Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 1, January – March 2014, p. 151 - 157

APPLICATION OF SILVER NANOPARTICLES AS ANTIFUNGAL AND ANTIAFLATOXIN B1 PRODUCED BY *ASPERGILLUS FLAVUS*

M. R. AL-OTHMAN[a], A. R. M. ABD El- AZIZ[a*], M. A. MAHMOUD[b], S. A. EIFAN[a], M. S. EL-SHIKH[a], M. MAJRASHI[c]
[a]*Botany and Microbiology Department, College of Science, King Saud University, Riyadh 1145, Kingdom of Saudi Arabia.*
[b]*Plant Pathology Research Institute, ARC, Giza, Egypt.*
Riyadh 2455, Kingdom of Saudi Arabia
[c] *King Abdulaziz City for Science and Technology (KACST), Riyadh, Kingdom of Saudi Arabia*.

In this study, we evaluated the effect of silver nanoparticles were biosynthesis by *Aspergillus terreus* (KC462061) on growth and aflatoxin production by Five isolates of *A. flavus* were isolated from nuts. TEM micrographs observed that the particles of AgNP saresperical in shape spherical or nearly sphericaland without significant agglomeration and the particle size ranges from 5 to 30 nm. Our results showed that all five *A. flavus* isolates were inhibited to various extents by different concentrations of silver nanoparticles but the best inhibition by 150 ppm with different significantly. In general, inhibition % of aflatoxin production at 50ppm ranged from 48.2 to 61.8%, at 100 ppm ranged from 46.1 to 82.2% whereas at 150ppm inhibition % reached to 100%. Scanning electron microscopy (SEM) was used to study antifungal activities of silver nanoparticles and to characterize the changes in morphology. SEM images indicate two different antifungal activities of AgNPs against *A. flavus*. AgNPs inhibited the growth of *A. flavus* by affecting cellular functions which caused deformation in fungal hyphae. In comparison, AgNPs cause reduce in spores number, malformation and hypertrophy, these effects lead to destroyed and damaged of spores. The aim of this work to study the possibility to use silver nanoparticles as an alternative to fungicides for controlling growth and aflatoxin production.

(Received November 20, 2013; Accepted January 22, 2014)

Keywords: Silver nanoparticles, Antiaflatoxin B1, Linear growth, SEM

1. Introduction

Mycotoxins are natural products and highly toxic secondary metabolites are produced by filamentous fungi found in cereals, dried fruits and nuts [1,2]. *Aspergillus* species is one of the largest and most important genera which produced aflatoxin [3]. AFB1 presents the highest toxic potential and is classified by the International Agency for Research on Cancer (IARC) as carcinogen, Group 1 [4]. Saudi Arabia is very high country in nuts consumption The worldwide yield in 2011 13584 (Hg/Ha) , production of nuts is 805681 tonnes (FAO, 2011). [5]. Silver is a non toxic, safe inorganic antimicrobial agent and is capable of killing about 650 type of microorganisms [6]. Silver has been described as being 'oligodynamic' because of its ability to exert a bactericidal effect at low concentrations [7]. Silver nanoparticles as an alternative to chemically manufactured pesticides without toxicity problems [8].

*Corresponding author: aabdelaziz@ksu.edu.sa

2. Materials and methods

Silver nanoparticles

Silver nanoparticle were producted using *Aspergillus terreus* (KC462061) and characterized by UV–Vis spectrophotometry, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) in our previous study [9]. We found all nanoparticles were 5-30 nm in size. And these nanoparticles were used for current study.

Anti-fungal activity of silver nanoparticles against *Aspergillus flavus* growth

The five toxigenic isolates of *A. flavus* utilised in the assays, were isolated from nuts. The antifungal activity method was performed according to [10]. Different concentrations (50, 100 and 150 ppm) of Silver nanoparticle were added to autoclaved potato dextrose agar (PDA), keeping one as Control (PDA without Silver nanoparticle). A disc (6 mm) of mycelia, taken from the edge of 7-day-old fungal cultures, was placed in the centre of each Petri dish containing the PDA culture medium containing silver nanoparticle and incubated at 25±2 °C. The efficiency of silver nanoparticle treatment was evaluated after control competed by measuring the fungi colonies diameters. each treatment replicated three times. The inhibition rate (%) was calculated by using the following formula:

$$\text{Inhibition } \% = \frac{R - r}{R} \times 100$$

Where R is radial growth of fungi in control plate and r is the radial growth of fungi in silver nanoparticle treated plates.

