

Review

The role of nitric oxide (NO) in plant responses to disturbed zinc homeostasis

Selahattin KONDAK^{a,b,*}, Árpád MOLNÁR^a, Dóra OLÁH^{a,b}, Zsuzsanna KOLBERT^a

^a Department of Plant Biology, University of Szeged, Közép fasor 52., 6726, Szeged, Hungary

^b Doctoral School of Biology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52., 6726, Szeged, Hungary

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ABSTRACT

Zinc (Zn) is an essential trace element for living organisms including plants, and sub- or supraoptimal amounts of available Zn induce stress responses. Nitric oxide (NO) signal molecule and its reaction products, the reactive nitrogen species (RNS) are involved in the regulation of numerous abiotic stress responses. Our knowledge regarding Zn deficiency is incomplete, thus in a preliminary experiment we showed that there is a correlation between the capability of mild Zn deficiency tolerance and the capability of root NO production. Additionally, in the case of severe Zn deficiency, the NO level responses proved to be species-dependent. Our computational analysis highlighted that among Arabidopsis Zn transporter proteins (ZIPs, MTPs, HMAs) there are numerous targets of NO-dependent S-nitrosation and tyrosine nitration indicating the regulatory role of NO in plant Zn transport. These observations support the putative role of NO in Zn deficiency responses, but further experimental confirmation is needed. Regarding excess Zn, the previously described oxidative stress processes have been supplemented by recent research which found that also RNS metabolism is affected and RNS-related signaling is increased in plants grown in the presence of supraoptimal Zn supply, but the alterations depend on the sensitivity of the plant species, the Zn concentration, and the duration of the treatment. According to the available data, the stress-relieving effect of exogenous NO is mediated by several mechanisms, such as the alleviation of oxidative stress due to the activation of antioxidants and the reduction of *in planta* Zn accumulation. Similar to the bulk form, nano-ZnO induces nitro-oxidative stress in plants in a way dependent on plant species, concentration, and particle size. Moreover, exogenous application of NO improves the performance of ZnO nanoparticle-treated plants by decreasing Zn ion accumulation, improving photosynthesis, and reducing oxidative stress due to the upregulation of antioxidants.

1. Introduction

Zinc (Zn) is an essential microelement which plays an important role in a number of physiological processes in living beings (Laemmli, 1970). Among the major biochemical functions displayed by Zn in plant cells, appropriate protein folding, catalytic and regulatory functions in enzymes have to be considered (Broadley *et al.*, 2007; Zheng *et al.*, 2021). The strong interaction between Zn and the ligands allows the folding of small protein domains, which is not possible in the absence of a metal (Clemens, 2021). Zn plays an important role in plant metabolism by influencing the activities of hydrogenase and carbonic anhydrase, in stabilization of ribosomal fractions and in synthesis of cytochrome. Plant enzymes activated by Zn are involved in carbohydrate metabolism, maintenance of the integrity of cellular membranes, protein synthesis,

regulation of auxin synthesis and pollen formation. The regulation and maintenance of gene expression required for the tolerance against environmental stresses are Zn-dependent (Yuvaraj and Subramanian, 2020). Zn binding sites can be found mainly in the Zn finger domain containing proteins, membrane lipids, and DNA/RNA molecules. Hence, protein metabolism, gene expression and membrane integrity depend to varying degrees on the maintenance of optimal Zn concentration in metabolically active pools (Broadley *et al.*, 2007). Therefore, plants require adequate amount of Zn derived primarily from the soil, where Zn²⁺ accounts for up to 50% of the soluble Zn fraction (Cakmak, 2002; Hacısalihoglu and Kochian, 2003, Noulas *et al.*, 2018). Roots absorb Zn from the soil solution primarily in the form of Zn²⁺ ions or complexes with organic acids (Palmgren *et al.*, 2008), and translocate it through the xylem into the above-ground plant parts. The uptake of Zn from the soil

* Corresponding author.

E-mail address: kondaksela@bio.u-szeged.hu (S. KONDAK).

is mediated by protein transporters of the ZIP (Zn Regulatory Transporter – Iron Regulatory Transporter like Protein) family identified in *Oryza sativa*, *Hordeum vulgare* and *Arabidopsis thaliana* (Milner *et al.*, 2013; Tiong *et al.*, 2014; Bashir *et al.*, 2013). These transporters can be found in plasma membranes of the cells, in the central cylinder and in the tonoplast (Milner *et al.*, 2013). The transport of Zn from rhizodermal and cortex cells into the xylem is performed by specialised protein transporters, HMA2 and HMA4 (Heavy Metal ATPase), located on plasma membrane of the vascular bundle cells in the root and shoot (Hussain *et al.*, 2004). Zn cations can also be transported through the extra-cellular apoplastic pathway in areas without fully developed Casparian strip (White *et al.*, 2002). The translocation of Zn ions through the shoot into the assimilation organ (mainly the seeds) happens in the phloem (Deinlein *et al.*, 2012).

If the phytoavailability of soil Zn is limited, plants may suffer from the consequences of Zn deficiency; however, plant species differently tolerate suboptimal Zn supply. Among crops, rice, bean, maize and grapes are highly sensitive to low Zn supply, lettuce, tomato and barley show medium sensitivity, whereas pea, carrot and alfalfa are tolerant species (Alloway, 2008). In general, sensitive species show symptoms like stunted growth, chlorosis and smaller leaves or spikelet sterility as the effect of short-term Zn limitation. Inadequate Zn supply can also adversely affect the quality of harvested products, and can intensify the susceptibility of plants to injuries caused by e.g. drought, high temperature, and viral, bacterial or fungal diseases (Ullah *et al.*, 2019; Cabot *et al.*, 2019). Suboptimal Zn content in the plant tissues negatively affects the capacity for water uptake and transport and also the synthesis of tryptophan which is a precursor of indole-acetic-acid (IAA) resulting in inadequate production of this phytohormone. Regarding the molecular mechanism of Zn deficiency response, Lilay *et al.* (2021) recently showed that the F-group bZIP transcription factors bZIP19 and bZIP23 function as Zn sensors by binding Zn²⁺ ions to a Zn-sensor motif thereby acting as major regulators of the Zn deficiency response in the model species *Arabidopsis thaliana*.

