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Investigation of titanium dioxide nanoparticles toxicity and uptake by plants

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Abstract. Nanoparticles (NP) are introduced in a growing number of commercial products and their production may lead to their release in the environment. Plants may be a potential entry point for NP in the food chain. Up to now, results describing NP phytotoxic effects and plant accumulation are scarce and contradictory.

To increase knowledge on titanium dioxide NP (TiO₂-NPs) accumulation and impact on plants, we designed a study on three plant species, namely wheat (*Triticum aestivum*), oilseed rape (*Brassica napus*) and *Arabidopsis thaliana*. These plants were exposed in hydroponics to a panel of well-characterized TiO₂-NPs, with diameters ranging from 12 to 140 nm, either anatase or rutile. Their accumulation in plant tissues is currently being assessed by complementary imaging techniques: scanning electron microscopy (SEM), transmission electron microscopy (TEM), micro-X-ray fluorescence (SR- μ -XRF) imaging and micro-particle induced X-ray emission (μ -PIXE) imaging. Moreover, the impact of TiO₂-NP exposure on germination rate, root elongation, dry biomass and evapotranspiration is evaluated. Preliminary results are presented here, with data collected on wheat plants exposed to 12 nm and 25 nm anatase TiO₂-NPs. These results show that TiO₂-NPs are taken up by plants, and do not significantly alter their germination and root elongation. These results underline the necessity of deeper evaluation of nanoparticle ecotoxicity, and particularly on their interaction with plants.

1. Introduction

Nanoparticles (NP) are introduced in a growing number of commercial products. Among them, TiO₂ anatase nanoparticles (TiO₂-NPs) are one of the most produced NPs in the world (see Nanowerk database, www.nanowerk.com). They are used as a pigment in paint, paper, ink and plastic. They are also introduced in cosmetics such as sunscreens for their UV protective properties (see Nanowerk database, www.nanowerk.com). Finally, they are presently used in pilot water-purification reactors, because of their ultraviolet irradiation-induced bactericidal effects (photocatalysis). For these reasons, these NPs will surely be released in the environment [1]. The knowledge of their potential effects on human health is rapidly increasing, however little is known on their potential toxicological effects on environment, i.e. destabilization of the ecosystems and trophic transfer, but also on their potential transfer to the food chain via plant ingestion. The literature relating NP toxicity and accumulation in plants is scarce; authors mostly relate NP inhibitory effects on seed germination and root elongation [2-4]. A few studies relate NP uptake by plant roots [3, 5], and root to shoot transfer [5]. Concerning TiO₂-NPs, TiO₂-based nanocomposites have recently been shown to be accumulated and translocated to the shoots of *Arabidopsis thaliana* [6]. These translocation studies are based on elemental analysis of the ionic form of metal in plants, but neither address the presence of metal as NPs, nor the spatial distribution of NPs throughout the plant. Actually, TiO₂-NP dissolution has been demonstrated to be low in aqueous solution [7], however its occurrence has never been tested in plant exposure solution and/or during NP transfer to the roots or from the roots to the shoots. Synchrotron radiation micro X-ray fluorescence (SR- μ -XRF), coupled to micro X-ray absorption spectroscopic (μ -XAS) analysis is the ideal method to address metal distribution after plant uptake, together with speciation analysis which would permit the identification of this metal as NPs. It has already been used to assess ionic metal internalization and distribution in plants [8].

We used a combination of complementary imaging techniques: scanning electron microscopy (SEM), transmission electron microscopy (TEM), micro-X-ray fluorescence (SR- μ -XRF) imaging and micro-particle induced X-ray emission (μ -PIXE) imaging, to assess TiO₂-NP accumulation in plants, and correlated their uptake with their impact on plant development. This study is currently under process in our laboratory with several plant models and several TiO₂-NPs varying in their diameter, crystalline phase and shape; we present here some preliminary results obtained on wheat (*Triticum aestivum*), exposed in hydroponics to 12 nm and 25 nm anatase TiO₂-NPs. These results show that TiO₂-NPs are taken up by plants, and do not significantly alter their germination and root elongation. These results underline the necessity of deeper evaluation of nanoparticle ecotoxicity, and particularly on their interaction with plants.

