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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