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Biosynthesis of copper nanoparticles using *Macrophomina phaseolina* and evaluation of its antifungal activity against *Fusarium verticillioides* and *Sclerotium rolfsii*

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Abstract
The conventional methods of disease management have deleteriously affected the non-target organisms and the environment as a whole. To overcome the aforesaid problems, an attempt was made to synthesize copper nanoparticles (CuNPs), presumptively benign to the environment and having enhanced efficacy against pathogens. A fungi *Macrophomina phaseolina* was exploited to synthesize copper nanoparticles (CuNPs). The generation of copper was apparent from a change in color of the reaction mixture. The absorbance maxima of the reaction mixtures by UV-Vis Spectroscopy was observed at 360 nm at four different concentrations. Transmission electron microscope (TEM) revealed spherical to irregular morphology of nanoparticles of size ranging from 7 to 89 nm for *M. phaseolina* mediated synthesis. Fourier transform infrared spectroscopy (FTIR) confirmed the presence of functional groups like amides, amine, and some alcohol groups. The *M. phaseolina mediated* CuNPs (MP-CuNPs) exhibited an inhibitory effect against *F. verticillioides* (causes stalk rot and ear rot in maize) and *Sclerotium rolfsii* (causes southern stem blight in vegetables and ornamental crops) rendering statistical significant inhibition at 20 and 50 ppm respectively.

Keywords: Nanoparticles, Nano fungicides, CuNPs, TEM, FTIR, UV-Vis, absorbance maxima, efficacy, biosynthesis

Introduction
Phytopathogens possess a major constrain in crop production rendering heavy loss every year. Often, epidemics are inevitable, leading to economic and environmental consequences, eventually affecting food security (Savary *et al.*, 2019) [30]. It has been estimated that Phytopathogens are responsible for about 12% of crop yield loss (Reeleder, 2003) [28]. The adoption of conventional methods to manage Phytopathogens has long been speculated for its numerous setbacks such as detrimental effects on non-target organisms and severe environmental consequences. Therefore, an utmost need has been realized for an alternate method to manage Phytopathogens to ensure a constant food supply for the incessantly growing population. Recently nanotechnology has emerged having profound applications in agriculture as well. And one such imperative application is the management of pathogens using nanoparticles as Nano-fungicides. Physical and chemical methods are well standardized and are well capable of producing nanoparticles of desired quantity of defined shape and size in a relatively short time, however, these methods have some major setbacks which cannot be ignored, such as they are complicated, outdated, costly, and inefficient and produce hazardous toxic wastes that are harmful, not only to the environment but also to human health (Li. *et al.*, 2012) [20]. Hence an avenue was needed for eco-friendly and efficient synthesis of nanoparticles. Propitiously "*Biosynthesis method*" satisfies almost all the criteria. Biosynthesis provides an advancement over chemical and physical methods as it is cost-effective and environment friendly and undoubtedly it doesn't require high pressure, energy, temperature, and toxic chemicals (Ramya *et al.*, 2012) [26]. Biosynthesis or green synthesis of Nanoparticles using microorganisms is an important area of research which is an emerging eco-friendly science of well-defined sizes, shapes, and controlled mono-dispersity (Ahmad *et al.*, 2003) [2]. Among micro-organisms, fungi are one of the highly preferred organisms as they have more tolerance and bioaccumulation ability, are efficient secretors of extracellular enzymes which makes it possible to obtain large scale production of enzymes and some other benefits are economic viability and ease of handling of biomass (Thakkar *et al.*, 2010) [34].

Furthermore, it requires simple nutrients and has intracellular metal uptake capabilities which makes it an impeccable candidate for the green synthesis of nanoparticles. Nevertheless, efforts need to be directed, to scale up the nanoparticles quantity through biosynthesis and to achieve more mono-dispersity. In the present investigation, CuNPs were synthesized using a *Macrophomina phaseolina* supernatant. The synthesized CuNPs were characterized by UV-Vis spectroscopy, Transmission electron microscope (TEM), and Fourier Transform Infrared (FTIR). Further, the synthesized CuNPs were evaluated for their efficacy against two important pathogens namely *Fusarium verticillioides* and *Sclerotium rolfsii.*

Materials and Methods

Culturing of fungus on PDA medium

Fungi procured from Maize pathology lab, IARI, New Delhi *viz.*, *Macrophomina phaseolina*, *Fusarium verticillioides*, and *Sclerotium rolfsii* were maintained on Potato Dextrose Agar medium at 27±1°C in BOD incubator.

Preparation of broth culture and extraction of supernatant

PDB and Czapek-Dox broth (CDB) culture of fungi was prepared**.** *M. Phaseolina* broth was incubated at 28 °C with a relative humidity of 80% at 150 rpm for 12-15 days. The broth was filtered through sterilized muslin cloth, which was further subjected to centrifugation at 8000 rpm for 10 min. To make the supernatant free of any spore, cell, or mycelial fragments, the supernatant was filtered again through Whatman paper (Axiva 100 R grade). The supernatant thus obtained was stored and observed for any growth. In case of re-growth, the supernatant was centrifuged and filtered again.

Preparation of copper sulphate ($CuSO_4$) solution

Copper sulphate of analytical grade (Copper sulphate extra pure AR, SRL) ($CuSO_4$) was used. To understand the effect of precursor concentration on the synthesis, four different concentrations of $CuSO_4$ solution *viz.*, 1, 3, 5, and 10 mM were taken into account. Distilled water (type III water) was used for the preparation of the precursor.

Biosynthesis of Copper nanoparticles

For synthesis, a 1:1 volume ratio of supernatant to $CuSO_4$ solution was used for 1 mM and 3mM $CuSO_4$ solution, 2:1 volume ratio for 5 mM of $CuSO_4$ solution, and 3:1 for 10 mM of $CuSO_4$. Different pH ranging from 5 to 10 were tested and based on the best-achieved result, pH 8.5 was understood as standard. The fungal supernatant and $CuSO_4$ solution, having pH adjusted to 8.5, were mixed and the mixtures were incubated at 28-30°C for 5-7 days at 200 rpm maintaining dark conditions. Control was maintained for each concentration devoid of fungal supernatant. Three replications of each sample were maintained.

