

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.710.144>

Green Synthesis of Zinc Nanoparticles using *Pseudomonas fluorescens* Extract and Their Antibacterial Activity against *Xanthomonas* spp.

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ABSTRACT

In the field of plant pathology, exploitation of metal nanoparticles in bacterial disease management is a novel and needy approach. Green synthesis of nanoparticles is most prioritized because of their safe, eco-friendly nature and cost effectiveness. A total of 16 fluorescent pseudomonad isolates were obtained from different crop rhizosphere in various locations. The isolates were screened for zinc utilization capabilities. Among the 16 isolates, one isolate (FPGrChHi-6) showed maximum zone of zinc utilization (30.00 mm) with bacterial growth (12.00 mm). Molecular characterization of isolate revealed that, isolate FPGrChHi-6 belongs to *Pseudomonas fluorescens*. Further, zinc nanoparticles (ZnNPs) were synthesized by using *P. fluorescens* extract. The green synthesized ZnNPs were characterized using various instruments. The UV-vis spectrophotometer analysis showed the prominent peak at 360 nm, which indicated the synthesis of ZnNPs. The characterization through particle size analyzer and atomic force microscope found that, the mean diameter of ZnNPs was found to be 21.40 nm with spherical to irregular in shape. ZnNPs showed phytotoxicity symptoms on tomato seedlings at 1500 ppm concentration and onwards. ZnNPs showed excellent antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas axonopodis* pv. *punicae*. Among tested concentrations, maximum inhibition zones were noticed against *Xap* (21.76 mm) and *Xoo* (15.67 mm) at 1000 ppm and 1250 ppm concentrations respectively.

Keywords

Zinc utilization, Zinc nanoparticles, *Pseudomonas fluorescens*, Antibacterial activity, *Xanthomonas* spp.

Article Info

Accepted:
10 September 2018
Available Online:
10 October 2018

Introduction

Nanotechnology is emerging as a revolutionary technology in the current era. It deals with the matter considered at nanoscale (1-100 nm) and their implication for the welfare of human beings. Materials reduced to

the nanoscale show some unusual properties which are different from what they exhibit on a macro scale, enabling unique systematic applications. Nanoparticles produced by a biogenic enzymatic process are far superior, in several ways, to those particles produced by chemical methods. Currently, there is a

growing need in using environmentally friendly nanoparticles that do not produce toxic wastes in their synthesis process. It is well known that biological entities like microorganisms and living cells are the best examples of machines that possess operating parts at the nano-scale level and perform a number of bio-chemical reactions ranging from generation of energy to extraction of targeted materials at a very high efficiency (Goodsell, 2004).

Bacterial blight of pomegranate (*Punica granatum* L.) caused by *Xanthomonas axonopodis* pv. *punicae* in recent years has become one of the most serious diseases in India. Under epidemic conditions blight resulted in yield losses up to 80 per cent (Benagi *et al.*, 2012, Sharma *et al.*, 2015). Similarly, bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) is major threat for the rice production in India. The disease in its severe form is known to result in yield loss ranges from 74-81 per cent in susceptible cultivar (Shivalingaiah *et al.*, 2013).

Presently, plant protection sectors ruling by only few antibiotics for management of bacterial plant diseases. Some of the bacterial pathogens are acquiring antibiotic resistance nature in recent days. Novel molecule is much needed to overcome these problems. Hence, zinc nanoparticles introduced in the field of plant pathology. Zinc oxide nanoparticles have been extensively used in agricultural and industrial sectors. ZnO is nontoxic and it is a strong antibacterial agent. ZnO nanoparticles have bactericidal effects on both gram-positive and gram-negative bacteria (Arabi *et al.*, 2012).

Considering the experimental evidence and emergence of nanotechnology in agriculture, an effort was made to expand in the field of plant pathology to manage plant diseases, bacterial blight of pomegranate and rice

through synthesis and exploitation of antibacterial nature of zinc nanoparticles.

