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RESEARCH ARTICLE

Endophytic fungi associated with *Zingiber cassumunar* Roxb. and its application in the synthesis of gold nanoparticles

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Abstract

Endophytic fungi are used as an environmentally safe alternative to chemicals for the synthesis of gold nanoparticles . Endophytic fungi associated with medicinal plants are promising candidates due to their ability to produce various bioactive compounds that can efficiently reduce and stabilize gold ions. Among the most commonly used traditional of medicinal plants in northeast India, *Zingiber cassumunar* Roxb. (Tekhao-yaikhu in Manipuri) is one of the most prominent ones. The endophytic fungi associated with *Z. cassumunar* Roxb were isolated and identified based on their morphological characteristics as well as the ITS regions of rRNA gene sequences. In this study, 31 endophytic fungal isolates were obtained from 60 healthy samples from the leafy and rhizomatous regions of *Z. cassumunar*. They were then grouped into 10 taxonomic groups based on the morphological characteristics. Higher endophytic colonization frequency (73 %) and isolation rate (0.73) were observed with the 60 leaf samples while the rhizome samples exhibited colonization frequency of 30 % and isolation rate of 0.30. The fungus *Colletotricum gloeosporioides* was observed to be the most abundant one with a colonization frequency of 36.6 % and isolation rate of 0.37. Analyses of the morphologically distinct isolates using internal transcribed spacer (ITS) sequences revealed 4 major clades - Sordariomycetes, Dothideomycetes, Eurotiomycetes and Polyporales. Evaluation of the endophytes for their ability to synthesize gold nanoparticles using mycelium-free extracts treated with aqueous chloroauric acid solution, *C. gloeosporioides* ZCL1 was observed to be the most promising for Au nanoparticle biosynthesis with the reduction of chloroauric acid within 6 h. UV-visible spectrum of the reaction mixture containing chloroauric acid and mycelium-free extracts showed a broad peak at around 520-580 nm. The formation of Au nanoparticles was confirmed using scanning electron micrograph. Further, transmission electron micrographs (TEM) showed anisotropic nanoparticles exhibiting different shapes such as spherical, pentagonal, triangular and hexagonal nanoparticles. The average size of Au nanoparticles was observed to be 28.5 nm ranging from 9-55 nm. The endophytic fungi *C. gloeosporioides* ZCL1 associated with *Z. cassumunar* is a promising candidate for environment friendly biosynthesis of Au nanoparticles, which have variety of applications in agriculture, as nano-based formulation of agrochemicals enhancing plant growth by improving nutrient uptake, deliveries, stress tolerance and disease resistance.

Key words: biosynthesis; diversity; endophytic fungi; gold nanoparticle; *Zingiber cassumunar*

Introduction

The Zingiberaceae plants are good sources of vegetables, food flavours, spices, dyes, condiments and also are widely used in various traditional medicines (1). The northeastern (NE) region of India, located in the Indo-Burma biodiversity hotspot region (2) harbours a rich and diverse flora and fauna including the members of the family Zingiberaceae, where 19 genera and about 88 species were documented (3). The state of Manipur situated between 23°50´N and 25°41´N latitude and between 93°2´E and 94°47´E longitude having a total area of 22327 km^2 with a rich diversity of various communities and traditional medicinal practices (4).

Among the commonly used medicinal plants having high economic values by the indigenous communities of NE India is a member of the Zingiberaceae family, *Zingiber cassumunar* Roxb. known as Tekhao-yaikhu in Manipur. The rhizomes of *Z. cassumunar* are used for the treatment of asthma, joint and muscle pain (5). Boiled leaf extract of the plant is used for treatment of asthma in Manipur and the rhizomes are also used for the treatment of indigestion or gas formation (6). Several bioactivities of the plant such as anti-inflammatory, anti-allergic, antioxidant activity with a broad range of potential biomedical and pharmacological application have also been reported (7,5).

The medicinal plants are known for providing a unique environment for harbouring several endophytes and producing novel pharmaceutically importance metabolites (8-10). Advances in molecular techniques and its applications has enhanced the sensitivity and specificity for the classification of microorganisms at diverse hierarchical taxonomic levels (11). The use of internal transcribed spacer (ITS) sequences of the 18S ribosomal has been the reliable technique for the accurate and reliable characterization of fungi (12, 13). The variations in the ITS sequences due to the fast rate of evolution between closely related species is widely used for the correct fungal

identification compared to the other highly conserved regions of the rRNA gene cluster (14, 15). Endophytic fungi are being explored for their potential to synthesise nanoparticles owing to its ease of synthesis, environmentally benign nature and greater stability of nanoparticles. Extracellular extracts of various fungal species are also utilized for the biosynthesis of metal nanoparticles with higher yields compared to the use of intracellular extracts (16). Fungi as a promising biosystem for the biosynthesis of gold (Au) nanoparticles have been reported by various workers such as *Trichothecium* sp. (17) and *Colletotrichum* sp. (18). The filamentous fungi like *Fusarium oxysporum* and *Neurospora crassa* were also reported to have produced nanosized Au-Ag bimetallic alloy (19, 20). The use of mycelia-free culture filtrate of a phytopathogenic fungus *Nigrospora oryzae* for the synthesis of Au nanoparticles (6–18 nm) by the bio reduction of chloroauric acid (21). Further, 25 fungal species have also been reported for their capability of Au nanoparticles biosynthesis has also been reported (22). However, very few of the fungal endophytes associated with ethno-medicinal plants have been investigated so far for the biosynthesis of Au nanoparticles in spite of the production of various natural products and possessing huge potential for exploitation in various applications (8, 23).

