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Proficiency of biosynthesized silver nanoparticles as a fungicide against selected damping off causing fungi

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Abstract

The present study aimed at studying the fungicidal properties of nano-sized silver particles (AgNPs), biosynthesized from *Nocardiopsis dassonvillei* at 10, 20, 40, 80 and 160 µg ml^{-1} against selected damping off causing fungi. Silver nanoparticles (AgNPs) were characterized by energy dispersive spectroscopy (EDS) and transmission electron microscopy (TEM). TEM micrographs revealed extracellular formation of spherical nanoparticles with an average size of 25.8 nm. All the tested fungi were highly inhibited by the biosynthesized AgNPs. Its effective concentrations inhibiting 50% of the fungal growth (EC_{50}) values on *F. solani*, *A. alternata*, *F. oxysporum* and *R. solani* were 1.25, 3.23, 4.3 and 9.2 µg ml^{-1}, respectively. The present study proved that biosynthesized AgNPs as a promising fungicide against the treated fungi.

Key words

Alternaria, Damping off, Fungicide, *Fusarium*, *Rhizoctonia*, Silver nanoparticles

Introduction

The incidence of fungal infection has increased and spread among human, animals and plants. Damping off disease is a serious problem caused by several soil-borne fungi infecting seedlings of most plants, causing death. Several studies have been carried out on the fungi responsible for damping off disease (Yaqub and Shahzad, 2005; Abdel-Fattah *et al.,* 2011; Alwathnani and Perveen, 2012). The most important means of protecting plants against phytopathogenic fungi is the use of synthetic fungicides that are poisonous with harmful effects on non-target biota; hence, through synthesis of fungicides at nano scale, non-hazardous and eco-friendly fungicides could be produced for protecting plants from fungal diseases (Pérez-de-Luque and Rubiales, 2009; Choudhury *et al.,* 2010). Thus, far insufficient studies on the potential of silver nanoparticles as fungicides are carried out and more research is urgently required (Roe *et al.*, 2008). Initial evidence suggest that silver, at nano-scale, possesses environmentally safe antifungal activity against plant pathogenic fungi when compared to synthetic fungicides (Park *et al.*, 2006). Comparing with chemical synthesis, the biological synthesis of nanoparticles is more effective and eco-friendly as use of toxic solvents and hazardous reducing agents in the chemical synthesis (Mukherjee *et al.*, 2008). Biosynthesis and characterization of silver nanoparticles (silver nano particle) by *Streptomyces* sp. BDUKAS10, isolated from mangrove sediment were reported (Sivalingam *et al.*, 2012).

Strong antifungal activity of silver nanoparticles inhibiting *Botrytis cinerea* growth has been reported (Oh *et al.*, 2006). *In vitro* and *in vivo* treatment with both silver ion and silver nanoparticles have been found to decrease the spread of disease by plant pathogenic fungi (Jo *et al.*, 2009). Silver nanoparticles significantly inhibited sclerotic germination of plant pathogenic fungi (Min *et al.,* 2009). In South Korea, silver nanoparticles are considered as effective antifungal agents against filamentous ambrosia fungi (Kim *et al.*, 2009). Silver nanoparticles have inhibitory effect on growth of *Fusarium culmorum* (Kasprowicz *et al.* 2010) and *F. oxysporum* (Musarrat *et al.* 2010). In light of the above, the present study aimed to biosynthesize silver

nanoparticles by *Nocardiopsis dassonvillei* and evaluate their efficiency on growth inhibition of *Alternaria alternata*, *Fusarium oxysporum, Fusarium solani* and *Rhizoctonia solani* as phytopathogenic fungi.

