Potential use and perspectives of nitric oxide donors in agriculture

Running title: Nitric oxide donors in agriculture

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Abstract

Nitric oxide (NO) has emerged in the last 30 years as a key molecule involved in many physiological processes in plants, animals and bacteria. Current research has shown that NO can be delivered via donor molecules. In such cases, NO release rate is dependent upon the chemical structure of the donor itself and the chemical environment. Despite NO's powerful signaling effect in plants and animals, the application of NO donors in agriculture is currently not achieved and research is mainly at the experimental level.

Technological development in the field of NO donors is rapidly expanding in scope, to include controlling seed germination, plant development, ripening, and increasing shelf life of produce. Potential applications in animal production have also been identified.

This concise review focuses on the use of donors that have shown potential biotechnological applications in agriculture. We provide insights into (i) the role of donors in plant production, (ii) potential use of donors in animal production, and (iii) future approaches to explore the use and applications of donors for the benefit of agriculture.

Keywords: Nitric oxide donors, plant production, animal production, sodium nitroprusside

Introduction

Nitric oxide (NO) has emerged in the last 30 years as a key molecule involved in many physiological processes. In animals, NO controls vascular tone, leukocyte adhesion and aggregation, inhibition of platelet, apoptosis, immune response, inflammation, tissue repair, neurotransmission and angiogenesis [1-5]. NO has also been described as anticancer agent and as a key molecule involved in wound repair [6].

In plant, endogenous production of NO is known since the 1970s, and extensive knowledge on the multiple effects of NO on different physiological and biochemical processes is available [7]. Emerging evidences suggest that NO function in plants has a more pervasive role during development than in the other kingdoms [8]. In plants, several different functions of NO have been extensively reviewed by Yu and collaborators (2014)[8]. NO is required for plant immunity [9,10], hypersensitive cellular death [11], and to cope with abiotic stresses [12,13]. Other functions include root hair gravitropic responses [14-16], iron homeostasis [17], and regulation and balance between auxin and reactive oxygen intermediates (ROIs) [8].

In prokaryotes NO acts as an antimicrobial or dispersal agent and it is involved in virulence of bacterial and fungal pathogens [18,19]. NO has been described as a component of the offensive strategy and developmental signal of hemi/biotrophic fungal and oomycete plant pathogens [20]. With reference to bacteria, pathogenic bacteria have evolved transcriptional regulatory systems that perceive NO gas and respond by reprogramming gene expression. NO acts as environmental cues that trigger the coordinated expression of virulence genes and metabolic adaptations necessary for survival within the host [21]. Genes involved in nitric oxide perception have been identified in both Gram-positive and Gram-negative bacteria showing a universal effect of NO-mediated genetic regulation [22,23].

Due to the universal effect of NO on living organisms, it is not surprising that such molecule has been used as a tool for biotechnological applications. Delivery of gaseous NO via fumigation has been demonstrated to alleviate some of the effects of abiotic stress on a wide range of fruits and vegetables [24]. However, it has to be noted that the application of NO as a gas on the industrial scale has several safety concerns: NO is a gaseous radical species, and its direct delivery, while possible, has significant limitations. Instead, it is safer and necessary to deliver NO using a reactive precursor [25].

In biotechnological applications, the delivery on NO is mainly mediated via donor molecules [2,26]. NO release rate is mediated by the chemical structure of the donor itself and the chemical environment including pH, light temperature and enzymatic reactions [2,27,28]. NO donors differ in the kinetics and intensity of the generated NO, in both *in vitro* and *in vivo* conditions [29]. In plant, the process of donor decomposition depends on numerous factors. For example, in S-nitrosothiols NO release rate is affected by plant metabolites, such as in the presence of reducing agents, i.e. ascorbic acid and reduced glutathione (GSH) [29,30]. Endogenous nitric oxide may be additionally stimulated or inhibited by live plant tissue, thus it is necessary to take into consideration these aspects when monitoring the amount of NO released by the donor [29].

As previously mentioned, light affects NO releasing rate, for example sodium nitroprusside (SNP) has been shown to be very photosensitive [29]. *In vivo* experiments supported the hypothesis that releasing of NO from SNP varies according with light penetration, with highest NO release in epidermal cells exposed to the light [29].

