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ORIGINAL ARTICLE

Impact of TiO_2 nanoparticles on *Vicia narbonensis* L.: potential toxicity effects

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Abstract This work was aimed to provide further information about toxicology of TiO_2 nanoparticles (NPs) on *Vicia narbonensis* L., considering different endpoints. After exposure to TiO_2 nanoparticle suspension (mixture of rutile and anatase, size <100 nm) at four different concentrations (0.2, 1.0, 2.0 and 4.0 ‰), the seeds of *V. narbonensis* were let to germinate in controlled environmental conditions. After 72 h, the extent of the success of the whole process (seed germination plus root elongation) was recorded as the vigour index, an indicator of possible phytotoxicity. After the characterisation of the hydric state of different materials, oxidative stress and enzymatic and nonenzymatic antioxidant responses were considered as indicators of possible cytotoxicity and to assess if damage induced by TiO_2 NPs was oxidative stress-dependent. Cytohistochemical detection of in situ DNA fragmentation as genotoxicity endpoint was monitored by TUNEL reaction. The treatments with TiO_2 NPs in our system induced phytotoxic effects, ROS production and DNA fragmentation. The nonenzymatic and enzymatic antioxidant responses were gradually and differentially activated and were able to maintain the oxidative damage to levels not significantly different from the control. On the other hand, the results of DNA fragmentation suggested that the mechanisms of DNA repair were not effective enough to eliminate early genotoxicity effects.

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Introduction

Nanotechnologies are already impacting worldwide in industrial and economic development, having extraordinary potential and promising applications, actually in all the productive sectors. TiO_2 nanoparticles (NPs), together with silver nanoparticles, carbon nanotubes and fullerenes, silica and zinc/zinc oxide nanoparticles, are among the top five NPs used in consumer products (Chuankrerkkul and Sangsuk 2008; Shukla et al. 2011; Gupta and Tripathi 2011). Indeed, due to their high stability, their high surface area, anticorrosive and photocatalytic properties, TiO_2 NPs are used in a broad range of products such as paints, papers, inks, coatings, plastics, food products, toothpaste, sunscreen and cosmetics, medicines and pharmaceuticals (Shi et al. 2013), in certain sectors of agriculture (Liu 2011) and in environmental cleanup technologies (Bhawana and Fulekar 2012).

With the increasing production of nanomaterials, there is a growing need to assess potential risks associated with their input into the environment and consequent interactions with living organisms. It is worth noting that recently, NPs have been included among the emerging contaminants by USEPA (2010). Studies on the environmental impact of NPs cannot be separated from evaluation of their effects on higher plants which, through their close relationship to substrates, are involved in the fate and transport of NPs in the environment (Ruffini Castiglione and Cremonini 2009; Miralles et al. 2012).

Some studies on animal models, employing multiple exposure routes, have revealed that TiO_2 NPs are more hazardous

than the fine particles of the same composition, being both cytotoxic and/or genotoxic (Valant and Drobne 2012; Shi et al. 2013). The composition of TiO_2 NPs is mainly characterised by a mix of anatase and rutile crystal forms. TiO_2 anatase seems to have a greater toxic potential than TiO_2 rutile (Sayes et al. 2006), inducing the formation of ROS after UV light irradiation (Xue et al. 2010). Therefore, ROS-mediated oxidative stress may be one of the possible events involved in the toxic effects of TiO_2 NPs, triggering cyto- and genotoxicity (Shi et al. 2013) and provoking apoptosis (Meena et al. 2012) and necrosis (Osano et al. 2003) in different animal systems.

For plants, the results are less clear and sometimes contradictory with respect to the animal kingdom, due to the limited number of information and of the published reports on this subject. Even if in some plant systems, TiO_2 NP treatments had shown positive effects on the growth of seedlings and/or any significant changes in growth, transpiration and water use efficiency (Zheng et al. 2005; Yang et al. 2006; Seeger et al. 2009; Song et al. 2013), in other plant systems, it came out that TiO_2 NPs provoked negative effects. Particularly in *Vicia narbonensis* and in *Zea mays*, treatments with TiO_2 NPs delayed germination progression, affected mitotic index and induced genotoxic effects (Ruffini Castiglione et al. 2011). Genotoxic effects and DNA damaging were also observed in *Nicotiana tabacum* and in *Allium cepa*; in the latter, TiO_2 NPs led to an increased lipid peroxidation and to oxidative stress (Ghosh et al. 2010).

