


Elucidating the interactions and phytotoxicity of zinc oxide nanoparticles with agriculturally beneficial bacteria and selected crop plants

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Abstract There is a growing interest in the use of bioinoculants to assist mineral fertilizers in improving crop production and yield. *Azotobacter* and *Pseudomonas* are two agriculturally relevant strains of bacteria which have been established as efficient bioinoculants. An experiment involving addition of graded concentrations of zinc oxide (ZnO) nanoparticles was undertaken using log phase cultures of *Azotobacter* and *Pseudomonas*. Growth kinetics revealed a clear trend of gradual decrease with *Pseudomonas*; however, *Azotobacter* exhibited a twofold enhancement in growth with increase in the concentration of ZnO concentration. Scanning electron microscopy (SEM), supported by energy-dispersive X-ray (EDX) analyses, illustrated the significant effect of ZnO nanoparticles on *Azotobacter* by the enhancement in the abundance of globular biofilm-like structures and the intracellular presence of ZnO, with the increase in its concentration. It can be surmised that extracellular mucilage production in *Azotobacter* may be providing a barrier to the nanoparticles. Further experiments with *Azotobacter* by inoculation of wheat and tomato seeds with ZnO nanoparticles alone or bacteria grown on ZnO-infused growth medium revealed interesting results. Vigour index of wheat seeds reduced by 40–50% in

the presence of different concentrations of ZnO nanoparticles alone, which was alleviated by 15–20%, when ZnO and *Azotobacter* were present together. However, a drastic 50–60% decrease in vigour indices of tomato seeds was recorded, irrespective of *Azotobacter* inoculation.

Keywords *Azotobacter* · Phytotoxicity · Nanoparticles · Vigour index · Zinc oxide

Introduction

The field of nanotoxicology has gained immense attraction over the last two decades, necessitating the need for a better understanding of its implications on routine daily activities. Any material that is reduced beyond the dimensions of 100 nm takes on a completely different set of properties and interactions (Oberdorster et al. 2005). Studies are being widely conducted on how nanoparticles of such dimensions interact with biological media and on the potential detriments to health that they can cause. Raffi et al. (2008) showed how silver nanoparticles can easily pervade cellular barriers and accumulate within biological systems. Research undertaken in this area has shown how metal and metal oxide nanoparticles pose a great threat due to their ability to initiate oxidation-mediated cell signalling pathways, which could potentially lead to harmful effects on biological systems (Raliya et al. 2015; Sil et al. 2014). There are positive applications too, as the invasive nature of metal oxide nanoparticles can greatly inhibit the proliferation of harmful pathogenic bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli* and even fungi such as *Botrytis cinerea* (Raffi et al. 2008; He et al. 2011; Xia et al. 2008; Raghupathi et al. 2011; Wu et al. 2004). Reports on the antibacterial activity of silver and zinc oxide nanoparticles in experiments with halophilic bacteria are also

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available (Khare et al. 2011), as well as their utility in the treatment of textiles using zinc oxide nanoparticles and nanofluids to prevent bacterial contaminations. Zhang et al. (2007) illustrated how zinc oxide nanoparticles infiltrated through the membrane assemblies and accumulated within the cytoplasm; this property of being able to pass through membrane barriers is being widely considered as the prime reason for its antibacterial and antifungal properties (Vijayaraghavan and Padmavathy 2008). Research carried out by the group of Yamamoto et al. (2008) illustrated the size-dependent antibacterial characteristics of ZnO nanoparticles, over a range of 0.1 to 1 μm . There is, however, very less research conducted so far on the physiological effects caused by the nanoparticle-sized infiltrations (particles having diameter <100 nm) and their sites of accumulation within bacterial cells. Bacteria belonging to genera *Azotobacter*, *Bacillus*, *Providencia* and *Pseudomonas* are known for their plant growth-promoting traits and ability to enhance crop production (Nain et al. 2010; Rana et al. 2011). The interaction between metal oxide nanoparticles such as ZnO and agriculturally beneficial bacteria such as *Azotobacter* and *Pseudomonas* could provide insights into the accumulation and mechanism of nanoparticle pervasion in biological systems.

Despite the availability of information on the kinetics of diffusion and infiltration of nanoparticles varying greatly on their shape, size and electrochemical nature (Xia et al. 2008), it is essential to understand the effect of nanoparticle dosages on the germination and growth of crops as well. Kumari et al. (2011) observed genotoxic effects of zinc oxide nanoparticles, which were illustrated by SEM images showing agglomeration of internalized nanoparticles. However, such effects are not investigated in other crop plants and nanoparticles are known to be selective in their toxicity responses.

The present investigation was aimed to understand the interactions of ZnO nanoparticles on bacterial growth, followed by their interplay with germination and growth of tomato and wheat seedlings. Different methods of visualization were studied and employed to analyse the internalization of ZnO nanoparticles inside *Azotobacter chroococcum*, including scanning electron microscopy (SEM) and elemental analysis using energy-dispersive X-ray spectroscopy (EDX).

Materials and methods

Chemicals

The culture medium ingredients were purchased from Hi Media Laboratories Pvt. Ltd., India. All the chemicals used were of analytical grade. The zinc oxide (ZnO) nanoparticles having an average size of 30 nm (mol. weight 81.38) was purchased from SRL, India.

