

Viewpoint

Nitric oxide-releasing nanomaterials: from basic research to potential biotechnological applications in agriculture

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Summary

Nitric oxide (NO) is a multifunctional gaseous signal that modulates the growth, development and stress tolerance of higher plants. NO donors have been used to boost plant endogenous NO levels and to activate NO-related responses, but this strategy is often hindered by the relative instability of donors. Alternatively, nanoscience offers a new, promising way to enhance NO delivery to plants, as NO-releasing nanomaterials (e.g., *S*-nitrosothiol-containing chitosan nanoparticles) have many beneficial physicochemical and biochemical properties compared to non-encapsulated NO donors. Nano NO donors are effective in increasing tissue NO levels and enhancing NO effects both in animal and human systems. The authors believe, and would like to emphasize, that new trends and technologies are essential for advancing plant NO research and nanotechnology may represent a breakthrough in traditional agriculture and environmental science. Herein, we aim to draw the attention of the scientific community to the potential of NO-releasing nanomaterials in both basic and applied plant research as alternatives to conventional NO donors, providing a brief overview of the current knowledge and identifying future research directions. We also express our opinion about the challenges for the application of nano NO donors, such as the environmental footprint and stakeholder's acceptance of these materials.

Main body of the text

Nitric oxide (NO) is widely recognized as a signaling molecule with a myriad of functions in plant growth, development, stress responses and interaction with beneficial microorganisms (Kolbert *et al.*, 2019, 2021), with an untapped biotechnological potential (Marvasi, 2017; Corpas *et al.*, 2020; Sun *et al.*, 2021). More than 20 years after the pioneer works in this field (Leshem & Haramaty, 1996; Gouvea *et al.*, 1997; Laxalt *et al.*, 1997; Delledonne *et al.*, 1998; Durner *et al.*, 1998), the proper delivery of NO to plant cells is still a challenge that hinders its use in natural field conditions. As NO is a gaseous free radical with a short half-life under aerobic conditions, the exogenous treatment with molecules that act as NO donors has been used as the main strategy to increase plant endogenous NO content and provoke NO biological effects (Seabra & Oliveira, 2016; Seabra *et al.*, 2022). Despite several NO donors being available, such molecules usually show properties that compromise the

desired signaling action of NO on target plants, because they have rapid degradation and are sensitive to environmental factors, release NO too quickly, and/or generate toxic by-products (Fig. 1a).

Nanotechnology is a novel approach that has been successfully used for a wide range of agricultural applications to modulate various processes such as seed germination, seedling development and growth, photosynthesis, hormonal balance, disease resistance and plant nutrition (Ahmad *et al.*, 2022; El-Shetehy *et al.*, 2021; Jiang *et al.*, 2021; and references therein). One of the most promising strategies is the nanoencapsulation of agrochemicals (*e.g.*, pesticides and fertilizers) to improve their delivery to plants (Usman *et al.*, 2020; Fincheira *et al.*, 2021). Briefly, the active ingredient is trapped into a nanomaterial that protects it from degradation and allows a sustained release. Due to the higher specific surface area and ability of nanomaterials to interact with cells compared to bulk materials, the nanoencapsulation enhances ingredient uptake by plant tissues and reduces its environmental losses, yielding higher efficiency and efficacy (Pascoli *et al.*, 2018; Jiménez-Arias *et al.*, 2020; Takeshita *et al.*, 2021). Thus, better effects of the agrochemical on the target organisms can be obtained with less frequent applications and lower doses, whereas the negative environmental impacts can be minimized.

Polymeric nanoparticles are used to encapsulate NO donors for biomedical purposes (Pieretti *et al.*, 2020). However, the application of nanoencapsulated NO donors in plants is much more recent and can bring enormous benefits to this field, including the sustained and localized NO release under varying environmental conditions and improved NO bioavailability in plant tissues (Fig. 1b). The few studies published up to now reporting the treatment of plants with nano NO donors provide exciting findings and many avenues to be further explored (Tables 1 and 2). Briefly, the nanoencapsulation of NO donors increased the NO delivery to plants due to a sustained NO release. As NO might be toxic at higher concentrations, there are more benefits with lower and prolonged NO doses, that are more easily attained by the use of nano NO donors.

