**RESEARCH PAPER**

# Biogenic silver nanoparticles as an antibacterial agent against bacterial leaf blight causing rice phytopathogen *Xanthomonas oryzae* pv. *oryzae*

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## Abstract

Silver nanoparticles (Ag NP) were produced utilizing leaf extract of rice cultivar Taichung native-1. Various factors like leaf extract, silver nitrate concentrations, and duration of autoclaving were standardized during synthesis. Nanoparticles were analyzed with UV–visible absorption spectroscopy (UV–vis), dynamic light scattering, zeta potential, X-ray diffraction and transmission electron microscopy techniques. The synthesis was noted at 0.4% extract, 0.6 mM silver nitrate, 30 min of autoclaving and NP formation was confirmed from 424 nm peak in UV–vis. NP showed zeta potential of $-27$ mV, face-centered cubic (fcc) crystal nature and sized around $16.5 \pm 5.9$ nm. Biogenic NP synthesized from susceptible rice variety were used as an antibacterial agent against phytopathogen *Xanthomonas oryzae* pv. *oryzae (Xoo)*, the causative agent of bacterial leaf blight (BLB) disease in rice. Antibacterial effect of Ag NP was evaluated using in vitro assays and in vivo efficacy under greenhouse conditions. Results confirmed effective inhibition of *Xoo* growth and colony formation by Ag NP and found to be the more powerful antibacterial agent. Besides, Ag NP treatment (10 µg/mL) caused an enhancement in seedling vigor index. Pots treated with Ag NP (15 µg/mL) in vivo in greenhouse showed disease severity of 26.6% and disease decrease over control of 49.2%, at a much lower NP concentration than earlier reported studies. Thus, the current report implies using the leaf extract synthesized Ag NP to control and BLB disease management in field conditions.

**Keywords** Bacterial leaf blight · Antibacterial · Leaf extract · Greenhouse · Phytopathogen · Rice · Silver nanoparticles · *Xanthomonas oryzae* pv. *oryzae*

## Key message

- Biogenic silver nanoparticles (Ag NP) were used as an antibacterial agent against bacterial leaf blight-causing rice phytopathogen *Xanthomonas oryzae* pv. *oryzae*.
- Based on MIC and MBC, NP was the powerful antibacterial agent concerning concentration and size.
- In vivo greenhouse studies showed disease severity of 26.6% and disease decrease over control of 49.2%, at a much lower concentration.
- Current report implies using the leaf extract synthesized Ag NP to control and BLB disease management in field conditions.

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## Introduction

Rice (*Oryza sativa* L.) occupies the second position among the cereal crops and serves as the staple food for nearly 3.8 billion world population in terms of dietary energy supply. It is cultivated in an area of 150 M ha and Asia is the maximum rice producer. In India, rice crop occupies 43.5 M ha area with 104.92 million tons production and 2393 kg/ha productivity [9]. In Telangana state, rice is cultivated in an area of 1.7 million hectares with 6.4 million metric tons production and 3685 kg/ha average productivity [9, 24, 25, 31].

In Telangana, various rice varieties such as RNR-15048, BPT-5204, MTU-1010, JGL-1798 and Tellahamsa are widely grown. They are very popular among the farmers due to their superior yield, desirable grain and cooking quality. Despite their popularity, they are susceptible to many pests and diseases like blast, bacterial leaf blight, sheath blight, brown spot, etc. For example, blast in rice is caused by *Magnaporthe grisea,* sheath blight by *Rhizoctonia solani* and brown spot by *Cochliobolus miyabeanus.* Among the diseases of rice, the bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* is one of the India's significant production constraints mainly in irrigated and rainfed lowland ecosystems. The high yielding rice cultivars grown under irrigated and surplus nitrogen fertilization are primarily affected by BLB disease. Being one of the most destructive diseases in *Kharif* (monsoon) season crop, it causes significant yield losses ranging from 20 to 50%. Especially in all rice growing Indian states such as Telangana, Andhra Pradesh, Kerala, Maharashtra, Chhattisgarh, Gujarat, Himachal Pradesh, Karnataka, Punjab, Haryana, Uttaranchal, Bihar, West Bengal, Tripura, Odisha, Assam, Tamil Nadu, Uttar Pradesh and Andaman and Nicobar islands. The BLB disease at farming level is controlled by spraying bactericides such as probenazole, jinggangmycin, streptomycin and streptocycline. As the chemical control of disease is not effective at the field level, it is managed through host plant resistance and nutrient management [6, 21, 24, 25, 28, 34].

The application of nanotechnology is emerging as an exciting area of science, especially in agriculture, as an alternative method for preventing and controlling various plant diseases [3]. Given the economic importance and unsatisfactory management of BLB in rice, considerable efforts have been made to develop alternative inhibition sources against *X. oryzae* pv. *oryzae*. Among the metal nanoparticles, the antibacterial action of silver nanoparticles is extensively studied. It is attributed to their characteristic physical, chemical and biological properties [26] in terms of its multiple modes of inhibitory action [15] and long-term protection via sustained silver release from

nanoparticles [41]. The effect of silver nanoparticles (Ag NP) on phytopathogenic bacteria such as *Pseudomonas syringae* pv. *syringae*, *Xanthomonas campestris* pv. *vesicatoria*; phytopathogenic fungi such as *Pythium ultimum*, *M. grisea*, *Colletotrichum gloeosporiodes, Botrytis cinerea, R. solani* [29] and cyanobacterial such as *Microcystis aeruginosa* [30] has been investigated.

The current study aimed at the biosynthesis of silver nanoparticles from the metal precursor silver nitrate using rice leaf extract as a reductant and stabilizer by autoclaving. It focuses on the various factor optimizations like leaf extract, silver nitrate concentrations; and autoclaving time during the synthesis. We have chosen autoclave based synthesis due to (i) non-stable and aggregated nature (within 24 h) of Ag NP synthesized at ambient temperature, (ii) inefficient synthesis at room temperature, (iii) production of inherently safe, microbe free Ag NP by autoclaving and (iv) stable and efficient synthesis by autoclaving. The possible bioactive substances of the leaf extract engaged in metal ion reduction and nanoparticle stabilization was quantified by standard spectroscopic methods. The nanoparticles were studied with UV–visible absorption spectroscopy, dynamic light scattering, zeta potential, X-ray diffraction and transmission electron microscopy techniques. Due to the abundance of potent reducing and stabilizing biomolecules in the leaf extract, the Ag NP with reasonably good size were produced at a much lower extract concentration, in comparison with earlier leaf extract based biosynthetic protocols.

The Ag NP fabricated with BLB susceptible rice variety, TN-1 were used as a bactericide against various TN-1 infecting *Xanthomonas oryzae* pv. *oryzae* isolates under in vitro conditions employing well diffusion, poisoned food and broth dilution techniques. The leaf extract biosynthesized silver nanoparticles were found to be a more powerful bactericide with reference to nanoparticle concentration and particle size. The applicability of the developed bactericide was also evaluated under greenhouse conditions for the management of BLB. In the current investigation, a disease severity of 26.6% and disease decrease over control of 49.2% were noted at a much lower concentration of nanoparticles than the previous studies.

## Materials and methods

### Materials

The seeds of rice variety Taichung native-1 (TN-1) were procured from Rice Research Centre, Agricultural Research Institute, Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad, India. Silver nitrate ($AgNO_3$) (Merck, Mumbai, India), nutrient broth (NB), nutrient agar (NA) (HiMedia, Mumbai, India),

streptocycline (Hindustan Antibiotics, Pune, India) and streptomycin sulfate (Sigma-Aldrich, Bengaluru, India) were employed in this study. The ultrapure water with 18.2 MΩ.Cm resistivity produced from Purelab Flex 3 water polishing unit (Elga, High Wycombe, England) was used for solution preparation. The plasticware, glassware and culture media were sterilized at 121 °C for 20 min in vertical autoclave (Obromax, Delhi, India).

