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Comparative Evaluation of Silver and Gold Nanoparticles for the Management of Bacterial Wilt of Potato Caused by *Ralstonia solanacearum*

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Abstract

Bacterial wilt of potato (*Solanum tuberosum* L.) is a devastating disease among potato crops across the world caused by soil-borne bacterium *Ralstonia solanacearum*, which leads to substantial economic and agricultural losses. The current study aimed to evaluate the antibacterial activity of silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) on *R. solanacearum* in *in-vitro* conditions. *In vitro* disc diffusion method revealed that out of five different concentrations of AgNPs and AuNPs, 5000 ppm causes the highest inhibition zone (mm) of 1.70 mm and 1.45 mm of *R. solanacearum*, respectively. Scanning Electron Microscope (SEM) analysis showed that these nanoparticles cause distortion of bacterial cell walls. Mode of action of AgNPs and AuNPs triggers the overproduction of superoxide radicals within bacteria that causes oxidative stress and structural damage leading to the disruption of cellular homeostasis, effectively killing the bacterium.

Keywords: AgNPs, AuNPs, Nanoparticles (NPs), *R. solanacearum*, Scanning Electron Microscope (SEM)

Introduction

Potato (*Solanum tuberosum* L.), often referred to as the "*King of Vegetables*," holds a significant place globally as a vital crop that supplies essential nutrients and is a key component of many recipes. As one of the world's most important foods, second only to rice, wheat and maize, it also makes a substantial contribution to global food security. Potatoes are an essential component to human nutrition since they are rich in carbohydrates, proteins, vitamins, minerals and dietary fiber (Kumar *et al.*, 2011). Meghalaya is a prominent potato producer in India and the crop plays a critical role in the state's food and agriculture economy. The East Khasi Hills region is the leader in production and contributes to 67% of the total production in the state (Kharumnuid *et al.*, 2020). Despite its importance, cultivation still faces many problems, especially diseases such as wilt caused by the bacterium *Ralstonia solanacearum*. Under favorable conditions of high humidity and temperature, this disease can lead to catastrophic yield losses, reaching 70% to 91% (Murthy *et al.*, 2021). The pathogen primarily targets the vascular system of the plants, obstructing water flow and resulting in rapid wilting and significant crop failure.

Existing management strategies for bacterial wilt rely heavily reliance on chemicals, which may be harmful to the environment as well as human health. This results in growing interest in sustainable and effective alternatives. In this aspect, nanotechnology provides promising solutions. Nanoparticles (NPs) possess a broad range of antimicrobial properties; particularly silver (Ag) and gold NPs, which may be used for the management of plant diseases. These NPs have unique physicochemical properties (small size and high surface area), which allows them to interact more with pathogenic microorganisms, potentially reducing the need for chemical pesticides.

Although several studies have demonstrated the antimicrobial potential of metallic nanoparticles against plant pathogens, comparative information on the antibacterial efficacy and mode of action of silver and gold nanoparticles against *Ralstonia solanacearum* causing potato bacterial wilt remains limited, particularly under Indian conditions. This research investigates the antimicrobial activity of AgNPs and

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AuNPs against various pathogenic bacteria, their mode of action as well as their effect on potato plant health with an aim to control bacterial wilt. This study aims to re-sustainable strategies for disease applicable with novel technology using nanotechnology integrated into agriculture that can help to preserve the potato crop as well as food security worldwide.

Materials and Methods

Samples were collected from a farmer's field in Pepbah village, East Khasi Hills district, Meghalaya, India (latitude 25.519865° N, longitude 91.93943725° E). Leaf, stems and soil samples were collected from solanaceous plants showing wilt disease symptoms. The collected stem samples were washed under running tap water to remove surface contaminants. An ooze test was carried out to confirm the pathogen by cutting the stem diagonally and placing them in distilled water. Samples that turned water turbid were selected for further analysis. *R. solanacearum*, a putative pathogen, was isolated through the dilution plate method. Morphological and biochemical characterization of the isolated *R. solanacearum* colonies were performed by the following tests:

1. Gram Staining: This technique was used to investigate the Gram reaction of the bacteria. Pure cultures were treated with crystal violet, iodine, ethanol and safranin; then examined under a microscope to differentiate between Gram-positive and Gram-negative bacteria.

