Research article

Nitric oxide alleviates cadmium- but not arsenic-induced damages in rice roots

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ABSTRACT

Nitric oxide (NO) has signalling roles in plant stress responses. Cadmium (Cd) and arsenic (As) soil pollutants alter plant development, mainly the root-system, by increasing NO-content, triggering reactive oxygen species (ROS), and forming peroxynitrite by NO-reaction with the superoxide anion. Interactions of NO with ROS and peroxynitrite seem important for plant tolerance to heavy metal(oid)s, but the mechanisms underlying this process remain unclear. Our goal was to investigate NO-involvement in rice (Oryza sativa L.) root-system after exposure to Cd or As, to highlight possible differences in NO-behaviour between the two pollutants. To the aim, morpho-histological, chemical and epifluorescence analyses were carried out on roots of different origin in the root-system, under exposure to Cd or As, combined or not with sodium nitroprusside (SNP), a NO-donor compound. Results show that increased intracellular NO levels alleviate the root-system alterations induced by Cd, i.e., inhibition of adventitious root elongation and lateral root formation, increment in lignin deposition in the sclerenchyma/endodermis cell-walls, but, even if reducing As-induced endodermis lignification, do not recover the majority of the As-damages, i.e., enhancement of AR-elongation, reduction of LR-formation, anomalous tissue-proliferation. However, NO decreases both Cd and As uptake, without affecting the pollutants translocation-capability from roots to shoots. Moreover, NO reduces the Cd-induced, but not the As-induced, ROS levels by triggering peroxynitrite production. Altogether, results highlight a different behaviour of NO in modulating rice root-system response to the toxicity of the heavy metal Cd and the metalloid As, which depends by the NO-interaction with the specific pollutant.

1. Introduction

Nitrogen monoxide (nitric oxide – NO) is a ubiquitous gaseous molecule involved in numerous animal and plant physiological processes, and it is also a mediator of plant development and response to abiotic/biotic stresses. Different environmental stresses rapidly induce NO-production, which, in turn, participates to the regulation of the plant responses. Several researches have highlighted the involvement of NO in the regulation of plant response to toxic elements, including cadmium (Cd) and arsenic (As) pollutants (Kopyra et al., 2006; Singh et al., 2017).

The NO-involvement in plant physiological/metabolic processes is due to its capability to modify numerous proteins, either directly through post-translational mechanisms, such as S-nitrosylation, nitration and nitrosylation, or indirectly by controlling the transcription of genes that encode proteins involved in stress responses (Fancy et al., 2017).

Various reports highlight that NO has an important role in reducing the damages in plant organs due to abiotic stresses by enhancing the activity of antioxidant enzymes. However, its role in the physiological processes depends on its cellular level. Indeed, at very low levels it functions as a signal molecule, on the contrary at higher levels becomes a stress-inducing molecule (Fancy et al., 2017).

Nitric oxide is a reactive nitrogen species (RNS) easily reacting in

the cell with a wide range of proteins, as transcription factors, messengers or receptors, but mainly with the reactive oxygen species (ROS). These last molecules are also induced by almost all abiotic stresses, including metal/metalloid stresses. One of the fastest reactions taking place in biological systems occurs between NO and the superoxide anion ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$) (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Corpas and Barroso, 2015). Peroxynitrite, an unstable anion belonging to the RNS family, was identified as a mediator of cell death in animal cells at the end of the last century. Only later it was demonstrated to have a positive role in the regulation of cell signalling transduction pathways, principally by post-translational modification of proteins through tyrosine nitration (Vandelle and Delledonne, 2011). Moreover, the $ONOO^-$ synthesis has been reported to reduce the cellular NO levels (Wulff et al., 2009). Now, it is well known that $ONOO^-$, at low levels, is not toxic for plant cells, but an increase in its cellular levels is associated with various stress responses, such as in Arabidopsis roots exposed to Cd (Corpas and Barroso, 2014). Similarly, it is possible that $ONOO^-$ levels may be enhanced in plants exposed to As, considering that the metalloid triggers NO and ROS levels in numerous plant species, including rice (Singh et al., 2017).

Cadmium and As soil pollution is of great concern because it prevents plant development by altering primary metabolic functions, by decreasing water and mineral nutrient uptake, and by inducing a general alteration in organ development, mainly in the root-system. Besides, the presence of Cd and/or As in the soil compromises the commercial value of the edible crops, and represents a potential risk to human health. Cadmium is present in the soil mainly as Cd^{2+}. It easily enters in the root cells using the transporters of the essential nutrients, thus competing with them, or through aquaporins (Przedpelska-Wasowicz and Wierzbicka, 2011). Arsenic is mostly present in the environment in two inorganic forms: arsenite [As(III)], and arsenate [As(V)] (Zhao et al., 2009). Organic forms are also possible. Arsenate, being an analogue of phosphate, enters the plant cells by the inorganic phosphate transport system (Meharg and MacNair, 1992), whereas arsenite uses the aquaporins of NIP subfamily (Bienert et al., 2008). In the root cells, As(V) is easily reduced to As(III) (Meharg and Hartley-Whitaker, 2002), with this reaction contributing to increase the cytosolic levels of ROS (Abbas et al., 2018).

The most serious plant damage caused by the toxic elements, which frequently characterize polluted soils, occurs in the root-system affecting all its components. Indeed, high Cd and As concentrations, reduce primary root (PR) elongation by altering stem cell niche definition and differentiation pathways, and by modifying lateral (LR) and adventitious (AR) root formation (Fattorini et al., 2017; Ronzan et al., 2018). Thus, the root-system is the first part of the plant which must display strategies to defend the rest of the organism from the pollutants (Fattorini et al., 2017; Ronzan et al., 2018).

Plants may activate defence strategies at molecular, cellular and physiological levels to cope with toxic elements. In any case, these mechanisms involve a complex signalling network that must guarantee perception and transmission of the stress signals for subsequently start a plethora of defence responses. Plants defend themselves from metal/metalloid toxicity either by keeping under control the cellular levels of free ions through exclusion, immobilization, chelation with binding thiol-peptides and vacuolar compartmentalization (Clemens et al., 2002; Tuli et al., 2010), or by reprogramming the entire plant metabolism to ensure to the cells to re-establish redox homeostasis (Farnese et al., 2016, and references therein).

In the last few decades, numerous studies have indicated that the interaction between RNS, in particular NO, and ROS is essential for plant tolerance to heavy metals/metalloids, however the mechanism on the base of this process remains unclear. In fact, based on their levels in the cells, ROS and NO may cause an oxidative/nitrosative stress or may act as signalling molecules (Farnese et al., 2016 and references therein).

Plants exposed to Cd toxicity can modify NO metabolism; however,

contradictory results have been reported regarding the impact of Cd^{2+} on endogenous NO production (Besson-Bard et al., 2009, and references therein). In fact, Cd can either increase NO levels, as reported in various plant species (Corpas and Barroso, 2014), or inhibit them (Rodríguez-Serrano et al., 2006). Moreover, exogenous treatments with a NO donor were shown to protect plant organs against the oxidative damage triggered by Cd^{2+}, by promoting ROS scavenging (Kopyra et al., 2006; Noriega et al., 2007).

It is also known that As, either as As(V) or As(III), provokes alterations in NO metabolism in various plants, with a significant increase in NO content, determining a nitro-oxidative stress (Singh et al., 2017; Rodríguez-Ruiz et al., 2019). Moreover, exogenous NO supplementation seems to limit As-induced damages in rice exposed to As(III) (Singh et al., 2017).

It is thus evident that NO has a crucial role in the regulation of biological processes activated by plants in response to metal(oid) toxicity, however the exact mechanism of NO signalling is still unclear.

One of the most widely used methods to supply NO to the plants, independently of their growth in soil, synthetic media or hydroponic systems, is the treatment with the NO donor compound sodium nitroprusside (SNP). It has been reported that rice plants exposed to Cd show an increased tolerance to heavy metal when supplied with SNP (Xiong et al., 2009). Similarly, the exogenous application of NO, through SNP treatment, alleviates As-induced toxicity in germinating seeds of mung bean (Ismail, 2012).

In light of literature information, the aim of the research was to deepen the knowledge on the involvement of NO in the organization of the rice root-system after exposure to Cd or As, investigating whether the behaviours of NO and of the NO-derived compounds peroxynitrite and superoxide anion, were different in the presence of either the heavy metal or the metalloid.

The results highlight that the exogenous treatments with the NO-donor SNP decreased the uptake of both Cd and As, enhance the NO-levels when combined with Cd or As. The enhanced cellular NO-content alleviates morphological and histological damages induced in the roots by Cd, but not those due to As. Finally, exogenous NO differently affects ROS and RNS cell balance in the roots exposed to either Cd or As.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *Oryza sativa* ssp. Japonica (cv. Nihonmasari) were surface sterilized with ethanol 70% (v/v) for 1.30 min, rinsed three times with Milli-Q water, soaked in a solution of 40% (v/v) sodium hypochlorite (5–9% active chlorine, Carlo Erba Reagents S.R.L., Milano, Italy) for 25 min, and again rinsed in sterile ultra-pure water for three times. Then, the seeds were sown in 11.4 × 8.6 × 10.2 cm phytatray-type vessels (Phytatray™, Sigma-Aldrich, Saint Louis, MO, USA) containing a half-strength Murashige and Skoog (MS, 1962), 0.1% sucrose and 0.8% agar, at pH 5.6–5.8 (Control medium). Media with the same composition of the Control medium, but containing either 25 μM NaAsO$_2$ [As(III) medium], or 100 μM Na$_2$HAsO$_4$·7H$_2$O [As(V)], or 100 μM CdSO$_4$, (Cd), with either or the NO donor Na$_2$[Fe(CN)$_5$NO]·2H$_2$O [sodium nitroprusside dehydrate (SNP), Sigma –Aldrich] at 50 μM, or the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [(cPTIO), Sigma-Aldrich] at 100 μM, were also prepared. Combined treatments with each pollutant with SNP and/or cPTIO, at the concentrations as above were also prepared. Ultra-pure water (Milli-Q) was used for all culture media, and the most of compounds were added before of autoclaving at 1 atm and 120 °C for 20 min.

