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PRODUCTION OF PLANT-GROWTH PROMOTING SUBSTANCES BY NODULE FORMING SYMBIOTIC BACTERIUM RHIZOBIUM SP. OS1 IS INFLUENCED BY CuO, ZnO AND Fe_2O_3 NANOPARTICLES

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ABSTRACT

Symbiotic nitrogen fixing rhizobia besides fixing atmospheric nitrogen also produces plant growth promoting substances such as indole acetic acids, siderophores, and cyanogenic compounds etc. However, the effects of nanomaterials on plant growth regulating substances synthesized by these bacteria are not reported. In this paper we have examined the impact of varying concentration of three metal oxide nanoparticles (MONPs) namely copper oxide (CuO), iron oxide (Fe_2O_3) and zinc oxide (ZnO) on growth behaviour and plant growth promoting activities of nodule forming bacterium Rhizobium sp. strain OS1. The three MONPs tested in this study differentially affected the levels of plant growth regulating substances in a dose dependent manner which varied with species of each nanoparticle. A maximum reduction in indole acetic acid, hydrogen cyanide, ammonia and siderophores, expressed by Rhizobium sp. OS1 was observed at 150 μgml^{-1} each of CuO, Fe_2O_3 and ZnO. Iron oxide did not show any toxicity to siderophores. At 50 μgml^{-1}, CuO induced the IAA production by 11% which decreased progressively with increasing concentrations. The synthesis of HCN and NH_3 was completely abolished when strain OS1 was grown with 150 μgml^{-1} of all nanoparticles. Unlike plant growth promoting substances, the production of exo-polysaccharide increased gradually with increasing concentration of each MONPs by rhizobial strain. This study suggests that the nanoparticles of different functional groups affect the physiological expression of rhizobial species differently and it further opens up a new vistas to better understand the impact of nanoparticles on symbiotic interaction between rhizobia and legumes.

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[I] INTRODUCTION

Recently, nanoscience has become one of the most promising fields of research with greater impact on economy and environment health. The research on nanomaterials: materials of 100 nm in at least one dimension, is likely to result in the production of huge number of new nano-products in the coming years. Considering the importance of nanotechnology, a greater attention has been paid on this industry which is expected to reach a market size of approximately 2.6 trillion dollars by 2015 [1]. In addition, nanotechnology is also likely to influence agricultural research especially in (i) the conversion of agricultural and food wastes to energy and other useful by-products through enzymatic nano-bio-processing (ii) disease prevention and treatment of plants using various nanomaterials [2] and (iii) reproductive science and technology. Despite these benefits, the increasing numbers of commercial products, from cosmetics to medicine and fertilizers to crop products are adding sufficient amounts of nanomaterials ultimately to soils. Such nanoparticles have however, been found highly resistant to degradation and persist in soil or water bodies. Nanomaterials for example carbon nanotubes [3, 4], graphene-based nanomaterials [5], iron-based nanoparticles [6], silver [7] and copper, zinc and titanium oxide nanoparticles [8, 9] have been reported to cause biologically undesirable toxic effects on both deleterious (DRMOs) and beneficial rhizosphere microorganisms [10-12] including *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus aureus* [13], *Pseudomonas chlororaphis* [14-18], *Pseudomonas putida* [11] and *Campylobacter jejuni* [19]. However, the reports on the effect of nanoparticles on secondary metabolites of microbes are conflicting. For example, Dimkpa et al. [16] in a recent study found that sub-lethal levels of CuONPs reduced the secretion of plant growth promoting substance siderophore in *P. chlororaphis* O6 whereas ZnO NPs increased the production of the fluorescent siderophore pyoverdine. Similarly, a contrasting effect of CuO and ZnO NPs on siderophores and IAA has also been reported by Dimpka et al. [18] suggesting that the effect of NPs on secondary metabolite production by bacterial populations cannot be generalized rather it is highly

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metabolite/nano specific and may vary from organisms to organisms. In addition, the nanomaterials are also affecting the human health very negatively [20-22] especially by damaging the macromolecules like DNA [23] and other cellular functions [24].

