Advanced Studies in Biology, Vol. 9, 2017, no. 2, 83 - 90
HIKARI Ltd, www.m-hikari.com
https://doi.org/10.12988/asb.2017.712

Impact of ZnO and Silver Nanoparticles on Legume- *Sinorhizobium* Symbiosis

Mahboobeh Nakhaei Moghaddam*, Azadeh Haddad Sabzevar and Zahra Mortazaei

Department of Biology, Mashhad Branch
Islamic Azad University
Rahnamaei 24, P.O. Box: 91735-413, Mashhad, Iran
*Corresponding author

Abstract

Rhizobia as useful soil bacteria can coexist with legume and fix nitrogen. Nanoparticles (NPs) are now widely used in various branches of science and silver (Ag) and zinc oxide (ZnO) NPs are the most widely used in this area. These particles finally enter in aquatic and terrestrial environments and probably have harmful effects on living organisms and environments. The aim of this study was to evaluate the effect of silver and ZnO NPs on *Sinorhizobium meliloti* and alfalfa symbiosis. *S. meliloti* bacteria were isolated from the alfalfa root nodules. Minimum inhibitory concentrations (MIC) of NPs were determined by agar dilution method. The effects of NPs on nodulation were studied by plant infection test in Jensen-nitrogen free agar medium. The gene expression of nitrogen fixation (*nif* gene) was evaluated in the presence of NPs by Real Time PCR. It was found that in the presence of NPs, the total length of treated plants and the number of nodules were decreased by increasing the concentration of NPs (1.25 to 10 μg/ml of AgNPs and 12.5 to 100 mg/ml of ZnO NPs) compared to the control plants ($p \leq 0.05$). *nif* gene expression was decreased in the presence of sub MIC concentration of NPs.

Keywords: *Sinorhizobium meliloti*, Alfalfa, Zinc oxide, Nanosilver, Symbiosis

Introduction

Although N_2 is the most abundant gas in Earth's atmosphere, cannot be used by plants. So, they need to the presence fixed nitrogen for their growth (Jensen *et al*., 2012). Biological fixation of molecular nitrogen from the atmosphere is one of the main sources of nitrogen increment in agricultural soils. Rhizobia are a group of Gram negative bacteria, normally found in soil, which induce nitrogen-fixing nodules on leguminous plants (Bradic *et al*., 2003, Haag *et al*. 2013). As a legume, alfalfa forms a symbiotic relation with nitrogen fixing bacteria belonging to *Sinorhizobium* (Bradic *et al*., 2003). Rhizobia and other soil microorganisms play a very important role in maintaining soil health, ecosystem functions and crop productivity (Mishra and Kumar 2009) and their use as biological fertilizers is more efficient and desirable than chemical fertilizers (Elboutahiri *et al*., 2010).
Nanoparticles (NPs), defined as particles that have a dimension of at least 100 nm or less, are becoming more and more prevalent in today's environment (Deng *et al*., 2009). Although many benefits of NPs has been clear for scientists, but their potential risks associated with their implementation has been less discussed (Colvin 2003). Some researchers have been reported some hazards of certain nanomaterials on the environment and organisms. ZnO NPs induce neural stem cell apoptosis in mouse (Deng *et al*., 2009) and silver nanoparticles (AgNPs) have been shown to be toxic to many different bacteria as tested in the laboratory (Dorobantu *et al*., 2015). NPs may also prove to be toxic to microorganisms in ground water and the soil, which would ultimately affect the entire food chain (Colvin 2003).
To evaluate hazardous and toxic environmental effects of NPs, the present study was conducted to investigate the effect of silver and ZnO NPs on *S. meliloti* nodulation in alfalfa roots.

Material and methods

Bacteria

Alfalfa plants were collected from agricultural field sites located in the most important alfalfa production area in Khorasan- Razavi province, in Iran. Rhizobia were isolated from homogenized nodules by culturing in yeast extract mannitol Agar (YEM) (Singh *et al*. 2008). Pure bacteria were re-streaked on YEM agar containing 0.0025% Congo red and were analyzed for colony morphology, Gram stain, motility and biochemical tests including catalase, oxidase and carbon source utilization. For confirmative identification of isolates, 50 ng of extracted DNA by Chen and Kuo method (Chen and Kuo 1993) was used to amplify a 1500 bp fragment of *16S rDNA* gene of *S. meliloti* with a pair of primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Shamseldin et al., 2008). Polymerase chain reaction (PCR) was performed in a 30 µl mixture of 0.33 µM of each primer, 1.25 mM of $MgCl_2$, 0.33 mM of dNTPs, and 0.2 µl of 5u/µl Taq DNA polymerase in a thermal cycler (Kyratec, Singapore). Temperature program was conducted as denaturation at 94°C for 45 sec,

annealing at 55°C for 45 sec and extension at 72°C for 90 sec. *Sinorhizobium meliloti* PTCC 1684 was used as positive control.

