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The effect of silver nanoparticles on the growth of nitrogen fixing and ACC deaminase producing bacteria isolated from sunflower rhizosphere

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ABSTRACT

Regarding the role of nitrogen fixing microorganisms in soil fertility and plant growth, the use of biological fertilizers is a potential process in agriculture. Regarding toxic effects of silver nanoparticles on soil beneficial bacteria, the purpose of this study was to isolate and identify free-living nitrogen fixing bacteria from sunflower rhizosphere and investigation of the toxic effect of silver nanoparticles on the growth of isolated bacteria. In order to isolate nitrogen fixing bacteria, soil samples were obtained from sunflower rhizosphere and cultured in nitrogen-free medium at 30˚C for 48 hrs. Then the production of 1-Aminocyclopropen-1-carboxylate (ACC) deaminase by the isolates was assayed by photometric method after growing in minimum DF medium containing ACC and aluminum sulfate. Phylogenetic identification of the selected bacteria was done using the amplification and sequence analysis of 16SrRNA gene. Finally, minimum inhibitory concentration (MIC) of colloidal suspensions of silver nanoparticles on the isolated bacteria was evaluated. The isolated bacteria from the soil samples of sunflower rhizosphere were included two species of nitrogen fixing bacteria with the ability for ACC deaminase production. Phylogenetic analysis of 16SrRNA gene resulted in identification of *Azotobacter nigricans* and an *Azorhizophilus pasali*. MIC of silver nanoparticles on both bacteria was evaluated as the concentration of 62.5 ppm. Silver nanoparticles with the concentration above 62.5 ppm had lethal effect on both studied nitrogen fixing strains. According to the importance of these bacteria in soil fertility and increasing utilization of silver nanoparticles in different industries, more studies on the dispersal of these nanoparticles in waters and waste waters and their penetration rates to agricultural environments seems necessary.

1. Introduction

Nanotechnology is a new technology that has recently entered the agricultural field. Soil bacteria are constantly affected by various environmental factors and Xenobiotic materials. Nanoparticles with various applications in different industries also affect the soil bacterial population (Babich et al., 1985). Among these particles, silver nanoparticles have become one of the most commonly used nanoparticles in consumed products due to its unique antimicrobial properties and low toxicity to mammalian cells. These particles are so small that can enter to the environment, penetrate to soils and cross the cell wall of bacteria. These particles are able to have lethal effects on soil

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beneficial bacteria (Mazumdar and Ahmed, 2011).

Biological fertilizers have has great potential use in agriculture. Biological fertilizers are not exclusively restricted to organic matter derived from resources such as animal fertilizers and vegetable supplements, but products derived from the activity of microorganisms such as ones that are involved in nitrogen fixation, and provision of phosphorus and other nutrients in the soil are also used (Franche et al., 2009; Çakmakçi et al., 2007). At the present, increasing cost of chemical fertilizers as well as the adverse environmental impacts caused by unsustainable use of these fertilizers is from the main problems which global agriculture is faced. In this regard, use of beneficial microorganisms in the soil, especially growth stimulating bacteria, has been increasing in various biological processes involved in plant growth and soil nutrient cycles. Among the most important bacteria found mainly in the vicinity of the root of plant species, *Azotobacter*, *Azospirilium, Enterobacter* and *Pseudomonas* can be mentioned (Luuml and Huang, 2010; Reinhardt et al., 2008; Zaied et al., 2003).

Azotobacter is Gram-negative aerobic bacterium from the family of Azobacteriaceae with relatively coarse cells and polymorphisms which are capable to form capsule and microcyst. These bacteria are from non-symbiotic nitrogen fixing organisms (Dobbelaere et al., 2003). Some of the beneficial activities of these bacteria that puts them in the group of plant growth promoters (PGPRs) are production of hormones which stimulate plant growth, especially auxins, the ability to dissolve organic and inorganic phosphates, and production of ionospheres, especially siderophores (Cocking, 2003).

In general, the fertility of soils reflects the diversity and distribution of microorganisms. Considering that nitrogen is one of the essential elements of plants, nitrogen fixing microorganisms provide the nitrogen required for plant use, by the process of nitrogen fixation.