Sterile water solutions of 50 μM SNP and 100 μM cPTIO were prepared by filtering (with a 0.22 μm pore filter) and added to the autoclaved media, when the temperature had reached a value of 45–50 °C.

The compounds and concentrations of As(V) and Cd were selected based on our previous data (Ronzan et al., 2018, 2019), while those of

As(III) and SNP were chosen based on preliminary experiments showing that NaAsO$_2$ or SNP concentrations higher than 25 μM and 50 μM, respectively, induced strong damages to the entire plant (Figs. S1 and S2), and concentrations of SNP lower than 50 μM, combined with CdSO$_4$ or NaAsO$_2$, did not induce evident morphological modifications with respect to the treatments with the pollutants only.

Thirty three seeds per treatment were sown on the media and kept in long-day conditions (14 h light/10 h dark) for 10 days at 210 μmolm^{-2}s^{-1} intensity of white light and 27 °C. No refresh of the medium was carried out during the culture period.

2.2. Morphological and histochemical analyses

After the growing period, the root-system of 30 seedlings per treatment was collected and analysed by measuring the mean fresh weight, the mean number and length of the embryonic crown roots, i.e., the embryonic adventitious roots (ARs), and the mean density of LRPs plus LRs.

The length and the number of the ARs were measured under a LEICA MZ8 stereomicroscope using Zeiss Zen 2.3 software from digital images captured with Zeiss AxioCam camera. The LRs were counted with a Leica DMRB optical microscope equipped with a Leica DC 500 camera, and the corresponding mean density expressed as mean number cm^{-1} (± SE).

To visualize lipid peroxidation a histochemical analysis was performed with the Schiff's reagent (109033 Merk Millipore) as described by Yamamoto et al. (2001). Briefly, the LRPs and LRs non-exposed (Control), or exposed to As(V), As(III) or Cd, combined or not with SNP, were stained with the Schiff's reagent for 60 min until red colour appeared. Then the roots were rinsed with a freshly prepared sulphite solution [0.5% (w/v) K$_2$S$_2$O$_5$ in 0.05 M HCl (Sigma-Aldrich)] to remove the extra stain, kept in a chloral hydrate solution (Cl$_3$CCH(OH)$_2$, Sigma-Aldrich, Saint Louis, MO, USA) (Weigel and Glazebrook, 2002), and observed with Nomarski optics applied to a LEICA DMRB microscope, equipped with a LEICA DC 500 camera. The image analysis was performed using LEICA IM1000 Image Manager Software (LEICA).

2.3. Elemental analysis

Thirty three seeds per treatments were sown in the absence or presence of SNP and in the absence or presence of Cd, As(III) or As(V), alone or combined with SNP. At day 10, the seedlings were harvested, desorbed in 20 mM Na$_2$-EDTA for 15 min to remove apoplastic Cd and As, and washed thoroughly three times with Milli-Q water. Then, the seedlings were separated into roots and shoots and oven dried at 70 °C for 72 h, weighed and subjected to a microwave assisted acid digestion for 30 min at 180 °C by using a HNO$_3$/H$_2$O$_2$ mixture (2:1, v/v). The digested solutions were then diluted to 50 mL with Milli-Q water and filtered with syringe filters (25 mm diameter, 0.45 μm pore size). Cadmium and As concentrations were determined by a quadrupole inductively coupled plasma mass spectrometer (ICP-MS, model 820-MS; Bruker, Bremen, Germany) equipped with a glass nebulizer (0.4 mL min^{-1}, Analytik Jena AG, Jena, Germany). External standard calibration curve was performed for Cd and As by serially diluting standard stock solution (1000 ± 2 mg L^{-1}, Exaxol Italia Chemical Manufacturers srl, Genoa, Italy). To control the nebulizer efficiency, rhodium was set at 5 μg L^{-1} as internal standard for all the measurements and was prepared from standard stock solution (1000 ± 2 mg L^{-1}, Panreac Química, Barcelona, Spain). The values of blanks, subjected to similar sample preparation and analytical procedures, were deducted from all measurements and the limits of detection (LODs; 0.018 μg L^{-1} for Cd and 0.022 μg L^{-1} for As) were set at 3 times the standard deviation (SD) of 10 replicate blank determinations. Standard deviations of the replicates were all below 20%. The used instrumental conditions and the performance of the method are detailed in Astolfi et al. (2018).

The obtained Cd and As concentrations (mg/kg) were divided by the dry weight of each sample.

The bioaccumulation factor (BF) and the translocation factor (TF) were calculated as the ratio of the total heavy metal/metalloid concentration in the shoot to that in the culture medium (BF), and the ratio of the total heavy metal/metalloid concentration in the shoot to that in the root (TF) (Rezvani and Zaefarian, 2011).

2.4. Detection of NO, O$_2^{\bullet-}$ and ONOO$^-$ in rice roots

Nitric oxide content was evaluated in LRPs and elongated LRs by using the specific NO-fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Sigma-Aldrich, Saint Louis, MO, USA), as previously described (Chen et al., 2015). Briefly, rice roots non-exposed (Control) or exposed to As(V), As(III) or Cd, combined or not with SNP or SNP plus cPTIO, were incubated with 10 μM DAF-FM DA in 20 mM HEPES-NaOH buffer (pH 7.5) for 30 min at 25 °C in the dark. After incubation, the roots were washed three times with fresh 20 mM HEPES/NaOH buffer to remove probe excess and immediately observed under a Leica DMRB optical microscope equipped with a Leica DC 500 camera (excitation 490 nm; emission 515 nm), and the fluorescence of elongated LRs quantified using ImageJ 1.52a software (https://imagej.nih.gov/ij). The fluorescence was not quantified in LRPs because they were frequently enclosed within the tissues of the parental root.

For O$_2^{\bullet-}$ detection, roots exposed or not to As(V), As(III) or Cd, alone or combined with SNP, were stained for 30 min with a solution of 0.5 mg mL^{-1} nitro blue tetrazolium (NBT; Roche Diagnostics Corp., Gmbh, Germany) in 10 mM Tris-HCl (pH 7.4). Nitro blue tetrazolium is a compound that is reduced by O$_2^{\bullet-}$ forming a purple/blue precipitate called formazan, and for this reason is used to study the intracellular production of the superoxide anion (Jones et al., 2007).

After having transferred the roots into distilled water to stop the reaction, they were kept in a chloral hydrate solution (Cl$_3$CCH(OH)$_2$) and observed with LEICA DMRB light microscope equipped with Nomarski optics. For peroxynitrite (ONOO$^-$) detection, the roots exposed or not to As(V), As(III) or Cd, alone or combined with SNP were incubated in a solution of 10 μM 3'-(p-aminophenyl) fluorescein (APF) (Invitrogen - Italy) dissolved in 10 mM Tris-HCl (pH 7.4) at 25 °C for 1h in the dark. After incubation, the roots were washed with the buffer for 15 min, then mounted on a slide, instantly observed under an optical microscope (excitation 490 nm; emission 515 nm), and the relative fluorescence quantified as described above for NO.

2.5. Histological and autofluorescence analyses

The apical region of five ARs (∼ 2.0 cm from the root tip) non-exposed (Control) or exposed to As(V), As(III) or Cd, alone or combined with SNP, were fixed in 70% (v/v) ethanol, dehydrated by an ethanol series, embedded in Technovit 7100 (Heraeus Kulzer, Germany), longitudinally and radially sectioned at 8 μm with a Microm HM 350 SV microtome (Microm, Germany), stained with 0.05% toluidine blue, and observed under a light microscope. To detect cell wall autofluorescence, root apical regions were transversally sectioned and observed under UV light, and the fluorescence quantified by measuring the average pixel intensity using ImageJ 1.52a software (https://imagej.nih.gov/ij).

