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The cytotoxic targets of anatase or rutile + anatase nanoparticles depend on the plant species

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Abstract

The potential toxicity of nanoparticles (NPs) is under debate. Information about TiO_2 NPs phytotoxicity is still limited partly due to the different TiO_2 NP forms that may be found in the environment. The present work investigated the impact of different TiO_2 NPs forms (rutile and anatase) on germination, growth, cell cycle profile, ploidy level, and micronucleus formation in *Lactuca sativa* (lettuce) and *Ocimum basilicum* (basil). Seeds were exposed to anatase (ana) or rutile + anatase (rut+ana) at concentrations 5 - 150 mg dm^{-3} for 5 d and after that different parameters were analyzed. Rut+ana showed high potential to impair germination and growth. On the other hand, ana alone showed a positive influence on seedling growth. Despite that, ana induced severe alterations in cell cycle dynamics. Regarding species, basil was more sensitive to TiO_2 NPs cytostatic effects (delay/arrest in G_0/G_1 phase), whereas in lettuce, TiO_2 NPs were more genotoxic (micronucleus formation increase). Finally, we propose that, besides germination and plant growth, cell cycle dynamics and micronucleus formation can be sensitive biomarkers of these NPs.

Additional key words: abiotic stress, cell cycle, genotoxicity, micronucleus, phytotoxicity, titanium dioxide.

Introduction

In the last years, the advancements in the nanotechnology resulted in a myriad of applications and the consequent increase of nanoparticles (NPs) release to the environment (Ma *et al.* 2015a, Shi *et al.* 2013). The interest on NPs is mostly due to their unique properties such as small size and large surface area to mass volume which enhance their reactivity, when compared to non-nanosized equivalents (Jiang *et al.* 2014a). Nevertheless, the release of NPs to the environment raises questions on their toxicity, with recent data demonstrating evident toxicity of metal NPs (*e.g.* Ag, ZnO) in microorganisms, animals, and in plants, though much less studied in this last group (Ma *et al.* 2015a, Srivastava *et al.* 2015). Therefore, the potential negative impacts of NPs to living organisms need to be fully characterized and understood, in order to minimize their impact and consequences (Ma *et al.* 2015a). Presently, the consequences of the presence of NPs in the environment on plant growth and yield are of great concern (Garcia-Sanchez *et al.* 2015). Thus, the effects of NPs on plants at physiological, biochemical, and molecular levels need to be further studied. Some of the reported consequences of NPs exposure are growth inhibition (Begum *et al.* 2014, Nair and Chung 2015) or delay (Cui *et al.* 2014a), reactive oxygen species (ROS) production (Jiang *et al.* 2014b, Nair and Chung 2015) and genotoxicity (Anjum *et al.* 2015, Bandyopadhyay *et al.* 2015, Chen *et al.* 2015, Ma *et al.* 2015b). However, plant responses to NPs exposure depend on plant species, NPs characteristics, and growth conditions. Nevertheless, despite NPs-induced phytotoxicity reported for several plant species, positive effects were also observed (Cui *et al.* 2014b, Tumburu *et al.* 2015).

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Abbreviations: Ana - anatase; MN - micronuclei; NP - nanoparticle; rut - rutile.
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One of the metallic NPs widely used in industry is titanium dioxide NPs (Shi *et al.* 2013). Titanium dioxide exists naturally in three forms: rutile (rut), anatase (ana), and brookite. Anatase (ana) is more reactive than rutile (rut) and generates reactive oxygen species (ROS) under UV radiation, which leads some authors to suggest that ana is more toxic than rut (Shi *et al.* 2013). On other hand, the nano-sized TiO_2 in comparison to the non-nano-sized TiO_2 presents higher photocatalytic capability (Ricci *et al.* 2013) and increased bioactivity (Shi *et al.* 2013). Nano-mixture of rut + ana (P25) is a white powder with hydrophilic character and it is more photocatalytic than pure ana (Hurum *et al.* 2003, Kurepa *et al.* 2010). The TiO_2 NPs may be found in food as antimicrobial agent and colorant, in cosmetics, in electronics, medicines, ceramic and metal materials, *etc*. (Weir *et al.* 2012, Jiang *et al.* 2014a, Cox *et al.* 2016). For example, rut + ana NPs are used as catalyst and photocatalyst in water remediation (Weir *et al.* 2012), whereas pure ana or rut are used as white pigments in food industry (Weir *et al.* 2012).

With the increased production of TiO_2 NPs, their release to the environment and the accumulation in soil and water also increases (Gottschalk *et al.* 2013, Sun *et al.* 2015). Predicted environmental concentrations indicate that nano TiO_2 accumulation in soil may have reached 450 μg kg^{-1} in some areas (Sun *et al.* 2015). Therefore, in the past few years several studies have been performed on the effects of TiO_2-NPs exposure on plants. Results are variable and show that TiO_2 NPs may become phytotoxic, but also may have neutral or positive effect on seed germination and plant growth, as in fennel, wheat, cucumber, onion, chickpea, and tomato (Seeger *et al.* 2009, Feizi *et al.* 2012, 2013, Larue *et al.* 2012a,b, 2014, Servin *et al.* 2012, Ardakani 2013, Song *et al.* 2013, Mohammadi *et al.* 2014, Pakrashi *et al.* 2014). Some studies reported non-specific effects of TiO_2 NPs, such as germination and growth inhibition (Castiglione *et al.* 2011, Clement *et al.* 2013), while others report cytotoxicity, such as increase in lipid peroxidation (Ghosh *et al.* 2010) or genotoxicity such as alterations in mitotic index, chromosomal aberrations, DNA damage, and micronucleus formation (Ghosh *et al.* 2010, Castiglione *et al.* 2011, Pakrashi *et al.* 2014). Furthermore, TiO_2 NPs were reported to be potentially hazardous to the *Rhizobium*-legume symbiosis system (Fan *et al.* 2014). On the other hand, TiO_2 NPs improve P uptake in lettuce plants (Hanif *et al.* 2015) and N assimilation in spinach (Yang *et al.* 2007), increase adhesion of beneficial bacteria on roots of rapeseed, and protect the plants against infection (Palmqvist *et al.* 2015).

