Biosynthesis of silver nanoparticle from pomelo (Citrus Maxima) and their antibacterial activity against acidovorax oryzae RS-2

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Abstract
Silver nanoparticles (AgNPs), synthesized with plant materials, are considered to be an emerging field of agriculture for their eco-friendly and outstanding antibacterial attributes. In this study, we synthesized AgNPs using pomelo (Citrus maxima) fruit extract as a biological capping and reducing material. The particle size was determined as 11.3–12.8 nm by using UV–vis spectrophotometer, TEM and x-ray diffraction analysis. UV–vis spectrophotometer analysis also confirmed the formation of AgNPs in colloidal solution and showed a maximum absorption at 426 nm. Fourier transform infrared spectra was used to analysed the involvement of biological molecule in AgNPs synthesis. The minimum inhibitory concentration of AgNPs against Acidovorax oryzae strain RS-2 was determined as 25 μg ml⁻¹ by agar well diffusion and bacterial growth assay. In addition, bacterial viability and swarming motility were significantly inhibited by AgNPs. Compared with the control, 25 μg ml⁻¹ of AgNPs lower bacterial biofilm formation up to 68.24%. The bacterial cell wall damaged by AgNPs was observed t TEM. Furthermore, AgNPs treatment resulted into the down regulation of expression of many type VI secretion system related genes, suggesting that AgNPs also have an effect on the virulence of bacteria. The overall conclusion of this study suggests that AgNPs can play an important role in controlling A. oryzae.

1. Introduction

Acidovorax oryzae (Ao) is a Gram-negative pathogenic bacteria that infects huge variety of crops including corn, oat, melon, millet, orchid, sugar cane and rice [1]. It causes seed born disease in rice which is known as bacterial brown stripe (BBS), which is responsible for causing heavy economic losses in many countries including China [2–5]. Due to the economic importance of BBS, it is necessary to have novel biocidal, disinfecting or antibacterial agents to complement conventional antibiotics [6].

Currently, bactericide is mainly used to prevent and control bacterial diseases. The rising anxiety about antimicrobial resistance, the intractable nature of biofilm related infections as well as due to high environmental pollution and bacterial resistance by the extreme use of chemicals in rice-growing countries across the world, a call for the development of alternative approaches to treat bacterial diseases. Nanoparticles have been considered as one of the evolving and promising platforms in this regard. Their unique physical and chemical properties may lead to precise and efficient interactions between them and bacteria. Among the antibacterial nanoparticles, silver nanoparticles (AgNPs) are the most thoroughly investigated ones and can kill both Gram-negative and
Gram-positive bacteria, which is also effective against drug-resistant species [7]. Given the unique physiochemical and biological properties, AgNPs are considered to be a good antibacterial, antiviral, antifungal agent and even have some important anti-inflammatory properties [8].

Nanotechnology is a new and an emerging field of science in which a great variety of metal nanoparticles have been synthesized. These nanoparticles have very small size which ranges from 1 to 100 nm and have large surface area which greatly increases its surface to volume ratio. Metals and metal oxides have attracted more attention over the past few years because they are safer to human and animals and can withstand tough environmental condition [9]. The inorganic antibacterial nanoparticles such as silver, gold, copper, CuO, TiO2 and ZnO are more stable and can withstand high pressure and temperature [10] and have extreme antibacterial activities [11]. Hence nanoparticle research has gained incredible interest especially use of silver nanoparticles has innumerable applications in biomedical sector with huge number of products already in market such as creams, bandage materials and wrapping materials [12]. Among all noble metal nanoparticles, AgNPs are used as antimicrobial agents in wound dressings [13]. It has been proven that AgNPs are useful in cancer diagnosis with antitumor and antioxidants properties [14, 15].

Physical and chemical methods have been used for AgNPs synthesis; however, these methods have many drawbacks such as they are generally time-consuming, expensive and environmentally harmful. Whereas green synthesis of nanoparticle is relatively much cheaper and environmentally safe [16]. Studies indicate that some proteins/enzymes which interact with the metal ions may play a major role in the biosynthesis process [17].

