Investigation of Antifungal Activity of Green-Synthesized Silver Nanoparticles on Phytopathogenic Fungi

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Abstract: Silver nanoparticles (SNPs) are nanoparticles of silver that are in the range of 1.0 and 100nm in size. They have unique antimicrobial properties which help in water sanitation and medical industries, but their potentials in agriculture have not been utilized. This study was conducted to investigate the effectiveness of SNPs synthesized using different concentrations of bitter leaf (*Vernonia amygdalina*) extracts in inhibiting the growth of three plant pathogenic fungi; *Fusarium oxysporum*, *F. solani* and *Cercospora canescens* isolated from diseased plants and identified using molecular method. Synthesized SNPs were characterized using UV-Vis absorption spectroscopy. The antifungal activity of SNPs synthesized at 0, 2, 5 and 10 mins using 0.10g/ml and 0.20g/ml of bitter leaf extracts were evaluated on the basis of colony formation by *in-vitro* assays. UV-visible spectroscopic analyses revealed rapid reduction of silver ions (Ag^+) by bitter leaf extracts where surface Plasmon absorption maxima were observed from the UV-vis spectra. The growth of pathogenic fungi on agar plates were significantly decreased or totally inhibited by treatment with the SNPs depending on the concentrations of the plant extracts and the time of reaction. SNPs synthesized with 0.20g/ml of the extract for 10 mins completely inhibited the growth of the tested fungal pathogens while other SNPs significantly reduced their growth (60 – 90% reduction). The results of this study indicated that the silver nanoparticles synthesized using bitter leaf extracts have potentials to be used as control agents against fungal plant diseases to replace the use of chemical fungicides.

Keywords: Silver nanoparticles, antifungal activity, *Vernonia amygdalina*, control, fungal pathogens

Introduction

Silver nanoparticles (Ag-NPs) are nanoparticles of silver that are in the range of 1.0 and 100nm in size. Silver nanoparticles have become the focus of intensive research due to a wide variety of potential applications in areas like catalysis, optics, antimicrobials, biomedical and electronic fields (Abdullah and Hamud, 2013; Awwad *et al*., 2013).

Silver nanoparticles can be synthesised by various methods like chemical and photochemical reactions in reverse micelles, thermal decomposition, electrochemical, sonochemical, microwave assisted process, and also by biological methods (Nethradevi *et al*., 2012). Among them, biological processes that are based on bacteria, fungus, bio-derived chemicals and plant extracts are considered as safe and economically sound for the nanomaterial fabrication (Mason *et al*., 2012; Abdullah and Hamud, 2013). Using 'green' methods in the synthesis of silver nanoparticles has become an alternative to conventional physical and chemical methods which are expensive and require the use of chemical compounds or organic solvents as reducing agents (Mason *et al*., 2012). A range of plant (leaf, flower, seed, tuber and bark) extracts have been investigated for their ability to efficiently synthesize silver nanoparticles. Bioreduction of Ag^+ ions to yield silver nanoparticles are caused by combinations of bio-molecules found in these extracts such as vitamins, enzymes/proteins, organic acids such as citrates, amino acids, and polysaccharides (Abdullah and Hamud, 2013).

In medical and industrial process, silver has effective inhibition on microbes. The most important application of silver and silver nanoparticles in medical industry is topical ointments to prevent infection against burn and open wounds (Ip *et al*., 2006). Silver nanoparticles are also used as antimicrobial and antibiotic agents when incorporated in proteins, nanofibres, first aid bandages, plastics, soap and textiles, in cell cleaning fabrics and as conductive filler. There is also an effort to incorporate silver nanoparticles into a wide range of medical devices including bone cement, surgical instruments, surgical masks and wound dressings (Nithya *et al*., 2011). Currently most of the applications of silver nanoparticles are in antibacterial/antifungal agents in biotechnology and bioengineering, textile engineering, water treatment and silver-based consumer products, but their potentials in agriculture have not been utilized. The present study was therefore conducted to investigate the effectiveness of green-synthesized silver nanoparticles using bitter leaf (*Vernonia amygdalina*) extracts in inhibiting the growth of plant pathogenic fungi with the aim of using these nanoparticles as control agents against

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fungal plant diseases to replace the use of chemical fungicides.

