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Research article

An exogenous source of nitric oxide modulates zinc nutritional status in wheat plants

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ABSTRACT

The effect of addition of the nitric oxide donor S-nitrosoglutathione (GSNO) on the Zn nutritional status was evaluated in hydroponically-cultured wheat plants (*Triticum aestivum* cv. Chinese Spring). Addition of GSNO in Zn-deprived plants did not modify biomass accumulation but accelerated leaf senescence in a mode concomitant with accelerated decrease of Zn allocation to shoots. In well-supplied plants, Zn concentration in both roots and shoots declined due to long term exposure to GSNO. A further evaluation of net Zn uptake rate (ZnNUR) during the recovery of long-term Zn-deprivation unveiled that enhanced Zn-accumulation was partially blocked when GSNO was present in the uptake medium. This effect on uptake was mainly associated with a change of Zn translocation to shoots. Our results suggest a role for GSNO in the modulation of Zn uptake and in root-to-shoot translocation during the transition from deficient to sufficient levels of Zn-supply.

1. Introduction

Plants require zinc (Zn) as a micronutrient for maintaining key physiological processes that influence growth and development. In soil, Zn^{2+} accounts for up to 50% of the soluble Zn fraction and constitutes the plant-available Zn. This fraction may be as low as 10^{-11}–10^{-9} M in calcareous soils thus limiting plant growth and making Zn the most common crop micronutrient deficiency in those environments (Cakmak, 2002; Hacisalihoglu and Kochian, 2003). In contrast, as the result of human activities or in "calamine" soils, the presence of high concentrations of Zn in the soil solution could compromise plant survival (Broadley et al., 2007). Chlorosis, reduced primary root growth as well as impairment of the antioxidant response, are common symptoms of Zn toxicity (Xu et al., 2010).

Among the major biochemical functions displayed by Zn in plant cells, appropriate protein folding as well as catalytic and regulatory functions in enzymes must be mentioned (Broadley et al., 2007). Zinc binding sites can be found mainly in zinc finger domain containing proteins, in membrane lipids and DNA/RNA molecules. Thus, protein metabolism, gene expression and membrane integrity depend at different degrees on the maintenance of Zn concentration in metabolically active pools within a narrow range (Broadley et al., 2007). In the leaves of most plants, Zn concentrations within the range of 15–20 mg Zn kg^{-1} DW have been reported as adequate to maintain physiological functions (Broadley et al., 2007). When Zn concentration drops below these values Zn deficiency symptoms become apparent, which include impaired stem elongation, reduced starch synthesis, root apex necrosis, interveinal chlorosis and "bronzing", as well as auxin deficiency-like responses, depending on the severity of Zn deficiency (Broadley et al., 2007). Another consequence of low Zn activity within tissues is the extensive oxidative damage, which exerts a negative impact on plant growth (Sharma et al., 2004). An increase in the levels of reactive oxygen species and a decrease in the activity of antioxidant mechanisms may lead to impairment of cellular functions in Zn-deficient plants (reviewed in Cakmak, 2000).

Plants are furnished with specific mechanisms that help them to maintain the concentration of Zn in shoots and roots within the optimal range, thus avoiding Zn-deficiency as well as Zn-toxicity

Abbreviations: AA, reduced ascorbate; DAB, 3,3′-diaminobenzidine; DHA, dehydroascorbate; GSNO, S-nitrosoglutathione; GSNOR, nitrosoglutathione reductase; NBT, p-nitro-blue tetrazolium chloride; NO, nitric oxide; NUR, net uptake rate; RAR, root accumulation rate; RSTR, root to shoot translocation rate.

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and also ensuring an adequate distribution of Zn between aerial and below ground parts of the plant (Sinclair and Krämer, 2012). The work performed over the last two decades illustrated that keeping Zn-homeostasis at those levels requires the concerted action of several transport systems including members of the ZIP family of transporters, CDF (Cation Diffusion Facilitator proteins), P-type ATPase (metal transporting ATPases), NRAMP (natural resistance-associated macrophage proteins), as well as other divalent cation exchange antiporters (Claus et al., 2012; Grotz et al., 2006; Hacisalihoglu and Kochian, 2003; Pedas et al., 2009).

The first point of control of Zn accumulation in plants involves the regulation of Zn-influx to roots which, according to classic studies, occurs through both high and low-affinity mechanisms (Broadley et al., 2007). A remarkable adaptive response early observed in wheat plants is that the capacity of roots to acquire zinc is considerably higher in plants exposed to Zn-deprivation than in plants grown at supra-optimal external Zn-concentrations (Hacisalihoglu et al., 2001). An important yet unexplored question is which signals are required to modulate Zn-acquisition during the transition from low to sufficient Zn-supply and vice versa.

Nitric oxide (NO) has been described as a small signalling molecule in plants (Durner and Klessig, 1999), taking part in several events throughout the whole life cycle, as well as in defence against biotic and abiotic stress. NO steady state concentration in a particular tissue is mainly maintained through synthesis and consumption mechanisms. Endogenous synthesis of NO in plants involves both reductive and oxidative pathways (Gupta et al., 2011), whereas it is consumed through reaction with target molecules, non-symbiotic haemoglobin and is indirectly influenced by the activity of S-nitrosoglutathione reductase (GSNOR) (Gupta et al., 2011). In addition, NO from the atmosphere or synthesized by soil microorganisms, can diffuse into plant tissues where its effects are exerted (Creus et al., 2005). Regulatory effects of NO in biological systems are mediated by protein reversible S-nitrosylation, metal nitrosylation, and Tyr-nitration (Reviewed in Simontacchi et al., 2013). NO reacts with transition metals, mainly Fe, leading to the formation of stable metal nitrosyl complexes related to the rescue of iron deficiency-induced chlorotic phenotypes and the increase of the labile iron pool after NO exposure (Graziano et al., 2002; Simontacchi et al., 2012). Experimental evidence showed increased NO levels in plants suffering from iron deficiency (Graziano et al., 2002), and a prominent role for NO during plant responses to the excess of copper and cadmium has been proposed (Rodríguez-Serrano et al., 2009; González et al., 2012).

Excess of Zn in the culture medium triggered an increase in NO synthesis evaluated *in vitro* in roots of *Solanum nigrum* seedlings, leading to reactive oxygen species accumulation and subsequent programmed cell death in root tips (Xu et al., 2010). This observation as well as the above mentioned role of NO in the control of metal homeostasis, prompted us to speculate on a possible role of NO on Zn accumulation during the transition from low to supra-optimal Zn supply. Accordingly, in this study we analysed the effect of GSNO over Zn acquisition and translocation in wheat plants.