Antimycotoxin activity of silver nanoparticles

The anti-aflatoxigenic efficacy of silver nanoparticle was determined using SMKY liquid medium (sucrose, 20 g; magnesium sulfate, 0.5 g; potassium nitrate, 3 g; yeast extract, 7 g; and distilled water, 1,000 ml) [11]. Three different concentrations of silver nanoparticle (50 ,100 and 150 ppm) were prepared and added to flasks, followed by inoculation with 6-mm diameter discs of the toxigenic *A. flavus* at 25 ± 2°C for 20 days) and the Control (SMKY without silver nanoparticle). After incubation, the content of each flask was filtered (Whatman, No. 1), and biomass of filtered mycelium was dried at 70°C for 4 days till their weights remains constant. Mycelial dry weights of treatments and control was determined and the filtrates were each treated three times with 50 ml of chloroform in a separatory funnel. The chloroform extracts were separated and dehydrated with anhydrous sodium sulfate and evaporated until dryness in a water bath at 50°C under vacuum. The residues were dissolved in 10 ml of methanol [10].

Morphological alterations

The treated fungi mycelia sections were collected, fixed with formaldehyde, washed with phosphate buffer solution and dehydrated with alcohol solution (30, 60, 80, 90 and 100%, maintaining the mycelia at 100%) and then submitted to critical point drying according to [12]. then the fungi mycelia were prepared for scanning electron microscopy (SEM) using JEOL (JSM-6380 LA) instrument.

Statistical analysis

All of the data from three independent replicate trials were sub-jected to analysis using Statistical Package for the Social Sciences (SPSS) 10.0 statistical software (Chicago, USA). The data are reported as the mean ± standard deviations, and significant differences between mean values were determined with Duncan's Multiple Range test ($p<0.05$), followed by one-way ANOVA.

3. Results and discussion

Silver nanoparticles used:

TEM measurements were used to determine the morphology and shape of nanoparticles.TEM micrographs (Fig. 1) observed that the particles of AgNP spherical in shape spherical or nearly sphericaland without significant agglomeration and the particle size ranges from 5 to 30 nm. In our previous study [9]

