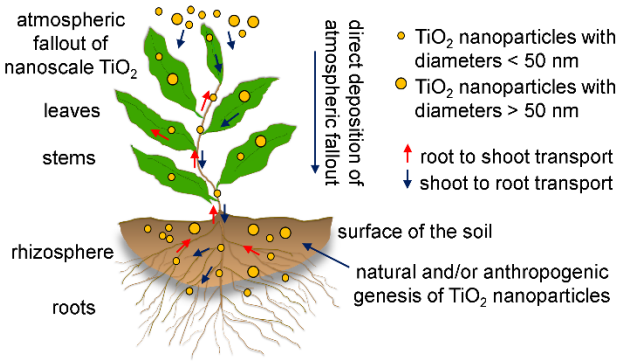
Graphical abstract



Size Fractionation of Titania Nanoparticles in Wild *Dittrichia Viscosa* Grown in a Native Environment

Bouchra Belhaj Abdallah[a,b], Irene Andreu[b], Abdelwaheb Chatti[b], Ahmed Landoulsi[a], and Byron D. Gates[b*]

[a] Biochemistry and Molecular Biology Unit, Faculty of Science of Bizerte, Carthage University, Jarzouna 7021, Tunisia

[b] Department of Chemistry and 4D LABS, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia, V5A 1S6, Canada

* Corresponding author: bgates@sfu.ca

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Abstract

We report a size fractionation of titania (TiO_2) nanoparticles absorbed from the environment and found within wild *Dittrichia viscosa* plants. The nanoparticles were isolated by extraction and isolation from distinct plant organs, as well as from the corresponding rhizosphere of wild, adult plants. The collected nanoparticles were characterized by scanning transmission electron microscopy coupled with energy dispersive X-ray spectroscopy (STEM-EDS). More than 1,200 TiO_2 nanoparticles were analyzed by these techniques. The results indicated the presence of TiO_2 nanoparticles with a wide range of sizes within the inspected plant organs and rhizospheres. Interestingly, a size selective process occurs during the internalization and translocation of these nanoparticles (e.g., foliar and root uptake), which favors the accumulation of mainly TiO_2 nanoparticles with diameters <50 nm in the leaves, stems, and roots. In fact, our findings indicate that among the total number of TiO_2 nanoparticles analyzed, the fraction of the particles with dimensions <50 nm were 52% of those within the rhizospheres, 88.5% of those within the roots, 90% of those within the stems, and 53% of those within the leaves. This significant difference observed in the size distribution of the TiO_2 nanoparticles among the rhizosphere and the plant organs could have impacts on the food chain, and further biologicals effects that are dependent on the size of the TiO_2.

Keywords: uptake, translocation, TiO_2 nanoparticles, natural environment, size distribution, soil, fate, plant behavior, internalization, *Dittrichia viscosa*

1. Introduction

There is a growing concern regarding the effects of some natural and engineered nanoparticles on the environment, and with regards to the resulting consequences on the ecosystems and the trophic chain.[1-4] Nanoparticles can end up in the environment as a result of disposal practices and/or consumer uses,[5] but also as a result of natural processes.[6,7,8,9] The properties of nanoparticles are strongly size dependent including some of their ecological and health effects.[10,11,12] There is, thus, an interest in understanding if nanoparticles can be taken up by plants from their natural environment, and if nanoparticles can be translocated to other parts of the plant as a function of their size. This information would be relevant, for example, in handling of phytoremediation waste where the aerial parts of the plants are harvested after soil decontamination, or in agricultural practices for human and livestock consumption. Understanding the size distribution of nanoparticles that have been internalized by different plant organs could, for example, enable the establishment of safe harvesting techniques to avoid exposure of the harvester to dangerous size fractions of specific phytoaccumulated nanoparticles. The distribution of particle sizes within plant organs would also have implications to the introduction of nanoparticles into the diets of livestock and/or humans consuming these plants.

Nanoscale titanium dioxide (TiO_2) is the most widely produced engineered nanomaterial worldwide with an estimated production of 10,000 tons in 2012.[13] Among its many applications, nanoscale TiO_2 is used as a pigment for painting, as an absorber of ultraviolet light in sunscreens, and as a food preservative.[14,15] Titanium dioxide nanoparticles can also be generated by natural processes such as volcanic eruptions or mineral weathering.[4,8,9] Although TiO_2 nanoparticles are currently deemed safe by both the EU and US,[16] there are concerns with regards to the long-term effects of manmade, nanoscale TiO_2 to ecosystems.[17,20] For example, Kaegi *et al*. reported the

leaching of TiO_2 nanoparticles from façade paints into aquatic environments (e.g., reaching quantities of 3.5 x 10^7 NPs/L).[21] Weterhoff *et al*. identified nanoscale TiO_2 in the effluents of wastewater treatment plants, which were being discharged into lakes, rivers, and streams.[22] In addition, Nowak *et al*., reported that biologically derived solids enriched with TiO_2 nanoparticles 273 to 342 mg/kg solids in New York City, 263 to 367 mg/kg solids in London, and 70 to 120 mg/kg solids in Shanghai),[23,24] which were disposed of by wastewater treatment facilities may cause high levels of exposure to TiO_2 if applied to agricultural soil. Concerns have also been raised with regards to human safety upon consumption of food with TiO_2 additives (e.g., skimmed milk, candy, ice cream, chewing gum).[25,27,28,29] It is estimated that in Great Britain, children under the age of 10 consume ~2 to 3 mg TiO_2/kg body weight/day, while adults consume ~1 mg TiO_2/kg body weight/day.[30] Nanoscale TiO_2 have been found to be carcinogenic to animals and the accumulation in the human body is a sufficient threat that the International Agency for Research on Cancer (IARC) classified TiO_2 nanoparticles as possibly carcinogenic to humans.[31] As nanoscale TiO_2 are likely to be broadly dispersed in the environment and pose unknown ecological risks, the French National Assembly re-evaluated the use of nanoscale TiO_2 and has decided to ban the use, import, and sale of products containing TiO_2 nanoparticles as food additives starting in 2020.[12]

