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Achieving Green Synthesis of Silver Nanoparticles by *Aspergillus ustus* ON076464 for Improving Immune Response and Vegetative Growth of Pepper Plant Towards Wilt Disease caused by *Fusarium oxysporum*

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Abstract

In light of climatical variations, fungal diseases increased, which led to heavy losses in economic crops. As a result of these concerns, the management of phytopathogenic fungi has emerged to be one of the most important global issues. Green-synthesized nanomaterials have a remarkable antimicrobial efficacy to be used as an alternate to harmful fungicides. Herein, this study focused on establishing the prospective effects of silver nanoparticles (AgNPs) synthesized by *Aspergillus ustus* ON076464 against phytopathogenic *F. oxysporum* to control wilt disease of pepper plant. The biosynthesized AgNPs were subjected to different characterization practices. The results evoked the ability of *Aspergillus ustus* filtrate to build up AgNPs in a spherical shape and dispersed without aggregation with a size average of 12.4 nm. Laser diffraction revealed that the particles obtained were monodispersed in a mixture having an average diameter of 17.20 nm. It is conceivable from the results that AgNPs at 100 µg/ml and 50 µg/ml are markedly effective against *F. oxysporum* exhibiting the highest antifungal action and minimum inhibitory concentration MIC respectively. The results of the scanning electron microscope (SEM) of *F. oxysporum* mycelium treated with AgNPs showed severe morphological destruction when compared to the nontreated hyphae. AgNPs at concentrations (50 and 25 µg/ml) highly gave a reduction in percent disease index by (25 and 37.5) and highly gave protection percent by (72.7 and 59.06 %) compared to the untreated infected plants. The application of AgNPs at concentrations (50 and 25 µg/ml) resulted in different responses regarding the photosynthetic pigments, total carbohydrates content, phenol, and total protein of *Fusarium*-infected plants. Consequently, this investigation presents a promising insight into fungi for AgNPs biosynthesis, and the application of these nanoparticles could effectively limit *Fusarium* wilt disease of pepper plants, as a biological and novel approach.

Keywords: AgNPs ; *Aspergillus ustus*; *Fusarium oxysporum* ; Antifungal activity; *Capsicum annuumL*; Disease index; Wilt disease.

1. Introduction

Around 44 million people over 38 countries are suffering from food insecurity, as reported by the Global Food Program [1]. Over the world, the quality and yield of agricultural commodities continue to be progressively declined globally each year owing to plant diseases. Plant diseases are indeed a global issue that affects the availability of food. Fungal plant diseases are considered one of the most dangerous pre and post harvested diseases that lead to the destruction of the crop in whole or in part beside to secretion mycotoxins [2-4]. Soil borne fungi as Fusarium are widely distributed and present in organic as well as conventional farming soils[5]. One of the most major and persistent wilt pathogens that affect agricultural commodities is Fusarium[6, 7]. Distinctive disease signs bring in vascular browning, a faint vein clearing on the outer part of the younger leaves, then the older leaves begin to droop downward marginal necrosis

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of remaining leaves followed by plant wilting and finally the death of the plant stunting, rising wilting, defoliation, and plant death [8, 9]. One of the most thoroughly researched plant diseases is that brought on by Fusarium spp., particularly Fusarium wilt (FW) in many crops. Pathogens of the genus Fusarium are reported to produce a variety of toxins, namely fumonisins, fusaric acid, that pose a serious threat to human health [10]. Plant health and resistance against plant stresses can be enhanced by biotic as well as abiotic natural products[11-13]. Several strategies have been implemented to control fungal diseases, involving biological control and chemical control [14, 15]. It has been found that applying fungicides is a faster way to treat Fusarium wilt, but fungicides are not eco-friendly and have a negative impact on people, microbes, and the environment because of their toxic residues[16], as a consequence, it has been noted that the fungus has genetically evolved resistance to the fungicides [17]. As a result, it's important to develop efficient control tactics that do not influence environmental safety [18]. In Egypt, current studies effectively encouraged the usage of natural agents as safety tactics for both humans and the environment on the way to managing F. oxysporum[19]. Nowadays, nanotechnology is indeed one of the new advancements that have made great progress over the past ten years and has attracted major scientific interest [20]. Sustainable and economic solutions for good agriculture with superior yield are obligatory; innovative nanomaterials could boost plant immunity and prevent plant diseases [21, 22]. Silver nanoparticles among many of the numerous sorts of metallic nanoparticles might be highlighted for their wide antimicrobial capabilities [23]. Production of Ag NP by fungi has several implications. They involve substantial-scale nanoparticle production using a simple process, excellent nanoparticle dispersion, and increased protein expression levels [24]. silver nanoparticles (AgNPs) increase antimicrobial activity and prevent infections from developing resistance. These characteristics make it a safe substitute for synthetic fungicides in the management of plant diseases [25]. According to reports, silver nanoparticles (AgNPs) stimulate plants' antioxidant defense mechanisms [26].Pepper, Capsicum spp. is a member of the family Solanaceae and is generally divided up into two groups, spicy and non-spicy, which are also known as hot and sweet pepper[27]. Due to its nutritious value, pepper is a prominent spice crop that is consumed all over the world. Therefore, the objectives of the recent study were (1) enhancement of pepper plant growth, (2) increasing the resistance of pepper plant alongside Fusarium wilt by AgNPs and the consideration of the induced systemic resistance (ISR) signs of regarded plants under greenhouse conditions, (3) providing the possibility of using these inducers as an innovative and safe method for effective control to combat this fungal disease.

2. Materials and methods

2.1. Chemicals and Reagents

Chemicals and reagents employed throughout this work were of analytical quality from Sigma-Aldrich Chemical Co (St. Louis Missouri, 63103, USA). Fungal media were purchased from Difco (United Kingdom). The solvents and reagents were used as supplied by the company. Deionized water has been used during the whole investigation.

2.2. Fungal strain

In the current study, the nanosilver producer fungus was isolated from Rhizosphere area of Egyptian soil samples collected from Helwan City, Cairo, Egypt.

2.3. Source of the fungal pathogen

Fusarium oxysporum f.sp.capsici was attained from the Regional Center for mycology and Biotechnology,Al-Azhar University (RCMB) then was recognized by pathogenicity assessment agreeing to [16]. The inoculum was organized corresponding to [28].

2.4. Identification of the fungal isolate

2.4.1. Morphological identification

Standardized methods for morphological characterization and species descriptions published in [29]were used. After 7 days ofculturing the fungal isolate on Potato Dextrose Agar (PDA), culture characteristics including color of colony, reverse pigments, texture, and appearance were observed and recorded.The isolate was also subjected to microscopic examination using a light microscope (Optika, Italy) [30, 31].

2.4.2. Molecular identification

Genetic identification of the fungal isolate was performed according to [32]. Genomic DNA of the nanosilver producer fungus was extracted using Biospin Extraction Kit (Bioer Technology Co., Ltd.,

Hangzhou, P. R. China), following the manufacturer's protocol. The ITS rDNA sequences were amplified using polymerase chain reaction (PCR) using forward and reverse primers of TCTGTAGGTGAACCTGCGG and TCCTCCGCTTATTGATATGC, respectively (synthesized by MWG-Biotech, Germany based on conserved regions of the eukaryotic rRNA gene [33]. The amplification was acheived in a thermocycler ABI GeneAmp 9700 (Applied Biosystems, USA), which was designed for an initial denaturation cycle at 94 °C for 5 minutes, then 40 cycles of denaturation for 1 min at 94 ˚C. The nucleotide sequences of the fungal isolate were submitted to GenBank for accession number. Next, the consensus sequences of a fungal isolate obtained from both ITS1 and ITS4 primers were first edited and subjected to BLAST search to assign putative identity with similar sequences using a database of NCBI http://blast.ncbi.nlm.nih.gov/Blast.cgi. The fungal isolate was then designed to its operational taxonomic unit (OTU) based on measures of sequence similarities, and inferences of phylogenetic trees. Sequences were then compared with other related sequences retrieved from GenBank using ClustalX [34], BioEdit[35], and Molecular Evolutionary Genetics Analysis (MEGA) software ver. 6.0 [36].

