



Original article

Copper nanoparticles: Green synthesis and managing fruit rot disease of chilli caused by *Colletotrichum capsici*

Krishnanand Shivanand Iliger^a, Tariq Ahmad Sofi^{a,*}, Nazir Ahmad Bhat^a, Farooq Ahmad Ahanger^a, Jagan Chandra Sekhar^a, Ahmed Zohier Elhendi^b, Asma A. Al-Huqail^c, Faheema Khan^c

^a Division of Plant Pathology, FoA and FoH, Sher-e-Kashmir University of Agricultural Sciences and Technology, Kashmir, India

^b National Plan for Science Technology and Innovation Unit, King Saud University, Riyadh, Saudi Arabia

^c Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:

Received 15 October 2020

Revised 1 December 2020

Accepted 1 December 2020

Available online 8 December 2020

Keywords:

Nanoparticles

Copper

Green synthesis

Eucalyptus

Mint

Chilli anthracnose

ABSTRACT

The present study was focused on synthesis and characterization of copper nanoparticles to evaluate their efficacy against fruit rot pathogen of chilli crop. The green synthesis of nanoparticles was carried out by using extracts of Eucalyptus and Mint leaves. The synthesis of copper nanoparticles was confirmed by XRD, PSA, SEM and TEM. The average size of these particles synthesized by Eucalyptus leaf extract (CuNP-E) ranged from 10 to 130 nm, while as size of Mint leaf extract synthesized particles (CuNP-M) ranged from 23 to 39 nm, thus confirming their nano size. These green synthesized copper nanoparticles were evaluated against *Colletotrichum capsici* where Carbendazim 50 WP @ 500 ppm and copper oxychloride 50 WP @ 2500 ppm served as standard checks. The mycelia inhibition of *Colletotrichum capsici* caused by copper nanoparticles was studied on PDA medium. CuNP-M @ 1000 ppm showed highest mycelial inhibition of 99.78% followed by 93.75% at 500 ppm and CuNP-E @ 1000 ppm compared to standard fungicides, carbendazim 50 WP @ 500 ppm (72.82%), and copper oxychloride 50 WP @ 2500 ppm (85.85%). The CuNP-M @ 500 ppm were significantly superior to carbendazim 50 WP @ 500 ppm and copper oxychloride 50 WP @ 2500 ppm, but was statistically at par with CuNP-E @ 1000 ppm. This shows effectiveness of much lower concentration of copper nanoparticles compared to conventional fungicides. In detached fruit method, nanoparticles applied before inoculation of pathogen showed better results with regard to incubation period, lesion number and lesion size than after inoculation of pathogen. The present study reveals a simple, convenient, non-toxic and cost-efficient technique for the synthesis of nanoparticles and their effectiveness against *Colletotrichum capsici*. CuNP-M first time synthesized and evaluated against *Colletotrichum capsici* performed better than CuNP-E.

© 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Colletotrichum capsici is among the most economically dominant plant pathogens. It causes anthracnose and fruit rot in chilli crop

Abbreviations: XRD, X-ray Diffractometer; PSA, Particle size analyser; SEM, Scanning Electron Microscope; TEM, Transmission Electron Microscope; PDA, Potato Dextrose Agar; CuNP-E, Copper, nanoparticles synthesis from Eucalyptus leaf extract; CuNP-M, Copper nanoparticles synthesis from Mint leaf extract.

* Corresponding author.

E-mail address: tariqsofi1@gmail.com (T.A. Sofi).

Peer review under responsibility of King Saud University.



thus reducing yield and quality of the plant products. The disease was first reported from United States in 1885 (Halsted, 1885) and afterwards from many parts of the world. In India it was first reported from Madras (Sydow, 1913) and subsequently from other states like Bihar, Assam, Tamil Nadu (Dastur, 1921; Choudhery, 1957). In terms of yield, losses upto 32% have been reported from USA (Doolittle, 1954) and 50% from India (Pakdevaraporn et al., 2005). Different practices are in vogue to manage this disease, but none except chemical management has shown satisfactory results with environmental risks. Among the different management technologies, nanotechnology is one newly emerging technology. Nanotechnology is shaping as a dynamic area of research in present day agriculture and allied subjects. Particles with a size ranging from 1 to 100 nm in not less than one dimension are usually referred to as nanoparticles (Hutchison, 2008). Nanoparticles reveal new or improved qualities based on specific features like morphology, size

and distribution. Among the nanomaterials metallic nanomaterials are emerging with greater importance by displaying eccentric and modified physico-chemical and biological properties compared to their equivalents of the macroscale (Mohammed Fayaz et al., 2011). Different types of approaches like chemical reduction (Prakash et al., 2009), electrochemical reduction (Zhang, 2008), chemical vapor deposition (Rao et al., 2006), thermal decomposition (Kim et al., 2006) solvothermal reduction (Tang et al., 2006) have successfully synthesized nanoparticles of metallic nature. However, aforementioned methods use toxic solvents, generate hazardous by-products and consume lot of energy. There is an increasing demand for cost-efficient, environmentally amiable and long lasting methods for nanoparticles synthesis. This has lead scientists to make use of biological structures as an alternate green synthesis approach. Use of plants, algae, fungi, bacteria and viruses for the production of efficient, non-toxic metallic nanoparticles has seen an increasing trend in the past. The use of *Azadirachta indica* (Neem) (Shankar et al., 2004), *Emblca officinalis* (Amla) (Ankamwar et al., 2005), Mangosteen leaf (Veerasamy et al., 2011), *Chenopodium album* (Dwivedi and Gopal, 2010) for synthesis of nanoparticles stand already reported. The studies carried out indicate that bio-molecules viz., proteins, phenols and flavonoids play a key role in reduction of ions to nano-scale and in capping of the nanoparticles (Arya, 2010). Copper has been used in inhibiting the growth of microorganisms for more than two centuries and reportedly decreased the microbial concentration by 99.9% (Krithiga et al., 2013; Subhankari and Nayak, 2013). Many reports suggest that copper nanoparticles exhibit broad antimicrobial activity against so many bacteria. Nano copper oxide (CuO) acts as inherent antimicrobial agent against *E. coli*, *Bacillus subtilis*, *Vibria cholera*, *Pseudomonas aeruginosa*, *Syphillis typhus*, and *Staphylococcus aureus* (Perelshtein et al., 2009; Akhavan and Ghaderi, 2010; Hassan et al., 2012; Padil and Černík, 2013). Copper oxychloride and copper sulphate are already in vogue throughout the world for the management of different diseases of apple caused by fungi. Use of copper as dormant spray against some diseases of stone and pome fruits is also in vogue in many countries including India. It is not applied during active phase of the plant because of phytotoxicity which can possibly be addressed by converting it into Nanoscale.

Till date no work has been carried out on the biosynthesis (Eucalyptus and Mint) of copper nanoparticles and their use against *Colletotrichum capsici*. In view of the above facts and importance of copper in management of plant diseases present study was carried out.

2. Materials and methods

2.1. Preparation of plant extracts

Eucalyptus (*Eucalyptus globulus* L.) leaves were collected from University of Agricultural Sciences, Dharwad, Karnataka whereas mint (*Mentha piperita*) leaves were collected from Jammu and Kashmir. The leaves were cleaned, dried under shade to remove moisture, powdered and stored for further use. 10 g of each powdered material was taken separately in a beaker containing 100 ml of de-ionized water and allowed to boil at 80 °C for 25–30 min under reflux condition and then cooled down to room temperature. Each solution was filtered through filter paper (Whatman No. 42) followed by centrifugation at 3000 rpm for 10 min for removal of heavy biomaterials. The clear solutions of plant extracts thus obtained were stored at 4 °C for further studies.

2.2. Synthesis of nanoparticles

In a prototypical synthesis of copper nanoparticles from eucalyptus and mint leaf extracts, 10 ml of each leaf extract was added

to 100 ml of 0.01 M CuSO₄·5H₂O aqueous solutions. The two mixtures were constantly stirred on a magnetic stirrer for 2 h. The suspended solutions were autoclaved followed by centrifugation at 5000 rpm for 25 min. The supernatants of both the solutions were decanted and residues were washed many times with de-ionized water. The above-mentioned processes were repeated several times to remove impurities (if any) from the copper nanoparticles. The precipitates so obtained were dried in an oven at 60 °C for 24 h.

