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RESEARCH ARTICLE

Green synthesis of silver nanoparticles from endophytic fungus *Colletotrichum* sp. with special emphasis on antibacterial, antioxidant and plant growth promoting potential

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Abstract

The creation of "microbial nanotechnology" for quick and large-scale manufacturing of nanoparticles would be possible with an effective biosynthesis method. *Plumbago zeylanica* L. is a medicinal plant from which the endophytic fungus *Colletotrichum* sp. was isolated and identified by 18S rDNA sequencing and microscopic studies. Cell filtrate of *Colletotrichum* sp. was employed to produce biological silver nanoparticles which acted as a reducing and stabilising agent during this process. The brown colour proved that silver nanoparticles (AgNPs) are being produced. In characterization of AgNPs, ultraviolet-visible spectroscopy revealed maximum absorption at 425 nm. From transmission electron microscopy and field emission scanning electron microscopic study the shape of the silver nanoparticle was found spherical with 10–30 nm diameters. The existence of elemental silver (peak)was verified by the silver signal in the energy-dispersive X-ray spectroscopy. Fourier-transform infrared analyses showed some major and minor shifts in the peaks of some chemical bonding. Synthesised AgNP had strong antibacterial action against pathogenic bacterial strains in 200 g/mL concentration and against *Pseudomonas aeruginosa* MTCC 741, it showed the highest inhibition. The AgNP concentration of 150 g/mL demonstrated the highest competency for antioxidant capabilities while ascorbic acid was used as the reference. Also, AgNP exhibited plant growth-promoting ability as in 40 mg/L concentration; it significantly enhanced the shoot and root growth, total soluble protein, sugar and indole acetic acid content of *P. zeylanica* L. This AgNP can be useful in pharmaceutical industries and agriculture as nano fertilizers for all these purposes.

Keywords

antibacterial and antioxidant activity; endophytic fungi; plant growth promotion; *Plumbago zeylanica* L.; silver nanoparticle

Introduction

Chemical, physical or biological methods can all be used to synthesize nanoparticles. Nanoparticles produced by biological means are inexpensive, dependable and biocompatible (1). The potential for microorganisms like bacteria and fungi to serve as bio-factories for the synthesis of metallic nanoparticles of gold, cadmium sulphide and silver has been investigated (2). Microorganisms or extracts from therapeutic plants are used to generate nanoparticles in the green synthesis process. A fungus uses the exposure of

its mycelium to produce the enzymes and metabolites necessary for the fungus to survive the solution of metal salt during the biosynthesis of metal nanoparticles. Solid metallic nanoparticles are produced by reducing the metal ions by the catalytic effect of the metabolites of the fungus and the extracellular enzymes released (3). In the biogenic manufacture of silver nanoparticles, the use of fungi as stabilising and reducing agents is appealing because they can produce vast amounts of proteins, have low toxicity of the reduces, are user-friendly and have robust productivity (4). Silver is a metal that has been used as an antimicrobial agent since ancient times because silver-based compounds are far cheaper than gold-based ones (5). Several applications of silver nanoparticles (AgNPs) in nanomedicine have been reported, including anti-microbial (6), antifungal (7), anti-cancerous (8), anti-plasmodial (9) and targeted drug delivery (10). According to a recent study (11), biomedical applications of nanoparticles in medical imaging, cancer treatment, drug delivery, disease diagnosis, treatment of pathogenic diseases (12), Parkinson's disease, Alzheimer's disease, etc., which are neurodegenerative diseases (13), and others have a significant positive impact on human life and health. As silver nanoparticles bear a very small size and large surface area with high reactivity, utilising NPs as fungicides or bactericides or as nanofertilizers lowers the issues in plant disease control (14). NPs have distinct physicochemical characteristics and the capacity to increase plant metabolism (15). To maximise agricultural output and reduce nutrient losses during fertilisation, newer applications of nanotechnology and nanomaterials must be explored. The release of nutrients, the ability to manage the release of hazardous chemical fertilizers that affect plant development, and the ability to increase target activity are only a few of the potential benefits of nano-fertilizers or nano-encapsulated nutrients for plants (16). According to reports, under some circumstances, plants may produce the natural mineralized nanoparticles needed for their development (17). Many studies have reported that increased plant growth, development and production were done by seed treatment using nanoparticles (18). In our study, we identified and isolated *Colletotrichum* sp. fungal endophytes from the medicinal plant *P. zeylanica* L., selected based on their plant growth-promoting characters, and synthesized silver nanoparticles from this endophyte by the green synthesis process. The healing herb 'Chitrak' (*P. zeylanica*) belongs to the Plumbaginaceae family. This plant grows throughout a large portion of India, mostly in West Bengal and the southern regions of India. Apart from India, this plant is also native to all tropical or subtropical realms. *P. zeylanica* is crucial to the pharmaceutical industry. It displays a wide spectrum of pharmacological properties, such as cytotoxic, hepatoprotective, anticancer, antifungal, anti-inflammatory, antidiabetic and antibacterial properties (19). For having all these medicinal properties, we selected this plant as our experimental material. Endophytes from medicinal plants are very effective in plant protection, plant growth promotion (20) and forming active silver nanoparticles (21). Synthesized AgNP was characterised using field emission scanning electron microscopy (FE-SEM), transmission electron microscopy (TEM), ultraviolet-visible spectroscopy (UV-vis), energy dispersive X-ray spectroscopy (EDX) and a fourier-transform infrared (FTIR) study, including its antioxidant and antibacterial activities. These synthesized nanoparticles were also tested for their ability to promote growth of *P. zeylanica*.

In our study, we aimed to synthesize silver nanoparticles using extraction of an endophytic fungal strain that may bear significant antioxidant, antibacterial, as well as plant growth elevating properties to boost the production of medicinally significant plant material, *P. zeylanica*, in healthy conditions that may be employed in the pharmaceutical industries. With all these properties, synthesized AgNP may be applied as nanofertilizers and nanopesticides in agriculture fields in the place of chemical fertilizers.

Materials and Methods

Collection of plant sample

For isolation of endophytic fungus, leaf samples of the *P. zeylanica* were selected and collected from the departmental garden of the Botany Department, University of Burdwan (Coordinates: 23.2393° N, 87.8512° E). The sample was submitted to the taxonomy laboratory of the Botany Department, University of Burdwan and identified by the taxonomy laboratory expert. A separate herbarium of this plant was deposited in the departmental herbarium library of the Botany Department, University of Burdwan and the accession no is BURD 12230.

Isolation of endophytic fungus

Isolation of endophytic fungus was done by collecting the leaf samples of *P. zeylanica*. Collected leaf samples were washed thoroughly with tap water and distilled water. Leaves were cut into 1 cm^2 blocks and surface sterilization was done aseptically using 0.1% sodium hypochlorite solution, 70% ethyl alcohol, sterilized distilled water and antibiotic (0.4 µg/mL of Streptomycin) water serially, each for 1 min. Next, the samples were incubated in an incubator at 28 ± 1°C in 3 replicates of Petri plates containing potato dextrose agar [potato infusion: 200 g/L, glucose: 20 g/L, agar: 20 g/L, pH 5.6 ± 0.2]. After 96 h, fungal colonies appeared and then the pure culture was done.