2.5. Biogenic synthesis of Ag NPs

Herein, the fungal cell filtrate was utilized in the biological synthesis of AgNPs. The fungus was grown-up in 100 ml Sterile glucose yeast peptone (GYP) medium. The medium contains (g/L): peptone, 10.0; glucose 20.0; yeast extract,5.0; agar-agar, 15.0 and distilled water to 1L [37] . Then the inoculated media were incubated at 28oC under shaking (180 rpm) for 72 hr. Next, the obtained fungal biomass was harvested by means of sterilized Whatman filter paper No 1 and washed with sterilized distilled water. Twenty grams of fresh, moist fungal biomass were treated with 100 ml of Milli-Q deionized water then incubated at 28oC, 180 rpm for 48 hr. according to[38] with minor modification. Following incubation, the filtrate was gathered, centrifuged at 5000 rpm for 10 min., then the collected supernatants were used to create AgNPs. The cell-free filtrate of the fungal isolate was mixed with 3mM AgNO3 solution (1:1) and incubated at 28oC on a shaker adjusted at 150 rpm for 72 h in dark conditions until the color changed. Under the same conditions of the experiment, a control without AgNO3 was performed [39, 40].

2.6. Characterization of AgNPs

The detailed characterization steps of the resultant AgNPs were done at the Egyptian Petroleum Research Institute- Nanotechnology Center, Cairo, Egypt. Analysis of the optical distinctive of the formulated AgNPs was achieved by observing the electron spectra employing a UV–Visible spectroscopy (T60U-UV–Vis-UK). The chemical structure of functional groups of AgNPs was analyzed using Fourier-transform infrared spectroscopy (ATR-FTIR, JASCO FTIR 4100 spectrometer) over a range of 4000–400 cm−1. For the size distribution of AgNPs, the DLS technique using (DLS-PSS-NICOMP 80-ZLS particle sizing system St. Barbara, California, USA) was used. Morphological characteristics of biosynthesized AgNPs involving size and shape were explored by means of a TEM (HRTEM JEM-2100Japan). Finally, the surface charge of AgNPs was determined by Zeta potential analyzer Nano ZS90 Zeta sizer (Malvern Instruments) equipped with He–Ne laser (633 nm, 5 Mw)[41, 42].

2.7. Evaluation of Ag NPs antifungal effectiveness against Fusarium oxysporum in vitro

The antifungal efficacy of biogenic AgNPs against Fusarium oxysporum was evaluated as described by [39, 43]using an agar well diffusion assay with minor modifications. Briefly, 20.0 ml of PDA medium was inoculated with 20.0 µl (106) of spore suspension of F. oxysporum and poured into Petri plates, stirred well together, and let to solidify. Next, holes of 4 -mm diameter were made in agar plates using a sterile Cork borer. The wells were filled with 100 µl of AgNPs (100µg/ml) , fluconazole and distilled water as a positive and negative control respectively) and maintained in a refrigerator for 1 hr. to enable consistent diffusion of the substances prior to Fusarium growth. Following incubation at 28°C for 72 hours, the plates were observed, and the zones of inhibition were detected [39].

2.8. Determination of minimum inhibitory concentration of AgNPs against Fusarium oxysporum

The method of agar well diffusion was used to test for performing the minimum inhibitory

concentration [39, 44]. Briefly, 0.1ml of the spore suspension was added on 20 ml PDA in petri-dish as inoculum. Four wells of 4 mm in diameter were made on the surface of cultured medium using sterilized cork borer and each well was filled with 100 µl of different concentrations AgNPs (25-50-100 and 200 µg/ml) the plates are allowed to stand in a refrigerator for one hr. for proper diffusion. Cultures were incubated at 28 ±2 ℃ for 3 days then the MIC was determined at the end of incubation.

2.9. Assessment of the Ag NPs action on Fusarium oxysporum morphology by Scanning Electron Microscope

Four-day-old fungal cultures of Fusarium oxysporum were usedto study the growth morphology.Potato dextrose agar medium (PDA) supplemented with (25 µg/ml) of AgNPs was used. Five millimeters disc of 5-day-old culture Fusarium was placed at the center of the Petri-dish containing 20 mL of PDA and incubated at 270C for 4 days. As a control, the PDA media devoid of AgNPs was used. 10 X 10 mm2 area of mycelial discs were cut off and stuck using 2.5% glutaraldehyde at 4 ℃ for 24 h. Next, the sections were then rinsed 3 times for one minute with Sorensen phosphate buffer. The samples were dehydrated gradually in different ethanol concentrations from 30 to 90% for 40 min. Then, the previous step was carried out three times for 30 minutes in 100% ethanol. The dehydrated samples were kept in aluminum samples on dual glue carbon conductive sticky tape, evaporated in a Quorum K850 critical point dryer, and then gold-coated in a Quorum Q150R S coater for one min. After all, the samples were seen by a scanning electron microscope (SEM) (JSM-IT 200; JEOL., USA) at an accelerating voltage of 10 Kv[4, 45].

2.10. Evaluation of bio fabricated silver nanoparticles on systemic resistance against wilt disease caused by Fusarium oxysporumf.sp. capsici.under pots condition

2.10.1. Pots experiment

Pepper seedlings (cultivar Capsicum annuum L.) of three weeks' age were purchased from the Agricultural Research Center (ARC), Giza, Egypt. F. oxysporum spore suspension was made in potato dextrose broth with a final concentration of 106 spores/ml. Matching to the method of [41] with minor modification, the pots were inoculated with 50 ml of F. oxysporum spore suspension one week prior to planting, then placed in the greenhouse at 30 oC with the soil kept moist until sowing. Later, the uniform pepper seedlings were moved into soil that had been inoculated with a fungus suspension in pots (20 cm in diameter) which contains two kg of a sand and clay combination (1: 3 wt/wt), at the Botanical Garden of Botany and Microbiology Department, Faculty of Science, Al-Azhar University. Six replicates of a fully random design were used to assemble the pots. One week after planting, the seedlings (healthy and infected) were treated with biogenic AgNPs three times (once every ten days) before and after flowering. Biogenic AgNPs was given through shoot foliar. The details of treatments include set as follows: T1-Control healthy, T2-Control infected with fusarium, T3-Healthy + 25 µg/ml AgNPs, T4- Infected + 25 µg/ml AgNPs, T5-Healthy + 50 µg/ml AgNPs, and T6-Infected + 50 µg/ml AgNPs. Disease development and severity were estimated. Once the plants were 45 days of planting, the plants were assessed for biochemical indicators intended for resistance assessment.

2.10.2. Disease symptoms and Disease index

The grades of symptoms were observed daily and extended for 60 days later on inoculation. The index of disease was evaluated matching to the methods of[15, 46]. The five-grade scale was used to assess the percent disease index (PDI), and the formula was as follows: PDI = (1n1+ 2n2 + 3n3 + 4n4)100/4nt, where n1;n4 represents the number of plants in each class and nt represents the total number of plants examined. The percent protection was calculated using the following formula: Protection % = A–B/A × 100%

where A=PDI in infected control plants and B=PDI in infected-treated plants.

2.10.3. Determination of phytochemicals

2.10.4. Estimation of photosynthetic pigments: Photosynthetic pigments were estimated according to the procedure outlined by [47].

2.10.5. Estimation of total soluble carbohydrate: Total soluble carbohydrate was estimated by the method depicted earlier by [48].

2.10.6. Determination of total proteins: Total proteins (mg/100 g of dry weight) were determined in accordance with the method of [49].

2.10.7. Determination of phenolic compounds:

Phenolic compounds determination (mg/100 g of dry weight) was conducted corresponding to the method outlined by[50]. Additionally, free proline concentrations (mg/100 g of dry weight) were assessed using the procedure of [51].

2.10.8. Estimation of oxidative enzymes activity

The method provided by [52] was used to measure the peroxidase activity.polyphenol oxidase activity was measured according to the method described by [53].

2.10.9. Biochemical genetic recognition:

2.10.10. Isozymes electrophoresis

Isozyme electrophoresis was accomplished in (100 mg fresh weight) of peeper leaf. Peroxidase (POD) isozyme in samples was calculated and evaluated according to [54]. The method of [55, 56] was used to compute the isozyme polyphenol oxidase (PPO).