Materials and Methods

Collection, isolation and characterization of fluorescent *Pseudomonas* isolates from different crops rhizosphere and locations

Soil samples were collected from the 16 crop rhizosphere of 13 locations (Table 1). The healthy plants in the vicinity of sick plants were collected by uprooting the plants, 80-100 g of rhizosphere soil was collected for isolation of fluorescent pseudomonads.

Isolation of fluorescent *Pseudomonas* was carried out by serial dilution technique. 10 g rhizosphere soil was transferred to 100 ml of sterile water blank then one ml of this suspension was made into serial dilution up to 10^{-5} , 10^{-6} and 0.1ml of this was placed on King's B medium to isolate the colonies. Plates were incubated at 28 °C for 48 hr. The individual colonies which showed bluish green fluorescent emission were selected and sub cultured. All the isolates were maintained on King's B medium.

All the isolates of fluorescent *Pseudomonas* were tested for growth assay at 4 °C and 42 °C temperature to differentiate between *Pseudomonas aeruginosa* and *P. fluorescens* (King and Phillips, 1978). The cultures were streaked on King's B medium and incubated at 4 °C and 42 °C temperature for 48 hr. Further growth of cultures was observed.

Coding of fluorescent pseudomonad isolates

The isolates were coded by using eight letters. First two letters indicated organism's name (Fluorescent Pseudomonad), third and fourth letters indicated the host rhizosphere (Ba-Banana, Ca-Castor, Ch-Chilli), fifth and sixth letters indicated the district from which

organism was isolated (Ch-Chitradurga, Dh-Dharwad) and last two letters indicated the village/location name (Ja- Jayasuvarnapura, Ha-Harihara).

Screening of fluorescent pseudomonad isolates for zinc utilization

Zinc dominated minimal media was prepared (KH₂PO₄ 2.5g/l, NaCl 2.5g/l, Glycerol 10 ml/l, ZnO 2.5 g/l, Agar 20g/L, Distilled water 1 l). The fluorescent pseudomonas cultures of different isolates were spotted on solidified media in Petri plates separately. The plates were incubated at 28 °C for 4 days. Further zone of zinc utilization was measured around the culture spotted area. The superior isolate having maximum zone of utilization was selected for synthesis of zinc nanoparticles.

Molecular characterization of efficient fluorescent pseudomonad isolate

The DNA of efficient fluorescent *Pseudomonas* isolate (FPGGrChHi-6) was extracted by using HiMedia bacterial genomic DNA extraction kit. Amplification of 16S rRNA gene from the genomic DNA of bacteria was done by using universal primer set, fD1 and rP2 primers (Liu *et al.*, 2006).

Synthesis of Pf-ZnNPs

P. fluorescens culture was allowed to grow as culture suspension in nutrient broth to attain the full growth (48 h). After incubation period, the culture was centrifuged (5,000 x g) for 30 min and supernatant was used for the biosynthesis of ZnNPs.

The synthesis protocol was modified from Getie *et al.*, 2017. Briefly, in 80 ml of hot distilled water, 125 mg of ZnO was dissolved and kept on magnetic stirring for 10 min. Pf-extract (10 ml) was added drop wise to ZnO solution under magnetic stirring condition. 1

ml of glycerol was added to the mixture followed by addition of 10 ml of Pf- extract and continued magnetic stirring for 24 h. Further, microwave irradiation treatment was done for 1 min at 800 W followed by ultrasonication for 30 min.

Characterization of Pf-ZnNPs

Synthesized nanoparticles were characterized to know the shape, size and other parameters using UV-Visible Spectrophotometer, Particle Size Analyser (PSA) and Atomic Force Microscope (AFM). For characterization under UV-Visible Spectrophotometer and PSA, 10 mg of Pf-ZnNPs was suspended in 100 ml of sterile distilled water and sonicated for 20 min for uniform distribution of nanoparticles. Then 3 ml of sonicated Pf-ZnNPs were subjected for PSA analysis (Nicomp, USA). Similarly size and shape of the nanoparticles were determined by using Atomic Force Microscope (Nanosurf AG, Swiss, Flex AFM, with 24bit C3000). For AFM characterization sonicated sample was coated on mica sheet and kept for overnight under hot air oven at 45 °C then characterized by AFM in dynamic mode by using NCLR-10 cantilever.

