3 Biotech (2017) 7:345
DOI 10.1007/s13205-017-0988-y

ORIGINAL ARTICLE

Effect of biosynthesized silver nanoparticles on native soil microflora via plant transport during plant–pathogen–nanoparticles interaction

Madhuree Kumari[1] · Shipra Pandey[1] · Shashank Kumar Mishra[1] · Chandra Shekhar Nautiyal[1] · Aradhana Mishra[1]

Received: 4 July 2017 / Accepted: 20 September 2017 / Published online: 23 September 2017

Abstract In this study, the interaction of biosynthesized silver nanoparticles (BSNP) with native soil via plant transport was assessed in model pathosystem of *Arabidopsis thaliana* and *Alternaria brassicicola*. Foliar application of 5 µg/mL of BSNP reduced number of spores of fungi to 2.2×10^5 from 7×10^5, while numbers of lesions got reduced to 0.9/leaf in treated plants compared to 2.9/leaf in pathogen-infected plant without altering soil pH, electric conductivity, soil organic carbon and soil microbial biomass carbon. Soil enzyme activities including dehydrogenase, acid and alkaline phosphatase, urease, β-glucosidase and protease did not alter significantly in BSNP-treated plants compared to control plants. Application of BSNP did not alter the number of cultivable bacteria, fungi and actinomycetes. Effect of BSNP on uncultured bacterial diversity was measured by DGGE analysis which revealed similar banding pattern in all different treatments except in *A. brassicicola*-infected (AB) and *A. brassicicola*-infected plants treated with silver nanoparticles (AB + BSNP) after 120 days. Although AB-infected plants exhibited a decrease in bacterial diversity, treatment of AB + BSNP after 120 days demonstrated maximum bacterial diversity. McIntosh, Shannon, and Simpson diversity indices were calculated based on carbon source utilization pattern by BIOLOG analysis, revealing no significant difference among all treatments in different time intervals. BSNPs have the potential to act as strong antimicrobial agent for plant disease management without altering the native soil microflora.

Keywords Silver nanoparticles · Bacterial community · Soil enzymes · DGGE · BIOLOG

Introduction

Due to the broad-spectrum antimicrobial activities, silver nanoparticles (AgNPs) are emerging as superior pathogen control agents in the field of plant disease management. After the emergence of resistant strains of pathogens and change in climatic conditions, they have the potential to take over the traditional means of disease management including use of disease-tolerant varieties and chemical control (Ocsoy et al. 2013). Previous studies have demonstrated foliar application of AgNPs to control the foliar plant diseases affecting aboveground parts of plants (Ocsoy et al. 2013; Mishra et al. 2014). Biosynthesis of nanoparticles has provided them an edge being an economic and eco-friendly approach (Mishra et al. 2014; Balakumaran et al. 2016; Kumari et al. 2016, 2017a).

Soil, living place of many creatures on earth, has always gained an important status especially when talked about agriculture. Being the sink for all agricultural practises, they are severely affected by all biotic and abiotic interactions of plants (Ignatova et al. 2015; Hosseini et al. 2017; Tan et al. 2017). Recently, studies are undergoing to assess the impact of engineered nanomaterials on physical, chemical and biological properties of soil, however, little is known about their possible effects (Dinesh et al. 2012; Mishra and Singh 2014). Hänschand and Emmerling (2010) demonstrated that AgNPs do not significantly alter

Electronic supplementary material The online version of this article (doi:10.1007/s13205-017-0988-y) contains supplementary material, which is available to authorized users.

✉ Aradhana Mishra
mishramyco@yahoo.com

[1] Division of Plant Microbe Interactions, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India

soil enzyme activities, but silver nanoparticles did increase basal respiration and metabolic quotients. In contrast, Peyrot et al. (2014) observed a decrease in soil enzymatic activities after the application of silver nanoparticles. Fate of nanoparticles in soil is largely dependent upon the soil organic matter and related compounds which can transform and modify the properties and transport of nanoparticles in environment negotiating their harmful effects in soil (Sillen et al. 2015; Rahmatpour et al. 2017). However, some studies have suggested the negative role of particles on soil microflora (Kumar et al. 2014). The effect of nanoparticles on soil microbial communities depends on the soil properties, coating of silver nanoparticles used, and exposure dose and time (Girilal et al. 2015; Maliszewska 2016).

The occurrence of an intricate relationship between "soil–plant systems" where any change in plant's interaction is bound to have an impact on soil system and vice versa; little is known about the impact of AgNPs on soils and investigations on AgNPs–plants interaction are still rare and in its rudimentary stage (Sillen et al. 2015; He et al. 2016). There is also little information available on how the nanoparticles interact with microbial communities in soil under field conditions via plant transport. Thus, the effect of foliar applications of AgNPs for the purpose of plant disease management on soil needs to be investigated.

The goal of this study is to investigate the effect of biosynthesized silver nanoparticles on native soil microflora via plant transport at different time intervals while acting as potent pathogen control agent in model pathosystem of *Arabidopsis thaliana* and *Alternaria brassicicola.* The specific objective of this study is to find out the role of BSNP in plant disease management while keeping focus on soil properties, soil enzymes, cultivable and non-cultivable population of native soil microflora.

Materials and methods

Nanoparticles

As previously described by Kumari et al. (2017b), spherical silver nanoparticles (2–5 nm) were earlier synthesized by authors from cell-free extract of *Trichoderma viride* (MTCC 5661) at specified conditions and concentration was adjusted to 5 µg/mL before use. Particles were filtered through 0.22-µ syringe Millipore filters and sonicated for 2 min before use.

Study site and experimental design

Arabidopsis thaliana seeds were sown (10 seeds) on garden soil of CSIR-NBRI ((80°59′E, 26°55′N; 132 m above sea level). The soil was clay loam with soil pH 7.8 as a normal agriculture soil (Srivastava et al. 2014). To minimize spatial heterogeneity, soil samples were collected from ten random sites and mixed thoroughly before filling the pots. Pots were transferred to temperature-controlled culture room (set at 22 °C) in continuous light conditions after 3 days of cold treatment and irrigated twice a week with water. After one month of germination, plants were divided into four groups with 15 replicates each: (1) control (CON), (2) plants sprayed with 5 µg/mL of BSNP (BSNP alone), (3) *A. brassicicola*-infected plants (AB alone), and (4) *A. brassicicola* + 5 µg/mL of BSNP (AB + BSNP). Control plants were sprayed with 1% gelatin, while BSNP and AB + BSNP treatments were sprayed with the BSNP until runoff. Two hours after treatment, AB and AB + BSNP treatments were inoculated with spore suspension of *A. brassicicola* (10^5 spores/mL). Plants were bagged with a transparent plastic bag and kept for 48 h. The efficacy of biogenic silver nanoparticles as pathogen control in normal garden soil was measured by counting number of lesions/leaf and number of spores/leaf after 48 h of infection.