## Synthesis of silver nanoparticles (Ag NP)

The seeds of the above rice variety were grown in earthen pots and the leaves were collected from 30-day-old seedlings. The leaves were thoroughly washed and cleaned with sterile ultrapure water. The leaves were finely cut into pieces, dried at 60 °C for 48 h and made into fine powder in a domestic mixer grinder. A 1% leaf extract stock solution was made by heating in a microwave oven for 2 min. With Whatman filter paper No 1 (25 μm), the solution was filtered, centrifuged (5, 500 g, 10 min) and the collected supernatant was further filtered with sterile 0.22 μm syringe filter (Millipore, Bengaluru, India). The obtained pale green colored extract was preserved at 4 °C and utilized for Ag NP production (Fig. 1). The soluble sugar [40], phenolic [38], protein [5] and chlorophyll [39] content of the extract was quantified with standard methods. The Ag NP were biosynthesized by autoclaving the AgNO$_3$ containing aqueous leaf extract solution at 121 °C and 103 kPa in a domestic pressure cooker for 30 min. The effect of an array of variables such as leaf extract concentration (0.1–0.5%), AgNO$_3$ concentration (0.2–1 mM) and autoclaving duration (10–60 min) on biosynthesis of Ag NP was optimized.

## Characterization of Ag NP

The fabricated Ag NP's absorption spectra were measured with UV–visible absorption spectrophotometer (Specord 200 Plus, Analytic Jena AG, Jena, Germany) against the corresponding autoclaved extract blanks at 300–800 nm. The zeta potential, z-average particle size and polydispersity index of the generated NP solutions were quantified with a zeta sizer (Nano ZS90, Malvern Panalytical, Malvern, UK). The X-ray diffraction pattern was obtained using monochromatic Cu Kα radiation ($\lambda = 1.5406$ Å) at 40 kV and 30 mA (Ultima IV diffractometer, Rigaku, Tokyo, Japan). The intensity data were collected from the glass slide deposited NP at 35–70° 2θ and scan rate of 1°/min. The NP's size and shape were captured at 200 kV with the transmission electron microscope (TEM) (Tecnai 20 G2 S-Twin, FEI, Eindhoven, Netherlands). The TEM samples were prepared by deposition and drying of nanoparticle suspension on carbon supported copper grid at ambient temperature. The NP solutions were powdered with benchtop cascade freeze dry system (Freezone 4.5L Plus, Labconco, Kansas City, MO, USA). The IR spectra of the powdered samples were recorded with FTIR spectrometer (Tensor 27, Bruker Optics, Ettlingen, Germany) at a wavenumber of 1000–4000 cm$^{-1}$.

## Antibacterial activity of biosynthesized Ag NP against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)

The pure cultures of *Xoo* named as NB-1, NB-2, NB-3, NB-4, NB-5 and NB-6, isolated from the infected rice leaves indicating characteristic BLB disease symptoms were used for studying the i*n vitro* and in vivo susceptibility towards Ag NP. The bacterial suspension of the isolates was obtained from the cultures which were grown in nutrient broth (NB) overnight and adjusted to 0.5 McFarland standard at 600 nm. The Ag NP biosynthesized at an optimal condition of 0.4% leaf extract, 0.6 mM AgNO$_3$ and 30 min of autoclaving were employed. Ag NP concentrations for testing were selected based on the antibacterial susceptibility assay and they were in the range of 2.5–15 μg/mL. The susceptibility studies were carried out in triplicate with all the six bacterial isolates.



**Fig. 1** The digital photographs of **a** 30-day-old seedlings of TN-1 rice cultivar, **b** dried leaf powder and **c** 1% aqueous leaf extract
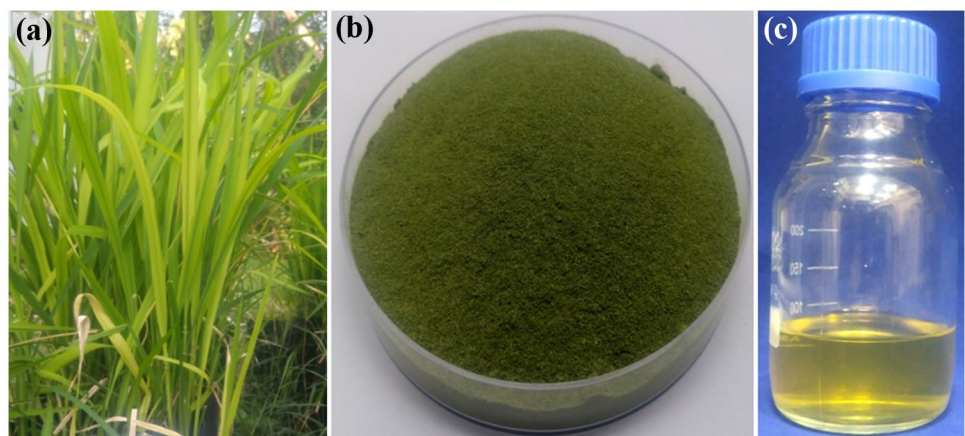
## Agar well diffusion method

Agar well diffusion method was used with nutrient agar (NA) plates inoculated with 100 µl of bacterial suspension ($10^6$ CFU/mL). A well of 13 mm diameter was made in the center of the plate and the wells were loaded with different amounts of Ag NP (5–15 µg). The corresponding positive and negative control plates were kept with streptomycin (10 µg) and rice leaf extracts (0.4%). The plates were incubated at $27 \pm 1$ ºC and the zone of bacterial growth inhibition was measured after 48 h of incubation. The inhibition zone was calculated by negating the well diameter from the total inhibition zone diameter and expressed in mm [16].

## Poisoned food technique

Agar dilution technique was used to study the dose-dependent antibacterial activity of the Ag NP-TN-1 [15]. The NA plates were supplemented with different concentrations of Ag NP (2.5–10 µg/mL) and inoculated with 100 µl of bacterial suspension ($10^6$ CFU/mL) by spread plating method. The corresponding positive and negative plates were kept with streptomycin (10 µg/mL) and leaf extract (0.4%). The plates were incubated at $27 \pm 1$ ºC for 48 h, monitored for bacterial colony formation and the number of surviving bacteria was counted. The bacterial growth inhibition (%) at different concentrations of Ag NP was calculated using the formula: The bacterial growth inhibition (%) = (number of colony forming units in control-number of colony forming units in treatment/number of colony forming units in control) × 100.

## Broth dilution method

The minimum inhibitory concentration (MIC) and minimal bacterial concentration (MBC) of Ag NP against the test bacterial isolates were determined with broth dilution method. Ag NP concentrations of (2.5–10 µg/mL) were added to glass tubes containing nutrient broth. The corresponding positive and negative plates were kept with streptomycin (10 µg/mL) and extract (0.4%). The NB tubes were inoculated with 100 µl of bacterial suspension ($10^6$ CFU/mL) and incubated at $27 \pm 1$ ºC in static mode for 48 h and observed for bacterial growth in terms of visual turbidity and colony count. The turbidity was determined through visual inspection and absorbance reading at 600 nm. The concentration with no increment in absorbance, in comparison with the initial absorbance was taken as MIC. The treated tubes with no turbidity and no increment in absorbance were plated on NA and the number of bacterial colonies was counted. The lowest concentration at which no colony formation on

NA plates was taken as MBC [15]. Further, the bacterial growth inhibition (%) at different Ag NP concentrations was calculated [18].

