

Antifungal evaluation studies of copper sulfide nano-aquaformulations and its impact on seed quality of rice (*Oryza sativa*)

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Abstract Surface capped copper sulfide nano-aquaformulations were prepared by in situ combination of copper ions with sulfide ions using sonochemical method, followed by microwave irradiations, in the presence of capping agents. Prepared nano-aquaformulations were characterized for particle size, morphology and optical properties. The in vitro antifungal evaluations studies indicated multifold efficacy against *Alternaria alternata*, *Drechslera oryzae* and *Curvularia lunata* in comparison to standard used. The in vivo seed treatment on discoloured paddy seeds showed the optimum results on application @ 7µg/ml for 2 h in case of citrate capped copper sulfide nanoformulation (NCuS₃). Significant reduction in seed rot and seedling blight was observed with favourable effect on germination and growth parameters at this concentration.

Keywords Copper sulfide · Nano-aquaformulations · Poisoned food technique · Phytopathogenic fungi · Mycelium growth

Introduction

The modern agriculture requisites the exploration of substances that exist in nature and are simple for nature to interpret them. There is continuous demand of eco-friendly

formulated safe bioactive substances in the agricultural world, because of toxic nature of the existing organic molecules, toxic organic formulations and development of resistance against them in the pathogens. Alternative safe water base formulations (<http://www.azom.com/article.aspx?ArticleID=13694>), are recognizably in demand, owing to increase in government regulations and restrictions, outrightly banning of products that have inherent toxicology and adverse environmental impact (Richardson 1997).

Copper (Cu) based fungicides are used world-wide as various formulations viz. Cupric sulphate, Bordeaux mixture, copper dugtdrazubium sulphate, copper oxochloride, copper zinc chromate, cupric carbonate, cuprous oxide, burgundy mixture, copper hydroxide and organo coppers. The toxicity of copper is largely due to its tendency to alternate between its cuprous, Cu(I) and cupric Cu(II) oxidation states (Santra 2014), which get finally detoxified in nature by sulfidation (Wang et al. 2013). There are various methodologies that are usually proposed and practised to detoxify copper (Santra 2014), prior to actual detoxification by nature. Efficacy of the copper compounds can also be considerably improved by reducing the particle size (Torgeson 1967), as it results in more surface area available per gram of product (Navarro et al. 2008), advocating exploration of nano-technological applications in agriculture. Water based aqueous formulations, consisting of water insoluble active ingredients dispersed in water, is one of the most pertinent methodologies for the development of green pesticides that have limited application for copper fungicides (<http://www.azom.com/article.aspx?ArticleID=13694>).

On nanotechnological front, studies on copper nanoparticles (Cu NPs) and copper oxide nanoparticles (CuO NPs) have been reported for phytopathological and phyto-applications. Nanotoxicological fronts for Cu

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fungicides are normally patented for methodologies meant for slow and controlled delivery of copper ions from the formulations. Santra (2014) invented copper–silica-based nanoformulation to treat citrus canker, inhibiting the growth of mold and mildew. A worth patent of the BASF company (Schneider et al. 2011) viz. polymer entangled CuNPs, showed improved efficiency against phytopathogenic fungus on vines (Gogos et al. 2012) in comparison to normal copper formulations (by 8%). CuNPs absorbed in chitosan hydrogel had positive effects on tomato growth and quality (Juarez-Maldonado et al. 2016). It is believed that the stimulatory effects of CuNPs are related to the induction of antioxidant activity (Fu et al. 2014). Copper nanoparticles have been found to increase germination percentage of wheat (*Triticum aestivum*) on seed treatment while severely reducing seedling vigor. CuO NPs have shown strong inhibitory effects on higher plants viz. *Lemna minor* (Guanling et al. 2016) and *Lactuca sativa* (Liu et al. 2016), at higher concentrations. Copper oxide nanoparticles are reported to induce DNA damage in agricultural and grassland plants. Significant plant growth inhibition has been reported for radish (*R. sativus*), perennial ryegrass (*L. perenne*), and annual ryegrass (*Lolium rigidum*) under controlled laboratory conditions (Atha et al. 2012). The most relevant study CuO NPs is on rice (*O. sativa*) showed that seed germination was not affected by CuO NPs at any of the investigated concentrations. At the concentration of 2000 mg L⁻¹, the root elongation (97.28% for rice), and the shoot length of maize was reduced by 30.98% (Ruttkey-Nedecky et al. 2017) by their applications. There is not literature on seed treatment of CuS in any form on any crop but still scanty investigation have been published on antimicrobial potential of CuS NPs in solid state (Chakraborty et al. 2016; Prabhavathi et al. 2015).