Due to the high volume at which these particles are produced and the subsequent risks for release into the environment,[18] there has been a reciprocal increase in published studies measuring nanoscale TiO_2 in the environment.[12,20] Given this level of production, potential concern, and general interest in nanoparticles of TiO_2 they are, therefore, a relevant system for use as a model to study the interactions between nanoparticles and natural plants.

Past studies have demonstrated that nanoparticles can be absorbed by and translocated within the tissues of different plant parts.[17-20,32-37] Zhu *et al*. reported the uptake of iron oxide nanoparticles by the roots of pumpkin plants, showing that these nanoparticles can reach the shoots and leaves.[38] Due to the difficulty in controlling all parameters involved in the study of nanoparticle uptake and translocation (e.g., plant growth conditions, as well as nanoparticle morphology, composition, and surface chemistry), most published studies on the interactions of plants and nanoparticles have been based on the use of controlled laboratory environments. In addition, most of these laboratory studies were achieved with plants grown hydroponically. Interactions between plants and TiO_2 nanoparticles specifically have been studied in the past, relying predominantly on hydroponic cultures of young plants.[17,18,39-42] An earlier study mimicked the exposure of plants to nanoscale TiO_2 found in the soil by spiking the soil with polydisperse TiO_2 nanoparticles (e.g., multiple particle morphologies, and diameters from 20 to 100 nm).[43] Wheat seeds germinated in this soil showed signs of toxicity. The authors observed a reduction of biomass and an inhibition of soil enzyme activities, which are known bioindicators of soil quality and health. The observed agglomeration of the TiO_2 nanoparticles in the soil medium was a limitation to the translocation of these nanoparticles to the aboveground biomass. The agglomeration also impeded a determination of the size distribution of these particles in the soil and within the plant because of the formation of large, dense aggregates. The internalization of TiO_2 nanoparticles was only studied for the roots. These TiO_2 nanoparticles were not able to enter the root cells of the wheat, but instead adhered to the surfaces of the periderm cells. Relatively few TiO_2 nanoparticles succeeded in penetrating through the root cell wall. These findings were recently confirmed as similar results were observed for a comparison of red clover and wheat plants.[44] The authors demonstrated a relatively low mobility and limited plant uptake of the TiO_2

nanoparticles by both red clover and wheat grown in controlled soil conditions. Upon analysis of their root cross-sections, it was demonstrated that TiO_2 nanoparticles with a nominal diameter of 145 ± 46 nm were located on the surfaces of the roots, but never inside of the root cells. In contrast, only a few Ti-containing nano-sized particles with diameters of 29 ± 9 nm were found inside the plant cells.[44] These prior studies represent an important step beyond the foundational studies performed through hydroponics and a step towards the study of TiO_2 nanoparticle transport within plants found in natural systems. It is, however, of great importance to extend these studies to analyzing processes that occur in the wild,[45] where each plant organ of a given species may internalize TiO_2 nanoparticles via different pathways.

Herein, we report an environmental study of the internalization of TiO_2 nanoparticles by fully grown, wild *Dittrichia viscosa* plants. Naturally occurring nanoscale TiO_2 were isolated from distinct plant organs, as well as from the soil fraction associated with the rooting zone of the plants. These nanoparticles were analyzed by scanning transmission electron microscopy (STEM) and energy dispersive X-ray spectroscopy (EDS) techniques. Electron microscopy images showing a large number of TiO_2 nanoparticles were analyzed to obtain an overall size distribution of the TiO_2 nanoparticles found within each plant organ and the soil samples. These results revealed a size dependent fractionation of TiO_2 particles during uptake either from the soil to the root and shoot, or from the air to the shoot and potentially the root as well. This work provides new insight into understanding the fate and transport processes for TiO_2 nanoparticles in native adult plants and rhizosphere systems occurring within the environment.

2. Experimental

2.1. Sampling of *Dittrichia viscosa* Plants

Wild *Dittrichia viscosa* plants and the soil surrounding their rooting zone (rhizosphere) were sampled from their native environment near the town of Menzel Bourguiba at the governorate of Bizerte in Tunisia (Latitude N 37°16′, Longitude E 9°52′). This site is highly industrialized and has been the focus of on-going studies by this research team for a number of years, and our previous studies indicated a relatively high level of Ti present in the soil in this region.[46] Three plant specimens were harvested in the spring to achieve a maximal growth of the plants.[47] The rhizosphere surrounding each of the harvested plants was collected to an average depth of 20 cm and air dried for 10 days. Each plant was thoroughly washed with water and bleach to remove bacteria, nanoparticles, and other debris attached to the outer surfaces of the plants.[48] After drying at room temperature, each plant organ and the rhizosphere samples were separately ground into a fine powder using a mortar and pestle, and sieved with 0.5 mm and 2 mm sieves, respectively. Additional experimental details can be found in the Supporting Information, Section S1.