Materials and Methods

Collection of plant materials

Infected cowpea plants were collected from College of Plant Sciences (COLPLANT) farm, Federal University of Agriculture, Abeokuta and taken to the laboratory. In the laboratory, the roots, stems and leaves of diseased samples were washed with sterile distilled water and cut into small pieces.

Fresh leaf samples of *Vernonia amygdalina* were collected from Obantoko, Abeokuta. The plant was identified at the Herbarium Unit of the Department of Pure and Applied Botany, Federal University of Agriculture, Abeokuta. The samples were collected by hand picking. The leaves were washed thoroughly with distilled water, cut into small pieces and then air-dried.

Isolation and Identification of Phytopathogenic fungi

The plant samples were surface-sterilized with 1% sodium hypochlorite solution for 3 min and rinsed with several changes of sterile distilled water. The sterilized samples were placed on peptone-pentachloro nitrobenzene agar (PPA) and potato dextrose agar (PDA) plates. The plates were incubated at room temperature for 7 days. Pure cultures of the isolates were obtained by sub-culturing on PDA plates. The pathogenicity of the fungal isolates was tested on the healthy seedlings of cowpea using the method described by Chehri *et al*. (2011). The phytopathogenic fungal isolates were phenotypically characterized by their cultural and morphological characteristics, and genotypically identified by gene sequencing method.

Preparation of Vernonia amygdalina extracts

The *V. amygdalina* extracts were made by boiling 10.0g and 20.0g of fresh leaves with 100.0ml of distilled water for about 10 minutes, to give concentrations of 0.10g/ml and 0.20g/ml respectively. The solutions were then removed from the heat source and left to cool to ambient temperature (approximately 25^{o}C). The extracts were then filtered through 0.25µm filter to remove any leaf matter and the resultant filtrates were used for synthesis of silver nanoparticles.

Synthesis of Silver Nanoparticles

The aqueous silver nitrate ($AgNO_3$) used for synthesis of silver nanoparticles was prepared by dissolving 0.03g of $AgNO_3$ in 250 ml of distilled water to give 1.0mM. Then 10 ml of each *V. amygdalina* extract was added into 40 ml of prepared aqueous solution of 1mM $AgNO_3$ for reduction of Ag^+ and kept on magnetic stirrer at 70^{o}C. Samples were collected at 0 min, 2 min, 5 min and 10 min of reaction time after the formation of silver nanoparticles, and used for antifungal analysis. The observed change in colour from colourless to transparent yellow and finally to a dark brown indicated the formation of silver nanoparticles.

Characterization of silver nanoparticles

Optical absorbance of the synthesized silver nanoparticles was performed using a UV-visible spectrophotometer between the wavelengths of 300 and 800 nm at a resolution of 1 nm.

In-vitro Antifungal Assay of Silver nanoparticles on phytopathogenic fungi

The phytopathogenic fungi used were isolates of *Fusarium oxysporum*, *F. solani* and *Cercospora canescens*. The antifungal activity of synthesized silver nanoparticles was examined on the basis of colony formation by *in-vitro* petri-dish assay. Each fungal isolate was grown in potato dextrose broth at 25±2^{o}C for 7 days. Each fungal biomass was homogenized for one minute. Spore suspensions were prepared using sterile distilled water to a concentration of 5×10^5 spores/ml using a haemocytometer. 1.0ml of each diluted spore suspension was spread on potato dextrose agar (PDA) supplemented with 5% of each synthesized silver nanoparticle. Three plates were prepared for each isolate and nanoparticle. The control plates were also prepared by inoculating PDA without silver nanoparticle with the diluted spore suspension of each isolate. The plates were incubated at 25^{o}C for 7 days. The number of colonies forming on plates was counted at 7 days of incubation and the average number of colonies on the plates was determined. The percentage growth inhibition (GI) was calculated as:

$$\% GI = \frac{C_o - C_S \times 100}{C_o}$$

C_o: Number of colonies on control plates

C_S: Number of colonies on synthesized silver nanoparticle plates

Results

Phytopathogenic fungal isolates

In this study, three phytopathogenic fungi were obtained from diseased cowpea plants. Based on their phenotypic and genotypic characteristics, these isolates were characterized as *Fusarium oxysporum*, *Fusarium solani* and *Cercospora marcescens* (Table 1).

