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RESEARCH PAPER

ANTIMICROBIAL ACTIVITIES OF BIO-SYNTHESIZED SILVER NANOPARTICLES ON STRAINS OF *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT
Silver nanoparticles (Ag-NPs) have been known to possess unique antimicrobial properties which help in medicine and water treatment, but their potentials in agriculture have not been fully utilized. This study was conducted to determine the inhibitory activity of bio-synthesized Silver nanoparticles on strains of Clavibacter michiganensis subsp. michiganensis, causative agents of canker disease of tomato plant. The Ag-NPs were synthesized using Cladosporium cladosporioides, leaf extracts of Azadirachta indica and Vernonia amygdalina. The synthesized silver nanoparticles were characterized using UV-visible absorption spectroscopy. The antibacterial activities of bio-synthesized Ag-NPs on strains of C. michiganensis subsp. michiganensis were evaluated using agar well diffusion method. UV-visible spectroscopic analysis revealed rapid reduction of silver ions by C. cladosporioides, A. indica and V. amygdalina extracts where surface Plasmon absorption maxima were observed at 400nm – 450nm from the UV-visible spectra. The in-vitro antibacterial activities revealed that Ag-NPs synthesized using V. amygdalina and A. indica extracts inhibited the growth of C. michiganensis subsp. michiganensis strains with zones of inhibition ranging from 15.70 mm - 24.70mm and 21.00mm – 30.00mm, respectively, while those synthesized using C. cladosporioides showed zones of inhibition ranging from 0.00mm – 12.00mm. Growth curves of C. michiganensis subsp. michiganensis strains in the presence of biosynthesized Ag-NPs showed inhibition of growths after 4 – 10 hours of exposure. The results of this study indicated that the bio-synthesized silver nanoparticles could be effective in controlling bacterial canker disease of tomato plant caused by strains of C. michiganensis subsp. michiganensis thereby reducing the toxic effects of chemical bactericides on important agricultural products.

Keywords: *Silver nanoparticles, inhibitory activity, canker, tomato, zones of inhibition*

INTRODUCTION

Metallic nanoparticles have received enormous attention from chemists, physicists, biologists, and engineers, but their potentials in agriculture have not been fully utilized. Silver nanoparticles are nanoparticles of silver that range between 1.0 and 100 nm in size. They are widely used in many pharmaceutical and biological applications because of their unique antimicrobial properties (Egger *et al*., 2009). Silver nano-

particles are being used as antimicrobial agents for burn treatment, prevention of bacterial colonization on catheters and elimination of microorganisms on textile fabrics (Chou *et al.,* 2005; Lui *et al.,* 2006). They are also capable of purifying drinking water, degrading pesticides and killing human pathogenic bacteria (Mohamed *et al*., 2014). Silver nanoparticles have also been reported to exhibit a strong cytoprotective activity towards human immunodeficiency virus (HIV) infections (Sun *et al.,* 2005).

There are diverse methods for nanoparticles formation in which biosynthetic green or biological methods are considered as safe and economically sound for the nanomaterial fabrication. The methods usually involve using medicinal plants and microorganisms such as fungi and algae to synthesize nanoparticles for pharmaceutical and biological applications. The methods are eco-friendly and cost effective as compared to the chemical and physical methods (Mohamed *et al*., 2014). Unlike physical and chemical methods that use toxic substances, biological methods make use of bacteria, fungi, bio-derived chemicals and plant extracts, and therefore, they are considered as safe, environmental friendly and cost effective for the synthesis of silver nanoparticles (Jose *et al.,* 2005; Mason *et al*., 2012; Abdullah and Hamid, 2013). The silver nanoparticles prepared using green methods have high surface area, a smaller size, high dispersion and show a strong bactericidal and antibiotic activity (Mohamed *et al*., 2014).

Bacterial canker disease is a serious disease of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). *C. michiganensis* subsp. *michiganensis* shows marginal necrosis on tomato leaves and stems, small dark spots on fruit surrounded with 'bird's-eye' spots. In seedlings, stunting, wilting and stem splitting are characteristics of the bacterial canker disease (Werner *et al.,* 2002; Agrios, 2007). Bacterial canker has caused serious losses to tomato crops by direct killing of the plants, most especially the young tomato plants, or by reducing the numbers of fruit and fruit size, leading to reduction in yields. The disease therefore causes great yield loss to the farmer.

