Nanoparticle (CdS) interaction with host (Sesamum indicum L.) – its localization, transportation, stress induction and genotoxicity

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ABSTRACT
The present study highlights the nanoimpact of cadmium sulfide quantum dots on a plant system (Sesamum indicum L.) encompassing uptake of nanoparticles (NPs), subsequent translocation following root to leaf transportation pathway using both water- and food-conducting elements and deposition in nucleus and cytoplasm with no preferential subcellular localization. Nanocrystal agglomeration, mucilaginous sheathing and vesicularization studied are the host toxicity minimization attempt. Cellular stress due to NPs is recorded in the form of elevated production of hydrogen peroxide and malondialdehyde. However, non-synchronous activation of ascorbate peroxidase-monodehydroascorbate reductase-glutathione reductase-glutathione S-transferase enzyme system contributes to failure of anti-oxidative response and persistence of stress environment. Flow cytometric assessment reveals changes in cellular metabolic event along with blockage of cell division at G1 phase and enhances apoptotic cell death. Nuclear internalization along with oxidative burst results in generation of DNA double-strand break which can be the focal point of genome alteration and subsequent gene mutation.

Introduction
Nanomaterials are the specialized nanodimensional class (at least one dimension <100 nm) of crystalline structures possessing high surface to volume ratio rendering unique optophysical, chemo-reactive and catalytic properties in the nanoscale region. Nanoparticle (NP)-mediated research is a promising multidisciplinary field enduring next generation potentiality due to its wide array of application in electronics (Delgado-Ramos 2014), photonics, cosmetics, energetics (Zhou et al. 2014), medicine, biotechnology (Al-Halafi 2014), agriculture (Halder et al. 2015a, 2015b), among others. However, geometric progression while utilizing the nanomaterials in consumable and manufactured products results in their indiscriminate release in eco-environmental inter-collegium mostly through industrial wastewater and various anthropogenic activities (Pillai et al. 2016), leading to environmental pollution (Nowack and Bucheli 2007). Soil acts as the ultimate sink for accumulation of the released NPs in the environment and also serves as the most effective exposure avenue to the rooted plant system (Remédios et al. 2012). Toxicity of nanomaterials is mostly assessed in animal models and cell lines (Bahadar et al. 2016) but rarely in plant species (Yang et al. 2017). Due to simplicity and cost-effectivity, plant species (a principle component of the ecosystem) are used as model for study of bio-nano interaction (also recommended by United Nation Environmental Programme, World Health Organization and US Environment Protection Agency) and is a significant aspect of NP-mediated research.

Potential assessment of phyto-NPs’ interaction is noteworthy as it undergoes bioaccumulation and subsequent bio-magnification in the food chain (Judy et al. 2011). Phyto-accumulated NPs are reported to induce DNA damages in higher plants and eventually trigger genome alteration (Santos et al. 2010; Ghosh et al. 2012), which has become the focal point of interest for future research in applied agricultural science and crop improvement. The present investigation highlights nano-bio interaction between metal-based semiconductor NPs (Cadmium sulfide, also known as quantum dots, band gap 2.54–2.65 eV (Soltani et al. 2012) and a plant system (Sesamum indicum L.; family: Pedaliaceae, important oil-yielding plant of commerce) considering the attributes like seed germination, seedling growth, NPs’ uptake, stress induction and plant defense responses, transport, vascular and intracellular localization, and assessment of genotoxicity including cell cycle damage. For the purpose, cadmium sulfide NPs (CdS-NPs) is wet-chemically synthesized and opto-physically (using UV-visible Spectrophotometer [UV vis], Fourier Transform Infra-red Spectrometer [FTIR], X-ray Diffractometer [XRD], Dynamic Light Scattering analyzer [DLS], Field Emission Scanning Electron Microscope [FESEM], Transmission Electron Microscope [TEM] and Photoluminescence Spectroscope [PL]) characterized for standard nano quality assurance. Moreover, the uptake of CdS-NPs in seedlings (detected in the form of Cd$^{2+}$ ions) is analyzed following the use of an Atomic Absorption Spectrometer (AAS).

