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The Antifungal Efficacy of Pre-Harvest Spraying With Organic Compounds and Nanoparticles on Aflatoxins Production on Stored Soybean Seeds

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ABSTRACT

This work was conducted to study the efficacy of pre-harvest spraying of soybean plants with some organic compounds [salicylic, succinic, oxalic, ascorbic acids and ethylenediaminetetraacetic acid (EDTA)] and some nanoparticles (NPs) e.g. Copper oxide (CuO), zinc oxide (ZnO) and magnesium oxide (MgO) for their antifungal activities against aflatoxins (AFs) producing fungi; *Aspergillus flavus* and *A. parasiticus* (*A. flavus* group) and AFs production after one year of storage. Laboratory and field trials were conducted at Etay El-Baroud Agricultural Research Station, El-Beheira governorate, Egypt, on soybean (*Glycine max* L.) cultivar Giza 21 during season 2017. The organic compounds were used at the concentrations of 25, 50, 75 and 100 mg/ml and NPs at the concentrations of 2.5, 5.0, 7.5 and 10 mg/ml. Generally, the radial growth inhibition increased with increasing the organic compounds and NPs concentrations. Soybean plants sprayed three times at bloom stage with organic compounds at 100 mg/ml and NPs at 10 mg/ml individually caused an increase in the weight of 100 seeds (g), a decrease in the disease incidence (%) and an increase in the seeds total protein content (%). The increment in seeds total protein content ranged between 53.2–2.87% compared with untreated control. After harvest (zero time), the organic compounds exhibited their reducing effect on AFs levels (B1, B2, G1 and G2) in seeds in the following order; EDTA< ascorbic acid< oxalic acid< salicylic acid< succinic acid with averages of 6.02, 9.00, 13.27, 29.92 and 31.46 ng/g, respectively. Also, examined NPs exhibited their reducing effect of AFs in the following order: CuO< MgO< ZnO with averages of 19.33, 42.87 and 58.57 ng/g, respectively. Under storage condition (95% relative humidity and 35 °C) for one year, the residual levels of AFs in artificially infected seeds and in non-infected seeds were non-detectable for B1, G1 and G2. However, oxalic acid treatment induced B2 generation (11.74 ng/g; average) in infected seeds and CuONPs induced generation (6.17 ng/g; average) in non-infected seeds.

Key words: soybean seeds; organic compounds; nanoparticles; aflatoxins; storage

INTRODUCTION

Soybean is an annual plant which belongs to family Fabaceae, its seeds are a major source of protein, free cholesterol oil **(Ash et al., 2006)**, carbohydrates and minerals **(Capeleti et al., 2005).**

Mycotoxins are produced by molds under bad storage conditions, especially seeds moisture content above 12%, 95% relative humidity (RH) and temperatures greater than 35 $^{\circ}$C which contribute to fungal growth. Aflatoxins (AFs) are food-borne secondary toxic fungal metabolites produced during the growth of *Aspergillus flavus* and *A. parasiticus* (*A. flavus* group) **(Criseo et al., 2001)**. Among 18 different types of identified AFs, major members are aflatoxins B1, B2, G1 and G2. B-group (B1-B2) and G group (G1-G2) are considered the most important ones distinguished by their fluorescent color under ultraviolet light due to containing cyclopentane and lactone rings, respectively. Aflatoxins (AFs) cause lipid peroxidation as well as oxidative damage to DNA, so they are well known hepatotoxic, mutagenic agents and hepatocarcinogenic, especially Aflatoxin B1 **(Ismaiel and Tharwat, 2014).**

Several compounds have a critical role in inducing systemic acquired resistance (SAR) in different plant tissues, so that it has been recorded as a promising approach to control *A. flavus* infection and reduce AFs production in pistachio fruits **(Panahirad et al., 2014).** Spraying ascorbic acid on soybean seeds decreased growth of different fungi in all storage periods **(El-Metwally et al., 2014).** Application of salicylic acid can protect plants from various diseases to get quality seeds **(Kuchlan et al., 2017).** Antifungal properties of Ethylenediaminetetraacetic acid (EDTA) were tested on the reduction of pulmonary aspergillosis *via* increasing AFB1 degradation **(Hachem et al., 2006).** On the other hand, **Hassan et al. (2015)** reported that oxalic acid gave low inhibition effect on *A. flavus*.

Nanotechnology approaches seem to be a promising, effective and low-cost way to minimize the harmful effects of mycotoxins. There are three main strategies for nanoparticles (NPs): mold inhibition, mycotoxin adsorption and reducing the toxic effect **(Horky et al., 2018).** Nanoparticles (NPs) may affect agricultural crops at biochemical, physiological and molecular levels through changes in mineral nutrition and photosynthesis **(Rizwan et al., 2017).** Among NPs, ZnO has gained more attention due to its special properties. Additionally ZnONPs can be used as antibacterial, antifungal and antiviral agents due to its toxic effect on organisms **(Hassan et al., 2015).** It was proved able to inhibit the mycelial growth of aflatoxigenic molds, particularly *A. flavus* and prevent AFs production **(Nabawy, 2015)**. Also, magnesium oxide (MgO) and nanocomposite MgO-SiO_2 were effective for adsorption of AFB1 **(Koper et al., 2002).** The present work was undertaken to investigate the antifungal potential of pre-harvest spraying with some organic compounds (salicylic, succinic, oxalic, ascorbic acids and EDTA) and some NPs

(copper oxide (CuO), zinc oxide (ZnO) and MgO) on *A. flavus* group growth and AFs production on stored soybean seeds.

MATERIALS AND METHODS

Examined Materials

The organic compounds (salicylic, succinic, oxalic, ascorbic acids and EDTA) were supplied by BDH Chemical Ltd. Poole, England. While, NPs (CuO, ZnO and MgO) were prepared in the laboratory of Nano-tech. Company, 6th October City, Egypt, according to **Lu et al. (2018).**

Nanoparticles (NPs) Characterizations

Powders of NPs were characterized at Faculty of Science, Alexandria University on Scanning Electron Microscopy (SEM) (JOEL, JSM 5300) with high resolution at an accelerating voltage of 120 Kev. An aliquot of them was coated on a copper grid and scanned for its size and shape. Electron Dispersive Analysis (EDA) was performed using X-ray Oxford detector unit (model 6647, England) equipped with SEM (JOEL, JSM 5300) to achieve the purity of prepared NPs.