Characterization of synthesized copper nanoparticles

UV-Vis spectroscopy was conducted. Readings of OD values of the mixtures were taken in a spectrophotometer (EPOCH L2 microplate reader) in the range of 240-800 nm wavelength to assess the reduction of copper ion size right from 0 h to 5 days at 24 h intervals. Three replications were maintained in each concentration. TEM was conducted to study the morphology of CuNPs. The sample was prepared on a 400 mesh carbon-coated copper grid. Prior to placing to the carbon grid, the liquid sample was sonicated for 40 minutes at room temperature then 1 drop of the sample was placed on a carbon-coated grid using a micropipette. The sample was then left for 2-3 seconds followed by staining the grid with 2% uranyl acetate and the sample was allowed to dry for an hour. Finally, the sample was observed under an electron microscope (Jeol 1011 (100 kV) Japan). To determine the functional group associated with the synthesized CuNPs, FTIR was conducted. The sample was prepared by adding 100 mg of spectral-grade KBr which was further pressed under the pressure of 6,000 kg cm^{-2} for almost 2 min which would yield a translucent KBr pellet. The pellet obtained was used for FTIR analysis (Nicolet 6700 FT-IR System, USA). The spectra of the sample were collected at a resolution and wavenumber accuracy of 4 and 0.01 cm^{-1}, respectively, and in total 32 scans were made (Borah *et al.*, 2014).

Efficacy evaluation of synthesized CuNPs

Biologically synthesized CuNPs were evaluated for their efficacy under *in vitro* conditions by following Poisoned Food Technique (Nene and Thapaliyal, 1979) [22] against *F. verticillioides* and *S. rolfsii* causing stalk rot/ear rot in maize and southern stem blight in vegetables and ornamentals crops, respectively. Different CuNPs concentrations ranging from 20 to 1000 ppm were used along with positive and negative control. As a negative control, Carbendazim 50WP @ 1g/lit and Hexaconazole 5 EC @ 1ml/lit was used for *F. verticillioides* and *S. roflsii*, respectively. The experiment was performed maintaining three replications for each treatment.

Statistical analysis

All the lab experiments were conducted by adopting a complete randomized design (CRD). The statistical analysis of the data generated in the experiments was performed by using the online software called OPSTAT (Sheoran *et al.*, 1998). Further, the data were converted by square root transformation and angular transformation using OPSTAT software. The least significance was calculated after analysis of variance (ANOVA) by the Duncan test or LSD at P=0 or a five percent level of significance.

Results

Biosynthesis of CuNPs

In the present investigation, *M. phaseolina* was exploited for the synthesis of copper nanoparticles. A color change in the supernatant was observed on the addition of four different concentrations of $CuSO_4$ solution (1mM, 3mM, 5mM, and 10mM) after 7 days which was apparent when compared with the first day color of the solution (Fig. 1). The light brown color of the reaction mixture turned into darker brown during the course of the reaction of 7 days. The color of the reaction mixtures turned deeper with the increase in concentrations of the precursor $CuSO_4$.