Microorganisms and growth conditions

A free living nitrogen fixer, *A. chroococcum* W5 and phosphorus-solubilizing bacterium *Pseudomonas striata* 27, were obtained from the germplasm of the Division of Microbiology, ICAR—Indian Agricultural Research Institute, New Delhi. Both these cultures are routinely used in biofertilizer preparations over the last 20 years and are being supplied both as pure cultures and inoculants by the Division of Microbiology, ICAR—Indian Agricultural Research Institute, New Delhi. The cultures were carefully grown on respective media agar slants and stored at 4 °C, with monthly subculturing.

A. chroococcum W5 culture was prepared by inoculating loopful samples carefully from slants into Jensen's nitrogen-free broth, while *P. striata* 27 was raised in Pikovaskaya's broth with tricalcium phosphate as insoluble P source. The cultures were incubated at 30 °C, with constant shaking (150 rpm) to prevent the settling of the mineral salts in the medium. The growth of the culture was monitored by counting the viable cells by a dilution plate method and maintained as 10^9 – 10^{10} CFU/mL.

Preparation of nanoparticle suspensions

Pre-weighed quantities of the zinc oxide nanoparticles were dissolved in autoclaved, ultrapure, deionized water. A range of nanoparticle suspensions were made in order to provide doses of 2 to 6 g/L (25–75 mmol/L). The suspensions were then sonicated for 15 min at 25 °C, to prevent aggregation, and further autoclaved at 103.42 kPa, to sterilize the nanoparticle suspensions.

Interaction of nanoparticles with the *Azotobacter* and *Pseudomonas* cultures

Inocula of *A. chroococcum* W5 and *P. striata* 27 were raised in the respective media for 48 h. Wells were created on plates filled with soft agar (1% agar w/v solution) and aseptically filled with different concentrations of nanoparticles from 25 to 75 mmol/L, along with the control (having no nanoparticle dosage). The cultures were then spread, plated carefully on the soft agar and observed for cell growth and inhibition, after 48 h incubation at 30 °C. Zones of inhibition were also measured to conclude about toxicity of nanoparticles. In order to ensure that the nanoparticle suspensions were efficiently dispersed, 1 mL of PEG-400 was added. Blank solutions containing pure nutrient media were tested using the well diffusion method against suspensions containing PEG-400 and a well-mixed, vortexed solution combining PEG-400 and ZnO nanoparticles. On observing the biocidal response of the PEG to the bacterial cultures, the suspensions were dispersed using only sonication and vigorous shaking.

In the second experiment, the previously used agar diffusion method was modified in order to ensure the complete exposure of the bacterial cultures to the nanoparticle doses. The nanoparticle doses were mixed into the respective agar-based growth medium and then poured into the petri plates, and the cultures were placed in the centre of the plates. The observations were noted for inhibition after 48 h of incubation at 30 °C.

Growth potential of *A. chroococcum* W5 and *P. striata* 27 cultures in the presence of different doses of the zinc oxide nanoparticle suspensions was also quantified in terms of optical density of culture suspension in a 96-well titre plate containing an equal volume of respective media. *A. chroococcum* W5 and *P. striata* 27 cultures were inoculated in series along with different doses of the nanoparticle suspensions. Continuous spectrometric measurements in terms of absorbance at 600 nm were recorded up to 26 h, at 30 °C in a growth kinetic analyser (Bioscreen growth analyser, Labsystems, Helsinki, Finland), which has a built-in software to generate the growth curves.

Assessment of the nitrogen fixing potential of *A. chroococcum* after inoculation with nanoparticle suspensions

Acetylene reduction activity (ARA) was measured as an index of nitrogenase activity or nitrogen fixing potential. The culture of *A. chroococcum* W5 was grown in Jensen's broth in Erlenmeyer flasks along with different doses of nanoparticles with an inoculum rate of 10% (v/v) in 50-mL Erlenmeyer flasks, taken in triplicate. The nanoparticle doses ranged from 25 to 75 mmol/L in concentration, and uninoculated medium was kept as control. Triplicate sets of flasks were incubated for 24 h at 30 °C, and ARA was measured on the 7th and 9th days of incubation. The plugs of flasks were replaced with rubber subaseals, and 1 mL of air was removed from the flasks and the same amount of acetylene was carefully injected in the flasks. After incubation, 0.1 mL of gas mixture from the flasks was injected into the preconditioned gas chromatograph having a flame ionization detector (model Bruker 450). Care was taken to make sure that the column temperature was 100 °C, with the injector and detector at a slightly higher temperature of 110 °C. A steady flow rate of nitrogen (N₂) gas was used as the carrier gas. The peak area and residence time values were noted for each of the different samples, and ARA activity was calculated as given in Prasanna et al. (2013).

Effect of nanoparticles on indole acetic acid (IAA) production by *Azotobacter*

A loopful of the bacterial inoculum was added to 5 mL of Jensen's media, amended with filter-sterilized tryptophan (100 µg/mL). One set of tubes were maintained wherein

zinc oxide nanoparticles were at a concentration of 50 mmol/L. The tubes were incubated for 5 days at 28 °C on a rotary shaker. The method of Gordon and Weber (1951) was used to determine the concentration of IAA, which was measured at 535 nm. The triplicate values were compared to a standard IAA curve and represented as micrograms per millilitre.