Application of biosynthesized AgNPs is of substantial interest in the field of agriculture based on their antioxidant and broad-spectrum antimicrobial activity. Biosynthesized AgNPs are environmentally friendly, biocompatible and cost-effective in nature. The nanoparticles exhibit different bactericidal mechanisms along with plant improvement properties [18]. It is therefore of keen interest to analyze the inhibitory effect of biosynthesized AgNPs against Acidovorax, which can be effectively used in the field of nanotechnology as a cost-efficient, eco-friendly and safe strategy. Therefore, we intended to produce AgNPs using fresh fruit extract of Pomelo and evaluate their antibacterial efficiency against Ao strain RS-2, pathogen of BBS.

Pomelo is a perennial plant having edible round fruits with various medicinal values including as an anticancer, antidiabetic, antibacterial and antioxidant agent. Crude organic extracts of leaves, seeds, pulp, fruits and roots of pomelo have been screened for some pharmacological activities and found to possess a large number of phytochemicals [19, 20], which provoked the idea of AgNPs reduction. Since the crude organic extracts of pomelo have been marked off for some pharmacological activities like pain killer, anti-inflammation, anticancer, antioxidant activity, anti-diarrheal and antibacterial, it can also reduce high cholesterol and helps in insulin regulation while the use of crude extracts from this plant has not yet been tested for AgNPs production [20–24]. Therefore, we have used the pomelo fruit extract for the production of a biodegradable, natural product AgNPs.

2. Materials and methods

2.1. Materials

Fresh fruits of pomelo i.e. yellow and pink (two different varieties) were collected from Zhejiang University Zijingang Campus local food street food shops. Silver nitrate (AgNO3) was acquired from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Ao strain RS-2 was collected from Plant Bacteriology Laboratory in Zhejiang University. The bacterial strain was routinely grown in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g of NaCl in 1000 ml ddH2O, pH = 7.0) at 30°C, 220 rpm.

2.2. Preparation of extracts from pomelo fruit

The fresh fruit extract of pomelo was prepared according procedure reported by [25] with minor modification. Briefly, the fresh fruits were thoroughly washed with ddH2O and then sliced into small pieces. The sliced fruits were grinded completely with the help of mortar and pestle after the fruits peel and seeds were removed. The resulting extract was passed through a muslin cloth and then filtered by Whatman paper and kept at 4°C until use.

2.3. Synthesis of AgNPs using pomelo fruit extracts

Synthesis of AgNPs was performed based on the method of [25] with minor modification. Briefly, 100 ml of 2 mM and 4 mM silver nitrate solution was prepared in conical flask. Then 25 ml of pomelo fruit extract from either pomelo yellow (PMY) or pomelo red (PMR) was added to 100 ml silver nitrate solution following by heating for 15 to 20 min at 70°C–80°C. After the colour changed from colourless to dark brown, the samples were kept at room temperature in dark for complete reduction of silver ions. The overnight samples were measured UV–vis spectral using UV-2550 Shimadzu Spectrophotometer (Shimadzu Corporation, Kyoto,
After repeated centrifugation and wash with distilled water, the AgNPs were freeze-dried using Alpha 1–2 LDplus (GmbH, Germany) yielding black coloured silver nano powder and stored at −80 °C for future use.

2.4. Characterization of the synthesized AgNPs

2.4.1. UV–Vis spectroscopy

The formation of AgNPs was confirmed by UV–vis spectra analysis according to the method used by [25]. The absorption was recorded by UV–visible spectrophotometer 2550 (Shimadzu Corporation, Kyoto, Japan) at a resolution of 1 nm in the wavelengths range 200–800 nm.

2.4.2. Fourier transform infrared spectroscopy

To see the effect of chemicals present in pomelo extracts on nanoparticle surface modification, fourier transform infrared (FTIR) spectroscopy was employed according to the method used by 25 through FTIR machine (Vector 22, Bruker, Germany) using ranges between 450 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹.

