Nitric oxide (NO) signalling in plant nanobiology: current status and perspectives

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Highlights

Here we discuss the role of nitric oxide (NO) in plant responses to nanomaterials like chitosan nanoparticles (NPs), metal-oxide NPs, nanotubes and NO-releasing NPs providing new insights in plant naNObiology.

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Abstract

Plant nanobiology as a novel research field provides scientific basis for the agricultural use of nanoparticles (NPs). Plants respond to the presence of nanomaterials by synthesizing signal molecules, such as the multifunctional gaseous nitric oxide (NO). Several reports have described the effects of different nanomaterials (primarily chitosan NPs, metal oxide NPs and carbon nanotubes) on endogenous NO synthesis and signalling in different plant species. Other works have demonstrated the ameliorating effect of exogenous NO donor (primarily sodium nitroprusside) treatments on NP-induced stress. NO-releasing NPs are more preferred alternatives to chemical NO donors and evaluating their effects on plants has recently begun. The accumulated literature data clearly indicate that endogenous NO production in the presence of nanomaterials or NO levels increased by exogenous treatments (NO-releasing NPs or chemical NO donors) exerts growth-promoting and stress-ameliorating effects in plants. Furthermore, a NP-based nanosensor for NO detection in plants has been developed, providing a new and excellent perspective for basic research and also for the evaluation of plants' health status in agriculture.

Abbreviations: catalase, CAT; 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, cPTIO; carbon nanotubes, CNTs; chitosan nanoparticles, CNPs; glutathione, GSH; hydrogen peroxide, H_2O_2; multi-walled carbon nanotubes, MWCNTs; nitrate reductase, NR; nitric oxide, NO; peroxidase, POX; peroxynitrite, $ONOO^-$; reactive oxygen species, ROS; single-walled carbon nanotubes, SWNTs; sodium nitroprusside, SNP; superoxide radical, $O_2^{\cdot-}$; superoxide dismutase, SOD; S-nitroso-glutathione, GSNO; S-nitroso-mercaptosuccinic acid chitosan nanoparticles, S-nitroso-MSA-CS NPs; S-nitrosothiol, SNO.

1. Introduction

Nanotechnology has been highlighted as a promising field of interdisciplinary research in the last decades. Its potential in developing sustainable agriculture is also getting attention nowadays. Indeed, agriculture practices can effectively be improved by the application of nanoparticles (NPs) as nanopesticides, nanoherbicides, nanofertilizers, nanosensors, and growth stimulants (Fraceto *et al.,* 2016; Shang *et al.,* 2019; Maksimović *et al.,* 2019). NPs are organic or inorganic materials with 1–100 nm size at least in one dimension (Ellenbecker and Tsai, 2015), which can have both natural (e.g. volcanic activity) and anthropogenic sources. Man-made nanoparticles can be synthetized as a by-product of industrial activities or as a deliberate product with particular properties for a specific purpose. Based on the core material, NPs can be divided into inorganic and organic NPs. Inorganic NPs include metals (e.g. Al, Bi, Co, Cu, Au, Fe, In, Mo, Ni, Ag, Sn, Ti, W, Zn), metal oxides (Al_2O_3, CeO_2, CuO, Cu_2O, In_2O_3, La_2O_3, MgO, NiO, TiO_2, SnO_2, ZnO, ZrO_2) and quantum dots. Organic NPs are liposomes, dendrimers, micelles, fullerenes, and carbon nanotubes (Khalid *et al.,* 2020).

As for crop production, low NP doses exert direct positive effects on seed germination and vegetative and reproductive growth of plants, as was experimentally verified by several studies in species like rice, wheat, tobacco, coffee, soybean etc. (reviewed in detail by Shang *et al*., 2019). At the same time, NP may cause toxic symptoms (stunted root and shoot growth, chlorosis, necrosis) in plants, and the toxicity depends on several factors like chemical composition, chemical structure, size, surface area and concentration of nanoparticles, duration of exposure, plant species, developmental phase and treatment conditions (Ruttkay-Nedecky *et al*., 2017; Singh *et al*., 2018; Sturikova *et al*., 2018).

Plants come into contact with NPs *via* both their shoot and root system. Available literature indicates that NPs can internalize leaf tissues through e.g. stomata, trichomes or hydathodes and enter root tissues *via* rhizodermis and lateral root junctions or wounds (Schwab *et al*., 2016; Ruttkay-Nedecky *et al*., 2017). Regarding the mechanism of NP internalization, several mechanisms have been proposed such as endocytosis, pore formation, carrier protein- or plasmodesmata-mediated entry or snorkelling (Schwab *et al*., 2016). However, NP uptake into plant tissues depends on factors like particle size, chemical composition, or morphology (Pérez-de-Luque, 2017). Beyond direct NP uptake, ion release is a further scenario for the interaction between metal NPs, metal oxide NPs and plants (Pérez-de-Luque, 2017).

Plants respond to environmental cues such as the presence of NPs by the synthesis of signal molecules. Among gaseous signal molecules (e.g. hydrogen sulphide, ethylene, carbon monoxide), nitric oxide (NO), having been extensively studied in the last forty years, has a prominent role (Kolbert *et al*., 2019). Its small size, redox properties and hydrophobic character allow its effective participation in the regulation of plant growth and development, as well as in stress responses. Nitric oxide present in the atmosphere and formed in the soil during nitrification and denitrification can be taken up by plants, but plants themselves produce NO using several oxidative and reductive metabolic pathways.

