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Effects of Nano Silver Oxide and Silver Ions on Growth of *Vigna radiata*

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Abstract Transformation of silver oxide nanoparticles (nano-Ag_2O) to silver nanoparticles (nano-Ag) and silver ions in environment is possible which might pose toxicity to plants and other species. The objective of this study was to study effects of nano-Ag_2O and silver ions on growth of Mung bean (*Vigna radiata*) seedlings. *V. radiata* seeds were exposed to nano-Ag_2O and silver ions (concentration range: 4.3×10^{-7}, 4.3×10^{-6}, 4.3×10^{-5}, 4.3×10^{-4}, and 4.3×10^{-3} mol/L) for 6 days. Root length, shoot length and dry weight of seedlings were found to decrease due to exposure of nano-Ag_2O and silver ions. These findings indicate silver ions to be more toxic to *V. radiata* seeds than nano-Ag_2O. Silver content in seedlings was found to increase with increasing concentrations of nano-Ag_2O and silver ions. Overall, findings of the present study add to the existing knowledge of phytotoxicity of silver-based nanoparticles of different chemical compositions to *V. radiata* seeds and need to be considered during use of nanoparticles-contaminated water for irrigation purposes.

Keywords Germination toxicity · Silver ions · Nano-Ag_2O · *Vigna radiata*

Nano-Ag_2O has been extensively used in various applications, such as data storage and photovoltaic cells, etc. (Ida et al. 2008). These nano-Ag_2O are amphoteric in nature and can give silver ions in acidic and neutral environments and AgO^- in basic environment. Studies have shown that nano-Ag_2O, nano-Ag and silver ions may inter-transform in the environment (Johnston et al. 1933; Lok et al. 2007; Kittler et al. 2010; Gallardo et al. 2012; Levard et al. 2012). As recent years have seen increased use of contaminated stream water as irrigation water, the use of nanoparticle-contaminated water is also a possibility. Thus, it becomes important to know toxic effects of different forms of silver on plants during irrigation activities. Effect of some forms of silver on plants have been studied in past (Table 1). For example, silver nanoparticles (nano-Ag), a priority nanoparticle (NP) as per the OECD designation, has been studied extensively for uptake and toxic effects on plants (Lee et al. 2012; El-Temsah and Joner 2012). Some studies have also compared toxic effects of nano-Ag and silver ions on plants for understanding toxicity mechanisms and found nano-Ag to be more toxic to seed growth than silver ions. Most of these investigations focused on studying effects of nano-Ag and silver ions on growth of seeds using different media, such as filter paper, agar media, soil media and hydroponic system. None of these studies focused on studying the effect of nano-Ag_2O on growth of seeds and plants, representing a knowledge gap. To fulfill this knowledge gap, our study aimed to understand effects of nano-Ag_2O and silver ions on growth of seeds as a first step. To illustrate the toxic effects and potential of silver uptake, *V. radiata* seedlings were selected as example seeds only. *V. radiata* were selected as this is one of the model specie routinely used in phytotoxicity tests under leguminosae family (OECD 1984; ASTM 2012; Lee et al. 2012). Findings of this study could add to the existing knowledge on phytotoxicity of different chemical forms of silver-based NPs to *V. radiata*.

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Table 1 Summary of studies focusing on toxic effects of silver-based nanoparticles (NPs) and silver ions on seeds

