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Attenuation of lead genotoxicity in *Glycine max* by adsorbent nanosized titanium dioxide using phenotypic, cytogenetic and DNA status bioassays

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ABSTRACT. Genotoxicity caused by heavy metals can negatively affect vital processes of crop plants, though remedial measures can be used to reduce such damage. We examined the possible stimulatory and phytotoxicity impacts of three nanosized titanium dioxide ($nTiO_2$) doses on soybean (*Glycine max*) and how adsorption of lead (Pb) onto the surface of $nTiO_2$ may attenuate the toxic action of Pb on soybean by comparing the toxicity of three Pb doses before and after the adsorption process. The exposure time was 48 h. Phenotypic parameters (seedling growth, phytotoxicity, tolerance and vigor indices), cytogenetic tests of pollen grain performance, and DNA status (using flow cytometry, comet assays and analysis of RAPDs) were used as bioassays to assess the effect of the treatments. The optimal $nTiO_2$ dose was 10 $mg.L^{-1}$ because it i) stimulated and accelerated seedling development parameters, fertility and germination of pollen grains, ii) increased nuclear DNA content and decreased the extent of DNA damage, and iii) generated the maximum number of amplified DNA bands as an indicator for appearances of new DNA bands (genes) more than the control. Doses of $nTiO_2$ higher and lower than the optimal dose resulted in a gradual decline in these parameters, especially the higher dose. The three

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doses of Pb induced notable inhibitory and genotoxic impacts on all biomarkers used, in a dose-dependent manner. We conclude that the powdered state of optimal dose (10 mg) had a good ability to adsorb Pb onto its surface and consequently mitigated its toxicity. This was evident through the significant amelioration of parameters of each biomarker after application of the three Pb adsorbate solutions on soybean seeds. Therefore, we suggest that stimulatory and adsorbent $nTiO_2$ dose may be used in the future to protect against heavy metal toxicity in economically important plants.

Key words: Adsorption process; Heavy metals; Nanoparticals; Soybean; Pollen grain performance; DNA bioassays

INTRODUCTION

Higher plant bioassays can detect a wide range of genetic damage, including gene mutations, chromosome damage, and DNA damage analysis. (Maluszynska and Juchimiuk, 2005; Iqbal et al., 2019). One of these studied plants, *Glycine max* (soybean), belongs to the family Leguminosae and has a diploid chromosomal set (2n = 40). The study of Blanco et al. (2017) demonstrated that soybean crops can incorporate and accumulate more potentially toxic metals, such as lead, than numerous other crops because of its high biomass and easy cultivation. It is used as a model plant material and suggested a preliminary screening test for genotoxic agents and environmental mutagens due to it has large number of genetic markers and/or database and are also highly suitable for testing the genotoxic agents (Vig, 1982).

In recent years, environmental pollution by heavy metals has significantly increased due to industrial, motor vehicle, agricultural, chemical fertilizer, geogenic, domestic effluent, pharmaceutical, and atmospheric sources. Therefore, the impact on ecological and global public health has been an increasing concern. Lead (Pb) is an anthropogenic pollutant and one of the most toxic heavy metals present in the environment known to pollute agricultural lands and consequently economical crop plants, even at low doses (Nas and Ali, 2018). They reported that Pb cause oxidative damages of protein and DNA by over production of reactive oxygen species ROS, which in turn may cause genotoxicity to plant cells. The excessive accumulation of lead in plant tissue not only strongly inhibits various vital growth processes such as seed germination and seedling development parameters but also DNA synthesis, pollen grains fertility and seed productivity (Pourrut et al., 2011). The effects of lead on DNA occur directly or indirectly, resulting in DNA damages leading to destabilization of the double helical structure of DNA, mismatches of the bases on nucleic acids, single-nucleobase lesions on DNA, single DNA strand breaks, and double DNA strand breaks and thereby affects horizontal DNA–DNA or DNA–protein links (Nas and Ali, 2018). The study of Pourrut et al. (2011) reported that lead may enter the nucleus and bind directly or indirectly to the DNA, causing disruption of DNA repair and replication mechanisms. They also evaluated DNA damage induced by lead in *Vicia faba* using comet assay and demonstrated that the genotoxic effects of lead (DNA breakage and micronucleus formation) may result from the overproduction of ROS induced by lead.

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Alternative strategy has been developed in order to reduce or ameliorate genotoxic effects of lead (Pb) on plant DNA. Fortunately, heavy metal ion speciation might be altered by nanoparticles (especially oxide NPs) as adsorbing pollutant agents, owing to their higher surface area and more active surface sites that can neutralize or reduce the toxic effects of heavy metals by changing their transport and bioavailability in natural systems (Bok-Badura et al., 2018). From these oxide NPs, nanosized titanium dioxide ($nTiO_2$) has been shown to aggregate and adsorb heavy metals on their surface as result of their remarkably high surface area, their photocatalysis ability, their aggregation behavior, their photocatalysis ability, their strong adsorption capacity and the presence of high affinity surface hydroxyl groups, and consequently, reduce their bio-availability and alter their toxic effects on plants (Bok-Badura et al., 2018). $nTiO_2$ has been used as an adsorbent material for the removal of heavy metals from water (Poursani et al., 2016). For example, the study of Yang et al. (2012) demonstrated that nTiO2 ($nTiO_2$) could reduce the free Cd^{2+} in toxic media, further lowering its bioavailability and toxicity to a green algae, *Chlamydomonas reinhardtii.*

Nanosized TiO_2 has been tested for its positive and stimulating potential on plants by promoting and improving seed germination and crop performance, especially at the appropriate dose due to its ability to penetrate the seed coat, resulting in increased water/nutrient absorption (Marchiol et al., 2016; Lyu et al., 2017). Only recently, the genetic implications of nanoparticle-induced positive changes have been validated through investigations on positive changes in gene expression, enhanced mRNA expression and protein level in spinach by nano-TiO_2 (Gao et al., 2006; Kole et al., 2013), indicating their potential use in crop improvement. This positive impact was linked with enhanced activities of enzymatic antioxidants, as well as reducing oxidative damages of DNA. The potential positive effects of $nTiO_2$ may provide a helpful approach for reducing the consumption of chemicals through agriculture by alleviating heavy metal environmental pollution.

On the contrary, nTiO2 at high doses can induce phytotoxicity and exhibit a negative impact on seed germination and plant growth (Feizi et al., 2013). The study of Ruffini Castiglione et al. (2014) concluded that plants can be harmed by TiO_2-NPs at high doses with influence on mitotic index and induced genotoxic effects, DNA fragmentation and reactive oxygen species (ROS) during its reaction with DNA.

It is important for detection of genotoxicity of various types of genotoxic agents on exposed and/or non-exposed crop plants, to understand the biological consequences of DNA damages and their molecular modes of action that lead to alterations or repair the genetic material by introducing number of bioassays that are commonly used to provide robust and reliable assessment of nuclear DNA damage in economic crop plants (Maluszynska and Juchimiuk, 2005). Some of the recent bioassays were flow cytometry, comet assay, and random amplified polymorphic DNA (RAPD).

DNA damage in germ cells (pollen mother cells and pollen grains) induced by accumulative genotoxicity and chromosomal aberrations can lead to heritable damage in subsequent generations, causing a reduction in fertility and vigor, seed yield, and quality of economic crop plants (Abdelhaliem et al., 2013). Therefore, pollen grains performance should be estimated as a useful tool to assess the biological effect of any genotoxic agent.

Recently, molecular cytogenetic techniques were introduced to allow analysis of genotoxicity, both at the chromosomal and nuclear DNA level (Maluszynska and Juchimiuk, 2005). Flow cytometry methodology (FCM) is a fast and accurate technique that has been tested theoretically with a number of different plant species for estimation minute

changes in nuclear DNA content (nDNA) and genome size, the gain or loss of a single chromosome chromosomal, DNA damage, and chemical adducts to DNA and DNA strand breakage to assess genotoxicity due to different genotoxic and mutagenic agents (Monteiro et al., 2010).

The comet assay, an alkaline version of the single-cell gel electrophoresis is another good and a new technique for assessing genotoxicity in eukaryotic plant cells because of its simplicity, quickness and sensitivity for the detection of various DNA lesions including strand breaks, base damage and alkali-labile sites in individual cells, induced by a variety of genotoxic agents (Bhat et al., 2011). The study of Rodriguez et al. (2011) compared the validity of the comet assay and FCM in evaluating genotoxicity tests in plants and demonstrated that the data provided by both techniques complement each other and presented high correlation in the detection of DNA damage.