2. Materials and methods

2.1. Plant material, growth conditions and treatments

Seeds of *Triticum aestivum* cv. Chinese Spring were surface-sterilized and germinated onto filter paper in dark. Two days after, seedlings were transferred to 0.7 L plastic pots filled with a nutrient solution (Moriconi et al., 2012) with the following composition: 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.5 mM H_3PO_4, 0.5 mM $MgSO_4$, 50 μM FeNaEDTA, 50 μM $CaCl_2$, 25 μM H_3BO_3, 2 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM H_2MoO_4 and 2.5 mM 2-(N-morpholino)-ethanesulfonic acid (MES) with or without 2 μM Zn provided as $ZnSO_4$, the pH was brought to 6.0 ± 0.1 by the addition of $Ca(OH)_2$. The solution was aerated continuously and completely renewed three times per week. The growth chamber conditions were set at 180 μmol m^{-2} s^{-1} photon flux density, 16 h light/8 h dark cycle, at 22 °C (Moriconi et al., 2012). In order to minimize the contamination with exogenous sources of Zn special care was given to the preparation of solutions employing ultrapure water and materials previously washed with acid solutions. Plants were harvested at days 10, 17, 21, 26 and 30 after sowing. For each harvest, shoots and roots were weighted and processed separately.

The NO donor S-nitrosoglutathione (GSNO) was synthesized by mixing equal volumes of GSH 140 mM and $NaNO_2$ 140 mM prepared in HCl 0.1 N, resulting in a red solution of GSNO 70 mM, being it immediately added to the nutrient solution to a final concentration of 100 μM each time that the nutrient solution was renewed. NO release from GSNO was assessed by electrochemical measurements with a WPI ISO NO Mark II electrode in the culture conditions.

2.2. NO detection by fluorescence microscopy

Roots were incubated for 30 min at 25 °C, in darkness, with 10 μM DAF-FM DA (Sigma–Aldrich) prepared in 10 mM Tris–HCl buffer (pH 7.4). Roots were then rinsed in the same buffer for 15 min. Measurements were performed at $\lambda_{excitation}$ 450–490 nm and $\lambda_{emission}$ 500–550 nm (Corpas et al., 2004) in the root tip and the zone 10 cm above. Fluorescence was monitored with an Olympus BX51 fluorescence microscope. Root sections incubated in 10 mM Tris–HCl buffer (pH 7.4) were used as controls to assess auto fluorescence levels.

2.3. SPAD index and transpiration rate

Chlorophyll content in the youngest fully expanded leaf was estimated using a portable SPAD 502 (Minolta, Konica Minolta Sensing, Inc.). The SPAD index reported per leaf section corresponds to the average of three measurements performed in the leaf tip three hours after the beginning of the light period. Transpiration rate was determined through the loss of water assessed by the change in pot mass between successive measurements (Graciano et al., 2005).

2.4. Ascorbic acid and glutathione content

The ascorbic acid content was measured by reverse phase HPLC. Samples were powered in liquid nitrogen, homogenized with trifluoroacetic acid (TFA) 3% and centrifuged at 4 °C, 13,000 *g* for 5 min. The supernatant was passed through a C-18 column (Eluted Bond C18 VARIAN™). Then, the partially purified samples were filtered and injected into an HPLC system (Shimadzu Co. LC-10Atvp solvent delivery module) equipped with a C-18 column (Varian Chromsep10034.6 mm) and detected at 265 nm (Shimadzu Co. UV–Vis SPD-10Avp detector). The elution conditions were flux of 0.5 ml min^{-1}, with 100 mM phosphate buffer pH 3.0 at 25 °C. Total ascorbate was measured after reducing dehydroascorbic acid (DHA) by mixing the partially purified sample and 100 mM phosphate buffer pH 7 in the presence of 5 mM dithiothreitol (DTT). The reaction was incubated for 5 min and stopped by adding TFA 3%. DHA content was calculated as the difference between total and reduced AA contents (Bartoli et al., 2006). For the measurement of glutathione, samples were powered in liquid nitrogen, ground in trichloroacetic acid 3% and centrifuged at 4 °C, 13,000 *g* for 10 min

and the supernatants used for the assay. Total glutathione (GSH + GSSG) was determined spectrophotometrically at 412 nm in the presence of 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.5 U ml^{-1} glutathione reductase and 0.2 mM NADPH. For quantification of oxidized glutathione (GSSG), 2 μl of vinylpyridine 95% were added to 100 μl of samples supernatant and incubated during 20 min at 25 °C in order to precipitate reduced glutathione. Then, the reaction mixture was centrifuged at 4 °C and 16,000 *g* for 5 min. The GSSG was determined in the supernatant by the reaction described before.

2.5. Reactive oxygen species in situ visualization

In order to localize O_2^- production, roots from intact plants were immersed for 40 min in 0.05% (w/v) p-nitro-blue tetrazolium chloride (NBT) in 100 mM potassium phosphate buffer pH 6.4 and then washed in the same buffer for 15 min. For H_2O_2 visualization, intact plant roots were incubated for 40 min in 2.5 mM 3,3′-diaminobenzidine (DAB) in 100 mM potassium phosphate buffer pH 6.4 and then washed in the same buffer for 15 min. The images for both chemical species were obtained with a Canon 6D 100 mm macro lens.

