Effects of extracellular polymeric substances on silver nanoparticle bioaccumulation and toxicity to *Triticum aestivum* L.

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**ABSTRACT**

The potential effects of extracellular polymeric substances (EPS) on the behavior and toxicity of silver nanoparticle (Ag-NPs) and silver sulfide nanoparticle (AgS-NPs) remains ambiguous. The interaction of EPS from *Bacillus subtilis* with AgS-NPs, metallic Ag-NPs, or ionic Ag, and the associated plant safety had been examined in this study. The biological impacts of Ag-NPs and AgS-NPs were Ag form-dependent and highly influenced by microbial EPS. Compared with metallic Ag-NPs, AgS-NPs exerted inert biological impacts, as revealed by 3.44 times lower Ag bioaccumulation in wheat (*Triticum aestivum* L.) seedlings and nearly reduce plant biomass when wheat was subjected to 1.0 mg-Ag L−1 of Ag-NPs and AgS-NPs with the transfer factors of 151.56–930.87 vs. 12.52–131.81, respectively. These observations were coincident with the low dissolved Ag ([Ag]diss) in the AgS-NPs treatment than the Ag-NPs treatment (114.0 vs. 0.0791, μg L−1). Compared with the enhanced toxicity of AgS-NPs to wheat, *Bacillus subtilis* EPS significantly alleviate the phytotoxicity of Ag-NPs, as revealed by the relative root elongation (7.15–45.40% decrease vs. 2.39–11.75% increase), and malondialdehyde (11.75% increase), and H2O2 (11.27–71.78% increase vs. 5.16–36.67% decrease) contents. These constraining plant responses of *B. subtilis* EPS are mainly caused by their complexation property with toxic Ag⁺ and nutrient elements for wheat stressed by Ag-NPs and AgS-NPs, respectively. Our findings highlight the importance of rhizospheric EPS in affecting the biogeochemistry and ecotoxicity of metal nanoparticles including Ag-NPs and AgS-NPs in agricultural systems.

1. Introduction

Over the few decades, silver nanoparticles (Ag-NPs) are commonly used in medical devices, coatings, textiles, food packaging, and cosmetics due to their strong antimicrobial properties (Ahamed et al., 2010; Keller et al., 2013; Wu et al., 2020). The subsequently released Ag-NPs and or ionic silver (Ag⁺) in surrounding environments is of increasing risk concerns (Dobias and Bernier-Latmani, 2013; Pourzahedi et al., 2017; Prajitha et al., 2019). Additionally, the phytotoxicity of Ag-NPs is also related to the aging process of Ag-NPs. Indeed, Ag-NPs are generally introduced into environments through the practices of wastewater effluent and land-application of biosolids and will be mainly transformed to chronic Ag-S nanoparticles (AgS-NPs) (Pradas del Real et al., 2016; Wang et al., 2016). Due to the low solubility and reactivity, Ag-S-NPs are considered to be less toxic than Ag⁺ and Ag-NPs (Liu et al., 2018). However, AgS-NPs could be directly taken up by plant roots and subsequently transferred to leaves without substantial transformation or dissolution (Wang et al., 2015b, 2017). Furthermore, more Ag⁺ is expected due to the hypochlorite oxidative or Fe³⁺-mediated dissolution of AgS-NPs in some natural environments, such as in surface water or aqueous systems with environmental levels of Fe³⁺ under the sunlight condition (Li et al., 2016b, 2017). These findings have demonstrated

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that the stability and bioavailability of the AgS S-NPs are governed by environmental conditions, suggesting the possible misestimation of eco-environmental safety of AgS S-NPs in previous studies. Compared with numerous toxicity studies of Ag-NPs, no attempts has been performed to compare the influence of biogenic organic matter such as extracellular polymeric substances (EPS) on the transformation behavior of Ag-NPs and AgS S-NPs. Therefore, elucidating the effects of these nanoparticles in intricate ecosystems will shed lights on the potential impact of AgS S-NPs and Ag-NPs in the environment and the phytotoxicity mechanisms.

