academicJournals

Vol. 7(19), pp. 1917-1923, 7 May, 2013
DOI: 10.5897/AJMR2013.5668
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro study of the antifungal efficacy of zinc oxide nanoparticles against *Fusarium oxysporum* and *Penicilium expansum*

Ramy S. Yehia[1]* and Osama F. Ahmed[2]

[1]Department of Botany, Faculty of Science, Cairo University, Giza 12613, Egypt.
[2]Medical Applications of Laser Department, Cairo University, Egypt.

Accepted 26 April, 2013

The antifungal efficiency of Zinc oxide nanoparticles (ZnO NPs) was investigated against two pathogenic fungal species, *F. oxysporum* and *P. expansum.* The two fungi were identified at molecular level by nuclear ribosomal DNA internal transcribed spacer (ITS) identities. They have been submitted to the GenBank with accession numbers of AB753032 and AB753033 for *F. oxysporum* and *P. expansum*, respectively. The antifungal activity of ZnO NPs was found to be concentration dependent. Hence, maximal inhibition of mycelial growth corresponded to the highest experimental concentration (12 mg L^{-1}), where 77 and 100% growth inhibition was observed for *F. oxysporum* and *P. expansum*, respectively. The effect of ZnO NPs on the mycotoxins fusaric acid and patulin production by *F. oxysporum* and *P. expansum*, respectively, was investigated using HPLC quantification. It was observed that ZnO NPs prevented both mycotoxins synthesis in a concentration dependent manner. Fusaric acid was reduced from 39.0 to 0.20 mg g^{-1} while patulin production was reduced from 14.2 to 1.10 mg g^{-1} in control and 12 mg L^{-1} ZnO NPs treated samples, respectively. The scanning electron microscopy (SEM) revealed obvious deformation in the growing mycelia treated with ZnO NPs in *F. oxysporum* which may be the cause of growth inhibition.

Key words: Zinc Oxide Nanoparticles (ZnO NPs), antifungal efficiency, *Fusarium oxysporum*, *Penicillium expansum.*

INTRODUCTION

Fungal growth and pathogenicity are the main cause of considerable economic loss during postharvest and handling of vegetable crop and fruits. *Fusarium oxysporum* is a cosmopolitan fungus that includes pathogenic and saprophytic members. The pathogenic members are best known for causing *Fusarium* wilt diseases of many economically important crops (Tripathi et al., 2009). Until now, synthetic fungicides are used as primary means to control *Fusarium* wilts. While *Penicillium expansum* causes severe postharvest fruit diseases including grey and blue mold even when the most advanced postharvest technologies are applied (Spadaro et al., 2004), it primarily causes the rot of stored apples and pears (Cabanas et al., 2009). Furthermore, *P. expansum* is regarded as the major producer of a mycotoxin, patulin, which is commonly found in rotting apples. U.S. Food and Drug Administration (FDA) limits patulin to 50µg/L in apple juices (Moake et al., 2006).

Today a number of synthetic fungicides have been found to cause adverse effects to humans and the environment

*Corresponding author. E-mail: Drramy4@hotmail.com.

and for this reason many of them have been banned. It is difficult to control fungal growth because fungi have developed resistance to many conventional fungicides such as benzimidazoles and dicarboximides (Elad et al., 1992). Moreover, these traditional fungicides are becoming ineffective due to the development of new physiological races of the pathogens (Ocamb et al., 2007). To overcome resistance, it is important to explore novel antifungal agents, which may replace current con-trol strategies. In recent years, nanoparticle (NP) mate-rials have received increasing attention due to their unique physical and chemical properties, which differs significantly from their conventional counterparts (Stoimenov et al., 2002).

Recent studies have demonstrated antimicrobial efficacy against bacteria, viruses and eukaryotic microorganisms of various NP materials, including silver (Kim et al., 2008; Kumar et al., 2008), copper (Cioffi et al., 2005), titanium dioxide (Kwak et al., 2001), magnesium, gold (Gu et al., 2003), alginate (Ahmad et al., 2006) and zinc oxide (Liu et al., 2009). Highly ionic nanoparticulate metal oxides such as MgO, CaO and ZnO nanoparticles (NPs) are unique in that they can be produced with high surface areas and with unusual crystal structures (Klabunde et al., 1996).

Compared to organic materials, inorganic materials such as ZnO possess superior durability, greater selectivity, heat resistance (Padmavathy and Vijayaraghavan, 2008) and the mineral element is essential to human health ZnO NPs also have good biocompatibility to human cells (Padmavathy and Vijayaraghavan, 2008). The antibacterial and antifungal activity of bulk ZnO powders has been already demonstrated (Yamamoto**,** 2001; Sawai and Yoshikawa, 2004).

