Use of a modified Allium test with nanoTiO2

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Extensive production and wide application of TiO2 nanoparticles has stimulated research on its potential biological effects on different groups of organisms but the interaction of TiO2 nanoparticles with higher plants remains poorly understood. We have studied the effect of TiO2 nanoparticles on Allium cepa using a modification of the conventional Allium test with nanoparticles suspended in distilled water as opposed to growth medium. Nanoparticulate TiO2 was found to have low toxic potential and the mitotic index was among the most sensitive measures of the effect of nano-TiO2. We conclude that modified Allium test is suitable to provide comparative data on the biological potential of a variety of nanoparticles and could be used in a tiered approach to nanotoxicity testing.

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1. Introduction

Nanosized TiO2 is one of the most frequently used nanoparticles and is expected in the near future to appear in the environment in large amounts. Only recently, the first report of an actual occurrence of manufactured TiO2 nanoparticles in the environment (Kaegi et al., 2008) and calculations of its expected environmental concentrations were made (Mueller and Nowack, 2008). In a parallel finding, the toxic potential of TiO2 nanoparticles in the absence of UV irradiation was confirmed (Baun et al., 2008; Lee et al., 2009).

There has been an exponential increase in data on the effects of TiO2 nanoparticles on different species but there is much less information on the effects of nanoparticles on plants as opposed to animals. Studies of the effects of TiO2 nanoparticles on plants provide information about positive, stimulative as well as negative impacts. Kim and Lee (2005) described that TiO2 nanoparticles in combination with UV-A radiation inhibits the photosynthetic activity of algae Anabaena, Microcystis and Melosira. Hund-Rinke and Simon (2006) in their study observed that nanosized TiO2 may cause growth reduction of the alga Desmodesmus subspicatus. TiO2 nanoparticles caused also growth inhibition of algae Pseudokirchneriella subcapitata (Warheit et al., 2007). On the other hand TiO2 nanoparticles at certain concentrations had a positive effect on the germination of aged spinach seeds and on the growth of shoots (Zheng et al., 2005). The presence of TiO2 nanoparticles also reduces oxidative stress caused by UV-B radiation (Lei et al., 2008) but Seeger et al. (2009) observed no effects of nanosized TiO2 on growth, transpiration and water use efficiency in willow trees. Asli and Neumann (2009) recently showed that TiO2 nanoparticles, by filling the space among cellulose microfibriles in the cell walls, may have a negative effect on leaf growth, transpiration and root hydraulic conductivity in maize seedlings. In plant nanotoxicity studies, parameters that are frequently measured are growth, seed germination and metabolic processes, such as photosynthesis. For TiO2 nanoparticles, 1 mg/L was reported to be the lowest concentration observed to affect transcriptional expression profiling of stress response genes (Wang et al., 2008).

The plants most widely used in general toxicity studies include some algal species, e.g. Pseudokirchneriella subcapitata (Warheit et al., 2007; Baun et al., 2008; Wang et al., 2008; Hartmann et al., 2010; Arujo et al., 2009) and Desmodesmus subspicatus (Hund-Rinke and Simon, 2006; Rosa et al., 2006; Tišler et al., 2009; Daus et al., 2010). Among plant tests, that employing Allium is one of the most widely used. This is a short-term test, which can assess genotoxicity of different chemicals suspended in a test solution. The Allium test has also been proposed as a standard assay for biomonitoring of environmental pollutants (Fiskešjö, 1985).

From published data it is becoming clear that there are some important differences between testing the effects of soluble chemicals and the toxic potential of nanoparticles. These differences relate to changing characteristics of nanoparticles in different test and biological media. Murdock et al. (2008) showed that the size of nanoparticles in media with salts or organic molecules is markedly different from that in distilled water. Different authors have pointed to the fact that it is sometimes
impossible to compare the nanotoxicity data because particles were suspended in different media (Murdock et al. (2008); Ahamed et al., 2008).

The aim of this work was to assess the toxicity of TiO₂ nanoparticles at different biological levels by analysing macroscopic and microscopic parameters in Allium cepa in relation to the concentration of nano-TiO₂, exposure time and lighting conditions. We also sought to evaluate the potential of the adapted Allium test for routine testing of the effects of nanoparticles in higher plants.

2. Materials and methods

2.1. Plant material

Two cultivars of onion (A. cepa L.), 'Stuttgarter Riesen' and 'Hollander yellow' were used in this study. Onion bulbs were stored at 4 °C prior to use. For the experiments only bulbs of 3–5 g in good condition were selected.

