

# Biosynthesis of Silver Nanoparticles by using Salt Tolerant *Trichoderma longibrachiatum*, Antifungal Activity and Impact on Plant Growth Promotion

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Received: 17 Jul 2025; Revised accepted: 26 Aug 2025

## Abstract

The biosynthesis of silver nanoparticles (AgNPs) using salt-tolerant *Trichoderma longibrachiatum* offers an eco-friendly and cost-effective approach to nanotechnology with significant agricultural applications and a promising alternative tool to control fungal diseases. In this study, *Trichoderma longibrachiatum* was utilized as a biological agent to synthesize AgNPs under optimized conditions. The process involved the reduction of silver nitrate (AgNO<sub>3</sub>) into AgNPs, confirmed by visual color change and spectrophotometric analysis. Characterization of the synthesized AgNPs was performed using UV-visible-spectroscopy showed maximum absorbance at 360 nm, Dynamic light scattering (DLS) supported that the Z-average size was 83.3 nm and 2.047 Pdl value. Zeta potential showed -66.2 mV with a single peak. X-ray diffraction (XRD) showed the spherical shape and crystalline nature, scanning electron microscopy (SEM) showed aggregation of small micro-nanoparticles, and Fourier-transform infrared spectroscopy (FTIR) showed bands at 3339.22 and 1635.37cm<sup>-1</sup>. The AgNPs synthesized through this bio-system approach were relatively stable up to 2 months after synthesis. The antifungal efficacy of the synthesized *Trichoderma longibrachiatum* - AgNPs lead to significant reduction in growth of major phytopathogenic fungi, with efficiencies reaching up to 56.66% against *F. oxysporum*, 44.44% against *R. solani* and 39.96%, against *A. alternata*. This study highlights the potential of salt-tolerant *Trichoderma longibrachiatum* as a novel biogenic source for AgNPs synthesis. Furthermore, research should be carried out for determination of the toxic effect of AgNPs before mass production and use of agricultural applications.

**Key words:** Silver nanoparticles (AgNPs), Salt-tolerant *Trichoderma longibrachiatum*, Phytopathogens, UV-visible spectroscopy, SEM, FTIR, Zeta potential, XRD, Antifungal activity

Nanotechnology has emerged as a promising field for developing eco-friendly and sustainable solutions in agriculture. Silver nanoparticles (AgNPs) have gained significant attention in various fields, including medicine, electronics, and environmental science due to their smallest size, High surface area, strong antimicrobial activity [1]. The biosynthesis of AgNPs using microorganisms offers an environmentally benign approach compared to chemical and physical methods [2]. Application of fungi for production of silver nanoparticles, which is the accent of the present paper. Production of silver nanoparticles through fungi has several advantages which include tolerance towards high metal nanoparticles concentration in the medium, easy management in large-scale production of nanoparticles, good dispersion of nanoparticle and much higher amounts of protein expressions. Compared to bacterial broth, fungal broth can be easily filtered by filter press or similar commonly used equipment, thus saving considerable investment costs for specialized equipment which may be needed for other methods. As a result, for large-scale production of nanoparticles fungi is preferred over other methods [3]. The synthesis of nanoparticles using *Trichoderma* is a type of biological (green) synthesis, which is an eco-

friendly alternative to conventional chemical methods. *Trichoderma* species produce bioactive compounds and enzymes that reduce metal ions to form nanoparticles with unique properties. *Trichoderma* Synthesized Nanoparticles includes, Silver Nanoparticles (AgNPs), Gold Nanoparticles (AuNPs), Zinc Oxide Nanoparticles (ZnO-NPs). *T. reesei* [4], *T. harzianum* [5], *T. hamatum* [6] and *T. koningii* [7] have been used for synthesis of metal nanoparticles. *Trichoderma longibrachiatum*, a salt-tolerant fungal species, has demonstrated the ability to biosynthesize AgNPs by reducing silver ions into nanoscale particles. This process involves Fungal cell free filtrate containing enzymes and reducing agents mixed with metal salt solution. Fungal bioactive metabolites reduce metal ions in to nanoparticles [8]. The use of salt-tolerant strains is particularly advantageous for applications in saline environments where conventional microbial systems may fail. The antimicrobial efficacy of these biosynthesized AgNPs has been tested against various phytopathogenic fungi, which are responsible for significant crop losses.