In agriculture, zinc compounds are mainly used as fungicides (Waxman, 1998). The 50% lethal dose (LD50) of oral toxicity for ZnO is relatively high reaching 240 mg/kg for rats (South, 2002). It is believed that smaller sizes of ZnO, correlated with its stronger antimicrobial activity (Yamamoto, 2001).

Preliminary studies show that the antibacterial activity of ZnO NPs might be related to the formation of free radicals on the surface of the NPs, and the damage to the lipids in bacterial cell membrane by free radicals, which consequently lead to the leakage and breakdown of bacterial cell membrane (Brayner et al., 2010; Reddy et al., 2007). However, to the best of our knowledge, the effect and mode of action of ZnO NPs on the growth of fungi such as *F. oxysporum* and *P. expansum* have not been studied. Therefore, in the current study the anti-fungal activity of ZnO NPs against two important plant pathogenicfungi,*F.oxysporum*and*P.expansum*wasinvestigated.

MATERIALS AND METHODS

Nanoparticle materials

ZnO NP suspensions with size of 70±15 nm were purchased from Sigma Aldrich. An aliquot of ZnO NP suspension was vacuum filtered through an aluminum oxide membrane filter with a 20 nm pore size and 25 mm OD (Anodisc; Whatman Inc., Clifton, NJ, USA), resulting in a NP-free solution. The composition of the NP-free solution was analyzed, and its effect on bacterial growth was examined. The original ZnO NP suspension (12 mg L^{-1}) and NP-free solution were then diluted with potato dextrose agar (PDA, containing per liter the extract from 200 g boiled potato, 20 g glucose and 20 g agar) to make a series of media containing ZnO NPs with concentrations of 0, 2, 4, 6, 8 and 12 mg L^{-1}, respectively and NP-free solution.

Isolation and identification of fungal isolate

Fungal samples were taken from different infected plant tissues. The pure culture was maintained on PDA media (Hi media Laboratories Ltd. Bombay, India) at 25±1°C. Identification of the fungal isolates was carried out by morphological and microscopic examination using the identification keys of Moubasher (1993) and Raper and Fennell (1985) followed by nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. The genomic DNA was isolated by CTAB extraction method using standard protocols (Sambrook et al., 1989). Internal transcribed spacer (ITS) regions were amplified using primers ITS1 and ITS4 with 5'-TCCGTAGGTGAACCTGCGG and 5'-TCCTCCGCTTATTGATATGC sequences (White et al., 1990). Amplified products were sequenced using ABI prism DNA sequencer by BigDye terminator method. The sequence obtained has been deposited in the GenBank database. The ITS sequence information was used to match the most closely retrieved fungal isolates with the NCBI BLAST program. The alignments of the ITS sequences were performed using ClustalW, and phylogenetic tree constructed with the maximum parsimony method and MEGA ver. 5 (http://www.megasoftware.net) (Felsenstein, 1985).

Antifungal test

F. oxysporum and *P. expansum* were cultured on PDA at 25°C. Antifungal tests were performed by the agar dilution method (Fraternale et al., 2003) with slight modifications. The autoclaved PDA media with ZnO NPs at concentrations of 0, 2, 4, 6, 8 and 12 mg L^{-1} and a NP-free solution were poured into the Petri dishes (9 cm diameter). A plug (1 cm) of fungal mycelia was taken from the edge of 7-day-old plate, placed in the center of each Petri dish and incubated at 25°C. The efficacy of ZnO NP treatment was evaluated at the time intervals of 2, 4, 6, 8, 10 and 12 days by measuring the diameter of fungal colonies. All tests were performed in triplicate and the diameters were expressed in centimeters.

Scanning electron microscopy (SEM)

SEM was used to examine morphological changes of *F. oxysporum* and *P. expansum* hyphae before and after treatment with ZnO NPs. Pieces of mycelial material cut from 7-day-old cultures were inoculated onto the PDA containing 12 mg L^{-1} ZnO NPs in comparison with control containing no ZnO NPs, followed by incubation for 12 days. Then, pieces of mycelia were cut from the edge of the fungal cultures, and directly subjected to SEM analysis under the environmental mode. SEM images were taken by FEI Quanta 600F Environmental SEM (FEI Company, Hillsboro, OR, USA) at a voltage of 7 or 10 kV and a pressure from 525 to 619 Pa.