In living organisms, endogenous NO synthesis may occur uniformly by the oxidation of reduced N compounds such as L-arginine, polyamines or hydroxylamine. Yet the synthesis of NO in higher plants is special, since it differs from all other living organisms (even from algae). In higher plants, L-arginine may be converted by the activity of a mammalian nitric oxide synthase- (NOS)- like enzyme or enzyme complex that has not been identified so far (Gupta *et al*., 2019). Oxidative degradation of polyamines can directly or indirectly result in the formation of NO, but the exact mechanism has not yet been elucidated (Wimalasekera *et al*., 2011), similarly to the process of NO release from hydroxylamine and salicylhydroxamate (Rümer *et al*., 2009). Additionally, NO is formed by the reduction of oxidized N compounds such as nitrate and nitrite, therefore it is connected to nitrate assimilation (Sanz-Luque *et al*., 2013). Earlier studies reported that NO production is associated with nitrate reductase (NR) activity in various plant tissues and diverse growth conditions (Hao *et al*., 2010; Mur *et al*., 2013; Lu *et al*., 2014; Medina-Andres *et al*., 2015). Recent evidences in *Chlamydomonas* indicate, however, that NR plays an indirect role in NO synthesis by providing electron source for the NO-forming nitrite reductase (NOFNiR), which might be a relevant mechanism also in higher plants (Chamizo-Ampudia *et al*., 2016; 2017). Beyond NR, the activity of the root-cell-specific nitrite:NO reductase (NiNOR, Stöhr *et al*., 2001) catalyses nitrite reduction-associated NO formation. Furthermore, NO and ATP formation *via* cytochrome c oxidase and/or reductase and possibly by alternative oxidase at the mitochondrial inner membrane was suggested (Stoimenova *et al*., 2007). Non-enzymatic processes like spontaneous nitrite reduction at acidic pH in the presence of ascorbate in cell walls can also be considered (Bethke *et al*., 2004).

Diverse reactions of NO in biological systems ensure its removal and the precise control of its steady-state level. Interactions of NO with molecular oxygen yield nitrite and nitrate, and the NO-phytoglobin reaction leads to the formation of nitrate (Perazzolli *et al*.,

2004; Hebelstrup *et al*., 2006). The conversion of NO into nitrate is also possible due to the activity of truncated haemoglobin THB1 receiving electron from NR (Sanz-Luque *et al*., 2013; Chamizo-Ampudia *et al*., 2017). Furthermore, the formation of S-nitrosothiols (SNO) such as S-nitrosocysteine (CysNO) or S-nitrosoglutathione (GSNO) in the reaction between NO and thiol- (SH)-containing proteins and peptides may influence steady-state NO levels, since SNOs are capable of NO liberation (Hogg, 2000; Stamler *et al*., 2001; Foster *et al*., 2003). The most abundant SNO is GSNO, which can non-enzymatically liberate NO or be reduced by the enzyme *S*-nitrosoglutathione reductase (GSNOR), yielding oxidized glutathione (GSSG) and ammonia (NH_3) resulting in NO removal (Barroso *et al*., 2006; Corpas *et al*., 2008b; Leterrier *et al*., 2011). Due to its stable character, GSNO may serve as a long-distance transport form of NO signal (Lindermayr, 2018; Begara-Morales *et al*., 2018). SNOs exert relevant biological functions such as transnitrosation of target proteins, by which NO signal perception is partly realized. The reversible reaction between GSNO and protein cysteine thiols leads to modifications in protein structure and activity and consequently in signal transduction. Reaction of NO with superoxide radical (O_2^-) produces peroxynitrite ($ONOO^-$, Beckman *et al*., 1990), which may be in turn scavenged by flavonoids, ascorbic acid, gamma tocopherols and enzymes with peroxynitrite reductase activity (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). $ONOO^-$ is indirectly responsible for nitration reactions in macromolecules like proteins, lipids and nucleic acids. Protein tyrosine nitration is an irreversible, possibly inactivating posttranslational modification, which may initiate the degradation of the target protein (Kolbert *et al*., 2017). In the case of nucleic acids, $ONOO^-$ (or nitrogen oxides) is responsible for the nitration of guanine and related nucleosides, nucleotides either in their free or DNA and/or RNA embedded form (Ihara *et al*., 2011), resulting in the formation of mainly 8-oxoguanine (8-Oxy-G) and 8-nitroguanine (8-NO_2-G). 8-NO_2-G incorporated in DNA may potentially be mutagenic or induce epigenetic changes; in RNA it may alter function and metabolism, and it may affect GTP-binding proteins and cGMP-dependent enzyme functions (Petřivalský and Luhová, 2020). In plant systems, nucleic acid nitration and its biological consequences are still poorly examined (Izbiańska *et al*., 2018; Andryka-Dudek *et al*., 2019). Recently, nitro-fatty acids (primarily nitro-linoleic acid and nitro-oleic acid) have been proposed as endogenous NO donors/reservoirs (Mata-Pérez *et al*., 2017; Vollár *et al*., 2020), which may liberate NO under specific circumstances and perform biological functions (Vollár *et al*., 2020) such as nitroalkylation of proteins (Aranda-Cano *et al*., 2019). Figure 1 gives an overview on the reactions and macromolecule modifications induced by NO and reactive nitrogen species.

As can be seen from the above, NO is a molecule that operates an extensive signalling network and regulates growth, development and stress responses in plants. It is therefore not surprising that plant physiological studies in association with nanomaterials have been involving NO in recent years. This review aims to give an overview about the current literature regarding plant nanobiology involving NO.

2. The involvement of NO in responses to nanomaterials

2.1. Nanomaterial-induced alterations of endogenous NO metabolism and signalling in plants

2.1.1. *NO is involved in chitosan nanoparticle-triggered innate immunity in plants*