2.11. Statistical analysis:

The data were examined by (ANOVA) variance and the variances amongst means were differentiated by the least significant difference (L.S.D) at a 5% degree of chance expending CO-state software[57] with the different letter demonstrating significant differences. Three replicates' means were used to calculate the values for the biochemical analysis [58].

3. Results and discussion

The objectives of the current study were the enhancement of pepper plant resistance versus Fusarium wilt by the biosynthesized AgNPs and the valuation of the ISR markers of regarded pepper plants under greenhouse conditions. Nevertheless, a set of tests have been conducted in succession to ascertain if induction of resistance was attained and if biogenic AgNPs guard pepper plants against F. oxysporum.

3.1.1. Identification of fungal isolate

According to the macroscopic and microscopic investigations, the AgNPs producer isolate was identified as Aspergillus ustus. The colony showed overhasty growth with fluffy, wooly whitish brown mycelium on PDA with the development of sporangia and sporangiospores. The reverse color was pale brown on PDA after 7 days as shown in fig.1.

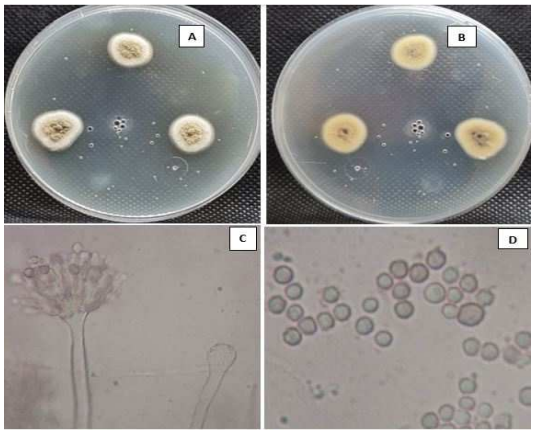

Figure 1: Culture characteristics (A= front and B= reverse) of Aspergillus ustus on PDA media after 7 days, C and D: microscopic features of fungal mycelia and spore under the light microscope (200 X).

3.1.2. Phylogenetic relationships

In the current study, the achieved Aspergillus ustus sequence was banked in Gene Bank with accession number (ON076464. Using MEGA software, version 10.1.6, the dendrogram was created using the utmost likelihood (ML) and neighbour-joining (NJ) method. The p-distance method was used to compute the evolutionary distances. (Fig.2).

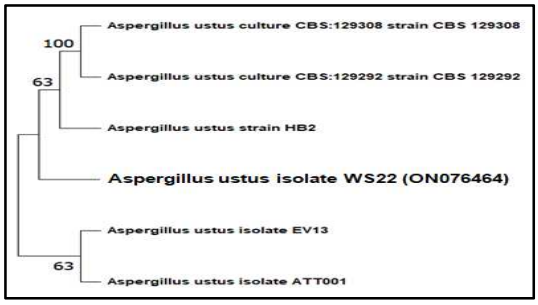

Figure. 2. Phylogenetic tree of Aspergillus ustus ON076464 in relative with international isolates

3.2. Synthesis and characterization of AgNPs

This research helps develop a practical, environmentally acceptable method for mycosynthesis of AgNPs that is affordable and feasible. The successful synthesis of AgNPs by Aspergillus ustus ON076464 was verified visually by means of color change from yellow to dark

reddish-brown and by SPR depiction using a UV–Vis spectrophotometer (fig 3a). The adjusting of color indicates that the Ag+ was reduced to Ag0. The extracellular filtrate of fungal cells was used in the current investigation instead of harmful and toxic solvents and chemicals for the mycosynthesis of AgNPs. This is the ideal illustration of AgNPs being produced in a green manner. A study by [59]. The finding of [40] evokedthat the ability of molds to absorb and reduce a heavy metal may provide microbe-based solutions for metal replacement or environmentally benign metal nanoparticle synthesis that are thought of as a corporate tactic and protection of the environment.

The mixture's absorption spectra were scanned over 200 to 800 nm. The UV–Vis. spectrum proved the maximum absorbance peak at 418nm which distinctive peak for AgNPs. The amino acids are crucial for the stability of nanoparticles because they provide a coating layer that keeps the particles from aggregating. The form and size of the particles may be related to the peak absorption area[60, 61]. The resulted strong peak at 418 nm signifies the AgNPs manufactured remained stable and greatly[62] dispersed. This absorbance resulted from the electron transfer on the surface of AgNPs as informed by. These findings were similar to those of researchers [63, 64] who recounted strong distinctive peaks at 440, 390–420, and 400 nm, individually.

In the current investigation, the size and morphology of biosynthesized AgNPs were examined using TEM. The findings demonstrated that AgNPs formed into spherical shapes and distributed throughout the sample without aggregating, with sizes ranging from 3.2 to 12.4 nm (Fig.3b). AgNPs' microbicidal force increases with decreasing size as a consequence of the extensive surface area exposed to microbial contamination[65]. This is also consistent with previous studies that report the tiny size of the AgNPs clarified the strength of their impact as an antimicrobial mediator. Silver nanoparticles have unique characteristics in their size, structure, and morphology that have enabled them to interact with a variety of microorganisms, animals, and plants[24, 66]. Additionally, it was publicized that the antimicrobial activity of AgNPs was size and shape-dependent and highly toxic in smaller sizes and even at a lesser concentration [67].

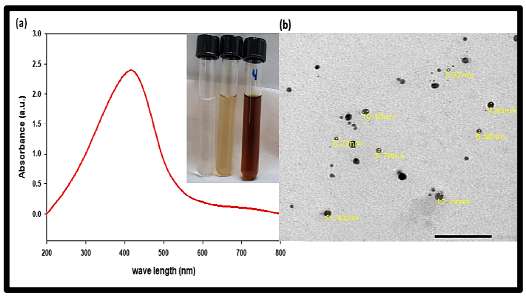

Figure.3: Characterization of Ag NPs synthesized by cell-free filtrate of Aspergillus ustus ON076464 (a) UV, (b) Transmission Electron Microscope (TEM)

To ascertain the participation of potential phytochemicals and biomolecules employed in the synthesis, FTIR spectroscopic analysis was carried out. The findings shown in Figure 4 showed that the acquired sample's FTIR spectrum displayed significant transmittance peaks. The peaks at 3454.81cm-1 could be assigned to a strong stretching vibration of the hydroxyl functional group according to the reports[24, 68].Interestingly, the peak that appeared in 2089.36cm-1could be assigned to C=C stretching alkyne monosubstituted[69]. The N–C=O amide bond of proteins direct to carbonyl stretch have being documented at the peak 1637.61 [70]. The peak appeared 1384.48 may be assigned to O-H bending group of phenols. The peak 671.12 corresponded to C=C alkene monosubstituted. More interestingly, a previous study indicated that the proteins and other metabolites made by fungi in the cell filtrate are accountable for the reduction of silver and also for capping of the AgNPs [64, 71, 72]

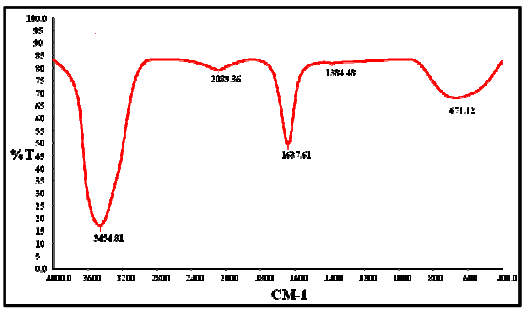

Figure 4. FTIR spectra of (AgNPs) synthesized by Aspergillus ustus ON076464

The outcomes have been verified with DLS examination as shown in (Fig 5a). Laser diffraction revealed that the particles obtained were monodispersed in a mixture having an average diameter of 17.20 nm. Polydispersity indexes (PDI) (numerical value representing the homogeneity of the sample) of the bio-

-synthesized AgNPs using *Aspergillus ustus* ON076464 were found to be 0.451. So, the sample is considered monodispersed (i.e., similar size), because PDI is below 0.7. Our findings were consistent with those obtained by [73] who disclosed that samples of NPs with very broad size distribution have polydispersity index values > 0.7. Additionally, our results evoked that, the zeta potential of (−29.5 Mv) with a distinct sharp peak was noted for AgNPs produced by *Aspergillus ustus* ON076464 signifying the presence of repulsion among the synthesized nanoparticles (Fig 5b). Our results concurred with those of [67] who found that the constancy of AgNPs is demonstrated by the negative charge of the AgNPs suspension resulting in repulsion and reducing their ability or tendency to form aggregate. More interestingly, previous studies indicated the polyphenolic metabolites generated by fungi in the solution utilized for AgNPs production may be the cause of the negative charge of AgNPs. Several fungi produce polyphenolic compounds as secondary metabolites during growth, and these phenols participate in capping during the synthesis of AgNPs [74-76].

