

Research Article

Antifungal and resistance-inducing effects of silver-reduced graphene oxide nanoparticles against *Pythium aphanidermatum* in cucumber**Hossein Vasighi Meraji, Sonbol Nazeri[✉]**

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Corresponding author[✉]snazeri@basu.ac.ir**Abstract****Keywords**Defense -related genes,
Pythium aphanidermatum,
Systemic acquired resistance,
qRT-PCR analysis

In the present study, silver-reduced graphene oxide nanoparticles (Ag-rGO) were synthesized, and their direct antifungal activity against *Pythium aphanidermatum* was evaluated, as well as their effectiveness in inducing resistance against damping-off in cucumber caused by *P. aphanidermatum*. The antifungal activity tests showed effective in vitro inhibition of *P. aphanidermatum* growth by Ag-rGO treatment at a concentration of 100 mg/L, with approximately 90% inhibition compared with the untreated control. Similar results were observed in the greenhouse and field experiments, with approximately 75–80% reduction in damping-off in cucumber plants treated with Ag-rGO (compared to untreated controls). Expression level of defense-related gene expression in SA and JA/ET signaling pathways was increased by 10-fold by treatment with Ag-rGO compared to untreated controls when determined by real-time RT-PCR assay (qRT-PCR). These results are the first to report that Ag-rGO could be a novel strategy for controlling *P. aphanidermatum*.

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Introduction

Species of *Pythium* are important oomycete pathogens responsible for damping-off disease in a wide range of economically important plant species. (Wu *et al.* 2020). *Pythium aphanidermatum* (Edson) Fitzpatrick is a serious plant pathogen that causes damping-off and root rot in a wide range of economically important agricultural crops worldwide. (Parveen *et al.* 2020). *Pythium aphanidermatum* causes damping-off, root rot, sudden death, stem rot and fruit rot under field conditions (Syed *et al.* 2020). Although conventional fungicides and oomycetocides are commonly used to manage diseases caused by *Pythium* spp., effective control remains challenging because of their broad host range, rapid production and germination of sporangia and zoospores under favorable conditions, and ability to survive in soil as persistent oospores (Martin 1990; Moorman 2015; Broders *et al.* 2007; Matthiesen *et al.* 2016). Extensive reliance on synthetic fungicides has caused serious environmental and human health problems (Beckerman *et al.* 2023; Wightwick *et al.*, 2010), and, as such, natural solutions have been investigated (Gupta, 2022). Silver nanoparticles have been proposed as a new strategy for controlling plant fungal pathogens (Nasrollahi *et al.* 2011). In recent decades, research has also shown increased use of synthesized silver nanoparticles produced by microorganisms such as bacteria, fungi, and plants (Mallmann *et al.* 2015; Zarowska *et al.* 2019). The green synthesis method for silver nanoparticles is thus considered a promising approach for controlling *P. aphanidermatum*, compared to chemical methods. However, the synthesis of Ag-rGO NPs relied on chemical reduction to achieve reproducibility. Developing nature-oriented and sustainable solutions to control this economically important disease is needed.

One of the innovative plant pathogen control methods that employs nanoparticles, nanomaterials, and their hybrid form (Rajwade *et al.* 2020). High surface-to-volume ratio nanomaterials facilitate effective interactions with plant pathogens, enabling control of various plant diseases through their antimicrobial activity (Mubeen *et al.* 2021).

Silver-reduced graphene oxide (Ag-rGO) nanoparticles have proven to be an effective new agent for fungal diseases due to their high antibacterial potential (Chen *et al.* 2019; Sedki *et al.* 2015). As very little is reported on Ag-rGO as an antifungal agent in plants, this study investigated it in light of its demonstrated effectiveness. Ag-GO, reported to exhibit strong antibacterial properties against bacterial spot of tomato (*Xanthomonas perforans*), induced bacterial membrane disorder and cell lysis with no phytotoxicity (Strayer *et al.* 2016). Similarly, Ag-GO also caused bacterial damage in *Xanthomonas oryzae* pv. *oryzae* (bacterial leaf blight of rice) through membrane destruction and leakage in cells (Kim *et al.* 2020). It was also shown that Ag-GO affects hyphal morphology and induces ROS production in *Fusarium graminearum*, demonstrating broad-spectrum activity (He *et al.* 2018). Ag-rGO possesses enhanced biological and antibacterial characteristics compared with Ag-GO in general; hence, as these agents primarily impact primary producers in environments, Ag-rGO nanoparticles were used for the study.

Herein, we described size- and morphologically controlled synthesis of Ag-reduced graphene oxide (Ag-rGO) nanoparticles and their in vitro antifungal activity against *P. aphanidermatum*. Furthermore, systemic acquired resistance induced by nanoparticles against damping-off was investigated in a greenhouse environment using cucumber plants. Expression patterns of important genes, including those regulating systemic acquired resistance and defense, were determined by quantitative real-time PCR (qRT-PCR). Moreover, the effect of the synthesized nanoparticle on the physiological properties of the plants was systematically determined.

Materials and methods

Chemicals

Analytical-grade Chemicals: All chemicals, such as AgNO₃ (silver nitrate), ammonia solution, and ascorbic acid, purchased from Sigma-Aldrich (Germany), were used without further purification. Mefenoxam was used as a fungicide and purchased

from Dhanuka Agritech Limited, India. The characterization techniques included Fourier-transform infrared spectroscopy (FTIR) using a Bruker Alpha-E instrument from Germany, Raman spectroscopy performed with a 910-nicolet instrument from the USA, X-ray diffraction (XRD) analysis conducted on a PW1730 instrument from PHILIPS Co. with a 2θ range of 5° to 80° , field-emission scanning electron microscopy (FESEM) using a JEOL JSM-6700 microscope, and X-ray photoelectron spectroscopy (XPS) performed with a BesTek (Germany) X-ray photoelectron spectrometer.