This work was aimed to provide further information about toxicology of TiO_2 NPs in *V. narbonensis*, considering some different endpoints. Oxidative stress, oxidative damage and enzymatic and nonenzymatic antioxidant responses were considered both as indicators of possible cytotoxicity and to assess if damage induced by NPs is oxidative stress-dependent. Cytohistochemical detection of in situ DNA fragmentation as genotoxicity endpoint was performed by TUNEL reaction.

Materials and methods

Material preparation

Seeds of *V. narbonensis* L. were washed over night in tap water, soaked in distilled water (control samples) and in TiO_2 nanoparticle suspension (treatment) at four different concentrations (0.2, 1.0, 2.0 and 4.0 ‰) for 24 h; then, all the samples were transferred into petri dishes and let to germinate until 72 h in controlled environmental conditions at 24 °C in the dark (imbibition). TiO_2 NPs (mixture of rutile and anatase, size <100 nm) were purchased from Sigma-Aldrich, USA. Sixty seeds for each treatment were utilised for germination tests and for root elongation capacity. At 72 h of germination, vigour index (VI) was calculated according to Abdul Baki and Anderson (1973) with the following formula:

$$\mathrm{VI} = \mathrm{Germination\%} \times \mathrm{Seedling\ Growth(mm)}.$$

Three days after treatment, roots were collected for physiological determinations (fresh materials or fixed in liquid nitrogen and stored at −20 °C until use). Moreover, five roots for each treatment were fixed in formalin–acetic acid–alcohol (FAA fixative), at room temperature for approximately 12 h for the TUNEL assay.

Determination of water content and of relative water content

Calculations of root fresh weight, dry weight and moisture content were based on weights determined before and after oven drying (100 °C) of root samples to constant weight. Water content percentage was estimated on the fresh weight basis. Root relative water content (RWC; Turner 1981, with minor modifications) was calculated by the formula:

$$\mathrm{RWC} = [(\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW})] \times 100,$$

where FW is fresh weight, DW is dry weight and TW is turgid weight.

Fresh weight was obtained by weighing the fresh roots. The roots were then immersed in water over night, blotted dry and then weighed to get the turgid weight. The roots were then dried in an oven at 100 °C to constant weight and reweighed to obtain the dry weight.

Hydrogen peroxide and lipid peroxidation

Hydrogen peroxide content of roots was determined according to Jana and Choudhuri (1982) spectrophotometrically at 410 nm, using titanium chloride in H_2SO_4 for peroxide detection. The amount of H_2O_2 in the extracts, calculated from a standard curve, was expressed as μmol g^{-1} DW.

The amount of lipid peroxidation products in roots was estimated by determining the malonyldialdehyde (MDA) content in the roots according to Hartley-Whitaker et al. (2001) with minor modifications as in Spanò et al. (2007). Freeze-dried roots were powdered and mixed with TBA reagent (10 %*w*/*v* trichloroacetic acid+0.25 %*w*/*v* thiobarbituric acid), heated (95 °C), cooled and centrifuged. Malonyldialdehyde (155 mM^{-1} cm^{-1} extinction coefficient) content was measured as specific absorbance at 532 nm and by subtracting the nonspecific absorbance at 600 nm (De Vos et al. 1989) and expressed as TBARS (TBA-reactive materials) in nmol g^{-1} DW.

Electrolytic conductivity method for membrane damage estimation

Membrane damage was estimated as in Spanò et al. (2002) with minor modifications. Five roots of uniform size, after a short washing, were incubated in deionised water, evacuated for 30 min and allowed stirring for 22 h at 4 °C. The conductivity of the aqueous solution was measured with a Jenway 4310 Conductivity Meter at 25 °C. Conductivity was also detected at 25 °C after boiling the test tube in a water bath for 1 h. The percentage injury was calculated as a percentage of membrane damage using the formula:

$$(C_1 - C_w)/(C_2 - C_w) \times 100$$

where C_1 is electro-conductance value of samples at the first measurement, C_2 is electro-conductance value after boiling and C_W is electro-conductance value of deionised water.

