Optimised green synthesis of copper oxide nanoparticles and their antifungal activity

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Abstract
Copper oxide (CuO) nanoparticles were synthesised from Ginkgo biloba L. leaf extract and several parameters that affected their formation were adjusted. The optimal synthesis system was ascertained as 10 ml leaf filtrate, 1 mM copper sulfate, and 80°C. The scanning electron microscope image showed that the synthesised CuO nanoparticles were shaped like a short rod. The antifungal effect of CuO nanoparticles against Bipolaris maydis was operated through agar well diffusion, mycelial growth, spore germination, and detached inoculation. At the concentration of 200 µg/ml, the suppression rate was up to 62.78%, and the spore germination was totally inhibited irrespective of suspension in a liquid or on detached maize leaf. The results are of great significance for comprehensive control of plant diseases, and it also provides a novel antimicrobial to assist or substitute chemical pesticides.

1 | INTRODUCTION

Nanoparticles that are defined as 1 to 100 nm in more than one dimension possess distinct characteristics compared with their bulk materials. In view of those properties, they are designated as footstones of future technology [1]. As an important member, metal nanoparticles have attracted more and more researchers’ attention. Lots of metal nanoparticles including silver (Ag), gold (Au), copper (Cu), zinc (Zn), and so forth were synthesised and applied in many fields such as industry, medicine, physics, chemistry, biology, agriculture, and so on [2–4].

It is reported that three main approaches were adopted to synthesise metal nanoparticles including physical, chemical, and biological methods [5]. With the concept of green chemistry deeply rooted in people’s minds, the biological synthesis method mediated by microorganisms or plant tissues has become more and more popular. It exhibits several advantages, compared with a physical or chemical method such as eco-friendly, low cost, low energy, high output, high stability, and so forth [6]. In recent years, copper oxide (CuO) nanoparticles were biosynthesised by plant extract of Bifurcaria bifurcate [7], Convulvulus pericicus [8], Stachys lavandulifolia [9], Gloriosa superb [10], and so forth.

Recently, CuO nanoparticles have caused more and more people’s concern owing to their broad applications including optics, electrics, sensotics, and other fields [11–13]. Pathogen control is never stopped whether it happened in medicine or agriculture, and the first choice of management is always chemical drugs. However, long-range, abundant, and unreasonable use of chemical drugs causes severe harm like environmental pollution, food safety, residue of pesticide, and drug resistance [14]. It is urgent to exploit novel approaches to resolve such problems. There were lots of articles that described the prominent antimicrobial effect of metal nanoparticles like silver [15–17], gold [18, 19], and copper oxide [20, 21]. Compared with the two heavy metals, copper was much cheaper and safer to the environment and living bodies, and it was also necessary for plant growth.

Although CuO nanoparticles were synthesised by several plant tissues before, Ginkgo biloba L. has not been used, and the optimised biosynthesis system has not been ascertained either. In addition, the antifungal activity of as-synthesised CuO nanoparticles against Bipolaris maydis was also carried out through multiple aspects including colony formation, inhibition zone, spore germination, and in vitro inoculation. These results would provide a novel fungistat with high efficiency and low toxicity for the comprehensive treatment of plant pathogens, and also lay the foundation for exploring nano-formulations to assist or substitute chemical pesticides.

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2 MATERIALS AND METHODS

2.1 Materials and reagents

G. biloba L. leaves were collected in Fengyang, China. Copper sulfate (CuSO₄) was ordered from Sinopharm Chemical Reagent Co., Ltd. (China). The single spore strain called B. maydis was conserved in the plant protection laboratory of our university.

2.2 Optimal green synthesis of CuO nanoparticles

Thoroughly washed and air-dried G. biloba L. leaves were ground into powder, 10 g powder was added into a blue cap bottle containing 100 ml deionised water, boiling for 35 min. Whatman no. 1 paper was filtrated for the first time and Millipore filter (φ = 0.22 µm) for another. CuO nanoparticles were biosynthesised by mixing 10 ml filtrate, 1 mM CuSO₄, and the total volume was set as 100 ml. Finally, the mixture was heated at 60°C until its colour changed. The optimisation process was performed including varied volumes (10, 20, 30, and 40 ml) of G. biloba L. leaf extract, different concentrations of CuSO₄ (1, 2, 4, and 8 mM), Pondus Hydrogenii (pH 3, 5, 7, 9, and 11), and processing temperatures (40, 50, 60, 70, and 80°C).

2.3 Characterisation of CuO nanoparticles

Such synthesised nanoparticles were characterised by UV-vis spectroscopy (TU-1950), scanning electron microscope (SEM, S-4800), energy dispersive X-ray (EDX), and X-ray diffraction (XRD).

2.4 Antifungal property of CuO nanoparticles

Antifungal property of CuO nanoparticles against B. maydis was carried out by mycelial growth, agar well diffusion, spore germination, and detached inoculation.

