Research Article

# Mycosynthesis, Optimisation and Characterization of Silver Nanoparticles by Endophytic Fungus Isolated from the Root of *Casuarina junghuhniana* Miq.

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**Received:** 07-01-2017; **Revised:** 28-02-2017; **Accepted:** 15-03-2017.

**ABSTRACT**

Endophytes are microorganisms which reside within the plant without causing any harm to its host. It is a source of various novel secondary metabolites which has a wide range of application including synthesis of nanoparticles. In the recent past, green synthesis of silver nanoparticles by developing an eco-friendly and reliable process has drawn much interest in the field of nanotechnology. Silver nanoparticles are used extensively in the field of research in agriculture and medicine. *Casuarina junghuhniana* Miq. (Casuarinaceae) is a multipurpose, non-leguminous tree which is rich in phytoconstituents like phenols, flavonoids, carbohydrates etc can withstand extreme edaphic and adverse climatic condition. Therefore, in the present study, endophytic fungi were isolated from the root of *Casuarina junghuhniana* Miq. and it was screened for extracellular biosynthesis of silver nano particles. Fungal isolate, CJN5, was found effective in the synthesis of silver nanoparticles. UV-Vis spectroscopic analysis showed absorption peak at 424nm which is a characteristic wavelength for silver nanoparticles. The synthesis of silver nanoparticle was optimized on the basis of different parameters like pH, temperature, concentration of silver nitrate solution and stability. Further, silver nanoparticles were characterized using FTIR and SEM-EDX analysis. The silver nanoparticles were assessed for its antimicrobial activity where maximum zone of inhibition was found against *Fusarium solani* (30±0.2mm) for fungal phytopathogen and *Bacillus subtitils* (22±0.4 mm) for bacterial pathogen. The effect of silver nanoparticle on germination, growth (shoot-root length) and biomass was studied using *Vigna radiata*. Further, the effect of silver nanoparticles on photocatalytic degradation of methylene blue was analysed. The results revealed that the biosynthesized silver nanoparticles can be effectively utilised for growth improvement and disease management of commercially important crop plants.

**Keywords:** Silver nanoparticles, *Casuarina junghuhniana*, optimization, characterization, germination.

## INTRODUCTION

Nanotechnology is an emerging field of science which deals with synthesis of different metal nano particles .[1] Nano particles can be produced by biological and non-biological methods. Biological synthesis is preferred in respect to non-biological method by the researchers as the latter is more expensive and release hazardous chemicals.[2] In biological system, various organisms like plants, bacteria, fungi and yeast involves in the synthesis of nano particle.[3] In comparison to various other metal nano particles, silver nano particles are known best since ancient times for its various application like medical, agriculture and textile industries.[4] The efficiency of nano particle depends mainly on its size , which in turn depends on its condition of synthesis ,i.e, concentration, reaction temperature and pH.[5]Silver particles are known to be more effective for its high surface area to volume ratio as a result of which large proportion of synthesized nano particles are in direct contact with its environment .[6]

Silver nano particles are synthesized by fungi by extracellular method.[7-8] Endophytic fungi have a symbiotic relationship with the host plant without causing any negative symptoms. Several researches proved that endophytic fungus are able to produce secondary metabolites similar to that of its host plant. In recent past it is found that endophytic fungi plays an important role in synthesis of nano particles as they are rich source of important enzymes and secondary metabolites. [9] Therefore, in the present study, the endophytic fungi were isolated from the root of *Casuarina junghuhniana* Miq .

*Casuarina junghuhniana* Miq. is an exotic, actinorhizal tree crop which belongs to family Casuarinaceae. It can withstand wide range of soil and climatic condition. It is a well-known multipurpose tree species which is extensively utilized for pulping in paper industries, in construction as poles and in soil reclamation. This plant is reported to be rich in many phyto constituents like alkaloids, carbohydrates, triterpenoids, phenols, flavonoids, tannins and steroids.[10]

Synthetic dyes are widely used in textile industries. The removal of the non-biodegradable harmful chemicals of the synthetic dyes from environment is a vital ecological problem. Several techniques such as flocculation, electro coagulation, redox treatment and UV light degradation are generally used for abating dye.[11] However, present situation demands improved technique to overcome the ineffectiveness of the existing process. In recent research, metal nano particles are reported to be an effective photo catalyst for degrading chemical complexes of synthetic dyes under visible light illumination.[12]

Several researches demonstrated that metal nano particle positively influence photosynthesis and nitrogen metabolism, thereby, improves plant growth at low concentration.[13] Germination of seeds is considered as most important phenomenon in agriculture because it is the first stage of the plant growth process. Application of nanotechnology in the field of agriculture is astonishingly increasing for which it is important to study the role of silver nano particle in seed germination.