This study delves into the potential of salt tolerant *Trichoderma longibrachiatum* in the biosynthesis of silver nanoparticles (AgNPs) and assesses its effectiveness against

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vital plant pathogens. The impact of *Trichoderma longibrachiatum* AgNPs on promoting plant growth is evaluated. Consequently, these nanoparticles aid plants in mitigating salt stress. The discoveries made here contribute to the advancement of sustainable and environmentally friendly strategies for disease management in agriculture.

## MATERIALS AND METHODS

### *Isolation and identification of fungal isolates*

Fungal isolates were collected from saline soil sample Vikhroli Mumbai during 2022 screened and purified using standard techniques and maintained on 3% and 4% potato dextrose agar (PDA) slant and stored at 4 °C until further use [9]. Fungal isolates were identified as *Trichoderma sp.* ST2 (4% salt) according to Morphological characteristics [10]. Further molecular identification was done by National Centre for Cell Sciences (NCCS) Pune using Internal Transcribed Spacer (ITS) region sequencing, providing precise taxonomic classification [11]. The *Trichoderma* species viz. *Trichoderma longibrachiatum* was screened for their ability to synthesize silver nanoparticles. The isolate was cultured in 4% PDA and incubated at 25 °C for 5 days and used for extracellular syntheses of silver nanoparticles. The fungal isolate; *F. oxysporum*, *R. solani* and *A. alternata* was obtained from pathology Department, Agricultural College, Latur confirmed and maintained on 4% potato dextrose agar (PDA) slant and stored at 4 °C until further use.

### *Fungal biomass preparation for AgNPs biosynthesis*

Two disks (5 mm in diameter) of each fungus were inoculated onto 100 ml of liquid medium containing (g/l) KH<sub>2</sub>PO<sub>4</sub> 7.0, K<sub>2</sub>HPO<sub>4</sub> 2.0, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.0, yeast extract 0.6, and glucose 10. The flasks were incubated in a shaker at 150 rpm at 25–28 °C. After 120 h of growth, the fungal mycelium took the shape of a circular mat. Then, the biomass was harvested by sieving through a Whatman filter paper no. 1, followed by extensive washing with Milli-Q deionized water to remove any adhering medium parts from the biomass.

### *Biosynthesis of silver nanoparticles*

A disc of mycelium of *Trichoderma longibrachiatum* was transferred to Potato dextrose Broth supplemented with 4% NaCl. The culture was incubated at 28 °C for 5 days after which a biomass was weighed (20g) and transferred to 100ml Milli-Q deionized water and incubated for 72h at 28 °C at 150 rpm. The sample was then filtered through Whatman filter paper no. 1, 50 ml of 1 mM AgNO<sub>3</sub> solution was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask, kept on a shaker at 150 rpm in dark [12]. The positive control is the only fungal biomass filtrate without the silver nitrate solution, and the negative control is the silver nitrate solution without cell-free filtrate. AgNPs were purified by centrifugation at 10,000 rpm for 10 minutes twice and collected for further characterization [13].

### *Characterization of silver nanoparticles*

#### *Ultraviolet-visible spectrophotometer analysis*

The formation of the reduced silver nanoparticles in colloidal solution was monitored by using a UV–visible double beam spectrophotometer (UV 19001, Shimadzu Dayanand Science college, Latur, India). The absorption spectra of the supernatants were taken between 300 and 800 nm, using a UV–visible double beam spectrophotometer. The deionized water was used as the blank.

#### *Fourier transform infrared (FTIR) spectroscopy*

The FTIR spectrum of the biosynthesized silver product was meticulously recorded using a Spectrum Two, NIOS<sub>2</sub> Main 00.02.0091, Perkin Elmer Spectrum, with a resolution of 4 cm<sup>-1</sup> attachment. All measurements were conducted within the spectral range of 450–4000 cm<sup>-1</sup>, maintaining this resolution throughout the analysis.

#### *Dynamic light scattering*

Horiba SZ-100, ver 2.70 Instruments Ltd., India, was used to determine the size distribution of particles by measuring dynamic fluctuations of light scattering intensity caused by the Brownian motion of the particles. The measurement gave the average hydrodynamic diameter of the particles, the peak values in the hydrodynamic diameter distribution, and the polydispersity index (PDI) that described the width of particle size distribution. All measurements were carried out in triplicate with a temperature equilibration time of 1 min at 25 °C. The data processing mode was set to high multi-modal resolution. The suspension of the synthesized silver nanoparticles was allowed to filter through a 0.22- $\mu$ m syringe driven filter unit or without filtration.

