Original Research Article

Screening Antimicrobial Potential of Copper Nanoparticles against *Pseudomonas fluorescens* and *Bacillus subtilis* and its Sustainability in Agriculture

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**A B S T R A C T**

Recent times copper nanoparticles (CuN) has gained attention due to its multifaceted action against several microbes. However, its impact on beneficial plant microbes has not much studied. To fill these knowledge gaps, copper nanoparticles were tested for antibacterial ability against well-known two plant biocontrol agents *Pseudomonas fluorescens* and *Bacillus subtilis* by an *in vitro* bioassay using disk diffusion methodology. Antibacterial activity of CuN was compared with botanicals like clove, pepper and standard antibiotics like ampicillin and streptomycin. Our study results revealed that CuN recorded higher effectiveness for both *P. fluorescens* and *B. subtilis* with maximum inhibition zone compared to others tested. Test results illustrate that, bio agents *P. fluorescens* and *B. subtilis* found to be sensitive to CuN. In conclusion, our research could help in determination of microbial strain specificity and to better understand nanoparticles usage for specific purposes. However, further research is needed to better understand CuN role, mechanisms and impact on beneficial plant microorganism in agriculture.

**Keywords**

Copper nanoparticles; antimicrobial ability, *Pseudomonas fluorescens*, *Bacillus subtilis*

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**Introduction**

Current advances and emergence of nanoscience or nanotechnology in last few decades showed rapid expansion, providing new opportunities in various fields include medical (Misra *et al.*, 2010; Mitra *et al.*, 2003; Liang *et al.*, 2008; Wang *et al.*, 2008), food industry (Caiyun *et al.*, 2005; Siegrist *et al.*, 2005), molecular diagnostics (Jain, 2003), paints, plastics and textiles, cosmetics, optical, electronic, mechanical and chemical fields etc. (Azam *et al.*, 2012; Mu and Sprando2010; Silva 2006; Goldhaber-Gordon *et al.*, 1997; Mnyusiwalla *et al.*, 2007). Furthermore, nanotechnology has gained a great deal of attention from the researchers in current times because of its multitasking in various fields.

In recent years increased chemical usage in management of insect pests and plant pathogens has impacted several environmental issues include environmental pollution (Dhaliwal *et al.*, 2010), pest resistance, pest resurgence and residual phytotoxicity problems etc. (Dhaliwal *et al.*, 2010). To reduce the environmental pollution impact caused by chemical pesticides or fungicide...
usage, there is an urgent need to develop an alternate strategy to reduce the concern. One such alternative could be use of metal nanoparticles. Metal nanoparticles has shown potent antibacterial capability against several microbes (Wang et al., 2017).

Metal nanoparticles (MNPs) with antibacterial properties have unique qualities than bulk materials include greater ratio of surface area to volume with larger low-coordinate atoms that are easily available for microbial membranes interactions and further in release of metal ions (Choi et al., 2008). Additionally, the bactericidal property of MNPs could be applied for surface coating, that can have wide application in various fields (Ruparelia et al., 2008).

Furthermore, nanoparticles due its smaller particle size proved to act as good antimicrobial activity (Jones et al., 2008). Moreover, the MNPs antibacterial activities depend mainly on two factors nanoparticle-physico chemical properties and bacteria type (Hajipour et al., 2012). Because of special characters MNPs possess, MNPs could be a potential and sustainable alternative in plant pathogen or plant disease management in agriculture sector. However, thorough research is needed on MNPs usage or application in agriculture field.

Some of the MNPs shown potential antibacterial properties include ZnO (Sinha et al., 2011), Ag (Morones et al., 2005; Ruparelia et al., 2008; Sinha et al., 2011), CuO (Ruparelia, et al., 2008, Azam et al., 2012), Al2O3 (Jiang et al., 2009), TiO2 (Jiang et al., 2009; Tsuang et al., 2008); NiO (Wang et al., 2010), Fe2O3 etc. (Azam et al., 2012). Despite MNPs are well known for its antimicrobial ability, while in agriculture usage or application must be cautious on MNPs impact or side effect on beneficial microorganisms.

Antimicrobial activity of nanoparticles has mostly documented on human pathogenic bacteria such as Escherichia coli (Ruparelia et al., 2008; Yoon et al., 2007) and Streptococcus aureus etc. (Baek and An, 2011). Further antibacterial activity of nanoparticles mostly depends on MNPs size, MNPs stability and MNPs concentration used in the growth medium. Further, bacterial growth was inhibited due to MNPs interactions with bacteria in growing medium (Raghupati et al., 2011). In comparison between bacterial cells and MNPs sizes, bacterial cells (size) are in micrometers while bacterial outer cell membranes pore (size) range are in nanometers. Whereas, nanoparticles size are much smaller than bacterial pore size. Because of MNPs unique ability of smaller size nature, helps MNPs in cell membrane crossing and interactions (Parisi et al., 2015).

