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The field of nanoscience is an emergent area of research in the agriculture sector owing to itswide spectrum of applications. Green synthesized nanoparticles are promising options as bacteriocides, in agricultural fields. Among the different metal nanoparticles, silver nanoparticles have been found to be maximally explored due to its high antimicrobial activity. The objectives of this study was to synthesize silver nanoparticles directly from the fruiting body, the pileus of the basidiocarp, of an edible mushroom. The pileus tissue of the milky mushroom, *Calocybe indica* was used for the green synthesis and the resulting silver nanoparticles were assayed for antibacterial efficacy against phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato*. The spherical biogenic silver nanoparticles showed a characteristic peak at 430 nm. The Scanning electron micrograph showed dispersed silver nanoparticles below 50 mm in radii. The silver nanoparticles showed concentration dependent strong antimicrobial activity against *P.syringae* as revealed by disc diffusion assays and growth kinetic studies. The mechanism behind antibacterial activity of these silver nanoparticles seem to be the disruption of the bacterial cell wall and loss of cell integrity as revealed by FE-SEM.

Keywords: Antibacterial, *Calocybe indica,* green synthesis, phytopathogen, *Pseudomonas syringae,* silver nanoparticles

INTRODUCTION

The vast discipline of nanotechnology is comparatively new to the contemporary science. This state-of-the-art technology primarily deals with synthesis, characterizations and application of the particulate matters with a size range of 1-100 nm, inorganic or organic in nature by origin (Khan *et al*. 2019). They are the particles which bridge the gap between bulk materials and elemental structures.Traditionally the fabrication of nanoparticles have been carried out by physical and chemical methods but these methods have been associated with many limitations such as high cost, low production, use of toxic chemicals etc. Consequently green synthesis or biological method of nanoparticles biosynthesis has emerged as an alternative tool for the sake of its sustainability.

The fabrication of nanoparticles through green synthesis method has been carried out using organisms of any biological entity (Gour *et al.* 2019). The advantages of green synthesis over physical and chemical method are cost effectiveness, high stabilization,eco-friendliness etc.

In recent years, the field of nanoscience has come up as a growing area of interest in agriculture sector owing to the widerange of applications (Shang *et al*. 2019). Due to the eco-friendly nature, there is greater inclination towards green synthesis methods for production of nanoparticles to be used in the control and management of phytopathogens,and thereby plant diseases. Nanoparticles can provide direct protection to crops and other plants from diseases by directly inhibiting or killing various phytopathogens and pests *viz.* bacteria, fungi, viruses, nematodes, aphids and other parasites (Henriquez *et al.* 2020). Among the different metal nanoparticles, silver nanoparticles (AgNPs) have been found to be maximally explored due to its high antimicrobial activity. Among different biological organisms, fungi and specially

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mushroom reservoirs have been utilized immensely for biogenic fabrication of AgNPs (Guilger-Casagrande and Lima. 2019;Al-Dbass*et al.* 2022). Previously it has been reported from our laboratory that AgNPs from cell-free filtrate of *Tricholoma crassum* (Ray *et al*. 2011) and *Macrophomina phaseolina* (Chowdhury *et al*. 2014) showed strong antimicrobial activity against pathogenic fungi and bacteria.

To ensure the food security of exploding global population, it is imperative to increase the yield potential of crop plants by reducing the loss of agricultural products as much as possible through plant disease management. Every year, the crop yield is severely hindered by several phytopathogens and insect pests (Flood, 2010). Among different notorious phytopathogenic bacteria, *Pseudomonas syringae* pv. *tomato* (Van Hall), causal organism of bacterial speck disease of tomato is responsible for significant yield losses and marketability of the crop (Pedley and Martin. 2003). Besides tomato, this bacterium can also infect the model plant *Arabidopsis thaliana* (Xin *et al*. 2013). Nanoparticles have the potential to be used as pesticides or protectant against various plant pathogens promoting high crop yield through plant disease management. Nanoparticles possess several advantages over raw chemical pesticides *viz.* target specific action, high shelf life, increase solubility and less leaching or toxicity (Hayles *et al.* 2017).

