RESEARCH   ARTICLE

Impact of Metal Oxide Nanoparticles on Beneficial Soil Microorganisms and their Secondary Metabolites

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ABSTRACT- In this study, the effect of ZnO and TiO$_2$-NPs on beneficial soil microorganisms and their secondary metabolite production was investigated. The antibacterial potential of NPs was determined by the growth kinetics of *Pseudomonas aeruginosa, P. fluorescens* and *Bacillus amyloliquefaciens*. Significantly decreased in the cell viability based on optical density measurements were observed upon treatment with increasing concentrations of NPs. While comparing the effect of the different concentrations of the NPs (200 μg/ml) on IAA production by different bacterial strains, ZnO nanoparticles showed greater inhibitory effect than TiO$_2$-NPs on IAA production by bacterial strains. The effect of Nanoparticles on phosphate solubilization was found inhibitory at 200 μg/ml. Treatment with ZnO shown concentration dependent enhancement in siderophore production by bacteriaby exposure to ZnO-NPs whereas TiO$_2$-NPs showed concentration dependent progressive decline for iron binding siderophore molecules. Reduction in antibiotic production by *P. aeruginosa* and *P. fluorescens* was noticed in the presence of ZnO and TiO$_2$ as compared to the control. The fluorescence of NADH released by *P. aeruginosa* was observed to be quenched in the presence of ZnO and TiO$_2$-NPs as compared to control. The present study highlights that the impact of nanoparticles on bacterial strains and the release of plant growth promoting substances by PGPR strains was dose dependent, which gives an idea about the level of toxicity of these nanoparticles in the environment. Therefore, the discharge of nanoparticles in the environment should be carefully monitored so that the loss of both structure and functions of agronomically important microbes could be protected from the toxicity of MO-NPs.

Key-words- MO-NPs, IAA, Phosphate Solubilization, Siderophore, PCA, NADH, ZnO-NPs, TiO$_2$-NPs

INTRODUCTION

Nanotechnology manipulates the enhanced reactivity of materials at the atomic scale for the advancement of various applications for humankind. Various metal oxide nanoparticles, due to their optical, electrical and magnetic properties [1] have numerous applications included sensors, catalysis, biomedical diagnostics and environmental remediation [2-4]. Since engineered nanoparticles (ENPs) released to the environment go down the soil, the effects of ENPs on soil processes and the organisms that carry them out should be grasped.

As present inadequate information is available on how these ENPs affect the soil microorganisms. They affect soil microorganisms via (i) A direct toxic effect, (ii) Changes in the bioavailability of toxins or nutrients, (iii) Indirect effects resulting from their interaction with natural organic compounds, and (iv) Interaction with toxic organic compounds which would increase or reduce their toxicity. Various reports demonstrated that metal oxide nanoparticles exhibit excellent antimicrobial activity against Gram-positive and Gram-negative bacteria [5]. The ENPs causes changes in the structure of the cell surface of the microorganisms that may finally lead to cell death [6]. Hence, it is clear that ROS production occurs in microorganisms and cause damage to the cell components [7]. Because of the antimicrobial efficacy of ZnO nanoparticles, they possess the potential to affect many aspects of food and agricultural systems, especially with the growing need to find alternative methods for formulating new type of safe and cost-effective antibiotics in

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controlling the spread of resisting pathogens in food processing environment [8,9]. It is thought that the adherenceof the particles on the surface of bacteria due to the electrostatic forces could be a mechanism of the antibacterial activity of ZnO particles [10]. Various mechanisms other than ROS production for e.g. zinc ion release, membrane dysfunction, and nanoparticles internalization could also be the possible mechanisms of the cell damage [11]. Nano-materials for example, carbon nanotubes [12,13], graphene-based nanomaterials [14], iron based nanoparticles [15], silver [16] and copper, zinc and titanium oxide nanoparticles [17,18] have been reported to cause biologically undesirable toxiceffects on both deleterious and beneficial soil microorganisms [19-21] including *Escherichia coli*, *B. subtilis*, and *Streptococcus aureus* [22], *P. chlororaphis* [23-26], *P. putida* [21] and *Campylobacter jejuni* [27]. Though, the contradictory reports have been found on the impact of nanoparticles on secondary metabolites produced by microorganisms. For example, in recent study, Dimkpa et al. [23] reported that sub-lethal levels of CuO-NPs reduced the siderophore production in *P. chlororaphis* O_6 whereas ZnO-NPs increased the pyoverdine siderophore production. Similarly, Brumfiel [28] reported a contrasting effect of CuO and ZnO-NPs on IAA production in which, there was an approximately 53% increase for cultures of *P. chlororaphis* O6 with CuO-NPs and an approximately 43% reduction with ZnO-NPs, signifying that the effect of NPs on secondary metabolite production by bacteria cannot be generalized rather it is highly nano specific and may vary from organisms to organisms. These findings are in agreement with results reported in another study done by Oves et al [29]. Nanomaterials are also affecting the human health very negatively [28,30,31] by damaging the DNA [32] and other cellular functions [33]. Therefore, to improve the soil ecosystem, a better understanding should be done on how nanomaterials affect microorganisms. Considering both the positive and negative aspect of the nanomaterials and lack of information of the impact of nanoparticles on the plant growth promoting activities of rhizobacteria, here, we examined the effect of ZnO and TiO₂nanoparticles on the growth characteristics and production of plant growth regulating substances by *P. aeruginosa, P. fluorescens and B. amyloliquefaciens.*

MATERIALS AND METHODS
Microbial Strains and culture conditions
Bacterial strains used in this study included the *P. aeruginosa, P. fluorescens* and *B. amyloliquefaciens,* which were obtained from the culture stocks of our laboratory, Dept. of Agricultural Microbiology, Aligarh. The strains were sub-cultured in Luria-bertani and Nutrient agar/broth and maintained on agar plates by sub-culturing. One set of these cultures was stored at -20ºC in 20% glycerol for long term preservation. All experiments were performed with the freshly grown cultures.