2.2. Extraction of Nanoparticles from Plant Organs and Soil Samples

A method of nanoparticle extraction was developed to isolate and concentrate the nanoparticle fraction found in each plant organ or soil sample. This method was adapted from our previous work.[46] The addition of a filtration step and process for increasing the concentration of isolated solids enabled a time-efficient analysis of the nanoparticle fractions by electron microscopy. Dry powders of the plant organs and rhizosphere were soaked for 48 h in a 1% w/v aqueous solution of sodium dodecyl sulfate (SDS) prepared in 18.0 MΩ·cm water. The SDS was used as a surfactant to assist in the release of nanomaterials from within these samples, such as

those attached to cell walls and other organelles. The suspensions obtained from this process were filtered through a medium sieve (1/16 inch mesh), and each of the filtered fractions were centrifuged at 3,000 rpm to further remove large debris. The supernatant of each fraction was passed through filters with a nominal pore size of 50 nm. These filters and their collected solids were air dried for storage until further analysis. The samples were rehydrated shortly before their analysis by electron microscopy as described below.

For the final step of the extraction process, each filter was soaked in 750 μL of high purity water (18.0 MΩ·cm) for 24 h. Each filter and its wash solution were subsequently agitated for 6 h using an orbital shaker operated at 250 rpm to further liberate solids adhered to the filter. The suspension was centrifuged at 300 rpm for 10 min at room temperature. The resulting supernatant was transferred to an Eppendorf tube for settling overnight at 4 °C to remove microscale or larger debris through decantation of the obtained supernatant. This supernatant obtained after the settling step was further centrifuged on a Beckman Optima Max ultracentrifuge at room temperature for 7 h at 80,000 rpm (347,027 *g*). The isolated solids were suspended in 500 μL of high purity water. The solids in this suspension were analyzed by STEM-EDS techniques.

Special care was taken to avoid cross-contamination of the samples, as well as to avoid the use of high energy processes, extreme temperatures, and changes in sample pH. These conditions were avoided to ensure minimal modifications to the existing nanoparticle fractions. Additional experimental details, including further analyses performed after each step of the nanoparticle purification, statistical analyses of each plant specimen, and controls for the extraction procedure can be found in the Supporting Information.

2.3. Characterization of the Isolated Nanomaterials

The size and shape of the TiO_2 nanoparticles were evaluated using an STEM operated at 200 kV (FEI Tecnai Osiris, FEI, Hillsboro, OR) and equipped with an energy dispersive X-ray spectroscopy (EDS) system (Bruker Nano, Super-X EDS). Each final suspension was drop-cast onto a separate Cu grid pre-coated with a Formvar-carbon film, and the solvent evaporated prior to the STEM-EDS analyses. Additional experimental details for these analyses can be found in the Supporting Information.

The specimens were imaged by STEM using a high-angle annular dark field (HAADF) detector. A series of EDS maps were acquired with a pixel dwell time of 500 μs. At least 15 EDS maps were analyzed per plant specimen, for a total of more than 60 separate elemental maps. These analyses were performed at magnifications between 10,000 and 60,000 times. Among these data sets, the elemental maps that contained Ti (about 30 maps per sample) were processed further to determine the morphology of the TiO_2 nanoparticles therein.

2.4. Image Processing and Calculations for Particle Size Distributions

The elemental maps obtained by EDS of the Ti-containing species were analyzed to calculate the dimensions of the TiO_2 nanoparticles. The processes used for this analysis are outlined in Figure 1. The background noise was removed using a map correction available through the Esprit software (Bruker, Germany, version 1.9.4.3329). The threshold of the signal intensity was optimized through ImageJ software (version 15.1k)[49] to create a binary image for each map. The threshold values for each image were selected to obtain the closest match between the features observed in the EDS map and those in the corresponding STEM-HAADF image (e.g., Figure 1C).

A watershed binary filter within ImageJ was used to separate the individual particles by segmentation. A particle analysis algorithm available in ImageJ was subsequently applied to obtain the projected area of each particle. This analysis excluded "particles" with dimensions <10 pixels in total area. These smaller "particles" were consistently associated with random noise observed in the images. The projected areas of the resulting particles were used to calculate the diameter of a circle with the same area, representing the 2D projection of a spherical particle. The calculated diameters are referred to throughout the manuscript as the equivalent diameter of the particles.

2.5. Statistical Analysis

To evaluate the variability in the dimensions of the TiO_2 nanoparticles between the rhizosphere and plant organs, we used a generalized linear model with a negative binomial distribution (glm.nb) to assess these datasets.[50] The results of this analysis are summarized in Table 1. To confirm any significance difference or similarity between each of the specific plant organs and the rhizosphere, we used the Tukey Honestly Significant Difference (HSD) test to perform a pairwise comparison of each size distribution of nanoscale TiO_2 particles (Table 2). Finally, we used the χ^2 test to investigate if the nanoscale TiO_2 particles with diameters <50 nm varied among the rhizosphere, leaves, stems, and roots. A separate analysis using the χ^2 test was applied to the nanoscale TiO_2 particles with dimensions >80 nm (Table 3). The χ^2 test was also used to examine the difference between distinct samples for each of the plant organs and the rhizosphere (Table S1).

