ARTIGOS

Synthesis of silver nanoparticles (AgNPs) by *Fusarium concolor* and inhibition of plant pathogens

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ABSTRACT

Almeida, A-S.F.; Corrêa Junior, A.; Bentes, J.L.S. Synthesis of silver nanoparticles (AgNPs) b**y** *Fusarium concolor* and inhibition of plant pathogens. ***Summa Phytopathologica,*** v.47, n.1, p.9-15, 2021.

The use of nanoparticles (NPs) in agriculture represents an important technological innovation, which can be employed in the production of nanofertilizers, nanocides or pesticides encapsulated in nanoparticles for controlled release. Silver NPs are recognized for their applications in agriculture, biotechnology and medicine. The objective of this paper was to study the synthesis of silver nanoparticles by endophytic *Fusarium concolor* obtained from guaraná leaves and seeds, as well as to evaluate the antifungal activity of silver nanoparticles on the *in vitro* control of the phytopathogens *Colletotrichum guaranicola, Colletotrichum* spp. and *Corynespora cassiicola*. Four endophytic *Fusarium concolor* isolates were used to produce silver nanoparticles. Characterization of NPs was performed by UV-Vis spectroscopy and scanning electron microscopy (SEM). The mycelial growth of phytopathogens in the presence of AgNPs was evaluated *in vitro*, evidencing antifungal activity, especially against *Colletotrichum guaranicola*, which suggests that biosynthesized silver nanoparticles can be used in the management of phytopathogens as an alternative to control diseases.

Keywords: nanotechnology; green synthesis; mycelial growth; biological control.

RESUMO

Almeida, A-S.F.; Corrêa Junior, A.; Bentes, J.L.S. **S**íntese de nanopartículas de prata (AgNPs) por *Fusarium concolor* e a inibição de fitopatógenos. ***Summa Phytopathologica,*** v.47, n.1, p.9-15, 2021.

O uso de nanopartículas (NPs) na agricultura, representa uma importante inovação tecnológica, utilizada na produção de nanofertilizantes, nanocidas ou pesticidas encapsulados em nanopartículas para liberação controlada. As NPs de prata são reconhecidas por suas aplicações na agricultura, biotecnologia, medicina. Este trabalho teve como objetivo estudar a síntese de nanopartículas de prata por Fusarium concolor endofíticos obtidos de folhas e sementes de guaranazeiro e avaliar a atividade antifúngica das nanopartículas de prata no controle in vitro dos fitopatógenos *Colletotrichum guaranicola, Colletotrichum* spp. e *Corynespora cassiicola*. Quatro isolados de *Fusarium concolor* endofíticos foram utilizados para a produção de nanopartículas de prata. A caracterização das NPs foi realizada por espectroscopia de UV-Vis e microscopia eletrônica de varredura (MEV). Foram avaliados crescimento mycelial dos fitopatógenos na presença das AgNPs in vitro e foi observado evidenciando atividade antifúngica principalmente contra o fitopatógeno *Colletotrichum guaranicola*, sugerindo a possibilidade de utilização de nanopartículas de prata biossintetizadas manejo de fitopatógenos como uma alternativa no controle de doenças.

Palavras-chave: nanotecnologia; síntese verde; crescimento mycelial; controle biológico.

Nanotechnology is a branch of science that involves the synthesis and processing of nanoscale materials and has attracted attention because of the impact nanostructured products can have on improving the life quality and preserving the environment due to their unique physicochemical properties (1, 33).

Several studies have demonstrated that metallic nanoparticles, especially silver nanoparticles (AgNPs), have a wide range of applications, e.g., as antimicrobial and antifungal agents, as well as in biomolecular detection, biological labeling and chemical catalysis (13, 32, 36).

The use of nanoparticles (NPs) in agriculture represents an important technological innovation, which can be employed in the production of nanofertilizers, nanocides or pesticides encapsulated in nanoparticles for controlled release (15), with consequent improvement in the productivity and product quality.

NPs can be synthesized by physical, chemical and biological methods. Physical and chemical methods require high energy input, usually involving the use of toxic substances with the generation of hazardous by-products. Synthesis by the biological route can be adopted to obtain NPs without generating toxic waste and with little impact on the environment (24, 33).