2.4.3. X-ray diffraction and EDS analysis

In order to assess the structure and composition of synthesized AgNPs we performed crystallographic analyses using x-ray diffraction (Siemens D5000, Germany) according to the method used by 25. In addition, the presence of Nano silver elements was confirmed by energy dispersive spectroscopy (EDS, Oxford Instruments, Oxford, UK) at 20 keV as described by [25].

2.4.4. TEM and SEM

Surface structure and surface morphology of AgNPs were analysed by SEM (SU8010, Hitachi, Japan) according to the method used by [25]. The size and morphology of AgNPs was characterized by TEM (JEOL JEM 100CX II) as described by.

2.5. Minimum inhibitory concentration (MIC).

The MIC of AgNPs against Ao was determined by adding the stock of AgNPs to the 5 ml half-strength LB broth with the final concentration of 0 μg ml⁻¹ to 25 μg ml⁻¹. A 50μl of bacteria cells of Ao strain RS-2 (~1 × 10⁸ CFU ml⁻¹) was inoculated into AgNPs solution of different concentrations, and then the samples were incubated at 30 °C for 12 h. The number of bacterial cells in the samples were calculated by reading the absorbance at 600 nm using Thermo Multiskan EX Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA). The experiments were carried out three times with six replicates for each treatment.

2.6. Agar well diffusion assay

The antimicrobial activity and inhibitory effect of AgNPs was investigated by agar diffusion technique against the Ao strain RS-2 according to the method used by. Briefly, 200 μl of overnight cultured Ao strain RS-2 was mixed with 5 ml half strength LB medium and then spread on the solid LB medium in a petri plate (~1 × 10⁸ CFU ml⁻¹). After the upper layer of agar medium was air dried, different concentrations of AgNPs (5 μg ml⁻¹, 15 μg ml⁻¹ and 25 μg ml⁻¹) were placed in equidistance to each other on the agar surface of the seeded medium and incubated at 30 °C for 24 h. The diameter of inhibition zone around the wells was measured. This experiment was repeated three times in the same manner in a completely randomized design.

2.7. Swarming motility assay

The effect of AgNPs on Ao strain RS-2 swarming motility was assessed on LB plates containing 0.7 percent (w/v) agar as described by [25]. Five microliters of overnight cultured Ao strain RS-2 were dropped in the centre of semisolid LB agar plate containing 25 μg ml⁻¹ AgNPs while the plates without AgNPs was used as control and then incubated at 30 °C for 3 d. To assess the swarming motility, the colony diameter of strain RS-2 was measured and the assay was repeated three times with six replications.

2.8. Biofilm inhibition assay

The capability of AgNPs to inhibit the biofilm formation of Ao strain RS-2 was examined using 96-well microtiter culture plates according to [27] with slight modification. Ao strain was grown in LB broth up to mid-exponential phase (~1 × 10⁸ CFU ml⁻¹), after that 100 μl of bacterial culture was added into each well with AgNPs concentration of 25 μg ml⁻¹, while sterile ddH₂O were used as a control instead of AgNPs. The plates were kept on static phase at 30 °C for 24 h. After that bacterial culture was discarded, washed slightly with sterile ddH₂O and dried for 1 h. The attached biofilm material was stained with 100 μl of 1% crystal violet (CV) and incubated for 30–45 min at room temperature. Then the unattached violet crystal solution was discarded and the plate was washed three times with sterile ddH₂O. After that the remaining CV stained solution was
solubilized by adding 150 μl of 33% acetic acid and its absorbance was measured at 570 nm using SPECTRAmax® PLUS384 Microplate Spectrophotometer. This experiment was repeated three times with six replicates for each treatment.