Soil sample collection

For soil nanoparticles interaction studies, soil was divided into three categories with five replicates of each treatment.

1. *Soil (0 h)* Control soil of all the four treatments were collected just after foliar spray of pathogen.
2. *Soil (48 h)* Soil samples of all the four treatments were collected 48 h after plants were pre-treated with BSNP when infection was clearly visible in AB alone.
3. *Soil (120 days)* Soil samples of all the four treatments were collected after decomposition of plant materials into the soil after 120 days.

Count of heterogeneous microbial population

Microflora associated with soil samples was determined by the culture enrichment technique (Mishra and Nautiyal 2009). Serial dilution of soil samples was prepared, and heterogeneous soil population comprising bacteria, actinomycetes and fungi was counted on nutrient agar, Ken-knight and Munaier's medium and Rose Bengal Chloramphenicol agar, respectively.

Soil properties

pH of soil samples of different stages were measured by pH meter (H1 2215 pH/ORP meter, Hanna instruments, USA) and electric conductivity (EC) was measured by conductivity cell (Orion, Thermo electron corporation, USA). Soil organic carbon (SOC) was estimated by modified Walkley–Black (modified-WB) method as described by Nelson and

Sommers (1996). SMBC was determined by fumigation–extraction method (Vance et al. 1987).

Soil enzyme activities

Different soil enzymes activities were estimated spectrophotometrically according to standard procedures reported in literature. Dehydrogenase activity was measured using the characteristics of TTC (2,3,5-triphenyltetrazolium chloride) reduction to TPF (triphenylformazan) as described by Mishra and Nautiyal (2009). Acid, alkaline phosphatase and β-glucosidase activities were estimated by the determination of *p*-nitrophenol released after the incubation of soil with *p*-nitrophenyl phosphate and *p*-Nitrophenyl-β-D-glucoside (PNG), respectively, for 1 h at 37 °C (Tabatabai 1994). Urease activity was measured using the protocols of Kandeler and Gerber (1988). The procedure was based on spectrophotometric determination of ammonia after incubation of the soil samples with urea solution. Protease activity was determined as previously described by Ladd and Butler (1972).

Microbial diversity using carbon source utilization pattern

Biolog Eco plates (Biolog, Inc., Hayward, CA, USA) were used to determine the carbon source utilization pattern of soil samples under different conditions according to protocol of Mishra and Nautiyal (2009). The rate of utilization of carbon source was indicated by the reduction of tetrazolium, a redox indicator dye, which changes from colourless to purple. Data were recorded for day 1–7 at 590 nm. Microbial activity in each microplate, expressed as average well colour development (AWCD), diversity and evenness indices, and principal component analysis (PCA) was determined. Statistical analyses were performed using SPSS 16.0 and Statistica 7.0.

DNA extraction and DGGE analysis of soil samples

For analysing the effect of biogenic silver nanoparticles (BSNP) on uncultured microflora of soil microbial community, denaturating gradient gel electrophoresis (DGGE) was carried as described by Khodakovskaya et al. (2012) with some modifications. Soil genomic DNA was extracted from 0.3 g of dry weight of each soil sample using PowerSoil® DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) and were further observed by agarose gel electrophoresis. 16S rRNA genes (V3 region) were amplified for conducting DGGE analysis using primers GC-clamp-357F (5′-TC TAC GGG AGG CAG CAG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′). The amplification was performed using a PCR machine (TC 3000, Genei, Bengaluru, India), adding 2.0 µl of Taq buffer, each of forward and reverse primers, deoxyribonucleoside triphosphate (0.1 mmol/L each) and 1 unit of Taq polymerase (Genei, Bengaluru, India). The condition of amplification was initial denaturation at 94 °C for 5 min, and then 30 cycles of denaturation (30 s, 94 °C), primer annealing (30 s, 55 °C), and extension (30 s, 72 °C), with a final extension step of 7 min at 72 °C. The amplified product was confirmed by 1.2% agarose gel electrophoresis and visualized with a Gel Doc system (BioRad, Hercules, CA, USA). Equal volume of amplified products from each sample were loaded on the DGGE gel with denaturing gradient ranging from 30 to 70% and the run was at 60 V for 16 h at 60 °C, using the Dcode system (BioRad, USA). The gels were run thrice to avoid any biological or technical variation. DGGE gel profiles were visualized by ethidium bromide staining and photographed using the Gel Doc system (BioRad, USA). Majority rule consensus tree with 500 bootstrap replicates was constructed using PAUP 4B (Sinauer associates, Sunderland, Massachusetts, USA).

Statistical analysis

The results were displayed as mean ± SD (±standard deviation). For statistical significance, mean ± SD of all groups were compared and analysis of variance (ANOVA) was performed using a statistical package, SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A probability of P value of ≤ 0.05 was taken to indicate statistical significance. Further, Duncan's multiple range test (DMRT) was used to identify the pairs of groups where the means are significantly different at $\alpha = 0.05$.

