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Extracellular biosynthesis of silver nanoparticles using terrestrial *Streptomyces* sp-SBU3 and its antimicrobial efficiency against plant pathogens

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Abstract: Plant pathogens cause many serious diseases to plants across the world and cause tremendous crop damage leads to economic loss to the farmers. Most of the plants possess disease resistance to many pathogens. However, some plants can harbor plant pathogens without any symptom development and reduce the crop yield drastically. In the present study we have isolated antagonistic actinomycetes from terrestrial red garden soil. The potential isolate was identified as *Streptomyces* sp.-SBU3 and used for extracellular synthesis of silver nanoparticles (Ag-NPs). The cell free culture supernatant was used for the synthesis of Ag-NPs by using 1mM silver nitrate ($AgNO_3$) solution. The colour of the reaction mixture was changed from pale to brown indicates the synthesis of silver nanoparticles. Silver nanoparticles were further confirmed by performing thin layer chromatography (TLC). A single separate band of silver nanoparticle was observed with the R_f value of 0.70. Silver nanoparticles have been known to have antimicrobial effects that can be used to control the plant disease. The synthesized Ag-NPs were evaluated for their antimicrobial activity against selected plant pathogens, which showed maximum activity against bacteria when compared to fungal pathogens. Silver nanoparticle synthesized using terrestrial *Streptomyces* sp.-SBU3 is a promising source of cost effective and eco-friendly antimicrobial agent for the management of plant diseases.
Key words: silver nanoparticles, antimicrobial activity, plant pathogens, *Streptomyces* sp.

Introduction

Plant diseases are the major challenging issues globally and life threatening problems caused by various agents which drastically affect crop yield. Agricultural production is reduced worldwide every year due to plant diseases caused by microorganisms[1]. Therefore millions of dollars have been invested and efforts to control these plant diseases. Plant pathogens are difficult to control because of their population variable in time, space, and genotype[2]. In recent years, environmental hazards caused by excessive use of pesticides and fungicides have been widely discussed. Therefore, scientists are searching for alternative measures against pesticides and fungicide to minimize the plant diseases. Currently, 20–25% of harvested crops worldwide are lost to pre- and post-harvest diseases and climatic change is expected to increase these losses. Climatic changes affect the activities and vigor of aerial and soil borne pathogens[3]. Some pathogens are become more active by capable of devastating crops and damaging yields because of their geographical ranges expand as a consequence of climate change[4].

Nanotechnology is the new and emerging field of biological research in the current scenario. The researchers focused on the synthesis of environmentally non toxic nanoparticles of different chemicals are being used. Plant extracts are routinely used for the synthesis of nanoparticles[5]. But microorganisms play a vital role

in metal ion remediation through metal reduction for the synthesis of nanoparticles and are being used as an antimicrobial substance[6,7]. Microbial enzymes and extracellular protein have reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles[8]. In this context, the study focuses on the production of silver nanoparticles (Ag-NPs) using soil *Streptomyces* species. The objective of the present investigation is to isolate *Streptomyces* from ground nut field. The isolated *Streptomyces* have been used for the extracellular synthesis of Ag-NPs and subjected to performing antibacterial efficiency against selected plant pathogens. Soil actinomycetes have the ability to reduce silver metal into silver ions by reduction reaction. Hence a preliminary attempt has been made for the synthesis of silver nanoparticles by terrestrial soil *Streptomyces* sp have been used as bioactive metabolites against selected plant pathogens.

Materials and Methods

Sample collection

Red garden soil sample was collected from ground nut (*Arachis hypogia* Linn) field from Aralvoimozhi, Kanyakumari District, Tamilnmadu, India. The soil sample was gently dried in a shady place and subjected for the enumeration of actinomycetes.