Identification of endophytic fungal isolate

The selected fungal isolate was identified by phenotypic characteristics as well as by modern molecular approaches of taxonomy like 18S rDNA sequencing and MALDI-TOF analyses. Both these molecular identification techniques were done by the National Centre for Microbial Resource (NCMR), Pune, India. The chosen fungus strain was located utilising the 18S rDNA sequencing technique. Genomic DNA of the isolated fungal strain was isolated using the standard phenol/chloroform extraction technique (22) and then the 18S rRNA gene was amplified using the primers 16F27 [5'-CCAGAGTTT GATCMTGGCTCAG -3'] and 16R1492 [5'-TACGGYTACCTTGTTACGACTT-3']. For cleaning the generated 18S rRNA gene PCR product, the PEG NaCl precipitation method was used. Then it was directly sequenced on an automated DNA sequencer (ABI® 3730XL). Using extra

internal primers, sequencing was done from both ends so that each location could be read at least twice. The laser gene package was used for assembly and the NCBI database for identification. The programme MEGA X (23) was used to create a phylogenetic tree using Jukes and Cantor's techniques based on the neighbour-joining method. 1000 bootstrap resamples of the data were used to determine the branch point's importance. This fungal strain was deposited at the NCMR, Pune and the accession no is MCC 9247. MALDI-TOF mass spectrometry was conducted in accordance with the established protocols (24). External calibration of the spectra was performed using a standard calibration mixture, which comprised *Escherichia coli* extracts supplemented with RNase A and myoglobin, as detailed in the Bruker Daltonik GmbH, Germany, instruction manual. Identification of strains was achieved using MALDI Bio typer 3.1 and Flex Analysis version 3.4 software from Bruker Daltonik GmbH, Germany. Strains demonstrating a log score of ≥1.7 in comparison to database entries were identified at the genus level, while those with a log score of ≥2.0 were classified at the species level.

Green syntheses of silver nanoparticles (AgNPs) from fungal cell extraction

Extracellular syntheses of AgNPs was done by following the previously described method (25). For preparation of fungal biomass for AgNP synthesis, selected endophytic fungal strain was inoculated in the broth media (100 mL) containing K_2HPO_4 (2 g/L), KH_2PO_4 (7 g/L), $MgSO_4$ $7H_2O$ (0.1 g/L), $(NH_4)_2SO_4$ (1 g/L), glucose (10 g/L), yeast extract (0.6 g/L) and kept in BOD incubator for 96 h at 28±1°C for making circular fungal mat. After the incubation period, fungal mats were collected by a sieving process through Whatman filter paper no. 1 and then the fungal mat was washed thoroughly with Milli-Q deionized water. For biosynthesis of AgNP, fungal biomass was kept in a shaker at 150 rpm in 100 mL Milli-Q deionized water in a covered flask aseptically for 72 h at 28±1°C. Filtration was done using Whatman filter paper no. 1 and after that, 50 mL of cell filtrate solution was mixed with freshly prepared 50 mL of 1 mM silver nitrate solution and kept in a shaker at 150 rpm in dark condition. After a few days, the colour of the mixture changed from light yellow to dark reddish brown by the reduction of metal ions. For the control set, silver nitrate solution without any cell filtrate solution was prepared.

Characterization of synthesized silver nanoparticles

Ultraviolet-visible spectrophotometric analyses

UV-vis spectrophotometric (UV-19001, SHIMADZU) analyses was done with the colloidal solution of reduced silver nanoparticles (25). Absorbance was taken from 300–700 nm. Distilled water was used as a blank sample.

FE-SEM and TEM analyses

The FE-SEM of synthesized AgNP from SL4 was conducted by the machine Sigma 300, ZEISS (26). The colloidal sample was fully dried by lyophilizer for FE-SEM analyses. For TEM (25) (JEM 1400+) analyses, a drop of lyophilized aqueous AgNPs was poured on the small copper grid coated with carbon and dried under vacuum conditions in a desiccator for 12 h. The dried TEM grid samples were loaded on a specimen holder. For the analyses of the shape, size and morphology of the synthesized AgNP, FE-SEM and TEM analyses were done.

Energy dispersive X-ray (EDX) spectroscopic

The ingredient element (Ag) of synthesized NPs was analysed using EDX spectroscopy (26) which was done by the machine Sigma 300, ZEISS, using the dried sample of the silver nanoparticle synthesized from fungal endophyte.

Fourier-transform infrared (FTIR) analyses of the silver nanoparticle

FTIR analyses was done (using the machine of Agilent Technologies) with the dried AgNP sample (25). After that, it was measured in the 650–4000 cm^{-1} range using an automated recording infrared spectrophotometer. It was done to identify the major vibrations involved in the reduction, capping, and stabilizing of the AgNP synthesized from the fungi *Colletotrichum* sp. It was recorded to identify the involved functional groups in the process of synthesis of the AgNP.

Antimicrobial activity of the nano-particle synthesized from fungal endophyte

The silver nanoparticles synthesized from endophytic fungal strain SL4 were examined for antibacterial activity. The indicator bacterial strain was Gram-positive (*Bacillus subtilis* MTCC 121) bacteria. The primary analyses of antibacterial activity was done following the agar well diffusion method (27). The Petri plates were filled with nutrient agar (NA) medium. These plates were inoculated with 0.2 mL of the bacterial suspension and spread uniformly with the help of a spreader. The AgNP (concentrations of 0, 50, 100, 150, 200 µg/mL) was synthesized from endophytic fungal isolate (SL4) and then placed in wells (formed by using a sterile cork borer) on NA media previously seeded with test organisms. The bacterial inoculated plates were parafilm sealed and incubated at 30°C for 48 h. After incubation, the presence of inhibition zones positively proved the antibacterial activity of the AgNP. After selecting the highest inhibition zone forming concentration of AgNP, further antibacterial activities were checked against some other pathogenic bacterial strains (*B. subtilis* MTCC 121, *Escherichia coli* MTCC 1667, *Listeria monocytogenes* MTCC 657, *Pantoea ananatis* MTCC 2307, *Salmonella typhimurium* MTCC 99, *Staphylococcus aureus* MTCC 96 and *P. aeruginosa* MTCC 741). For negative control, distilled water and for positive control, the antibiotic streptomycin was used. All were done in triplicates. Average values were taken for results. Inhibition zones were calculated using Equation 1.

$$\text{Inhibition zone} = \pi (R1 - R2)(R1 + R2) \quad \text{.....}$$

(Eqn. 1)

Where, R1 = Radius of well + inhibition zone; R2 = Radius of well

Antioxidant activity of synthesized silver nanoparticle

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (28), hydrogen peroxide scavenging activity (29), hydroxyl radical (OH^-) scavenging activity (29) and superoxide (O^-_2) radical scavenging activity (29) of synthesized nanoparticles were checked to ascorbic acid. Different concentration gradients (50, 75, 100, 125 and 150 µg/mL) of AgNP were used to check its competency and measured through the UV-Vis spectroscopic method. The percentage of the activity of these enzymes was expressed using Equation 2 (28, 29).

$$\% \text{ of Antioxidant enzyme scavanging activity} = \frac{(Ec - Es)}{Ec} \times 100$$

……..(Eqn. 2)

Where, the absorbance of the control is presented by Ec and the absorption of AgNPs/Ascorbic acid is presented by Es.