The effects of TiO_2 NPs exposure are greatly dependent on TiO_2 NPs size, shape, and concentration, on plant species and growth conditions (Hawthorne *et al.* 2012, Larue *et al.* 2012a, Miralles *et al.* 2012). Furthermore, NP form also seems to play an important role on its phytotoxicity, but the available information is still very limited (Silva *et al.* 2016). Since so many different effects of TiO_2 NPs were observed and since they are dependent of many other variables, it is not yet possible to define TiO_2 NPs toxicity mechanisms.

Having in mind that rut + ana mixture is mostly composed by ana (80 - 90 %) and is more photocatalytic than pure ana, we hypothetise that both forms present different phytotoxicity degrees, which may be species dependent. Based on our previous work (Silva *et al.* 2016) and on the work of Larue *et al.* (2012a) who demonstrated for wheat no phytotoxic differences between ana *versus* rut, we compared the phytotoxic effects of rut + ana *versus* ana on seedlings of two species *Lactuca sativa* and *Ocimum basilicum*. Several parameters were analyzed including germination, seedling growth rates, cell cycle profile, and micronucleus formation to identify most sensitive endpoints to these NPs.

Materials and methods

Nanoparticles dispersion and characterization: TiO_2-NPs (powder) were acquired from *Sigma Aldrich* (St. Louis, MO, USA) with a purity ≥ 99.5 % in two forms: anatase (ana) and rutile + anatase (rut+ana; about 20:80, aeroxide P25). According to the manufacturer rut+ana NPs have length of 21 nm and surface area of 35 - 65 m^2 g^{-1}, ana NPs have length < 25 nm and surface area of 45 - 55 m^2 g^{-1}. Stock suspensions (1 g dm^{-3}) were prepared in *Milli-Q* (*Synergy, Merck Millipore*, Darmstadt, Germany) water and then sonicated with a probe for 30 min with 16 W output to avoid aggregation. The physicochemical characterization of these NPs was reported in previous paper (Silva *et al.* 2016) and is summarized in Table 1 Suppl. NPs were then dispersed in agarized ultra-pure water. Briefly, an appropriate volume of NPs stock was added to heated (50 °C) *Milli-Q* water with 0.8 % (m/v) agar to obtain final concentrations of 5, 10, 50, 100, and 150 mg dm^{-3} TiO_2. For germination, 20 cm^3 of melted agarized water with/without NPs was transferred to Petri dishes and allowed to solidify at 4 °C.

Plants and growth conditions: *Ocimum basilicum* L. and *Lactuca sativa* L. seeds were surface disinfected using sodium hypochlorite (20 %, v/v), rinsed in *Milli-Q* water and placed on Petri dishes with 20 cm^3 of agarized water (on average 30 seeds per dish) containing 0, 5, 10, 50, 100, and 150 mg dm^{-3} ana or rut+ana TiO_2 NPs and allowed to germinate for 5 d in the dark at 24 °C. Then, the germination rate, seedling biomass, and shoot and root lengths were assessed. For germination rate determination, three Petri dishes were screened and for growth measurements three to six seedlings from three different dishes were used.

DNA content and cell cycle analysis: For flow cytometry studies, nuclear suspensions of root apices

(5 - 6 pools of 5 - 8 apices from three different Petri dishes) were obtained by chopping the roots in Woody Plant Buffer (Loureiro *et al.* 2007) and processed according to Silva *et al.* (2016). Nuclei were stained with 50 µg cm^{-3} propidium iodide (PI, *Sigma,* St. Louis, USA) and 50 µg cm^{-3} of RNAse (*Sigma*) was added to the suspension to avoid PI staining of RNA. Nuclei were then analyzed in a *Coulter EPICS-XL* flow cytometer (*Coulter Electronics*, Hialeah, FL, USA) equipped with an air-cooled argon-ion laser (15 mW operating at 488 nm). Prior to analysis, the instrument was checked for linearity and the settings were kept constant throughout the experiment. For each sample, the number of analyzed nuclei was approximately 5 000. The percentage of nuclei in each phase of the cell cycle (G_0/G_1, S, and G_2) and ploidy were analyzed using the *FlowJo* software (*Tree Star*, Ashland, OR, USA).

Micronucleus assays: For micronuclei (MN) assessment, root apices were fixed in Carnoy's solution (methanol + acetic acid, 3:1) and stored at 4 °C. The apices were then hydrolyzed in 1 M HCl at 70 °C for 7 min, washed in water and stained with PI. MN were scored in a fluorescent microscope *Eclipse 80i* and images were acquired with a digital camera; software *NIS-Elements F 3.00 SP7* (*Nikon*, Tokyo, Japan). For each condition 2 apices from three different plates were counted for MN. From each apex 1 000 cells were scored.

Statistical analysis: The comparison between the treatments was made using a one-way *ANOVA*, followed by Holm-Sidak multiple comparison procedure. Data normality distribution was tested and when normality failed data were transformed or Kruskal-Wallis one-way *ANOVA* on Ranks test was used followed by Dunn's multiple comparison procedure. The statistical significance was set at $P < 0.05$.

Results

Seed germination rate under control conditions was in an average of 94 ± 7 % for lettuce and of 88 ± 8 % for basil. Germination rates were not significantly affected by TiO_2 NP exposure in both species (Fig. 1). Nevertheless, for lettuce a trend for decrease in germination rate under rut+ana exposure was observed. In both species, rut+ana inhibited at a higher extension germination rate than ana (at 50 and 100 mg dm^{-3} for lettuce and 50 mg dm^{-3} for basil). Furthermore, the decreases of germination induced by rut+ana were more severe in lettuce (-13 % to -27 %) than in basil (-0.5 % and -7 %).

