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Article

Effect of Synthetic Metal Oxide Nanomaterials on *Xanthomonas citri* growth

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ABSTRACT

Xanthomonas citri infecting commercially important crop citrus lemon is a resistant pathogen causing huge crop loss. The study is aimed at identifying synthetic metal oxide nanomaterials as a solution for *X. citri* eradication. Citrus canker causing fungus *X. citri* was isolated from infected citrus lemon fruits. The inhibitory effect of titanium dioxide (TiO_2), copper oxide nanoparticles (CuO), zinc oxide nanoparticles (ZnO), and FeO nanomaterials against the isolated *X. citri* was studied. ZnO inhibited the growth of *X. citri* with a clear zone of growth inhibition in contrast TiO_2, FeO, and CuO did not exhibit growth inhibition against *X. citri*. The inhibitory effect of ZnO was confirmed with optical density measurement of *X. citri* growth curve, ZnO prevented *X. citri* in reaching Log page of its growth curve. The results of this work will be in addition to the present knowledge of nanomaterials applications in agriculture. The study will be beneficial to the farmers who are facing a difficulty in eradication of *X. citri* infecting commercially important crop citrus lemon.

Key words: Citrus canker, Citrus lemon, Synthetic metal oxide Nanomaterials, *Xanthomonas citri*.

1. INTRODUCTION

The bacteria *Xanthomonas citri* is a Gram-negative rod-shaped bacillus having antibiotic resistance and a leading pathogen, with reports saying difficulty in controlling its infection – canker disease in citrus plants. Disease can be identified with lesions of canker on leaves, stem, and fruits. The canker disease leads to huge loss to farmers due to reduced or no production of fruits.

Copper is a micronutrient having significant role in plant health and nutrition. Copper nanoparticles are known antimicrobial agents. Synthetic metal oxide nanoparticles are significant in inhibiting the growth of pathogenic microorganisms [1]. Copper oxides can kill the bacteria by contact called contact killing. As the antibiotic resistance of bacteria is increasing, the copper oxide nanomaterial is gaining much attention. It is proved to kill methicillin-resistant *Staphylococcus aureus* superbug) and pure copper is highest in its antimicrobial activity [2]. Copper oxide is explored for its antibacterial and anticancer properties.

Depending on their concentration, iron (Fe) nanoparticles can have either a positive or a negative impact on the bacterial growth. A research group at National Institute of Technology, Rourkela, prepared iron oxide nanoparticles (IONP) of 10–20 nm diameter with magnetite like atomic arrangement by coprecipitation method and negative surface potential (n-IONP) with positively charged chitosan molecule coating. These particles due to chitosan molecule coting which can reverse the positive surface potential IONP and enhance ROS production were found to be good antimicrobial agents against *Bacillus subtilis* and *Escherichia coli* [3].

Nanometer range zinc oxide (ZnO) exhibits significant antimicrobial activities, nano-sized ZnO can enter inside the cell and interact with bacterial surface and/or with the bacterial core and subsequently inhibits distinct bactericidal mechanisms [4]. ZnO nanoparticle (ZnO-NP) has antibacterial activity against carbapenem-resistant *Acinetobacter baumannii*. The proposed mechanism of the action of ZnO involves the production of reactive oxygen species, which can elevate membrane lipid peroxidation that may result in membrane leakage of reducing sugars, DNA, proteins, and results in cell death [5]. Sanford School of Medicine, USA, reported that the antimicrobial efficiency increases with decrease in particle size of ZnO nanoparticles [6].

Titanium dioxide (TiO_2) is considered as safe antimicrobial compound due to some of its advantages such as photocatalytic property, chemically stable nature, non-toxic, and inexpensive. Several studies indicated that this metal oxide exhibits excellent antibacterial properties against a broad range of both Gram-positive and Gram-negative bacteria. These properties were significantly improved by TiO_2 nanoparticles (TiO_2 NPs) synthesis. Modified TiO_2 with reduced graphene oxide reported to exhibit antimicrobial properties [7].

The present study is aimed in addressing the citrus canker bacterial disease in citrus lemon with metallic oxide nanoparticles as a solution. The analysis will result in identifying the potential metal oxide nanomaterials useful as antimicrobial agents that can be safely used in agriculture for crop protection. The results can be applicable for further study and application for eliminating other pathogens.

