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Role of Green Synthesized Zno Nanoparticles as Antifungal against Post-Harvest Gray and Black Mold of Sweet Bell Pepper

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ABSTRACT

In this work, green ZnO nanoparticles were synthesized using leaves extract of olive (GS ZnO-NPo) and marjoram (GS ZnO-NPm). The antifungal activity of both green synthesized ZnO nanoparticles against Botrytis cinerea and Alternaria alternate, the cause of postharvest gray and black mold of pepper fruits diseases, was tested in vitro. The results of GS-ZnO were compared with those of chemically synthesized ZnO nanoparticles (ZnO-NPc).Corporating GS ZnO-NPm at concentration of 400µg/ml to PDA medium achieved the highest inhibition to the radial growth of both pathogenic fungi, followed by GS ZnO-NPo then ZnO-NPc. The evaluation disease management of gray and black mold of pepper fruits (artificially inoculated with the tested two fungi) showed significant reduction to the appearance of both diseases of pepper fruits treated with any of the two green synthesized ZnO nanoparticles compared with chemically synthesized ZnO-NPc and control. Application of green synthesized ZnO nanoparticles is promising as an alternative to synthetic fungicides for management of postharvest diseases.

Keywords: *Antifungal potential, Black mold, Gray mold, green synthesis, pepper, ZnO nanoparticles .*

INTRODUCTION

Sweet bell pepper (Capsicum annuum L.) is one of the most commercially important crops of the world. From the nutritional point of view, peppers have a high content of vitamins, rich in flavonoids phytochemical, and an important antioxidants, Serrano et al. (2010). However, the fruits damage rapidly during handling and storage. Postharvest diseases of vegetables cause great losses in food production. Peppers are susceptible to several fruit rots such as gray mold, caused by the fungus Botrytis cinerea, is usually the second worst postharvest disease of peppers. Small spots are formed on wounded areas of the fruit surface, and then enlarge and water-soaked lesions covered with a gray mold appear. Optimum temperatures for growth of the fungus are between 18 to 23°C. Moreover, black mold, caused by the fungus Alternaria alternata, firstly affects mature fruit that have been injured. Symptoms at first begin as small, circular, slightly deep spots associated with cracks or other surface injuries. The spots enlarge into larger lesions, which may show alternating light and dark brown concentric zones then heavy fungal spore production occurs. The seeds and inner fruit walls covered with black mold due to the fungal infection and growth.

Most losses of pepper fruits production result from fungal diseases, especially B.cinerea and A. alternata. There is therefore a serious need to identify novel and safer solutions to reduce postharvest fungal losses. Alternative strategies to control the diseases are needed that are safe, effective, and economical. Another great property of ZnO nanoparticles is that they can be used to enhance food preservation.

The nanotechnology developments were evaluating for their potential to promote food safety and the ZnO nanoparticles have chance to be used as an antimicrobial agent, which has non-toxic nature. In addition, other great properties of the ZnO nanoparticles can be used in food nanotechnology as a food preservation material Chitra & Annadurai (2013).

Plant extracts and soil microorganisms are significant as bioagents and used in the synthesis of nanoparticles. An important part of bio-nanotechnology is evolution of functional and eco-friendly processes of synthesis of nanoparticles Khan & Tanveer (2014), Gunalan et al. (2012) repotrted that green ZnO nanoparticles display more promoted biocidal activity against different pathogens when compared to chemical ZnO nanoparticles. Also, the efficiency of nanoparticles was increased with increasing the concentration of the ZnO particle applied in solution, the time of treatment and the method of synthesis. In addition, green synthesis of metal and metal oxide nanoparticles has been a highly appealing research area over the last decade. Many kinds of natural extracts, i.e. biocomponents like plant, fungi, yeast, bacteria, and plant extract have been used as efficient resources for the synthesis and/or manufacture of materials, sing et al. (2018).

The aim of this work is evaluating the efficacy of green synthetize of ZnO nanoparticles compared with metal ZnO nano-particles on inhibition the liner growth of B. cinerea and A. alternata in vitro. Also, management of postharvest gray and black mold diseases on pepper fruits.

