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Role of hematin and sodium nitroprusside in regulating *Brassica nigra* seed germination under nanosilver and silver nitrate stresses

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ABSTRACT

Silver nanoparticles (AgNPs) are one of the most widely used nanomaterials, although the mechanisms of AgNP toxicity in terrestrial plants is still unclear. We compared the toxic effects of AgNPs and $AgNO_3$ on *Brassica nigra* seed germination at physiological and molecular levels. Both AgNPs and $AgNO_3$ inhibited seed germination, lipase activity, soluble and reducing sugar contents in germinating seeds and seedlings. These reductions were more pronounced in AgNP treatments than $AgNO_3$ treatments. Application of 200–400 mg/L both AgNPs and $AgNO_3$ increased transcription of heme oxygenase-1. However, at 800, 1600 mg/L, AgNPs or $AgNO_3$ suppressed HO-1 expression. At 400 mg/L, AgNPs or $AgNO_3$-induced inhibitory effects on seed germination and were ameliorated by the HO-1 inducer, hematin, or NO donor, sodium nitroprusside (SNP). Additionally, 4 μM hematin and 400 μM SNP were able to markedly boost the HO/NO system. However, the addition of the HO-1 inhibitor (ZnPPIX) or the specific scavenger of NO (cPTIO) not only reversed the protective effects conferred by hematin, but also blocked the up-regulation of HO activity. In addition, hematin-drived NO production in *B. niger* seeds under AgNPs was confirmed. Our results at physiological and molecular levels suggested that AgNPs were more toxic than $AgNO_3$. Based on these results, for the first time, we suggest that endogenous HO is needed to alleviate AgNPs-induced germination inhibition, which might have a possible interaction with NO.

1. Introduction

Nanoparticles (NPs), also called particulate nanomaterials, are defined as particles that are smaller than 100 nm. The small size, structure and surface characteristics of nanoparticles confer novel physical and chemical properties that are not shared by bulk particles of the same material (Stampoulis et al., 2009). Silver nanoparticles (AgNPs) are one of the most important nanomaterials in consumer product inventories due to their known antimicrobial properties and usefulness in personal care products, food service, building materials, medical instruments, and textiles (Park et al., 2010). Many reports have demonstrated that AgNPs are highly toxic to bacteria, fish, algae and other organisms (reviewed by Anjum et al., 2013; Navarro et al., 2008). However, it remains difficult to fully understand the mechanism of AgNPs toxicity in terrestrial plants because of the limited number of studies. Given that the increase in production of commercial AgNPs may have potential negative impacts on ecosystems, our knowledge regarding the toxicity of AgNPs must be broadened to predict the environmental and human health risks associated with these materials. The understanding plant response to AgNPs stress is important and also a fundamental part of making crops stress-tolerant. Nevertheless, the mechanisms and signaling pathway of response to these materials in plants are not fully understood.

Heme oxygenases (HOs, EC 1.14.99.3) catalyze the oxidative conversion of heme to biliverdin (BV) with a concomitant release of carbon monoxide (CO) and free iron (Fe^{2+}). BV is then converted to bilirubin by the biliverdin reductase (Shekhawat and Verma, 2010). Recently, it has been known that HO plays an important role in a number of physiological processes such as dormancy break and seed germination (Dekker and Hargrove, 2002; Xu et al., 2006; Liu et al., 2007), growth and developmental regulation, stomatal closure, and adaptation responses to environmental stresses (Han et al., 2007, Song et al., 2008; Xie et al., 2008; Xuan et al., 2008). Li et al. (2012) reported expression of *HO-1* was induced significantly by heavy metal Hg. They showed over expressing *HO-1* in transgenic *Brassica juncea* increased the plant resistance to Hg toxicity.

Abbreviations: AgNPs, silver nanoparticles; CO, carbon monoxide; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; HO, heme oxygenase; NO, nitric oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside; ZnPPIX, zinc protoporphyrin

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Hemin and hematin belong to heme (ferroprotoporphyrin IX) compounds. Recently, it was observed that hemin and hematin induced HO-1 expression in wheat (Xu et al., 2011), and rice germinating seeds (Liu et al. 2007). The application of the exogenous HO-1 inducer, hematin and a CO aqueous solution dose-dependently alleviated the inhibition of wheat seed germination and seedling growth under osmotic stress, both of which were partially due to the induction of antioxidant metabolism as well as the degradation of storage reserve (Liu et al., 2010). However, the information about hematin-induced effects on the alleviation of nanoparticles-induced seed germination inhibition has not been investigated yet.

The favorable effects of HO are similar to some behaviors reported for nitric oxide (NO), an important gaseous signaling molecule recently uncovered in plants (Palavan-Unsal and Arisan, 2009). NO as well as HO was reported to be an important positive regulator of both dormancy break and seed germination (Bethke et al., 2004, Zhang et al., 2005) or act as a signal transduction molecule in various responses against biotic and abiotic stress (Amooaghaie and Nikzad, 2013; Arasimowics and Floryszak-Wieczorek, 2007; Beligni and Lamattina 2001; Zheng et al., 2009). For example, exogenous NO induced tomato tolerance to copper toxicity through antioxidant enzyme activity (Hu et al., 2007). The relationship between NO signaling and HO response in plants has been demonstrated by some investigators. For example, guard cell closure by HO-driven CO requires participation of NO (Song et al., 2008). However, whether the mitigation of seed germination inhibition induced by nanosilver stress is closely associated with alterations of endogenous HO-1 expression and the relationship between HO and NO in seed germination under nanosilver has not yet been established.

