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Effect of silver nanoparticles on the growth of *Colletotrichum* spp. and *Nigrospora* sp.

Dragoni-Rosado J[1], González-Mederos A[1], Sambolín-Pérez C[2]

[1]*Department of Science and Technology, Inter American University of Puerto Rico, San Germán Campus 00683, USA*
[2]*Institute of Sustainable Biotechnology, Inter American University of Puerto Rico, Barranquitas Campus 00794, USA*

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Abstract

The widespread use of agrochemicals to control phytopathogen fungi may harm crop, environment, and human health. Silver nanoparticles (AgNPs) are proposed as an alternative antifungal agent against phytopathogenic fungi. However, the effects of silver nanoparticles in different concentrations need to be more evaluated. In this study, five environmental endophytic fungi from banana leaves were isolated and classified by molecular analysis. These fungi were cultivated and purified in two culture media (PDA and SDA) using three concentrations (16, 50, and 100 ppm) of AgNPs - 15 nm. Results showed that AgNPs are good antifungal agents for some fungal species. The growth of *Colletotrichum* isolate 3, *Colletotrichum* isolate 4, and *Nigrospora* isolate was reduced when exposed to 50 and 100 ppm of AgNPs. Furthermore, the growth of *Colletotrichum* isolate 1 and *Colletotrichum* isolate 2 was not affected by the concentrations used in this experiment. The effect of AgNPs depends on the fungal species and the concentration of the nanoparticles. This research demonstrated that the use of AgNPs effectively controls the growth of some fungal species. However, as observed in our results, these particles are not specific. For these reasons, more comprehensive analysis of microbial communities' behavior is needed before recommending the use of AgNPs as an antifungal agent.

Keywords – agriculture – antifungal – environment – nanotechnology

Introduction

Phytopathogens cause significant losses in agriculture due to being fast spreading within the crops, producing damages in the leaves and roots (González 2001, Juárez-Becerra et al. 2010, Rizwana et al. 2022) and affecting the quality of the products (Pérez-Rojas et al. 2015). So far, producers have used several practices such as crop rotation, the removal of the affected areas, and agrochemicals to reduce the effect of phytopathogens (Africa Soil Health Consortium 2015). The application of agrochemicals such as fungicides on crops is one of the widely and frequently used methods to improve crop production (Gunjal 2020). For example, bananas are one of the most consumed and economically essential crops globally (Martínez-Solórzano & Rey-Brina 2021). They are also one of the most affected crops by phytopathogenic microorganisms, causing considerable economic losses. For this reason, the use of agrochemicals in banana plantations for the prevention of phytopathogens is common. However, the widespread use of those chemicals may harm the crop, environment, and human health (FAO 2017, Rizwana et al. 2022).

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Corresponding Author: Jason Dragoni-Rosado – e-mail – jason.dragoni@gmail.com

Therefore, a new kind of agricultural pesticide is being developed using nanotechnology to reduce this negative impact (Worral et al. 2018). Metal-based nanoparticles have been proposed as carriers of fertilizer and even pesticides (Bhattacharyya et al. 2014). Among those, silver nanoparticles (AgNPs) are used to preserve fruits due to their antimicrobial properties and ability to inhibit the growth of phytopathogenic fungi (Kim et al. 2012, Mendes et al. 2014, Mahdizadeh et al. 2015). Aguilar-Méndez et al. (2011) reported that the phytopathogen *Colletotrichum gloeosporioides* showed a significant decrease in its growth when it is exposed to 56 ppm of 24 nm AgNPs while growth inhibition of phytopathogenic fungi *Phomopsis* spp. isolated from soybean seeds was obtained when exposing it to 270 ppm of 52 nm AgNPs (Mendes et al. 2014). Ouda (2014) reported that the growth of phytopathogens *Alternaria alternata* and *Botrytis cinerea* was affected when using 15 ppm of 38 nm AgNPs. Kim et al. (2012) reported that the 18 phytopathogenic fungi used in their experiment presented different growth patterns when exposed to 25 nm AgNPs.