Therefore they play an important role in soil fertility (Tejera et al., 2005). These bacteria are effective on cell growth by nitrogen fixation as well as production of thiamine, riboflavin, nicotine, indole, acetic acid and gibberellin (Eleiwa et al., 2012). Many nitrogen fixing bacterial species such as *Azospirillum*, *Gluconacetobacter*, *Herbaspirillum*, *Azoarcus*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Azorhizobium*, *Beijerinckia* and *Azotobacter* known as two types of entophytic or free organisms in the root and stem of non-chickpeas plants (Cocking, 2003; Martyniuk and Martyniuk, 2003).

In the present study nitrogen fixing bacteria were isolated from the vicinity of sunflower root and molecularly identified. Then the ability of ACC deaminase activity by the isolates was evaluated. Finally, considering the importance of the role of nitrogen fixing bacteria in soil for plant growth and lack of sufficient information about the effect of silver nanoparticles on these bacteria, the sensitivity of the isolated bacteria to various concentrations of silver nanoparticles was investigated in laboratory conditions (Monica and Cremonini, 2009; Mishra and Kumar, 2009).

2. Materials and Methods

2.1. Soil samples

Agronomic soil samples were randomly collected from 0-30 cm depths of rhizosphere in sunflower farms located in west of Isfahan (Includes: Falavarjan, Kelishad and Shaher Abrisham villages), Iran. Samples were transferred to the laboratory in plastic bags in cool condition and kept in 4°C until the isolation of bacteria (Canli and Atli, 2003).

2.2. Isolation of nitrogen fixing bacteria

In order to isolate nitrogen fixing bacteria from soil samples, different dilutions (10^{-1}-10^{-10}) were prepared and inoculated by pour plating into nitrogen lack mannitol agar medium (Reinhardt et al., 2008) containing 0.5 g magnesium sulfate, 3 g calcium carbonate, 0.02 g Sodium Molybdate, 18 g agar and distilled water up to the total volume of 1000 ml. The cultured media were incubated at 30°C for 48 hrs and the bacterial colonies evaluated for macroscopic and microscopic characteristics. In order to better isolation, culturing was repeated in three stages.

2.3. Screening isolates for the ability to produce ACC deaminase

The ability of isolates to use ACC as the sole source of nitrogen was determined by using modified Gilick et al., method. To this purpose, 50 µl of the cultured isolates in TSB were transferred to 20 ml of minimum DF medium (Reinhardt et al., 2008) containing 3 mM ACC in addition to 2 g per liter aluminum sulfate, and incubated for 48 hrs at 28°C and 180 rpm. Then the light absorption of the medium was measured at the wave length of 450 nm (Williams et al., 2005).

2.4. Identification of the isolates

Conventional microbiological methods including biochemical tests as well as molecular sequencing of 16SrRNA gene were used to identify the isolates (Ueda et al., 1995).

2.5. Colony PCR

Polymerase chain reaction (PCR) was done by using universal primers including 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5' ACGGCTACCTTGTTACGACTT 3') for identification of selected nitrogen fixing bacteria (Reinhardt et al., 2008). The protocol which was used for gene amplification included 30 cycles: 95ºC (1 min), 50ºC (30S), 72ºC (1 min) followed by one step of 72ºC for 10 min in the PCR reaction containing reaction buffer (1X), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), each primer (0.4 µM) and 2×10^7 bacterial cells. The amplified fragment was evaluated and confirmed by 1% agarose gel electrophoresis in 1x TBE buffer and sequenced. The results were evaluated and corrected by Mega 7.1 software. The sequences were aligned with recorded sequences in gene bank for identification of bacterial species. Phylogenetic trees were plotted by using Test Neighbor-Joining Tree Bootstrap 1000 program.