Laboratory Experiment

Five organic compounds (salicylic, succinic, oxalic, ascorbic acids and EDTA) were prepared at concentrations 0, 25, 50, 75 and 100 mg/ml **(Attitalla and Brishammar, 2002)**, also three NPs (CuO, ZnO and MgO) at 0, 2.5, 5.0, 7.5 and 10 mg/ml **(Wani and Shah, 2012)** individually were tested for their ability to control the radial growth of *Aspergillus flavus* group as the AFs producing fungi**.** Nanoparticles were prepared in deionized water and sonicated for enough time to avoid aggregation complex before media preparation. All chemicals were thoroughly mixed with Potato Dextrose Agar (PDA) medium just before pouring in the dishes to make the final appropriate concentrations. Fungal disc (5 mm diameter) was taken from the growing margins of 7 days old PDA fungal culture. Check treatment was prepared by growing the tested fungi on PDA medium free from the tested compounds. Three replicates were made for each treatment. Plates were then incubated at 27 $^{\circ}$C for 7 days. Fungal radial growth was measured and the inhibitory concentration for each chemical was recorded and used in the field experiment.

Field Experiment

Under field conditions at Etay El-Baroud Agricultural Research Station, El-Beheira governorate, soybean seeds cultivar Giza 21were sown in the first of June 2017, in 4 m long rows and 0.60 m apart and four rows represented a replicate. Seeds were inoculated with Okadeen (*Rhizobium japonicum*) and sown in hills 20 cm apart on one side of each ridge at a rate of 3 seeds/hill. All treatments were replicated three

times and arranged in a randomized completely block design (RCBD). The pre-harvest spraying of organic compounds was at the concentrations 100 mg/ml, and 10 mg/ml of NPs to evaluate their effects on disease incidence and aflatoxins levels on stored seeds. The above mentioned compounds were sprayed on soybean plants three times; 1st spray at bloom stage, 2nd and 3rd after two weeks each **(Soltan et al., 2016).** Deionized water was used as a control. After 120 days from sowing, seeds of all treatments were harvested and packed in paper bags. The effect of the previous treatments on weight of 100 seeds (g), percentage of disease incidence, total seeds protein content and levels of AFs pre and post storage were evaluated.

Effect of Organic Compounds and NPs Pre-harvest Spraying on:

Weight of 100 seeds (g)

At soybean harvesting time (120 days from sowing), samples of soybean seeds were collected randomly and the average weight of 100 dry seeds from each treatment were recorded.

Disease incidence caused by A. flavus group

Detection of infected soybean seeds with *A. flavus* group was carried out according to the International Seed Testing Association **(ISTA, 1996)**. Forty five seeds from each treatment were tested using the standard blotter. Five seeds were plated in 9 cm diameter Petri-dish containing three layers of water-soaked blotters using sterilized tap water. The plates were incubated at 20±2 $^{\circ}$C for 7 days. Plates were divided to three replicates (3 plates/replicate) and examined under a stereoscopic binocular microscope (6-50X) for the presence of *A. flavus* group mycelial growth **(Singh et al., 1991).**

Seeds total protein content

Kjeldahl method was used for determination of total protein content in soybean seeds **(Francis et al., 2015)** at the Environmental and Food Biotechnology Laboratory, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City.

Aflatoxin(s) production levels

Soybean seeds preparation

Aflatoxin(s) produced by *A. flavus* group were determined immediately after harvest (zero time) and after one year from storage. Two hundreds g of soybean seeds for each treatment were divided into two groups, the first group (100 g) was artificially inoculated with *A. flavus* group using a mixture of 100 ml of aqueous spore suspension at a concentration of $3x10^6$ spores/ml and 100 g of talc powder and mixed thoroughly with seeds at the rate of 10 g/treatment and the other (100 g) was

stored without inoculation. Each treatment was packed in a sterile cloth bag and stored at 95% RH and 35 °C.

Determination of aflatoxin(s) levels

Extraction procedure

Ten g of grinding seeds were mixed with 20 ml of acetonitrile: methanol (40:60 v/v), stand for 30 min at 25 °C and shacked for the same time. One g of Sodium chloride (NaCl) and 4 g of Magnesium Sulfate ($MgSO_4$) were added and shacked for 10 min. Subsequently, an aliquot (2 ml) of supernatant was evaporated to dryness and rainsed with 0.5 ml acetonitrile 10%. Derivatization process was done by using trifluoroacetic acid (TFAA) under incubation for 15 min. After cooling, the supernatant was subjected to liquid chromatographic measurement **(Choochuay et al., 2018).**

High performance liquid chromatography (HPLC)

The measurement was done by using HPLC, Agilent Technologies 1260 Infinity equipped with fluorescence detector. The analytical column used was C_{18} (3.9 × 25 mm with 5 μm particle size). The column was maintained at 40 °C. Analysis was run at a flow rate of 1 ml/min by an isocratic mobile phase using a mixture of acetonitrile: methanol: water (15:15:70 v:v:v) for 20 min. The detection was employed under excitation and emission wavelength 360 and 440 nm, respectively. Chromatograms were displayed with standard of B1, B2, G1 and G2 through class VP-LC software.

Method validation

Fortified aflatoxin-free samples were analyzed during the method development as well as verify limits of detection (LODs) and recovery percentage. For the study of recoveries, standards were added to analytic-free samples at two levels prior to the extraction step and the spiked samples were analyzed by the method described above. All experiments were performed in duplicates under the same experimental conditions. The LOD was calculated as the lowest concentration of AFs giving a single response 3 times greater than the average of the baseline noise obtained from 10 independent blank samples.

The methods and instruments were fully validated as a part of laboratory quality assurances systems (**ISO/IEC, 1990**). The codex committee's criteria for quality assurance were followed to determine the performance of toxic materials.

Statistical Analysis

The obtained data were analyzed as RCBD. Data were tested using the general linear model (GLM) procedure of the statistical analysis system (SAS) (version 9.3)

and means were compared using the least significant difference (LSD) at $P < 0.05$ **(Steel and Torrie, 1960).**

RESULTS AND DISCUSSION

Nanoparticles Characterization

Examined NPs exhibited characteristic spherical shape with size in ranges; 20-40, 20-30 and 20-35 nm for MgO, ZnO and CuO, respectively, as visualized in SEM images Figure 1(a, b and c). EDA pattern for elemental analysis is plotted in the same figure (a1, b1 and c1) displaying the dominance of Mg, Zn and Cu percentages; 100, 97 and 100% of the total contents.

