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Genotoxicity and Molecular Response of Biotechnological Agent *Trichoderma harzianum* as a Result of Silver Nanoparticles Application

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Silver nanoparticles possess unique physical, chemical, and biological properties which find myriad applications but the random application of silver nanoparticles may arise many problems particularly on benefits biotechnological agent *T. harzianum* which serves as bio-control and growth promoting agents for many crop plants. The results indicated that AgNPs possess antifungal properties against *T. harzianum* and the antifungal activity increased with increasing their concentrations. Electrophoretic analysis was performed to determine the effect of AgNPs on *T. harzianum* protein profile. Protein migration in this study demonstrated that nanoparticles caused changes in the protein profiles. Two bands completely disappeared after treatment with 100 and 200 ppm of AgNPs with molecular weights 140, 27 and 21.0 kDa. The genotoxicity exhibited by AgNPs was demonstrated by DNA fragmentation post treatment particularly at high concentrations of the nanoparticles. The fragmented bands increased with increasing AgNPs concentrations. 2, 5 and 7 fragmented bands with different molecular bands were detected at 50,100 and 200 ppm of AgNPs respectively. Three bands with molecular weight 1000,900 and 750 bp were detected at 100 and 200 ppm of AgNPs. Also, the result showed that AgNPs treated fungal cells both accumulated more intracellular glucose and trehalose than the compound-untreated cells. Also, the result showed that *T. harzianum* treated with AgNPs increased extracellular glucose and trehalose than the compound-untreated cells as a result of cell wall damage.

Keywords: Genotoxicity, molecular response, *Trichoderma harzianum,* silver nanoparticles.

Nanomaterials are defined by their small size (< 100 nm) and their novel physical, chemical and biological properties, which are progressively applied on numerous research and economical fields. However, the rapid progress in nanoscience has not been accompanied by enough information regarding its toxicity. In the past ten years, particularly the past three years, a large number of scientific papers have been published in an attempt to understand various aspects of the hazards of silver nanoparticles (AgNPs). Several reviews have also dealt with the exposure, environmental fate, and *in vivo* and *in vitro* toxicities of AgNPs (Lynch and Dawson 2008; Reidy *et al.*, 2013; Yu *et al.*, 2013). The unique properties of AgNPs make them ideal for numerous technologies, including biomedical, optical materials, optical, and antimicrobial applications (Kim *et al.,* 2007; Choi *et al*., 2008).

Trichoderma harzianum used in several application, it has the ability to minimize the severity of fungal and bacterial plant diseases by inhibiting plant pathogens through their high antagonistic and mycoparasitic potential (Rosa *et al.*, 2012; Hussein *et al.,* 2017). Moreover, *T. harzianum* can solubilize several plant nutrients such phosphorus compounds (Janardan *et al.*, 2011), Certain *Trichoderma* spp. have beneficial

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impacts on plant growth and decrease sensitive to both biotic and a biotic stresses (Elshahawy *et al.*, 2017).

The antimicrobial effects of silver have been known for numerous years. Over the past several years, the use of silver or silver salts as key components to control microbial proliferation has become increasingly popular (Quadros and Marr 2011). As a broad-spectrum antimicrobial agent, AgNPs are widely used in medical and consumer products, including household antiseptic sprays and antimicrobial coatings for medical devices (Faunce and Watal 2010; Zhi-Kuan *et al.*, 2016). Hwang *et al.* (2012) demonstrated for the first time that AgNPs promotes apoptosis in *Candida albicans* through phosphatidylserine exposure. AgNPs may also cause DNA damage and damage replication ability (Morones *et al.*, 2005). Shunmugadevi and Palanisamy (2016) cleave DNA of *Escherichia coli* completely compared to untreated DNA with AgNPs. As a result, it can be said that the ability of these compounds for the DNA cleavage may be considered as a major reason for the inhibitory effect of them on the growth of the pathogenic organisms. According to Gopinath *et al*., (2010) AgNPs can interact with membrane proteins and activate signaling pathways, leading to repress cell proliferation. Cytotoxic and/or genotoxic effect of AgNPs and the ability to induce various chromosomal aberrations in mammalian cells, bacteria and root meristematic cells of different plants were reported in numerous studies (Patra *et al*., 2007; Kumari *et al*., 2009; Sahar *et al*., 2014). In addition, treatment with an AgNPs suspension may decrease the replication ability of bacterial DNA and inactivate the cellular proteins (Feng *et al*., 2000).

