Tikrit Journal for Agricultural Sciences (2024) 24 (2): 161-177
DOI: https://doi.org/10.25130/tjas.24.2.12

IRAQI Academic Scientific Journals
العراقية المجلات الأكاديمية العلمية
TJAS
Tikrit Journal for Agricultural

ISSN:1813-1646 (Print); 2664-0597 (Online)
Tikrit Journal for Agricultural Sciences
Journal Homepage: http://www.tjas.org
E-mail: tjas@tu.edu.iq

Characterization of biologically synthesized copper nanoparticles by *Aspergillus carbonarius* and evaluation of their activity in reducing the pathogenic stress of tomatoes infected with *Fusarium oxysporum*

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KEY WORDS:
Copper nanoparticles, *Aspergillus carbonarius*, *Fusarium oxysporum*, pathogenic stress markers, Rot root disease , Tomato cultivars

Received: 30/01/2024
Revision: 09/04/2024
Proofreading: 18/04/2024
Accepted: 21/04/2024
Available online: 31/06/2024

ABSTRACT

This study was conducted to evaluate the efficiency of the fungus *Aspergillus carbonarius* in the synthesis of copper nanoparticles (CuNPs) from copper chloride solution, with the characterization of these nanoparticles and the demonstration of their effectiveness in inhibiting the pathogenic fungus *Fusarium oxysporum* causing tomato root rot disease. The results indicated that the treatment of copper chloride solution at 150 mM with *A. carbonarius* filtrate led to the production of CuNPs which characterized by a color change from blue to green, with the highest absorbance observed at 300 nm. X-ray diffraction (XRD) results revealed the crystalline structure with peaks at angles 28.47286, 47.43258, and 56.20644 ° corresponding to (111), (220), and (311) planes, respectively, matching the JCPDS card number 01-081-1841. Scanning electron microscopy images showed cubic, pyramidal, and prismatic shapes with dimensions ranging from 15 - 84.291 nm. In laboratory experiment, CuNPs inhibited the growth of the pathogenic fungus, with an inhibition zone of 1.6 cm, compared to copper chloride which recorded 0.9 cm. In the field, , the treatment of CuNPs led to an increase in the stress resistance indicators, reaching the highest values for CuNPs when combined with the fungicide Medazim in the American tomato cultivar. The concentrations of total phenols and glutathione in this treatment were 2.56 mg/g and 0.322 µg/g, respectively. The lowest concentration of proline was recorded as 4.31 µg/g in the treatment involving CuNPs against the fungal infection. CuNPs with the fungicide medazim, significantly outperformed other treatments, registering lowest disease severity at 12.22% in the American cultivar, compared to the highest disease severity was recorded in the treatment with the pathogenic fungus alone, reaching 91.16% in the Dutch cultivar. CuNPs significantly increased tomato fruit weight, both in healthy and infected plants. The American, Turkish, and Dutch tomato cultivars infected with the pathogenic fungus and treated with under + medazim showed higher productivity, reaching 6432.55, 6334.34, and 6305.73 g.plant^{-1}, compared to the lowest productivity in the treatment of these cultivars with the pathogenic fungus, reaching 1008.34, 977.45, and 833.78 g.plant-1, respectively.

توصيف جسيمات النحاس النانوية المحضرة احيائيا بواسطة الفطر *Aspergillus Carbonarius* وتقييم فعاليتها في تقليل الإجهاد المرضي للطماطة المصابة بالفطر *Fusarium oxysporum*

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الخلاصة

أجريت هذه الدراسة لتقييم كفاءة الفطر *Aspergillus carbonarius* في تخليق جسيمات النحاس النانوية (CuNPs) من محلول كلوريد النحاس، مع توصيف هذه الجسيمات النانوية وبيان فعاليتها في تثبيط الفطر الممرض *Fusarium oxysporum* المسبب لمرض تعفن جذور الطماطة. أشارت النتائج إلى أن معاملة محلول كلوريد النحاس بتركيز 150 ملي مولر براشح الفطر *A. carbonarius* أدى إلى إنتاج CuNPs التي تميزت بتغير اللون من الأزرق إلى الأخضر، مع أعلى امتصاصية لوحظت عند 300 نانومتر. كشفت نتائج حيود الأشعة السينية (XRD) عن التركيب البلوري مع قمم عند الزوايا 28.47286 و47.43258 و56.20644° المقابلة للمستويات (111) و(220) و(311) على التوالي، والتي تتوافق مع رقم بطاقة JCPDS 01-081-1841. أظهرت صور المجهر الإلكتروني الماسح أشكالاً مكعبة وهرمية ومنشوريه بأبعاد تتراوح بين 15 - 84.291 نانومتر. مختبريا، ثبطت CuNPs نمو الفطر الممرض *F. oxysporum* ، حيث بلغت منطقة التثبيط 1.6 سم، مقارنة بكلوريد النحاس الذي سجل 0.9 سم. حقليا، ادت معاملة CuNPs الى ارتفاع مؤشرات مقاومة الشد المرضي وبلغت اعلى تلك المؤشرات عند المعاملة ب CuNPs مع المبيد الفطري ميدازيم في صنف الطماطة الامريكي اذ بلغت تراكيز الفينولات الكلية و الكلوتاثيون 2.56 ملغم/غم و0.322 مايكروغرام .غم -1 على التوالي، وادنى تركيز للبرولين 4.31 مايكروغرام .غم -1 في معاملة الفطر الممرض بالنحاس النانوي. سجلت أدنى شدة للمرض بنسبة 12.22٪ في الصنف الأمريكي، مقارنة بـأعلى شدة اصابة في المعاملة بالفطر الممرض وحده حيث بلغت 91.16% في الصنف الهولندي. أدت CuNPs إلى زيادة وزن ثمار الطماطة بشكل ملحوظ، سواء في النباتات السليمة أو المصابة. أظهرت أصناف الطماطة الأمريكية والتركية والهولندية المصابة بالفطر الممرض والمعاملة بـ CuNPs + ميدازيم اعلى إنتاجية بلغت 6432.55، 6334.34، 6305.73 غم.نبات-1، مقارنة بأقل إنتاجية في معاملة هذه الأصناف بالفطر الممرض اذ بلغت 1008.34، 977.45، 833.78 غم.نبات-1 على التوالي.