Synthesis of Ag-rGO

GO was synthesized via the modified Hummers' method (Dat *et al.* 2020). A GO dispersion (50 ml; 5 mg/ml) in ddH₂O was prepared. 30 ml solution of AgNO₃/NH₃ (20 mg/ml) was added to GO solution and sonicated for 20 min at 600 W. 2 g of ascorbic acid (a friendly reducing agent with non-gaseous by products) was added in three steps stirred at 90 °C for 3 h; this step reduces Ag ion and oxygen content in the functional group of GO simultaneously. The obtained Ag-rGO nanocomposite was washed a few times with ddH₂O and ethanol after centrifugation and filtration in order to produce powder.

Pythium aphanidermatum isolates and inoculum Preparation

The *Pythium aphanidermatum* isolates used in this study were previously isolated and identified from diseased cucumber plants collected during earlier studies (unpublished data). For *P. aphanidermatum* propagation, a rice-bran medium was prepared by autoclaving (1.5 atm, 20 min) 100 g rice bran, 200 g sand, and 100 mL water in 500 mL flasks. Sterile flasks were inoculated with mycelial discs from PDA cultures and incubated (25–28 °C, 7 days). assessed 45 days post-planting using Khalifa's (1987) method.

$$\% \text{Post_emergence damping-off} = \frac{\text{No. of dead seedlings}}{\text{No. of sown seeds}} \times 100$$

Field conditions: In this experiment, sandy clay

Effect of Ag-rGO against P. aphanidermatum under laboratory conditions

Ag-rGO antifungal activity against *P. aphanidermatum* was assessed using a modified method (Fraternal *et al.* 2003). Sterile liquid PDB medium was amended with Ag-rGO (25, 50, and 100 µg/mL) and Metalaxyl-M (mefenoxam) (400 mg/mL; concentrations based on preliminary screening—data not shown) and poured into petri dishes. Untreated PDA served as a control. 0.5-cm discs from 7-day-old fungal cultures were placed centrally, and dishes were incubated ($25 \pm 2^\circ\text{C}$). After about 7 days (or when control colonies reached full growth), colony diameters were measured, and percent growth inhibition was calculated using the Vincent formula (Vincent 1947). Each treatment was replicated three times.

Effect of Ag-rGO against damping off pathogen

Greenhouse conditions: Greenhouse experiments were conducted under controlled day/night conditions (25–27/16–18 °C), with relative humidity maintained at approximately 60% during the day and 70% at night. Sandy loam soil was sterilized by a 5% formalin treatment followed by 7 days of air drying (Wang *et al.* 2011). Sterile soil (3 kg/pot, 20-cm diameter) was inoculated with *P. aphanidermatum* rice-bran culture (3% w/w soil) one week prior to planting 14-day-old DP-164 cucumber seedlings. Seedlings were root-dipped (2 h) in Ag-rGO (100 µg/L) or mefenoxam (300 mg/L) solutions, then planted. Ten mL of respective solutions were applied to the soil around each plant's roots. Foliar spray treatments were performed using the same concentrations and formulations as those applied via soil drench. Controls received distilled water treatments (root dip, foliar spray, soil irrigation). Each treatment was replicated three times. Disease incidence was assessed 45 days post-planting using Khalifa's (1987) method. Soil was used, treated with a 5% formalin solution to completely sterilize and eliminate potential contaminants, ensuring that disease development resulted exclusively from the introduced *P. aphanidermatum* inoculum. The soil was then left exposed to open air for 7 days to dry completely,

following the protocol provided by Wang *et al.* (2011). After this phase, sterilized soil was transferred into 20 cm diameter spots (3 kg soil each) and maintained under field conditions. The aim was to create a uniform environment free of any competing pathogens for the fungus under study. Before planting the seedlings, the soil was artificially inoculated with the fungus *P. aphanidermatum*. For this purpose, rice husk culture of the fungus was used, and the inoculum level was adjusted to constitute 3% of the soil weight. This was done one week prior to planting to allow adequate establishment of the fungus in the soil. The soil was kept moist until planting to ensure suitable conditions for fungal growth and to prevent drying and soil damage. Cucumber seedlings of the DP-164 cultivar, which had been grown for 14 days under greenhouse conditions, were used in this experiment. The roots of these seedlings were immersed in various solutions for 2 hours. Some roots were placed in a solution of Ag-rGO at a concentration of 100 µg/L, while others were placed in a solution of the fungicide Metalaxyl-M (mefenoxam) at a concentration of 300 mg/L. Following this stage, the seedlings were planted in the pots. To examine the effects of Ag-rGO and mefenoxam, the respective solutions were injected into the soil around the cucumber roots. For each plant, 10 mL of the solution was injected into the soil around the roots. In the control group, the seedlings' roots were immersed in distilled water, their foliage was sprayed with it, and the soil around the roots was irrigated with it. All treatments were carried out in three replications to ensure accurate results.

$$\% \text{Pre emergence damping-off} = \frac{\text{No. of non-emerged seeds}}{\text{No. of sown seeds}} \times 100$$

$$\% \text{Post-emergence damping-off} = \frac{\text{No. of dead seedlings}}{\text{No. of sown seeds}} \times 100$$

To compare the progress of damping-off under pre- and post-emergence conditions and to ensure it could be compared with the standard previously described for cucumber (Khalifa, 1987), damping-off was assessed at 15 and 45 days after transplanting. In addition to assessing damping-off

progress at a systemic level, further granular detail of symptoms was obtained using the 0–4 visual assessment rating system proposed by Saldajeno Hyakumachi (2011) 45 days after transplantation: 0, no visible symptoms; 1, slight wilt/slight discoloration in the root system; 2, moderate wilt/moderate browning in the root system; 3, severe wilt with the most necrosis occurring in roots and stem; 4, dead plants. In addition to the damping-off severity index, the general cucumber growth impact resulting from Ag-rGO and mefenoxam treatments was evaluated by measuring several quantitative physiological and morphological parameters, including plant height, number of leaves, total chlorophyll content, and plant fresh and dry weight. Reproductive yield was evaluated by harvesting marketable cucumbers daily for 45 days after transplantation, and by recording the average number and weight per plant.