Moreover, nanoparticles as well as AgNPs are also known to induce oxidative stress in microbes which will eventually lead to the killing of microbes. It has been previously reported that increased reactive oxygen species (ROS) production due to AgNPs damage membranes, forming free radicals with a powerful microbiocidal action (Wu *et al*., 2014). Study on AgNPs interactions with the phosphate groups and nitrogenous bases in DNA was earlier reported (Sheikpranbabu *et al.*, 2010). Some investigations showed that OH ion is active structures and the strongest antimicrobial elements; these free radicals attack to interior DNA of fungus and bacterium, and cause apoptosis in cells (Naghsh *et al*., 2012). Another mechanism of antifungal activity of AgNPs was reported (Keuk-Jun 2009; Siddhanta *et al.*, 2016), where the analysis of glucose and trehalose release, during AgNPs exposure, suggests that it may be one of several intracellular components released during membrane disruption by AgNPs. This study aimed to explore the genotoxic and DNA-damaging potential of AgNPs using bioapplicable agent *T. harzianum* to avoid AgNPs using in pest control in the presence of this fungus.

MATERIAL AND METHODS

Biotechnological agent and culture conditions

Biotechnological agent *Trichoderma harzianum* was used a commercially available product (Plant guard produced by El-Nasser Com. Egypt).The rate of mycelial growth was measured in colonies grown in Petri dishes containing PDA supplemented with different concentration (50,100 and 200 ppm) of AgNPs (<100nm) at 30°C for different incubation periods. The average diameter of each colony was measured daily to record the growth and growth inhibition %.

Protein gel-electrophoresis

Five grams of each dried fungus treated with AgNPs or untreated were ground in a volume of 0.1 ml sample buffer (sodium dodecyl sulfate) cracking solution. Extracts were added in 1.5 cm eppendorf centrifuge tube according to Laemmli (1970). Homogenates were heated at 95°C for 5 min then briefly centrifuged at 12,000 rpm to pellet cellular debris. The resulting supernatants (total protein extracts) were stored at -70°C until analysis by Polyacrylamide Gel Electrophoresis. The extract was separated by electrophoresis on 1mm thick 12.5% acrylamide slab gels. Gels were stained with Coomassie blue at Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt.

DNA fragmentation of *T. harzianum*

T. harzianum cultivated at different concentrations of AgNPs at 30°C for 6 days. At the end of the incubation, the harvested, mycelia were treated with 200 µl cell lysis buffer (50 mM TrisHCl, pH 8.0, 10 Mm ethylene diamine tetra acetic acid, 0.1 M sodium chloride, and 0.5% sodium dodecyl sulphate) for 1 h at 37°C. The

lysate was incubated with 0.2 mg/ml proteinase K at 40°C for 2 h. After completion of incubation, the sample was centrifuged for 10 min at 10,000 rpm. The aqueous portion, containing the DNA was transferred to new Eppendorf tube. DNA fragmentation assay was performed on 1% agarose gel (Vahdati and Sadeghi, 2013). Genomic DNA was loaded into the slots of 1% agarose gel containing 1 µg/ml Ethidium bromide, at a constant power supply of 80 volts for one hour. The gel was visualized under UV transilluminator and documented.

Determining released glucose and trehalose

T. harzianum after cultivation in Potato Dextrose Broth Medium was harvested, washed three times with PBS, and then 5 ml of the *T. harzianum* sores suspension (2 x 10^4 spores), containing different concentrations 50, 100 and 200 ppm of AgNPs, were incubated for 5 hrs at 30°C in Phosphate buffered saline (PBS). The control was incubated without AgNPs. The spores were settled by centrifugation (at 12,000 rpm for 15 min). The supernatants were transferred to a new tube. Released glucose and trehalose-containing supernatants were added to 0.05 units of trehalase. After 1 hr of enzymatic reaction at 37°C, the reaction suspension was mixed with water and 16% DNS reagent (3,5-dinitrosalicylic acid 1%, NaOH 2%, sodium potassium tartrate 20%) was added. For the reaction of glucose with the DNS reagent, the mixture was boiled for 5 min and cooled. Color formations were measured at 525 nm. The Intracellular trehalose was measured according to a modified previously described method (Pedreno *et al.*, 2007).