Effect of nanoparticles on seed germination

Zinc oxide nanoparticle suspensions were prepared such that they would give doses of 10, 25, 50 and 75 mmol/L concentrations in each petri dish having 25 mL of soft agar (1% w/v). The seeds of tomato (variety *Bombyx*) and wheat (variety HD 2967) were obtained from the Centre for Protected Cultivation and Division of Agronomy, IARI, New Delhi. Triplicate samples of the seeds were washed with mercuric chloride solution (0.01%) for 5 min and subsequently washed with two rounds of deionized water. The soaked seeds were then carefully placed on the petri dishes containing different doses of nanoparticle + agar suspensions. One set of petri dishes contained the seeds placed on pure agar; the second treatment was of seeds placed on ZnO-infused agar suspension. The third treatment was with the seeds dipped in *A. chroococcum* W5 culture. The seeds were incubated at 30 °C for 48 h, and root plumule length was measured, along with the germination percentages. The seed vigour was calculated by using the following formula (Zucconi et al. 1981):

$$\text{Vigour index} = (\text{Average plumule length} + \text{Average root length}) \times \% \text{ Germination}$$

Scanning electron microscopy and energy-dispersive X-ray analysis

Aseptically withdrawn bacterial cultures grown with different nanoparticle dosages were centrifuged at 10000 rpm for 10 min. The pellets were then washed with phosphate buffer (0.1 M, pH 7.2). The samples were then fixed with the Karnovsky's fixative fluid at 4 °C for 8 h. The fixed samples were then washed in a graded series of 30, 50, 70, 90, 95 and 100% acetone solutions. The dehydrated samples were then placed on aluminium stubs and subsequently coated with silver, under optimized vacuum conditions. The SEM observations were recorded using a scanning electron microscope (EVO Ma10 Zeiss EVO 40 EP, Carl Zeiss, Germany). EDX analysis was also performed to quantify the amount of zinc nanoparticles adsorbed by the bacterial culture, since the electron microscope was also equipped with EDX.

Results

Growth and viability of bacterial cultures on exposure to ZnO nanoparticles

Log-phase cultures of *P. striata* 27 and *A. chroococcum* W5 were inoculated in respective growth media on which nanoparticles (concentrations of 25–75 mmol/L) had been aseptically placed in wells, using the standard well diffusion assays. Qualitative analysis of the interaction of the nanoparticles with the P27 and W5 cultures, using PEG-400 surfactant and the standard agar-based growth medium, revealed no biocidal response. In order to elucidate the scale of interaction in a fully dispersed environment, further kinetic studies were conducted using the growth analyser. In this experiment, measured doses of the nanoparticle suspension were added to a 96-well titre plate, along with blank and control sets, to visualize their interaction with *P. striata* 27 and *A. chroococcum* W5 cultures. With increasing doses of the nanoparticle suspensions, the *P. striata* 27 strain showed a decrease in population as evidenced by the difference in optical density (Fig. 1a). The continuous monitoring based on spectrophotometric measurements of the growth kinetic analyser confirmed the trend of the interaction of ZnO with the *P. striata* 27 strain. However, the *A. chroococcum* W5 strain exhibited an increase in the optical density with the increasing nanoparticle concentration (Fig. 1b). As observed from the kinetic growth analyser data, there was a steep fall in the viability of *P. striata* 27 cells on exposure to higher doses of ZnO nanoparticles; therefore, further studies were undertaken only using *A. chroococcum* W5 culture. All of the observations for the changes in the optical density were taken relative to the initial absorbance of the solutions.

Scanning electron microscopy and energy-dispersive X-ray analysis

SEM analyses illustrated a gradual increase in the agglomerate/biofilm formation by the *Azotobacter* cells which was observed with the increase in the concentration of ZnO nanoparticles (25–75 mmol/L), leading to the bacterial cells forming distinct globular structures (Fig. 2a–d). The energy-dispersive X-ray (EDX) analysis of the samples depicts the elemental analysis of the samples and illustrates the intracellular presence of Zn, which increases with an increase in the concentration of nanoparticles (from 30.2 to 42.88%). There were clear peaks for the organic matter as well as those for zinc present in the oxidized state as part of the ZnO inoculant, thereby confirming the presence of the nanoparticles within the sample (Table 1). Supplementary Fig. 1

provides details of the elemental analysis, as obtained from EDX spectra of these samples.

Further focus was directed on how the plant growth-promoting (PGP) traits of *A. chroococcum* W5 were influenced by their interactions with the ZnO nanoparticle suspensions.

Effect of nanoparticles on metabolic activities of *Azotobacter*

The *A. chroococcum* W5 culture was grown with ZnO nanoparticles for 7–10 days, and nitrogen fixing potential was measured as ARA. Initial experiments with incubation time with the substrate acetylene for 4–6 h did not show any specific trend in the ARA of the bacterial samples; further studies were undertaken with the incubation time enhanced to 24 h. The samples from the 7-day-old treatments showed a gradual increase in ARA from 25 to 50 mmol/L concentrations, followed by a slight decrease at 75 mmol/L. In terms of percent change over control (JM + Az), an enhancement of 36.8 followed by 72% was observed at 25 and 50 mmol/L concentrations (Fig. 3a). However, the nitrogen fixing potential at 75 mmol/L concentration was 35.8% higher than in the control, indicating a threshold for ARA at 50 mmol/L concentration of the ZnO nanoparticles. An interesting trend was observed for the interaction of ZnO nanoparticles with the *A. chroococcum* W5 cultures in 9-day-old cultures, wherein only a minor reduction over the control, ranging from 2.8–4.8, was recorded at 25–50–75 mmol/L concentrations. This illustrates the adaptation of the culture to ZnO nanoparticles by the 9th day, leading to significant differences, with respect to control culture (without ZnO nanoparticles). It can be hypothesized that the gradual development and increase in the extracellular sheath produced by *A. chroococcum* W5 resulted in the increase in ARA. An increase of 40–70% over the control (no nanoparticles) was recorded in 7-day-old cultures. There was an average of only ~5% reductions, as compared to the control (no nanoparticles) experiment after 9 days of incubation.