2.6. Statistical analysis

Statistical analysis was performed using one-way ANOVA test followed by Tukey's post-test through GraphPad Prism 8 software. All experiments were performed in three biological replicates with very similar results. Data from the second replicate were shown, if not otherwise specified in the figure legend.
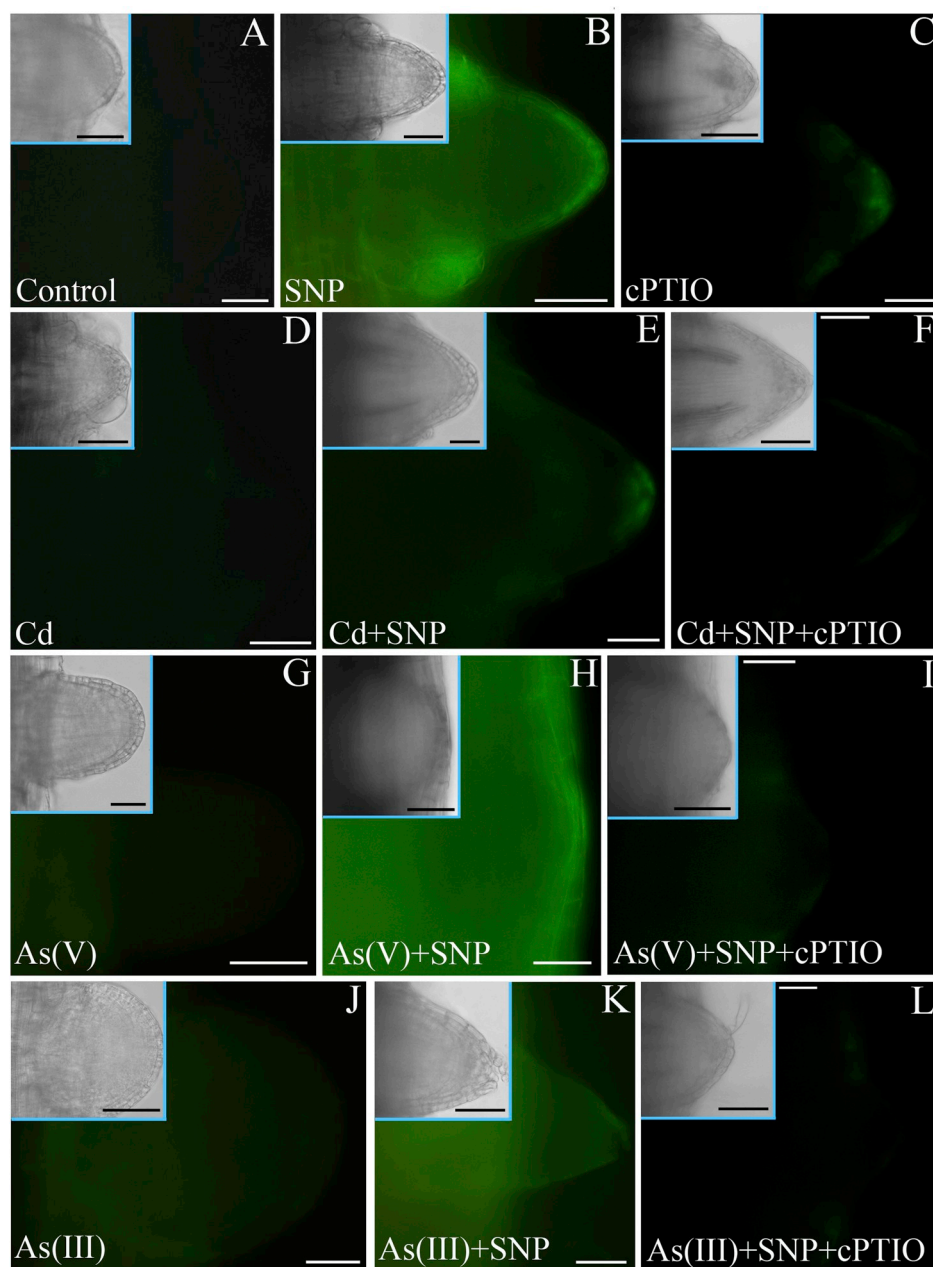
Fig. 1. Nitric oxide (NO) epifluorescence signal in rice lateral root primordia (LRPs). **A-L**, NO signal in bright green colour in LRPs not treated (**A**, Control) or treated with 50 μM Na$_2$[Fe (CN)$_5$NO]·2H$_2$O (**B**, SNP), 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (**C**, cPTIO), or with 100 μM CdSO$_4$ (**D**, Cd), 100 μM Na$_2$HAsO$_4$·7H$_2$O [**G**, As(V)] or 25 μM NaAsO$_2$ [**J**, As(III)] alone or combined with SNP (**E**, Cd + SNP; **H**, As(V) + SNP; **K**, As(III) + SNP) or with SNP and cPTIO (**F**, Cd + SNP + cPTIO; **I**, As(V) + SNP + cPTIO; **L**, As(III) + SNP + cPTIO). LRPs taken from 10-days-old seedlings loaded with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). Insets in **A-L** show the same LRPs under white light. Bars = 30 μm (A, C,E-F,K-L) and 50 μm (B,D,G-J and Insets). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Sodium nitroprusside treatment increases the NO levels in rice roots when combined with cadmium or arsenic

The epifluorescence analysis carried out on roots exposed to the NO-donor sodium nitroprusside (SNP) showed an increase in intracellular NO levels in rice roots, which was confirmed by a strongly reduction of NO signal when the roots were exposed to the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Lateral root primordia (LRPs) formed on the adventitious roots (ARs) of seedlings grown in the Control medium, i.e., those not treated with Cd or As or SNP or cPTIO, showed a weak NO signal (Fig. 1A). On the contrary, the signal increased in elongated lateral roots (LRs), especially in their apex and in the elongation region (Fig. 2A,M). The treatment with SNP significantly (P < 0.01) reinforced NO-signal both in the LRPs and in the LRs (Fig. 1 B and 2B,M). The NO scavenger strongly (P < 0.001) reduced NO signal both in LRPs and LRs in comparison with Control and SNP treatments (Fig. 1C and 2C,M).

To check whether Cd and As changed the NO levels, the roots exposed to the heavy metal or the metalloid, either as As(III) or As(V), combined or not with SNP, alone or with cPTIO, were also treated with the NO-fluorescent probe. The combined treatment of each pollutant with both SNP and cPTIO was carried out to confirm that the epifluorescence signal was due to NO and not to other compounds that are known to be possibly released by SNP (Besson-Bard et al., 2009). Surprisingly, Cd alone strongly (P < 0.001) reduced the NO-signal in both LRPs and LRs in comparison with the Control (Fig. 1D and 2D,M). The treatment with Cd plus SNP weakly increased it (Fig. 1E and 2E,M). When cPTIO was combined with Cd plus SNP NO fluorescence was considerably reduced in LRPs and LRs (P < 0.001) in comparison with Cd plus SNP treatment (Fig. 1F and 2F,M). Arsenic, either as As (III) or As(V), slightly, but not significantly, decreased the NO-signal in LRPs and LRs in comparison with the Control (Fig. 1G,J and 2G,J,M). The combined treatments of arsenate or arsenite and SNP increased the NO levels, in comparison with As alone in both LRPs and LRs (P < 0.01),
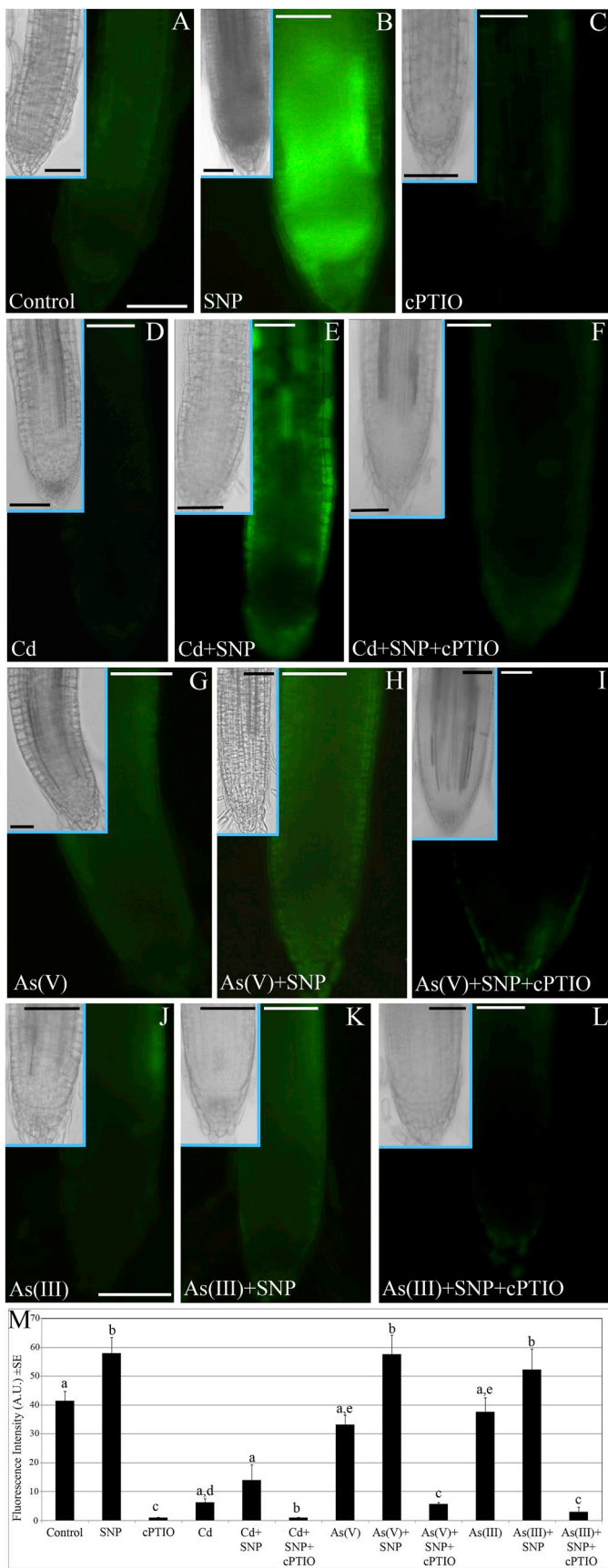
Fig. 2. Nitric oxide (NO) epifluorescence (A–L) signal in rice lateral roots (LRs) and its quantification (**M**). **A-L**, NO signal in bright green colour in lateral roots (LRs) not treated (**A**, Control) or treated with 50 μM Na$_2$[Fe(CN)$_5$NO]·2H$_2$O (**B**, SNP), 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (**C**, cPTIO), or with 100 μM CdSO$_4$ (**D**, Cd), 100 μM Na$_2$HAsO$_4$·7H$_2$O [**G**, As (V)] or 25 μM NaAsO$_2$ [**J**, As(III)], alone or combined with SNP (**E**, Cd + SNP; **H**, As(V) + SNP; **K**, As(III) + SNP) or with SNP and cPTIO (**F**, Cd + SNP + cPTIO; **I**, As(V) + SNP + cPTIO; **L**, As(III) + SNP + cPTIO). LRs taken from 10-days-old seedlings were loaded with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). Insets in **A-L** show the same LRs under white light. Bars = 30 μm (C-D,F,I-L) and 50 μm (A-B,E,G-H and Insets). **M**, Mean values of NO fluorescence intensity (± SE) in LRs measured using ImageJ 1.52a software and expressed in arbitrary units (AUs). Letters a,b and c show statistical differences, at least at P < 0.05 level, among Control, SNP and cPTIO treatments or among the treatments with the same pollutant. Letter d shows statistical differences, at least at P < 0.05 level, between Cd or As alone treatment compared to Control. Letter e shows statistical differences, at least at P < 0.05 level, compared to Cd. Columns followed by the same letter within the Control, SNP and cPTIO treatments or within the treatments with the same pollutant are not different. N = 30. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
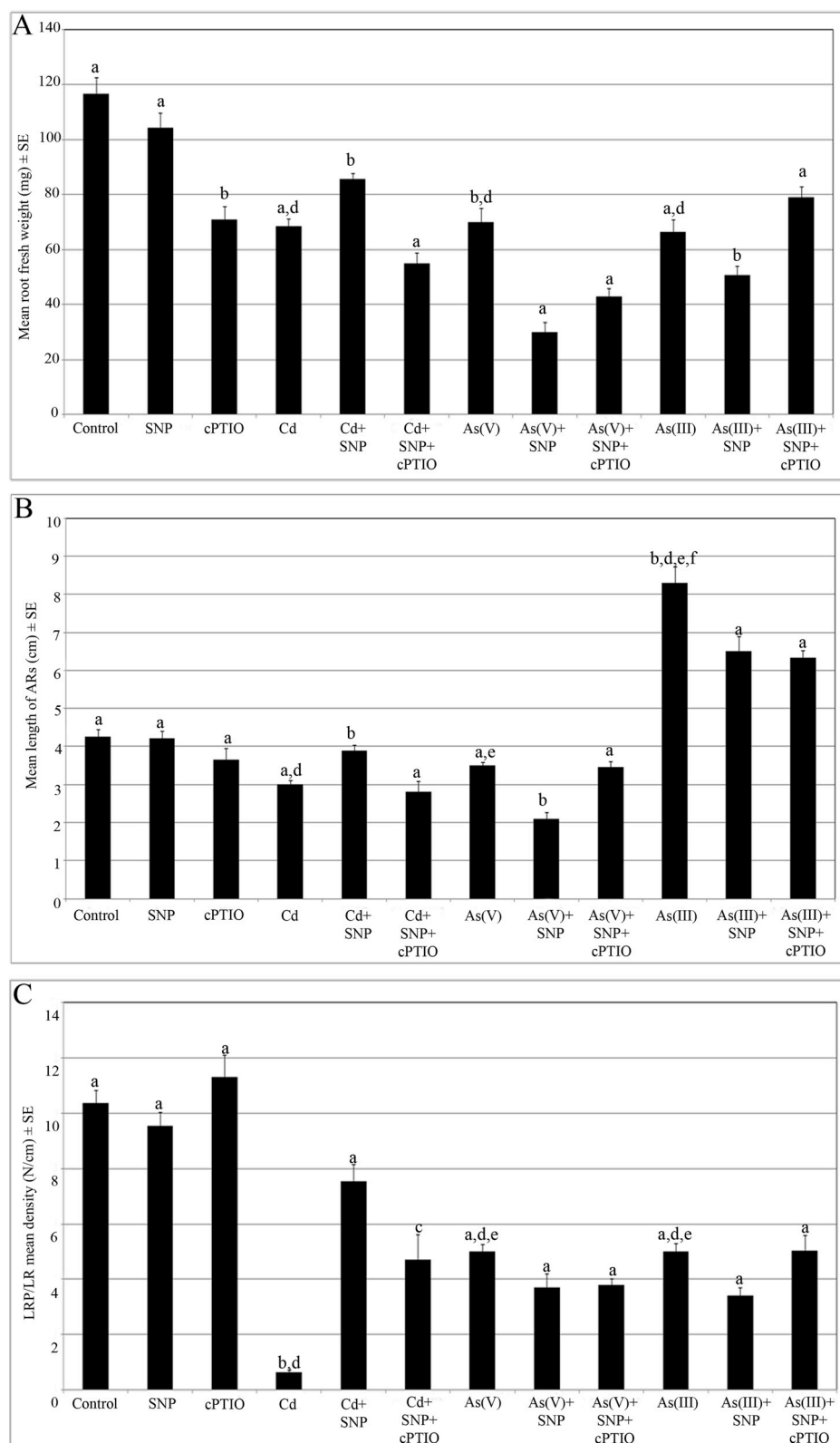
Fig. 3. Mean values (± SE) of rice root fresh weight (**A**), adventitious root (AR) length (**B**) and density of lateral root primordia (LRPs) and elongated lateral roots (LRs) (**C**) in seedlings not exposed (Control) or exposed to 50 μM Na$_2$ [Fe (CN)$_5$NO]·2H$_2$O (SNP), 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), or to 100 μM CdSO$_4$ (Cd), 100 μM Na$_2$HAsO$_4$·7H$_2$O [As(V)] or 25 μM NaAsO$_2$ [As(III)] alone or combined with SNP (Cd + SNP, As(V) + SNP, As(III) + SNP) or with SNP and cPTIO (Cd + SNP + cPTIO, As(V) + SNP + cPTIO, As(III) + SNP + cPTIO) for 10 days. Letters a, b, c show statistical differences, at least at P < 0.05 level, among Control, SNP and cPTIO treatments or among the treatments with the same pollutant. Letter d shows statistical differences, at least at P < 0.05 level, between Cd or As alone treatments compared to Control. Letter e shows statistical differences, at least at P < 0.05 level, compared to Cd. Letter f shows statistical differences, at least at P < 0.05 level, compared to As (V). Columns followed by the same letter within the Control, SNP and cPTIO treatments or within the treatments with the same pollutant are not significantly different. N = 30.

