CdSe/ZnS Quantum Dots trigger DNA repair and antioxidant enzyme systems in *Medicago sativa* cells in suspension culture

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**Abstract**

**Background:** Nanoparticles appear to be promising devices for application in the agriculture and food industries, but information regarding the response of plants to contact with nano-devices is scarce. Toxic effects may be imposed depending on the type and concentration of nanoparticle as well as time of exposure. A number of mechanisms may underlie the ability of nanoparticles to cause genotoxicity, besides the activation of ROS scavenging mechanisms. In a previous study, we showed that plant cells accumulate 3-Mercaptopropanoic acid-CdSe/ZnS quantum dots (MPA-CdSe/ZnS QD) in their cytosol and nucleus and increased production of ROS in a dose dependent manner when exposed to QD and that a concentration of 10 nM should be cyto-compatible.

**Results:** When *Medicago sativa* cells were exposed to 10, 50 and 100 nM MPA-CdSe/ZnS QD a correspondent increase in the activity of Superoxide dismutase, Catalase and Glutathione reductase was registered. Different versions of the COMET assay were used to assess the genotoxicity of MPA-CdSe/ZnS QD. The number of DNA single and double strand breaks increased with increasing concentrations of MPA-CdSe/ZnS QD. At the highest concentrations, tested purine bases were more oxidized than the pyrimidine ones. The transcription of the DNA repair enzymes Formamidopyrimidine DNA glycosylase, Tyrosyl-DNA phosphodiesterase I and DNA Topoisomerase I was up-regulated in the presence of increasing concentrations of MPA-CdSe/ZnS QD.

**Conclusions:** Concentrations as low as 10 nM MPA-CdSe/ZnS Quantum Dots are cytotoxic and genotoxic to plant cells, although not lethal. This sets a limit for the concentrations to be used when practical applications using nanodevices of this type on plants are being considered. This work describes for the first time the genotoxic effect of Quantum Dots in plant cells and demonstrates that both the DNA repair genes (*Tdp1β, Top1β* and *Fpg*) and the ROS scavenging mechanisms are activated when MPA-CdSe/ZnS QD contact *M. sativa* cells.

**Keywords:** CdSe/ZnS quantum dots, 3-Mercaptopropanoic acid, Plant cells, Medicago sativa, Cytotoxicity, Genotoxicity

**Background**
Nanoparticles offer many technological solutions since they are valuable as carriers, coaters, repellents, screens and conductors. Nanoparticles may also be useful as nanosensors, cell-imaging devices and smart delivery systems and appear to be promising devices for application in the agriculture and food industries.

While the full potential of new nanomaterials is still far from being explored, their impact on living systems shows that different type of toxic effects may be imposed, depending on the type and concentration of nanoparticle as well as the time of exposure, among other factors. Nanotoxic effects have been detected at relatively high, in many cases unrealistic, particle concentrations and associated with cell death, but subtler effects that arise at lower concentrations without necessarily causing cell death also need to be considered. In particular, a number of mechanisms were envisaged underlying the ability of nanoparticles to cause DNA...
damage [1]. It was found that quantum dots can damage DNA by factors such as surface coatings [2].

Quantum dots (QD) are fluorescent semiconductors extensively used in biological studies [3]. CdSe-core QD are often used for these studies because they are easily prepared, have size tunable properties, a narrow emission band and a broad absorption spectrum. They can be coated with ZnS in order to protect the core from oxidation and other degradation processes that could release Cd ions into the medium [4]. 3-Mercaptopropanoic (MPA) coated CdSe/ZnS quantum dots (MPA-CdSe/ZnS QD) are readily prepared by the ligand exchange/phase transfer method [5]. They are small and stable water soluble QD due to the carboxyl groups [6] and this facilitates their uptake by biological systems. The mercapto group of the MPA provides a suitable ligand for attachment to the Lewis acidic Zn atoms on the QD. However, this is a relatively weak bond and some dissociation could occur [7].

In a previous study, we showed that Medicago sativa cells growing in suspension culture accumulated MPA-CdSe/ZnS-QD in the cytosol and particularly in the nucleus 8 [4]. This accumulation induced the production of undifferentiated ROS in a dose dependent manner and it was shown that a maximum concentration of 10 nM should be cyto-compatible [8]. We also showed that cell suspension cultures exposed to 100 nM of MPA-CdSe/ZnS-QD during 48 hours did not show any noticeable production of superoxide radicals (O$_2^\cdot$), and the production of H$_2$O$_2$ was far less than 10 nM, if any [8].

Little information has been found in the literature on the expression and activity of plant detoxifying enzymes and DNA repairing enzymes in response to contact with nanoparticles. Plants respond to toxicity by producing ROS that trigger the activation of ROS scavenging mechanisms. These mechanisms include the superoxide dismutase (SOD) enzyme, the water-water cycle, the ascorbate-glutathione cycle (AGC), the glutathione peroxidase cycle and the catalase (CAT) enzyme [9]. Most reports on the effect of Cd on the activity of these enzymes indicate that there is a decrease or no variation in the activity of these enzymes when plants were subjected to concentrations in the micromolar range [10].