#### *X-ray diffraction (XRD)*

The crystal phase information of the biosynthesized AgNPs was characterized from 10 to 80°C in 2 $\theta$  by an X-ray diffractometer with CuK  $\alpha$  radiation (Shimadzu XRD 6000, India) [14].

#### *Scanning electron microscopy (SEM)*

For the SEM analysis, the samples were dried and mounted on a special stub or holder. The prepared sample is loaded into the SEM's vacuum chamber. the operating voltage (typically 1-30 kV), focusing the electron beam, and selects the imaging mode. The surface morphology of the nanoparticles was examined by SEM (YCIS Satara).

#### *Antifungal activity of silver nanoparticles (AgNPs)*

The antifungal activity of the synthesized AgNPs was tested by Poisoned food method [15] against the pathogenic fungi: *A. alternata*, *F. oxysporum*, and *Rhizoctonia solani*. Fungal spores at a concentration of 10<sup>5</sup> spores/ml were incubated in potato dextrose broth supplemented with 4% NaCl and sterile, distilled water as a control at 28 °C for 24 h in a shaker (120 rpm). A 25- $\mu$ l aliquot of the spore suspension was spread on PDA. Wells of 3 mm diameter were made on PDA plates by using cork borer. AgNPs (75 mL) were poured in each well, after incubation at 27 °C for 7 days, the diameter of colony (radial growth) was measured in millimeter. Four replicates of the trials were done.

#### *Pot experiment*

Full Soybean and chickpea seeds were surface-sterilized with 10% sodium hypochlorite for 10 min, and further treated with warm water at 50 °C for 2 h. The seeds were wrapped into wet sterile gauze, then put into an incubator at 30 °C for germination. The treated seeds were sown in plastic pots filled with about 500 g soil with 4% NaCl. After 5 days of cultivation at 28 °C AgNPs- solution (100 mg L<sup>-1</sup>), 10<sup>6</sup> CFU mL<sup>-1</sup> *T. longibrachiatum* spore suspension, AgNPs- solution (100 mg L<sup>-1</sup>) + 10<sup>6</sup> CFU mL<sup>-1</sup> *F. oxysporum* were watered into different pots, respectively. The control including water and *F. oxysporum* were watered with 50 mL sterile water. There were 5 pots at every treatment, in triplicate. 20 days after inoculation, the incidence and disease index were calculated basing on the wilt status and root infestation of the plant. At the same time,

the plant root and stem length, and number of leaves were measured to evaluate the development and growth of the Soybean and chickpea seedlings.

#### Statistical analysis

Statistical analyses were conducted using SPSS for Windows, Version 22.0 All the experimental data were presented as the mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) was conducted to compare the differences of the means. Dunnett's test was performed for multiple comparisons, and the Student's t-test was performed for each dataset, comparing with the control data. The statistical significance for all tests was considered at a probability level of 0.05 ( $P < 0.05$ ) or 0.01 ( $P < 0.01$ ).

## RESULTS AND DISCUSSION

#### Biosynthesis of silver nanoparticles

The fungal strain *T. longibrachiatum* reduced silver salt to silver nanoparticles as Noticed by visual observation of the fungal filtrate. The fungal filtrate exhibited a gradual Change from yellow to brown indicating formation of AgNPs. The colour of the culture filtrate with silver nitrate solution changed to intense brown after 48 hours of incubation, while the control (Without silver nitrate salt) did not exhibit any colour change (Fig 1) and by measuring the ultraviolet-visible spectrophotometer of the solution by periodic sampling of aliquots (1 ml) of the aqueous component. The formed silver nanoparticles were subjected to different instrumental analytical techniques for characterization.