Therefore, a better understanding of how nanomaterials affect microorganisms is likely to improve the environment health including soil ecosystem. Even-though the symbiotic nitrogen fixers in general are reported to transform atmospheric nitrogen to usable forms of N, the effect of nanoparticles on secondary metabolites of rhizobia is not known. Considering both the positive and negative aspect of the nanomaterials and lack of information on the impact of nanoparticles on the plant growth promoting activities of rhizobacteria, here, we examined the effects of metal oxide nanoparticles like CuO, Fe_2O_3 and ZnO on the growth characteristics and production of plant growth regulating substances by symbiotic nitrogen fixing bacterium *Rhizobium sp*. OS1.

[II] MATERIALS AND METHODS

2.1. Rhizobial strains

A total of 20 rhizobial strains were recovered from the rhizosphere of chickpea (*Cicer arietinum*) plants grown in the experimental fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India. About one gram of soil samples was serially diluted using 50 mM phosphate buffer and spread over yeast extract mannitol agar (YMA) plates amended with Congo-red dye. The plates were incubated at 30±2℃ for three days. The rhizobial strains were characterized biochemically and morphologically. Further, most promising plant growth promoting rhizobial isolate was characterized molecularly by 16S rDNA sequence analysis. The *Rhizobium*-chickpea specificity was further determined by nodulation test [25] using chickpea as a host legume plant.

2.2. Synthesis of metal oxide nanoparticles

In the present investigation, metal oxide nanoparticles were synthesized using metal nitrates. For this, the nitrates of Zn, Cu and Fe and citric acid were dissolved in distilled water with a molar ratio of 1:1. The solutions were stirred with magnetic stirrer at 100^0C. Stirring continued for approximately 2h until the gels were formed. Thereafter, gel was allowed to burn at 200 ^{0}C. A light fluffy mass produced as a result of combustion was annealed further at 400 ^{0}C for one hour to achieve the respective crystalline metal oxide nanoparticles [26].

2.3. Nanoparticles suspension

In this study, 20 nm size metal oxide nanoparticles (MONPs) were used. All metal oxide nanoparticles namely, copper oxide (CuO), iron oxide (Fe_2O_3) and zinc oxide (ZnO) were obtained from the Department of Applied Physics, Excellence centre of Nanoscience, Aligarh Muslim University, Uttar Pradesh, India. For stock solution preparation, 12.5, 25, 50, 100 and 150 mg MONPs were mixed with 100 ml. ultrapure water in 250 ml capacity flask and vigorously stirred for 15 min. After sonicating the sample for 30 min. the resulting suspension was collected and stored at 4 ℃ for further studies.

2.4. Tolerance level of nanoparticles

The rhizobial strains were tested for their sensitivity/tolerance to chemically and functionally diverse MONPs CuO, Fe_2O_3 and ZnO, by agar plate dilution method. The freshly prepared agar plates were amended separately with increasing concentrations (0 to 300 μgml^{-1}) of nanoparticles. Plates were incubated at 30±2℃ overnight to check the sterility of the medium. Later, plates were spot inoculated with loopful rhizobial culture (10 µl of 108 cells ml^{-1}). Plates were incubated at 30^0 (SE=2℃) for three days and the highest concentration of each nanoparticle supporting rhizobial growth was defined as the maximum tolerance level (MTL).

2.5. Growth pattern

For the determination of growth pattern, a 0.5 ml of the culture (10^8 cells ml^{-1}) of freshly grown *Rhizobium sp*. OS1 was inoculated into 100 ml yeast extract mannitol broth medium containing 0 (control) to 150 μgml^{-1} nanoparticles of CuO, Fe_2O_3 and ZnO, separately. The cultures were incubated at 30±2℃ on a rotary shaker with 120 r/min. At regular intervals, the optical density was measured at 600 nm using a spectrophotometer (Spectronic 20, USA). The rhizobial growth curve was obtained by plotting the optical density as a function of time.