2.9. Live/dead cell staining

Live/dead staining method was used to detect damage or intact membrane in bacterial cells exposed to AgNPs according to the method used by [25]. BacLight bacterial viability kit (Invitrogen) was used for this purpose. According to the description of the kit’s protocol, live bacteria was used as positive control while the dead bacteria samples treated by isopropanol as negative control. So bacterial cells were separated into three tubes each containing 1 ml of bacterial cells and treated separately as positive control, negative control and AgNPs treatment. Fluorescence in the sample was then detected using the Olympus inverted confocal microscope as described previously [28].

2.10. Observation of cell morphology using TEM

Ao strain RS-2 was prepared for TEM analysis according to [29]. Briefly, 1 ml suspension of Ao strain RS-2 (∼1 × 10⁸ CFU ml⁻¹) was added with AgNPs to a final concentration of 25 μg ml⁻¹. The bacterial suspension without AgNPs served as control. Bacterial cultures were incubated in shaker at 160 rpm and 30 °C for 4 h. The bacterial cells were washed with 0.1 M phosphate-buffered saline (PBS), followed by fixing with 2.5% glutaraldehyde on a glass slide. The samples were then dehydrated with a series of 50%, 70%, 80%, 90%, 95% and 100% ethanol solutions for TEM observation.

2.11. Gene expression analysis of type VI secretion system (T6SS) related genes using qRT-PCR

The selection of these T6SS genes based on previous studies which were conducted on Ao strain RS-1 and RS-2 [30]. Briefly, AgNPs with a final concentration of 25 μg ml⁻¹ was added to 4 ml LB media containing bacteria in log phase and incubated for 1 h, while the same quantity of ddH₂O was added to bacteria culture as control. After the bacteria was harvested, total RNA was extracted using RNA extraction kit (Sangon Biotech Shanghai Co Ltd), followed by synthesizing the cDNA using Goldenstar™ RT6 cDNA Synthesis Mix TSK313S (TsingKe). The PCR reaction for cDNA synthesis was 50 °C for 1 min; followed by 40 cycles at 95 °C for 10 s and 34 °C for 15 s. Finally, the target gene expression was calculated using the 2^−ΔΔCt. qRT-PCR primers of T6SS genes are listed in table 1.

2.12. Statistical analyses

The experimental data was analysed by SPSS software package SPSS (IBM SPSS statistics 21, Georgia, USA) and the mean values of all the treatments were grouped as LSD (least significant difference) tools. The data represented shows the average value with standard error of minimum three values of each independent experiment.

| Table 1. List of oligonucleotide primers for quantitative real-time PCR used in this study. |
| Gene names | Nucleotide sequences (5′–3′) |
|------------|-----------------------------|
| 16S rDNA   | F-TTGGCGGTCCCTGCTTTCTCAT    R-CGGTAAACGTTCTTCCGATGCT |
| clpA       | F-CAGATGATCCGATCTGCGCC      R-GCCCATGTCCAGCGGAATAGA |
| clpB       | F-GCAGGCAGGAAAGCAAGAG       R-GCCAGGAAACGGAACAGG |
| impJ       | F-TCCAGAGTGCAGAAGACA        R-GACCCAGGTTGGAAAGA |
| Lip        | F-GCGATGCCGAGATCTCGATACCTT  R-TCCCTGCCACCGTGATGCT |
| pppA       | F-AGATCCACGGGGGACCATT       R-TTCTCTGCGTCTGAGACAT |
| vgrG1      | F-ATCCGATGAAAAAGAACTC       R-AAATGATGCCCTGCTGCT |