Laboratory Experiment

Data presented in Table (1) show that, all the tested organic compounds (salicylic, succinic, oxalic, ascorbic acids and EDTA) at their concentrations significantly decreased the radial growth of *A. flavus* group. This is in close with the findings of **Wu et al. (2011)** whose demonstrated effects of succinic acid on the growth and conidia germination of *Fusarium oxysporum* f. sp. *niveum*, as well as on salicylic acid on the growth of *Aspergillus flavus* **(Panahirad et al., 2014);** oxalic acid on *A. flavus* **(Hassan et al., 2015);** EDTA on *Rhizoctonia solani* **(Rani et al., 2016)** and ascorbic acid on *A. parasiticus* **(Dana et al., 2018).** The reduction of the radial growth of the tested fungi increased with increasing the concentration. In case of succinic and oxalic acids, the radial growth was completely inhibited at concentration of 75 mg/ml. At the same time, all the tested organic compounds at 100 mg/ml completely inhibited the fungal growth. Generally, oxalic acid decreased the fungal growth with an average of 3.56 cm followed by succinic acid and EDTA with averages of 3.62 and 4.62 cm, respectively. This finding is in agreement with that obtained by **Hassan et al. (2015),** where oxalic acid at (5 and 10%) gave low inhibition effect of *A. flavus* (7.71 and 8.54%), respectively. In contrast, oxalic acid at concentrations up to 20 mM did not induce any inhibition in radial growth of *F. oxysporum* f.sp. *lycopersici* either in solid or liquid media used at *in vitro* experiments **(Attitalla and Brishammar, 2002).** In case of succinic acid, **Aderiye et al. (1998)** reported that the inhibition percentage of germination of viable fungal spores of *Botryodiplodia theobromae* by 0.01% succinic or citric acids ranged between 51.6 and 58.1%, respectively. EDTA antifungal properties were mainly tested on yeasts **(Kubo et al., 2005).** Other investigations stated its antifungal effects on *Candida albicans* and *A. fumigates* **(Hachem et al., 2006).** Some mechanisms have been suggested to explain the inhibitory mode of organic acids, where **Kang et al. (2003)** reported that organic acids decreased pH value, this may influence the growth by acidifying the cell, which will consume a great amount of energy to

maintain the intracellular pH homeostasis. pH affects the permeability of the cell membrane and on the enzymes that are active in degrading the substrate **(El-Kadi, 2003; Hauka et al., 2005).** On the other hand, organic acids caused the death by the susceptible organisms act on the plasmic membrane by neutralizing its electrochemical potential and increasing its permeability **(Dalie et al., 2010).** The decreasing of pH resulted a greater concentration of protons and increasing the diffusion of acid across the plasmic membrane and the cytoplasm **(Lopez et al., 2012; Palaez et al., 2012).**

Also, data presented in Table (2) show that, all the tested NPs (CuO, MgO and ZnO) at the tested concentrations significantly decreased the radial growth of *A. flavus* group. The radial growth decreased with increasing the NPs concentrations. The antimicrobial activity of the NPs is known to be a function of the surface area in contact with the microorganisms, because large surface area enhances their interaction with the microbes to carry out a broad range of probable antimicrobial activities **(Martinez-Gutierrez et al., 2010).** It can be noticed that, MgO and ZnONPs completely inhibited the radial growth at 5 and 7.5 mg/ml, respectively. Also all the tested NPs at 10 mg/ml completely inhibited *A. flavus* group radial growth. Generally, MgO, ZnO and CuO decreased the fungal growth with averages of 2.66, 3.38 and 4.56 cm, respectively. In this respect, different types of NPs like Cu, Zn, iron (Fe) **(Hassan et al., 2013)** Mg and gold (Au) **(Gu et al., 2003)** were reported to have antimicrobial effect. The inhibitory effect of NPs may be due to release of extracellular enzymes and metabolites that serve as an agent for their own survival when exposed to stress from toxic materials and temperature variations **(Pere-de-Luque and Diego, 2009).** It is also may be due to suppression of enzymes and toxins produced by the fungi for pathogenesis **(Vahabi et al., 2011).**

Effect of organic compounds and nanoparticles pre-harvest spraying on:

Weight of 100 seeds (g)

Data presented in Table (3) show that, soybean plants sprayed with the tested organic compounds and NPs individually caused significant increase in the weight (g) of 100 seeds in relative to the control treatment except in case of oxalic acid treatment. EDTA treatment had the highest 100 seeds weight (g) with an average of 21.29 g followed by ascorbic acid and ZnONPs with averages of 19.24 and 18.91 g, respectively. Compared to the control treatment, these increments are 22.43, 10.64 and 8.74%, respectively. In this respect, **Kuchlan et al. (2017)** reported that foliar spraying of salicylic acid showed positive effect on soybean seed yield and seed health. Also, **El Mantawy (2017)** revealed that, applications of ascorbic acid were very effective to improve sunflower (*Helianthus annuus* L.) growth characters in relative to the control treatment. **Nofal et al. (1990)** found that, growth of cotton, corn, bean, pea and sunflower increased by organic acids external treatment,

especially succinic acid. Moreover, the positive effect of ascorbic acid on growth parameters may be due to its stimulatory effect on many physiological processes, such as respiration activities, cell division and many enzymes activities as reported by **Youssef et al. (2015).** In case of NPs treatments, **Parmer (2016)** revealed that the foliar application of ZnONPs (500 ppm) significantly enhanced groundnut pod yield.