Most studies have focused on plant protection against abiotic stress, but NO-releasing nanomaterials could also be used for the induction of plant response to biotic stress and for modulating plant growth and development. As potential and practical applications in agriculture, we would suggest the use of nano NO donors in seed and seedling priming for improving germination and early growth (specially under limiting conditions), in saplings for enhancing stress tolerance, in

micropropagation for improving plantlet development and hardening, and in floriculture and fruit post-harvest for increasing the shelf life of flowers and fruits.

As there are different ways to supply nano NO donors to plants (*e.g.*, seed treatment, soil amendment, leaf and fruit spraying, and cell culture), the entry route of the nanocarrier into the plant is variable and thus may result in different responses (Fincheira *et al.*, 2020; Pereira *et al.*, 2021). In addition, several NO donors can be used such as *S*-nitrosothiols (SNOs), NONOates, diazeniumdiolates, sodium nitroprusside (SNP), Roussin's black salt, metal nitrosyl complexes, and nitro fatty acids (Mata-Pérez *et al.*, 2016), as well as molecules that induce endogenous NO synthesis by plants, such as organic nitrate/nitrate, nitrite, polyamines, and *L*-arginine (Pissolato *et al.*, 2020; Silveira *et al.*, 2021b; Seabra *et al.*, 2022). Another important factor to be explored is the nature of nanomaterials, which may vary in size, morphology, chemical composition, surface charge, and presence of functional groups in their surface (Fig. 1c), promoting significant variation in the biological effects of NO-releasing nano formulations. By changing the nanoparticle characteristics, its adhesion and absorption by plants, cell internalization, and short- and long-distance translocation can be altered to obtain a more efficient nano formulation to a given purpose (Avellan *et al.*, 2021; Zhang *et al.*, 2021). The development of stimuli-responsive nanocarriers is another possibility as well as nanomaterials with binding motifs/functional groups, allowing a better targeting to specific tissues/cells/organelles (Pieretti *et al.*, 2020; Liang *et al.*, 2021). These strategies are extensively explored in biomedical applications but poorly explored in plant science (Seabra *et al.*, 2014).

The few pioneer studies of nano NO donors in plants have explored only chitosan nanoparticles, which is a cost-efficient and eco-friendly biopolymer (Oliveira *et al.*, 2016; Lopes-Oliveira *et al.*, 2019; do Carmo *et al.*, 2021; Silveira *et al.*, 2021a). However, there are many other biodegradable, biocompatible polymers obtained from renewable sources to be used, such as lignin, alginate, cellulose and zein (Darder *et al.*, 2020; Urzedo *et al.*, 2020; Low *et al.*, 2021). In addition to polymeric nanoparticles, there are different classes of nanomaterials for NO incorporation (via nanoencapsulation or surface functionalization) as liposomes (Suchyta & Schoenfisch, 2015), metal/metal oxide nanoparticles coated with organic matrix (Santos *et al.*, 2016; Pieretti *et al.*, 2021), and carbon-based nanomaterials (Tanum *et al.*, 2019, Jin *et al.*, 2021) (Fig. 1b). Another possibility is the application of NO gas allied to nanoporous materials, such as metal organic frameworks (MOFs)

and zeolites, that adsorb NO gas and release it upon contact with moisture (Seabra & Durán, 2010). These nanomaterials have been used only for biomedical applications. They have a limited amount of NO loading in addition to fast NO release, high cost and difficult storage (McKinlay *et al.*, 2013).

