Original Article

Garlic Mediated Green Synthesis of Silver Nanoparticles as Antifungal Agents against *Magnaporthe oryzae*

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ABSTRACT

Background: Plant elements such as carbohydrates, lipids, flavonoids, polyphenols, enzymes, terpenoids, and alkaloids are used as reducing substance in the green production of silver nanoparticles. The strategy proved to be highly straightforward, cost-effective, and practical. **Materials and Methods:** The synthesis of nanoparticles was validated using optical inspection, in which the yellow colour solution became brown. UV-visible spectroscopy, XRD, FTIR analysis, and SEM were used to further characterise the material. **Results:** Transmission electron microscopy have shown that the silver nanoparticles size was between 10–46 nm (SEM). Size of silver nanoparticles was found to be between 10–46nm approximately as determined by transmission electron microscopy (SEM). Well diffusion method demonstrated the antifungal activity of AgNPs on *Magnaporthe oryzae* with the zone of inhibition of 5 and 11mm when 12.5 and 25µg/ml of AgNPs was used respectively. Lowest inhibitory concentration was found to be 5.2. **Conclusion:** The leakage of reducing sugars and proteins was used to explore the mode of action of nanoparticles' antifungal activity, indicating that AgNPs were able to reduce membrane permeability.

Keywords: *Allium sativum*, *Magnaporthe oryzae*, Silver nanoparticles, Conidia germination inhibition assay, Colony growth inhibition assay.

INTRODUCTION

The fungus *Magnaporthe oryzae* is the reason of rice blast disease (RBD) in rice cultivars.[1] During the whole development cycle, the rice blast pathogen grows in the nodes, leaves, collars, necks, panicles, seeds, and roots.[2] The harm caused by the rice blast assault mechanism has been discovered to be heavily impacted by environmental conditions. Although conidia do not germinate in direct sunshine, gloomy circumstances and humidity helps the development of the illness throughout the year in the air.[3] Their life cycle lasts 7 to 14 days,[4-6] after which they damage plants in 15 to 20 days, resulting in yield losses.[7]

Diseases caused by insects, bacteria, fungus and other pathogens in the environment have been the greatest challenge to agriculture since its inception.[8,9] Phytopathogenic fungi cause numerous diseases in agriculture.[10] Phytopathogenic fungi are now mostly managed with inexpensive and readily available chemical agents.[11,12] However, because of their widespread usage, they have resulted in environmental contamination, animals and human illnesses and ecological imbalances.[13,14] Furthermore, the use of chemical agents has resulted in fungus acquiring greater resistance to chemical compounds, making them stronger.[15,16] Currently, friendly and good alternatives for the environment are being used to control phytopathogen fungi, such as biological control,[17,18] plant extracts[19] and essential oils.[20,21] Such alternatives have been beneficial and are therefore considered as a good choice.

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Nanomaterials, employed effectively in other industries such as energy, health, and electronics, are another newly researched and applied method in agriculture.[22,23] Nanomaterials have grown in importance as a result of their distinct physicochemical features from bulk materials.[24,25] Chemical, physical and green approaches are the methods that may be used to make AgNPs. Several research organisations have recently implemented green plant-based reduction approaches, which are also regarded safe, easy and cost-effective protocols.[26] The presence of naturally occurring biomolecules such as proteins, enzymes, tannins, phenols, sugars, and flavonoids in the green technique was the key benefit. The existence of naturally occurring biomolecules such as proteins, enzymes, tannins, phenols, sugars, and flavonoids, which may be employed safely as reducing and stabilising agents to generate stable nanometals, was the major benefit of the green technique.[27,28] Due to their simplicity of handling and synthesis, nanoparticles provide environmentally friendly, efficient, and contemporary solutions for the control of phytopathogenic diseases that may be utilised as biomanufacturing agents.[29,30] Metal nanoparticles are thought to be a promising option for controlling phytopathogenic fungus in agriculture. Several metal nanoparticles (Ag, Cu, Se, Ni, Mg, and Fe) produced and tested as antifungal agents to date.[31]

