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Research Article

Effect of Metal Oxide Nano-particles on *Alternaria polianthi* Infecting *Polyanthes tuberosa*

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Abstract

Agave amica synonym *Polyanthes tuberosa* or Tuberose commonly called as Rajanigandha in India, is one of the most important tropical bulbous commercial ornamental plant. It is cultivated in tropical and subtropical areas, for its intensely fragrant flowers. Many fungal bacterial and viral diseases has been found to infect tuberose during various stages of plant growth. Fungal species *Alternaria polianthi* infects the plant causing leaf spot disease leading to crop loss. Nanoparticles are rising with latest research for its antimicrobial properties. Inhibitory effect of synthetic metal oxide nanoparticles CuO, ZnO and Fe3O4 against the growth *Alternaria polianthi*, isolated from infected leaves of the Tuberose was tested. In the study CuO exhibited good growth inhibition against the tested fungus. Other nano materials ZnO had less inhibitory effect and FeO was unable to inhibit the growth of this fungus. The CuO nanoparticles were found to be effective in killing *Alternaria polianthi* which can be further studied for utilization as fungicide against the growth of *Alternaria polianthi*.

Keywords: Nanoparticles; Tuberose; fungicide; *Alternaria*

Abbreviations

SDA: Sabouraud Dextrose Agar; PDA: Potato Dextrose Agar; MRSA-Super Bug: Methicillin Resistant *Staphylococcus aureus*; CuO-NPs: Copper Oxide Nanoparticles; ZnO-NPs: Zinc Oxide Nanoparticles; FeO-NPs: Iron Oxide Nanoparticles

Introduction

The fungus *Alternaria polianthi* causing leaf spot disease is a major pathogen of intensely fragrant flowering perennial plant *Agave amica* formerly *Polyanthus tuberosa*, a tropical ornamental bulbous flowering plant commonly called Tuberose. It's a small herb with tuber like corn. Stems are linear narrow with inflorescence spike. The flowers are elongated waxy funnel shaped. Flowers are also used for extraction of natural flower oil [1]. In humid or high rainfall conditions, the fungal infection starts at the leaf tip as a brown spot which increases in size and spreads. The number of brown round or oval spots increase with time. The disease progresses to blight like symptoms [2]. The fungi affect the growth of plant and flower production.

Synthetic metal oxide nanoparticles can be synthesized in laboratory by chemical process. The resulting synthetic metal oxide nanoparticles reported for having good antimicrobial properties depending on the size, shape and surface structure. Copper is a well-known antimicrobial agent with significant microbial inhibitory ability. It is also essential for plant health and nutrition. The antimicrobial activity of copper oxide nanoparticles got global attention due to its wide application in inhibiting the growth of pathogenic microorganisms. It is considered as ancient metal and natural ingredient for killing bacteria by contact killing. Copper has the ability to kill 99.9% of bacteria in two hours in contact with its surface allowing the elimination of pathogenic bacteria [3]. Copper is capable of inhibiting the bacteria having antibiotic resistance like methicillin resistant *Staphylococcus aureus* (MRSA-super bug) [4]. Copper is a significant micronutrient supporting plant growth and health. Nanoparticles of Copper are known for their antimicrobial property [5]. Copper is continued for discovering its abilities in inhibiting pathogenic bacteria and also in cancer research for cancerous cells growth inhibition [6]. Antimicrobial properties

of copper on *Candida albicans* and yeast *Saccharomyces cerevisiae* by mechanism of contact killing was tested. Rapid and extensive cytoplasmic membrane damage of yeast was reported after exposure to copper surfaces. Synthetic copper nanoparticles of 8 nm size having antifungal activity tested against *Alternaria alternata*, *Aspergillus flavus*, *Fusarium solani* and *Penicillium chrysogenum* with Minimum inhibitory concentration of 40 to 80 mg l^{-1} and identified that particle size is an important factor [7]. Antifungal activity of copper nanoparticles was also reported against *Candida* species.

