



Sub-lethal doses of widespread nanoparticles promote antifungal activity in *Pseudomonas protegens* CHA0

Shams Tabrez Khan^{a,*}, Javed Ahmad^b, Maqsood Ahamed^c, Alexandre Jousset^d

^a Department of Agricultural Microbiology, Faculty of Agriculture, Aligarh Muslim University, Aligarh, India

^b Zoology department, College of Science, King Saud University, Riyadh, Saudi Arabia

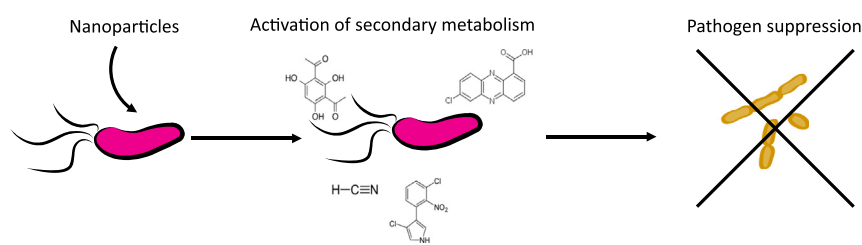
^c King Abdullah Institute for Nanotechnology, King Saud University, Riyadh 11451, Saudi Arabia

^d Department of Ecology and Biodiversity, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

HIGHLIGHTS

- Nanoparticles in soil impact the activity of plant-beneficial microbiota.
- We tested if nanoparticles affect the antifungal activity of a biocontrol *Pseudomonas*.
- Sublethal doses of nanoparticles stimulated antifungal activity.
- This study provides new tools to enhance disease suppression by soil microbes.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanoparticles are widely used as antimicrobial compounds. At sub-lethal concentrations, they may also stress microbes, potentially inducing antibiosis. Here we assess whether nanoparticles can serve as an enhancer of antibiosis in beneficial microbes. Several host-associated bacteria can suppress pathogens, providing therefore a first line of defense against diseases. In the present study, we assessed whether nanoparticles stimulate the antifungal activity of *Pseudomonas protegens* CHA0, a model plant-associated bacterium, against the ascomycete yeast *Candida albicans*. We synthesized and characterized four of the most common nanoparticles, namely Ag, SiO₂, TiO₂, and ZnO, with an average size of 25, 11, 25 and 35 nm, respectively. The dose-dependent effect of these nanoparticles on the growth of *Pseudomonas protegens* CHA0 was assessed. Ag, SiO₂, TiO₂, and ZnO nanoparticles inhibited the growth of *Pseudomonas protegens* by 100, 22, 15 and 15%, respectively at a concentration of 250 µg/mL. We then selected sub-lethal dose (500 ng/mL) and assessed whether the same nanoparticles stimulated the production of antifungal compounds inhibiting *C. albicans*. Incubating the bacteria in the presence of nanoparticles led to a four-fold increase in antifungal activity. We finally show that nanoparticles induce the expression of the *prm* operon, responsible for the production of antifungal compound pyrrolnitrin, within hours after nanoparticle exposure. This study shows that nanoparticle application may be a valuable tool to stimulate the antifungal activity of fluorescent pseudomonads, potentially assisting the development of future sustainable disease control strategies.

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1. Introduction

Engineered nanomaterials (ENMs) have unique and industrially important properties due to their nanoscale size. The global production of these ENMs is increasing rapidly as a consequence of their increasing use in various commercial products (Keller et al., 2013; Piccinno et al.,

* Corresponding author at: Department of Agricultural Microbiology, Faculty of Agriculture, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India.

E-mail address: tabrez.ag@mail.amu.ac.in (S.T. Khan).