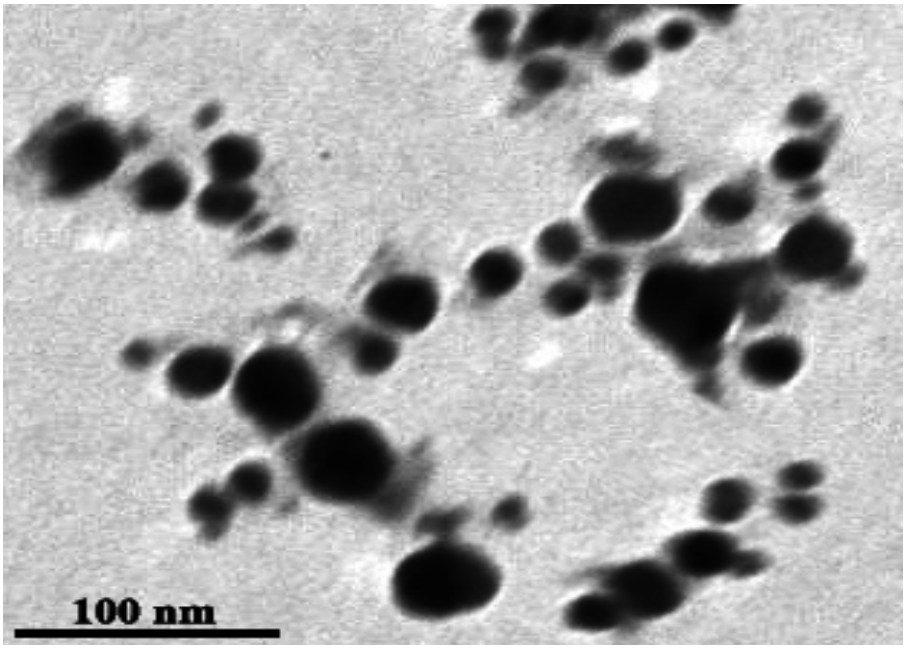

Fig. 1. Transmission Electron Microscopy (TEM) images of synthesized silver nanoparticles by Aspergillus terreus (KC462061).

Anti-fungal activity of silver nanoparticles against *Aspergillus flavus* growth

Our results showed that the effect of silver nanoparticles on growth diameter of fungal. All five *A. Flavus* isolates were inhibited to various extents by different concentrations of silver nanoparticles but the best inhibition by 150 ppm with different significantly (fig. 2, Table 1) Data followed by the same letter are not significantly different, whereas those followed by different letters are significantly different at P≤0.05). In general, inhibition % at 50ppm ranged from 8.9 to 17.4%,at 100 ppm ranged from 43.3 to 54.8% whereas at 150ppm inhibition % reached to 86.3 %. [13] observed in the antifungal activity of the silver nanoparticle, chitosan nanoparticles and silver /chitosan nanocomposite, upon the mycelial growth and zone of inhibition of the Aspergillus sp showed significant inhibition effectiveness reached to 94%. [10] found that treatments with ZnO-NPs lead to reduce on conidia production and fungi growth. Significant inhibition in mycelial growth of *Colletotrichum* supplied with 100 ppm silver nanoparticles on PDA was reported by [14]

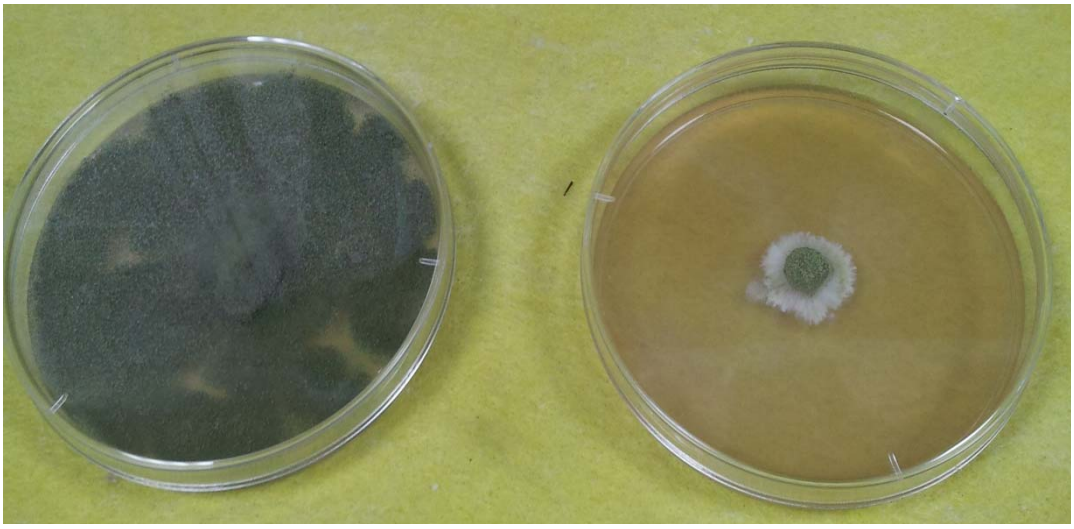

Fig. 2. A. flavus without treatment (A) , (B) with 150ppm of silver nanoparticles after 5 days

Table 1. Effect of three different concentrations (ppm) silver nanoparticles on linear growth (cm.) of five isolates of A. flavus .