Results and discussion

Disease assessment in garden soil

The role of BSNPs in plant disease management in garden soil was assessed by counting number of spores and lesions after 48 h of inoculation of silver nanoparticles (Fig. 1a). Plants treated with AB + BSNP demonstrated 68.57 and 68.96% reduction in spore and lesion count, respectively, compared to the *A. brassicicola*-infected plants (Fig. 1b, c). 48 hours after the application of BSNP, the number of fungal spores per leaf was reduced to 2.2×10^5 per leaf from 7×10^5 per leaf, while the number of lesions per leaf was also reduced to 0.9 per leaf in BSNP-treated plants from 2.9 per leaf in AB control (Fig. 1b, c). BSNPs were capable to act as a potential pathogen control agent in normal garden soil conditions. Previous studies have also demonstrated the potential of silver nanoparticles as an

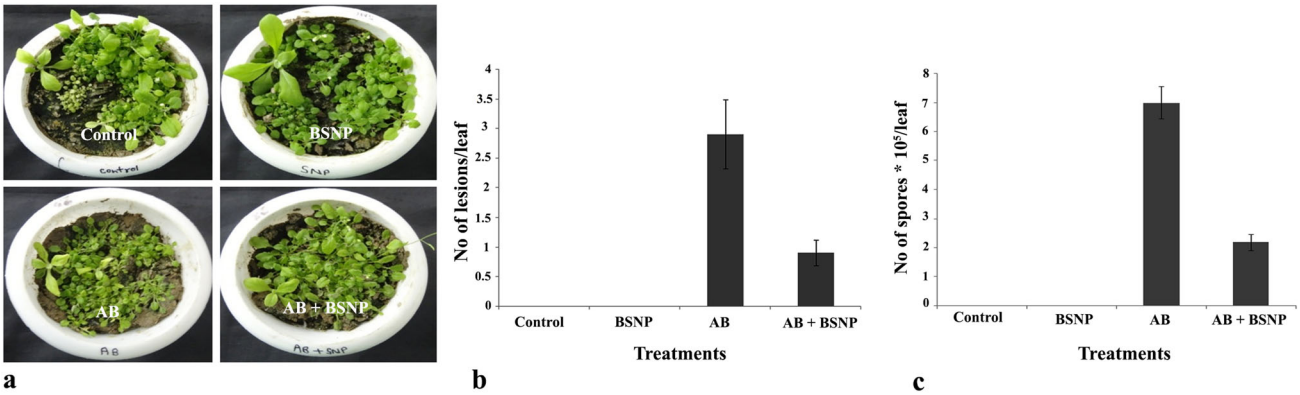

Fig. 1 Reduction in (**a**) disease severity (**b**) number of lesions (**c**) number of spores after pre-treatment of silver nanoparticles

efficient control agent against plant pathogens (Ocsoy et al. 2013; Balashanmugam et al. 2016).

Enumeration of culturable heterogeneous microbes

Soil extracts demonstrated very little differences in terms of total heterotrophic microbial counts (Table 1). Application of BSNP did not significantly alter the number of bacteria recovered from the soil in comparison with non-treated controls. Similarly, no significant variation in the number of fungi and actinomycetes recovered from the soil of BSNP-treated plants in comparison with non-treated controls at any time point was observed. Native microflora governs a number of activities that maintain soil health but may be disturbed by anthropogenic activities, such as use of pesticides and effluents. Enumeration of cultivable heterogeneous microbial population can directly provide evidence about soil vigour (Janvier et al. 2007). These results indicate that foliar application of BSNP over plants did not affect the number of native microflora recovered from soil.

Soil properties

Foliar application of BSNP did not significantly alter soil pH in comparison with non-treated control at any time point. In general, there was also no significant difference in EC among all four treatments (Table 2). However, EC of soil collected from soil of AB-inoculated plants did decline to 227.6 µS/cm. Soil pH and EC are very commonly used to determine soil health because they have potential to alter the soil microflora and enzymatic activity (Xiu-Mei et al. 2008). In this study, foliar application of BSNP did not significantly impact soil pH and EC.

Soil organic carbon (SOC) and soil microbial biomass carbon (SMBC) are two important parameters which describe soil health. SOC represents the carbon stored within soil which consists of carbon sources, such as decayed plant and animal materials. In this experiment, no significant difference in SOC was observed among all treatments in any of the time points (Fig. 2a). Similarly, SMBC is affected by agricultural practises and environment stresses in agricultural ecosystems (Singh et al. 2016). It also demonstrated no significant impact of BSNP

Table 1 Heterogeneous microbial population of bacteria, fungi and actinomycetes in soil at different time points (0 h, 48 h and 120 days) after different treatments viz. control, plants sprayed with biosynthesized silver nanoparticles (BSNP), *A. brassicicola*-infected plants (AB), and *A. brassicicola*-infected plants sprayed with BSNP (AB + BSNP)

Treatments	Bacteria (log_{10} CFU/g)			Fungi(log_{10} CFU/g)			Actinomycetes(log_{10} CFU/g)		
	0 h	48 h	120 day	0 h	48 h	120 day	0 h	48 h	120 day
Control	5.14^{b}	5.14^{b}	5.07^{b}	2.16^{b}	2.16	2.17^{b}	5.06^{b}	5.07^{b}	5.01^{c}
BSNP	5.09^{b}	5.12^{b}	5.1^{b}	2.14^{b}	2.13^{b}	2.17^{b}	5.06^{b}	5.04^{b}	5.01^{c}
AB	5.12^{b}	5.18^{a}	5.07^{b}	2.14^{b}	2.17^{b}	2.21^{a}	5.08^{b}	5.06^{b}	5.01^{c}
AB + BSNP	5.11^{b}	5.18^{a}	5.08^{b}	2.16^{b}	2.14^{b}	2.14^{b}	5.05^{b}	5.03^{a}	5.00^{c}

Values are the means of five replicates for each treatment. Means sharing different alphabets "a", "b" differ significantly from each other at $p \leq 0.05$

Table 2 pH and EC of soil at different time points (0 h, 48 h and 120 days) after four treatments, control, plants sprayed with biosynthesized silver nanoparticles (BSNP), *A. brassicicola*-infected plants (AB), and *A. brassicicola*-infected plants sprayed with BSNP (AB + BSNP)

Treatments	pH			EC (μS/cm)		
	0 h	48 h	120 day	0 h	48 h	120 day
Control	7.79[a]	7.79[a]	7.8[a]	297.4[a]	298.4[a]	297.4[a]
BSNP	7.79[a]	7.74[a]	7.8[a]	297.4[a]	298.8[a]	297.9[a]
AB	7.79[a]	7.75[a]	7.9[a]	297.4[a]	296.2[a]	227.6[c]
AB + BSNP	7.79[a]	7.73[a]	7.85[a]	297.4[a]	298.6[a]	280.7[b]

Values are the means of five replicates for each treatment. Means sharing different alphabets "a", "b" differ significantly from each other at $p \leq 0.05$