Copper sulfide (CuS) is one of the most detoxified form of copper (Baek 2017; Baek and Kim 2017; Guo et al. 2013; Wang et al. 2013), natural, thermally stable (Rao et al. 2016), water insoluble (Guo et al. 2013) still retaining the biopotential of copper (Chakraborty et al. 2016; Prabhavathi et al. 2015). It is relatively safe and non-toxic behaviour to humans (Guo et al. 2013) and limited literature on synthesis and biopotential on water dispersed CuS nanoparticles prompted us to prepare the novel surface protected formulations of nano-copper sulfide in aqueous media, for the evaluation of mycotoxic potential against various agriculturally important phytopathogenic fungi. To best of the author's knowledge it is the first reporting on in vitro myotoxicity along with on seed germination and seed quality parameters, on discoloured rice seeds, by application.

Materials and methods

Instrumentation used

Absorption spectra of nanoparticles were recorded in aqueous, using UV-1800 Shimadzu Double-beam Spectrophotometer. The measurements were carried out using quartz cell in the wavelength range of 200–800 nm. The morphology and size of nanoparticles were recorded in Hitachi Transmission Electron Microscope Hi-7650 at an accelerated voltage of 200 kV by casting a drop of particle solution onto a 200-mesh carbon coated copper grid from EMN laboratory, Punjab Agricultural University, Ludhiana.

Synthesis of copper sulfide nano-aquaformulations (NCuS₁₋₃)

30 ml of copper nitrate trihydrate (Cu(NO₃)₂·3H₂O) solution (0.001 M), prepared in ethylene glycol, was added dropwise to 30 ml of sodium sulfide aqueous solution (0.001 M), during sonication, along with a pinch of cetyltrimethylammonium bromide (CTAB) (surfactant). The solution was irradiated with microwaves for 30 s and allowed to cool down at the room temperature. Different capping agents viz polyvinyl pyrrolidone (PVP), 4-aminobutyric acid (GABA) and tri-sodium citrate (0.3 g) dissolved in 20 ml distilled water was added to above prepared solution during sonication, which was continued for another 15 min, to get surface stabilized copper sulfide nanoparticles NCuS₁₋₃, respectively.

The prepared NCuS₁₋₃ (0.0005 M, 35.85 µg/ml) were retained as such as stock solution, which was further diluted by adding autoclaved water, as and when required.

Antifungal evaluation of NCuS₁₋₃

Antifungal activity of copper sulfide nano-aquaformulations (NCuS₁₋₃) was tested against pathogenic fungi of rice viz. *Alternaria alternata*, *Drechserla oryzae* and *Curvularia lunata* at different concentration viz. 5, 6, 7, 8, 9, 10, 15 µg/ml by poisoned food technique (Nene and Thapliyal 1993). The cultures of phytopathogenic fungi were obtained from Seed Pathology laboratory of Punjab Agricultural University, Ludhiana. These concentrations were further tested on potato dextrose agar (PDA) and the results were validated on the test fungi.

In vitro studies (poisoned food technique method)

PDA medium was prepared using 250 g peeled potato, 20 g dextrose and 20 g agar in 1 l water which was then

autoclaved for half an hour. 36 ml of PDA media was taken in the round bottom flask to which 14 ml of stock solution (35.85 $\mu\text{g/ml}$) of the test formulation was added to give the resulting 10 $\mu\text{g/ml}$ of NCuS-PDA concentrate. The contents of the flask were poured aseptically into the Petri plates. Similarly, 7 $\mu\text{g/ml}$ and lower concentration up to 5 $\mu\text{g/ml}$ were prepared using normality equation. Test samples, however, was replaced by equal amount of PDA only in the control set. After solidification of the medium a 3 mm culture disc of mycelium, the test fungus was aseptically placed to each Petri plate and was incubated at 25 ± 1 °C. The efficacy of NCuS_{1–3} treatment was evaluated at the time intervals of 3, 5, 7 and 9 days by measuring the diameter of fungal colonies. The average diameter of fungal colonies was measured on 9th day after inoculation. The percent mycelial growth inhibition was recorded using the following formula:

$$\text{Percent radial growth inhibition} = \frac{\text{Radial growth of fungal colony in control} - \text{Radial growth of fungal colony in treatment}}{\text{Radial growth of fungal colony in control}} \times 100$$

The antifungal activity was expressed in terms of percent radial growth inhibition. The ED₅₀ and ED₉₀ value (effective dose at which 50 and 90% inhibition of fungal growth occurred) were calculated from percent radial growth inhibition.