2. Methods

2.1. Nanoparticles, characterization and suspension

For TiO₂-NP dispersion studies and impact studies on plant development, we used commercial Evonik Aeroxide® P25 TiO₂-NPs. The NPs used in plant uptake experiments were synthesized by laser pyrolysis in Laboratoire Francis Perrin (LFP) [9], then annealed under air at 400°C during 3 h. Physico-chemical characterization of these NPs has been reported in two previous studies [10, 11]. Briefly, LFP NPs are 95% anatase and 5% rutile. Their mean diameter is 12 ± 3 nm, they are spherical-shaped and their specific surface area, measured according to Brunauer, Emmett and Teller (BET) [12], is 82 ± 4 m².g⁻¹. Their point of zero charge, measured by assessment of zeta potential at increasing pH, is 6.4. Evonik P25 nanoparticles are 75% anatase and 25% rutile. Their mean diameter is 25 ± 7 nm, they are spherical-shaped and their specific surface area, measured according to Brunauer, Emmett and Teller (BET) [12], is 46 ± 1 m².g⁻¹. Their point of zero charge is 7.

These TiO₂-NPs were suspended in ultrapure water and sonicated using a high energy probe, during 30 mn, at 4°C, in pulsed mode (1 s on, 1 s off). Agglomeration status was evaluated using photon correlation spectroscopy (PCS) using a Zetasizer 3000HS (Malvern). Z-average values are given in the result section.

2.2. Plant exposure and sample preparation for imaging

For imaging, seeds were germinated on sand, and their roots were directly immersed in the NP suspension (100 mg.L^{-1}), and placed in a growth chamber under controlled conditions: day/night photoperiod: 16/8 h; temperature (day/night), $24/20 \pm 1^\circ\text{C}$ and relative humidity (day/night), 70/75%. After 7 days, plant roots were thoroughly rinsed with deionised water. For μ -XRF, SEM and μ -PIXE analysis, plant roots and leaves were cryofixed, immediately embedded in Tissue Tek OCT™ resin (Agar Scientific, Essex, UK) and cross sections were cut and freeze-dried.

The distribution of Ti, P, Cl and Ca in plant thin sections were mapped using synchrotron radiation μ -X-ray fluorescence (SR- μ -XRF), images presented in this study were recorded on LUCIA beamline (SOLEIL synchrotron, France). The spot size was $3 \times 3 \mu\text{m}^2$. XAS spectra were recorded between 4.95 and 5.05 keV, *i.e.* around Ti X-ray absorption edge (4.966 Kev). On each sample, at least 10 spectra were collected, *in situ* on map locations containing high concentrations of Ti. These spectra were then averaged and compared to XAS spectra collected on Ti standards: 12 nm, 25 nm and 140 nm TiO_2 anatase NPs, 20 and 50 nm TiO_2 rutile NPs. Spectrum was then normalized to the second-order polynomial to be equal to one, with Athena software [13]. SEM image were acquired on a Zeiss-Supra 55VP Field Emission Gun Scanning Electron Microscope (FEG-SEM) equipped with a X-ray Energy Dispersive Spectrometer (EDS), operated at 15 kV, at ICMMO, Orsay, France.

2.3. Nanoparticle impact on plant development

For NP impact on plant development assessment, 10 seeds were germinated in Petri dishes in deionised water containing 10 to 100 mg/L NPs. According to US-EPA guidelines (US-EPA, 1996), seeds with roots longer than 5 mm were considered germinated. For root elongation experiment, after 7 days, roots were measured on a picture thanks to ImageJ software.