A rapid emergence and re-emergence of multi-drug resistant pathogens with their antibiotic resistance profile has caused a great threat to environment and mankind .[9] Nanotechnology can be used effectively to combat this problem as nanoparticles having higher surface to volume ratio and decreasing size imparts its catalytic reactivity and antimicrobial activity .[14]

The objective of the present study is to screen endophytic fungus for its ability to bioreduce silver nitrate solution to silver nanoparticles and assess its bio efficacy for its effective use in the field of environment, agriculture and pharmaceutical industry.

## Materials and Methods

### Collection of plant sample:

The root samples of *Casuarina junhuhniana* Miq. were collected from State Forestry Research Institute, Neyveli, and Tamil Nadu. Identification and authentication of the plant sample was confirmed at Botanical Survey of India (BSI), Coimbatore, and TamilNadu [Confirmation I.D No: BSI/SRC/5/23/2015/Tech/2154].

### Isolation and identification of endophytic fungi

The plant root samples were washed thoroughly in running tap water to remove adhered soil particles and debris. It was then rewashed in distilled water. The root samples were sterilized using the modified method of Fisher *et al* (1993) and were individually inoculated in Potato Dextrose Agar (PDA) medium supplemented with Streptomycin (100 mg/L).[15] The inoculated Petri plates were incubated at 37˚C (room temperature) for 3 days. On appearance of fungal colonies, individual colonies were sub cultured in PDA plates and were stored at 4˚ C. CJN5, dominant fungal endophyte was identified using molecular tool.

### Molecular Identification

Molecular phylogenetic tree of fungal isolate CJN5 was determined using 18S rRNA sequencing. Extraction of DNA was carried out using NucleoSpin, Plant II Kit (Macherey-Nagel). In this study two primers NS1(forward sequence) and NS4(reverse sequence) were used. The PCR amplification of the isolated DNA was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Bio systems). Gel electrophoresis was carried out using 1% agarose to analyse the size of amplified PCR product. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 .[16]The DNA sequence was analysed using BLASTn (Nucleotide Basic Local Alignment Search tool) from the facility of National Centre for Biotechnology Information (NCBI). The evolutionary relationship of the fungus was studied using BLAST. The tree was constructed using MEGA5 Software.[17] The evolutionary history was inferred using the neighbouring-joining methods.

### Extracellular synthesis of silver nano particles

The isolated fungi, CJN5, was grown in 250ml Erlenmeyer flask containing 100ml Potato Dextrose Broth (PDB) for a period of 7 days incubated at 28±0.5˚C under shaking condition. After the period of incubation, the fungal biomass was harvested by filtration and was thoroughly washed in distilled water to remove any traces of adhered media component. 10gm of washed fungal biomass was weighed and suspended in 100ml of sterilized distilled water in 250ml conical flask and was incubated for 3 days at 28±0.5°C under shaking condition. After 3 days, the cell filtrate was obtained by removing the mycelial biomass through filtration. The cell filtrate was mixed with aqueous solution of $AgNO_3$ of 1mM concentration for reduction.[18]

### Optimization studies of silver nano particles [19]

a) **Effect of $AgNO_3$ concentration**
Synthesis of silvernano particles is dependent on its substrate concentration. The different concentration of cell filtrate to $AgNO_3$ solution, i.e, 1:1, 1:2, 1:3 was studied. The optimum concentration for the biosynthesis of silvernano particles were determined by UV visible absorption spectroscopy.

b) **Effect of pH**
pH is reported in having a prominent influence on growth and production of enzyme by fungus which is essential for biosynthesis of silver nano particles. Different pH of 3,5,7,9 and 11 was adjusted to study the influence of pH on production of silver nanoparticles. The absorbance of the resulting mixture was measured spectrophotometrically.

c) **Effect on temperature**
Temperature plays a significant role in all bioreactions. Optimization study was carried out at different temperature such as 20 ˚C, 40˚C and 60˚C for the production of silver nano particles. The absorption of the resulting solution was analysed using UV Visible Spectroscopy.

d) **Stability**
The optimized reaction solution was stored in dark condition at room temperature and the stability of the synthesised silver nano particles was assessed periodically for 45 days using UV Visible spectral analysis.