We used glm.nb[51] and visreg[52] packages in MASS to assess and plot the relationships between the dimensions of the TiO_2 nanoparticles and their source (e.g., rhizosphere, leaves, stems,

and roots). These analyses were carried out using the R software environment for statistical computing (version 3.6.2).[53]

3. Results and Discussion

Nanoparticles of TiO_x were found in each of the samples isolated from the wild *Dittrichia viscosa* and their corresponding rhizospheres. Representative STEM and EDS micrographs of these nanoparticles extracted from the stems of *Dittrichia viscosa* are shown in Figure 1. The overlap of the EDS signals for the Ti and O can be seen in additional data provided in the Supporting Information (Figure S9). Additional STEM and EDS micrographs of samples isolated from the other plant parts and soil samples can also be found in the Supporting Information (Figures S8, S10, and S11). For each sample isolated from the plant organs and soil, an analysis of the EDS data indicated that the Ti signal was not co-localized with other metals within the detection limits of the EDS system. These results suggest that the Ti-containing nanoparticles were not alloyed or mixed with other metals. The average pH of the soil was neutral to slightly alkaline (pH ≤8), and the pH inside the plant organs is expected to be slightly acidic.[54] In this pH range, the expected stoichiometry of Ti and O in a solid form is titanium dioxide, or TiO_2, also known as titania.[55] The TiO_2 can crystallize in two distinct phases, anatase and rutile, with different toxicity profiles and properties.[17,18,39,44] The classification of the detected TiO_2 nanoparticles according to their crystalline phase is beyond the scope of this work. Larue *et al*. conducted hydroponic studies with TiO_2 nanoparticles on lettuce and wheat species.[17,39] They did not find chemical differentiation or further speciation of the TiO_2 nanoparticles within the plant tissues upon application to the leaves or after transport to the roots. Additional published studies also agreed with these results. For example, cross-sections performed on cucumber tissues confirmed that the translocation of TiO_2 nanoparticles from the soil to the above ground fraction of the plants happen

without biotransformation of the titania.[56,57] These results are consistent with our findings. The TiO_2 nanoparticles had a consistent composition across the samples collected from various plant organs or found within the soil. These results suggest that the TiO_2 nanoparticles did not dissolve or otherwise transform during their transport between the various regions of the wild *Dittrichia viscosa*.

These TiO_2 nanoparticles exhibited a non-faceted, smooth morphology, but their shapes were diverse. The majority of the particles were nearly spherical. No differences were observed in the shapes of the TiO_2 nanoparticles isolated from specific plant organs and the rhizosphere. Analysis of the dimensions of the TiO_2 nanoparticles found in the rhizosphere and isolated from each plant organ of the wild *Dittrichia viscosa* did, however, reveal distinct differences. The trends therein are more clearly observed through histograms of their size distributions (Figure 2 and Figure 3). A summary of measurements performed on this data is found in Table 1. For all of the samples analyzed, the average and median equivalent particle diameters were <80 nm. All of the samples had a large standard deviation, close to the value of the average equivalent diameter. The size distributions were similar for the TiO_2 nanoparticles isolated from the rhizosphere and the leaves. These nanoparticles were, in general, larger than those particles found within the roots and stems of the wild *Dittrichia viscosa*. The TiO_2 nanoparticles found in the roots and stems had similar size distributions.

A preliminary statistical analysis was used to evaluate the variability in the size distribution of nanoscale TiO_2 particles between the samples isolated from the plant organs and those in the rhizosphere using a generalized linear model. A significant difference in the dimensions of the nanoscale TiO_2 particles ($P < 0.05$) was revealed while comparing those particles isolated from the rhizosphere to the particles from the roots and the stems of *Dittrichia viscosa* (Table 1). This

analysis also identified that the nanoscale TiO_2 particles isolated from the leaves and those isolated from the rhizosphere exhibit a similarity in their distributions ($P = 0.13$, which is > 0.05). To further confirm any similarity or difference between the particles isolated from each of the plant organs and the rhizosphere of *Dittrichia viscosa*, a pairwise comparison was performed using the Tukey HSD test (Table 2). A distinct difference in the size of nanoscale TiO_2 ($P < 0.05$) was observed between the samples obtained from the rhizospheres and those from the stems (Tukey HSD: $P < 0.0001$; Figure 2 and 3), and between those from the rhizosphere and the roots (Tukey HSD: $P < 0.0001$; Figure 2 and 3). A significant difference in particle size was also observed between those particles isolated from the leaves and the stems (Tukey HSD: $P < 0.0001$; Figure 2 and 3), and between those from the leaves and the roots (Tukey HSD: $P < 0.0001$; Figure 2 and 3). The results also indicated a lack of significant difference in the size distribution of nanoscale TiO_2 between the rhizosphere and leaves (Tukey HSD: $P = 0.27$, which is > 0.05; Figure 2 and 3) and between the stems and the roots (Tukey HSD: $P = 0.94$, which is > 0.05; Figure 2 and 3). These results confirmed the similarities in the datasets that can also be observed in comparing the results within Figure 2 and within Figure 3. But the results of these analyses also confirmed that the particle size distributions observed for the samples collected at the outermost regions of the plant were statistically distinct (e.g., between the rhizosphere and roots, and between the leaves and stems). It also confirmed distinct results in the particle size distributions observed when comparing the outermost regions of the plant to those from plant organs at the opposite end of the plant (e.g., between the rhizosphere and stems, and between the leaves and roots). Differences in the dimensions of the nanoscale TiO_2 isolated from different plant organs and the soil could result from the biosynthesis of TiO_2 nanoparticles or, more likely, differences in the uptake and transport

mechanisms of the various plant organs. Uptake and transport pathways could limit the dimensions of the nanoparticles that are allowed to pass into or through the plant.