Of great importance is also the potential neutralization/toxicity of the donors once depleted from the nitric oxide. Some of them may release toxic, active compounds during their decomposition. Plant and animal toxicity of by-products needs to be more fully confirmed, especially as subsequent reactions between decomposition products [31].

Different NO releasing-platforms have been extensively reviewed [3,25,32-36]. Examples are: nanoparticles [37,38], silica gel [39,40], hydrogels, xerogels, dendrimers [41-44] and small molecular weight donor molecules [37,38]. Several reviews summarize the role small donors or nanocarriers for nitric oxide delivery affecting plant physiological processes [45,46].

Due to the wide literature on the fundamental features of NO signaling in plants and animal, this mini review only focuses on the use of donors that have shown potential biotechnological applications in agriculture. Use of donors in field treatment has not yet been applied, but a number of potential applications have been identified. This review provides insights on (i) the potential role of donors in plant production (Table 1), (ii) potential use of donors in animal production (Table 2), and (iii) future approaches to explore the use and applications of donors for the benefit of agriculture.

NO donors for controlling seed vigor and dormancy.

Breaking dormancy involves tightly controlled signaling pathways that are important to maximize growth and crop yield. Selection against dormancy has been always behind any domestication effort [47]. In some cases, the aim of removing dormancy has not been achieved, and in others, it has gone too far resulting in susceptibility of pre-harvest sprouting [47]. Mechanical and chemical strategies have been employed to reduce seed dormancy, such as abrasion of seed or exposure to H_2SO_4 or NaOCl. However, less aggressive molecules may find application in this context [48]. As reviewed, SNP can find application to improve germination of seeds, also considering that NO is a signaling molecule active at very low concentrations ($nmolL^{-1}$ or $pmolL^{-1}$) and a minimal quantity would be required for an effective treatment.

When seed dormancy was studied in *Amaranthus retroflexus* (seeds can only germinate over a limited, high temperature range) exposure to SNP showed that relative dormancy of seeds was significantly released. Interestingly, dormancy was reverted by using NO specific scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), confirming that NO signaling pathway plays a role in the dormancy release and germination of *A. retroflexus* seeds [46,49].

Interesting data about germination are also available for *Malus domestica* (apple), which has an important commercial value on the market. In order to germinate, apple seeds must undergo a 3-

month long cold stratification. A pre-treatment with SNP resulted in an increase of 60% in germination of dormant apple embryos (when compared with the untreated controls), and this effect has been associated with marked increases in H_2O_2 and $O_2^{\bullet}$ concentrations in the embryos at early germination stages. Not-dormant embryos germinated well and young seedlings grown from non-dormant embryos did not exhibit any morphological anomalies, such as asymmetric growth [50]. However, further research should be conducted to clarify occurrence of anomalies in yield and quality.

Nitric oxide was also identified to foster induction of new rootlets in *Panax ginseng* [51]. NO released by SNP and S-Nitroso-N-acetyl-DL-penicillamine (SNAP) was shown to activate NADPH oxidase activity, resulting in higher number of new rootlets in the adventitious root explants. NO supplied through the donor would enhance antioxidant enzymatic activity reducing H_2O_2 levels, lipid peroxidation, modulation of ascorbate and non-protein thiol concentrations in the adventitious roots [51]. Interestingly, as complementary approach, the NO scavenger (PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide) was used to reveal the contribution of NO on the formation of new rootlets. The authors showed a significant decline in number of new rootlets under PTIO treatment [51]. Concluding, low seed vigor and dormancy were controlled by treating seeds with NO donors, in particular SNP [48]. The use of nitric oxide donors may find potential application in reducing long dormancy and improve germination rate.

NO donors for controlling salt stress

Seed germination is affected by salt stress. Twenty percent of the world's cultivated land and nearly half of all irrigated lands are currently affected by salinity [52]. High salinity conditions can cause plant death or decreased productivity at the whole-plant level [53]. The complex regulatory processes of salt stress involve control of water flux and cellular osmotic adjustment, balance of cellular ion homeostasis which ultimately has impact on the cellular energy supply and redox homeostasis [53-55].