2. Potassium Hydroxide (KOH) Test: The bacterial culture was placed on a glass slide and a drop of 3% KOH solution was added. The bacteria were classified as Gram-positive or Gram-negative based on whether a slimy thread was present or absent.

3. Kovac's Oxidase Test: This test was aimed at identifying the cytochrome oxidase enzyme in the bacteria. A positive result was indicated by the color change to blue of the tetramethyl-p-phenylenediamine dihydrochloride discs within 10 to 60 seconds after the application.

4. Catalase Test: The catalase test was performed to observe the production of catalase enzymes by the bacteria since these breaks down hydrogen peroxide to oxygen and water. The presence of bubbles was a positive result.

5. Simmons Citrate Agar Test: This biochemical test was used to determine the bacterium's ability to use citrate as a sole carbon source. The medium color changed from green to blue which signified a positive result.

6. Triphenyl Tetrazolium Chloride (TZC) Media Test: This test was used to distinguish between virulent and avirulent colonies of *R. solanacearum*. The virulent colonies were pink or light red with a red center, while the avirulent colonies were smaller and whitish in color.

7. Starch Hydrolysis Test: The hydrolysis of starch by the bacteria was determined on nutrient agar which contained 1% soluble starch. After incubation, plates were treated with iodine. The production of a clear zone around the line of bacterial growth was a positive result.

Silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) were purchased from Sisco Research Laboratories Pvt. Ltd. A stock solution of 5000 ppm from AgNPs and AuNPs were prepared by sonicating nanoparticles (NPs) for 10 minutes in a sonicator followed by continuous stirring on a magnetic hot plate for 30 minutes to ensure a proper mixing and NPs were confirmed *via* UV-Vis absorption spectroscopy at 340 nm and 540 nm, respectively.

Efficacy of AgNPs and AuNPs against *R. solanacearum* was tested *in vitro* at five concentrations, 5000, 4000, 3000, 2000 and 1000 ppm using the disk diffusion technique (Tendencia, 2004). A comparison was carried out with the standard antibiotics, *i.e.*, streptomycin sulphate @ 500 ppm (Table 1). The control plate without AuNPs and antibiotics was maintained with three replications. All plates were incubated at 28±1 °C in a BOD incubator (Equitron Medica Pvt. Ltd.) for 48 hours. The diameter of the inhibition zones was measured in mm after 48 hours of incubation. The experiment was conducted in a Completely Randomized Design (CRD) with three replications. Data were subjected to analysis of variance (ANOVA) and treatment means were compared at the 5% level of significance.

Table 1: *In-vitro* efficacy of AgNPs against the *R. solanacearum* by disc diffusion method

Treatment Combinations	Inhibition zone (mm)
T_1: AgNPs @ 5000 ppm + *R. solanacearum*	1.70b*
T_2: AgNPs @ 4000 ppm + *R. solanacearum*	1.65^b
T_3: AgNPs @ 3000 ppm + *R. solanacearum*	1.25^c
T_4: AgNPs @ 2000 ppm + *R. solanacearum*	0.00^d
T_5: AgNPs @ 1000 ppm + *R. solanacearum*	0.00^d
T_6: Control (*R. solanacearum*)	0.00^d
T_7: Streptomycin sulphate @ 500 ppm + *R. solanacearum*	3.33^a

*Data are the mean of three replications

Table 2: *In-vitro* efficacy of AuNPs against the *R. solanacearum* by disc diffusion method

Treatment Combinations	Inhibition zone (mm)
T_1: AuNPs @ 5000 ppm + *R. solanacearum*	1.45
T_2: AuNPs @ 4000 ppm + *R. solanacearum*	1.32
T_3: AuNPs @ 3000 ppm + *R. solanacearum*	1.10
T_4: AuNPs @ 2000 ppm + *R. solanacearum*	0.00
T_5: AuNPs @ 1000 ppm + *R. solanacearum*	0.00
T_6: Control (*R. solanacearum*)	0.00
T_7: Streptomycin sulphate @ 500 ppm + *R. solanacearum*	3.49