CdS-NPs possessing narrow band gap of 2.5 eV (Khataee et al. 2014) are reported to induce toxicity in algae (Jagadeesh et al. 2015), higher plants (Khataee et al. 2014), microbes (Hossain and Mukherjee 2013), animals (Liu et al. 2014) and cell lines (Chen et al. 2012; Munari et al. 2014). However, due to their wide array of application in biomedical sciences...
(Reyes-Esparza et al. 2015), photocatalytic (Torimoto et al. 2011; Kumbhakar et al. 2017) and electronic (Khan et al. 2016) devices, anti-microbial agents (Kumbhakar et al. 2017) among others, they are used as source material in the present investigation. The objective of using S. indicum in the present investigation is due to its worldwide cultivation for its oil-yielding property (Moazzami et al. 2006). The species is designated as ‘Queen of the oil seeds’ (Bedigian and Harlan 1986) possessing high percentage of unsaturated fatty acids (85.0%) and lignan (~1%; Hanzawa et al. 2013). Furthermore, the species can be cultivated both as irrigated and rainfed crop in India (Gopinath et al. 2011) and therefore bears immense significance in genetic studies. CdS-NPs’ interaction with S. indicum can shed light on protecting the important natural resource against the said environmental pollutant.

Methods

Germplasm

Seed stock of Sesamum indicum L. var. B-67 (moisture content: 7.40%) was collected from Pulse and Oil Seed Research Station, Govt. of West Bengal, Berhampore, India.

CdS-NPs’ synthesis

CdS-NPs were synthesized following the wet-chemical co-precipitation technique adopted earlier by Halder et al. (2015b). For the purpose, cadmium acetate dihydrate [(CH3COO)2Cd·2H2O; 0.01 M, 4 mL] and sodium sulfide [Na2S; 0.01 M, 4 mL] reaction kinetics was regulated within sodium dodecyl sulfate (SDS; 10.0%, 50 mL) capping environment under vigorous stirring.

Bulk CdS was prepared following the procedure similar to NPs without employing the capping agent.

Characterization

Opto-physical characterization of the synthesized CdS-NPs was performed by measuring its visible (UV-Vis Spectrophotometer- Shimadzu UVPC-1601) and infra-red (FTIR-FT/IR-6300) absorption efficiency, X-ray diffraction ability (XRD- Shimadzu-LabX), dynamic light scattering intensity (DLS – Delsa™ Nano C, Beckman Coulter) and nanoscale geometry [Scanning (SEM – JEOL JSM 7600F) and Transmission (TEM – JEOL JEM 2100HR) Electron Microscopy]. For UV-Vis Spectroscopy, ethanol mounted CdS-NPs were analyzed using ethanol as reference. Powdered CdS-NPs were diluted (1:100) in 1% Hydrogen peroxide (H2O2) generated in seedlings was estimated following Evans blue (in 0.2%, w/v) staining method (Tamás et al. 2004). For the purpose, surface cleaned fresh roots were dipped in 0.2% (w/v) Evans blue solution overnight at room temperature (20°C ± 1°C). Stained roots were washed in ddH2O until no stain eluted from the roots and the root tips were excised and incubated with moist filter paper (Whatman filter paper No. 1). Radical emergence was considered as an index of germination. Randomly selected 25 seedlings (10 days old) from each treatment along with controls were measured using a stereo microscope.

Seedling accumulation of NPs

About 0.22 g seedlings from each (six replicas) treatment (including bulk CdS and dry control) were taken for AAS analysis. Out of the six replicas, three were maintained as seed-coat containing seedlings (SCR), whereas in the rest, seed-coats were removed and seedlings were washed with ddH2O (seed-coat devoid replica-SDR). Initial dry mass was converted to dry ash following heating in a muffle furnace (400°C for 6 h). Ash samples were subsequently digested in tri-acid mixture (sulfuric acid:nitric acid: perchloric acid = 3:3:1) followed by volume reduction to 5 mL (by heating at 80°C) under a fume hood. Crystal clear acid digested samples were made up to 25 mL by ddH2O and analyzed in an AAS (Agilent Technologies; 326.1 nm for Cd2+) for detection of accumulated CdS-NPs in the form of cadmium ions (Cd2+).

Estimation of root cell viability

Root cell viability was determined following Evans blue (in 0.2%, w/v) staining method (Tamás et al. 2004). For the purpose, surface cleaned fresh roots were dipped in 0.2% (w/v) Evans blue solution overnight at room temperature (20°C ± 1°C). Stained roots were washed in ddH2O until no stain eluted from the roots and the root tips were excised and incubated in N, N-dimethyl formamide (500 µL) for 24 h at 4°C. After incubation, Evans blue released from the root tips was estimated spectroscopically at 600 nm. A control set was uniformly analyzed.