2.6. Susceptibility determination of the isolates to silver nanoparticles

For this purpose, concentrations of 0.47, 0.94, 1.87, 3.75, 7.5, 15.62, 31.25, 62.5, 125 and 250 ppm of silver nanoparticles (10-15 nm diameters, spherical shape, purity of 99.9%, mineral in nature, and wet synthesis method in liquid phase alternation) were prepared in Muller Hinton broth and the number of 1.5×10^6 bacterial cells was inoculated to each dilution of the silver nanoparticle. After incubation at 30°C for 24 hrs, the growth of bacteria was evaluated based on the absorbance of cultured bacteria in the wavelength of 625 nm. The lowest concentration with any turbidity observation, considered as minimum inhibitory concentration (MIC). For detection of minimum bactericidal concentration (MBC), cultured Muller Hinton broth which considered as MIC as well as the cultured medium containing one higher concentration of silver nanoparticle were inoculated to Muller Hinton Agar (MHA; Merck) and incubated at 30°C for 24 hrs. The concentration in which bacterial colonies were not grown, considered as MBC (Lin and Xing, 2007).

2.7. Statistical analysis

All data were analyzed by using the statistical package for social sciences (SPSS v.19). Data analysis was conducted for each factor on based model ANOVA.

3. Results

3.1. The isolated bacteria

The isolated bacteria from rhizosphere of the sunflower plant were detected as 2 closely related isolates of *Azotobacter nigricans* and one isolate of *Azorhizophilus paspli* based on macroscopic, microscopic, biochemical and molecular analysis. *Azotobacter nigricans* created medium size spherical shiny mucosal and pale colonies which turned to brown color by time passed. *Azorhizophilus paspli* created very small spherical shiny mucosal and colorless colonies. Both isolates colonies were soluble in water and both isolates produced capsule and were catalase positive. The microscopic view of the isolates is shown in figure 1.

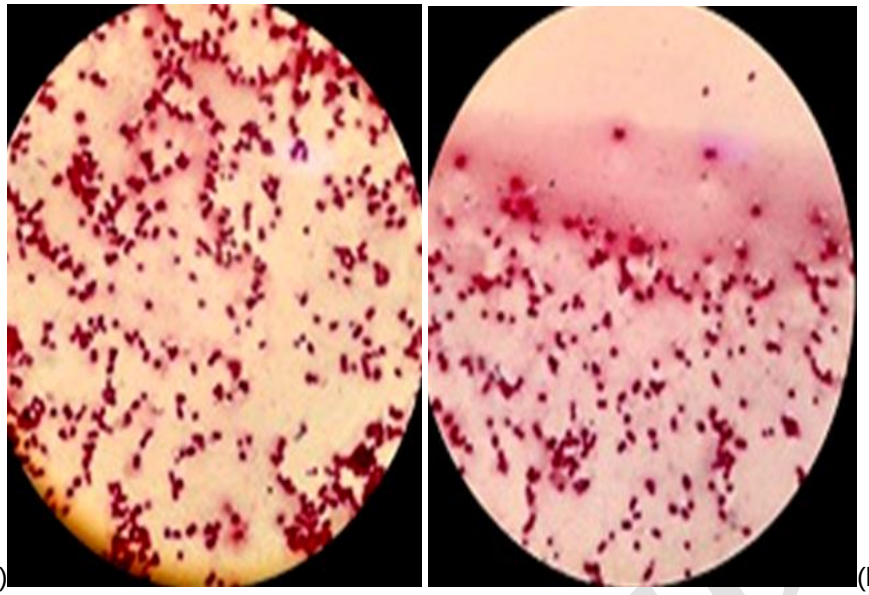
(a)  (b)

Figure 1. (a) Microscopic view of *Azotobacter nigricans* **(b)** *Azorhizophilus pasali*. indicates Gram-negative cocci (1000X magnification)