Extraction and determination of proline, ascorbate and glutathione

Proline concentration was determined according to the method of Bates et al. (1973) with minor modifications, as in Spanò et al. (2013). Roots were homogenised with 3 % sulfosalicylic acid. The supernatant was incubated with glacial acetic acid and ninhydrin reagent (1:1:1) and boiled in a water bath at 100 °C for 60 min. After cooling the reaction mixture, toluene was added, and the absorbance of toluene phase was read at 520 nm. Calculations were made on the base of a standard curve, and proline content was expressed as μmol g^{-1} DW.

Ascorbate (AsA) and dehydroascorbate (DHA) extraction and determination were performed according to Kampfenkel et al. (1995) with minor modifications. Total ascorbate (AsA + DHA) was determined at 525 nm after reduction of DHA to AsA by dithiothreitol. Dehydroascorbate content was estimated on the basis of the difference between the total ascorbate and AsA value. Calculations were made on the base of a standard curve and ascorbate content was expressed as mg g^{-1} DW.

Glutathione was extracted and determined according to Gossett et al. (1994). Total glutathione (GSH + GSSG) was determined by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase recycling procedure. The reaction was monitored as the rate of change in absorbance at 412 nm, and GSSG was determined after removal of GSH from the sample extract by 2-vinylpyridine derivatisation. Calculations were made on the basis of a standard curve, and content was expressed as nmol g^{-1} DW.

Enzyme extraction and assays

Roots were ground in liquid nitrogen with a mortar and pestle. Extraction was made according to Spanò et al. (2013) at 4 °C. For ascorbate peroxidase, 2 mM ascorbate was added to the extraction medium. The extracts were stored in liquid nitrogen until use. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was determined following the decrease in absorbance at 290 nm as ascorbate was oxidised (Nakano and Asada 1981). Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to Navari-Izzo et al. (1997) by coupling its reaction with that of GR. The activity was determined by following the oxidation of NADPH at 340 nm. Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi (1984) by monitoring the decomposition of H_2O_2 at 240 nm. Guaiacol peroxidase (POD, EC 1.11.1.7) activity was determined as described by Arezki et al. (2001) using as substrate 1 % (*v*/*v*) guaiacol. Enzymatic activity was determined following guaiacol oxidation by H_2O_2 at 470 nm.

All enzymatic activities were determined at 25 °C and expressed as U g^{-1}protein. Protein measurement was performed according to Bradford (1976), using BSA as standard.

All spectrophotometric determinations were made using a Shimadzu UV mini-1240 Spectrophotometer.

DNA fragmentation (TUNEL assay)

To detect in situ DNA fragmentation, we utilised the TUNEL assay, which foresee the incorporation of fluorescein-labelled dUMP at 3'-hydroxyl termini (fragmentation sites) using terminal deoxynucleotidyl transferase. FAA fixed roots were paraffin embedded, sectioned (10 μm) with a microtome and mounted on poly-L-lysine coated slides for TUNEL assay. After incubation in proteinase K (Sigma-Aldrich, USA) for 15 min and two washes in 1× PBS, manufacturer's instructions of the Apoptosis Detection System Kit (Promega, WI) were followed. Co-staining with DAPI (Sigma-Aldrich, USA) was used to visualise all nuclei.

Positive controls were treated with 2 μg/ml DNase I (Sigma) for 10 min at room temperature. Samples that were treated with a reaction mix without terminal deoxynucleotidyl transferase were used as negative controls.

Slides were examined with Axio Observer.Z1, Zeiss Microscopy, Jena, (Germany) equipped with Axiocam MRc5 (Zeiss MicroImaging, Göttingen, Germany). The experiment was conducted twice. The examination of tissues for the TUNEL positivity was performed in a blind fashion.

Results

Vigour index and water content

Vigour index (Table 1) was not significantly affected by NP treatment at concentrations up to 2 ‰. The treatment with the highest concentration caused a significant decrease of the value of this parameter, declining from around 1,400–1,500 to the value of 1,180.

