Pharm Res (2020) 37:246
https://doi.org/10.1007/s11095-020-02966-x

RESEARCH PAPER

Enhanced Biosynthesis Synthesis of Copper Oxide Nanoparticles (CuO-NPs) for their Antifungal Activity Toxicity against Major Phyto-Pathogens of Apple Orchards

H. Ahmad[1] • K. Venugopal[1] • A. H. Bhat[2] • K. Kavitha[3] • A. Ramanan[4] • K. Rajagopal[5] • R. Srinivasan[4] • E. Manikandan[1,6,7,8]

Received: 1 June 2020 / Accepted: 27 October 2020

ABSTRACT

Purpose The present study made an attempt to develop copper nanoparticles (Cu-NP) with antifungal property using green synthesis method. Copper oxide nanoparticles (CuO-NPs) botanically synthesized using Neem leaf extract (*Azadirachta indica* A. Juss) were characterized by using different techniques like; UV–visible spectrophotometry, FTIR, XRD, SEM and TEM.

Methods Materials were chosen the disease free and fresh *Azadirachta indica* A. Juss were collected and identified at Center of Biodiversity and Taxonomy. The plant samples were vigorously washed with distilled water then shade dried followed by sterilization with 0.1% mercuric chloride for 20 s and again it was washed with distilled water. 15 g powder form of plant material was added to 200 ml double distilled, CO_2 free and deionized water and kept in shaker at 80°C and 1500 rpm for six hours. After agitation, the extract was separated by regular centrifugation at 10,000 rpm followed by filtration by using whatmann filter paper. The final volume of 100 ml of supernatant was collected as pure extract and stored in cool place for further use.

Results The final results confirm a significant inhibition of CuO-NPs for the test fungi. Additionally, CuO-NPs demonstrated an enhanced effect when combined with Neem leaf extract. A total of 20–30% improvement in activity was noticed after combination, which correlates with commonly used synthetic fungicides. The toxicity results reveal that *A. indica* extract and their combined fractions with CuO-NP were less toxic to the test seeds of experimental plant while as bulk Cu followed by biosynthesized CuO-NPs influenced the germination rate as compared to control pots.

Conclusions The study drops a concern of research and offers a promising route of developing Copper based green fungicides that can help to combat with modern issues of synthetic fungicides. An average size of 80 ± 15 nm monoclinic cupric oxide (CuO) and cubic cuprous oxides (Cu_2O) nanocrystals that existed in mixed form were successfully developed.

KEY WORDS biomedical activity · electron microscopic · green route · nanoparticles · spectroscopic assay

✉ H. Ahmad
hhiillaallbiotech@gmail.com

✉ E. Manikandan
maniphysics@gmail.com

[1] Department of Biotechnology, Vels Institute of Science Technology & Advanced Studies (VISTAS), Pallavarm Chennai, Tamil Nadu (TN) 600117, India

[2] Division of Plant Pathology, SK University of Agriculture Science and Technology Srinagar, Jammu & Kashmir 190006, India

[3] Department of Microbiology, Madras Christian College (MCC), Tambaram Chennai, TN 600059, India

[4] Tamil Nadu State Council for Science & Technology (TNSCST), DOTE Campus Chennai, TN 600025, India

[5] Department of Botany (Plant Biology and Plant Biotechnology) Ramakrishna Mission Vivekananda College, Chennai, India

[6] Department of Physics, Thiruvalluvar University College for Arts & Science (TUCAS), Thennangur, (Affiliated: Thiruvalluvar University, Vellore), Thennangur, Tamil Nadu 604408, India

[7] UNESCO-UNISA AFNET in Nanosciences/Nanotechnology, College of Graduate Studies, University of South Africa, Muckleneuk Ridge PO BOX 392, Pretoria, South Africa

[8] Avanz Bio Private Ltd., East Tambaram, MES Road, Near to MCC College Chennai, Tamil Nadu 600059, India

INTRODUCTION

Phytosynthesis of surface plasmon resonance (SPR) based noble metal and oxide nanoparticles (NPs) offers a perspective to enrich their application in various activities like textile industry, biosensing, photocatalysis, electronics, laundry additives, room sprays, dyes, water cleaners, medicine, cancer biology

and food technology. The study of metal nanoparticle applications in agriculture is the matter of interest because this field is already concerned with the use of heavy metals as pesticides (1). Thus, the use of nanotechnology procedure will of course help to reduce the heavy metal quantity in pesticides after they are built in a way that all the required properties are possessed (2–5). However, Nanoparticles provide a significant means for the growth of pesticides in a controlled manner with high site specificity that can help to reduce collateral damage (1). Phytosynthetic route of nanoparticle synthesis has many advantages over other biological processes as it is helpful the way it eliminates the total development of cell culture maintenance and also it is easy to utilize this process for mass production of nanoparticle synthesis (6,7). Copper nanoparticles (Cu-NPs) with significant antimicrobial properties have been previously synthesized using plant extract (8–12). On the other hand, the use of Neem leaf extract (*Azadirachta indica* A. Juss) as biogenic source for nanoparticle synthesis (13) and a suitable pesticide agent has also been reviewed (14–20).

While considering the pesticide role of copper compounds (like copper oxychloride), copper nanoparticles as well as Neem extract were focused in the present study for their evaluation of individual and synergistic way of how the formula can be useful to generate a bio-pesticide with less quantity of heavy metals.

METHODOLOGY

Plant Extract Preparation

The disease free and fresh leaves of *Azadirachta indica* A. Juss were collected within the main campus of Vels University-Chennai, and was identified at Center of Biodiversity and Taxonomy, Department of Botany, University of Kashmir (India). The plant sample was vigorously washed with distilled water then shade dried followed by sterilization with 0.1% mercuric chloride for 20 s and again it was washed with distilled water. 15 g powder form of plant material was added to 200 ml double distilled, CO_2 free and deionized water and kept in shaker at 80°C and 1500 rpm for six hours (53). After agitation, the extract was separated by regular centrifugation at 10,000 rpm followed by filtration by using whatman filter paper. The final volume of 100 ml of supernatant was collected as pure extract and stored in cool place for further use.

Phytosynthesis of Copper Oxide Nanoparticles (CuO-NPs)

Copper oxide nanoparticles were synthesized according to the procedure provided recently by Abboud, Y. *et al* (21). 10% plant extract was added to 1 mM copper(II) sulfate solution in the ratio of 2:1. The solution was kept for shaking (2000 rpm) at 70 to 90°C for 24 h. The color change was monitored time to time. Deep blue solution was seen to gradually become colorless changed to brick red coloration followed by dark solution. This color change indicates formation of CuO nanoparticles (21).

Detection of CuO-NPs by UV–Vis Spectrophotometer

The primary detection of absorption spectrum of CuO-NPs was done by using UV–vis spectrophotometer (UV-260 Shimadzu) observable in 200–700 nm range to check for surface resonance plasmon absorption transitions. The measurements were conducted with highly diluted solutions.

Characterization of CuO-NPs by Different Methods

FT-IR experiments were done using IR spectrophotometer (Bruker D8). All samples were examined and compare molecular chemical functional groups of vibrational bonds of neem extract and CuO-NPs by using KBr pellet in the range of 400–4000 cm^{-1}. X-Ray Diffraction (XRD) instrument (Bruker D8) was operated at 40 kV (30 mA) with Cu K_α radiation in θ to 2θ degree configurations. Further, the SEM (scanning electron microscope) studies (JEOL JSM-5600) were performed for characterization of shape of CuO-NPs by placing the samples on carbon tape coated standard grid. TEM images (Tecnai G2–20) were observed to get the accurate morphology with size and shape of nanoparticles. Colloidal nanoparticles were drop was put onto carbon-coated copper grid for TEM investigation.