Materials and Methods

Preparation of ZnO Nanoparticles

Green Synthetic ZnO

Zinc acetate dihydrate (98%) was obtained from ACROS Organics. Distilled water was used throughout the experiments. 0.2 M of zinc acetate dihydrate (98 % from ACROS Organics) was dissolved in 70 mL of distilled water and stirred for few minutes. 30 mL of plant extract was mixed homogenously with the already prepared zinc acetate solution. The reacted solution was dried at 60 °C overnight to yield pale-white ZnO nanoparticles, which were finally calcined at 100 °C for 1 h and preserved in air-tight vials for further studies. Senthilkumar & Sivakumar (2014)

Metal ZnO

Zinc oxide was prepared by dissolution of zinc acetate in ammonia solution ; the resulted solution was stirred at 50° C, left at this temperature for 24 h and then filtered. The resultant zinc hydroxide precipitate was washed several times with deionized water. The produced solid was dried at 80 oC, in stagnant air, using an electric drier . The dried material was then calcined isothermally in an electric furnace at 500° C for 4h, Bardhan et al. (2007)

Fungal Isolates

Two fungi i.e., Botrytis cinerea and Alternaria alternata were isolated from different diseased pepper fruits that showed the symptoms of gray and black mold, respectively. Small sections (1mm) were excised from a range of lesions, incubated on water agar. Single hyphal tips were transferred onto PDA medium in 9 cm Petri-dishes and incubated at 25±1°C. The isolated fungi were identified depending on their morphological features and the description of Jarvis (1980) and Simmons (2007).

Preparation of Olive and Marjoram Extracts

Leaves of olive (Oleae uropaea) and marjoram (Origanum majorana) plants were collected and washed under tape water, dried in microwave for two minutes then grinded by mill. Plant powder of olive and marjoram was weighed and amended to PDA medium, each alone, before sterilization to make three concentrations, i.e. 200, 300 and 400 µg/ml medium. Five replicates were used for each plant extract.

Effect of Plant Extracts on the Mycelial Growth of the Two Pathogenic Fungi

The antifungal test was performed by mix plant extract of any of the two tested plants before sterilizing PDA medium, Disk (5mm in diameter) of 5day-old fungal cultures was placed in the center of each Petri- dish, and incubated at 25± 1° C. Cultures of plant extract-free medium under the same growth conditions were used as control. The experimental data were recorded when the fungal growth covered all plate area in the control treatment. The linear growth was measured when the plates of the control treatment covered with the fungal growth. Inhibition percentage of the mycelial growth of the tested pathogen was calculated by the following formula

$$\%\ \text{Growth inhibition} = \frac{\text{control radial growth} - \text{treatment radial growth}}{\text{control radial growth}} \times 100$$

Effect of Both Green Synthesized and Metal Zno Nanoparticles on the Mycelia Growth of the Two Pathogenic Fungi

The antifungal test was performed by mixing ZnO nanoparticles before sterilizing PDA medium with three concentrations, i.e. 200, 300 and 400µg/ml medium. Five replicates for each treatment were prepared. A disk (5mm in diameter) of 5day-old fungal culture of any of the two tested fungi was placed in the center of each Petri-dish, and incubated at 25±1°. Cultures of nanoparticle-free medium were used as control. The linear growth was measured when the plates of the control treatment covered with the fungal growth. Inhibition percentage of the mycelial growth of the tested pathogen was calculated as mentioned before.

Inoculation and Treatment Procedures

The isolates were routinely grown on PDA medium for 4 days at 25±1°C in the darkness. The spores were obtained from the surface of the PDA cultures and suspended in 5 ml of sterile distilled water containing 0.1% v/v Tween 20. Spore suspension was filtered through four layers of sterile cheese cloth to remove mycelial fragments. A hemocytometer was used to calculate the number of the fungal spores. The concentration of the spore suspension of A. alternata was adjusted to 1×104 conidia/ml. and for B. cinerea to 1×105 conidia/ml.

