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RESEARCH ARTICLE

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In vitro effects of copper nanoparticles on plant pathogens, beneficial microbes and crop plants

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Abstract

Copper-based chemicals are effectively used as antimicrobials in agriculture. However, with respect to its nanoparticulate form there has been limited number of studies. In this investigation, *in vitro* tests on effect of copper nanoparticles (CuNPs) against plant pathogenic fungi, oomycete, bacteria, beneficial microbes *Trichoderma harzianum* and *Rhizobium* spp., and wheat seeds were conducted. Integration of CuNPs with non-nano copper like copper oxychloride (CoC) at 50 mg/L concentration each recorded 76% growth inhibition of the oomycete *Phytophthora cinnamomi in vitro* compared to the control. CuNPs also showed synergistic inhibitory effect with CoC on mycelial growth and sporulation of *A. alternata*. *Pseudomonas syringae* was inhibited at 200 mg/L of CuNPs. CuNPs were not significantly biocidal against *Rhizobium* spp. and *Trichoderma harzianum* compared to CoC. Evaluation of the effect of CuNP on wheat revealed that rate of germination of wheat seeds was higher in presence of CuNPs and CoC compared to control. Germination vigor index, root length, shoot dry weight and seed metabolic efficiency of wheat were negatively affected. At low concentration, CuNPs promoted the growth of the plant pathogenic fungi *Botrytis fabae, Fusarium oxysporum* f.sp. *ciceris, F.oxysporum* f.sp. *melonis, Alternaria alternate* and *P. syringae*, and sporulation of *T. harzianum*. Synergistic effect of CuNPs and CoC in inhibiting *P. cinnamomi* offers a possibility of developing new fungicide formulation for better control of the oomycetes. Non-biocidal effect of CuNPs against beneficial microbes indicates its potential use in the agri-ecosystem.

Additional keywords: *Phytophthora cinnamomi*; *Trichoderma*; *Pseudomonas*; *Rhizobium*; wheat.

Abbreviations used: APS (average particle size); CoC (copper oxychloride); CuNPs (copper nanoparticles); DAI (days after inoculation); SME (seed metabolic efficiency); SSA (specific surface area); WAI (weeks after inoculation).

Authors' contributions: Conceived and designed the experiments, analysed the data and wrote the paper: SB and APL. Performed the experiments: SB. Contributed reagents/materials/analysis tools: APL.

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Introduction

Increased research in nanotechnology during the last years has led to the development of several nanomaterials for using in multiple industrial applications, such as medicine, food industry, textile, cosmetic, etc. Agriculture has been one of the most recent disciplines to join the nanotech race (Pérez-de-Luque & Hermosín, 2013). Nanoparticles have attracted special attention, due to their different properties compared with bulk materials. Indeed, several nanoparticles are being explored these days for their antimicrobial effects (Ren *et al*., 2009; Jia *et al*., 2012), which can be beneficial or harmful, depending on the context. Silver nanoparticles are the most widely studied and used as general antimicrobials (Morones *et al*., 2005; Banik & Sharma, 2011). However, copper nanoparticles (CuNP) have also been reported as effective antimicrobials in several studies (Cioffi *et al*., 2005; Ren *et al*., 2009; Jia *et al*., 2012).

Copper is as an essential trace element with distinct biological roles to play in all organisms, including plants, animals or microorganisms, when present in small amounts. In addition, copper based compounds have been reported to be one of the first fungicides used for plant disease management (Johnson, 1935). Bordeaux

mixture with a composition of copper sulphate, lime and water is regarded as the first fungicide discovered, used to control grapevine downy mildew disease caused by *Plasmopara viticola*, an oomycete pathogen (Millardet, 1886). Suspension of finely divided metallic copper was reported to have marked fungicidal activity as well (Large, 1943). Various copper compounds are recommended and are widely used for control of plant diseases caused by bacteria as well (Janse, 2005). Even organic agriculture still relies on copper for control of some diseases (Dorn *et al*., 2007).

Reports of antimicrobial effects of CuNPs are mainly focussed on microbes of human importance (Yoon *et al*., 2007; Raffi *et al*., 2010) or food industry (Cioffi *et al*., 2005; Jia *et al*., 2012), whereas those on agriculturally important plant pathogens and/or beneficial microbes are scarce. There has been little systematic study on the various biological components of the agro-ecosystem involving plants, plant pathogens and beneficial microbes together and addressing both the opportunities and threats of any nanoparticles. Therefore, in the present investigation, commercially available CuNPs (average particle size or APS =50 nm) were tested *in vitro* against a wide range of organisms encompassing plant pathogenic and non-pathogenic fungi and bacteria, and wheat (*Triticum aestivum* L.) seeds of cultivar 'Gazul'. The aim of this study was to evaluate if application of copper in the form of nanoparticles can be used against some of the phytopathogens found in field crops, and its putative negative effects on beneficial organisms and crop plants.

Material and methods

Microbiological material

The microbiological materials used were plant pathogenic fungi *Botrytis cinerea*, *B. fabae*, *Colletotrichum acutatum*, *Fusarium oxysporum* f. sp. *ciceris*, *F. o*. f. sp. *lycopersici*, *F. o*. f. sp. *melonis*, *Verticillium dahliae*, *Verticillium albo-atrum* and *Alternaria alternata*; an oomycete (*Phytophthora cinnamomi*), one phytopathogenic bacterium (*Pseudomonas syringae*); one fungal biocontrol agent (*Trichoderma harzianum*) and three species of *Rhizobium* viz., *Rhizobium etli*, *Rhizobium meliloti*, *Rhizobium tropici* that fix atmospheric nitrogen in symbiotic association with roots of leguminous crops. Table 1 shows their collection sources and references.

All the fungal species and *P. cinnamomi* were retrieved and maintained in PDA medium (20 g/L dextrose, 4 g/L potato extracts, 15 g/L agar). *P. syringae* was maintained in Nutrient Broth/Agar I medium (5 g/L beef extract, 10 g/L peptone, 5 g/L NaCl , 15 g/L agar made up according to supplier instructions), and *Rhizobium* spp. Were kept in TY medium (0.5 g/L $CaCl_2$, 5 g/L tryptone, 3 g/L yeast extract, 15 g/L agar).

Suspension of copper nanoparticles

All the above mentioned microorganisms were tested against different doses of commercial CuNPs, provided by M.K. Impex Corp., Ontario L5N 6X1, Canada (product MKN-Cu-050, CAS number 7440-50-8, 99.9% purity, 50 nm APS, 12 m^2/g of specific surface area (SSA)). Required quantity of CuNPs was weighed and added to a sterile test tube containing 10 mL of sterile distilled water. The tube was held in an ultrasonic bath (USC Series 'TH'; Device type USC 900; 45kHz ultrasound frequency) for 60 s to evenly disperse the CuNPs. The nanoparticle dispersion was added to sterile, melted microbial media and stirred before plating.

The commercial form ('Ossirame 50 wg', ManicaCobre S.L.; 50% a.i) of the compound copper oxychloride (CoC) was used as positive control and as counterpart of CuNPs for comparison. The Petri plates without any addition of CuNPs or CoC served as negative control. CuNPs and CoC were mixed at various proportions given in 'Treatment' section below to study their synergistic effect as well.

Treatments

Initially, all the fungal materials including the oomycete *P. cinnamomi* were tested against either copper formulation (CuNPs and CoC) at 100 mg/L in the growth media. Subsequently, *P. cinnamomi* was grown on the medium containing 50 mg/L of both CuNPs and CoC, to check their synergistic effect *in vitro*. The plant pathogenic fungus *A. alternata* was further exposed to concentrations of 100, 200 and 400 mg/L of CuNPs alone and in combination with CoC (CuNP:CoC 50:50, 100:100, 200:200, and 400:400 mg/L) to test its growth and sporulation behaviour. Similarly, growth and sporulation of the biocontrol fungus *T. harzianum* was tested in a medium containing 10, 100 or 1000 mg/L of CuNPs.