**Characterization of silver nano particles:**

**a) UV Vis Spectral analysis**

Synthesis of silver nano particle was initially determined by visual observation of colour change from pale yellow to brownish/dark brown. Further, sharp peak of UV Visible spectrum (UV 1650 PC Shimadzu) ranging between 390-440nm confirms the presence of silver nano particles.

**b) FTIR analysis**

The synthesized silver nano particles were air dried at room temperature, subjected to FTIR analysis to determine the different functional groups present in the sample. FT-IR spectral system (Shimadzu, IR Affinity 1, Japan), equipped with a DLATGS detector with a mirror speed of 2.8mm/sec. scan range: from 400-4000$cm^{-1}$ with a resolution of 4$cm^{-1}$ was used for this analysis. The dried samples were finely grounded using potassium bromide (KBr) in 1:10 ratio. The IR pellet was recorded in the region 4000-400 $cm^{-1}$ and the functional group of the biosynthesized silver nano particle was recorded.

**c) SEM and EDX analysis**

The synthesized silver nano particles were centrifuged for 10min at 10,000g. The obtained pellet was washed three times to remove any traces of silver nitrate. The silver nano pellet was then air dried and used for further analysis. The size and shape of the silver nano particles were observed using SEM (Scanning Electron Microscope) and elemental analysis were carried out with the help of EDX (Energy-Dispersive X–ray spectroscopy) (FEI Quanta FEG 200).

**Application of bio synthesized Silver nano particles**

***Antimicrobial activity***

Antimicrobial activity was carried out using different concentration (50µg and 100µg) of synthesised silver nano particle against fungal phyto pathogens, namely, *Fusarium solani, Fusarium oxysporum, Curvularia lunata, Alternaria alternata, Rhizoctonia solani* and *Macrophomina phaseolina* and bacterial pathogens like *Bacillus subtilis* and *Pseudomonas aeruginosa.* This assay was carried out using well diffusion method following standard method.[20]PDA and NA medium were used for this assay. Gentamycin (100µg) and Carbendazim (100µg) were used as positive control .Further, the effect of1mM $AgNO_3$ against the phytopathogens were also studied. Triplicates were maintained for all the samples. The plates were incubated for 24hrs at 37˚C for bacteria and 48 to 72hrs at room temperature for fungal pathogens. Zone of inhibition was observed and measured.

**Photocatalytic degradation**

Photocatalytic degradation of methylene blue dye was evaluated using biosynthesized silver nano particles.[21] This experiment was carried out under sun as chief source of light.[22] Prior to the experiment 10mg of methylene blue was added to 1000ml of double distilled water and used as stock solution. In 100ml of methylene blue dye solution, 10mg of bio synthesized silver nano particles were added. A control was maintained without silver nano particles in the reaction mixture, kept in shaker in about 30min, following which it was kept under direct sun light and absorbance was recorded at specific time interval; aliquot of 3ml suspension were filtered in whatmann filter paper 1 and used to evaluate the photocatalytic degradation of dye. The absorbance spectrum of the treated methylene blue dye was subsequently measured using UV Visible spectrophotometer at 660nm. Percentage of dye degradation was calculated by following formula

$$\% \text{ of decolorization} = \frac{C_0 - C}{C_0} \times 100$$

**Seed Germination**

Seeds of *Vigna radiata* (mung) were purchased from local market. These seeds were washed thoroughly in deionised water. It was then surface sterilized in 5% sodium hypochlorite solution for 10 min.[23] The surface sterilised seeds were soaked in distilled water for 2 hrs following which it was soaked in series of silver nano particles suspension (0.5mg/l, 1mg/l, 1.5mg/l) for 2 hrs.10 treated seeds per treatment were transferred in its respective petriplate containing sterilized filter paper, 5ml of test solution was further added to it. Triplicates were maintained. Germination percentage of the silver nano treated seeds, growth (root-shoot length) and biomass was recorded after 12 days.

**Statistical Analysis**

The data presented are the mean value of three replicates. Promotion of germination and growth (root-shoot length) of *Vigna radiata* was determined by statistical significance using Student's *t* – test.

## RESULTS AND DISCUSSION

Endophytic fungi, CJN5, isolated from the root of *Casuarina junghuhniana* Miq. was identified based on its morphological characteristics and microscopic study of the fungal spores in our previous study. A phylogenetic tree was constructed with the help of neighbour-joining algorithm for further identification of CJN5 sequence obtained (Fig: 1). On the basis of high sequence percentage (>100%), endophytic fungus was assigned to its respective species, i. e, *Aspergillus tamaraii*.

