ESRF	**Experiment title:** Investigation of TiO_2 nanoparticles uptake and distribution in *Pisum sativum* plants cultivated in sludge-amended agricultural soil	**Experiment number**: EV189
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Introduction

The spread of engineered nanoparticles (ENPs) in the environment is becoming a worldwide problem. Soils serve as primary sink for the accumulation of ENPs, mainly due to their presence in sewage sludge and biosolid (Bs) from wastewater treatment plant (WWTP), plant protection products, potentially contaminated surface water and, nanomaterial products becoming waste at the end of their life cycle. Recent studies revealed that NPs may enter the food web or cause direct toxicity to the plants or the soil microbial community. Currently, the recycle of Bs in agricultural soils is stimulated due to the increasing amount of its production and organic matter and nutrients' supply to the soil-plant system, being identified as one of the best environmental management practice for this kind of waste. TiO_2 NPs, the most produced nanostructures worldwide, are being widely used in fertilizers, pesticides, sunscreens, cosmetics, paints, cements, batteries, textiles, etc., and, consequently, they have the major fate in sewage systems, landfilling and receiving waters. Apparently, there is no consensus on the response of plants to TiO_2 NPs exposure, then the need to assess their impact in real soil matrixes and also under the common soil amendment practices, such as the use of Bs from WWTP. In order to investigate the impact of commercial TiO_2 NPs in a Bs-amended agricultural soil, this study focused on the crop *Pisum sativum* growing under two different concentrations of the different crystal forms of TiO_2 [applied singularly or in a mixture (Mix 1:1 ratio) as anatase (A) and rutile (R) NPs, and as bulk material (B)]. The aim of this study was to use μ-XRF and μ-XANES techniques available at ID21 beamline to evaluate the uptake and distribution/localization of TiO_2 NPs in the roots, their possible speciation inside the plant tissue and the identification of preferential uptake of crystalline phases.

Material and Methods

The soil was sampled in an agricultural field, and a dewatered sludge qualified for use in agricultural soil (Bs), was obtained from a WWTP in Pisa, Italy. Commercial TiO_2 NPs (powder) as anatase or rutile at 99.9% of purity, both having a mean size of 30 nm and bulk TiO_2 with about 300 nm were used. Nano/bulk particles suspensions were obtained by sonication for 30 min. in continuous mode. Prior the addition to soil, Bs was spiked with the nano/bulk particles suspensions by mixing for 24hs, at appropriate concentration, to obtain a loading in soil of 80 and 800 mg kg^{-1}. Then, spiked-Bs was thoroughly mixed with the soil and left to equilibrate for a month. Ten different soil treatments were prepared and distributed in pots, where the pea plants grew in controlled conditions for 30d. Pea roots were collected, washed, immediately cryo-embedded in OCT resin and shipped to ESRF. Thin-section of about 25 μm of pea roots were prepared using the cryomicrotome available at the ID21 beamline. Roots from the highest TiO_2 treatments (**A800, R800, Mix800 and B800**) and from the controls (**C1**: -Bs -NPs; **C2**: +Bs -NPs) as well as a Bs (powder sample) were analysed at ID21. Ti elemental maps (μ-XRF) under cryogenic conditions (-160°C) were recorded for at various step-sizes (20, 2, 0.5 μm^2) and dwell-times (150, 130, 100 ms) with an incident energy of 5.1 keV. Ti K-edge μ-XANES spectra (4.95 – 5.10 keV) were recorded in fluorescence yield detection mode on Ti spots of the maps for the identification of crystalline phases and eventual Ti speciation.