Table 1 Vigour index, calculated as seedling length (mm) *x* germination % and water content, relative water content (RWC), lipid peroxidation (TBARS), electrolytic conductivity, content of hydrogen peroxide (H_2O_2), proline, ascorbate and glutathione in roots from seeds treated with different concentrations of TiO_2 NPs. Data are means (±SE)

Treatments	Control	0.2 ‰	1 ‰	2 ‰	4 ‰
Vigour index	1,476.62±88.10a	1,570.57±77.81a	1,475.80±85.93a	1,417.79±73.26a	1,180.05±73.29b
H_2O (%)	92.30±1.66a	93.90±0.32a	95.90±1.69a	94.03±0.42a	93.30±0.36a
RWC (%)	82.80±0.91a	83.36±1.16a	84.26±0.72a	83.66±1.86a	79.16±2.10a
TBARS (nmol g^{-1} DW)	523.87±9.77a	586.67±9.46a	554.84±18.06a	557.42±40.06a	557.42±0.00a
Electrolytic conductivity (%)	16.87±1.24a	21.31±0.52a	17.97±0.44a	22.72±3.16a	18.67±1.50a
H_2O_2 (μmol g^{-1} DW)	25.86±0.27b	35.82±1.55ab	46.31±6.80a	28.81±3.44b	26.63±1.98b
Proline (μmol g^{-1} DW)	79.50±3.41b	104.65±3.03a	96.84±2.98a	68.13±3.29c	100.86±1.96a
Total ascorbate (mg g^{-1} DW)	3.34±0.26b	2.43±0.29bc	5.43±0.46a	1.99±0.25c	2.50±0.08bc
Asa/DHA	0.34±0.04b	0.54±0.11b	0.41±0.11b	1.24±0.35a	0.57±0.12b
Total glutathione (nmol g^{-1} DW)	2,877.90±89.12c	3,199.98±43.55c	5,770.05±133.01a	3,727.4±178.65b	3,182.10±145.79c
GSH/GSSG	2.02±0.29a	2.51±0.19a	1.64±0.31a	2.29±0.08a	1.92±0.13a

Different letters indicate significant differences ($p<0.05$)

There were no significant differences in water content, always higher than 92 %, and in relative water content among the different treatments and in comparison with control (Table 1).

Membrane damage and hydrogen peroxide

Membrane damage, indirectly assessed as leaching and lipid peroxidation, was about the same in all materials (Table 1). The content of hydrogen peroxide progressively increased, from 25.86 μmol g^{-1} DW in control roots to 46.31 μmol g^{-1} DW in 1 ‰ treatment, and then decreased reaching values not significantly different from control (Table 1).

Proline, ascorbate and glutathione

Proline content was significantly higher in materials under NP exposure than in control with the exception of roots from 2 ‰ treated seeds. The concentration of this amino acid increased from 79.50 μmol g^{-1} DW of the control to values around 100 μmol g^{-1} DW in 0.2, 1 and 4 ‰ treated materials. In 2 ‰ treated seeds, the minimum value (68.13 μmol g^{-1} DW) of this protective molecule was recorded (Table 1). Both ascorbate and glutathione reached the maximum values (5.43 mg g^{-1} DW and 5,770.05 nmol g^{-1} DW, respectively) in 1 ‰ treatment, that however was characterised by a reducing power of ASA/DHA and GSH/GSSG couples not significantly different from other materials. Ascorbate reducing power showed the highest value (1.24) in 2 ‰ treatment, despite the content of this antioxidant molecule had a minimum value (1.99 mg g^{-1} DW) just in this material (Table 1).

Antioxidant enzymes

The trend of antioxidant enzyme activities varied considerably in the different treatments and in comparison with control, without following a precise pattern (Fig. 1). In particular, APX had not significantly different values of activity (in the range 314.26–355.46 U g^{-1} protein) in control, 0.2 and 2 ‰ TiO_2 NP treatments. The lowest activity was recorded in 1 ‰ treatment (171.79 U g^{-1} protein), while roots from 4 ‰ NP-treated seeds were characterised by the highest value of activity (438.90 U g^{-1} protein) of this H_2O_2-scavenging enzyme. GPX showed in 4 ‰ treatment the maximum activity, significantly higher than that of control roots. This material had in turn a significantly higher activity than the other treatments, all characterised by similar GPX activity level. The other treatments were characterised by lower and not significantly different values of activity. Catalase activity, not significantly different in comparison with the control until 1 ‰ treatment, progressively increased with increasing of NP concentration, reaching the highest value of activity (47.38 U g^{-1} protein) in 4 ‰ treatment. POD activity had similar values in control and 1 ‰ treatment and was significantly higher in 0.2, 2 and 4 ‰ treatments, with activities not significantly different from each other.