Expression of defense-related genes by qRT-PCR analysis

For sampling and RNA extraction, the required Plant material was excised and quickly preserved at -80°C (two replications per plant) (Elsharkawy *et al.* 2012). After extraction, the total RNA concentration was measured with a NanoDrop spectrophotometer, and the RNA was converted to cDNA using the Thermo Scientific Fermentas kit (#EP0451). Target genes including LOX1, PAL1, Actin, -1,3-glucanase, catalase, and PR1 were chosen to test the defense-inducing activities of the synthesized nanomaterial in this study because the literature reported that they play roles in salicylic acid- and jasmonic acid-mediated defense responses and in plant responses to reactive oxygen species. Actin was used as a housekeeping gene for relative gene expression calculations, and all qRT-PCR experiments were run in triplicate. The mean Ct value was used to calculate the relative expression in terms of 2-Ct. The mRNA expression levels of these targeted genes were measured by qRT-PCR using gene-specific primers (Table 1). Quantitative RT-PCR analysis was performed on the 7500 Real-Time PCR system (Applied Biosystems, USA). Raw threshold cycle numbers were analyzed using

ABI PRISM 7500 software, and the mean values were determined. The expression relative to the housekeeping gene Actin was computed using the

2-Ct method (Livak & Schmittgen 2001). Primers used in this RT-PCR are described below.

Table 1. Gene primers.

Gene	Forward primer (5'-----3')	Reverse primer (5'-----3')	Ref.
LOX1	CTCTTGGGTGGTGGTGTTTC	TGGTGGGATTGAAGTTAGCC	[31]
PAL1	ATGGAGGCAACTTCCAAGGA	CCATGGCAATCTCAGCACCT	[32]
Actin	AGTATTGTTGGTCGTCCCG	TCAGTGAGAAGAACTGGGTGTTTC	[33]
β -1,3-Glucanase	AAATCCCAGCTCTCCAACAT	AACAGGGATGTGTAGAACCT	[34]
Catalase	AGCGCTCACCTGGTTAAGAAT	ACCAGTGGGATCCTGTCTAAC	[34]
PR1-1a	CTCAAGACTTCGTCGGTGTCCA	CGCCAGAGTTCACTAGCCTAC	[35]

Data analysis

The results of the experiments were analyzed using the statistical software XLSTAT PRO (Addinsoft, France). Experimental results under greenhouse conditions were obtained through a factorial experiment in the Complete Randomized Design (CRD). Statistical analysis was performed using Analysis of Variance (ANOVA) to test for

significant differences among the mean values of the different treatment groups. Then followed by Fisher's Least Significant Difference (LSD) test for determining the number of groups that are statistically different. The level of statistical significance adopted in the analysis was 0.05. That is, those that have a probability less than 0.05 would be considered significant.

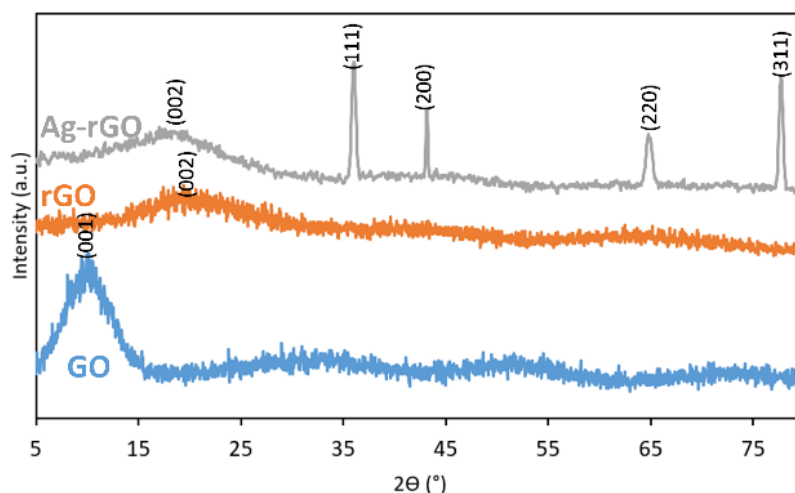


Figure 1. The XRD patterns of Ag-rGO, rGO, and GO.

Results and Discussion

Characterization of Ag-rGO

XRD analysis

To better investigate the structure of the Ag-rGO nanocomposite, X-ray diffraction (XRD) analysis was performed at a 2θ angle range of 5° to 80° . Figure 1 shows the XRD patterns of the Ag-rGO composite, reduced graphene oxide (rGO), and graphene oxide (GO). In the XRD spectrum of GO, only a sharp peak at $2\theta = 10^\circ$ was observed, corresponding to the (001) plane. Moreover, the rGO in the XRD spectrum showed a broad peak at $2\theta = 23^\circ$, corresponding to the (002) plane (Kumar *et*

al. 2019). In contrast, when Ag-rGO composite was synthesized, peaks corresponding to (111), (200), (220), and (311) planes arose in the XRD pattern at $2\theta = 38, 44, 64,$ and 77° , respectively. These observations confirmed the face-centered cubic crystalline structure of the Ag-rGO composite (JCPDS #04-0783). The results indicate the presence of silver nanoparticles in the synthesized composite. Moreover, the broad peak at $2\theta = 23^\circ$ in the Ag-rGO spectrum indicates the presence of reduced graphene oxide in the composite's final structure (Chen *et al.* 2019).