The IAA production potential of *A. chroococcum* W5 in the presence of ZnO nanoparticle suspension (50 mmol/L concentration) was evaluated in the presence and absence of tryptophan (Fig. 3b). There was a marked difference of over 90% for the IAA production upon addition of ZnO nanoparticles compared to the *A. chroococcum* W5 culture grown in Jensen's medium (JM). The addition of tryptophan (Trp) to the suspension resulted in a 45% increase in IAA production. Subsequently, on addition of ZnO nanoparticles to the mixture of tryptophan-amended culture of *A. chroococcum* W5 in Jensen's medium, there was an

Fig. 1 Growth kinetic analyser-based studies on the interaction of ZnO nanoparticles. **a** *Pseudomonas striata* 27. **b** *Azotobacter chroococcum* W5

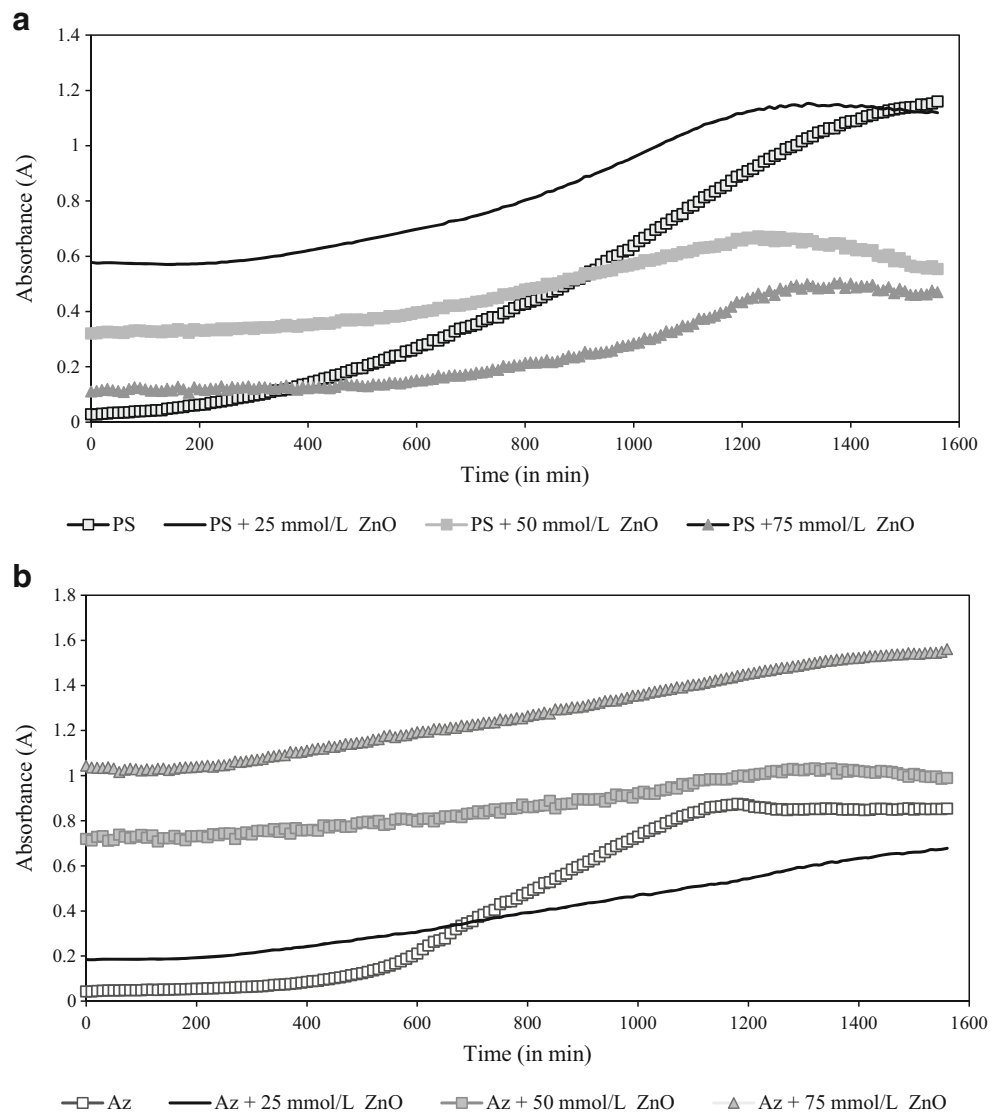


Fig. 2 Scanning electron microscopy (SEM) images illustrating the biofilm formation by *Azotobacter chroococcum* W5, as a result of interactions with different concentrations of ZnO nanoparticles. Clockwise, **a–c** 25, 50 and 75 mmol/L ZnO. **d** Magnified view of the globular biofilm of *Azotobacter*-ZnO nanoparticle interactions