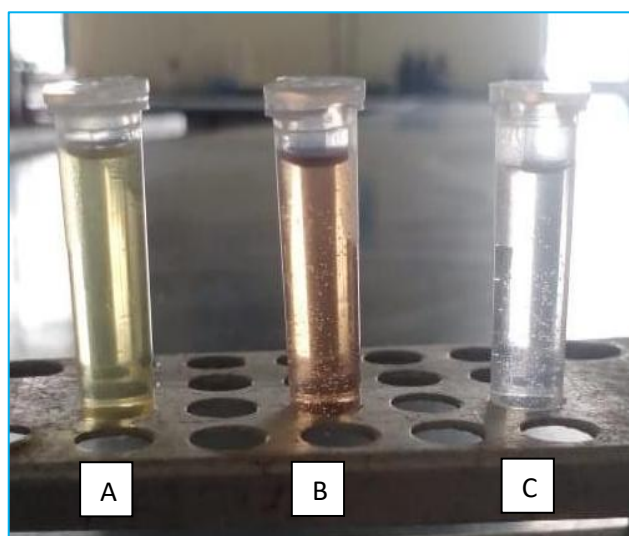


Fig 1 (A) Culture filtrate of fungus *Trichoderma longibrachiatum* (B) Biosynthesis of silver nanoparticle color change reaction after exposure to AgNO<sub>3</sub> solution for 48 h (C) AgNO<sub>3</sub> 1 mM solution

#### Characterization of AgNPs

##### Ultraviolet-visible spectrophotometer analysis

UV-visible spectrum of the biosynthesis of silver nanoparticles using the fungi of *T. longibrachiatum* showed a peak at 420 nm corresponding to the plasmon absorbance of silver nanoparticles for the tested fungi *T. longibrachiatum*. UV absorption spectra are essential way to monitor the formation and stability of metallic nanoparticles in aqueous solutions, such as the spectral response to nanoparticles depend on their diameter. The peak echo of the plasmon moves to longer wavelengths. It expands as the diameter increases [16]. Therefore, the aqueous biosynthesis of silver ions can be successfully observed through UV-VIS rays, which are

sensitive to many factors, such as particle shape, size, and interaction with the medium when synthesized the sample was subjected to UV spectroscopy [17]. There is a relation between the particle size and the plasmon peak, as the particles became larger when the Plasmon peak shifts to longer wavelengths. Values for Ag particle size and Plasmon maxima that were reported with [18] indicated that UV spectra were in lower wavelengths (384–414), with 10- to 14-nm particle size. This event clearly indicated that the reduction of ions occurs extracellularly through reducing agents released in to solution by *T. longibrachiatum*. The control treatment without silver ions showed no color change when incubated under same conditions. The color change observed could be recognized to the surface Plasmon resonance of deposited AgNPs. The increase in the color intensity of culture filtrate was due to an increase in the number of nanoparticles formed from the reduction of silver ions present in the aqueous solution.

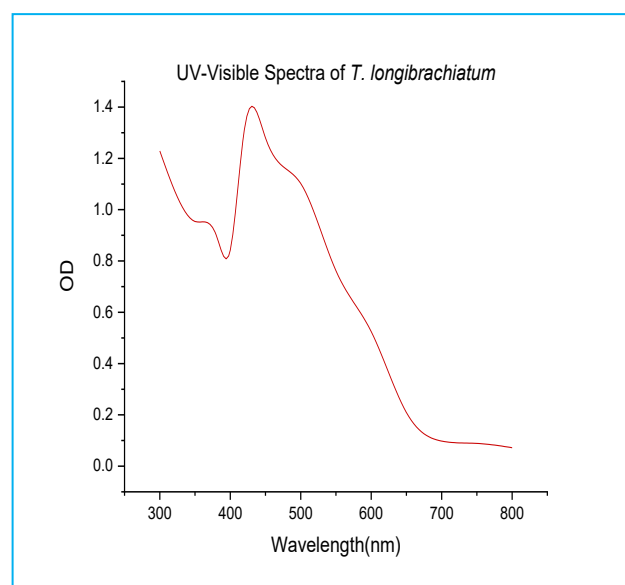


Fig 2 UV-visible absorption spectra of extracellularly synthesized AgNPs by *Trichoderma longibrachiatum* (MF276664)

#### Scanning electron microscopy (SEM)

Scanning electron Microscopy (SEM) revealed the formation of monodispersed spherical shape, although a few irregular shapes were also observed. The particle size ranged from 20-85nm. The nanoparticles observed minimal agglomeration, indicating the presence of capping agents secreted by *Trichoderma longibrachiatum*, such as proteins or secondary metabolites, which stabilize the particles [19] individual nanoparticles are grouped in to clusters (Fig 3). The morphological features are important because the shape and size of nanoparticles significantly influence their biological activity, including Antimicrobial and plant growth promoting effects [20].