The natural biopolymer chitosan has been reported to induce disease resistance in plant-pathogen systems. The beneficial effects of chitosan on the plant immune system can be further improved by using its nanoparticle form (CNP). The deacetylation degree and the molecular weight of chitosan can be modulated to achieve different physicochemical properties. Nano-chitosan has different size, surface area, ion structure, lower phytotoxicity but better bioactivity, biocompatibility, and biodegradability as compared to chitosan. Due to these beneficial properties, CNPs as pesticides have potential for agricultural applications. Nitric oxide has long been known as a regulator of pathogen defence responses in plants (Durner *et al*., 1998; Delledonne *et al*., 1998; Wang *et al*., 2009; Yun *et al*., 2011; Trapet *et al*., 2015; Skelly *et al*., 2019). Recently, Chandra *et al*., (2017) examined the involvement of the NO signal in CNP-triggered innate immunity in tea (*Camellia sinensis)*. In this study, leaves of *Camellia* were subjected to spherical CNPs (0.001%) with an average diameter of 90 nm. The nano form of chitosan showed more intense bioaccumulation in tea leaves compared to regular chitosan, which may be the reason for the greater inducing effect of the former on defence enzymes like peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), superoxide dismutase (SOD) and catalase (CAT). The amount of phenolic components (e.g. gallic acid, epichatechin) and the expression of defence-related genes (e.g. genes involved in flavonoid biosynthesis or antioxidant mechanisms) was increased to a higher extent by CNP compared to chitosan, supporting the view that CNP is an effective inducer of plant defence. Both CNP and chitosan treatments induced an increase in NO level in tea leaves, and NO scavenging by the application of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or the mammalian NOS inhibitor L-N^G-nitro arginine methyl ester (L-NAME) notably mitigated the inducer effect of CNP on

defence-related enzymes and genes and also on secondary metabolite production, indicating that CNP-induced NO accumulation is an essential contributor to the development of innate immunity.

Based on the previously observed anti-fungal properties of CNPs (Saharan *et al.*, 2015; Manicandan and Sathiyabama, 2016; Sathiyabama and Parthasarathy, 2016), Siddaiah *et al.* (2018) studied the involvement of the NO signal in the CNP-triggered immune response of pearl millet against downy mildew. In contrast to the previous study, where the CNP solution was applied to the leaves of a healthy plant, seeds were incubated with CNP solutions of different concentrations, and the positive effect of CNP on germination and seedling viability was demonstrated. In pre-treated and then fungal-infected pearl millet seedlings, CNP seed treatment was shown to increase systemic resistance. This CNP-induced systemic resistance was mainly achieved by activating defence enzymes (e.g. PAL, PPO, POX, SOD, CAT) and by enhancing the transcription of corresponding genes as well as the pathogenesis-related 1 and 5 (PR1 and PR5) genes. Although the NO-inducing effect of CNP was not demonstrated in this work, NO quenching significantly inhibited the enhancement of the above defence processes by CNP, demonstrating the role of NO in the antifungal effect of CNP.

Further studying the involvement of NO in the development of CNP-induced pathogen defence is a promising research direction, as the results may contribute to improving the agricultural use of CNPs.

2.1.2. *Carbon nanotubes-promoted stress tolerance involves NO signalling*

Carbon nanotubes (CNTs) are characterized by large specific surface area, high electrical conductivity, thermal stability and hydrophobicity, and due to these desirable features CNTs are manufactured in high quantities worldwide. With the remarkable advancement of nanotechnology, carbon nanotubes have been heavily used for numerous applications in different areas of the plant system. Recently, the interest in applying CNTs to crops for agricultural purpose is constantly growing, since CNTs have a potential to be utilized as directed delivery systems for pesticides, fertilizer and other chemical compounds. The properties of CNTs are influenced by their structure. Different CNTs (e.g. single-walled carbon nanotubes, SWCNTs; multi-walled carbon nanotubes, MWCNTs) have distinct properties and application potentials (Sinha and Yeow, 2005; Sinha *et al.*, 2006; Saifuddin *et al.*, 2013; Eatemadi *et al.*, 2014; Sarangdevot and Sonigara, 2015).

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It is known that MWCNTs have positive effects on germination, biomass production, and stress tolerance in several plant species (Mondal *et al*., 2011; Wang *et al*., 2012; Khodakovskaya *et al*., 2013; Lahiani *et al*., 2013; Tiwari *et al*., 2014; Martínez-Ballesta *et al*., 2016; Hatami *et al*., 2017). Similarly, NO has been proven to intensify tolerance in multiple plant-stress systems (reviewed in Feigl and Kolbert, 2020). The first research showing a correlation between CNPs and NO was published by Karami and Sepehri (2018a), who reported that sodium nitroprusside (SNP) and MWCNTs, used either separately or together exert beneficial effects on barley germination under control conditions and also during drought or salt stress. The authors concluded that NO promotes the beneficial effect of MWCNTs on seed germination and ameliorates the adverse effect of high MWCNT doses. However, this study did not investigate the putative effect of MWCNT on endogenous NO levels, and did not provide evidence for the involvement of the NO signal in MWCNT-induced salt and drought tolerance. These gaps in knowledge have been filled in by the comprehensive study of Zhao *et al*., (2019), who studied MWCNT-induced salt tolerance and the involvement of NO in it in rapeseed (*Brassica napus*) and thale cress (*Arabidopsis thaliana*). It was observed that MWCNTs are internalized into plant cells and are translocated from root to shoot in *Brassica* seedlings. Moreover, the application of MWCNTs could effectively mitigate growth inhibition induced by salt, and resulted in high NO levels in roots. Reduction of the NO level by cPTIO in MWCNT-subjected plants terminated the beneficial effect of the nanoparticles on seedling growth. Using pharmacological treatments and mutant analyses (*nia1/2* and *noa1 Arabidopsis* with reduced NO levels), the authors suspected that NR may be partially involved in NO production during MWCNT-induced salt tolerance. As for the mechanism of NO action, the study proved that salt-triggered and MWCNT-alleviated oxidative stress depends on the presence of NO in *Brassica* roots. Additionally, MWCNT-induced NO accumulation may activate antioxidant enzymes, as suggested by the fact that cPTIO negatively affects MWCNT-enhanced activities and gene expressions of APX and SODs. The authors also observed that disturbed ion homeostasis under salt stress was improved by the MWCNT-NO pathway. These results were strengthened by genetic experiments using NO-deficient *Arabidopsis* lines. The authors conclude that NR-dependent NO is, at least partially, required for MWCNT-triggered salt tolerance *via* re-establishing redox and ion homeostasis. Additionally, the same research group recently reported that MWCNT exposure of tomato seedlings induced lateral root (LR) formation and concomitant NO production (Cao *et al*., 2020). Similarly to *Brassica* seedlings (Zhao *et al*., 2019), MWCNTs were also absorbed by tomato roots, as MWCNTs were demonstrated by TEM to

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be associated with the cell wall of root cells. When NO was scavenged by cPTIO, MWCNT-induced LR formation was significantly inhibited, indicating that the NO signal is necessary for the beneficial effect of MWCNT on LR emergence of tomato. Further results indicated that MWCNT-induced NR activity may be responsible for endogenous NO production in tomato roots (Cao *et al*., 2020).