Considering other naturally available biological resources, fungi are more efficient and more suitable for the synthesis of metallic particles at a nanometer scale, compared to plants and other microorganisms. The fungal mycelium can withstand harsh environments in bioreactors or chambers and is easier to manipulate and be manufactured in the biosynthesis processing (34, 36).

Fungi provide a wide variety of bioactive secondary metabolites

with unique structures that could be explored for their AgNPs biosynthesis ability to develop an efficient process for the environment. This study aimed to investigate the synthesis of AgNPs by *Fusarium concolor* Reinking, endophytes obtained from guaraná (*Paullinia cupana* var. *sorbilis* (Mart.) Ducke) leaves and seeds, as well as to evaluate the antifungal activity of AgNPs *in vitro* to control: *Colletotrichum guaranicola* Albuq., the causal agent of anthracnose, an important disease affecting guaraná plants (8); *Colletotrichum* sp., which cause anthracnose in *Capsichum chinense* Jacrd fruits, reaching 100% disease incidence, and *Corynespora cassiicola* (Berk. & Curt.), an important aerial pathogen of tomato in Amazonas State (AM), Brazil.

MATERIAL AND METHODS

Obtaining isolates and cultivation

The endophytic isolates of *Fusarium concolor* CCCT 17.43 and CCCT 17.29 were obtained from guaraná leaves, and the isolates CCCT 17.109 and CCCT 17.111 were obtained from seeds collected from guaraná fields in Manaus and Maués-AM. Isolation was performed according to Alfenas & Mafia (4), monosporic cultures were obtained (14) and the isolates were preserved based on the method of Castellani (7). Reactivation of the isolates was performed in 8cm-diameter Petri plates containing PDA (potato, 200 gL^{-1}, dextrose, 20 gL^{-1}, 17 gL^{-1} agar), at laboratory temperature (± 26°C), for seven days.

Biosynthesis of silver nanoparticles

After colony growth, the isolates were cultured in triplicate in a 250mL Erlenmeyer containing 100 mL PD (Potato-Dextrose) liquid medium, to which ten 5mm-diameter culture discs were added and kept on an orbital shaker at 120 rpm, at laboratory temperature (± 26°C), for seven days (11, 28). The metabolic extract was separated from the mycelial mass by filtration through Whatman no. 1 filter paper, sterilized by 0.22µm porosity membrane filtration. The recovered metabolic extract received 50 µL of a 1 mM solution of silver nitrate ($AgNO_3$). The reaction solution was kept at 120 rpm on the orbital shaker, at laboratory temperature (± 26°C), in the dark, for nine days (9, 28, 33).

The reduction in silver ions was monitored by visual inspection of the solution and the absorption measurement through the UV-Visible spectrum by aliquot sampling (1.5 mL) of the reaction solution. UV-Vis spectroscopy measurements were recorded on a double-beam spectrophotometer (Shimadzu - model UV-1601 PC) operated at the resolution of 1 nm, between 300 and 800 nm, at three different times, after 72h, 144h and 216h incubation.

Shaking was interrupted when there was no increase in the maximum absorption peak of silver nanoparticles and simultaneous color change in the filtrate incubated with the 1mM silver solution, indicating bio-reduction of silver ions to nanoparticles. The filtered metabolite extract without silver nitrate solution was used as control (9, 28, 33).

Purification of silver nanoparticles

To obtain purified silver nanoparticles (AgNPs), a 1mL aliquot was withdrawn from the reaction solution and added to the gel filtration chromatography column.

The used column was composed of Sephadex G-75 gel and the adopted buffer was Sodium Citrate buffer, pH 6.0 (Citric Acid + Sodium Citrate) (35).

Transmission electron microscopy

Color changes in the reaction mixtures were adopted as evidence of silver nanoparticle formation. Samples (1.5 mL) were taken from vials containing the already purified reaction solution, and the absorbance spectrum was measured within the 300-800 nm range. (28).

For observations under a scanning electron microscope (SEM), 10 µL of each reaction solution containing AgNPs were deposited on a carbon tape coated stub and allowed to dry at laboratory temperature (± 26°C) for 72 hours. Images were obtained under a FEG scanning electron microscope with FIB Nanofabrication system with field emission electron gun, model - Quanta FEG 3D FEI.

