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Pandurang R Divte
Department of Plant Biotechnology, V.D. College of Agricultural Biotechnology, VNMKV, Latur, Maharashtra, India

Sarika Shende
Department of Plant Biotechnology, V.D. College of Agricultural Biotechnology, VNMKV, Latur, Maharashtra, India

Omkar M Limbalkar
Division of Genetics, ICAR-Indian Agricultural Research Institute, Pusa, New Delhi, India

Rohan A Kale
Department of Plant Biotechnology, V.D. College of Agricultural Biotechnology, VNMKV, Latur, Maharashtra, India

Correspondence
Pandurang R Divte
Department of Plant Biotechnology, V.D. College of Agricultural Biotechnology, VNMKV, Latur, Maharashtra, India

Characterization of biosynthesised copper nanoparticle from *Citrus sinesis* and *in-vitro* evaluation against fungal pathogen *Colletotrichum capsici*

Pandurang R Divte, Sarika Shende, Omkar M Limbalkar and Rohan A Kale

Abstract
Nanotechnology, a new emerging and fascinating field of science which permits advanced research in many areas with novel applications in the field of biotechnology and agriculture. In present studies, copper nanoparticles (CuNPs) biosynthesized from orange *(citrus sinesis)* juice act as a reducing agent, mixed with copper sulphate solution and boiled at 60-80 °C upto deposition of nanoparticles on inner wall of aluminium vessel. Later, the synthesized CuNPs were characterized by using UV-Vis spectrophotometer, Fourier transform infrared spectroscopy (FTIR), Transmission electron microscopy (TEM), Particle size analyzer and Malvern zeta sizer. The size of CuNPs were 1-3 nm with an average size of 2nm analyzed using TEM, Particle size analyzer measured the total concentration of CuNPs were 20.91 particles/frame and 0.81E8 particles/ml. The zeta potential value of biosynthesized CuNPs was -4.55 mV. CuNPs solution of different concentration like 30, 50, 70, 100, 150, 200 ppm used for testing antifungal activity against *Colletotrichum capsici*. We found that, *Colletotrichum capsici* was more susceptible to the action of copper nanoparticle at higher concentration than lower concentration. The maximum activity of CuNPs was found against *Colletotrichum capsici* at concentration 200 ppm (28.00±081mm diameter), while the minimum activity was found at concentration 30 ppm (9.75± 2.06 mm diameter). The results of the disc diffusion method revealed that copper nanoparticles possess potential antifungal activity. In future, biosynthesized CuNPs could be useful for formulations of agriculturally important pesticides for the effective control of chilli anthracnose.

Keywords: Copper nanoparticles (CuNPs), Characterization, *Colletotrichum capsici*, Growth inhibition, antifungal activity

Introduction
Chilli (*Capsicum annuum* L.), is an important cash crop grown in India and is grown for its pungent fruits, which are used as both green and ripe (later in the dry form) to impart pungency to the food (Bosland and Votava, 2003) [3]. India is the world's largest producer, consumer and exporter of chilli and contributes about 25 per cent to total world production. Chilli constituents are important for nutritional value, flavor, aroma, texture and colour. Chillis are low in sodium and cholesterol free, rich in vitamin A and C and are a good source of potassium, folic acid and vitamin E (Marin *et al.*, 2004) [12].

The most destructive disease in chilli crop is anthracnose which is caused by *Collectrochum capsici*. The pathogen is both external and internal seed borne with its total contamination up to 96 per cent has been reported already (Kulkarni, 1990) [8]. Kim *et al.* in 2004 [7] has been reported that different species of *Collectrochum* are damaging to different parts of the chilli plant. The disease has been reported to occur in three phases, which are seedling blight or damping off, leaf spot and die back. The fungus is distributed throughout the tropics and very commonly occurs in chilli growing areas. *C. capsici* appeared to be the most severe, being able to infect a range of *Capsicum* species and its resistant genotypes (Taylor, 2007) [20]. Chilli anthracnose usually develops under high humid condition when rain occurs after fruits starts to ripen and reported losses at this stage can be up to 84 per cent (Thind and Jhooty, 1985) [22]. The total yield losses due to this disease in chilli were estimated to occur in the range of 15-60 per cent (Narasimhudu and Balasubramanian, 2002) [17]. Anthracnose or fruit rot reduces fruits dry weight and quantities of capsacin and oleoresin (Mistry *et al.*, 2008) [14] and affected fruits

are deformed, white in colour and lose their pungency. Ultimately, the diseased fruit shrivels and dries up (Than *et al.,* 2008) [21].

