

Modulatory effect of zincoxide nanoparticles on gamma radiation-induced genotoxicity in *Vicia faba* (Fabeaceae)

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ABSTRACT. Gamma radiation is commonly used to disinfect agricultural products to increase shelf-life. However, this may exert adverse effects on plant growth, development, fertility, and crop production due to oxidative stress and cellular damage. Post irradiation protection using nanoparticles could reduce or reverse deleterious effects after exposure to ionizing radiation. We monitored the effect of zinc oxide nanoparticles (ZnO NPs at 500, 2000, 4000 mg/L) on *Vicia faba* grown from seeds treated with gamma rays (20, 50, 100 Gy). Phenotypic (seed germination, percentage of inhibition, seedling growth) and cytogenetic markers (chromosomal behavior in mitosis, meiosis and pollen grains) along with ultrastructural changes in the chloroplasts and nuclei (transmission electron microscopy) were assessed. At 20 Gy radiations, ZnO NPs had no effect on the final germination percent; however, at 100 Gy and post-treatment with 4000 mg/L of ZnO NPs, a substantial reduction occurred. While vegetative growth and fruit production increased with 500 and 2000 mg/L ZnO NPs, all three doses of gamma rays induced reduction. ZnO NPs provoked a significant increase in the mitotic index of root meristems compared with the control and gamma radiation. A radioprotective effect of ZnO NPs in the mitotic-meristematic root tips of *V. faba* was observed. The degree of mutagenic efficiency and pollen grain sterility was dose-dependent. Chloroplasts and nuclei treated

with higher concentrations of ZnO NPs (4000 mg/L) and the three doses of gamma rays showed adverse ultrastructural changes. An amelioration or modulation of these changes was observed post irradiation with 500 and 2000 mg/L ZnO NPs. ZnO NPs at 500 and 2000 mg/L concentrations had protective effects through the reduction of adverse effects of all doses of gamma rays at the phenotypic, cytogenetic, and cellular ultrastructure levels. Additional studies are warranted to explore ZnO NPs as potential nano-irradiation protective agents.

Key words: *Vicia faba*; Gamma rays; ZnO nanoparticles; Phenotypic effects; Cytogenetic effects mitotic index

INTRODUCTION

The application of ionizing radiation such as gamma irradiation has revolutionized the field of agricultural science by increasing seed conservation time, reducing pathogen propagation and increasing the shelf life of various agricultural products (Melki and Salami, 2008). However, ionizing radiation is a potent DNA-damaging agent that interacts with cellular DNA by producing free radicals that induce lesions in irradiated cells (Jagetia and Rao, 2011) and therefore increases the incidence of cell cycle disturbances, aberrant mitoses and cell death.

Amongst the methods to improve the quality of agriculture products and for other innovative purposes, nanotechnology occupies a prominent position (Nair et al., 2010). In the previous few decades, nanoparticles (NPs) have received extraordinary consideration because of their one of a kind properties and useful applications in agribusiness. Based on the core material, they can be comprehensively isolated into inorganic and organic NPs. Inorganic NPs incorporate metals (Al, Bi, Co, Cu, Au, Fe, In, Mo, Ni, Ag, Sn, Ti, W, Zn), metal oxides (Al_2O_3 , CeO_2 , CuO , Cu_2O , In_2O_3 , La_2O_3 , MgO , NiO , TiO_2 , SnO_2 , ZnO , ZrO_2) and quantum dots, while fullerenes and carbon nanotubes are organic NPs (Rajput *et al.*, 2018), although their use in agriculture is a fairly recent practice (Nair et al., 2010; Mahajan et al., 2011; Rico et al., 2011). Indeed, an evaluation of a variety of nanomaterials (NMs), mostly metal-based (MBNMs) and carbon-based (CBNMs), for their absorption, translocation, accumulation, and importantly, effects on growth and development in an array of crop plants indicated their potential for improving agricultural production (Nair et al., 2010; Rico et al., 2011). These reviews included non-consequential or negative effects on plant growth and development along with positive morphological effects, including enhanced germination percentage and rate, length of roots and shoots and their ratio, and vegetative biomass of seedlings in many crop plants.

Due to the deleterious effects and oxidative stress of gamma radiation on economically important crop plants, strategies should be developed in order to protect or reduce the risk of this type of irradiation negatively affecting yield and quality. Higher plant bioassays using *Vicia faba* are an important and integral part of test batteries used in detecting genotoxic contamination in the environment (Uhl et al., 2003). We examined the possibility of reducing radiation damage through the use of ZnO NPs, owing to their inherent surface properties that influence aggregation behavior (Lin and Xing, 2008). Moreover, the electron clouds that surround NPs could have high reactivity with radiolytic

free radicals and reactive oxygen species (ROS) (Bhatia, 2008). Interestingly, Dhoke et al. (2013) recorded a pronounced increase in root and shoot length as well as accumulation of biomass in ZnO NPs treated plants. The interaction of Ag NP or TiO₂ NPs with ionizing radiation (X-rays) was shown in an in vitro test system with DNA damage induction and repair as end-points (Zhang et al., 2009).

While DNA damage has both genotoxic and cytotoxic effects, it is highly likely that DNA damage in agronomically important plants plays a significant role in the “aging” of seed stocks and perennial crops. The acceleration of the ageing processes and the induction of degenerative diseases are causally related to DNA alterations. DNA damage in germ cells may also lead to heritable mutations in the offspring and cause a reduction in fertility (Uhl et al., 2003). It is therefore important to examine the oxidative stress and mutagenic potential of various types of genotoxic and mutagenic agents in crop plants to understand their phenotypic consequences. We evaluated the cytotoxic and genotoxic risk of gamma radiation in *V. faba* plants and assessed the possible stimulatory or inhibitory influence of ZnO NPs in modulating or inhibiting these effects.

MATERIAL AND METHODS

Fava bean seeds (*V. faba* variety Hsawi 2) were obtained from the College of Food Science and Agriculture, Department of Plant Production, King Saud University. Fresh and healthy uniformly sized seeds were surface sterilized with 2.5 % calcium hypochloride and air dried. Seeds were divided into four groups: no treatment (control), seeds treated with three concentrations of NPs (500, 2000 and 4000 mg/L), seeds irradiated with three doses of gamma rays (20, 50, 100 Gy), and seeds treated with gamma rays + ZnO NPs. Sixteen treatment groups were thus obtained: Control, N500, N2000, N4000, R20, R50, R100, R20/N500, R20/N2000, R20/N4000, R50/N500, R50/N2000, R50/N4000, R100/N500, R100/N2000, and R100/N4000. Seeds from each group were analyzed for phenotypic parameters and cytogenetic changes. All experiments were performed in triplicate. All chemicals were obtained from Medical Land – Mark EST for trading, Salehiya Trading EST and Sigma Chemical Company (#S-8394).