The present study was designed to understand the diversity and the phylogenetic relatedness of the culturable endophytic fungi associated with *Z. cassumunar* and their biosynthetic potential of Au nanoparticles.

Materials and Methods

Sampling

Field surveys and sampling was undertaken in Imphal East district of Manipur state and collection of the samples was done during August-September. Healthy looking matured *Z. cassumunar* plants were carefully collected and maintained under greenhouse conditions for further studies. The endophytic fungi were isolated from freshly collected leaves and rhizomes to be used for further experiments.

Isolation of endophytic fungi

Endophytic fungi associated with the *Z. cassumunar* leaf and rhizome parts were isolated with protocols laid out by Strobel et al., (9) with slight modifications. First the plant materials were cleansed with running tap water to washed off adhering particles and then the parts were excised with a sterile blade into blocks of approximately 5×5 mm sizes. This is then followed by surface sterilization in 70 % ethanol for 1 min and then treating with 4 % sodium hypochlorite solution for 3 min. The final rinsing was done thrice with sterile distilled water and the excess water was blot dried on a sterile blotting paper. The dried excised plant parts were inoculated on petri-plates containing Potato Dextrose Agar (PDA) supplemented with 100 µg/mL of ampicillin under aseptic conditions and incubated at 25 °C. Emergence of fungal growth were monitored and recorded daily and the emerging fungal hyphae was aseptically sub-cultured onto a fresh PDA plate for obtaining pure isolates. The pure cultures were inoculated on PDA slants and incubated at 25 °C for 5-7 days. The pure fungal isolates obtained were then inoculated into a sterilized test tube containing sterile distilled water and stored at 4 °C until use.

Morphological characterization of endophytic fungi

Morphological characterization of the isolated endophytic fungal isolates was carried out to tentatively identify up to genus level based on the morphological and microscopic observations. After growing the fungi for five to seven days in PDA medium, fungal isolates were examined macroscopically. Morphological features such as colony colour (front and reverse) and size were observed. Microscopic examination was achieved through staining with lactophenol cotton blue and examining using a light microscope (24).

Molecular characterization

DNA extraction, PCR amplification and sequencing

Genomic DNA of the fungal isolates was extracted using CTAB method with minor modifications. Around 10 mg of mycelia from young culture was scooped into a 2 mL tube and disrupted using a sterile glass rod and the 400 µL of extraction buffer containing 200 mM Tris-HCl (pH 8.5), 25 mM ethylene diaminetetra acetic acid (EDTA) (pH 8.0), 200 mM NaCl, 0.5 % sodium dodecyl sulfate and 2 % CTAB was added. The tube was then incubated in a water bath at 65 ºC for 45 min, cooled down to room temperature and 120 µL of 0.5 M sodium acetate (pH 5.8) was added followed by slight vortexing of the mixture. The mixture was then incubated at for 10 min at -20 ºC followed by centrifugation for 10 min at 10000 rpm. The supernatant was then pipetted into a fresh 1.5 mL tube and an equal volume of chloroform:phenol:isoamyl alcohol (24:23:1) was added, centrifuged at 10000 rpm for 10 min. The aqueous layer was pipetted out into a fresh 1.5 mL tube and an equal volume of ice-cold isopropyl alcohol was added followed with centrifugation at 10,000 rpm for 10 min. The supernatant was discarded and the DNA pellet was collected then washed with 200 µL of ice-cold 70 % ethanol followed by centrifugation at 13000 rpm for 5 min. Again, the supernatant was discarded and the DNA pellet was air dried and dissolved in TE buffer. All the extractions were done in duplicate for each sample. The extracted DNA was PCR-amplified using the primers for ITS regions (12) in a reaction mixture containing 1×PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTP, 1U Taq polymerase, 0.5 pM each of the forward and the reverse primers. The PCR was programmed with an initial denaturation at 94 ºC for 5 min followed by 35 cycles of denaturation at 94 ºC for 1 min, annealing temperature at 52 ºC for 1 min, extension at 72 ºC for 1 min and final extension for 10 min 72 ºC respectively. The resulting PCR-amplification products of around 550 bp were then purified and sent for sequencing at Eurofins Pvt. Ltd. (Bangalore, India).

Phylogenetic analysis of sequences

The ITS sequences obtained from the PCR products were then compared with the National Centre for Biotechnology Information (NCBI) databases using Basic Local Alignment Search Tool (BLAST) initially to determine the phylogenetic neighbours from the non-redundant nucleotide database (25). Further, Molecular Evolutionary Genetics Analysis (MEGA version 7.0) software was used for phylogenetic analyses (26) by aligning the sequences of identified phylogenetic neighbours with the sequences of representative strains using Clustal W based on the neighbour-joining method (27) for generating the phylogenetic tree using a p-distance matrix with 1000 bootstrap replications iterations (28). The ITS region

of the isolated endophytic fungal sequences was the submitted to NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Biosynthesis of gold nanoparticles

Biosynthesis of Au nanoparticles using the isolated endophytic fungal cultures were carried out in 500 mL Erlenmeyer flasks containing 100 mL potato dextrose broth medium with continuous shaking on a rotary shaker maintained at 28 ± 2 °C and 200 rpm for 96 hr. After 96 hr of culture, mycelia were harvested and washed thrice with sterile distilled water under aseptic conditions. Then 10 g fresh weight of the harvested mycelial masses were a resuspended in a sterilized 250 mL Erlenmeyer flask containing 100 mL of sterile distilled water and incubated for 24 h at 28 ± 2 ºC with constant shaking at 120 rpm. After the incubation, the cell filtrates were harvested by passing through a Whatman no.1 filter paper then mixed with 0.5 mM aqueous chloroauric acid solution in a 500 mL Erlenmeyer flask and incubated at room temperature under dark conditions. A flask containing cell-free filtrate without chloroauric acid solution was used as a control. All the experiments were conducted with 3 replicates. The bio-reduction of the Au^{3+} ions to Au nanoparticles in solutions was monitored by periodic visual observation and then measured for the UV-VIS spectra of the solution.