Antimicrobial activity

The isolates of the phytopathogenic fungi *C. guaranicola, Colletotrichum* sp. and *C. cassiicola* were assigned by the Laboratory of Microbiology and Phytopathology of the Federal University of Amazonas - UFAM, where all *in vitro* tests were performed. The isolates were kept in microtubes containing sterile distilled water, at laboratory temperature (± 26ºC).

Reactivation of the isolates was carried out in PDA culture medium (200 gL^{-1} potato, 20 gL^{-1} dextrose, 17 gL^{-1} agar) in a Petri plate of 70 x 15 mm diameter, and culture medium containing the colony of the isolate was deposited on the center of each plate. For fungal growth, the Petri plates were kept at room temperature during seven days. Pathogenicity test was previously carried out, confirming the pathogenic viability of isolates.

The antifungal activity of AgNPs was evaluated according to the methodologies proposed by Bautista-Banõs et al. (5) and Guo et al. (17).

To verify the effect of AgNPs on the mycelial growth of phytopathogens, 100µL, 500µL and 1000µL aliquots of each reaction solution were distributed on the surface of the PDA culture medium in Petri plates with the aid of a Drigalsky's handle. After two hours, a 0.5cm-diameter disc of the culture medium containing the pathogen was peeled onto the center of the Petri plates, at laboratory temperature (± 26°C), until the colonial growth of one of the treatments occupied the entire plate. Control constituted of PDA Petri plates with mycelium culture discs and 1000 µL sterile distilled water.

Experiments were conducted separately for each phytopathogen, in a completely randomized design, with three doses of NPs (100, 500 and 1000 µL), and five replicates.

Mycelial growth was daily evaluated by measuring the longitudinal and transverse diameter of the fungal colony with a digital caliper. The radial growth (RG) and the daily radial growth of the fungus was calculated according to Fortí (12). The percentage of mycelial growth inhibition (PIC) was calculated according to Hillen et al. (18)

The obtained results were previously subjected to normality and homogeneity tests of variances. In accordance with the principles of normality and homoscedasticity, data were subjected to analysis of variance and, when F was significant ($P < 0.05$), means of the dependent variables were tested and adjusted to mathematical models of first and second polynomial regression. The criteria for choosing the regression models were the highest determination coefficient and the model's significance. Analyzes were performed in SPSS 26.0 and the graphs in SigmaPlot 12.0.

RESULTS AND DISCUSSION

Characterization of silver nanoparticles

F. concolor isolates (CCCT 17.43, CCCT 17.29, CCCT17.109 and CCCT 17.111) were cultivated in triplicate in PD medium to obtain the

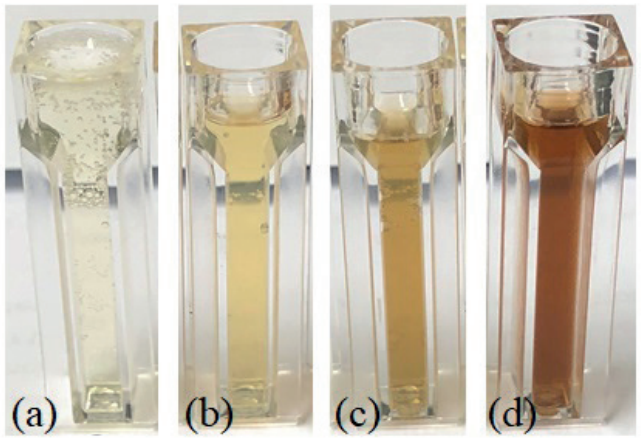

Figure 1. Color change in the culture medium and presence of silver nanoparticles (AgNPs) over time. (a) time zero; (b) 72h; (c) 144h; (d) 216h.

fungal filtrates that were used to evaluate the conversion capacity of AgNPs. After exposure of the PD medium to silver nitrate, the color of the solution changed, as shown in Figure 1.

The color of the synthesized AgNPs changed to reddish brown within 216h incubation at room temperature. According to Link & El-Sayed (22), this color is manifested in the dispersion of nanoparticles due to absorption of photons associated with surface plasmon resonance (SPR). The physical origin of strong light absorption by noble metal nanoparticles is the electron oscillation induced by interaction with the electromagnetic field. The electric field of a wave induces the polarization of electrons in relation to the ionic nucleus of the spherical nanoparticle, creating a difference in the charge on the NP surface. Thus, a dipole oscillation is produced for all electrons with the same phase. When the frequency of the electromagnetic field becomes resonant with the movement of electrons, there is strong absorption of electrons, which originates the observed color.

