



Co-exposure to titanium dioxide nanoparticles does not affect cadmium toxicity in radish seeds (*Raphanus sativus*)



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ABSTRACT

Recent developments on environmental fate models indicate that as *nano waste*, engineered nanomaterials (ENMs) could reach terrestrial ecosystems thus potentially affecting environmental and human health. Plants can be therefore exposed to ENMs but controversial data in terms of fate and toxicity are currently available. Furthermore, there is a current lack of information on complex interactions/transformations to which ENMs undergo in the natural environment as for instance interacting with existing toxic compounds. The aim of the present study was to assess the behavior and biological effects of titanium dioxide nanoparticles (n-TiO₂) (Aeroxide P25, Degussa Evonik) and its interaction with cadmium (CdCl₂) in plants using radish seeds (*Raphanus sativus* L. *Parvus*) as model species. Radish seeds were exposed to n-TiO₂ (1–1000 mg/L) and CdCl₂ (1–250 mg/L) alone and in combination using a seed germination and seedling growth toxicity test OECD 208. Percentage of seed germination, germination index (GI) and root elongation were calculated. Cell morphology and oxidative stress parameters as glutathione-S-transferase (GST) and catalase activities (CAT) were measured in radish seeds after 5 days of exposure. Z-Average, Pdl and Z-potential of n-TiO₂ in Milli-Q water as exposure medium were also determined. DLS analysis showed small aggregates of n-TiO₂, negative Z-potential and stable Pdl in seed's exposure media. Germination percentage, GI and root length resulted affected by n-TiO₂ exposure compared to controls. In particular, n-TiO₂ at 1 mg/L and 100 mg/L did not affect radish seeds germination (100%) while at concentration of 10 mg/L, 200 mg/L, 500 mg/L, and 1000 mg/L a slight but not significant decrease of germination % was observed. Similarly root length and GI resulted significantly higher in seeds exposed to 10 mg/L and 200 mg/L compared to 1 mg/L, 100 mg/L, 500 mg/L, 1000 mg/L and control ($p < 0.05$). On the opposite, CdCl₂ significantly abolished germination % and GI compared to control seeds and a concentration dependent decrease on root elongation was observed against controls ($p < 0.05$). As well, significant decrease of germination %, GI and root elongation was observed in seeds co-exposed to n-TiO₂ and CdCl₂ at the highest concentrations (1000 mg/L n-TiO₂ and 250 mg/L CdCl₂) compared to co-exposed seeds at low concentration (1 mg/L n-TiO₂ and 1 mg/L CdCl₂) and controls ($p < 0.05$). Root elongation significantly increase compared to control at the lowest co-exposure concentration ($p < 0.05$). Similarly at intermediate concentrations of 10 and 100 mg/L in co-exposure conditions, n-TiO₂ did not affect CdCl₂ toxicity. Concerning antioxidant enzymes, a significant increase of CAT activity in seeds exposed to single high n-TiO₂ concentration (1000 mg/L) was observed while n-TiO₂ (1 mg/L), CdCl₂ (1 and 250 mg/L) and co-exposure resulted significantly decreased compared to controls ($p < 0.05$). Regarding GST activity, a slight increase in seeds exposed to 1000 mg/L n-TiO₂ but no significantly was observed, however both n-TiO₂ and CdCl₂ alone (1 and 250 mg/L, respectively) or in combinations caused a significant decrease in GST activity ($p < 0.05$). Therefore, overall data support the hypothesis that the presence of n-TiO₂ do not affect the toxicity of CdCl₂ at least at the highest concentration (100 and 250 mg/L) in radish seeds. Morphological alterations in nuclei, vacuoles and shape of radish root cells were observed upon single Cd exposure and not abolished in the presence of n-TiO₂. Nevertheless, although n-TiO₂ seems not to reduce Cd toxicity at high concentration (up to 250 mg/L), interactions cannot be excluded based on obtained results.