On the other hand, the RAPD fingerprinting technique, a PCR-based molecular marker technique, is a fundamental tool, being a simple, rapid, and low-cost assay used to detect genotoxins that induce a wide range of DNA damage to plants (point mutations, inversions, deletions). It can also be used to describe similarities and diversification between samples by the appearance of new unique bands, disappearance of polymorphic bands, and variation in band intensities (Abdelhaliem and AL-Huqail, 2016; Pal, 2016)

Taking the above into consideration, the present study aimed: 1) to evaluate possible stimulatory or inhibitory influences of $nTiO_2$ as well as phytotoxicity of heavy metal lead (Pb) on soybean seeds; 2) to test the efficiency of the stimulatory (optimal) dose of $nTiO_2$ as an adsorbent material to neutralize and alleviate the phytotoxic action of lead on economic crop plant soybean, and; 3) to apply $nTiO_2$ in agriculture for protection of economic crops and to increase agricultural production in future.

MATERIAL AND METHODS

Plant material

Soybean seeds (*Glycine max* variety “Hodgson”) were obtained from the King Saud University, College of Food Science and Agriculture, Department of Plant Production, Riyadh, Saudi Arabia. Fresh and healthy uniformly sized seeds were divided into four groups. Seeds of each group were surface sterilized in a 1% v/v solution of sodium hypochlorite by gentle magnetic stirring for 10 min, then rinsed three times with deionized water and air dried. The first group of seeds did not receive any further treatment to serve as the control. The second group was treated with $Pb(NO_3)_2$ solutions, the third group with $nTiO_2$, and the fourth group was treated with Pb adsorbate solutions after adsorption process.

Preparation of lead nitrate solutions

White powdered lead nitrate (purity: 99%, molecular weight 331.20; Sigma Chemical Co., Sigma, MO, USA), was used in this study. The experimental treatments included three doses (75, 100, and 150 $mg.L^{-1}$) that were freshly prepared by dissolving $Pb(NO_3)_2$ in deionized water and adjusting their pH to 5.5 with HNO_3 before being used as treatments for soybean seeds. These doses were in the range between doses reported in

other studies [doses were in the range between doses reported in other studies as (Oladele et al., 2014)]. Distilled water was used as an untreated control (without Pb).

Preparation of $nTiO_2$ suspension

Fine-particulate nTiO2 (AEROXIDE® TiO_2 P25, Sigma Chemical Co., Germany), had a high specific surface area of 35–65 $m^2.g^{-1}$; average primary particle size, 21 nm, and; purity, ≥99. $nTiO_2$ was suspended directly in filter-sterilized double distilled water. Small magnetic bars were placed in the suspension to stir and avoid aggregation followed by sonication on ice by ultrasonic vibration at 450 W, 40 kHz (UP100H Ultrasonic processor, Hielscher Ultrasound Technology, Germany), for 30 min and vigorous vortexing to obtain homogeneous suspensions (5 min), when required. A triplicate hydroponic experiment was conducted to determine the positive and negative effects of the three $nTiO_2$ doses (5, 10, and 20 $mg.L^{-1}$) on soybean seeds.

The selected doses were in the range between doses reported in other studies (Feizi et al., 2013) and distilled water (DI) without $nTiO_2$ was used as control. To better understand the characters of the selected nanosized titanium dioxide ($nTiO_2$), the current study used transmission electron microscope (TEM) (JEOL JEM-2010, Japan, operated at 80 kV).

Characterization of TiO_2 in dispersion solutions

The TiO_2 nanoparticle suspension was characterized for size and dispersity. Laser doppler velocimetry (LDV) was performed using a Malvern Nanosized-ZS ZEN3600 (Worcestershire, United Kingdom) for the characterization of zeta potential. The particle size distributions were determined based on number, volume, and scattering intensity.

Treatments of soybean seeds

Viability and uniformity in size soybean seeds were used in this study. A batch hydroponic experiment was conducted in triplicate to determine the effects of 48 h exposure of $nTiO_2$ (5, 10, and 20 $mg.L^{-1}$) and Pb (75, 100, and 150 $mg.L^{-1}$) doses on seedling growth parameters of soybean seeds, alongside the control. Twenty surface-sterilized soybean seeds were placed on sterilized petri dishes and soaked in 30 mL of each dose of $nTiO_2$ or Pb alongside untreated samples under the laboratory conditions. The solution level was maintained to avoid changes in exposure dose. After all treatments, treated and untreated soybean seeds were washed three times with distilled water and used for analyses by different bioassays.

Phenotypic parameters of soybean seedling growth, phytotoxicity, tolerance, and vigor indices

After all treatments, each replicate (corresponding to treated and untreated soybean seeds) was washed three times with distilled water and sown immediately in earthenware pots (30 cm high x 20 cm diameter) containing soil obtained from topsoil in the field and grown in a greenhouse for 35 days until reaching the seedling stage.

Triplicates of 10 seedlings were randomly sampled, and the following seedling growth parameters were recorded: mean lengths (cm) of root, shoot, and seedlings. The percentage phytotoxicity of shoot and root of seedlings was calculated following the formula given by Mishra and Choudhuri (1999). The mean root/ shoot ratios, leaf parameters (based on the number of leaves per plant), leaf surface area (cm^2), and dry weight (g) of root and shoot systems were also measured. The tolerance index (TI) and seedling vigor index (VI)I were computed based on the study of Vashisth and Nagarajan (2010) as shown in the following four equations:

% Phytotoxicity of root = Root length of control - Root length of treatment ×100 (Eq. 1)

% Phytotoxicity of shoot = Shoot length of control - Shoot length of treatment ×100 (Eq. 2)

Tolerance index (TI)% = Root length of treatment / Root length of control ×100 (Eq. 3)

Vigor index (VI)I = Seedling length (cm) × % of Germination (Eq. 4)

Cytogenetic analyses of pollen grains (PGs) of mature anthers

Triplicates of treated and untreated soybean seedlings mentioned above left for 45 days until reached the flowering stage. Ten large-sized flower buds from ten plants for each treatment dose, plus the controls, were collected, then fixed immediately in Carnoy's fixative for 24 h and then stored in ethanol (70%, v/v) at 4°C until they were used for cytogenetic analyses of the pollen grains. Finally, mature anthers containing PGs were stained using alcoholic hydrochloric acid-carmine smear for one week as described in the study of Abdelhaliem et al. (2013). PGs anomalies were examined using certain PGs parameters. PGs which took red stain and had a regular outline were considered as fertile, while empty and unstained (colorless) ones were sterile (Abdelhaliem et al., 2013). The following PGs parameters were recorded: the final percentage of morphological homogeneity (MH) and absolute pollen viability (APV) were calculated using the formula used by Tosun and Koyuncu (2007) while and Pollen germination (PGs) percentage was calculated according to (Visser et al., 2007) as shown in the following equations:

The final percentage of morphological homogeneity (MH):

$$MH\% = \frac{(\text{No.of normal shaped PGs}) - (\text{No.of aborted PGs})}{\text{total number of pollen field}} \times 100 \quad \text{(Eq. 5)}$$

Absolute pollen viability (APV):

$$APV\% = \frac{\%\ \text{stained PGs} \times \%\ \text{Germinated PGs}}{100} \quad \text{(Eq. 6)}$$

Germination percentage of PGs :

$$\text{Pollen germination}\ \% = \frac{\text{No.of germinated PGs}}{\text{Total No.of PGs}} \times 100 \quad \text{(Eq. 7)}$$

Molecular cytogenetic biomarkers for detection DNA status

Treated and untreated soybean seeds with $nTiO_2$, Pb, and Pb adsorbate solutions were used instead of leaves in this study to avoid the accumulation of staining inhibitors within leaves (Sliwinska et al., 2009).