RESULTS AND DISCUSSION

The inhibitory effect of AgNPs to living organisms has been investigated in a number of studies. But many studies failed to describe the behavior of nanoparticles in the particular biological media. Therefore the purpose of our study was to investigate the toxicity AgNPs to commercial biocontrol agent *T. harzianum*. AgNPs treatment with 50,100 and 200 ppm concentration resulted in about 29.48, 66.66 and 73.33% inhibition percentage of *T. harzianum* respectively at 6 days of incubation period (Table 1 and Figure 1). Although the growth increased at 8 days but the inhibition percentage of growth increased this may due to resistance of *T. harzianum* to AgNPs. Therefore, the results suggested that maximum inhibition was obtained at treated with 200 ppm concentration of AgNPs at all tested incubation periods. Similarly, a recent publication showed that AgNPs had antifungal effects against fungi (Abdlghany 2013, Sahar 2014; Zhi-Kuan *et al*., 2016, Abdlghany *et al*., 2017).

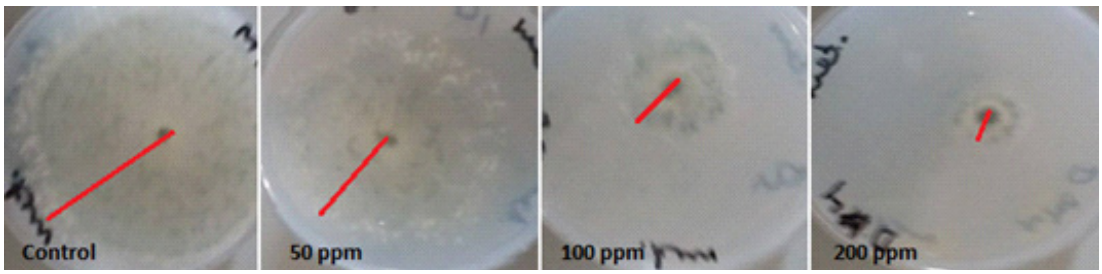

Fig. 1. Growth of *T. harzianum* exposure to different concentrations of AgNPs at 6 days incubation period

Table 1. Growth and inhibition % of *T. harzianum* exposure to AgNPs at different incubation periods

Incubation period (Day)	AgNPs concentration ppm							
	Control		50		100		200	
	Growth	Inhibion %	Growth	Inhibion %	Growth	Inhibion %	Growth	Inhibion %
2	1.2±0.2	0	0.8±0.8	33.33	0.0±0.0	100	0.0±0.0	100
4	4.5±0.5	0	3.2±0.4	28.88	2.2±0.4	51.11	1.4±0.4	68.88
6	7.8±0.2	0	5.5±0.5	29.48	2.5±0.5	66.66	2.0±0.1	73.33
8	8.2±0.4	0	7.0±0.2	14.63	2.8±0.2	65.85	2.4±0.7	70.73

SDS-PAGE gel electrophoresis was carried out to monitor the change in gene expression of *T. harzianum* exposed to different concentration to AgNPs (Figure 2). The average total number of bands per lane induced in *T. harzianum* was 18 with molecular weight ranging from 150 to 12.5 kDa (Table 2). There were numerous protein bands present were detected in treated and in untreated *T. harzianum* by AgNPs . Only two bands completely disappeared after treatment with 100 and 200 ppm of AgNPs with molecular weights 140, 27 and 21.0 kDa (Table 2). The amount of expression of proteins in each treatment changed compared with the control, where the band intensity of each treatment was changed as compared with their control (Figure 2).These results agreed with a previous study observed that nanometer-sized silvers possess different properties, which might come from structural and physiological changes (Nel_*et al*., 2006; Sahar 2014). It is evident that AgNPs directly interacts with macromolecular structures of living cells and exerts an active influence on their metabolism. They can also cause damage to the nuclear DNA by altering the chemical structure of the nucleotide bases and the deoxyribosyl backbone (Cooke *et al.*, 2003). The cytotoxic effects of silver are the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA.