الكلمات الدالة؛ جسيمات النحاس النانوية، *Aspergillus carbonarius*، *Fusarium oxysporum*، مؤشرات الإجهاد المرضي، مرض تعفن الجذور ، أصناف الطماطة.

INTRODUCTION

Fusarium oxysporum, a plant pathogenic fungus, is responsible for a spectrum of diseases affecting various plant families crops. Being soil-borne, this fungus exhibits prolonged persistence in the soil for multiple years (AL-Qaissi *et al.,* 2021). The disease instigated by *Fusarium oxysporum* is recognized as Fusarium wilt, primarily targeting the plant's vascular system and inducing symptoms like wilting, yellowing, and eventual plant demise. The fungus gains entry through the roots, colonizing the xylem vessels and obstructing the transport of water and nutrients, leading to substantial yield losses (wbester and weber ,2007;Doilom *et al* ,2018 ; AL-Qaissi *et al.,* 2021).

Management strategies for Fusarium wilt encompass the utilization of disease-resistant cultivars, adopting crop rotation practices, and the application of fungicides. However, once a plant is infected, effective treatment becomes challenging, necessitating the removal of infected plants from the field to forestall the disease's dissemination. Copper nanoparticles (Cu-NPs) have demonstrated significant potential as an antifungal agent for the management of plant diseases. Their effectiveness extends across a broad spectrum of plant pathogens, encompassing fungi and bacteria, and has been associated with concurrent enhancements in plant growth and productivity (Taran *et al* .2017;Allosh,2020). The

mechanism of action attributed to Cu NPs involves the infliction of damage to the cell walls and membranes of the pathogen, thereby disrupting essential cellular processes and impeding its growth. Furthermore, Cu NPs have been reported to trigger plant defense mechanisms, including the generation of reactive oxygen species (ROS) and the induction of pathogenesis-related (PR) proteins. These defense responses play a pivotal role in enabling plants to resist infections and recuperate from diseases(Ismail ,2019 ;Omar *et al* ,2019).

The present investigation is directed towards the mycosynthesis of copper nanoparticles from $CuCl_2$ by *Aspergillus carbonarius* and evaluation of their activity in reducing the pathogenic stress of three tomatoes cultivars infected with *Fusarium oxysporum*.

MATERIALS AND METHODS

Source of the fungi

The pathogenic fungus, *Fusarium oxysporum* isolate Abier-6 (ON624357.1) and *Aspergillus carbonarius* (ON624347.1) were used in this study. The fungi were molecularly identified according to the previous study (AL-Qaissi et al., 2023) .

Preparation of fungal filtrate of *A. carbonarius*

A volume of 100 ml of the autoclaved Sabouraud Dextrose Broth was inoculated with a 0.5-centimeter-diameter disc obtained from a young colony (5 days old) of *A. carbonarius*. The inoculated broth was then incubated at a temperature of 25°C for 5 days, with continuous shaking applied daily. Following the completion of fungal colony growth, the culture medium underwent filtration to collect the fungal filtrate, which was subsequently separated from the biomass using Whatman No.1 filter paper. The filtrate was centrifuged at 6000 rpm for 10 minutes, after which the liquid phase was carefully withdrawn. This liquid component was then utilized for the mycoynthesis of copper nanoparticles.

Mycosynthesis of copper nanoparticles

Copper nanoparticles were synthesized by treating the fungal filtrate of *A. carbonarius* with the $CuCl_2$ at a concentration of 150 mM, then it was incubated in the dark for 24 hours at room temperature (AL-Qaissi et al., 2023).

Detection of the copper nanoparticles (CuNPs)

Heterochromia test

Following the incubation period, an observation of color transformation from blue to either yellow or brown was recorded (Madkour ,2019).

Spectroscopy test

The absorbance (wavelength 200-600 nm) of copper nanoparticles synthesized by *A. carbonarius* filtrate were determined using a uv-vis spectrophotometer.

X-ray diffraction (XRD)

CuNPs were analyzed using X-ray diffraction (XRD) with a Cu-Kα radiation source in an X'pert high device at a wavelength (λ) of 1.54060. Then, the crystallite size of the synthesized CuNPs was measured using the Scherrer equation (Narayanan and Sakthivel, 2011):

$D = 0.94y/ \beta \cos \emptyset$

Where:

D represents the crystallite size of the different particles.