For this research, *Colletotrichum* spp. and *Nigrospora* sp. that have been associated with diseases in banana plants were isolated and preliminarily identified. Some studies isolated these species as endophytic fungi from the foliar tissue of banana leaves (Zakaria & Aziz 2018). Also, the AgNPs concentrations used in this research were selected using those investigations where the growth of the phytopathogens fungi was substantially inhibited. Mahdizadeh et al (2015) obtained a total inhibition of the growth of fungi such as *Sclerotinia* sp. and *Pythium* sp. using 16 ppm. Pulit et al (2013) obtained a 90**%** growth inhibition of *Cladosporium* sp. using 50 ppm of AgNPs. Kim et al (2012) obtained a total inhibition of some phytopathogenic fungi (e.g., *Botrytis* sp., *Cladosporium* sp., *Fusarium* spp., among others) using 100 ppm. Therefore, this study evaluated the effect of AgNPs -15 nm on endophytic fungi isolated from banana leaves in Maricao, Puerto Rico.

Materials & Methods

Fungi isolation

The banana leaf samples were collected at the municipality of Maricao, Puerto Rico, on a private farm dedicated to the cultivation of several types of bananas. The samples were stored in a sterile bag (Whirl-Pack**™**) and refrigerated to 4 °C before laboratory analysis. All procedures for isolation, purification, and sub-culture of isolated fungi were carried out in a bacteriological cabinet (EACI Envirco**:** Model 10557) to avoid sample contamination.

To isolate endophytic fungi, the banana leaf surfaces were prepared following the procedure reported by Salgado-Salazar & Cepero (2005). A consecutive immersion of the leaf was performed for one minute in 70**%** v/v ethanol, three minutes in sodium hypochlorite (0.8**%** v/v), one minute in 70 **%** v/v ethanol, and five washes for a minute in sterile distilled water. Once disinfected, the leaf was cut to a standard size of 5 mm^2 and the tissues were placed for 10 minutes in Petri dishes with a sterilized paper towel to allow them to dry (Castellanos et al. 2011). Disinfected leaf tissues were placed in Petri dishes with potato dextrose agar (PDA - 39 $g \cdot l^{-1}$, Actero) or sabouraud dextrose agar (SDA - 65 $g \cdot l^{-1}$, HiMedia) and incubated at 25 ºC (GCA Corp**:** Model Precision) for five days (Castellanos et al. 2011). A total of 20 fungal isolates were obtained in SDA and PDA.

Fungi purification

To purify each isolated fungi, a piece of their hyphae was cut using a sterilized scalpel knife. Then the mycelia were transferred to a Petri dish with fresh culture media, either SDA or PDA. The fungi were incubated at 25 °C for five days and, the macroscopic characteristics such as mycelium (front and back), texture, and elevation were recorded. This subcultivation process was carried out to guarantee a pure isolate.

Characterization and selection of fungal isolates

The pure isolates were grown in moist chambers to differentiate them by their production of

mycelia and spores. Moist chambers were prepared using a glass rod (V form) in a Petri dish, and a slide (previously sterilized) was placed on top. A block of the culture media (SDA or PDA) was placed on the slide, which was inoculated with the fungi and protected with a cover glass. Then, 1 mL of sterile distilled water was added to the Petri dish and incubated for 5 days at 25 °C. After incubation, the cover glass was removed and placed in a clean slide with a drop of lactophenol (Lactophenol Cotton Blue - BBL™). The type of mycelium and conidia presence was observed and recorded through the microscope (40X). According to their morphology, five different isolates were selected for the study.

Fungi identification

Fresh isolates of fungi were prepared in culture media SDA or PDA prior to DNA extraction. This process was performed with extraction kit Zymo Research Company (ZR fungal/bacterial miniprep DNA™). Electrophoresis was performed using a 0.8 **%** agarose gel stained with ethidium bromide to visualize the product of the extraction. Regions 1 and 2 of the Internal Transcriber Spacer (ITS) were amplified by a polymerase chain reaction (PCR) using primers ITS1 and ITS4 (White et al. 1990). The PCR product was visualized by using a 1.0 **%** agarose gel stained with ethidium bromide.

PCR products for each fungus were sequenced in the Sequencing and Genomics Laboratory at the University of Puerto Rico in Rio Piedras, P.R., using the ABI DNA sequencer 3130xl (Applied Biosystems, USA). The sequences were obtained using the primers ITS1 and ITS4, the reverse complement was determined with Biology Workbench, the contig sequence with BioEdit (7.2.5 version), and the results were compared with the information in the BLAST database. The ITS sequences were used to perform a molecular phylogenetic analysis using the maximum likelihood method conducted in MEGA7.

