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Nitric oxide-releasing chitosan nanoparticles alleviate the effects of salt stress in maize plants

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ABSTRACT

Nitric oxide (NO) is a signaling molecule involved in plant response to various abiotic stresses. However, the application of NO donors in agriculture is hampered by the instability of these compounds. Despite the successful uses of NO-releasing nanoparticles for biomedical purposes and the variety of nanomaterials developed as carrier systems of agrochemicals, the potential applications of nanocarriers for NO delivery in plants have not yet been tested. Herein, we report the synthesis and characterization of chitosan nanoparticles (CS NPs) containing the NO donor S-nitroso-mercaptosuccinic acid (S-nitroso-MSA). The efficiency of these NO-releasing NPs in mitigating the deleterious effects of salinity on maize plants was compared to that of the non-encapsulated NO donor. The NPs were synthesized through ionotropic gelation process, and mercaptosuccinic acid (MSA), the NO donor precursor, was encapsulated into CS NPs (91.07% encapsulation efficiency). Free thiol groups of MSA-CS NPs were nitrosated, leading to S-nitroso-MSA-CS NPs (NO-releasing NPs). The incorporation of S-nitroso-MSA into CS NPs allowed a sustained NO release. Treatments of salt-stressed maize plants with S-nitroso-MSA-CS NPs resulted in a higher leaf S-nitrosothiols content compared to that of free S-nitroso-MSA. Moreover, S-nitroso-MSA-CS NPs were more efficient than was the free NO donor in the amelioration of the deleterious effects of salinity in photosystem II activity, chlorophyll content and growth of maize plants because the protective action of the nanoencapsulated S-nitroso-MSA was achieved at lower dosages. Overall, these results demonstrate the positive impact of S-nitroso-MSA nanoencapsulation in increasing NO bioactivity in maize plants under salt stress.

1. Introduction

Soil salinity is one of the main abiotic stress factors that limit crop productivity, affecting nearly one-fifth of the worldwide cultivated lands [1]. In addition to primary salinization caused by natural processes, the areas subjected to secondary salinization have increased in recent decades due to anthropogenic actions, such as improper irrigation methods and deforestation [2]. The negative effects of soil salinity in plants are related to the specific toxicity of ions, such as Na^+ and Cl^-, as well as to the low osmotic potential of soil solution, which results in water deficiency in plant cells [3]. Plants submitted to salt stress have been shown to present an increased production of reactive oxygen species, reduced efficiency of photosystem II, decreased stomatal conductance and accelerated leaf senescence with a decrease in the content of photosynthetic pigments. Thus, salinity impairs CO_2 assimilation, leading to reduced growth rates and productivity [3].

Nitric oxide (NO) is a gaseous signaling molecule involved in several processes of plant growth and development, as well as in plant response to abiotic stresses [4–6]. Exogenous NO application has been shown to mitigate the adverse effects of salt stress in diverse plant species [7–13]. The involvement of NO in response to salinity has been further substantiated by the higher sensitivity of NO-deficient mutants to salt stress [14,15]. In salt-stressed plants,

Abbreviations: CS, chitosan; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; MSA, mercaptosuccinic acid; NO, nitric oxide; NP, nanoparticle; rETR, photosystem II relative electron transport rate; RGR, relative growth rate; RSNO, S-nitrosothiol; S-nitroso-MSA, S-nitroso mercaptosuccinic acid; SNP, sodium nitroprusside.

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NO modulates gene expression and protein function, inducing the antioxidant defense system, chlorophyll biosynthesis, osmolyte accumulation and H^+-ATPase activity [7,8,12,16,17]. NO has also been shown to reduce the negative effects of salinity on photosynthesis and the growth of plants subjected to salt stress [7,12,16], thereby emerging as a molecule with potential uses in agriculture [18].

However, the direct treatment of plants with NO is technically difficult, given the gaseous nature of NO [19]. Additionally, NO has a short *in vivo* half-life (1–5 s), rapidly reacting with oxygen and cellular components, which makes necessary a constant delivery of NO to the tissues [20–24]. Low-molecular-weight NO donors, such as S-nitrosothiols (RSNOs) and sodium nitroprusside (SNP), have been widely used for the investigation of biological functions of NO both in plants and in animal models [12,15,18,21–28]. Despite this, NO donors are generally unstable, and their decomposition rates are increased by high temperatures and light exposition, leading to a rapid NO release, which may potentially result in toxic effects and reduce the efficacy of the signaling molecule [20,22].

In this scenario, the entrapment of NO donors in nanomaterials has emerged as a strategy that could protect these molecules from decomposition/degradation and allow a controlled NO release, thereby extending its period of action [20,22–24,29]. Nanomaterials present distinct physico-chemical characteristics compared to the same material at the macroscopic scale, with an increase in the superficial area [30,31]. A variety of nanomaterials have been used as carrier systems of bioactive compounds for agricultural applications, such as fertilizers and pesticides [31–33]. However, the potential applications of nanocarriers for NO delivery in plants have not yet been tested [18], despite their successful use for biomedical purposes [20–24].