The bacterium *P. syringae* was tested against 50, 100, 200 and 400 mg/L of CuNPs alone and also in combination with CoC (CuNP:CoC 50:50, 100:100, 200:200, and 400:400 mg/L) *in vitro*. The three *Rhizobium* spp. (*R. tropici*, *R. etli* and *R. meliloti*) were tested against lower concentrations of CuNPs alone (25, 50, 100, 200 mg/L) and in combination with CoC (CuNP:CoC 25:25, 50:50, 100:100 mg/L). The Petri plates without any addition of CuNPs or CoC served as negative control.

Table 1. Materials used in the present study.

	Reference no.	Source[1]
Botrytis fabae (isolated from *Vicia faba*)	-	[a]
B. cinerea (isolated from *Vicia faba*)	-	[b]
Alternaria alternata (isolated from *Capsicum annuum*)	CECT 20560	[c]
Colletotrichum acutatum (isolated from *Vaccinium corymbosum*)	CECT 20411	[c]
Fusarium oxysporum f. sp. *ciceris* (isolated from *Cicer arietinum*)	-	[d]
F. oxysporum f. sp. *lycopersici* (isolated from *Lycopersicon esculentum*)	CECT 2866	[c]
F. oxysporum f. sp. melonis (isolated from *Cucumis melo*)	CECT 20474	[c]
Trichoderma harzianum (isolated from unknown host)	CECT 2423	[c]
Verticillium albo-atrum (isolated from *L. esculentum*)	CECT 2693	[c]
V. dahliae (isolated from *C. melo*)	CECT 2884	[c]
Phytophthora cinnamomi (isolated from *Quercus ilex*)	-	[e]
Pseudomonas syringae (isolated from *L. esculentum*)	CECT 126	[c]
Rhizobium etli CFN42T (isolated from *Phaseolus vulgaris*)	-	[f]
Rhizobium meliloti 1021 (isolated from *Medicago truncatula*)	-	[f]
Rhizobium tropici 890T (isolated from *P. vulgaris*)	-	[f]
Copper nanoparticles (CuNP)	-	[g]
Copper oxychloride (CoC)	-	[h]

[1] [a]: IFAPA Alameda del Obispo collection, kindly provided by Dr. C. Ávila. [b]: IFAPA Alameda del Obispo collection, kindly provided by Dr. A. Villegas. [c]: Colección Española de Cultivos Tipo, Parc Cientific Universitat de Valencia. [d]: Race 5, kindly provided by Dr Chen, Washington State University, Pullman, WA, USA. [e]: A2-type isolate, provided and certified by NBT laboratories (New Biotechnics, Sevilla, ISO 9001/17025). [f]: Estación Experimental Zaidín, Granada, Spain, kindly provided by Prof. Eulogio Bedmar. [g]: M.K. Impex Corp., Ontario L5N 6X1, Canada (product MKN-Cu-050, CAfS number 7440-50-8, 99.9% purity, APS (average particle size) = 50 nm, SSA (specific surface area) =12 m^2/g). [h]: 'Ossirame 50 wg', ManicaCobre S.L.; 50% a.i.

To know the effect of CuNPs on germination and other growth behaviours of wheat seeds, seeds cv. 'Gazul' were grown in presence of 1, 5, 10, 25, 50 and 100 mg/L of CuNPs in aqueous suspension *in vitro*.

Inoculation and growing of microorganisms

Six millimetre diameter mycelial discs were cut from the leading edge of three days old fungal cultures and used for inoculation of all the fungi and *P. cinnamomi* in Petri dishes containing the corresponding culture media (PDA) and the different treatments.

For the plant pathogenic bacterium *P. syringae*, one bacterial colony of ~2 mm diameter from two-day old culture was picked and transferred to 10 mL sterile distilled water in a sterile test tube. The tube was shaken manually to obtain a bacterial suspension. The suspension thus made, was serially diluted to 10^{-3} concentration of the original and 50 µL of the final dilution was transferred to each Petri dish prepared two days ahead with the corresponding culture medium (Nutrient Broth/Agar I) and treatments. The bacterial inoculum was spread on the medium surface with a sterile glass spreader. A similar protocol was followed for *Rhizobium* spp., except that the colony diameter varied based on the type of species and the culture medium (TY medium).

The cultures of all the fungi, the oomycete *P. cinnamomi* and all the bacteria under study were grown in a BOD incubator maintained at 25°C.

Measurement of microorganism development

The growth of all the fungi and the oomycete *P. cinnamomi* was scored measuring radial mycelial growth in Petri dishes with a digital calliper, seven days after inoculation. Bacterial growth was measured by counting and recording the number of colonies appearing on the medium surface after 3-6 days, depending on the species. In addition, *T. harzianum* and *A. alternata* were further assessed for sporulation. In the case of *T. harzianum*, spore production was recorded every day, beginning two days after inoculation. For *A. alternata*, plates were kept in alternate light and dark cycles seven days after inoculation and observations on spore production were measured 7, 14 and 21 days after inoculation.

Wheat germination and initial growth

Wheat seeds of the cultivar 'Gazul' were used for the experiment. For each replication under various treatments 20 seeds were weighed initially. A germination test was conducted in 85 mm disposable Petri dish in the presence

of aqueous suspension of CuNPs and CoC separately. Twenty seeds were placed in each plate containing 20 mL of aqueous suspension of either CuNPs or CoC, at concentrations of 1, 5, 10, 25, 50 and 100 mg/L. Sterile distilled water was used as a negative control. Observation of seed germination was made every 24-hour interval until the 5th day. A seed was regarded as germinated when both radicle and plumule emerged with a length larger than 2 mm (Sikder & Paul, 2010).

On the 5th day, root and shoot lengths for all the seedlings were measured. Roots and shoots were excised carefully from each seedling and dried in hot air oven at 70°C for 72 hours. The remaining seeds after separating the roots and shoots were also oven-dried. Dry weight was recorded for all of them.

Analysis

For all the assays, every treatment and concentration for each of the microorganisms was replicated three times. All the experiments were conducted using a randomized experimental design and repeated at least twice. For the wheat experiments, three replications (thus 60 seeds altogether in one treatment) were performed for each treatment using a randomized design. ANOVA and HSD were calculated using Statistix 8.0 for Windows.

The percent inhibition of fungal growth *in vitro* was calculated using the following formula (Lamsal *et al*., 2011):

$$Percent\ inhibition = \frac{R - r}{R} \times 100$$

where R =radial growth of the fungi in control plate, *r*= radial growth of the fungi in treatment plate.

The rate of germination was calculated according to the following formula proposed by Krishnasamy & Seshu (1990):

$$Rate\ of\ germination\ (\%) = \frac{Number\ of\ seeds\ germinated\ at\ 48\ hours}{Number\ of\ seeds\ germinated\ at\ 120\ hours} \times 100$$

Co-efficient of germination and vigor index was calculated using the formula given by Copeland (1976):

$$Co\text{-}efficient\ of\ germination = \frac{100 \times (A_1 + A_2 + \cdots + A_n)}{A_1T_1 + A_2T_2 + \cdots + A_nT_n}$$

$$Vigor\ Index = \frac{A_1}{T_1} + \frac{A_2}{T_2} + \cdots + \frac{A_n}{T_n}$$

where *A* = number of seeds germinated, *T* = time (days) corresponding to *A*; and *n* = no. of days to final count.

Seed metabolic efficiency (SME) was calculated using the following formula (Rao & Sinha, 1993):

$$SME = \frac{Shoot\ dry\ weight + Root\ dry\ weight}{Seed\ material\ respired\ (SMR)}$$

SMR was calculated as follows (Sikder & Paul, 2010):

$$SMR = SDW - (SHW + RTW + RSW)$$

where *SDW* = seed dry weight before germination, *SHW* = shoot dry weight, *RTW* = root dry weight, *RSW* = remaining seed dry weight.