Reference/plants	Experimental conditions	EC_{50} in mg/L[a]		
		Root length	Shoot length	Dry weight
Silver ions				
Our study/*Vigna radiata*	(Petri dish and filter paper) 0, 0.1, 1, 10, 100, 1000 mg/L	109.98 ± 98[b] (using log-logistic curve)	122 ± 58 (using log-logistic curve)	144 ± 69.41 (using log-logistic curve)
Pokhrel and Dubey 2013/*Zea Maize*	(Petri dish and filter paper) 0, 0.1, 1, 10, 50, 100, 200, 500 mg/L	200	Not studied	Not studied
Lee et al. 2012/ *Phaseolus radiatus*	(Agar Media) 0, 5, 10, 20, and 40 mg/L	4.45	4.45	458.72
Nano-Ag_2O				
Our study/*Vigna radiate*	(Petri dish and filter paper) 0, 0.07329, 0.7329, 7.329, 73.29, 732.9 mg/L	307.466 ± 176.89 (using log-logistic curve)	208.8 ± 25.67 (using log-logistic curve)	167.91 ± 19.73 (using log-logistic curve)
Nano-Ag				
Lee et al. 2012/ *Phaseolus radiates*	(Agar Media) 0, 5, 10, 20, and 40 mg/L	>2000 mg/L	2000 mg/L	Not studied
Pokhrel and Dubey 2013/*Zea Maize*	(Filter paper) 0, 0.05, 0.1, 1, 3.7, 36.7, 73.4 mg/L	<1 mg/L	Not studied	Not studied
El-Temsah and Joner 2012/Flax seeds	(Petri dish with soil) 0, 20, 40, 60, 80 and 100 mg/L	Not studied	20 mg/L	Not studied

[a] Concentration giving 50 % effect response; the approximate values obtained either directly from data (this study) or from data reported in graphs of different published studies

[b] Average value ± one standard error

Materials and Methods

Nano-Ag_2O was purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India (CAS No. 20667-12-3). The particle size of NPs suspension were determined using dynamic light scattering (DLS)-based particle size analyzer (Nicomp 380 ZLS-Particle sizing system). The NPs were dispersed in double distilled water and ultrasonicated (100 W, 33 ± 3 kHz) for 60 min. Suspension pH was found to vary between 8 and 9. Average zeta potential of the NPs suspension was found to be −0.57 mV. For preparing silver ion solutions, silver nitrate (CAS No. 7761-88-8) (Merck, India) was dissolved in double distilled water. The seeds of *V. radiata* (Variety: SML-668) were purchased from National Seed Corporation (NSC), New Delhi, India. These seeds were kept in dry place at room temperature prior to use. *V. radiata* seeds were first sterilized in 3 % H_2O_2 for 30 min to ensure surface sterility and then rinsed thoroughly with double distilled water several times. Then the seeds were allowed to germinate in wet cotton in an incubator with a controlled temperature of 25 ± 5°C for 24 h in the dark (ASTM 2012). Subsequently, the seeds were checked for germination. Only healthy and uniform seedlings were selected for further study.

Previously, equal mass concentrations of nanoparticles and ions have been used in toxicity studies which might not give same molar concentrations of nanoparticles and ions. For example, (Lin and Xing 2008) used same mass concentration for both nano-ZnO and zinc ions (Concentration range: 10, 20, 50, 100, 200, and 1000 mg/L) which might not give same zinc ion concentrations from two different sources of zinc. To address this issue, we exposed seeds to same initial molar concentrations of nano-Ag_2O and Silver ions. Nano-Ag_2O and silver ions were dissolved in double distilled water to make suspensions of five different concentrations: 4.3×10^{-7}, 4.3×10^{-6}, 4.3×10^{-5}, 4.3×10^{-4}, and 4.3×10^{-3} mol/L (here the concentrations values for nano-Ag_2O is 0.1, 1, 10, 100 and 1000 mg/L and for silver ions is 0.073, 0.733, 7.33, 73.29, and 732.9 mg/L). The selected concentration range represents exposure concentration values on a log-scale as recommended by OECD for range finding tests (OECD 2003). This study selected a broad range of concentration to understand toxic effects due to exposure of low initial nanoparticles concentrations (<1 mg/L) and high initial nanoparticles concentrations (>1 mg/L). Some of these concentrations indicate reported nanoparticle concentrations in wastewater effluent and streams (values in ng/L to µg/L range) (Mueller and Nowack 2008). High concentrations of nanoparticles and ions were used as studies have observed detectable toxic effects with exposure of high concentration of nanoparticles (1000–4000 mg/L) (Rico et al. 2011). The nanoparticles suspension was ultrasonicated (100 W, 33 ± 3 kHz) for 1 h before use. Silver ion concentrations in five prepared nanoparticle suspensions (in terms of moles/L) were observed to be $0.0001 \pm 3.51 \times 10^{-5}$, $0.001 \pm 3.01 \times 10^{-4}$, $0.032 \pm 4.9 \times 10^{-3}$, $0.20 \pm 2.81 \times 10^{-2}$, and $2.33 \pm 3.74 \times 10^{-1}$ for 4.3×10^{-7}, 4.3×10^{-6}, 4.3×10^{-5}, 4.3×10^{-4}, and 4.3×10^{-3} mol/L nanoparticles concentrations, respectively.