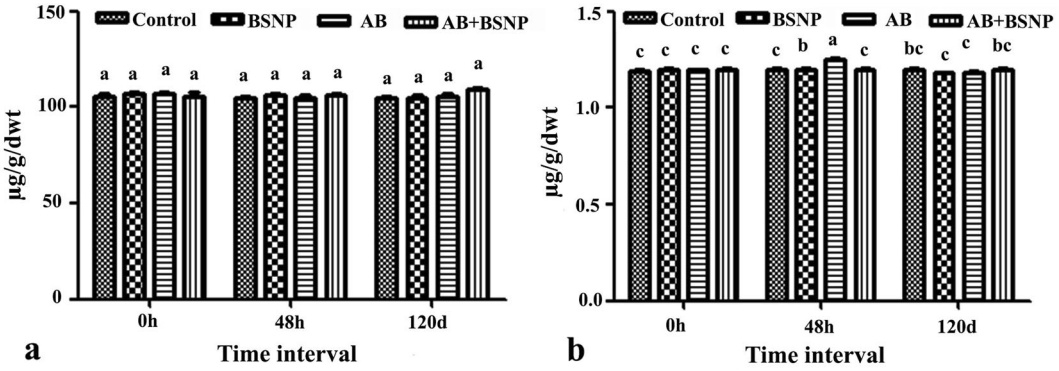

Fig. 2 (**a**) Soil microbial biomass carbon (SMBC) and (**b**) soil organic carbon (SOC) at different time intervals of 0, 48 h and after decomposition (120 days) after spray of nanoparticles over plants. Values are the means of three replicates. Means sharing different alphabets "a", "b" differ significantly from each other at $p \leq 0.05$

in soil microbial biomass in BSNP-treated soil samples (Fig. 2b).

Soil enzyme activities

Dehydrogenase

Soil dehydrogenase (EC 1.1.1) depicts the oxidoreductase activity occurring in soil, directly indicating overall soil microbial activity (Wolińska and Stępniewska 2012). Thus, dehydrogenase activity was measured in the presence and absence of BSNP (Fig. 3a). Initially, dehydrogenase activity of soil was 93.66 mg TPF g^{-1} soil h^{-1} at 0 h which increased slightly at 48 h. Though the activity was similar in control, BSNP and AB + BSNP (106.5, 106.6 and 105.6 μg TPF g^{-1} soil h^{-1}, respectively), an increase in dehydrogenase activity was observed in AB-inoculated control (134.3 μg TPF g^{-1} soil h^{-1}). Inoculation of *A. brassicicola* could have altered microbial population and caused the leakage of nutrients from plants (Naseby et al. 2000), thereby increasing the redox activity. The activity of AB + BSNP soil was nearer to control at 0 and 48 h. However, at 120 days, dehydrogenase activity of AB + SNP was significantly higher than AB alone but lower than non-treated control indicating its ability to reduce damage caused by pathogen. As the BSNPs were sprayed on plant leaves, soil enzyme activity was also assessed after complete decomposition of plants into the soil after 120 days of pathogen infection. After decomposition, activity of control (165.6 μg TPF g^{-1} soil h^{-1}) and BSNP (161.1 μg TPF g^{-1} dwt h^{-1}) were significantly comparable to each other indicating that no microbial activity was altered due to nanoparticles after 120 days, while at 0 and 48 h, dehydrogenase activity of BSNP and AB + BSNP-treated soil were significantly closer to control. While 1.39-fold reductions in AB were observed in comparison with control, AB + BSNP demonstrated 0.6-fold increase in activity compared to pathogen control (AB).

Protease and urease

Soil microflora are the main source of protease and urease activity in soil (Sardans and Penuelas 2005; Rejsek et al. 2008). Initially, no significant difference in protease activity was detected among the treatments, except for

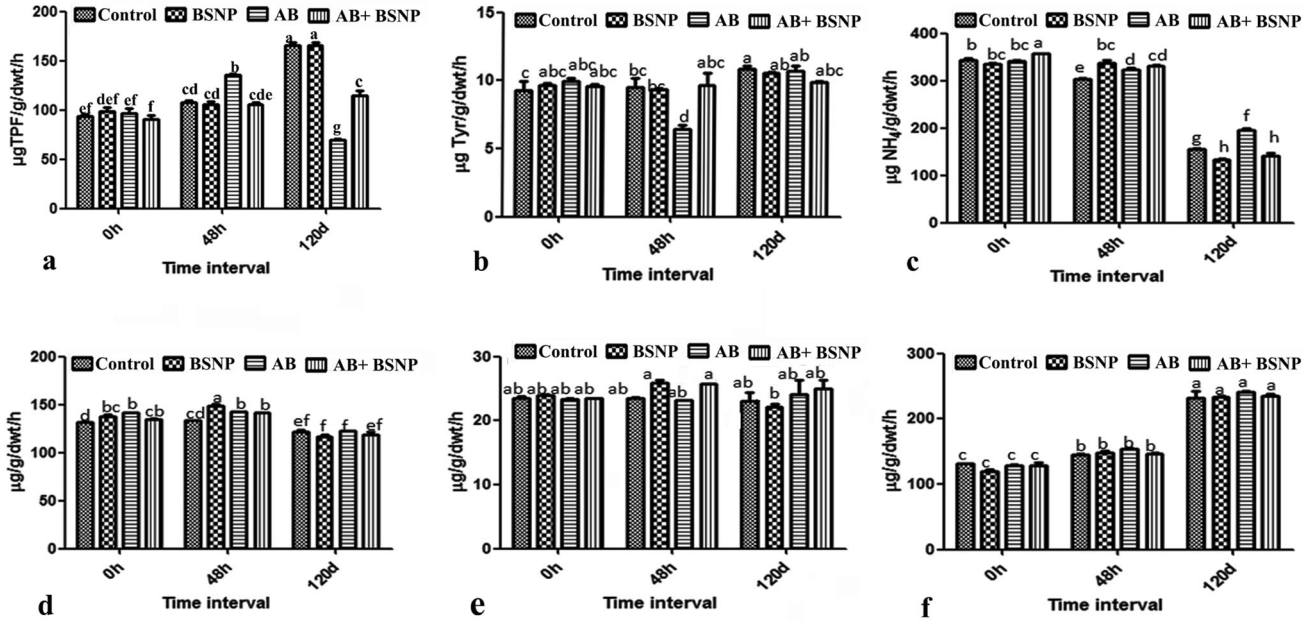

Fig. 3 Soil enzyme activities of (**a**) dehydrogenase (**b**) protease (**c**) urease (**d**) acid phosphatase (**e**) alkaline phosphatase (**f**) β-glucosidase at different time intervals 0, 48 h and after decomposition (120 days) after spray of nanoparticles over plants. Values are the means of three replicates. Means sharing different alphabets "a", "b" differ significantly from each other at $p \leq 0.05$