## Greenhouse evaluation of Ag NP

Ag NP's efficacy against BLB disease of rice under in vivo greenhouse conditions was determined by clip inoculation technique (International Network for Genetic Evaluation of Rice and Institute 1996; [11]. The leaves of rice cultivar BPT-5204 grown for 45 days in earthen pots were clip inoculated and kept under polythene bags in a moist chamber at 90% relative humidity and 25–28 ºC for expression of BLB disease symptoms [21]. A total of two sprays of Ag NP of different concentrations (2.5–15 µg/mL) were applied at an interval of 30 days, after the first appearance of disease symptoms. The corresponding positive and negative control pots were kept with streptocycline (15 µg/mL) and sterile ultrapure water. The observations for BLB incidence were drawn on five randomly selected plants and the disease severity (%) and disease decrease over control (%) were recorded using 0–9 scale at 15 days (60 days after sowing) and 45 days (90 days after sowing), after clip inoculation.

## Seed germination

The healthy seeds of rice cultivars BPT-5204 (BLB susceptible) and RP Bio-226 (BLB resistant) were surface sterilized with 0.5% sodium hypochlorite for 10 min and rinsed several times with sterile ultrapure water. The seeds were soaked in Ag NP (0–10 µg/mL) for 30 min, washed with sterile ultrapure water and blot dried. Ten seeds were placed on sterile filter paper lined petriplates moistened with 5 mL of sterile ultrapure water. These plates were covered with lids and incubated under dark at 25 °C. The seed germination was monitored for 5 days and data on germination (%), seedling length and dry weight were collected. The seedling vigor index (SVI) I and II were calculated based on the equations: SVI I = germination (%) × seedling length (cm) and SVI II = germination (%) × seedling dry weight (g), respectively [7].

All the experiments were carried out in triplicate and the data were presented as mean ± standard deviation (SD). The statistical significance of the results was determined at $p < 0.05$ with one-way analysis of variance (ANOVA) using statistical package OPSTAT.

## Results and discussion

A facile, autoclave mediated synthetic method for Ag NP was designed with rice leaf extract. The Ag NP were generated by autoclaving the $AgNO_3$ supplemented extract

solutions at 121 °C and 103 kPa in a domestic pressure cooker for different periods. The extract mediated Ag NP formation was visualized through a color change from faint yellow to intense brown (Fig. 2). The observation was found to be following the Ag NP synthesis by the aqueous solution of gum kondagogu, *Cochlospermum gossypium* [16]. The various factors including the concentrations of leaf extract (0.1–0.5%), $AgNO_3$ (0.2–1 mM), and autoclaving time (10–60 min) on nanoparticle formation were studied for obtaining the stable preparation. The autoclaving process generates sterile and safe Ag NP in aqueous medium towards antimicrobial applications [15]. The synthesis optimization was studied through recording the characteristic UV–vis absorption spectra of the synthesized Ag NP between 300 and 800 nm. The mean particle size and polydispersity index (PDI) of the produced NP were further utilized to establish the optimal synthesis conditions.

## Characterization of biosynthesized Ag NP

The produced Ag NP were characterized using an array of technical tools such as UV–visible absorption spectroscopy, dynamic light scattering, zeta potential, X-ray diffraction, transmission electron microscopy and Fourier transform infrared spectroscopy.

## UV–visible absorption spectroscopy (UV–vis)

At 0.6 mM $AgNO_3$ and 30 min of autoclaving, the synthesis was carried out at a varying extract concentration (0.1–0.5%). The absorption band intensity increased with a raise in extract concentration from 0.1 to 0.4% and remained more or less at 0.5% (Fig. 2a), indicating the increment in nanoparticle synthetic efficiency with an enhancement in extract concentration. Concomitantly, the nanoparticle solution's color intensity is increased with increase in extract concentration [Inset of (Fig. 2a)]. Additionally, the reduction was monitored at a fixed extract concentration of 0.4% and 30 min of reaction time; by varying the $AgNO_3$ concentration (0.2–1 mM). The reduction was augmented with a raise in $AgNO_3$ concentration from 0.2 to 1 mM. The high absorbance intensity at higher $AgNO_3$ concentration is due to enhanced functional group oxidation by the silver ions (Fig. 2b). The respective spectra were matched with the intensities of NP solution colors [Inset of Fig. 2b]. However, further optimization studies were carried out with 0.6 mM $AgNO_3$, as the Ag NP synthesized at higher concentrations of metal precursors (0.8 and 1 mM) were precipitated within 24 h. Then, the reduction was followed at 0.6 mM $AgNO_3$ and 0.4% extract by changing the autoclaving time (10–60 min). The reduction rate increased from 10 to 50 min and continued more or less at 60 min (Fig. 2c). It is further corroborated from the respective nanoparticle solution colors [Inset of Fig. 2c]. In the UV–vis spectra, a solitary



**Fig. 2** The UV–vis absorption spectra of biogenic Ag NP generated at changing **a** concentrations of leaf extract (0.1–0.5%) at 0.6 mM $AgNO_3$ and 30 min of autoclaving. Inset: Respective colors of NP solutions; **b** concentrations of $AgNO_3$ (0.2–1 mM), at 0.4% extract and 30 min of autoclaving. Inset: Respective colors of NP solutions; and **c** autoclaving period (10–60 min) at 0.4% extract and 0.6 mM $AgNO_3$. Inset: Respective colors of NP solutions
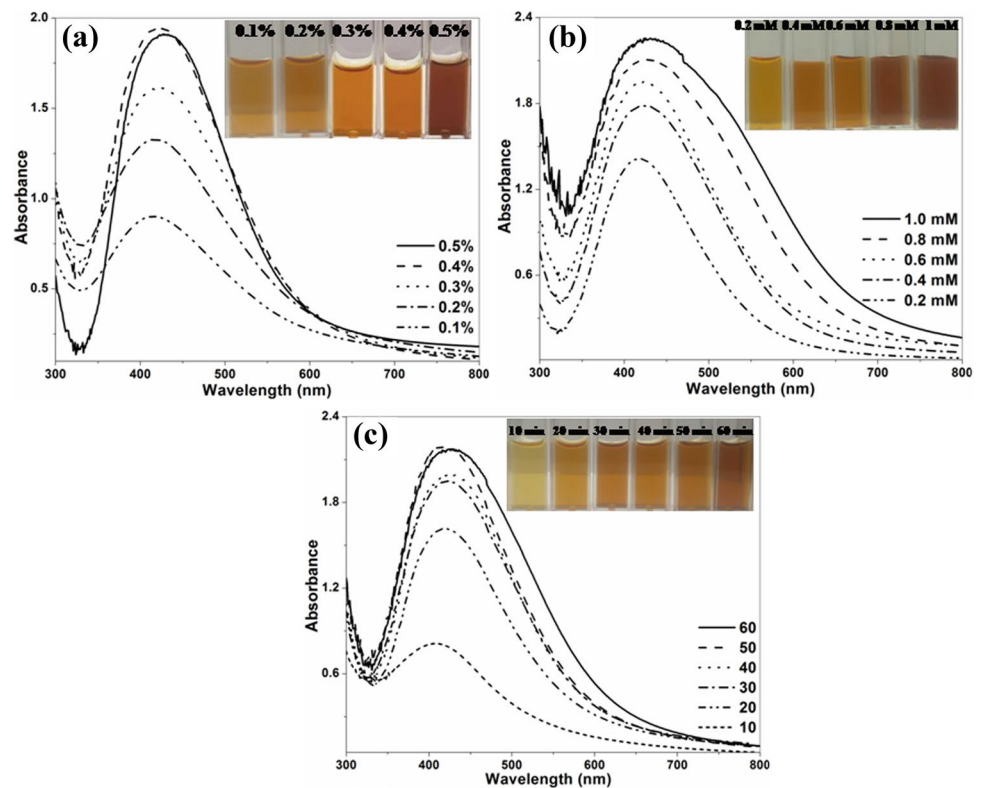
dominant peak with absorption maximum in 409–433 nm range was noted in the present study was corresponding to typical surface plasmon resonance (SPR) of Ag NP. With a raise in extract concentration from 0.1 to 0.3%, the SPR peak value shifted from 414 to 425 nm. The peak value decreased to 422 nm at 0.4% extract and increased to 427 nm at an elevated extract concentration of 0.5%. Based on the SPR peak position, further optimizations were carried out with 0.4% extract by changing the $AgNO_3$ concentration (0.2–1 mM) at 30 min of autoclaving. It was noted that the SPR peak shifted from lower (418 nm) to higher wavelength (433 nm), with an increase in metal precursor concentration. At an optimal concentration of 0.6 mM $AgNO_3$, the SPR peak was noted at 422 nm. The Ag NP synthesized at higher metal precursor concentrations (0.8 and 1 mM) precipitated in 24 h. Further, at 0.4% extract and 0.6 mM $AgNO_3$, the SPR peaks were noted by changing the reaction time (10–60 min). As autoclaving time increased, the SPR peak was shifted to higher wavelength (409–429 nm) and the particle size enhanced concomitantly leading to particle precipitation. At optimized conditions of 0.4% extract, 0.6 mM $AgNO_3$ and 30 min of reaction time, the SPR peak was noted at 422 nm. The results are in tune with Ag NP synthesis using gum tragacanth (*Astragalus gummifer*) [12].