Effect of Both Green Sensitized and Metal ZnO Nanoparticles on Management of Gray and Black Mold of Pepper Fruits

Fruits of two cvs. Cannon or 58(red) and La Rica (yellow) were surfaced sterilized with 2% sodium hypochlorite for 2 min. then washed with sterilized water, and air-dried. The surface of the fruit was prepared for inoculation by inflicting 1-mm deep wound with sterilized cork borer (4 mm). Then the fruits dipped in ZnO nanoparticles solution at concentration of 400µg/ml for the two types of ZnO nanoparticles for 30 min, then inoculated with the pathogen *B. cinerea* by placing 5 µl of spore suspension (1×10^5 conidia/ml) on the wound, or with the pathogen *A. alternata* by placing 5 µl of spore suspension (1×10^4 conidia/ml) on the wound. Fruits dipped in sterile distilled water only were served as control. Each treatment consisted of 10 fruits with three replications and the experiment was repeated twice . The treated fruits were incubated in plastic boxes at 25°±1C for 6 days.

Statistical Analysis

Data obtained were statistically analyzed with complete randomized block and spilt designs according to Steel and Torrie (1981). Software program, COSTAT version 6.311 was used to perform the analysis of complete randomized block design and means were compared using Duncan`s test significant at P= 0.05 level. (Duncan, 1955).

RESULTS

Characterization of Metal and Green Synthesized ZnO Nanoparticles

The TEM image in Fig.(1) clearly shows that the metal ZnO nanoparticles diameter ranged from approximately 11.1 to 15.5 nm with approximately spherical shape. characterizations of the prepared ZnO nanoparticles in Fig.(2), XRD pattern of the prepared ZnO, it displays the dominant diffraction lines at 2Θ values of 31.21, 34.11 and 36.32° corresponding to 200 and 210 planes of cubic phase of ZnO.

In the IR spectrum of olive and Marjoram as green synthesized ZnO nanoparticles are shown in Fig. (3). The band at 3435 cm–1 is ascribed to O–H groups in phenols, alcohol and water and N–H in amines. The peak bear at 2923 cm–1 is according to the CH in alkanes. The sturdy band at 1605 cm–1 is assigned to the C=C in the aromatic rings and C=O in polyphenols. The protein amide-I C–N appears in the band at 1397 cm–1. The C–O stretching in amino acid shows in the band at 1034 cm–1. The weak band at 819 cm–1 is associated to the C–H bending. Thus from the IR spectrum, it can be deduced that the used plants extract are loaded by polyphenols, proteins, carboxylic acids, and amino acids. The involvement of these biomolecules in the preparation and stabilization (capping) of the ZnO NPs is confirmed from the IR spectrum. Finally, the three new peaks appearing at 680, 514 and 468 cm–1 in the IR spectrum are the characteristic peaks of ZnO NPs molecules. Obviously, the presence of a great number of phenolic groups of molecules is responsible for accomplishing the reduction process. Also, the amino acids and amide linkages in protein are accountable for the stabilization of the ZnO nanoparticles.

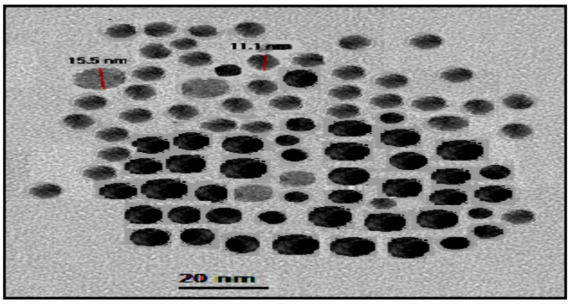

Fig1. *TEM image of the prepared nanoparticles*

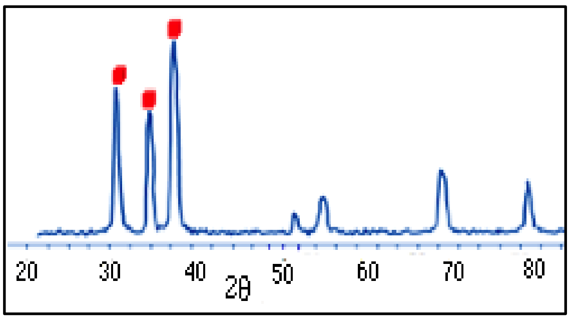

Fig2. *XRD characterizations of the prepared ZnO nanoparticles XRD pattern of the prepared ZnO , it displays the dominant diffraction lines at2Θ values of 31.21, 34.11 and 36.32° corresponding to 200 and 210 planes of cubic phase of ZnO*

Effect of Green Synthesized and Metal ZnO Nanoparticles on the Mycelial Growth of B. Cinerea and A. Alternata

Effect on the Mycelial Growth of B. Cinerea

Result shown in Table (1) show the effect of leaves powder of olive and marjoram as well as the metal and green synthesized of ZnO on the mycelial growth of *B. cinerea*, after incubation at 25°±1°C. at concentrations 200, 300 and 400µg/ml. The different concentrations of the three kinds of ZnO nanoparticles caused significant reduction to the linear growth of *B. cinerea* compared with control treatment Fig.(4).