The possibility to join NO-releasing nanomaterials with other bioactive compounds used as plant growth regulators or compounds able to increase the endogenous NO production is an approach deserving attention in further studies (Silveira *et al.*, 2021b; Pissolato *et al.*, 2020). In any experiment, it is very important to define the proper controls to differentiate the effects of NO donor from those of the nanocarrier, as many nanomaterials (including chitosan) may induce dose-dependent responses that are beneficial to plant growth and defense (Kumaraswamy *et al.*, 2018; Malerba & Cerana, 2018). For instance, it has been recently demonstrated that the application of copper oxide nanoparticles increased the endogenous *S*-nitrosothiol levels of lettuce (*Lactuca sativa* L.) seedlings (Pelegrino *et al.*, 2020; Kohatsu *et al.*, 2021). Thus, the nanomaterial *per se* can potentiate the effect of the NO donor in modulating the plant NO homeostasis.

Although significant advance has been achieved in the use of NO-releasing nanomaterials in plants, there are key questions that remain unresolved (Fig. 1d):

- a) *What is the fate of the nanomaterials and the NO donor in plant tissues?* Tracking the nanomaterial and the NO donor in plant tissues is essential to understand the mechanisms of interaction among them, thus providing information on how we could design smarter nanocarriers and how they could be employed to manipulate cellular processes (Avellan *et al.*, 2017, 2021). This approach could also provide information about the best application strategy (including type and frequency/number of treatments) to guarantee an efficient and effective NO delivery. The local and systemic impacts of free and nano NO donors on plant NO homeostasis should also be addressed, as the nanomaterials can enhance the translocation and distribution of the delivered compounds inside plants, mediating systemic responses (Avellan *et al.*, 2019; Lowry *et al.*, 2019; Takeshita *et al.*, 2021). Then, the development of reliable protocols to accurately track NO/NO derivatives and nanomaterials in plant tissues is required. For example, fluorescently-labelled nanomaterials (Bombo *et al.*, 2019) can be used together with NO_x detection dyes (*e.g.*, diaminofluorescein- and diaminorhodamine-based probes) to localize simultaneously both entities in plant tissues by confocal microscopy. The binding of rare metallic elements (such as gadolinium) is another way to track the nanomaterial inside plants

(Zhang *et al.*, 2021). In this sense, the use of magnetic nanoparticles that allows directing them to a specific plant organ with the use of a magnet could be an alternative such as has been used in medical applications (Sola-Leyva *et al.*, 2020). The knowledge about the fate of nanomaterials *in planta* is also important to verify whether potentially harmful nanomaterials and/or their by-products could accumulate in edible parts of the plants.

b) *What is the extent of NO signaling in plants upon supplying NO-releasing nanomaterials in comparison to conventional NO donors?* Given that the NO-triggered cellular effects depend on the kinetics release and the chemical nature of the NO donor, as well as on the subcellular localization of NO, it should be evaluated whether the use of NO-releasing nanomaterials would allow a better specificity of NO effects and if the target biomolecules in local and systemic tissues are changed. The subcellular site of NO production and its spatial and temporal diffusion are key parameters of NO specificity (Hess *et al.*, 2005; Umbreen *et al.*, 2018); thus, the cellular distribution of NO derived from nanoformulations is an important point to follow. Several omics approaches (*e.g.*, proteomics, metabolomics and transcriptomics) would be very much useful to uncover the underlying processes and signaling induced by NO-releasing nanomaterials.

c) *What is the extent of the impacts of NO-releasing nanomaterials to the environment and to organisms that interact with plants, such as insects, microorganisms and humans?* Ecotoxicological assays with bioindicator organisms in terrestrial and aquatic systems (including mesocosm approaches) are necessary to verify the safety of nano NO donors to the environment and plant-interacting organisms (Tortella *et al.*, 2020). In addition, microbiome and functional analyses of the soil may indicate the effects of NO-releasing nanomaterials on soil microbiota. As NO is also an important signaling molecule for microorganisms (Astuti *et al.*, 2018), it could be hypothesized whether the effects of NO-releasing nanomaterials on plants could be mediated by changes in microbial activity (particularly for soil treatment). The possible impacts of nano NO donors to human beings who will consume fruits and vegetables obtained from plants treated with these nanomaterials should be also investigated. It is noteworthy that, due to the short half-life of released NO and its derivatives, the low doses of NO donors applied to plants are likely to have much lower impact on NO metabolism in humans in comparison to nitrate/nitrite present in plant food. In addition, the treatment of plants with NO donors allied to nanomaterials has the potential to improve the nutritional