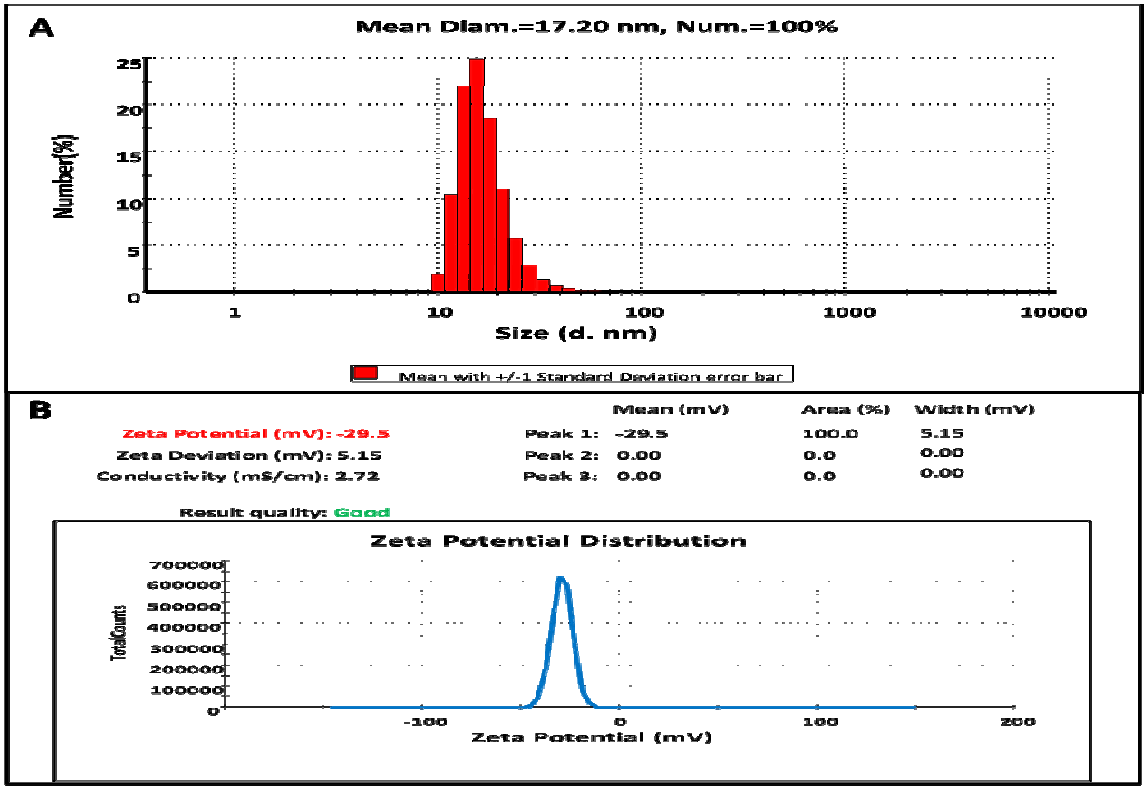

Figure 5: (a) DLS, (b) Zeta potential of Ag NPs synthesized by *Aspergillus ustus* ON076464

3.3. Antifungal efficacy of Ag NPs against *Fusarium oxysporum*

In the present investigation, biosynthesized AgNPs (100 µg/ml) demonstrated positive antifungal efficacy against *F. oxysporum* with an inhibition zone of 29 mm. as shown in Fig. 6a in comparison with fluconazole as a positive control with an inhibition zone of 26 mm. Moreover, the results proclaimed that biologically synthesized AgNPs exert minimum inhibitory concentration at 50 µg/ml giving 23 mm ZOI. Nowadays, biosynthesized metal nanoparticles have become widely employed to combat fungi that cause plant diseases [77, 78]. Acording to the studies of[40, 41, 79] the antimicrobial efficacy of AgNPs may arise from a lack of replication activity that renders the pathogens' cellular proteins and enzymes inactive. Several studies have shown that due to their antimicrobial properties, AgNPs synthesized from

fungi have got marvelous attention and are widely used in agriculture, exhibiting strong potential against plant pathogenic insects, fungus, bacteria, and viruses[80-83] . A recent investigation reported that AgNPs work by generating reactive oxygen species and free radicals, which damage cell walls, nucleic acids, proton pumps, lipids, and proteins through denaturation[84] . As a result, they modify the permeability of the cell membrane, leading to cell death. Additionally, it was announced that the AgNPs antimicrobial activity was the size and shape reliant[85].

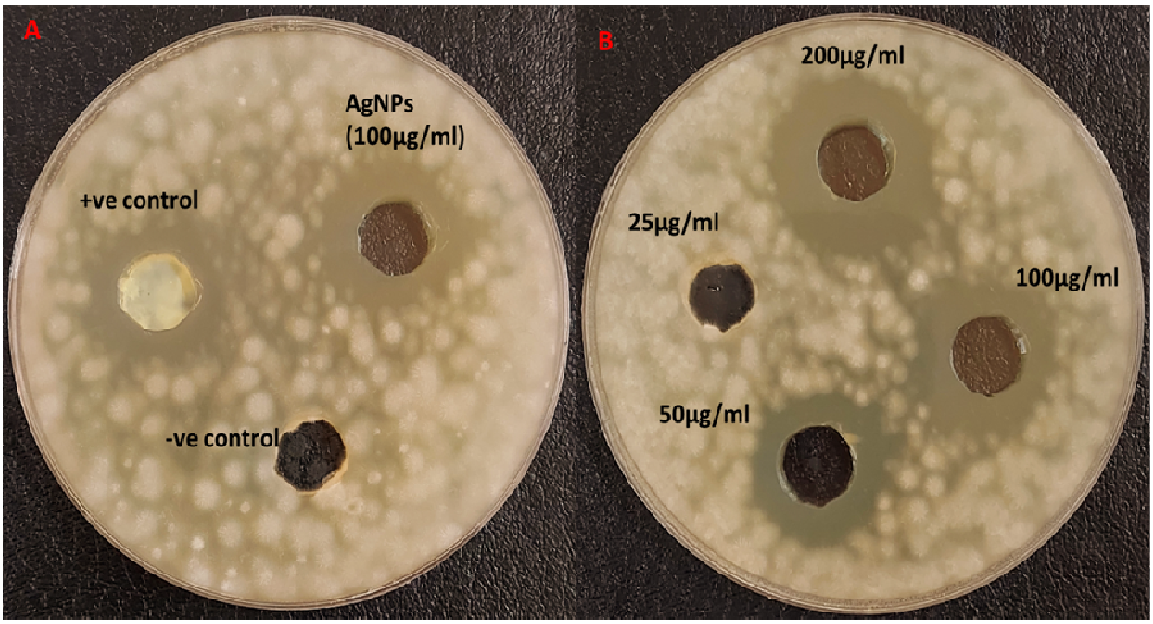

Figure 6 (a): Antifungal activity of Ag NPs against *Fusarium oxysporum*, (b)MIC

3.4. Clarification of AgNPs impact on *Fusarium oxysporum* morphology by the Scanning Electron Microscope

In the current investigation, Figure 7 depicts the morphological modifications to the mycelium for the AgNPs treatments and the controls. The filamentous mycelium on the control exhibits a smooth outer surface, which is a typical structural trait. In contrast, the AgNPs treatment causes the mycelium to become a rough, significantly distorted and lose its smoothness. This suggests that the cell wall has been severely damaged, which fosters the leakage of cellular materials and then hyphal shrinkage. On the other hand, the morphological alterations on the conidia for the controls and the AgNPs treatment were also noted. The control sample shows typical appearance characteristic, which incorporates a soft outward surface on the fusiform shaped conidia. In conflict, the AgNPs treatment promote deformation of the conidia which appeared rough and shrinked.

