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RAPD Analysis of Genotoxic Effects of Nano–Scale SiO_2 and TiO_2 on Broad Bean (*Vicia Faba* L.)

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Growing use of oxide nanoparticles has driven their access to the natural environment, including biological interactions within the ecosystem, despite a lack of knowledge about their genotoxic potential on exposed plant tissue. In this study, seeds of broad beans (*Vicia faba* L.), one of the major carbohydrate food sources as well as an ecotoxicological model plant, were treated with three concentrations (25, 50 and 75 mg/L) of two different types of nanoscale materials (n–), n–SiO_2 and n–TiO_2, to assess their safe use. Seeds were soaked in n–SiO_2 and n–TiO_2 each for 24 h, then germinated in peat moss for two weeks. DNA was isolated from leaves for RAPD (Random Amplified Polymorphic DNA) profile analyses. Results revealed a concentration dependent genotoxic effect for n–SiO_2 (however it seems to maintain genetic material stability) and high genotoxic effect for n–TiO_2.

Key words: broad bean, genotoxicity, GTS, Nano–scale materials, RAPD

INTRODUCTION

Nanoscale materials grew rapidly and have been used profusely not only in technological products but also in products for daily use, which stimulates some apprehension especially their influence on directly affected organisms. These materials can exert genotoxicity by direct and indirect mechanisms (Mehrian and Lima, 2016) showing a mutagenic effect upon eukaryotic (plant, animal and human) cells despite on–going debate on their toxic action (Golbamaki *et al*., 2015). Since plants serve as producers, forming the basal, most critical trophic level of the food chain in the ecosystem, understanding how nanoscale materials affect them has become an urgent necessity. Due to the small size, large surface area and ability to generate reactive oxygen species (ROS) (Tumburu *et al*., 2014; Elespuru *et al*., 2018), nanomaterials could manipulate DNA resulting in different types of chromosomal aberrations (Kumari *et al*., 2009; Singh *et al*., 2009; Galal and El–Samahy, 2012). Nano–SiO_2 (n–SiO_2) and nano–TiO_2 (n–TiO_2) affect plant genetic material negatively depending on concentration, particle size and structure (Castiglione *et al*., 2016; Khan and Ansari, 2018; Thabet *et al*., 2019).

RAPD (Random Amplified Polymorphic DNA) is a PCR (Polymerase Chain Reaction) based technique and a relatively quick, easy, efficient, reliable and sensitive method that can identify a wide range of damaged DNA and genetic mutations. Therefore, RAPD can be applied to studies of genotoxicity by detecting differences in genomic materials if they occur in primer specific DNA sequences (Williams *et al*., 1990; Atienzar and Jha, 2006; Cenkci *et al*., 2009; Aboulila and Galal, 2019). Efficient RAPD analysis also depends on the purity of the template DNA (Sharma *et al*., 2010). Here, to evaluate the genetic effects of n–SiO_2 and n–TiO_2, RAPD analysis was performed to detect DNA variations induced in *V. faba* cells after treatment with different concentrations (25, 50 and 75 mg/L) compared to untreated control.

MATERIALS AND METHODS

Nano–scale materials

Nano–scale silicon dioxide (n–SiO_2) and titanium dioxide (n–TiO_2) (anatase) were purchased from Nanotech Egypt Co., Egypt. For visualization purposes, osmium coating was applied to these nano–scale materials and observations were made under a scanning electron microscope (SEM) (SU8000, Hitachi Hitechnologies, Japan) at the Center of Advanced Instrumental Analysis, Kyushu University, Japan. The particle sizes measured of n–SiO_2 and n–TiO_2 were 119.1 ± 2.8 and 283.6 ± 15.9nm, respectively (mean ± SD, n = 10 for each). Both n–SiO_2 and n–TiO_2 were dissolved in double distilled water by sonication for 30min before being used to make concentrations of 25, 50 and 75mg/L.