Fungal Culture

Three pathogenic fungal species of apple orchards namely *Alternaria mali*, *Diplodia seriata* and *Botryosphaeria dothidea* were obtained from Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology, Srinagar, India. The fungal cultures were grown at normal temperature and then maintained at low temperature onto PDA slants. All fungal growth inhibition experiments were done on PDA media previously poisoned with test fractions. For proper dispersal, the media was shaken well at 150 rpm and then incubated at 25°C for 72 h.

Assessment of Anti-Fungal Activity of CuO-NPs

CuO-NPs were evaluated against *Alternaria mali, Diplodia seriata* and *Botryosphaeria dothidea* for estimation of their anti-fungal activity by using modified food poisoning technique (FPT) as described by some researchers (22). A volume of 0.5 ml of each 0.05, 0.10, 0.25, and 1 mg per milliliter concentrations were aseptically poured into the petriplates loaded with 9.5 ml of molten PDA and were thoroughly mixed to achieve a uniform solution. In control, Mancozeb a commercial fungicide was used. In order to take comparative studies, copper

sulphate and plant extract were also evaluated along with. An inoculum disc (5 mm) of each test fungi (slashed from a growing mycelium) was inoculated at the center of the media and was incubated at 25 ± 2°C. Using micro-scale, the average size (diameter) of the growing mycelium was measured on the 10th day after incubation and percent mycelial growth inhibition (PMGI) was measured using the formula;

Mycelial growth inhibition (%) = $(g^c - g^t)/g^c \times 100$
where, g^c = growth of mycelial colony in control and g^t = growth of mycelial colony in treatment.

Statistical Analysis

All experiments were done in triplicate and the resulting fungal growths were analyzed as mean ± standard deviation ($n = 4$).

Microscopic Observation of Fungi Treated with Nanoparticles

Light Microscopy

To investigate the effect of nanoparticles on the growth of mycelia, fungal mass was collected by hand picking using sterilized cork borer from the surface of petri plates containing *Diplodia seriata* culture developed on PDA media treated with 50 ppm CuNP synthesized by using *A. indica*. Concurrently, the fungal plates treated with tap water were preserved as control samples. Morphology of the fungal cells was observed under a florescence light microscope equipped with a digital camera (23).

Scanning Electron Microscopy (SEM) of Fungal Mycelia

In order to see microscopic effect of nanoparticles on test fungi, healthy hyphae of test fungi grown on PDA plates were sprayed with 50 ppm of CuO-NPs solution synthesized by using *A. indica*, and incubated for 7 days. The specimen was washed with distilled water following dehydration in a graded ethanol arrangement up to 100%. SEM studies of treated and untreated test fungi were carried out by using scanning electron microscope (JEOL JSM 5600). The sample was placed on a carbon-coated copper grid operating at 80 kV for SEM studies (8).

Phytotoxicity Study of Copper Oxide Nanoparticles (CuO-NPs)

Efficacy of CuO-NPs, their bulk material (ion solution) along with leaf extract of *A. indica* were investigated for their impact on seed and root parameters of *Vigna radiate* (seed germination test) and *Allium cepa* (Allium test) respectively while considering the methodology described earlier (24,25).

Preparation of Suspension Solutions

The equivalent masses of bio-synthesized CuO-NPs, their bulk materials (i.e. ion solutions of Cu salt) and leaf extract of *A. indica* were ground and dissolved in DDW to obtain 0.1 mg/mL, 1.0 mg/mL and 2.5 mg/mL concentrations at room temperature under aseptic conditions. To obtain uniform solutions, the samples were dispersed by ultrasonic vibration (100 W, 40 kHz) for 30 min and manually agitated prior to use (26).

Seed Germination Inhibition Test

The seeds germination inhibition test of nanoparticles was carried out on seeds of *Vignaradiata* (Fabaceae) as test plant. Healthy seeds of *Vignaradiata* (weighing 47.82 ± 1.50 mg) were obtained from agricultural out let of Chennai city and were identified by using standard procedure. A voucher specimen of the test seeds was deposited at Department of Biotechnology, Vels University (Voucher No. VUCC0037).

Seed Surface Sterilization

Sterilization was carried out by soaking the healthy seeds in 10% sodium hypochlorite for 10 min and then rinsed thoroughly with DI-water to remove adsorbed Sodium hypochlorite present on the outer coat of the seeds (USEPA, 1996). The seeds were air-dried for several days and were then stored at room temperature (25).

Seed Viability Test

Seed viability is the most fundamental condition for Seed germination. 100 seeds were sterilized and germinated in a petri plate with moist cellulose paper and was incubated in dark for 48 h. This was done in triplicates and the numbers of seeds germinated were counted. The average germination rates of seeds were greater than 90% as shown by a preliminary study. Seeds were kept in dry place in the dark under room temperature before use.

Seed Germination Assay

The effect of copper nanoparticles and their corresponding bulk materials along with aqueous extract of *A. indica* were tested for their impact on seed germination and root elongation of *Vigna radiate* seeds. For germination study, seeds were placed in Petri dishes (35 mm × 10 mm); each dish was amended with 3 mL of copper nanoparticle solution, bulk copper solution and plant extract. The seeds were placed per dish and 1 cm or larger distance between each seed (24,27). For the elongation assay, seeds were pre-germinated and those with a radical of approximately 0.5 mm were

selected. There were five replicate dishes for each treatment. The seeds were allowed to grow for five days in the dark under room temperature, until more than 80% of the control seeds had germinated and when the roots reached up to 20 mm in length in control, the germination process was halted and percentage of seed germination was calculated along with control seeds.

Allium Test

Test Plant

Healthy bulbs of *Allium cepa var. aggregatum* were obtained from agricultural out let of Chennai city and were identified by using standard procedure. A voucher specimen of the test plant was deposited at Department of Biotechnology, Vels University (Voucher No. VUCC0036).

Toxicity Assay

The *Allium* toxicity assay was conducted according to the procedure described by Akdeniz and Ali (28). Healthy bulbs of *Allium cepa var. aggregatum* (200–250 g) were carefully un-scaled and cultivated on top of test tubes filled with DDW at room temperature (28 ± 0.5°C) and water supply was maintained in every 24 h. When the roots reached 2–3 cm in length the DDW was exchanged with treatments (suspension solutions of copper nanoparticles, corresponding bulk copper suspension and test plant extract) of different concentrations as discussed early. Test tubes filled with tap water were considered as control. There were five replicate test tubes for each treatment. The test tubes were kept in an incubator at 24 ± 2°C. After 72 h the roots were counted and their lengths were measured for each onion. The interaction time was 4 h in accordance with a protocol standardized by Fiskesjo (29). The data was collected as mean ± SD (standard deviation) of triplicate samples with 10 seeds in each trail for *Vigna radiate* seeds. While as the values were given as mean ± SD (standard deviation) of triplicate samples with four bulbs in each trail for *A. cepa*.

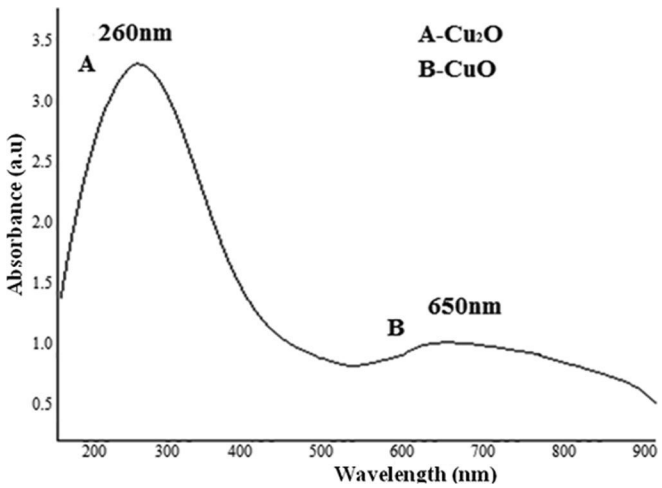

Fig. 1 UV-visible spectroscopy of phyto-synthesized Copper nanoparticles using *A. indica* leaf extract.