Because of their antioxidant, antibacterial, and anticancer qualities, as well as their biocompatibility, ease of manufacture, cheap cost and non-toxicity, Ag nanoparticles have been intensively studied in a variety of scientific domains.[32,33] Ag nanoparticles have been the most used nanoparticles for controlling phytopathogenic fungi due to their features and strong antifungal activity.[34,35]

The size distribution, shape, content, crystallinity, agglomeration and surface chemistry of the nanoparticles all have an impact on their antifungal activity.[36,37] Small nanoparticles, for example, favour the surface area to volume ratio, which may enhance their antifungal efficacy.[38] These parameters may be adjusted and regulated by synthesis pathways, as is well known.[39,40] It has also demonstrated that the production technique might influence antifungal action, as metal precursors or surfactants are sometimes difficult to remove from nanoparticles. As a result, synthesis residues can alter the surface chemistry of nanoparticles, hence influencing their antifungal effectiveness.[41]

However, it has been demonestrated that smaller nanoparticles, between 10 and 30 nm, have stronger antifungal activity.[42-49] The reason is the smaller nanoparticles enter or damage the pathogen's cell membrane more quickly, uniting the fungal hyphae and mycelium and deactivating the pathogens.[50] Nonetheless, despite the bigger size has a higher antifungal ability, it is slower to penetrate the pathogen's membrane, causing damage to mycelium and spores or inhibiting fungal development.[51] While the greater size has a strong antifungal potential, it penetrates the pathogen's membrane more slowly, causing harm to mycelium and spores or inhibiting fungal development.[52-54]

Various plant extracts, such as garlic, ginger, onion, curry leaf, and palak, have been employed in the green production of nanoparticles. Garlic (*Allium sativum* L.), which is widely used even as a spice, food, and traditional medicine, has also been demonestrate to have antimicrobial and antioxidant properties. Garlic extract is a rich source of phenolics and flavonoids, which has an important role in the reduction process for the synthesis of metal nanoparticles.[55] Furthermore, garlic extract has shown as an effective bio-reducing agent for the production of AgNPs.[56]

In this study, Garlic cloves extract was used to reduce silver nitrate to silver nanoparticles, and X-ray diffraction (XRD), UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), Scanning electron microscope (SEM), and were used to characterise the synthesised nanoparticles. Antifungal property of the produced nanoparticles was tested against pathogenic fungus *M. oryzae*. Antifungal property was evaluated by agar well diffusion method, conidia inhibition assay and colony growth inhibition.

MATERIALS AND METHODS

Garlic cloves were purchased from local market stores and peeled off and were sundried for 4–5 days so as to remove moisture completely. Dried cloves were pulverized to fine powder and stored in dry container for further use. Silver nitrate ($AgNO_3$) was purchased from HiMedia Ltd., Bangalore. All the reagents used in the study were of analytical and molecular grade. For the experiment, all of the solutions were prepared fresh with deionized water and maintained in the dark to avoid any photochemical reactions.

Micro-organisms: Antifungal activity of green synthesized silver nanoparticles was studied against *Magnaporthe oryzae*. Fungal culturesisolated from the infected paddy seeds. Oatmeal agar was used to maintain the strain. The fungal culture grown in the laboratory was rechecked for the specific conidia to confirm the strain (Figure 1).

Green Synthesis of silver nanoparticles: AgNPs were synthesized by reduction of silver nitrate with garlic

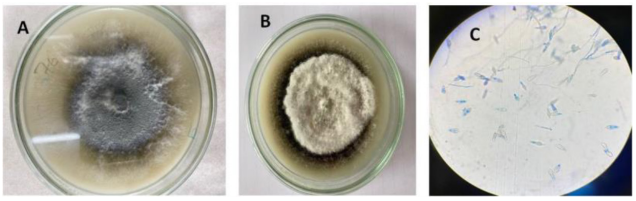

Figure 1: Image of oat meal agar plate showing *Magnaporthe oryzae* culture. A, B: *Magnaporthe oryzae* culture; C: *Magnaporthe oryzae* conidia under (100x) microscope.

extract by the following method. 200ml de-ionized water was added to 20g of garlic powder prepared in the previous section. The contents were then kept on magnetic stirrer for one hour at 40°C and filtered with Whatman No.1 filter paper and finally centrifuged at 10000rpm for 20min. The filtrate was then evaporated in rotary evaporator and about 50mg of extract was then suspended in 40ml of deionized water. Equal volume of 0.5mM $AgNO_3$was added to the suspension incubated for 2 days at 50-70°C with constant stirring on magnetic stirrer. pH of the solution was maintained at 10.