Characterization of gold nanoparticles

Visual observation

The changes in colour of the mycelium free filtrates incubated with 0.5 mM chloroauric acid solution, were visually observed to evaluate the bio reduction of Au^{3+} ions to Au nanoparticles.

UV-Visible spectroscopy analysis

UV–Vis absorbance analysis of the Au nanoparticles formed in the reaction mixture was carried out by pipetting out 1 mL aliquot at different time intervals for 48 hr to evaluate the Au nanoparticles at a resolution of 1 nm between 200-800 nm by a CARY-100 BIO Varian Inc., USA make UV-VIS Spectrophotometer (29).

Electron Microscopy analysis

The biosynthesizes Au nanoparticles solution was characterised using Scanning Electron Microscopy and Transmission Electron Microscopy (30).

Scanning Electron Microscopy analysis (SEM)

For SEM analysis, the resulting Au nanoparticle solution was dropped and coated on a thin glass film and observed using the Scanning Electron Microscope (JSM-6360, JEOL).

Transmission electron microscopy (TEM) analysis

For TEM, the synthesized Au nanoparticles were casted by dropping on carbon coated copper grids and then vacuum dried in a desiccator. TEM micrography was performed using JEOL JSM 100CX TEM instrument (Jeol, Japan) by running at an accelerating voltage of 200 kV. Selected area Electron Diffraction (SAED) pattern analysis of the Au nanoparticles was also performed.

Data analysis

For the analyses of the isolated endophytic fungi, the isolation rate (IR) and fungal richness of a sample was determined by dividing the number of isolates from tissue segments by the total number of segments (31). Calculation of the colonization frequency (CF %) of the endophytic fungal isolates was carried out using the following formula (32).

$$CF\% = \frac{N_{col}}{N_t} X100 \qquad \text{(Eq. 1)}$$

Where, N_{col} indicates the number of segments colonized by each endophyte and N_t indicates the total number of segments observed.

Average size, size distributions and standard deviations of the synyhesized Au nanoparticles were calculated by averaging 100 particles from the TEM images using ImageJ software (https://imagej.nih.gov/ij/).

Results

Isolation and morphological grouping of fungal endophytes

In the present study, a total of 31 endophytic fungi were isolated from 60 healthy leaves and rhizome samples *Z. cassumunar*. The fungal isolates were tentatively identified based on their macroscopic as well as the microscopic parameters. Some of the fungal isolates could not be identified as they failed to sporulate under the assay conditions used in the present study. The fungal isolates which failed to sporulate and having only mycelial structures were designated as mycelia sterilia. The various endophytic fungi with different colonization frequency (CF %) and isolation rates (IR) as shown in the Table 1. Leaf samples showed higher CF (73 %) and IR (0.73) than the rhizomes with the CF (30 %) and IR (0.30) values indicating the fungal endophytes were more prevalent on leaf tissues than the rhizome tissues. The most abundant isolated endophytic fungi group was observed with *Colletotricum*

Table. 1 Colonization frequency (%) and isolation rate of fungal endophytes isolated from *Zingiber cassumunar*

Endophytic fungi	Number of isolates		Colonization frequency (%)		Isolation rate (IR)	
	Leaves	Rhizomes	Leaves	Rhizomes	Leaves	Rhizomes
Colletotrichum gloeosporioides gloeosporioides	11	-	36.6	-	0.37	-
Colletotrichum sp.1	2	-	6.7	-	0.07	-
Colletotrichum sp.2	1	-	3.3	-	0.03	
Colletotrichum sp.3	2	-	6.7	-	0.07	-
Curvularia lunata	3	-	10	-	0.10	-
Mycelia sterilia 1	2	-	6.7	-	0.07	-
Mycelia sterilia 2	1	-	3.3	-	0.03	-
Phialophora sp.	-	6	-	20	-	0.20
Mycelia sterilia 3	-	3	-	10	-	0.10
Total	22	9	73	30	0.73	0.30

(36.7 %) while the isolation rate was observed with *C. gloeosporioides* (0.37).