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Estimation of variation in nuclear DNA content and genome size by flow cytometry

Nuclear DNA content and genome size of soybean nuclei were estimated using a rapid and simple protocol as described by Arumuganathan and Earle (1991). Fifty mg of germinated soybean seeds were chopped into <0.5 mm pieces on ice using a sharp razor blade after adding 1 mL of solution A (14.3 mL $MgSO_4$ buffer (ice-cold), 15 mg dithiothreitol (Sigma, D-0632), 300 µL fluorochrome stain, propidium iodide (PI) stock, and 375 µL Triton X-100 stock) in plastic petri dishes. The pieces were then homogenized using a mortar for isolation of nuclei from treated and untreated seeds. The solution of homogenized nuclei was filtered through a nylon cloth (50 µm mesh size), centrifugated at 15,000 *g* for 15 to 20 s and finally the supernatant discarded. The pellet was resuspended in 200 µL of solution B (3 mL Solution A, 7.5 µL RNAase (DNAase free), 3.0 µL of human leucocytes (HLN)). The relative fluorescence of nuclei was measured using flow cytometry which was conducted in the immunology laboratory at King Khalid Hospital. The measurements of relative fluorescence intensity of nuclei stained with propidium iodide (PI) was performed on a linear scale and, typically, at least 5000 nuclei were analyzed for each sample. The histogram of relative DNA content was obtained after flow cytometric analysis of stained nuclei of soybean. Fresh HLN (2C = 7.0 pg) served as internal standards for PI flow cytometric analysis. The analysis compared the mean position of the peaks due to soybean nuclei with the mean peak position of the internal standard. Fluorescence ratios (2C DNA content/ sample, relative to the standard, were used to calculate DNA content (in picograms, pg) and genome size (in mega base pairs, Mbp) according to the following formulae used by Dolezel et al. (2003):

$$\text{2C DNA content (pg)} = \frac{\text{(Sample Peak mean x Standard DNA content (7)}}{\text{Standard Peak mean}} \quad \text{(Eq. 8)}$$

where: the symbol (C) (the DNA content of the haploid set of chromosomes) while the equivalent number of base pairs (genome size) was calculated assuming that 1 pg DNA is equivalent to 0.965×10^9 bp or 965 Mbp according to (Dolezel et al., 2003).

Comet assay method for the detection of damage in the nuclear DNA of treated soybean

Isolation of nuclei and single cell gel electrophoresis (Comet assay) were carried out and described as in the study of Abdelhaliem and Al-Huqail (2016).

Isolation of nuclei

After removal of the seed coat from embryonic tissues, the latter were placed in a small petri dish containing 200 µL of cold 400 mM Tris-HCl buffer, (pH 7.5) on ice. Using a razor blade, the seed was gently sliced into small pieces to release nuclei into the buffer under yellow light. Each slide was previously coated with 1% agarose. After heating to the normal melting point (NMP), the slide was dried and covered with a mixture of 55 µL of nuclear suspension and 55 µL of low melting point (LMP) agarose (1% prepared with phosphate-buffered saline) at 40°C and sealed with a cover slip. The slide was placed on ice

for at least 5 min, and then the coverslip was removed. Then, 110 µL of LMP agarose (0.5%) was placed on the slide and coverslip was mounted again. After 5 min on ice, the coverslip was removed.

Single cell gel electrophoresis (SCGE)

The slides containing soybean nuclei were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) and incubated for 15 min. Electrophoresis was performed at 16 V, 300 mA for 30 min at 4°C. Then the gels were neutralized by washing three times in 400 mM Tris-HCl (pH 7.5) and stained with ethidium bromide (20 µg/mL) for 5 min. After staining, the gels were dipped in ice-cold distilled water and immediately analyzed.

Imaging and analysis software

DNA damage in 50 randomly selected nuclei on each slide were analyzed and assessed qualitatively and quantitatively by visual scoring or by fluorescence microscopy with an excitation filter of 546 nm, a barrier filter of 590 nm, and a computerized CCD camera digital image analysis system (Komet Version 3.1. Kinetic Imaging, Liverpool, UK). Tail moment (TM, fraction of migrated DNA multiplied by some measure of tail length) became a common descriptor along with tail length (µm) and percentage of DNA in the tail, tail DNA (TD%, relative percentage of DNA in the comet tail), were used as parameters of DNA damage.

RAPD- PCR for detecting damage in treated soybean DNA

Isolation of Genomic DNA

Isolation of genomic DNA from untreated and treated germinated soybean seeds with $nTiO_2$, Pb, and Pb adsorbate solutions were accomplished using the hexadecyl trimethyl ammonium bromide (CTAB) method as described in the study of Abdelhaliem and Al-Huqail (2016).

RAPD-PCR analysis and agarose gel electrophoresis

RAPD-PCR analysis and agarose gel electrophoresis were conducted as described in the study of Abdelhaliem and Al-Huqail (2016). A total of twenty random DNA oligonucleotide primers (10 mer) were independently used in the PCR reactions (UBC, University of British Columbia, Canada), with some modifications. Only four RAPD primers (P-12, 14, 15, and 19) succeeded to generate reproducible amplified DNA products.

Band scoring and data analysis

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Analysis of RAPD bands based on alterations in the number of amplified DNA bands, their size, intensity as well as loss or gain of DNA bands were performed by Bio One D++ software (Vilber Lourmat, France). Any change observed in RAPD profiles of treated soybean DNA was scored. The presence and absence of each DNA band was determined by making a binary matrix (1 if present and 0 if absent) for each sample. Polymorphic DNA bands (unique and non-unique) and monomorphic bands were also scored. DNA polymorphism in RAPD biomarker profiles included the loss of a normal band and the appearance of a new band compared with the control samples were evaluated.

Adsorption process

Preparation of $nTiO_2$ adsorbent

The powdered state (10 mg) of positive and stimulatory $nTiO_2$ dose (10 $mg.L^{-1}$) that called (optimal dose) was selected to be adsorbent dose based on its vigorous stimulatory action on phenotypical parameters (seedling growth) and fertility, germination and viability of soybean pollen grains. This was carried out for testing its adsorbent capacity to adsorb Pb onto its surface, which aimed to neutralize and minimize lead toxicity on soybean seeds.

Preparation of Pb adsorbate solutions

Adsorption of Pb onto the surface of powdered $nTiO_2$ was carried out from the solutions of three Pb doses (75, 100, and 150 $mg.L^{-1}$) which prepared and used previously.

Absorption process

Adsorption process was carried out according to (Pena et al., 2005) with some modifications. Three replicates of 10 mg powdered $nTiO_2$ was used as an adsorbent for each dose of Pb (75, 100, and 150 $mg.L^{-1}$) in separate 250 mL Pyrex glass Erlenmeyer flasks. The adjustment of the pH to 7 ± 0.1 in each flask was achieved by adding hydrochloric acid and sodium hydroxide at room temperature (21 - 25°C). Samples were then stirred using a magnetic stirrer to mix for 24 h. After that, the three flasks were shaken at 150 rpm in a reciprocating shaker and kept in dark at 25 ± 1°C and then they were stirred in a VORTEX-Gene™ for 10 s and incubated for 2 min at room temperature. Then, the mixture was centrifuged twice for 35 min at 14,000 rpm using a high-speed centrifuge (Hermle Z323, Germany) and the supernatant (the liquid phase) was collected and named ‘Pb adsorbate solution’.

Treating soybean seeds with three Pb adsorbate solutions

Under the laboratory conditions, a completely randomized design was conducted in triplicate to apply the three Pb adsorbate solutions on soybean seeds.

Twenty fresh, healthy uniformly sized and surface sterilized soybean seeds were placed in sterilized petri dishes and soaked in Pb adsorbate solutions separately for 48 h alongside untreated seeds (control) which were exposed only to distilled water. All solutions were added to the Petri dishes and run at the same time. The treatment of Pb adsorbate solutions in addition to both treatments of $nTiO_2$ and Pb were tested on soybean seeds at the same time alongside the control. All the same biomarkers and analyses used above for $nTiO_2$ and Pb treatments were applied to the seeds treated with three doses of Pb adsorbate solutions compared to that of the equivalent Pb doses before adsorption.

Statistical analyses

Each experiment in this study was carried out in triplicate. The data obtained were expressed as means ± standard deviation (SD) and statistically analyzed by using one-way ANOVA. The P-value level was set at 0.05.

