Mycosynthesis of silver nanoparticles by *Aspergillus flavus* : characterization and antifungal activity

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The present study aimed to mycosynthesize silver nanoparticles (AgNPs) by *Aspergillus flavus* and to assess their antifungal activity. The presence of AgNPs was certified by colour change from yellow to brown and by the maximum absorbance at 420 nm, because of Surface Plasmon resonance. Transmission Electron Microscopy images revealed the aproximately spherical shape of AgNPs, dimensions ranging between 3.3 and 40 nm, crystalline structure and a good dispersion. AgNPs presented antifungal activity against *Aspergillus ochraceus* and *Penicillium expansum* but totally inhibited *Fusarium oxysporum*. Minimum inhibitory concentration (MIC) varied from 7.5 (*Penicillium expansum* and *Fusarium oxysporum*) to 12.5 µg/mL (*Aspergillus ochraceus*).

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1. Introduction

Nanotehnology is a modern research domain worldwide, focused on synthesis of nanoparticles (NPs) with new desired physicochemical properties for multiple applications, following ecofriendly processes that avoid hazardous and toxic chemicals previously used in chemical synthesis [1; 2].

Among various NPs, the most intense research reffers to silver nanoparticles (AgNPs) [3; 4]. New protocols developed consider the use of biological methods as green synthesis ways to obtain AgNPs with appropriate shape, sizes and high monodispersion [5; 6].

In the last years, silver nanoparticles have become one of the most explored nanostructures in nanotechnology applications for various domains.

Research results revealed that microbe-mediated NPs synthesis is low costing, energy saving nanotechnology that offers simultaneous benefits for human/animal health and environment than non-green approaches [7; 8; 9].

Metalic nanoparticles of biogenic origin are synthesized by microorganisms, especially imperfect fungi and ascomycetes, using their excellent potential to produce many bioactive compounds and their various natural metabolic pathways [10].

The synthesis of metalic nanoparticles is possible by either intracellular or extracellular mechanisms of enzymatic reduction of metalic ions to their elemental forms.

Extracellular route was used in biosynthesis of AgNPs mediated by various fungal species such as: *Aspergillus terreus* [11], *Aspergillus* spp. [12], *Trichoderma asperellum* [13], *Trichoderma viride* [14; 6], *Penicillium fellutanum* [15], *Cladosporium cladosporioides* [16], *Aspergillus flavus* [7], *Fusarium scirpi* [17].

Many fungal species are already cultivated on a large scale in so called “nanofactories” for producing high biomass and massive amounts of extracellularly secreted metabolites [18; 19; 20]. These biomolecules are useful for the reduction of metal ions, as well as capping agents of metal nanoparticles, providing a good dispersion, better particle size, shape, stability and improved antimicrobial activity [21].

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Recent results from literature concerning the antifungal activity of mycosinthesized AgNPs are presented in Table 1.

Table 1. Fungal species utilized for extracellular biosynthesis of AgNPs with antifungal activity.

Synthesizer (Fungal species)	Antifungal activity (Test fungi)	References
Trichoderma longibrachiatum	*Alternaria alternata, Pyricularia grisea, Fusarium verticillioides, Helminthosporium oryzae, Penicillium glabrum*	[22]
Aspergillus niger	*Penicillium digitatum, Fusarium oxysporum, Aspergillus flavus*	[23]
Penicillium duclauxii	*Bipolaris sorghicola*	[24]
Chaetomium globosum	*Fusarium oxysporum* f.sp. *lycopersici*	[25]

The most recent applications of AgNPs biosynthesized by using microorganisms belonging to the group of fungi refers to various domain from nanomedicine [26; 27], agriculture [28], environment protection, food industry [29; 30; 31; 32; 33].

The aim of the present paper was to present the results of the research carried out on extracellular biosynthesis of silver nanoparticles mediated by culture filtrate of fungus *Aspergillus flavus* and to assess their antimicrobial activity for further practical application.

2. Experimental

2.1. Fungal isolates and culture media

Pure cultures of fungi representing 4 isolates of various origins have been used in this experiment.

Aspergillus flavus isolated from maize rhizosphere was utilised for extracellular synthesis of AgNPs. *Aspergillus ochraceus* isolated from tomato rhizosphere (in Buzau greenhouse), *Fusarium oxysporum,* isolated from growth substrate in greenhouse (Sanjai Farm) and *Penicillium expansum* isolated from infected apple were the test fungi to assess the antifungal activity of AgNPs with biogenic origin.