Plant growth-promoting traits of silver nanoparticles synthesized from fungal endophyte

The role of the biosynthesized AgNP on the growth of *P. zeylanica* was assessed by following the previously described method with slight modification (30). Seeds of *P. zeylanica* were disinfected with 4% sodium hypochlorite for 15 min and washed with distilled sterile water three times. Sterilized seeds were incubated in a growth chamber in Petri plates on sterilized moist blotting paper [RH 70–80%, temp 27±2°C, illumination 270 mE/m^2/s (for 96 h)]. After that, seeds were incubated with synthesized AgNP (0, 20, 40 and 60 mg/L conc.) at 27±2°C for 12 h. The control seeds were treated with distilled water. The germinated seeds were grown on pots in aseptical conditions. Selected pots were 30 cm in diameter and 15 cm in depth. The soil of the Burdwan district was collected, crushed, air -dried and sieved through a 2 mm sieve. Crushed soil was mixed with sterilized sand at a 3:1 ratio. All pots were sterilized in an autoclave, followed by the application of 10% formaldehyde solution. After sterilization, all pots were thoroughly washed with sterilized autoclaved water. All experiments were done in triplicate sets. Seed germination and seedling survival percentage were measured and calculated by Equations 3 and 4.

$$\text{Germination percentage } (\%) = \frac{\text{Total no.of seed germinated}}{\text{Total no of seeds in all replicates}} \times 100$$

……..(Eqn. 3)

$$\text{Seedling survival } (\%) = \frac{\text{Total no. of seedling survived after 20 days of treatment}}{\text{Total no. of seeds in all replicates}} \times 100$$

.....(Eqn. 4)

After the selection of the concentration of the synthesized silver nanoparticles for plant growth promotion, two sets of plants were prepared. After 35 days of sowing, 40 mg/L AgNP were sprayed on a T2 set of plants.

T1 = Control set

T2 = Plants treated with 40 mg/L AgNP synthesized from SL4 endophyte

Samples were taken after 60, 120 and 180 days after sowing to analyze the plant performance in terms of growth parameters and photosynthetic pigments.

Estimation of chlorophyll (chlorophyll a, chlorophyll b and total chlorophyll)

Total chlorophyll estimation of seedlings was done following the acetone extraction method of Arnon (31). 3 g of fresh leaves from each set were taken and cut into minute pieces. The pieces were homogenized with acetone for 30 min. Then the absorbance of the samples was estimated at 663 nm and 645 nm. The chlorophyll content of the plant sets was studied following the Equations 5, 6 and 7.

$$\text{Chl a (mg/gFW)} = (12.7\,A663 - 2.69\,A645) \times \text{total volume of filtrate}/1000 \times \text{tissue weight}$$

.....(Eqn. 5)

$$\text{Chl b (mg/gFW)} = (22.9\,A645 - 4.68\,A663) \times \text{total volume of filtrate}/1000 \times \text{tissue weight}$$

.....(Eqn. 6)

$$\text{Total chl (mg/gFW)} = (20.2\,A645 + 8.02\,A663) \times \text{total volume of filtrate}/1000 \times \text{tissue weight}$$

.....(Eqn. 7)

Estimation of growth parameters (shoots and roots)

Shoot growth parameters like shoot length (cm), shoot fresh weight and dry weight (g), root length (cm), root fresh weight and dry weight (g) were measured for each set (T1, T2) on 60, 120 and 180 days of germinating plants (23).

Estimation of total soluble sugar, total soluble protein, total indole acetic acid

Total soluble sugar estimation was done following the method of (23) by using an anthron reagent. Plant samples (1 g leaf) of each set were taken and homogenized in a mortar pestle and then centrifuged at 10000 RPM for 10 min. Then, 1 mL supernatant of these centrifuged samples was mixed with 1 mL 5% phenol solution and after that 5 mL of concentrated H_2SO_4 was added to these mixtures separately. The reaction mixtures were left for 20-30 min. Finally, absorbance was taken with respect to the blank set at 490 nm of wavelength.

Total soluble protein estimation was done by using the method of Bradford (32). Coomassie brilliant blue reagent was used for this method. 100 mg of the leaf sample of each set was dropped in a boiling water bath after mixing with 10 mL of 80% ethanol for one min. After that, cooling was done at room temperature. The mixture was homogenized with 80% ethanol using a pestle and mortar. The homogenate was centrifuged at 6000 rpm for 10 min. The residue was re-extracted with 10 mL of 5% perchloric acid. Re-extraction was done with this residue after mixing with 5 mL of 1 N NaOH and was kept in warm water for 20–30 min at 40–50°C to dissolve. After 30 min the mixture was centrifuged at 6000 rpm for 10 min. For protein sources, this supernatant was stored in cool conditions.

About 0.7 mL of distilled water was added with 0.3 mL of this extract and 5 mL of coomassie brilliant blue G-250 dye was added to this reaction mixture. This reaction mixture was shaken in a vortex at room temperature. Optical density (OD) was measured at 595 nm. The blank was prepared with 5 mL of dye in 1 mL of distilled water. The protein content of the samples was calculated against a standard curve of bovine serum albumin.

For estimation of total soluble indole acetic acid (IAA), 1 g of plant leaf sample from each set of plants was homogenized in 10 mL of ice-cold 50 mM sodium phosphate buffer (pH 6.8±0.2) containing 0.1 mM ethylenediamine tetra-acetic acid (EDTA), 0.1 mM phenyl methane sulfonyl fluoride (PMSF) and 1% polyvinyl poly-pyrrolidone phosphate (PVPP). This mixture was centrifuged at 10000 rpm for 15 min. 2 mL of supernatant from each sample was mixed with 3–4 drops of ortho-phosphoric acid. The mixture was added to 2 mL of freshly prepared Salkowsky reagent (1% 0.5 M $FeCl_3$ in 35% perchloric acid). These reaction mixtures were kept in dark condition for 20 min. Transformation of these reaction mixtures into reddish pink colour proved the production of IAA. The absorbance of these reaction mixtures was taken at 533 nm (33).

Results

P. zeylanica (Fig. 1a and b) was identified from the departmental herbarium library and then a separate herbarium was deposited in the library of the Department of Botany, Burdwan University. The deposition number or the accession number is BURD 12230, presented in Fig. 2. From the leaf sample, several fungal endophyte strains were obtained and selected one (SL4) based on their plant growth-promoting traits like IAA production, phosphate solubilization, ammonia production, etc. SL4 showed the best potency for these purposes and was identified by its morphological characteristics, microscopic characteristics and 18S rDNA sequencing.

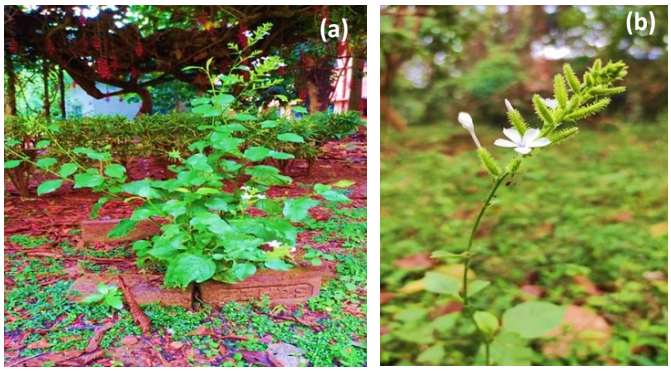

Fig. 1. Images of the plant *P. zeylanica* (**a)** the plant body, and (**b)** the flowering part of the plant.