In addition to insufficient Zn supply, Zn overload adversely affects the physiological processes of plants as well. The optimal level of Zn required for the majority of crops falls between 30 and 200 µg Zn g⁻¹ dry weight Marschner (2012). Zn concentrations above this range cause damage, and the threshold of Zn toxicity highly varies among the plant species (Kaur and Garg, 2021). The excess Zn-induced growth inhibition is partly due to direct effects caused by the accumulation of Zn ions in cells. Such effects include inhibition of cell division and elongation, decreased cell viability, secondary cell wall thickenings (e.g. lignification, pectin and callose deposition), and inhibition of auxin transport (reviewed by Kaur and Garg, 2021). Moreover, surplus Zn influences plant growth due to indirect effects like the limitation of water and nutrient transport. Inhibition of water movement by elevated Zn supply can be explained by recent observations that excess Zn reduces the expression of aquaporin (PIP) genes both in the root and shoot of plant species like lettuce, barley, and *Brassica rapa* (reviewed by Kaur and Garg, 2021). Excess Zn competes with other ions (magnesium, manganese, copper, iron etc.) for binding sites in absorption regions of roots (Tewari *et al.*, 2008) resulting in modified nutrient homeostasis in Zn-stressed plants. Additionally, surplus Zn can alter nutrient uptake also by influencing the activity of membrane transporters and ion channels as well as causing the depolarization of root cell membranes, thus increasing membrane permeability (Bazihizina *et al.*, 2014). Further evidences support that excess Zn levels influence the distribution of nitrogen, phosphorus, potassium, magnesium, calcium, iron etc. in the plant body (Sagardoy *et al.*, 2009; Garg and Singh, 2018). Excess Zn affects photosynthesis due to multiple events (e.g. reduced leaf area, disrupted photosynthetic electron transport, limited stomatal conductance, enhanced respiration). According to Kaur and Garg (2021), the main reason of Zn toxicity on photosynthesis can be considered as the disturbed mineral nutrition (mainly iron, manganese, magnesium). Beyond the above, Zn is able to indirectly cause secondary oxidative

stress by replacing other essential metal ions in their catalytic sites thus disrupting metabolism (Schützendübel and Polle, 2002), and/or by hindering electron transport chains. During Zn-triggered oxidative stress, reactive oxygen species (ROS), such as superoxide anion (O₂•⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (•OH) are commonly generated as revealed by several authors (e.g. Morina *et al.*, 2010; Jain *et al.*, 2010). Overproduction of ROS negatively affects plant cells at multiple levels, as it causes damage to the genetic material, the protein pool, and lipids. Regarding Zn-induced genotoxicity, the assumed mechanisms involve irregular scattering of chromosomes, formation of anaphasic bridges inability of broken chromosomes to recombine, inadequate chromatid separations, and micronucleus formation (Ola-dele *et al.*, 2013; Sidhu, 2016). At the proteome level, excess ROS directly affects proteins either by oxidation of amino acid side chains or by reactions with aldehydic products of lipid peroxidation or glycosylation yielding to carbonyl groups in the protein molecule (Madian and Regnier, 2010). Modified proteins can form aggregates or can be targets for degradation. Regarding excess Zn-triggered lipid modifications, elevated lipoxigenase activity may stimulate lipid peroxidation thus influencing membrane integrity and permeability (Goodarzi *et al.*, 2020).

Beyond ROS, also reactive nitrogen species (RNS) can affect lipids, proteins and nucleic acids. The main representative of RNS is nitric oxide (NO) which is a redox active, gaseous molecule being present in cells in rapidly interchangeable forms such as nitric oxide radical (•NO), nitroxyl anion (NO⁻), nitrosonium cation (NO⁺). Endogenous NO production in higher plants uses nitrite as the major substrate (Astier *et al.*, 2018), while in algae, similar to animals, NO is primarily derived from L-arginine (Astier *et al.*, 2021) indicating that reductive pathways of NO formation have become dominant during the evolution of terrestrial plants (Fröhlich and Durner, 2011). Additionally, NO formed in the atmosphere by natural and anthropogenic activities (Chaparro-Suarez *et al.*, 2011) and in soils NO derived mainly from nitrification and denitrification also contributes to NO levels of the plant (Medinets *et al.*, 2015, reviewed by Ma *et al.*, 2020). Based on the evidences obtained in different plant systems, the perception of NO signal happens mainly at the proteome level, through NO-dependent post-translational modifications (PTMs) of specific protein targets. From the reaction of NO with glutathione, S-nitrosoglutathione (GSNO) is formed which is the main substance mediating protein S-nitrosation reversibly affecting cysteine (Cys)-containing proteins causing activation or inactivation due to the formation of SNO groups (Hess *et al.*, 2005). Protein S-nitrosation has been established as a significant route by which NO transmits its ubiquitous cellular function (Hess *et al.*, 2005; Spadaro *et al.*, 2010; Astier and Lindermayr, 2012). The other biologically relevant NO-dependent PTM is tyrosine (Tyr) nitration mediated by peroxynitrite (ONOO⁻). During the reaction, a nitro-group is attached to the aromatic ring of Tyr yielding 3-nitrotyrosine thus irreversibly modifying the protein structure. In case of most plant proteins examined in detail, tyrosine nitration causes activity loss and possibly assigns the proteins for degradation (Kolbert *et al.*, 2017). Beyond proteins, NO/RNS affects fatty acids and nucleic acids by nitration reactions. According to recent literature, nitro-fatty acids (e.g. nitro-linolenic acid, nitro-oleic acid) seem to exert signal functions and act as NO donors (Begara-Morales *et al.*, 2021), while nitro-nucleotides (e.g. 8-nitro-cGMP, 8-nitro-guanine) may mediate signaling, affect transcription or contribute to damage by inducing mutagenic effects (Petrivský and Luhová, 2020). Through the above molecular mechanisms, NO is an integral regulator in a wide range of physiological processes such as abiotic stress responses (Fancy *et al.*, 2017), symbiotic interactions (Berger *et al.*, 2019), vegetative-reproductive development (Sánchez-Vicente *et al.*, 2019), stomatal movements (Van Meeteren *et al.*, 2020), photosynthesis (Lopes-Oliveira *et al.*, 2021), and defence mechanisms against phytopathogens (Lubega *et al.*, 2021; Jedelská *et al.*, 2021). Of the abiotic effects, heavy metal stresses (e.g. cadmium, lead, aluminum, arsenic) have been studied in detail in relation to NO/RNS signaling. However,

the role of NO signaling in plants with altered Zn homeostasis is barely known, especially in case of Zn deficiency.

The aim of this review is to discuss the available literature on the relationship between Zn deficiency/excess and NO metabolism/signaling, and to support the involvement of NO/RNS in Zn-deficient plants by presenting data from own analysis.

2. The role of NO in Zn-deficient plants

Only one research article is available that examines the role of NO in the regulation of Zn nutritional status of plants. Buet *et al.* (2014), evaluated the effect of the addition of the NO donor GSNO on the Zn nutritional status in hydroponically-cultured wheat plants (*Triticum aestivum* cv. Chinese Spring). Given the well-known role played by NO in the control of the homeostasis and utilization of iron (Graziano and Lamattina, 2007; Tewari *et al.*, 2021), they explored whether this signal molecule could play a similar role for Zn. In their work, it is evidenced that the exogenous addition of NO does not influence the utilization efficiency of Zn to generate fresh biomass following long-term Zn-deprivation and 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 stresses as well as in diverse physiological processes (Jasid *et al.*, 2009; Bruand and Meilhoc, 2019), and could be associated with a rapid NO-related decrease of Zn allocation to shoots. Additionally, the authors observed that the addition of GSNO to Zn-deprived plants doesn't modify biomass accumulation but accelerated leaf senescence in a mode concomitant with accelerated decrease of Zn allocation to shoots. They also observed in well-supplied plants, that Zn concentration in both roots and shoots declined due to long term exposure to GSNO. The further evaluation of net Zn growth rate during the recovery of long-term Zn-deprivation unveiled that enhanced Zn accumulation was partially blocked when GSNO was present in the growth medium. It can be assumed that this effect on uptake is mainly associated with a change of Zn translocation to shoots. The results of this study suggest a role for GSNO in the modulation of uptake and root-to-shoot translocation of Zn during the transition from deficient to sufficient levels of Zn supply.