treatment of AB at 48 h (Fig. 3b). There was no significant difference in BSNP-treated and control soil in all the three time intervals. A significant decrease was observed in all the four treatments with respect to the time point (Fig. 3c). There was a decrease in urease activity at decomposition stage at 120 days post-treatment of BSNP, while an increase was observed in soil of AB treatment.

Acid and alkaline phosphatase

Phosphatase activity in soil denotes the soil fertility and plays a key role in soil ecosystem (Dick et al. 2000). The average acid phosphatase activity in all four treatments was 140 μg g^{-1} dwt h^{-1} at 48 h and decreased to 120 μg g^{-1} dwt h^{-1} at 120 days post-treatment (Fig. 3d). Furthermore, no significant difference was found in alkaline phosphatase activity (Fig. 3e). During the entire time period, among all the treatments, the activity ranged from 23 to 25 μg g^{-1} dwt h^{-1}.

β-Glucosidase

β-Glucosidase activity is commonly used as an indicator of soil quality because it depicts the ability of soil to degrade cellulose (Turner et al. 2002). There was no significant difference in β-glucosidase activity among any of the treatments (Fig. 3f). However, β-glucosidase activity did begin to increase at 48 h after treatment and reached an average of 215 μg g^{-1} dwt h^{-1} at 120 days. This increase in β-glucosidase activity at 120 days may have been caused by an increase in the amount of cellulose to the soil due to decomposition of plant material. The fate of SNPs in soil is regulated by many factors including surface structure, composition, and morphology of SNP and presence of clay minerals and organic matter in the soil (Dinesh et al. 2012; Peyrot et al. 2014). Prior studies have revealed that the toxicity of nanoparticles is reduced by the binding of nanoparticles with organic and inorganic sulphur and aggregation in soil (Levard et al. 2013; Wang et al. 2016). Low concentration of BSNP provided enhanced protection of plants towards *A. brassicicola* without altering the native soil properties.

Microbial diversity using carbon source utilization pattern

To monitor the effect of foliar application of BSNP on microbial activity and community composition in soil system, a community level approach based on carbon source utilization pattern by soil microflora was used (Mishra and Nautiyal 2009; Khan et al. 2012; Maliszewska 2016). The activity of soil microflora was assessed by AWCD which continued to increase for entire time duration for all the four treatments (Fig. 4). However, the AWCD was higher in AB treatment after 48 h and in decomposition stage after 120 days compared to control. These results might indicate the strong influence of *A. brassicicola* on native soil microbial community. Carbon

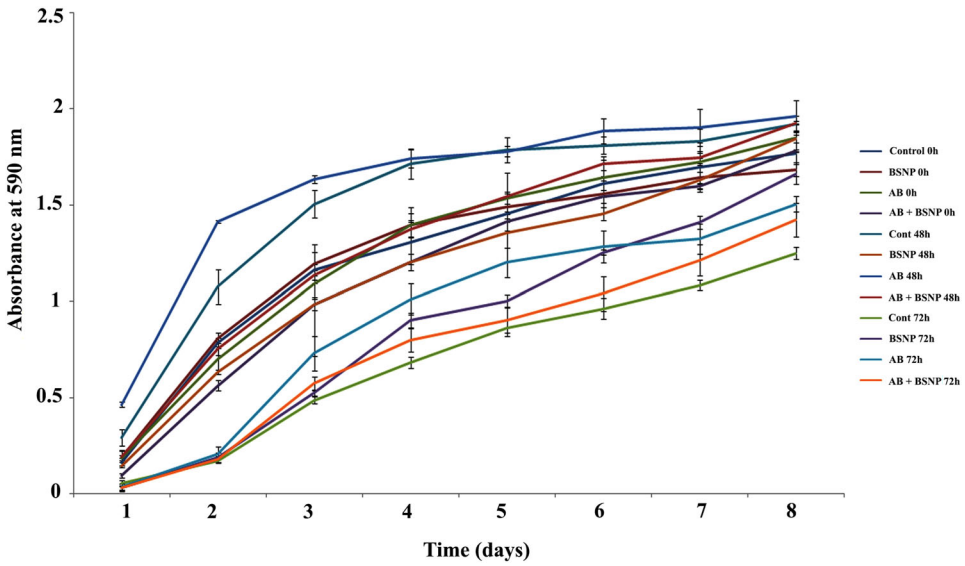

Fig. 4 Average well colour development (AWCD) based on substrate utilization pattern on Biolog Eco plates by soil samples of four treatments at different time intervals

utilization pattern for all carbon sources were also similar for BSNP and in control (Fig. S1). However, the carbon source utilization of D-xylose, D-galactouranic acid, L-phenyl alanine, tween 80 and glycogen was lower in the treatment of BSNP in comparison with control. Carbon source utilization pattern of AB-infected soil was completely different for most of the carbon sources in comparison with control. Biolog is a successful strategy to assess microbial community structure, functional capabilities of the microbial population, and metabolic efficacy of communities (Garland 1996). Biolog has proved its candidature to study the microbial communities from diverse habitats, such as freshwater, sea water, coastal lagoon, soil, rhizosphere, phyllosphere, groundwater, activated sludge reactors, and compost (Garland 1996; Khan et al. 2012). However, the effect of nanoparticles on metabolic capabilities on soil microbial communities needs to be investigated.

