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Evaluation of the effects of nitric oxide-releasing nanoparticles on plants

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Abstract. Nowadays, there are several commercially available products containing nanostructured materials. Meanwhile, despite the many benefits that can be obtained from nanotechnology, it is still necessary to understand the mechanisms in which nanomaterials interact with the environment, and to obtain information concerning their possible toxic effects. In agriculture, nanotechnology has been used in different applications, such as nanosensors to detect pathogens, nanoparticles as controlled release systems for pesticides, and biofilms to deliver nutrients to plants and to protect food products against degradation. Moreover, plants can be used as models to study the toxicity of nanoparticles. Indeed, phytotoxicity assays are required to identify possible negative effects of nanostructured systems, prior to their implementation in agriculture. Nitric oxide (NO) plays a key role in plant growth and defense, and recently, several papers described the beneficial effects due to application of exogenous NO donors in plants. The tripeptide glutathione (GSH) is an important anti-oxidant molecule and is the precursor of the NO donor, S-nitrosoglutathione (GSNO). In this context, the present work investigates the effects of different concentrations of alginate/chitosan nanoparticles, containing either GSH or GSNO, on the development of two test species (*Zea mays* and *Glycine sp.*). The results showed that the alginate/chitosan nanoparticles present a size average range from 300 to 550 nm with a polydispersity index of 0.35, and encapsulation efficiency of GSH between 45 - 56%. The NO release kinetics from the alginate/chitosan nanoparticles containing GSNO showed sustained and controlled NO release over several hours. Plant assays showed that at the concentrations tested (1, 5 and 10 mM of GSH or GSNO), polymeric nanoparticles showed no significant inhibitory effects on the development of the species *Zea mays* and *Glycine sp*., considering the variables shoot height, root length, and dry mass. Therefore, these nanoparticles seem to have promissing uses in agriculture, and might be potencially used as controlled release systems applied by the foliar route.

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1. Introduction

In recent years, there have been major advances in nanotechnology, which is now used in diverse areas such as biomedicine, pharmaceuticals, agriculture, amongst others [1,2]. Examples include the use of carbon nanotubes in the production of electronic devices [3], metallic nanoparticles (such as silver) as bactericidal agents [4], and a wide range of polymeric nanoparticles used as carrier systems for active agents [5,6]. Many products containing nanostructured materials are now commercially available, including paints, cosmetics, and electronic goods. Meanwhile, despite the many benefits that can be obtained from this technology, it is still necessary to understand the mechanisms in which nanomaterials interact with the environment, and to obtain information concerning their possible toxic effects.

In agriculture, nanotechnology has been used in various ways, with the aim of improving yields and the quality of products [7]. Applications include the use of nanosensors to detect pathogens, nanoparticles as controlled release systems for pesticides, and biofilms to deliver nutrients to plants and protect food products against degradation [1]. The use of nanoparticles as carrier systems for agrochemicals can improve the bioavailability and effectiveness of active agents, enabling lower dosages to be safety used and reducing damage to the environment [8].

Plants can be used as models to study the toxicity of nanoparticles and obtain information concerning possible interactions and effects of these nanosystems on processes such as germination, metabolism, and plant growth [9,10]. Phytotoxicity assays are required in order to identify any possible negative effects of nanostructured systems, prior to their implementation in agriculture [8].

The free radical nitric oxide (NO) plays important role in plant defense and growth [11, 12]. It has been reported that administration of exogenous NO donors can break seed dormancy, improve plant greening and germination, regulate iron homeostasis, and improve plant tolerance to salinity, metal toxicity, temperature and drought stress [12]. However, small molecular weight NO donors, such as S-nitrosothiols (RSNOs) are thermally and photochemically unstable [13]. Among the NO donors, S-nitrosoglutathione (GSNO) is an important RSNO, which spontaneously releases NO through the cleavage of S-N bound [14]. In order to increase the thermal and photochemical stability of NO donors, RSNOs have been incorporated into polymeric matrices, such as in alginate/chitosan nanoparticles [6]. The encapsulation of NO donors in nanoparticles is able to control the release of therapeutic amounts of NO, thus improving its beneficial effects [6].