Results

The cutting of roots samples at the cryomicrotome available at ID21 permitted an *in situ* selection of the best cut sample for the mapping and the spectra analysis at low temperatures. Micro-XANES spectra of reference materials (**Fig 1(a) -** pure anatase and pure rutile) permitted, the comparison, in the measured conditions, with spectra in roots and Bs. A previous acid extraction of Ti from **Bs** (not spiked with NPs) showed a concentration of about 750 mg kg^{-1} (dw basis). At ID21, μ-XANES spectra of the powdered Bs allowed the identification of both anatase and rutile crystalline phases **(Fig 1(b))** and also a not well defined Ti coordination (distorted octahedral or tetrahedral Ti sites) **(Fig 1(c))**. Titanium elemental maps (μ-XRF) performed on **C1 control roots** showed Ti spots mainly localized in the epidermis, however μ-XANES on selected spots resulted not exploitable. Moreover, Ti elemental maps on **C2 control roots** showed also Ti spots mostly localized in the epidermis, with some other spots found in the cortex and very few in the vessel (**Fig 2(A), spots a-d**). **Figure 2(B)** showed the μ-XANES spectra on the selected spots, where the main crystalline phase in epidermis and cortex was identified as anatase **(spots b, d, respectively)**, although also a not well defined Ti coordination was found in other spots from cortex and vessel **(spots a, c, respectively)**. This result is an indication of the presence of natural anatase and other Ti coordination in the biosolid or soil, since this last is also a natural source of titanium (Waychunas G.A., Am. Mineral. (1987) 72, 89-101).

Micro-XRF maps of **A800 roots** (**Fig. 3(A), spots a-d**) showed Ti spots mainly localized in the epidermis, although they were also found in the cortex. Moreover, μ-XANES spectra (**Fig. 3(B)**) showed that anatase was mainly found in the epidermis (**spectra a, b)** and in the cortex (**spectra d),** while in few other spots of epidermis a not well defined Ti coordination (**spectra c)** were also observed, probably present in the growth media before the spiking with 100% anatase NPs.

Similarly, in **R800 pea roots** Ti spots were mainly localized in the root epidermis, with less spots in the cortex. Rutile was the main crystalline phase identified in epidermis (**Fig. 4(A), spots b-d; Fig. 4(B), spectra b-d)** and in the cortex (**Fig. 4(A), spot e; Fig. 4(B), spectrum e)**. Interestingly, some anatase was also found in the epidermis (**Fig. 4(A), spot a; Fig. 4(B), spectra a**) which could be attributed to its existence in the growth matrix before 100% rutile NPs spiking.

The same pattern of Ti localization was found in **Mix800 and Mix80 roots**: Ti spots were mainly present in the epidermis, less in the cortex and few in the vessel (**Fig. 5(A), spot (a) in Mix80** and **Fig. 5(B), spots (b, c) in Mis800**). From the exploitable μ-XANES spots, surprisingly it was possible to identify only anatase as the most common crystalline phase and any rutile in the samples exploited. Although anatase could also be partially originated from the un-spiked growth media as in C2, this result is a good indication that anatase was internalized inside the root tissues, moved to the vascular system and was translocated to the upper parts in a preferential way, since in these roots anatase and rutile NPs were both equally spiked in the Bs. This result confirm the findings on the previous experiment (EV140), where roots grown in a composted soil spiked with a mixture of both phases showed the predominance, in the root tissues, of the anatase respect to the rutile phase. The maps of roots grown in bulk TiO_2 treatment (**B800**) have the same Ti localization (**Fig. 6(A)**, epidermis>cortex>vessel), where anatase was the unique crystalline form found in the epidermis (**Fig. 6(B), spots a-e**). However, due to beamtime end it was not possible to investigate the spots in the cortex or vessel (marked as red circle in Fig. 6(A)).

Conclusion

Generally, elemental maps in cross sections of pea roots showed that Ti was mainly localized in the root epidermis, independently of the crystalline phase (anatase or rutile) or the particle size of TiO_2 present/spiked in the biosolid (nano: 30 nm or bulk: 300 nm). Moreover, most of the crystalline phases internalized in the epidermis of R800 and A800 were found to be rutile and anatase, respectively, even if it was not possible to exclude the presence of the other phase since three different phases could be identified in the biosolid (Fig.1A). Less Ti spots were found localized in the cortex or vessel of roots, however anatase was identified in cortex/vessel of A800 and rutile in cortex/vessel of R800. Roots of Mix800 or Mix80 showed anatase as the main crystalline phase, suggesting that it was preferentially adsorbed and translocated trough the roots when equally present in the growth media. Although any synchrotron analysis could be performed in the upper parts of plants, data gives indication of NPs' translocation to the aerial parts although in reduced amount respect to that adsorbed in the roots, confirming the chemical analysis of Ti in shoots and roots separately, which showed Ti concentration about 40 times lower in the upper parts than in the roots.