For exposure of seeds to suspensions, 6 mL volumes of nano-Ag_2O and silver ions were added to the petri dishes. Control petri dish was treated with 6 mL of double distilled water alone (i.e., without nano-Ag_2O and silver ions). The petri dishes were placed in an incubator in the dark at 25 ± 5°C (ASTM 2012) for 6 days. All experiments were conducted in triplicates at three different occasions. After 6 days, all seeds were found to be germinated for the concentrations till 4.3×10^{-4} mol/L of nano-Ag_2O as well 4.3×10^{-4} mol/L of silver ions. At the maximum exposure concentration of 4.3×10^{-3} mol/L for nano-Ag_2O and silver ions, only 60–70 % of the seeds were germinated. All the germinated seedlings were washed with double distilled water. The effects on root length, shoot length and dry weight were used to understand toxic effects of nano-Ag_2O and silver ions on seedlings. These parameters have also been used in previous toxic studies (Lin and Xing 2007, 2008; Lee et al. 2012; Pokhrel and Dubey 2013);ASTM, 2012). Root length, shoot length and dry weight of washed seedlings were measured to calculate percentage reduction with regards to control. Root length and shoot length were measured using ruler (value ±0.05 cm). Dry weight was measured after oven drying of washed seeds at 70°C for 24 h (ASTM 2012; Cui et al. 2014). Silver ion uptake in exposed seedlings was calculated and expressed as moles of silver/g dry weight of shoot of *V. radiata* seeds. For determining silver content in exposed seedlings, acid digestion was performed (Allen et al. 1986) and then silver ion analysis was conducted using atomic absorption spectrometer (model 4141, ECI). In addition, size of suspended nano-Ag_2O particles in double distilled water (expressed as Z-average diameter) was measured at 0th and 6th day of the study. The nano-Ag_2O was found to settle in vessel within 15 min. Z-average diameter value of particles in suspension was found to decrease in 6 days (size: 681.74 ± 15.65 nm at 0th day vs. 218.34 ± 2.21 nm at 6th day) (p value <0.05).

All the experimental data were statistically analyzed and tested for a 0.05 level significance test ($\alpha = 0.05$). Normality of the data was tested using Shapiro–Wilk normality test in the R software (R version 3.1.2 (2014-10-31)). F-tests was conducted for testing homogeneity of samples, showed that data were normally-distributed and samples had equal variance. Further, the difference of effects of concentrations of nano-Ag_2O and silver ions were analyzed using an Analysis of Variance (ANOVA) method and Tukey's *Honest Significant Difference* (HSD) test using the Data Analysis Tool Pack of Microsoft Excel (MS Excel 2013). A comparison of toxicity and uptake of nano-Ag_2O with that of silver ions was done by comparing their effective concentrations for giving 50 % toxic effect (i.e., EC_{50}) for different endpoints. Towards this, log-logistic dose–response model (Seefeldt et al. 1995) was first fit to data on toxic effects of nano-Ag_2O and silver ions using a "DRC" package of the "R" software. The obtained EC_{50} values of nano-Ag_2O and silver ions from model fitting were compared using "comped" function of the "DRC" package.