Gamma irradiation (IR)

Seeds were soaked in distilled water for 12 h at room temperature (25°C) and packed in high-density polyethylene bags and then irradiated with Gamma rays (Cobalt ⁶⁰ radiation source) from unit Gammacell 220 No. 246; at the Research Center, College of Sciences, King Saud University. Non-irradiated seeds served as controls.

Preparation of ZnO NPs Suspensions

ZnO NPs suspensions were prepared from analytical grade ZnO NPs (size 50 nm and purity 99.9%) using nano powder (purchased from M K Impex Corp, Canada) by weighing and dispersing them in deionized Milli-Q water with a mechanical stirrer. Small magnetic bars were placed in the suspension for stirring to avoid aggregation; this was followed by sonication on ice by ultrasonic vibration at (450 W, 40 kHz) for 30 min, and vigorous vortexing to obtain homogeneous suspensions.

Morphological characterization of the ZnO NPs

The ZnO nanoparticle size distribution was determined through transmission electron microscopy measurements (JEOL JEM 1011, Japan, operated at 80 kV) and images were captured using Scion Image processing software and characterized using scanning electron microscopy (Hitachi S-415A electron microscope at 25 kV).

Treatments and germination of *V. faba* seeds

Seeds were arranged randomly in three sets (20 seeds each) and exposure treatments were performed for 24 h. After treatments, seeds were washed with distilled water and germinated on sterilized cotton wool saturated with distilled water placed in sterilized Petri dishes in an incubator at 25°C until roots reached 1.5-2 cm and then used for determination of seed germination and cytogenetic analyses.

Determination of seed germination parameters

The number of germinated seeds was recorded after the radical reached 2 mm long, post 7 and 15 days. Emergence of radical was taken as an index of seed germination. The germination parameters taken into consideration were the final germination percent (FGP) and inhibition of seed germination was evaluated as below:

The final germination percent (FGP):

$$\text{FGP} = \frac{\text{Number of germinated seeds after 5 days} \times 100}{\text{Total number of seeds}} \quad (\text{Eq. 1})$$

The inhibition of seed germination:

$$\% \text{ of Inhibition} = \frac{\text{control} - \text{treated}}{\text{control}} \times 100 \quad (\text{Eq. 2})$$

Cytogenetic analyses for detection of chromosomal damage in mitotic-meristematic root tip analysis

Actively growing roots (1.2- 2 cm) were cut and root tips immediately fixed in fresh cold Carnoy's fixative (3:1 v/v absolute ethyl alcohol: glacial acetic acid) for 24 h, and then stored in 70% ethyl alcohol in the refrigerator until use for cytogenetic analyses. Fixed root tips were hydrolyzed and mitotic chromosomes were stained; the slides were prepared according to standard Feulgen squash technique. Briefly, fixed root tips were washed with distilled water and hydrolyzed in 1N HCl, washed with distilled water and stained with Leucobasic fuchsine solution. Deeply stained root tips were squashed in acetic acid, dehydrated in absolute ethyl alcohol, mounted in Euporal, dried and visualized at 40x magnification.

The following observations were calculated:

Mitotic index (MI)

MI was calculated as the average number of dividing cells from different root tips for each treatment using the following equation:

$$\text{Mitotic index (MI)} = \frac{\text{No. of dividing cells}}{\text{Total No. of cells examined}} \times 100 \quad (\text{Eq. 4})$$

where;

Total Number (No.) of cells = number of dividing cells + number of non-dividing cells.

Percentage of total abnormalities:

This was calculated as follows:

$$\% \text{ of total abnormalities} = \frac{\text{No. of abnormal cells} \times 100}{\text{Total No. of dividing examined cells}} \quad (\text{Eq. 5})$$

Percentages of different types of abnormalities:

This was calculated as follows:

$$\% \text{ of abnormal type } (\times) = \frac{\text{No. of abnormal type}}{\text{Total No. of abnormal cells}} \times 100 \quad (\text{Eq. 6})$$

Where;

(\times) is any type of mitotic abnormalities

Cytogenetic analyses for detection chromosomal damage in meiotic-pollen mother cells (PMC) and pollen grains (PGs):

After planting the treated and untreated seeds, 10 flower buds from 10 plants for each treatment condition were collected at maturity and fixed immediately in Carnoy's fixative and stained using an acid-carmines smear method. Cytogenetic analyses of PMCs of six randomly selected flower buds were scored for 1st and 2nd meiotic anomalies.

The types and frequency of aberrations were scored at the first and second meiotic divisions using the below formulae:

Percentage of total abnormalities:

$$\% \text{ of total abnormalities} = \frac{\text{No. of abnormal PMCs} \times 100}{\text{Total No. of PMCs}} \quad (\text{Eq. 7})$$

For PGs, the pollen fertility test was carried out using the same acid-carmines stain of mature anthers. PGs that took stain and had a regular outline, were considered as fertile, while empty and unstained ones were considered sterile. Unstained PGs of *V. faba* plants with acid-carmines stain for each treatment was used as the index for determination of non-viability or sterility.

$$\% \text{ of total abnormalities} = \frac{\text{No. of non-stain PGs only} \times 100}{\text{Total No. of PGs}} \quad (\text{Eq. 7})$$

$$\text{Total No. of PGs} = \text{No. stained PGs} + \text{no. unstained PGs} \quad (\text{Eq. 8})$$

Changes in chloroplasts and nucleus structure of fava beans by Transmission Electron Microscopy

Seeds were treated as above and planted along with untreated seeds (control) under field conditions for a period of five weeks. Actively growing leaf cells were harvested and immediately prepared for TEM (JEOL - JSM-1011 LV) with an accelerating voltage of 15 KV according to the method used by the Electron Microscopic Unit, Central Laboratory, Faculty of Science, King Saud University, as follows: The leaf tissue was fixed using buffered 2.5% glutaraldehyde overnight in a refrigerator, washed with phosphate buffer pH = 7.2 and fixed again using buffered 1% osmium tetroxide overnight in a refrigerator. Dehydration was carried out in a series of concentrations of ethanol. Samples were embedded in a resin mixture from SPI (SPI-Pon™ - Araldite® Epoxy Embedding Kit) and blocks were cut using a Leica UC6 ultra microtome with a section thickness of from 70-80 nm. Staining of specimen was done with Aura's uranyl acetate and lead citrate.

Statistical analyses

Estimation of phenotypic parameters was carried out based on seed germination parameters represented as final germination percent (FGP) and inhibition percentage of seed germination (%inhibition) and seedling growth parameters at vegetative stages (30 days after planting) represented as plant height (cm), number of leaves per plant, and leaf surface area (cm²) in addition to fruiting stage (45 days from planting) represented as plant height (cm), number of leaves per plant, leaf surface area (cm²), and number of green pods per plant.