2.6. Indole acetic acid assay

Indole-3-acetic acid (IAA) synthesized by *Rhizobium sp*. OS1 strain was quantitatively estimated by the method of Gordon and Weber [26], later modified by Brick et al. [27]. For this activity, the rhizobial strains were grown in Luria Bertani broth (gl^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5). Luria Bertani broth (100 ml) having a fixed concentration of tryptophan (100 μgml^{-1}) was treated with 0, 12.5, 25, 50, 100 and 150 μgml^{-1} of CuO, Fe_2O_3 and ZnO, separately. A 0.1 ml (10^8cells ml^{-1}) of rhizobial strains was inoculated into each separate flask and was incubated for three days at 30℃ (SE=2℃) with shaking at 120 r/min. After three days, five mil culture from each treatment was removed and centrifuged (5433) for 15 min. and an aliquot of 2 ml supernatant was mixed with 100 µl of orthophosphoric acid and 4 ml of Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) and incubated at 30^0C (SE=2℃) in darkness for 1 h. The absorbance of pink colour was read at 530 nm. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard.

2.7. Siderophores assay

The *Rhizobium sp*. OS1 strain was further tested for siderophores production using Chrome Azurol S (CAS) agar medium following the method of Alexander and Zuberer [28]. Chrome Azurol S agar plates supplemented with 0, 12.5, 25, 50,100 and 150 μgml^{-1} of each nanoparticles were prepared separately and spot inoculated with loop full fresh culture (10 µl of 108 cells ml^{-1}) and incubated at 30℃ (SE=2℃) for three days. Development of yellow to orange halo around the bacterial growth was considered as positive indicator of siderophores-biosynthesis. The production of siderophores by the test strains was further detected quantitatively using Modi medium (K_2HPO_4 0.05%; $MgSO_4$ 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1% and NH_4NO_3 0.1%) [29]. Modi medium amended with 0, 12.5, 25, 50, 100 and 150 μgml^{-1} of each metal oxide nanoparticles was inoculated with 108 bacterial cells ml^{-1} and incubated at 30 ℃(SE=2℃) for three days. Catechol type phenolates were measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride-ferricyanide reagent of Hathway [29]. Ethyl acetate extracts were prepared by extracting 20 ml of supernatant three times with an equal volume of the solvent at pH_2. Hathway's reagent was prepared by adding one milliliter of 0.1 M ferric chloride in 0.1 NHCl to 100 ml of distilled water, and to this, was added 1 ml of 0.1M potassium ferricyanide [30]. For the assay, one volume of the reagent was added to one volume of the sample and the absorbance was determined at 560 nm for salicylic acid (SA) with sodium salicylate as a standard and at 700 nm for

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dihydroxy phenols with 2,3-dihydroxy benzoic acid (DHBA) as a standard.

2.8. Assay of hydrogen cyanide and ammonia

Hydrogen cyanide (HCN) production by *Rhizobium sp*. OS1 strain was detected by the method of Bakker and Schipper [30]. For HCN production, rhizobial strains were grown on an HCN induction medium (gl^{-1}: tryptic soy broth 30; glycine 4.4 and agar 15) supplemented with 0, 12.5, 25, 50, 100 and 150 μgml^{-1} of each metal oxide nanoparticles at 30℃(SE=2℃) for five days. Further, 100 µl of 108 cells ml^{-1} of freshly grown culture of *Rhizobium sp*. OS1 strain was spread on Petri plates. A disk of Whatman filter paper No. 1 dipped in 0.5% picric acid and 2% Na_2CO_3 was placed at the lid of the Petri plates. Plates were sealed with parafilm. After four days incubation at 30℃ (SE=2℃), red/orange brown colour of the paper indicating HCN production was observed. For ammonia (NH_3) assessment, the *Rhizobium sp*. OS1 strain was grown in peptone water with 0, 12.5, 25, 50, 100 and 150 µg/ml of each metal oxide nanoparticles and incubated at 30℃(SE=2℃) for five days. One milliliter of Nessler reagent [potassium iodide 50 g; distilled water (ammonia free) 35 ml; mixed with saturated aqueous solution of mercuric chloride until a slight precipitate developed; potassium hydroxide 400 ml; diluted the solution to 1000 ml with ammonia free distilled water; allowed it to stand for one week, decanted supernatant liquid and stored in a tightly capped amber bottle] was added to each tube and the development of yellow colour indicating ammonia production was recorded following the method of Dye [31].