**Mycosynthesis of silver nano particles**

The efficiency of mycosynthesis of silver nano particles by fungal isolate, *Aspergillus tamaraii*, was initially evaluated by observing the colour change of the reaction mixture from pale yellow to dark brown colour while the control test tube remained unchanged after 24hrs of incubation (Fig 2). Development of dark brown colour is due to Surface Plasmon Resonance (SPR) exhibited by the nano particles and this is the unique characteristic of silver nano particles.

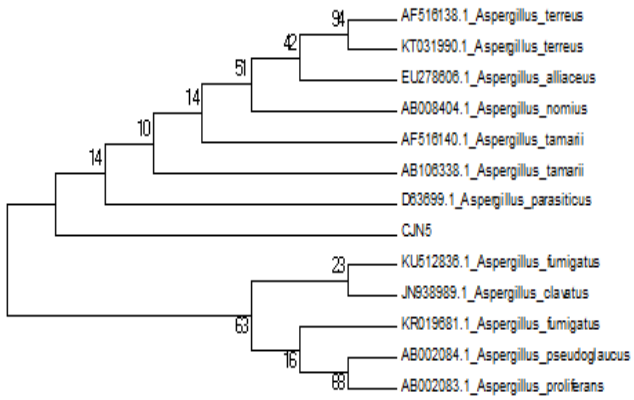



**Figure 1:** Phylogenetic tree of fungus CJN5

The mechanism in the production of silver nano particles by fungi might be due to the trapping of $Ag^+$ ions at the surface of the fungal cells and thereby reduction of $Ag^+$ ions by the enzymes produced in the fungal system .[24] Extracellular enzymes like napthoquinons and anthroquinons may also promote the reaction process. In *Fusarium oxysporum*, NADPH dependent nitrate reductase and shuttle quinine extra cellular process involves in the synthesis of silver nano particles.[25]Extracellular synthesis of silver nano particles has advantage over intracellular process as it neither involve in lysis of fungal cells nor purification of nano particles .[26]

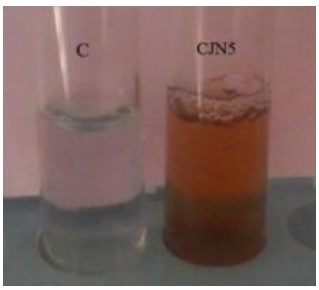



**Figure 2:** Visual observation of synthesis of silver nano particles**.**

**Optimization studies of silver nano particles**

Growth of fungus and production of its metabolites depends vastly on environmental conditions. Optimization of physical parameters not only enhances the growth but also improves the product yield.[27] The growth conditions such as pH, temperature, and concentration of silver nitrate solution (1mM) directly related to the enzymatic activity which affects the biosynthesis of silver nano particles.

a) Concentration ratio of fungal filtrate and silver nitrate solution: Different concentration of fungal filtrate and silver nitrate solution was optimised for the maximum production of silver nano particles. It was found that maximum synthesis was obtained at 1:1 ratio ( fungal filtrate :silver nitrate solution).The effect of different concentration ratio on the biosynthesis of nano particles were studied using UV visible spectrum, maximum peak at 420nm was obtained at 1:1 ratio (Fig 3). At other concentrations, there was a shift in the peak with difference in intensity.

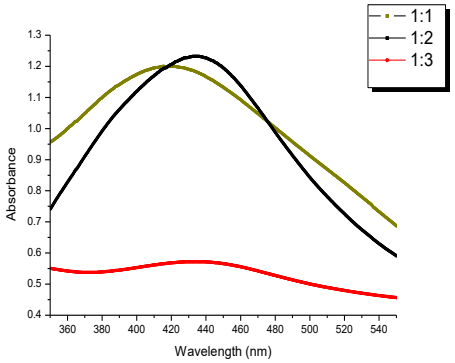



**Fig-3:** UV spectrum for the effect of different concentration ratio in nano particle synthesis

b) Effect of pH: pH plays a vital role in the synthesis of silver nano particles. In the present study, the reaction was adjusted with five different pH. Maximum synthesis of silver nano particles occurred at neutral pH (pH 7) with the formation of reddish brown colour in the reaction mixture. UV Visible spectrum showed ƛmax at 423 nm at neutral pH.No colour change was observed at acidic pH (pH 3 and 5). However, at alkaline condition, reddish brown colour appeared but subsequent shift in the peak was found (Fig:4).At acidic pH , protein structure gets denatured and becomes inactive, therefore, aggregation of the nano particles was observed .[28] It can be thus concluded that protein secreted by *Aspergillus tamaraii* in the solution for the capping of silver nano particles is stable at pH 7, but not at acidic condition. Similar results were reported by Dattu Singh *et al* showing maximum absorbance at 425nm at neutral pH using *Penicillium* sp isolated from *Curcuma longa*.[19]