Nanotechnology, a new emerging and fascinating field of science, permits advanced research in many areas, and nanotechnological discoveries could open up novel applications in the field of biotechnology and agriculture (Carmen *et al*., 2003) [5]. Nanomaterials (NMs) are materials with at least one external dimension in the size range from approximately 1-100 nanometers (Nair *et al*., 2010) [16]. The focused integration of bio and nano techniques for biological synthesis of NMs, known as bionanotechnology, has emerged from nanotechnology. Nobel metal colloids have the optical, catalytically electromagnetic properties which are dependent on size and shape of the nanoparticles. In top down synthesis, nanoparticles are produced by size reduction from a suitable starting material (Meyers *et al*., 2006) [13]. In bottom up synthesis, the nanoparticles are built from smaller entities, for example by joining atoms, molecules and smaller particles (Mukherjee *et al*., 2001) [15]. Biological synthesis combines biological principles i.e., reduction/oxidation by microbial enzymes or plant phytochemicals with physical and chemical approaches to produce nano-sized particles (Ball *et al*., 2002) [1]. The plant extract is simply mixed with a solution of the metal salt at room temperature. The reaction is complete within minutes. Nanoparticles of silver, gold and many other metals have been produced this way (Li *et al.*, 2011) [10]. The nature of the plant extract, its concentration, the concentration of the metal salt, the pH, temperature and contact time are known to affect the rate of production of the nanoparticles, their quantity and other characteristics (Dwivedi and Gopal, 2010) [6].

In general, in most of studies commonly used nanoparticles are of gold, silver and copper but out of them, copper nanoparticles are cheaper, easily available than gold and silver. Biosynthesis of cu nanopartilces has many application in agricultural field (Ranjitham *et al*., 2015) [18]. Cao in 2004 [4] identified that copper play a crucial role in inhibiting the microbial growth. Fungicides synthesized from copper generate reactive hydroxyl radicles which damage macromolecules as well as biomolecules and important role in fungal disease resistance particular in crop plants (Borkow and Gabbay, 2005) [2]. Therefore, the copper nanoparticles can be used for suppressing the activity of fungal pathogen on crops. In this endeavor, the present study was undertaken to observe the effect of copper nanoparticle against the *Collectrochum capsici*.

Material and Methods

Microorganisms and cultivation of fungal cultures

Pure culture of microbial samples of *Colletotrichum capsici* was collected from Department of Plant Pathology, College Of Agriculture, Latur and maintained on potato dextrose agar (PDA) media at 26-28ºC to evaluate antifungal potential of bio syntesized copper nanoparticles.

Composition of Potato Dextrose Agar media

Potato dextrose agar (PDA) medium was used to culture *Colletotrichum capsici*. The 200 gm peeled potato slices boiled and filtered using muslin cloth, 20 gm of dextrose and agar powder was added and final volume made up to 1 liter. This was then autoclaved at 121ºC and 15-psi pressure for 15-20 minutes. Fungal cultures were incubated on potato dextrose agar (PDA) medium at 26-28ºC for 7-10 days.

Extraction of Orange juice (*Citrus sinensis*)

The fruits were washed and peeled off. The fruit juice was extracted by squeezing the fruits. The juice was then filtered by muslin cloth and centrifuged at 8000 rpm for 10 min. The clear supernatant of orange fruit juice extract was used as reducing agent for synthesis of copper nanoparticles.