Nanoparticles Used
Technical grade (99% pure), Zinc oxide (ZnO) and Titanium dioxide (TiO₂) NPs were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Evaluation of antibacterial activity of metal oxide NPs
Time-dependent growth inhibition assay
The effect of ZnO and TiO₂-NPs on the growth of *P. aeruginosa*, *B. amyloliquefaciens* and *P. fluorescens* strains was assessed by the optical density (OD $_{600nm}$) measurements of treated and untreated bacteria. The aliquots of 100 µl each from freshly grown cultures of *P. aeruginosa, P. fluorescens* and *B. amyloliquefaciens* were inoculated in 5 ml Luria Bertani in glass test tubes maintaining aseptic conditions. NPs were added to achieve the increasing concentrations of 100–1600 µg/ml. Un-treated cells were used as a control. The treated and untreated samples (100 µl each) were transferred to a microtitre plate and incubated at 37^0C. The absorbance was measured at 600 nm at regular intervals of 2 h by use of a microplate reader (Thermo scientific Multiskan EX, REF 51118170, China) and growth curve were plotted as O.D Vs time.

Mechanism of interaction of NPs with bacterial cell
Scanning electron microscopy (SEM) and NPs bacterial cell interactions
Scanning electron microscopic analysis was carried out using fine powder of the NPs on a carbon tape in JSM 6510 LV scanning electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 15 kV. Also, the interaction studies for observing the morphological changes in *P. aeruginosa, B. amyloliquefaciens* and *P. fluorescens* cells upon treatment with MO-NPs and untreated control cells were performed by use of SEM. Briefly, the samples containing untreated and treated bacterial cells were deposited on a Millipore filter (Millipore). Bacterial cells (10^6 CFU/ml) were treated with 200 µg/ml MO-NPs for 12 h at 37°C and centrifuged at 3000 rpm for 10 min. The pellets were washed with phosphate-buffered saline (PBS) three times and pre-fixed with 2.5% glutaraldehyde for 1 h at 4°C. The pre-fixed cells were washed with PBS two times and post-fixed with 1% osmium tetroxide for 1 h at 25°C. After three successive washes with PBS, the samples were dehydrated by sequential treatment with 30, 50, 70, 80, 90 and 100% of ethanol for 10 min each. The cell biomasses were then fixed and the coated samples were observed under scanning electron microscope (SEM) (JEOL, Tokyo, Japan) at an accelerating voltage of 10-15 kV.

Transmission electron microscopy (TEM) and NPs bacterial cell interactions
Electron micrographs of control and MO-NPs treated bacterial cells were taken using an FEI Technai G2 TEM instrument operated at an accelerating voltage of 200 kV,

as described by Ansari et al. (2014b) [35]. In order to assess the interaction of MO-NPs with bacteria, the cells were grown in 20 ml LB medium at 35 ± 2°C for 24 h. The culture was centrifuged at 12,000 rpm for 10 min and pellet was suspended in 1X PBS. The suspension containing bacterial cells (10^{6} CFU/ml) was then treated with 200 µg/ml of MO-NPs and incubated at 37°C with shaking at 150 rpm for 6 h. Control without NPs was run in parallel. The suspension was then centrifuged and the cell pellet was washed thrice with 1X PBS and pre-fixed with 2.5% glutar aldehyde for 1 h at 4°C. The cells were recovered by centrifugation at 12,000 rpm for 5 min and the pellet was again washed thrice with PBS. Cells were fixed in 1% osmium tetraoxide for 1 h. After three washes with 1X PBS, the fixed specimens were dehydrated by immersing in a series of graded acetone (30, 50, 60, 70, 80, 90, 95, and 100 % water/acetone mixtures) for 5 min each. Dehydrated specimens were embedded in white resin overnight. Ultra thin sections (50–70 nm thickness) were cut with a microtome diamond knife, stained with 2 % uranyl acetate (Sigma-Aldrich, USA) and counter stained with 2 % lead citrate (Sigma-Aldrich, USA). The sections were mounted on carbon coated copper grids and finally, the internal structures of cells were observed by use of an HR-TEM (Technai, FEI, Electron Optics, USA) at an accelerating voltage of 120 keV.

Effect of MO-NPs on secondary metabolites produced by bacterial strains
Effect of nanoparticles on IAA production
IAA was quantitated according to the method of Bric et al. [35] in inoculated nutrient broth without and with tryptophan (50, 100, 250 and 500 µgmL^{-1}) at 28 ± 2°C. For IAA inhibition assay, NPs in the concentration range of 50–400 µgmL^{-1} was added to the tubes containing strains in nutrient broth supplemented with tryptophan (500 µgmL^{-1}) and processed for IAA measurements on 4th day.

Effect of nanoparticles on phosphate solubilization
The soluble phosphate released was quantitated following the method of King [36]. For determining the nanoparticles mediated inhibition of inorganic phosphate solubilization, the NPs in concentration range of 50–400 µgmL^{-1} was added to the tubes containing strains in Pikovskaya's liquid medium and processed after 5th day of incubation for phosphate measurements.

Effect of nanoparticles on Siderophore production
CAS liquid assay for the quantitative estimation was performed in accordance with the original protocol (Schwyn and Neilands [37]. For determining the nanoparticles impact on siderophore production, 200 and 400 µgmL^{-1} NPs concentration was added to the tubes containing medium and test strains and processed for siderophore detection.

Effect of nanoparticles on antibiotic production
The phenazine-1-carboxylic acid (PCA) production in bacterial cell culture was determined by the following method of Mavrodi *et al.* [38]. For determining the nanoparticles impact the nanoparticles at concentrations 100 and 200 µg/ml to the flasks containing cell culture of bacterial strains grown at 37^{0}C in modified LB medium (LB +1 mM tryptophan) and processed for further measurements following the standard method.

