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Full Length Article

Optimized Biosynthesis and Antifungal Activity of *Osmanthus fragrans* Leaf Extract-mediated Silver Nanoparticles

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Abstract

With the development of nanoscience and nanotechnology, increasing research focuses on synthesis, characterization and application of silver nanoparticles. Reported here is the synthesis of silver nanoparticles using *Osmanthus fragrans* leaf extract, as well as several optimized synthesis parameters, including quantity of fern leaves, concentration of $AgNO_3$, pH, and regulation of reaction temperature. Antifungal activity of silver nanoparticles on *Bipolaria maydis* (AH-B06) was measured through colony formation and inhibition zone methods. The optimal biosynthesis system contained 5 g of fern leaf and 1 mM $AgNO_3$ at pH 9.0 and 60°C. Under said conditions, the resulting synthesized nanoparticles were nearly spherical, with an average size of 20 nm. In tests, silver nanoparticles synthesized at different concentrations of $AgNO_3$ inhibited colony formation to varying degrees, resulting in different inhibition zones. No colony formed on PDA plates at $AgNO_3$ concentrations at or over 2 mM, while quantity of herbal component was unchanged. Regarding inhibition zones, diameters increased gradually at $AgNO_3$ concentrations from 1–8 mM, while antifungal effect reached maximum at 5 g of herbal component. Results of this study offer a new approach and novel material for comprehensive control pathogenic fungi on plants. As well, the presented method and material have potential application for screening novel fungistats with high efficiency and low toxicity, thus offering a green chemistry answer to a worldwide need.

Keywords: Biosynthesis; Silver nanoparticles; Antifungal activity; *Bipolaria maydis*

Introduction

Silver was recognized as an effective disinfectant and wound dressing in the 19th century. Since then, antimicrobial effects of silver has been evaluated extensively (Oloffs *et al.*, 1994; Russell and Hugo, 1994). Developments in nanoscience and nanotechnology have allowed reduction of materials to nanoscale level, yielding preferable, scale-sensitive physical, chemical, optical and other properties (Osuwa and Anusionwu, 2011).

Based on different components, nanoparticles can be classified into several species: ceramic, carbon, polymer, metal, etc. Metallic nanoparticles, especially silver nanoparticles, have drawn the attention due to their useful biomedical, electronic, optical, physical and chemical properties (Zhang and Wang, 2010; Yan *et al.*, 2013). Preparation of silver nanoparticles can be achieved through physical, chemical and biological methods. The first two named methods are energy-intensive, require complex operation, and are highly toxic, which qualities conflict with green chemistry principles (Albrecht *et al.*, 2006). Thus, researchers increasingly turn their eyes to biosynthesis as an environment-friendly method. Many living entities are used to synthesize silver nanoparticles, such as bacteria (Kalimuthu *et al.*, 2008; Gurunathan *et al.*, 2009), fungi (Vigneshwaran *et al.*, 2007; Huang *et al.*, 2013) and plants (Hebbalalu *et al.*, 2013; Velmurugan *et al.*, 2014; Kathiraven *et al.*, 2015). Among these living entities, plants are considered ideal organisms, due to speed of production, low cost, high efficiency and superior stability. In recent years, various plants have been reported to synthesize silver nanoparticles, including a *Eucalyptus* hybrid (Dubay *et al.*, 2009), *Moringa oleifera* (Prasad *et al.*, 2011), *Brassica rapa* (Narayanan and Park, 2014) and *Aristolochia indica* (Shanmugam *et al.*, 2016).

Plant disease control is a perpetual problem affecting agricultural economics worldwide, which a new era endows with new connotation. Exploration is imperative for new agents with low toxicity and high efficiency to replace existing highly-toxic pesticides. Accordingly, more and more attention has been paid to research on silver nanoparticles as antimicrobial agents against organisms that include bacteria (Li *et al.*, 2010; Li *et al.*, 2011), fungi (Jo *et al.*, 2009; Kim *et al.*, 2009; Gull *et al.,* 2015), and viruses (Elechiguerra *et al.*, 2005; Lara *et al.*, 2010).

In this research, *Osmanthus fragrans* leaf extract was applied to rapid and simple biosynthesis of silver nanoparticles. In addition, optimization of silver nanoparticle production was achieved through adjustment of several parameters, like quantity of leaf, concentration of $AgNO_3$,

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pH and reaction temperature. The biosynthetic AgNPs were characterized using UV-Vis spectrophotometry, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Finally, antifungal activity of silver nanoparticles was measured for effect on colony formation and inhibition zone.