Disease incidence caused by A. flavus group

Results in Table (4) show the effect of tested compounds foliar sprayed on soybean plants concern the disease incidence with *A. flavus* group on produced seeds. In case of the tested organic compounds, all of them decreased the disease incidence significantly with variable degrees in relative to control treatment. Succinic and oxalic acids had the best effect in reducing the disease incidence with averages of 75.00 and 50.06%, respectively. **Xiang et al. (2017)** reported that oxalic and succinic acids inhibited the sporangia yield and zoospores release rate of *Phytophthora parasitica* var. *nicotiana* in any concentration. **Askarne et al. (2011)** cleared that, EDTA completely inhibited mycelial growth and sporulation of *Penecillium italicum* at only 0.02 M. Salicylic acid cause several physiological changes such as making granulation of cytoplasm, damage of cytoplasmic membrane and inhibition or inactivation of extracellular and intracellular enzymes due to their indirect influence in modifying the expression of some specific genes **(Cowan, 1999).**

In case of the tested NPs, MgO had the best effect in reducing the disease incidence followed by CuO and ZnO with averages of 75.00, 50.06 and 50.06%, respectively, compared with untreated control. Different types of NPs like Cu, Zn and Fe **(Nabawy, 2015)** were reported to have antimicrobial effect. Also, **Habibi et al. (2017)** showed that, the mean Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values of MgONPs for the species of *A. flavus, A. fumigatus, A. niger* and *A. parasiticus* were 10.1 and 10.31 mg/ml, respectively and these values for CuONPs were 10.25 and 10.08, respectively. On the other hand, the antimicrobial effect of ZnO was reported to occur by 2 ways. The first is the formation of H_2O_2 on the surface of ZnO due to the possible formation of hydrogen bond between hydroxyl group of cellulose molecules of fungi with oxygen atom of ZnO leading to inhibition of the microbial growth, while the second is the release of Zn^{+2} that causes damages of cell membrane **(Moraru et al., 2003).** Cytotoxicity of CuONPs results not only from the small size of the particles, high specific surface value and close interaction with microbial membranes but also from formation of leached cuprum-peptide complexes leading to several-fold increase in reactive oxygen intermediate (ROI) generation, cell viability decrease and general biomass growth suppressed **(Gunawan et al., 2011).** Sometimes, the cell molecules are destroyed and this mechanism occurs in such ways that the Cu bind with the

phosphate groups that are part of the structural backbone of DNA molecules. As a result, this mechanism may lead to the separation of the double helix **(Meyer, 2001).**

Seeds total protein content

Data presented in Table (5) show that, soybean plants sprayed with the tested organic compounds and NPs individually caused an increase in seeds total protein content (%). In case of the tested organic compounds, EDTA had the first grade followed by oxalic acid with averages of 38.5 and 38.1%, respectively. The increment in soybean seeds total protein content ranged between 53.2 – 2.87% compared with untreated control. **Goli et al. (2012)** reported that, the treatment with salicylic acid spraying to three-week-old soybean plants increased protein from 2.9% to 3.4%. Also, spraying of ascorbic acid on sunflower (*Helianthus annuus* L.) cultivar Sakha 53 caused significant promotion of seeds protein content with comparison to the untreated plants **(El Mantawy, 2017)**. Plants sprayed with the tested NPs produced seeds containing higher in total protein content than that in organic compounds treatments. Plants sprayed with ZnONPs produced seeds had the highest protein content with an average of 56.1% followed by MgO and CuO with averages of 53.2 and 45.9%, respectively. This finding is in agreement with that reported by **Kabata-Pendias and Pendias (1999)** who mentioned that Zn is contained in the structure of 200 enzymes and transcriptional factors which have important roles in protein and carbohydrate synthesis. Treatment of cluster bean with foliar sprays of ZnONPs at (10 mg/L) caused a significant increase in total soluble leaf protein (27.1%) **(Raliya and Tarafdar, 2013).**

Aflatoxin(s) Production Levels

At zero time

Aflatoxin(s) produced by *A. flavus* group were determined directly after harvest (zero time). The concentrations of individual AFs; B1, B2, G1 and G2 were detected in all treated samples compared with control (Table 6). Both organic compounds and NPs altered AFs levels. Ascorbic acid treatment exhibited the lowest potent induction for B1 (17.55 ng/g), but B2 was not detectable in case of salicylic and oxalic acids treatments. Both of them as well as ascorbic acid treatment had non-detectable levels of G1. In respect to G2, it can be noticed that oxalic acid treatment only had non-detectable level. The organic compounds exhibited their reducing effect of AFs levels in the following order; EDTA< ascorbic acid< oxalic acid< salicylic acid< succinic acid with averages of 6.02, 9.00, 13.27, 29.92 and 31.46 ng/g, respectively. Regarding induction by NPs, CuO treatment had the least level for B1 (19.33 ng/g) and with respect to B2 and G1, it can be noticed that (CuO and MgO) and (CuO and ZnO) treatments had non-detectable levels of AFs. In case of G2, ZnONPs treatment had the least level with an average of 29.38 ng/g. Examined

NPs exhibited their reducing effect of AFs in the following order: CuO< MgO< ZnONPs with averages of 19.33, 42.87 and 58.57 ng/g, respectively.

Aflatoxin(s) residue levels in artificially infected soybean stored seeds

The residual levels of AFs (ng/g) in artificially infected seeds under storage period for one year are listed in Table (7). All the tested organic compounds and NPs in addition to control treatment had non-detectable levels of B1, G1 and G2, but in case of B2, oxalic acid treatment had the least level followed by salicylic acid and EDTA with averages of 11.74, 12.65 and 14.75 ng/g, respectively. In respect of NPs, CuO treatment had lower level than MgO and ZnONPs with an average of 12.94 ng/g.

Aflatoxin(s) residue levels in non- infected soybean stored seeds

In comparative with non-infected stored seeds, B2 production (ng/g) is listed in Table (8). All treatments altered B2 production compared with untreated control, except ZnONPs treatment which enhanced B2 generation with value of 20.0 ng/g. The inhibition potent of examined compounds was in the following order; CuONPs> MgONPs> EDTA> succinic acid> salicylic acid> oxalic acid> ascorbic acid with values; 6.17, 8.61, 8.64, 9.30, 10.64, 10.65 and 12.03 ng/g, respectively.

EDTA is recognized as an antimicrobial agent who disrupts the membrane integrity and as a potentiator of other lethal agents **(Oita, 2003). Abrunhosa and Venancio (2008)** demonstrated that Na_2EDTA can significantly inhibit the growth rate of black *aspergilli* isolated from grapes and reduce the ochratoxin A produced by the ochratoxigenic isolates. The finding data arising non-detectable of AFs after storage period is accordance with that stated previously, where some acids and their derivatives have the ability to react with lactone groups of B1 and G1 and with non-aromatic double bonds present in AFs derivative. On the other hand, some alkaline and oxidant agents such as peroxides or oxyradicals are reactive with non-conjugated double bonds of AFs **(Borrell and Gimeno, 2002).**

CONCLUSION

In vitro present results clearly exhibited that succinic and oxalic acids completely inhibited the radial growth of *A. flavus* group at the concentration of 75 mg/ml. At the same time, all the tested organic compounds completely inhibited the fungal growth at 100 mg/ml. Also, it can be noticed that, MgO and ZnO NPs completely inhibited the radial growth at 5 and 7.5 mg/ml, respectively. All the tested NPs at 10 mg/ml completely inhibited the radial growth.