The antibacterial property of Iron oxide (FeO) nanoparticles changes with concentration. Iron nanoparticles of 10-20 nm with chitosan coated surface were proved to have good antimicrobial properties against *Bacillus subtilis* and *Escherichia coli* [8]. ZnO nanoparticles can interact with bacterial surface and enter in to the cell and inhibit various bacterial mechanisms [9]. ZnO-NP produce Zn^{2+} ions and reactive oxygen species which can damage the cytoplasmic membrane. Bacteria reported having inhibited by the action of ZnO include *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* [10]. The antimicrobial properties of nanoparticles increases with decrease in nanoparticle size. ZnO nanoparticles of 30nm which can damage membrane and lead to leakage of cell contents were able to inhibit carbapenem resistant *Acinetobacter baumannii* [11]. Synthetic Titanium dioxide (TiO_2) nanoparticles have antifungal and antibacterial properties [12]. TiO_2 by production of reactive oxygen species inhibits fungal growth by membrane destruction.

Many commercial crops get infected with fungi and result in crop loss. Less research has been carried out analyzing the antifungal properties of synthetic metal oxide nanoparticles against plant pathogens. Few reports indicate the significance of inhibitory role of nanoparticles against plant pathogens [13]. It is said that Nano technology is now an essential tool to be used for detection and diagnosis of plant disease. Nano particles can be used directly as fungicide or as a carrier for chemical fungicides. It can reduce quantity of chemicals usage and minimize chemical toxicity due to abundance usage of chemical pesticides. Some of the nano materials such as chitosan are ecofriendly being biodegradable and also effective in killing plant pathogenic fungi [14]. The present study is aimed in addressing the *Alternaria* leaf spot disease, *Polianthes* infection in *Polianthes tuberosa* with metallic oxide nanoparticles as a solution. The metal oxide capable of inhibiting the fungal growth will be screened by fungal growth inhibitory activity. The analysis will result in identifying the potential metal oxide nanoparticles useful as antimicrobial agents that can be further studied and utilized in agricultural field for crop protection. The results can be applicable for further study and application of synthetic metal oxide nanoparticles in eliminating *Alternaria polianthi* pathogen.

Materials and Methods

Collection of infected plants

All the infected plant samples were collected from Mettamedapalli and Vellatoor villages of Kadapa district, Andhra Pradesh, India. Infected plant material was collected by identifying the disease symptoms in the fields with the fungal infections. Infected *Polianthes tuberosa* plant was collected and infected plant parts like leaves and flowers were used for isolation of *Alternaria polianthi* causing leaf spot disease.

Isolation of plant pathogen

The fungus after isolation was maintained as pure culture on Sabouraud dextrose agar (SDA) and Potato dextrose agar (PDA) (Himedia). Isolation of plant pathogens: 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 Potato dextrose agar medium (PDA) for fungal isolation. PDA plates were kept at room temperature. For isolation of fungi on 4^{th} day of inoculation fungi growing on the edges of the infected plant parts were taken and sub cultured in to fresh PDA media. Fungi *Alternaria polianthi* causing leaf spot disease was isolated from infected leaves of *Polianthes tuberosa*. The fungus was identified by slide culture method and observing fungal mycelia under microscope [15]. The identified fungus was used for further experiments.

Simple microscopic observation of fungus

A drop of Lacto phenol cotton blue was placed on the slide. Using a sterile inoculating needle, a small portion of fungus grown from the pure culture plate was picked and placed onto a drop of lacto phenol cotton blue on the slide. The preparation was covered with a cover slip and examined under low and high-power objectives. The shape, types of spores, nature of the growth was observed and recorded. The fungus was cultured by pure culture technique [16] and preserved at 4^0C.

Inhibitory effect of Metals and Metal oxide nanoparticles

The inhibitory activity of the metal and metal oxide nanoparticles was studied by agar diffusion method. Preparation of nanoparticles for testing antimicrobial properties: Copper oxide nanoparti-