Agricultural associations have devised several means of controlling such disease by means of using herbicide and biological means but they are not effective most of the time because different strains of bacterial canker disease are beginning to show up. Therefore, the study was undertaken to investigate the inhibitory activity of bio-synthesized silver nanoparticles using *Cladosporium cladosporioides,* leaf extracts of *Azadirachta indica* and *Vernonia amygdalina* on strains of *Clavibacter michiganesis* subsp. *michiganensis* with the aim of preventing the growth and development of bacterial canker disease of tomato.

MATERIALS AND METHODS

Collection of plant materials

Fresh leaf samples of *Azadirachta indica* and *Vernonia amygdalina* were collected from FUNAAB campus and Obantoko, Abeokuta. The plants were identified by a Plant Taxonomist and deposited to the Herbarium of the Department of Pure and Applied Botany, Federal University of Agriculture, Abeokuta with voucher numbers of FUNAABH-0083 and FUNAABH-0084 for *A. indica* and *V. amygdalina* respectively. The samples were collected by hand picking. The leaves were washed thoroughly with distilled water, cut into small pieces and then air-dried.

Cladosporium cladosporioides used for the synthesis of silver nanopaticles was collected from the Department of Microbiology, Federal University of Agriculture, Abeokuta, Nigeria.

Bacterial strains and growth conditions

Three virulent strains of *Clavibacter michiganesis* subsp. *michiganensis* were used for this study. The strains are designated as *Clavibacter michiganensis* subsp. *michiganensis* F001, *C. michiganensis* subsp. *michiganensis* F002 and *C. michiganensis* subsp. *michiganensis* F005 which were previously isolated from symptomatic tomato plants, tested for pathogenicity, and then identified by phenotypic and molecular methods. These strains were grown on nutrient agar supplemented with 1 % D-glucose and incubated at 30 ^{o}C for 24 hours.

Preparation of *Vernonia amygdalina* and *Azadirachta indica* extracts

The *V. amygdalina* and *A. indica* extracts were made by boiling 20.0 g and 40.0 g of fresh leaves of each plant with 100.0 ml of distilled water for about 10 minutes, to give concentrations of 0.20 g/ml and 0.40 g/ml respectively. The extracts were then removed from the heat source and left to cool to ambient temperature (approximately 25 ^{o}C). The extracts were then filtered through 0.25 µm filters to remove any leaf matter and the resultant filtrates were used for synthesis of silver nanoparticles.

Synthesis of silver nanoparticles from leaf extracts

The aqueous silver nitrate ($AgNO_3$) used for synthesis of silver nanoparticles was prepared by dissolving 0.03 g of $AgNO_3$ in 250 ml of distilled water to give 1.0 mM. Then 10.0 ml of each leaf extract was added into 40.0 ml of prepared aqueous solution of 1mM $AgNO_3$ for reduction of Ag^+ and kept on magnetic stirrer at 70 ^{o}C until the formation of silver nanoparticles. The observed change in colour from colourless to transparent yellow and finally to a dark brown indicated the formation of silver nanoparticles.

Synthesis of silver nanoparticles from *Cladosporium cladosporioides*

Cladosporium cladosporioides hyphae were inoculated in potato dextrose broth and placed in a shaker incubator at 150 rpm for 48 hours. After incubation, the biomass was filtered using a Whatman filter paper to separate the fungal hyphae from the potato dextrose broth and washed with sterile distilled water to remove the growth medium. The resulting fungal hyphae were inoculated in sterile distilled water and placed in a shaker incubator for 48 hours at 150 rpm. The biomass was filtered again and the aliquot of the filtrate was taken. Ten milliliter (10.0 ml) of the aliquot of the filtrate was mixed with 40.0 ml of 1.0 mM silver nitrate solution in an Erlenmeyer flask and agitated on magnetic stirrer at 25 °C for 72 hours in dark until the formation of silver nanoparticles.

