**ORIGINAL PAPER** 



### Antibacterial and biofilm inhibition activity of biofabricated silver nanoparticles against *Xanthomonas oryzae* pv. *oryzae* causing blight disease of rice instigates disease suppression

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#### Abstract

Antimicrobial activity of silver nanoparticles (AgNPs) has been well documented in earlier studies. As their efficient role in combating phytopathogens has begun recently, there is a huge scope to explore their effectiveness in agriculture. Considering the strong antifungal activity of biosynthesized AgNPs (as reported in our previous study), our main aim is to elucidate their antibacterial activity against bacterial plant pathogens to authenticate their wide range of agricultural applications. The present manuscript highlights the potential role of biosynthesized AgNPs against *Xanthomonas oryzae* pv. *oryzae* (Xoo) causing disastrous sheath blight disease of rice worldwide. We observed strong antibacterial activity of biosynthesized AgNPs (size ~ 12 nm) against Xoo at 20, 30 and 50  $\mu$ g/mL concentrations. The significant inhibitory impact of AgNPs on biofilm formation by Xoo was noted even at the lower dose of 5  $\mu$ g/mL (p=0.001). Maximum biofilm inhibition (p=0.000) was caused at 50  $\mu$ g/mL concentration of AgNPs in comparison to control. Furthermore, disease suppression by biosynthesized AgNPs was authenticated under greenhouse conditions. Foliar spray of AgNPs significantly reduced the blight symptoms in rice sheaths as shown by 9.25% DLA (% Diseased leaf area) as compared to 33.91% DLA in Xoo inoculated rice plants. Altogether, our data suggest that biosynthesized AgNPs based nanoformulation can be applied for successful management of blight disease of rice. In addition, the antibiofilm strategies instigated by AgNPs can be exploited against a wide range of bacterial phytopathogens. In light of rapidly emerging antibiotic-resistant microbial strains, the current work provides an alternate effective platform for the application of nanoformulation for augmenting sustainability in the agriculture.

Keywords Silver nanoparticles · Xanthomonas oryzae pv. oryzae · Biofilm · Rice · Nanoformulation

#### Introduction

*Xanthomonas oryzae* pv. *oryzae* (Xoo) causes devastating leaf blight disease in rice around the world. This is one of the most serious disease of rice causing 70% yield loss under favorable conditions (Mew et al. 1993; Zhou et al. 2013;

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Chien et al. 2019). Though Xoo has a wide host range, the rice-Xoo pathosystem is more critical as Xoo colonizes vascular tissues producing deadly symptoms (Zhang and Wang 2013). Interestingly, more than 30 races of Xoo have been identified based on their potential to infect different rice cultivars (Adhikari et al. 1999; Mishra et al. 2013).

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The pathogenicity of Xoo is predominantly determined by several factors such as type III-secretion dependent effectors, siderophores, motility, extracellular enzymes, extracellular polymeric substances (EPS), biofilm formation, etc. (Ray et al. 2000; Hu et al. 2007; He et al. 2010; Xue et al. 2018). Biofilm formation by plant pathogenic bacteria is considered as one of the most important virulence triggering factors. Several plant pathogenic bacteria including *Xanthomonas*, Xylella, Erwinia and others, have been reported to cause severe plant disease by colonizing xylem vessels through biofilm formation (Castiblanco and Sundin 2016). Likewise, previous studies also provide a link between biofilm formation and virulence of Xoo in rice either through mutational analysis or other methods (Yu et al. 2015; Sahu et al. 2018). In addition, the phenomenon of quorum sensing (QS) regulates the virulence of Xanthomonas sp. by affecting motility, biofilm formation, chemotaxis, EPS formation, etc. (He and Zhang 2008; He et al. 2010; Shi et al. 2015; Barel et al. 2015). The QS mechanism facilitates the virulence of pathogenic bacteria by triggering their population density that functions as a community for causing disease. The bacterial population receives signals through these autoinducers and they further adjust their cell density and gene expression in order to function in unison (Rutherford and Bassler 2012; Polkade et al. 2016). The expression of pathogenicity factors in several plant pathogenic bacteria viz. Pseudomonas syringae (blights, cankers, and diebacks), Erwinia carotovora (soft rot erwinia), Ralstonia solanacearum (vascular wilt disease), Xanthomonas campestris (black rot of cruciferous plants), Agrobacterium tumefaciens (crown gall), etc. is primarily reliant on QS mechanisms (Whitehead et al. 2001; von Bodman et al. 2003; Dwivedi et al. 2017). The rpf gene cluster is reported to play vital role in determining the virulence of Xanthomonas by regulating DSF (diffusible signaling factor) signaling (He et al. 2010; Ryan and Dow 2011) as validated by mutational analysis suggesting diminished virulence via disruption of QS pathway, biofilm formation, and other virulence factors (Cho et al. 2013; Huang et al. 2013).