Materials and Methods

Isolation of *Nocardiopsis dassonvillei* from soil : Soil samples were collected from Eldrieh, Riyadh, Saudi Arabia and stored in sterile air locked polyethylene bags at 4 ºC. A suspension of soil sample was inoculated on starch casein agar medium supplemented with antibiotics such as cycloheximide (40 g l^{-1}), nystatin (30 g l^{-1}) and nalidixic acid (10 g l^{-1}). The plates were incubated at 30 ºC until the appearance of colonies with a tough leathery texture, dry or folded appearance and branching filaments with aerial mycelia. Pure colonies were isolated and subcultures were carried out by striking particular isolate directly onto ISP-2 agar medium.

Extraction of DNA andamplification of 16S rDNA and its sequencing : Total genomic DNA of the actinomycete isolate was extracted according to Smoker and Barnum (1988) with slight modification. The 16S ribosomal DNA gene was amplified by PCR using universal primer pair; F27, 5'-AGAGTTTGATCMTGGC TCAG-3' and R1492, 5' TACGGYTACCTTGTTACGACT-3'). The amplified products were analysed by GATC Biotech, European Custom Sequencing Centre, D-51105 Cologne, Germany. DNA sequence analysis was then performed by BLAST network services at the National Center for Biotechnology Information (Thompson *et al.,* 1997).

Biosynthesis and characterization of silver nanoparticles: Biosynthesis of nanoparticles was carried out according to Li *et al.* (2012). The isolate was further cultured on ISP-2 medium (pH 7.2) and grown for 72 hr at 30 ºC at 220 rpm. Cell filtrate was obtained with 10,000 rpm centrifugation for 10 min at 4 °C. For bio-reduction process to occur, $AgNO_3$ (Qualigens 99.8%) was added at 1 mM to 100 ml cell filtrate and incubated at 30 °C in dark for 48 hr, followed by initial observation of colour change in the bio-reduction process. UV–visible spectrometric analysis was performed on Lambda 35 UV-VIS spectrophotometer at 210-800 nm wave length range. The bio-transformed products present in cell-free filtrate after 72 hr of incubation were freeze-dried and diluted with distilled water. For energy dispersive spectroscopy (EDS), a sample was prepared on copper substrate by drop coating silver nanoparticles which was carried out using a JEOL (JSM-6380 LA) equipped with scanning electron microscope. Transmission electron microscope was performed on a JEOL (JEM-1010) instrument, with an accelerating voltage of 80 kV, after drying a drop of aqueous silver nano particle on the carbon-coated copper TEM grids. Samples were dried and kept in vacuum in desiccators before loading them onto a specimen holder. The particle size distribution of silver nanoparticles was evaluated using Image J 1.45s software.

Antifungal activity of silver nano particle : Screening of antifungal effect of the microbiologically synthesized silver nano particle was performed using radial growth test according to the conventional method (Torgeson, 1967) at 10 µg ml^{-1}. In the disk inhibition zone method, Capex-Dox medium was inoculated with freshly prepared cells of *F. solani, A. alternata*, *F. oxysporum* and *R. solani.* Treated phytopathogenic fungi were brought from the Plant Pathology Laboratory, College of Food and Agricultural Sciences, King Saud University and were grown on *Capex*-Dox *medium*. Control was concurrently carried out by dipping the sterilized disks into water before placing in the centre of Petri-dishes. The treatments were replicated three times. After incubation at 28^{0}C for 96 hr, the antifungal effect of biosynthesized silver nanoparticles was determined by measuring the inhibition zone compared with the control treatment.

On the other hand, 25 ml of *Capex*-Dox *medium* was inserted with silver nano particle at 10, 20, 40, 80 and 160 µg ml^{-1} in 100 ml in sterile conical flasks. A *Capex*-Dox *medium* without silver nano particle was kept as control. Fresh fungal inoculum was placed on the medium surface. Three replicates were used for each treatment and incubated at 26°C until the un-treated fungus hyphal growth filled the medium surface (seven days inoculation). The grown fungi were filtered, dried instantly and their weights were measured. Percentage inhibition of the hyphal growth were also calculated (Topps and Wain, 1957).