Plant infection test

Isolates were tested for symbiotic relationship on Jensen-nitrogen free agar slants (Truchet et al. 1985) using sterile test tubes containing N-free medium added with 0.8% agar. Alfalfa seeds variety Hamedani were surface sterilized before planting and 5 seeds were sown in each test tube. Each tube was inoculated with 1 ml of broth culture (containing 10^8 cells/ml) at developing root hair time. Plants were watered with nitrogen free nutrient solution (Tham and Tham 2007). A set of test tubes fertilized with seeds infected by standard strain (*Sinorhizobium meliloti* Persian Type Culture Collection 1684) and seeds without infection were included as controls. Isolates that were able to nodulate at least three plants, were selected for further steps.

Nanoparticles and antibacterial assay

NPs were purchased from US Research Nanomaterials, Inc. (USA) with purity over 99%. The average size of ZnO NPs and AgNPs were ~20 nm. The diameter of NPs was confirmed by using transmission electron microscope.

The minimum inhibitory concentration (MIC) of NPs was determined by agar dilution method in 24-well micro titer plates. Different concentrations of NPs were prepared in wells by using molten YEM agar at a double concentration, NPs stock solution and distilled water. 0.01 ml of every bacterial suspension, equivalent to McFarland tube No. 0.5 (10^8 CFU/ml) was inoculated on the agar of every well and plates were incubated at 29^{o}C for 48 hour. Minimum inhibitory concentration was defined as the lowest concentration of NPs that inhibited the growth of bacteria.

Effect of NPs on *Sinorhizobium*- legum symbiosis

Experiments were carried out by using plant infection test as described earlier, but in the presence of different concentration of nanoparticles; 0, 1.25, 2.5, 5, 10 µg/ml for AgNPS and 0, 12.5, 25, 50, 100 µg/ml for ZnO NPs.

Real Time PCR

Gene expression of *nif* (nitrate fixing) gene, a gene contributing in biological nitrogen fixation was evaluated in the presence of sub MIC concentration of NPs (5 µg/ml of AgNPs and 25 µg/ml of ZnO NPs). For this purpose, RNA was extracted from nodules (after freezing in liquid nitrogen) of treated and control alfalfa plants co-existed with one of the isolates by RNX-Plus Solution (Sina Gen Co., Iran). 8 µl of extracted RNA was analyzed by electrophoresis in 1% (w/v) gel agarose. Purification of extracted RNA was carried out by DNase (Fermentas Co. Canada) according to the company instruction. The Vivantis kit (Malaysia) was used for cDNA synthesis. To ensure the cDNA synthesis, PCR was conducted by using designed (Allele ID version 6) specific primers of *nif*A1 (5′-CCT TGC AAG AGC ATT CCT TC-3′) and *nif*A2 (5′-TCT TTG ACC TGG CGA GAG TT-

3′) which amplified a 160 bp fragment. Reaction mixtures contained 0.4 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.25 µl Taq DNA polymerase (5 U/µl) (Fermentas-Lituania) in a final volume of 25 µl. PCR was carried out in a thermal cycler (Kyratec- Korea) in 94°C for 5 minutes, and 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds. PCR product was analyzed by electrophoresis in 1% (w/v) gel agarose along with a 100 bp ladder standard (Fermentas, Lithuania), negative and positive (*Sinorhizobium meliloti* PTTC 1684) controls. 2 µl of diluted (3:1) cDNA, 10 µl of SYBR green PCR basic solution (Bivard Co., USA), 7 µl of distilled water and finally 25 µM of each primer were entered into the reaction in wells of 96 micro titer plate. The specific *16S rDNA* gene of *Sinorhizobium* was selected as housekeeping gene. The primers (5′F-AGGCGGCTCACTGGTCTG-3′ and 5′R-AGGCGGAATGTTTAATGCGTTAC-3') were designed based on *16S rDNA* Gene Bank sequence using Allele ID software.