The lack of extensive research on the relationship between NO signaling and Zn deficiency prompted us to compare endogenous NO levels in roots of Zn deficient plant species showing different sensitivity to Zn supply. We detected NO levels in the root tips of moderately sensitive wild-type *Arabidopsis thaliana* and *Brassica napus*, highly sensitive *Zea mays* and tolerant *Pisum sativum* (Fig 1). In *Arabidopsis* and *Brassica* both belonging to the *Brassicaceae* family, mild Zn deficiency (Zn/10) caused significantly increased NO levels in the root tips; however, the severe Zn limitation (Zn0) led to NO accumulation only in *Brassica napus*. Similar to this, the root tips of the highly sensitive *Zea mays* produced more NO compared to control as the effect of Zn/10 treatment, but the severe Zn limitation resulted in decreased NO levels compared to control in this species. *Pisum sativum* tolerates Zn deficiency and shows different NO responses compared to the sensitive species: both rates of Zn limitation significantly reduced NO levels in the root tips. These preliminary observations indicate that there is a correlation between the capability of mild Zn deficiency tolerance and the capability of root NO production. In case of severe Zn deficiency (which rarely happens in nature), the NO level responses seems to be species-dependent.

To further support the involvement of NO signaling in regulating plant Zn status, we analyzed the putative NO-dependent post-translational modifications of Zn transporter proteins using computational prediction. S-nitrosation and tyrosine nitration of 28 *Arabidopsis* Zn transporter proteins belonging to the ZIP family (ZIP1-12), MTP family (MTP1,3,4,5,6,7,8,9,10,11,12) and HMA family (HMA1-5) were predicted. Amino acid sequences in FASTA formats were downloaded from UNIPROT (<https://www.uniprot.org>) and the analysis was performed by selected computational tools freely available online (Kolbert and Lindermayr, 2021).

2.1. S-nitrosation

The mechanism of S-nitrosothiol formation is an important issue for understanding the biological actions of NO. For the indication of S-nitrosation sites in the proteins, GSP-SNO 1.0 (<http://sno.biocuckoo.org/>, Xue *et al.*, 2010), iSNO-PseAAC (<http://app.aporc.org/iSNO-PseAAC/>, Xu *et al.*, 2013a), iSNO-AAPair (<http://app.aporc.org/iSNO-AAPair/>).

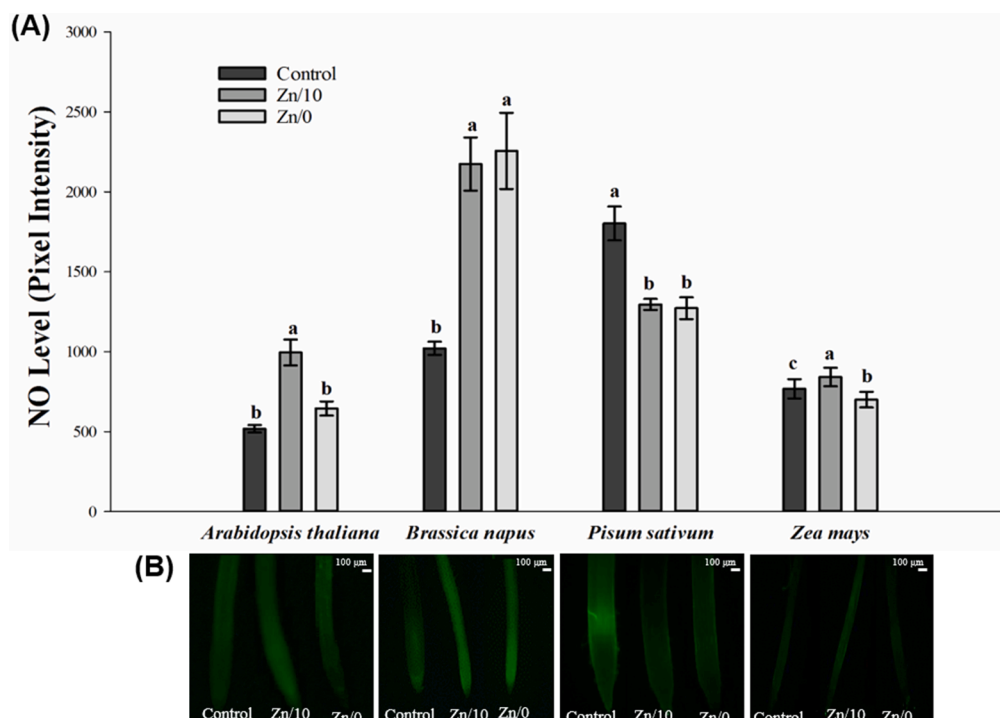


Fig 1. (A) NO levels (pixel intensity of DAF-FM-related fluorescence) in root tips of Zn-deficient wild-type *Arabidopsis thaliana*, *Brassica napus*, *Pisum sativum* and *Zea mays*. Plants were grown in full (0.5 μ M ZnSO₄, Control), in mildly Zn-deficient (0.05 μ M ZnSO₄, Zn/10) and in severely Zn-deficient (0 μ M ZnSO₄, Zn/0) nutrient solution (*Brassica napus*, *Pisum sativum*, *Zea mays*) or agar-solidified medium (*Arabidopsis thaliana*). The treatment period was 7 days (*Arabidopsis thaliana*) or 15 days (*Brassica napus*, *Pisum sativum*, *Zea mays*). Data are presented as mean \pm SE. Significant differences were determined using Duncan's test and indicated by different letters (n=10-15, p \leq 0.05). (B) Microscopic images showing DAF-FM-stained root tips of Control, Zn/10- and Zn/0-exposed wild-type *Arabidopsis thaliana*, *Brassica napus*, *Pisum sativum* and *Zea mays* seedlings. Bar = 100 μ m.