Brassica nigra is tolerant towards the heavy metals (Angelova and Ivanov, 2009). It is possible that this plant be tolerant to metal nanoparticles and could be a model plant for study about mechanisms of metal nanoparticles tolerance. Thus, the aim of the present study was to elucidate the toxic effects of AgNPs on the seed germination, lipase activity and seedling growth of *B. nigra* by comparing with the toxicity of $AgNO_3$. In addition, for the first time, the effects of hematin and SNP on AgNPs-induced seed germination and seedling growth inhibition were studied, and hypothesis of a possible signaling link between HO and NO responsible for the alleviation of AgNPs-induced damages was further investigated.

2. Materials and methods

2.1. Chemicals

Hematin as an HO-1 inducer (Liu et al., 2010; Xuan et al., 2008) and sodium nitroprusside (SNP) as NO donor (Bethke et al., 2004), zinc protoporphyrin IX (ZnPPIX) as a potent inhibitor of HO-1 (Liu et al. 2010; Xuan et al., 2008), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) as a specific NO scavenger purchased from Sigma (St Louis, MO, USA).

Seeds were obtained from the medicinal plant research center, Isfahan, Iran.

The AgNPs were purchased from Shanghai Huzheng Nanotechnology Co. LTD (AGS-WMB1000C, Shanghai, China), and the particle diameter was approximately 40 nm according to the manufacturer's data. Upon addition to the medium, the AgNPs were dispersed by ultrasonic vibration (100 W, 40 kHz) for 30 min. To avoid aggregation of the particles suspensions were continuously mixed using a magnetic stirrer before use.

2.2. Plant materials, growth condition and treatments

B. nigra seeds were sterilized with 2% (v/v) sodium hypochlorite solution for 5 min and were washed thoroughly with distilled water. In the first experiment, seeds were germinated in covered 9 cm Petri dishes at various concentrations (0, 50, 100, 200, 400, 800, 1600 mg/L) AgNPs or at the same concentration of Ag^+ (from $AgNO_3$) to compare the toxicity of AgNPs and Ag^+ (from $AgNO_3$). *B. nigra* seeds germinated in a culture room at (25 ± 1) °C under a 12 h light/dark cycle at a light intensity of 300 μmol/m^2 s.

In the second experiment, for evaluating of the best hematin and SNP concentrations, surface-sterilized seeds were presoaked for 2 h at various concentrations of hematin (0, 1, 2, 4 and 8 μM) and SNP (0, 100, 200, 400, 800 μM), then washed thoroughly with distilled water and were transferred to Petri dishes containing distilled water (Control), 400 mg/L of AgNPs and 400 mg/L Ag NO_3.

For evaluating whether AgNPs or Ag^+-induced seed germination inhibition are related to endogenous HO and NO, the effects of the HO-1 inducer (4 μM hematin, H) and its potent inhibitor (50 μM ZnPPIX), NO donor (400 μM SNP) and its specific scavenger (100 μM cPTIO) also were investigated on seed germination, lipase activity, oil content, total soluble sugar and reducing sugar in germinating seeds. Based on two preliminary experiments, 400 mg/L of AgNPs or $AgNO_3$, 4.0 μM hematin and 400 μM SNP were used in the third experiment. Seeds were presoaked for 2 h in these treatments, then thoroughly washed with distilled water and were placed in Petri dishes containing of distilled water (Control), 400 mg/L of AgNPs or $AgNO_3$ and incubated in a similar growth chamber according to above described conditions. These solutions were renewed each day. After 24 h exposure to AgNPs or $AgNO_3$ the seeds were sampled from various treatments then immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Germination analysis

Germination tests were performed on three replicates of 150 seeds each. There were 50 seeds in each Petri dish. Seeds were considered to have germinated when the emerging root was approximately half the length of the seeds. Seed germination energy (GE, %), germination rate (GR), Means of germination time (MGT) and Time (in days) to obtain 50% germination (referred to as T50) were calculated as described in the Association of Official Seed Analysis, 1983, using the following formulas:

GE = number of germinated seeds after 3 days/number of total seeds

Germination rate $(GR) = (G/T)$

where, G=number of total germinated seeds; and T=number of total seeds in experiment.

Mean germination time $(MGT) = (GtDt/G)$

where Gt=number of germinated seeds at day t; Dt=day t; and G=germinated seeds.

$$T_{50} = [(t_2 - t_1) \times 50\% + (p_2 t_1 - p_1 t_2)]/p_2 - p_1$$

where t_1=time at which the germination percentage is less than 50%, t_2=time at which the germination percentage is more than 50%, and p_1 and p_2 are the measurements of germination percentage occurring at t_1 and t_2, respectively.

Seed germination was recorded daily for 10 days, and final germination percentages were calculated. Root and shoot length were measured 10 days after the time of radicle protrusion. The vigor index and relative shoot length were determined by the