Antifungal Activity of Silver Colloidal Nanoparticles against Phytopathogenic Fungus (*Phomopsis* sp.) in Soybean Seeds

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***Abstract*—**Among the many promising nanomaterials with antifungal properties, metal nanoparticles (silver nanoparticles) stand out due to their high chemical activity. Therefore, the aim of this study was to evaluate the effect of silver nanoparticles (AgNPs) against *Phomopsis* sp. AgNPs were synthesized by silver nitrate reduction with sodium citrate and stabilized with ammonia. The synthesized AgNPs have further been characterized by UV/Visible spectroscopy, Biophysical techniques like Dynamic light scattering (DLS) and Scanning Electron Microscopy (SEM). The average diameter of the prepared silver colloidal nanoparticles was about 52 nm. Absolute inhibitions (100%) were observed on treated with a 270 and 540 µg ml^{-1} concentration of AgNPs. The results from the study of the AgNPs antifungal effect are significant and suggest that the synthesized silver nanoparticles may have an advantage compared with conventional fungicides.

***Keywords*—**Antifungal activity, *Phomopsis* sp., Seeds, Silver Nanoparticles, Soybean.

I. Introduction

SOYBEAN is the most important oilseed cultivated in the world and currently Brazil is the world's second biggest producer of soybeans. Brazil's 2012/13 soybean crop, 27 million ha of soybean were planted, which corresponds to almost half (52%) of the total area of field crops in the country, reaching a total production of 81.5 million metric tons and a mean yield of 2939 kg/ha [1].

However, agricultural production is reduced worldwide every year due to plant disease caused by pathogenic fungi and millions of dollars have been empowered to control these plant diseases [2]. Thus, growth of fungal pathogens is the primary cause of considerable economic loss during postharvest handling of seeds [3].

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Among the various fungi carried by soybean seeds and responsible for its low quality, *Phomopsis* sp. stands out. On soybean, *Phomopsis* sp., is a phytopathogen responsible for several diseases, cause seed decay, stem blight and stem canker, resulting in significant yield and quality losses [4], [5]. For instance, these diseases were responsible for yield losses of 80% to 100% in Brazil, depending on the pathogen involved [6].

Moreover, a strong negative relationship between the incidence of *Phomopsis* sp. seed infection and soybean germination, vigor, and emergence have been reported in numerous studies [7]-[10].

The application of silver nanoparticles as antifungal agents has become more common with the advances of the technology, making its production more economical [11]. One of the potential applications in which silver can be applied is in management of plant diseases. Since silver displays multiple modes of inhibitory action to microorganisms [12], it may be use and application for controlling various plant pathogens in a relatively safe way compared to synthetic fungicides. Until now, limited research provided some evidence of the applicability of silver for controlling plant diseases [13].

In the present work, we evaluate the antifungal activity of silver colloidal nanoparticles against one important *in vitro* phytopathogenic fungus, *Phomopsis* sp. The inhibitory properties of silver nanoparticles were also examined.

II. Materials and Methods

A. Synthesis and Characterization of Silver Colloidal Nanoparticles

Silver nanoparticles were synthesized by means of the Turkevich method [14] and were prepared according to the procedure reported by Gorup et al. [15]. Solutions were prepared with deionized water obtained from a commercial Millipore Elix 3 Water Purification system. Silver nanoparticles were prepared by the reduction of silver nitrate ($AgNO_3$) (99.0% Sigma-Aldrich, USA) solutions with sodium citrate ($Na_3C_6H_5O_7$) (99% Synth, Brazil). A volume of 200 ml of an aqueous solution of silver nitrate (5.0×10^{-3}mol l^{-1}) was heated and stirred gently with a magnetic Teflon-coated bar. When the silver nitrate solution reached 90 ºC, 5 ml of an aqueous solution of sodium citrate (0.3 mol l^{-1}) preheated to 90ºC was added. After 12 min of reaction, the system became yellow, indicating the formation of silver nanoparticles. The