Characterization of nanoparticles: Synthesized AgNPs were then analysed for spectral analysisusing a UV–Vis spectrophotometer (Shimadzu UV1800) at the resolution of 1nm with wavelength range from 200 to 800nm for each sample. Both control and experimental solution of the synthesized AgNPs were subjected to FTIR analysis (Perkin-Elmer FTIR-1600, USA) in the range of 450–4000 cm^{-1} at a resolution of 4cm^{-1}. The crystalline structure of the synthesized AgNPs was further studied with X-ray diffraction system. The diffraction pattern was recorded with the scanning mode on a Rigaku Miniflex II operated at about 30kV with a current of 15mA and Cu/Ka radiation in the range of 3°–80° in 2hr angles. Standard powder was used along with samples. The morphology of the synthesized particles was characterized by scanning electron microscopy (SEM, Hitachi S-5500) at an accelerating voltage of 2.0kV. Nanoparticle tracking and analysis (NTA) was done for the study of the particle size and distribution using LM-20 (NanoSight Ltd. UK). NTA usually aids in separating the particles population by size and intensity simultaneously determining their Brownian motion.

Antifungal Activity of AgNPs: Antifungal activity against *Magnaporthe oryzae* was conducted through agar well diffusion method, colony growth assay and through conidia germinationfollowing the medium as described by Huang (2020).

Agar well diffusion assay: Zone of inhibition formed on an agar plate reflects the antifungal property of AgNPs. An agar-discs or cylinder from previously grown culture was cut aseptically with a 5mm diameter sterile cork borer and plated on a series of OMA agar plates. About 20μL of synthesized AgNPs was added onto the well made at equidistant points. Bavistin (20mg/ml) was used as positive control. The plates were then incubated at 28°C for 7 days.

Colony Growth Assay: The synthesized AgNPs after oven drying were suspended in sterile water and the concentration was 10mg/mL.5mL of diluted AgNPs were added to 50mL of OMA medium at 55°C. Nanoparticles were added at two different concentrations of 12.5 and 25μg/mL separately. The control was maintained with 5mL of sterile de-ionized water without silver nanoparticles. Bavistin (20mg/ml) was used as positive control. A fungal culture disc was taken with a cork borer was placed at the centre of each OMA plate and then incubated at 28°C for 7days.

Conidia Germination inhibition assay: Germination assay was followed according to the procedure of Weidong Huang (2020). In brief, the density of *Magnaporthe oryzae* conidia was diluted to 10^6spores /mL using a neubauer chamber. Synthesized AgNPs and conidial suspension of the test fungus were mixed at a ratio of 1:9 (by volume) and the AgNPs were used at varying concentrations of 12.5, 25, 50, 100 and 200µg/mL. Conidial suspension without AgNPs was used as negative control and Bavistin 20mg/ml) was used as positive control. The tubes were incubated at 25°C for about 12 to 48hr.

Statistical Analysis

All the experiments were performed in triplicates and expressed as the mean ± standard deviation. Statistical analysis was performed using ANOVA to determine the variance and relation ($p \leq 0.05$).

RESULTS

The brown colour of AgNPs in water is widely known and it results from the activation of surface plasmon vibrations in the metal nanoparticles. Initial detection was by visual observation where in specific colour change i.e. to dark brown colour of the mixture was observed on constant stirring (Figure 2A, 2B). UV-visible spectroscopy, scanning electron microscopy, X-ray diffraction and FTIR spectroscopy were used to demonstrate the production of AgNPs.