Statistical analysis : Antifungal activity was carried out in triplicate and all data (average of the three values) were analysed using Statistical Analysis Software programme (Cary, 1988). EC_{50} values as g ml^{-1} (the effective concentration caused 50% inhibition in the hyphal growth) were determined for each fungus using Probit Analysis Method (Finney, 1971).

Results and Discussion

The 16S rRNA gene sequencing showed 1400 nucleotides. GB, the isolate was found to have a similarity of 99% with *Nocardiopsis dassonvillei* (Accession No: KP148313) identifying the isolate as *Nocardiopsis dassonville*ess_am2. The results also showed that after mixing with silver nitrate, the cell filtrate changed incolour, indicating biosynthesis of silver nano-particles by *Nocardiopsis dassonvillei* cell filtrate. The role of NADH dependent nitrate reductase from fungi in silver nanoparticles biosynthesis has recently been reported (Kumar *et al.,* 2007; Bai *et al.,* 2011); however, different NADH-dependent reductases may also be produced by *Nocardiopsis dassonvillei*. The results showed different sizes of nanoparticles, mostly spherical in shape, but aggregated to a lesser extent (Fig. 1, 2). There were four distinct sizes of silver nanoparticles synthesized by *Nocardiopsis dassonvillei*. The average size of silver

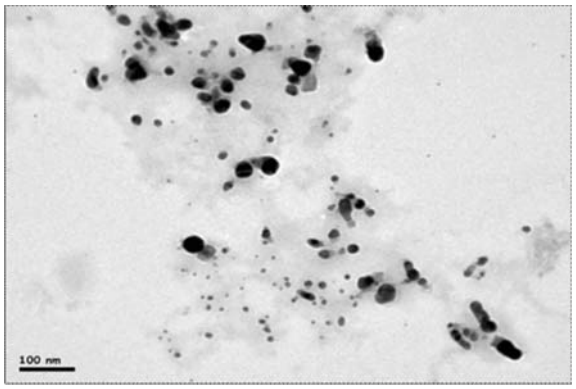

Fig. 1 : Biosynthesized silver nanoparticles under transmission electron microscope at scale bar = 100 nm

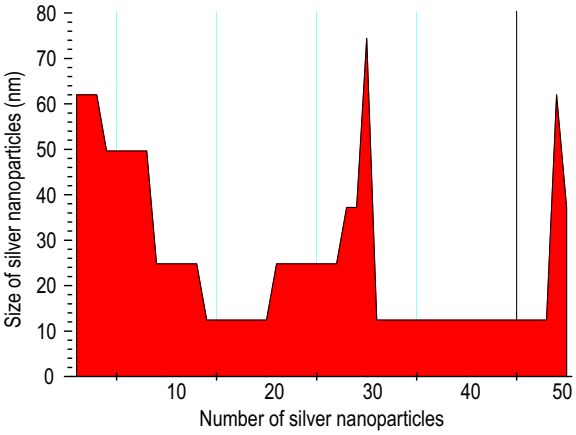

Fig. 2 : Distribution of silver nanoparticles sizes in the microscopically observed nano-sample, using ImageJ 1.45S software

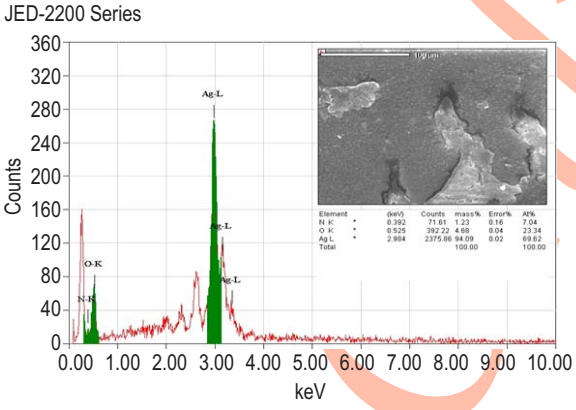

Fig. 3 : Elemental analysis of biosynthesized silver nanoparticle sample by energy dispersive X-ray spectroscopy

