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EVALUATION OF ANTIFUNGAL ACTIVITY OF ZINC OXIDE NANOPARTICLES AGAINST *ALTERNARIA SOLANI* THE CAUSAL AGENT OF TOMATO EARLY BLIGHT DISEASE

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ABSTRACT

One of the multifunctional inorganic nanoparticles is zinc oxide due to its effective antifungal activity. This study was designed to evaluate the antimicrobial activities of ZnO nanoparticles prepared by each of chemical and biological fabrication methods against *Alternaria solani* using the agar well-diffusion method. To characterize the changes in morphology of fungal hyphae treated with ZnO NPs Scanning Electron microscopy (SEM) was applied. Results proved that by increasing particle dose the effectiveness of nanoparticles was increased. Electron micrographs of the treated pathogen showed that the hypha lost their smoothness and appeared swollen, crumbled and shrunken. Results of application under greenhouse conditions demonstrated that 12.5 and 50 ppm of chemically fabricated ZnONPs and biologically fabricated ZnONPs, respectively, were the most effective due to their significant decrease in the percentage of diseased severity up to 21.25 and 25.50%, respectively, 14-days post pathogen inoculation compared to the control treatment. As a conclusion, the results could be suggested that ZnONPs could be considered as an effective fungicide in agricultural and food safety applications.

Key words: Inorganic nanoparticles, Chemical and biological fabrication, Zinc oxide nanoparticles, *Alternaria solani*, Antifungal activity..

INTRODUCTION

The tomato plant (*Solanum lycopersicum L.*) has been recorded in Egypt and many regions of the world as the second most important crop in terms of cultivation range, after potatoes **(Celma *et al*., 2009** and **Akhtar *et al*., 2016)**.This is because it is rich in vitamins, essential amino acids, minerals, dietary fiber and sugars **(Noonari *et al*., 2015).** Given that the world population is expected to reach nearly 8 billion people in 2025 and 9 billion people in 2050, increasing agri-cultural productivity to feed these numbers must be taken into consideration to face the needs of this growing and rapid growth (**Sekhon**, **2014).**

In addition, food security will continue to be threatened by crop loss due to pests and attacks of pathogens represented by fungi, bacteria, viruses and insects **(Bebber and Gurr, 2015)**.It has been proven that the tomato crop is always exposed to bacterial, fungal, viral and nematode diseases, and early blight caused by *Alternaria solani* is consi-dered one of the most dangerous diseases that affect the tomato crop and reduce its productivity by 80% **(Khan *et al*., 2012** and **Malik *et al*., 2014**). What makes this disease difficult to control is the uantities of secondary inoculum, other than the diversity of pathogenic fungal isolates, the activity of the disease-cycle stage for long periods, and the wide host range that it can infect. In order to resist early blight and reduce or prevent the losses it causes, tomato fields must be sprayed extensively with fungicides. Which has proven its rapid effect in reducing the spread of the disease, but it may represent health and environmental risks. Hence, there is an urgent need to use new, more efficient technologies to combat pathogens without environmental risks **(Bramhanwade *et al.*, 2016)**. The field of nanotechnology has brou-ght about a significant change in all aspects of human life so that the expected applications of nanotechnology in industries such as agriculture, medicine, cosmetics, electronics and textiles have gained momentum in recent times **(Jain *et al*., 2020)**. Due to the small size of the nanoparticles and the high surface-to-volume ratio, the nano-particles exhibit antifungal, anticorrosive and antibacterial proper-ties because they enhance their interaction with microbes to perform broad-spectrum antimicrobial activities due to their large surface area. The antimicrobial activities of ZnO against fungi were quantitatively evaluated in a culture media **(Rajiv *et al.*, 2013; Sardella *et al.*, 2017; Jamdagni *et al.*, 2018; Nandhini *et al.*, 2019; Pariona *et al.*, 2020** and **González-Merino *et al.*, 2021)**. Mainly enhancing the antimicrobial potential of ZnO NPs is

disruption of membrane structure, release of reactive oxygen species and hydrogen peroxide **(Lipovsky *et al.*, 2011**and **Sinha *et al.*, 2011)**.