Biosynthesis of copper nanoparticles using orange fruit juice (*Citrus sinensis*)

For the synthesis of copper nanoparticles, 200 ml of orange juice was mixed with 200 ml aqueous solution of 100mM copper sulphate (1:1 ratio of fruit juice and copper solution) and stirred continuously for 2 min at 30ºC. Reaction takes place rapidly which was indicated by the change in colour of the solution from turbid orange to light green. The solution was mixed thoroughly, poured into aluminium vessel for the further reduction reaction and gradually heated to boiling (60-80 °C). The primary detection was carried out by visual observation. The change in colour of the precursor solution from light green to bluish green with time and deposition of reddish shiny brown coloured precipitation on the inner surface of the vessel provides evidence of CuNPs synthesis. The upper brown coloured dry matter was scrapped from the vessel and quantity of nanoparticles dissolved in water and kept overnight at room temperature for better dissolution.

Characterization of copper nanoparticles

Absorption spectra of copper nanoparticle were measured using a Hitachi U-2900 UV-Vis Spectrophotometer at resolution on 1nm from 200 nm to 800 nm wavelength range. The shape, size, stability, elemental composition and capping of copper nanoparticles were determined by Fourier transform infrared spectroscopy (FTIR), Transmission electron microscopy (TEM) and Malvern zeta sizer. UV-Vis spectral analysis was done by using UV-Visible Hitachi U-2900 spectrophotometer. The reduction of pure Cu+ ions was monitored, the UV-V is spectrum of the reaction medium after diluting a small aliquot of the sample into deionized water. 100 µl of sample was pipette into a test tube and diluted with 10 ml of deionized water and subsequently analyzed at room temperature.

In fourier transform infrared spectroscopy (FTIR), to remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, the residual solution of 100 ml after reaction was centrifuged at 5000 rpm for 10 min. The supernatant was again centrifuged at 10000 rpm for 60 min and the pellet was obtained. This is followed by redispersion of the pellet of Cu-NPs into 1 ml of deionized water. Thereafter, the purified suspension was freeze dried to obtain dried powder. The dried powder was analyzed for FTIR (Shimadzu) spectrum. The Transmission electron microscopy (TEM) analysis of the sample was done using PHILIPS- CM 200 instrument operated at an accelerating voltage of 200 kV with resolution of 0.23 nm. A drop of the solution was placed on carbon coated copper grid and later exposed to infrared light (45 minutes) for solvent evaporation. In malvern zeta sizer, the particle size and zeta potential were determined by using the Malvern zeta sizer (Model; ZEN3600) as such without dilution.

Antifungal Activity of Biosynthesized copper Nanoparticle

The antifungal activity of nanoparticles was tested against fungal pathogen *Colletotrichum capsici*. The Potato dextrose agar (PDA) agar plates were prepared for evaluation of antifungal activity of copper nanoparticles. Antifungal activity was evaluated by well diffusion method, by creating

wells with the help of well borer on agar medium. Fungal spore suspension was prepared in distilled water and 100 µl spore suspension was spread on the PDA media. 50µl biosynthesized copper nanoparticle solution with 30, 50, 70, 100, 125, 150, 200 ppm concentration were filled in wells which were punched out in PDA media. Similarly, 50µl $CuSO_4.5H_2O$ solution (100 mM) and orange juice were also added in wells to measure the control activity. The plates were incubated at 26-28ºC for 3-4 days and observed for zone of inhibition.

Evaluation of antifungal potential

Antifungal potential of copper nanoparticles with 30, 50, 70, 100, 125, 150, 200 ppm concentration were evaluated by recording the diameter of zone of inhibition against *Colletotrichum capsici*.

Results and Discussion

Anthracnose disease caused by *Colletotrichum capsici* is one of the major and baffling diseases of the chilli (*Capsicum annuum*) causing considerable quantitative and qualitative losses in yield. The losses estimated to be tune of 15-60 per cent in the country (Narasimhudu and Balasubramanian, 2002) [17]. The disease was also reported to occur and cause yield losses up to 50-55 per cent in the Marathwada region of the Maharashtra state. Present studies on the *C. capsici* of chili was undertaken on the aspects *in vitro* evaluation of effect of Cu nanoparticles on *C. capsici.* The results obtained on all these aspects are presented in the following paragraphs.