2. EXPERIMENTAL

2.1. Plant Material

The infected plant samples were collected from Mittamedapalli and Vellatur villages of Kadapa district. From the infected citrus fruits,

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we have isolated a bacteria *X. citri* causing canker disease in citrus lemon. Isolated organisms were maintained for experiments on nutrient agar (NA) (HiMedia). The metal oxide nanomaterials used in the experiments copper oxide (CuO) nanoparticle, FeO, ZnO, and TiO_2 were analyzed for their antimicrobial properties against these pathogens by agar diffusion. CuO were synthesized using copper nitrate and copper chloride [8]. ZnO nanoparticles were prepared using zinc nitrate hexahydrate and sodium chloride [9]. Iron oxide nanoparticles were prepared using ferrous chloride precipitation method using isobutanol and ammonium hydroxide [10]. The synthesized metal and metal oxide nanomaterials were used for testing the antibacterial activity against *X. citri*.

2.2. Isolation of Plant Pathogens

Infected plant material was collected by identifying the disease symptoms in the fields. Infected citrus fruits twigs and leafs were collected for isolation of *X. citri*. The infected plant parts were washed with 0.1% mercuric chloride followed by two washes with distilled water to remove surface microorganisms. Small pieces of infected plant parts were placed on NA for bacterial isolation. NA medium plates were kept at 39°C in an incubator. The plates were observed for growth and bacterial cultures were subcultured after 24 h. The sub-cultured bacteria were conformed as pure culture by microscopic observation. The isolated pure culture of bacteria was used for further experiments.

2.3. Microscopic Observation of Bacteria

To observe the growth of bacteria under light microscope, Gram staining [11] was performed using crystal violet and safranin as primary and counter stains, respectively, and bacteria were observed under 45× and 100× objective lens.

2.4. Biochemical Tests

Biochemical tests including KOH test, H_2O_2 test, starch hydrolysis test, Simmons citrate test, methyl red and Voges–Proskauer test, gelatin liquefaction test, oxidase test, and nitrate reduction test were performed [12].

2.5. Scanning Electron Microscopic (SEM) Observation of Bacteria

Bacteria colony was taken with the inoculation loop under sterile conditions under inoculation chamber and spread on a glass slide and air-dried before observing under SEM.

2.6. Inhibitory Effect of Synthetic Metal Oxide Nanomaterials

The bacterial growth inhibitory activity of the synthetic metal oxide nanomaterials was studied by agar diffusion method and optical density measurement of *X. citri* growth curve.

2.7. Preparation of Nanomaterials for Testing Antimicrobial Properties

Different concentrations of TiO_2, CuO, ZnO, and FeO test samples were prepared by taking 25 mg, 50 mg, 75 mg, and 100 mg of each nanomaterial in test tubes and lyophilized in 1000 µl of distilled water and maintained separately with proper labeling. Freshly prepared test solutions were used for antibacterial assay.

2.8. Bacterial Growth Inhibition by Agar Diffusion Method

Metals and metal oxides were tested for their antibacterial activity by agar diffusion method. One hundred microliters inoculum of freshly grown bacterial cultures in nutrient broth was incubated for 18–24 h which was spread as lawn culture on 100 mm Petri plates containing NA. Wells of 6 mm diameter were cut into agar media making a well with the help of cup borer. Ten microliters and 20 µl of each nanomaterial TiO_2, CuO, ZnO, and FeO at different concentrations (25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL) were poured in to the wells with the help of micropipette. The plates were sealed and incubated for 24 h at 37°C and were observed for zones of growth inhibition in mm. The experiment was performed in triplicates in aseptic conditions and means of diameter of inhibition zone were calculated.

2.9. Bacterial Growth Inhibition by Optical Density Measurements

Bacterial growth inhibition was measured by taking 100 µl of freshly grown bacterial cultures (incubated for 18–24 h) inoculated in to 10 ml of nutrient broth and mixed with 500 µl of each nanomaterial TiO_2, CuO, ZnO, and FeO at concentration of 100 µg/mL and incubated at 37°C for 24 h and changes in bacterial growth were observed by taking OD values at 600.

3. RESULTS AND DISCUSSION

3.1. Isolation of Plant Pathogens

Citrus canker disease on plant leaves and fruits was observed in field and the infected fruits and leaves were collected for pathogen isolation (Figure 1).

3.2. X. citri pure culture

Isolated bacteria were cultured on NA and yellow-colored colonies with entire margin were selected and subcultured on to fresh plates with NA. The plates were tested for single isolated colonies and pure culture was maintained by picking single colony and inoculation into fresh NA media and NA broth.

3.3. Microscopic Observation of Bacteria

A smear was prepared from subcultured plate on fresh clean glass slide and Gram staining was performed for microscopic observation of *X. citri*. Pink-colored single rod-shaped bacteria were observed (Figure 2). Pink-colored rod-shaped bacteria were observed without any other bacterial growth and confirmed as pure culture of *X. citri*.