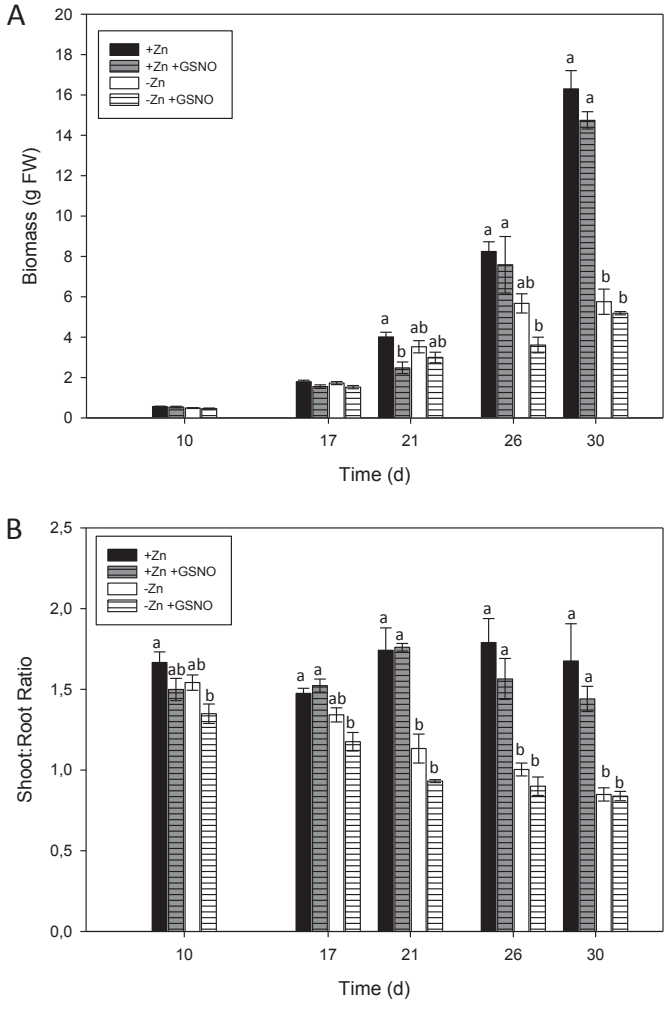

Fig. 1. Effect of GSNO over growth of Zn-well supplied and long-term Zn-deprived wheat plants. Plants were grown from germination until day 30 in nutrient solution with (■) or without (□) 2 μM Zn. When appropriate 100 μM GSNO was applied in the nutrient solution three times a week in control (striped grey bars) or Zn-deprived plants (striped white bars). **A**. Total biomass accumulation, fresh weight per plant, **B**. Shoot:Root biomass ratio. Data correspond to the mean obtained in 3 experiments ($n = 4$). Error bars correspond to SE. Different letters indicate significant different values ($P < 0.05$).

2.6. Zinc measurement

Before the harvest procedure roots were washed in nutritive solution without Zn for 3 min. Shoots and roots were weighted and collected in glass vials. Samples were reduced to ashes at 550 °C during 6 h. Ashes were suspended in nitric acid and analysed for zinc in an AAnalyst 100 atomic absorption spectrometer (Perkin–Elmer). The percentage of Zn accumulation in shoot was calculated as the relation between shoot and total Zn-content in plant multiplied by 100.

2.7. Measurements of Zn uptake and translocation in Zn-recovery experiments

In order to estimate net uptake (NUR), root accumulation (RAR) and root-to-shoot translocation (RSTR) Zn rates, 17-day-old plants grown as above described were transferred to pots containing a complete modified Hoagland's solution, including 2 μM $ZnSO_4$, in the presence or absence of GSNO 100 μM for 48 h. Net uptake (NUR) and accumulation rates in roots (RAR) and shoots (RSTR) were calculated as follows:

$NUR = (Q_{tf} - Q_{ti})(W_r{}^*t)^{-1}$ where Q_{tf} and Q_{ti} are the total content of Zn in plants after and before the treatment, respectively, being t the duration of the treatment (48 h) and W_r the root fresh weight at the end of the experiment.

$RSTR = (Q_{sf} - Q_{si})(W_r{}^*t)^{-1}$ where Q_{sf} and Q_{si} correspond to the final and initial content of Zn in shoots, respectively.

$RAR = NUR - RSTR$.

2.8. Statistical analyses

The mean value obtained for plants grown in the same pot was considered as a single replicate. A minimum of 4 replicates was obtained for each treatment in each experiment. Data was analysed by ANOVA. The means were compared by the post-hoc Tukey test at a significance level of 0.05.

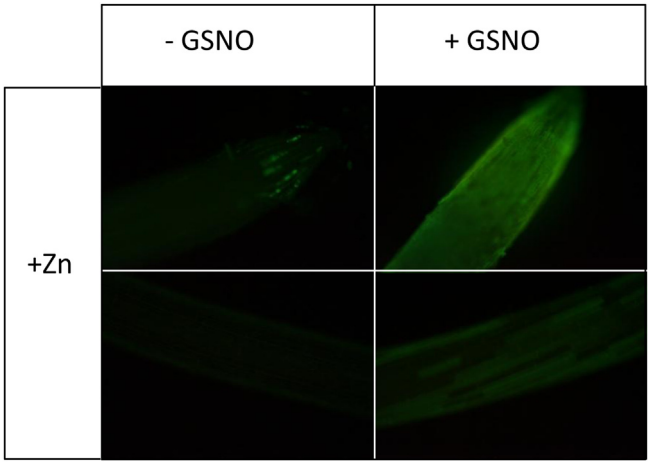

Fig. 2. Nitric oxide detection in roots of 17 day-old wheat plants employing DAF-FM DA and fluorescence microscopy. GSNO was added to the culture solution at final concentration of 100 μM, being measurements performed 3 h later. Root sections (tip and the 10 cm above zone) loaded with 10 μM DAF-FM DA for 30 min and washed for 15 min, were observed ($\lambda_{excitation}$ = 450 – 490 nm and $\lambda_{emission}$ = 500 – 550 nm). Images are representative of three independent experiments.

Table 1
Effect of NO on Zn utilization efficiency indicators in Zn-deprived plants.

Treatment	Zn use efficiency (g FW μmol^{-1})	Zn utilization efficiency (g^2 FW μmol^{-1})	Zn productivity (g FW μmol^{-1} d^{-1})	Zn accumulated productivity (g FW μmol^{-1} d^{-1})
−Zn	65 ± 3	122 ± 9	6.9 ± 0.4	12.5 ± 0.9
−Zn + GSNO	70 ± 4	115 ± 9	7.0 ± 0.4	12.4 ± 0.9

Plants were grown from germination until day 17 in a nutrient solution without (−Zn). GSNO was applied in the culture solution three times a week. Utilization efficiency indicators for whole plants were calculated as described by Moriconi and Santa-Maria (2013). Data correspond to the average of 3 experiments with SE. No statistical significant differences were found between treatments ($P < 0.05$).

3. Results

3.1. Effect of long term GSNO treatment in plants grown at different Zn supplies

Wheat plants (*T. aestivum* cv. Chinese Spring) were allowed to grow in a complete nutrient solution, containing 2 μM $ZnSO_4$ (control plants) or in the same medium without addition of Zn (Zn-deprived plants). The effect of external Zn availability on plant growth was firstly analysed. After 30 days of growth in a solution without Zn-addition plants showed a lower biomass as compared to control plants (Fig. 1A). This decrease of biomass accumulation was parallelled by a decrease of the shoot:root ratio after day 21 of Zn-deprivation (Fig. 1B).