λ is the wavelength of the X-rays.

β represents the peak width at half-height.

θ is the angle at which diffraction occurs.

Scanning Electron Microscope (SEM): Copper nanoparticles were examined using a scanning electron microscope at the University of Technology/Iraq - Electron Microscope

Unit. Imaging was done using a field-emission scanning electron microscope (FESEM) F-7600JSM manufactured by JEOL Japan Ltd.

Inhibitory effect of CuNPs against *F. oxysporum* (*In Vitro*)

Poisonous plate method described by Hassan and Ajaj (2021) was used for the test of the inhibitory effect of CuNPs against *F. oxysporum*. $CuCl_2$ solution at the same concentration was also test for comparison.

Preparation of Spore Suspension for *F. oxysporum*

A 5-day-old culture of the pathogenic fungus *F. oxysporum*, exhibiting full growth, was utilized for the preparation of a spore suspension. To this culture, 10 mL of sterile distilled water was added. Subsequently, the spores were harvested from the culture using sterilized brushes. The spore suspension was collected, and the process was repeated by adding another 10 mL of sterile distilled water, followed by the collection of spores. The spore count in the aqueous suspension was determined to be 10^8 cfu/ml by Countess™ Cell Counting Chamber Slide.

Field Experiment

Tomato Cultivation and the Infection by the Pathogenic Fungus *F. oxysporum*

Three tomato cultivars, namely Dutch, American, and Turkish, were planted on October 8, 2022. The induction of infection by *F. oxysporum* was carried out in the diseased treatments and their interactions. This was achieved by immersing tomato seedling roots in the spore suspension of the fungus before planting.

Ten days after planting, on October 18, 2022, CuNPs solution and the fungicide Medazim (25 ml/ 10 L of water prepared according to the instructions of the manufacturer JU AGRI Sciences Pvt. Lit./ India) were applied by injecting 10 mL of each solution in the rhizosphere of tomato cultivars , additionally, 10 mL (5 mL of CuNPs solution + 5 mL of Medazim) was added in the binary treatment. Crop service operations were carried out, through manual weed removal and regular irrigation.

Estimating plant tolerance markers to disease stress

Phenolic Compounds Extraction

Phenolic compounds were extracted following the method described by Parekh and Chanda (2006). 100g of plant root powder were weighed and mixed with 500 mL of 90% ethyl alcohol. The flasks were left for 24 hours with the stirring. Subsequently, the extract was filtered using three layers of medical gauze followed by filtration through Whatman No.1 filter paper in a Buchner funnel connected to a Vacuum apparatus. The filtered solution was concentrated using a Vacuum Rotary Evaporator to remove alcohol, resulting in a dense liquid. The concentrated extract was then spread on polystyrene dishes, dried at laboratory temperature, collected in glass bottles, and stored in the refrigerator (at 4°C) until use. In the case of liquid extracts, 1 gram of the dried powder was dissolved in 10 mL of distilled water.

Total Phenols Estimation

To 1 ml of the extract, 1 mL of 0.05 N HCL, 1 mL of Folin-Ciocalteu reagent, 10 mL of distilled water, and 2 mL of 4% sodium hydroxide (NaOH) solution were added. The absorbance was measured at 515 nm. To determine phenolic concentrations, a standard curve of catechol (Mahadevan and Sridhar, 1986) was employed.

Estimation of Proline

The proline concentration was determined according to the method described by Bates (1973). Briefly; The plant leaves, weighing 100 mg, were crushed in 5 mL of a 3% aqueous solution of sulfosalicylic acid. To 2 mL of this extract, 2 mL of ninhydrin solution (1.25

grams of ninhydrin in a mixture of 30 mL concentrated acetic acid and 20 mL orthophosphoric acid) along with 2 mL of glacial acetic acid were added. The mixture was thoroughly mixed. The tubes were placed in a 100 C hot water bath for an hour, during which a brownish-red color appeared. Afterward, the tubes were cooled by placing them in an ice bath. 4 mL of toluene was added, and the absorbance was measured at 520 nm using a Spectrophotometer. The proline content in the samples was determined based on a standard curve for pure proline.

Estimation of glutathione

The spectrophotometric method described by Rahman et al. (2016) was used for glutathione (GSH) assay which involves oxidation of GSH by the sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. The reaction mixture consisted of 0.5 mL of plant extract (prepared by homogenized 1 g of plant roots in 2.5 mL of distilled water), to which 0.25 mL of phosphate buffer at pH 6.8 and 0.5 mL of DTNP (prepared by dissolving 0.8 g in 1 liter of phosphate buffer) were added. The mixture was left to stand for 5 minutes, then absorbance of the mixture was measured at 412 nm. The concentration of glutathione was determined from the standard curve prepared from known concentrations of glutathione.

Estimation of Plant Infection Severity by *F. oxysporum*

The severity of plant infection was assessed according to the McKinney (1923) equation, following the guidelines provided by Gray (1978) as follows: 0 = No evidence of infection (plant is healthy), 1 = Slight brown discoloration on the roots and yellowing of a limited number of leaves., 2 = Complete root discoloration with widespread yellowing of leaves. , 3 = Discoloration extends from the roots to the bases of the stems, 4 General plant death.