Preparation of plant samples

Vicia faba seeds were grown following Thabet *et al*. (2019). Briefly, seeds were surface sterilized with 2.5% sodium hypochlorite (NaOCl), then washed by distilled water three to four times followed by immersing in distilled water for 6 h. The seeds were then soaked for 24 h in n–SiO_2 and n–TiO_2, at three concentrations (25, 50 and 75 mg/L) each as well as in distilled water as a control (0 mg/L). After the treatment, the seeds were thor-

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oughly washed with distilled water. Ten seeds per replication (three replications per treatment) were allowed to grow in pots supplied with peat moss.

DNA extraction and RAPD–PCR

Individual plant samples were collected from each treatment and mixed to form a combined sample. Total genomic DNA (4–6 μg) was extracted from young healthy leaves by using the DNeasy Plant Mini Kit (QIAGEN GmbH, Germany). Polymerase chain reaction (PCR) was performed using 1 μl of the extracted genomic DNA in a 10 μl reaction mixture containing 5 μl 2X PCR Master mix solution [(i–TaqTM) iNtRON Biotechnology, Shanghai, China], 1 μl (20 μM) of one of 14 decamer arbitrary random primers (Bio Basic Inc., Canada) (Table 1), and made up to 10 μl with sterile ddH2O. The PCR amplification was performed according to Williams *et al*. (1990) in a thermal cycler (Perkin–Elmer, GeneAmp 2400, USA). PCR amplified products were differentiated on 1.5% agarose gel against a known DNA molecular marker: L1 (O'GeneRuler DNA Ladder Mix, Carlsbad, California, USA) or L2 (1Kb plus DNA ladder, TIANGEN, Taiwan).

Table 1. The nucleotide sequences of the primers used for RAPD analysis

Primer name	Sequence (5´→3´)
OPA–09	GGGTAACGC
OPA–20	GTTGCGATCC
OPB–01	GTTTCGCTCC
OPB–05	TGCGCCCTTC
OPB–06	TGCTCTGCCC
OPB–07	GGTGACGCAG
OPB–08	GTCCACACGG
OPB–11	GTAGACCCGT
OPB–12	CCTTGACGCA
OPB–14	TCCGCTCTGG
OPH–01	GGTCGGAGAA
OPH–03	AGACGTCCAC
OPH–04	GGAAGTCGCC
OPH-05	AGTCGTCCCC

Estimation of total polymorphism and genomic template stability

DNA polymorphism was analyzed for each treatment as the percentage of polymorphic bands compared to control and genomic template stability (GTS) was calculated as follows:

$$GTS\ (\%) = (1 - \alpha/n) \times 100$$

where α is the number of polymorphic bands detected in each treatment and n is the number of total bands detected in the control (Cenkci *et al*., 2010; Qari, 2010). By definition, GTS is the highest in the control (= 1). Polymorphism observed in the RAPD profile included disappearance of a band(s) and appearance of a new band(s) in each treatment compared to the control profile (Atienzar *et al*., 2000; Luceri *et al*., 2000).

Estimation of band sharing index

Band sharing index (BSI) indicates resemblance between two samples using the following equation (Savva, 2000):

$$BSI = 2S / (A + B)$$

where S is the number of shared bands between two samples, A is the number of bands in the control and B the number of bands in the respective samples.

Estimation of primer polymorphism and polymorphic information content

The polymorphic information content was calculated as

$$PIC = 2fi \times (1 - fi)$$

where PIC is the polymorphic information content of the primer and fi is the frequency of band present (Roldan–Ruiz *et al*., 2000; Aboulila *et al*., 2019).

RESULTS

Total polymorphism

The number of amplified bands (a total of 113) in untreated control ranged from 4 (OPB–05) to 13 (OPA–09) bands (Fig. 1, Table 2). Also, the lowest number of polymorphic bands (17) detected at 25 mg/L n–SiO_2 recorded the lowest polymorphism (15.04%), whereas the highest number (47) detected at 25 and 50 mg/L n–TiO_2 corresponded to the highest polymorphism (41.59%). Nano–TiO_2 treatments generated higher numbers of new polymorphic bands whilst n–SiO_2 recorded noticeably the highest number of disappeared bands (33) at 75 mg/L compared to other treatments. These changes in band number illustrate that n–SiO_2 showed dose dependent increased polymorphism whereas n–TiO_2 showed equal (higher than other treatments) polymorphism at 25 and 50 mg/L.