Assessment of oxidative stress

Measurement of hydrogen peroxide

Hydrogen peroxide (H2O2) generated in seedlings was estimated following Brennan and Frenkel (1977). About 0.2 g frozen seedlings (7 days old) were homogenized in 5 mL chilled acetone and filtered using Whatman No. 42 filter paper. The reaction mixture was constituted using filtrate, titanium reagent (20% titanium IV chloride in concentrated hydrochloric acid v/v) and concentrated ammonia solution in 2:1:1 ratio. Reaction mixtures were centrifuged at

Bulk CdS was oven dried (80°C ± 1°C) and the powdered samples were used for size measurement under a stereo microscope (Stereo Zoom® Leica S8APO).

Treatments

Dry seeds were exposed to CdS-NPs (1.0, 2.0 and 4.0 µg mL−1, 3 and 6 h durations). Dry seeds and seeds treated with bulk CdS (4.0 µg mL−1, 6 h – the highest employed dose was used for assessment) were kept as controls. Two hundred seeds were treated in each lot.

Germination and seedling growth

One hundred seeds from each treatment (along with dry and bulk CdS controls) were allowed to germinate in Petri plates lined with moist filter paper (Whatman filter paper No. 1). Radical emergence was considered as an index of germination. Randomly selected 25 seedlings (10 days old) from each treatment along with controls were measured using a stereo microscope.
7500 rpm for 30 min. Precipitates were dissolved in 2 N sulfuric acid. Absorbance was recorded at 415 nm. H$_2$O$_2$ content was measured from treated samples in comparison to controls using the prepared standard curve.

**Quantification of malondialdehyde**
Malondialdehyde (MDA) concentration in seedlings was measured according to Heath and Packer (1968). About 0.2 g of 7-day-old seedlings from each treatment was homogenized in 80% ice-chilled ethanol and centrifuged (5000 rpm for 15 min). Clear supernatants were transferred to vials containing trichloroacetic acid (20%) – thiobarbituric acid (5%) solution. Mixtures were heated in a water bath at 90°C for 1 h. Samples were then cooled in an ice bath and centrifuged. Supernatants obtained from treated and control samples were used to read absorbance in 532 and 600 nm.

**Study of antioxidant responses**

**Analysis of ascorbate peroxidase**
For measurement of ascorbate peroxidase (APX) (EC 1.11.1.11) kinetics, about 0.4 g of frozen seedlings (10 days old) were homogenized in extraction buffer (50 mM potassium phosphate buffer; pH 7.8) consisting of 4 mM ascorbate, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT) and 2% (w/v) polyvinylpyrrolidione (PVP). Enzyme extraction was materialized in a cold room under 4°C environment. Total soluble protein content in seedlings was measured following Lowry et al. (1951). APX activity was measured following Nakano and Asada (1981). For the purpose, about 1.8 mL reaction mixtures were prepared by addition of 800 µL potassium phosphate buffer (50 mM, pH 7.0), 200 µL EDTA (1 mM), 760 µL ascorbate (1 mM) and 40 µL H$_2$O$_2$. Oxidation reaction was initiated by introducing 100 µL of enzyme extract into the pre-reaction mixture. Hydrogen peroxidase-mediated ascorbate oxidation kinetics was estimated by measuring decrease in absorption efficiency of the reaction mixture at 290 nm (% CE%B5 = 2.8 mM$^{-1}$ cm$^{-1}$). Ascorbate peroxidase action kinetics was represented as µM ascorbate oxidation under unit time and unit protein concentration (min$^{-1}$ mg$^{-1}$ protein).

**Extraction and estimation of glutathione S-transferase**

**Extraction**
Cryopreserved (−20°C for 24 h) 10-day-old seedlings (0.4 g) were ground in 1.5 mL ice-chilled 50 mM potassium phosphate buffer (pH 7.8; containing 2 mM EDTA, 2 mM DTT and 2% PVP). The homogenate was centrifuged (15,000 rpm for 30 min at 4°C) and clear upper aqueous phase was used for quantification of enzyme activity.