FTIR spectra (Figure 2) showed characteristic

peaks for oxygen-containing functional groups in graphene oxide (GO): OH (3400 cm^{-1}), C=O (1750 cm^{-1}), COC- (1215 cm^{-1}), and C-O (1000 cm^{-1}). C=C and CO-H peaks (~ 1530 and 1400 cm^{-1}) were also observed. In rGO, OH, C=C, and COC₂- peak intensities decreased, and C-O, COH, and CO₂- peaks disappeared, indicating reduction of

oxygenated groups. Ag-rGO FTIR spectra resembled rGO but with slightly reduced peak intensities due to AgNP attachment (Kumar *et al.* 2019). This slightly reduced intensity corresponded to a significant decrease in specific surface area (Section 3.1.5).

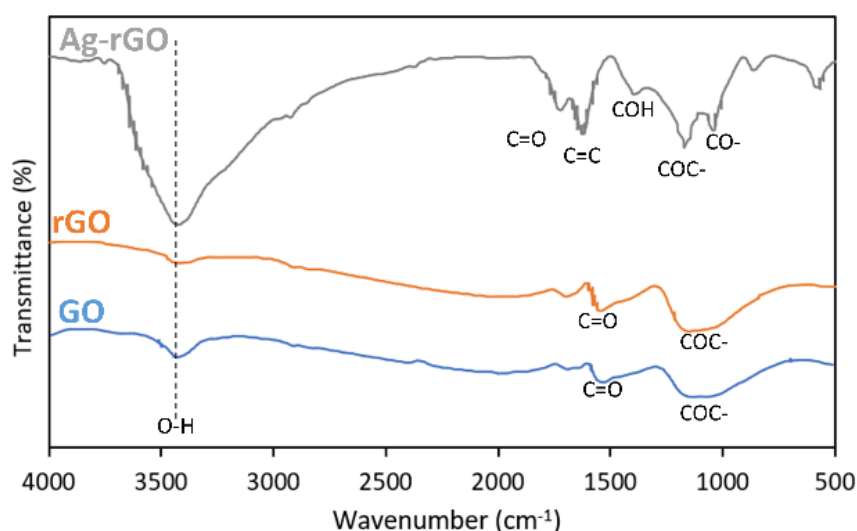


Figure 2. The IR spectra of Ag-rGO, rGO, and GO.

In this material, there exist two main bands around 1300 cm^{-1} called the D band and around 1585 cm^{-1} called the G band (Figure 3). The ratio of intensities of the two bands (ID/IG) represents the number of defects or disorderliness in the graphitic layers. The ID/IG of pristine rGO was found to be 0.99, whereas for the composite, which is synthesized by the addition of silver nanoparticles and termed as Ag-rGO composite, it increases to 1.28, which denotes the creation of a lot of defects in the material, and it indicates the creation of a number of dislocations in this material because of silver nanostructures present in the new composite.

In simple terms, the addition of silver nanoparticles modifies the graphene sheets and causes structural damage. These defects could increase the effective surface area, thereby promoting the adsorption of molecules/ions. Defects help slow the release of ions (Ag^+) that cause damage to the cell walls and cell membranes of bacteria and fungi. Moreover, graphene can be used as a supporting substrate for silver nanoparticles. Graphene plays a role in stabilizing silver nanoparticles and hindering their agglomeration, resulting in greater stability of the Ag-rGO nanocomposite and extended activity (Chen *et al.* 2019).

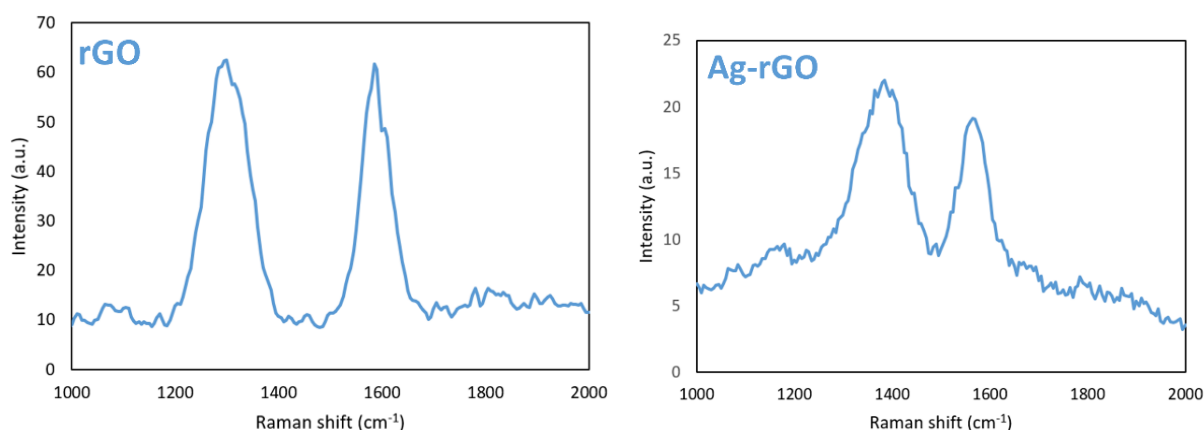


Figure 3. The Raman spectra of Ag-rGO and rGO.

SEM images showed that GO had a wrinkled sheet-like structure. After AgNP reduction and incorporation, a rougher rGO surface with uniformly dispersed spherical AgNPs was observed (Figure 4). EDS mapping showed relatively uniform distribution of C, O, and Ag. However,

SEM images showed some areas of high AgNP density. This aggregation affected particle size measurements (Figure 4). EDS analysis revealed 20.22% Ag (indicating AgNP attachment to rGO), 57.21% C, and 42.79% O, consistent with FTIR and XRD results.

