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Comet Assay Technique Value as a Response on Zinc Oxide Nanoparticales in *Trigonella foenum-graecum* Plant

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Abstract
The research was chosen to evaluate the genotoxicity effects of using zinc oxide nanoparticles (ZnO NPs) on plants, due to their increased use in the environment without knowing the upper and lower limits of use. Therefore, the fenugreek plant (*Trigonella foenum-graecum*), known to be widespread in the Iraqi environment, was chosen. ZnO NPs were added at different concentrations (0, 300, 200, 100) mg/L, and plant seeds were grown in nutrient solution for six months. Genotoxicity was evaluated by comet assay analysis to determine DNA damage as well as total protein, and Malondialdehyde (MDA) were investigated to ensure the integrity of the membranes. The laboratories of the Ecology Department at the College of Science, University of Kufa for the year 2022-2023 were used to conduct experiments. DNA damage according to the values of some indicators related to the Comet Assay technique showed a significant increase ($P \leq 0.05$) in the tail length parameter in *T. foenum-graecum* plants when exposed to a concentration of 300 mg/L of ZnO NPs. In *T. foenum-graecum* plants, the highest percentage of DNA damage was found at a concentration of 100 mg/L. Also, the highest percentage of damage in Head Diameter and Tail Area parameters at a concentration of 300 mg/L with significant value at $P \leq 0.05$.

Total protein showed the highest value at a concentration of 200 mg/L ZnO NPs. The treatment of 300 mg/L caused a negative effect led to an increase in content of MDA. Therefore, it is recommended to use a concentration of 200 mg / L as a fertilizer. This indicates the variability in the response of the studied plant to the doses of ZnO NPs according to different plant species.

Keywords: *Trigonella foenum-graecum*, Comet Assay, Malondialdehyde (MDA), Total Protein, ZnO NPs

Introduction

Synthetic nanomaterials (often deadly) substances were used more in agriculture, food, and pharmaceuticals in the 20th century. "Green" therapies are essential due to environmental degradation and associated health impacts (Owied *et al*., 2023) [15]. Instead of increasing agricultural output through the widespread use of synthetic pesticides and fertilizers, now have a return to more traditional farming methods (Adrees *et al*., 2020) [1]. Review and prospective analysis of the effects of ZnO nanoparticles on plants is very important because Zinc (Zn) is a mineral that is necessary for all living things, including people and plants, influences plant metabolism and expansion (Ahmed *et al*., 2021) [2]. More Than its role in a wide range of enzymatic events, metabolic processes, and redox reactions, it is crucial for iso-transphosphorylases, RNA polymerases, and DNA polymerases (Guda *et al*., 2016) [9]. Zinc is required for the production of tryptophan, cell division, preservation of membrane structure, and photosynthesis and a crucial factor in protein synthesis (Elena *et al*., 2019) [6]. As a result of their accumulation in plant tissues, NPsZnO can have a negative effect on crop development and production (Rahi *et al*., 2023) [16]. Lower concentrations of NPs ZnO enhanced wheat seed germination (Guda and Semysim, 2022) [7]. Onions' root growth and dry weight increased significantly after NP administration (Guda *et al*., 2020). ZnO nanoparticles, on the other hand, perform critical functions in plants in reducing the damaging effects of ROS on cell organelles (Kralova *et al*., 2021) [12]. In addition to their well-known negative effects, ROS species are also known to activate several defensive mechanisms by activating the cell signaling cascade and influencing the expression of many genes (Muthik *et al*., 2019). Plants, on the other hand, have enzymatic and non-enzymatic antioxidant mechanisms that are continually on the hunt for damaging reactive oxygen species. By increasing antioxidant enzymes and osmole, amino acid, and free nutrient accumulation (Duea and Guda, 2023). This study of genotoxicity in *T. foenum-graecum* is considered as an evaluation of plants in tolerance of different concentrations of zinc oxide nanoparticles and knowledge of the upper limits that can be tolerated by plant cells without damage to DNA, as well as to choose the best concentration in the manufacture of fertilizers to reduce the accumulation of nanomaterials in the

environment.