Birla et al. (6), using PD medium to produce AgNPs with the fungus *Phoma glomerata* (Corda) Wollenw. & Hochapfel., visualized a color change from transparent yellow to brown; similarly, Vahabi et al. (33) used GC medium (glucose and casein hydrolysate) to produce AgNPs with the fungus *Trichoderma reesei* E.G. Simmons and reported a color change from transparent yellow to yellowish-brown after 72h incubation, indicating the formation of AgNPs in the mixture.

The light absorption profile was obtained from scanning at 300 to 800 nm since, at the nanoscale, silver is known to have its maximum absorption ranging from 400 to 670 nm (25). The isolates that presented color change had plasmon band characteristic of AgNPs, which was the first evidence that the silver formed in all samples was in the nanoparticulate form.

The absorption spectrum in the UV-Vis region of AgNPs of the reaction mixtures indicates that the surface plasmon resonance peak (SPR) and absorbance are maximal for CCCT 17.109 (405.82 nm, 1.83) (Figure 2a), CCCT 17.111 (404.91 nm, 1.01) (Figure 2b), CCCT

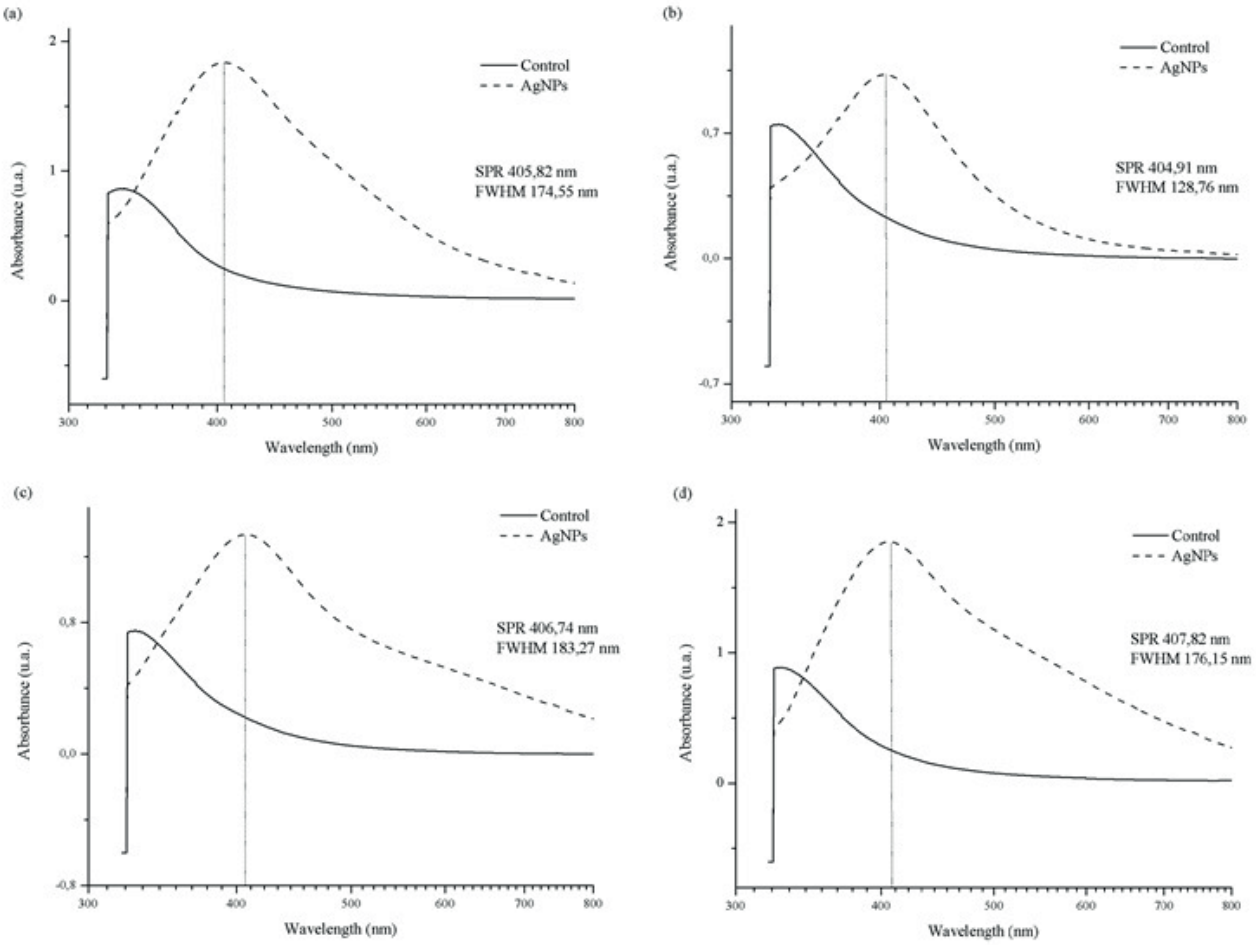

Figure 2. Absorption spectrum for the synthesis of silver nanoparticles (SPR) by fungi. (a) CCCT 17.109; (b) CCCT 17.111; (c) CCCT 17.43; (d) CCCT 17.29.

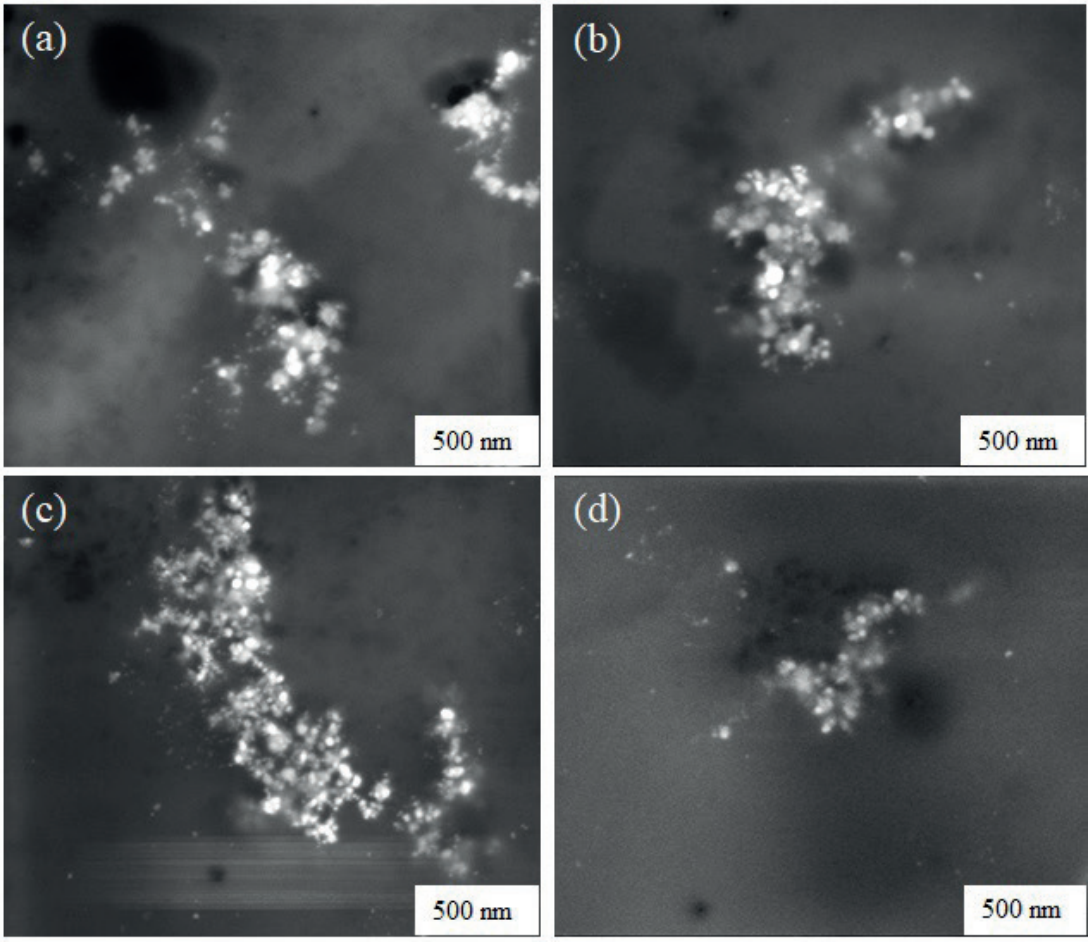

Figure 3. Scanning electron microscopy images of silver nanoparticles synthetized by fungi. (a) CCCT 17.109; (b) CCCT 17.111; (c) CCCT 17.43; (d) CCCT 17.29.