2.3. Characterizations of synthesized nanoparticles

The preliminary detection of copper nanoparticles formation was observed by visual colour change. The characterization of nanoparticles was further carried by X-ray diffraction (XRD), Particle Size Analyzer (PSA), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). X-ray diffraction studies of copper nanoparticles was carried out by X-ray Diffractometer (XRD) (Bruker d8 Advance) supplied with Cu K α radiation source using Ni as a filter with a setting of 40kv/30 mA. Particle diameter was computed according to Debye-Scherrer equation ($D = K\lambda/\beta \cos\theta$). Particle size analysis was ascertained by sub-micrometer PSA using dynamic light-scattering technique (DLS). PSA was measured in the range of 1–5000 nm at a scattering angle of 173°. Three-dimensional photon cross-correlation method was employed for the concurrent measurement of PSA and stability. Viscosity of dispersion medium was 0.894 ± 1 mPa.s. Aqueous dispersion of synthesized nanoparticles was carried out under sonication at 30 kHz for 30 min and used for obtaining PSA curve. Horiba SZ100 machine was used for the study. The surface morphological features such as shape, particle size and composition of nanoparticle were measured using a JEOL JSM-5600 model (Scanning Electron Microscope). Size and morphology of nanoparticles was determined by using TEM. An aliquot of aqueous nanoparticle suspension was transferred on to an amorphous carbon coated copper grid followed by drying and analysis was carried out using Tecnai G2 20 TEM operated at voltage of 120 kV.

2.4. Evaluation of nano-particles

Evaluation of nano-particles was carried out under controlled conditions through poison food technique (Sharvelle, 1961) (*in-vitro* evaluation) and detached fruit technique (Manzoor et al., 2013) (evaluation on detached chilli fruit) with some modifications. In both the cases carbendazim 50 WP (500 ppm) and copper oxychloride 50 WP (2500 ppm) served as standard checks while as water served as control.

2.5. In vitro evaluation of nanoparticles (Poison food technique)

Evaluation of copper nanoparticles was carried out at 25, 50, 100, 150, 250, 500 and 1000 ppm concentrations while as carbendazim 50 WP and copper oxychloride 50 WP were evaluated at 500 and 2500 ppm respectively. The investigation was implemented with 3 replications for each treatment under Completely Randomized Design (CRD).

The nanoparticle suspensions were added to the molten PDA media with 25, 50, 100, 150, 250, 500 and 1000 ppm concentrations. The poisoned media was then poured in sterilized petriplates under aseptic conditions. Fungicides served as standard checks and in control only water was used. 4 mm mycelial discs taken from 10 days old culture were put in the center of each plate followed by incubation at 27 ± 1 °C for 10 days. Observations on mycelial diameter of *C. capsici* were recorded after ten days of incubation. The diameter of the mycelial colony was measured in four directions and their average was recorded. Growth inhibition (%) was calculated by using formula as below:

$$I = \frac{C - T}{C} \times 100$$

where

I = Inhibition (%); C = Growth in control (mm); T = Growth in treatment (mm)

2.6. Evaluation of nanoparticles on detached chilli fruits (Detached fruit technique)

The experiment was carried out on detached chilli fruits under controlled laboratory conditions. The best three concentrations obtained from the results of poison food technique of each nanoparticle were assayed against *C. capsici* on detached fruits. Two sets of detached fruits were maintained. In one set nanoparticles were sprayed at 48 h before-inoculation of the pathogen and in other set nanoparticles were sprayed 48 h after-inoculation of pathogen. The experimental setup was kept up to sixteen days for recording the observations on incubation period, lesion number and size of lesions. Fungal conidia were collected from ten days old culture of *C. capsici*. The culture plates were flooded with sterilized distilled water and conidia were scraped gently with camel hair brush. Conidial mass thus obtained was collected into a beaker and filtered for further studies. The concentration of conidial suspension was later adjusted at 1×10^6 spores/ml.

Healthy fruits from susceptible chilli cultivar Pusa Jwala were evaluated under the study. Fruits were first surface sterilized for 30 s in 0.1 percent mercuric chloride and washed thoroughly with distilled sterilized water. The surface sterilized fruits were then air dried on sterilized blotter paper. Test fruits were divided in two sets each for nano-particle application 48 h before spore (*C. capsici*) inoculation and 48 h after spore inoculation. All the fruits were given pin-pricks at five specific points and then sprayed with spore suspension of 10^6 spores/ml. The treated fruits were incubated in moist chamber at 27 ± 1 °C for 16 days. The fungicides served as standard check while as sterilized distilled water (SDW) was applied in case of control. Three replications of each treatment were maintained and experiment carried under Completely Randomized Design.

2.7. Data analysis

The data of experiments conducted for characterization of nanoparticles was analyzed with the help of origin 6.1 and Image J software. The statistical evaluation of biological data was carried out through Statistical Package for Agricultural Research Workers (OPSTAT). Before analysis appropriate transformations of the data were carried out as suggested by Gomez and Gomez (1984).

3. Results

3.1. Synthesis of copper nanoparticles

Copper sulfate solution (aqueous), blue in colour changed to brown color within 2 h after addition of leaf extract of Eucalyptus (Fig. 1A) depicting the presence of Cu nanoparticles in the solution. The Cu nanoparticle solution was further autoclaved to increase the intensity of nanoparticles in the solution. After autoclaving, brown color changed to dark brown color. In case of the mint extract, the aqueous blue coloured copper sulfate pentahydrate solution changed to greenish black within 2 h, indicating the synthesis of nanoparticles. Upon autoclaving, the intensity of the col-

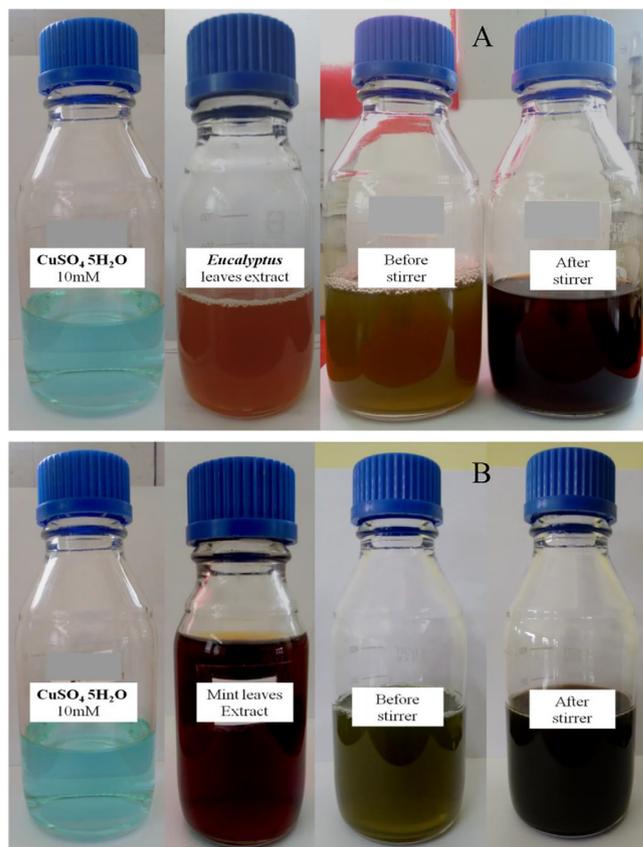


Fig. 1. Colour indication of synthesis of copper nanoparticles from (A) eucalyptus and (B) mint leaf extract.

our increased, which was directly proportional to the reaction time and reduction of copper ions (Fig. 1B).