Materials and Methods

Fungal Isolates

Bipolaria maydis was isolated from infected maize leaves among regions of Anhui province, China. Single-spore isolates were preserved in the Laboratory of Plant Protection at the University of Science and Technology of Anhui. A single-spore isolate (AH-B06) was selected for this research in view of its high sensitivity to Tebuconazole, an efficient reagent for controlling *B. maydis*.

Preparation of Plant Extract

The leaf extract was prepared as follows: *O. fragrans* leaves were thoroughly washed in sterile water and cut into small pieces, followed by drying on a clean bench. Pre-selected quantities of leaves (1, 2, 5, 10, 15 and 20 g) were added to 100 mL of deionized water separately, then heated at 90°C for 30 min. The resulting extracts were filtered through Whatman No. 1 paper and reserved for further experiments.

Optimal Synthesis of Silver Nanoparticles

Biosynthesis of silver nanoparticles was achieved by adding the filtrate to deionized water at the ratio of 1: 9 (v: v), followed by reaction with $AgNO_3$ under varying temperature. Formation of silver nanoparticles was defined by color change in the reaction liquid. In order to determine optimal reaction parameters, an optimization process was performed that applied various quantities of *O. fragrans* leaf (1, 2, 5, 10, 15 and 20 g), concentrations of $AgNO_3$ (1, 2, 4, and 8 mM), hydrogen potentials (pH 1, 3, 5, 7 and 9) and reaction temperatures (4, 20, 40, 60 and 80°C). Silver nanoparticles obtained under various conditions were determined by use of UV-Vis absorption spectra.

Characterization of Silver Nanoparticles

Obvious solution color change was observed after incubation for 15 min. UV-Vis spectroscopy (TU-1950) determined silver nanoparticles, while TEM analysis (JEM-2100 F), field-emission SEM (S-4800) were also performed and energy-dispersive X-ray analysis (EDX) measured the purity of silver nanoparticles.

Antifungal Activity of Silver Nanoparticles

On the basis of colony formation and inhibition zone effect, the antifungal activity of silver nanoparticles was examined. To test colony formation, AH-B06 spores were adjusted to 10^6/mL by use of a counting chamber. Next, various silver nanoparticles and spore suspensions were added to sterile centrifuge tubes at the ratio of 1:9 (v:v), while sterile water and Tebuconazole (20 μg/mL) served as control. Finally, 100 μL of the above-described solution was spread evenly on PDA plates, then incubated at 30°C for 2–3 d. Following the inhibition zone method, 100 μL of spore suspension was spread uniformly on PDA plates separately. Following this, 10 μL of different treatments including silver nanoparticles, *O. fragrans* filtrate, sterile water and Tebuconazole (20 μg/mL) were added to the sterile filter paper discs (5 mm*5 mm), evenly distributed on said PDA plates. After standing for 5 min, the primed plates were incubated at 30°C for 2–3 days. Three replicates were performed for each treatment.

Results

Biosynthesis of Silver Nanoparticles

As shown in Fig. 1, the color of diluted filtrate containing 1 mM $AgNO_3$ changed from white to light brown after heating at 60°C for 15 min, indicating formation of silver nanoparticles. UV-Vis spectroscopy indicates that maximum absorbance is at 460 nm (Fig. 1c), corresponding to the surface plasmon resonance of silver nanoparticles.

Optimal Biosynthesis of Silver Nanoparticles

Effect of varying of *O. fragrans* leaf quantity: The solution color turned from light brown to deep brown as the herbal component increased from 1 g to 20 g (Figs. 2a–f). Maximum absorbance peak increased, to a degree, from 1–5 g of leaf, while peak decreased dramatically at 10 g (Fig. 2g). Results suggest that 5 g is optimum quantity of *O. fragrans* leaf for this process. The following optimization experiments refer to this quantity, except in case of special illustration.

Effect of different concentrations of $AgNO_3$: The solution color darkened with increased $AgNO_3$ concentration (Fig. 3a–d). However, absorbance peak maximum decreased sharply from 2.56 to 0.75 as concentration of $AgNO_3$ increased from 1–8 mM, which result does not agree with previous study (Iravani and Zolfaghari, 2013). Said discrepancy may be due to use of different plant species. Hence, optimal concentration of $AgNO_3$ is regarded as 1 mM.