Genomic template stability

Presence and absence of bands in a given sample was used to estimate GTS, as a qualitative measurement of DNA alterations in RAPD profile. With increasing n–SiO_2 concentration a decrease in *V. faba* GTS was observed, however n–TiO_2 at 25 and 50 mg/L recorded an equal effect on the genome stability (58.41%) (Fig. 2). This indicates that *V. faba* genome was more stable for n–SiO_2 than for n–TiO_2. Furthermore, n–SiO_2 increased numbers of both intensity–changed bands. It was noticeable that n–SiO_2 at 75 mg/L decreased both band numbers and intensity when the common event arising in the DNA patterns treated by n–SiO_2 was low intensity bands (Fig. 3).

Band sharing index

The band sharing index illustrates similarity among samples; here the first sample is the control. The high-

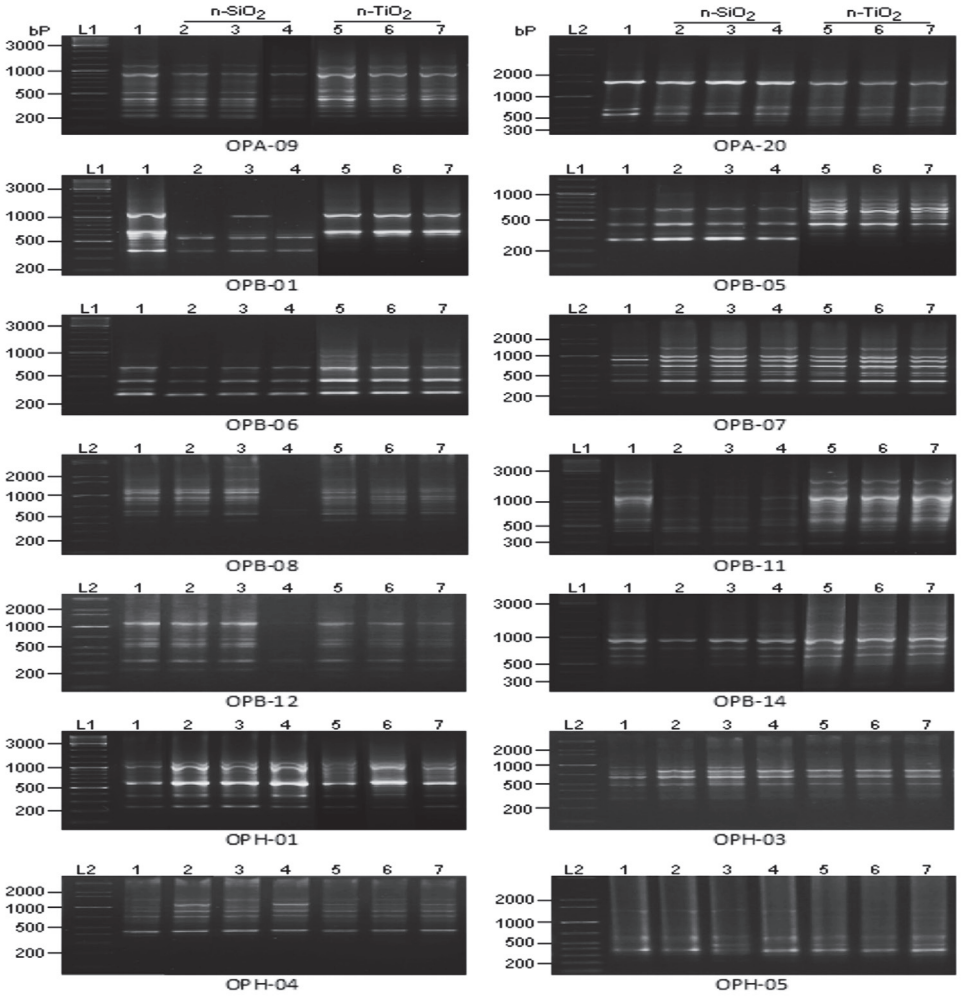

Fig. 1. Amplification pattern of *Vicia faba* DNA with 14 RAPD primers. L1 and L2: reference DNA ladders (molecular markers). Lane 1: control, lanes 2, 3 and 4: treated with n–SiO_2 (25, 50 and 75 mg/L, respectively), and lanes 5, 6 and 7: treated with n–TiO_2 (25, 50 and 75 mg/L, respectively).