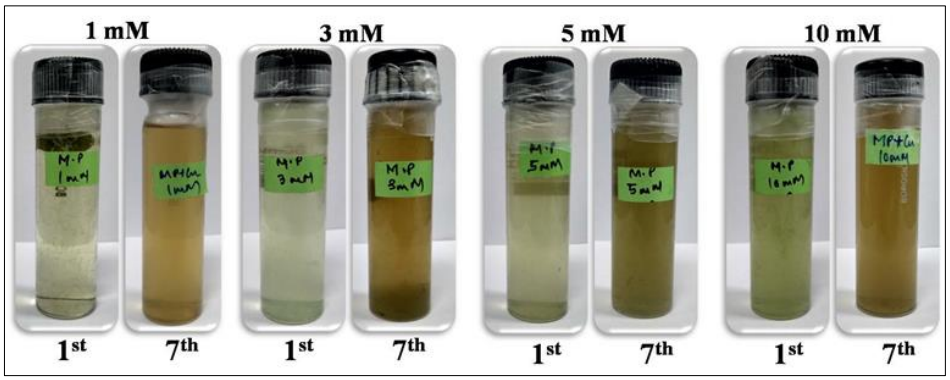
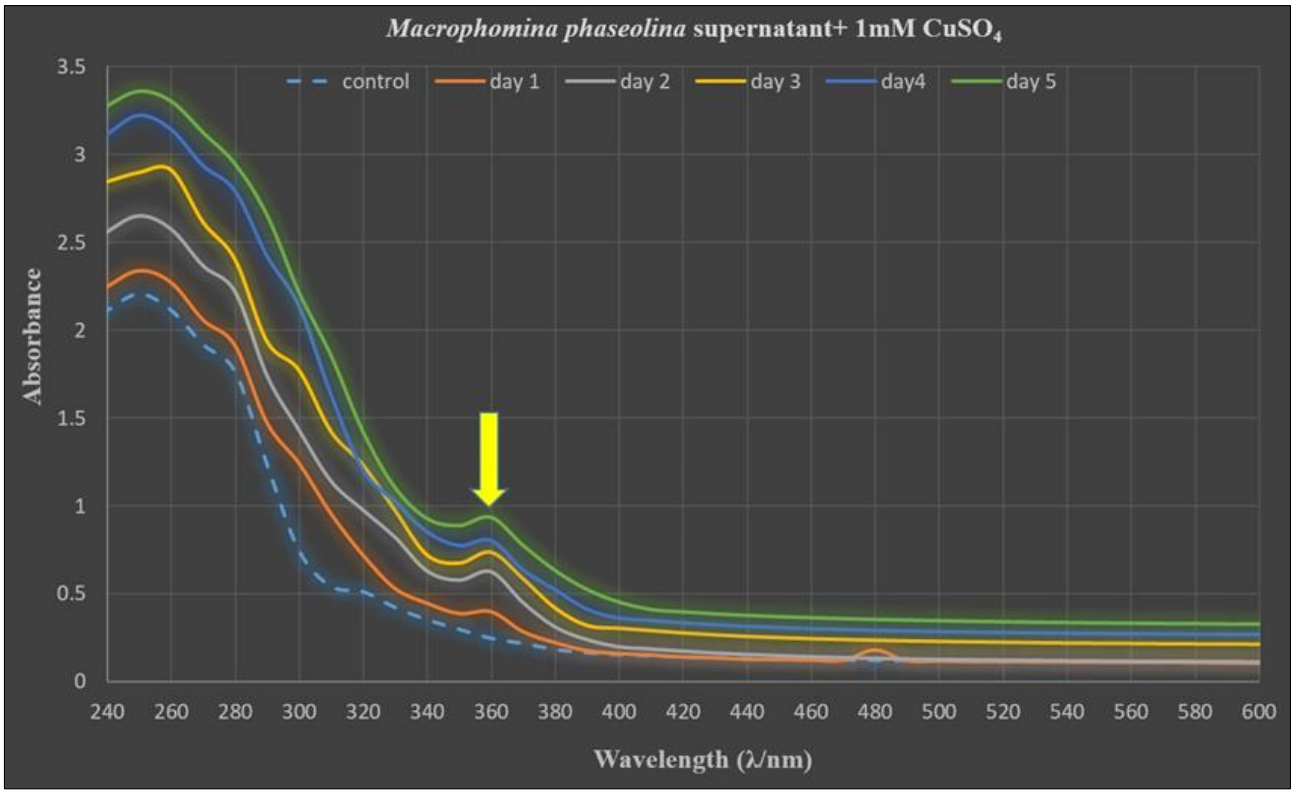
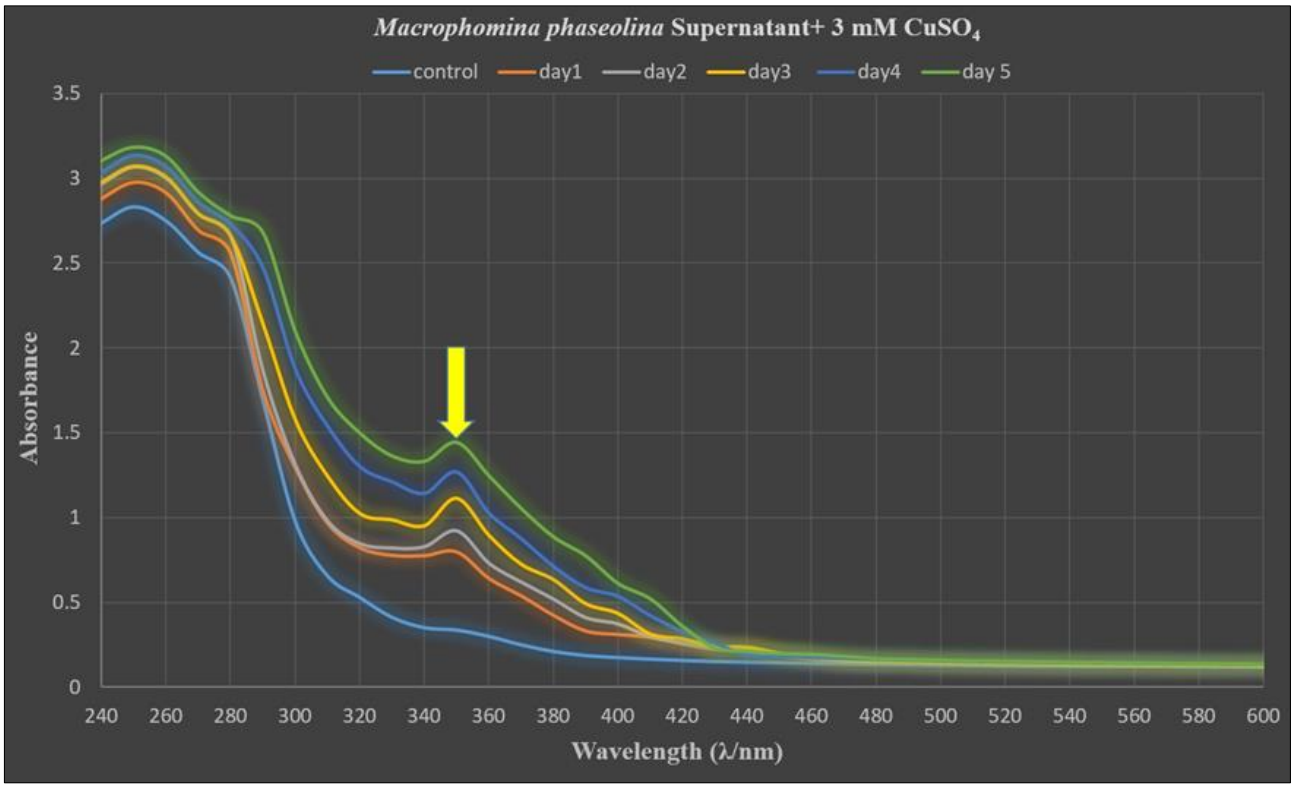