Mycotoxins estimation

Fusaric acid Toxin of Fusarium oxysporum

The production and extraction of fusaric acid was performed accor-

Figure 1. Phylogenetic analysis of *Penicillium* spp based on the gene sequences showing the relationship of *P. expansum* to other *Penicillium* spp. The phylogenetic tree was constructed based on internal transcribed spacer sequences using the maximum parsimony method on MEGA ver. 5.

according to method described by Bacon et al., 1996. Separation was carried out by using HPLC (Shimadzu, Japan) with SPD-10 AD UV spectrophotometric detector and LCI 100 integrator on a reversed phase column RP-18 Licrospher 100, 5 µm particle size (250 × 4 mm). The mobile phase (HPLC quality) used in the chromatographic run consisted of methanol (A), water (B) and 1% dipotassium hydrogen phosphate in water adjusted to pH 7.25 with concentrated phosphoric acid (C). Elution conditions reported by Amalfitano et al. (2002) were slightly modified and started with A: B: C (55:10:35), which was changed according to a linear gradient over 20 min. to A: B: C (70:10:20); with restoring the initial conditions over 5 min. The column was re-equilibrated under these conditions for 15 min before starting the next run. The flow rate was 1 ml/min, and 20 µL aliquots of samples were injected for analysis. Detection was carried out at 268 nm, and HPLC calibration curves were performed using solutions of both standards in methanol in the range of 0.10 to 1.00 µg. Samples for HPLC analysis were prepared as follows: fungal culture filtrates (10 ml) were extracted with methanol (5 ml), the methanol suspensions were passed through disposable filters and aliquots (maximum 20 µl) were analyzed.

Patulin Toxins of Penicillium expansum

Patulin extraction was illustrated by Fred and MCcalla, 1969. Analysis of Patulin was performed on a model 'HP1050' HPLC equipped with UV detector. Separation and determination of patulin was performed on RP18 (ODS) column (length 250mm).

RESULTS AND DISCUSSION

Amplification and sequencing of fungal rRNA genes resulted in 620 and 750 bp long nucleotide sequence, which have been submitted in NCBI GenBank (Accession Numbers: AB753032 and AB753033) for *F. oxysporum* and *P. expansum*, respectively. The sequences were compared using BLAST algorithm and the closely related sequences were selected, which confirmed that isolated strains exhibited 98 and 97% similarity with *F. oxysporum* and *P. expansum*, respectively. A phylogenetic tree was generated using the maximum parsimony method and MEGA ver. 5 (Figures 1 and 2).

ZnO NPs are usually present in a form of agglomerates during its manufacturing process (Zhang et al., 2007). Two methods, ultrasonication and addition of dispersants, are often used to break down NP agglomerates. Commonly used dispersants include polyvinylpyrolidone, polyethylene glycol, and other chemicals (Brayner et al., 2006). Figures 3 and 4 represent the growth of *F. oxysporum* and *P. expansum* cultivated on PDA containing different con-centrations of ZnO NPs (0, 2, 4, 6, 8 and 12 mg L^{-1}) and incubated at 25°C for 12 days. Generally, the use of ZnO NPs suspension efficiently inhibited fungal growth of both *F. oxysporum* and *P. expansum*. The average mycelial growth inhibition in *F. oxysporum* has been ranged from 19.3 to 77.5% as ZnO NPs concentration increase from 2 to 12 mg L^{-1} (Figure 3). For *P. expansum*, the reduction rate of fungal growth significantly varied from 25.3 to 100% as the concentration of ZnO NPs increased from 2 to 12 mg L^{-1} with a near complete inhibition at 6 mg L^{-1} (Figure 4). The efficacy of ZnO NPs was found to be con-centration dependent in both two fungal species. These results suggest that ZnO NPs may disrupt and damage the conidia of fungi. Consequently, the growth was deeply inhibited.

P. expansum proved to be more sensitive than *F. oxysporum* to the ZnO NPs treatment. Hence, different antifungal effects may result from different growth morphologies of fungi. *P. expansum* tends to grow more densely on the surface of the medium than *F. oxysporum,* so it has a larger contact area with ZnO NPs than *F. oxy-*