Effect of different reaction temperature: Increased reaction temperature is necessary for silver nanoparticle production. Within the same reaction time (15 min), the solution at 4°C was yellowish white (Fig. 4a), which indicates sparse formation of silver nanoparticles. As reaction temperature increased from 4°C to 80°C, the solution darkened gradually (Fig. 4a–e). As shown in Fig. 4f, although the absorbance peak increased markedly from 20°C to 80°C, maximal absorbance occurred at 60°C, not 80°C. As a result, 60°C is regarded as optimal reaction temperature.

Effect of varied pH: The degree of acidity or alkalinity (pH)

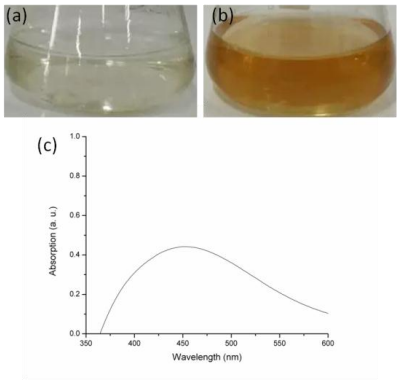

Fig. 1: Biosynthesis of silver nanoparticles, based on 1 g *O. fragrans* leaf: (a) and (b), color change of diluted filtrate before and after addition of 1 mM $AgNO_3$ at 60 °C; (c), UV-Vis spectrum of silver nanoparticles

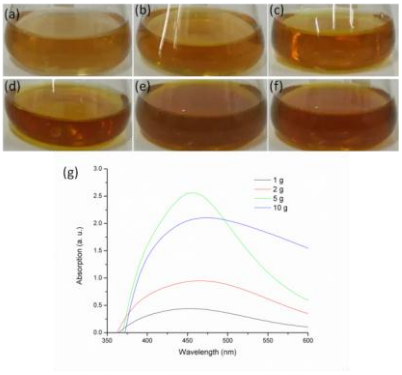

Fig. 2: Effect of varied quantity of *Osmanthus fragrans* leaves on silver nanoparticle formation: (a–f) showing 1, 2, 5, 10, 15 and 20 g, respectively of herbal component; (g) UV-Vis spectra of silver nanoparticles for varied leaf dosages

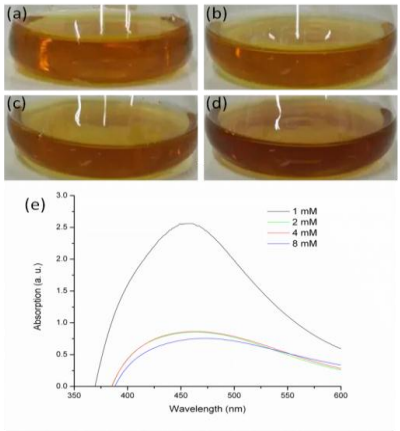

Fig. 3: Effect of varied $AgNO_3$ concentrations on silver nanoparticle formation: (a–d), respective $AgNO_3$ concentrations = 1, 2, 4 and 8 mM; (e), UV-Vis spectra of silver nanoparticles for varied amounts of $AgNO_3$

of solution is an important parameter influencing production of silver nanoparticles. In acidic solution (pH 3 and 5), the color was white or yellowish white (Fig. 5a), while the absorbance peak was not obvious (Fig. 5b). However, the solution became dark at pH 7 or higher (Fig. 5a). As well, the corresponding absorbance peak increased significantly, reaching maximum at 9 pH (Fig. 5b). As a result of the above, 9 pH is regarded as the optimal solution condition for synthesis.

Characterization of Silver Nanoparticles

TEM analysis: Characterizations of shape, dispersal level, size and size distribution of silver nanoparticles appear in Fig. 6. Synthesized silver nanoparticles were spherical or near-spherical with favorable dispersal behavior (Fig. 6a). In order to define particle size and size distribution, 200 typical silver nanoparticles were selected from several TEM micrographs, in which average particle size was about 20 nm. 52.5% of particles distributed between 10 ~ 20 nm, while 42.5% fell between 20 ~ 30 nm and 5% between 30 ~ 40 nm (Fig. 6b).