These moulds were deposited in the culture collection of the Laboratory of Soil Biology from National Research & Development Institute for Soil Science, Agrochemistry and Environment, Bucharest. Microbial strains were maintained on slants of Czapek Agar (CzA) (Merck KGaA Germany) for fungi at 4ºC.

2.2. Biogenic synthesis of silver nanoparticles

Extracellular synthesis of AgNPs was carried out by mixing 50 ml cell free supernatant from 48 hours liquid culture of *Aspergillus flavus* filtered through membrane filter (0.2 µm pore dimension) with 50 ml aqueous solution of 1 mM silver nitrate ($AgNO_3$). The reaction mixture was incubated in Erlenmeyer flasks on orbital shaker (200 rpm) at 28±2^oC in the dark for 5 days. [34].

Along with experimental flask was run another flask with cell free supernatant without $AgNO_3$, as control.

2.3. Visual analysis and spectrophotometric characterization of silver nanoparticles

Visual observation of colour changing towards yellowish to brown in reaction mixture after adding $AgNO_3$, consequently to completion of reaction, was carried out. This phenomenon was produced by excitation of Surface Plasmon resonance of silver nanoparticles [35].

1ml aliquots of AgNPs solution incubated in dark for 24 hours were taken in quartz cuvette for spectrophotometrically reading the optical density (O.D.) to the Carl-Zeiss Jena Spectrophotometer (wavelength ranging between 400 and 500 nm against deionized water as blank).

2.4. Transmission electron microscopy (TEM) analysis

The size, shape and dispersion of silver nanoparticles mycosynthesized were analyzed by Transmission Electron Microscopy (TEM) using a JEM – 1400 (Jeol) microscope operated at an accelerating voltage of 80kV.

The samples were prepared for imaging by drop-coating silver nanoparticles solution on carbon-coated copper TEM grid (40 μm x 40 μm mesh size). Grids were air-dried, then loaded on specimen holder [36] and TEM images were taken at various magnifications.

The size of AgNPs was calculated as average value of two perpendicular diameters for nanoparticles assumed as circular or as average of minor and major axes [37].

2.3. Antifungal activity of silver nanoparticles

The antifungal activity of biogenic AgNPs was assessed against the mycotoxigenic test fungi: *Aspergillus ochraceus*, *Fusarium oxysporum* and *Penicillium expansum.* Fungal strains were previously activated in Potato Dextrose Agar (PDA) at 25°C for 5 days.

Antifungal activity of biosynthesized AgNPs was assayed by agar well diffusion method [38]. 30μl AgNPs suspension were added in 6 mm diameter wells bored in PDA Petri plates, previously inoculated with 10^3 CFUs/mL fresh pure culture suspensions belonging to the test fungi.

Identic quantities of distilled water and of $AgNO_3$ added in wells served as negative, respectively positive controls.

Petri plates were incubated at 28±2°C for 5 to 7 days and monitored for the antifungal activity of AgNPs with biogenic origin, by measuring the diameter of growth inhibition halos.

2.4. Determination of Minimum Inhibitory Concentration (MIC)

A serial dilution of the AgNPs solution was prepared within a desired range: 300, 200, 100, 50, 25, 12.5 and 7.5 μg/mL and distributed into the agar wells from Petri plates with PDA medium.

The Petri plates were incubated at 28ºC for 5 days.

The MIC value is defined as minimum concentration of AgNPs that inhibits the growth of test microorganism [39].

The experiment was conducted in triplicate and the results represent the mean values of three replicates. The derived data were compared using one-way ANOVA analysis of variance for $P<0.05$, according to Student test.

3. Results

3.1. Visual analysis and spectrophotometric characterization of silver nanoparticles

Extracellular synthesis of AgNPs was carried out by using cell-free culture filtrate of *Aspergillus flavus* for reducing $AgNO_3$ to silver nanoparticles.

The formation of AgNPs was visually confirmed by gradually colour change from pale yellow to brown (Fig.1a), due to bioreduction of Ag^+ to Ag^0 mediated by enzyme nitrate reductase from cell free culture filtrate belonging to fungus *Aspergillus flavus,* after 24 h incubation with $AgNO_3$. No colour change was evidenced for the control without silver ion Ag^+.

Spectrophotometric analysis of the biosynthesized AgNPs was carried out at various wavelengths (400-500 nm) and maximum absorption peak registered occured at 420 nm, within 24 h (Fig.1b).