Determination of phytotoxicity range of ZnNPs on tomato seedlings

The characterized ZnNPs were tested to know their phytotoxicity range on tomato seedlings from 1 to 2,000 ppm concentrations under glasshouse conditions with corresponding checks. Tomato seedlings of Arka Vikas variety raised in coco peat tray for twenty days were transferred to small pots filled with sterilized soil and allowed for few days for better establishment. Later phytotoxicity of green synthesized ZnNPs were tested at 1 to 2,000 ppm on 30 days old tomato seedlings of by spraying method under glasshouse condition. Observations were recorded on 1, 3,

5 and 7 days after spraying for expression of various symptoms of phytotoxicity (hyponasty, epinasty, vein clearing, stunting, scorching, necrosis and chlorosis).

Isolation and characterization of *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas axonopodis* pv. *punicae*.

Diseased specimens were collected from rice and pomegranate plants. Infected tissue showed typical characteristic symptoms and isolated by standard procedures by using nutrient agar medium. Pure virulent cultures of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Xanthomonas axonopodis* pv. *punicae* (*Xap*) were isolated from the plants were maintained on nutrient agar medium at 4 °C. *X. o.* pv. *oryzae* and *X. a.* pv. *punicae* were tested on rice (BPT-5204) and pomegranate (Bhagwa) respectively for pathogenicity under glass house conditions.

The DNA of both plant pathogenic bacteria was extracted by using HiMedia bacterial genomic DNA extraction kit. Amplification of 16S rRNA gene from the genomic DNA of bacteria was done by using universal primer set, fD1 and rP2 primers. Amplified products were checked on 1.4 per cent agarose gel.

The amplified products were sent for sequencing to know the nucleotide sequence and further the sequences were analyzed in NCBI BLAST software to confirm the species name of the isolated organism.

Evaluation of Pf-ZnNPs against *Xanthomonas* spp.

Characterized nanoparticles were evaluated under *in vitro* against *Xanthomonas* spp. by standard paper disc method at different concentrations (10-1250 ppm). Loop full of (72 h old) of *X. axonopodis* pv. *punicae* and *Xanthomonas oryzae* pv. *oryzae* were

multiplied on nutrient broth. Pathogenic suspension of 300 µl was spread on nutrient glucose agar, sterilizing filter paper discs (Whatman No. 1) measuring 5 mm diameter were soaked for 10 min in different concentrations of ZnNPs. Dipped discs placed on the surface of media seeded with bacterial cultures separately. The sterilized filter paper discs were dipped in sterilized water/antibiotics/microbial extract/bulk ZnO served as control checks. The inoculated plates were incubated at 26 ± 2 °C. Four replications were maintained for each treatment. Observation was recorded by measuring the diameter of the inhibition zone around the filter paper disc.

Results and Discussion

The development of noble eco-friendly nanoscaled particles is a challenge as these are safe and eco-friendly in their synthetic processes. The microbes produce several hydrolytic enzymes and pigments which play an important role in reduction of metal ions. Zinc oxide exhibits antibacterial activity that increases with decreasing particle size. Nanoparticle synthesis is an important component of rapidly growing research efforts in nanoscale science and engineering (Bansal *et al.*, 2004).

Collection, isolation and characterization of fluorescent *Pseudomonas* isolates

Sixteen fluorescent *Pseudomonas* isolates were collected from the rhizosphere soil of 16 crops (Table 1). All the isolates showed Gram-negative reaction, rod shaped and pigment production was seen in King's B medium under UV light (Fig. 1). In growth assay study, all the isolates were grown at 4 °C but not showed any growth at 42 °C. The results revealed that, all isolates were not belonged to *Pseudomonas aeruginosa* group. The similar results were observed by (Singh *et al.*, 2015).