***In vivo* studies using rolled paper towel method**

To get insight into the effect of NCuS seed treatment on various pathological and physiological aspects, fungi infested discoloured rice seeds were used and seedling symptoms test of treated as well untreated seeds were performed using rolled paper towel method (ISTA 1999). Hundred seeds of rice (discoloured as well as healthy) were treated with the test formulation of NCuS @ 5–10 $\mu\text{g/ml}$ and captan @ 0.25% seeds by dipping the seeds in test and standard fungicide solutions for 1 h. The seedlings were evaluated after 10 days and data were recorded on seed germination, root length (RL), shoot length (SL), number of rotten seeds and blighted seedlings. Vigour index was calculated using formula given by Abdul-Baki and Anderson (1973).

$$\text{Vigour index} = \text{Dry weight} \times \text{percent germination}$$

Results and discussion

Surface protected copper sulfide nanoparticles (NCuS_{1–3}) in aqua-dispersed form were synthesized by sonochemical irradiation method. Cu²⁺ ions from Cu(NO₃)₂ in ethylene glycol (HOCH₂CH₂OH) solution was made to combine with S²⁻ from sodium sulfide (Na₂S) solution, while sonication, forming copper sulfide nanoparticles, which were stabilized using different stabilizing agents viz. polyvinyl pyrrolidone (PVP) (Fig. 1a), 4-aminobutyric acid (GABA) (Fig. 1b) and tri-sodium citrate (Fig. 1c).

Characterization of copper sulfide nanoaqua-formulation (NCuS_{1–3})

The morphological, particles size and optical properties of synthesized surface protected copper sulfide nanoparticles

(CuS-NPs) in aqua-dispersed form were studied by Transmission Electron Microscopy (TEM) and UV–Visible spectrophotometric studies. Morphology of CuS prepared was independent of the capping agent used with slight variation in the size. Shape analogy indicated from TEM morphograph showed distorted spherical to oval shapes of CuS nanoparticles with slight aggregations. The particle sizes were least in NCuS₃ (Fig. 2d), followed by NCuS₂ (Fig. 2c) and NCuS₁ (Fig. 2b). The average particle size was in the range from 8 to 14 nm (Fig. 3). The trends of optical properties were in consonance with tem observations. The λ_{max} values in all the cases were lying in the range of 218–220 nm (Fig. 4) indicating the similarity in average size of the particles in all the samples. These values were slightly blue shifted, in comparison to the naked CuS nanoparticles ($\lambda_{\text{max}} = 230$ nm), which is due to the combined effect of size reduction (Agnitotri et al. 2014) due to capping agents. The size range and the optical properties of NCuS_{1–3} are given in Table 1.

To best of the author's knowledge, in situ synthesis of copper sulfide as nanoaqua-dispersed formulation form have no reporting previously. However, prepared CuS NPs in solid form have been reported using different sulfide sources viz. sodium sulfide (Huang et al. 2017; Riyaz et al. 2016; Ayodhya et al. 2016; Shamsipur et al. 2013; Castillon-Barraza et al. 2011; Ding et al. 2010), sodium thiosulfate (Nafees et al. 2015; Kundu and Pradhan 2013;