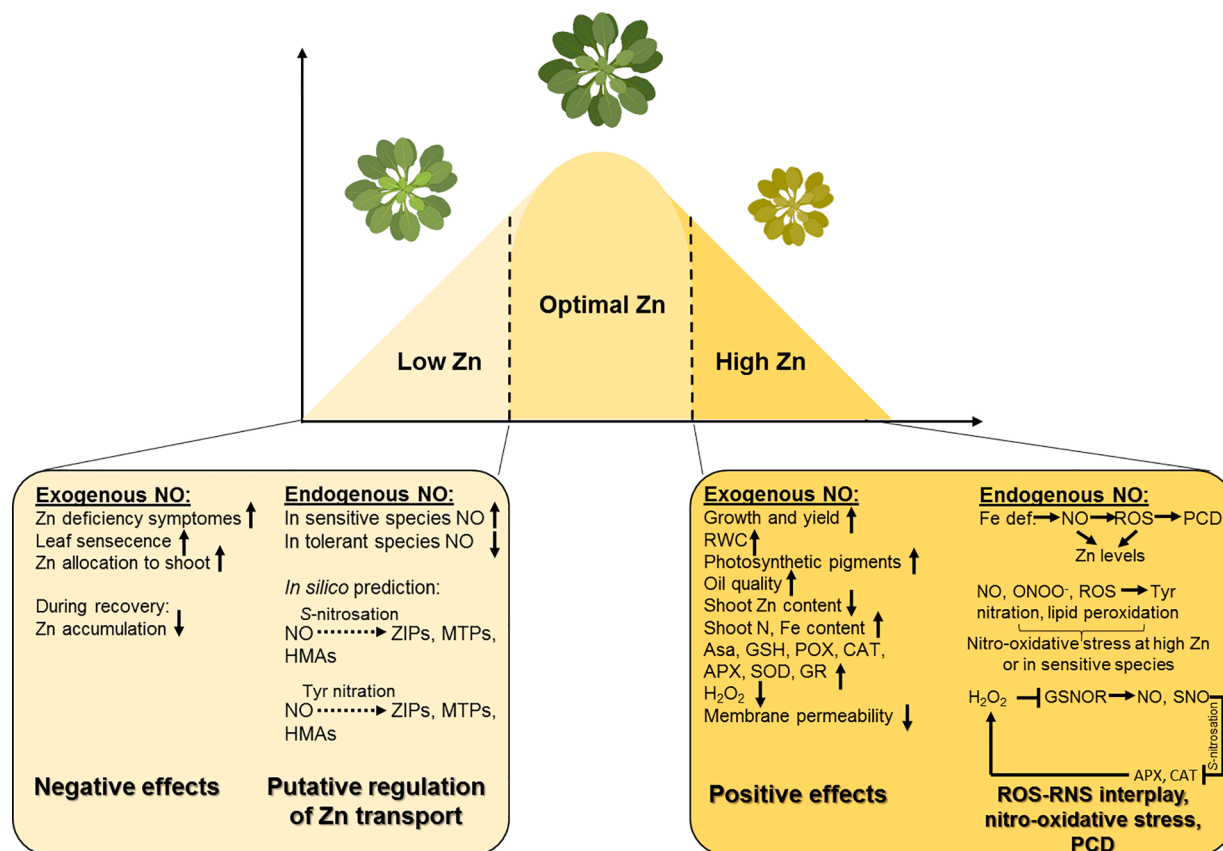


Fig 2. Schematic representation of the known effects of exogenous and endogenous NO in plants with low or high zinc (Zn) concentrations. In Zn-deficient plants, exogenous NO exerts negative effects, while endogenous NO may regulate Zn transporters through S-nitrosation and tyrosine (Tyr) nitration. In case of supraoptimal Zn supply, exogenous NO treatment caused positive effects like improving water, mineral and ROS balance. Excess Zn induces the endogenous production of NO, ONOO⁻ and ROS resulting in secondary nitro-oxidative stress and cell damage. Surplus Zn triggered H₂O₂ autoregulates its own level involving NO-dependent S-nitrosation. See further details in the text. Abbreviations: NO, nitric oxide; ZIPs, Zinc Regulatory Transporter-Iron Regulatory Transporter like Proteins; MTPs, Metal Tolerance Proteins; HMAs, Heavy Metal ATPases; RWC, relative water content; N, nitrogen; Fe, iron; Asa, ascorbate; GSH, glutathione; POX, peroxidase; CAT, catalase; APX, ascorbate peroxidase; SOD, superoxide dismutase; GR, glutathione reductase; H₂O₂, hydrogen peroxide; ROS, reactive oxygen species; PCD, programmed cell death; ONOO⁻, peroxyntirite, GSNOR, S-nitrosoglutathione reductase; SNO, S-nitrosothiol.

O-AApair/, Xu *et al.*, 2013b) and DeepNitro (<http://deepnitro.renlab.org/>, Xie *et al.*, 2018) tools were used.

Based on the predictions presented in Table 1, most of the ZIP proteins (with the exception of ZIP5) contains one or more Cys residues (Cys are indicated by the letter C and the position within the protein sequence is indicated by numbers in Table 1) which are putative targets of S-nitrosation. The total number of target Cys residues in 12 ZIPs is 102 predicted by the four tools. Regarding MTP transporters, there are several cases when the prediction tools did not indicate target sites, but collectively one or more software tools predicted one or more S-nitrosation sites in every examined MTPs. In contrast, the five HMA transport proteins were predicted to contain in total 146 putative S-nitrosation target sites according to the four applied tools (Table 1).

2.2. Tyrosine nitration

Tyrosine is a moderately hydrophilic aromatic amino acid, which is therefore often on the surface of the protein and thus subject to modifications. For analyzing the possible tyrosine nitration sites in Zn transporter proteins, the results of GPS-YNO₂ 1.0 (<http://yno2.biocuckoo.org/>, Liu *et al.*, 2011), iNitro-Tyr (<http://app.aporc.org/iNitro-Tyr/>, Xu *et al.*, 2014), PredNTS (<http://kurata14.bio.kyutech.ac.jp/PredNTS/>, Nilamyani *et al.*, 2021) and DeepNitro have been compared and presented in Table 2, where the Tyr residues are indicated by the letter Y and the positions within the protein sequence are indicated by numbers. Similar to S-nitrosation, ZIP proteins are possible

targets of tyrosine nitration as well, but the total number of target Tyr residues is moderate (64 in 12 ZIPs with 4 software tools). In transporters belonging to MTP and HMA families, the predictions indicated several possible Tyr residues as targets for nitration (78 in 11 MTPs and 76 in 5 HMAs by using 4 software tools, see Table 2).

Although, the prediction software tools gave highly different results as for the target sites due to their different algorithms, the results that the amino acid sequences of transporters in all three protein families contain several putative sites for S-nitrosation and tyrosine nitration suggest that NO may regulate Zn transport through affecting transport proteins at the post-translational level. It has to be noted that computational prediction cannot substitute laboratory work but can provide a starting point for experimental verification (Kolbert and Lindermayr, 2021).

3. The role of NO in excess Zn-exposed plants

3.1. Nitro-oxidative stress in Zn-stressed plants

Nitrosative stress as the consequence of RNS accumulation in plant cells involves nitrative macromolecule modifications such as protein, lipid and nucleic acid nitration, and protein S-nitrosation (Corpas *et al.*, 2007, 2011). Moreover, the metabolism and signaling of RNS is tightly connected to ROS generation, signaling and macromolecule modifications, therefore the term nitro-oxidative stress has been proposed by Corpas *et al.* (2013) and reported to occur under diverse stress

Table 1

List of Arabidopsis Zn transporter proteins in which the S-nitrosated sites were computationally predicted using GSP-SNO 1.0, iSNO-PseAAC, iSNO-AAPair, DeepNitro software. Target cysteines are indicated by the letter C and the position within the protein sequence is indicated by numbers.