UV-Vis spectroscopy was used to characterise the AgNPs, which were then tested for antifungal efficacy. The wavelength scale was set to 200–800nm and the solution was scanned in that range. The maximum absorbance was recorded at 440nm, which is typical

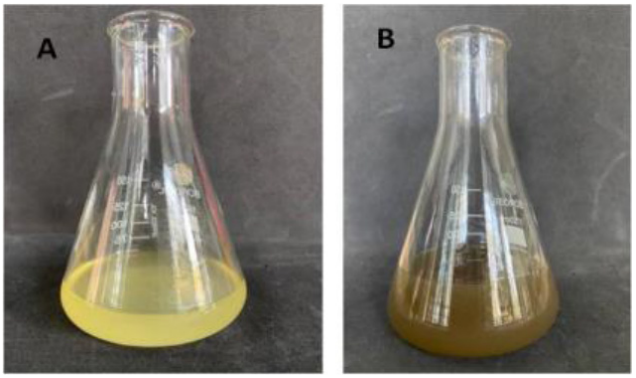

Figure 2: Image showing the green synthesis of AgNPs (0.5mM) with garlic extact. A: yellow colour (control); B: Colour change to dark brown after synthesis of AgNPs.

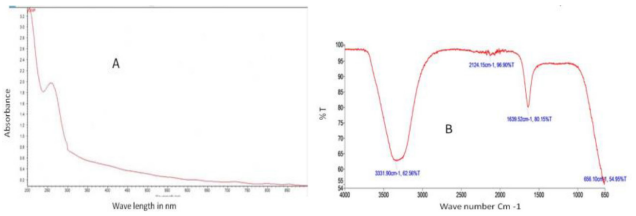

Figure 3: A: Image showing the UV spectra at 280nm. B: Image showing the FTRI spectra observed for the synthesized AgNPs.

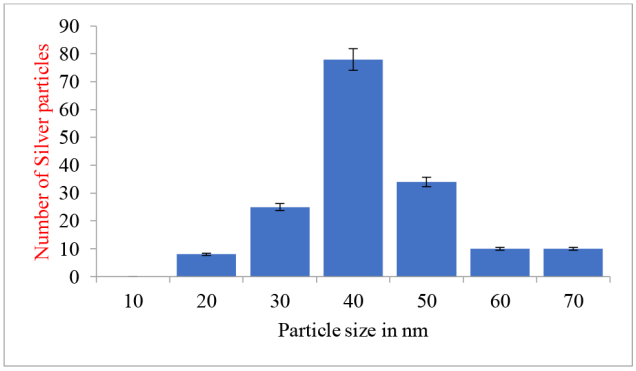

Figure 4: Graph showing the total size distribution of AgNPs as viewed from SEM. All the values are average of triplicates expressed as number ±SD.

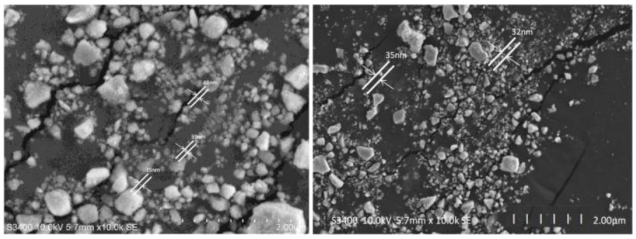

Figure 5: HR-SEM analysis of synthesized AgNPs showing spherical shape and size of nanoparticles ranging between 10 – 46nm.

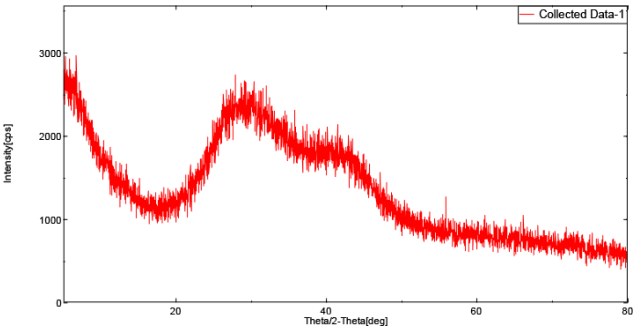

Figure 6: Image showing the XRD spectra of AgNPs?

for silver nanoparticles as reported by Bahuguna *et al.* (2016). The curve demonstrates that the absorbance of silver nitrate and seed extract increases as the incubation duration (30 min, 45min and 1hr) increases.