MATERIALS AND METHODS

Preparation of zinc oxide nanoparticles: In this work, zinc oxide nanoparticleswere prepared using wet-chemical co-precipitation and biological method as described by **Koutu *et al.*, (2016).** Method described by **Shende *et al.*, (2016)** was used for bio reduction of zinc acetate dihydrate to zinc oxide. The physicochemical characterization of ZnO nano-particles was performed by applying XRD and TEM.

Source of early blight pathogen: The plant pathogenic fungal isolate *Alternaria solani* (NCBI GenBank accession number KY312035.1) was used.

The effectiveness of fabricated ZnO nanoparticles against *A. solani* in vitro:The method of **Mohamed (2018)** was applied to examine the antifungal activity of synthesized ZnO NPs in *in vitro* conditions by poison food essay. In this respect, chemically and biologically fabricated zinc oxide nano-particles (cZno NPs and bZno NPs) at 12.5, 25, 50, 100, 200 and 400 ppm were tested in *in vitro* against *A. Solani* (**Min *et al.*, 2009**). Control treatments were made by inoculating a disc of *A. solani* onto PDA plate without adding any nanoparticles. The rate of radial growth, the following equation was used to calculate the inhibition percentage compared to the control (**Singh and Tripathi, 1999**). These treatments were carried out in triplicate and the experiment was repeated three times.

Identification of morphological and ultra-structural damage to *A. solani:* To investigate the morphological alterations of *A. solani* hyphae treated with ZnO NPs scanning electron microscopy was applied. Pieces of mycelial material cut from 7-day-old cultures were inoculated onto the PDA containing 100 ppm ZnO NPs and the control followed by incubation for 5days at 25°C. Then, mycelia were cut from the fungal colonies' edges, and directly examined by SEM under the environmental mode.

Evaluation of ZnO NPs nanoparticles for controlling tomato early blight infection under greenhouse conditions: The present experiment was carried out on tomato plants (hybrid Janaa) in pots (30 cm Ø) under greenhouse conditions of the Plant Pathology Institute, Agricultural Research Center (ARC), Giza, during the growing season 2019.

Pottery pots (30 cmØ) were filled with a sterilized soil, and the seedlings were transferred to greenhouse (28°C/22°C day/night). Four weeks old tomato seedlings were transplanted into Pottery pots (30 cmØ) containing sterilized soil. Three seedlings per pot were transplanted. Pots were kept under a greenhouse at a temperature of 22-25°C, relative humidity about 85-90% and irrigated periodically. Each treatment was replicated as six pots. The canopy of tomato plants was sprayed individually with either of the tested chemically and biologically fabricated zinc oxide nano-particles **(cZno NPs and bZno NPs)** at different concentrations *i.e.*, 12.5,25,50,100, 200 and 400ppm, at one and seven days before inoculation at rate 30 mL per each plant. Twenty-four hours after last treatment, plants were inoculated with mycelial suspension of *A. solani* 5×10^5 cfu/mL. The inoculum of *A. solani* was prepared by culturing on PDA plates and incubation for 15 days at 25°C and 12 h light, and then 10 mL of sterile d.H_2O was added to each plate and colonies were carefully scrapped with a sterile fine brush. The resulting mycelial suspen-sion was adjusted to 5×10^5 cfu/mL.