RESULTS AND DISCUSSION

Nanosized Titanium Dioxide characterization

Figure 1 shows the TEM images of purified $nTiO_2$ dispersion in the applied three doses at ×100,000 magnification. TEM observation showed homogenous dispersion with fine particles at the 5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ doses but with appropriate large size without any agglomerations. This is in contrast to the 20 $mg.L^{-1}$ $nTiO_2$ dose which was observed to have high levels of agglomerations with non-homogeneous dispersion. The zeta potential of the $nTiO_2$ suspensions was determined and scored at -10.3, -10.8, and -5.01 mV for $nTiO_2$ doses 5, 10, and 20 $mg.L^{-1}$, respectively (Figure 1D-F). The variation in zeta potential may be attributed to the dispersion of the three $nTiO_2$ doses.

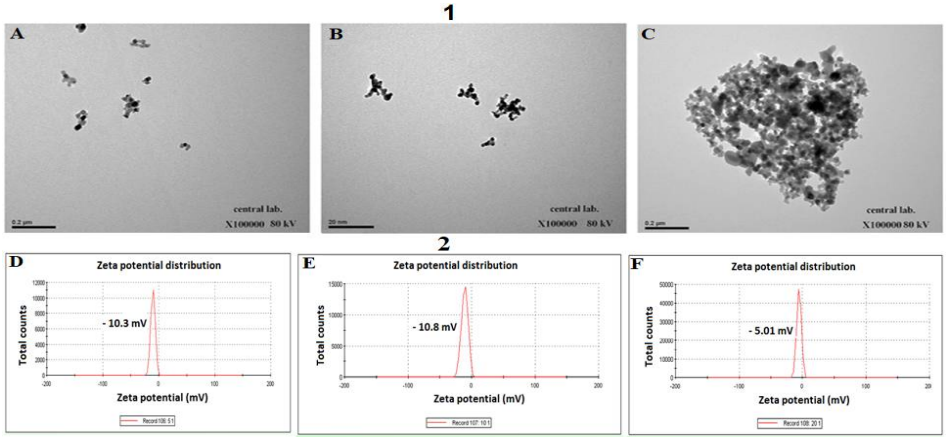

Figure 1. Characterization of TiO_2 nanoparticles (1A-C) typical transmission electron microscope images represent size distribution at magnification powers ×100,000; (2D-F) zeta potential characterization of nTiO2 in their applied doses (A) 5 $mg.L^{-1}$ (B) 10 $mg.L^{-1}$, and (C) 20 $mg.L^{-1}$.

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Phenotypic parameters of seedling growth

Soybean seedling growth parameters 35 days after planting untreated and treated soybean seeds following the three treatments ($nTiO_2$, Pb, and Pb adsorbate solutions) were estimated and used as parameters of seedling development (Table 1).

Table 1. Parameters of soybean seedling growth affected by $nTiO_2$, Pb and Pb adsorbate solutions 35 days after planting treated and untreated (control) germinated seeds.

Treatments	Dose mg.L^{-1}	Seedling growth parameters ±SD									
		Mean Lengths (cm)			Phytotoxicity %		Mean root /shoot ratios	Seedling vigor index (VI)	Tolerance index (TI)	Leaf parameters	
		Root	Shoot	Seedling	Root length	Shoot length				No of Leaves / plants	Leaf surface area /plant (cm^2)
Control	0.00	43.03±1.60	25.67±1.16	69.00±1.09	00.00	00.00	1.69 ±0.05*	7210.56	100	13.33±1.16*	14.40±0.10*
$nTiO_2$	5.00	43.68±2.08*	24.67±2.31*	68.35±2.20*	5.71±1.53	10.84±0.65	1.77 ±0.20*	6883.57	95.59	12.00±1.00*	13.00±1.00*
	10.00	47.67±3.06	27.67±0.57*	75.34±2.11	00.00	-00.14±0.15	1.67 ±0.06*	8243.69	108.73	16.67±1.53	14.63±0.15
	20.00	41.00±1.00*	22.00±2.65*	63.00±1.83*	11.50±1.08	17.51±1.21	1.86 ±0.23	5249.49	89.85	11.0±1.00*	12.90±0.03
	75.00	35.33±1.53*	19.33±1.35*	54.66±1.44*	23.74±1.40	30.14±1.77	1.83 ±0.07*	3794.50	75.70	10.33±0.58*	11.70±0.10*
	100.00	33.67±1.53*	17.67±1.53*	51.34±1.50*	27.32±1.10	36.10±0.52	1.91 ±0.11	3279.60	72.14	9.68±0.58*	9.23±0.06*
Pb	150.00	30.67±3.06*	15.83±0.76*	46.50±1.87*	44.28±0.88	54.79±1.47	1.94 ±0.15	2065.07	65.71	8.00±1.00*	5.70±0.05*
Pb adsorbate solutions	75.00	42.00±1.00*	25.50±2.52*	67.50±1.67*	9.35±1.15	10.84±1.67	1.70 ±0.21	6888.96	97.99	12.00±0.03	13.90±0.30*
	100.00	41.67±2.08*	22.33±1.53*	64.00±1.08*	12.23±1.23	19.29±2.35	1.82±0.17	6040.00	94.14	11.33±0.58*	13.63±0.55*
	150.00	39.00±2.65*	19.67±1.16*	58.67±1.91*	15.43±1.19	28.91±1.34	1.98 ±0.24	5400.25	88.57	9.68±0.58*	10.70±0.10*

*The mean difference is significant at the 0.05 level.

Seedling vigor (VI) I and tolerance index (TI) varied between the applied treatments, with each treatment having a specific effect. $nTiO_2$ 10 mg.L^{-1} dose showed a significant increase in (VI) I and highest TI, reaching a value of 8243.69 and 108.73% respectively when compared to that of untreated control which reached the value of 7210.56 for (VI) I and 100.00% for TI. However, (VI) I and TI decreased significantly in seeds treated with the highest Pb dose (150 mg.L^{-1}), which reached the values of 2065.07 and 65.71% respectively. After the adsorption process, the negative effects on (VI) I and TI were ameliorated and instead increased in the treatments of the three Pb adsorbate solutions over their values before adsorption. The maximum values of (VI) I and TI were 6888.96 and 97.99% respectively of 75 mg.L^{-1} Pb adsorbate solution compared to their values of the same Pb dose before adsorption compared to their values of the same Pb dose before adsorption (3794.5 and 75.70, respectively) (Table 1).

The 10 mg.L^{-1} $nTiO_2$ dose exhibited stimulatory effects, increasing the value of all leaf parameters based on mean number of leaves/plant and leaf surface area when compared to that of the untreated leaves and contrary to the other two $nTiO_2$ doses. However, the different Pb doses had significant adverse effects on leaf parameters based on dose dependency compared to that of the $nTiO_2$ doses. The three Pb adsorbate solutions showed good amelioration in all leaf parameters when compared to that of the equivalent doses of Pb before adsorption.

The percentage phytotoxicity of root and shoot lengths varied among the different treatments. The percentage phytotoxicity at optimal 10 mg.L^{-1} $nTiO_2$ dose reached 0.00% and -0.14±0.15%, for roots and shoots, respectively, while the lower and higher doses of $nTiO_2$ (5 mg.L^{-1} and 20 mg.L^{-1}, respectively) showed gradually increasing phototoxicity percentages compared to that of the untreated control (which showed no evidence of toxicity) (Table 1).

Meanwhile, the percentage phytotoxicity of shoot and root lengths significantly increased with the increasing Pb dose, with the maximum phytotoxicity values reaching 44.28 ± 0.88% and 54.79 ± 1.47%, respectively, for the higher Pb dose (150 mg.L^{-1}) compared with that of the untreated seedlings and that of the highest $nTiO_2$ dose. Most interestingly, the three doses of Pb adsorbate solution showed a significant reduction in phototoxicity percentage of root and shoot lengths reached the values of 9.35 ± 1.15% and 10.84 ± 1.67%, respectively, recorded at the 75 mg.L^{-1} Pb adsorbate solution treatment, which were 23.74 ± 1.40% and 30.14 ± 1.77%, respectively (Table 1).

The data obtained from seedling growth parameters indicate that three Pb adsorbate solutions increased the seedling growth parameters (mean seedling lengths, (VI) I, (TI) and leaf parameters) and significantly decreased phytotoxicity of Pb compared to equivalent Pb doses used before adsorption process (Table 1). The best amelioration and attenuation of Pb was at adsorbate solutions of 75 mg.L^{-1} Pb dose compared to equivalent of this dose before adsorption.