The use of donors have found a few encouraging applications to cope with salt stress. In peppers, the application of SNP has been shown to alleviate the oxidative damage caused by salt stress, which was mainly achieved by means of enhancing anti-oxidative capability in pepper seedlings [56]. Studies in barley (*Hordeum vulgare*) also confirm the advantageous application of SNP during 50 mM NaCl salt stress response [53]. Barley leaves exposed to 50 μM SNP alleviated the damage of salt stress reflected by decreased ion leakage, malendialdehyde, carbonyl, and hydrogen peroxide content. In addition exposure to SNP increased the activities of superoxide dismutases, ascorbate peroxidases, and catalases [52,53,57]. SNP has also been used to pre-treat seed to enhance seed germination of wheat in high salinity (*Triticum aestivum* L., cv. Huaimai 17). Seeds were exposed to 0.1 mM SNP plus 300 mM NaCl for 20 h before germination, which increased germination rate, weights of coleoptile and radicle when compared with NaCl alone. As factors contributing to such plant development, authors identified that SNP enhanced seed respiration rate, ATP synthesis, soluble sugar content and decreasing starch content. In addition the treatment increased the activities of superoxide dismutase and catalase and decreased the release rate of malondialdehyde, hydrogen peroxide (H_2O_2), and superoxide anions ($O_2^{\bullet-}$) in the mitochondria [58].

NO donors for controlling heavy metal stress

Most of the heavy metals exert their toxicity with two principal mechanisms: as redox active or not-redox active mechanisms [59]. Autoxidation of redox active metals such as Fe^{2+} or Cu^{+} may results in $O_2^{\bullet-}$ formation and subsequently in H_2O_2 and $OH^{\bullet}$. The toxicity mechanisms of not-redox active metals are due to their ability to bind to oxygen, nitrogen and sulphur atoms [59].

Copper is an essential micronutrient for plants and it is present in soil. However, copper poses toxicity at high concentrations possibly by inducing oxidative stress [60]. With the increase of copper stress, the germination percentage of seeds decreases gradually [60]. Pre-treatment of wheat seeds with SNP significantly improved wheat seeds germination and alleviated oxidative stress caused by copper toxicity. Treated seeds retained higher amylase activities when compared with the un-treated controls. Authors identified that seed-pretreatment with SNP stimulated the activities of superoxide dismutase and catalase, decreased the activities of lipoxygenases, sustained a lower level of malondialdehyde, and interfered with hydrogen peroxide excessive accumulation compared with the control, thereby enhancing the antioxidative capacity of wheat seeds under copper stress [61].

Oxidative stress induced by iron was also modulated by exposing sorghum seedlets (*Sorghum bicolor* (L.) Moench) to SNP or diethylenetriamine NONOate (DETA NONOate) [62]. Authors showed that incubation of seeds with 1 $mmolL^{-1}$ SNP protected against oxidative damage to lipids and maintained membrane integrity. The content of the siderophore deferoxamine–Fe (III) complex significantly increased in homogenates of sorghum embryonic axes excised from seeds incubated in the presence of 1 mM SNP or1 mM DETA NONOate as compared to the control (SNP 19±2 nmol Fe g^{-1} fresh weight (fw), DETA NONOate 15.2±0.5 nmol Fe g^{-1} fw, and Control 8±1 nmol Fe g^{-1}, fw). The data presented by Jasid and collaborators (2008) showed that in exposed sorghum embryonic axes, membranes and proteins were preserved from oxidative damage during the initial steps of development. The treatment seemed to exert a double effect in sorghum by increasing iron availability and preventing its toxicity [62].

Use of SNP was effective in the protection of wheat roots from Cadmium-induced oxidative damage [63]. Cadmium is also present in the environment and it can induce oxidative stress in plants. Pal Singh (2008) and co-workers identified that SNP has protective role against cadmium toxicity [63]. 50 or 250 μM cadmium alone or in combination with 200 μM SNP were delivered hydroponically on grown wheat roots for 24 h. Supplementation of SNP in presence of cadmium significantly reduced the Cd-induced lipid peroxidation, H_2O_2 content and electrolyte leakage in wheat roots [63]. SNP supply with cadmium also decreased activities of scavenging enzymes, such as superoxide dismutase, guaiacol peroxidase, catalase, and glutathione reductase [63].