Among the treated materials, 1 ‰ treatment was characterised by low activities of all the studied enzymes while the highest values of activity were characteristic of roots from seeds treated with the highest NP concentration.

TUNEL assay

The fragmentation of genomic DNA leads to an increase in the number of DNA molecules with 3'-hydroxyl termini and as a consequence of the green fluorescent signal.

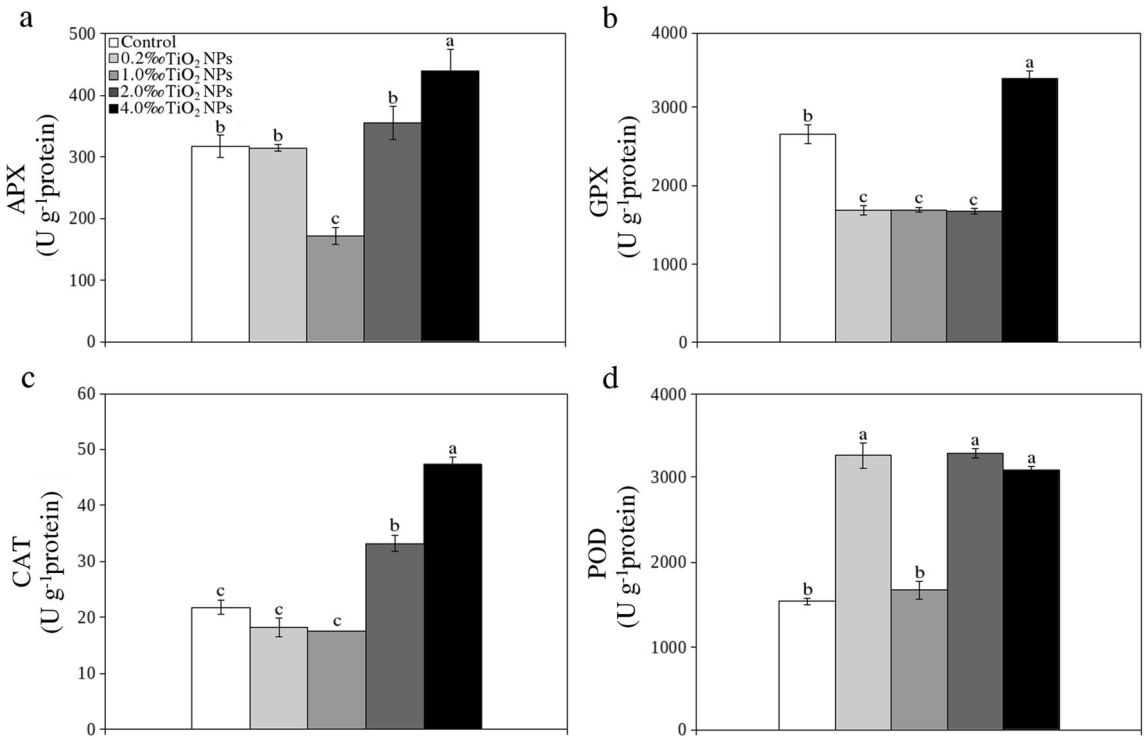

Fig. 1 Activity of ascorbate peroxidase (APX) (**a**), glutathione peroxidase (GPX) (**b**), catalase (CAT) (**c**), guaiacol peroxidase (POD) (**d**), in roots of *V. narbonensis* plants treated with 0 (control), 0.2, 1.0, 2.0 and 4.0 ‰ TiO_2 NPs. Values are means of triplicate and *vertical bars* indicate SE. Different *letters* denote significant differences at $P<0.05$

TUNEL-positive signals were observed in some xylem vessels and in root cap cells in all the analysed samples belonging to each treatment, with an increase of the signal intensity with the increasing concentrations of TiO_2 NPs (Fig. 2). Moreover, following TiO_2 NP treatments, a concentration-dependent DNA fragmentation was evidenced, also in cortical cells and at meristem level. Examining the root anatomy, the histo-anatomical structure of root tip did not appear particularly affected to shrinking and/or other peculiar symptoms, apart from an early development of the vascular bundles, especially observed in the 2.0 and 4.0 ‰ treated samples (Fig. 2).