Screening of fluorescent *Pseudomonas* isolates for zinc utilization on zinc dominated minimal media revealed that, among tested 16 isolates FPGGrChHi-6 isolate was found to be superior in zinc utilization (30.00 mm zone of utilization and 12.00 mm of bacterial growth) followed by FPCoHaRa-3 isolate (20.00 mm zone of utilization and 9.50 mm of bacterial growth) compared to other isolates (Table 2).

Four isolates (FPPiKaKv-11, FPPoChJa-12, FPSuDhAc-15 and FPToBeNi-16) lacked zinc utilization capacity (Fig. 2).

The results are in agreement with Anuradha and Syed, (2016) who identified the variation in zinc utilization. Hence, FPGGrChHi-6 isolate was selected for molecular characterization.

Further identification of species of fluorescent pseudomonad isolate through 16S rRNA sequence analysis revealed that, the isolate FPGGrChHi-6 belonged to *Pseudomonas fluorescens*.

The results were similar with Trivedi *et al.*, (2015). The FPGGrChHi-6 was isolated from groundnut rhizosphere of Hiriya taluka and Chitradurga district. Further, this isolate was selected for synthesis of zinc nanoparticles.

Synthesis and characterization of Pf-ZnNPs

The eco-friendly synthesis of zinc nanoparticles was challenged and needed effort for successful exploitation of nanoparticles in the field of plant pathology. The integration of nanotechnology in management of plant disease management solves the many risks in the area of plant protection. Exploitation of green synthesized nanoparticles is required for combating of bacterial diseases of plants.

Zinc nanoparticles were successfully synthesized using extract of *Pseudomonas*

fluorescens with magnetic stirring and microwave treatments. The white uniform dispersion of reaction mixture (ZnO + Pf-extract) indicated the successful synthesis of zinc nanoparticles. Similar observation was recorded by Getie *et al.*, (2017). The extracellular enzymes, volatiles and oxidative compounds released from microbes acts best reducing agents of metal compounds in to nanoparticles.

The other secondary metabolites like proteins also serve as capping agents for nanoparticles (Kundu *et al.*, 2014). Extracellular secretion of enzymes offers the advantage to obtain pure, monodisperse nanoparticles, Some microorganisms can survive and grow even at higher metal ion concentration due to their resistance to the metal.

The mechanisms involve: efflux systems, alteration of solubility and toxicity via reduction or oxidation, biosorption, bioaccumulation, extra cellular complexation or precipitation of metals results in nanoscale reduction (Husseiny *et al.*, 2007); Harish kumar and Savalgi (2016).

Characterization of synthesized nanoparticles was done using UV- vis spectrophotometer, particle size analyzer and atomic force microscope. The promising peak at 370 nm in UV- vis spectrophotometer analysis initially confirmed synthesis of zinc nanoparticles (Fig. 3). Similar peak was noticed by Malaikozhundan *et al.*, 2017, wherein they reported that, absorption peak at 373 nm was characteristic of ZnONPs. The particle size analyzer results revealed that, zinc nanoparticles has mean diameter of 21.40 nm with uniform size distribution (Fig. 4). The atomic force microscope analysis revealed that, nanoparticles were spherical to irregular in shape (Fig. 5). Similar results were observed by Nagarajan and Kuppasamy (2013); Harish kumar and Savalgi, (2016).

Table.1 Coding of fluorescent pseudomonad isolates collected from different crops rhizosphere