## Mean particle size and polydispersity index (PDI)

With a raise in extract concentration, the mean particle size decreased from 224 to 120 nm up to 0.4% and increased at 0.5% (324 nm). The PDI increased from 0.28 to 0.36, with a raise in extract concentration from 0.1 to 0.3%. It reached a minimum value of 0.27 at 0.4% and then again increased to 0.46 at 0.5% extract (Fig. 3a). Depending on the data, the particle size distribution was evaluated at different$AgNO_3$ (0.2–1 mM), 0.4% leaf extract and 30 min of autoclaving. With a rise in metal precursor concentration from 0.2 to 0.6 mM, the particle diameter decreased from 151 to 120 nm. Then, it increased at higher $AgNO_3$ concentrations and reached to 252 nm at 1 mM $AgNO_3$. The PDI reduced to a minimum value of 0.27 at 0.6 mM and enhanced to 0.52 at 1 mM $AgNO_3$ (Fig. 3b). In addition, the particle size distribution was studied at variable reaction time (10–60 min) at 0.4% leaf extract and 0.6 mM $AgNO_3$. As the reaction time enhanced from 10 to 30 min, the particle size diminished from 221 to 120 nm. Then, with a raise in time from 40 to 60 min, the size enhanced in parallel from 177 to 196 nm. However, the PDI reduced to a minimum value of 0.27 at 30 min and further elevated at 60 min (0.31) (Fig. 3c). From the UV–vis spectra and zeta sizer results, the optimal reaction conditions for reduction were 0.4% leaf extract, 0.6 mM $AgNO_3$ and 30 min of autoclaving and the produced Ag NP were labeled as Ag NP-TN-1. The corresponding average particle diameter and zeta potential values were 120 nm and
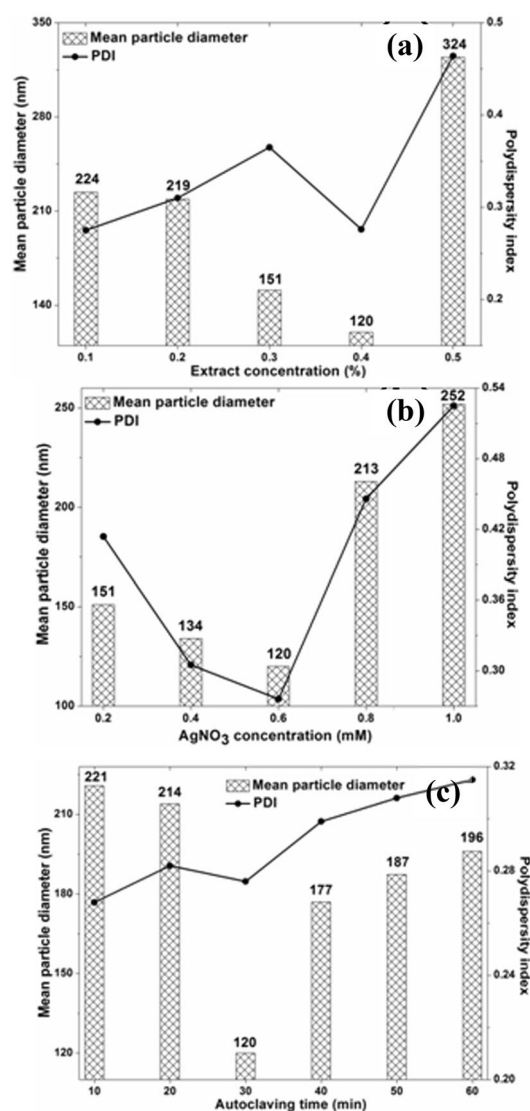


**Fig. 3** The z-average particle diameter and PDI of Ag NP at varying **a** leaf extract concentration at 0.6 mM $AgNO_3$ and 30 min of autoclaving, **b** $AgNO_3$ concentration at 0.4% extract and 30 min of autoclaving and **c** autoclaving time at 0.4% extract and 0.6 mM $AgNO_3$

−27.4 mV (Fig. 4). The results are in line with palladium nanoparticle synthesis using gum ghatti (*Anogeissus latifolia*) [14].

## X-ray diffraction (XRD)

Ag NP's XRD pattern showed four discrete diffraction peaks at 38.3º, 44.5º, 64.7º and 77.9º, respectively. They were corresponding to (111), (200), (220) and (311) planes from face-centered cubic crystal structure of metallic silver (Fig. 5). The quantified corresponding interplanar spacing ($d_{hkl}$) and lattice constant values (2.33, 2.03, 1.44 and 1.22Aº; and 4.05 Aº) matched well with the standard metallic silver values
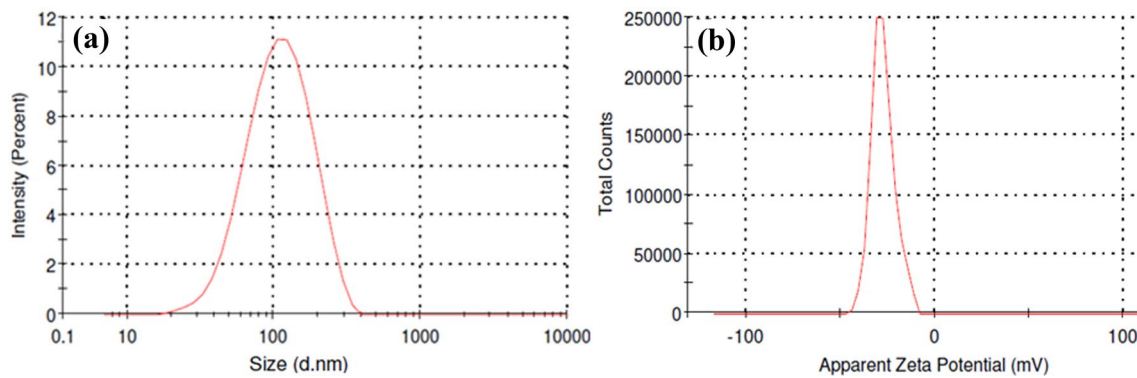
**Fig. 4** The **a** particle size distribution and **b** zeta potential of Ag NP-TN-1
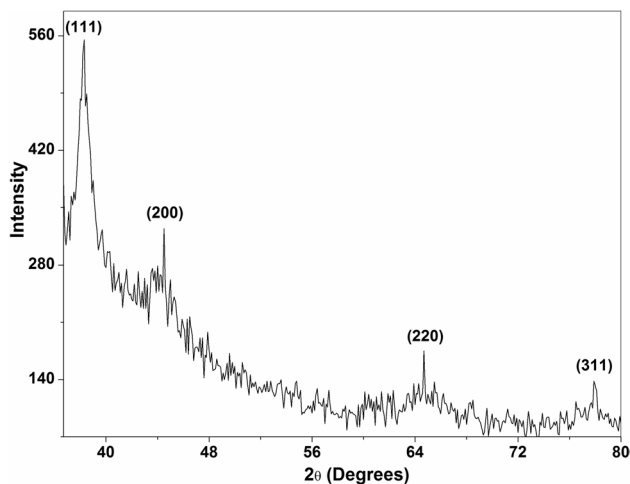