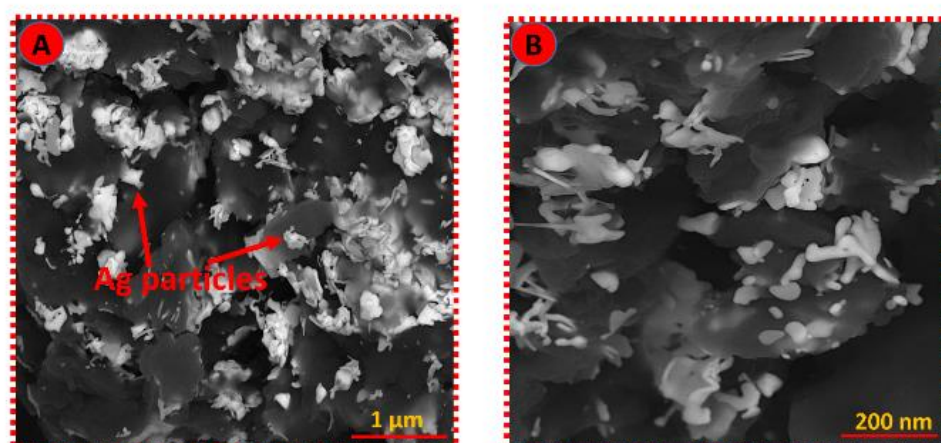


Figure 4. The SEM images of Ag-rGO.

The chemical structure analysis of the surface of the Ag-rGO has been conducted by the X-ray photoelectron spectroscopy (XPS) as presented in Figure 5. High-resolution X-ray photo-emission spectra were taken to observe the major elements (C1s, Ag3d, O1s) that have been present at the surface of Ag-rGO (Fig. 4). High resolution spectrum for carbon (C) consist three peaks located at binding energies of 283 eV, 284 eV, 285 eV with respect to their corresponding C-C, C-O and C=O bonds. Similarly, for oxygen (O1s), there were two

clearly visible peaks that appeared in binding energies of 533 eV and 537 eV, which were assigned to O-H and C=O functional groups. This provided evidence of rGO in the composites. Finally, for the metal part (Ag), two clearly resolved peaks of Ag3d at 365 eV and 373 eV (Ag3d5/2 and Ag3d3/2) confirmed the formation of silver nanoparticles on the rGO substrate. Thus, combining the two studies, we are now sure that the target composite has been fabricated.

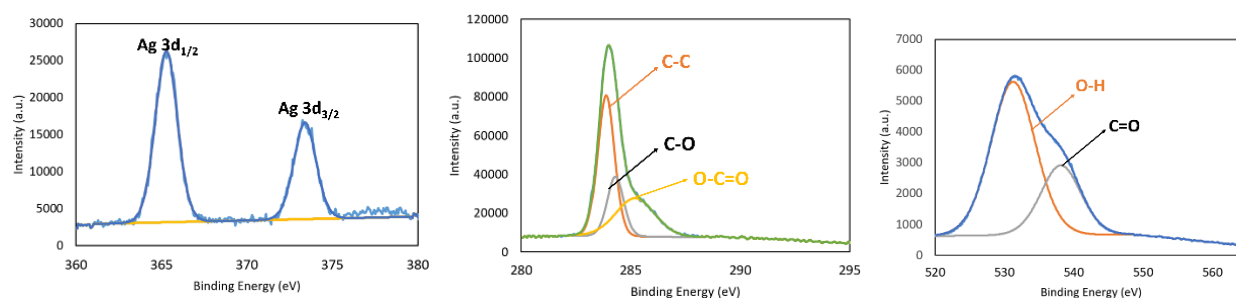


Figure 5. The XPS analysis of Ag-rGO.

Antifungal activity of Ag-rGO against *P. aphanidermatum*

Laboratory conditions: This study also demonstrated that Ag-rGO nanoparticles significantly inhibited the growth of *P. aphanidermatum*. The fungal inhibition rate showed a direct relationship with increasing Ag-rGO concentration - higher concentration led to greater

inhibition (Table 2, Figure 6). Analysis of the results revealed that even at low concentrations, Ag-rGO significantly inhibited *P. aphanidermatum* growth. At 25, 50, and 100 mg/L, the inhibitory effect of Ag-rGO was compared with a recommended fungicide at 400 mg/mL, indicating that the Ag-rGO compound showed somewhat better inhibition than the fungicide even at lower concentrations.



Figure 6. Effect of Ag-rGO and mefenoxam on *Pythium aphanidermatum* under laboratory conditions after 7 days; (a) control, (b) 25 mg L⁻¹, (c) 50 mg L⁻¹, (d) 100 mg L⁻¹ of Ag-rGO, and (e) 400 mg L⁻¹ of mefenoxam.

Table 2. Growth inhibition percentages of *P. aphanidermatum* after treatments by Ag-rGO and mefenoxam.

Treatments	Concentration (mg/L)	Growth inhibition Percentage
Control	-	0.00
	25	21.9 ± 1.13 ^c
Ag-rGO	50	49.8 ± 1.81 ^b
	100	88.1 ± 1.14 ^a
mefenoxam	400	89.8 ± 1.36 ^a

Different letters show significant differences using the Fisher's LSD test at 0.05 ≤ P. Each mean value was obtained from three replications.

Greenhouse conditions: The results presented in Table 3 and Figure 7, demonstrate that both Ag-rGO nanoparticles and a conventional fungicide (mefenoxam) significantly reduce seedling mortality and disease incidence in cucumber plants compared to the control group. Furthermore, there is no statistically significant difference between Ag-

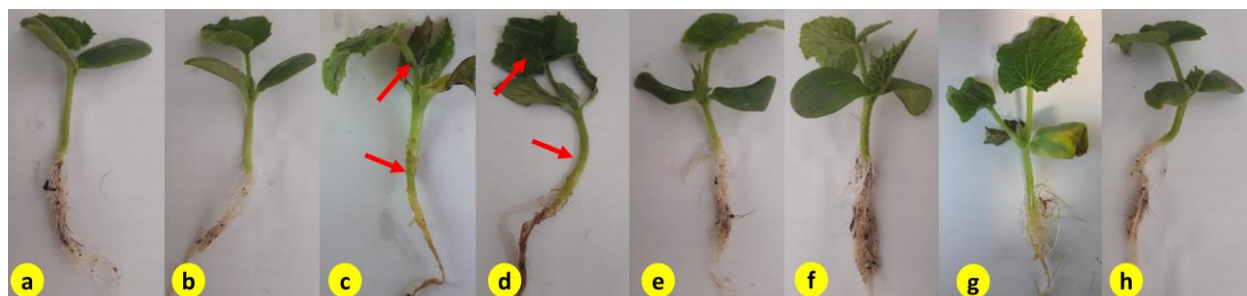
rGO and mefenoxam in terms of reducing seedling mortality. However, it is important to note that the Ag-rGO concentration was lower than that of mefenoxam. Additionally, based on the results of controlling damping off disease incidence, soil enrichment treatments were more effective than soil spray treatments.