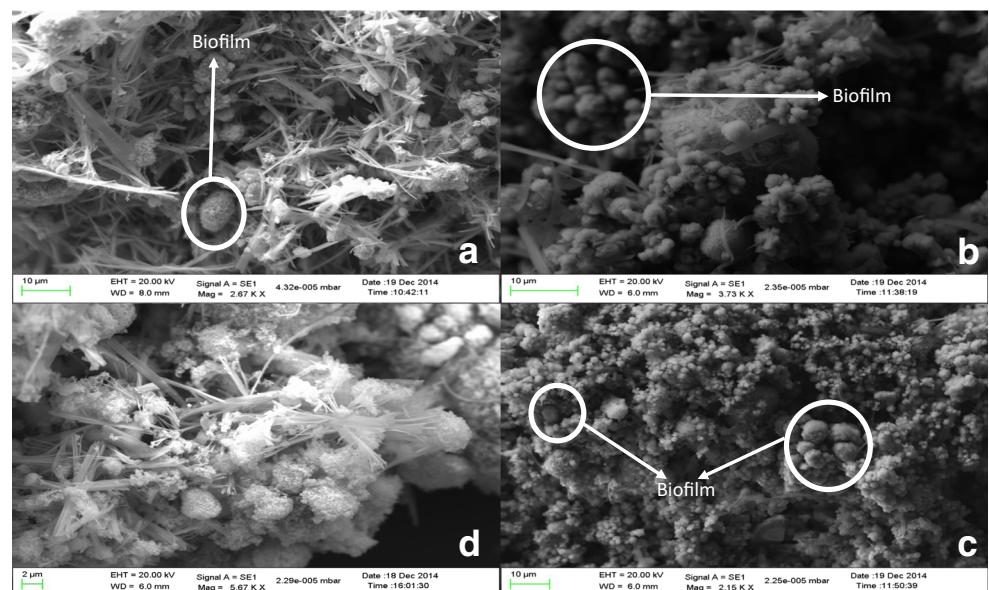
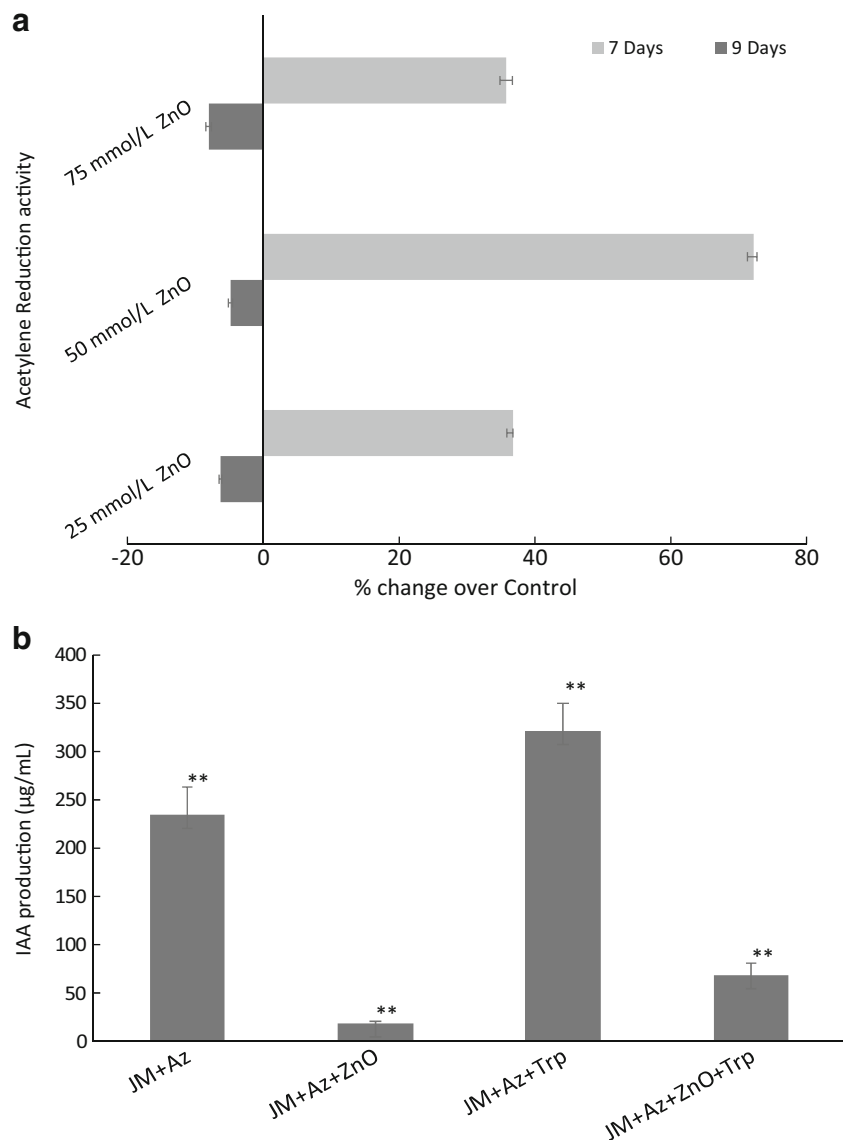


Table 1 Atomic and mass percentage of zinc (intracellular) present in the samples

Nanoparticle dosage	Atomic % in sample	Mass % in sample
25 mmol/L	10.90	30.20
50 mmol/L	15.65	41.36
75 mmol/L	16.88	42.88

80% decrease in the IAA production. Although the addition of ZnO reduced IAA production tenfold, and in tryptophan amended cultures by 80%, ZnO + Trp led to 85% enhancement, over the treatment in which ZnO alone was added to the bacterial cultures. All of the different samples showed statistically significant differences in the IAA production as indicated by double asterisks, with $p = 0.05$.