Statistical Analysis

Data analysis for infection test (including total length plant and number of nodules) was performed using analysis of variance (ANOVA) program of SPSS version 16 statistical software. Duncan test was used to evaluate the significance of differences and a p value ≤ 0.05 was considered significant.

Results

In this study, 10 isolates of *Rhizobium* were collected from nodules of alfalfa roots. Colonies of bacteria were mucoid, convex with smooth edges and white to cream. All bacteria were catalase and oxidase positive, motile, and able to utilize mannitol, maltose and sucrose. Bacteria had the ability to grow in the presence of Congo red and absorb the reagent. 6 of 10 isolates were able to nodulate at least three plants in infection test and were selected for further steps. A fragment of 16S rDNA gene with a size of approximately 1500 bp (base pair) was detected in all isolates by PCR. Figure 1 shows the gel electrophoresis of PCR products on 1.5% gel agarose.

Electron-microscopy and particle size techniques showed that NPs had an average size of about 20 nm. The MIC of AgNPs (1.3±0.5 mg/ml) was less than the MIC of ZnO NPs (391.7±131.9 µg/ml) against *S. meliloti* isolates. So the effect of AgNPs against tested bacteria was more than ZnO NPs. NPs had a bacteriostatic effect against *S. meliloti* isolates.

In experiments of symbiotic plants with bacteria, it was found that by increasing the concentration of AgNPs and ZnO NPs, the total length of treated plants was decreased compared to the control plants (plants in the absence of NPs) ($p \leq 0.05$). Figures 2 and 3 display the effect of silver and ZnO NPs on the total length of the symbiotic plants with *S. meliloti*. Also by increasing the concentration of NPs, the number of nodules was decreased. Nodules in symbiotic plants, in the presence of 5 and 10 µg/ml of AgNPs and 50 and 100 µg/ml ZnO NPs were significantly de-

creased compared to the control plants ($p \leq 0.05$). If the nodules were formed, their size was smaller than the control plants. Presence of *nif* gene was detected in all isolates by using PCR and specific primers. Figure 4 shows a DNA band with the size of about 160 bp. RNA extracting from the nodules and Real Time PCR showed that the expression of *nif* gene in the presence of sub MIC concentration of NPs significantly decreased compared to the control plants.

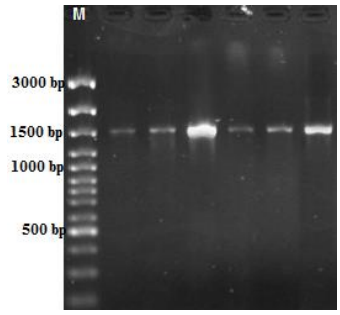

Figure 1. Gel electrophoresis of 16S rDNA gene PCR product of *Sinorhizobium* isolates by rD1 and fD1 primers on 1.5% gel agarose (M: DNA marker, bp: Base pair)

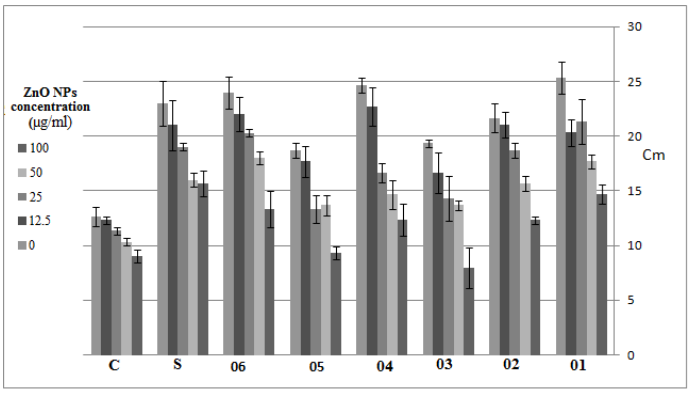

Figure 2. The mean (±SE) of total length of treated plants in the presence of different concentration of ZnO NPs compared with the control plants