Control treatments were in form of treated plants with Bioxan72% fungicide pre inoculation with the tested early blight pathogen, infected plants only with the tested early blight pathogen without spraying of any treatment pre inoculation and un-sprayed plants with any one of the tested treatments and without inoculation of the tested pathogen. The artificial infestation with *A. solani* was carried out as foliar spraying of pathogen suspension. Plastic bags were placed over the inoculated plants for 48 hours to maintain a high relative humidity and promote fungal infection **(Derbalah *et al.*, 2018).** Disease severity was determined at 7, 14 and 21 days post inoculation based on the percentage of leaf area covered with early blight lesions. Infected plants were rated using the modified scale (0 -10) of **James, (1971**) disease severity (%) was calculated using the equation suggested by **Townsend** and **Heuberger** (**1943).**

RESULTS AND DISCUSSION

Characterization of fabricated nanoparticles

The chemically fabricated ZnO NPs nano-particles and biologically fabricated bZnO NPs exhibited a crystalline structure with hexagonal structure of the wurtzite. The morphology of cZnO NPs presented a predominantly spherical shape with the average primary size of 5- 68.8 nm. While, the resultant bZnO NPs were spherecal in shape with a particle size ranged from 8.92-37.9 nm.

The effectiveness of cZnO NPs nanoparticles against *A. solani*in vitro: Data in **Table-1** and

Table-2 illustrated in **Figure-1** and **Figure-2** represent the growth As-3 isolate of *A. solani* that cultivated on PDA containing different concentrations of cZnO NPs and bZnO NPs and incubated at 25°C for 8 days. The use of ZnO NPs inhibited the fungal growth, and the inhibition average of mycelial growth was ranged from 13.61 to 53.06 % for cZnO NPs as ZnO NPs when the concen- tration was increased from 12.5 to 400 ppm. Regarding bZnO NPs, the reduction rate of fungal growth was varied from 14.44 to 47.78% when the concentration of ZnO NPs raised up to 400 ppm instead of 12.5 ppm. The best reduction percentage of *A. solani* growth was obtained at 400 ppm of each of cZnO NPs and bZnO NPs.

These results agree with the findings of **Rajiv *et al.*, (2013)** who reported that the maximum zone of inhibition was observed in 25 µg/mL of 27±5 nm size ZnO NPs against *Aspergillus flavus* and *Aspergillus niger*. **Yehia and Ahmed (2013)** noted that ZnO NPs antifungal activity was concentrated dependent. In the same direction, **Jamdagni *et al.*, (2018)** showed that synthesized ZnO NPs were found to be effective against a number of phytopathogens belonging to genera *Alternaria, Aspergillus Botrytis, Fusarium* and *Penicillium* with the lowest MIC value recorded as 16 g/mL. Moreover, **Nandhini *et al.*, (2019)** showed that the treatment with 50 ppm of ZnO NPs resulted in the plasmolysis of the *Sclerospora graminicola* zoospore and an inhibition of spore germination. **Dimkpa *et al.*, (2013)** reported that the ZnO NPs' antifungal action against *Fusarium graminearum* seems to be fungistatic rather than fungicidal.

Some investigators **(Lipovsky *et al.*, 2011; Sinha *et al.*, 2011; Lv *et al.*, 2012; Mudun –kotuwa *et al.*,2012** and **Jalal *et al.*, 2018)** reported that one proposed mechanism of antifungal effect of ZnO-NPs is the action of ROS, which produced by the nanoparticles, and /or Zn^{2+} on N-acetylglucosamine or b-1,3-D-glucan synthase. While **Mosquera-Sánchez *et al.*, (2020)** indicated that the mechanisms of action of nanoparticles on fungi are not well understood, they can be physical or chemical in nature. Physical mechanisms depend mainly on the size and surface characteristics of NPs.

Table-2: Effect of biologically fabricated bZnO-NPs at various concentrations on hyphal diameter growth of *A. solani* isolate.

Tested treatment	Conc. (ppm)	Linear growth (mm)	Fungal growth inhibition (%)
Biologically fabricated zinc oxide nanoparticles (bZnuO NPs)	**12.5**	77.00	14.44
	25	75.00	16.67
	50	68.50	23.89
	100	51.33	42.96
	200	47.50	47.22
	400	47.00	47.78
Control		90.00	0.00
L.S.D at 5%		**0.26**	

Table-1: Effect of chemically fabricated cZnO-NPs at Various concentrations on hyphal diameter growth of *A. solani* isolate.