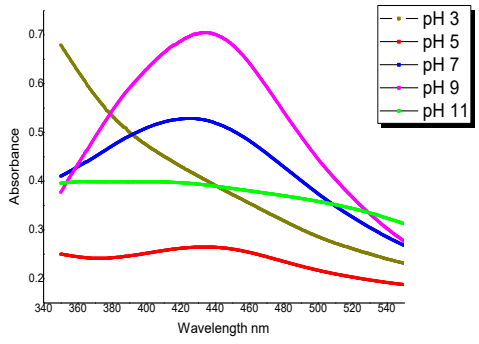



**Figure 4:** UV spectra for effect of different pH in nano particle synthesis.

c) Effect of temperature:
Temperature is considered to be an essential factor affecting synthesis of silver nano particles. The different temperature was maintained for the production of silver nano particles. Maximum

synthesis was found a 40°C having a sharp peak at 430nm (Fig: 5). Temperature is reported to play a prime role in the synthesis of nano particles as it can be manipulated.[19]

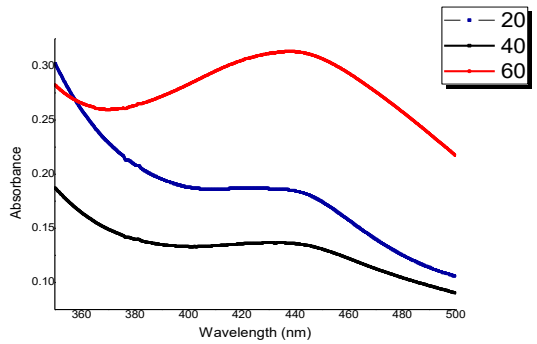



**Figure 5**: UV spectra for the effect of different temperature in nano particle synthesis (T1=20°C, T2= 40°C, T3=60°C)

d) Stability:

Stability of the synthesized silver nano particles is an important factor. In the present study, the synthesised silver nano particles were found stable till 45th day. The UV Visible spectrum of biosynthesised silver nano particles showed peak at 432 nm on 45th day(Fig: 6). Proteins might play a role in forming a coat covering the metal nano particles. The capping of silver nano particles is necessary for preventing agglomeration of nano particles and thereby it attains stability.[29] Silver nano particles produced by the endophyte *Fusarium oxysporum* was reported to be stable for 60 days .[30]

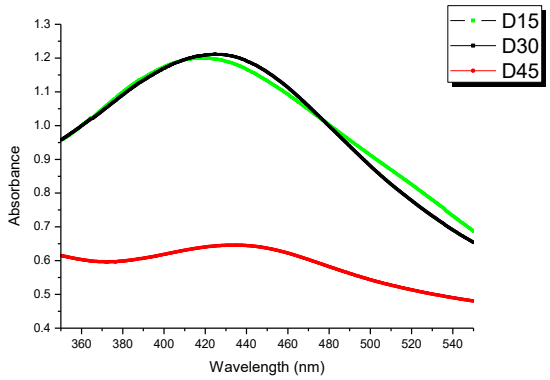



**Figure 6**: UV spectra for synthesized nano particle synthesis at different days interval

**Characterization of silver nano particles**

a) UV Spectral analysis: The UV visible spectra of the filtrate treated with 1mM $AgNO_3$ revealed surface plasmon absorption band at 424nm (Fig: 7). Characteristic surface plasmon resonance reveals the formation of silver nano particles falls within the range 390-440nm .[14]

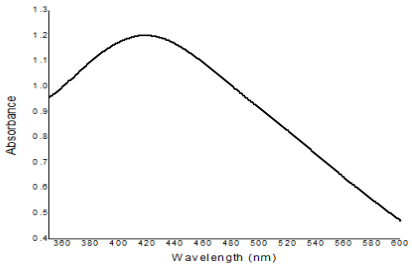



**Figure 7**: UV-Vis spectra recorded after 24hrs of reaction, showed peak at 424nm.

b) FTIR analysis: FTIR analysis was carried out to characterize the nature of capping ligand which stabilizes the synthesized nano particles formed by bioreduction process.[14] FTIR spectra of mycosynthesized silver nano particle is shown in Fig: 8. FTIR spectra showing peak at 3265$cm^{-1}$ was due to the presence of hydroxyl group. The sharp peak at 2925.17$cm^{-1}$ was due to vibration of aliphatic group. The IR band at 2351.33$cm^{-1}$ assigned as P-H (phosphine) group. Sharp peak at 1654.03$cm^{-1}$ shows the presence of amide group. Similarly, peak at 1031.96$cm^{-1}$ reveals the presence of primary aliphatic amine. The data reveals that the involvement of various compounds and functional groups were responsible in the biosynthesis of silvernano particles.

