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Antifungal Activity of Silver and Copper Nanoparticles on Two Plant Pathogens, *Alternaria alternata* and *Botrytis cinerea*

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ABSTRACT

In this study, we investigated the antifungal activity of silver (AgNPs), copper (CuNPs and silver/copper (Ag/CuNPs) nanoparticles against two plant pathogenic fungi *Alternaria alternata* and *Botrytis cinerea*., which have been responsible for the damage of a large number of plants. Metals nanoparticles were applied at various concentrations to determine antifungal activities *in vitro*. The application of 15 mg L^{-1} concentration of silver nanoparticles produced maximum inhibition of the growth of fungal hyphae. We also assessed the effectiveness of combining the silver and copper nanoparticles. Microscopic observation revealed that nanoparticles caused a damage effects on fungal hyphae and conidia. In addition to Ag-nanoparticles had a detrimental effect on sugar, protein, n-acetyl glucosamine and lipid of culture filtrate and cell wall components of both plant pathogens.

Key words: Metal nanoparticles, silver nanoparticles, antifungal activity, action mechanism, *Alternaria* sp., *Botrytis cinerea*

INTRODUCTION

In order to control various phytopathogenic fungi, agrochemicals have been used for a long time. Widespread use of agrochemicals has certainly decreased the outbreak of fungal diseases but at the same time has contributed to the development of resistant pathogens (Lamsal *et al*., 2011a). Moreover, such chemicals can be lethal to beneficial microorganisms in the rhizosphere and useful soil insects and they may also enter the food chain and accumulate in the human body as undesirable chemical residues (Bartlett *et al*., 2002). With the emergence and increase of microbial organisms resistant to multiple antibiotics and the continuing emphasis on health-care costs, many researchers have tried to develop new and effective antimicrobial reagents that do not stimulate resistance and are less expensive (Lamsal *et al*., 2011a).

Currently, the metallic nanoparticles are thoroughly being explored and extensively investigated as potential antimicrobials. The antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms, the small size and the high surface to volume ratio i.e., large surface area of the nanoparticles enhances their interaction with the microbes to carry out a broad range of probable antimicrobial activities (Martinez-Gutierrez *et al*., 2010).

One of the potential applications of silver is in management of plant diseases. Silver displays multiple modes of inhibitory action against microorganisms; therefore, it may be used with relative

safety for control of various plant pathogens (Jung *et al*., 2010; Lamsal *et al*., 2011b; Kim *et al*., 2012). Silver is known to attack a broad range of biological processes in microorganisms including cell membrane structure and functions (Pal *et al*., 2007). Silver also inhibits the expression of proteins associated with ATP production (Yamanaka *et al*., 2005). Understanding of biological processes on the nanoscale level is a strong driving force behind development of nanotechnology (Taton *et al*., 2003; Gajjar *et al*., 2009).

In this study, we have demonstrated the inhibitory action of silver, copper and silver/copper nanoparticles against two plant pathogens; *Alternaria alternata* and *Botrytis cinerea* under different concentrations in growth medium, as well as a trial for understand the control mechanisms of those metallic nanoparticles on the two tested plant pathogens.

MATERIALS AND METHODS

Metal nanoparticles: Three different types, silver (AgNPs), copper (CuNPs) and silver-copper (Ag/CuNPs) nanoparticles which were provided by Chemistry Department, Science Faculty, Taif University, KSA. TEM showed that the particle in single Ag on NaY zeolite was spherical with particle size of ~38 nm. However, its shape changed to rode shape after alloying with Cu with diameter of ~20 nm and length of ~0.3 μm. Metal nanoparticles were diluted at different concentrations of (i.e., 1, 5, 10 and 15 mg L^{-1}) using distilled water. All solutions were stored at 4°C until further use (Kim *et al*., 2012).

Microorganisms and culture conditions: *Alternaria alternata* and *Botrytis cinerea* have been isolated from infected tomato and strawberries fruits, respectively. They were purified and identified according to Gilman (1967). The selected fungal isolates are commercially important and cause various diseases on vegetables, fruits and crop plants. These fungi were grown on Potato Dextrose Agar (PDA) for further experimentation.

