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Myco-fabrication of Silver Nanoparticles from Endophytic fungus *Epicoccum nigrum* Ehrenb. ex Schlecht: A Novel Approach for Sustainable Plant Disease Management

Sudhir S. Shende[1,2,†], Dilip V. Hande[3,†,*], Pramod U. Ingle[2], Rahul Bhagat[4], Patrycja Golinska[5], Mahendra Rai[2,6], Tatiana Minkina[1], and Aniket K. Gade[2,5,7,‡]

[1]*Academy of Biology and Biotechnology, Southern Federal University, Stachki Ave, 194/1, Rostov-on-Don - 344 090, Russian Federation.*
[2]*Nanobiotechnology Laboratory, Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati - 444 602, Maharashtra, India.*
[3]*Shri. Pundlik Maharaj Mahavidyalaya, Nandura Rly., Buldhana - 443 404, Maharashtra, India.*
[4]*Department of Biotechnology, Government Institute of Science, Chhatrapati Sambhajinagar - 431 004, India.*
[5]*Department of Microbiology, Nicolaus Copernicus University, Torun, 87-100, Poland.*
[6]*Department of Chemistry, Federal University of Piaui (UFPI), Teresina, Brazil.*
[7]*Department of Biological Sciences and Biotechnology, Institute of Chemical Technology, Nathalal Parekh Marg, Matunga, Mumbai - 400 019, Maharashtra, India.*
† These authors contributed equally and share first authorship.

Corresponding authors Email: dvhande@gmail.com; ak.gade@ictmumbai.edu.in

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ABSTRACT

Resistance against fungicides or antibiotics in plant pathogens is nowadays a greater challenge to the scientific community, as most of the pathogenic strains survive unless treated with anti-agents. Therefore, there is a need to develop sustainable approaches that could manage the diseases in plants. Silver nanoparticles (AgNPs) have received attention due to their eco-friendly fabrication methods and interesting antimicrobial properties. This study reported the myco-fabrication of AgNPs by a novel biogenic approach using an extract of endophytic fungus *Epicoccum nigrum* Ehrenb. ex Schlecht isolated from *Dioscorea bulbifera* (L.) leaves. The primary detection was done visually and by UV-Vis spectrophotometric analysis. The color change in the reaction solution from pale yellow to brown indicated the formation of myco-AgNPs. The UV-Vis spectral analysis revealed a typical surface plasmon resonance (SPR) at 447 nm. Nanoparticle tracking and analysis (NTA) demonstrated a mean size of 51 nm with a standard deviation (SD) of 22 nm and a concentration of 5.4×10^9 particles/mL. The zeta potential value was found to be -8.70 mV. FTIR spectroscopy revealed the presence of functional groups, stabilizing the myco-AgNPs that corresponded to the proteins from the fungal extract. TEM analysis showed spherical-shaped AgNPs with an average size range of 20-30 nm. The results suggest that the endophytic fungal extract is capable of synthesizing myco-AgNPs. The synthesized myco-AgNPs could be used in the formulation of novel nano-products for sustainable disease management of agricultural crops.

Keywords: Agriculture, *Epicoccum nigrum*, Myco-AgNPs, Nanobiotechnology

INTRODUCTION

Agriculture is a backbone of global wealth and most of the population is dependent on it for their livelihood. The plant pathogens pose a greater challenge, as they acquire resistance against fungicides or antibiotics and most of the pathogenic strains survive after treatment. Therefore, there is an urgent need to develop sustainable approaches that could manage the diseases in plants to prevent yield losses. Nanotechnology (NT) is getting fascination as a new generation of advanced technology, withstanding novel methods to mitigate technological problems and their solutions (Rai and Golińska 2021; Shende *et al*., 2021a; Rai *et al*., 2023). The manipulation of materials at the nanoscale and their application has become an important part of nanobiotechnology (NBT), where the biological resources such as plants, fungi, bacteria, etc. are used to fabricate nanoparticles (NPs) (Rai *et al.*, 2021). At nanometer size, NPs showed a distinct improvement in their properties because of a larger surface-to-volume ratio (Saleha *et al*., 2023; Gaikwad *et al*. 2013; Shende *et al*., 2021b). In recent years biogenically synthesized NPs are extensively applied and are used in most of the agricultural products that are employed for crop plants, such as nano-fungicides, nano-pesticides, etc. to combat the diseases. These approaches are considered as, sustainable, and novel approaches to use and to control the plant diseases and decrease the individual losses. The use of fungi for the synthesis of NPs as biological system is a termed as 'Myconanotechnology' and it was first coined by Rai *et al*. in 2009. Fungi are a splendid source of secondary metabolites such as amines, proteins, alkenes, phenols, flavonoids, lipids, terpenes, and many more. These secondary metabolites are explored for their ability to share electrons with ionic entities such as metal ions, e.g., silver (Ag^+), copper ion ($Cu^{+/2+}$), gold (Au^+), zinc (Zn^{2+}), etc. (Gade *et al*., 2014; Shende *et al.*, 2017; Rai *et al.*, 2023). The release or sharing of electrons stabilizes the ions and abolishes their charge. Therefore, the