Molecular characterization

Further identification of the isolated fungal samples upto species level was conducted by sequencing of the 550 bp ITS PCR products and analysis of ITS1-5.8S ITS2 rDNA regions. The ITS region of the 10 morphologically distinct endophytic fungal isolates coded as *Colletotrichum gloeosporioides* ZCL1, *Colletotrichum* sp.1 ZCL2, *Curvularia lunata* ZCL3, *Colletotrichum* sp.2 ZCL4, *Colletotrichum* sp.2 ZCL6, Mycelia sterilia1 ZCL7, *Colletotrichum* sp.3 ZCL8, Mycelia sterilia2 ZCL10 and *Phialophora* sp. ZCR1 and Mycelia sterilia3 ZCR2 were sequenced and characterized. The ITS sequences were aligned with nucleotide BLAST search tool for identifying the similar sequences from the NCBI-GenBank databases. The BLAST search revealed the sequence similarity of the isolated fungi to the closest sequences in the databases varying from 96 % to 100 %. Based on high similarity percentage (>98.7 %) the isolates were assigned to a particular species (Table 2). The ITS characterized gene sequences were then submitted to NCBI GenBank and their assigned GenBank accession numbers are listed in Table 2. The ITS region sequence analysis of the isolated fungi indicated that isolates having different morphology does not always belong to different species. Morphologically different endophytic fungal isolates ZCL1 and ZCL8 which are identified as different species are revealed to be same species, *Colletotrichum gloeosporioides*. Similarly, ZCL2 and ZCL6 which have different morphological characteristics are also found to be *C. musae*.

Phylogenetic analysis and taxonomic grouping of endophytic fungi

The generated rDNA ITS sequences-based phylogenetic tree with bootstrap values of the endophytic fungi isolated from *Z. cassumunar* and the sequences retrieved from GenBank database (indicated by database code) are shown in Fig 1. The maximum composite likelihood method-based genetic distances were computed in the units of the number of base substitutions per site (33). Based on the molecular phylogenetic analysis of the endophytes, it is revealed that the medicinal plant used in the study is associated with rich endophytic fungal diversity. The resulting clear phylogenetic clustering of the endophytic fungal isolates in the same branch

Table 2. Molecular identification, closest match and GeneBank accession numbers of the morphologically distinct fungal endophytes isolated from *Zingiber cassumunar* on the basis of ITS gene sequences

Fungal isolates	Molecular identification		
	Closest match with Genbank accession number	Similarity percentage	Accession number
Colletotrichum gloeosporioides ZCL1	*Colletotrichum gloeosporioides* (KM203611)	99	KX347472
Colletotrichum sp. ZCL2	*Colletotrichum musae* (DQ453986)	99	KX347473
Curvularia lunata ZCL3	*Curvularia lunata* (KP901305)	100	KX347474
Colletotrichum sp. ZCL4	*Glomerella acutata* (GQ924900)	99	KX347475
Colletotrichum sp. ZCL6	*Colletotrichum musae* (DQ453986)	99	KX347476
Mycelia sterilia1 ZCL7	*Cercospora gerberae* (KC776160)	99	KX347477
Colletotrichum sp.3 ZCL8	*Colletotrichum gloeosporioides* (KM203611)	99	KX347478
Mycelia sterilia2 ZCL10	*Cercospora gerberae* (KC776160)	99	KX347479
Phialophora sp. ZCR1	*Phialophora cyclaminis* (KM520038)	99	KX347480
Mycelia sterilia3 ZCR2	*Phanerochaete* sp. (KP135022)	96	KX347481