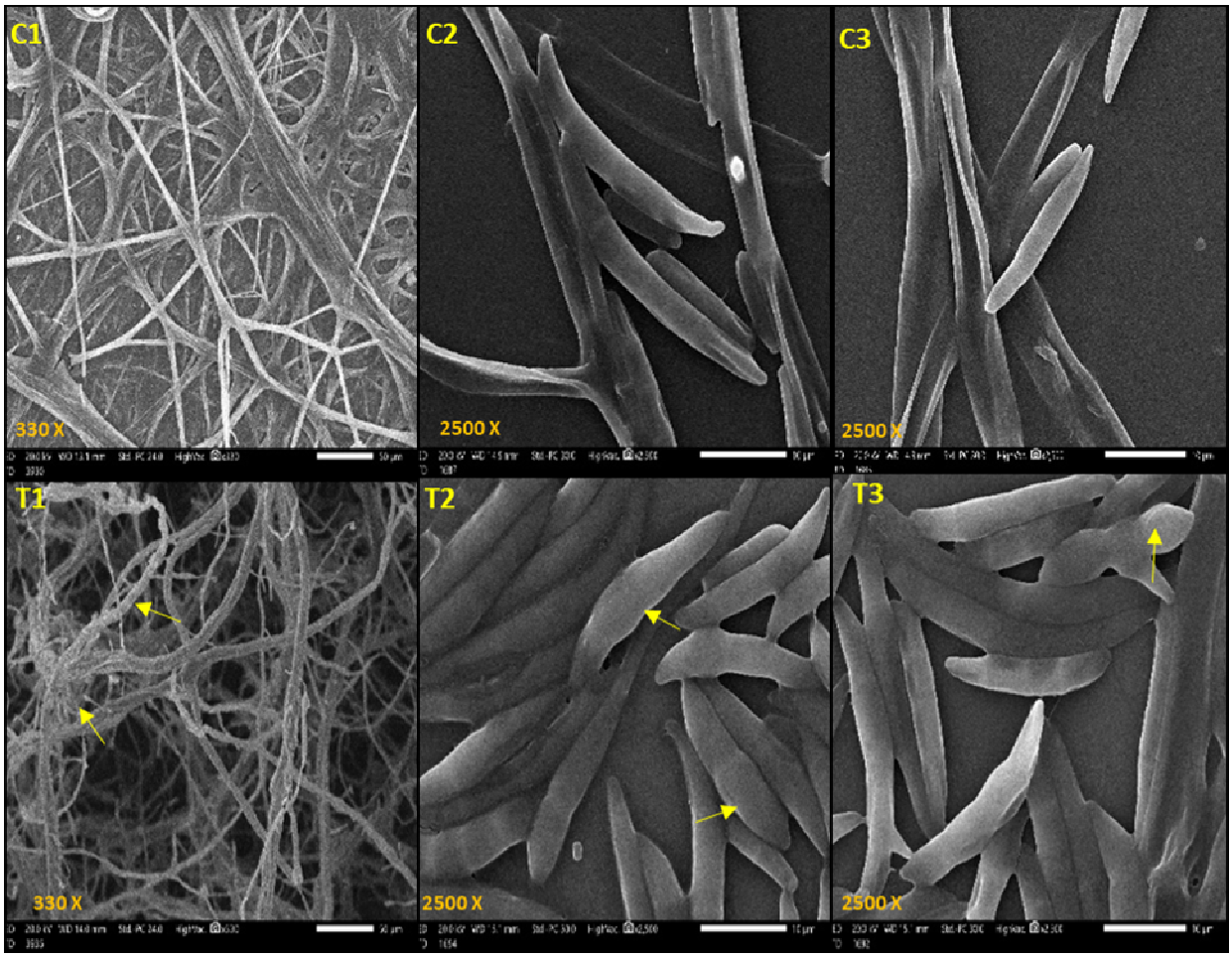

Figure 7: SEM photo showing the (C): non-treated of*Fusariumoxysporum* hyphae, and (T) treated with silver nanoparticles where the hyphae seem broken and deformed

3.5. Disease index and protection percent

The data listed in table (1) point to that *Fusarium oxysporum f.sp.capsici*commenced severe infection in pepper plants, where the severity of wilt disease reached 91.6% compared to healthy controls where no wilt symptoms developed (Fig. 8). Improving plant defenses against diseases can be accomplished by applying biological or abiotic substances externally to plants [86, 87].

By focusing on the effect of biosynthesized AgNPs on infected plants, it was found that each of the concentration (50 and 25 µg/ml) highly gave a reduction in percent disease index by (25 and 37.5) and highly gave protection percent by (72.7 and 59.06 %) compared to the infected untreated controls. Moreover, a decrasing of disease index ratio and vastly increased protection against infection by *F. oxysporum* isthe initial marker to detect the occurrence of SR in plants through treatment with AgNPs extracts. Our result was in agreement with [39]as they reported that biosynthesized AgNPs significantly reduced wilt triggered by *F. oxysporum* equated with untreated infected control plants.

Table 1.

Effect of AgNPs on wilt disease of *Capsicum annuum* L. cv. Castle rock II PVP under pots conditions

Treatment	Disease symptoms Classes					DI (disease index) (%)	Protection (%)
	0	1	2	3	4		
Control healthy	6	0	0	0	0	0	-
Control Infected	0	0	0	2	4	91.6	0
Infected + 25 µg/ml Ag NPs	1	2	2	1	0	37.5	59.06
Infected+ 50 µg/ml Ag NPs	2	2	2	0	0	25	72.7

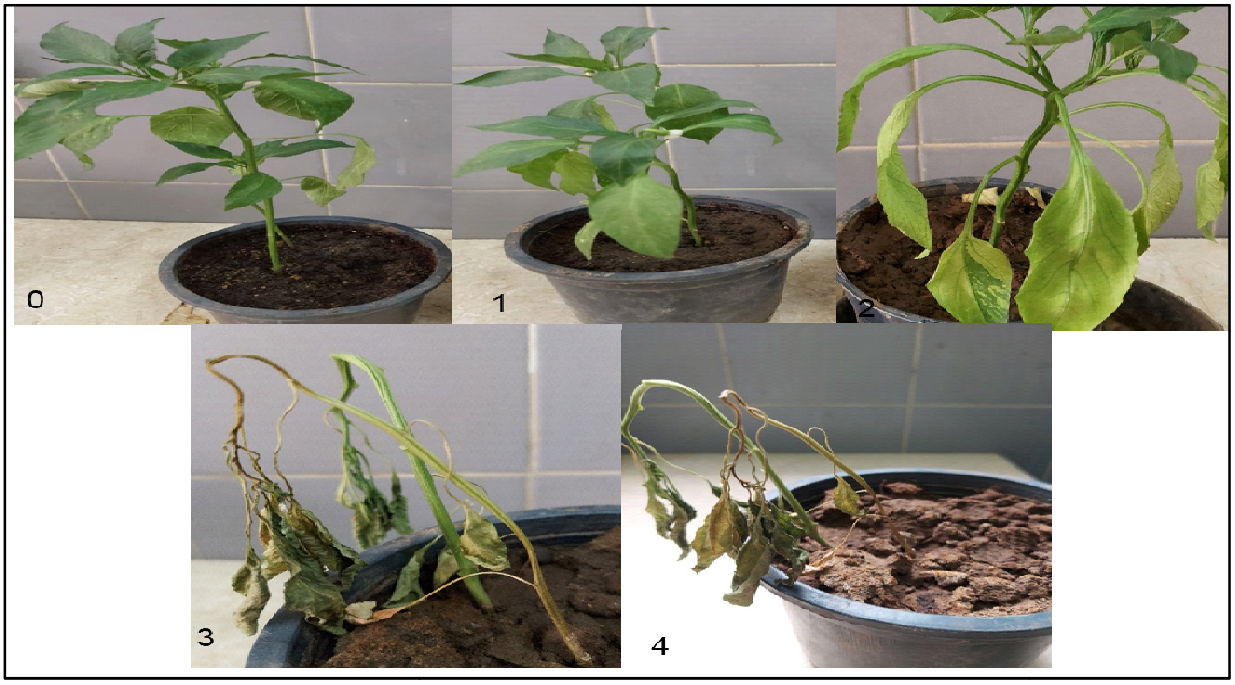

Figure 8. Symptoms grade of wilt disease instigated by *Fusarium oxysporum f.sp.capsici*on pepper plants 0: (no symptoms) 1: (slight yellow of lower leaves), 2: (moderate yellow plant), 3: (wilted plant) and 4: (plants severely cramped and damaged)