properties of precursor bulk counterparts change significantly, and it converted to nanoscale materials.

In the present study, the secondary metabolites from the cell free extract of endophytic fungus *Epicoccum nigrum* Ehrenb. Ex. Schlecht. isolated from the leaves of host plant *Dioscorea bulbifera* (L.) or Karanda (Marathi name), was exploited to reduce and to stabilize Ag^+ ions to convert them into AgNPs (hereafter as myco-AgNPs). The detection and characterization of myco-AgNPs was done by various instrumental techniques and their further applications in plant disease management are described in further sections.

MATERIALS AND METHODS

The endophytic fungus *E. nigrum*. was isolated on potato dextrose agar (PDA) from host plant *D. bulbifera* and identified by morpho-taxonomically under microscope for the spore structure and conidial morphology. Then the isolated fungus *E. nigrum*. was cultured on potato dextrose broth for seven days or until the sufficient fungal growth was observed in the form of fungal mycelial mat. The mycelium was separated and thoroughly washed thrice with sterilized distilled water. The fugal mycelium was then suspended in sterile distilled water for 24 hours in the conical flask on constant stirring at 120 rpm in an orbital shaker at room temperature. The extracellular secondary metabolites are secreted from fungal mycelium due to stressed condition into the water. The fungal biomass was separated by simple filtration through nitrocellulose filter membrane to obtain cell free extract. The fungal extract obtained was exploited to fabricate myco-AgNPs from silver nitrate ($AgNO_3$) by adding 1 mM stock solution in a fungal extract and kept it in sunlight for 15-20 minutes. The color change in the reaction mixture was observed to confirm the formation of NPs and primarily detected by UV-Vis spectrophotometry (Shimadzu UV-1900, Japan) (Gade *et al*., 2014). The colloidal suspension of myco-AgNPs was further used for characterization by nanoparticle tracking and analysis (NTA) (Malvern's Nanosight LM 20, UK), Zeta potential analysis (Malvern's Zetasizer NanoZS-90, UK), Fourier Transform Infrared (FT-IR) spectroscopy (BrukerOptics, GmbH, Germany), and transmission electron microscopy (TEM) and selected area electron diffraction (SAED) studies.

RESULTS AND DISCUSSION

The endophytic fungus *E. nigrum* was isolated on the PDA from leaves of host plant *D. bulbifera* (L.) or Karanda (Marathi name) which is commonly known as the air potato/air yam/bitter yam/cheeky yam/potato yam/vine yam, species of true yam of Dioscoreaceae family (**Figure 1A**). **Figure 1B, C** demonstrated the morpho-taxonomic observation of fungal culture, which showed that the fungal hyphae were filamentous, septate, hyaline, smooth, and turned to brown on maturation (de Lima Fávaro *et al*., 2011; Braga *et al*., 2018). The conidiophores were short, originated in clusters on hypha, repeatedly branched, visible as dense masses, claviform, 1-3 septate, hyaline, smooth, 8-11.5 µm, terminally produce a single dark gangliospore.

Figure 1: A, Host plant *Dioscorea bulbifera* (L.) or Karanda (Marathi name); **B**, Mycelium and Conidiophores of *Epicoccum nigrum* Ehrenb. Ex. Schlecht; **C**, Mature gangliospore.