Genotoxic effects have been reported when nanoparticles interact with living systems. Silver nanoparticles exhibited cytotoxicity by decreasing the mitotic index in a dose dependent manner in root tips of Allium cepa [11]. It was also reported that Cd damaged nucleoli in root tip cells of A. cepa [12] and altered the synthesis of RNA and inhibited ribonuclease activity in rice [13]. It was demonstrated that 0.5 nM of non-coated CdSe/ZnS QD cause DNA fragmentation and nicking in cell-free systems [14]. Very high doses of Mercaptoacetic CdSe-QD undoped and doped with cobalt induce genotoxicity in mouse tissues [15], but so far there are no reports on the genotoxicity of MPA-CdSe/ZnS QD on plant cells.

In this work, the cyto- and genotoxic effects of MPA-CdSe/ZnS QD in Medicago sativa cells in suspension culture were analyzed. It was shown that some of the ROS scavenging mechanisms are active at the cellular level, preventing the accumulation of some specific ROS when cells were exposed to these QD. Moreover, it was demonstrated that extensive DNA damage occurs when a 100 nM solution of MPA-CdSe/ZnS QD is placed in contact with plant cells and that the expression of the DNA repair genes Top 1 and Tdp is activated by the stress imposed by this type of nanoparticles.

**Results and discussion**

**MPA-CdSe/ZnS QD trigger the activity of antioxidant enzymes**

Knowing that plants exposed to high temperatures increase their anti-oxidant activity [16-18] we have established a positive control for the triggering of anti-oxidant enzymes. For that, we used a heat shock treatment that involved exposing the cell cultures to 50°C for 20 minutes. Under these conditions, an increase of about 50% in the activity of SOD, CAT and GR was recorded (Figure 1a, b and c).

SOD activity increased 12%, 27% and 88% when M. sativa cells were exposed to 10, 50 and 100 nM of MPA-CdSe/ZnS QD respectively (Figure 1a). The interaction of MPA-CdSe/ZnS QD with plant cells triggers SOD activity, and this may explain why, in a previous study [8], we could not detect O$_2^\cdot$ accumulation when M. sativa cells were exposed to MPA-CdSe/ZnS QD. Within a cell, SODs constitute the first line of defence against ROS, catalyzing the dismutation of O$_2^\cdot$. Oxygenation activation may occur in different compartments of the cell where an electron transport chain is present, such as the cytosol, mitochondria, chloroplasts, peroxisomes and glyoxysomes [19].

CAT activity increased by 8%, 16% and 72% of CAT when M. sativa cells were exposed to 10, 50 and 100 nM of MPA-CdSe/ZnS QD respectively (Figure 1b). This significant increase in the activity of CAT suggests a constant detoxification of H$_2$O$_2$ when M. sativa cells are exposed to MPA-CdSe/ZnS QD. Catalase is active only at relatively high H$_2$O$_2$ concentrations. Low levels of H$_2$O$_2$ are eliminated by ascorbate peroxidases (APX) and other peroxidases with the aid of various reducing metabolites such as ascorbate and glutathione [20].

Glutathione reductase (GR) activity increased by 5% and 23% when M. sativa cells were exposed to 50 and 100 nM solutions of MPA-CdSe/ZnS QD respectively (Figure 1c), while 10 nM MPA-CdSe/ZnS QD induced a GR activity that was not significantly different from the control.

It is possible that the increase in the activity of SOD, CAD and GR could be due to the liberation of Cd ions following MPA-CdSe/ZnS QD degradation. We did not
observe this degradation. Kirchner et al. [4] showed that poisoning of normal rat kidney fibroblasts by MPA-CdSe/ZnS particles due to the release of Cd$^{2+}$ ions only starts at concentrations of around 6 μM of surface Cd atoms. Neither 1 nor 10 μM Cd ions inhibited the growth of Tobacco BY2 cells in cell suspension cultures and 100 μM Cd induces a decrease of SOD and CAD [21]. In fact, a decrease, and not an increase, of the activity of antioxidant enzymes has been associated with Cd toxicity in different plant species [22-25].

As seen for other oxidative stresses in plants, plant cells respond to the presence of quantum dots by mobilizing ROS scavenging mechanisms to protect the cells from activated oxygen forms. This activation seems to be dose dependent and serves to prevent the accumulation of $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$ when cells are exposed to MPA-CdSe/ZnS QD concentrations between 10 and 100 nM.

MPA-CdSe/ZnS QD induce DNA damage in exposed plant cells

Four different versions of the Comet assay were used to estimate the range and type of genotoxicity imposed by MPA-CdSe/ZnS QD in *M. sativa* cells: the neutral version, useful to assess DNA double strand breaks (DSBs); the alkaline/neutral version (A/N) that detects mainly DNA

![Figure 1](http://www.biomedcentral.com/1472-6750/13/111)
single strand breaks (SSBs); the A/N version followed by an enzymatic treatment with formamidopyrimidine DNA glycosylase (FPG), to evaluate the extent of purine base oxidation; and the A/N version followed by the enzymatic treatment with Endonuclease III (EndoIII) to determine the amount of oxidized pyrimidine bases. The two enzymes remove the oxidized bases and generate a DNA strand break at the position of the excised base that can be detected via the comet assay [26].