Table 1: Phytopathogenic fungi from infected cowpea plants

Genotypic identification	Source	% similarity	Accession number
Cercospora canescens strain CCA19	Leaf	99.0	AY266164.1
Fusarium solani f.sp phaseoli strain T1	Stem/root	100.0	AF150481.1
F. oxysporum f.sp phaseoli strain YN091019-4	Stem/root	99.0	HM756256.1

UV-vis spectra analysis

The addition of *V. amygdalina* leaf extracts to silver nitrate ($AgNO_3$) solution resulted in colour change of the reaction mixtures with respect to time of reaction (Plate 1). The colourless solution turned to yellow immediately the silver nitrate and extracts were mixed together, indicating the initial formation of silver nanoparticles. Colour changes were further observed at 2 minutes from faint yellow to dark brown indicating the formation of silver nanoparticles. As time elapsed, the solution eventually became deep dark brown at 10 minutes, as a result of the increasing concentrations of silver nanoparticles as well as the particles' growth in size. There was no significant change in colour beyond 10 minutes, indicating the completion of the reduction reaction. The colour changes arise from the excitation of Surface Plasmon vibrations with the silver nanoparticles.

The UV-vis spectra of the reaction mixtures are shown in Figure 1. The Surface Plasmon resonance (SPR) of the synthesized silver nanoparticles produced the peaks centered near 400nm and 450nm for silver nanoparticles synthesized using 0.10g/ml and 0.20g/ml of *V. amygdalina* leaf extracts respectively. These indicated the reduction of silver nitrate into silver nanoparticles using *V. amygdalina* leaf extracts.

10 mins 5 mins 2 mins 0 min

Plate 1: Photograph of reaction mixtures (V. amygdalina leaf extract and silver nitrate solution) at different time

Fig. 1: UV-vis spectra of synthesized silver nanoparticles from different concentrations of *V. amygdalina* leaf extracts

Inhibition of phytopathogenic fungal isolates by silver nanoparticles

All synthesized silver nanoparticles showed various levels of inhibition on colony formation of *Fusarium oxysporum, Fusarium solani* and *Cercospora marcescens* depending on the concentrations of *V. amygdalina* leaf extracts used for Ag-NPs synthesis and time of reaction (Figures 2, 3 and 4). As concentration of the leaf extract and reaction time increased, colony formation decreased. The total inhibition (100%) was recorded with Ag-NPs synthesized using 0.20g/ml of *V. amygdalina* leaf extracts for 10 min against all tested pathogens. However, other silver nanoparticles, except those synthesized at 0 min, showed considerable inhibition of 60% - 90% against the tested fungal pathogens. Silver nanoparticles synthesized at 0 min showed the lowest percentage inhibition (10.0%) of the pathogens. From this study, it was observed that silver nanoparticles synthesized using *V. amygdalina* leaf extracts showed good inhibitory activity against the fungal pathogens of cowpea plants and could be used as control agents against fungal plant diseases to replace the use of chemical fungicides.