Exposure to the AgNPs

Fresh cultures were transferred by pressing the stock culture colony to form a disk that contained the organism mycelia, using the top of a sterile Pasteur pipette. The produced disk, with a diameter of 4 mm, was aseptically transferred to the center of Petri dishes prepared with the culture media (SDA or PDA) with the different concentrations (Table 1) of AgNPs (15nm) (Fig. 1) purchased from US Research Nanomaterial Inc., Houston, TX and incubated at 25 °C (Kim et al. 2012, Mendes et al. 2014). After 72 hours of incubation, the radial growth of the fungi was measured with a calibrated ruler, and these measures were taken daily until the growth of the colonies reached the edge of the Petri dishes.

Table 1 Concentrations of AgNPs used in this experimentation.

AgNPs concentrations	References
0 ppm (control)	-
16 ppm	Mahdizadeh et al. (2015)
50 ppm	Pulit et al. (2013)
100 ppm	Kim et al. (2012)

The standard deviation of the average diameter (three replicates) of the colonies was calculated. A *t*-test (with a 95 % confidence level) was conducted to determine a significant difference between the growth in the three concentrations of AgNPs. SPSS® software (IBM Corp., USA) was used to compare the growth diameter grown in the different concentrations of AgNPs vs. the control group.

Fig. 1 – Transmission electron microscope image of the AgNPs (15nm) used for this experiment (https**:**//www.us-nano.com/inc/sdetail/892)

Results

In the isolation process, 20 fungal isolates were obtained in SDA and PDA. According to the similarities in their morphology, five different isolates were selected for the study. Four *Colletotrichum* and one *Nigrospora* species were preliminary identified. The sequences were compared using the BLAST database of the National Library of Medicine. Table 2 presents the isolate, query cover, percent identity, GenBank accession number, and the assigned code for all the isolates in this experiment.

Table 2 Fungal isolates.

Isolate	Query Cover	Percent Identity	GenBank Accession Number and Reference	Assigned Code
Colletotrichum isolate 1	99%	99%	KM520013.1 (Lai 2014)	*C1*
Colletotrichum isolate 2	98%	99%	GQ407097.1 (Sim et al. 2009)	*C2*
Colletotrichum isolate 3	62%	99%	KC790971.1 (Sharma 2013)	*C3*
Colletotrichum isolate 4	86%	99%	FJ527880.1 (Gao & Guo 2008)	*C4*
Nigrospora isolate	99%	99%	HQ608152.1 (Rodrigues 2011)	*N1*

A phylogenetic tree was developed using the fast-minimum evolution method, considering the ITS sequences, and comparing them with fungi associated with banana plant diseases (Fig. 2). This analysis showed a close relationship between the four *Colletotrichum* spp. (*C1*, *C2*, *C3*, *C4*) and *Colletotrichum karsti*, a species associated with anthracnose in bananas (Huang et al. 2021). The isolate *N1* had a close relationship with *Nigrospora oryzae* isolated as an endophytic fungus from banana leaves (Zakaria & Aziz 2018) and *N. vesicularifera* reported as a phytopathogen in sugarcane (Raza et al. 2019).

