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Pure anatase and rutile + anatase nanoparticles differently affect wheat seedlings

Sónia Silva [a, *], Helena Oliveira [a], Sandra C. Craveiro [b], António J. Calado [b], Conceição Santos [c]

[a] Department of Biology and CESAM, Laboratory of Biotechnology & Cytomics, University of Aveiro, 3810-193 Aveiro, Portugal
[b] Department of Biology and GeoBioTec Research Unit, University of Aveiro, 3810-193 Aveiro, Portugal
[c] Department of Biology, Faculty of Sciences & GreenUP / CITAB - UP, University of Porto, Rua Campo Alegre, 4169-007 Porto, Portugal

HIGHLIGHTS

- TiO_2-NPs exposure induced cyto- and genotoxity.
- TiO_2-NPs toxicity in wheat was formulation dependent.
- Rutile + anatase formulation showed higher cytotoxicity and genotoxicity than anatase.
- MN test appears to be a good endpoint to assess NP toxicity in plant species.

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ABSTRACT

TiO_2-nanoparticles (TiO_2-NPs) are increasingly released to the environment. The present work investigates the cytotoxicity, genotoxicity and uptake of TiO_2-NPs in *Triticum aestivum*. Wheat seeds were exposed to 5–150 mg L^{-1} of anatase (ana) or rutile + anatase (rut + ana) TiO_2-NPs for 5 d. After exposure, germination and growth rates were determined. Cytotoxic effects were evaluated by changes in the cell cycle dynamics and in the membrane integrity. Genotoxicity was assessed by ploidy mutations and DNA-damage, and by mitotic abnormalities. NP uptake was analyzed by Energy Dispersive X-ray Spectroscopy (EDS). Ana-TiO_2 revealed higher toxicity regarding the rate of germination, but no negative effects were detected concerning growth. Although roots and shoots showed no EDS-detectable levels of Ti, despite cyto- and genotoxicity was observed in ana and rut + ana-NPs exposed roots. Cell cycle profile was formulation dependent with rut + ana presenting a higher capability to induce a cell cycle arrest at G_0/G_1. Both formulations induced genotoxic effects by increasing micronucleated cells: for rut + ana a dose-dependent response is evident and seems to be more genotoxic than ana at lower concentrations. Rut + ana also increased membrane permeability. The observed higher cytotoxicity of rut + ana may be explained by the higher photoactivity of this mixture. Overall, these data indicate that during germination, TiO_2-NPs induce severe cyto/genotoxic effects, which are dependent on the TiO_2-NP formulation.

1. Introduction

Organisms have always been exposed to nanoparticles (NPs) since their formation and release to the environment may occur naturally (Nowack and Bucheli, 2007). However, in the past few decades, the applications of engineered NPs has augmented leading to a dramatic increase of their release into the environment, justifying their classification as emerging contaminants by the Organization for Economic Co-operation and Development (OECD) (2012) and Environmental protection Agency (EPA) (2010a).

Titanium dioxide-NPs (TiO_2-NPs) are widely used in many daily products, such as paints, papers, cosmetics, food, and as photocatalyst in environmental technology (Nowack and Bucheli, 2007; Weir et al., 2012; Li et al., 2013). The nano-sized TiO_2 presents

Abbreviations: Ana, Anatase; CV, Coefficient of variation; EDS, Energy Dispersive X-ray Spectroscopy; FCM, Flow cytometry; NPs, Nanoparticles; PI, Propidium iodide; Rut, Rutile.

* Corresponding author.
E-mail addresses: soniasilva@ua.pt (S. Silva), holiveira@ua.pt (H. Oliveira), scraveiro@ua.pt (S.C. Craveiro), acalado@ua.pt (A.J. Calado), csantos@fc.up.pt (C. Santos).

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incremented capabilities, such as higher photocatalytic activity when compared with the non-nano-sized TiO_2 (Ricci et al., 2013). TiO_2-NPs exist in three allotropic forms: anatase (ana), rutile (rut) and brookite (Ricci et al., 2013). Rut is used as a pigment, it has ultraviolet light absorbing properties (Menard et al., 2011) and high thermodynamic stability, whereas ana is used for its superior photocatalytic properties (Ricci et al., 2013). Furthermore, nano-formulations of both rut + ana have higher photoactivity than each phase alone (Hurum et al., 2003; Kurepa et al., 2010). The effects of NPs, including TiO_2-NPs on the environment and on human health are raising great concern (Bhatt and Tripathi, 2011; Maurer-Jones et al., 2013). NPs can reach plants by contaminated wastewater treatment plant effluents and sludge, contaminated water, direct application, contaminated soils, or atmospheric fallout (EPA, 2010b). Even anticipating a conservative market penetration of TiO_2-NPs of only 10% (EPA, 2010b), corresponding predicted environmental concentrations may reach 24.5 μg/L in water and 1030 μg Kg^{-1} (~1 ppm) in soil. According the EPA predictions (Boxall et al., 2007), these values raise to ~500 ppm when the market penetration is estimated to reach 50%.