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silver colloidal nanoparticles were stabilized by adding 5 ml of a previously prepared aqueous solution of ammonia (NH_3 1.4 mol l^{-1}). To characterize the silver colloidal nanoparticles suspension, UV/Visible absorption spectroscopy (Spectrophotometer Shimadzu MultSpec-1501, Shimadzu Corporation, Tokyo, Japan) and Scanning Electron Microscopy (SEM-FEG, JSM 6701F, high resolution: 1 nm-15 kV, 2.2 nm-1 kV, JEOL, Tokyo, Japan) at an acceleration voltage of 2.0 kV. The synthesized silver colloidal nanoparticles were filtered through a 0.22 µm syringe driven filter unit and the size of the distributed silver nanoparticles measured by using the principle of dynamic light scattering (DLS), using a Nano Size Particle Analyzer (ZEN 3600 MALVERN UK) in the range between 0.6 nm to 6.0 µm, under the following conditions: particle refractive index 1.390, particle absorption coefficient 0.002, water refractive index 1.330, viscosity 0.8872 cP, equilibration time of 2 minutes and temperature 25ºC. Fifteen measurement cycles of 10 seconds each were taken and the average was done by using software (DTS, Ver. 5.00 from Malvern).

B. Biological Material and Growth Conditions

One phytopathogenic fungus, *Phomopsis* sp. (MES 1154), was obtained from the culture collection at the Embrapa Soybean (Brazilian Enterprise for Agricultural Research, Londrina, Paraná, Brazil). *Phomopsis* sp. was cultured on Potato Dextrose Agar (PDA) plates at 25ºC in the dark, for 7 days.

C. In vitro Antifungal Assays

PDA was used to test the antifungal activity of synthesized silver nanoparticles. Media were prepared, autoclaved at 121ºC for 15 min and allowed to cool. When media achieved 50ºC, 5 ml of silver nanoparticle colloidal suspension at different dilution rates were poured into media and then, 20 ml aliquots plated into 90 mm petri dish. Controls plates were similarly prepared with 5 ml of aqueous solutions of sodium citrate (0.3 mol l^{-1}), NH_3 (1.4 mol l^{-1}) and amphotericin B (1 µg ml^{-1}). The influence of the stabilizers ($Na_3C_6H_5O_7$ and NH_3) used for synthesizing the AgNPs was also tested. A solution of amphotericin B was used as positive control.

In vitro antifungal assays were performed on five different concentrations (i.e., 5, 50, 180, 270, 540 µg ml^{-1}) of silver nanoparticles. Five ml of AgNPs having different concentrations was poured into growth media prior to plating in a petri dish (90 × 15 mm). Media containing silver nanoparticles was incubated at 25ºC. After 24h of incubation, agar plugs of uniform size (diameter, 8 mm) containing fungi were inoculated simultaneously at the center of each petri dish containing silver nanoparticles, followed by incubation at 25ºC for in the dark, for 7 days. All the experiments were performed in triplicate with three repetitions.

D. Data Analysis

After incubation of fungus on PDA culture medium containing silver nanoparticles, radial growth of fungal mycelium was recorded. Radial inhibition was calculated when growth of mycelia in the control H_2O plate reached the edge of the petri dish. Equation (1) was used for calculation of the inhibition rate (%) [2].

$$Inibition\ rate\ (\%) = \frac{R-r}{R} \quad (1)$$

where R is the radial growth of fungal mycelia on the control H_2O plate and r is the radial growth of fungal mycelia on the plate treated with AgNPs [2].

E. Statistical Analysis

All statistical analyses were performed with the General linear mixed models using the MIXED procedure in SAS (SAS Institute, Inc., Cary, NC, USA). Means were compared by analysis of variance followed by the Tukey-Kramertest. The level of significance all analyzes was of 0.05 ($P<0.05$).