Table 2 and Figure 2 show the effect of three doses of $nTiO_2$; lead (Pb), Pb adsorbate solutions on soybean pollen grains (PGs) performance. The data obtained illustrated that values of absolute pollen grain viability (APV) were significantly increased at the optimal nTiO2 dose, much higher than control PGs (10 mg.L^{-1} $nTiO_2$, 55.48 ± 1.21%; control, 43.14 ± 1.50%).

Table 2. Pollen grains (PGs) performance generated from untreated and treated soybean seeds with $nTiO_2$, Pb and Pb adsorbate solutions.

			Pollen grains performance ±SD				
Treatments	**Dose mg.L^{-1}**	**Total No. PGs**	**% Morphological homogeneity (MH)**	**% Sterility**	**% non-viable PGs**	**% germinated pollen tube**	**Absolute pollen viability (APV)**
Control	0.00	1000	95.40± 0.02	2.80± 0.12	1.70± 0.25	45.93± 0.20	43.14±1.50
	5.00	1000	92.50± 0.22	12.75 ±0.30	1.60 ±0.45	27.23±1.34	23.76±2.4
$nTiO_2$	10.00	1000	97.50± 0.10*	0.10± 0.03*	0.00 ±0.50*	50.99± 0.80	55.48±1.21*
	20.00	1000	87.20± 0.15	21.41±0.21	2.10 ±0.10	16.28± 0.98	13.20±0.98
	75.00	1000	77.20 ± 0.08	50.48±0.01	4.80 ±1.30	8.34± 0.25	4.80±0.57
Pb	100.00	1000	41.70± 0.30*	69.74±0.11	5.60 ±0.33	5. 25± 0.55	2.52±1.72
	150.00	1000	22.60 ± 0.11*	83.07±1.21*	6.50 ±052*	2. 25± 0.55	0.89±2.10*
	75.00	1000	96.20± 0.10*	7.32±0.08	1.15 ±0.40	38.08± 0.11	39.67±1.45*
Pb adsorbate solutions	100.00	1000	82. 90± 0.14	18.32±0.08	2.40 ±0.25	24.08± 0.11	19.67±1.45
	150.00	1000	54.10± 1.20	32.62±0.12	3.70 ±1.30	18.37± 1.25	12.38±0.98

*The mean difference is significant at the 0.05 level.

The obtained results also showed that morphological homogeneity (MH%) of germinated pollen tube and germination index (GI) had the same trend of the above APV parameter which improved after exposure to 10 mg.L^{-1} $nTiO_2$ dose but were reduced by the other $nTiO_2$ doses (5 and 20 mg.L^{-1}); however, their effects were less than those of the Pb doses which were more toxic on these PGs parameters in a dose-dependent manner, as shown in (Table 2).

However, the sterility of pollen grains (PGs) was found to be dose-dependent as its increased as Pb doses increased, compared to that of untreated plants. The maximum value of PGs sterility was 83.07 ± 1.21% from the 150 mg.L^{-1} Pb dose, whereas the minimum value was 1.10 ± 0.03% from the 10 mg.L^{-1} $nTiO_2$ dose, which was less than the sterility value of untreated samples (2.80 ± 0.12%). This indicates that the 10 mg.L^{-1} $nTiO_2$ dose

was more effective in reducing the PGs sterility and increasing the fertility and viability of soybean PGs.

Meanwhile, the three Pb adsorbate solutions resulted in a significant reduction of PGs sterility, ameliorating the PGs viability, and increasing their fertility compared to that of the equivalent Pb doses before adsorption process (Table 2 and Figure 2).

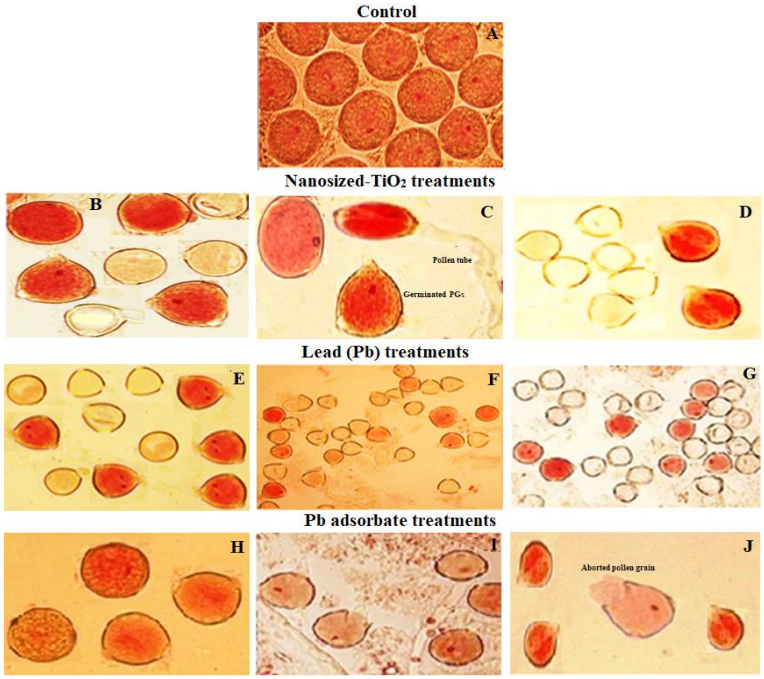

Figure 2. The most pronounced PGs parameters of soybean treated with $nTiO_2$, Pb, and Pb adsorbate solutions alongside control PGs. A) Fertile stained pollen grains of the control group; B) Fertile colored PGs and colorless empty sterile PGs at 5 $mg.L^{-1}$ nTiO2 dose; C) Full germinated pollen tube and fertile colored germinated PGs at 10 $mg.L^{-1}$ $nTiO_2$ dose; D) Fertile stained PGs and sterile empty PGs at 20 $mg.L^{-1}$ $nTiO_2$ dose; E) Small fertile stained PGs and sterile empty PGs at 75 $mg.L^{-1}$ Pb dose; F) Few small germinated pollen tube with numerous sterile empty PGs at 100 $mg.L^{-1}$ Pb dose; G) Few very small germinated pollen tube with numerous sterile empty PGs at 150 $mg.L^{-1}$ Pb dose, and; H-J) Variable range of fertile colored PGs treated with Pb adsorbate solutions based on the equivalent Pb dose prior to the adsorption process. Note: red PGs refer to fertile PGs while empty and colorless refer to sterile PGs.

Estimation of variations in nuclear DNA content and genome size by flow cytometry

A detailed description of the action of $nTiO_2$, Pb, and Pb adsorbate solutions were focused on nuclei isolated from soybean seeds to estimate the alteration of nDNA content and genome size of treated nuclei alongside untreated samples by flow cytometry (FCM) as well as genome size per the chromosome number of soybean (Table 3 and Figure 3). The obtained results showed an increase in nuclear DNA content of soybean after being treated with two $nTiO_2$ doses (5 and 10 $mg.L^{-1}$) which reached the value of 9.00 ± 0.012 and 9.62 ± 0.002 pg, respectively, when compared to that of the nuclei isolated from untreated soybean seeds (8.48 ± 0.006 pg). Meanwhile, the nuclear DNA content of the 20 $mg.L^{-1}$ $nTiO_2$ dose decreased relative to the control group and reached 8.14 ± 0.005 pg.

Table 3. Nuclear DNA content and genome size of nuclei isolated from untreated and treated soybean seeds with nanosized- TiO_2, Pb and Pb adsorbate solutions.

