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Original Article

Ultrastructures of silver nanoparticles biosynthesized using endophytic fungi

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ABSTRACT

Three endophytic fungi *Aspergillus tamarii* PFL2, *Aspergillus niger* PFR6 and *Penicllium ochrochloron* PFR8 isolated from an ethno-medicinal plant *Potentilla fulgens* L. were used for the biosynthesis of silver nanoparticles. Scanning and transmission electron microscopic analysis were performed to study the structural morphology of the biosynthesized silver nanoparticles. The electron microscopy study revealed the formation of spherical nano-sized silver particles with different sizes. The nanoparticles synthesized using the fungus *A. tamarii* PFL2 was found to have the smallest average particle size (3.5 ± 3 nm) as compared to the nanoparticles biosynthesized using other two fungi *A. niger* PFR6 and *P. ochrochloron* PFR8 which produced average particle sizes of 8.7 ± 6 nm and 7.7 ± 4.3 nm, respectively. The energy dispersive X-ray spectroscopy (EDS) technique in conjunction with scanning electron microscopy was used for the elemental analysis of the nanoparticles. The selected area diffraction pattern recorded from single particle in the aggregates of nanoparticles revealed that the silver particles are crystalline in nature.

1. Introduction

Nanoparticles are the clusters of atoms in the size range of 1–100 nm. In this size range, materials often develop useful attributes that are distinct from the properties of the bulk material. Metal particles in the nanometre size exhibit unique physical properties that are different from both the ion and the bulk material. Their uniqueness arises specifically from higher surface to volume ratio which results in increased catalytic activity due to morphologies with highly active facets; hence, the nanosize materials are more advantageous than their bulk materials. The enhanced reactivity of nanomaterials can also be attributed to their large number of edges, corners, and high-energy surface defects [1–3]. They have tremendous applications in the area of catalysis, opto-electronics, diagnostic biological probes, display devices and photo electrochemical applications due to their unique size-dependent optical, electrical and magnetic properties [4–9].

There has been a rapid increase in microbes that are resistant to conventionally used antibiotics [10]. This has resulted in an inevitable and urgent need for development of novel antimicrobial agents. It has been known since ancient times that silver and its compounds are effective antimicrobial agents [11–13]. Compared with other metals, silver exhibits higher toxicity to broad spectrum of microorganisms while it exhibits lower toxicity to mammalian cells [14]. Silver ion has been known to be effective against a broad range of microorganisms including antibiotic-resistant strains [15]. Silver nanoparticles with higher surface to volume ratio compared to common metallic silver have shown better antimicrobial activity.

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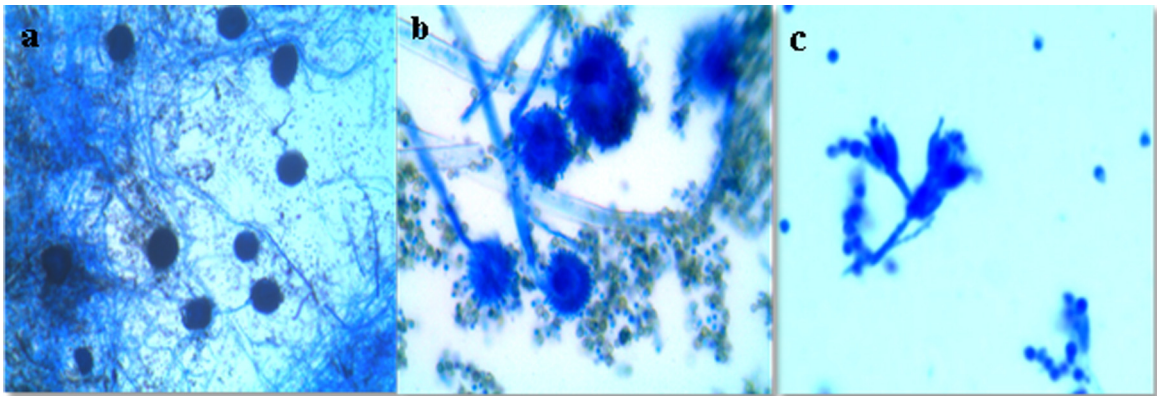

Fig. 1. Reproductive structures of the endophytic fungal isolates as seen under compound microscope: (a) PFL2, (b) PFR6 and (c) PFR8.

Most importantly silver nanoparticles are also non-toxic to the mammalian cells at low concentrations [16]. Silver nanoparticles have been known for a long time but have not been given due attention [17]. Nanoparticles can disturb functions of cell membranes such as permeability and respiration. The silver nanoparticles get attached to the cell membrane and also penetrate inside the bacteria. Inside the bacterial cells silver nanoparticles can disturb the functions of sulfur-containing proteins and phosphorus-containing compounds such as DNA by effectively reacting with them leading to the inhibition of enzyme functions [18,19]. The nanoparticles bind to proteins and DNA and damage them by inhibiting replication. Thus the silver nanoparticles interrupt the respiratory chain and cell division leading to cell death [20–22]. In addition, complex action mechanisms of metals decrease the probability of bacteria developing resistance to them [23]. Thus, one of the promising approaches for overcoming antibiotic resistance of microorganisms is the use of silver nanoparticles.