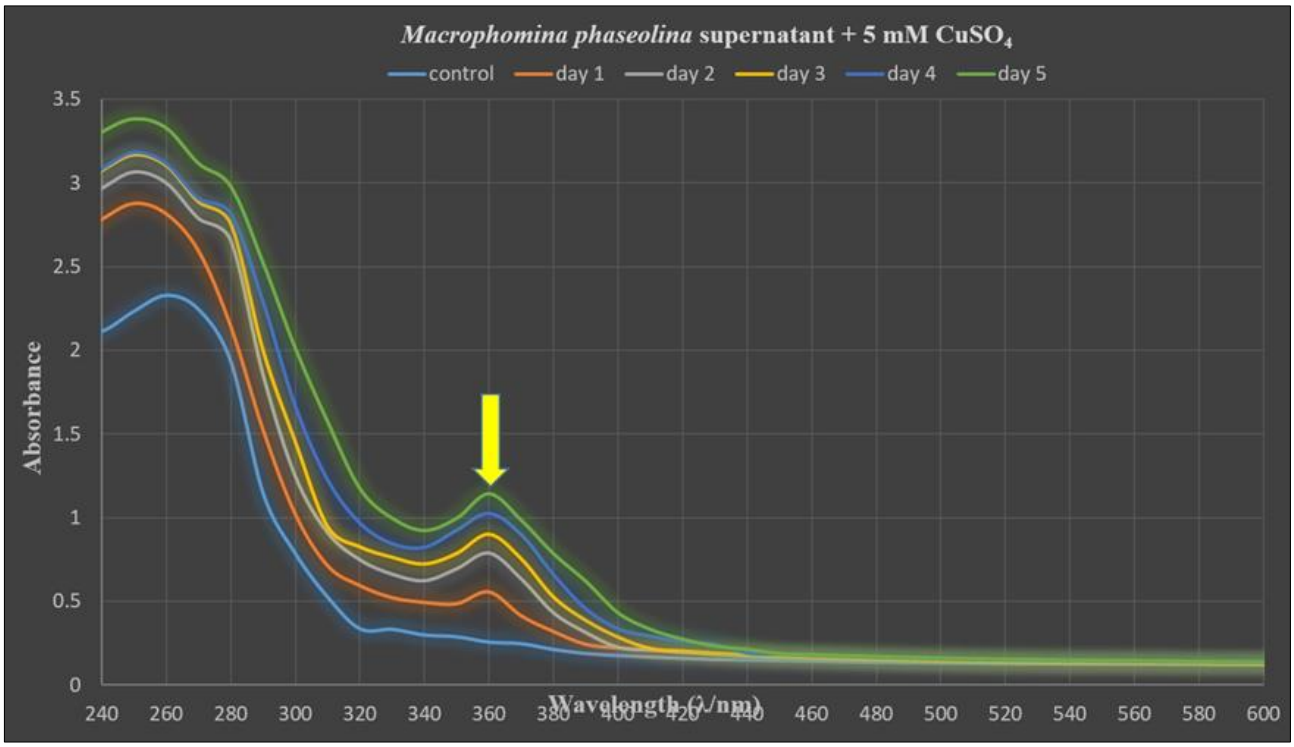
Fig 1: Change in colour of reaction mixture after 7 days incubation of *Macrophomina phaseolina* supernatant+ 1, 3, 5 & 10 mM of $CuSO_4$ solution *1st = First day: 7th = Seventh day*

Characterization of synthesized CuNPs

UV-Vis spectroscopy revealed that when fungi (supernatants) under the investigation were used as a reducing agent in the reaction mixture, it resulted in a progressive increase in the characteristic absorption peak with an increase in the reaction time (Fig. 2). Reading of the reaction mixture for absorbance was taken with 24 hrs intervals for consecutive 5-6 days. *M. phaseolina* supertanant and $CuSO_4$ reaction mixture exhibited a plasmon resonance band at 360 nm at all four concentrations. The spectrum confirms the formation of CuNPs over time. A transmission electron microscope study revealed the morphology of synthesized CuNPs which were apparently monodisperse (Fig. 3). CuNPs were spherical, and ellipsoidal in shape with sizes ranging from 7-19 nm, 10-25 nm, 11-28, and 14-89 nm respectively. With the increase of $CuSO_4$ concentration, aggregation appeared to be more and the size of nanoparticles also increased correspondingly. At a lower concentration of precursor, the dispersity was well pronounced. Fourier Transform infrared spectroscopy determined the functional group associated with the formed CuNPs, which possibly are the reason for stability and mono-dispersity (Fig. 4). The spectrum of FTIR measured for *M. Phaseolina* mediated CuNPs showed bands at 3314, 1642 1398.68, 1110, and 1192.04 cm^{-1}. The bands obtained at 3314 and 1642 cm^{-1} are due to the N-H stretching of secondary amines and C-NH_2 bending vibrations of primary amines, respectively. The peak at 1728 corresponds to the C=O stretching of aldehyde. The bands observed at 1398.68, 1110, and 1192.04 cm^{-1} correspond to the OH, bending of a primary alcohol, C=O stretching of a secondary alcohol, and C=O stretching of tertiary alcohol, respectively.




Macrophomina phaseolina Supernatant+ 3 mM $CuSO_4$
control
day1
day2
day3
day4
day 5
Absorbance
Wavelength (λ/nm)


Macrophomina phaseolina supernatant + 5 mM $CuSO_4$
control
day 1
day 2
day 3
day 4
day 5
Absorbance
Wavelength (λ/nm)