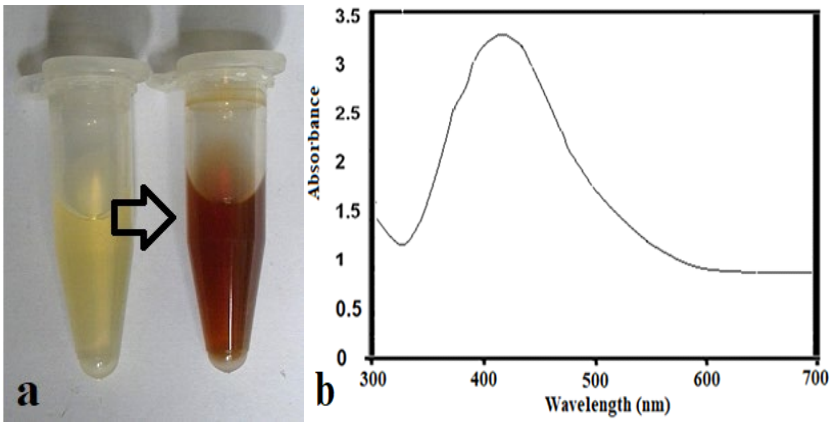

Fig. 1. a) Colour change of reaction mixture from pale yellow to brown after addition of $AgNO_3$ (1mM) to culture filtrate of Aspergillus flavus; b) Spectrophotometric analysis of mycosynthesized AgNPs.

3.2. Transmission electron microscopy (TEM) analysis of AgNPs

Transmission Electron Microscopy (TEM) images were examined to assess the size, shape and dispersion of silver nanoparticles of biogenic origin.

TEM aspect of silver nanoparticles mycosynthesized using culture filtrates of *Aspergillus flavus* revealed the aproximately spherical shape and a relative good dispersion without the tendency of forming significant agglomerations (Fig.2).

Silver nanoparticles presented clear parallel strips (lattice fringes), confirming their crystalline structure.

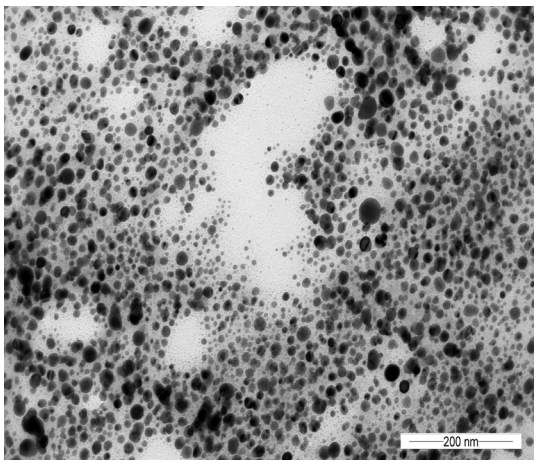

Fig. 2. TEM image of silver nanoparticles synthesized using culture filtrate of Aspergillus flavus

The histogram of AgNPs size distribution is depicted in Figure 3. Nanoparticles extracellularly synthesized using culture filtrate of *Aspergillus flavus* presented dimensions ranging between 3.3 and 40 nm, with highest and relatively similar frequency for categories 10-15 nm and 15-20 nm.

Frequency distribution revealed that dimensions of biogenic AgNPs were preponderant below 20 nm (72.54%).

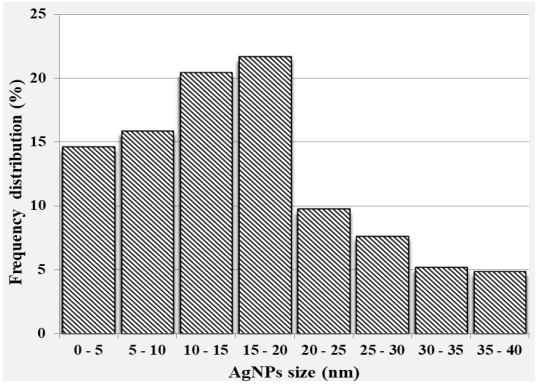

Fig. 3. The particles size distribution of AgNPs synthesized with culture filtrate of Aspergillus flavus

3.3. Antifungal activity of silver nanoparticles

The antifungal activity of biogenic AgNPs was tested using agar well diffusion method against the following mycotoxigenic test fungi: *Aspergillus ochraceus, Fusarium oxysporum* and *Penicillium expansum*.

The values of inhibition zone diameter and MIC are summarized in Table 2.