up to levels comparable to those observed in the SNP alone treatment (Fig. 1H,K and 2H, K,M). Again the addition of cPTIO to arsenate or arsenite plus SNP significantly reduced the NO fluorescence in LRPs and LRs in comparison with As(V) and As(III) alone or combined with SNP (Fig. 1I,L and 2I,L-M).

3.2. Nitric oxide alleviates Cd-induced morphological damages in rice root but not those due to As

A morphological analysis was carried out on the root system of rice seedlings, treated or not with Cd or As, and with or without SNP or cPTIO. The NO scavenger, cPTIO, has been used in the morphological analysis to corroborate NO role in the alleviating root alterations due to

toxic elements.

The results show that SNP did not affect the morphological parameters analysed, i.e., the root fresh weight, the mean length of the ARs, the mean density of the LRs, also including the LRPs, in comparison to the Control (Fig. 3).

The root fresh weight significantly (P < 0.01) decreased in the Cd, As(III) and As(V) treatments in comparison with the Control (Fig. 3A). The highest levels of NO, due to the treatment with its donor, resulted into a significant (P < 0.05) increase in root fresh weight when SNP was combined with Cd, but in a weight reduction when SNP was combined with As(III) or As(V), mainly (Fig. 3A). A significant reduction of the fresh weight was observed in the presence of cPTIO alone in comparison with Control and SNP treatments, and when cPTIO was combined with Cd and SNP (Fig. 3A). The NO scavenger combined with both As(V) and As(III) and SNP increased fresh weight in comparison with the metalloid plus SNP treatments (Fig. 3A).

Cadmium and As(III), alone or combined with SNP, did not affect the mean AR number in comparison with the Control. Arsenate alone did not change the mean number of ARs also, whereas the combination of SNP with As(V) significantly (P < 0.01) reduced it (Fig. S3).

The mean AR-length was significantly (P < 0.01) reduced by Cd alone, on the contrary As(V) alone did not changed it, and As(III) strongly (P < 0.01) enhanced it in comparison with the Control (Fig. 3B). The NO-donor induced a significant (P < 0.01) recovery of the length of the ARs exposed to Cd, but was not able to restore the AR length when combined with the arsenate, because a further and significant (P < 0.01) reduction in length was instead observed (Fig. 3B). The combined treatment of As(III) and SNP also reduced the AR length in comparison with the As(III) alone, i.e., the treatment causing the highest AR elongation (Fig. 3B). When cPTIO alone was added to the culture media a weak and not significant reduction of AR length was observed in comparison with Control and SNP treatments (Fig. 3B). NO scavenger added to Cd and SNP significantly reduced AR length in comparison with Cd plus SNP (Fig. 3B). The cPTIO combined with As(V) plus SNP restored AR length in comparison with arsenate plus SNP. On the contrary, it did not change AR length when added to As(III) plus SNP with respect to the last treatment (Fig. 3B).

The mean density of the LRPs/LRs was strongly and significantly reduced by As(V), As(III), and mainly by Cd, in comparison with the Control, and when SNP was combined with the heavy metal a significant (P < 0.001) recovery of the LRP/LR density, in comparison with Cd alone, was observed (Fig. 3C). On the contrary, the NO donor added to As(V) or As(III) did not change LRP/LR density (Fig. 3C). The cPTIO alone did not change LRP/LR density in comparison with Control and SNP (Fig. 3C). On the contrary it significantly reduced root density when combined with Cd plus SNP in comparison with heavy metal plus NO donor treatment and did not significantly change root density when combined with both As forms and SNP (Fig. 3C).

3.3. Nitric oxide reduces the Cd-induced lipid peroxidation, but not the as-induced peroxidation, in rice roots

The staining with Schiff's reagent highlights the lipid peroxidation of the membranes through an intense red colouring (Yamamoto et al., 2001). In the Control, the faint red colour localized at the base of the LRPs and LRs showed the presence of weak membrane peroxidation (Fig. 4A–B). The SNP treatment increased the red signal, but it remained localized at LRPs and LRs base (Fig. 4C–D). The heavy metal treatment moderately increased the lipid peroxidation signal at the base of LRPs, but also caused the appearance of the red signal in the root meristem (Fig. 4E). The Cd-treated LRs showed an intense red colour which was extended along the organ and was higher than with SNP alone (Fig. 4D and F). When SNP was combined with Cd, the lipid peroxidation signal was reduced in both the LRPs and LRs (Fig. 4G–H). The LRPs and LRs formed in the presence of either As(V) or As(III) similarly showed an intense red colour at their base. The presence of

SNP did not apparently changed the signal suggesting the incapability of exogenous SNP to counteract the lipid peroxidation caused by As in the membranes of the LRs, in particular (Fig. 4I-P).