Fig. 3 Influence of ZnO nanoparticles on the functional attributes of *Azotobacter chroococcum* W5 in terms of **a** percent decrease in nitrogen fixing potential, measured as acetylene reduction assay (ARA), over control (Jensen's medium grown *Azotobacter* culture) and **b** IAA production ($\mu\text{g}/\text{mL}$)



Tripartite interactions of nanoparticles with *Azotobacter* and seed germination in wheat and tomato

Comparative performance of the treatments with wheat seeds revealed that the vigour index exhibited a clear trend of decrease, with the increase of the ZnO nanoparticle dosage (Fig. 4a). Treatments in which the seeds were treated with 10 mmol/L ZnO and *A. chroococcum* W5 recorded a stimulatory response; however, at higher concentrations, a gradual decrease was recorded. In the present investigation, the seeds treated with nanoparticle suspensions showed an approximately 30% decrease (with respect to the control) in the vigour indices, as compared to the seeds having no treatment at all. There was a clear 10% increase in the vigour index of the wheat seeds with *A. chroococcum* W5 inoculation, as compared to those with no inoculation at all. Approximately 20%

increase in the vigour indices of the seeds having bioinoculants was recorded to those treated with only nanoparticle suspensions. An important observation recorded was the protective role of *A. chroococcum* W5 in alleviating the toxic effects and promoting the growth of wheat seedlings.

In the experiment with tomato seeds, a drastic reduction was recorded when seeds were treated with ZnO suspensions. A reduction of over 60% in the vigour indices was observed upon being treated with the nanoparticle suspensions (Fig. 4b). However, co-inoculation with *A. chroococcum* W5 led to stimulation at 25 and 50 mmol/L concentrations, as compared to 10- and 75-mmol/L doses. The *A. chroococcum* W5-inoculated seeds have shown 15% more growth and an overall effect of approximately 50% more growth in the samples which were also treated with the ZnO nanoparticle suspensions. Statistically significant differences were observed for the vigour indices of the non-inoculated (with *Azotobacter*) seeds to those which were inoculated.

Discussion

Nanotechnology provides innovative means to develop novel formulations which can be effectively utilized for improving crop productivity. It is well known that plants represent a trophic level most prone to accumulation of trace elements or nanoforms, as they are directly in contact with air, water and soil (Gordon and Weber 1957; Misra et al. 2012). Metal-based nanoparticles or ions are known for their ecotoxicological and phytotoxic effects, impairing plant growth and development (Ma et al. 2010). However, several nanoparticles, including those of zinc, are essential for the proper growth of plants and can be useful at low concentrations. Zinc is an essential nutrient which plays a significant role in biological processes, not only as integral components of enzymes but also in the major metabolic pathways of the cell (Mousavi Kouhi et al. 2015; Pokhrel and Dubey 2013). Our earlier investigations have revealed the role of microbes in biofortification of wheat (Rana et al. 2012). Tarafdar et al. (2014) have developed zinc nanofertilizers, which improved growth and yields of pearl millet. The influence of nano-ZnO on mung bean and chickpea seedlings was investigated using a plant agar method, which revealed that seedling roots absorbed ZnO nanoparticles, leading to enhanced length and biomass of root and shoots (Mahajan et al. 2011). Palmqvist et al. (2015) showed that TiO₂ nanoparticles played an important role in adhesion of bacteria on the roots of plants.

The major aim of the present investigation was to identify the key interactions taking place between zinc oxide nanoparticles and the bacterial cultures, commonly used as bioinoculants (Nain et al. 2010; Rana et al. 2012). By observing their effect on the vigour and germination levels, one could surmise about the toxicological effects of ZnO