Table 2. Number of polymorphic bands and polymorphism percentage deduced by RAPD profiles of *Vicia faba* after treatment with different concentrations of n–SiO_2 and n–TiO_2

Primer	Control bands	n–SiO_2						n–TiO_2					
		25 mg/L		50 mg/L		75 mg/L		25 mg/L		50 mg/L		75 mg/L	
		a	b	a	b	a	b	a	b	a	b	a	b
OPA–09	13	0	2	1	2	1	4	3	4	3	4	3	3
OPA–20	7	2	0	2	2	0	0	2	0	2	0	2	0
OPB–01	7	0	4	0	2	0	3	1	1	1	1	1	1
OPB–05	4	0	0	1	0	0	0	11	0	11	0	11	0
OPB–06	7	0	3	0	1	0	3	2	1	2	1	2	1
OPB–07	9	1	0	1	0	1	0	1	0	1	0	1	0
OPB–08	11	0	0	2	0	0	9	2	1	3	1	2	2
OPB–11	10	1	1	1	1	3	3	4	0	3	0	3	0
OPB–12	9	0	0	0	0	0	6	2	1	2	0	1	1
OPB–14	7	0	1	0	0	0	1	3	0	3	0	3	0
OPH–01	8	1	0	1	1	1	2	1	2	2	2	1	1
OPH–03	6	0	0	0	0	0	1	0	2	0	2	0	2
OPH–04	7	1	0	1	0	1	0	1	0	1	0	1	0
OPH–05	8	0	0	1	1	0	1	1	1	1	1	1	1
Total	113	6	11	11	10	7	33	34	13	35	12	32	12
Polymorphic bands (a+b)		17		21		40		47		47		44	
Polymorphism (%)		**15.04**		**18.58**		**35.40**		**41.59**		**41.59**		**38.94**	

a: appearance of new band(s) compared to the control, b: disappearance of band(s) compared to the control.