17.29 (406.74 nm, 1.33) (Figure 2c), and CCCT 17.43 (407.82 nm, 1.84) (Figure 2d).

The AgNP spectra show UV-Visible absorption between 350 and 500 nm, as recorded in Figure 2. The maximum absorbance value is an indication of the number of NPs present in the colloidal solution. The increased absorbance in the UV-Vis as a function of the synthesis time demonstrates a gradual increase in the concentration of AgNPs in the reaction solution (29).

The reaction solution of the fungal isolate from seeds CCCT 17.109 has the maximum absorption wavelength greater than that of CCCT 17.111 (427.68>410.06) and the highest half-width (174.55>128.76). These values suggest that as the particle size increases, the surface plasmon band becomes wider and shifts to larger wavelengths (23). In the reaction solution of the isolate CCCT 17.109 there are possibly larger particles than in the reaction solution of CCCT 17.111, i.e., there was greater aggregation and growth of NPs.

According to Sharma et al. (31), peaks ranging from 380 to 400 nm show yellow gold coloration and characteristic smaller particles, while Albernaz (3) commented that color ranging from reddish-brown to dark-brown varies with the size of AgNPs.

The maximum absorbance and the half-height width of the plasmon band are known to depend on different factors, including average size, size distribution, shape and nature of the medium in which NPs meet.

NPs of smaller diameter tend to have the wavelength of absorption maximum shifted to the ultraviolet light spectrum region, while an increase in the mean size of the NPs is followed by a shift towards the region of the light spectrum of red (16).

The presence of AgNPs in the medium was confirmed by SEM (Figure 3).

Antifungal activity

Reduced mycelial growth was observed for all phytopathogens in the presence of AgNPs solution (Table 1), especially the isolate CCCT 17.111 for *C. guaranicola*; CCCT 17.29 for *Colletotrichum* sp., and CCCT 17.109 for *C. cassiicola* (Figure 4).

Mycelial growth inhibition was proportional to the increase in AgNPs in the culture media for *C. guaranicola* and *Colletotrichum* sp.; there was no statistical difference between the applied doses in the evaluation of *C. cassiicola* mycelial growth.

The AgNPs of CCCT 17.109 isolate at the dose of 100 µL presented a lower PIC, corresponding to 5.93% mycelial growth inhibition for *C. guaranicola*, followed by CCCT 17.43 isolate, showing 6.90% inhibition.

The AgNPs of CCCT 17.111 showed 41.12% mycelial growth inhibition for *C. guaranicola*.

The PIC for *Colletotrichum* sp. and *C. cassiicola* had no differences when AgNPs at 500 µL of CCCT17.109 were used, corresponding to 19.81% and 19.19%, respectively.

Table 1. Colony diameter (CD) and percentage of mycelial growth inhibition (PIC) for *Colletotrichum guaranicola*, *Colletotrichum* sp., and *Corynespora cassiicola*, exposed to different doses of silver nanoparticles (AgNPs) synthesized by *Fusarium conolor*.