Due to the inevitable reaction with AgS S-NPs and Ag-NPs, natural organic matter (NOM) will greatly affect their transport, stabilization, dissolution, bioaccumulation, bioavailability and phytotoxicity in the environments (Xu et al., 2019). EPS, the complex macromolecular substances from various microbial activities, are mainly consist of polysaccharides, proteins, nucleic acids (Huangfu et al., 2019). Because of the high content of functional groups, EPS can effectively bind heavy metals and prevent their toxic metals from contacting directly with the cells (Sheng et al., 2010; Kang et al., 2014). Additionally, the phenol group and hemiacetal reducing terminal in polysaccharides may be related to the reducing ability of EPS towards high-oxidation-state metals including Ag+ (Cao et al., 2011; Zhang et al., 2016). The tolerance of bacterial or algal cells to heavy metals and nanoparticles is also enhanced by the EPS. For instance, EPS from Escherichia coli are capable of increasing its survival ratio by 4.3 and 1.6 times for Cu2+ and Co2+, respectively, due to the complexation of metal-EPS, which decreased the cellular absorbability of metals (Shou et al., 2018). EPS from a green alga (Chlorella pyrenoidosa) could facilitate the TiO2 nanoparticles aggregation and sedimentation, limiting algal cell internalization of TiO2 nanoparticles (Gao et al., 2018). However, information regarding the comparative effect of EPS on the behavior and toxicity of Ag-NPs and AgS S-NPs to plants remains limited. Furthermore, the phytotoxicity of Ag-NPs to wheat Triticum aestivum L. and the relevant Ag+ dissolution were clearly mitigated by EPS from a representative Gram-negative bacterium, Pseudomonas putida (Li et al., 2016a). On the contrary, dissolved organic matter (DOM) had been reported to causes more copper ions released from copper nanoparticles and might pose a higher toxicity to organisms (Wang et al., 2015a). These contrasting effects have highlighted the importance of organic composition in regulating the dissolution and toxicity of metal nanoparticles. In addition to cell wall compositions, the chemical compositions and prosperities of bacterial EPS are largely species-dependent, particularly for Gram-negative versus Gram-positive cells. For example, EPS from a Gram-positive bacterium, Bacillus valismortis sp., were rich in polysaccharides (Li et al., 2020); while EPS from a novel Gram-negative bacterium, Acidithiobacillus sp. Ksh, contained more protein-like substances (Vardanyan et al., 2020). EPS from Bacillus subtilis (Gram-positive) were less aliphatic and hydrophobic than EPS from two Gram-negative bacteria, E. coli and P. putida, respectively (He et al., 2015; Chen et al., 2021). The higher contents of polysaccharides and proteins from B. subtilis EPS than P. putida EPS provided more binding sites for heavy metals, accounting for the higher decrease percentage of Cd adsorption on B. subtilis cells compared with P. putida cells (51.4% versus 9.7%) caused by EPS removal (Wei et al., 2011). The important role of EPS in regulating AgNPs phytotoxicity had already been confirmed for Gram-negative P. putida cells by our previous study (Li et al., 2016a), while the role of Gram-positive bacterial EPS toward the phytotoxicity of AgNPs or AgS S-NPs is still unknown.

The representative Gram-positive bacterium, B. subtilis, was used to investigate the influence of its EPS on the phytotoxicity and associated mechanisms of AgS S-NPs, Ag-NPs, and Ag+ to an important cereal crop, Triticum aestivum L. in this study. B. subtilis is a common plant growth-promoting rhizobacteria, exhibiting protective effects for plants against abiotic and biotic stresses (Rais et al., 2004; Lastochkina et al., 2017). Transmission electron microscope (TEM) and energy-dispersive spectroscopy (EDS) were used to probe visually the formation and element compositions of EPS-nanoparticles complex. The key structural components of EPS involved in their reactions with nanoparticles were characterized by three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy and Fourier-transform infrared spectroscopy (FTIR).

2. Materials and methods

2.1. Nanoparticles and characterization

AgS S-NPs were synthesized by the reported method using AgNO3 and elemental sulfur (Wang et al., 2015b). Briefly, 42.0 mg of sulfur was dissolved in 50 mL warm ethanol (~60 °C) by bath sonication (KQ-3200, Kunshan, China), then added droplets to 500 mL AgNO3 solution (1 mM) containing 500 mg polyvinylpyrrolidone under magnetic stirring. The reaction was performing for 5 h at 60 °C under dark. AgS S-NPs were concentrated by centrifugation (10,000 g, 30 min) and purified with deionized (DI) water for 3 times. Then AgS S-NPs stock was suspended in ultrapure water and stored at 4 °C under dark prior to use. PVP-coated silver nanoparticles (Ag-NPs) were purchased from XFNANO Materials Technology Ltd. (Nanjing, China).

The morphology and elemental composition of the AgS S-NPs and Ag-NPs were examined with TEM-EDS (Hitachi HT-7700, Japan). Hydrodynamic size and zeta (ζ) potential of Ag-NPs (1 mg L−1, pH 6.0 ± 0.1) and AgS S-NPs (300 mg L−1, pH 6.0 ± 0.1) were characterized using a zetasizer (Nano ZS, Zen 3700, Malvern Instruments, UK). All nanoparticle stocks were ultrasonicated for 10 min prior to use to achieve a uniform particle distribution.

Dissolved Ag, defined as the concentration of ionic Ag dissolved from Ag-NPs and AgS S-NPs in nutrient medium, was quantified by inductively coupled plasma mass spectrometry (ICP-MS, Agilent, 7700x, USA) analysis. Prior to ICP-MS analysis, 4 mL suspension was centrifuged at 5000 g for 20 min to removal nanoparticles using Amicon Ultra-4 centrifugal ultrafiltration filter (3 kDa, Millipore, Billerica, USA), and the filtrate was digested with HNO3 and H2O2.

