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ORIGINAL RESEARCH

Differential tolerance of *Trichoderma harzianum* and *Rhizoctonia solani* towards silver nanoparticles: potential for agricultural applications?

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Abstract. In the previous study, we examined the effect of silver nanoparticles (AgNPs) on beneficial soil fungus including *Trichoderma harzianum* (T22), and pathogenic soil-borne fungus, *Rhizoctonia solani* (AG3-PT). The result exhibited that *T. harzianum* (T22) is tolerance towards AgNPs. On the other hand, the pathogenic fungi, *R.solani* (AG3-PT), is more sensitive to AgNPs. *T. harzianum* is well known as biocontrol agent to suppress *R. solani*. Therefore, in this study we investigated the combination of *T. harzianum* (T22) and AgNPs at low concentration to control two strains of *R. solani* (AG3-PT and AG2-1). The effect of AgNPs at two different levels (20 mg L^{-1} and 50 mg L^{-1}) was examined over the growth of the two strains of *R. solani* and *T. harzianum* (T22) using dual culture technique. The results shows that this combination have a potential to reduce colony growth of *R. solani* (AG2-1) at higher AgNPs concentration. However, it was not the case for *R. solani* (AG3-PT). It can be concluded that AgNPs toxicity depend on several factors including species strain and the size of AgNPs particle.

Keywords: AgNPs, biocontrol, fungi, plant pathogenic control technique

INTRODUCTION

Silver nanoparticles (AgNPs) are extensively used in many fields, including agriculture, due to their antimicrobial characteristics [1–5] . We studied the effect of AgNPs on both plant pathogenic fungi and antagonist fungi. Our previous study showed that the growth of *Rhizoctonia solani*, a plant pathogenic fungi, affected by AgNPs at 20 mg L^{-1} and 50 mg L^{-1} [6]. In contrast, *Trichoderma harzianum*, antagonist fungi, is more tolerant as AgNPs only affected the growth of the fungus (as measured by colony diameter) at a very high level (600 mg L^{-1}) [7] . These findings imply the possibility to combine *T. harzianum* and AgNPs at low concentration to control plant pathogens. This could potentially has two outcomes. First, the presence of AgNPs could reduce the ability of *T. harzianum* to control pathogens. Otherwise, the AgNPs and *T. harzianum* could work synergistically to reduce pathogens growth even further.

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Trichoderma species has been combined with other fungicide and microorganisms to induce their efficacy [8–12]. Kumar et al. [12] claimed that the combinations of *Trichoderma* species with other bacteria or fungus were frequently more effective in controlling plant disease than the use of a single *Trichoderma* as biocontrol [12]. Strains combination has been also shown more effective in controlling plant disease. For example, combination of *T. harzianum* and *T. asperellum* reduced disease severity caused by *Fusarium* [13]. To the best of our knowledge, there has been no previous work on the combination of AgNPs and *T. harzianum* to control *R. solani*.

T. harzianum is well known as biological control agent and the most common antagonist fungi used to control *R. solani* by attacking the mycelium and produced antibiotic [14–16]. *R. solani* mainly targets plants' underground parts

but it is also able to infect the above ground parts of plants. The pathogen is most commonly associated with "damping-off."

Antagonist mechanisms by *T. harzianum* including competition over space and nutrition, mycoparasitism, antibiotic (toxin) and enzyme production. Competition over space and nutrition is the most well-known mechanism and can be easily studied by plating both fungi on agar medium known as dual culture technique. Therefore, the method was employed to study the effect of AgNPs on the ability of *T. harzianum* to control two strains of *R.solani* (AG3-PT, AG2-1) using same AgNPs concentration (20 mg L^{-1} and 50 mg L^{-1}) as studied by Oktarina et al. [6].

METHODOLOGY

AgNPs analysis

Silver nanoparticles were obtained from M K Impex Corp. Mississauga, Canada. AgNPs were characterised, in terms of structure and particle size distribution. The structure of AgNPs was analysed using JEOL 2100F FEG TEM at Durham University. To prepare samples for images, a dilute suspension of AgNPs were dropped on a 300 mesh Cu grid with Lacey carbon film and then dried over air. The behaviour i.e. aggregation of AgNPs, in growth media was observed using light microscopy. AgNPs were introduced to Potato Dextrose Agar (PDA) media before autoclaving at 121°C for 15 minutes. Prior to plating into 90 mm petri plates, sterile media were thoroughly swirled. Once the media had set, a small amount of AgNPs containing media was placed onto a microscope slide and observed under microscope. The particle size distribution of AgNPs was determined by a Dynamic Light Scattering (DLS) technique using a Zetasizer Nano S. One ml of sample was used for analysis in plastic cuvettes. The suspension was prepared by suspending the AgNPs in DI water (10 ml) with further dilution. The AgNPs were sonicated for 5 min immediately prior to making the DLS measurements [17].