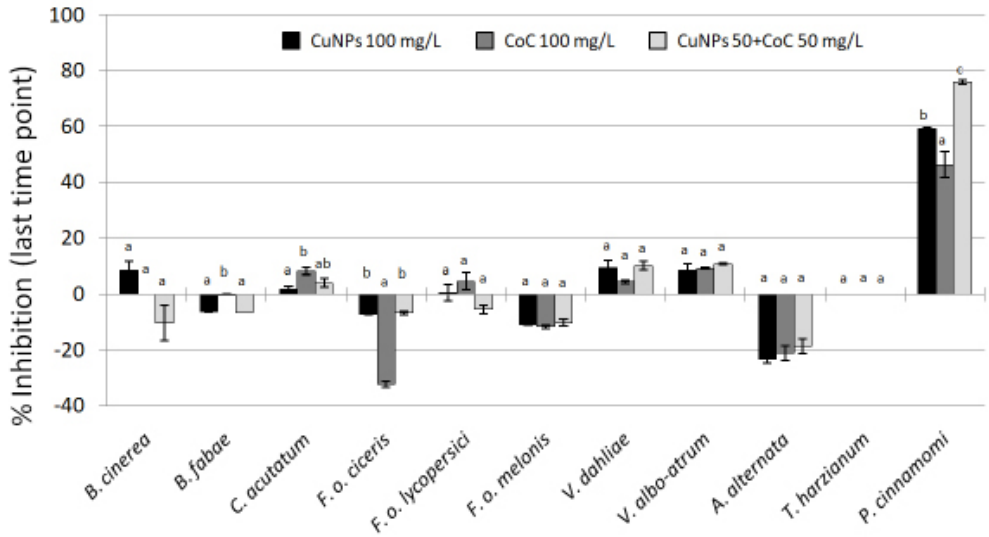
Results

Effect of nanocopper on fungi

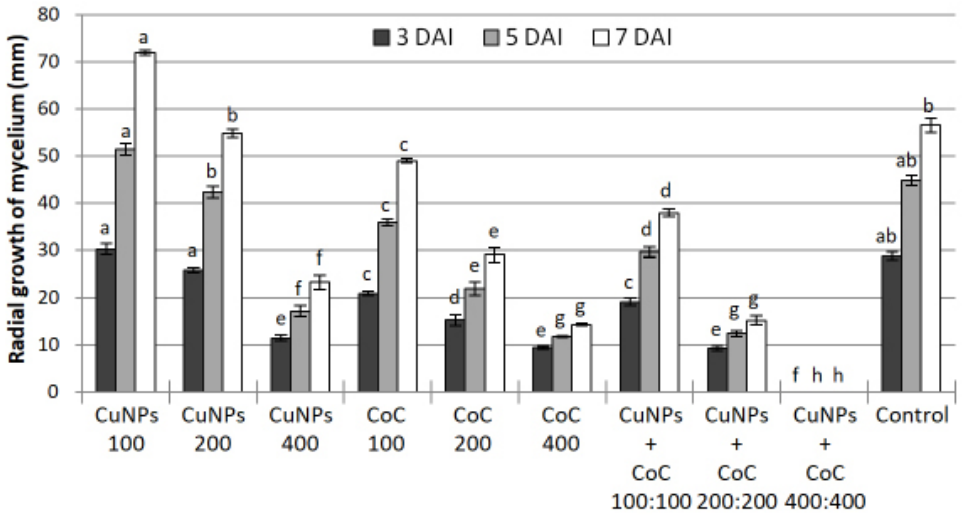
CuNPs were not effective in inhibiting the mycelial growth of most of the tested fungi (Fig. 1A). However, significant inhibition was observed for the oomycete *P. cinnamomi,* against which CuNPs recorded near 60% growth inhibition compared to 46% with a commercial formulation of CoC at a dose of 100 mg/L. More interestingly, a synergistic effect of the two products could be observed resulting in inhibition of 76% when they were mixed at 50 mg/L each (Fig. 2A). Other plant pathogenic fungi showed variable responses for growth inhibition and growth promotion with CuNPs, CoC and their combinations. Growth reduction of the fungal pathogens *B. cinerea*, *C. acutatum*, *V. dahliae* and *V. albo-atrum* by CuNPs was observed but at low level (less than 10%) (Fig. 1A). Enhancement of mycelial growth of plant pathogenic fungi *B. fabae*, *F. oxysporum* f. sp. *ciceris*, *F. oxysporum* f. sp. *melonis* and *A. alternata* was recorded to various degrees by CuNPs at 100 mg/L with the highest being in *A. alternata* (23%). The highest degree of growth promotion (32%) was observed in *F. oxysporum* f. sp. *ciceris* in CoC treatment compared to only 7% with CuNPs (Fig. 1A).

Regarding *A. alternata*, the pathogen was subjected to further experiment with CuNPs at higher doses. It is revealed from Fig. 2B that 800 mg/L dose of CuNPs completely inhibited mycelial growth of *A. alternata*. Interestingly, a synergistic effect of CoC and CuNPs was observed with complete inhibition at 400 mg/L dose of each of them combined (Fig. 1B). The synergistic inhibitory effect was more pronounced on *A. alternata* in terms of sporulation. Spores were not detected in Petri dishes treated with 400 mg/L CuNPs, nor in dishes treated with 200 mg/L of both CoC and CuNPs (Fig. 1C). There was a significant difference in the number ofspores formed (0.6, 2.4 and 4.1) with CoC and CuNPs

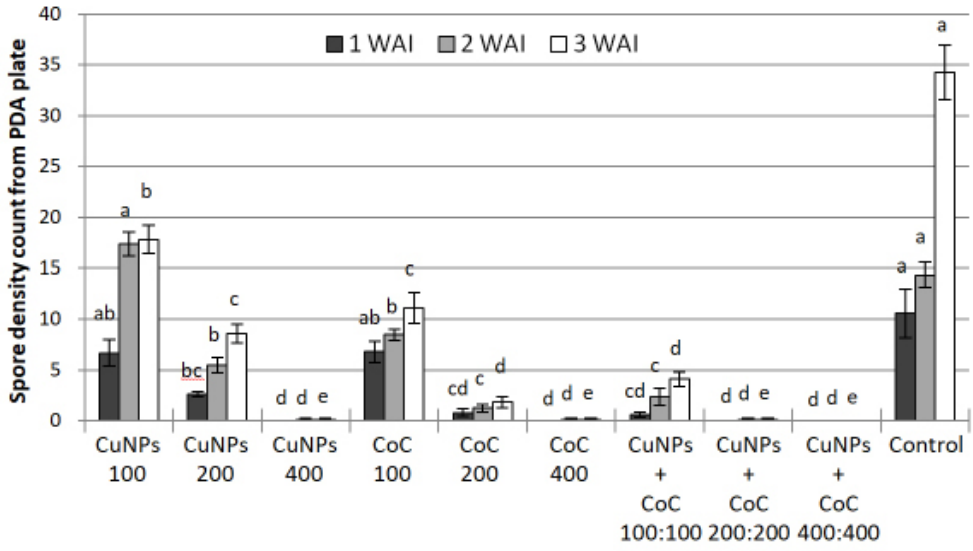
A


CuNPs 100 mg/L
CoC 100 mg/L
CuNPs 50+CoC 50 mg/L
% Inhibition (last time point)
B. cinerea
B. fabae
C. acutatum
F. o. ciceris
F. o. lycopersici
F. o. melonis
V. dahliae
V. albo-atrum
A. alternata
T. harzianum
P. cinnamomi