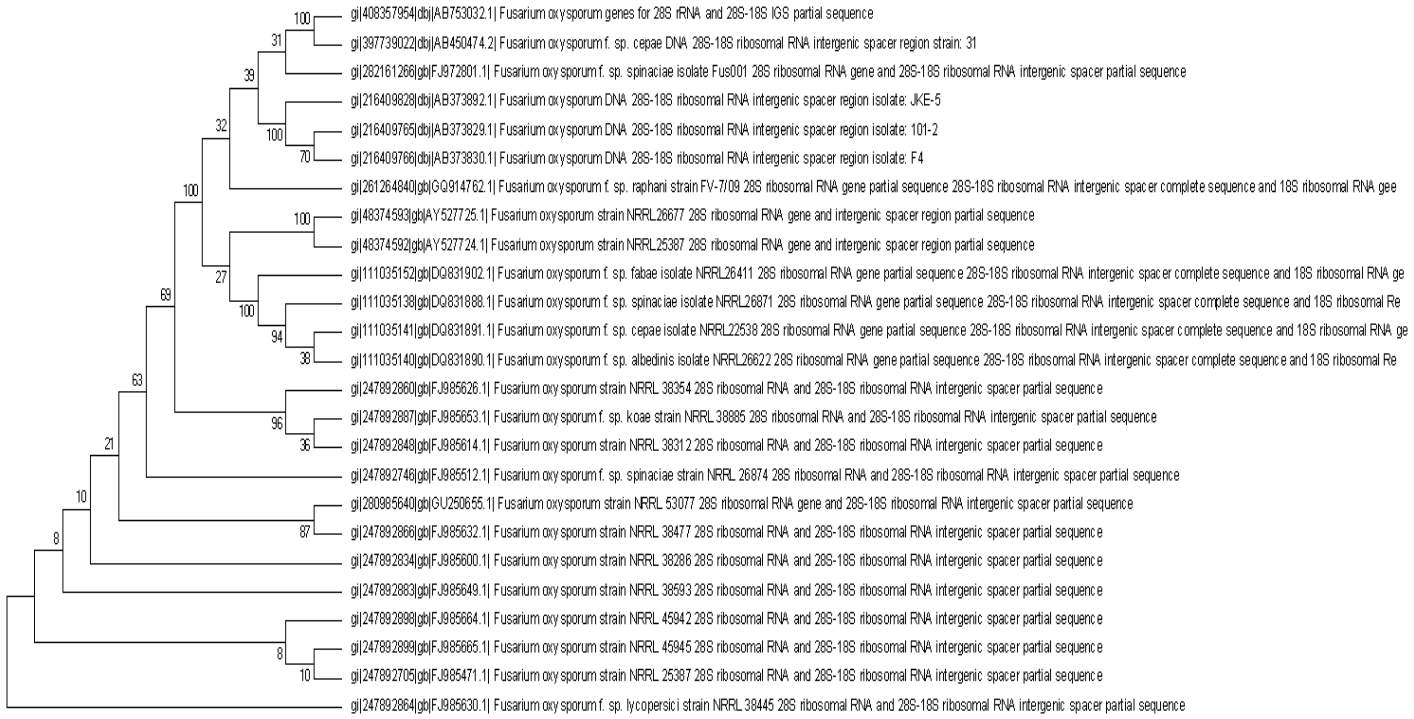

Figure 2. Phylogenetic analysis of *Fusarium* spp based on the gene sequences showing the relationship of *F. oxysporum* to other *Fusarium* spp. The phylogenetic tree was constructed based on internal transcribed spacer sequences using the maximum parsimony method on MEGA ver. 5.