The following parameters were used for determination of cytotoxicity and genotoxicity: (i) the mitotic index (MI), calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as a percentage; (ii) chromosomal aberrations were used as endpoints for determination of cytogenetic effects and (iii) micronuclei (MN) were scored in mitotic and interphase cells per cell (% of MN). For the most frequent abnormalities, microphotographs were taken at 1000 X magnification under oil immersion, using a Leica 2000 phase contrast light digital microscope with camera (Olympus Japan). Data for germination parameters, MI and percentage of chromosomal abnormalities were calculated as a measure of variation of these parameters between different roots of the same treatment. The percentages of both mitotic index and chromosome aberrations were evaluated. Each experiment was carried out in triplicate. Data was expressed as means \pm standard error (SE). The data was analyzed using one way ANOVA; $P < 0.05$ was considered significant.

RESULTS

Morphological characterization of ZnO NPs

The presence of ZnO nanoparticles, their size distribution and morphological confirmation of characterization was made with TEM (Figure 1) and SEM, respectively.

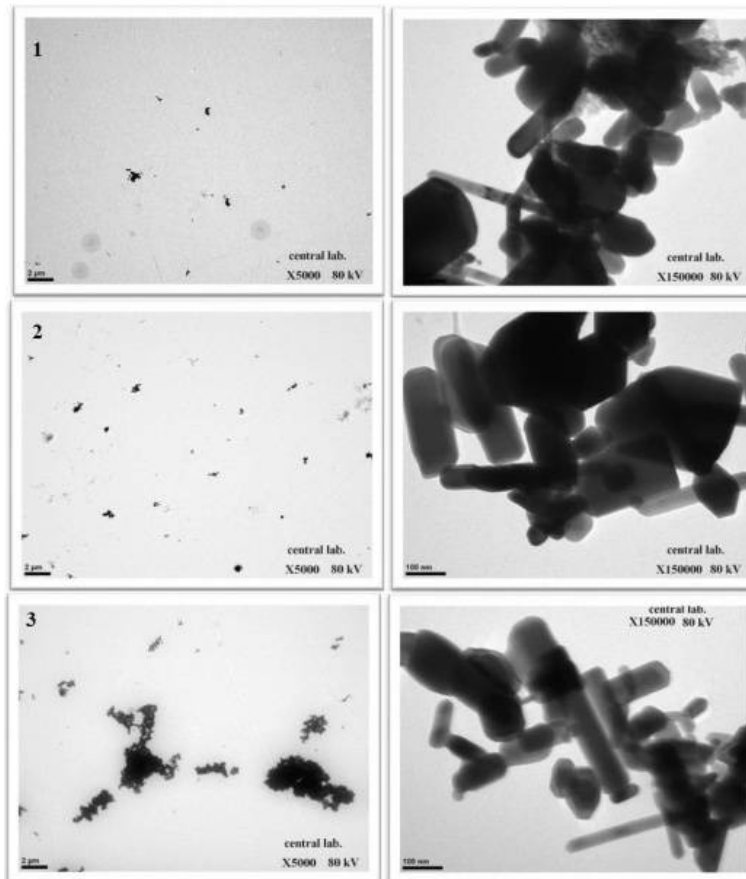


Figure 1. Transmission Electron micrographs showing dispersion of ZnO NPs at three concentrations: 1- 500 mg/L, 2- 2000 mg/L, 3- 4000 mg/L

Phenotypic parameters based on seed germination and seedling growth

Seed germination parameters

All three concentrations of ZnO NPs, 20 Gy of gamma radiation and a combination of these had no significant effect on the FGP after five days of exposure compared to controls. On the other hand, FGP was decreased to 55 and 35% after gamma irradiation with 50 and 100 Gy respectively. An improvement in FGP post treatment with two concentrations (500 and 2000 mg/L) of ZnO NPs was observed, but not with 4000 mg/L. After 15 days, treatment with the two concentrations (500 and 2000 mg/L) of ZnO NPs and post treatment with 20 Gy irradiation had no significant effect on FGP; seeds maintained their germination capacity compared to the control (100%). After three doses of gamma radiation (20, 50 and 100 Gy), FGP decreased with increasing doses to 70, 20, and 10%, respectively, with improvement in post treatments with two concentrations (500 and 2000 mg/L) but not with 4000 mg/L (Table 1).

Table 1. Seed germination parameters affected by the treatments.

| Treatments ZnONPs (mgL ⁻¹) and gamma rays (Gy) | | Percentages of seed germination parameters \pm SE | | | |
|--|-----------|---|---------------|----------------------|---------------|
| | | After 5 days | | After 15 days | |
| | | FGP% | Inhibition% | Seedling emergence % | Inhibition% |
| 1. | Control | 100 | 0 | 100 | 0 |
| 2. | N500 | 100 | 0 | 100 | 0 |
| 3. | N2000 | 100 | 0 | 100 | 0 |
| 4. | N4000 | 100 | 0 | 95 \pm 2.88 | 5 \pm .577 |
| 5. | R20 | 100 | 0 | 70 \pm 15.2 | 30 \pm 2.88 |
| 6. | R20/N500 | 100 | 0 | 100 | 0 |
| 7. | R20/N2000 | 100 | 0 | 100 | 0 |
| 8. | R20/N4000 | 100 | 0 | 70 \pm 15.2 | 30 \pm 0.00 |
| 9. | R50 | 55 \pm 2.8 | 25 \pm 2.08 | 20 \pm 2.88 | 80 \pm 2.88 |
| 10. | R50/N500 | 60 \pm 2.8 | 40 \pm 6.36 | 30 \pm 2.88 | 70 \pm 0.33 |
| 11. | R50/N2000 | 75 \pm 12.0 | 45 \pm 5.00 | 40 \pm 8.66 | 60 \pm 1.15 |
| 12. | R50/N4000 | 35 \pm .00 | 65 \pm 4.09 | 10 \pm 2.88 | 90 \pm 3.93 |
| 13. | R100 | 15 \pm 5.70 | 65 \pm 5.48 | 10 \pm 5.77 | 90 \pm 5.92 |
| 14. | R100/500 | 20 \pm 2.8 | 80 \pm 5.77 | 20 \pm 2.88 | 80 \pm 5.77 |

ZnO NPs: Zinc Oxide nanoparticles; FGP: Final germination percent

Seedling growth parameters at vegetative (30 days) and fruiting stages (45 days):

An increase in most vegetative and fruiting criteria, represented by plant height, number of leaves per plant, leaf surface area, and number of green pods/plant, was observed when treated with 500 and 2000 mg/L ZnO NPs but not with 4000 mg/L. The three doses of gamma rays gave decreasing parameters with increasing dose. Interestingly, ZnO NPs post treatment resulted in amelioration of the adverse effects of gamma ray treatment. For plant height, the highest values of 20.68 and 29.00 cm were recorded at a ZnO NPs concentration of 2000 mg/L. The lowest values of 13.13 and 17.67 cm were recorded at a gamma ray dose of 100 Gy compared with the values in untreated samples, which reached 18.67 and 21.00 cm at vegetative and fruiting stages, respectively. For the number of leaves/plant, the highest numbers (21.3 and 44.67) were recorded at a ZnO NPs concentration of 2000 mg/L, while the lowest values (8.67 and 14.00) were recorded at a gamma rays dose of 100 Gy compared with the values in untreated samples at vegetative and fruiting stages (18.67 and 34.33, respectively). A similar trend was observed for leaf surface area and number of green pods (Table 2).