3.6. Morphological indicators:

In the current study, results listed in table (2) showed that vegetative growth (shoot and root length) were significantly lower in the *F. oxysporum*-infected pepper plants than they were in the healthy control plants. This sharp decrease in all morphological characteristics can be explained by an imbalance in growth hormones, disorders of respiration, transport and storage processes within the vascular bundles as a result of intracellular

oxidative blasts [15, 88-90]. These results are in agreement with [91]who reported that plant infection with *F. oxysporum* led to wilting and stunting due to weak roots, reduced root size and failure of water uptake and nutrient transport through the vascular carrier system. Moreover, data in table 2 illustrated that the application of AgNPs demonstrated significant improvement in all vegetative characters (shoot length, root length, and number of leaves) when compared with healthy untreated pepper plants (control) as it improved shoot length by (13, 56.5%), root length (93.9, 379.23 %). also, leaves number by (45, 22.11 %), respectively. Similar studies demonstrated the growth-stimulating effects of AgNPs [92-94] . Moreover, it was mentioned that silver may be a growth stimulator for plants [95] . It is worth mentioning that AgNPs-treated pepper seedlings had higher shoots length comparing with the control ones [39].

Table 2.

Effect of tested biosynthesized silver nanoparticles on shoot and root lengths and number of leaves per plant

Treatments	Shoot length (cm)	Root length (cm)	Number of leaves per plant
Control healthy	18±0.38[b]	9.55 ±0.11 [b]	29.91±0.73[c]
Control infected	11.5±0.15[d]	1.83 ±0.01[e]	19.58±0.96[e]
Healthy +25 µg/ml Ag NPs	19.11±0.38[a]	12.44 ±0.40[a]	39.5±0.43[b]
Healthy + 50 µg/ml Ag NPs	19.32±0.25[a]	12.28 ±0.30[a]	53.83±0.44[a]
Infected + 25 µg/ml Ag NPs	13±0.29 [c]	3.55 ±0.11[d]	28.41±6.50 [c]
Infected + 50 µg/ml Ag NPs	18±0.38[b]	8.77 ±0.29[c]	23.91±0.8[d]
LSD at 0.05	0.98	0.75	8.36

Data were stated as means ± standard error of triplicates The distinct alphabetic superscripts in the same column are differ significantly ($p < 0.05$) built on Tuky multiple range analysis.

3.7. Photosynthetic pigments:

The data represented in fig. (9 a) proved that the chlorophyll a and b have being dramatically reduced in plants infected with *F. oxysporum* by (29.36% and 51.48%), separately. While, in plants exposed to *F. oxysporum*infection content of carotenoids increased by 36.7 % when being compared with healthy control plants. In this regard, plant infection with *Fusarium* affects the photosynthesis process by causing oxidative brusts within cells and inhibiting respiratory enzymes, which leads to the plant's inability to gain light efficiently to do photosynthesis[15, 96]. The production of reactive oxygen (ROS), which damages chlorophyll and inhibits plant photosynthesis, may be to blame for the reduction in photosynthetic pigments [97]**.**The application of tested AgNPs (25 µg/ml or 50 µg/ml) on either (healthy or infected) plants enhanced the photosynthetic pigments as chlorophyll (a) and chlorophyll (b) in comparison with plants treated with AgNPs (25 µg/ml). Moreover, it was found that a significant increase in carotenoid content in the applied AgNPs (50 µg/ml) by (74.53%) in comparison with the infected control. Numerous studies have previously shown that the foliar application of AgNPs induces the synthesis of chlorophyll [98-100] . Improvement of chlorophyll and enhancement of photosynthesis efficiency after foliar AgNP application have been demonstrated previously in leaves of pepper plants[92, 93, 101,

102]**.** The activation of the photosynthetic pigments may be owing to bioactive metabolites production by *A.ustus*through physicochemical and biological characteristics of AgNPs. Our results in harmony with the findings of [103]they noted that *Asperagillus*Sp.resulted in advancement of photosynthetic pigment in plants .

3.8. Osmolytes (soluble sugar, soluble protein) contents

Herein, the infection with *F. oxysporum*decreased both the amount of soluble carbohydrates by 28.10% and soluble protein by 40.8% compared to the healthy control. These results were in accord to previous investigation [15, 104], which showed a sharp decrease in the carbohydrate and protein content of plants infected with *F. oxysporum*, but caused a sharp rise in proline contents. Furthermore, the result showed that the application of AgNPs (25 or 50 µg/ml) has an inductive effect on the contents of total carbohydrates, and protein, on both healthy and infected pepper plants. The results evoked that AgNPs at concentrations (25 µg/ml and 50 µg/ml) enhanced the total carbohydrate by (19.61,28.17%), soluble protein by (38.38 and 46.51%) respectively in comparison with infected untreated pepper plants (control) as illustrated in fig.9 b. Our results show that when affected plants were handled with AgNPs, the levels of osmolytes (soluble sugar and soluble protein) increased dramatically, as previously explained by [105, 106]**.**As reported by [107] these soluble carbohydrates function as metabolic signaling by triggering specific or hormonal crosstalk transduction routes, which change the way of genes expression. When plants are exposed to pathogens, their defensive mechanisms are activated, which accounts for the increase in soluble proteins [108]**.**