Several management strategies are being applied to cope with the severe damage caused by Xoo. The prevalent methods for disease control include the use of disease resistant variety, chemicals and antibiotics (Mansfield et al. 2012; Singh et al. 2017). However, these management practices have certain limitations, for instance, development of antibiotic resistance in Xoo, toxic impacts of chemicals on the environment and suppression of disease resistance genes during the evolution of sub-populations of rice cultivar (Mac-Manus et al. 2002; Yasmin et al. 2017). Consequently, an alternative approach of biological control has been employed for successful disease management due to its cost-effectiveness and environment friendly nature. Application of antagonistic microbes such as *Bacillus* and *Pseudomonas* sp. against Xoo can reduce the disease incidence up to 90% (Montano et al. 2014). Given that biofilm formation by pathogens is an important mechanism for causing disease, the use of biomolecules to impair biofilm formation could provide an efficient alternative way to restrain the pathogenicity of Xoo. Some studies have suggested the efficiency of plant oil, niclosamide drug and nitrogen sources to reduce the virulence of Xoo via disruption of biofilm formation (Singh et al. 2017; Ham and Kim 2018; Sahu et al. 2018). Likewise, harnessing the possibility of nanotechnological approach against Xoo can also provide an effective alternative way to combat this pathogen challenge. Considering the well-known antimicrobial property of silver nanoparticles (AgNPs) against broad range of phytopathogens (Mishra et al. 2014, 2017a, b; Mishra and Singh 2015a; Singh et al. 2018), the current study highlights the antibacterial and biofilm inhibitory activities of biosynthesized AgNPs against Xoo causing blight of rice. The present study is the extension of our previous study demonstrating broad range antifungal efficacy of biosynthesized AgNPs by Stenotrophomonas sp. BHU-S7 (Mishra et al. 2017a). We hypothesized that biosynthesized AgNPs might provide effective control measures against bacterial phytopathogens by diminishing virulence factors such as cell density, biofilm formation, etc. Moreover, we also aim to highlight that plant disease controlling efficiency of AgNPs is not restricted to fungi only but this can also be used against bacterial pathogens pointing towards their wide-range applications in the agricultural sector.

#### Materials and methods

#### **Bacterial strains and growth conditions**

The biosensor strain, *Chromobacterium violaceum* ATCC 12472 (hereafter, referred as *C. violaceum*) culture was maintained in Luria–Bertani (LB) medium under shaking conditions at 30 °C. The pathogenic strain Xoo, causing blight of rice was grown and maintained in Nutrient Agar at 30 °C. The bacterial strain *Stenotrophomonas* sp. BHU-S7 culture was grown and maintained on Nutrient Agar (NA) at 30 °C. Details about isolation, characterization and 16S identification of *Stenotrophomonas* sp. BHU-S7 have already been reported in our previous study (Mishra et al. 2017a).

### Biosynthesis of AgNPs using *Stenotrophomonas* sp. BHU-S7

AgNPs were biosynthesized using *Stenotrophomonas* sp. BHU-S7 following the protocol described in our earlier report. Briefly, culture supernatant obtained by centrifugation of 48 h old culture of *Stenotrophomonas* sp. BHU-S7

incubated under shaking conditions at 30 °C was exposed to 1 mM silver nitrate solution followed by incubation in dark under shaking conditions at 30 °C for biosynthesis of AgNPs. After visualizing colour change of the supernatant solution from yellow to brown, the biosynthesized nanoparticles were collected by centrifugation at 14,000 rpm for 30 min followed by thorough washing with sterilized distilled water and freeze drying the resulting pellet. The optical, structural, morphological, elemental, functional and thermal characterization of the biosynthesized AgNPs was also done as described earlier (Mishra et al. 2017a).

