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Non-Dependency of *In Vitro* Fungicidal Efficiency of Copper Nanoparticles against *Fusarium oxysporum* upon Particle Size

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Abstract

Although many papers have consented that the smaller the size of nanoparticles, the higher their efficiency, this paper sheds light on one potential exception of this rule. The paper shows that the *in vitro* antifungal efficiency of copper nanoparticles against the fusarium wilt pathogen, *Fusarium oxysporum*, isolated from the infected date palm, *Phoenix dactylifera* L., is not size-dependent; instead, as it was found that larger copper nanoparticles have better *in vitro* antifungal efficiency against the fungal pathogen than smaller ones. Copper nanoparticles were synthesized via chemical reduction method at two different pH values, 6.5 and 10.5. Dynamic light scattering was used to measure their particle sizes, which were 345.1 nm and 278.1 nm, respectively. Transmission Electron Microscopy was used to figure out the shapes of nanoparticles, which were polygonal and spherical, respectively. Poison food essay was used to test their *in vitro* inhibition efficiencies against the fusarium wilt pathogen, *F. oxysporum*, isolated from the infected date palm, *Phoenix dactylifera* L., which were 46% and 19%, respectively; at the same concentration. Ultimately, the paper has proposed and discussed a potential reason beyond these unexpected findings, which relies upon the larger surface area to volume ratio of the polygonal copper nanoparticles compared to the spherical copper nanoparticles. The paper concluded that, despite their larger size, polygonal copper nanoparticles have better *in vitro* antifungal efficiency than spherical copper nanoparticles against *F. oxysporum* isolated from the infected date palm, *Phoenix dactylifera* L. at the same concentration.

Keywords: Antifungal efficiency; *Fusarium oxysporum*; Polygonal copper nanoparticles; Spherical copper nanoparticles

Introduction

A significant portion of the crop production can be lost due to phytopathogenic infections, including but not limited to fungal, bacterial, viral and nematodal infections, in addition to the insect pests, this portion accounts for 14.1% of the total losses affect crops from various sources of infections [1]. On the other hand, many phytopathogens have developed resistance against a lot of traditional chemicals used to control such phytopathogens [2-14], which in turn spur farmers to use larger quantities of such chemicals to in order to control the more resistive pests, which result in dangerous health consequences and more pollution hazards on the environment. So, it is hoped that new technologies, such as nanotechnology, may provide more efficient, cost-effective, and eco-friendly nanocides for controlling such pathogens.

Nanotechnology is considered one of the most promising technologies that may revolutionize the agricultural sector via it`s versatile potential applications regarding many agricultural challenges such as climate changes, fertilization efficiency, sustainable agriculture, and food demands [15]. In the agricultural field, nanotechnology has a broad range of applications, including but not limited to growth promotion and nutrition supplement using nanofertilizers [16], plant protection against phytopathogens and treatment of plant diseases using nanocides [17].

Among different types of nanoparticles, metal nanoparticles have attracted much attention due to their unique catalytic, optic, electronic and magnetic properties compared to their bulk counterparts [18,19]. In this regard, copper nanoparticles, as one of the transition metals, has a very promising application in many different fields such as catalytic degrading of organic dyes, including rose Bengal and methylene blue [20], as a conductive ink [21] and in the antimicrobial applications [22-27]. Particularly, it was shown that the antifungal efficacy of copper nanoparticles was stronger than many other metal nanoparticles including Al, Fe, Mn, Ni and Zn, [28].

Many crops are subjected to infection with different diseases caused by many soil-borne pathogenic fungi which may cause considerable losses in the productivity of the infected plant. Investigations showed that among these pathogens the fungal pathogen *F. oxysporum*, which cause fusarium wilt disease, is considered one of the most common and most virulent one, as it has a wide range of hosts, including but not limited to sugarcane, legumes, tomato, potato, pepper, bananas, oil palm and many other species; and may lastly cause death of the infected plant [29,30].