Microorganism and culture media

Actinomycetes were isolated from soil sample by spread plate method using Starch Casein Agar (SCA) Hi-media Private Limited, Mumbai, India in sterile distilled water supplemented with antibiotics (Nalidixic acid 20µg ml^{-1}; Nystatin 25µg ml^{-1}; Cycloheximide 100µg ml^{-1}) to minimize the gram negative bacteria, fungal and yeast contaminants respectively[9] since actinomycetes are filamentous gram positive bacteria. The inoculated agar plates were incubated at 28^{o}C for 7 days. After incubation period morphologically different typical powdery colony was picked and sub-cultured on ISP2 (International *Streptomyces* project) medium and stored at 4^{o}C for further studies.

Screening of antagonistic actinomycetes

Antagonistic activity of actinomycete was detected using Modified Nutrient Agar (MNA) medium containing glucose 5g, peptone 5g, beef extract 3g, sodium chloride 5g, agar 18g, distilled water 1000 ml was used. The final pH of the medium was adjusted to 7.0. The isolate was cross streaked with the size of 4-5mm on MNA medium across the plate and incubated at 30^{o}C for 5 days still to get ribbon like growth. The test pathogens (10^{6} cells) of bacteria such as *Xanthomonas* sp., *Agrobacterium* sp., *Bascillus campestris*, *Erwinia amylovora, Pseudomonas campestris* and fungus *Fusarium oxysporium* was streaked perpendicular to already grown *Streptomyces* sp. separately. Triplicates were maintained for each test organism and each isolate. The inoculated plates were incubated at 37^{o}C for 24 h for bacteria and at 22^{o}C for 72 h for fungal pathogens. The zone of inhibition was measured for each test organism and expressed as good, moderate, weak and no activity. Bioactive metabolites production was determined in terms of their antimicrobial spectrum. The potential candidate strain was subjected to species level identification and further adopted for extracellular biosynthesis of silver nanoparticles.

Identification of actinomycete

The potential isolate was identified based on the morphological, physiological, reverse side melanin pigment and biochemical characteristics on ISP2 media recommended by Shirling and Gottileb[10].

Biosynthesis of silver nanoparticles (Ag-NPs)

The *Streptomyces* isolate showed promising antimicrobial spectrum is subjected for the synthesis of silver nanoparticles. The *Streptomyces* sp.-SBU3 was inoculated in ISP2 broth and incubated on thermo control Orbital shaker at 28^{o} C for 3- 5 days. Then the culture was filtered using What man No.1 filter paper in an aseptic condition. Ten gram (wet weight) of cell biomass was transferred into the Erlenmeyer flask containing 100ml of sterile double distilled water and kept on thermo control Orbital shaker at 28^{o} C for 48 hours. Then the culture was centrifuged at 12000 rpm for 5 minutes. The cell free culture supernatant was used for the synthesis of silver nanoparticles. 1mM silver nitrate ($AgNO_3$) solution was prepared in Milli Q water and stored in brown bottle. Ten milliliter of cell free culture supernatant was transferred into the reaction vessel containing 100ml of

1mM $AgNO_3$ solution. The color of the reaction mixture was changed from pale to brown indicates the synthesis of silver nanoparticles. This solution was maintained in a dark place at room temperature for further antimicrobial spectrum and its characterization studies.

Determination of Antimicrobial assay

The synthesized silver nanoparticles were tested to determine the antimicrobial activity against selected bacterial and fungal plant pathogens by agar cup method on Muller Hinton Agar (MHA) and Rose Bengal Agar (RBA) respectively[11]. Overnight broth culture of each test pathogen was uniformly swabbed on the solidified agar plate and 3cup of center to center equal distance with the size of 6mm were made using sterile gel puncture. Silver nanoparticle of 10µl and 20µl samples was poured into first two cup. In third cup 20µl of cell free supernatant was used as negative control. The bacterial test pathogens were incubated at 37^{o}C for 24 hours and fungal pathogen was incubated at 30^{o}C for 48 hours. Triplicates were maintained for each test pathogens to calculate the mean standard deviation. After incubation period the different level of zone of inhibition was measured and expressed in mm in diameter.