2.2. EPS extraction and characterization

Bacillus subtilis, a typical soil bacterium isolated from a metal-contaminated municipal waste in Wuhan, China (Fang et al., 2011), was used for EPS extraction with our previous method (Li et al., 2016a). Briefly, B. subtilis was inoculated in the Luria broth (10 g L−1 tryptone, 5 g L−1 yeast extract, 5 g L−1 NaCl, pH 7.4) and cultivated on a horizontal vibrator (30 °C, 150 rpm) for 24 h to reach the stable growth phase. Cell solution was centrifuged (5000 g, 4 °C, 10 min) to remove bacterial cells, followed by an additional centrifugation (10,000 g, 4 °C, 20 min) to remove other cell residues. The supernatant was mixed with ice-cold ethanol at a volumetric ratio of 3 : 1 (ethanol: EPS supernatant) for 48 h, and the raw B. subtilis EPS were obtained by centrifugation (8000 g, 10 min). The pellet was further dialyzed by spectral/Por 7 regenerated cellulose membrane for 3 d to remove small molecular weight impurities by replacing DI water three times per day. The purified B. subtilis EPS were freeze-dried and stored at −20 °C for later experiments.

The total organic carbon (TOC) and zeta potential of B. subtilis EPS were determined by a TOC analyzer (TOC-LCPH, Shimadzu, China) and Zetasizer (Nano ZS, Zen 3700, Malvern Instruments, UK), respectively. Total nitrogen (TN), total phosphorus (TP), polysaccharides and protein contents were measured by the alkaline potassium persulfate ultraviolet spectrophotometric method, molybdenum blue spectrophotometric method (Johnes and Heathwaite, 1992; Li et al., 2016b), phenol-sulfuric acid method and comassie brilliant blue method (Chen et al., 2015), respectively. The nucleic acids content were measured by the diphenylamine colorimetric method with the standard of calf thymus DNA (Jia et al., 2017).
2.3. Toxicity assays and Ag uptake experiment

Root elongation test. The wheat (Triticum aestivum L., Yang line 16) seeds, purchased from Lixiahe Agricultural Research Institute of Jiangsu Province, Yangzhou, China, were sterilized in 0.5% NaClO solution for 10 min and then thoroughly washed for three times with DI water.

To explore the 48-h root elongation toxicity test, sterilized seeds were germinated at 28 ± 1°C on wet filter paper for 48 h in the dark with 80% relative humidity. Afterwards, 10 uniform wheat seedling with 1.5 cm root length were selected and transplanted to a nutrient medium (0.25 mM KNO₃, 0.25 mM MgSO₄·7H₂O, 0.25 mM Ca(NO₃)₂·4H₂O, 0.08 mM KH₂PO₄, 2 mM Morphotelinehasulamic acid, pH 6.0 ± 0.1) amended with different concentrations of AgNO₃ (0, 0.05, 0.1, 0.25, 0.5 and 1.0 mg L⁻¹), Ag-NPs (0, 0.05, 0.2, 0.5, 1.0 and 2.0 mg L⁻¹) or AgS-NPs (0, 0.5, 1.0, 5.0, 10.0 and 15.0 mg L⁻¹). There were four parallel trials for each treatment. After 48-h exposure, two longest roots of each seedling were measured, and a total of 20 measurements from 10 seedlings in each replicate were averaged. To assess the role of B. subtilis EPS on the toxicity of Ag, seedlings were exposed to the fixed median effective concentrations (EC₅₀) of Ag⁺, Ag-NPs, and AgS-NPs with a series of concentrations of B. subtilis EPS (0, 10, 20, 50 and 100 mg L⁻¹, based on mass concentration). After harvest, the wheat seedlings were rinsed with 1-cysteine (10 mM, pH 8.0) and DI water to remove loosely-bound Ag ions or nanoparticles, and then dried and digested with 10 mL HNO₃ and 1 mL H₂O₂. Silver contents were determined by ICP-MS.

Malondialdehyde (MDA) analysis. MDA levels in wheat roots were measured by a thiobarbituric acid (TBA) reactive substances assay (Jambunathan, 2010) and assessed as lipid peroxidation. Briefly, frozen tissues were homogenized with 5 mL phosphate buffer solution (0.05 M NaH₂PO₄·Na₂HPO₄ and centrifuged (5000 g, 4°C, 10 min). The supernatant was sufficiently mixed with a mixture of trichloroacetic acid (5%) and TBA (0.5%), heated in a water bath at 95°C (10 min), and subjected to UV spectrophotometric analysis at 532 and 600 nm (Cary 60 UV–Vis spectrophotometer, Agilent Technologies).

Hydrogen peroxide (H₂O₂) analysis. H₂O₂ contents were determined by a public protocol (Li et al., 2020). Briefly, 0.5 g of fresh roots were homogenized with acetone, centrifuged (5000 g, 4°C, 10 min), and added with titanium sulfate and concentrated ammonia hydroxide. After additional centrifugation (5000 g, 10 min), the precipitate (a peroxide–titanium complex) was washed with acetone for three times (to remove plant pigment), dissolved in sulfuric acid and then measured at 415 nm (Cary 60 UV–Vis spectrophotometer, Agilent Technologies, USA).