Biosynthesis of Cu nanoparticles

Green synthesis of copper nanoparticles was achieved in aqueous solution using orange juice extract as reducing agent. When the fruit juice extract of orange was mixed with copper sulphate solution, the colour of aqueous extract was changed within 5 min, which turns faint orange to light green within an hour (Fig.1.A, B). The properly mixed solution was transferred to aluminium vessel and gradually boiled at temperature 60-80 °C till colour changes to brown (Fig.1C, D). The brown colour formation in aluminium vessel was indication of Cu nanoparticles synthesis and synthesized Cu nanoparticles deposited on inner wall of aluminium pot (Fig.1.D). These biosynthesized Cu nanoparticles were further characterized for analysis of size and shape.

Characterization of biosynthesized of Cu nanoparticles

The synthesized copper nanoparticles were characterized by UV-Vis spectrophotometer, FTIR, TEM, Particle size analyzer and Malvern zeta sizer. The most common technique for characterization of metal nanoparticle is UV-Vis spectroscopy, which is used for analysis of intensely coloured colloidal dispersions having surface plasmon absorption. Krithiga *et al.* (2013) [19] reported that CuNPs presence by visual appearance and UV-visible spectroscopy technique, which is commonly used to characterize metal nanoparticles to give a convenient signature for indication. The plasmon bandwidth increases with decreasing size in the intrinsic region (mean diameter smaller than 25 nm) and also increases with increasing size in the extrinsic size region (mean diameter larger than 25 nm) (Link and Sayed, 1999) [11]. The reduction of copper ion to obtain copper nanoparticles on exposure to orange juice extracts was observed by colour change and detected by UV-Visible spectroscopy. Absorption spectra of copper nanoparticles formed in the reaction has sharp absorbance peak at 550-800 nm by using UV-Vis spectrophotometer which can be attributed to absorption by nano size copper particle confirming the presence of copper nanoparticles. The synthesized nanoparticles were characterized and showed maximum absorbance (1.4**)** at 653 nm by using orange juice extract as reducing agent (Fig. 2.A). Therefore, copper nanoparticles synthesized by using orange juice was used for further characterization and evaluation of antifungal potential.

FTIR analysis of copper nanoparticles was performed and results are shown in (Table1. and Fig.2.B). FTIR analysis of the copper nanoparticles revealed the presence of various compounds. The functional groups were detected on the basis of bond formation. The FTIR spectra showed absorption bands in regions 3416.07- 609.68 cm^{-1}. The analysis confirms different number of functional groups from the spectrum (Table 1). The FTIR analysis of the copper nanoparticles revealed the presence of various compounds. FTIR spectrum of copper nanoparticles suggested that copper nanoparticles were surrounded by different organic molecules such as terpenoids, alcohols, ketones, aldehydes and carboxylic acid (Kulkarni *et al*., 2013) [9]. Vasudev and Pramod in 2013 [24] reported that use of FTIR analysis to identify the capping, reducing and stabilising capacity of leaf extract from copper nanoparticles. The absorbance bands help in identifying resultant groups responsible for formation of nanoparticle carbonyl groups from amino acid residues and peptides of proteins showed strong affinity for binding with metals suggesting that the protein could act as an encapsulating agent and hence, can also protect nanoparticles agglomeration.

The size and shape of green synthesized copper nanoparticles were analyzed using Transmission Electron Microscopy (TEM) and particles were spherical in shape and well dispersed (Fig.3.B). The TEM image of copper nanoparticles are shown in Fig.3.A, B, C. The size of CuNPs was 1-3 nm with an average size of 2nm (Fig.3. A). Selected area electron diffraction (SAED) patterns of the copper nanoparticles synthesized using orange juice extract were investigated using HRTEM. The corresponding SAED of copper nanoparticle patterns sample showed spotty ring patterns without any additional diffraction spots. SAED patterns were recorded and the obtained Debye-Sherrer rings (Fig.3.C)

The biosynthesis of copper nanoparticles structure analyzed to determine the size and concentration of the nanoparticles by employing nanoparticle tracking analyzer measurements. The total concentration of CuNPs was 20.91 particles/frame and 0.81E8 particles/ml (Fig.4.A). Green synthesized copper nanoparticles which denotes strong single in the copper and confirmed the formation of nanoparticles. Hence, it is worth studying the antifungal behavior of synthesized copper nanoparticles of 25 nm average particle size.