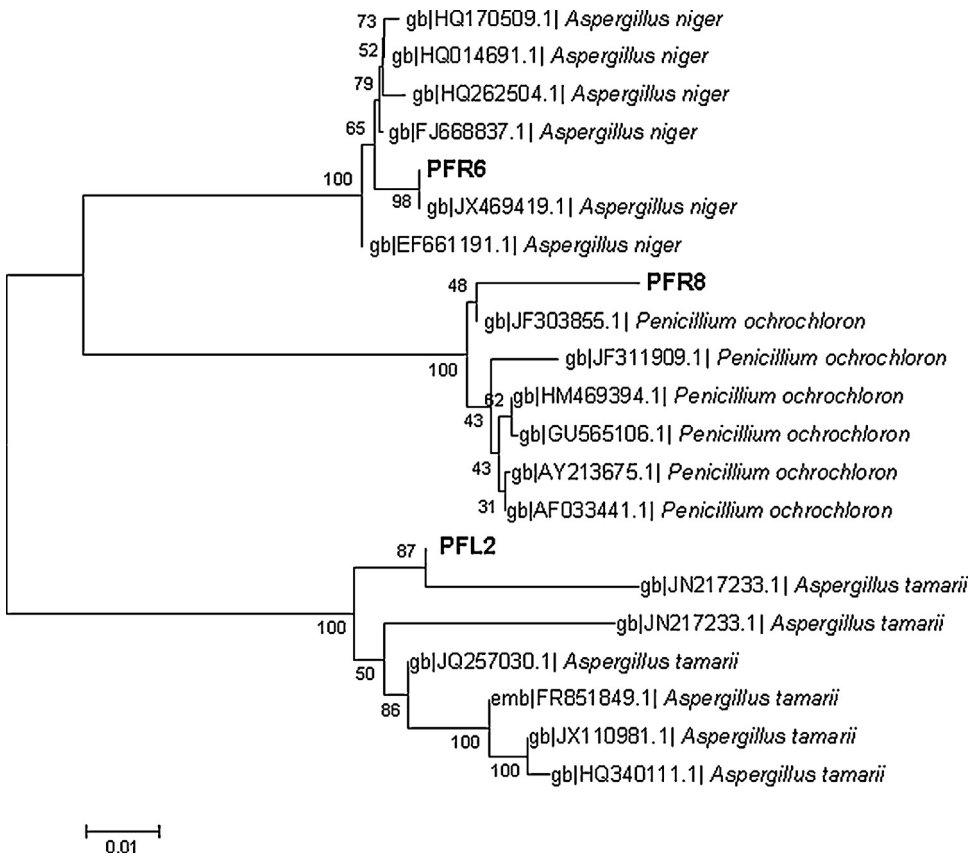

Fig. 2. Phylogenetic relationships between the three endophytic fungi and the ITS sequences of closely related fungal strains retrieved from NCBI GenBank.