It is unlikely that the TiO_2 nanoparticles found in the wild *Dittrichia viscosa* were biosynthesized by the plant due to the low solubility of Ti compounds in the pH range found inside plants. These conditions would impede the microenvironment within the plant organs from reaching a high-enough concentration of Ti to nucleate and grow nanoparticles of TiO_2.[39,41,58] Nanoparticles of TiO_2 were also present in the rhizosphere, indicating that there is a natural source of TiO_2 nanoparticles immediately available to the plants. The TiO_2 nanoparticles found in the plant organs were most likely accumulated from the environment surrounding the plants. Several processes for nanoparticle uptake and translocation in plants have been reported in the literature.[10,19,37,45] The most widely studied uptake process is root absorption.

The roots of *Dittrichia viscosa* are the main organ to provide nutrients to the plant through absorption from the water and soil. Several studies have demonstrated the absorption of nanoparticles through plant roots for a number of different terrestrial plant species.[38,59,60] The specifics of the uptake mechanisms for nanoparticles are still under study, but the current consensus is that uptake is dependent on both the plant species and the physicochemical properties of the nanoparticles.[19] Several hydroponic studies with a range of plant species and nanoparticles of different compositions have shown that root uptake of the nanoparticles can be size selective. For example, the roots of *Arabidopsis thaliana* grown in a hydroponic culture with silica nanoparticles added to the growth medium at a pH of 5.8 exhibited more uptake for 50-nm diameter nanoparticles than for either 14-nm or 200-nm diameter particles over a period of 6 weeks.[61] Uptake of and toxicity towards these nanoparticles were found to be affected by the pH of the growth medium. *Nicotiana xanthi* grown hydroponically in the presence of 3.5-nm and 18-

nm diameter gold nanoparticles showed a preferential uptake of the smaller particles.[62] To the best of our knowledge, no previous studies have described the uptake of TiO_2 nanoparticles by the roots of wild *Dittrichia viscosa*. Size selective root uptake processes could depend on plant species, as well as on growth conditions.

A size-selective uptake of TiO_2 nanoparticles could explain the differences in size distributions observed between particles found in the rhizosphere and those within the roots of the *Dittrichia viscosa* (Table 2 and Figure 2). In particular, very few nanoparticles with an equivalent diameter >80 nm were found within the samples obtained from the roots (0.3%) and the stems (1%), while nanoparticles of that size range were identified in about 8% of the particles isolated from the rhizosphere and 9% from the leaves. The amount of nanoscale TiO_2 particles with diameters >80 nm isolated from the plant organs relative to those isolated from the rhizosphere reflected the significant difference observed from statistical analyses that are summarized in Table 3. The data in Figure 2 indicates that most of the TiO_2 nanoparticles internalized by the plants had diameters <50 nm. Among the nanoparticles internalized within the stems, 90% had a diameter <50 nm. About 88.5% of the TiO_2 nanoparticles in the roots, 53% of these particles in the leaves, and 52% of those in the rhizosphere had diameters <50 nm. Although each of the plant organs and the rhizosphere predominantly contained nanoscale TiO_2 particles with diameters <50 nm, there are significant differences in the relative amounts of particles internalized by the plant and those found in the rhizosphere. These results were statistically confirmed using a χ^2 test, which is reported in Table 3. The results in Figure 2 and Table 3 indicate a size-dependent uptake mechanism by *Dittrichia viscosa* that excludes larger nanoparticles from the roots and favors the uptake of smaller nanoparticles, with equivalent diameters <50 nm. These results agree with those

found in the literature for other plant species and for nanoparticles of other compositions but extends this knowledge to wild plants grown in their autochthonous environment.

The presence of TiO_2 nanoparticles in the roots with equivalent diameters <10 nm indicates that there could be another uptake mechanism at play. This size fraction was not present in an appreciable amount within the rhizosphere. It is possible that before and/or during root uptake some agglomerates of small TiO_2 nanoparticles present in the soil could become dispersed by the effect of root exudates and/or pH fluctuations and taken up by the roots. Such aggregates of small particles were, however, not found in the STEM micrographs of samples associated with the soil. These analyses suggested that the fraction of TiO_2 nanoparticles with an equivalent diameter <10 nm could be internalized by the plant through a pathway other than root associated uptake. Although small nanoparticles could also diffuse through the root cuticle, or enter through young/damaged tissues, the low proportion of <10 nm TiO_2 nanoparticles found in the rhizosphere indicates that, although these mechanisms might be present, they probably pose a relatively minor pathway of entry into the plant for this size of nanoparticle.

An additional pathway for nanoparticle entry into the aerial parts of the plant is through foliar uptake from atmospheric fallout. Although this mechanism has been traditionally less studied, in recent years this has been shown to be a relevant mechanism for nanoparticle uptake.[19] Nanoparticles are suspected to enter the leaves primarily through stomata. Similar to the case of root uptake, the exact mechanism of foliar uptake is not yet clear and seems to depend on both plant species and the type of nanoparticles.[19,63] Nanoparticles of TiO_2 with diameters of 4 nm and 150 nm can be internalized by lettuce leaves through uptake via stomata.[39] In another study, gold nanoparticles of different shapes and sizes were applied to watermelon leaves via drop-casting or the generation of an aerosol.[64] It was found that through either of these methods the nanoparticles

under study were internalized by the leaves and subsequently translocated to the stems and roots, but in different amounts. Plants and vegetables grown near metal smelters can exhibit high foliar levels of heavy metals, possibly due to foliar uptake of these metals in a nanoparticle form.[65] There is some evidence showing that the foliar uptake of particles is also a size-dependent process, favoring the uptake of smaller nanoparticles.[66]

Although no air sampling was performed during the period of growth and harvesting of the *Dittrichia viscosa*, it is expected that the atmosphere contains a higher relative percentage of small nanoparticles in contrast to larger nanoparticles than that found in the soil. The smaller nanoparticles likely remain airborne for a longer period of time than that observed for the larger nanoparticles.[67] This differentiation could lead to a higher concentration of small nanoparticles relative to the larger nanoparticles that are present in the atmosphere than the relative concentrations found in the soil. It is, thus, possible that the TiO_2 nanoparticles with an equivalent diameter of 10 nm or less found in the leaves of the analyzed plants, as well as in their stems and roots, were internalized through foliar uptake from airborne soil dust.