2.4.1 Colony growth inhibition

CuO nanoparticles powders were obtained by centrifugation at 8000 rpm for 15 min with deionised water and ethyl alcohol separately followed by drying at 80°C. The concentration of CuO nanoparticles was prepared as 10 mg/ml with sterile water. Then, diluted CuO nanoparticles (5 ml) were added to potato dextrose agar (PDA) medium (45 ml) at about 60°C, adjusting the ultimate concentration of CuO nanoparticles at 12.5, 25, 50, 100, and 200 µg/ml. The PDA medium containing an equal volume of sterile water was set as control. A fungus disk (φ = 8 mm) was transferred to the middle of each PDA plate and then incubated at 28°C for 7 days [16].

2.4.2 Agar well diffusion assay

The inhibition zone generated on the PDA plate showed the antifungal property of CuO nanoparticles (it was modified by the previous report [22]). About 100 µl of spore suspension (10⁶/ml) was evenly smeared on cold PDA plates. Then, wells with a diameter of 8 mm were drilled and followed by dripping 30 µl of different concentrations (25, 50, 100, and 200 µg/ml) of CuO nanoparticles, taking isometric sterile as control. The plates were cultivated for 2–3 days at 28°C after 5 min standing.

2.4.3 Spore germination inhibition

The density of B. maydis spore was adjusted to 10⁶/ml. CuO nanoparticles and spore suspension were mixed at 1:9 (v/v), making the ultimate concentration of CuO nanoparticles at 12.5, 25, 50, 100, and 200 µg/ml. Spore suspension without CuO nanoparticles was controlled. The mixture was incubated for 12 h at 25°C [16].

2.4.4 Detached inoculation

Maize leaves (Denghai 618, DH618) were thoroughly washed using sterile water, their surface was sterilised by dipping in 75% (v/v) ethyl alcohol and 1% (v/v) sodium hypochlorite for 1 min separately. Then, leaves were immersed in sterile water for 2 min in order to eliminate solution residue. Finally, a mixture (15 µl) of CuO nanoparticles and spore suspension produced as 2.3.3 was added on air-dried leaves that was fixed on cold mediums, and isometric spore suspension without CuO nanoparticles was set as control. Processed leaves were incubated for 3–5 days at 28°C [16].

3 RESULTS AND DISCUSSION

3.1 Green synthesis of CuO nanoparticles

The reduction of CuSO₄ (Cu²⁺) into CuO nanoparticles could be identified by the solution colour changed from pale yellow to red-brown in the existence of leaf extract and CuSO₄ as shown in Figure 1(a), while the solution of G. biloba L. leaf extract without CuSO₄ kept its primary colour after heating at 60°C. The UV-vis spectrum displayed that there was a strongest absorbance at 398 nm (red line), which is consistent with the surface plasmon resonance of CuO nanoparticles [7, 10, 11]. Nevertheless, there was no strong absorption for leaf filtrate alone in the range of 250–500 nm (black line).
3.2 Optimal biosynthesis system confirmation

It is demonstrated that *G. biloba* L. leaf extract has the ability to synthesise CuO nanoparticles, and the volume of extract affected their formation. As shown in Figure 2(a), the maximum absorbance at 398 nm ($A_{398}$) was measured based on different volumes (10–40 ml) of leaf extract. $A_{398}$ decreased with increasing extract volume, and the optimal extract volume was ascertained as 10 ml. As the only chemical reagent used in the synthesis process, CuSO$_4$ plays an important role, and it is necessary to optimise its concentration to achieve eco-friendly and economical production. $A_{398}$ of CuO nanoparticles differed at various concentrations, and the maximum appeared at 1 mM (Figure 2(b)). Thus, the optimal concentration of CuSO$_4$ was determined as 1 mM. Besides plant extract and CuSO$_4$, pH is another important parameter that influences the formation of CuO nanoparticles. Figure 2(c) showed that $A_{398}$ decreased sharply when the solution turned from acid to alkaline (3 to 11), and the optimal pH was ascertained as 3. In addition, it is found that appropriate temperature facilities the formation of nanoparticles. $A_{398}$ increased frequently as the temperature enhanced from 40 to 80°C (Figure 2(d)), and the optimal temperature was determined as 80°C. The result is of positive significance for synthesis nanoparticles at low energy consumption.
3.3 | SEM and EDX assay

Figures 3(a)–(d) show that the morphology of synthesised nanoparticles are mainly short rod-shaped, and there are no obvious aggregations appearing on the substrate. The EDX analysis indicated there were strong signals of Cu and O that confirmed the elemental components of CuO nanoparticles. While the other signals like Si and C might be attributed to *G. biloba* L. leaf extract or the element of the substrate (Figures 3(e)–(f)). Previous articles reported that the morphology of CuO nanoparticles mainly was near-spherical or polyhedral [7, 10, 23, 24], while the shape of nanoparticles synthesised by *G. biloba* L. leaf extract changed to a short rod; the reason might be different plant species, diverse synthesis procedures, or others.