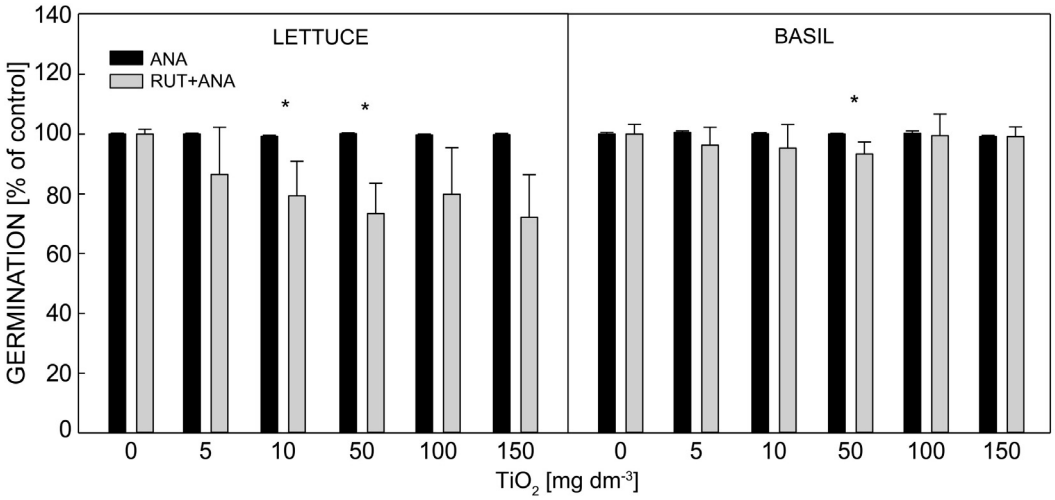

Fig. 1. Germination rates after exposure to 0, 5, 10, 50, 100, and 150 mg dm^{-3} anatase (ANA) or rutile + anatase (RUT+ANA) TiO_2 NPs. Means ± SDs, $n = 3 \times 30$, asterisks denote significant differences ($P \leq 0.05$) between RUT+ANA and ANA.

Concerning growth, basil seedlings exposed to NPs had a total length similar to controls (Fig. 2), showing that none of the NPs forms significantly affected this parameter. Nevertheless, basil seedlings exposed to ana presented higher variation in length (-7.5 to 13.6 % of controls) than those exposed to rut+ana (0.9 to 7.8 % of control). Significant differences between forms were observed at 5 mg dm^{-3} in shoot length. Regarding lettuce, different forms led to distinct responses: rut+ana did not influence seedling length, whereas ana stimulated length in all concentrations (Fig. 2). On the other hand, the effect induced on organs length was dependent on the concentration. In particular, seedlings exposed to 5 mg dm^{-3} ana showed a total length stimulation of 48.5 % (regarding untreated seedlings, $P < 0.05$). Shoot length increased at 5, 10, 50, and 100 mg dm^{-3} ana (by 84.6, 68, 43.7, and 60.3 % respectively), whereas root length increased only in seedlings exposed to

100 mg dm^{-3} ana (by 26 %).

The effect of TiO_2 NPs on plant biomass was also species and NP form dependent. Compared to the fresh mass of the control plants (basil: 7.6 ± 3.37 mg and lettuce: 12.6 ± 3.83 mg; Fig. 3), seedlings exposed to ana showed a trend to increase biomass, which was only significant in basil (Fig. 3). On the other hand, when exposed to rut+ana there was a decrease in seedling biomass, this response being mostly evident in lettuce (Fig. 3). The decrease in lettuce biomass ranged from 3.6 to 34.3 % in whole seedlings, from 13.3 to 31.7 % in shoots and from 21.9 to 45 % in roots. The impairment in lettuce biomass was mainly detected in roots and when seedlings were exposed to 5, 10, and 100 mg dm^{-3} rut+ana. Under 5 mg dm^{-3} rut+ana both lettuce shoots and roots were negatively affected. In basil, root biomass decreased only when seedlings were exposed to 150 mg dm^{-3} rut+ana (Fig. 3).

The histogram of control roots was the typical diploid with dominant G_0/G_1. All exposed roots showed similar diploid histogram and no occurrence of aneuploidisation nor polyploidisation was detected (Fig. 1 Suppl.). Nevertheless, cell cycle profile of the different populations was affected by TiO_2 NPs (Fig. 4). Rut+ana exposed roots of both lettuce and basil did not present significant differences when compared to the control roots regarding cell cycle dynamics. Contrarily, ana increased the relative percentage of the subpopulation of cells in G_0/G_1 phase and decreased the relative percentage of cells in S phase in basil roots (Fig. 4, and 1 Suppl.), with similar responses at all concentrations. At 5 mg dm^{-3} ana, basil roots also showed a decrease of the relative percentage ($P < 0.05$) of cells in G_2. Comparing the two forms, roots of both species showed distinct cell cycle profiles: roots showed higher percentage of cells in G_0/G_1 phases and less number in S phase under ana than under rut+ana.