In this context, our present report deals with extracellular biosynthesis of AgNPs using tissue filtrate directly from the pileus tissue of basidiocarps of *Calocybe indica* Purkayastha and Chandra. These nanoparticles were characterized with spectrophotometry and scanning electron microscopy. These were finally assayed for antibacterial activity against the plant pathogenic bacteria *Pseudomonas syringae* pv. *tomato* in terms of growth inhibition, inference of growth kinetics and cell damage.

MATERIALS AND METHODS

Growth conditions for fungus and bacterium

Fresh basidiocarps of *Calocybe indica* were collected from cultivation center of Narendrapur, Kolkata, used for AgNPs biosynthesis (Fig.1 A). The plant pathogenic bacterium, *Pseudomonas syringae* pv. *tomato* was grown and maintained in Luria Bertani Agar medium (LBA) used for antibacterial assay.

Biosynthesis of silver nanoparticles

1.0 g of pileus tissue from fresh basidiocarps of *C. indica* was chopped into tiny pieces and agitated with 10 ml of deionized water at 28°C for 48 hr in an orbital shaker at 50 RPM. The collected extract was filtered through Whatman filter paper No. 1. This tissue filtrate (pH 7) was incubated with 1 mM solution of silver nitrate ($AgNO_3$) and agitated at 37°C in the dark for 3 hr. The reaction mixture was observed regularly for change of color of the mixture.

Characterization of silver nanoparticles
UV-visible spectroscopy

After production of the AgNPs, the suspension was analyzed using Hitachi U-2000 spectrophotometer range between 350 and 600 nm. An absorbance was plotted to get the absorbance spectra. The peak of the curve was marked.

Scanning electron microscopic (SEM) observation of nanoparticles

Morphological analysis of biosynthesized AgNPs was done using SEM. 10 µl nanoparticles suspension was used to make a thin film on a glass stub and was vacuum dried. It was subjected to SEM using Carl Zeiss EVO 18, Germany.

Antibacterial and bacterial growth kinetic assay

These assays were done according to Chowdhury *et al.* (2014). For disc diffusion assay, a dilution series of AgNPs was made with 25, 50, 75, and 100% v/v using stock suspension of nanoparticles of concentration 50.25 mg/L with double distilled sterile water. The concentration of AgNPs in stock suspension was calculated according to Chowdhury et al. (2014). 25 µl of fresh overnight grown bacterial cultures of *P.*

syringae were spread on LBA plate. Sterilized paper discs of 5 mm diameter with sterile double distilled water (control) and four increasing concentration of AgNPs suspension (25, 50, 75, and 100% v/v of stock AgNP suspension) were placed on plates and incubated for 48 hr at 28 °C. Diameter of inhibition zones was observed after incubation period. Bacterial growth kinetics assay was performed according to Basu *et al*. (2018). The O.D. value of the control and nanoparticles treated (50% v/v of stock AgNP suspension) bacterial culture was recorded at 600 nm for regular intervals (0, 2, 4, 6, 12 and 24 hr post inoculation). This was used to prepare the growth curve of nanoparticles treated bacterial culture and was compared with that of the untreated control.

Analysis of morphologyof bacterial cell by field emission-scanning electron microscope

Fresh overnight grown culture of *P.syringae* were treated with 50% v/v of stock AgNP suspension (Stock suspension concentration is 50.25 mg/L), for 6 hr at 28 °C. The untreated control and AgNPs treated bacterial cultures were centrifuged and the pellet was washed to obtain cells. Then the samples were prepared and observed under FE-SEM (JEOL JSM-7600F;JEOL Ltd., Japan) according to protocol of Han *et al*. (2021).

Statistical analysis

All experimental data were analyzed according to our previous publication (Chowdhury et al., 2017). All analysis were performed with three independent experiments with at least three replicates each and values represented as the mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) with different letters indicating significant difference between treatments at $p < 0.05$, according to Duncan's multiple range test (DMRT), using a software package, SPPS (version 16, 2007).