As NO donor S-nitrosoglutathione (GSNO) was used, which is a nitrosothiol that has been proposed as an endogenous carrier and storage form of NO in biological systems (Corpas et al., 2013). The kinetic of NO release from GSNO was here assessed by electrochemical measurements (ISO NO Mark II, WPI electrode). This procedure indicated that the concentration of NO in a 100 μM GSNO aerated nutrient solution reached a maximum of 0.6 μM after 5 h. An important question in experiments based on the use of NO donors is whether or not addition of the donor actually leads to a change of endogenous NO levels. In order to explore this issue, fluorescence associated to the NO tracer DAF-FM DA was also measured. By exploring two different zones of roots, an increase of the endogenous NO level assessed three hours after the addition of GSNO to the medium was confirmed (Fig. 2).

The addition of this compound to the culture solution did not significantly affect the accumulation of total biomass (shoot + root) neither in control nor in Zn-deprived plants (Fig. 1A). Consistently, measurements of Zn utilization efficiency made through the use of four different algorithms (Moriconi and Santa-Maria, 2013) indicated that the presence of GSNO in the culture solution did not exert a marked effect on Zn utilization (Table 1).

Senescence, as estimated by chlorophyll content decay (Uauy et al., 2006), was evaluated in leaves through the measurement of the SPAD index, which is related with total chlorophyll content when measurements are performed under constant irradiance. In this regard, exposure to GSNO led to an early decrease of the SPAD

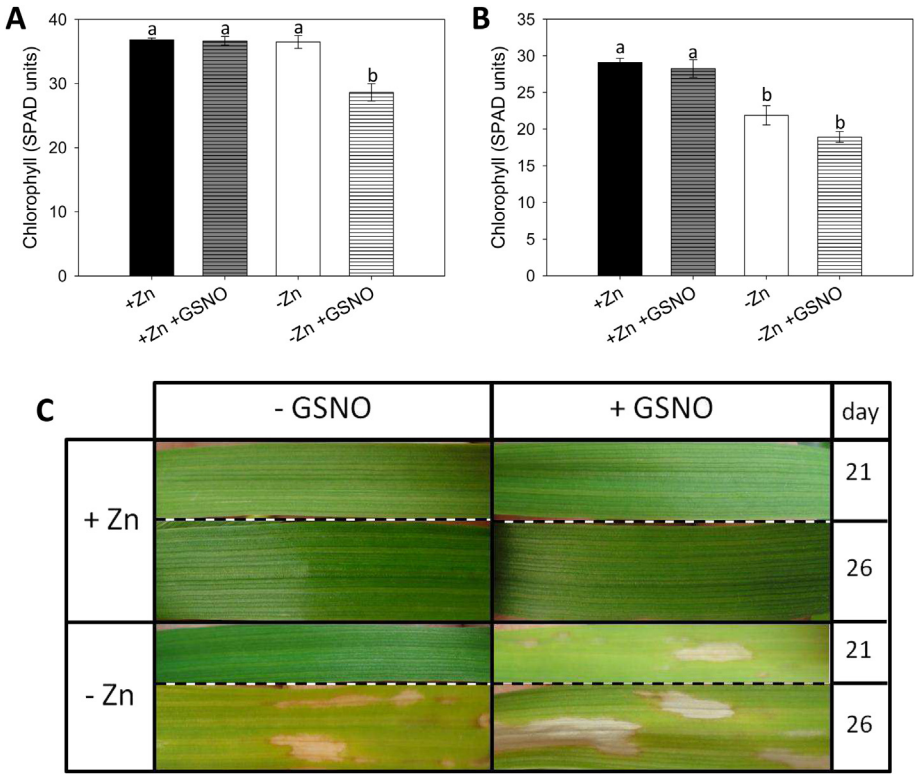

Fig. 3. Effect of Zn deprivation and GSNO addition on chlorophyll content and necrotic spots visualization. **A**. SPAD index measured in the youngest fully expanded leaf from control (+Zn) and Zn-starved plants (−Zn) after 21 days of growth in the presence or absence of 100 μM GSNO. **B**. SPAD index measured after 26 days. Data correspond to the average of 2 experiments with SE. **C**: Representative images of youngest fully expanded wheat leaves from control and Zn-starved plants taken after 21 and 26 days of growth with or without 100 μM GSNO.