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3. Results and discussion

3.1. Characterization of AgNPs

3.1.1. UV–vis spectroscopy

After the addition of 2 mM and 4 mM silver nitrate solution, the colour of pomelo fruit extracts changed from colourless to reddish brown after heating at 60 °C–80 °C for 10 min (figure 1(a)), indicating the reduction of Ag ions by pomelo fruit extracts. As it is shown in figure 1(b), AgNPs showed maximum absorption at 426 nm by recording the surface plasmon resonance using UV–visible spectroscopy, which is consistent with previous studies [32–34]. Two different types of pomelo fruit extracts including PMY and PMR with 2 mM and 4 mM AgNO₃ solution, respectively, were determined for UV–vis spectroscopy. The results showed that the surface plasmon resonance absorption band for PMY with 4 mM AgNO₃ solution is better than that of the other three samples. The surface plasmon resonance absorption band is due to the combined effect of vibration of free electrons of metal ions and resonance of light waves [35]. Therefore, PMY with 4 mM AgNO₃ was used for further characterization and function analysis. With the help of mie scattering theory, the particle size has been calculated as 12.8 nm.

3.1.2. FTIR analysis

FTIR spectrum of biosynthesized AgNPs showed 9 peaks which located at about 378, 2921, 2851, 1640, 1536, 1396, 1063, 534 and 466 cm⁻¹ as shown in figure 2. Peak at 3361.02 cm⁻¹ represent O-H stretching for alcohols and phenols which may be present in pomelo fruit extracts [36]. Peak at 2921 and 2851 cm⁻¹ shows C-H
Amide I and amide II regions of proteins are represented by peaks at 1640 and 1536 cm$^{-1}$ [38]. Peak at 1396 cm$^{-1}$ corresponds to C–N bonds stretching while peak observed at 1063 cm$^{-1}$ may be due to stretching of C–O and C–O–C [39]. Absorption peaks shown at 534 and 466 cm$^{-1}$ represents strong C–Cl stretching in halogen compounds [40, 41]. This result confirms the binding ability of carbonyl group of proteins which can bind metal ions and act as a capping material for AgNPs, which prevents cluster formation and helps to stabilize the medium [12, 40]. It is obvious from our results that biological molecules are responsible for the formation and stabilization of AgNPs.

3.1.3. TEM analysis and XRD
Conduction of TEM experiment verified the formation of nanocrystalline silver particles, as shown in figure 3(a). These nanocrystalline silver particles are spherical shaped and well dispersed in the medium. The XRD pattern of biosynthesized AgNPs using pomelo fruit extract was done to confirm the cubic structure of AgNPs. Some extra peaks were also found at 21.77, 27.7, 32.12, 46.08, 54.8 and 81.28, representing AgCl according to the XRD report. $\theta = 37.1^\circ$, 44.428$^\circ$, 64.36$^\circ$, and 77.27$^\circ$, that can be ascribed to the (111), (200), (220), and (311) planes of silver, respectively. These results indicate that particles are very well dispersed in the medium and coated with biological molecules, which are consistent with previous reports [42–44]. The particle size calculated by TEM and XRD pattern through rietveld refinement were 12.7 nm and 11.3 nm, respectively, which are similar to the results from UV–Vis.

3.1.4. SEM analysis and EDX
Surface morphology of the pomelo fruit extracts mediated synthesized AgNPs is determined by SEM (figure 4(a)). It showed that biosynthesized AgNPs were mostly spherical [45]. Moreover, EDX analysis was carried out to confirm the presence of the silver element in the synthesized AgNPs. As shown in figure 4(b), the broad peak at 3 keV represents a reduction of Ag$^+$ to Ag$^0$ which is in agreement with previous studies [46, 47].

3.2. In vitro antibacterial activity of AgNPs against Ao strain RS-2
The antagonistic activity of AgNPs was determined against Ao strain RS-2 by agar well diffusion assay. The inhibitory effect of AgNPs was observed in the form of inhibition zones. It was found that inhibitory effect of AgNPs was increased with increase of the amount of AgNPs. Indeed, the diameters for inhibition zones were 1.8, 2.0 and 2.4 cm when 15, 20, 25 $\mu$g ml$^{-1}$ AgNPs were added in the wells, respectively (figures 5(a) and (b)). The MIC of AgNPs to RS–2 was determined using 96 well plate reader after incubation at 30 $^\circ$C for 24 h. As shown in figure 6, the MIC of biosynthesized AgNPs to RS-2 was 25 $\mu$g ml$^{-1}$, and the growth of bacteria decreased with the increase of AgNPs concentration, which is consistent with previous reports [48–50].