*Data are the mean of three replications

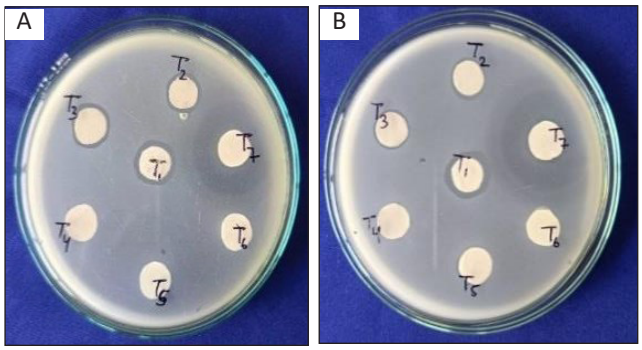

Figure 1: *In-vitro* efficacy of AgNPs and AuNPs against *R. solanacearum*: A) AgNPs at different treatment combinations; B) AuNPs at different treatment combinations

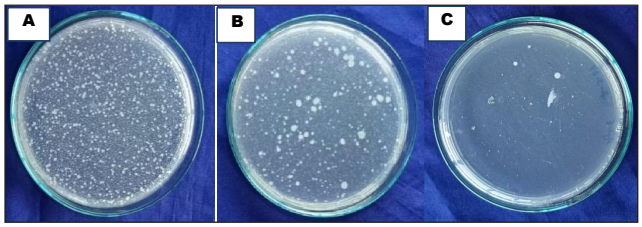

Figure 2: Microbial populations recorded at 24 hours of plating on NA media: A) 10^{-5} bacterial suspension (Control); B) AuNPs @ 5000 ppm + 10^{-5} bacterial suspension; C) AgNPs @ 5000 ppm + 10^{-5} bacterial suspension

Mode of Action of AuNPs and AgNPs on R. solanacearum

To study the mode of action of AuNPs and AgNPs on *R. solanacearum* three flasks were prepared containing nutrient broth, bacterial suspension and AgNPs and AuNPs. The flasks were shaken for 24 hours and then serial dilutions were performed with 10^{-5} dilutions factor. The plates were incubated in a BOD incubator for 48 hours and colonies were counted by a colony counter.

Mode of Action of AuNPs and AgNPs on R. solanacearum under Electron Microscope

The sample preparation was done using the protocol followed by Santiago *et al.* (2019). Bacterial cells were cultured in nutrient broth (NB) media and AgNPs and AuNPs (5000 ppm/ 0.5 ml) was added in culture tubes and the other was not treated (control); then incubated at 28 °C for two days. The cell of bacteria treated with NPs and untreated were applied to Formvar-coated Cu (400 mesh), film preparation was performed. The cells were then treated with a series of graded ethanol for dehydration followed by gold sputtering for SEM imaging. SEM analysis was performed at North-Eastern Hill University, Shillong.

Mode of Action of AuNPs and AgNPs on the Formation of ROS (Reactive Oxygen Species) in R. solanacearum

The study investigated the effects of silver and gold nanoparticles on the production of ROS, more specifically the superoxide radical (O_2^-), in *R. solanacearum* in an experiment using NBT (nitroblue tetrazolium) performed, according to the protocol given by Javvaji *et al.* (2020). To the best concentration of silver and gold nanoparticles suspension, 0.5 ml was introduced in 5 ml of the bacterial suspension culture and 1 ml NBT (0.1%) culture, 10 mM sodium azide and 50 mM sodium phosphate buffer (pH 7) were also introduced in the culture. The bacterial culture was allowed to grow for 24 hours and the sediment formed was examined under the microscope.

Results and Discussion

The bacterium *Ralstonia solanacearum*, responsible for bacterial wilt of potato, was successfully isolated on nutrient agar (NA) medium from infected potato roots and soil shows the smooth, circular, raised and dirty-white colony with pungent smell covering the entire Petri plate within 24 hours. However, colony characteristics of *R. solanacearum* have also been reported by Khetmalas (1984) and Tahat and Sijam (2010). The Gram staining reaction revealed that the bacterial cells were Gram-negative in reaction. This observation was further confirmed by the KOH test, in which the bacterial culture produced characteristic strands of viscid material upon treatment with 3% KOH on a glass slide, indicating a positive reaction for Gram-negative bacteria. Comparable observations for *R. solanacearum* grown on nutrient agar medium have also been reported by Chaudhry and Rashid (2011). Similarly, the test was positive for Kovac's oxidation Test, Catalase, Simmon's citrate agar test and negative for starch hydrolysis test. In triphenyl tetrazolium chloride (TZC) media, colonies of bacteria appeared fluidal whitish with a pink center. TZC medium is widely used for the selective identification of *Ralstonia solanacearum* during isolation (Kelman, 1954). On TZC medium, virulent colonies

typically appear fluidal, irregular and creamy white with a characteristic pink to red centre, whereas avirulent colonies are smaller, dark red and non-fluidal, thereby facilitating differentiation between the two colony types (Klement, 1990; Rahman *et al.*, 2010).