Concentrations (ppm)	Linear growth (cm.)									
	F1	I %	F2	I %	F3	I %	F4	I %	F5	I %
Control	9.00±0.00a	0	9.00±0.00a	0	9.00±0.00a	0	9.00±0.00a	0	9.00±0.00a	0
50	7.43±0.23b	17.4	8.13±0.13b	9.7	7.97±0.03b	11.4	8.20±0.21b	8.9	7.10±0.10b	21.1
100	5.10±0.06c	43.3	4.07±0.12c	54.8	4.93±0.23c	45.2	5.07±0.18c	43.7	5.00±0.06c	44.4
150	2.03±0.03d	77.4	1.90±0.12d	78.9	2.60±0.23d	71.1	1.90±0.10d	78.9	1.23±0.15d	86.3
LSD	0.396		0.348		0.538		0.474		0.303	

*I %= inhibition percentage

Values in the same column followed by (±) are significantly different (P = 0.05). The presented data are the mean (n = 3) ± standard error of three replicates. Data followed by the same letter are not significantly different at P≤0.05, whereas those followed by different letters are significantly different at P≤0.05).

Antimycotoxin activity of silver nanoparticles

Our results in Table 2 showed that the effect of silver nanoparticles on production of aflatoxin B1. All concentrations lead to decreasing in aflatoxin B1 compared with control. The inhibition was increased by increased concentration, the best inhibition noticed by 150 ppm. In general, inhibition % at 50ppm ranged from 48.2 to 61.8%, at 100 ppm ranged from 46.1 to 82.2% whereas at 150ppm inhibition % reached to 100%. The production of AFB1 *A. flavus* grown on ZnO-NPs was smaller when compared to controls [10]. [15] reported that nontoxic metal oxides such as magnesium oxide and calcium oxide in nanocrystalline nanoparticle formulations are effective on Aflatoxin B1. nanocomposite MgO-SiO2 was an effective adsorption of aflatoxin and adsorption characteristics of this nanocomposite depends on the concentration of aflatoxin [15] .

Table 2. Effect of silver nanoparticles with three different concentrations (ppm) on aflatoxin B1 (ppb) produced by five isolates of Aspergillus flavus

Concentrations (ppm)	aflatoxin B1									
	F1	I %	F2	I %	F3	I %	F4	I %	F5	I %
50	14.2	48.2	30.1	56.6	28.4	53.6	20.1	49.1	7.1	61.8
100	7.5	72.6	21.6	68.8	18.3	70.0	14.2	64.1	3.3	82.2
150	3.6	86.9	16.7	75.9	13.4	78.1	7.3	81.5	0.0	100.0
Control	27.4	0.0	69.3	0.0	61.2	0.0	39.5	0.0	18.6	0.0

*I %= inhibition percentage

Morphological alterations

Due to reduction of their growth and ability to produce mycotoxins, we supposed that morphological changes on treated fungi could occur. To evaluate them, treated fungi hyphae at the highest silver nanoparticles concentration (150 ppm) after 5 days were examined to SEM. Treatment of fungal hyphae with silver nanoparticles showed damage such as: deformations in mycelial growth and the shape of hyphal walls, unusual bulges and ruptures (fig.3). SEM examination confirmed the fungi cell membrane rupture resulting in possible reduction of the enzymatic activity of the micro-organism due to Zn-compounds treatments. ZnO-NPs lead to damaged on hyphae and spores of *C. gloeosporioides* [14]. Changes in the structure of the fungus were also observed using ZnO-NPs against *P. expansum* and *B. cinerea* [16], also [17] confirm changes and rupture of the fungal cell membrane in *F. verticillioides*, *A. flavus* and *P.citrinum* due to gold NPs. Physical, chemical stresses and antifungal compounds, have been reported to trigger necrosis or apoptosis-like cell death in fungi [18]. While study on the effect of AgNP on the fungal spores were observed damage such as reduce in spores number (fig.4a), malformations (fig.4b) and hypertrophy (fig.4c) these effects lead to destroyed and damaged of spores. This aspect is great important because Aspergillus reproduction involves mainly formation of spores, which are resistant to desiccation and favourable to dispersion [19].