nanoparticles was 25.8nm. The most prevalent size of distribution in nanoparticles was 12nm. Saifuddin *et al.* (2009) biosynthesised silver nanoparticles from *Bacillus subtilis* using microwave radiation. Nanoparticle sizes of 20–30 nm produced by *Streptomyces* culture was also reported (Sadhasivam *et al.,* 2010; Maged *et al.,* 2014). Different size distribution of nanoparticles may be due to difference in reductase produced by *Nocardiopsis dassonvillei* or the effects of other proteins coating the nanoparticles. The size distribution of nanoparticles is very important for application purposes and smaller nanoparticles are more effective as antimicrobials against pathogens (Maged *et al.* 2014; Elechigurra *et al.,* 2005).

The results of energy dispersive spectroscopy for silver nanoparticles confirmed the presence of elemental silver signal. As shown in Fig.3, spectrum of nanoparticles sample showed mainly silver particles (94.1%) with only minor amount of other elements (5.9%) and these results is in agriment with results of Magudapathy *et al.* (2001). Signals for N and O indicated the presence of proteins as a capping material on the surface of silver nanoparticles.

The obtained results showed that the biosynthesized fungicide affected linear growth of tested fungal isolates with significant variation. The growth of all the fungal isolates were highly inhibited once they treated with biosynthesized silver nanoparticle at concentations of 10, 20, 40, 80, 160 µg ml^{-1} (Table 1 and Fig. 4) and these results are agreeing with results of Ali *et al*. 2015, Pulit *et al.* 2013 and Kim *et al.* 2012. Silver nanoparticles were most effective against *F. solani* followed by *A. alternata*, *F. oxysporum* and *R. solani*. In addition, the results indicated a significant difference in the inhibition rate at 10 and 20 µg ml^{-1}, while at 40, 80 and 160 µg ml^{-1}, no significance was noted. The effective concentration inhibited 50% of hyphal growth (EC_{50}) against *F. solani*, *A. alternata*, *F. oxysporum* and *R. solani* and was 1.25, 3.23, 4.3 and 9.2 µg ml^{-1}, respectively. Recently, more efforts have been focused on research into eco-friendly methods for producing fungicides at nanoscale.

Regarding the mode of action of silver nanoparticles, swollen fungal hyphae, aggregation of cellular content, cell vacuolation and cell disintegration symptoms were observed (Fig.5). This could be due to high density at which the solution was able to saturate and cohere to the fungal hyphae. silver nano particle may also affect cell membrane and DNA, leading to the observed changes in hyphal morphology. Silver ions affects DNA its ability to replicate (Yamanaka *et al.*, 2005) making it lose by deactivating gene expression.

In conclusion, this study indicated *Nocardiopsis dassonvillei* as a suitable candidate for biosynthesis of silver nanoparticle at an average size of 25.8nm.using silver nitrate. Furthermore, it was concluded that silver nanoparticle have a pronounced inhibitory effect on the fungal species responsible for

Table 1 : Effect of biosynthesized silver nanoparticles against damping off responsible fungi shown as percent inhibition and EC_{50} values

Treated fungi	Inhibition % at different concentrations						EC_{50}(95% C.L)	Slope± SE	c^2
	Control	10	20	40	80	160			
F. solani	0	96.7	100	100	100	100	1.25		
F. oxysporum	0	77.3	84	93	100	100	4.3(2.5 – 7.2)	1.71± 0.076	4.6
R. solani	0	60	75	100	100	100	9.2(7.5 –11.1)	2.95± 0.143	10.8
A.alternata	0	85	90	95	100	100	**3.23**(1.0 – 2.4)	1.51±0.095	3.2

The tested concentrations and EC_{50} values are in µg ml^{-1} Degree of freedom = 3