#### Dynamic scattering analysis of AgNPs

The DLS study was conducted in order to detect the particle size distribution of AgNPs synthesized from filtrate (Fig 4). The data of DLS supported that the Z-average (r.nm) was 83.3. The average size of the synthesized AgNPs was 26.8 nm and 2.047 PdI value. The obtained single peak indicated that the quality of the synthesized silver nanoparticles was good (Fig 4a). One peak was obtained with a wide base, indicating that variable sizes of AgNPs were found in the solution. Zeta potential value of the mycosynthesized AgNPs recorded -66.2mV (Fig 4 b-c).

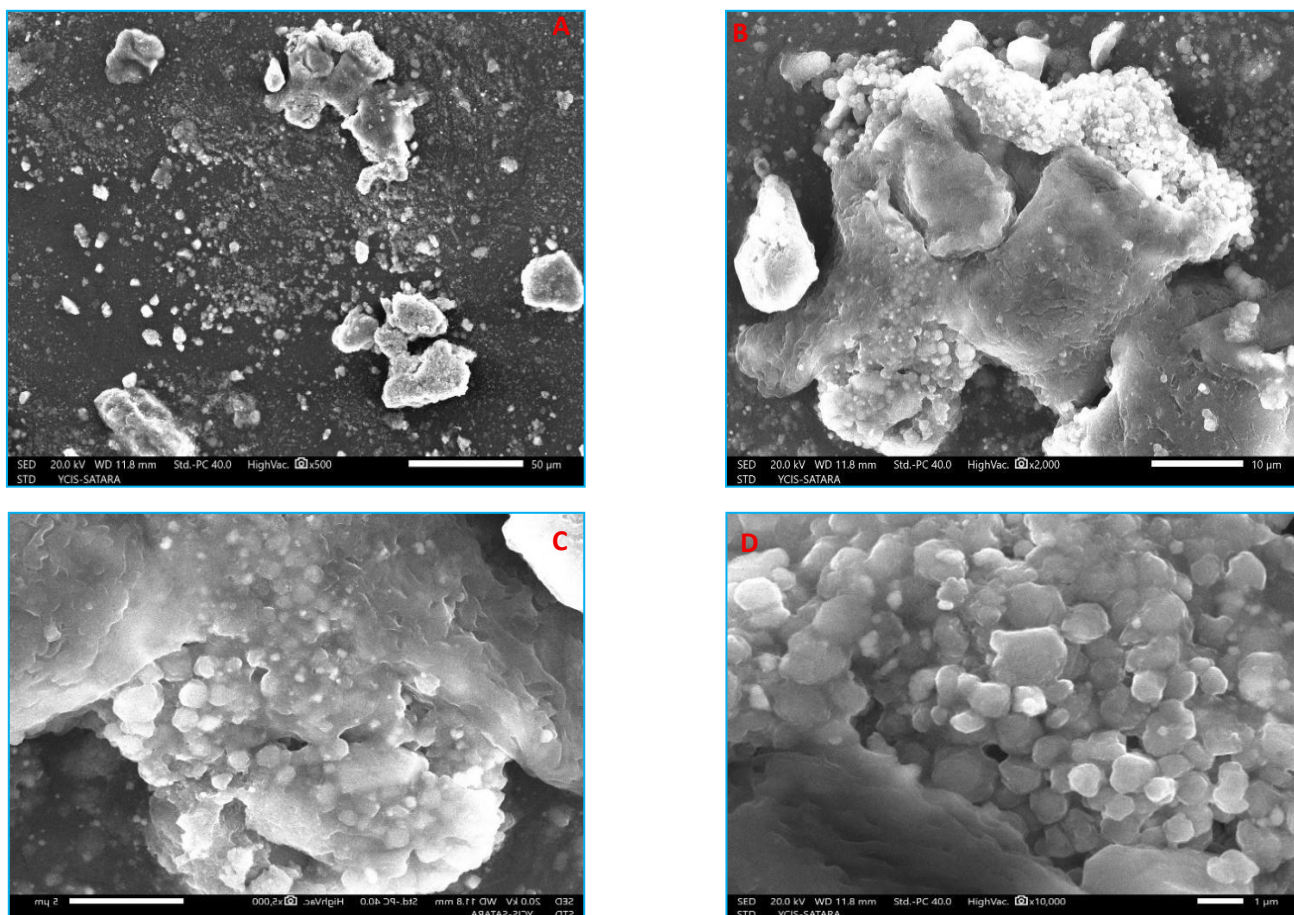


Fig 3 Scanning Electron Micrograph of *T. longibrachiatum* AgNPs. (a)50µm image (b) 10µm image (c)5µm image (d)1µm Instrument JSM-IT200, YCIS Satara