c)

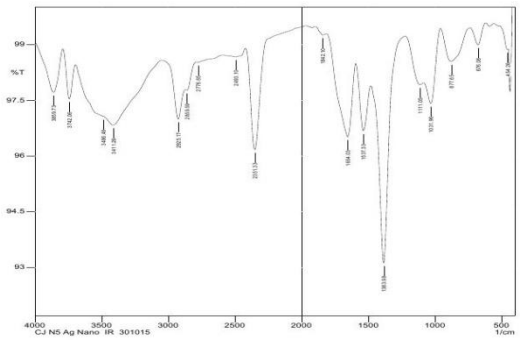
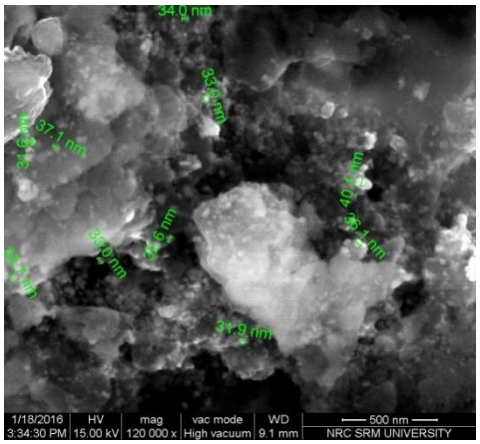


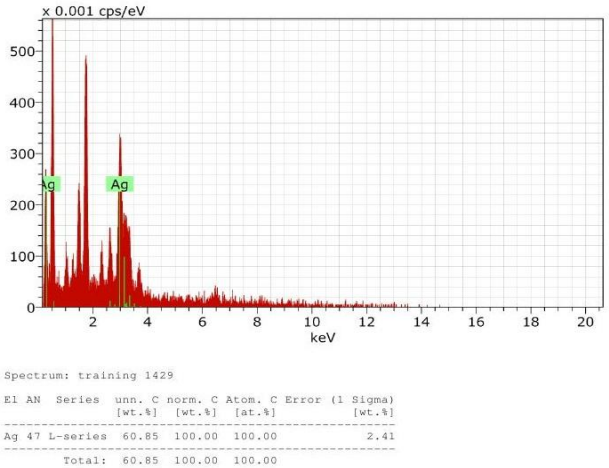

**Figure 8:** FTIR spectrum of synthesized silver nano particles.

d) Scanning Electron Microscopy (SEM) and EDS study: Scanning electron microscopy (SEM) is a commonly used technique for characterization of nanoparticles, i.e, to study the physical morphology of the particles. In the present study, SEM micrograph revealed that synthesised nano particles are mostly spherical in structure. The size of the silver nano particles was found in range from 31 to 40 nm (Fig: 9a). The varying size of the nano particles is due to the slow reduction of silver nitrate to silver nano particle.[14] The EDX profile of the biosynthesised silvernano particles revealed sharp peak at silver region 3KeV which confirms the presence of the elemental silver (Fig 9b). This absorption spectrum is typical for silvernano particles because of surface

plasmon resonance therefore confirming successful synthesis of silvernano particles using endophytic fungal extract.




a




b

**Figure 9:** SEM image and EDX spectrum of synthesized silver nano particles

**Antimicrobial activity**

Among varied type of metal nano particles like copper, zinc, gold, titanium, silver, silvernano particles are found to be the most efficient as they have antimicrobial properties against bacteria, viruses, fungi.[31-32]In this study, microbial activity was carried out against fungal pathogens such as *Curvularia lunata*, *Macrophomina phaseolina*, *Alternaria alternata*, *Alternaria solani*, *Fusarium solani*, *Fusarium oxysporum*, *Rhizoctonia solani* and bacterial pathogens like *Bacillus subtilis* and *Pseudomonas aeruginosa* (Fig: 10). However, at 100µg concentration maximum zone of inhibition was found against *Fusarium solani* (30±0.2 mm) followed by *Fusarium oxysporum* (26±0.2 mm), *Alternaria solani* (22±0.4 mm), *Rhizoctonia solani* (20±0.3 mm), *Curvularia lunata* (12±0.2 mm), *Alternaria alternata* (12±0.3 mm) and *Macrophomina phaseolina* (5±0.2 mm). In case of bacterial phytopathogen, *Bacillus subtilis* showed higher zone of inhibition (22±0.4 mm) than *Pseudomonas aeruginosa* (15±0.2 mm).