Fungi	Isolate	CD (cm)				PIC (%)			
		Doses				Doses			
		0 µL (Control)	100 µL	500 µL	1000 µL	0 µL (Control)	100 µL	500 µL	1000 µL
C. guaranicola	CCCT 17.109	0.52 a	0.50 a	0.41 c	0.35 d	0.0 a	5.93 b	19.32 c	30.13 d
	CCCT 17.111	0.52 a	0.48 b	0.38 c	0.29 e	0.0 a	7.90 b	24.75 c	41.12 e
	CCCT 17.43	0.52 a	0.47 b	0.37 c	0.34 d	0.0 a	8.04 b	25.95 c	32.64 d
	CCCT 17.29	0.53 a	0.49 b	0.39 c	0.41 c	0.0 a	6.90 b	23.58 c	21.13 c
		CV% 5.84				CV% 31.65			
Colletotrichum sp.	CCCT 17.109	0.34 a	0.24 c	0.27 b	0.23 c	0.0 a	27.66 b	19.81 b	31.18 b
	CCCT 17.111	0.36 a	0.26 b	0.24 c	0.22 c	0.0 a	24.49 b	30.86 b	36.54 b
	CCCT 17.43	0.36 a	0.22 c	0.25 c	0.24 c	0.0 a	36.20 b	28.36 b	31.45 b
	CCCT 17.29	0.37 a	0.28 b	0.28 b	0.23 c	0.0 a	22.82 b	21.44 b	33.90 b
		CV% 9.70				CV% 34.86			
C. cassiicola	CCCT 17.109	0.36 a	0.23 b	0.28 b	0.23 b	0.0 a	23.82 b	19.19 b	33.97 b
	CCCT 17.111	0.36 a	0.27 b	0.25 b	0.24 b	0.0 a	23.63 b	28.22 b	32.40 b
	CCCT 17.43	0.36 a	0.26 b	0.25 b	0.25 b	0.0 a	26.85 b	28.50 b	28.50 b
	CCCT 17.29	0.37 a	0.29 b	0.29 b	0.26 b	0.0 a	20.06 b	19.67 b	27.19 b
		CV% 10.76				CV% 40.99			

Means followed by the same letter do not differ statistically from each other. Scott-Knott test was applied at 5% probability level.

For *C. guaranicolla*, the percentage of mycelial growth inhibition was best fit to the linear model, depending on the doses, while for the remaining pathogens there was no adjustment to a model.

Although there is no report in the literature about the antifungal effect of AgNPs on the mycelial growth of *C. guaranicola, Colletotrichum* sp. and *C. cassiicola,* some studies have presented satisfactory results for the effect of other AgNPs on different fungi. According to Rai (27), AgNPs present a broad-spectrum action against fungi, especially those of the genus *Candida*, which was confirmed by Segala et al. (30), who evaluated the effect of AgNPs on the growth of *Candida albicans* (C.P. Robin) Berkhout and observed that they provided total inhibition of the growth of this phytopathogen.

Petica et al. (26) also reported antifungal activity of AgNPs on species like *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. Kim et al. (20), studying the *in vitro* control of *Raffaelea* sp., observed that the antifungal effect of AgNPs significantly inhibited the fungi depending on the applied dose (0, 5, 10 and 25 ppm), so that the higher the dose, the lower the growth of fungal hyphae, while harmful effects of AgNPs were observed on conidial germination.

Aguilar-Méndez et al. (2) evaluated the antifungal effect of AgNPs on the *in vitro* control of *Colletotrichum gloeosporioides* (Penz.) Penz. Sacc., a fungus causing anthracnose in several fruits, and observed that the mycelial growth decreased in a dose-dependent manner, while inhibition of the fungus reached almost 90%.

Kim et al. (21), assessing the *in vitro* control of eighteen species of the phytopathogenic fungi *Eucalyptus* spp., observed that total inhibition of the growth of most pathogens was obtained at the concentration of 100 ppm NPs, as is the case for *Fusarium* sp., to which AgNPs presented higher inhibition potential. In addition, the concentration of 10 ppm can result in the lowest inhibition rate, which was 12.7% for the fungus *Glomerella cingulata* Stoneman, indicating that the antifungal activity of these AgNPs depends on their concentration.

A number of studies have suggested that silver ions react with groups of proteins and play an essential role in bacterial and fungal inactivation, inhibiting respiratory chain enzymes or interfering with membrane permeability (10).

Studies by Jung et al. (19) showed the activity of AgNPs on *Escherichia coli* (Migula) Castellani and Chalmers, and *Staphylococcus aureus* Rosenbach. AgNPs rupture the membranes of bacteria, causing a massive loss of intracellular potassium and decreasing ATP levels. Both effects may culminate in cell viability loss.

These studies must be carried out separately for each pathogen, since the fungicidal action of AgNPs, as shown by several researchers (20), is associated with the applied concentration of this nanoparticle, as well as with the analyzed pathogen.

The present results suggest the possibility of using biosynthesized AgNPs in the control of phytopathogens as an alternative to collaborate to reducing the use of agrochemicals of high toxicity.