In vitro efficacy of AgNPs at different concentrations (5000, 4000, 3000, 2000 and 1000 ppm) against *R. solanacearum* was evaluated using antibiotics, *i.e.*, streptomycin sulphate (500 ppm) and a control for comparison. Out of different treatment concentrations, AgNPs at 5000 ppm demonstrated the most significant antibacterial effect, producing a 1.70 mm zone of inhibition, followed by 4000 ppm and 3000 ppm with an inhibition diameter of 1.65 and 1.25 mm, respectively (Table 1, Figure 1A). Similarly, for AuNPs, highest inhibition was recorded at 5000 ppm with an inhibition diameter of 1.45 mm. This was followed by 4000 ppm and 3000 ppm with an inhibition diameter of 1.35 and 1.10 mm, respectively (Table 2, Figure 1B). Antibiotics showed the highest inhibition zone and no zone was found for the treatment of 1000 ppm, 2000 ppm and control in case of Ag as well as Au NPs. Dilbar *et al.* (2023) reported that all treatments tested against *R. solanacearum* were concentration dependent, *i.e.*, the activity decreased with a decrease in the treatment concentration. It was previously reported by Das and Dutta (2021) that AgNPs at 200 ppm were more inhibitative (73.39%) as compared to AuNPs at 200 ppm (60.83%) in the radial growth of *Rhizoctonia solani*.

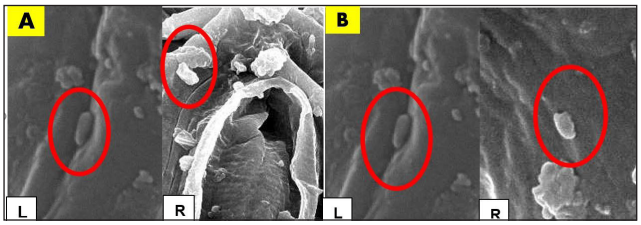

Figure 3: Scanning electron microscopic images showing distortion of the bacterial cell wall of *Ralstonia solanacearum* following treatment with: A) silver nanoparticles and B) gold nanoparticles [L: Untreated *R. solanacearum*, R: Distortion of cell wall treated with *R. solanacearum*]

The application of AgNPs and AuNPs against *R. solanacearum* at the concentration of 5000 ppm resulted in significant reduction of *R. solanacearum* growth, with AgNPs exhibiting a more pronounced antibacterial effect than AuNPs. The maximum inhibitory effect of AgNPs, was recorded with the bacterial count of 6.78 cfu ml^{-1}, compared to AuNPs (7.20 cfu ml^{-1}) and the control (7.70 cfu ml^{-1}) (Figure 2). According to Lok *et al.* (2006), AgNPs showed their antibacterial mechanism as an interaction with thiol groups on proteins, which in turn, disrupts essential enzymes and hinders bacterial metabolism. Additionally, AgNPs can penetrate bacterial cells and alter the integrity of bacterial membranes by increasing their permeability, resulting in cell content diffusing outwards (Feng *et al.*, 2000). The effect of AuNPs was slightly less effective in inhibiting the growth of *R. solanacearum* than AgNPs, which was also reported by Dykman and Khlebtsov (2012). According to Li *et al.* (2010), mode of action of AuNPs is thought to involve their binding to the bacterial cell membranes with resultant cell wall destruction and inhibition of ATP production or other metabolic processes. AuNPs have been shown to affect the expression of genes involved in bacterial stress responses, further contributing to their antibacterial activity as reported by Gupta *et al.* (1999).