Protein name	Accession number	Total number of Cys	Predicted by GPS-SNO 1.0 (2010) (medium threshold)	Predicted by iSNO-PseAAC (2013)	Predicted by iSNO-AAPair (2013)	Predicted by DeepNitro (2018) (medium threshold)
ZIP1	At3g12750	10	C4, C6, C16	C6, C249	C98, C116, C249	non
ZIP2	At5g59520	6	C21	C21, C67, C210	non	non
ZIP3	At2g32270	8	non	C157, C235	C38, C86, C243	non
ZIP4	At5g48390	21	C257, C300, C742, C812	C156, C385, C491, C519, C535, C630, C665, C782	C156, C257, C300, C491, C921	C156, C257, C300, C385, C921
ZIP5	At1g05300	6	non	non	non	non
ZIP6	At2g30080	10	C4, C17, C92, C207	C159, C230	C207	non
ZIP7	At2g04032	8	C7, C238	C7, C46	C37, C121, C153, C314	C46
ZIP8	At5g45105	11	C41, C184, C220, C329	C41, C90, C220, C243, C329, C337	C41, C90, C116, C184, C220, C230, C243, C296, C329	C41
ZIP9	At4g33020	4	C217	C217, C240, C328	C217	non
ZIP10	At1g31260	7	C237	C34, C42, C237, C260	C42, C117, C237, C260	non
ZIP11	At1g55910	5	C52, C128, C182	C52, C128, C182, C277	non	non
ZIP12	At5g62160	6	C337	C15, C32, C66, C251	C251	non
MTP1	At2g46800	6	non	C296, C362	non	non
MTP3	At3g58810	7	C331	C81, C331, C356	C331	C52
MTP4	At2g29410	7	C7	non	C7	C338
MTP5	At3g12100	4	C288, C327	C151	C257	non
MTP6	At2g47830	6	non	C15, C98	C98, C373	C98
MTP7	At1g51610	5	C355	C67, C196	non	non
MTP8	At3g58060	3	C6	C6, C244	C244	non
MTP9	At1g79520	2	non	C400	non	non
MTP10	At1g16310	2	non	C269, C427	non	non
MTP11	At2g39450	5	non	C54	non	C316
MTP12	At2g04620	3	non	C243	C58	non
HMA1	At4g37270	17	C395, C490	C91, C130, C142, C485, C655, C726	C428, C655, C726	C655
HMA2	At4g30110	33	C17, C18, C279, C683, C905	C17, C235, C279, C305, C319, C361, C486, C683, C744, C745, C768, C769, C784, C785, C799, C800, C829, C830, C886, C905	C18, C279, C305, C349, C361, C683, C704, C744, C745, C785, C886	C235, C279, C486, C744
HMA3	-	20	C23, C285, C384, C690	C159, C241, C285, C325, C367, C384, C492, C690, C751	C241, C285, C311, C355, C367	C241, C285, C384, C492
HMA4	At2g19110	58	C289, C315, C692, C1037, C1078, C1122	C245, C279, C289, C371, C661, C692, C714, C755, C782, C783, C799, C800, C835, C836, C851, C852, C865, C870, C907, C908, C941, C960, C1019, C1025, C1026, C1051, C1078, C1079, C1122, C1130, C1146	C28, C315, C359, C371, C661, C714, C799, C817, C836, C1037	C245, C289, C496, C714, C865, C941, C1130
HMA5	At1g63440	11	C10, C65, C131, C969	C10, C65, C131, C343, C617, C657, C968, C969	C62, C65, C619, C968	C10, C726

conditions (Corpas *et al.*, 2021). The excess Zn-triggered imbalance in NO and ROS homeostasis and the occurrence of nitro-oxidative stress has been documented in different experimental systems.

In the earliest paper, Xu *et al.* (2010) observed that *Solanum nigrum* roots accumulate NO in response to zinc chloride (ZnCl₂) treatment. The elimination of NO using cPTIO (2-4-carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide) or L-NAME (N^G-nitro-L-arginine methyl ester) prevented Zn-induced H₂O₂ and O₂^{•-} production by reducing NADPH oxidase (NOX) activity, and increasing catalase (CAT) and ascorbate peroxidase (APX) gene expression and activity. This suggests that NO is needed to increase ROS production in Zn-stressed roots. Using DAPI and TUNEL assays, NO scavenger treatment was

shown to decrease the rate of Zn-induced cell death in the root tips of Sorghum suggesting the involvement of NO accumulation in Zn-triggered programmed cell death (PCD). Further results indicated that the produced NO acts in modifying root development (primary root elongation, lateral root emergence and root hair formation) which may lead to better excess Zn tolerance of Sorghum. An additional interesting result of Xu *et al.* (2010) was that the NO production in Zn-treated *S. nigrum* roots was partially linked to Zn-induced iron deficiency. According to these results, NO is involved in long-term Zn tolerance by inducing PCD together with ROS in the root tips and thus modifying the root system in Sorghum.

Similar experimental setup was applied by Duan *et al.* (2015) where

Table 2

List of Zn transporter proteins in which nitration sites were computationally predicted using GSP-YNO₂ 1.0, iNitro-Tyr, PredNTS, DeepNitro software. Target tyrosines are indicated by the letter Y and the positions within the protein sequence are indicated by numbers.