Effect of nanoparticles on bacterial extracellular enzyme viz. NADH
Fluorescence spectra of the supernatants obtained from the untreated bacterial culture and those treated with 200 and 400 µg/ml ZnO and TiO$_2$-NPs was measured in 1 cm path length cell by use of Shimadzu spectrofluorophotometer, model RF5301PC (Shimadzu Scientific Instruments, Japan) equipped with RF 530XPC instrument control software, at ambient temperature. The excitation and emission slits were set at 5 nm each. The emission spectra were recorded in the wavelength range of 300–600 nm and the excitation wavelength was set at 280 nm.

STATISTICAL ANALYSES
Data were expressed as a mean$\pm$S.D. for the values obtained from at least two independent experiments done in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Holm-Sidak method, multiple comparisons versus control group (Sigma Plot 10.0, USA). The level of statistical significance chosen was *$p < 0.05$, unless otherwise stated.

RESULTS AND DISCUSSION
Growth pattern of bacterial strains in the presence of MO-NPs
The study of the growth pattern of bacterial strains (*P. aeruginosa, P. fluorescens and B. amyloliquefaciens*) was studied while growing the strains in the absence and presence of MO-NPs at concentration ranging from 100-1600 µg/ml. Bacterial growth was monitored after every two hours. Growth curves showed that the bacterial growth decreased with increasing concentration of MO-NPs (Fig. 1). Yamamoto [39] found that the higher concentration and the larger surface area can obtain the better antibacterial activity. Bacterial growth inhibition may be caused by the interaction of MO-NPs with the bacterial membrane, causing pitting of the cell wall, dissipation of the proton motive force, and consequently the cell death [40]. Also, MO-NPs have also been reported to interact with bacterial DNA, leading to the DNAs replication damage [41,42]. These interpretations agree with those of the previous work reported by Baek and An [22]. Jones *et al.* [43] also determined the detrimental effect of ZnO against broad spectrum microorganisms.
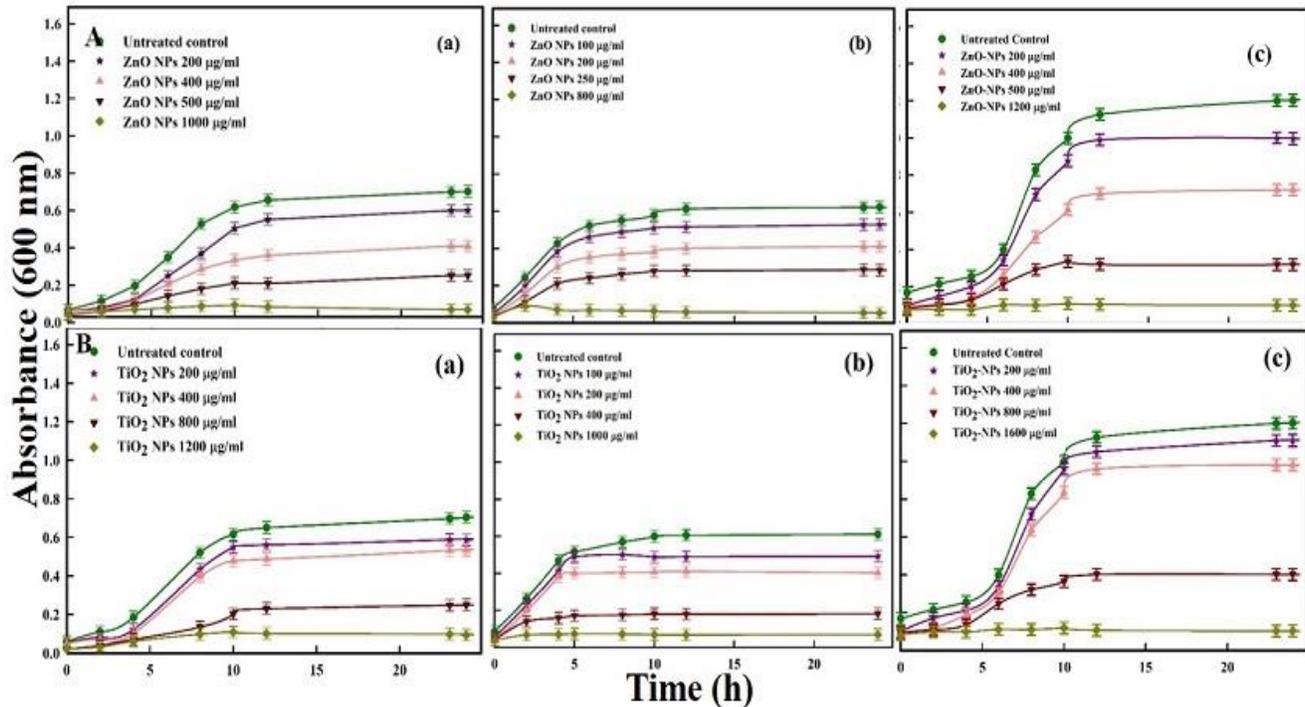
Fig. 1: Growth analysis curves of (a) *P. aeruginosa* (b) *B. amyloliquefaciens* and (c) *P. fluorescens* through optical density (OD$_{600}$ nm) measurements at different (A) ZnO and (B) TiO$_2$ concentrations

Nanoparticles bacterial interactions

Exposure of *P. aeruginosa, P. fluorescens* and *B. amyloliquefaciens* cells with 200 µg/ml MO-NPs for 6 h resulted in significant interaction of NPs with bacterial cell surface. The NPs binding to the cellular targets and consequent structural alterations in the cell envelope have been observed by SEM and HR-TEM analysis (Fig. 2 & Fig. 3). The SEM images explicitly revealed the normal morphology of the bacteria, and clustering of NPs around the cells (Fig. 2). The NPs were present all over the cells and some NPs penetrate the cell membrane and become intracellular as shown in HR-TEM micrographs (Fig. 3).

The untreated control of *P. aeruginosa*, and *P. fluorescens* cells show a moderate size Gram-negative bacillus with the normal internal structures and multilayered cell surface consisting of an outer membrane, a peptidoglycan layer in the periplasmic space, and a cytoplasmic membrane whereas the untreated control of *B. amyloliquefaciens* shows a moderate size Gram-positive bacillus with the normal internal structures and cell surface consisting of a cytoplasmic membrane and a peptidoglycan layer. However, the MO-NPs treated cells exhibited a severely damaged membrane with multiple pits and gaps. The HR-TEM images revealed that the attachment of NPs to the outer membrane of the cell, which further leads to cell breakage due to membrane fragmentation and results in cell lysis.