Treatments	Dose $mg.L^{-1}$	Mean 2C nDNA content (pg)	1C genome size (Mbp)	Chromosome number	Genome size/ Chromosome number
Control	0.00	8.48±0.006	8183.20		204.58
	5.00	9.00±0.012	8685.00		217.13
nTiO2	10.00	9.62±0.002	9283.30		232.09
	20.00	8.14±0.005	7855.10		196.38
	75.00	8.02±0.023	7739.30	2n = 40	193.49
Pb	100.00	7.48±0.008	7218.20		180.46
	150.00	7.24±0.040	6986.60		174.67
	75.00	9.23±0.004	8906.95		222.68
Pb adsorbate solutions	100.00	9.15±0.003	8829.75		220.75
	150.00	8.81±0.001	8501.65		212.54

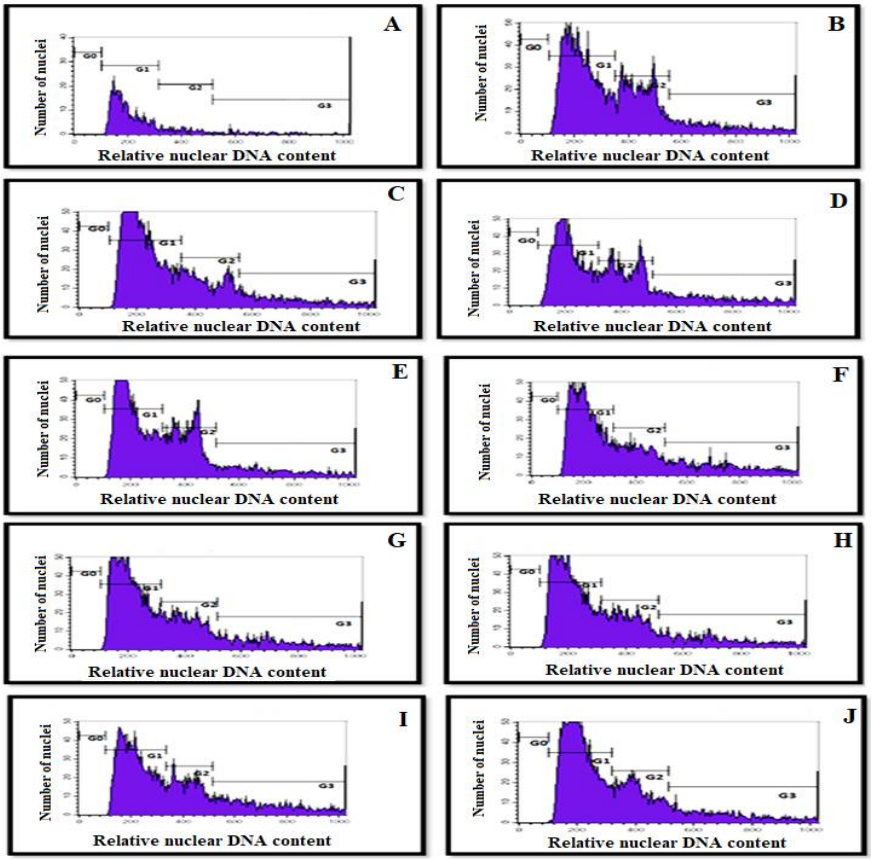

Figure 3. Flow cytometric analyses of nuclei isolated from untreated and treated soybean seeds which chopped and then stained with propidium iodide (A-J). 2C nDNA content (pg) estimated at Gap_1 (G_1) of interphase using peak position of the internal standard Human leucocytes (HLN) (2C nuclear DNA content 7.0 pg) as internal standard. (A) control, (B-D) $nTiO_2$ doses, (E-G) Pb doses , and (H-J) Pb adsorbate solutions, respectively.

In contrary, the nuclei of the Pb-treated seeds showed the maximum decrease in nuclear DNA content at the 150 $mg.L^{-1}$ Pb dose of 7.24 ± 0.040 pg compared to that of the untreated nuclei, which reached 8.48 ± 0.006 pg. Furthermore, soybean nuclei treated with one of the three Pb adsorbate solutions showed amelioration and improvement in nDNA content values. The nDNA content of the 75 and 100 $mg.L^{-1}$ Pb adsorbate solution reached the values of 9.23 ± 0.006 pg and 9.15 ± 0.003 pg, respectively, well over the value of that of the untreated nuclei of 8.98 ± 0.0067 pg and in comparison to the values of nDNA content at the equivalent Pb doses used before adsorption (8.02 ± 0.023 pg and 7.48 ±0.008 pg, respectively) (Table 3).

Based on the conversion of 1 pg = 965 Mbp, the genome sizes of treated soybean nuclei were estimated. Genome size ranged from maximum value of 9283.30 Mbp for the 10 $mg.L^{-1}$ $nTiO_2$ dose to a minimum value of 6986.60 Mbp at 150 $mg.L^{-1}$ Pb dose, relative to that of the nuclei from untreated seedlings of 8183.2 Mbp.

On the other hand, the Pb adsorbate from aqueous solution of 75 and 100 $mg.L^{-1}$ Pb doses showed an increase in genome size to 8906.95 Mpb and 8829.75 Mpb, respectively, to be larger than that of the untreated soybean of 8665.7 Mpb and of the equivalent Pb doses (7739.30 Mpb and 7218.20 Mpb, respectively) (Table 3 and Figure 3).

Single cell gel electrophoresis (SCGE) or comet assay for detection of DNA damage

Table 4 and Figure 4(A-J) show remarkable variation in the extent of DNA damage in soybean nuclei exposed to three treatments of $nTiO_2$, Pb, and Pb adsorbate solutions alongside the untreated one. This variation reflects the specific action of these treatments on nDNA.

Table 4. Extent of nuclear DNA damage identified by single-cell gel electrophoresis (SCGE) of nuclei isolated from untreated and treated soybean seeds with nanosized- TiO2, Pb and Pb adsorbate solutions.

Treatments	Dose $mg.L^{-1}$	Tail (damaged DNA) %	Head (undamaged DNA) %	Tail length (µm)	Tailed DNA %	Tail Moment Unit
Control	0.00	4	96	1.88	1.54	3.61
$nTiO_2$	5.00	9	91	2.66	2.51	6.68
	10.00	2	98	1.06	1.30	3.54
	20.00	11	89	3.11	3.16	10.83
Pb	75.00	15	85	5.73	5.57	31.92
	100.00	17	83	6.21	6.43	39.93
	150.00	20	80	6.89	6.41	44.17
Pb adsorbate solutions	75.00	5	95	2.11	2.30	4.81
	100.00	7	93	2.68	2.49	6.67
	150.00	8	92	2.88	2.71	7.80

The 10 $mg.L^{-1}$ $nTiO_2$ dose showed minimum DNA migration (Tailed 2%) with tail length of 1.06 µm, tailed DNA % of 1.30, and tail moment unit of 3.54 (Figure 4C), less than the DNA migration exhibited by the nuclei of untreated soybean which reached (Tailed 4%) with tail length of 1.88 µm, tailed DNA % of 1.54, and tail moment unit of 3.61 (Figure 4A). This indicates that this $nTiO_2$ dose could reduce nDNA damage and protect

DNA while the other $nTiO_2$ doses induced nDNA damage in a dose-dependent pattern (Table 4 and Figure 4B and D).

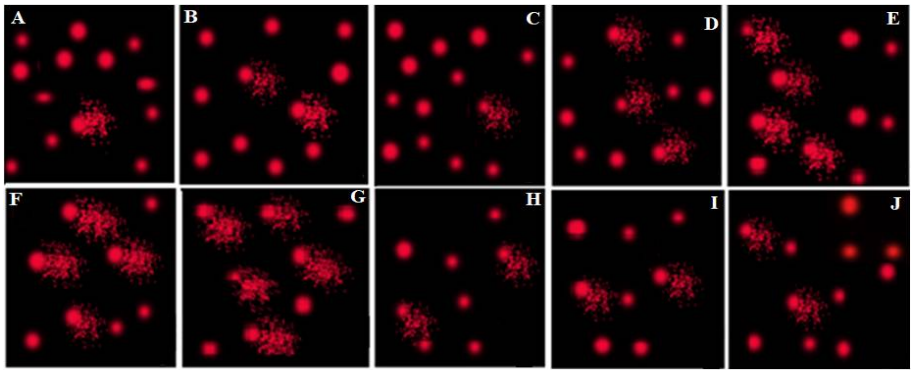

Figure 4. Comet images prepared by single cell gel electrophoresis (SCGE) show the variable extent of nuclear DNA damage in the nuclei isolated from treated and untreated soybean seeds. The images A-J represent control (A) $nTiO_2$ doses (B-D), Pb doses (E-G), and Pb adsorbate solutions (H-J), respectively.

On the contrary, the 150 $mg.L^{-1}$ Pb dose scored the maximum DNA migration (Tailed 20%) with tail length of 6.89 µm, tailed DNA % of 6.41 and tail moment unit of 44.17, indicating that the Pb treatment increase nDNA damage and was very toxic to the nDNA of soybean cells (Figure 4E-G) .

