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Composition effect of Cu-based nanoparticles on phytopathogenic bacteria. Antibacterial studies and phytotoxicity evaluation.

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Abstract

Nanotechnology can offer new possibilities to the agrochemical sector providing plant protection products that can reduce the quantities and frequency of their use and getting maximum effect on the target pest. Herein, copper based nanoparticles (Cu-based NPs) of different composition have been hydrothermally synthesized in the presence of the biocompatible surfactants polyethylene glycol (PEG1000), tetracethylene glycol (TEG) and polysorbate 20 (Tween 20) and tested in vitro and in vivo on plant pathogenic bacteria strains. The obtained NPs, Cu@Tween20, Cu@TEG, Cu₂O@TWEEN20 and CuO@PEG1000 with sizes 46, 39, 12 and 18 nm respectively were evaluated in vitro as antibacterial agents, against three phytopathogenic Gram negative bacterial strains, Erwinia amylovora, Xanthomonas campestris and Pseudomonas syringae. Cu NPs were found the most potent in the in vitro studies against all bacteria strains studied, with the lowest MIC value- <3 μg/mL for E. amylovora and MBC value 100 μg/mL for P. syringae always in comparison with the wide used conventional pesticide Kocide 2000 35 WG tested at the registered dose of 1000 μg/mL. Further investigation was performed on testing the antibacterial activity of Cu@TWEEN20 and Cu₂O@TWEEN20 nanoparticles in vivo in pot experiments under greenhouse conditions on bean plants (P. vulgaris). The penetration of the nanoparticles,
labelled with Alizarin Red S, was investigated in plant tissues under a fluorescence microscope while their possible impact on plant photosynthesis and growth has been estimated. Cu-based NPs found to penetrate the plant tissues without causing any toxicity.

**Keywords:** Copper Nanoparticles, *E. amylovora, X. campestris, P. syringae*, Antibacterial, Pot experiment

1. Introduction

The wide range of nanotechnology applications in agriculture includes also pesticides for pest and disease management, and provides new techniques for crop disease control. [1,2] Recently, several metal and/or metal oxide inorganic nanoparticles (NPs), such as Ag, Cu, Au, ZnO, TiO\(_2\), etc have been evaluated *in vitro* and *in vivo* as antimicrobial agents against a range of Gram negative and Gram positive bacteria with promising results, while the effectiveness of nanosilver (Ag NPs) has led to commercialization. [3] However, benefits and potential risks of NPs as nanopesticides is an under-explored area in the research community. There are several parameters that govern the effectiveness of the NPs since the size, the composition, the shape, the presence of organic coating on the surface of the metal core are crucial. Taking into account the plethora of microorganisms that cause severe damages to many crops, overall, research is a demanding and multidisciplinary task. [4]

Copper has been used against fungi and bacteria in traditional agriculture since 1882 (Bordeaux mixture). Since the biological activity of all copper fungicides is based on free ions of divalent copper and their high insolubility, they need to be applied in high doses, which fact raises serious concerns about consequent environmental impact.[5] Also, copper-resistant bacteria pathogens have become prevalent [6] and have reduced the efficacy of copper-based pesticides.[7] The increased activity of NPs that is attributed to the small size (<100 nm) and the large active surface in comparison to both bulk and atomic / molar compounds comes to resolve this problem. Therefore, by applying an active ingredient as NPs formulation could achieve the same effectiveness with lower doses. Moreover, it is stated that NPs are much more soluble than bulk
materials, which allow them to interact closer with solvent molecules and show faster dissolution. [8] Our previous results suggest that Cu-based NPs are more effective against Phytophthora infestans than the commercial fungicides (Kocide 2000, Kocide Opti, Cuprofix disperss and Ridomil Gold Plus) when were applied at lower formulated product and active ingredient rate while no deleterious effect on tomato plants were observed. [9] Metallic copper (Cu), cupric oxide (CuO) and cuprous oxide (Cu$_2$O) NPs reveal their effectiveness and specificity towards a broad spectrum of microorganisms. [10,11] Aside from the nanosize effect, which is mainly presented in literature, a comparative study of Cu-based NPs is essential in order to evaluate the effect of different composition on the bacterial growth. It still is a matter of concern though, which form of copper is more suitable, since it is linked to the flexible Cu(I)/Cu(II) redox activity. Comparative studies between CuO and Cu NPs against bacteria suggest that Cu NPs inhibit more successfully a number of bacterial species. Therefore, metallic Cu NPs may be a promising antibacterial agents for future use. Towards this goal the most challenging task is the synthesis of stable metallic Cu NPs, since they undergo rapid oxidation to Cu$^{2+}$ ions in air or in aqueous media.[12] Though the mechanisms behind the biocidal activity of metallic nanostructures are not yet fully understood, [13] possible reasons include a better electron transfer and a more direct interaction between the Cu NPs and the bacteria strain, leading to enhance penetration and rupture of the bacterial membrane accompany with cell enzyme malfunction and eventually cell death. [14] However, light irradiation can lead to excited electron–holes pairs in CuO NPs, and the bacteria’s inactivation could be mostly attributed to a photocatalytic process.

In continuing our studies on Cu-based NPs, the antibacterial activity of coated Cu-based NPs of different compositions was tested against three bacteria species, Erwinia amylovora, Xanthomonas campestris, Pseudomonas syringae in comparison with the registered and widely used conventional copper-based pesticide Kocide 2000 35 WG. These three Gram- bacterial strains are common members of epiphytic bacterial communities [15] and have been selected as they are of great importance in the field of agriculture causing severe economic losses and still remain difficult to control. [16] Moreover, strains of P. syringae and X. campestris, are ice nucleation active (INA) and can initiate frost damage to plants. [17] On that basis, PEGylated Cu-based NPs were hydrothermally synthesized in the presence of the biocompatible surfactants polyethylene glycol (PEG1000), tetraethylene glycol (TEG) and polysorbate 20 (Tween 20) in
order to avoid oxidation of the metal core and to protect plant cells from possible increased NPs toxicity. [18,19] Moreover, polyols perform stealth properties increasing the antibacterial efficacy of the nanoparticles. [20,21] The resulted Cu@TEG, Cu@TWEEN20, CuO@PEG1000 and Cu$_2$O@TWEEN20 have been characterized as it is widely accepted that the intrinsic characteristics of the NPs affect the microorganisms and their biological activity. The obtained nanoparticles were evaluated in vitro by agar well diffusion and microwell dilution bioassay and in vivo at pot experiment on bean plants (P. vulgaris L.) against the phytopathogenic bacteria. In order to investigate the protective effect of copper nanoparticles on plants the nanoparticles were applied prior to the bacteria strain. Their impact on plant photosynthesis and growth was further investigated. Finally, the nanoparticles were transformed into fluorescent probes by post-synthetic methods and their penetration in plant tissues, was observed under a fluorescence microscope.

