control), and Cr(VI) (200 µM) individually or in combination with SNP or $_{L}$-NAME (500 µM). Values are given as the means ± SE of six replicates ($n = 6$). Bars followed by different letters indicate significant differences ($p \leq 0.05$). Log_{10} normalisation using logarithmic transformation of data

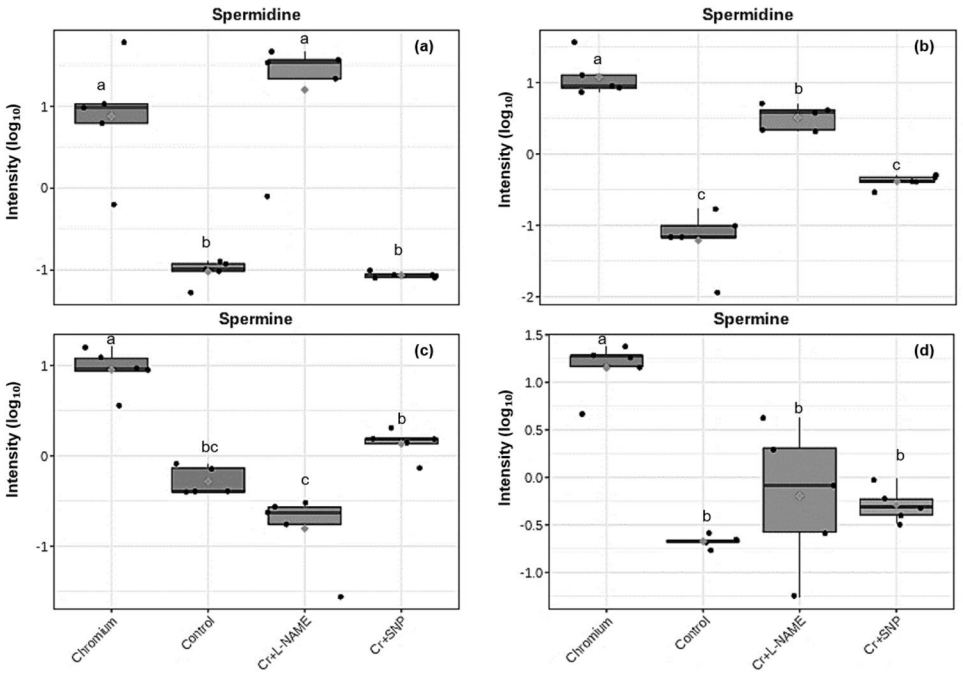

Fig. 8 Accumulation of spermidine (**a, b**) and spermine (**c, d**) in radicles (**a**, **c**) and epicotyls (**b**, **d**) of 9-day-old maize seedlings treated by H_2O (control), and Cr(VI) (200 µM) individually or in combination with SNP or $_L$-NAME (500 µM). Values are given as the means ± SE of six replicates ($n = 6$). Bars followed by different letters indicate significant differences ($p \leq 0.05$). Log_{10} normalisation using logarithmic transformation of data

and c) and epicotyls (430%, compared to control, for Spd as an example) (Fig. 8b). However, SNP addition suppressed Cr-increased Spd and Spm levels in epicotyls and particularly in the radicles (Fig. 8). This would indicate that SNP-mitigation of Cr toxicity does not involve enhancement of PAs. Therefore, we hypothesize that the higher accumulation of endogenous NO may limit NO biosynthesis pathways mediated by PAs. In contrast, Groppa et al. (2008) reported that exogenous NO enhances PAs accumulation and *visa-versa* in Cd-stressed plants. In line with this, Nahar et al. (2016) suggest an important cross-protection mechanism between NO and PAs to modulate the antioxidant defence systems in Cd-stressed bean plants.