3. Preliminary results and discussion

3.1. Characterization of TiO_2 -NPs and dispersion in aqueous solution

In order to obtain properly dispersed and stable TiO_2 -NP suspensions, sonication was applied to TiO_2 -NP powders dispersed in water at 10 g.L^{-1} . For this study, TiO_2 -NPs from Evonik (Aeroxide® P25) were used. Optimal TiO_2 -NP concentration for proper measure was evaluated, and concentrations of 50-1000 mg.L^{-1} were found to be appropriate, whereas at lower concentrations, the Zetasizer was not able to properly measure the mean hydrodynamic diameter (Table 1). At concentrations higher than 1000 mg.L^{-1} , the suspension is too opaque to obtain a proper measure.

Table 1. Mean hydrodynamic diameter of nanoparticle agglomerates^a

Concentration (mg.L^{-1})	10	50	100	500	1000	2000
Diameter (nm)	140 0.6	24.2	38.7	43.1	66.6	12.5
Standard deviation	333. 4	11.1	15.4	22.6	26.1	7.3

^aMean hydrodynamic diameter was determined by PCS after a 30 mn sonication, in water, using high energy probe. TiO_2 -NP concentration in these suspensions ranged from 10 to 2000 mg.L^{-1} .

When diluted in Hoagland medium, these suspensions of TiO_2 -NPs immediately agglomerated. To prevent agglomeration, various dispersing agents were tested. The obtained suspensions are presented in Figure 1, which shows that NP agglomeration occurs in most conditions. Mean hydrodynamic diameters of TiO_2 -NP agglomerates are presented in Table 2.

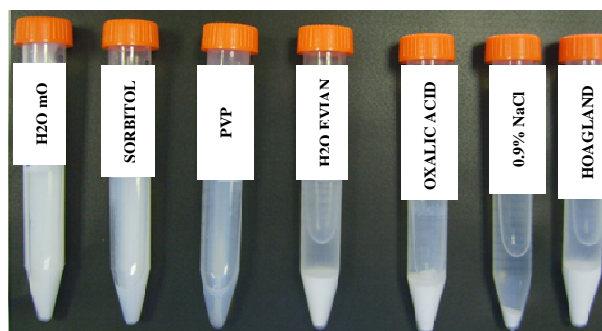


Figure 1. TiO₂-NP suspensions, use of a dispersing agent. From left to right: TiO₂-NPs sonicated in water, sorbitol, 1 g.l⁻¹ PVP, Evian water, oxalic acid, 0.9% NaCl, or sonicated in water and then diluted in Hoagland medium.

Table 2. Mean hydrodynamic diameter of NP agglomerates when using a dispersing agent^a

Dispersing agent	20 mM oxalic acid	1 g.l ⁻¹ PVP	10 g.l ⁻¹ PVP	1 mM sorbitol	0.9% NaCl	Evian water
After sonication	1536	46	245	41	1000	1668
Hoagland	1234	896	41	853	1126	746

^aMean hydrodynamic diameter was determined by PCS at 50 mg/L after a 30 mn sonication, in water containing a dispersing agent (oxalic acid or polyvinylpyrrolidone (PVP) or sorbitol or in physiologic serum (0.9% NaCl) or Evian water). Diameters are determined directly after sonication, or after sonication and dilution in Hoagland medium.

As regards these dispersion results, and considering that we aimed at exposing plants in a well-dispersed TiO₂-NP suspension, we chose to expose plants in TiO₂-NPs prepared in water without addition of any dispersing agent, which would not increase suspension quality.

3.2. Impact of TiO₂-NPs on plant development

The impact of TiO₂-NP exposure on plant development was evaluated on wheat seeds and plantlets, exposed to Evonik (Aeroxide® P25) TiO₂-NPs. Exposure did not induced any statistically significant modification in wheat seed germination (Figure 2B), nor in root elongation (Figure 2C). However, during root development, TiO₂-NPs strongly stuck onto roots and agglomerated in exposure medium (water, Figure 2A).