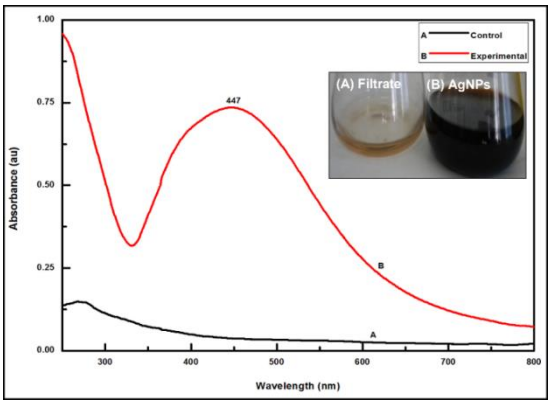

Figure 2: UV-Vis spectral analysis. **A**, Filtrate of *Epicoccum nigrum* Ehrenb. Ex. Schlecht extract (Control); **B**, colloidal myco-AgNPs showing absorption maxima at about 447 nm.

Conidia was found to be non-septate, rounded, and pale in color. Mature gangliospore was brown/golden brown/black to olivaceous colored and they are pyriform or globose or sometimes of irregular angular shape, septate, verrucose, muriform,14.5-3.9 µm in diameter. Mature conidia showed multiple vertical and transverse septa. The cell free fungal extract when reacted with $AgNO_3$ (1 mM) it turned colorless to brown coloration of reaction solution which depicts the formation of

myco-AgNPs (**Figure 2A, B**). The primary detection by spectrophotometric analysis revealed the absorption maxima for surface plasmon resonance (SPR) of synthesized myco-AgNPs at 447 nm as shown **Figure 2**).

The further characterization of myco-AgNPs by NTA system exhibited the average size of 51 nm, with a mode value of 38 nm and standard deviation of 22 nm (**Figure 3A**), and the 2D distribution showed major concentration below 100 nm with particle concentration of 5.4×10^9 particles/mL of myco-AgNPs (**Figure 3B**). Zeta potential measurement was found to be an average value of -8.70 mV with a deviation of 5.74 mV (**Figure 3C**). FT-IR measurements of experimental sample were carried out to identify the possible functional groups in fungal extract responsible to reduce Ag^+ ions, capping and efficient stabilization of NPs. The FT-IR spectrum of myco-AgNPs in the capping layer of *E. nigrum* stabilized. The strong broad band at 3403 cm^{-1} for –OH stretching (aliphatic hydroxyl group) and maybe for –N-H stretching (amide I or II groups), 2919 cm^{-1} for C–H stretching (aliphatic C–H), weak band at 1654 cm^{-1} for C=C (Alkene), sharp band at 1377 cm^{-1} for C–N stretching (aromatic amines) (**Figure 3D**). There are functionalization of hydroxyl, carbonyl, and amide groups at the respective banding positions, suggesting that these groups are accomplished binding to metal and possibly form metal NPs (i.e., capping AgNPs) to prevent agglomeration and to, thus, stabilize them (Gade *et al*., 2014; Abdel-Hafez *et al*., 2017). **Figure 3E** depicts the TEM micrograph and SAED pattern of myco-AgNPs which showed an average size in the range of 20-30 nm with spherical-shaped NPs. The SAED pattern demonstrated the crystalline nature of myco-synthesized AgNPs (**Figure 3E**).

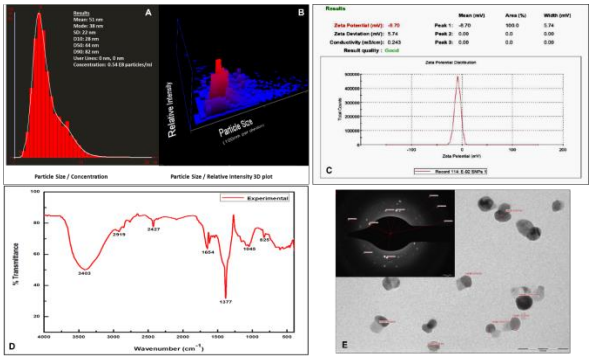

Figure 3: Characterization of myco-AgNPs. **A**, NTA analysis; **B**, 2D distribution; **C**, Zeta potential measurement; **D**, FTIR spectrum; **E**, TEM micrograph (inset SAED pattern).