Results and Discussion

Figure 1 shows the effects of concentrations of nano-Ag_2O and silver ions on root length, shoot length and dry weight of *V. radiata* seedlings. The lengths of root and shoot decreased with increasing concentrations of nano-Ag_2O as well as silver ions (Fig. 1a, b). Brown tips and necrosis were detected in the roots of *V. radiata* seedlings indicating toxic effects of nano-Ag_2O and silver ions. Same effects were also seen in previous studies (Lee et al. 2008; Ma et al. 2010). The minimum concentration giving significant reduction in the root length of *V. radiata* seedlings was found to be 4.3×10^{-4} mol/L for both nano-Ag_2O as well silver ions (reduction value: 34 % ± 6 % for nano-Ag_2O and 41 % ± 4 % for silver ions as compared to control; p value < 0.05). Maximum reduction was noticed at highest exposed concentration of 4.3×10^{-3} mol/L for nano-Ag_2O and silver ions (reduction value: 83.79 % ± 3 % for nano-Ag_2O and 85.09 % ± 4 % for silver ions as compared to control; p value <0.05). At 4.3×10^{-3} mol/L, *V. radiata* seeds showed almost no growth with underdeveloped roots and shoots. The trends of toxic effects of nano-Ag_2O and silver ions on shoot growth were found to be similar to that of root growth. The minimum concentration giving significant reduction in shoot length of *V. radiata* seedlings was found to be 4.3×10^{-3} mol/L for both nano-Ag_2O as well as silver ions (reduction value: 84.17 % ± 3 % and 87.27 % ± 2 % for nano-Ag_2O and silver ions, respectively; p value <0.05). Both nano-Ag_2O and silver ions had noticeable inhibitory effects on dry weight with increasing concentration. The minimum concentration giving significant reduction in dry weight of *V. radiata* seedlings (Fig. 1c) was found to be 4.3×10^{-5} mol/L (reduction value: 15.96 % ± 3 % for nano-Ag_2O and 26 % ± 6 % for silver ions as compared to control; p value < 0.05). At 4.3×10^{-3} mol/L,