III. Results and Discussion

A. Synthesis and Characterization of Silver Colloidal Nanoparticles

In this work, the silver nanoparticles were characterized by UV-Vis spectroscopy studies. The absorption spectrum of the colloidal suspension displayed in Fig. 1 shows a well-defined Plasmon band centered at approximately 425 nm, specific characteristic of absorbance peak for silver nanoparticle colloidal suspensions. The symmetrical shapes of Plasmon band in Fig. 1 confirmed the colloidal stability and sharp particle size distribution. Furthermore, the silver colloidal nanoparticles were stabilized using NH_3 in order to prevent aggregation. NH_3 plays an important growth moderating role, making it possible to stabilize metallic silver nanoparticles, since free silver ions, which are responsible for particle growth and the formation of new nuclei, are trapped by the formation of diammine silver (I) complexes [16], [15].Such results are in a good agreement with the previously published paper by Kvitek et al. [17] considering the impact of the stabilization of AgNPs on their antimicrobial activity.

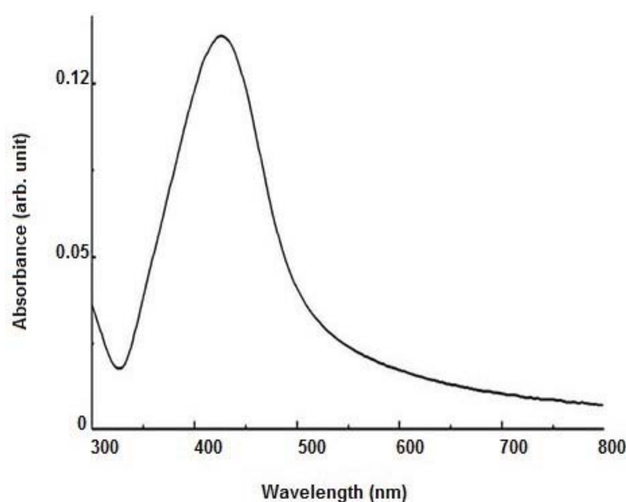

Fig. 1 UV–Vis absorption spectrum of silver nanoparticle colloidal suspensions prepared by reduction of silver nitrate with sodium citrate stabilized using ammonia

As shows in Fig. 2, the data analysis of Dynamic Light Scattering (DLS) supported that the average size of the synthesized nanoparticles are 51.92 nm and 0.093 PDI value and the obtained single peak indicated that the quality of the synthesized silver nanoparticles that's great [18]. Poly dispersity index is a parameter to define the particle size distribution of nanoparticles obtained from photon correlation spectroscopic analysis. It is a dimensionless number extrapolated from the autocorrelation function and ranges from a value of 0.01 for mono dispersed particles and up to values of 0.5–0.7. Samples with very broad size distribution have polydispersity index values >0.7 [19].The polydispersity index values obtained in this study further corroborates the observations made, with lower values being observed for relatively mono disperse systems.