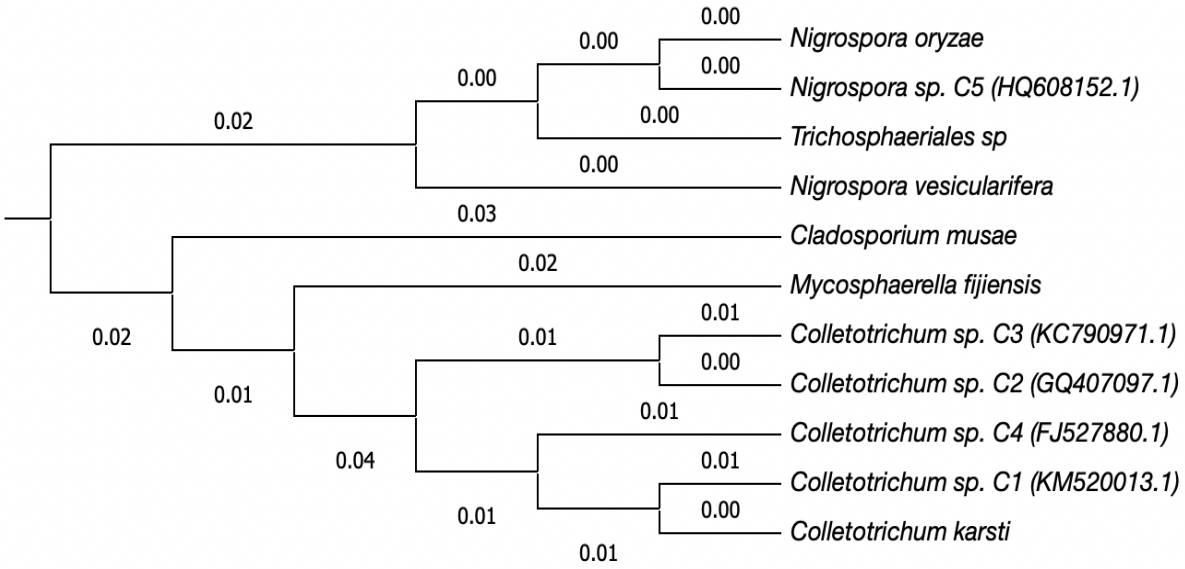

Fig. 2 – Phylogenetic tree using the fast-minimum evolution method showing the relationships between the five isolates used for this research and other common banana tree phytopathogens.

When exposed to the different concentrations of AgNPs (16, 50 and 100 ppm), the growth of some isolates of *Colletotrichum* and *Nigrospora* was affected. A reduction of the fungal mycelia was observed when the isolate was exposed to an increment of AgNPs concentration. For *C3* at day five of incubation, the AgNPs affected their growth at 50 and 100 ppm if compared with 0 and 16 ppm in PDA and SDA (Fig. 3).

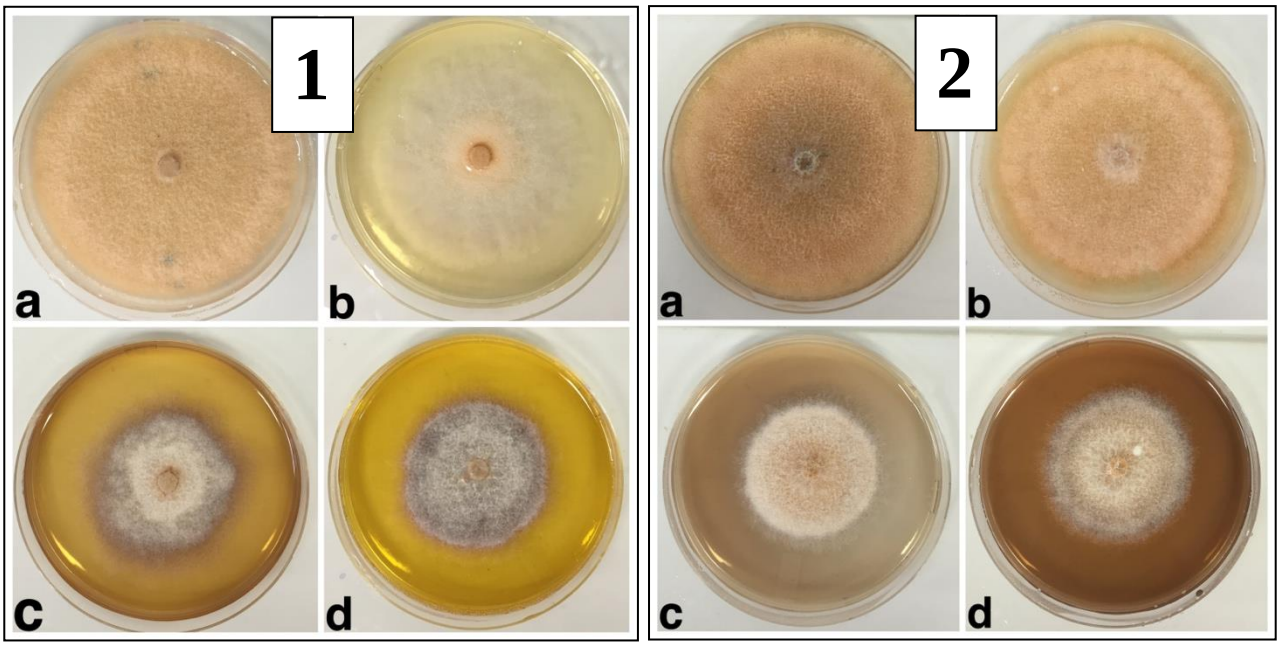

Fig. 3 – Isolate *C3* exposed to different concentrations of AgNPs (a = 0 ppm, b = 16 ppm, c = 50 ppm, d = 100 ppm) in PDA (1) and SDA (2) at five days.