2. Materials and Methods

2.1 Materials

All the reagents were of analytical grade and were used without any further purification. The utilized products were the following: Copper (II) nitrate trihydrate Cu(NO$_2$)$_3$·3H$_2$O (Merck, ≥99.5%, M = 241.60 g/mol), hydrazine hydrate N$_2$H$_4$·H$_2$O (Merck, about 100%, M = 50.06 g/mol), polyethyleneglycol 1000 (Merck, M = 950 g/mol), and polyoxyethylene (20) sorbitan laurate (Tween 20) (TCI, >97%, M = 1200 g/mol), Kocide 2000 35 WG (Efthymiadis agrochemicals, copper hydroxide, concentration in metallic copper 35% w/w).

For bacterial growth: Erwinia amylovora, Xanthomonas campestris were grown on Nutrient Agar medium, and Pseudomonas syringae on King’s medium B. [22] The latter medium supplemented with 100 μg/mL rifampicin (KBR) were used for the bioassay. All media were supplemented with 100μl Natamycin (C$_{33}$H$_{47}$NO$_{13}$). Measurements of bacterial growth were conducted in Liquid Nutrient Broth Media.

Alizarin Red S (C$_{14}$H$_{7}$NaO$_7$S), for the fluorescence microscopy study, was purchased from Sigma-Aldrich.
2.2 Bacterial strains

Three different bacterial strains were used in experimentation: *Pseudomonas syringae pv syringae*, *Erwinia amylovora* strain BPIC 980, and *Xanthomonas campestris* strain BPIC 908. The first strain was kindly provided by S. Lindow from UC Berkeley USA, [23] while the two latter strains by Benaki Phytopathological Institute, Greece. Strains were grown on Nutrient agar (NAG) and King’s medium B (KBR).

2.3 Preparation of Cu-based NPs

All the synthetic routes followed the same procedure of hydrothermal synthesis in autoclaves under autogenous pressure. For the preparation of the samples CG1 and CG3, two sets of experiments were carried out at 120°C and 160°C in the presence of TWEEN20 respectively, while for the samples CG2 and CG4, TEG and PEG1000 have been used at 120°C and 160°C respectively. Reaction parameters such as time (4 h), ratio of precursor to reducing agent (1:1) as well as precursor to surfactant ratio (1:1) kept the same in all the experiments. Experimental results are summarized in Table 1.

In a typical synthesis (e.g. sample CG1), Cu(NO$_3$)$_2$·3H$_2$O 2 mmol (0.5 g) was dissolved in 6 ml deionized water to obtain a blue solution. Then 2 mmol (0.06216 g) of N$_2$H$_4$·H$_2$O were added dropwise to the solution and the color changed from blue to yellow, indicating the reduction of Cu(II). Afterwards, 2 mmol (2.5 g) TWEEN20 was added. The resulting solution was stirred thoroughly and then transferred into a 23 mL Teflon-lined stainless-steel autoclave. The crystallization was carried out under autogenous pressure at temperature of 120 °C for 4 h. Then the autoclave was cooled naturally at room temperature and after centrifugation at 5000 rpm, the supernatant liquids were discarded and a black-brown precipitate was obtained and washed with ethanol, at least three times, to remove the excess of ligands and the unreacted precursors.

2.4 Copper ionic release

A Perkin Elmer Optima 3100 XL axial viewing inductively coupled plasma atomic emission spectrometer (ICP-AES) was used by the appropriate choice of wavelength for Cu: 324.752 nm. The release of Cu$^{2+}$ ions from the Cu, Cu$_2$O and CuO NPs into deionized (DI) water was studied by suspending 10 mg of NPs in 100 mL DI water and sonicating for 10 min. The suspensions
were kept in a rotary shaker under the same conditions as in the bioassays and residual Cu$^{2+}$ concentration in the aqueous phase was determined by ICP-AES after 24, 48, 96 h respectively.

2.5 Dynamic light scattering and zeta potential measurements
Hydrodynamic diameters were determined by electrophoretic measurements, carried out at 25 °C by means of a VASCO Flex™Particle Size Analyzer NanoQ V2.5.4.0 dynamic light scattering (DLS), while ζ-potentials were determined by electrophoretic measurements, carried out at 25 °C by means of a NanoZetasizer, Nano ZS Malvern apparatus. All the samples (CG1-CG4) have been measured at a concentration of 100 μg/mL. To remove unwanted coarse particles that may originate from drying agglomerates or dust contamination, the dilution mediums were filtered through a 0.2 micron membrane.