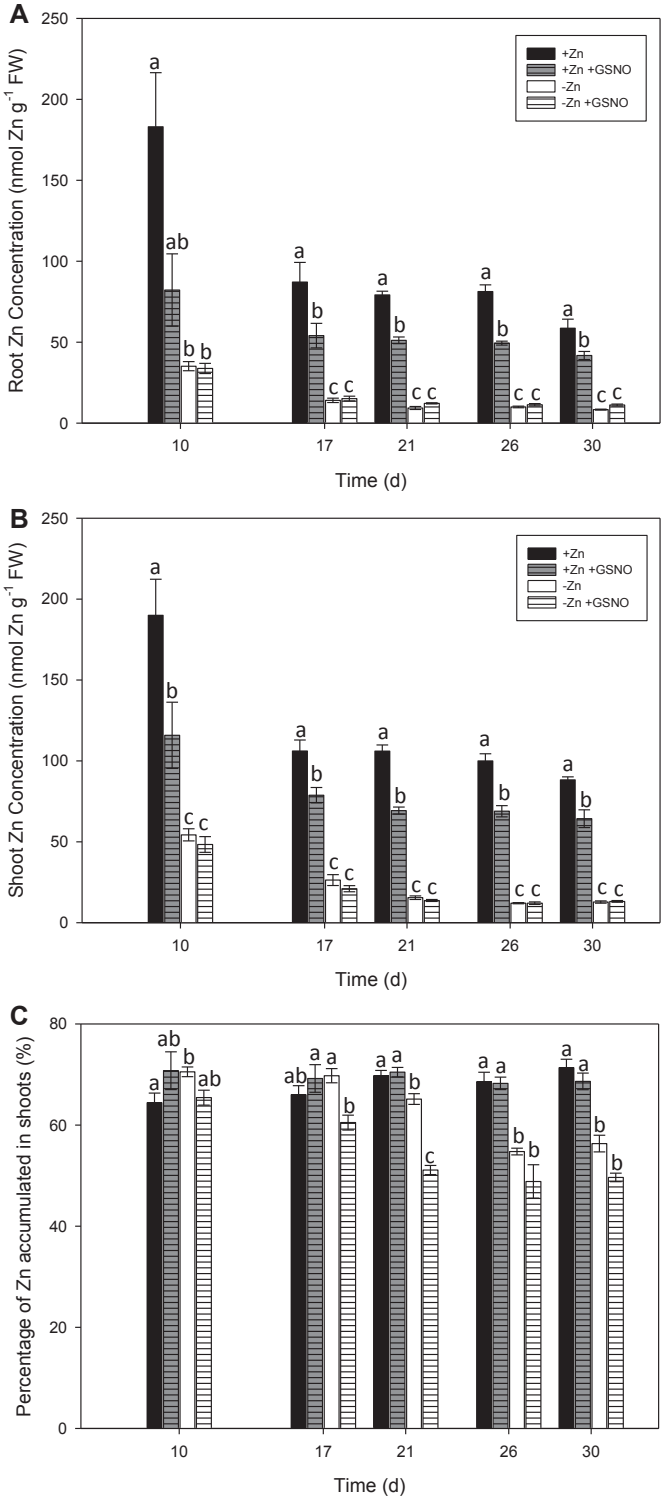

Fig. 4. Effect of GSNO on Zn concentration in Zn-well supplied and long-term Zn-deprived wheat plants. Plants were grown from germination until day 30 in a medium with (■) or without (□) 2 μM Zn, 100 μM GSNO was applied to the nutrient solution three times a week in well-supplied (striped grey bars) or Zn-deprived plants (striped white bars). **A**. Zn concentration in roots, **B**. Zn concentration in shoot, **C**. Percentage of Zn accumulated in shoots referred to the content of Zn in the whole plant. Data correspond to the mean obtained in 3 experiments ($n = 4$). Error bars correspond to SE. Different letters indicate significant different values ($P < 0.05$).

index (21 d of treatment), relative to that measured in plants deprived of Zn in the absence of NO donor (Fig. 3A and B). Consistently, visible necrotic spots, which are characteristic of Zn deficiency, were observed after 26 days in Zn-starved plants grown without GSNO but with GSNO present, necrotic spots were clearly observed after 21 days of Zn-deprivation (Fig. 3C).

3.2. Addition of GSNO affects Zn concentration in plant tissues

The concentration of Zn in shoots and roots of plants growing in the presence or the absence (no addition) of Zn in the culture medium was analysed. As expected, Zn concentration in both plant organs was lower in plants growing without Zn-addition as compared to the control plants, throughout the study's duration (Fig. 4A and B). The presence of 100 μM GSNO in the nutrient solution did not affect the concentration of Zn in shoots and roots of Zn-deprived plants (Fig. 4A and B). On the other hand, for plants grown in the presence of 2 μM Zn a clear effect of the NO donor was observed. In this case, the concentration of Zn in roots and shoots sharply decreased relative to that observed for Zn-well supplied plants grown in the absence of GSNO (Fig. 4A and B). In Zn-well supplied plants exposure to the NO donor did not lead to changes in Zn-partitioning between shoots and roots (Fig. 4C). Instead, in plants deprived of Zn, allocation of this element to shoots tends to decrease, being this process accelerated by the presence of the NO donor (Fig. 4C).

Since exogenously applied NO affected Zn concentration, we explored the possibility that Zn availability in the medium could reciprocally affect NO homeostasis in plants. In order to obtain an assessment of the possible changes of endogenous NO levels after long-term growth in the conditions here assayed, the NO sensitive fluorescent probe DAF-FM DA was used. Roots from 17-day old plants were employed, as at this time Zn-deprived plants showed low Zn content while no major differences in total biomass or shoot: root ratio were observed. Since no clear differences were detected in the fluorescence attributable to NO between plants exposed long-term to Zn-deficiency and those grown at 2 μM Zn (data not shown), this issue remained unsolved.

NO and reactive oxygen species interact mainly through the diffusion-controlled reaction between NO and O_2^-. The amount of O_2^- in root tips, assessed through NBT staining, was higher in Zn depleted plants as compared to control roots, as it was previously described (Cakmak, 2000; Sharma et al., 2004), and the presence of the NO donor decreased the detection of this reactive oxygen species, particularly in Zn-deprived plants (Fig. 5A). DAB staining was employed to analyse H_2O_2 generation, showing a differential pattern of accumulation between +Zn and −Zn roots, which was strongly affected by the addition of GSNO (Fig. 5B).

3.3. Addition of GSNO modulates the accumulation of Zn during the recovery from Zn-deprivation

Results above indicate that the presence of an NO donor in the culture solution modulates long-term accumulation of Zn. An important aspect is whether or not the NO donor could modulate the transition of Zn accumulation that takes place when Zn supply is suddenly modified. In order to study the acclimation response, plants grown for 17 d under conditions of Zn-scarcity were transferred to a complete nutrient solution for 48 h (recovering period), and net Zn uptake (ZnNUR) and Zn root to shoot translocation (ZnNTR) rates were measured. ZnNUR from a 2 μM Zn solution was almost four times higher for plants previously grown under Zn-deficiency than for plants kept since germination at 2 μM Zn (Fig. 6), indicating that long-term Zn-deprivation up-regulates the potential ZnNUR which is in agreement with previous observations (Hacisalihoglu et al., 2001). We explored the possibility that the

presence of the NO donor either during the Zn deprivation period (17 d) and/or during the recovering period (48 h) would affect ZnNUR. The addition of GSNO during the long-term Zn deprivation period (but not during the recovery period) did not affect ZnNUR, as compared to plants long term exposed to Zn deprivation (Fig. 6A). However, the presence of GSNO only during the recovery period (48 h) strongly affected ZnNUR (Fig. 6A). Furthermore, the magnitude of ZnNUR when the NO donor was included in the culture solution during the whole experiment (deprivation + recovery periods) was similar to that observed when NO was present only during the recovery period. These results indicate that the increase of Zn-uptake is suppressed in a reversible manner by the effective presence of the NO donor.