Disease Severity (%) = (Sum of (Lesion Grade × Number of leaves)) / (Highest Grade × Total Number of leaves) × 100

The plant productivity

The fruit weight (g / plant) was recorded for each individual plant. Subsequently, the average yield was calculated by taking the mean of the weights of the first three fruits harvested.

Statistical Analysis

The laboratory experiments were carried out according to the Completely Randomized Design (CRD), while the field experiment was designed according to the Randomized Complete Block Design (RCBD) The analysis of variance was achieved by SPSS program. The comparison of means was executed based on the Least Significant Difference (LSD) and Duncan multiple test at the 0.05 probability level (Al-Rawi and Khalaf Allah 1980**).**

RESULTS AND DISCUSSION

Fig. 1 showed the change of copper chloride color from blue (Fig.A) to green(Fig.B) , induced by the treatment with the *A. carbonarius* filtrate, serves as an indicative marker for the synthesis of copper nanoparticles. This phenomenon is attributed to the reduction of copper ions (Cu^{2+}) present in the copper chloride solution to copper nanoparticles. The presence of the fungal filtrate acts as a reducing agent, facilitating the conversion of copper ions to metallic copper. The color change is associated with the surface plasmon resonance phenomenon exhibited by nanoparticles. Copper nanoparticles exhibit unique optical properties, and the shift in color from blue to green is a characteristic feature of the formation of copper nanoparticles (Chin et al., 2010; Buazar et al., 2019). Studies have indicated that changing the reaction conditions, such as temperature and incubation duration, as well as the

concentration of the solution, affects the reaction speed and the shape of the nanoparticles formed (Tao et al., 2008; Salama et al., 2021).

Figure (1) Color change of the fungal filtrate of *A. carbonarius* indicating the occurrence of biosynthesis of copper nanoparticles (A: copper chloride solution not treated with the fungal filtrate, B: copper chloride solution treated with the fungal filtrate)

Figure (2) presents the spectral analysis of CuNPs resulting from the treatment of copper chloride solution ($CuCl_2$) with the fungal filtrate of *A. carbonarius*. The obtained results revealed the highest peak at 300 nm, while the untreated copper chloride solution ($CuCl_2$) exhibited the highest peak at 400 nm. The spectral analysis technique at ultraviolet and visible wavelengths is employed to verify the occurrence of the biosynthesis process of copper nanoparticles utilizing the fungal filtrate of *A. carbonarius*. The highest absorbance at wavelengths between 250-300 nm serves as an indicator for the synthesis of copper nanoparticles (AL-Dabbagh, 2022). Our findings align with previous studies by Verma et al., 2010; Fatma et al., 2017; and Taran et al., 2017, who reported peaks for biosynthesized copper nanoparticles using *Aspergillus* spp. ranging from 250 to 300 nm.

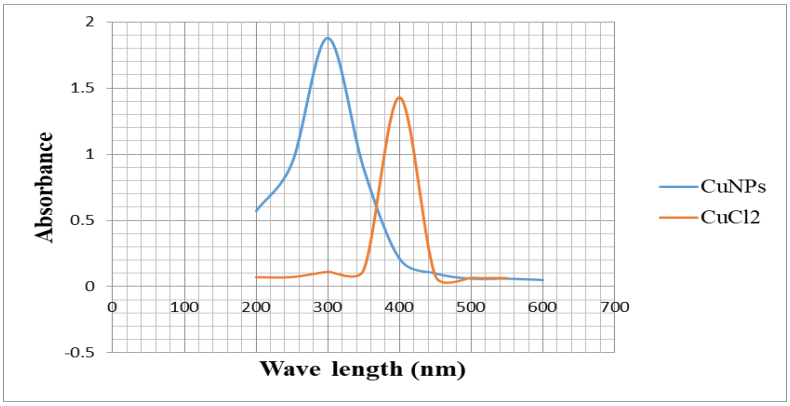

Figure (2) The spectral analysis of CuNPs resulting from the treatment of (CuCl2) with the *A. carbonarius* filtrate, compared to the untreated copper chloride solution.

The X-ray diffraction (XRD) pattern

As shown in Figure (3), the diffraction peaks of the CuNPs. The crystalline structure is observed with peaks corresponding to (111), (220), and (311) planes at angles of 28.47286, 47.43258, and 56.20644 degrees, respectively. These values are in agreement with the JCPDS card number 01-081-1841. The calculated average nanoparticle size is reported as 42.3 nm (Table 1)

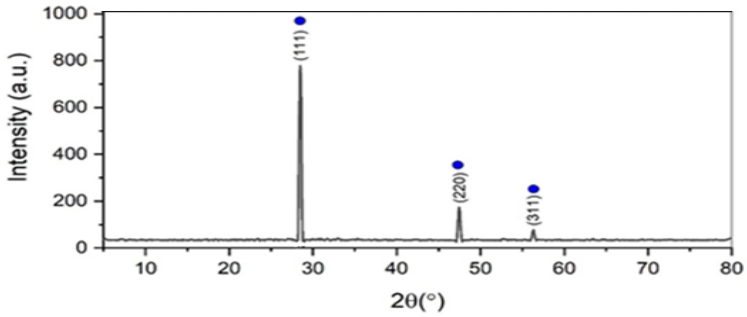

Figure 3. X-ray diffraction pattern of the synthesized CuNPs by *A. carbonarius*.

Table 1. X-ray diffraction measurements of the synthesized CuNPs by A. carbonarius.