Materials and Methods

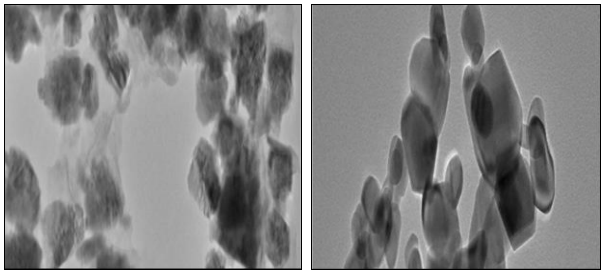
Seed Cultivated

The seeds of plants (T. foenum-graecum) were sterilized in 10% (v/v) sodium hypochlorite solution for 10 min and then washed with sterile deionized water, then soaked in a suspension of ZnO (NPs) at different concentrations (0, 100, 200 and 300 mg/L). Then put in glass jars, with filter papers on the bottom (ten seeds per dish) (Guda *et al*., 2020); Then, ten mL of deionized water as a control treatment or different concentrations of NPs suspensions were added to the Hoagland's solution (Image 1). They were kept, after being closed with adhesive tape, in an incubator at 25 °C. After 3 days, 10 ml of ZnO NPs suspension was added. After three weeks of growth, plants harvested and the ZnO NPs phytotoxicity study. A container were arranged in a completely random design in four replicates for each treatment. Plants were grown in a supplemental illumination in addition to natural daylight provided by sodium-vapour lamps. Temperature control maintained a night temperature of 15 °C and a daytime temperature of 25 °C at minimum, The pH measurement is also maintained within the range (6.5-7.5) for the nutritional solution (Guda *et al*., 2020).

Image 1: The studied plants treated with different concentrations of ZnO NPs suspension

Nanoparticles Characterization

TEM observations showed that it concentrationisted of 50-150 nm particles in a hexagonal lamellar main axis and 20-70 nm in a secondary axis (Image 2 A, B). A significant portion of the particles stuck together in loose agglomerations of 1000–2000 nm size (Image 2, B). In the ZnO NP colloidal solution, the particles were scattered more. The particle size range was from 10 to 150 nm. The highest frequency of particle sizes examined ranged from 15 to 40 nm. The globular shaped particles predominantly, with a size of between 15 and 40 nm, primary individual (A) and aggregate (B) (Hassan, 2022) [11].



(A) (B)

Image 2: Transmission electron microscope image of ZnO NP at magnification of (A) = 3 *105 X (aggregate) and (B) = 10* 104 (Individual primary)

1. Comet Assay Studies

Evaluation of genotoxicity and measurement of DNA damage caused by ZnO NPs. The method of Singh *et al.* (2023) [17], Four weeks after the plant was exposed to ZnO NPs. Plants of each container were collected at different concentrations to determine the dry fraction. The dried plants were ground into a homogeneous powder. The comet research was performed on plant samples collected from the leaves and dry parts of the plant. 15 µl of cell suspension (~15,000 cells) was mixed with 85 µl of 0.5% low collapsed point agarose (LMPA) and mixed. On a cover, a layer of 200 µl normal and 100 µl LMPA, respectively, was covered. After gel solidification, the slides were immersed in lysing solution for 60 min at 4 °C containing 2.5 M NaCl, 100 mM Na2-EDTA, 10 mM MgCl pH 10% DMSO and 1% Triton X 100. For acid deconvolution Nuclear loop 35. C for 25 minutes to decode DNA. Next, the electrocautery was connected to a power source and set for 60 minutes. 70 volts. To read excess alkali, slides were gently washed for 10 min at 4 °C with neutral solution (0.4 M Tris, pH 7.4) and stained with 75 µl ethidium bromide (10–20 µg/mL). Tested using ZEISS technology.