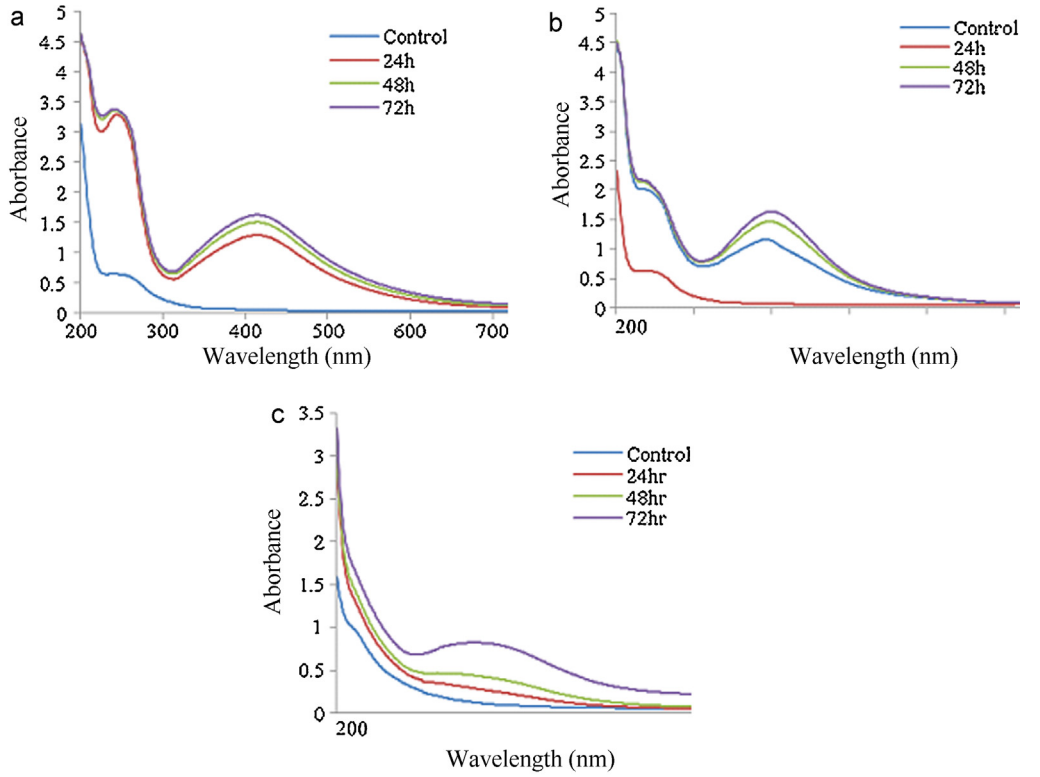

Fig. 3. UV–vis absorption spectrum at different time intervals for silver nanoparticles biosynthesized using (a) *Aspergillus niger* PFR6, (b) *Penicillium ochrochloron* PFR8 and (c) *Aspergillus tamarii* PFL2.

Owing to the fact that the endophytic fungi provide a broad variety of bioactive secondary metabolites with unique structures they could be the explored for their ability to biosynthesis of silver nanoparticles to developed an efficient environment friendly process. Few studies on the biosynthesis of silver nanoparticles using endophytic fungi like *Amylomyces rouxii, Penicillium* sp., *P. janthinellum, Pestalotia* sp., *Aspergillus clavatus, A. concius*, *Epicoccum nigrum* and *Phomopsis* sp. have shown the proficiency of the endophytic fungi to synthesize silver nanoparticles possessing antimicrobial activity [24–31]. In the present study, an attempt has been made to investigate endophytic fungi associated with an ethnomedicinal plant *Potentilla fulgens* L. for their ability to synthesize silver nanoparticles and the ultrastructure and size distribution of the biosynthesized nanoparticles were studied using electron microscopy techniques.

2. Materials and methods

2.1. Isolation and characterization of endophytic fungi

The endophytic fungi were isolated according to the method described by Strobel et al. [32] with minor modifications. The plant material was gently rinsed in running water to remove any adhering dirt. The samples were then cut into 2 mm blocks and were surface sterilized with 70% ethanol for 1 min. Then the samples were soaked in 4% sodium hypochlorite solution for 3 min, and then rinsed with 70% alcohol for 1 min. The final rinsing was done with sterile distilled water and blot dried on sterile filter paper. The excess water was dried and the surface sterilized explants were then inoculated onto PDA plates. The plates were periodically observed for fungal growth. The fungus growing out from the plant explants was then sub-cultured in PDA plates. Three morphologically distinct endophytic fungi isolated from an ethno-medicinal plant *P. fulgens* L. were used for the biosynthesis of silver nanoparticles (Fig. 1 a–c).

The fungal isolates were characterized based on the analysis of the phylogenetic relationship of the isolates using internal transcribed spacer (ITS) region gene sequences. Amplification of ITS (ITS1, 5.8S and ITS2) region using primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ [33] were performed using a 9700 Gold thermal cycler (Applied Biosystems, UK) under the following conditions: initial denaturation at 94 °C for 1 min, annealing temperature at 52 °C for 1 min and extension at 72 °C for 1 min with an initial denaturation and final extension for 5 and 10 min at 94 and 72 °C, respectively. For sequencing, the amplified ITS products were purified using QIAquick Gel Extraction Spin Kit (QIAGEN, Germany). The purified PCR products were bi-directionally sequenced using the forward and reverse primers. Sequencing of the ITS gene products were performed using the Big DyeTerminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing of ITS region of fungal rDNA resulted in approximately 564 bp long