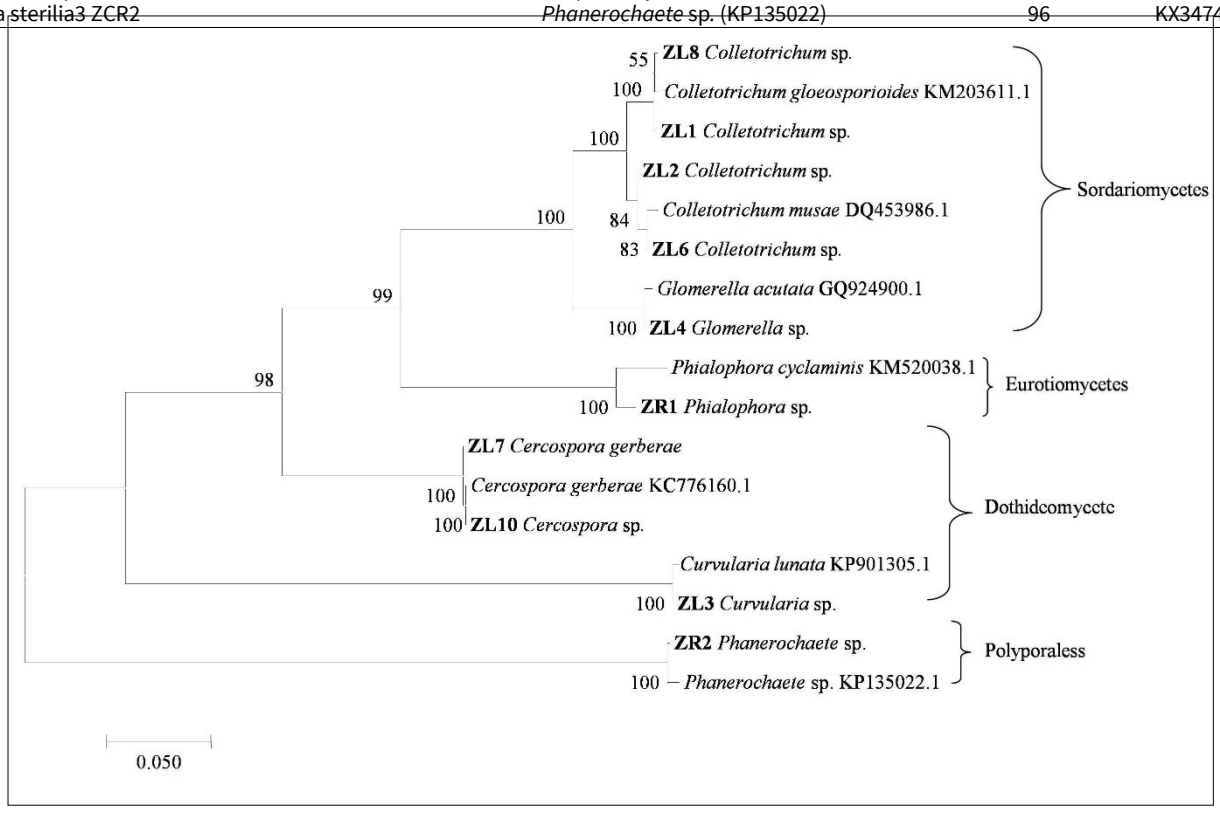

Fig. 1. Phylogenetic relationship of the endophytic fungi isolated from *Z. cassumunar* Roxb. and closely related fungal strains retrieved from NCBI GenBank based sequences of the ITS1-5.8S-ITS2 of rDNA region using Neighbour-joining method.

also showed four major clades: Sordariomycetes with 2 genera of the order Glomerallales; Dothideomycetes including two genera belonging to 2 orders Pleosporales and Capnodiales); Eurotiomycetes represented by genus *Phialophora* belonging to the order Chaetothyriales and Polyporales represented by genus *Phanerochaete*. Thus, the phylogenetic analysis revealed that the endophytes from *Z. cassumunar* Roxb. consists of 5 orders (Glomerallales, Chaetothyriales, Capnodiales, Pleosporales and Phanerochaete) which were distributed in 6 genera and 7 species (Table 3). Sordariomycetes was observed to be the dominant class and the genus *Colletotrichum* was found to be the dominant group of fungal endophytes.

Biosynthesis of gold nanoparticles

Fungal endophytes isolated from the ethnomedicinal plant *Z. Cassumunar* were tested for biosynthesis of Au nanoparticles by treating the mycelium-free filtrate of the fungal isolates with the chloroauric acid solution. After treating the mycelia-free extracts of the endophytic fungal isolates with chloroauric acid solutions were checked for any visible colour change. Among the isolates, the extract of fungal endophyte *Colletotrichum gloeosporioides* ZCL1 treated with chloroauric acid solution exhibited a gradual change of the solution from light yellow to purple colour when incubated with chloroauric acid solution within 6 h of incubation (Fig. 2). Thus, the appearance of a violet colour in the solution containing the extract of *Colletotrichum gloeosporioides* ZCL1 was a clear indication of the formation of Au nanoparticles in the reaction mixture due to excitation of surface plasmon vibrations in the Au nanoparticles. This indicates that the endophytic fungi, *Colletotrichum gloeosporioides* ZCL1, isolated from *Zingiber cassumunar* has the biological potential biological to synthesise Au nanoparticles which can be further explored for the mass production. The absorbance of the biosynthesized nanoparticles characterized by the UV-Vis spectrophotometer at different time interval with a broad peak was observed between 500 and 580 nm after 6 h and steadily increased with maximum intensity at 42 h (Fig. 3).

The characteristic SEM information of the sample topography such as the surface features, morphology, shape and size of the synthesised Au nanoparticles are shown in Fig 4. TEM measurements showed the presence of various shapes (spherical, pentagonal, triangular and hexagonal) and sizes of Au nanoparticles (Fig. 5). The particle size histogram of the Au nanoparticles is shown in Fig 6. The maximum number of Au nanoparticles was with the size ranging from 20-25 nm and whereas the overall particle size ranged from 9 to 55 nm with an average size of 25.8 nm. For the triangular nanoparticles, dimension corresponds to edge lengths; for hexagonal and pentagonal profile, dimension corresponds to the distance between opposite sides and for spherical, the core diameter of the particles. The crystalline nature of the nanoparticles can be confirmed by circular ring pattern through SAED pattern with the characteristic polycrystalline ring pattern for a face-centered-cubic structure (Fig. 7).

Discussion

The NE region of India is known for harbouring vast number of unique and diverse floral and faunal members. The rich biodiversity is also used in a variety of traditional medicinal uses such as the *Z. cassumunar.*

In the present work, the fungal endophytes associated with the ethnomedicinal plant *Z. cassumunar* of NE region of India were identified. From 60 segments of plant materials a total of 31 fungal isolates were obtained. Based on the morphological analysis the 31 isolated fungal endophytes are grouped into 10 taxonomic groups. The fungal endophytes were analyzed from two plant parts, namely, leaf and rhizome which are widely used in traditional treatment of many diseases and ailments. Colonization rate of endophytic isolations from leaf segments were greater (73 %) than isolations from rhizome (30 %). Among the fungal genera *Colletotrichum* was isolated more often than any other fungal endophytes, *Colletotrichum gloeosporioides* being the most dominant fungal species. In our study, the diversity of fungal endophytes in the leaf and the rhizome was found to be different.