2.9. Exo-polysaccharides production

Exo-polysaccharide (EPS) produced by the *Rhizobium sp*. OS1 was determined as suggested by Mody et al. [32]. For this, the bacterial strains were grown in 100 ml capacity flasks containing yeast extract mannitol broth medium supplemented with 10% sucrose and treated with 0, 12.5, 25, 50, 100 and 150 μgml^{-1} of each metal oxide nanoparticles. Inoculated flasks were incubated for five days at 30 ℃(SE=2℃) on rotary shaker at 120 r/min. Culture broth was spun (5433 g) for 30 min., and EPS was extracted by adding three volumes of chilled acetone (CH_3COCH_3) to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying at room temperature. Each individual experiment was repeated three times.

2.10. Statistical analysis

The experiments were repeated three times using the same treatments. The difference among treatment means was compared by high range statistical domain (HSD) using Tukey test ($p \leq 0.05$).

[III] RESULTS AND DISCUSSION

3.1. Nanoparticles-tolerance and identification of bacterial strain

Symbiotically nitrogen fixing bacterial strain *Rhizobium sp*. OS1 demonstrated a variable MONPs tolerance and when tested exhibited different PGP activities under in vitro environment. The MTL values of CuO, Fe_2O_3 and ZnO was 160, 190, and 155 μgml^{-1}, respectively. The variation in tolerance to certain MONPs tested in this study could probably be due to differences in the interaction of physiological products of rhizobial strain with varying species of MONPs. Also, the genetic makeup of this strain might have played an important role in tolerating the MONPs. The toxicity of nanoparticles has however, been reported to be affected by many factors including types and chemical composition of metals, size of particles [33], shape of particles [34], surface charge [6] and bacterial strains [19]. In similar studies, bacteria *Cupriavidus metallidurans* and *E. coli* have been found highly sensitive to higher concentrations of metal oxide nanoparticles such as TiO_2 or Al_2O_3 NP [35]. In a follow up study, Khan et al. [36] isolated nanoparticles tolerant bacterium *Aeromonas punctata* from sewage environment which was able to tolerate 200 µg/ml AgNPs while Li et al. [37] in a similar study identified a Gram negative bacterium *Pseudomonas putida* which could tolerate >500 µgml-1 zinc oxide nanoparticle. Of the total 20 rhizobial strains recovered from the chickpea rhizosphere and identified using morphological and biochemical tests and nodulation test, nanoparticle tolerant strain OS1 was further characterized by amplification of ribosomal 16S rDNA sequence. The resulting base sequence was compared with those of some related organisms by BLASTn analysis. An 1189 bp sequence demonstrated 99% similarity with *Rhizobium* species. The sequence was deposited in the EMBL database under accession numbers HE663761 so that it remains in the public domain.

3.2. Growth pattern of rhizobial strain in the presence of MONPs

The growth pattern of *Rhizobium* species was observed while growing strain OS1 in the presence of 0 (control) to 150 µgml-1 nanoparticles of CuO, Fe_2O_3 and ZnO, added separately to nutrient broth medium. The variation in rhizobial growth was monitored after every two hours intervals. The bacterial growth decreased with increasing concentration of each individual MONPs **[Supplementary figure–1]**. Among the tested MONPs, CuO at 150 μgml^{-1} was highly toxic and decreased the bacterial growth by 68 % compared to rhizobial strain OS1 grown in MNOPs free medium. While comparing the effect of only 150 µgml-1 of different MNOPs on rhizobial growth, the order of toxicity was: CuO>ZnO>Fe_2O_3. Although, the exact mechanism as to how the MNOPs kills the bacterial cell is not conclusively known but the inhibition may be caused by the interaction of MNOPs with the bacterial membrane causing pitting of the cell wall, dissipation of the proton motive force, and consequently the cell death [38]. In addition, nanoparticles have also been reported to interact with bacterial DNA leading to the DNAs replication damage [39, 40]. These observations agree with those of the previous work reported by Beak and An [13] where CuO NPs was found most inhibitory against *E. coli*, *B. subtilis*, and *S. aureus* than ZnO. In a similar report, Jones et al. [10] also determined the detrimental effect of ZnO against broad spectrum microorganisms.