Tested treatment	Conc. (ppm)	Linear growth (mm)	Fungal growth inhibition (%)
Chemicall y fabricated zinc oxide nano-particles (cZnO NPs)	**12.5**	77.75	13.61
	25	76.13	15.42
	50	74.25	17.50
	100	47.50	47.22
	200	44.00	51.11
	400	42.25	53.06
Control		90.00	0.00
L.S.D at 5%		**0.37**	

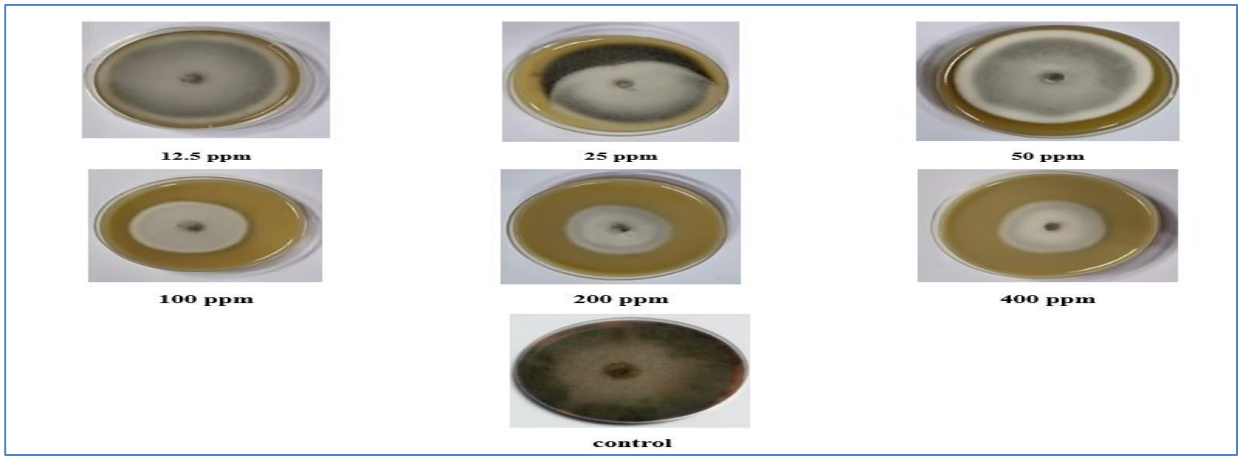

Fig.-1: Effects of chemically fabricated cZnO-NPs nano-particle sat various concentration on A. soloni isolate hyphal diameter growth.