In this regard, the fusarium wilt infection begins, under suitable environmental conditions, by the germinating spores or by the fungal mycelia, which penetrate the plant's lateral roots. After penetration, the mycelium continues spreading through the vascular vessels of the infected plant and producing its microconidia. The microconidia flow upward into the sap stream and germinate where the flow of the sap stops. Finally, the spores and the mycelia plug the vascular vessels of the infected plant, which in turn hinders the plant from up-taking and translocating nutrients, which results eventually in wilting the leaves, and death of the whole plant [31,32].

This paper shed light on an approach to enhance the antifungal efficiency of copper nanoparticles against the fusarium wilt pathogen, *F. oxysporum*, as one of the agricultural applications of metal nanoparticles.

In this regard, copper nanoparticles have been known with their antifungal effect against *Fusarium* sp. [33]. But there is a dire need to enhance this antifungal efficiency of copper nanoparticles, so as to

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minimize the number of nanoparticles required to affect the fungal pathogen, and hence minimizing the environmental pollution and the accumulation of nanoparticles in the treated plant.

This paper has proposed a trial to enhance the antifungal effect of copper nanoparticles against fusarium wilt pathogen, *F. oxysporum*, isolated from the date palm, *Phoenix dactylifera* L., via maximizing their surface area to volume ratio by varying the reaction conditions at which copper nanoparticles were synthesized.

Materials and Methods

All chemicals used in the following experiments were the analytical grade of purity and were used without further purification.

Chemicals used for synthesis of copper nanoparticles

Copper sulfate pentahydrate was obtained from Elnasr Pharmaceuticals Co., Egypt. L - Ascorbic Acid (99.0% pure) was obtained from Alpha Chemika Co. Egypt. and Cetyltrimethylammonium bromide (CTAB) (99.0% pure) was obtained from Rashmi Diagnostics.

Synthesis of copper nanoparticles

Copper nanoparticles were synthesized according to the chemical reduction method [34]. A simple modification of the method was done, in which 1.1 g of CTAB and 1.94 g of L-ascorbic acid were dissolved in 80 mL of deionized water (solution A); also, 0.25 g of copper sulfate pentahydrate was dissolved in 10 mL of deionized water (solution B) and 0.08 g of sodium hydroxide was dissolved into another 10 mL of deionized water (solution C).

The pH of solution A was adjusted at 6.5 (in the 1st trial) or at 10.5 (in the 2nd trial), and the solution was heated to 85°C. After that, solutions B and C were simultaneously added dropwise to the solution A under stirring. The reaction continued for 30 min, till the reaction mixture developed a reddish-brown color.

In this regard, it is noteworthy that the pH value decreases as the reaction proceeds due to the consumption of hydroxyl anions by copper cations according to the following mechanism [35]:

$$Cu^{2+} + 2OH^- \rightarrow Cu(OH)_2 \quad (1)$$

$$Cu(OH)_2 + C_6H_8O_6 \rightarrow Cu + C_6H_6O_6 + 2H_2O \quad (2)$$

So that, I simply modified the original method [34] so as to maintain the pH value constant throughout the reaction. this modification was done through adding sodium hydroxide solution (solution C) with as twice molarity as that of the copper sulfate pentahydrate simultaneously with copper sulfate pentahydrate (solution B) to compensate the consumed hydroxyl anions, since two hydroxyl anions are consumed to react with one copper cation.

After finishing the reaction, the synthesized nanoparticles were collected by centrifugation at 3000 rpm for 10 minutes, washed twice with deionized water and twice with ethanol and dried for further characterization and application.

Characterization of copper nanoparticles

Copper nanoparticles were suspended in deionized water for Uv-vis spectroscopy using Helios Gamma Spectrophotometer, which used to determine the characteristic surface Plasmon resonance of the synthesized copper nanoparticles. Dynamic Light Scattering (Zeta sizer nano series (Nano ZS), Malvern, UK) was used to measure particles sizes. Transmission Electron Microscope (Tecnai G20, Super twin, double tilt, FEI, Netherland) was used to figure out the shapes of the synthesized copper nanoparticles.