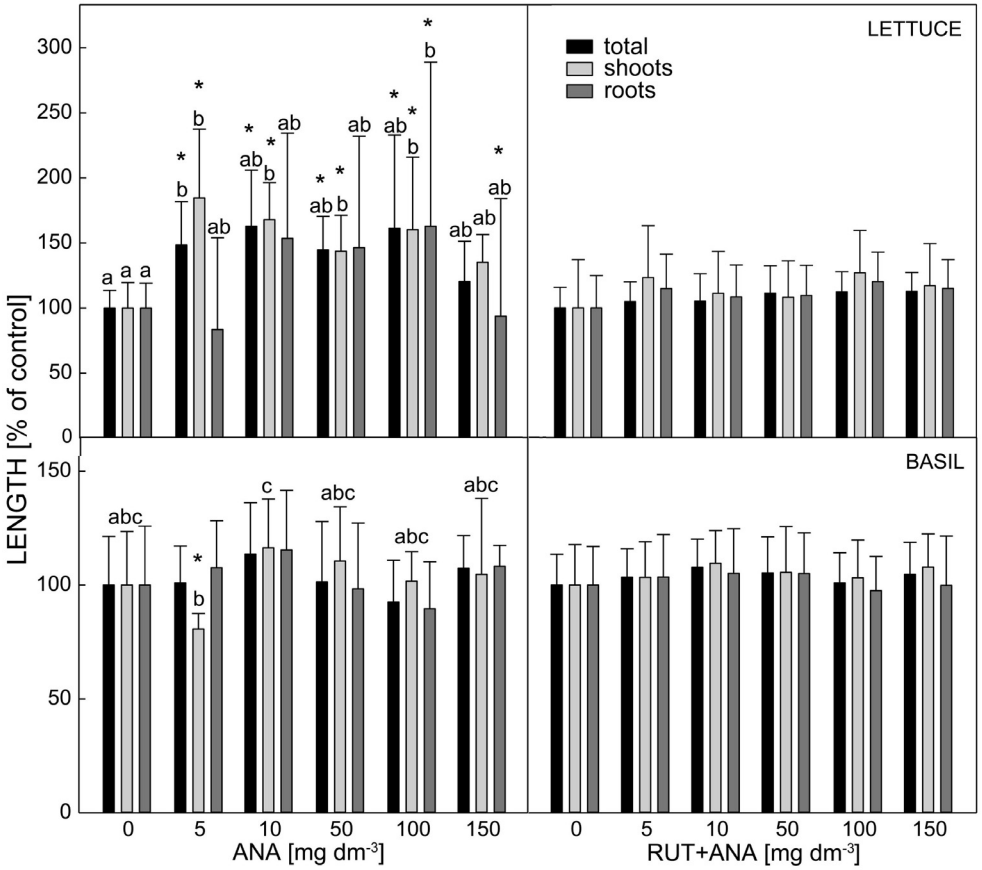

Fig. 2. Seedling length after exposure to anatase (ANA) or rutile + anatase (RUT+ANA) TiO_2 NPs. Total length in control plants was 2.3 ± 0.22 cm for basil and 3.6 ± 1.06 cm for lettuce. Means ± SDs, $n \approx 5 \times 3$, *asterisks* denote significant differences ($P \leq 0.05$) between RUT+ANA and ANA, and different letters denote significant differences between ANA and control .

Lettuce control roots presented on average 1.3 ± 2.45 ‰ cells with micronuclei and only in this species TiO_2 NPs induced MN formation (Fig. 2 Suppl.): in roots exposed to ana the number of cells with MN increased under 150 mg dm^{-3} (7.75 ‰ cells), whereas in rut+ana after the exposure to 50 mg dm^{-3} (13.25 ‰ cells). Nevertheless, a trend to increase the frequency of MN was detected in all rut+ana concentrations and in 50 - 150 mg dm^{-3} ana. Comparing NP forms, rut+ana enhanced the number of MN at lower concentrations

(5 and 10 mg dm^{-3}), whereas at the highest concentration (150 mg dm^{-3}) the number of MN was higher when roots were exposed to ana. Basil controls presented undetectable MN and the seedlings exposed to both NP forms were not affected in this parameter (Fig. 2 Suppl.).

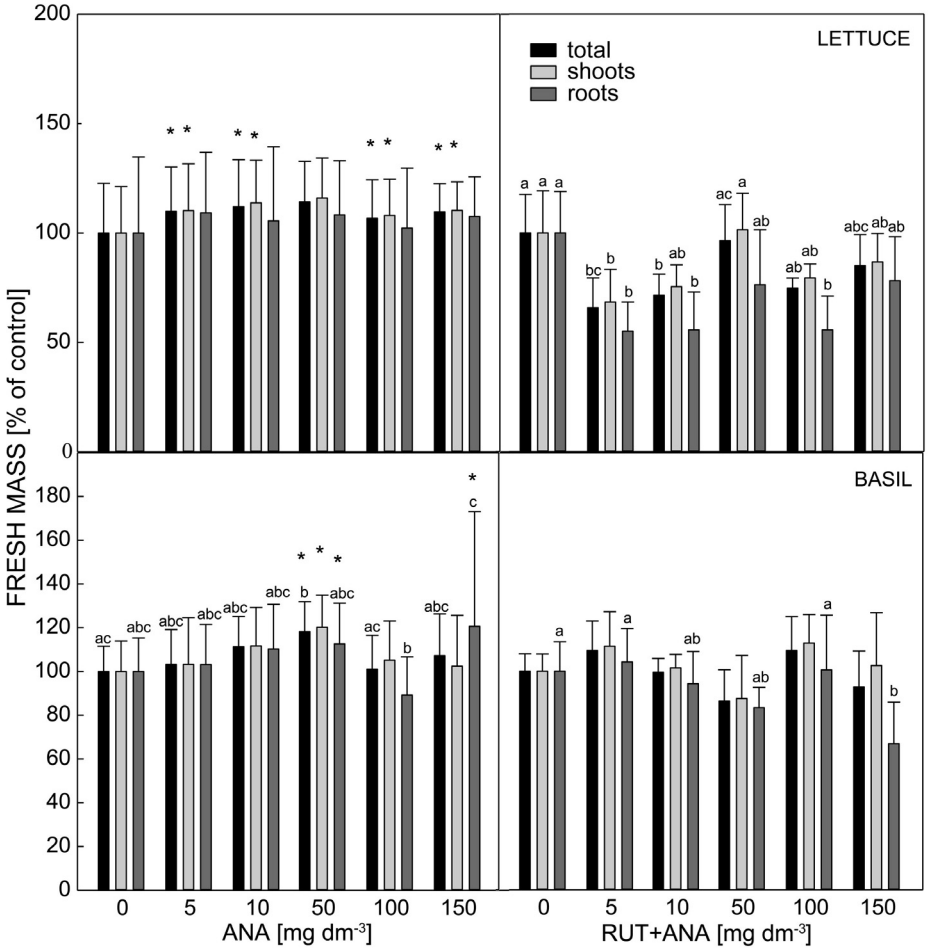

Fig. 3. Seedling fresh mass after exposure to anatase (ANA) or rutile + anatase (RUT+ANA) TiO_2 NPs. Control plant fresh mass was 7.6 ± 3.37 mg and 12.6 ± 3.83 mg in basil and lettuce, respectively. Means ± SDs, $n \approx 5 \times 3$, asterisks denote significant differences ($P \leq 0.05$) between RUT+ANA and ANA, and different letters denote significant differences between NPs and control.

Discussion

Different endpoints to evaluate NPs phytotoxicity have been proposed, with germination rates being among the most widely used (Feizi *et al.* 2013, Fan *et al.* 2014, Parveen and Rao 2015, Tumburu *et al.* 2015). Using the germination endpoint, we demonstrated that the doses 50 and/or 10 mg dm^{-3} TiO_2 NPs allowed a distinction between forms; rut+ana having more severe effects than ana. However, within the same TiO_2 NP form, no significant changes were found between doses. Germination is not a specific response, but rather an ultimate response dependent on multiple genetic, biochemical, and physiological responses. This complex interdependence may justify the low levels of reproducibility found in germination tests for NP toxicity (Barrena *et al.* 2009, Sanchez *et al.* 2011). Therefore, these assays support that germination tests may not correctly reflect NPs phytotoxicity and should be complemented with other analyses.