From these results it can be seen that the beneficial effects of MWCNT on stress tolerance and root development are associated with endogenous NO signalling; however, further research is needed to better understand the molecular details of the MWCNT-NO signal pathway.

2.1.3. *NO signalling contributes to the phytotoxicity of metal-oxide nanoparticles*

Recent reviews (Khan *et al*., 2017; Marslin *et al*., 2017) have already discussed that some of the metal oxide (ZnO, Fe_3O_4) NPs may provoke oxidative stress in plant cells, whereas others containing basically non-essential metals (e.g. TiO_2 or Al_2O_3) can act positively on plant growth or stress tolerance. Nonetheless, there are only few data about the impact of metal oxide NPs on the homeostasis of reactive nitrogen species (RNS), especially NO. Here we overview some cases showing the diverse influences of these NPs depending on the metallic component.

Regarding essential metals like zinc (Zn) there are two considerable publications. Chen *et al*. (2015) reported that elevated NO content was detected both in roots and shoots of rice after ZnO NP application (250 mg L^{-1}), but NO generation was more explicit when 10 μM SNP was also added. The elevated endogenous NO due to SNP application diminished the ZnO NP-induced toxicity symptoms including root and shoot growth inhibition or reactive oxygen species (ROS) overproduction. This study suggests that the involvement of NO in enhancing ZnO NP tolerance is based on its cross-talk with ROS and the antioxidant defence system. Recently Molnár *et al*. (2020a) investigated rapeseed (*Brassica napus*) and Indian Mustard (*Brassica juncea*) seedlings exposed to ZnO NPs (~8 nm, 25 or 100 mg/L). Whereas the low dose of ZnO NP had positive effects, the higher concentration (100 mg/L) was toxic to both species. ZnO NPs elevated $O_2^{.-}$ content in the root tips due to the increased activity of NADPH oxidase, and hydrogen peroxide (H_2O_2) homeostasis was also altered. In more tolerant *B. juncea* exposed to 25 mg/L ZnO NP, the tissue level of GSNO significantly decreased and the endogenous NO level increased, but there was no evidence to show that the relationship between NO and GSNO levels might be affected by ZnO NPs. Since the changes

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of oxidative stress parameters were similar in both species, the authors suppose that the difference between the ZnO NP tolerances of the two *Brassica* species is more likely related to nitrosative than to oxidative signalling. Using ZnO NPs with larger size (~45 nm, 25 or 100 mg/L), Molnár *et al.* (2020b) detected cell wall modifications in *B. napus* where the lack of the nitrosative response was associated with ZnO NP tolerance.

Another microelement, cobalt (Co) in the form of metal oxide was also examined. In the study of Faisal *et al.* (2016) cobalt oxide nanoparticles (Co_3O_4 NPs) were reported to cause phytotoxicity expressed in retarded root elongation, and this kind of NP can massively adsorb to the root surface (Ghodake *et al.*, 2011). In this study, eggplant (*Solanum melongena*) seeds treated with Co_3O_4 NPs (1.0 mg/ml) for 7 days exhibited lower germination rate and root growth compared to the control. Additionally, in protoplasts derived from the root, endogenous NO content was shown to be elevated by all NP treatments. Since several studies have demonstrated that NO participates in cell death induction due to the disturbance of mitochondrial functions and ROS overproduction, it is not surprising that Co_3O_4 NPs cause stunted root development.

In the paper of Saquib *et al.* (2016) the impact of ferric oxide nanoparticles (Fe_2O_3 NPs) on radish (*Raphanus sativus*) was analysed. The application of Fe_2O_3 NPs provoked root shortening and reduced the seed germination rate due to the increased level of reactive ROS and NO. A dose-dependent induction of the antioxidant enzymes like CAT, SOD and glutathione (GSH) as well as lipid peroxidation were also demonstrated. These results suggest that metal oxide NPs containing essential microelement may cause severe nitro-oxidative damage in plants.

At the same time, metal oxide NPs incorporating non-essential metals like aluminium (Al) or titanium (Ti), seem to be beneficial for plants, even under stress conditions. When *Arabidopsis thaliana* was exposed to 98 μM Al_2O_3 NPs, the NO content in roots showed no changes compared to the control, whereas ionic Al ($AlCl_3$) at 196 μM concentration resulted in significant inhibition of root growth accompanied by NO accumulation (Jin *et al.*, 2017). Moreover, previously Poborilova *et al.* (2013) used tobacco BY-2 cell suspension culture as plant cell model, and exposed it to Al_2O_3 NPs (10, 20, 50 and 100 μg mL^{-1}) for 12–96 h. The levels of RNS (endogenous NO) and ROS (H_2O_2 and O_2^-) showed time- and dose-dependent enhancement. Besides, elevated malondialdehyde (MDA) production was observed, which resulted in plasma membrane damage and, finally, programmed cell death. Nanomaterial-induced NO production in different plant species and experimental systems is summarized in Table 1.