3.2. Characterization of synthesized nano-particles

The crystalline nature of copper nanoparticles was corroborated by X-ray diffraction (XRD) ornamentation. XRD pattern of copper nanoparticles formed by bioreduction of copper ions using eucalyptus (*Eucalyptus globulus* L.) leaf extract is presented in Fig. 2A. The observed diffraction peaks of CuNP-E at $2\theta = 43.36^\circ$, 50.41° , and 74.20° correspond to (1 1 1), (200) and (220) planes of metallic copper. The mean particle diameter of CuNP-E was 67 nm (Table 1). In the bioreduction of copper ions using mint (*Mentha piperita*) leaf extract the observed diffraction peak at $2\theta = 43.220$, 50.250 and 74.060 confirming the metallic copper (Fig. 2B). The mean particle diameter of CuNP-M as revealed by XRD was 27.5 nm (Table 1).

The particle size distribution (PSD) of CuNP-E and CuNP-M is presented in Fig. 3A and Fig. 3B respectively. According to PSA analysis size of CuNP-E ranged from 60 to 75 nm with an average size of 65 nm while as CuNP-M size ranged from 36 to 50 nm and the average size recorded was 45 nm (Table 2).

The SEM images (Fig. 4A & B) of CuNP-E and CuNP-M show cluster shaped morphology and size more than 100 nm. The average size for CuNP-E ranged from 0.5 to $3.2 \mu\text{m}$ with an average of $1.53 \mu\text{m}$ while as the average size for CuNP-M ranged from 0.47 to $1.63 \mu\text{m}$ with an average of $1.13 \mu\text{m}$ (Table 2).

Typical TEM image (Fig. 5A) of CuNP-E shows triangular shaped nanoparticles poly-disperse in nature without any bio-molecules

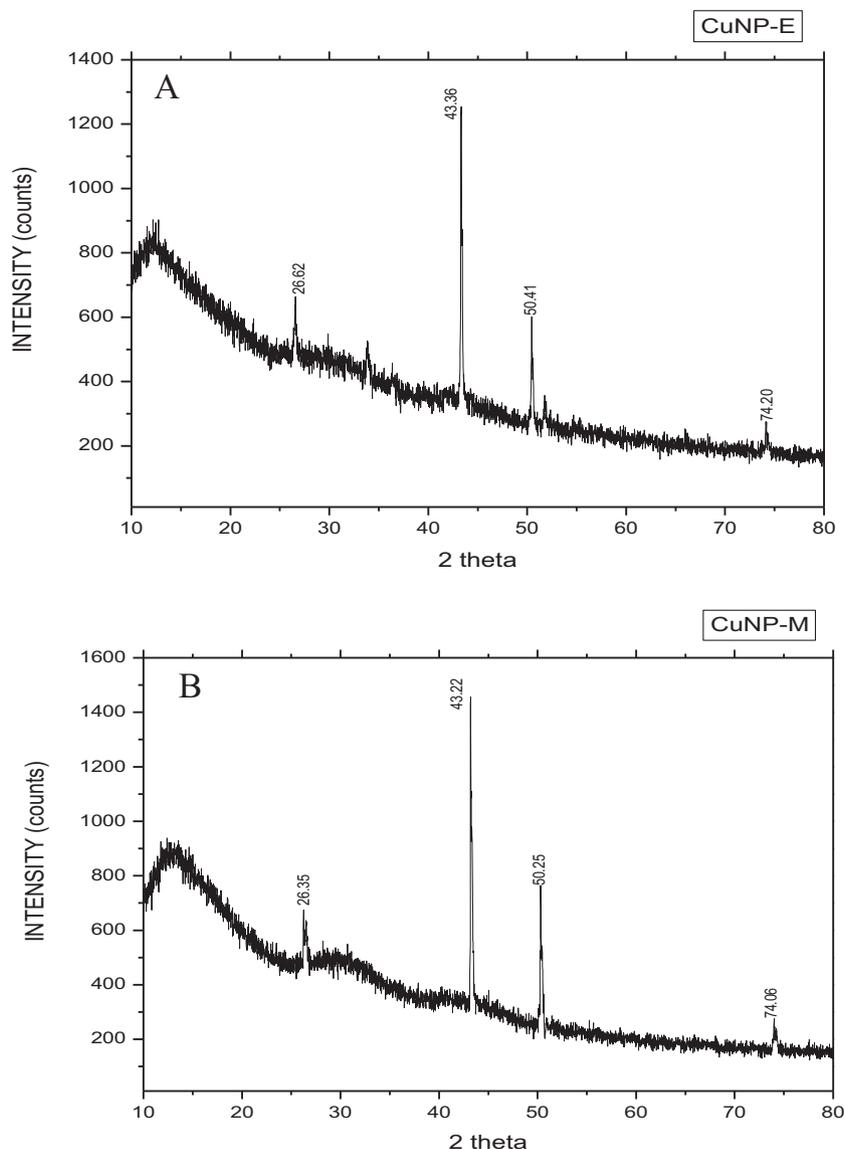


Fig. 2. XRD peaks of copper nanoparticles synthesized from (A) eucalyptus and (B) Mint leaf extract.

Table 1
Crystalline size of copper nanoparticles as revealed by XRD.

| Nanoparticles | Extract used | FWHM (Degree) | | Average crystalline size (nm) | |
|---------------|--------------|---------------------------|--------------------------|-------------------------------|------|
| | | Range (10 ⁻³) | Mean (10 ⁻³) | Range | Mean |
| CuNP-E | Eucalyptus | 1.60 – 2.99 | 2.40 | 46 – 85.5 | 67.0 |
| CuNP-M | Mint | 3.27 – 9.50 | 3.80 | 15 – 42.5 | 27.5 |

FWHM: Full Width at Half Maxima of XRD peak.

around them. The size of CuNP-E ranged from 10 to 100 nm with an average of 42 nm (Table 2). CuNP-M were cluster shaped (Fig. 5B) and size ranged from 23 to 39 nm with an average of 33 nm (Table 2). The analysis of the TEM images is a useful tool for measuring the size and shape of the nano-particles.

3.3. Evaluation of synthesized copper nanoparticles

In the present investigation, the copper nanoparticles were evaluated by two methods i.e., Poison food technique and detached

fruit method. In both methods, copper nanoparticles (@ 1000 ppm showed significantly better results in comparison to standard fungicides and control.

3.4. Effect of nanoparticles on mycelial growth of *C. capsici* (Poison food technique)

The impact of copper nanoparticles on mycelial growth of *C. capsici* is presented in table 3. Perusal of the data reveals significantly decreased mycelial growth of *C. capsici* with increase in the nanoparticle concentration. Lowest growth (0.25 mm) was recorded in CuNP-M @ 1000 ppm followed by 4.75 mm both in CuNP-E @ 1000 ppm and CuNP-M @ 500 ppm. CuNP-E @ 500 ppm (11.25 mm) was statistically at par with Copper oxychloride 50WP @ 2500 ppm (11.25 mm) but was significantly superior to Carbendazim 50 WP @ 500 ppm (20.75 mm). The data reveals that both the treatments (CuNP-E and CuNP-M) at all the concentrations and both the standard checks (Copper oxychloride and Carbendazim) significantly reduced the mycelial growth compared to control where mycelial growth of 76.0 mm was recorded. Data further reveals that copper nanoparticles (CuNP-E and CuNP-M)