In the higher concentrations of AgNPs (50 and 100 ppm), the isolates *C4* and *N1* (Fig. 4) show a low growth rate compared with the control *(*0 ppm*)* and the other isolates after six days of incubation in both culture media PDA and SDA.

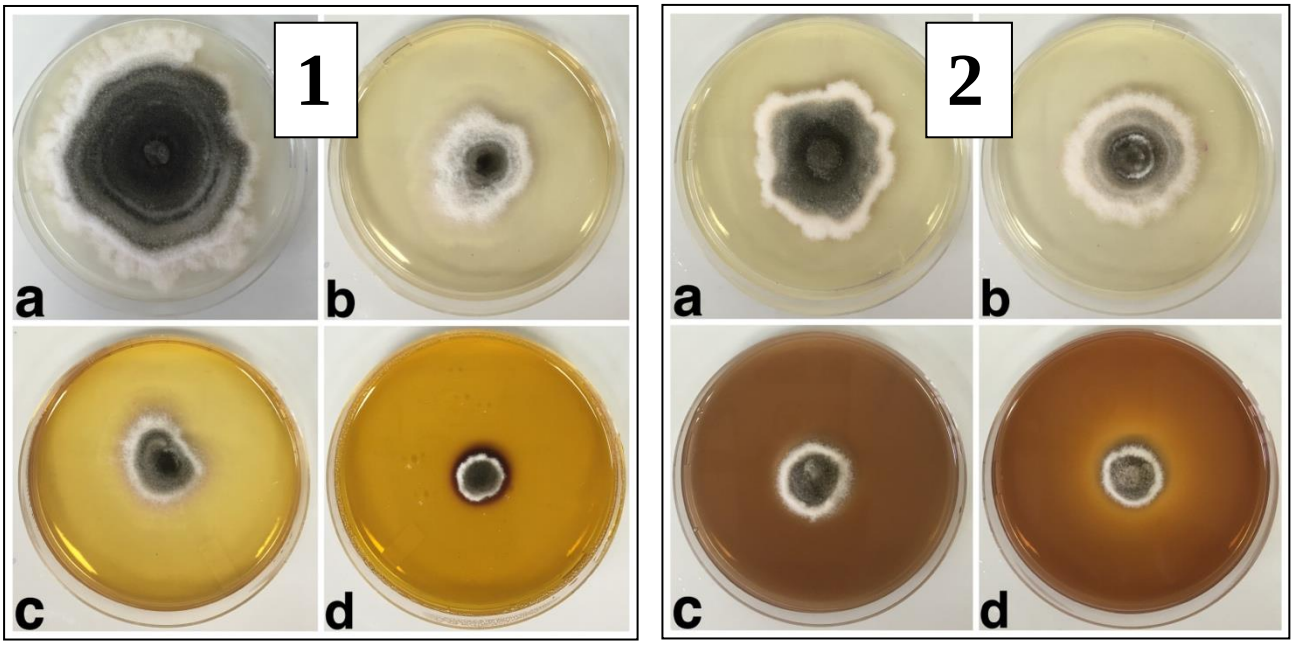

Fig. 4 – Isolate *N1* exposed to different concentrations of AgNPs (a = 0 ppm, b = 16 ppm, c = 50 ppm, d = 100 ppm) in PDA (1) and SDA (2) at six days.

For isolates *C1* (Fig. 5) and *C2*, a different result was observed when exposed to 16, 50 and 100 ppm AgNPs in PDA and SDA. In higher AgNPs concentrations, a visible mycelia reduction was not observed during the incubation.

