Can nano-silver products endanger beneficial soil fungi?

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Abstract. Silver nanoparticles (AgNPs) are widely used in industry due to their anti-microbial properties. Despite of its beneficial, they may potentially affect the activity of beneficial soil microorganisms. *Trichoderma harzianum* was used in this to investigate the effect of AgNPs on beneficial soil fungi. Colony diameter and spore production of *T. harzianum* were observed in the presence of three levels of AgNPs (200, 600, 1000 mg L⁻¹) in growth media. The results showed that the application of AgNPs at high levels reduced colony diameter and spore production of the tested fungi. The findings indicate that AgNPs have the potential to damage beneficial soil fungi.

1. Introduction

Silver nanoparticles (AgNPs) are widely used in industry due to their anti-microbial properties. Common applications include the use of AgNPs for antimicrobial coatings on textiles, electronics and biomedical devices [1] [2] [3]. Recently, researchers have proposed the use of AgNPs in agriculture to control plant pathogenic fungi such as *Rhizoctonia solani*, *Bipolaris sorokiniana*, *Magnaporthe grisea*, and *Colletotrichum* sp. [4] [5] [6].

The use of AgNPs to control plant pathogen fungi does raise concerns as they may potentially affect the activity of beneficial soil microorganisms. The nanoparticles applied will end up in the soil and may become toxic to microorganisms. In bacteria, silver ions inhibit cell growth and multiplication by breaking through the cell wall, disrupting respiration and binding [7]. Antifungal activity of AgNPs damage fungal membrane integrity by interrupting the structure of the cell membrane and inhibiting the normal budding process [8].

The genus *Trichoderma* is one of a range of beneficial fungi that live in soil as saprophytes. In particular, *Trichoderma* has the ability to decompose organic matter [9]. Furthermore, *Trichoderma* species also have capability to live on other fungi and this property makes them well known as biocontrol agent against a wide range of plant pathogens. The potential of *Trichoderma* species as a biocontrol agent was first demonstrated by Weindling in the early 1930s [10] and has been used to control many plant pathogenic fungi since then. *Trichoderma* suppress plant pathogen by (1) coiling and penetrating the pathogen hypha [11], (2) produce toxins and enzyme (chitinase and/or glucanases) to destroy pathogen cell wall integrity [12] [13], (3) compete for space and nutrients [14] [15] [16]. Today, several species of *Trichoderma* have been produced commercially as biological fungicides.

Very little work has been done to study the impact of AgNPs on *Trichoderma* sp. Most studies have only focused on the use of AgNPs to control fungal plant pathogens. Due to the ecological
importance of *Trichoderma* in soil and as biocontrol, the current work investigates the effect of AgNPs exposure on colony diameter and spore production of *T. harzianum* *in vitro*.

2. **Materials and Methods**

2.1. **The impact of AgNPs on *T. harzianum* colony diameter**

The AgNPs used in the experiment were obtained from M K Impex Corp. Mississauga, Canada (MKN-Ag-020). Three levels of AgNPs (200, 600, and 1000 mg L$^{-1}$ of AgNPs) were added to Czapek Dox Agar (CDA), before autoclaving at 121 °C for 15 minutes. Sterile media containing AgNPs were swirled thoroughly before being plated into 90 mm petri dishes. Once the media had set, a 3 mm plug of 7-day-old *T. harzianum* culture was placed in the centre and incubated at 24 °C. The growth of *T. harzianum* was observed by measuring the colony diameter at 24 hours intervals until control plates were fully covered by hyphae (4 days). Control plates were prepared without AgNPs. All experiments were carried out in triplicate.

2.2. **The impact of AgNPs on *T. harzianum* spore production**

The spores number produced by *T. harzianum* at 1000 mg AgNPs L$^{-1}$ was counted on the 7th, 14th, 21st, and 28th day after inoculation. The spores were harvested by pouring sterile distilled water on the culture. One ml of spore suspension was transferred to a clean 1.5 ml microfuge tube. Dilutions were made up to $10^{-3}$, depending on density of spores. Twenty µL of the spores dilution were dropped on the centre of haemocytometer and observed under light microscope. Spores from five random squares (0.04 mm$^2$ each square) were counted as the sample. To calculate the number of spores per ml suspension, equation below was used:

$$\frac{\text{number of spores counted in } 5 \text{ squares}}{5} \times 25 \times 10^4 \times \text{dilution factor}$$

The number of spores on control plates were also counted. The spore count was carried out in triplicate.