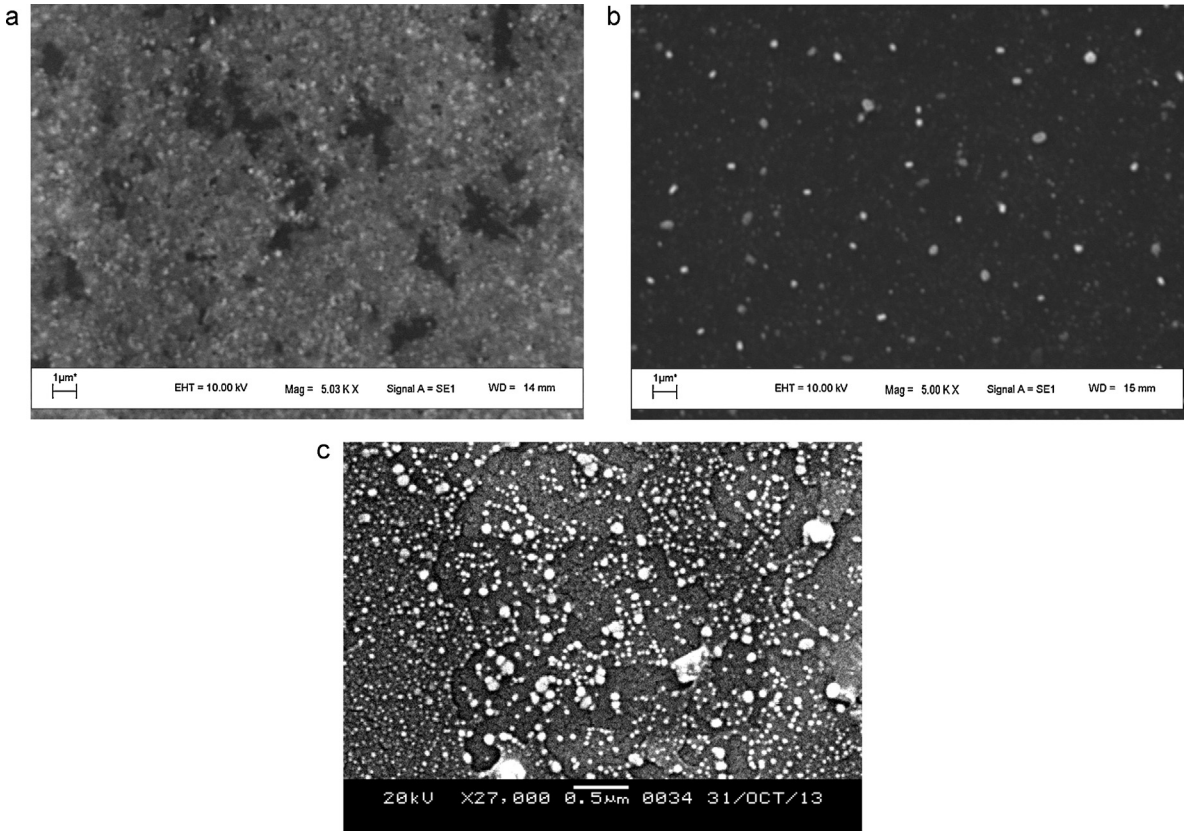

Fig. 4. SEM micrographs of the biosynthesized silver nanoparticles using endophytic fungi (a) *Aspergillus tamarii* PFL2, (b) *Aspergillus niger* PFR6 and (c) *Penicillium ochrochloron* PFR8.

nucleotide sequences. The nucleotide sequences were used for the identification of the fungal isolates. Molecular Evolutionary Genetics Analysis (MEGA version 5) software was used for phylogenetic analyses [34]. The closest homologues to the sequences were selected and the multiple sequence alignments were carried out using the ClustalW program in the MEGA 5 software. Phylogenetic tree was constructed using the neighbour joining algorithm with 1000 bootstrap replications. Neighbour-joining tree based on ITS gene sequences was constructed to show the phylogenetic relationships between fungal isolates used for biosynthesis of silver nanoparticles and the closely related strains retrieved from NCBI GenBank.

2.2. Biosynthesis of silver nanoparticles

For the synthesis of silver nanoparticles, the biomass of each endophytic fungal isolates were grown aerobically in potato dextrose broth containing infusion of 250 g potato and 20 g dextrose per litre of distilled water. The inoculated flasks were incubated on orbital shaker at $25 \pm 2\,^{\circ}C$ and agitated at 120 rpm for 96 h. The biomass was harvested after incubation by filtering through filter paper followed by repeated washing with distilled water to remove any medium component from the biomass. 10 g (wet weight) was brought in contact with 100 mL of sterilized double distilled water for 48 h at $25 \pm 2\,^{\circ}C$ in a 250 mL Erlenmeyer flask and agitated again at 120 rpm. After the incubation, the cell filtrate was obtained by filtering it through Whatman filter paper No. 1. The filtrates were treated with 1 mM silver nitrate (Sigma Aldrich) solution in an Erlenmeyer flask and incubated at room temperature in dark. Control containing cell-free filtrate without silver nitrate solution was also run as standard.

2.3. UV–visible spectroscopy analysis

Change in colour of the mycelium free filtrate incubated with 1 mM silver nitrate solution visually observed over a period of time indicates the bioreduction of silver ions to silver nanoparticles. The silver nanoparticles formed in the mycelium free fungal filtrate were monitored by sampling of aliquots (1 mL) at different time intervals. Absorption measurements were carried out on UV–visible spectrophotometer (CARY-100 BIO UV–vis spectrophotometer; Varian Inc., USA) at a resolution of 1 nm between 200 and 800 nm ranges.