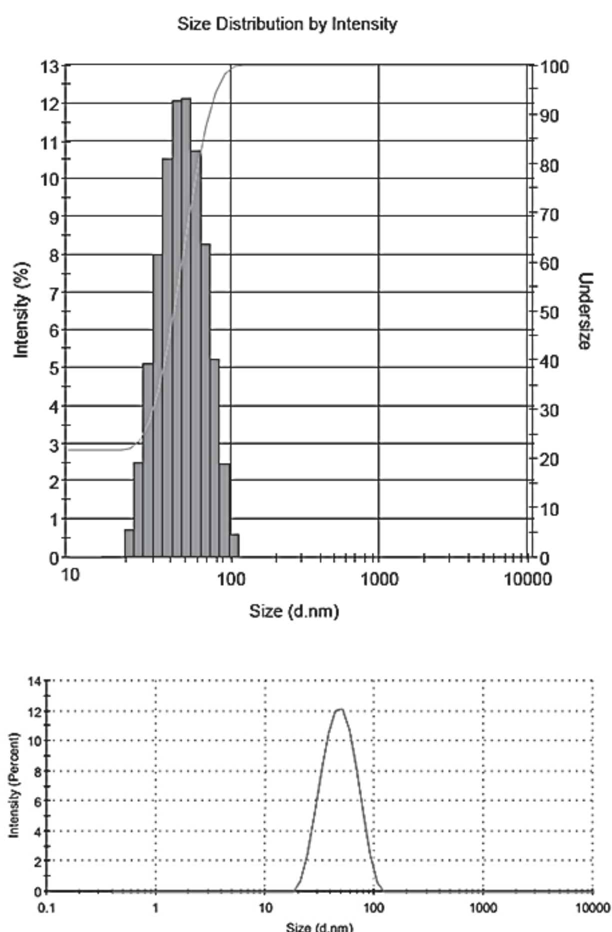

Fig. 2 Size distribution of AgNPs determined by the DLS technique

This band (425 nm) has been commonly assigned to nanoparticles having a spherical or spheroidal shape [20]. SEM images (Fig. 3) indicated that the nanoparticles were well formed, spherical and dispersed, with mean diameters of 52 nm. These results confirmed that the morphology of the nanoparticles was typically spherical (Fig. 3) in contrast to the work of Klaus et al. [21] who observed triangular, hexagonal and spherical morphologies.

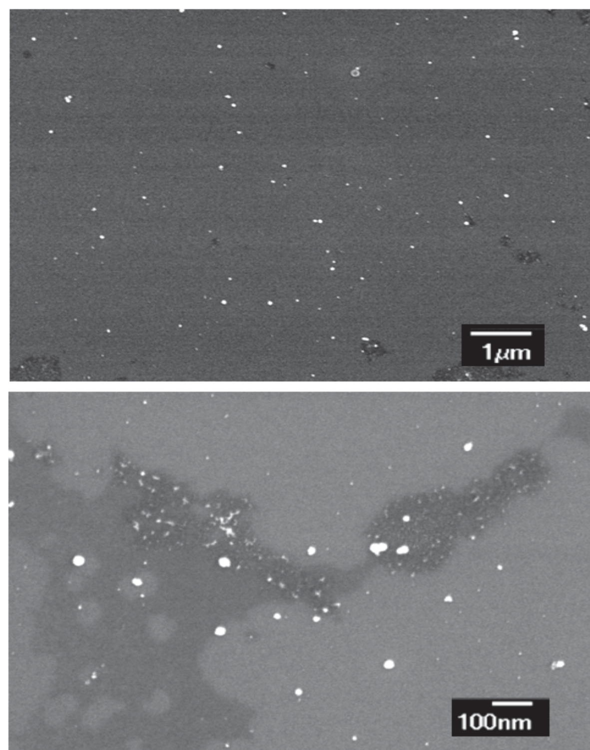

Fig. 3 Scanning Electron Microscopy (SEM) images of silver nanoparticles (AgNPs) with ~52 nm, stabilized with ammonia

B. In vitro Antifungal Assays

Results presented in this study confirm that silver nanoparticles colloidal have significant inhibitory effects and antifungal activity on colony formation from mycelia of *Phomopsis* sp. *in vitro*. The growth rate of strain in the presence of the tested silver nanoparticles and other compounds are summarized in Table I.

TABLE I
PROPERTIES INHIBITORY RATE (%) ON DIFFERENT CONCENTRATIONS OF SILVER NANOPARTICLES AGAINST *PHOMOPSIS* SP., COMPARED TO CONTROL (H_2O)

Compounds	Radial growth (cm day^{-1})**	Inhibition rates (%)*
H_2O	4.26±0.032^a	0
$Na_3C_6H_5O_7$ 0.3 mol ml^{-1}+ NH_3 1.4 mol ml^{-1}	4.24±0.08^a	0
Amphotericin B 1 μg ml^{-1} ***	4.14±0.05^b	2
AgNPs 5 μg ml^{-1}	3.98±0.06^c	8
AgNPs 50 μg ml^{-1}	3.78±0.11^d	11
AgNPs 180 μg ml^{-1}	0.65±0.18^e	90
AgNPs 270 μg ml^{-1}	0.00±0.00^f	100
AgNPs 540 μg ml^{-1}	0.00±0.00^f	100

*Inhibition rates were determined based on nine replicates of each experiment, inhibition rate of control = 0%;
**Values are the mean of nine replicates of each ± standard deviation (SD). Data marked with different letters are significantly different from respective control at $P < .0001$ for the Tukey-Kramer test;
***Control positive.

