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Research article

Engineered nickel oxide nanoparticles affect genome stability in *Allium cepa* (L.)

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ABSTRACT

Indiscriminate uses of engineered nickel oxide nanoparticles (NiO-NPs) in heavy industries have ushered their introduction into the natural environment, ensuing novel interactions with biotic components of the ecosystem. Though much is known about the toxicity of NiO-NPs on animals, their phytotoxic potential is not well elucidated. NiO-NP hinders intra-cellular homeostasis by producing ROS in excess, having profound effect on the antioxidant profile of exposed animal and plant tissues. In the present study, bulbs of the model plant *Allium cepa* were treated with varying concentrations of NiO-NP (10 mg L^{-1} - 500 mg L^{-1}) to study changes in ROS production and potential genotoxic effect. The data generated proved a concomitant upsurge in intracellular ROS accumulation with NiO-NP dosage that could be correlated with increased genotoxicity in *A. cepa*. Augmented *in situ* ROS production was revealed through DCFH-DA assay, with highest increase in fluorescence (70% over control) in bulbs exposed to 125 mg L^{-1} NiO-NP. Effect of NiO-NP on genomic DNA was studied through detailed analyses of RAPD profiles which allows detection of even slightest changes in DNA sequence of treated plants. Significant differences in band intensity, loss and appearance of bands as well as genomic template stability and band sharing indices of treated plants revealed increased vulnerability of genomic DNA to NiO-NP, at even lowest concentration (10 mg L^{-1}). This is the first report of NiO-NP induced genotoxicity on *A. cepa*, which confirms the nanoparticle as a potent environmental hazard.

1. Introduction

Intensification in synthesis and application of engineered nanoparticles (ENPs) have augmented their large scale production and usage in various industrial and commercial projects in the last decade (Mueller and Nowack, 2008). From fast moving consumer goods (FMCG) sector to hard core semiconductor and electrical industry, ENPs now-a-days find use in myriad novel avenues (Zhang et al., 2012). The estimated worth of ENP industry worldwide might stood at 2.4 billion USD in 2016 (Global Markets for Nanocomposites, Nanoparticles, Nanoclays, and Nanotubes, 2012). Among these commercially exploited ENPs, metallic ENPs are most predominantly used and hence are extensively studied. Mostly oxides, these metal nanoparticles can occur simultaneously in nature, though wide-scale production in recent times, have raised concerns regarding their impact and association with the environment (Wiesner et al., 2006). Reports suggest that metallic ENPs can interact with and affect plants adversely. Genotoxic and cytotoxic outcomes consequent to exposure of metal ENPs like, Ag_2O, ZnO, CeO_2 and TiO_2, are known in a variety of plants, including *Arabidopsis thaliana*, *Allium cepa*, *Cucurbita pepo*, *Vicia faba*, etc. (Kaveh et al., 2013; Ghosh et al., 2010; Zhang et al., 2012; López-Moreno et al., 2010). Nickel oxide nanoparticle (NiO-NP) has gained prevalence in the last few years, being used capaciously in the stainless steel and electrical industry, in making coin and artificial jewelleries etc. (Salimi et al., 2007; Rao and Sunandana, 2008). Large scale production and uses have raised apprehensions regarding their release into the environment and the consequences of their interaction with the biotic components in particular (Gong et al., 2011). Research on direct interaction of bulk nickel oxide with higher animals and humans helped identify it as a carcinogen, effective at even low concentrations (Oyabu et al., 2007). The interaction and effect of NiO-NP on plants, however, is relatively less studied. Since plants are the primary part and most critical component of the food chain in the ecosystem, understanding how NiO-NPs affect them becomes an urgent necessity.

RAPD (Random Amplified Polymorphic DNA) is a PCR (Polymerase Chain Reaction) based technique, and relatively quick, easy and efficient for studying subtle differences in genomic DNA bands (Williams et al., 1990). The technique was originally extensively used for species

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classification, genetic mapping and phylogeny analyses, etc. (Powell et al., 1996; Bartish et al., 2000; Ranade et al., 2001). When used in surveying genomic DNA for evidence of DNA damage and mutation, RAPD has the potential to form the bases of novel biomarker assays for the detection of DNA damage and mutational events (e.g. rearrangements, point mutation, small insertions or deletions of DNA and ploidy changes) in cells of bacteria, plants, invertebrate and vertebrate animals (Savva, 1996, 2000; Atienzar et al., 2000), thus qualifying as the technique of choice for most genotoxicity researchers. Detection of genotoxic effect using RAPD involves the comparison of profiles generated from control (unexposed) and treated (exposed) DNA. The resultant amplified DNA amplification profiles may differ due to band shifts, missing bands or the appearance of new bands.

Allium cepa is the model system for studying ecotoxicity potential of any chemical or xenobiotic agent, and as a test system has been used since the 1940s to evaluate genotoxicity. Its ease of growth, wide availability, genomic plasticity, low cost and easy handling makes *A. cepa* the ideal organism to study genotoxicity, mutagenecity and cytotoxicity (Leme and Marin-Morales, 2009). It was also adopted by the International program on plant bioassays (IPPB) for evaluation of environmental pollutants (Ma, 1999). In the present study, RAPD technique was used to investigate genotoxicity of various concentrations of NiO-NP suspensions (10 mg L^{-1} to 500 mg L^{-1}) in *Allium cepa*. To the authors' knowledge, this is the first report of utilization of RAPD profiling for toxicity assessment after NiO-NP on either plants or animal systems.