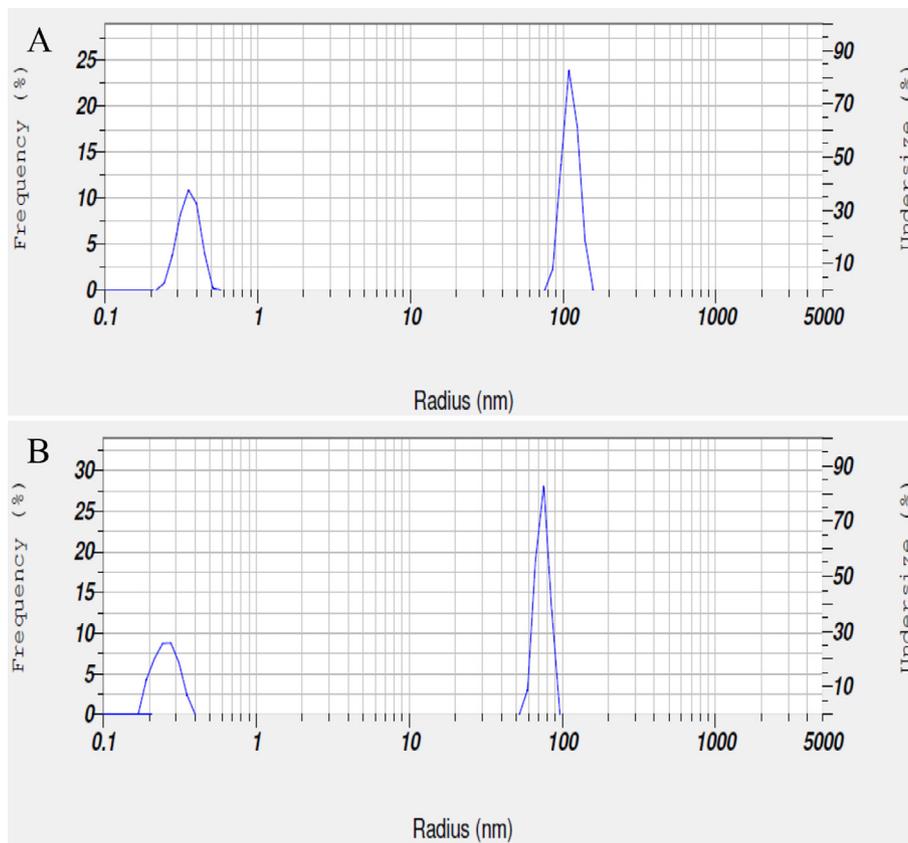


Fig. 3. PSA peaks of copper nanoparticles synthesized from (A) eucalyptus and (B) mint leaf extract.

Table 2
Shape and size of copper nanoparticles as revealed by PSA, SEM and TEM.

| Method | Nanoparticles | Shape of NPs | Particle size Range (nm*/µm**) | Average size (nm*/µm**) |
|--------|---------------|--------------|--------------------------------|-------------------------|
| PSA | CuNP-E | – | 60–75* | 65* |
| | CuNP-M | – | 36–50* | 45* |
| SEM | CuNP-E | Cluster | 0.5–3.2** | 1.53** |
| | CuNP-M | Cluster | 0.47–1.63** | 1.13** |
| TEM | CuNP-E | Triangular | 10–100* | 42* |
| | CuNP-M | Cluster | 23–39* | 33* |

at 250 ppm and lower concentration were not as effective as the standard checks (Copper oxychloride and Carbendazim) (Table 3). Mycelial growth inhibition percentage of *C. capsici* under *in vitro* condition is shown in Fig. 6. Highest inhibition (99.78%) of mycelial growth was recorded in CuNP-M @ 1000 ppm followed by 93.75% in CuNP-E @ 1000 ppm and CuNP-M @ 500 ppm. The data in Fig. 6 further reveals that CuNP-E @ 500 ppm inhibited 85.19% growth of mycelium which was statistically at par with copper oxychloride 50 WP @ 2500 ppm but statistically superior to Carbendazim 50 WP @ 500 ppm which recorded growth inhibition of 72.69%.

3.5. Evaluation of nanoparticles on detached chilli fruits (Detached fruit method)

3.5.1. Incubation period

In the present study copper nanoparticles showed significantly better results compared to standard checks and control. Studies on incubation period of *C. capsici* (Fig. 7A) reveals that CuNP-M @ 500 and 1000 ppm applied “before-inoculation” and CuNP-M @ 1000 ppm applied “after-inoculation” inhibited the disease com-

pletely and there was no question of any incubation period. However, disease first appeared in control after an incubation period of three days only. Perusal of the data reveals that even though other treatments could not completely inhibit the disease but prolonged the incubation period. It is also evident from the data that application of nanoparticles 48 h prior to the pathogen inoculation enhanced incubation period more than when these were applied 48 h after pathogen inoculation. The CuNP-E @ 1000 ppm and CuNP-M @ 250 ppm applied before pathogen inoculation prolonged the incubation period up to 8 days in each case. Similarly, CuNP-E @ 1000 ppm and CuNP-M @ 500 ppm applied “after-inoculation” prolonged the incubation period up to 7 days. In case of standard fungicides (Carbendazim 50 WP @ 500 ppm, Copper Oxychloride 50 WP @ 2500 ppm) incubation period of the pathogen remained same (4 days) for both before and after inoculation of pathogen, but was significantly lower than the nanoparticles (Fig. 7A).

3.5.2. Lesion number

The number of lesions on fruits observed after 16 days of incubation varied significantly among treatments. Perusal of the data

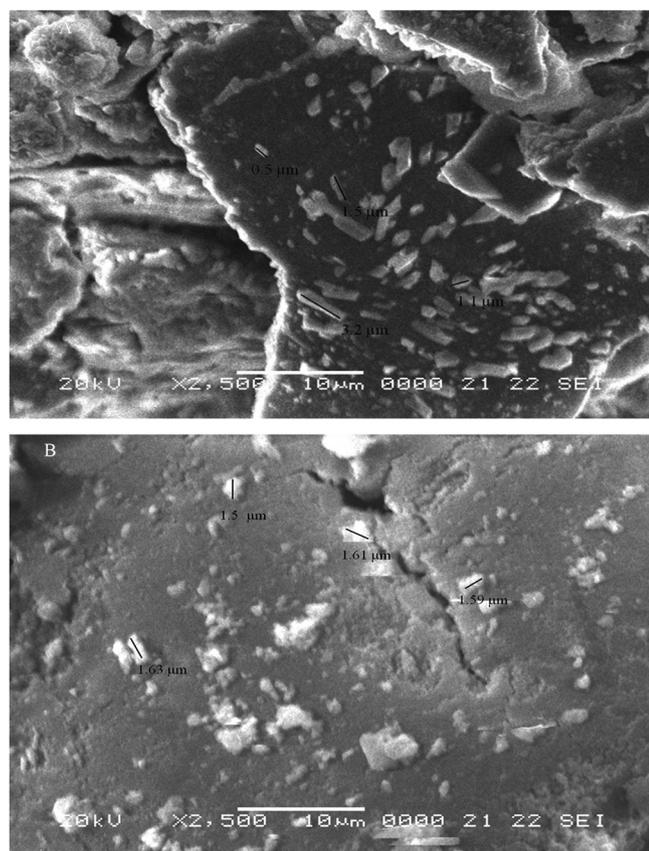


Fig. 4. SEM Image of copper nanoparticles synthesized by (A) eucalyptus and (B) mint leaf extract.

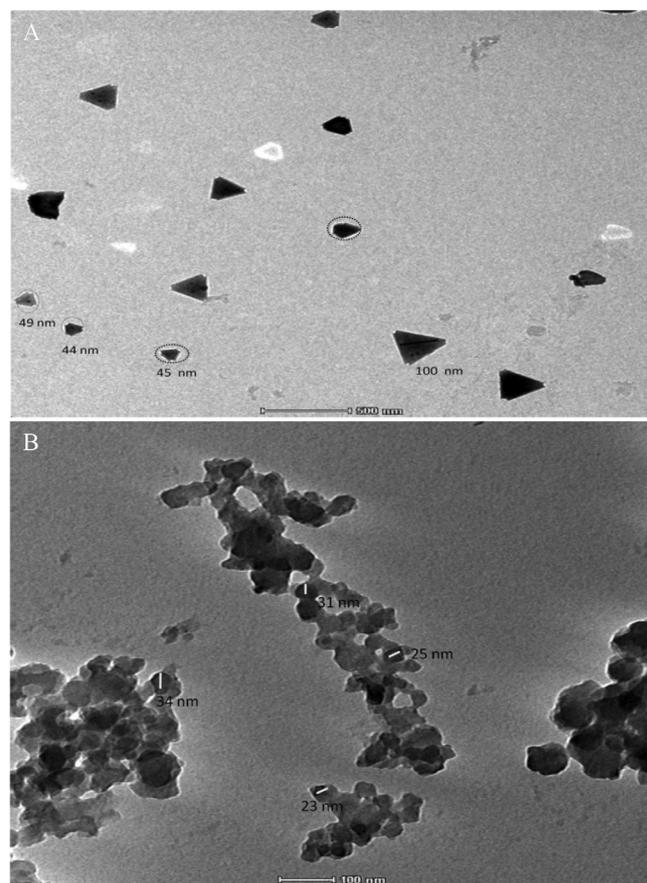


Fig. 5. TEM Image of copper nanoparticles synthesized by (A) eucalyptus and (B) mint leaf extract.