Identification of isolated endophyte

Morphological characteristics

Colony morphology was recorded on the 4th day and found that colonies were 80 mm in diameter, margin regular and aerial mycelium was whitish grey and short; in contrast, the reverse was light yellow with black spots presented in Fig. 3.

Fig. 2. Herbarium sheet of our collected specimen of *P. zeylanica* with accession number.

Fig. 3. Colony morphology of SL4.

Microscopic characteristics

A microscopic study found that the colour of the hyphae is hyaline to brown, long and branched. Appressoria was found abundantly. They were irregular-shaped and the colour was medium brown to pale. Cells that formed conidia were found hyaline and clavate to cylindrical in shape. Conidia transformed into orange masses after 10 days. Conidia were cylindrical; both ends were round, aseptate, hyaline, straight and guttulate. The size of the conidia was 9.51 - 13.77 (11.63) × 2.94 - 4.72 (3.90) µm. Perithecial Ascoma was found with an ostiolate. Ascoma was dark brown and obpyriform to sub-globose in shape. In measurement, Ascoma was 771 - 759 µm in height and 609 - 729 µm in width. Ascospores were aseptate, but at maturity, 1 septa was formed which was median. Asco-

spores were cylindrical to fusoid in shape, hyaline in colour, straight to slightly curved, rounded at both ends, aseptate, 1-septate at maturity, septum median, 9.05–14.82 (12.28) × 3.01–4.15 (3.61) µm (Fig. 4).

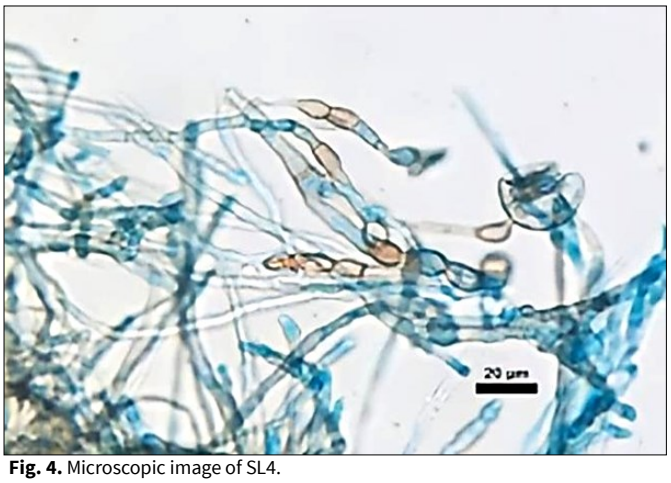

Fig. 4. Microscopic image of SL4.

18S rDNA sequencing and MALDI-TOF analyses

In 18S rDNA sequencing, SL4 showed similarity with three strains of *Colletotrichum* sp., with an overall 98–99% similarity (Table 1). A 1254 base pair long 18S rDNA sequence was used to construct a phylogenetic tree using MEGA 7 software. The tree showed SL4 forming a clade with *Colletotrichum* sp. B11 (FJ517761.1), confirming its genus identity presented in Fig. 5(a). Strain SL4 was deposited in NCMR, Pune and the accession number was MCC 9247. MALDI-TOF analyses of ribosomal proteins revealed that strain SL4 is classified within the genus *Colletotrichum* sp., as shown in Fig. 5(b). The log value exceeding 1.7 confirmed its identification at the genus level as *Colletotrichum* sp.

Table 1. Identification of SL4 based on 18S rDNA sequencing MALDI-TOF analyses.

Molecular identification by 18S rDNA		
Strain No.	**Closest Neighbour***	**% Similarity**
SL4	*Colletotrichum salicis* RB157	98.96
	Colletotrichum lupini RB173	98.57
	Colletotrichum simmondsii RB179	98.57

MALDI-TOF analyses of ribosomal protein			
Identification of the strain (best match)	**MALDI score of the strain (best match)**	**Identification of the strain (second match)**	**MALDI score of the strain (second match)**
Colletotrichum lupini RB173	1.723	*Colletotrichum lupini* RB173	1.711

Characterization of silver nanoparticle

The silver nanoparticles were synthesized by using the extraction of the fungal endophyte SL4 and were characterized using different methods. As the colour of the solution started to change from yellow to brown after 96 h of dark condition, absorbance in the UV-vis spectrophotometer was measured from the 4th day and it was maximum on the 12th day. Absorbance was measured continuously in 2

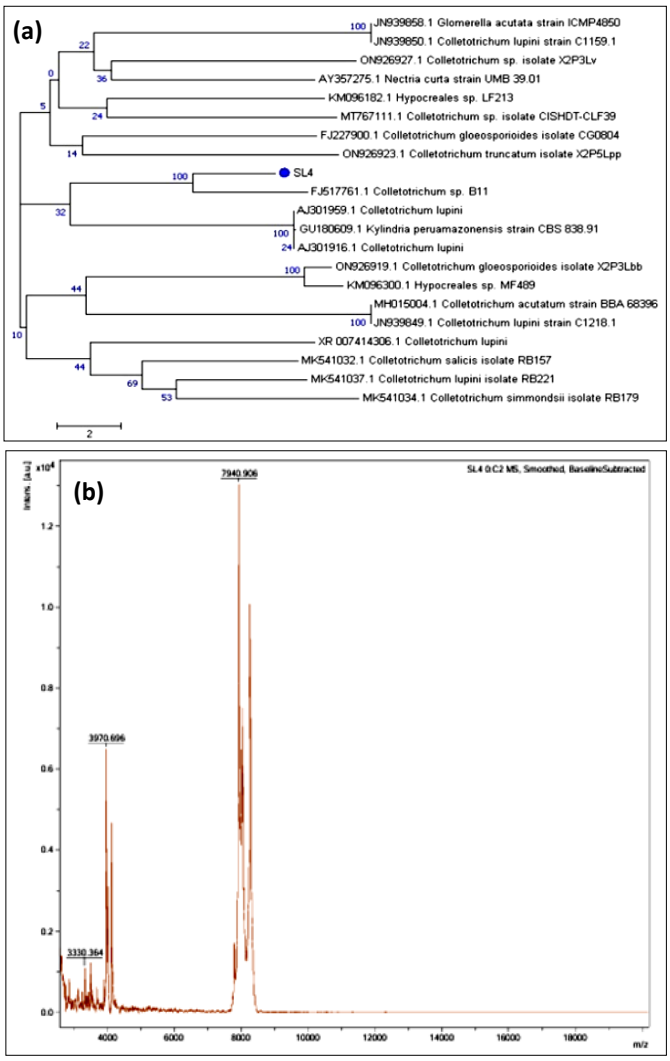

Fig. 5. (**a**) Phylogenetic tree of SL4 showing the position of *Colletotrichum* sp. (**b**) MALDI-TOF spectra analysis of the ribosomal protein of SL4.

days intervals and the value was highest on the 12th day. The absorbance peak was near 425 nm presented in Fig. 6. Colour was unchanged for the control solution. In FE-SEM (Fig. 7) and TEM (Fig. 8) analyses, it was proved that the synthesized nanoparticles were spherical in shape and size varied between 10–30 nm. Elemental silver was present in the nanoparticles, which was proved from EDX (Fig. 9) analysis, where the silver peak was found at 3 keV due to localized surface plasmon resonance. Using IR spectrophotometer equipment, FTIR analyses was done to determine the presence of functional groups, which is presented in Fig 10. The functional groups were present with