2.6 Characterization of Cu-based NPs
X-ray powder diffraction (XRD) measurements were performed on a Philips PW 1820 diffractometer at a scanning rate of 0.050 per 3 sec, in the 2θ range from 10–90°, with monochromatized Cu Ka radiation (λ=1.5406 nm). Thermogravimetric analysis (SETA-RAM SetSys-1200) was carried out in the range from room temperature to 800 °C at a heating rate of 10 °C min$^{-1}$ under N$_2$ atmosphere. Infrared spectra (280–4000 cm$^{-1}$) were recorded on a Nicolet FTIR 6700 spectrometer with samples prepared as KBr pellets. (Table 2) [42]

2.7 In vitro antibacterial bioassay
The in vitro antibacterial activity of the Cu-based nanoparticles was estimated using both, the agar well diffusion and the microwell dilution bioassay. For the agar well diffusion bioassay, the surface of each agar plate was inoculated with 100 μl of fresh culture of each tested strain, always at its exponential growth phase (10$^6$ CFU) and sterilized water solutions (20 μl) of NPs (Cu@TWEEN20, Cu@TEG, Cu$_2$O@TWEEN20 and CuO@PEG1000 NPs) were added in the well at two tested doses (10 μg/mL and 100 μg/mL) while the commercial pesticide (Kocide 2000 35 WG) was used as reference, at the recommended dose (1000 μg/mL). Each treatment had four replicates, and each bioassay was performed twice. Antibacterial activity was determined from the zone of growth inhibition reported in millimeter (mm), formed around each well after incubation for 48h at 22-24 °C. Quantitative evaluation of antibacterial activity was
further elucidated by determination of Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values for the tested compounds by applying a microwell dilution bioassay. Fresh overnight bacterial cultures in NG broth, at two different inoculum sizes (10^4 and 10^6 CFU) were used, in order to investigate the effectiveness of antibacterial activity at different pressure of bacterial infection. The bioassay was performed in 96-well microliter plates and different concentrations of Cu NPs were applied as described earlier. Briefly, solutions of Cu NPs in (100 μl) water at different concentrations (0, 3, 6, 12, 25, 50 or 100 μg mL) as well as the reference Kocide 2000, at two doses (3 and 100 μg/mL) were added in 96-well plate and each well was then inoculated with either Pseudomonas syringae, Erwinia amylovora or Xanthomonas campestris fresh overnight cultures (100 μl). Bacterial inhibition was recorded by measuring the optical density (OD) at 600 nm after incubation for 24h at 22-24 °C. The MIC value was assigned to the lowest NPs concentration that totally inhibited bacterial growth. The MBC value was determined according to Karamanoli et al. 2000, to the lowest concentration of NPs at which no bacterial growth was recorded in the inoculated plates following 48h incubation at 22–24 °C. Each treatment had six replicates, and each bioassay was run twice. The antibacterial activity of the surfactants was also tested against the three bacterial strains, by applying a microwell dilution bioassay.

2.8 In vivo antibacterial bioassays on greenhouse-grown plants

Individual P. vulgaris plants, were grown in plastic pots (10 cm in diameter) containing a sand–peat mixture under control conditions (16-24°C T, 55-65 % RW, 16 day cycle). When bean plants reached the stage of second fully developed trifoliate three plants per treatment were randomly assigned and sprayed with the tested Cu@TWEEN20 NPs at doses of 200, 150, and 100 μg/mL and Cu₂O@TWEEN20 NPs at doses of 250, 200 and 150 μg/mL to runoff. Plants treated with a) Kocide 2000 b) Control - Bacteria c) Control - H₂O were kept as negative and positive controls respectively. 24h after NPs treatment, plants were inoculated with aqueous bacterial suspensions of a rifampicin-resistant P. syringae fresh culture (10^6 CFU) to run-off with an air pressure atomizer. Plants were then placed in a moist chamber. Bacterial populations were evaluated 1 hr after spraying (0 time), then at 24 h intervals over a 96 h period. The leaf washings were inoculated on KBR plates and bacteria were counted 48 hr after incubation at
24°C. Spontaneous rifampicin-resistant mutants of *P. syringae* had been obtained as described earlier. [24]

**2.9 Cu-based NPs impact on plant photosynthesis and growth**

In an attempt to assess possible effects of Cu-based NPs on plant characteristics, photosynthetic efficiency of PSII (quantum yield (QY)), chlorophyll content index (CCI= % transmittance at 931 nm/ % transmittance at 653 nm) and net photosynthetic rate (Anet, mol m$^{-2}$s$^{-1}$) were determined on the second fully developed trifoliate (counting from the apex) of the treated bean plants, 24 hrs after Cu-based NPs application. Shoot length of treated plants was also recorded at different sampling points 24, 48 and 96h respectively.

**2.10 Fluorescence microscopy**

In order to investigate the possible penetration of the nanoparticles into the tissues of *P. vulgaris* plants, Cu@TWEEN20 and Cu$_2$O@TWEEN20 nanoparticles were labelled with Alizarin Red S and sprayed onto the leaves. The nanoparticles were mixed with Alizarin red S (50mM) and shaked at 100 rpm for 24h at room temperature. Then the samples were centrifuged at 8000 rpm for 1h and after that the precipitates were washed with deionized water (5 times). Dark red powders of NPs were obtained. For the foliar application of the labeled Alizarin red S - NPs to the leaves the nanoparticles were mixed with water to form doses of 150 μg/mL in both cases. Three untreated plants were kept as (a) control, three were treated with (b) Cu@Alizarin Red S and three with (c) Cu$_2$O@Alizarin Red S. The labeled nanoparticles were sprayed on the leaves of bean plants on doses of 150 μg/mL for both compositions. Plain Alizarin Red S was dissolved in deionized water and sprayed on the leaves as well. After 24h the leaves were inoculated with the bacteria *P. syringae* fresh culture. The plant tissues were examined under a fluorescent microscope (longitudinal and cross sections).

**2.11 Statistical analysis**

The antibacterial bioassays were designed at a completely randomized pattern in a split -plot arrangement, with the the Cu-based nanoparticles, dosage and the sampling time as independent factors. Each combined factor had three replicates in all parameters except for the CCI and QY that had 9 replicates. The analysis of variance (ANOVA) and the Duncan test performed on the
results were attained by the statistical package SPSS (version 22). Multiple average comparisons, graphs and data processing were performed in EXCEL.