Antifungal activity: Antifungal activity of nanoparticles was carried out on PDA media. It were supplemented with 0, 1, 5, 10 and 15 mg L^{-1} of silver (AgNPs), copper (CuNPs) and silver/copper nanoparticles (Ag/CuNPs), separately, an agar plug of 8 mm diameter containing the tested fungi was inoculated simultaneously at the center of each Petri dish and incubated at 28±2°C. Growth diameter was measured after 2, 3, 5 and 6 days of incubation, each treatment were replicated.

Scanning electron microscopy (SEM): The hyphae morphological changes of *Alternaria alternata* and *Botrytis cinerea* by silver nanoparticles (Ag-NPs), copper nanoparticles (Cu-NPs) and silver/copper nanoparticles (Ag/Cu-NPs) at 15 mg L^{-1} were observed with a Scanning Electron Microscope (SEM). Fungal isolates were prepared by cutting the agar after 7 days incubation periods, fixed for a minimum of 3 h in 2.5% (v/v) glutaraldehyde (100 mM phosphate buffer solution, pH 7.2) and then fixed in1% (w/v) osmium tetra oxide for 1 h. The agar blocks were dehydrated through a graded series of ethanol and ethanol: isoamyl acetate. The agar blocks on grid were dried with a critical-point drier using liquid CO_2 and coated with gold-coater for 5 min. The coated samples were observed under JSM-5600 LV with accelerating voltage of 10 kV.

Antifungal effect of silver nanoparticles on culture filtrate and cell wall components of plant pathogens: For assessing the antifungal activity of silver nanoparticles on fungal components in both treated culture media and cell wall, the two fungi

Alternaria alternata and *Botrytis cinerea* were grown on treated and untreated PDA media for 5 days. The changes of culture and fungal cell wall components under the effect of 15 mg L^{-1} AgNPs were compared with control. The total sugars (Miller, 1959); N-acetyl glucosamine, NAG (Chen and Johnson, 1983); total protein (Lowry *et al.*, 1951) and total lipids (Knight *et al.*, 1972) were measured in both culture filtrate and fungal cell walls in treated and untreated plant pathogens.

RESULTS

Antifungal activity of nanoparticles: The inhibition effect of AgNPs, CuNPs and Ag/CuNPs at different concentrations was estimated against *Alternaria alternata* (Table 1, Fig. 1a) and *Botrytis cinerea* (Table 2, Fig. 1b) for different incubation periods. In all cases, a higher inhibition

Table 1: Antifungal activities of silver, copper, silver/copper nanoparticles against *Alternaria alternata* at different concentrations (mg L^{-1}) and for different incubation periods

		Fungal growth diameter (mm)											
Incubation periods		Silver nanoparticles (Ag NPs)				Copper nanoparticles (Cu NPs)				Silver/copper nanoparticles (AglCu NPs)			
(days)	Control	1	5	10	15	1	5	10	15	1	5	10	15
2	28.5±0.5	27.0±0.0	26.0±1.0	20.0±0.0	18.0±0.0	27.5±0.5	26.0±0.0	27.0±0.2	27.0±0.1	28.5±0.5	28.5±0.5	28.0±1.0	21.5±0.5
3	38.5±0.5	33.5±0.5	33.0±2.0	25.5±0.5	21.5±0.5	38.0±0.0	38.0±0.5	37.0±0.0	38.5±0.5	33.0±0.0	38.5±0.5	38.5±0.0	30.0±0.5
5	64.5±0.5	59.0±0.2	58.5±0.5	32.5±0.5	29.5±0.5	64.5±0.5	64.5±0.5	62.5±0.5	62.5±0.5	68.0±1.0	67.5±0.5	68.5±0.5	43.0±1.0
6	80.0±0.0	77.0±0.0	67.0±0.2	38.0±0.0	32.5±0.5	80.0±0.0	80.0±0.0	70.5±0.0	69.0±0.0	79.0±0.0	78.0±0.0	78.0±0.0	49.5±0.0