Fusarium oxysporum strain

The fungal strain was obtained from the Microbiological Resources Center; Ain Shams University; Cairo; Egypt.

Investigation of the antifungal efficiency of copper nanoparticles: Poison food essay was used to investigate the antifungal effect of the synthesized copper nanoparticles. The fungus was inoculated on Potato Dextrose Agar (PDA) media containing 300 ppm of copper nanoparticles synthesized at pH 6.5 and copper nanoparticles synthesized at pH 10.5; Then, incubated in dark at 25°C for 1 week. Radial growth was measured, and the inhibition percentage was calculated relative to the control, in which fungus was inoculated on copper nanoparticles free Potato Dextrose Agar media, according to the following equation [36]:

$$Fungicidal\ Efficacy\ (\%) = \frac{C-T}{C} \times 100 \quad (3)$$

Where, C is the radial growth of mycelia in control (in cm), T is the radial growth of mycelia in treatments (in cm) (Copper nanoparticles - containing Potato Dextrose Agar media)

Statistical analysis

SPSS 22 software was used at $P \leq 0.05$ to distinguish between the fungicidal efficacies. Each treatment was conducted in triplicate, and the whole experiment was repeated twice [37].

Results

UV-vis spectroscopy

UV-vis spectroscopy for both copper nanoparticles synthesized at pH 6.5 and pH 10.5 exhibited the characteristic plasmonic resonance bands at 589 nm and 584 nm, respectively.

Particle size distribution

Dynamic Light Scattering (DLS) showed the particle size

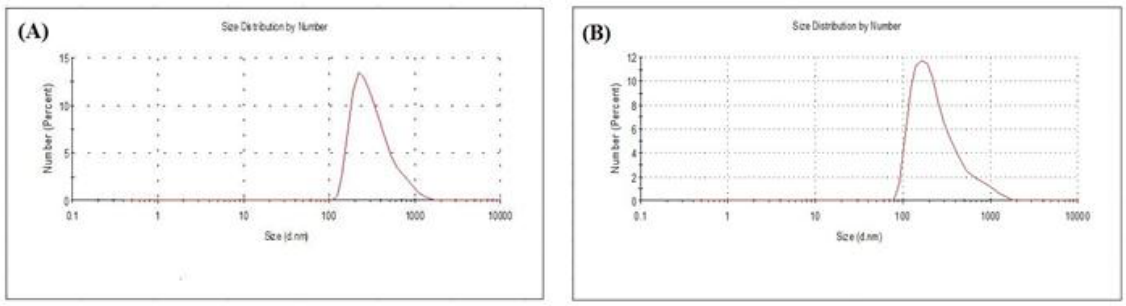

Figure 1: Particle size distribution of copper nanoparticles synthesized at pH 6.5 (A) and copper nanoparticles synthesized at pH 10.5 (B).

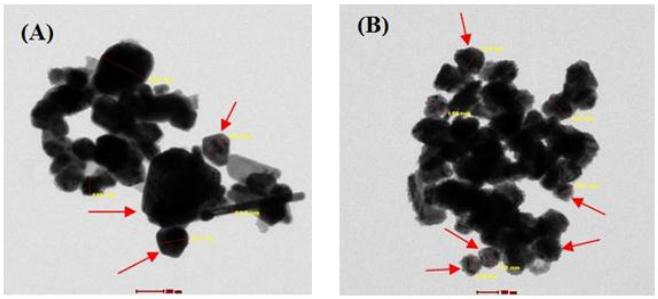

Figure 2: TEM micrographs of polygonal copper nanoparticles synthesized at pH 6.5 (A) and spherical copper nanoparticles synthesized at pH 10.5 (B).