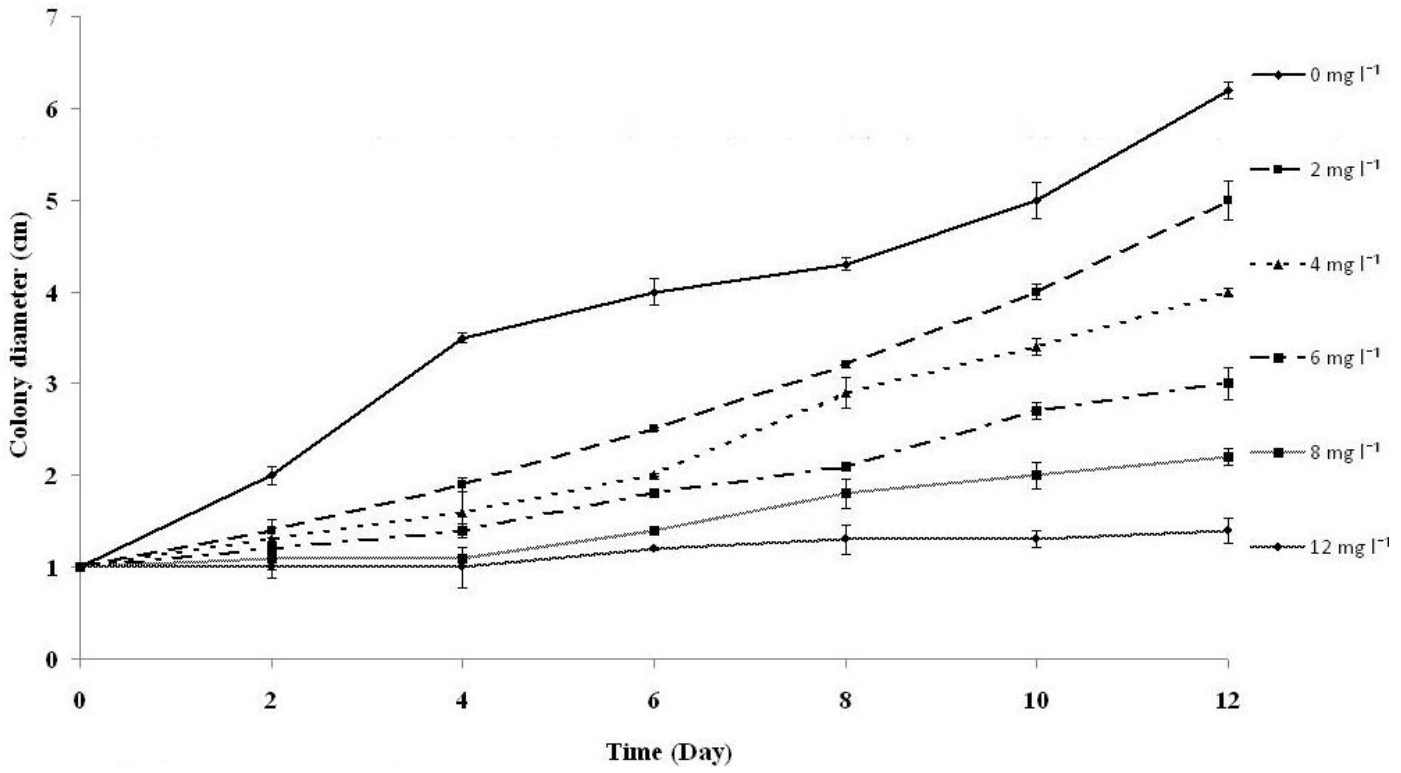

Figure 3. Effect of ZnO NPs on the colony diameter of *F. oxysporum*.

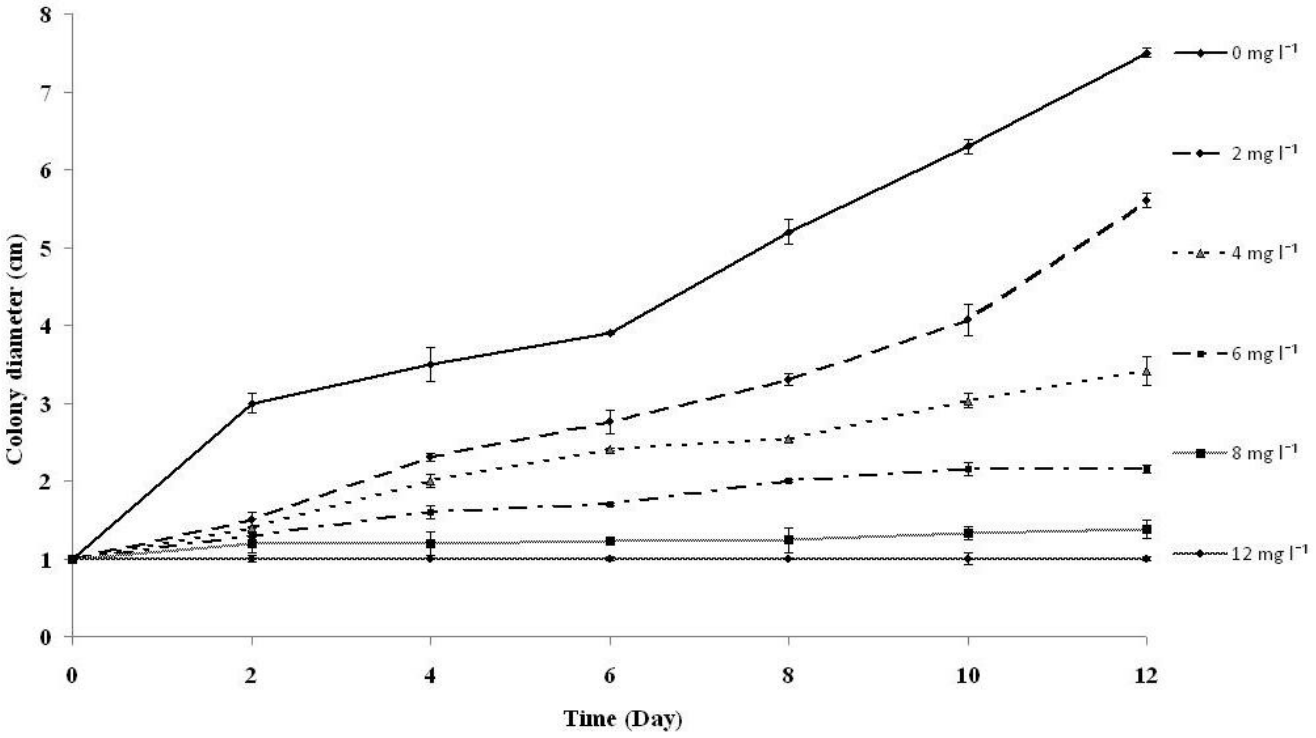

Figure 4. Effect of ZnO NPs on the colony diameter of *P. expansum.*

oxysporum. Another possible reason for the difference in antifungal efficacy among fungi could be the constitutive tolerance of each fungus to ZnO NPs.