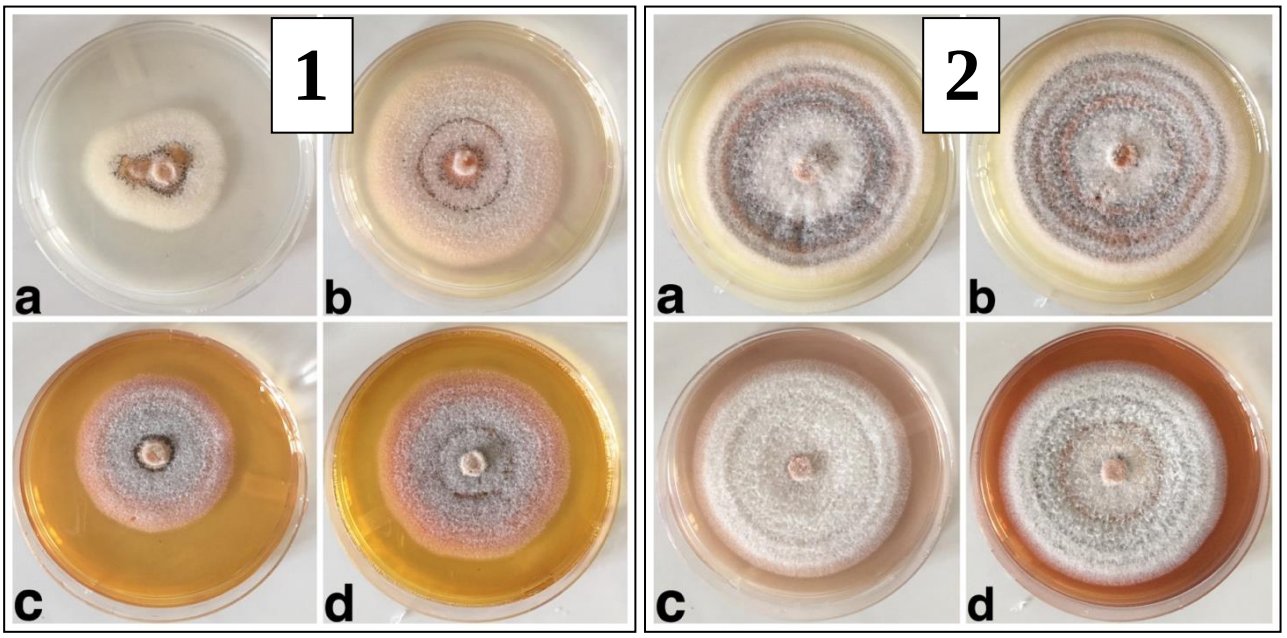

Fig. 5 – Isolate C1 exposed to different concentrations of AgNPs (a = 0 ppm, b = 16 ppm, c = 50 ppm, d = 100 ppm) in PDA (1) and SDA (2) at seven days.

Table 3 shows the average diameter of the colony *(*mm*)* for all the fungi exposed to the different concentrations of AgNPs in PDA and SDA. The isolate *C3* in PDA showed a significant decrease in their growth when exposed to concentrations of 16 ppm, 50 ppm and 100 ppm (compared with the control group). A similar result was obtained for *C3* in SDA at 50 ppm and 100 ppm. Compared with the control group in both culture media, the 50 and 100 ppm concentrations significantly impact *C4* and *N1*. In contrast, *C1* and *C2* showed no significant decrease when

exposed to the same concentrations of AgNPs. These results agree with Fig. 3-5, which observed an effect in their mycelia in some isolates.

Table 3 Average colony diameter (mm) of the isolates at different AgNPs concentrations.

Isolates/ Culture Media	Colony diameter (mm)			
	0 ppm (control)	16 ppm	50 ppm	100 ppm
*C*1 PDA	51.7 ± 16.2^{a}	57.5 ± 12.3^{a}	52.8 ± 8.4^{a}	57.3 ± 10.4^{a}
*C*1 SDA	74.8 ± 2.3^{a}	76.8 ± 2.0^{a}	54.8 ± 14.6^{b}	60.7 ± 13.2^{b}
*C*2 PDA	52.5 ± 9.2^{a}	51.0 ± 9.6^{a}	61.8 ± 8.4^{a}	59.7 ± 8.9^{a}
*C*2 SDA	69.3 ± 7.7^{a}	64.7 ± 8.8^{ab}	60.7 ± 5.5^{b}	69.5 ± 4.4^{ab}
*C*3 PDA	80.8 ± 5.0^{a}	56.8 ± 5.7^{b}	52.8 ± 11.3^{b}	32.8 ± 7.9^{c}
*C*3 SDA	78.8 ± 2.9^{a}	66.2 ± 6.9^{b}	35.7 ± 3.5^{b}	35.7 ± 7.0^{c}
*C*4 PDA	60.3 ± 6.1^{a}	56.8 ± 3.8^{a}	47.0 ± 7.0^{b}	45.7 ± 3.1^{b}
*C*4 SDA	65.5 ± 2.9^{a}	61.2 ± 5.4^{a}	58.5 ± 7.8^{b}	59.5 ± 4.3^{b}
*N*1 PDA	70.7 ± 11.5^{a}	61.3 ± 13.4^{b}	57.3 ± 12.8^{c}	20.2 ± 11.5^{c}
*N*1 SDA	45.2 ± 7.4^{a}	41.7 ± 4.4^{a}	30.5 ± 2.7^{b}	22.8 ± 3.4^{c}