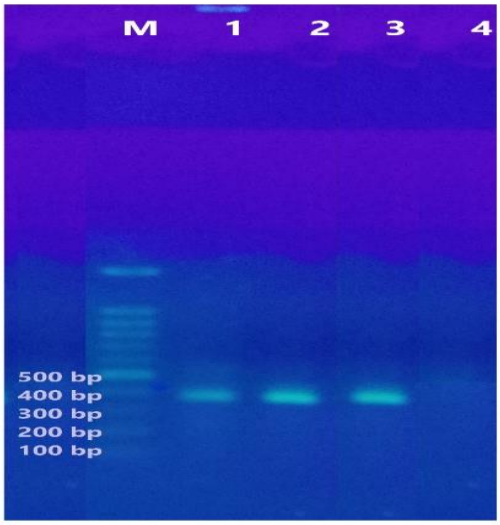


Figure 2. Agarose gel electrophoresis if the amplified fragment in 16SrRNA gene. M: 100 Kb DNA size marker, 1, 2 and 3: bacterial isolates, 4: negative control

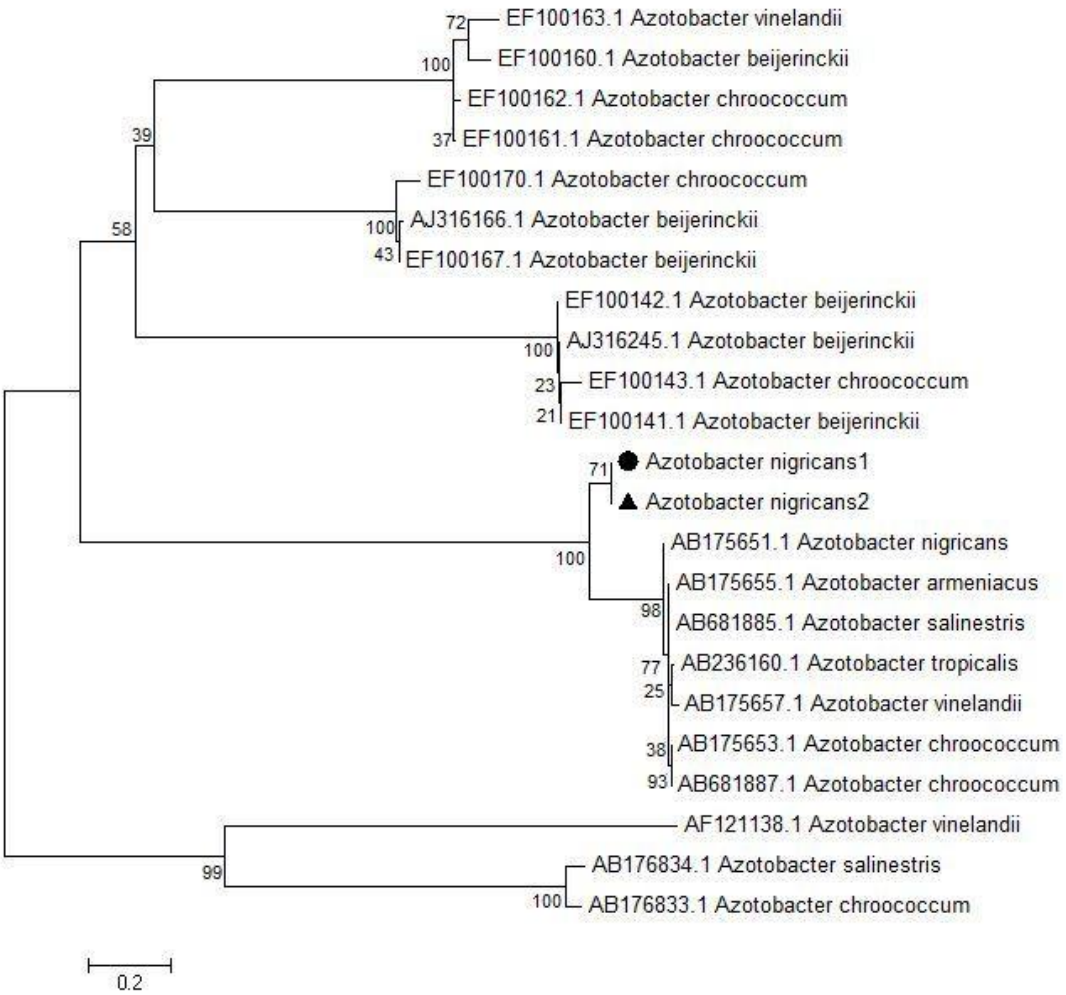


Figure 3. The phylogenetic positions of two isolates represents related strains of *Azotobacter nigricans*.