2Theta (degree)	Hkl	FWHM (Deg.)	theta 2 (Rad)	FWHM (Rad.)	C.S. (nm)	Matched by	Phase
28.47286	111	0.15744	0.248	0.003	52.04	1841-081-01	Cubic
47.43258	220	0.23616	0.414	0.004	36.73		
56.20641	311	0.23616	0.49	0.004	38.12		
					42.3		

Figure (4) shows the shapes of CuNPs, which appeared in varying shapes between prismatic, rhombic, and cubic. Figure (5) also shows that the dimensions of these particles reached a range of 84.291 - 15.023 nm. Electron microscopy is a powerful tool for studying nanoscopic dimensions, which are characterized by structures ranging from mesoscopic dimensions (about 100 nm) down to individual atoms and clusters (Su, 2013) . According to our results, the CuNPs prepared in this study was within the standard nanoparticle range. The present results agree with Devaraja et al., (2016). The nanoparticle shape was varied from prismatic, rhombic, and cubic, this may be depend on the concentration of reducing agents, according to Mott et al., (2007), spherical shapes formed in lower concentrations and other shapes such as pentagons, cubes, tetrahedra, and elongated forms in higher concentrations. The formation of certain initial core shapes and the difference in the relative rates of growth on different crystal facets were proposed to explain the shape formation.

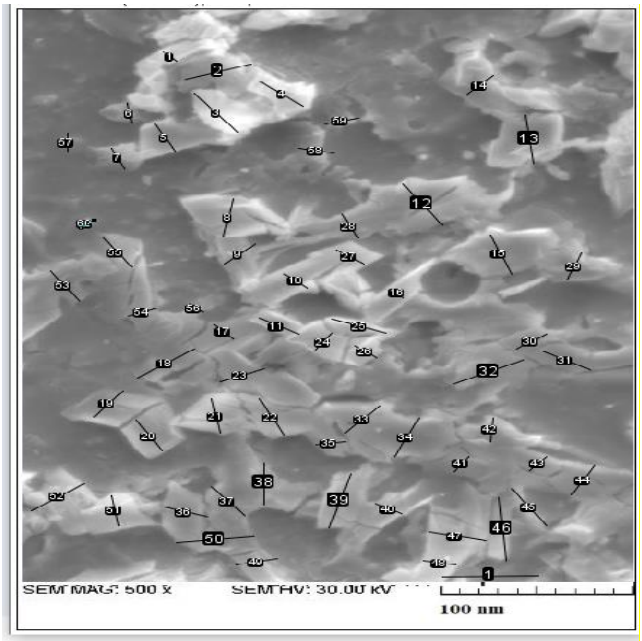

Figure (4) Scanning Electron Microscope image of CuNPs,

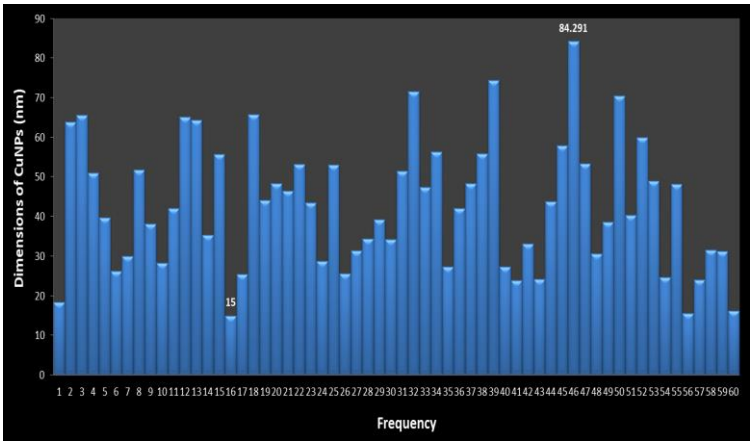

Figure (5) Frequency of CuNPs dimensions

Figure (6) illustrates the significant superiority of CuNPs synthesized from copper chloride at a concentration of 150 mM using the *A. carbonarius* filtrate over an equal concentration of copper chloride solution in inhibiting the growth of *F. oxysporum*. The inhibition zone reached 1.6 cm for CuNPs, while copper chloride recorded 0.9 cm, compared to the control (distilled water) that showed no inhibition of the pathogenic fungus.

The superiority of CuNPs in inhibiting pathogenic fungi may be due to the nanoparticles have a higher surface area compared to bulk materials. This increased surface area allows for more interactions with fungal cells, enhancing the efficacy of the nanoparticles in disrupting cellular structures and functions. In addition, nanoparticles can penetrate the cell walls and membranes of fungi more effectively due to their small size. This improved penetration allows nanoparticles to target intracellular structures, disrupting vital cellular processes and leading to the death of the pathogenic fungi. The results agreed with the studies of Ponmurugan et al. (2016), who demonstrated the role of copper nanoparticles as effective antifungals against red root rot disease in tea plants, and Khatami et al. (2019), who reported that the use of copper nanoparticles with dimensions of 80 nm inhibited the growth of the fungus *F. solani* by 90 percent.% .