Furthermore, the data obtained in the present study demonstrated evidence of the reduction in nDNA damage by Pb adsorbate solutions based on equivalent Pb dose-dependent effect seen before adsorption. The clear amelioration of nDNA content was observed from the adsorbate 75 $mg.L^{-1}$ solution of Pb which scored DNA migration (Tailed 5%) with tail length of 2.11 µm, tailed DNA % of 2.30%, and tail moment unit of 4.81 (Figure 4H-J) compared to the equivalent Pb dose without adsorption which scored DNA migration (Tailed 15 %) with tail length, 5.73 µm; tailed DNA %, 5.57, and; tail moment unit, 31.92).

RAPD-PCR bioassay for detection of alterations and DNA damage

RAPD-PCR analysis was employed in the current study to evaluate the extent of the DNA alterations in soybean treated with the different doses of $nTiO_2$, Pb, and Pb adsorbate solutions alongside untreated samples. Each treatment in addition to each primer used exhibited distinctive quantitative and qualitative alterations in electrophoretic banding pattern of treated DNA compared with each other and with the untreated controls.

Twenty random primers were used for the RAPD analysis, in which only four primers (P-12, P-14, P-15, and P-19) succeeded to produce clear reproducible DNA bands and gave satisfactory results with many alterations in the RAPD profiles. The alterations in DNA banding pattern based on changes in number of amplified DNA bands, band sizes (bp), bands intensities, appearance of new bands (unique bands) and disappearance of some bands (polymorphic bands) are shown in (Table 5) and (Figure 5).

In total, three hundred and fifty-six (356) reproducible DNA bands were scored after using the four primers (with an average of 89.00 bands/primer), resulting in a

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reproducible set of DNA bands with variable sizes that were specific for each primer on gel electrophoresis. Of these, 189 polymorphic bands were obtained with value of 53.09%, out of which 143 bands were unique (40.17%) and 46 bands non-unique (12.92%) while monomorphic bands were absent. The total values of polymorphism generated by four primers were 100% because of the absence the monomorphic bands for these primers. On the other hand, the highest number of gene products (115 bands; 32.31%) were generated by primer-12 while the lower number of 70 bands (19.66%) were generated by primer-15 (Table 5).

Table 5. RAPD-PCR amplification products of DNA isolated from soybean seeds treated with nTiO2, Pb, and Pb adsorbate solutions using four random primers. Lane 1, control; lanes 2-4, nanosized TiO_2 doses (5, 10, and 20 $mg.L^{-1}$, respectively); 5-7 Pb doses (75, 100, and 150 $mg.L^{-1}$, respectively), and; (8-10) three Pb adsorbate solutions.

			Total number of scorable bands in each Lane										Total bands		Unique		Non - Unique		Polymorphic bands		
Primer code	Primers sequences (5′ → 3′)	Amplicon Lengths (bp)	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	No	%	No	%	No	%	Polymorphic No	Polymorphic %	Polymorphism%
12	CAC CGT ATC C	950-200	14	12	14	13	11	9	7	13	11	11	115	32.31	51	44.35	13	11.30	64	55.65	100
14	CTT CCC CAA G	983-274	12	10	8	9	8	7	8	9	10	7	88	24.72	26	29.55	9	10.23	35	39.77	100
15	CAT CCG TGC T	885-075	7	5	13	5	7	6	5	8	6	8	70	19.66	37	52.86	8	11.43	45	64.29	100
19	CTG GGG ACT T	900-100	8	10	11	9	7	9	6	8	8	7	83	23.31	29	34.94	16	19.28	45	54.23	100
Overall total			41	37	46	36	33	31	26	38	35	33	356	100	143	40.17	46	12.92	189	53.09	100
% of total bands in each Lane			11.52	10.39	12.93	10.11	9.27	8.71	7.30	10.67	9.83	9.27									

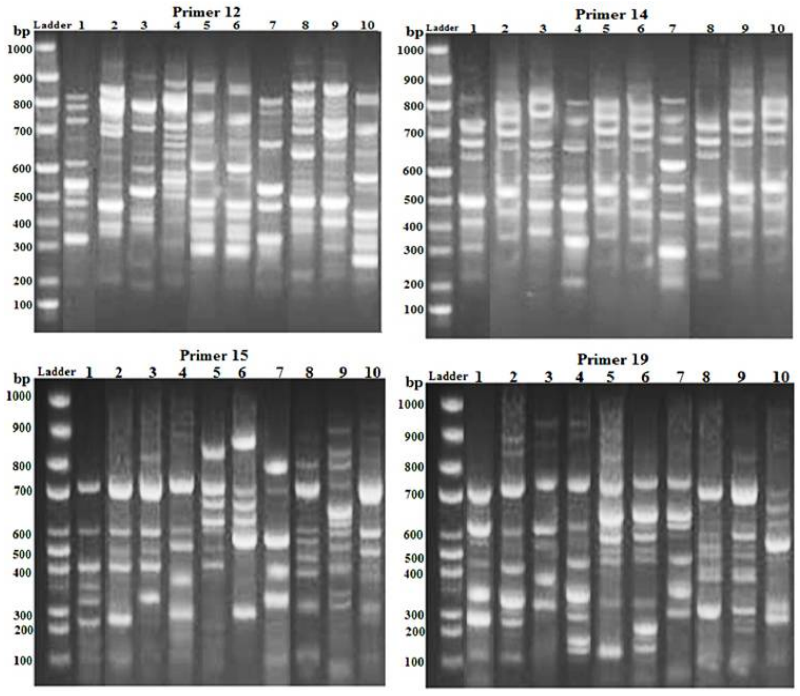

Figure 5. RAPD profiles of genomic DNA of soybean generated by four random decamer primers. The samples 1-10 represent control (1) nTiO2 doses (2-4), Pb doses (5-7), and Pb adsorbate solutions (8-10), respectively.

Furthermore, the maximum number of gene products (46 bands; 12.93%) was observed in the soybean treated with the 10 mg.L^{-1} nTiO2 dose while the minimum number of bands (26; 7.30%) was at the 150 mg.L^{-1} Pb dose compared to the number of gene products of the untreated samples which reached 41 bands (11.52%), as shown in (Table 5).

The results of RAPD analysis were performed by considering the bands that appear in the control sample as the criterion of the judgment. A RAPD polymorphism included disappearance of a control band and appearance of a new one following treatment.

The three Pb adsorbate solutions showed an increase in the number of amplified DNA bands based on equivalent Pb dose-dependent effects. It reached 38 DNA bands (10.67%) at the adsorbate 75 mg.L^{-1} solution of Pb compared to the equivalent Pb dose without adsorption which scored 33 DNA bands (9.27%) (Table 5 and Figure 5).

DISCUSSION

The results obtained in this study demonstrated that all parameters of soybean seedling growth were significantly stimulated and improved upon exposure to 10 mg.L^{-1} $nTiO_2$ dose being higher than those of the untreated samples. Therefore, this dose was considered as optimal, while the two $nTiO_2$ doses higher and lower than this dose induced a variable reduction in these growth parameters compared to that of the optimal dose. The significant effect of $nTiO_2$ on soybean seedling growth parameters may be attributed to their small particle size and their degree of homogeneous dispersion, which may influence their speed of penetration into the seed coat.

The positive and promoting action of the optimal $nTiO_2$ dose (10 mg.L^{-1}) on growth of soybean seeds may due to the creation pores in seed coats during its penetration and consequently, enhancing the absorbance and uptake of water into seeds and cells or may be due to increasing some important enzymes such as nitrate reductase in the soybean seeds or by stimulating transcription of plant genes responsible for the accumulation of water channel proteins (aquaporins) in soybean tissues (Marchiol et al., 2016). Additionally, interpretation the promoting action of this optimal dose may be due to promotion of the antioxidant system in soybean cells causing increased strength and resistance to oxidative stress by reducing ROS which may hasten seed germination and improve early seedling growth (Dolatabadi et al., 2016). In this respect also, Mittler (2017) reported that $nTiO_2$ at certain doses can regulate and promote plant growth and enhance its tolerance by modulating ROS-dependent signalling pathway(s), which is dependent upon enhancing activities of enzymatic antioxidants and consequently, reducing oxidative damages of DNA.