Table 2. Seedling growth parameters affected by applied treatments.

| Treatments ZnONPs(mgL ⁻¹) and gamma rays (Gy) | | Seedling growth parameters \pm SE | | | | | | |
|---|------------|-------------------------------------|------------------------------|---|-------------------------|--------------------------|--|----------------------------|
| | | Vegetative stage (30 day) | | | Fruiting stage (45 day) | | | |
| | | Plant height (cm) | No. of leaves / plant | Leaf surface area (cm ²) | Plant height (cm) | No. of leaves / plant | Leaf surface area(cm ²) | No. of green pods/plant |
| 1. | Control | 18.67 \pm .88 | 18.67 \pm 0.68 | 17.55 \pm .58 | 21.00 \pm 1.16 | 34.33 \pm 1.28 | 21.33 \pm 4.16 | 4.00 \pm 1.00 |
| 2. | N500 | 20.00 \pm 1.73 | 20.0 ⁺ \pm 5.13 | 25.00 \pm .00 | 24.67 \pm 4.93 | 43.00 \pm 0.94 | 28.00 \pm 1.00 | 4.33 \pm 0.58 |
| 3. | N 2000 | 20.68 \pm 0.88 | 21.3 ⁺ \pm 4.09 | 27.00 \pm .00 | 29.00 \pm 5.57 | 44.67 \pm 2.52 | 32.33 \pm 1.53 | 4.67 \pm 0.58 |
| 4. | N4000 | 18.00 \pm 0.58 | 17.67 \pm 2.33 | 16.27 \pm .26 | 20.00 \pm 2.00 | 27.33 \pm 1.53 | 21.00 \pm 1.73 | 3.33 \pm 0.58 |
| 5. | R20 | 14.33 \pm 0.33 | 14.00 \pm 0.58 | 13.67 \pm .06 | 20.00 \pm 1.00 | 31.00 \pm 1.00 | 20.33 \pm 2.51 | 4.33 \pm 2.08 |
| 6. | R20/N500 | 20.33 \pm 1.76 | 15.00 \pm 1.00 | 21.50 \pm .27 | 22.33 \pm 2.08 | 32.67 \pm 2.58 | 19.33 \pm 2.03 | 4.00 \pm 1.00 |
| 7. | R20/N2000 | 19.67 \pm 1.45 | 19.00 \pm 0.00 | 21.00 \pm .46 | 25.00 \pm 0.00 | 35.67 \pm 2.03 | 24.00 \pm 4.58 | 5.00 \pm 1.00 |
| 8. | R20/N4000 | 18.33 \pm 2.73 | 12.67 \pm 2.60 | 17.10 \pm .08 | 20.33 \pm 4.04 | 25.33 \pm 3.51 | 14.33 \pm 2.89 | 3.33 \pm 0.58 |
| 9. | R50 | 13.33 \pm 1.88 | 9.33 \pm 0.333 | 12.00 \pm .39 | 19.67 \pm 2.51 | 20.67 \pm 1.96 | 18.00 \pm 1.00 | 3.30 \pm 0.577 |
| 10. | R50/N500 | 17.67 \pm 2.96 | 16.00 \pm 1.359 | 14.60 \pm .46 | 26.33 \pm 6.43 | 28.67 \pm 1.53 | 16.00 \pm 4.00 | 5.00 \pm 0.00 |
| 11. | R50/N2000 | 18.00 \pm .58 | 17.33 \pm 3.33 | 16.67 \pm .16 | 27.0 \pm 0.00 | 34.33 \pm 2.08 | 18.67 \pm 1.29 | 5.33 \pm 0.58 |
| 12. | R50/N4000 | 14.00 \pm 0 .00 | 12.33 \pm 1.36 | 11.00 \pm .00 | 21.33 \pm 5.03 | 18.00 \pm 18.52 | 17.33 \pm 1.53 | 4.33 \pm 0.58 |
| 13. | R100 | 13.13 \pm 3.22 | 8.67 \pm 0.68 | 10.50 \pm .73 | 17.67 \pm 8.02 | 14.00 \pm 1.79 | 11.00 \pm 1.00 | 3.00 \pm 0.00 |
| 14. | R100/500 | 16.00 \pm 1.00 | 11.33 \pm 2.404 | 14.20 \pm .01 | 21.00 \pm 3.61 | 25.33 \pm 2.16 | 18.00 \pm 1.61 | 4.00 \pm 1.00 |
| 15. | R100/N2000 | 16.50 \pm 0.29 | 14.00 \pm 2.08 | 16.93 \pm .11 | 24.67 \pm 2.52 | 32.33 \pm 1.53 | 18.67 \pm 0.87 | 4.50 \pm 1.000 |
| 16. | R100/N4000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

ZnO NPs: Zinc Oxide nanoparticles; * P < 0.05; **P < 0.01

Cytogenetic effects on mitotic – meristematic root tips:

Vicia faba root meristems grown from seeds treated with ZnO NPs showed a significant increase ($p < 0.01$) in MI compared to other treatments. The ZnO NPs at 2000 mg/L caused a significant stimulation of mitotic activity compared to the other two concentrations. A significant reduction in mitotic activity in a dose-dependent pattern was observed following exposure to gamma radiation ($p < 0.001$). Cytogenetic changes in *V. faba* root-tip mitotic cells varied from stimulation of MI due to ZnO NPs action to reduction of the adverse effects caused by gamma radiation. Additionally, ZnO NPs post treatment of irradiated seeds resulted in amelioration in mitotic activity (Table 3).

All treatments induced a variable range of mitotic abnormalities compared to non-irradiated samples. However, all concentrations of ZnO NPs induced fewer mitotic abnormalities than gamma rays and showed clear radio protection of mitotic-meristematic root tips of *V. faba* against adverse effects of gamma radiation.