#### Antibacterial activity of biosynthesized AgNPs against Xoo

The phytopathogenic strain Xoo was grown overnight in a conical flask containing 20 mL of Nutrient Broth (NB) media at 30 °C on a rotatory shaker (140 rpm). After incubation, an aliquot of 100  $\mu$ L from freshly grown culture was spread over NA Petriplate. Wells were prepared with a sterile cork borer and filled with 50  $\mu$ L of different concentrations of AgNPs (10, 20, 30 and 50  $\mu$ g/mL). Another well filled with 50  $\mu$ L of sterile water served as control. After this, the plate was incubated overnight at 30 °C and the inhibitory impact was observed by the formation of a zone of inhibition around treated wells.

#### **Greenhouse experiment**

The greenhouse experiment under natural light and temperature conditions was conducted to evaluate disease suppression activity of biosynthesized AgNPs during rice-growing season. Firstly, rice seedlings were grown separately and then transplanted to the plastic pot (13 cm length and diameter) containing 700 g sandy loam soil, after 2 weeks of growth. The following treatments were maintained with 5 replicates each: control (C), pathogen control (Xoo inoculation) (P) and Xoo+AgNPs (PA). After one week of transplantation, plants in C and PA set were given foliar spray treatment with water and AgNPs suspension (25 ppm) respectively. After 2 days, plants in P and PA set were clip inoculated with Xoo inoculation (10<sup>6</sup> CFU mL<sup>-1</sup>). After 15 days of treatment plant parameters (plant height, dry weight and % diseased leaf area) were recorded for 15 plants (3 plants from each replicate). Disease suppression was measured using bacterial blight lesion length parameter by calculating % Diseased leaf area (% DLA) as follows:

# Biofilm formation inhibition by biosynthesized AgNPs

In vitro biofilm assay was performed using crystal violet staining method as described earlier (Mishra and Nautiyal 2012). Test tubes containing 5 mL of Nutrient broth (NB) media were prepared and autoclaved. Thereafter, fresh stock of biosynthesized AgNPs was prepared in sterile MQ water and added to test tube containing autoclaved 5 mL NB medium at different concentrations (5, 10, 25, 50 µg/mL). Control NB tubes without AgNPs amendment were also maintained. Hereafter, all the supplemented and non-supplemented culture tubes were inoculated with 50 µL of overnight grown culture of Xoo followed by inoculation of 0.1 mL of each suspension into the wells of a PVC microtitre plate. In total, eight replicates were maintained for each treatment. The plate was further sealed with parafilm to prevent contamination followed by incubation at 28 °C for 48 h. Post incubation, the plate was inverted to remove the contents and flooded with 1% crystal violet for 10 min for staining of the biofilm. Excess stain was removed by destaining with 95% ethanol. The amount of biofilm formed was quantified by recording the absorbance at 590 nm.

# Quorum quenching (QQ) activity by biosynthesized AgNPs

The QQ potential of the biosynthesized AgNPs was assessed in C. violaceum culture (Giménez-Bastida et al. 2012). Briefly, C. violaceum culture was grown overnight in Erlenmeyer flasks containing LB medium at 27 °C for 16-18 h. The fresh stock of biosynthesized AgNPs was prepared in sterile MQ water and added to test tube containing autoclaved LB medium (5 mL) at different concentrations (5, 6, 7, 8, 9, 10 µg/mL). Control LB tube without AgNPs was also maintained. Hereafter, all supplemented and non-supplemented culture tubes were inoculated with 50 µL of overnight grown culture of C. violaceum and further incubated in a rotary shaker for 24 h. One mL of culture was centrifuged at 10,000 rpm for 5 min and supernatant was discarded. To the pellet, 1 mL of DMSO (Dimethyl sulfoxide) was added and vortexed vigorously for 30-60 s to completely solubilize violacein pigment. The solution was again centrifuged at 10,000 rpm for 5 min in order to remove the cells and to get a transparent solution for recording absorbance. The violacein content was quantified using UV-Vis spectrophotometer at a wavelength of 585 nm. This experiment was repeated three

% Diseased leaf area (% DLA) = (Total lesion length of the test sample/Total leaf length of the test sample) × 100

times in triplicate. In order to ensure QQ effects of the biosynthesized AgNPs, bacterial cell counts were also calculated as CFU/mL after the incubation period.