3.2. The effect of silver nanoparticles on the isolated strains

All three isolates did not grow in the presence of silver nanoparticle concentrations above 62.5 ppm based on turbidity assessment of broth culture media containing different concentrations of silver nanoparticles. Therefore this concentration considered as MIC. Also all three isolates do not grow in agar culture media containing 125 ppm silver nanoparticle. Therefore this concentration considered as MBC.

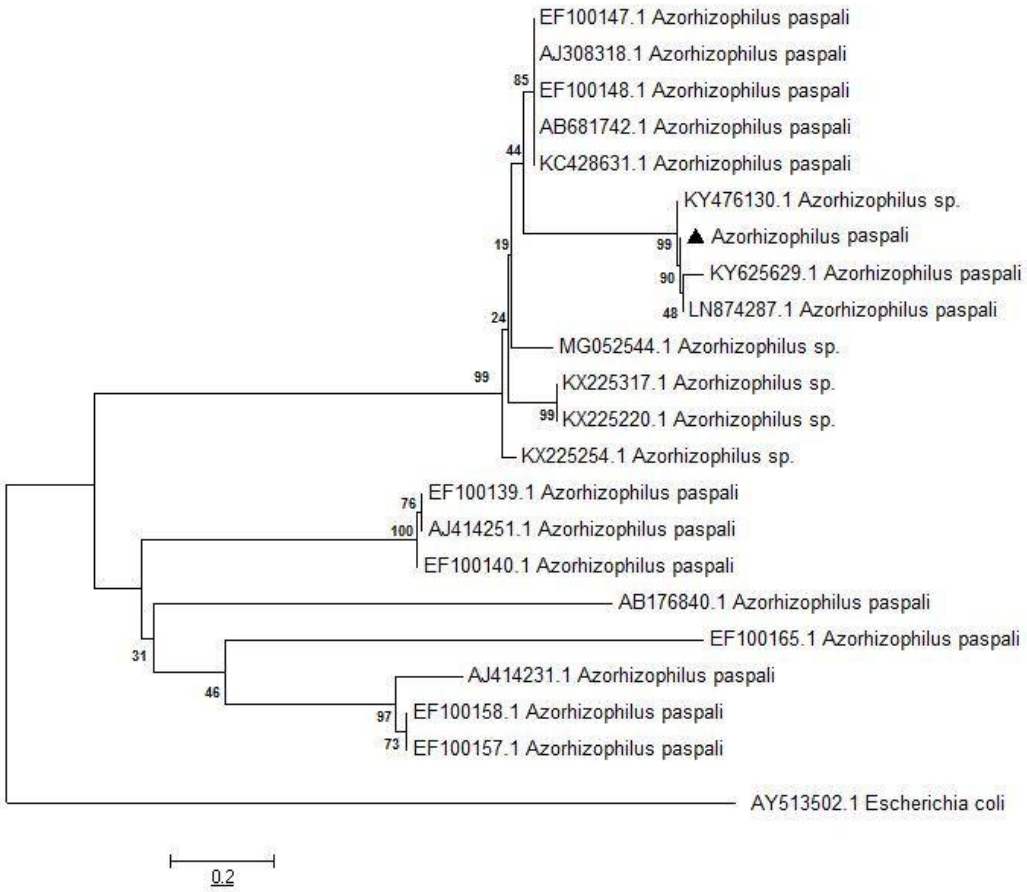


Figure 4. The phylogenetic position of third isolate represents a strain of *Azorhizophilus pasali.*