The synthesized silver nanoparticles were subjected to FTIR analysis for further characterization. FTIR analysis showed two peaks at 1760 and 3324. For the size of AgNPs nanopartcile tracking analysis (NTA) analysis by the NanoSight (LM-20) was carried out (Figure 3). The NTA images showed most of the synthesized NPs are having an average size diameter of 46nm which was calculated on the basis of Brownian motion of particles (Figure 4). The shape and the size of the produced AgNPs were also investigated using SEM. The AgNPs were primarily scattered and more or less spherical in form. The particles ranged in size from 10 to 40nm (Figure 5). XRD results demonestrated that the synthesized particles are face-centred round structures of silver (Figure 6).

Antifungal activity: *In vitro* antifungal property of synthesized AgNPs with commercially available antifungal agent was done against *Magnaporthe oryzae*. AgNPs showed remarkable activity against *Magnaporthe oryzae* when compared to positive control (Bavistin @ 20mg/ml). The diameter for the AgNPs was found to be 5 and 11mm for 12.5 and 25μg/mL, respectively. The results are satisfactory and significant when compared to positive control at 25μg/mL (14mm) (Figure 7 and 8). Similar inhibition zones were observed with Tricyclozole (30mm for 3.0µg/ml chemical used in controlling blast disease) at different concentrations.[57] Silver nanoparticles synthesized by the *Allium sativum* (garlic) extract showed maximum antimicrobial activity against both bacteria and fungus.[58] Many of the species of *Candida* were found to be susceptible with silver nanoparticles,[59] confirmed the potential antagonistic activity of silver nano particles against several fungal pathogens.[60] Their experiments reported that this anti fungal activity is due to loss of membrane integrity leading to electrolyte leakage. Similarly present results

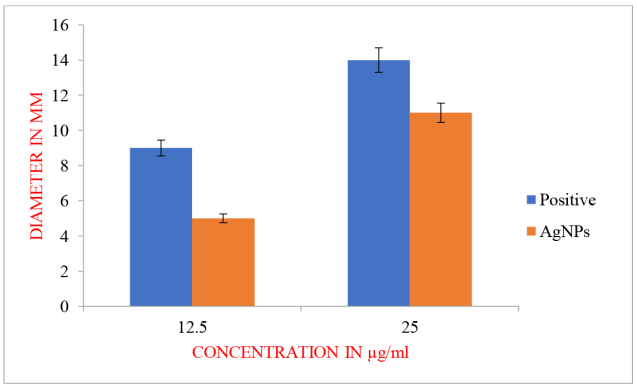

Figure 7: Formation of the zone of inhibition observed in well diffusion assay. Bavistin was used as positive control. All the values are the average of triplicates and are expressed as number ±SD.

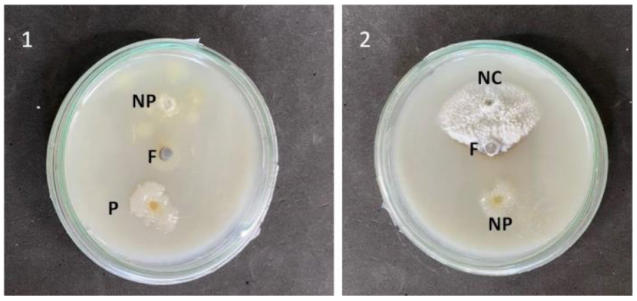

Figure 8: Images showing the Antifungal activity of AgNPs (NP) against *Magnaporthe oryzae*. Inhibition zones are measured in mm. 1: Nanoparticles and positive control (P) against fungal strain. 2: Nanoparticles and negative control (NC, deionized water) against fungal strain.

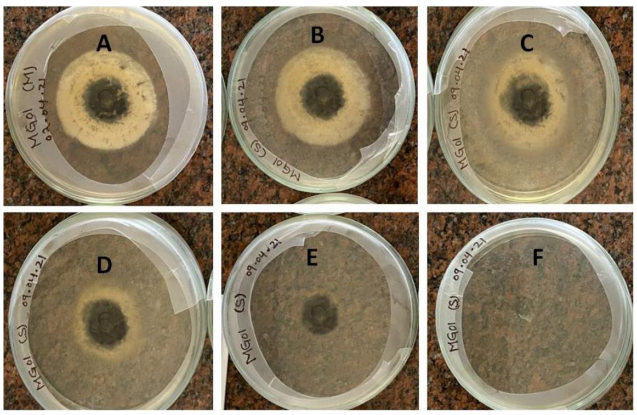

Figure 9: Image of the plates showed *in vitro* colony inhibition assay. Varying concentrations of the nanoparticles are added to the plates. A: control; B: 12.5µg/mL; C: 25µg/mL; D: 50µg/mL; E: 100µg/mL; F: 200µg/mL.