Fig. 1 Physical representation of **a** NCuS₁, **b** NCuS₂, **c** NCuS₃

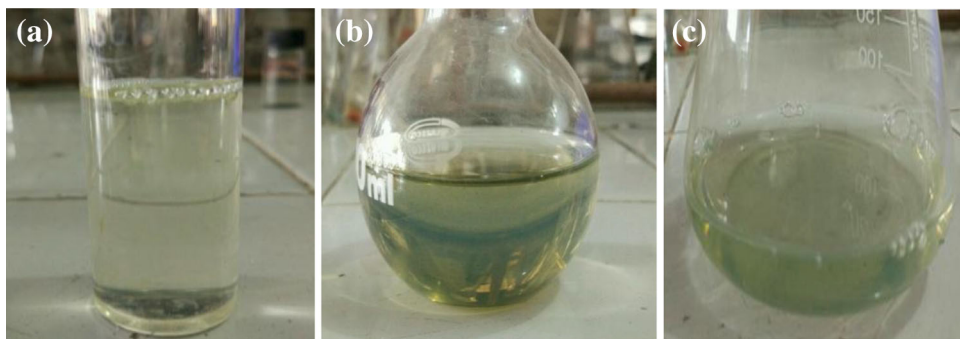


Fig. 2 TEM analysis of **a** naked CuS, **b** NCuS₁, **c** NCuS₂, **d** NCuS₃

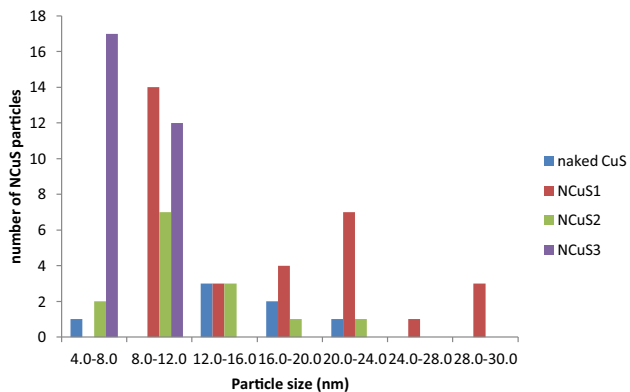
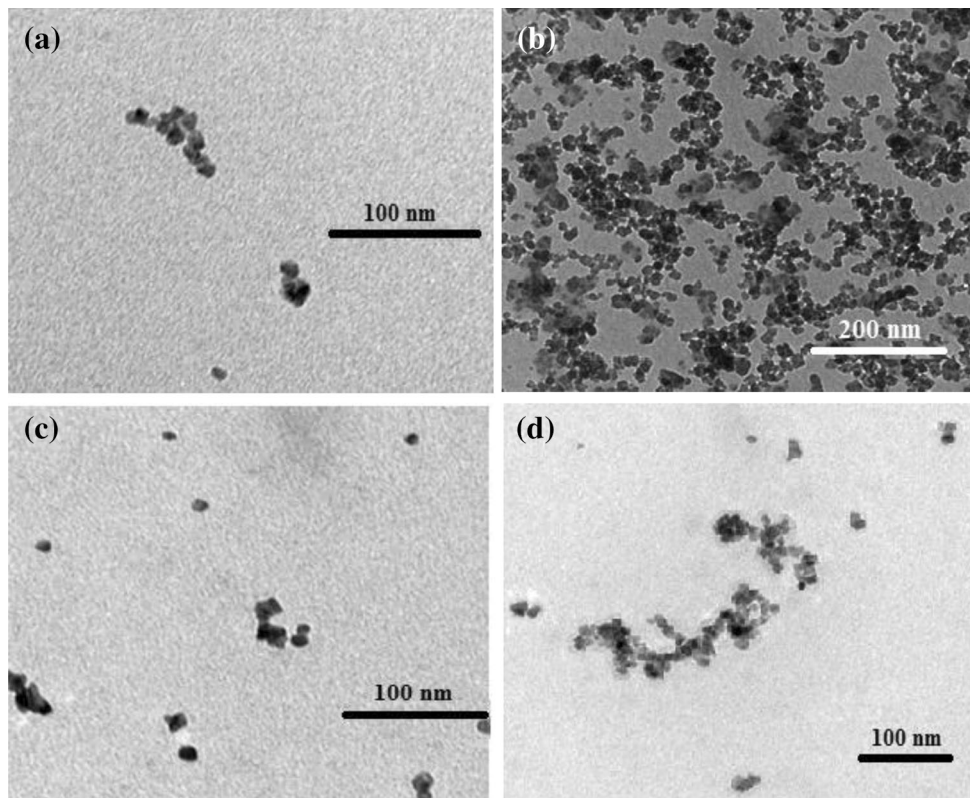