RESULTS

UV–Visible Analysis

The initial detection of CuO-NPs was seen as a color change from deep blue to colorless solution and then changed slowly to reddish and finally to dark black which happens due to phenomenon of Surface Plasmon Resonance. The surface plasmon resonance of CuO-NPs revealed a well absorption (resonance) peak at about 260 nm and a weak absorbance at about 650 nm shown in Fig. 1 which confirms the formation of cuprous oxide nanoparticles (Cu_2O) and cupric oxide (CuO) nanoparticles respectively.

Characterization of CuO-NPs by Different Methods

Microscopic Analysis

The morphology and size of synthesized CuONPs was elucidated with the help of SEM and TEM. Scanning electron micrographs shown in Fig. 2 confirm CuO-NPs have irregular shaped clusters form produced in large number. Transmission electron microscopy focused further insight of the morphology and size of CuO-NPs. Fig. 3 shows TEM image recorded from the CuO-NPs. Upon the analysis of data obtained from TEM, the average size of nanoparticles was confirmed to be approximately 80 ± 15 nm.

X-Ray Diffraction (XRD)

The formation of Copper nanoparticles was further confirmed by XRD examination as mentioned in Fig. 4. Two distinct crystalline forms of mono-clinic cupric oxide (CuO) and cubic cuprous oxides (Cu_2O) nanocrystals existed. CuO and Cu_2O were identified as confirmed with the standard peaks with 2θ values. The diffraction peaks for CuO exist at 32.8°, 35.9°,39.1°, 46.3°, 49.1°, 52.9°, 58.7°, 66.6°, 68.3° and 72.6° which are indexed at (−111), (002), (111), (−112), (−202), (020), (202), (−311), (113), and (311) respectively while as peaks for Cu_2O exist at 29.4°, 36.8°, 42.1°, and 61.9° were monitored and assigned as (110), (111), (200), and (220) respectively. These results display that the amount of cupric oxide (CuO) was greater than that of cuprous oxides (Cu_2O). The relative higher intensities at 32.8°, 35.9° and 46.3° for cupric oxide indicate their crystalline nature and bulk proportion in the total mixture. In order to estimate the average particle size of CuO-NPs, Scherrer's formula was used.

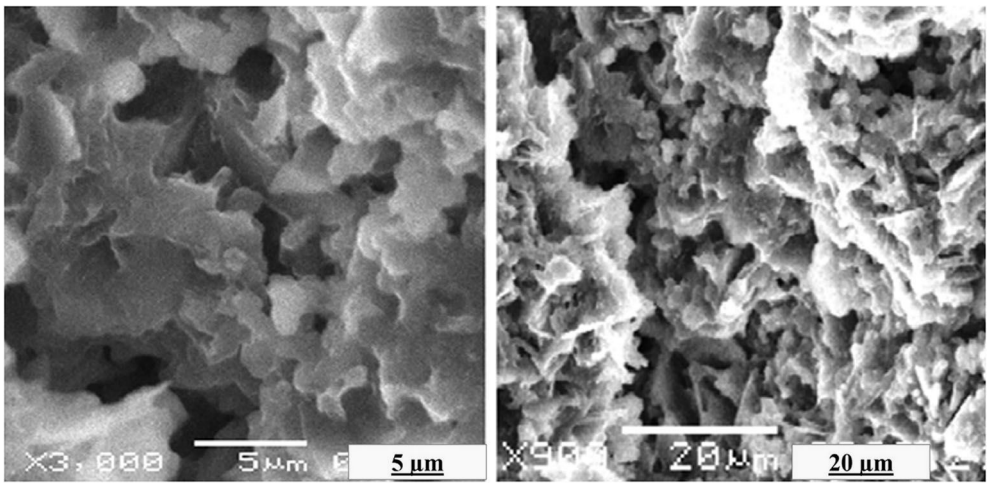

Fig. 2 SEM images of phyto-synthesized Copper nanoparticles using *A. indica* leaf extract.

$$D = \frac{k\lambda}{\beta\cos\theta} \quad (1)$$

where, D = diameter, k = constant (equals 1), λ = wavelength of X-ray source (0.1541 nm), β is the full width at half maximum (FWHM) and θ, the half diffraction angle. After calculation, the average particle size of cupric oxide and cuprous oxide obtained from XRD was found to be approximately 80 ± 15 nm, further compared with TEM analysis.

Fourier-Transform Infrared Spectroscopy (FT-IR)

IR measurements of aqueous neem extract and dried CuO-NPs (Fig. 5) were carried out to find out the related bio-molecules mainly responsible for reduction, capping and stabilization of the bio-reduced CuO-NPs. Our FTIR study revealed the presence of absorption peaks mainly at 3408, 2910, 1719, 1615, 1037 and 680 cm^{-1}. While the phytochemistry of *A. indica*is compared, different views can be drawn about the formation of additional peaks in synthesized nanoparticles. Terpenoids from *A. indica* are identified as strongest hydroxyl peaks at 3408 cm^{-1}, α, β-unsaturated ketone band at 1719 cm^{-1}, olefinic band at 1615 cm^{-1}. Secondary alcohol functional bands at 1037 cm^{-1}, bands at 2910 cm^{-1} attribute to aliphatic functional group of C–H stretching and bending modes as seen earlier by some researchers (30,31). The absorption peak at 1719 cm^{-1} can be due to presence of CH_3CO (j = 2.05 Hz) previously analyzed using NMR study of nimanol which contributes to the major part of active components of Neem leaf extract (32). Moreover, the absorption peaks at 3408, 1615, 1037 cm^{-1} observed after synthesis correspond to O-H, C=C and C-O stretches respectively (21). However, the broad absorption bands of 1719 cm^{-1} (CH_3CO group) which lie between 2800 and 4000 cm^{-1} mainly ascribed to O-H and C − O groups situated at the surface of copper oxide nanostructure crystals.

Anti-Fungal Assay

Impact of CuO-NPs on Mycelial Growth of Test Fungi

Anti-fungal activity of CuO-NPs synthesized using *A.indica* was determined against three plant pathogenic fungi viz., *Alternaria*

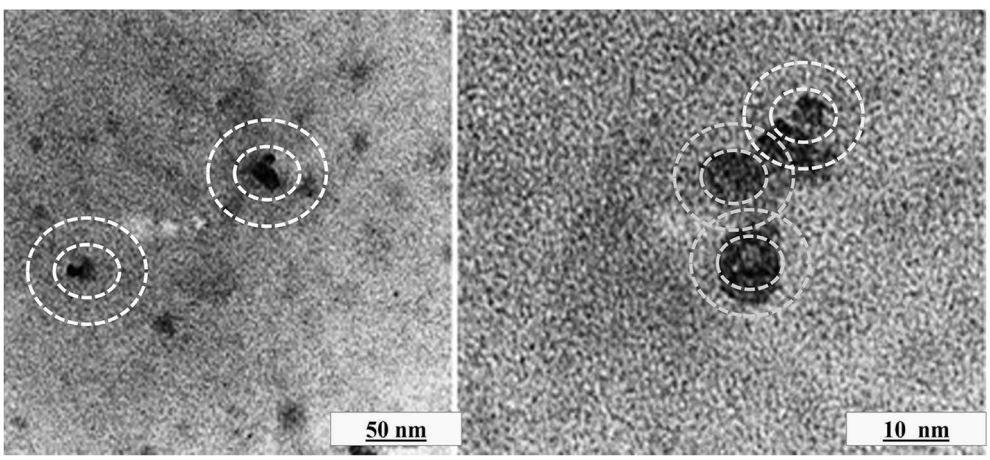

Fig. 3 TEM images of phyto-synthesized Copper nanoparticles using *A. indica* leaf extract.

