

Control of Bacterial Blight Disease of Pomegranate Using Silver Nanoparticles

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Abstract

Successful cultivation is difficult to pomegranate growers due to *Xanthomonas axonopodis* pv. *punicae* which cause bacterial blight disease to pomegranate has become serious threat. However, everyone is looking for solution; this problem is resolved by synthesizing silver nanoparticles by the reduction of silver ions in aqueous solutions with the help of green synthesis method. In the present study the causative agent was isolated from the infected pomegranate fruits and was tested with silver nanoparticles which are synthesized from leaf extracts of Tulsi, Neem, Tridax and Drumstick at variable concentrations and it was found that silver nanoparticle containing Tulsi extract with 15 mM concentration showed maximum zone of inhibition (22 mm), then Tridax (20 mm), Neem (19 mm) and Drumstick (18 mm) while at 1 mM concentration it shows minimum. The bioreduction was tested by using UV-Vis Spectrophotometer. This study showed that by using synthesized silver nanoparticles we successfully controlled the growth of *Xanthomonas axonopodis* pv. *Punicae*. They can be used in the field of medical as well as in the management of plant diseases.

Keywords: Bacterial blight; *Xanthomonas axonopodis*; Silver nitrate; Nanoparticles; Antimicrobial activity

Introduction

Pomegranate is important fruit crops which are cultivated in arid and semiarid regions of the world; however is gaining lot of attention of total world over due to its high nutritional and economics values [1,2]. In addition pomegranate is used in several purposes as diarrhea, ulcers [3], diabetes [4], male infertility [5], and antiparasitic agent [6]. In the recent year pomegranate cultivation is creating problem due to bacterial blight disease *Xanthomonas axonopodis* Pv. *Punicae* which shows on leaves, stems as well as on fruits and reduce crop yield up to 60-80%. This disease could not be effectively controlled by antibiotics, chemicals [7-9]. Now a day, Nanoscience is rapidly growing field with great application for generating nanoparticles and biosynthesis of silver nanoparticles by using medicinal plants in agriculture due to their diverse effect [10]. Silver nanoparticles have great importance in the field of medical due to its broad spectrum application in the medical field [11,12]. The synthesis of nanoparticles by biological method show better results than the chemical methods and is easily scaled up for large scale synthesis and cost effective [13,14]. The use of plant extract for the synthesis of nanoparticles is adventitious, because it does not require the processes like intracellular synthesis, purification steps and the maintenance of microbial cell cultures. In the present study the causative agent was isolated from the infected pomegranate fruits and to control of this disease silver nanoparticles were synthesized from leaf extracts of Tulsi, Neem, Tridax, and Drumstick at variable concentrations (1 mM, 5 mM, 10 mM and 15 mM). However, the plant extracts are potentially effective than microorganisms with respect to stabilization by provide capping agents for the silver nanoparticles, prevent the aggregation of nanoparticles and free from toxic chemicals [15]. Antimicrobial activity of silver nanoparticles depends mostly on the relative surface area; even though; the smaller silver nanoparticles can have greater toxic potentials [16-18]. However, the results were confirmed and characterized by UV-Visible spectroscopy and Antimicrobial activity of silver nanoparticles was evaluated against bacterial blight disease *Xanthomonas axonopodis* Pv. *Punicae* for diagnosis, treatment, control and to manage disease which will be beneficial to farmers as well as further studies must be conducted to verify if the bacteria develop resistance towards the nanoparticles or not.

Materials and Methods

Sample collection of infected fruits

The infected fruits showing the typical symptoms of bacterial spot were collected from farmer's fields of major pomegranate growing areas of Baramati in Pune district. The fruits showing typical symptoms like yellowish brown spots, decolouration, minute water soaked lesions, cankers lesions and irregular yellow and brown patches.