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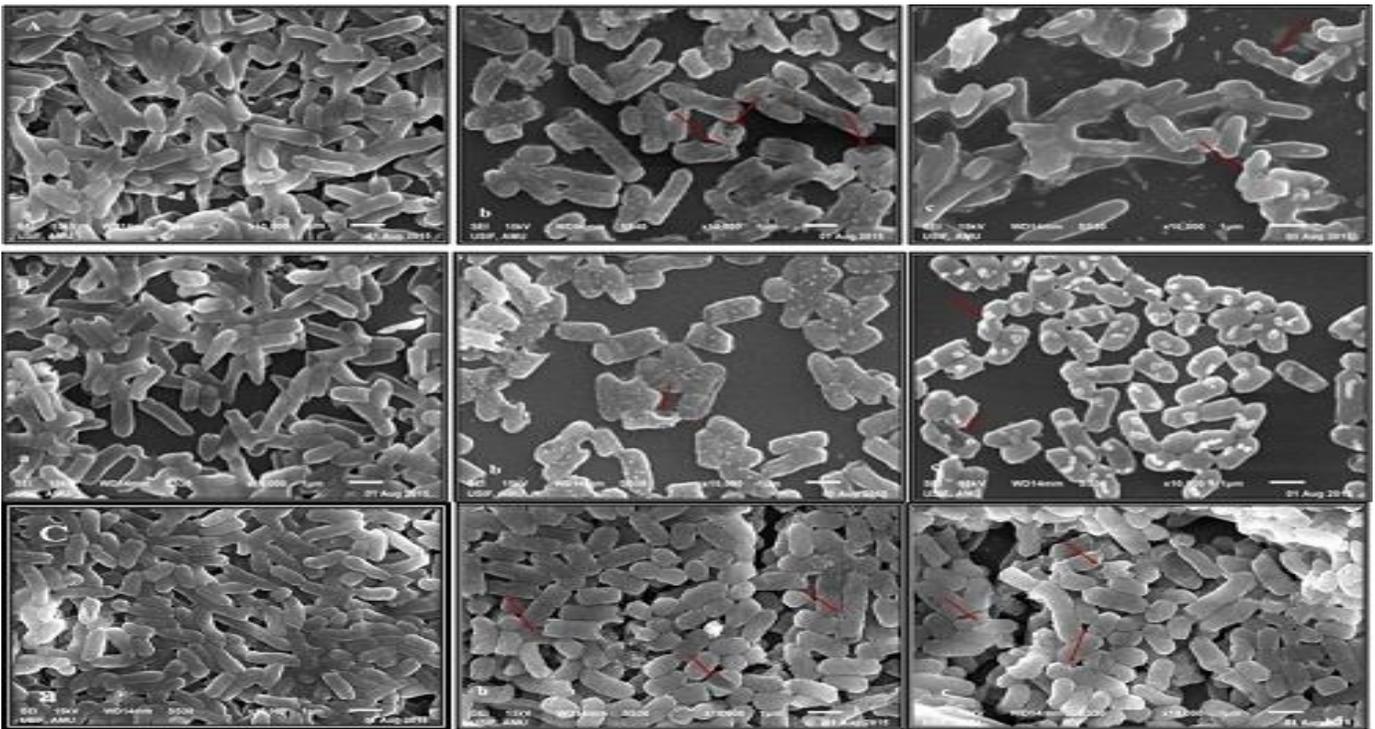
Fig. 2: SEM micrographs of (A) *B. amyloliquefaciens* (B) *P. aeruginosa* and (C) *P. fluorescens* cells. Representative images show the cell biomass, sputter coated with a thin layer of gold (Sputter coater Polaron SC7640) using a scanning electron microscope (Carl Zeiss EVO 40, Germany) at an accelerating voltage of 15 kV. (a) Depict the untreated control cells (b) & (c) Depict cells treated with TiO_2-NPs and ZnO-NPs respectively
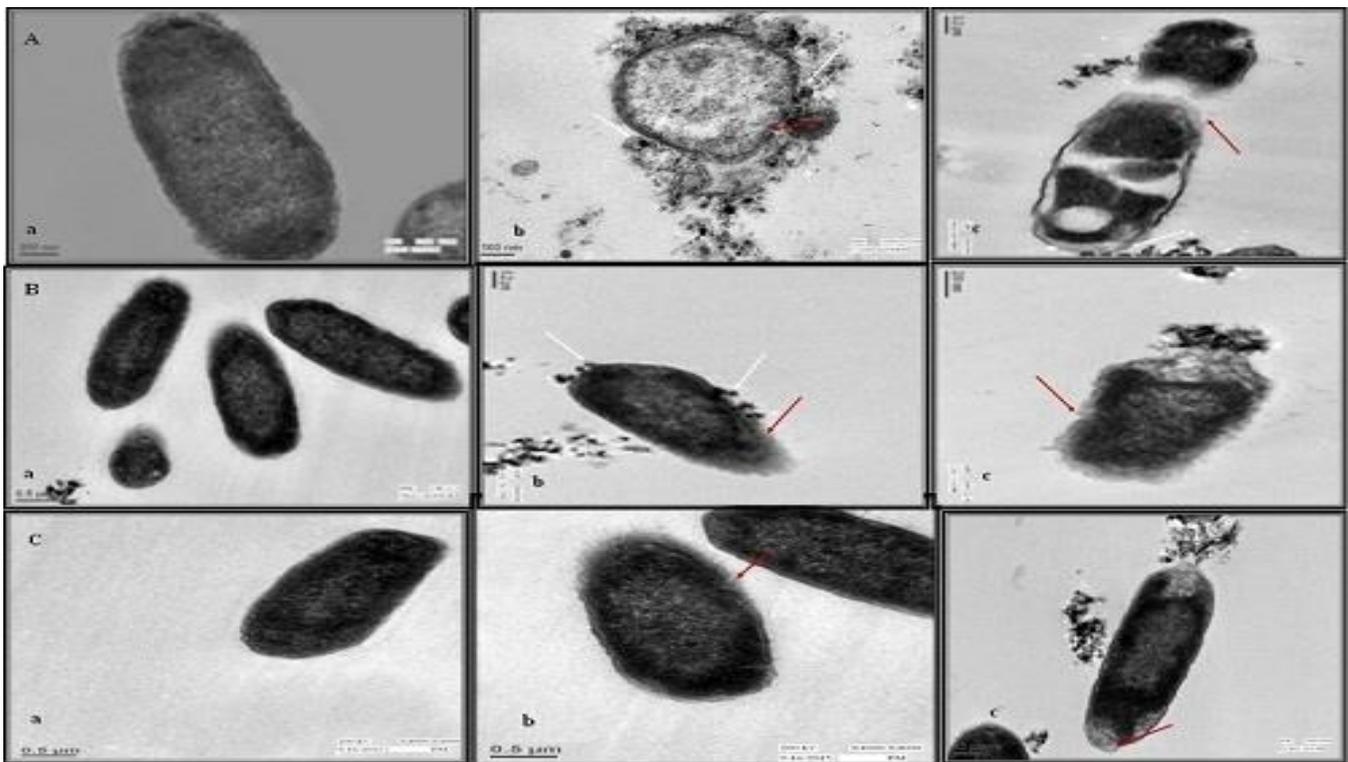
Fig. 3: HR-TEM images of (A) *B. amyloliquefaciens* (B) *P. aeruginosa* and (C) *P. fluorescens* cells. The panels   depict the representative HR-TEM images of ultrathin sections of the cells. (a) Untreated cell (b) and (c) cells treated with TiO_2 and ZnO-NPs at a magnification of 15,000 X, observed through the HR-TEM (Technai, FEI, Electron Optics, USA) instrument at an accelerating voltage of 200 kV. White arrow indicates NPs attachment on membrane, red arrow showed partially damaged membranes at various sites