Further examples of reduced toxicity of lead and cadmium has also been described by Kopyra and Gwóźdź (2003) in lupin (*Lupinus luteus* L. cv. Ventus) seed germination [64]. Pretreatment of lupin seedlings for 24 h with 10 µM SNP resulted in efficient reduction of the detrimental effect of lead, cadmium and sodium chloride. In agreement with literature, the inhibitory effect of heavy metals on root growth was accompanied by increased activity of superoxide dismutase, peroxidase and catalase [64]. Similarly in rice, application of 30 µM SNP counteracted partly 100 µM cadmium toxicity by reducing H_2O_2 and malondialdehyde contents of Cd-exposed seedlings. SNP markedly stimulated the activities of superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase and catalases. With reference to accumulation, Cd accumulation in seedlings was also significantly reduced by SNP [65].

On the basis of current literature, it can be reasonably assumed the protective effect of NO in stressed seeds and roots may be at least partly due to the stimulation of antiradicals mechanisms and/or direct scavenging of the superoxide anion [64]. NO donors could be used to improve soil management practices or seed preparation for sustainable use in salt or heavy metal affected soils in future applications [53].

Wound healing

Nitric oxide donors could also find biotechnological applications in wound healing. Wounding is a special type of stress that plants encounter during pathogens attack. Plants have evolved constitutive and induced defense mechanisms to properly respond to wounding and prevent infections [66]. After the wound, oligogalacturonides play a pivotal role in eliciting defense responses, including production of ROS, pathogenesis-related proteins, nitric oxide, phytoalexins, glucanase, chitinase, and callose that protect plants against pathogen infections [66,67]. Endogenous NO plays a pivotal role in plant responses to wounding. Studies in pelargonium leaves (*Pelargonium peltatum L.*) showed the central role that NO plays in NO-mediated lignification and callose deposition during wound healing [68]. NO caused marked increase in H_2O_2 level accompanied by time-dependent inhibition of catalase and ascorbate peroxidase activity. NO/H_2O_2 ratio restricted the depletion of the low-molecular weight antioxidant pool (i.e. ascorbic acid and thiols) and was positively correlated with sealing and reconstruction in injured pelargonium leaves leading by lignin formation and callose deposition [68].

París and coworkers showed that SNP can be applied to speed the wound healing response of potato leaves [69]. Deposition of the cell-wall glucan callose was induced by the application of SNP, and such induction was additive to the wound-induced callose production. Exposure to SNP showed an accumulation of wound-related phenylalanine ammonia-lyase enzyme [69]. In another study, SNP has also been used to control cellulose synthesis in tomato (*Solanum lycopersicum*) roots [70]. Nitric oxide affected cellulose content in roots in a dose dependent manner: pmolL^{-1} of SNP increased cellulose content in roots while higher concentrations of nmolL^{-1} of SNP had the opposite effect: In addition, the expression of tomato cellulose synthase (SICESA) transcripts SICESA1 and SICESA3 levels were repressed by increasing SNP concentrations [70].

The above mentioned experimental evidences show the possible positive effect that NO donor may promote in restoration of wounded tissue through stabilization of the cell redox state and stimulation of the wound scarring processes [68,71]. In terms of agricultural applications, SNP might potentiate the healing responses in plants leading to a rapid restoration of the damaged tissue via wound-induced callose and cell wall cellulose production [69,70].

Ripening

Of great interests are a few studies aimed in understanding the contribution of NO donors to the ripening process. Gaseous NO in *Prunus persica* (peach) affects the differential accumulation of proteins involved in ripening and senescence, consequently the action of SNP has been investigated to control ripening processes in plants [72]. In a study by Hu and coworkers (2014) [73], mangos 'Guifei' treated with SNP exhibited a delay in ripening evidenced by the reduction of metabolic cascades typically involved in the ripening process such as softening, flesh yellowing, changes in soluble solid contents, titratable acidity, peaks of the respiration rate and ethylene production [73]. SNP treatment also increased total phenolics, flavonoids and lignin. [73].