Isolation of disease causing bacteria from infected fruit part

Infected Fruits samples are collected from orchard. Ooze test is performed by cutting 1 cm of the infected portion of the fruit and the outer surface was removed and sterilized with 0.1% HgCl₂ solution for 1 minute and are 3 times washed with sterile distilled water. Then the infected portion was squeezed gently with a sterile scalpel to get a suspension in a sterile test tube containing 3 ml of sterilized saline. The suspension was serially diluted and plated in sterilized petriplates containing YDC Agar medium (Yeast extract-10 g/L, Dextrose-20 g/L, Calcium carbonate- 20 g/L, Agar-25 g/L, pH 6.5). Inoculated plates were incubated at 30°C for 72 hours. After the incubation period, light yellow colored bacterial colonies were developed resembling *Xanthomonas* sp. further Cultures were screened based on biochemical characterization according to Bergey's manual of Determinative Bacteriology (Figure 1).

Purification of bacterial culture

The suspected bacterial colonies were picked up with the help of sterilized inoculated loop and streaked onto the surface of YDC Agar. The inoculated plates were incubated at 30°C for 72 hours and the

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observations were made for the development of well separated light yellow colored bacterial colonies. The purified bacterial colonies were streaked on nutrient agar slants and stored at 4°C in refrigerator and also in sterile distilled water taken in small culture tubes, by suspending 2-3 loops full of the bacterial culture for future use.

Identification of causal organism

The morphological characteristics such as cell shape, gram staining (Table 1) and Biochemical test (Table 2) was performed for the identification of causative agent of oil spot disease of the isolate.

Synthesis of silver nanoparticles by using plant extracts

Around 5 g of fresh tulsi, tridax, drumstick and neem leaves were collected from the Bio-village, VSBT of VPASC College, Baramati. All the veins were removed and leaves were crushed in deionized water. After crushing the extract was filtered using whatman's filter paper no.1. The 2 ml and 5 ml of extract filtrate was collected and then added in various concentrations of silver nitrate solution (1 mM, 5 mM and 10 mM, 15 mM). This reaction was kept under dark condition for more than a week. Tubes were monitored after every 24 hours and reactions showing precipitation of silver particles were discarded. After 15 days

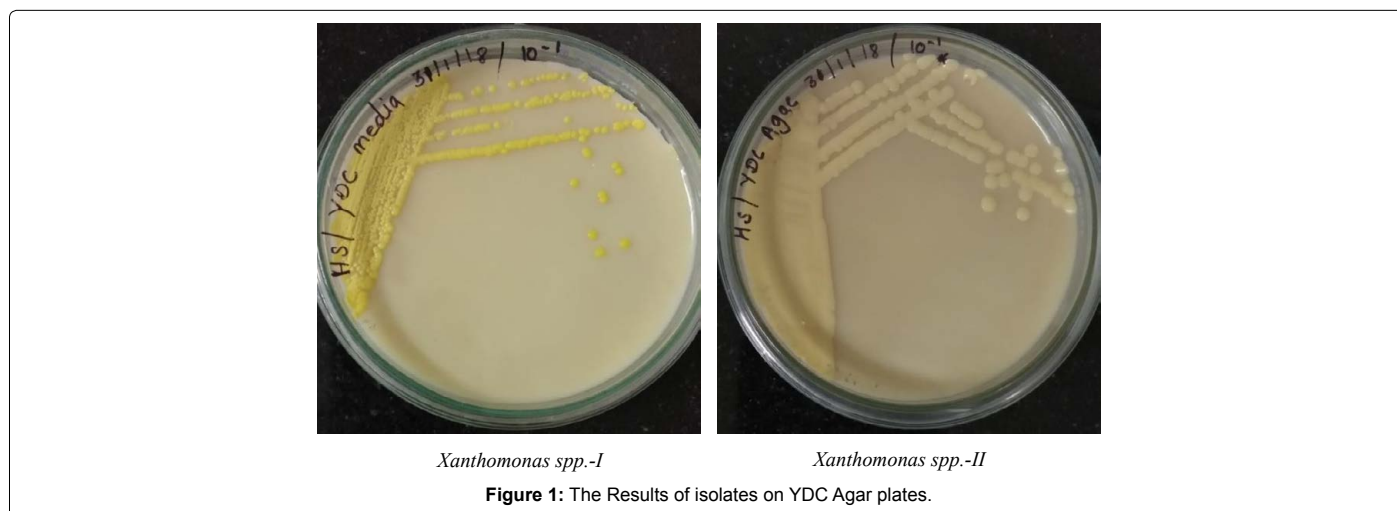
reaction the color of the solution of silver nanoparticles was analyzed by UV visible spectroscopy (Figure 2). Reaction showing peaks at around 390-430 nm was chosen for further study (Table 3). This solution was centrifuged at 10,000 rpm for 10 minutes, and the precipitate of silver nanoparticles was washed in methanol three times. The resulting pellet was dried in oven at 65°C for 24 h and then collected in tubes for further use.