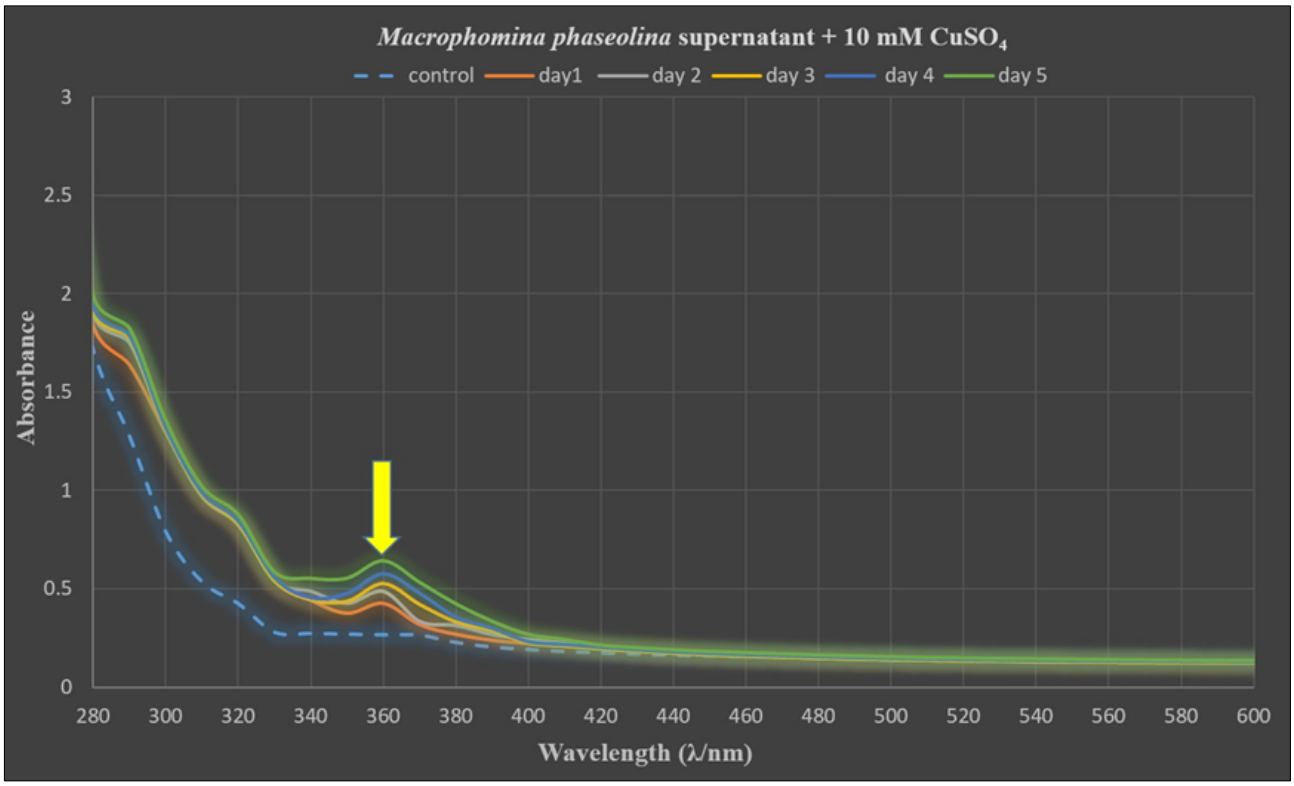


Fig 2: UV-Vis spectrum as a function of time of reaction in a solution containing *Macrophomina Phaseolina* supernatant and $CuSO_4$

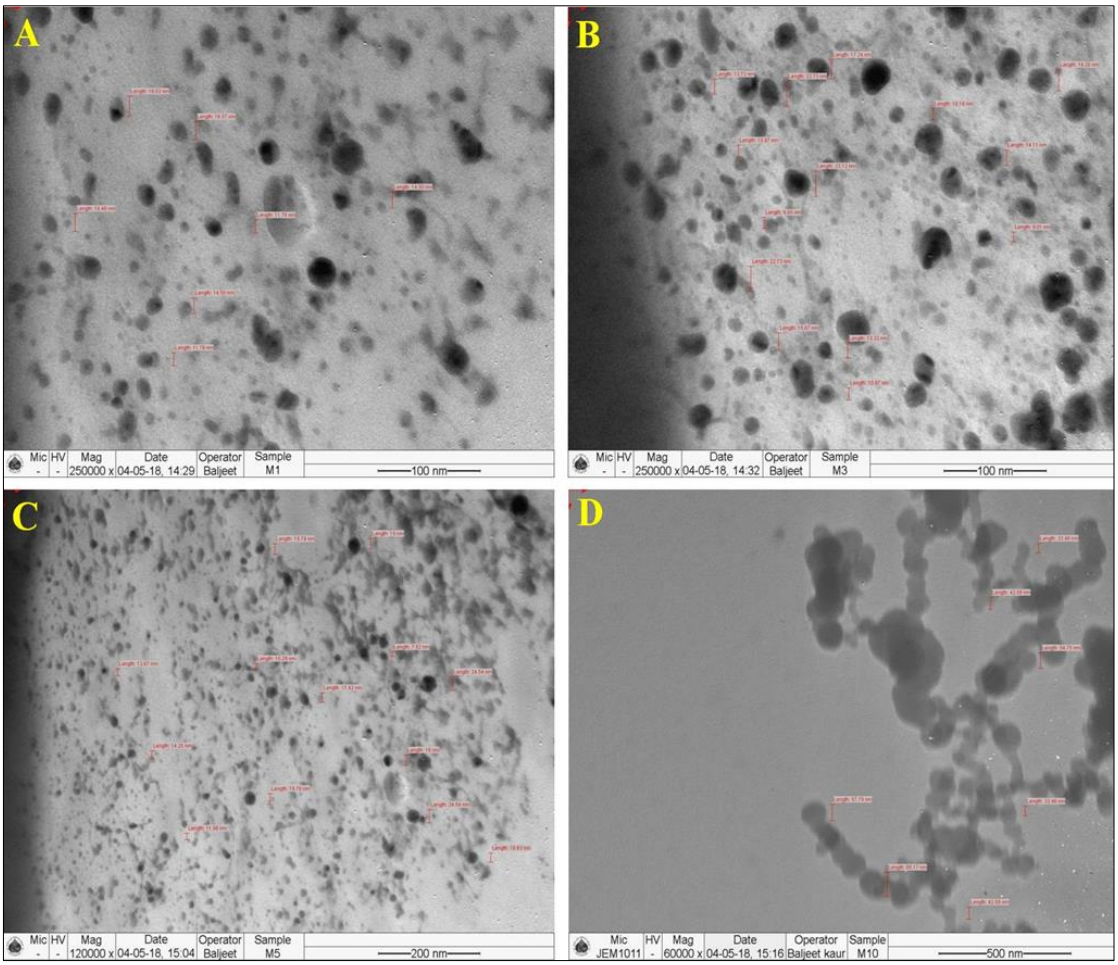


Fig 3: TEM images of CuNPs synthesized using the supernatant of *Macrophomina phaseolina* and $CuSO_4$: (A) 1mM, (B) 3mM, (C) 5 mM (D) 10 mM