Characterization of bio-synthesized silver nanoparticles

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

In-vitro* antibacterial activity of bio-synthesized silver nanoparticles against strains of *clavibacter michiganensis* subsp. *michiganensis

The antibacterial activity of bio-synthesized silver nanoparticles was determined using agar well diffusion method. An overnight culture of each strain grown in nutrient broth supplemented with 1% D-glucose at 28 ^{o}C was diluted to a turbidity equivalent to 0.5 Mcfarland standard (1.5×10^8 cfu/ml) with a sterilized normal saline (0.85 % NaCl solution). The cell suspension was then flooded on the surface of the nutrient agar supplemented with 1 % D-glucose (NGA) plates and the plates were allowed to dry. A sterile cork borer of diameter 7.0mm was used to make wells on the agar plates. Each well was filled with 50µl of each synthesized silver nanoparticles and the control wells were filled with sterile distilled water. All these assays were carried out in triplicates. The plates were left for one hour to allow the silver nanoparticles to diffuse in the agar and then incubated at 30 ^{o}C for 48 hours, without inversion. The antibacterial activity was determined by measuring the clear zones around the wells. The diameters (mm) of the zones of inhibition were recorded.

Determination of the growth kinetics of bacterial strains exposed to bio-synthesized silver nanoparticles

The growths of *C. michiganensis* subsp. *michiganensis* strains treated with AgNPs were studied using the method described by Mohamed *et al*. (2014) with little modifications. Ten milliliter (10.0 ml) of nutrient broth supplemented with 10 % D-glucose was inoculated with 100 µl of a fresh and standardized bacterial strain. These cultures were then supplemented with 300 µl of each bio-synthesized silver nanoparticles and control cultures were treated with sterile distilled water. The bacterial cultures were incubated at 30 ^{o}C with agitation at 150 rpm. The bacterial growth rates and concentrations were determined by measuring the optical density using spectrophotometer at wavelength of 600 nm at 2 hours interval. The growth curves

were obtained by plotting optical density against time.

Statistical analysis

Means and standard deviations were calculated using standard statistical methods. Analysis of antibacterial activity was done using Duncan's Multiple Range test at 5 % level of significance.

RESULTS

UV-vis spectra analysis

The addition of *A. indica* and *V. amygdalina* leaf extracts to silver nitrate ($AgNO_3$) solution resulted in colour change of the reaction mixtures. The colourless solutions turned to yellow immediately the silver nitrate and extracts were mixed together, indicating the initial formation of silver nanoparticles. Colour changed from faint yellow to dark brown indicating the formation of silver nanoparticles and finally became deep dark brown due to increase in concentrations of silver nanoparticles formed. The colour changes arose from the excitation of surface plasmon vibrations with the silver nanoparticles. Similarly, the addition of the fungal filtrate (*Cladosporium cladosporioides*) to silver nitrate solution, followed by agitation at 25 ^{o}C led to colour change from pale yellow to deep dark brown on reduction of silver ions (Ag^{+}). The appearance of deep dark brownish colour in solution containing fungal biomass was a clear indication of the formation of silver nanoparticles in the reaction mixture. In addition, it was observed that the reduction of silver ions (Ag^{+}) into silver nanoparticles started immediately the extracts and fungal filtrate mixed with silver nitrate solution, and reductions were completed at 10 minutes, 30 minutes and 72 hours for extracts of *A. indica, V. amygdalina* and *Cladosporium cladosporioides* filtrate, respectively. The UV-vis spectra of the reaction mixtures are shown in Figs 1- 3. The Surface Plasmon resonance (SPR) of the bio-synthesized silver nanoparticles produced the peaks centered near 400 nm and 450 nm for silver nanoparticles synthesized using 0.20 g/ml and 0.40 g/ml of *A. indica* and *V. amygdalina* leaf extracts respectively, and 400 nm for *C. cladosporioides* filtrate.