2012). The indiscriminate and uncontrolled use of the ENMs is resulting in their continuous influx and accumulation in various environments (Keller and Lazareva, 2014; Keller et al., 2013). This has led to an increased concern regarding the potential environmental and health risks associated with ENMs. SiO₂, TiO₂, and ZnO are three main nanoparticles accumulating in landfills and soil in significantly high quantities every year (Keller and Lazareva, 2014; Keller et al., 2013). The fate of these nanomaterials in the soil, their influence on soil microbial communities, and their interaction with higher organisms need serious investigations. A few studies on the subject reveal that nanomaterials cause significant changes in microbial communities favoring the growth of some bacteria while retarding the growth of beneficial soil bacteria like *Rhizobium* (Ge et al., 2012; Liu et al., 2015; Zhai et al., 2016). At the same time, due to their strong impact on several species, the importance of nanoparticles and their persistence in soil make them more than potential pollutants. Instead, they might work as a tool to enhance microbial community functioning. Soil microbes are involved in several processes linked to soil fertility and improving their activity may allow developing sustainable pathogen management strategies.

Improving microbial functionality is particularly relevant in the context of sustainable agriculture. Plant roots are in association with dense microbial communities that play an important role in plant protection against diseases. Several bacteria can produce antimicrobial compounds that will prevent the growth of potential pathogens (Compant et al., 2005; Schmidt et al., 2015). Such antimicrobial compounds include a range of molecules including polyketides, cyclic lipopeptides, and have the potential to affect virtually any known pathogen (Haas and Defago, 2005). However, in several soils, secondary metabolite production by soil microbes is too low, leaving plants exposed to diseases (Imperiali et al., 2017).

In the present study, we assess whether nanoparticles may stimulate antifungal activity in beneficial, root-associated bacteria. We tested the effect of different nanoparticles on the growth of *Pseudomonas protegens* CHA0 and their ability to suppress the fungal pathogen *Candida albicans*. Nanoparticles can stress bacteria already at low, sub-lethal doses. As stressed bacteria may overexpress secondary metabolite production as a defense strategy (de Carvalho and Fernandes, 2010), we expected that nanoparticles may help improve pathogen inhibition.

2. Materials and methods

2.1. Synthesis and characterization of nanomaterials

The Ag nanoparticles (later: NPs) were synthesized using conventional chemical reduction method (Wang et al., 2005), while the TiO₂ NPs were synthesized using simple sol-gel method (Dalvandi and Ghasemi, 2013). ZnO NPs were also prepared via sol-gel chemistry (Raja et al., 2008). Silica (SiO₂) nanopowder (product no. 4850MR) was purchased from Nanostructured and Amorphous Materials, Inc. (Houston, Texas, USA). Crystal nature of these NPs was characterized by X-ray diffraction (XRD) using PANalytical X'Pert X-ray diffractometer (Spectris plc, England) equipped with a Ni filter using Cu K α (λ = 1.54056 Å) radiations, as an X-ray source. Morphology and size of our samples were determined using transmission electron microscopy (TEM; JEM-2100F, JEOL) analysis at an accelerating voltage of 200 kV.

2.2. Microbial strains and media

Pseudomonas protegens CHA0 was originally isolated from tobacco roots and is a model organism for plant-microbes interactions and fungal pathogen suppression (Oberhansli et al., 1991). In addition to the wild-type, we used a *prnA*-GFP reporter fusion allowing us to peer into the expression of the *prn* operon, linked to the production of the broad spectrum antifungal compound pyrrolnitrin (de Werra et al., 2011). Bacteria were kept in frozen glycerol stocks at -80°C . prior to experiments, bacteria were grown on Luria Broth (LB) or on nutrient

yeast broth or agar (NYB; peptone 10 g, beef extract 10 g, NaCl 5 g, yeast extract 5 g) with or without appropriate antibiotics as and whenever required.

We used the model pathogenic yeast *Candida albicans* ATCC 10145 as target organism. This organism is commonly used as a reference for toxicity assays thanks to its ability to grow in high throughput experiments. It was grown on Sabouraud dextrose broth and agar (dextrose 40 g, peptone 10 g and agar 16 g per liter).