Similarly at 50µg concentration, maximum zone of inhibition was found against *Fusarium* sp (20±0.2 mm) followed by *Rhizoctonia solani* (18±0.4 mm), *Alternaria solani,* (16±0.4 mm), *Curvularia lunata* (7±0.3 mm), *Alternaria alternata* (5±0.2 mm) and *Macrophomina phaseolina* (2±0.4 mm). Unlike 100µg concentration, *Pseudomonas aeruginosa* showed higher zone of inhibition (13±0.3 mm) than *Bacillus subtilis* (11±0.4 mm).

The toxicity of the silver nano particles against microorganisms is because of free silver ions.[14] There are many proposed mechanism discussed by various researchers but the exact mechanism has not been elucidated. However, it is believed that silver ions cripples the enzyme that metabolise oxygen, which is necessary to sustain life, thus it stops oxygen metabolization. This suffocates the fungi and bacteria leading to cell death .[33]

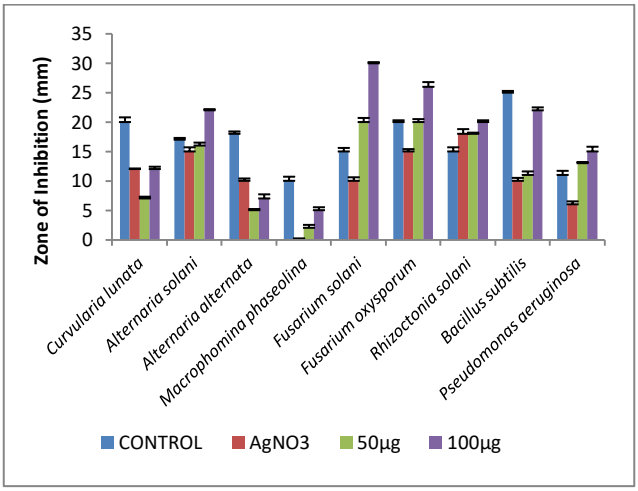
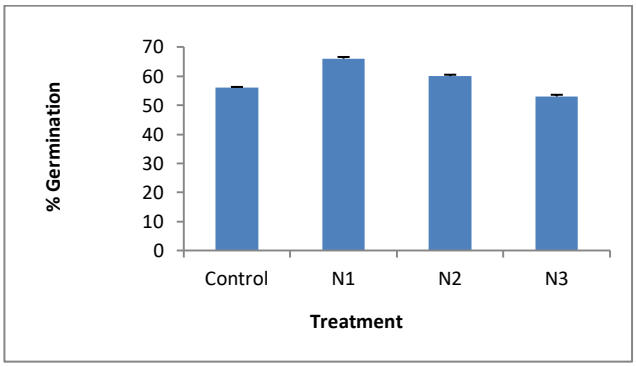



**Figure 10:** Antimicrobial activity of silver nano particles against different phytopathogens

**Germination study**

Nanotechnology is considered to be one of the promising upcoming solutions to the problems in food and agriculture industry. Seed germination is considered to be a suitable foundation to plant growth, development and yield.[23] In this study, among the three different concentration (N1-0.5mg/l; N2-1mg/l; N3- 1.5mg/l), N1 showed highest germination percent (66%) followed by N2 (60%) and N3 (53%) (Fig-11a). Germination percentage of both N1 and N2 treatment was found significant over control. Growth (root-shoot length) and biomass was found maximum for N2 (15.6mm and 88.9mg) followed by N1 (11.5mm and 78.6mg) (Fig: 11 b & c). This result concludes that both N1 and N2 can be used as an effective bioinoculant as it has a significant effect on the growth and biomass of *Vigna radiate* ($p > 0.05$) when compared with the control.