3. Results and Discussion

3.1 Synthetic aspects and characterization of Cu-based NPs
The impact of synthetic conditions (precursors, temperature, time, pH solvent, and presence/absence of surfactant) on the composition, size and shape of Cu-based NPs has been defined before by us and others. [7,9,25,26,27] Based on our previous results and in an attempt to model composition relationships for biological applications, the preparation of Cu-based NPs achieved under hydrothermal processes in a close system (autoclave) in the presence of the biocompatible surfactants polyethylene glycol 1000 (PEG 1000), tetraethylene glycol (TEG) and polysorbate 20 (Tween 20). In that vein, in the presence of Tween 20, Cu and Cu$_2$O NPs have been isolated at 120$^\circ$C / 160$^\circ$C and 4h reaction time respectively, whereas in case of implementing TEG and PEG 1000, Cu and CuO NPs have been prepared at 120$^\circ$C / 160$^\circ$C and 4h reaction time respectively. Surfactants/capping molecules are crucial especially in the case of Cu-based NPs, [43] as they do not act only as dispersant for avoiding agglomeration of the samples, [28] but also as a protective layer for preventing surface oxidation, since Cu(I/II) flexible redox behavior [29] while hydrophilicity and different charge on the surface of the NPs can be provided by altering the organic molecules. [44] As the influence of NPs on green plants depends on its size, shape, chemical properties and chemical milieu of subcellular sites that are accumulated, [30] our study was focused on three different surfactants, which are non-ionic (Tween20, PEG1000 and TEG) and have gained increasing attention in the field of nanomedicine on account of being biocompatible. It is important also to mention that as synthesis took place in a close system the physicochemical characteristics of the NPs through composition, size, shape and surface chemistry is effectively controlled (under the specific
conditions) and products are reproducible with a prospect for further use in the agrochemical sector.

The XRD patterns of samples CG1-CG4, are given in Fig1. Samples CG1 and CG2 exhibit Bragg reflections that correspond to the crystal structure of fcc Cu, with the major peak (111), (JCPDS Card pdf.85-1326). Basic peaks of Cu are (111), (200), and (220). By using MDI’s Jade software, lattice parameters for the samples CG1 and CG2 were calculated and found to be $a = 3.612 \, \text{Å}$ and $3.608 \, \text{Å}$ respectively which are very close to the theoretical value for metallic copper ($3.615 \, \text{Å}$). The sample CG3 distinctly indexed to the cubic (Pn3m) crystal structure of Cu$_2$O (JCPDS card no. 05-0667) with basic peaks at (111), (200), and (220) while the sample CG4 exhibited the monoclinic structure of CuO (ICDD, JCPDS card No. 80-1916) with basic peaks of (111), (200), (112), (220). The lattice parameters for the sample CG3 were found to be $a = 4.29 \, \text{Å}$ while for the sample CG4 $a = 4.74 \, \text{Å}$, $b = 3.42 \, \text{Å}$, $c = 5.13 \, ^\circ \text{Å}$, which are in good agreement with the bulk lattice parameter of Cu$_2$O ($4.27 \, \text{Å}$) and CuO ($a = 4.68 \, \text{Å}$, $b = 3.42 \, \text{Å}$, $c = 5.12 \, \text{Å}$) respectively. The average crystalline sizes of the NPs were determined by measuring the full width at half-maximum (FWHM) of the most intense peaks and using the Scherrer equation. The XRD sizes were calculated at 46, 39, 12 and 18 nm for the samples CG1, CG2, CG3 and CG4 respectively (Table 2).

FTIR spectra of the samples CG2 and CG4, with the corresponding patterns of TEG and PEG1000 are given in Fig. 1S(B). Peaks at 2930 and 2850 cm$^{-1}$ confirm the asymmetric and symmetric stretch of the –CH$_2$ groups, while the broadband around 1610–1630 cm$^{-1}$ can be attributed either to hydrogen bonding amongst neighboring organic molecules and/or to glycolate derivatives indicating the presence of oxidized species of the polyols on the surface of the NPs. The bands around 1340 cm$^{-1}$ and 1470 cm$^{-1}$ are associated with (C-H) and (C-H$_2$) bending modes respectively. Additionally, bands at 1240, 1100 and 947 cm$^{-1}$ correspond well to the CH$_2$-O-CH$_2$ wagging (gauche), twisting and rocking modes, respectively. Finally, in the case of sample CG2 no clear peaks appear in the region of the spectra below 650 cm$^{-1}$, verifying the metallic nature of the as synthesized NPs. For the samples CG1 and CG3, Fig. 1S(A), the bands at 1628 and 1381 cm$^{-1}$ are attributed to asymmetric (v$_{as}$) and symmetric (v$_s$) stretching vibrations of laurate while the strong peak at 1105 cm$^{-1}$ is characteristic of stretch vibration of CH$_2$-O-CH$_2$. In the case of CG3 the peak at 625 cm$^{-1}$ is assigned to the vibrations of Cu(I)–O bonds.
The amount of residual surface capping agents of the samples CG1-CG4 (Fig. 2S) was evaluated by thermogravimetric analysis over a temperature range as high as 900 °C under nitrogen atmosphere. For all samples, the decomposition of the surfactants starts at around 200 °C and finishes right after 400 °C. In the case of CG1 and CG2 samples, after 350 °C both of them gained weight indicating the oxidation of the metallic copper. Also, CG3 at nearly 300 °C gained weight indicating the oxidation of copper from Cu$_2$O to CuO. The cumulative organic content was estimated 32 wt %, 3 wt %, 25 wt % and 27 wt % for the samples CG1, CG2, CG3 and CG4 respectively. "Based on the obtained TGA results, the number of surfactant molecules per one NP have been estimated.[25] The calculations for samples CG1-CG4 were performed under assumption that the density of the nanoparticles (6.5 g/cm$^3$) is the same and their shape is totally spherical. The molar masses of the surfactants are 1227.54, 150.17 and 1000 g/mol for TWEEN20, TEG and PEG1000 respectively. As a result the number of ligands that are bound on the NPs was calculated (Eq. 1) and found 520.04, 242.87, 7.21 and 32.2 for CG1, CG2, CG3 and CG4 respectively, indicating the thickness of the organic coating around the metal core.

$$N = \frac{\omega N_A \rho \frac{4}{3} \pi R^3 \times 10^{-23}}{M}$$ (1)

Where N is the number of ligands on each particle, R is the mean radius of the Cu- based NPs, ρ is the density of the NPs, NA is Avogadro’s number, M is the molar mass of surfactant molecules (g/mol) and ω is the mass loss in percent (%).