**Estimation**
Super oxide dismutase (SOD): Total SOD (TSOD: EC 1.15.1.1) reactivity was measured as per Beyer and Fridovich (1987) using nitroblue tetrazolium (NBT). About 1000 µL of pre-reaction mixture was prepared comprising 880 µL potassium phosphate buffer (50 mM, pH 7.8), 50 µL L-methionine (10 mM), 30 µL NBT (60 µM), 30 µL Triton X-100 (0.05%) and 20 µL extract. Reaction was initiated by addition of 0.005% riboflavin and exposure of reaction mixture in 20 W fluorescent lamp for 7 min. Sample absorbance was recorded at 560 nm. SOD reactivity was expressed as unit per mg (U mg$^{-1}$) of protein comprising 1 unit equal to 1$_{50}$ (50% inhibition of NBT reduction mediated by SOD). Glutathione reductase (GR): GR (EC 1.8.5.1) activity was monitored as per Carlberg and Mannervik (1985). For the GR assay, 900 µL of pre-reaction solution (750 µL of 200 mM potassium phosphate buffer at pH 7.0 consisting 2 mM EDTA, 75 µL of 2 mM NADPH and 75 µL of 20 mM oxidized glutathione) was mixed with 100 µL enzyme extract. GR reaction kinetics was estimated using extinction coefficient of NADPH as 6.2 mM$^{-1}$ cm$^{-1}$. Monodehydroascorbate reductase (MDAR): MDAR (EC 1.6.5.4) activity was assessed as per Miyake and Asada (1992) and was represented as µM NADPH oxidized min$^{-1}$ mg$^{-1}$ protein. MDHA generated by ascorbate oxidase was quantified spectroscopically (265 nm). For the purpose, reaction mixture was constructed using 340 µL HEPES-KOH buffer (50 mM, pH 7.6), 60 µL NADPH (0.1 mM), 560 µL ascorbate (2.5 mM), 20 µL enzyme extract.

**Intra-seedling NPs’ transport**
Intra-seedling NPs’ transport was visually observed using Scanning Electron Microscope (EVO LS 10, Zeiss). For the purpose, 7-day-old seedlings from treatments were cryopreserved (−80°C, Haier, DW-86L628) overnight. Longitudinal and transverse sections were made through cryo-hardened seedlings (using a platinum-edged razor blade) and immediately subjected to palladium–platinum (pd–pt) coating for SEM observations.

**NPs’ localization in the tissue system**
The auto-fluorescence property (emission maxima: 530–550 nm) of CdS-quantum dots (QDs) was utilized for its localization in the tissue system. Thin transverse hand sections of treated 7-day-old seedlings were mounted on a grease-free glass slide. A glycerine–ethanol mixture (5:3) was used as quenching media to inhibit false fluorescence activity of the samples. Sections were excited using near UV-blue filter monochromatic source (430 nm) and subsequently observed under 550 nm emission filter.
Intracellular localization
Intracellular localization of CdS-NPs was studied following detection of auto-fluorescent signals from the quantum dots localized within the cells. For the purpose, germinating roots of 2 mm length were cut, enzymatically digested (cellulase: pectinase: 4:1 in phosphate buffer; pH 6.8) and hydrolyzed (Chloroform: Glacial acetic acid: 1 N Hydrochloric acid: ddH2O = 2:2:1:5) using a spirit lamp for 15 min. Hydrolyzed root tips were then excised, stained with fluorochrome (Hoechst 33342 Sigma Aldrich; 0.01%) and mounted on a clean grease-free glass slide. Gentle pressure was applied on the cover glass for uniform squashing of the root tip tissues and visualized under fluorescent microscope (Magnus-MLXi, excitation: 430 nm; emission: 550 nm).

Samples for a confocal laser scanning microscopic (CLSM) study were prepared following the identical procedure employed for fluorescent microscopic observation. The only exception adopted is the double fluorescence staining of the samples using Hoechst 3342 and acridine orange (SRL, India). For detection of fluorescent signals, three distinct laser excitation–emission (excitation/emission: 361/430, 430/550 and 561/632; AOBS TCS-SP2, LEICA) filters were used.