In this context, our group has successfully encapsulated low-molecular-weight NO donors in chitosan nanoparticles (CS NPs) for different biomedical applications, including antimicrobial activities against resistant bacteria [29] and protozoa [23] and toxicity towards several tumorigenic cell lines (unpublished work). Herein, we report the synthesis and characterization of CS NPs containing S-nitroso-mercaptosuccinic acid (S-nitroso-MSA), a low-molecular-weight NO donor that belongs to the class of RSNOs. S-nitroso-MSA-CS NPs were used as spontaneous NO donors in maize plants submitted to salt stress. For this, the efficiency of these NO-releasing NPs in mitigating the deleterious effects of salinity on the growth and physiological parameters of maize plants was compared to that of the non-encapsulated NO donor. To the best of our knowledge, this is the first report to evaluate the protective effects of NO-releasing NPs in plants under high salinity.

2. Materials and methods

2.1. Materials

Sodium tripolyphosphate (TPP), mercaptosuccinic acid (MSA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), sodium nitrite ($NaNO_2$), and phosphate buffer saline (PBS, pH 7.4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, acetone, sodium chloride (NaCl) and nutritive solution reagents were obtained from Labsynth (Diadema, SP, Brazil). Chitosan (87.4% deacetylation) was purchased from Polymar (Fortaleza, CE, Brazil). The aqueous solutions were prepared using analytical-grade water from a Millipore Milli-Q Gradient filtration system.

2.2. Synthesis of CS nanoparticles containing MSA

CS nanoparticles (CS NPs) were prepared by ionotropic gelation [34,35]. Briefly, CS (2.6 mg mL^{-1}) and MSA (2.7 mM) were mixed through magnetic stirring in an aqueous solution of acetic acid (0.175 M) for 90 min. TPP aqueous solution (0.6 mg mL^{-1}) was added dropwise in the CS/MSA suspension, obeying the volumetric proportion of 3 CS/MSA:1 TPP. The final mixture was stirred for a further 45 min at room temperature, yielding a suspension of CS NPs containing MSA (final concentration of MSA 1000 μM), henceforth referred to as MSA-CS NPs.

2.3. Determination hydrodynamic size, size distribution and zeta potential

The average hydrodynamic diameter (% by number), size distribution, polydispersity index (PDI), and zeta potential of MSA-CS NPs were evaluated by dynamic light scattering (DLS) using a Nano ZS Zetasizer (Malvern Instruments Co, UK) [36]. Measurements were performed in three independent experiments at 25 °C using a fixed angle of 173° in disposable folded capillary zeta cells with a 10 mm path length in aqueous suspension.

2.4. Encapsulation efficiency of MSA in CS NPs

The encapsulation efficiency of MSA in CS NPs was determined by the titration of the free thiol group of MSA with DTNB, as previously described [21,29,37,38]. To separate free MSA from encapsulated MSA in CS NPs, 500 μL of aqueous suspension of MSA-CS NPs was filtered through a Microcon centrifugal filter device (MWCO 10,000, Millipore). Then, 260 μL of free MSA solution, eluted from the centrifugal filter device, was added to 1.2 mL of 0.7 mM DTNB in PBS aqueous solution (pH 7.4) containing 10.3 mM EDTA. DTNB rapidly reacts with thiol groups, forming 2-nitro-5-thiobenzoate anion (TNB^{-2}), which presents the characteristic electronic absorption band at 412 nm ($\varepsilon = 14.15\ M^{-1}\ cm^{-1}$) [37,39]. The measurements were performed in triplicate using the UV–vis spectrophotometer Agilent 8453 (Palo Alto, CA, USA).

2.5. Nitrosation of MSA leading to S-nitroso-MSA

Free thiol groups of encapsulated MSA (1000 μM) in CS NPs were nitrosated by adding equimolar amounts of sodium nitrite ($NaNO_2$) (1000 μM) in acidified medium (pH = 3), leading to the formation of S-nitroso-MSA-CS NPs. The final solution was stirred for 60 min, protected from light, in an ice bath. Similarly, free S-nitroso-MSA (non-encapsulated S-nitroso-MSA) was prepared through the nitrosation of the free MSA by adding an equimolar amount of $NaNO_2$. The formation of S-nitroso-MSA either free or encapsulated in CS NPs was confirmed by the appearance of the characteristic S-NO group absorption bands at 336 nm ($\varepsilon = 980.0\ M^{-1}\ cm^{-1}$) or at 545 nm ($\varepsilon = 18.4\ M^{-1}\ cm^{-1}$) using the UV–vis spectrophotometer Agilent 8453 (Palo Alto, CA, USA) [37,40].

2.6. NO release profiles from free S-nitroso-MSA and from S-nitroso-MSA CS NPs

The kinetics of NO release from free S-nitroso-MSA and from S-nitroso-MSA-CS NPs were determined by monitoring the spectral changes at 336 nm ($\pi \rightarrow \pi^*$ transition), which are associated with S-N bond cleavage and free NO release [41,42]. Kinetic data were collected in 30 min intervals at room temperature for 12 h of monitoring, and MSA-CS NPs were used as negative control group. The initial concentration of S-nitroso-MSA (free or in CS NPs suspension) was 1000 μM. The quantity of NO released over time was calculated according to the quantity of S-nitroso-MSA that decomposed [42,43]. This calculation was based on the fact that the decay of the absorption band of S-nitroso-MSA at 336 nm can be