A range of results regarding TiO_2 NP influence on germination have been published: ana exposure had a positive effect in *Linum usitatissimum* (Clement *et al.* 2013) and in *Arabidopsis thaliana* (Tumburu *et al.* 2015), but no effect was observed in *Triticum aestivum* cv. Courtaud (Feizi *et al.* 2012, Larue *et al.* 2012a). Moreover, in the same species cv. Artur germination was negatively affected (Silva *et al.* 2016). Concerning rut+ana, several species, including *L. sativa* (Song *et al.* 2013), do not present alterations in germination (Kurepa *et al.* 2010, Castiglione *et al.* 2011, Larue *et al.* 2012b, Feizi *et al.* 2012, 2013, Song *et al.* 2013, Silva *et al.*

2016) and enhancements are only detected in *Foeniculum vulgare* (Feizi *et al.* 2013). In *Nicotiana tabacum* TiO_2 NPs impaired germination (authors did not refer which form was used) (Frazier *et al.* 2014), but the high concentration applied (2.5 %) was not environmentally relevant. In summary, the current state of art, points to few effects (which may possibly be indirect) of TiO_2 NPs on germination of most plant species. Nevertheless, this available information is clearly limited and more studies need to be done.

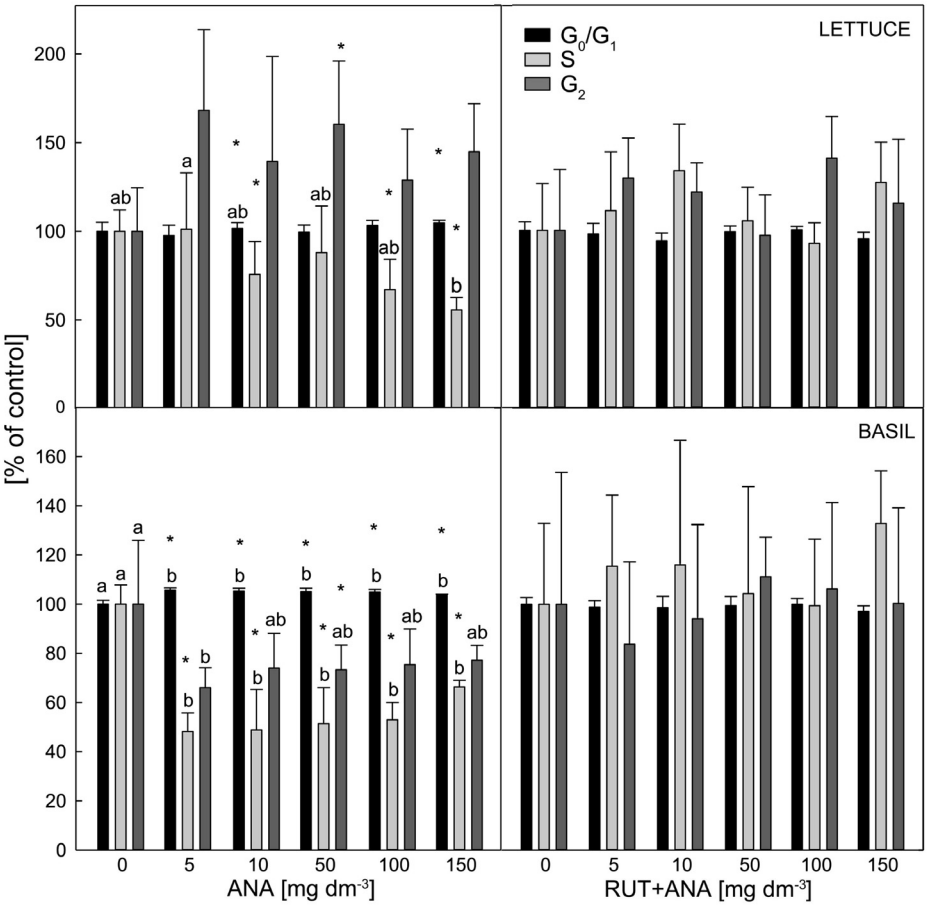

Fig. 4. Root cell cycle profile of seedlings grown in the presence of anatase (ANA) or rutile + anatase (RUT+ANA) (5, 10, 50, 100, 150 mg dm^{-3}). Means ± SDs, $n = 6 \times 3$, *asterisks* denote significant differences ($P \leq 0.05$) between RUT+ANA and ANA, and different letters denote significant differences between control and seedlings exposed to ANA.

Similarly to germination, plant growth is also dependent on complex of biochemical/cytological processes, being eventually affected by toxic effects of contaminants in a non-specific manner. Thus, growth related parameters should be complemented with other more sensitive endpoints. In this research, seedling growth parameters (biomass and length) showed different responses to TiO_2 NPs in a species dependent manner. The rut+ana negatively affected shoot and/or root biomass in both species, whereas ana had in general a stimulatory effect: *a*) in lettuce only ana stimulated length regarding the control, showing higher values for both biomass and length than rut+ana; *b*) in basil ana also had stimulatory effects, though at less extension than in lettuce. So, ana can improve plant growth at a certain range of concentrations. Ana growth benefits may be due to increasing P bioavailability (Hanif *et al.* 2015) and/or by reducing N_2 to NH_3, as reported for deficient soils (Yang *et al.* 2007). Nevertheless, TiO_2 NP impact on seedling growth is dependent on NP size: the smaller NPs the higher is the impact on plant growth (Larue *et al.* 2012a).

The stimulatory effects of ana on plant growth has been demonstrated previously for other species including *A. thaliana* (Tumburu *et al.* 2015), *Spinacia oleracea* (Yang *et al.* 2007), and in *T. aestivum* (Larue *et al.* 2012a, Silva *et al.* 2016). Moreover, Tumburu *et al.* (2015) reported up-regulation of genes related to growth and development. Nevertheless, *L. usitatissimum* (Clement *et al.* 2013) show a decrease in both biomass and root length after ana exposure. On the other hand, neutral (Feizi *et al.* 2012, 2013, Larue *et al.* 2012b, Clement *et al.* 2013, Song *et al.* 2013) and negative (*L. sativa*) (Song *et al.* 2013) effects of rut+ana on plant growth have been demonstrated, though concentration of 5 000 mg dm^{-3} is of limited environmental relevance

(Song *et al.* 2013).