3.4. Biochemical Tests for X. citri

Biochemical tests were done for pure culture of *X. citri*. KOH, H_2O_2, starch hydrolysis test, Simmons citrate test, MR-VP test, and gelatin liquefaction tests were positive, oxidase test, nitrate reduction tests were negative (Table 1).

Figure 1: Citrus canker disease on lemon fruit.

Table 1: Biochemical tests for *Xanthomonas citri*

Entry	Biochemical test	Result
1	KOH	+
2	H^2O^2 test	+
3	Starch hydrolysis test	+
4	Simmons citrate test	+
5	MR-VP test	+
6	Gelatin liquefaction test	+
7	Oxidase test	-
8	Nitrate reduction test	-

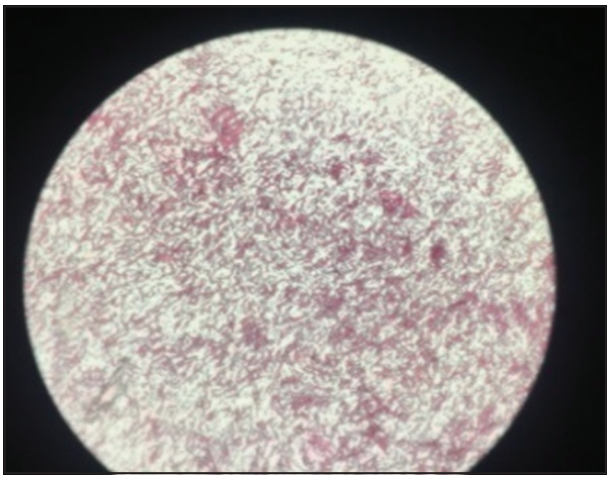

Figure 2: Pink-colored rod-shaped bacteria – *Xanthomonas citri* under (100×).

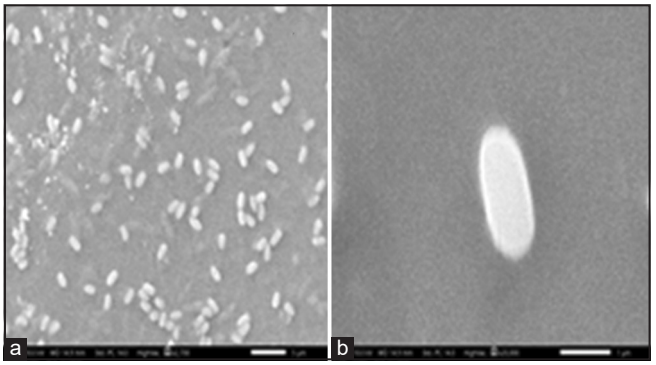

Figure 3: (a) *Xanthomonas citri* under SEM. (b) *Xanthomonas citri* single bacillus.

3.5. SEM observation of bacteria

X. citri colony was taken with inoculation loop in sterile conditions under inoculation chamber and spread on a glass slide and air-dried before observing under scanning electron microscope (SEM). Rod-shaped bacteria with 1 µm diameter and 2 µm length were observed (Figure 3).

3.6. Inhibitory Effect of Synthetic Metal Oxide Nanomaterials

The inhibitory effect of TiO_2, CuO, ZnO, and FeO nanomaterials was tested against the isolated *X. citri*. Ten microliters and 20 µl of each nanomaterial TiO_2, CuO, ZnO, and FeO at different concentrations (25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL) were poured into the wells with the help of micropipette. The plates were sealed and incubated for 24 h at 37°C and were observed for zones of growth inhibition in mm. The experiment was performed in triplicates in aseptic conditions and means of diameter of inhibition zone were calculated (Table 2). Only ZnO exhibited good antibacterial activity against *X. citri* with clear zone of growth inhibition (Figure 4b) compared to control (Figure 4a). Similar results as mentioned, ZnO were good inhibitors of Gram-positive and Gram-negative bacterial growth [13]. In this study, ZnO had good antibacterial property. This results are significant as *Xanthomonas* is a resistant bacteria. Tested nanomaterials TiO_2, CuO, and FeO could not inhibit *Xanthomonas* growth (Figure 4c-e). Our study could explore the usage of ZnO nanoparticles against the growth of *X. citri*.