In this context, the present work therefore investigates the effects of different concentrations of alginate/chitosan nanoparticles containing either reduced glutathione (GSH) (an important anti-oxidant molecule and the precursor of the NO donor, GSNO) or GSNO on the development of two test species (*Zea mays* and *Glycine* sp.), in order to obtain information on the toxicity of the nanoparticles, with a view to the possible use of this nanocarrier system in agricultural applications.

2. Methods

2.1. Synthesis of alginate/chitosan nanoparticles containing GSH and GSNO

Alginate/chitosan nanoparticles (at ratio 0.75) were prepared using the ionic gelation method, as previous described [6,15]. Briefly, 0.266 g of chitosan was added to 10 mL of water containing 0.092 mL of acetic acid. After 24 h of magnetic stirring, 10 mL of water was added to the chitosan solution, and the final solution was homogenized for more 24 h. Required amounts of GSH were added to the chitosan solution and homogenized for 30 min. A volume of 1.0 mL of chitosan/GSH solution of was dropwise in 200 mL of alginate solution (50 µg/mL) at pH 4,0. This process led to the preparation of alginate/chitosan nanoparticles containing GSH in the following concentrations: 1, 5 and 10 mmol/L). In order to obtain GSNO-containing alginate/chitosan nanoparticles, equimolar amounts of sodium nitrite ($NaNO_2$) related to GSH were added to the nanoparticles. The final solution was homogenized for 8 h, protected from the light, and used immediately.

2.2. The average size and size distribution for polymeric particles in aqueous medium

The average size for GSH-containing alginate/chitosan nanoparticles were measured using photon correlation spectroscopy (PCS) (Nano ZS Zetasizer, Malvern Instruments Co.) at 25°C in polystyrene cuvettes with a 10 mm path length.

2.3. GSH encapsulation efficiency in alginate/chitosan nanoparticles

The encapsulation efficiency of GSH in alginate/chitosan nanoparticles were measured by the UV–vis method, as already described [6]. Briefly, free GSH was separated from polymeric nanoparticles by ultracentrifugation, by using a Microcon centrifugal filter device containing ultrafiltration membranes (MWCO 10,000, Millipore). The amount of free GSH in the ultrafiltrates was measured by titration with a thiol-reacting 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB), based on the absorbance band at 412 nm (ε = 14.15 mmol $L^{-1}cm^{-1}$) of the 2-nitro-5-thiobenzoate anion, which is generated in the reaction of GSH with DTNB. The percentage of GSH encapsulation was determined by the described equation:

(%) = (mass of GSH encapsulated/mass of GSH total) x 100 (Eq. 1)

2.4. Kinetics of GSNO decomposition with free NO release

The kinetics for NO release from GSNO in alginate/chitosan nanoparticles were monitored by following the spectral changes at 336 nm, which is associated with S-N bond cleavage and free NO release [16] by using an Uv-Visible spectrophotometer (Agilent 8453). The kinetics were monitored at 35°C for 24 h. The amount of NO released was calculated from the amount of GSNO decomposed, as previous described [13]. The initial rates of NO release through GSNO decomposition were determined through linear regression of the curve slopes.

2.5. Phytotoxicity of GSH or GSNO-containing alginate/chitosan nanoparticles

Phytotoxicity assays were conducted using *Zea mays* and *Glycine* sp. collected in the field, in the municipality of São Miguel Arcanjo (São Paulo, Brazil). The seeds were sown in pots with upper and lower diameters of 12.5 and 9.3 cm, respectively, and heights of 9.3 cm. The pots were filled with 600 g of the Carolina Soil plant substrate. Ten seeds of the separate species were used in each pot, followed a fully randomized 7 x 3 experimental design. The pots were kept in a plant house, and at the pre-emergence stage (7 days after sowing) the nanoparticle suspensions containing GSH or GSNO (at concentrations of 1, 5, and 10 mmol/L) were sprayed. Deionized water was used as a control treatment. The plants were left for another 7 days after application of the treatments, and were then collected and analysed. Measurements were made of the heights of the aerial parts (cm) and the lengths of the roots (cm), after which the plants were placed in a drying cabinet at 60°C, for 7 days, and then weighed to determine their dry masses (g). The data obtained were presented as means and standard deviations, and statistical analysis of the differences between treatments was performed using analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test