Table 3. Effect of Ag-rGO and mefenoxam against damping off disease in cucumber under greenhouse conditions.

Treatments	Foliar spray		Soil Drench	
	Post-emergence damping off	Disease incidence	Post-emergence damping off	Disease incidence
Control	47.37 ± 0.86 ^a	8.69 ± 0.73 ^a	47.37 ± 0.86 ^a	8.69 ± 0.73 ^a
Ag-rGO (100 µg/L)	14.11 ± 0.36 ^b	3.24 ± 0.21 ^b	10.18 ± 0.21 ^b	2.48 ± 0.27 ^b
Mefenoxam (300 mg/L)	13.98 ± 0.22 ^b	3.11 ± 0.27 ^b	10.01 ± 0.19 ^b	2.41 ± 0.18 ^b

Different letters show significant differences using the Fisher's LSD test at 0.05 ≤ P.

Each mean value was obtained from three replications.

**Figure 7.** Morphological appearance and observed symptoms in cucumber plants: (a,b) control, (c,d) fungus-infected, (e,f) fungus-infected including 100 mg/L of Ag-rGO, and (g,h) fungus-infected including 400 mg/L of mefenoxam.

Field conditions: In addition, the field study (Table 4) demonstrated that both Ag-rGO nanoparticles and mefenoxam fungicide significantly reduced disease incidence and post-emergence mortality in cucumber plants during the

growing season compared to the control group. The fungicide was more effective at controlling cucumber damping-off during the growing season than Ag-rGO.

Table 4. Effect of Ag-rGO against damping off disease in cucumber under field conditions.

Treatments	Post-emergence damping off	Disease incidence
Control	48.63 ± 1.24 ^b	10.24 ± 1.11 ^b
Ag-rGO (100 µg/L)	10.34 ± 0.86 ^c	5.24 ± 0.58 ^c
Mefenoxam (300 mg/L)	8.86 ± 0.79 ^b	4.13 ± 0.51 ^a

Different letters show significant differences using the Fisher's LSD test at $p \leq 0.05$.

Each mean value was obtained from three replications

Effect of Ag-rGO application on cucumber growth characters

Other physiological and productive parameters. To continue evaluating the influence of Ag-rGO nanoparticles on cucumber plants, all growth parameters were assessed for plants and fruits (Table 5). Such parameters were plant height, total chlorophyll content, fresh and dry weights, the average number of fruits per plant⁻¹, the average fruit weight (g), and the yield per plant⁻¹. In

practical cases, this study demonstrated a similar effect and a positive correlation across all parameters considered for Ag-rGO nanoparticles and the mefenoxam fungicide compared with the control group. The cucumbers treated with Ag-rGO and mefenoxam show taller plants and increased yield, including the number of fruits, average fruit weight, and yield. This effect on growth was almost as strong as that of mefenoxam, despite using only a low dose.

Table 5. Effect of Ag-rGO and mefenoxam on growth characters of cucumber under field conditions.

Treatments	Plant height (cm)	Total chlorophyll (SPAD)	Leaves number/plant	Fresh weight/plant (g)	Dry weight/plant (g)	Fruit number/plant	Average weight/fruit (g)	Yield/plant (kg)
Control	321.8 ± 6.21 ^b	26.2 ± 0.98 ^b	21.1 ± 1.13 ^b	19.9 ± 0.23 ^b	2.6 ± 0.24 ^b	11.6 ± 1.16 ^b	68.3 ± 1.85 ^c	1.9 ± 0.61 ^b
Ag-rGO (100 µg/L)	358.1 ± 5.68 ^a	38.4 ± 0.95 ^a	37.9 ± 0.25 ^a	36.4 ± 0.27 ^a	4.3 ± 0.24 ^a	36.1 ± 0.93 ^a	89.6 ± 1.12 ^b	4.1 ± 0.24 ^a
Mefenoxam (300 mg/L)	358.8 ± 5.71 ^a	40.1 ± 0.96 ^a	37.6 ± 0.28 ^a	41.1 ± 0.17 ^a	4.9 ± 0.26 ^a	41.2 ± 0.95 ^a	94.1 ± 1.98 ^a	4.2 ± 0.46 ^a

Different letters show significant differences using the Fisher's LSD test at 0.05 ≤ P.

Each mean value was obtained from three replications.

Effect of Ag-rGO on the expression of defense-related genes

Pathogenesis-related genes (PRGs, including LOX1, PAL1, Actin, β -1,3-glucanase, Catalase, and PR1) in the cucumber treated with Ag-rGO showed considerably higher levels one day after inoculation of the damping-off pathogen than those of controls

(Figure 8). These results display that all PRGs in the Ag-rGO treatment group were significantly up-expressed, among which β -1,3-glucanase had the highest expression level. These increased expression levels indicate that Ag-rGO has the potential to regulate cucumber plant defense against pathogens.