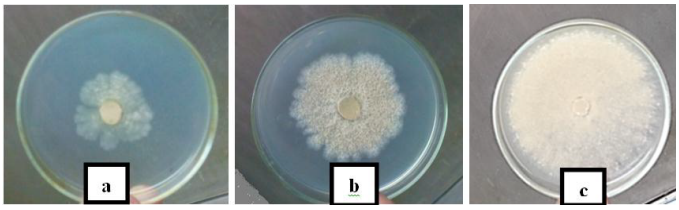

Figure 3: Mycelia growth on Potato Dextrose Agar media containing Copper nanoparticles synthesized at pH 6.5 (A), pH 10.5 (B) and free from any copper nanoparticles (control) (C).

distributions for both copper nanoparticles synthesized at pH 6.5 and pH 10.5, with the average particle sizes 345.1 nm and 278.1 nm, respectively; as shown in Figures 1A and 1B.

Transmission electron microscopy

Transmission Electron Microscopy (TEM) was used to figure out the shape of Copper nanoparticles. Figures 2A and 2B show the transmission electron micrographs of copper nanoparticles synthesized at pH 6.5 and pH 10.5, respectively. Transmission electron micrographs revealed that the Copper nanoparticles synthesized at pH 6.5 were nearly polygonal, while that synthesized at pH 10.5 were nearly spherical.

Assessing the *in vitro* antifungal efficiencies of copper nanoparticles synthesized at pH 6.5 vs. copper nanoparticles synthesized at pH 10.5

Also, the *in vitro* antifungal efficiencies of copper nanoparticles synthesized at pH 6.5 and pH 10.5 were investigated. Poison Food essay revealed that 300 ppm of copper nanoparticles synthesized at pH 6.5 inhibited the mycelia growth of the fungal pathogen, *F. oxysporum*, by 46%. On the other hand, the same concentration of copper nanoparticles synthesized at pH 10.5 inhibited the mycelia growth by only 19%, as shown in Figures 3A-3C.

Statistical analysis at $P \leq 0.05$ showed that the *in vitro* fungicidal efficacy of copper nanoparticles synthesized at pH 6.5 was significantly higher than that of copper nanoparticles synthesized at pH 10.5 against *F. oxysporum* isolated from date palm.

Discussion

Firstly, Copper nanoparticles were successfully synthesized via the chemical reduction method and confirmed by exhibiting their characteristic surface plasmonic resonance. In this regard, Copper nanoparticles usually have a characteristic resonance band in the range 560 nm-570 nm [38]; this band is shifted toward longer wavelengths in case of larger particles [38], which toke place in this case.

Furthermore, it was clear that increasing the pH value at which copper nanoparticles were synthesized affects particle characteristics including size, shape and hence the antifungal efficiency. Firstly, increasing the pH value from 6.5 to 10.5 oriented the particles to smaller sizes; this may due to faster nucleation rate than the growth rate of the particles at higher pH value. Also, higher pH value developed roughly spherical particles, while lower pH value resulted in almost polygonal particles. Finally, the *in vitro* antifungal efficiency, which is the net of these characteristics, of the copper nanoparticles was significantly enhanced with the polygonal shape of particles despite being larger in their size.

The enhanced *in vitro* antifungal efficiency of copper nanoparticles synthesized at pH 6.5 can mainly be attributed to its relatively larger surface area to volume ratio (SAVR) as compared with the spherical copper nanoparticles synthesized at pH 10.5; this is because the polygonal shapes usually exhibit larger SAVR than the spherical shapes [39].

In this regard, a simple calculation of the approximate surface area to volume ratio of the spherical copper nanoparticles with particle size 278.1 nm will be as follow [39]:

$$SAVR = \frac{3}{r} \quad (4)$$

As shown in Figure 4A, where r is the radius of the spherical particle, which in this case is 139.05 nm. i.e. SAVR will be $3/(139.05 \times 10^{-9}) = 2.16 \times 10^{7}\ m^{-1}$.