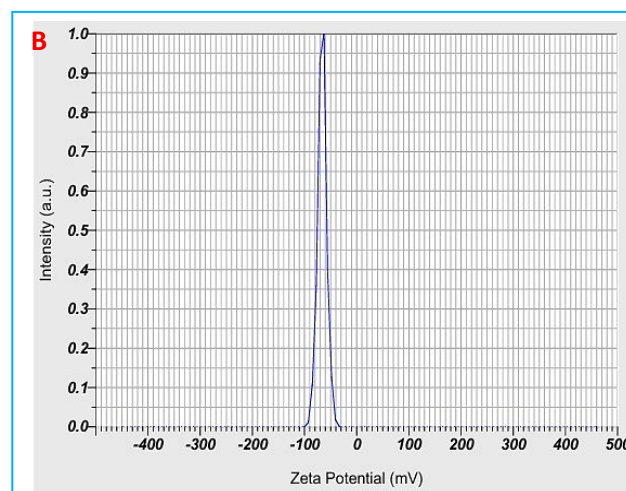
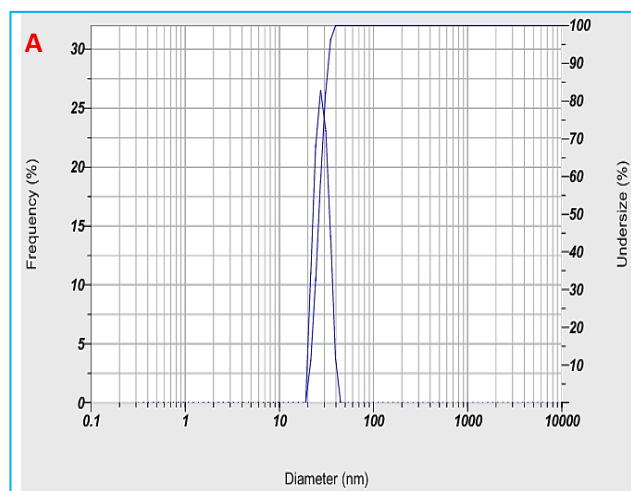


Fig 4 (a) DLS analysis of silver nanoparticles synthesized using *Trichoderma longibrachiatum* filtrate (b) Zeta potential of *Trichoderma longibrachiatum* synthesized nanoparticle (c) Zeta potential value of the mycosynthesized AgNPs recorded -66.2mV

Peak No.	Zeta Potential	Electrophoretic mobility
1	-66.2 mV	-0.000512 cm <sup>2</sup> /Vs
2	... mV	... cm <sup>2</sup> /Vs
3	... mV	... cm <sup>2</sup> /Vs
Zeta Potential (Mean)		: -66.2 mV
Electrophoretic mobility Mean		: -0.000512 cm <sup>2</sup> /Vs

AgNPs biosynthesized by *Trichoderma longibrachiatum* were nearly polydispersed with the size ranges from 26.8nm to 83.3nm the value of zeta potential was -66.6mV with a single peak signifying the presence of repulsion among the synthesized nanoparticles (Fig 4b) When the particles have low zeta potential values than there will be no force to prevent the particles from coming together and flocculating [21]. The biosynthesized AgNPs were found to have negative zeta

potential, which indicates the repulsion among the green synthesized silver nanoparticles and increase the stability of the formulation. It is evident that the AgNPs are polydispersed in nature, due to its high negative zeta potential; thus, the electrostatic repulsive force between them results in the prevention of agglomeration of the nanoparticles and is also very much helpful for long term stability in the solution [22-23].

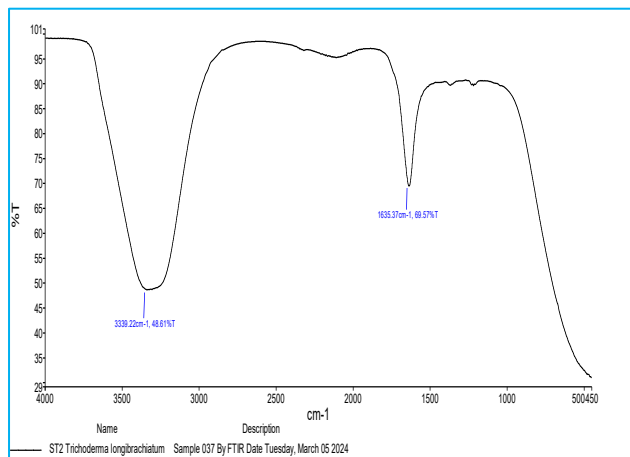


Fig 5 Fourier transform infrared spectroscopy (FTIR) spectrum of AgNPs synthesized by reduction of Ag<sup>+</sup> ions by *Trichoderma longibrachiatum*