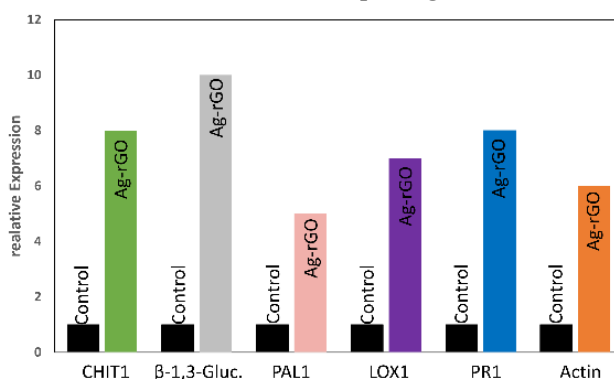


Figure 8. Relative expression of LOX1, PAL1, Actin, β -1,3-glucanase, Catalase, and PR1 genes in leaves of cucumber plants treated with Ag-rGO.

Discussion

This study gives clear evidence: silver-reduced graphene oxide nanoparticles fight damping off disease caused by *P. aphanidermatum*. Lab experiments show how these tiny particles stop the fungus from growing - an effect noticed before with related compounds. Past reports, including those on yeasts and skin-related fungi, mention the same slowdown in fungal activity when exposed to Ag-rGO. Unlike bulky clusters in usual options such as mefenoxam, these newly formed particles are far smaller, which may help them move more freely into problem areas. Tiny shapes possibly slide into fungal cell walls, maybe making it easier to block growth. Because they are small, Ag-rGO bits hit microbes harder, especially when more surface shows up. Silver spreads wide and even on each bit - this layout may be key to stopping germs fast. Clear patterns appear under tests using water-thinned fungus parts, done by adjusting standard lab steps. Methods came from updated clinical protocols seen in newer studies. Papers by Chen, Gu, and others back these clues (Gu *et al.* 2016; Chen *et al.* 2019). Before, scientists tested materials with silver using regular techniques. These showed fungus growth and spore start times got longer when treated (Gottardo *et al.* 2013). Small bits do more

inside organisms, thanks to how tiny they are and unique features unlike bulk stuff. Yet, the way Ag-rGO blocks *P. aphanidermatum* remains partly unknown. Past studies still confirm silver particles beat back various fungi - Kumar, Gu, and Hsueh found this repeatedly (Gu *et al.* 2016; Hsueh *et al.* 2019; Kumar *et al.* 2019). One thought jumps out from Gottardo's results: the particles could interfere with cell functions, warping thin fungal threads in the process. Here's how it likely goes, matching pretty much what Gottardo talked about earlier (Gottardo *et al.* 2013). Chen and colleagues (2019) went another way entirely - crafting Ag-rGO with a distinct process while probing its effect on microorganisms, backing their angle with fresh data. What stood out in their work? The silver-infused rGO hits bacteria hard. The punch comes mostly from tiny silver bits hidden throughout the structure, turning it into an effective germ fighter. They did not jump ahead; instead, trials unfolded using *E. coli*, one typical gram-negative strain. They did not stop there. There was another test with simply plain reduced graphene oxide alone to ensure it functioned properly on its own, and this proved one thing clearly. The silver mixture went through the cells' outer layers much better than plain rGO alone. It turns out that the silver may

facilitate the graphene material in somewhat spreading those cell membranes apart, and that opening them up makes them leaky. Inside chaos begins as normal processes go off track. Over time, especially with ongoing contact, bacterial life shuts down completely. Still, nobody fully understands every stage yet. To move forward carefully, researchers want clearer views of how particles and frameworks interact within nanocomposites. Clearer data on safety matters a lot, particularly under farm conditions. Where broad deployment is concerned, performance tests then quickly become quite necessary. Ecosystem impacts need thorough examination before expanding beyond current limits. Doing much better research into what the day-to-day impacts are may lead to more thought put into decisions for the next big jump.

The present study investigates the efficacy of Ag-rGO nanoparticles in inducing systemic resistance in cucumber plants against the pathogen *P. aphanidermatum*, one of the primary causes of damping-off in cucumber plants. This approach can be considered a novel strategy for plant protection, particularly for crops, as it activates the plant's natural defense system to prevent diseases. To evaluate the effect of Ag-rGO on controlling damping off, seedling mortality after germination and disease incidence were measured in treated and untreated plants. The results demonstrate the remarkable ability of Ag-rGO to suppress damping off in cucumber plants. This desirable efficacy can be attributed to the nanoscale size of Ag-rGO, which facilitates penetration into plant tissues or even fungal cells (Chen *et al.* 2019). Moreover, a comparison between soil drench and foliar spray methods revealed that soil drench with Ag-rGO was more effective in controlling damping off. This is likely due to the direct and faster contact of Ag-rGO with soil-borne pathogens in the soil drench method compared to the foliar spray method, where contact with the pathogen is more limited and indirect. Greenhouse studies conducted in this research indicate that Ag-rGO induces systemic resistance in cucumber plants. This induced resistance likely occurs through the activation or stimulation of plant defense-related genes. Other studies have revealed

that many genes expressed in plant defense responses have become more susceptible to plant signals like Ethylene (ET), Jasmonic acid (JA), and salicylic acid (SA). In an effort to better understand how Ag-rGO may aid in plant defense, the impact on the expression of cucumber plant defense related genes was also evaluated. When Ag-rGO nanoparticles were supplied to the soil, the expression of defense related plant genes increased in a way that boosted plant ability to resist *Pythium aphanidermatum*.