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Stress tolerance improvement by the application of metal oxide NPs can be a future perspective in agriculture. Barley was exposed to salt stress (100 or 200 mM NaCl), and the potentially positive impact of titanium dioxide (TiO_2) NPs at 500, 1000 and 2000 mg kg^{-1} (pot experiment) was tested (Karami and Sepehri, 2018b); moreover, exogenous NO was added in the form of SNP (100 µM). TiO_2 NPs at all concentrations had a beneficial effect on plant growth and photosynthetic activity in salt-stressed plants. SNP itself also improved the activity of antioxidant enzymes like SOD, CAT and APX, whereas TiO_2 together with SNP proved to be effective in decreasing MDA and H_2O_2 levels, which are the indicators of oxidative stress induced by salinity. In cadmium-stressed wheat the joint application of SNP and TiO_2 NPs could moderate the negative effect of Cd on seed germination and seedling growth, suggesting their promising potential in the alleviation of the negative effects induced by Cd stress (Faraji *et al.*, 2018). This theory was further reinforced by the observation that the combined application of exogenous NO and TiO_2 NPs was able to protect wheat seedlings against oxidative stress induced by drought (Faraji and Sepehri, 2020). In this study 100 µM SNP with 2000 mg/kg TiO_2 NP reversed seedling growth inhibition, and increased the amount of total soluble proteins and SOD activity, together with photosynthetic activity, leading to reduced H_2O_2 content and lipid peroxidation under drought stress. Additionally, the application of 15 mg L^{-1} TiO_2 NPs to drought-stressed bean (*Vicia faba*) induced NR activity and consequently increased the endogenous NO level in the seedlings (Khan *et al.* 2020). This higher NO level fortified the enzymatic (SOD, CAT) and non-enzymatic (ascorbate and GSH) antioxidant defence system and attenuated the generation of H_2O_2, O_2^- and lipid peroxides. Based on the above studies exogenous NO and TiO_2 NPs have a mutually reinforcing, positive effect (summarized in Table 2), which could be a powerful tool to help plants cope with abiotic stressors; however, these results should be confirmed by examining other metal oxide NPs and NO donors.

2.2. Protective effect of exogenous chemical NO donors on nanoparticle-induced stress in plants

Exogenously applied NO (mainly in the form of SNP) is well known to be able to alleviate the negative effects of various abiotic stresses, including high concentrations of elements (heavy metals included) (Terrón-Camero *et al.*, 2019), although very little is known about the protective effect of exogenous NO on NP-induced stress in plants. So far, only three studies have dealt with the topic in question, all of them using SNP as a NO donor agent.

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Chen *et al*. (2015) examined the effect of SNP on ZnO nanoparticle-stressed rice (*Oryza sativa* L.) seedlings, and found that 10 μM SNP was able to effectively reduce toxicity symptoms. Exogenous NO was able to overturn the ZnO NP-induced growth inhibition, by the reduction of Zn accumulation. Moreover, SNP mitigated ROS accumulation by the elevation of GSH level and SOD activity and reversing the ZnO NP-induced decrease in POX, CAT and APX activities. In agreement with the activity results, gene expression of the above-mentioned antioxidant enzymes was upregulated by SNP under ZnO NP stress. Moreover, NO overproducer (*noe1*) and deficient (*noa1*) rice lines were also tested, proving that high NO content can increase ZnO NP tolerance by upregulating the gene expression of antioxidant enzymes.

Tripathi *et al*. (2017a) also studied the effect of ZnO NP, but on wheat (*Triticum aestivum* L.) seedlings, and found that 100 μM SNP successfully enhanced their ZnO NP tolerance through two mechanisms. Firstly, exogenous NO lowered Zn content in the vascular tissues, resulting in reduced oxidative stress and lipid peroxidation. Secondly, in the background of decreased oxidative stress, upregulation of the enzymes (APX, glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MHAR)) of the ascorbate-glutathione cycle was observed, resulting in an enhanced ascorbate/dehydroascorbate and reduced/oxidized glutathione ratio, providing a higher protection against ZnO NP-induced oxidative stress.

Comparing the two similar studies, it is conspicuous that there was a ten-fold difference in the effective SNP concentrations, despite working with hydroponically-grown seedlings in both experimental setups. This difference may be due to differences in treatment conditions. Namely, rice plants were subjected to both SNP and ZnO NPs at the same time (Chen *et al*., 2015), whereas wheat plants were treated with SNP for 24 hours prior to NP supplementation (Tripathi *et al*., 2017a).

In the third and last study, also by Tripathi *et al*. (2017b) the effect of exogenous NO on silver nanoparticle (Ag NP)-induced stress in pea (*Pisum sativum* L.) seedlings was studied, and it was found that 100 μM SNP was able to effectively decrease the negative effects induced by Ag NP. Similarly to the previous studies, exogenous NO was able to decrease Ag accumulation, oxidative stress and lipid peroxidation caused by Ag NP stress. NO supplementation improved photosynthetic activity together with the enzymatic (APX, SOD, GR, DHAR) and non-enzymatic (total ascorbate and GSH content) antioxidant defence system. It was also found that SNP treatment was able to ameliorate Ag NP-related morphological toxicity symptoms in leaves, such as abnormal parenchymatic differentiation

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and epidermis development, and also in roots, where Ag NP-inhibited root hair formation was reversed by NO supplementation.

Based on the (scant) information available, exogenous NO in the form of SNP supplementation protects plants from the consequences of NP-induced stress (summarized in Table 2). Based on the results, at least two main mechanisms of NO action can be assumed. Nitric oxide decreases metal uptake (liberated from the NPs) and reduces oxidative stress through the upregulation of both enzymatic and non-enzymatic antioxidant capacity. The molecular mechanisms of NO effects on metal uptake and antioxidants like *S*-nitrosation or protein nitration are still not known and need to be further elucidated. Although, these mechanisms may be similar to the much better studied effects of exogenous NO on plants subjected to "standard" heavy metal stress (reviewed by Terrón-Camero *et al*., 2019).

3.   <u>NO-releasing nanoparticles and their effects on plants</u>

Although SNP is widely used as supported by the research presented above, the reliability of such chemical NO donors in plant biology is limited by their putative side effects and instability. The production and use of NO donor molecules in the form of NPs can bring a breakthrough in this area. Such NO-releasing NPs have already been extensively studied in clinical research (Zhou *et al*., 2016; Xu *et al*., 2019), whereas in plants, so far, only a few reports describe their effects.