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1. Introduction

Nanotechnology is rapidly growing and plays an important role in our daily life. Consequently, there is a worldwide increased concern toward the impact and risk associated to the release of engineered nanomaterials/nanoparticles (ENM/Ps) into the environment by their direct usage and/or from products disposal (Boxall et al., 2007). Titanium dioxide (TiO₂) is one of the most manufactured metal oxide-NP (n-TiO₂) worldwide used in various consumer products (Nowack and Bucheli, 2007) and produced in such high quantities that its release into the environment may cause not only considerable levels of exposure but also entering into the food chain (Hou et al., 2013). However, n-TiO₂ behavior in soil and its effects on plants have been investigated so far but contradictory findings have been reported (Gogos et al., 2012). The effects of n-TiO₂ on growth parameters in various crop species have been documented by numerous studies and differential responses to n-TiO₂ were observed. For instance, n-TiO₂ (up to 5000 mg/L; particle diameter 27 nm) do not affect seed germination (near to 100%) on kidney bean (*Phaseolus vulgaris* var. *humilis*), lettuce (*Lactuca sativa* L.) and oil rape (*Brassica campestris* ssp. *napus* var. *nippo-oleifera* Makina) but was shown to be up taken into tissues of seeds. Absorption rates of n-TiO₂ were up to 12.8 mg/kg in hydroponic system and in pot experiment from 4 to 10 mg/kg approximately (Song et al., 2013b). Again, no effects on seed germination have been reported on wheat (*Triticum aestivum* spp.) up to 1000 mg/L n-TiO₂ suspension (particle diameter 12 and 25 nm) (Larue et al., 2011). Neither seed germination nor root elongation were reported upon exposure of n-TiO₂ up to 2000 mg/L (particle diameter 21 nm) in asian rice (*Oryza sativa* L.) and maize (*Zea mays* L.) seeds from 2 h up to 7 days (Yang et al., 2015) and up to 1000 mg/L (particle diameter 30 nm) healthy root were reported (Asli and Neumann, 2009). Furthermore, no significant effect on root elongation was confirmed after testing different concentrations of n-TiO₂ on wheat seeds (Feizi et al., 2012). More recent studies reported an increase in seed germination on *Arabidopsis thaliana* upon exposure to n-TiO₂ at lower concentrations (up to 500 mg/L; 33 nm) (Tumburu et al., 2015). Moreover some positive effects such as increase in weight, chlorophyll synthesis and plant growth and seed germination were reported by Zheng et al. (2005) upon n-TiO₂ up to 6.0% in spinach (*Spinacia oleracea*). An increase in photosynthesis due to the activation of photochemical reaction of chloroplast and chloroplast protecting against long time illumination has been also reported in spinach (Hong et al., 2005).

In terms of responses at cellular levels, neither ROS nor oxidative stress enzymes including catalase (CAT), glutathione reductase (GR), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and lipid peroxidation resulted affected upon n-TiO₂ exposure on kidney bean, lettuce, and oil seed rape germinated seeds (Song et al., 2013a). Koc et al. (2014) reported no significant effect after 24 h on anti-oxidative enzymes or lipid peroxidation expression levels in onion roots (*Allium cepa* L.) upon n-TiO₂ exposure up to 1000 µg/L (particle diameter, 25 nm). Beside the absence of significant effects upon exposure to n-TiO₂ in specific crops, some negative effects have been reported. Vicia (*Vicia narbonensis* L.) and maize seeds showed a decrease on seed germination compared to control after 72 h of exposure to n-TiO₂ (Castiglione et al., 2011). Moreover long-term exposure period (66 days) to n-TiO₂ (10–1000 mg/L; particle diameter 25 ± 3.5 nm) significantly affect chlorophyll content on roots and leaves of tomato (*Solanum lycopersicum* L.) (Raliya et al., 2015). In addition, malondialdehyde (MDA) and hydrogen peroxide contents and electrolyte leakage showed a significant decrease after spraying n-TiO₂ (5 mg/L; particle diameter 100 nm) after 12 and 16 days (Mohammadi et al., 2014). Moreover, in cucumber (*Cucumis sativus*) a decrease in APX and an increase in CAT activities were reported upon n-TiO₂ exposure up to 750 mg kg⁻¹ (Servin et al., 2013).