Results above could be dissected into two components: Zn-accumulation in roots and in shoots, respectively. It was found that the rate of Zn-accumulation in roots during the recovering period was not influenced by the presence of the NO donor either before or during the exposure to a 2 μM Zn solution (Fig. 6C). On the other hand, translocation rate of Zn to the aerial part essentially resembles the pattern above described for ZnNUR (Fig. 6B) indicating that modifications of ZnNUR driven by NO involve an effect of this compound on the net movement of Zn to the shoot. Since differences in transpiration rate can potentially affect long distance movements of nutrients and consequently their concentration in the aerial part, it was evaluated in plants growing under control conditions, plants without Zn supply, and in both groups exposed to GSNO 100 μM. The transpiration rate was 0.21 ± 0.01 nmol H_2O h^{-1} cm^{-2} for control plants being non-significant differences observed among treatments. This result highlights that the effect of GSNO might be attributed essentially to a regulatory effect of NO on long-distance transport of Zn that does not depend on transpiration rate.

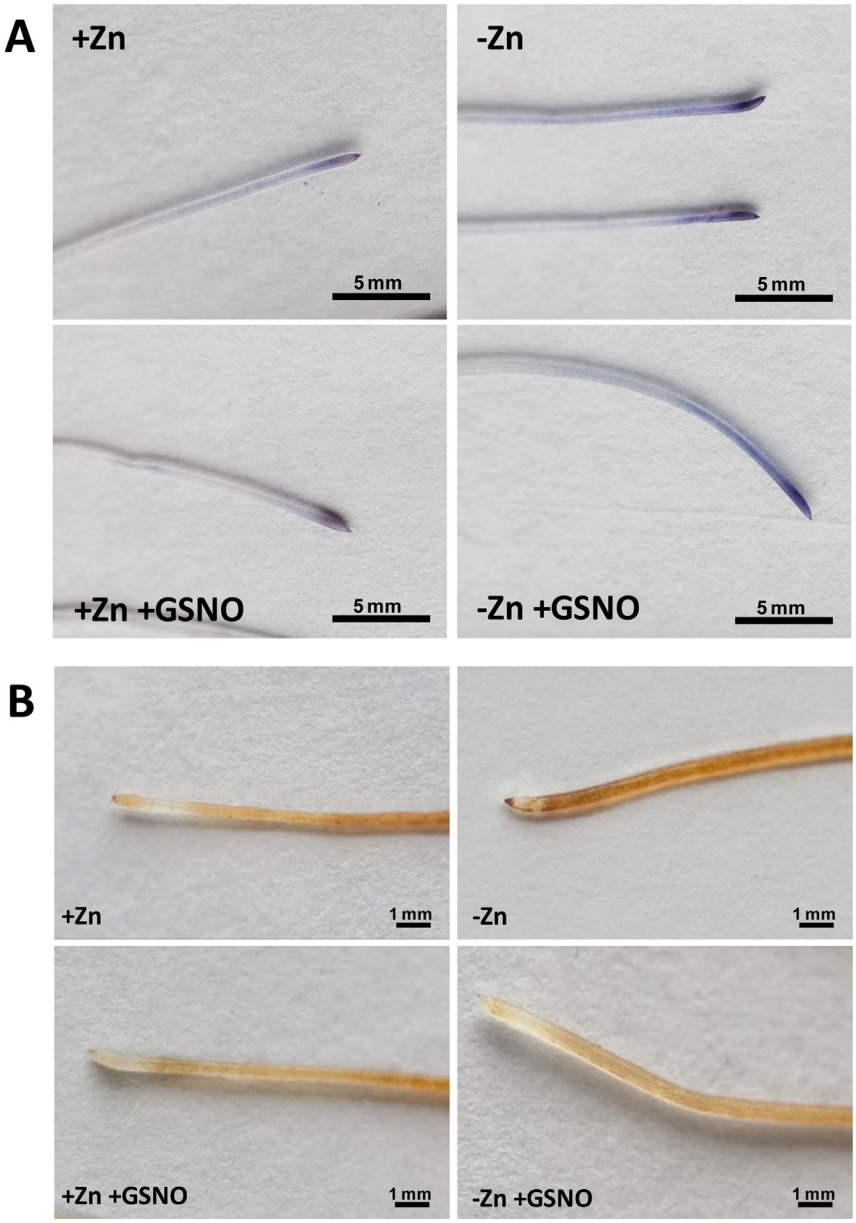

Fig. 5. Effect of Zn deprivation and GSNO addition on reactive oxygen species generation. Plants were grown from germination until day 17 in a medium with (+Zn) or without (−Zn) 2 μM Zn, 100 μM GSNO was applied in the nutrient solution three times a week in well-supplied (+Zn + GSNO) or Zn-deprived plants (−Zn + GSNO). **A.** Visualization of O_2^- employing NBT staining. **B.** Visualization of H_2O_2 after incubation with DAB.