3.7. Bacterial Growth Inhibition by Optical Density Measurements

Bacterial growth inhibition was measured by taking 100 µl of freshly grown bacterial cultures

in 10 ml nutrient broth and mixed with 500 µl of each nanomaterial CuO and ZnO at concentration of 100 µg/mL and incubated at 37°C for 24 h. One hundred microliters of freshly grown bacterial cultures into 10 ml nutrient broth were maintained as control. OD values were taken at 600 every 2 h for a period of 24 h. ZnO exhibited inhibition of *Xanthomonas* growth compared to control and culture mixed with CuO. Log phase and stationary phase of *Xanthomonas* growth was observe only in control and CuO treated culture. ZnO treated *Xanthomonas* culture did not reach log phase of bacterial growth curve (Figure 5). This results indicate the inhibitory effect of ZnO on *X. citri* growth.

Control (*Xanthomonas*) – log phase (2–10 h) and stationary phase (10–24 h); CuO treated *Xanthomonas* culture – log phase (2–10 h) and stationary phase (10–24 h); ZnO treated *Xanthomonas* culture – no significant growth (2–24 h).

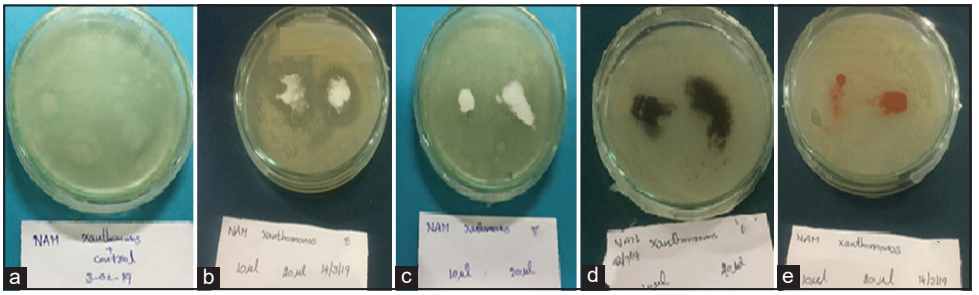

Figure 4: (a) Control: *Xanthomonas* growth on nutrient agar. (b) Effect of ZnO (100 µg/mL) on *Xanthomonas* growth (zone of growth inhibition 2.5 and 3 cm). (c) Effect of titanium dioxide (100 µg/mL) on *Xanthomonas* growth (no zone of growth inhibition). (d) Effect of CuO (100 µg/mL) on *Xanthomonas* growth (no zone of growth inhibition). (e) Effect of FeO (100 µg/mL) on *Xanthomonas* growth (no zone of growth inhibition)

Table 2: Effect of nanomaterials on *Xanthomonas*

Nanomaterials	Conc. µg/mL	Zone of growth inhibition	Conc. µg/mL	Zone of growth inhibition
TiO_2	25	0	50	0
FeO	25	0	50	0
ZnO	25	2±0.57	50	5±1.52
CuO	25	0	50	0
TiO_2	75	0	100	0
FeO	75	0	100	0
ZnO	75	20±2	100	30±3
CuO	75	0	100	0

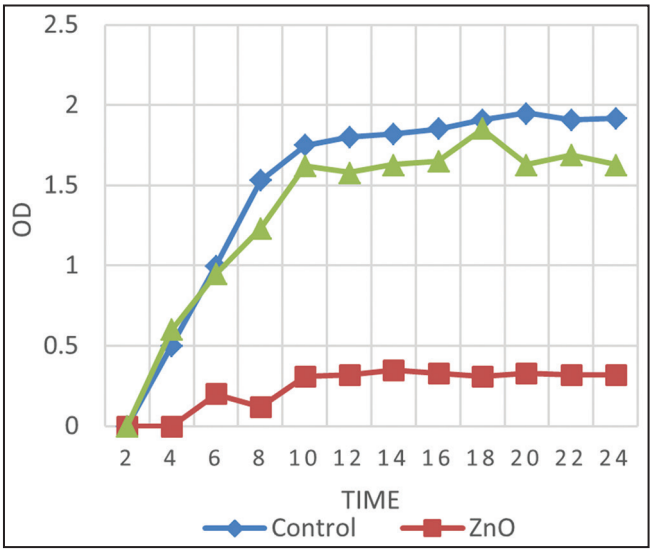

Figure 5: *Xanthomonas* growth curve.

4. CONCLUSION

The results of the project were in addition to the present knowledge of nanomaterials. The inhibitory effect of the ZnO nanomaterials is a direct approach in eradication of resistant plant pathogenic bacteria *Xanthomonas*. The study will be beneficial to the farmers who are facing difficulty in eradication of *Xanthomonas* infecting commercially important crop citrus lemon.

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