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3.3. Plant growth promoting activities of rhizobial strain

3.3.1. Siderophore production under MONPs-stress

Siderophore, a low molecular weight iron chelating peptide released by majority of Gram negative bacterial strains is one of the most important biological traits that provide protection to plants by making the accessibility of iron difficult to phytopathogens. On the other hand, iron is essentially required by bacteria for its effective metabolic activity. However, there is acute scarcity of iron due to its ability of insolubility at neutral to alkaline pH [41]. In order to challenge this elemental problems, majority of bacteria belonging to both Gram positive and Gram negative groups have evolved mechanism wherein they secrete siderophores to scavenge iron in iron deficient environment [42-45]. This exceptional quality of bacteria to secrete chemically diverse class of siderophores is likely to help bacterial communities to survive better in an iron-deficient environment. Therefore, considering the importance of siderophore, we determined the synthesis of secondary metabolites: siderophores, by the MONPs tolerant *Rhizobium sp*. OS1 using CAS agar plates treated differently with varying concentration of the nanomaterials **[Supplementary table– 2]**. *Rhizobium sp*. OS1 produced a 13 mm orange zone, an indicator of siderophore production, on MNOPs free CAS agar plate. The size of the zone was however decreased significantly ($p \leq 0.05$) when MONPs were added to CAS agar plates. Generally, the effects of MONPs were less at lower concentrations of each MONPs which became more pronounced at the higher rates of MONPs. Accordingly, when the effect of varying concentrations of MNOPs on siderophore production were compared, a maximum of 61% decrease in zone size was recorded at 150 μgml^{-1} each of CuO and ZnO over control while any tested concentration of Fe_2O_3 did not show inhibitory effect on siderophore production **[Supplementary table– 2]**. After qualitative analysis, the production of siderophores was quantitatively assayed. In this regard, the SA and DHBA type siderophores were detected in the culture supernatant of the *Rhizobium sp*. OS1, grown in the Modi medium devoid of nanomaterials **[Supplementary figure– 2a, 2b]**. A nanomaterial concentration dependent progressive decline for both iron binding molecules was observed. Indeed, the reduction in siderophores quantity was greatly influenced by the species of MONPs. Of the three MONPs, ZnO at 150 μgml^{-1} was most toxic for the generation of both SA (17.3 μgml^{-1}) and DHBA (9.5 μgml^{-1}) and decreased SA and DHBA synthesis significantly ($p \leq 0.05$) by 49% and 56%, respectively, over control. Furthermore, while comparing the effect of 150 μgml^{-1} each of ZnO and CuO, the ZnO reduced the production of SA and DHBA by 12 and 17% respectively over CuO. Like the qualitative assay, there was no effect of Fe_2O_3 nanomaterial even at 150 µg/ml on SA and DHBA secretion by the strain OS1. In agreement to our findings, Dimkpa et al. [14] observed that the sub-lethal 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.

3.4. Impact of MONPs on indole-acetic acid production

The IAA synthesized by the *Rhizobium sp*. OS1 under different concentrations of MONPs was variable **[Supplementary table– 2]**. Generally, the IAA was produced both under normal and MONPs treated and Rhizobium inoculated LB medium but the level of IAA under MONPs was considerably lower compared to those detected in conventional (untreated) medium. For example, *Rhizobium sp*. OS1 produced a significant ($p \leq 0.05$) amount of IAA (43 μgml^{-1}) when grown in medium devoid of MNOPs. Interestingly, the IAA production decreased with increasing concentration of each MNOPs except CuO NPs which induced the synthesis of IAA by 11% up to 50 μgml^{-1}; IAA decreased substantially thereafter. While comparing the impact of higher tested concentration (150 μgml^{-1}) of the three MNOPs, ZnO nanomaterials had greatest deleterious effect on IAA and reduced it by 79% which was 44 and 25% for CuO and Fe2O3, respectively relative to the control. Among different concentrations used in this study, ZnO at 150 μgml^{-1} maximally declined the IAA by 67 % compared to those observed for CuO (33%) and Fe_2O_3 (34%), assayed at 50 μgml^{-1}. While calculating the average value of MONP toxicity on IAA synthesis by strain OS1, ZnO was most toxic (mean value 24.9 μgml^{-1}) and reduce the IAA production by 42% compared to control (43 μgml^{-1}). Based on this, the order of MONP toxicity increased in the order: $ZnO > Fe_2O_3 > CuO$. In a recent study, Dimkpa et al. [15] observed 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 ($P \leq 0.05$) by 34% in a 48h grown culture while ZnO NPs reduced IAA levels by 79% 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.