Sl. No	Crop (Rhizosphere)	District	Taluk	Location/Village	Isolate code for
					Fluorescent <i>Pseudomonas</i>
1	Banana	Chitradurga	Hosadurga	Jayasuvarnapura	FPBaChJa-1
2	Chilli	Davanagere	Harihara	Harihara	FPChDaHa-2
3	Cotton	Haveri	Ranebennur	Ranebennur	FPCoHaRa-3
4	Fingermillet	Tumkur	Chikkanayakanahalli	Kenkere	FPFiTuKe-4
5	Ginger	Uttarkannada	Sirsi	Sirsi	FPGiUtSi-5
6	Groundnut	Chitradurga	Hiriyur	Hiriyur	FPGrChHi-6
7	Maize	Dharwad	Dharwad	UAS campus	FPMaDhAc-7
8	Niger	Chitradurga	Molakalmur	Molakalmur	FPNiChMo-8
9	Onion	Kolar	Malur	Malur	FPOnKoMa-9
10	Paddy	Koppal	Ganagavathi	Ganagavathi	FPPaKoGa-10
11	Pigeon pea	Kalaburgi	Kalaburgi	KVK	FPPiKaKv-11
12	Pomegranate	Chitradurga	Hosadurga	Jayasuvarnapura	FPPoChJa-12
13	Sesamum	Raichur	Manvi	Kallur	FPSeRaKa-13
14	Soyabean	Dharwad	Dharwad	UAS campus	FPSoDhAc-14
15	Sunflower	Dharwad	Dharwad	UAS campus	FPSuDhAc-15
16	Tobacco	Belagavi	Nippani	Nippani	FPToBeNi-16

Table.2 Zinc utilization by fluorescent pseudomonad isolates

Isolates	Zone of utilization (mm)	Bacterial growth (mm)
FPBaChJa-1	13.00 (3.74)*	11.00 (3.46)
FPChDaHa-2	16.00 (4.12)	10.50 (3.39)
FPCoHaRa-3	20.00 (4.58)	9.50 (3.24)
FPFiTuKe-4	15.00 (4.00)	9.50 (3.24)
FPGiUtSi-5	11.00 (3.46)	9.50 (3.24)
FPGrChHi-6	30.00 (5.57)	12.00 (3.61)
FPMaDhAc-7	12.00 (3.61)	9.50 (3.24)
FPNiChMo-8	11.00 (3.46)	9.50 (3.24)
FPOnKoMa-9	15.00 (4.00)	10.50 (3.39)
FPPaKoGa-10	15.00 (4.00)	12.50 (3.67)
FPPiKaKv-11	0.00 (1.00)	12.00 (3.61)
FPPoChJa-12	0.00 (1.00)	12.00 (3.61)
FPSeRaKa-13	16.00 (4.12)	11.50 (3.54)
FPSoDhAc-14	18.00 (4.36)	11.00 (3.46)
FPSuDhAc-15	0.00 (1.00)	12.00 (3.61)
FPToBeNi-16	0.00 (1.00)	11.50 (3.54)
FPIOFDh	0.00 (1.00)	12.00 (3.61)
S.Em.±	0.03	0.02
C.D. @ 1%	0.13	0.09

* Transformed value $\sqrt{x+1}$

Table.3 *In vitro* efficacy of *Pseudomonas fluorescens* based Zinc nanoparticles (Pf-ZnNPs) against *Xanthomonas* spp.

Treatments	Concentration (ppm)	Zone of inhibition (mm)	
		<i>Xap</i>	<i>Xoo</i>
Pf- ZnNPs	10	3.00 (2.00)*	0.00 (1.00)
	25	9.00 (3.16)	0.00 (1.00)
	50	11.00 (3.46)	8.33 (3.06)
	100	13.33 (3.79)	10.00 (3.32)
	250	19.33 (4.51)	11.67 (3.56)
	500	20.33 (4.62)	13.67 (3.83)
	1000	21.67 (4.76)	14.67 (3.96)
	1250	21.67 (4.76)	15.67 (4.08)
<i>P. fluorescens</i> extract	2000000	0.00 (1.00)	0.00 (1.00)
Bulk ZnO	1250	0.00 (1.00)	0.00 (1.00)
Streptocyclin	500	25.33 (5.13)	19.67 (4.55)
Water control	-	0.00 (1.00)	0.00 (1.00)
S. Em. ±		0.04	0.03
C.D. @ 1%		0.15	0.12

* Transformed value $\sqrt{x + 1}$

Fig.1 Fluorescence emission by *P. fluorescens* (FPGGrChHi-6) under UV-light (254 nm)