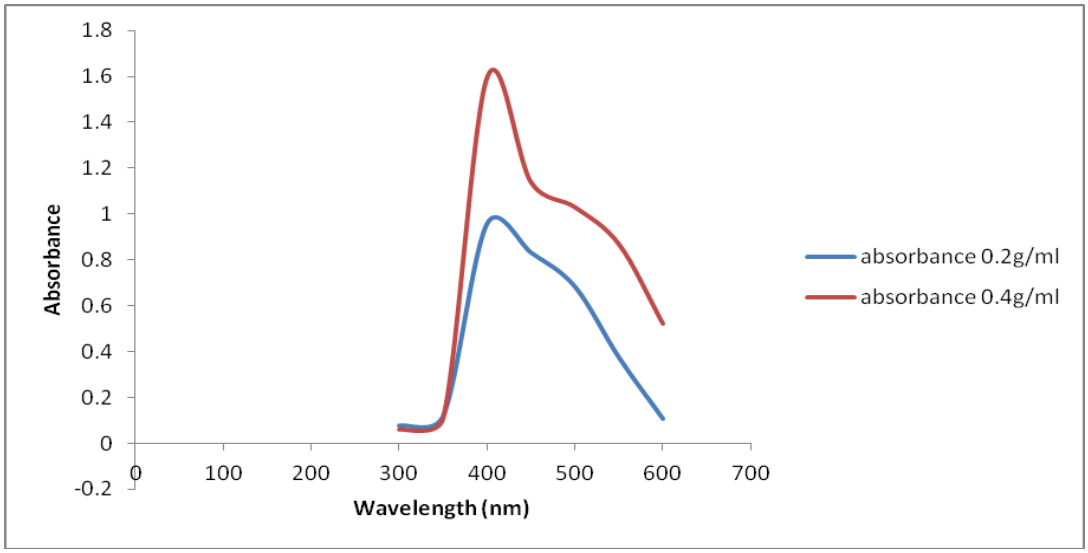

Fig. 1: UV-vis spectra of bio-synthesized silver nanoparticles from different concentrations of *A. indica* leaf extracts

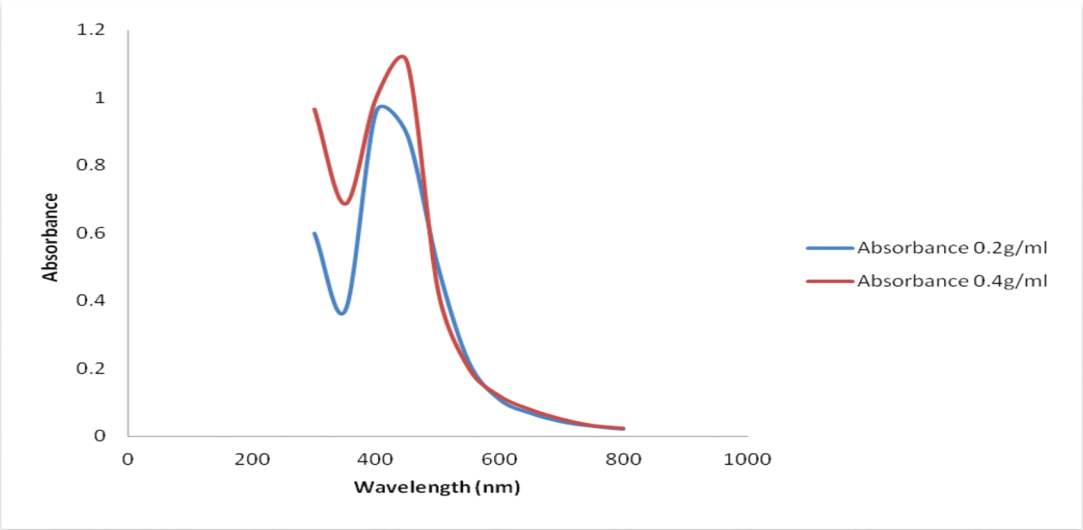

Fig. 2: UV-vis spectra of bio-synthesized silver nanoparticles from different concentrations of *V. amygdalina* leaf extracts