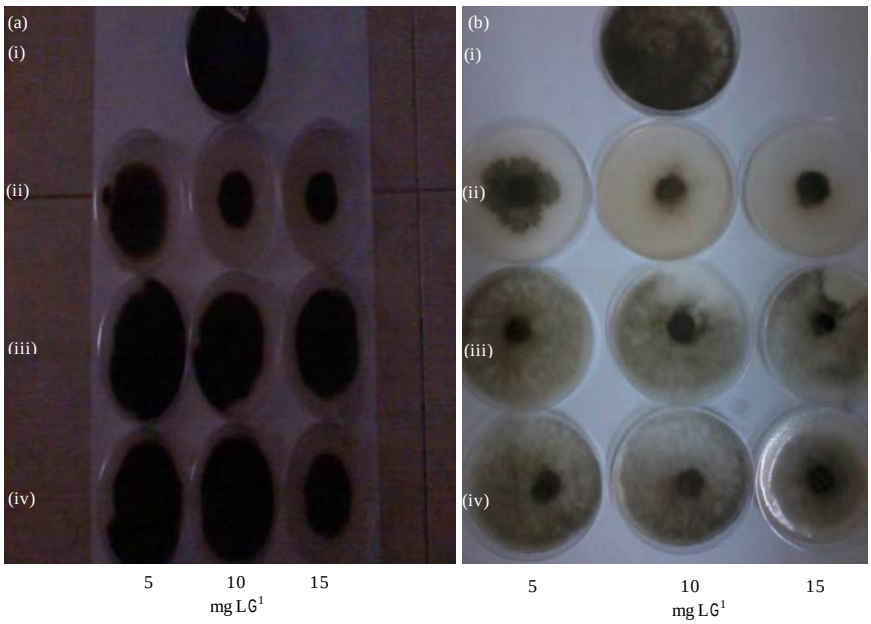

Fig. 1(a-b): Antifungal activities of (i) Control, (ii) Ag-NPs, (iii) Cu-NPs and (iv) Ag/Cu-NPs nanoparticles against (a) *Alternaria alternata* and (b) *Botrytis cinerea* at different nanoparticles concentrations (5, 10, 15 mg L^{-1}) after 7 days of incubation periods

Table 2: Antifungal activities of silver, copper, silver/copper nanoparticles against *Botrytis cinerea* at different nanoparticles concentrations (mg L^{-1}) and for different incubation periods

Incubation periods (days)	Fungal growth diameter (mm)												
	Control	Silver nanoparticles (Ag NPs)				Copper nanoparticles (Cu NPs)				Silver/copper nanoparticles (Ag/Cu NPs)			
		1	5	10	15	1	5	10	15	1	5	10	15
2	34.0±1.0	29.5±0.5	25.0±0.0	28.0±2.0	21.0±0.0	29.0±0.5	25.5±0.5	25.0±0.0	25.0±0.0	34.0±0.0	34.5±0.5	33.0±1.0	25.0±0.0
3	52.0±1.0	39.0±0.5	34.0±2.0	35.5±3.0	29.5±0.5	45.0±2.0	45.5±0.5	45.0±1.0	45.0±0.0	52.0±0.5	52.5±0.5	53.5±0.5	34.0±1.0
5	79.0±0.0	79.0±0.0	51.5±0.5	45.0±0.0	35.5±0.5	79.0±0.0	79.0±0.0	79.0±0.0	75.0±0.5	79.5±0.5	78.5±0.5	78.5±0.5	56.0±1.0
6	85.0±0.0	81.0±0.0	55.0±0.0	49.0±0.0	40.0±0.0	85.0±0.0	82.0±0.0	82.0±0.0	81.0±0.0	85.0±0.0	85.0±0.0	85.0±0.0	62.0±1.0

of fungal growth was recorded at a concentration of 15 mg L^{-1} of three tested nanoparticles and the two fungi showed growth inhibition with the increment of incubation time. AgNPs treatment with 15 mg L^{-1} concentration resulted in about 59.3 and 52.9% inhibition percentage with *Alternaria alternata* and *Botrytis cinerea*, respectively. The lowest level of inhibition against both fungi was observed with a concentration 15 mg L^{-1} of CuNPs treatments. While, addition of copper with silver to form a mixture of Ag/Cu nanoparticles lower the inhibition precentage to level 38.12 and 27.0% against *Alternaria alternata* and *Botrytis cinerea*, respectively compared with AgNPs treatment at a concentration 15 mg L^{-1}. Therefore, the results suggested that maximum inhibition was obtained in both fungal isolates treated with 15 mg L^{-1} concentration of silver nanoparticles.