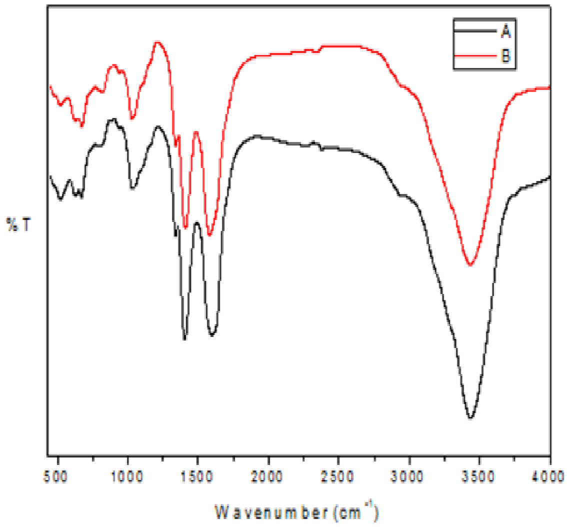

Fig3. *Infrared spectrum of the green synthesized ZnO nanoparticles, (A) olive and (B) Marjoram as green synthesized ZnO nanoparticles.*

However, the reduction in the linear growth was gradually increased by increasing the used concentrations. At 400 µg/ml concentration green synthesized ZnO nanoparticles GSZnO-NPm achieved the highest inhibition followed by green synthesized ZnO nanoparticles GS ZnO-NPo then metal ZnO nanoparticles ZnO-NPc, being 87.0, 79.3 and 69.8%, respectively. Meanwhile, leaves powder of olive and marjoram resulted in the lowest inhibition, being 8.4 and 15.6%, respectively.

Effect on the Mycelial Growth of A. Alternata

Result presented in Table(2) show the effect of leaves powder of olive and marjoram as well as the metal and green synthesized of ZnO on the mycelial growth of *A. alternata*, 4 days after incubation at 25°±1°C. at concentrations 200, 300 and 400µg/ml. The different concentrations of ZnO nanoparticles caused significant reduction to the linear growth of the causal fungus compared with control treatment. In addition, the reduction in the linear was gradually increased by increasing the used concentrations. At 400 µg/ml concentration green synthesized ZnO nanoparticles GSZnO-NPm resulted in the highest inhibition followed by the green synthesized ZnO nanoparticles GSZnO-NPo then metal ZnO nano-particles ZnO-NPc, being 74.2, 68.8 and 65.4%, respectively Fig. (5). Meanwhile, leaves powder of olive and marjoram achieved the lowest inhibition, being 8.3 and 9.7%, respectively. Result of Tables 1 and 2, indicate, in general, that the fungus *B. cinerea* was more sensitive to the three kinds and concentrations of ZnO nanoparticles (GSZnO-NPm, ZnO-NPo and ZnO-NPc) than the fungus *A. alternata*.