The zeta potential is related to the surface charge density and high magnitudes of zeta potential denote stability of the nanoparticles in suspension. The zeta potential value of biosynthesized copper nanoparticles was about 4.55 mV (Fig.4.B).

Antifungal activity and *in vitro* evaluation against fungi *Colletotrichum capsici*

The biosynthesized Cu which deposited on inner wall of aluminium vessel scrapped, made solution of different concentration such as 30, 50, 70, 100, 150, 200 ppm and kept it overnight at room temperature for better dissolution. Later, used for testing antifungal activity against fungi *Colletotrichum capsici*. Viet and co-workers in 2016 reported that 9 day incubation at 450 ppm concentration of CuNPs

showed 93.98% fungal growth inhibition under *in vitro* study. These nanoparticles were assessed for their antifungal activity against *Colletotrichum capsici*. The antifungal activity of the biologically synthesized, Cu nanoparticles at different concentration was done using well diffusion method. The result of the present findings revealed that fungi, *Colletotrichum capsici* is more susceptible to the action of copper nanoparticle at higher concentration (Fig.5.). All the treatment shown growth inhibition of *Colletotrichum capsici* fungi and zone of inhibition was measured (Table 2). The highest zone of inhibition was observed at 200 ppm concentration of Cu nanoparticles which shown 28.00±081 mm diameter of zone inhibition, however, 30 ppm concentration of CuNPs was shown minimum growth inhibition with 9.75± 2.06 mm diameter of zone inhibition. On other hand, we observed that 70 ppm concentration of CuNPs showed higher zone of inhibition than 100 ppm concentration of CuNPs (Table 2, Fig.5). The nanoparticles of Cu showed antifungal activity against tested fungal culture of *Colletotrichum capsici* at different concentrations with effective zone of inhibition, 30 ppm (9.75± 2.06 mm), 50 ppm (12.5± 0.57 mm), 70 ppm (17.00±0.081 mm), 100 ppm (15.75±0.95 mm), 125 ppm (18.75±0.95 mm), 150 ppm (20.75±0.95 mm), 175 ppm (24.05±1.29 mm), 200 ppm (28.00±081 mm) (Table 2.). The results of the disc diffusion method shown that copper nanoparticles possess potential antifungal activity and in comparative study it was found significant as compared to control. Control activity of copper sulphate and orange juice extract was also tested. Orange juice extract showed negligible antifungal activity due to their acidic property where as copper sulphate did not shown any activity as compared to copper nanoparticles (Fig.5, Table 2).

Table 1: FTIR peaks of orange copper nanoparticle and their respective assigned functional groups.

Sr. No.	FTIR Peaks (per centimeter)	Functional Groups
1.	3426.07	Amine or amide N-H
2.	2928.05	Alkyl sp^3 C-H
3.	1665.63	Amide C = O
4.	1410.80	C-C stretching of aromatic ring structure, Scissoring and bending of alkanes.
5.	1063.94	C-O stretching of alcohol, ether, esters, carboxylic acid

Table 2: Effect of copper nanoparticles on growth inhibition of *Colletotrichum capsici*.

Sr. No.	Copper Nanopaticles (ppm)	Diameter of zone of inhibition (mm)
1.	30	9.75± 2.06
2.	50	12.5± 0.57
3.	70	17.00±0.081
4.	100	15.75±0.95
5.	125	18.75±0.95
6.	150	20.75±0.95
7.	200	28.00±081
8.	Orange Juice	4.00±081
9.	$CuSO_4.5H_2O$	-