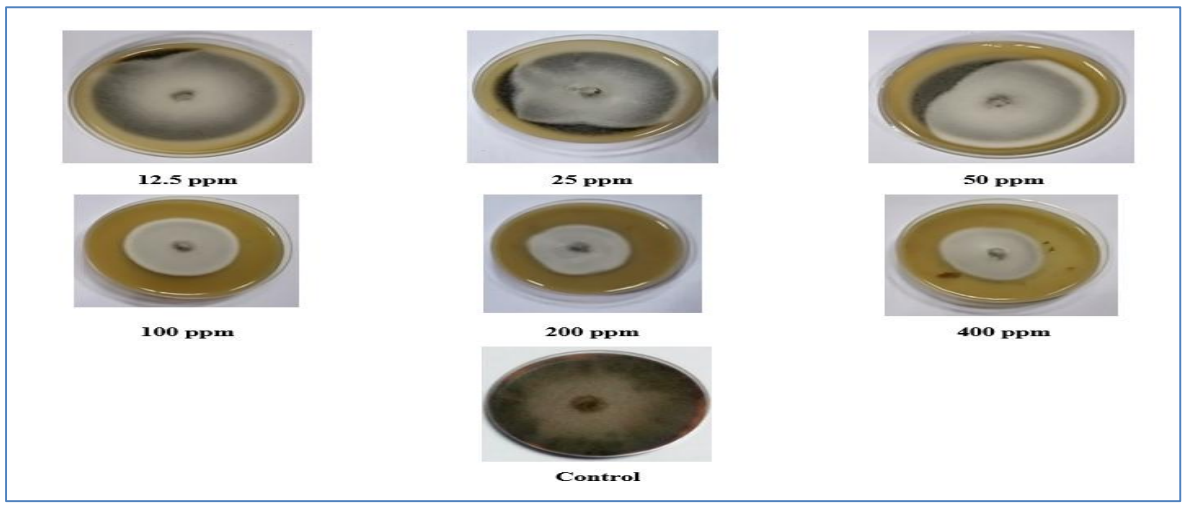

Fig.- 2: Effects of biologically fabricated bZnO-NPs nano-particles at various concentrations on *A. solani* isolate hyphal diameter growth.

Identification of morphological and ultra-structural damage to *A. solani* isolate due to zinc oxide nanoparticles treatment using scanning electron microscopy: The ultra-structure of *A. solani* hyphae before and after expo-sure to zinc oxide nanoparticles was studied in order to better understand the zinc oxide nano-particles' control mechanism. Electron micro-graphs (**Figure-3**) showed that the disruption of cell integrity, especially the vegetative parts, could be clearly observed, and the physical changes described relative to the control. The images of mycelia obtained from the edge of *A. solani* culture showed that hypha with typical net structure and smooth surface were observed. Post treatment with 100 ppm of chemically fabricated cZnO NPs, the hypha crumbled, shrunken, appeared swollen, and lost their smoothness. This indicated that ZnO NPs caused growth inhibition in *A. solani* may be due to deformation in the structure of fungal hypha. Results are in agree-ment with those reported by **Yehia and Ahmed (2013)** and **Sardella *et al.*, (2017).** They reported that SEM micrograph and raman spectra demons-trated that ZnO NPs produced deformation in fungal hyphae and inhibited the growth of conidiophores and conidia. Similar observations were reported by **Ahmad *et al.*, (2020)** and supported the experimental results.

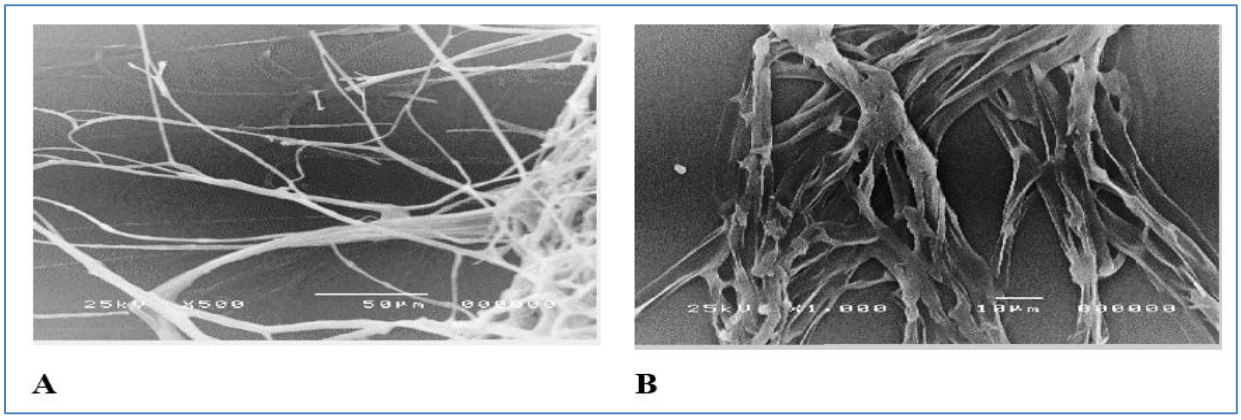

Fig. -3: Scanning electron microscopy images of *A. solani* without (A) or with (B) the treatment of chemically fabricated zinc oxide nanoparticles (100 ppm).