attributes of vegetables (Pelegrino *et al.*, 2021). It is noteworthy that, as the NO concentration required for signaling in plants is low, a high dilution ratio of the stock nano formulations is usually carried out (do Carmo *et al.*, 2021), thus minimizing the exposition of the plant/environment/human to the nanomaterial and NO derivatives. Efforts for augmenting the amount of the NO donor incorporated into the biocompatible nanomaterials (that generally have a safe profile) are welcomed, which would allow even higher dilution rates.

d) *What would be the social acceptance of this approach?* Once demonstrated the beneficial vs. negative effects of the application of NO-releasing nanomaterials, we believe that the public and societal opinion about the use of this technology is a vital point to be considered, to avoid a rejection similar to that happened with genetically modified organisms and classical chemical-based pesticides. Proper actions for scientific dissemination and continuous dialogue with stakeholders (companies, farmers, consumers, governmental and non-governmental agencies) should not be put aside by researchers, thus ensuring an effective communication about the significant benefits of this technology in view of its costs and potential issues. A detailed cost-benefit analysis would be an important future research area to help establish the potential financial advantage associated with this nascent technology. In addition, for a proper acceptance of NO-releasing nanomaterials, the shelf life of the formulation should be taken into account, and this aspect depends on the nature of the NO donor and the nanomaterial. For instance, in the case of *S*-nitrosothiol-loaded chitosan nanoparticles, the non-nitrosated formulation can be transported at room temperature and stored at refrigeration for up to one month, but there are many strategies to be tested in order to improve their durability. Last but not least, it is noteworthy that there is no appropriate international regulatory framework of nanomaterials in the agri-food sector, which might pose severe limitations to their application and public acceptance (Sodano, 2018; Allan *et al.*, 2021).

In summary, despite of extensive applications in nanomaterials, only minor progress has been achieved in plant science by using NO-releasing nanomaterials. Basic and applied research is still required to understand their mode of action in plants and to safely translate this technology to field applications. To this end, not only a scientific investigation of the detailed effects of nano NO donors on plants is mandatory, but also a realistic evaluation of (i) the advantages in terms of costs and benefits of this approach, (ii) the industrial interests, (iii) the farmers' and consumers' perception and

acceptance of this new technology, and (iv) the economic viability of this strategy in the whole production chain of agricultural products. To achieve this huge challenge, we believe that collaboration among multidisciplinary teams with skills in chemistry, material sciences, biology, agronomy, ecotoxicology, food engineering and socio-economy is fundamental. We expect that this Viewpoint opens new avenues for this exciting and promising approach that would contribute to the development of precision agriculture.

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Author contribution

ABS, ZK and HCO conceived the manuscript. ABS, NMS, RVR, JCP, ZK and HCO wrote the manuscript draft. NMS and JCP prepared the figure and tables. ABS, NMS, RVR, JCP, JBB, FJC, JMP, JTH, MP, KJG, DW, GJL, JD, CL, AM, ZK and HCO discussed and revised the manuscript. ABS and HCO contributed equally.

Data availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Key words: nanomaterial, nanoparticle, nanotechnology, nitric oxide (NO), NO donor, S-nitrosothiol

Figure legend

Figure 1. Schematic representation of (a) major classes of nitric oxide (NO) donors applied in plants, (b) major types of nanomaterials to carry and delivery NO, (c) properties of NO-releasing nanomaterials, and (d) major challenges for the use of NO-releasing nanomaterials in plants.