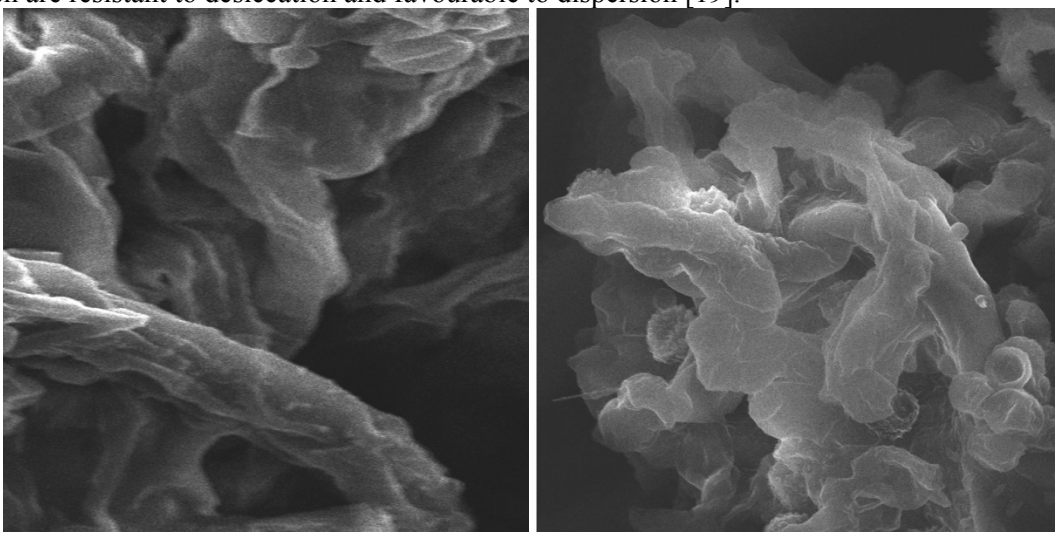

Fig. 3. SEM images of A. flavus hyphae treated with 150ppm of silver nanoparticles (A and B)

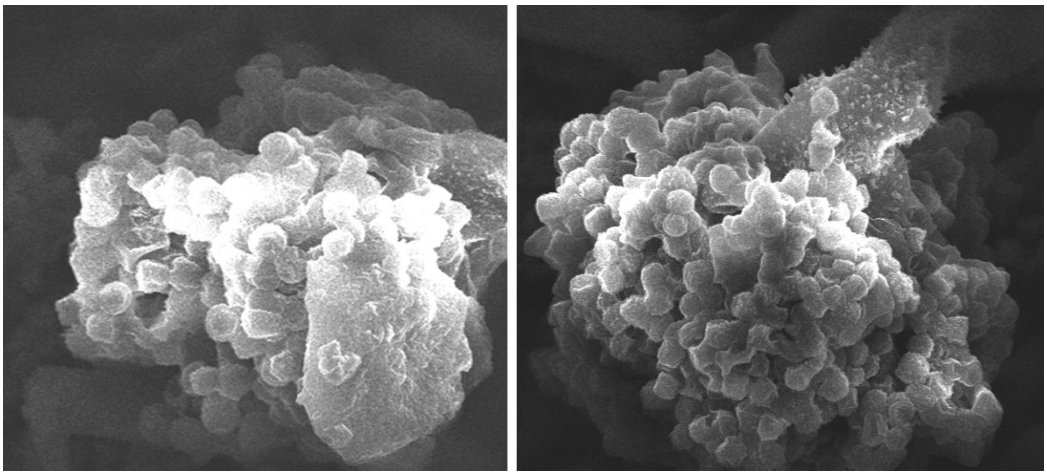

Fig. 4a SEM images of A. flavus without (A) or with (B) 150ppm of silver nanoparticles (reduce in spores number)