Though many methods involving chemical and physical processes have been developed, biological methods using natural resources such as bacteria, fungi, and plants have appeared as economical, safe, stable, sustainable, and effective alternatives for the biogenic synthesis of AgNPs. In this study, *E. nigrum* was isolated and identified as a prevalent endophytic fungus associated with host plant *D. bulbifera* or Karanda (Marathi name). Host plant *D. bulbifera* is commonly known as the air potato/air yam/bitter yam/cheeky yam/potato yam/vine yam, species of true yam of Dioscoreaceae family. Karanda is native to Africa, Southern Asia, India, Sri Lanka, in Sinhalese it is called 'Guvan Arthaapal'. Maldives, in Dhivehi it is known as 'Mathivah', China, Japan, the Philippines, and Indonesia and northern Australia. The plant and bulbils are generally used as a folk remedy to treat conjunctivitis, diarrhea, and dysentery among other ailments. (https://en.wikipedia.org/wiki/Dioscorea_bulbifera#cite_note-WCSP-1).

Endophytic fungi are endosymbiont, and ubiquitous those enhances host plant growth, increase nutrient acquisition, and improve the plant's ability to tolerate abiotic stresses, such as drought, improve resistance to insects, plant pathogens, and herbivores. Fungi are found in almost all the plants species, that is they appear to be ubiquitous. In fact, very few studies still observed the presence of a plant species devoid of endophytes (Promputtha *et al*., 2007; Abdel-Hafez *et al*., 2017). With respect to this, many investigators emphasized the isolation of *E. nigrum* as endophyte from many crops (Martini *et al*., 2009; de Lima Favaro *et al*., 2012). Endophytic fungus *E. nigrum* is considered as one of the worthiest fungal species that produce bioactive secondary metabolites (Wang *et al*., 2010; Musetti *et al*., 2011). *E. nigrum* was a source of natural compounds with biological properties including antithrombosis, antiviral, antioxidants, antifibrosis, and antihypertensive activities (Zhang *et al*., 2007; Wang *et al*., 2010; Abdel-Hafez *et al*., 2017). In the study by Abdel-Hafez *et al*., (2017), the authors have evaluated the potential of *E. nigrum* to make fascinating bioactive secondary metabolites. In their investigation, the authors isolated and purified the antioxidant compound curvularin from the ethyl acetate extract of *E. nigrum*, that has been previously reported as an extracellular metabolite produced by many fungal species from genera like *Alternaria* (Robeson and Strobel, 1981), *Curvularia* (Musgrave, 1956; Dai *et al*., 2010), and *Penicillium* (Kobayashi *et al*., 1988; Meng *et al*., 2013). Additionally, they employed this curvularin compound for the myco-fabrication of AgNPs and observed the color change in the reaction solution to reddish brown and dark-brown due to the excitation of the SPR effect corroborated with the present study. This observation agreed with the procedures of preparation of metal NPs (Shende *et al*., 2017). The UV-Vis spectral analysis revealed a strong absorbance peak at 447 nm at different time intervals which is characteristic for SPR of myco-AgNPs corresponding to the 425 nm (Abdel-Hafez

et al., 2017) and hence indicates the formation of myco-AgNPs. The UV-Vis absorption spectra also showed good stability of the formed myco-AgNPs after being stored for one month at room temperature which are well dispersed in solution without any agglomeration. Furthermore, the myco-AgNPs capped with biomolecules and curvularin compounds were characterized by NTA, Zeta potential analysis, FT-IR spectroscopy, TEM and SAED studies. The results showed the formation of spherical myco-AgNPs with an average size of 51 nm by NTA and 20-30 nm by TEM analysis, which corroborated with the findings by Gade *et al*., (2014). The FT-IR results suggested that hydroxyl, amide, and carbonyl functional groups from proteins, and possibly curvularin compounds are responsible for the reduction of the Ag^+ ions and stabilizing myco-AgNPs as mentioned in the study by Abdel-Hafez *et al*., (2017). Moreover, this study recommends that the functional groups from proteins and possibly curvularin compounds may play two roles in the biosynthesis and stabilization of myco-AgNPs in aqueous medium. This study recommends conventional mechanism of myco-AgNPs synthesis by the biomolecules from *E. nigrum* according to the presence of amide, hydroxyl, and carbonyl groups in proteins and curvularin compounds. During the process of reduction, the hydroxyl groups were tending oxidized to carbonyl groups and Ag^+ ions were reduced through free electron produced to form myco-AgNPs.