3. Results and Discussion

3.1. Synthesis of GSH and GSNO-alginate/chitosan nanoparticles

The mixture of alginate and chitosan polymers in acidified aqueous solution led to the formation of nanoparticles due to the strong electrostatic interactions of the anionic alginate chain with the cationic chitosan chain [15]. In aqueous solution, the hydrodynamic diameter of the GSH-alginate/chitosan nanoparticles were found to be in the range of 300 to 550 nm with a polydispersity index of 0.35, which are in accordance with our previous results [6,15]. The encapsulation efficiency of GSH at concentrations of 1, 5 and 10 mmol/L in alginate/chitosan nanoparticle solutions were found to be 45

± 2%, 45 ± 3% e 56 ± 5%, respectively. The nitrosation of GSH in acidified alginate/chitosan nanoparticle solution was performed by the addition of equimolar amount of sodium nitrite ($NaNO_2$). In acidified aqueous solution, sodium nitrite will form nitrous acid (HNO_2), which is the nitrosating agent, leading to the formation of GSNO, according to Equation 2. In this work, GSH-containing nanoparticles were nitrosated *in situ*, leading to the formation of GSNO-nanoparticles.

$$GSH + HNO_2 \rightarrow GSNO + H_2O \quad \text{(Eq. 2)}$$

3.2. Kinetics of NO release from free GSNO and GSNO encapsulated in nanoparticles

The kinetics for NO release from GSNO (1, 5 and 10 mol/L) encapsulated in alginate/chitosan nanoparticles were monitored through the spectral changes at 336 nm. The decrease in this absorption band corresponds to GSNO decomposition with free NO release. This calculation is based on the GSNO absorption band decay at 336 nm, solely associated with homolytic cleavage of the S-N bond and NO release in accordance with Equation 3 [13]. The end products of GSNO decomposition, either free or encapsulated, are NO and oxidized glutathione (GSSG) [16]:

$$2\ GSNO \rightarrow 2\ NO + GSSG \quad \text{(Eq. 3)}$$

Figure 1 shows the NO release profile from GSNO encapsulated in alginate/chitosan nanoparticles, at different concentrations, as indicated in the Figure, at 35°C, over 24 h.

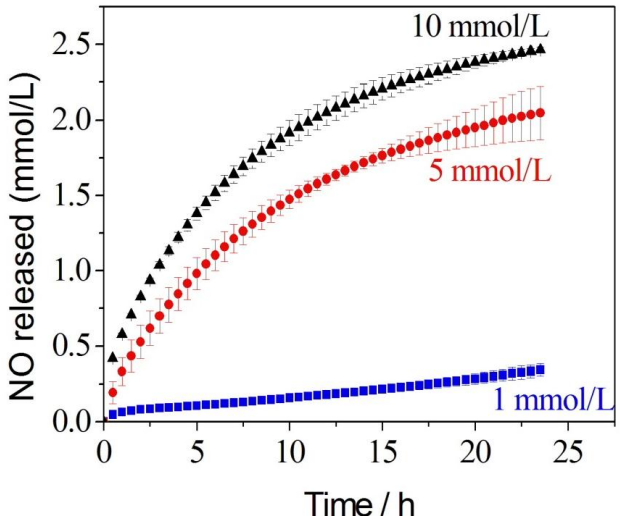


Figure 1. NO release profile from GSNO-containing alginate/chitosan nanoparticles at 1, 5 and 10 mmol/L, at 35°C.