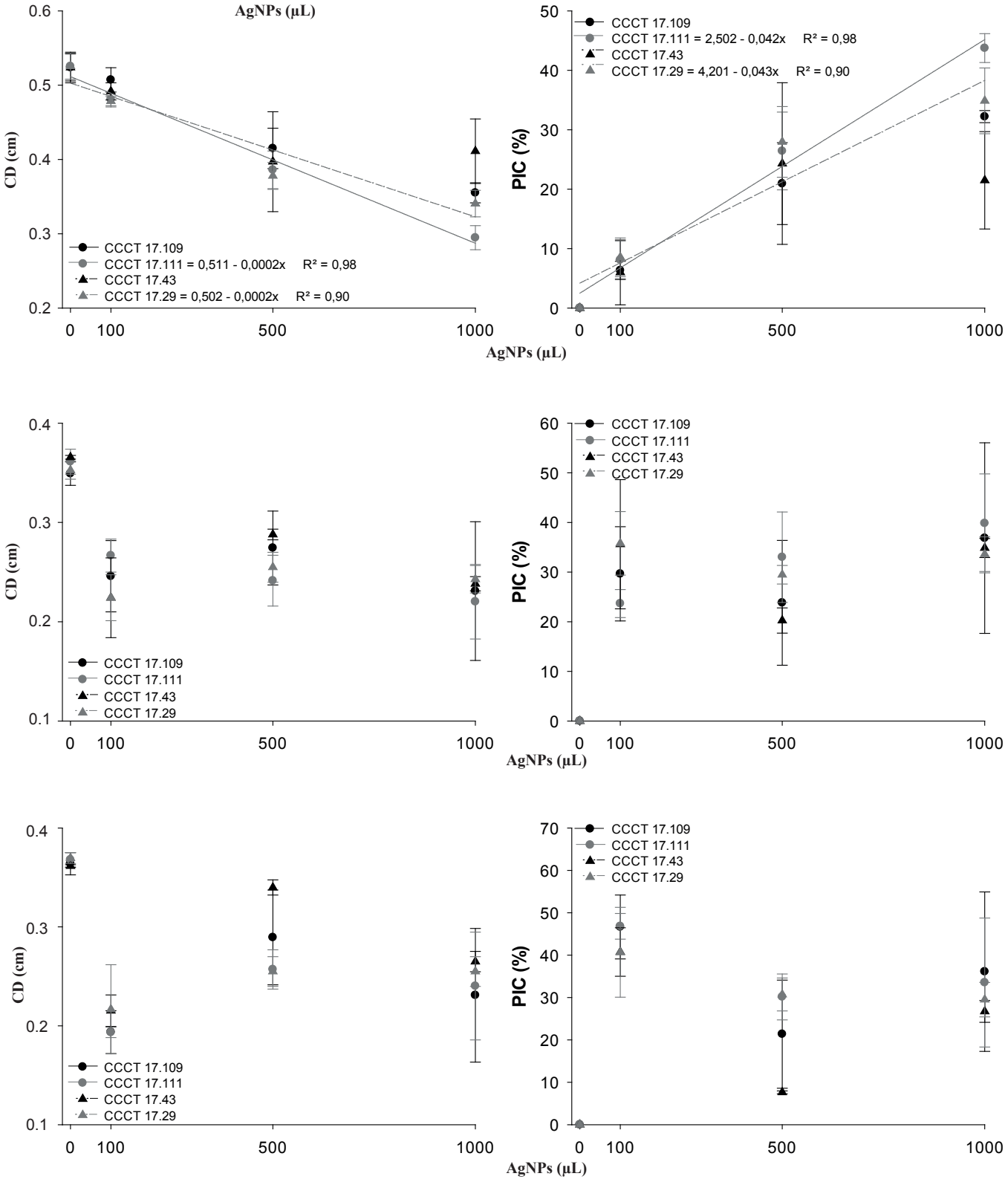

Figure 4. Colony diameter (CD) and percentage of mycelial growth inhibition (PIC) for (a) *Colletotrichum guaranicola*, (b) *Colletotrichum* sp. and (c) *Corynespora cassiicola* exposed to different doses of silver nanoparticles (AgNPs) synthesized by *Fusarium concolor*.

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