Figure 2A shows the A/N Comet assay histogram distribution of cell suspension cultures added to 10, 50 and 100 nM solutions of MPA-CdSe/ZnS QD, or heated at 50°C for 20 minutes. Figure 2B shows images of comets that represent the five classes used for visual scoring. The control shows that 77% of the comets fall into class 1 and 2 while only 4% fall into classes 3 and 4. This may be considered a basal level of damage that may reflect the impact of protoplastization. Except for the 10 nM concentration, an increment of MPA-CdSe/ZnS QD induces an increase in the frequency of comets in the higher classes. When cells are exposed to 100 nM MPA-CdSe/ZnS QD, 78% of the comets fall into class 2 and 13% and 8% of the comets fall in class 3 and 4, respectively. Heat-treated cells present 25% of comet frequencies in classes 0, 1 and 2 and 10% in class 3 and 4. These results show that stressed plant cells undergo DNA SSBs and that increasing concentrations of MPA-CdSe/ZnS QD increment the amount of damage.

The results of the four variants of the Comet assay were plotted together in Figure 3. Strikingly, contact with 10 nM of MPA-CdSe/ZnS QD induced an increase in the number of DSBs when compared to the control, contrasting with the results obtained for the single strand break analysis. At the highest MPA-QD concentration tested purine bases were more oxidized than the pyrimidine ones.

![Figure 2](image_url)
The accumulation of SSBs and oxidative induced base lesions can lead to DSBs, considered the most lethal type of DNA oxidative damage [26]. Compared with other types of DNA damage, DSBs are intrinsically more difficult to repair and as little as one DSB lesion in the DNA can kill the cell if the lesion deactivates a critical gene. SSBs and oxidatively induced DNA base lesions are known to block DNA transcription and replication processes, resulting in accelerated cytotoxicity and genomic instability [26]. It seems that even 10 nM MPA-CdSe/ZnS QD may induce DNA double strand breaks in plant cells, being potentially deleterious and that the increment of nanoparticles induces an increase in genotoxicity.

MPA-CdSe/ZnS QD up-regulate DNA repair and antioxidant defence genes

Oxidative DNA damage is typically associated with the accumulation of 7,8-dihydro-8-oxoguanine (8-oxo-dG), an oxidized form of guanine. The 8-oxo-dG is highly mutagenic frequently inducing mispairs with the incoming dAMP during DNA replication and causing G:C to T:A transversions. The Base Excision Repair (BER) is responsible for recognizing and excising damaged bases by a multi-step process using different enzymes, such as DNA glycosylases, AP endonucleases or DNA ligases. Formamidopyrimidine DNA glycosylase (FPG) is a DNA glycosylase/AP lyase enzyme involved in the repair of oxidized purines such as 8-oxo-dG and imidazole-ring opened purines (FapyA, FpyG) [27]. Initially the presence of FPG was considered a unique feature of prokaryotes, but recently it has also been detected in plants [28-30].

The transcript accumulation of the FPG gene was evaluated in M. sativa cell suspension cultures exposed to 10, 50 and 100 nM MPA-CdSe/ZnS QD. Changes in the expression levels of the FPG gene were observed during the induced treatments (Figure 4). An up-regulation was observed when the higher QD concentrations (50 and 100 nM) were used (0.7-fold and 2.0-fold, respectively), which is in agreement with the Comet results: cells tend to respond to a genotoxic effect by increasing the expression of FPG to increase the enzyme activity.

Tyrosyl-DNA phosphodiesterase (Tdp1) is a key enzyme that hydrolyzes the phosphodiester bond between the tyrosine residue of DNA topoisomerase I (topo I) and the DNA 3'-phosphate, and thus it is involved in the repair of topoisomerase I – mediated DNA damage [31]. Macovei et al. [32] reported on the presence of a Tdp1 gene family (Tdp1α and Tdp1β) in M. truncatula and demonstrated its involvement in oxidative stress responses while Lee et al. [33] isolated Tdp1-depleted Arabidopsis mutants that exhibited a dwarf phenotype and cell death events, suggesting that this enzyme plays a decisive role during plant development.

The accumulation of transcripts of the β isoforms of Tdp1 and Top1 was evaluated in M. sativa cell suspension cultures added to 10, 50 and 100 nM MPA-CdSe/ZnS QD. Results (Figure 4) show an increase in the transcript accumulation of both Tdp1β and Top1β mRNAs. In the case of Tdp1β, a 2.0-fold increase was observed at 10 nM and 50 nM, while the addition to 100 nM Cd induced a 7.0-fold transcript accumulation. The expression of the Top1β gene did not show a significant change when 10 nM and 50 nM of Cd were added while when 100 nM was added a 4.0-fold increase was observed.

This is the first time that the expression of genes of DNA repair enzymes has been evaluated with nanoparticles in contact with plant cells. The over accumulation of transcripts of FPG, Tdp1β and Top1β shows these
nanoparticles are exerting a genotoxic effect that the cells try to counteract by increasing the expression of these genes. This is corroborated by the data obtained from the Comet assays, that show that even 10 nM of MPA-CdSe/ZnS QD may induce a genotoxic response by plant cells. The fact that the expression of APX and SOD genes is also up-regulated by the nanoparticles (Figure 4), mostly at the highest concentrations, is in agreement with the results obtained for the antioxidant enzyme activities during a previous study [8]. The balance between ascorbate peroxidase and superoxide dismutase activity in cells is considered to be crucial for determining the steady-state level of reactive oxygen species [34]. These enzymatic antioxidant defences protect the cells by directly scavenging hydrogen peroxide and superoxide radicals, converting them into less reactive species [35].