2.3. Antibacterial activity of synthesized nanoparticles

Pseudomonas protegens CHA0 was grown in Luria broth at 30°C and adjusted to 10^5 cells/mL. Aliquots of 500 μL from this culture were added to 4.5 mL of sterile NY Agar containing different concentrations (125, 250 and 500 $\mu\text{g/mL}$) of nanoparticles (Ag, SiO₂, TiO₂, and ZnO) for 14 h at 30°C . NYB without any nanoparticle was used as a control. Bacterial density was assessed by serial dilution plating after 12 h incubation at 30°C and expressed as colony forming unit (CFU) per mL of culture on NY Agar.

2.4. Measurement of the *prnA*-GFP reporter fusion expression

The influence of the sub-lethal concentrations of the NPs on the expression of the *prn* operon, coding for the major antifungal compound pyrrolnitrin (de Werra et al., 2011), was assessed using a *prnA*-GFP reporter fusion, in which GFP is fused to the promoter region of the *prnA* gene, the first pyrrolnitrin biosynthetic gene of the *prn* operon. GFP signal is therefore proportional to the expression of the target gene (de Werra et al., 2011). A 10 μL aliquote from an overnight preculture was added to 96-wells microtiter plates containing 90 μL of LB medium containing sublethal concentrations of NPs (500 ng/mL). Wells without any nanoparticles were taken as control. Plates were sealed and incubated at 30°C for 24 h on a rotary shaker (50 rpm). Growth (OD₆₀₀) and GFP signal (Excitation 485 nm, Emission 538 nm) were recorded at zero, six, twelve and twenty-four hours using a Fluoroskan Ascent fluorometer (Thermofisher Scientific). Gene expression was expressed as the ratio between GFP signal intensity to OD₆₀₀. Relative fluorescence was integrated over the whole growth period with the `audpc()` function from the R package `agricolae`.

2.5. Influence of sub-lethal concentrations of NPs on the antifungal activity of *Pseudomonas protegens*

Bacteria were grown as described above, in NYB medium containing 500 ng/mL of nanoparticles (Ag, SiO₂, TiO₂, and ZnO) at 30°C overnight on a rotary shaker (150 rpm). Cultures were centrifuged at 12,000 rpm, for 15 min at 4°C , to remove bacteria and nanoparticles. Supernatants were transferred to a sterile 15 mL Falcon tube and filtered using 0.22 μm Millipore filter.

Candida albicans was grown overnight at 30°C on a rotary shaker in Sabouraud dextrose broth (SDB). An aliquot of 10 μL from this culture was transferred in 96 well plates containing 90 μL of sterile SDB. Aliquots of 10 μL of spent medium prepared above were also added to the wells. Two controls were used: The first control, SDB medium supplemented with 10 μL of NYB containing 500 ng/mL of nanoparticles (Ag, SiO₂, TiO₂, and ZnO), served to account for the direct inhibitory effect of the nanoparticle on the growth of *C. albicans*. The second control, the spent medium of *P. protegens* CHA0 grown in NYB without any added nanoparticles, was used as a baseline for the production of antifungal compounds in absence of nanoparticles. Plates were sealed and incubated at 30°C on a rotary shaker with slow shaking (50 rpm) for 9 h. OD₆₀₀ was recorded at an interval of 1.5 h for 9 h. Growth expressed as the area under the growth curve using the `audpc()` function in the R package `agricolae` (<https://CRAN.R-project.org/package=agricolae>).

2.6. Statistical analyses

All statistical analyses were carried out in R 3.2 (R core development team). Responses of bacterial growth, antifungal activity, and gene expression were assessed with a one-way ANOVA followed by a Tukey HSD test ($\alpha = 0.05$), using nanoparticle identity (factor, five levels) as independent variable. One separate analysis was performed per dependent variable. Dose-dependent response of bacteria was assessed with a two-ways ANOVA addressing the interactive effects of nanoparticle concentration (continuous, log-transformed) and nanoparticle identity (factor, five levels). For the bacterial growth assays 30 replicates were set up for each treatment, spread on five plates. As we did not detect major plate effects, further experiments were set up with six replicates for each treatment.