Fungal isolates preparation

The *T. harzianum* strain employed in this study was obtained from Koppert B.V., The Netherlands, under the name Trianum-P (T22). Using a sterile loop, the powder form of *T. harzianum* was transferred onto a PDA plate and incubated at 24ºC for further use. Two strains of *R. solani* (AG3-PT and AG2-1) isolates, kindly provided by James Woodhall (Parma Research and Extension Center, University of Idaho, Parma, USA), were reisolated on fresh PDA plate and incubated at 24ºC for further use.

Dual culture technique

The effect of AgNPs on *T. harzianum* ability to suppress two strains of *R. solani* (AG3-PT and AG2-1) growth was investigated using a dual culture approach. Two levels of AgNP concentration (20 and 50 mg L^{-1}) were prepared by mixing AgNPs powder with PDA before autoclaving at 121 ºC for 15 minutes. Prior to plating into 90 mm petri plates, sterile media were thoroughly swirled. Once the media had set, a 3 mm agar disc of a 7-day-old culture of *T. harzianum* (T22) was placed at the edge of petri dishes (90 mm diameter). The same size and age of another agar disc of *R. solani* (AG3-PT) was similarly placed on the media but on the opposite end and incubated at 24 ºC. Antagonistic activity was assessed by measuring diameter of both the pathogen and *T. harzianum* (T22) colonies daily until the plate was covered entirely by hyphae (5 days). Same technique was applied to *T. harzianum* (T22) against *R. solani* (AG2-1). Control plates were prepared without AgNPs. All experiments were repeated three times.

Statistical analysis

All presented data are the mean value of three replicates. The data were analysed statistically using One-way Analysis of Variance (ANOVA) in Minitab. Significant differences between mean values were determined using Least Significant Difference (LSD) (P=0.05).

RESULTS AND DISCUSSION

Figure 1 shows that AgNPs concentration at 20 and 50 mg L^{-1} weaken antagonistic ability of *T. harzianum* (T22) significantly on the fifth day of observation. The combination of AgNPs and *T. harzianum* do not show synergism effect on *R. solani* (AG3-PT) growth. Researchers reported that *Trichoderma* species produces metabolites and enzymes for their own survival when contact with AgNPs [18,19]. Harmful metal ions were transformed to non-toxic metallic AgNPs during catalytic action (18). As the result AgNPs became less toxic to *R. solani* (AG3-PT).

The combination of *T. harzianum* (T22) and AgNPs seems to be more promising to control *R. solani* (AG2-1). Figure 2 shows that the growth of *R. solani* (AG2-1) was restricted significantly when cultured with *T. harzianum* (T22) at 50 mg L^{-1} on the 4^{th} and 5^{th} day of observation. The finding suggest that silver sensitivity appears to vary among fungal strains within the same species. *Rhizoctonia* species are classified as anastomosis groups, which are

taxonomically separate (AGs). To date, there are 13 identified AGs belonging to the *R. solani* species complex, and these can be further categorised based on the pathogenicity, biochemistry, and genetic marker of the organism [20] . Min et al. [21] reported that 7 mg L^{-1} of AgNPs inhibited the growth of *R. solani* (AG5) significantly due to the irregular shape of the hyphal walls which they were prone to collapse. Similarly, Elgorban et al. [22] reported that six different strains of *R. solani* (AG-1, AG2-2, AG-5, AG-6, AG-10, and AG-4-HGI) are sensitive to low levels of AgNPs.

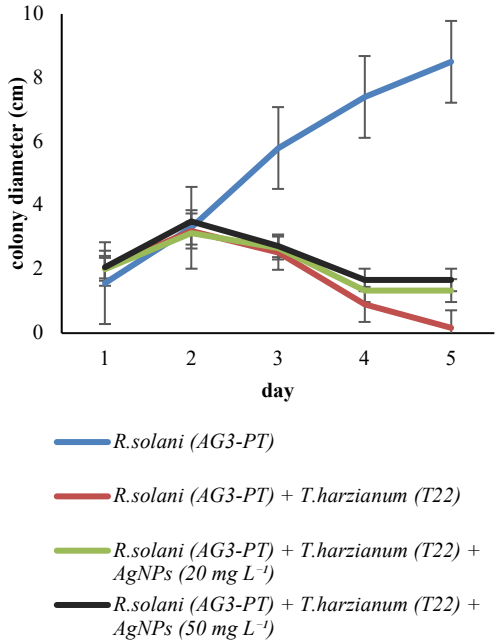


Figure 1. Colony diameter of *Rhizoctonia solani* (AG3-PT) with and without *Trichoderma harzianum* (T22) at 0, 20 and 50 mg L^{-1} of AgNPs. The colony diameter was measured daily for 5 days. Data represent means of three replicates with standard error.

A morphological change was observed on *T. harzianum* (T22) colony when cultured at 20 and 50 mg L^{-1} of AgNPs in the growth media (Figure 3. only shows *T. harzianum* (T22) colony at 20 mg L^{-1}). The mycelia of *T. harzianum* (T22) appeared thinner when grown on AgNPs contaminated media. When contact with heavy metal morphological changes are commonly observed among fungi. For example, Lima et al. [23] observed change on mycelia morphology of *T. harzianum* when contact with cadmium. Similarly, heavy metal affected the morphologies of whole fungal colony of *T. viride* and *Rhizopus arrhizus* (24). The phenomenon is potentially a defence mechanism of fungi in the presence of heavy metals.