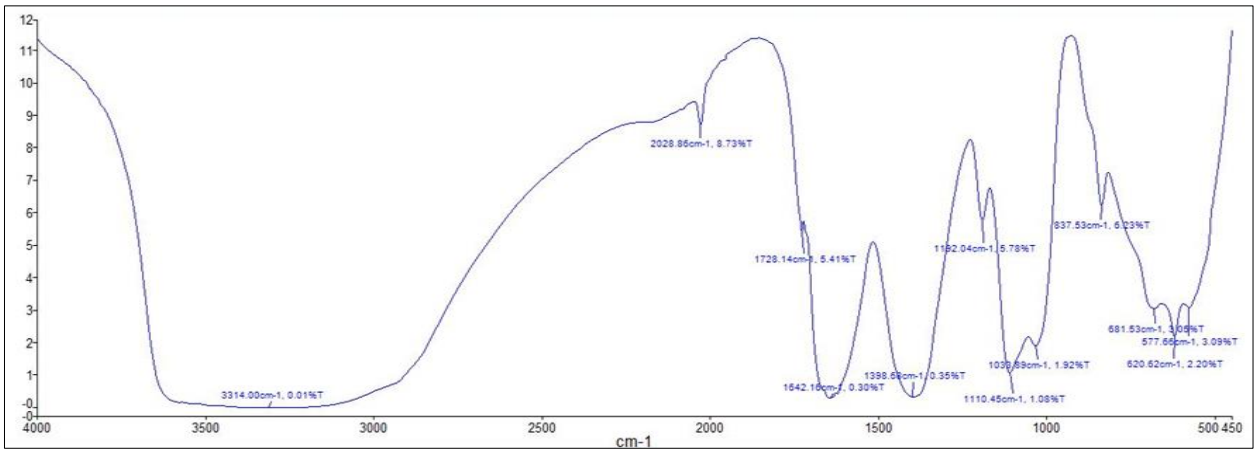
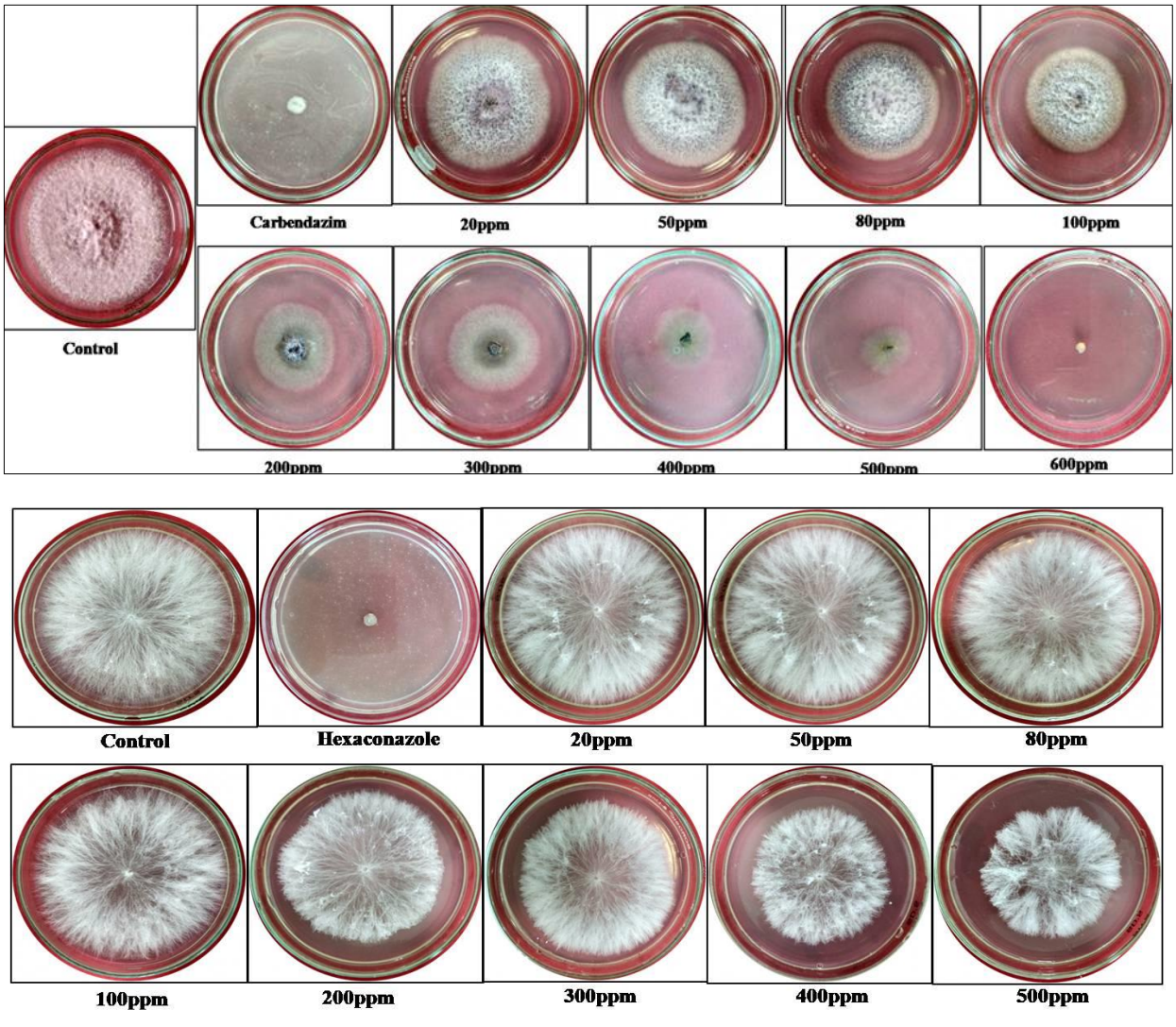


Fig 4: FTIR spectrum recorded from reaction mixtures of *Macrophomina phaseolina* supernatant and CuNPs