2.4. Spectral analysis

The B. subtilis EPS (1000 µg mL⁻¹) sample containing AgNO₃ (0.1 mg L⁻¹), Ag-NPs (0.3 mg L⁻¹) or AgS-NPs (12 mg L⁻¹) was mixed with cold ethanol (1 : 3 vol), respectively. After settling for 48 h at 4°C, the precipitate was centrifuged (10,000 g, 20 min). The freeze-dried samples mixed with KBr (1 : 100, w/w) were acquired by FTIR spectrometer (Nicolet 380, Thermo Fisher Scientific Inc., USA) from 400 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹.

The EEM fluorescence spectroscopy was used to characterize the fluorescent EPS components of B. subtilis EPS. All samples were measured by a luminescence spectrophotometer (F-7000 FL, Hitachi, Japan) with DI water as the blank. The EEM spectra were recorded at 5 nm sampling intervals with the excitation wavelength from 200 nm to 500 nm, and the emission spectrum was scanned from 200 nm to 500 nm at a scan rate of 2400 nm min⁻¹. Excitation and emission of slits were maintained at 5 nm.

2.5. Characterisation of NPs and EPS reaction

Nanoparticles and B. subtilis EPS suspension samples were captured on carbon formvar copper grids. B. subtilis EPS solutions (1000 mg L⁻¹) containing 100 mg L⁻¹ Ag-NPs or 50 mg L⁻¹ AgS-NPs were incubated for 48 h to prepare nanoparticle-EPS complexes. Several drops of nanoparticle-EPS complexes were added to copper grids and held for 15 min for vacuum drying for TEM analysis.

The possible dissolution of nanoparticles caused by EPS was also examined by mixing B. subtilis EPS (0, 10, 20, 50, 100 mg L⁻¹) and Ag-NPs (2 mg L⁻¹) or AgS-NPs (15 mg L⁻¹) in the nutrient solutions. After 12/24/48 h incubation, 4 mL suspension was centrifuged at 5000 g for 20 min to remove nanoparticles using Amicon Ultra-4 centrifugal ultrafiltration filter (3 kDa, Millipore, Billerica, USA), and the filtrate was digested and quantified dissolved Ag contents by ICP-MS.

2.6. Data analysis

The relative root elongation (RRE, %) and EC₅₀ were calculated and fitted using Eqs. (1) and (2), respectively (Haanstra et al., 1985; Fu et al., 2016, 2018; Li et al., 2016a):

\[
\text{RRE} = \frac{R_{100} - R_{0}}{R_{100}} \times 100
\]

\[
\text{RRE} = \frac{100}{1 + e^{k-x(MC_{50})}}
\]

where R₅₀ (cm) represented the mean root length (RL) in the presence of toxicants (i.e., AgNO₃, Ag-NPs or AgS-NPs), RLₐ (cm) represented RL at the time of seedling transfer to the exposure medium, and RLₐ (cm) represented RL in the nutrient solutions without toxicants. a is the slope parameter indicating the inhibition ratio, and x is the natural logarithm of the toxicant concentration.

Differences between treatments were determined using one-way ANOVA (SPSS 19.0), followed by a Student–Newman–Keuls (S–N–K) test at a significance of p < 0.05. Data was present as mean ± SD (n = 3–4).

3. Results and discussion

3.1. Nanoparticles and EPS characterisation

Both Ag-NPs and AgS-NPs were in spherical shape with particle sizes of 19.3 ± 0.5 nm, 67.3 ± 2.2 nm, respectively (Fig. S1). Ag-NPs (1 mg L⁻¹, pH 6.0 ± 0.1) and AgS-NPs (3 mg L⁻¹, pH 6.0 ± 0.1) in DI water were homodisperse with an average hydrodynamic size of 96.7 ± 0.5 nm and 147 ± 3.9 nm (Fig. S1), respectively.

The TOC, TP and TN contents of B. subtilis EPS were 387.0 ± 25.5, 9.1 ± 2.7 and 57.4 ± 0.4 mg g⁻¹ of, respectively. B. subtilis EPS contained comparable TOC and TN, but significantly less TP than P. putida EPS (p < 0.05) (Li et al., 2016a), which was consistent with the fact that lipid layers in cell walls of the Gram-negative P. putida cells were much thicker than those for the Gram-positive B. subtilis cells. In addition, the purified B. subtilis EPS were predominantly composed by proteins and polysaccharides (87.3 ± 8.5, 227.2 ± 8.0 mg g⁻¹, respectively), followed by less content of nucleic acids (13.6 ± 2.1 mg g⁻¹). Proteins and polysaccharides contents of B. subtilis EPS in this study were around half of our previously reported values for P. putida EPS (Li et al., 2016a). Furthermore, B. subtilis EPS and P. putida EPS were reported to have comparable levels of polysaccharides (437.6 versus 532.2 mg/g, respectively), but differ largely in protein contents (249.1 versus 152.2 mg g⁻¹ protein, respectively) under the same experimental conditions and procedures (He et al., 2015). But, an inverse tendency was also reported for EPS from B. subtilis and P. putida cultured in the same way with EPS extracted from the cationic exchange resin (Wei et al., 2011). These results demonstrated that the yield and biochemical composition of EPS are not only dependent on bacterial species, but also vary from cultural and extractions. B. subtilis EPS and P. putida EPS might, therefore, affect differently on the transform and phytotoxicity of Ag-NPs and AgS-NPs. The zeta (ζ) potential of B. subtilis EPS (1000 mg L⁻¹) containing 100 mg L⁻¹ Ag-NPs or 50 mg L⁻¹ AgS-NPs was incubated for 48 h to prepare nanoparticle-EPS complexes. Several drops of nanoparticle-EPS complexes were added to copper grids and held for 15 min for vacuum drying for TEM analysis.