Scanning electron microscopy (SEM): In order to elucidate the inhibition effect of silver, copper and silver copper nanoparticles on the two fungal isolates *Alternaria alternata* and *Botrytis cinerea*. Both healthy and treated fungal hyphae grown on PDA plates, were observed by SEM. The microscopic observation revealed that the three types of nanoparticles clearly damaged the hyphae of *Alternaria alternata* compared with control (Fig. 2a). Fungal mycelia were damaged due to the effect of silver nanoparticles and it showed deformities in conidia (Fig. 2b).

In Cu nanoparticles treatment, many hyphae were collapsed and there was metal precipitations on a hyphal walls (Fig. 2c). Also in Ag/Cu nanoparticle treatments, the layers of hyphal walls were also tore off and there were damaged hyphae in addition to low sporulation was observed (Fig. 2d).

In order to visualize the microscopic effect of the three nanoparticles treatments on Botrytis cinerea, healthy fungal hyphae grown on PDA plates (Fig. 3a) were compared with other plates that were supplemented with 15 mg L^{-1} of Ag, copper and Ag/Cu nanoparticles separately and then observed under an electron microscope (Fig. 3b-d). Hyphae in control appear to have remained intact (Fig. 3a), while there were damage to the surface of the fungal hyphae was observed, which could have caused the release of internal cellular materials, resulting in shrinkage of the hyphae. In addition to the deformities in conidia (Fig. 3d).

Antifungal effect of silver nanoparticles on culture filtrate and cell wall components of plant pathogens: Antifungal activity of silver nanoparticles on different fungal filtrate and cell wall components of two plant pathogens were investigated (Table 3). In *Alternaria alternata* the changes of released component in culture filtrate and cell wall components after incubation for 5 days were studied, there was found that a great reduction in both total sugar and total protein