Evaluation of fungicidal efficacy of synthesized CuNPs

The CuNPs synthesized by *M. phaseolina* (MP-CuNPs) were evaluated at 20 to 600 ppm concentrations for the fungicidal property against *F. verticillioides* and at 20 to 1000 ppm against *S. rolfsii* (Table. 1). MP-CuNPs provided statistically significant inhibition in radial growth of *F. verticillioides* from 20 ppm (20.95%) onwards and complete inhibition (100%) was perceived at 600 ppm which was at par with the standard fungicide carbendazim (1000 ppm). A comparison of the efficacy of all tested concentrations of MP-CuNPs (20 to 600 ppm) exhibited a significant difference in percent growth inhibition among them. In the case of *S. rolfsii*, significant growth reduction was observed from 50 ppm of MP-CuNPs (3.33%) onwards as compared with the absolute control (Fig. 5). However, the highest concentration i.e. 1000 ppm of MP-CuNPs showed significantly 67.14% inhibition against *S. rolfsii*.



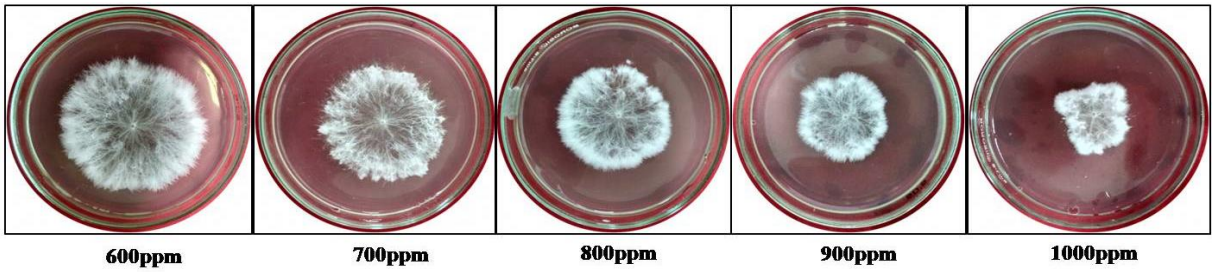


Fig 5: Radial growth inhibition of *Fusarium verticillioides* (i) and *Sclerotium rolfsii* (ii) *in vitro* exposed to *Macrophomina phaseolina* mediated copper nanoparticles (MP-CuNPs)

Table 1: Effect of biologically synthesized CuNPs on the growth of pathogenic fungi

***Fusarium verticillioides* (MP- CuNPs)**			***Sclerotium roflsii* (MP- CuNPs)**		
Treatment	**Radial growth (cm)****	**Inhibition (%)**	**Treatment**	**Radial growth (cm)****	**Inhibition (%)**
Control	7.00	0.00(0.00)#	Control	7.00	0.00(0.00)#
Carbendazim @1000 ppm	0.00	100.00 (90.00)	Hexaconazole @1000 ppm	0.00	100.00 (90.00)
*CN20 ppm	5.53	20.95 (27.22)	$Copper oxy. @1000 ppm	7.00	0.00 (0.00)
CN 50 ppm	5.10	27.14 (31.38)	*CN 20 ppm	7.00	0.00 (0.00)
CN 80 ppm	4.66	33.33 (35.24)	CN 50 ppm	6.76	3.33 (10.46)
CN 100 ppm	4.26	39.04 (38.65)	CN 100 ppm	6.25	10.71 (19.08)
CN 200 ppm	3.60	48.57 (44.16)	CN 200 ppm	5.83	16.66 (24.08)
CN 300 ppm	3.80	45.71 (42.52)	CN 300 ppm	5.60	20.00 (26.54)
CN 400 ppm	2.70	61.42 (51.59)	CN 400 ppm	5.23	25.23 (30.13)
CN 500 ppm	1.66	76.19 (60.77)	CN 500 ppm	5.00	28.57 (32.29)
CN 600 ppm	0.00	100.00 (90.00)	CN 600 ppm	4.46	36.19 (36.96)
CN 700 ppm	-	-	CN 700 ppm	3.70	47.14 (43.34)
CN 800 ppm	-	-	CN 800 ppm	4.00	42.85 (40.87)
CN 900 ppm	-	-	CN 900 ppm	3.23	53.80 (47.16)
CN 1000 ppm	-	-	CN 1000 ppm	2.03	67.14 (55.00)
C.D. (5%)	0.48	1.50	C.D (5%)	0.14	1.43
C.V.	15.12	1.89	C.V.	1.78	2.81

*****CN: Copper nanoparticles, synthesized using *Macrophomina phaseolina* supernatant (MP-CuNPs)
******Data are the mean of three replications
$Copper oxychloride (Blitox)
#Data within parentheses are Angular transformed value