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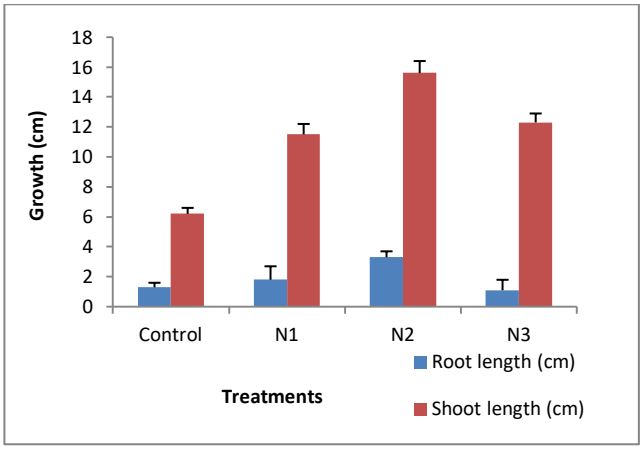


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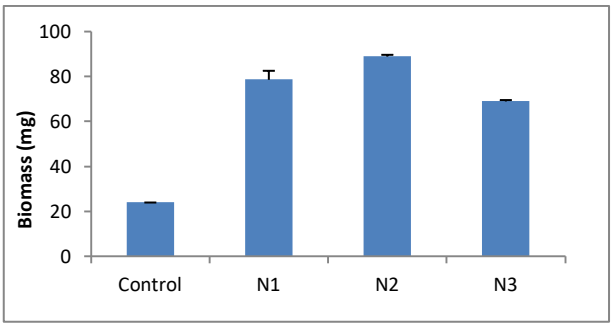


c

C: Control
N1: 0.5mg/l
N2: 1mg/l
N3: 1.5mg/l

**Figure 11:** Effect of silvernano particle on a) Germination b) Growth c) Biomass using *Vigna radiata.*

**Photocatalytic degradation:**

Photocatalytic degradation of methylene blue dye was studied using biosynthesised silver nano particles by solar irradiation technique in different time intervals. Dye degradation was assessed by analysing the colour change from dark blue to light blue after 12 hrs of reaction time. The intensity of the absorption peak at 660nm for methylene blue decreased gradually with increase in time.The photocatalytic degradation of methylene blue is shown in Fig: 12. The percentage of dye degradation was found to be 89.26% after 36hr. It is reported that photocatalytic degradation of dye using silvernano particles is strongly dependent on the structure, size, morphology and crystallographic nature of nano particles.[34] Further, Kansal *et al* (2006) reported that dye decolorisation takes place at much faster rate in solar light than in any other irradiation techniques.[35] The photocatalytic activity of silver nano particles in visible light may be because of excitation of surface plasmon resonance which is nothing but oscillation of charge density propagating at the interface between dielectric medium and metal.[36] Similar results were reported on using silvernano particles synthesized from *Hypnea musciformis*.[37]

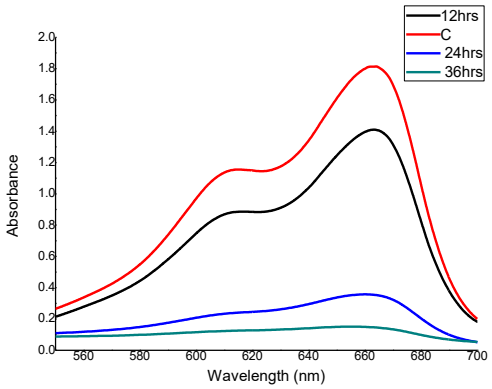


**Figure 12:** The absorption spectra of aqueous solution of methylene blue treated with synthesized silver nano particles.

## CONCLUSION

The present study revealed the potent isolate *Aspergillus tamaraii* can be effectively used in green synthesis of silver nano particles. Optimization parameters for better synthesis of silver nano particles was found at concentration ratio 1:1, neutral pH , temperature 40°C and stability up to 45days. Therefore, biogenic nano silver can be effectively used in agriculture as it acts as a promising antimicrobial agent to protect the economically important crop plant against harmful phytopathogens. Further, it can also enhance the growth and yield, therby it can be highly beneficial to farmers. Silver nano particles can also be used intensively in biomedical research and for dye degradation in textile industry.

**Acknowledgement:** The authors thank Mrs. Prema Sampathkumar, Associate Professor and Head, the faculty members and non-teaching staff of the Department of Plant Biology and Plant Biotechnology, Dr. Mrs A. Nirmala, Principal, Ethiraj College for Women (autonomous) Chennai 600008, for their valuable support and encouragement throughout the entire period of research. We would also like to express our thanks for the facilities extended by the Central Instrumentation Centre, Ethiraj College for Women. Sincere Thanks to State Forest

Research Institute, Kolapakkam, Chennai-48 and SRM University for extension of facilities.

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**Source of Support:** Nil, **Conflict of Interest:** None.