In vivo, soybean plants sprayed three times at bloom stage with the tested organic compounds at 100 mg/ml and NPs at 10 mg/ml individually caused an

increase in the weight of 100 seeds (g), a decrease in the disease incidence (%) and an increase in the seeds total protein content (%) compared with untreated control.

After harvest (zero time) EDTA and CuONPs treatments had the best effect in reducing AFs levels (B1, B2, G1 and G2) in soybean seeds with averages of 6.02 and 19.33 ng/g, respectively. Under storage condition (95% RH and 35 °C) for one year, the residual levels of AFs in artificially infected seeds and in non- infected seeds, all the tested organic compounds and NPs treatments had non-detectable levels of B1, G1 and G2. In case of B2, oxalic acid treatment which infected with *A. flavus* group and CuONPs treatment which non-infected had the least levels with averages of 11.74 and 6.17 ng/g, respectively.

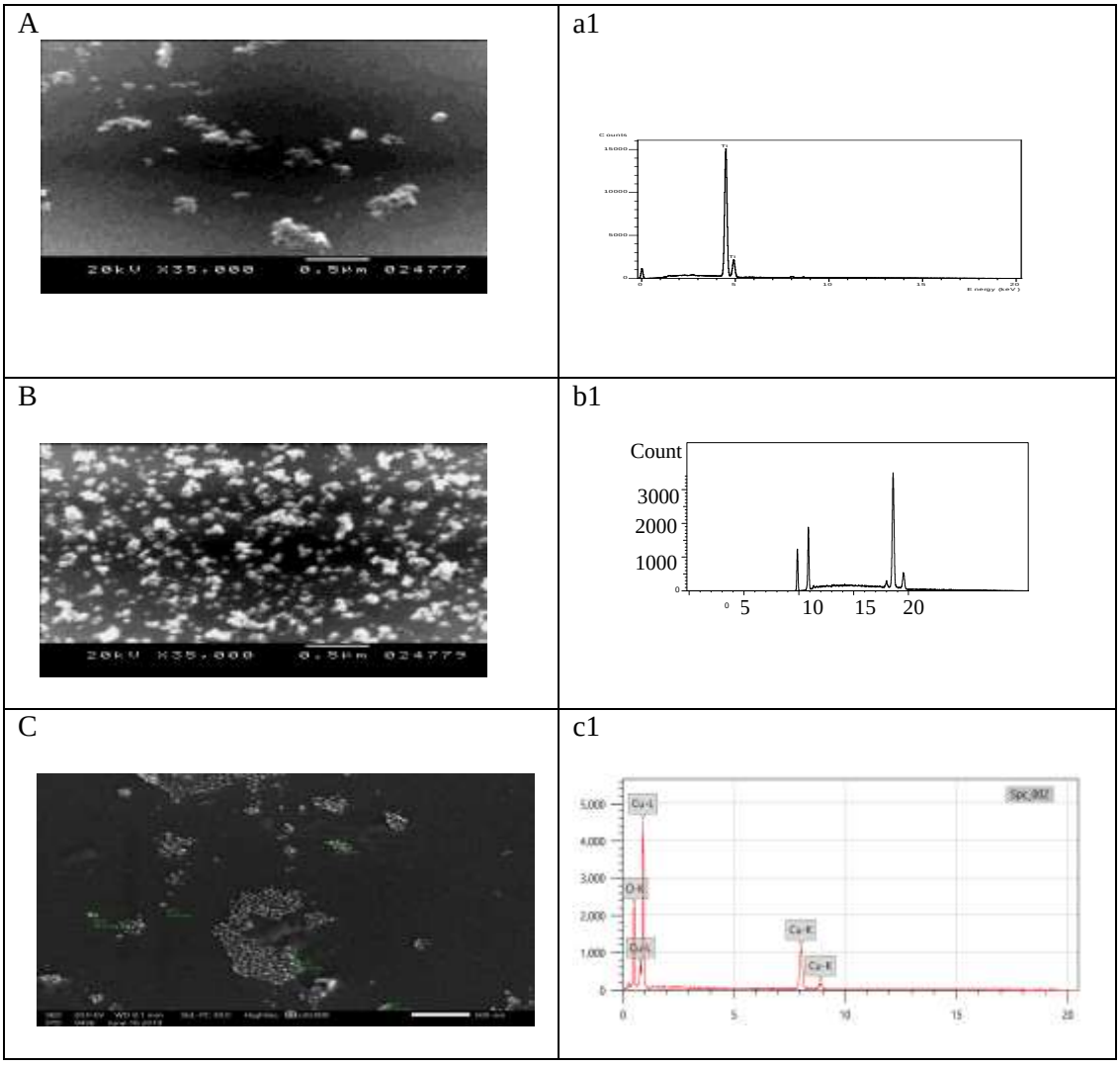

Fig (1): Characterization of NPs using SEM visualized at 35.000x for (A) MgO, (B) ZnO and (C) CuO. EDA patterns of (a1) Mg, (b1) Zn and (c1) Cu purities, respectively, in prepared particles.

Table (1): Effect of different concentrations of organic compounds on radial growth (cm) of *Aspergillus flavus* group on PDA medium, incubated at 27 °C for 7 days.

Organic compound	Concentration (mg/ml) 0	25	50	75	100	Mean
Salicylic acid	9.0	7.2±0.17^{a}	5.1±0.34^{a}	2.3±.35^{a}	0.0	4.72±0.17^{A}
Succinic acid	9.0	6.3±0.15^{b}	2.8±0.47^{b}	0.0±0.47^{b}	0.0	3.62±0.22^{B}
Oxalic acid	9.0	5.9±0.29^{b}	2.9±0.44^{b}	0.0±0.47^{b}	0.0	3.56±0.24^{B}
Ascorbic acid	9.0	7.3±0.21^{a}	4.9±0.27^{a}	2.1±0.28^{a}	0.0	4.66±0.15^{A}
EDTA	9.0	6.9±0.06^{a}	5.0±.30^{a}	2.2±0.31^{a}	0.0	4.62±0.14^{A}
Mean	9.00^{A}	6.72±0.88^{B}	4.14±0.03^{C}	1.32±1.03^{D}	0.00^{E}	

Table (2): Effect of different concentrations of some NPs on radial growth (cm) of *Aspergillus flavus* group isolate on PDA medium, incubated at 27 °C for 7 days.