Table 3. Number of total examined cells, total mitotic cells, mitotic index (MI) and percentage of chromosomal aberrations and types and frequency of abnormalities due cytogenetic effects of different applied treatments.

| Treatments ZnO NPs (mgL ⁻¹) and gamma rays (Gy) | MI \pm SE | No. of Abn. cells | % of Total Abnormal. \pm S.E. | Types and frequency of abnormalities in mitosis | | | | | Interphaseabnorm. | | | |
|---|------------------------|-------------------|---------------------------------|---|-----------|-----------|-----------------|---------|-------------------|---------------|----------------|---|
| | | | | Stickiness | Disturbed | Fragments | Lag. \pm free | Bridges | Micro-nucleus | Micro-nucleus | Multi-nucleate | |
| 1-Control | 14 \pm | 6 | 4.00 \pm .58 | 4.00 | -- | - | - | - | - | - | - | - |
| 2-N500 | 16 \pm | 31 | 18.6 ^{**} \pm .88 | 51.00 | 30.00 | 9.30 | - | 9.70 | - | .10 | - | - |
| 3-N 2000 | 16.7 \pm | 31 | 18.6 ^{**} \pm 1.20 | 64.50 | 22.80 | 3.20 | 3.20 | 6.50 | - | .60 | - | - |
| 4- N4000 | 15.6 \pm | 31 | 19 ^{***} \pm 1.20 | 65.60 | 19.70 | - | - | 14.70 | - | 3.00 | - | - |
| 5-R20 | 12.6 \pm | 30.5 | 24 ^{***} \pm 1.15 | 49.00 | 26.60 | 6.50 | 4.90 | 13.00 | - | .10 | - | - |
| 6-R20/N500 | 13 \pm 0 | 29 | 22 ^{**} \pm 1.201 | 51.70 | 20.60 | 6.80 | 13.70 | 6.80 | - | 1.40 | 0.20 | - |
| 7-R20/N2000 | 15.6 \pm | 24 | 15 ^{**} \pm 1.86 | 44.50 | 22.00 | 4.00 | 8.00 | 8.00 | 12.50 | .30 | - | - |
| 8- R20/N4000 | 15 \pm 0 | 32 | 21 ^{**} \pm 1.86 | 50.00 | 25.00 | 9.00 | 16.00 | - | - | .20 | - | - |
| 9- R50 | 11 \pm | 30.6 | 27.6 ^{**} \pm 0.33 | 34.60 | 26.60 | 16.00 | 9.80 | 13.00 | - | - | - | - |
| 10-R5/500 | 11.6 \pm | 29.5 | 25 ^{**} \pm .58 | 52.50 | 13.50 | 10.30 | 17.00 | 6.70 | - | 2.00 | 0.50 | - |
| 11-R50/2000 | 14 \pm | 35 | 25 ^{**} \pm 1.53 | 51.40 | 25.70 | 2.80 | 5.70 | 5.70 | 8.50 | 3.00 | 0.40 | - |
| 12-R50/4000 | 12.6 \pm | 34.5 | 27 ^{***} \pm 0.67 | 53.60 | 23.00 | 5.70 | 5.70 | 5.70 | 5.70 | 2.80 | 0.30 | - |
| 13-R100 | 3 ^{***} | 21.5 | 71.6 ^{**} \pm 1.85 | 48.80 | 23.00 | 9.00 | 13.90 | - | - | 3.80 | 0.50 | - |
| 14-R100/500 | 8 ^{**} \pm | 39 | 44 ^{**} \pm 0.58 | 46.00 | 25.60 | 15.00 | 5.00 | 5.00 | 2.50 | 7.00 | 0.50 | - |
| 15-R50/2000 | 8 ^{***} \pm | 31.5 | 39 ^{**} \pm 2.96 | 41.00 | 25.00 | 9.50 | 6.00 | 11.00 | 6.00 | 6.00 | 2.00 | - |
| 16-R100/4000 | 6 ^{***} \pm | 24.5 | 53 ^{**} \pm 1.86 | 40.80 | 26.50 | 16.00 | 16.00 | - | - | 3.00 | 0.50 | - |

Number of total examined cells was 1000 for every condition; ZnO NPs: Zinc Oxide nanoparticles; MI: Mitotic index; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

The frequencies of mitotic abnormalities induced by ZnO NPs (500, 2000 and 4000 mg/L) were 18.60, 18.60, and 19.00%, respectively, whereas, the frequencies of mitotic abnormalities induced by gamma rays (20, 50 and 100 Gy) were 24.00, 27.60, and 71.60%, respectively. ZnO NPs post treatment of irradiated seeds reduced the frequency of mitotic abnormalities after each post treatment of each irradiation dose.

The types and percentages of chromosomal aberrations in treated *V. faba* cells showed a dose-dependent pattern response (Table 3). The most frequent types of mitotic abnormalities induced by ZnO NPs were stickiness, chromosomal disturbances, and bridges (Figure 2); whereas, stickiness, chromosomal fragmentations, chromosomal disturbances, bridges, and micronuclei were the most frequent types of mitotic abnormalities induced by gamma rays. In contrast, the most frequent types of mitotic abnormalities induced post treatment with ZnO NPs were lagging and free

chromosomes. Percentages of abnormalities in the interphase stage ranged from 7-10% for micronuclei and 2-20% for multinucleate cells (Table 3 and Figure 2).

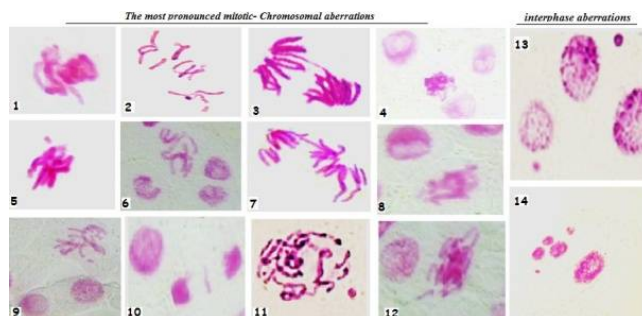


Figure 2. The most pronounced mitotic- chromosomal aberrations shown in meristematic root tips of *V. faba* treated with three concentrations of ZnO NPs (from 1-4) and three doses of Gamma irradiation (from 5 - 8), and ZnO NPs post treatment after irradiation (from 9 - 14). 1-Stickiness in metaphase, 2-Disturbed chromosomal metaphase , 3-Bridge in anaphase, 4-Sticky prophase, 5-Sticky metaphase with chromosomal fragment. 6-Disturbed chromosomal metaphase, 7- Anaphase bridge with disturbed chromosome in one pole. 8- Sticky metaphase. 9- Fragment chromosomes in metaphase 10- Laggard chromosome between two poles of telophase. 11- Prophase with micronuclei 12-Sticky anaphase with free chromosome, 13-Interphases with micronuclei, 14- Interphases with multinucleate cells.

Cytogenetic effects on Meiotic PMCs and PGs:

The types and frequencies of anomalies in meiotic PMCs were linearly a function of treatment exposure dose. The maximum value of meiotic-PMC abnormalities was (45.90%) at 100 Gy, whereas the highest concentration of ZnO NPs (4000 mg/L) induced 11.60% abnormalities. The most frequent abnormalities induced by various treatments were stickiness and chromosomal disturbances, while micronuclei and fragments were the most frequent types of PMCs abnormalities in post-treated samples (Figure 3).