RESULTS AND DISCUSSION

Biosynthesis of silver nanoparticles

The tissue filtrate of pileus of *C.indica* was utilized for synthesis of AgNPs as described in methods. Fig.1B shows that fungal tissue filtrate was almost colourless (tube "a") and 1mM solution of $AgNO_3$ without fungal filtrate was pale white (tube "c"). After incubation of fungal cell free tissue filtrate with 1 mM $AgNO_3$ solution underwent a distinct colour change of the mixture to brown within 3 hr indicating the extracellular biosynthesis of AgNPs (tube "b").The colour of nanoparticles solution persisted even after 72 hrs indicating dispersed and stable nature of the AgNPs in the suspension. Cell free fungal filtrate has been known to Cause bioreduction and stabilization of precursor metal salt to nanoparticles in extracellular suspensions, where synthesized nanoparticles could be collected easily (Rodríguez-Serrano *et al.* 2020). Fungal mediated nanoparticles synthesis is considered more advantageous than bacterial or Actinomycetes mediated fabrication; this is due to the higher production of reducing enzymes by fungal biomass in culture media (Guilger-Casagrande and Lima. 2019).

UV-VIS spectroscopy of silver nanoparticles

UV-VIS spectrum of the biosynthesized AgNPs is presented in Fig.1C**.** Here the absorbance peak shows symmetric single band with peak maximum at 430 nm for the AgNPs produced over 24,48 and 72 hrs of incubation of cell filtrate with $AgNO_3$. The steady increase in peak intensity without any shift as a function of reaction time indicate longitudinal excitation of surface plasmon resonance (SPR), typical of stable silver nanoparticles (Chowdhury et al. 2014).

Morphological study of the AgNPs with Scanning Electron Microscopy

The morphology of the AgNPs observed under Scanning Electron Microscopy (SEM) (magnification x 50000) revealed that the AgNPs were spherical in shape and polydispersed in nature (Fig.1D). The nanoparticles were not aggregated suggested stabilization of the particles.

Antibacterial activity of the AgNPs against plant pathogenic bacteria

The AgNPs exhibited strong antibacterial activity against plant pathogenic bacterium *Pseudomonas syringae* pv. *tomato* in a dose-

