Ecotoxicology and Environmental Safety 88 (2013) 48–54

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Effect of silver nanoparticles on *Oryza sativa* L. and its rhizosphere bacteria

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ARTICLE INFO

*Article history:*
Received 26 July 2012
Received in revised form
22 October 2012
Accepted 24 October 2012
Available online 20 November 2012

*Keywords:*
Silver nanoparticle
*Bacillus*
*Oryza sativa* L.
Environmental toxicity

ABSTRACT

Silver nanoparticles (AgNPs) are widely used as antibacterial and antifungal agents in agriculture. Nevertheless, these nanoparticles with newborn properties pose a potential risk to the environment, Due to contact with crops and bacteria that are beneficial to the soil. This study is based on the examination of the phytotoxic effects of AgNPs on *Oryza sativa* L. and some of its rhizosphere bacteria, by physiological and biochemical assays. In order to study the complex interaction of the AgNPs life expectancy that are mixed with culture medium, the incubation time for the fresh mixture, 7, 14 and 21 days old of AgNPs, on the seedlings growth was investigated. Results indicated that plant's response to the treatment with AgNPs affected on the cell wall, and that with an increase in its concentration (up to 60 μg/mL). The obtained results of transmission electron microscopy (TEM) exhibited that those particles not only penetrated the cell wall, but they could also damage the cell morphology and its structural features. AgNPs treatment up to 30 μg/mL accelerated root growth and at 60 μg/mL was able to restrict a root's ability to grow. The 30 μg/mL treatment had significant effect on root branching and dry weight. In contrast, shoot growth was more susceptible to the effects of AgNPs treatment. The root content for total soluble carbohydrates and starch demonstrated that despite stable starch content, total soluble carbohydrates showed the tendency to significantly decline in response to AgNPs. However, induction of root branching and photosynthetic pigments can attributed to AgNPs stress based on evidence from the production of the reactive oxygen species (ROS) and local root tissue death. Nine isolates of the genus *Bacillus* selected and identified according to morphological and chemotaxonomic methods. The AgNPs treatment revolutionized the populations of bacteria as *Bacillus thuringiensis* SBURR1 was totally eliminated, and *Bacillus amyloliquefaciens* SBURR5 became the most populated one. Images from an electron microscope and the leakage of reducing sugars and protein through the bacterial membrane, similarly confirmed the "pit" formation mechanism of the AgNPs. Moreover the hypothesis from the growth curve study demonstrated that AgNPs may damage bacterium cell wall and transform them to protoplasts.



## 1. Introduction

Recent achievements in nanotechnology have increased their applications in several fields (Asharani et al., 2008). Silver nanoparticles (AgNPs), (Rai et al., 2009), or their composite forms, (Yasa et al., 2012) are potent antibacterial agents. Researchers have claimed that using an AgNPs colloidal solution is a safe and efficient method to preserve and treat agents of disease in agriculture (Kim et al., 2008; Rahman Nia, 2009). The toxicity of the Ag ionic form is well understood. However, comprehension of the toxicological impact of AgNPs is more complicated that it is estimated due to uncertainties in the amounts of aggregation or exposure (Schreurs and Rosenberg, 1982; Mirzajani et al., 2011). Mueller and Nowack investigated the life-cycle model of engineered AgNPs released into the environment. The study reported that, may is the process applied that governs the toxicity of AgNPs in circumstances that are the result of a combination of influences such as the nature, concentration and the exposure path of nanoparticles as well as the environment, biological and the functional ecology of the involved organism (Mueller and Nowack, 2008). Furthermore, it has been proclaimed that environmental water content plays an important role in the stability of AgNPs, as well as the rate of aggregation or ion release. Proper hydraulic conductivity is essential for the mobility and encounter of AgNPs to plant roots and rhizobacteria (Bystrzejewska-Piotrowska et al., 2009; Fabrega et al., 2011). An appropriate risk assessment of the effect of AgNPs requires documentation, which is not currently available. Still it is enough to justify attempts to

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http://dx.doi.org/10.1016/j.ecoenv.2012.10.018

evaluate the risks. The primary step to understand the impact of applying AgNPs to the environment, is to study their penetration and transportation.