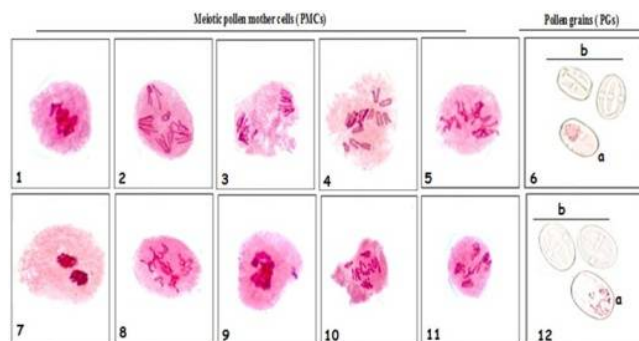


Figure 3. The most pronounced meiotic- chromosomal aberrations shown in PMCS and pollen grains of *Vicia faba* treated with three concentration of -ZnO NPs (from 1 - 6) and three doses of gamma rays (from 7 - 12), each separately and ZnO NPs post treatments of irradiated seeds. 1- Sticky metaphase I. 2- Anaphase II with lagging chromosome, 3-Anaphase I with lagging chromosome, 4 - Disturbed anaphase I, 5- Disturbed metaphase II, 6 - a-fertile PG with the genetic material, b -sterile PG without the genetic material. 7 - Sticky Metaphase I, 8- bridge in Anaphase I, 9 -. Sticky Anaphase I with chromosomal fragment, 10- Multipolar telophase II with fragment, 11 - anaphase II with two laggard chromosomes, 12- a-fertile PG. with the genetic material. b- sterile PG without the genetic material.

Tripolar phases were pronounced with irradiation doses 50 and 100 Gy, and post treatment of irradiated seeds with ZnO NPs reduced the frequency of mitotic abnormalities (Table 4 and Figure 3). On the other hand, pollen grain sterility was dose-dependent compared to controls. The maximum value of PG sterility was 43% at irradiation dose 100 Gy, whereas the maximum value of PG sterility was 30% at a ZnO NPs concentration of 4000 mg/L; this indicated that the irradiation exposure dose was more effective in reduction of PGs fertility than ZnO NPs concentrations. On the other hand, the ZnO NPs post treatments improved the values of PGs fertility, demonstrating protection against the adverse effects of gamma radiation (Table 4 and Figure 3).

Table 4. Number and frequencies of total meiotic abnormalities and percentage of different types of chromosomal aberrations in meiotic PMCs due cytogenetic effects of applied treatment.

| Treatments | Total no. of abnormal PMCs Scored | % of abnormal PMCs \pm SE | Types and frequency of meiotic abnormalities of PMCs | | | | | | Pollen Grains (PGs) Mean % of sterile PGs \pm SE |
|---------------|-----------------------------------|-----------------------------|--|-----------|-------------------------|----------------|--------|----------|---|
| | | | Stickiness | Disturbed | Micronuclei + Fragments | Lagging + Free | Bridge | Tripolar | |
| 1. Control | - | 0.00 | - | - | - | - | - | - | 0.00 |
| 2. N500 | 83 | 8.50 \pm .289 | 48.00 | 36.00 | - | 15.60 | - | - | 6.3 \pm 3.75 |
| 3. N2000 | 103 | 11.00 \pm 1.53 | 46.00 | 39.00 | 6.00 | 8.80 | - | - | 17.60 \pm 2.96 |
| 4. N4000 | 113 | 11.60 \pm 1.20 | 54.00 | 34.30 | 2.00 | 9.70 | - | - | 30.00 \pm 0.20 |
| 5. R20 | 170 | 18.00 \pm 2.08 | 44.00 | 35.00 | 11.70 | 8.80 | - | - | 12.66 \pm 0.72 |
| 6. R20/500 | 153 | 14.80 \pm 1.01 | 52.70 | 30.70 | 16.60 | - | - | - | 11.33 \pm 0.33 |
| 7. R20/2000 | 156 | 15.00 \pm 2.31 | 47.00 | 25.60 | 18.00 | 6.40 | 3.00 | - | 10.50 \pm 0.76 |
| 8. R20/4000 | 228 | 21.00 \pm 1.59 | 44.00 | 29.00 | 16.00 | 8.00 | 3.00 | - | 12.00 \pm 0.57 |
| 9. R50 | 388 | 27.80 \pm 3.98 | 50.30 | 38.00 | - | - | - | 11.70 | 13.00 \pm 0.58 |
| 10. R50/500 | 210 | 21.00 \pm 1.00 | 42.60 | 38.40 | 14.00 | - | - | 5.00 | 15.00 \pm 0.98 |
| 11. R50/2000 | 240 | 24.00 \pm 2.31 | 46.00 | 23.00 | 21.00 | 10.00 | - | - | 13.00 \pm 2.03 |
| 12. R50/4000 | 280 | 28.00 \pm 2.88 | 46.50 | 27.00 | 18.00 | 5.50 | - | 3.00 | 20.50 \pm .763 |
| 13. R100 | 459 | 45.90 \pm 3.20 | 45.50 | 24.50 | 20.00 | - | - | 10.00 | 43.00 \pm 0.64 |
| 14. R100/500 | 293 | 29.00 \pm 6.35 | 49.00 | 35.00 | 16.00 | - | - | - | 31.60 \pm 0.88 |
| 15. R100/2000 | 306 | 30.60 \pm 8.08 | 53.00 | 31.00 | 10.00 | - | - | 6.00 | 41.60 \pm 2.41 |

Changes in chloroplasts and nuclei ultrastructure

Chloroplasts of untreated *V. faba* leaves were elongated with a regular arrangement of grana stacks, a well-developed grana and intergranal thylakoids, a large number of starch granules and few plastoglobuli (Figure 4a). On the other hand, different concentrations of ZnO NPs and gamma rays alone induced significant changes in external shape and the internal structure of the chloroplasts, ranging from swollen thylakoids, destructive thylakoid membranes, inducing osmiophilic droplets (plastoglobuli), the accumulation or degradation of starch grains, and formation of electron-dense dark particles inside and between grana and stromal thylakoids (Figure 4b). Untreated nuclei were clear and showed a typical organization with a clear double membrane nuclear envelope and a pronounced nucleolus. Additionally, euchromatin and small patches of heterochromatin had a regular distribution throughout the nucleoplasm. Nuclei treated with ZnO NPs at 4000 mg/L showed deformation and degradation of most nuclear membranes due to the accumulation of nanoparticles (Figure 5). They had large patches of heterochromatin and euchromatin but

without a nucleolus. Similarly, various concentrations of gamma rays caused changes in the chloroplast and nuclei ultrastructure, causing changes in morphology and deformation/degradation of most membranes. Treated chloroplasts and nuclei with 500 and 2000 mg/L concentrations of ZnO NPs showed a clear nuclear and chloroplast ultrastructure compared to untreated ones, while ZnO NPs at 4000 mg/L and three doses of gamma rays resulted in greater adverse effects in chloroplasts and nuclei ultrastructure, leading to large-scale changes compared to untreated controls. Additionally, there was amelioration in the damage to the ultrastructure of chloroplasts and nuclei of *V. faba* irradiated seeds treated with 500 and 2000 mg/L of ZnO NPs; however 4000 mg/L concentration produced adverse effects in chloroplasts and nuclei ultrastructure.