**Fig. 1** Inhibitory impact of different concentrations of biosynthesized AgNPs on cells growth of *Xanthomonas oryzae* pv. *oryzae*. Red arrows indicate zone of inhibition caused by AgNPs loading in well

#### **Statistical analysis**

All statistical analyses were performed using SPSS statistical package (SPSS Version 16.0). Results for quorum quenching, biofilm inhibition and greenhouse experiment were expressed as mean  $\pm$  standard deviation from all replicates. Analysis of variance (ANOVA) was performed using one-way ANOVA followed by a posthoc Duncan's Multiple Comparison Test to analyze the significant difference among treatments. A *p* value of  $\leq 0.05$  was considered statistically significant.

#### Results

### Antibacterial property of biosynthesized AgNPs against Xoo

As reported in our previous study, the biosynthesis of AgNPs was successfully achieved using *Stenotrophomonas* 

sp. BHU-S7. The biosynthesized AgNPs showed spherical conformation with an estimated size to be 12.7 nm. Antibacterial potential of biosynthesized AgNPs against Xoo was assessed by the formation of a zone of inhibition. Figure 1 demonstrates that the inhibitory dose of AgNPs was found to be in the range of 20–50  $\mu$ g/mL. The maximum zone of inhibition (12 mm) was reported at 50  $\mu$ g/mL concentration of AgNPs followed by 8 mm and 6 mm zone of inhibition at 30 and 20  $\mu$ g/mL AgNPs concentration, respectively.

### Biosynthesized AgNPs mediated inhibition of biofilm formation by Xoo

Biofilm formation is an important factor in plant pathogenesis and AgNPs mediated hindrance in the biofilm formation by Xoo could be the probable mechanism for disease suppression as shown in our greenhouse experiment results. As expected, we observed dose-dependent inhibition in the biofilm formation caused by biosynthesized AgNPs application (Fig. 2). The significant inhibitory impact was observed even at the lower dose of 5 µg/mL of AgNPs with p value of 0.001 as compared to control. Maximum inhibition (p = 0.000) was caused at 50 µg/mL concentration of AgNPs in comparison to control. Interestingly, the biosynthesized AgNPs in the concentration range of 5-25 µg/mL exhibited biofilm inhibition activity without affecting bacterial cell viability as validated by CFU method. The minimum CFU value of 4.64  $Log_{10}$  CFU mL<sup>-1</sup> was recorded in the treatment having 25 µg/mL AgNPs showing significant antibacterial and antibiofilm activity compared to the control set which showed 7.94 Log<sub>10</sub> CFU mL<sup>-1</sup>. However, maximum biofilm inhibition was observed in the treatment having 50 µg/mL AgNPs which was due to Xoo cells death that is justified more as bactericidal activity.





p = 0.000) was observed in P set as compared to control

showing %DLA of 1.72%. Whereas, in PA set %DLA was significantly decreased to 9.25% (p = 0.000) as compared to P set. In addition to disease suppression, AgNPs treat-

ment also improved plant growth while pathogen inoculation

caused a significant reduction in plant growth parameters as compared to control. Pathogen attack caused 41 and 61%

significant reduction in plant height and plant dry weight

respectively, as compared to control (Fig. 3).

## In vivo disease suppression activity of biosynthesized AgNPs

The foliar spray treatment of biosynthesized AgNPs was observed to suppress blight disease of rice significantly. The pathogen (Xoo) inoculation resulted in severe disease symptoms. The data on %DLA clearly indicate the successful management of blight disease by AgNPs application. Due to pathogen attack, a significant increase in %DLA (33.91%,

Fig. 3 Effect of biosynthesized AgNPs treatment for suppression of blight disease of rice in pot experiment under greenhouse conditions. Bar graphs are showing effect of different treatments on plant height (cm), dry weight (g) and DLA (%). Data are mean  $\pm$  SD (= 15) and different letters indicate significant differences among treatments according to Duncan's multiple range test at p  $\leq 0.05$ 



Fig. 4 a Inhibition of violacein production by AgNPs during cells growth in LB media. **b** Photograph showing violacein content (recorded at 585 nm) as influenced by different concentration of AgNPs and **c** Bar diagram showing inhibitory impact on violacein production and cells growth with increasing concentration of AgNPs. Different letters indicate significant differences at  $p \le 0.05$ 



#### QQ potential of biosynthesized AgNPs

The QS mechanism facilitates the virulence of pathogenic bacteria by triggering their population density that functions as a community for causing disease. Hence, we also made an attempt to study the QQ potential of biosynthesized AgNPs used in this study in order to gain insight into the underlying mechanisms for virulence suppression. However, we cannot directly link this QQ potential with Xoo virulence suppression as QS in Xoo is mainly regulated by diffusible signaling factor (DSF) family signals. Here, we used *C. violaceum* strain that is used for screening AHL mediated quorum sensing activity. We, therefore, believe that this data can be useful for making interpretation for phytopathogenic bacteria exhibiting AHL-mediated QS system.