It can be observed that NO is spontaneously released from GSNO-containing alginate/chitosan nanoparticles. The kinetic curves show an initial burst of NO release in the first 5 hours, followed by a progressively increase at lower rates. A sustained NO release is observed for at least 24 h. The NO-release profile increased with the increase of initial GSNO concentrations in the nanoparticles, due to the auto-catalytic effect, as previous described [13,14]. Initial rates of NO released were calculated from the kinetic curves of Figure 1. The initial rates of NO release from GSNO-nanoparticles were found to be 0.0057 ± 0.0010; 0.3088 ± 0.0066 and 0.5270 ± 0.0035 mmol/Lh for GSNO at 1, 5 and 10 mmol/L, respectively. As expected for GSNO decomposition with NO release, the initial rates increase with the initial concentration of GSNO. The NO release from GSNO-containing alginate/chitosan nanoparticles is reported to occur mainly through diffusion process over the pores or wall and disintegration of the hydropolymeric structure [15].

3.3. Phytotoxicity of GSH or GSNO-nanoparticles

Figure 2 shows the mean values obtained for *Zea mays* shoot lenght and root length.

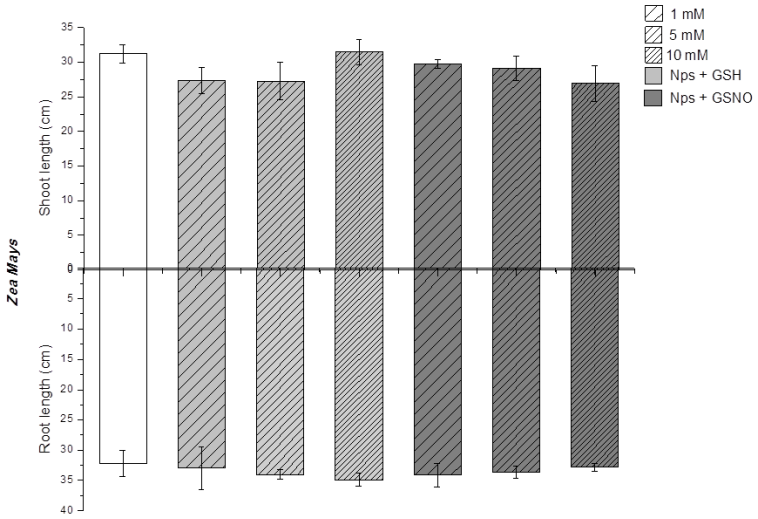


Figure 2. Shoot and root length for *Zea mays* after 7 days of daily treatment with GSH- or GSNO-alginate/chitosan nanoparticles (GSH or GSNO concentrations 1, 5 and 10 mM), as indicated in the Figure. The plants were left for another 7 days after application of the treatments, and were then collected and analysed.

Similarly, Figure 3 shows the mean values obtained for *Glycine* sp shoot length and root length after 7 days of treatment.

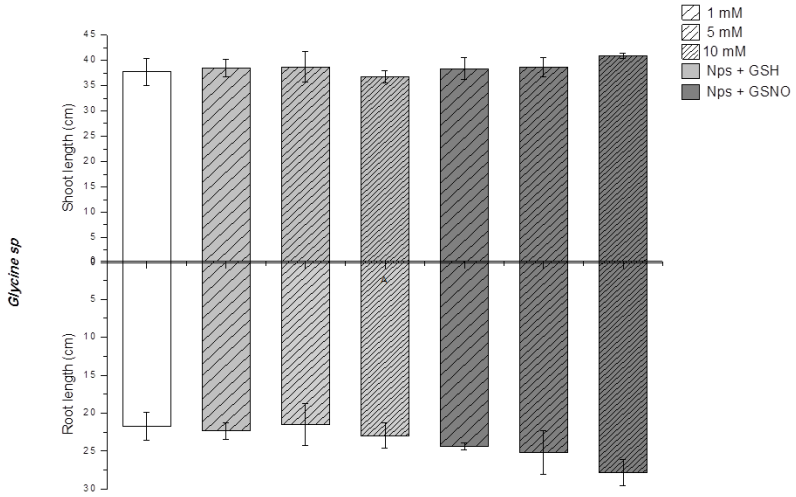


Figure 3. Shoot and root length for *Glycine* sp after 7 days of daily treatment with GSH- or GSNO-alginate/chitosan nanoparticles (GSH or GSNO concentrations 1, 5 and 10 mM), as indicated in the Figure. The plants were left for another 7 days after application of the treatments, and were then collected and analysed.