Thin Layer Chromatography (TLC) of silver nanoparticles

Silver nanoparticles were characterized by thin layer chromatography (TLC). A pre-coated silica gel plate of 0.25 mm thickness (Merck, India) was used to identify the silver ions. For the development of chromatogram, chloroform and acetic acid (1:1 v/v) was used as a mobile phase. The activated TLC plate was observed to elucidate the presence of Ag-NPs. The eluted spot was visualized in the iodine elution chamber for the conformation and stability of Ag-NPs. The retention factors (R_f) value of silver nanoparticles was measured and calculated by the following formula.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Chemicals and culture media

All chemicals and culture media ingredients were obtained from Hi-media Laboratories Private Limited, (Mumbai, India) to execute the present investigation.

Statistical analysis

The antimicrobial efficiency results were calculated as mean diameter of zone of inhibition in mm ± standard deviation (mean ± SD).

Results and Discussion

Environment contains diverse group of beneficial microorganisms which are useful to industry medicine[12,13] and agriculture[14]. Terrestrial counterpart contain rich source of microorganism producing novel bioactive metabolites. Streptomycetes have received important attention in recent years because of their bioactive compound for industrial applications[15]. Nowadays many researchers focused on biosynthesis of silver nanoperticles from microorganisms and used as bioactive compounds against various bacterial and fungal plant pathogens[16-18]. The antagonistic activity of teresitrial actinomycetes was screened against selected plant pathogens. In the present study, out of 6 isolates screened,4 isolates showed antagonistic activity against all the tested plant pathogens. All the isolate exhibited antagonistic activity against all the tested bacterial plant pathogens. Isolate SBU3 showed promising antagonistic activity against all the tested bacterial and fungal pathogens(Table 1).

Table 1 Screening of antagonistic actinomycetes against selected plant pathogens

Isolates	**Bacterial pathogens**					**Fungal pathogen**
	Xs	As	Bc	Ea	Pc	Fo
Isolate SBU1	+++	++	+++	++	++	+
Isolate SBU2	++	++	++	++	++	-
Isolate SBU3	+++	+++	+++	+++	+++	++
Isolate SBU4	+++	++	+++	++	++	+
Isolate SBU5	++	+	++	++	+	-
Isolate SBU6	+++	++	++	++	++	+

Xs - *Xanthomonas sp.*, As - *Agrobacterium sp.,* Bc - *Bascillus campestris,* Ea - *Erwinia amylovora,* Pc - *Pseudomonas campestris* , Fo- *Fusarium oxysporium*
("+++" - good activity; "++" - moderate activity; "+" – weak activity; "-" - no activity)

The potential strain is subjected to species level identification based on the morphological, physiological biochemical characteristics. The potential candidate isolate was identified and named as *Streptomyces* sp.-SBU3 is depicted in Table 2. The isolate posses long filamentous structure. Aerial mycelium was white with 21-50 numbers of terminal spores. The *Streptomyces* could grow up to 30^{o}C. LL-diaminopimelic (LL-DAP) was present in the cell wall along with glycine. That indicates the cell wall chemo-type-1, which is the characteristic of *Streptomyces* species. Melanin pigment and reverse side colour was negative for the potential candidate strain.

Table 2 Morphological and biochemical characteristics of potential *Streptomyces* sp.-SBU3 strain

Cultural characteristics	**Test result**
True mycelium	Present
Facultative anaerobe	+
Spores in aerial mycelium	+
Spores in substrate mycelium	-
No. of spores on aerial mycelium	21 – 50
Shape of spores	Globes
Spore motility	motile
Cell wall type	1
DAP isomer	LL-DAP
Spore chain morphology	Recti-flexibles
Spore surface	Smooth
Aerial mycelium colour	white
Reverse side colour	-
Melanin pigment	-
Growth at 30^{0}C	+
pH	7.2 ±0.2

DAP – Diaminopimelic acid; +: denotes Positive; - : denotes Negative

In the present research the potential candidate strain *Streptomyces* sp.SBU3 have been used for the biosynthesis of silver nanoparticles. It was observed that the culture supernatant has a pale colour before the addition of silver nitrate solution. The culture supernatant contact with silver ions the reaction starts with in few minutes and the colour of the reaction mixture became brown after 30 minutes on completion of the reaction indicates the formation of silver nanoparticles. The color change of reaction mixture was caused by the surface plasmon resonance of Ag nanocrystals in the visible spectral region[19].