Once inside the plant (via the roots or leaves), the nanoparticles can translocate to other organs within the plants. Indeed, nanoparticles were found in the plant stems. The TiO_2 nanoparticles with equivalent diameters <10 nm were found in each of the plant organs, but not within the rhizosphere (Figure 2). These results indicated that this nanoparticle fraction is highly mobile. It is likely that these nanoparticles were translocated from the leaves to the roots. Regardless of the transport pathway (root to shoot and shoot to root transfers), the size distribution obtained in the stem of the *Dittrichia viscosa* suggests that the stems and roots exclude particles with an equivalent diameter greater than 80 nm. This size fraction, when internalized by the leaves, was prevented from reaching the vascular system and being translocated within the plant. It is also

possible that the larger nanoscale TiO_2 observed in the leaves were not actually internalized, but only adhered strongly to the outer surfaces of the leaves. In either case, there was a lack of >80-nm diameter TiO_2 in the roots and stems. These results suggest that the larger particles did not adhere to the outer surfaces of the roots or stems of the plant, and were not transported to these organs through internal mechanisms. The presence of these larger particles in the leaves and rhizosphere confirms the existence of size-selective mechanisms for the translocation of TiO_2 nanoparticles in naturally grown plants.

The TiO_2 nanoparticles found in the wild *Dittrichia viscosa* could have a natural or an anthropogenic origin. Nanoparticles can be naturally generated from the weathering of minerals,[9] or formed by growth from metal precursors.[68] Titanium dioxide nanoparticles are widely manufactured, and incorporated into many consumer products, such as sunscreens, toothpastes, and paints.[14,15] Tools or machine parts coated with TiO_x could result in the generation of nanoparticles from mechanical processes during their use. Nanoparticles of TiO_2 can also be released into the environment during waste disposal, such as through some forms of incineration used by industrial furnaces for metal-recycling.[59,69] Both natural and anthropogenic TiO_2 nanoparticles, like most nanoparticles, can be transported hundreds of kilometers through the atmosphere or hydrosphere.[7,70] Accurately determining the source of TiO_2 nanoparticles found in the environment can, therefore, be a very challenging task,[67] and is beyond the scope of the present study.

The processes described for extracting and isolating the nanoparticles were used to concentrate the nanomaterials that were naturally present in the plant organs and the rhizosphere of wild *Dittrichia viscosa*. Traditionally the preparation of plant specimens for TEM analysis involves a laborious process of fixation, embedding, and ultramicrotoming of plant tissues.[17,18,39]

These preparation methods require the use of potentially toxic fixatives and stains. Additionally, the analysis of sections of plant specimens can require several fields of view during the TEM analyses to obtain a sufficient number of nanoparticles as needed to estimate particle size distributions. A relatively larger number of samples would need to be analyzed by TEM due to the relatively low concentrations of nanoparticles in each plant tissue. In contrast, our analysis increases the concentration of nanoparticles in each S/TEM field of view, which reduces the time required for these analyses. In addition, our sample extraction and isolation procedures are environmentally friendly. Although the analysis of intact tissues by ultramicrotomy provides important insights into the spatial distribution of nanoparticles within plant organs and cell organelles,[17,39,71] this type of analysis lacks the throughput required for evaluating subtle differences in, for example, nanoparticle size. A relatively small number of samples can be analyzed by sectioning within a reasonable time frame,[44] such that this approach will not be able to determine differences in particle size with the necessary confidence. This approach also does not extend to the analysis of soil samples. Our process of nanoparticle extraction enabled the analysis of a relatively large number of nanoparticles. Three plant specimens were analyzed with at least 300 nanoparticles per plant organ or rhizosphere for a total of over 1,200 nanoparticles analyzed by these methods. This approach was sufficient to characterize differences in the distribution of particle size between these samples.

To the best of our knowledge, this is the first study of the uptake of nanoparticles by plants and its associated size distribution within wild plants. Previously published studies have focused on controlled, hydroponic conditions, but the need for assessing nanoparticle exposure under more realistic settings for environmental studies has been highlighted before.[45] The increasing production and release of engineered nanoparticles into the environment can have long-term

effects on the ecosystem, which can span all stages of the trophic chain. It is important to understand the processes of nanoparticle uptake and their size distribution within different plant organs. For example, the health effects of nanoparticles are typically size dependent.[10] The present study demonstrates that a size-selective shoot and foliar uptake of TiO_2 nanoparticles occurs naturally in the environment, and that the internalized nanoparticles can be translocated to other organs in *Dittrichia viscosa* depending on their size. Nanoparticles of TiO_2 with equivalent diameters >80 nm are not taken up by the roots but are present in the leaves. Their translocation to other plant organs is impaired by the mechanisms of nanoparticle transport. On the other hand, nanoparticles with equivalent diameters <10 nm are efficiently translocated from the leaves to the roots, even when this size fraction is not observed in the rhizosphere. The combination of controlled laboratory experiments and the type of field experiments performed in the current study will enable the environmental and agricultural communities to continue to build a more complete picture of the interactions of nanoparticles with plants. These efforts are necessary to better understand the ecological effects of nanoparticle uptake and transport.