Table 2. Antifungal agar well diffusion activity of biogenic AgNPs and MIC

Fungal isolate	**Inhibition zone – $AgNO_3$** (mm)	**Inhibition zone – biogenic AgNPs** (mm)	**Inhibition zone – H_2O**	**MIC** ($\mu g x mL^{-1}$)
Aspergillus ochraceus	10c[1]	15.5b	w.i	12.5a
Penicillium expansum	12.5b	15b	w.i	7.5b
Fusarium oxysporum	18a	25a (t.i.)	w.i	7.5b

[1]The values in a column followed by the same letter are not significantly different for $P< 0.05$ (Student test)
w.i.-without inhibition, t.i.-total inhibition

Data revealed that biogenic AgNPs had a higher activity than $AgNO_3$ against all test fungi. No inhibition zone was observed around the wells with H_2O as negative control.

AgNPs presented a similar antifungal activity against growth of *Aspergillus ochraceus* and *Penicillium expansum,* with inhibition zone diameters of 15.5 mm and, respectively 15 mm, as evidenced in micrographs from Figure 4 and Figure 5.

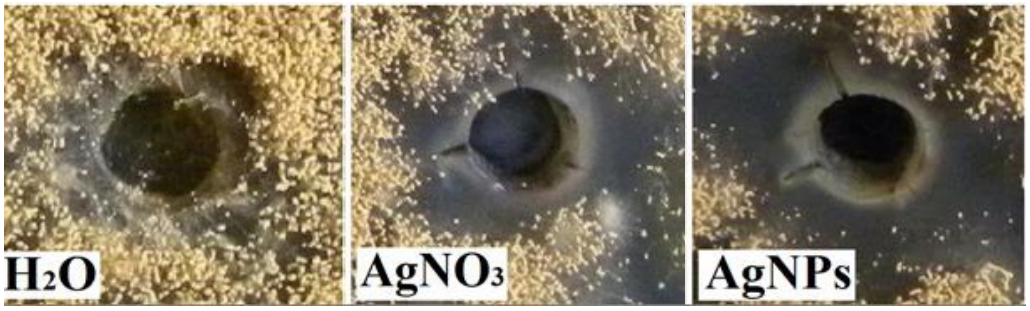

Fig. 4. Antifungal activity of mycosynthesized AgNPs against A. ochraceus comparatively with $AgNO_3$ and H_2O

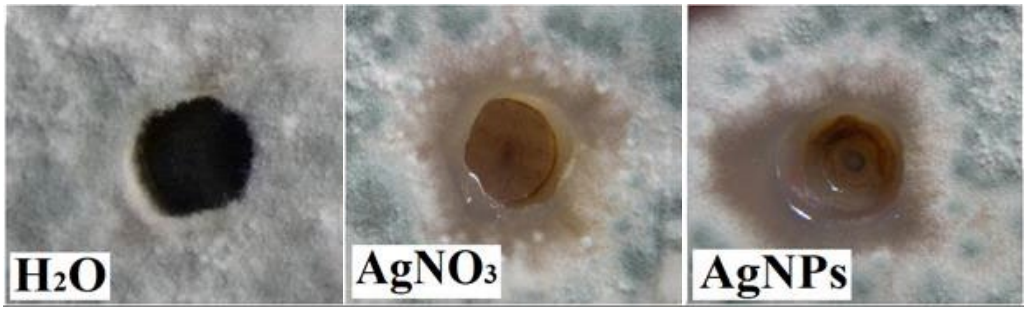

Fig. 5. Antifungal activity of mycosynthesized AgNPs against P. expansum comparatively with $AgNO_3$ and H_2O.

Fusarium oxysporum was significantly more susceptible to biogenic AgNPs, with 25 mm clear halo (total inhibition zone) around the well (Figure 6).

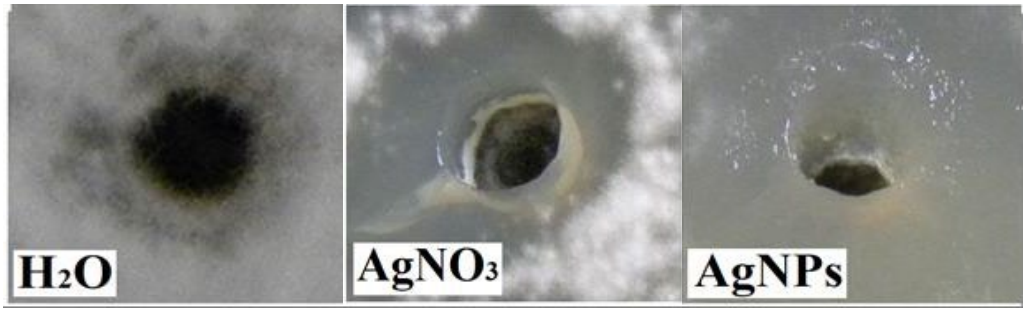

Fig. 6. Antifungal activity of mycosynthesized AgNPs against F. oxysporum comparatively with $AgNO_3$ and H_2O.