The uptake of NPs by plants remains a crucial topic of discussion. Regarding TiO_2-NPs, Ti was found in different species as: *Lycopersicum esculentum* (Song et al., 2013a), *Phaseolus vulgaris*, *Triticum aestivum* and *Rumex crispus* (Larue et al., 2012b; Jacob et al., 2013), *Cucumis sativus* (Servin et al., 2012, 2013). Contrarily, no TiO_2-NPs uptake was detected in *Lemna minor* (Li et al., 2013). Larue et al. (2012b) suggested that both rut and ana phases can be uptaken by wheat plants but only accumulated in roots when primary particle diameter is below 140 nm. So, it seems established that plants can uptake TiO_2-NPs. Nevertheless, since the exposure conditions greatly vary, it remains difficult to compare results and draw conclusions. The size of TiO_2-NPs is unlikely to be the only factor important for Ti uptake and toxicity. The period of exposure and the plant growth stage are expected to significantly affect Ti uptake and published works differ greatly regarding these parameters. In some of them, authors exposed plant seeds to TiO_2 for only 48 h (Servin et al., 2012, 2013; Song et al., 2013a), whereas in other works plants or seedlings were exposed for 1–4 weeks (Larue et al., 2012b; Servin et al., 2012; Jacob et al., 2013) or even for 150 d (Servin et al., 2013). Concerning seedlings/plants, most of them were already 6–15 d old (Larue et al., 2012b; Servin et al., 2012; Jacob et al., 2013) when they were subjected to TiO_2-NPs.

TiO_2-NPs effects on plants are very variable. Positive responses, such as shoot and root growth increments, have been reported (Larue et al., 2012b; Feizi et al., 2013, 2012), but also negative effects, as genotoxicity (Castiglione et al., 2011; Pakrashi et al., 2014) and germination inhibition (Clement et al., 2013). Other studies reported no alterations in the studied parameters (Seeger et al., 2009). Taking all together, literature shows that TiO_2-NPs may have different accumulation patterns or phytotoxicity and that their effects on plant species are dependent on suspension properties (Hawthorne et al., 2012; Larue et al., 2012a).

Since TiO_2-NP formulations have distinct properties and capabilities, in the present work we focused on studying the influence of nano-TiO_2 formulation upon inducing seedling phytotoxicity in wheat, an important crop and a model by OECD test guidelines (1984). Furthermore, comparative evaluations of toxicity of different TiO_2 formulations are limited and most studies reported on wheat focus on TiO_2-NPs uptake (Feizi et al., 2012; Larue et al., 2012a, b) and/or effects on germination/growth rates (Jacob et al., 2013; Larue et al., 2012b).

Using wheat as model species, the present study aimed at comparing the toxicity of two different TiO_2 formulations under realistic estimations (Boxall et al., 2007): pure ana and rut + ana [Aeroxide P25; which has as principal component ana (80%)]. The toxicity was evaluated at a critical stage of seedling development. Considering the scarce information available regarding comparative effects of ana vs. rut + ana, we hypothesized that exposed seedlings would be differently affected. In order to cover all the secondary stages that comprehend the primary germination stage, according to Zadoks growth scale, the exposure was performed during 5 d. For that, wheat seeds were exposed to five different concentrations of both ana and rut + ana NPs and the following endpoints were analyzed: a) germination and growth rates; b) root cell cycle profile; c) root genotoxicity; d) NPs uptake and translocation by X-ray EDS. For better control of TiO_2-NPs agglomeration or aggregation and to avoid NPs deposition, a plant agar test was employed. This is the first comprehensive study that compares the toxicity of ana and rut + ana formulations under the same conditions.

2. Material and methods

2.1. NP dispersion and characterization

TiO_2-NPs were purchased from Sigma Aldrich (St. Louis, MO-USA) with a purity ≥99.5%. Two formulations were used: anatase (ana) and rutile + ana (rut + ana) (Aeroxide® P25: about 20:80). According to the manufacturer rut + ana NPs have 21 nm and 35–65 m^2 g^{-1} surface area whereas ana NPs present <25 nm and 45–55 m^2 g^{-1} surface area. Stock suspensions (1 g L^{-1}) were prepared in Mili-Q water and sonicated for 30 min at 16 W output using an ultrasonicator with a probe (Vibra Cell, Sonics®, Newtown, USA). The stock suspensions were used to prepare the final concentrations in agar (0.8%): 5, 10, 50, 100 and 150 mg L^{-1}, by adding the appropriate volume of NP stock to heated agarised Mili-Q water at 50 °C. For seeds exposure, 20 mL of melted agarized water with/without NPs was transferred to Petri dishes and rapidly solidified at 4 °C in order to minimize alteration of size and agglomeration values.

The stock suspensions of 1 g L^{-1} and the final concentrations of 5, 50 and 150 mg L^{-1} were used to determine NPs hydrodynamic size and zeta potential using dynamic light scattering (DLS) (Zetasiser Nano, Malvern Instruments, Malvern, UK). NPs size was corroborated in a Hitachi SU-70 scanning electron microscope (SEM, Hitachi High-Technologies Corp., Tokyo, Japan). For SEM analyses, one drop of TiO_2-NPs suspensions (up to100 mg L^{-1}) was placed on graphite foil and observed after drying.

2.2. Plant material and growth conditions

Seeds of *T. aestivum* L. cv. Arthur were surface-disinfected using sodium hydrochlorite (20% v/v), rinsed in Mili-Q water and germinated for 5 d in Petri dishes containing agarised NPs suspension (30 seeds per dish). Germination took place in the dark at 24 °C. After the exposure period, the germination rate, seedling growth (root and shoot) and fresh weight were assessed. For germination rate determination three Petri dishes from three independent assays were used and for growth evaluation three seedlings from three different Petri dishes were used.

2.3. Membrane degradation

Loss of plasma membrane integrity in roots was measured spectrophotometrically by Evans blue staining, were cells with damaged membranes incorporate the stain. The roots were incubated in 0.05% (w/v) Evans blue for 90 min at room temperature and then rinsed in running water (30 min). Root tips (0.5 cm) were selected and macerated with 600 μL 50% methanol (v/v) and 1% sodium dodecyl sulfate (w/v) for 30 min at 60 °C. The extract was