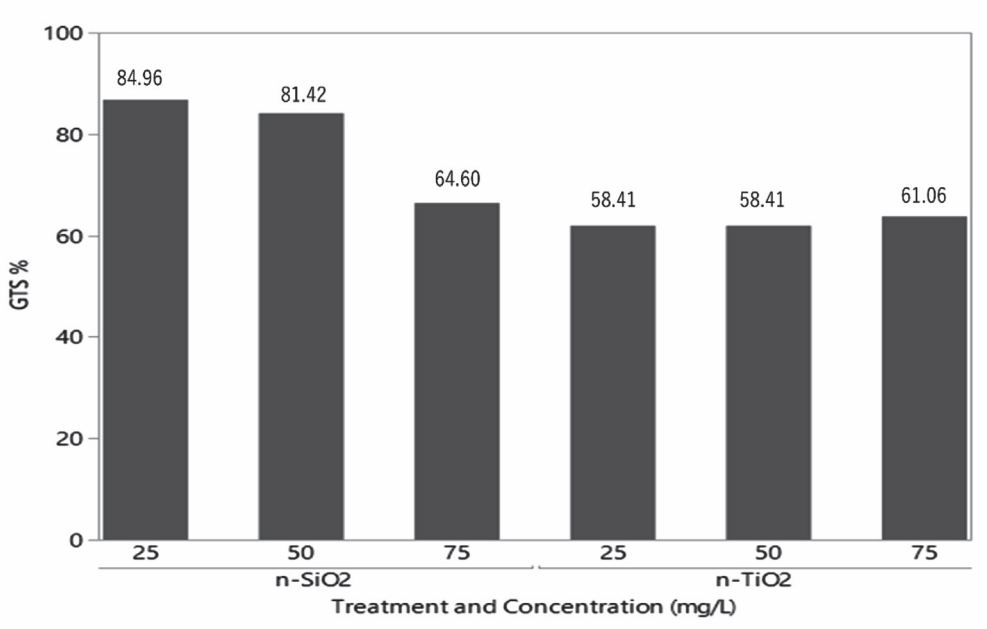

Fig. 2. Genomic template stabilities (GTS) in *Vicia faba* plants after treatment with different concentrations of n–SiO_2 and n–TiO_2.

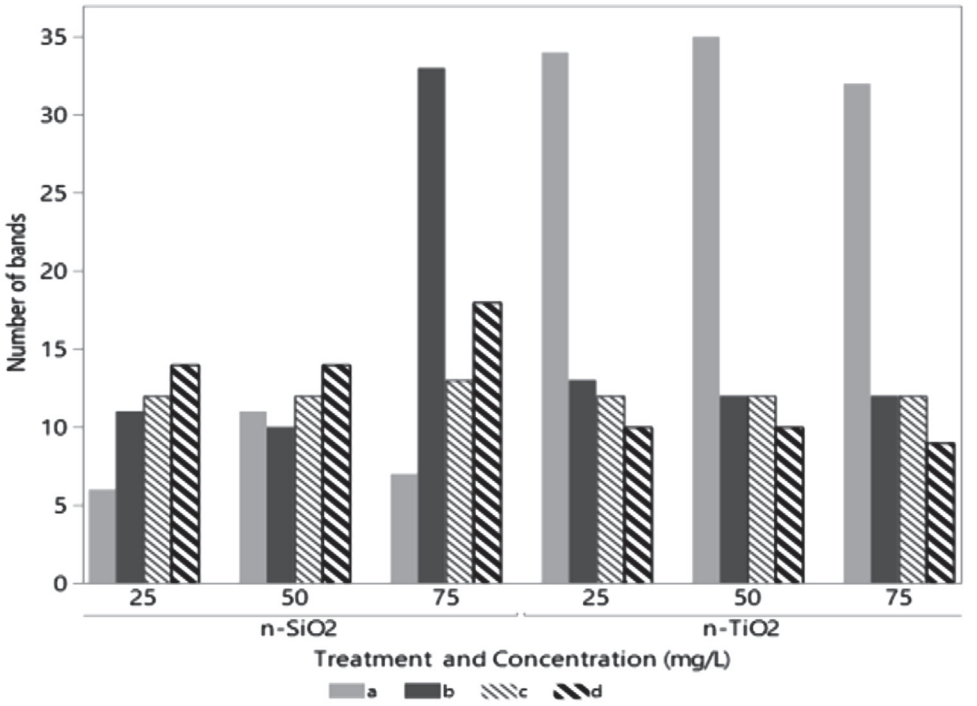

Fig. 3. Changes in DNA–RAPD profile of *Vicia faba* treated with different concentrations of n–SiO_2 and n–TiO_2; a: appearance of new bands, b: disappearance of bands, c: increase in band intensity, d: decrease in band intensity.

est BSI value (0.92) was recorded at 25 mg/L n–SiO_2 (Table 3). Nano–SiO_2 maintained the similarity of bands whilst n–TiO_2 recorded lower values. Nano–SiO_2 showed a concentration dependent decreasing effect on BSI value. Generally, n–SiO_2 exhibited an alleviated effect compared to n–TiO_2.

Primer polymorphism and PIC value

Eight out of 14 primers used as RAPD markers (OPA–09, OPB–01, OPB–05, OPB–06, OPB–08, OPB–11, OPB–12 and OPH–01) can be selected for future studies based on the high polymorphism (> 50%) and PIC (> 0.20) values. Results showed that 158 bands were amplified with an average of 11.29 bands per primer, 53.80% of them were polymorphic. The OPB–08 primer induced the highest polymorphism (87.50%), whereas the OPH–04 induced the lowest polymorphism (12.50%). The PIC values ranged from 0.02 (OPB–07) to 0.36 (OPB–05) with an average value of 0.20 (Table 4).