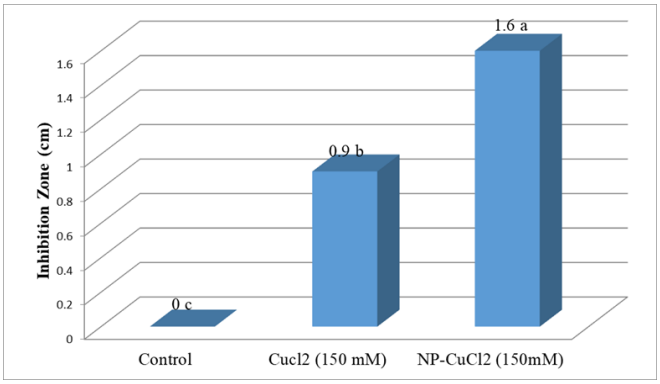

Figure 6: The inhibitory effect of CuNPs compared to copper chloride solution against the pathogenic fungus *F. oxysporum*.

Table 2 illustrates the impact of CuNPs on total phenols in three tomato cultivars under the stress of root rot caused by *F. oxysporum*. The table reveals a significant enhancement in the production rate of total phenols in healthy plants treated with CuNPs, reaching 1.73 mg·g^{-1} in the American cultivar compared to the lowest phenolic content of 1.34 mg·g^{-1} in the control treatment of the Dutch cultivar. Upon infection with the pathogenic *F. oxysporum*, the treatment with CuNPs combined with the fungicide medazim showed a significant superiority over all treatments, recording 2.56 mg·g^{-1}, compared to the lowest total phenols 2.12 and 2.13 mg·g^{-1} (with no significant differences between them) in the disease-infected treatments for the Turkish and Dutch cultivars, respectively.

Our findings align with Savithramma et al. (2011), who reported that CuNPs stimulate plant growth rates by increasing the production of secondary metabolites such as alkaloids and phenols, crucial in inducing plant systemic resistance. Numerous studies have highlighted the role of nano-sized particles of various synthesized metals in enhancing the production of secondary metabolites by interacting with plant cell wall components, rapidly entering plant cells, and directly affecting physiological and biochemical processes in different plants (Jumma and Yahya, 2021). One of the significant applications of nano-sized particles is their ability to stimulate specialized metabolic processes to cope with environmental stress and pathogenic plant stress. They lead to the production of various plant metabolic compounds, such as alkaloids, phenylpropanoids, and total phenols. Das et al. (2018) demonstrated that treating plants and microorganisms with nano-sized particles (NPs) improved the photosynthetic representation, resulting in increased production of phenols acting as antioxidants that scavenge reactive oxygen species (ROS), the main factor in secondary metabolic changes in plants. These changes activate plant defense mechanisms by stimulating an increase in secondary metabolism (Franklin et al., 2008). Studies have shown that nano-sized particles of copper, silver, and gold have the ability to enhance the accumulation of phenols and flavonoids in the callus cultures of *Prunella vulgaris* and *Momordica charantia* (Chung et al., 2018).

Table (2) Effect of CuNPs and the fungicide medazim on the total phenols (mg/g) of three tomato cultivars under the influence of root rot disease caused by *F. oxysporum*

Treatments	Tomato Cultivars			Average of treatments
	Dutch	American	Turkish	
Control (Healthy plants)	1.34	1.60	1.44	1.46
Healthy plants + CuNPs	1.51	1.73	1.51	1.58
Pathogenic fungus, *F. oxysporum*	2.13	2.26	2.12	2.17
F. oxysporum+ CuNPs	2.18	2.36	2.22	2.25
F. oxysporum+ Medazim	2.16	2.27	2.15	2.19
F. oxysporum+ CuNPs+ Medazim	2.38	2.56	2.42	2.45
Average of cultivars	2.002	2.19	2.04	
LSD $_{0.05}$	Treatments; 0.10 , Cultivars; 0.12 Treatments x Cultivars;0.15			

Table 3 presents the impact of CuNPs on proline production in three tomato cultivars under disease stress. The table demonstrates a significant enhancement in proline production rates in healthy plants treated with CuNPs, reaching 3.46 $\mu g \cdot g^{-1}$ in the Dutch cultivar compared to the lowest proline content of 3.29 $\mu g \cdot g^{-1}$ in the untreated control of the American cultivar. Upon infection with the pathogenic fungus *F. oxysporum*, the medazim treatment showed a significant superiority, recording the highest proline level of 6.18 $\mu g \cdot g^{-1}$ in the Dutch cultivar. In contrast, the treatment with CuNPs combined with medazim exhibited the lowest proline content at 4.62 $\mu g \cdot g^{-1}$ in the American cultivar, compared to the highest proline level of 7.16 $\mu g \cdot g^{-1}$ in the Dutch cultivar infected with the pathogenic fungus. According to these results, it is clear that pathological stress leads to an increase in proline production. Proline is considered one of the most important amino acids synthesized in plants as a response to unfavorable environmental conditions and pathological stresses. This amino acid plays a crucial role as a protective response in plants, accumulating due to the inability of plant tissues to construct and break down proteins. Numerous studies indicate that proline accumulation in plant leaf cells helps alter the osmotic pressure of plant tissue, increasing the plant's ability to draw water from the soil. Therefore, proline serves as a reservoir for metabolic substances within the cell (Roosens et al., 1998; Shtereva et al., 2008; Lotfi et al., 2010). Proline works to protect the cell membrane from the negative effects of high concentrations of metal ions and improves various physiological processes, including cell division, elongation, and increased leaf surface area, enhancing carbon assimilation efficiency and increasing dry matter (Al-Khateeb, 2002).