Positive controls treated with DNAse I showed a general fluorescence involving all the nuclei (Fig. 2f), while in negative controls, the omission of terminal deoxynucleotidyl transferase led to a lack of signal (data not shown).

Discussion

Inhibition of germination and alterations of growth are sometimes reported as effects of NPs (Ruffini Castiglione and Cremonini 2009; Song et al. 2012). Above all, literature data on the biological effects of TiO_2 NPs, especially on plant kingdom, have provided contradictory results, ranging from positive to no adverse or negative effects. In studies about the effect of TiO_2 NPs on spinach (Zheng et al. 2005; Hong et al. 2005), a stimulation of growth was recorded in the presence of the lower NP concentrations. More recently, in oilseed rape, lettuce and kidney bean, it has been shown that TiO_2 NPs were not toxic to the three plant species (Song et al. 2013). On the contrary, in *V. narbonensis* and *Zea mays*, TiO_2 NPs gave rise to alterations in seed germination and early plant development (Ruffini Castiglione et al. 2011), in accordance to what was found for *Nicotiana tabacum* (Ghosh et al. 2010) and for *Linum usitatissimum* (Clement et al. 2013).

In our work, the extent of the success of the whole germination process in *V. narbonensis* after TiO_2 NP treatments was initially recorded as vigour index, an indicator of possible phytotoxicity. A detriment of VI, which summarises all the information on the impact of a potential toxic compound on seed germination and root length, revealed a phytotoxic effect of TiO_2 NPs on *V. narbonensis* only at the highest concentration treatment (4 ‰ NPs). This was in agreement with previous data about growth inhibition effects on *V. narbonensis* (Ruffini Castiglione et al. 2011).

Many stress conditions induce in plants the development of ROS, such as superoxide, hydrogen peroxide and hydroxyl radicals (Azevedo Neto et al. 2006; Cruz de Carvalho 2008; Lei et al. 2008), important signalling molecules (Mittler 2002) that however, when accumulate at critical level, can induce damages to cells and metabolism resulting in oxidative stress