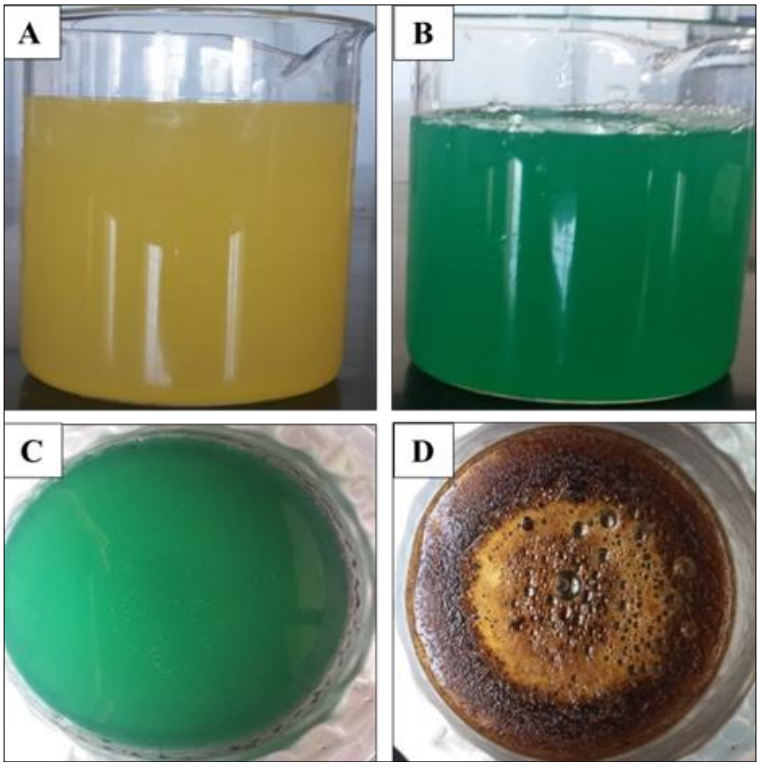

Fig 1: A. Orange juice extract, **B.** Change in colour from faint orange to light green after addition of copper sulfate solution, **C.** Copper sulfate solution with orange juice in aluminium vessel before boiling, **D**. Deposited CuNPs on the inner wall of aluminium vessel after synthesis

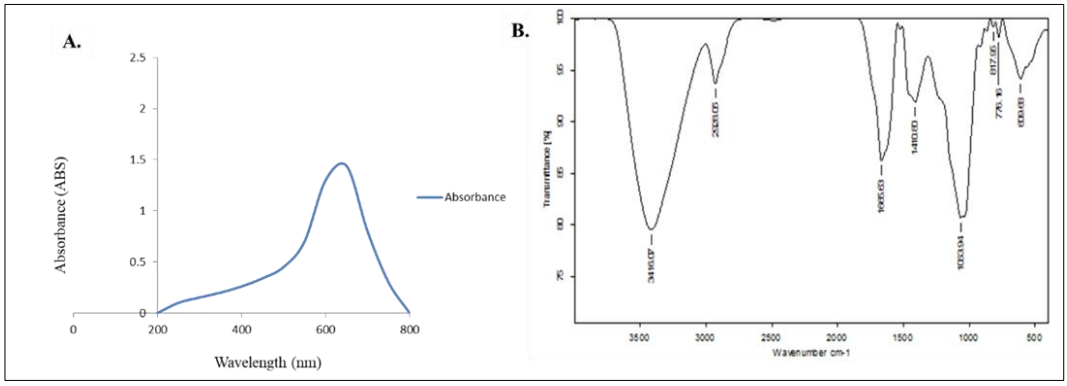

Fig 2: A. UV-visible absorption spectra of biosynthesized copper nanoparticles, B. FTIR spectra of biosynthesized copper nanoparticle by orange juice

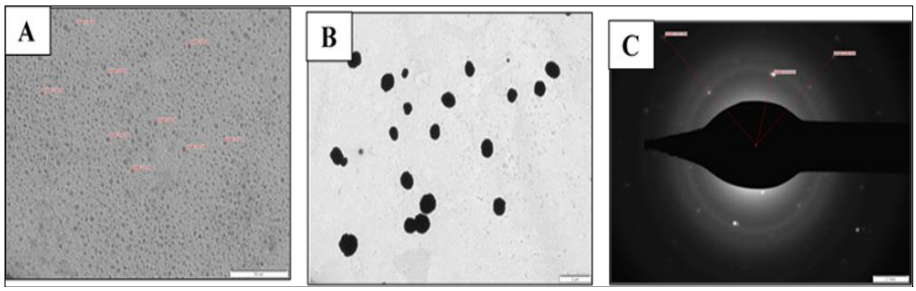

Fig 3: TEM image of Biosynthesized copper nanoparticle. A. Different size of CuNPs, B. Spherical well dispersed CuNPs, C. Selected area electron diffraction (SAED) patterns of the copper nanoparticles.