Assessment of genotoxicity
Comet assay
Genotoxic potentiality of CdS-NPs was assessed using alkaline comet assay (Pourrut et al. 2015). For the purpose, fresh nucleoli were isolated (from each treatment along with bulk CdS and dry controls) by gentle chopping (60 times min⁻¹ for 3 min) of 7-day-old seedlings (0.5 g) in ice cold (4°C) Tris-HCl buffer (600 µL g⁻¹ tissue) and kept for 30 min under refrigeration (4°C). Low amount (100 µL) of chilled nucleoli suspensions were transferred to equal volume of low melting point agarose (LMP) (0.8%) and warmed at 38°C for maintaining liquidity of the mixture. About 80 µL of LMP-nucleoli was pipetted over a normal melting agarose (NMP)-coated grease-free glass slide and was allowed to spread uniformly by a cover glass (20 mm x 10 mm). Agar solidification was performed under refrigeration (4°C) for 15 min. After removal of the cover glass another LMP (0.5%) coating (80 µL) was employed forming a base NMP-LMP + nucleoli-LMP trilayer. Subsequent lysis was performed by emerging the slides in a lysing buffer (2.5M NaCl, 20 mM tris-HCl, 100 mM EDTA, 2% Triton X-100 and 10% DMSO) for 2 h at 4°C in the dark. Lysed nucleoli were rinsed (by ddH2O) and subjected to electrophoresis (electrophoresis buffer: 1 mM EDTA, 300 mM NaOH, pH > 13.0) at 300 mA (25 V) for 30 min over an ice-bed. After completion of electrophoresis, slides were neutralized in ddH2O and stained in acridine orange (0.02%) and assessed under a compound microscope (Magnus-MLXi) with fluorescent attachment (Frean RG3; excitation/emission: 450 nm/560 nm). Percent of tail DNA in comet micrographs was estimated using Komet 4 Gel analyzing software. Comet slides were prepared in triplicate for each treatment and data across replicas (50 cells/replica) were pooled and represented.

DNA isolation
DNA isolation was performed from treated seedlings (along with dry and bulk CdS controls) following modified CTAB extraction protocol. Isolated DNA from each treatment was loaded in 1.8% agarose gel (ethidium bromide was mixed in 10 µg mL⁻¹ w/v ratio) in 1X TAE buffer (4.84 g Tris base, 1.14 mL glacial acetic acid, 0.2 mL 0.5 M EDTA, volume made up to 1000 mL) and run at 100 V at 4°C. Gel plates were observed and photographed under a UV-transilluminator (BioTech).

Assessment of cell cycle inhibition
Cell cycle inhibitory effect of NPs’ treatment was studied using flow cytometry. For the purpose, fresh nuclei were isolated from 7-day-old seedlings (Galbraith et al. 1983) followed by incubation with 50 µg mL⁻¹ propidium iodide (simultaneously with RNase – 50 µg mL⁻¹) and studied under Fluorescence assisted cell sorter (BD FACS Verse; excitation: 488 nm; emission: 527 nm; Argon laser). Obtained data were analyzed using BD FACS Suite™ software. Control samples were uniformly studied.

Statistical analysis
Duncan’s Multiple Range Test was performed (using IBM SPSS Statistics software) for the parameters namely oxidative stress, antioxidant enzyme assay and comet assay to assess significant variations (p < .05), if any, between the mean values. Furthermore, CD at 5% level was computed for seedling length for assessment of significant variation between/among doses of treatments.

Results and discussion
Characterization of CdS-NPs
UV-Vis absorption plot of CdS-NPs in the visible spectrum demonstrates a well-defined absorption edge toward a 470 nm spectrum band along with higher absorption efficiency at the lower wavelength region (blue shifting pattern – Figure 1(a)) which is in good agreement of decreasing size of the nanocrystals. Infra-red transmission pattern shows multiple inverse absorption peaks in 400–420 (Cd–S bonding), 580–750 (S–S bonds vibration), 1020–1080 (S–O stretching), 2240–2380 (S–H vibration) and 3100–3460 (inter molecular hydrogen bond) cm⁻¹ region of the spectrum (Figure 1(b)). X-ray diffractogram of the powdered CdS-NPs reveals the occurrence of prominent broad triplet peaks at the 2θ values of 43.3°, 52.1° and 74.8° corresponding to the index of scattering from (1 1 1), (2 2 0) and (3 1 1) crystal faces respectively (Figure 1(c)) which are in good conformity of the cuboid geometry of the studied NPs. Existence of broad diffraction peaks is in agreement with decreasing size of the quantum crystals corroborating UV-vis absorption data. Mean CdS-nanocrystal size estimated from Scherrer equation is 8.76 nm. The DLS result demonstrates the prevalence of unit size nanometric quantum dots in the prepared suspension. Average hydrodynamic diameter of CdS-NPs from DLS analyzer is measured to be 1.2 nm (range: 1.0–4.7 nm) with 0.23 polydispersity index (pdi) (Figure 1(d)). Field emission electron scanning of CdS-nanocrystal surface visually confirms the cubic shaped morphology with very minute undulating surface topology (Figure 1(e)). TEM analysis shows solid internal core structure ranges from 1.6 to 9.7 nm (1.9 ± 0.8 nm). Photoluminescence spectral
analysis reveals green (500–530 nm) fluorescence emission efficiency of the prepared CdS-NPs. Results suggest that characterization of CdS-NPs is in good agreement with standard nanocrystal geometry.