Effect of MO-NPs on secondary metabolites produced by bacterial strains

Impact of MO-NPs on Indole Acetic Acid Production

The IAA produced by the bacterial strains under different concentrations of ZnO and TiO$_2$-NPs were variable. Generally, the IAA was produced both under normal and MO-NPs treated bacterial strains inoculated in NB medium, but the level of IAA under NPs was considerably lower compared to those detected in untreated medium. For example, *Pseudomonas aeruginosa, P. fluorescens,* and *B. amyloliquefaciens* produced a significant amount of IAA (23.76$\pm$0.60 , 34.30$\pm$1.0, 29.88$\pm$1.01 µgml^{-1}) when grown in medium devoid of NPs. Interestingly, the IAA production decreased with increasing concentrations of each ZnO and TiO$_2$-NPs . While comparing the effect of different concentrations of the NPs (200 µg/ml) on IAA production by different bacterial strains, ZnO nanoparticles showed a greater deleterious effect than TiO$_2$-NPs on IAA production by *Pseudomonas aeruginosa, P. fluorescens,* and *B. amyloliquefaciens* and reduced it by 29.38% (p<0.05), 18.2% (p<0.05), and 58.37% (p<0.05), which was 19.53% (p<0.05), 12.37% and 47.19% (p<0.05), for TiO$_2$, respectively, relative to the control (Fig. 4). Based on this, the order of NPs toxicity is as follows: ZnO> TiO$_2$. Also, in a recent study, Dimkpa *et al.* [24] found that the amendment of the medium with CuO and ZnO-NPs modified IAA levels from those of the control plant growth promoting Gram negative bacterium *P. chlororaphis*. They found that CuO-NPs increased the IAA production significantly in a 48 h grown culture while ZnO-NPs reduced IAA levels relative to the control. The extent of inhibition of IAA formation caused by the ZnO-NPs was however reduced by co-addition of CuO-NPs; the amount of IAA released by bacterial cell grown with mixture of NPs was closer to that of cells grown solely with CuO-NPs. Interestingly, the *P.chlororaphis* cells exposed to sub-lethal concentration of CuO-NPs was found to metabolize tryptophan more aggressively than cells grown with ZnO-NPs. The differential effects of NPs on bacterial metabolisms thus suggest that the nanoparticles may have a specific target in bacterial population.
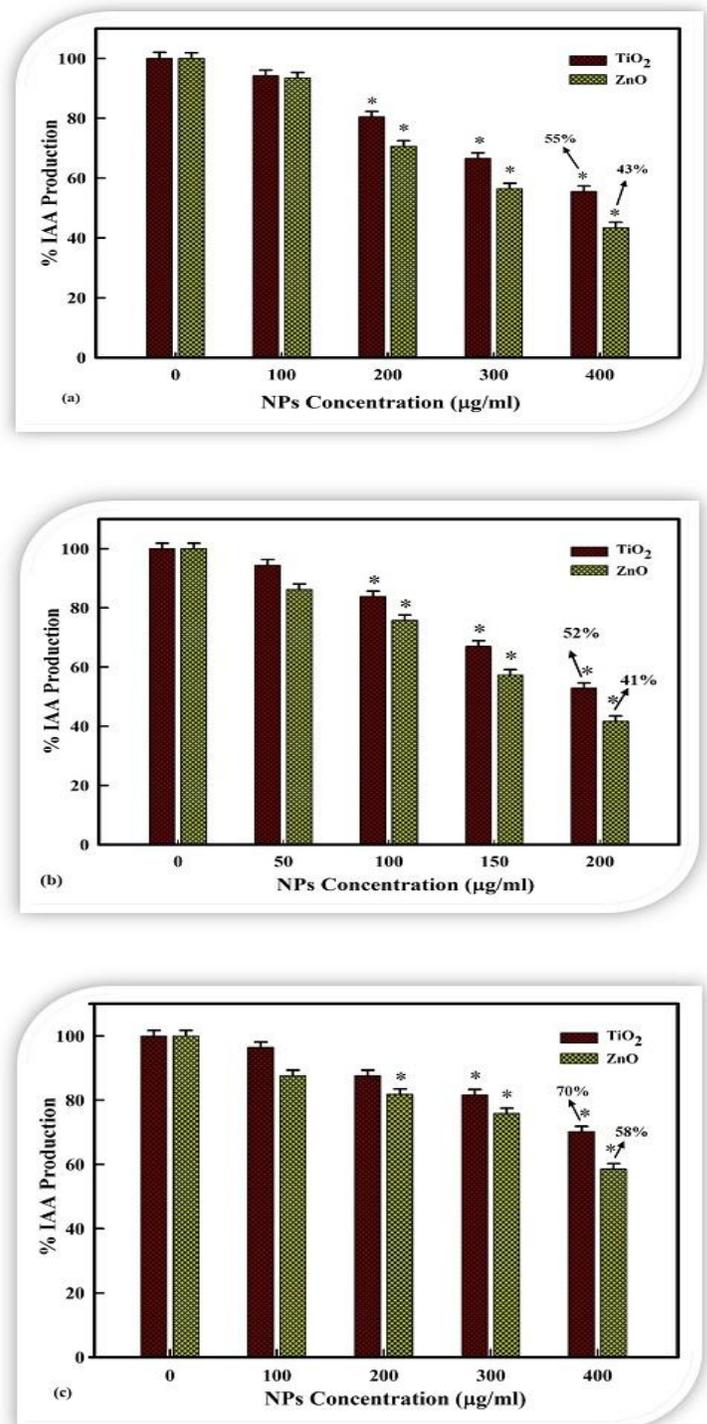
Fig. 4: MO-NPs concentration dependent inhibition of IAA activity of (a) *P. aeruginosa* (b) *B. amyloliquefaciens* and (c) *P. fluorescens* (*p<0.05)