First, in 2015, Pereira *et al*. prepared and applied on plants GSNO-containing alginate/chitosan nanoparticles with a hydrodynamic diameter of 300–550 nm. As for NO releasing capacity, the NPs resulted in a NO burst in the first five hours, then caused further increase in NO in the next 24 hours. The rate of NO release was proportional to the concentration of GSNO-containing alginate/chitosan nanoparticles. At a concentration of 10 mmol/L, NPs released approx. 2.5 mmol/L NO within 24 hours. However, the NPs produced did not have a significant effect either on soybean (*Glycine max*) or on maize (*Zea mays*), which on the one hand means that the NPs are non-toxic, and on the other hand draws attention to the fact that it is worth examining the effects in a wider concentration range to explore their assumed positive effect related to stress response/tolerance and their transport and fate in different plant species.

In the first relevant study, Oliveira *et al*., (2016) used the low-molecular weight NO donor, S-nitroso-mercaptosuccinic acid (S-nitroso-MSA) belonging to the class of RSNOs. S-nitroso-MSA was encapsulated by chitosan, yielding S-nitroso-MSA CS NPs with a

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hydrodynamic diameter range between 20 and 56 nm. In the first 12 hours, approx. 70-80 μM NO was liberated by 1000 μM S-nitroso-MSA CS NPs, which is much less than the amount of NO liberated by free S-nitroso-MSA. Maize plants were exposed to NaCl plus S-nitroso-MSA CS NPs in sand culture. S-nitroso-MSA CS NPs (100 μM) further increased salt-triggered elevation of SNO content in maize leaves, which in turn ameliorated the growth reduction, photosynthetic inhibition and chlorophyll loss induced by salt stress. The ineffectiveness of NPs containing non-nitrosated MSA suggests that the salt stress-ameliorating effect of S-nitroso-MSA-CS NPs is due to the released NO. The authors noted that the uptake, translocation and accumulation of S-nitroso-MSA-CS NPs in plant tissues needs to be studied in the future.

In a recent study, GSNO was encapsulated in CS NPs, and the resulting GSNO CS NPs with a hydrodynamic size ~104 nm were shown to release NO *in vitro*, although the rate of NO liberation was approx. 50% less than in case of free GSNO (Silveira *et al*., 2019). This indicates that encapsulation prevents GSNO from transient decomposition. Interestingly, when applied on sugarcane plants, both the free and the NP-form of GSNO increased the SNO level to a similar extent in the leaves. These observations emphasize that GSNO CS NPs have more advantageous properties (enhanced stability with similar NO-liberating capacity) than free GSNO. Sugarcane plants were exposed to polyethylene glycol (PEG)-induced drought decreasing CO_2 assimilation, transpiration, PSII-related photosynthetic capacity, relative water content, chlorophyll concentration as well as biomass production. Plants sprayed with free GSNO or GSNO CS NPs showed an improvement in the above-mentioned parameters, indicating that exogenous GSNO (both free and NP form) positively regulates drought stress tolerance of sugarcane plants. There was no significant difference between the effects of free GSNO and the NP form except for the root/shoot ratio, where the GSNO CS NPs caused a greater increase, suggesting its potential use in agricultural/cultivation methods.

In their recent study, Lopes-Oliveira *et al*., (2019) prepared S-nitroso-MSA CS NPs with 35-40 nm hydrodynamic size according to their previous method (Oliveira *et al*. 2016). Two-phased NO release was observed *in vitro,* where the first NO burst occurred after 15 min in light and after 50 min in the dark and the second phase resulted in a steady-state NO level. Similarly to previous observations, the NO-releasing capacity of S-nitroso-MSA CS NPs was lower than that of free S-nitroso-MSA. Treatments with 2 mM S-nitroso-MSA CS NPs, free S-nitroso-MSA or MSA NPs were applied *via* the growth substrate on *Heliocarpus popayanensis* and *Cariniana estrellensis* seedlings cultivated in an outdoor nursery. The

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concentration of SNO increased significantly only in the case of *C. estrellensis* leaves, although MSA NPs also caused SNO level increase, which makes the NO specificity of the NP effect uncertain. Additionally, the treatments did not modify SNO levels in the leaves but increased most of the observed growth parameters in *H. popayanensis.* As for *C. estrellensis*, none of the treatments affected growth despite the S-nitroso-MSA CS NP-triggered SNO increase. This indicates the lack of a connection between SNO/NO levels and growth induction. Regarding photosynthesis, S-nitroso-MSA CS NPs were ineffective in both species. Furthermore, a slight reduction in phenolics and a moderate increase in H_2O_2 level was observed in S-nitroso-MSA CS NPs-treated H. *popayanensis*, whereas other parameters showed no relevant modifications as a result of NO-releasing NP treatment. According to the authors, S-nitroso-MSA CS NP treatment may be a powerful strategy to develop seedling acclimation. However, it is important to highlight that S-nitroso-MSA CS NPs were not effective in increasing SNO levels in all cases, the growth-promoting effect was species-dependent and there was no correlation between SNO levels and growth induction.

The results available so far will need to be supplemented in the future, but based on the above, it can be concluded that encapsulation of NO donors provides better stability against thermo- and photolysis, better storage, and the NPs are able to control the release of NO *in vitro* within a similar order of magnitude but to a lesser extent than the free NO donors. Treatment of plants (*via* foliar spray or *via* the root system) in most cases demonstrably increases SNO levels and alleviates stress-induced damages in the plant species studied so far (summarized in Table 3). Therefore, it is necessary to further investigate and critically evaluate these promising combinations of NO donors and nanomaterials prior to use.