**Fig. 5** The XRD pattern of Ag NP demonstrating face-centered cubic (fcc) crystal structure

(JCPS PDF 04–0783). The nano-dimension of the Ag NP was corroborated from the broadened diffraction peaks. Thus, the diffraction pattern obtained in the current study is as per the results obtained with Ag NP synthesized using the leaf extract of *Hibiscus cannabinus* [4].

## Transmission electron microscopy (TEM)

The morphology and particle size of the Ag NP were characterized using TEM. The electron micrographs at various magnification scales show polydisperse nature and spherical particle shape (Fig. 6). The particle size ranged from 5 to 33 nm and the mean particle size derived from the corresponding particle diameter distribution was about $16.5 \pm 5.9$ nm (Fig. 6e). The crystalline nature of the produced Ag NP was supported from the concentric ring and intermittent dot pattern of selected area electron diffraction (SAED). These rings could be ascribed to (111), (200), (220) and (311) diffraction planes of face-centered cubic silver

(Fig. 6f). Additionally, it was further established from the XRD pattern.

The particle size distribution and mean particle size of the Ag NP synthesized with plant leaf extract depend upon the extract, $AgNO_3$ concentrations, and other reaction conditions such as temperature and reaction time. Hence, the NP's achieved average size in the present report was compared with various biogenic Ag NP produced by employing leaf extracts from different plants. With several extract-based methodologies, the reaction rate is slow and the reduction time ranged from 24 to 48 h [2, 22]. Notably, the final concentration of the extract in the reaction mixture was about 0.4%, which was lower than many other leaf extracts [1, 18, 19, 32]. This observation supports the presence of potent reducing and stabilizing compounds in the rice leaf extract. With many leaf extracts, produced nanoparticle size was larger [1, 17–19, 32] in comparison with 16.5 nm-sized Ag NP produced in the current study. Thus, the Ag NP-TN-1 in the present study were reasonably good in size, compared with many other leaf extract-based biosynthetic methods.

## Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the leaf extract-synthesized Ag NP and leaf extract were taken to check the extract's functional groups which mediated the NP's reduction and capping (Fig. 7). The key absorbance bands in the extract's IR spectrum were at 3135, 2362, 1634, 1401, 1274, 1123 and 994 $cm^{-1}$, respectively. The extensive band at 3135 $cm^{-1}$ could be correlated to O–H group stretching vibrations. The peak at 2362 $cm^{-1}$ could be linked to different carbonyl species. The peak at 1634 $cm^{-1}$ was from symmetric carboxylate stretch and amide I from the carbonyl stretch of protein's amide linkage. The dominant band at 1401 $cm^{-1}$ might be ascribed to distinct asymmetrical carboxylate stretch. The bands noted at 1274 and 1123, and 1002 $cm^{-1}$ represent C–O stretch of corresponding polyol and alcoholic groups.
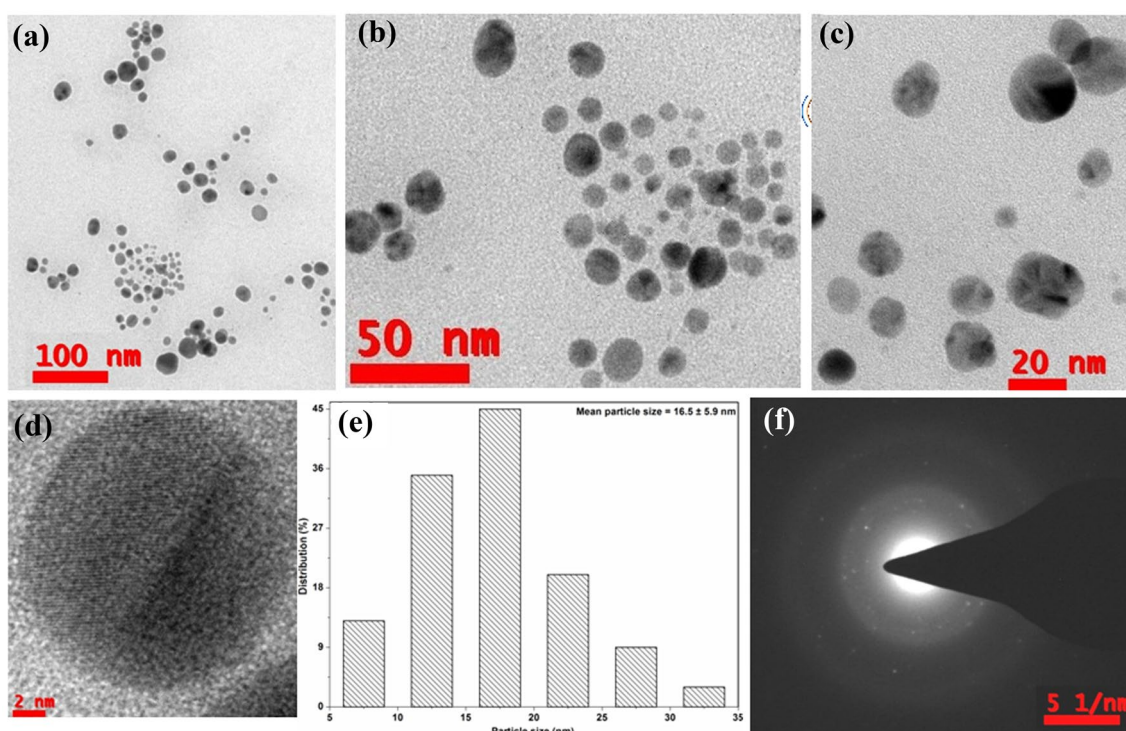
**Fig. 6** The TEM images of Ag NP-TN-1, at **a** 100 nm, **b** 50 nm, **c** 20 nm, **d** 2 nm, **e** histogram of particle size distribution and **f** respective SAED pattern
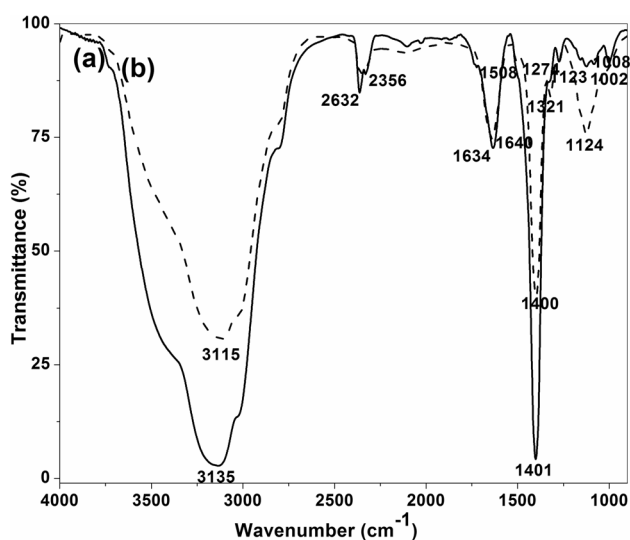