The genus *Colletotrichum* has been isolated from numerous plant species from various regions of the world either as symptomatic pathogens or asymptomatic endophytes as they are capable of associating as mutualistic or commensal organisms with the host plants (34). Numerous *Colletotrichum* spp. were reported for its association with endophytic fungal communities in a large variety of plants such as *Camptotheca acuminate* (35), *Phaseolus vulgaris* (36), *Corchorus capsularis* L. (37) and *Citrus sinensis* (38). Fungi often show pronounced morphological variability which is useful for

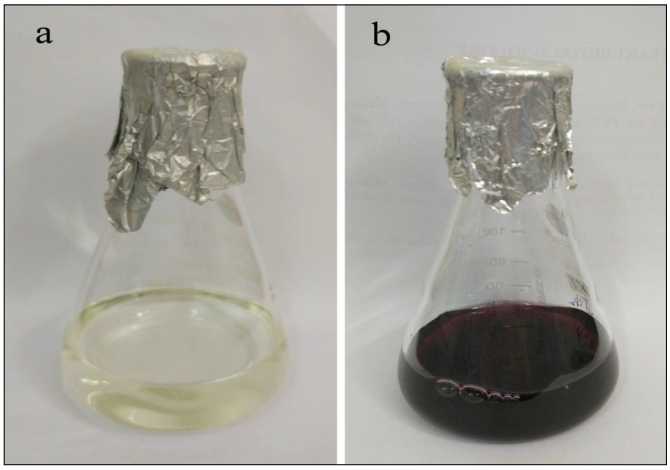

Fig. 2. Colour change observed in mycelia free fungal extract of *Colletotrichum gloeosporioides* ZCL1 treated with $HAuCl_4$ solution, a) before incubation b) after incubation.

Table 3. Taxonomic grouping of endophytic fungi isolated from the ethnomedicinal plant *Zingiber cassumunar*

Division	Class	Order	Species
Ascomycota	Sordariomycetes	Glomerallales	*Colletotrichum gloeosporioides, Colletotrichum musae, Glomerella acutata*
	Dothideomycetes	Pleosporales	*Curvularia lunata*
		Capnodiales	*Cercospora gerberae*
	Eurotiomycetes	Chaetothyriales	*Phialophora cyclaminis*
Basidiomycetes	Polyporales	Phanerochaete	*Phanerochaete* sp.

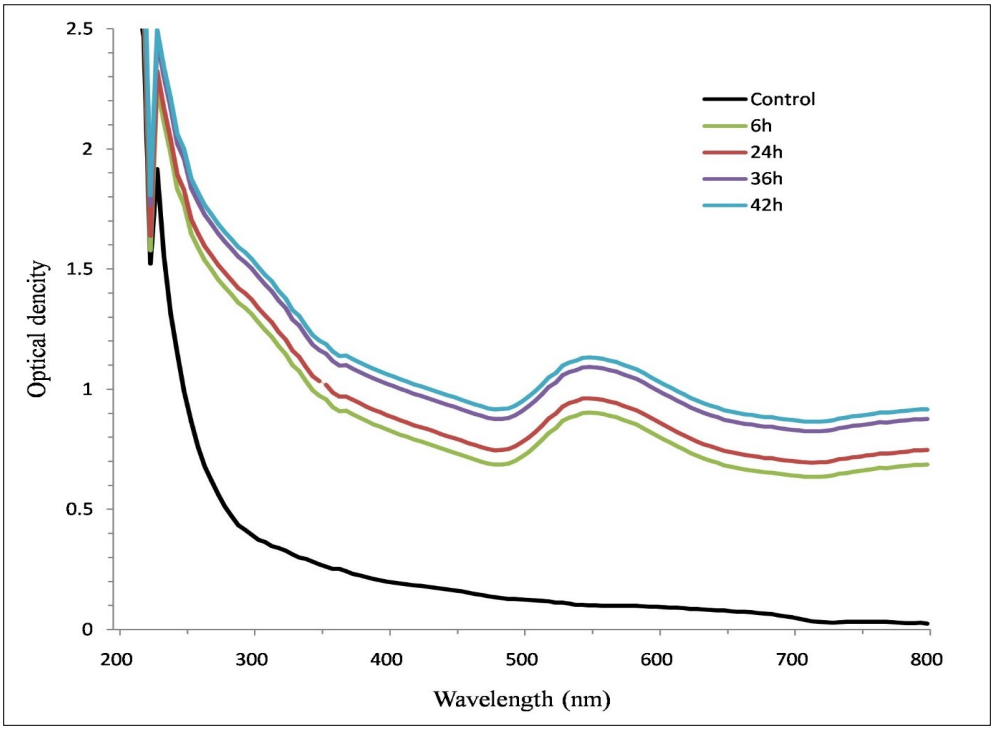

Fig. 3. UV-Vis absorption spectrum at different time intervals of Au nanoparticles synthesized by *Colletotrichum gloeosporioides* ZCL1.

morphological characterization (39-41) however, many fungi are anamorphic yet have high level of genetic variation (42-44). Morphologically indistinguishable or cryptic species also have been described earlier (45).

Fungi is promising for industrial Au nanoparticles biosynthesis because for their easy handling, larger protein secretion then bacteria, high biomass yield and accessibility for scale up (22). Several researchers have reported the ability of various endophytic fungal species for Au nanoparticles biosynthesis. A marine endophytic fungus *Penicillium citrinum* was reported to biosynthesize the Au nanoparticles with the size range of 60-80 nm (46). Biosynthesis of Au nanoparticles using endophytic fungus *Fusarium oxysporum* isolated from *Azadirachta indica* (47). In our study, based on the bio reduction ability of the chloroaurate ions into Au nanoparticles, *Colletotrichum gloeosporioides* ZCL1 was found to be potential one which could be further explored for the mass production. The Au nanoparticles were synthesized from Au^{3+} ions using the mycelia-free filtrate of the fungal isolate indicated by the appearance of violet colour and the Au nanoparticles surface plasmon band is known to occur in the range 520–560 nm in an aqueous medium (48). The sharp absorption peak at 280 nm observed was due to the strong absorption of peptide bonds in filtrate indicating the presence of aromatic acid residues in the protein interaction with the Au^{3+} ions due to the involvement of aromatic amino acids like tyrosine and tryptophan in the reduction of Au^{3+} ions (49). The reduction of the Au^{3+} ions is known to occurs due the reductases released by the fungus into the solution involving nicotinamide adenine dinucleotide, reduced form (NADH) and NADH-dependent nitrate reductase enzyme leading to the formation of metal nanoparticles. Such observations with the proteins forming a coat covering the metal nanoparticles i.e. capping of Au nanoparticles to prevent agglomeration of the particles and stabilizing in the medium. Au nanoparticles biosynthesis has also been successfully demonstrated using extracellular soluble protein extract of fungi *Fusarium oxysporum* (50). In *R. capsulata*, species specific NADH