Conclusions

Although not lethal, concentrations as low as 10 nM of MPA-CdSe/ZnS QD may be cytotoxic and genotoxic to plant cells. This sets a limit for the concentrations to be used when carrying out experiments on plants using nanoparticles of this type.

As previously reported [8], when in contact with the plant cell suspensions, some nanoparticle aggregation was observed. At 10 nM this occurrence is small, but is amplified at higher concentrations. Aggregation may mask an even higher level of stress caused by these nanoparticles at higher concentrations than 10 nM, preventing their absorption into cells.

*M. sativa* cells responded to the oxidative stress caused by the addition of MPA-CdSe/ZnS QD by activating their antioxidant enzyme systems. In this study, three antioxidant enzymes: SOD, CAT and GR were activated within 48 hours of MPA-CdSe/ZnS QD exposure, preventing over-accumulation of H$_2$O$_2$ and O$_2$–, as shown previously [8]. Higher concentrations of MPA-CdSe/ZnS QD may induce the accumulation of ROS that are able to damage the plasma membrane, mitochondria and nucleus.

Cells adapt to the imposed stress by up-regulating antioxidant and/or repair systems. This may protect them against damage to some extent, or sometimes even over-protect them; the cells are then resistant to higher levels of oxidative stress imposed subsequently [36].

This is the first report on the genotoxic effects of MPA-CdSe/ZnS QD in plant cells and demonstrates that both the DNA repair genes (*Tdp1β*, Top1β and *FPG*) and the ROS scavenging mechanisms are activated when these QD interacts with *M. sativa* cells.

Methods

**Synthesis and characterization of QD**

3-Mercaptopropanoic acid coated CdSe/ZnS QD were synthesized, solubilised and characterised according to Miguel et al. [5]. In brief, MPA-CdSe/ZnS QD were obtained by the phase transfer method and the resultant water-soluble QD were purified and concentrated using a Sartorius Vivaspin 6 tube (cut-off 10KDa) at 7500 g.

For the characterisation of the synthesized CdSe/ZnS core-shell QD, Transmission Electron Microscopy (TEM)
was used. Low-resolution images were obtained using a JEOL 200CX traditional TEM operating at an acceleration voltage of 200 kV. Dynamic Light Scattering (DLS) analysis was performed using a Zetasizer Nano ZS dynamic light scatterer from Malvern Instruments. The water-soluble QD had a hydrodynamic diameter of 13.5 nm and zeta potential of -46.5 mV. The concentration of the stock solution was determined as in [5] using the spectrophotometric method of Yu et al. [37,38]. Appropriate dilution of this stock solution afforded the solutions used in this study.

**Cell suspension culture treatments**

*M. sativa* cell suspension cultures previously established [8] were used and maintained in an orbital shaker at 110 rpm (Innova 4900, New Brunswick Scientific, Germany) in the dark at 24°C. A MPA-CdSe/ZnS QD stock solution was added to the cell suspension cultures at day 3 of culture (beginning of exponential phase) to obtain the different final concentrations (0, 10 nM, 50 nM and 100 nM). After 48 hours of incubation cells were harvested for RNA or enzyme extraction and frozen at -80°C or used directly for the Comet assays. Cell suspension cultures heat-treated at 50°C for 20 min were used as an abiotic stress control.

**Antioxidant enzyme activity**

**Enzyme extraction**

The following steps were carried out at 4°C unless otherwise stated. The *in vitro* cultured *Medicago sativa* cells (about 500 mg of fresh weight) were homogenized in a mortar with 2 mL of 100 mM Tris–HCl buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol, 0.2% Triton X-100 and 2% (w/v) insoluble PVPP. The homogenate was centrifuged at 12000 g for 30 min and the supernatant was stored in separate aliquots at -80°C, for CAT, GR, SOD and protein quantification.

For the enzyme assays three types of controls were used: a stress control (heated cells), a control with no treatment and a negative control consisting of a boiled extract of the non treated cells (inactivated enzyme).

**Protein quantification**

Protein concentration was quantified spectrophotometrically at 595 nm according to the Bradford method [39] with BSA as a standard.

Protein quantification and all enzyme activities were measured using an Ultrospec 4000 UV/Visible Spectrophotometer (Pharmacia Biotech).

**Quantification of Superoxide Dismutase activity**

Total SOD activity was quantified according to the modified method described by Rubio et al. [40], measuring the increase in absorbance at 550 nm for 2 minutes (10 seconds interval) in a 1 mL solution containing 0.5 mM xanthine, 0.05 mM ferricytochrome-C, 0.1 mM EDTA, 0.01U of xanthine-oxidase and 0.05 mL of enzyme extract in 100 mM potassium phosphate buffer (pH 7.5). The enzymatic activity was estimated as the quantity of enzyme necessary for the inhibition of 50% of ferricytochrome-C reduction per minute under the assay conditions [41]:

\[
\text{Units/mg protein} = \left(\frac{\% \text{ inhibition}}{50\%}\right) \times \left(\frac{1}{v}\right) \text{mg of total protein}
\]

Where: % inhibition = \((\Delta \text{Abs control} - \Delta \text{Abs sample})/\Delta \text{Abs control} \times 100\); 50% = inhibition of the rate of cytochrome C reduction; \(v\) (volume of enzyme extract) = 0.05 mL