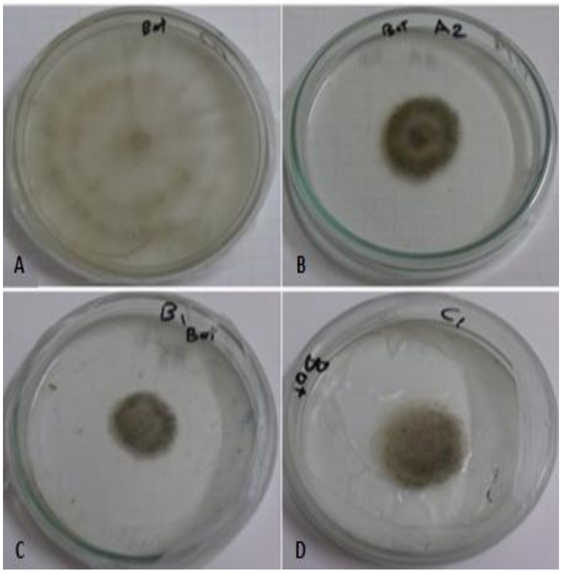

Fig4. *Effect of ZnO nanoparticles on the mycelial growth of B. cinerea,on potato dextrose agar medium, at 400µg/ml; (A) Without nanoparicles, (B) Green synthesized ZnO nanoparticls using olive (GSZnO-NPo), (C) Green synthesized ZnO nanoparticles using marjoram (GSZnO-NPm), and (D) metal ZnO nanoparticles (ZnO-NPc)*

Effect of ZnO Nanoparticles on Gray and Black Mold Diseases on Pepper Fruits

Data presented in Table (3) show the effect of metal and green synthesized ZnO nanoparticles at concentration 400 µg/ml on incidence of gray and black mold diseases on two cvs of pepper fruits, i.e. Cannon(red) and La Rica(yellow). In case of gray mold, green synthesized ZnO nanoparticles of marjoram (GSZnO-NPm) resulted in the highest reduction to the disease appearance % followed by green synthesized ZnO nanoparticles of olive (GS ZnO-NPo) then metal ZnO nanoparticles (ZnO-NPc), being 46.67, 50.0 and

65.0 %, respectively on the average, Compared with control (100% Disease appearance). In addition, black mold disease caused by A. alternate recorded the same trend with gray mold, being 40.0, 58.35 and 82.0%, respectively on the average.

Table1. *Effect of ZnO nanoparticles and plant powder of olive and marjoram on the mycelial growth of B. cinerea, 4 days after incubation at 25 ± 1̊C*

Treatments	Tested concentrations µg/ml					
	200		300		400	
	Lg(mm)	R%	Lg(mm)	R%	Lg(mm)	R%
Olive leaves powder	85.0^{b}	5.6	83.3^{b}	7.4	82.4^{b}	8.4
Marjoram leaves powder	80.8^{c}	10.2	78.5^{c}	12.8	76.0^{c}	15.6
ZnO-NPc[1]	30.0^{d}	66.7	28.3^{d}	68.6	27.2^{d}	69.8
GSZnO-Npo[2]	23.3^{e}	74.1	19.3^{e}	78.6	18.6^{e}	79.3
GSZnO-NPm[3]	14.9^{f}	83.4	12.5^{f}	86.1	11.7^{f}	87.0
Control	90.0^{a}	----	90.0^{a}	-----	90.0^{a}	----

Values within same column followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Lg =linear growth, R% =growth reduction (%). 1. ZnO-NPc: ZnO metal nanoparticls. 2. .GSZnO-NPo: Green synthesized of ZnO nanoparticls using olive. and 3.GSZnO-NPm:Green synthesized of ZnO nanoparticls using marjoram.

Table2. *Effect of ZnO nanoparticles and plant powder of olive and marjoram on the mycelial growth of A. alternata, 4 days after incubation at 25 ±1̊C*

Treatments	Tested concentrations µg/ml					
	200		300		400	
	Lg(mm)	R%	Lg(mm)	R%	Lg(mm)	R%
Olive leaves powder	87.5^{b}	2.8	85.0^{b}	5.6	82.5^{b}	8.3
Marjoram leaves powder	85.0^{c}	5.6	82.7^{c}	8.1	81.3^{c}	9.7
ZnO-NPc[1]	33.3^{d}	63.0	32.6^{d}	63.8	31.1^{d}	65.4
GSZnO-Npo[2]	33.0^{e}	63.3	29.5^{e}	67.2	28.1^{e}	68.8
GSZnO-NPm[3]	31.2^{f}	65.3	24.6^{f}	72.7	23.2^{f}	74.2
Control	90.0^{a}	----	90.0^{a}	-----	90.0^{a}	----

Values within same column followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

Lg= linear growth, R% =growth reduction (%). 1. ZnO-NPc: ZnO metal nanoparticls. 2. GSZnO-NPo: Green synthesized of ZnO nanoparticls using olive.and 3. GSZnO-NPm: Green synthesized of ZnO nanoparticls using marjoram.