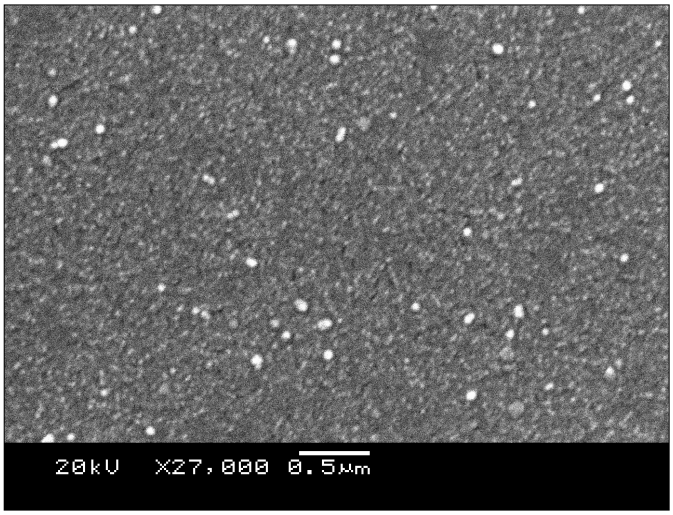

Fig. 4. SEM micrograph of Au nanoparticles biosynthesized using endophytic fungi *Colletotrichum gloeosporioides* ZCL1.

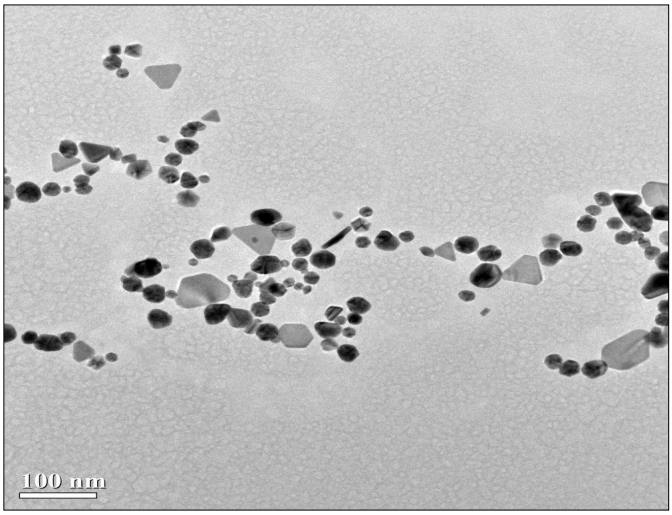

Fig. 5. TEM micrograph of Au nanoparticles biosynthesized using endophytic fungi *Colletotrichum gloeosporioides* ZCL1.