The colloidal stability of the samples in water is an important issue for bio applications. [46] All the synthesized nanoparticles CG1-CG4 were dispersed in water without any further modification and were considered hydrophilic. By the means of DLS measurements, their hydrodynamic size was determined at 172, 176, 197 and 163 nm, respectively (mean size provided by intensity and numbers measurements) (Fig. 3S). Aggregation hindrance, uniform dispersity and stability of the suspensions are indicated by DLS measurements while correlate well with previous studies of our group on PG, TEG and PEG 1000. [31] Additionally, the ζ-potential values of the aqueous suspensions of samples CG1, CG2, CG3 and CG4 were found – 11.1, + 11.8 and - 14.5 and - 20 mV, respectively. However, only values of ± 30 mV can affect the antibacterial activity of the NPs. [45]
3.2 Copper ion release measurements

In general, the biological activity of the NPs is strongly affected not only by their composition and morphology (size, shape), but also by the ionic release from the NPs into the medium. Though the mechanisms behind the biocidal activity of metallic/metal oxide nanostructures are not yet fully understood [32] the dissolution of ions from the metal core is believed to be a partial factor and the amount of toxicity is dependent on the number of copper ions that can be released. [33] In order to investigate the stability of the Cu-based NPs in aqueous suspensions, the ionic release into the aqueous phase was estimated at 24, 48 and 96 h respectively (Table 3). The concentration of ions released in the case of sample CG1 was 2.4-2.8 μg/mL, for CG2 1.3-1.9 μg/mL, for CG3 0.4-1.1 μg/mL and for CG4 0.8-2 μg/mL. No significant release has been indicated through time. Earlier studies show that copper ion concentrations above 20 mg/L can inhibit bacterial growth. [34] These results are a further evidence that surfactants effectively protect the core. Thus, the ionic contribution of NPs to the bactericidal activity might be considered negligible, whereas the nanosized effect is more pronounced.

3.3 In vitro experiments

An initial evidence of the antibacterial activity of the NPs was recorded by employment of the agar well diffusion method. According to the results, all the nanoparticles inhibited the bacteria growth while the surfactants (TWEEN 20, TEG and PEG1000) didn’t perform antibacterial activity against *E. amylovora*, *X. campestris* and *P. syringae* *in vitro*. Cu@TWEEN20 and Cu@TEG NPs showed the highest inhibition zones among the tested NPs (Table 4) against *Pseudomonas syringae* (Fig. 4s). It is worth to mention that the commercial pesticide Kocide 2000 35 WG was applied at the registered dose 1000 μg/mL and the antibacterial zone was found similar to those of the nanoparticles even though the NPs were applied at lower doses, 10 μg/mL and 100 μg/mL. (Table 4).

The antibacterial activity of the most potent composition of copper, Cu@TEG and Cu@TWEEN20 NPs was further evaluated in broth dilution tests estimating MIC and MBC values (Table 5). In the case of *E. amylovora* lowest MIC and MBC values were found for Cu@TEG <3 μg/mL and 12 μg/mL respectively (Table 5A). In the case of *X. campestris* and *P.
syringae, Cu@TWEEN20 MIC values were estimated at 75 and 100 μg/mL respectively while MBC was found 100 μg/mL for both bacterial strains (Table 5B,C). The MIC values of Cu@TEG and Cu@TWEEN20 were found similar indicating that neither the different type and percentage of the surfactant nor the surface charge \cite{35,36} (-11.1 mV for Cu@TWEEN20 NPs and +11.8 mV for Cu@TEG NPs) affect the antibacterial activity of the nanoparticles. \cite{37} The lowest MIC and MBC values were found against E. amylovora <3± 0.1 indicating the sensitivity of the specific bacterial strain to the NPs. The MIC and MBC values on all bacteria strains were found lower than the registered dose of the widely used conventional commercial pesticide Kocide 2000 35 WG (1000 μg/mL). Moreover, although the bacterial inhibition was assessed at two different inoculums sizes (10^4 and 10^6 CFU) for each bacterial strain, no significant changes were observed on the antibacterial properties of the Cu-based NPs.

3.4 In vivo antibacterial bioassays on greenhouse-grown plants

Cu@TWEEN20 and Cu_2O@TWEEN20 NPs were selected as the most potent compositions according to the in vitro results and were further evaluated in in vivo trials. Also, in order to exclude any involvement of the organic layer, we selected NPs prepared with the same surfactant. The in vivo studies were performed on bean plants (P. vulgaris), in a pot experiment, under controlled conditions against a P. syringae strain as it represents an important potential phytopathogenic organism worldwide. In all tested doses Cu@TWEEN20 NPs exhibited antibacterial activity, since the bacterial population reduced at least one order of magnitude 24h after treatment (Fig. 2). In particular, bacterial growth was totally inhibited after 48h when Cu@TWEEN20 NPs was applied at the highest dose (200 μg/ml) indicating that this concentration was the most effective against P. syringae. Notably, this concentration was at least 5 times lower than the registered dose of Kocide 2000 35 WG (1000 μg/mL). On the contrary, when Cu_2O@TWEEN20 applied on bean plants inoculated with P. syringae the bacterial growth reduced only one to two orders of magnitude compared to the control and this activity was similar to the activity of the commercial pesticide (Kocide 2000 35WG) treatment (Fig. 3). Higher doses of Cu_2O NPs were not employed in this bioassay as no promising results were obtained in the in vitro tests compared to Cu NPs. In previous studies of our team against P. infestans among Cu/Cu_2O nanocomposite and Cu_2O NPs the second was the most effective. This result indicates the specificity of activity of different compositions of copper depending on the
type of the organism studied. In this study, compositions tested had a stable performance after 48h. Optical density (OD 600) was decreased with time and after 48 hours a time-stable antibacterial activity was observed. Cu@TWEEN20 NPs were found more effective against *P. syringae* compared to Cu$_2$O@TWEEN20 NPs. This behavior can be attributed to the presence of the metallic core which can be considered more bioreactive than the oxides. The Gram- bacteria and the metallic nanoparticles act as good electron acceptors, both contributing to the electron transfer and rupture of the bacteria membrane. [7]