He et al. (2011) found that ZnO NPs at concentrations greater than 3 mmol L^{-1} can significantly inhibit the growth of *Botrytis cinerea* and *P. expansum*. Sawai and Yoshikawa, (2004) reported the minimum inhibitory concentration of bulk ZnO powder against *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger,* and *Rhizopus stolonifer* was over 100 mg ml^{-1} (~1.2 mol L^{-1}) by an indirect conductimetric assay (Sawai and Yoshikawa, 2004). ZnO NPs show great enhancement in the antimicrobial activity due to their unique properties such as large surface area. However, Kasemets et al. (2009) found nano and bulk ZnO were of comparable toxicity against *S. cerevisiae*.

To investigate the mechanism by which ZnO NPs affect the growth of *F. oxysporum* and *P. expansum*, SEM analysis was employed to examine the structural changes of fungal samples after ZnO NPs treatment. PDA media containing 12 mg L^{-1} ZnO NPs were prepared, and fungal samples were then inoculated onto the PDA plate and incubated at 25°C for 12 days.

Figure 5 shows the images of mycelia obtained from the edge of *F. oxysporum* and *P. expansum* culture. In the control (untreated sample), hypha with typical net structure and smooth surface were observed. After treatment with 12 mg l^{-1} ZnO NPs, the hypha lost their smoothness and appeared swollen and crumbled (Figure 5). This indicated that ZnO NPs growth inhibition in *F. oxysporum* and *P. expamsum* may be due to deformation in the structure of fungal hypha. SEM has been successfully used to assess morphological changes of microbial cells induced by ZnO NPs (Brayner et al., 2006; Zhang et al., 2007) and fungal hyphae treated with other chemicals (Sharma and Sharma, 2008; Yen et al., 2008). Some studies proposed that ZnO NPs may cause structural changes of microbial cell membrane, causing cytoplasm leakage and eventually the death of bacterial cells (Sawai and Yoshikawa, 2004; Brayner et al., 2006). Endo et al. (1997) have reported that the inhibition of bud growth by Ag-NPs correlates with membrane damage. This report suggests that Ag-NPs inhibit the normal budding process, probably through the destruction of membrane integrity.

F. oxysporum and *P. expansum* are well-known as mycotoxins producers. Table 1 revealed that the production of mycotoxins by the tested fungi gradually decreased with the increase of ZnO NPs concentrations to reach a minimum value at the highest concentration 12 mg L^{-1}. The total mycotoxins at 12 mg L^{-1} were recorded to be 0.20 and 1.1 mg g^{-1} as compared to 39.0 and 14.2 mg g^{-1} in control, in cases of *F. oxysporum* and *P. expansum*, respectively. This inhibition of mycotoxin synthesis may be attributed to inactivation of certain enzymes in the biosynthesis pathway of these toxins by ZnO NPs.