Among ripening processes, increase in soluble sugars and synthesis of secondary metabolites are important factors that support fruit's taste. Further applications of donors can be also found in herbal medicine. In *Ginkgo biloba*, for example, SNP treatments have increased soluble sugar, proline and secondary metabolite [74-76].

Post harvested shelf life

Consumers judge the quality of fresh fruit based on the appearance and firmness at the time of the purchase [77]. Maturity stages ultimately dictate the shelf life and fruit qualities [77]. A comprehensive review on the applications of NO gas and donors to cope with postharvest stress of fruits, vegetables and ornamentals is available by Wills and coworkers [77]. In this paragraph, only applications of NO donors in extending produces' shelf life have been reviewed.

Post-harvest strawberries and mushrooms were exposed to diethylenetriamine/nitric oxide (DETANO), a solid NO-donor compound, in order to extent fruit shelf-life. The treatment was found to quantitatively liberate NO in the presence of a range of acidic substances including citric acid[24]. According to the authors, a solid mixture of DETANO, citric acid and wheat starch (added as a filler and moisture absorbent) at the ratio of 1:10:20 was found to be stable for at least six months when stored in dry air. When the dry mixture was placed in a container with strawberries or mushrooms, the moisture released by the produce activated the mixture, resulting in a similar extension of postharvest life as achieved by direct fumigation with the nitric oxide gas. The author proposed a commercial use of such compounds via tablets or sachets.

Use of DETANO was also reported to inhibit browning in apple slices [78,79]. Fresh-cut apples (*Malus domestica* Borkh. 'Granny Smith') were dipped in a DETANO solution and the development of surface browning was examined during subsequent storage at 0°C and 5°C. Authors found that dipping in the DETANO solution inhibited the development of browning, considering the solution was slightly acidic buffered. Optimal treatment to delay browning was the dipping of slices in 10 mg/L DETANO dissolved in a phosphate buffer at pH 6.5. The extension in post-harvest life achieved by DETANO was about 170% (compared to untreated samples) and the extension in post-harvest life compared to water-dipped slices was about 100%. Interestingly, 'Granny Smith' apple slices exposed to DETANO solution before storage at 5 °C showed lower level of total phenolics, inhibition of polyphenolic oxidase activity, reduced ion leakage and reduced rate of respiration but did not show significant effect on ethylene production or lipid peroxide level as measured by malondialdehyde and hydrogen peroxide levels [79]. A comprehensive review of the applications of NO gas and donors to cope with postharvest stress of fruits, vegetables and ornamentals has recently highlighted by Wills and coworkers [24].

Co-application of nitric oxide donors with fertilizers.

To our knowledge only one work is available on co-application on NO donors and fertilizers, showing perhaps potential applications. Co-application of SNP into a controlled release fertilizer or sprayed on leaves to supply NO was recently used to cope with iron deficiency stress in peanut (*Arachis hypogaea* Linn) grown on calcareous soils. Under such conditions, iron deficiency reduces plant growth and chlorophyll content. Iron homeostasis represents an important topic in the plant mineral nutrition, since iron is an essential cofactor for fundamental biochemical activities [80,81]. Iron can be deficient under alkaline and oxidative conditions [81]. An interconversion between different redox forms based on the iron and NO status of the plant cells might be the core of a metabolic process driving plant iron homeostasis [82]. 5.63 mg SNP and 18.90 mg $FeSO_4$ per g of fertilizer were applied in conjunction with 150 g Kg^{-1} nitrogen, 150 g Kg^{-1} P_2O_5, and 150 g Kg^{-1} K_2O. The treatment improved peanut growth and alleviated leaf interveinal chlorosis when SNP was co-applied in presence of iron. The photochemical efficiency and photochemical maximum efficiency of photosystem II (PSII) increased when compared with the not treated. Minimum fluorescence yield decreased under NO-treated condition, which supported the protective effect of NO on PSII in peanut leaves. SNP treatment increased the activities of antioxidant enzymes, and reduced malondialdehyde accumulation [83].