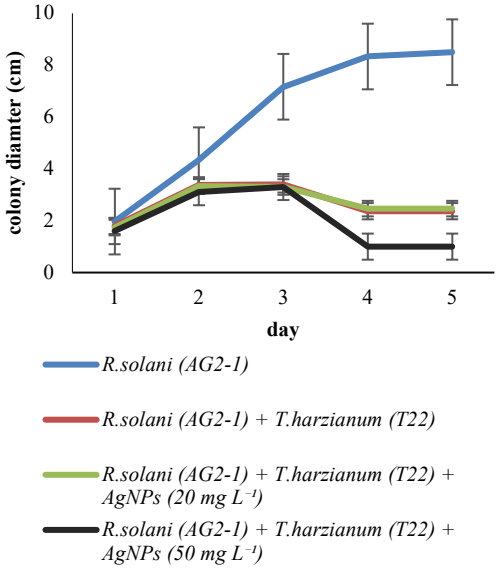


Figure 2. Colony diameter of *Rhizoctonia solani* (AG2-1) with and without AgNPs at 20 and 50 mg L^{-1} and *Trichoderma harzianum* (T22) presence in growth media. The colony diameter was measured daily for 5 days. Data represent means of three replicates with standard error.

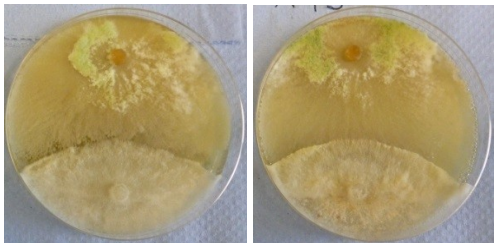
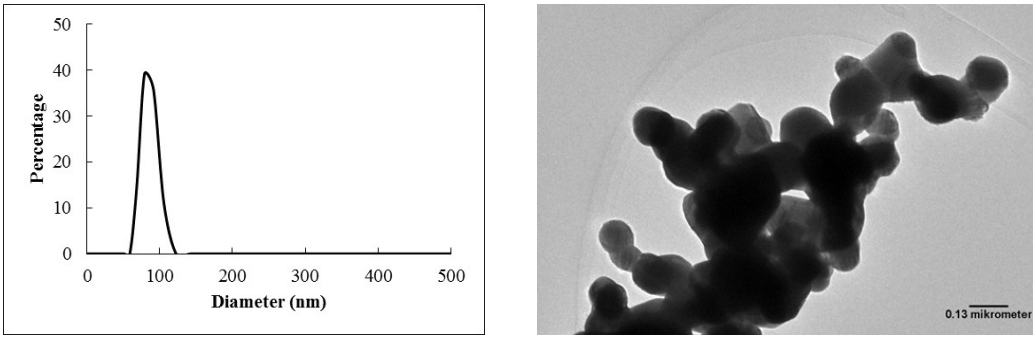


Figure 3. Morphological change on *T. harzianum* (T22) mycelia when cultured with *Rhizoctonia solani* (AG3-PT) at 20 mg L^{-1} (A) and *Rhizoctonia solani* (AG2-1) at 20 mg L^{-1} (B)

Antimicrobial activity of AgNPs also depends on their size. The smaller the size the more toxic nanoparticle are because they potentially release many more Ag^{+} ions that dominate the microbial activity [25]. AgNPs analysis revealed that the size of AgNPs applied in this study was much larger than the nanoparticles used in previous studies. Min et al. [21] reported that AgNPs at 7 mg L^{-1} with average size of 4-8 nm inhibited the growth of AgNPs on *R. solani* (AG5) significantly. While AgNPs with average size 18-34 nm employed by Gavanji et al. [26] on *T. harzianum*. Figure 4A shows the average diameter used in this study to be in the range of 60-120 nm. In addition, microscopic observation of AgNPs (20 mg L^{-1}) in growth media showed aggregation of AgNPs



(A) (B)

Figure 4. Silver nanoparticles anylised by DLS and TEM. DLS showing that the diameter of silver nanoparticles varied from ~ 60 to ~120 nm (A) TEM image showing AgNPs present as aggregates (B)

(Figure 4B) which presumably reduces their toxicity by reducing available surface area. However, the behaviour of NPs in natural environment is completely different to the behaviour in laboratory media [27] . In natural environment there are factors affecting aggregation of NPs including particles concentration, pH, and organic matter [28] .

CONCLUSION

In summary, the current investigation confirmed that the combination of AgNPs and *T. harzianum* has a potential to work synergistically to reduce plant pathogens growth. However, it depends on several factors including the size of the AgNPs and strain of the pathogen. Further work is required on the ability of AgNPs to control *R. solani* growth in a soil environment and on the potential effects of AgNPs contamination on the biocontrol mechanisms of *T. harzianum*.

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