The inhibitor $_L$-NAME was used to confirm the involvement of NO in the PAs metabolism. Cr + $_L$-NAME treatments led to a decrease in Spm levels which further exacerbated with Cr + SNP in radicles (Fig. 8c) and slightly accumulated in epicotyls (Fig. 8d) as compared with other treatments. By contrast, Spd showed higher amounts (more than 7 and 1.8 × fold in radicles and epicotyls, respectively, as compared to its endogenous levels under Cr + SNP; Fig. 8a and b). This PAs misbalance between Spd and Spm may be connected to the lower NO levels by the addition of $_L$-NAME. In our case, PAs may play a dual role; as antioxidant compounds and controller of NO biosynthesis. A similar finding was reported by Wang et al. (2020); PAs concentration in tea roots showed different trend by applying exogenous NO scavenger and inhibitor. Based on these results we can assume an existing relationship between the biosynthesis of NO and its intracellular amount. This balance should be maintained to avoid NO toxicity effects (Fig. 9).

Conclusion

In maize seedlings, the NO-induced Cr toxicity alleviation could involve (i) the suppression of NADPH oxidase generated $O_2 \cdot^-$ in order to reduce oxido-nitrosative stress, (ii) the attenuation of H_2O_2, MG and Pro accumulation, and (iii) the build-up of cellular GSNO pool acting to prevent cellular disruption through de-regulated protein S-nitrosylation. NO may regulate its own cellular levels by modulating PAs production (one of the several NO-generating pathways in plants) and preventing GSNO decomposition (considered as a NO reservoir in cells). 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.

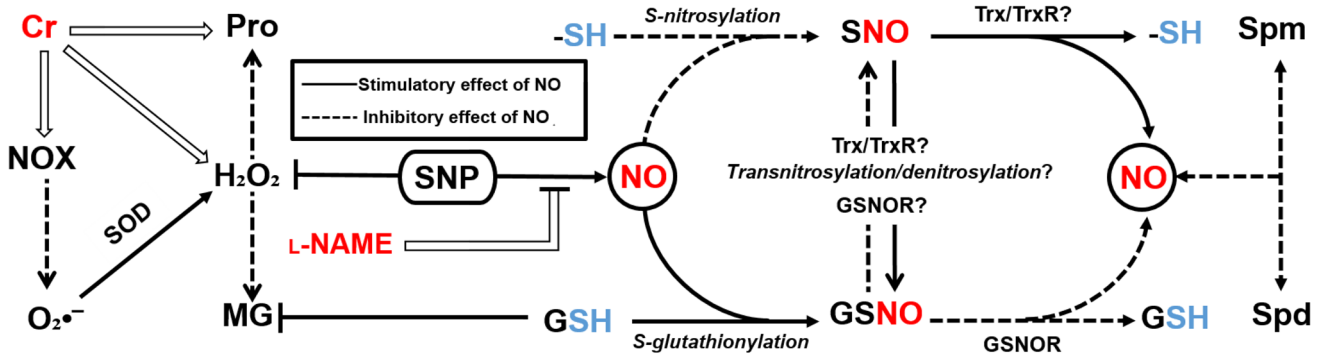

Fig. 9 Schematic diagram representing the protective mechanisms mediated by nitric oxide (NO−) in maize seedlings under Cr stress. Chromium (Cr) affects seedling growth through the production of hydrogen peroxide (H_2O_2), methylglyoxal (MG) and proline (Pro). Sodium nitroprusside (SNP) application improves NO cellular levels, which counteracts anion superoxide ($O_2\cdot^-$) accumulation by enhancing superoxide dismutase (SOD) activity and suppressing NADPH oxidase (NOX) activity. Nitric oxide, has particular importance in the regulation of S-nitrosylation processes by decreasing the S-nitrosothiols (SNO) levels but S-nitrosoglutathione reductase (GSNOR) appears to not greatly modulate SNO levels. As a result, denitrosylation is probably mediated by the thioredoxin (Trx)/thioredoxin reductase (TrxR) system rather than GSNOR. The beneficial effects of NO were not linked to polyamines (PAs) production despite it being one of the several NO-generating pathways in plants by involving L-arginine. However, spermidine (Spd) and spermine (Spm) levels were reduced by SNP application, which might be a feedback mechanism to modulate NO production. This was supported by the addition of $_L$-NAME which can reverse the protective mechanisms of SNP by reducing endogenous NO levels (Color figure online)

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Author Contributions OK performed the experiments and wrote the manuscript. MBM helped in data analysis. AC helped in designing the experiment. LAJM and WD revised and improved the final version. All authors approved the manuscript.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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