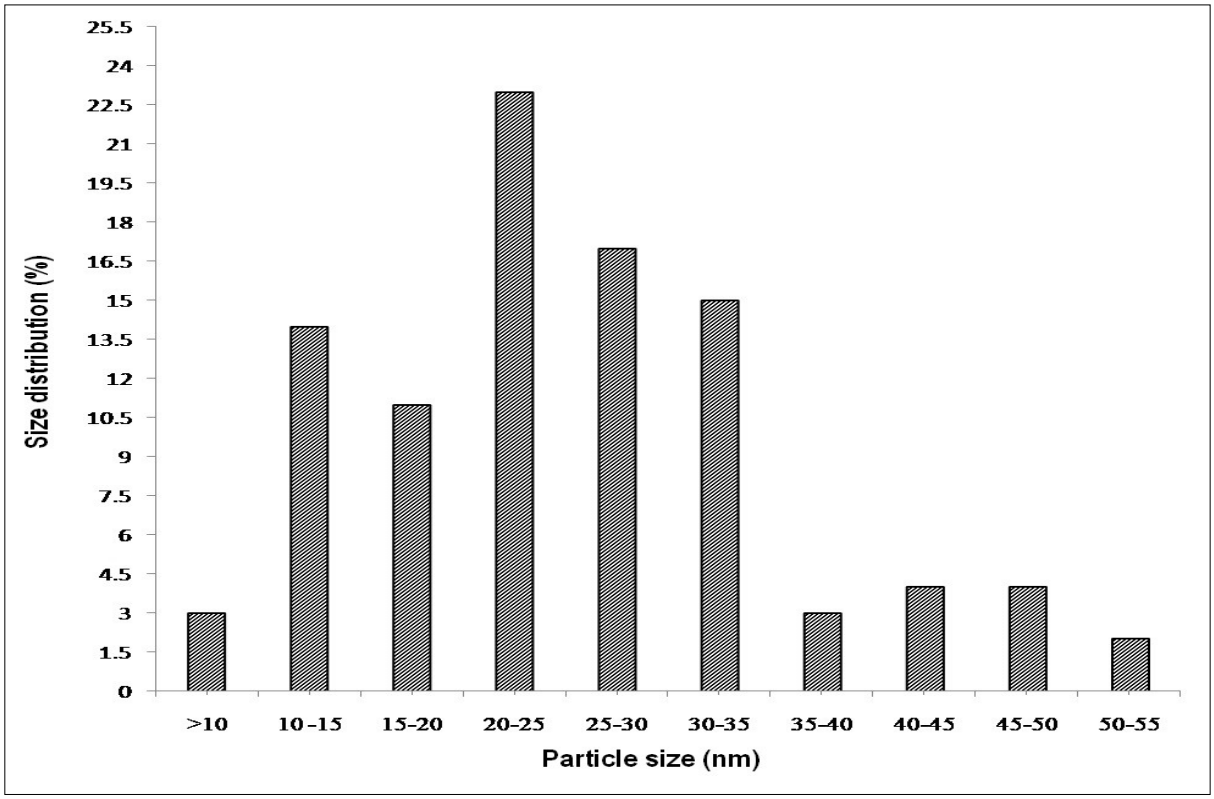

Fig. 6. Particle size distribution pattern histogram of Au nanoparticles biosynthesized using endophytic fungi *Colletotrichum gloeosporioides* ZCL1.

dependent reductases were reported to be involved for bio reduction of Au^{3+} ions to carry out the synthesis of Au nanoparticles (51). However, the specific protein(s) and mechanisms involved in the NADH dependent reduction of Au^{3+} ions is not known (52). To understand and established mechanistic aspects of microbial mediated Au nanoparticles synthesis required further in-depth study. SEM image analysis confirmed the formation of Au nanoparticles. TEM analysis revealed that the synthesized nanoparticles are anisotropic with the majority of them having pentagonal shape. The Au nanoparticles are found to have an average size of 28.5nm (Fig. 5). Triangular shaped nanoparticles were frequently observed in the TEM images. The edges of the triangles are truncated in most cases and extremely thin which can be seen from the increased contrast in the region of overlap of triangular particles as evident in Fig 5. In an earlier study, the biosynthesis of Au nanoparticles which are spherical in shape from aqueous Au^{3+} ions with geranium endophytic fungus *Colletotrichum* sp. (18), however, our present study revealed the synthesized nanoparticles exhibited anisotropic structures. The biosynthesis of nanoparticles using fungi and other microorganisms are safer and eco-friendlier (53). The mechanism involved in the synthesis of Au nanoparticles using *Colletotrichum gloeosporioides* ZCL1 need to be further investigated to gain higher production rates and desired morphology.

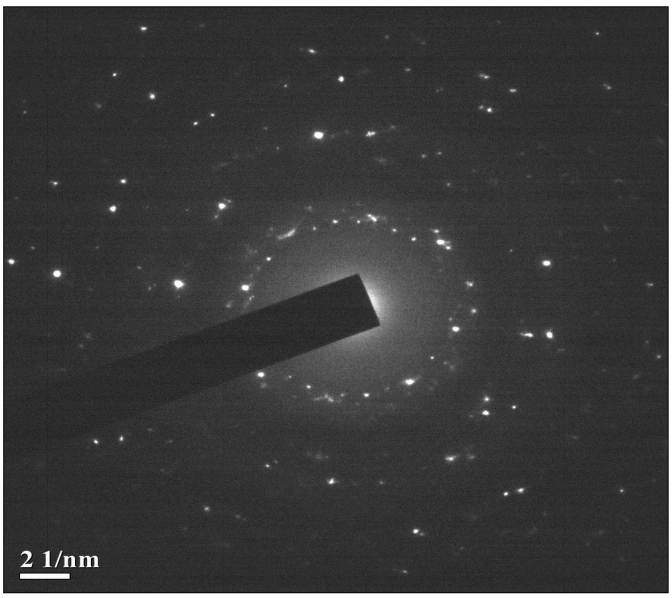

Fig. 7. The selected area diffraction (SAED) pattern Au nanoparticles biosynthesized using endophytic fungi *Colletotrichum gloeosporioides* ZCL1.

Conclusion

The present study showed that the ethno-medicinal plant *Z. cassumunar* of NE region of India, is an ecological niche for diverse endophytic fungi. A total of 31 fungal isolates were obtained from 60 healthy leaves and rhizomes. Fungal isolates are identified based on morphological analysis as well as ITS sequence analysis and are grouped into 10 taxonomic groups. Colonization rate of endophytic isolations from leaf segments were greater (73 %) than isolations from rhizome (30 %). Among all the fungal species, *Colletotrichum gloeosporioides* was found to be the most dominant fungal species.

Our study also reveals that, *Colletotrichum gloeosporioides* ZCL1 isolated from *Z. cassumunar* has great potential for bio reduction of the chloroaurate ions into Au nanoparticles. The biosynthesized Au nanoparticles were characterized by various analytical techniques and were found to have particle size ranged from 9 to 55 nm with an average size of 25.8 nm. This study suggests that endophytic fungi associated with the ethno-medicinal plants can be explored and efficiently used for the biosynthesis of Au nanoparticles. It is necessary to

screen and study the various groups of endophytic fungi for eco-friendly synthesis and also scale up of their mass production is immensely desired for future applications.

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Authors' contributions

SDL carried out the experiments, analyzed the data and prepared the draft of the manuscript. RT was responsible for the overall designing and guiding the experiments, analysis of data and finalizing of the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interests regarding the publication of this paper.

Ethical issues: None

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