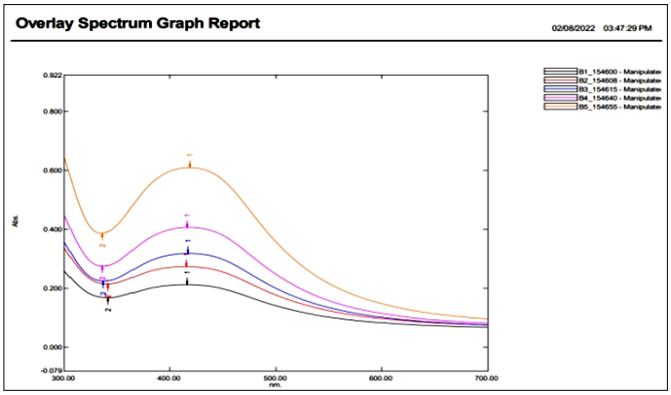

Fig. 6. UV-vis spectroscopic image of AgNP synthesized from endophytic fungal strain SL4. B1, B2, B3, B4, B5 denotes day 4, 6, 8, 10, 12 of absorption, respectively.

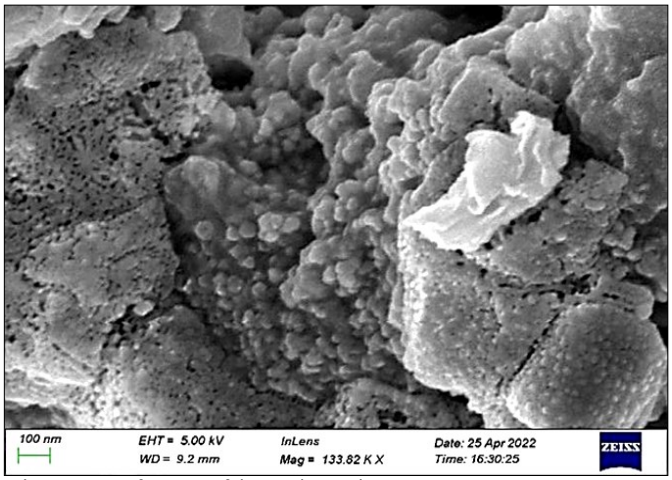

Fig. 7. Image of FE-SEM of the synthesized AgNP.

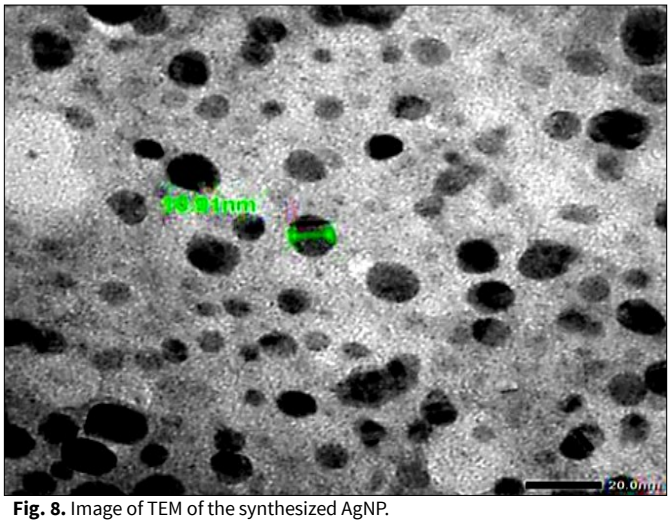

Fig. 8. Image of TEM of the synthesized AgNP.

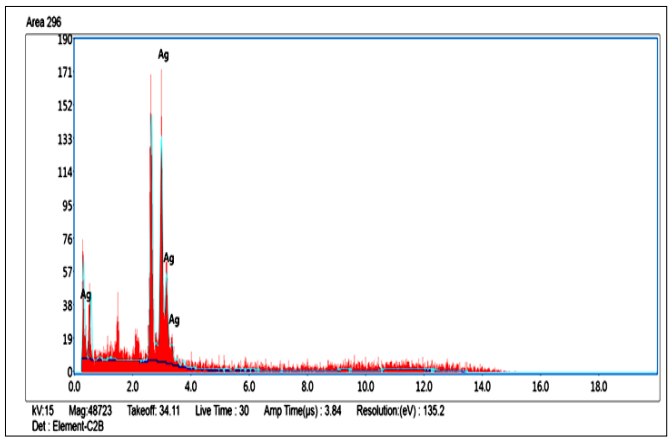

Fig. 9. EDX spectroscopic image of synthesized AgNP.

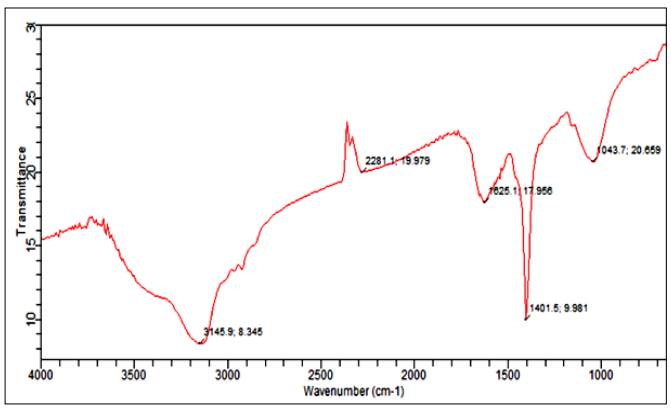

Fig. 10. FTIR analyses of synthesized AgNP.

absorbances of 8.345, 19.979, 17.956, 9.981 and 20.659 in the peak at 3145.9, 2281.1, 1625.1, 1401.5 and 1043.7 cm^{-1}, respectively. The absorbance peaks were assigned the functional groups of O-H stretching of alcohol, N=C=O stretching of isocyanate, C=C stretching of alkene, S=O stretching of sulphate, CO-O-CO stretching of anhydride and N-H stretching of amine.

Antibacterial activity and antioxidant activity of silver nanoparticles

In antibacterial activity, the AgNP showed the best potency against *B. subtilis* MTCC 121 in 200 µg/mL concentration, which is shown in Fig. 11. Synthesized AgNP also showed good potency against all the other pathogenic bacterial strains mentioned so far (Fig. 12, 13), but it was highest against the case of *P. aeruginosa* MTCC 741. This AgNP also proved its competency in antioxidant properties when ascorbic acid worked as standard. 150 µg/mL concentration of AgNP showed the highest competency for antioxidant properties. In the case of DPPH, H_2O_2 and O^{2-} scavenging activity, this synthesized AgNP showed less competency, but in the case of OH^- scavenging activity, AgNP showed better competency than ascorbic acid (Fig. 14).