Impact of MO-NPs on Phosphat solubilization

The ZnO and TiO₂were found to inhibit the phosphate solubilizing activity of the bacterial strains *P. aeruginosa, P. fluorescens, B. amyloliquefaciens*. An estimated 35.6%, 35.61%, 57.6% at 200 μgmL^{-1} ZnO-NPs ($p < 0.05$) and 19.53%, 12.37%, 47.19% at 200 μgmL^{-1} TiO₂ NPs ($p < 0.05$) reduction in phosphate solubilization has been noticed for *P. aeruginosa, P. fluorescens,* and *B. amyloliquefaciens* as compared to the control (Fig. 5).

Impact of MO-NPs on Siderophore production

Siderophore is a low molecular weight iron chelating peptide that provides protection to plants by making the accessibility of iron difficult to phytopathogens. Considering the significance of siderophore in scavenging iron in iron deficient environment [44-47] and help bacterial communities survive better in an iron-deficient environment, we determined the production of secondary metabolites: siderophores, by bacterial strains i.e *P. aeruginosa, P. fluorescens, and B. amyloliquefaciens* used in our study, using CAS liquid assay, treated with different concentrations of the nanoparticles. CAS liquid assay for the quantitative estimation showed the production of siderophores. In this regard, siderophores were detected in the culture supernatant of the bacterial strains, grown in the medium devoid of nanomaterials. ZnO showed concentration dependent enhancement in Siderophore production by *P. aeruginosa, P. fluorescens,* as well as *B. amyloliquefaciens* by exposure to ZnO-NPs, whereas TiO₂-NPs shown concentration dependent progressive decline for iron binding siderophore molecules as shown in Fig. 6. In agreement with our findings, Dimkpa *et al.