2.4. Electron microscopy analysis

The morphology of the biosynthesized silver nanoparticles was studied using electron microscopy technique. In order to carry out SEM analysis, silver nanoparticles solutions were centrifuged for 20 min at 10,000 rpm and drop

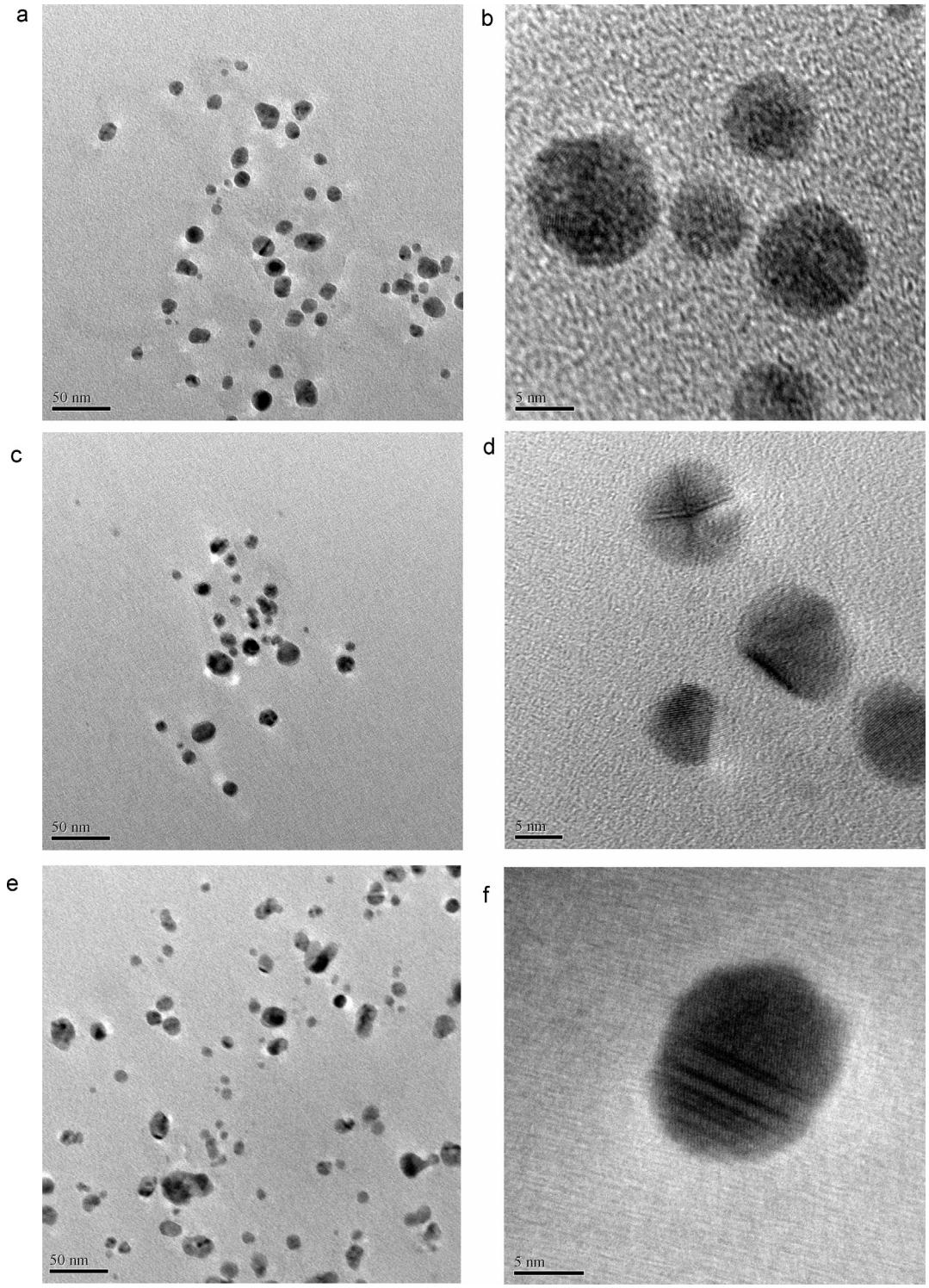

Fig. 5. TEM micrographs of the biosynthesized silver nanoparticles using endophytic fungi (a) *Aspergillus tamarii* PFL2, (c) *Aspergillus niger* PFR6 and (e) *Penicillium ochrochloron* PFR8; HrTEM micrographs of the biosynthesized silver nanoparticles using endophytic fungi (b) *Aspergillus tamarii* PFL2, (d) *Aspergillus niger* PFR6 and (f) *Penicillium ochrochloron* PFR8.