The antimicrobial activities of silver nanoparticles were investigated against selected bacterial and fungal pathogens. The zone of inhibitions of tested plant pathogens around each cup is represented in Table 3. The utmost antimicrobial activity was noticed against bacterial pathogens such as *Bacillus campestris* (26±2mm) followed by *Xanthomonas* sp (20±2mm), *Agrobacterium sp* (18±2mm), *Pseudomonas campestris* (17±2mm), *Erwinia amylovora* (16±2mm) and least activity against *Fusarium oxysporium*(15±2mm). Among the pathogens tested bacterial plant pathogens are more susceptible than the fungal pathogens. From the results

it is clearly reveald that *Streptomyces* sp used for the synthesis of silver nanoparticles showed wide spectrum of antimicrobial activity against all the chosen plant pathogens.

Table 3 Antimicrobial efficiency of silver nanoperticles (Ag-NPs) against plant pathogens

Test pathogens	Zone of inhibition in mm in diameter		
	Control	Concentration of silver nanoperticles	
	(20µl)	(10µl)	(20µl)
Bacterial pathogens			
Xanthomonas sp	NA	17±2	20±2
Agrobacterium sp	NA	16±2	18±2
Bacillus compestris	NA	22±2	26±2
Erwinia amylovora	NA	14±2	16±2
Pseudomonas compestris	NA	15±2	17±2
Fungal pathogens			
Fusarium oxysporium	NA	12±2	15±2

Each value is the mean ± SD of three individual estimates; NA- No activity

The biosynthesis of silver nanoparticles from *Streptomyces* sp. is highly susceptible to tested plant pathogens. Silver nanoparticles synthesized from different strains of *Streptomyces* sp showed highest zone of inhibition against both gram positive and gram negative bacterial pathogens[20-22]. This report was corresponding with the present study against gram positive and gram negative bacterial plant pathogens. Moreover in this research bacterial pathogens are more susceptible than the fungal plant pathogens by the silver nanoparticles. This result is supported by earlier finding that the silver nanoparticles synthesized using *Fusarium oxysporium* was more susceptible to *Staphylococcus aureus* and *Escherichia coli* than *Candida albicans*[23].

Synthesized silver nanoparticle was characterized and confirmed by performing thin layer chromatography (TLC). The retention factors (R_f) value was calculated based on the mobility and size of the nanoparticles. A single ideal band of silver nanoparticle was observed with R_f value of 0.70.The use of nano-sized silver particles as antimicrobial agents has become more common as technological advances make their production more economical. One of the potential applications in which silver can be utilized is in management of plant diseases. Since silver displays multiple modes of inhibitory action to microorganisms[24], it may be used for controlling various plant pathogens in a relatively safer way compared to synthetic fungicides[25]. In the present study it is proved that the silver nanoparticle synthesized from *Streptomyces* sp.-SBU3 seems to be a potent antimicrobial agent against the selected plant pathogens. It is recommended that silver nanoparticles synthesized using *Streptomyces* sp isolated from terrestrial soil could be effectively used for the management of plant diseases. Further characterization study is under progress by FTIR, TEM and AFM to characterize the size and stability of the nanoparticles.

Conclusion

This research revealed that the importance of silver nanoparticles synthesized using terrestrial soil *Streptomyces* sp.- SBU3 and that may useful to control plant diseases caused by bacterial and fungal pathogens. The biosynthetic silver nanoparticle would have an increased importance in agriculture industry in the current scenario again further research on the above said aspects may be undertaken for commercial applications. Thus, bionanoparticles obtained from *Streptomyces* sp.-SBU3 seems to be a potential source of antimicrobial agent by arresting the growth and metabolic activities of various plant pathogenic microorganisms. This study is suggested that the possible use of silver nanoparicle as an alternative safe, non toxic, cost effective and eco-friendly antimicrobial agent to control the plant pathogens to improve the crop productivity of our demanding requirements.

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