2. Measurement of Total Protein

Total proteins were extracted and estimated according to method of (Bishop *et al*., 1985) [3]. Protein content estimation by weight 0.5 g of fresh plant leaves were crushed with 6 ml of phosphate buffer solution (pH = 5.6) in a cold mortar with a pestle, then conducted by the centrifuge process strongly 15,000 r/min for 15 min, (2 ml) of the protein extract were added to 8 ml of the Bayourat reagents. Then leave for half an hour. The absorbance of protein samples was measured at 555 nm by using a spectrophotometer. Using bovine albumin as the standard curve. A content of total protein was calculated using standard curve.

3. Malondialdehyde (MDA) Content Determination in Plants

It was estimated according to (Kramer *et al*., 1996) [13]. Reaction mixture was consisting of 1 ml of crude extract with 2 ml of 0.6%. Thiobarbituric acid (TBA), while supernatant was taken to the spectrophotometer to read at (450, 532, 600) nm. MDA content was calculated by the following equation:

$$MDA\mu mol \bullet g-1=[6.45\times(A532-A600)-0.56\times A450]\times R/W$$

Where:

- A532: Absorbance at wavelength 532 nm,
- A600: Absorbance at wavelength 600 nm,

- A450: Absorbance at wavelength 450 nm,
- R: Total reaction volume (3 ml),
- W: crude extract of sample.

Statistical Analysis

The results were analyzed statistically based on the complete randomized design and the Least Significant Difference (LSD) test and the Duncun test to replicate between means and at a probability level ($p \leq 0.05$). DNA damage (comet assay) and the means of all data were separated by the difference in the length of the comet resulting from migration.

Result

Genotoxicity of ZnO (NPs) in plant

Comet assay test

The explanation of double- and single-strand breaks, alkaline-dismutable sites, incomplete repair sites, cross-links, and repair in single cells is of great relevance for the DNA damage detection using single-cell gels. A flexible and accurate approach for determining DNA damage and DNA repair capability at the single-cell level is the comet test. Plants exposed to various concentration of ZnO NPs used as a plant fertilizer may experience DNA damage and genotoxicity. The damage in the DNA was classified into four categories, according to the degree of the damage is as shown in the (Image 3).

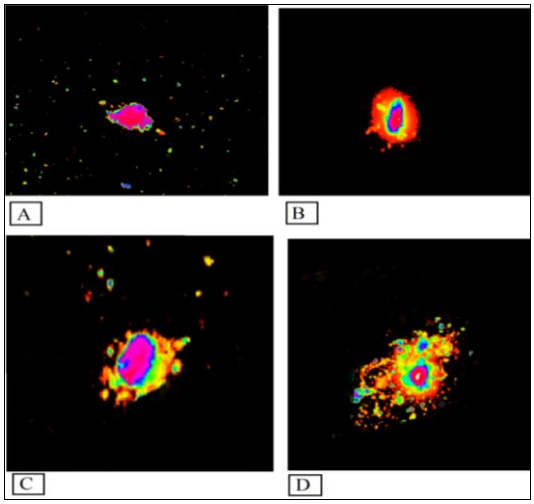


Image 3: Comet image analysis by comet score software, scoring categories for comet assay (a and b: normal to low DNA damage, c: medium DNA damage, d: high DNA damage

Estimated DNA Damage in Studied Plants

The extent of DNA damage in the studied plant was estimated through some damage indices related to the comet assay which include (tail length µm), (comet length µm), (head diameter µm) and (tail area µm) shown in Table 1 and Fig 1.