Applications of Myco-AgNPs

In recent years, exploration of AgNPs for various biological and environmental applications has become one of the most important attributes of NBT because of their remarkable physicochemical properties and use in wide-ranging applications. Also, they have demonstrated great commercial applications, since they have great chemical stability, catalytic activity, conductivity, and antimicrobial potential. The anti-potential of AgNPs has been highlighted in several recent research that implementing their application for controlling the growth and spread of infectious pathogens such as resistant strains against fungicides and antibiotics, since the AgNPs introduction efficiently diminishes plant diseases instigated by a spectrum of bacteria and fungi in modern agricultural practices. The antimicrobial activity of AgNPs to plant pathogenic organisms may be identified as their properties to inhibit or exclude the pathogen's growth deprived of damaging adjoining plant tissues (Panja *et al*., 2021). AgNPs exist to be the most fascinating of metal NPs that demonstrate acceptable biological activity (Zhang *et al*., 2016). At specific concentrations, AgNPs exhibited a broad range of advantageous effects on plants (Mehmood, 2018) and prevent the growth of related fungal pathogens (Haroon *et al*., 2019). Several publications reported that AgNPs have shown the boosting in seed germination (Parveen and Rao, 2015), growth triumph (Jasim *et al*., 2017), vegetative growth, shoot induction, and proliferation (Saha and Gupta, 2018), and increased pigmentation (Latif *et al*., 2017) by preventing the pathogens attack and disease hinderance in plants. With recent examples, Tariq *et al*. (2022) comprehensively reviewed the current advancement made in the AgNPs synthesis by biogenic methods and deliberates their potential applications as in various antifungal, antibacterial, antinematic, and antiviral from a plant disease perspective. In line with this, we propose the application of myco-AgNPs in sustainable plant disease management to agriculture. Myco-AgNPs are prospective NPs for the successful inhibition of pathogen growth and plant disease management. They could also be used in the disease diagnosis, in the development of nano-fungicides, nano-pesticides, etc., development of agri-products, and post-harvest disease management of agriculture crops as shown in **figure 4**.

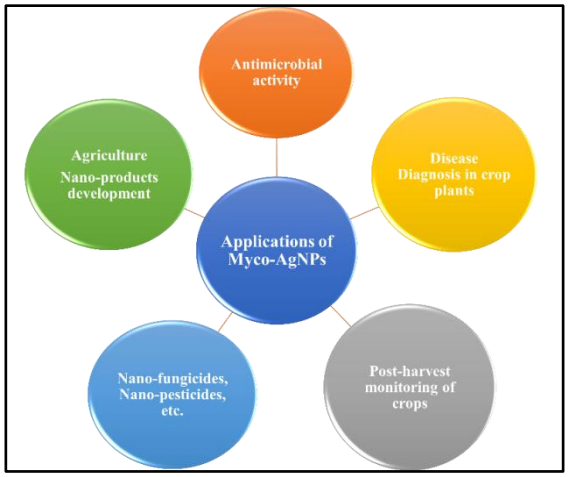

Figure 4: Applications of myco-AgNPs in sustainable plant disease management

The application of myco-AgNPs produced by *E. nigrum*, Elkhateeb and Daba (2019) reported the action mechanisms demonstrated by *E. nigrum* as a biocontrol agent differs from reducing host stem disease severity index and growing along the fungal pathogen hyphae and inducing its lysis (Herzner *et al*., 2013), or through causing degradation of the pathogen protoplast, malformation in its hyphae, and leakage of cytoplasm (Ramos *et al*., 2013). The production of different antimicrobial and bioactive compounds by *E. nigrum* such as polyketide, flavipin, and other, causing growth inhibition of numerous phytopathogenic fungi (Bamford *et al*., 1961; Madrigal *et al*., 1991; de Lima Fávaro *et al*., 2011; Dzoyem *et al*., 2017; Braga *et al*., 2018).

Alternatively, epicolactone isolated from *E. nigrum* has antifungal activity and can induce root growth (Musetti *et al*., 2011; Li *et al*., 2013; Li *et al*., 2017). All those studies support using *Epicoccum* species in different host plants as a safe biological control agent and encourage deep investigations for further understanding of the physiological and molecular aspects of this interaction (de Lima Fávaro *et al*., 2012; Xiao *et al*., 2013; Ye *et al*., 2013). From the above discussion it is clearly suggested that these bioactive compounds capped to the synthesized myco-AgNPs, which synergistically affect disease management in the plants.

CONCLUSION

The present study concluded that the cell free extract of endophytic fungus *Epicoccum nigrum* Ehrenb. Ex. Schlecht. isolated from the leaves of host plant *Dioscorea bulbifera* (L.) could be used to synthesize stable myco-AgNPs which is an easy, rapid, cost-effective, and sustainable approach.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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