The possible dissolution of nanoparticles caused by EPS was also examined by mixing B. subtilis EPS (0, 10, 20, 50, 100 mg L⁻¹) and Ag-NPs (2 mg L⁻¹) or AgS-NPs (15 mg L⁻¹) in the nutrient solutions. After 12/24/48 h incubation, 4 mL suspension was centrifuged at 5000 g for 20 min to remove nanoparticles using Amicon Ultra-4 centrifugal ultrafiltration filter (3 kDa, Millipore, Billerica, USA), and the filtrate was digested and quantified dissolved Ag contents by ICP-MS.
3.2. Ag bioaccumulation and phytotoxicity to wheat

As illustrated in Figs. 1A and S3, the RRE values of the wheat were significantly reduced for the AgNO₃ (25.73–89.28%) and Ag-NPs (13.51–86.92%) with a dose-dependent effect, but less decreased (6.17–59.86%) by Ag₂S-NPs. The variation of fresh biomass was consistent with RRE results. The fresh biomass of the root and shoot treated with 1.0 mg L⁻¹ AgNO₃ was reduced by 49.26% and 48.47%, 45.59% and 39.58%, 6.62% and 2.66% compared to the control, respectively (Fig. 1B). At the biochemical level, MDA and H₂O₂ contents were used to assess lipid peroxidation levels in wheat roots. Both the MDA and the H₂O₂ contents increased significantly relative to the control group after exposure to a series of concentrations of AgNO₃, Ag-NPs and Ag₂S-NPs (Fig. 1C). It should be noted that, at similar silver exposure levels (e.g., 1.0 mg L⁻¹), the MDA and the H₂O₂ contents of Ag₂S-NPs exposure were lower than AgNO₃ and Ag-NPs treatments, suggesting that Ag₂S-NPs was less deleterious. These results are consistent with earlier studies and suggesting an Ag species-dependent and dose-dependent growth inhibition toward wheat seedlings. Although Ag₂S-NPs is extremely inert and insoluble, exposure to Ag₂S-NPs could reduce the growth of wheat (Wang et al., 2017), and ionic Ag is more toxic than metallic Ag-NPs (Dimkpa et al., 2013).

The total silver including all silver that was associated with the wheat (externalized and internalized) after rinsed with DI water. Significant differences (p < 0.05) were observed for Ag accumulation in wheat roots and shoots exposed to a series of concentrations of AgNO₃, Ag-NPs with a concentration-dependent increase. Although the concentration of Ag₂S-NPs in nutrient solution was 10-fold higher than that of AgNO₃ or Ag-NPs, the concentrations of Ag in the wheat roots treated with Ag₂S-NPs were prominently lower (p < 0.05) than the other two treatments (1322.92 ± 64.76, 234.23 ± 54.86 and 68.18 ± 16.98 μg g⁻¹ DW) for 1.0 μg-Ag mL⁻¹ of AgNO₃, Ag-NPs, and Ag₂S-NPs treatment. Moreover, compared with ionic Ag or metallic Ag-NPs, Ag₂S-NPs exert inert biological impacts, as revealed by lower (19.40 or 3.44 times) Ag bioaccumulation in plants at equivalent exposure concentrations (1.0 mg L⁻¹) (Fig. 2). The transport factors (TFs) defined as the ratios of Ag concentrations in roots/shoots to those in exposure solution was calculated to evaluate the transport potential of Ag⁺, Ag-NPs, and Ag₂S-NPs from nutrient solutions to plant. The results showed that Ag transport potential depended on both the Ag species and concentrations. Overall, the TFs for the wheat followed in the order of AgNO₃ > Ag-NPs > Ag₂S-NPs, suggesting an Ag form-dependent impact on Ag accumulation in wheat (Fig. 2). The TFs values decreased with an increasing exposure concentration, which was in line with other studies (Wu et al., 2020), but there was an exception in the AgNO₃ treatment. For AgNO₃ treatment, the TFs values increased by nearly 228% when the exposure concentration increased from 0.25 to 1.0 mg L⁻¹.

3.3. Ag bioaccumulation and phytotoxicity to wheat affected by EPS

As shown in Figs. 3A and S4, EPS apparently mitigated the inhibition of root elongation of wheat in AgNO₃ treatment group, but only slightly in Ag-NPs group at the EC₅₀ levels of Ag²⁺ and Ag-NPs (0.1 and 0.3 μg mL⁻¹). However, in Ag₂S-NPs treatment at EC₅₀ level (12 μg mL⁻¹), the RRE decreased with the increase of EPS concentrations, implying the enhanced toxicity of Ag₂S-NPs by EPS to wheat. Compared with the root elongation, the biomass of wheat was less affected by EPS (Fig. 3B). Moreover, the RRE was inhibited by the EPS concentration over 50 mg L⁻¹ for both in AgNO₃ and Ag-NPs treatments (Fig. 3A). Because NOM can affect the stability and biotransformation of nanoparticles in aqueous environment (Li et al., 2018b), the enhanced effect of EPS on wheat roots growth was probably not caused by its potential role as a growth matrix, but due to the complexation property of EPS with Ag ions or form EPS-NPs complex (Glenn and Klaine, 2013; Li et al., 2018a).