SEM and EDX analysis: When illuminated by SEM, many white particles appeared dispersed on the substrate, which represent silver nanoparticles (Fig. 7a). Peaks at 3 keV indicate the existence of elemental silver, while other peaks may be attributed to the Cu grid and elements of *O. fragrans* leaf extract (Fig. 7b).

Antifungal Activity of Silver Nanoparticles

Inhibition of colony formation: As shown in Fig. 8, silver nanoparticles significantly inhibited colony formation of AH-B06; the colony decreased to some extent when compared with silver nanoparticles containing 1 mM $AgNO_3$. No colony appeared on the plates at $AgNO_3$ concentrations of 2 mM, which effect parallels that of Tebuconazole at 20 μg/mL. Quantity of herbal component was not varied in the above cases.

Measurement of inhibition zone: Fig. 9 illustrates strong antifungal activity of silver nanoparticles against *B. maydis* (AH-B06), where appear obvious inhibition zones on each plate, of quantity of *O. fragrans* leaves or various concentrations of $AgNO_3$. Worth noting is that antifungal effect on AH-B06 exceeded that of Tebuconazole at 20 μg/mL.

In order to quantify the resulting inhibition zone, the diameter of each inhibition zone sample was measured separately (Table 1). On the whole, 5 g *O. fragrans* leaf-mediated silver nanoparticles present the best inhibition effect. Inhibition zone diamet–ers increased gradually along with increasing concentration of $AgNO_3$, reaching maximum at 8 mM $AgNO_3$.

Discussion

With the progress of nanoscience and nanotechnology,

Table 1: Diameter of inhibition zone in condition of different silver nanoparticles

Different quantities of leaves (g)	*O. fragrans* leaves-mediated silver nanoparticles Diameter of inhibition zone (mm)				
	Tebuconazole	1 mM	2 mM	4 mM	8 mM
1	8.7±0.47	7.8±0.85	10.0±1.47	10.2±0.62	10.0±1.41
2	6.8±1.93	7.5±0.41	9.7±0.47	11.7±1.31	13.3±1.25
5	7.3±0.85	11.3±0.24	11.8±0.24	12.5±0.41	12.7±0.47
10	7.0±0.82	7.3±0.47	8.3±0.85	9.2±0.62	10.5±0.71
15	7.0±0.82	6.0±0.82	6.8±0.62	9.8±1.43	14.0±0.82
20	6.2±0.24	6.3±0.62	9.3±0.47	9.3±0.62	11.2±0.24

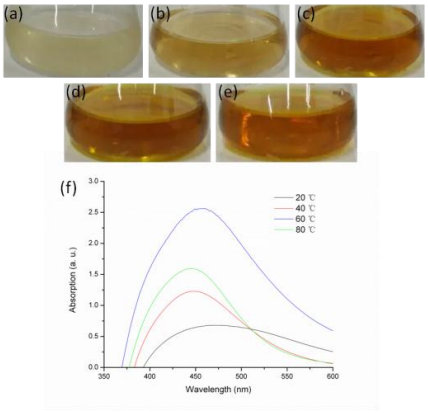

Fig. 4: Effect of different reaction temperature on silver nanoparticle formation: (a–e) show respective reaction temperatures of 4, 20, 40, 60 and 80°C; (f) UV-Vis spectra of silver nanoparticles for various reaction temperatures

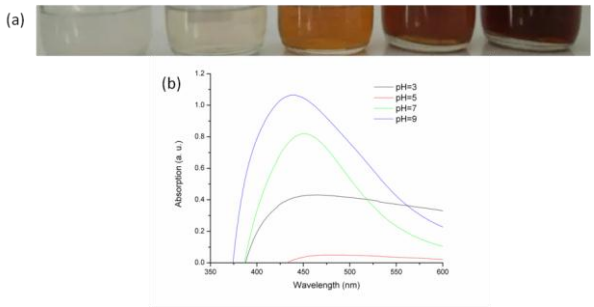

Fig. 5: Effect of pH on silver nanoparticle formation: (a) respective pH levels of 3, 5, 7, 9, and 11 from left to right; (b) UV-Vis spectra against various pH

growing numbers of researchers are focusing on silver nanoparticles, conducting related investigations on synthesis, characterization, as well as medical sterilization and antimicrobial application. Synthesis of silver nanoparticles by living entities, such as plants, is a practice in accord with green chemistry principles, possessing not only high purity, low toxicity, speed, accessibility, but also capacity for large-scale preparation.