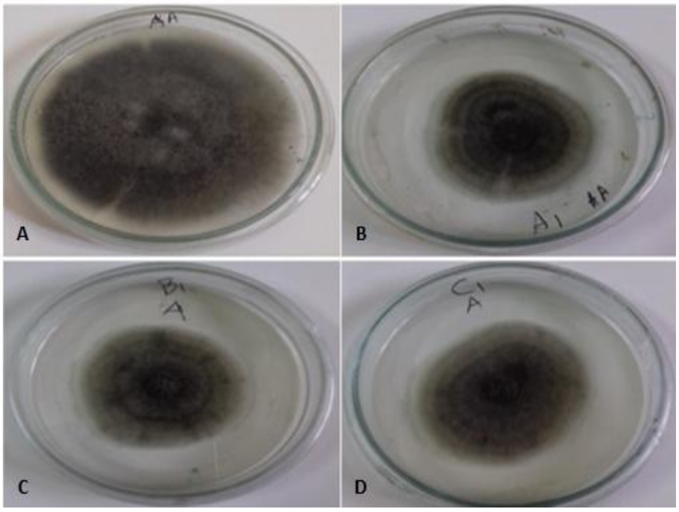

Fig5. *Effect of ZnO nanoparticles on the mycelial growth of A. alternata,on potato dextrose agar medium, at 400 µg/ml; (A) Without nanoparicles, (B) Green synthesized ZnO nanoparticles using olives (GSZnO-NPo), (C) Green synthesized ZnO nanoparticles using marjoram (GSZnO-NPm), and (D) metal ZnO nanoparticles (ZnO-NPc).*

Table3. *Effect of metal and green synthesized ZnO nanoparticles at 400 µg/ml on appearance of gray and black mold diseases on the fruits of two pepper cvs.*

Nanoparticles	% Disease appearance of gray mold (*B. cinerea*)			% Disease appearance of black mold (*A.alternata)*		
	Cannon (red)	La Rica (yellow)	Mean	Cannon (red)	LaRica(yellow)	Mean
ZnO-NPc[1]	60.0	70.0	65.0	86.7	77.3	82.0
GSZnO-NPo[2]	33.3	66.7	50.0	50.0	66.7	58.4
GSZnO-NPm[3]	26. 7	20.0	46. 7	23.3	56.7	40.0
Control	100	100	100	100	100	100
Mean	55.0	64.2	------	65.0	75.2	-----
L.S.D. at 5% for:	Nanoparticles (N) =		3.8			3.4
	Disease appearance (D) =		4.5			4.6
	N x D =		4.2			3.8

1 .ZnO-NPc: ZnO metal nanoparticls.2.GSZnO-NPo:Green synthesized of ZnO nanoparticls using olive.and 3.GSZnO-NPm:Green synthesized of ZnO nanoparticls using marjoram.

Discussion

For immobilize and modify the biomolecules we need some features such as high biocompatibility, fast electron transfer kinetics, and to be non-toxic material all this features found in Zinc oxide (ZnO),Kumar *et al.* (2008). A mong a kinds of semiconducting materials, zinc oxide is opulent in nanostructures and it has the ability to produce into a diversity of morphologies Wahab *et al.* (2008). Presently, the importance of plant-mediated biological synthesis of nanoparticles due to its eco-friendliness, simplicity, and extensive antimicrobial activity, Saxena et al. (2010 and Khandelwal *et al.* (2010). Gardea-Torresdey *et al.* (2002) and Gardea-Torresdey *et al.* (2003) have been reported the biosynthesis of zinc oxide nanoparticles by using Aloe vera and gold nanoparticles by using alfalfa. the physical and chemical production of zinc oxide nanoparticles need toxic chemicals and extreme environment, the higher percentage of phenolic group of molecules are responsible for completing the reduction process. The stabilization of the ZnO nanoparticles due to the amino acids and amide linkages in protein, Agarwal *et. al*, (2017).