#### FTIR spectroscopy analysis

This analysis determines the portion bindings with AgNPs. FTIR spectroscopy was carried out on AgNPs synthesized after 96 h of incubation with AgNO<sub>3</sub> as shown in (Fig 5). FTIR spectrum revealed bands at 1635.37 and 3339.22 cm<sup>-1</sup>. A band at 1634.92 cm<sup>-1</sup> corresponded to the bending vibrations of the amide I and amide II bands of portions [24] while their corresponding stretching vibrations of primary amines were observed at 3339.22 cm<sup>-1</sup>. FTIR results revealed that secondary structures of proteins had not been affected as a consequence of reaction with silver ions or binding with AgNPs [25]. FTIR spectroscopic study confirmed that amino acid and peptides form a coat covering the silver nanoparticles. The presence of the signature peaks of amino acids supports the presence of proteins in cell-free filtrate as observed in Un-visible Spectra. It is well known that nanoparticle – protein interactions can occur either through free amino groups or cysteine residues in proteins and via the electrostatic attraction of negatively charged carboxylic groups in enzyme proteins [26]. The carbonyl group of amino acid residues and peptides had strong ability to bind silver [27]. The proteins present over the surface of nanoparticle act as a capping agent.

#### XRD analysis

XRD analysis demonstrated that the emission peaks at 2θ values of 27.2, 36.0, 38.2, 44.2, 64.6, and 77.5 corresponded to the silver crystal planes (110), (101), (111), (200), (220) and (311) respectively, confirming the crystalline nature of AgNPs. The XRD pattern thus clearly illustrates that the synthesized AgNPs were crystalline in nature as well as colloidal form. The line broadening of peaks was related to the presence of small particles in the medium. A few unassigned peaks were also observed in the vicinity of the characteristic peaks (Fig 6). The unassigned peaks were recorded in the XRD pattern and they could be due to the crystallization of the bio-organic phase that occurs on the surface of the nanoparticles [28].

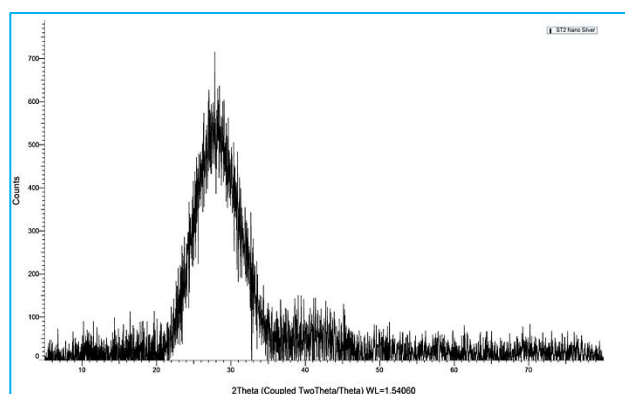


Fig 6 XRD analysis of AgNPs synthesized by *T. longibrachiatum*

#### Antifungal activities of biosynthesized AgNPs

The antifungal activity of biosynthesized AgNPs against the investigated three Fungal species is shown in (Table 1). The biosynthesized AgNPs of *Trichoderma longibrachiatum* gave the highest inhibition zone by average mm against *Fusarium oxysporum* relative to the control treatment, 16.6 On the other hand, *T. longibrachiatum* gave the least antifungal effect with the other two investigated phytopathogenic fungi species being 10 and 14 mm on the average, respectively compared with control for each fungus. Earlier workers investigated the antifungal activity of different biosynthesized AgNPs and stated that Nano materials play an important role in suppressing each of the tested fungi if produced by microorganisms [29-30].