As the concentrations of silver nanoparticles increases, colony formation was reduced. Silver antifungal activity to reduce the formation of colony was observed after 6 hours of incubation.

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The effect of all concentrations of silver nanoparticles in the mycelial growth of fungi tested after 7 days of incubation can be observed in the Figs. 4 and 5.

The results showed that solutions of $Na_3C_6H_5O_7$ and NH_3, used as controls, did not exhibit any antifungal activity on the *Phomopsis* sp. assayed.

No significant inhibition on growth rates was registered for compound amphotericin B (control positive) at concentration of 1 µg/ml. The data obtained are in agreement with the previously published results by Hawser and Douglas. These authors concluded that of the antifungal agent tested (amphotericin B) showed much less activity against *Candida albicans* [22].

The lowest level of inhibition was observed against *Phomopsis* sp. treated with 5 and 50µg ml^{-1} concentrations of AgNPs, these treatments inhibited only 0.28 and 0.48 cm, respectively, after 7 days of growth compared to the treatment with deionized water ($P<0.0001$). However, lower amounts of these concentrations of AgNPs (5 and 50 µg ml^{-1}) increase the lag time, since visible mycelia only appear on the first day of incubation.

In addition, greater than 90% inhibition was observed against *Phomopsis* sp. treated with a 180 µg ml^{-1} concentration of AgNPs, has the antifungal activity significantly ($P<0.0001$), exhibited the highest reductions in the radial growth of fungal mycelia.

Absolute inhibitions (100%) were observed on treated with a 270 and 540 µg ml^{-1} concentration of AgNPs.

According to the variance analysis, the interaction between treatments and the daily growth of the fungus was significant ($P<0.0001$), so the analysis proceeded with the adjustment cubic model to the data and the comparison of the curves by the Tukey-Kramer showed that only the curves of the control treatments $Na_3C_6H_5O_7 + NH_3$ and H_2O are not significantly different ($P = 0.3368$), the other comparisons are significantly different (values less than 0.0001).

For several decades, silver (Ag^+) has been studied for use in disinfection of various harmful microorganisms [23], [24].

In most cases, inhibition increased as the concentration of AgNPs increased. This could be due to the high density at which the solution was able to saturate and cohere to fungal hypha and to deactivate plant pathogenic fungi. Reports on the mechanism of inhibitory action of silver ions on microorganisms have shown that upon treatment with Ag^+, DNA loses its ability to replicate [25], resulting in inactivated expression of ribosomal subunit proteins, as well as certain other cellular proteins and enzymes essential to ATP production [26]. It has also been hypothesized that Ag^+ primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain [27], [28], resulting in loss of cellular integrity and osmotic culminating in acute toxicity to the cells [29]. Fairly recently, transcriptomic analysis of *Saccharomyces cerevisiae* exposed to AgNPs confirmed the potential damage to the cell wall and transmembrane proteins and up regulation of cell-wall-strengthening genes in surviving cells [30].

Thus, AgNPs exerted potent antifungal effects on fungus tested *in vitro*, probably through destruction of membrane integrity [2]. Due to their antifungal properties, silver nanoparticles attach and anchor to the surface of the cell. This interaction causes structural changes and damage, markedly disturbing vital cell functions, such as permeability, causing pits and gaps, depressing the activity of respiratory chain enzymes, and finally leading to cell death [31]-[36].