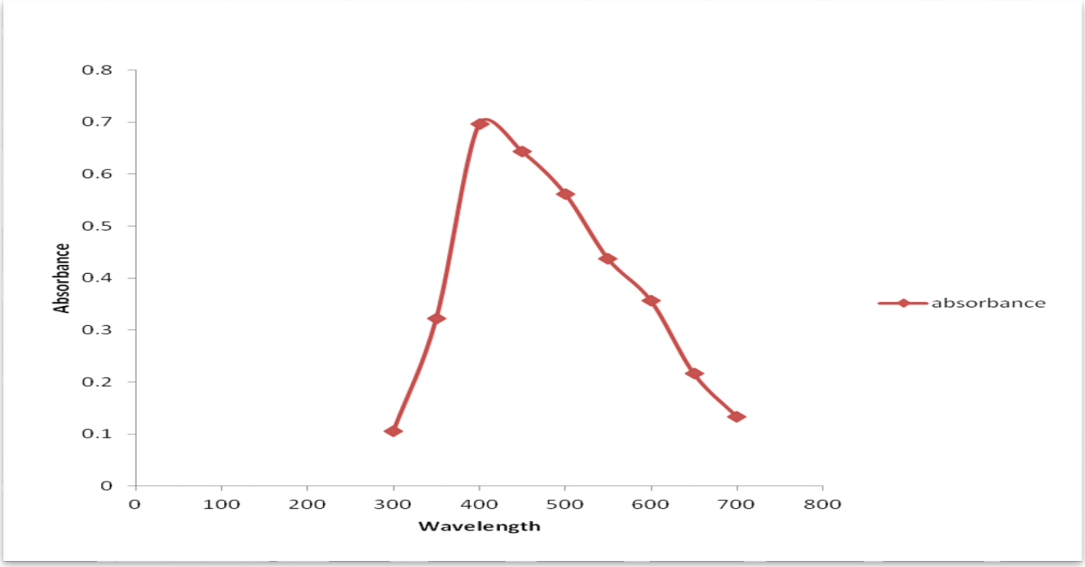

Fig. 3: UV-vis spectra of bio-synthesized silver nanoparticles from *C. cladosporioides* filtrate

Antibacterial activities of bio-synthesized silver nanoparticles against *clavibacter michiganensis* subsp. *michiganensis strains*

The antimicrobial activities of bio-synthesized silver nanoparticles are shown in Table 1. The silver nanoparticles synthesized using leaf extracts of *A. indica* and *V. amygdalina* exhibited higher antibacterial activity against *C. michiganensis* subsp. *michiganensis* strains than those synthesized using *C. cladosporioides* filtrate. Silver nanoparticles synthesized using *C. cladosporioides* filtrate showed no inhibition against *C. michiganensis* subsp. *michiganensis* F002, but showed strong inhibition against *C. michiganensis* subsp. *michiganensis* F001 and *C. michiganensis* subsp. *michiganensis* F005. On the other hand, silver nanoparticles synthesized using leaf extracts of *A. indica* showed higher inhibitory activity than those synthesized using leaf extracts of *V. amygdalina.* Also, as concentrations of the leaf extracts increased, the zones of inhibition increased. The silver nanoparticles synthesized using 0.4 g/ml leaf extracts showed significant and effective antimicrobial activity against *C. michiganensis* subsp. *michiganensis* strains when compared with those synthesized using 0.2g/ml leaf extracts and *C. cladosporioides* filtrate (Table 1). Thus, these results indicated that biosynthesized silver nanoparticles could be used to control the disease caused by strains of *C.*

Growth curves of *clavibacter michiganensis* subsp. *michiganensis* strains treated with different biosynthesized silver nanoparticles

The growth curves of *C. michiganensis* subsp. *michiganensis* strains exposed to different biosynthesized silver nanoparticles are shown in Figs 4, 5 and 6. In the control media, the bacterial growth curves were found to increase rapidly indicating that the bacterial strains reached the exponential phases rapidly. On the other hands, the growth curves of the strains treated with biosynthesized silver nanoparticles initially increased and then decreased. The growths of the bacterial strains exposed to silver nanoparticles synthesized using 0.2 g/ml and 0.4 g/ml leaf extracts were inhibited after 6 hours and 4 hours respectively while the growths of *C. michiganensis* subsp. *michiganensis* F001 and *C. michiganensis* subsp. *michiganensis* F005 were inhibited after 8 and 10 hours respectively when exposed to silver nanoparticles synthesized using *C. cladosporioides* filtrate. Silver nanoparticles synthesized using *C. cladosporioides* filtrate had no effect on the growth of *C. michiganensis* subsp. *michiganensis* F002.