Supporting Information

The Supporting Information for this article is available via the internet free of charge. Additional experimental details, additional SEM, STEM, and EDS data supporting protocols for nanoparticle isolation, dynamic light scattering data on the isolated particle fractions, additional data on the identification of individual titania nanoparticles, and additional statistical analyses of the results.

Abbreviations

DLS	Dynamic Light Scattering
EDS	Energy Dispersive X-ray Spectroscopy
HAADF	High-Angle Annular Dark-Field
SEM	Scanning Electron Microscopy
STEM	Scanning Transmission Electron Microscopy
TEM	Transmission Electron Microscopy
TiO_2	Titanium Dioxide

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FIGURES

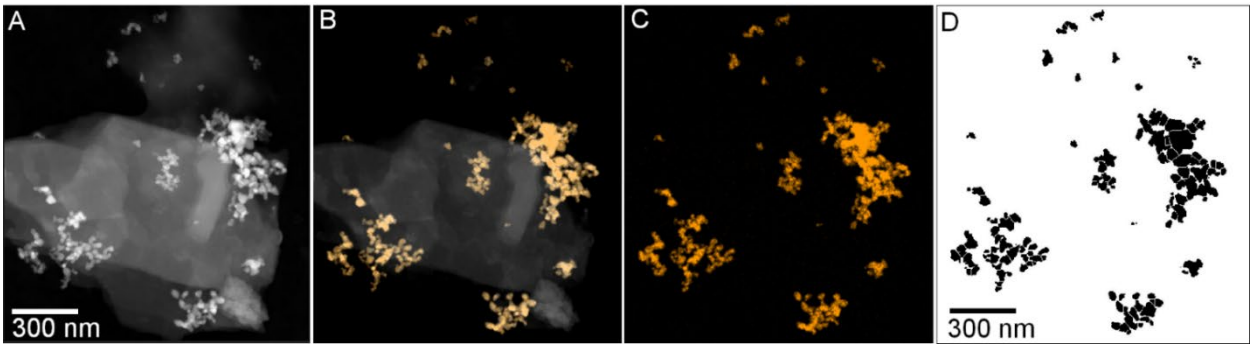

Figure 1. Representative micrographs of TiO_2 nanoparticles extracted from the stems of *Dittrichia viscosa*. (A) Scanning transmission electron microscopy (STEM) image of these particles obtained using a high-angle annular dark-field (HAADF) detector. (B) An energy dispersive X-ray spectroscopy (EDS) map of Ti containing species (orange) overlapped with the HAADF image (gray). (C) The EDS map of only the Ti species for the same region of the sample in (A). (D) A binarized image of the Ti containing nanoparticles used for the determination of their dimensions.