On the other hand, the toxic action of $nTiO_2$ at high dose (20 mg.L^{-1}) on soybean seedling growth may be interpreted based on accumulation of TiO_2 nanoparticles on external surfaces of soybean seeds causing clogging of the pores which may interrupt the uptake of water though the seed coat and leading to adverse and negative action, such as increasing cellular oxidative stress, accumulation of harmful reactive oxygen species (ROS), and reduction of the antioxidant defense system which lead to significant damage to membranes and cellular macromolecules (such as DNA and proteins) and consequently, disturbance of several metabolic cycles during early seedling growth (Dolatabadi et al., 2016). In this respect, the results match the study of Ruffini-Castiglione et al. (2011) which showed reduction and alteration in development growth of *Zea mays* L. and *Vicia narbonensis* L. after treatment with higher $nTiO_2$ concentrations.

Meanwhile, the three Pb doses had adverse and toxic effects on soybean seeds by the reduction of seedling growth parameters, even in small doses. This may be due to the ability of Pb to produce oxidative stress by increasing ROS in the soybean cells that affects plant metabolic processes leading to inhibition of important enzymes required for cell division and seedling growth, as explained by Pourrut et al. (2011).

Pollen germination and growth of pollen tubes are, in principle, necessary for fertilization and seed formation in flowering plants, and good fruit set, and high crop yield depend on healthy pollen grains (Abdelhaliem et al., 2013). Pollen staining tests are among the most reliable and widely used pollen viability tests; however, in some cases pollen grain germination tests are necessary to observe the realized viability of pollen grains. In the current study, the cytogenetic investigations indicated that soybean pollen grain performance showed a significant stimulation and increase at the optimal $nTiO_2$ dose (10 $mg.L^{-1}$) in comparison to that of the untreated samples, while the other two $nTiO_2$ doses showed gradual reduction depending on dose when compared to that of the untreated sample. This study interpreted induction and increasing of viability, fertility and germination of soybean pollen grains by the optimal $nTiO_2$ dose may be due to the antioxidant defense system of pollen grains which protects their DNA from oxidative damage by ROS without too much damage. This indicates the potential use of this dose in crop yield improvement. On the contrary, the remained $nTiO_2$ doses especially high dose induced genotoxic and oxidative action in soybean PGs. These actions may be due to the induction of oxidative damage in these PGs by production of the free radical oxygen that lead to higher frequency of chromosomal aberrations and DNA damage which in turn can affect the vigor, pollen grains fertility and likely to persist in seeds yield or even longer due to the accumulative genotoxicity, as explained by Abdelhaliem et al. (2013).

On the other hand, the three Pb doses showed a strong reduction in soybean PGs performance in dose dependent manner. The high Pb dose showed inhibition of pollen germination and pollen tube growth leading to the highest percentage of sterility of pollen grains. This may be due to the induction of high ROS in pollen mother cells which may cause induction of structural changes in the DNA, such as chromosomal rearrangement, strand breaks, base deletions, pyrimidine dimers, cross-links and base modifications, mutations, and other genotoxic effects, in accordance with (Pourrut et al., 2011). The reduction of pollen grain germination and inhibited viability may be due to decreased effectiveness of pollination and fertilization, consequently affecting the quantity and quality of seed set.

The current study showed remarkable variations in the extent of DNA damage experience by the nuclei of soybeans treated with $nTiO_2$ and Pb. The increase in nuclear DNA content and genome size as well as minimum DNA migration or DNA damage scored at the optimal $nTiO_2$ dose as illustrated by flow cytometry and comet assay, respectively, may be due to increased antioxidant activity of soybean cells by this dose leading to the scavenging of free radicals, providing nDNA with adequate resistance and protection against oxidative damage.

On the contrary, the three Pb doses resulted in an obvious reduction in nuclear DNA content and genome size and induced an increase in migration and damage of soybean DNA when comparison to that of the untreated samples. This may be due to the ROS generated by these toxic treatments attacking DNA and producing lesions via base deletion, pyrimidine dimers, cross-links, DNA strand breaks, chain breaks, modification of

carbohydrate parts and nitrogenous bases by oxidation (Nas and Ali, 2018). Therefore, Pb could induce different types of DNA damage in exposed nuclei which might be the cause for the arrest at the G2/M checkpoint. On the other hand, the Pb treatments, especially the highest dose may increase chromosomal sticky which led to chromosomal and DNA damage and consequently, a reduction in nuclear DNA content which resulted in unequal distribution of the DNA in the daughter cells. This DNA damage may influence the expression of several genes and, consequently, the synthesis of specific proteins that control many metabolic processes in soybean cells such as plant development, cell cycle, pollen grains fertility, fertilization, and seed formation.

The RAPD-PCR used in this study scored a wide range of DNA damage and specific-level qualitative and quantitative alterations in amplified DNA profiles. Different sizes of DNA bands may be interpreted as score separated loci based on the presence (amplification) and absence (non-amplification) of DNA segments by RAPD-PCR. The highest number of amplified DNA bands (45) generated by four random RAPD primers scored at optimal $nTiO_2$ dose compared to 41 DNA bands for untreated DNA. The increase in the number of amplified DNA bands (genes) at this dose may be attributed to gene expression of some genes for synthesis of proteins such as water channel proteins (aquaporins) that essential for cell division, plasma membrane formation, and promotion of water transport leading to activation the cellular metabolic processes (Lyu et al., 2017). The increase may also be explained on the basis that this optimal $nTiO_2$ dose likely caused a large addition of nitrogenous bases or insertion of the amplified regions at the genomic level.

Alterations the characteristic DNA banding pattern generated by RAPD analysis observed at the highest dose of $nTiO_2$ and three Pb doses may be explained based on the biological way by which each of these treatments interacts with DNA or by the production of ROS induced genotoxic DNA damage. This ROS may induce structural changes in the DNA, which may reflect the structure of the genomic DNA due to altering the distance between two annealing sites and deleting an existing site or as a reflection of the variation in gene expression (Dhakshanamoorthy et al., 2011). This study observed that the characteristic changes in the RAPD profiles ranged from the gain of DNA bands (unique DNA band), which may be due to addition or insertions and transposition of some genes induced by the treatments, to loss of DNA bands (polymorphic DNA band) and intensities of DNA bands which may be due to large deletions of amplified DNA regions or breaks in double-strand of the DNA molecule or a single base change in the genomic DNA which was induced by other treatments (Dhakshanamoorthy et al., 2011).

Data obtained in this study illustrated that the application of Pb adsorbate solutions on soybean seeds induced a significant increase and improvement of seedling growth parameter and alleviation of phytotoxic action of Pb on pollen grains and DNA, resulting in amelioration and increasing the viability and fertility of PGs and protection of DNA from oxidative damage comparable to that of treated soybean seeds with three Pb doses used before adsorption process. Consequently, when Pb adsorbate solutions were applied to soybean seeds, this may have induced the capacity of antioxidant

defense system in soybean cells, reducing free radicals and the subsequent genotoxic action of Pb.

CONCLUSIONS

In conclusion, we demonstrated that the optimal dose (10 $mg.L^{-1}$) of $nTiO_2$ induced positive and stimulatory actions on soybean cells as evaluated by bioassays used while other doses induced negative actions, especially at the highest doses. Thus, the study suggests that this optimal $nTiO_2$ dose should be utilized to develop ecofriendly and effective 'nano-fertilizers'.

The data obtained from phenotypic parameters, cytogenetic test of PGs, and DNA bioassays (flow cytometry, comet assay, and RAPD-PCR) indicated that powdered state (10 mg) of optimal $nTiO_2$ dose exhibited a good adsorbent affinity for adsorbing Pb onto its surface, leading to mitigation of Pb and consequently, increased vitality and tolerance resistance of soybean plant to Pb toxicity and improved the viability and fertility of PGs and protected DNA from damage when applied to soybean. This will open an avenue for using $nTiO_2$ to protect crops from heavy metal toxicity.

To the best of our knowledge, this type of study has not been conducted until now. We recommend developing an efficient $nTiO_2$ adsorbent for Pb or any heavy metal as a widely applicable means in agriculture for the protection of economically important crop plants, increasing the capacity of seeds to express their vital functions, and improving their yields, particularly after selecting optimal and adequate doses of both adsorbent and adsorbate necessary for success in the adsorption process.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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