3.4. Determination of Minimum Inhibitory Concentration (MIC)

The values from Table 2 indicate that minimum inhibitory concentration (MIC) varied from 7.5 μg/mL for *Penicillium expansum* and *Fusarium oxysporum* to 12.5 μg/mL for *Aspergillus ochraceus*.

The last fungal species was more resistant to biogenic silver nanoparticles and a higher concentration was needed to inhibit its growth than in the case of *Penicillium expansum* and *Fusarium oxysporum*, where a lower concentration inhibited their growth.

3. Discussions

Silver nanoparticles were obtained by green synthesis using cell-free culture filtrate of fungus *Aspergillus flavus* and aqueous solution of 1mM $AgNO_3$.

The presence of AgNPs was certified by colour change from pale yellow to brown due to bioreduction of Ag^+ to Ag^0 mediated by fungal enzyme nitrate reductase from cell free culture filtrate of *Aspergillus flavus.* Spectrophotometric analysis of the biosynthesized AgNPs revealed that maximum absorption peak occured at 420 nm, within 24 h, in concordance with previous statements in literature [40] that the maximum absorbance exhibited by silver nanoparticles is in the range 400-500 nm, because of Surface Plasmon resonance. *Trichoderma reesei* mediated extracellular biosynthesis of AgNPs with diameters ranging from 5 nm to 50 nm and tendency to agglomerate into colloids of varying sizes (between 10–1000 nm). The strong surface plasmon resonance was centered at about 414–420 nm, this peak being characteristic for colloidal silver [41].

Results are in concordance with literature reporting extracellular biosynthesis of silver nanoparticles mediated by a wide range of fungal species: *Cladosporium cladosporioides* [16], *Penicillium chrysogenum* [42], wasp nest soil fungus, *Penicillium italicum* [43], *Penicillium cyclopium* [44], *Aspergillus flavus* [45], *Aspergillus niger*, *Trichoderma gamsii*, *Rhizopus arrhizus*, *Penicillium aurantiogriseum* and *Verticillium chlamydosporium* [46], *Aspergillus melleus* [47].

Data regarding extracellular biosynthesis of AgNPs mediated by various microorganisms revealed implication of exoenzyme nitrate reductase and proteic compounds, acting as capping agents, in reducing silver ions and formation of nanoparticles [48; 49].

TEM images of AgNPs mycosynthesized using culture filtrates of *Aspergillus flavus* revealed their aproximately spherical shape, crystalline structure and a relative good dispersion, without significant agglomerations.

Similar TEM images, with clear parallel strips (lattice fringes) indicating the polycrystalline nature of silver nanoparticles biosynthesized with culture filtrates belonging to various microorganisms (bacteria, fungi) are presented in literature [50]. Other authors [51] confirmed the highly crystalline nature of the biosynthesized nanoparticles using Selected-Area Electron Diffraction (SAED).

AgNPs extracellularly synthesized using culture filtrate of *Aspergillus flavus* presented dimensions ranging between 3.3 and 40 nm, with highest and relatively similar frequency for categories 10-15 nm and 15–20 nm. 72.54% of biogenic AgNPs presented dimensions below 20 nm. Other authors [52] reported extracellular synthesis of AgNPs with dimensions of 9–41nm mediated by fungus *Trichoderma asperellum*. According to literature, a high frequency of AgNPs with small dimensions is related to a more intense antimicrobial activity (due to the larger surface of contact with a pathogen) than in the case of the AgNPs with larger diameter [53].

Our results for antifungal activity performed using agar well diffusion assay with mycotoxigenic fungi revealed that biogenic AgNPs had a higher activity than $AgNO_3$ against all test fungi, in close agreement with previous studies [54].

Results are similar with research on extracellular mycosynthesis of AgNPs mediated by isolates of *Aspergillus flavus* that revealed their antimicrobial activity against various multidrug resistant bacteria and pathogenic fungi [7; 55]. Researchers, also described synthesis of AgNPs using various fungal strains: *Aspergillus caespitosus* [56], *Penicillium purpurogenum* [57], *Epicoccum nigrum* [58].