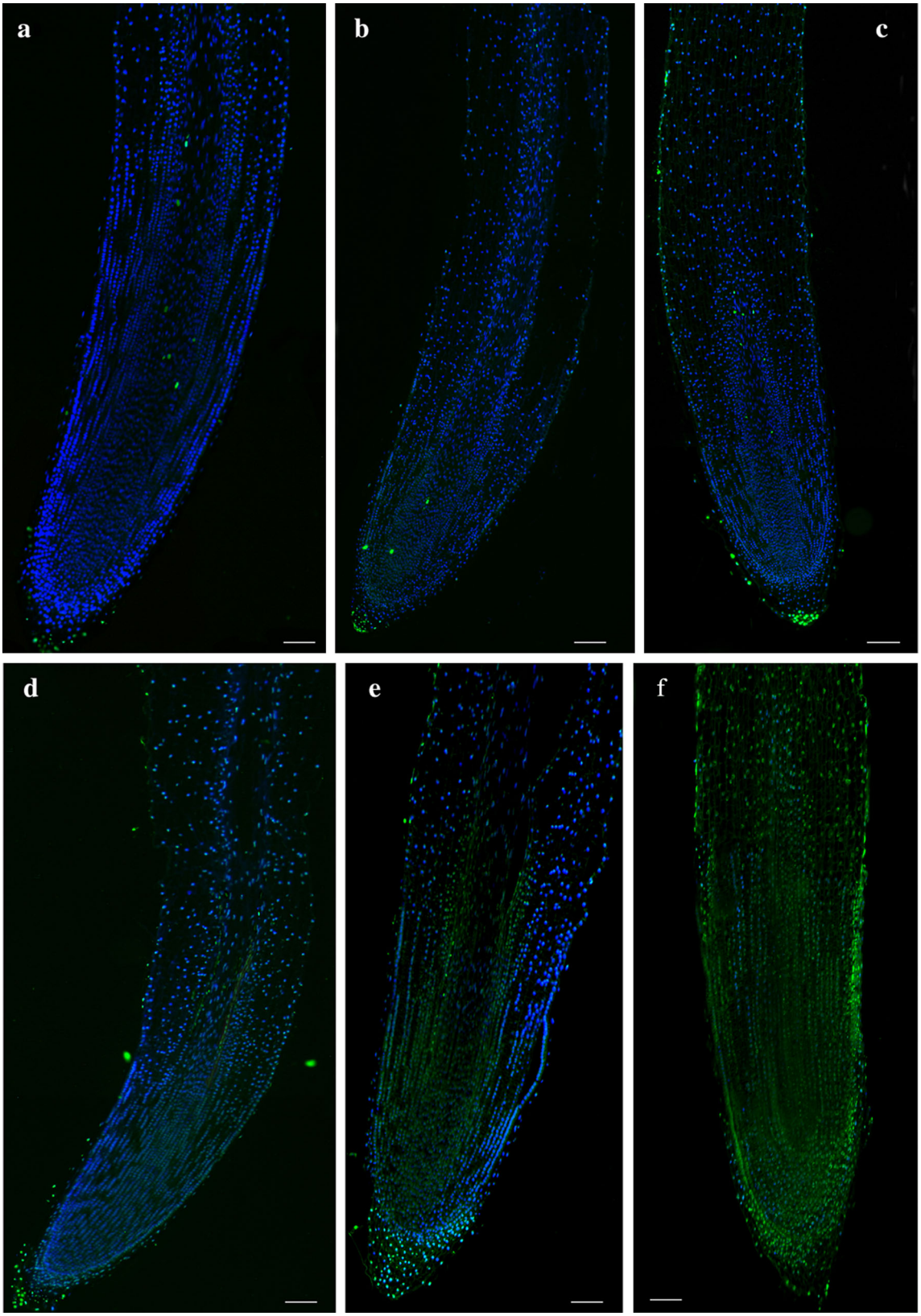

Fig. 2 Longitudinal sections of *V. narbonensis* root tips following TUNEL reaction. **a** Control root. **b** 0.2 ‰ TiO_2 NPs. **c** 1 ‰ TiO_2 NPs. **d** 2 ‰ TiO_2 NPs. **e** 4 ‰ TiO_2 NP-treated roots. **f** Positive control treated with DNase I. *Bars*=100 μm

(Parida and Das 2005). Past studies on the ability of NPs to induce oxidative stress have given contrasting results, and both increase (Zhao et al. 2012) and decrease (Sharma et al. 2012) in hydrogen peroxide content have been recorded. In the present study, treatment with TiO_2 NPs caused a significant increase in hydrogen peroxide content in a concentration-dependent manner until 1 ‰ treatment. This was in accordance with data on *Zea mays* treated with CeO_2 NPs, where in early stages of growth plants suffered from a concentration-dependent oxidative stress, and there was an increased accumulation of H_2O_2 in many different tissues (Zhao et al. 2012). However, in treatments with higher NP concentrations, the content of this molecule decreased, reaching values not significantly different from the control. Interestingly, the increase in concentration of this ROS never induced lipid peroxidation, and membrane damage was never higher than in control roots, in accordance with the lack of negative impact on membranes already reported for *Vicia faba* treated with altered TiO_2 nanocomposites (Foltête et al. 2011) and for *Zea mays* treated with CeO_2 NPs (Zhao et al. 2012). Actually, to counteract the damaging effects of ROS, plants have evolved multiple antioxidant defence mechanisms including both enzymes and low molecular weight molecules. The lack of impact of hydrogen peroxide on membranes could indicate a good ability of *V. narbonensis* to deal with nanoparticle-induced stress through an adequate antioxidant response. The importance of the different antioxidants changed in a nanoparticle concentration-dependent way. The activity of antioxidant enzymes had an irregular pattern, and with the exception of POD, showed a u-shaped trend, already recorded in plants treated with NPs (Rico et al. 2013), with values lower or not significantly different from those of control until 1 ‰ treatment. In 0.2 ‰ treatment, the antioxidant machinery was barely activated, and only proline and POD were significantly higher than in the control. In 1 ‰ treatment, enzymatic activities were at their minimum value, and the defence was antioxidant molecule-dependent. Proline was assisted in its protective role by ascorbate and glutathione that reached their highest concentrations just in this treatment. The lack of enzymatic activation in plants treated with our lower nanoparticle concentration was not surprising and has been already reported in literature for wheat treated with CuO and ZnO NPs (Dimkpa et al. 2012). In 2 and 4 ‰ treatments, there was a progressive activation of antioxidant enzymes that reached their maximum value of activity in roots from plants treated with our highest nanoparticle concentration. Under this treatment, only proline concentration remained at its highest value.