Size of the bulk CdS measured from stereomicroscopy is 15.8 µm ± 4.8.

**Seed germination and seedling growth**

Assessment of seed germination and seedling growth provides a preliminary understanding about the trends of plant growth and development under NPs-affected environment. Compared to controls, germination frequency reduces in treatments (excepting 1.00 µg mL\(^{-1}\), 3 h). Seedling length also decreases significantly (p < .05) at higher doses (4.00 µg mL\(^{-1}\), 3 h) and duration (6 h) of treatments than controls (Supplementary Table 1). Reduction in the studied physiological attributes in treatments in relation to controls suggests inhibitory effect of CdS-NPs. Such effect is the primary indication of toxicity in the cellular system. Engineered NPs are reported to exert both positive (Hojjat and Hojjat 2015) as well as negative (Lee et al. 2010; Ma et al. 2010; Kumbhakar et al. 2016; Das et al. 2017) effects on the said physiological attributes in higher plants. Negative effects of NPs on the physiological attributes are the consequences of NP-mediated up-regulating stress-responsive gene expression (Khodakovskaya et al. 2009). However, the effects may vary based on particle size, concentration, chemical composition, water and nutrient uptake, genotype sensitivity among others (Ma et al. 2010; Dehkourdi and Mosavi 2013; Masaroviová and Králová 2013; Hao et al. 2016; Elizabeth et al. 2017).
Seedling accumulation of CdS-NPs

Accumulation of CdS-NPs in seedling ash is dose dependent suggesting possible passive uptake of nanomaterials. For such energy-efficient diffusion, particle size and surface chemistry are significant factors (Raliya et al. 2016). Out of the two experiments conducted, SCR exhibit higher amount of accumulation than SDR (Figure 2) thereby suggesting immediate counter-acting barrier nature of seed-coat. Entry of NPs into seeds through intercellular spaces of parenchymatous tissue facilitates its diffusion to cotyledons (Lee et al. 2010; Ma et al. 2010).

Root cell viability

NPs-generated reactive oxygen species (ROS) are indicated by excess Evans blue uptake in root tissue. Evans blue test shows progressive loss of root cell viability following CdS-NPs treatment in respect to controls (dry and bulk CdS controls) (Figure 3(a,b)) suggesting root growth inhibitory effect of the studied NPs. The dye specifically binds to damaged cell membrane and determines the extent of damage induced by NPs (Ikegawa et al. 2000). Plants under NPs stress initiate root cell wall lignifications resulting in reduction of root cell viability (Passardi et al. 2005). Such cell viability reduction acts as a possible contributing factor to seedling length reduction in NPs-treated materials.

Oxidative stress

Accumulation of H2O2 in treatments is dose dependent (Figure 3(c)) compared to controls suggesting the potentiality of CdS-NPs to generate ROS. Surface-dependent properties of NPs including highly reactive particle interface (Buzea et al. 2007), surface-bound free radicles (Knaapen et al. 2004), among others are reported as the direct physical contributing factors in peroxide species generation. Increase in MDA concentration is found directly proportional to CdS-NPs treatments and that with accumulated H2O2, indicating lipid peroxidation in treated seedlings. Enhanced decomposition of polyunsaturated fatty acid leads to over-production of MDA causing cell membrane phospholipid breakdown (Weismann and Binder 2012) and subsequent membrane permeability for larger-sized particles. Therefore, increase in MDA concentration provides a favorable path for progressive intracellular accumulation of CdS-NPs.