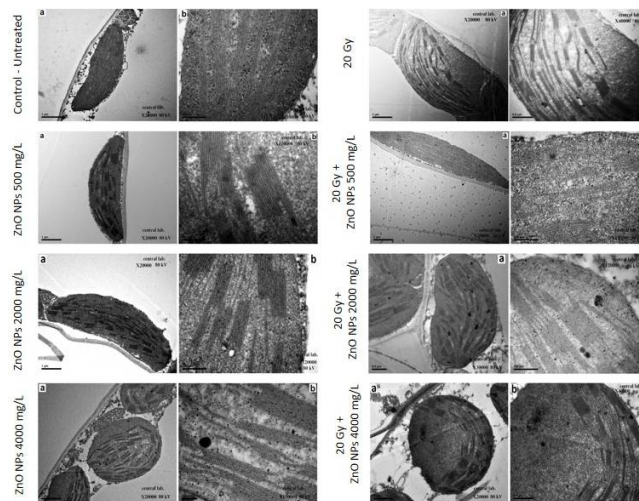


Figure 4a. Electron micrographs of treated chloroplasts of *Vicia faba* after treatment with ZnO NPs and gamma rays at different magnifications a-X20000, and b- X120000

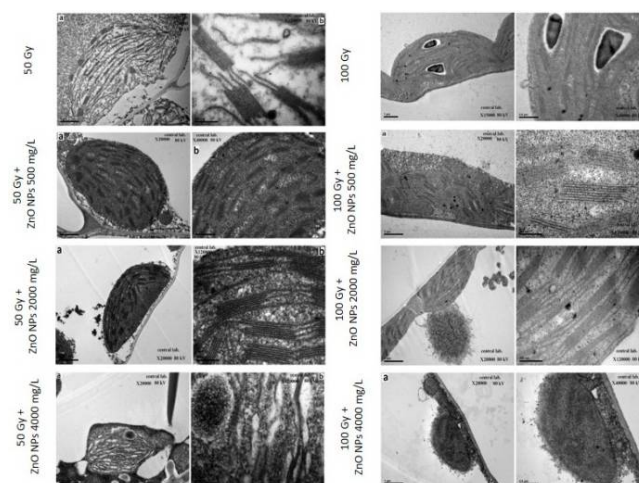


Figure 4b. Electron micrographs of treated chloroplasts of *Vicia faba* post-treated with ZnO NPs and gamma rays at different magnifications a-X20000, and b- X120000

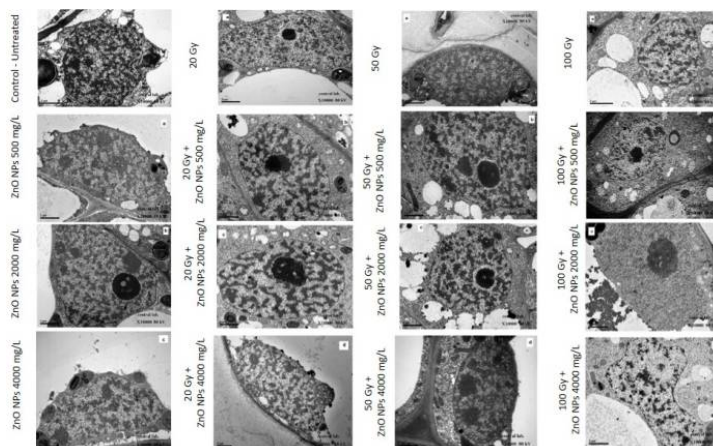


Figure 5. Electron micrographs of treated nuclei of *Vicia faba* chloroplasts after treatment with ZnO NPs and gamma rays at different magnifications a-X20000, and b- X120000

DISCUSSION

Morphological characters (phenotype) and the extent of chromosomal damage due to various xenobiotics play a role in plant survival and development (Kionget *et al.*, 2008). We evaluated the effects of ZnO NPs, gamma radiation, and their combination on phenotypic parameters and cytogenetic changes induced in fava beans. While, low concentrations of NPs and low doses of radiation did not significantly alter the germination of seeds, higher doses reduced seed germination parameters. These results were in accordance with Melki and Marouani (2009) who reported no significant difference in germination and survival percentage of irradiated and non-irradiated wheat seedlings at low doses. The effects of gamma rays on germination may be due to the activation of RNA or protein synthesis (Abdel-Hady *et al.*, 2008), alterations in hormonal signaling networks or increased anti-oxidative capacity (Wi *et al.*, 2007).

In contrast, similar to the results in our study, high-dose irradiation has been found to cause growth inhibition, which can be ascribed to cell cycle arrest at the G2/M phase (Preuss and Britta, 2003). Gamma radiation reduced seedling growth and caused an extensive variety of morphological and chromosomal changes in the fava bean. Likewise, it caused the development of abnormal leaflets, flowers and pollen grains. The most widely recognized chromosome abnormalities in the mitotic cells were stickiness, lagging and chromosome breaks along with disturbed polarity (Alghamdi *et al.*, 2018). Similar results were observed by Melki and Marouani, 2009, and Wi *et al.*, 2007 who reported a significant increase in plant growth at lower doses of irradiation but not at higher doses. Indeed, an improvement of 18 and 32% in root number and root length, respectively, was observed in hard wheat at a dose of 20 Gy (Melki and Marouani 2009). Our study results are also in agreement with those of Chaudhuri (2002), who found that at lower doses the germination percentage was not significantly different from control. However, at higher doses, it decreased, possibly due to a reduction in the levels of endogenous growth regulators Kiong *et al.* (2008), auxin destruction, changes in the ascorbic acid content and physiological and biochemical disturbances (Shah *et al.*, 2008).

The ability of higher concentrations of ZnO NPs (4000 mg/L) to also cause phenotypic alterations could be due to the induction of oxidative stress, with consequent overproduction of reactive oxygen species that lead to less germinability or inhibition and reduction in plant growth (Noreen and Ashraf, 2009). Tripathi et al. (2017) demonstrated ameliorative impacts of nitric oxide against ZnO NPs actuated phytotoxicity in wheat seedlings. Nitric oxide manages the amassing of Zn and maintains the normal cellular functioning of the glutathione-ascorbate cycle, thus decreasing damage caused by ZnO NPs. We found that ZnO NPs could ameliorate the effects of oxidative stress induced by gamma rays at lower concentrations (500 mg/L and 2000 mg/L) but not at the highest concentration (4000 mg/L). These results confirm the findings of Gowayed and Kadasa, 2016 who have shown that the oxidative stress induced by the heavy metal Cd was alleviated by treatment with ZnO NPs through a significant increase in antioxidant enzymes.