2.2. Characterisation of TiO₂ nanoparticles

The nanoparticles of TiO₂ used in this study were obtained from Sigma-Aldrich, Germany. The manufacturer guaranteed a primary particle size of 15 nm, 99.7% purity of the sample and anatase crystalline structure. Nanoparticles were suspended in distilled water by vigorous shaking and the following suspensions were prepared: 0, 0.1, 1, 10, 100, and 1000 μg/mL.

Transmission electron microscopy (TEM), dynamic light scattering (DLS), Brunauer-Emmett-Teller (BET) and X-ray powder diffraction (XRD) analyses were used to characterize the nanoparticles in this study.

For TEM, dispersions of nanoparticles were applied to carbon-coated grids, dried at room temperature and examined with a 200 kV field emission transmission electron microscope (Philips CM 100, Koninklijke Philips Electronics, Eindhoven, The Netherlands).

Dispersions of nanoparticles at 10, 100 and 1000 μg/mL were inspected by DLS using a 3D DLS-SLS spectrometer (LS Instruments, Fribourg, Switzerland). This enables the determination of hydrodynamic radii of particles in extremely turbid systems. The mean values for all three parameters were calculated and statistical analysis was performed as described below.

2.3. Experimental set-up

We conducted two series of experiments. In the first, onions were exposed to nanoparticle suspensions for 24 and 72 h in normal lighting conditions. In the second, the plants were exposed to nanoparticle suspensions for the same periods in the presence of artificial UV-A radiation provided by a Sylvania light bulb blacklight (wavelength of 632.8 nm) as the light source and scattering was measured at an angle of 90°.

Suspensions of samples, dried and degassed with nitrogen prior to analysis, were examined using BET analysis (Tristar 3000, Micrometritics Co., Norcross, GA, USA) to obtain information about the surface area of solid material.

Titanium dioxide samples were monitored by X-ray powder diffraction (XRD) using a Bruker AXS D4 Endeavor diffractometer (Karlsruhe, Germany) with Cu-Kα, radiation and a Sol-X energy dispersive detector within the angular range 20° < 2θ < 80° with a step size of 0.04° and a collection time of 3 s.

2.4. Statistical analysis

The differences in the measured macroscopic parameters and mitotic index between treated and control groups were tested applying the non-parametric Mann-Whitney test. All calculations were done using STATGRAPHICS Plus 4.0 statistics software. The level of significance was accepted at p ≤ 0.05. Genotoxicity (frequency of chromosome aberrations and micronuclei) was statistically analysed by Student’s t-test and ANOVA due to low occurrence of parameters (GraphPad Prism). The level of significance was accepted at p ≤ 0.05. Symbols in the box plot represent centils (whiskers: +) and the mean value ( ■ ); box plot contains 50% of all values.

3. Results

In our study we analysed macroscopic parameters (number and average length of roots and total length of the root system for each bulb) and microscopic parameters (mitotic index, shares/ portions of mitotic phases, chromosome aberrations and micronuclei) in the root meristem of A. cepa incubated in a suspension of TiO₂ nanoparticles for 24 h or 72 h. To minimise the effect of medium on nanoparticles, we adapted the test protocol with normal lighting conditions. The growth medium was replaced daily. We compared the effect of distilled water with that of tap water on the activity of the root tips.

2.3.2. Microscopic parameters

Macroscopic parameters were measured after 72 h of exposure. The roots were cut at their base and the number counted and the length measured. The length of all roots per bulb was summarised and expressed as the total length of the root system. The mean values for all three parameters were calculated and statistical analysis was performed as described below.

Ten roots of each bulb were fixed in a freshly prepared mixture of absolute ethanol and glacial acetic acid (3:1 v/v) for 24 h at 4 °C and then stored in 96% ethanol at −20 °C until subsequent use in microscopic analysis.

2.3.3. Microscopic parameters

Observations of microscopic parameters were made after 24 h and 72 h exposures to TiO₂ nanoparticles. Three of ten fixed root tips were used from each bulb (15 root tips for each concentration) to use prepare slides for microscopic analysis. Except in the case of 72 h exposure to nanoparticles in normal lighting conditions, 9 or 10 root tips of each concentration were used to provide microscopic slides (exact numbers are given in the Results section).