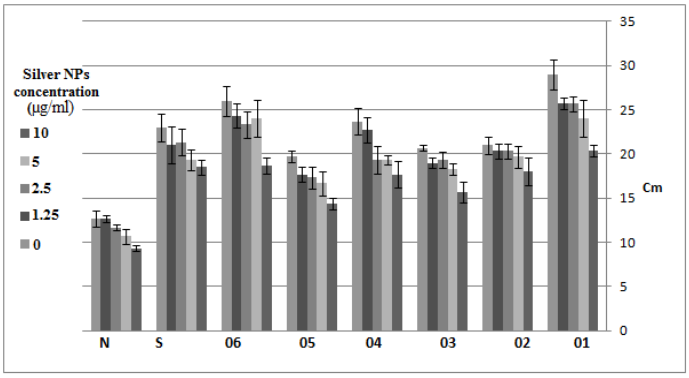

Figure 3. The mean (±SE) of total length of treated plants in the presence of different concentration of AgNPs compared with the control plants

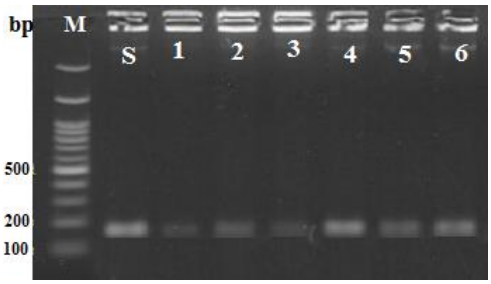

Figure 4. Gel electrophoresis of PCR products of *nif* gene for *S. meliloti* isolates in this study (S: Standard strain, 1-6: Isolates, M: DNA Marker)

Discussion

NPs are now widely used in many fields of science. Silver and ZnO NPs are the most widely used in this area. Few studies have investigated the toxic and environmental effects of direct and indirect exposure to NPs (Colvin 2003). Soil microorganisms play a very important role in maintaining soil health, ecosystem functions and crop productivity (Mishra and Kumar 2009) and it is necessary to evaluate the effects of metal NPs on soil bacteria.

Tested NPs in our study affected the legume- *Rhizobium* symbiosis by the deduction of the number and the size of nodules and the depression of expression of *nif* gene. Also by increasing the concentration of NPs, the total length of treated plants was decreased. AgNPs showed the effects on symbiosis in lower concentration than ZnO NPs.

Results of a study showed the toxicity function of AgNPs on the plant itself or on faba bean-*R. Glomus* symbiosis. The germination declined by 40% when exposed to AgNPs with the size of 5 to 50 nm at concentration 800 µg/kg soil. Silver NPs (800 µg/kg soil) considerably retarded the process of nodulation and nitrogenase activity in *R. leguminosarum* bv. *viciae* symbiosis with faba bean. AgNP in this concentration resulted in detectable alterations including the intracellular deterioration of cytoplasmic components by means of autophagy and disintegration of bacteroids in root nodule cells, which could physiologically mimic programmed cell death (Abd-Alla *et al*., 2016). Mishra and Kumar (2015) showed that the effect of ZnO NPs on nitrogenase activity in legumes depends on nano-ZnO concentration and exposure time. Nitrogenase activity in cluster bean, green gram and cowpea roots increased after dipping in solution containing 1.5 µg/ml ZnO NPs, but decreased in roots dipped in solution containing 10 µg/ml ZnO NPs. Fan and colleagues in a report in 2013 stated that Nano-TiO_2 contamination in the environment is potentially hazardous to the *Rhizobium*-legume symbiosis system. Cultured *R. leguminosarum* bv. *viciae* 3841 was also impacted by exposure to nano-TiO_2, resulting in morphological changes to the bacterial cells. Furthermore, *Rhizobium*-legume symbiosis was affected by nano-TiO_2 exposure, such that root nodule development and the subsequent onset of nitrogen fixation were delayed (Fan *et al*., 2015).

Conclusions

The results of this study showed that the existence of ZnO and Ag NPs in the environment can affect the legume- bacteria symbiosis, reduce the number and size of nodes and decrease the expression of *nif* gene. In this study, AgNPs had a greater inhibitory effect than ZnO NPs and at lower concentrations inhibited the growth plants and coexistence.

Acknowledgements. We gratefully acknowledge financial support from the Islamic Azad University, Mashhad Branch, Iran.