Well diffusion method used to assess the antifungal potential of the spherical 3–10 nm AgNPs biosynthesized using *Alternaria* sp. cell free culture filtrate, against four pathogenic strains (*Fusarium oxysporum*, *Fusarium moniliforme*, *Fusarium tricinctum* and *Alternaria* sp.) revealed maximum inhibition zones of 21.6 ± 1.5 mm and 21.3 ± 1.0 mm at 100 μl concentration from the stock solution of AgNPs for *Fusarium oxysporum* and *Alternaria* sp., respectively [31].

Recent study [59] reported the efficiency of spherical silver bio-nanoparticles (AgNPs), with uniform distribution and size between 28 and 45 nm, synthesized in secondary metabolic products of *Penicillium pedernalens* 604 EAN, in inactivating of the *Aspergillus fumigatus, A. parasiticus*, *A. flavus* var. *columnaris* and *A. aculeatus* airborne spores, as a function of AgNPs volume (1–10 μL/mL).

Data from the present research are confirmed by other literature results [60] showing that silver nanoparticles eco-friendly synthesized by using soil-isolated fungal strains of *Phoma sp., Chaetomium globosum* and *Chaetomium sp.* were sphaerical in shape, with average size between 12.26 and 70.24 nm and displayed strong antimicrobial activities due to interaction with cellular components of test-microorganisms.

Anisotropic AgNPs synthesized through plant pathogen *Bipolaris nodulosa* presented significant antimicrobial potential against bacterial and fungal pathogens [61]. Extracelullarly synthesized 120 nm AgNPs using *Aspergillus terreus* presented antifungal activity against *Aspergillus fumigatus* by severe destructions of its genetic material [62].

Biogenic AgNPs with diameters of 1–22 nm and spherical shape, obtained using cell free extract of *Epicoccum nigrum* presented antifungal activity against pathogenic, mycotoxigenic or food spoilage species *Candida albicans, C. parapsilosis, C. tropicalis, C. krusei, Cryptococcus neoformans, A. fumigatus, A. flavus, F. solani* and *Sporothrix schenckii* [58], authors reporting

MIC:1 μg/mL. Recent studies [32] reported extracellular mycosynthesis of spherical 20–60 nm AgNPs mediated by *Alternaria alternata,* with antimicrobial activity against *C. albicans* and *Phoma glomerata* at 20 μL dose.

AgNPs extracellularly synthesized using culture filtrate of *Aspergillus flavus* showed significant antimicrobial properties against mycotoxigenic test fungi and they could be recommended as a potential source for the preparation of new environment friendly antifungal agents. Similar results [63] demonstrated the antifungal and anti-mycotoxin efficacy of biogenic AgNPs produced by *Fusarium chlamydosporum* and *Penicillium chrysogenum* at non-cytotoxic doses.

Other results on the role of fungi in production of nanoparticle-mediated fungicides evidenced their importance for phytopathogens management using non-toxic products of nanotechnology [64].

Also, recent research integrated various myconanoparticles into fertilizers, fungicides, herbicides and pesticides and discussed their beneficial impact on farming, because NPs may promote the release of secondary metabolites of microorganisms and plants [65; 66]. These results are important for new applications in stress management and crop protection [67].

4. Conclusion

Green synthesis of silver nanoparticles was accomplished by using culture filtrate of *Aspergillus flavus* and aqueous solution of 1mM $AgNO_3$.

The presence of AgNPs was certified by colour change of reaction mixture from yellow to brown.

Spectrophotometric analysis of the biosynthesized AgNPs revealed that maximum absorption peak occured at 420 nm, within 24 h, because of Surface Plasmon resonance.

TEM aspect of silver nanoparticles mycosynthesized using culture filtrates of *Aspergillus flavus* revealed the aproximately spherical shape and a good dispersion without the tendency of forming significant agglomerations.

AgNPs presented parallel lattice fringes confirming their crystalline structure.

Nanoparticles extracellularly synthesized using culture filtrate of *Aspergillus flavus* presented dimensions ranging between 3.3 and 40 nm, with highest and relatively similar frequency for categories 10-15nm and 15-20 nm.

Frequency distribution revealed that the biogenic AgNPs dimensions were preponderant below 20 nm (72.54%).

These biologically synthesized AgNPs showed significant antimicrobial properties against mycotoxigenic test fungi, so we suggest that they could be recommended as a potential source for the preparation of new environment friendly antifungal agents.

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