3.3. Effect of incubation time on antibacterial activity
For the sake of investigation to know whether incubation time influences the antibacterial activity of AgNPs, we conducted an experiment of growth kinetics with Ao strain RS-2. As shown in figure 7, the cell number of strain RS-2 increased with an increase in incubation time. Indeed, the addition of AgNPs caused 19.3%, 49.3% and 52.7% reduction in the OD600 values of Ao strain RS-2 after 4, 6, and 8 h of incubation, respectively, compared...
This result revealed that the antibacterial activity of AgNPs against Ao strain RS-2 was affected by the incubation time, which is consistent with previous studies [50–52].

### 3.4. Swarming motility assay

In order to investigate the effect of AgNPs on bacterial motility, we performed the swarming motility assay of Ao strain RS-2. As shown in figures 8(a) and (f), the diameter of bacterial colony was 1.70, 1.83, 1.93 and 2.13 cm after 24, 28, 72 and 96 h, respectively, incubating in the absence of AgNPs, while the diameter of bacterial colony was found to be 0.85, 0.90, 1.10 and 1.30 cm after 24, 28, 72 and 96 h, respectively, incubating in the presence of 25 μg ml⁻¹ AgNPs. It is obvious that AgNPs had a significant inhibitory effect on the swarming motility of Ao strain RS-2 regardless of the incubation time. As the swarming motility has been reported to be frequently linked with bacterial growth, propagation, and virulence [53], the inhibitory effect of AgNPs is partly due to the inhibition of swarming motility of Ao strain RS-2 by AgNPs.

### 3.5. Biofilm inhibition assay

In order to know whether AgNPs interferes with biofilm of Ao strain RS-2, we performed an experiment of biofilm formation with Ao strain RS-2 (figure 9). The results showed that RS-2 biofilm in the absence of AgNPs was 0.116 while in the presence of AgNPs it was 0.037 which indicates a decrease of up to 68.24%. Bacterial biofilm is believed to play an important role in the virulence of pathogenic bacteria by engaging several mechanisms such as increasing the potential to survive in harsh conditions and inadequate nutrient availability, stimulating host colonization and developing resistance to plant defense reactions [54]. Moreover, our previous study found that exopolysaccharide (EPS) production is a key component of the biofilm complex which is strongly linked with virulence factors in Ao strain RS-1 [5]. As biofilm helps in protecting bacteria from external attacks and plays an important role in bacterial survival during infections [55], the interfering with biofilm formation will largely decrease the virulence of pathogenic bacteria.
3.6. Live/dead cell analysis
In order to observe the bacterial integrity, live/dead staining method was used to see damage and intact membranes in bacterial cells exposed to 25 μg ml⁻¹ AgNPs. Bacterial cells were stained with a combination of...
red and green dye, and all bacteria having intact cell membranes showed green fluorescence (figure 10(b)), whereas bacteria with damaged cell membranes showed red fluorescence (figure 10(a)). Interestingly, bacterial cells indicated a mixture of red and green fluorescence when exposed to AgNPs (figure 10(c)). However, the number of cells was highly reduced in the presence of AgNPs which indicated an inhibition of bacterial growth and replication, which is consistent with previous studies [56, 57]. Therefore, it seems that AgNPs has a bacteriostatic as well as bactericidal effect on Ao strain RS-2.