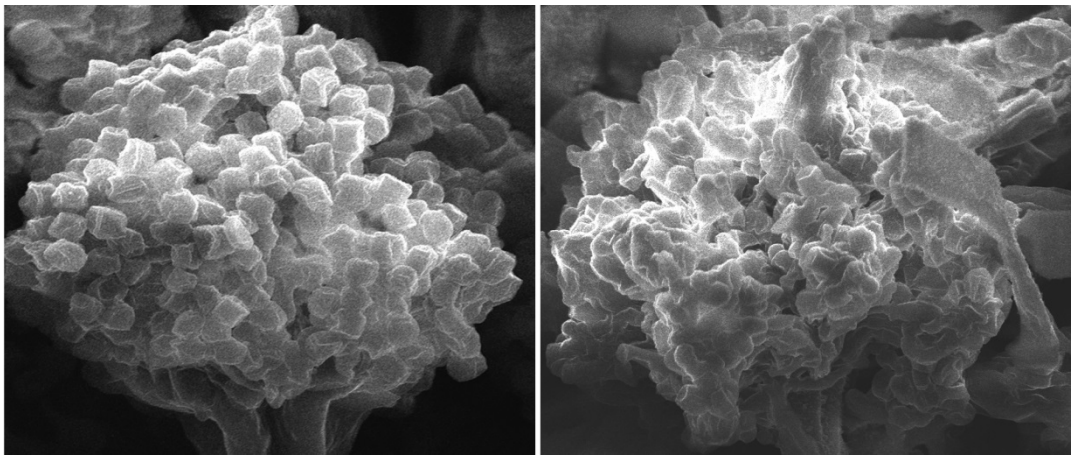

Fig. 4 b. SEM images of A. flavus spores (A) without treatment, (B) with 150ppm of silver nanoparticles (malformation)

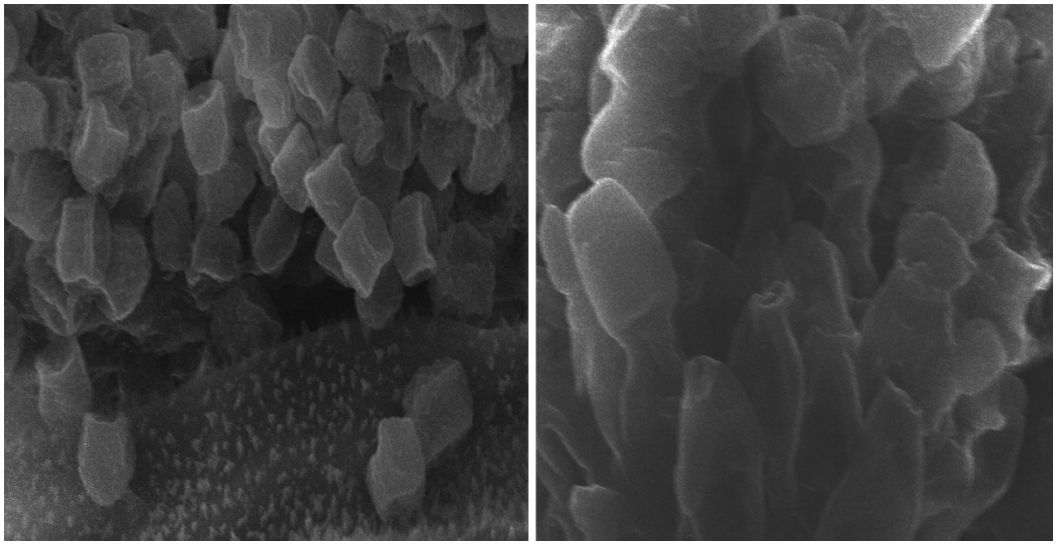

Fig. 4 c. SEM images of A. flavus spores (A) without treatment, (B) with 150ppm of silver nanoparticles (hypertrophy)

4. Conclusion

The possibility to use silver nanoparticles as an alternative to fungicides for controlling growth and aflatoxin production, in this study, we evaluated the effect of silver nanoparticles were biosynthesis by *Aspergillus terreus* (KC462061) on growth and aflatoxin production by *A. flavus*. Our results showed that all *A. flavus* isolates were inhibited to various extents by different concentrations of silver nanoparticles but the best inhibition by 150 ppm with different significantly. AgNPs inhibited the growth of *A. flavus* by affecting cellular functions which caused deformation in fungal hyphae. In comparison, AgNPs cause reduce in spores number, malformation and hypertrophy, these effects lead to destroyed and damaged of spores.