Growth is the result of cell division and elongation. Therefore, alterations in cell cycle profile may give valuable information about plant growth potential and about genotoxicity of a contaminant. Concerning cell cycle profile, we demonstrate here that TiO_2 NP forms strongly influence responses of both species. Distinct cell cycle dynamics has been also recently reported by our group in wheat seedlings exposed to rut+ana *versus* ana, supporting again that NP form influence plant´s responses (Silva *et al.* 2016). It becomes clear that ana exposure induced cell cycle delay/arrest in G_1 phase. Furthermore, basil showed higher susceptibility to NPs exposure regarding cell cycle dynamics than lettuce or wheat (Silva *et al.* 2016). Studies on the effects of TiO_2 NPs on cell cycle dynamics in plants are very limited, but the effects seem to be dependent on species and TiO_2 form.

Genotoxicity was reported for several NPs in different organisms including plants. In particular, DNA damage induced by several metal NPs (Ag, ZnO, CeO, and TiO_2) was described for diverse plant species (Ghosh *et al.* 2010, Lopez-Moreno *et al.* 2010, Panda *et al.* 2011, Moreno-Olivas *et al.* 2014, Vannini *et al.* 2014). MN test has been reported to be a reliable and sensitive biomarker of NP genotoxicity in plants (Handy *et al.* 2012). The MN test detects genotoxic damage in interphase cells as a result of aneugenic (whole chromosome) or clastogenic (chromosome breakage) damage. Using this biomarker, it is evident that the exposure to TiO_2 NPs induced genotoxicity in *L. sativa* roots and observations indicate that MN formation may be a result of clastogenic damages. Concerning MN induction, lettuce was more sensitive to TiO_2 NPs than basil. In lettuce, rut+ana induced MN formation at lower concentrations followed by a decrease at higher doses, whereas ana increased MN formation only at the highest rut+ana concentration. Thus, rut+ana was more genotoxic at lower concentrations than ana. Also, in wheat a dose-dependent response was observed and rut+ana was more genotoxic than ana at lower concentrations (Silva *et al.* 2016). In the present work, ana induced DNA-damage only at higher concentrations (150 mg dm^{-3}). Therefore, it was clear that the TiO_2 NP effects on DNA were species, form, and concentration dependent. Similarly, in *Allium cepa* (Ghosh *et al.* 2010, Pakrashi *et al.* 2014), *Vicia narbonensis*, and *Zea mays* (Castiglione *et al.* 2011) TiO_2 NPs exposure induces MN formation. On the other hand, in *Vicia faba*, altered TiO_2 nanocomposites do not stimulate MN formation (Foltete *et al.* 2011). The different degrees of toxicity found for both forms (with rut+ana showing higher tendency to induce genotoxicity) may be justified by the different electro-physical properties of the rut+ana, which shows higher surface energy and reactivity (Bourikas *et al.* 2014).

In conclusion, ana showed a stimulatory effect on plant growth, whereas rut+ana presented higher ability to induce germination and growth impairments. Also, TiO_2 NPs were cytotoxic and genotoxic to plants but the targets or mechanisms of toxicity were form and species dependent. Basil was more sensitive to TiO_2 NPs regarding cell cycle but did not show genotoxic effects, whereas lettuce was more resistant to TiO_2 NPs induced cytotoxicity but more sensitive to its induced genotoxicity. Both ana and rut+ana induced MN formation, but only ana impaired cell cycle. These different mechanisms of toxicity may be justified by the different physical properties of the two TiO_2 NPs. We also stress the urgency of defining better assay parameters and techniques for determining NP phytotoxicity. The MN test showed to be a promising endpoint to assess TiO_2 NPs phytotoxicity as a complement to germination and growth parameters.

References

Anjum, N., Adam, V., Kizek, R., Duarte, A., Pereira, E., Iqbal, M., Lukatkin, A., Ahmad, I.: Nanoscale copper in the soil-plant system – toxicity and underlying potential mechanisms. - Environ. Res. **138**: 306-325, 2015.

Ardakani, A.: Toxicity of silver, titanium and silicon nanoparticles on the root-knot nematode, *Meloidogyne incognita*, and growth parameters of tomato. - Nematology **15**: 671-677, 2013.

Bandyopadhyay, S., Plascencia-Villa, G., Mukherjee, A., Rico, C.M., Jose-Yacamán, M., Peralta-Videa, J., Gardea-Torresdey, J.: Comparative phytotoxicity of ZnO NPs, bulk ZnO, and ionic zinc onto the alfalfa plants symbiotically associated with *Sinorhizobium meliloti* in soil. - Sci. total Environ. **515**: 60-69, 2015.

Barrena, R., Casals, E., Colon, J., Font, X., Sanchez, A., Puntes, V.: Evaluation of the ecotoxicity of model nanoparticles. - Chemosphere **75**: 850-857, 2009.

Begum, P., Ikhtiari, R., Fugetsu, B.: Potential impact of multi-walled carbon nanotubes exposure to the seedling stage of selected plant species. - Nanomaterials **4**: 203-221, 2009.

Bourikas, K., Kordulis, C., Lycourghiotis, A.: Titanium dioxide (anatase and rutile): surface chemistry, liquid-solid interface chemistry, and scientific synthesis of supported catalysts. - Chem. Rev. **114**: 9754-9823, 2014.

Castiglione, M.R., Giorgetti, L., Geri, C., Cremonini, R.: The effects of nano-TiO_2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. - J. Nanoparicle. Res. **13**: 2443-2449, 2011.

Chen, J., Liu, X., Wang, C., Yin, S., Li, X., Hu, W., Simon, M., Shen, Z., Xiao, Q., Chu, C., Peng, X., Zheng, H.: Nitric oxide ameliorates zinc oxide nanoparticles-induced phytotoxicity in rice seedlings. - J. Hazard. Mater. **297**: 173-182, 2015.

Clement, L., Hurel, C., Marmier, N.: Toxicity of TiO_2 nanoparticles to cladocerans, algae, rotifers and plants – effects of size and crystalline structure. - Chemosphere **90**: 1083-1090, 2013.