Numerous researches have been done to progress nanoparticles as an antimicrobial because of the rising microbial resistance towards antibiotics and common pesticides. In this regard, *in vitro* and *in vivo* antimicrobial studies showed that the green and metallic nanoparticles effectively obstruct both pathogenic fungi. Singh *et al.* (2018) mentioned that the antimicrobial efficiency of the metallic nanoparticles depends upon two significant parameters: (a) material used for the synthesis of the nanoparticles and (b) the size of the particle.

As per the literature survey there is limited studies carried out on antifungal activity of green synthesis of ZnO nanoparticles compared with the same metal ZnO nanoparticles. In the present study, Metal and green synthesized, ZnO nanoparticles of the three concentrations (200,300,400µg/ml) antimicrobial activity of resulted in different degrees of inhibition towards both fungal pathogens, i.e. B.cinerea and A. alternata . The existence of inhibition zone obviously indicated that the mechanism of the biocidal action of ZnO nanoparticles included high rate of production surface oxygen species with disruption of the membrane and at last lead to the death of pathogens. Interestingly, the type of the pathogen, synthesis method and the concentrations of ZnO nanoparticles influnce on the percent of the inhibitionthe. Rizwan, *et al.* (2010) indicated that the growth inhibition has also been increased consistent with increasing the concentration of ZnO nanoparticles because of suitable diffusion of nanoparticles in the agar medium. Metal and both green synthesized ZnO nanoparticles showed antimicrobial activity against both pathogens and among the concentrations tested the highest inhibition was observed on Botrytis cinerea then A. alternata at 400µg/ml. Previous works have been reported the antifungal activity of ZnO nanoparticles against B. cinerea. They suggested that the cell functions of B. cinerea was influenced by ZnO nanoparticles due to increasing the productivity of nucleic acids through the stress reply in fungal hyphae leads to the death of cells, He *et al.* (2011).

In the present study, the effect of the green synthesized ZnO nanoparticles of olive and marjoram on the mycelial the growth of both *B. cinerea* and *A. alternata* was evaluated. ZnO nanoparticles of Marjoram leaves powder achieved at 400 µg/ml resulted in the highest inhibition to the linear growth of both fungi followed by ZnO nanoparticles of olive leaves powder. Meanwhile only Marjoram leaves powder and olive leaves powder achieved the lowest inhibition to the linear growth of both fungi. (Bozin et al., 2006) found antifungal effect of the essential oil of origanum on Candida albicans. Also, Chishti *et al.* (2016) indicated that Origanum vulgare had significant inhibitory effect against four fungal species, *i.e. Aspergillus fumigaitus*, *Candida albicans*, *Pencillium cryogeneum* and *Saccharomyces cereviceae* included in the study, which may find its application in future research for the therapy, food and pharmaceutical industry. Metal ZnO nanoparticles and both green synthesized ZnO nanoparticles were evaluated on gray and black mold disease for two cvs. of pepper, *i.e.* Cannon (red) and LaR (yellow).

All treatments showed significant reduction to disease appearance compared with the control. The two green synthesized ZnO nanoparticles achieved the highest effect on reduction of disease appearance for gray and black mold compared with metal ZnO nanoparticles, It has been found that in case of gray mold, green synthesized ZnO nanoparticles of marjoram showed the lowest disease appearance flowed by green synthesized ZnO nanoparticles of olive. Meanwhile, metal ZnO nanoparticles achieved the highest disease appearance. Meanwhile, in case of black mold, green synthesized ZnO nanoparticles of marjoram showed the lowest disease appearance flowed by green synthesized ZnO nanoparticles of olive. Meanwhile, metal ZnO nanoparticles achieved the highest disease appearance. Metal and green synthesized ZnO nanoparticles of margoram and olive were more effective on controlling gray mold the black mold, respectively

CONCLUSION

In this study, green synthesized ZnO nanoparticles at a concentration of 400µg/ml were more effective than metal ZnO nanoparticles on controlling gray and black mold in pepper fruits caused by B. Cinerea and A. alternata, respectively. Moreover, green synthesized ZnO nanoparticles of marjoram were more effective than green synthesized ZnO nanoparticles of olive. The inhibitory effect was obvious on the linear growth of both studied fungi and disease appearance of gray and black mold in pepper fruits. Application of green synthesized ZnO nanoparticles is promising as an alternative to synthetic fungicides for management of postharvest gray and clack mold diseases on pepper fruits.

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