Current challenges impacted by climate change, food security motivated researchers in engage and exploration of new areas of science, one such discovery is investigation on nanotechnology as new tool in crop improvements for the agricultural sector. However, despite of numerous reports on nanotechnology advantages in recent times, still there is a lot of uncertainty in research and understanding (Parisi et al., 2015).

Among MNPs, copper nanoparticle is one such metal that has potential antimicrobial activity against several microbial species (Azam et al., 2012). Among various MNPs, copper oxide (CuO) has unique properties such as photoconductive and photothermal applications (Rakhshani et al., 1986), electric, catalytic, optical, nanofluid and photonic. Furthermore, CuN compared to other metal nanoparticles are cheaper, release Cu ions readily, has greater penetrating ability, cause cell wall disruption and nucleic acid damage (Yoon et al., 2007; Raffi et al., 2010; Rispoli
et al., 2010), however the exact mechanism of CuN antimicrobial potentiality is still not clear.

Moreover, CuO nanoparticles, can be prepared from several plant extracts include Aloe vera (Kumar et al., 2015), Tabernaemontana divaricate (Sivaraj et al., 2014), tea leaf and coffee powder extracts (Sutradhar, et al., 2014), gum karay (Padil and Cernik, 2013) and brown algae, Bifurcaria bifurcate (Abboud et al., 2014). Furthermore, Cu and CuO nanoparticles showed potential antimicrobial against several human pathogenic organisms include Bacillus subtilis, E. coli, Vibria cholera, Syphilis typhus, Pseudomonas aeruginosa and Staphylococcus aureus (Akhavan and Ghaderi, 2012; Hassan et al., 2012; Stoimenov et al., 2002), Vibrio cholerae non.0139 and Shigella dysenteriae 1 (Sutradhar, et al., 2014).

We considered Cu nanoparticles for our study because of its unique characteristics and multi potent action against several microbes. Further investigated to better understand the CuN effect on beneficial microbes when applied in agriculture, we framed the experiment to determine Cu nanoparticles effect on two beneficial bioagents include Bacillus subtilis (gram-positive) and Pseudomonas fluorescens (gram-negative) bacterial strains. The objective of this study was to compare the bactericidal effect of copper nanoparticles with botanicals and other commercial antibiotics on beneficial microbial strains and to study the sensitivity of bioagents towards CuN.

**Materials and Methods**

All the experiments were carried out at Department of Plant Pathology, College of Agriculture, Rajendranagar, Hyderabad, India. Two bioagents, Pseudomonas fluorescens and Bacillus subtilis were procured from Directorate of Oil Seeds Research, Rajendranagar, Hyderabad. Nano copper from Osmania University, Hyderabad, India (fig. 2). Botanicals and antibiotics from local market, Hyderabad, India. Efficacy of copper nano, botanicals and antibiotics were evaluated against bacterial bioagents under *in vitro* condition by disk diffusion assay (Ruparelia et al., 2008). Method followed summarized in the flow chart in the fig.1.

**Isolation of bacterial bioagents**

The procured bioagent bacterial cultures P. fluorescens and B. subtilis were cultured on nutrient agar medium (NA). The inoculated plates were incubated at 28 ± 2°C for one week and were isolated and identified and were used for the further studies.

**Disk diffusion assay**

Disk diffusion method was used to evaluate *in vitro* antibacterial potentiality of copper nanoparticles against two bacterial strains.

**Antibacterial activity**

The bacterial suspension (10^4-10^5 CFUml^-1) was applied on nutrient agar medium surface uniformly.

To determine the antibacterial effect standard paper disk of uniform size (6mm diameter) were impregnated in 5 mgml^-1 of copper nanoparticles, botanicals like clove, pepper and antibiotics like ampicillin and streptomycin, each disk containing 100µg.

Sterile water impregnated disks as a control. The nanoparticle amended filter paper was dried for approximately 1 hr. These disks were then placed on to the inoculated nutrient agar medium (4 disks per plate) containing bacteria.
**P. fluorescens**

Tested with copper nanoparticles, botanicals such as clove and pepper impregnated on sterile Whatman No. 1 filter paper disks was used. Not compared with standard antibiotics.

**B. subtilis**

Copper nanoparticles, botanicals such as clove, pepper and antibiotics like ampicillin and streptomycin impregnated on sterile Whatman No. 1 filter paper disks were used.