* [23] observed that the sublethal level of CuO-NPs decreased the production of the fluorescent siderophore pyoverdine (PVD) by *P. chlororaphis* which could probably be due to the impairment of the genes involved in PVD secretion, whereas ZnO-NPs increased the production of the fluorescent siderophore pyoverdine by *P. chlororaphis.*
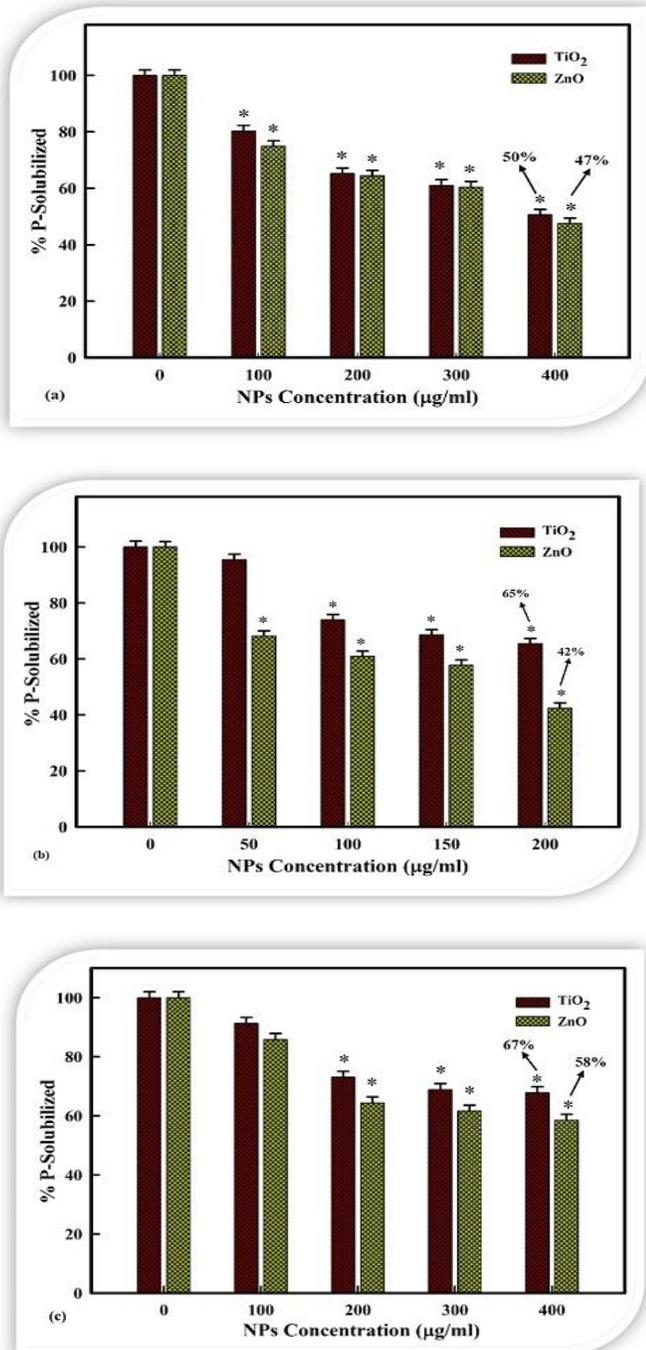
Fig. 5: MO-NPs concentration dependent inhibition of inorganic phosphate solubilization by (a) *P. aeruginosa* (b) *B. amyloliquefaciens*, (c) *P. fluorescens* in liquid Medium
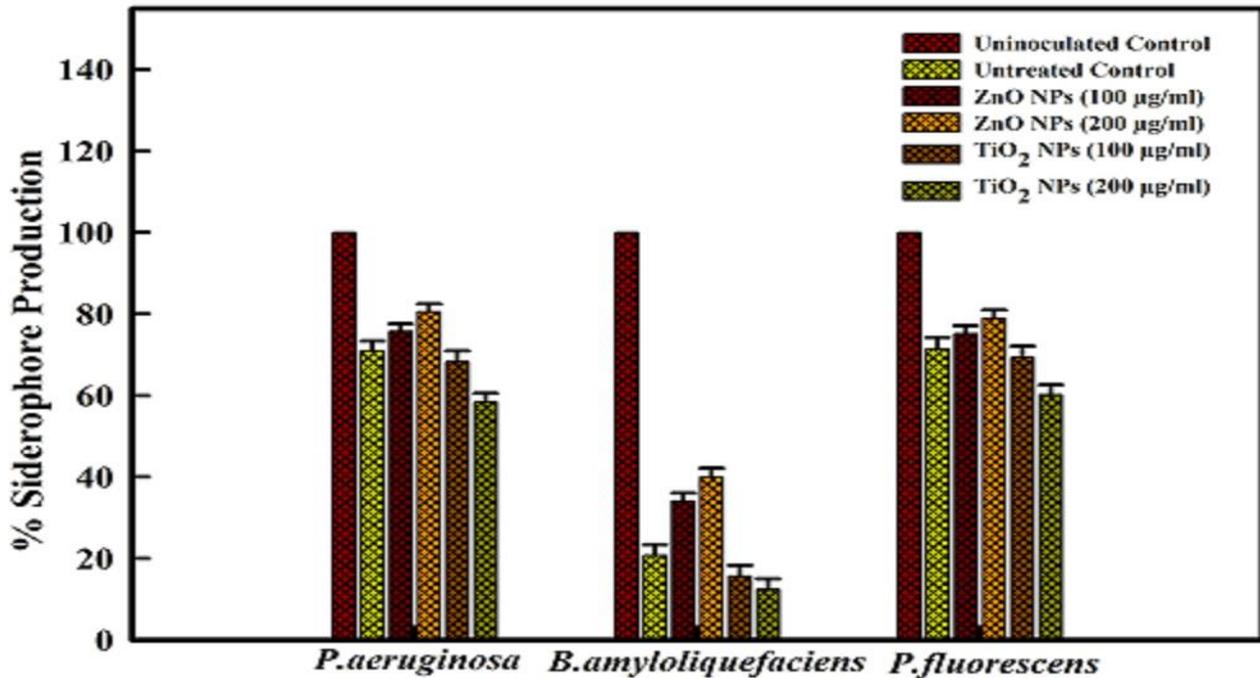
Fig. 6: Siderophore production by bacterial strains in presence of varying concentrations of MO-NPs

Impact of MO-NPs on Antibiotic production

The ZnO and TiO_2 were found to inhibit the Antibiotic production by bacterial strains *P. aeruginosa*, as well as *P. fluorescens*. An estimated 35.23%, and 20.05% reduction in Antibiotic production by *P. aeruginosa* has been noticed at ZnO and TiO_2 concentration of 200 μgmL^{-1} as compared to the control (Fig. 7a) Also, *P. fluorescens* showed 26.42% and 16.67% reduction in antibiotic production at ZnO and TiO_2 concentration of 200 μgmL^{-1} as compared to the control (Fig. 7b).
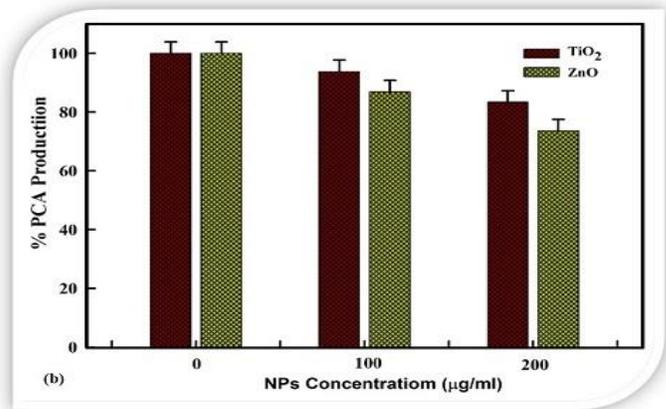
Fig. 7: Histogram showing % PCA production by (a) *P. aeruginosa* and (b) *P. fluorescens* in absence and presence of 100 and 200 µg/ml MO-NPs
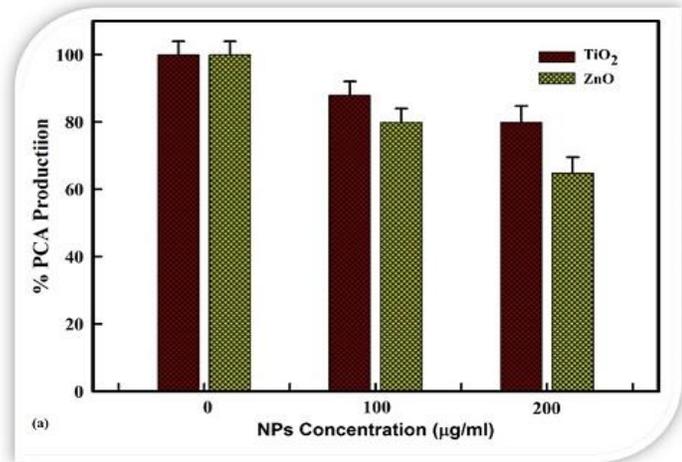
Effect of MO-NPs on bacterial extracellular enzyme viz. NADH