4. NO-detection in plants with nanoparticle-based sensors

The other relevant methodological problem in plant NO research is quantification of the free radical within plant tissues. The most common method available to most laboratories is microscopic detection of NO by diaminofluorescein probes (Kojima *et al*., 1998), but this approach does not provide quantitative results. The development of NO-specific nanosensors can make progress on this issue due to their favourable characteristics such as being non-destructive, minimally invasive, and capable of real-time analysis (Iverson *et al*., 2018). However, only one study has been published to date in which a smart NP-based sensor detecting NO has been applied in plants (Giraldo *et al*., 2014). Previously, 3,4-

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diaminophenyl-functionalized dextran (DAP-dex) wrapped in single-walled carbon nanotubes (SWNTs) was synthetized, and it was observed that the near-infrared fluorescence of SWNT$_{DAP-dex}$ is rapidly, directly and selectively quenched by NO (Kim *et al*., 2009). It was shown that SWNT penetrates lipid bilayers and internalizes chloroplasts, which made it possible to sense chloroplast-localized NO by SWNT. *Arabidopsis* leaf was infiltrated with NO-sensing SWNTs and was excited by epifluorescence microscope following the addition of dissolved NO solution. Based on the degree of fluorescence quenching, the level of NO could be estimated. Such nanosensors allow the translation of plant chemical signals (e.g. NO) into digital information that can be monitored by electronic devices in real time. Smart plant sensors can be used for the evaluation of the health status of plants in order to improve plant productivity, and therefore they can have a great potential in agricultural practices (Giraldo *et al*., 2019).

5. Conclusion and future perspectives

Diverse types of nanomaterials, e.g. chitosan NP, nanotubes, metal-oxide NP, and NO-releasing NP promote NO production within the plant body. In some cases, NR was associated with NP-induced NO production. In general, endogenous NO has a positive effect by activating the antioxidant system (enzymatic and non-enzymatic) and contributing to the beneficial effect of nanomaterials by eliciting immune response, by enhancing tolerance in plants exposed to abiotic stress or by promoting growth and development. Several studies focus on the ameliorating effect of chemical NO donors on NP phytotoxicity. In these cases, NO has been observed to exert its effect both by inducing the antioxidant system and reducing metal uptake (Figure 2). Overall, nanoscience in plant systems is a novel research field. The few available literature data need to be expanded by molecular studies. The molecular mechanism of NO signalling (e.g. *S*-nitrosation, tyrosine nitration, lipid nitration etc.) behind the effects of NPs on plant physiology need to be closely investigated by future studies. From a practical point of view, testing of NO-releasing NPs on plants is highly relevant, as those can replace chemical NO donors both in plant research and in possible agricultural applications. Equally important is that NO-specific nanosensors may promise methodological development in plant research and in nano-agriculture, thus their testing in plants needs to be continued.

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Author Contribution

ZSK: Conceptualization, Visualization, Writing – original draft, Writing – review & editing; RSZ: Writing – original draft; GF: Writing – original draft; ZK: Writing – review & editing; AR: Writing – original draft, Writing – review & editing.

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Table 1 Nanomaterial-induced NO production in different experimental systems. Abbreviations: CNP, chitosan nanoparticle; MWCNT, multiwalled carbon nanotube; ZnO NPs, zinc-oxide nanoparticles; Co_3O_4 NPs, cobalt oxide nanoparticles; Fe_2O_3 NPs, ferric oxide nanoparticles.

Type of NP	Characteristics of NP (average diameter, length, form)	Plant treatment conditions	Plant species	Reference
CNP	~ 90 nm, spherical	0.01% for 24h *via* excised leaves	tea *(Camellia sinensis)*	Chandra et al. 2017
MWCNT	6-12 nm, 1-9 μm	20 mg/L for 5 days *via* agar-solidified MS medium	rapeseed *(Brassica napus)* seedlings	Zhao et al. 2019
		5 mg/mL for 24 hours *by* incubating the seedlings in treatment solutions	thale cress *(Arabidopsis thaliana)* seedlings / tomato *(Solanum lycopersicum)* seedlings	Cao et al. 2020
ZnO NPs	~30 nm	250 mg/L for 3 days *via* nutrient solution	rice *(Oryza sativa)* seedlings	Chen et al. 2015
ZnO NPs	~8 nm, spherical	germination in the presence of 25 or 100 mg/L	Indian mustard *(Brassica juncea)* root	Molnár et al. 2020
Co_3O_4 NPs	~21 nm, polyhedral	2 hours-long seed treatment, 0.25, 0.5 or 1 mg/mL	eggplant *(Solanum melongena)* root protoplasts	Faisal et al. 2016
Fe_2O_3 NPs	~22-26 nm, polyhedral	2 hours-long seed treatment, 0.5 or 1 mg/L	radish *(Raphanus sativus)*	Saquib et al. 2016
Al_2O_3 NPs	~5 μm	10, 20,50 100 μg/mL for 96 hours	tobacco *(Nicotiana tabacum)* BY2 cell suspension	Poborilova et al. 2013

Table 2 Ameliorating effects of exogenous chemical NO donors applied alone or in combination with nanoparticles on stresses. Abbreviations: MWCNTs, multiwalled carbon nanotubes; SNP, sodium nitroprusside; TiO2 NPs, titanium dioxide nanoparticles; ZnO NPs, zinc oxide nanoparticles; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; LPO, lipid peroxidation; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; AsA, ascorbate; GSH, glutathione; POX, peroxidase; GR, glutathione reductase; DHAR, dehydroascorbate reductase; Ag NPs, silver nanoparticles.