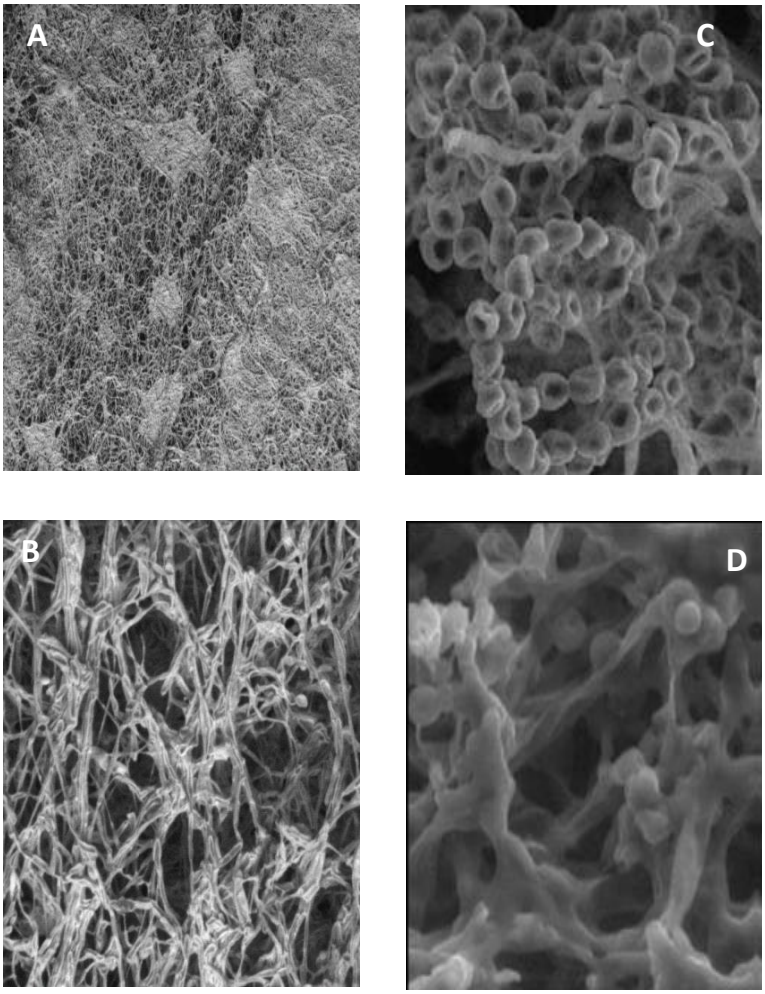

Figure 5. Scanning electron microscopy images of *F. oxysporum* and *P. expansum* without (A and C) or with (B and D) the treatment of ZnO NPs (12 mgl L^{-1}).

Table 1. Effect of ZnO nanoparticles at different concentrations on production of mycotoxins (mg g^{-1} dry mass) by *Fusarium oxysporum* and *Penicillium expansum*.

ZnO nanoparticle conc. (mg L^{-1})	*Fusarium oxysporum*	*Penicillium expansum*
	Fusaric acid	Patulin
0	39.00[a]	14.20[a]
2	35.02[b]	12.32[b]
4	13.00[c]	9.56[c]
6	10.51[d]	4.73[d]
8	6.32[e]	4.15[e]
12	0.20[f]	1.10[f]

[a],significant difference (P<0.05) according to Duncan test.

ACKNOWLEDGEMENT

The author is grateful to Prof. Tahany M. A. Abd-El Rahman, professor of microbiology, Department of Botany, Faculty of Science, Cairo University, for her continuous encouragement and for revising the manuscript.

REFERENCES

Ahmad Z, Pandey R, Sharma S, Khuller, GK (2006). Alginate nano-particles as antituberculosis drug carriers: formulation development, pharmacokinetics and therapeutic potential. Indian J. Chest Dis. Allied Sci. 48:171-176.

Amalfitano C, Pengue R, Andolfi A, Vurro M, Zonno MC, Evidente, A (2002). HPLC analysis of fusaric acid, 9,10-dehydrofusaric acid and their methyl esters, toxic metabolites produced by weed pathogenic *Fusarium* species. Phytochem. Anal. 13:277-282.

Bacon CW, Porter JK, Norred WP, Leslie JF (1996). Production of Fusaric Acid by *Fusarium* Species. Appl. Environ. Microbiol. 62(11):4039-4043.

Brayner R, Dahoumane SA, Claude Yéprémian C, Djediat C, Meyer M, Alain Couté A, Fiévet F (2010). ZnO nanoparticles: synthesis, characterization, and ecotoxicological studies. Langmuir 26:6522-6528.

Brayner R, Ferrari-Iliou R, Brivois N, Djediat S, Benedetti MF, Fievet F (2006). Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano. Lett. 6:866-870.

Cabanas R, Abarca ML, Bragulat MR, Cabanes FJ (2009). *In vitro* activity of imazalil against *Penicillium expansum*: comparison of the CLSI M38-A broth microdilution method with traditional techniques. Int. J. Food Microbiol. 129:26-39.

Cioffi N, Torsi L, Ditaranto N, Tantillo G, Ghibelli L, Sabbatini L (2005). Copper nanoparticle/polymer composites with antifungal and bacteriostatic properties. Chem. Mater. 17:5255-5262.

Elad Y, Yunis H, Katan T (1992). Multiple fungicide resistance to benzimidazoles, dicarboximides and diethofencarb in field isolates of *Botrytis cinerea* in Israel. Plant Pathol. 41:41-46.

Endo M, Takesako K, Kato I, Yamaguchi H (1997). Fungicidal action of aureobasidin A, a cyclic depsipeptide antifungal antibiotic against *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. 41:672-676.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Fraternale D, Giamperi L, Ricci D (2003). Chemical composition and antifungal acivity of essential oil obtained from *in virto* plants of *Thymus mastichina* L. J. Ess. Oil Res. 15:278-281.

Fred AN, McCALLA TM (1969). Patulin Production by *Penicillium urticae* Bainier in Batch Culture. Appl. Microbiol. 17(2):193-196.

Ganley RJ, Newcombe G (2006). Fungal endophytes in seeds and needles of *Pinus monticola*. Mycol. Res. 110:318-327.

Gu H, Ho PL, Tong E, Wang L, Xu B (2003). Presenting vancomycin on nanoparticles to enhance antimicrobial activities. Nano. Lett. 3:1261-1263.

He L, Liu Y, Mustapha A, Lin M (2011). Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. Microbiol. Res. 166:207-215.

Kasemets K, Ivask A, Dubourguier HC, Kahru A (2009). Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast *Saccharomyces cerevisiae*. Toxicol In Vitro. 23:1116-1122.