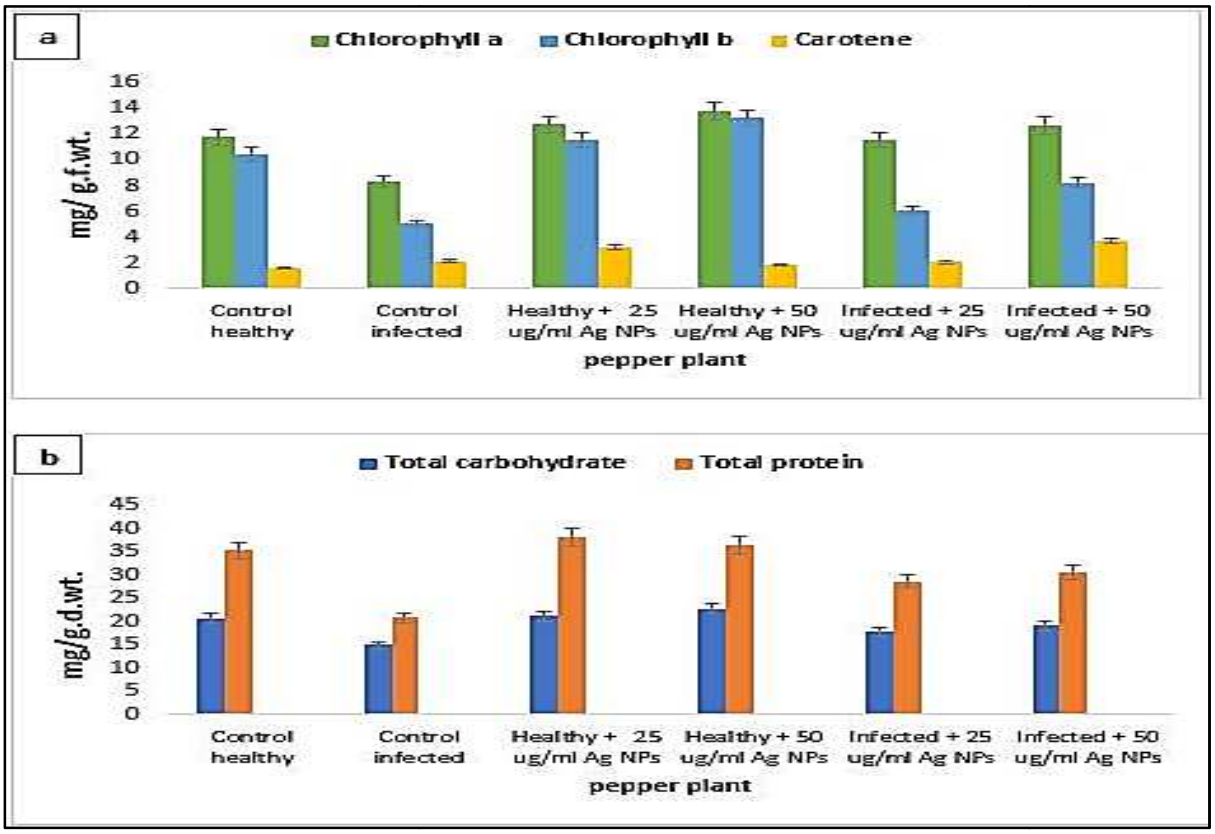

Figure 9: Effect of *A.ustus*bio-synthesized AgNPs on the metabolic indicators of (Capsicum annuum L. cv. Castle rock II PVP): (a) photosynthetic pigment, (b) soluble carbohydrate, and total protein

Data in fig (10) illustrated that,*F. oxysporum*, initiate a distinct considerable increase in the content of total phenols and free proline ininfected pepper plants by (84.1% and 94%) respectively.

Application of (50 µg/ml and 25 µg/ml) AgNPs show a substantial increase in total phenols and free proline. The key to a plant's immune response is phenolic chemicals, which also play a role in the activation of defense genes, the generation of phytoalexins and phytoanticipins, the creation of structural barriers, and the control of pathogenicity[15, 109, 110].The obtained results are in perfect harmony with the results obtained by studies of[111, 112]who noticed an increase in the phenolic content of the metallic NP-treated plants. This rise up in phenolic content accounted for antifungal efficacy against *F. oxysporum* via a number of different mechanisms, including rupture of the fungal cell, the release of intracellular protein and carbohydrate, inhibition of ATP synthesis, and oxidative damage[113]**.** Many phenols are respected as inhibitors of pre-infection, supplying the plant with specific level of essential resistance against pathogenic microorganism [114]**.** Therefore, cell wall lignification and phenol metabolism are engaged in and have conclusion for several cellular of ecological process and whole plant, that may supply plants the immunity against negative agents [115].The proline (amino acid) may work as potent scavenger of reactive oxygen species, and this prevents the promotion of cell death [21, 110, 116]. Similar reports have explained that exposure to AgNPs has been shown to improve the antioxidative defense system and proline content of some crops as documented in several reports by [117-119]**.**

First most essential preventative enzymes in biotic stress response are peroxidase (PO) and polyphenol oxidase (PPO) [21, 87, 120] . The activity of both POD and PPO were higher in the infected plants and in the plants treated with AgNPs at concentrations (50 µg/mL, and 25 µg/ mL). Antioxidant enzymes improving activity is strong and clear evidence of plant resistance against disease[104, 121] . Healthy pepper plants treated with silver nanoparticles exhibited an increase in Peroxidase activity by (52.9 and 67.6%) when compared with healthy control. While treated infected pepper plants showed an increase in Peroxidase activity by (14.16 and 25.8%) respectively when compared with infected control.Additionally, a significant increase in polyphenol oxidase (PPO) activity by (75.2%) was recorded as illustrated in fig. (11). Furthermore, treatment with silver nanoparticles resulted in the induction of polyphenol oxidase (PPO) activity in both healthy and infected plants. Peroxidase (PO) or polyphenol oxidase (PPO) has been provoked by bio-control agent strains in different plants[122, 123] . Agreeing to our results, AgNPs significantly increased oxidative stress and antioxidant enzyme activity compared to control plants., which is reliable with[39] .

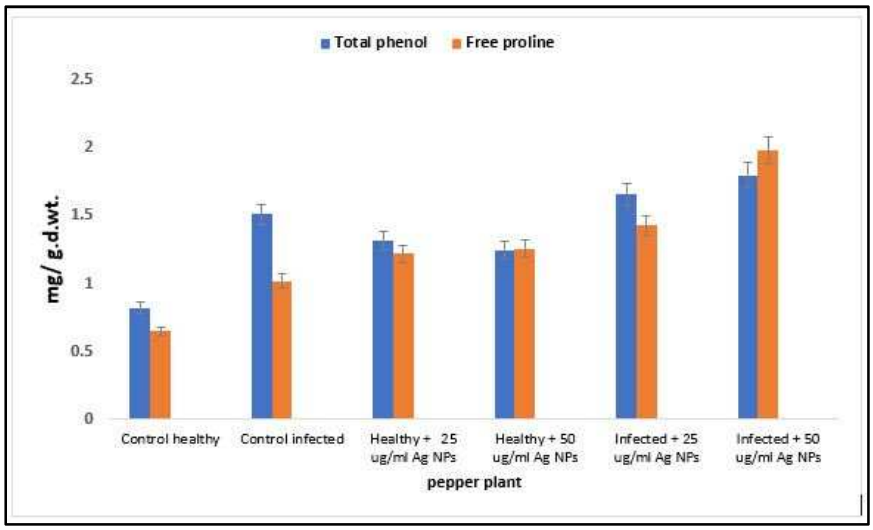

Figure 10: Effect of *A.ustus*bio-synthesized AgNPs on the metabolic indicators of (Capsicum annuum L. cv. Castle rock II PVP): total phenols and free proline

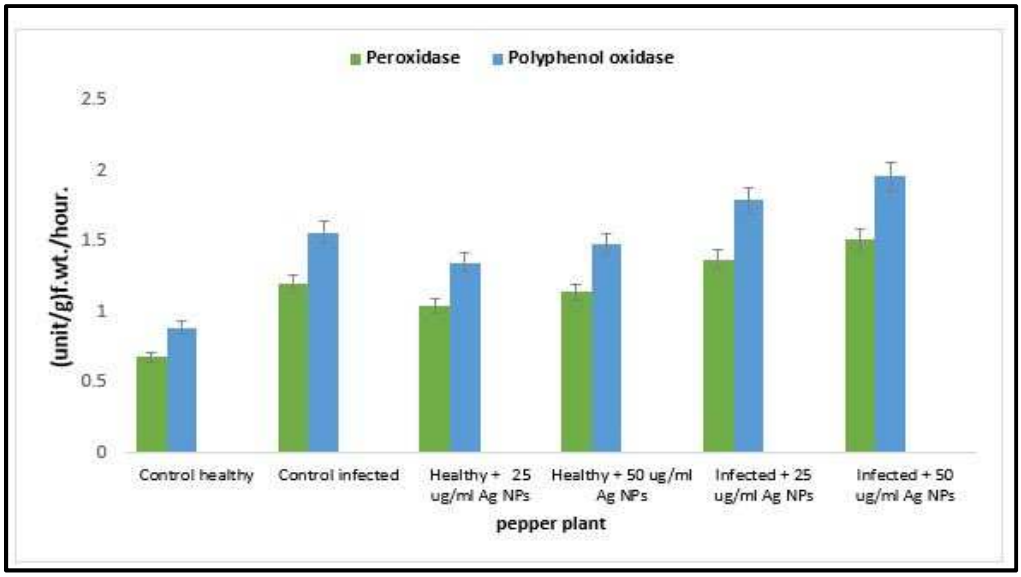

Figure 11: Effect of *A.ustus* bio-synthesized AgNPs on the oxidative enzymes activities in the infected and healthy plants