Stress ameliorating treatments	Stressor	Plant species	Effects	Reference
MWCNTs (500, 1000, 2000 mg/kg) +SNP (100 µM)	100 or 200 mM NaCl	*barley (Hordeum vulgare)*	improved photosynthesis, chlorophyll content, relative water content, increased SOD, CAT, APX, proline content, reduced LPO, H_2O_2	Karami and Sepehri 2018a
TiO$_2$ NPs (500, 1000, 2000 mg/kg) + SNP (100 µM)	100 or 200 mM NaCl	*barley (Hordeum vulgare)*	increased SOD, CAT, APX, reduced LPO, H_2O_2	Karami and Sepehri 2018b
TiO$_2$ NPs (50, 1000, 2000 mg/L) + SNP (100 µM)	50 or 100 mM CdCl$_2$	*wheat (Triticum aestivum)*	improved germination and biomass production	Faraji et al. 2018
TiO$_2$ NPs (2000 mg/kg) + SNP (100 µM)	drought by limited water supply	*wheat (Triticum aestivum)*	improved growth, photosynthesis, SOD activity, decreased LPO and H_2O_2	Faraji and Sepehri 2020
10 µM SNP	~30 nm ZnO NPs, 250 mg/L for 3 days	*rice (Oryza sativa)*	improved growth, reduced Zn accumulation, mitigated ROS accumulation, increased GSH, SOD, POX, CAT, APX enzyme activities and gene expression	Chen et al. 2015

100 µM SNP	~5-20 nm, spherical ZnO NPs, 100 or 200 µM for 7 days	*wheat (Triticum aestivum)*	reduced Zn accumulation, upregulated enzymes of AsA-GSH cycle	Tripathi et al. 2017a
100 µM SNP	~20 nm, spherical Ag NPs, 1000 or 3000 µM for 15 days	*pea (Pisum sativum)*	improved photosynthesis, improved enzymatic (APX, SOD, GR, DHAR) and non-enzymatic (AsA, GSH) defence, ameliorated morphology in leaves and roots	Tripathi et al. 2017b

Table 3 Effects of NO-releasing nanoparticles (NO NPs) on different plant species. Abbreviations: GSNO, S-nitrosoglutathione; S-nitroso-MSA CS NP, S-nitroso-mercaptosuccinic acid chitosan nanoparticles; SNO, S-nitrosothiol; GSNO CS NP, S-nitrosoglutathione chitosan nanoparticles; PEG, polyethylene glycol;

Type of NO NPs	*In vitro* NO liberation	Tested plant species	Effects	Reference
GSNO alginate/chitosan, 300-550 nm, 1,5 or 10 mM	from 10 mmol/L NP ~2.5 mmol/L NO after 24 hours	soybean *(Glycine max)* maize (*Zea mays)*	no effects on biomass production compared to control	Pereira et al. 2015
S-nitroso-MSA CS NP, ~20-56 nm, 100 µM	from 1000 µM NP ~70-80 µM NO after 12 hours	NaCl-exposed maize (*Zea mays)*	increased leaf SNO content, improved growth and photosynthesis, increased chlorophyll content	Oliveira et al. 2016
GSNO CS NP, ~104 nm, 100 µM	from 1 mmol/L NP ~100 µmol/L after 3 days	PEG-exposed sugarcane (*Saccharum* spp.)	increased SNO content, improved CO_2 assimilation, transpiration, PSII activity, relative water content, chlorophyll content, biomass production	Silveira et al. 2019
S-nitroso MSA CS NP, ~35-40 nm, 2 mM	from 2 mM NP ~1.6 mM NO after 50 min in the light	*Heliocarpus popayanensis Cariniana estrellensis*	increased SNO content in *C. estrellensis*, but the observed growth promoting effects could not be associated with the NO releasing capacity	Lopes-Oliveira et al. 2019

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Figure legends

Figure 1. Reactions and signalling of NO in plant cells resulting in regulation of growth, development and stress responses. See explanations in the text. Scavenging reactions are indicated by grey arrows. Putative consequences are indicated by dashed arrows.

Figure 2 The effects of endogenous and exogenous NO in nanoparticle-exposed plants. Enhanced NO production due to NP (chitosan NPs, nanotubes, NO NPs) or chemical NO donor treatments exerts beneficial effects such as participating in pathogen defence, contributing to salt tolerance and promoting plant growth. On the other hand, NO accumulation in plants exposed to metal-oxide NPs contributes to toxicity *via* macromolecule damage (e.g. protein nitration) and cell death.

Figure 1

Growth, development, stress responses
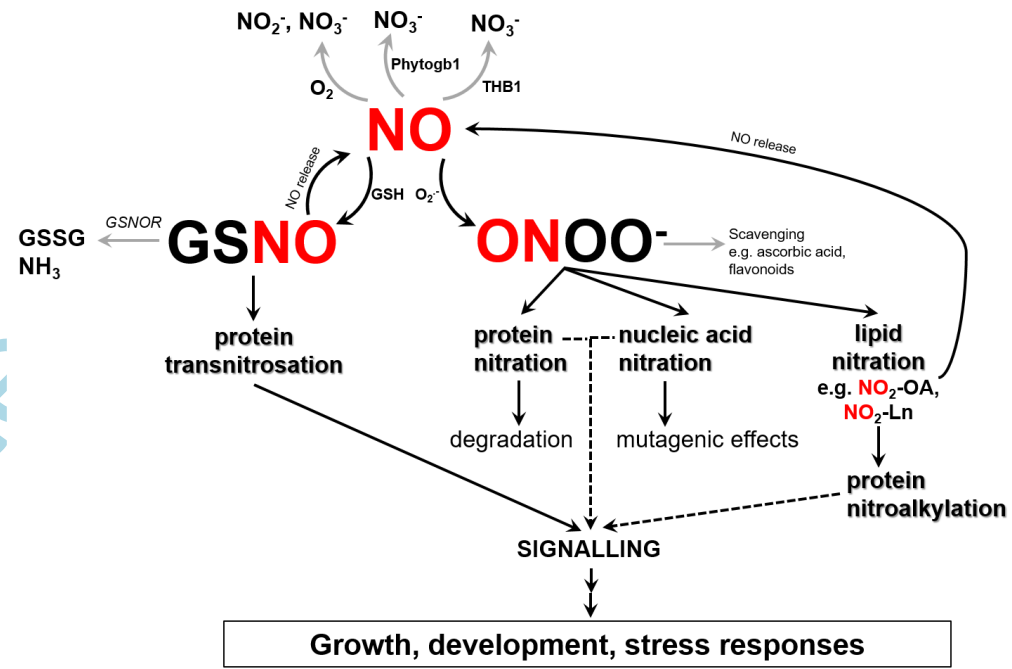
Figure 2