3.3. Estimation of EPS, HCN and NH_3 under MONPs stress

Among the PGP substances which indirectly facilitates the growth of plants and released by bacterial populations including *Rhizobium* was investigated further in this study by exposing *Rhizobium sp*. OS1 to MONPs. The release of one such compound for example EPS increased significantly ($p \leq 0.05$) when strain OS1 was exposed to progressively increasing

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concentration of each MONPs added separately to nutrient broth amended with a fixed rate of dextrose ($10gl^{-1}$). Among all the tested MONPs, a more pronounced stimulatory effect on EPS secretion was detected at 150 μgml^{-1} CuO which increased the EPS by 90% compared to control **[Supplementary table– 2]**. While calculating the mean effect of all concentration of each MONPs, CuO NPs was found as a strong inducer of EPS synthesis and enhanced the EPS production by 58% which was followed by Fe_2O_3 (46%) and ZuO (29%). The EPS secreted by bacterial strains has been considered one of the important metabolic traits that protect bacterial cells from the nuisance of adverse environment [46]. It was therefore, very much likely that the rhizobial cells tested in this investigation might have been masked by the toxicity of MONPs by secreting EPS. Moreover, the EPS is reported to protect bacteria against desiccation, dehydration, phagocytosis and phage attack besides supporting N_2 fixation by preventing high oxygen tension. In our previous work we have also observed a similar enhanced production of EPS by rhizobial strains when grown with metal ion [44] and fungicides [47]. Comparable observation on the effects of nanoparticles on EPS secretion by bacterial strain is also reported Wu et al. [48].

The other mechanism by which rhizobacteria protects the growing plants from pathogen attack involves the direct killing of parasites by producing cyanogenic compound HCN [49]. Consequently, the synthesis of such compounds was examined here. Interestingly, the concentrations of CuO, Fe_2O_3 and ZnO up to 100 µg/ml did not affect negatively the HCN and ammonia synthesis by the *Rhizobium sp*. OS1 **[Supplementary table– 2]** while concentration greater than 100 μgml^{-1} of each MONPs had a strong inhibitory effect. In agreement to our report, [45-47] observed the release of cyanogenic compounds like HCN by the rhizobacterial strains into the rhizosphere. The ammonia released by the rhizobacterial strain plays a signaling role in the interaction between rhizobacteria and plants and also increase the glutamine synthetase activity [50]. Therefore, it seems probable that MONPs employed in this study might have inhibited the functioning of the enzymes participating in different metabolic pathways of PGP traits such as SA, DHBA and IAA of the *Rhizobium sp*. OS1 leading to a differential losses in the production of PGP substances.

[IV] CONCLUTION

In conclusion, we have established that the application of nanoparticles differentially modified the production of plant growth promoting substances under in vitro conditions in the nodule forming Gram-negative bacterium *Rhizobium sp*. OS1. Generally, the release of plant promoting substances by rhizobial strain was dose dependent and nanomaterial specific. 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 MONPs. This study also provides a base line data to further understand the symbiotic interaction between legumes and rhizobia in a nanoparticles contaminated environment.

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CONFLICT OF INTEREST

No conflict of interest in the form of either financial or commercial is involved in any way with the present study.

FINANCIAL DISCLOSURE

No financial sponsor in the form of person, institution or organization is involved in the present work.