Table 3. Band sharing indices (BSI) in *Vicia faba* DNA after treatment with different concentrations of n–SiO_2 and n–TiO_2

Primer	*A*	n–SiO_2									n–TiO_2								
		25 mg/L			50 mg/L			75 mg/L			25 mg/L			50 mg/L			75 mg/L		
		S	*B*	*A+B*	*S*	*B*	*A+B*	*S*	*B*	*A+B*	*S*	*B*	*A+B*	*S*	*B*	*A+B*	*S*	*B*	*A+B*
OPA–09	13	11	11	24	11	12	25	9	10	23	9	12	25	9	12	25	10	13	26
OPA–20	7	7	9	16	5	8	15	7	8	15	7	9	16	7	9	16	7	9	16
OPB–01	7	3	3	10	5	5	12	4	4	11	6	7	14	6	7	14	6	7	14
OPB–05	4	4	4	8	4	5	9	4	4	8	4	15	19	4	15	19	4	15	19
OPB–06	7	4	4	11	6	6	13	4	4	11	6	8	15	6	8	15	6	8	15
OPB–07	9	9	10	19	9	10	19	9	10	19	9	10	19	9	10	19	9	10	19
OPB–08	11	11	11	22	11	13	24	2	2	13	10	12	23	10	13	24	9	11	22
OPB–11	10	9	10	20	9	10	20	7	10	20	9	13	23	10	13	23	10	13	23
OPB–12	9	9	9	18	9	9	18	3	3	12	8	10	19	9	11	20	8	9	18
OPB–14	7	6	6	13	7	7	14	6	6	13	7	10	17	7	10	17	7	10	17
OPH–01	8	8	9	17	7	8	16	6	7	15	6	7	15	6	9	17	7	8	16
OPH–03	6	6	6	12	6	6	12	5	5	11	4	4	10	4	4	10	4	4	10
OPH–04	7	7	8	15	7	8	15	7	8	15	7	8	15	7	8	15	7	8	15
OPH–05	8	8	8	16	7	8	16	7	7	15	7	8	16	7	8	16	7	8	16
Total	**113**	**102**	**108**	**221**	**103**	**115**	**228**	**80**	**88**	**201**	**99**	**133**	**246**	**101**	**137**	**250**	**101**	**133**	**246**
BSI			0.92			0.90			0.80			0.80			0.81			0.82	

A, number of bands in control. *S*, number of shared bands between control and treatment. *B*, number of bands in treatment.

Table 4. Number of total amplified bands, polymorphic bands, percentage of polymorphism and polymorphic information content (PIC) values of the primers used for RAPD profile

Primer name	Number of bands		Polymorphism (%)	PIC value
	Total	Polymorphic		
OPA-09	17	10	58.82	0.27
OPA-20	11	5	45.45	0.15
OPB-01	8	5	62.50	0.23
OPB-05	15	11	73.33	0.36
OPB-06	9	5	55.56	0.25
OPB-07	10	1	10.00	0.02
OPB-08	16	14	87.50	0.28
OPB-11	16	9	56.25	0.21
OPB-12	11	8	72.73	0.24
OPB-14	10	5	50.00	0.20
OPH-01	12	7	58.33	0.24
OPH-03	6	2	33.33	0.16
OPH-04	8	1	12.50	0.03
OPH-05	9	2	22.22	0.10
Total	158	85	53.80	2.74
Average	11.29	6.07		0.20