Another important aspect that comes up in their study is the reduction in antifungal properties of Ag-rGO from in vitro environment (which leads to almost 90% inhibition) to the greenhouse and field (around 75-80% inhibition). This difference is largely due to the environmental variables. In the lab, all environmental factors - temperature, humidity, illumination, etc - are kept in range for optimum conditions, which in turn allows for good interaction between nano- and pathogen elements. But compared to those in the laboratory, environmental factors such as temperature, humidity, light intensity, wind, and other microorganisms in greenhouses and fields significantly affected the performance of nanoparticles (Kah & Hofmann 2014; Ma *et al.* 2010). In contrast, compared with highly controlled laboratory conditions, greenhouse environments, and especially open-field conditions, environments are subject to greater environmental variability, including fluctuations in temperature, humidity, light intensity, wind, and interactions with other microorganisms, which can influence nanoparticle performance (Kah & Hofmann 2014). Additionally, soil and growth media may adsorb or aggregate the nanoparticles, reducing their accessibility to the pathogen. The accessibility of nanoparticles to the pathogen also plays a critical role in the efficacy of Ag-rGO. In vitro, nanoparticles are uniformly dispersed in the medium, allowing direct and consistent interaction with the pathogen (Dimkpa *et al.* 2013). But in greenhouse and field conditions, Ag-rGO can bind to soil particles or organic materials, resulting in its accumulation within the soil or on leaf surfaces (Tourinho *et al.* 2012), thereby restricting its exposure to pathogens. The

nanoparticles can be washed away by irrigation water, leached deep into the soil, reducing their availability and efficacy. In vitro, it's exposed to fewer interacting environmental factors than in greenhouse or field conditions (Lowry *et al.* 2012), and its greater stability may lead to better efficacy in vitro, whereas temperature changes, humidity, and microbial activity can affect its stability in greenhouse or field conditions and its antifungal efficacy.

Expression of LOX1, PAL1, Actin, -1,3-glucanase, Catalase and PR1 genes in the leaves of cucumber plants treated with and without Ag-rGO. Cucumber plants with and without treatment Ag-rGO, have their expression profile in genes as follows. Comparison with untreated plants. Gene expression level in the plants treated by Ag-rGO resulted in higher expression in each gene. At minimum, the level of gene expression was at least 5 times higher than untreated plants. Higher level of gene expression: -1,3-glucanase gene which encodes enzymes able to digest glucans present in fungal cell walls. The increased expression, similarly to chitinase gene, contributes to increase cucumber's ability to defense towards fungi. Lower level of gene expression: PAL1 gene encodes a polyphenol oxidase which is also responsible for synthesis of plant's phenolic compound that is used for plant's defence against environment and pathogenesis attack. Conclusion The result study in question suggest Ag-rGO as a potential agent to prevent cucumber damping-off without harmful effects for environment like many chemical fungicides used Nowadays, safe, and cost-effective.

The current research suggests that the combined use of a two-stage treatment of silver-reduced graphene oxide (Ag-rGO) can successfully manage damping-off disease in cucumber. It includes a preliminary treatment with Ag-rGO prior to onset of disease in the first step, for elevating plant

resistance with systemic resistance induction and increasing expression of defense genes, and an intervene treatment with Ag-rGO after onset of disease in the second step, aiming to control disease spreading and decrease symptom severity. Therefore, these two preventive and intervening steps are potentially used for a better control of damping-off diseases induced by *Pythium aphanidermatum*.

Physiologically, appropriate suppression of damping-off caused by Ag-rGO could lead to some changes to the growth properties of cucumber plants. Two underlying reasons could justify such phenomena: (1) activation of plant defense systems that provide the plant systemic resistance to the pathogenic microorganisms, and (2) directly mitigating the disease symptom development. Ag-rGO nanocomposite may suppress the growth of soil-borne pathogens such as *Pythium* species and induce the expression of defense-related proteins and the production of metabolic products to reinforce plant defenses. With lower disease incidence, pressure plants have more metabolic energy for development and may result in some positive alterations on growth properties like the longest root length, and fresh and dry biomass.

The results of this study further showed that the amount of Ag-rGO required for effective damping-off control (100 μ L of the treatment solution) was lower than the application rate of the conventional chemical fungicide mefenoxam (400 mg/L). Therefore, despite the relatively high economic value of silver, the use of very small amounts of Ag-rGO may reduce concerns related to cost, toxicity, and environmental impact. In addition, the wet chemical synthesis method employed in this study provides a simple, low-cost, and high-yield approach for Ag-rGO production at low temperatures, which supports its potential for practical use.

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اثرات ضدقارچی و القاکننده مقاومت نانوذرات گرافن اکسید احیاشده با نقره علیه *Pythium aphanidermatum* در خیار

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چکیده

در این مطالعه سنتز نانوذرات اکسید گرافن احیا شده با نقره (Ag-rGO)، فعالیت ضد قارچی مستقیم Ag-rGO علیه *Pythium aphanidermatum* و اثربخشی آنها در القای مقاومت در برابر مرگ گیاهچه در خیار ناشی از *P. aphanidermatum* بررسی شد. فعالیت ضد قارچی با استفاده از غلظت ۱۰۰ میلی‌گرم در لیتر Ag-rGO ارزیابی شد که مهار قابل توجهی در شرایط آزمایشگاهی در رشد *P. aphanidermatum* (حدود ۹۰٪ کاهش در مقایسه با شاهد تیمار نشده) نشان داد. آزمایش‌های گلخانه‌ای و مزرعه‌ای کاهش بروز مرگ گیاهچه در گیاهان خیار تیمار شده با Ag-rGO (حدود ۷۵-۸۰٪ کاهش، در مقایسه با شاهد تیمار نشده) را نشان داد. علاوه بر این، تجزیه و تحلیل واکنش زنجیره‌ای پلی‌مراز در زمان واقعی (qRT-PCR) نشان داد که تیمار Ag-rGO بیان ژن‌های مرتبط با دفاع درگیر در مسیرهای سیگنالینگ اسید سالیسیلیک (SA) و اسید جاسمونیک/اتیلن (JA/ET) را تا ۱۰ برابر (در مقایسه با شاهد تیمار نشده) افزایش می‌دهد. در نهایت، این مطالعه اولین گزارشی است که پتانسیل Ag-rGO را به عنوان یک استراتژی جدید برای کنترل *P. aphanidermatum* در خیار نشان می‌دهد.

کلمات کلیدی: ژن‌های مرتبط با دفاع، مقاومت اکتسابی سیستمیک، *Pythium aphanidermatum*، qRT-PCR