Cox, A., Venkatachalam, P., Sahi, S., Sharma, N.: Silver and titanium dioxide nanoparticle toxicity in plants: a review of current research. - Plant Physiol Biochem **107**: 147-163,

2016.

Cui, D., Zhang, P., Ma, Y., He, X., Li, Y., Zhang, J., Zhao, Y., Zhang, Z.: Effect of cerium oxide nanoparticles on asparagus lettuce cultured in an agar medium. - Environ. Sci. Nano **1**: 459-465, 2014a.

Cui, D., Zhang, P., Ma, Y., He, X., Li, Y., Zhao, Y., Zhang ,Z.: Phytotoxicity of silver nanoparticles to cucumber (*Cucumis sativus*) and wheat (*Triticum aestivum*). - J. Zhejiang Univ. **15**: 662-670. 2014b.

Fan, R., Huang, Y., Grusak, M., Huang, C., Sherrier, D.: Effects of nano-TiO_2 on the agronomically-relevant *Rhizobium*-legume symbiosis. - Sci. total Environ. **466**: 503-512, 2014.

Feizi, H., Kamali, M., Jafari, L., Moghaddam, P.: Phytotoxicity and stimulatory impacts of nanosized and bulk titanium dioxide on fennel (*Foeniculum vulgare* Mill). - Chemosphere **91**: 506-511, 2013.

Feizi, H., Moghaddam, P., Shahtahmassebi, N., Fotovat, A.: Impact of bulk and nanosized titanium dioxide (TiO_2) on wheat seed germination and seedling growth. - Biol. Trace Element Res. **146**: 101-106, 2012.

Foltete, A.S., Masfaraud, J.F., Bigorgne, E., Nahmani, J., Chaurand, P., Botta, C., Labille, J., Rose, J., Ferard, J.F., Cotelle, S.: Environmental impact of sunscreen nanomaterials: ecotoxicity and genotoxicity of altered TiO_2 nanocomposites on *Vicia faba*. - Environ. Pollut. **159**: 2515-2522, 2011.

Frazier, T., Burklew, C., Zhang ,B.: Titanium dioxide nanoparticles affect the growth and microRNA expression of tobacco (*Nicotiana tabacum*). - Funct. integr. Genomics **14**: 75-83, 2014.

Garcia-Sanchez, S., Bernales, I., Cristobal, S.: Early response to nanoparticles in the *Arabidopsis* transcriptome compromises plant defence and root-hair development through salicylic acid signalling. - BMC Genomics **16**: 341-357, 2015.

Ghosh, M., Bandyopadhyay, M., Mukherjee, A.: Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: plant and human lymphocytes. - Chemosphere 81: 1253-1262, 2010.

Gottschalk, F., Sun, T., Nowack, B.: Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies. - Environ. Pollut. **181**: 287-300, 2013.

Handy, R.D., Van den Brink, N., Chappell, M., Muhling, M., Behra, R., Dusinská, M., Simpson, P., Ahtiainen, J., Jha, A.N., Seiter, J., Bednar, A., Kennedy, A., Fernandes, T.F., Riedike, M.: Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? - Ecotoxicology **21**: 933-972, 2012.

Hanif, H., Arshad, M., Ali, M., Ahmed, N., Qazi, I.: Phyto-availability of phosphorus to *Lactuca sativa* in response to soil applied TiO_2 nanoparticles. - Pak. J. agr. Sci. **52**: 177-182, 2015.

Hawthorne, J., Musante, C., Sinha, S.K., White, J.C.: Accumulation and phytotoxicity of engineered nanoparticles to *Cucurbita Pepo*. - Int. J. Phytoremediat. **14**: 429-442, 2012.

Hurum, D.C., Agrios, A.G., Gray, K.A., Rajh, T., Thurnauer, M.C.: Explaining the enhanced photocatalytic activity of Degussa P_{25} mixed-phase TiO_2 using EPR. - J. Phys. Chem. B **107**: 4545-4549, 2003.

Jiang, C., Jia, J., Zhai, S.: Mechanistic understanding of toxicity from nanocatalysts. - Int. mol. Sci. **15**: 13967-13992, 2014a.

Jiang, H., Qiu, X., Li, G., Li, W., Yin, L.: Silver nanoparticles induced accumulation of reactive oxygen species and alteration of antioxidant systems in the aquatic plant *Spirodela polyrhiza*. - Environ. Toxicol. Chem. **33**: 1398-1405, 2014b.

Kurepa, J., Paunesku, T., Vogt, S., Arora, H., Rabatic, B.M., Lu, J.J., Wanzer, M.B., Woloschak, G.E., Smalle, J.A.: Uptake and distribution of ultrasmall anatase TiO_2 Alizarin Red S nanoconjugates in *Arabidopsis thaliana*. - Nano Lett. **10**: 2296-2302, 2010.

Larue, C., Castillo-Michel, H., Sobanska, S., Trcera, N., Sorieul, S., Cecillon, L., Ouerdane, L., Legros, S., Sarret, G.: Fate of pristine TiO_2 nanoparticles and aged paint-containing TiO_2 nanoparticles in lettuce crop after foliar exposure. - J. Hazard. Mater. **273**: 17-26, 2014.

Larue, C., Laurette, J., Herlin-Boime. N., Khodja. H., Fayard. B., Flank. A.M., Brisset. F., Carriere. M.: Accumulation, translocation and impact of TiO_2 nanoparticles in wheat (*Triticum aestivum* spp.): influence of diameter and crystal phase. - Sci. Total Environ. **431**: 197-208, 2012a.

Larue, C., Verones,i G., Flank, A.M., Surble, S., Herlin-Boime, N., Carriere, M.: Comparative uptake and impact of TiO_2 nanoparticles in wheat and rapeseed. - J. Toxicol. Environ. Health A **75**: 722-734, 2012b.

Lopez-Moreno, M.L., De la Rosa, G., Hernandez-Viezcas, J.A., Castillo-Michel, H., Botez, C.E., Peralta-Videa, J.R., Gardea-Torresdey, J.L.: Evidence of the differential biotransformation and genotoxicity of ZnO and CeO_2 nanoparticles on soybean (*Glycine max*) Plants. - Environ. Sci. Technol. **44**: 7315-7320, 2010.