cles (CuO-NPs) were synthesized as per the procedure of Kankanit Phiwdanga [17] using Copper nitrate and Copper chloride. CuO-NPs were synthesized by precipitation using ($CuCl_2$) and (Cu $(NO_3)_2.3H_2O$) as precursors dissolved in 100 ml deionized water to form 0.1 M solutions. While stirring Na OH solution (0.1 M) was used to adjust pH to 14. Black precipitate was formed was washed with deionized water in absolute ethanol repeatedly till pH reached 7. The precipitates were then kept for drying at 80°C for 16 h in hot air oven. Calcination of the dried precipitate was done at 500°C for 4 h. Zinc oxide nanoparticles (ZnO-NPs) were prepared by using zinc nitrate hexa hydrate and Sodium chloride by wet chemical method [18]. Zinc oxide nanoparticles (ZnO-NPs) were synthesized using zinc nitrate and sodium hydroxide as precursors. A 0.5M aqueous ethanol solution of zinc nitrate (Zn (NO3)2·4H2O) was kept for constant stirring for one hour. A 0.9M aqueous ethanol solution of sodium hydroxide (Na OH) was prepared in the same way with stirring for one hour. After complete dissolution of zinc nitrate, 0.9M Na OH aqueous solution was added slowly for 45 min with constant stirring. After complete addition of sodium hydroxide the beaker was sealed and the reaction was allowed to proceed for 2 hours and allowed to settle for overnight. Solution was centrifuged for 10 min and supernatant was removed. Precipitated ZnO NPs were washed three times with deionized water and ethanol to remove the by-products which were bound with the nanoparticles. The formed ZnO NPs were then dried at 60°C for 1hour. Iron oxide nanoparticles (FeO-NPs) were prepared by using ferrous chloride precipitation method using isobutanol and ammonium hydroxide using hydrothermal method [19]. The iron oxide (Fe_3O_4) nanoparticles were synthesized by using 0.25g of Iron Chloride Tetra hydrate ($FeCl_2.4H_2O$) in 25.5 ml of deionized water. The solution has sonicated for 30 minutes for better dispersion. Then 2.5 ml of ammonia solution was added in a drop wise manner with stirring for 30 minutes. The complete solution was transferred to autoclave and allowed for hydrothermal treatment at 130°C per 3h. It is washed for two to three times with deionized water. It is dried overnight at 373k in a hot air oven.

Antifungal assay

The following samples were prepared for antifungal assay:

- CuO NPs - 50 mg of CuO was taken and lyophilized in 500 micro liters of distilled water.
- ZnO NPs- 50 mg of ZnO was taken and lyophilized in 500 micro liters of distilled water.
- FeO NPs- 50 mg of Fe_3O_4 was taken and lyophilized in 500 micro liters of distilled water.

Metal oxide nanoparticles CuO, FeO and ZnO were analyzed for their antimicrobial properties against these pathogens by agar diffusion method (20). Freshly grown fungal culture in potato dextrose broth incubated for 48 hours in a shaker incubator at 30°C was used for testing antifungal activity. A 100 µl fungal inoculum was spread as lawn culture on 100 mm petri plates containing Potato dextrose agar. Wells of 6 mm diameter was cut into agar media making a well with the help of cup-borer. A 100 µl of each nanomaterial was poured in to the wells with the help of micropipette. A well with distilled water was maintained as control. The plates were sealed and incubated for 24 hours and observed for zones of growth inhibition and measured. The experiment was performed in triplicates in aseptic conditions and means of diameter of growth inhibition zone was calculated.

Results and Discussion

Collection of infected plants

Infected plant material in the fields with the fungal infections was collected by identifying the disease symptoms (Figure 1).

Figure 1: Infected leaves of Polyanthes tuberosa.

Isolation of plant pathogen

For isolation of fungi infected plant leaf was taken and infected leaf part was identified and cut in to small pieces of about 0.5 to 1 cm. Small pieces of infected plant parts were washed in sterile distilled water and subsequent washing in 0.1% mercuric chloride and two washes in distilled water. The series of washes was carried out in lids of petri plates. Surface sterilized infected plant parts were placed on PDA medium and incubated for 3 days. On 3rd day of inoculation fungi growing on the edges of the infected plant parts was taken (Figure 2) and sub cultured on to fresh PDA media. Fungus *Alternaria polianthi* (Figure 3), causing leaf spot disease was identified by colony morphology after sub-culturing. The pure culture was maintained for further studies.