Effects of ENPs on plants may depend to different parameters such as core composition, agglomeration, shape, size of ENPs and source of

plant species (Nowack and Bucheli, 2007). For instance, exposure of n-TiO₂ in the condition of modified surface with Alizarin red S and sucrose showed that nano-conjugates pass cell walls and accumulate in distinct sub cellular compartment in *A. thaliana* (Kurepa et al., 2010). Concerning the possible trophic transfer of n-TiO₂ in plants, several studies reported accumulation and transfer to leaves, fruits, and seeds (Ma et al., 2010; Nair et al., 2010). Study on wheat seeds exposed to n-TiO₂ showed that NPs size plays a role on effects in plants. Based on their findings, n-TiO₂ with diameter size lower than 140 nm can accumulate in plant tissue. In addition, n-TiO₂ with diameter size of lower than 36 nm could transfer in plants without changing of diameter size in translocation process (Larue et al., 2012).

Up to date no study has been performed by coupling n-TiO₂ exposure with other toxic contaminants known to affect seed germination and root growth. Considering the fate of n-TiO₂ in soils from its presence in sewages, it might be probably end up in soils at certain concentrations and able to interact with other co-existing contaminants as for instance heavy metals. The main aim of the present study was to assess n-TiO₂ interaction with cadmium in plants by using radish seeds as model species. Radish is categorized as a robust plant because of existing a hard coat around seeds that it is a protective layer in germination stage against entering contaminant in presence of environmental stress (Koul, 2000). In some studies, radish seeds resistance against n-TiO₂ exposure has been documented. For example, radish seeds exposed to n-TiO₂ showed an increase of root length and shoot at 100 mg/L concentration. Previous studies have illustrated the effects of testing different kinds of ENPs suspensions or heavy metal solutions on radish seeds. For instance, testing several NPs as Al₂O₃ and ZnO as well as Al and Zn showed no changes on seed germination even at high concentration (2000 mg/L) (Wu et al., 2013). Also, any effects on radish seed germination was observed upon exposure of 200 mg/L CeO₂ (Trujillo-Reyes et al., 2013). However, nAg have been reported to affect growth and nutrient content in radish germination (Zuverza-Mena et al., 2016). Cadmium (Cd) is one of the non-essential heavy metals and is one of the relevant causes for soil contamination by anthropogenic activities (Toppi and Gabbriellini, 1999). Heavy metals in plants are responsible for changes in photosynthesis activities, accumulation of other nutrient elements, root system damaging, stoma function, enzymatic activities and plant growth (Dell'Amico et al., 2008; Singh et al., 2007). Cd is a mobile element in soil and water that because of geochemically can be up taken by plants (Krishnan et al., 2014). In living cells, Cd up taking depend to exposure duration and concentration is a major cause of cell death (Vitória et al., 2001). Study on black gram (*Vigna mungo* L.) plant showed that Cd in the form of cadmium chloride (CdCl₂) has negative effects on normal growth by reducing major morphological parameters such as germination rate, root length, shoot length, fresh and dry weight, total number of leaves and leaf area (Krishnan et al., 2014).

2. Materials and methods

2.1. Nanoparticles stock preparation and characterization in exposure media

Nanosized Titanium Dioxide powder (n-TiO₂), namely Aeroxide P25 © (declared purity of 99.9%) was kindly provided from Eigenmann & Veronelli (Milan, Italy). According to the manufacturer, P25 consist of an 82–18% anatase - rutile crystal structure mixture with a specific surface area (BET) of 50 ± 15 m²/g and pH of 3.5–4.5. A stock suspension of 2 g L⁻¹ n-TiO₂ was prepared by dispersing the powder in Milli-Q water, followed by 45 min of sonication (100 W, 50% on/off cycle in an ice-cool bath), with a probe sonicator HD 2070 Bandelin Electronic (Berlin), and stored up to 24 h. n-TiO₂ solutions were freshly prepared prior to use by diluting the stock solution with Milli-Q water (1, 10, 100, 200, 500 and 1000 mg/L) and bath-sonicating for 15 min. Size distribution of n-TiO₂ suspended in Milli-Q was

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