(Fig. 7B) reveals that CuNP-M @ 500 and 1000 ppm applied “before-inoculation” and CuNP-M @ 1000 ppm applied “after-inoculation”, inhibited the disease completely and there were no lesions on fruits. Highest number of lesions (5/fruit) was recorded in control. The standard fungicides, Carbendazim 50 WP @ 500 ppm and Copper oxychloride 50 WP @ 2500 ppm recorded four lesions/fruit when applied “before-inoculation” of pathogen and 4 and 5 lesions/fruit respectively when applied “after-inoculation” of pathogen. The data further reveals that CuNP-E @ 1000 ppm recorded single lesion/fruit and CuNP-M @ 250 ppm recorded 3 lesions/fruit, when applied “before-inoculation” of pathogen while as CuNP-E @ 1000 ppm and CuNP-M @ 500 ppm each recorded three lesions/fruit when applied “after-inoculation” of pathogen. In case of “after-inoculation” of pathogen CuNP-E @ 250 and 500 ppm were not as effective as Carbendazim 50 WP @ 500 ppm but were at par with Copper oxychloride 50 WP @ 2500 ppm. In case of “before-inoculation” of pathogen CuNP-E @ 500 ppm was statistically at par with Carbendazim 50 WP @ 500 ppm and Copper oxychloride 50WP @ 2500 ppm. The results indicate that nanoparticles applied before inoculations are significantly better than after inoculation of the pathogen (Fig. 7B).

3.5.3. Lesion size

CuNP-E and CuNP-M at three different concentrations and standard check fungicides (Carbendazim 50 WP, Copper oxychloride 50 WP) before and after pathogen inoculation significantly lowered lesion length as compared to control (Table 4). The results showed significantly better inhibition of *C. capsici* can be realized when nanoparticles are sprayed before inoculation of pathogen as compared to after inoculation. The results distinctly indicate that the nanoparticles are significantly better than conventional fungicides

in reducing the lesion size of *C. capsici* on chilli fruit. Perusal of the data (Table 4) reveal that CuNP-M @ 500 and 1000 ppm applied “before-inoculation” and CuNP-M @ 1000 ppm applied “after-inoculation” inhibited the disease completely and there were no lesions on fruits. Maximum lesion length of 65.30 mm was recorded in control where only water was applied and was statistically inferior to all the treatments. The standard fungicides, carbendazim 50WP @ 500 ppm recorded 13.50 mm and copper oxychloride 50WP @ 2500 ppm 15.50 mm lesion length on fruits when applied “before-inoculation” of pathogen. Whereas carbendazim 50 WP @ 500 ppm and copper oxychloride 50 WP @ 2500 ppm recorded lesion length of 22.30 mm and 22.70 mm respectively when applied “after-inoculation” of pathogen. The data further reveals that CuNP-E @ 1000 ppm and CuNP-M @ 250 ppm recorded lesion length of 5.26 and 6.30 mm respectively when applied “before-inoculation” of pathogen. Whereas CuNP-E @ 1000 ppm and CuNP-M @ 500 ppm recorded 7.50 mm and 10.50 mm respectively, when applied “after-inoculation” of pathogen. Among all the treatments CuNP-E @ 250 ppm applied “before-inoculation” of pathogen was not as effective as the recommended fungicides. The data presented in Table 4 reveals that CuNP-E @ 500, 1000 ppm and CuNP-M @ 250, 500 and 1000 ppm recorded lesion length of 12.70, 5.26, 6.30, 0.00 and 0.00 mm respectively and were significantly superior to carbendazim 50 WP @ 500 ppm and copper oxychloride 50 WP @ 2500 ppm in after inoculation of pathogen. The data further reveals that CuNP-E and CuNP-M @ 250, 500, 1000 ppm recorded lesion length 21.70, 17.70, 7.50, 14.80, 10.50 and 0.00 mm respectively and were significantly superior to standard fungicides.

Table 3
Effect of nanoparticles on mycelial growth of *Colletotrichum capsici* after 10 days of incubation.

| Concentration Treatments | Mycelial diameter (mm) | | | | | | |
|-----------------------------|--|--------|---------|---------|---------|---------|----------|
| | 25 ppm | 50 ppm | 100 ppm | 150 ppm | 250 ppm | 500 ppm | 1000 ppm |
| CuNP-E | 63.50 | 50.42 | 47.50 | 38.25 | 32.83 | 11.25 | 4.75 |
| CuNP-M | 61.42 | 45.25 | 38.58 | 30.92 | 27.83 | 4.75 | 0.25 |
| Carbendazim 50 WP* | 20.75 | 20.75 | 20.75 | 20.75 | 20.75 | 20.75 | 20.75 |
| Copper oxychloride 50 WP** | 11.25 | 11.25 | 11.25 | 11.25 | 11.25 | 11.25 | 11.25 |
| Control | 76.00 | 76.00 | 76.00 | 76.00 | 76.00 | 76.00 | 76.00 |
| Mean | 46.58 | 40.73 | 38.82 | 35.43 | 33.73 | 24.80 | 22.60 |
| CD p ≤ 0.05 | | | | | | | |
| Treatments 0.44 | Concentrations 0.52 Treatment × Concentration 1.18 | | | | | | |

* 500 ppm.

** 2500 ppm.

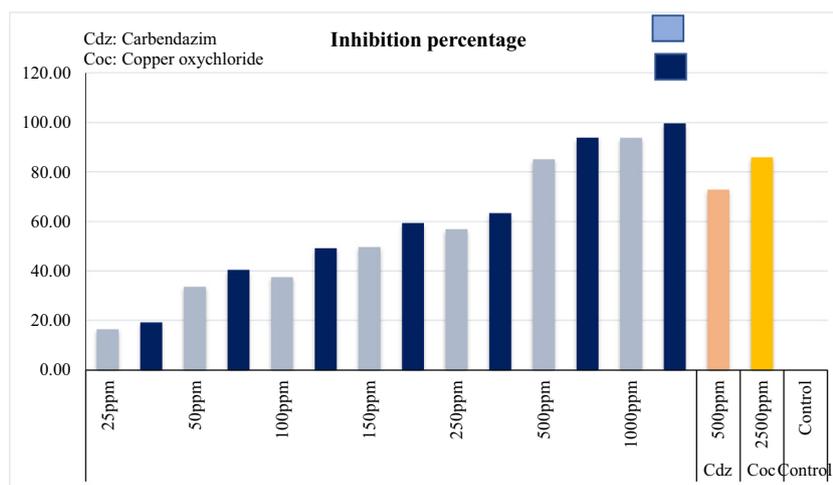


Fig. 6. Mycelial growth Inhibition (%) of *C. capsici* by copper nanoparticles.

4. Discussion

Chilli an important spice cum vegetable crop faces various biotic challenges and fruit rot of chilli caused by *Colletotrichum capsici* is of considerable importance in India (Chauhan et al., 2014). Species of *Colletotrichum* are among the predominant plant pathogens throughout the world. They cause economically major diseases in a broad range of hosts including vegetables, fruits cereals and legume crops (Bailey et al., 1992). In major chilli growing areas of India the disease incidence ranges from 66 to 84% with a yield loss between 12 and 50 percent (Thind and Jhoo, 1985; Bagri et al., 2004; Sharma et al., 2005). A perusal of literature reveals that the disease is important in Kashmir and no work has so far been carried on synthesis and testing of nanoparticles against this disease. Keeping the importance of the disease and novelty of nanotechnology in view, present studies were undertaken to synthesize nanoparticles and test against fruit rot of chilli (*Colletotrichum capsici*).