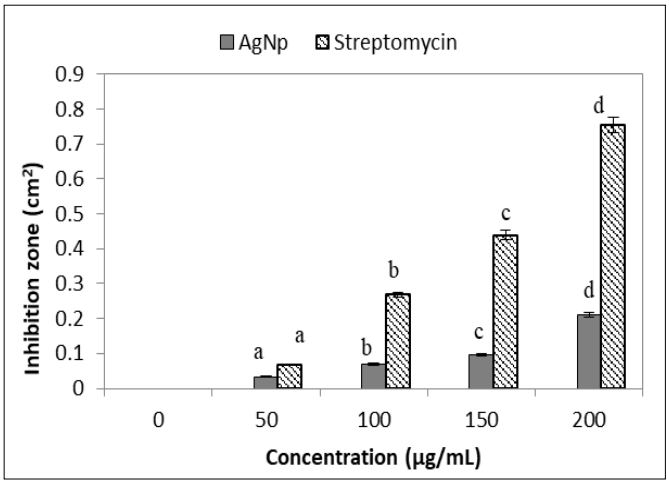

Fig. 11. Antibacterial activity of AgNP against *B. subtilis* MTCC 121 in different concentration.

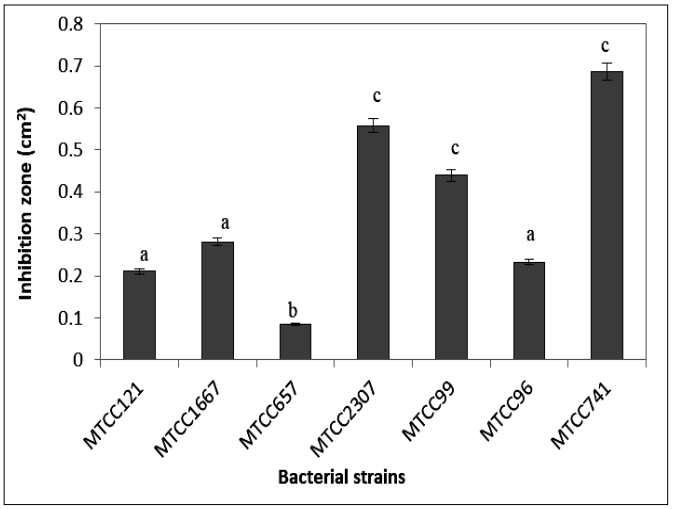

Fig. 12. Antibacterial activity of synthesized AgNP against different pathogenic bacterial strains *B. subtilis* MTCC 121, *E. coli* MTCC 1667, *L. monocytogenes* MTCC 657, *P. ananatis* MTCC 2307, *S. typhimurium* MTCC 99, *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 741.

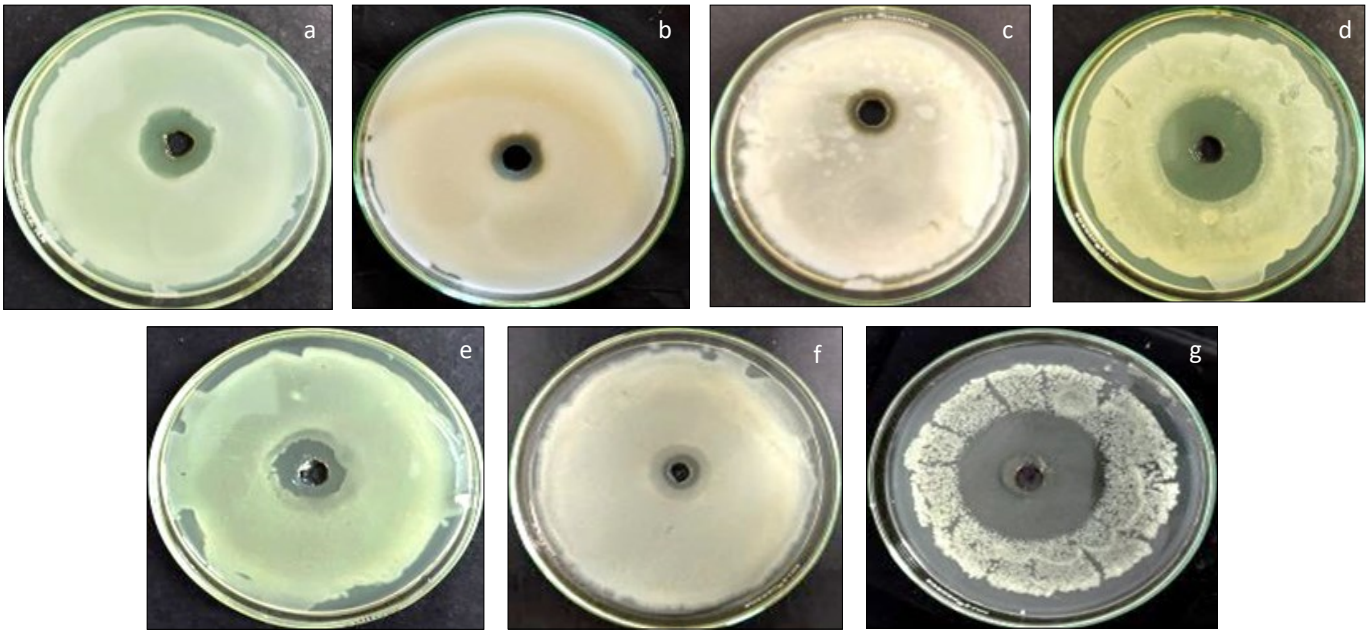

Fig. 13. Inhibition zones in antibacterial activity of AgNP against different pathogenic bacterial strains: **(a)** *B. subtilis* MTCC 121, **(b)** *E. coli* MTCC 1667, **(c)** *L. monocytogenes* MTCC 657, **(d)** *P. ananatis* MTCC 2307, **(e)**, *S. typhimurium* MTCC 99, **(f)**, *S. aureus* MTCC 96, and **(g)** *P. aeruginosa* MTCC 741.

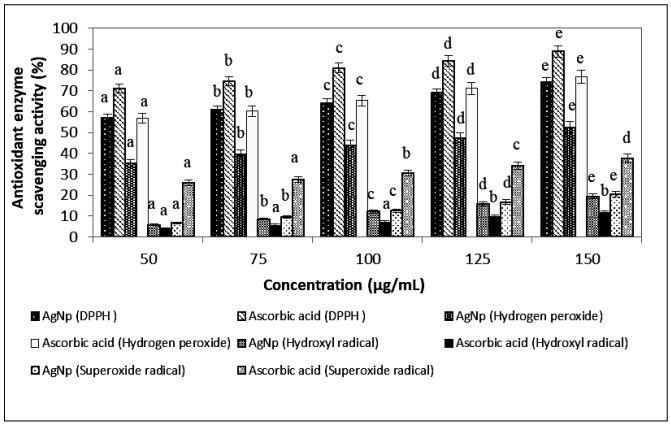

Fig. 14. Antioxidant activity of synthesized AgNP.

Plant growth promoting activity of silver nanoparticle

In plant growth promotion, germination percentage and seedling survival percentage of the *P. zeylanica* were highest in 40 mg/L concentration of the AgNP presented in Fig. 15. In most of the growth parameters, like chlorophyll content, shoot growth parameters, the plant set (T2) treated with synthesized AgNP, showed better result than the control set (T1) in 120 days of germinating plants and after that, these growth parameters started decreasing (Fig. 16, 17). In the case of root growth parameters, the T2 set showed better results than the T1 set in 180 days of germinating plants shown in Fig. 18. In some other growth parameters like total sugar, total protein and total IAA content, the T2 set showed better potency than T1 set in 120 days of germinating plants and in 180 days all these parameters began declining outcomes (Fig. 19, 20).