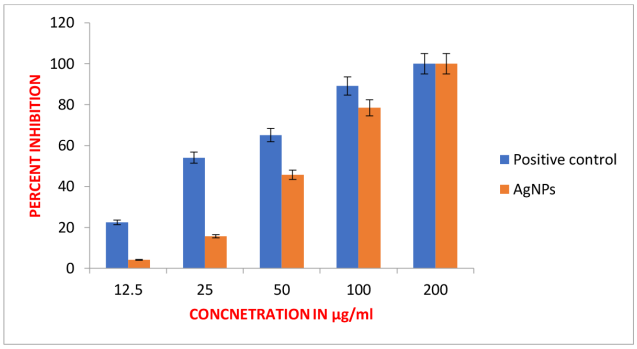

Figure 10: Graph showing the percent colony growth inhibition of *M. oryzae*. All the values are average of triplicates and values are expressed as value ±SD.

are in accordance to these reports suggesting the possible role of the green synthesized AgNPs in the loss of membrane integrity.

Colony Growth Inhibition: *Magnaporthe oryzae* colonies were significantly inhibited by AgNPs. The colony diameter of the control was about 14.5cm where it gradually got diminished with increasing concentration of AgNPs. The diameter descended to its minimum value (5.1cm) at 200µg/mL. The inhibition rate was shown to be in the range of 4.11% - 100% for 12.5-200µg/mL, respectively (Figure 9 and 10).

The application of four concentrations of silver nanoparticles to *M. grisea* culture exhibited considerable reduction of both hyphal development and the number of colonies generated in a dose-dependent manner under lab conditions. The silver nanoparticles delayed and greatly inhibited fungal growth at low concentrations, according to radial growth measurements.[61] found that secondary metabolites extracted from *C. elatum* showed antifungal activity against *P. oryzae* with an ED_{50} of 231ppm.[62]

Conidia Germination Inhibition: Because fungi need a large number of germinated conidia to successfully infect plant tissue, inhibiting conidia germination is critical in reducing or preventing the formation of plant diseases. Controlled growth with optimum temperature and humidity showed 93% germination rate for conidia in the absence of AgNPs. The percentage was found to diminish with increase in concentration of AgNPs (12.5, 25, 50, 100 and 200µg/mL). It was shown that the conidia were completely inhibited or not seen at 200µg/mL. The results are based on present agar well diffusion method. The percent inhibition for positive control was shown to be in the range of 87% at 12.5µg/mL to zero percent at 200µg/mL. Synthesized nanoparticles are found to be inhibited at the range of 90% at 12.5µg/mL to zero percent at 200µg/mL (Figure 11).

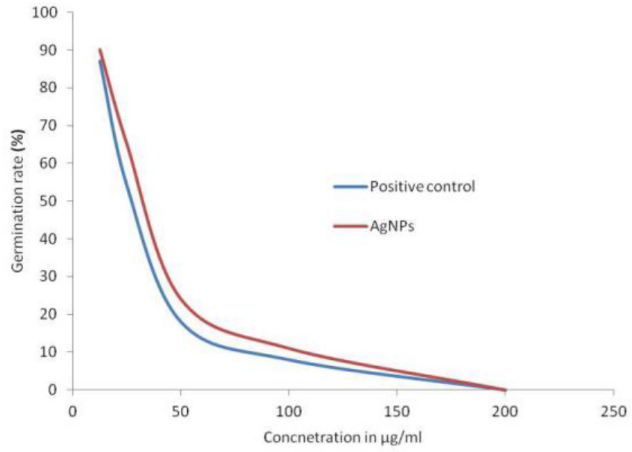

Figure 11: Graph showing the conidial inhibition with the treatment of AgNPs against *Magnaporthe oryzae* All the values are average of triplicates and the values are expressed as value ±SD.