Fig. 3 Particles size distribution of NCuS

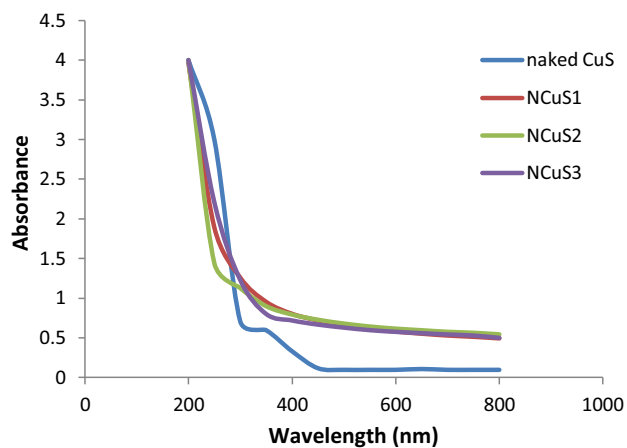


Fig. 4 UV-Vis spectra of NCuS

Table 1 Size range and absorption data of prepared samples of NCuS_{1–3}

| S. No. | Sample name | Copper source | Sulfide source | Capping agent | Absorption peak (nm) | Range of particle size (nm) | Average size (nm) |
|--------|-------------------|----------------|----------------|---------------|----------------------|-----------------------------|-------------------|
| 1. | Naked CuS | Copper nitrate | Sodium sulfide | – | 230 | 7–22.5 | 14 |
| 2. | NCuS ₁ | Copper nitrate | Sodium sulfide | PVP | 218 | 10–30 | 12 |
| 3. | NCuS ₂ | Copper nitrate | Sodium sulfide | GABA | 219 | 4.5–22 | 10 |
| 4. | NCuS ₃ | Copper nitrate | Sodium sulfide | Citrate | 220 | 4–12 | 8 |

Table 2 Percent radial growth inhibition of the test fungi used against different samples of NCuS_{1–3} on the 9th day of inoculation

| Samples | Concentration (µg/ml) | Percent radial growth inhibition | | |
|---------------------|-----------------------|----------------------------------|------------------|------------------|
| | | <i>A. alternata</i> | <i>D. oryzae</i> | <i>C. lunata</i> |
| NCuS ₁ | 3 | 12 | 11 | 19 |
| | 5 | 32 | 27 | 33 |
| | 7 | 58 | 41 | 57 |
| | 10 | 79 | 61 | 78 |
| | 15 | 100 | 100 | 100 |
| NCuS ₂ | 3 | 19 | 27 | 9 |
| | 5 | 29 | 40 | 20 |
| | 7 | 48 | 57 | 34 |
| | 10 | 76 | 77 | 56 |
| | 15 | 100 | 100 | 100 |
| NCuS ₃ | 3 | 37 | 37 | 32 |
| | 5 | 45 | 50 | 44 |
| | 7 | 64 | 76 | 62 |
| | 10 | 99 | 97 | 91 |
| | 15 | 100 | 100 | 100 |
| Captan ^a | 200 | 35 | 40 | 39 |
| | 300 | 58 | 60 | 62 |
| | 400 | 73 | 75 | 71 |
| | 500 | 87 | 90 | 84 |

^a Standard fungicide (*N*-trichloromethyl-thio-4-cyclohexene-1,2-dicarboximide)

Yang et al. 2013), thiourea (Han et al. 2016; Priyadharshini and Rajagopal 2015; Nafees et al. 2015) and thioacetamide (You et al. 2017). The size of nano CuS have been found to be in consonance with the reported literature. The λ_{\max} values of capped samples (NCuS_{1–3}) were lower than the naked copper sulfide nanoparticles (Ajibade and Botha 2016; Riyaz et al. 2016; Priyadharshini and Rajagopal 2015; Nandipha 2015; Suja et al. 2013; Hosseini et al. 2012) as per our results. The values of λ_{\max} were liable to change with the amount and type of capping used for metal nanoparticles. With the increase in level of capping the size reduces and in turn, the λ_{\max} value get relatively blue shifted (Rani 2016), which is the most probable

explanation for the altered optical properties of nanoparticles when capped.