Antioxidant enzyme responses

Nano-bio interaction documents differential response by the different class of antioxidant enzymes (Figure 4(a–e)). Increase in APX reaction kinetics in NPs-challenged seedlings demonstrates oxidative defense response of the plant system to maintain a favorable redox state by constructing reduced ascorbate pool (Smirnoff 2011). However, quantification of MDAR indicates no concomitant increase in enzymatic kinetics suggesting disproportionate activity of APX:MDAR enzyme system. GR also shows no significant enhancement at the cellular level. Such ground-level GR reactivity inevitably contributes to sensitivity of the plant system toward NPs-induced oxidative stress. Dose-dependent increase in total SOD (tSOD) activity indicates NPs-mediated stimulatory effect. Such upliftment in tSOD kinetics suggests successful functioning of second line of defense against ROS. GST exhibits weak upliftment in its activity and it is studied only at 2.0 and 4.0 µg mL−1, 6 h doses. Apart from the free radical scavenging enzymes, increase in GST activity promotes the thiol-dependent ROS scavenging system thereby minimizing peroxide toxicity (Zagorchev et al. 2013). In the

![Figure 2. Graphical representation of CdS-NPs accumulation in seedlings (SCR: seed-coat containing seedlings; SDR: seed-coat devoid seedlings).](image)

![Figure 3. (a–c) Dose-dependent accumulation of Evans blue ((a) seedlings; (b) bar histogram) and (c) MDA and H2O2 in seedlings.](image)
present investigation, differential defense enzyme activity possibly leads to the failure of APX-MDAR-GR synchrony resulting in persistence of intracellular stress environment.

**NPs’ transport in seedlings**

SEM analyses reveal transportation of CdS-NPs mostly in agglomerated form through cortex and vascular elements. EDX scanning identifies cadmium (Cd) and sulfur (S) as the elemental nature of the crystal aggregates. Partial prevalence of carbon (C) and hydrogen (H) indicates polysaccharide-rich mucilaginous sheathing of CdS-nanoparticles (Figure 5(a–c)). Two probable transportation routes of NPs in the plant system namely root to leaf (López-Moreno et al. 2010) and leaf to root (Hong et al. 2014; Laruea et al. 2014) are reported. In the present study, the mechanism of NPs’ translocation is possibly through vascular elements to various aerial parts demonstrating ‘root to leaf’ transport. Mucilage shielding of NPs during transport is hypothesized to be a primary *in vivo* defense mechanism against mechanical damage exerted by the penetrated NPs. For NPs’ intake and subsequent transportation, mucilage, cell wall composition, xylem segmentation, casparian strips are the important contributing factors (Schwab et al. 2015).

**NPs’ localization in the tissue system**

Fluorescent signals (Figure 6(a,b)) due to the auto-fluorescence property of CdS-NPs exhibit random distribution of the NPs. NPs are detected in cortical tissues as well as in water- and food-conducting elements. Lv et al. (2015) reported that biologically internalized NPs invade the vascular cylinder through lateral root junction where metabolically active root tip cells along with rudimentary casparian strips provide passage for spatial distribution of NPs.

**Intracellular localization**

Conventional fluorescence and CLSM studies reveal randomized particle distribution in the cytoplasmic matrix along with
Figure 5. (a–c) Transverse and longitudinal sections showing agglomerated CdS-NPs and (d) EDX spectrum confirming the presence of CdS-NPs.

Figure 6. (a–f) Autofluorescence of CdS-NPs. (a–b) Transverse section of seedlings showing localization of CdS-NPs in both corticular and vascular regions and (c–f) intracellular localization of CdS-NPs.

Figure 7. (a–f) Confocal microscopic images showing intracellular localization of CdS-NPs. (a) Under visible light; (b) under acridine orange; (c) Hoechst 33342 specific filters respectively; (d) autofluorescence of CdS-NPs; (e) merged image of Hoechst 33342 and acridine orange and (f) merged image of acridine orange and autofluorescing CdS-NPs.
their deposition in vesicles (Figure 6(c–f)). Under Confocal microscope, Hoechst 33342 and acridine orange provide blue (specific to cell outline) and red (nuclear specific) emission signal respectively. CdS-NPs-specific sample excitation demonstrates random positioning of the auto-fluorescing particle in the nucleus along with partial prevalence in the cytoplasm (Figure 7(a–f)). Vesicular compartmentalization of NPs assumed to be a cellular toxicity minimization attempt, facultatively expressed in the NPs-stressed biological system. Cellular internalization of NPs is either due to ballistic diffusion and sedimentation (Geiser et al. 2005; Limbach et al. 2005; Rothen-Rutishauser et al. 2006) or ion channel gated transportation (Kim et al. 2006; Wong et al. 2006; Zhang and Monteiro-Reviere 2009) or by different endocytic processes (Kuhn et al. 2014). Lin and Xing (2008) reported the cell wall as a limiting factor for potential entry of NPs in a plant cell. Plant cells possessing few nanometer pore diameter (Carpita et al. 1979) are impenetrable to larger-sized NPs. However, in the present study, nanocrystal size minimization to less than 2 nm (average 1.4 nm from DLS analyzer) possibly surpasses the physical barrier of the cell wall during cellular accumulation.