In this study the leaf extract act as a capping and stabilizing agent and copper sulphate pentahydrate act as a reductant chemical. In the current study copper nanoparticles were synthesized using leaf extracts of Eucalyptus and Mint. While synthesizing copper nanoparticles solution colour changed from blue to dark brown within 2 h by adding eucalyptus extract to copper sulphate and from blue to greenish black by adding mint extract to the copper sulphate solution. Kulkarni et al. (2015) also reported copper nanoparticle synthesis from *Eucalyptus* sp., where colour of aqueous copper sulphate extract solution changed from blue to pale yellow.

Crystalline nature of copper nanoparticles was validated by XRD device. XRD pattern of copper nanoparticles was achieved through copper ion bioreduction by eucalyptus (*Eucalyptus globulus* L.) and mint (*Mentha piperita*) leaf extracts. The averages crystalline size was 67.0 and 27.5 nm respectively. The observed diffraction peaks of CuNP-E at $2\theta = 43.360, 50.410, 74.200$ and CuNP-M diffraction peak at $2\theta = 43.220, 50.250, 74.060$. The diffraction peaks of nanoparticles presented similarity with copper nanoparticle synthesized from khat (*Catha edulis*) and castor oil (*Ricinus communis*) as reported by G/Egziabher (2012). Dash and Balto (2011) also reported similarity in diffraction 2θ peak by synthesizing copper nanoparticles through wire explosion methods. More or less same results of XRD were observed in the copper nanoparticle synthesized by chemical reduction method (Viet et al., 2016).

The particle size distribution (PSD) analysis of CuNP-M showed different sizes with average size of 45 nm and mean particle size of CuNP-E as 65 nm.

The SEM results showed cluster shaped CuNP-E with a diameter of 1.53 μm while as CuNP-M had particle size of 1.13 μm and cluster in shape. Kulkarni et al. (2015) showed synthesis of copper nanoparticles by using leaf extract of *Eucalyptus* and found that copper nanoparticles bond with bio-molecules of leaf extracts. Awwad (2015) reported sulphur nanoparticles synthesis from *Albizia julibrissin* fruit extracts with spherical shaped particles. The SEM image reveals that the products mainly consist of copper nanoparticles crowded together with bio-molecules of plant extract. The larger size shown by SEM may possibly be due to agglomeration of nanoparticles and biomolecules.

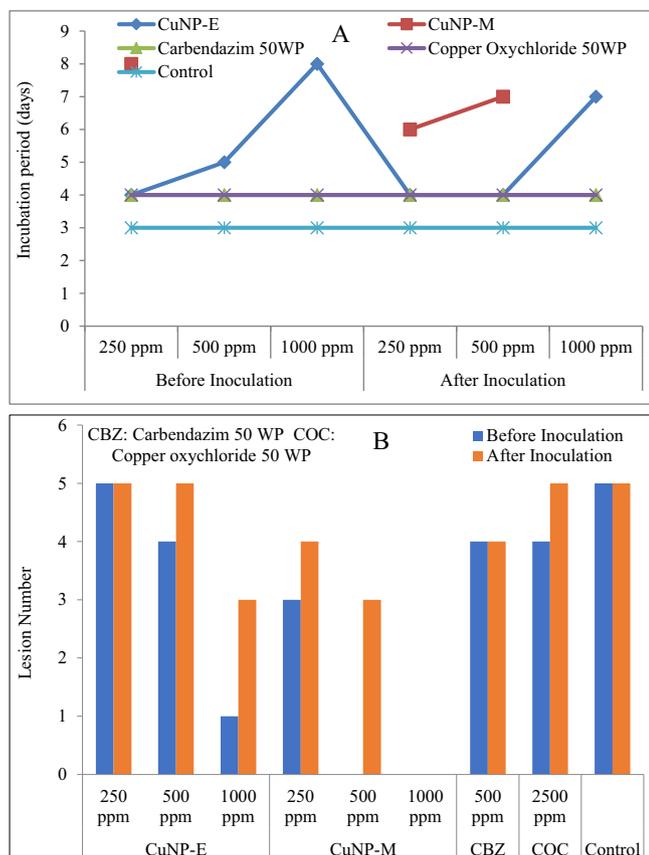


Fig. 7. Effect of copper nanoparticles on (A) incubation periods of *C. capsici* and (B) lesion number caused by *C. capsici* under detached fruit method.

A higher resolution study with TEM analysis showed shape and size of the synthesized nanoparticles. TEM image of CuNP-E revealed triangular shape with 42.0 nm average size and CuNP-M cluster in shape with 33.0 nm average size. The different studies have shown different shapes and sizes through different methods. The difference in shape and size in the present study may possibly be because of the different plant extracts used. Hariprasad et al. (2016) reported spherical morphology of copper nanoparticles synthesized from *Arevalanata* leaf extract. Khodashenas and Ghorbani (2019) reported triangular shape of silver nanoparticles produced by reduction method. Suleiman et al. (2013) reported that sulphur nanoparticles synthesized by water-in-oil micro emulsions

technique were spherically shaped as proved by their TEM analysis. The copper nanoparticles were poly disperse in shape.

In modern Agriculture nanoparticles are the upcoming arsenals against plant pathogens and green synthesized nanoparticles have shown great promise for disease control (Lamsal et al., 2011a; Surega et al., 2015; Viet et al., 2016). In the present investigation two nanoparticles were evaluated along with standard fungicides under poison food technique. The nanoparticles inhibited mycelial growth of *C. capsici* at various concentrations. Three best concentrations of each nanoparticle under poison food technique were evaluated by detached fruit method. All the synthesized nanoparticles significantly inhibited growth of *C. capsici* compared to standard fungicides and control.

CuNP-M @ 1000 ppm proved significantly superior in inhibiting mycelial growth (0.25 mm), followed by CuNP-E @ 1000 ppm (4.75 mm) which was significantly less than the mycelial growth in carbendazim 50 WP @ 500 ppm (20.75 mm) and copper oxychloride 50 WP @ 2500 ppm (11.25 mm). All treatments showed significantly superior results as compared to control. The CuNP-M showed better results than CuNP-E possibly because of smaller size. Viet et al. (2016) reported antifungal properties of chemically synthesized copper nanoparticles against *Fusarium sp.*, under *ex vivo* conditions. All the nanoparticles inhibited mycelial growth of *C. capsici* excellently at 1000 ppm as compared to control. The CuNP-M were highly effective in inhibiting mycelial growth @ 1000 ppm (99.78%), followed by CuNP-E @ 1000 ppm (93.75%). The standard fungicides copper oxychloride 50 WP @ 2500 ppm reported 85.19% followed by carbendazim 50 WP @ 500 ppm 72.69% growth inhibition. The findings of the present study are in agreement with Umer et al. (2012); Ramy and Osama (2013); Surega et al. (2015) who reported antifungal properties of plant mediated silver and copper nanoparticles against *Alternaria alternata*, *Fusarium oxysporum* and *Penicillium expansum*.

The results indicate that the growth of *C. capsici* is checked by all the concentrations of nanoparticles. The results further reveal better inhibition of test fungus can be achieved when nanoparticles are applied “before-inoculation” of pathogen as compared to “after-inoculation”. The results clearly confirm that the nanoparticles have the excellent quality to arrest growth of *C. capsici*. Lamsal et al. (2011b) reported better effectiveness of silver nanoparticles against pepper anthracnose when applied before disease appearance than after symptoms.