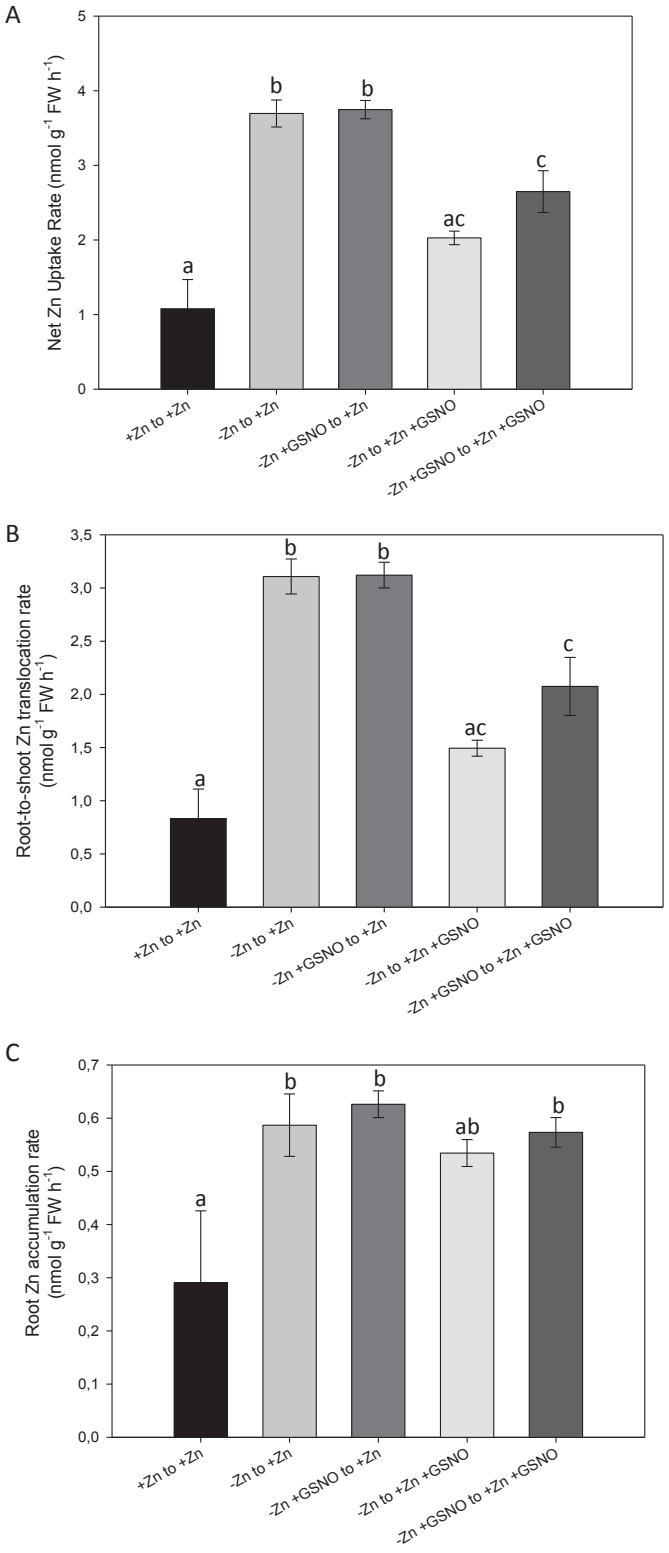

Fig. 6. Effect of GSNO on Zn uptake and translocation in wheat plants. Symbols are as follows: +Zn to +Zn, corresponds to plants grown in complete solution during 17 d and transferred to a complete solution for 48 h; −Zn to +Zn, corresponds to plants grown in the absence of Zn during 17 d and transferred to a complete solution for 48 h; −Zn + GSNO to +Zn, corresponds to plants grown in the absence of Zn and the presence of 100 μM GSNO during 17 d and transferred to a complete solution for 48 h; −Zn to +Zn + GSNO, corresponds to plants grown in the absence of Zn during 17 d and transferred to a complete solution for 48 h in the presence of 100 μM GSNO; −Zn + GSNO to +Zn + GSNO, corresponds to plants grown in the absence of Zn and the presence of 100 μM GSNO during 17 d and transferred to a complete solution for 48 h in the presence of 100 μM GSNO. **A**. Net Zn uptake rate (ZnNUR), **B**. Net Zn translocation rate (RSTR), **C**. Root Zn accumulation rate (RAR). Data correspond to the mean obtained in 2 experiments ($n = 9$). Error bars correspond to SE. Different letters indicate significant different values ($P < 0.05$).

Induction or repression of the activity of several ion transport systems has proved to involve the control of the redox status. Therefore, the effect of the NO donor on the redox balance was studied after the 48 h period of recovery from Zn-deprivation. In roots, the content of total glutathione in plants exposed for 17 d to Zn-deprivation followed by 48 h of Zn supply was not affected by the addition of GSNO, either during the long term Zn-deprived period or in the 48 h of recovery (Table 2). In addition, the presence of the oxidized form of glutathione was negligible in all the analysed samples. The content of the reduced form of ascorbate in roots tended to increase by the presence of GSNO during the Zn-supply recovering period as compared to plants always kept in absence of the NO donor (Table 2). Plants exposed to the presence of GSNO either before or during the recovery of Zn-deprivation exhibited a significantly higher ratio of reduced ascorbate relative to the total ascorbate content (Table 2).

4. Discussion

The availability of Zn in soils extends over a very wide scale that includes deficient and highly toxic extremes (Broadley et al., 2007). Under such a broad range of external Zn availability, maintenance of plant tissue Zn-concentration within a narrow range, particularly in metabolically active subcellular compartments, is a prerequisite for survival and growth in both low and high Zn environments (Hall, 2002). Low Zn availability affects crop yield and food production worldwide, which has led to the idea of making a more efficient use of zinc in agricultural soils (Takkar and Walker, 1993). Several processes could be involved in determining the efficiency in the acquisition and utilization of Zn in a particular crop species, including mechanisms that modify Zn bioavailability in the soil solution, uptake by roots, root to shoot translocation, subcellular compartmentalization as well as the utilization of Zn to generate plant biomass (Hacisalihoglu and Kochian, 2003). Given the well-known role played by NO in the control of the homeostasis and utilization of iron (Graziano and Lamattina, 2007), we explored whether this small signalling molecule could play a similar role for Zn. In this work evidence is offered that, for wheat, the exogenous addition of NO does not influence the efficiency of utilization of Zn to generate fresh biomass following long-term Zn-deprivation and that this molecule exerts a negative effect on plant performance by accelerating senescence and the appearance of Zn-deficiency symptoms. This effect contrasts with the antioxidant and anti-senescent role of NO in plants exposed to different types of stress as well as in diverse physiological processes (Jasid et al., 2009), and could be associated with a rapid NO-related decrease of Zn-allocation to shoots.

Results here reported support the notion that exogenous addition of GSNO, which is parallelled by the subsequent increase of NO levels within tissues, contributes to modulate the acquisition of Zn in wheat plants grown at supra-optimum, non-toxic, Zn concentrations. Acquisition of Zn by wheat plants has been early studied by several authors (Broadley et al., 2007). A seminal observation made during the course of those studies was that the influx of Zn from a definite diluted Zn concentration is considerably lower in plants grown in Zn-sufficient than in a Zn-deprived media (Hacisalihoglu et al., 2001). The signals involved in that process remain essentially unknown. Results here reported introduce NO as a possible player in the regulation of net Zn-uptake during the

Table 2
Antioxidant status in roots of wheat plants after a 48 h recovery of Zn-deprivation.