A. DNA damage in T. *foenum-graecum*

The results indicate that there is clear DNA damage in the T. foenum-graecum plant when the plant was treated with zinc nanoparticles. The statistical results indicated that there was a clear significant change (LSD; $P \leq 0.05$), between different concentration of ZnO NPs. The Low comet tail highest values were recorded in 100 mg/L, reaching (56.52 µm) and lower values in both 200 mg/L and 300 mg/L, reaching 28.13 µm in 200 mg/L and 26.7 µm in 300 mg/L treatments. also found that the medium comet tail, highest values in the control, reaching (42.9 µm) > in 100 mg/L (39.13 µm) > in 200 mg/L (31.25 µm) > in 300 mg/L (23.3 µm), while High comet damage highest values were recorded in 300 mg/L, where it reached (50 µm), and lower values in both control reached (4.67 µm) and other 100 mg/L, reached (40.63 µm) Fig 1.

T. *foenum-graecum* plants showed the highest DNA damage according to the values of some indicators related to the comet assay technique at a concentration of 300 mg/L, the comet length at a concentration of 300 mg/L showed the highest value among the treatments with a value of 56.2 µm and in Head Diameter its value was 8.19 µm and in the parameter Tail Area µm it was 0.47 µm at the same concentration with a significant value at $P \leq 0.05$ table (1).

The lowest values for Comet length, Head Diameter, and Tail Area parameters were 26.5µm, 2.04µm, and 0.06µm, respectively, in the 200 mg/L treatment with a significant value at $P \leq 0.05$ Table (1).

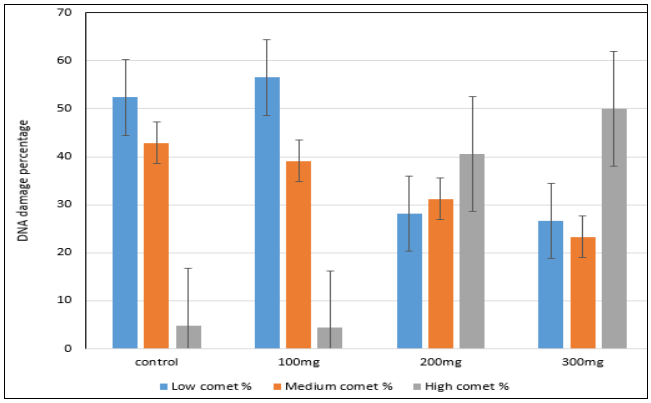


Fig 1: DNA damage values of comet tail indicators related to the Comet Assay technique in *T. foenum* plants

Table 1: Values of some indicators related to the Comet Assay technique in T. *foenum* plants

Comet assay markers	Control	100 mg/L of ZnO NPs	200 mg/L of ZnO NPs	300 mg/L of ZnO NPs
Comet length µm	44± 0.4 a	47 ± 8 b	26.5 ± 3 a	56.2 ± 5.7 c
Head Diameter µm	3.4 ± 0.1 a	7.05 ± 1 b	2.04 ± 0. 1 a	8.19 ± 2 C
Tail Area µm	0.11 ±0.0 a	0.46 ± 0.02 b	0.06 ± 0.01 a	0.47 ± 0.08 b

Different letters mean a significant difference

Table 2: ANOVA values of some indicators related to the Comet Assay technique in T. foenum plants

		Sum of Squares	df	Mean Square	F	Sig.
Low4	Between Groups	2232.839	3	744.280	258.960	.000
	Within Groups	22.993	8	2.874		
	Total	2255.832	11			
Medium4	Between Groups	729.942	3	243.314	496.462	.000
	Within Groups	3.921	8	.490		
	Total	733.862	11			
High4	Between Groups	5362.776	3	1787.592	838.945	.000
	Within Groups	17.046	8	2.131		
	Total	5379.823	11			

Table 3: Multiple Comparisons values of some indicators related to the Comet Assay technique in T. foenum plants