Table 4 indicates a significant increase in glutathione production with CuNPs, both in of healthy plants and the infected plants. The results revealed, at the level of healthy plants, that the highest glutathione value was observed in the American cultivar treated with CuNPs, reaching 0.238 $\mu g \cdot g^{-1}$, followed by the Turkish cultivar at 0.235 $\mu g \cdot g^{-1}$ for the same treatment. In comparison, the untreated Dutch and Turkish varieties recorded values of 0.223 $\mu g \cdot g^{-1}$. The interaction results between varieties and treatments of pathogen-infected plants showed that the highest glutathione value was in the American cultivar. It significantly outperformed the Turkish and Dutch varieties in all treatments, registering the highest glutathione value in the CuNPs and medazim treatment at 0.322 $\mu g \cdot g^{-1}$, compared to the lowest glutathione values in the treatment with the pathogenic fungus *F. oxysporum* alone, which were 0.274, 0.262, and 0.256 $\mu g \cdot g^{-1}$ for the American, Turkish, and Dutch tomato cultivars, respectively.

Table (3) Effect of CuNPs and the fungicide medazim on the proline (µg·g^{-1} fresh wt.) of three tomato cultivars under the influence of root rot disease caused by *F. oxysporum*

Treatments	Tomato Cultivars			Average of treatments
	Dutch	American	Turkish	
Control (Healthy plants)	3.36	3.29	3.34	3.33
Healthy plants + CuNPs	3.46	3.35	3.38	3.40
Pathogenic fungus, *F. oxysporum*	7.16	6.03	6.81	6.67
F. oxysporum+ CuNPs	4.76	4.31	4.72	4.60
F. oxysporum+ Medazim	6.18	4.88	5.67	5.58
F. oxysporum+ CuNPs+ Medazim	4.93	4.62	4.88	4.81
Average of cultivars	4.75	4.28	4.62	
LSD $_{0.05}$	Treatments;0.13 , Cultivars; 0.11 Treatments x Cultivars; 0.17			

The current results are consistent with a previous study that indicated the positive role of glutathione in reducing disease severity in *Arabidopsis thaliana* plants infected with the pathogenic fungus *Alternaria brassicicola* (Datta et al., 2022). Pathogenic fungi have developed multiple antioxidant defenses to cope with the host-derived oxidative stress. Glutathione is a major antioxidant that can prevent cellular damage caused by various oxidative stressors (Wangsanut and Pongpom, 2022). It was shown that over-expression of GSH biosynthesis genes and increased GSH level can induce the expression of different disease-responsive genes, thus imparting stress tolerance (Datta et al., 2015; Datta et al., 2022). The results have indicated that an increase in glutathione concentration serves as a trait that enhances the plant's resistance to biotic stress. The elevated levels of glutathione in the treatment with the pathogenic fungus compared to healthy plants can be attributed to biotic stress induced by the pathogenic fungus. This stress is caused by its direct impact on the activation of oxygen radicals, influencing the electron transport chain, causing membrane damage, and increasing lipid peroxides. Consequently, the plant responds by increasing the synthesis of antioxidant compounds and materials, including antioxidant enzymes such as glutathione, to withstand biotic stress. This results in an elevated glutathione content in the plant (Noctor *et al* .,2012). The inhibition process of the pathogenic fungus by CuNPs, through their impact on the proteins and DNA of the fungus, which prevents the replication and duplication of the fungus's DNA strand, positively contributed to preventing any biotic stress. This led to a balance in the glutathione content in the plant (Elgorban et al., 2016).

Table (4) Effect of CuNPs and the fungicide medazim on the glutathione (µg·g^{-1} fresh wt.) of three tomato cultivars under the influence of root rot disease caused by *F. oxysporum*

Treatments	Tomato Cultivars			Average of treatments
	Dutch	American	Turkish	
Control (Healthy plants)	0.223	0.226	0.223	0.22
Healthy plants + CuNPs	0.231	0.238	0.235	0.23
Pathogenic fungus, *F. oxysporum*	0.256	0.274	0.262	0.26
F. oxysporum+ CuNPs	0.302	0.317	0.309	0.31
F. oxysporum+ Medazim	0.278	0.298	0.290	0.29
F. oxysporum+ CuNPs+ Medazim	0.303	0.322	0.314	0.31
Average of cultivars	0.27	0.28	0.28	
LSD $_{0.05}$	Treatments; 0.003 , Cultivars; 0.007 Treatments x Cultivars; 0.009			

Table 5 illustrates the impact of CuNPs on the severity of infection in three tomato cultivars under the influence of infection with the pathogenic fungus *F. oxysporum*. CuNPs + medazim significantly outperformed, registering significantly lower infection severity, with 12.22% in the American cultivar, followed by the fungicide treatment at 17.44%. In contrast, the highest infection severity was recorded in the treatment with the pathogenic fungus alone, reaching 91.16% in the Dutch cultivar. The results showed there was no observable disease impact in the treatment of healthy plants with CuNPs.