Table 2. Protein bands (kd) detected at different concentrations of silver nanoparticles

Band No.	Molecular weight of protein bands (kd) at different concentrations of			
	Control	50 ppm	100 ppm	200 ppm
1	12.5	12.5	12.5	12.5
2	14.8	14.8	14.8	14.8
3	16.7	16.7	16.7	16.7
4	18.5	18.5	18.5	18.5
5	19.3	19.3	19.3	19.3
6	21.0	21.0	-	-
7	25.0	25.0	25.0	25.0
8	24.0	24.0	24.0	24.0
9	27.0	27.0	27.0	27.0
10	35.0	35.0	35.0	35.0
11	45.0	45.0	45.0	45.0
12	50.0	50.0	50.0	50.0
13	60.0	60.0	60.0	60.0
14	72.5	72.5	72.5	72.5
15	75.0	75.0	75.0	75.0
16	100	100	100	100
17	140	140	-	-
18	150	150	150	150

Agarose electrophoresis of genomic DNA from fungus exposed to silver nanoparticles revealed fragmented DNA compared to control (Figure 3). Agarose gel electrophoresis showed

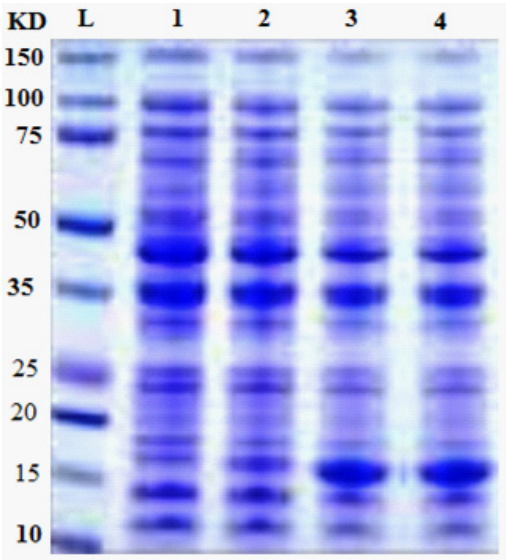

Fig. 2. Protein profile of *T. harzianum* at different concentrations of silver nanoparticles

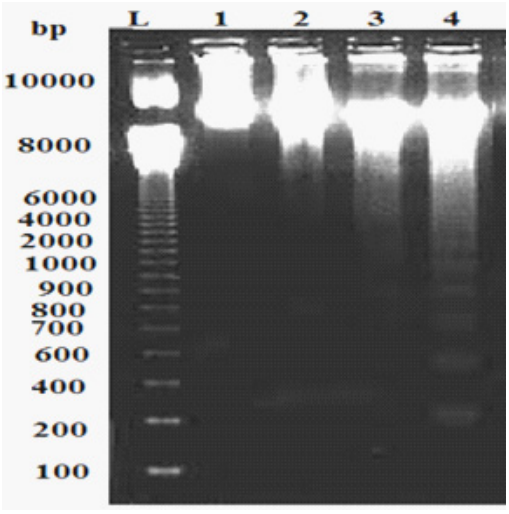

Fig. 3. DNA Fragmentation Assay of *T. harzianum* at different concentrations of AgNPs. M: Ladder; 1: Control; 2:50 ppm; 3: 100 ppm; 4:200 ppm AgNPs

Table 3. DNA Fragmented Bands of *T. harzianum* at different concentrations of AgNPs