**Quantification of Catalase activity**

Total CAT activity was measured as described in [42]. Briefly, the decrease in absorbance was measured at 240 nm for 2 minutes (10 seconds intervals), in a 1 mL solution containing 10 mM H₂O₂ in 50 mM phosphate buffer (pH 7.5). CAT enzymatic activity was defined as the consumption of 1 μmol H₂O₂ per minute per ml at room temperature, under the assay conditions, according to the following equation:

\[
(\Delta \text{Abs}/\Delta T) \times (1/\varepsilon) \times (1/L) \times (1/v) / \text{mg of total protein}
\]

Where \(\varepsilon\) H₂O₂ = 0.00394 μmol⁻¹ mm⁻¹; \(L = 10\) mm; \(v = 0.037\) mL.

**Quantification of Glutathione reductase activity**

GR activity was quantified based on the increase in absorbance at 412 nm (10 seconds interval during 2 minutes) when 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB) was reduced by GSH [43]. The 1 mL reaction mixture contained 100 mM potassium phosphate buffer at pH 7.5, 1 mM EDTA, 0.75 mM DTNB, 0.1 mM NADPH and 1 mM GSSG. The components of the reaction mixture were added in the stated order and the reaction was initiated by the addition of GSSG. The activity of the enzyme was expressed in U/mL*mg protein wherein unit activity is the amount of enzyme which reduces 1 mM of GSSG per minute at 24°C under assay conditions:

\[
(\Delta \text{Abs}/\Delta T) \times (1/\varepsilon) \times (1/L) \times (1/v) / \text{mg of total protein}
\]

Where \(\varepsilon\) GSSG = 0.62 mL μmol⁻¹ mm⁻¹; \(L = 10\) mm; \(v = 0.05\) mL.
Comet assay

**Protoplast preparation**

Cells from the suspension culture were pelleted by centrifugation at 1000 rpm for 10 min and incubated with a protoplastization solution consisting of 10 mM MES buffer pH 5.8, 10 mM CaCl\_2, 0.4 M mannitol, 1% Macerozyme and 1% Cellulase (for about 1 g of cells 5 mL enzymatic solution was added) at room temperature in the dark for 3–4 hours under gentle agitation. After incubation the protoplasts were sieved through a 90 μm mesh without applying pressure.

200 μL of protoplasts were mixed with 200 μL of 0.75% LMP agarose (at 3°C) and 80 μL aliquots were placed on a microscope slide previously coated with 0.75% agarose. A 22×22 mm glass cover slip was placed on each gel and the slides were allowed to set on ice for a few minutes, the coverslips were then removed. The slides were marked as “control” (protoplasts from cultures with no treatment), “heat treated” (protoplasts treated for 20 min at 50°C), “10 nM, 50 nM or 100 nM” (protoplasts from cultures treated with one of the three QD concentrations), “buffer” (protoplasts from cultures treated with one of the three QD concentrations plus enzyme buffer), “FPG” (protoplasts from cultures treated with one of the three QD concentrations plus FPG enzyme) and “Endo III” (protoplasts from cultures treated with one of the three QD concentrations plus Endo III enzyme).

**Alkaline unwinding/neutral electrophoresis**

The modification of the comet assay described by Angelis et al. [44] employs various combinations of neutral and alkaline solutions immediately prior to and during electrophoresis. Exposure of DNA to highly basic conditions prior to electrophoresis under neutral and alkaline solutions immediately prior to the electrophoresis facilitates the detection of double-strand breaks and crosslinks. Under these conditions the total DNA damage is much less pronounced than under alkaline conditions [45].

In brief, slides marked as “control”, “heat treated” and “10 nM, 50 nM or 100 nM” were lysed in the Coplin jar for 1 hour at 4°C in 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris–HCl pH 7.5. The samples were then neutralized by dipping in a 0.4 M Tris–HCl, pH 7.5 solution, 3 times for 5 minutes at 4°C. The samples were then neutralized by dipping in a 0.4 M Tris–HCl, pH 7.5 solution, 3 times for 5 minutes at 4°C. The slides were transferred to the electrophoresis tank and placed in TBE (pH 8) for a few minutes and then electrophoresed for 10 min at 25 V, 10 mA at 4°C. After being electrophoresed they were fixed in ethanol 70% 2x5 min and left to dry overnight. 20 μL of 1 μg/mL DAPI was placed on each gel and covered with a coverslip, and scored after 5 min.

**Neutral incubation/neutral electrophoresis**

DNA unwinding and electrophoresis at neutral pH (pH 7–8) facilitates the detection of double-strand breaks and crosslinks. Under these conditions the total DNA damage is much less pronounced than under alkaline conditions [45].

In brief, slides marked as “control”, “heat treated” and “10 nM, 50 nM or 100 nM” were lysed in the Coplin jar for 1 hour at 4°C in 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris–HCl pH 7.5. The samples were then neutralized by dipping in a 0.4 M Tris–HCl, pH 7.5 solution, 3 times for 5 minutes at 4°C. The slides were then electrophoresed in TBE 10 min at 25 V, 10 mA. They were fixed, stained as above and scored.