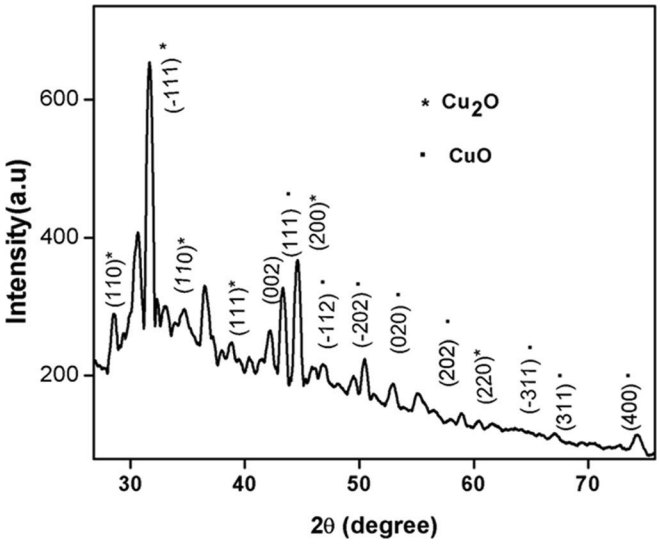

Fig. 4 XRD pattern of phyto-synthesized Copper nanoparticles using *A. indica* leaf extract.

mali, *Diplodia seriata* and *Botryosphaeria dothidea* dominant in apple orchards of Kashmir valley in India. The results of their food poisoning technique reveal that all the fungi were inhibited by total fractions like; (i) Aqueous neem extract, (ii) Copper sulphate, (iii) CuO-NPs, and combination of (i) and (iii) as is evident from Table I. The rate of growth reduction was directly proportional to the concentration of tested fraction in the medium. *Alternaria mali*was the most altered fungi followed by *Botryosphaeria dothidea*. Above 40% of mycelial growth inhibition was seen for concentration of 1 g/ml of all the fractions (Plate.1-a). Moreover, Copper nanoparticles showed enhanced activity when used in combination with the Neem extract even at lower concentrations (Plate.1-b). The active compounds in neem extract which are supposed to be main chemicals taking part in reduction of Cu^{+2} ions to Cu^{0} remain unaltered while mixing with CuO-NPs. Thus, CuO-NPs along with these active compounds act upon the target fungi individually. This phenomenon of action provides more aberration sites in fungi to kill it.

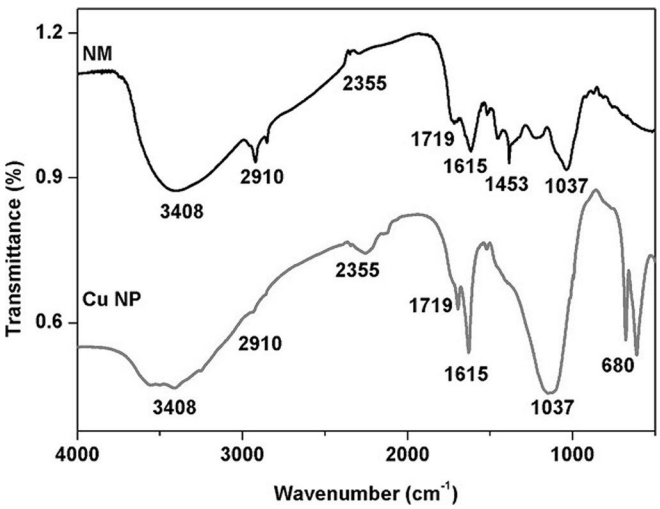

Fig. 5 IR spectra of phyto-synthesized Copper nanoparticles and *A. indica* leaf extract.

Microscopic Observation of the Effect of the CuO-NPs Fractions on Mycelial Growth of Test Fungi

Light Microscopy

The effect of the CuO-NPs synthesized using *A. indica* upon fungal growth of test fungi is shown in Fig. 6. The mycelia of non-treated cultures were abundant. On the other hand, the treated fungal cells show remarkable decrease of mycelial growth. The microscopic study reveals presence of mycelial fragments or conidia spores for test fungi. The formation of conidial spores by pathogenic fungi indicates the final stage of their life cycle because of occurrence of toxic environment that is unsuitable for their growth and development.

Scanning Electron Microscopy (SEM)

The microscopic observation of impact of nanoparticles on growing hypea revealed that nanoparticles clearly damaged hyphae of test fungi (Fig. 7), while hyphae treated with water appeared to remain intact (Fig. 7A, a). In the treatment of nanoparticles (Fig. 7B, b), the shape of hyphal walls turned abnormal while some layers of hyphal walls were tearing off and damaged and many hyphae were collapsed.

Phytotoxicity Study of Copper Nanoparticles

The effect of coppernanoparticles synthesized by using *A.indica* extract and corresponding bulk material used for synthesis, on growth parameters was determined using test plants. *Vigna radia*, a dicot plant and *Allium cepa*, a monocot plant was selected for toxicity observations. The results of seed germination rate of treated samples demonstrate a difference in comparison with control (Fig. 8). The treatment concentration and time factor influenced the germination rate of test seeds. Among all fractions, Cu ion solution showed highest toxicity for seed germination, which was also dose dependent. While as *A. indica* extract had least effect on germination activity of seeds. Seeds treated with CuNP solution were suppressed for germination that was dose dependent. At higher concentrations (100 $\mu g.ml^{-1}$), CuO-NPs influenced the germination rate and touched up to 65%, which was increased up to 80% when CuO-NPs were used in combination with *A. indica* extract at the same concentrations. The overall results reveal that *A. indica* extract and their combined fractions with CuO-NPs were less toxic to the test seeds while as bulk Cu followed by biosynthesized CuO-NPs influenced the germination rate as compared to control pots.

Table I Percent mycelial growth inhibition of various metallic fractions and aqueous neem extract

Fractions	Alternaria mali				Diplodia seriata				Botryosphaeria dothidea			
	C_4	C_3	C_2	C_1	C_4	C_3	C_2	C_1	C_4	C_3	C_2	C_1
NE	++	++	+	+	++	++	+	–	++	++	+	+
$CuSO_4$	+++	++	++	++	+++	+++	++	+	+++	+++	++	+
Cu NP	+++	+++	+++	++	+++	+++	+++	++	++++	+++	++	++
CuNP + NE	++++	++++	+++	++	++++	+++	+++	++	++++	++++	+++	++
Mancozeb	++++	++++	++++	+++	++++	+++	+++	+++	++++	++++	+++	++

Key; C_1, C_2, C_3 and C_4 denote 0.01, 0.25, 0.5 and 1 g/ml are concentrations respectively; NE = Neem leaf extract and NP = Nanoparticle; −, no antifungal activity; +, scantly antifungal activity with PMGI value 25%; ++, modest antifungal activity with PMGI value 25–50%; +++, strong antifungal activity with PMGI value 50–75%; ++++, very strong antifungal activity with PMGI value 75–100%

The results of *Allium* test also produced substantial variation among treatments and control (Fig. 9). All the growth parameters had a significant effect by treatments which was dose dependent. However, in case of bulk Cu solution root number was reduced with increasing concentration while as root and shoot length was consequently increased with increase in concentration (Fig. 10). The reason may be because of elicitor nature of this metal. The results put a further insight that the treatments of combination of CuO-NPs and plant extract has less effect on the growth parameters compared to control.