3.4. Nitric oxide decreases Cd and As uptake in rice seedlings

In order to investigate the effects of exogenous NO on Cd or As uptake and translocation, the accumulation of the heavy metal and of the metalloid was evaluated in the roots and shoots of the seedlings. Arsenic and Cd were mainly accumulated in the roots, and As, both as As(III) and As(V), was taken up more than Cd (Fig. 5A–B). The treatment with SNP significantly (P < 0.01) reduced the accumulation in the roots of both pollutants (Fig. 5). The transport of Cd to the shoot was low, and the co-presence of SNP furtherly and significantly (P < 0.01) reduced it (Fig. 5A). Even the As was transported to the shoot at very low amounts, independently from the SNP presence (i.e., 6.41, 4.96, 12.06 and 9.11 mg/kg for As(III), As(III) plus SNP, As(V) and As(V) plus SNP, respectively) (Fig. 5B). The evaluation of the translocation factor (TF) and the bioaccumulation factor (BF) of both the elements, taken alone or combined with SNP, showed that the NO donor did not affect the translocation capability of the heavy metal and the metalloid from the root to the shoot, overall highlighting a significant role of NO in the reduction of the uptake of these elements in rice.

3.5. Exogenous NO differently changes ROS and RNS cell balance in roots exposed to Cd or As

To verify whether the root morphological alterations were due to an unbalance of ROS/RNS levels induced by the heavy metal/metalloid, and whether exogenous NO was able to restore the ROS/RNS balance, the superoxide anion ($O_2^{\cdot-}$) and the peroxynitrite ($ONOO^-$) contents were evaluated in LRPs and LRs under all treatments for monitoring ROS and RNS, respectively.

Both the root types showed a weak NBT-staining in the apex in the Control treatment (Fig. 6A–B), which increased after SNP treatment (Fig. 6C–D). Cadmium alone induced further increase in $O_2^{\cdot-}$ levels in both LRPs and LRs (Fig. 6E–F), but mainly in the latter ones (Fig. 6F). The presence of SNP combined with Cd reduced the NBT-monitored superoxide anion, but only in the LRs (Fig. 6G–H).

In the treatments with As(V) or As(III) alone, the $O_2^{\cdot-}$ signal was localized mainly in the apical region of the LRP, (Fig. 6I, M). However, in the LRs, the $O_2^{\cdot-}$ signal occurred in spots localized mainly in differentiating vascular system (Fig. 6J, N), and, surprisingly, when As (both species) was combined with SNP the level of the superoxide anion strongly increased in both LRPs or LRs (Fig. 6K–L, O–P).

The presence of peroxynitrite ($ONOO^-$) was shown by a weak fluorescent signal already in the Control treatment (Fig. 7A–B, Q), and the fluorescence was significantly (P < 0.01) reinforced in the presence of SNP (Fig. 7C–D, Q). The exposure to Cd alone significantly (P < 0.01) increased the ONOO- signal both in LRPs and LRs, in comparison with the Control (Fig. 7E–F, Q), and when Cd was combined with SNP, the peroxynitrite signal did not change significantly (Fig. 7G–H, Q). Also the treatments with both arsenic species alone did not significantly change the peroxynitrite signal in comparison with the Control in both LRPs and LRs (Fig. 7I–J, M-N, Q). The presence of SNP combined with As did not affect significantly the fluorescent signal (Fig. 7K-L, O–P, Q). Taken together, the evaluation of the $O_2^{\cdot-}$ and ONOO- levels in roots exposed to Cd or As combined with SNP demonstrated that NO reduces ROS levels due to Cd-treatment by triggering peroxynitrite production (Fig. 6E–H and 7E-H, in comparison, and 7Q). On the contrary, NO does not reduce $O_2^{\cdot-}$ levels induced by the As treatments in LRP/LR cells (Fig. 6I-P), with this leading to very high levels of $O_2^{\cdot-}$ and low levels of $ONOO^-$ (Fig. 6I-P and 7I-Q).
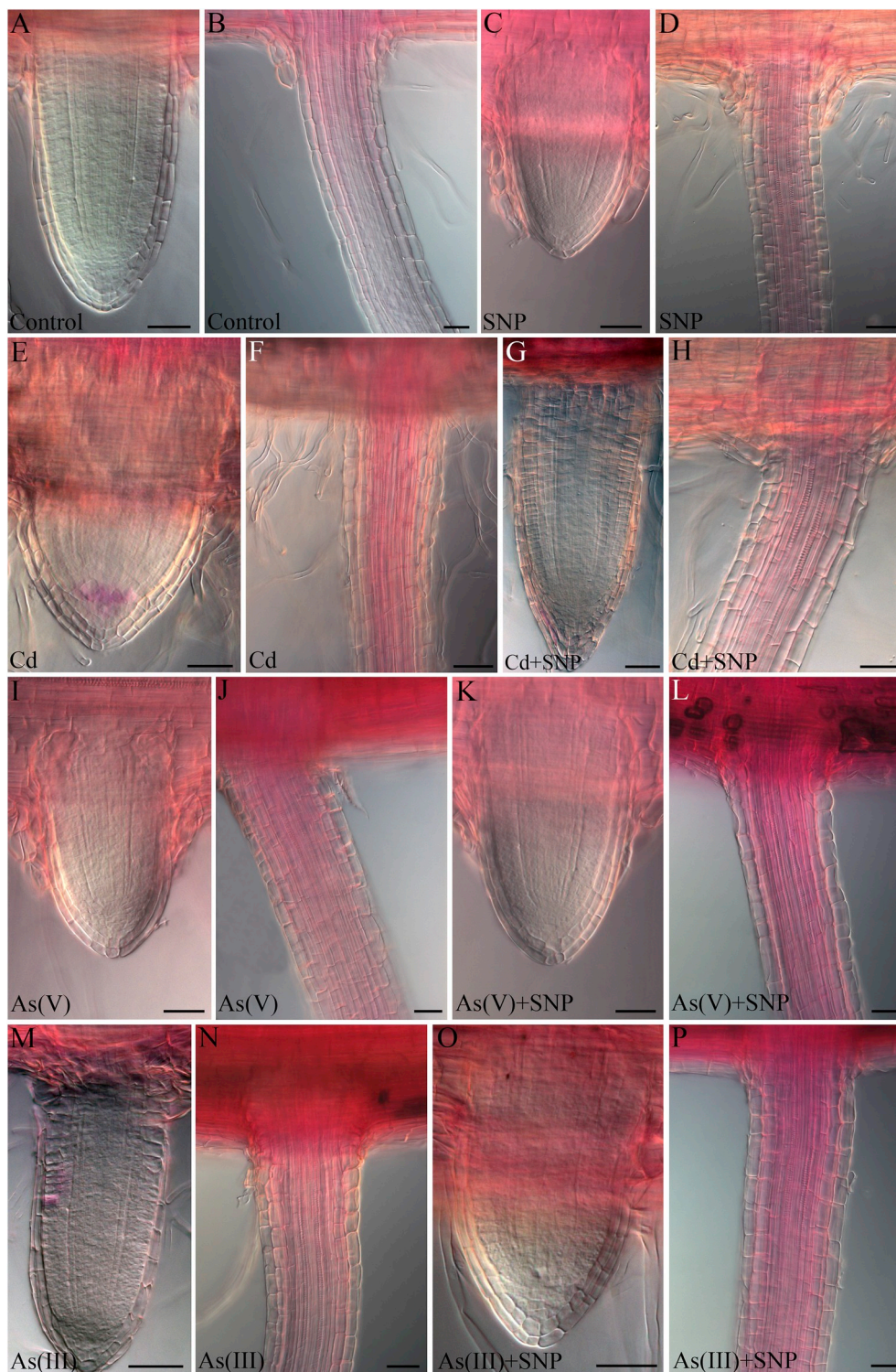
Fig. 4. Lipid peroxidation detected as red colour after Schiff's reaction in lateral root primordia (LRPs, A,C,E,G,I,K,M and O) and elongated lateral roots (LRs, B,D,F,H,J,L,N and P) of rice seedlings treated or not for 10 days with 100 μM CdSO$_4$ (Cd), 100 μM Na$_2$HAsO$_4$·7H$_2$O (As(V)) or 25 μM NaAsO$_2$ (As(III)) alone or combined with 50 μM Na$_2$ [Fe(CN)$_5$NO]·2H$_2$O (SNP). Bars = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. Nitric oxide reduces the histological alterations induced by Cd, but not all those induced by As

We deepened the investigation on the role of NO in the root alterations induced by Cd and As through histological and autofluorescence analyses on roots treated or not with Cd or As combined or

not with SNP.