Nanoparticle	Concentration (mg/ml) 0	2.5	5.0	7.5	10.0	Mean
CuO	9.0	6.9 ±0.46^{a}	4.8±0.86^{a}	2.1±.49^{a}	0.0	4.56±0.36^{A}
MgO	9.0	4.3±0.46^{c}	0.0±0.84^{c}	0.0±0.25^{b}	0.0	2.66±0.30^{B}
ZnO	9.0	5.6±0.00^{b}	2.3±0.02^{b}	0.0±0.25^{b}	0.0	3.38±0.05^{C}
Mean	9.0^{A}	5.60±0.7^{B}	2.37±^{C}	0.70±1.0^{D}	0.00^{E}	

Table (3): Effect of tested chemicals individually sprayed on soybean plants three times at bloom stage on the weight of 100 seeds (g).

Treatment	Mean of 100 seeds weight (g)	Increase (%)
Organic compound (100 mg/ml):		
Salicylic acid	18.26±0.16^{cd}	5.00±1.28^{de}
Succinic acid	18.62±0.03^{bc}	7.07±0.54^{bc}
Oxalic acid	17.88±0.29^{de}	2.82±2.05^{e}
Ascorbic acid	19.24±0.19^{b}	10.64±0.72^{b}
EDTA	21.29±0.91^{a}	22.43±4.89^{a}
Nanoparticle (10 mg/ml):		
CuO	18.46±0.09^{cd}	6.15±0.87^{cde}
ZnO	18.91±0.07^{bc}	8.74±0.05^{bc}
MgO	18.44±0.10^{cd}	6.04±0.91^{cde}
Control	17.3±0.50^{e}	---

Table (4): Effect of tested chemicals individually sprayed on soybean plants three times at bloom stage on disease incidence of produced soybean seeds infected by *Aspergillus flavus* group.

Treatment	Disease incidence (%)	Reduction (%)
Organic compound (100 mg/ml):		
Salicylic acid	13.34±1.05[b]	25.06±7.73[c]
Succinic acid	4.45±2.10[d]	75.00±9.93[a]
Oxalic acid	8.89±0.53[c]	50.06±1.11[b]
Ascorbic acid	13.34±1.05[b]	25.06±7.73[c]
EDTA	13.34±1.05[b]	25.06±7.73[c]
Nanoparticle (10 mg/ml):		
CuO	8.89±0.53[c]	50.06±1.11[b]
ZnO	8.89±0.53[c]	50.06±1.11[b]
MgO	4.45±2.10[d]	75.00±9.93[a]
Control	17.80±2.62[a]	---

Table (5): Effect of tested chemicals individually sprayed on soybean plants three times at bloom stage on seeds total protein content (%).

Treatment	Seeds total protein content (%)	Increase (%)
Organic compound (100 mg/ml):		
Salicylic acid	36.9±1.72[c]	5.73±5.80[c]
Succinic acid	35.9±2.07[c]	2.87±6.81[c]
Oxalic acid	38.1±1.30[c]	9.17±4.58[c]
Ascorbic acid	36.4±1.90[c]	4.30±6.31[c]
EDTA	38.5±1.15[c]	10.32±4.18[c]
Nanoparticle (10 mg/ml):		
CuO	45.9±1.46[b]	31.52±3.32[b]
ZnO	56.1±5.07[a]	60.75±13.65[a]
MgO	53.2±4.04[a]	52.44±10.71[a]
Control	34.9±2.43[c]	---

Table (6): Aflatoxins residue levels (ng/g) in soybean seeds after plant treatments with different chemicals three times at bloom stage (zero time).

Treatment	Aflatoxins residue levels (ng/g)				Mea
	B1	B2	G1	G2	
Organic compound (100					
Salicylic acid	104.53±2.0	ND	ND	15.16±0.	29.9
Succinic acid	20.15±0.09	31.61±0.87	14.43±0.26	59.63±0.	31.4
Oxalic acid	53.07±1.05	ND	ND	ND	13.2
Ascorbic acid	17.55±0.31	8.66±0.46[c]	ND	9.77±0.3	9.00
EDTA	20.10±0.07	3.23±0.06[d]	0.68±0.04[d]	0.07±0.0	6.02
Nanoparticle (10 mg/ml)					
CuONPs	40.69±0.33	ND	ND	36.62±0.	19.3
ZnONPs	144.32±1.9	60.56±1.45	ND	29.38±0.	58.5
MgONPs	116.16±1.0	ND	6.62±0.44[b]	48.69±1.	42.8
Control (untreated)	10.46±0.13	8.31±0.17[c]	1.42±0.05[c]	58.62±1.	19.7

Table (7): Aflatoxins residue levels (ng/g) in artificially infected soybean seeds with *A. flavus* group after one year of storage under conditions (95% RH and 35 °C)

Treatment	Aflatoxins residue levels (ng/g)			
	B1	B2	G1	G2
Organic compound (100 mg/ml)				
Salicylic acid	ND	20.69±0.46[a]	ND	ND
Succinic acid	ND	12.65±0.16[d]	ND	ND
Oxalic acid	ND	11.74±0.39[d]	ND	ND
Ascorbic acid	ND	21.80±0.34[a]	ND	ND
EDTA	ND	14.75±0.38[c]	ND	ND
Nanoparticle (10 mg/ml)				
CuONPs	ND	12.94±0.16[d]	ND	ND
ZnONPs	ND	21.62±0.01[a]	ND	ND
MgONPs	ND	17.70±0.39[b]	ND	ND
Control (un treated)	ND	16.81±0.32[b]	ND	ND

Table (8) : Aflatoxins residue levels (ng/g) in non-infected soybean seeds after one year of storage under conditions (95% RH and 35 °C).

Treatment	Aflatoxins residue levels (ng/g)			
	B1	B2	G1	G2
Organic compound (100 mg/ml)				
Salicylic acid	ND	10.64±0.37[d]	ND	ND
Succinic acid	ND	9.30±0.36[e]	ND	ND
Oxalic acid	ND	10.65±0.35[d]	ND	ND
Ascorbic acid	ND	12.03±0.14[c]	ND	ND
EDTA	ND	8.64±0.29[e]	ND	ND
Nanoparticle (10 mg/ml)				
CuONPs	ND	6.17±0.07[f]	ND	ND
ZnONPs	ND	20.00±0.40[a]	ND	ND
MgONPs	ND	8.61±0.64[e]	ND	ND
Control (untreated)	ND	13.64±0.38[b]	ND	ND

*Each value in all tables represents the mean of duplicates ± SE. The same letters indicate no significant different at 0.05 levels. ND=non-detectable.