In vitro antifungal evaluation of NCuS_{1–3}

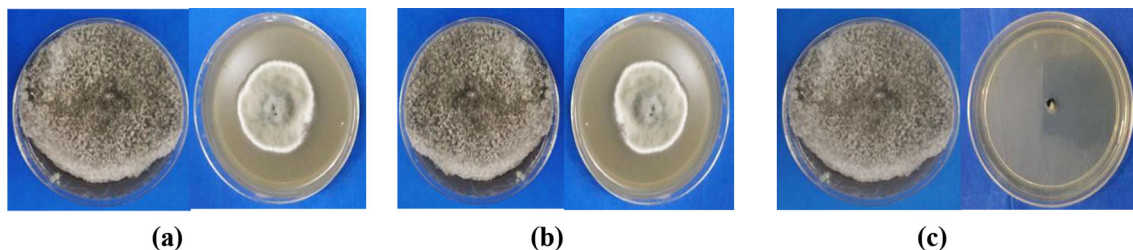
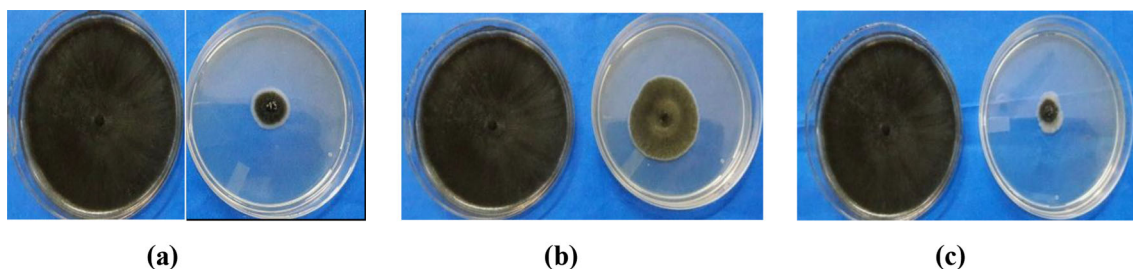
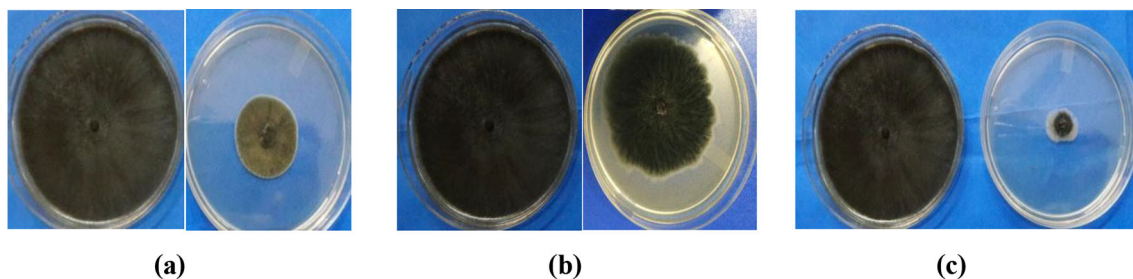
The antifungal evaluation study of NCuS having different capping agents viz PVP, GABA and citrate at different concentrations viz 15, 10, 7, 5 and 3 µg/ml, was done using poisoned food technique against three economically important phytopathogenic fungi (*Alternaria alternata*, *Drechslera oryzae* and *Curvularia lunata*). The results were expressed in terms of percent radial growth inhibition, at different test concentrations (Table 2), from which ED₅₀ and ED₉₅ values were calculated using polo software programme (Table 3). Captan (*N*-trichloromethyl-thio-4-cyclohexene-1,2-dicarboximide) was used as standard fungicide against the test fungi. The Table 2 clearly implicates the remarkably high bioactivity of copper sulfide nanoformulations. The zone of inhibition was equivalent to standards at much lower concentrations of NCuS in all the test samples against all the test fungi. NCuS₃ was most effective against all the test fungi with > 90% inhibition at 10 µg/ml on 9th day of inoculation. Radial growth inhibition of test fungi at concentration of 10 µg/ml are shown in Figs. 5, 6, 7. The more comprehensive interpretation came out to be in the form of ED₅₀ and ED₉₀ values. The comparative account revealed ED₅₀ and ED₉₀ values lower than 11.0 and 14 µg/ml, respectively. These values are multifold lower than the standard fungicides, captan used against the respective fungi. NCuS₃ was the best against *D. oryzae* with ED₅₀ and ED₉₀ of 5.0 and 7.0 µg/ml, respectively. Against *A. alternata*, NCuS₃ was ED₅₀ and ED₉₀ of 5.5 and 8.3 µg/ml, respectively. NCuS₃ nearly followed the same trend against *C. lunata*, with ED₅₀ and ED₉₀ of 6.0 and 8.0 µg/ml, respectively.

Practically, no literature is available on the antifungal evaluation of study of NCuS as aqua formulations. However, limited reported literature on CuS NPs in solid state revealed appreciable bio potential of CuS NPs. The synthesized nanoparticles (CuS-I and CuS-II) possibly gets adsorbed on the surface of the cell wall of microorganisms to disturb the respiration process, leading to breakdown of the permeability barrier of the cell and resulting in

Table 3 Antifungal activity of NCuS_{1–3} in terms ED₅₀ values (µg/ml)

| Samples | <i>A. alternata</i> | | <i>D. oryzae</i> | | <i>C. lunata</i> | |
|---------------------|---------------------|------------------|------------------|------------------|------------------|------------------|
| | ED ₅₀ | ED ₉₀ | ED ₅₀ | ED ₉₀ | ED ₅₀ | ED ₉₀ |
| NCuS ₁ | 6.7 | 9.6 | 8.0 | 10.0 | 8.0 | 10.5 |
| NCuS ₂ | 8.3 | 11.0 | 7.0 | 9.5 | 11.5 | 14.0 |
| NCuS ₃ | 5.5 | 8.3 | 5.0 | 7.0 | 6.0 | 8.0 |
| Captan ^a | 240 | 430 | 250 | 390 | 270 | 405 |