Treatment with AgNPs	Molecular Weight of Fragmented Bands	
Ladder		10 K bp
Control		9600 bp
50 ppm	Band 1	9100 bp
	Band 2	8200 bp
100 ppm	Band 1	8900 bp
	Band 2	7800 bp
	Band 3	1000 bp
	Band 4	900 bp
	Band 5	750 bp
200 ppm	Band 1	8800 bp
	Band 2	7900 bp
	Band 3	1000 bp
	Band 4	900 bp
	Band 5	750 bp
	Band 6	500 bp
	Band 7	300 bp

intact DNA bands with the untreated DNA (control), where no significant damage occurred. In contrast, fungs treated with different concentrations of silver nanoparticles showed a dose-dependent induction of DNA strand break, characterized by increased DNA fragmentation (Figure 3). In the present study, the genotoxicity exhibited by AgNPs was demonstrated by DNA fragmentation post treatment particularly at high concentrations of the AgNPs. The fragmented bands increased with increasing AgNPs concentrations. 2, 5 and 7 fragmented bands with different molecular bands were detected at 50,100 and 200 ppm of AgNPs respectively. Three bands with molecular weight 1000,900 and 750 bp were detected at 100 and 200 ppm of AgNPs (Table 3). Such genotoxic activities of nanoparticles were reported earlier (Supriyo *et al.*, 2014) where degree of DNA degradation was directly proportional to the concentration of AgNPs. DNA smear of the NPs treated fungus showed that the AgNPs treated cells exhibited extensive double strand breaks of various sizes, thereby yielding a smear like appearance (lane 2,3 and 4), while the DNA of control cells exhibited no breakage (lane 1). DNA fragmentation assay revealed that the AgNPs showed genotoxic effect in a dose dependent manner. These results suggest

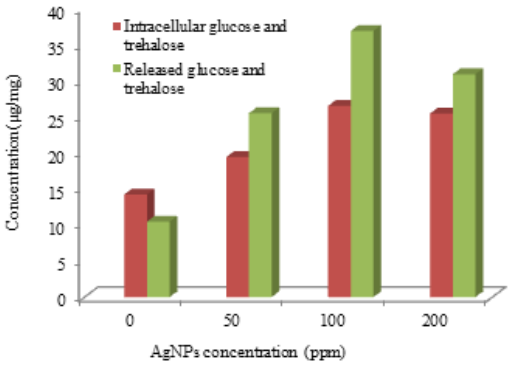

Fig. 4. Trehalose and glucose concentrations (ig/mg) detected in *T. harzianum* exposed to different concentrations of AgNPs

that the biocidal effect of AgNPs may occur by direct chemical damage to DNA. These results are in accordance with the findings by Sobhy *et al.*, (2012), showing action of AgNPs on DNA of *Alternaria solani*. Recently, Mukesh (2016) reported that NPs caused damage of *E. coli* genomic DNA in a dose dependent manner. Sobhy *et al*. (2012), stated that he mechanism of AgNPs antifungal activity may be related to damaging the membrane lipid bilayer, leading to intracellular ion efflux resulting in cell death. Also, accumulation of AgNPs in the cell nuclei and interaction with DNA may lead to cell death.

In the current study, measuring the glucose and trehalose released was used to assess the ability of AgNPs to disturb the integrity of the plasma membrane of fungal cells. Kim *et al.*, (2009) reported that AgNPs exhibited potent antifungal effects, probably through destruction of membrane integrity. The result showed that AgNPs treated fungal cells both accumulated more intracellular glucose and trehalose than the compound-untreated cells (Figure 4). The rate of accumulation increased at highest concentration of AgNPs and reached to maximum (26.50 µg/mg) at 100ppm of AgNPs. At the same time, fungal cells also increased extracellular glucose and trehalose than the compound-untreated cells. Release of glucose and trehalose may explained as a result of cell wall damage, such changes were reported in previous studies (Elbein *et al.*, 2003; Kim *et al.*, 2009; Niazi *et al.*, 2011). Recently Siddhanta *et al.,* (2016) found that perturbation of membrane by AgNPs leads to the generation of

glucose and trehalose which indicate that they are the intracellular components of the membrane.

CONCLUSION

In the present study AgNPs have exhibited antifungal and genotoxic effect on *T. harzianum* and therefore this paper suggest that AgNPs, because of the harmful effects on environment, should be applied less in the presence this biotechnological agent *T. harzianum*.

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