DISCUSSION

Exposure to nanoscale materials involves phytotoxicity (Ghosh *et al*., 2010; Hatami *et al*., 2014) and genotoxicity (López–Moreno *et al*., 2010; Thabet *et al*., 2019) in plants which is related to the ability to cause oxidative stress. Nano–SiO_2 (Tripathi *et al*., 2017) and n–TiO_2 (Song *et al*., 2013; Laware and Raskar, 2014; Tumburu *et al*., 2014; Koce, 2017) uptake into the plant cell often involves free radical (unstable molecules with free outer electrons) development that ends up in the oxidative destruction of macromolecules such as proteins and nucleic acids generating DNA modifications and/or enzyme disruption (Droge, 2002). Plants exposed to nanoscale materials show cytotoxic and genotoxic effects, including a change in mitotic index and increase in chromosomal aberration (Kumari *et al*., 2009; Yang *et al*., 2015; Khan and Ansari, 2018), indicating genomic damage.

Nano–SiO_2 had a concentration dependent toxic effect on the genetic material as recorded in *Glycine max* (Elsadany *et al*., 2015) at 250–450 mg/L and in *V. faba* at 75–225 mg/L (Thabet, 2015) and at 25–75 mg/L (Thabet et al., 2019). Nano–SiO_2 is also known to regulate expression of genes involved with salt stress on *Solanum lycopersicum* (Almutairi, 2016) and increases lignin gene expression in *Avena sativa* (Asgari *et al*., 2018). A study reporting the interaction of n–SiO_2 with algae showed that n–SiO_2 coated alumina were less toxic to *Pseudokirchneriella subcapitata* than bare n–SiO_2 (Van Hoecke *et al*., 2008).

Our results demonstrate that n–TiO_2, by inducing new amplified bands, generated high polymorphism and low GTS, indicating that n–TiO_2 had a genotoxic effect on *V. faba* DNA. A genotoxic effect of n–TiO_2 has been recorded with *Arabidopsis thaliana* at 100 mg/L (Landa *et al*., 2012), *Cucurbita pepo* at 50 mg/L (Moreno–Olivas *et al*., 2014), *Triticum aestivum* at 5–150 mg/L (Silva *et al*., 2016), *Zea mays* at 1000–3000 mg/L (Mutlu *et al*., 2018) and *V. faba* at 25–75 mg/L (Thabet et al., 2019). Nano–TiO_2 can activate antioxidant enzymes (Xue *et al*., 2010) or regulate genes involved mainly in responses to biotic and abiotic stress (Landa *et al*., 2012). Furthermore, n–TiO_2 has been linked to mutagens as it could induce DNA breakage (Petković *et al*., 2011; Moreno–Olivas *et al*., 2014). Also, structure and

concentration controls its genotoxic effect as anatase/rutile structure had a dose–dependent response and was more genotoxic than an anatase structure at lower concentrations (Silva *et al*., 2016).

Generally, mutations are responsible for the appearance of new PCR product visible on agarose gel if they occur at the same locus in at least 10% of cells (Atienzar *et al*., 2000) and/or large deletions. Disappearance of bands may be associated with point mutations and/or complex chromosomal rearrangements caused by genotoxic chemicals (Atienzar and Jha, 2006). Changes observed in DNA profiles such as modifications in band intensity and loss of bands may be due to changes in oligonucleotide priming sites leading to genomic rearrangements, and less likely due to point mutations or DNA damage in the primer binding sites which can block or reduce the efficiency of DNA polymerization in PCR reaction (Liu *et al*., 2005; Gao *et al*., 2010).

CONCLUSION

This work demonstrated toxic effects of n-TiO_2 and n-SiO_2 on *V. faba* DNA and these are in line with our previous cytological and developmental studies. Data on uptake and translocation mechanisms of nano-scale materials are needed to understand mechanism and to inhibit potential toxic influences of such materials on the plant.

AUTHOR CONTRIBUTIONS

O. A. Galal and M. F. M. El–Samahy conceived the original idea. O. A. Galal designed and directed the experiment, and supervised the work. O. A. Galal and A. F. Thabet contributed to the experiments, the data analysis, preparation of the figures and tables, and the interpretation of the results. A. F. Thabet wrote the manuscript and conducted the literature search. Both O. A. Galal and M. Tuda did critical revision of the earlier versions of the manuscript. All authors approved the final version.

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