4. Discussion

Sufficient nutrients supply is one of the constraints on the potential yield of arable crops. Human beings have long been thinking about overcoming soil nutrients limitations using organic fertilizers (plant debris and animal wastes), but because of difficulties in large scale production of these fertilizers along with increasing world population which need more agricultural products, second-generation of fertilizers i.e chemical fertilizers were replaced from conventional agriculture to present time. New types of chemical fertilizers with different formulation and amount of nutrients are proposed each year (Jeun et al., 2004). Increased consumption of chemical fertilizers, in turn has created new problems in the environment, leading to thinking about more reliable alternatives. Therefore, the use of organic fertilizers in agriculture has been proposed and lead to organic farming. In the next step, the third generation of fertilizers known as bio-fertilizers entered agriculture and makes the world optimism about future of sustainable agriculture. Bio-fertilizers contain a surface covered with a heap of one or more beneficial soil microorganisms and/or their metabolic products. Bacteria form colonies in the area around the root or internal parts of the plant and stimulate host plant growth by different ways (Mazumdar and Ahmed, 2011; Holt, 1994).

In present, the use of soil biotechnology has been taken into consideration with the aim of using the potential of beneficial soil organisms in order to produce maximum crop, while considering improvement of soil quality and environmental safety (De Freitas et al., 1997). The use of microorganisms, especially plant growth-promoting bacteria, has been increasing in the various biological processes involved in plant growth and soil nutrient cycles (Zaied et al., 2003). For example, inoculation of corn by *Azospirillium* has been effective on nitrogen metabolism (Ribaudo et al., 2001). Also corn stem and root weight increasing (Kapulnik et al.,

1982) and leaf area expanding (Gholami et al., 2009) have been reported by inoculation of growth-promoting bacteria. Dobbelaere et al. (2003) observed that plants inoculated with a variety of auxin producing bacteria had more prolonged and branched roots than the control. The most important mechanism of plant growth stimulation by *Rhizobium* strains is production of indole-induced phytohormones (IAA), which results in better root growth which leads to increased water and nutrient uptake by the plant and results increased plant growth and reduces the amount of chemical fertilizers requirement (Gholami et al., 2009).

In the present study two strains of nitrogen fixing bacteria (*Azotobacter nigricans* and *Azorhizophilus paspali*) were isolated from rhizosphere of sunflower plant in nitrogen lacked mannitol agar and identified based on the sequence of 16SrRNA gene. Also the ability of the isolates to produce ACC deaminase was screened. In different studies nitrogen fixing bacteria have been successfully isolated in nitrogen lacked mannitol containing media (Luuml and Huang, 2010; Tejera et al., 2005; Ueda et al., 1995).

Nanotechnology is one of new technologies that have recently entered the agricultural field because of its beneficial effects on plant growth. For instance, Salehi and Tamaskani (2008) showed that treatments with 50 mg L^{-1} silver and alumina nanoparticles increased germination percentage as well as stem and root length, and ultimately improved wheat establishment, but the positive effect of this nanoparticle on seedling growth did not result in establishment of salinity stress conditions (Salehi and Tamaskani, 2008; Riahi-Madvar et al., 2012). Lu et al. (2002) observed that the combination of TiO and SiO nanoparticles increased the activity of nitrate reductase in soybeans and induced the ability to absorb and use water and fertilizers (Lu et al., 2002). Williams et al. (2005) showed increasing germination, plant dry weight, chlorophyll content, rubisco enzyme activity and photosynthesis rate in spinach in the presence of nanoparticles. The beneficial effects of silver nanoparticles on saffron plants under water stress have also been reported (Rezvani et al., 2012). On the other hand, heavy metals in the form of an element, ion or nanoparticles, are environmental pollutants for atmosphere when use in industries, and for soil by using chemical fertilizers or urban sewages in agriculture. In contrast to some organic pollutants that could convert to non-toxic materials, metals remain intrinsically persistent in nature and irreversibly enter the soil (Bruins et al., 2000; Canli and Atli, 2003; Chen et al., 2009). Some of these metals, such as iron, zinc, copper, magnesium, potassium, calcium, sodium and potassium, in a small amounts are micronutrients essential for the growth of plants, animals, and even humans, while the excess amounts of them in the soil causes metabolic disorders and ultimately inhibits the growth of most plants and microorganisms species (Ashraf and Ali, 2007; lu et al., 2002). One of the most important toxicity mechanisms of heavy metals such as zinc and silver is production of free radicals and oxidative stress. Many researchers have shown that nanoparticles have low stimulatory effects and in high concentrations, inhibitory effects on plant growth. Mazumdar and Ahmed reported that silver nanoparticles, in addition to chemical effects, may have an effect on the structure of enzymes in different stages of photosynthesis (Mazumdar and Ahmed, 2011).