Protein name	Accession number	Total number of Tyr	Predicted by GPS-YNO ₂ (2011) (medium threshold)	Predicted by iNitro-Tyr (2014)	Predicted by PredNTS (2021)	Predicted by DeepNitro (2018) (medium threshold)
ZIP1	At3g12750	5	non	Y151	Y287	non
ZIP2	At5g59520	12	non	Y127, Y266	non	non
ZIP3	At2g32270	5	non	non	Y53, Y153	non
ZIP4	At5g48390	25	Y459, Y581, Y662, Y676, Y918, Y925	Y662, Y824, Y870, Y918, Y925	Y138, Y219, Y459, Y472, Y494, Y506, Y527, Y662, Y676, Y678, Y855, Y859, Y870, Y918, Y925	Y859, Y870, Y925
ZIP5	At1g05300	5	non	non	Y48, Y107, Y146	non
ZIP6	At2g30080	6	non	non	Y142	non
ZIP7	At2g04032	7	Y3, Y8, Y191	Y3	Y3, Y8, Y191, Y348	non
ZIP8	At5g45105	6	non	Y74, Y150, Y191, Y249	Y150, Y191	non
ZIP9	At4g33020	6	non	non	non	non
ZIP10	At1g31260	7	Y202	Y202	Y40, Y347	non
ZIP11	At1g55910	8	non	Y293, Y306	Y293, Y306	non
ZIP12	At5g62160	5	non	Y49	Y49, Y144	non
MTP1	At2g46800	6	Y382	Y263	Y263, Y382, Y387	non
MTP3	At3g58810	5	non	non	Y376	non
MTP4	At2g29410	5	non	Y239	Y239, Y322, Y363, Y366	non
MTP5	At3g12100	15	Y392	Y34, Y253, Y375, Y392	Y25, Y34, Y71, Y174, Y176, Y392	non
MTP6	At2g47830	10	Y345	Y137, Y345	Y66, Y124, Y137	non
MTP7	At1g51610	10	Y19	Y177	Y90, Y258, Y353, Y382, Y414	Y90, Y382, Y414
MTP8	At3g58060	15	Y5, Y73, Y96	Y96, Y294	Y5, Y72, Y73, Y96, Y255, Y350	non
MTP9	At1g79520	12	Y60, Y74	Y253, Y278	Y60, Y73, Y74, Y132	non
MTP10	At1g16310	13	Y6, Y101	Y201, Y280, Y305	Y6, Y87, Y100, Y101, Y159	non
MTP11	At2g39450	16	Y381, Y383	Y244, Y283, Y383	Y122, Y308, Y314, Y381, Y383	non
MTP12	At2g04620	7	non	Y98	non	non
HMA1	At4g37270	14	Y259	Y288, Y366, Y421, Y654	Y121, Y421, Y587, Y654	Y532
HMA2	At4g30110	19	Y10, Y179	Y10, Y157, Y474, Y504, Y506	Y10, Y267, Y308, Y309, Y378, Y465, Y474, Y705, Y925	Y10, Y378, Y504, Y506
HMA3	-	15	non	Y16	Y273, Y314, Y315, Y514, Y531, Y712	Y16, Y512, Y514
HMA4	At2g19110	19	Y20, Y189	Y20, Y48, Y1150	Y20, Y133, Y277, Y318, Y319, Y388, Y475, Y715, Y733, Y928, Y1150	Y1150
HMA5	At1g63440	21	Y20, Y486	Y20, Y248, Y358, Y384, Y421, Y976	Y20, Y173, Y296, Y297, Y384, Y421, Y486, Y688, Y709, Y976	Y924

high concentration of Zn (3 mM) was applied together with the NO scavenger cPTIO and the NOX inhibitor diphenylene iodonium (DPI) in order to reveal the involvement of NO and ROS and their interaction in excess Zn-triggered cell death. The elimination of NO in Zn-treated plants significantly lowered Zn levels in roots and leaves, and led to the further inhibition of root growth compared to plants treated with Zn alone. Meanwhile, Zn stress in combination with DPI lowered the Zn level and the length of the roots, but did not affect the Zn content in leaves, in comparison with single Zn treatment. These results indicate that changes of NO and ROS levels might play an important role in root growth and Zn accumulation in wheat seedlings. The authors suggested that the moderate increase of NO production might play important roles in alleviating Zn-induced inhibitory effects on root growth in wheat seedlings, and that the high endogenous NO levels might be toxic and causes further shortening of the roots in wheat seedlings treated with Zn plus DPI. Similar to the results of Xu *et al.* (2010), Duan *et al.* (2015)

revealed that the increased NO content induced by Zn stress is partly responsible for the elevated H₂O₂ levels in the roots. Furthermore, NOX inhibition revealed that this enzyme may be associated with the regulation of NO and ROS production as well as with the modulation CAT, APX, peroxidase (POD), and superoxide dismutase (SOD) activities in excess Zn-exposed wheat.

In the work of Feigl *et al.* (2015) the correlation between ROS, RNS production/signaling and excess Zn tolerance was examined in the root system of *Brassica napus* and *Brassica juncea*. In the roots of *Brassica juncea*, which possesses better Zn resistance, only a slight ROS formation, a moderate activation of antioxidant enzymes (SOD, APX) and no remarkable lipid peroxidation were observed, which reflect the lack of a serious Zn-induced oxidative stress. However, the significant production of RNS (NO and ONOO⁻) and the occurrence of protein nitration reveal a Zn-triggered secondary, nitrosative stress in this species. It was also observed, that as a result of Zn exposure, nitro-oxidative stress occurred

in the more sensitive *Brassica napus* as a consequence of ROS and RNS accumulation, lipid peroxidation and protein tyrosine nitration. Their data reveal the existence of a relationship between ROS and RNS metabolism under Zn stress and the contribution of nitro-oxidative stress to Zn sensitivity. This research also suggests that sensitivity to Zn is determined by the level of oxidative rather than by the nitrosative processes in *Brassica* species.

When the shoot system of *Brassica* species exposed to longer term of Zn stress (14 days) was examined, it was observed that the relative Zn tolerance of *B. napus* shoot was associated with slight alterations of the protein nitration pattern (Feigl *et al.*, 2016). In contrast, more moderate increase in protein nitration compared to *B. juncea* was revealed in the root system of *B. napus* suffering more severe damages by excess Zn. The data revealed that the proteomes of *Brassica* organs react differentially to Zn exposure, since modification of the nitration pattern in the shoot proteome occurred, while a general increment in the root nitroproteome could be observed. An important conclusion of these data can be that the significant alteration of protein nitration pattern is coupled with enhanced Zn sensitivity of the *Brassica* shoot system and the general intensification of protein nitration in the roots is associated with relative Zn tolerance (Feigl *et al.*, 2016).

In a recent paper, the growth-inducing and growth-limiting Zn concentrations on the early development of root architecture and the nitro-oxidative signaling were studied in *Brassica napus* grown in soil-filled rhizotron system (Feigl *et al.*, 2019). The high concentration of Zn exposure caused increased rate of protein tyrosine nitration due to the imbalance in ROS and RNS levels, and the pattern of protein nitration was not changed by Zn supply compared to the control. The observed nitro-oxidative stress was accompanied by significant changes in the cell wall composition and decrease in the cell proliferation and viability in the root cells, due to the notable Zn accumulation and disturbed microelement homeostasis. Interestingly, the authors observed that low Zn concentrations caused reorganisation in the tyrosine nitration pattern of the root proteome, while the ROS and RNS homeostasis was undisturbed and the viability loss and limitation of proliferation of the root tips' cells was slighter, due to the lower Zn uptake. These results suggest that depending on its amount Zn triggers different root growth responses accompanied by distinct changes in the pattern and strength of tyrosine nitration/nitro-oxidative stress.