Antimicrobial analysis

Biosynthesized Silver nanoparticles were tested for antimicrobial activity against isolated oil spot disease causing bacteria. Freshly prepared 100 µl of the isolated pathogen was spread on the sterile nutrient agar plates. By using cork borer a hole was made in the petriplates. Then 100 µl of each suspension of milli-Q water as a control, silver nanoparticles, silver nitrate as positive control was added into the wells. Plates were incubated at 37°C for 24 hours, and then the zone of inhibition was measured in mm (Figures 3, 4 and Table 4).

Result and Discussion

The yellow and pale yellow coloured colonies were observed on the YDC Agar medium. The morphological characters of the isolate were



Isolate	Size	Shape	Colour	margin	elevation	consistency	Opacity
<i>Xanthomonas spp.-I</i>	2 mm	Circular	Yellow	Regular	Elevated	sticky	Opaque
<i>Xanthomonas spp.-II</i>	3 mm	Circular	Pale yellow	Regular	Elevated	Moist	Opaque

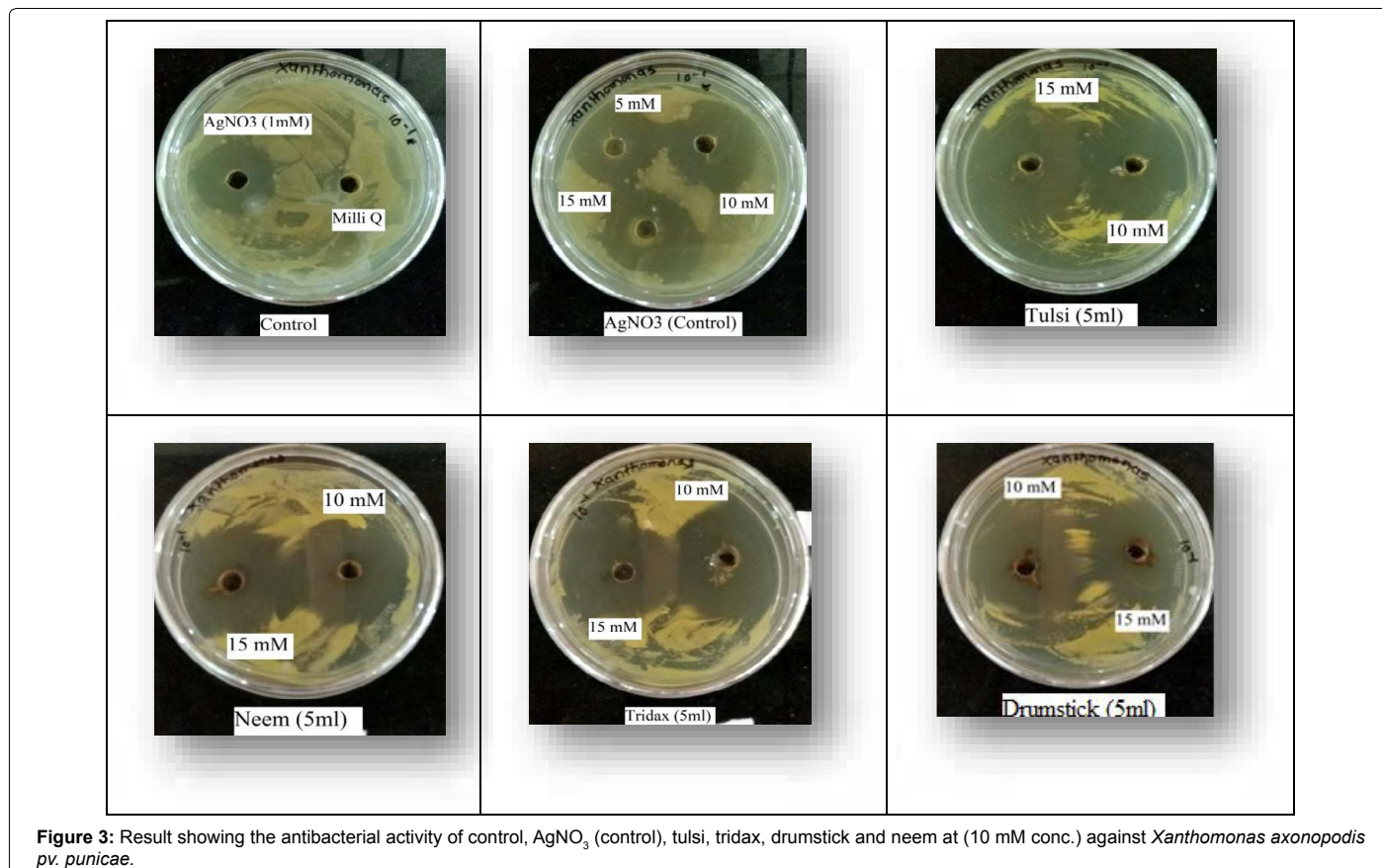
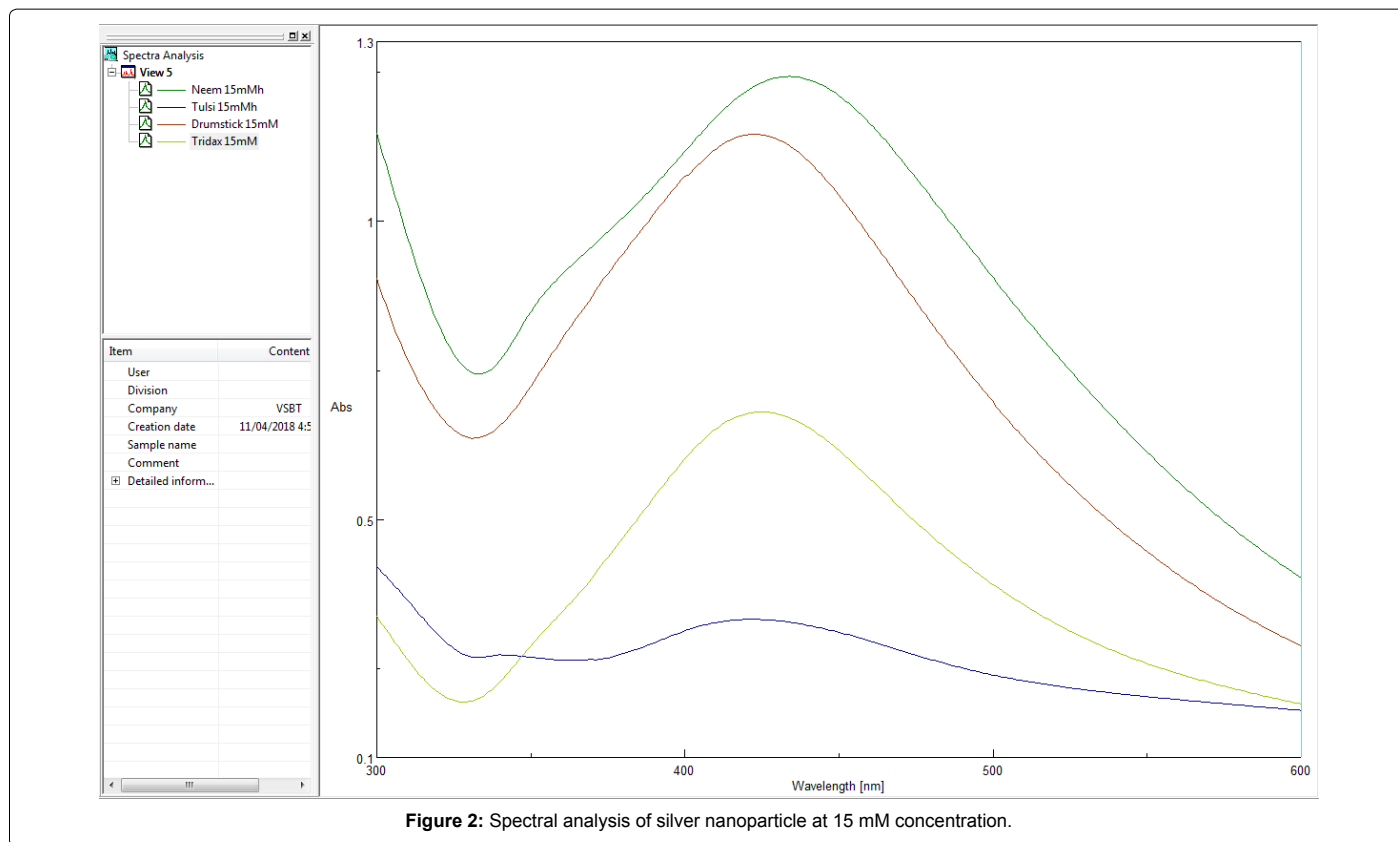
Table 1: Colony characters of isolated pathogens.