The treatments were further grouped in four, taking average of different time intervals and further PCA analysis was done. PCA plot showed that the four treatments were distributed separately on PC axes depicted by Factor 1 (84.7% variation) and Factor 2 (10.27% variation) (Fig. 5). The PCA results demonstrated that maximum change in microbial community structure of soil occurred in the treatment of AB, while AB + BSNP and BSNP were grouped nearer to control. The results obtained were similar with the earlier finding of Khan et al. (2012) where the tomato pathogen was grouped separately in PCA plot compared to control, while inoculation of biocontrol *Paenibacillus lentimorbus* B-30488^{r} was grouped nearer to control.

No significant differences among four treatments were noted for the samples collected using the McIntosh,

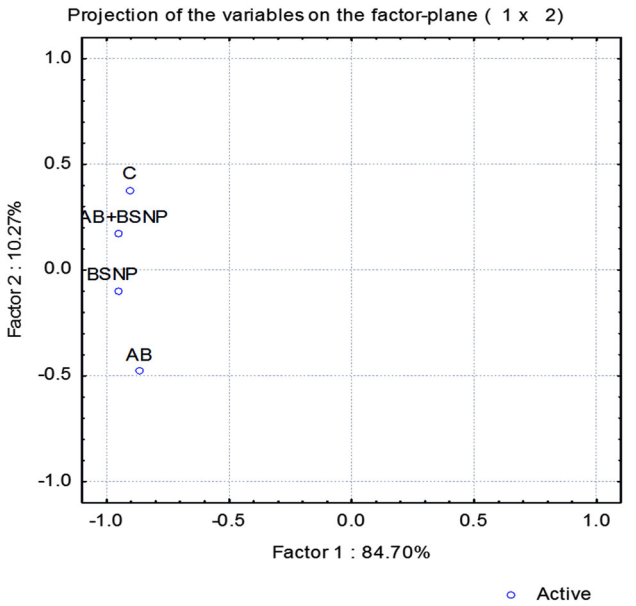

Fig. 5 Principal component analysis (PCA) of carbon source utilization pattern on Biolog Eco plates of the treatments: control (C), plants sprayed with biosynthesized silver nanoparticles (BSNP), *A. brassicicola*-infected plants (AB), and *A. brassicicola* + biosynthesized silver nanoparticles (AB + BSNP) in the soil microflora. PCA was performed using Statistica 7.0

Shannon, and Simpson indices (Table 3). These evenness and diversity indices are used to evaluate species diversity in a given population. The McIntosh, Shannon, and Simpson indices are alpha diversity indices based upon the statistical analysis of proportional species abundances. These results demonstrate that foliar application of BSNP of plant disease control did not alter the microbial diversity in soil at this concentration even after complete decomposition of plants into the soil.

Table 3 Diversity/evenness indices based on carbon utilization by microflora of *Arabidopsis* soil in four treatments Control, plants sprayed with biosynthesized silver nanoparticles (BSNP), *A. brassicicola*-infected plants (AB), and *A. brassicicola*-infected plants sprayed with BSNP (AB + BSNP)

	Control	BSNP	AB	AB + BSNP
Shannon diversity index	3.08 ± 0.038[a]	3.14 ± 0.032[a]	3.12 ± 0.045[a]	3.11 ± 0.013[a]
Shannon evenness index	0.91 ± 0.006[a]	0.92 ± 0.006[a]	0.92 ± 0.009[a]	0.91 ± 0.004[a]
Simpson diversity index	0.97 ± 0.002[a]	0.98 ± 0.001[a]	0.98 ± 0.002[a]	0.97 ± 0.002[a]
McIntosh diversity index	0.93 ± 0.006[a]	0.94 ± 0.003[a]	0.94 ± 0.007[a]	0.93 ± 0.005[a]
McIntosh evenness index	0.94 ± 0.004[a]	0.94 ± 0.002[a]	0.95 ± 0.005[a]	0.94 ± 0.004[a]

Values are the means of three replicates. Means sharing different alphabets "a", "b" differ significantly from each other at $p \leq 0.05$

DGGE analysis

Foliar application of BSNP will ultimately move to soil through plant transport and run off, so it becomes important to assess their interaction with native uncultured soil microflora. DGGE studies were carried out to compare different microbial communities residing in native soil before and after interaction with silver nanoparticles at different time intervals. Figure 6a shows the DGGE profile of different treatments at 0 h, 48 h and 120 days after spraying BSNP over *A. thaliana* leaves. No significant difference in banding pattern was observed among all four treatments at 48 h. After 120 days, banding pattern of control and BSNP were similar to the pattern observed earlier at 0 and 48 h, while AB and AB + BSNP depicted some differences in their bands. In Fig. 6a, arrows indicate the differential bands observed in AB and AB + BSNP. While number of bands demonstrating different bacterial communities decreased in AB treatment, AB + BSNP demonstrated maximum number of bands. Cluster analysis also revealed 100% similarity in all the treatments except for AB and AB + BSNP after 120 days. AB showed similarity of 59% with control, while AB + BSNP had 67% of similarity with control (Fig. 6b). The intensity of bands constantly increased in control with increase in time intervals depicting increase in bacterial population. The intensity of bands in AB after 48 h and 120 days decreased significantly which indicates that the pathogen had a negative impact on uncultured flora of soil. However, the intensity of BSNP and AB + BSNP was comparable to control.

Few studies have explored the interaction between nanoparticles with uncultured soil microflora and to demonstrate equal role of concentration of particles and nature of soil, clay and organic matter in assessing their effects on native microbial communities (Schlich and Hund-Rinke 2015; Berry et al. 2016). Klitzke et al. (2015) proposed that soil with higher organic matter content and clay enhance aggregation and sorption of nanoparticles, but the mechanism remains unknown. In this study, 5 µg/mL