3. Results

3.1. Synthesis and characterization of nanomaterials

Nanoparticles of Ag, SiO₂, TiO₂, and ZnO were synthesized using the protocols detailed in materials and methods. Crystalline nature of the synthesized nanomaterials their shapes and sizes were determined using XRD and TEM analysis. The XRD spectra of all the synthesized nanomaterials except for SiO₂-NPs suggest the crystalline nature of the synthesized nanomaterials (Fig. S1). While no spectra were obtained for SiO₂ NPs indicating the amorphous nature of this material. The shapes of these NPs as observed under TEM are shown in Fig. S2. Ag- and ZnO-NPs were almost spherical in shape, while SiO₂-NPs are hexagonal in shape. Anatase TiO₂ NPs were also slightly hexagonal to oval in shape. Ag, SiO₂, TiO₂, and ZnO exhibited an average size of 25, 11, 25 and 35 nm, respectively.

3.2. Antimicrobial activity of synthesized nanoparticles

All the tested NPs inhibit the growth of *Pseudomonas protegens* CHA0 ($F_{3,376} = 432.0$, $p < 0.0001$). Inhibition was dose-dependent ($F_{1,376} =$

890.7, $p < 0.0001$) and the dose-response depended on the nanoparticle type ($F_{3,376} = 178.6$, $p < 0.0001$, Fig. 1). Silver nanoparticles showed the strongest growth inhibition, followed by zinc oxide, silicon dioxide, and titanium dioxide nanoparticles (Fig. 1).

3.3. Influence of nanoparticles on *Pseudomonas protegens* CHA0 antifungal activity

The assessed nanoparticles affected antifungal activity. Ag and ZnO nanoparticles induced the strongest inhibition of *Candida albicans* by spent bacterial supernatant (Fig. 2). It is worth noting that these two nanoparticles were also the two most toxic against the bacteria, supporting the idea that sublethal stress may serve to enhance antibiotics production.

3.4. Effect of nanoparticles on *prnA*-GFP expression

The tested nanoparticles further affected the expression of the *prn* operon, responsible for the production of the broad spectrum antifungal compound pyrrolnitrin ($F_{4,43} = 35.1$, $F < 0.0001$). In particular, SiO₂ and Ag nanoparticles stimulated *prnA*:GFP expression by 25–30% under the tested conditions (Fig. 3), while TiO₂ and ZnO nanoparticles had no significant effect on gene expression.

4. Discussion

Application of beneficial microbes providing protection against pathogens may replace antibiotics and pesticides as the main tool of disease management strategy. However, in order to work, we need to control the expression of microbial traits linked to disease suppression. In the context of plant growing in field conditions, bioaugmentation of the root-associated microbial communities with beneficial microbes producing useful antibiotics has long been regarded as a straightforward strategy to reduce pesticide input (Bahroun et al., 2017). However, introduced microbial species often fail to express the traits linked to disease suppression. This can be enhanced by the addition of sublethal