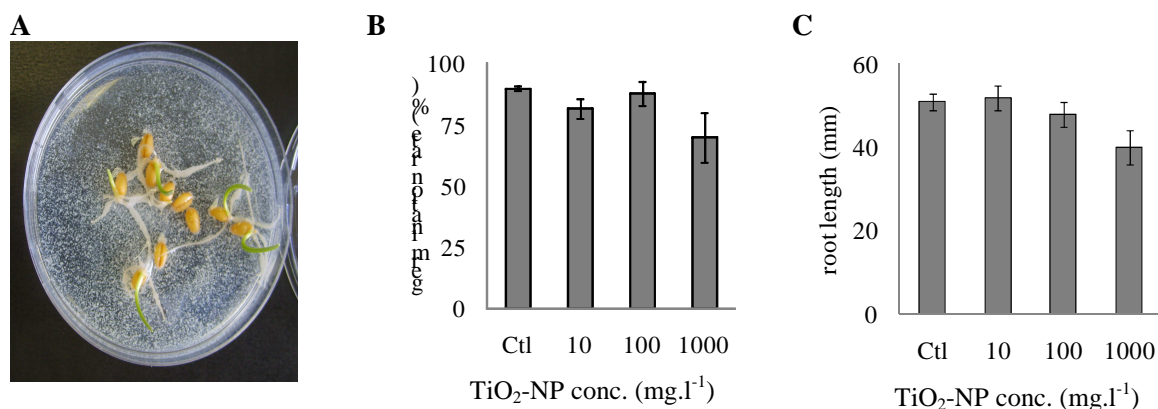


Figure 2. Seed germination test. Wheat seeds were exposed to Aeroxide® P25 TiO₂-NPs (A), and number of germinated seeds was counted (B), then the mean length of roots was measured (C).

3.3. TiO₂-NP accumulation in plants

Elemental analysis, in scanning mode, enabled to draw images of P content, representative of plant tissues, and of Ti content, representative of TiO₂-NPs, in plant roots and leaves after exposure. An example is presented in Figure 3, mapped on wheat plant roots after exposure to anatase, 12 nm TiO₂-NPs. We used these nanoparticles rather than Evonik nanoparticles because they were smaller, and thus would more probably be taken up by plants. Their agglomeration status and impact on plant development was close to that of Evonik nanoparticles (not shown). After 7 days of exposure, Ti was observed in the parenchyma of wheat roots (Figure 3, arrows), but also in the vascular cylinder, demonstrating that TiO₂-NPs were transferred from the exposure suspension to vegetal tissues.

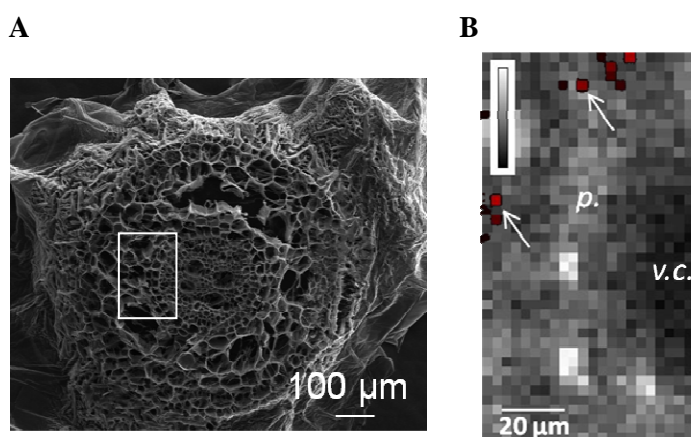


Figure 3. Ti distribution in wheat roots. Transversal sections of a root analyzed by SEM (A) and by μ -XRF image of a region related to the white square in A (B) after a 7-day exposure to 12 nm anatase TiO₂-NPs. In B: maps of P distribution (gray) and Ti distribution (red, clusters are also indicated by arrows) are superimposed. SEM image was recorded in ICMMO and μ -SR-XRF image on LUCIA beamline, SOLEIL synchrotron.