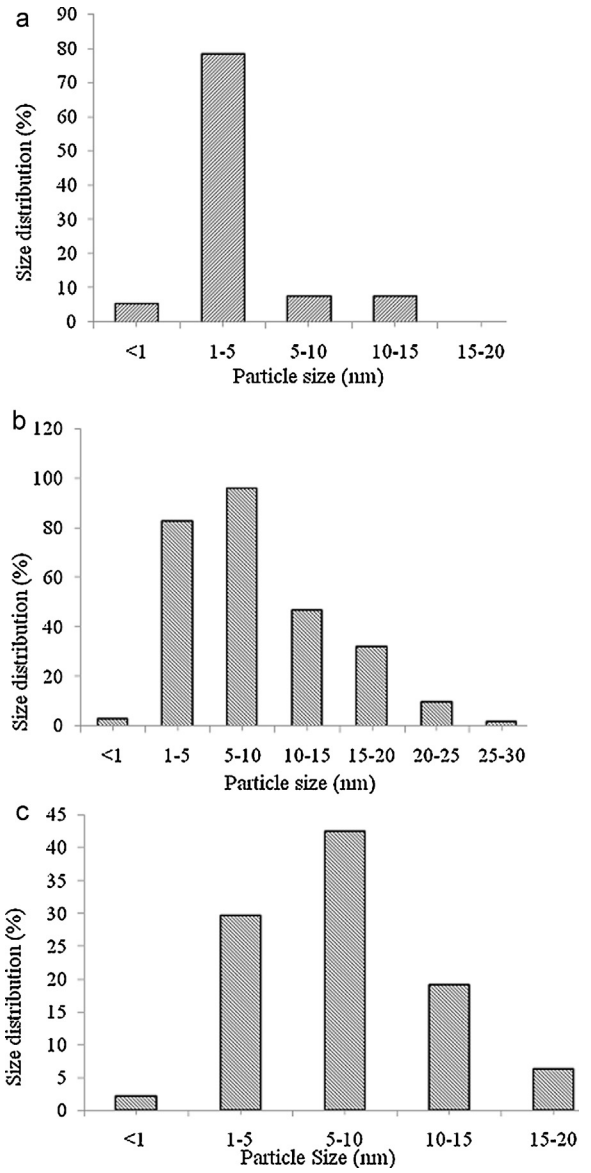

Fig. 6. Particle size distribution histogram of silver nanoparticles synthesized using (a) *Aspergillus tamarii* PFL2, (b) *Aspergillus niger* PFR6 and (c) *Penicillium ochrochloron* PFR8.

coated on to thin glass film. Samples were then examined using the Scanning Electron Microscope (JSM-6360, JEOL). Compositional analysis and the conformation of presence of elemental silver was carried out through Energy dispersive X-ray Spectroscopy (EDS) using the SEM equipped with an EDAX attachment (Oxford, London).

For TEM analysis, a drop of biosynthesized silver nanoparticles solutions was placed on the carbon coated copper grids and kept overnight under vacuum desiccation. The carbon coated copper grids were then loaded onto a specimen holder. Transmission electron micrographs of the samples were taken using the JEOL JSM 100CX TEM (Jeol, Japan) operated at an accelerating voltage of 200 kV. Selected area Electron Diffraction (SAED) of the nanoparticles was also analyzed using the Transmission Electron Microscope. Particle size distributions and average silver core diameter were calculated for each nanoparticle sample by averaging 200 particles from the TEM images using ImageJ software (National Institutes of Health, USA).

3. Results

3.1. Characterization of endophytic fungi

The reproductive structures of the fungi were filamentous with globose tip as observed under the compound microscope (Fig. 1). A phylogenetic tree was constructed using the neighbour-joining algorithm with 1000 bootstrap replications for the identification of the endophytic fungi using the sequences obtained from the molecular marker used (Fig. 2). Based on high similarity percentage (>98.7%) and clear phylogenetic clustering in the same branch, the three endophytic fungi were assigned to their respective species, i.e., *Aspergillus tamarii* PFL2, *Aspergillus niger* PFR6 and *Penicllium ochrochloron* PFR8. The ITS gene sequences of all the selected fungal isolates were submitted to NCBI GenBank and accession numbers obtained (JX469419, KF358720 and JX469420).

Silver nitrate ($AgNO_3$), upon incubation with the filtrates, turned dark brown colour, while the control flasks remained as such during the 72 h incubation period. The generation of dark brown colour is due to the surface plasmon resonance (SPR) exhibited by the nanoparticles and is typical of the silver nanoparticles. The UV–vis spectra of the mycelium free filtrates and the filtrate treated with 1 mM $AgNO_3$ at different time interval are presented in Fig. 3 a–c. The UV–vis spectrophotometer analysis of the fungal filtrates obtained for the isolates namely, *A. tamarii* PFL2, *A. niger* PFR6 and *P. ochrochloron* PFR8 treated with $AgNO_3$ showed a characteristic surface plasmon absorption band at 419, 430 and 430 nm, respectively. All the treated samples showed increased absorbance with increasing time of incubation, which attained maximum intensity on the third day (72 h) (Fig. 3a–c). Beyond 72 h of incubation, no further increase in intensity was recorded indicating complete reduction of silver ions by the fungal culture filtrates. Apart from this, an absorption peak was also observed in the UV region corresponding to 280 nm indicating presence of amino acid residues. The peak at 280 nm is attributed to the tryptophan and tyrosine residues present in the protein. This observation indicates the presence of proteins secreted by fungus in the cell-free filtrate.