DISCUSSION

The study shows that leaf extract of neem provides an economical and preeminent way for synthesis of Copper nanoparticles of apposite shape and size. These Copper nanoparticles (CuO-NPs) were confirmed by color change, UV-vis and XRD analysis reports as described by previous authors (21,33–34). An average sized 80 nm cuprous oxides (Cu_2O) and cupric oxide (CuO) nanocrystals were generated using neem leaf extract at normal conditions. Such findings were previously reported for biological synthesis of copper nanoparticles (21,35,36,and) some others suggest that colloidal mixture of synthesized nanoparticles has a combination of nanocrystals each of Cu_2O and CuO (36–38,and). The morphology and shape of CuO-NPs was further analyzed by SEM and TEM microscopy. The bio-synthesized CuO-NPs using plant extracts were previously characterized using SEM and TEM by several investigators (8–11,39). The crystalline nature of copper nanoparticles was further confirmed by XRD examination. Our results of XRD reports are in agreement with previous literatures (21,40–42). Moreover, the XRD results were in agreement with the UV–visible spectroscopy. FT-IR reports corroborate the presence of different absorption peaks accountable for nanoparticle configuration. As early investigated (43) some of the absorption peaks of CuO nanoparticles lie in the range of 500–700 cm^{-1} and well sighted from Fig. 5, the major peak observed to be around 680 cm^{-1} is the stretching of Cu-O. The plane (202) seen by XRD study correspond this peak (680 cm^{-1}) which shows the Cu-O stretching and thus confirm the formation of copper oxide nanostructures.

These nanoparticles had a significant bio-activity against pathogenic fungi like; *Alternaria mali, Diplodia seriata* and

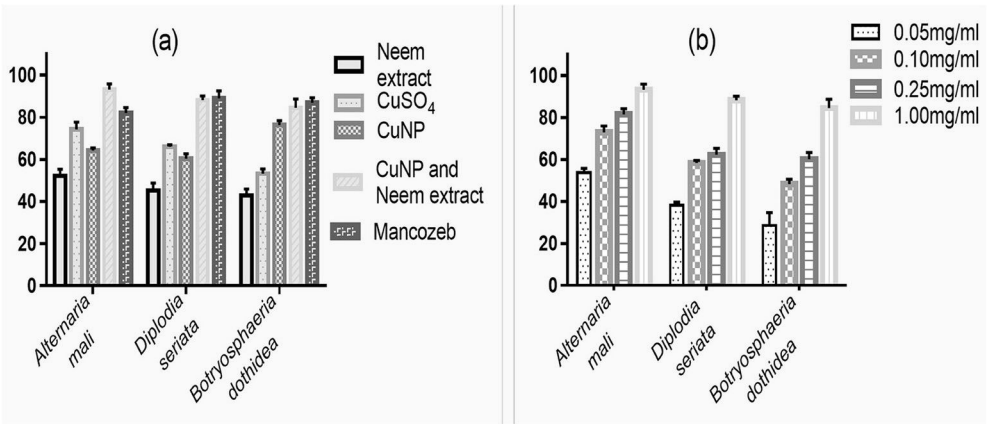

Plate I Percent Growth Inhibition of Pathogenic fungi Exposed to, (**a**) Nanoparticles and Corresponding Materials in 1 g/ml conc. (**b**) Only CuNP+Neem extract

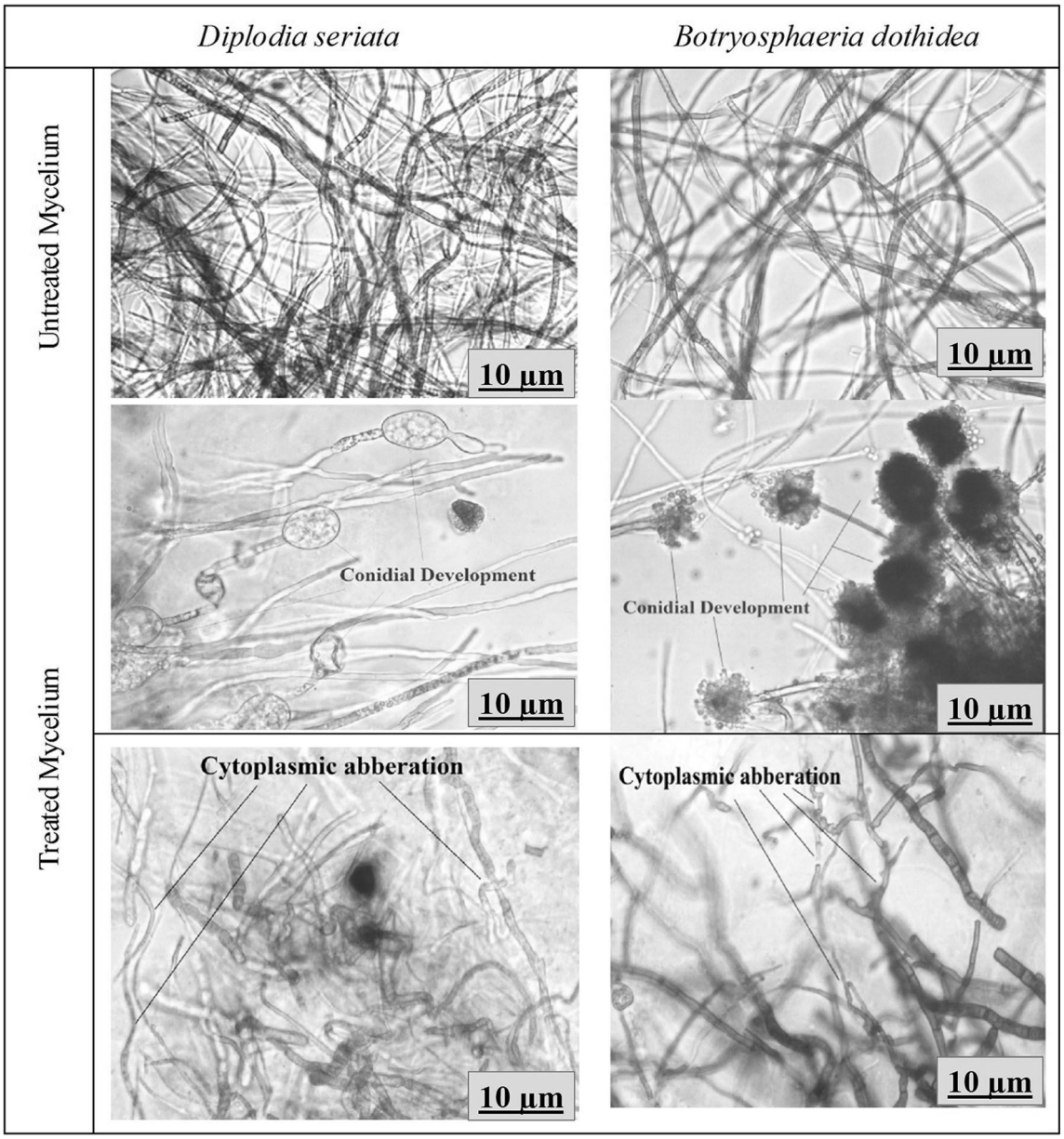

Fig. 6 Morphology of fungi before and after treatment of CuO-NP, observed in light microscope with 40X magnification.