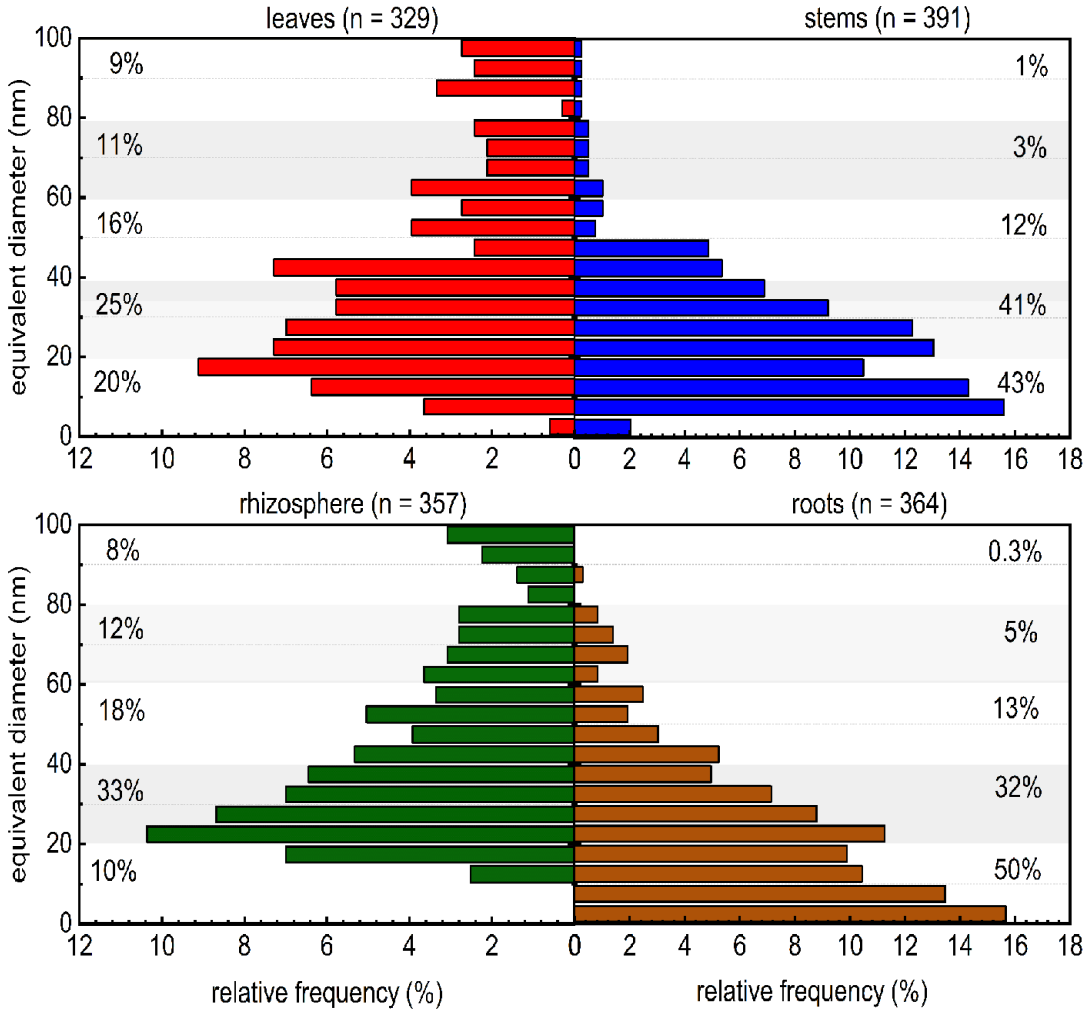

Figure 2. Estimated size distributions of TiO_2 nanoparticles isolated from the rhizosphere, roots, stems, and leaves of wild *Dittrichia viscosa*. The relative amounts of nanoparticles found within each 20 nm step size for equivalent particle diameter are indicated by the associated percentage values reported on each plot.

773 **Table 1.** Results from analyzing the size distributions of nanoscale TiO_2 particles isolated from
774 wild *Dittrichia viscosa*.

soil or plant organs	rhizosphere	leaves	stems	roots
total particle count (n)	357	329	391	364
mean diameter ± SD [*] (nm)	70 ± 58	63 ± 56	26 ±17	24 ± 20
P	0.000 [†]	0.13 [††]	0.000 [††]	0.000 [††]

[*] SD are the errors reported as one standard deviation from the calculated mean values for the diameters of the TiO_2 nanoparticles. [†] *P* value of the reported significance between the rhizosphere and all the plant's organs as determined using a preliminary statistical analysis with a generalized linear model. [††] *P* values of the reported significance between each of the plant's organs and the rhizosphere as determined using a preliminary statistical analysis with a generalized linear model.

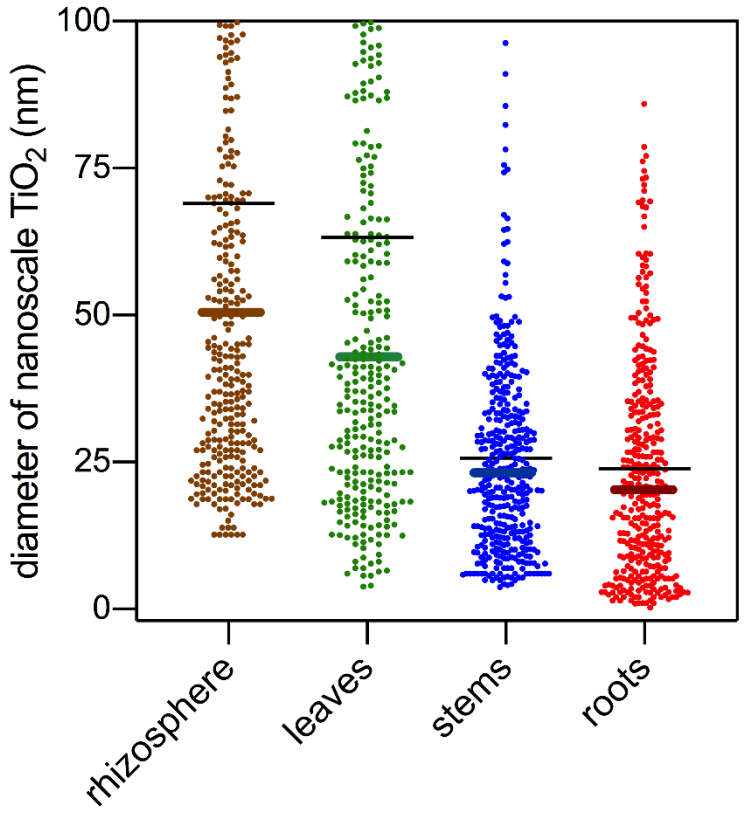

Figure 3. Plots of diameter of nanoscale TiO_2 <100 nm counted in the rhizosphere (n = 357), leaves (n = 329), stems (n = 391), and roots of *Dittrichia viscosa* (n = 364), their corresponding mean equivalent diameter (thin, black line) and median equivalent diameter (thicker, colored lines).

803

804 **Table 2.** Results from a Tukey HSD test to examine differences in the dimensions of nanoscale

805 TiO_2 isolated from the various plant organs and the rhizosphere of *Dittrichia viscosa*.

soil or plant organs	Rh vs L	Rh vs St	Rh vs R	L vs St	L vs R	R vs St
P †	0.272	0.000	0.000	0.000	0.000	0.938

† *P* values are the calculated significance between the size distributions for the nanoscale TiO_2 resulting from the

807 analyses of the leaves (L), stems (St), roots (R), and the rhizosphere (Rh) of *Dittrichia viscosa*.

827 **Table 3.** Analyses for statistical significance in the differences observed in the size distributions
828 of nanoscale TiO_2 particles with equivalent diameters < 50 nm, and those with equivalent diameters
829 >80 nm between the plant's organs and the rhizosphere of wild *Dittrichia viscosa.*

variables	χ^2	degrees of freedom (df)	*P* †
TiO_2 nanoparticles with diameters <50 nm	19.078	3	0.000
TiO_2 nanoparticles with diameters >80 nm	13.632	3	0.003

† *P* values for the reported significance (tested by χ^2) for the distributions in size of nanoscale TiO_2 with equivalent
831 diameters <50 nm and separately for those with equivalent diameters >80 nm as compared between the collective
832 results for the plant's organs and the rhizosphere of *Dittrichia viscosa*.
833