2.3. **Statistical analysis**

The data on colony diameter, spore production, and spore viability were statistically analysed for Analysis of Variance (ANOVA) on Minitab 17. Significant differences between mean values were determined using Least Significant Different (P=0.05).

3. **Results and Discussion**

The colony diameter of *T. harzianum* was measured every 24 hours for four days, when the plate was covered fully by hyphae. Three levels of AgNPs (200, 600 and 1000 mg L$^{-1}$) were used to determine the level that affected colony diameter of *T. harzianum* that grown on CDA. Figure 1 shows that AgNPs at 200 mg L$^{-1}$ had no effect on the growth of *T. harzianum*. The colony diameters of the fungi growth on AgNPs contaminated media were equal to control (without AgNPs).
Figure 1. Colony diameter of *Trichoderma harzianum* grown on CDA at 200 mg L\(^{-1}\). The colony diameter was measured every 24 hours for four days.

However, higher levels of AgNPs (600 and 1000 mg L\(^{-1}\)) decreased the colony diameter of *T. harzianum* (Figure 2 and Figure 3). A significant reduction, around 30%, was noticed on the last day of observation for both AgNPs levels.

Figure 2. Colony diameter of *Trichoderma harzianum* grown on CDA at 600 mg L\(^{-1}\). The colony diameter was measured every 24 hours for four days.
Figure 3. Colony diameter of *Trichoderma harzianum* grown on CDA at 1000 mg L\(^{-1}\). The colony diameter was measured every 24 hours for four days.

*T. harzianum* growth used in this study was affected by AgNPs at high levels (600 and 1000 mg AgNPs L\(^{-1}\)). Other reports revealed that AgNPs at lower level reduce the colony diameter of *T. harzianum*. [17] reported that 25 mg L\(^{-1}\) of AgNPs inhibited colony growth of *T. harzianum* and *T. viride* by 50%. Similarly, [18] reported 90% growth inhibition of *T. harzianum* at 10 mg L\(^{-1}\). These findings suggest that sensitivity to silver is likely to be different between fungal strains of the same species but this should be tested further using similar growth conditions.

Another possible explanation for the resistance of *T. harzianum* to AgNPs is the ability of *Trichoderma* species to produce nanoparticles when exposed to metal ions. This ability is thought to be a detoxification mechanism e.g. toxic soluble metal ions are reduced to elemental nanoparticles which are less toxic. For example, a previous study showed that *T. asperellum* [19] and *T. viride* [20] produced AgNPs as by-product of their metabolism. [21] revealed that *T. reesei* produce enzyme and metabolites for their survival when the mycelium was exposed to silver nanoparticles. In this process the toxic metal ions were reduced to the non-toxic metallic AgNPs through the catalytic effect of the extracellular enzyme and metabolites of the fungus.
The fact that *T. harzianum* survived in media that contaminated with AgNPs at very high level, lead the research to study the spore production of the fungi. The spore of *T. harzianum* at 1000 mg AgNPs L\(^{-1}\) was harvested and counted at different age (7, 14, 21, 28 days after inoculation). Figure 4 indicates that spore production of *T. harzianum* was reduce significantly with the presence of AgNPs in growth media. The number of spores was reduced around 25% on the 7\(^{th}\), 14\(^{th}\), and 28\(^{th}\) day. The lowest number of spores was produced by *T. harzianum* on the 21th day.

This study revealed that spore production was affected by the presence of AgNPs. [22] stated that AgNPs damage the hyphae of *Aspergillus flavus* and reduce the number of spores produced. [23] reported that *T. harzianum* growth and spore production reduced as heavy metal concentration in the media used was increased. Researchers reported that heavy metal rupture of fungal cell membrane [24], change in the structure of fungus [25], damage the shape of hyphal walls, malformations and hyperthropy resulting in possible damage of the spores [22].

4. Conclusion
*T. harzianum* colony diameter and spore production were affected by the presence of AgNPs at high level. The study also revealed that when comparing results from different studies on the effect of AgNPs on fungal growth it is important to take into account that fungal strains of the same species can show markedly different responses sensitivity to AgNPs. For instance, the growth of *T. harzianum* that sensitive to low level of AgNPs may affected by the application of AgNPs as fungicide to control plant pathogenic fungi. However, *T. harzianum* that resistance to AgNPs at higher level might has a potential as bioremediation agent. Further study is required to verify this hypothesis.

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