Sr. No	Characteristics	<i>Xanthomonas spp.-I</i>	<i>Xanthomonas spp.-II</i>
1	Methyl Red Test	-	-
2	Voges Proskauer Test	-	-
3	Indole Test	-	-
4	Catalase Test	+	+
5	Oxidase Test	-	-

Table 2: Biochemical tests for identification of isolates.

Common name of plants used for synthesis of AgNP's	Scientific name	λ-max of AgNP's
Neem	<i>Azadirachta indica</i>	434 nm
Tulsi	<i>Ocimum tenuiflorum</i>	425 nm
Drumstick	<i>Moringa oleifera</i>	423 nm
Tridax	<i>Tridax procumbens</i>	425 nm

Table 3: Common Name of Plants used for synthesis of AgNP's, Scientific Name and their Spectral analysis of extracts was carried to find out λ-max of nanoparticles. λ-max for nanoparticles isolated from different plants listed below.



studied (Table 1). This isolate was characterized by the biochemical tests and the causative agent may belongs to *Xanthomonas spp.*

Biochemical characteristics of isolates

The biochemical characters of isolate *Xanthomonas spp.-I*, *Xanthomonas spp.-II* (Scheme 1). Identification of isolates was done by comparing various colony morphological and biochemical tests according to Bergy's manual.

Synthesis of silver nanoparticle

As the leaf extracts were added to different concentrations of aqueous silver nitrate solution, the colour of the solution changed from

faint light to yellowish brown to reddish brown and finally to colloidal brown indicating AgNP formation. The completion of reaction between leaf extract and AgNO₃ was observed. Absorption spectra of AgNPs formed in the reaction media has absorption maxima in the range of 420 to 460 nm.

UV visible spectral analysis

UV visible spectral analysis has given in Figure 2.

Anti-microbial activity of extract

The antimicrobial activity shows most rapid bioreduction in 5 ml extract of Tulsi, Neem, Drumstick and Tridax of 15 mM concentration

Organism	<i>Xanthomonas spp.-I</i>		<i>Xanthomonas spp.-II</i>	
	12 mm		10 mm	
Sample	Leaf extract	AgNP's	Leaf extract	AgNP's
Drumstick	6 mm	18 mm	7 mm	15 mm
Neem	5 mm	19 mm	5 mm	16 mm
Tridax	8 mm	20 mm	7 mm	19 mm
Tulsi	8 mm	22 mm	7 mm	17 mm

The results are average of three replicates.

Table 4: Antibacterial activity of plant extract which were treated with AgNO₃ and AgNPs against *Xanthomonas axonopodis pv. punicae* showing zone of inhibition (mm).