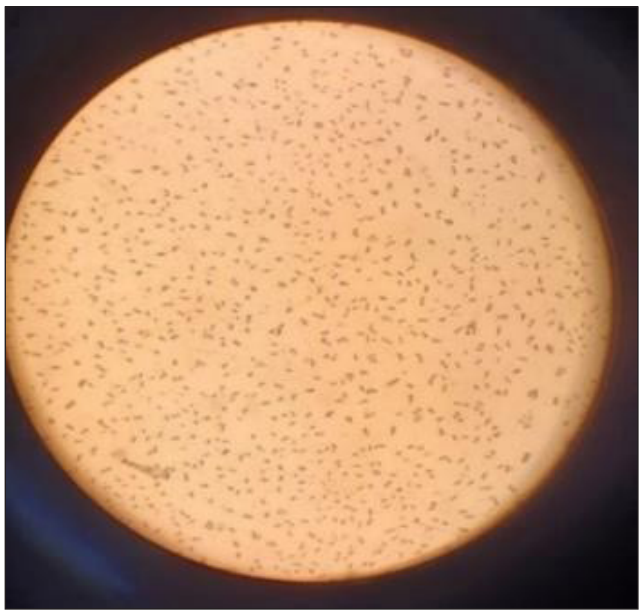

Figure 4: Nitroblue tetrazolium (NBT) assay showing intracellular superoxide radical generation in *Ralstonia solanacearum* following nanoparticle treatment (40X magnification)

The SEM results revealed that untreated *R. solanacearum* cells maintain a typical, undisturbed cell wall structure, suggesting their viability and functionality. In contrast, the treatment with AgNPs and AuNPs results in pronounced distortions of the cell wall, which is indicative of significant cellular damage (Figure 3). The similar observation was reported by Talarska *et al.* (2021) and Hovhannisyan *et al.* (2023), AgNPs are known to attach to bacterial cell walls, penetrate the cells and disrupt essential cellular processes, suggesting severe membrane disruption that ultimately resulted in bacterial cell death through mechanisms such as the generation of reactive oxygen species (ROS) and interference with metabolic functions. AgNPs tend to release silver ions that are directly linked to their antibacterial activity. These ions interact with proteins and DNA within cells, disrupting electron transport chains and inhibiting DNA replication (Talarska *et al.*, 2021). This mechanism is supported by Hovhannisyan *et al.* (2023), the formation of pores in the bacterial membrane, causing osmotic shock and ultimately leading to cell lysis. Bacterial cells were also examined for ROS generation by measuring superoxide radical levels after treatment with 5000 ppm concentrations of AgNPs and AuNPs. This resulted in a marked color change in the bacterial suspensions, indicative of elevated oxidative stress levels in *R. solanacearum* treated with both AgNPs and AuNPs compared to the untreated control (Figure 4).

No colour change was observed in the untreated control, indicating that nanoparticle exposure induced superoxide radical formation. The increased production of superoxide radicals serves as a primary marker for oxidative stress, resulting in significant cellular damage and ultimately bacterial cell death (Sharma *et al.*, 2012). AgNPs produce these radicals by generating ROS through decomposition of hydrogen peroxide (H_2O_2) or reaction with molecular oxygen (O_2) (Gurunathan *et al.*, 2009). An excessive accumulation of these radicals within bacterial cells can lead to oxidative damage to vital structures such as cell membrane, genetic material and proteins, leading to the disruption of cellular homeostasis and the eventual death of the bacterium (Kim *et al.*, 2007).

Conclusion

The present investigation demonstrated that both silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) possess significant antibacterial activity against *Ralstonia solanacearum*, the causal agent of bacterial wilt in potato. Among the two nanoparticles, AgNPs exhibited comparatively greater antibacterial efficacy, as reflected by larger inhibition zones, more pronounced suppression of bacterial growth and greater disruption of bacterial cell integrity. Scanning electron microscopic observations confirmed severe morphological alterations in the bacterial cells following nanoparticle treatment, while enhanced production of reactive oxygen species indicated that oxidative stress was an important mechanism underlying their antibacterial activity. The findings suggest that metallic nanoparticles, particularly AgNPs, have considerable potential as eco-friendly alternatives for the management of bacterial wilt by effectively suppressing the pathogen through multiple antibacterial mechanisms. Nevertheless, comprehensive greenhouse and field evaluations, together with detailed assessments of environmental safety, phytotoxicity and economic feasibility, are essential before their large-scale integration into sustainable potato disease management programmes.

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Ethical Statement

The authors declare that no generative artificial intelligence tools were used in drafting or preparing this manuscript. The manuscript was written, interpreted and revised solely by the authors, who take full responsibility for its content.

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