It is known that Cd and As induce extensive damages in rice AR primary structure during LR-formation (Ronzan et al., 2018). To verify if the increased intracellular NO levels resulted into a reduction in these damages, a histological analysis was carried out in the AR region forming the LRPs, and the lignification in cell walls detected and
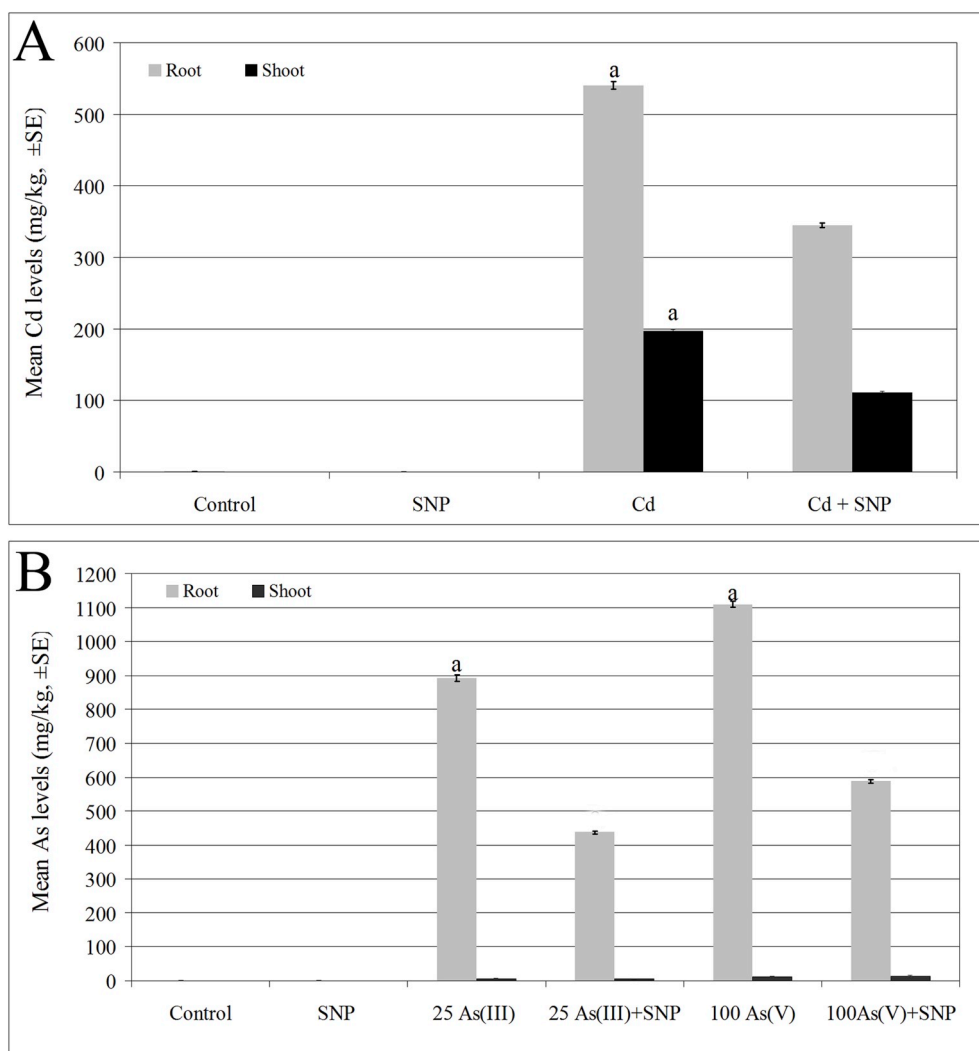
Fig. 5. Cadmium (**A**) and Arsenic (**B**) accumulation in roots and shoots of rice seedlings treated or not for 10 days with 100 μM CdSO$_4$ (Cd), 100 μM Na$_2$HAsO$_4$·7H$_2$O (As(V)) or 25 μM NaAsO$_2$ (As(III)) or 50 μM Na$_2$ [Fe(CN)$_5$NO]·2H$_2$O (SNP) alone or combined. Letter a shows statistical difference, at least at P < 0.05 level, in comparison to the same treatment with/without SNP, for the same organ. Mean of tree biological replicates.

quantified (Fig. 8).

The histological analysis showed that the SNP-treated roots, were characterized, as the Control ones, by regularly differentiated epidermis, exodermis, sclerenchyma layer, cortical parenchyma, endodermis and vascular bundles (Fig. 8A–B, D-E). The autofluorescence analysis showed that the sclerenchyma cells were mildly lignified (Fig. 8C, F, small arrows, and S), while the differentiated endodermis cells did not show regular lignin deposition in the cell walls (Fig. 8C, F, arrowheads, and S). The Cd-alone-treatment induced a precocious aerenchyma formation and a strong thickening of the sclerenchyma cell walls due to a higher lignin deposition (Fig. 8G–I and S). Also the endodermic cells were characterized by lignin deposition (Fig. 8I, arrowheads), and the significant increase of lignin in the cell walls of sclerenchyma and endodermis was also confirmed by the increase of the lignin autofluorescence signal in both tissues (Fig. 8S). The combined treatment of Cd and SNP significantly reduced cell wall lignification in the sclerenchyma (Fig. 8J-L), with the lignin autofluorescence signal decreasing up to values comparable to the Control roots Fig. 8S).

Considering that As(V) and As(III) induced similar histological alterations in root, here we show the images of arsenate-exposed roots only. The As-treatment determined an increase in the sclerenchyma and endodermis cell wall thickening, similarly to Cd, and a related enhancement in lignin autofluorescence (Fig. 8M–O, S). Moreover, As also

induced precocious aerenchyma formation, in addition to an anomalous proliferation of the sclerenchyma cells (Fig. 8M–N, arrows), and an alteration of the exodermis (Fig. 8M). The NO-donor combined with As induced the roots to differentiate cells with a reduced lignin deposition, and a reduced autofluorescence signal (Fig. 8P–R, S), but did not counteract the As-caused anomalous cell proliferation (Fig. 8P–Q, arrows).

These results demonstrated that the NO reduces the pollutant-caused increase in the cell wall lignification of the sclerenchyma and endodermis, the tissues committed to protect the root from the toxicity of the heavy metal and metalloid, however, does not counteract the anomalous cell proliferation caused by As.

4. Discussion

The present results highlight a role of NO in modulating the rice responses to the toxicity of the heavy metal Cd and the metalloid As, which differs between the two pollutants. In fact, an increased intracellular NO level in rice roots occurred as a consequence of the application of the exogenous NO-donor SNP combined with each pollutant, alleviates the morphological and anatomical damages, and restores ROS/RNS cell balance in the presence of Cd, but it is not able to restore some damages, and the oxidative system imbalance, caused by
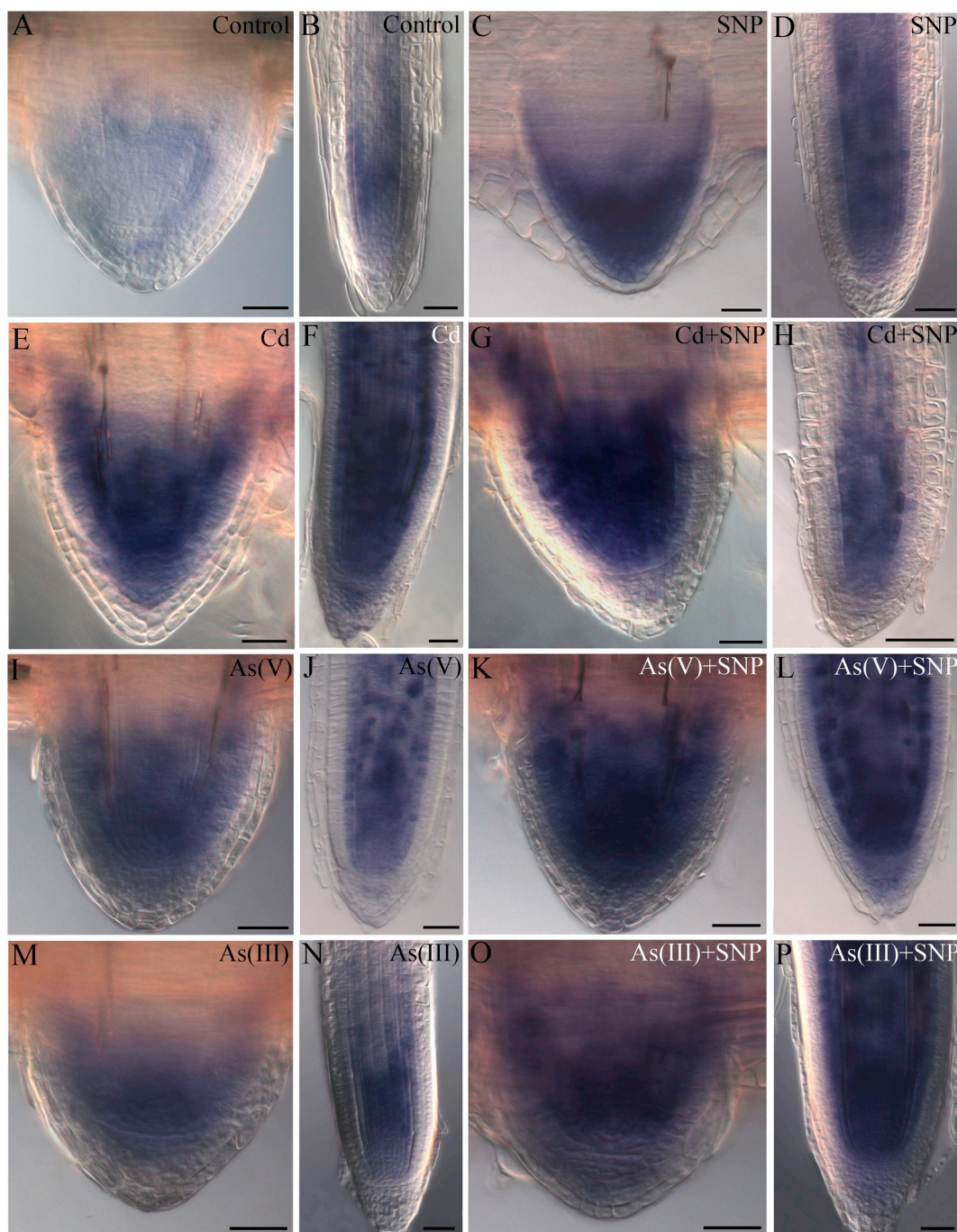
Fig. 6. Superoxide anion (blue colour) detected by NBT staining in lateral root primordia (LRPs; **A,C,E,G,I,K,M and O**) and elongated lateral roots (LRs; **B,D,F,H,J,L,N** and **P**) of rice seedlings treated or not (Control) for 10 days with 100 μM CdSO$_4$ (Cd), 100 μM Na$_2$HAsO$_4$·7H$_2$O (As(V)) or 25 μM NaAsO$_2$ (As(III)) or 50 μM Na$_2$ [Fe(CN)$_5$NO]·2H$_2$O (SNP) alone or combined with 50 μM Na$_2$ [Fe(CN)$_5$NO]·2H$_2$O (SNP). Bars = 30 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

As.