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Supplementary Materials (As supplied by authors)

Supplementary Table: 1. General features of the *Rhizobium sp.* OS1

Characteristics	*Rhizobium* sp. OS1
Accession number	HE663761
Morphology	
Shape	Short rod
Gram reaction	_
Biochemical reaction	
Citrate utilization	_
Indole	+
Methyl red	+
Nitrate reduction	+
Voges Proskaur	+
Oxidase	_
Catalase	+
Carbohydrate utilization	
Glucose	+
Lactose	_
Mannitol	+
Sucrose	+
Enzymatic Hydrolysis	
Starch	+
Gelatin	_
Maximum tolerance level to MONPs	
CuO	160 µgml-1
Fe_2O_3	190 µgml-1
ZnO	155 µgml-1

“

+” and “-” sign indicates positive and negative reaction results, respectively

Supplementary Table: 2. Plant growth promoting activities of the *Rhizobium* sp. OS1 influenced by MONPs

MONPs	Concentration (µgml-1)	IAA (µgml-1)	EPS (µgml-1)	Siderophore [zone on CAS agar (mm)]	NH3	HCN
Control	0	43.0±1.0g	18.3±1.0a	13.0±1.0e	+	+
CuO	12.5	46.0±1.0h	21.6±1.5b	12.0±1.0d	+	+
	25	46.6±1.5h	25.3±1.0c	11.0±1.5d	+	+
	50	48.0±1.0h	29.6±1.5d	9.7±1.5c	+	+
	100	40.0±2.0g	33.3±1.5e	8.0±1.5b	+	+
	150	32.1±2.0e	35.0±1.5f	5.0±1.0a	-	-
	Mean	42.5	28.9	9.1		
Fe_2O_3	12.5	42.3±1.5g	20.1±2.5a	13.0±1.0e	+	+
	25	41.0±1.0g	23.0±1.5b	13.0±1.0e	+	+
	50	36.3±1.5f	28.3±1.0c	12.0±1.0d	+	+
	100	29.3±2.3c	30.3±2.1d	11.0±1.0d	+	+
	Mean	34.6	26.7	12.0		
ZnO	12.5	37.3±2.0f	18.6±1.5a	11.0±1.0d	+	+
	25	31.0±1.0e	20.2±1.5a	10.0±1.5c	+	+
	50	27.6±1.5d	24.5±1.0c	9.0±1.0c	+	+
	100	20.0±1.0b	25.0±2.0c	7.0±1.0b	+	+
	150	9.1±1.0a	29.1±1.5d	5.0±1.0a	-	-
	Mean	24.9	23.4	8.4		
	***F* value**	156.73	48.56	16.11		
	LSD	2.484	2.237	1.922		

± indicate standard deviation, + indicates positive and - indicates negative

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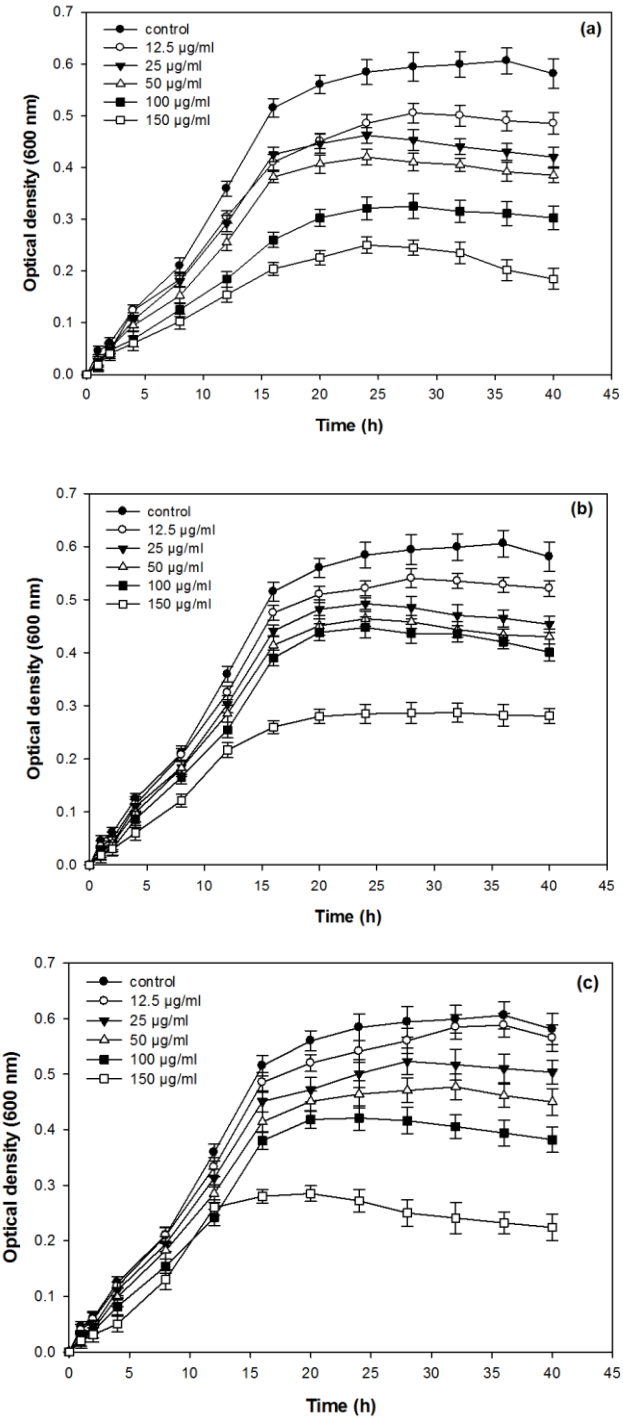