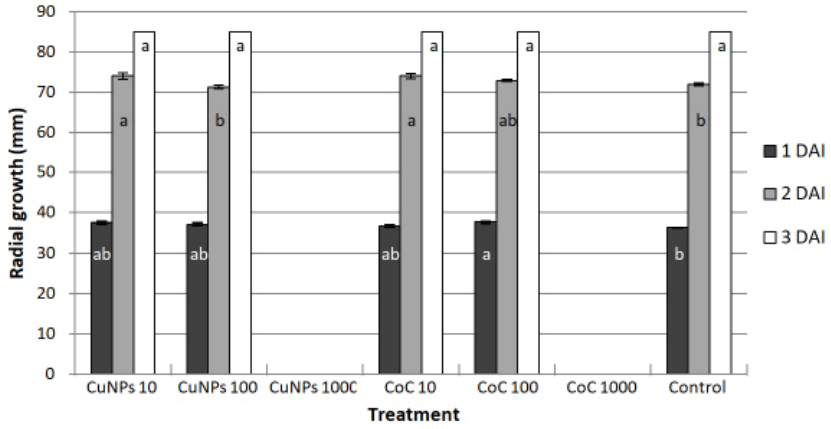
B


3 DAI
5 DAI
7 DAI
Radial growth of mycelium (mm)
CuNPs 100
CuNPs 200
CuNPs 400
CoC 100
CoC 200
CoC 400
CuNPs + CoC 100:100
CuNPs + CoC 200:200
CuNPs + CoC 400:400
Control

C


1 WAI
2 WAI
3 WAI
Spore density count from PDA plate
CuNPs 100
CuNPs 200
CuNPs 400
CoC 100
CoC 200
CoC 400
CuNPs + CoC 100:100
CuNPs + CoC 200:200
CuNPs + CoC 400:400
Control

D **Figure 1.** continued.



E

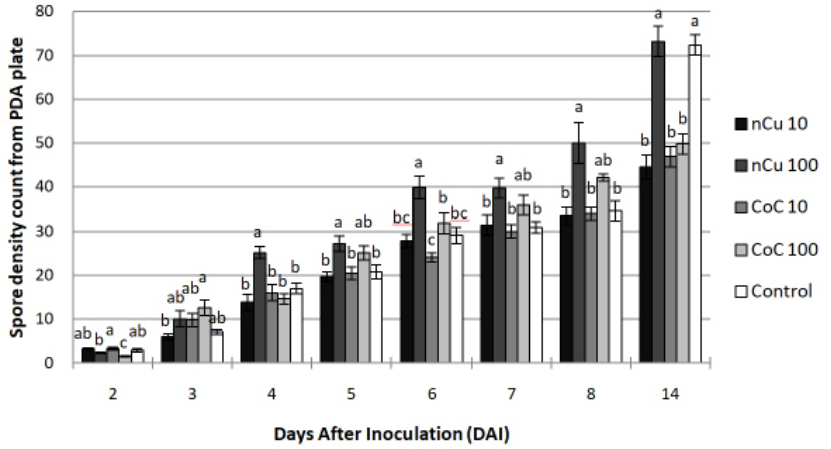


Figure 1. Effect of copper nanoparticles (CuNPs) on fungi. (A) Percentage of growth inhibition of different phytopathogenic fungi in presence of CuNPs and copper oxychloride (CoC). (B) Radial growth of *Alternaria alternata* in presence of CuNPs and CoC. (C) Effect of CuNPs and CoC on sporulation of *A. alternata.* (D) Radial growth of *Trichoderma harzianum* in presence of CuNPs and CoC. (E) Effect of CuNPs and CoC on sporulation of *T. harzianum*. WAI, weeks after inoculation. Bars represent SE. (A): Data with the same letter within the same species or strain are not significantly different (HSD, $p<0.05$). (B, C, D, E): Data with the same letter within the same days after inoculation (DAI) are not significantly different (HSD, $p<0.05$).

treatment (100 mg/L each) compared to control (10.6, 14.4, 34.3) at one, two and three weeks after inoculation respectively (Fig. 1C).

The biocontrol fungus *T. harzianum* was not inhibited at all in terms of mycelial growth (Fig. 1A). However, earlier and higher rate of sporulation of *T. harzianum* were recorded in CuNPs (100 mg/L) treated Petri dishes till 8 days after inoculation (DAI) (Fig. 1E), though the final sporulation count at 14 DAI scored a spore density which is not statistically different from that of control plates. CoC treatment also induced higher sporulation of the fungus till 8 DAI. The mycelial growth of *T. harzianum* was completely inhibited only at 1000 mg/L of CuNPs and CoC when tested separately (Figs. 1D, 2C).

Effect of nanocopper on bacteria

Growth of *P. syringae* was completely inhibited *in vitro* with CuNPs at 200 mg/L (Fig. 3). At lower doses, CoC showed more inhibitory effect than CuNPs at 100 mg/L concentration. However, significantly higher numbers of growing colonies of *P. syringae* were observed at 50 and 100 mg/L concentrations of CuNPs as well as CoC, compared to control plates. Six to eight-fold higher number of colonies of *P. syringae* was observed in growth media treated with CuNPs at concentrations lower than 200 mg/L compared to control. The synergistic effect of CuNPs and CoC was prominent when tested in the combination of 50 mg/L each. Further, combined 100 mg/L concentration of both CuNPs and CoC completely stopped *P. syringae* growth *in vitro*.

Colonies of the three *Rhizobium* spp., *R. etli*, *R. meliloti* and *R. tropici*, appeared six, five and three DAI, respectively, due to the differences in their growth traits. The three *Rhizobium* spp. responded differently to the CuNPs and CoC treatments. It appears from the data (Fig. 4) that copperF is not inhibitory to *R. tropici*

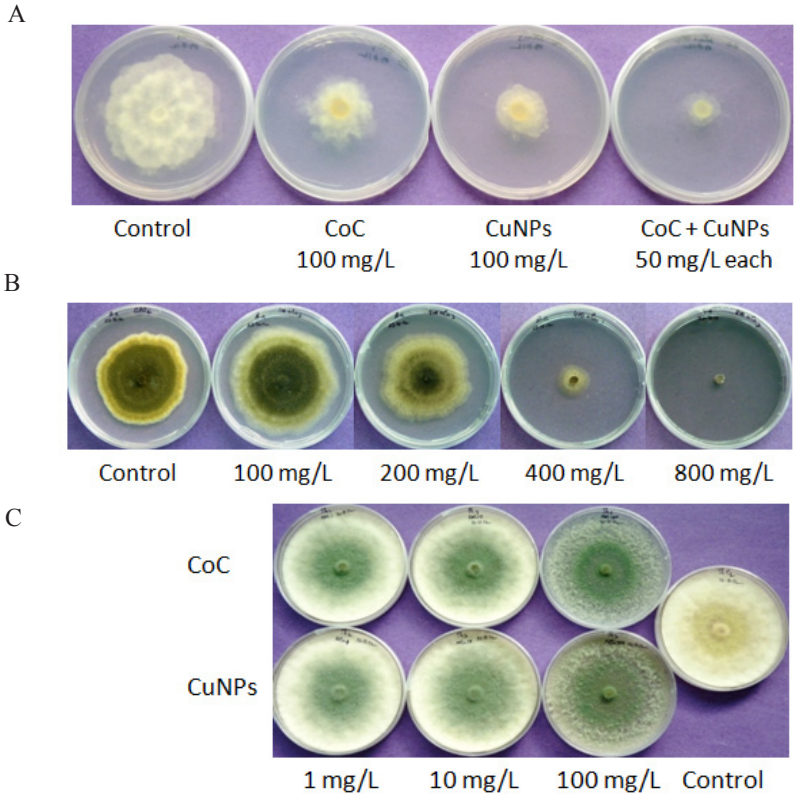


Figure 2. Effect of nanocopper on the growth of several fungi. (A) Growth inhibition of *Phytophthora cinnamomi* 7 days after inoculation (DAI). (B) Differential growth of *Alternaria alternata* in presence of copper nanoparticles (CuNPs). (C) Growth of *Trichoderma harzianum* and Cu accumulation in mycelia in presence of CuNPs and copper oxychloride (CoC).

growth up to 200 mg/L. *R. etli* was inhibited completely only at 200 mg/L CoC but not in the presence of CuNPs (Fig. 5). In contrast, *R. meliloti* was inhibited at a lower concentration of 100 mg/L CoC and 50 mg/L of either CuNPs and CoC (Fig. 6), showing more sensitivity to copper compared with the other two species.