**Scoring for DNA damage**

Visual image analyses of DNA damage were carried out in accordance with the described protocol [46]. Slides were examined at 200 X magnification on a Nikon Eclipse TE2000-S (Japan) inverted microscope equipped with a HMX-4 100 W Mercury lamp and UV excitation filter. One hundred randomly selected non-overlapping nucleoids were analyzed by visual inspection giving each comet a value of 0–4 according to the degree of damage. Examples of images of nuclei falling into the different classes are seen in Figure 2B. Two or three slides were evaluated per treatment and each treatment was repeated at least twice.

Images were acquired with an Evolution MP 5.1 megapixel digital CCD Color Camera (Media Cybernetics) controlled by Image Pro Plus 5.0 software (Media Cybernetics).

For the lesion-specific enzymes the standard procedure was used, including a control slide (incubated with buffer alone) in parallel with the slide treated with the enzyme, and to subtract the mean Comet score of the control from the mean score of the slide treated with the enzyme. Net enzyme-sensitive sites constituted the measure of the oxidized bases concerned.

**Real time quantitative Polymerase Chain reaction**

**RNA extraction**

The RNA extraction protocol was based on the protocol developed by Chang et al. [47] with some modifications.
Frozen cells were ground in the presence of liquid nitrogen in a mortar and the powder was transferred to a 2 mL microcentrifuge tube. The extraction buffer containing 2% CTAB, 2% PVP, 100 mM Tris–HCl pH 8, 25 mM EDTA, 2 M NaCl, 0.5 g/L spermidine and 2% β-mercaptoethanol (added just before use) was heated at 65°C for 10 min in a water bath. 900 µL of this extraction buffer was added to each sample and quickly mixed and vortexed vigorously. Samples were incubated for 15 min at 65°C, then placed on ice for 5 min and 900 µL of chloroform:isoamyl alcohol (CIA) (24:1) was added. Each sample was vigorously vortexed until a unique liquid phase was observed and again placed on ice for 5 min. Samples were then centrifuged for 15 min at 20000 g. The CIA extraction was repeated 3 times. The final combined supernatant was placed into a new microcentrifuge tube and 65 µL of 4 M NaOAc (pH 5.2) and 1500 µL of ethanol were added. Each sample was mixed by inversion and allowed to precipitate at -20°C for one hour. The samples were then centrifuged for 30 min at 20000 g and the supernatant was carefully decanted. 250 µL of 70% ethanol at 4°C was then added, the mixture centrifuged and the supernatant again discarded. An additional washing with absolute ethanol at 4°C was carried out. The pellet was dried and resuspended in 50 µL of Milli-Q water and 50 µL of 12 M LiCl added and left to precipitate overnight at -20°C. It was then centrifuged 1 hour at 20000 g and the supernatant discarded. The residue was subsequently washed with 70% ethanol and, finally, stored in absolute ethanol. Total RNA was quantified in the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and quality assessed by agarose gel electrophoresis. Only the samples with purity (A260/280 ratio) between 1.8-2.0 were used for qPCR.

cDNA synthesis and real time quantitative Polymerase Chain Reaction (qPCR)

The total RNA was reversely transcribed into cDNAs using the iScript cDNA Synthesis Kit (Bio-Rad), as indicated by the supplier.

The high degree of sequence identity and remarkably conserved genome structure and function between Medicago truncatula (barrel medic) and M. sativa (alfalfa) provides the opportunity to use the model legume M. truncatula as a surrogate [48-50] to design the oligonucleotide sequences of Tdp1β, Top1β, FPG, SOD and APX genes. Primers were designed using the Real-Time PCR Primer Design, Gene-Script software, (https://www.genscript.com/ssl-bin/app/primer) covering their highly conserved motifs. The primer sequences used are listed in Table 1. The ELF1α gene was used as a reference for the qPCR reactions [51].

qRT-PCR was carried out in a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Australia) by adding 10 µL of SsoFast EvaGreen Supermix (Bio-Rad), 200 ng of cDNA, 0.5pmol of each primer and water to a final volume of 20 µL. After one initial incubation step at 95°C for 30 sec, amplification was performed for 40 cycles with the following profile: denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec and extension at 72°C for 10 sec. Fluorescence data were collected during the extension (72°C) step and the specificity of PCR products was confirmed by performing a melting temperature analysis at temperatures ranging from 55°C to 95°C at intervals of 0.5°C. The PCR products were subsequently run on a 2.5% agarose gel to confirm the presence of a unique band with the expected size. The resulting PCR efficiency and Ct (Treshold Cycle) were used for transcript quantification. The Pfaff method [52] was used for the relative quantification of the transcript accumulation. For all the tested genes and treatments, three independent replicates were performed.

Statistical analysis

All results are presented as the mean ± standard deviation (SD). The One Way ANOVA test of significance was used to compare the different conditions by Tukey Test (VasserStat Website for Statistical Computation, http://vassarstats.net).

Abbreviations

CAT: Catalase; DSBs: Double strand breaks; Endo III: Endonuclease III; FPG: Formamidopyrimidine DNA glycosylase; GR: Glutathione reductase; M&S: Murashige & Skoog; MPA: 3-Mercaptopropanoic acid; QD: Quantum Dots; ROS: Reactive oxygen species; SOD: Superoxide dismutase; SSBs: Single strand breaks; Tdp: Tyrosyl-DNA phosphodiesterase; Top: Topoisomerase.
Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ARS, ASM and AM participated equally in the execution of the experiments and wrote the first draft of the manuscript. CM supervised the QD synthesis and contributed to the revision of the manuscript. AB, AO and PF participated in the design and coordination of the study, contributed to the interpretation of data and revision of the manuscript. All authors read, participated in the writing of and approved the final manuscript.