3.2. Electron microscopy analysis

The electron microscopic observations confirmed the formation of the silver nanoparticles in all the three samples analyzed. SEM and TEM measurements were used to determine the morphology of the synthesized nanoparticles. The scanning electron micrograph revealed the morphology of the biosynthesized silver nanoparticles to be more or less spherical (Fig. 4a–c). TEM micrographs provided further insight into the morphology and particle size distribution profile of the mycosynthesized silver nanoparticles. The analysis of data obtained from TEM micrographs

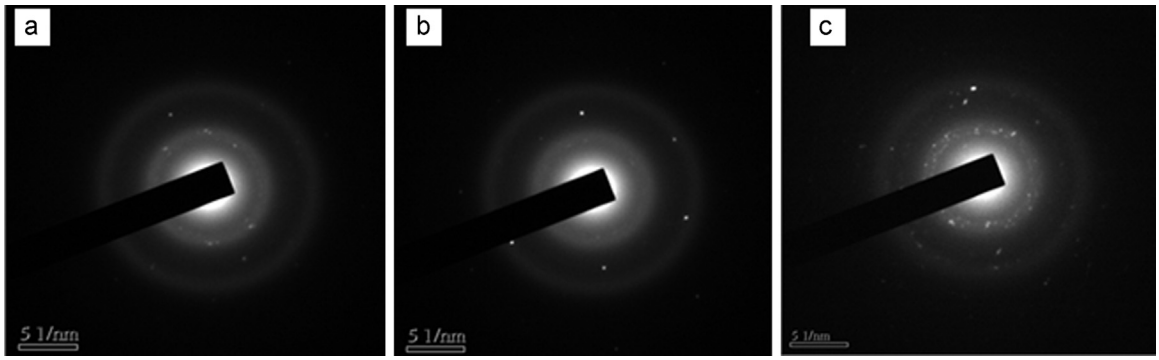

Fig. 7. SAED pattern of the biosynthesized silver nanoparticles using endophytic fungi (a) *Aspergillus tamarii* PFL2, (b) *Aspergillus niger* PFR6 and (c) *Penicillium ochrochloron* PFR8.

of silver nanoparticles synthesized using three different fungal isolates also confirmed the formation of spherical nanoparticles with differing sizes (Fig. 5a–c). These pictures show individual silver particles as well as a number of aggregates. Under observation of this image in an optical microscope, these assemblies were found to be aggregates of silver nanoparticles in different size range. The nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping agent. The particle size histograms of the mycosynthesized silver nanoparticles after analysis using ImageJ software have been shown in Fig. 6a–c. Among all the samples, it has been observed that *A. tamarii* PFL2 were able to synthesize nanoparticles of smallest particle sizes with the average size of 3.5 ± 3.3 nm followed by *P. ochrochloron* PFR8 (7.7 ± 4.3 nm) and *A. niger* PFR6 (8.7 ± 6 nm).

SAED patterns recorded for single particle in the aggregates of all the nanoparticles samples corresponded to a characteristic polycrystalline ring pattern for a face-centred-cubic structure (Fig. 7a–c). This finding revealed