Effect of CuNPs on wheat

CuNPs affected wheat seed germination, root length, shoot length, root and shoot dry weight of wheat seedlings in all the concentrations. The rate of germination, co-efficient of germination and germination vigour index of the wheat seeds germinated in presence of CuNPs are presented in Fig. 7. Results showed that for the three germination traits, CuNPs significantly influenced only germination vigour index. Overall, rate of germination of wheat seeds (Fig. 7A) showed tendency to a higher value than control in presence of CuNPs and CoC. However, with increasing concentration of CuNP and CoC the rate of germination did not show a clear increasing or decreasing tendency nor significant differences with control. On the other hand, germination vigour index of wheat seeds was recorded to be gradually declining with increasing concentration of CuNPs and CoC (Fig. 7C).

Shoot length was more or less unaffected in presence of CuNPs and CoC, with no significant differences (Fig. 8A). It was the root that was most influenced, showing significant reduction in length (Fig. 8B) at doses as low as 5 mg/L of CuNPs (16.7 mm compared to 31.5 mm in control).

Shoot dry weight showed no significant differences, and only a tendency in reduction was observed over 25 mg/L concentration of CuNPs (Fig. 8C), but again a more pronounced effect was observed in root dry weight (Fig. 8D) which was significantly lower at 10 mg/L of CuNPs compared to CoC. A trend of gradual inhibition of root length and root dry weight was observed with increasing concentration of CuNPs as well as CoC.

Seed metabolic efficiency (SME) was calculated to reflect on the reserved food material of seeds utilized in process of germination and seedling growth in presence of CuNPs (Fig. 9). No significant reduction with respect to the control was observed, but again a tendency to reduction appeared with the highest concentrations.

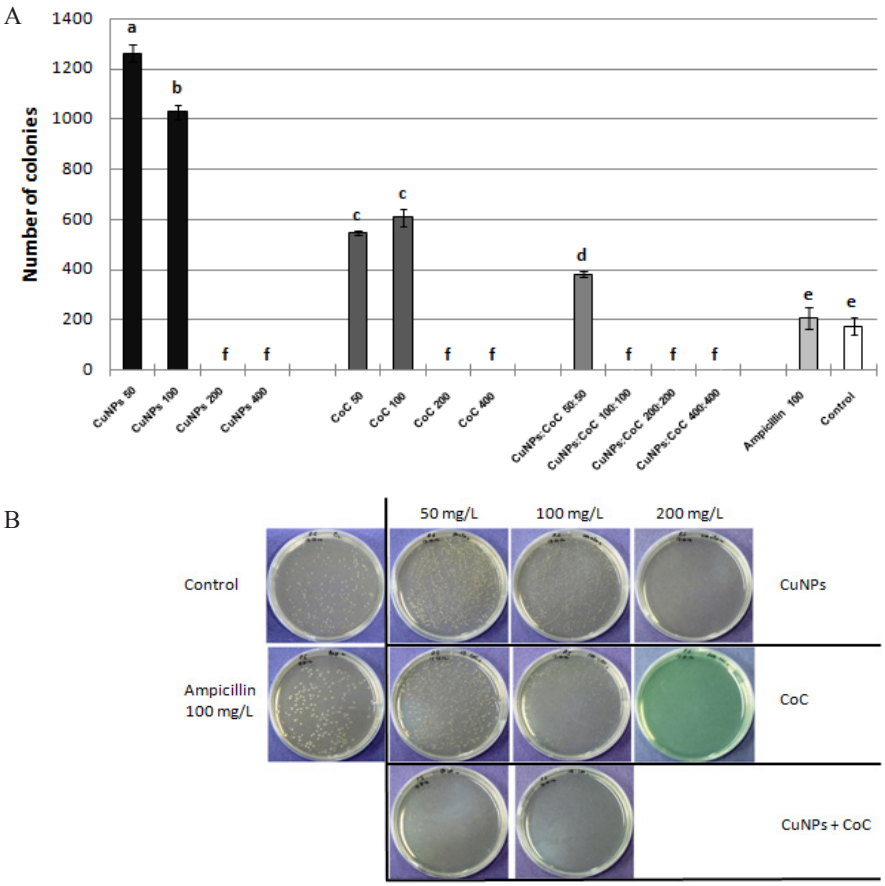


Figure 3. Effect of copper nanoparticles on *Pseudomonas syringae in vitro*. (A) Number of colonies under different treatments and concentrations (mg/L). (B) Growth of *P. syringae* colonies in Petri dishes. CuNPs, copper nano particles; CoC, copper oxychloride. Bars represent SE. Data with the same letter are not significantly different (HSD, $p<0.05$).

Discussion

P. cinnamomi and various fungi

Most of the research on antimicrobial effects of metal nanoparticles in general have revolved around human pathogenic microbes, both bacteria and fungi, due to their importance in human health, whereas little information is available on plant pathogens.

The present investigation was carried out to know the antimicrobial potential against harmful plant pathogens and, at the same time, to address the toxicity issues of CuNPs with regard to plant and beneficial microbes. Copper is required by all living organisms as a trace element and it is part of several enzymes and electron carriers in oxidative electron transport chain. *In vitro* growth promotion of certain plant pathogens by CuNPs is not surprising at all and stimulation of mycelial growth by copper at high concentrations has been previously reported (Englander & Corden, 1971). However, the very high growth inhibition of *P. cinnamomi* (oomycetes) in contrast with the other plant pathogenic fungi (Ascomycetes and mitosporic fungi) tested in the present study may be due to its pseudofungus nature and differences in cell wall and membrane composition (Alexopoulos *et al*., 2002). The cellulosic nature of cell wall of oomycete pathogens (Alexopoulos *et al*., 2002) coupled with absence of ergosterol in *P. cinnamomi* cell membranes (Pérez-de-Luque *et al*., 2011) may contribute to the higher copper sensitivity than the ergosterol-containing plant pathogenic fungi, which have chitin as a chief constituent of the cell wall (Alexopoulos *et al*., 2002). For example, in the yeast *Saccharomyces cerevisiae,* copper toxicity has been found to be dependent on plasma membrane fatty acid composition (Avery *et al*., 1996). In addition, physiological and metabolic differences could contribute to such susceptibility, in the same way that all those factors could be responsible for specific lack of sensitivity of oomycetes against common antifungals (Pérez-de-Luque *et al*., 2011). Indeed, heavy metal toxicity (which includes copper)