Discussion

Nanotechnology a blooming science has a wide application in various fields such as electronics, medical, cosmetics, packaging, biotechnology, etc. (Thakkar *et al.,* 2010) [34]. In agriculture it has been used for the development of new varieties using nanoparticles (NPs) as a mutagen, as a slow release of nanomaterial assisted fertilizers, bio-fertilizers, and micronutrients for their efficient use; delivery of nanoncides-pesticides encapsulated in nanomaterials for control release (Ghormade *et al.*, 2011) [12], etc. Recently nanoparticles use as fungicides have attracted the eyes of researchers around the globe owing to their serviceable and beneficial properties. The manifold enhanced activity of converted nanoparticles can be attributed to a small size (1- 100 nm) and large surface area which allows them to manifest new characters or new chemical and physical properties superior to their precursor (Nel *et al.*, 2006 [21], Shobha *et al.,* 2014[31]). In the last two decades, extensive efforts have been directed to improve agriculture productivity by deploying nanotechnology. In the present investigation, the extracellular synthesis approach was followed for the synthesis of CuNPs using the fungal supernatant. The fungus under the study was found to be an excellent candidate for copper nanoparticle synthesis. The change in color of the reaction mixture over time was observed, which is the first and foremost indication confirming the formation of nanoparticles. The color changes due to the excitation of surface plasmon vibrations (Slistan *et al.,* 2005 [32]; Ramteke *et al.,* 2012 [25]; Henglein *et al.,* 1993 [14]). *M. phaseolina* mediated synthesis of CuNPs led to a change in color from light yellowish to brown. Honary *et al.* (2012) [15] also observed the brown color of the final reaction mixture from the pale yellow color using copper oxide as a precursor and *Penicillium aurantiogriseum*, *P. citrinum,* and *P. waksmani* as a reducing agent. The final color in all aforesaid cases became more intense with the increase in the concentration of $CuSO_4$. In general, CuNPs absorb light in the range of 250 to 360 nm (Din *et al.*, 2017) [9]. The absorbance maxima achieved in *M. Phaseolina* mediated synthesis of copper nanoparticles is in impeccable agreement with the statement given by Din *et al.,* 2017 [9]. The absorption peaks obtained from the supernatants of the fungal source are due to the excitation of surface plasmon resonance (SPR) which depends on the particle size as well as the refractive index of the solvent, or in other words, it is due to the inter-band transitions excitation of metallic particles which is the characteristics properties of metals (Creighton *et al.,* 1991) [6]. In all aforesaid studies, a progressive increase in absorbance was observed with the increase in reaction time (24 h interval) that confirmed the synthesis of CuNPs. After 6 days of incubation, the increase in absorbance halted, this might be an indication of the reaction mixtures reaching equilibrium. The copper nanoparticles size was below 100 nm i.e. 7 to 89 nm as observed under the Transmission electron microscope. However, with the increase in the concentration of precursor

the size of particle increased. This gives an idea that a higher concentration of precursor is not suitable for nanoparticles synthesis or perhaps the reducing agent present in the fungal supernatant is not strong enough for the reduction of a very high concentration of precursor. Fungi such as *Aspergillus niger* (Noor *et al.,* 2020) [23], *Stereum hirsutum* (Cuevas *et al.*, 2015) [7], *Aspergillus fumigatus* (Ghareib *et al.,* 2018) [11], *Trichoderma harzianum* (Consolo *et al.*, 2020) [5], etc. have been exploited which could synthesize CuNPs below 100 nm size. FTIR analysis of the reaction mixture revealed the presence of various functional groups. The band indicates the presence of various proteins and biomolecules on the surface of synthesized copper nanoparticles. The presence of alcohol groups that is due to copper ion binding with the O-H group (Noor *et al.,* 2020) [23] could possibly be the reason for the reduction of particle size. There are several reports in which alcohol like ethanol, ethylene glycol, diethylene glycol, isopropyl alcohol, etc. has been used as a reducing agent to synthesize CuNPs. For instance, ethylene glycol was used by Zhu *et al.* (2004) [38] and Ramyadevi *et al.* (2012 [27]) for the synthesis of NPs. The use of diethylene glycol was also proved to be efficient enough to synthesize CuNPs (Park *et al.*, 2007[24]). Some workers considered the presence of diverse biomolecules *viz.*, carboxyl group, aldehydes, primary secondary amine, a band I, II, and III of amides as a possible tool for reduction of particle size as well as capping agent (Kamil *et al.,* 2017 [17]; Hulkoti *et al.,* 2014 [16]). The presence of primary amine, secondary amine, aliphatic amine, and amide groups are responsible for providing stability to the synthesized CuNPs by acting as a capping agent. The findings of the current investigation are consistent with the results of Gole *et al.* (2001) [13], who concluded that proteins have the ability to bind with nanoparticles either through free amine groups or cysteine residues which is basically due to the electrostatic attraction of negatively charged carboxylate groups in enzymes which confer stability to the synthesized copper nanoparticles. Thokala *et al.* (2018) [35] reported band corresponded to amine groups (NH stretching) responsible for stabilizing the AgNPs. The MP-CuNPs exhibited statistically significant inhibition from 20 ppm and 50 ppm onwards against *F. verticillioides* and *S. rolfsii*, respectively. Complete inhibition of *F. verticillioides* was observed at 600 ppm. The enhanced fungicidal activity is due to its reduced size or high surface area to volume ratio and its ability to disrupt enzymes by binding to sulfydryl amino and carboxyl groups of amino acids. By their small size, CuNPs even disrupt the DNA helix of the microbes (Shobha *et al.,* 2014) [31]. Furthermore, CuNPs are also found to be affecting membrane integrity and membrane lipids (Santo *et al.*, 2008) [29]. Pertaining to the findings of the present investigation, the same kind of effectiveness of CuNPs was reported by Umer *et al.* (2012) [36] against *F. oxysporum, Alternaria alternata, Curvularia lunata*, and *Phoma destructiva*. Viet *et al.* (2016) [37] reported the efficacy of CuNPs @ 450 ppm against *Fusarium* sp. Bramhanwade *et al.* (2015) reported the effectiveness of CuNPs against *F. oxysporum* and *F. equiseti* and Kanhed *et al.* (2014) [18] against *Phoma destructiva, C. lunata, A. alternata*, and *F. oxysporum* where they perceived the effectiveness of CuNPs better than the commercial fungicides.

Conclusion

The fungi *M. phaseolina* was confirmed to be an excellent candidate for the extracellular synthesis of copper nanoparticles. However, an increase in the concentration of precursors hampers NPs formation leading to particle aggregation and loss of monodispersity. As confirmed by the TEM, CuNPs of average size ranging from 7- 89 nm were achieved manifesting spherical to irregular shape, reasonably monodisperse. FTIR determined the presence of functional groups responsible for the reduction and stabilizing of the particles. The biologically synthesized CuNPs exhibited statistically better control of pathogens under the study than the commercial fungicides. Therefore the adoption of such technology will greatly help in managing Phytopathogens due to its enhanced efficacy and also would thwart the development of fungicidal resistance in phytopathogens. Environmental pollution due to pesticides is a major problem. Since nanofungicides are required in traces to cover a large area and to manage pathogens, it will highly limit the usage of pesticides and assuredly have a lower impact on the environment.

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