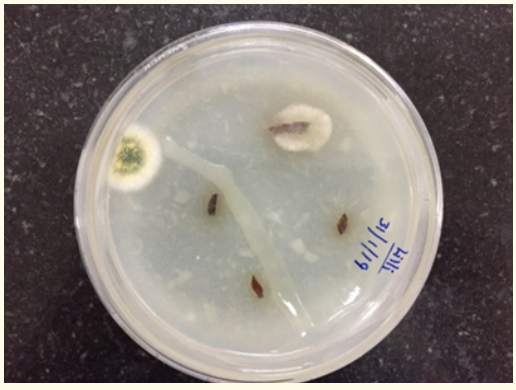
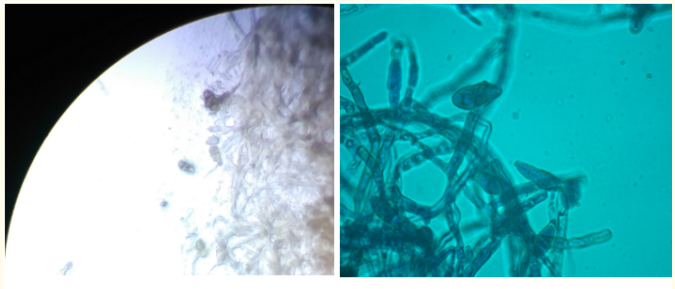


Figure 2: Fungi growing from edges of infected leaf parts.

Figure 3: The sub cultured fungus on PDA plate.

Simple microscopic observation of fungus

Small portion of mycelia from PDA plates after 4 days of growth was taken with sterile needle and placed on a slide mixed with a drop of lacto phenol cotton blue and observed and identified based on fungal mycelia under microscope (Figure 4).



(a) (b)

Figure 4: Fungus observed under microscope: (a) 45X (b) 100X stained with lacto phenol cotton blue.

Inhibitory effect of Metals and Metal oxide nanoparticles

The inhibitory effect of CuO, ZnO and FeO nanomaterials against *Alternaria polianthi* was studied. Among all the tested nanoparticles only CuO exhibited good growth inhibition against *Alternaria polianthi.* Evidenced with a clear zone of growth inhibition with 50 and 100 µl concentration (Table 1).

S. No	Metal oxide Nanoparticle Antimicrobial activity			
	NP (50 µl)	Zone (mm)	NP (100 µl)	Zone (mm)
1	CuO	5.3 ± 0.6	CuO	12 ± 0.39
2	ZnO	1.2 ± 0.4	ZnO	3.5 ± 0.23
4	FeO	0	FeO	0

Table 1: Effect of synthetic metal oxide nanoparticles on *Alternaria polianthi* growth. The zone of growth inhibition is mean of three replicates with standard deviation.

Some studies demonstrated the ability of ZnO and CuO nano particles at nano size have high antimicrobial activities against gram positive bacteria, gram negative bacteria and plant pathogenic fungal strains [21]. In our study CuO was inhibiting the growth of *Alternaria polianthi* and the zone of growth inhibition increased with increase in concentration of nano particle. Some reports demonstrated Copper Oxide Nanoparticles at concentration of 200 ppm and 400 ppm caused alteration in ultrastructure of *Fusarium incarnatum* and the inhibition efficiency against *Fusarium incarnatum* increased as the CuO nano particle concentration increased [22]. Antifungal property of CuO-NPs was effective when compared with ZnO-NPs having little growth inhibition. FeO had no effect on growth of tested fungus. Some studies reported CuO-NPs have the potential in agriculture for application as fungicides, insecticides and fertilizers [23]. They noted in their work that CuO-NPs with particle size of 28 nm prepared using Serratia Sp. ZtB29 strain were having anti-bacterial and antifungal activity against *Xanthomonas sp. and Plant Pathogenic fungus Alternaria sp.* The present study can indicate that CuO-NPs can be utilized after further studies in agriculture for inhibiting the growth of *Alternaria polianthi* infecting *Polianthes tuberosa*.

Conclusion

The results of the project supports the present knowledge about antimicrobial properties of Nanoparticles and the possibility of their application in agriculture. The inhibitory effect of the synthetic CuO nanoparticles is a direct approach in eradication of

resistant bacteria and fungi. Further the study is beneficial to the farmers who are facing a difficulty in eradication of pathogens infecting commercially important crops.

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