All the inoculated treatment plates were incubated at 35°C for 24 hrs. Each treatment was replicated thrice. Average inhibition zone diameter (mm) surrounding the discs was measured.

**Statistical Analysis**

The experiment was Completely Randomized (CRD). The data obtained was transformed and was statistically analyzed by SAS-9.4 (SAS Institute, Cary, NC). Significant differences were further analyzed by the mean separation test by Least square means (LSD) (Table1.)

**Results and Discussion**

The antibacterial activity of copper nanoparticles was investigated on two bioagents include *Pseudomonas fluorescens* (gram negative bacterium) and *Bacillus subtilis* (gram-positive bacterium) using the inhibition zonediameter (mm) in disk diffusion test. Generally, the diameter of inhibition zone (DIZ) reveal susceptibility of microorganism to the treatment. Microbes exhibiting larger DIZ reflect susceptibility to the disinfectant or treatment, whereas with smaller DIZ are considered as resistant strains. In our results we noted that disks with copper nanoparticles were surrounded by a larger DIZ (18mm) compared to botanicals such as clove and pepper against in both *P. fluorescens* and *B. subtilis* tested (fig. 2 and 3). Additionally, when tested with antibiotics like ampicillin and streptomycin against *B. subtilis*, copper nanoparticles were surrounded by a larger DIZ compared with others (fig. 4 and 5). The copper nanoparticles impregnated disks showed high effectiveness compared to other impregnated disks on both the strains of bacteria.

When tested on *P. fluorescens*, copper nanoparticles recorded larger DIZ followed by clove and least was by pepper (fig. 3). In *B. subtilis*, copper nanoparticles (CuN) recorded larger DIZ followed by antibiotics ampilicin and streptomycin, botanical clove and least was by pepper (fig. 4 and 5). Results are summarized in the fig. 6.

The test copper nanoparticles were found effective on both *P. fluorescens* and *B. Subtilis* compared to others tested. Thus, our results put forth that CuN have potential inhibitory property towards target bacteria. We also noted that beneficial microbes i.e. the two bioagents tested were sensitive to CuN. Although our results show that CuN has antimicrobial potentiality, it clearly illustrates from our results that in agriculture application or usage, special caution needed, as CuN could be deleterious to beneficial microbes present in soil or beneficial microbes present in plant ecosphere etc. In summary according to our investigation output clarifies that beneficial bioagents tested are sensitive to CuN. Our results of antibacterial property of CuN are in accordance with the results of Ruparelia *et al.*, 2008; Bogdanovic *et al.*, 2014; Cioffi *et al.*, 2005; Yoon *et al.*, 2007. Similarly, CuN showed antibacterial activity (DeAlba-Montero *et al.*, 2017) against several microbes include *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (Bogdanovic *et al.*, 2014).
**Fig.1** Diagrammatic representation of disk diffusion assay followed in the study

- **Bacterial suspension** ($10^4$-$10^6$ CFU/ml) applied uniformly on the surface of nutrient agar plates respectively.
- **Standard paper disk** of uniform size (6mm diameter) were impregnated in 5 mg/ml of the materials, each disk containing 100μg.
- Sterile water impregnated disks as control.
- Disks were then placed on to the inoculated nutrient agar medium (4 per plate).
- Inoculated plates - incubated at 35°C for 24h.
- Average diameter of inhibition zone surrounding discs was measured.
- Results were taken based on 3 replication.

**Fig.2** Copper nanoparticle powder

**Fig.3** Comparison of copper nanoparticles versus botanicals efficacy on bioagent *Pseudomonas fluorescens*
**Fig. 4** Comparison of copper nanoparticles versus standard antibiotics efficacy on bioagent *Bacillus subtilis*

**Fig. 5** Comparison of copper nanoparticles versus botanicals efficacy on bioagent *Bacillus subtilis*
**Fig.6** Graphical representation of comparison between copper nanoparticles versus botanicals versus standard antibiotics effect on bioagents *Bacillus subtilis* and *Pseudomonas fluorescence*

| Treatment | Inhibition zone (mm) | Mean | LSD |
|-----------|----------------------|------|-----|
| CopperNano | a a                  |      |     |
| Ampicillin | b                    |      |     |
| Streptomycin | NT                 |      |     |
| Clove     | e c                  |      |     |
| Pepper    | f d                  |      |     |
| Control   |                      |      |     |

*Mean of three replications, means followed by the same letter in a column are non-significant, at 0.05 level of significance according to LSD. Highest mean is assigned the letter A. NT= not tested, Bs *Bacillus subtilis* and Pf *Pseudomonas fluorescence.*

Likewise, Azam *et al.*, 2012 recorded that ZnO and CuO nanoparticles showed greatest antibacterial ability against gram-negative bacteria and gram-positive compared to Fe$_2$O$_3$ nanoparticles.