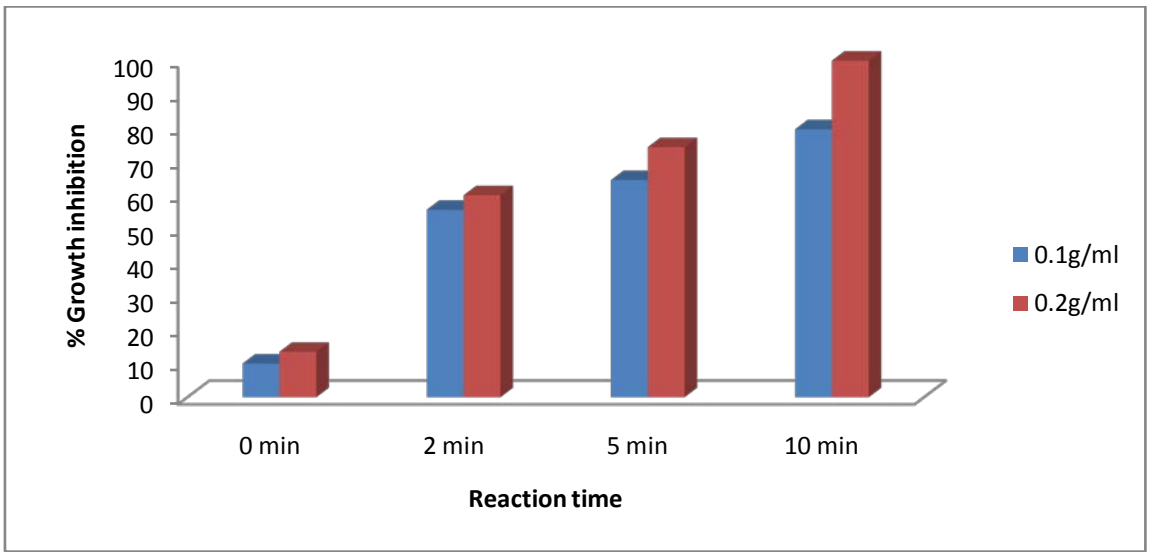

Fig. 2: Effect of synthesized silver nanoparticles on growth inhibition of *Fusarium oxysporum*

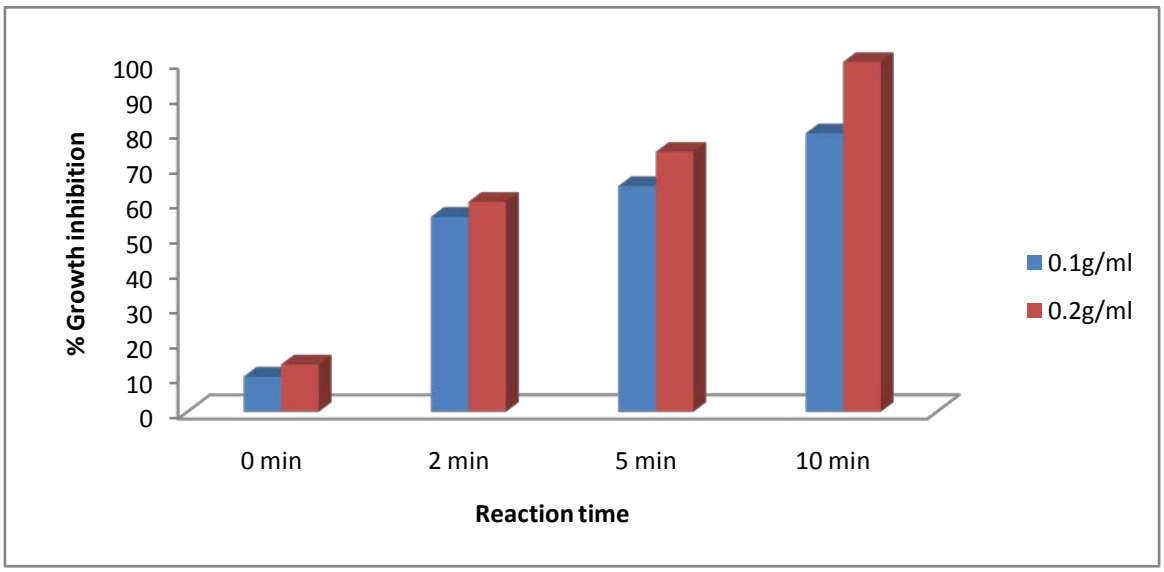

Fig. 3: Effect of synthesized silver nanoparticles on growth inhibition of *Fusarium solani*