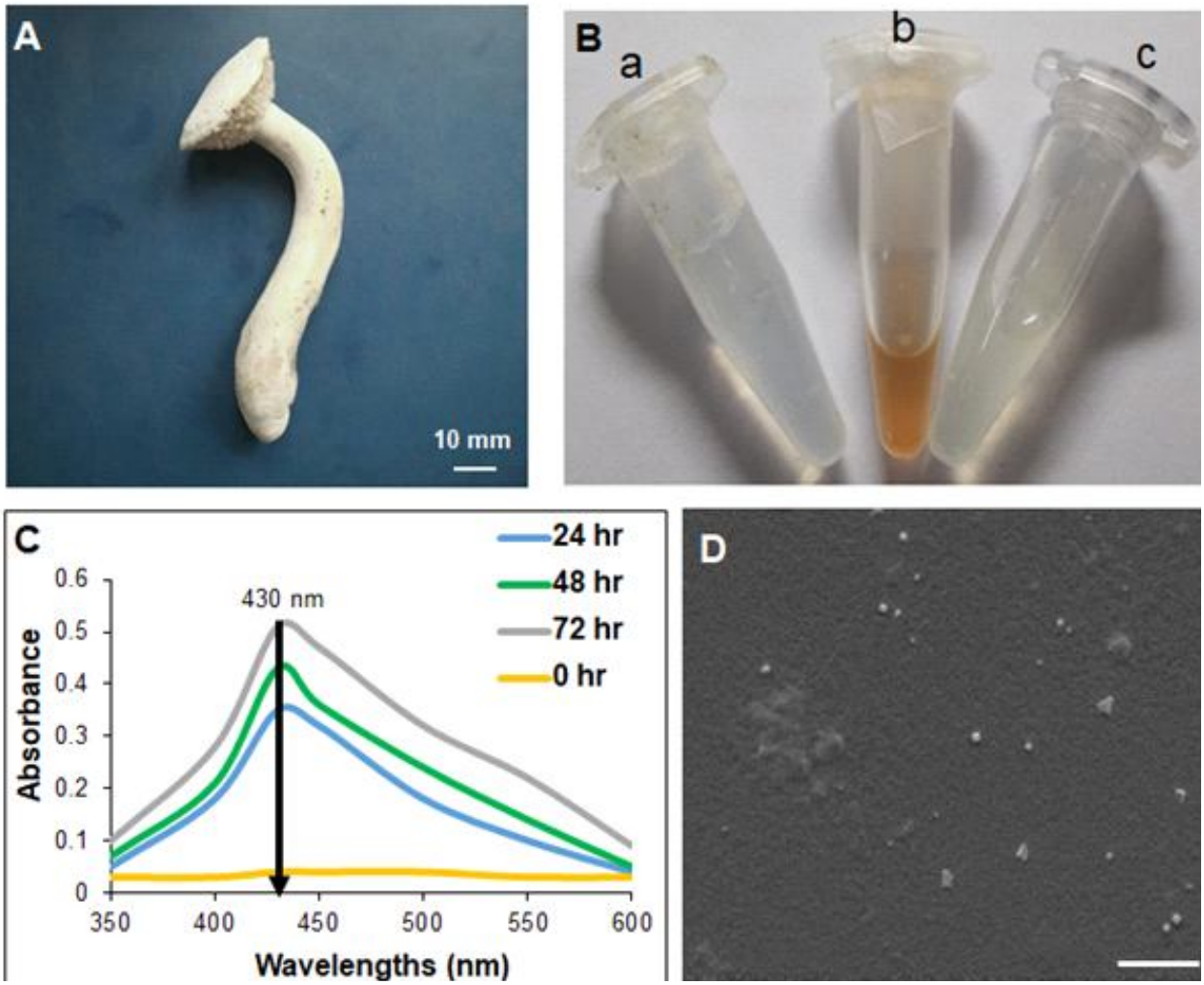

Fig.1: Biosynthesis of silver nanoparticles (AgNPs) using tissue filtrate of pileus of *Calocybe indica*, spectroscopic analysis and SEM characteristics**.** (A) Basidiocarp of *C. indica* as the source of the reducing enzymes for green synthesis of the AgNPs. (B) Colour change during reaction. a. Tissue filtrate of pileus of *C. indica*, b. 1 mM silver nitrate ($AgNO_3$) with tissue filtrate for 3 hr at 37°C showing brown colour indicating synthesis of AgNPs, c. 1 mM solution of $AgNO_3$. (C) UV-VIS spectra of the biosynthesized AgNPs. Solid arrow shows absorption peak at 430 nm. (D) Scanning electron microscopic (SEM) image showing particles distribution of biosynthesized AgNPs in a field. (Bar = 200 nm).

dependent manner. The antibacterial activity of the AgNPs was analysed using paper discs with increasing amount of nanoparticles suspension i.e., 25, 50, 75 and 100% v/v of stock AgNP suspension(Stock suspension concentration is 50.25 mg/L). The AgNPs were inhibitory to bacteria even at the lowest concentration and the inhibition zones increased proportionately with increasing concentration of AgNPs (Fig. 2A). Inhibition zones of *P.syringae* were found to be a function of the amount of AgNPs, as depicted in Fig.2B. The exact mode of action of AgNPs in retardation of phytopathogens' growth is not thoroughly known. TEM and SEM studied revealed that NPs could attach with bacterial cell wall and disrupt its normal functioning (Ali *et al.* 2020). As cell wall protects the inner content of the cell from various external stresses, abnormalities in its normal activities may lead to cell instability (Abdel-Aziza *et al.* 2020). Additionally, NPs induced oxidative stress and increased ROS production which in turn damaged several components of cells essential for survival (Fu *et al*. 2014). Antimicrobial activities of silver nanoparticles from other fungal sources like *Macrophomina phaseolina* (Chowdhury *et al*. 2014) and *Tricholoma crassum* (Ray *et al.* 2011) gave similar observations.