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REFRENCES

Abrunhosa L. and Venancio A. (2008). *In vitro* Antifungal Effect of EDTA Disodium Salt in Tested Black *Aspergilli*. *Asian J. of Biochem*., **3**, 176-181.

Aderiye B., Laleye S. and Ojo B. (1998). Toxicity of citric and succinic acids on the pycnidiospores of *Botryodiplodia theobromae*. *Folia Microbiol*., **43**, 147-150.

Ash M., Livezey J. and Dohlman E. (2006). Soybean backgrounder. Electronic Outlook Report from the Economic Research Service, USDA.

Askarne L., Talibi I., Boubaker H., Serghini M., Boudyach E. and Ait Ben Aoumar A. (2011). Effects of organic acids and salts on the development of *Penicillium italicum*; The causal agent of citrus blue mold. *Plant Pathol. J.* **10**, 99-107.

Attitalla I. and Brishammar S. (2002). Oxalic acid elicited resistance to fusarium wilt in *lycopersicon esculentum* Mill. *Plant Protec. Sci*., **38**, (1), 128-131.

Borrell J. and Gimeno G. (2002). Micotoxinas en los alimentos: medidas de prevención y detoxificación. *Selecciones Avícolas*, **1**, 567-571.

Capeleti I., Ferrarese M., Krzyzanowski F. and Ferrarese F. (2005). A new procedure for quantification of lignin in soybean (*Glycine max* (L.) Merrill) seed coat and their relationship with the resistance to mechanical damage. *Seed Sci. Technol*., **33**, 511-515.

Choochuay S., Phakam J., Jala P., Maneeboon T. and Tansakul N. (2018). Determination of Aflatoxin B_1 in Feedstuffs without Clean-Up Step by High-Performance Liquid Chromatography. *Int. J. of Ana. Chem*., 8 pages.

Cowan M. (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev*. **12**(4), 564-582.

Criseo G., Bagnara A. and Bisignano G. (2001). Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group . *Lett. Appl. Microbiol*., 33 (4) : 291-295.

Dalie D., Deschamps A. and Forget F. (2010). Lactic acid bacteria-potential for control of mold growth and mycotoxins: A review, *Food Control*, **21**, 370-380.

Dana Maryam A., Kordbacheh P., Ghazvini R., Moazeni M., Nazemi L. and Rezaie S. (2018). Inhibitory effect of vitamin C on *Aspergillus parasiticus* growth and aflatoxin gene expression. *Curr. Medi. Mycol*. **4**(3), 10-14.

El-Kadi S. (2003). Studies on the microbial production of citric acid, M. Sc. Thesis, Agric. Microbiol. Dept., Fac. of Agric., Mansoura Univ., Egypt.

El Mantawy R. (2017). Physiological role of antioxidants in improving growth and productivity of sunflower under different sources of nitrogen fertilizers**.** *Egy. J. of agron*. **39**: 167-177 (2017).

El-Metwally M., El-Saidy Aml E. and Abd El-Hai K. (2014). Role of some vitamins in decreasing soybean seed deterioration during storage. *Res. J. Seed Sci.* **7**(4), 97-115.

Francis A., Nnamani Emmanuel V., Eze Chukwuebuka G. (2015). Estimation of protein content in soyabeans consumed in Abakaliki, Ebonyi State, Nigeria. *J. Pharma. Biol. Sci*. **10**(1), 8-12.

Goli M., Pande M. and Bellaloui N. (2012). Effects of chelating agents on protein, oil, fatty acids, and minerals in soybean seed. *Agric. Sci.,* **3**, 517-523.

Gu H., Ho P., Tong E., Wang L. and Xu B. (2003). Presenting vancomycin on nanoparticles to enhance antimicrobial activities. *Nano letters*, **3**(9), 1261-1263.

Gunawan C., Teoh W., Marquis C. and Amal R. (2011). Cytotoxic origin of copper (II) oxide nanoparticles: comparative studies with micron-sized particles, leachate, and metal salts, *ACS Nano,* **5**(9), 7214–7225.

Habibi N., Gheisari H., Aminlari M. and Sedaghati F. (2017). The study of antifungal activities of magnesium oxide and copper oxide nanoparticles against different species of *Aspergillus*. J. of Fasa Univ. of Medi. Sci., **7**, 317-327.

Hachem R., Bahna P., Hanna H., Stephens L. and Raad I. (2006). EDTA as an adjunct antifungal agent for invasive pulmonary aspergillosis in a rodent model. *Antimicrob. Agents Chemother*., **50**, 1823-1827.

Hassan A., El-Shafei Howayda M. and Mahmoud H. (2013). Effect of zinc oxid nanoparticles on the growth of some mycotoxigenic moulds. *J. Studies in Chem. Process Technol. (SCPT), American Society of Science and Engineering*, **1**(3), 16-25.

Hassan A., Oraby Noha H., El-Dahshan E. and Ali M. (2015). Antimicrobial potential of iron oxide nanoparticles in control of some causes of microbial skin affection in cattle. *Europ. J. Acad. Ess*., **2**(6), 20-31.

Hauka F., El-Sawah M., Kassem M. and El-Kadi S. (2005). Factors controlling citric acid production by some of *Aspergillus niger* strains. *Int. Conf. on Microbiol. and Biotechnol.*, In favor of man and environment in Africa and Arab region. Fac. Agric., Mansoura Univ.

Horky P., Skalickova S., Baholet D. and Skladanka J. (2018). Review, Nanoparticles as a solution for eliminating the risk of mycotoxins. *Nanomaterials*, **8**, 727-748.

Ismaiel A. and Tharwat N. (2014). Antifungal activity of silver ion on ultrastructure and production aflatoxin B1 and patulin by two mycotoxigenic strains, *Aspergillus flavus* OC1 and *Penicillium vulpinum* CM1. *J. Mycol. Méd.,* **24**, 193–204.

ISTA (International Seed Testing Association) (1996). International rules for seed testing rules, Seed Sci. Technol., **24**, 1-335.

Kabata-Pendias A. and Pendias H. (1999). Biogeochemistry of trace elements, *Polish Scientific Publishing Company*, Varsov.