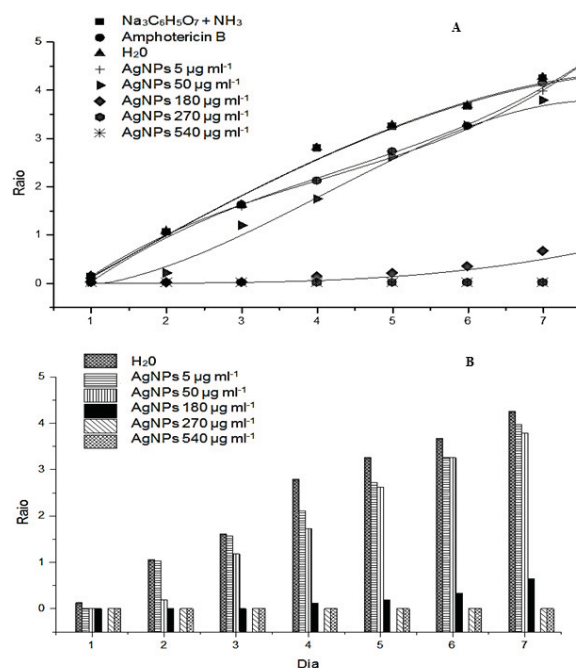

Fig. 4 Daily radial growth of fungal mycelium when *Phomopsis* sp. was cultivated in PDA medium supplemented with different concentrations of AgNPs, after 7 days of incubation at 25°C

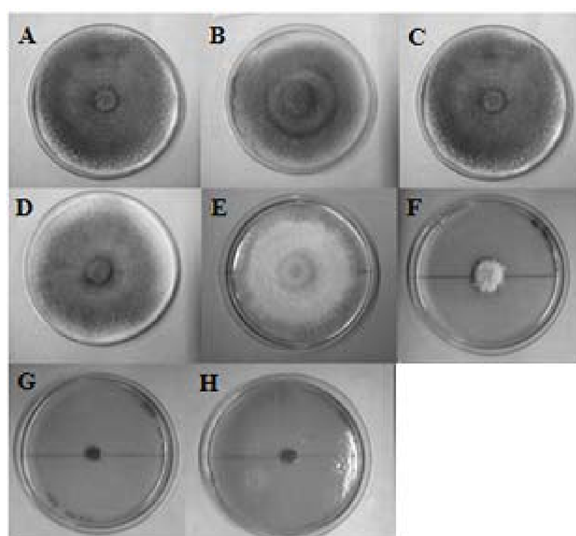

Fig. 5 Effect of AgNPs and other compounds on the mycelia growth of tested *Phomopsis* sp. after 7 days of incubationat 25°C. (**A**) H_2O, (**B**) $Na_3C_6H_5O_7 + NH_3$, (**C**) Amphotericin B (positive control), (**D**) AgNPs5 µg ml^{-1}, (**E**) AgNPs50 µg ml^{-1},(**F**) AgNPs180 µg ml^{-1},(**G**) AgNPs270 µg ml^{-1} and (**H**) AgNPs540 µg ml^{-1}

IV. Conclusion

The results indicated that the antifungal effect of silver nanoparticles is a promising alternative technique against the traditional use of conventional fungicides.

In conclusion, silver nanoparticles with sizes of ∼52nm possess significant antifungal properties against *Phmopsis* sp., and the inhibitory effects increase as the concentrations of silver nanoparticles increase.

Moreover, silver nanoparticles were stabilized with ammonia and this agent does not interfere with the *in vitro* antifungal activity of nanoparticles.

Silver nanoparticles at concentration greater than 180 µg ml^{-1} are significantly inhibiting the growth *Phomopsis* sp.

In our work, silver nanoparticles at concentration 270 µg ml^{-1}, completely inhibited the tested strain.

In summary, AgNPs exerted potent antifungal effects on fungi tested *in vitro*, probably through destruction of membrane integrity; therefore, it was concluded that AgNPs have considerable antifungal activity.

These results suggest that silver nanoparticles could be used as an effective fungicide in agricultural applications against phytopathogenic fungi in soybean plants and seeds. Furthermore, silver nanoparticles can be used in the preparation of new formulations like pesticides and fungicides for applications in management of plant diseases.

Therefore, more investigations about field applications and *in vivo* are needed.

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