^a Standard fungicide (*N*-trichloromethyl-thio-4-cyclohexene-1,2-dicarboximide)

**Fig. 5** Antifungal activity against *A. alternata* **a** NCuS₁, **b** NCuS₂, **c** NCuS₃**Fig. 6** Antifungal activity against *D. oryzae* **a** NCuS₁, **b** NCuS₂, **c** NCuS₃**Fig. 7** Antifungal activity against *C. lunata* **a** NCuS₁, **b** NCuS₂, **c** NCuS₃

interference with, the normal cell process inhibiting further growth of the microorganisms (Chakraborty et al. 2016). The antimicrobial and antifungal activities of pyrolytically synthesized CuS NPs were found dependent on morphology of NPs (Chakraborty et al. 2016). These results were contradictory to our results, which were concentration dependent (when used in the concentration lower than 15 µg/ml) and independent of morphology and size of NPs in the average size range 8–14 nm.

***In vitro* seed treatment**

The seeds were treated with different concentrations of NCuS₁ and NCuS₃. The different concentrations used were 6–10 µg/ml and the seeds were dipped into the formulations for different time intervals starting from 1 to 6 h, the results indicated that dipping into NCuS₃ for 2 h inflicted the best results to inhibit the fungal mycoflora (Fig. 8) at minimum concentration of @ 7µg/ml.

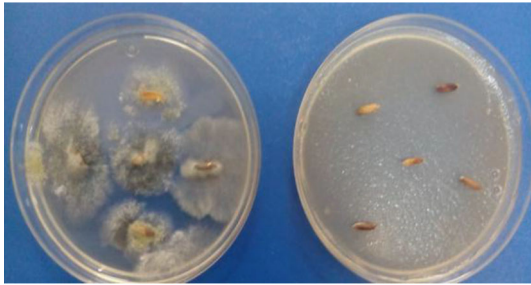


Fig. 8 Inhibition the fungal mycoflora at 7µg/ml on treatment by NCuS₃ for 2 h

In vivo evaluation of NCuS₃

In vitro studies indicated that the most active value for maximum inhibition of fungal growth lies below 10 µg/ml. So different concentrations viz. 5, 6, 7 and 8 µg/ml were tested for seed treatment studies. There was a decrease of seed rot and seedling blight with favourable effect on seed quality parameters such as seed germination, shoot length, root length and vigour index of seedlings at low concentrations. Reduction in seed rot and seedling blight was 60 and 83.3%, respectively, when the seeds were treated with 7 µg/ml NCuS₃ (Fig. 9) which was significantly higher than percentage reduction in seed rot (50%) and seedling blight (73.3%) in captan treated seeds. Root length of the seedlings was also significantly increased in NCuS₃ treated seed as compared to untreated control. Increase in shoot length was also comparable with captan treated seeds and it was significantly more than the untreated control. Vigour index was also maximum (11.06) in case of NCuS₃ at 7 µg/ml as compared to 10.53 obtained in captan treated seeds. At concentrations lower than 7 µg/ml, the seed rot and seed blight increased with decreased in vigour index and other vital parameters. At concentration ≥ 8 µg/ml the

phytotoxicity was observed with significant reduction in germination (Table 4).

The comparison of the results with Cu²⁺, copper (Cu) nanoparticles and copper oxide (CuO) nanoparticle reflects the edge of NCuS over other nano copper analogues. Cu²⁺ inflict high phytotoxic effects on seed application studies and toxicity was more than the chemically synthesized CuO nanoparticles (Saif et al. 2016). The results of germination enhancement were consistent with the increased germination on the application of CuO nanoparticles on *Brassica napus* (Atha et al. 2012) and *Triticum aestivum* (Farhat et al. 2015). The severe toxicity was further observed following severe reduction in seedling vigour, root length and shoot parameters by CuO and Cu nanoparticles. The increased germination in our results were at the concentrations as low as 7 µg/ml which were parallel with our previous *in vitro* results. Since sulfur and copper, both carry antifungal activities and their combination is much more effective than other nanoanalogues of copper. Side by side strong, thermally stable and non-hydrolysable linkage between Cu²⁺ and S²⁻ due to soft acid-soft base interaction makes release of Cu²⁺ ions (Wang et al. 2013) in aqueous media difficult, which is the main cause of lower toxicity copper toxicity in NCuS.