The scavenger isozymes (POD and PPO) were detected using native PAGE. The results (Fig. **12** and 13) showed the appearance of 4 POD and 4 PPO isozymes in pepper plants leaves. For POD isozymes, Application of AgNPs at 50 µg/ml on infected plants displayed awfully overexpressed POD that evoked 4 strong bands Rf (0.307, 0.479, 0.634 and 0.779), followed by AgNPs at 25 µg/ml that recorded 3 bands 2 of them are strong band at Rf (0.307, 0.479) and 1 moderate band at Rf (0.634). Regarding PPO isozymes, it was established that treating of infected plants with AgNPs at 50 µg /ml resulted in greatly overexpressed PPO, which was highlighted by 4 bands, 1 of which was strong at Rf strong bands Rf (0.562), and 3 intermediate bands at Rf (0.161, 0.411and 0.625), followed by AgNPs at 25 µg/ml that recorded 3 band 2 of them moderate band at Rf (0.161,0.411) and 1 faint band at Rf (0.562). Nanoparticles that function as stressors or promoters improved plant antioxidant defense mechanisms and improved plant tolerance by scavenging reactive oxygen species (ROS) [41, 124]**.**As known, isozyme variation discloses the biochemical entity of resistant genes to physiological changes in organisms [125, 126]**.**In this work, there were distinct changes among infected plants without inducers and plants treated with inducers in terms of both enzyme activity and composition.According to prior research, the activities of examined isozymes were often higher in tested plants treated with AgNPs than they were in untreated plants. This could be a role in the induction of SAR against *Fusarium oxysporum*. Additionally, biotic inducers boosted several PR-proteins such polyphenol oxidase and isozymes of peroxidase [7, 12, 127]**.** From the above information we can conclude that AgNPs play significant role in induction of systemic resistant SR by stimulation of pathogenesis related protein [15, 128].

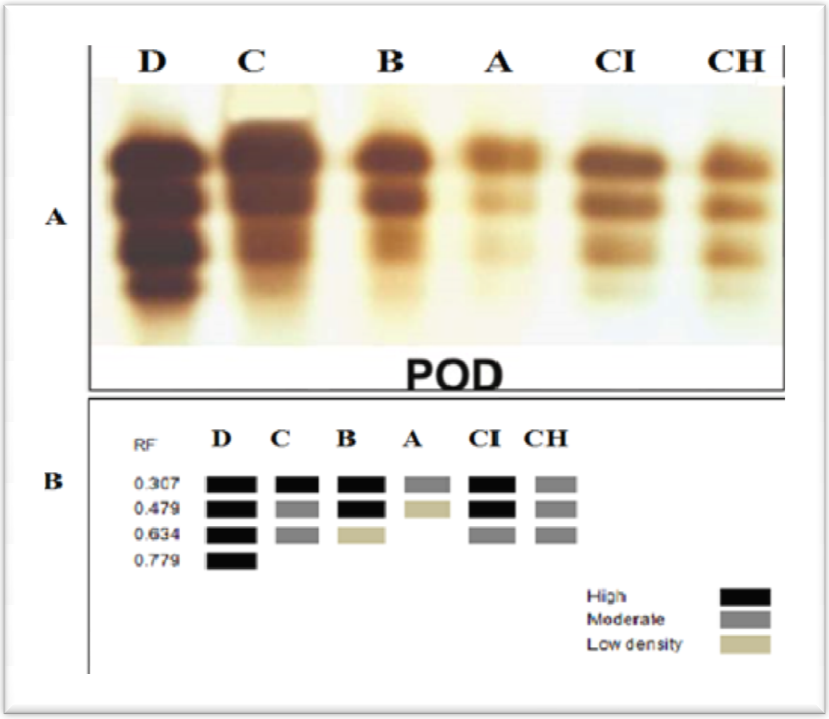

Figure 12: Isomers of POD in response to *F. oxysporum* infected Capsicum annuum L. cv. Castle rock II PVP **treated with Ag NPs at (25 µg/mlor 50 ug/ml).** Ch = Healthy. CI= Infected with ***F. oxysporum***: A = Healthy +25 µg/ml AgNPs, B= Healthy + 50 µg/ml Ag NPs. C= Infected + 25 µg/ml Ag NPs, D = Infected + 50 µg/ml Ag NPs.

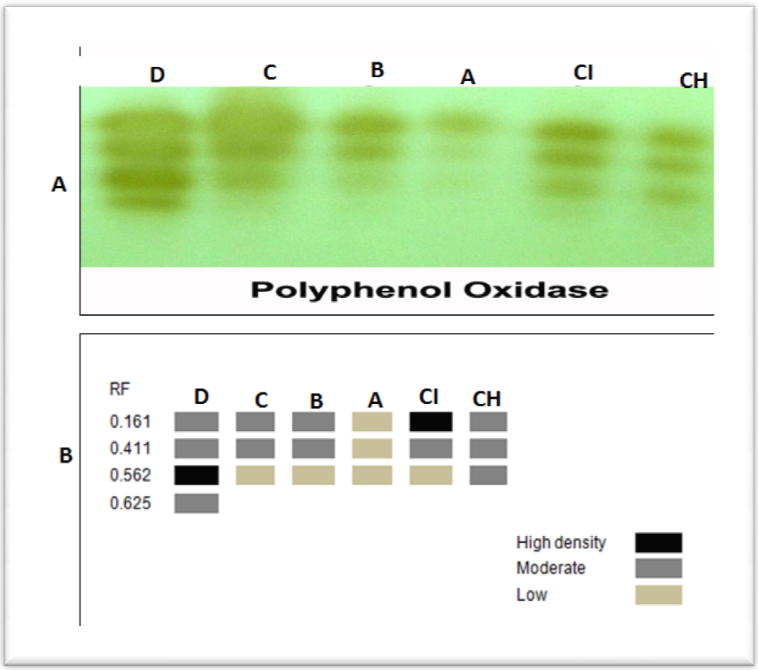

Figure 13: Isomers of PPO in response to *F. oxysporum* infected Capsicum annuum L. cv. Castle rock II PVP treated with Ag NPs at (25 µg/mlor 50 ug/ml). Ch = Healthy. CI= Infected with *F. oxysporum* A = Healthy +25 µg/ml Ag NPs, B= Healthy + 50 µg/ml Ag NPs. C= Infected + 25 µg/ml Ag NPs, D = Infected + 50 µg/ml Ag NPs.

4. Conclusion:

In conclusion, an eco-friendly green approach was utilized for production of AgNPs by *Aspergillus ustus* ON076464. The Results evoked the ability of *Aspergillus ustus* filtrate to build up AgNPs in a spherical shape and dispersed without aggregation with a size average of 12.4 nm. Based on information reported herein, it is possible to inferred that synthesized AgNPs can be used as a talented and effective potential fungicide mediator alongside *F. oxysporum* both in vitro and in vivo. Application of AgNPs at concentrations (50 and 25 µg/ml) highly gave a reduction in percent disease index by (25 and 37.5) and highly gave protection percent by (72.7 and 59.06 %) compared to the untreated infected plants. Moreover, The application of AgNPs at the same concentrations resulted in different responses regarding the photosynthetic pigments, total carbohydrates content, phenol, and total protein of *Fusarium*-infected plants.Moreover, the utilization of biosynthesized AgNPs actually has the ability to combat *Fusarium* wilt in pepper plants by constraining the pathogen, improving ROS removal via more strong antioxidant defense systems, and enhancing phenotypic, molecular, and enzymatic features. The results of this study pave the foundations for further investigation into the efficiency and toxicity of metal nanoparticles as well as the chemical adjustments they undergo in response to biotic stress.

5. Abbreviations

DI: disease index
FTIR: Fourier-transform infrared spectroscopy
GYP: Glucose yeast peptone medium
ISR: Induced systemic resistance.
MIC: Minimum inhibitory concentration
PAGE: Poly Acrylamide Gel Electrophoresis
PCR: polymerase chain reaction
PDA: Potato dextrose agar medium
PDI: percent disease index
PO: Peroxidase
PPO: polyphenol oxidase
ROS: Reactive oxygen species
SEM: Scanning Electron Microscope
SR: Systemic resistant
TEM: Transmission Electron Microscope

6. Conflicts of interest

"There are no conflicts to declare".

7. Formatting of funding sources

Authors state no funding involved.

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