Violacein pigment production is indicative of AHL mediated quorum sensing signaling in *C. violaceum* 12472. In the current study, biosynthesized AgNPs were found to inhibit quorum sensing signaling as was evident by reduced production of violacein pigment. Figure 4a and b represents the dose-dependent lowering of violacein content in C. violaceum 12472 culture with steadily increasing concentrations of the biosynthesized AgNPs. The above data may be correlated to the plausible attenuation of AHL production responsible for the regulation of violacein biosynthesis. As compared to control, significant inhibition (p = 0.000)was observed in violacein content under the influence of different concentrations of AgNPs. Maximum attenuation (79.89%) was observed at 10 µg/mL (p=0.000) while 5 µg/ mL (p=0.000) of biosynthesized AgNPs displayed minimum inhibition (22.34%) of violacein production (Fig. 4c). The non-supplemented control expressed maximum violacein production due to absence of any form of inhibitors. Furthermore, to ensure that biosynthesized AgNPs mediated



**Fig.5** A hypothetical model illustrating biosynthesized AgNPs mediated suppression of virulence factors in *Xanthomonas oryzae* pv. *oryzae* leading to inhibition of disease progression in plants. Abbre-

viations: bsAgNPs (biosynthesized AgNPs), Anti-B (antibacterial), Anti-QS (anti-quorum sensing), BI (biofilm inhibition)

QQ effect is not caused by bacterial cells death, we also calculated bacterial cell counts. As shown in Fig. 4c, the viable cells count was not affected by AgNPs treatment at concentration range of  $5-10 \ \mu g/mL$ .

#### Discussion

Nanotechnology-based approaches have provided a new dimension to plant disease management practices due to their high efficiency and immense potential to minimize excessive use of agrochemicals (Elmer and White 2018; Worrall et al. 2018). AgNPs showing promising antimicrobial properties stand as strong nano-weapon against a myriad of phytopathogens including fungi, bacteria, nematodes, etc.

(Ocsoy et al. 2013; Cromwell et al. 2014; Mishra and Singh 2015a). As described in our previous study, the biosynthesized AgNPs proved enormous application in agriculture owing to their antagonistic potential against soil-borne and foliar phytopathogenic fungi (Mishra et al. 2017a). In addition, we, therefore, tested the antibacterial potential of AgNPs against Xoo to propose their broad range application in agriculture especially for plant disease management.

Xoo displays a typical mode of infection where it enters rice leaves through open wounds or hydathodes and causes systemic infection after attacking xylem vessels (Niño-Liu and Ronald 2006). Recently, Xue et al. (2018) identified a key role of EAL domain protein i.e. phosphodiesterase *EdpX1* in stimulating virulence phenotypes in Xoo by promoting EPS production and biofilm formation. Likewise, previous reports have documented that Xoo produces rpf gene cluster regulated diffusible signaling factor (DSF) family signals for quorum sensing, biofilm formation and virulence enzyme production (Feng et al. 2009; Deng et al. 2010; Wang et al. 2016). The *rpf* gene cluster is reported to be well conserved in all xanthomonads (Lee et al. 2006) and contains 8 genes such as *rpfA* to *rpfH* (Cho et al. 2013). These genes have been reported to play a significant role in DSF production (*rpfB* and *rpfF*), histidine kinase (*rpfC* and rpfG), expression of virulence activities (rpfC and *rpfG*) (Tang et al. 1996; Dow et al. 2000; He et al. 2007; Feng et al. 2009). As evident from previous literature, *rpfB*, xrvA, thiG, histidine utilization pathway (hut) genes have been identified to regulate virulence in Xoo and promotes disease incidence in rice (Feng et al. 2009; Yu et al. 2015; Wang et al. 2016; Liang et al. 2018). Further, the importance of biofilm formation for triggering disease symptoms by Xoo has been recognized earlier (Han et al. 2008; Kim et al. 2009; Xue et al. 2018). In view of these reports, we postulate that biosynthesized AgNPs mediated disruption of biofilm formation by Xoo possibly reduced disease occurrence in rice. Our attempt to confirm the disease suppressing effects of biosynthesized AgNPs is not limited to in vitro studies. Greenhouse experiment provided better evidence for their effectiveness against sheath blight disease in rice. There was a significant reduction in %DLA in AgNPs treated rice sheaths which confirms the antibacterial role of AgNPs application against Xoo. This observation may also be correlated to the fact that the presence of silver ions within the plant interior may directly penetrate bacterial pathogen membrane thereby inducing oxidative burst along with simultaneous inhibition of DNA replication and suppressing virulence genes expression (Mishra and Singh 2015b; Liang et al. 2017). The dual antibacterial and antibiofilm activities of biosynthesized AgNPs are sufficient in providing a protective shield to rice plants against Xoo infection. According to Sharma et al. (2012), an optimal concentration of metal nanoparticles correlates with the improved quantum efficiency and higher chlorophyll content in the treated seedlings apart from an augmented induced systemic resistance status in the host. Hence, our subsequent focus must be on optimizing the environment relevant concentrations of biosynthesized AgNPs for safe use in agroecosystem.