Kang H., Park Y. and Go S. (2003). Growth inhibition of a phytopathogenic fungus, *Colletotrichum* species by acetic acid, *Microbiol. Res*., **158**, 321–326.

Koper O., John S., Marchin L., Klabunde K., Stoimenov P. and Bohra L. (2002). Nanoscale powders and formulations with biocidal activity toward spores and vegetative cells of *Bacillus* species, viruses, and toxins. *Current Microbiol*., **44**, 49-55.

Kubo I., Lee S. and Ha T. (2005). Effect of EDTA alone and in combination with polygodial on the growth of *Saccharomyces cerevisiae*. *J. Agric. Food Chem.*, **53**,1818-1822.

Kuchlan P., Kuchlan M. and Husain S. (2017). Effect of foliar application of growth activator, promoter and antioxidant on seed quality of soybean. *Legume Research*, **40**(2), 313-318.

Lopez E., Garcia H. and Malo A. (2012). Organic acids as antimicrobials to control *Salmonella* in meat and poultry products, *Food Research International*, **45**, 713-721.

Lu P., Fu W., Huang S., Lin C.,Ho M., Chen Y. and Cheng H. (2018). Methodology for sample preparation and size measurement of commercial ZnO nanoparticles. J. of food and drug analysis, **26**, 628-636

Martinez-Gutierrez F., Olive P., Banuelos A., Orrantia E., Nino N., Morales Sanchez E., Ruiz F., Bach H. and Av-Gay Y. (2010). Synthesis, characterization and evaluation of antimicrobial and cytotoxic effect of silver and titanium nanoparticles. *Nanomed.: Nanotechnol. Biol. Med.*, **6**, 681-688.

Meyer W. (2001). Coping with resistance to copper/silver disinfection. *Water Engin. Manag*., **148**:11.

Moraru C., Panchapakesan C., Huang Q. and Takhistov P. (2003). Nanotechnology: A new frontier in food science. *Food Technology*., **57** (12), 24-29.

Nabawy Gehan A. (2015). Effect of metal element nanoparticles in the growth of aflatoxogenic *A. flavus* and aflatoxin production in feed, Master thesis in veterinary science (Microbiology), Faculty of Veterinary Medicine, Cairo University.

Nofal M., Rabie M., Khalid K. and Mohamed A. (1990). Growth and some micro-nutrients content of lettuce plants as applied by seed soaking and foliar spray of

ascorbic and citric acid. Proc. of Soil Fertility and Foliar Fertilization. *Conf., Giza, Egypt*, Paper No. **3**, pp.14 – 15.

Oita S. (2003). Synergistic bactericidal effect of α-purothionin and chelating agents for gram-negative food-poisoning bacteria. *Bull. Natl. Agric. Res. Cent*., **2**, 59-66.

Panahirad S., Zaare-Nahandi F., Mohammadi N., Alizadeh-Salteh S. and Safaie N. (2014). Effects of salicylic acid on *Aspergillus flavus* infection and aflatoxin B1 accumulation in pistachio (*Pistacia vera* L.) fruit. *J. Sci. Food Agric*., **94**, 1758–1763.

Parmer S. (2016). Effect of ZnO nanoparticles on germination, growth and yield of groundnut (Arachis hypogaea L.) Dept. soil sci. Agric. Chem. College of Agric. Anand Agric. Univ., pp. 122.

Pelaez A., Catano C., Yepes E., Villarroel R., Antoni G. and Giannuzzi L. (2012). Inhibitory activity of lactic and acetic acid on *Aspergillus flavus* growth for food preservation. *Food Control*, **24**, 177-183.

Pere-de-Luque A. and Diego R. (2009). Nanotechnology for parasitic plant control. *Pest Manag. Sci.*, **65,** 540 – 545.

Raliya R. and Tarafdar J. (2013). ZnO nanoparticle biosynthesis and its effect on phosphorous-mobilizing enzyme secretion and gum contents in clusterbean (Cyamopsis tetragonoloba L.) *Agric Res*., **2**, 48-57.

Rani M., Raj S., Dayaman V., Kumar M., Dua M. and Johri A. (2016). Functional characterization of a hexose transporter from root endophyte *Piriformospora indica*. *Front Microbiol.*, **7**, 1083**.**

Rizwan M., Ali S., Qayyum M., Ok Y., Adrees M., Ibrahim M., Zia-Ur-Rehman M., Farid M. and Abbas F. (2017). The effect of metal and metal oxide nanoparticles on the growth and physiology of globally important food crops: a critical review. *J. Hazard Mater.*, **322**(Pt A), 2–16.

Singh K., Frisvad J., Thrane U. and Mathur S. (1991). An illustrated manual on identification of some seed borne *Aspergilli*, *Fusaria*, *Pencillia* and their mycotoxins. AiO Tryk as Odense, Denmark., pp: 133.

Soltan H., Mohamed Naglaa T. and Abo Rehab M. (2016). Influence of preharvest spraying with the chemical salts on grey mold disease and keeping quality of table grapes during storage. *J. of Phytopathol. and Pest Manag*., **3**(2), 26-34.

Steel R. and Torrie J. (1960). Principles and procedures of statistics. (with special reference to the biological sciences.) McGraw-Hill Book Company, New York, Toronto, London, 481 S., 15 Abb.; 81 s 6 d.

Vahabi G., Ali M. and Karimi S. (2011). Biosynthesis of silver nano-particles by fungus *Trichoderma reesei*. *Insci. J.*, **1**(1), 65–79.

Wani A. and Shah M. (2012). A unique and profound effect of MgO and ZnO nanoparticles on some plant pathogenic fungi. *J. Appl. Pharmaceut. Sci*., **2**(3), 40-44.

Wu J., Gao S., Tang L., Hou P., Gao J. and Chen F. (2011). The traits, oil content and correlation studies of seed and kernel in *Jatropha curcas* L. *Afric. J. of Agric. Res.,* **7**(10), 1487-1491.

Xiang B., Yuan Z., Wen Y., Ge W., Li X. and Feng L. (2017). Effect of phenolic and organic acid on growth of *Phytophthora parasitica* var. *nicotiana*. Southwest China *J. Agric. Sci*,. **30**(6), 1364-1368.

Youssef E., Ahmed M., Badr N., Shafaby M. and Gamal El-Din K. (2015). Alleviation of drought effect on sunflower (*Helianthus annus* L.) c.v Sakha-53 cultivar by foliar spraying with antioxidant. *Middle East J. Agric. Res*., **4**(4), 794-801.