DISCUSSION

Silver nanoparticles (Ag-NPs) have become an important application of many fields including microbiology due to their antimicrobial activities. Thus, the results of this present study rev-

Table 1: Antibacterial activities of biosynthesized silver nanoparticles on *Clavibacter michiganensis* subsp. *michiganensis* strains

Test strains	Zones of Inhibition (mm ± S.D)				
	Vernonia amygdalina		*Azadirachta indica*		*C. cladosporioides*
	0.20 g/ml	0.40 g/ml	0.20 g/ml	0.40 g/ml	
C. michiganensis F001	16.20±0.01[a]	24.70±0.1[a]	21.00±0.08[a]	30.00±0.07[a]	12.00±0.1[a]
C. michiganensis F002	16.50±0.3[a]	18.00±0.12[b]	21.60±0.11[a]	27.70±0.02[a]	0.00±0.00[c]
C. michiganensis F005	15.70±0.15[a]	22.40±0.02[a]	21.10±0.06[a]	28.20±0.02[a]	8.00±0.01[b]

Note: Means with different superscripts along the columns are significantly different (P < 0.05)

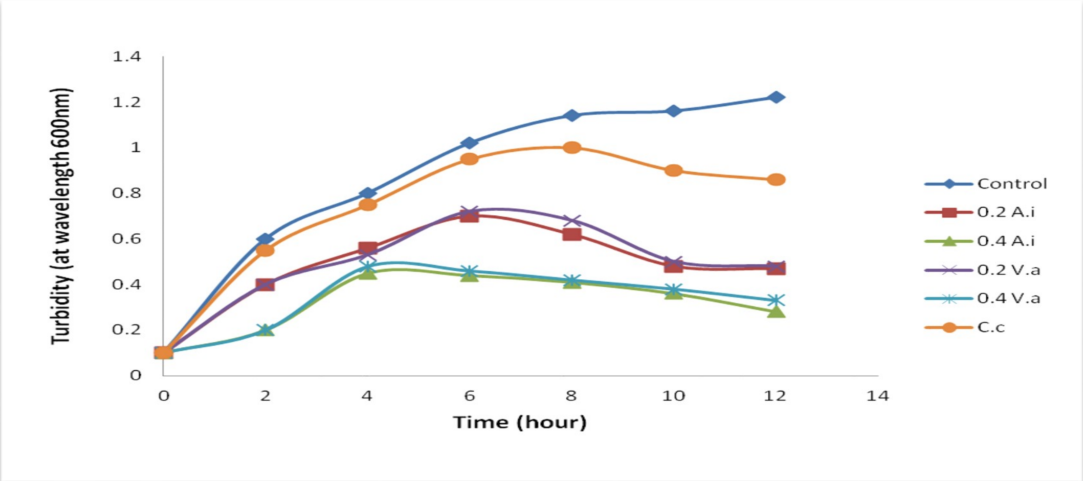

Fig. 4: Growth curves of *Clavibacter michiganensis* F001 exposed to different biosynthesized silver nanoparticles

Note: 0.2 A.i: Silver nanoparticles synthesized using 0.2g/ml Azadirachta indica leaf extract
0.4 A.i: Silver nanoparticles synthesized using 0.4g/ml Azadirachta indica leaf extract
0.2 V.a: Silver nanoparticles synthesized using 0.2g/ml Vernonia amygdalina leaf extract
0.4 V.a: Silver nanoparticles synthesized using 0.4g/ml Vernonia amygdalina leaf extract
C.c: Silver nanoparticles synthesized using C. cladosporioides