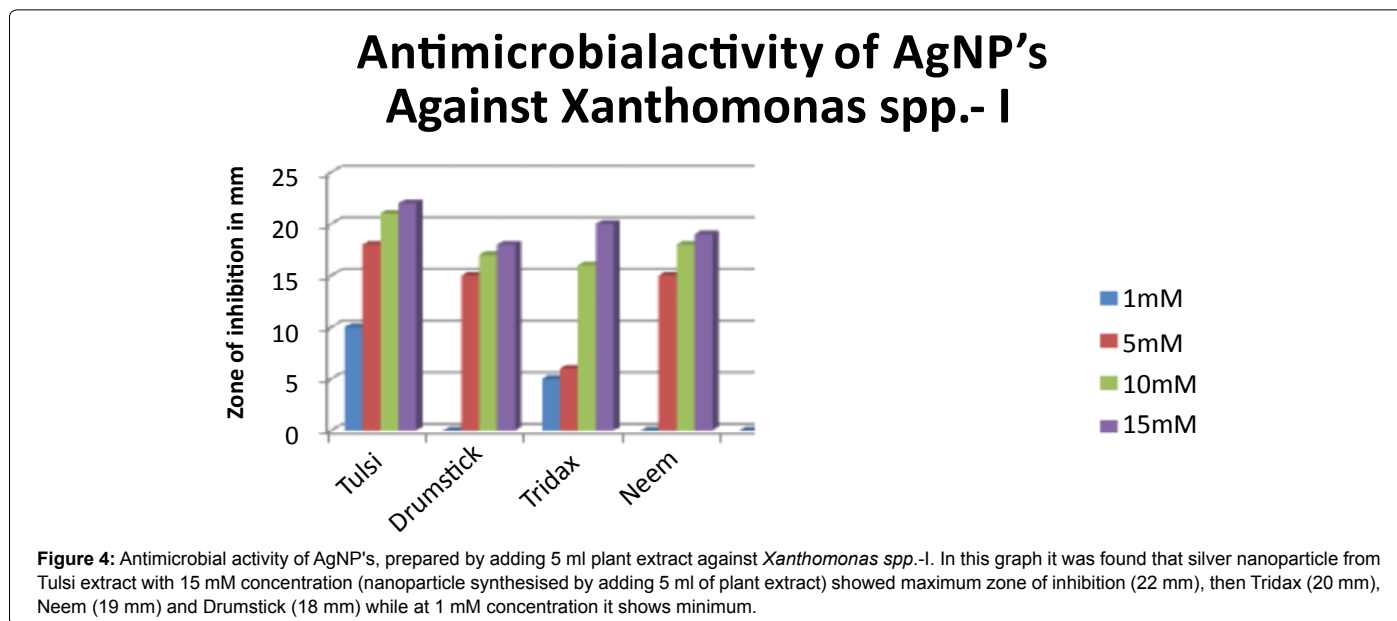


Figure 4: Antimicrobial activity of AgNP's, prepared by adding 5 ml plant extract against *Xanthomonas spp.-I*. In this graph it was found that silver nanoparticle from Tulsi extract with 15 mM concentration (nanoparticle synthesised by adding 5 ml of plant extract) showed maximum zone of inhibition (22 mm), then Tridax (20 mm), Neem (19 mm) and Drumstick (18 mm) while at 1 mM concentration it shows minimum.

Isolate	Gram nature		Motility
<i>Xanthomonas I</i>	Gram Negative Coocci		Motile
<i>Xanthomonas</i>	Gram negative Rod		Motile

Scheme 1: Colony characteristics.

of silver nitrate solution. Results obtained are shown in Table 2. The inhibition zones obtained indicates maximum antibacterial activity of the prepared test sample.

Conclusion

Xanthomonas axonopodis pv. *punicae* which cause bacterial blight disease to the Pomegranate that affects around 80% of the production. The current scenario, Nanotechnology provides a good platform to overcome the problem and resolved it with help of synthesizing silver nanoparticles from leaf extracts of Tulsi, Neem, Tridax and Drumstick at variable concentrations. And the causative agent from the infected fruits was treated with silver nanoparticles at variable concentrations. In this study it was observed that silver nanoparticles with the concentration 15 mM showed highest antimicrobial activity and minimum at 5 mM. This study showed that synthesized silver nanoparticles having bactericidal potential and successfully controlled the growth of *Xanthomonas axonopodis* pv. *punicae*. Therefore, silver nanoparticles would be used as powerful weapons against *Xanthomonas axonopodis* pv. *punicae* even at very lower concentrations.

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