**Assessment of genotoxicity**

Genotoxic potentiality of CdS-NPs estimated from tail DNA percentage following alkaline comet assay (Pourrut et al. 2015) exhibits a dose-dependent relationship. The damaging effect is higher in 6 h treatments than 3 h (Figure 8(a–f)). Bulk CdS also shows 12.7% tail DNA. Qualitative evaluation of DNA damaging effect of CdS-NPs following DNA laddering assay is corroborated with quantitative DNA damage data obtained from single-cell gel electrophoresis. Higher doses of treatment show severity in DNA double-strand break by maximizing genomic DNA fragmentation and subsequent uniform smearing along

![Figure 8. (a–f) Comet photomicrographs (% tail DNA) in control (a) and in treatments (b–f).](image)

![Figure 9. DNA laddering profile.](image)
the path of DNA migration (Figure 9). Oxidative stress due to NPs-generated superoxide radicals in cellular environment causes disturbances in ionic homeostasis resulting in oxidation of purine molecules (Pakrashi et al. 2014) and subsequent DNA damage (Xi et al. 2004). However, extent of damage is attributed to physical properties of NPs including particle size, surface to volume ratio, stability, aggregation tendency, electro-chemical properties (Gao et al. 2006; Liu et al. 2010; Kim et al. 2011; Rico et al. 2011; Gaiser et al. 2012; Panda et al. 2017) among others.

**Flow cytometry**

Flow cytometric study in CdS-NPs’ treatments in relation to control (Figure 10(a)) demonstrates shifting of the dominant cellular metabolic phase (Figure 10(b–d)), G1 phase blockage...
(Figure 10(c)) and apoptosis (Figure 10(c–d)). At the initial doses of NPs’ treatments, the metabolic peak shifted toward the S/G2 segment of the cell cycle. Such cellular metabolic peak shifting is assumed to be the consequence of in vivo cellular attempt to overcome NPs-generated toxicity in the subcellular environment. However, higher dose of NPs documents complete blockage of cellular event at the G1 phase resulting in inhibition of cell cycle and subsequently affecting cell growth and morphogenesis. Apoptotic cell death is found to enhance in terminal (4.0 µg mL\(^{-1}\), 3 and 6 h) and penultimate (2.0 µg mL\(^{-1}\), 6 h) doses of treatments (Figure 10(e)). Results highlight that NPs-mediated stress and genotoxicity are the possible contributing factors for the deviation of cellular events and enhanced apoptosis.

Conclusions

Phyto-NPs’ interaction raises few key questions and they are: (1) Is the uptake of NPs only through passive way in the plant system or does any plant-specific accumulation gateway prevail? (2) Is a similar oxidative stress signaling pattern followed in other plant species in relation to NPs-mediated environment? (3) Is there any deleterious effect that exists on the photosynthetic efficiency and photo assimilation product translocation as the NPs transport utilizes both water- and food-conducting path of the plant system? (4) Which of the mechanisms of interaction dominates — indirect stress generating responsiveness or direct physical interaction or both act as parallel directional forces exerting toxicity? (5) How do DNA binding motifs of NPs potentially alter the genomic profile? (6) Are the genomic alterations only structural or any epigenetic interactions prevailing as an additive factor? (7) What is the ultimate fate of NPs in the cellular system? among others. However, experimental novelty using the next generation sequencer (NGS) can throw more light on nano-bio interaction specifically in understanding the role of NPs interacting with DNA and subsequent genome alteration in the form of gene mutation.

The work performed significantly highlights the higher plant–nanoparticle interactions encompassing physiological, cellular and genotoxic aspects which can be helpful in understanding the adverse effects of environmentally released engineered NPs on the most important component of the ecosystem as well as anticipating subsequent toxicity minimization attempts potentially activated by the phyiological system.

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