Fig. 1. Relative root elongation (RRE, A), plant biomass (B) and biochemical assays (MDA content and H₂O₂ content, C) of wheat seedlings after 48 h of exposure to AgNO₃ (0, 0.05, 0.1, 0.25, 0.5 and 1.0 mg L⁻¹), Ag-NPs (0, 0.05, 0.2, 0.5, 1.0 and 2.0 mg L⁻¹) or Ag₂S-NPs (0, 0.5, 1.0, 5.0, 10.0 and 15.0 mg L⁻¹). Values are mean ± SD (Four replicates with 10 seedlings per replicate).
which finally relieved the phytotoxicity of AgNO$_3$ and Ag-NPs.

The oxidative damage of wheat root was quantitatively assessed by MDA and H$_2$O$_2$ assays (Fig. 3C). MDA contents were significantly decreased upon increasing *B. subtilis* EPS concentrations for AgNO$_3$ and Ag-NPs groups, but increased slightly by 100 mg L$^{-1}$ *B. subtilis* EPS. On the contrary, for Ag$_2$S-NPs treatment, the MDA content increased with the increasing levels of *B. subtilis* EPS up to 100 mg L$^{-1}$ (Fig. 3C). The variation tendency of H$_2$O$_2$ contents was generally consistent with that for MDA content. Similarly, it was also reported that NOM could mitigate the seed germination and root elongation of rice stressed by AgNO$_3$/Ag-NPs (Huang et al., 2020). These findings suggest that *B. subtilis* EPS could alleviate the toxicity of AgNO$_3$ and Ag-NPs, but aggravate the toxicity of Ag$_2$S-NPs.

As shown in Fig. 4 and S5, the concentration of Ag accumulated in roots and shoots after 48 h exposure decreased by *B. subtilis* EPS. For example, the accumulated total Ag in roots treated with AgNO$_3$, Ag-NPs and Ag$_2$S-NPs was decreased by 27.73%, 61.15% and 22.40%, respectively, in the presence of EPS (0, 10, 20, 50 and 100 mg L$^{-1}$). The TFs of Ag decreased with the increase of *B. subtilis* EPS concentration (Fig. 4). The significant decrease of Ag-NPs or Ag$_2$S-NPs absorbed on wheat roots
Fig. 4. Ag accumulation in wheat root and the associated Ag transfer factors (TFs) after 48 h of exposure to AgNO₃ (0.1 mg L⁻¹), Ag-NPs (0.3 mg L⁻¹) or Ag₃S-NPs (12 mg L⁻¹) in the presence of EPS (0, 10, 20, 50 and 100 mg L⁻¹). The TFs are defined as the ratios of Ag concentrations in roots to those in exposure solution. Values are mean ± SD (Four replicates with 10 seedlings per replicate). Different letters represent statistical differences among treatments at p < 0.05.

by B. subtilis EPS may be resulted from the negative charge feature of B. subtilis EPS, which is involved in the formation of EPS-nanoparticles or EPS-Ag complexes. Free Ag⁺ in Ag-NPs solution was reported to be unavailable Ag-NOM complexes (Gunsolus et al., 2015; Li et al., 2018a). It should be notable that B. subtilis EPS are capable of reducing Ag accumulation, but enhancing phytotoxicity of Ag₃S-NPs to wheat seedlings. These contrasting effects of B. subtilis EPS are a result of the metal-binding capacity of B. subtilis EPS, which decrease the bioavailability of toxic Ag ions as well as nutrients (e.g., Ca, Mg, Fe ions).

The overall tendency of RRE values, MDA and H₂O₂ contents, Ag accumulation affected by B. subtilis EPS in this study are consistent with our previous observations for P. putida EPS (Li et al., 2016a). Considering the difference in EPS biochemical compositions between these two studies, this consistent change for B. subtilis EPS and P. putida EPS suggest that the transformation and Ag-NPs toxicity to wheat are insignificantly affected by relative contents of different EPS biochemical compositions. More specifically, proteins play more important roles in altering Ag-NPs dissolution and phytotoxicity and coordinating with Ag⁺ than polysaccharides because proteins contain more active sites include carboxyl, carbonyl and amide groups, and therefore are more chemically active for metal ions.

3.4. Interaction mechanism between Ag and EPS

As shown in Fig. 5A, two typical peaks of EEM fluorescence spectra were observed in pristine EPS, which were located at Ex/Em (excitation/emission) wavelengths of 235–280/340 nm and 205–215/225–490 nm, respectively. These peaks indicated that both EPS and Ag-NPs toxicity to wheat. EPS and Ag-NPs toxicity to wheat. These contrasting effects of B. subtilis EPS are a result of the metal-binding capacity of B. subtilis EPS, which decrease the bioavailability of toxic Ag ions as well as nutrients (e.g., Ca, Mg, Fe ions).