2. Material and method

2.1. Plant materials and growth condition

Seeds of *Allium cepa* (var. Nasik Red), were procured from Suttons Seeds Pvt. Ltd., Kolkata, germinated and grown into healthy plants in the Experimental Garden, Department of Botany, University of Calcutta. Fresh, healthy bulbs, grown under controlled condition, were obtained which were put on wet bed made of moist, sterilised sand and kept in a growth chamber at 23 ± 2 °C in dark for rooting. Healthy uniformly rooted bulbs were selected for treatment with various concentrations of NiO-NP suspension (10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 62.5 mg L^{-1}, 125 mg L^{-1}, 250 mg L^{-1}, 500 mg L^{-1}) for 24 h; double distilled ultrapure water and 0.4 mM EMS solution were used as negative and positive controls respectively. After treatment, roots were excised from the bulbs and stored at −80 °C for DNA extraction. However, fresh root tips were used for fluorescent staining and ROS quantification using DCFH-DA.

2.2. Characterisation of nickel oxide nanoparticle

Engineered NiO-NP, obtained from Sigma Aldrich, (St. Louis, USA) [Product code 637130, Molecular weight: 74.69, EC Number: 215-215-7, Pubchem Substance ID 24882831, < 50 nm particle size (TEM), 99.8% trace metal basis] was used in the present study. NiO-NP was directly suspended in deionized, ultrapure water (DI-water) and dispersed by ultrasonic vibration at 60 W, 40 kHz for 45 min to produce seven different concentrations as follows: 10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 62.5 mg L^{-1}, 125 mg L^{-1}, 250 mg L^{-1} and 500 mg L^{-1}, which were directly used for treating the onion bulbs.

Characterisation of NiO-NP was done using Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM). Estimation of morphology and size of NiO-NPs was based on the observations from TEM, performed on a Field Emission Transmission Electron Microscope (JEM-2100F, JEOL, Japan) at 200 keV. NiO-NP ultrastructure was also examined using AFM (Bruker AXS Instruments, USA, Model-Innova). The topographical images were obtained in tapping mode at a resonance frequency of 218 kHz.

2.3. Qualitative estimation of ROS generation

Qualitative estimation of ROS generation was performed by 2′,7′-dichloroflourescein diacetate (DCFH-DA) staining. 15 root tips from each set of NiO-NP concentration were immersed in 0.25 μM solution of the dye for 30 min (Faisal et al., 2013), then washed and viewed under a confocal microscope (1X CLSM 81, Olympus, Japan). Images were captured using software version, Flouview FVV 1000.

2.4. Quantitative estimation of ROS generation

Quantitative estimation of ROS was done according to Gouazè et al. (2001) using 2′,7′-dichloroflourescein diacetate (DCFH-DA) assay. The treated and untreated root tips were freshly crushed in 1X Phosphate Buffered Saline (PBS, pH- 7.4) and centrifuged at 10000 g at 4 °C. The supernatant was immediately mixed with 0.25 μM DCFH-DA solution and incubated at 37 °C for 30 min. ROS associated fluorescence was measured at excitation and emission wavelengths of 495 nm and 525 nm respectively in a spectrofluorometer (F-7000, Hitachi, Japan). The experiment was repeated thrice to assure credibility of the result.

2.5. DNA extraction and RAPD procedures

For both the controls and NiO-NP treated samples, 100 mg of the root tissue were used for total genomic DNA extraction. CTAB protocol of Doyle and Doyle (1987) was used with slight modifications (Bhadra and Bandyopadhyay, 2015).

Qualitative integrity of the extracted DNA was studied by Gel Electrophoresis in 1.5% agarose (medium EEO) gel prepared in 0.5 X TBE buffer. Electrophoresis was performed using Tris-borate-EDTA running buffer system (45 mM Tris-base, 45 mM boric acid and 0.5 M EDTA), at 60 V for 1 h. A 100 bp ladder was used to check the size of the DNA bands in the gel. The gel, after being stained with ethidium bromide (Thermo Fisher Scientific) was visualized with a UV Transilluminator (Life Technologies, E-Gel manager) and photographed using Gel Capture software.

PCR protocol was modified after Ghosh et al. (2016). In brief, PCR was performed in a reaction mixture of 25 μl containing100 ng of genomic DNA, 100 pmol of decanucleotide primer, 100μMdNTPs (Applied Biosystems, USA), 1.5 U Taq DNA polymerase (Merck, India) and 10X reaction buffer (containing 10 mM Tris-HCl, pH 8.3 and 15 mM $MgCl_2$) (Merck, India). 15 decamer primers (Table 1) were chosen initially for the study, and all sets were amplified thrice, however some of them did not amplify consistently and thus amplification data from 9 primers were finally included in the study.

Amplification was conducted by following the program:

Table 1
List of primers used in the study and their sequences.

Serial no.	Primer	Sequence of primer
1.	**OPA04**	**5′-AATCGGGCTG-3′**
2.	**OPA18**	**5′-AGGTGACCGT-3′**
3.	OPA10	5′-GTGATCGCAG-3′
4.	OPB01	5′-GTTTCGCTCC-3′
5.	**OPB07**	**5′-GGTGACGCAG-3′**
6.	OPB11	5′-GTAGACCCGT-3′
7.	**OPD02**	**5′-GGACCCAACC-3′**
8.	**OPE07**	**5′-AGATGCAGCC-3′**
9.	OPG19	5′-GTCAGGGCAA-3′
10.	**OPJ12**	**5′-GTCCCGTGGT-3′**
11.	**OPL03**	**5′-CCAGCAGCTT-3′**
12.	**OPM05**	**5′-GGGAACGTGT-3′**
13.	OPM14	5′-AGGGTCGTTC-3′
14.	OPN06	5′-GAGACGCACA-3′
15.	**OPZ03**	**5′-CAGCACCGCA-3′**

*The primers in bold produced reproducible result.