Examining the molecular processes of the relationship between excess Zn and NO signaling in Arabidopsis, Kolbert *et al.* (2019) found that the GSNOR reductase (GSNOR) enzyme-deficient *gsnor1-3* mutant shows a high degree of Zn tolerance relative to the wild-type (WT) and to GSNOR enzyme overproducing 35S:FLAG-GSNOR1 line. Interestingly, in *gsnor1-3*, Zn loading did not lead to increased NO and SNO levels (in contrast to WT), which may be the consequence of GSNOR-independent, NADPH-dependent thioredoxin reductase-associated SNO level regulation. In the WT, surplus Zn caused down-regulation of GSNOR at the post-translational level. When Zn-induced H₂O₂ accumulation in the WT was limited by external addition of glutathione, the loss of GSNOR activity was abolished, demonstrating a direct role for H₂O₂ in the inactivation of GSNOR by Zn excess. Increased NO and SNO production as the effect of Zn exposure in WT resulted in increased S-nitrosation. Among the few S-nitrosation target proteins identified by mass spectrometry, APX1 is worth mentioning; and total APX activity was decreased in Zn-exposed plants. A similar change was observed in total CAT activity, and the CAT3 enzyme was identified as an S-nitrosation target. Regarding protein nitration, it was shown that in the WT and 35S:FLAG-GSNOR1, excess Zn intensified it, while in *gsnor1-3*, a slight decrease in Zn-induced protein nitration occurred compared to control. The main conclusion of this work is that a regulatory mechanism operates in which Zn-induced H₂O₂ causes an increase in NO/SNO levels through GSNOR enzyme inactivation and thus intensifying S-nitrosation of ROS-regulating enzymes (e.g. APX, CAT) and decreasing their activity. This means that H₂O₂ affects its own levels in a self-regulatory process involving SNO signaling in Arabidopsis plants exposed to

excess Zn.

From these studies it can be concluded that surplus Zn induces NO production which in turn increases ROS levels through modifying antioxidant enzymes and triggers cell death. At the same time, excess Zn-triggered H₂O₂ affects NO levels via the inactivation of GSNOR enzyme. Regarding the molecular mechanisms of NO's action, Zn stress-induced imbalances in ROS/RNS homeostasis result in S-nitrosation of certain proteins and it also intensifies protein nitration, which is a major indicator of nitro-oxidative stress. Moreover, excess Zn-induced nitro-oxidative stress occurs in the plant proteome in a species-, organ-, concentration-, and time-dependent manner.

3.2. The effects of exogenous NO on excess Zn tolerance

Treatment of plants with exogenous NO causes an increase in the NO content of the tissues, which has a number of beneficial effects during various stresses (Nabi *et al.*, 2019; Zhou *et al.*, 2021) including Zn overload.

Kaya (2016) examined the effects of exogenous NO (in the form of 100 µM sodium nitroprusside, SNP) on dry matter production and mineral nutrients of maize grown in the presence high Zn concentrations. It was observed that foliar NO treatment can partially overcome the deleterious effects of Zn stress on growth, relative water content, chlorophyll content and membrane permeability of maize plants. Additionally, NO donor lowered Zn content in the leaf and root and increased leaf and root nitrogen content and leaf iron concentration in the Zn-exposed plants resulting in an improved nutritional status in the presence of high external Zn concentrations.

In a more detailed study, Akladios and Mohamed (2017) examined the effects of toxic levels of Zn on growth, yield components, photosynthetic pigments, enzymatic and non-enzymatic antioxidants, protein patterns and fatty acid composition of harvested seeds in order to explore the possible role of exogenous application of NO (20 µM SNP) in the alleviation of Zn toxicity in sunflower plants. Supplying of the NO donor improved growth (plant height, head diameter) and yield (e.g. number of seeds per head, 1000-seed weight) parameters, increased the contents of photosynthetic pigments, ascorbic acid and glutathione, and activated antioxidant enzymes (e.g. SOD, APX, glutathione reductase [GR]). NO donor treatment also improved sunflower oil quality due to the enhancement of unsaturated fatty acid contents. Interestingly, NO application altered Zn distribution in the plants, since it increased Zn concentration in roots and limited Zn accumulation in shoots compared to plants treated with Zn alone. These suggested for the first time that exogenous administration of NO acts as an inducer of the antioxidant system and also able to regulate Zn uptake and translocation thus improving vegetative growth, yield and oil quality in Zn-exposed sunflower.

The antioxidative role of NO donors in Zn-stressed plants was supported by Nasiri-Savadkoobi *et al.* (2017) who treated *Plantago major* L. plants with 100 and 200 µM SNP as exogenous NO donor in addition to Zn exposure (0, 100, 300 and 500 µM). Their study proved that exogenous NO at low concentration (100 µM) increased Zn tolerance in *Plantago* by intensifying antioxidant enzyme activities (POD, CAT, APX, SOD) and decreasing H₂O₂ content thus preventing Zn-induced oxidative stress. The authors indicated that NO efficiently scavenges ROS and stabilizes membranes in *Plantago* plants exposed to Zn stress.

The above studies found that exogenous application of NO exerts its beneficial role via (1) improving water status, (2) mineral nutrient homeostasis, and (3) antioxidant defense leading to better performance of plants under excess Zn conditions. Antioxidant enzymes like SOD, CAT, APX etc. may be regulated by NO mainly at the post-translational level due to S-nitrosation and/or tyrosine nitration (Clark *et al.*, 2000; Ortega-Galisteo *et al.*, 2012; Holzmeister *et al.*, 2015, reviewed in Begara-Morales *et al.*, 2016), however the above studies did not evidence that.

3.3. The role of NO in nano Zn-treated plants

Due to the development of nano-industry the release of nanoparticles into the environment is constantly increasing, thus nowadays there is a strong emphasis on studying the effects of nanoparticles on physiological processes of plants. When handled properly, Zn-oxide nanoparticles (ZnO NPs) can be an efficient fertilizer for increasing Zn content of plants (Sun *et al.*, 2020, Dimpka *et al.*, 2020). However, ZnO NPs can be released in the environment where sessile plants are particularly effected by their toxic effects. Plants may come in contact with ZnO NPs both through their shoot and root systems. In the presence of plant roots, ZnO NPs release Zn ions (López-Moreno *et al.*, 2010) which are absorbed by the roots with specific transporters (Milner *et al.*, 2013). Internalization of ZnO NPs smaller than the cell wall pores (5–30 nm) may also happen (Fleischer *et al.*, 1999; Nair *et al.*, 2010) as well as the decomposition of larger NPs into smaller ones. Additional mechanisms of NP uptake into plant cells such as endocytosis, pore formation and transporter proteins-mediated internalization have been suggested (Pérez-de-Luque, 2017; Lv *et al.*, 2019). Within the root tissues, ZnO NPs move *via* symplastic pathway involving plasmodesmata, even though their root to shoot translocation has not been convincingly evidenced yet (Wang *et al.*, 2013; Lv *et al.*, 2015; Singh *et al.*, 2018a,b; Ahmed *et al.*, 2021).

Oxidative stress signaling due to ROS overproduction is known to be partly responsible for ZnO NP toxicity, although the few available literature data was recently supplemented by Molnár *et al.* (2020a) who examined nitro-oxidative processes in ZnO NP (~8 nm)-exposed *Brassica* seedlings. Their results revealed that ZnO NP supplementation disturbs $O_2^{\bullet-}$ and H_2O_2 metabolism and modifies ROS-related enzymes (NOX, SOD, APX) and non-enzymatic antioxidants (ascorbate, glutathione) in a similar manner in both species. Moreover, ZnO NP supplementation resulted in altered RNS levels (NO, ONOO⁻, GSNO) in both *Brassica* species. The more tolerant *B. juncea* responded to ZnO NPs (100 mg/L) with enhanced NO, ONOO⁻ and GSNO levels compared to the sensitive *B. napus*. As for the protein-level consequences of nitro-oxidative signaling, it was revealed that the rate of ZnO NP-induced protein carbonylation was similar in *Brassica* species, whereas the sensitive species showed increased rate of protein nitration compared to the relatively tolerant *Brassica* species. These results reflected to the nitro-oxidative stress inducing capacity of ZnO NPs and to the relationship between ZnO NP tolerance and nitrosative signaling in *Brassica* seedlings.