### 3.5 Fluorescent analysis – Alizarin Red S labeled Cu-based NPs

The penetration of Cu@Alizarin Red S and Cu$_2$O@Alizarin Red S into the plant vessels was investigated by converting the nanoparticles into fluorescent probes by post-synthetic methods, producing Alizarin Red S NPs derivatives before their application on leaves. The labeled nanoparticles were sprayed on the leaves of bean plants on doses of 150 $\mu$g/mL for both compositions. After 24h the leaves were inoculated with *P. syringae* fresh culture. The plant tissues were examined under a fluorescent microscope after 48h. The nanoparticles have the capability of entering plant cells other than as individual particles but as aggregates. When solely Alizarin Red S was applied to plant tissues no fluorescent signal was observed. This is in agreement with previous studies, in which a strong fluorescent signal was only detected for nanoparticles modified with Alizarin Red S. [47] In the plants sprayed with Cu@Alizarin Red S NPs accumulation of the fluorescent aggregates in the pith and associated cells of the vascular tissue was recorded (Fig. 4B,E). In the case of the plants sprayed with Cu$_2$O@Alizarin Red S NPs the nanoparticles were observed in both the pith and xylem-phloem vessels (Fig. 4C,F). It has been reported that the carbon-iron nanoparticles were capable of penetrating living plant tissues and migrating to different regions of the plant, although movements over short distances seemed to be done more easily. [38] Even though it is well known that the cuticle (the primary barrier) repels all polar substances, the foliar application increases the uptake of NPs and bypasses it. [34] The nanoparticles enter through the stomatal pore of the leaf as well, which is >100 nm in diameter and this presents a relatively large gap for the weak cuticle charge to sustain complete anion repulsion. [39] The subsequent transport of Cu-based NPs from shoot to root is then achieved by plant's vascular systems. [40] The nanoparticles were transported by
both the apoplast (through the wall) and the symplast (cell to the cell, mediated by plasmodesmata). The hydrodynamic size of the NPs is calculated to 172 and 197nm for Cu and Cu$_2$O NPs respectively. Through the apoplast pathway larger particles (~200 nm) can move and through the symplastic pathway smaller particles (<50 nm). [39] Moreover, after the observation of a germinated bean seed on treated plants after 96h of the application of Cu@Alizarin Red S NPs, no bacteria or aggregates of Cu-based NPs were observed (Fig. 5). These findings indicate that nanoparticles penetrate foliage and translocate through the plant tissues, taking advantage of the plant’s natural transportation mechanisms.

3.6 Cu-based NPs impact on plant photosynthesis and growth

Possible impact of the NPs on the treated plants functions was assessed by recording photosynthetic parameters during experimentation, including chlorophyll content index (CCI), quantum yield, CO$_2$ assimilation, and shoot height changes (Table 6). Those parameters are in need to be carried out, in order to evaluate any adverse effects on photosynthesis other physiological and biochemical processes in plants after Cu and Cu$_2$O NPs application. Excess toxicity induces adverse effects on plants photosynthesis including reduction of CO$_2$ assimilation and stomatal conductance and decelerating activity of photosystem II (PSII). [41] Every healthy plant is often able to maintain a high steady-state concentration of active PSII. Although Cu is an essential element for many biological processes of the plant, doses above the required level can be toxic. Chlorophyll parameter is very important for plant photosynthesis and is highly affected by copper deficiency and toxicity. Published studies have shown that high amounts of copper causes decreased photosynthesis due to altered photochemical reactions in photosystem II (PSII) and damages to plant growth. [41]

According to the measurements, CCI values ranged between 22 to 16 both on plants treated with Cu and Cu$_2$O NPs confirming the proper biofunction of the plants. Both compositions of the NPs did not cause any chlorophyll content reduction, whereas Kocide 2000 35 WG treatment exhibited the lower CCI among samples, indicating a negative impact of this agent on chlorophyll.

According to quantum yield and CO$_2$ assimilation measurements, no difference were recorded between the treated and untreated leaves indicating no significant impact of any treatment on
these processes. When toxicity of copper occurs, it leads to slowing down of CO₂ assimilation and lower quantum yield rates, as reported by Cassana et al. [41]

Moreover, in the case of the plants treated with Cu NPs at the dose of 150 μg/mL exhibit a significant plant height increase (4.3±0.23 cm) (Table 6A). As was previously mentioned copper is a micronutrient for plants but in doses above the required level it can affect plant’s growth. So the dose of 150 μg/mL seems to have enhanced plants growth by also having bacteriostatic effect against P. syringae. On plants treated with Cu₂O NPs we have observed a dependent development (height) with time and dose (Table 6B)

To our knowledge, biological indicators of toxicity in bean plants treated with nanoparticles, such as measurements of chlorophyll content, quantum yield, CO₂ assimilation and plant growth, in pot experiments (in vivo) are very few or doesn’t exist.

4. Conclusion

Nanotechnology provides new antimicrobial agents in the sector of agriculture that can reform the modern agricultural farming system. Applications of these nanomaterials can add tremendous value in the current scenario of a global food scarcity. Hence, seeking for less toxic and environmentally acceptable antimicrobial agents, Cu@TEG, Cu@Tween20, Cu₂O@Tween20 and CuO@PEG1000 NPs have been prepared and tested against three Gram(-) bacterial strains Erwinia amylovora, Pseudomonas syringae and Xanthomonas campestris. These phytopathogenic species have been selected as they cause serious problems to crops while the number of bactericides in the market is limited. Among the different forms/compositions of the Cu-based NPs, Cu@TEG and Cu@TWEEN20 NPs were found the most effective in vivo and in vitro. The lowest MIC values were found for Cu@TEG NPs at <3 μg/mL for Erwinia amylovora indicating the sensitivity of this bacterial strain. However, for both Pseudomonas syringae and Xanthomonas campestris, MIC values were estimated at 100 μg/mL for Cu@TEG and Cu@TWEEN20. These values are significant lower than the registered dose of the wide used conventional Cu-based pesticide Kocide 2000 35 WG, that was found for these species at 1000 μg/mL. Importantly, the NPs didn’t perform any permanent damage to the plant tissues while they didn’t affect the plant photosynthesis and growth. The present findings give rise to Cu NPs to be proposed as effective alternatives in the agricultural section while further studies in this area are under progress.
REFERENCES