**Fig. 7** The FTIR spectra of powdered **a** leaf extract and **b** biogenic Ag NP

Ag NP spectrum showed characteristic absorbance bands at 3115, 2356, 1640, 1508, 1400, 1321, 1124 and 1008 cm$^{-1}$, respectively. A vast peak shift was observed for NP from 3135 to 3115 cm$^{-1}$ suggesting silver ion binding with extract's hydroxyl groups. A demarcated shift in the polyolic

(1274–1321 cm$^{-1}$) and alcoholic groups (1002–1008 cm$^{-1}$) confirms the metal ion reduction coupling with hydroxyl and carbonyl group oxidation. It is worth noting that the NP exhibit peak shift in the amide I linkage (1634–1640 cm$^{-1}$), validating the NP capping by extract proteins. Thus, the abundance of diverse biomolecules such as chlorophyll (1.6 ± 0.1 μg/mL), soluble sugars (240.4 ± 1.3 μg/mL), proteins (17.2 ± 0.5 μg/mL) and phenolics (126 ± 0.1 μg/mL) in the extract was also proved from the biochemical compositional analysis. These observations are in accordance with the earlier report on Ag NP produced with leaf extract of *Dendrophthoe falcata.* The water-soluble substances such as reducing sugars, phenolics and proteins mediated the synthesis and subsequent stabilization of Ag NP [13]. The leaf extract biomolecules in *Pedalium murex,* including proteins, flavonoids, alkaloids, steroids, rosins and saponins acted as reducing agents for Ag NP [1].

## Evaluation of antibacterial activities of the biosynthesized Ag NP

For checking the antibacterial activity, various assays were employed using Ag NP with an average particle size of 16.5 nm, synthesized with 0.4% extract and 0.6 mM AgNO$_3$ by 30 min of autoclaving.

## Agar well diffusion method

In the agar well diffusion assay, the wells were loaded with different amounts of Ag NP (5–15 μg). After 48 h of incubation at 27 ℃, growth control was observed in plates loaded with varying amounts of Ag NP (Fig. 8). The zone of inhibition (ZOI) values increased linearly with a raise in Ag NP amount from 5 to 15 μg in all the *Xoo* isolates. At the highest tested Ag NP amount of 15 μg, the ZOI values for NB-1, NB-2, NB-3, NB-4, NB-5 and NB-6 isolates were $9 \pm 1.4$, $8.5 \pm 1.5$, 10, $7 \pm 1.4$, $6.5 \pm 0.7$ and 6 mm, respectively. The data show that the isolates NB-3 and NB-1 were more sensitive towards Ag NP than the remaining isolates. Among the isolates, the NB-6 and NB-5 were more resistant to Ag NP tested range. The positive control, streptomycin was used as a known reference compound for comparing the antibacterial action of Ag NP. As assumed, streptomycin (10 μg) loaded positive control plates exhibited highest ZOI

values for the respective strains. The presence of any antibacterial compounds in the extract was detected using it as a negative control. While, the extract (0.4%)-loaded negative control plates did not show any ZOI, suggesting no antibacterial action (Table 1). Based on the one-way ANOVA test carried out between different Ag NP concentrations and *Xoo* strains, the ZOI values noted at 10 μg are on par with streptomycin. The ZOI values observed in the current study were compared with previous antibacterial studies carried out with biosynthesized Ag NP against various phytopathogenic *Xanthomonas* strains. The Ag NP synthesized with extract of marine algae *Padina pavonica* showed a ZOI value of 10.3 mm against cotton pathogen *X. campestris* pv. *malvacearum* at 11 μg [35]. The *Vernonia cinerea* leaf extract biosynthesized Ag NP showed an inhibition zone of 13 mm against *X. campestris* pv. *malvacearum* at 16 μg [36]. A separate report on *Azadirachta indica* leaf extract synthesized Ag NP, a ZOI of 21.5 mm was noted against *X. oryzae*



**Fig. 8** The observed inhibition zones with *Xoo* NB-6 isolate at different amounts of Ag NP, **a** 0, **b** 5, **c** 7.5, **d** 10, **e** 15 μg; and **f** streptomycin (10 μg)
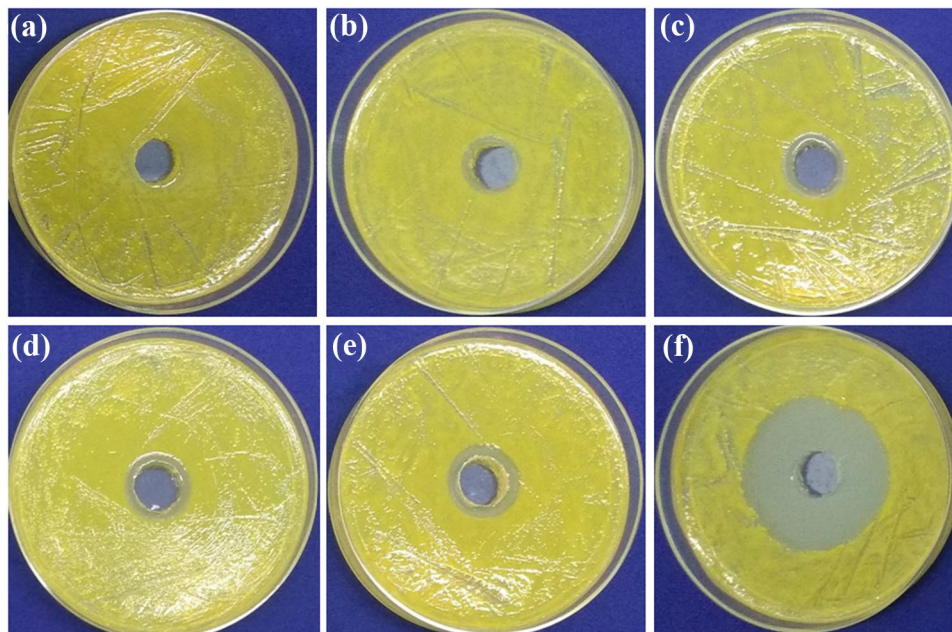
**Table 1** The zone of inhibition values noted with different *Xoo* isolates in well diffusion assay at varying amounts of Ag NP (5–15 μg), antibiotic streptomycin (10 μg) and leaf extract (0.4%)