The reduction in root and shoot growth at higher doses may be attributed to the accumulation and uptake of ZnO NPs to reach toxic levels in the roots. On the other hand, individual ZnO NPs treatments, and post treatments with lower concentrations (2000 and 500 mg/L) along with low doses of gamma rays (20 Gy) stimulated the germination of seeds and increased the growth of seedlings; Ghosh et al. (2016) studied cytotoxicity, genotoxicity and biochemical effects of ZnO NPs (85 nm) in three crop plants (*Allium cepa*, *Nicotianatabacum*, and *V. faba*). In the root meristems of *Allium cepa*, ZnO NPs were found to cause chromosome abnormalities, including micronucleus formation, DNA strand breaks, and cell-cycle disturbance at the G₂/M checkpoint. In *V. faba* and *Nicotianatabacum*, they produced intracellular ROS, a result also supported by Kiong *et al.*, (2008) and Melki and Marouani (2009). Lower doses of ZnO NPs (2000 mg/L) led to 100% germination of seeds, similar to the observations of Lin and Xing (2007) who reported that ZnO NPs at these concentrations did not affect seed germination of radish, rape, ryegrass, lettuce and cucumber, implying that the toxicity of ZnO NPs is species specific and also size dependent. A study by Zafar *et al.* (2016) showed significant inhibition of germination of *Brassica nigra* seeds. Similar results in morphological parameters were observed in cowpea (Gnanamurthy *et al.*, 2013) and sesame (Anbarasan and Raj, 2013).

We found a dose-dependent effect on the mitotic cell cycle of meristematic cells in *V. faba* roots. Mitotic index was used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the mitotic phase of the cell cycle. The reduction in mitotic activity due to gamma irradiation could be due to the arrest of mitotic cycle at the G₁ phase (El-shazly and El-Sheikh, 2000) or an inhibition of DNA synthesis and nucleoproteins or a blockage at the G₂ phase of the cell cycle, preventing the cell from entering mitosis (Sousa and Viccini, 2011).

The inhibition of mitosis indicated that gamma rays have mutagenic effects on embryonic roots of *V. faba*. Interestingly, Kumari *et al.* (2011) demonstrated that ZnO NPs can be a clastogenic/genotoxic and cytotoxic agent; these results are contrary to the findings of our study. While an increase in MI may mean disordered cell proliferation and formation of tumors (Campos *et al.*, 2008), it could also be due to shortening of the duration of mitotic cycle and enhancement of the interphase cells to enter the subsequent division stages or by inducing the synthesis of DNA in dividing cells (Haroun, 2010).

Cytological examination of dividing cells revealed dose-dependent chromosome irregularities, including chromosome stickiness, chromosome bridges, lagging, free

migration of chromosomes, chromosomal fragments, and micronuclei. Sticky behavior of chromosomes due to xenobiotic exposure could be attributed to alterations in the physicochemical properties of DNA, protein or both (Celik and Aslanturk, 2009). Another abnormality observed in this study was chromosome bridges and lagging chromosomes, which could be linked to stickiness of chromosomes. Bridges and laggards with or without fragments were found both at anaphase and telophase; bridges without fragments were found at higher concentrations of the mutagens. Both single and double bridges were found, but multiple bridges were not rare. Multiple bridges were mostly found at anaphase and single bridges at telophase (Bhatet *et al.*, 2007). Spindle disturbance is another dominant abnormality recorded after all treatments. We also found that mitotic micronuclei and chromosomal fragmentation were most pronounced common aberrations in some treatments. While micronuclei are formed as a consequence of chromosomal fragments (Maluszynska and Juchimiuk, 2005), fragments at metaphase may be due to the failure of broken chromosomes to recombine (Agarwal and Ansari, 2001).

Our study was also novel in its evaluation of cytogenetic changes in PMCS and PGs. Not only did the study of meiotic behavior of mutagenized plant provide a reliable indicator for estimating the type of effects of mutagens, the studies on PGs indicated alterations in the quantity and quality of pollen produced by a plant in response to mutagens (Boff and Schifino-Wittmann, 2002). All treatments demonstrated interference with meiotic PMCs and PGs, possibly due to the induction of oxidative damage, a higher frequency of chromosomal aberrations and DNA damage which that affect vigor, fertility and yield (Uhl *et al.*, 2003). ZnO NPs and gamma rays at higher concentrations may lead to the induction of structural changes in DNA (Ahmad *et al.*, 2008), which can affect many metabolic processes, such as plant development, cell cycle, fertilization, and seed formation (Agrawal *et al.*, 2009). Perhaps the chromosomal aberrations due to meiosis impairment compromised pollen fertility, resulting in an increased frequency of pollen sterility, although survival of abnormal pollen grains may produce imbalanced progeny bearing some peculiar genetic traits (Kumar and Dwivedi, 2012).

The assessment of changes in the ultrastructure of internal organelles shows the nature of modifications caused by xenobiotics. Chloroplasts and nuclei are remarkably complex and sensitive to developmental changes, environmental effects, and genetic lesions. An impairment of photosynthetic function owing to distortion of nuclear membrane, chloroplast swelling, thylakoid dilation, and rupture of the chloroplast outer membrane reported in this study are consistent with the findings of Sreedhar *et al.* (2013). Furthermore, an accumulation of starch grains a high dose (100 Gy) of gamma irradiation could be related to elevated levels of cytosolic sugars because of loss of secretory Golgi activity or even blockage of amylase transport from the Golgi to the chloroplast or due to inhibition of amylolytic enzymes (Hummel *et al.*, 2010). An increase in the osmiophilic droplets (plastoglobuli) could be a response to oxidative stress and serve as an indicator of destructive processes in chloroplasts under abiotic stress and represent adaptive modifications (Austin *et al.*, 2006).

The toxic effects of metal nanoparticles on plants could with a consequence of chemical toxicity or due to stress or stimuli caused by the surface, size and/or shape of the particle. On the other hand, research has also shown the positive effects of metal and metal oxide nanoparticles on the growth of higher plants (Singh *et al.*, 2018). Consistent with the findings our current study, it was also reported by Mahajan *et al.* (2011) that the

accumulation and uptake of ZnO NPs is dependent on exposure concentrations. They Also found that at certain concentrations, the seedlings displayed good growth compared to the control, and beyond that, retardation in growth was observed.

CONCLUSIONS

We found that ZnO NPs at 500 and 2000 mg/L concentrations were positive or stimulatory for the plant, while higher concentrations 4000 mg/L were inhibitory. Gamma rays affected mitosis and meiosis and pollen grains of *V. faba* at higher doses of 50 and 100 Gy. The modulation or reduction of adverse effects of all doses of gamma rays at the cellular, cytogenetic, biochemical and molecular levels were apparent when post treated by ZnO NPs at concentrations of 500 or 2000 mg/L. This will possibly open an avenue for the potential use of low doses of ZnO NPs as “nano-irradiation protective agents”. However, additional studies are needed to firmly establish the positive effects of low doses of nanoparticles on crops.

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CONFLICTS OF INTEREST

The authors report no conflicting interests with respect to this study.

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