Multiple Comparisons

LSD

Dependent Variable	(I) ID	(J) ID	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Low4	Control	T1	1.23532	1.38422	.398	-1.9567-	4.4273
		T2	27.13649*	1.38422	.000	23.9445	30.3285
		T3	28.59482*	1.38422	.000	25.4028	31.7868
	T1	Control	-1.23532-	1.38422	.398	-4.4273-	1.9567
		T2	25.90117*	1.38422	.000	22.7091	29.0932
		T3	27.35950*	1.38422	.000	24.1675	30.5515
	T2	Control	-27.13649-*	1.38422	.000	-30.3285-	-23.9445-
		T1	-25.90117-*	1.38422	.000	-29.0932-	-22.7091-
		T3	1.45833	1.38422	.323	-1.7337-	4.6504
	T3	Control	-28.59482-*	1.38422	.000	-31.7868-	-25.4028-
		T1	-27.35950-*	1.38422	.000	-30.5515-	-24.1675-
		T2	-1.45833-	1.38422	.323	-4.6504-	1.7337
Medium4	Control	T1	4.27282*	.57160	.000	2.9547	5.5909
		T2	12.42057*	.57160	.000	11.1024	13.7387
		T3	20.33724*	.57160	.000	19.0191	21.6554
	T1	Control	-4.27282-*	.57160	.000	-5.5909-	-2.9547-
		T2	8.14775*	.57160	.000	6.8296	9.4659
		T3	16.06441*	.57160	.000	14.7463	17.3825
	T2	Control	-12.42057-*	.57160	.000	-13.7387-	-11.1024-
		T1	-8.14775-*	.57160	.000	-9.4659-	-6.8296-
		T3	7.91667*	.57160	.000	6.5985	9.2348
	T3	Control	-20.33724-*	.57160	.000	-21.6554-	-19.0191-
		T1	-16.06441-*	.57160	.000	-17.3825-	-14.7463-
		T2	-7.91667-*	.57160	.000	-9.2348-	-6.5985-
High4	Control	T1	-2.89713-*	1.19185	.041	-5.6455-	-.1487-
		T2	-38.46778-*	1.19185	.000	-41.2162-	-35.7194-
		T3	-47.84278-*	1.19185	.000	-50.5912-	-45.0944-
	T1	Control	2.89713*	1.19185	.041	.1487	5.6455
		T2	-35.57065-*	1.19185	.000	-38.3191-	-32.8222-
		T3	-44.94565-*	1.19185	.000	-47.6941-	-42.1972-
	T2	Control	38.46778*	1.19185	.000	35.7194	41.2162
		T1	35.57065*	1.19185	.000	32.8222	38.3191
		T3	-9.37500-*	1.19185	.000	-12.1234-	-6.6266-
	T3	Control	47.84278*	1.19185	.000	45.0944	50.5912
		T1	44.94565*	1.19185	.000	42.1972	47.6941
		T2	9.37500*	1.19185	.000	6.6266	12.1234

*. The mean difference is significant at the 0.05 level.

Fig 2 shows comparison between the percentages of indicators related to the Comet Assay technique in T. foenum-graecum plants. The Comet length parameters between the different concentrations, showed the control constitutes 25%, in 100 mg /L constitutes 27%, and 200 mg / L constitutes 15%, which is the lowest percentage and the highest percentage for comparison was in the concentration of 300 mg / L with a value of 32 %. As for the Head Diameter, the highest percentages are in 300 mg / L, equivalent to 40%, and the lowest percentages are in 200 mg / L, with a value of 10% of DNA. And Tail Area, the highest percentages were in 300 mg / L, equivalent to 43%, and the lowest percentages were in 200 mg / L, with a value of 5% of DNA, as in Fig 2.