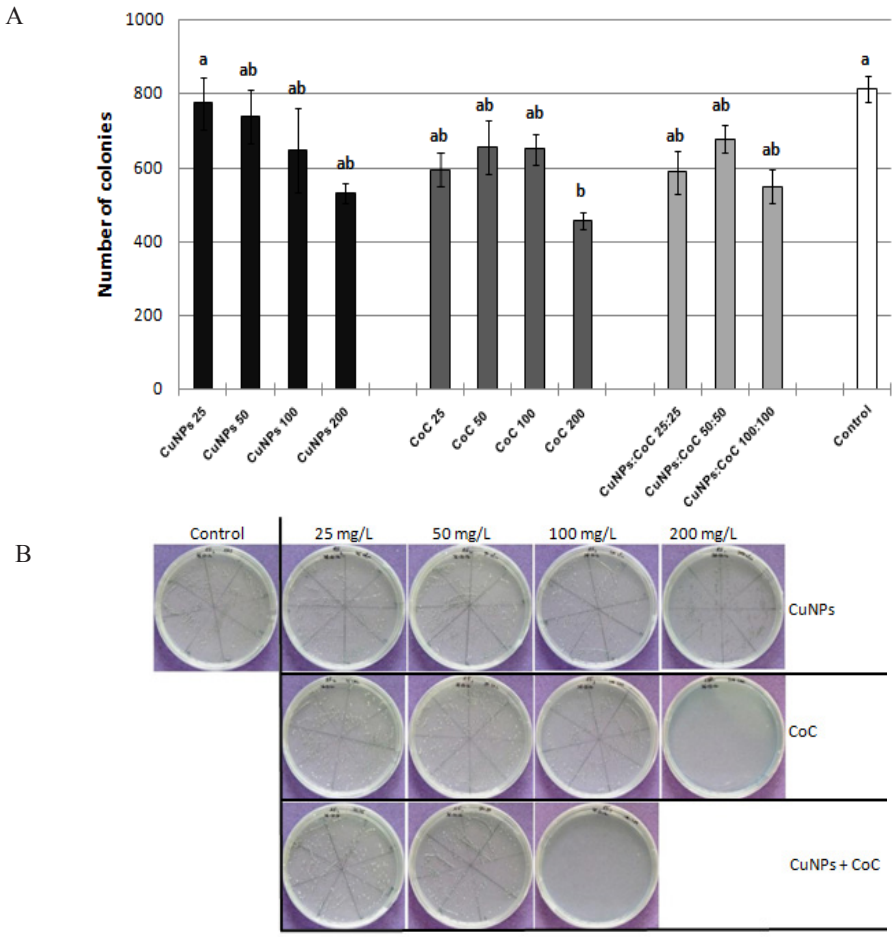


Figure 4. Effect of copper nanoparticles on *Rhizobium tropici in vitro.* (A) Number of colonies under different treatments and concentrations (mg/L). (B) Image showing the growth of *R. tropici* colonies in Petri dishes. Bars represent SE. Dara with the same letter are not significantly different (HSD, $p<0.05$).

can be due to alteration or blocking of enzymes, inhibition of macromolecule synthesis, or disruption of cellular and organellar membranes (Arduini *et al*., 1995; Denkhaus & Salnikow, 2002).

Alternaria alternata has been recently described as tolerant to high levels of copper metal ion in the medium (Al Abboud & Alawlaqi, 2011), so it agrees with the results we have found in which only the highest levels of CuNPs affect its mycelial growth. The work by Al Abboud & Alawlaqial (2011) indicates how sporulation rate decreases with increasing concentration of copper in the medium, and that fatty acid composition of membranes is severely affected.

Regarding *T. harzianum*, there are reports about the ability of this genus to accumulate copper as a layer in the cell wall surface during the first stages of growth (Anand *et al*., 2006). This would explain the green colour observed in the central (and older) parts of the mycelium (Fig. 2C). Such a mechanism allows the fungus to remove the copper from the medium, leaving a less toxic environment for further growth (Anand *et al*., 2006), which probably explain why sporulation is not affected later.

An interesting phenomenon observed during the experiments was the synergistic effect between CuNPs and CoC for inhibition of mycelial growth and/or sporulation. More specific studies are needed to elucidate the exact nature of this effect, but a possible explanation could be that co-adjuvants present in the commercial formula of CoC would prevent agglomeration of CuNPs (acting as capping/stabilizing agent) and help CuNPs to penetrate into fungal cells better, increasing their toxicity. Several capping/stabilizing agents of biological origin (Shobha *et al*., 2014; Singh *et al*., 2015) and chemical nature (Guzman *et al*., 2009) are reported in the literature.

It is known that reactivity, and consequently effectiveness of nanoparticles depends on their size. It is regarded that the antimicrobial effect of metal nanoparticles is due to their small size and high surface

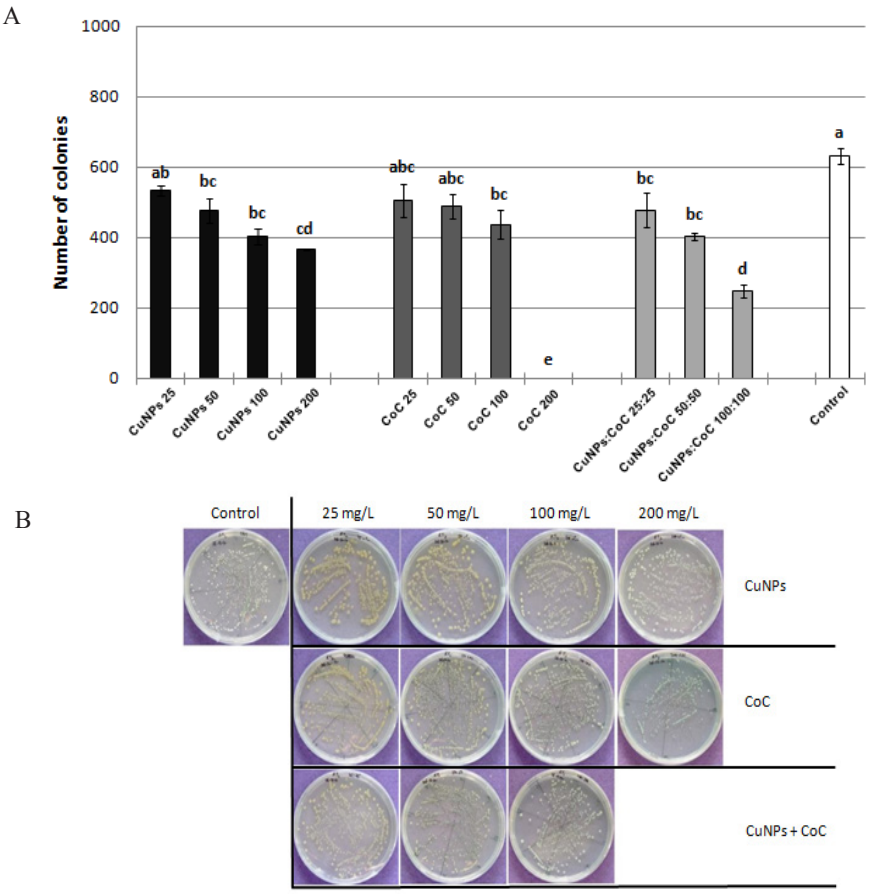


Figure 5. Effect of copper nanoparticles on *Rhizobium etli in vitro*. (A) Number of colonies under different treatments and concentrations (mg/L). (B) Image showing the growth of R. meliloti colonies in Petri dishes. Bars represent SE. Data with the same letter are not significantly different (HSD, $p<0.05$).

to volume ratio as well as the release of metal ions in solution (Morones *et al*., 2005). Finely divided metallic copper of size 1 µm was more effective against *Phytophthora infestans*, the potato blight pathogen, than that of size 2-5 µm (Large, 1943). Similarly, smaller sized CuNPs are expected to be more microbicidal than their larger counterparts. Commercial grade CuNPs (APS 50 nm) were used in this experiment, which do not ensure uniform size distribution of nanoparticles. The key for increasing their effectiveness in the future may be in research with smaller and uniformly sized CuNPs.

P. syringae

CoC, as a copper compound, is antibacterial (Janse, 2005). But in the present experiment, CoC at doses lower than 200 mg/L was found to have a growth promotion effect on *P. syringae*. Appearance of significantly very high numbers of *P. syringae* colonies was seen in CuNPs and CoC treated media plates compared to control plates in spite of inoculation with same quantity of inoculum (and therefore same number of bacterial cells in the aliquot). This reflects the fact that the control plate does not support the complete growth of all the bacterial cells inoculated, and the growth medium is at least copper deficient. The supply of copper in the form of nanoparticles and CoC promotes the growth of the bacteria.