Values with different letters are significantly different at 95**%** confidence level.

Discussion

AgNPs toxicity depends on their concentration (Alananbeh et al. 2017) and fungal species. In this research, the AgNPs have shown the ability to inhibit the growth of several fungal isolates from banana leaves. Differences in results may be related to the nanoparticles concentration or species studied in this investigation. The effect of the AgNPs concentration appears to be an essential variable since the fungi responded differently per nanoparticle concentration. Abdelmalek & Salaheldin (2016) reported a growth inhibition of the phytopathogenic fungi *Alternaria alternata*, *Penicillium digitatum* and *Alternaria citri* when exposed to 50 ppm of 10 ± 5 nm AgNPs. For isolated fungi from wastewater, Alananbeh et al. (2017) found higher inhibition rates when using 100 ppm of 15 ± 3 nm AgNPs. In addition, Lamsal et al. (2011) evaluated the effect of AgNPs (8 nm) on six *Colletotrichum* species at different concentrations. They observed that the growth of *C. acutatum* and *C. gloeosporioides* is inhibited when exposed to 100 ppm of AgNPs, while *C. higginsianum* did not present a significant decrease in its growth when it is exposed to that concentration. Also, Rizwana et al (2022) concluded that using green nanoparticles in high concentrations (80 ppm), a substantial reduction is obtained in the mycelium and sporulation of the fungi *Alternaria alternata*, *A. brassicae*, *Fusarium solani,* and *F. oxysporum*. Our results were similar; the isolates *C3*, *C4*, and *N*1 were affected by 50 and 100 ppm AgNPs, whereas *C1* and *C2* did not present a significant decrease when compared with the control (0 ppm).

Not all fungi are affected by the presence of AgNPs in the culture media. Therefore, the type of fungus to be studied is another factor that influences the results of this experiment and others previously reports. An example would be Rizwana et al (2022), who used five species of fungi, but only *Trichoderma harzianum* did not show a decrease in growth after being exposed to silver nanoparticles. Due to this variability in the antifungal effects of AgNPs, authors such as Bruna et al. (2021) have recommended manipulating the properties of nanoparticles (e.g., size) to optimize their capabilities against microorganisms. Nanoparticle size is another factor that affects the inhibition capabilities of the AgNPs. In this experiment, 15 nm nanoparticle decreases some fungi's growth (*C3*, *C4*, and *N1*). Min et al. (2009) reported growth reduction of phytopathogenic fungi *Rhizoctonia solani*, *Sclerotinia solani,* and *Sclerotinia minor* when using 7 ppm of 8 nm AgNPs. Another study reported an enhanced antimicrobial capability of the AgNPs due to their small size (Osonga et al. 2020). The behavior of different organisms exposed to AgNPs may indicate that the particle must be small enough to penetrate the organism's cell wall to cause growth inhibition. It is a critical parameter that affects the antimicrobial properties of AgNPs (Akpinar et al. 2021). Lamsal et al. (2011) explain that it is essential to reduce the particle size to improve their biocompatibility with the microorganisms.

Conclusions

This work demonstrated that the use of AgNPs is effective in controlling the growth of some fungal species. Based on the results, it can be inferred that the 15nm AgNPs could act similarly with these genetically close fungi. However, the antifungal activity will depend on the type of microorganism and the concentration of the nanoparticles. For these reason, more comprehensive analyses of microbial communities' behavior are needed before recommending the use of AgNPs as an antifungal agent.

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