Fig.2 Zinc utilization by fluorescent pseudomonad isolates

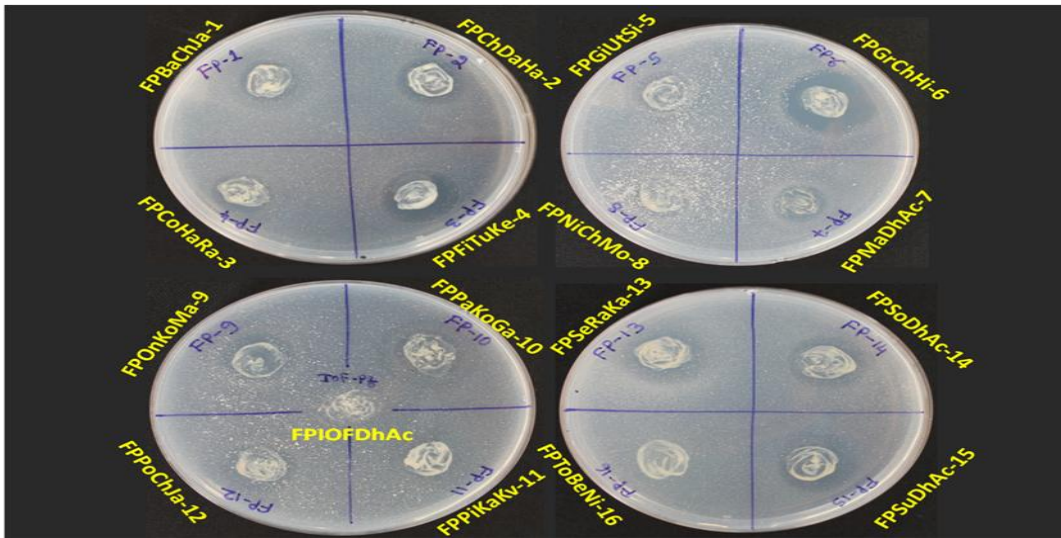


Fig.3 UV-Visible spectral graph of Pf-ZnNPs (Peak at 370 nm)

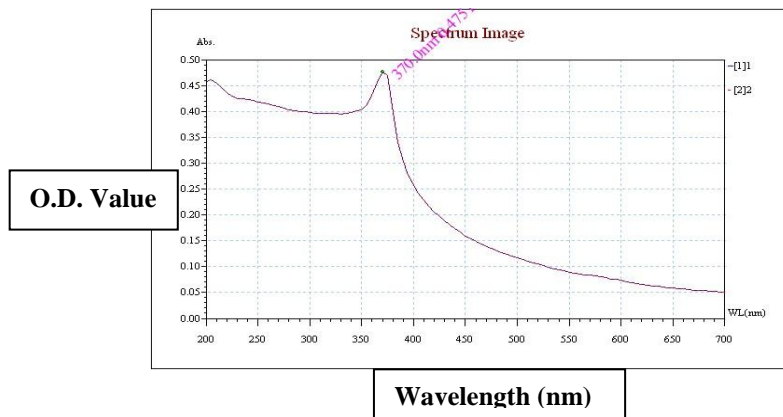


Fig.4 Characterization of Pf-ZnNPs under particle size analyzer (21.40 nm)