[16] Zgurskaya HI, Llöpez CA, Gnanakaran S. Permeability Barrier of Gram-Negative Cell Envelopes and Approaches To Bypass It. ACS infect. diseases. 2015, 1, 512-522


Figure captions

Fig. 1 XRD patterns of the samples CG1- CG4

Fig. 2 Bacterial growth of *P. syringae* on bean plants treated with different doses of Cu@TWEEN20 NPs, LSD= 0.686

Fig. 3 Bacterial growth of *P. syringae* on bean plants treated with different doses of Cu2O@TWEEN20 NPs, LSD= 1.072

Fig. 4 Confocal laser-scanning microscopy (cross A, B, C, and longitudinal D, E, F sections) of *P. vulgaris* tissues infected by *P. syringae* strain and treated with Cu@Alizarin Red S (B, E), and
Cu$_2$O@Alizarin Red S (C and F) compared to untreated (A, D). The green arrows indicate the healthy green stem of the plant, the blue arrows indicate the tissues colonized by *P. syringae* and the red arrows indicate the presence of the labelled Alizarin Red S nanoparticles within the plant tissues.

Fig. 5 *P. vulgaris* bean seed germinated from plants after 96h of the treatment with labeled Cu@Alizarin Red S NPs.

Table 1 Summary of experimental results and main characteristics of samples CG1-CG4. Emphasized samples have been selected for further structural and biological characterization.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Surfactant</th>
<th>Time (h)</th>
<th>Temperature (°C)</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG1</td>
<td>TWEEN20</td>
<td>4</td>
<td>120</td>
<td>Cu</td>
</tr>
<tr>
<td>CG3</td>
<td>TWEEN20</td>
<td>4</td>
<td>160</td>
<td>Cu$_2$O</td>
</tr>
<tr>
<td>CG2</td>
<td>TEG</td>
<td>4</td>
<td>120</td>
<td>Cu</td>
</tr>
<tr>
<td>CG4</td>
<td>PEG1000</td>
<td>4</td>
<td>160</td>
<td>CuO</td>
</tr>
</tbody>
</table>

Table 2 Summary of experimental results and main characteristics of Cu-based NPs samples (tested for the antibacterial activity).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Composition</th>
<th>% Surfactant</th>
<th>Size by XRD (nm)</th>
<th>Size by DLS (nm)</th>
<th>ζ potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG1</td>
<td>Cu@ TWEEN20</td>
<td>32± 3%</td>
<td>46± 2</td>
<td>172± 12</td>
<td>-11.1± 1</td>
</tr>
</tbody>
</table>
Table 3 Copper ion release measurements of Cu-based NPs (μg/mL)

<table>
<thead>
<tr>
<th>Sample</th>
<th>24h</th>
<th>48h</th>
<th>96h</th>
</tr>
</thead>
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<tr>
<td>Cu@TWEEN20</td>
<td>2.4±0.2</td>
<td>2.6±0.3</td>
<td>2.8±0.6</td>
</tr>
<tr>
<td>Cu@TEG</td>
<td>1.3±0.8</td>
<td>1.6±0.5</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>Cu₂O@TWEEN20</td>
<td>0.4±0.8</td>
<td>0.6±0.1</td>
<td>1.1±0.8</td>
</tr>
<tr>
<td>CuO@PEG1000</td>
<td>0.8±1</td>
<td>0.8±0.2</td>
<td>2.0±0.3</td>
</tr>
</tbody>
</table>

Table 4 Size of the inhibition halo (cm), in a disk diffusion bioassay following exposure of bacterial strains to 10 μg/mL and 100 μg/mL of the Cu-based NPs

<table>
<thead>
<tr>
<th>Sample</th>
<th>10 μg/mL</th>
<th>100 μg/mL</th>
<th>10 μg/mL</th>
<th>100 μg/mL</th>
<th>10 μg/mL</th>
<th>100 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erwinia amylovora</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu@TWEEN20</td>
<td>2±0.05</td>
<td>3±0.02</td>
<td>1.2±0.01</td>
<td>2.1±0.02</td>
<td>1.2±0.03</td>
<td>2.2±0.01</td>
</tr>
<tr>
<td>Cu@TEG</td>
<td>2.1±0.03</td>
<td>3.2±0.03</td>
<td>1.3±0.03</td>
<td>2.2±0.01</td>
<td>1.2±0.03</td>
<td>3.1±0.03</td>
</tr>
<tr>
<td>Cu₂O@TWEEN20</td>
<td>1.8±0.01</td>
<td>2.7±0.03</td>
<td>1.15±0.02</td>
<td>2±0.02</td>
<td>1±0.02</td>
<td>2±0.02</td>
</tr>
<tr>
<td>CuO@PEG1000</td>
<td>1±0.02</td>
<td>2±0.01</td>
<td>1±0.01</td>
<td>1.9±0.03</td>
<td>0.7±0.01</td>
<td>1.4±0.02</td>
</tr>
<tr>
<td>Kocide 2000 35 WG</td>
<td>4±0.06</td>
<td>3±0.04</td>
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<td></td>
<td></td>
<td>4±0.05</td>
</tr>
</tbody>
</table>

Table 5 Minimum Inhibition Concentration (MIC) and Minimum Bactericide Concentration (MBC) (μg/mL) of Cu@TEG and Cu@TWEEN20 against phytopathogenic bacteria E. amylovora (A), X. campestris (B) and P. syringae (C), calculated 24 h after inoculation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (μg/mL)</th>
<th>MBC (μg/mL)</th>
<th>MIC (μg/mL)</th>
<th>MBC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu@TEG</td>
<td></td>
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</tr>
<tr>
<td>Cu₂O@TWEEN20</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