Kim KJ, Sung WS, Moon SK, Choi JS, Kim JG, Lee, DG (2008). Antifungal effect of silver nanoparticles on dermatophytes. J. Microbiol. Biotechnol. 18:1482-1484.

Kim KJ, Sung WS, Suh BK, Moon SK, Choi JS, Kim JG (2009). Antifungal activity and mode of action of silver nano-particles on *Candida albicans*. Biomaterials 30:6333-6340.

Klabunde KJ, Stark J, Koper O, Mohs C, Park D, Decker S (1996). Nanocrystals as stoichiometric reagents with unique surface chemistry. J. Phys. Chem. 100:12142-12153.

Kumar PK, Vemula PM, Ajayan G, John D (2008). Silver-nanoparticle-embedded antimicrobial paints based on vegetable oil. Nat. Mater. 7:236-241.

Kwak SY, Kim SH, Kim SS (2001). Hybrid organic/inorganic reverse osmosis (RO) membrane for bactericidal anti-fouling. 1. Preparation and characterization of TiO_2 nanoparticle self-assembled aromatic polyamide thin-film-composite (TFC) membrane. Environ. Sci. Technol. 35:2388-2394.

Liu Y, He L, Mustapha A, Li H, Lin M (2009). Antibacterial activities of zinc oxide nanoparticles against *Escherichia coli* O157:H7. J. Appl. Microbiol. 107:1193-1201.

Moake MM, Padilla-Zakour OI, Worobo RW (2006). Comprehensive review of patulin control methods in foods. Food Sci. Technol. 4:8-21.

Moubasher AH (1993). Soil Fungi in Qatar and other Arab Countries. 1st Edn., Centre of Scientific and Applied Research, University of Qatar, Doha, Qatar, Pp 566.

Ocamb CM, Hamm PB, Johnson DA (2007). Benzimidazole resistance of *Fusarium* species recovered from potatoes with dry rot from storages located in the Columbia basin of oregon and Washington. Am. J. Potato. Res. 84:169-177.

Padmavathy N, Vijayaraghavan R (2008). Enhanced bioactivity of ZnO nanoparticles–an antimicrobial study. Sci. Technol. Adv. Mater. 9:1-7.

Raper K, Fenell D (1985). The genus *Aspergillus*. Williams and Wilkins, Baltimore, Maryland.

Reddy KM, Feris K, Bell J, Wingett DG, Hanley C, Punnoose A (2007). Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems. Appl. Phys. Lett. 90:213902-213905.

Sambrook J, Fritschi EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press, New York.

Sawai J, Yoshikawa T (2004). Quantitative evaluation of antifungal activity of metallic oxide powders (MgO, CaO and ZnO) by an indirect conductimetric assay. J. Appl. Microbiol. 96:803-809.

Sharma N, Sharma S (2008). Control of foliar diseases of mustard by *Bacillus* from reclaimed soil. Microbiol. Res. 163:408-413.

South DB (2002). Inorganic pesticides. In: Pimentel D, editor. Encyclopedia of pest management. Marcel Dekker, Inc., New York: pp. 395-397.

Spadaro D, Garibaldi A, Martines GF (2004). Control of *Penicillium expansum* and *Botrytis cinerea* on apple combining a biocontrol agent with hot water dipping and acibenzolar-S-methyl, baking soda, or ethanol application. Postharvest Biol. Technol. 33:141-151.

Stoimenov PK, Klinger RL, Marchin GL, Klabunde JS (2002). Metal oxide nanoparticles as bactericidal agents. Langmuir 18:6679-6686.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

Tripathi A, Sharma N, Sharma V (2009). *In vitro* efficacy of *Hyptis suaveolens* L. (Poit.) essential oil on growth and morphogenesis of *Fusarium oxysporum* f.sp. gladioli (Massey) Snyder and Hansen. World J. Microbiol. Biotechnol. 25:503-512.

Waxman MF (1998). The agrochemical and pesticide safety handbook. CRC Press; pp. 616.

White TJ, Bruns T, Lee S, Taylor J (1990). PCR Protocols: A Guide to Methods and Applications, ed. Innis MA, Gelfand DH, Shinsky JJ, White TJ, Academic Press, San Diego, pp. 315-322.

Yamamoto O (2001). Influence of particle size on the antibacterial activity of zinc oxide. Int. J. Inorg. Mater. 3:643-646.

Yen TB, Chang HT, Hsieh CC, Chang ST (2008). Antifungal properties of ethanolic extract and its active compound from *Calocedrus macrolepis var. Formosana* (Florin) heartwood. Bioresour. Technol. 99:4871-4877.

Zhang L, Jiang Y, Ding Y, Povey M, York D (2007). Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). J. Nanoparticle Res. 9:479-489.