Sodium nitroprusside is the most commonly NO donor used in *in vitro* plant cultures and, although it also releases other compounds than NO, we exclude that the effects observed in the presence of SNP treatment are due to these other compounds. In fact, as shown by the epifluorescence analysis, the increase of the fluorescence signal after treatment of SNP alone or combined with each pollutant was specific for NO, because it was almost completely suppressed by the application of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazo-line-1-oxyl-3-oxide (cPTIO) (Figs. 1 and 2).

In the last decade, numerous researches have demonstrated the

involvement of NO in the defence responses against toxic elements (Besson-Bard et al., 2009; Silveira et al., 2015; Kharbech et al., 2017, just to name a few). In particular, it is emerging that a correct cellular balance between NO and ROS is of fundamental importance to allow plants to counteract the toxicity induced by the most dangerous and common soil pollutants, such Cd or As (Farnese et al., 2016, and references therein). Most research attributes a positive role to NO in alleviating the toxicity of heavy metals/metalloids, however some researches also report that an increase in endogenous levels of NO can intensify the dangerous effects of the toxic metals (Besson-Bard et al., 2009; Yuan and Huang, 2016). Our results show that Cd alone
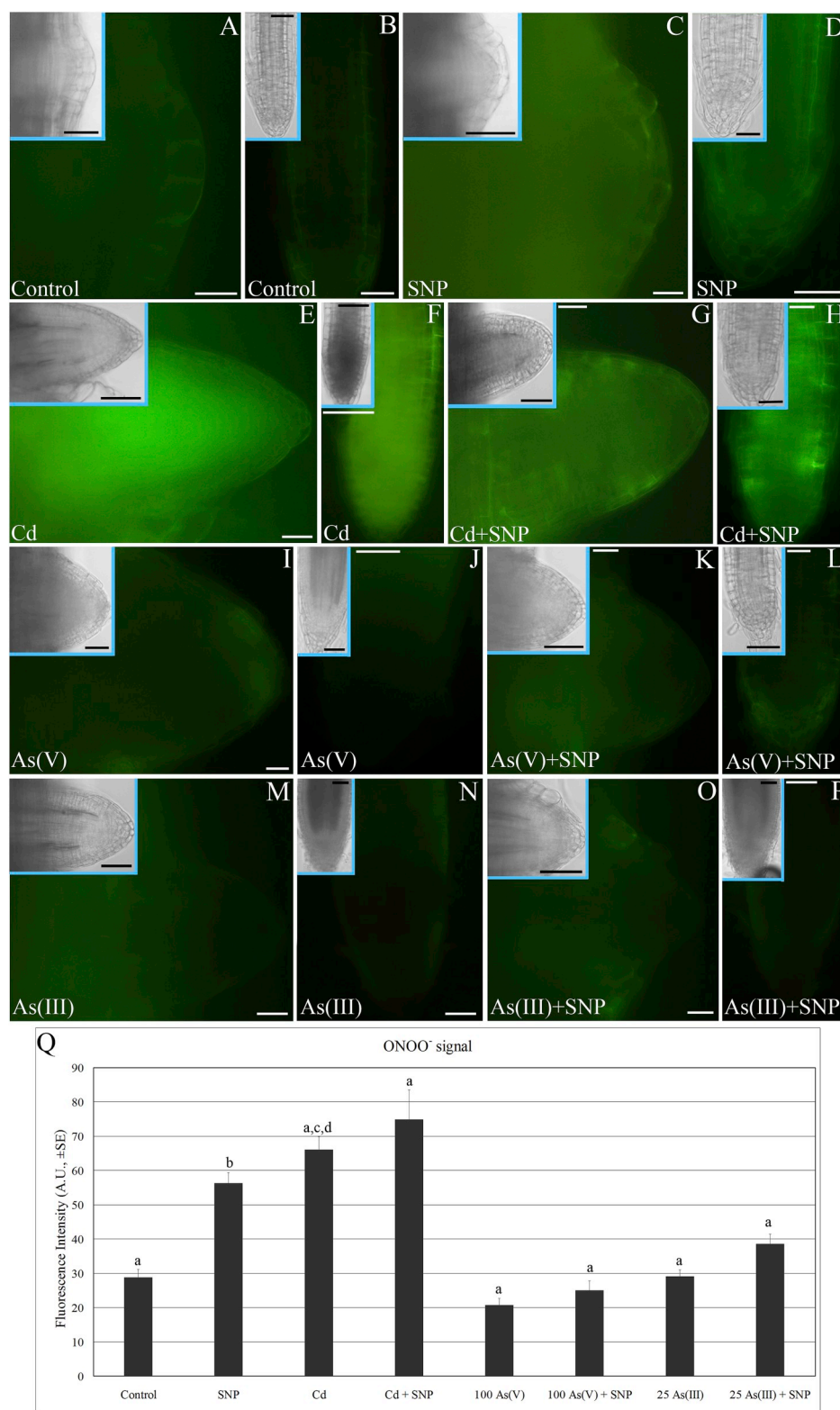
Fig. 7. Peroxynitrite levels (bright green colour) in rice lateral root primordia (LRPs; **A,C,E,G,I** and **K,M** and **O**) and elongated lateral roots (LRs; **B,D,F,H,J, L, N** and **P**) treated or not with 100 μM CdSO$_4$ (Cd), 100 μM Na$_2$HAsO$_4$·7H$_2$O (As(V)) or 25 μM NaAsO$_2$ (As(III)) alone or combined with 50 μM Na$_2$ [Fe(CN)$_5$NO]·2H$_2$O (SNP). LRPs and LRs taken from 10-days-old seedlings were loaded with 3'-(p-aminophenyl) fluorescein (APF). Insets in A-P show the same organs under white light. Bars = 20 μm (A-E, G-I, K-M, O Inset in D) and 50 μm (F, J, N,P and Insets in A-C, E-P). **Q**, Mean of ONOO$^-$ fluorescence intensity (± SE) in LRPs/ LRs measured using ImageJ 1.52a software and expressed in arbitrary units (AUs). Letters a and b show statistical differences, at least at P < 0.05, between the same treatments with or without SNP. Letter c shows statistical differences, at least at P < 0.05 level, between Cd or As alone treatments compared to Control. Letter d shows statistical difference, at least at P < 0.05 level, in comparison with both As treatments. N = 30. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significantly decreased the NO levels in rice root-system (Figs. 1 and 2), and this is in accordance with previous results obtained in *Medicago truncatula*, *Pisum sativum* and *Oryza sativa* after exposition to heavy metals (Xiong et al., 2009; Xu et al., 2010 and Rodríguez-Serrano et al., 2006). In particular, Rodriguez-Serrano and co-workers (2006) attribute the reduction of NO levels to a prolonged exposure to high Cd concentrations that could inactive the enzymes involved in NO-synthesis (NOS-like enzymes). The same mechanism might be active in rice

seedlings, because we exposed them to high Cd (100 μM of CdSO$_4$) for a long period (10 days). Thus, it is possible that the nearby absence of NO in rice root cells exposed to Cd alone could not allow the activation of endogenous appropriate strategies to withstand the heavy metal toxicity. On the contrary, the exposure to As, and mainly to As(III), did not significantly reduce root NO levels compared to the Control-treatment conditions, and this could be positively correlated with root morphology alterations less than those caused by Cd, and even opposite,
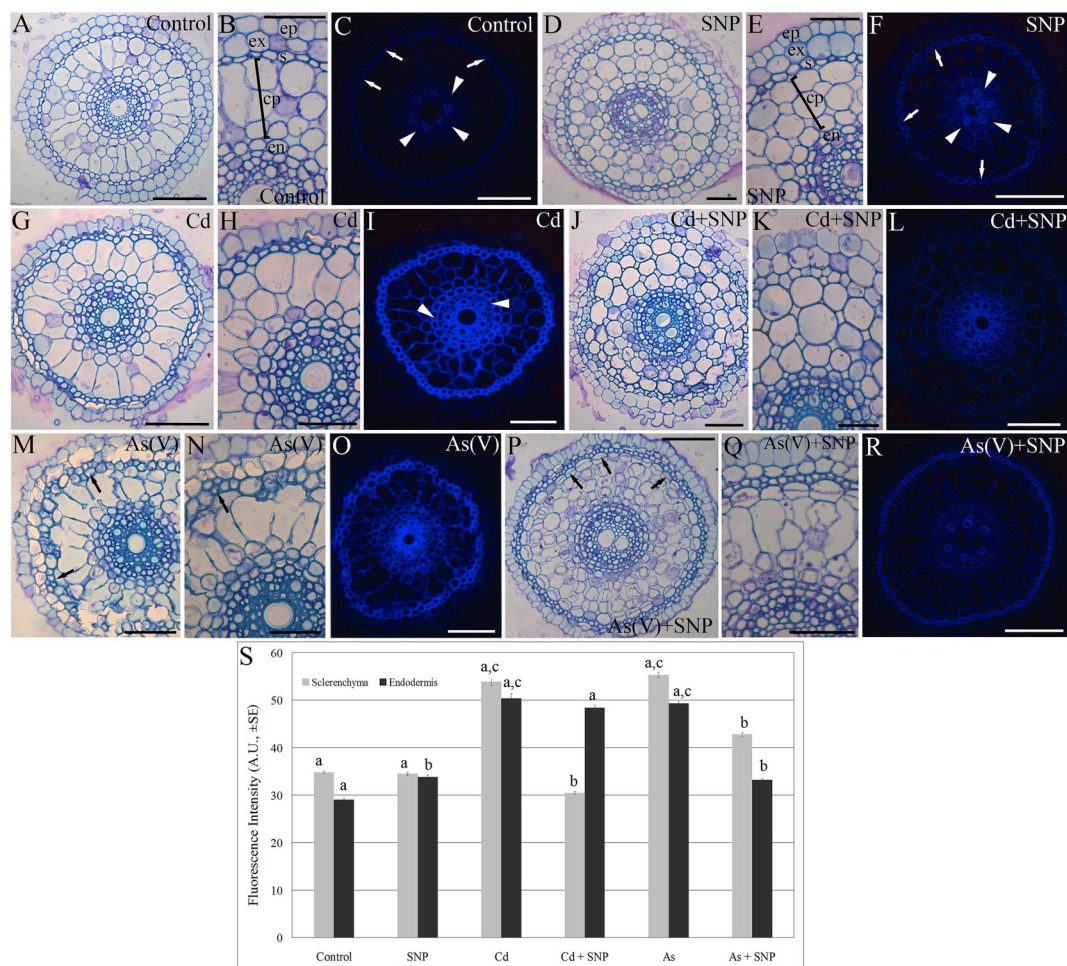
Fig. 8. Transverse sections of ARs at 2.0 cm from the root tip taken from rice seedlings treated or not for 10 days with 100 μM CdSO₄ (Cd), 100 μM Na₂HAsO₄·7H₂O (As(V)) alone or combined with 50 μM Na₂ [Fe(CN)₅NO]·2H₂O (SNP). **A-B, D-E, G-H, J-K, M-N** and **P-Q** light microscope images of sections stained with toluidine blue. **C,F,I, L,O** and **R** images showing lignin autofluorescence (bright blue colour) in sclerenchyma and endodermis cell walls. Bar = 50 μm (B, E, H, K, N, Q) and 100 μm (A, C-D, F-G, I-J, L-M, O–P, R). cp, cortical parenchyma; ep, epidermis; ex, exodermis; s, sclerenchyma layer; en, endodermis. **S,** mean values (± SE) of lignin autofluorescence intensity in sclerenchyma and endodermis measured using ImageJ 1.52a software and expressed in arbitrary units (AUs). Letters a and b show statistical differences, at least at P < 0.05 level, for the same tissue and treatment, with/without SNP. Letter c shows statistical differences, at least at P < 0.05 level, for the same tissue in comparison with Control. Columns followed by the same letter within the same treatment with/without SNP are not significantly different N = 30. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