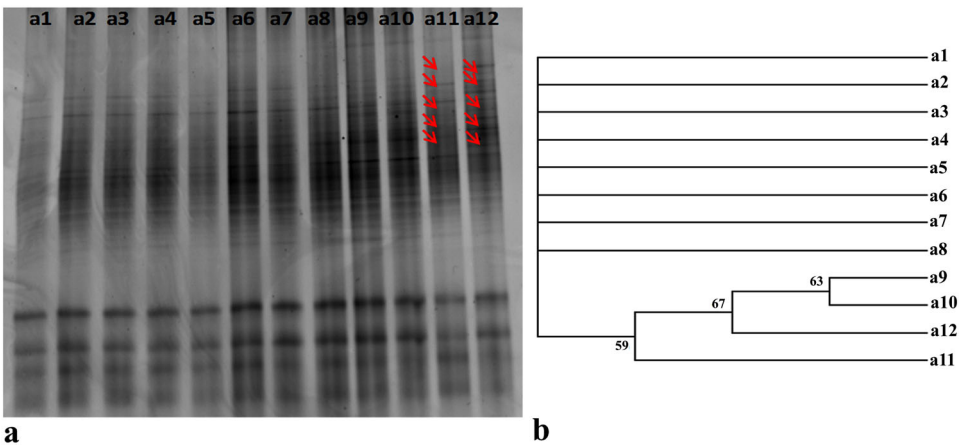

Fig. 6 **a** DGGE gel profiles obtained from soil samples under different treatments: Control (C), plants sprayed with biosynthesized silver nanoparticles (BSNP), *A. brassicicola*-infected plants (AB), and *A. brassicicola* + biosynthesized silver nanoparticles (AB + BSNP) at different time point. Arrows indicate differential bands obtained. **b** Generated dendogram with bands obtained in DGGE gel profile. a1 control (0 h), a2 BSNP (0 h), a3 AB (0 h), a4 AB + BSNP (0 h), a5 control (48 h), a6 BSNP (48 h), a7 AB (48 h), a8 AB + BSNP (48 h), a9 control (120 days), a10 BSNP (120 days), a11 AB (120 days), a12 AB + BSNP (120 days)

of BSNP did not alter the native soil microflora. Under soil conditions, agglomeration of nanoparticles is favoured (Mishra et al. 2014; Rahmatpour et al. 2017) and their toxicity is reduced which makes them safe for their innumerable applications in agriculture.

Conclusions

In conclusion, we found that foliar application of 5 µg/mL of BSNP did not alter the cultured and uncultured soil microflora even after decomposition of plants into soil. The biosynthesized particles acted as a potent antifungal agent causing 60–70% decrease in disease severity in model pathosystem of *A. thaliana* and *A. brassicicola.* No effect on the heterogeneous population of microbes, soil properties and soil enzymes were observed at different time intervals. BIOLOG studies revealed that AWCD continued to increase for entire time duration for all the four treatments; however, the AWCD was higher to some extent in AB treatment after 48 h and in decomposition stage compared to control. No significant differences among treatments were noted for the samples collected using the McIntosh, Shannon, and Simpson indices indicating that silver nanoparticles treatment as pathogen control on plant did not alter the microbial diversity in soil. DGGE studies revealed that there was a significant decrease in bacterial diversity after 120 days of *Alternaria* infection. In contrast, the application of BSNPs did not result in significant differences in uncultured bacterial population in comparison with the control in any of the time point. Fate of nanoparticles in soil largely depends upon the concentration and coating of particles and soil properties. Clay and organic matters can cause nanoparticles to aggregate which significantly diminishes the toxicity. Though the effect of multiple applications and long-term use of nanoparticles as plant disease management still needs to be assessed, their use as foliar spray to combat plant pathogen doesn't alter the native microflora in the soil.

Acknowledgements This study was partially funded by network project of Council of Scientific and Industrial Research (CSIR) "Root SF BSC0204". MK thanks CSIR for awarding her Senior Research Fellowship (SRF).

Compliance with ethical standards

Conflict of interest Authors do not declare any conflict of interest.

References

Balakumaran MD, Ramachandran R, Balashanmugam P, Mukeshkumar DJ, Kalaichelvan PT (2016) Mycosynthesis of silver and gold nanoparticles: optimization, characterization and antimicrobial activity against human pathogens. Microbiol Res 182:8–20

Balashanmugam P, Balakumaran MD, Murugan R, Dhanapal K, Kalaichelvan PT (2016) Phytogenic synthesis of silver nanoparticles, optimization and evaluation of in vitro antifungal activity against human and plant pathogens. Microbiol Res 192:52–64

Berry TD, Clavijo AP, Zhao Y, Jafvert CT, Turco RF, Filley TR (2016) Soil microbial response to photo-degraded C60 fullerenes. Env Poll 21:338e345

Dick WA, Cheng L, Wang P (2000) Soil acid and alkaline phosphatase activity as pH adjustment indicators. Soil Biol Biochem 32:1915–1919

Dinesh R, Anandaraj M, Srinivasan V, Hamza S (2012) Engineered nanoparticles in the soil and their potential implications to microbial activity. Geoderma 173:19–27

Garland JL (1996) Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biol Biochem 28:213–221

Girilal M, Krishnakumar V, Poornima P, Fayaz AM, Kalaichelvan PT (2015) A comparative study on biologically and chemically synthesized silver nanoparticles induced Heat Shock Proteins on fresh water fish *Oreochromis niloticus*. Chemosphere 139:461–468

Hänschand M, Emmerling C (2010) Effects of silver nanoparticles on the microbiota and enzyme activity in soil. J Plant Nutr Soil Sci 173:554–558

He S, Feng Y, Ni J, Sun Y, Xue L, Feng Y, Yu Y, Lin X, Yang L (2016) Different responses of soil microbial metabolic activity to silver and iron oxide nanoparticles. Chemosphere 147:195–202

Hosseini F, Mosaddeghi MR, Hajabbasi MA, Mamedov AI (2017) Effects of endophyte-infected and non-infected tall fescue residues on aggregate stability in four texturally different soils. Geoderma 285:195–205

Ignatova LV, Brazhnikov YV, Berzhanova RZ, Mukasheva TD (2015) Plant growth-promoting and antifungal activity of yeasts from dark chestnut soil. Microbiol Res 175:78–83

Janvier C, Villeneuve F, Alabouvette C, Edel-Hermann V, Mateille T, Steinberg C (2007) Soil health through soil disease suppression: which strategy from descriptors to indicators. Soil Biol Biochem 39(1):1–23

Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol Fert Soil 6:68–72

Khan N, Mishra A, Nautiyal CS (2012) *Paenibacillus lentimorbus* B-30488^r controls early blight disease in tomato by inducing host resistance associated gene expression and inhibiting *Alternaria solani*. Biol Control 62:65–74

Khodakovskaya MV, De Silva K, Biris AS, Dervishi E, Villagarcia H (2012) Carbon nanotubes induce growth enhancement of tobacco cells. ACS Nano 6(3):2128–2135