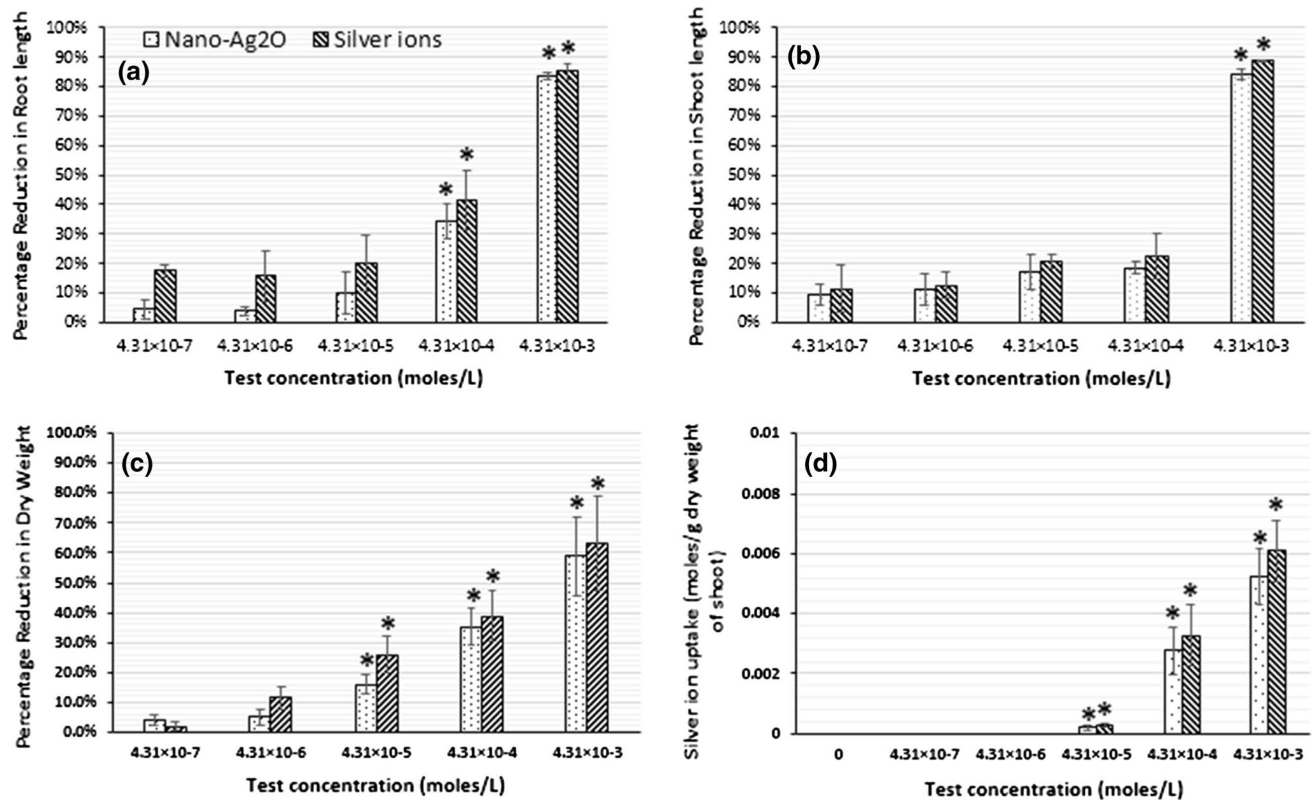

Fig. 1 Effects of nano-Ag_2O nanoparticles (NPs) and silver ions on *V. radiata* seedlings: **a** root length, **b** shoot length **c** dry weight and **d** silver ion uptake (moles/g dry weight of shoots (n = 3) (1 mol Silver ions = 231.75 g/L of Ag_2O NPs and 169.87 g/L of Silver ions). Significant difference as compared to control was marked with "*asterisk*" ($p < 0.05$) (*RL* root length, *SL* shoot length, *NPs* Nanoparticles)

maximum reduction in dry weight was found (reduction value: 63.18 % ± 16 % and 59 % ± 13 % for nano-Ag_2O and silver ions respectively; p value < 0.05). The dose–response model fitting of data on toxic effects of nano-Ag_2O and silver ions on growth of *V. radiata* showed that log-logistic model described observed data well. The obtained EC_{50} values of nano-Ag_2O and silver ions for toxic effects on growths of root and shoot and biomass growth are presented in Table 1. A comparison of the EC_{50} values of nano-Ag_2O and silver ions indicated that nano-Ag_2O are lesser toxic to growth of *V. radiata* than silver ions.

Previously, although studies have focused on effects of exposure of nano-Ag and silver ions on germination of seeds, none of them has studied effects of nano-Ag_2O on *V. radiata*. However, studies have compared toxicities of nano-Ag and silver ions to seeds. The findings of present study was compared with that of studies using nano-Ag and silver ions. The literature review indicates that some studies have reported that nano-Ag is more toxic than silver ions. For example, (Pokhrel and Dubey 2013) studied the effects of silver ions and nano-Ag on the seeds of *Brassica oleracea* in petri dishes and filter papers and observed nano-Ag to be more toxic than silver ions (50 % reduction in root length of *Brassica oleracea* happened at 1 mg/L nano-Ag and at 150 mg/L silver ions). On the contrary, some studies have reported silver ions to be more toxic to seeds than nano-Ag. For example, the toxicity of silver was found to be 18 times higher than nano-Ag (in terms of EC_{50}) (Navarro et al. 2008).The present study reports silver ions to be more toxic to *V. radiata* than nano-Ag_2O. The difference in observations of toxicities of nano-Ag, nano-Ag_2O and silver ions could be attributed to difference in structure, size and shape of nanoparticles and ions and their interaction with seeds (Rico et al. 2011). However, this aspect needs to be studied in detail.