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The overall tendency of RRE values, MDA and H₂O₂ contents, Ag accumulation affected by B. subtilis EPS in this study are consistent with our previous observations for P. putida EPS (Li et al., 2016a). Considering the difference in EPS biochemical compositions between these two studies, this consistent change for B. subtilis EPS and P. putida EPS suggest that the transformation and Ag-NPs toxicity to wheat are insignificantly affected by relative contents of different EPS biochemical compositions. More specifically, proteins play more important roles in altering Ag-NPs dissolution and phytotoxicity and coordinating with Ag⁺ than polysaccharides because proteins contain more active sites include carboxyl, carbonyl and amide groups, and therefore are more chemically active for metal ions.

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ring of gray material was observed around the nanoparticles surface (Fig. S6) due to the interaction and complexation between B. subtilis EPS and Ag-NPs or AgS-NPs, which was similar with DOM-Ag-NPs complexes (Ding et al., 2019). EDS results demonstrated the presence of oxygen on EPS-Ag-NPs and EPS-AgS-NPs complexes (Figs. S1 and S6), suggesting the adsorption of B. subtilis EPS on EPS-Ag-NPs and EPS-AgS-NPs complexes. B. subtilis EPS were primarily consisted of polysaccharides and proteins as well as some other organic matter (such as fulvic and humic acids) and were negatively charged over the environmental pH values (3–8, Fig. S2). The EPS-Ag-NPs and EPS-AgS-NPs complexes were also therefore negatively charged, and not easily be accessed to the negatively charged root cell membrane surfaces of plant due to electrostatic repulsion (Wang et al., 2018). The formation EPS-nanoparticles complexes will thus reduce the bioavailability of nanoparticles (Ding et al., 2019), which also support the alleviated phytotoxicity of Ag-NPs in this study (Gao et al., 2012; Huang et al., 2020).

3.5. Impact of EPS on Ag-NPs/AgS-NPs dissolution

The oxidative dissolution of Ag-NPs in environments is of increasing concerns because the released Ag$^+$ is the major contributor to the toxicity of Ag-NPs or AgS-NPs. In simple solutions without other oxidants or reductants, Ag-NPs dissolution proceeds as in the following stoichiometry (Eq. (3)) (Liu and Hurt, 2010). Moreover, the dissolution of AgS-S-NPs may primarily proceed through the following equations (Eq. (4)) in oxidative environments (Li et al., 2017).

$$2\text{Ag}_\text{aq}^+ + \frac{1}{2}\text{O}_2\text{aq} + 2\text{H}_\text{aq}^+ \rightarrow 2\text{Ag}_\text{aq} + \text{H}_2\text{O}_\text{aq}$$ (3)

$$\text{Ag}_\text{S-NPs} + \text{H}_2\text{O}_2 + \text{OH}^- \rightarrow \text{Ag}_\text{aq}^+ + \text{SO}_4^-$$ (4)

As shown in Fig. 6A, the dissolved Ag concentrations ([Ag]$_\text{diss}$) gradually increased with the increase levels of Ag-NPs in the absence of B. subtilis EPS during 48 h experimental period. For B. subtilis EPS treatments, the [Ag]$_\text{diss}$ values decreased significantly with increasing concentrations of B. subtilis EPS, which was in line with decreasing level of [Ag]$_\text{diss}$ for Ag-NPs with P. putida EPS (Li et al., 2016a). For example, the [Ag]$_\text{diss}$ reached its maximum (~180.0 μg L$^{-1}$) value after 48-h exposure in the medium solely containing Ag-NPs (2.0 mg L$^{-1}$). And the [Ag]$_\text{diss}$ value decreased to 65.1 μg L$^{-1}$ for the similar level of Ag-NPs with 100 μg mL$^{-1}$ B. subtilis EPS. Regarding AgS-NPs (Fig. 6B), due to the low solubility of AgS ($K_{sp} = 6 \times 10^{-51}$) (Lowry et al., 2012; He et al., 2019), the dissolution ratio of AgS-NPs was closed to 0% even in the scenario with 15 μg mL$^{-1}$ AgS-NPs. This observation was coincident with the phenomenon that AgS-S-NPs were less toxic than AgNO$_3$ or Ag-NPs even though wheat seedlings were exposed to 15 times higher concentrations of AgS-S-NPs than Ag-NPs and AgNO$_3$. An insignificant decrease in the [Ag]$_\text{diss}$ value was observed for B. subtilis EPS-AgS-NPs treatments (Fig. 6B).

Although B. subtilis EPS addition reduced insignificantly the dissolution of AgS-S-NPs, the dissolution ratio of AgS-S-NPs as low as 0%, B. subtilis EPS was thus unlikely to affect the phytotoxicity of Ag-S-NPs in terms of Ag$^+$ dissolution. Since plants can uptake AgS-S-NPs directly without substantial transformation (Wang et al., 2017), the observed phytotoxicity of AgS-S-NPs was caused by their nano effects (e.g. reactive oxygen radicals) rather than the released Ag$^+$ (Li et al., 2018b).