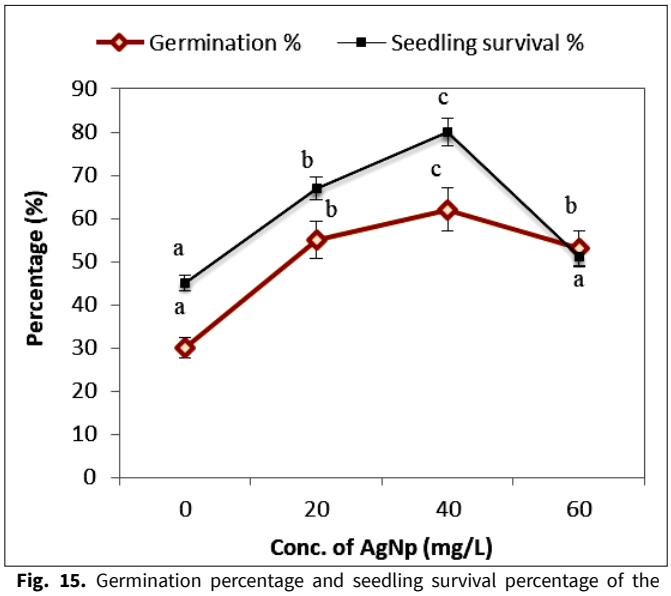

Fig. 15. Germination percentage and seedling survival percentage of the seed *P. zeylanica* using different concentrations of AgNP.

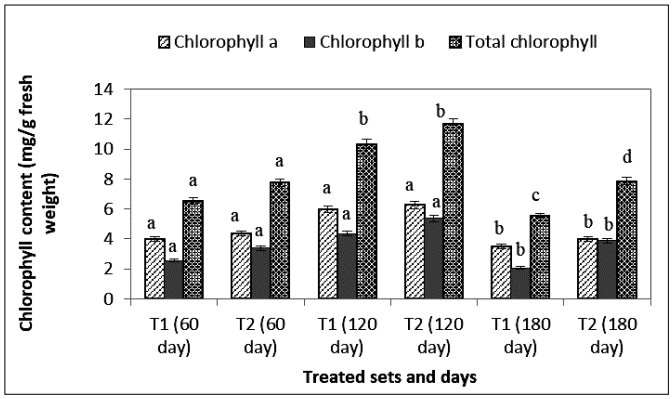

Fig. 16. Chlorophyll content of all the sets in 60 days, 120 days and 180 days seedling.

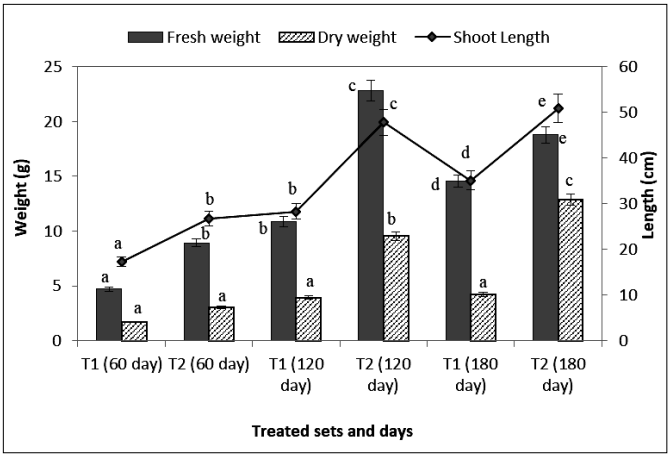

Fig. 17. Shoot growth parameters of all the sets in 60 days, 120 days and 180 days seedling.

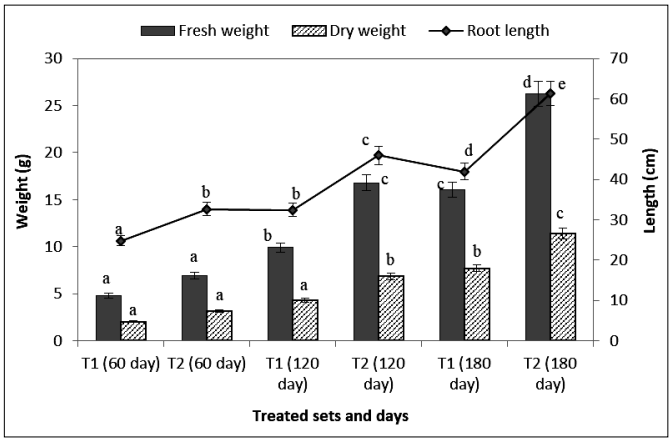

Fig. 18. Root growth parameters of all the sets in 60 days, 120 days and 180 days seedling.

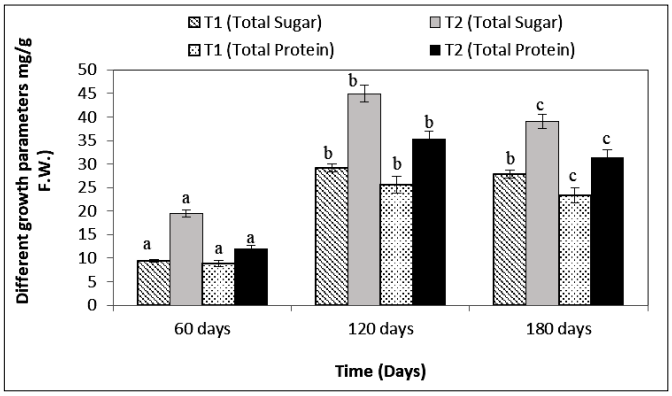

Fig. 19. Primary metabolite content (total sugar, total protein) of all the sets in 60 days, 120 days and 180 days seedling.

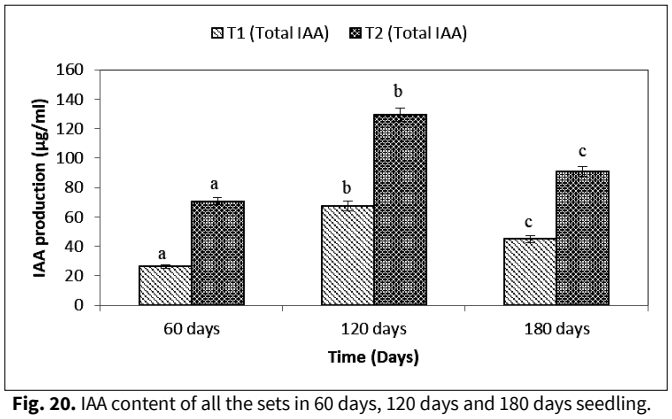

Fig. 20. IAA content of all the sets in 60 days, 120 days and 180 days seedling.

Discussion

P. zeylanica is a very important ethno-medicinal plant having tremendous medicinal properties (19). In the current work, the synthesis of AgNPs is described utilizing the extraction of fungal endophyte *Colletotrichum* sp. that was isolated from *P. zeylanica*. The fungal endophyte *Colletotrichum* sp. was isolated from leaf samples of the *P. zeylanica* and was identified by microscopic study, MALDI-TOF analyses and 18S rDNA sequencing methods. Previous studies proved that *Colletotrichum* sp., which are endophytes and have roles like antimicrobial activities, generally are non-pathogenic and help in plant growth promotion (34, 35). Under phosphate-deficient circumstances, the endophytic fungus *Colletotrichum* sp. colonizes the roots of *Arabidopsis* plants and transmits the macronutrient phosphorus to its host and this process increases plant growth and fertility (34). *Colletotrichum siamense*, an endophytic fungus, boosted the biomass of tomato plants (35).