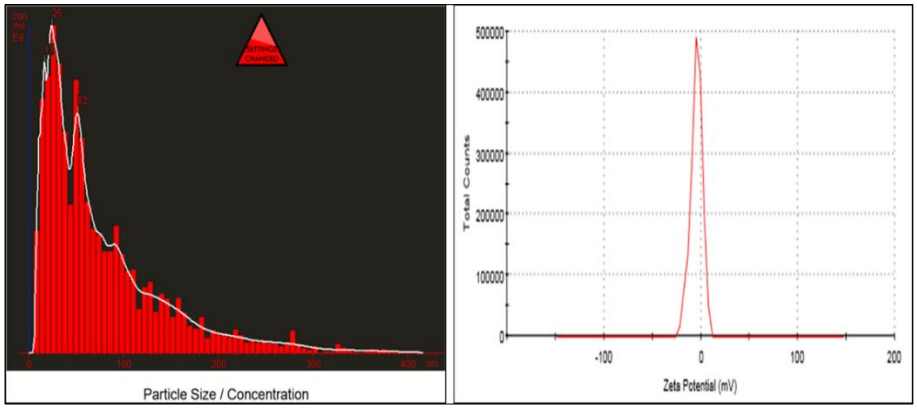

Fig 4: A. Particle size spectra of biosynthesized copper nanoparticle, B. Zeta potential of copper nanoparticle by zeta size analyzer

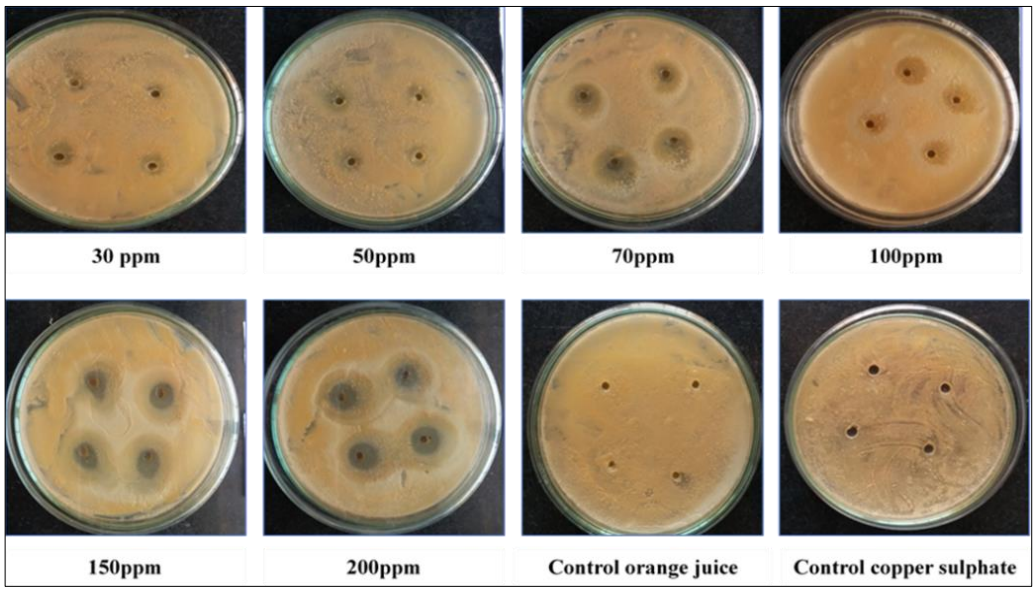

Fig 5: The fungi *Colletotrichum capsici* showed growth inhibition at 50, 70, 100, 150, 200 ppm concentrations and control as orange juice and copper sulphate.

Conclusions

In conclusions, these nanoparticles were synthesized and showed significant antifungal activity against a fungal pathogen *Colletotrichum capsici* at 150 and 200 ppm concentrations. The various effective concentrations of CuNPs showed good significant results against *Colletotrichum capsici.* The biosynthesized cupper nanoparticles in this study will presumably useful in formulation of various biopesticides and ecological feasible effective management strategy against *Colletotrichum capsici*. Further study in this respect may help to synthesize effective formulations than day today used.

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