Using chemically synthesized ZnO NPs with bigger size (~45 nm), Molnár *et al.* (2020b) studied cell wall remodeling and ROS/RNS signaling in roots of *Brassica napus* and *Brassica juncea* seedlings. Interestingly, in contrast to the smaller ZnO NPs (Molnár *et al.* 2020a) in this system, *B. napus* proved to be relative tolerant over *B. juncea*, which indicates that the plant tolerance against nano ZnO depends on the nanoparticle size. The higher degree of ZnO NP tolerance of *B. napus* may be linked to reduced Zn ion accumulation in the roots due to cell wall modifications (lignin, suberin deposition, pectin accumulation, increase in cell-wall peroxidase activity). Furthermore, nano ZnO supplementation caused elevations in $O_2^{\bullet-}$, H_2O_2 , ONOO⁻, NO, and GSNO levels suggesting the intensification of nitrosative signaling in this species. In *B. napus*, the levels of ROS was increased by ZnO NP treatments, but the levels of RNS and tyrosine nitration were unmodified by ZnO NPs. This indicates a clear correlation between phytotolerance against ~45 nm ZnO NPs and nitrosative signaling.

Using rice mutants, Chen *et al.* (2015) studied the correlation between endogenous NO content and ZnO NP tolerance. Compared to the WT rice, the growth limitations and the decrease in gene expression of CATA, CATb, APX and POD were more severe in the NO-deficient *noa1*, whereas the NO overproducer *noe1* rice mutant showed improved tolerance against nano ZnO due to increased expression of antioxidant genes compared to the WT rice plant. Beyond the internal changes of NO metabolism and signaling, Chen *et al.* (2015) investigated the

ameliorating effect of exogenous NO (SNP 10 μ M) on ZnO NP phytotoxicity in rice as well. Their results indicate that SNP causes a notable reduction in Zn accumulation, ROS production and lipid peroxidation in rice plants supplemented with ZnO NPs. The mechanism behind the protective role of NO against ZnO NPs-induced oxidative stress proved to be the NO-dependent improvement of the antioxidant system, since exogenous NO treatment increased the activities and gene expressions of POD, CAT, APX.

Moreover, Tripathi *et al.* (2017) treated wheat plants with SNP (100 μ M) and nano ZnO simultaneously and focused their research on the antioxidant system. Based on the results the authors proposed two ways through which NO regulates ZnO NPs toxicity. NO decreases excess Zn accumulation in the vascular tissues, resulting in milder oxidative stress. Moreover, NO supplementation up-regulates enzymes of the ascorbate-glutathione cycle (APX, GR, monodehydroascorbate reductase, dehydroascorbate reductase) and non-enzymatic antioxidants (ascorbate, glutathione), which further improve the protection of wheat seedlings against ZnO NPs-triggered oxidative stress.

In a recent study, Bhat *et al.* (2021) applied foliar SNP (100 μ M) treatment as exogenous NO to nano-ZnO-treated (<100 nm) *Brassica juncea* and observed that NO improves germination, vigor index, biomass production, and chlorophyll contents of ZnO NP-exposed seedlings. Similarly, net photosynthetic rate, stomatal conductance, transpiration, carbonic anhydrase, nitrate reductase activities and proline content showed better values in the presence of NO donor treatment compared to plants treated with nano ZnO alone. Furthermore, NO donor treatment resulted in enhanced activities of the examined antioxidant enzymes (SOD, CAT, POD) in ZnO NP-exposed *Brassica* plants.

Based on the low number of studies in the field, ZnO NP exposure causes nitro-oxidative stress processes in plants which due to disturbed ROS and RNS homeostasis and protein modifications contributes to phytotoxicity. Plant tolerance against ZnO NPs shows species-dependence, and also depends on the size and concentration of the nanoparticles being present in the medium. Moreover, exogenous application of NO is able to improve the performance of ZnO NP-treated plants by decreasing Zn ion accumulation, improving photosynthesis, and reducing oxidative stress due to the upregulation of antioxidants.

4. Conclusion and future perspectives

The role of NO signaling in plant responses to suboptimal and supraoptimal Zn supply has been supported by some research in recent years as summarized in Fig. 2. The result that GSNO modulates Zn uptake and root-shoot translocation during the recovery from Zn deficiency is supplemented by own preliminary observations. We found that there is a correlation between the capability of mild Zn deficiency tolerance and the capability of root NO production. Additionally, in the case of severe Zn deficiency, the NO level responses proved to be species-dependent. Furthermore, our computational analysis highlights that among Arabidopsis Zn transporter proteins (ZIPs, MTPs, HMAs) there are numerous targets of NO-dependent S-nitrosation and tyrosine nitration. This indicates the regulatory role of NO in plant Zn transport. The alterations in NO and ROS homeostasis, as well as the proteome- and genome-level consequences in Zn-deficient plants are highly unknown, thus the following questions remain to be answered: how suboptimal Zn supply alters RNS metabolism and gene expression, and what is the role of RNS-dependent post-translational modifications in plant responses to inadequate Zn supply.

Exogenous application of NO is beneficial for plants grown with supraoptimal Zn supply. The stress relieving effects of NO are mainly achieved by the positive regulation of water and mineral status and by the induction of the antioxidant system. Exogenous NO acts as an antioxidant due to the activation of antioxidant enzymes such as SOD, CAT, APX etc. *via* S-nitrosation and/or tyrosine nitration. Although, the ameliorating effects of exogenous NO in case of suboptimal and supraoptimal Zn supply need to be better understood. The effects of several

NO donors (e.g. GSNO, or NO-releasing NPs) are needed to be revealed and molecular approaches (mutant and transgenic plants) have to be used.

Among the endogenous processes caused by excess Zn, first the role of NO and ROS in PCD was elucidated, and further works confirmed the changes in RNS homeostasis and in protein nitration. The degree of excess Zn-triggered secondary nitro-oxidative stress correlates with the tolerance of plant species. Therefore, nitro-oxidative processes can be good indicators for Zn stress. Moreover, nitro-oxidative signaling occurs in a time- and concentration-dependent manner as the effect of Zn overload. The in-depth understanding of the processes will require „omics” approaches in the future. Furthermore, understanding the physiological effects of nano ZnO is a novel field of research, but a more thorough understanding of the molecular details involving mutants is still the task of future research.

Collectively, the molecular-level understanding of plant responses to sub- and supraoptimal Zn supply is relevant from agricultural, human nutritional and ecotoxicological point of views. Therefore, such kind of studies have to be the task for the future, and should involve research on NO metabolism and signaling.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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