The fixed roots were hydrolysed in 5 M HCl, stained with Feulgen reagent and washed in SO₂-water to remove excess dye (Dolenc Kocic et al., 2001). Each root tip was sectioned in the F0 and F1 regions. The F0 region was prepared by cutting the first millimetre behind the root cap and contained only meristematic cells. The following F1 section was prepared by cutting the second millimetre of the root tip, containing dividing and elongating cells of the root tip (Fig. 1). Squash preparations of each section were prepared in a drop of 45% acetic acid. The slides were mounted in DPX Mounting Medium (Fisons, UK) and dried in the dark for at least 3 days. The slides were analysed with a Motic BA210 light microscope at 1000× magnification. For each slide, the mitotic index, portion of mitotic phases, the presence and frequency of chromosome aberrations (fragments, anaphase bridges, c-mitosis) and of micronuclei were determined (Fig. 2). The mitotic index was calculated as the number of mitotic cells divided by the total number of cells. A minimum of 1000 cells was counted in each slide.

Fig. 1. Light micrograph of regions F0 and F1 in Allium cepa root tip.
A. cepa by exposing the bulbs to distilled water instead of tap water.

3.1. Characterisation of TiO₂ nanoparticles

Transmission electron microscopy reveals the shape and the size of nanoparticles. Dynamic light scattering analysis was used to assess the size of aggregates in suspensions of nanosized TiO₂. The average value of the hydrodynamic radius of TiO₂ nanoparticles in distilled water dispersion was 870 nm.

The Brunauer–Emmitt–Teller (BET) method was used to assess the surface area of TiO₂ nanoparticles used. The surface area of TiO₂ was 144 m²/g and thus the size of TiO₂ nanoparticles were approximately 10 nm.

X-ray powder diffraction (XRD) confirmed that the 10 nm TiO₂ nanoparticles were in anatase crystal form.

3.2. Preliminary experiment

In the preliminary experiments we assessed effect of distilled water on A. cepa by comparing the bulbs grown in distilled water with those growing in tap water, the latter being usually used as a negative control (Fiskesjö, 1985). The median value of the mitotic index in the F0 region was 6.38 in the roots growing in tap water and 3.00 in the roots growing in distilled water (p = 0.007, N = 5). In F1 region median value of mitotic index was 4.61 in the roots growing in tap water and 2.49 in the roots, which were grown in distilled water (p = 0.012, N = 5). Thus the mitotic index of the roots grown in distilled water was in both regions (F0 and F1) statistically significantly reduced when compared with the roots grown in tap water. Distilled water is a less favourable growing medium, but since nanotoxicity test results are given in relative terms, i.e. expressed as differences from the control, we used distilled water as a suspension medium for nanoparticles. Distilled water was already used as negative control in Allium test in several ecotoxicological studies (Kumari et al., 2009, Yildiz et al., 2009).

3.3. Macroscopic parameters

After 72 h of exposure to TiO₂ nanoparticles, the numbers of roots per bulb, the average length of roots and total length of the root system of each bulb were measured. Macroscopic parameters in bulbs exposed to TiO₂ nanoparticles were not statistically significantly different from those in a control group (Fig. 3).

3.4. Microscopic parameters

3.4.1. Mitotic index

When onions were exposed to five different concentrations of TiO₂ nanoparticle suspensions for 24 h in normal lighting conditions, we observed a slight increase of the mitotic index in both regions (F0 and F1) of treated root tips as compared to negative controls (Figs. 4a and b). A statistically significant increase of mitotic index in the region F0 was observed only at concentrations of 0.1 mg/mL TiO₂ (p = 0.047, N = 15) and 1000 µg/mL TiO₂ (p = 0.042, N = 15) (Fig. 4a). In the F1 region of the treated root tips a statistically significant increase of mitotic index was observed at a concentration of 1000 µg/mL of TiO₂ nanoparticles (p = 0.05, N = 15) (Fig. 4b).

When onions were exposed to both TiO₂ nanoparticles and UV-A radiation for 24 h, we did not observe any effect on the mitotic index in the regions F0 and F1 of the roots (Figs. 4c and d). In general, the mitotic index was lower in F1 region compared to F0 region due to different structure of root tip regions. F1 region consists from meristematic as well as elongating cells, which arrest in interphase and do not divide while cells in F0 region actively divide.