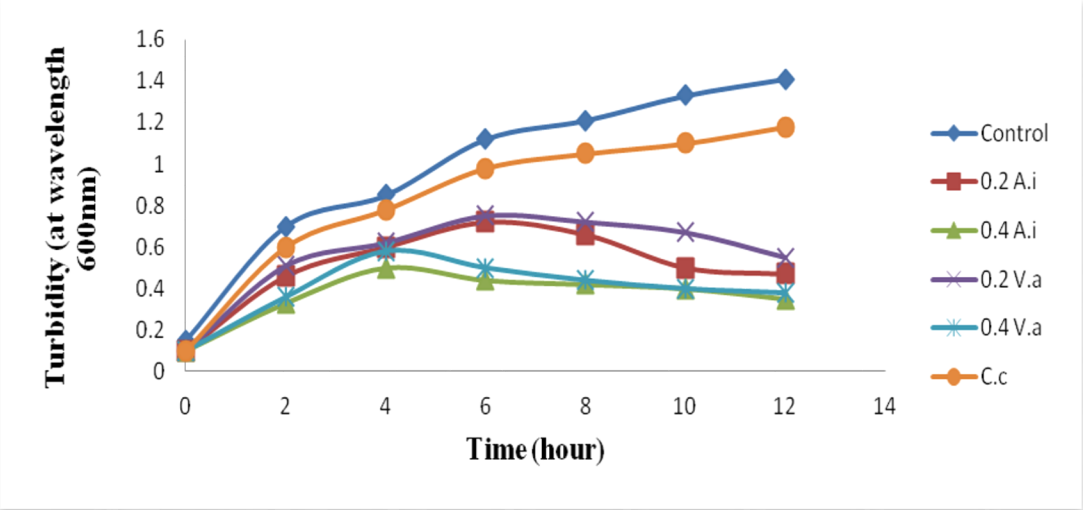

Fig. 5: Growth curves of *Clavibacter michiganensis* F002 exposed to different biosynthesized silver nanoparticles

Note: 0.2 A.i: Silver nanoparticles synthesized using 0.2g/ml Azadirachta indica leaf extract
0.4 A.i: Silver nanoparticles synthesized using 0.4g/ml Azadirachta indica leaf extract
0.2 V.a: Silver nanoparticles synthesized using 0.2g/ml Vernonia amygdalina leaf extract
0.4 V.a: Silver nanoparticles synthesized using 0.4g/ml Vernonia amygdalina leaf extract
C.c: Silver nanoparticles synthesized using C. cladosporioides

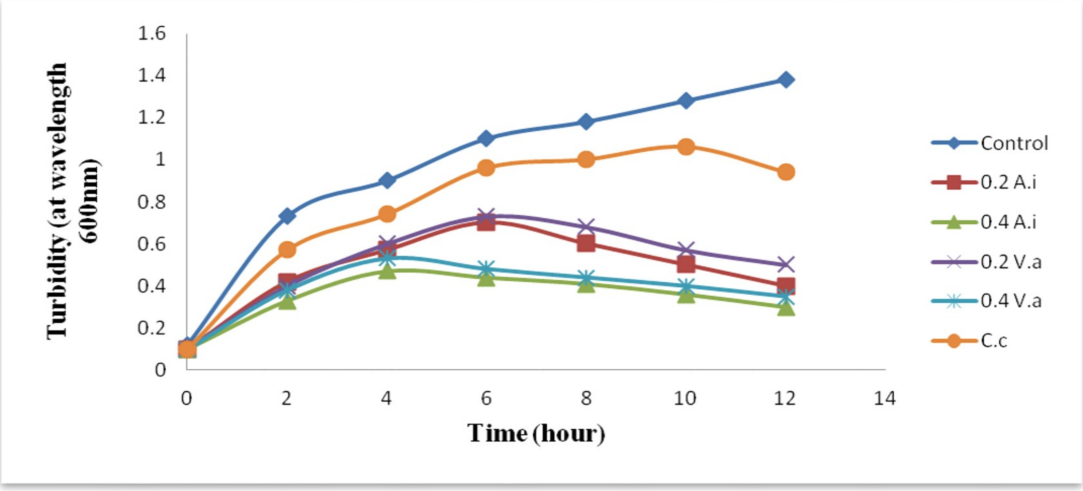

Fig. 6: Growth curves of *Clavibacter michiganensis* F005 exposed to different biosynthesized silver nanoparticles