The CuNP-M showed complete inhibition of pathogen on fruits @ 1000 ppm in both applications and @ 500 ppm applied “before-inoculation” of pathogen. Nanoparticles showed better results in enhancing the incubation period of pathogen on fruits among all

Table 4
Effect of copper nanoparticles on lesion size caused by *C. capsici* chilli under Detached fruit method.

| Treatments | Lesion size (mm) | | | | | | | |
|--|--|-----------------|-----------------|------------------------|---|-----------------|-----------------|------------------------|
| | Nanoparticles applied before inoculation | | | | Nanoparticles applied after inoculation | | | |
| | 250 ppm | 500 ppm | 1000 ppm | Mean | 250 ppm | 500 ppm | 1000 ppm | Mean |
| CuNP-E | 20.20 (4.60) | 12.70 (3.69) | 5.26 (2.32) | 12.72 (3.53) | 21.70 (4.76) | 17.70 (4.32) | 7.50 (2.66) | 15.63 (3.91) |
| CuNP-M | 6.30 (2.70) | 0.00 (1.00) | 0.00 (1.00) | 2.10 (1.56) | 14.80 (3.97) | 10.50 (3.39) | 0.00 (1.00) | 8.43 (2.78) |
| Carbendazim 50 WP (500 ppm) | 13.50 (3.80) | 13.50 (3.80) | 13.50 (3.80) | 13.50 (3.80) | 22.30 (4.83) | 22.30 (4.83) | 22.30 (4.83) | 22.30 (4.83) |
| Copper oxychloride 50 WP (2500 ppm) | 15.50 (4.06) | 15.50 (4.06) | 15.50 (4.06) | 15.50 (4.06) | 22.70 (4.86) | 22.70 (4.86) | 22.70 (4.86) | 22.70 (4.86) |
| Control | 65.30 (8.14) | 65.30 (8.14) | 65.30 (8.14) | 65.30 (8.14) | 65.30 (8.14) | 65.30 (8.14) | 65.30 (8.14) | 65.30 (8.14) |
| Mean | 24.16 | 21.40 | 19.91 | 21.82 | 29.36 | 27.70 | 23.56 | 26.87 |

CD $p \leq 0.05$
Inoculation Time **0.14** (Treatments × Concentrations × Inoculation time) 0.57

Figures in parenthesis are square root transformed values.

the treatments because nanoparticles acted as protectants in “before-inoculation” and as eradicant in “after-inoculation” applications. The nanoparticles were evaluated on detached fruits regarding the number of lesions and lesion size. Treatment with nanoparticles “before-inoculation” of pathogen showed better results than “after-inoculation” of pathogen. CuNP-M showed complete inhibition of pathogen on fruits in both the applications at 1000 ppm and @ 500 ppm, when applied “before-inoculation” of pathogen. All the nanoparticles were significantly superior over the standard fungicides in both applications. By and large, pre-inoculation treatments of nanoparticles as well as fungicides were superior over post-inoculation treatments. However, nanoparticles at concentrations as low as 500 ppm and 1000 ppm were more effective than standard fungicides tested. Nanoparticles not only prolonged the incubation period but also reduced the number and length of lesions. The possible better results of the nanoparticle may be because of their size. Applications and success of nanoparticles in other areas has been proved and use of nanoparticles for control of plant diseases is in infancy. Present investigation is a beginning in this direction under Kashmir conditions.

5. Conclusion

Present study demonstrates simple, advantageous and green method for the synthesis of copper nanoparticle by employing plant extracts of eucalyptus (*Eucalyptus globulus* L.) and mint (*Mentha piperita*). The reduction of the metal ions through plant extracts leads to the development of well-defined copper nanoparticles.

Nanoparticles were evaluated at different concentrations for their effectiveness against *C. capsici*. CuNP-M proved to be highly efficacious in limiting the mycelial growth of the test fungus. With the increase in concentration of nanoparticles from 25 to 1000 ppm mycelial growth of test fungus decreased significantly. Nanoparticles also proved to be highly efficacious in limiting the pathogen on detached fruits. Comparatively better results were observed when nanoparticles were applied “before-inoculation” than “after-inoculation” of the pathogen. The CuNP-M nanoparticles with 500 and 1000 ppm completely inhibited growth of pathogen on fruits when applied “before-inoculation” of pathogen. All the nanoparticles showed better results than the standard fungicides and control in both the methods of “poison food technique” and “detached fruit technique”.

6. Declarations

Ethics approval: Not Applicable

Consent to participate: All authors consent to participate in this manuscript

Consent for publication: All authors consent to publish this manuscript in Saudi journal of Biological Science

Availability of data and material: Data will be available on request to corresponding or first author

Code availability: Not Applicable

Authors' contributions: Krishnanand Shivanand Iliger, Tariq Ahmad Sofi, Nazir Ahmad Bhat drafted and performed the experiments. Farooq Ahmad Ahanger, Jagan Chandra Sekhar analyzed the data. Asma A. Al-Huqail, Ahmed Zohier Elhendi, Faheema Khan did the statistical analysis and revised the manuscript. All authors read and approve the same for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors acknowledge the support and are thankful to Centre director and director of UGC-DAE-Consortium for Scientific Research Indore Centre MP India for permission to work at the centre. Authors also acknowledge and are grateful to Dr. Mukul Gupta for XRD, Dr. D.M. Phase for SEM and Dr. N. P. Lalla for TEM studies at Indore centre. The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP-2020/186), King Saud University, Riyadh, Saudi Arabia.

References

- Akhavan, O., Ghaderi, E., 2010. Cu and CuO nanoparticles immobilized by silica thin films as antibacterial materials and photocatalysts. *Surf. Coat. Technol.* 205, 219–223. <https://doi.org/10.1016/j.surfcoat.2010.06.036>.
- Ankamwar, B., Damle, C., Ahmad, A., Sastry, M., 2005. Biosynthesis of gold and silver nanoparticles using *emblica officinalis* fruit extract, their phase transfer and transmetalation in an organic solution. *J. Nanosci. Nanotechnol.* 5, 1665–1671. <https://doi.org/10.1166/jnn.2005.184>.
- Arya, V., 2010. Living Systems: eco-friendly nanofactories. *Dig. J. Nanomater. Biostruct. (DJNB)*, 5.
- Awwad, A.M., 2015. Novel approach for synthesis sulfur (S-NPs) nanoparticles using *Albizia Julibrissin* fruits extract. *Adv. Mater. Lett.* 6, 432–435. <https://doi.org/10.5185/amlett.2015.5792>.
- Bagri, R., Choudhary, S., Rai, P., 2004. Management of fruit rot of chilli with different plant products. *Indian Phytopathol.* 57, 107–109.
- Bailey, J., O'connell, R., Pring, R., Nash, C., 1992. Infection strategies of *Colletotrichum* species. In: *Colletotrichum: Biology, Pathology and Control*, CAB International, Walling Ford, UK, pp. 88–120.
- Chauhan, Y., Patel, R., Chaudhary, R., Rathod, N., 2014. Efficacy of different fungicides for the management of chilli anthracnose caused by *Colletotrichum capsici*. *The Bioscan* 9, 399–402.
- Choudhery, 1957. Studies on the development and control of fruit rot of chillies. *Indian Phytopathol.* 10, 55–62.
- Dash, P.K., Balto, Y., 2011. Generation of nano-copper particles through wire explosion method and its characterization. *Res. J. Nanosci. Nanotechnol.* 1, 25–33. <https://doi.org/10.3923/rjnn.2011.25.33>.
- Dastur, J.F., 1921. Dieback of chillies in Bihar. *Imperial Dep. Agronomy India* 11, 129–144.
- Doolittle, S.P., 1954. Disease of Peppers. In: *Plant Disease. Year book of Agriculture*, U. S. D. A., pp. 466–469.
- Dwivedi, A.D., Gopal, K., 2010. Biosynthesis of silver and gold nanoparticles using *Chenopodium album* leaf extract. *Colloids Surf. Physicochem. Eng. Asp.* 369, 27–33.
- G/Egziabher, H.M., 2012. Biosynthesis of Copper Nanoparticles using Some Plant Leaf Extracts, Their Characterization and Anti-bacterial Activity in Physical Chemistry. Haramaya University.
- Gomez, K.A., Gomez, A.A., 1984. *Statistical Procedures for Agricultural Research*. John Wiley & Sons.
- Halsted, B.D., 1885. A new anthracnose of peppers. *Bull. Torrey Bot. Club* 18, 14–15. <https://doi.org/10.2307/2477422>.
- HariPrasad, S., Bai, G.S., Santhoshkumar, J., Madhu, C., Sravani, D., 2016. Green synthesis of copper nanoparticles by *Areva lanata* leaves extract and their antimicrobial activities. *Int. J. Chem-Tech. Res.* 9, 98–105.
- Hassan, M.S., Amna, T., Yang, O.B., El-Newehy, M.H., Al-Deyab, S.S., Khil, M.-S., 2012. Smart copper oxide nanocrystals: synthesis, characterization, electrochemical and potent antibacterial activity. *Colloids Surf. B. Biointerfaces* 97, 201–206. <https://doi.org/10.1016/j.colsurf.2012.04.032>.
- Hutchison, J.E., 2008. Greener nanoscience: a proactive approach to advancing applications and reducing implications of nanotechnology. *ACS Nano* 2, 395–402. <https://doi.org/10.1021/nn800131j>.
- Khodashenas, B., Ghorbani, H.R., 2019. Synthesis of silver nanoparticles with different shapes. *Arab. J. Chem.* 12, 1823–1838. <https://doi.org/10.1016/j.arabj.2014.12.014>.
- Kim, Y.H., Lee, D.K., Jo, B.G., Jeong, J.H., Kang, Y.S., 2006. Synthesis of oleate capped Cu nanoparticles by thermal decomposition. *Colloids Surf. Physicochem. Eng. Asp.* 284–285, 364–368. <https://doi.org/10.1016/j.colsurfa.2005.10.067>.
- Krithiga, N., Jayachitra, A., Rajalakshmi, A., 2013. Synthesis, characterization and analysis of the effect of copper oxide nanoparticles in biological systems. *Ind. J. Ns.* 1, 6–15.
- Kulkarni, V., Suryawasanshi, S., Kulkarni, P., 2015. Biosynthesis of copper nanoparticles using aqueous extract of *Eucalyptus sp.* plant leaves. *Curr. Sci.* 109, 255–257.
- Lamsal, K., Kim, S.-W., Jung, J.H., Kim, Y.S., Kim, K.S., Lee, Y.S., 2011a. Inhibition effects of silver nanoparticles against powdery mildews on cucumber and pumpkin. *Mycobiology* 39, 26–32. <https://doi.org/10.4489/MYCO.2011.39.1.026>.
- Lamsal, K., Kim, S.W., Jung, J.H., Kim, Y.S., Kim, K.S., Lee, Y.S., 2011b. Application of silver nanoparticles for the control of *colletotrichum* species in vitro and pepper