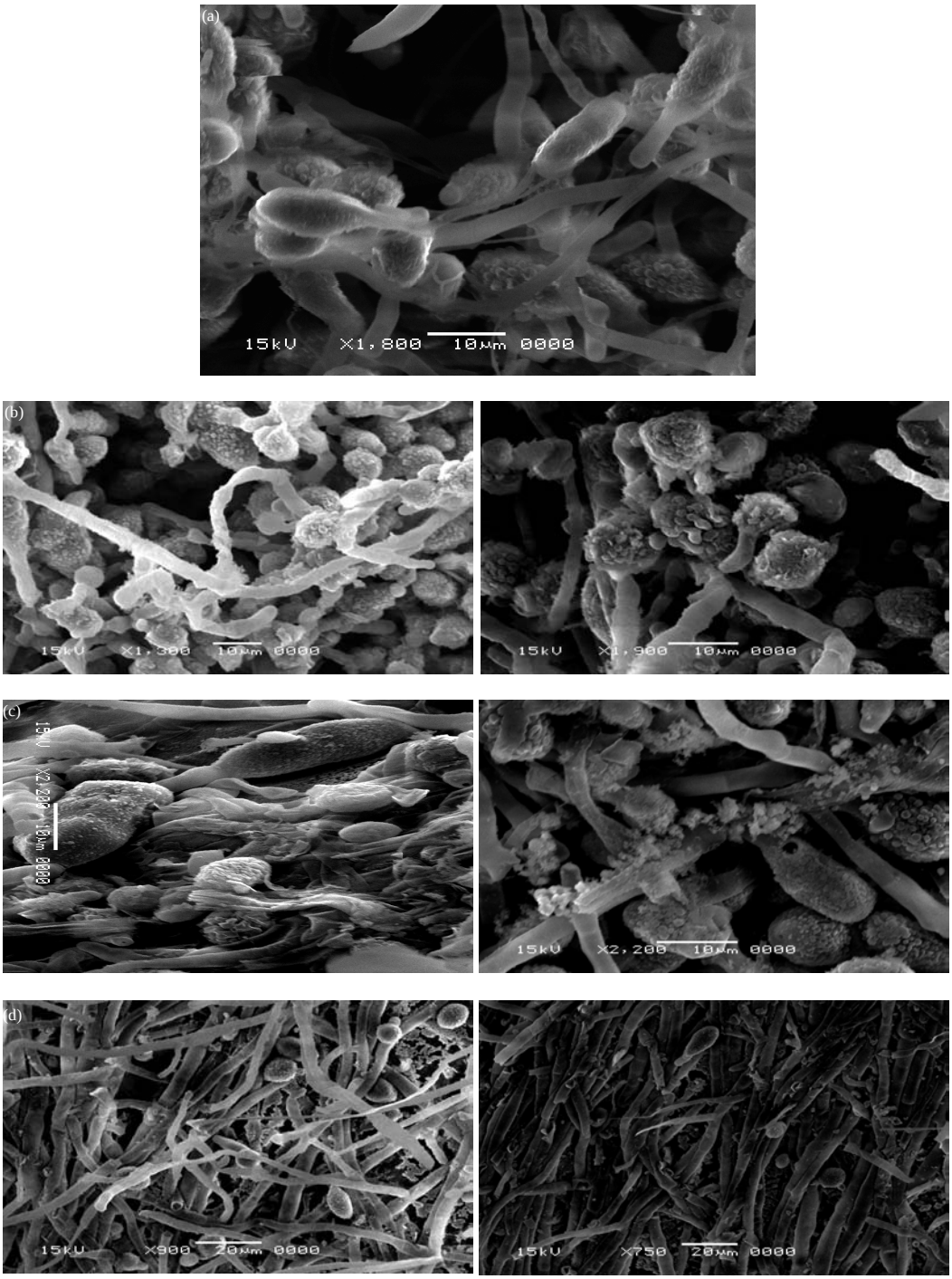

Fig. 2(a-d): Antifungal effect of silver nanoparticles on culture filtrate and cell. Scanning electron microscopy of hyphae of *Alternaria alternata* treated with silver, copper or copper/silver nanoparticles. Fungal hyphae grown on potato dextrose agar plates as (a) Control or supplemented with 15 mg L^{-1}, (b) Silver, (c) Copper or (d) Silver/copper nanoparticle solution, respectively, Photos were taken at seven days after the incubation period