The increased severity of infection in plants treated with the pathogenic fungus alone is a result of the fungal attack on plant tissues, feeding on them, and subsequently increasing the density of fungal hyphae within plant tissues, especially in the vascular bundles. This leads to the obstruction of water and nutrient absorption, affecting various growth indicators. Additionally, the toxins released by the pathogenic fungus contribute to the breakdown of plant cell walls (McGovern et al., 2015). The severity of infection decreased in all experimental treatments, with the lowest severity recorded in treatments involving CuNPs. The effectiveness of CuNPs in reducing infection severity can be attributed to their high penetration capability through the fungal cell wall, leading to the distortion and breakdown of fungal hyphae, resulting in their collapse and death, or inhibiting their growth. Furthermore, these nanoparticles affect fungal cell walls, plasma membranes, and nucleic acids, preventing proton movement through cell membranes, thereby inhibiting fungal growth and causing their demise (Sirelkhatim et al., 2015; Ahmed et al., 201 6; Ahmed et al., 2021; Datta et al., 2022).

Table (5) Effect of CuNPs and the fungicide medazim on the severity of infection (%) caused by *F. oxysporum* in three tomato cultivars

Treatments	**Tomato Cultivars**			**Average of treatments**
	Dutch	**American**	**Turkish**	
Control (Healthy plants)	0	0	0	0
Healthy plants + CuNPs	0	0	0	0
Pathogenic fungus, *F. oxysporum*	91.16	88.43	90.34	89.98
F. oxysporum+ CuNPs	19.66	15.88	16.79	17.44
F. oxysporum+ Medazim	21.88	17.44	19.60	19.64
F. oxysporum+ CuNPs+ Medazim	16.56	12.22	14.65	14.48
Average of cultivars	20.28	17.72	19	
$LSD_{0.05}$	Treatments; 1.11 , Cultivars; 1.13			
	Treatments x Cultivars; 1.18			

The results of Table (6) demonstrate that CuNPs significantly increased the fruit weight in both healthy and fungus-infected plants compared to the control and the medazim fungicide treatment alone. In healthy plants treated with CuNPs, the highest fruit weight reached 6686.33 $g.plant^{-1}$ in the American tomato cultivar. The American, Turkish, and Dutch cultivars infected with the pathogenic fungus *F. oxysporum* and treated with CuNPs, along with the medazim, showed higher productivity, reaching 6432.55, 6334.34, and 6305.73 $g.plant^{-1}$, respectively, compared to the lowest productivity in the control of these cultivars with the pathogenic fungus, which reached 1008.34, 977.45, and 833.78 $g.plant^{-1}$, respectively. The final plant productivity resulting from the effect of CuNPs, according to our current study, was attributed to the superior content of tomato cultivars in terms of positive stress indicators, including phenols, proline, and glutathione. Additionally, there was a significant reduction in the severity of infection with the pathogenic fungus in the CuNPs treatment for healthy plants and in the CuNPs treatment with the medazim for the infected plants. The superior performance of CuNPs combined with the medazim can be attributed to a synergistic effect between them. The inhibitory effectiveness of CuNPs against the pathogenic fungus, as demonstrated in this study, combined with the antifungal activity of the

medazim, resulted in a more effective joint impact on inhibiting the pathogenic fungus. Nanoparticles often exert their antifungal effects through multiple mechanisms, such as oxidative stress, disruption of cell membranes, interference with cellular signaling, and damage to genetic material. This multifaceted approach reduces the likelihood of fungi developing resistance. Similar synergistic effects between fungicides and nano or non-nano organic materials have been reported in previous studies (Hassan and Ajaj, 2021; Hassan et al., 2022a). The American tomato cultivar outperformed other cultivars in productivity and positive stress tolerance indicators. This may be attributed to the fact that the American cultivar is a genetic composition that differs from other cultivars in some genes whose gene expression reflects physiological traits. This aligns with previous studies that have shown that different plant cultivars have varied effects on the tolerance of stress caused by different fungal pathogens (Hassan et al., 2022b; Mohamed and Hassan, 2023).

Table (6) Effect of CuNPs and the medazim on the yield of three tomato cultivars (g.plant^{-1} under the influence of root rot disease caused by *F. oxysporum*

Treatments	**Tomato Cultivars**			**Average of treatments**
	Dutch	**American**	**Turkish**	
Control (Healthy plants)	6203.32	6393.11	6228.26	6274.89
Healthy plants + CuNPs	6409.81	6686.33	6501.12	6532.42
Pathogenic fungus, *F. oxysporum*	833.78	1008.34	977.45	939.86
F. oxysporum+ CuNPs	6290.73	6478.59	6341.35	6370.22
F. oxysporum+ Medazim	5881.12	6062.55	5800.34	5914.67
F. oxysporum+ CuNPs+ Medazim	6305.73	6432.55	6334.34	6357.54
Average of cultivars	5671.47	5864.27	5713.17	
LSD $_{0.05}$	Treatments; 34.5 , Cultivars; 39.59 Treatments x Cultivars; 42.57			

CONCLUSION

It can be concluded from this study that CuNPs biosynthesis by the fungus *Aspergillus carbonarius* was successful. At the laboratory level, CuNPs inhibited the growth of the pathogenic fungus *F. oxysporum* greater than copper chloride solution. At the field level, CuNPs in combination with the fungicide Midazim demonstrated high efficiency in increasing indicators of resistance to biotic stress and reducing the severity of infection caused by the fungus *F. oxysporum* , this reflected positively on the increased productivity of the studied tomato cultivars.

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