Evaluation of ZnO NPs nano-particles for controlling tomato early blight infection under greenhouse conditions: Results in **Table-3** and **Table-4** show that, all treatments with the biologically and chemically fabricated ZnO-NPs significantly reduced the early blight disease under greenhouse conditions. In this respect, the highest reduction percentage of disease severity was obtained from using chemically fabricated ZnO-NPs at 12.5 ppm and biologically fabricated ZnO-NPs at 50 ppm where they reduced the disease severity to be 21.25 and 25.50%, respectively at 14-day post inoculation with the pathogen. On the other hand, all tested zinc oxide nanoparticles at 100, 200 and 400 ppm were moderately effective in controlling the early blight infection under

greenhouse conditions where chemically fabricated ZnO-NPs and biologically fabricated ZnO-NPs at 400 ppm were the lowest effective treatments where it reduced disease severity to be 41.50, 39.35% respectively.

These findings are consistent with those of **Elamawi *et al*., (2016)** who demonstrated that rice seed soaking treatment and foliar spray five days prior to inoculation with ZnO NPs led to a reduction in brown spot infection percent-age without a significant difference in ZnO NPs concentrations. Foliar spray two days post-inoculation with lower concentrations of ZnO NPs (10 and 25 ppm) reduced the percentage of brown sized ZnONPs and control.

Table-3: Effect of application different concentrations of chemically cZnO NPs on early blight disease severity caused by *A. solani* under greenhouse conditions.

Zinc oxide nanoparticles	Conc.(ppm)	Disease severity% at days		Mean
		7d	14d	
Chemically fabricated **zinc oxide nanoparticles**	**12.5**	20.00	21.25	20.63
	25	27.50	24.75	26.13
	50	23.67	25.50	24.59
	100	31.5	39.5	35.50
	200	37.5	43.5	40.50
	400	38.5	44.5	41.5
Fungicide		**13.2**	**15.1**	**14.15**
Infected control		**61.50**	**66.20**	**63.85**
Control		**3.50**	**5.45**	**4.48**

L.S.D at 5%	
Conc.	**11.16**
Time	**N.S**
Conc. X Time	**N.S**

Table-4 Effect of application different con centrations of biologically fabricated bZnO NPs on early blight disease severity caused by *A. solani* under green- house conditions

Zinc oxide nanoparticles	Conc.(ppm)	Disease severity% at days		Mean
		7d	14d	
Biologically fabricated **zinc oxide nanoparticles**	**12.5**	31.25	35.00	33.13
	25	22.50	31.25	26.88
	50	20.00	25.50	22.75
	100	30	35	32.50
	200	35	40	37.50
	400	37.5	41.2	39.35
Fungicide		**13.2**	**15.1**	**14.15**
Infected control		**61.50**	**66.20**	**63.85**
Control		**3.50**	**5.45**	**4.48**

L.S.D at 5%	
Conc.	**11.51**
Time	**4.03**
Conc. X Time	**N.S**

spot infection.**Nandhini *et al*., (2019)** found that the appl-ying ZnO NP as a seed treatment and foliar spray reduced downy mildew incidence by 35% compared to the untreated control.**Siddiqui *et al*., (2018)** reported that ZnO nano-particles (0.1 mg/mL) showed antifungal efficacy against *Alternaria alternate* and *Fusarium oxysporum*f. sp. *Lentis*on lentil plants. Moreover, **Hassan *et al*., (2009)** reported thatthe evaluation disease management of gray and black mold of pepper fruits revealed significant reduction to the appearance of both diseases of pepper fruits treated with any of the two green synthesized ZnO NPs400µg/mL compared with chemically synthe-

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