Concerning possible genotoxic effects due to NPs several data are available in the literature for living organisms (Singh et al. 2009; Remedios et al. 2012; Ghosh et al. 2010; Giorgetti et al. 2011). DNA fragmentation, as genotoxicity endpoint, has been demonstrated after TiO_2 NP treatments both in animal (Trouiller et al. 2009) and in plant systems (Ghosh et al. 2010; Ruffini Castiglione et al. 2011) by means of different experimental approaches such as comet assay, micronuclei assay, γ-H2AX assay, and DNA laddering assay. Among various strategies for detecting in situ DNA damages, we have employed the TUNEL assay on histological sections of roots, to recognise at cellular and tissue level, both single- and double-strand DNA breaks.

TUNEL-positive nuclei were found in the tissues and structures of the root that, as a rule, undergo developmental PCD processes, namely differentiating tracheary elements and root cap cells (Kuriyama and Fukuda 2002). In addition, we have observed in the same tissues an intensification of the signal with the increasing concentrations of TiO_2 NPs, especially in the tracheary elements that were precociously differentiating in comparison to what happened in the control.

Furthermore, it was worth noting a concentration-dependent DNA fragmentation following TiO_2 NP treatments also in cortical cells and especially at meristem level. This result was in accordance with previous cytological evidences on *V. narbonensis* and on *Zea mays*, where concentration-dependent genotoxicity, in terms of chromosomal aberrations and micronuclei formation, were observed (Ruffini Castiglione et al. 2011).

It has been reported that TiO_2 NPs may induce DNA damage and fragmentation indirectly, via oxidative stress, through generation of reactive oxygen species (Ghosh et al. 2010; Shukla et al. 2011). The ROS produced can indirectly induce degradation of macromolecules and DNA cleavage (Trouillier et al. 2009). Hydrogen peroxide and other ROS have been recognised to be also key modulators of DNA fragmentation as well as of other biological processes such as growth, development and stress adaptation (Gechev et al. 2006). As we found an increase in hydrogen peroxide content only until 1 ‰ treatment, in our case, the signal transduction networks controlling DNA fragmentation would seem only partly related to oxidative stress, given the adequate recorded antioxidant response of the plant. Therefore, we have to assume that other mechanisms, ROS-independent, were induced by the treatments with TiO_2 NPs, through other effectors and/or other signals leading to genomic DNA cleavage. Moreover, in this regard, it was significant that TiO_2 NPs could directly exert chemical interactions with phosphate groups of DNA, interfering with the structure and the function of genomic DNA, as suggested by Zhu et al. (2007). In addition, it is worth noting that in our experimental system, different DNA fragmentation mechanisms probably act at the same time: from one hand, a fragmentation related to developmental PCD processes, namely in differentiating tracheary elements and root cap cells, from the other a DNA damage more strictly dependent on the nanoparticle treatments, demonstrated in other animal and plant systems, whose nature is still not yet fully elucidated but which does not necessarily lead to cell death.

In conclusion, the treatments with TiO_2 NPs in our system induced phytotoxic effects, ROS production and DNA fragmentation. The nonenzymatic and enzymatic antioxidant responses were gradually and differentially activated and were able to maintain the oxidative damage to levels not significantly different from the control. On the other hand, the results of DNA fragmentation have shown that genomic DNA of *V. narbonensis* is particularly susceptible to these NPs, suggesting that the mechanisms of DNA repair were not effective enough to eliminate early genotoxicity effects. The persistence of DNA damage, apparently, did not cause a severe impact on *V. narbonensis*, able to trigger an active and adequate antioxidant response to the stress induced by NPs in root apex.

Conflict of interest The authors declare that they have no conflict of interest.

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