Table 1 Antifungal activities of AgNPs of *Trichoderma longibrachiatum* compared with AgNO<sub>3</sub> and filtrate control on 3% PDA plates incubated at 28 °C for 7 days

Fungi	Inhibition zone diameter (mm) <i>T. longibrachiatum</i> AgNPs	Inhibition Zine diameter (mm) AgNO <sub>3</sub> control	Filtrate control
<i>Fusarium oxysporum</i> I	16	12	-
<i>Fusarium oxysporum</i> II	15	11	-
<i>Fusarium oxysporum</i> III	19	12	-
Mean	16.6	12	0.0
<i>Rhizoctonia solani</i> I	11	8	-
<i>Rhizoctonia solani</i> II	10	8	-
<i>Rhizoctonia solani</i> III	9	7	-
Mean	10	8	0.0
<i>Alternaria alternata</i> I	14	10	-
<i>Alternaria alternata</i> II	13	9	-
<i>Alternaria alternata</i> III	15	10	-
Mean	14	10	0.0

**Pot assay:** Experiments were performed to determine the effect of the *Trichoderma longibrachiatum* biogenic silver nanoparticles on the germination and seedling growth of

soybean and chickpea (Fig 7) exposure of soybean and chickpea seeds to the biogenic AgNPs did not result in any significant differences compared to the negative control [31-32].

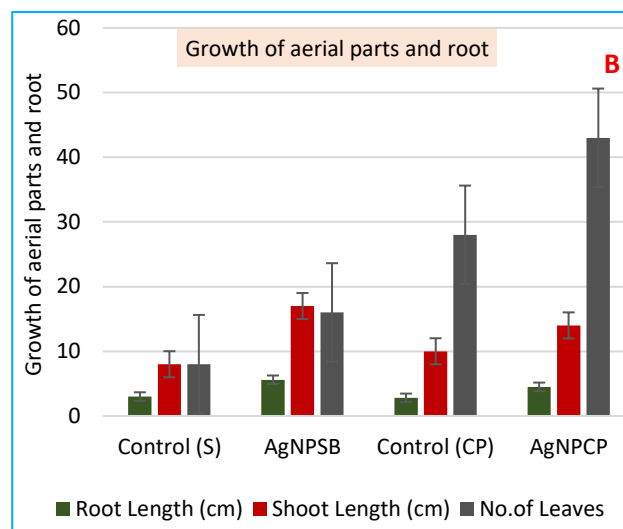
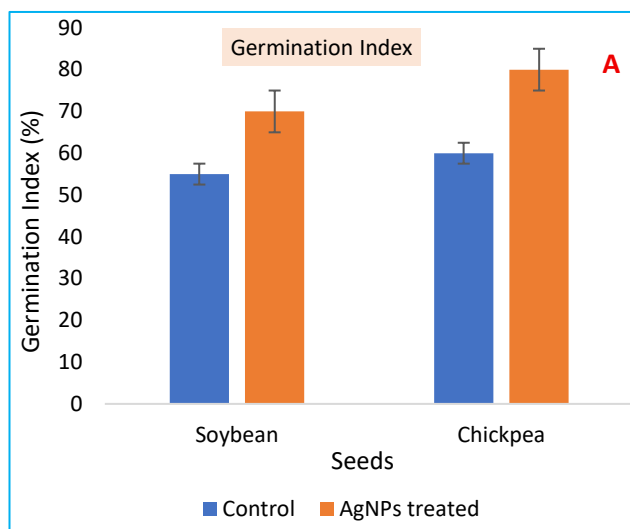


Fig 7 Effect of *T. longibrachiatum* AgNPs on plant growth promotion

(A) Germination index of AgNPs treated seeds of both soybean and chickpea were more effective than non-treated (Control)

(B) Growth of aerial parts and root was more effective in AgNPs treated pots than control pots

## CONCLUSION

The present study successfully demonstrated the eco-friendly biosynthesis of silver nanoparticles (AgNPs) using the salt-tolerant fungus *Trichoderma longibrachiatum*. Characterization of the synthesized nanoparticles confirmed their nanoscale size, stability, and morphology. The prepared AgNPs showed the spherical shape of cubic crystal nanoparticles with size ranges from 26 to 83 nm and many functional group moieties. The biologically synthesized AgNPs exhibited significant antifungal activity against *F. oxysporum* (16.6mm) than *A. alternata* (14mm) and *R. solani* (10mm),

suggesting their potential as an alternative to chemical fungicides. Moreover, the nanoparticles enhanced chickpea and soybean plant growth parameters, including seed germination, root-shoot length, and formation of number of leaves, highlighting their role in promoting plant development under saline conditions. These findings indicate that *T. longibrachiatum*-mediated AgNPs are promising biocontrol and biofertilizer agents, contributing to sustainable agriculture by combining disease suppression and growth enhancement in saline soil. Further field trials and toxicity assessments are recommended to validate their large-scale application and environmental safety.

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