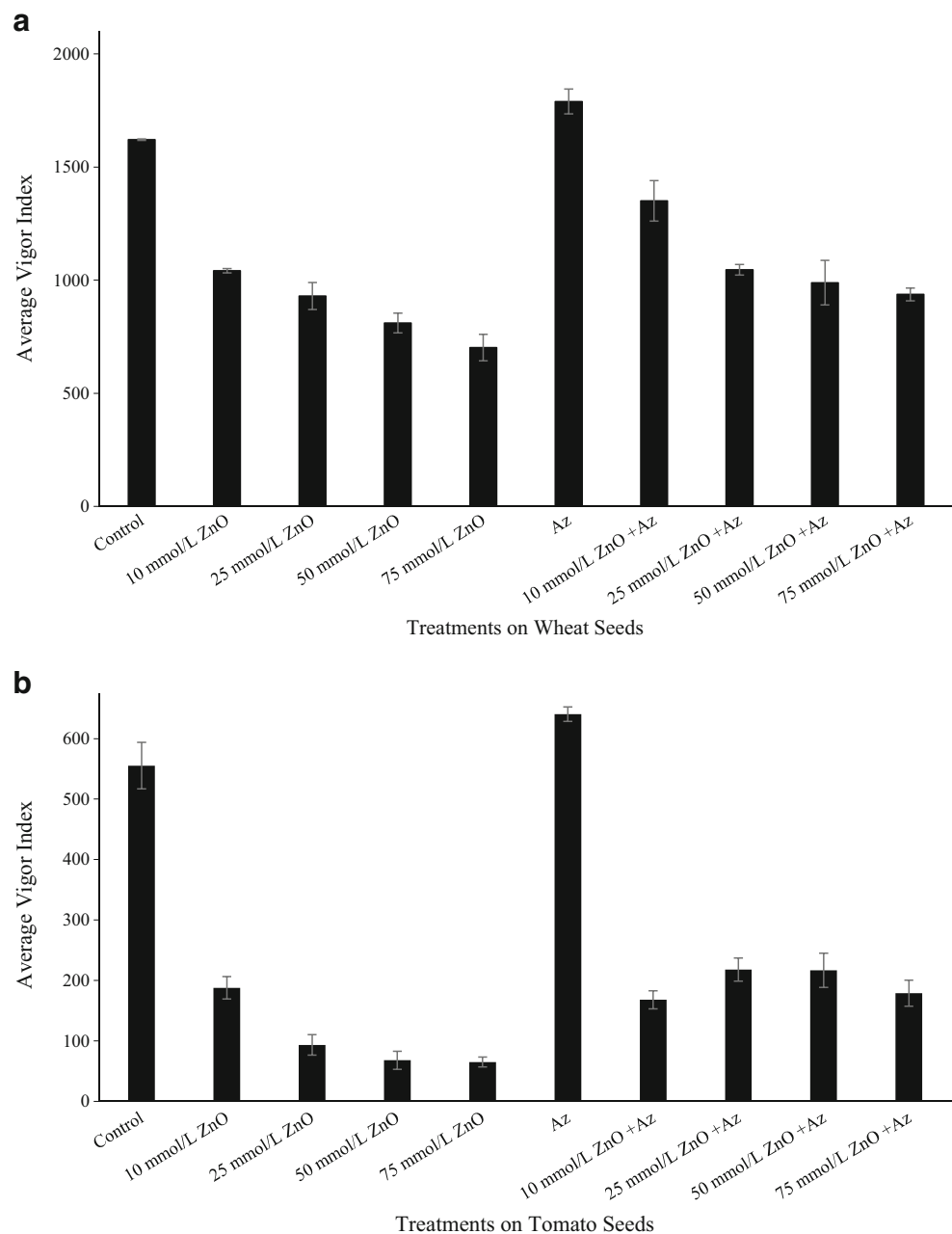
nanoparticles, which are known to be a common constituent of mineral fertilizers. The aforementioned interactions could be characterized on the cause-effect basis, through biochemical tests and scanning electron microscopy, to visualize the physiological effect of the nanoparticle suspensions on the bacteria. The ZnO nanoparticle suspensions were sonicated and prepared in autoclaved, deionized water to prevent any salting. Care was taken to avoid the usage of any solvents for the preparation of the suspensions, as the toxicity of the solvents would later interfere in the biological interactions.

Modifications were made to the standard well diffusion agar assay for better visualization of growth/inhibition as well as better solubility and diffusion of the nanoparticles. For assays in which the concentrations were greater than 75 mmol/L nanoparticle concentrations, there was no growth observed. The nanoparticle suspension was mixed with agar, which proved to be a novel and effective way of exposing the bacteria to the nanoparticle surfaces. In order to minimize the heterogeneity of the nanoparticle suspensions, these solutions were mixed well prior to inoculation. The bacterial cultures showed clear zones of inhibition on these plates, which were directly proportional to the concentration of the nanoparticle suspensions (data not shown). These preliminary experiments were an important component of the process of understanding the scale of interactions occurring in the inoculation experiments. Kinetics of the interaction using the growth analyser revealed the threshold levels of the nanoparticle formulations and the effective incubation periods.

EDX spectra revealed that Zn particles were present in the microscopic sections of samples with *A. chroococcum* W5. This is significant and essential for proving the interactions of ZnO with *Azotobacter* and the intracellular presence of the metal oxide nanoparticles, because the harsh washing and sample preparation steps which represent integral components of SEM analyses can adversely affect the presence of such nanoparticles. Studies conducted by Lin and Xing (2008) showed that ZnO nanoparticles have the tendency to get internalized within the cells. In many such cases, their electrochemical difference with the cell potential gets reduced, and nanoparticle accumulation within the cytoplasm is observed (Auld 2001; Reed et al. 2012).

SEM studies revealed the presence of globular structures, which are not only a characteristic of *A. chroococcum* W5 but also a common observation in biofilm-forming organisms, which can be interpreted as a mechanism of adaptation to stress. It is important to note the presence of zinc in three oxidative states, namely zinc ion, Zn¹⁺ and Zn²⁺ states (Hermann et al. 2014; Reed et al. 2012). It has been shown that the highly negatively charged bacterial surface electrostatically attracts the positively charged nanoparticles. The possible presence of ZnO aggregates present on the samples at the resolutions taken for the sample interaction images is also visible. Microscopic imaging revealed a “tunnelling-like effect” in root tissues exposed to ZnO nanoparticles (Ma et al. 2010; Pokhrel and Dubey 2013). With further studies, such as

Fig. 4 Effect of nanoparticles and *Azotobacter chroococcum* W5 inoculation on the germination, expressed in terms of vigour indices of **a** wheat seeds and **b** tomato seeds



those conducted by Mousavi Kouhi et al. (2015), the locations of the majority of each of the states can be possibly determined, so as to give an in-depth electrochemical analysis of bacteria-nanoparticle interactions.