A
<table>
<thead>
<tr>
<th></th>
<th>inoculums size $10^4$ CFU</th>
<th>inoculums size $10^6$ CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cu@TEG</strong></td>
<td>$&lt;3 \pm 0.1^d$</td>
<td>$12 \pm 1.2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$12 \pm 1.0$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$25 \pm 1.2$</td>
</tr>
<tr>
<td><strong>Cu@TWEEN20</strong></td>
<td>$6 \pm 0.1$</td>
<td>$12 \pm 1.0$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$12 \pm 0.9$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$12 \pm 1.1$</td>
</tr>
<tr>
<td><strong>KOCIDE 2000 35 WG</strong></td>
<td>$&gt;3 \pm 1.2$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$&lt;100 \pm 1.3$</td>
<td>$&gt;3 \pm 2.4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$&lt;100 \pm 4.0$</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inoculums size $10^4$ CFU</td>
<td>inoculums size $10^6$ CFU</td>
<td>inoculums size $10^4$ CFU</td>
<td>inoculums size $10^6$ CFU</td>
</tr>
<tr>
<td><strong>Cu@TEG</strong></td>
<td>$100 \pm 1.6^d$</td>
<td>$100 \pm 2.00$</td>
<td>$100 \pm 2.3$</td>
<td>$100 \pm 2.0$</td>
</tr>
<tr>
<td><strong>Cu@TWEEN20</strong></td>
<td>$75 \pm 1.9$</td>
<td>$100 \pm 0.02$</td>
<td>$100 \pm 1.2$</td>
<td>$100 \pm 4.0$</td>
</tr>
<tr>
<td><strong>KOCIDE 2000 35 WG</strong></td>
<td>$1000 \pm 10.0$</td>
<td>-</td>
<td>$1000 \pm 23.0$</td>
<td>-</td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>inoculums size $10^4$ CFU</td>
<td>inoculums size $10^6$ CFU</td>
<td>inoculums size $10^4$ CFU</td>
<td>inoculums size $10^6$ CFU</td>
</tr>
<tr>
<td><strong>Cu@TEG</strong></td>
<td>$100 \pm 1.8^d$</td>
<td>$100 \pm 2.0$</td>
<td>$100 \pm 4.6$</td>
<td>$125 \pm 1.5$</td>
</tr>
<tr>
<td>Treatment</td>
<td>Chlorophyll content index</td>
<td>Quantum Yield</td>
<td>CO$_2$ assimilation</td>
<td>Plant height increase</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------</td>
<td>---------------</td>
<td>---------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Control - Bacteria</td>
<td>22.3$^a$</td>
<td>0.7$^a$</td>
<td>5.04$^a$</td>
<td>1.8$^a$</td>
</tr>
<tr>
<td>Control - H$_2$O</td>
<td>19.6$^{ab}$</td>
<td>0.7$^a$</td>
<td>4.82$^a$</td>
<td>1.5$^a$</td>
</tr>
<tr>
<td>Cu -200 μg/mL</td>
<td>22.5$^a$</td>
<td>0.7$^a$</td>
<td>4.55$^a$</td>
<td>2.7$^c$</td>
</tr>
<tr>
<td>Cu -150 μg/mL</td>
<td>22.0$^a$</td>
<td>0.7$^a$</td>
<td>4.11$^a$</td>
<td>4.3$^d$</td>
</tr>
<tr>
<td>Cu -100 μg/mL</td>
<td>22.5$^a$</td>
<td>0.7$^a$</td>
<td>4.34$^a$</td>
<td>1.5$^a$</td>
</tr>
<tr>
<td>Kocide 2000 35 WG</td>
<td>16.2$^b$</td>
<td>0.7$^a$</td>
<td>4.10$^a$</td>
<td>2.2$^b$</td>
</tr>
</tbody>
</table>

*α = 0.05

Table 6 Measurements of Chlorophyll Content Index (CCI), Quantum Yield (QY), CO$_2$ Assimilation (A) and Plant Height Increase (PHI) of bean plants 24h after Cu@TWEEN20 (A) Cu$_2$O@TWEEN20 (B) treatment.

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chlorophyll content index</th>
<th>Quantum Yield</th>
<th>CO$_2$ assimilation</th>
<th>Plant height increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - Bacteria</td>
<td>22.3$^a$</td>
<td>0.7$^a$</td>
<td>4.4$^a$</td>
<td>1.8$^b$</td>
</tr>
<tr>
<td>Control - H$_2$O</td>
<td>19.6$^{ab}$</td>
<td>0.7$^a$</td>
<td>4.7$^a$</td>
<td>1.5$^{ab}$</td>
</tr>
<tr>
<td>Cu$_2$O -250 μg/mL</td>
<td>20.7$^a$</td>
<td>0.7$^a$</td>
<td>4.8$^a$</td>
<td>2.0$^d$</td>
</tr>
<tr>
<td>Cu$_2$O -200 μg/mL</td>
<td>19.4$^{ab}$</td>
<td>0.7$^a$</td>
<td>4.2$^a$</td>
<td>2.0$^c$</td>
</tr>
<tr>
<td>Cu$_2$O -150 μg/mL</td>
<td>23.6$^a$</td>
<td>0.7$^a$</td>
<td>4.5$^a$</td>
<td>1.0$^a$</td>
</tr>
<tr>
<td>Kocide 2000 35 WG</td>
<td>16.2$^b$</td>
<td>0.7$^a$</td>
<td>4.1$^a$</td>
<td>2.2$^{cd}$</td>
</tr>
</tbody>
</table>

*α = 0.05
Fig. 1 XRD patterns of the samples CG1- CG4
Fig. 2 Bacterial growth of *P. syringae* on bean plants treated with different doses of Cu@TWEEN20 NPs, LSD = 0.686

Fig. 3 Bacterial growth of *P. syringae* on bean plants treated with different doses of Cu$_2$O@TWEEN20 NPs, LSD = 1.072
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