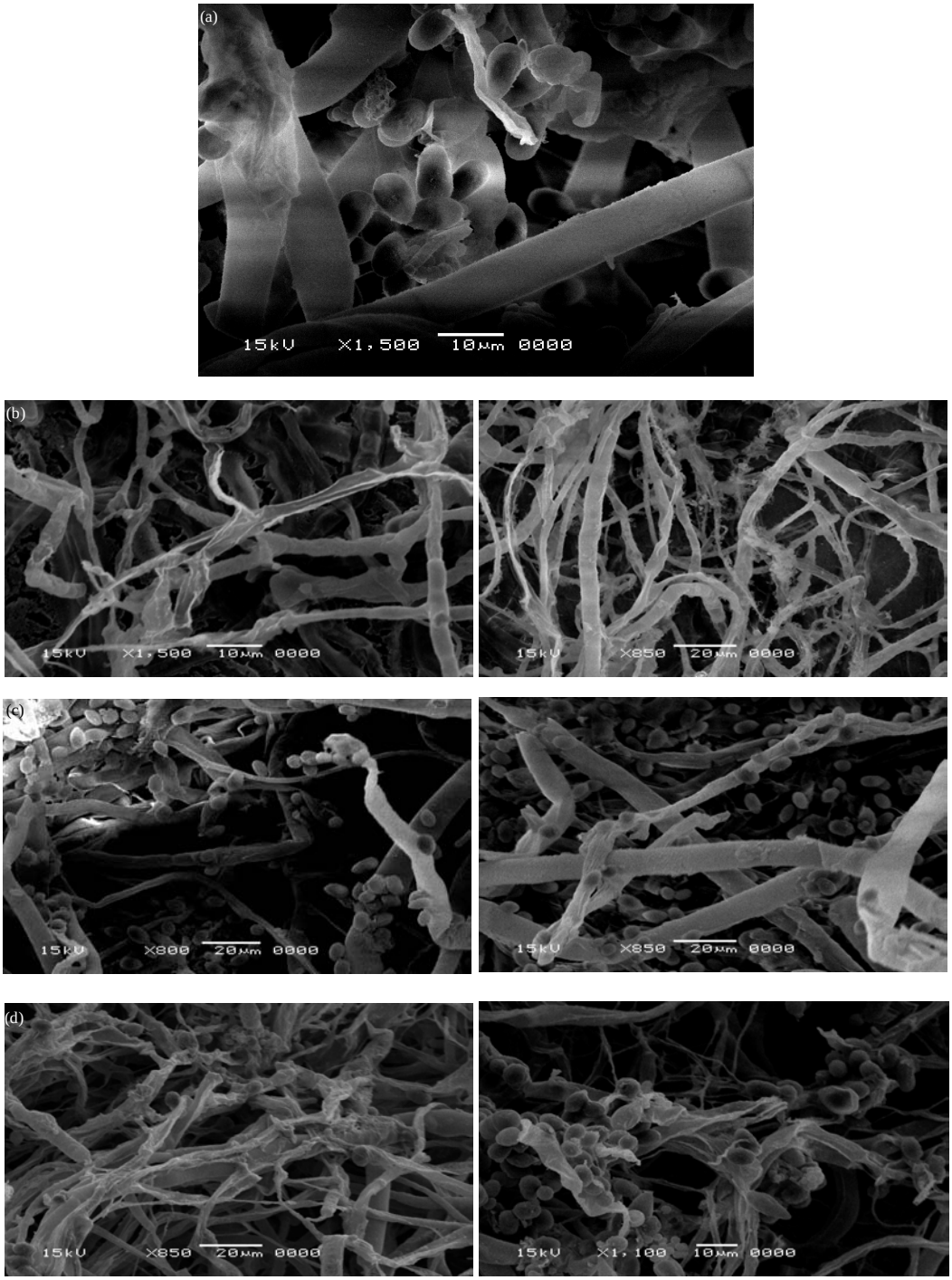

Fig. 3(a-d): Antifungal effect of silver nanoparticles on culture filtrate and cell. Scanning electron microscopy of hyphae of *Botrytis cinerea* treated with silver, copper or copper/silver nanoparticles. Fungal hyphae grown on potato dextrose agar plates as (a) Control or supplemented with 15 mg L^{-1}, (b) Silver, (c) Copper or (d) Silver/copper nanoparticles, respectively, Photos were taken at seven days after the incubation period

Table 3: Antifungal activity of silver nanoparticles on different fungal filtrate and cell wall components of both plant pathogens

		Component released (mg mL^{-1})						
		In filtrate			In cell wall			
Plant pathogen	Treatment	Total sugar	Total protein	Total lipids	Total sugar	Total protein	Total lipids	NAG (µg mL^{-1})
Alternaria alternata	Control	612±1.2	18.50±1.2	9.10±0.4	770±1.2	20.5±0.0	7.35±0.0	10.88±0.0
	Ag NPs	104±1.5	4.90±0.0	7.17±0.5	148±1.4	5.1±0.0	19.30±0.5	8.40±0.0
Botrytis cinerea	Control	638±1.5	20.90±0.0	16.10±0.0	812±3.5	17.9±0.0	9.86±0.0	15.41±0.0
	Ag NPs	644±2.3	7.91±0.0	28.50±1.2	664±2.3	9.8±0.0	15.20±0.0	7.06±0.0

while a slightly reduction in lipids compared with control. In concern to cell wall components, there were also reduction in sugar, protein and N-acetyl glucoseamine (NAG) while there was a distinctive increasing in lipids.

Silver nanoparticles effect on different components of cultural filtrate and cell wall in *Botrytis cinerea* was also studied. There was a highly reduction in total protein of culture filtrate and total protein and NAG of cell wall, while there were a great increasing in total lipids for culture filtrate and cell wall.