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References
1. Karlsson H: The comet assay in nanotoxicology research. Anal Bioanal Chem 2010, 398:651–666.
2. Walling MA, Novak JA, Shepard JR: Quantum dots for live cell and in vivo imaging. Int J Mol Sci 2009, 10:4241–491.
3. Biju V, Itoh T, Ishikawa M: The impact of CdSe/ZnS quantum dots in cells of Medicago sativa in suspension culture. Int J Mol Sci 2010, 11:3313–338.
4. Miguel AS, Maycock C, Oliva A: Synthesis and functionalization of CdSe/ZnS QD using the successive ion layer adsorption reaction and mercaptopropionic acid phase transfer methods. In Nanoparticles in Biology and Medicine. Methods in Molecular Biology Volume 906. Humana Press; 2012:143–155.
5. Medintz II, Uyeda HT, Goldman ER, Mattoussi H: Quantum dot bioconjugates for imaging, labelling and sensing. Nat Mater 2005, 4:435–446.
6. Bruchez M, Moronne M, Gin P, Weiss S, Alivisatos AP: Semiconductornanocrystals as fluorescent biological labels. Science 1998, 281(5385):2031–2036.
7. Santos A, Miguel AS, Maycock C, Oliva A: Synthesis and functionalization of CdSe/ZnS QD using the successive ion layer adsorption reaction and mercaptopropionic acid phase transfer methods. In Nanoparticles in Biology and Medicine. Methods in Molecular Biology Volume 906. Humana Press; 2012:143–155.
8. Santos A, Miguel AS, Maycock C, Oliva A: The impact of CdSe/ZnS quantum dots in cells of Medicago sativa in suspension culture. J Nanobiotechnol 2010, 8:24.
9. Apel K, Hess H: Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 2004, 55:373–399.
10. Smeets R, Ruitert G, Semame B, del Belleguitt F, van Sanden S, Vangronsveld J, Cuyper S: Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. Environ Exp Bot 2008, 63:1–31–38.
11. Kuniya M, Mukherjee A, Chandrasekaran N: Genotoxicity of silver nanoparticles in Allium cepa. Sci Total Environ 2009, 407:2483–2526.
12. Liu D, Wang W, Zhang W, Zhai L: Evaluation of metal ion toxicity on root tip cells by the Allium test. J Plant Sci 1995, 43:125–133.
13. Shah K, Dubey RS: Effect of cadmium on RNA level as well as activity and molecular forms of ribonuclease in growing rice seedlings. Plant Physiol Biochem 1995, 33:577–584.
14. Green M, Howman E: Semiconductor quantum dots and free radical induced DNA nicking. Chem Commun 2005, 1:121–123.
15. Khalil WK, Girgis E, Emam AN, Mohamed MB, Rao KV: Genotoxicity evaluation of nanomaterials: DNA damage, micronuclei, and 8-hydroxy-2-deoxyguanosine induced by magnetic doped CdSe quantum dots in male mice. Chem Res Toxicol 2011, 24(5):640–650.
16. Larkindale J, Mishkind M, Werling E: Plant responses to high temperature. In Plant Abiotic Stress. Edited by Jenks MA, Hasegawa PM. Blackwell Publishing Ltd; 2005:100–144.
17. Xue D-W, Jiang H, Hu J, Zhang X-Q, Guo L-B, Zeng D-L, Dong G-J, Sun G-C, Qian C: Characterization of physiological response and identification of associated genes under heat stress in rice seedlings. Plant Physiol Biochem 2012, 61:46–53.
18. Zhao F-Y, Liu W, Zhang S-Y: Effects of plant growth and antioxidant system to the combination of cadmium and heat stress in transgenic and non-transgenic rice. J Integr Plant Biol 2009, 51(10):942–950.
19. Ascher R, Erturk N, Heath L: Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Bot 2002, 53:1331–1341.
20. Ghechev T, Breusegem F, Stone J, Deney L, Laloi C: Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. Bioessays 2006, 28:1091–1101.
21. Mohammad ML, Eiji O, Yasuaki S, Yoshiyuki M: Exogenous proline and glycinebetaine increase antioxidant enzyme activities and confer tolerance to cadmium stress in cultured tobacco cells. J Plant Physiol 2009, 166(15):1587–1597.
22. Guo B, Liang Y, Zhu Y: Does salicylic acid regulate antioxidant defense system, cell death, cadmium uptake and partitioning to acquire cadmium tolerance in rice? J Plant Physiol 2009, 166(1):29–37.
23. Milone MT, Sgheri C, Clijsters H, Navari-Izzo F: Antioxidative responses of wheat treated with realistic concentration of cadmium. Environ Exp Bot 2003, 50(3):265–276.
24. Rodríguez-Serrano M, Romero-Puertas M-C, Zabalza A, Corsa FJ, Gómez M, del Río LA, Sandalio LM: Cadmium effect on oxidative metabolism of pea (Pisum sativum L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. Plant Cell Environ 2006, 29(8):1532–1544.
25. Sandalio LM, Daluzzo HC, Gómez M, Romero-Puertas MC, del Río LA: Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot 2001, 52(364):2125–2126.
26. Petersen EJ, Nelson BN: Mechanisms and measurements of nanomaterial-induced oxidative damage to DNA. Anal Bioanal Chem 2010, 398:813–850.
27. Dzdzorek M: Base-excision repair of oxidative DNA damage by DNA glycosylases. Mutat Res 2005, 591:45–59.
28. Murphy T, George A: A comparison of two DNA base excision repair glycosylases from Arabidopsis thaliana. Biochim Biophys Acta 2005, 1729(3):469–472.
29. Scortecci K, Lima A, Cavallaro F, Silvi U, Agnez-Lima L: Batistuzzo de Medeiros S: a characterization of a MutM/FPG ortholog in sugarcane - a monocot plant. Biochim Biophys Acta 2007, 1761(5):1045–1060.
30. Macovei A, Balestrazzi A, Confalonieri M, Fae M, Carbonera D: New insights on the barrel medic MROGG1 and MtFPG functions in relation to oxidative stress response in planta and during seed imbibition. Plant Physiol Biochem 2011, 49(9):1040–1050.
31. Yang S, Burgin A, Huzenga B, Robertson C, Yao K, Nash H: A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and proteins. J Biol Chem 2003, 278(20):18998–19005.
32. Smeets K, Cuyper S, Lambrechts A, Semame B, Hoet P, Laere A, Vangronsveld J: Induction of oxidative stress and antioxidative mechanisms in Phaseolus vulgaris following Cd application. Plant Physiol Biochem 2005, 43:437–444.
33. Scandalios J: Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defences. Bras J Med Biol Res 2005, 38:995–1014.
34. Halliwell B: Reactive species and antioxidants. redox biology is a fundamental theme of aerobic life. Plant Physiol 2006, 141:312–322.