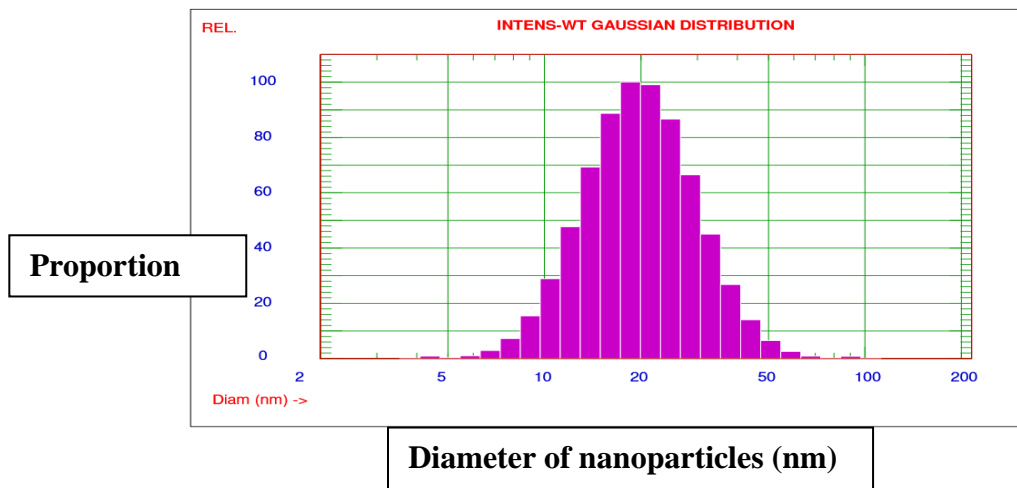


Fig.5 Characterization of Pf-ZnNPs under atomic force microscope

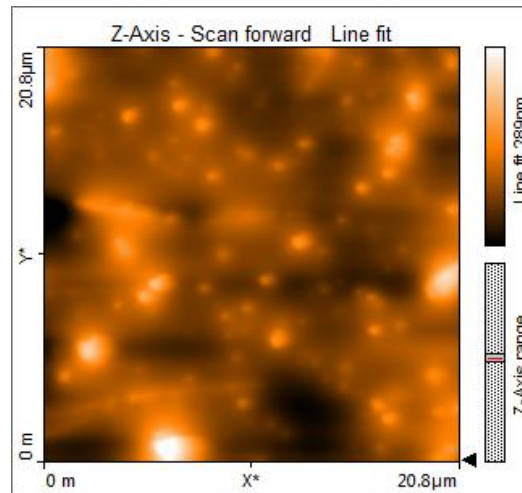
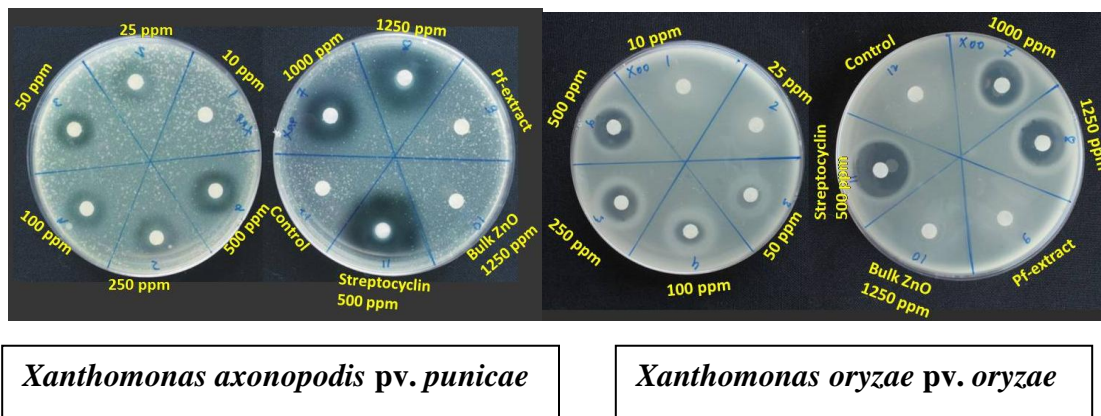


Fig.6 *In vitro* efficacy of Pf-ZnNPs against *Xanthomonas* spp.



The phytotoxicity studies of Pf-ZnNPs on tomato revealed that, the toxicity symptoms were observed on tomato leaves at above 1500 ppm concentration with characteristic necrotic lesion and leaf drooping symptoms on seven days after inoculation.

The concentration from 1 -1250 ppm were safest concentrations for foliar application on plants against diseases. Hence, concentrations upto 1250 ppm were used for evaluation against pathogens.

The present results are in agreement with Prakash and Ching (2016), wherein they reported the phytotoxicity of ZnONPs on

wheat seedlings at higher concentration (>400 mg/L).