Note: 0.2 A.i: Silver nanoparticles synthesized using 0.2g/ml Azadirachta indica leaf extract
0.4 A.i: Silver nanoparticles synthesized using 0.4g/ml Azadirachta indica leaf extract
0.2 V.a: Silver nanoparticles synthesized using 0.2g/ml Vernonia amygdalina leaf extract
0.4 V.a: Silver nanoparticles synthesized using 0.4g/ml Vernonia amygdalina leaf extract
C.c: Silver nanoparticles synthesized using C. cladosporioides

ealed that Ag-NPs synthesized using the leaf extracts of *Azadirachta indica* and *Vernonia amygdalina* exhibited strong antibacterial activities against strains of *Clavibacter. michiganensis* subsp. *michiganensis* when compared with those synthesized using *C. cladosporioides* and they may be effective in controlling or reducing the incidence of canker disease caused by these pathogens. Though, there are no studies to our mind on antimicrobial activity of silver nanoparticles on *Clavibacter michiganensis* subsp. *michiganensis*, the results of this study corroborated that of Oloyede *et al.* (2016) who reported that green-synthesized silver nanoparticles effectively inhibited the growth of *Fusarium oxysporum, Fusarium solani* and *Cercospora marcescens in-vitro.* Similar reports were made by Mohamed *et al.* (2014) that green-synthesized silver nanoparticles exhibited good antimicrobial activity against many Gram-negative bacteria such as *Escherichia coli*, *Serratia* sp and *Pseudomonas aeruginosa*. The effectiveness of the biosynthesized Ag-NPs on strains of *Clavibacter michiganensis* subsp. *michiganensis* were different, depending on the type of biological agent used, concentrations of leaf extracts and the strains of the pathogens. In this study, *C. michiganensis* subsp. *michiganensis* F002 showed more tolerance to biosynthesized Ag-NPs than other strains. This observation is in agreement with previous reports that stated that antimicrobial activity of silver ions was different depending on microbial species (Takai *et al*., 2002).

Though the exact mechanism of the antimicrobial activity of silver nanoparticles is still unknown, it has been reported that the activity could be due to the ability of Ag-NPs to attach to the surface of the cell membrane leading to disturbance of permeability and respiration functions of the cell (Slawson, 1992; Mohamed *et al*., 2014). Similarly, silver nanoparticles could affect some proteins and phosphate lipids as well as inducing collapse of membrane, resulting in cell decomposition and eventual

death (Abdullah and Hamid, 2013). Other mechanisms reported include uptake of free silver ions followed by disruption of adenosine triphosphate (ATP) production and DNA replication, formation of reactive oxygen species and direct damage to cell membranes (Mohamed *et al*., 2014). These actions could result in the destruction of pathogenic microorganisms without any detrimental effects on the host tissues.

CONCLUSION AND RECOMMENDATIONS

In conclusion, biosynthesis of silver nanoparticles was obtained by green method using leaf extracts of *Azadirachta indica* and *Vernonia amygdalina*, and filtrate of *Cladosporium cladosporioides*. The study revealed that these leaf extracts and fungal filtrate could be used as effective stabilizing reducing agents for the synthesis of AgNPs. The biosynthesized AgNPs showed strong antibacterial activities against virulent strains of *Clavibacter michiganensis* subsp. *michiganensis* implying that these AgNPs could be effectively used to control these pathogens. These bio-synthesized silver nanoparticles are eco-friendly and may be less toxic to plants, humans and animals than synthetic biocides. Hence, bio-synthesized silver nanoparticles have wide applications in controlling plant diseases.

Further research need to be carried out to determine the effectiveness of bio-synthesized silver nanoparticles in controlling bacterial canker disease of tomato caused by strains of *Clavibacter michiganensis* subsp. *michiganensis* in the fields. Also, it is important to assess the impacts of bio-synthesized silver nanoparticles on the environment and human health when applied in the fields.

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