- anthracnose disease in field. *Mycobiology* 39, 194–199. <https://doi.org/10.5941/MYCO.2011.39.3.194>.
- Manzoor, A.P., Razdan, V.K., Sofi, T.A., 2013. Comparative study of different fungi associated with fruit rot of chilli and screening of chilli germplasm against *Colletotrichum capsici*. *Int. J. Agric. Crop Sci.* 5 (7), 723–730.
- Mohammed Fayaz, A., Girilal, M., Rahman, M., Venkatesan, R., Kalaichelvan, P.T., 2011. Biosynthesis of silver and gold nanoparticles using thermophilic bacterium *Geobacillus stearothermophilus*. *Process Biochem.* 46, 1958–1962. <https://doi.org/10.1016/j.procbio.2011.07.003>.
- Padil, V.V.T., Černík, M., 2013. Green synthesis of copper oxide nanoparticles using gum karaya as a biotemplate and their antibacterial application. *Int. J. Nanomed.* 8, 889.
- Pakdevaraporn, P., Wasee, S., Taylor, P.W.J., Mongkolporn, O., 2005. Inheritance of resistance to anthracnose caused by *Colletotrichum capsici* in *Capsicum*. *Plant Breed.* 124, 206–208. <https://doi.org/10.1111/j.1439-0523.2004.01065.x>.
- Perelshtein, I., Applerot, G., Perkash, N., Wehrsuetz-Sigl, E., Hasmann, A., Guebitz, G., Gedanken, A., 2009. CuO–cotton nanocomposite: Formation, morphology, and antibacterial activity. *Surf. Coat. Technol.* 204, 54–57. <https://doi.org/10.1016/j.surfcoat.2009.06.028>.
- Prakash, N., Jayapradeep, S., Sudha, P., 2009. Investigation of antimicrobial properties of silver and copper nanoparticles encapsulated in chitosan. In: *Conference Proceedings 1st International Conference on Nanostructured Materials and Nanocomposites*, pp. 6–8.
- Ramy, S.Y., Osama, F.A., 2013. In vitro study of the antifungal efficacy of zinc oxide nanoparticles against *Fusarium oxysporum* and *Penicillium expansum*. *Afr. J. Microbiol. Res.* 7, 1917–1923. <https://doi.org/10.5897/ajmr2013.5668>.
- Rao, L., Reddy, N.K., Coulombe, S., Meunier, J.-L., Munz, R.J., 2006. Carbon nanotubes as nanoparticles collector. *J. Nanopart. Res.* 9, 689–695. <https://doi.org/10.1007/s11051-006-9175-5>.
- Shankar, S.S., Rai, A., Ahmad, A., Sastry, M., 2004. Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using Neem (*Azadirachta indica*) leaf broth. *J. Colloid Interface Sci.* 275, 496–502. <https://doi.org/10.1016/j.jcis.2004.03.003>.
- Sharma, P.N., Kaur, M., Sharma, O.P., Sharma, P., Pathania, A., 2005. Morphological, pathological and molecular variability in *Colletotrichum capsici*, the cause of fruit rot of chillies in the subtropical region of North-western India. *J. Phytopathol.* 153, 232–237. <https://doi.org/10.1111/j.1439-0434.2005.00959.x>.
- Sharville, E.G., 1961. The nature and use of modern fungicides. Burges publication co. Minnesota, USA, pp. 308.
- Subhankari, I., Nayak, P., 2013. Antimicrobial activity of copper nanoparticles synthesised by ginger (*Zingiber officinale*) extract. *World J. Nano Sci. Technol.* 2, 10–13.
- Suleiman, M., Al Ali, A., Hussein, A., Hammouti, B., Hadda, T.B., Warad, I., 2013. Sulfur nanoparticles: synthesis, characterizations and their applications. *J. Mater. Environ. Sci.* 4, 1029–1033.
- Surega, R., Ramakrishnan, S., Anita, B., Gunasekaran, K., Nakkeeran, S., 2015. Antifungal effect of plant mediated silver nanoparticles against *Fusarium oxysporum f sp lycopersici*. *Int. J. Trop. Agric.* 33, 157–160.
- Sydow, H., 1913. *Vermicularia capsici*. *Ann. Rev. Mycol. Berlin* 11, 329.
- Tang, X., Ren, L., Sun, L., Tian, W., Cao, M., Hu, C., 2006. A solvothermal route to Cu₂O nanocubes and Cu nanoparticles. *Chem. Res. Chin. Univ.* 22, 547–551. [https://doi.org/10.1016/s1005-9040\(06\)60159-1](https://doi.org/10.1016/s1005-9040(06)60159-1).
- Thind, T., Jhooty, J., 1985. Relative prevalence of fungal diseases of chilli fruits in Punjab. *Indian J. Mycol. Plant Pathol.* 15, 305–307.
- Umer, A., Naveed, S., Ramzan, N., Rafique, M.S., 2012. Selection of a suitable method for the synthesis of copper nanoparticles. *NANO* 7, 1230005.
- Veerasamy, R., Xin, T.Z., Gunasagaran, S., Xiang, T.F.W., Yang, E.F.C., Jeyakumar, N., Dhanaraj, S.A., 2011. Biosynthesis of silver nanoparticles using mangosteen leaf extract and evaluation of their antimicrobial activities. *J. Saudi Chem. Soc.* 15, 113–120. <https://doi.org/10.1016/j.jscs.2010.06.004>.
- Viet, P.V., Nguyen, H.T., Cao, T.M., Hieu, L.V., 2016. *Fusarium* antifungal activities of copper nanoparticles synthesized by a chemical reduction method. *J. Nanomater.* 2016, 1–7. <https://doi.org/10.1155/2016/1957612>.
- Zhang, J.-Z., 2008. Anthracnose of persimmon caused by *Colletotrichum gloeosporioides* in China. *Asian Aust. J. Sci. Biotechnol.* 2, 50–54.