As described in several publications, Ti pre-edge features are characteristic of the crystalline phase and size of TiO₂-NPs. Anatase displays a typical triplet feature (A1, A3 and B peaks), with a weak shoulder on the low-energy side of the central A3 peak (A2) [14]. The intensity of A2 peak is related to the distortion of the octahedral TiO₆ unit, particularly the distortion observed on the surface of NP. The intensity of this peak is thus related to the size of the particle: the smaller the NP, the higher this A2 peak [15]. Meanwhile, the intensity of the A1 peak also decreases as the particle size decreases [16]. Analysis of this pre-edge region may then inform on changes in the crystalline phase of NPs after cellular internalization, and on their dissolution. In the present experiment, no modification of these pre-edge features was observed after plant internalization of anatase TiO₂-NPs (Figure 4), meaning that Ti is still in the nanoparticulate TiO₂ chemical form inside plants, and that their crystalline phase did not change. Moreover the size of NPs remained constant after plant internalization meaning that no partial dissolution of NPs occurred.

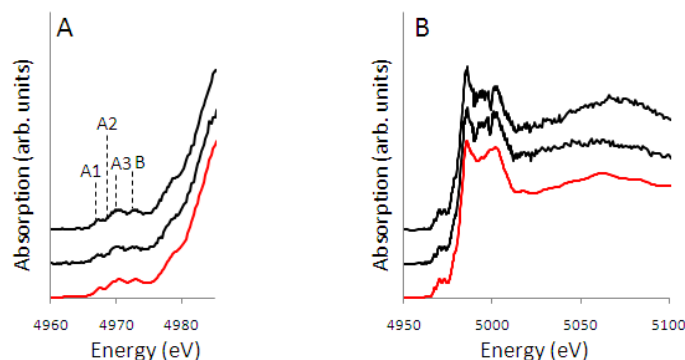


Figure 4. XANES spectra of Ti in plant roots exposed to TiO₂-NPs. Plants were exposed for 7 days to anatase TiO₂-NPs. Spectra were recorded at the Ti K-edge (4.966keV) at room temperature, locally on two Ti-rich regions determined on μ -SR-XRF maps (black spectra), and compared to the spectrum of a reference compound (red spectrum). A: pre-edge feature; B: XANES spectra. Image recorded on LUCIA beamline, SOLEIL synchrotron.

In the post-edge region, the oscillations show some differences in the samples as compared to reference compounds. However these spectra are noisy and their interpretation is difficult. Still, these changes may be due to surface modifications of TiO₂-NPs exposed to root exudates or plant fluids. A future experiment will aim at studying these changes which may occur in these conditions.

4. Conclusion

We determined sonication conditions enabling to properly disperse anatase NPs, the conclusion is that dispersion has to be performed in water, by ultra-sonication during 30 min. In these conditions, dilution in Hoagland medium leads to agglomeration, and none of the dispersing agent studied permits to maintain a stable dispersion.

Plant exposure to anatase, 25 nm NPs neither affect their seed germination rate, nor their root elongation. However 12 nm anatase NPs are efficiently taken up by plant roots and localize in the parenchymal region and vascular cylinder. Their nutrient solution-to-root transfer do not induce their dissolution, nor localized change in their crystalline structure.

μ PIXE experiment are under process and will permit to have a global view of the perturbations in major elements homeostasis which may be caused by TiO₂-NP uptake. Moreover, on all these plant samples, transmission electron microscopic observations will now enable to evaluate if isolated NPs are taken up by plants, or if these NPs are grouped into clusters. The present results prove that TiO₂-NPs can be transferred to plants by their roots in case of an accidental release in the environment, and consequently may enter the food chain.

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