Figure 1d shows trends of silver uptake in *V. radiata* seedlings with concentrations of nano-Ag_2O and silver ions. The minimum concentration giving significantly different uptake of silver in shoot was found at 4.3×10^{-5} mol/L concentration level for both nano-Ag_2O and silver ions (p value < 0.05). At the highest exposure concentration, silver uptake in *V. radiata* seedlings was found to be 0.0053 ± 0.0009 mg/g dry weight of shoot for nano-Ag_2O and 0.0060 ± 0.001 mg/g dry weight of shoot for silver ion, respectively. Higher uptake of silver ions was observed during exposure of seeds to silver ions than exposure of seeds to nano-Ag_2O (p value <0.05). Observed silver uptake of *V. radiata* seedlings during exposure of seeds to nano-Ag_2O was compared with that of published studies on exposure of seeds to nano-Ag as no study is currently available on uptake of nano-Ag_2O to *V. radiata* seedlings. Similar to our observations of increased silver uptake with increasing nano-Ag_2O concentrations, (Lee et al. 2012) also noticed increased accumulation of nano-Ag in *Phaseolus radiatus* and *Sorghum bicolor* grown in agar medium (Lee et al. 2008) also reported higher uptake of silver ions mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*) during exposure of nano-Ag than that during exposure of silver ions. The observed difference in uptake of silver ions by *V. radiata* seeds during exposure of seeds to nano-Ag_2O and silver ions could be due to differences in availability of silver ions for uptake, stability of nanoparticles in water, and interaction of nanoparticles with seeds. Some studies have reported effects of these factors on uptakes of ions and nano-Ag by seeds (Rico et al. 2011). However, effects of these factors on uptake of nano-Ag_2O by *V. radiata* seedlings need to be studied in detail.

Findings of this study showed that nano-Ag_2O is toxic to *V. radiata* seedlings. Nano-Ag_2O was not found to be toxic to *V. radiata* at low concentration but to be toxic at high concentration. The findings of toxicities at low and high concentrations of nano-Ag_2O on *V. radiata* provided information on its possible impacts on *V. radiata* during use of wastewater- contaminated with different concentrations of nano-Ag_2O. As low concentrations of nanoparticles are generally expected in natural water, irrigation of seeds with natural water containing nano-Ag_2O might not result in significant toxic effects on growth of seeds. However, irrigation of seeds with raw wastewater and/or industrial wastewater containing high concentrations of nano-Ag_2O might result in toxic effects on growth of seeds.

Overall, the findings of this study add information to the existing knowledge of toxicity of silver-based NPs of different chemical forms and silver ions to seeds. However, the findings are limited to exposure of equal molar concentrations of nano-Ag_2O and silver ions to *V. radiata* seeds during germination stage only. Detailed studies using same initial silver ion concentrations are also required to understand role of particulate and ionic forms on toxicity and uptake. As *V. radiata* seeds represent seeds of leguminosae family in the OECD list (OECD 1984), observed findings indicate that probably nano-Ag_2O might be toxic to other seeds of the leguminosae family as well. However, this aspect need to be confirmed experimentally as toxicity varies with seed type, NP type, and exposure conditions. For example, (Ma et al. 2010) reported that root growth depends on plant species and nanoparticles types and (Wu et al. 2012) showed that toxicity to seeds depend on surface area-to-volume ratio of seeds. Further research is needed to study the uptake during full growth of the plant. This is an important data gap for conducting human health risk assessment due to consumption of nanoparticles-contaminated food (Singh and Kumar 2014).

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