| *Xoo* isolate | Zone of inhibition (mm) | | | | | |
|---|---|---|---|---|---|---|
| | Antibacterial agent (μg) | | | | | |
| | Ag NP | | | | Streptomycin | Extract |
| | 5 | 7.5 | 10 | 15 | 10 | 0.4% |
| NB-1 | $4.0 \pm 1.4$ | $6.0 \pm 0$ | $7.5 \pm 0.7$ | $9.0 \pm 1.4$ | $35.0 \pm 0.4$ | 0 |
| NB-2 | $4.5 \pm 0.7$ | $5.5 \pm 0.7$ | $8.0 \pm 1.4$ | $8.5 \pm 1.5$ | $37.0 \pm 0$ | 0 |
| NB-3 | $4.5 \pm 0.7$ | $7.0 \pm 0$ | $7.5 \pm 0.7$ | $10.0 \pm 0$ | $33.0 \pm 0$ | 0 |
| NB-4 | $2.8 \pm 0.7$ | $4.1 \pm 0$ | $6.5 \pm 0$ | $7.0 \pm 1.4$ | $30.0 \pm 0.6$ | 0 |
| NB-5 | $3.0 \pm 0$ | $4.0 \pm 0$ | $5.5 \pm 0.7$ | $6.5 \pm 0.7$ | $28.0 \pm 0$ | 0 |
| NB-6 | $2.5 \pm 0.7$ | $3.5 \pm 0.7$ | $4.0 \pm 1.4$ | $6.0 \pm 0$ | $27.0 \pm 0$ | 0 |

pv. *oryzae* at 20 μg [25]. The Ag NP biosynthesized with leaf extracts of tulsi, *Tridax*, neem and drumstick showed ZOI values of 22, 20, 19 and 18 mm, respectively, at an elevated amount of 160.5 μg against pomegranate bacterial leaf blight pathogen *X. axonopodis* pv. *punicae* [37]. Thus, the noted ZOI values are either comparable or better than the reported studies.

## Poisoned food technique

Further, the agar dilution method was employed to demonstrate the dose-dependent antibacterial activity of Ag NP (2.5–10 μg/mL). After 48 h of incubation at 27 °C, growth inhibition was noted in plates amended with different concentrations of Ag NP, in terms of bacterial colony formation (Fig. 9). The inhibition (%) of *Xoo* growth increased with a raise in Ag NP concentration from 2.5 to 10 μg/mL, for all the isolates. Even at a lower concentration of 2.5 μg/mL, the inhibition (%) ranged from 70.3 to 75.6. At an elevated concentration of 10 μg/mL, all the isolates were inhibited entirely. The obtained results at 10 μg/mL are on par with the positive control streptomycin (10 μg/mL), based on one-way ANOVA at 95% confidence. As expected, the negative control leaf extract did not show any growth inhibition (Table 2).

## Broth dilution method

The growth inhibition effect of Ag NP ((2.5–10 μg/mL) against the *Xoo* isolates was measured by minimum inhibitory concentration (MIC). The MIC values of *Xoo* isolates were 5, 7.5, 5, 7.5, 7.5 and 7.5 μg/mL for NB-1, NB-2, NB-3, NB-4, NB-5 and NB-6, respectively. The minimum bactericidal concentration (MBC) values were 7.5 μg/mL for all the isolates, which is line with the streptomycin based on the critical difference value obtained with one-way ANOVA.



**Fig. 9** The dose-dependent inhibition of bacterial colony formation with *Xoo* NB-3 isolate at different concentrations of Ag NP, **a** 0, **b** 2.5, **c** 5, **d** 7.5, **e** 10 μg/mL; and **f** streptomycin (10 μg/mL) in poisoned food technique
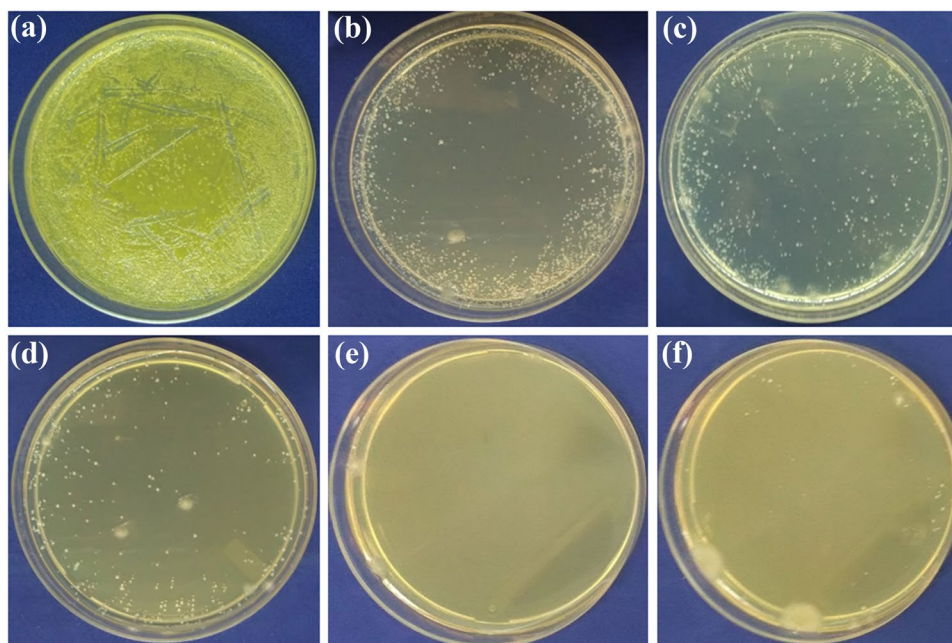
**Table 2** The effect of variable concentrations of Ag NP on growth inhibition (%) of *Xoo* isolates in solid agar medium

| *Xoo* isolate | Growth inhibition (%) | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | Antibacterial agent (μg/mL) | | | | | |
| | Ag NP | | | | Streptomycin | Extract |
| | 2.5 | 5 | 7.5 | 10 | 10 | 0.4% |
| NB-1 | 71.0 ± 2.8 | 80.6 ± 1.9 | 92.6 ± 1.2 | 99.99 ± 1.1 | 99.99 ± 0.5 | 0 |
| NB-2 | 71.0 ± 2.4 | 80.6 ± 2 | 93.7 ± 1.6 | 99.99 ± 1.4 | 99.99 ± 0 | 0 |
| NB-3 | 73.6 ± 2.2 | 83.3 ± 1.8 | 95.6 ± 1.2 | 99.99 ± 1.1 | 99.99 ± 0 | 0 |
| NB-4 | 70.3 ± 2.1 | 79.0 ± 2.1 | 90.0 ± 1 | 99.99 ± 0 | 99.99 ± 0.2 | 0 |
| NB-5 | 74.3 ± 2.1 | 83.0 ± 1.9 | 95.0 ± 1.7 | 99.99 ± 0.9 | 99.99 ± 0.6 | 0 |
| NB-6 | 75.6 ± 2.6 | 84.3 ± 2 | 96.1 ± 1 | 99.99 ± 0.5 | 99.99 ± 0 | 0 |

A lower concentration of 2.5 µg/mL showed an inhibition (%) ranging from 58 to 69 (Table 3), suggesting the antibacterial efficacy of biosynthesized Ag NP towards *Xoo* isolates in liquid broth as well as solid agar assays. The MIC values observed in the current study were much lesser in comparison with the earlier reports. The *Ulva fasciata* extract biosynthesized Ag NP of 40 nm size showed a MIC value of 40 µg/mL against cotton bacterial leaf blight pathogen *X. campestris* pv. *malvacearum* [33]. A MIC value of 80 µg/mL was noted against *X. campestris* pv. *malvacearum* with *Vernonia cinerea* leaf extract produced Ag NP of 5–50 nm [36]. Thus, the Ag NP biosynthesized using rice leaf extract was a more powerful antibacterial agent regarding Ag NP concentration and particle size.