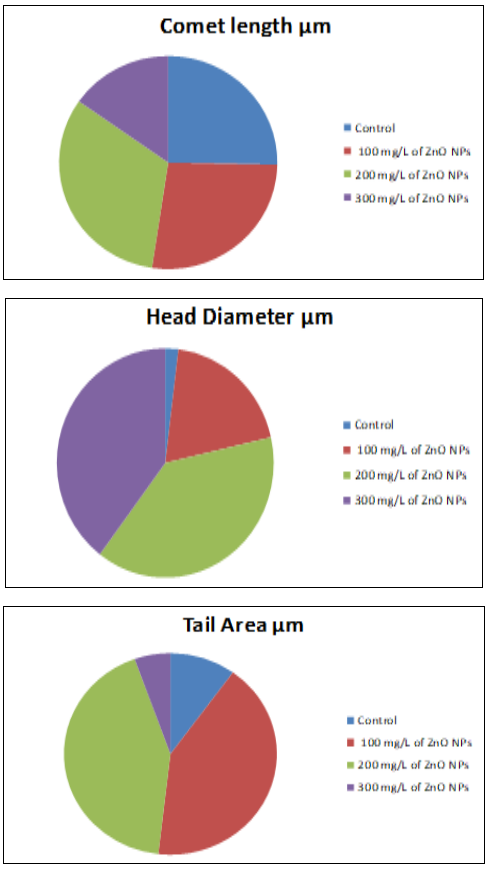


Fig 2: Comparison between some indicators related to the Comet Assay technique in T. *foenum-graecum* plants

Malondialdehyde (MDA)

Malondialdehyde in *Trigonella foenum*

The maximum MDA contents in T. foenum-graecum was 71.466 mg/L in the control group and the minimum was 18.963 mg/L in the 200 mg/L treated group Fig 3. The study showed a non-significant ($p < 0.05$) decrease in MDA activity in T. foenum-graecum.

The peroxidation of membrane lipids, of which MDA is an indication, is one of the most detrimental impacts of free radicals and their byproducts in plant cells. MDA is a sensitive diagnostic marker of oxidative stress in cells and the byproduct of lipid oxidation. One of the key pathways in oxidative stress cell damage is lipid peroxidase.

MDA is a significant unsaturated fatty acid oxidation byproduct. The increase in MDA content in T. foenum-graecum is an indication that the concentration is not the ideal and destroys the cell wall, as it was found that 200 mg/L is the ideal concentration Fig 3.

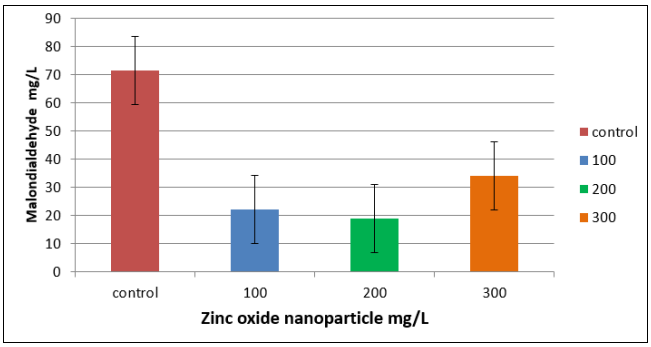


Fig 3: Comparison of Malondialdehyde contents in T. *foenum-graecum* plants

Conclusions

The genotoxic response of the studied plant to different concentrations of ZnO NPs differed as T. foenum-graecum plant showed the highest DNA damage according to the values of some indices related to the comet assay technique, comet length µm, at a concentration of 300 mg/L. However, in µm head diameter and µm tail area, the highest parameters were shown at a concentration of 300 mg/L with significant value at $P \leq 0.05$.

Total protein showed the highest value at a concentration of 200 mg/L ZnO NPs in T. foenum-graecum plant. This means stimulating the production of new proteins that may be cellular resistance or addition, and this indicates the reinforcing effect of these concentrations.

The strength of the antioxidant response indicates that a concentration of 200 mg/L stimulates vegetation and can be used in the manufacture of agricultural fertilizers and the production of phytodrugs. According to this study, this concentration in the environment is not harmful to the ecosystem.

Nanoparticles of zinc at a rate of 300 mg/L entered the structure of cell walls and damaged cell membranes. The 300 mg/L treatment produced a negative effect that increased the MDA content.

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