3.7. Bacterial cell morphology
TEM of Ao strain RS-2 was performed in order to analyze the surface morphology of the cells treated with AgNPs. The morphology of bacterial cells without treatment were typical rod-shaped bacterial forms (figure 11(a)), while the cells became extremely abnormal after treated with AgNPs (figure 11(b)). As we can see from figure 11(b), the cell wall and cell membrane became wrinkle and broken after treated with AgNPs, by contrast, the cell wall and cell membrane stayed intact. TEM micrographs showed intensive as well as extensive damage to cell wall which resulted in leakage of cell nucleic acid and proteins causing bacterial death. The high antibacterial activity may be due to the interaction of silver cation with bacterial cell wall which increase membrane permeability. AgNPs can also inhibit DNA synthesis and induce apoptosis in E. coli. [38]. Qayyum et al reported that AgNPs could induce bacterial cells to produce ROS, which might be the cause of cell lysis or distortion of bacterial membranes [59]. Therefore, it is rational to assume that antimicrobial activity of AgNPs can be ascribed to the damage of the cell membrane and the leakage of cytoplasm of Ao strain RS-2.
Figure 8. Effect of biosynthesized AgNPs on the swarming motility of * Ao RS-2*. Bacterial colony for determination of swarming motility (a) and colony diameter at different incubation time (b). The concentration of AgNPs is 25 μg ml⁻¹. Error bars represent standard error of the mean (n = 6). Bars tagged with same letter(s) are not significantly different (P > 0.05).

Figure 9. Effect of AgNPs (25 μg ml⁻¹) on biofilm formation of * Ao RS-2* after 24 h incubation at 30 °C for 24 h without agitation. Error bars represent standard errors of the means (n = 6). Bars tagged with different letter(s) are significantly different (P > 0.05).

Figure 10. Live/dead cell staining study of * Ao RS-2* cells treated with 25 μg ml⁻¹ of AgNPs for 4 h. Cells were stained using BacLight bacterial viability kit (Invitrogen, Carlsbad, CA, United States), and pictured by fluorescence microscope. Green fluorescence shows live bacteria with intact membranes while red fluorescence shows dead bacteria with damaged membranes. (a) Live bacteria in positive control (without AgNPs); (b) Dead bacteria in negative control (95% ethanol treatment); (c) Bacteria treated with AgNPs.
3.8. Expression of T6SS related genes

Our previous study showed that T6SS played an important role in the pathogenicity of Ao strain RS-2 [25]. In order to study the mechanism by which AgNPs reduce the virulence of Ao strain RS-2, we conducted qRT-PCR assay to determine T6SS genes expression under the treatment of AgNPs. In this experiment, RNA samples were extracted from Ao strain RS-2 treated with or without AgNPs, followed by cDNA synthesis and qPCR. As shown in figure 12, the treatment of AgNPs caused a significant decrease in expression of T6SS genes of Ao strain RS-2 compared to the control. Indeed, there was a 7.14-, 15.52-, 4.54-, 8.77-, 11.49-, 7.66- and 7.68-fold decrease for clpB, clpA, lip, impJ, pppA, icmF and vgrG1, respectively, compared to the control. This result indicated that AgNPs may reduce the virulence of Ao strain RS-2 by down-regulating the expression of T6SS genes. It has been reported that the secretion of Hcp protein, an important effector protein of T6SS, of Ao strain RS-2 was reduced by treating with AgNPs [25]. Since T6SS plays an important role in many aspects such as pathogenicity, quorum sensing and environmental adaptability of bacteria, AgNPs can be widely used to prevent diseases caused by plant pathogenic bacteria by inhibiting the T6SS [60].

4. Conclusion

In conclusion, we successfully synthesized the AgNPs using pomelo fruit extracts with a straightforward reproducible approach. The biosynthesized AgNPs was characterized by UV-Vis, SEM, TEM, FTIR and XRD analysis. Furthermore, the inhibition effect of AgNPs against Ao strain RS-2 was tested by multiple methods, indicating that the antibacterial activity of AgNPs may be attributed to inhibiting bacterial growth, swarming motility and biofilm formation as well as damaging bacterial integrity and down-regulating the expression of T6SS genes. Altogether, the biosynthesized AgNPs have a potent bactericidal effect against the pathogen of BBS which can be widely used in agricultural production.

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