Fig. 1: Impact of varying concentrations of metal oxide nanoparticles (a) CuO (b) Fe2O3 and (c) ZnO on the *Rhizobium sp.* OS1 grown in nutrient broth medium at different time intervals.

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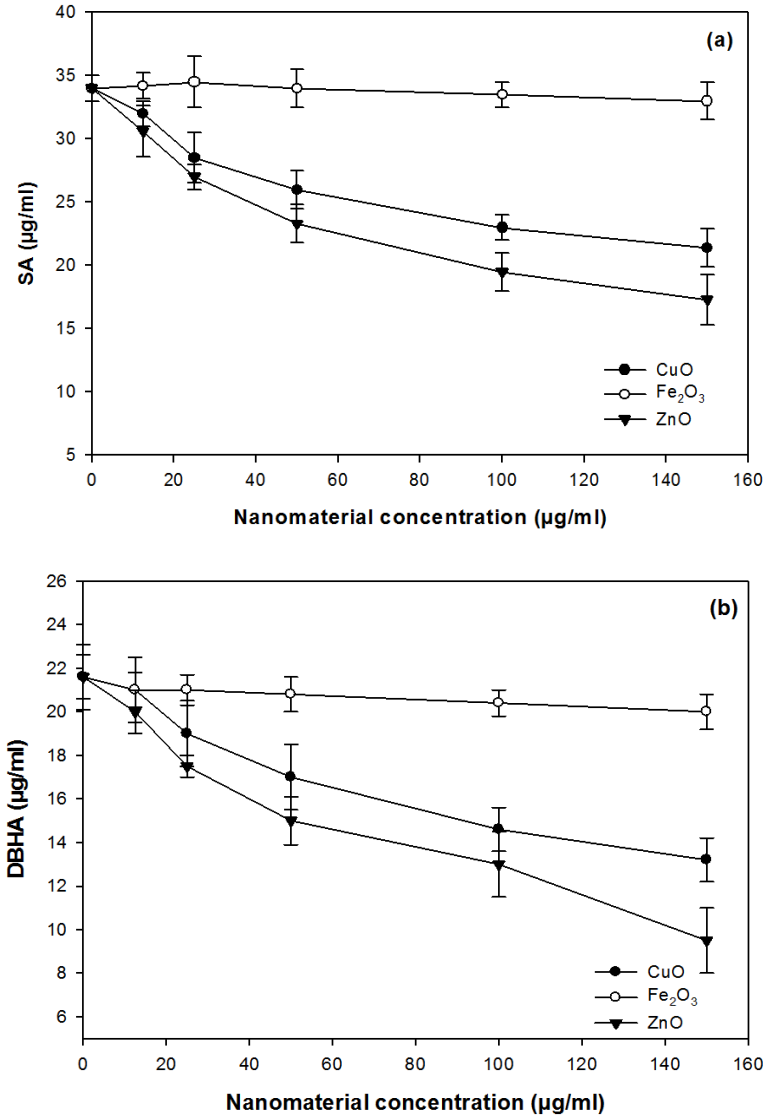

Fig. 2: Salicylic acid (a) and 2,3-dihydroxy benzoic acid (b) production by *Rhizobium* OS1 at varying concentration of MONPs