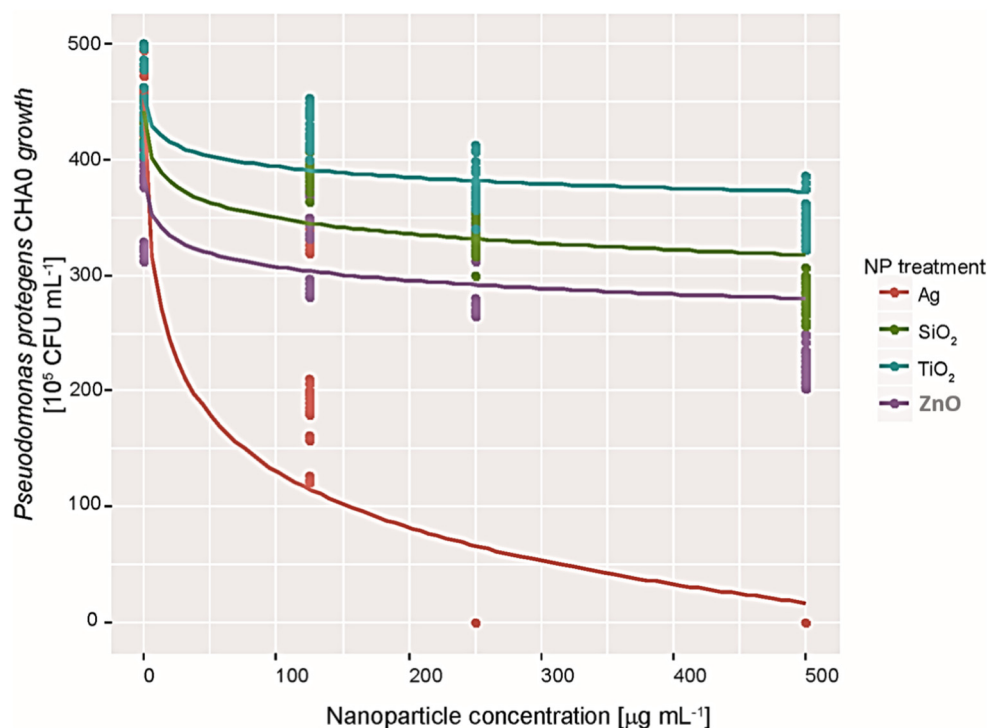


Fig. 1. Effect of nanoparticles exposure on the growth of *Pseudomonas protegens* CHA0. Bacteria were grown in NYB broth supplemented with various concentrations of nanoparticles. In each treatment, growth is summarised as the area under the growth curve over 48 h.

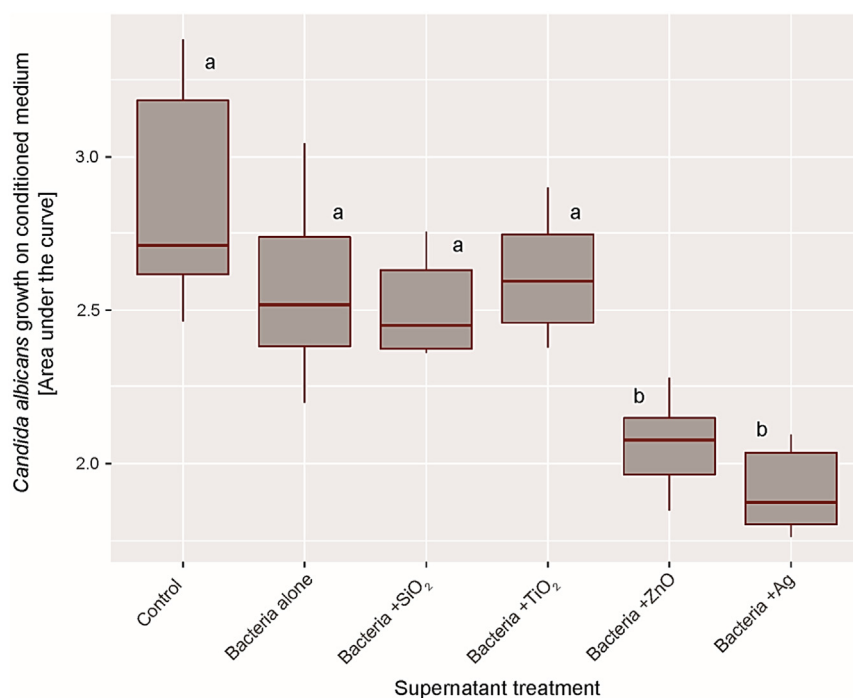


Fig. 2. Effect of nanoparticles on the antifungal activity of the bacteria *Pseudomonas protegens* CHA0, assessed as the growth of the yeast *Candida albicans* in a culture medium supplemented with the spent supernatant of a bacterial culture pre-incubated in absence or presence of a sublethal dose (500 ng/mL) of the different nanoparticles. Control treatment received fresh culture medium. Categories with different letters differ significantly from each other based on a Tukey HSD test ($\alpha = 0.05$).