Conclusion

Overall, NCuS have shown broad spectrum of activities against phytopathogenic fungi. The results of bioactivity were deca-fold better than the standard fungicide captan used. Copper sulfide is natural, non toxic and have been reported to get metabolised in human body to cure various ailments (Guo et al. 2013). Moreover copper and sulfur belongs to essential micro and macro nutrient for the living

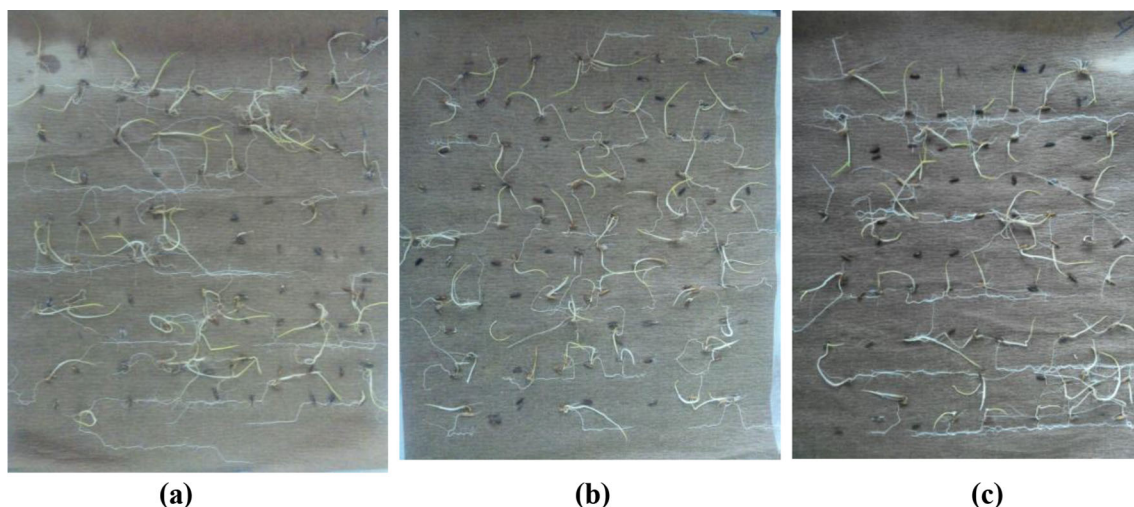


Fig. 9 The effect of NCuS₃ on seed treatment of discoloured paddy seeds at 7 µg/ml **a** control, **b** captan, **c** NCuS₃ treatment

Table 4 Effect of seed treatment with Captan and NCuS₃ on seed rot, seedling blight and seed quality parameters of healthy and infected rice seeds

| Treatment | Seed used | Seed health and seed quality parameters | | | | | | | | |
|-----------------------------|----------------------------------|---|--|-------------------|-----------------|-------------------|--------------|---------------------------|---------------------|----------------------------------|
| | | Germination (%) | Root length (cm) | Shoot length (cm) | Dry weight (mg) | Vigour index (VI) | Seed rot (%) | Reduction in seed rot (%) | Seedling blight (%) | Reduction in seedling blight (%) |
| NCuS ₃ | Discoloured @ 5µg/ml | 77 | 7.98 | 2.20 | 0.12 | 9.24 | 19 | 52.5 | 7 | 76.6 |
| | Discoloured @ 6µg/ml | 80 | 7.99 | 2.50 | 0.11 | 8.80 | 18 | 55 | 6 | 80 |
| | Discoloured @ 7µg/ml | 79 | 8.97 | 2.80 | 0.14 | 11.06 | 16 | 60 | 5 | 83.3 |
| | Discoloured @ ≥ 8µg/ml | < 50 | Seedling mortality due to phyto-toxicity | | | | | | | |
| Captan (standard fungicide) | Discoloured @ 0.25% ^a | 81 | 8.61 | 2.78 | 0.13 | 10.53 | 20 | 50 | 8 | 73.3 |
| Water | Healthy | 82 | 10.55 | 3.36 | 0.17 | 13.94 | 3 | Nil | Nil | Nil |
| Untreated (control) | Discoloured | 50 | 4.12 | 1.20 | 0.10 | 5.0 | 40 | Nil | 30 | Nil |

^a Standard dose for seed treatment

kingdom. Their multi times effectivity against the test fungi in comparison to the standards, along with non-poisonous effects, eco-friendly water based nature, low cost of production and easy synthesis demands their further exploitation in agricultural world. Their effect on seed quality further advocates their exploration and proper implementation as seed treatment agent which could significantly invigorate and accelerate seed germination and seedling growth.

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