However, CuNPs at 200 mg/L completely prevented the growth of *P. syringae*. It has been described that CuNPs of size 12 nm interact with the cell wall of Gram-negative bacterium *E. coli* and form cavities in the wall (Raffi *et al*., 2010). Opposite electrical charge of CuNPs make them adhere to the bacterial cell wall resulting in certain reduction reaction (Raffi *et al*., 2010). In *P. syringae*, being a gram-negative bacterium, a similar mechanism of action might be inhibiting the bacterial growth. Disruption of plasma membrane is one of the mechanisms of action of copper in microorganisms (Englander & Corden, 1971; Hoshino *et al*., 1999). It seems that binding of copper ions to

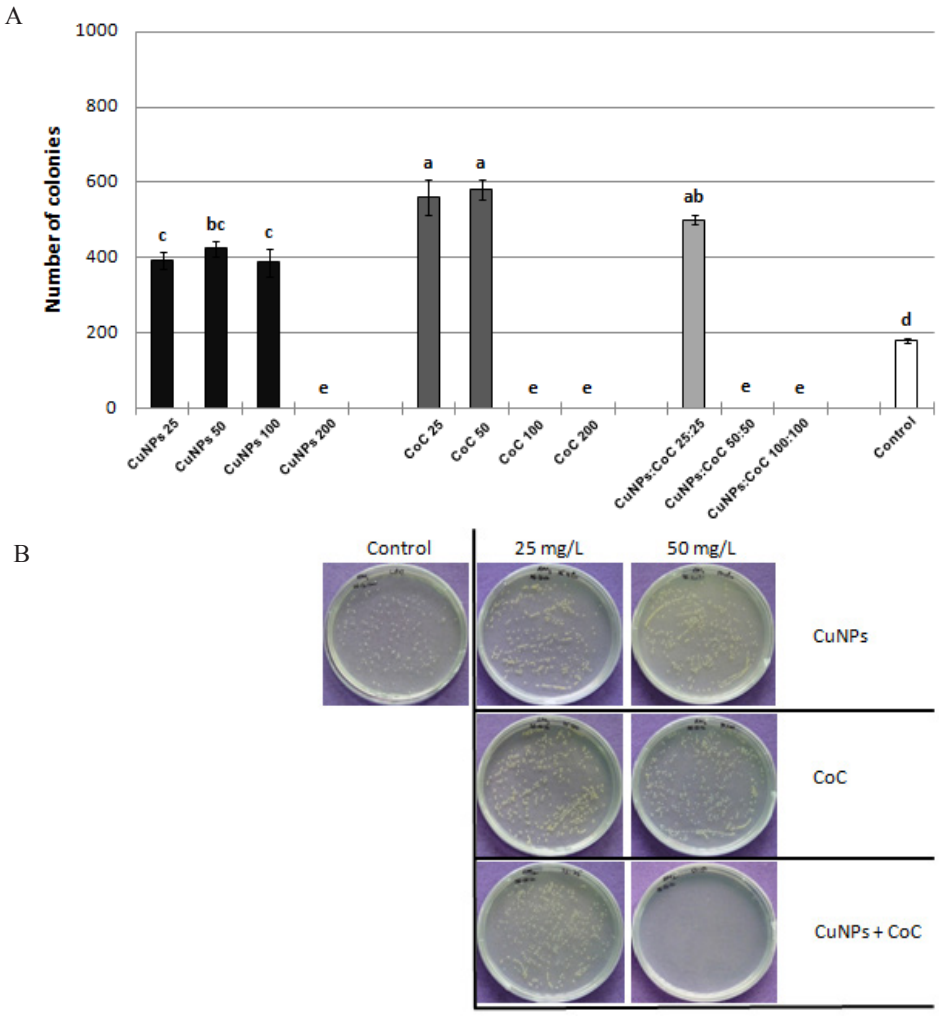


Figure 6. Effect of copper nanoparticles on *Rhizobium meliloti in vitro.* (A) Number of colonies under different treatments and concentrations (mg/L). (B) Image showing the growth of *R. meliloti* colonies in Petri dishes. Bars represent SE. Data with the same letter are not significantly different (HSD, $p<0.05$).

the surface of bacterial cells plays an important role in the bactericidal activity (Hoshino *et al*., 2000). Antimicrobial effect of copper mediated by contact killing has been reported even from a dry metallic copper surface (Grass *et al*., 2011) or copper alloys (Hong *et al*., 2012).

Rhizobium spp.

Similarly to the results with *T. harzianum*, the present investigation revealed that CuNPs are not potent biocidal agents against the tested beneficial bacteria (*Rhizobium* spp.), and in one case (*R. meliloti*), even promote bacterial multiplication. The differential sensitivity of the three *Rhizobium* spp. towards CuNPs also reflects the differences existing among them in terms of physiology. The complete inhibition of *R. meliloti* at 200 mg/L CuNPs while the other two species (*R. etli* and *R. tropici*) recorded abundant colony growth provide further scope to unravel the mechanism of action of CuNPs. In addition, commercial grade CoC also recorded different responses against the *Rhizobium* spp., with complete halt of *R. meliloti* development at 100 mg/L CoC. Therefore, application of copper compounds in soil for leguminous crops needs careful consideration, because variations in the tolerance against this heavy metal can be found in soil populations, and in the long term they can be affected, altering their presence and population structure in the soil (Laguerre *et al*., 2006).

Wheat (Triticum aestivum)

Germination of wheat seeds was not severely affected by the presence of CuNPs, and the behaviour

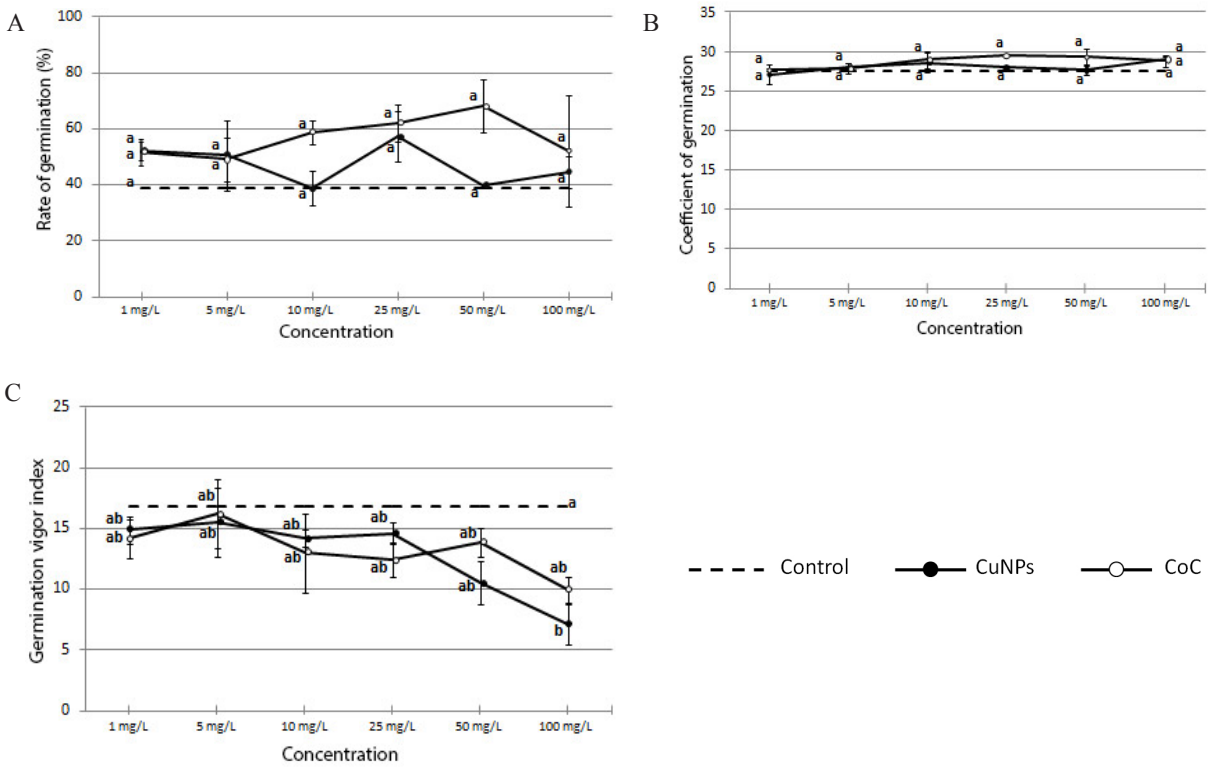


Figure 7. Effect of copper nanoparticles on germination of wheat seeds. (A) Rate of germination in presence of CuNPs and CoC. (B) Coefficient of germination in presence of CuNPs and CoC. (C) Germination vigor index in presence of CuNPs and CoC. Bars represent SE. Data with the same letter are not significantly different (HSD, $p<0.05$).

was similar to the treatment with the commercial CoC. Only the vigor index was affected by both treatments, leading to a decrease when concentration increased. This indicates that treated seeds, despite germinating at a similar rate as untreated seeds, produce less vigorous seedlings. This agrees also with the data regarding root development. It was clear that root development was more severely affected than shoot development in presence of CuNPs and CoC. This is due to the direct contact of wheat roots with the nanoparticles in the aqueous medium. Probably, the excess of copper ions released from the nanoparticles altered the root physiological elongation processes adversely leading to inhibited root growth and thereby affecting shoot development and dry weight. Indeed, inhibition of root growth by the presence of copper has been attributed to interferences with cell elongation (Arduini *et al*., 1995) and cell division (Jiang *et al*., 2001) by inducement of chromosomal aberrations, abnormal mitosis, damage to cell wall and plasma membrane integrity. It is discernible from the data presented that an inhibitory effect of CuNPs on the shoot was recorded at higher concentrations only when the roots were severely affected, so probably the effect on the shoot could be due to a low root development and not to a direct copper effect. At low concentration, shoot growth occurred at par with the control treatment.