The effect of Fe and silver nanoparticles on the growth of bean and sweet corn showed that this substance has a concentration-dependent inhibitory effect on plant growth (Kapulnik et al., 1982; Racuciu et al., 2008). Therefore exposure of plants to nanoparticles at low concentrations can be the best treatment and it seems to be a good alternative to micronutrient fertilization; but in high concentrations they could have toxic effect.

Babich and Stotzky (1985) conducted a research on the effect of zinc on the soil bacteria. The results of their study showed that zinc with the concentration of 2 mM reduced the activity of soil bacteria (Babich et al., 1985). Cevik and Karaca (2003) investigated the sensitivity of soil bacteria to zinc during a pot experiment. The results showed that the bacteria were sensitive to zinc concentrations above 50 ppm (Cevik and Karaca, 2006). In other study conducted by Shakibaie et al. (2008), the effect of zinc was investigated on mutation and viability of bacteria. Non-resistant bacteria were found to be sensitive to concentrations of more than 30 ppm of zinc. Also Rajapaksha et al. (2004) in the study on short-lived effect of the metal on soil microorganisms showed that increasing the amount of zinc and cupper caused

a decrease in the number of soil bacteria (Rajapaksha et al., 2004; Shakibaie et al., 2008). By estimating the sensitivity of the isolated bacteria to silver nanoparticles in the present study, both nitrogen fixing isolated strains were sensitive to the concentrations above 62.5 ppm of silver nanoparticles. This result is close to the effect of zinc on soil bacteria in above studies.

Malakootian et al. (2011) investigated the sensitivity of urban sewage bacteria to nanoparticles. It was shown that the studied bacteria did not show sensitivity to nanoparticles with the concentration of 80 ppm. Also the concentration of 100 and 1000 ppm resulted in the elimination of respectively 36% and 84% of sewage bacteria (Malakootian and Toolabi, 2010). These results are different from the results obtained from silver nanoparticles in the present study. This difference in the susceptibility of bacteria could be due to differences in the sampling sites, the diversity of the studied microorganisms, and the type, size and shape of metal compound used (Jeun et al., 2004; Seif Sahandi et al., 2011). The combined effect of bacteria and nanoparticles on the growth of sunflower seedlings was investigated and it was shown to increase the fresh and dry weight of the plant (Çakmakçi et al., 2007; Malakootian and Toolabi, 2010). Phytohormones are from the most important growth regulators with inducing impact on plant metabolism. These hormones also have vital effect on the stimulation of plant defense responses against abiotic stresses. These stresses included cool, heat, drought, heavy metals and salinity (Egamberdieva et al., 2017). Çanli et al. reported that the most important mechanism of stimulation by rhizobia strains is related to the production of indole phytohormone (IAA) and the high ACC deaminase strength, which resulted in better root growth, followed by increased absorption of water and elements. The increasing nutrients (K, P and N) uptake leads to increased plant growth and this could reduce the amount of chemical fertilizers used (Canli and Atli, 2003; Wu et al., 2005). In the present study, both isolated nitrogen fixing strains, *Azotobacter nigricans* and *Azorhizophilus paspali* have the advantage to produce ACC deaminase which would help sunflower plant to undergo environmental stresses including heavy metals.

Conclusion

According to the results of present study, the concentrations upper than 62.5 ppm of silver nanoparticles was inhibited the growth of *Azotobacter nigricans* and *Azorizophilus pasali* isolated from the rhizosphere of sunflower plant. Therefore, according to the probable lethal effect of silver nanoparticles on other beneficial soil bacteria, increasing excessive consumption of silver nanoparticles as micronutrient fertilizers in arable crops should be monitored by agricultural health organizations.

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Refereces

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