i.e., an increase in AR elongation instead of a reduction, as in fact observed (Figs. 1–3).

We show that the exogenous application of SNP increases the intracellular NO content in the roots, both when combined with Cd, and with arsenite or arsenate (Figs. 1 and 2). The increase in NO-levels in the roots exposed to Cd and SNP, even if slight, seems sufficient to alleviate the damages caused by the heavy metal, according to what has been reported for *M. truncatula* and tomato (Cui et al., 2010; Xu et al., 2010). In fact, when Cd was combined with SNP, all the morphological parameters showed an improvement compared to Cd alone (Fig. 3).

Sodium nitroprusside, combined with both the inorganic forms of As, induced very high levels of NO in the rice roots already starting from the early stages of LRP formation (Figs. 1 and 2). This probably does not allow the plant to improve the development of the root-system, differently from what reported for *var*. Pusa Basmati of rice under different culture conditions (Praveen and Gupta, 2018). In fact, it is known that NO must be present in the cells at low levels to act as a positive mediator of plant responses to abiotic stresses, including those induced by metals (Terrón-Camero et al., 2019). At the same time, too high levels of NO are known to accentuate cell suffering by inducing nitrosative stress, because they can trigger the nitrosylation of key proteins of the cellular metabolism (Molassiotis and Fotopoulos, 2011).

Cadmium or As stress induces ROS formation as shown by present results (Fig. 6), in accordance with many other Authors. An increased ROS production is widely known to determine oxidative stress in plant cells, and damages in the plant as a whole. Thus, cellular levels of ROS must be kept under control by enzymatic and non-enzymatic antioxidant systems in order to protect the plant against the oxidative damages (Gill and Tuteja, 2010). As above reported, the heavy metal and the metalloid also directly interfere with NO-metabolism either increasing or decreasing its levels (Arasimowicz-Jelonek et al., 2011). Nitric oxide, at specific concentrations, in turn activates enzymatic and non-enzymatic antioxidant systems to scavenge ROS, thus protecting cells against the oxidative damage. In our conditions, it is possible that the strong reduction of NO induced by Cd reduces the ROS scavenging activity mediated by the signal molecule. By contrast, useful NO levels for activating the antioxidant system can be restored after adding SNP to the heavy metal. In line with these observations, the Schiff's reagent, that highlights the presence of lipid peroxidation in the membranes, moderately stained the base of the LRPs and LRs in the Cd treatment, but the staining decreased in the Cd plus SNP treatment (Fig. 4). Arsenic *per se* slightly reduced NO levels and, after adding SNP to the metalloid, the NO-levels became too high to counteract its toxicity. This hypothesis is also sustained by the results obtained with Schiff's

reagent. In fact, in our conditions a more intense signal of lipid per-oxidation was observed in association with high NO levels, i.e., in the treatments with arsenate or arsenite combined with SNP (Fig. 4).

An active interplay between ROS and RNS modulates plant responses to environmental stresses, and it is crucial for the plant to keep the ROS/RNS levels below the values that trigger the oxidation/nitrosylation reactions in the cells. This interaction is based on different mechanisms, but the most recurrent and immediate one is the reaction between the ROS $O_2^{\bullet-}$ and NO to form the $ONOO^-$, a RNS, to which an important role in the signalling processes has been given in recent years (Molassiotis and Fotopoulos, 2011). However, very high levels of peroxinitrite induces nitro-oxidative stress (Corpas and Barroso, 2014, and references therein). The present results show that Cd alone or, mainly combined with SNP, increased peroxinitrite formation, and this is consistent with the low NO levels, and the reduction of the superoxide anion, simultaneously observed in the roots (Figs. 1, 2, 6 and 7). In our conditions, and particularly when Cd was combined with SNP it is possible that a favourable balance between ROS and RNS levels is achieved and this allows rice plant to counteract the heavy metal toxicity.

In the presence of As(V) or As(III) alone and above all, combined with SNP, $ONOO^-$ levels remained rather low. On the contrary, the levels of $O_2^{\bullet-}$ were high (Figs. 6 and 7). As reported, in the root cells a reaction converts As(V) to As(III) and this contributes to increase the cytosolic levels of ROS (Meharg and Hartley-Whitaker, 2002; Abbas et al., 2018). It is therefore possible that the higher ROS levels induced by As in comparison with Cd alter the ROS/RNS ratio so much that the NO-donor becomes unable to rebalance it, and to mitigate the damages due to As.

It is therefore clear that, for NO to have a positive role in alleviating metal-induced toxicity, a perfect cellular balance must be achieved between ROS and RNS, with $ONOO^-$ acting as an effector of NO-mediated signalling, as also previously suggested (Vandelle and Delledonne, 2011). However, the cellular ROS/RNS balance depends on the culture conditions, but, above all, on the concentrations and on the exposure times to the toxic elements. In fact, our results showing that, at the present concentrations and exposure times, the seedlings absorbed less Cd or As when exposed to each pollutant plus SNP (Fig. 5), suggesting that a lower Cd or As uptake is the main responsible for the decrease in oxidative stress. Our observations that SNP significantly reduced Cd and As uptake are in accordance with the data obtained on wheat and rice (Singh et al., 2008; Praveen and Gupta, 2018; Kaya et al., 2020), but in contrast with those reported by some other Authors, e.g. by Besson-Bard et al. (2009) in Arabidopsis. It is possible that in rice, similarly to what happens in wheat, the SNP supply protects the plant against the membrane damage, as also here supported by Schiff's reagent data, by promoting the activity of membrane transporters that should limit the entrance of harmful Cd/As in the root cells (Singh et al., 2008) in favour of an improvement in the absorption of ions useful to mineral nutrition, such as Ca^{2+} and K^+, overall sustaining the positive role of NO in alleviating damages caused by high levels of toxic elements.

The present results also showed that Cd alone induces a strong increase in lignin deposition in the cell walls of the sclerenchyma and endodermis in the AR region committed to form LRPs (Fig. 8). This can be considered as a strategy to limit the entry of the heavy metal into the cortical (sclerenchyma/endodermis) and vascular cells, similarly to what is observed in the fronds of *Pteris vittata* exposed to Cd (Ronzan et al., 2017). Moreover, the SNP plus Cd treatment resulted into the development of roots with a reduction in lignin deposition in the sclerenchyma. This might be positively linked with the effects of exogenous (SNP-derived) NO on the heavy metal uptake (Fig. 5). In fact, increased NO-levels significantly reduced Cd-levels in the roots, with a consequent lower Cd content, and rebalance of ROS/RNS ratio, possibly responsible for the recovery of the correct root anatomy. The As-treatments also increased lignin deposition in the cell walls of the

sclerenchyma and endodermis, also triggering anomalous sclerenchyma proliferation. When SNP was combined with each As form, lignifications occurred at a lower level, but no reduction in the anomalous cell proliferation was induced, even if As uptake was reduced (Fig. 8). Collectively our results suggest that the exogenous NO was not enough to limit As toxicity, but enough to limit Cd toxicity.

5. Conclusions

In conclusion, the results highlight that NO differently affects the responses of rice root-system to the toxicity of Cd and As. In fact, increased cellular levels of NO alleviate root damages induced by Cd by improving the entire root-system, but do not improve the root-system ability to counteract As toxicity. The explanation of this different behaviour is probably attributable to the NO-ability to restore the ROS/RNS cellular balance, differently altered by the two pollutants. Of course, further researches are needed to shed light on the full mechanisms governing NO action in pollutant environments.

CRediT authorship contribution statement

D. Piacentini: Conceptualization, Data curation, Formal analysis, Methodology, Funding acquisition. **M. Ronzan:** Formal analysis, Methodology. **L. Fattorini:** Formal analysis, Methodology. **F. Della Rovere:** Formal analysis, Funding acquisition. **L. Massimi:** Formal analysis. **M.M. Altamura:** Conceptualization, Data curation, Supervision, Writing - original draft, Writing - review & editing. **G. Falasca:** Conceptualization, Data curation, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.04.004.

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