Botryosphaeria dothidea (Table I) prevailing in apple orchards of Kashmir valley in India. Neem extracts which used as bio-controls (14–16) showed a significant activity against all fungi because of presence of active compounds like tetra terpenoids and phenolics in it (17–20) purified polyphenolic flavonoids like; Quercetin and ß-sitosterol, from neem fresh leaves found that content of major compounds such as 6-deacetylnimbin, azadiradione, nimbin, salannin and epoxy-azadiradione were the active compounds when bio-assayed on many pathogenic fungi (44). A considerable value of percent mycelial growth inhibition (PMGI) was seen against these fungi. 60% PGMI value of CuO-NPs and 100% of their combination of neem extract with CuO-NPs at constant concentrations (Plate 1). Such findings support the concept of synergistic effect where the total effect is approximately the summation of individual fractions (45,46). Our results confirm that the synthesized CuO-NPs had significant anti-fungal activity against pathogenic fungi of apple which may be attributed to their extremely large surface area. In addition, the CuO-NPs provide better contact with microorganisms for their activity. On the other hand, Cu ions released subsequently may also damage the DNA by binding with them this can lead to total damage of the helical structure by cross-linking within and between the nucleic acid strands as suggested by some researchers (47). Our microscopic study on treated fungi shows remarkable decrease of mycelial growth and formation of conidial spores

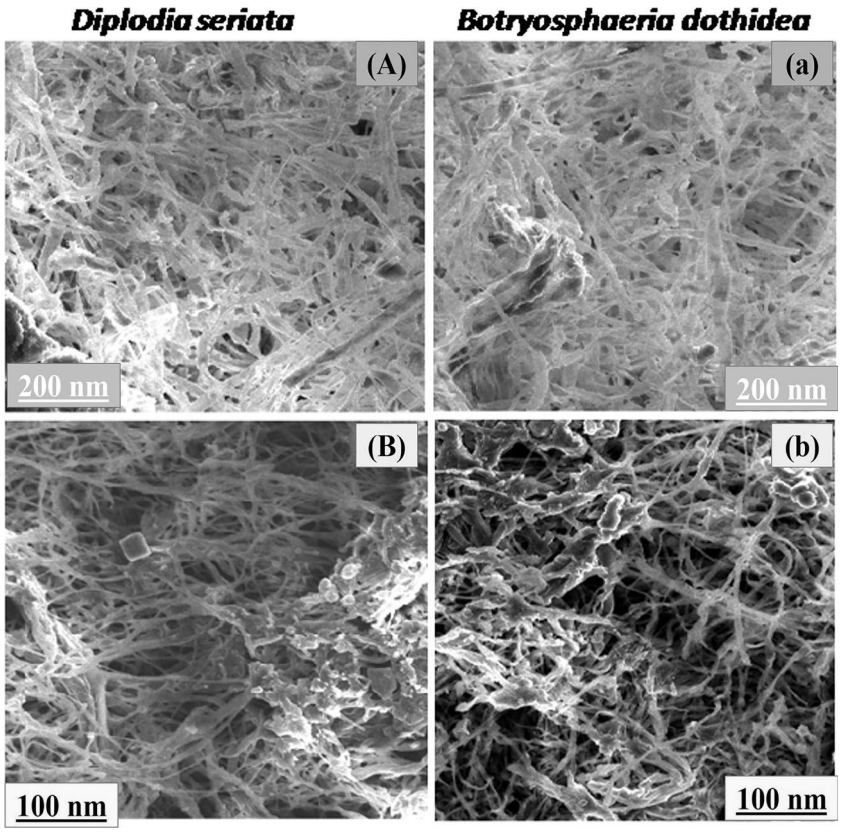

Fig. 7 SEM images of; (**A**) untreated, (**B**) treated *Diplodia seriata* mycelium; (**a**) untreated, and (**b**) treated *Botryosphaeria dothide*.

by pathogenic fungi which indicates the final stage of their life cycle because of occurrence of toxic environment that is unsuitable for their growth and development. Our results are in accordance with the study upon pathogenic fungi conducted (8) where they saw presence of mycelial fragments and conidia spores in case of treated fungi. Some of the researchers also predict that Copper ions inside microbial cells may also disrupt biochemical processes (48). Our previous reports with iron nanoparticles also showed the same types of findings (49). However, the exact mechanism needs overall attention for their further studies.

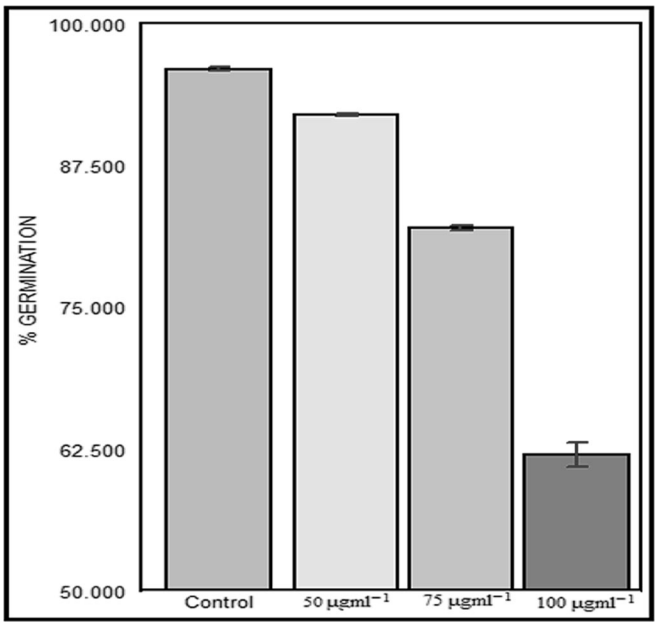

Fig. 8 The germination rate of seeds soaked within 50 μg.ml^{-1}, 75 μg.ml^{-1} and 100 μg.ml^{-1} copper oxide nanoparticle suspension. Significant difference was marked with lower case letters ($p < 0.05$).

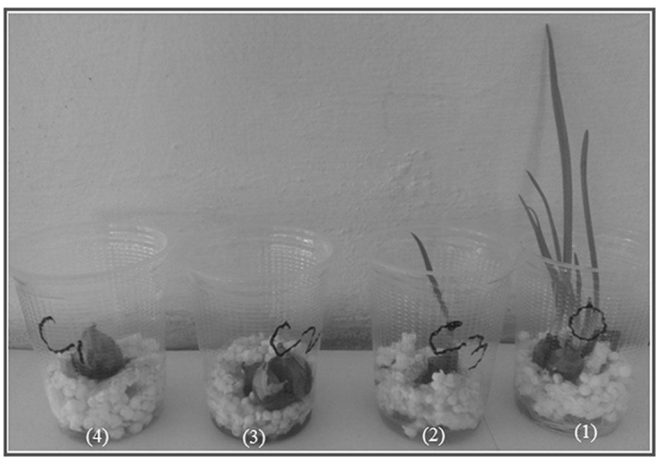

Fig. 9 Phyto-toxicity of CuO-NPs observed in *Allium* test at different concentrations.