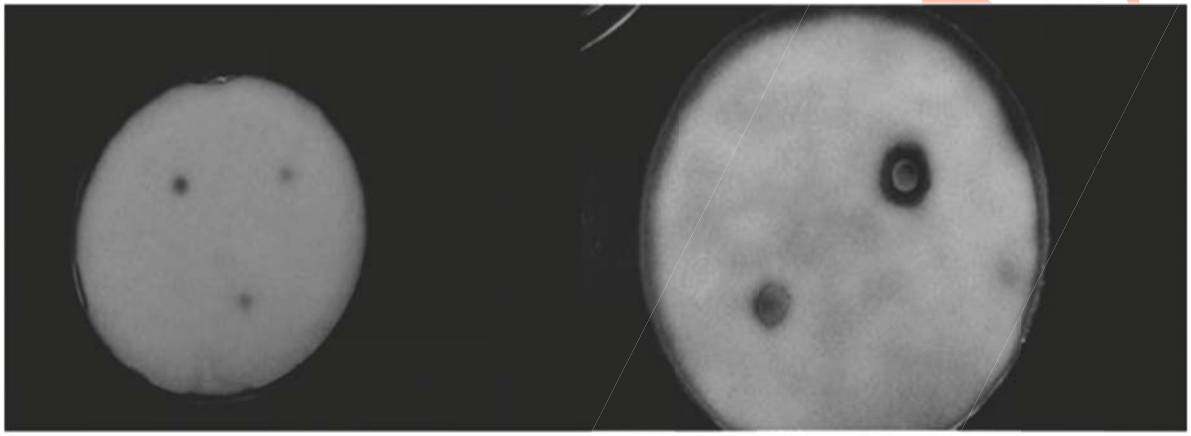

Fig. 4 : Antifungal activity of biosynthesized silver nanoparticles on *F. solani* (inhibition zone) using agar diffusion method

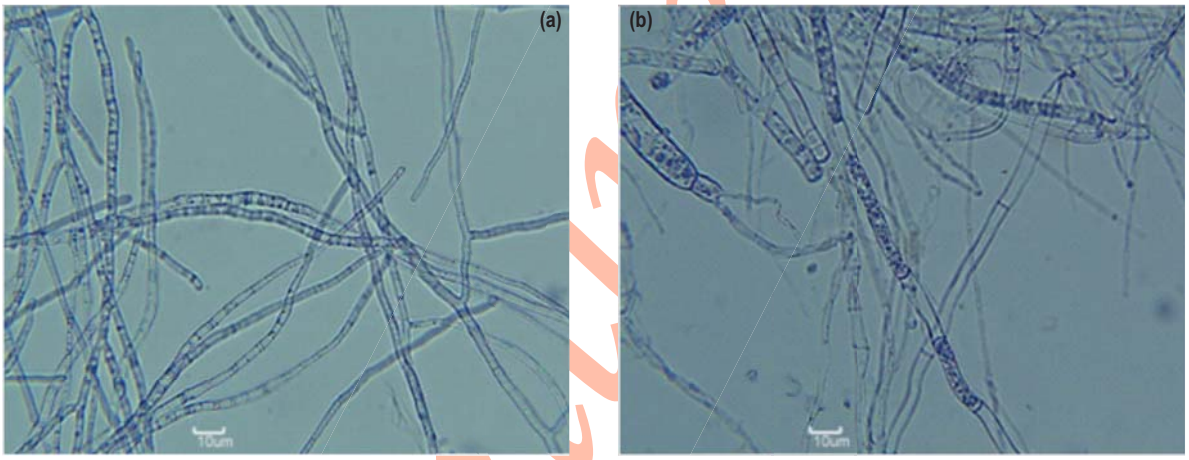

Fig. 5 : Effect of silver nanoparticles on (a) control hyphae and (b) silver nano particle treated *F. oxysporum* hyphae (Scale bar = 10 µm)

damping off disease, suggesting potential role as a fungicide against *F. solani*, *F. oxysporum*, *R. solani* and *A. alternata*.

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