References

[1] M.H. Abd-Alla, N.A. Nafady, D.M. Khalaf, Assessment of silver nanoparticles contamination on faba bean-*Rhizobium leguminosarum* bv. *viciae-Glomus aggregatum* symbiosis: Implications for induction of autophagy process in root nodule, *Agriculture, Ecosystems & Environment*, **218** (2016), 163–177. https://doi.org/10.1016/j.agee.2015.11.022

[2] M. Bradic, S. Sikora, S. Redzepovic, Z. Stafa, Genetic identification and symbiotic efficiency of an indigenous *Sinorhizobium meliloti* field population, *Biotechnology*, **41** (2003), 69-75.

[3] W.P. Chen, T.T. Kuo, A simple and rapid method for the preparation of Gram negative bacteria genomic DNA, *Nucleic Acids Research*, **21** (1993), 2260. https://doi.org/10.1093/nar/21.9.2260

[4] V.L. Colvin, The potential environmental impact of engineered nanomaterials, *Nature Biotechnology*, **21** (2003), 1166-1170. https://doi.org/10.1038/nbt875

[5] X. Deng, Q. Luan, W. Chen, Y. Wang, M. Wu, H. Zhang, Z. Jiao, Nanosized zinc oxide particles induce neural stem cell apoptosis, *Nanotechnology*, **20** (2009), 115101. https://doi.org/10.1088/0957-4484/20/11/115101

[6] N. Elboutahiri, I. Thami-Alami, S.M. Udupa, Phenotypic and genetic diversity in *Sinorhizobium meliloti* and *S. medicae* from drought and salt affected regions of Morocco, *BMC Microbiology*, **10** (2010), 15. https://doi.org/10.1186/1471-2180-10-15

[7] R. Fan, Y.C. Huang, M.A, Grusak, C.P, Huang, D.J. Sherrier, Effects of nano-TiO_2 on the agronomically-relevant *Rhizobium*-legume symbiosis, *Science of the Total Environment*, **466-467** (2014), 503-512. https://doi.org/10.1016/j.scitotenv.2013.07.032

[8] A.F. Haag, M.F. Arnold, K.K. Myka, B. Kerscher, S. Dall'Angelo, M. Zanda, P. Mergaert, G.P. Ferguson, Molecular insights into bacterial development during *Rhizobium*-legume symbiosis, *FEMS Microbiology Reviews*, **37** (2013), 364-383. https://doi.org/10.1111/1574-6976.12003

[9] E. Jensen, M. Peoples, R. Boddey, P.M. Gresshoff, H. Hauggaard-Nielsen, B.J.R. Alves, M.J. Morrison, Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review, *Agronomy for Sustainable Development*, **32** (2012), 329-364. https://doi.org/10.1007/s13593-011-0056-7

[10] L.S. Dorobantu, C. Fallone, A.J. Noble, J. Veinot, G. Ma, G.G. Goss, R.E. Burrel, Toxicity of silver nanoparticles against bacteria, yeast, and algae, *Journal of Nanoparticle Research*, **17** (2015), 172-185. https://doi.org/10.1007/s11051-015-2984-7

[11] V.K. Mishra, A. Kumar, Impact of metal nanoparticles on plant growth promoting rhizobacteria., *Digest Journal of Nanomaterials and Biostructures*, **4** (2009), 587-592.

[12] A. Shamseldin, M. Sadowsky, M. El-Saadani, C. Sun An, Molecular biodiversity and identification of free living *Rhizobium* strains from diversity Egyptian soil as assessed by direct isolation without trap hosts, *American-Eurasian Journal of Agriculture*, **4** (2008), 541-549.

[13] B. Singh, R. Kaur, K. Singh, Characterization of *Rhizobium* strain isolated from the roots of *Trigonella foenumgraecum* (Fenugreek), *African Journal of Biotechnology*, **7** (2008), 3671-3676.

[14] I. Tham, Effects of nitrogen on nodulation and promiscuity in the *Acacia mangium* rhizobia relationship, *Asian Journal of Plant Sciences*, **6** (2007), 941-948. https://doi.org/10.3923/ajps.2007.941.948

[15] G. Truchet, F. Debelle, J. Vasse, B. Terzaghi, A.M. Generone, C. Rosenberg, J. Batut, F. Mallet, J. Denarie, Identification of *Rhizobium meliloti pSYM 2011* region controlling the host specificity of root hair curling and nodulation, *Journal of Bacteriology*, **164** (1985), 1200-1210.

Received: February 8, 2017; Published: March 11, 2017