Table 1. Nitric oxide (NO)-releasing nanoparticles and their effects on plants under diverse physiological and environmental conditions.

NPs	Species	Conditions	Application/ dosage	Responses*	Reference
SN-MSA CNPs	Maize (<i>Zea mays</i> L.)	Salinity	Soil application (50 and 100 μ M)	(+) leaf <i>S</i> -nitrosothiol content (-) photochemical damage (+) chlorophyll content (+) growth	Oliveira et al., 2016
GSNO CNPs	Sweet cherries fruit (<i>Prunus avium</i> L. cv. Hongdeng)	Fruit storage (postharvest)	Immersed fruits (60 mM)	(-) fruit weight loss (-) respiration rate (-) ethylene production (+) soluble solids content (-) oxidative stress (+) antioxidant response	Ma et al., 2019
SN-MSA CNPs	Neotropical tree seedling (<i>Heliocarpus popayanensis</i> Kunth)	Seedlings submitted to acclimation under full sun	Soil application (2 mM)	(+) growth	Lopes-Oliveira et al., 2019
	Neotropical tree seedling [<i>Cariniana estrellensis</i> (Raddi) Kuntze]			(+) leaf <i>S</i> -nitrosothiol content	
GSNO	Sugarcane	Drought	Foliar supply	(+) leaf CO ₂ assimilation	Silveira et al., 2019

CNPs	(<i>Saccharum</i> spp. cv. IACSP94-2094)		(100 μ M)	(+) root:shoot ratio	
GSNO CNPs SNAC CNPs SN-MSA CNPs	Sugarcane (<i>Saccharum</i> spp. cv. IACSP95-5000)	Drought	Foliar supply (100 μ M)	(+) leaf gas exchange during the recovery period (+) <i>S</i> -nitrosothiol content (+) chlorophyll content (-) growth inhibition (-) oxidative stress	Silveira et al., 2021a
SNP CNPs				(+) oxidative stress not effective in mitigating water deficit	
SN-MSA CNPs	Neotropical tree seedling (<i>Heliocarpus popayanensis</i> Kunth)	Drought	Soil application (200 μ M)	(+) <i>S</i> -nitrosothiol content (+) root hair formation (+) leaf gas exchange (+) leaf relative water content (-) oxidative stress (+) antioxidant response	do Carmo et al., 2021

Abbreviations: CNPs, chitosan nanoparticles; GSNO, *S*-nitrosoglutathione; SNAC, *S*-nitroso-*N*-acetylcysteine; SN-MSA, *S*-nitroso-mercaptoposuccinic acid; SNP, sodium nitroprusside.

*(+) and (–), respectively, mean increases and decreases in a given trait or process.

Table 2. Description of the different nanoencapsulated nitric oxide (NO) donors*.

Nanoparticles	NO release light/dark (% in 8 h)	Estimated cost for 50 mM solution** (US\$ per liter)	Encapsulation efficiency (%)	By-products
SN-MSA-CNPs	94.0±2.3/94.1±0.9	5.9	99.8	Oxidized mercaptosuccinic acid
GSNO-CNPs	64.6±0.7/17.7±0.8	63.7	99.7	Oxidized glutathione
SNAC-CNPs	66.1±3.1/26.3±3.7	8.8	99.5	Oxidized <i>N</i> -acetylcysteine
SNP-CNPs	***	8.0	7.8	Cyanide, ferrocyanide and ferricyanide

Abbreviations: CNPs, chitosan nanoparticles; GSNO, *S*-nitrosoglutathione; SNAC, *S*-nitroso-*N*-acetylcysteine; SN-MSA; *S*-nitroso-mercaptosuccinic acid; SNP, sodium nitroprusside.

* Based on Silveira et al. (2021a).

**Prices may vary among chemical companies and countries.

***It was not possible to accurately verify the NO release profile from SNP-CNPs.