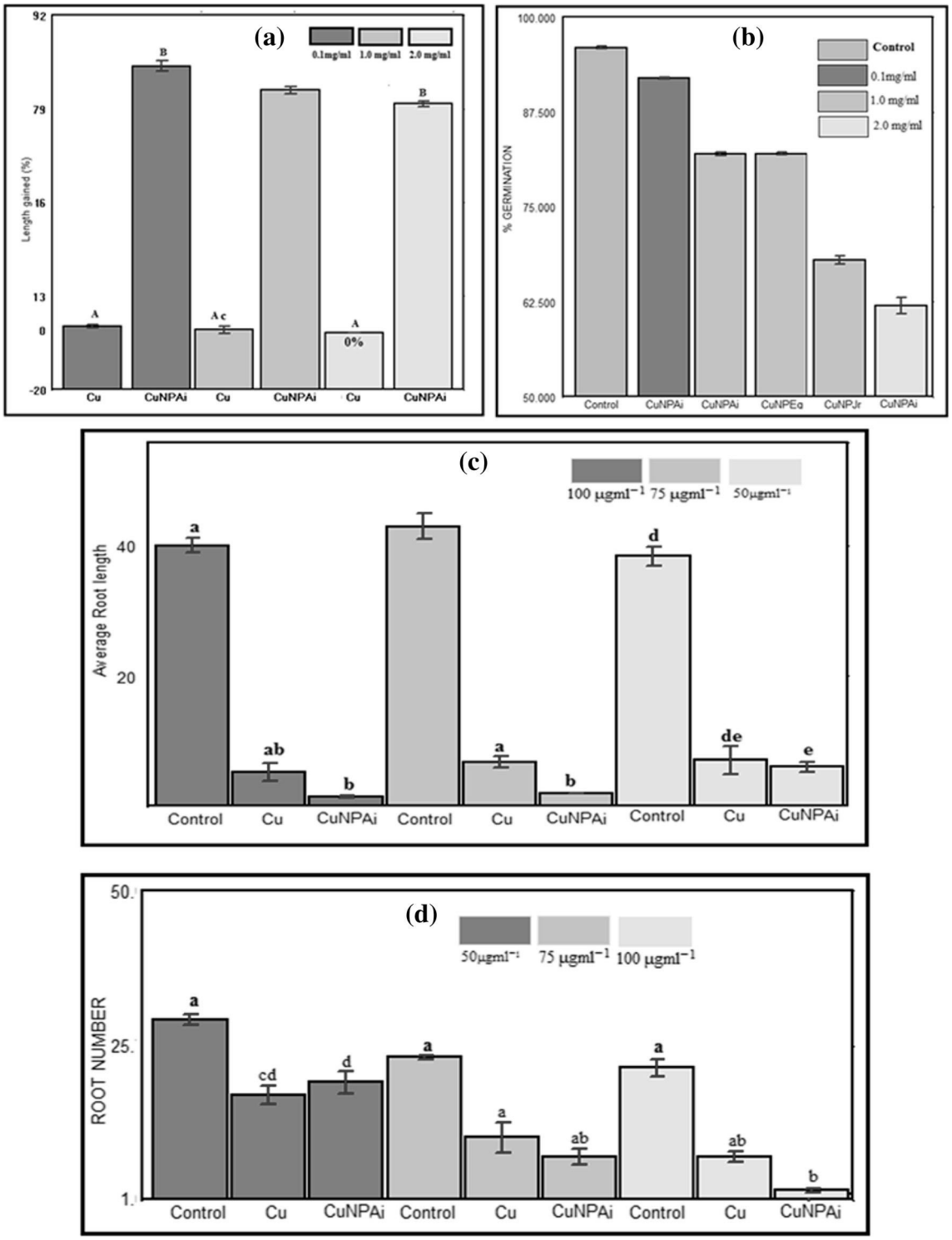

Fig. 10 Phyto-toxicity of CuO-NPs observed for (**a**) root Length at 0.1, 1.0 and 2 and (**b**) % germination of Mong seeds (*V. radiata*); (**c**) Root Length and (**d**) Root number of onion plants (*A. cepa*).

Phytotoxicity study of copper nanoparticles were further carried out using test plants like, *Vigna radia*, a dicot plant and *Allium cepa*, belonging to monocotyledons. Root tips frequently used for cytogenetic studies in the past five decades were from *Allium cepa* and *Vicia faba* (50–51), which are considered to be excellent materials for clastogenicity studies of physical and chemical agents. In addition, the seeds of *Vigna radiate* have long been used in

phytotoxicity studies (52). The overall results reveal that *A. indica* extract and their combined fractions with CuNP were less toxic to the test seeds while as bulk Cu followed by biosynthesized CuO-NPs influenced the germination rate as compared to control pots. More research is needed to be done in this regard in order to develop biogenic CuO-NPs pesticide formula that can be ideal for fungal pathogen control in orchards.

Acknowledgments and Disclosures. The authors are thankful to Dr. V. Ganesan, Centre Director UGC-DAE Consortium for Scientific Research, Indore Centre, India, for providing us a chance to conduct various analytical measurements with special thanks to Dr. T. Shripathi, Dr. D.M. Phase, Dr. N.P. Lalla, Dr. Mukul Gupta, Dr. U.P. Deshpande for their assistance at the cUGC DAE centre. We also thank to Dr. G.H. Bhat, HOD and Dr. V.K. Ambardar, Associate professor, Division of Plant Pathology, SK University of Agricultural Sciences and Technology, Srinagar, India for availing the lab facilities to conduct anti-fungal assessment experiments and present & future financial funding aid from TNSCST, DOTE campus, Chennai, India.