Characterization of *Xanthomonas* spp. of rice and pomegranate

Colonies of *X. o. pv. oryzae* formed on nutrient agar were creamy-yellow with copious slime, raised and convex. Colonies of *X. a. pv. punicae* were pale yellow to dark yellow and later turned to metallic yellow colour. These pure cultures of pathogens were used for proving pathogenicity. After glasshouse inoculation, typical symptoms of bacterial blight of pomegranate *i.e.*, water soaked lesions with yellow hollow developed

on the surface leaves. In rice, inoculated plants showed characteristic symptom of bacterial blight *i.e.*, water-soaked streaks that spread from the leaf tips and margins. These characteristic symptoms confirmed the virulent nature of pathogens. The similar observations were made previously by Shankara *et al.*, (2017) and Giri *et al.*, (2011).

The pathogens were further confirmed at molecular level by using 16s rRNA analysis. The amplified products has 1500 bp in length and sequencing followed by BLAST analysis of these amplicons confirmed the pathogens of pomegranate and rice as *X. axonopodis* pv. *punicae* and *Xanthomonas oryzae* pv. *oryzae* respectively. The similar results were observed by Mondal *et al.*, (2012): Shivalingaiah *et al.*, (2012). These pathogenic cultures were further used for *in vitro* experiments.

Evaluation of Pf-ZnNPs against *Xanthomonas* spp.

Zinc nanoparticles are known to possess antibacterial activity. When the particle size of zinc oxide decreased, antibacterial activity increased. Further, characters of nanoparticles also changed (Bansal *et al.*, 2004). Hence, by exploiting this property of ZnNPs, experimentation was done to suppress the growth of the plant pathogenic bacteria. The synthesized ZnNPs were evaluated against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (bacterial blight of rice) and *Xanthomonas axonopodis* pv. *punicae* (*Xap*) (bacterial blight of pomegranate) under *in vitro* condition. Among the tested concentrations of Pf-ZnNPs, maximum growth inhibition of bacterium was noticed against *Xap* (21.76.mm) at 1000 ppm and *Xoo* (15.76 mm) at 1250 ppm (Fig. 6). The bulk ZnO (1250 ppm) and Pf-extract (2000000 ppm) did not possess any antibacterial effect on both bacteria (Table 3).

The antibiotic Streptocyclin showed maximum zone of inhibition (19.67 mm for *Xoo* and 25.33 mm for *Xap*) at 500 ppm concentration (Table 3). The results are in accordance with Poovizhi and Krishnaveni, (2015) where, they also reported the antibacterial activity of zinc oxide nanoparticles against *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus*.

Zinc oxide nanoparticles are antibacterial and inhibit the growth of microorganisms by permeating into the cell membrane. The oxidative stress damages lipids, carbohydrates, proteins, and DNA (Kelly *et al.*, 1998). The antibacterial activity of ZnO nanoparticles is due in part to their electrostatic interaction with cell surfaces which shows that on contact with bacteria, the cytotoxic actions of ZnO nanoparticles ruptures the lipid bilayer of bacterium, resulting in leakage of the cytoplasmic substances (Singh *et al.*, 2012).

The zinc utilization capacity of *Pseudomonas fluorescens* was exploited for successful synthesis of zinc nanoparticles. The ZnNPs were toxic to the tomato seedlings at higher concentration (>1500 ppm). The best concentrations for evaluation against pathogen were determined (1-1250 ppm). Green (microbial) synthesized ZnNPs were successfully inhibited the growth of both bacterial plant pathogens (*Xap* and *Xac*). *In vitro* efficiency of ZnNPs suggests that, integration of ZnNPs in management of bacterial diseases may be considered as best alternative to antibiotics in managing the bacterial plant diseases.

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How to cite this article:

Vinay, J.U., V.B. Nargund, S. Jahagirdhar, R.R. Patil and Hegde, R.V. 2018. Green Synthesis of Zinc Nanoparticles using *Pseudomonas fluorescens* Extract and Their Antibacterial Activity against *Xanthomonas* spp. *Int.J.Curr.Microbiol.App.Sci*. 7(10): 1280-1291.
doi: <https://doi.org/10.20546/ijcmas.2018.710.144>