## Greenhouse evaluation of Ag NP

Ag NP's (2.5–15 µg/mL) efficacy towards control and management of BLB disease in rice under the greenhouse environment was determined with clip inoculation method. At 60 days after sowing, the pots treated with Ag NP of 2.5, 5, 7.5, 10 and 15 µg/mL showed disease severity of 32.1, 31.3, 28.8, 28.6 and 26.6%, respectively. The streptocycline (15 µg/mL) treated pots recorded a disease severity of 24.5%. The highest disease severity of 44.5% was recorded in the untreated control. The results indicate significant difference among the treatments in

terms of disease intensity, after applying the first spray. A similar trend with 34.8, 31.8, 30.7, 29.3 and 28.1% of disease severity was observed 90 days after sowing at respective concentrations. The % disease decrease over control (DDC) of Ag NP (49.2%) at 90 days after sowing is comparable with streptocycline (59.7%) at 15 µg/mL concentration, based on one-way ANOVA (Table 4). The obtained results were compared with earlier in vivo greenhouse studies for managing diseases caused by fungal and bacterial phytopathogens. In a report on rice blast disease causing fungal pathogen *M. grisea*, disease severity of 35.9% was noted at 100 µg/mL concentration with Ag NP (20–30 nm) foliar spray [6]. The DNA directed Ag NP effectively decreased the disease incidence of bacterial spot disease (20%) caused by *X. perforans* in tomato at 100 µg/mL concentration in the greenhouse [27]. The foliar spray of thymol based nanoemulsion (600 µg/mL) containing 80–150 nm sized spherical droplets significantly lowered the disease severity (3.3%) and enhanced% efficacy of disease control (95.4%) of bacterial pustule in soybean caused by *X. axonopodis* pv. *glycine* in pot experiments carried out in a net house [20]. In the current investigation, disease severity of 26.6% and DDC of 49.2% was noted at a lower concentration of 15 µg/mL of 16.5 nm sized Ag NP. Thus, the present study implicates the possible utilization of leaf extract synthesized Ag NP in controlling the BLB disease of rice in field conditions.

**Table 3** The bactericidal concentration of Ag NP on growth inhibition (%) of *Xoo* isolates in liquid broth medium

| *Xoo* isolate | Growth inhibition (%) | | | | | |
|---|---|---|---|---|---|---|
| | Antibacterial agent (µg/mL) | | | | | |
| | Ag NP | | | | Streptomycin | Extract |
| | 2.5 | 5 | 7.5 | 10 | 10 | 0.4% |
| NB-1 | 65 ± 2.7 | 91.5 ± 2.0 | 99.99 ± 0 | 100 ± 0 | 100 ± 0 | 0 |
| NB-2 | 63 ± 3.1 | 82.8 ± 2.0 | 99.99 ± 0.4 | 100 ± 0 | 100 ± 0 | 0 |
| NB-3 | 69 ± 2.1 | 92.8 ± 1.8 | 99.99 ± 0 | 100 ± 0 | 100 ± 0 | 0 |
| NB-4 | 64 ± 3.0 | 88.1 ± 2.4 | 99.99 ± 0 | 100 ± 0 | 100 ± 0 | 0 |
| NB-5 | 59.5 ± 3.0 | 80.1 ± 2.7 | 99.99 ± 0.6 | 100 ± 0 | 100 ± 0 | 0 |
| NB-6 | 58 ± 2.9 | 84.3 ± 2.6 | 99.99 ± 0.5 | 100 ± 0 | 100 ± 0 | 0 |

*Values are mean ± SD for $n = 3$ at $p < 0.05$

**Table 4** The effect of different concentrations of Ag NP on disease severity (%) and % disease decrease over control of bacterial leaf blight disease of rice caused by *Xanthomonas oryzae* pv. *oryzae* under greenhouse conditions

| Parameter | Treatment (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ag NP | | | | | Streptocycline | Control |
| | 2.5 | 5 | 7.5 | 10 | 15 | 15 | 0 |
| % disease severity 60 days after sowing | 32.1 ± 1.6 | 31.3 ± 1.9 | 28.8 ± 2.4 | 28.6 ± 2.6 | 26.6 ± 3.1 | 24.5 ± 3.5 | 44.5 ± 1.8 |
| % disease severity 90 days after sowing | 34.8 ± 0.8 | 31.8 ± 1.0 | 30.7 ± 0.7 | 29.3 ± 1.4 | 28.1 ± 1.7 | 22.3 ± 3.6 | 55.5 ± 3.2 |
| % disease control | 37.2 ± 1.4 | 42.6 ± 1.2 | 44.6 ± 0.9 | 47.1 ± 1.2 | 49.2 ± 1.1 | 59.7 ± 3.0 | 0 |

**Table 5** The effect of different concentrations of Ag NP on seedling vigor index of rice cultivars

| Parameter | BPT-5204 | | | RP Bio-226 | | |
|---|---|---|---|---|---|---|
| | Ag NP (µg/mL) | | | Ag NP (µg/mL) | | |
| | 0 | 5 | 10 | 0 | 5 | 10 |
| SVI I | 407.6 | 513 | 738 | 569 | 639 | 759 |
| SVI II | 7.4 | 11.1 | 12.2 | 8.1 | 9.1 | 11.6 |

## Seed germination

The seedling vigor index (SVI) I and II values of BLB susceptible (BPT-5204) and resistant (RP Bio-226) rice cultivars were found to enhance with an increase in Ag NP concentration, in comparison with hydroprimmed seeds. The SVI I values for BPT-5204 were found to be 407.6 and 738 for hydroprimmed and Ag NP-treated (10 µg/mL) seeds, respectively. For RP Bio-226, the respective SVI I values were 569 and 759. The hydroprimmed and Ag NP (10 µg/mL) treated seeds of BPT-5204 variety showed SVI II values of 7.4 and 12.2, respectively. The corresponding SVI II values were 8.1 and 11.6 for RP Bio-226 cultivar (Table 5). Compared with hydroprimmed seeds, the Ag NP treatment at 10 µg/mL caused an enhancement in SVI I and II values by 1.8 and 1.6; and 1.3 and 1.4 times for BPT-5204 and RP Bio-226 varieties, respectively. These findings are in accordance with previously carried out investigations on seed germination with Ag NP [23] and titanium dioxide nanoparticles ($TiO_2$ NP) [8] with rice and wheat, respectively. The augmentation in seed germination and SVI could be due to an enhancement in water uptake and α-amylase activity from better penetration of nanoparticles into the seed [7, 8, 23].

## Conclusions

The Ag NP biosynthesis was carried out with the leaf extract of TN-1, the most susceptible rice variety towards BLB. The produced biogenic Ag NP were applied as an antibacterial agent against various *Xoo* isolates under in vitro conditions. Further, the method meets the requirements of green chemistry and can be utilized for the bulk commercial production of cost effective Ag NP. Due to the abundance of potent reducing and stabilizing biomolecules in the leaf extract, the Ag NP with reasonably good size were produced at a much lower extract concentration, in comparison with earlier leaf extract based biosynthetic protocols. Based on the MIC and MBC values, the Ag NP were a more powerful antibacterial agent regarding Ag NP concentration (5–7.5 µg/mL) and size (17 nm). The applicability of Ag NP was also evaluated under greenhouse conditions for the management of BLB. Disease severity of 26.6% and DDC of 49.2% was noted at a much lower Ag NP concentration (15 µg/mL) than

the previous studies. Thus, the present report demonstrates the Ag NP's effective utilization against *Xoo* under in vitro and in vivo conditions, implicating its field application to control and manage BLB disease in rice. Further studies such as in vivo evaluation of Ag NP against other bacterial and fungal phytopathogens, possible bactericidal mechanism and Ag NP toxic effects on the food chain, environment and human health are envisaged.

## Declarations

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