5. Acknowledgements

The authors would like to extend their sincere appreciation to the Deanship of the Scientific Research at King Saud University for its funding of this research through the Research Group Project no. RGP-VPP-269.

6. References

[1] P. Sekar, N. Yumnam, K. Ponmurugan, Adv. Biotech, **12**, 15 (2008).
[2] Munirah F. Al-Gahtani, Monira R. Al-Othman, M. A. Mahmoud,Abeer R. M. Abd El-Aziz, African Journal of Microbiology Research **7**, 3151 (2013).
[3] J.I. Pitt, R.A. Samson, Stud Mycol, **59**, 67 (2007).
[4] IARC. International Agency for Research on Cancer: Monograph on the Evaluation of Carcinogenic Risk to Human, France: Lyon., 56, 257 (1993).
[5] FAO http://faostat.fao.org/site/339/default.aspx, (2011).
[6] S.H. Jeong, S.Y. Yeo, S.C. Yi, J. Mat. Sci., **40**, 5407 (2005).
[7] S.L.Percivala, P.G. Bowler, D. Russell, J. Hosp. Infect., **60**, 1 (2005),
[8] Y.K. Jo, B.H. Kim, G. Jung, Plant Dis. **93**, 1037 (2009).
[9] Abeer R. M. Abd El- Aziz, Monira R. Al-Othman, S. A. Eifan, M. A. Mahmoud, M. Majrashi, Digest Journal of Nanomaterials and Biostructures, **8**, 1215 (2013).
[10] G. D. Savi, A. J. Bortoluzzi, V. M. ScusseInternational Journal of Food Science and Technology, **48**, 1834 (2013).
[11] Abeer R. M. Abd El- Aziz, Monira R. Al-Othmana, S. A. Alsohaibani, M. A. Mahmoud, S. R. M. Sayed, Digest Journal of Nanomaterials and Biostructures, **7**, 1491(2012).
[12] D. Bray, Critical point drying of biological specimens for scanning electron microscopy. Springer Protocols, **13**, 235 (2000).
[13] P. Kaur, R. Thakur, A.S. Choudhary, Intern. J. of Scientific & Technol. Res., **1**(6), 83 (2012).
[14] K. Lamsal, S. W. Kim, J. H. Jung, Y. S. Kim, K. S. Kim, Y. S. Lee, Application of Silver Nanoparticles for the Control of *Colletotrichum* Species *In Vitro* and Pepper Anthracnose Disease in Field Mycobiology, **39**, 194 (2011).
[15] O. B. Koper, S. John, L. G. Marchin, K. J. Klabunde, P. Stoimenov, L. Bohra, Nanoscale Powders and Formulations with Biocidal Activity Toward Spores and Vegetative Cells of *Bacillus* Species, Viruses, and Toxins, Current Microbiology , **44**, 49 (2002).
[16] L.Y. Liu, A. Mustapha, M. Lin, Antifungal activity of zinc oxide nanoparticles against Botrytis cinerea and Penicillium expansum. Microbiological Research, **166**, 207. (2011).
[17] G.D. Savi, M.M.S. Paula, J.C. Possato, T. Barichello, D. Castagnaro,V.M. Scussel, Journal of Nano Research, **20**, 11 (2012).
[18] A. Sharon, A. Finkelstein, N. Shlezinger, I. Hatam, Fungal apoptosis: function, genes and gene function. FEMS Microbiology Reviews, **33**, 833 (2009).
[19] R. J.B. Pinto, A. Almeida, S. C.M. Fernandes, C. S.R. Freire, A. J.D. Silvestre, C. P.l Neto, T. Trindade, Antifungal activity of transparent nanocomposite thin films of pullulan and silver against *Aspergillus niger* ,Colloids and Surfaces B: Biointerfaces **103** ,143, (2013).