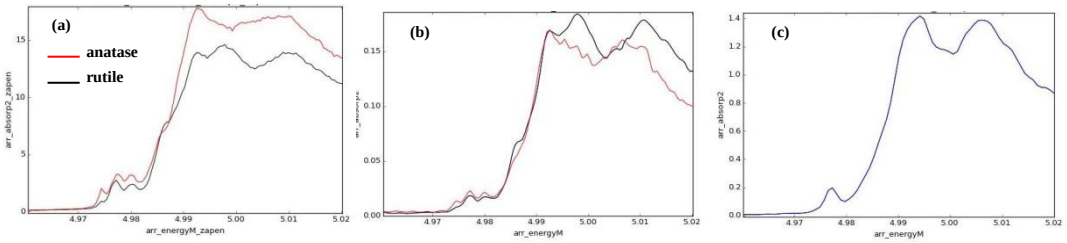

Fig 1. Micro-XANES spectra zoomed in the region of interest of (a) anatase and rutile reference materials; (b) and (c) spots on Bs powder sample.

Fig 2. C2 root:
(A) Micro-XRF
(B) micro-XANES on spots (a-d) zoomed in the region of interest
(RGB = Ti, Ca, P)

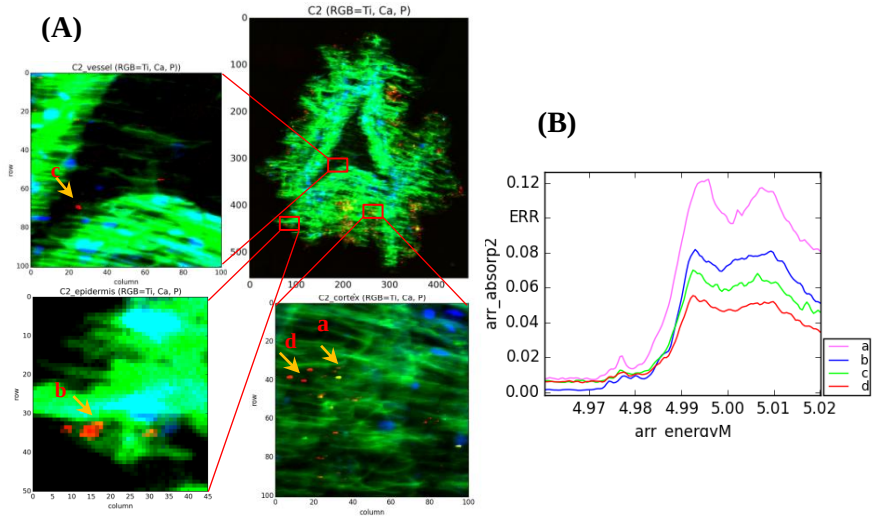

Fig 3. A800 root:
(A) Micro-XRF
(B) micro-XANES on spots (a-d) zoomed in the region of interest
(RGB = Ti, Ca, P)