On the other hand, a simple calculation of the approximate surface area to volume ratio of the polygonal copper nanoparticles with particle size 345.1 nm, assuming that the particle is a polygonal pyramid and 2x=I=345.1 nm, will be as follow [39]:

$$SAVR = \frac{3}{h} + 2\sqrt{3}\sqrt{\frac{3}{4h^2} + \frac{1}{S^2}} \quad (5)$$

As shown in Figure 4B, where S is the pyramid side and h is the pyramid height. $\boldsymbol{S}$ can be calculated from the formula [39]

$$x = \frac{\sqrt{3}}{2}s \quad (6)$$

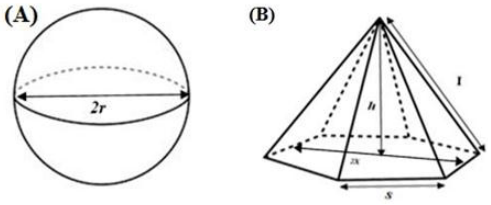

Figure 4: Diagram of a spherical nanoparticle (A) and polygonal pyramid nanoparticle (B).

Where, x is the perpendicular length from the center of the base to one of the sides. And h can be calculated from Pythagorean Theorem,

$$h = \sqrt{l^2 - x^2} \quad (7)$$

Thus,

$$SAVR = \frac{3}{298.86\times10^{-9}} + 2\sqrt{3}\sqrt{\frac{3}{4\left(298.86\times10^{-9}\right)^2} + \frac{1}{\left(199.24\times10^{-9}\right)^2}} = 3\times10^{7}\,m^{-1}$$

From the previous approximate calculations, it is very clear that the SAVR of copper nanoparticles synthesized at pH 6.5 is larger than that of copper nanoparticles synthesized at pH 10.5.

The previous analysis suggests a correlation between the SAVR of copper nanoparticles and their antifungal efficiency; this can draw our attention to the importance of maximizing the SAVR of copper nanoparticles not only by minimizing particle size, but also by adopting such reaction conditions which result in polygonal shapes rather than spherical ones.

Another suggested reason beyond the better *in vitro* antifungal efficiency of the larger copper nanoparticles may be that the targeted entities in the fungal pathogen have a comparable size to that of larger copper nanoparticles, i.e. the higher degree of matching between the larger copper nanoparticles and the size of the targeted entities in the fungus increased the probability of affecting such entities.

Thus, a further research is required to investigate the exact reason(s) beyond the higher toxicity of some larger nanoparticles than their smaller counterparts. Such cases are present in the literature with other types of nanoparticles [40-42].

Conclusion

Conclusively, I do emphasize that the *in vitro* antifungal efficiency of copper nanoparticles against *F. oxysporum* isolated from date palm is not dependent upon particle size; rather it may depend upon their surface area to volume ratio, or on matching between the particle size and the targeted entities in the fungal pathogen.

Also, the paper concluded that for enhancing the *in vitro* antifungal efficiency of copper nanoparticles developed for use as a fungicide against the fusarium wilt pathogen, *F. oxysporum*, it may be more effective to adopt such synthesis conditions that result in larger polygonal shapes of the particles instead of smaller spherical ones.

The importance of our findings can be embodied in proposing a trial to enhance the *in vitro* antifungal efficiency of copper nanoparticles, so as to minimize the number of nanoparticles required to affect the fungal pathogen, and hence minimizing the environmental pollution and the accumulation of nanoparticles in the host plant.

Finally, and Honestly speaking, although copper nanoparticles which were synthesized at pH 6.5 demonstrated a better *in vitro* antifungal efficiency against the fungal pathogen, *F. oxysporum*, than copper nanoparticles which were synthesized at pH 10.5, this may present a higher toxicity side effects on the other beneficial micro flora in case of using such nanoparticles as a fungicide to control fusarium wilt disease. This may require the addition of suitable supplemental biofertilizers in order to compensate the affected flora.

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