concentrations of nanomaterials as demonstrated in this study. This enhancement of the activity can be due to the general stress induced by the nanoparticles in bacteria (Khan et al., 2016; Saccà et al., 2014; Wang et al., 2017), which may turn on the biosynthetic genes for secondary metabolites like pyrrolnitrin, which we used as reference gene in the present study. Note that bacteria produce several antimicrobial compounds that may work in synergy with each other (Dubuis et al.,

2007). Future studies addressing metabolomics level changes in response to nanoparticles may help understand better their impact on microbial interactions and community functioning. To the best of our knowledge, this is the first report on the nanomaterials mediated induction of antimicrobial activity. Furthermore these NPs, for example, ZnO NPs can survive in the soil long enough to support a crop (Collins et al., 2012). It has been demonstrated that ZnO NPs can persist in soil for a duration of 160 days (Collins et al., 2012).

In the present study, we show that nanoparticles can, at a low concentration, stimulate antifungal activity in bacteria. While at high concentration nanoparticle show an indiscriminate inhibition of bacteria and fungi akin, at low concentration they barely affected bacterial growth but stimulated their ability to suppress the yeast *Candida albicans*. This has two consequences. From an environmental management perspective, our results illustrate that nanoparticle pollution can affect microbe-microbe interactions at concentrations much lower than the threshold for direct toxicity. This calls for a more careful monitoring of nanoparticle contamination and a re-evaluation of their environmental impact. Increasing negative interactions between microbes may completely change community function, potentially stabilizing it but also bearing a risk of complete collapse (Becker et al., 2012; Coyte et al., 2015). From a very different perspective, this study also shows that nanoparticles may be included in future biotechnological applications to stimulate the activity of beneficial microbes, be they naturally occurring or co-added with the nanoparticles.

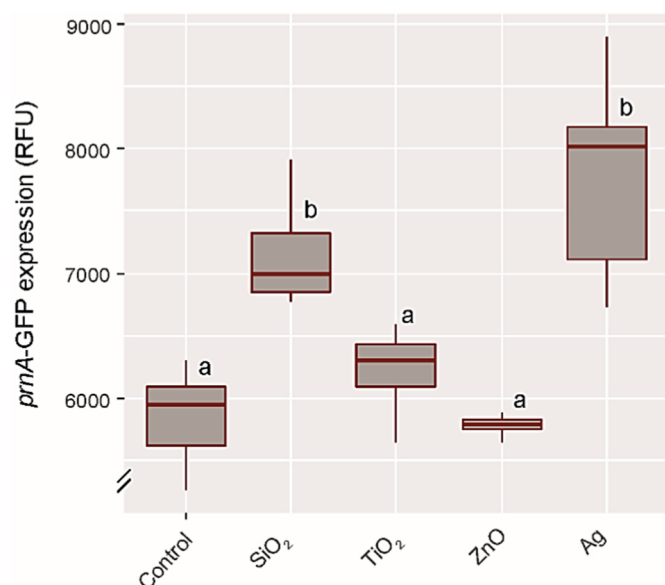


Fig. 3. Effect of incubation of the bacteria *Pseudomonas protegens* CHA0 with sublethal doses of nanoparticles (500 ng/mL) on the expression of the *pm* operon, linked to the production of the broad spectrum antifungal compound pyrrolnitrin. Gene activity was measured using a *pmA*-GFP reporter fusion and expressed as relative fluorescence unit. Categories with different letters differ significantly from each other based on a Tukey HSD test ($\alpha = 0.05$).

Conflict of interest

Authors don't have any conflict of interest whatsoever.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.01.257>.

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