Loureiro, J., Rodriguez, E., Dolezel, J., Santos, C.: Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. - Ann. Bot. **100**: 875-888. 2007.

Ma, C., White, J., Dhankher, O., Xing, B.: Metal-based nanotoxicity and detoxification pathways in higher plants. - Environ. Sci. Technol. **49**: 7109-7122, 2015a.

Ma, Y., Zhang, P., Zhang, Z., He, X., Li, Y., Zhang, J., Zheng, L., Chu, S., Yang, K., Zhao, Y., Chai, Z.: Origin of the different phytotoxicity and biotransformation of cerium and lanthanum oxide nanoparticles in cucumber. - Nanotoxicology **9**: 262-270, 2015b.

Miralles, P., Church, T.L., Harris, A.T.: Toxicity, uptake, and translocation of engineered nanomaterials in vascular plants. - Environ. Sci. Technol. **46**: 9224-9239, 2012.

Mohammadi, R., Maali-Amiri, R., Mantri, N.: Effect of TiO_2 nanoparticles on oxidative damage and antioxidant defense systems in chickpea seedlings during cold stress. - Russ. J. Plant Physiol. **61**: 768-775, 2014.

Moreno-Olivas, F., Gant, V., Johnson, K., Peralta-Videa, J., Gardea-Torresdey, J.: Random amplified polymorphic DNA reveals that TiO_2 nanoparticles are genotoxic to *Cucurbita pepo*. - J.. Zhejiang Univ. **15**: 618-623, 2014.

Nair, P.M.G., Chung, I.M.: The responses of germinating seedlings of green peas to copper oxide nanoparticles. - Biol. Plant. **59**: 591-595, 2015.

Pakrashi, S., Jain, N., Dalai, S., Jayakumar, J., Chandrasekaran, P., Raichur, A., Chandrasekaran, N., Mukherjee, A.: *In vivo* genotoxicity assessment of titanium dioxide nanoparticles by *Allium cepa* root tip assay at high exposure concentrations. - Plos ONE **9**: e87789, 2014.

Palmqvist, N., Bejai, S., Meijer, J., Seisenbaeva, G., Kessler, V.: Nano titania aided clustering and adhesion of beneficial bacteria to plant roots to enhance crop growth and stress management. - Sci. Rep. **5**: 10146-10158, 2015.

Panda, K.K., Acharya, V.M.M., Krishnaveni, R., Padhi, B.K., Sarangi, S.N., Sahu, S.N., Panda, B.B.: *In vitro* biosynthesis

and genotoxicity bioassay of silver nanoparticles using plants. - Toxicol. *in Vitro* **25**: 1097-1105, 2011.

Parveen, A., Rao, S.: Effect of nanosilver on seed germination and seedling growth in *Pennisetum glaucum*. - J. Cluster. Sci. **26**: 693-701, 2015.

Ricci, P.C., Carbonaro, C.M., Stagi, L., Salis, M., Casu, A., Enzo, S., Delogu, F.: Anatase-to-rutile phase transition in TiO_2 nanoparticles irradiated by visible light. - J. Phys. Chem. **117**: 7850-7857, 2013.

Sanchez, A., Garcia, A., Espinosa, R., Delgado, L., Casals, E., Gonzalez, E., Puntes, V., Barata, C., Font ,X.: Acute toxicity of cerium oxide, titanium oxide and iron oxide nanoparticles using standardized tests. - Desalination **269**:136-141, 2011.

Seeger, E., Baun, A., Kastner, M., Trapp, S.: Insignificant acute toxicity of TiO_2 nanoparticles to willow trees. - J. Soils Sediments **9**: 46-53, 2009.

Servin, A., Castillo-Michel, H., Hernandez-Viezcas, J., Diaz, B., Peralta-Videa, J., Gardea-Torresdey, J.: Synchrotron Micro-XRE and Micro-XANES confirmation of the uptake and translocation of TiO_2 nanoparticles in cucumber (*Cucumis sativus*) plants. - Environ. Sci. Technol. 4**6**: 7637-7643, 2012.

Shi, H., Magaye, R., Castranova, V., Zhao, J. Titanium dioxide nanoparticles: a review of current toxicological data. - Part. Fibre Toxicol. **10**: 15-48, 2013.

Silva, S., Oliveira, H., Craveiro, S., Calado, A.J., Santos, C.: Pure anatase and rutile+anatase nanoparticles differently affect wheat seedlings. - Chemosphere 151: 68-75, 2016.

Song, U., Shin, M., Lee, G., Roh, J., Kim, Y., Lee, E.: Functional analysis of TiO_2 nanoparticle toxicity in three plant species. - Biol. Trace Element Res. **155**: 93-103, 2013.

Srivastava, V., Gusain, D., Sharma, Y.: Critical review on the toxicity of some widely used engineered nanoparticles. - Ind. Eng. Chem. Res. **54**: 6209-6233, 2015.

Sun, T.Y., Conroy, G., Donner, E., Hungerbuhler, K., Lombi, E., Nowack, B.: Probabilistic modelling of engineered nanomaterial emissions to the environment: a spatio-temporal approach. - Environ. Sci. Nano **2**: 340-351, 2015.

Tumburu, L., Andersen, C., Rygiewicz, P., Reichman, J.: Phenotypic and genomic responses to titanium dioxide and cerium oxide nanoparticles in *Arabidopsis germinants*. - Environ. Toxicol. Chem. **34**: 70-83, 2015.

Vannini, C., Domingo, G., Onelli, E., De Mattia, F., Bruni, I., Marsoni, M., Bracale, M.: Phytotoxic and genotoxic effects of silver nanoparticles exposure on germinating wheat seedlings. - J. Plant Physiol. **171**: 1142-1148, 2014.

Weir, A., Westerhoff, P., Fabricius, L., Hristovski, K., Von Goetz, N.: Titanium dioxide nanoparticles in food and personal care products. - Environ. Sci. Technol. **46**: 2242-2250, 2012.

Yang, F., Liu, C., Gao, F., Su, M., Wu, X., Zheng, L., Hong, F., Yang, P.: The improvement of spinach growth by nano-anatase TiO_2 treatment is related to nitrogen photoreduction. - Biol. Trace Elem. Res. **119**: 77-88, 2007.