Klitzke S, Metreveli G, Peters A, Schaumann GE, Lang F (2015) The fate of silver nanoparticles in soil solution—sorption of solutes and aggregation. Sci Total Environ 535:54–60

Kumar N, Palmer GR, Shah V, Walker VK (2014) The effect of silver nanoparticles on seasonal change in Arctic tundra bacterial and fungal assemblages. PLoS One 9(6):e99953

Kumari M, Mishra A, Pandey S, Singh SP, Chaudhry V, Mudiam MKR, Shukla S, Kakkar P, Nautiyal CS (2016) Physico-Chemical condition optimization during biosynthesis lead to development of improved and catalytically efficient gold nanoparticles. Sci Rep 6:27575

Kumari M, Pandey S, Giri VP, Bhattacharya A, Shukla R, Mishra A, Nautiyal CS (2017a) Tailoring shape and size of biogenic silver nanoparticles to enhance antimicrobial efficacy against MDR bacteria. Microb Pathog 105:346–355

Kumari M, Shukla S, Pandey S, Giri VP, Bhatia A, Tripathi T, Kakkar P, Nautiyal CS, Mishra A (2017b) Enhanced cellular internalization: a bactericidal mechanism more relative to biogenic nanoparticles than chemical counterparts. ACS Appl Mat Intefaces 9:4519–4533

Ladd JN, Butler JHA (1972) Short-term assays of soil proteolytic enzyme activities using proteins and di-peptide derivatives as substrates. Soil Biol Biochem 4:19–30

Levard C, Hotze EM, Lowry GV, Brown GE (2013) Environmental transformations of silver nanoparticles: impact on stability and toxicity. Environ Sci Technol 46(13):6900–6914

Maliszewska I (2016) Effects of the biogenic gold nanoparticles on microbial community structure and activities. Ann Microbiol 66:785–794

Mishra A, Nautiyal CS (2009) Functional diversity of the microbial community in the rhizosphere of chickpea grown in diesel fuel-spiked soil amended with *Trichoderma reesei* using sole-carbon-source utilization profiles. World J Microbiol Biotechnol 25:1175–1180

Mishra S, Singh HB (2014) Biosynthesized silver nanoparticles as a nanoweapon against phytopathogens: exploring their scope and potential in agriculture. Appl Microbiol Biotechnol 99(3):1097–1107

Mishra A, Kumari M, Pandey S, Chaudhry V, Gupta KC, Nautiyal CS (2014) Biocatalytic and antimicrobial activities of gold nanoparticles synthesized by *Trichoderma* sp. Biores Technol 166:235–242

Naseby DC, Pascual JA, Lynch JM (2000) Effect of biocontrol strains of *Trichoderma* on plant growth, *Pythium ultimum* populations, soil microbial communities and soil enzyme activities. J Appl Microbiol 88:161–169

Nelson DW, Sommers LE (1996) Methods of soil analysis. Part 3. Chemical methods. Soil Science Society of America, American Society of Agronomy, Madison, pp 961–1010

Ocsoy I, Paret ML, Ocsoy MA, Kunwar S, Chen T, You M, Tan W (2013) Nanotechnology in plant disease management: DNA-directed silver nanoparticles on graphene oxide as an antibacterial against *Xanthomonas perforans*. ACS Nano 7(10):8972–8980

Peyrot C, Wilkinson KJ, Desrosiers M, Sauvé S (2014) Effects of silver nanoparticles on soil enzyme activities with. and without added organic matter. Environ Toxicol Chem 33(1):115–125

Rahmatpour S, Shirvani M, Mosaddeghi MR, Nourbakhsh F, Bazarganipour M (2017) Dose–response effects of silver nanoparticles and silver nitrate on microbial and enzyme activities in calcareous soils. Geoderma 285:313–322

Rejsek K, Formanek P, Pavelka M (2008) Estimation of protease activity in soils at low temperatures by casein amendment and with substitution of buffer by demineralised water. Amino Acids 35:411–417

Sardans J, Penuelas J (2005) Drought decreases soil enzyme activity in a Mediterranean *Quercus ilex* L. forest. Soil Biol Biochem 37:455–461

Schlich K, Hund-Rinke K (2015) Influence of soil properties on the effect of silver nanomaterials on microbial activity in five soils. Environ Poll 196:321–330

Sillen WMA, Thijs S, Abbamondi RG, Janssen J, Weyens N, White JC, Vangronsveld J (2015) Effects of silver nanoparticles on soil microorganisms and maize biomass are linked in the rhizosphere. Soil Biol Biochem 91:14e2

Singh AK, Rai A, Singh N (2016) Effect of long term land use systems on fractions of glomalin and soil organic carbon in the Indo-Gangetic plain. Geoderma 277:41–50

Srivastava PK, Gupta M, Pandey A, Pandey V, Singh N, Tewari SK (2014) Effects of sodicity induced changes in soil physical properties on paddy root growth. Plant Soil Environ 60:165–169

Tabatabai MA (1994) Soil enzymes. In: Mickelson SH, Bigham JM (eds) Methods of soil analysis, part 2. Microbiological and biochemical properties. SSSA, Madison

Tan Y, Cui Y, Li H, Kuang A, Li X, Wei Y, Ji X (2017) Rhizospheric soil and root endogenous fungal diversity and composition in response to continuous *Panax notoginseng* cropping practices. Microbiol Res 194:10–19

Turner BL, Hopkins DW, Haygarth PM, Ostlec N (2002) β-Glucosidase activity in pasture soils. Appl Soil Ecol 20(2):157–162

Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. Soil Biol Biochem 19:703–707

Wang Z, Zhang L, Zhao J, Xing B (2016) Environmental processes and toxicity of metallic nanoparticles in aquatic systems as affected by natural organic matter. Environ Sci Nano 3:240–255

Wolińska A, Stępniewska Z (2012) Activity in the soil environment "dehydrogenases". In: Canuto RA (ed) Dehydrogenase. Intech publishing group, Croatia, pp 183–210

Xiu-Mei L, Qi L, Wen-Ju L, Yong J (2008) Distribution of soil enzyme activities and microbial biomass along a latitudinal gradient in farmlands of Songliao plain, Northeast China. Pedosphere 18(4):431–440