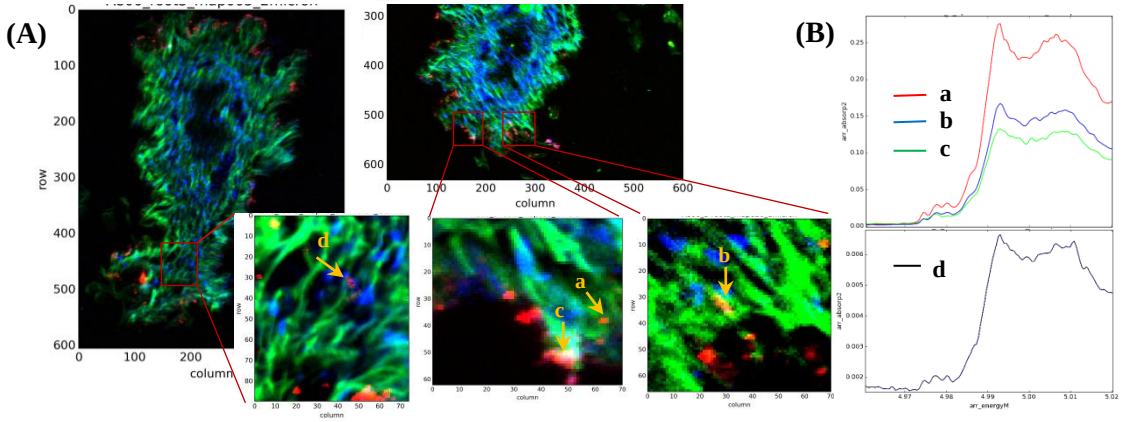

Fig 4. R800 root:
(A) Micro-XRF
(B) micro-XANES on spots (a-e) zoomed in the region of interest
(RGB = Ti, Ca, P)

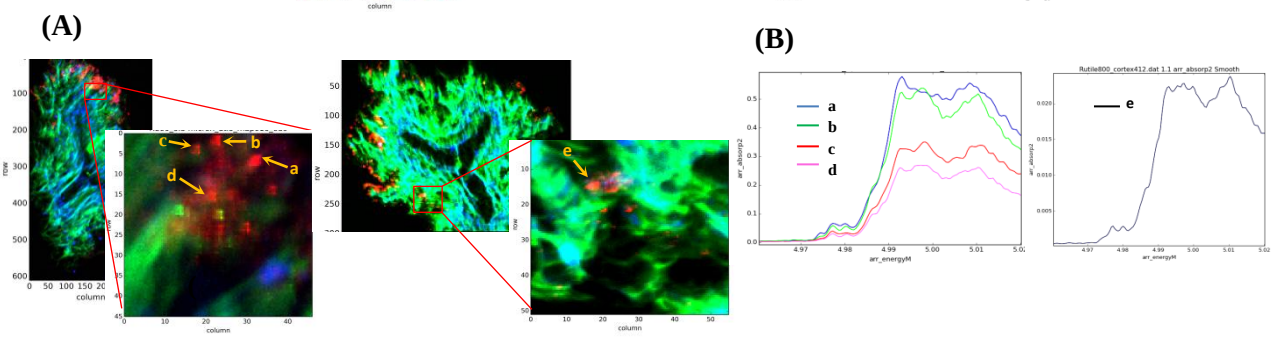

Fig 5. (A) μ-XRF and μ-XANES on spot (a) of Mix80 root; (B) μ-XRF and μ-XANES on spots (b-vessel, c-cortex) of Mix800 root
(RGB = Ti, Ca, P)

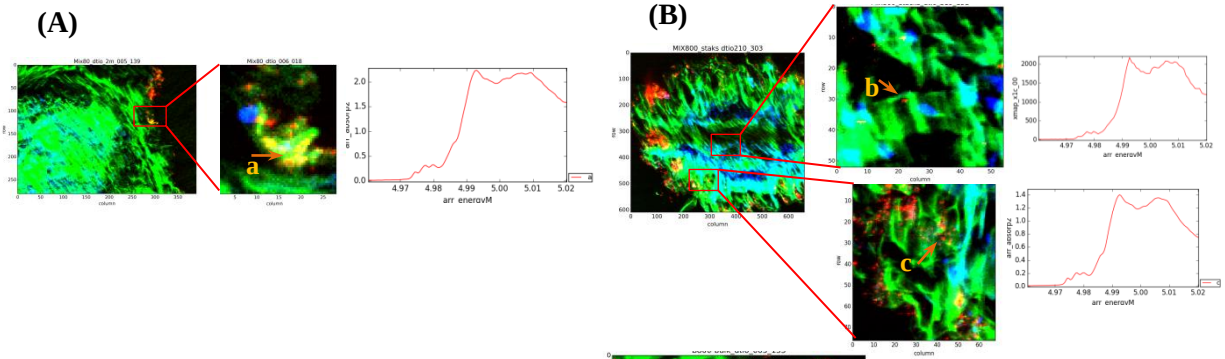

Fig 6. B800 root:
(A) Micro-XRF maps and (B) micro-XANES on spots (a-e) zoomed in the region of interest
(RGB = Ti, Ca, P)