In this research, *O. fragrans* leaves were used in biosynthesis of silver nanoparticles for the first time. In order to discover the optimal system, several parameters that influence synthesis of silver nanoparticles were regulated

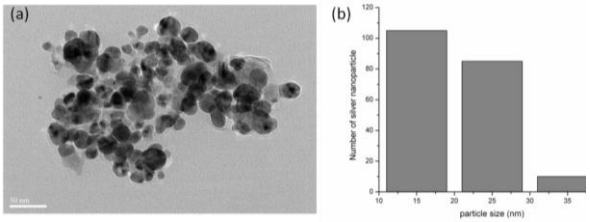

Fig. 6: TEM micrograph (a) and size distribution of silver nanoparticles (b)

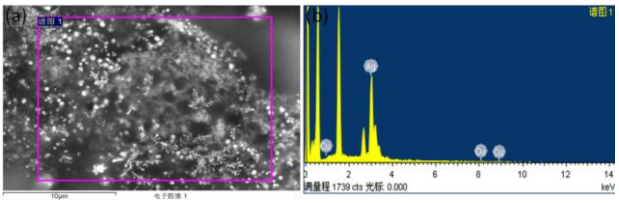

Fig. 7: SEM micrograph (a) and EDX spectroscopy (b) of silver nanoparticles

one-by-one, such as quantity of leaf component, concentration of $AgNO_3$, pH, and reaction temperature. During the optimization process, quantity of leaves and concentration of $AgNO_3$ were found to have peak ideal concentrations, while pH and reaction temperature also exhibited defined ideal limits. Results herein provide a point of reference for synthesis of silver nanoparticles using other plants.

Silver nanoparticles possess not only the general characteristics of other nanoparticles, but also have excellent antimicrobial activity. Antifungal assay shows that *O. fragrans* leaf-mediated silver nanoparticles present excellent inhibition activity on *B. maydis* (AH-B06) when compared with Tebuconazole (a highly-efficient fungicide), and that the optimal leaf quantity is 5 g. Furthermore, inhibition effect appears to be closely related to concentration of $AgNO_3$.

Conclusion

Biosynthetic silver nanoparticles may efficiently serve an important role for integrative control of plant pathogenic fungi. In addition, unreasonable use of pesticides exacerbates the growing crisis presented by common drug-resistant pathogens. The need is urgent for new agents to resolve the pathogen resistance issue, and silver nanoparticle types appear to be valuable candidates. Further research should focus on

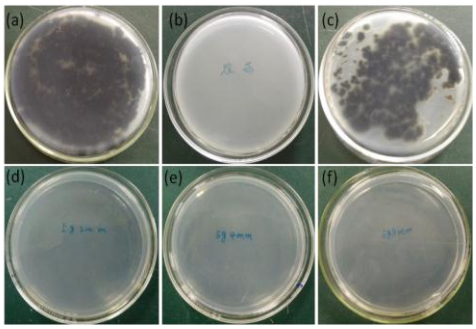

Fig. 8: Colony formation of AH-B06 on PDA plates: (a) AH-B06 with sterile water, (b) AH-B06 with Tebuconazole (20 µg/mL), (c)-(f) AH-B06 with different concentrations of $AgNO_3$ at 1, 2, 4 and 8 mM, respectively

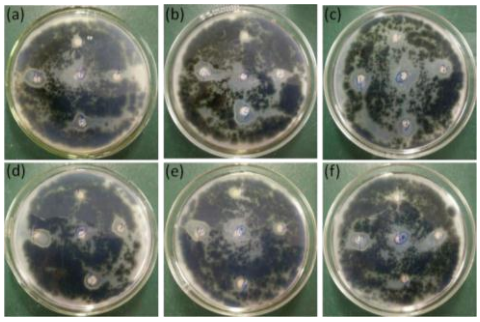

Fig. 9: Inhibition zones created by *O. fragrans* leaf-mediated silver nanoparticles on AH-B06: (a–f) showing respective quantities of *O. fragrans* leaves was 1, 2, 5, 10, 15, and 20 g. In each plate shown, the middle disk is Tebuconazole at 20 µg/mL, while positions 1–4 around the perimeter of each plate is silver nanoparticles at $AgNO_3$ concentrations (1, 2, 4 and 8 mM, respectively)

the antifungal activity of silver nanoparticles on other prominent fungal, bacterial, and drug-resistant plant pathogens.

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