Reducing potential of the supernatant was endorsed by measuring the native fluorescence of extracellular nicotinamide adenine dinucleotide (NADH) released by bacterial strains *P. aeruginosa, P. fluorescens* in culture medium (Fig. 8). The fluorescence of NADH released by *P. aeruginosa* was observed to be quenched by 55.4, and 85.4% with the addition of 200 and 400 µg/ml ZnO-NPs, whereas 26.1 and 70.6% quenching was observed with the addition of 200 and 400 µg/ml TiO_2-NPs respectively. Also, the fluorescence of NADH released by *P. fluorescens* was observed to be quenched by 38.3, and 80.6% with the addition of 200 and 400 µg/ml ZnO-NPs, whereas 19 and 41.5% quenching was observed with the addition of 200 and 400 µg/ml TiO_2-NPs respectively. These results signify the interaction of metal NPs with NADH. NADH is known

to play a fundamental role in the conversion of chemical energy to useful metabolic energy [48,49]. It is a well-known reduced coenzyme in redox reaction, and can be used as reducing agents by several enzymes [50,51]. Thus, it was concluded that the reducing activity of strains culture supernatant is mainly attributed to soluble redox active agents like NADH, besides the already known NADH dependent reductases.
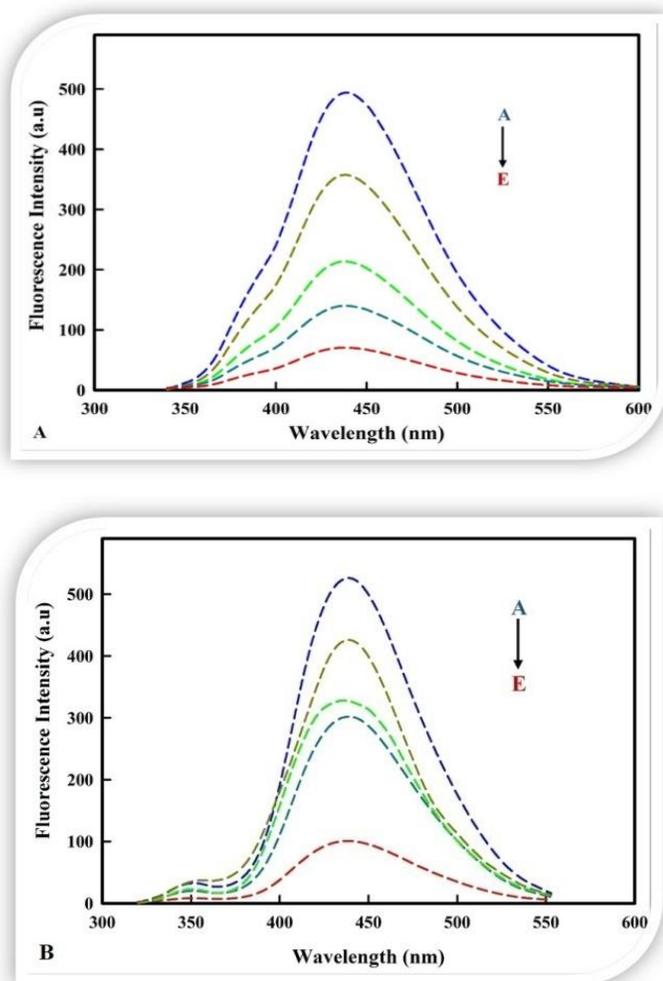
Fig. 8: NADH fluorescence of bacterial supernatant of (A) *P. aeruginosa* and (B) *P. fluorescens* alone and after treatment with NPs

The arrow represents the fluorescence quench of spectra A–E, where A is untreated control supernatant, and spectra B to E represent the supernatant treated with 200 μg/ml TiO₂-NPs, 200 μg/ml ZnO-NPs, 400 μg/ml TiO₂-NPs and 200 μg/ml ZnO-NPs respectively

CONCLUSIONS

Engineered nanoparticles (ENPs) are finding increasing applications in various biomedical and industrial areasdue to their high surface area and larger catalytic property and will inevitably enter natural ecosystems, with soils predicted to be a substantial sink. This study was carried out to enlighten the understanding of the undefined aspects of nanotoxicology which elucidated the deleterious effect

of nanoparticles on agriculturally important bacterial strains and established that the application of nanoparticles differentially modified the function and plant growth promoting activities under *in-vitro* conditions in *P. aeruginosa*, *P. fluorescens*, *B. amyloliquefaciens*. Nanoparticles under study (ZnO, TiO₂) exhibited dose dependent effect on PGPR strains which gives an idea about the level of toxicity of these nanoparticles in the environment. The toxicity of ZnO-NPs was found to be higher than TiO₂-NPs. Damage to cell wall of bacteria was observed at low concentration of ZnO-NPs than TiO₂-NPs, indicating their relative toxicity. The discharge of nanoparticles in the environment should be carefully monitored so that the loss of both structure and functions of agronomically important microbes could be protected from the toxicity of MO-NPs. Further investigation on the nanoparticle toxicity on microbes and microbe-plant interaction under microcosm and natural conditions are needed for better understanding of the phenomenon.

ACKNOWLEDGMENT

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