Perspectives on the use of nanoparticles releasing nitric oxide in produce and crop industry

The application of NO releasing nanoparticles in produce and crop industry is still at a preliminary stage. To our knowledge, liposomes or chitosan nanoparticles capable of mediating NO release have not been used in agriculture [45]. Polymeric nanoparticles have been proposed as cytotoxic agents to treat plant parasites [45].

Formulation of dendrimers has also attracted attention for increasing the efficacy of active chemicals in agriculture [45]. Dendrimers are synthetic polymers with branching structure that rely on supramolecular properties which are new dimensions for targeting biofilms featuring drug encapsulation, binding and delivery to the target site [84,85]. Dendrimers act as a platform for NO transport and delivery but their application in agriculture is still not explored [3,32].

Finally, the donor S-nitrosoglutathione (GSNO) encapsulated in alginate/chitosan nanoparticles might be potentially used as controlled release systems applied via foliar route [86].

Perspective on the use of NO donors in livestock industry and dairy production

Only few NO donors currently show potential applications in livestock industry and dairy production. Indeed, current literature refers mainly to the use of donors for the study of the NO-mediated response on cellular physiology. Extensive bibliographical research in this field has shown that only a few papers support potential applications.

Donors could find applications in the treatment and prevention of bovine mastitis [87,88]. Alginate/chitosan or chitosan/sodium tripolyphosphate were used to encapsulate the NO-releasing molecule mercaptosuccinic acid (MSA) generating S-nitroso-MSA-alginate/chitosan particles [88]. *Staphylococcus aureus* and *Escherichia coli* isolated from subclinical and clinical bovine mastitis were killed by using up to 125 µg/mL of S-nitroso-MSA-alginate/chitosan particles. Indeed, the results indicated that NO-releasing polymeric particles may be an interesting approach to combating bacterial antibiotic resistances [88].

NO donors could also find application in cow reproduction. Preliminary experiments with SNP showed that up to 100μmolL^{-1} of SNP differentially modulated oviductal contraction in Holstein cows depended on the type of muscular strips. Results showed the estrous phase-dependent changes related to the NO metabolic cascades could be of physiological importance to the oviduct for secretory and ciliary functions involved in gametes and embryo(s) transportation during reproduction [89].

A similar experiment aimed to understand the role of NO in reproduction showed the contribution of the donor NOC-18 which induced the release of spermatozoa from the oviductal epithelia. As complementary approach sperm oviduct interaction was reversed by the addition of 30 μg/ml hemoglobin, a NO scavenger [90].

A few studies are available on the role of NO donors to the control of livestock weight gain [91]. In these experiments, 50 mg/day of diethylenetriamine-NO (DETA) supplemented to lactating sows increased their production performance and growth of the nursing piglets. Body weights and backfat thickness of sows, as well as body weights of piglets were measured at 0, 7, 14, and 21 days of lactation. Significant weight gain in the treatment (40.5 kg) greater than the not treated (36.5 kg) was achieved up to 21 days of lactation. Dietary DETA supplementation to lactating sows showed an improved growth of nursing piglets possibly by enhancing nutrient outputs in milk due to increased blood flow across the mammary gland [91]. On the contrary, SNP treatment has not shown the same effect of nutrient uptake in chickens. SNP intraperitoneally administered to chicks did not show any significant change in the nutrient uptake. Authors concluded that in chicken, NO concentrations above physiological levels was not an important factor in the regulation of food intake [92].

Conclusion

The use of NO donors in agriculture is still in its infancy and applications are only at the experimental level. However the technology of NO donors is promising, in particular when used as an additive agent. The advantage of using NO donors is the extremely low effective concentrations (picomolar or nanomolar). In addition, donors have recently been proposed as dispersant agents to reduce biofilm biomass of pathogen such as *Salmonella*, pathogenic *Escherichia coli* and *Listeria* from materials of industrial interests [26,93-95].

Therefore, NO donors could be used to obtain multiple effects during the same application, from controlling bacterial pathogens to production. Controlling animal health and safety in dairy production, for example, could be another interesting future application to exploit the potential of NO donors.

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