Treatment		Glutathione	AA	DHA	AA/(AA + DHA) (%)	AA/DHA
17 d	**48 h**					
−Zn	+Zn	160 ± 2[a]	87 ± 15[a]	53 ± 4[a]	51 ± 6[a]	1.6 ± 0.2[a]
−Zn + GSNO	+Zn	145 ± 7[a]	119 ± 5[ab]	39 ± 2[a]	76 ± 1[b]	3.05 ± 0.09[b]
−Zn	+Zn + GSNO	147 ± 5[a]	128 ± 3[b]	47 ± 4[a]	73 ± 2[b]	2.7 ± 0.1[b]

Wheat plants were grown for 17 days under conditions of no Zn addition with or without 100 μM GSNO, then transferred to the correspondent treatment for 48 h. AA, reduced ascorbate, DHA, oxidized ascorbate. Concentrations are in nmol g^{-1} FW. Data correspond to the average of 2 experiments with SE. Different letters means significant differences between treatments.

recovery from Zn-deprivation. Experiments carried out with plants exposed to Zn deficiency for 17 d and then transferred to a medium with optimal Zn availability indicated that net Zn uptake rate was lowered by the presence of GSNO in a reversible manner. Interestingly, the rate of Zn accumulation in roots remained unaffected by the presence of GSNO, while Zn net translocation was sharply down-regulated by the presence of 100 μM GSNO in the nutrient solution during the recovery period. This result indicates that net long-distance transport of Zn to shoots is a target for the action displayed by GSNO. This might implicate an effect on several processes including the balance between influx and efflux in the root cells involved in Zn-capture, the radial transport of Zn, its delivery to xylem vessels and its recirculation via phloem. NO could modulate the activity of the transporters specifically involved in these processes via post-translational modifications, such as nitrosylation and nitration, of the transport proteins themselves and/or of those that participate in the regulation of their activity. An interesting fact here observed is that the effect of GSNO was relieved when this compound was subtracted from the culture solution, being this observation consistent with a non-permanent effect of GSNO on the transport systems determining net Zn-uptake. Since changes in redox status can be elicited by NO (Patel et al., 1999) and have been reported to occur during the course of Zn-deficiency (Oteiza, 2012; Sharma et al., 2004), we explored whether or not changes in redox status match changes in Zn-uptake during recovery from Zn-deprivation. The pattern of glutathione accumulation was not affected after Zn recovery either in presence or absence of GSNO. Previous studies pointed out a possible role for NO in the regulation of glutathione synthesis. In this regard after 12 h exposure to 1 mM GSNO the levels of γ-*ecs* and *gshs* transcripts coding for enzymes involved in the referred process were increased in roots of *Medicago truncatula*, being an increase of GSH content observed after 1 mM sodium nitroprusside (SNP) exposure (Innocenti et al., 2007). Treatment of wheat plants with 0.1 mM SNP lead to a 5-fold increase in GSH content (Groppa et al., 2008). However, glutathione pool was not-significantly affected after exposure of tobacco cv BY-2 cells to 0.5 mM SNP (de Pinto et al., 2002). These divergent observations could be probably related with the nature of NO donor and the experimental conditions. On the other hand, increases in the content of AA relative to total ascorbate (AA + DHA) observed when GSNO was applied along with, or following, Zn-deprivation support the hypothesis that this compound plays an antioxidant role in the roots of plants exposed to different Zn-supplies. Noticeably this pattern differs from that displayed by Zn-NUR during the recovery of Zn-deprivation, indicating that observed changes in the antioxidant response may be not sufficient to explain the reversible changes in Zn-acquisition driven by the presence of GSNO.

Since the biological action of NO, from endogenous synthesis or exogenously applied, largely depends on its interaction with proteins through nitration or S-nitrosylation (Batthyány et al., 2005; Martínez-Ruiz et al., 2004), an alternative and yet unexplored possibility is that the effect here observed rests on the S-nitrosylation or Tyr-nitration of a specific set of proteins. The increased generation of peroxynitrite due to the simultaneous presence of high levels of NO and O_2^- might contribute to protein nitration events. Examples of post-translational modifications of proteins by NO include the modulation of auxin signalling through S-nitrosylation of the TIR1 auxin receptor (Terrile et al., 2012), and the inactivation of MnSOD through nitration (MacMillan-Crow et al., 1996). It has been shown that S-nitrosylation of NADPH oxidase, AtRBOHD, at Cys 890 impairs its ability to generate O_2^- limiting the hypersensitive response (Yun et al., 2011). *In vitro* S-nitrosylation of catalase and glycolate oxidase has been also observed, suggesting that the level of H_2O_2 could be affected under abiotic stress where increased levels of S-nitrosylated proteins were reported (Ortega-Galisteo et al., 2012). Further studies should be carried out in order to analyse NO-dependent post-translational modification of proteins involved in Zn-acquisition or translocation.

A major role of NO in plant adaptation was described first in pathogen defence and later for a wide spectrum of biotic and abiotic stresses (Mur et al., 2013). Noticeably, a key participation of NO has been described in plants suffering from both excess and deficiency of iron. In tomato plants exposed to Fe deficiency the presence of NO was required for the accumulation of mRNAs coding for major components of the iron transport machinery (Graziano and Lamattina, 2007). In *S. nigrum*, it has been reported that endogenous NO is produced as a consequence of an excess of Zn (Xu et al., 2010). Here no clear difference was observed in NO levels between Zn-deprived and Zn-well supplied 17-day-old wheat plants through the use of DAF-FM DA, which constitutes a highly sensitive methodology. Since the steady state concentration of NO in a tissue depends on the balance of its kinetics of synthesis, through different pathways, or release from cellular storage compounds and the rate of consumption by cellular targets, this finding cannot rule out alterations in generation rates or decay mechanisms leading to a constant steady state concentration. It has been described that Zn deficiency induces O_2^- production, mainly through a NADPH oxidase associated to the plasma membrane (Cakmak and Marschner, 1988), thus it is possible that in our experimental conditions the removal of NO through reaction with O_2^- could contribute to explain the absence of NO differences when DAF-FM DA was used for imaging NO by fluorescence microscopy.

On the other hand, it is worth mentioning that in *S. nigrum* the addition of L-NAME, an inhibitor of NO synthesis through an arginine dependent route (Xu et al., 2010), led to a decrease of Zn-accumulation suggesting that NO could favour hyper-accumulation of this element. In the same way it has been reported that in *Arabidopsis thaliana,* NO contributes to cadmium toxicity, favouring Cd^{2+} uptake, by initiating a cellular pathway resembling that activated upon iron deprivation (Besson-Bard et al., 2009). These results clearly differ to those here reported. We hypothesized that this difference could be attributed either to differences between monocot and dicots and/or to a differential

effect of NO accumulation in the transition from deficient to sufficient Zn-levels as compared to the transition from sufficient to toxic levels.

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Contributions

Agustina Buet has conducted the experiments, performed the statistical analysis and contributed to data interpretation. Jorge I. Moriconi made ion measurements and contributed to data analysis. Guillermo Santa María contributed to the experimental design, data interpretation and manuscript writing. Marcela Simontacchi designed the experiments, interpreted data and wrote the manuscript.

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