For bacteria, growth inhibition can be attributed to an uptake of AgNPs followed by the disruption of ATP production and DNA replication that causes direct damage to cell membranes (Mirzajani et al., 2011; He et al., 2011). Generation of reactive oxygen species (ROS) also contributes to growth inhibition from contact with AgNPs contact (He et al., 2011). Generation of ROSs by AgNPs provides compelling evidence that these species are responsible for the strong activity of AgNPs (He et al., 2011). Moreover, recent reports have demonstrated that AgNPs make a connection with both peptide and glycan ports of a cell wall and generate a pit on it (Mirzajani et al., 2011; Li et al., 2010). Similar to bacteria, plant systems also respond to AgNPs. Lin and Xing reported that root epidermis and the root cap of *Lolium perenne* (ryegrass) were broken in the presence of metallic nanoparticles (Lin and Xing, 2008). Besides this, research on nanoparticle penetration and transportation in *Cucurbita pepo* (pumpkin) concluded that carbon coated magnetic nanoparticles are capable of penetrating into living plants tissue and migrating to diverse regions of a plant (Corredor et al., 2009). Therefore, the main question in using AgNPs as a disease preventing fertilizer is as follows: "What is beyond the antimicrobial effects of AgNPs on the plant and the soil flora?" and " How are the soil microflora and their population may affected by AgNPs application?"

Research has reported on soil ecosystems subjected to short- or long-term exposure of toxic compounds (Wang et al., 2007). However, there are numerous bacteria in soil communities with an outstanding ability to adapt to a polluted environment via various mechanisms; and these mechanisms, specific to strains of bacteria, are unavailable to other organisms (Fabrega et al., 2011; He et al., 2011; Filip, 2002). Consequently, polluted soil becomes inhabited with new kinds of microflora settel in polluted soil; some members alter significantly, while others disappear from an environment. In comparison with the bacteria, there are limited toxicology studies on the effects and mechanisms of nanoparticles on higher plants. Several negative effects on growth and development of plants have been demonstrated, suggesting that microorganisms suffer adverse reactions from nanoparticles (Ruffini Castiglione and Cremonini, 2009).

Reports have shown that growing plants in an aqueous medium containing AgNPs can absorb, move and even accumulate these particles in their tissue faster. A suspension of AgNPs was used in rice (*Oryza sativa* L.) paddy field as an antibacterial and antifungal fertilizer in Iran. Rice is one of the most important food crops in the world and the aim of this study was to investigate the effects of AgNPs on the rice crop's root, and the *bacillus* genus of its micro flora.

## 2. Materials and methods

### 2.1. Chemicals

Nutrient agar (NA) and Mueller-hinton broth (MHB) media were purchased from Merck Co. (Darmstadt, Germany). The homemade silver nanoparticle colloidal suspension (AgNPs) was prepared and characterized according to the previously reported method (Mirzajani et al., 2011). Briefly, colloidal AgNPs with spherical morphology and distinct diffraction peaks of crystalline plans of cubic including (1 1 1), (2 0 0), (2 2 0) and (3 1 1), have a maximum absorbance ($\lambda_{max}$) at 426 nm. Their size was 18.34 nm (X99) with the homogeneity of their size within the range of 0.1–1000 nm. The Chu (N6) salt mixture (20 ×) medium was obtained from Nihon Pharmaceutical Co., Ltd. (Kyoto, Japan). Bovine serum albumin (BSA), Bradford solution (5 ×), sulphuric acid, and resazurin were purchased from Sigma-Aldrich Co. (Steinheim, Germany). In all procedures, Milli-Q HPLC grade water was used. Dimethylsulphoxide (DMSO), phenol, ethanol, acetone, sodium chloride, hydrochloric acid and sodium hydroxide were purchased from Merck Co. (Darmstadt, Germany).

### 2.2. Plant material and growth conditions

Seeds of *O.* sativa L. cv. IR651 were gifted from the Rice and Citrus Research Institute (RCRI), Agricultural Science and Natural Resources University, Sari, Iran. The seedlings were prepared on petri dishes (ID: 7 cm), which were filled with 10 mL of sterile water, at 27 °C and darkness for 7 days. Plant seedlings (six per bottle) were transferred to glass bottle (10 cm diameter and 20 cm depth) each containing 110 mL of N6 (+0.4 percent agar) growth cultivation media. They were grown in a phytotrons maintained at a thermo period of 27 ± 0.5 °C, photoperiod of 16 h, relative humidity 60 percent, and a photon flux density of 220 μM/m$^2$/S. Plants were irrigated by AgNPs colloidal solution in the concentration ranges 0.30–60 μg/mL three replications. After 7 days of treatment, plants were washed with water and were kept at −80 °C before analysis. In order to investigate the complex interaction of the AgNPs life expectancy of its mixture with culture media, the incubation time for the fresh mixture of AgNPs, 7, 14 and 21 days old, on the seedlings growth were studied.