http://www.biomedcentral.com/1472-6750/13/111
37. Yu WW, Qu LH, Guo WZ, Peng XG: Experimental determination of the extinction coefficient of CdTe, CdSe, and CdS nanocrystals. Chem Mater 2003, 15(14):2854–2860.
38. Yu WW, Qu LH, Guo WZ, Peng XG: Experimental determination of the extinction coefficient of CdTe, CdSe and CdS nanocrystals (vol 15, pg 2854, 2003). Chem Mater 2004, 16(5):560–570.
39. Bradford M: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 1976, 72:248–254.
40. Rubio MC, González EM, Minchin FR, Webb KJ, Arrese-Igor C, Ramos J, Becana M: Effects of water stress on antioxidant enzymes of leaves and nodules of transgenic alfalfa overexpressing superoxide dismutases. Physiol Plantarum 2002, 115:531–540.
41. McCord J, Fridovich I: Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969, 244:6049–6055.
42. Aebi HE: Catalase. In Methods of enzymatic analysis, Vol III. Edited by Bergmeyer US. Germany: Verlag Chemie; 1983:273–277.
43. Shanker A, Djanaguiraman M, Sudhagar R, Chandrashekar C, Pathmanabhan G: Differential antioxidative response of ascorbate-glutathione pathway enzymes and metabolites to chromium speciation stress in green gram (Vigna radiata (L.) R.Wilczek. cv CO 4) roots. Plant Sci 2004, 166:1035–1043.
44. Angelis KJ, Dusinská M, Collins AR: Single cell gel electrophoresis: detection of DNA damage at different levels of sensitivity. Electrophoresis 1999, 20(10):2133–2138.
45. Azqueta A, Shaposhnikov S, Collins A: Detection of oxidised DNA using DNA repair enzymes. In The Comet assay in toxicology. Edited by Dhawan A, Anderson D. Royal Society of Chemistry; 2008:57–78.
46. Collins A: The comet assay for DNA damage and repair. Mol Biotechnol 2004, 26:249–261.
47. Chang S, Puryear J, Cairney J: A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol 1993, 11:113–116.
48. Boldon F, Marie D, Brown S, Kodorosi A: Genome size and base comparison in Medicago sativa and M. truncatula species. Genome 1994, 37:264–270.
49. Choi H, Kim D, Uhm T, Limpens E, Lim H, Mun J, Kalo P, Pennmetsa R, Seres A, Kulikova O, Roe B, Boseling T, Kiss G, Cook D: A sequence based genetic map of Medicago truncatula and comparison of marker colinearity with M. sativa. Genetics 2004, 166:1463–1502.
50. Yang S, Gao M, Xu J, Despande S, Lin S, Roe BA, Zhum H: Alfalfa benefits from Medicago truncatula: the RCT1 gene from M. truncatula confers broad-spectrum resistance to anthracnose in alfalfa. Proc Natl Acad Sci USA 2008, 34:12164–12169.
51. Kadar K, Wandrey M, Czechowski T, Gaertner T, Scheible W, Stitt M, Torres-Jerez I, Xiao Y, Rediman J, Wu H, Cheuny F, Town C, Udvardi M: A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in Medicago truncatula. Plant Methods 2008, 4:18.
52. Pfaff M: A new mathematic al model for relative quantitation in real-time RT-PCR. Nucleic Acids Res 2001, 29:2002–2007.

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