REFERENCES

1. Remya N, Saino HV, Baiju GN, Kumar DS. Nanoparticulate material delivery to plants. Pl Sci. 2010;179:154–63.
2. Green JM, Beestman GB. Recently patented and commercialized formulation and adjuvant technology. Crop Prot. 2007;26:320–7.
3. Wang L, Li X, Zhang G, Dong J, Eastoe J. Oil-in-water nanoemulsions for pesticide formulations. J Colloid Inter Sci. 2007;314:230–5.
4. Boehm AL, Martinon I, Zerrouk R, Rump E, Fessi H. Nanoprecipitation technique for the encapsulation of agrochemical active ingredients. J Microencapsul. 2003;20:433–41.
5. Tsuji K. Microencapsulation of pesticides and their improved handling safety. J Microencapsul. 2001;18:137–47.
6. Song JY, Kim BS. Rapid biological synthesis of silver nanoparticles using plant leaf extracts. Bioprocess Biosyst Eng. 2009;32(1):79–84.
7. Krishnaraj C, Jagan E, Rajasekar S, Selvakumar P, Kalaichelvan PT, Mohan N. Synthesis of silver nanoparticles using *Acalypha indica* leaf extracts and its antibacterial activity against water borne pathogens. Coll Surf B Biointer. 2010;76:50–6.
8. Lee HJ, Lee G, Jang NR, Yun JH, Song JY, Kim BS. Biological synthesis of copper nanoparticles using plant extract. Nanotech-2011 (NSTI Publ). 2011;1:371–4.
9. Gopalakrishnan K, Ramesh C, Ragunathan V, Thamilselvan M. Antibacterial activity of Cu2O nanoparticles on *E. coli* synthesized fromTridaxprocumbensleaf extract and surface coating with polyaniline. Digest J NanomatBiostruct. 2012;7:833–9.
10. Sangeetha G, Rajeshwari S, Rajendran V. Aloe barbadensis miller mediated green synthesis of mono-disperse copper oxide nanoparticles: optical properties. Spectrochima Acta Part A. 2012;97:1140–4.
11. Harne S, Sharma A, Dhaygude M, Joglekar S, Kodam K, Hudlikar M. Novel route for rapid biosynthesis of copper nanoparticles using aqueous extract of Calotropisprocera L. latex and their cytotoxicity on tumor cells. Colloids Surf. 2012;B. 95:284–8.
12. Haverkamp RG, Marshall AT, Agterveld D. Pick your carats: nanoparticles of gold–silver–copper alloy produced in vivo. J Nanopart Res. 2007;9:697–700.
13. Zaheer K, Javed IH, Akthar AH. Shape-directing role of cetyl Trimethyl ammonium bromide in the green synthesis of Ag-nanoparticles using Neem (*Azadirachta indica*) leaf extract. Coll and Surf B: Biointer. 2012;95:229–34.
14. Akhtar M, Mahmood I. Control of root-knot nematode *Meloidogyne incognita* in tomato plans by seed coating with suneem and neem oil. J Pestic Sci. 2007;22:37–8.
15. Amadioha AC. Controlling rice blast *in vitro* and *in vivo* with extract of *Azadirachta indica*. Crop Prot. 2000;19:287–90.
16. Michereff M, Torres JB, Andrade LNT, Nunes MUC. Effect of some biorational insecticides on *Spodoptera eridania* in organic cabbage. Pest Manag Sci. 64:761–7.
17. Devakumar C, Dev S, Randhawa NS, Parmar BS, editors. In Chemistry. Neem Research and Development. New Delhi: Society of Pesticide Science, SPS Publication; 1993. p. 63–96.
18. Carol SA. Publication of the Forest, Farm, and Community Tree Network. (FACT Net) FACT. 1998 98–01. FAO: https://www.fao.org/publications.
19. Tegegene G, Pretorius JC, Swart WJ. Antifungal properties of *Agapanthus africanus* extracts against plant pathogens. Crop Prot. 2007;7(27):1052–60.
20. Govindachari TR, Suresh G, Gopalakrishnan G, Banumathy B, Masilima S. Identification of antifungal compounds from the seed oil of *Azadirachta indica*. Phytoparasitica. 2008;26(2):1–8.
21. Abboud Y, Saffaj T, Chagraoui A, El Bouari A, Brouzi K, Tanane O, *et al.* Biosynthesis, characterization and antimicrobial activity of copper oxide nanoparticles (CuONPs) produced using brown alga extract (*Bifurcaria bifurcata*). Appl Nanosci. 2014;4:571–6.
22. Uddin N, Rahman A, NazimUddin A, SohelRana M, Akter R, Chowdhury AMMA. Antioxidant, cytotoxic and antimicrobial properties of ethanol extract. Int J Biol Med Res. 2010;1(4):341–6.
23. Lee SH, Chang KS, Su MS, Huanga YS, Jang HD. Effects of some Chinese medicinal plant extracts on five different fungi. Food Cont. 2007;18:1547–54.
24. Daohui L, Baoshan X. Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. Envir Pol. 2007;150:243–50.
25. Dimitrios S, Saion KS, Jason C. White Assay-Dependent Phytotoxicity of Nanoparticles to Plants. Environ Sci Technol. 2009;43:9473–9.
26. Anita KP, Ashley B, LaBethani M, Paul BT. Genotoxicity of silver nanoparticles in Vicia faba: a pilot study on the environmental monitoring of nanoparticles. Int J Environ Res Public Health. 2012;9:1649–62.
27. Yang L, Watts DJ. Particle surface characteristics may play an important role in phytotoxicity of alumina nanoparticles. Toxicol Lett. 2005;158:122–32.
28. Duygu A, Ali O. Antimitotic effects of the biopesticide oxymatrine. Caryo. 2011;64(1):117–20.
29. Fiskesjo G. The Allium test as a standard in environmental monitoring. Hereditas. 1985;102:99–112.
30. Magne AO, Culioli G, Valls R, *et al.* Polar acyclic diterpenoids from *Bifurcaria bifurcata*. Phytoch. 2005;66:2316–23.
31. Culioli G, Daoudi M, Mesguiche V, Valls R, Piovetti L. Geranylgeraniol-derived diterpenoids from the brown alga *Bifurcaria bifurcata*. Phytoch. 1999;52:1447–54.
32. Mahmoud DA, Hassanein NM, Youssef KA, AbouZeid MA. Antifungal activity of different neem leaf extracts and the nimonol against some important human pathogens. Braz J Micro. 2011;42:1007–16.

33. Krithiga N, Jayachitra A, Rajalakshmi A. Synthesis, characterization and analysis of the effect of copper oxide nanoparticles in biological systems. Ind J Ns. 2013;1:6–15.
34. Natarajan K, Selvaraj S, Murty VR. Microbial production of silver nanoparticle. Digest J Nanomat Biostr. 2010;5:135–40.
35. Borgohain K, Murase N, Mahamuni S. Synthesis and properties of Cu_2O quantum particles. J Appl Phys. 2002;92:1292–7.
36. Yin M, Wu CK, Lou YB, Koberstein JTC, Zhu YM, O'Brien S. Copper oxide nanocrystals. J Am Chem Soc. 2005;127:9506–11.
37. Rahman A, Ismail A, Jumbianti D, Magdalena S, Sudrajat H. Synthesis of copper oxide nanoparticles by using Phormidiumcyanobacterium. Indo J Chem. 2009;9:355–60.
38. Swarnkar RK, Singh SC, Gopal R. Optical characterizations of copper oxide nanomaterial. In: Proceedings of the ICOP International Conference on Optics and photonics. Chandigarh: CSIO; 2009.
39. Guajardo-Pacheco J, Morales-Sanchez JE, Gonzalez-Hernandez J, Ruiz F. Synthesis of copper nanoparticles using soybeans as a chelant agent. Mat Lett. 2010;64:1361–4.
40. Kooti M, Matouri L. Fabrication of nanosized cuprous oxide using Fehling's solution. Trans F Nanotechnol. 2010;17:73–8.
41. Srivata M, Singh J, Mishra RK, Ojha AK. Electro-optical and magnetic properties of monodispersed colloidal Cu_2O nanoparticles. J Alloys Comp. 2013;555:123–30.
42. Volanti DP, Keyson D, Cavalcante LS, Simões AZ, Joya MR, Longo E, *et al*. Synthesis and characterization of CuO flower-nanostructure processing by a domestic hydrothermal microwave. J Alloys Comp. 2008;459:537–42.
43. Thekkae P, Černík. Green synthesis of copper oxide nanoparticles using gum karaya as a biotemplate and their antibacterial application. Int J Nanomed. 2013;8:889–98.
44. Hummelbrunner LA, Isman MB. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). J Agric Food Chem. 2011;49:715–20.
45. Bhuiyan MKR, Hassan E, Isman MB. Growth inhibitory and lethal effects of some botanical insecticides and potential synergy by dillapiol in *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae). J Plant Dis Prot. 2001;108:82–8.
46. Pavela R, Vrchotová N, Sera B. Growth inhibitory effect of extracts from *Reynoutria* sp. plants against *Spodoptera littoralis* larva. Agrociencia. 2008;42:573–84.
47. Kim JH, Cho H, Ryu SE, Choi MU. Effects of metals ions on the activity of protein tyrosine phosphate VHR: highly potent and reversible oxidative inactivation by Cu2+ ion. Arch Biochem Biophys. 2001;382:72–80.
48. Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic Bio Med. 1995;18:321–36.
49. Rather HA, Rajagopal K, Shah AH, Bhat AH, Krishnan V. Study of bio-fabrication of Iron nanoparticles and their fungicidal property against phytopathogens of apple orchards. IET Nanobiotech. 2017;11(3):230–5.
50. Grant WF. Chromosome aberration assays in *Allium*. A report of the US Environmental Protection Agency gene-Tox Program. Mut Res. 1982;99:273–91.
51. Ma TH. *Vicia* cytogenetic tests for environmental mutagens. A report of the US Environmental Protection Agency Gene-Tox Program. Mutat Res. 1982;99:259–71.
52. Kumar VL, Singhal A. Germinating seeds of the mung bean, *Vigna radiata* (Fabaceae), as a model for the preliminary evaluation of cytotoxic effects of drugs. Biocell. 2009;33(1):19–24.
53. Vats S. Effect of initial temperature treatment on phytochemicals and antioxidant activity of Azadirachta indica a. Juss Appl Biochem Biotechnol. 2016;178(3):504–12. https://doi.org/10.1007/s12010-015-1890-x.