Figure 4 shows the values obtained for the dry masses of the specimens of *Zea mays* and *Glycine* sp after the GSH or GSNO-nanoparticles treatment.

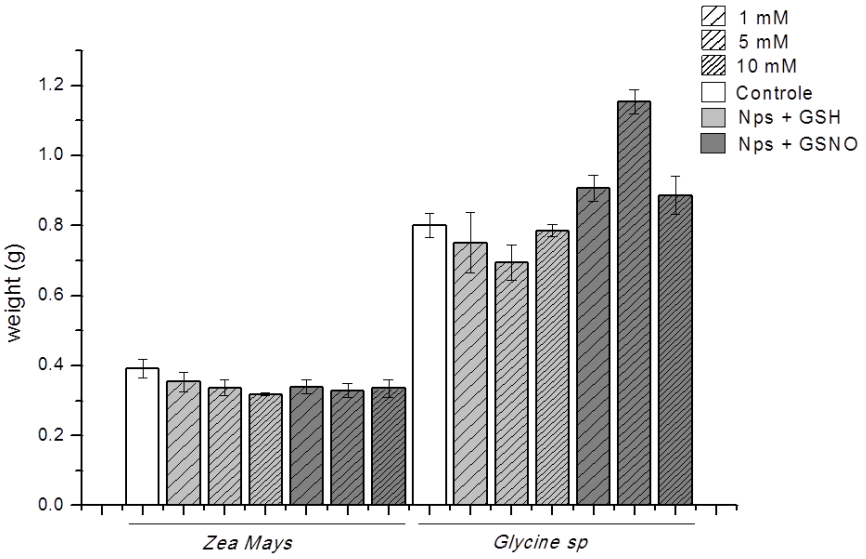


Figure 4. Dry masses for *Zea mays* and *Glycine* sp after 7 days of daily treatment with GSH- or GSNO-alginate/chitosan nanoparticles (GSH or GSNO concentrations 1, 5 and 10 mM), as indicated in the Figure. The plants were left for another 7 days after application of the treatments, and were then collected and analysed.

For all the concentrations tested, GSH- or GSNO-containing alginate/chitosan nanoparticles had no significant effect on the growth and development of *Zea mays* and *Glycine* sp., in terms of shoot height, root length, and dry mass. It should be noted that the increase of dry mass for *Glycine* sp upon treatment with GSNO-nanoparticles (Figure 4) were not statistically significant.

Figure 5 shows photographs of the tested plants 7 days after exposure to GSH- or GSNO-nanoparticles treatment, at the highest tested GSH or GSNO concentration 10 mmol/L, together with photographs of the corresponding controls (plants treated with deionized water).

The use of nanoparticles as carriers for active agents in agricultural applications shows considerable potential. These systems can help to improve the stability of the active agents, increase their effectiveness, and at the same time reduce the possibility of environmental contamination [1,17,18]. Despite these potential benefits, studies aimed to further investigate the effects of nanomaterials on plants remain scarce. It should be noted that characteristics of nanoparticulate systems including particle size, composition, and physicochemical properties might influence their effects during different stages of plant development [19]. In particular, alginate/chitosan are known as biocompatible and biodegradable system ideal for carrying and delivering important molecules in agriculture with no toxic effects to the environment [17,18].

On the other hand, phytotoxicity studies have shown that nanoparticles of zinc oxide and cobalt can influence the development and morphology of the species *Allium cepa* [20]. It has also been found that nanoparticles of nickel oxide can induce oxidative stress, mitochondrial dysfunction, and necrosis and apoptosis in tomato root cells [21]. Exposure of Arabidopsis thaliana to gold nanoparticles resulted in increased germination rates, greater plant development, and higher antioxidant activity [22]. Other types of nanomaterials, such as carbon nanotubes, have been found to increase germination rates and tissue development in tobacco and tomato plants [23].