The green method of synthesizing AgNPs is attractive because of the use of non-toxic chemicals, low cost, environmental friendliness and suitability for pharmaceutical and biological applications (1). It is known from the previous study that nanoparticles can be created extracellularly at a nanometric scale where the extracted enzymes from the fungal filtrate decrease silver ions and synthesize elemental silver (Ag^0) (4). Here, AgNP was synthesized by using the extraction of the isolated fungal endophyte *Colletotrichum* sp. The extract of the endophytic fungi altered the colour of the solution from bright yellow to dark brown when it was dissolved in silver nitrate solution. The solution's dark brown hue demonstrated that silver nitrates had been converted into silver nanoparticles. UV-visible spectroscopy was done to detect surface plasmon resonance bands reflecting modification of the filtrate's colour (36). Furthermore, the UV-vis spectrophotometer (300–700 nm) indicated the creation of silver nanoparticles from silver nitrate. The creation of silver nanoparticles was verified by the absorbance peak at 425 nm, which was seen because of localized surface plasmon resonance. The bands in UV-vis exhibit a range of absorbance wavelengths between 400 and 450 nm, with the presence of bigger nanoparticles indicated by an absorbance peak at a longer wavelength (29). From the TEM and FE-SEM studies, the shape of the synthesized silver nanoparticle was found spherical and the size varied between 10 to 30 nm. From the previous study, it was found that the TEM study revealed the size of the biologically (especially from fungus) synthesized silver nanoparticle ranges from 1–30 nm with spherical shape (25, 37). From the previous SEM study, maximum size of the synthesized silver nanoparticle from the biological approach was found to be 30 nm (38). The existence of metallic silver in silver nanoparticles biosynthesized with extracts of endophytic fungus in EDX analysis was demonstrated by a significant Ag signal at 3 keV. *Talaromyces* sp. reduced silver nanoparticles having an optical absorption characteristic peak at 3 keV were analyzed using EDX (39). Synthesized AgNP's FTIR measurements showed distinct absorption peaks that corresponded to several phytochemical functional groups of O-H stretching of alcohol, N=C=O stretching of isocyanate, C=C stretching of alkene, S=O stretching of sulphate, N-H stretching of amine and CO-O-CO stretching of anhydride.

The antibacterial activity of synthesized AgNP was checked by measuring the inhibition zones against some pathogenic bacterial strains. Among all the pathogenic strains, this AgNP showed the highest antibacterial activity at 200 µg/mL concentration against *P. aeruginosa* MTCC 741 by creating the largest inhibition zone. Silver nanoparticles synthesized from the endophytic fungus *Talaromyces purpureogenus* isolated from *Taxus baccata* showed antibacterial activity against pathogenic bacterial strains, which were gram-positive and gram-negative bacteria. DPPH, H_2O_2, OH^- and O^{2-} scavenging activity was tested to assess the antioxidant capability of the synthesized AgNPs

with respect to ascorbic acid. This synthesized AgNP showed the highest competency in the case of OH^- scavenging activity. Silver nanoparticles synthesized from the endophytic fungus *T. purpureogenus* isolated from *T. baccata* also showed antioxidant activity, especially for DPPH (39). As the synthesized AgNP has both antibacterial and antioxidant activities, it may be applied for disease control and stress release of plants in agriculture fields. Few studies have been conducted to assess the potential of biogenically synthesized silver nanoparticles for the management of pests and phytopathogenic microbes in the agriculture field. Silver nanoparticles made from several endophytic fungus species have been shown to have use in agriculture and pest management as they have the capability of antibacterial and antioxidant activities (4). Of having antibacterial and antioxidant properties, this synthesized nanoparticle may take a useful role in medical purposes. The unique producer of variously shaped silver nanoparticles with potent antibacterial and antioxidant properties from the isolated endophytic fungus *T. purpureogenus*, showed potency for biomedical and medicine-related industrial applications (39).

AgNP (40 mg/L) helped in growth promotion like shoot growth, root growth, chlorophyll content, germination percentage, seedling survival percentage, etc., in the plant *P. zeylanica*. Based on the application of silver nanoparticles, growth parameters like shoot, root growth parameters increased in the plants, *Vigna sinensis* and *Brassica juncea* (40,41). AgNPs can greatly enhance plant height, fresh and dry weight and chlorophyll concentrations in wheat (*Triticum aestivum*) when compared to the control (41). By applying this AgNP in 40 mg/L concentration, total protein, total sugar and total IAA content increased in the *P. zeylanica*. When the growth and chemical characteristics of *T. aestivum* were examined in relation to biosynthesized AgNPs, it was discovered that there was a considerable increase in shoot length, fresh and dry weight, chlorophyll, carbohydrate and protein contents. *Phaseolus vulgaris* showed a considerable improvement in growth parameters and physiological response when AgNPs were applied (42, 43). The studied silver nanoparticles synthesized from *Colletotrichum* sp. may be used as nanopesticides and nanofertilizers in the agriculture fields as they have growth-promoting properties, antibacterial properties, as well as antioxidant properties. AgNPs are essential to agriculture since they have several uses that are critical to raising agricultural yield. Additionally, they function as nanopesticides, giving the target plants a suitable dosage without releasing extra pesticides into the environment (44, 45). By releasing nutrients to the plants gradually, nanofertilizers help to avoid excessive nutrient loss. AgNPs are a great tool for safely controlling pests since they are used for non-toxic, efficient pest management (44). As the particles have a higher surface area than traditional ones, nanomaterials can aid in this situation by allowing the agrochemical to release gradually. In addition to its numerous special qualities, silver nanoparticles may be employed as nanofertilizers by helping plants absorb more nutrients more efficiently (46, 47).

Conclusion

In conclusion, it can be stated that the endophytic fungus *Colletotrichum* sp. from the plant *P. zeylanica* is efficient in synthesizing silver nanoparticles in the presence of $AgNO_3$ solution in the green synthesis process. Since this synthesized silver nanoparticle has both antibacterial and antioxidant activity, this synthesized AgNP can be used in the pharmaceutical industry and for plant protection in agricultural fields under various biotic and abiotic stresses. The plant, *P. zeylanica*, exhibited significant growth promotion in response to this synthesized silver nanoparticle. Thus, it can be utilized as environmental friendly nanofertilizers in agricultural fields instead of dangerous chemical fertilizers. After taking all of these into account, it can be said that this specific AgNP can be utilized in the pharmaceutical industries as well as in agricultural industries as nanofertilizers and nanopesticides in place of chemical fertilizers and chemical pesticides.

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Authors' contributions

Every author has contributed. The first author SR and the corresponding author SD have framed the research work. SR wrote the manuscript and completed the most laboratory works with the help of the second author BM and the third author NP. The graphic figures were created by the second author BM. The manuscript was arranged by the third author NP, while the corresponding author SD supervised and reviewed every work. Statistical analyses, data checking and evaluation was done by SR, BM and NP. The manuscript was approved by every author.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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