Several phytopathogenic bacteria induce diseases through QS strategy, where virulence factors are triggered only when the bacterial population achieves a certain cell density (Helman and Chernin 2015). Disruption of this QS system by QQ phenomenon has been recognized as a potential way to control plant diseases as QQ reduces the virulence of a pathogen by diminishing the cell–cell communication without killing the bacteria (Dong et al. 2000; Chen et al. 2013). In this regard, this strategy may be considered as far more effective means of biocontrol owing to the rapid emergence

of antibiotic resistant strains (Rajesh and Rai 2014). Furthermore, QQ offers a minimum selective pressure on the target microbes, hence the development of resistance towards this strategy is practically minimum (Defoirdt et al. 2010). In our study, we also observed anti-QS potential of AgNPs that could act as an extra armament against bacterial phytopathogens. Our case study on Xoo and anti-QS potential of biosynthesized AgNPs are representing two different aspects in this paper and are not linked with each other. Since we used bioindicator strain of C. violaceum for confirming anti-QS potential, we therefore, suggest that this approach could provide an effective way of managing phytopathogens producing AHL signals such as Pseudomonas syringae, Erwinia carotovora, Bacillus thuringienesis (LaSarre and Federle 2013). Hitherto, studies pertaining to QQ potential of metal nanoparticles as an effective tactic against phytopathogenic bacteria are very scarce. Thus, our preliminary study is providing a better clue to implement this approach for managing plant diseases by overcoming difficulties of antibiotic resistance and the hazards of using chemicals. Attenuation of AHL signaling molecules by AgNPs would have resulted into reduced violacein pigment production in C. violaceum that is indicative of anti-QS phenomenon.

#### Conclusions

This study highlights antibacterial, anti-biofilm and anti-QS properties of biosynthesized AgNPs indicating their promising applications in agriculture for managing plant diseases caused by bacterial pathogens (Fig. 5). Moreover, our case study on the interaction between AgNPs and Xoo indicates successful management of sheath blight disease in rice under greenhouse condition. The biosynthesized AgNPs exhibited strong antibacterial and biofilm inhibition activity against Xoo at a low concentration range which will contribute to achieving sustainability in agriculture. There is a constant demand for novel products to combat pathogen challenges in agriculture. With the help of advanced nanotechnology, plant disease management practices have witnessed a remarkable growth by virtue of unique properties of nanoparticles. Hence, we believe that the outcome of this study will be helpful for developing nanoformulations for fighting against plant diseases. The effectiveness of AgNPs as antibiofilm and anti-QS agents might provide a constructive approach to designing management practices against a broad range of phytopathogenic bacteria as these two are key factors responsible for bacterial pathogenesis.

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Author contributions HBS and SM conceived and designed the experiments; SM performed the experiments and analyzed data; SM and SR wrote the paper; YXD and HBS executed the article editing. LFF contributed to the critical reading of the manuscript. All authors read and approved the final manuscript.

#### **Compliance with ethical standards**

Conflict of interest The authors have no conflict of interest to declare.

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