### 2.3. Physiological analysis and assay for the growth quality of O. sativa L

Roots and shoots fresh weights (immediately after harvesting the plant) and dried masses (72 h in an oven at 60 °C) were measured. The influence of AgNPs treatment on the plant growth quality was evaluated using the dry weight (DW). Therefore, the total soluble sugar and starch content, were determined according to the previously reported method, (Hansen and Moller, 1975). Briefly, 100 mg of dried root powder was extracted using ethanol 80 percent for 60 min. The supernatant and the residue were dried and monitored for the total soluble sugar and starch content, respectively. Five milliliter of HCl 1.1 percent was added to each and the mixtures were heated in a water bath (97 ± 2 °C) for 30 min. Thereafter, 1 mL of heated samples was mixed with 5 mL of ice-cold anthrone reagent (72 percent sulphuric acid containing 0.2 percent anthrone) and heated again for 11 min. The solutions absorbance was measured at 630 nm. Moreover, the total photosynthetic pigments content of the shoots were determined according to Lichtenthaler, 1987 method. In brief, 20 mg of oven dried (at 60 °C for 24 h) shoot samples were grounded and extracted using dimethylsulfoxide (DMSO) at 70 °C for 60 min. The extract's absorbances were measured at 470, 646 and 663 nm. The chlorophyll a (Chl a) and b (Chl b) contents, the ratio among them and the carotenoids content were determined following the equations proposed by Lichtenthaler (1987).

### 2.4. Microorganism, isolation and identification

Rice (*O. sativa* L.) rhizosphere (the soil near the surface of the root) bacteria were collected from a paddy farm at Sari, Mazandaran Province, Iran in April, 2010. The soil samples were dried at 30 °C and were passed through a sieve (1.2 mm mesh) to remove large pieces of debris and vegetation. The bacteria were originally isolated by plating the 100 μL suspension of soil dilutions (in the range of $10^{-1}$–$10^{-5}$) in sterile saline solution (0.85 percent NaCl) on nutrient agar and were then incubated at 36 ± 0.5 °C for 24 h. The developed colonies were counted in plates and the average number of colonies per three plates was determined. Individual colonies of bacteria which varied in shape and color were picked up and purified by streaking on nutrient agar. The purified strains were identified by microscopic examination, Gram staining, biochemical assay (Schaad et al., 2001) and 16S rRNA gene sequencing. The identified bacteria were preserved on nutrient agar at 4 °C and recultured every month. For long term storage, all strains were kept at −80 °C using a Cryobank system (Mast Diagnostica GmbH, Germany).

To identify *Bacillus* isolates, 16S rRNA Gene Sequence analysis was performed according to the previously reported method (Lee et al., 2010). Concisely, the gene, encoding 16S rRNA, was amplified by PCR using universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-TACCTTGTTACGACTT-3′), subsequently the amplified nucleotide product was sequenced and similar sequences were identified using online BLAST in NCBI nucleotide database [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. A multiple alignment and a phylogenetic tree were obtained using the CLUSTAL X 2.0 (Larkin et al., 2007) and MEGA 4 (Kumar et al., 2008) software packages, respectively.

Additionally, for better identification of the rice rhizosphere, some physiological properties of the *Bacillus* (SBURR) isolates from their nearest phylogenetic neighbors, were studied (Larkin et al., 2007; Shivaji et al., 2009; Baranyi and Roberts, 1994; Yoon et al., 2003). In addition, for the detection of *Bacillus thuringiensis* from *Bacillus cereus* isolates, the presence of genes (*cry* genes) coding for the insecticidal toxins was studied. Briefly, in order to detect the subgroups of *cry* genes, universal primers as follow: *cry*1 (forward 5_-CAT GAT TCA TGC GGC AGA TAA AC-3_; reverse 5_-TTG TGA CAC TTC TGC TTC CCATT-3_), *cry*2 (forward 5_-GTT ATT CTT AAT GCA GAT GAA TGG G-3_; reverse 5_-CGGATAAAATAATCTGG-GAAATAGT-3_), *cry*3 (forward 5_-CGT TAT CGC AGA GAG ATG ACA TTA AC-3_; reverse 5_-CAT CTGTTG TTT CTG GAG GCA AT-3_) and cry 4 (forward 5_-GCA TAT GATGTA GCG AAA CAA GCC-3_; reverse 5_-GCG TGA CAT ACC CAT TTCCAG GTC C-3_) were used according to the protocol laid out by Ben-Dov et al. (1997).