Conclusions

The present study gives a broader view of biocidal effects of CuNPs that showed hitherto unknown effects on some agriculturally important microorganisms. In general terms, CuNPs could be used as an alternative to commercial copper (such as CoC), and even as an additive enhancing the effect of commercial products. However, as it should be done in the case of other copper applications in fields (and agrochemicals in general), special care should be taken in controlling dosage and frequency of treatments in order to avoid further environmental problems. Additionally, since growth promotion effects of CuNPs and CoC was observed in the case of some plant pathogens such as *A. alternata* and *P. syringae* at certain concentrations, they should be applied with caution. An inhibitory concentration of CuNPs applied in field conditions may be washed or leached by natural processes in the course of time and the soil concentration would be reduced to a level which may promote the growth of the surviving target pathogens.

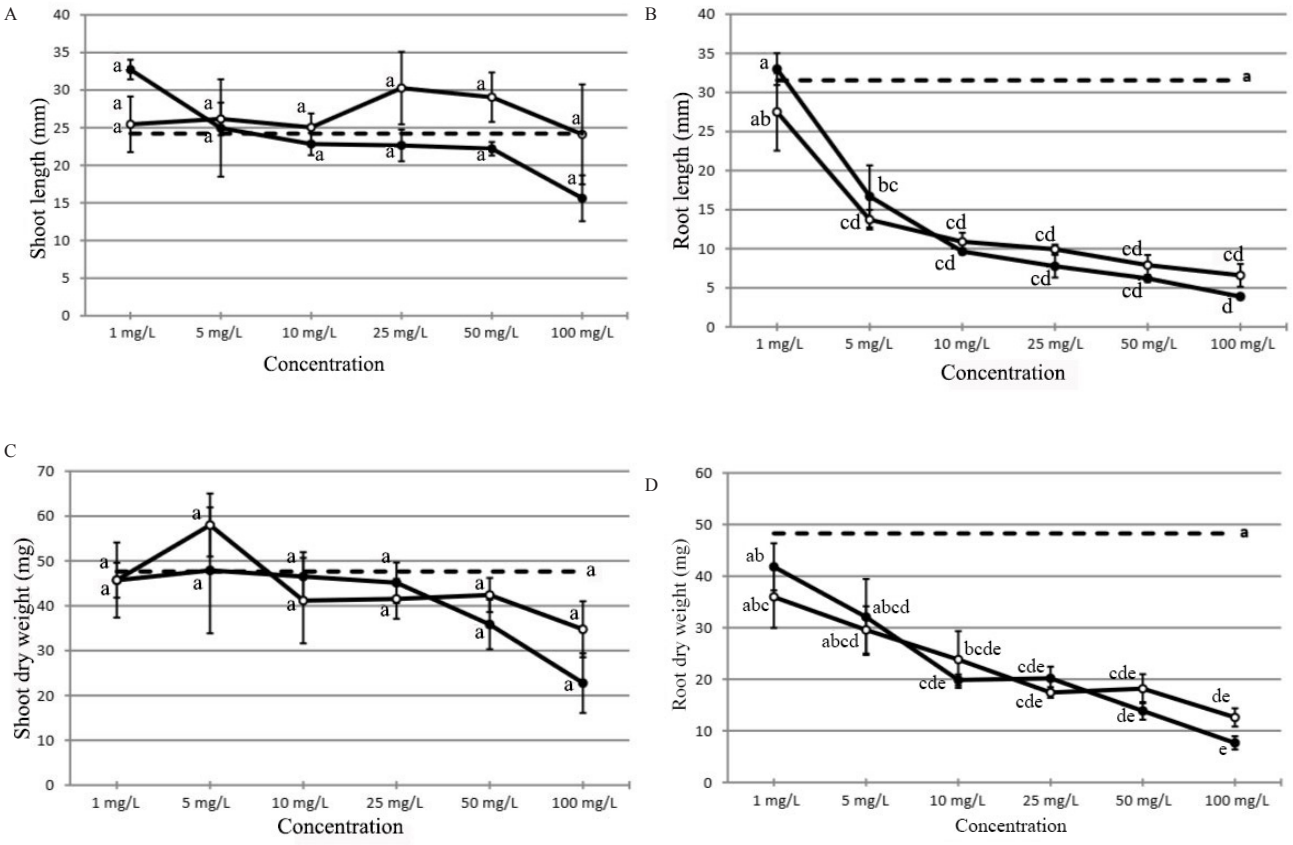


Figure 8. Effect of copper nanoparticles on shoot and root of wheat seedlings. (A) Shoot length of 5 days old seedlings grown in presence of CuNPs and CoC. (B) Root length of 5 days old seedlings grown in presence of CuNPs and CoC. (C) Shoot dry weight of 5 days old seedlings grown in presence of CuNPs and CoC. Bars represent SE. Data with the same letter are nor significantly different (HSD, $p<0.05$).

The negligible negative or even stimulating effect on beneficial microbes is encouraging and opens the opportunity for exploiting uses of CuNPs further, increasing effectiveness of commercial pesticides through the observed synergistic effect. It may be possible to find out the best ecologically harmless combination of CuNPs and present day copper fungicides for better plant disease management.

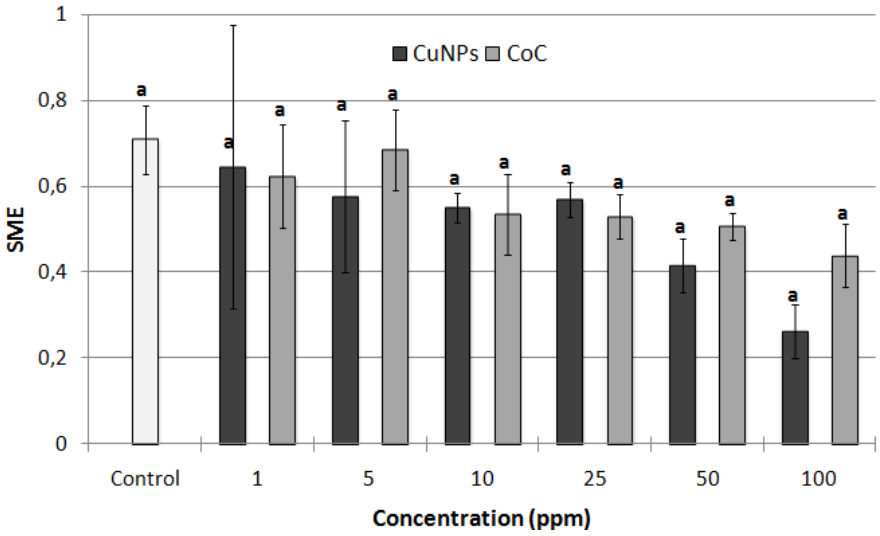


Figure 9. Seed metabolic efficiency (SME) of wheat seedlings grown in presence of CuNPs and CoC. Bars represent SE. Data with the same letter are not significantly different (HSD, $p<0.05$).

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