The sustained ARA values are reflective of the acclimatization of the bacteria to the nanoparticle exposure, and its ability to reduce acetylene efficiently in its biofilm (stressed) state was more efficient, even at high concentrations. Continuous adaptation is known to result in moderate levels of acclimatization (Vu et al. 2009; Chopra 2007; Lin and Xing 2008). Therefore, the effect of tryptophan had a stimulatory role, even in the presence of ZnO nanoparticles. The presence of the polysaccharides, present as mucilage and biofilm

produced by *A. chroococcum* W5, as a response to the stress induced by the ZnO nanoparticles can help in alleviating the toxicity. Research conducted by He et al. (2011) on *Penicillium* and *Botrytis* strains, to study their interaction with ZnO nanoparticles, showed that their surface chemistry stressed out the cells into forming protective sheaths, which would help the cells adapt to the harsh environment produced by the nanoparticle suspensions. Raliya and Tarafdar (2013) reported that ZnO NPs induced a significant increase in the plant biomass, root and shoot growth and root area. Earlier studies by Dhas et al. (2014) observed that ZnO and Ag NPs were toxic to a number of bacteria tested and attributed this to the disruptions in cell membrane and increased permeability,

finally culminating in cell death. In our investigation, the presence of extracellular mucilage and biofilm formation may have provided a significant barrier to the entry of nanoparticles, resulting in low toxicity, which is supported by the observations of Dimkpa et al. (2011). Also, the exopolysaccharides (EPS) or extracellular polysaccharides (ECP) are known to provide abundant binding ligands for metal ions released from nanoparticles, preventing cell membrane damage by the nanoparticles.

Phytotoxicity of nanoparticles to vascular plants is known to be more intense in hydroponically grown plants, as compared to the soil system. As systematic studies on seed germination and plant vigour are scanty, the phytotoxicity of ZnO in two important crop species—wheat and tomato—was also evaluated. Lee et al. (2008) demonstrated the utility of the plant agar test for the phytotoxicity of nanoparticles, and in the present investigation, a modification involving the use of ZnO-infused agar suspension was used. Earlier studies using silver nanoparticles and wheat seeds had shown that the major effect was on the growth and developmental processes. The toxicity observed has been attributed to the generation of reactive oxygen species or damage to the cell membrane of the nanoparticles entering the pores of the seeds when they are germinating and preventing the intracellular functioning due to their metal oxide charges (Ma et al. 2010; Gorczyca et al. 2015). Nanoparticles may also bring about a stimulation of photosynthesis (Miralles et al. 2012; Lin and Xing 2007). Studies conducted by De La Rosa et al. in 2013 suggested that zinc nanoparticles, particularly Zn²⁺ ions, show a very strong effect on the germination of tomato seeds in a positive trend upto a low concentration of 2–5 mmol/L, beyond which the seed germination is detrimentally effected. Research conducted by Mallevre et al. (2014) also suggested that the *Pseudomonas* strain showed susceptibility towards apoptosis in the presence of increasing doses of ZnO nanoparticle concentrations. There is concurrence with their discussion that, depending on the size of the nanoparticles considered, as well as the porosity of the media worked on, the interactions can vary greatly.

The stimulation of growth in *Azotobacter* can be possibly explained due to its ability to form biofilms or aggregates of cells, which is well documented, along with its ability to secrete a mucilaginous extracellular sheath that protects the cells from the environment (Dalton and Postgate 1969). Aggregation has been known to increase with higher concentration of nanoparticles, indicative of the role of stress on the induction of the protection mechanism, resulting in enhanced growth. Intracellular zinc may also play a beneficial role in the metabolism, triggering growth and multiplication as it is an essential nutrient and component of enzymes (Vijayaraghavan and Padmavathy 2008). Research conducted by Vu et al. (2009) showed how extracellular polysaccharide-producing bacteria are more common components of biofilms (Dimkpa et al. 2011). Extracellular polysaccharides (EPS) are known to be one of the main contributors for

the hardiness and high proliferation rate of *Azotobacter* cultures. Pokhrel and Dubey (2013) suggested the supplementation of germination data with anatomical studies, and future studies need to analyse the interesting trend observed in the experiments, which may possibly be related to the differences in the requirements for micro/macronutrients of the two different crops, as well as signalling mechanisms involved in response to the *Azotobacter* cells in the environment.

Scanty literature is available on understanding the interactions of nanoparticles, microorganisms and plants. Our investigation clearly illustrated the positive effects of selected agriculturally beneficial microorganisms in alleviating the toxicity of nanoparticles. This emphasizes the need for more research probing into the signalling mechanisms and mechanisms involved in such tripartite interactions.

Future strategies need to focus on understanding the interactions of bacteria with nanoparticles, which also serve as useful micronutrients for microorganisms and plants.

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Authors' contributions AB contributed to the experimental conception, design and its implementation, along with the acquisition of data and its interpretation, besides drafting the manuscript. RT and AS were also involved in the experimentation, acquisition of data and analyses. SS provided critical suggestions for improving the manuscript. RP and LN provided valuable inputs to the editing of the manuscript, particularly in writing the discussion.

Compliance with ethical standards

Conflict of interest All authors declare that there is no conflict of interest.

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