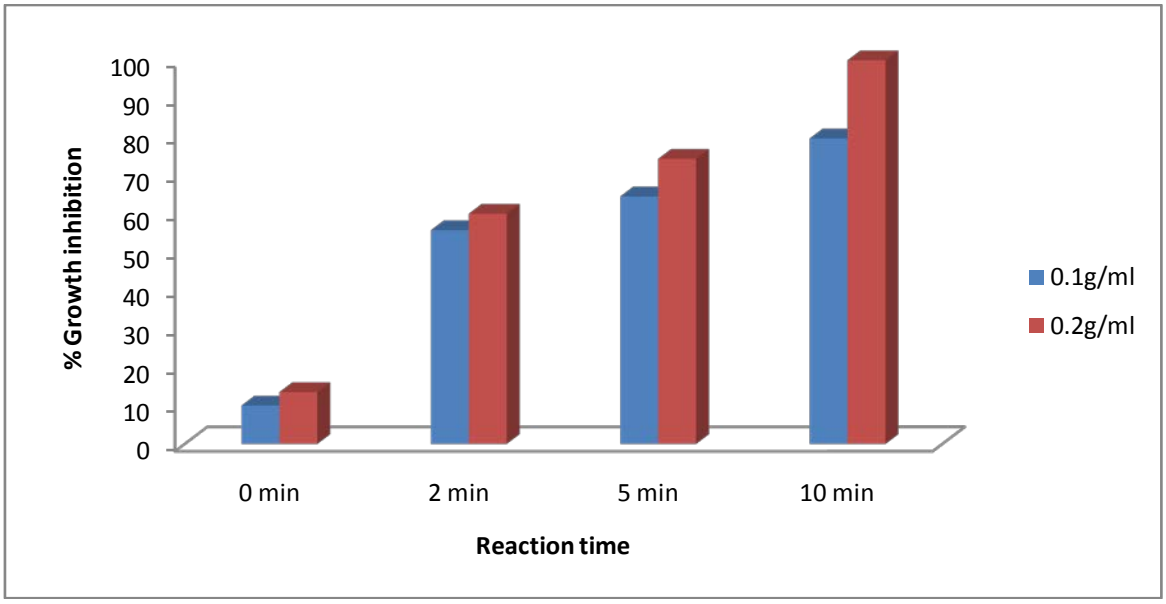

Fig. 4: Effect of synthesized silver nanoparticles on growth inhibition of *Cercospora canescens*

Discussion

Management of fungal diseases in food crops with safe and cheap methods is economically important in sustainable agriculture. Recently, more efforts have been given to develop safe management methods that pose less damage to humans and animals as well as solving the problems posed by synthetic fungicides. Nano silver is a powerful and natural antimicrobial agent that has been proven highly effective in fighting a whole range of microbes. Thus, the present study showed that silver nanoparticles synthesized using *V. amygdalina* leaf extracts successfully inhibited the growth of *Fusarium oxysporum, Fusarium solani* and *Cercospora marcescens in-vitro* and they may be effective in controlling or reducing cowpea diseases caused by these fungal pathogens. Though, there are few studies on antifungal activity of silver nanoparticles on plant pathogenic fungi, the results of this study corroborated that of Jo *et al*. (2009) who reported that silver ions and nanoparticles effectively reduced the colony formation of *Bipolaris sorokiniana* and *Magaporthe grisea.* In addition, the sensitivities of the tested fungal pathogens in this study to synthesized Ag-NPs were different, depending on the concentrations of leaf extracts and reaction time. *Cercospora marcescens* showed more tolerance to synthesized Ag-NPs than those of *Fusarium oxysporum* and *Fusarium solani* at different reaction time and leaf extracts' concentrations. This is in agreement with previous reports that stated that antimicrobial activity of silver was different depending on microbial species (Takai *et al*., 2002).

The antifungal activity of synthesized silver nanoparticles could be due to the ability of Ag-NPs to directly attach to and penetrate the cell membrane to kill spores. The silver nanoparticles have also been reported to disable some enzymes needed by the microbes for their respiration and growth without causing corresponding harm to host enzymes (Abdullah and Hamid, 2013). The silver nanoparticles also cause permeability of outer membrane, resulting in the leakage of cellular materials. Ag-NPs could also affect some proteins and phosphate lipids, and induce collapse of membrane leading to decomposition and eventually death. All these actions of silver nanoparticles result in the destruction of disease-causing microorganisms without any detrimental effects on the surrounding host tissue.

Conclusion and Recommendations

In conclusion, since *Vernonia amygdalina* is readily available, the active nano compound from this plant can be prepared by the farmers and used effectively for controlling the growth of fungal plant pathogens. These nanoparticles may be less toxic to humans and animals than synthetic fungicides. This method also provides an important benefit of avoiding the development of resistance, which is normally associated with the use of chemical fungicides for controlling many plant fungal diseases. Hence, green-synthesized silver nanoparticles have wide applications in agriculture.

Further research need to be conducted to determine the application of silver nanoparticles for controlling of fungal pathogens in the fields. At the same time, the environmental impact of silver nanoparticles when applied in the fields is important to assess their impacts on environments and human health.

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