3.4 | XRD analysis

The XRD pattern of synthesised CuO nanoparticles is shown in Figure 4. It indicates the existence of CuO with a monoclinic crystalline system. The reflections at 34.28, 37.54, 47.56, 52.32, 57.14, 60.34, 65.10, 66.86, 71.28, and 73.86$^\circ$ (2$\theta$) shows the main reflections of CuO that are related to (111), (002), (−202), (002), (111), (202), (113), (−222), (020), (−311) planes in monoclinic crystalline system [7, 8].

3.5 | Mycelial growth inhibition

Figure 5 shows colony of *B. maydis* that was prominently restrained by CuO nanoparticles. The colonial diameter
TABLE 1  Diameter of inhibition zone in the presence of CuO nanoparticles

| Concentration of CuO nanoparticles (µg/mL) | Diameter of inhibition zone (mm) |
|------------------------------------------|----------------------------------|
| 12.5                                     | 8.6 ± 0.38                       |
| 25                                       | 10.2 ± 0.65                      |
| 50                                       | 11.2 ± 0.58                      |
| 100                                      | 15.0 ± 0.66                      |
| 200                                      | 18.3 ± 0.78                      |

of control measured by cross-over method was 6.63 cm, and it diminished gradually as the concentration of CuO nanoparticles increased. The diameter dropped to a minimum (2.97 cm) at 200 µg/ml. The suppression ratio induced by varied CuO nanoparticles concentrations (12.5–200 µg/ml of B. maydis was around a range of 1.13%–62.78%.

3.6 | Agar well diffusion assay

It exhibited tight relevance between inhibition zone diameter and their antifungal effect. Table 1 showed the data of the inhibition zone under the condition of CuO nanoparticles, and it became large as the concentration of CuO nanoparticles increased. It reached 14.0 and 18.3 mm when the concentration of CuO nanoparticles was 100 and 200 µg/ml. It is reported that the paper disk diffusion approach was also applied to determine the inhibition zone besides the agar well diffusion method, and it seemed smaller owing to less volume of nanoparticles [7, 9, 25].

3.7 | Spore germination inhibition

Sufficient germinated spores are crucial for fungi to infect plant tissues successfully, so it is the critical point to restrain spores germination for plant diseases control. Figure 6 shows that CuO nanoparticles could inhibit spores germination of B. maydis significantly. Under proper humidity and temperature, the spore germination rate of control was 89.2%, while it dropped radically with different concentrations of CuO nanoparticles, and the spores were restrained completely at 200 µg/ml.

3.8 | Detached inoculation analysis

Detached inoculation was carried out to assay the antimicrobial effect of CuO nanoparticles, and an apparent lesion occurred on the maize leaf that was inoculated with spore suspension alone (Figure 7(a)), while the leaf inoculated with a mixture
of spore suspension and CuO nanoparticles (200 µg/ml) appeared healthy as a regular leaf (Figure 7(b)). Besides, the relevant microscopy imaging displayed lots of germinated spores through tissues of the whole leaf (Figure 7(c)); however, it was inhibited absolutely for the spores inoculated on maize leaf in the presence of 200 µg/ml of CuO nanoparticles (Figure 7(d)).

4 | DISCUSSION

It is common that nano-scaled metal and metal oxides are synthesised through several methods and applied in various domains in consideration of their unique properties. As an important metal oxide, it was proved that CuO nanoparticles exhibited prominent antimicrobial activity [20, 21]. However, the optimised synthesis process of CuO nanoparticles is reported rarely. The parameters that affect their formation varied; Naika et al. reported that the maximum absorbance peak enlarged along with increasing extract volume of G. superba L. [10], which is inconsistent with our experiment based on different plant species.

The antimicrobial effect of CuO nanoparticles against bacteria and fungi was proved in previous reports [21, 23], the same CuO nanoparticles exhibited different antimicrobial activity against various pathogens [20], and it also varied for CuO nanoparticles synthesised by different approaches against the same pathogen [7, 10]. In consideration of the external performance and microscopic examination, spores of B. maydis were restricted to germinate and extend on maize leaf by CuO nanoparticles. The comparable consequence was described before the Ag nanoparticles inhibited the spores of Curvularia lunata [16] and Bipolaris sorokiniana [26] germinated and infected maize and wheat leaf.

5 | CONCLUSION

B. maydis is a crucial plant pathogen that induces serious leaf disease on maize worldwide. It is a huge challenge to formulate a highly efficient and environmental-friendly approach to control hazards. In this research, G. biloba L. leaf extract was applied to synthesise CuO nanoparticles, and the optimised green synthesis process was also confirmed. It is demonstrated that such biosynthesised CuO nanoparticles exhibited a significant antifungal effect against B. maydis through several measurement indices. The results provide a novel approach for the comprehensive management of phytopathogens as well as decrease the dosage of toxic chemical pesticides and minimise the occurrence of drug resistance. Management of CuO nanoparticles including surface charge modification, acid-base adjustment, assembling behaviour would be conducted to assay their effect on antifungal activity in the near future.

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