Antibacterial property of CuN against *Pseudomonas* spp. was documented by Longano *et al.*, 2012. Similarly, Kumar *et al.*, 2015 recorded that CuN exhibited enhanced antibacterial activity against *P. fluorescens* even at lower concentrations i.e. above 20 μg/ml (Kumar *et al.*, 2015). In our studies we found that *P. fluorescens* was sensitive to CuN. Possibly the reason for *P. fluorescens* sensitivity towards CuN could be attributed by the fact *P. fluorescens* is a gram-negative bacterium consists of special cell membrane structure (Shahmiri *et al.*, 2013).

Sensitive of *B. subtilis* to copper nanoparticles in our results are in agreement with Yoon *et al.*, 2007, who reported that copper nanoparticles showed greater antibacterial than silver nanoparticles on *B. subtilis* and *E. coli*. Probable reason for *B. subtilis* susceptibility to CuN could be attributed to the fact that gram-positive bacterial cell wall consists of thick peptidoglycan layer (Scott and Barnett, 2006) and bacterial cell surface with higher amounts of amines and carboxyl groups (Shahmiri *et al.*, 2013). However, size of CuN are much smaller that cell pore size. Additionally, Azam *et al.*, 2012 recorded in their findings that gram-positive bacteria showed higher sensitivity than gram-negative towards nanoparticles.

The antibacterial property of CuN could be attributed by several factors, firstly due to ready release of copper ions in the medium (Cioffi *et al.*, 2005) or by direct nanoparticles interaction on bacterial cell membrane (Ruparelia *et al.*, 2008). Another possibility could be released ions could have attached to cell wall of that bacteria finally leading to cell
membrane disruption (Chatterjee et al., 2014) or thereby leading to protein denaturation, disruption of biochemical pathway (Kim et al., 2000; Stohs and Bagchi, 1995; Azam et al., 2012). Further released copper ions inside the bacterial cells, get bonded and cross linked to nucleic acid molecule strands internally, resulting in helical structure disorganization (Azam et al., 2012), nucleic acid degradation (Chatterjee et al., 2014), bacterial cell filamentation, reactive oxygen species generation, protein oxidation, lipid peroxidation (Chatterjee et al., 2014) cell killing and ultimately cell death (Yu-sen et al., 2000). The nanoparticles (NPs) can change or manipulate bacteria microenvironment and generate reactive oxygen species and increase NPs solubility, that finally cause bacteria death (Heinlaan et al., 2008).

The toxicity MNPs ions also depends on the properties of heavy metals. Further MNPs toxicity are in relation to the microbial colony size, colony number and the concentration of nanoparticles (NPs) used (Baek and An, 2011). Furthermore, other factors such as nanoparticle diffusion ability may also influence on its effect on target bacteria or microorganism. Nevertheless, further research is needed to confirm the process. However, the mechanism involved for CuN antimicrobial ability has not been fully understood.

Botanicals tested clove, pepper showed an inhibition effect on the both bacteria tested, however we noted differential inhibition rates. Pepper was highly effective on B. subtilis compared to P. fluorescens. While clove was highly effective on P. fluorescens compared to B. subtilis. However, clove and pepper both have bactericidal properties that have already been documented by many researchers (Nascimento et al., 2000; Karsha and Lakshmi, 2010).

Antibiotics, ampicillin and streptomycin showed effective against B. subtilis, and both were on par with each other with inhibition rate and were inferior than CuN in inhibition rate. The antibacterial property of standard antibiotics ampicillin and streptomycin already has been well established by many research scholars (Schatz et al., 1944; Dyas and Wise, 1983).

In conclusion, in our studies we found that copper nanoparticles have a greater antimicrobial affinity against both bacteria P. fluorescens and B. subtilis, in comparison with botanicals like clove, pepper and commercial antibiotics ampicillin and streptomycin. Susceptibility of the bioagents tested P. fluorescens and B. subtilis to copper nanoparticles was recorded. Our study could help to determine microbial strain specificities and provide knowledge on better application of metal nanoparticles for specific purposes. However, further research needed to better understand the relationship between the nanoparticle size and microbe susceptibility index. Before commercialization of CuN, detailed research and comparative study needed to understand CuN impact on beneficial microorganisms when applied in agriculture.

**Future work**

Detailed research should be conducted on metal nanoparticles implications on different beneficial bioagents before utilization in agricultural application. Further metal nanoparticles effect on different bacterial and fungal plant pathogens must be investigated by following effective, inexpensive and eco-friendly technology usage.

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