DISCUSSION

Management of fungal diseases on food crops and ornamental plants is economically important. Recently, more efforts have been given to develop safe management methods that pose less danger to humans and animals and have focused on overcoming deficiencies of synthetic fungicides. The current investigation showed that metals nanoparticles with low toxicity and a broad spectrum of antimicrobial activity were also very effective for reducing plant diseases caused by spore producing phytopathogenic fungi. Results presented in this study investigate the inhibitory effect of silver, copper and copper/silver nanoparticles on growth of *Alternaria alternata* and *Botrytis cinerea*. The sensitivities of the two fungal species to silver, copper and silver/copper nanoparticles were different. The obtained results of the antifungal activity clearly reveal that the growth of *Alternaria alternata and Botrytis cinerea* were inhibited at different concentrations of silver and silver/copper nanoparticles. The growth of both plant-pathogenic fungi was highly influenced at 15 mg L^{-1} AgNPs. This study was the first to demonstrate that the inhibitory effect of silver, copper and a mixture of them against *Alternaria alternata* and *Botrytis cinerea*. Previous reports stated that, antimicrobial activity of silver was different depending on microbial species (Galeano *et al*., 2003; Takai *et al*., 2002). Silver nanoparticles can significantly delay mycelial growth in a dose-dependent manner *in vitro* (Min *et al*., 2009; Aguilar-Mendez *et al*., 2011). silver nanoparticles may directly attach to and penetrate the cell membrane to kill spores, although penetration of silver nanoparticles into microbial cell membranes is not completely understood (Hwang *et al*., 2008).

The highest antifungal properties were observed in the case of treatment with 15 mg L^{-1} silver, silver/copper and copper nanoparticles, respectively. Therefore, the microscopic observation revealed that the three types of nanoparticles clearly damaged the hyphae of *Alternaria alternata* and *Botrytis cinerea* compared with control. Microscopic observation revealed that silver nanoparticles caused detrimental effects not only on fungal hyphae but also on conidial germination (Lamsal *et al*., 2011a). There were also, disrupting in the structure of the cell membrane and inhibiting the normal budding process of *Candida albicans* due to the destruction of the membrane integrity (Kim *et al*., 2009).

In concern to, the antifungal effect of silver nanoparticles on culture filtrate and cell wall components of both plant pathogens under this study. There was a reduction in total protein of culture filtrate and total protein and NAG of cell wall for both fungi, while there were an increasing in total lipids for culture filtrate and cell wall. These results agreed with a previous study observed that nanometer-sized silvers possess different properties, which might come from morphological, structural and physiological changes (Nel *et al*., 2006). Silver is known to attack a broad range of biological processes in microorganisms including the alteration of cell membrane structure and functions (Pal *et al*., 2007). Also, silver ions are known to produce reactive oxygen species via their reaction with oxygen, which is detrimental to cells, causing damage to proteins, lipids and nucleic acids (Hwang *et al*., 2008). It was also, observed that the Sulfur-NPs reduced the total lipid content with unusual accumulation of Saturated fatty acids-SFAs, which is atypical for the membrane lipid biosynthetic cascade (Choudhury *et al*., 2012).

The present study also suggests that silver nanoparticles was more effective than copper/silver or copper nanoparticles for the control of both plant pathogens *Alternaria alternata* and *Botrytis cinerea*. Thus, silver nanoparticles prepared by a cost effective method has great promise as an antimicrobial agent and antifungal activity and it has a great potential for use in controlling spore-producing fungal plant pathogens.

In summary, little is known regarding the effects of silver, copper or a mixture of them on phytopathogenic fungi, because most studies have focused on bacterial and viral pathogens in animals. Here, we evaluated the antifungal activity of these nanoparticles against the fungal phytopathogens *Alternaria alternata* and *Botrytis cinerea*. Our data clearly demonstrated that the Ag-nanoparticles strongly inhibited fungal growth and development and damaged cell walls compared with cu-nanoparticles and Ag/Cu-nanoparticles. These results suggest the possibility of using silver nanoparticles to eradicate phytopathogens. Several parameters will require evaluation prior to practical application, including phytotoxicity and antimicrobial effects in situ and development of systems for delivering particles into host tissues that have been colonized by phytopathogens.

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