Exposure of onions to the test suspensions of TiO₂ nanoparticles for 72 h in normal lighting conditions, produced a statistically significant decrease of the mitotic index in the F1 region after exposure to the highest concentration of nanoparticles, 1000 µg/mL TiO₂ (p = 0.036, N = 10), when compared to negative control (Fig. 5b). We did not detect statistically significant differences between the treated and negative control.
roots in the F0 region (Fig. 5a). When onions were exposed to both TiO$_2$ nanoparticles and UV-A radiation for 72 h, a statistically significant decrease of mitotic index occurred again in the F1 region after exposures to 10 and 100 $\mu$g/mL of nanosized TiO$_2$ ($p=0.02$, $N=15$ and $p=0.00004$, $N=15$) (Fig. 5d). In the F0 region we did not observe any effect on the mitotic index after exposure to TiO$_2$ nanoparticles (Fig. 5c).

### 3.4.2. Mitotic phase shares

In parallel with mitotic index, each sample was also analysed for the mitotic phases of nuclei in the dividing cells of regions F0 and F1. The data obtained suggest that the presence of TiO$_2$ nanoparticles in test suspensions after 24 h exposure did not affect the course of mitosis in either UV-A irradiated bulbs or those without UV-A irradiation (Figs. 6a and b). The share/portion

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**Fig. 3.** Macroscopic parameters (number of roots, average length of roots and total length of root system) after 72 h of exposure to TiO$_2$ nanoparticles: (a–c) in normal lighting conditions and (d–f) in presence of UV-A radiation. 1 mM EMS was used as negative control.
of each mitotic phase found by investigation of the divided nuclei in treated roots was similar as in the roots of the negative control. After 72 h of exposure to TiO$_2$ nanoparticles in test suspensions, the course of mitotic phases in root meristem also was not significantly affected. However, some statistically significant differences in normal lighting and under UV-A irradiation were observed in shares of mitotic phases when treated plants were compared to negative controls (Figs. 7a and b). Otherwise we did not observe a consistent trend of differences in the shares of mitotic phases.

### 3.4.3. Genotoxicity

The genotoxic effects of tested TiO$_2$ nanoparticles were analysed by recording the type and the frequency of chromosome aberrations and the presence of micronuclei. It appears that the frequencies of aberrations and abundance of micronuclei were low, in the same range as in the negative controls, irrespective of exposure duration and concentrations of nanoparticles and UV-A radiation (Table 1). This indicates that TiO$_2$ nanoparticles did not cause any significant genotoxic effects either under UV-A irradiation or under normal lighting conditions.

### 3.5. Comparison of results between negative and positive control groups

Measurement of macroscopic parameters showed statistically significant differences between negative and positive control groups. Although the numbers of roots per bulb in the negative and positive control groups did not differ, the roots of positive control bulbs preteated with EMS, were however significantly shorter and this is further reflected in significant reductions in the total length of the root system.

Microscopic analysis revealed minor reductions in the mitotic index after 24 h of exposure to EMS while after 72 h of exposure, regardless of lighting conditions, reduction of mitotic index and changes of shares of mitotic phases were significant. Results of genotoxicity analysis showed a statistically significant increase in the formation of micronuclei in EMS treated plants.

### 4. Discussion

Nanosized TiO$_2$ failed to affect measured macroscopic parameters (number of roots, average length of roots or total length of the root system) of *A. cepa* after exposure to a range of concentrations of TiO$_2$ nanoparticles (0.1–1000 µg/mL) suspended in water for 72 h (Fig. 3). We did not observe significant effects on shares of mitotic phases, as well as on chromosome aberrations and micronuclei incidence after exposure to nanosized TiO$_2$ for 24 or 72 h. These parameters are obviously not sensitive enough to show any differences among differently treated groups with nano-TiO$_2$. However, TiO$_2$ nanoparticle exposure significantly affects the mitotic index in root tips.

Our results obtained on macroscopic parameters are in agreement with those of Seeger et al. (2009) who found no statistically significant differences in growth of willow trees after exposure to TiO$_2$ nanoparticles in the range of concentrations of
1–100 mg/L TiO₂ for 235 h. Other studies however have reported toxicity of nanosized TiO₂ on growth of algae at concentrations up to 380 mg/L (Hund-Rinke and Simon, 2006; Warheit et al., 2007; Arujo et al., 2009; Hartmann et al. 2010) and this indicates either different sensitivity to nanosized TiO₂ of disparate plant species or differing behaviour of nanoparticles in various suspension media.