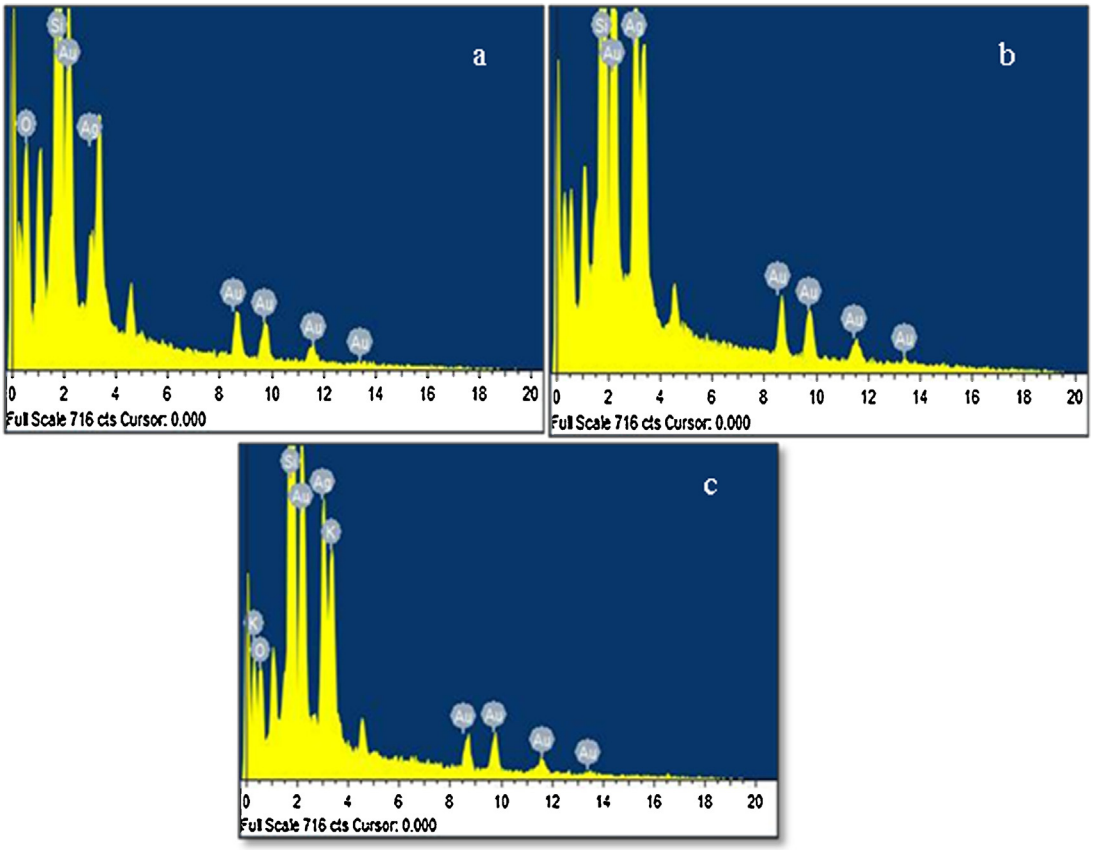

Fig. 8. EDX spectra of silver nanoparticles biosynthesized using endophytic fungi (a) *Aspergillus tamarii* PFL2, (b) *Aspergillus niger* PFR6 and (c) *Penicillium ochrochloron* PFR8.

the nanoparticles synthesized were crystalline in nature. In the analysis by energy dispersive X-ray spectroscopy of the silver nanoparticles, the presence of elemental metal signal was confirmed. The EDS profiles of the nanoparticles showed peaks in silver region at approximately 3 keV confirming presence of elemental silver (Fig. 8a–c), which is typical for the absorption of silver nanoparticles due to surface plasmon resonance confirming silver nanoparticles biosynthesized successfully using endophytic fungal extracts.

4. Discussion

The present study was aimed to assess the endophytic fungi isolated from the ethnomedicinal plants *P. fulgens* L. for their ability for extracellular reduction of silver ions to form nanoparticles. The formation on silver nanoparticles in fungal filtrate was characterized using UV–vis spectrophotometer based on its characteristic surface plasmon resonance. Fungal cell filtrate treated with silver nitrate solution (1 mM) are known to show sharp peak at around 420 nm with high absorbance [35] which supports the finding of the absorbance peaks observed at 419, 430 and 430 nm indicating the synthesis of nanoparticles by the selected fungal isolates obtained from the ethnomedicinal plant. Apart from this, the absorption peaks at around 208 nm was assigned to the strong absorption of peptide bonds in filtrate indicating the presence of aromatic acid such as tryptophan and tyrosine residues in the protein [36]. This observation indicates the release of proteins into the filtrate which suggests possible mechanism for the reduction of silver ions present in the solution. The reduction of the Ag^+ ions occurs due the reductases released by the fungus into the solution. Previous studies have indicated that nicotinamide adenine dinucleotide, reduced form (NADH) and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles [37,38]. The proteins could most possibly play a role in forming a coat covering the metal nanoparticles i.e. capping of silver nanoparticles to prevent agglomeration of the particles and stabilizing in the medium [39].

SEM is the most widely used technique for characterizing the nanoparticles in terms of physical morphology of the particles. SEM micrographs suggest that biosynthesized silver nanoparticles are almost spherical in structure. EDS analysis confirmed the presence of elemental silver in the samples. TEM provided insight into the morphology and particle size distribution profile of the silver nanoparticles. TEM analysis confirmed the synthesis of spherical silver nanoparticles in the reaction mixture. The nanoparticles synthesized by *A. tamarii* PFL2, *A. niger* PFR6, *P. ochrochloron* PFR8 were found to be in different size ranges. SAED helped to identify crystal structures and to examine crystal defects of nanosized particles. SAED patterns of the nanoparticles samples showed characteristic polycrystalline ring pattern for a face-centred-cubic structure.

The present finding elucidates that the mycosynthesized silver nanoparticles using the endophytic fungi isolated from traditionally used ethno-medicinal plants *P. fulgens* L. possess unique and fascinating optical properties that can be exploited for their utility in various technological applications as they offered different particle sizes and also are polycrystalline in nature.

5. Conclusion

The findings of the present study leads to the conclusion that the three endophytic fungi *A. tamarii* PFL2, *A. niger* PFR6 and *P. ochrochloron* PFR8 were found to be reduce the silver ions to silver nanoparticles. The electron microscopic study revealed the biosynthesized nanoparticles were spherical with different particle sizes. The silver nanoparticles synthesized using the endophytic fungus *A. tamarii* PFL2 with the average size 3.5 ± 3 nm was found to be smallest in terms of average particle diameter as compared to the rest of the biosynthesized silver nanoparticles. Thus, the endophytic fungi *A. tamarii* PFL2, *A. niger* PFR6 and *P. ochrochloron* PFR8 isolated from the medicinal plant *P. fulgens* L. offers promising scope as fungal nano-factories for their eco-friendly and sustainable production.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

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