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Mechanism of toxicity and transformation of silver nanoparticles: Inclusive assessment in earthworm-microbe-soil-plant system

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ABSTRACT

Long term and inclusive toxicity studies encompassing soil, plants, and organisms are rare in literature for AgNPs. This study examines AgNP behavior in soil-plant system through 72 weeks long soil experiment, earthworm response, and plant metabolic analysis. AgNP exposed earthworms did not show reproductive failure; yet high oxidative stress and reduced protein synthesis led to significant weight loss. Such stress was highest with $AgNP_{50}$ exposure. Correspondingly, the 50 ppm exposure of AgNP was capable to reduce nutrient availability and microbial growth in soil. Contrary to previous reports, we demonstrated that dissolution rate of AgNP increased with time in soil. Dynamic Light Scattering and UV-VIS assessments exhibited concentration and time dependent agglomeration of AgNP in soil and aqueous media. Moreover, lab based experiments in aqueous medium revealed that significant reduction in silver availability was due to formation of Ag_2S or Ag_3PO_4; which also greatly affected the P and S availability. Although the vegetative growth of tomato was normal, AgNP (10 mg kg^{-1}) treatment markedly upset the fruit yield. The 10 mg kg^{-1} AgNP exposure significantly enhanced oxidative stress and Ag uptake in plants; consequently, retarded N-assimilating enzyme (glutamate synthase, glutamine synthetase, and nitrate reductase) activity by suppressing their genes in plants. Eventually, photosynthesis and CO_2 assimilating efficiency were severely disrupted. These assays were vital to appreciate the true toxicity and are not well attended in most of the studies with AgNPs.

1. Introduction

World's land and water resources are considerably exposed to silver nanomaterials, since silver (Ag) is the most widely used nanomaterial (Rejeski, 2009; Lee et al., 2012). An estimate has shown that the exposure levels of silver nanoparticles (AgNPs) were likely to be 1581 ng kg^{-1} h^{-1} for the contaminated lands of Europe (Gottschalk et al., 2009). Therefore, it is important to derive mechanistic interpretations through focused as well as holistic experimentations to ascertain the true impacts of AgNPs on soil environment.

The behavior and effects of nanoparticles (NPs) in soil-plant systems are rather unpredictable because of influence of numerous factors (inherent soil chemistry, soil porosity, water retention capacity, size of NPs, coating materials, time, and level of exposure) (Dinesh et al., 2012; Goswami et al., 2017). Studies have revealed concentration driven agglomeration property of engineered nanomaterials in soil greatly influences microbial diversity, nitrogen metabolism, photosynthesis, and plant growth (Li et al., 2017; Yang et al., 2017). However, AgNP toxicity to plant have been more severe in soil-less media than in soil (Lee et al., 2012; Musante and White, 2012; Dimkpa et al., 2013). Contrarily, AgNP exposure has been reported to promote root nodulation and shoot growth in plants (Lee et al., 2012; Pallavi et al., 2016). However, to which extent the AgNP exposure disturbs molecular functions in plants and how AgNP affects soil quality is a least attended question.

The unique features of AgNPs (aggregation/agglomeration, dissolution, dispersibility, charge, surface area, and surface chemistry) greatly modify the stability and migration of the nanomaterials within soil systems; which may also alter the physico-chemical character of the contaminated soils (Anjum et al., 2013). As such, the distinctive

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physico-chemical properties of AgNPs are likely to affect microbial diversity in soil (El Badawy et al., 2011; Lee et al., 2012; Levard et al., 2012; Pallavi et al., 2016). However, to the best of our knowledge, there is no report about the AgNP-nutrient (N, P, K, and S) interactions in plant based soil media.

Earthworms are vital indicators of soil health; thus can be dependable subjects for ecotoxicity assessment. Distinct harmful effects of AgNP on earthworm survival, growth, and fecundity have been reported earlier (Shoults-Wilson et al., 2011a,b; Heckmann et al., 2011). Conversely, AgNP exposure may not affect earthworm growth depending on the composition of the rearing substrates and level of exposure (Barua et al., 2013). Nevertheless, apparent growth and reproduction patterns hardly explain the internal metabolic adjustment in earthworm body to accommodate the stress.

Hence, in this research we addressed the following questions: a) Is bioavailability of AgNP/Ag^+ in soil governs phytotoxicity?; b) How earthworms manage the AgNP induced stress?; c) How AgNP interferes with soil biochemical environment?; and d) To what extent AgNP influences photosynthesis and nutrient assimilation in plants?. Various concentrations of plant leaf extract mediated AgNP (Barua et al., 2013, 2017) were incorporated in a typical alluvial soil and the long term changes in soil quality and microbial health were studied. Moreover, the biotic stress of AgNP exposure was assessed in earthworms (*Eisenia fetida*) and plant (*Lycopersicon esculentum*) models based on some critical metabolic functions (oxidative stress, N-assimilation, regulation in gene expression, and photosynthesis).