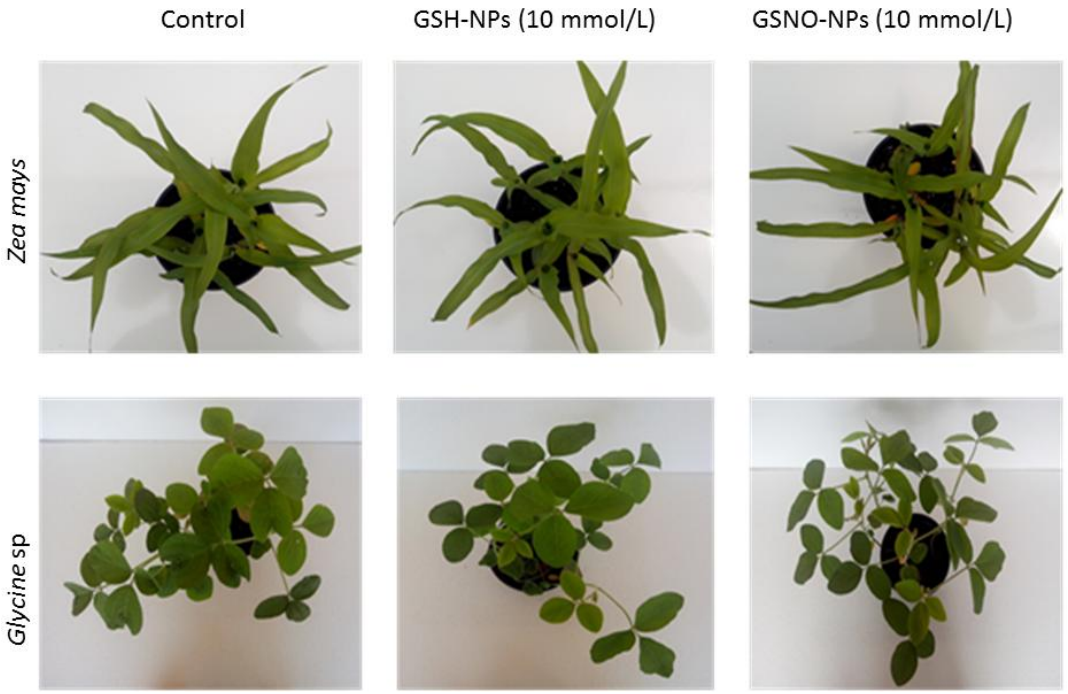


Figure 5. Photographs of *Zea mays* and *Glycine* sp. specimens, 7 days after application of the GSH- or GSNO-alginate/chitosan nanoparticles, at the highest test concentration (10 mmol/L of GSH or GSNO). Photographs of control specimens (treatment with deionized water) are provided for comparison.

In the present work, polymeric nanoparticles of alginate/chitosan containing GSH or GSNO (1, 5 and 10 mol/L) showed no significant effects on the development of the species *Zea mays* and *Glycine* sp, considering the variables shoot height, root length, and dry mass. These formulations therefore seem to have potential for use in agriculture as sustained release systems, although further work will be needed to investigate a wider range of concentrations and/or plant species.

4. Conclusions

This work describes the preparation and characterization of GSH- or GSNO-alginate/chitosan nanoparticles and their impact in plants. Nanoparticulate carrier systems have many potential applications in agriculture, where they can be used to transport a range of agrochemicals, including substances used to control pests and diseases. They can also be used in packaging to improve the quality of agricultural products. The present study showed that polymeric nanoparticles containing GSH or GSNO had no significant inhibitory effects on the development of two plant species (*Zea mays* and *Glycine* sp.), and could therefore be used commercially as controlled release systems applied using the foliar route.

5. Acknowledgements

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