In our study, some microscopic parameters proved to be more sensitive than macroscopic parameters. Short term exposure (24 h) caused a slight stimulatory effect on the mitotic activity of the root tips in both regions (F0 and F1) under normal lighting conditions (Figs. 4a, b). After 72 h of exposure to TiO₂ nanoparticles in normal lighting conditions, no effect was observed in the F0 region and some reduction of mitotic index was detected in the F1 region (Fig. 5b). Kumari et al. (2009) also recorded a concentration dependant decrease in the mitotic index when A. cepa roots were treated with manufactured silver nanoparticles, but our study failed to reproduce the concentration dependency. The different response to silver nanoparticles is probably due to the mode of action of nanosized TiO₂, which differs from that of silver nanoparticles. The toxic potential of silver nanoparticles could be ascribed to the dissolution of silver ions off the surface of particles, which has no parallel in the case of nano-TiO₂ (Navarro et al., 2008).

When bulbs were exposed to TiO₂ nanoparticle suspensions and UV-A radiation for 24 h none of the TiO₂ concentrations used proved to be stimulatory (Figs. 4c, d). However, after 72 h of exposure to TiO₂ and UV-A radiation, inhibition of mitotic index was detected in some cases in the same range as in positive control (Fig. 5d) indicating that meristem region is affected. Several studies on animal cell lines have reported increased toxicity of nanosized TiO₂ in presence UV radiation (Dunford et al., 1997; Reeves et al., 2008; Zhu et al., 2009). UV radiation enhances the toxic potential of anatase TiO2 nanoparticles through additional reactive oxygen species formation (Zhu et al., 2009) but there are no similar studies published on UV-irradiated nanosized TiO2 and higher plants.

Other microscopic parameters such as shares of mitotic phases, frequency of chromosome aberrations and micronuclei were not affected by nanosized TiO₂. Studies on different cell lines reported an increase in DNA damage when cells were exposed to TiO₂ nanoparticles at concentrations up to 380 μg/mL (Reeves et al., 2008; Karlsson et al., 2009; Falck et al., 2009; Di Virgilio et al., 2010). Different organisms or cells lines are expected to have different sensitivity to nanoparticles or the effect is modulated by the suspension of the nanoparticles (Navarro et al., 2008; Singh et al., 2009).

Some other authors have also confirmed that, like parameters at lower levels of biological complexity, macroscopic parameters such as growth are less sensitive to exposure to TiO₂ nanoparticles. For example, Hund-Rinke and Simon (2006) confirmed negative effects of nanosized TiO₂ on photosynthetic activity and reproduction of algae, but they did not observe any effects on the growth of algae.

In the work presented here we have selected two exposure durations, 24 and 72 h. On the basis of the results obtained, we conclude that the exposure period may play a role in interpreting biological reactivity of nanoparticles. The same concentration of
TiO₂ nanoparticles, which stimulated division of cells in root meristem after a brief exposure had no effect, or was in some cases inhibitory when bulbs were exposed for longer periods (72 h). A similar conclusion regarding the exposure period was also reached in in vivo study of nanosized TiO₂ toxicity on Daphnia magna (Zhu et al., 2010).

The tested concentrations selected in our study are generally higher than those expected in the environment. Mueller and Nowack (2008) predicted that the environmental concentrations of TiO₂ nanoparticles are expected to be 0.7–16 μg/L in water and 0.4–4.8 μg/kg in soil. Such environmental concentrations are potentially not harmful to Allium. Whether or not these data could be extrapolated to other higher plants and environmental conditions are questions that require further research. However, the major contribution of this current study is that the modified Allium test has been successfully applied to an assessment of the effects of nanoparticles suspended in a nutrient deprived medium. This is significant because the effects of nanoparticles vary with the medium in which they are suspended. It has been shown that particle size in suspension media can be dramatically different from that in distilled water (Murdock et al., 2008), and this may alter the response of exposed plants.

5. Conclusion

The tested concentrations of TiO₂ nanoparticles up to 1000 μg/mL were not toxic to A. cepa in 24 or 72 h of exposure but a conclusion that there were no observed effect concentrations (NOEC) could not be drawn. After 24 h of exposure under...
normal lighting conditions or under UV-A radiation, TiO₂ nanoparticles had a slight stimulatory effect on the mitotic activity of root meristem. After 72 h of exposure the effects of nanosized TiO₂ in some of the exposed groups was slightly inhibitory regardless of the lighting conditions. Our data show that the response of Allium roots was duration as well as concentration dependent.

The adapted Allium test could be used to provide comparative data on the biological potential of nanoparticles. By testing the effects of nanoparticles in a distilled water medium, we minimised the impact of medium on the behaviour of nanoparticles in a suspension. As a consequence, such a biological test has a potential used also for other nanoparticles where the effects of medium is expected to influence their behaviour.

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