2. Experimental

2.1. The source and basic properties of AgNP

Polymer assisted Silver nanoparticles was prepared by following the previously reported protocol (Barua et al., 2013). At first, aqueous leaf extracts of *Thuja occidentalis* was prepared by stirring 0.2 g of ground leaves at 50 °C for 20 min in 50 ml of de-ionized water followed by filtration through muslin cloth. Simultaneously, a solution mixture of 0.01 M $AgNO_3$ and 5% (*w/v*) poly(ethylene glycol) (PEG) was prepared; in which 2 ml of *Thuja* leaf extract was added in drop by drop. The formation of AgNP could be identified by gradual change in colour from black to dark brown. Here, the *Thuja* leaf extract was used to reduce Ag^+ of $AgNO_3$ into Ag^0; while PEG was used to provide steric stabilization of the nanomaterial through electrostatic interaction (Barua et al., 2013). The whole process was carried out in neutral condition (pH 7). According to the analytical evidences silver was present in zero valent form (Ag^0). The size of the synthesized material was recorded between 7 and 14 nm with an average hydrodynamic diameter of 9.8 ± 0.15 nm (Barua et al., 2013). The Silver concentration in the liquid phase was determined as 2.16 mg ml^{-1} and confirmed through Atomic Absorption Spectrophotometry.

2.2. Earthworm fecundity, body weight, Ag accumulation, oxidative stress enzymes, and histological analysis

Non-clitellated, juvenile specimens of *Eisenia fetida*, weighing about 300–450 mg were used for the study and undergone gut evacuation. Then, the gut evacuated adult specimens were inoculated into a urine-free cow dung based substrates (2 kg) @ 10 worms kg^{-1}. The experiment was conducted for 120 days during spring season. About 40–50% moisture was maintained by sprinkling water at an interval of 2–3 days. Earthworm count and body weight were recorded at an interval of 10 days till 120th day.

The accumulation of Ag in earthworm intestines was measured. Earthworm specimens were collected at 30th days and 120th day. Then, the collected specimens were washed, and kept overnight in a moist filter paper for gut cleaning. The sacrificed earthworms were digested in di-acid mixture [$HClO_4$: HNO_3 (1:6)] and the Ag concentration was determined by Atomic Absorption Spectrophotometry (AAS) (Lab India AA 7000) (Berman, 1980).

Simultaneously, a group of untreated and AgNP (10 mg kg^{-1}) treated earthworms were gut cleaned, killed by freezing, and used for histological assay (Sharma and Satyanarayan, 2011). Initially, fixation of earthworm tissues were done in Bouin's fluid for a period of 24 h, then the tissues were dehydrated in graded alcohol solution from 30% to 100% and then for 10 min in xylene solutions and embedded in paraffin. Microtome cutter were used to maintain a fine section of 5 μm thickness and mounted in albumin coated slides. Hematoxylin-eosin staining technique was used for slide staining. Finally the prepared slides were observed in high resolution microscope.

In addition, activity of catalase (Aebi et al., 1974), reduced glutathione (GSH) (Ellman, 1959), glutathione peroxidase (GPx) (Clair and Chow, 1996), glutathione S transferase (GST) (Nimmo et al., 1979), and total protein content (Lowry et al., 1951) were determined in both treated and un-treated earthworms.

2.3. Soil spiking by AgNP

A typical alluvial (typic endoaquepts) soil was collected from nearby agricultural fields in Sonitpur, Assam, India (Lat.: 26.7008°N; Long.: 92.8303°E). Availability of N, P, and K in the soil was 158.9 ± 1.5 mg kg^{-1}, 32.6 ± 1.5 mg kg^{-1}, and 110.5 ± 0.5 mg kg^{-1} respectively; the soil had a pH of 5.5 ± 0.1 and microbial biomass carbon (MBC) of 43.8 ± 1.7 μg g^{-1}; urease and phosphatase activity in the soil was recorded as 13.38 ± 0.48 μg g^{-1} and 5.74 ± 0.2 μg g^{-1} respectively. Collected soil samples were then air dried, ground in an agate mortar, and screened through 2 mm mesh sieve. Subsequently, the whole soil batch was subdivided into 2 kg sub-samples to accommodate all different concentrations and their 5 replicates. Then, three various concentrations (10, 25, and 50 mg kg^{-1} of dry soil) of AgNP in dispersed form was spiked to the soil samples. The AgNP treated soil samples were thoroughly mixed for uniform distribution of the added materials and the incubation was carried out for 72 weeks within an ambient temperature range of 20–35° C. Moisture content was maintained at 45% water holding capacity (WHC) through sprinkling water at 2–3 days interval during the study period. The treatments combinations were as detailed below:

Treatments	Abbreviations
Control	C
AgNP 10 ppm [mg kg^{-1}(soil)/mg L^{-1}(aqueous media)]	$AgNP_{10}$
AgNP 25 ppm [mg kg^{-1}(soil)/mg L^{-1}(aqueous media)]	$AgNP_{25}$
AgNP 50 ppm [mg kg^{-1}(soil)/mg L^{-1}(aqueous media)]	$AgNP_{50}$

Soil samples were periodically drawn at: 0 day, 4, 8, 12, 24, 48, 60, and 72 weeks for analysis of various physico-chemical attributes [pH, easily mineralizable-N (minz. N), available-P (Avl P), available-K (Avl K), microbial biomass carbon (MBC), and microbial biomass N (MBN)] (Page et al., 1982). The activity of soil enzymes (urease and phosphatase) was assessed periodically (Tabatabai and Bremner, 1969, 1972). We also measured the total bacterial counts and bacterial biomass in the treated soil samples (Parmer and Schmidt, 1964; Kim et al., 2012). Moreover, the change in DTPA extractable Ag along with the fractional variations of different bound and labile forms of Ag were enumerated (Lindsay and Norvell, 1978; Tessier et al., 1979).

2.4. Behavior of AgNP in aqueous media

2.4.1. AgNP-P^H interaction: batch experiment no. 1

Solutions of different pH (4, 7, and 9) were prepared with diluted