DISCUSSION

Our reports suggest of the potential role of the antifungal activity of the green synthesized silver nanoparticles. Silver nanoparticles synthesized by the *Allium sativum* (garlic) extract showed maximum antimicrobial activity against both bacteria and fungus.[58] Many of the species of *Candida* were found to be susceptible with silver nanoparticles,[59] confirmed the potential antagonistic activity of silver nano particles against several fungal pathogens.[60] Their experiments reported that this anti fungal activity is due to loss of membrane integrity leading to electrolyte leakage. Similarly present results are in accordance to these reports suggesting the possible role of the green synthesized AgNPs in the loss of membrane integrity.

The application of four concentrations of silver nanoparticles to *M. grisea* culture exhibited considerable reduction of both hyphal development and the number of colonies generated in a dose-dependent manner under lab conditions. The silver nanoparticles delayed and greatly inhibited fungal growth at low concentrations, according to radial growth measurements.61 found that secondary metabolites extracted from *C. elatum* showed antifungal activity against *P. oryzae* with an ED_{50} of 231ppm.[62]

In field testing on cucumbers and pumpkins, the treatment of 100ppm silver nanoparticles exhibited the greatest inhibition rate both before and after the disease onset. In addition, *in vivo* experiments revealed that 100ppm silver nanoparticles inhibited the development of fungal hyphae and conidial germination the most.[63] The present study is focused on the extracellular synthesis of silver nanoparticles (AgNPs) using culture supernatant of an agriculturally important bacterium, *Serratia* sp. BHU-S4 and demonstrates its effective application for the management of spot blotch disease in wheat. Similar reports were made and the findings were confirmed in the green house, where bsAgNPs administration drastically decreased *B. sorokiniana* infection in wheat plants.[64]

CONCLUSION

Magnaporthe oryzae is a serious phytopathogen that has caused severe blast infections in rice all over the world. Controlling that illness in an environmentally sustainable manner is a huge task. In this investigation, garlic clove extract-derived AgNPs showed strong antifungal efficacy against *M. oryzae*. In conclusion, the production of silver nanoparticles using garlic clove extract is a straight forward, safe and one-step technique. These extracts operate as a reducing agent in the production of nanoparticles. The method does not require any chemical reagents or surfactant templates, giving the bioprocess the advantage of being ecologically benign. These findings not only offer a novel strategy to plant pathogen management, but they also significantly reduce or eliminate drug resistance. In the near future, modifications to AgNPs such as surface charge, acid-base property and aggregation behaviour will be carried out to assess their impact on AgNPs' antimicrobial activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AgNPs: Silver Nanoparticles; **NTA:** Nanoparticle tracking and analysis; **SEM:** Scanning Electron Microscopy; **FTIR:** Fourier Transform Infrared Spectroscopy; **XRD:** X-Ray Diffraction; ***M. oryzae*:** *Magnaporthe oryzae*; **RBD:** Rice Blast Disease.

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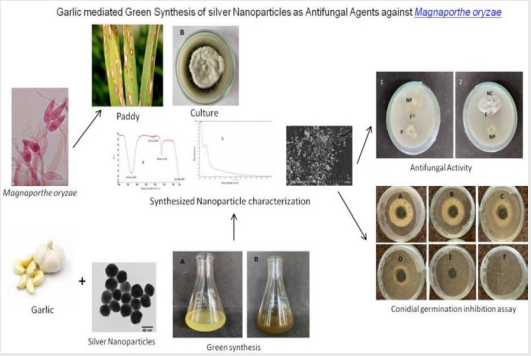
PICTORIAL ABSTRACT



SUMMARY

Many natural compounds are used and being tried as reducing agents to green synthesize silver nanoparticles which could be economical and more effective in terms of biological activities. Keeping in line with this aim, we tried to green synthesize silver nanoparticles using Garlic extract and used against the most potential plant pathogen *Magnaporthe oryzae*. We green synthesized, characterized and studied for antifungal activity. We found significant antifungal activity against *Magnaporthe oryzae*. The synthesized nanoparticles were of 20nm and might be the possible role of these AgNPs is they could be acting as leaking agents for the membrane. This could have lead to the growth inhibition of the fungus.

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