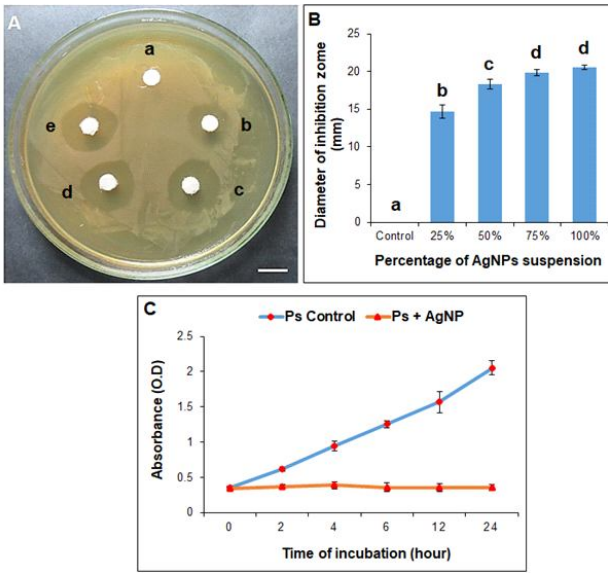

Fig.2: Assay of antibacterial properties of the silver nanoparticles against plant pathogenic bacteria *Pseudomonas syringae* pv *tomato***.** (A,B) Plate and corresponding graph showing disc-diffusion assay of the AgNPs with increasing inhibition zones for *P. syringae*. All experiments were done with increasing amounts of AgNPs on paper discs; clock-wise from top: a: control; b: 25%; c: 50%; d: 75%, and e: 100% v/v AgNP stock suspension (Conc 50.25 mg/L). Data are means ± SE of three replicates. Different letters indicate statistically significant differences among the samples (Bar = 1 cm). (C) Inhibitory effect of AgNPs on the growth kinetics of bacteria. Absorbance data for bacterial growth of *P. syringae* without (control) or with nanoparticles for 0, 2, 4, 6, 12, and 24 hrs postinoculation showing significant inhibitory effect on the growth kinetics of the bacteria.

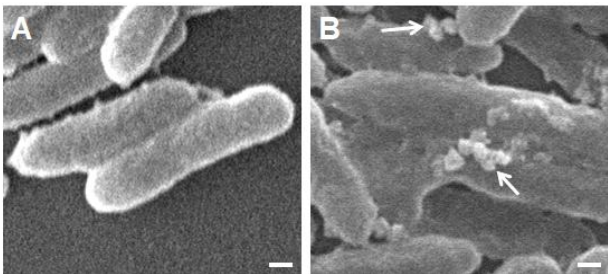

Fig.3: Effect of the silver nanoparticles on cell morphology of *Pseudomonas syringae***.** FE-SEM micrographs of *P. syringae* cells in (A) untreated control set showing healthy cells with intact cell membranes, (B) set treated with the green synthesized AgNPs showing loss of integrity of the cells, swelling and aggregation. Arrows shows the location of the AgNPs. (Bar = 200 nm).

Effect of the AgNPs on the kinetics of P.syringae growth

The growth kinetics of the bacteria *P.syringae* (Fig.2C) was clearly suppressed by the addition of the AgNPs. Growth of *P.syringae* showed inhibition of growth within 2 hr post-inoculation with slightly less optical density readings at all subsequent time points compared to the control, due to partial dissolution of the bacterial cells. This observation of reduced growth rate of bacterial cells was due to antimicrobial activity of silver nanoparticles.

Effect of the AgNPs on bacterial cell morphology

Normal *P. syringae* bacteria are rod shaped with clear smooth outlines (Fig. 3A). The bacteria when incubated with the AgNPs showed damaged cells, with irregular outline, and loss of integrity (Fig. 3B). The cells also showed aggregation. Recent reports revealed that biogenic AgNPs from *Phyllanthus emblica* (Masum*et. al.* 2019) and *Citrus maxima* fruit extract (Ali*et al*. 2020) showed *in vitro* antibacterial activity against rice bacterial brown stripe pathogen *Acidovorax oryzae*. These biogenic AgNPs also inhibited biofilm formation, swarming motility ability and disrupt bacterial cell wall and cell membrane as observed in TEM.

CONCLUSION

It can be concluded that biogenic AgNPs from pileus tissue filtrate of *C. indica* exhibited strong antibacterial activity against bacterial pathogen of tomato, *P.syringae.* The AgNPs showed concentration dependent inhibition of bacterial growth and division kinetics. The mechanism behind antibacterial activity of AgNPs seemed to be the disruption of cell wall and destruction of cell integrity.

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