In this study, it was observed that B. subtilis EPS could aggravate AgS-NP toxicity without significantly decreasing the dissolved Ag.

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Fig. 6. Dissolved Ag concentrations and dissolution ratio from Ag-NPs (0, 0.05, 0.2, 0.5, 1.0 and 2.0 mg L$^{-1}$, A) and AgS-S-NPs (0, 0.5, 1.0, 5.0, 10.0 and 15.0 mg L$^{-1}$, B) in the absence and presence of EPS within 48 h of incubation in the nutrient solution. The initial concentration of Ag-NPs and AgS-S-NPs were 2.0 and 15.0 mg L$^{-1}$. Values are mean ± SD (n = 3). Different letters indicated a significant difference at $p < 0.05$. 

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concentrations. According to the hard and soft acids and bases (HSAB) theory (Pearson, 1968), proteins in B. subtilis EPS were abundant in soft base function groups, which preferentially coordinated with the soft acid Ag⁺ and less complexed with hard acids, Fe³⁺, Ca²⁺ and Mg²⁺ in the nutrient media for AgNO₃ and Ag-NPs treatments. While, B. subtilis EPS will generally bind to Fe³⁺, Ca²⁺ and Mg²⁺ for the Ag₃S-NPs treatment, where Ag⁺ is far less than AgNO₃ and Ag-NPs treatments (Fig. 6), reducing the bioavailability of nutrient elements. Thus, it is reasonably concluded that B. subtilis EPS might exacerbate the nano effects of Ag₃S-NPs or affect the uptake of nutrient elements such as Fe³⁺, Ca²⁺, and Mg²⁺ by wheat, finally inhibiting plant growth in Ag₃S-NPs treatment.

4. Conclusion and environmental significance

The contrasting effect of microbial EPS on the phytotoxicity and accumulation of Ag₃S-NPs, metallic Ag-NPs by the crop, Triticum aestivum L. were compared in this study. The Ag⁺ is more toxic than Ag-NPs or Ag₃S-NPs toward wheat. The B. subtilis EPS are capable of alleviating Ag-NPs and Ag₃S-NPs phytotoxicity but enhancing the phytotoxicity of Ag₃S-NPs, which is caused by the interaction between EPS and Ag-NPs or Ag₃S-NPs via amide groups, hydroxyl, carboxyl of protein-like and tryptophan-like substances, forming EPS-nanoparticle complexes. Moreover, dissolution of Ag-NPs and Ag₃S-NPs is also inhibited by B. subtilis EPS. In addition to toxic Ag⁺, B. subtilis EPS also coordinate with nutrients metals, reduce their bioavailability, hindering the growth of wheat stressed by Ag₃S-NPs in the presence of B. subtilis EPS.

In comparison with our previous results for P. putida EPS (Li et al., 2016a), similar influences are observed for B. subtilis EPS and P. putida EPS on Ag⁺ dissolution and uptake and phytotoxicity of Ag₃S-NPs to wheat seedlings. Indeed, the major biochemical compositions of B. subtilis EPS and P. putida EPS were comparative (437.6 versus 532.2 mg g⁻¹ polysaccharides and 249.1 versus 152.2 mg g⁻¹ protein, respectively) in the same study (He et al., 2015). These similar results suggest that EPS from the representative Gram-positive and Gram-negative, B. subtilis and P. putida cells, will affect analogously the transform and phytotoxicity of Ag-NPs in the agricultural system. Both P. putida and B. subtilis are omnipresent rhizosphere bacteria and are often reorganized as soil dweller or soil colonizer (Earl et al., 2008; Volke et al., 2020). Bacillus and Pseudomonas species can also grow on the root surface, forming biofilm with large yields of EPS on crop roots (Earl et al., 2008; Marvasi et al., 2016; Wheatley and Poole, 2018). The formation of B. subtilis biofilm is an important survival strategy for B. subtilis and crops against abiotic and biotic stresses (Bais et al., 2004; Lastochkina et al., 2017). Similarly, P. putida is favorable to enhance the phosphorous uptake, growth, and yield of wheat under greenhouse and field conditions (Zabihi et al., 2011), and alleviate biotic stress of Parthenium hysterophorus in wheat (Mishra and Nautiyal, 2012). Because of these results and similar effects of B. subtilis EPS and P. putida EPS on dissolution and toxicity of Ag-NPs, the ubiquitous presence of rhizospheric biofilm EPS will reduce Ag-NPs dissolution and Ag⁺ uptake by crops, alleviate Ag-NPs phytotoxicity. Similarly to B. subtilis EPS, P. putida EPS is expected to enhance Ag₃S-NPs toxicity to crops. Findings of this study have highlighted the critical role of EPS in governing the transformation and toxicity of Ag-NPs and Ag₃S-NPs in rhizosphere, and shed helpful lights on the ecotoxicity and health safety of metal nanoparticles including Ag-NPs and Ag₃S-NPs via food chains in agricultural environments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.


